Development 133, 785-790 doi:10.1242/dev.02260

# Association of tracheal placodes with leg primordia in *Drosophila* and implications for the origin of insect tracheal systems

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Adaptation to diverse habitats has prompted the development of distinct organs in different animals to better exploit their living conditions. This is the case for the respiratory organs of arthropods, ranging from tracheae in terrestrial insects to gills in aquatic crustaceans. Although *Drosophila* tracheal development has been studied extensively, the origin of the tracheal system has been a long-standing mystery. Here, we show that tracheal placodes and leg primordia arise from a common pool of cells in *Drosophila*, with differences in their fate controlled by the activation state of the *wingless* signalling pathway. We have also been able to elucidate early events that trigger leg specification and to show that cryptic appendage primordia are associated with the tracheal placodes even in abdominal segments. The association between tracheal and appendage primordia in *Drosophila* is reminiscent of the association between gills and appendages in crustaceans. This similarity is strengthened by the finding that homologues of tracheal inducer genes are specifically expressed in the gills of crustaceans. We conclude that crustacean gills and insect tracheae share a number of features that raise the possibility of an evolutionary relationship between these structures. We propose an evolutionary scenario that accommodates the available data.

**KEY WORDS: Tracheae, Appendage primordia, Gills, *wingless* signalling, *Distal-less* (*Dll*), *buttonhead* (*btd*), *trachealess* (*trh*), *ventral veinless* (*vvl*)**

## INTRODUCTION

A crucial step in the colonization of terrestrial habitats has been the evolution of specialized respiratory organs that allow efficient gas exchange and minimize water loss. The main respiratory organs in insects are the tracheae, comprising a network of tubes that bring air deep into the body (Snodgrass, 1935; Mill, 1985). In *Drosophila*, as in other insects, the tracheal system is a complex tubular network that arises from the tracheal placodes, clusters of ectodermal cells that appear on either side of thoracic and abdominal embryonic segments. Tracheal cells are specified by the activity of a set of 'tracheal inducer genes' that includes the transcription factors *trachealess* (*trh*) and *ventral veinless* (*vvl*), whose expression is controlled by genes that specify positional cues along the embryonic body axes (Isaac and Andrew, 1996; Wilk et al., 1996; Boube et al., 2000). The cells of each cluster invaginate and migrate in a stereotypic pattern to form each of the primary tracheal branches (Manning and Krasnow, 1993).

In recent years, many of the genes that are required for the specification of the tracheal cells have been identified (Ghabrial et al., 2003). However, not much attention has been given to the evolutionary origin of these cells. It is believed that in the common ancestors of all arthropods, specialised parts of appendages had a major role in respiration and osmoregulation, acting as gills (Brusca and Brusca, 1990; Budd, 1996). Indeed, this close association between respiratory organs and appendages is maintained currently in many crustaceans, which are the closest living relatives of insects (Regier and Shultz, 1997; Boore et al., 1998; Mallatt et al., 2004).

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**Accepted 21 December 2005**

To investigate the origin of tracheal cells, we have asked whether these may also arise in association with the cells that give rise to appendages in a present day insect like *Drosophila*. We have found that indeed the tracheal placodes and leg primordia arise from a common pool of cells in *Drosophila*, and that the decision between these two fates is controlled by the activity of the *wingless* signalling pathway. By manipulating the genetic program that controls leg specification, we have been able to show that, even in the abdomen, tracheal primordia develop in close association with cryptic appendage primordia. These results point to a close relationship between the tracheal and leg fates, and suggest some interesting similarities with the appendage-associated gills of aquatic crustaceans. To investigate these similarities further, we have cloned homologues of the tracheal inducer genes and studied their expression patterns in two divergent groups of crustaceans. We argue that crustacean gills and insect tracheae, hitherto considered to be independent systems for gas exchange, may share a number of features in their developmental origin and specification.

## MATERIALS AND METHODS

### Fly strains

We have used *wg*CX4 as a *wg* null allele. The 1-eve-1 line (Perrimon et al., 1991) is a *lacZ* insertion in the *trh* gene. Expression of *btd* and *Dll* was induced with the UAS/GAL4 system (Brand and Perrimon, 1993), using a UAS-*btd* line (Schock et al., 1999) and a UAS-*Dll* line (Gorfinkiel and Guerrero, 1997), and a *btd*-Gal4 line as a driver (Estella et al., 2003). To induce ectopic expression of *wg*, we used a *nullo*-Gal4 (from W. Gehring, Basel, Switzerland) and a UAS*wg* line (Lawrence et al., 1995). We used *ptc*-GAL4 (Speicher et al., 1994) to drive the expression of UAS-TCF$^{DN}$ (van de Wetering et al., 1997) and UAS-Arm* (Pai et al., 1997) in *Drosophila* embryos.

### *Drosophila* immunostaining and in situ hybridisation

We used the following primary antibodies: a mAb2A12 monoclonal antibody (1:5-1:10, from the Developmental Studies Hybridoma Bank, University of Iowa), which recognises an epitope from the lumen of the

tracheal tree, and an antibody specific for β-gal (Cappel, 1:2000). Embryos were stained according to standard protocols using the Vectastain Elite ABC kit. For immunofluorescence, we used secondary antibodies Alexa488-conjugated goat anti-rabbit (1:200) and Alexa594-conjugated goat anti-mouse (1:200), both from Molecular Probes. Whole-mount in situ hybridisation was carried out with *trh* and *btd* anti-sense RNA probes, following the method of Tautz and Pfeifle (Tautz and Pfeifle, 1989), with minor modifications. For immunofluorescence, we used *trh* anti-sense RNA probes following the procedure described by Wilkie and Davis (Wilkie and Davis, 1998). For antibody labelling followed by in situ hybridisation, we followed the procedure described by Manoukian and Krause (Manoukian and Krause, 1992). Photographs were taken using Nomarski optics or a SP1 Leica confocal microscope.

#### Preparation of embryonic cuticle

For the analysis of embryonic cuticle, late embryos were removed from the chorion and vitelline membrane, and mounted in a mixture of Hoyer's medium (van der Meer, 1977) and lactic acid (1:1).

#### *Artemia*, *Parhyale* and crayfish immunostaining and in situ hybridisation

We initially cloned a fragment of *vvl* from *Artemia*, by PCR from cDNA generated from larval RNA with degenerated oligonucleotides designed from the *Drosophila vvl* sequence. An antibody against the Vvl protein of *Artemia* was generated by injecting rabbits with a His-tagged fragment of the Vvl protein [amino acids 241 to 386 of the previously published sequence (Chavez et al., 1999)]. The serum was then affinity purified on a nickel column. We initially cloned a fragment of *vvl* and *trh* from *Parhyale hawaiensis* by PCR from a cDNA library kindly provided by Nipam Patel (University of California, Berkeley), with degenerated oligonucleotides designed from the *Drosophila vvl* and *trh* sequences. We obtained a 240 bp fragment of the *Parhyale vvl* gene that we used to clone the full-length cDNA from the library. We also obtained a *Parhyale trh* fragment of around 700 bp that encompasses the region of the HLH, the PAS-1 and the PAS-2 domains (corresponding to amino acids 100 to 550 in *Drosophila*). In situ hybridization in *Parhyale* was carried out using a protocol provided by Nipam Patel; the protocol is available upon request. For immunostaining in crayfish embryos, we used the 4D9 monoclonal antibody for Engrailed (Patel et al., 1989) and a polyclonal antibody for Nub/Pdm (Averof and Cohen, 1997). Immunohistochemical staining was carried out as described by Patel (Patel, 1994).

## RESULTS AND DISCUSSION

### Tracheal placodes arise in close proximity to the leg primordia in *Drosophila*

The *Drosophila* tracheal system has a clearly metameric origin, arising from clusters of cells, on either side of each thoracic and abdominal segment, that express the tracheal inducer genes *trachealess* (*trh*) and *ventral veinless* (*vvl*) (de Celis et al., 1995; Isaac and Andrew, 1996; Wilk et al., 1996) (Fig. 1B). Conversely, the leg precursors can be recognized as clusters of cells that express the *Distal-less* (*Dll*) gene, on either side of each thoracic segment; these will give rise both to the Keilin's Organs (KOs, the rudimentary legs of the larvae) and to the three pairs of imaginal discs that will give rise to the legs of the adult fly (Cohen, 1993).

To investigate whether there is a direct physical association between the leg and tracheal primordia, we examined *Drosophila* embryos co-stained for the expression of *trh* and early markers of leg primordia. Although *Dll* is one of the most commonly used markers for the leg primordia, it is not the earliest gene required for their specification. Instead, a couple of related and apparently redundant genes, *buttonhead* (*btd*) and *Sp1*, act upstream of *Dll* in the specification of these primordia (Estella et al., 2003). Examining the specification of tracheal cells with respect to *btd* expression, we observe that tracheal cells appear in close apposition to *btd*-expressing cells, from the earliest stages of their appearance (by stage 9/early stage 10, Fig. 1E,F). Interestingly, unlike *Dll*, *btd* is initially expressed both in the thoracic and abdominal segments, and its expression is restricted to the thoracic segments later, under the influence of the BX-C genes (Estella et al., 2003). Thus, the cells of the respiratory system in *Drosophila* always arise in close proximity to the cells that are fated to give rise to the legs.

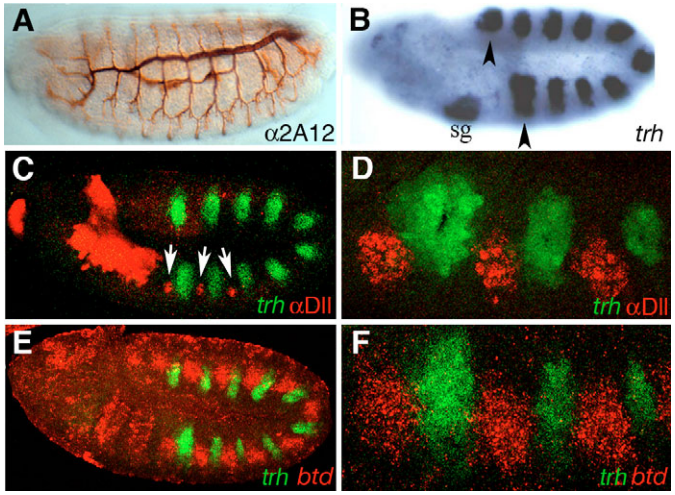


**Fig. 1. *Drosophila* tracheal and leg primordia derive from closely associated populations of cells.** (**A**) The *Drosophila* tracheal system visualized by the 2A12 antibody. (**B**) The tracheal system arises from 10 tracheal placodes (arrowheads on first and last placode) that express the *trh* gene; *trh* is also expressed in the salivary glands (sg). (**C**) Staining for *trh* in the tracheal placodes, and *Dll* in cephalic structures and leg primordia (arrows) in a stage 11 embryo. (**D**) Higher magnification view, showing the proximity of thoracic tracheal placodes and leg primordia. (**E**) Staining for *trh* expression (by means of an enhancer trap insertion in the gene) and *btd* expression in a stage 9/10 embryo. (**F**) Higher magnification view, showing the close apposition of *trh*- and *btd*-expressing cells.

### Induction of Keilin's organs in the abdomen: tracheal primordia are associated with cryptic appendage primordia in the abdominal segments

To fully endorse this conclusion it is necessary to show that the *btd*-expressing cells in the abdomen correspond to cryptic leg primordia. This may be a key point because, although many of the genes required for leg development are already known, it has not yet been possible to induce leg development in abdominal segments (except by transforming these segments into thoracic ones). In particular, although the *Dll* promoter contains BX-C binding sites that repress its expression in the abdominal segments (Vachon et al., 1992), no ectopic appendage has been reported by misexpressing *Dll* in the abdomen. These observations have lead to some doubts as to whether a leg developmental program is at all compatible with abdominal segmental identity.

As the initial expression of *btd* in the abdominal segments is downregulated by the BX-C genes, we reasoned that sustained expression of *btd* might overcome the repressive effect of the BX-C genes and force the induction of leg structures in the abdomen. To test this, we used a *btd*-GAL4 driver to drive *btd* expression, expecting that the perdurance of the GAL4/UAS system would ensure a more persistent expression of *btd* in its endogenous expression domain. We never obtained any sign of ectopic *Dll* expression or KOs in the abdominal segments, but we observed

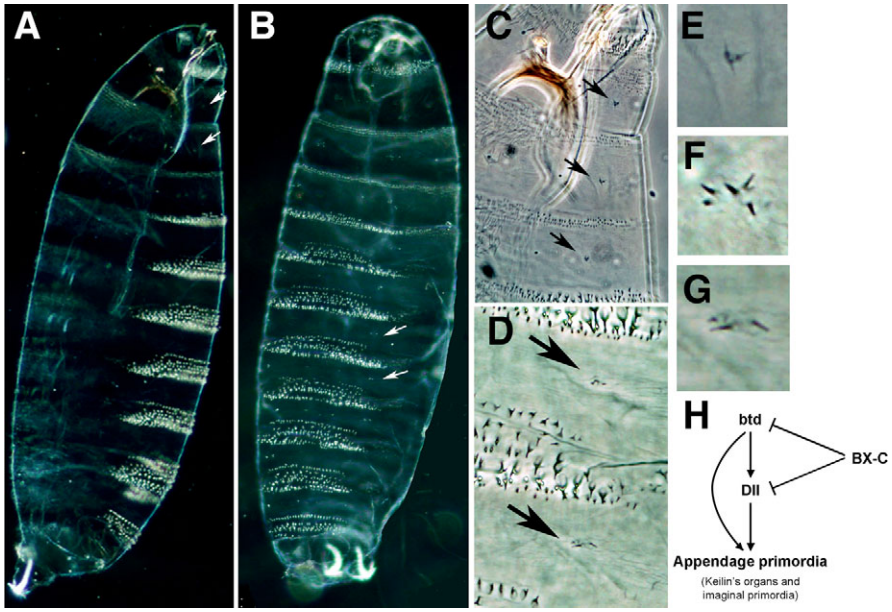


**Fig. 2. Induction of leg structures in the *Drosophila* larval abdomen.** (**A**) Lateral view of a wild-type first instar larval cuticle. Arrows point to Keilin's Organs (KOs), the rudimentary larval legs that appear in the thoracic segments. (**B**) Ventral view of a cuticle upon ectopic expression in the abdomen of *Dll* and *btd*. Arrows point to KOs that develop in otherwise normal abdominal segments. (**C**) Detail of A showing a wild-type thoracic KO (arrows). (**D**) Detail of B, showing the KO developing in abdominal segments (arrows; abdominal segments 4 and 5; more than 85% of the scored abdominal hemisegments show these KOs; *n*=20 embryos). (**E**) High magnification of a wild-type KO. (**F**) High magnification of a thoracic KO upon *btd* overexpression. (**G**) High magnification of an abdominal KO upon *btd* and *Dll* overexpression. KOs upon *btd* overexpression have more than the three hairs seen in the wild type. (**H**) Scheme of the proposed interactions giving rise to appendage primordia.

that the increased expression of *btd* had an effect on the KOs of the thoracic segments, which had more sensory hairs than the three normally found in wild-type KOs (Fig. 2F). Thus, on its own, *btd* seems unable to overcome BX-C repression of leg development.

One possibility would be that the BX-C genes could suppress appendage development in the abdomen by independently repressing both *btd* and *Dll* in this region. To assess this possibility, we used the same *btd*-GAL4 driver to simultaneously induce the expression of both *btd* and *Dll*. Under these circumstances, we observe that KOs develop in otherwise normal abdominal segments (Fig. 2B,D,G); as in the previous experiment, the newly formed KOs have more than three sensory hairs. These results suggest that expression of *btd* and *Dll* in the *btd*-expressing abdominal primordia is sufficient to induce the development of leg structures in the abdomen, overcoming the repressive effect of the BX-C genes. Furthermore, these results demonstrate that these clusters of *btd*-expressing cells in the abdomen are indeed cryptic leg primordia. These results clearly show that tracheal cells are specified in close proximity to the leg primordia, in both thoracic and abdominal segments.

## A leg-tracheal equivalence group: *wingless* signalling provides a genetic switch for the specification of leg versus tracheal fate

Previous results have shown that the leg primordia are specified straddling the segmental stripes of *wingless* (*wg*) expression in the early embryonic ectoderm (Cohen et al., 1993), whereas tracheal cells are specified in between these stripes (de Celis et al., 1995). To investigate whether *wg* might play a role in determining the fate of these primordia, we studied what happens when the normal pattern of *wg* expression is disrupted. We find that, in *wg* mutant embryos, *trh* and *vvl* from the earliest stages of their expression are no longer restricted to separate clusters of cells; instead larger patches of expression add up to a continuous band of cells running along the anteroposterior axis of the embryo (Fig. 3C) (de Celis et al., 1995), while *btd* expression is suppressed in this part of the embryonic ectoderm (Fig. 3D) (Estella et al., 2003). Conversely, ubiquitous expression of *wg* suppresses *trh* expression (Fig. 3E), while causing an expansion of *btd* expression along the embryo (Fig. 3F,L). Restricted activation or inactivation of the *wg* pathway by the expression of a constitutive form of *armadillo* or a dominant-negative form of *dTCF*, respectively, are also able to specifically induce or repress *trh* and *btd* expression (Fig. 3G-J). *trh*/*vvl* and *btd* seem to respond independently to *wg* signalling and there is no sign of cross-regulation among them, as *btd* expression is normal in *trh vvl* double mutants, and *trh* and *vvl* expression is normal in mutants for a deficiency uncovering *btd* and *Sp1* (data not shown).

The role of *wg* as a repressor of the tracheal fate is further illustrated by looking at the behaviour of transformed cells: the clusters of cells that have lost *btd* expression and gained *trh* and *vvl* expression in *wg* mutant embryos begin a process of invagination that is characteristic of tracheal cells (Fig. 3K). Furthermore, these cells also express the *dof* (*stumps* – FlyBase) gene, a target gene of both *trh* and *vvl* in the tracheal cells (Boube et al., 2000) (data not shown). Although further development of these cells is hard to ascertain because of gross abnormalities in $wg^{-}$ embryos, these results indicate that they have been specified as tracheal cells. Thus, *wg* appears to act as a genetic switch that decides between two mutually exclusive fates in this part of the embryonic ectoderm: the tracheal fate, which is followed in the absence of *wg* signalling; and the leg fate, which is followed upon activation of the *wg* pathway (Fig. 3M). Given that there are no cell lineage restrictions setting apart the cells of the tracheal and leg primordia (Meise and Janning, 1993), these two cell populations could be considered as a single equivalence group, with the differences in their fate controlled by the activation state of the *wg* signalling pathway.

## Crustacean homologues of tracheal inducer genes are expressed in appendage-associated gills

A link between respiratory organs and appendages is also found in many primitively aquatic arthropods, like crustaceans, where gills typically develop as distinct dorsal branches (or lobes) of appendages called epipods (Brusca and Brusca, 1990). Following our observations, which suggest a link between respiratory organs and appendages in *Drosophila*, we decided to examine whether

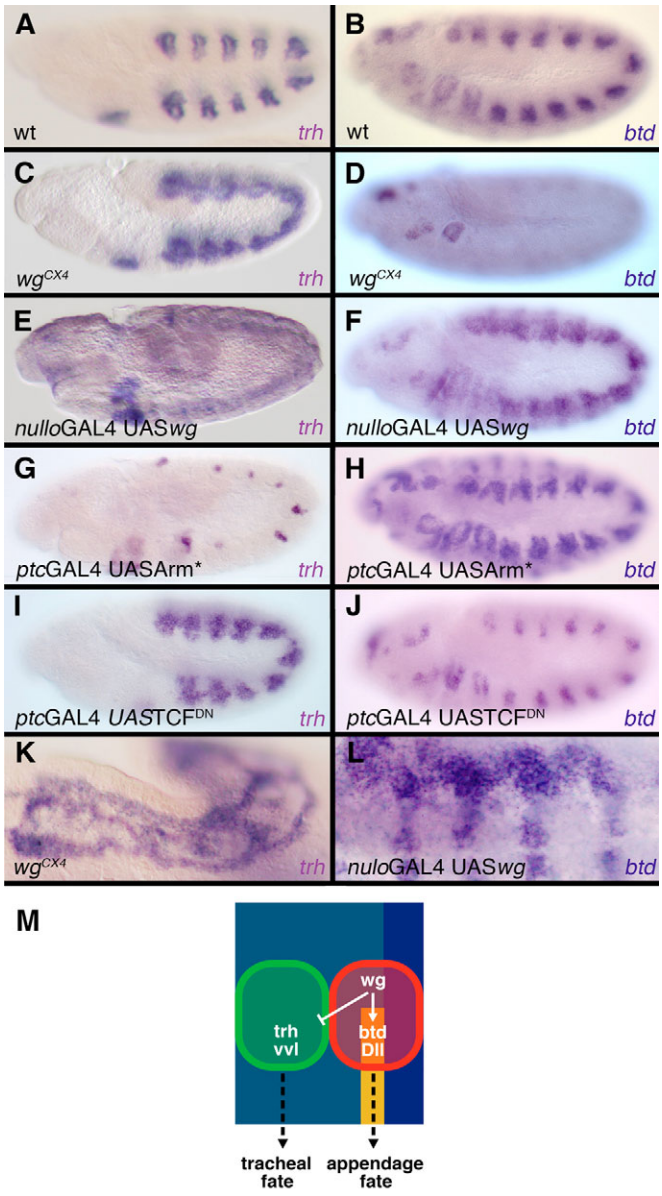


**Fig. 3. *wg* signalling provides a genetic switch for the specification of leg versus tracheal primordia.** (**A**,**B**) Wild-type expression pattern of *trh* in the salivary glands and tracheal placodes, and of *btd* in cephalic segments and cell clusters in thoracic and abdominal segments. (**C**,**D**) In a *wg* mutant, *trh* expression is expanded along the anteroposterior axis, whereas *btd* expression is abolished in the thoracic and abdominal segments. (**E**,**F**) Conversely, upon ectopic expression of *wg*, *trh* expression in the tracheal placodes is suppressed and *btd* expression is expanded. (**G**,**H**) Restricted ectopic activation of the *wg* pathway reduces the domains of *trh* expression and expands those of *btd* expression. (**I**,**J**) Restricted inactivation of the *wg* pathway expands *trh* expression and reduces *btd* expression. All embryos are at stage 11. (**K**) Detail of *wg* mutant at a somewhat later stage, showing that the ectopic *trh*-expressing cells begin to invaginate. (**L**) Detail of an embryo at germ band extension upon ectopic expression of *wg* at germ band retraction, indicating that expansion of *btd* occurs only in part of the embryonic ectoderm, while the segmental pattern persists in the central nervous system. (**M**) Schematic representation of the role of *wg* (transcribed in the yellow domain) in promoting appendage and repressing tracheal fates.

further similarities could be found between insect tracheal cells and crustacean gills. Specifically, we considered whether homologues of the tracheal inducing genes might have a role in the development of appendage-associated gills in crustaceans.

We used RT-PCR to clone fragments of the *vvl* and *trh* homologues from *Artemia franciscana* and from *Parhyale hawaiensis*, representing two major divergent groups of crustaceans (members of the branchiopod and malacostracan crustaceans, respectively). In the case of *Artemia vvl*, we cloned a fragment that corresponds to the *APH-1* gene previously reported by Chavez et al. (Chavez et al., 1999) and generated an antibody for immunochemical staining in developing *Artemia* larvae. We observe that *Artemia* Vvl is initially absent from early limb buds; it becomes weakly and uniformly expressed while the limb is developing its characteristic branching morphology, and becomes strongly upregulated in one of the epipods as its cells begin to differentiate (Fig. 4A,B). Uniform weak expression persists in mature limbs, but expression levels in the epipod are always significantly higher. The *trh* homologue from *Artemia* has previously been studied by Mitchell and Crews (Mitchell and Crews, 2002), and its expression appears to be restricted to the same epipod as Vvl. Similarly, we have cloned homologues of *vvl* and *trh* from *Parhyale hawaiensis* and have studied their expression by in situ hybridization. Both genes are specifically expressed in the epipods of developing thoracic appendages (Fig. 4C-E). Besides epipods, the *Artemia trh* and *vvl* homologues are also expressed in the larval salt gland, an organ with osmoregulatory functions during early larval stages of *Artemia* development (Chavez et al., 1999; Mitchell and Crews, 2002).

## Implications for the origin of insect tracheal systems

What is the significance of the two *Drosophila* tracheal inducer genes being specifically expressed in crustacean epipods/gills? One possibility is that the expression of these two genes was acquired independently in insect tracheae and in crustacean gills. Alternatively, tracheal systems and gills may have inherited these expression patterns from a common evolutionary precursor, perhaps a respiratory/osmoregulatory structure that was already present in the common ancestors of crustaceans and insects.

The latter possibility is considered unlikely by conventional views, because of the structural differences between gills and tracheae (external versus internal organs, discrete segmental organs versus fused network of tubes), and the difficulty to conceive a smooth transition between these structures. Yet, analogous transformations have occurred during arthropod evolution: tracheae can be organized as large interconnected networks or as isolated entities in each segment (as in some apterygote insects), invagination of external respiratory structures is well documented among groups that have made the transition from aquatic to terrestrial environments (terrestrial crustaceans, spiders and scorpions), and conversely evagination of respiratory surfaces is common in animals that have returned to an aquatic environment (tracheal gills or blood gills in aquatic insect larvae) (Snodgrass, 1935; Mill, 1985; Brusca and Brusca, 1990). A very similar (but independent) evolutionary transition is, in fact, thought to have occurred in arachnids, where gills have been internalised to give rise to book lungs, and these in turn have been modified to give rise to tracheae in some groups of spiders (Lankester, 1885; Purcell, 1910; Damen et al., 2002). Thus, a relationship between insect tracheae and crustacean gills is plausible.

A particular type of epipod/gill has also been proposed as the origin of insect wings (Wigglesworth, 1976; Kukalova-Peck, 1983), a hypothesis that has received support from the specific expression

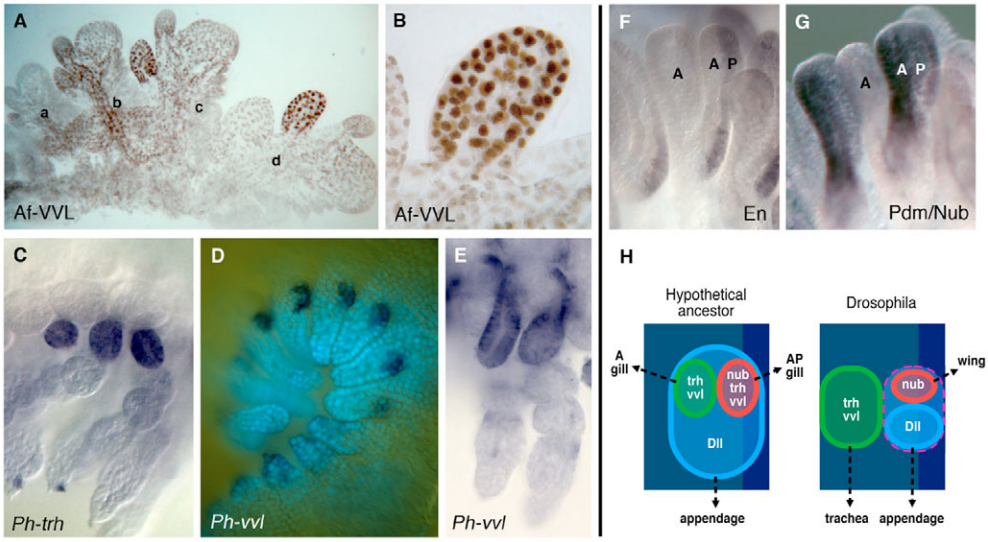


**Fig. 4. Similarities and hypothetical relationships between surfaces in crustaceans and insects.** (**A**) Expression of Vvl in thoracic appendages of *Artemia franciscana*. Four appendages of increasing maturity are shown, immunochemically stained with an anti-Vvl antibody: (a) onset of Vvl expression; (b) early uniform Vvl expression; (c) the beginning of Vvl upregulation in the distal epipod/gill; and (d) higher levels of Vvl in the distal epipod of near-mature appendages. (**B**) Higher magnification of mature epipod, showing high levels and nuclear localization of Vvl. (**C-E**) Expression of *trh* and *vvl* in thoracic appendages of *Parhyale hawaiensis*, visualized by in situ hybridization. (C) Expression of *Parhyale trh* in thoracic appendages, showing strong staining in epipods but no staining in the endopods/legs. (D) Expression of *Parhyale vvl* at an early stage of appendage development, showing specific expression in the primordia of epipods. Cells are visualized by nuclear staining with DAPI. (E) Expression of *Parhyale vvl* at a late stage of appendage development. (**F**) Engrailed staining in the distal epipods/gills of the crayfish *Pacifastacus leniusculus*. The anterior gill comprises exclusively non-*en*-expressing cells (labelled A), whereas the posterior gill comprises both non-*en*- and *en*-expressing cells (labelled AP). (**G**) Nubbin/Pdm staining in the same epipods/gills in crayfish. Nub is expressed only in the posterior gill (labelled AP). (**H**) Summary of hypothetical relationships between insect tracheae and crustacean gills. Colour code is cyan for appendages/legs (expressing *Dll* in all arthropods), red for AP gills and insect wings (straddling the AP boundary, and expressing *nub* and *vvl/trh*), and green for A gills and insect tracheae (comprising anterior cells only, and expressing *vvl* and *trh*, but not *nub*).

of the *pdm/nubbin* (*nub*) and *apterous* (*ap*) genes – that have wing-specific functions in *Drosophila* – in a crustacean epipod (Averof and Cohen, 1997). In fact, the *Artemia nub* and *ap* homologues are expressed in the same epipod as *trh* and *vvl*, raising questions as to the specific relationship of this epipod with either tracheae or wings. A resolution to this conundrum becomes apparent when one considers the different types of epipods/gills found in aquatic arthropods, and their relative positions with respect to other parts of the appendage.

The primary branches of arthropod appendages, the endopod/leg and exopod, develop straddling the anteroposterior (AP) compartment boundary, which corresponds to a widely conserved patterning landmark in all arthropods (Martinez-Arias and Lawrence, 1985; Patel et al., 1989a; Basler and Struhl, 1994; Damen, 2002). Different types of epipods/gills, however, differ in their position with respect to this boundary. For example, in the thoracic appendages of the crayfish, some epipods develop spanning the AP boundary [visualized by *engrailed* (*en*) expression running across the epipod], whereas others develop exclusively from anterior cells (with no *en* expression; Fig. 4F). Given that wing primordia comprise cells from both the anterior and posterior compartments, wings probably derived from structures that were straddling the AP boundary. Conversely, given that tracheal primordia arise exclusively from cells of the anterior compartment (anterior to *en* and even *wg*-expressing cells) (de Celis et al., 1995), it seems probable that tracheal cells evolved from a population of cells that was located in the anterior compartment. In this respect, it is interesting to note that the former type of epipods express *nub*, whereas the latter do not (Fig. 4G).

In summary, we would like to suggest that the ancestors of arthropods had specific areas on the surface of their body that were specialized for osmoregulation and gas exchange. Homologues of *trh* and *vvl* were probably expressed in all of these cells and played a role in their specification, differentiation or function. Some of these structures were probably associated with appendages, in the form of epipods/gills or other types of respiratory surfaces. A particular type of gill, straddling the AP compartment boundary, is likely to have given rise to wings (Averof and Cohen, 1997), whereas respiratory surfaces arising from anterior cells only may have given rise to the tracheal system of insects. Confirmation of this hypothetical scenario may ultimately come from the discovery of new fossils, capturing intermediate states in the transition of insects from an aquatic to a terrestrial lifestyle.

We are grateful to Tijana Copf for immunochemical stainings in *Artemia*, to David Aldridge for collecting crayfish, to Nipam Patel for a cDNA library and protocols on *Parhyale*, and to Michael Akam and Jean-Paul Vincent in whose labs some experiments were carried out. We also thank C. Estella, G. Morata and N. Patel for providing flies, antibodies and libraries, and the people in our labs for help and critical comments on the manuscript. This work was supported by grants from the CIRIT of the Generalitat de Catalunya and the Ministerio de Ciencia y Tecnología (to J.C.), and by the Marie Curie Research Training Network 'ZOONET' (to M.A.).

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