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Présentée et soutenue par

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Titre : *The role of two members of the Ly6 superfamily in the organization of septate junctions during Drosophila melanogaster development*

JURY

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“It is not the strongest of the species that survives… nor the most intelligent that survives. It is the one that is the most adaptable to change.”

"We can allow satellites, planets, suns, universe, nay whole systems of universe to be governed by laws, but the smallest insect, we wish to be created at once by special act.”

Charles Darwin

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For my parents, I am going to dedicate my thesis …

CONTENT

SUMMARY

Author: Assia HIJAZI

Title: The role of two members of the Ly6 superfamily in the organization of septate junctions during *Drosophila melanogaster* development.

PhD supervisors: Dr. Fernando Roch and Dr. Lucas Waltzer

The Ly6 superfamily is a large family of genes present in most metazoan genomes, including 45 members in Humans. These genes mainly encode for extracellular glycoproteins attached to the cell membrane by a GPI anchor (Glycosylphosphatidylinositol), but also for soluble ligands. They are characterized by the presence of an extracellular domain, called Ly6 domain, whose structure is provided by 8 to 10 cysteines present in conserved positions. The great variability exhibited by the Ly6 primary sequences allows these proteins to exert highly divergent roles. Although their function has been elucidated in various organisms, we still know very little about their potential roles during animal development. During my PhD, I used the *Drosophila* model system to extend our knowledge about the functions of these proteins in a developmental context. Our work has permitted the identification of 36 members of the Ly6 superfamily in *Drosophila melanogaster*, and I have characterized at a functional level two of these genes during development. Phenotypic analysis of mutants for these two genes, called *boudin* and *coiled*, has shown that both of them are required for tracheal morphogenesis and organization of septate junctions in epithelial tissues. Septate junctions are cell adhesion structures analogous to vertebrate tight junctions. They allow epithelia to perform their barrier function and regulate the passage of solutes and ions through the paracellular space. Septate junctions in *Drosophila* are similar to the vertebrate paranodal junctions, present at the contact between axons and Schwann cells, and our results show that *boudin* and *coiled* are also required for the organization of septate junctions in the fly nervous system. On the other hand, we have shown that the protein Boudin is able to diffuse from one cell to another to regulate septate junction formation. This non cell autonomous mode of action had never been described for proteins involved in septate junction organization. Studying the diffusion mechanisms and the trafficking of Boudin is important to better understand how this protein performs its function. Finally, another challenge will be to identify functional partners of Boudin and Coiled to elucidate the molecular mechanisms by which these proteins control the maintenance and the organization of septate junction structures.

Key words: Ly6 superfamily, Drosophila development, septate junctions, paracellular barrier, paranodal junctions.

Discipline: Cell biology, Genetics and Development.

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RÉSUMÉ

AUTEUR : HIJAZI Assia

Titre : Etude du rôle de deux gènes de la superfamille Ly6 dans l'organisation des jonctions septées au cours du développement de *Drosophila melanogaster*

Directeurs de thèse : Dr. ROCH Fernando et Dr. WALTZER Lucas

La superfamille Ly6 est une famille de gènes présente dans le génome de la plupart des métazoaires, y compris l'Humain. Ces gènes codent principalement pour des glycoprotéines attachées à la membrane par une ancre GPI (Glycosylphosphatidylinositol), mais aussi pour des ligands solubles. Les membres de cette famille se caractérisent par la présence d'un domaine extracellulaire, appelé domaine Ly6, dont la structuration est assurée par 8 à 10 cystéines présents dans des positions conservées. La grande variabilité du reste de la séquence des protéines Ly6 leur permet d'exercer des fonctions divergentes, hautement spécialisées. Même si certaines fonctions des protéines Ly6 ont été élucidées chez divers organismes, on connait très peu sur leurs rôles potentiels pendant le développement animal.

Durant ma thèse, j'ai utilisé la drosophile comme système modèle afin d'étendre nos connaissances sur les fonctions de ces protéines dans un contexte développemental. Notre travail a permis d'identifier l'ensemble des 36 membres de la superfamille Ly6 chez *Drosophila melanogaster*. J'ai étudié plus particulièrement le rôle de deux membres de cette famille au cours du développement. La caractérisation fonctionnelle des mutants pour ces deux gènes, appelés *boudin* et *coiled*, a montré qu'ils sont tous les deux requis pour la morphogenèse trachéale et l'organisation des jonctions septées dans les tissus épithéliaux. Les jonctions septées sont des structures d'adhérence cellulaire, analogues aux jonctions serrées des vertébrés. Elles permettent aux épithéliums d'exercer leur fonction de barrière paracellulaire qui régule le passage des solutés et des ions. Les jonctions septées de la drosophile sont aussi similaires aux jonctions paranodales des vertébrés, présentes au contact entre axones et cellules de Schwann, et nos résultats montrent que *boudin* et *coiled* sont également requis pour l'organisation des jonctions septées dans le système nerveux. D'autre part, nous avons montré que la protéine Boudin est capable de diffuser d'une cellule à l'autre pour réguler la formation des jonctions septées. Ce mode d'action «cellulaire non-autonome » n'avait jamais été décrit pour des protéines qui participent à l'organisation des jonctions septées. L'étude du mode de

diffusion et du trafficking de Boudin permettra de mieux comprendre comment cette protéine exerce sa fonction. Enfin, un autre challenge sera d'identifier les partenaires fonctionnels de Boudin et Coiled pour élucider les mécanismes moléculaires par lesquels ces protéines contrôlent le maintien et l'organisation des jonctions septées.

Mots-clefs: la superfamille Ly6, développement de la drosophile, jonctions septées, barrière paracellulaire, jonctions paranodales.

Discipline: Biologie cellulaire, Génétique et Développement.

Intitulé et addresse du laboratoire

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LISTS OF ABBREVIATIONS

&

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Abbreviations list

AChE: Acetylcholinesterase

ARP2/3: Actin-Related Protein-2/3

ARS: Actin Rich structures

ASSET: Accelerated Segment Switch in Exons to alter Targeting

Babo: Baboon

BBB: Blood Brain Barrier

Bou: Boudin

CAMs: Cell Adhesion Molecules

Caspr: Contactin-associated protein

CD59: Cluster of differentiation 59

Cold: Coiled

Cont: Contactin

Cor: Coracle

D-Contactin: Drosophila-Contactin

Dlg: Discs Large

ECM: Extracellular Matrix

EM: Electron Microscopy

Fas III: Fasciclin III

FBS: Foetal Bovine Serum

FERM: 4.1 Ezrin, Radixin, Moesin

FITC: Fluorescein Isothiocyanate

GFP: Green Fluorescent Protein

GPCR: G-protein coupled receptor

GPI: Glycosylphosphatidylinositol

Ig L1-CAM: Immunoglobulin cell adhesion molecules

IgLON: Immunoglobulin LAMP, OBCAM and Neurotrimin

JAMs: Junctional Adhesion Molecules

Lach: Lachesin

Ly6: Lymphocyte Antigen 6

Ly6/uPAR/CD59: Lymphocyte Antigen 6/urokinase Plasminogen Activator/Cluster of Differentiation 59

Lynx-1: Ly6/neurotoxin-like protein 1

MAC: Membrane Attack Complex

MDCK: Madin-Darby Canine kidney

MAGI: Membrane-Associated Guanylate kinase with Inverted domain orientation

MAGUKs: Membrane-Associated Guanylate Kinase

Mega: Megatrachea

Mtf: Melanotransferrin

MUPP1: Multi-PDZ domain protein 1

Na^+/K^+ ATPase: Sodium/Potassium Adénosine Triphosphatase

nAChR: nicotinic Acetylcholine Receptors

NCP1: Neurexin IV/ Caspr/Paranodin1

NF-155: Neurofascin 155

NlLynx-1: *Nilaparvata lugens* Lynx-1

NlLynx-2: *Nilaparvata lugens* Lynx-1

NMR: Nuclear Magnetic Resonance

Nrg: Neuroglian

Nrx IV: Neurexin IV

Nrv2: Nervana 2

PAF: Paraformaldehyde

Pdcyst-rich: *Pocillopora damicornis* cystein-rich

PDZ: PSD-95 (Postsynaptic density protein 95)/Discs large/ZO-1

PSJ: Paranodal Septate Junctions

Put: Punt

Rtv: Retroactive

S2: Schneider-2

Sax: Saxophone

Sinu: Sinuous

SJ: Septate Junctions

SPG: Subperineurial Glia

SSS: Sleepless

TevP: Tobacco mosaic virus protease

TFD: Three-Finger Domain

TGF-β: Transforming Growth Factor-βeta

TJ: Tight Junctions

Tkv: Thickveins

TRITC: Tetra methyl Rhodamine Iso Thio Cyanate

uPA: urokinase-type Plasminogen Activator

uPAR: urokinase-type Plasminogen Activator Receptor

Vari: Varicose

Wit: Wishful thinking

ZA: Zonula Adherens

ZOs: Zonula Occludens

ZO-1: Zonula Occludens-1

ZO-2: Zonula Occludens-2

ZO-3: Zonula Occludens-3

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Introduction

Supplementary results:

I. INTRODUCTION

Part 1

I. Biological barriers, a hallmark of life

In his wonderful essay “What is life?” appeared in 1944, the physicist E. Schrödinger enquired about the essential features that define living organisms and mark the transition between life and the inanimate matter. He found that one of the essential properties of living beings is that, despite their active metabolism, they are extremely organized structures capable of standing for a considerable amount of time against the universal course towards thermodynamic equilibrium. How do these “entropy islands” manage to temporarily avoid decay? He found a satisfactory explanation stating that life has the unique property of extracting order from the external environment, incorporate it into its structure and then release waste products in a constant exchange that, as he liked to put it, feeds the organism with negative entropy. Although Schrödinger did not venture into these grounds, it follows that such entropy islands need to be separated from the surrounding environment by some kind of barrier, which becomes thus one of the fundamental features of living organisms. So, it is tempting to propose that without efficient barriers, life simply would not be possible.

The most obvious and universal type of barrier existing in living organisms is the cell membrane that surrounds the protoplasm of each cell and separates the ordered intracellular components from the chaotic external environment. However, this cell barrier is selectively permeable, which means that is able to regulate the passage of different substances that enter and exit the cell, assuring the transport of materials needed for cell survival.

During evolution, multicellular organisms have in turn developed highly specialized cell types, the epithelial cells, whose main function is to act as barriers. However, epithelia not only insulate organisms from the external environment, but also allow body subdivision into physiologically distinct compartments, opening the way to both organogenesis and evolution of complex body plans. Thus, a crucial function of epithelia during animal development is to maintain the unique composition of different body compartments and to regulate the passage of different materials through the space separating adjacent cells, providing a control over which substances are allowed to enter or leave a particular tissue.

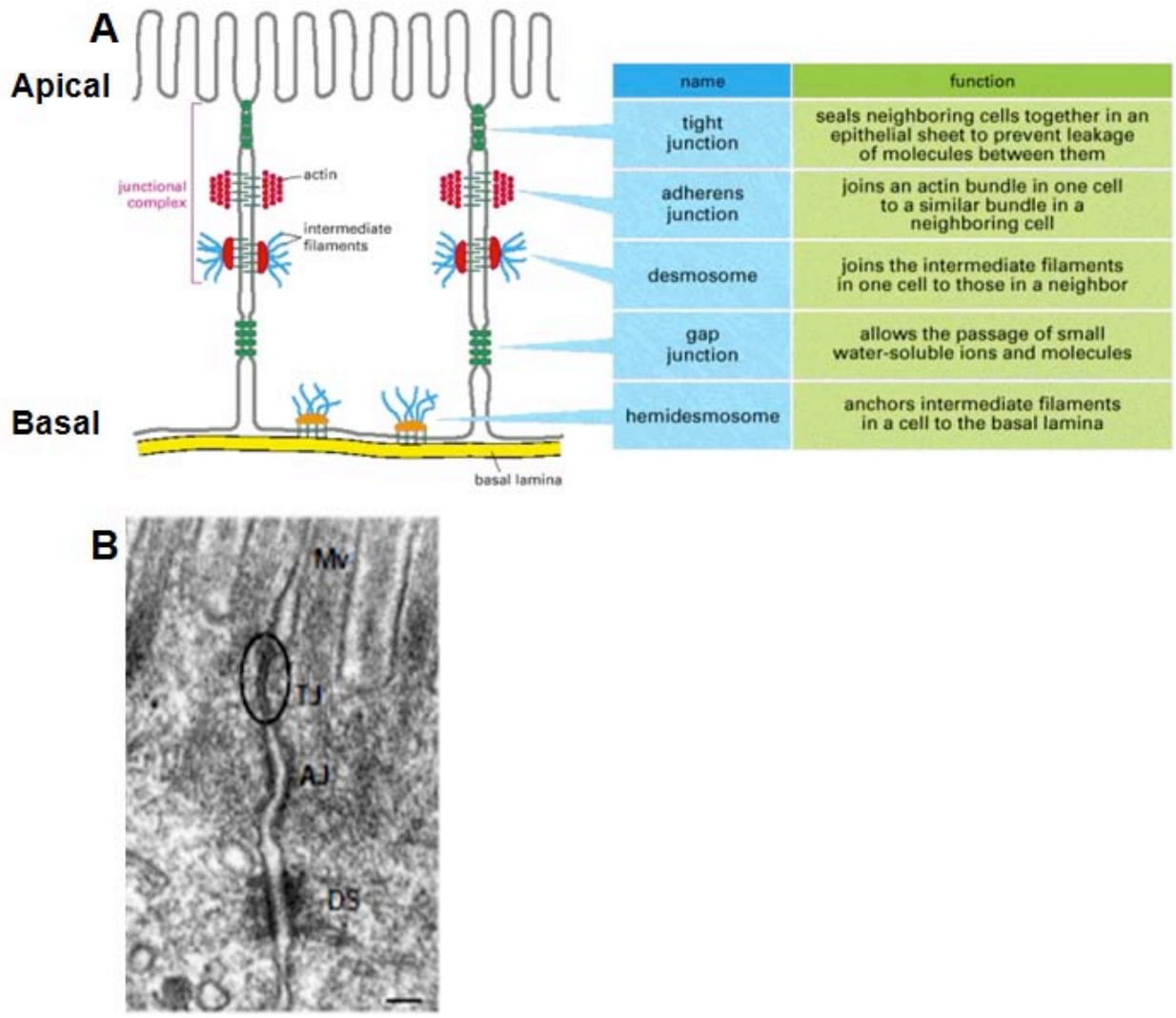

Fig 1. Junctional cell complexes in vertebrate epithelial cells. (A) Schematic drawing of intestinal epithelial cells. Membrane epithelial cells display three types of cell adhesion complexes: 1) Occluding junctions embodied by the tight junctions in vertebrates and localized in the most apical part of the cell). 2) Anchoring junctions (including adherens junctions, desmosomes and hemidesmosomes). 3) Gap junctions. Apical cell domain is at the top, basal cell domain is at the bottom. Respective function established by different cell junction complexes is mentioned in the table, on the right. (**B)** Electron microscopy image showing different junctional complexes of mouse intestinal epithelial cells. (Mv, microvilli; TJ, tight junction, circled in black and localized most apically; AJ, adherens junction localized just below the TJ; DS, desmosome.) Scale bar, 200 nm. **Ref. panel A:** Alberts B. et al., 2002. Mol Biol of the Cell, Garland Science, 4th edition. **Ref. panel B:** (Tsukita et al., 2001).

How epithelial cells are organized and assembled and how they accomplish their physiological "barrier" function remains a fundamental issue in animal development, and has been for decades the object of multiple studies.

Interestingly, the concept of epithelial cell barrier has evolved over time, as different experiences and novel observations have accumulated. In fact, initial experiences performed on epithelial tissues using vital dyes, revealed a distinct region at the apical end of the lateral cell membrane, referred to as the "terminal bar." This structure was thought to constitute an absolute barrier totally blocking the passage between cells. However, by the early 20th century, it became clear that some materials, like macrophages and water could indeed cross epithelia through the paracellular space, which is the space available between contiguous cells of the same epithelium. Current understanding shows that specialized cell junctions essentially form a selective permeability barrier across epithelial cells and behave as gates regulating the passage of solutes, ions and even small molecules from one side of the epithelium sheet to the other (Tsukita et al., 2001; Knust and Bossinger, 2002). But, which is the material basis for these selective gates?

II. Cell junctions: a material basis for the barriers in multicellular organisms

The solution that multicellular organisms have adopted to respond to the need for efficient paracellular barriers is to build up specialized cell adhesion contacts charged with this specific task. In fact, not all the known types of adhesion contacts contribute to the formation of paracellular barriers.

In general terms, epithelial cell junction complexes have been classified into three groups that assume different, albeit often related functions (Müller and Bossinger, 2003). We can distinguish (Fig 1):

- Sealing or occluding junctions, such as zonula occludens or tight junctions, which maintain the selective barrier of epithelia (Schneeberger and Lynch 1992; Anderson et al., 1993).

- Anchoring junctions such as adherens junctions (Niessen and Gottardi, 2008) and desmosomes (Holthofer et al., 2007; Garrod and Chidgey, 2008) which keep cells mechanically attached to each other's by joining specific cytoskeleton components of

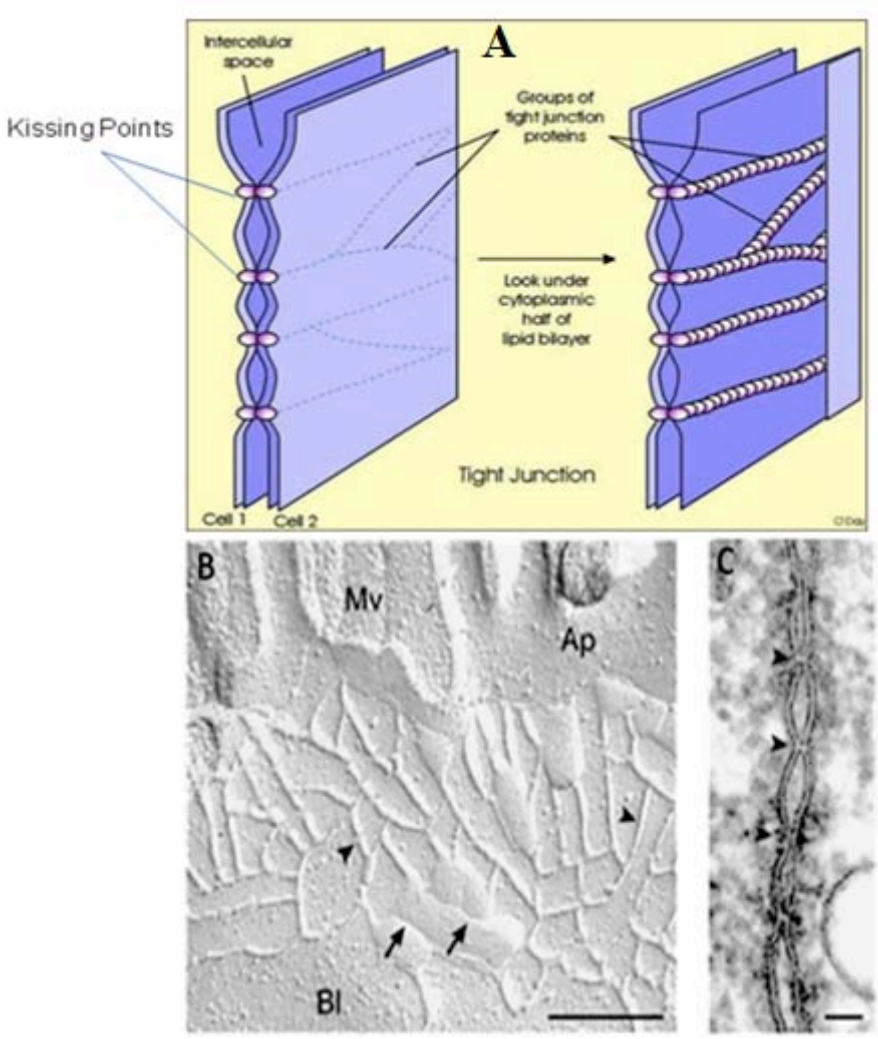

Fig 2. (A) Cartoon of a tight junction strand. At tight junctions, tightly aligned rows of proteins, localized under cytoplasmic half of lipid bilayer, permit to join the tight junction strand in the apposed membranes, sealing the association between adjacent cells. This serves to block the movement of materials through the intercellular space, by forming the so-called kissing points. **(B) Structure of tight junctions.** Freeze-fracture replica electron microscopic image of intestinal epithelial cells. Tight junctions appear as a continuous, anastomosing particle fibrils, forming strands (arrowheads) with complementary vacant grooves (arrows). (Mv, microvilli; Ap, apical membrane; Bl, basolateral membrane.) Scale bar, 200 nm. (**C) Ultrathin transmission electron microscopy section of tight junction structures**. This electron micrograph shows that at kissing points of tight junctions (arrowheads), the intercellular space is obliterated. Scale bar, 50 nm. **Ref. panels B and C:** (Tsukita et al., 2001).

the adjacent epithelial cells, or the hemidesmosomes (reviewed by Jones et al., 1994) (Fig 1), permitting to attach the cytoskeleton of epithelial cells to the extracellular matrix.

- Communicating junctions, such as gap junctions which are channels that mediate communication of chemical or electrical signals between cells that are in direct contact with each other's (Bennett et al., 1991; Kumar and Gilula, 1996).

In my thesis, I will particularly focus on the "sealing junctions" that control the paracellular flow of water, nutrients, ions, growth factors and even cells. However, this is not their only role, as they assure at the same time other important functions. For instance, they share with anchoring junctions the capacity to mediate cell adhesion and communication between adjacent cells.

III. Sealing junctions: general structure and particular features

Sealing junctions characterized in so far can be grouped in three main categories: the vertebrate tight junctions (TJ), the invertebrate septate junctions (SJ) and the paranodal septate junctions (PSJ), found in both vertebrates and invertebrates.

a) Tight junctions

The irruption of electron microscopy applied to biology permitted the discovery of the two main types of sealing junctions that we can recognize in extant organisms. The first to be identified were the tight junctions or zonula occludens, which are vertebrate-specific type of cell adhesion complexes and were discovered by M.G. Farquhar and G.E. Palade in 1963. Tight junctions, firstly resolved in the electron microscope as tightly associated regions between membranes of adjacent cells, localize to the most apical part of the lateral cell membrane, just above the adherence junctions of the polarized epithelial cells (Fig 1, see also Fig 6). As their name implies, TJ constitute a site where the outer leaflets of the membranes of two contacting cells come very close or tight (Fig 2 B, C).

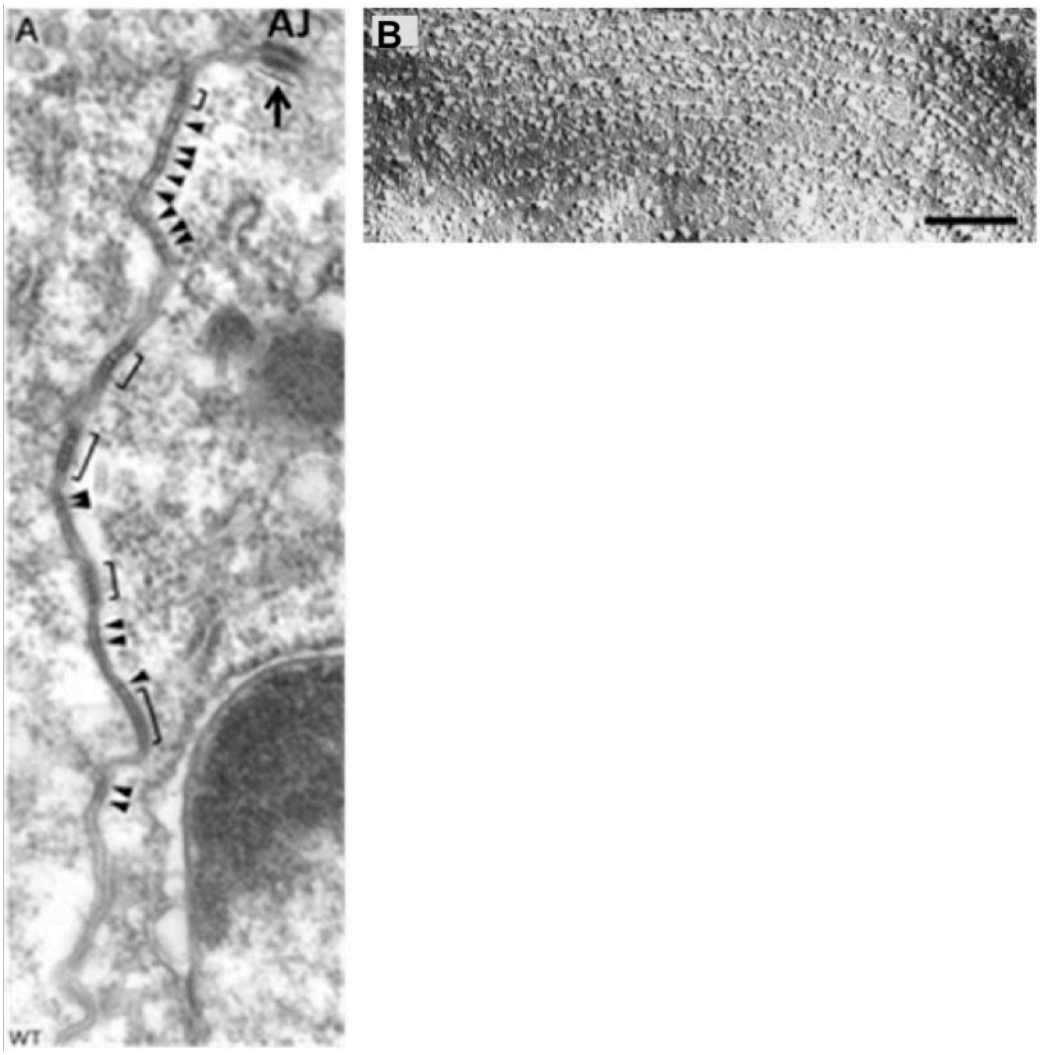

Fig 3. (A). TEM of *Drosophila* epidermis from late stage 17 wild-type embryo, showing the apical adherens junctions (AJ, arrow) and the lateral septate junctions. Laterally, interacting plasma membranes are joined by septa, arranged in parallel rows with a regular periodicity. Brackets indicate clustered groups of septa, and arrowheads point to individual septa. **(B). Freeze fracture replica of septate junction structures in arthropod epithelia**, showing the parallel arrays of intramembrane rounded particles, forming septa between adjacent cell membranes. Scale bar represent 100 nm. **Ref panel A**: (Wu et al., 2004). **Ref. panel B:** (Furuse and Tsukita, 2006).

Basically, the key structure in TJs is called “TJ strand”, a belt-like region in which two apposing membranes lie close together (Fig 2, A). The TJ strands are localized within the plasma membrane, as shown by freeze-fracture replica electron microscopy (Staehelin, 1973) (Fig 2, B). Each TJ strand is tightly associated with an equivalent strand situated in the opposing membrane of an adjacent cell to form a paired strand. The sites of contact of the two structures are called “Kissing Points” that can be visualized in ultrathin sections, as regions in which the intercellular space is obliterated (Fig 2 A, C) (Farquhar and Palade, 1963; Tsukita et al., 2001).

The TJ strands are composed at least by 40 different proteins, whose function is not only restricted to the maintenance of a paracellular seal. In fact, the TJ structural complexity reflects the contribution of some its components to other interrelated cell process, like the maintenance of cell polarity (Cereijido et al., 1998) in which TJ participate by limiting the diffusion of proteins and lipids within the membrane, ultimately keeping the apical and basolateral regions of the plasma membrane as separated domains (Cereijido et al., 2008).

However, this is not their only associated function, as TJ are also known to participate in signalling (Izumi et al., 1998; Ebnet et al., 2008), cell cycle control (Tsukita et al., 2008), vesicle trafficking (Yeaman et al., 2004) and even transcriptional regulation (Balda and Matter, 2003).

b) Septate junctions

Septate junctions were described for the first time by R.L. Wood in 1959 (Wood, 1959) as “septate desmosomes”, during his electron microscopy (EM) observations of Hydra epithelial cells. Commonly found in invertebrate epithelia, their name is due to their ladder-like appearance visible in electron microscopy cross-sections (Fig 3). In sections perpendicular to the cell surface, the septa appear as regularly spaced electro-dense crossbars spanning the space existing between the opposed membranes of adjacent cells (Fig 3), that are separated by a constant distance of approximately 15-20 nm (Tepass et al., 2001, also reviewed by Furuse and Tsukita, 2006).

It only became clear that these structures were responsible for the formation of the paracellular barrier when the paracellular diffusion of electron-dense dyes was studied in EM sections. In fact, in an intact epithelium the diffusion of dyes like the Lanthanum is precisely stopped at the

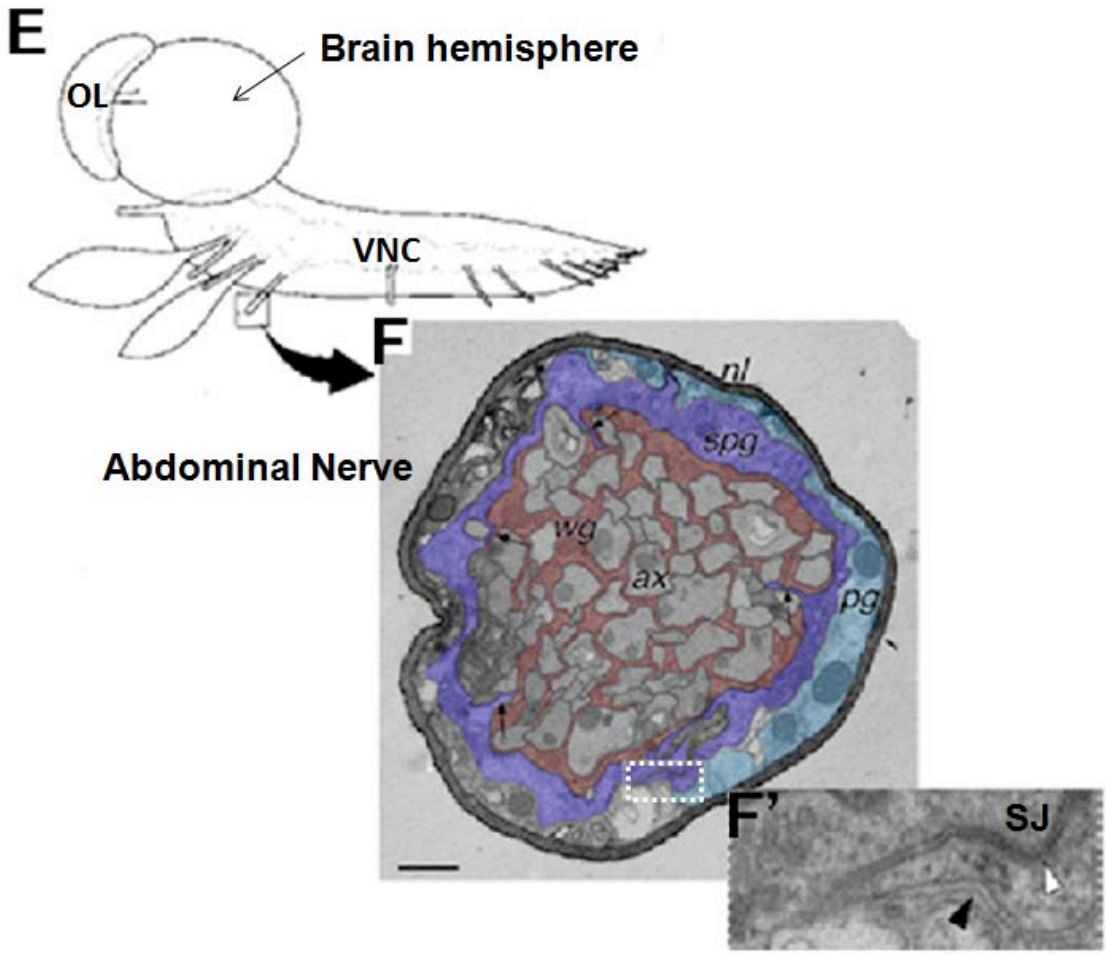

Fig 4. Glial layers of the peripheral nervous system in Drosophila larvae. (E) Cartoon of the CNS dissected out from Drosophila third larval instar and oriented laterally. OL (optic lobe), VNC (Ventral nerve cord). **(F)** Cross-section of larval peripheral abdominal nerve observed in electron. Three glial cell layers are present in the larval nerve: axons (ax) are ensheathed by wrapping glial (wg) cells (shown in pink) and the overlying subperineurial glia (spg) which appears as a flattened layer (in purple). SPG itself is surrounded by the perineurial glia (pg) in green, ensheathed by the neural lamella which covers the nervous system. **(F')** Close up of the white boxed area in F, showing a part of the adjacent subperineurial and perineurial glia. septate junctions (SJ) appears only in the intercellular space between subperineurial glial cells (white arrowhead), whereas they are missed between perineurial glial cells (black arrowhead). **Fig adapted from** (Stork et al., 2008).

SJ level. The implication of the SJ in the barrier function has also been confirmed in *Drosophila* by morphological analysis of mutants in which intercellular septa are missing and that consistently present defective barriers (Baumgartner et al., 1996; Lamb et al., 1998).

Interestingly, the ultrastructural features of the SJ seem well conserved in the multiple species in which their presence has been reported, which range from chordates (Rosenbluth, 1995; Banerjee et al., 2006) to porifera, where SJs have been observed between sclerocyte cells that secrete the spicules of the calcareous sponge *Sycon ciliatum* (Ledger et al., 1975).

However, some structural heterogeneity exists. In insects, Flower and Filshie (Flower and Filshie, 1975) distinguished two different subtypes of septate junctions, based on their characteristic appearance in tangential views: the “pleated” and the “smooth” septate junctions. The pleated SJ, found in ectoderm derived epithelia and in glial sheets, form the typical ladders like electron-dense septa described above (Tepass et al., 2001). In contrast, the smooth SJs are only found in endodermal derivatives, like the midgut and lack the regular arrays of septae (Green et al., 1983).

In invertebrates and particularly in insects, SJ can be easily recognized in most ectodermally derived epithelia. However, very similar adhesion structures have also been observed in the insect nervous system, where they contribute to the formation of a sealing barrier that protects and isolates the neurons from their surrounding environment.

More in detail, a series of electron microscopy observations performed on the nervous system of *Drosophila* have shown that septate junctions are present at the cell contacts between specialized types of glial cells, the so-called subperineurial glial (spg) cells, which surround and ensheath axon fascicles of central and peripheral nervous system (Stork et al., 2008) (Fig 4). These connections have an essential role for the maintenance of the Blood Brain barrier (BBB) organization, a physiological barrier protecting the nervous system from the high potassium (K^+) concentration present in the hemolymph but also regulating the entry of other molecules inside the nervous system (Bainton et al., 2005; Schwabe et al., 2005; Stork et al., 2008).

c) Paranodal septate junctions

Interestingly, structures morphologically very similar to the invertebrate SJ have also been observed in the nervous tissues of vertebrate organisms: the so-called paranodal septate

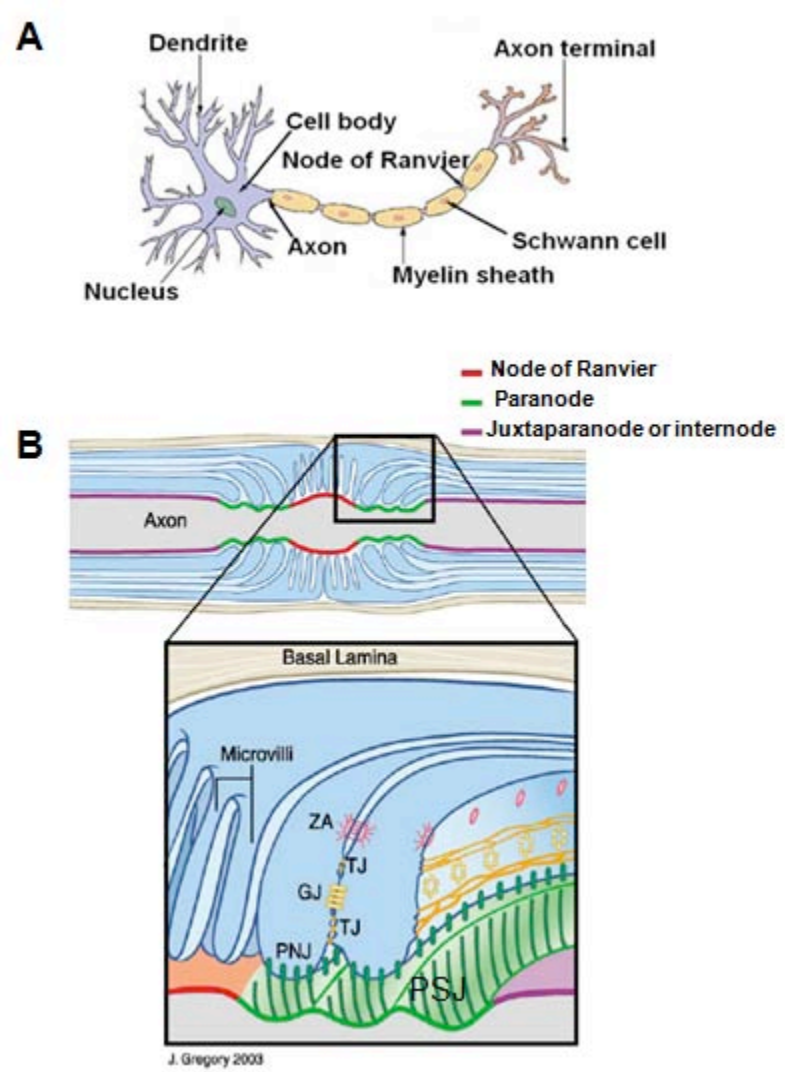

Fig 5. Membrane domain organization of the vertebrate myelinated axons. (A) Cartoon of a myelinated neuron. Myelinating Schwann cells ensheath all axon surfaces excepting nodes of Ranvier to induce the "saltatory conduction" of action potentials. (**B)** Schematic representation of myelinated axon shows three distinct compartments or axon domains: the node of Ranvier (in red), the paranode (in green) and the juxtaparanode or internode (in purple). In nodes, axons are in contact with Schwann cell microvilli of glial cells, but they are in contact with paranodal myelinated loops in paranodal domain (fig B, close up), whereas internodal domains are ensheated with myelinated axons. Paranodal septate junctions PSJ (presented by the hatched green lines) are formed between paranodal loops and junctional axon domain. Paranodal loop cells are polarized presenting tight junctions (TJ) that provide paracellular barrier between the periaxonal space and the loops, gap junctions (GJ) permit direct communication between loops, and adherens junctions (AJ) that promote attachment between adjacent loops. Such organization of paranodal myelinated loop cells is very similar to that of polarized epithelial cells. **Fig adapted from:** (Salzer, 2003).

junctions (Brophy, 2001). This denomination is due to their presence in the paranodal axon domains of the myelinated nerves, at the interface between glial myelinated Schwann cell loops and axonal membranes (Fig 5, B).

It has been recognized that myelinated axons of the central and peripheral nervous systems (Fig 5, A) are compartmentalized into three functionally distinct domains: the nodes of Ranvier, the paranodes, and the juxtaparanodes or internodes (Fig 5, B) (reviewed by Salzer et al., 2003).

Paranodes, located on both sides of Ranvier nodes are the site of attachment of the axonal membrane to the terminal loops of myelinating glial cells, which spiral around the axon, forming a series of septate-like junctions (Fig 5, B). This axo-glial paranodal junction plays three important physiological roles. First, it provides electrical insulation, allowing saltatory conduction of the nerve impulses from one node of Ranvier to the next node. Second, it restricts the lateral mobility of axonal membrane proteins and channels, organizing a fence within the axonal membrane that separates Na^+ channels present at the unmyelinated node of Ranvier from K^+ channels present under the glial cells, in the juxtaparanode (Bhat, 2003; Bhat et al., 2001). Third, it also provides adhesion and putative intercellular communication between axons and Schwann cells at the level of the axon-glia contact domains.

Thus, paranodal septate junctions establish a physical Blood Brain Barrier (BBB) between the neuron and the ensheathing glial cells by preventing the unregulated exit into the blood of neurotransmitters and other substances emanating from nerve cells, and at the same time blocking the passage of blood material into the nerves (Bellen et al., 1998). They are also likely to contribute to a bi-directional signalling between axons and glial cells and seem to play important roles in the process of myelination, as defects in the organization of paranodal septate junctions have been associated with several neuropathies and dysmyelinating disorders (Griffiths et al., 1996).

We have seen that both epithelial septate junctions and paranodal septate junctions play an equivalent role to that of tight junctions, but differ strikingly in morphological terms. Another remarkable difference between SJ and TJ, as far as epithelial tissues are concerned, is that the position along the lateral cell membrane of these structures is different. In fact, SJ are placed just below the adherens junctions and not above, as TJ are (Fig 6), suggesting that these adhesion structures are completely different, both in composition and in evolutive origin. In fact, for a long time, they have been envisaged as analogous structures rather than homologous ones.

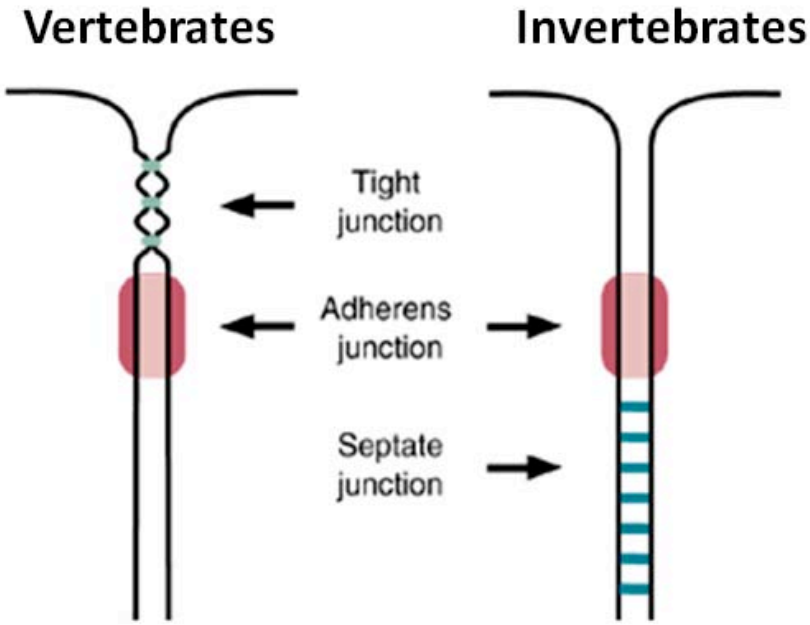

Fig 6. **Localization of tight and septate junctions, respectively in vertebrate and invertebrate epithelial cells.** In vertebrates, tight junction (TJ) structures, where adjacent cell membranes join together in a specific sites (shown in green), are localized in the most apical part of epithelial cells, just above the adherens junctions (in pink, left hand panel). Whereas in invertebrates, tight junctions analogous, called septate junction (SJ), are situated just below adherens junctions (in pink, right hand panel) and are characterized by a ladder-like septa (in green) spanning the intercellular epithelial space. **Ref. Fig:** (Tepass, 2003).

However, the detailed characterization of their multiple components has shown that these structures share more similarities than previously thought, a feature that also applies to the paranodal septate junctions. So, how are these junctional complexes organized at the molecular level?

IV. Molecular organization of the sealing junctions

a) Molecular organization of TJ

After the first electron microscopy observations, cell biologists began to focus in the study of TJ, to unmask the nature of its molecular components. Contrary to what was initially thought, the nature of the TJ strands is not predominantly lipidic, but made of protein complexes arranged like beads that span the paracellular space. In fact, at the level of the TJ, the adjacent plasma membranes are hold together by rows of transmembrane junctional proteins. On the one hand, the extracellular domains of these proteins directly interact with one another to occlude the intercellular space and create a seal (Schneeberger and Lynch, 1992; Gumbiner, 1993; Anderson and van Itallie, 1995). On the other hand, the intracellular part of these transmembrane proteins associates with a set of cytosolic components that anchor the strands to the actin cytoskeleton. In this way, the tight junctions allow the cytoskeletons of adjacent cells to join together. At the molecular level, the different components of the tight junctions can be broadly separated in three different groups:

- The Claudins: tetraspan membrane proteins, like Occludin, which are allegedly responsible for the selective barrier function (Fig 7 A, B).

- A large set of different single-pass transmembrane proteins that can be collectively designed as junctional adhesion molecules (JAM) (Fig 7, C).

- Multiple cytoplasmic adaptor proteins, that form the so called tight junction plaque and include the Zonula Occludens ZO-1, ZO-2 and ZO-3, all belonging to the MAGUKs (Membrane-Associated Guanylate Kinase) family and other proteins containing a PDZ domain (PSD-95/Discs large/ZO-1) which interact specifically with the

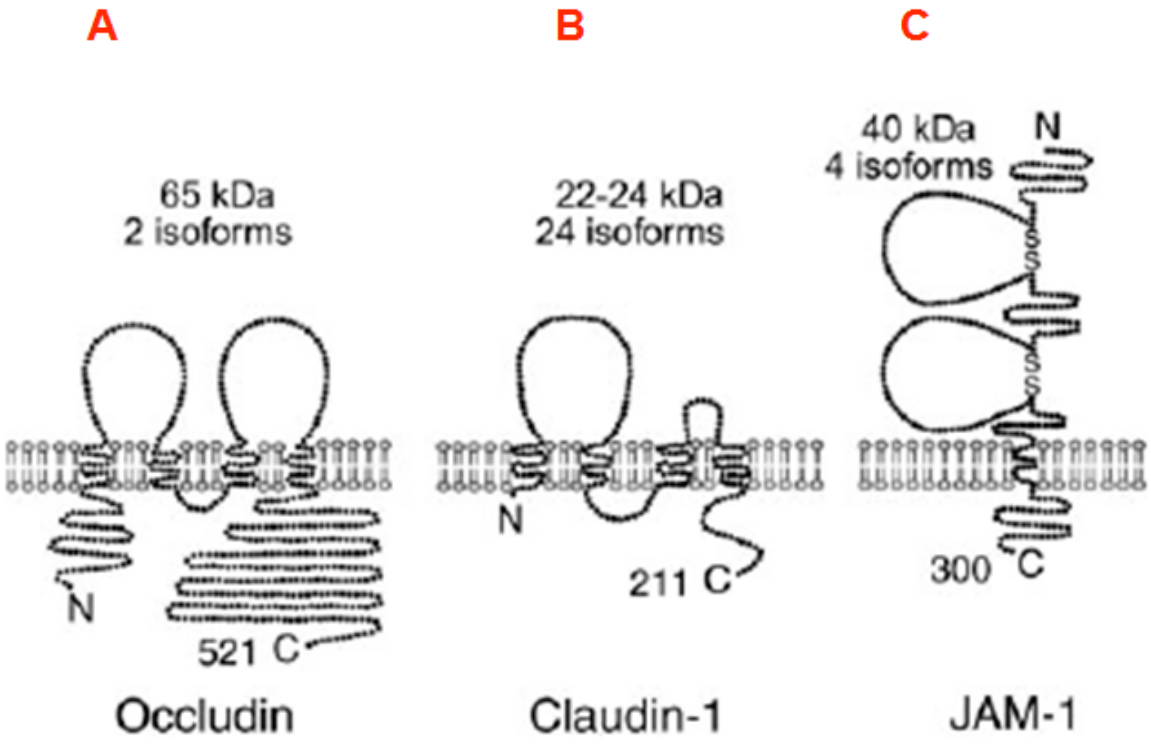

Fig 7. Topology of tight junction integral membrane proteins. (A) Occludin has four transmembrane domains with two extracellular loops, one short intracellular loop with amino and carboxy terminal cytoplasmic domains. (**B)** Claudin-1 also has four transmembrane domains, but does not display sequence similarity to Occludin. Note that the cytoplasmic tail of claudin-1 is shorter than that of Occludin. Different Claudin members show variability in the aminoacid composition of their extracellular loops. **(C)** Junctional adhesion molecule 1 (JAM-1) also known by JAM-A has a single transmembrane domain; its extracellular domain contains two immunoglobulin-like loops that are formed by disulphide bonds. The first amino terminal loop is known to mediate homologous interaction with JAM-1 molecule, present in the adjacent cell membrane. Molecular weight, isoform and aminoacid number of each molecule are also mentioned. **Ref. Fig**: (Schneeberger and Lynch, 2004).

cytoplasmic domains of Occludin, Claudins and JAM and form a scaffold capable of recruiting other signalling proteins and cytoskeleton components to the TJs.

Occludin derives its name from the latin word ‘‘occludere’’, to close, and represents the first example of integral membrane proteins specifically found in TJ strand (Furuse et al., 1993; Ando-Akatsuka et al., 1996). *Occludin* encodes for a membrane protein with four transmembrane domains, a topology that generates two extracellular loops, one intracellular loop and two amino- and carboxy-terminal cytoplasmic domains (Fig 7, A). Although Occludin is specifically localized at the tight junctions, its physiological function is still unclear. In fact, epithelial tissues deficient for Occludin do not show obvious defects at the level of the TJ and the paracellular diffusion barriers seem functional (Saitou et al., 1998). However, Occludin knock-out mutant mice display clear phenotypes, such as growth retardation, male sterility, and a tendency to develop gastritis, suggesting that the digestive apparatus barrier function could be impaired (Saitou et al., 2000). The presence of functional tight junctions in these mutants has stimulated the study of other membrane proteins present in the TJ complex, leading to the discovery of the key role played by Claudins in paracellular barriers.

Claudins, also named from the latin “claudere”, to close, have emerged as key components of the TJs and are thought to have a direct function in barrier and tight junction strand formation (Inai et al., 1999; McCarthy et al., 2000). They form a large multigene family with approximately 24 members in human and mice (Van Itallie and Anderson, 2006; Furuse and Tsukita, 2006; Angelow et al., 2008), but recently they have also been identified in insects (Wu et al., 2004). Claudins are also tetraspan proteins, sharing a similar topology to that of Occludin (Fig 7, B). Claudins are known to interact in a homo- and heterophilic way in the plane of the membrane (Furuse et al., 1999; Blasig et al., 2006), but also with the Claudins of the adjacent cells (Furuse et al., 1999), thereby sealing the cell junctions. Ectopic expression of Claudins in fibroblasts results in the formation of tight junction-like structures, indicating that Claudin expression has a capital role in driving tight junction formation. Interestingly, the observed profusion of Claudin paralogues seems to provide a molecular basis for the different selective properties exhibited by different epithelial barriers. Indeed, manipulations altering the type of Claudin expressed in a tissue seem to have a direct impact on paracellular ion and/or size selectivity (Van Itallie et al., 2001; Nitta et al., 2003). It seems that these selective properties reside in the extracellular loops of Claudins that contain several electrically

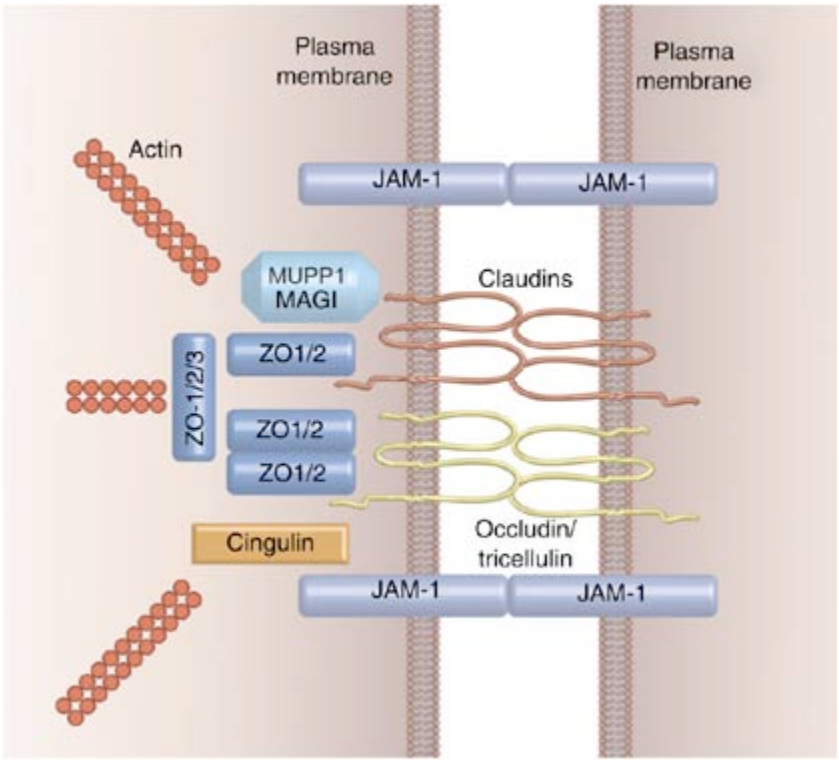

Fig 8. Schematic representation of the basic molecular components of tight junctions in epithelial cells. Claudins, Occludin and the junctional adhesion molecules (JAMs) are the most prominent components. Within the cytoplasm many first-order adaptor proteins, including the scaffolding proteins Zonula Occludens 1, 2 and 3 (ZO-1–3) bind to the cytoplasmic tail of intramembrane proteins and provide a direct link to the actin-based cytoskeleton. Among the second-order adaptor molecules, Cingulin is shown. Signaling and regulatory proteins include multi-PDZ-protein 1 (MUPP1) and MAGI (membrane-associated guanylate kinase with inverted orientation of protein–protein interaction domains).

charged residues. In fact, it is known that different members of the Claudin family exhibit a large variability of isoelectric points and, for instance, point mutations altering the charge in the first extracellular loop of Claudin-15, expressed in the mammalian polarized Madin-Darby canine kidney cells (MDCK), result in changes of barrier ion specificity (Colegio et al., 2002). Thus it is now widely accepted that barrier specificity is largely due to the type of claudin(s) present at the tight junctions (Anderson et al., 2004; Furuse and Tsukita, 2006).

JAMs or junctional adhesion molecules are the second type of integral membrane proteins localized at TJ. They belong to a family of single-span transmembrane proteins characterized by the presence of immunoglobulin extracellular domains (Fig 7, C) (Martin-Padura et al., 1998; Ebnet et al., 2004). For instance, in humans, the family consists of four closely related molecules called JAM-A, -B, -C and JAM-4 (Ebnet et al., 2004). Differing from Claudins, their expression in fibroblasts does not induce the formation of tight junctional strands, suggesting that they may play a subsidiary role in TJ assembly. As their name implies, JAM's main contribution seems to be mediating cell adhesion. In fact, they are supposed to hold together the two opposing membranes of the tight junction thanks to their capacity to interact in an homophilic and heterophilic way (Keiper et al., 2005). However, it seems that this is not their only function, as they also mediate interactions with a wide range of cytoplasmic proteins. In particular, they are thought to play a role in the regulation of the cell polarity, as JAM-C is necessary for the recruitment of the cell polarity complexes PAR6, Cdc42, PKCl and PATJ during mammalian spermatid differentiation (Gliki et al., 2004).

Scaffolding proteins: The incorporation and association of the transmembrane proteins Occludin, Claudins, and JAMs in tight junctional strands requires the local clustering of these proteins in a particular membrane region. Although direct interactions between Occludin, Claudins, and JAMs may contribute to their clustering, this process mainly relies on the scaffolding properties of their cytoplasmic binding partners.

An important group of tight junctional scaffolding molecules are the zonula occludens proteins ZO- 1, ZO-2 and ZO-3 (Fig 8). These proteins belong to the MAGUK family and are characterized by the presence of three N-terminal PDZ domains, an SH3 domain followed by a catalytically active guanylate kinase domain. These proteins can interact directly with Occludin, Claudins and JAMs via their PDZ domains (Furuse et al., 1994; Haskins et al., 1998; Itoh et al., 1999; Ebnet et al., 2000), whereas their C-terminus can associate with filamentous actin, thus providing a direct link with the actin cortex (Fig 8) (Fanning et al.,

1998; Wittchen et al., 1999). In fact, their interaction with actin could be essential for their localization at the level of the TJ, as it has been shown for the ZO-1 protein (Fanning et al., 2002). In addition, ZO-1 has been shown to form homodimers and also heterodimers with either ZO-2 or ZO-3, a property that could contribute decisively to the clustering of the TJ components. Along this line, it has been shown recently that both ZO-1 and ZO-2 are essential for Claudin clustering, strand formation and barrier maintenance (Umeda et al., 2006).

The ZO proteins are not the only PDZ-motif adaptors present in TJ. Several proteins, such the Multi-PDZ domain protein 1 (MUPP1) and the membrane-associated guanylate kinase with inverted domain orientation (MAGI) proteins have been shown to interact with one or more integral membrane TJ components (Fig 8) (Schneeberger and Lynch, 2004). However, it is unclear if these molecules are part of a structural core essential for tight junctions stability or if they serve a subsidiary regulatory function, as TJ are dynamic structures whose properties change in different cellular and physiological situations. For instance, Cingulin, a non-PDZ tight junctional plaque protein, also interacts with ZOs, JAMs, and actin via its head domain, whereas its central domain is required for homodimerization and can interact with myosin. As such, this protein may be an important regulator of tight junctional dynamics during actomyosin contraction (Clayburgh et al., 2005).

As we have seen, the general molecular organization of tight junctions stands on a group of transmembrane proteins (Occludin, various Claudins and JAMs) whose C-terminal cytoplasmic sequences present high affinity for scaffolding proteins, mostly containing one or more PDZ domains. The study of invertebrate septate junctions has shown that their structural logic is very similar to that of TJ, and that both complexes are formed by similar types of molecules.

b) Molecular organization of SJ

Basically, the most detailed molecular dissection of invertebrate septate junction (SJ) has been carried out in *Drosophila.* In this organism, many SJ components have been identified and it has been found that some of them play equivalent molecular functions to known vertebrate TJ proteins. Moreover, in many cases the septate and tight junction components appear to be clear homologous proteins that share the same organization and domain composition.

As in vertebrates, the internal logic of SJ allows to classify its components in three groups.

First, the *Drosophila* SJs present **transmembrane proteins** like the Claudins, supposed to maintain the barrier function of these structures. For instance, three fly Claudins displaying the characteristic topology of these proteins have been described: Megatrachea (Mega) (Behr et al., 2003), Sinuous (Sinu) (Wu et al., 2004) and Kune Kune (Kune Kune) (Nelson et al., 2010). However, Occludin seems to be absent in this insect.

Second, the *Drosophila* SJ complex is also composed by a group of **cell adhesion molecules** that includes both transmembrane proteins, such as Neurexin IV (Baumgartner et al., 1996), Gliotactin (Genova and Fehon, 2003; Schulte et al., 2003), Fasciclin III (Woods et al., 1997), Neuroglian (Hortsch et al., 1995; Genova and Fehon, 2003) and the Na^+/K^+ ATPase pump (Paul et al., 2003)), and also Glycosylphosphatidylinositol (GPI) anchored cell membrane proteins, such as Lachesin (Llimargas et al., 2004), Contactin (Faivre-Sarrailh et al., 2004) and Melanotranferrin (Tiklová et al., 2010).

The third type of SJ proteins consists of **scaffolding adaptor molecules** found at the cytoplasmic side of the membrane. These proteins include ZO homologous proteins with different PDZ proteins, like Dlg (Woods et al., 1991) and Varicose (Wu et al., 2007), founding members of the MAGUK family. Many other fly scaffolding components, notably Coracle, a cytoskeletal linker belonging to the 4.1, Ezrin, Radixin, Moesin (FERM) protein domain family (Fehon et al., 1994; Lamb et al., 1998) and Scribble (Bilder et Perrimon, 2000), a protein with leucine-rich repeats (LRRs) and PDZ domains, known to regulate cell polarity, are also associated with vertebrate tight junctions (D'Atri et al., 2002), thus pointing on the molecular and functional conservation existing between tight and septate junction structures.

In *Drosophila* polarized cells, the different SJ components appear clustered in a membrane region placed below the adherens junctions. Multiple observations have shown that in mutant background for one of the SJ components, the other components appear systematically mislocalized and found uniformly distributed along the lateral membrane (Genova and Fehon, 2003; Faivre Sarrailh et al., 2004; Llimargas et al., 2004; Moyer et al., 2008; Tiklova et al., 2010 …). These observations have lead to the notion that the different components of SJ are

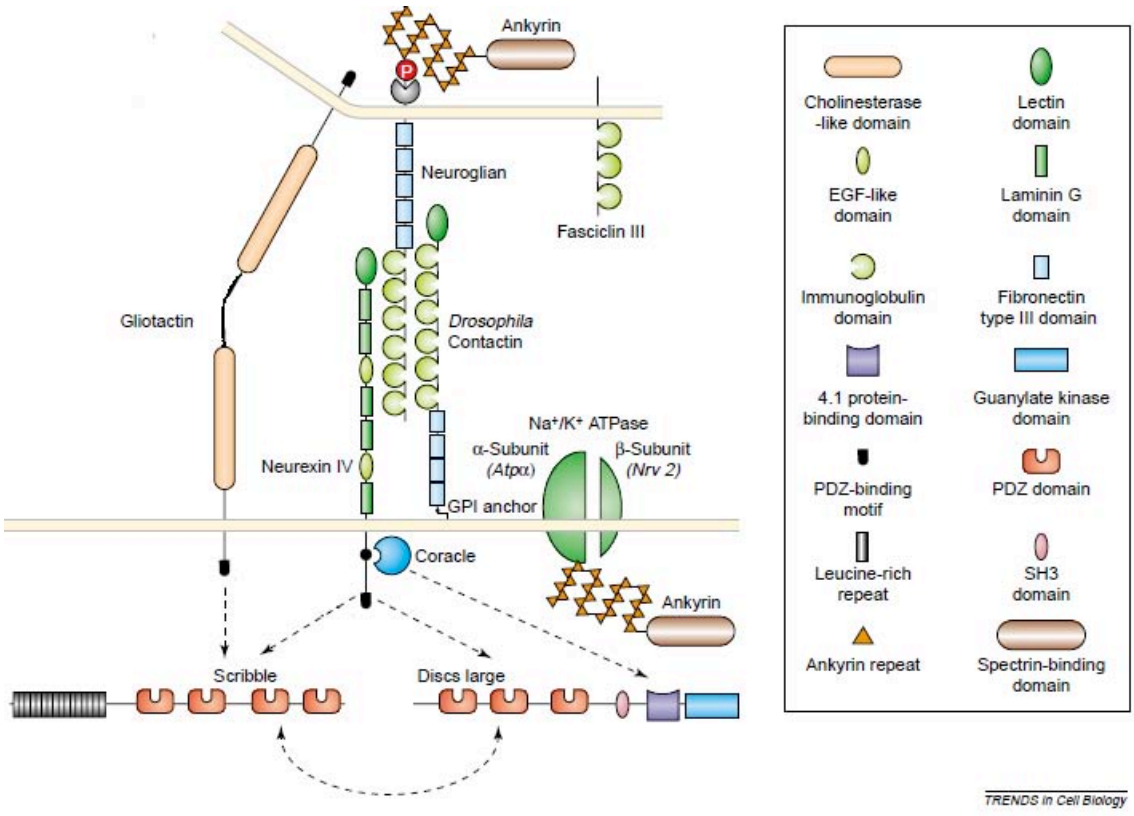

Fig 9. Schematic representation of septate junction molecular complex between *Drosophila* epithelial cells. Different protein interactions are shown between septate junction components. The cell adhesion molecules NeurexinIV, D-Contactin and Neuroglian interact together to form a tripartite complex, also present in vertebrate PSJ. Coracle and NeurexinIV also form an interdependant complex with Neuroglian and the Na^+/K^+ ATPase pump. Other hypothetical interactions are supposed to occur between the cytoplasmic PDZ-binding domains of Neurexin IV with the PDZ domains of Scribble or Discs large, as well as interactions between Coracle and the 4.1-protein-binding domain of Discs large, but also between Gliotactin and the PDZ domains of Scribble and between the PDZ domains of Scribble and Discs large. These interactions are indicated by broken arrows; however they still need to be demonstrated. These molecules, with the exception of the homophilic cell adhesion molecule Fasciclin III and the *Drosophila* ankyrins, have now been demonstrated to be essential for the function of septate junctions in the epithelia and nervous system of *Drosophila*. **Ref. adapted from:** *(*Hortsch M and Margolis B, 2003).

interdependent for their clustered localization and therefore for the organization of the whole SJ complex.

Moreover, genetic and biochemical studies have unveiled a complex network of interactions between some of the SJ components (Fig 9). For example, it has been shown that the FERM protein Coracle (Cor) interacts with the cytoplasmic tail of Nrx IV (illustrated in Fig 9) and, accordingly, the clustered localization of Cor is lost in *NrxIV* mutants (Ward et al., 1998). Further studies based on immunoprecipitation experiments have also indicated that Cor and Nrx IV are found in an interdependent complex with the Na^+/K^+ ATPase pump and the transmembrane protein Nrg (Genova and Fehon, 2003). In addition, it has been shown that the cytoplasmic tail of Nrx IV binds to the PDZ domain of the scaffolding MAGUK protein Varicose (Wu et al., 2007). Finally, biochemical experiments indicate that MTf, a conserved transferrin family of GPI anchored iron-binding protein, also interacts with Nrx IV, Cont and Nrg complex (Tiklová et al., 2010).

However, even though more and more interactions between septate junction components become apparent, further studies will be required to understand how the SJ complex is assembled and how its integrity is maintained during development. In particular, the dynamic of the interactions established between SJ components has hardly been explored. For instance, it has been shown that in mutants for Nrx IV, Contactin protein seems unable to reach the plasma membrane and is seen instead accumulating in intracellular vesicles (Faivre-Sarrailh et al., 2004). This finding suggests that SJ complexes may preassemble en route to the membrane, but we still know little about how the trafficking of the different septate junction components is organized and how these proteins are addressed to a particular region of the cell membrane. These questions have just begun to be analyzed in a seminal study focusing on the MTf SJ component (Tiklová et al., 2010). This work has shown that before SJ formation, the MTf protein is uniformly distributed along the lateral membrane, then enters different endosomal compartments (Rab5 positive early endosomes and Rab11 positive recycling endosomes), and is finally reshipped to the apical part of the lateral membrane where it forms a cluster at the level of the SJ. This suggests that membrane recycling plays an active role in the initial clustering of the SJ components, but it could also participate in SJ maintenance during development and/or in physiological regulation of paracellular barrier activity. Indeed, the endocytosis of SJ components could be a recurrent way to regulate the properties of barriers, as many studies in the vertebrate TJ have demonstrated (Utech et al., 2010).

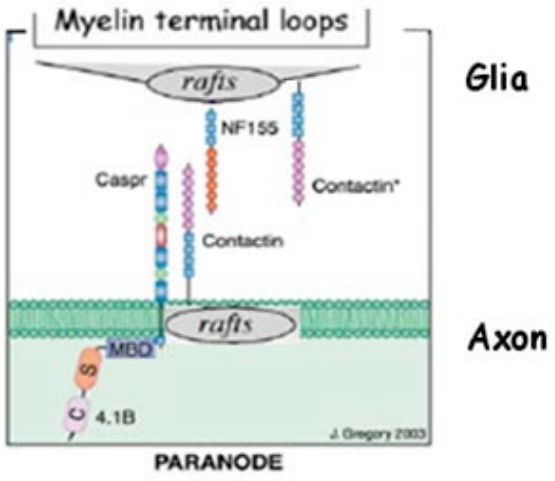

Figure 10. Schematic model describing molecular interactions at the paranodal region of myelinated axons. A cis complex of adhesion molecules Caspr (contactin-associated protein) and Contactin, present in the lipid raft domain of the axon, are interacting together. Caspr binds in its cytoplasmic region to protein 4.1B, a member of the 4.1 family of cytoskeletal and cytoplasmic adaptor proteins. Caspr/Contactin complex interacts with the glial protein, NF155 (Neurofascin 155), anchored to lipid rafts of the myelinated terminal loops membrane. **Fig. adapted from**: (Labasque and Faivre-Sarrailh, 2010) and (Salzer, 2003).

c) Molecular organization of paranodal septate junctions

Septate junctions display extraordinary similarities with the vertebrate paranodal septate junctions (PSJ) not only at the morphological level, but also at the molecular one, as they share many different membrane adhesion molecules and cytoplasmic adaptors.

The structural core of the PSJ is composed by members of the Immunoglobulin cell adhesion molecules (Ig L1-CAM) family, including Caspr/Paranodin (NCP1), F3/Contactin and Neurofascin 155 (NF-155), which are the respective homologues of Drosophila Neurexin IV, D-Contactin and Neuroglian. These proteins are thought to be associated with lipid rafts in the glial membrane domains, and their insertion in these membrane microdomains seems important for the function of paranodal junctions (Fig 10) (Maier et al., 2007), also reviewed by (Labasque and Faivre-Sarrailh, 2010). It is known that the Neurexin-type Caspr/Paranodin protein interacts with GPI-anchored Contactin in a cis-configuration (Peles et al., 1997). In paranodal junctions, this complex binds by its cytoplasmic tail to the scaffolding 4.1B protein (Denisenko-Nehrbass et al., 2003), also present in invertebrate SJ and that acts as an important linker between the membrane proteins and the cytoskeletal network. Finally, this complex mediates cell adhesion by a trans interaction with the Neurofascin-155 present in the opposite membrane (Fig 10) (Charles et al., 2002). The interaction between these PSJ proteins is required for the organization of the proper axon functions and the nerve potential action conduction (Bhat et al., 2001; Boyle et al., 2001). In Paranodal region, Claudins play an intriguing role, as they are also present, but appear associated with the formation of TJ between myelinated loops surrounding axons (Poliak et al., 2002).

Despite recent advances, we are still far from having understood the cellular and molecular mechanisms controlling the assembly and maintenance of the PSJ, and their specific functions in the vertebrate nervous system. However, the picture emerging from the studies comparing TJ, SJ and PSJ at the molecular level indicates that strong parallelisms exist between invertebrate and vertebrates at the level of their structural components (see Table 1), pleading for an ancient common origin for all of the extant sealing junctions.

This consideration also implies that studies focusing on the insect septate junctions could be specially informative to shed light on the molecular complexity, the assembly mode and the interactions existing between the different components of paranodal septate junctions.

Drosophila molecule	Vertebrate homolog	Gene family	Protein domains	Functions
Neuroglian (NRG)	Neurofascin 155	L1 family	Ig- and FnIII type domains	Cell-adhesion molecule
Fasciclin III	?	?	Ig-domains	Cell-adhesion molecule
Dcontactin	Contactin	F11/Contactin family	Ig- and FnIII type domains	GPI-anchored cell-adhesion molecule
Neurexin IV	Caspr/Paranodin	Neurexin family	Laminin G and EGF domains	Heterophilic adhesive molecule
Gliotactin		Neuroligins/Electrotactins	Noncatalytic cholinesterase-like molecule	Heterophilic adhesive molecule
Na^+/K^+ ATPase	Na^+/K^+ ATPase	Na^+/K^+ ATPases		Two subunit ion pump ATPase
Coracle (COR)	Protein 4.1	4.1 proteins	FERM protein	Linker protein
Drosophila ankyrins	AnkyrinG	Ankyrin family	Ankyrin and spectrin binding domains	Linker molecules to membrane skeleton
Discs large (DLG)	hDLG/Sap97	MAGUK family	SH3, PDZ and guanylate kinase domains	Linker protein
Scribble	*hscrib1*	LAP family	Leucine-rich repeats and PDZ domains	Linker protein

Table 1. Molecular components of Drosophila SJ components and their vertebrate counterparts. Abbreviations: Ig, immunoglobulin; EGF, epidermal growth factor; FERM, Band 4.1 ezrin radixin moesin homology; FnIII, fibronectin III; GPI, glycosyl-phosphatidylinositol; LAP, leucine-rich repeat and PDZ-containing; MAGUK, membrane-associated guanylate kinase; PDZ, PSD-95 DLG ZO-1. In addition to these proteins in Drosophila, diverse other molecules (lachesin, Melanotransferrin,..) have been described after for their roles in SJ organization. Even though, some of them have vertebrate homologous, however, no evidence has yet been reported concerning their contribution to the vertebrate PSJ organization. No vertebrate homolog of the Drosophila Fasciclin III protein has been identified and, therefore, it remains uncertain whether Fasciclin III belongs to a separate gene family. **Ref. :** (Hortsch and Margolis 2003).

V. *Drosophila*: a model system to study sealing junctions

The overall goal of my PhD project is to contribute to the characterization of new septate junction components and the study of the molecular mechanisms involved in their assembly and maintenance during development, using *Drosophila melanogaster* as a paradigm.

This insect presents multiple advantages for this kind of studies, as its development is relatively simple and well understood, it is cheap and easy to handle and is well suited for genetic studies. In addition, its genome is entirely sequenced and extremely well annotated. The study of gene function is greatly facilitated by the availability of mutants for many genes, which allow rapid phenotypic characterizations. In addition, the information obtained in this model system can often be extrapolated to other organisms, as about 60% of fly genes have mammalian homologues. Furthermore, 75% of genes thought to be involved in human diseases are also found in flies.

Studies carried out in *Drosophila* have contributed to the identification of many septate junction components, taking advantage of straight forward genetic approaches. This has been possible mainly because in this organism, phenotypes denoting defects at the level of the septate junction can be scored after morphological analysis of a simple tissue, the embryonic tracheal system.

The tracheas, the respiratory system of insects, are formed by a complex network of interconnected epithelial tubes that allow transport of oxygen and other gases throughout the organism. Tracheal morphogenesis begins at an early embryonic stage (stage 10), by the singularization of tracheal placodes, epithelial sacs of 80 cells present from the second thoracic segment to the eighth abdominal segment of the body embryo (Fig 11 A, arrowheads). These ectodermal cells invaginate at stage 11 and form the tracheal pits (stage 12) (Fig 11, B). After this stage, tracheal tree formation occurs without cell divisions. First, the cells migrate internally in a distinct pattern, and then they fuse with tracheal cells from adjacent segments to form a continuous tubular network, after having undergone sequential sprouting of primary, secondary, and terminal branches. In late embryonic stages, when branching is completed, the tracheal network consists of a dorsal trunk supporting different ramified branches, running all over the body of the *Drosophila* embryo (Fig 11, D) (Affolter and Caussinus, 2008).

Drosophila mutants for different septate junction components consistently display a characteristic phenotype in their dorsal tracheal trunks, which acquire a convoluted shape and

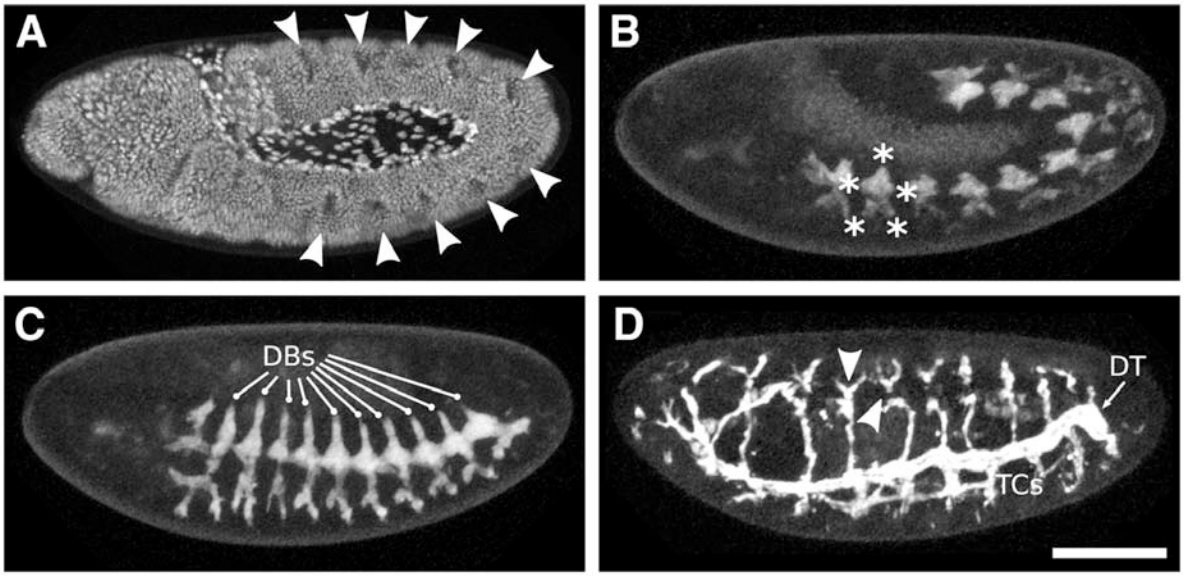

Fig 11. Branching morphogenesis of the *Drosophila* tracheal system during embryonic development. (A) Stage 11 of embryonic development. Tracheal cells invaginate (arrowheads) and form tracheal pits during the initial phase of germ band retraction. **(B)** Stage 12 of embryonic development. Tracheal pits extend branches in stereotyped directions (asterisks). **(C)** Stage 14 of embryonic development showing tracheal branches elongation. **(D)** Late stage 15 of embryonic development. Branches are fusing to form an interconnected network of tracheal tubes (arrowheads). DBs, dorsal branches; DT, dorsal trunk; TCs, transverse connectives. Anterior is to the left and dorsal to the top. Scale bars: 100 μm. **Ref. Fig:** (Affolter and Caussinus, 2008).

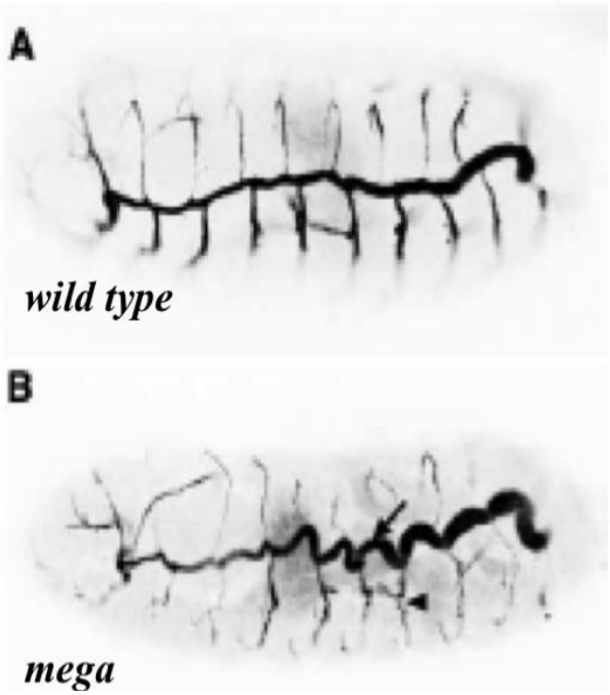

Fig 12. Septate junction components are required for tracheal morphogenesis. Stage 15 of *wild type* **(A)** and *megatrachea* (*mega*) homozygous mutant **(B)** embryos labeled for the tracheal lumen antigen marker, using 2A12 antibody. *mega* embryo displays a tortuous dorsal trunk (arrow) and a tortuous transverse connective (arrowhead) compared to the *wild type*. **Ref. Fig** (Behr et al., 2003).

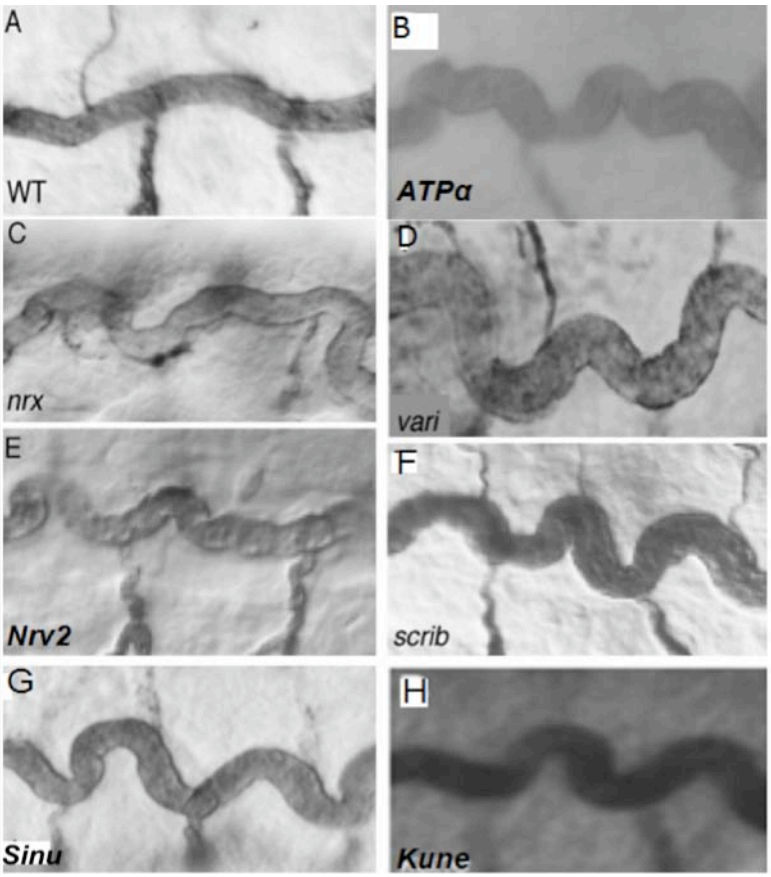

Fig 13. Septate junction genes are required for tracheal tube size control. SJ genes mutations induce similar tracheal defects, mostly marked by an elongated and convoluted tracheal dorsal trunk shape, with and extended and enlarged lumen width **(B-H)**, compared to the wild type tracheal dorsal trunk **(A)**. Abbreviations: WT, Wild Type; *ATPα*, Na^+/K^+ATPase α subunit; *nrx*, neurexin IV; *vari*, varicose; *Nrv2*, Nervana2; *scrib*, scribble; *Sinu*, Sinuous; *Kune*, kune kune. **Ref. adapted from :** (Wu et al., 2004) ; (Behr et al., 2003) ; (Paul et al., 2007).

present an enlarged lumen width, a phenotype that becomes apparent when compared to the straight dorsal trunk shape of wild type embryos (Fig 12-13). These defects can be easily detected, thanks to tracheal luminal markers like the 2A12 monoclonal antibody that specifically labels the lumen of *Drosophila* tracheal system (Fig 12). These tracheal morphological phenotypes are often correlated with a defect in the paracellular barrier formed by the tracheal epithelium. In a wild type background, these cells form SJs acting as a barrier that selectively regulates the passage of solutes and ions in and out of the tracheal lumen. Injections of a fluorescently labelled dextran dye (10 kDa) in live embryos are commonly used as a tool to monitor barrier integrity, allowing the unambiguous identification of mutants affecting paracellular barrier integrity (Fig 14).

An intriguing question is why the integrity of the SJ seems to be required for the tracheal morphogenesis and the control of lumen size. Some studies examining this issue have shown that the tracheal lumen is a highly organized structure containing a fibrous chitin matrix that is present during lumen morphogenesis and has a critical role for determining lumen length and diameter (Tonning et al., 2005). Interestingly, many mutations affecting SJ components seem to affect the secretion into the tracheal lumen of both chitin and a series of matrix-modifying enzymes, like Vermiform and Serpentine (Wang et al., 2006; Wu et al., 2007). These observations indicate that the organization of the luminal chitin matrix has an important role in determining tube size and morphology, as confirmed by the analysis of mutants in which chitin synthesis is compromised (Devine et al., 2005; Moussian et al., 2006).

However, there are probably other mechanisms at play. For example, a recent study has shown that the interplay between SJ proteins like Yurt and Cor modulates the dimensions of the apical surface of tracheal cells by interacting with the cell polarity regulator Crumbs (Laprise et al., 2010). This activity of Yurt and Cor specifically impinging on the tracheal cell architecture could control the tracheal tube size independently of the luminal secretion of the matrix components (Wang et al., 2006; Wu et al., 2007).

During my PhD, I took advantage of the *Drosophila melanogaster* model system to identify two new genes required for SJ formation. These two genes code for two distinct membrane proteins that however contain a similar extracellular domain, the Ly6 domain. The analysis of mutants for both genes has shown that they present all the classical phenotypes seen in mutants for septate junction components. However, the participation of Ly6 proteins in SJ formation

Fig 14. Septate junction integrity is required for epithelial paracellular barrier in *Drosophila*. (a-c) Live stage 16 embryos are injected into their body cavity with fluorescent 10kda dextran dye. In SJ mutants like *ATPα* and *Cor* ^{1} for example, the paracellular barrier between tracheal cells is disrupted and the dye easily penetrates into the tracheal lumen, contrary to wild type embryo where the dye is completely excluded from the tracheal lumen (trachea is delimited by white dotted lines). **Ref. Fig:** (Paul et al., 2007).

was not expected, because this Ly6 module was not previously known to be involved in the formation of sealing junctions.

What are Ly6 domain proteins and what do we know about them?

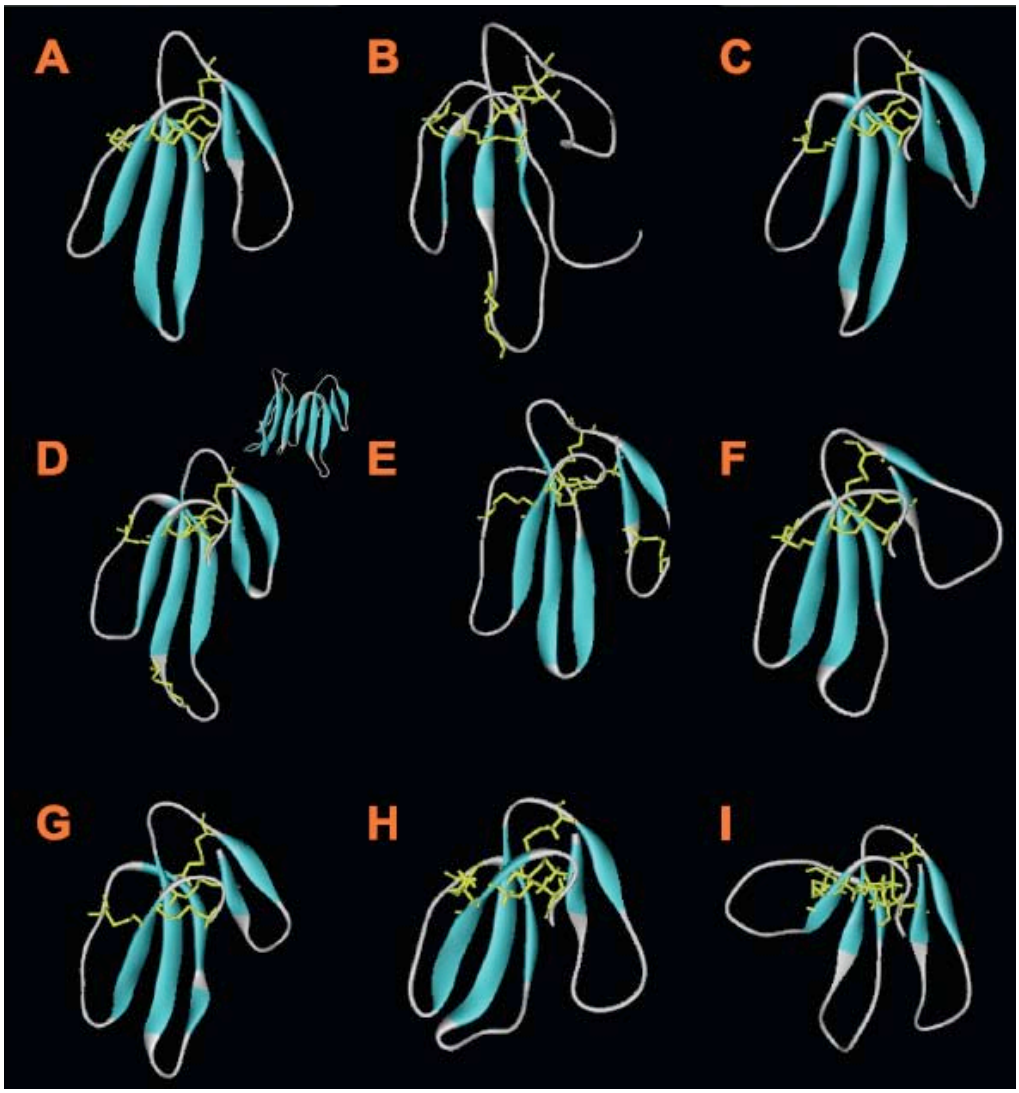

Fig 15. Ly6/TFD proteins share comparable tridimensional structure. **(A-I).** Crystallographic representation of TFD proteins shows that they share comparable structures. The core of the TFD proteins contains from 4 to 5 conserved disulphide bridges (in yellow) established between cysteines. Three loops or 'fingers' protrude from the inner core, hence their denomination "three finger proteins". These sheeted loops are numbered right to left as loop I, II and III, respectively. We can remark that the length of loops is variable from one molecule to another. **Ref. Fig:** (Kini, 2002).

Part 2

The Ly6/ three-finger domain (TFD) family of proteins

I. Structural conformation of Ly6 proteins

Proteins of the Ly6 superfamily are defined by a small motif called Ly6 domain, three-finger domain or Ly6/CD59/uPAR domain (Ploug and Ellis, 1994). This structural module is characterized by a pattern of 8-10 cysteines found in stereotyped positions. Besides these cysteines, the Ly6 primary sequences are highly variable, although they always result in comparable three-dimensional structures (Tsetlin, 1999 and Kini, 2002) (Fig 15). In fact, after the first example was identified in the sea-snake toxin erabutoxin-b (Low et al., 1976), many studies using X- ray crystallography and nuclear magnetic resonance (NMR) have shown that all the Ly6 proteins share a similar structural and tridimensional organization. The Ly6 motif is characterized by a twisted array of anti-parallel beta-sheets composed of five short strands. This central core is stabilized by four to five disulphide bridges established between the conserved cysteines and supports three prominent loops, resembling the outstretched fingers of a hand, whence their three-finger domain denomination (Fig 15).

II. Most Ly6 glycoproteins are anchored to the cell membrane by a GPI anchor

The Ly6 is an extracellular module that is never found in combination with other extracellular domains, thus constituting the hallmark of a large superfamily of cell surface glycoproteins. This superfamily includes both glycosylphosphatidylinositol (GPI)-anchored and soluble proteins. They are firstly synthesized as precursors containing an N-terminal signal peptide that leads their entry into the lumen of the rough endoplasmic reticulum. Most Ly6 precursors have also a hydrophobic carboxy-terminal region that is cleaved after addition of a GPI anchor. This GPI moiety maintains the mature proteins attached to the cell membrane (Fig 16).

However, some Ly6 proteins, and in particular the different snake toxins, lack this hydrophobic C-terminal region and behave as secreted factors, which is envisaged as a secondary adaptation to their specific function as venoms.

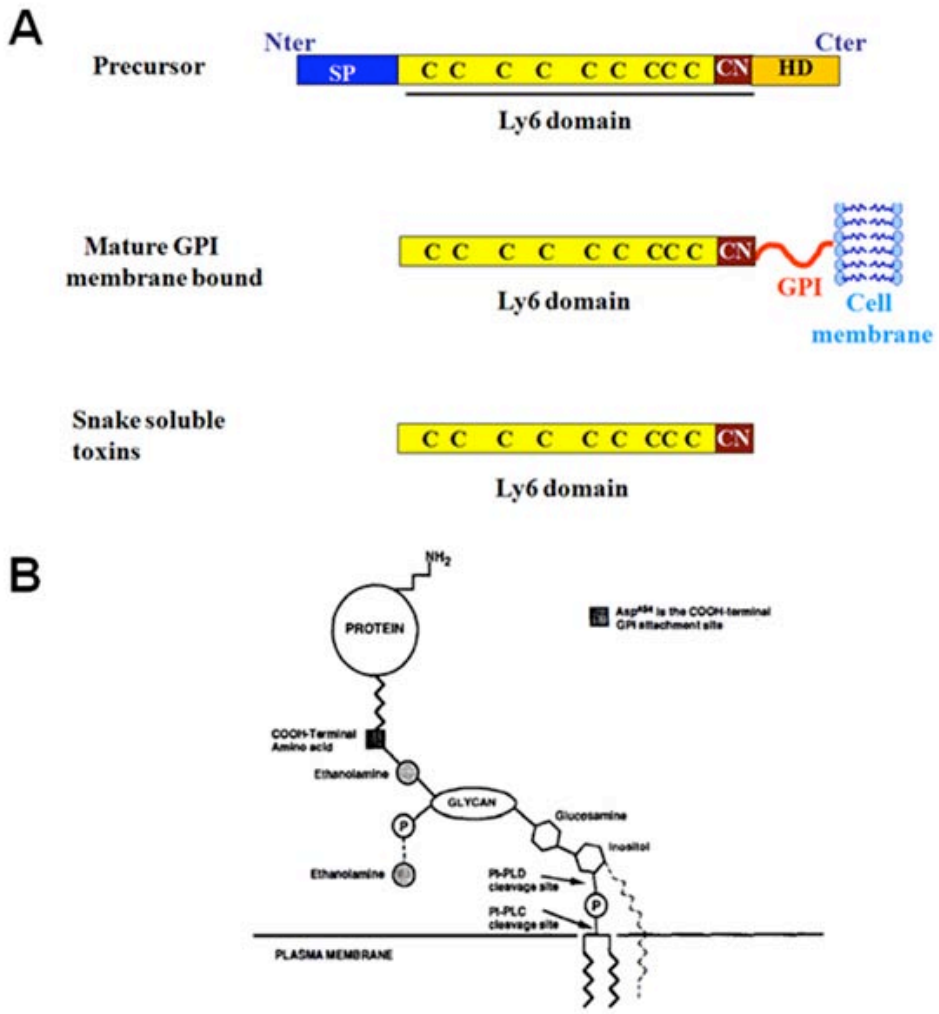

Fig 16. (A) General structure of Ly6/TFD proteins. Precursor form of Ly6/TFD proteins, produced in the endoplasmic reticulum, contains an amino-terminal signal peptide (SP), the Ly6 domain which presents 8-10 stereotyped cysteines and a hydrophobic Carboxy terminal domain, with a conserved asparagine (N) situated after the last cystein of the Ly6 motif. (N) is the site of GPI anchoring. Mature proteins lack the amino and terminal domains and are anchored to the outer leaflet of the cell membrane through the GPI anchor moiety. In parallel, snake toxins TFD proteins are synthesized as soluble proteins, not tethered to the cell membrane. **(B) General structure of a typical GPI anchored protein**. GPI anchored proteins are linked by their carboxy-terminus asparagine through a phosphodiester linkage of phosphoethanolamine to a trimannosyl-non-acetylated glucosamine core. Terminal end of non-acetylated glucosamine is linked to phosphatidylinositol (PI). PI is then anchored by another phosphodiester linkage to the outer leaflet cell membrane through its hydrophobic region. GPI membrane proteins can be soluble, by phosphatidylinositol-phospholipase C (PI-PLC) or Phosphatidylinositol–phospholipase D (PI-PLD) enzymatic cleavage. These enzymes specifically hydrolyze phosphodiester bond of phosphatidylinositol, permitting the release of GPI proteins from the cell membrane. **Ref. Fig:** (Udfriend and kodukula, 1995).

III. Characteristic features of the Ly6 domain proteins

The Ly6 motifs behave as protein-protein interaction domains and exhibit a series of properties intimately related to their particular architecture. Their most striking feature is their sequence plasticity that consists in a remarkable capacity to accommodate for multiple aminoacid substitutions without perturbing the protein general folding. This plasticity has allowed in turn a high sequence divergence and a rapid rate of evolution that is probably also the main reason for the high number of gene duplicates coding for Ly6 proteins that can be observed in most animal genomes.

These general principles are nicely illustrated by examples coming from the multiple detailed studies that have been conducted with the three-finger snake venom toxins. The key feature of these toxins is that, despite their common organization, they display a high variability, not only at the level of their loop sequences (Fig 17), which are the main sites of interaction, but also in other surface exposed regions (Ohno et al., 1998). In fact, diversity in the number of their disulfide bonds (Servent et al., 1997), specific post-translational modifications like glycosylation (Osipov et al., 2004) and the possibility of forming dimers (Osipov et al., 2008) (see Fig 15, E) can also have an important impact on their binding properties.

It has been shown that related TFD venom toxins can bind to disparate targets such as the peripheral site of acetylcholinesterase (AChE), that is blocked by the mamba Fasciculins (Eastman et al., 1995), the muscle nicotinic acetylcholine receptors (nAChR), the classic target of α-bungarotoxins (Tsetlinet al., 1999; Changeux et al., 1990), or the L-type calcium channels, that are blocked by the calciseptins, (De Weille et al., 1991; Albrand et al., 1995).

In turn, their capacity to achieve high specificity is clearly illustrated by the selectivity of the muscarinic toxins from Dendroaspis (Mamba) venom, which target with much higher affinity the muscarinic acetylcholine receptors than the nicotinic subtypes (Karlsson et al., 2000; Jerusalinsky et al., 1994).

Snake toxins are not only very specific, but also bind to their targets with high affinity, with typical inhibition constants (Ki) in the pico or nanomolar range (Chiappinelli, 1991). Perhaps not surprisingly, site-directed mutagenesis of determinant residues inside the loops of some TFD induces a significant decrease in the affinity of these proteins for their targets (Pillet et al., 1993). These observations illustrate how strong adaptive pressure can finely tune the properties of these plastic structures. Interestingly, it has been shown that non-synonymous nucleotide substitutions (leading to change in amino acid residues) are more common than synonymous

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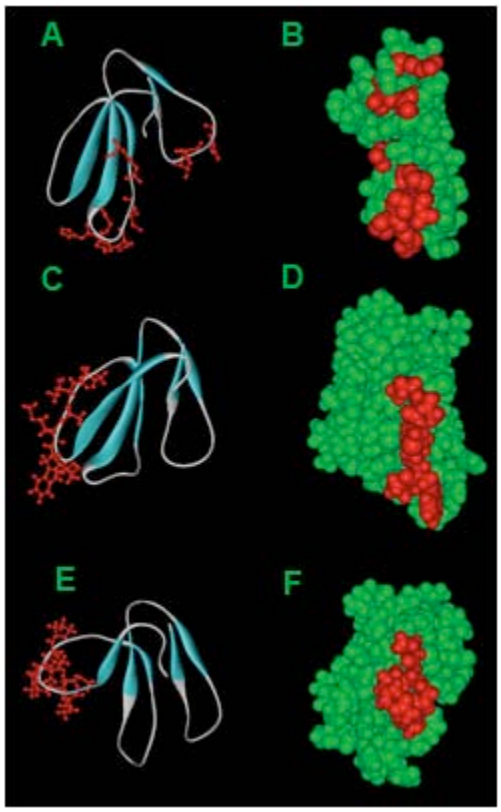

Fig 17. The Ly6/TFD protein loops contain molecular determinants that mediate the interaction with their specific site receptors. Functional sites of TFD proteins like snake toxin fasciculin **(A, B)** calciseptine **(C, D)** and dendroaspin **(E, F)** are represented in red. The interaction site of fasciculin is found in loops I and II, whereas that of calciseptine and dendroaspin is only found in loop III. **(A, C, E)** the residues involved in interaction are shown as stick models (red). **(B, D, F)**. Space-filling Corey–Pauling–Koltun (CPK) models, showing in red the functionally important residues mediating interactions with the specific receptors. **Ref. Fig:** (Kini, 2002).

nucleotide substitutions (not producing change in amino acid residues) in the protein coding regions of TFD, when these are compared to their respective introns and non-coding (UTRs) regions (Chang et al., 2000; Gong et al., 2000; Fujimi et al., 2003). This observation has lead to the idea that a high rate of point substitutions accelerated the evolution of TFD toxins. But, is this the only mechanism responsible for the high functional diversification of these toxins?

Different studies analyzing the whole gene structure of these toxins and their evolution have shown that TFD domains have undergone a particular mode of accelerated evolution, called Accelerated Segment Switch in Exons to alter Targeting or ASSET (Doley et al., 2009). Briefly, comparison of the coding sequences of related toxin genes revealed the presence of sequence blocks within the TFD that are nearly identical in two or more proteins and that are found next to other blocks that are not conserved (i.e., are protein specific) or are shared with other proteins, as if they have been shuffled during evolution. Interestingly, these LEGO-like blocks seem to correspond to equivalent parts in the tridimensional structure, suggesting that these dramatic protein rearrangements do not perturb the overall stability of the TFD domain, while they can provoke radical changes in their binding specificity.

Obviously, gene duplication has played key role in the genesis of this diversity, and several successive duplication events involving the ancestral gene(s) of extant TFD toxins have allowed in different species the evolution of a broad arsenal of paralogous genes displaying highly divergent functions.

Not surprisingly, the members of the Ly6/three finger domain superfamily have also been found in the genomes of different vertebrate organisms, where they carry out physiological functions very different to those of toxins. Below, I will briefly describe some of the better characterized members of this superfamily in vertebrates and their respective functions.

IV. The roles of the Ly6 superfamily members in vertebrates

TFD proteins, firstly discovered in snake toxins, were subsequently reported in multiple vertebrate organisms, including Humans, whose genome encodes for 45 members of this family (Galat, 2008). These proteins include the 12 TGF-β/Activin receptors, whose ectodomains (their extracellular ligand binding motifs) adopt also a snake three-finger fold (Greenwald et al., 1999). However, while the role of the TGF-β receptors in cell signalling has been studied in great detail, comparatively much less is known about the function of the other members of this

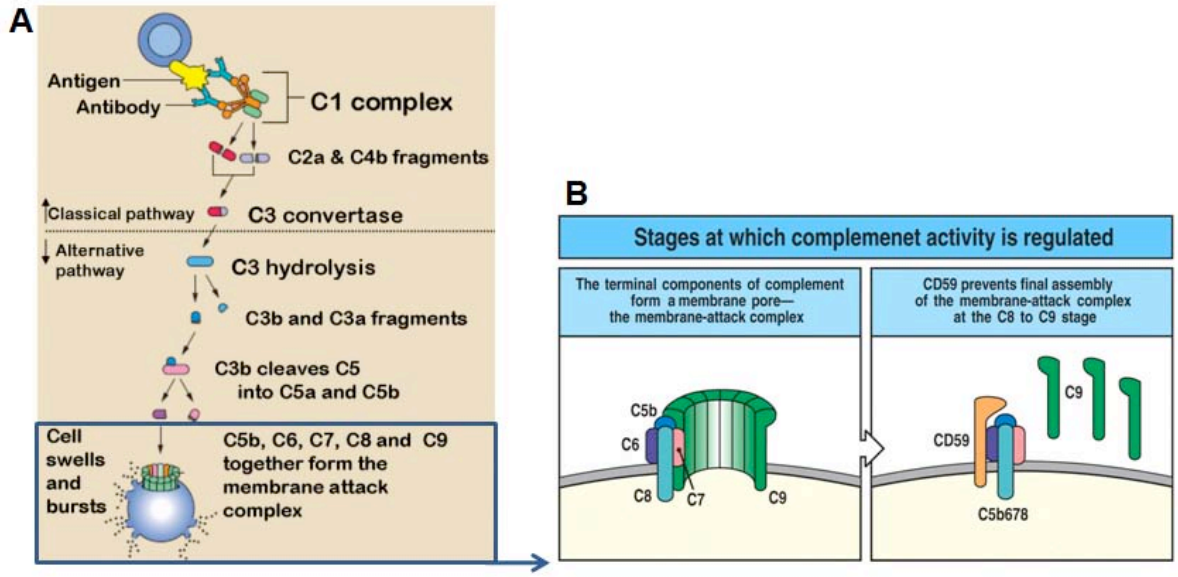

Fig 18. **A. Complement system cascade pathway**. Complement system is a proteolytic cascade in blood plasma. The terminal components of the complement cascade elements: C5b, C6, C7, C8 and C9 form the Membrane attack complex (MAC) (Inset in A and **B**, left panel). MAC formation is initiated by the cleavage of C5 protein into C5a and C5b. Sequential addition of C6, C7, C8, and C9 to C5b leads to the formation of the membrane attack complex (MAC) which, when inserted into the lipid bilayer, form transmembrane pores and induces cell lysis. MAC is the stage where the protectin CD59 is active. This complement regulatory factor binds the complement factors, C8 and C9, within the C5b6-9 complex to prevent final assembly of the MAC and inhibit polymerization of the final C9 membrane pore (**B**, right panel), thus protecting cells from lysis.

superfamily. Among these, the proteins of the Ly6 complex, the CD59 factor and the uPAR receptor, all at the origin of the Ly6/CD59/uPAR denomination, are some of the best characterized at the functional level.

The *ly6* (lymphocyte antigen 6 complex) locus was firstly identified in the mouse, and codes for different TFD antigens expressed at the surface of multiple cell types, including most of the immune system cells (granulocytes, lymphocytes, etc...). This has allowed their widespread use as markers for different subpopulations of immune cells, both in mice and humans. In addition, they are up-regulated in some tumoral situations, and provide useful markers for malignancy diagnosis and promising targets for cancer immunotherapy (Bamezai et al., 2004). Current evidence suggests that they fulfill roles in signalling and lymphocyte T activation, although their precise mode of action is not fully understood.

More information is available about the molecular function of **CD59 (or Cluster of Differentiation 59)**, a cell surface glycoprotein that is expressed in different human cell types, including erythrocytes (red blood cells), leukocytes (white blood cells), and different epithelial cell types. This protein acts as a regulatory element of the complement system cascade, preventing the formation of the membrane attack complex (MAC) (Fig 18), and therefore protecting the cells from complement mediated lysis (Davies et al., 1989). This is essential to restrain the effect of the complement cascade activation to targeted cells, while sparing the surrounding tissue. Its molecular partners are the terminal complement cascade elements (C8 and C9) and its presence directly inhibits the formation of the complement lytic pore (Fig 18).

Interestingly, CD59 has been also found in spermatozoids, although is not produced by these cells. Instead, it is synthesized by prostatic cells and reaches the sperm travelling associated to “prostasomes”, membranous secreted vesicles carrying a GPI anchored form of CD59 (Rooney et al., 1993). The presence of CD59 allows spermatozoids to elude complement attack triggered by the antibodies present in the female reproductive tract.

This case shows that these proteins can be shared by different cell populations and that their extracellular traffic can have an important physiological impact, adding a supplementary level of complexity to their functional properties.

The uPAR receptor (or urokinase Plasminogen Activator Receptor) is a GPI-anchored protein containing three contiguous three-finger motifs. uPAR levels are low in physiologically normal conditions, but are up-regulated in invasive tumor cells and also in migrating

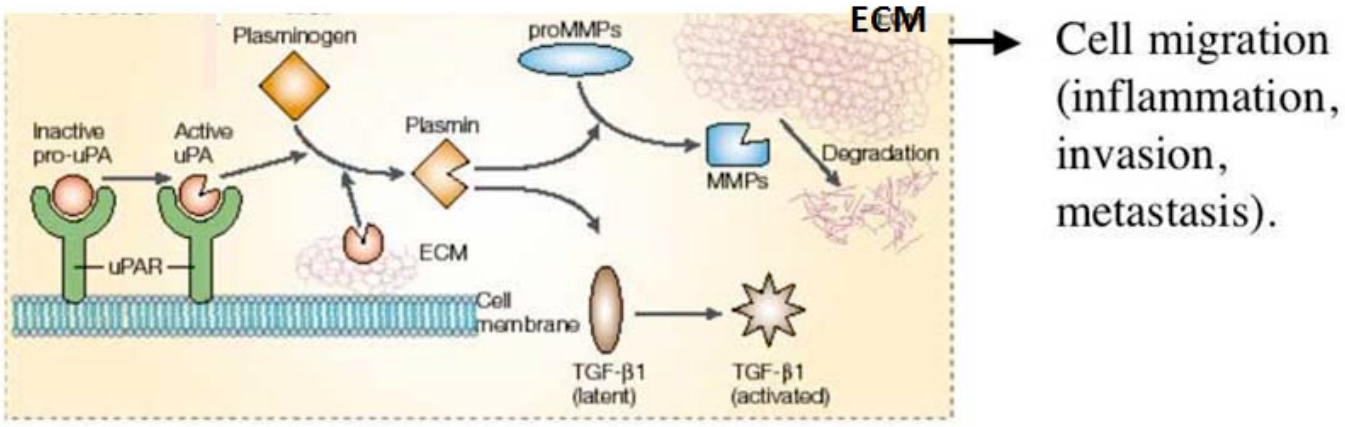

Fig 19. Schematic representation of the uPA/uPAR complex function. The urokinase Plasminogene Activator (uPA) binds to its specific receptor uPAR, present at the cell membrane to be activated. Activated uPA converts the plasminogen proenzyme into plasmin, a serine protease protein that activates other families of proteases, such as the matrix metalloproteinases (MMPs), to promote extracellular matrix degradation, and thereby cell migration or metastasis. In addition, activated plasmin and uPA contributes to the activation of latent growth factors (like TGF-β1) and induce cell proliferation and overgrowth. **Ref. Fig adapted from: (**Blasi and Carmeliet, 2002).

keratinocytes during wound healing, indicating that it could have a role in cell mobility (Romer et al., 2001). This role is mediated by its ligand, the serine protease urokinase-type Plasminogen Activator (uPA). Upon binding to its receptor (uPAR), uPA activates the plasminogen and converts it into plasmin, which in turn degrades the extracellular matrix (ECM), thus facilitating cell mobility but also cancer metastasis (Fig 19). However, uPAR is also known to interact with vitronectin, a component of the ECM (Wei et al., 1994), Integrins (Wei et al., 1996) and G-protein coupled receptors (Resnati et al., 2002) thus mediating both cell adhesion and signal transduction.

These few examples show that the physiological roles of vertebrate Ly6 proteins can be very diverse and that they can interact with a broad range of different partners. They also clearly indicate that the three-finger proteins can act in very different cellular contexts and have been probably co-opted during evolution into very diverse cellular tasks.

Another example, provided by the **Lynx-1** (**Ly6/neurotoxin-like protein 1**), furnishes an interesting illustration of how this co-option into novel processes could occur. This three-finger protein presents structural similarities with the snake venom toxins, and it has been shown to act as a physiological endogenous modulator of nicotinic acetylcholine receptors (nAChR) in the mammalian central nervous system (Miwa et al., 1999). Lynx-1 binds specifically to the nAChR (Ibañez-Tallon et al., 2002) and regulates its activity by reducing its sensibility to the acetylcholine neurotransmitter. It has been proposed that this endogenous neurotoxin-like cell surface molecule could incarnate an ancestral non-toxic form of the different snake toxins targeting the nAChR, which subsequently derived into novel poisons targeting other substrates.

Although the relationship between Lynx-1 and the snake toxins remains hypothetical, as convergent evolution may also explain their similar modes of action, this example illustrates how natural selection could drive co-option of three finger proteins into novel biological processes, providing a plausible scenario for their evolutive radiation and functional diversification.

V. The Ly6 module is also present in invertebrate genomes

The Ly6 motif must be an ancient module, because it is present throughout the animal kingdom, from cnidarians to vertebrates. For instance, the Pdcyst-rich protein of the coral *Pocillopora damicornis*, a member of this family, has been identified as a factor playing a role

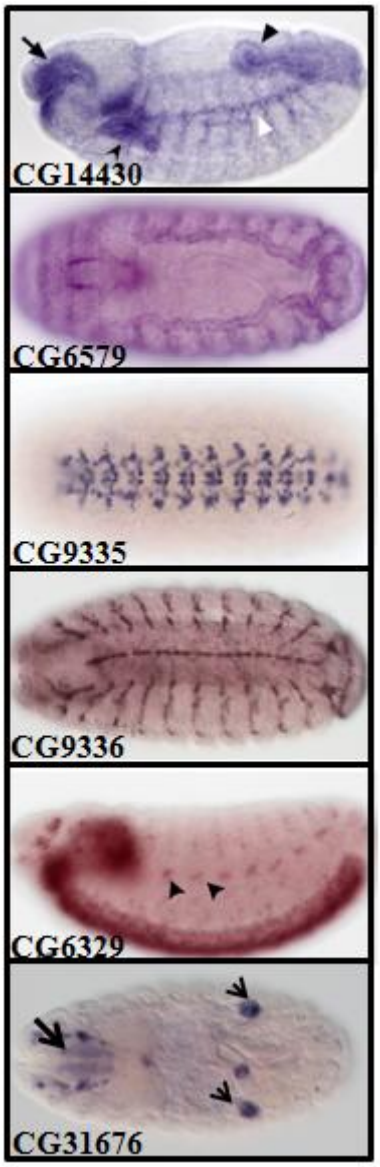

Fig 20. Expression patterns of Ly6 genes in *Drosophila* embryos. In situ hybridizations were realized on stage 16 embryos. These genes show various expression patterns and are expressed in different tissues. CG14430 (*boudin*) and CG6579 are expressed in ectodermal derivatives, other genes are expressed in the nervous system: CG9335 in the ventral nerve cord, CG6329 in the whole CNS and a subset of PNS neurons (black arrowheads), CG9336 in the midline and the PNS glia. CG31676 transcripts are mainly detected in mesodermal gonad cells (black arrowheads) and in the pharyngeal epithelial cells (black arrow).

in the relationships tying the coral algal symbionts and its cnidarian host (Vidal-Dupiol et al., 2009). Ly6 proteins have also been reported in nematodes, where the *Caenorhabditis elegans* Odr-2 protein is required for the function of some olfactory neurons (Chou et al., 2001). Finally, it has also been identified in insects, where the proteins NlLynx-1 and NlLynx-2, found in the planthopper *Nilaparvata lugens*, appear to also modulate the activity of nAChRs in this insect (Liu et al., 2009).

However, the exploration of the diverse functions of invertebrate Ly6 family members has just began, we know very little about the potential implication of these proteins in processes like morphogenesis and development.

In *Drosophila*, and besides the five TGF-β receptors (*tkv*, *babo*, *sax*, *put* and *wit*) that carry a Ly6 domain, little was known about Ly6 proteins. Its only characterized members are the products of a gene called *retroactive* (*rtv*), required for cuticle organization and chitin extracellular matrix assembly (Moussian et al., 2005) and of another gene called *sleepless* (*sss*), required for the regulation of the circadian rhythm (Koh et al., 2008; Wu et al., 2010).

My PhD supervisor Fernando Roch carried out a systematic search in the *Drosophila* genome for proteins containing a Ly6 domain. Using as a diagnostic criterion the presence of a typical set of 8 to 10 cysteines in stereotyped positions, he identified a large family of 36 new Ly6 genes in the fly genome. Preliminary characterization of the expression patterns of some of these genes showed that they are present in a wide variety of tissues during embryogenesis (Fig 20), consistently with the idea that they may be implicated in multiple developmental processes.

When I arrived in the laboratory as a PhD student three years ago, my project main goal was the functional characterization of two members of this fly family, for which lethal loss of function mutations were available. At the beginning, we focused in the study of the CG14430 gene that we have subsequently called *boudin (bou)*, due to the tracheal defects observed in these mutant embryos. In fact, in these animals the tracheal dorsal trunk resembles a string of sausages, *boudin* being the name of a black French sausage.

In a second time, we also analyzed the role of the CG2813 gene that surprisingly displayed also very similar "*boudin-like*" phenotypes. Eventually, this gene has been called *coiled* (*cold*) by another group that identified the same phenotypes independently (Nilton et al., 2010).

We found that *bou* and *cold* genes have similar expression patterns and are both required for the organization of septate junction components in embryonic and larval epithelial tissues. Furthermore, our results also revealed that both are required for blood brain barrier maintenance in neural embryonic tissues.

ARTICLE 1

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

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

Assia Hijazi, Wilfried Masson, Benoit Augé, Lucas Waltzer, Marc Haenlin and Fernando Roch*

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

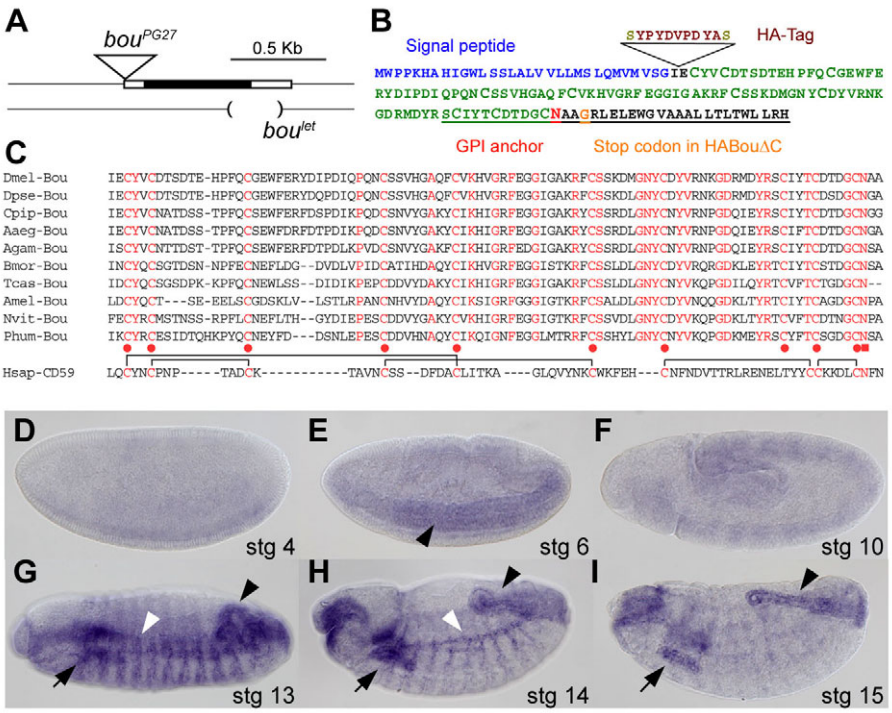

Fig. 1. *bou* codes for a conserved Ly6 protein. (**A**) The *bou* locus, indicating the *bou*^{PG27} and *bou*^{let} DNA lesions. (**B**) Bou precursor features: the signal peptide (indigo), the Ly6 domain (green) and the putative GPI anchor site (Asn125, red). The HA-tag position (brown) and the HA-BouΔC 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*^{I1} 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*^{I1} 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*^{I1} 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*^{I1} 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

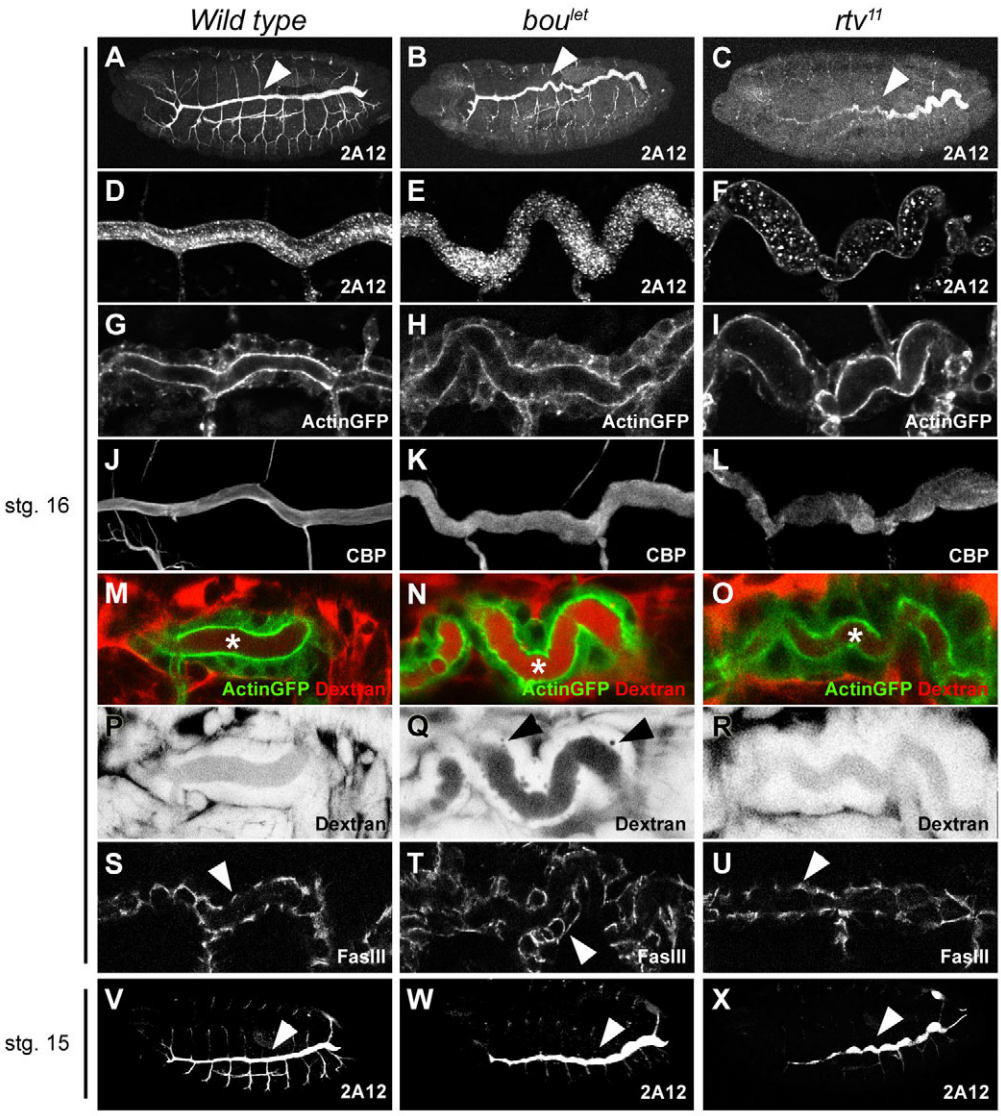

Fig. 2. *bou* and *rtv* regulate tracheal morphogenesis by different mechanisms. Projections (A-C, J-L and V-X) or single confocal sections (D-I and M-U) of embryonic trachea. (**A-I**) At stage 16, the dorsal trunk of both *bou*^{let} and *rtv*^{11} mutants displays an enlarged width and a convoluted 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

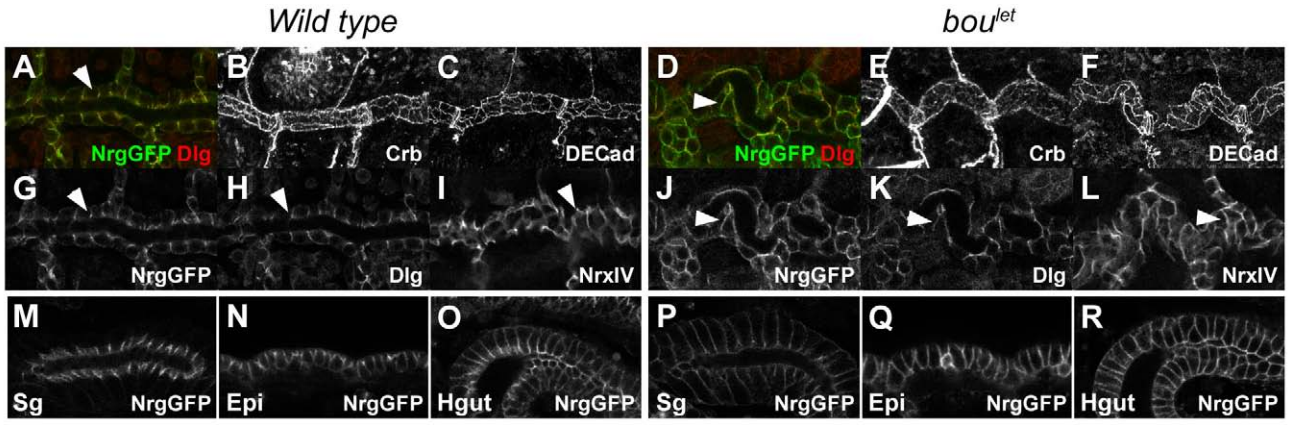

Fig. 3. *bou* is required for septate junction organisation in embryonic ectodermal derivatives. (**A-L**) Confocal sections of stage-16 tracheal dorsal trunks of wild-type and *bou*^{let} embryos stained as indicated. NrgGFP (G,J, green in A,D), Dlg1 (H,K, red in A,D) and Nrx-IV (I,L) distribute along 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

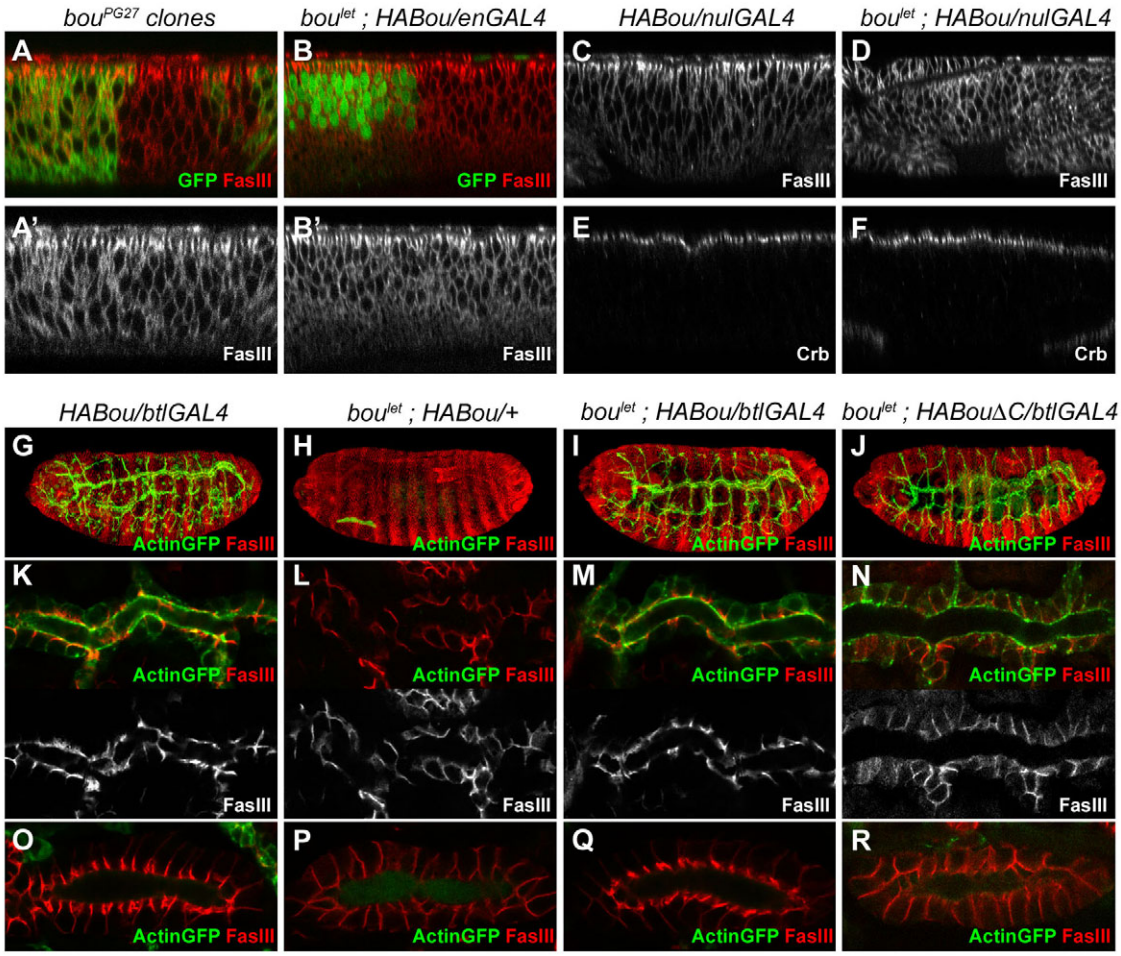

Fig. 4. *bou* SJ phenotypes are not cell-autonomous. (A-F) Confocal *z*-sections through the pouch of third-larval-instar wing discs. (**A**,**B**) Fas3 (red; white in A′, B′) is correctly localised in all the cells of mosaic wings containing GFP$^+$ *bou*^{PG27} mutant clones (A, green) or *bou*^{let} 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.

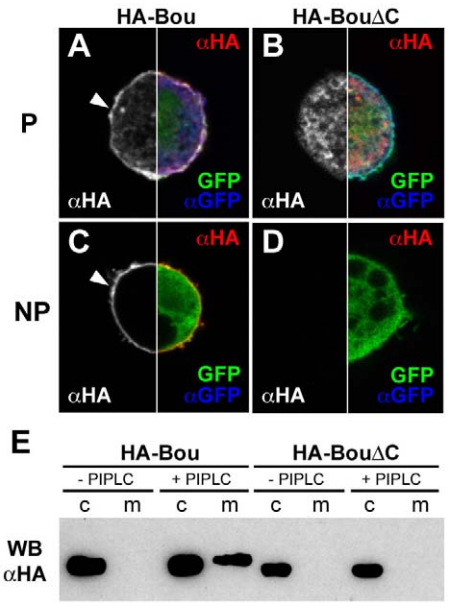

Fig. 5. Bou is a GPI-anchored membrane protein. (**A-D**) *Drosophila* S2 cells co-expressing Moesin-GFP (green, right panels) and HA-Bou (A,C) or HA-BouΔC (B,D). Staining with anti-HA (white, left panels; red, right panels) and anti-GFP (blue, right panels) was carried out in permeabilising (A,B) or 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

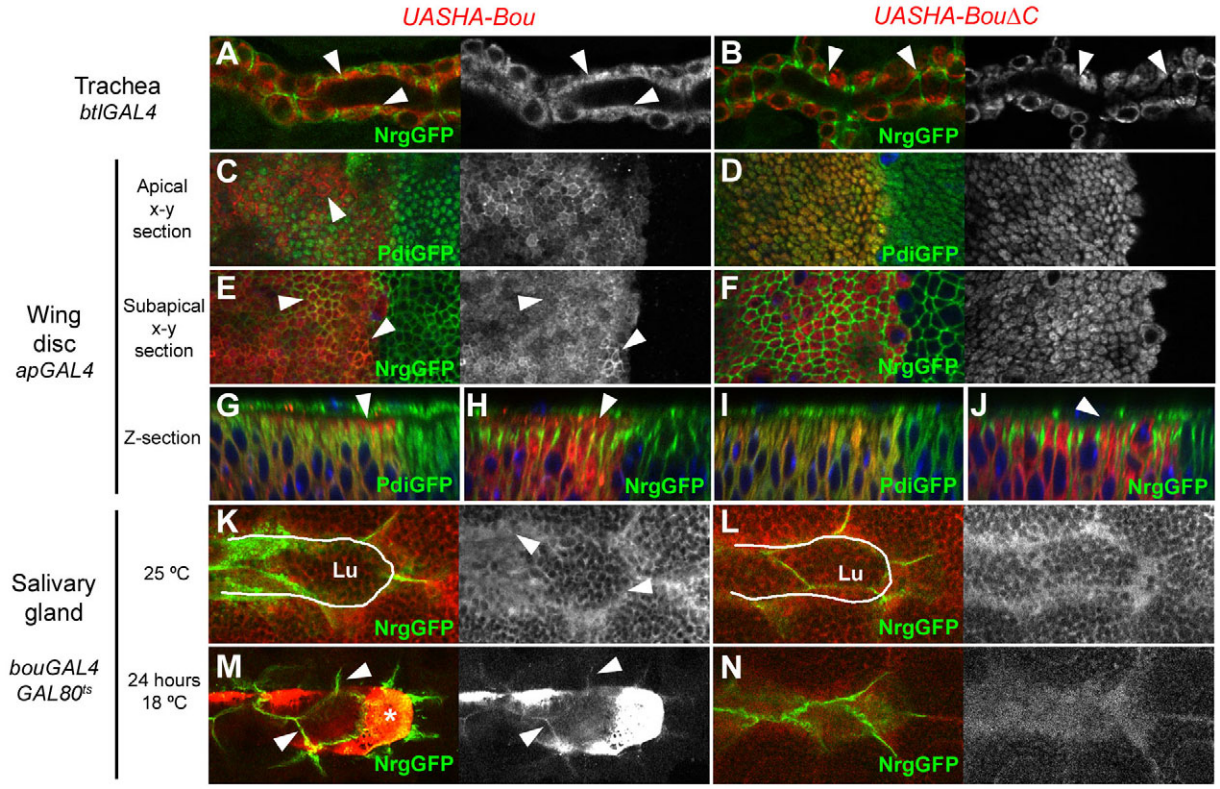

Fig. 6. HA-Bou is found in the membrane and is stabilised in SJ membrane areas. (**A-J**) Confocal sections showing HA-Bou and HA-BouΔC distribution (red) in tracheal cells (A,B) and wing discs (C-J) counterstained with NrgGFP or PdiGFP (green). HA-Bou overlaps with NrgGFP in membrane areas (A,E,H, arrowheads) 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,

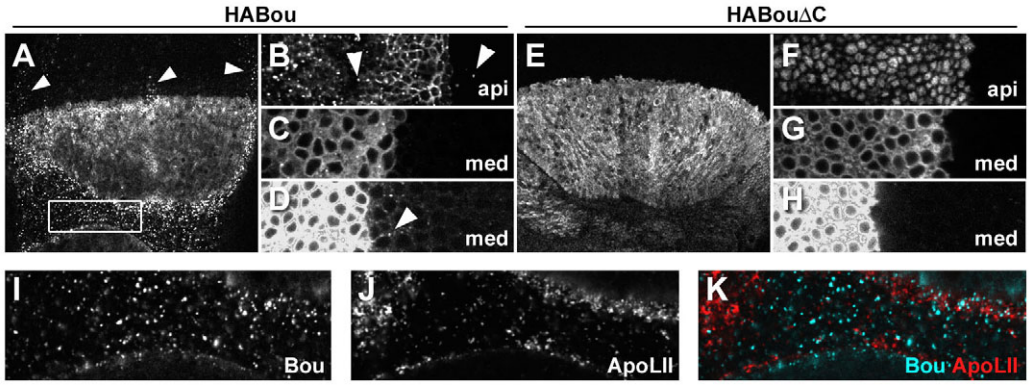

Fig. 7. The HA-Bou protein is present in extracellular particles. (**A-H**) Single confocal *x/y* sections of third-larval-instar wing discs stained with anti-HA antibody. HA-Bou accumulates apically in extracellular particles (A,B, arrowheads) and intracellular vesicles found in the medial regions of *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.

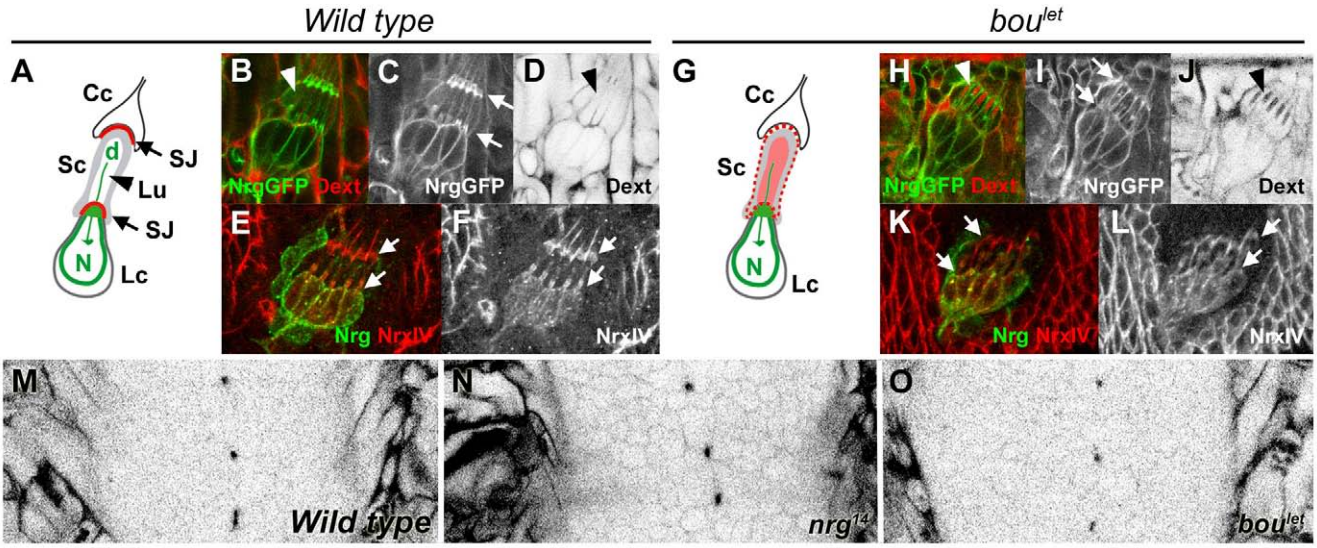

Fig. 8. The *bou* gene is required for blood-brain barrier maintenance in a subset of neural tissues. (**A-L**) Single confocal sections showing chordotonal organs of wild-type (A-F) and *bou*^{let} (G-L) stage-16 embryos. (A,G) Schematic representations of a single chordotonal organ unit. Injected dextran (red, B,H; black, D,J) diffuses into the lumen (arrowheads) of *bou*^{let} but not wild-type chordotonal organs. NrgGFP (green) and Nrx-IV (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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DEV033845 Supplementary Material

- **Supplemental Figure S1**

Fig. S1. Manual alignments of the 10 conserved cysteines present in *Drosophila* Ly6/uPAR domains, allowing visualisation of the characteristic distances existing between these residues. When multiple domains of the same protein are considered, they have been numbered in the same order as they appear on each protein.

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)

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
```

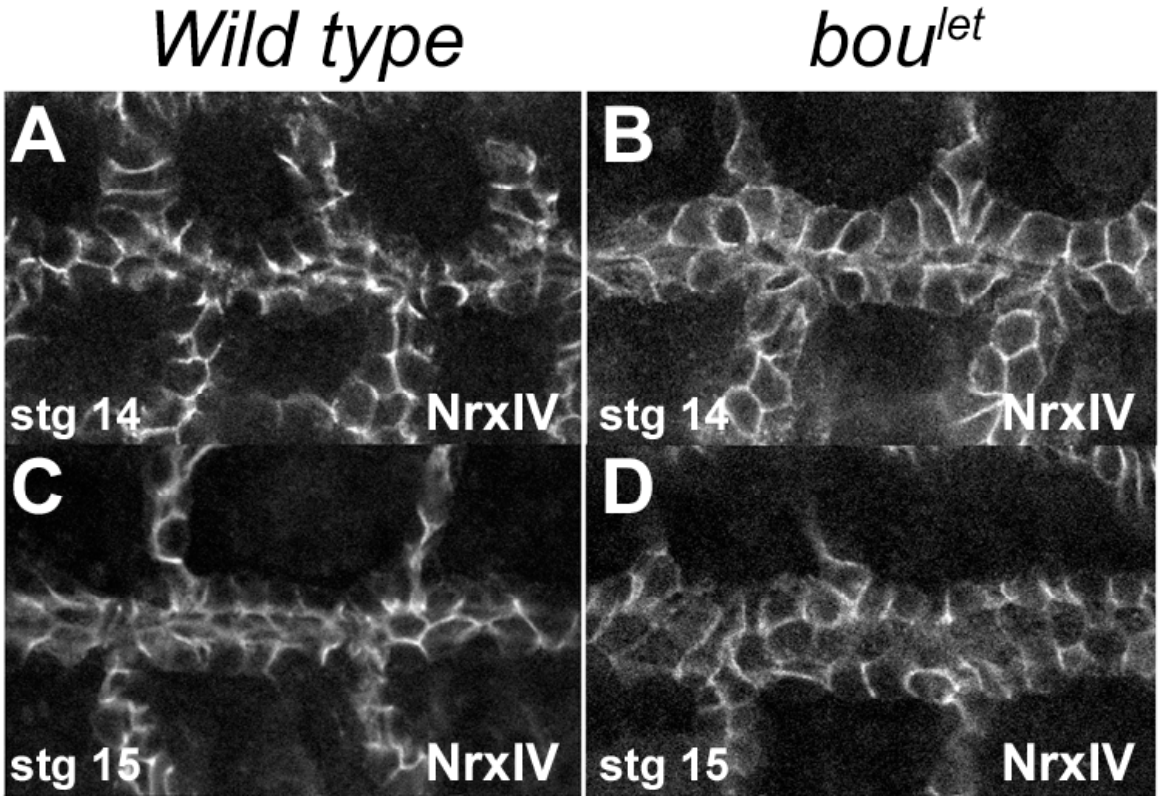
Type II receptors

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

DEV033845 Supplementary Material

- **Supplemental Figure S2**

Fig. S2. *bou* is involved in early establishment of tracheal septate junctions. (**A-D**) Tracheal dorsal trunks stained with NrxIV. At stages 14 and 15, this marker is already delocalised along the tracheal lateral cell membrane of bou^{let} embryos (B, D).


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

ARTICLE 2

The Ly6 protein Coiled is required for septate junction and blood brain barrier organisation in *Drosophila*

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PLoS one

The Ly6 Protein Coiled Is Required for Septate Junction and Blood Brain Barrier Organisation in Drosophila

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Abstract

Background: Genetic analysis of the *Drosophila* septate junctions has greatly contributed to our understanding of the mechanisms controlling the assembly of these adhesion structures, which bear strong similarities with the vertebrate tight junctions and the paranodal septate junctions. These adhesion complexes share conserved molecular components and have a common function: the formation of paracellular barriers restraining the diffusion of solutes through epithelial and glial envelopes.

Methodology/Principal Findings: In this work we characterise the function of the *Drosophila cold* gene, that codes for a protein belonging to the Ly6 superfamily of extracellular ligands. Analysis of *cold* mutants shows that this gene is specifically required for the organisation of the septate junctions in epithelial tissues and in the nervous system, where its contribution is essential for the maintenance of the blood-brain barrier. We show that *cold* acts in a cell autonomous way, and we present evidence indicating that this protein could act as a septate junction component.

Conclusion/Significance: We discuss the specific roles of *cold* and three other *Drosophila* members of the Ly6 superfamily that have been shown to participate in a non-redundant way in the process of septate junction assembly. We propose that vertebrate Ly6 proteins could fulfill analogous roles in tight junctions and/or paranodal septate junctions.

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Introduction

The proteins of the Ly6 superfamily are an ancient feature of metazoan genomes, as genes coding for the Ly6 motif have been identified in a wide variety of animal clades, ranging from cnidarians [1] to vertebrates [2,3]. The Ly6 domains are small extracellular modules of about 100 residues characterised by presence of 4–6 pairs of cysteines placed in stereotypical positions [4]. These conserved residues form internal disulphide bridges that stabilise the conformation of the motif, but the rest of the protein sequence can vary to a remarkable extent. Despite this variability, these proteins adopt upon folding comparable three-dimensional structures, that are characterised by an internal hydrophobic core supporting three protruding fingers [4]. Indeed, these architectural motifs are often referred to as Three Finger Domains (TFD). The Ly6 module is present in both soluble and GPI anchored membrane proteins but is never observed in combination with other extracellular motifs. Due to its plasticity, it has been co-opted into many different biological processes, where it participates as a protein-protein interaction domain binding specifically to a wide variety of molecular partners [5,6].

The *Drosophila* genome codes for 45 proteins belonging to the Ly6 superfamily [7]. Further illustrating the versatility of the Ly6 module, three of these genes have been analysed at a functional level and have been found to participate in distinct developmental tasks, namely the assembly of the chitin extracellular matrix (*retroactive*) [8], the regulation of circadian rhythms (*sleepless*) [9] and the organisation of cell adhesion junctions (*boudin*) [7]. Thus, *Drosophila* represents an attractive system where to pursue genetic studies identifying the multiple physiological roles of these proteins.

We have analysed the role of another member of the fly Ly6 superfamily, the gene *CG2813/coiled* (*cold*) [10]. We show that *cold* mutants display similar phenotypes to those seen in *bou* alleles [7], indicating that *cold* is essential for the organisation of the insect septate junctions (SJ). These invertebrate adhesion structures contribute both to the maintenance of cell contacts and the establishment of paracellular barriers preventing the unregulated passage of ions and solutes through epithelial layers and glial sheaths [11]. The *Drosophila* SJ have received considerable attention because they share with the vertebrate tight junctions not only a common role but also several conserved components, suggesting that they could be homologue structures [11,12]. In addition, there are also striking parallelisms at the functional and molecular level between the insect SJ and the vertebrate paranodal septate junctions [13], which are adhesion structures formed at the axon-Schwann cells contact areas on both sides of the nodes of Ranvier [14]. Thus, studying the *Drosophila* SJ is a way to identify

new components of these multi-molecular adhesion complexes and to understand the general mechanisms controlling their assembly.

In this work we show that *cold* is specifically required for the organisation of the SJ in both epithelial tissues and in glial cells, where its activity is required for maintenance of the blood brain barrier. We present evidence suggesting that the *cold* product could behave as a membrane component of the septate junctions and we show that this gene, differing from *bou*, is required in a cell autonomous way.

Results

Cold is expressed in ectodermal derivatives and in a subset of glial cells

To begin the functional characterisation of new members of the *Drosophila* Ly6 superfamily, we searched in public stock collections for potential mutants affecting their activity and focused in the analysis of the *CG2813* gene, for which three different putative mutants are available for genetic studies (see below). While we were preparing this manuscript, a study reported an analysis of *CG2813* mutants and named this locus as *coiled* (*cold*) [10]. Thus, thereafter we will refer to *CG2813* as to *coiled*. The *cold* gene codes for a single Ly6 domain, whose primary sequence appears to be well conserved among insects. In fact, a *cold* orthologue can be recognised in several available fully sequenced insect genomes (Fig. 1B and data not shown). This indicates that, in contrast with other Ly6 *Drosophila* paralogues, which are found exclusively in the drosophilid lineage [7], the *cold* product could be part of an ancient genetic network common to all insects. Genetic analysis of three independent mutant lines carrying PiggyBac insertions in different regions of the *cold* locus (Fig. 1A) revealed that all of them behave as recessive embryonic lethal alleles belonging to a single complementation group. These observations indicate that *cold* function is essential for embryonic development. Consistently, remobilisation of the $PBac^{f05607}$ transposon restored fly viability both in homozygosis and in heteroallelic combinations, suggesting that this insertion is responsible for the observed lethality.

To begin the analysis of *cold* function, we studied by *in situ* hybridisation the embryonic distribution of its transcript. In early embryos, we detected an uniform signal corresponding to a *cold* mRNA maternal contribution (Fig. 1C–D). After cellularisation, *cold* is expressed in the ectoderm and at low levels until stage 11, when its transcript begins to accumulate in the fore and hindgut primordia (Fig. 1E). By stage 13, *cold* is expressed at high levels in epithelial derivatives, including the tracheal network, the fore and hindgut and the salivary glands (Fig. 1F). We also detected a weak expression in the embryonic epidermis and, at late stages, in cells associated with the nerve tracks exiting the ventral cord (Fig. 1I). Furthermore, we monitored the expression of YFP (Yellow Fluorescent Protein) in embryos carrying the $PBac^{1001277}$ protein trap insertion, which is placed in the first *cold* intron and produces an in frame YFP fusion with the Cold protein (Fig. 1A). The expression pattern of this protein matches the observed distribution of the *cold* transcript, as we observed Cold-YFP in all epithelial derivatives by stage 13 (Fig. 1G–H). At later stages, this fusion protein was also detected in a subset of Repo-positive glial cells [15] seen both at the surface of the ventral cord and in close association with the nerve tracks (Fig. 1J–K). Thus, these observations indicate that *cold* could have a role not only in epidermal tissues but also in glial cells.

Cold is required for tracheal morphogenesis and SJ organisation

We examined embryos homozygous for the $cold^{f05607}$ insertion in search of visible phenotypes. For this, we focused on the development of the tracheal network, a tissue where *cold* is expressed at high levels. Staining of the embryonic tracheal system with the 2A12 luminal marker revealed that the overall organisation and branching pattern of this tubular network was preserved in *cold* mutants (Fig. 2C–D). However, both the shape and the length of the tracheal dorsal trunk segments were abnormal in this mutant (Fig. 2A–D′). Differing from the wild type, this structure appeared in stage 15 *cold* embryos as a

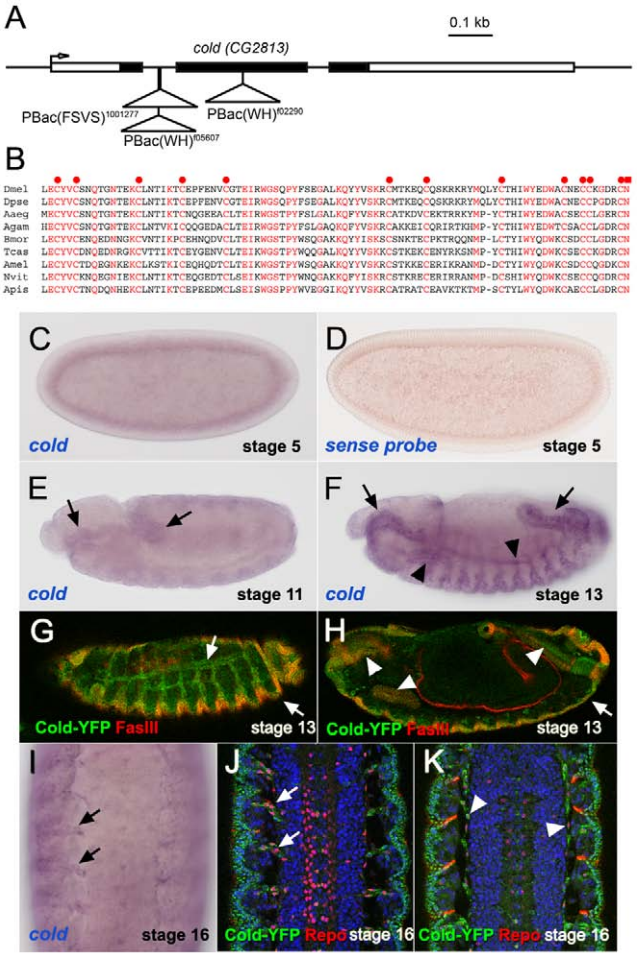

Figure 1. Structure and expression pattern of the *cold* gene. (A) *coiled/CG2813* genomic region, showing the localisation of the *cold PBac* insertions used in this work. (B). Protein sequence alignment corresponding to the Ly6 domain of different insect Cold orthologues. Invariant residues are shown in red. The 12 invariant cysteines and C-terminal asparagines, are marked respectively by red circles and a red square. See Materials and Methods for the species full names. (C,E,F) *In situ* hybridisation showing the *cold* mRNA distribution at different embryonic stages. Note at stage 5 an uniform signal corresponding to a maternal transcript and progressive accumulation of *cold* mRNA in the foregut and hindgut primordia at stage 11 (arrows), and in foregut, hindgut (arrows), trachea and salivary gland (arrowheads) of stage 13 embryos. (D) No signal was observed using a *cold* sense RNA probe. (G,H) Stage 13 $PBac^{1001277}$ embryos expressing a YFP-Cold fusion protein and stained for FasIII. The YFP signal was detected in the trachea and epidermis (white arrows) and in salivary gland, fore and hindgut (white arrowheads). (I) Ventral cord of a stage 16 embryo revealing *cold* transcript accumulation in cells associated with the nerve tracks (arrows). (J,K) Stage 16 embryos stained for Repo (red) and the YFP-Cold fusion protein (green), which is expressed in a subset of glial cells placed in the ventral cord surface (arrowheads) and associated with the exiting nerves (arrows).
doi:10.1371/journal.pone.0017763.g001

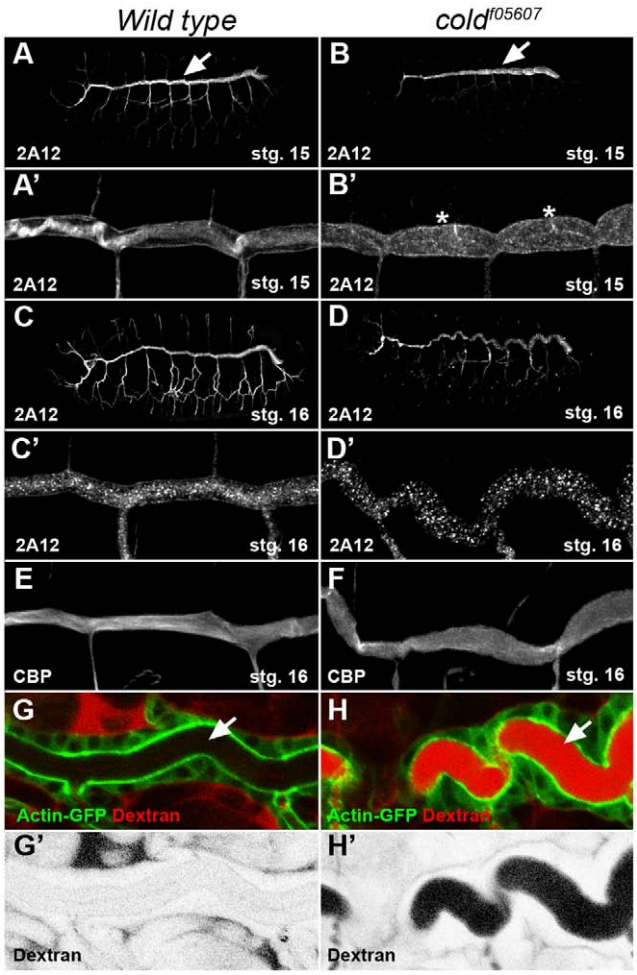

Figure 2. The tracheal morphology and paracellular barrier integrity are perturbed in *cold* embryos. (A–D) Projections of confocal stacks corresponding to wild type and *cold*^{f05607} embryos staged as indicated and stained for the 2A12 tracheal luminal antigen. The same trachea are shown at higher magnification in panels A′–D′. At stage 15, the morphology of the tracheal dorsal trunk (arrows) is affected and displays a series of cysts (asterisks) visible in *cold* mutants. By stage 16, the dorsal trunk adopts a convoluted shape. (E–F) Projections of confocal stacks showing the tracheal dorsal trunk stained with fluorescent chitin binding probe (CBP). In the wild type, the chitin cable displayed a fibrous structure that was lost in *cold* embryos of the same stage. (G,H) Single confocal sections showing a view of the dorsal tracheal trunk marked by ActinGFP (green) and corresponding to stage 16 live embryos injected in the hemolymph with rhodamine 10 kDa dextran (red). (G′,H′) show a greyscale negative image of the red channel shown in G,H.
doi:10.1371/journal.pone.0017763.g002

succession of bulging cysts (Fig. 2A–B′) and, by stage 16, an abnormally convoluted tube (Fig. 2C–D′). Staining of the tracheal chitin cable with a fluorescent chitin binding probe (CBP) [16], showed that its fibrous structure was disorganised in the mutant embryos and chitin was deposited as an amorphous material in the tracheal lumen (Fig. 2E,F). These phenotypes are highly reminiscent of mutants affecting the formation of the septate junctions (SJ) [12], and prompted us to test whether the paracellular barrier preventing solute passage through epithelial layers is functional in *cold* embryos, a diagnostic character for mutants involved in SJ organisation [17]. We monitored the ability of a 10kDa fluorescent dextran dye injected into the body cavity to diffuse into the tracheal lumen of *cold* live embryos. In sharp contrast with the wild type controls, we observed a rapid diffusion (<30 minutes) of this soluble dye into the lumen of stage 16 *cold* mutant trachea (Fig. 2G–H′), showing that *cold* is essential in this tissue for organisation of a functional paracellular barrier.

We then studied in the same mutant background the subcellular distribution of Nrg-GFP [18] and Dlg [19], two SJ markers. The localisation of Nrg-GFP was perturbed in all epithelial tissues examined in *cold* mutant embryos (Fig. 3A–H). Instead of accumulating in the most apical part of the cells, Nrg-GFP appeared homogenously distributed along their lateral side in the trachea, the salivary glands, the hind-gut and the epidermis (Fig. 3A–H). The localisation of Dlg was affected in a similar way, but we observed that a portion of this protein was still accumulating in the apical part of the cells, suggesting that apico-basal polarity is not completely lost in the *cold* embryos (Figs. 3A–H and 4F′–G′). Consistently with this idea, the localisation of E-Cadherin and Crumbs, respectively apical junction and cell polarity markers [20,21] was not altered in *cold* mutant trachea, suggesting that this gene is not required for cell polarity or assembly of other adhesion structures (Fig. 3I–L).

However, the *cold*^{f05607} homozygous embryos could still contain some wild type product supplied maternally and capable of masking its requirements during early establishment of cell polarity, as observed with other genes required for SJ assembly such as *coracle*, *NrxIV*, *yurt* and the Na^+/K^+ *ATPase* [22]. To address this issue, we generated embryos in which the *cold* maternal contribution was missing, taking advantage of the FLP-DFS (FLP-recombinase-dominant female sterile) technique [23]. Embryos lacking the *cold* maternal contribution but rescued paternally with a wild type allele did not show any phenotype and survived into adulthood (Fig. 4C and data not shown). In contrast, mutant embryos lacking both maternal and zygotic contributions died during embryogenesis but did not display obvious morphological defects and were indistinguishable from embryos lacking only the *cold* zygotic contribution (Fig. 4A–D). Notably, they exhibited similar defects in SJ organisation, as revealed by staining with antibodies against FasIII (Fig. 4E–I) and Dlg, which was still seen accumulating in the most apical part of the salivary gland cells in both types of embryos (Fig. 4E′–I′). Since *boudin*, another member of the Ly6 gene superfamily, is also required for SJ organisation [7], we wondered whether some degree of genetic redundancy could exist between *cold* and *bou*. The overall morphology of embryos either double or single mutant for these genes was identical and the distributions of FasIII and Dlg were also indistinguishable in the three mutant backgrounds (Fig. 4G–I′). Thus *cold* and *bou* do not show redundant activities during embryogenesis.

Altogether, these results indicate that the tracheal morphological phenotypes and the defects observed in the paracellular barrier of *cold* mutant trachea are likely to result from a specific defect in the assembly or maintenance of the epithelial SJ.

Cold is required for blood brain barrier organisation

Insect pleated SJ are not exclusive of epithelial cells and are also seen at the cell contacts existing between certain types of glial cells [24]. In the embryonic ventral cord, the presence of SJ in the subperineural glia is essential for the formation of the so called blood-brain barrier, a physiological fence essential for brain insulation from the hemolymph [25]. Given that *cold* is expressed in surface glial cells, we monitored if dye injected into the hemolymph could penetrate into the ventral cord of *cold* mutants 22 hours old, when the blood-brain barrier is fully established [26]. Our results show that a 10 kDa dextran dye readily diffused into the ventral cord of *cold* mutant embryos, whereas it was efficiently

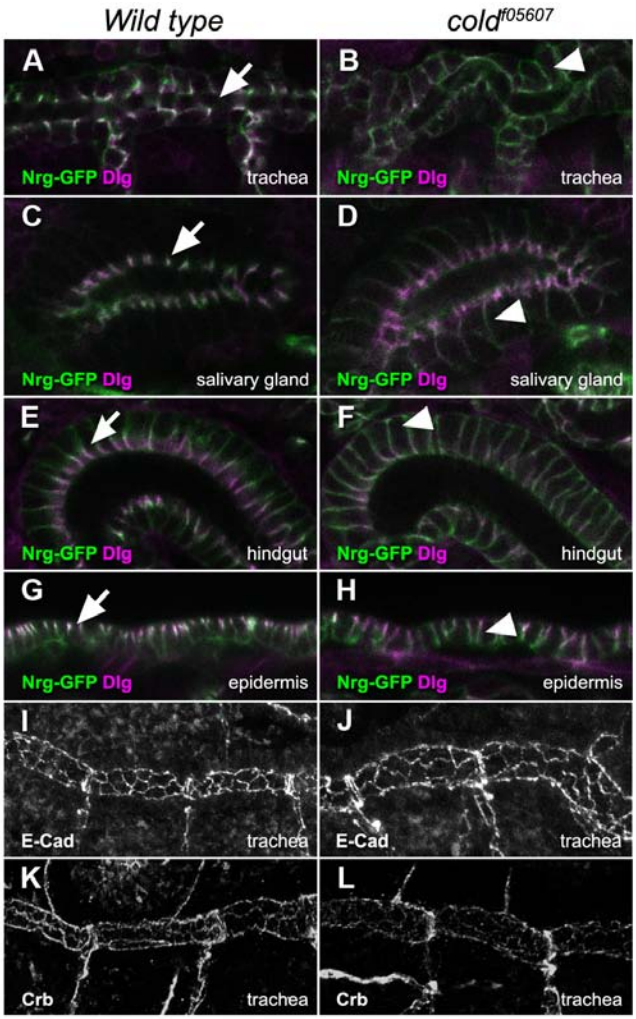

Figure 3. *cold* is required for the organisation of epithelial septate junctions. (A–H) Single confocal sections showing the subcellular localisation of the Nrg-GFP (green) and Dlg (magenta) proteins in different epithelial tissues belonging to stage 16 wild type and *cold*^{f05607} embryos. In wild type embryos, Nrg-GFP and Dlg accumulated in the apical part of these epithelia (arrows). In the mutant embryos, the Nrg-GFP protein appeared uniformly distributed in the lateral part of the cells (arrowheads), whereas some apical accumulation of Dlg protein was still visible. (I–L) Projections of confocal stacks representing the trachea of wild type or *cold*^{f05607} embryos, stained as indicated. E-cadherin and Crumbs localised to the apical part of the tracheal cells in both genetic backgrounds.
doi:10.1371/journal.pone.0017763.g003

excluded from this structure in wild type embryos of the same age (Fig. 5A–B). We also analysed dye diffusion in the peripheral nervous system lateral chordotonal organs, which are insulated from the hemolymph by a specific set of glial cells [27,28]. We observed that the injected dye diffuses into the lumen of the chordotonal organs in stage 17 *cold* embryos (Fig. 5G), whereas SJs established between the cap, scolopal and ligament cells (Fig. 5F) [28] prevented dye intake in the wild type controls (Fig. 5C). In fact, the SJ markers Nrg-GFP and NrxIV were not seen accumulating at the contact regions between these cells in a *cold* mutant background (Fig. 5D–E′ and H–I′). These observations demonstrate that maintenance of an efficient paracellular barrier and proper distribution of SJ markers depends on the activity of *cold* in the nervous system.

The *cold* gene is autonomously required for SJ organisation

Previous analysis of the *bou* Ly6 gene, which is also required for SJ formation, indicates that the mutant phenotypes for this gene are not cell autonomous, as presence of wild type neighbour cells can rescue the SJ defects seen in *bou* mosaic embryos [7]. To test whether this feature also applies to the *cold* phenotypes, we directed expression of a FLAG tagged form of Cold in the tracheal cells of a *cold* mutant embryo, using the *btlGAL4* driver. The SJ marker FasIII delocalisation phenotype observed in *cold* trachea was fully rescued by FLAG-Cold expression (Fig. 6A–A′). This indicates that this protein is fully functional and further confirms that the *cold* gene is responsible for the observed tracheal phenotypes. However, unlike the Bou protein, targeted Cold expression in the trachea did not restore proper FasIII localisation in other tissues, like the salivary gland (Fig. 6A″). We further confirmed the full autonomy of the *cold* requirements by monitoring FasIII distribution in the hindgut of mutant embryos expressing FLAG-Cold under the control of the *engrailedGAL4* driver, which is only expressed in the dorsal half of this epithelial tube [29]. In fact, FasIII appeared correctly localised only in the cells expressing the FLAG-Cold protein (Fig. 6C–C″). Thus, our results show that *cold* rescuing activity is neither able to diffuse from tissue to tissue (like in the case of *bou*) nor between neighbour cells belonging to the same epithelium. In line with these results, we found that *cold* is also autonomously required for proper SJ maintenance in the epithelial cells that form the imaginal discs. Indeed, in mosaic third larval instar wing discs containing large *cold*^{f02290} *Minute*$^{+}$ clones, we observed that the *cold* mutant cells fail to accumulate the FasIII marker in their most apical part, unlike the surrounding wild type cells (Fig. 7A,A′). Consistently with the idea that *cold* is not involved in cell polarity maintenance, we also observed that a small amount of Dlg and normal levels of Crumbs are present in the most apical part of the mutant cells (Fig. 7B–C′).

Cold is present at the membrane and stably associates with regions containing SJ

Lacking specific antibodies against Cold, we took advantage of our FLAG-Cold fusion protein to analyse its subcellular distribution in wing disc epithelial cells, using *apterousGAL4* as a driver. The FLAG-Cold was detected inside the cells, where we observed extensive co-localisation with the endoplasmic reticulum marker Pdi-GFP [30] (Fig. 8A–A″). We also detected a slight concentration of FLAG-Cold in the most apical part of the cells, in a region free of endoplasmic reticulum that could correspond to the plasma membrane, as it contained low levels of Nrg-GFP (Fig. 8B–C″). Yet, the FLAG-Cold protein did not obviously accumulate in the membrane regions displaying the highest levels of Nrg-GFP and harbouring the SJ (Fig. 8B–C″). In addition, we observed in more basal regions internal vesicles containing FLAG-Cold which do not stain positively for Nrg-GFP (Fig. 8D–D″). Thus, although FLAG-Cold is not preferentially associated with the SJ, our observations indicate that part of this protein could be present in the plasma membrane, as it is the case in S2 cultured cells. In fact, FLAG-Cold is readily detected at the cell surface of non permeabilised S2 cells that were stained in conditions that prevent antibody access to the interior of the cell (Fig. 8F–F″). In permeabilised cells, we also observed presence of FLAG-Cold in internal vesicles, as previously noticed (Fig. 8E–E″) [10].

These observations are consistent with the idea that FLAG-Cold could be a membrane protein cycling between internal compartments and the plasma membrane, but we reasoned that the high

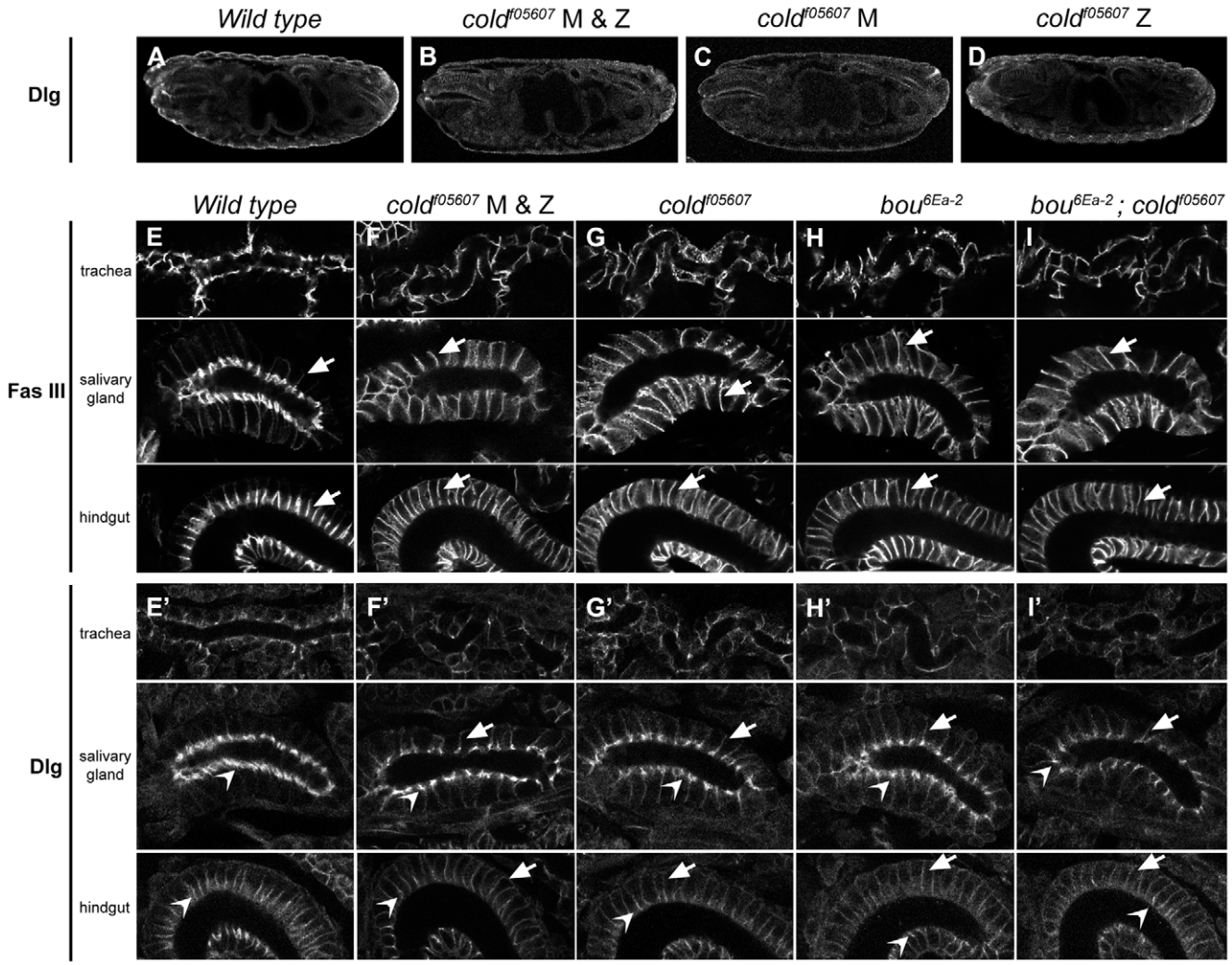

Figure 4. *cold* is not required for establishment of epithelial cell polarity. (A–D) Single confocal sections showing the overall morphology of either wild type embryos or mutant combinations lacking the *cold* maternal contribution (*cold* M), the zygotic one (*cold* Z) or both (*cold* M & Z), all stained for Dlg. (E–I′) Subcellular distribution of FasIII and Dlg in trachea, salivary gland and hindgut of stage 16 embryos of the indicated genotypes. Notice FasIII uniform distribution in the lateral membrane of the mutant tissues (E–I, arrows). A portion of Dlg (E′–I′) was redirected to the lateral membrane in the mutant tissues (arrows), although apical accumulation was still visible in the salivary gland and hindgut cells (open arrowheads). doi:10.1371/journal.pone.0017763.g004

levels of FLAG-Cold produced in these experiments could saturate the cell, obscuring its potential accumulation in particular subcellular compartments. To analyse the localisation of this protein in a less saturated background, we took advantage of the GAL80^{ts} repressor to switch down FLAG-Cold production during development [31]. Accordingly, we expressed this protein in presence of the *tubGAL80^{ts}* repressor in third larval instar salivary glands, using *bouGAL4* as a driver [7]. In larvae growing at 25°C, we detected high levels of FLAG-Cold in the lumen and in the cell bodies of the salivary gland cells, but no preferential accumulation at the SJ level (Fig. 9A,A′). Then, we switched-off FLAG-Cold expression by shifting the fly cultures to 18°C 40 hours prior to dissection. In these conditions, the FLAG-Cold protein was still seen in the salivary gland lumen, but could also be detected at low levels in a lateral cell region containing the FasIII SJ marker (Fig. 9B,B′). This weak staining was not observed in control glands lacking the *bouGAL4* driver (Fig. 9C,C′), indicating that upon expression, a small portion of FLAG-Cold seems stably associated with SJ-containing regions.

Discussion

Is *cold* specifically required for SJ organisation?

The profusion of *Drosophila* Ly6 paralogues (45 members) and the variety of their expression patterns [7] suggest that mutants for these genes could *a priori* display the most various phenotypes, as it is the case for the three fly Ly6 genes characterised in so far: *rtv* [8,16], *sss* [9,32] and *bou* [7]. However, a recent report pointed out that three other *Drosophila* Ly6 proteins, Coiled, Crooked and Crimpled participate in a non redundant way in the same process as Bou: the organisation of epithelial septate junctions [10]. Our genetic characterisation of *cold* mutants further confirms that this gene is required for SJ organisation in epithelial tissues, and shows by direct comparison with *bou* mutants that both elicit undistinguishable phenotypes. Still, besides their diagnostic set of 10 cysteines, the primary sequences of Bou, Coiled, Crooked and Crimpled are remarkably different, making impossible to predict a common molecular role. Given their structural divergences and the versatility of the Ly6 domain, they could in principle bind to

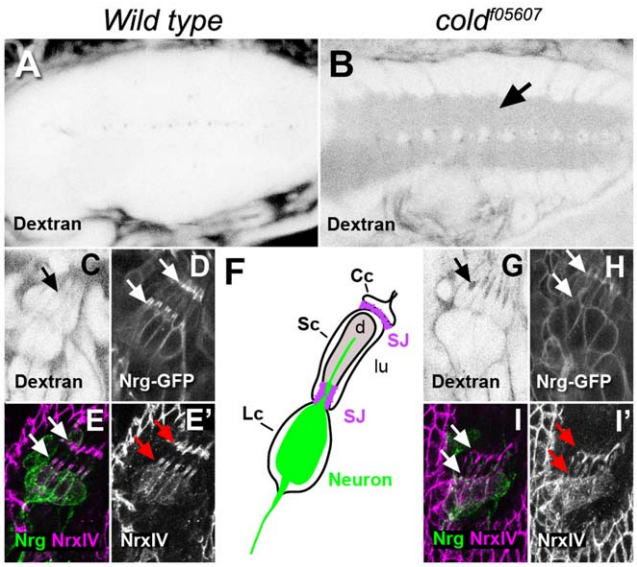

Figure 5. The *cold* gene is required for blood-brain barrier organisation. (A–B) Single confocal sections taken at the level of the ventral cord and showing in negative the distribution of 10 kDa rhodamine dextran injected into the body cavity of live stage 17 embryos. Notice dye penetration in the neuropile region of the *cold* mutant (arrow). (C,G) Confocal sections taken at the level of the lateral chordotonal organ of dextran injected stage 16 live embryos, showing dye accumulation in the lumen of these organs in the *cold* mutant (arrows). (D,H) Distribution of Nrg-GFP in the chordotonal organ of stage 16 live embryos, showing accumulation of this protein in the junctions existing between cap and scolopal cells (arrows) and ligament and scolopal cells (arrowheads). Notice that Nrg-GFP accumulation was lost in the mutant embryos. (E,I) Projections of confocal stacks showing the distribution of NrxIV (magenta) and the BP104 antibody-reactive Nrg neural isoform (green) in the lateral chordotonal organs of stage 17 embryos. (E′,I′) correspond to the magenta channel shown in E,I. The NrxIV SJ marker was not properly localised at the level of the cell junctions in the mutant embryos (white and red arrows). F. Cartoon representing the cellular composition of one single scolopal unit. Cc, cap cell; Sc, scolopal cell; Lc, ligament cell; d, dendrite; lu, lumen; SJ, septate junctions.
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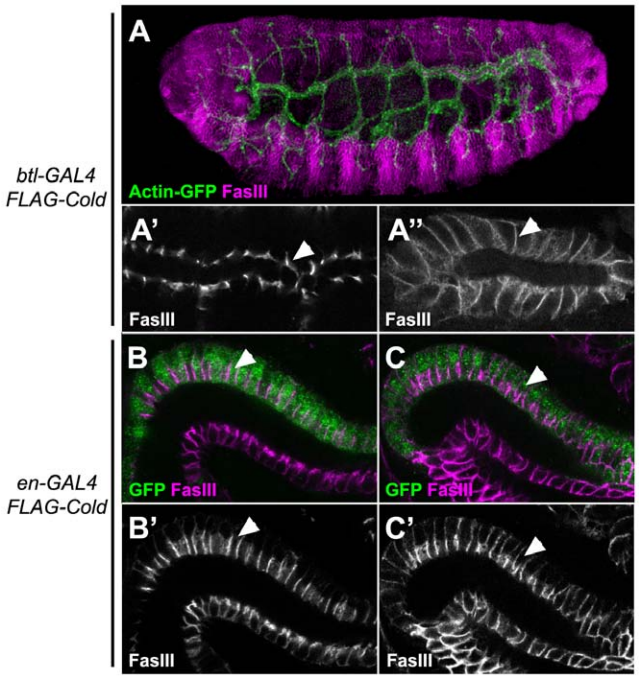

Figure 6. The embryonic *cold* SJ phenotypes are cell autonomous. (A) Projection of a confocal stack showing a stage 16 *cold*^{f05607} homozygous embryo stained for FasIII (magenta) and expressing FLAG-Cold and Actin-GFP (green) proteins under the control of *btlGAL4*. (A′,A″) Single confocal sections at a higher magnification of the trachea dorsal trunk (A′) and the salivary gland (A″) of the same embryo, showing the distribution of the SJ marker FasIII. Notice the delocalisation of this marker in the salivary gland (arrowhead) and its normal distribution in the trachea (arrowhead). (B–C′) Distribution of FasIII (shown in magenta in B,C and in greyscale in B′,C′) in the hindgut of stage 16 embryos expressing FLAG-Cold in the *engrailedGAL4* territory, marked by GFP (green). In wild type embryos, expression of FLAG-Cold in the *en* cells (green) did not affect the localisation of the FasIII marker in the apical portion of the lateral membrane (arrowheads) (B,B′). In *cold*^{f05607} homozygous embryos normal apical accumulation of FasIII was only observed in the *en* cells (green, arrowheads).
doi:10.1371/journal.pone.0017763.g006

different molecular partners. Our analysis of *bou cold* double mutant embryos indicates that at least these two genes do not exert redundant functions during SJ assembly and cell polarity establishment. Thus, the available data are coherent with the idea that these proteins have non-exchangeable roles, despite their similarities at the phenotypic level. Interestingly, the four Ly6 genes implicated in the organisation of the fly SJ have highly conserved orthologues in other insects, such as the honey-bee [7], pleading for a hardwired role of these proteins in SJ assembly.

Our analysis of embryos lacking both the *cold* zygotic and maternal contributions indicates that this gene is unlikely to have a role during the establishment of cell polarity. Thus, while some SJ components such as Yurt, NrxIV, Coracle and the Na^+/K^+ ATPase are also necessary for this process [22], the activity of Cold seems dispensable. Hence, it seems that this protein would participate in a genetic module whose role is solely required for the assembly of SJ. It will be interesting to test whether this also applies to the three other Ly6 genes affecting SJ organisation, as a differential requirement could provide hints facilitating the recognition of their specific partners.

A recent genetic screen identified *cold* as a gene required in the embryonic epidermis for efficient reestablishment of epithelial integrity upon injury [33]. This observation indicates that the integrity of the whole SJ adhesion complex could be required for wound healing or, alternatively, that *cold* may have a specific role in this process. Further analysis of the role of the SJ adhesion structures during epithelial repair will allow to clarify this issue. In any case, this observation shows that upcoming functional analysis of the *Drosophila* Ly6 proteins is likely to contribute to a better understanding of many developmental and physiological processes in which this versatile module has been co-opted.

Is Cold a SJ component?

Previous studies in S2 cells pointed out that a Cold tagged version expressed in these cells accumulates in endocytic vesicles [10]. We have further analysed the localisation of a functional FLAG-Cold fusion protein, both in S2 cells and in developing tissues containing SJ. Our findings are consistent with the idea that Cold, predicted to be GPI anchored by the bigPI software [34], is associated not only with the endoplasmic reticulum and internal vesicles but also with the plasma membrane. In the salivary glands, we observed that a small amount of FLAG-Cold was stably associated with SJ containing regions, as if making part of a complex localising to this membrane compartment. Interestingly, a similar accumulation has been observed with a HA-Bou tagged version in the same tissue

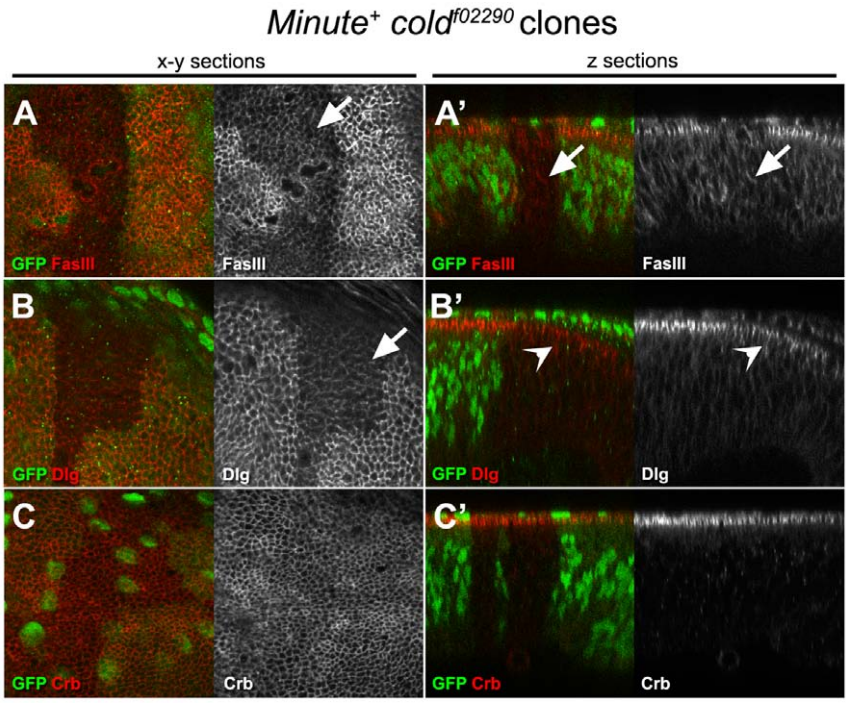

Figure 7. The *cold* gene is autonomously required in the wing disc for SJ organisation. (A–C′) Confocal pictures of third larval instar wing imaginal discs containing M^+ $cold^{f02290}$ clones generated in first instar larvae and stained as indicated. The homozygous *cold* mutant cells lacking GFP (green) can be distinguished from the surrounding GFP positive heterozygous tissue. A–C correspond to optical planar x-y sections taken at the level of the SJ and A′–C′ show optical z-sections of the same discs. The planar views revealed abnormally low levels of FasIII and Dlg (red in left panels, greyscale in right panels) in the apical part of the *cold* mutant cells (arrows). The z planes show that Dlg was found accumulating in a wider apical domain (open arrowhead), whereas FasIII was distributed uniformly along the lateral part of the cells (arrow). A normal Crb apical distribution (red in left panel, greyscale in the right panel) was observed in *cold* mutant cells.
doi:10.1371/journal.pone.0017763.g007

[7]. It is thus possible that a small subset of both proteins could contribute to the organisation of the SJ by interacting with each other and/or with other SJ components. However, it is premature to conclude that Cold function is circumscribed to the lateral membrane region containing the SJ, as other valid alternatives exist. In fact, the FLAG-Cold protein was also seen in other subcellular compartments, and it is difficult to ascertain where it exerts its primary activity. For instance, it has been shown that the SJ component NrxIV is re-localised to internal vesicles in *cold* mutant embryos [10]. Although this phenotype is also observed in embryos lacking known SJ components such as Coracle and Nrv2 [10], the Ly6 proteins could indeed play a role in the vesicular trafficking of these proteins or in their preassembly into larger macro-complexes en route to the membrane. As the intracellular traffic seems to play a key role in the early assembly of the SJ [35], it will be interesting to compare the paths followed by Ly6 proteins and known SJ components during the formation of these structures. Concerning the traffic of the Ly6 proteins themselves, it is clear that Cold differs from Bou in two related aspects: it behaves in a cell autonomous way and we have not found any evidence indicating that this protein could travel from cell to cell. Thus, although these proteins may meet at the level of the SJ or in other subcellular compartments, these observations implicate that they do not always traffic together. It will be interesting to analyse whether this differential behaviour provides a rationale for their non-exchangeable roles.

A conserved role for Cold in the nervous system?

Our results show that *cold* is expressed in a subset of glial cells and is required for organisation of the blood-brain-barrier in the *Drosophila* nervous system. Thus, it is possible that vertebrate members of the Ly6 superfamily could fulfil an analogous role in the formation of the paranodal junctions existing in the contact areas between axons and Schwann cells [14]. The high variability observed in the Ly6 domains primary sequence precludes identification of vertebrate orthologues corresponding to the *Drosophila* genes. However, the genetic networks in which these proteins are implicated could be better conserved, as insect SJ and paranodal junctions share a significant number of components [36]. Thus, future functional studies in *Drosophila* and vertebrates may reveal analogous roles for apparently unrelated Ly6 proteins, as it is the case for the four *Drosophila* Ly6 members participating in SJ assembly.

Materials and Methods

Sequence analysis

We used the TBLASTN program to search for *cold* orthologues in insect genome databases using the BLAST search program [37] and the ClustalW program to create the alignments. The species considered are Dmel, *Drosophila melanogaster*; Dpse, *Drosophila pseudobscura*; Aaeg, *Aedes aegypty*; Agam, *Anopheles gambiae* (Diptera). Bmor; *Bombyx mori* (Lepidoptera). Tcas, *Tribolium castaneum* (Coleoptera). Amel, *Apis mellifera*; Nvit, *Nasonia vitripennis* (Hymenoptera). Apis, *Acyrthosiphon pisum*, (Hemiptera).

Genetics

Full definitions of the stocks used can be found in Flybase [38], and include the *cold* alleles $P(WH)Bac^{f05607}$ and $P(WH)Bac^{f02290}$ and the strains $P(GawB)bou^{PG27}$, bou^{6ea-2}, $P(PTT\text{-}GA)Nrg^{G00305}$,

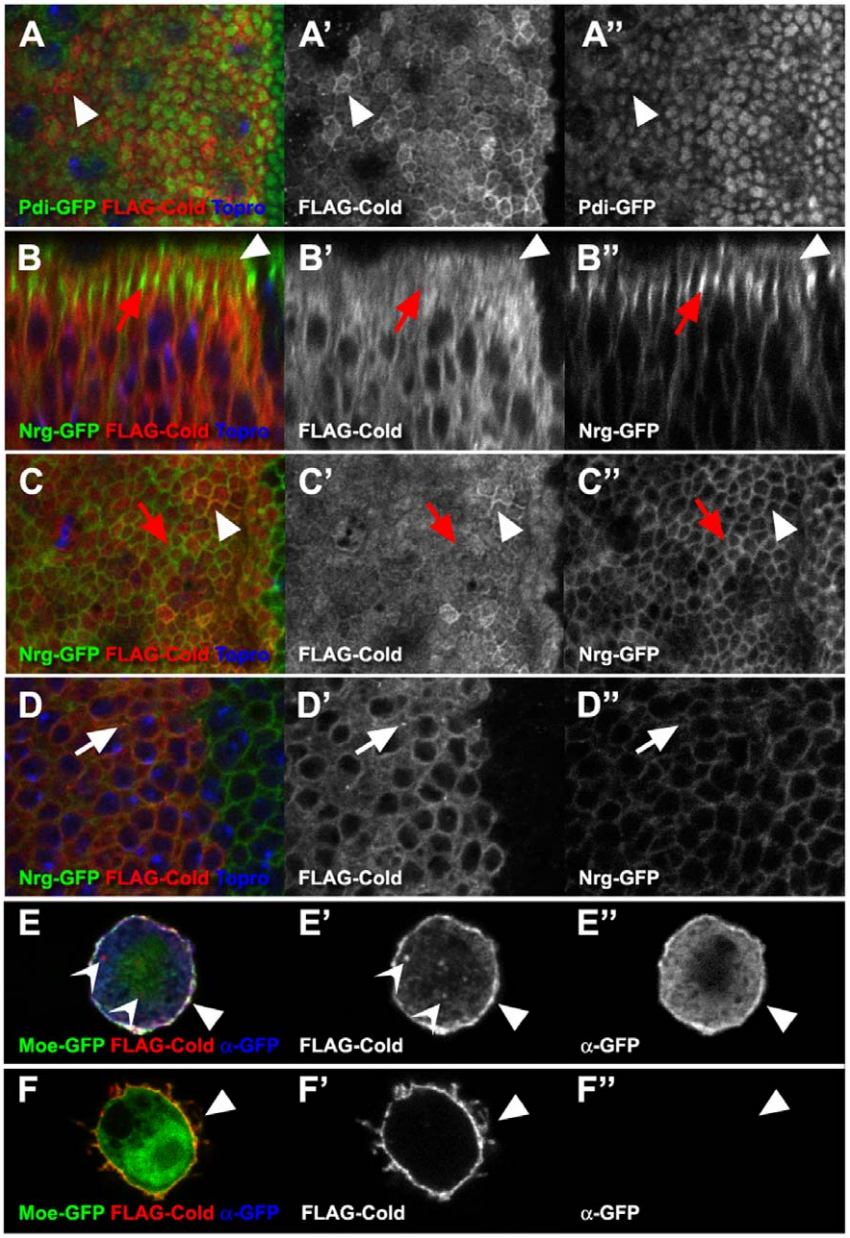

Figure 8. FLAG-Cold subcellular localisation in the wing epithelium and in S2 cells. (A–D″) Confocal images of third larval instar wing discs expressing the FLAG-Cold protein (shown in red in left panels, greyscale in the mid panels) in the *apGAL4* domain. The A–A″ and C–C″ show x-y planar views of the apical part of the epithelium, whereas D–D″ shows a more basal region and B–B″ a z-section. Accumulation of FLAG-Cold was seen in a cell apical region containing weak levels of Nrg-GFP (B–B″ and C–C″ white arrowheads) and no Pdi-GFP (A–A″, white arrowheads). Regions containing high levels of Nrg-GFP and corresponding to the SJ did not show FLAG-Cold accumulation (B–B″ and C–C″, red arrows). FLAG-Cold was also seen in internal vesicles (D–D″, white arrows). E–F″ Confocal images of S2 cells expressing FLAG-Cold (red in left panel, greyscale in the mid panel) and Moesin-GFP (GFP fluorescence shown in green, left panel). In permeabilised cells (E–E″), both FLAG-Cold and Moesin-GFP were detected by antibodies in the cell interior and at the membrane (anti-GFP shown in blue, left panel and in greyscale, right panel). In these conditions, we also observed internal vesicles accumulating FLAG-Cold (open arrowheads). In non permeabilised cells (F–F″), FLAG-Cold was detected at the membrane (arrowhead), whereas the cell interior was not accessible to the antibodies.
doi:10.1371/journal.pone.0017763.g008

P(PTT-un1)Pdi$^{74\text{-}1}$, *P(GawB)ap*^{md544}, *btlGAL4 UASActinGFP*, *P(en2.4-GAL4)*^{e16E} *UASGFP* and *tubGAL80*^{ts}. *The PBac(FSVS-1)*^{1001277} insertion was generated by the CPTI (http://www.flyprot.org/). The *FM7c-ActinLacZ*, *CyO-wglacZ* and *CyO KrGA-L4UASGFP* balancers were used for embryo genotyping. All experiments were carried out at 25°C, except the temperature shifts at 18°C, which where done 40 hours before dissection in cultures containing third larval instars of the *bou*^{PG27}*/+; UASFLAG-Coiled/+; tubGAL80*^{ts}*/+* genotype. The *cold* embryos lacking both the maternal and the zygotic contributions were recovered in the progeny of *hsFLP/+; P(ovoD1-18)*^{2La} *P(ovoD1-18)*^{2Lb} *FRT40A/cold*^{f05607} *FRT40A* females heat shocked two times for 1 hour at 37°C during larval stages and mated to *cold*^{f05607}*/CyO-wglacZ* males. The somatic *Minute*$^{+}$ *cold* clones were induced 48 hours after egg laying by 1 hour heat shock at 37°C in *w hsFLP*; *cold*^{f02290} *FRT40/M(2)24F*^{1} *ubiGFP FRT40A* larvae.

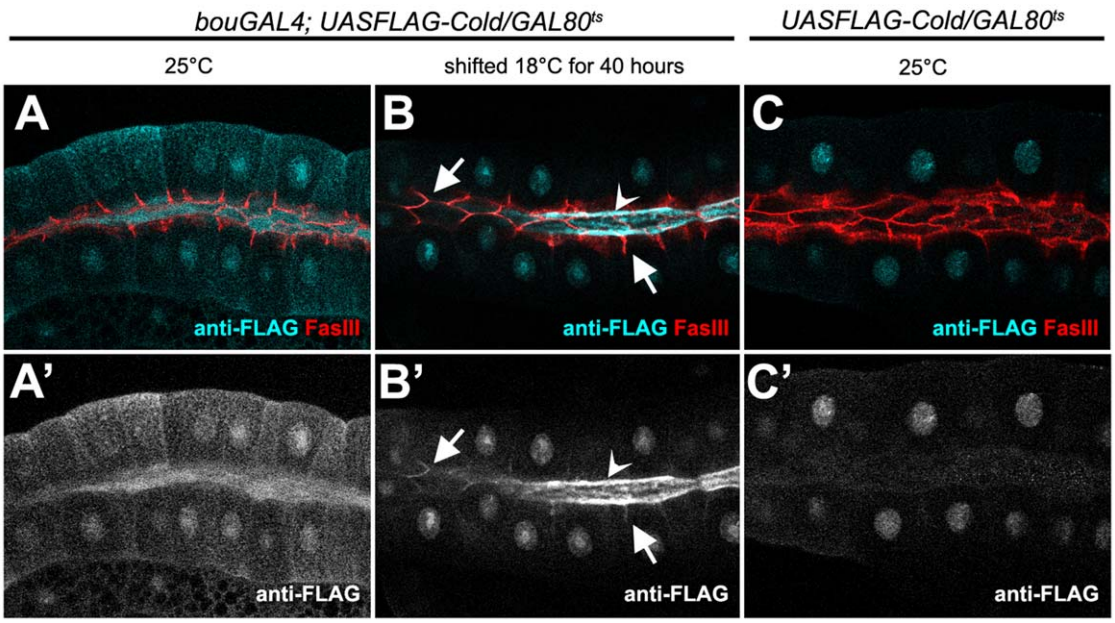

Figure 9. FLAG-Cold is stably associated with the SJ in larval salivary glands. (A–C′) Confocal single sections of salivary glands belonging to third instar larvae carrying the *UASFLAG-cold* construct and the *tubulinGAL80^{ts}* repressor. (A,A′) At 25°C and in presence of the *bouGAL4* driver, FLAG-Cold (cyan in upper panels, greyscale in lower panels) was present at high levels in the salivary gland lumen and the cell body. (B,B′) After 40 hours at 18°C the cell body staining disappeared but FLAG-Cold persisted in the salivary gland lumen (open arrowhead) and in lateral cell regions (arrows) accumulating FasIII (seen in red). A nuclear staining was also observed but it corresponds to a unspecific interaction of the FLAG antibody, as it was also present in larvae not carrying the *bouGAL4* driver (C,C′).
doi:10.1371/journal.pone.0017763.g009

Dye injection

Dye diffusion into trachea and chordotonal organs was analysed by injecting 10 mg/ml 10 kDa rhodamine-Dextran (Molecular Probes) with a micromanipulator into the body cavity of dechorionated stage 16 live embryos [17]. Diffusion into the nerve cord was studied in 22 hours old embryos. Samples were visualised within 20–30 minutes after injection with a Leica SP2 confocal microscope.

Molecular biology

The FLAG tag coding sequence DYKDDDDK, flanked in each side by one A residue was introduced in frame by PCR within the Coiled coding region after the E25 residue, using specifically designed oligonucleotides and the LD16147 (DRGC) *coiled* cDNA as template. The construct was then sequenced and subcloned into pUAST [39] for generation of transgenic flies or pAc5.1 (Invitrogen), for cell transfections.

Cell culture

Cell culture, transfections and antibody stainings were carried out as in Koh et al. [32]. S2 cells co-transfected with pAcDMoe-GFP (kind gift from F. Payre, CBD, Toulouse, France) and pAcFLAG-Cold were fixed and stained in either permeabilising (PBS, 0.1% Triton-X100) or non-permeabilising (PBS) conditions.

Immunohistochemistry

In situ hybridisation with clone LD16147 sense and antisense riboprobes were performed according to [40]. Embryos and larval tissues were fixed for 20–30 minutes in PBS 4% paraformaldehyde. Blocking, washings and over night incubation with primary and secondary antibodies was carried out in 0.1% Triton-X100 0.1% BSA. Primary antibodies included mouse anti-ßGal 1/100 (Promega), rabbit anti-ßGal 1/1000 (Cappel), mouse anti-FLAG 1/200 (Covance), rabbit anti-FLAG 1/100 (Sigma) rabbit anti-GFP 1/500 (Torrey), anti-NrxIV 1/100 (kind gift of H. Bellen), rat anti-Crb 1/500 (kind gift of U. Tepass), and monoclonals anti-2A12 1/10, 4F3 anti-Dlg 1/100, DCAD2 anti-DECad 1/20, BP104 anti-Nrg 1/100, 7G10 anti-FasIII 1/30, all from DSHB. Secondary FITC and TRITC conjugated antibodies and streptavidin were diluted 1/200 (Molecular Probes). We also used CBP-FITC 1/100 (NEB). Samples were mounted in Vectashield (Vector) and visualised with a LeicaSP2 confocal microscope.

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Author Contributions

Conceived and designed the experiments: AH MH LW FR. Performed the experiments: AH MH LW FR. Analyzed the data: AH MH LW FR. Wrote the paper: FR.

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III. SUPPLEMENTARY

UNPUBLISHED RESULTS

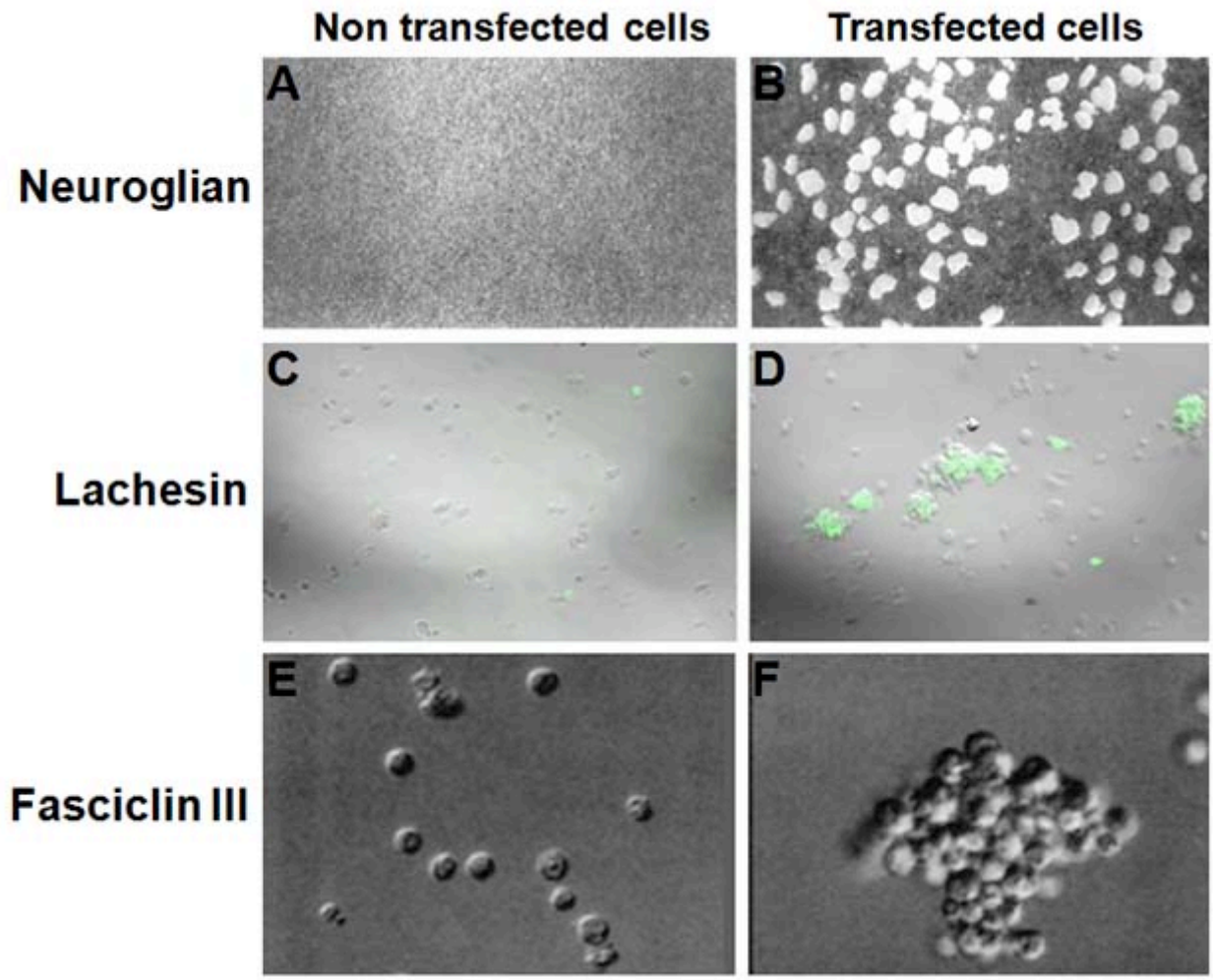

Fig 1. The Septate junction components Neuroglian, Lachesin, and Fasciclin III behave as homophilic adhesion molecules in S2 aggregation assays. Whereas *Drosophila* S2 cells do not form aggregates in control experiments **(A, C, E)**, cells expressing Neuroglian, Lachesin, and Fasciclin III form cell aggregates **(B, D, F)**. **Ref Fig (Hortsch et al., 1995; Strigini et al., 2006 & Snow et al., 1989).**

I. Could *Drosophila* Ly6 proteins Boudin and Coiled mediate cell adhesion?

Several known septate junction components, like Neuroglian (Hortsch et al., 1995), Lachesin (Llimargas et al., 2004; Strigini et al., 2006) and Fasciclin III (Snow et al., 1989), are thought to behave as "sticky" adhesion molecules and are suggested to be required on both cellular sides of the junctional complex to maintain contacts between contiguous cells.

Given that both Boudin (Bou) and Coiled (Cold) are predicted to be membrane proteins, they could also act as components of the SJ complex and mediate cell adhesion.

One commonly used way to disclose the roles of membrane proteins in cell adhesion is to perform aggregation assays with transfected Schneider-2 (S2) cells, a *Drosophila* cell line that normally does not form aggregates in culture. In fact, using this approach it has been found that S2 cells expressing Neuroglian, Lachesin and Fasciclin III aggregate to each other, suggesting that these molecules mediate homophilic adhesive interactions (Fig 1).Thus, we decided to investigate whether over-expression of Bou and Cold could enhance the adhesive properties of S2 cells in aggregation assays.

As expected, S2 cells transfected with a control pAcGFP plasmid appeared as isolated cells (Fig 2, A), whereas GFP positive cells co-expressing the homophilic adhesion molecule Neuroglian (Nrg) formed big clumps of aggregated cells (Fig 2, B). We also observed that GFP positive cells expressing high levels of either HA-Bou or FLAG-Cold behaved as wild type cells and did not aggregate (Fig 2, C, D) (transfection of 0.2 µg to 1.5 µg of either pAcHA-Bou or pAc Flag-Cold in $3x10^6$ cells). We could confirm by antibody staining that both of these tagged-protein are present in the membrane of these transfected S2 cells (Hijazi et al., 2009; Hijazi et al., 2011). Thus, the presence in the membrane of these proteins does not alter the adhesive capacities of these cells.

We also examined the possibility that Bou and Cold could enhance cell adhesion by interacting with each other. For this, we monitored cell aggregation after incubating cells expressing HA-Bou together with cells expressing FLAG-Cold (Trans-interaction test) and, in a separate experiment, cells co-expressing both proteins (Cis-interaction test). In both cases, we did not observe formation of cell aggregates, indicating that expression of these two molecules is not sufficient to trigger S2 cell adhesion (Fig 3).

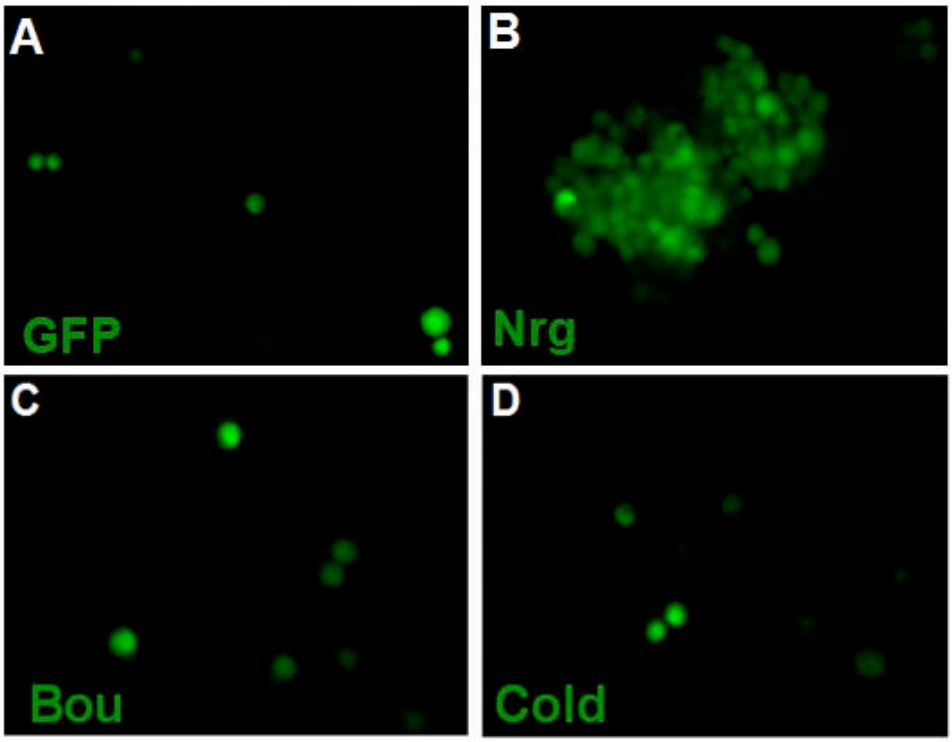

Fig 2. Overexpression of Bou or Cold does not elicit aggregation in S2 cells. (A) *Drosophila* S2 cells transfected with pAcGFP do not form aggregates after 2 hours of incubation. **(B)** Cotransfection of pAcGFP with pAcNrg elicits the formation of large clumps of aggregated cells. Cells transfected with pAcHA-Bou **(C)** or pAcFLAG-Cold **(D)** behave as normal cells and appear isolated.

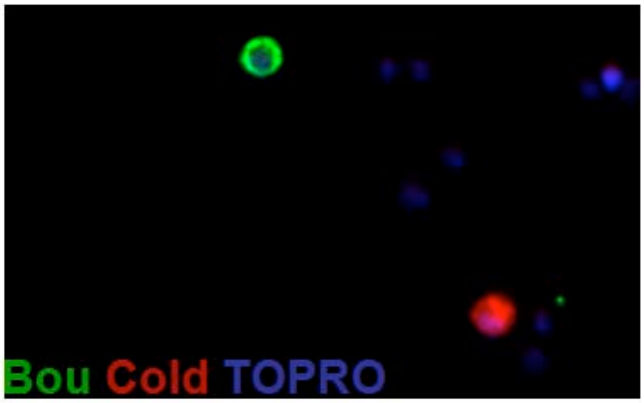

Fig 3. Overexpression of Bou and Cold does not elicit aggregation in S2 cells. Separate cultures of S2 cells were transfected with pAcHA-Bou and pAcFLAG-Cold, mixed together and incubated for two hours. Immunostaining with anti-HA and anti-Flag antibodies reveals that cells expressing Bou and Cold do not aggregate together.

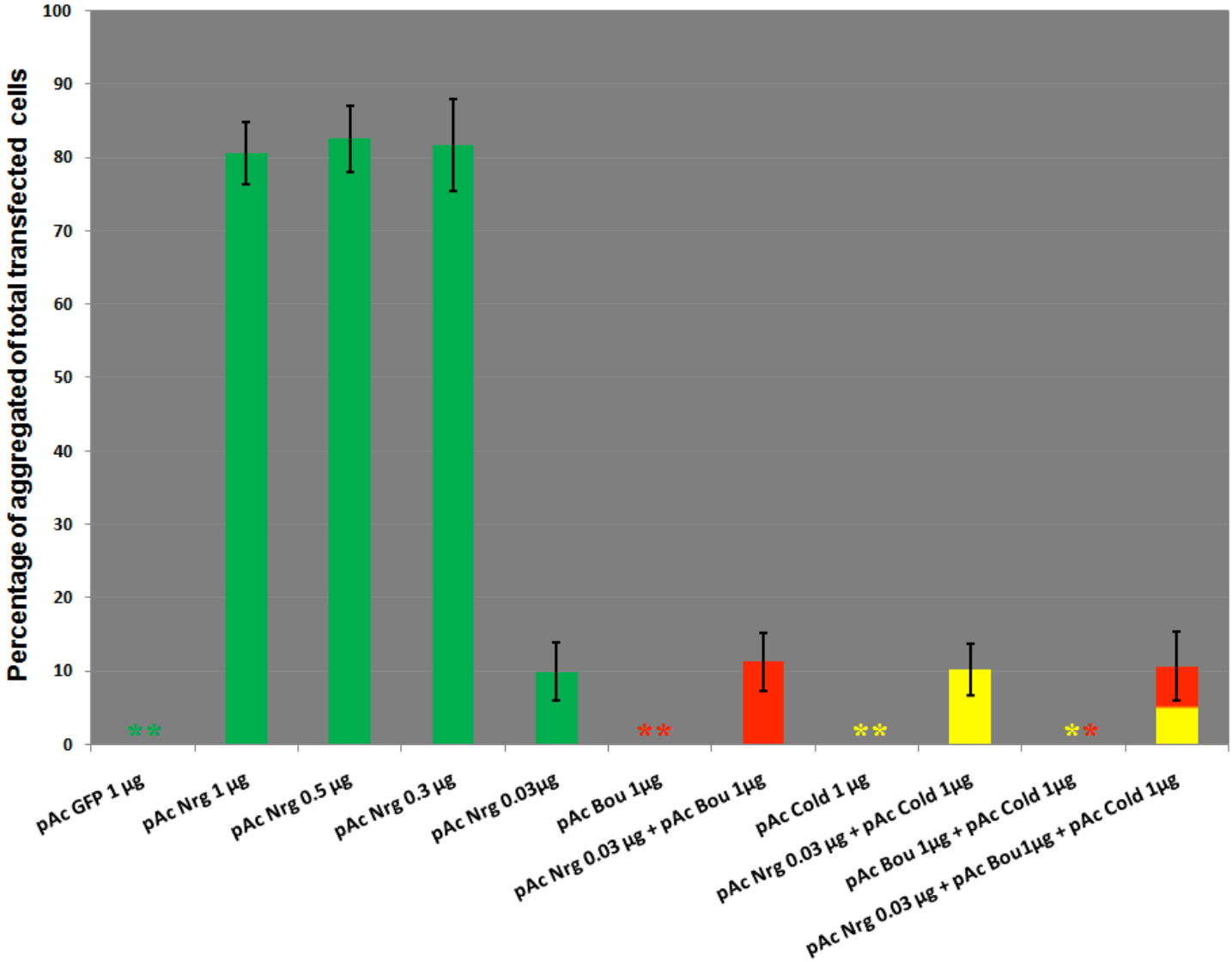

Fig 4. Expression of HA-Bou and FLAG-Cold does not enhance Nrg mediated aggregation. The graph shows the percentage of aggregated S2 cells observed after transfection with different amounts of Nrg (green bars) or in co-transfection experiments with HABou (red), FLAG-Cold (yellow) or both (red + yellow). The amounts of plasmids transfected in $3x10^6$ cells are indicated. The values correspond to the mean obtained in three independent experiments constituted each by three replicas. Error bars reflect the standard deviation. Whereas high levels of Nrg result in aggregation of 80% of the cells, a dose of 0.03 µg results in a 10% of cell aggregation. In presence of this dose of Nrg, the overexpression of HA-Bou, FLAG-Cold or both does not modify significantly the aggregation rate. pAc GFP is used as a negative control (aggregation = 0%; green asterisks) and is co-transfected with all samples to visualize transfected cells. The overexpression of HA-Bou (red asterisks), Flag-Cold (yellow asterisks) or both (red and yellow asterisks) do not elicit cell aggregation.

II. Could *bou* and *cold* act cooperatively with the adhesion molecule Neuroglian?

The results described above indicate that over-expression of HA-Bou and FLAG-Cold is not sufficient to mediate cell adhesion. However, one possibility is that these molecules could act as cofactors of other adhesion molecules, like Nrg. In our assays, expression of high levels of Nrg triggers the aggregation of 80% of the cells (transfection of 1µg of pAcNrg in $3x10^6$ cells) (Fig 4). In contrast, the expression of lower amounts of Nrg (0.03 µg of pAcNrg in $3x10^6$ cells) consistently resulted in the aggregation of ± 10% of the cells in three independent experiments, each containing three different replicas (Fig 4). We thus tested whether the percentage of aggregation observed when Nrg quantity becomes a limiting factor could be increased by co-expressing HA-Bou, FLAG-Cold or both in the same cells. For this, we co-transfected 1 µg of pAcHA-Bou and/or pAcFLAG-Cold with 0.03 µ g of pAcNrg. However, none of these combinations appeared to modify the aggregation levels induced by Nrg alone (Fig 4). Similar results were obtained using a range of HA-Bou and/or FLAG-Cold plasmid varying from 0.1 to 1.5µg (data not shown). Therefore, it seems that in these assays, neither HA-Bou nor FLAG-Cold are limiting for Nrg-mediated cell adhesion.

Materials and Methods

Cell aggregation experiments

Confluent S2 cells grown in a Schneider medium (Gibco) supplemented with FBS (Foetal Bovine Serum) 10%, were adjusted to a concentration of 10^6cells/ml and transfected in 6-well culture plates with pAc GFP, pAc Neuroglian, pAc HA-Bou, and/or pAc Flag-Cold, following the protocol of (Koh et al., 2008). As described by (Hortsch et al., 1995), 48 hours after transfection, cells were placed on a rotating platform and rocked at 110rpm for 2 hours at room temperature, then fixed with PAF 4% in PBS for 30 min, transferred to eppendorfs tubes and centrifugated at 800 rpm for 3 min. Pellets were washed twice with PBS 3% FBS and centrifugated as above. Then, cells were gently resuspended in Vectashield, mounted and imaged with a confocal Zeiss Inverted microscope. Since cell clusters of five cells were occasionally observed in both untransfected and in pAc GFP mock-transfected S2 cells, only cells belonging to aggregates bigger than five cells were considered. Eight fields were counted per transfected condition. For each field, the ratio of aggregated cells versus the total number of cells was quantified using Image J. The final values represent the means obtained from 3 separated experiments performed in triplicates.

Immunohistochemistry on S2 cells

HA-Bou and FLAG-Cold vectors were transfected in separate S2 cell cultures, as described above and mixed together 48 hours after transfection. After 2 hours of incubation, S2 cells were fixed for 30 min, washed in PBS 1X, permeabilized with Schneider 10% FBS, 0.1 % Triton and incubated over night at 4°C with the primary antibodies mouse anti-HA (Covance) 1:100 and the rabbit anti-Flag (Clontech) 1:100. After washing twice with Schneider 10% FBS 0.1 % Triton, cells were incubated for 45 minutes at room temperature with secondary FITC and TRITC conjugated antibodies 1:400 (Molecular probes), mounted and imaged on a Zeiss Inverted microscope.

IV. DISCUSSION

&

PERSPECTIVES

a) Discussion

The discovery of the Ly6 family of proteins in *Drosophila melanogaster* offers the possibility to use this attractive model system to carry out basic research and better understand the multiple roles of these proteins during development. My PhD work has contributed to a functional characterization of two newly described members of this family, *boudin* (*bou*) and *coiled* (*cold*). Our study has shown that both genes are expressed in epithelial tissues following a very similar temporal profile. Consistently, we have shown that they are both required for a common process during the development of epithelial tissues: the proper organization of the septate junction adhesion structures.

I. The role of *bou* and *cold* in epithelial septate junction organization

In the *Drosophila* epithelia, whose cells are characterized by a marked apico-basal polarization, cell contacts are mainly mediated by an apically located adhesion belt called Zonula Adherens (ZA), a structure that forms early in development during the cellular blastoderm stage (stage 5). In contrast, the pleated SJ junctions, which also mediate cell adhesion, appear just below the ZA at stage when cell polarity is already established, midway through embryogenesis (stage 14) (Tepass and Hartenstein, 1994; Tepass, 1997).

Our analysis of *bou* and *cold* mutants indicates that these two Ly6 genes are specifically required for SJ organization in both embryonic and larval epithelial tissues. We propose that the phenotypes in SJ organization and barrier maintenance observed in the mutants reflect the direct contribution of *bou* and *cold* to the maintenance of this structure and are not an indirect consequence of defects in other processes such as ZA formation or cell polarity establishment. Supporting this hypothesis, our observations show that in *bou* and *cold* mutant epithelial cells the sub-apical cell marker Crumbs and the ZA marker E-Cadherin are properly localized, suggesting that cell polarity and ZA integrity are preserved in these mutants.

We also studied the distribution of the SJ marker Dlg in *bou* and *cold* mutant embryos. The Dlg protein has been described as a tumor suppressor gene, and is known to regulate apicobasal cell polarity in most epithelial cells (Woods et al., 1994; Woods et al., 1996). In *bou* and *cold* mutants, Dlg localization is affected and a fraction of this protein is seen homogenously distributed along the lateral membrane of the mutant cells, in a similar way to

other SJ components such as Nrg, NrxIV and FasIII. However, and differing from these SJ markers, the apical localization of Dlg is not completely lost, and a portion of this protein is still seen accumulated in an apical domain.

Despite the observed Dlg mislocalization, we failed to detect cell polarity defects in both *bou* and *cold* mutant embryos and also in double mutant combinations for these genes. In addition, and although Dlg localization is similarly affected in *cold* mutant cells belonging to imaginal wing disc mosaics, the *cold* mutant patches do not develop the typical overgrowths observed in Dlg loss of function (Woods and Bryant, 1991; Woods and Bryant, 1994). Thus, this ensemble of observations is coherent with the idea that the role of *bou* and *cold* is circumscribed to SJ assembly and that these genes do not participate in epithelial cell polarity establishment. The particular repartition of Dlg protein observed in the cell membrane of *bou* and *cold* mutant embryos could then reflect the duality of the Dlg mode of action in both SJ organization and apico-basal cell polarity. One pool of Dlg could depend for its proper localization on the integrity of SJ, whereas a second pool could still carry its normal function during cell polarity maintenance. Interestingly, detailed genetic analysis of different SJ components, such as *coracle, NrxIV, yurt* and the *Na+/K+ ATPase* has recently shown that they also play a dual role, acting first during apico-basal polarity establishment and subsequently in SJ formation (Laprise et al., 2009). It seems that Yurt participation during the establishment of cell polarity is contributed by the maternal genome, as this early requirement is not evident in *yurt* zygotic mutants. As our characterization of *bou* and *cold* is based in the analysis of zygotic mutants, we wondered if the maternal contribution of *cold* could mask a requirement for this gene during early establishment of the cell polarity, as it has been shown for *yurt* (Laprise et al., 2009). We found that embryos lacking both the maternal (M) and the zygotic (Z) contribution of *cold* did not display phenotypes additional to those observed in *cold* zygotic mutants, neither at the SJ level nor in epithelial morphology. Thus, these observations confirm that the *cold* product only obvious function is to participate in SJ assembly. However, we still have to test if embryos lacking both the zygotic and maternal contribution of *bou* behave similarly. Furthermore, the *bou* and *cold* double zygotic mutants present the same SJ defects observed in the separate single mutants and no additional obvious phenotypes, suggesting that these genes do not exert redundant functions in other processes and could act in the same genetic pathway during SJ organization.

Do the two Ly6 proteins *bou* and *cold* participate in SJ maintenance or are they required for their early assembly? At stages 15 and 16, when pleated SJ are well established, we observe that many SJ markers such as Nrg, FasIII, NrxIV, Contactin and Coracle are mislocalised along the lateral cell membrane of *bou* and *cold* mutants (Hijazi et al., 2009, Hijazi et al., 2011 and unpublished observations). Interestingly, we observed that *bou* and *cold* are also required by stage 14, when pleated SJ begin to form (Tepass and Hartenstein, 1994), because a clear mislocalisation of the NrxIV marker is already observed at this stage. Thus, it seems that these genes are required for the early establishment of SJ. However, little is known about how SJs are assembled and it is difficult to predict how the two Ly6 proteins could contribute to this process. This issue has just begun to be explored in a study focusing on the early trafficking of a SJ component, the GPI-anchored MTf membrane protein. It has been shown that at stage 13, before the establishment of SJ structures (Tiklová et al., 2010), MTf is present at the cell membrane but is not distributed in a polarized manner, being uniformly found all along the lateral part of the cells. It is only at a later time (stage 15-16) that the MTf distribution is restricted to a more apical localization and a proper accumulation on the SJ domain is detected (Tiklová et al., 2010). This transition seems to depend on MTf intracellular trafficking. Indeed, this protein is also found accumulated at stage 13 in cytoplasmic puncta that correspond to early and recycling endosomes and it has been shown that trafficking through these intracellular compartments is required for MTf redistribution and clustering in the apical part of the cell lateral membrane. Moreover, other components like Coracle, Gliotactin and the Claudin Sinuous could similarly become redistributed during septate junction maturation, since they have been transiently detected in intracellular puncta at stage 13, but not at stage16, when they are seen principally at the apical part of the membrane (Tiklová et al., 2010). These observations suggest that the trafficking of SJ components could be essential for the assembly of mature SJ complexes. However, how SJ components transport and targeting to specific cell membrane domains is controlled needs to be studied in more detail.

In our work, we have studied the localization of different SJ components, such as Nrg, FasIII, NrxIV, Cont and Cor during late embryonic stages in wild type and in both *bou* and *cold* mutant backgrounds. In the mutants, SJ integrity is affected and these SJ components appear mislocalized along the lateral cell membrane. This phenotype could result from a defect in the clustering of these components, which, in absence of the two Ly6 proteins, are now free to diffuse along the lateral membrane. In fact, a series of elegant *in vivo* studies have shown that

in embryos mutant for one of the three cell adhesion molecules (CAMs) NrxIV, Nrg or Cont, the mobility of the two other SJ components is increased in the lateral cell membrane (Laval et al., 2008). Thus, the observed phenotypes in *bou* and *cold* mutants are consistent with the idea that the products of these genes, predicted to be GPI-anchored membrane proteins, could behave as SJ components and participate in the lateral clustering of the SJ junctional complex.

However, a recent study has shown in the epidermis of stage 16 *cold* mutant live embryos that the SJ component NrxIV is not only mislocalised along the membrane but is also detected in cytoplasmic puncta, suggesting that a fraction of this protein is remobilized into internal vesicles in this mutant background. Interestingly, a similar phenotype has been detected in mutants lacking other SJ components, such as the Coracle adaptor and the Nrv2 β-subunit of the Na^+/K^+ ATPase pump (Nilton et al., 2010), but not in a mutant for Gliotactin, a cell membrane protein. These results suggest that SJ components can traffic intracellularly during late stages when particular SJ components are missing, indicating that vesicle traffic participates to the abnormal distribution of SJ components seen in *cold* and *coracle* mutants. However, it is difficult to establish at this stage whether these phenotypes are a consequence of a direct role for Cold, Nrv2 and Cor in cell trafficking rather than an indirect effect of defects in the SJ apical clustering, and it is also possible that these proteins actually have a role in both processes.

Clarifying this issue will certainly require more live imaging studies, as our NrxIV localization analysis carried out on fixed *cold* mutant embryos of the same stages failed to detect a significant remobilization of this protein to intracellular vesicles. In fact, it is possible that the use of detergents in common immunohistochemistry protocols affects the integrity of internal membrane compartments and masks the potential accumulation of some SJ components in cytoplasmic puncta, thus preventing their visualization. In addition, we do not know if SJ components cycle during SJ maintenance, and, although the SJ complex seems a stable structure in late stages (Laval et al., 2008), cellular traffic could have a more important role than previously thought. Future analysis of the dynamic localization of SJ proteins in *cold* and *bou* mutant live embryos during development will allow to better understand the cellular role of these genes during SJ biogenesis.

II. Are Bou and Cold new septate junction components?

One possibility is that both Bou and Cold, putative membrane proteins, could be SJ components and participate directly in their assembly or maintenance. All septate junction components described in so far appear accumulated in the apico-lateral part of epithelial cells. In order to investigate whether Bou and Cold accumulate at the SJ level, we tried to characterise their subcellular localization and their respective distributions at the cell membrane. Unfortunately, immunization of rabbits with synthetic peptides corresponding to these proteins did not allow the production of antibodies able to detect the endogenous products of the *bou* and *cold* genes.

To circumvent this limitation, we generated transgenic flies carrying constructs allowing the expression of HA-Bou and FLAG-Cold tagged forms under the control of an inducible UAS promoter. The overexpression of HA-Bou with different drivers, such as a *bouGAL4*, *engrailedGAL4* and *patchedGAL4* rescue the embryonic lethality of *bou* mutants, giving rise to viable adult flies. Moreover, the distribution of SJ markers such as FasIII, in *bou* mutant embryos rescued by HA-Bou expression appears normal, suggesting that the HA-Bou protein has a wild type activity. We also obtained adult *cold* mutant flies by expressing a FLAG-Cold fusion under the control of the *patchedGAL4* driver, and again, expression of this protein restores FasIII normal distribution in the embryonic epidermal tissues of *cold* mutants. Therefore, our strategy of inserting a tag between the putative signal peptide and the TFD domain of Bou and Cold Ly6 precursors seems to permit the production of functional proteins.

We have firstly studied the subcellular localization of these tagged forms in transfected *Drosophila* S2 cells. Analysis of their distribution, in permeabilizing or non permeabilizing conditions indicated that a fraction of HA-Bou and FLAG-Cold is found at the outer leaflet of the plasma membrane, as expected for putative GPI-anchored proteins.

Moreover, we have shown that in *Drosophila* S2 cells at least a fraction of the HA-Bou protein is anchored to the cell membrane by a GPI moiety, which can be released from the plasma membrane by the specific enzymatic activity of PLPC. However, the observation of transfected S2 cells also revealed that a substantial amount of both HA-Bou and FLAG-Cold is found inside the cell, where it could be associated to internal membranes and trafficking vesicles.

Secondly, we examined the localization of these tagged proteins *in vivo* during development focusing on different epithelial tissues. Immunostainings done on the imaginal wing disc have shown that, as in S2 cells, an important fraction of the HA-Bou and FLAG-Cold is found in the interior of the cells. We observed in the same tissue that both proteins are distributed in a homogenous fashion along the cell body, but we detected a slight accumulation only in the most apical part of the disc epithelial cells, above the SJ. In fact, comparison of the localizations of our tagged proteins with the SJ marker Nrg GFP revealed that a fraction of HA-Bou and FLAG-Cold is found at the membrane, including septate junction areas, but we did not detect any obvious accumulation at this level. This result is intriguing, because all proteins required for SJ organization are mostly found in SJ areas. One possibility is that HA-Bou and FLAG-Cold could act in other domains of the membrane, for instance restricting the movement of the SJ components and therefore contributing to their clustering in a specific domain.

Alternatively, sustained production of these Ly6 proteins in our experiments could saturate the cells and mask potential accumulation sites that are relevant to understand their cellular function. This might well be the case as by switching-off HA-Bou or FLAG-Cold expression in the larval salivary gland cells, we could show that the remnant of both proteins seemed stabilized at the level of the SJ domains, where they clearly co-localize with SJ components like Nrg and Fas III. This observation raises the possibility that small amounts of HA-Bou and FLAG-Cold proteins, acting at the level of the SJs could be sufficient to maintain their normal organization.

Actually, other explanations are consistent with the available observations. Notably, in the wing and in the trachea of stage 16 embryos, we detect accumulation of both HA-Bou and FLAG-Cold in internal vesicles, and it is tempting to speculate that their primary activity is to regulate the intracellular trafficking of SJ components. However, this localization may simply reflect the fact that these proteins have to reach the membrane following a secretory pathway, and their forced expression may also result in abnormal accumulation in some intercellular compartments.

Development of efficient antisera and the use of immuno-electron microscopy will probably be required to elucidate if Bou and Cold are localized at SJ level and participate in the formation of intercellular septae. In addition, identification of their molecular partners would provide important hints to unveil their particular mode of function during SJ assembly.

III. Are Bou and Cold cell adhesion molecules?

Some SJ components act in certain physiological contexts as adhesive molecules without necessarily forming septate junctions. For example, NrxIV controls the adhesive properties of cardial and pericardial cells in the embryonic heart of Drosophila (Yi et al., 2008). In the central nervous system, this molecule also mediates interactions with Wrapper (a protein of the midline glia), permitting the ensheathment of commissural axons (Stork et al., 2009; Banerjee et al., 2010). The adhesive properties of NrxIV have been also shown in S2 cells, where this protein equally mediates cell adhesion with Wrapper (Wheeler et al., 2009). So, SJ membrane proteins can mediate cell adhesion both in vivo and in S2 cells. We have expressed Bou and Cold to high levels in S2 cells, to test their ability to elicit cell adhesion. Our results suggest that a putative homophilic or heterophilic interaction between Bou and Cold is not sufficient to elicit cell adhesion in this system. In addition, overexpression of these proteins is not capable to enhance cell aggregation mediated by the adhesion molecule Nrg. Thus, whereas these results argue against the idea that Bou and Cold directly mediate cell adhesion, they do not allow to rule out the possibility that they act as cofactors of other adhesion proteins.

In fact, we do not know to what extent S2 cells aggregation allows reproducing the complex interactions that take place in vivo in cell adhesion junctions. For instance, overexpression of Nrg efficiently triggers cell aggregation in S2 cells, but it is thought that at the level of the SJ, this membrane protein mainly interacts in cis with the NrxIV and the Cont proteins of the opposite cell to form a tripartite complex. Interestingly, both Nrx IV (Wheeler et al., 2009) and Cont (Faivre-Sarrailh et al., 2004) seem to be expressed by S2 cells, suggesting that in this case S2 cells do reproduce the in vivo situation. Data from transcriptome analyses suggest that Bou and Cold are also expressed by S2 cells and thus it would be interesting to knock down their expression by RNAi to test whether their presence is required for Nrg mediated aggregation.

In addition, we cannot exclude the possibility that an adhesive role of Bou and Cold could become evident in presence of other molecules that are not expressed in S2 cells at sufficient levels. For instance, Bou and Cold could enhance the aggregation activity of other septate junction adhesion molecules such as Fasciclin III and Lachesin, that are thought to mediate homophilic cell adhesion and whose ectopic expression can trigger cell aggregation in S2 cells (Snow et al., 1989; Strigini et al., 2006).

IV. Autonomous versus non autonomous requirements and the mode of action of *cold* and *bou*

Our results indicate that both in embryonic and larval epithelial tissues the activity of *bou* can operate in a non cell autonomous fashion to organize septate junctions. Indeed, the overexpression of HA-Bou specifically in the tracheal system of *bou* mutant embryos rescues SJ organization in tracheal cells, but also in all ectodermal derivatives where Bou activity is required. In addition, we have shown in larval wing disc that this HA-tagged Bou version can travel from cell to cell, possibly associated to extracellular particles. Therefore, one straightforward way to explain the non autonomous action of Bou is that it diffuses from cell to cell. Our observations also suggest that, at least in the embryo, this protein could diffuse from tissue to tissue, possibly trough the hemolymph, although we have failed to reveal this phenomena by immunostainings. Finally, in the larval wing disc, clones of *bou* mutant cells have no SJ defects, consistent with the idea that *bou* requirement for SJ organisation has been rescued by the surrounding wild type cells. In contrast, the *cold* gene, which codes for a very similar protein and elicits indistinguishable phenotypes, acts in clear-cut autonomous way and we did not detect extracellular diffusion of the Flag-Cold protein.

Interestingly, this “non-cell autonomous” mode of action of *bou* has not been previously described for genes participating in septate junction organization. In order to better understand how Bou could diffuse, we have begun studying the nature of HA-Bou positive vesicles that we detect in the wing discs. In mammals, the GPI-bound Ly6 member CD59, a cell-surface glycoprotein protecting host cells from the complement system attack, has been found to travel in the human serum, directly associated with HDL apolipoproteins (Väkevä et al., 1994). Interestingly, recent studies have reported the crucial role of Drosophila lipoproteins as vehicles for the movement of lipid-linked morphogens (like Wingless and Hedgehog) and also GPI-linked proteins (Panáková et al., 2005). However, our observations indicate that HA-Bou is not associated with apolipophorin II particles (the insect equivalent of vertebrate apolipoproteins) (Hijazi et al., 2009), which are readily detected in the wing disc in the extracellular space, as the HA-Bou positive particles. This result suggests that Bou diffuses in a different way. The protein CD59 can also travel with its intact GPI coupled to membranous vesicles called prostasomes that are specifically produced by the prostatic gland cells (Rooney et al., 1993). It is envisageable that the *Drosophila* larval wing epithelium produce vesicles similar to these prostasomes, which we propose to call ‘boudosomes’.Whether these particles resemble exosomes or other known membranous vesicles remains to be explored.

Another open question is whether the HA-Bou GPI anchor is required for its incorporation into these particles and for its extracellular diffusion. In fact, cleavage of the GPI anchor, mediated by the activity of phospholipases, could allow extracellular release of the Bou TFD. We tried to explore this possibility getting inspiration from the snake toxins, which lack a GPI anchor and are efficiently secreted. We designed a Bou protein lacking its C-terminal end, which corresponds to the presumptive sequences required for GPI moiety addition. Unfortunately this defective protein is not capable of reaching the cell membrane and is neither functional nor capable of leaving the cell. Therefore, it seems that the deleted C-terminal sequences are required for the internal trafficking of the Bou precursor but deciphering the role of the GPI anchor will require other strategies.

Still the most interesting issue that should be addressed in the future is to understand if Bou diffusion is essential for its function during SJ organization or it is just an added feature that is independent of its main function. As judged by *in situ* hybridisation, *bou* realm of expression accurately matches the tissues in which we have detected a *bou* genetic requirement, so in principle Bou could act in these tissues without leaving the cell. Interestingly, *cold* which codes for a very similar protein and elicits indistinguishable phenotypes, acts in clear-cut autonomous way. Furthermore, we did not detect extracellular diffusion of the Flag-Cold protein, suggesting that either this protein does not possess a GPI-anchor, which is still a possibility, or that presence of this lipidic moiety is not the only factor determining the ability of Ly6 proteins to exit the cell.

V. Functions of *cold* and *bou* in neural tissues

Our results indicate that the activity of the Ly6 gene *cold* is essential for the maintenance of paracellular barrier and septate junction organization in different embryonic and larval epithelial tissues. We have shown as well that the paracellular barrier is compromised in *cold* mutants at the level of the ventral cord and the chordotonal organs of the peripheral nervous system. This result was somehow expected, since similar defects have been reported both in epithelial and neural tissues in mutants for many SJ components, reflecting the strong structural similarities existing between the SJ of both tissues.

Many different organisms, including vertebrates, have developed efficient systems to isolate the brain from the rest of the body fluids and tightly regulate the composition of ions, proteins, hormones and nutrients that have access to the neuronal tissues. In *Drosophila*, the blood brain

barrier (BBB) is established by SJ formed between a subpopulation of glial cells, called subperineurial glia, that form a covering sheet protecting axon projections and the ventral cord (Stork et al., 2008). We have detected *cold* expression in a subset of glial cells that could correspond to the subperineurial population, suggesting that the BBB increased permeability observed in *cold* mutants could be due to defects in the glial SJ. However, analysis at the electron microscopy level of the SJ structure of *cold* mutants will be required to confirm this hypothesis, as other valid alternatives exist. For instance, it has been shown in the *Drosophila* ventral cord that the Moody G-protein coupled receptor (GPCR) is required for maintenance of an efficient BBB function, suggesting that GPCR signalling contributes to SJ formation. However, in *moody* mutants the formation of SJ is not completely abrogated in the subperineurial glial contact regions, and only a reduction in the total number of septae has been reported (Schwabe et al., 2005). Actually, *moody* BBB defects could be consecutive to the overall perturbations of the subperineurial cells structure, as in this mutant background cells fail to interdigitate as much as they do in a wild type context (Schwabe et al., 2005). Interestingly, a recent study has shown that mutants for different components of the actin-related protein-2/3 (Arp2/3) complex, which regulate the actin cytoskeleton organization, show similar structural defects to those seen in *moody* mutants, including an impaired BBB (Hatan et al., 2011). Moreover, a careful structural characterization of the subperineurial cell contacts has shown that *moody* signalling triggers the formation of a series of specialized actin rich structures (ARS). In fact, it has been proposed that *moody* contribute to the maintenance of the BBB by controlling actin cytoskeleton organization. As many known SJ components, such as Neuroglian, also appear to be associated with the ARS (Hatan et al., 2011), it could be worth to evaluate if Cold exerts its activity at the level of these structures.

Our work indicates that, differing from *cold*, *bou* is not essential for the organization of the BBB in the central nervous system, although it is required at the level of the chordotonal organs. This conclusion comes from analysis of diffusion of a 10 KDa fluorescent dye into the ventral cord of live embryos at stage 17, when the brain is already protected by an organized barrier preventing penetration of this molecule (Carlson and Hilgers, 1998; Carlson et al., 2000). However, it has been shown that dyes as small as the FITC-dextran molecules (3 KDa) are efficiently excluded from the fly adult brain (Mayer et al., 2009), and not all the SJ mutants exhibit the same degree of permeability. For instance, it has been reported that a 70 kDa dye is excluded from the brain in some mutants (like *moody*) and not others (like *nrxIV* and *sinu*), whereas smaller dyes (10 kDa) easily penetrated into the brain of all of them (Stork et al., 2008). Thus, the use of smaller dyes may reveal BBB also in the ventral cord of *bou* mutants.

Another possibility is that a permeability defect might only became manifest in *bou* mutants at later stages, arguing for different temporal requirement for *bou* in the CNS and the PNS. In fact, both SJ types do not mature at the same moment: an efficient barrier is first established in the peripheral nervous system (stage 16) and later on in the ventral cord (stage 17) (Banerjee and Bhat 2007; Schwabe et al., 2005).

Nonetheless, our results may also genuinely reflect a different requirement for *bou* in the peripheral *versus* the central nervous systems. In this respect, it seems plausible that the SJ formed by different cell types (epithelial, peripheral nervous system or central nervous system) display different permeability thresholds or selective properties, and thus rely on a different molecular and structural organization. The differential requirement for *bou* could reflect this heterogeneity; raising the exciting possibility that *bou* belongs to a molecular circuit involved in the modulation of the selective properties of paracellular barriers.

In any case, our results show that two *Drosophila* Ly6 proteins could play an essential role at the level of the nervous system in glial tissues. Could other Ly6 proteins carry out similar function in vertebrates? It has been shown that F3/Contactin, NF155/Neurofascin and Caspr/Paranodin are conserved proteins with a role in the organization of PSJ (Maier et al., 2007; Labasque and Faivre-Sarrailh 2010). These proteins have clear orthologues in *Drosophila* (respectively, Contactin, Neuroglian and Neurexin IV) that are all involved in SJ formation in the subperineural glia. This conservation between flies and vertebrates SJ might thus extend to members of the Ly6 family.

The human genome codes for 45 Ly6 members, playing multiple roles in processes such cell adhesion, migration and signalling. Many, if not all of these 45 genes have orthologues in other mammals, notably in mice and rats, which are suitable model systems for research in neurobiology. The high variability of the Ly6 domain precludes the identification of vertebrate orthologues corresponding to the *Drosophila* Ly6 genes. Although evidence are still lacking in vertebrates, further investigations may reveal that some Ly6 proteins carry out functions analogous to those of Cold and Bou in the organization of PSJ. Also, a better understanding of the mode of action of these two proteins in *Drosophila* and the identification of their molecular partners will add more information permitting further evaluation of this hypothesis.

As far as the arthropods lineage is concerned, there are chances that the function of *cold* and *bou* might be conserved, at least in this phyletic group. Our comparative analysis of the full complement of Ly6 coding genes present in *Drosophila* and in the hymenopteran *Apis*

mellifera shows that *bou* and *cold* orthologues are present in the genome of both insects, which is not the case for most other *Drosophila* Ly6 proteins (Hijazi et al., 2009). In addition, we have been able to identify orthologues for these two genes not only in multiple insect genomes (Hijazi et al., 2009; Hijazi et al., 2011), but also in the genomes of other arthropods, like the crustacean *Daphnia pulex* and the chelicerate *Tetranychus urticae* (Roch F., unpublished results). These observations are consistent with the idea that *bou* and *cold* make part of an ancient conserved molecular network operative at least in the arthropod lineage.

VI. Multiple roles for 4 Ly6 genes in SJ organization?

The high degree of variability observed in the TFD domains of Ly6 proteins has been thought to be at the origin of their capacity to bind a broad spectrum of targets and to accomplish multiple biological functions. Mutants for four different *Drosophila* Ly6 genes, *boudin, cold, crooked* and *crimpled* show indistinguishable phenotypes and they are likely to participate in a non-redundant manner in the same process, the formation of epithelial SJ (Hijazi et al, 2009; Nilton et al., 2010; Hijazi et al., 2011). However, besides their diagnostic set of cysteines, these four proteins are highly divergent at the level of their primary sequences and their direct comparison does not permit to predict a common molecular role. Interestingly, the four Ly6 genes have highly conserved orthologues in other insects, such as the honey-bee (Hijazi et al., 2009). Thus, it is possible that although they participate in the same biological process, they could operate via completely different mechanisms. Moreover, their non-redundant roles could have diverged early in insect evolution. Supporting this line of thought, our data concerning the localization of Bou and Cold indicate that at least a fraction of these proteins is present at the cell membrane, whereas the Crooked protein seems to mainly concentrate in cytoplasmic puncta (Nilton et al., 2010). In addition, at least some of these Ly6 proteins have acquired specific properties, as *boudin* can act in a non cell autonomous way whereas the other genes act in a strictly cell autonomous manner. This observation indicates that, at least in the case of Bou, these proteins do not always traffic together in the same cell compartments.

However, we still lack enough data to propose a comprehensive model explaining the mode of action of these four Ly6 proteins during SJ organization. Upcoming studies will allow clarifying if these genes act in completely independent ways or cooperate together to organize the assembly of the SJ structures.

b) Perspectives

Our results show that *bou* and *cold* are required for the organization of septate junctions. Nevertheless, we still ignore if the products of these two genes act as components of the septate junction complex or participate in their assembly by another mechanism. Future studies focusing in this question, and in the long run, permitting the identification of the Bou and Cold molecular partners will greatly benefit from a more detailed characterization of the subcellular localization of these two proteins.

I. Studying in vivo the localization and the cellular trafficking of Bou and Cold

As all GPI anchored proteins, Bou and Cold are supposed to be inserted into lipid raft membrane microdomains, whose presence in *Drosophila* tissues has been postulated for a long time (Rietveld et al., 1999). In principle, the organization of these microdomains could be extremely sensitive to the presence of detergents commonly used in immunohistochemistry techniques. To bypass this potential technical problem, we decided to generate fluorescent versions of Bou and Cold, using the mCherry and Citrine tags. Expression of these proteins in live tissues will allow to better characterize the distribution of Bou and Cold, and in particular their intracellular trafficking and membrane localization. In addition, they will also allow studying the diffusion of the Bou protein in fully physiological conditions. We expect that these tools could be more adapted for studying the nature of vesicles transporting Bou from cell to cell and to understand how this protein exits the cell and enter the adjacent ones. The use of different fluorescent markers for the main endosomal compartments will also help to clarify the cellular routes followed by Ly6 proteins during SJ assembly.

Although our results suggest that Bou travels associated with extracellular vesicles, we still ignore if Bou diffuses with an intact GPI-anchor. It is also possible that the GPI-anchored form is cleaved by cellular phospholipases at the cell surface and then released as a soluble form, like the snake toxins. To explore this possibility, we will generate chimeric proteins where a fluorescently tagged version of the Bou Ly6 domain is coupled to a transmembrane protein domain, like the Nrg transmembrane region. We plan to insert between these two domains the consensus cleavage site of the tobacco mosaic virus protease (TevP), so that the tagged Ly6 domain could be released from the membrane upon co-expression of the TevP protease (Brankatschk and Eaton, 2010). This tool might be instrumental to test if Bou diffuses as a secreted factor, and if this form of Bou is functional (*i.e.* capable of contributing to SJ organization in a non cell autonomous way).

II. Identification of Bou and Cold molecular partners by phenotypic analysis and biochemical approaches

Phenotypic analysis: Basically, our studies have shown that in *bou* and *cold* mutants, all the SJ components tested in so far appear distributed homogeneously along the lateral cell membrane instead of clustering in the apico-lateral part of epithelial cells. However, we have not analyzed the localization of other known SJ components, like the *Drosophila* Claudins, the Lachesin protein and the different Na^+/K^+ pump subunits, which are also potential partners of the Ly6 proteins. It has been shown in the case of the Contactin SJ component that this protein is retained in intracellular vesicles in *NrxIV* mutants, but not in other mutant backgrounds (Faivre-Sarrailh et al., 2004). Thus, we plan to extend the phenotypic characterization of the *bou* and *cold* phenotypes using a larger battery of markers and, when possible, analysis of live embryos. In particular, these studies will be not only focused on the late stages of development but also on the early phases of development in which the SJ become established. We hope in this way to progress in the identification of particular relationships between Ly6 proteins and already known SJ components, pointing to potential couples of interacting proteins.
In addition, the study of the localization of the different fluorescent Bou and Cold forms in live embryos could be also carried out in different mutant backgrounds, to provide further opportunities of detecting particular interactions.

Biochemical approach: The identification of potential Bou and Cold SJ partners by detailed phenotypic characterizations would serve as prime criteria allowing the choice of candidate genes for more in depth biochemical studies, permitting to establish which are the molecular partners of the Ly6 proteins. In this sense, we envisage using co-immunoprecipitation tests to detect physical interactions, and we are envisaging the generation of new efficient antisera against Bou and Cold that will allow their detection in wild type contexts to ease the biochemical experiments.

In the long run, we expect that these strategies will provide a satisfactory answer to a series of questions that this PhD work has permitted to formulate: which are the cellular and molecular contributions of the Ly6 proteins, Bou and Cold, to the cell barrier function and the SJ formation?

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Title: The role of two members of the Ly6 superfamily in the organization of septate junctions during *Drosophila melanogaster* development.

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Abstract

The Ly6 superfamily is a large family of genes present in most metazoan genomes, including 45 members in Humans. These genes mainly encode for extracellular glycoproteins attached to the cell membrane by a GPI anchor (Glycosylphosphatidylinositol), but also for soluble ligands. They are characterized by the presence of an extracellular domain, called Ly6 domain, whose structure is provided by 8 to 10 cysteines present in conserved positions. The great variability exhibited by the Ly6 primary sequences allows these proteins to exert highly divergent roles. Although their function has been elucidated in various organisms, we still know very little about their potential roles during animal development. During my PhD, I used the *Drosophila* model system to extend our knowledge about the functions of these proteins in a developmental context. Our work has permitted the identification of 36 members of the Ly6 superfamily in *Drosophila melanogaster*, and I have characterized at a functional level two of these genes during development. Phenotypic analysis of mutants for these two genes, called *boudin* and *coiled*, has shown that both of them are required for tracheal morphogenesis and organization of septate junctions in epithelial tissues. Septate junctions are cell adhesion structures analogous to vertebrate tight junctions. They allow epithelia to perform their barrier function and regulate the passage of solutes and ions through the paracellular space. Septate junctions in *Drosophila* are similar to the vertebrate paranodal junctions, present at the contact between axons and Schwann cells, and our results show that *boudin* and *coiled* are also required for the organization of septate junctions in the fly nervous system. On the other hand, we have shown that the protein Boudin is able to diffuse from one cell to another to regulate septate junction formation. This non cell autonomous mode of action had never been described for proteins involved in septate junction organization. Studying the diffusion mechanisms and the trafficking of Boudin is important to better understand how this protein performs its function. Finally, another challenge will be to identify functional partners of Boudin and Coiled to elucidate the molecular mechanisms by which these proteins control the maintenance and the organization of septate junction structures.

Key words: Ly6 superfamily, Drosophila development, septate junctions, paracellular barrier, paranodal junctions

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