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Evolutionary origin of the insect wing via integration of two developmental modules

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SUMMARY Insect wing is a key evolutionary innovation for insect radiation, but its origins and intermediate forms are absent from the fossil record. To understand the ancestral state of the wing, expression of three key regulatory genes in insect wing development, *wingless* (*wg*), *vestigial* (*vg*), and *apterous* (*ap*) was studied in two basal insects, mayfly and bristletail. These basal insects develop dorsal limb branches, tracheal gill and stylus, respectively, that have been considered candidates for wing origin. Here we show that *wg* and *vg* are expressed in primordia for tracheal gill and stylus. Those primordia are all located in the lateral body region marked by down-regulation of early segmental *wg* stripes, but differ in their dorsal--ventral position, indicating their positions drifted within the lateral body region. On the other hand, *ap* expression was detected in terga of mayfly and bristletail. Notably, the extensive outgrowth of the paranotal lobe of apterygote bristletail developed from the border of *ap*-expressing tergal margin, and also expressed *wg* and *vg*. The data suggest that two regulatory modules involving *wg*--*vg* are present in apterygote insects: one associated with lateral body region and induces stick-like dorsal limb branches, the other associated with the boundary of dorsal and lateral body regions and the flat outgrowth of their interface. A combinatorial model is proposed in which dorsal limb branch was incorporated into dorsal--lateral boundary and acquired flat limb morphology through integration of the two *wg*--*vg* modules, allowing rapid evolution of the wing.

INTRODUCTION

Evolutionary novelties are newly acquired, functionally adaptive structures, which have been of critical importance in the diversification of animal forms (Müller and Wagner 1991). The insect wing is a marked novelty that has contributed to insect radiation, and a number of hypotheses have been proposed to explain the wing origin (Kukalová-Peck 1978; Grimaldi and Engel 2005). The hypotheses can be categorized into two classes based on whether the wing is regarded as an essentially novel structure or a modified old structure (Jockusch and Ober 2004; Grimaldi and Engel 2005). Paranotal hypothesis is one of the major ones of the former class and proposes that the wing originated as de novo extension of the thoracic tergum, or paranotal lobe (Crampton 1916; Snodgrass 1935; Hamilton 1971). Limb branch hypothesis represents the later class and proposes that the wing emerged as a modification of the pre-existing dorsal limb branch (Wigglesworth 1973, 1976; Kukalová-Peck 1983; Averof and Cohen 1997).

Each hypothesis has merit and demerit, and opinions have been divided among experts. The paranotal hypothesis is consistent with the flatness and position of the wing in the tergum/pleuron boundary, but the origin of the set of muscles and articulations that allow flapping movement of the wing is unclear (Kukalová-Peck 1978). On the other hand, the limb branch hypothesis allows tracing the candidate origin of wing to branched limbs of crustacean-like ancestors, but explains neither the flatness nor dorsal location of the wing that are crucial elements for the aerodynamic properties for flight (Kukalová-Peck 1978; Jockusch and Ober 2004). Moreover, neither hypothesis has provided compelling explanation for the rapid emergence of the wing in the insect lineage that left no trace of fossil records of intermediate forms of its evolution. Recent study of crustaceans reporting expression of wing-related genes in dorsal gills of branched limbs has supported the limb branch hypothesis (Averof and Cohen 1997). However, this finding cannot be considered as a definitive proof for the limb branch hypothesis, because the forms and functions of crustacean gills and insect wings are different and

the phylogenetic distance between insects and crustaceans is quite far. We approached these questions by comparing the developmental origins of the wing in higher insects and the dorsal limb branches in basal insects.

The insect wing is a nonsegmented, sheet-like outgrowth composed of double-layered epithelia (Fig. 1A) (Snodgrass 1935). The wing hinge is connected to the lateral side of the thoracic tergum (dorsal body wall), where direct flight muscles are attached and control flapping flight. The ventral side of the wing base is supported by the wing process located at the upper end of the pleuron (lateral body wall), and the thoracic leg is flanked by the pleuron and sternum (ventral body wall) (Fig. 1A) (Snodgrass 1935). Thus, the insect thorax is subdivided into dorsal, lateral, and ventral territories, and the wings and leg outgrowths take place at dorsal/lateral and lateral/ventral boundaries, respectively (Fig. 1A).

In *Drosophila*, dorso-ventral subdivisions of the segment begins in embryonic stages when the stripe of *wingless (wg)* expression is repressed in the lateral region (Baker 1988), distinguishing the *wg*-negative lateral body region from the *wg*-expressing dorsal and ventral region (Fig. 1B). Two imaginal discs are specified from a common cell cluster in the mesothoracic segment (Est.12, 15); one is located at the lateral/ventral boundary (ventral disc), and the other is located in the lateral region (dorsal disc) (Cohen et al. 1993; Goto and Hayashi 1997; Kubota et al. 2003). Thus, in *Drosophila*, the segment is subdivided into three dorso-ventral regions in the embryonic stages before the specification of leg and wing primordia.

Ventral discs maintain discontinuous *wg* expression and give rise to the pleuron and the leg (Fig. 1B) (Couso et al. 1993). In the dorsal discs at second instar (L2), *wg* is activated again and induces the wing pouch (Ng et al. 1996). As the wing pouch grows in size, *apterous* (*ap*) subdivides the dorsal disc into dorsal and ventral compartments that form a straight interface in the wing pouch where *wg* and *vestigial* (*vg*) are expressed and specify the wing margin that organizes wing pouch outgrowth (L2 early, L3) (Williams et al. 1991; Couso et al. 1993; Diaz-Benjumea and Cohen 1993; Kim et al. 1995, 1996; Ng et al. 1996). Furthermore, a stripe of *wg* expression appears in the future tergum (L2 early, L3). Thus sequential activation of *wg* in the dorsal discs corresponds to specification and flat outgrowth of the wing pouch, and patterning of the tergum.

The tracheal gill in mayfly (basal Pterygota, Ephemeroptera (Ogden and Whiting 2003) is a filamentous or lamellate respiratory organ in aquatic nymphs, which is articulated to the coxopodites of the first–seventh abdominal limbs (Fig. 1A) (Snodgrass 1935). The stylus in bristletail (Apterygota, Archaeognatha (Sturm and Machida 2001)) is a rod-like outgrowth articulated to the coxopodites of the meso- and metathoracic limbs of fourth instar nymphs and later stages (Machida 1981; Sturm and Machida 2001). Abdominal stylus

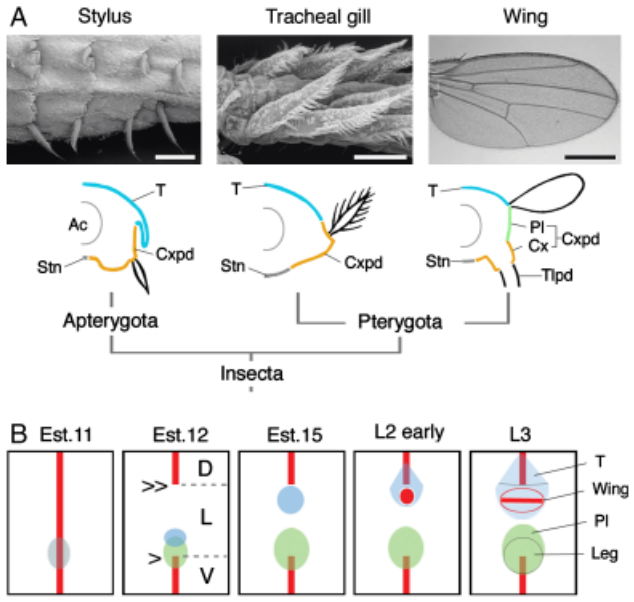

Fig. 1. Dorsal branches of insect limbs and wing development in *Drosophila*. (A) Morphologies of stylus in *Pedetontus unimaculatus*, tracheal gill in nymph of *Ephoron eophilum*, and wing in *Drosophila melanogaster*. Shown below are cross-sectional views of segments in each insect (dorsal up), highlighting their morphological features. Ac, alimentary canal; Cx, coxa; Cxpd, coxopodite (orange); Pl, pleuron (green); Stn, sternum; T, tergum (blue); Tlpd, telopodite. Scale bars: 500 μm for stylus and wing, 100 μm for tracheal gill. (B) Lateral views of *Drosophila* mesothoracic segments from embryonic stage 11 (Est.11) to third larval stage (L3) (based on references (Williams et al. 1991; Couso et al. 1993; Kim et al. 1996; Ng et al. 1996; Shirras and Couso 1996; Goto and Hayashi 1997; Kubota et al. 2003), dorsal up, anterior left). *wg* expression (red line) demarcates three dorsoventral territories. Dorsal disc (wing disc, blue) and ventral disc (leg disc, green) outgrowths take place near the dorsal/lateral (≫) and lateral/ventral (>) boundaries, respectively. Dorsal and ventral discs are superimposed on the larval body segments.

develop in the second–ninth abdominal segments of embryos, that attaches to the coxopodite with direct muscles and functions as an abdomen-supporting organ (Fig. 1A) (Machida 1981; Sturm and Machida 2001). Both of these organs have been proposed to be origins of the insect wing based on morphological similarities, such as the arrangement of direct muscles (Wigglesworth 1973, 1976). However, no embryological comparison has been available so far to verify this model.

In order to gain a better understanding of the ancestral segmental ground plan underlying the emergence of the wing, we examined the primordia of the tracheal gills in the mayfly *Ephoron eophilum* and the styli in the bristletail *Pedetontus unimaculatus*, using *wg, vg*, and *ap* as markers for key signaling activity for induction and outgrowth of the wing primordium (Williams et al. 1991, 1993; Cohen et al. 1992; Couso et al. 1993; Kim et al. 1996; Ng et al. 1996; Ohde et al. 2009) (supporting information Figs. S1 and S2).

MATERIALS AND METHODS

Animals

Adults of *P. unimaculatus* and *E. eophilum* were collected from their natural habitats in Japan. Rearing and egg collection were performed as described previously (Machida 1981; Aoyagi et al. 1998). Both species have a long embryonic period with diapause (about 9 months for *Pedetontus* and 7 months for *Ephoron*). The eggs were periodically dissected to obtain embryos at appropriate stages, according to previous studies (Machida 1981; Tojo and Machida 1997). For expression analysis, *Pedetontus* and *Ephoron* embryos were fixed overnight in 8% paraformaldehyde (PFA) in PBS at 4°C, and in 4% PFA for 2 h at room temperature (RT), respectively. *Ephoron* nymphs were dissected at the midline and fixed overnight in 4% PFA at RT. For SEM, both species were fixed in 2% glutaraldehyde in PBS for 24 h at 4°C, and postfixed in 1% OsO_4 for 2 h at RT.

Cloning and phylogenetic analysis

Homologs of *wg*, *vg*, and *ap* were isolated from embryonic cDNAs of *Pedetontus* and *Ephoron* by PCR using the following degenerate primers:

5′-AYCGMMGMTGGAAYTGCYCNAC-3′ (primer 1),
5′-ACYTCGCARCACCARTGGAANGTRCA-3′ (primer 2) and
5′-CARCACATYARRTCRCAKCCRTCRAC-3′ (primer 3) for *wg*,
5′-ATGTAYSRIGCITAYTAYCCITAYYTITA-3′ and
5′-SWRTTCCARAAISWIGGIGGRAARTT-3′ for *vg*, and
5′-GGIAAYATHTAYTGYAARRAIGAYTAYTA-3′ and
5′-CKIGCRTTYTGRAACCAIACYTG-3′ for *ap*. Accession numbers are AB439847 (*Pedetontus wg*), AB439845 (*Pedetontus vg*), AB486006 (*Pedetontus ap*), AB439848 (*Ephoron wg*), AB439846 (*Ephoron vg*), and AB486007 (*Ephoron ap*). Multiple alignments and phylogenetic analyses (see supporting information) were performed using online version 6 of MAFFT (Katoh and Toh 2008). Trees were drawn using TreeView software (Page 1996). Phylogenetic relationships were deduced by the neighbor-joining method (Saitou and Nei 1987) and support values (>50) for branches were determined by performing 1000 bootstrap repetitions.

In situ hybridization and immunostaining

Whole mount in situ hybridizations using digoxigenin-labeled riboprobes were performed as described previously (Niwa et al. 2000) with slight modifications. Before hybridization, the specimens were treated for 10 min with proteinase K solution of the following concentrations: 30 μg/ml for *Pedetontus* embryos, 2 μg/ml for *Ephoron* embryos, and 20 μg/ml for *Ephoron* nymphs. Hybridizations were performed at 65°C in *Pedetontus* and at 60°C in *Ephoron*, respectively. Sense probes were used as negative controls. Immunostaining protocol was as described previously (Niwa et al. 2000). Rabbit anti-Distal-less (Dll) antibody (a gift from S. B. Carroll) was used at 1:200, and Rabbit anti-aPKC (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at 1:100. Secondary antibody conjugated to Alexa Fluor 488 goat anti-rabbit (Invitrogen, Carlsbad, CA, USA) was used at 1:200.

RESULTS

Expression of *wg* and *vg* genes in primordia of tracheal gill

The tracheal gills of *Ephoron* appear at the lateral sides of abdominal segments (A1–A7) of the second instar nymphs (Aoyagi et al. 1998). We examined *wg* expression in the tracheal gill from embryonic stages to the second nymphal stage. *wg* was initially expressed in a stripe pattern in each body segment, similar to other insect embryos (Fig. 2, A, B, and E) (Niwa et al. 2000). As embryogenesis progressed, the *wg* stripe disappeared in the lateral region of all segments (Fig. 2, C and E), and punctate *wg* expression later reappeared near the dorsal side of the *wg*-negative lateral region (Fig. 2, D and E). Expression of *vg* was detected simultaneously in the same region where *wg* was re-expressed (Fig. 2, F and G). Those cells continued to coexpress *wg* and *vg* in gill-possessing abdominal segments (A1–A7) of the first instar nymph (Fig. 2, H, I, K, and L), and then everted in the second instar (Fig. 2, J and M), suggesting that they are embryonic primordia of the tracheal gill. At the nymphal stages, *wg* and *vg* were expressed weakly in the margin of the growing tracheal gill (Fig. 2, J and M). No expression of *wg* or *vg* was detected in the corresponding region of the thoracic segments of embryos and early nymphs, reflecting the appearance of the wing bud during late nymphal stages (Aoyagi et al. 1998).

Expression of *wg* and *vg* genes in primordia of stylus

In *Pedetontus*, the limb primordium initially forms as a bulky protuberance expressing Dll, a master regulator of arthropod limb development (Panganiban et al. 1997; Angelini and Kaufman 2005), before being split into a massive medial ventral sac and a lateral rod-like stylus (Fig. 3, J–M) (Machida 1981). Similar to the pattern of *wg* expression in *Drosophila* and *Ephoron*, *Pedetontus wg* was also initially expressed as continuous stripes in all body segments (Fig. 3, A and N), and then repressed in the lateral regions of the abdominal segment (Fig. 3, B and N). In the *wg*-negative region that corresponds to the dorsal–proximal side of the limb primordium, *wg* was subsequently re-expressed in the cells where Dll expression is sustained and the future stylus protrudes (Fig. 3, C, K, and N). In contrast to *wg*, *vg* was initially expressed in the distal region of the developing ventral sac, and later in the primordium of the stylus (Fig. 3, F–H). During subsequent stages, the *vg* signal in the ventral sac was restricted to the dorsal side before finally disappearing, whereas distal expression in the developing stylus was continuously observed (Fig. 3, H and I).

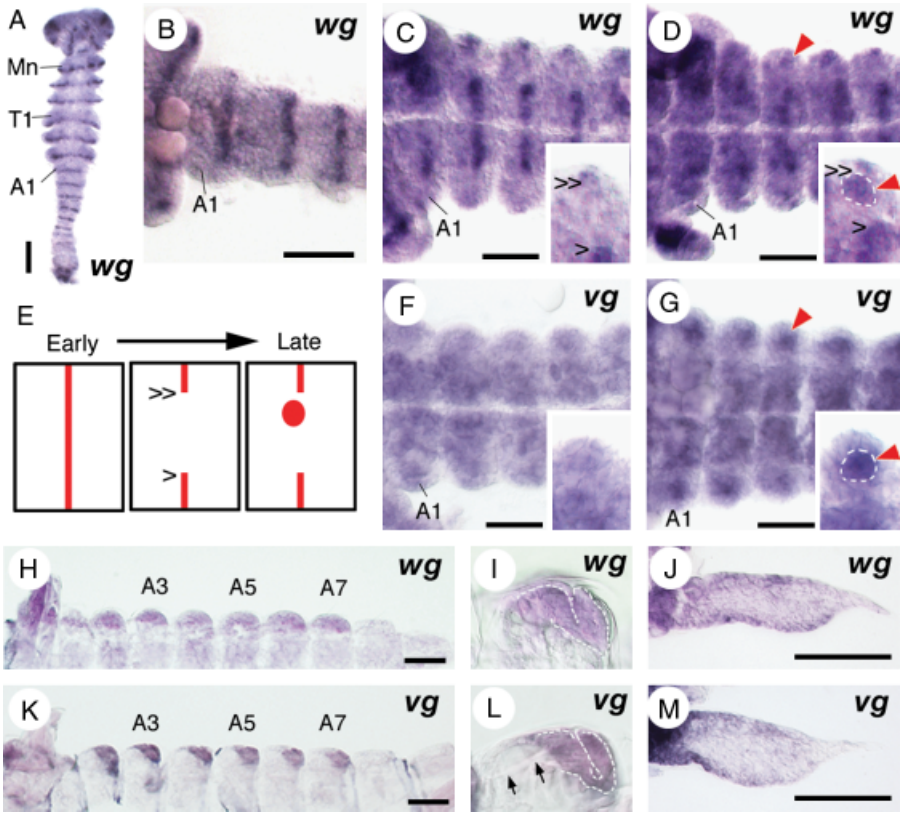

Fig. 2. Expression of *wg* and *vg* in mayfly, *Ephoron eophilum*. (A) Segmental expression of *wg* in early embryo. Orientation: anterior is up. Mn, mandibular segment; T1, first thoracic segment; A1, first abdominal segment. (B–D) Abdominal expression of *wg* before katatrepsis (B), at early katatrepsis (C), and at late katatrepsis (D). (E) Schematic diagrams showing changes in *wg* expression (red) in abdominal segments. Reactivation of *wg* (arrowheads in D, solid circle in E) occurs near the dorsal–lateral boundary (≫). (F and G) Intense expression of *vg* (arrowheads) appeared in lateral region of abdominal segments corresponding to the *wg* reactivation domain (compare insets in C, D, F, G showing higher magnification views of hemisegments). Signals around midline are probably neural expression of *vg*. (H–M) Expression of *wg* (H–J) and *vg* (K–M) in developing tracheal gills during nymphal stages. (H and K) Dorsal view of the gill-possessing abdominal segments (A1–A7) of first instar nymphs. (I and L) Higher magnification views of folding tracheal gill primordia (dashed white line) at lateral sides of abdominal segments (H and K). Arrows indicate where muscles are attached to the tracheal gill. (J and M) Growing tracheal gills in second instar nymphs. Embryos and nymphs (except for A, J, and M) are oriented with anterior side to the left. Scale bars: 50 μm.

Expression of wing-related genes in terga of basal insects

The coexpression of *wg* and *vg* in stylus, tracheal gill and wing specification may support the limb branch hypothesis of wing origin. However, stylus and tracheal gill do not resemble the sheet-like form of the wing and *wg* and *vg* expressions in those organs showed only limited patterns of regional specification (Figs. 2, J and M, and 3, E and I). On the other hand in *Drosophila*, *wg* and *vg* are expressed intensely at the dorsoventral boundary in the wing pouch and activate margin outgrowth (Fig. 1B) (Kim et al. 1996; Ng et al. 1996). To explore the origin of margin-dependent flat outgrowth, we examined the expression of the *ap* gene that confers dorsal compartment identity and specifies the wing margin in the wing primordium in *Drosophila* (Cohen et al. 1992; Williams et al. 1993; Kim et al. 1996; Ng et al. 1996).

In *Ephoron*, *ap* expression was detected in the dorsal ectoderm of the thoracic and all abdominal segments (Fig. 4, A–F). This expression pattern was distinct from that of *vg* that was restricted to internal cells in A1–A7 (Fig. 2, G and K). We concluded that in *Ephoron, ap* expression corresponds to the future tergum, but not the gill primordium. In *Pedetontus, ap* was not detected in the stylus primordium (Fig. 4, I and J). On the other hand, *ap* was intensely expressed in the dorsal region of the segment corresponding to the future tergum (Fig. 4, G–J). In thoracic segments, the ectodermal *ap* expression formed a clear boundary within the tergum (Figs. 3Q and 4, H, K, M, N). During later stages, *wg* and *vg* expression gradually became concentrated at the boundary (Figs. 3, S and T, and 4, L and N, and data not shown), where the cells are aligned along the margin (Fig. 3, U and V). This boundary region corresponds to the lateral lobe margin of the thoracic tergum (paranotum), which extends broadly and protects the lateral side of the body (Kukalová-Peck 1978; Sturm and Machida 2001).

DISCUSSION

Lateral body region is a common ground for induction of wing, tracheal gill, and stylus

Down-regulation of the early *wg* stripe in the lateral region of the segment was observed not only in *Drosophila*, *Ephoron*, *Pedetontus*, but also in the other insects such as *Gryllus* (Niwa et al. 2000) and *Tribolium* (Bolognesi et al. 2008), suggesting that the three dorsal–ventral subregions demarked by *wg* are

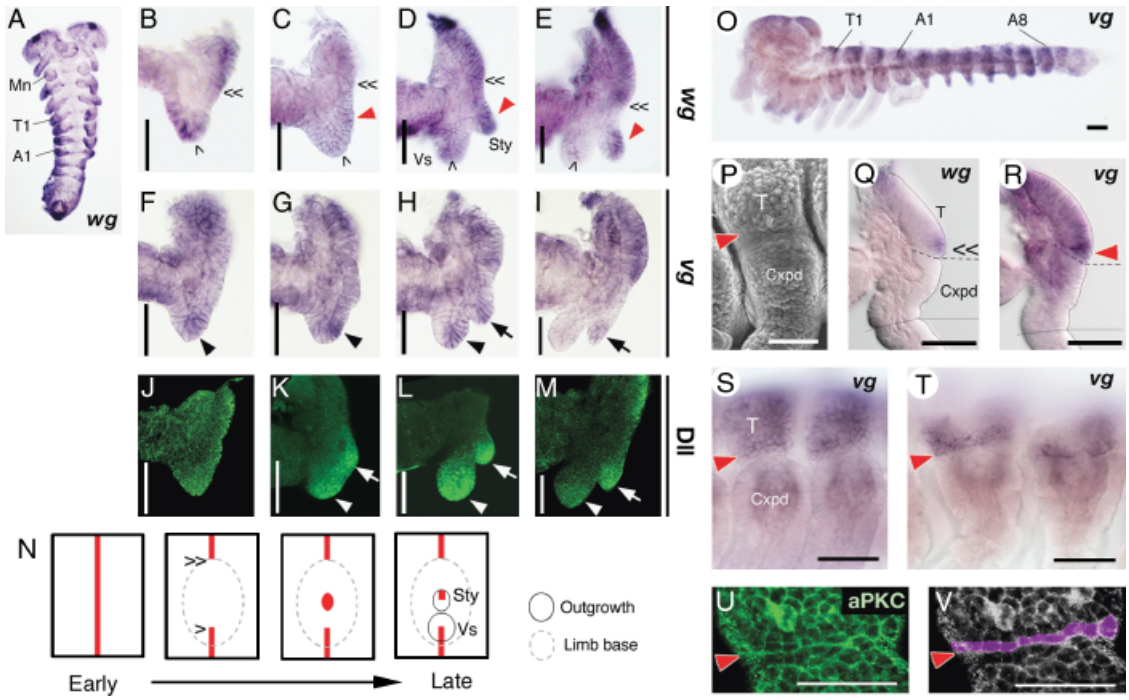

Fig. 3. Expression of *wg* and *vg* in jumping bristletail, *Pedetontus unimaculatus*. (A) Segmental expression of *wg* in early embryo. Orientation: anterior is up. (B–M) Cross-sectional view of abdominal hemisegments showing expression of *wg* (B–E), *vg* (F–I), and Dll (J–M) in developing abdominal limbs, ventral sac (Vs, arrowheads) and stylus (Sty, arrows). Reactivation of *wg* (red arrowhead in C) appears at the lateral body region, and later localizes to the dorsal side of developing stylus (red arrowheads in D and E). In addition to the expression at developing limbs, intensity of *wg* and *vg* signals at dorsal body region are slightly increased at late stage (E and I). The Dll expression at dorsal body region is non-specific signal (M). Orientation: dorsal side of segment (proximal end of limb) is up, midline of segment (ventral side of limb) is left. (N) Schematic diagrams of lateral view of developing abdominal segments showing changes in *wg* expression (red). Orientation: dorsal is up, anterior is left. (O) Lateral view of late embryo showing expression of *vg* in thoracic and abdominal terga. A8, eighth abdominal segment. Orientation: dorsal is up, anterior is left. (P) SEM image showing the tergum-coxopodite boundary in mesothoracic segment at same embryonic stage (O). (Q and R) Cross-sectional view of mesothoracic hemi-segments showing expression of *wg* (Q) and *vg* (R) in ventral margin of developing tergum. Higher magnification views around the boundary between tergum and coxopodite (dotted line) are shown in Fig. 4, K and L. Orientation: dorsal side of segment (proximal end of limb) is up, midline of segment (ventral side of limb) is left. (S and T) Lateral view of *vg* expression in meso-, metathoracic segments at early (S) and late (T) stages. (U and V) Expression of subapical marker aPKC (Tepass et al. 2001) showing the cellular arrangement around the tergum–coxopodite boundary in mesothoracic segment at same stage (T). A row of cells is aligned along the ventral margin of tergum (pseudo-colored magenta in V). Arrowheads: ventral margin of developing tergum. Scale bars: 50 μm.

coextensive across insect species. Each subregion closely corresponds to the morphological dorsal, lateral, and ventral body walls of the *Drosophila* abdomen (Shirras and Couso 1996), suggesting that the dorsal–ventral subregions may be used as a stable framework for mapping the positions of various appendages across insect species (Fig. 5, left).

In all three cases of dorsal limb-branch formation examined here, *wg* and *vg* were coexpressed in the early stages of their specification, and their expression domains were located in the lateral region. These similarities suggest that *wg* and *vg* act as a common inductive signal of the stylus, tracheal gill and wing, and the competence for induction is restricted to the lateral region. Consistently, the prospective lateral body wall in the proximo-dorsal region of the *Drosophila* leg disc, where endogenous *wg* is not expressed, is permissive for transformation into wing disc in response to ectopic *wg* (Maves and Schubiger 1998). Furthermore, in *Drosophila* embryos, sustained expression of *wg* in the lateral region suppresses dorsal disc (wing disc) specification (Kubota et al. 2003), implying that *wg* must be turned off once to become competent for further specification of dorsal appendage. These findings suggest that the absence of *wg* expression in the lateral body region provides a relatively open field for *wg* reactivation to induce dorsal limb branches.

Close examination revealed variation in the position of dorsal limb branches. The stylus primordia emerged at a distance from both the dorsal/lateral and lateral/ventral boundaries (Fig. 3, C and N), whereas the tracheal gill primordium was located much closer to the dorsal/lateral boundary (Fig. 2, D and E). Because there are no reported cases of duplicated appearances of these dorsal branches observed among insect species, the variability in the position indicates an evolutional

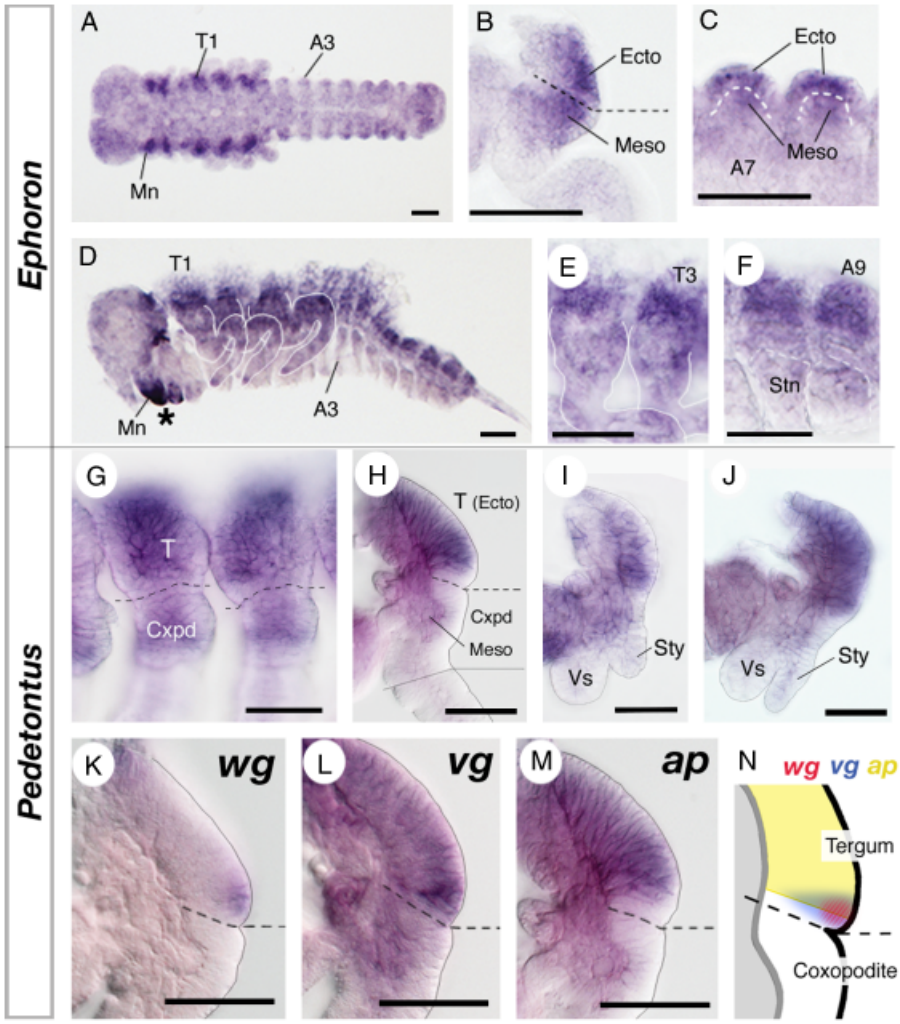

Fig. 4. Expression of *ap* in the mayfly and jumping bristletail. (A–F) *Ephoron eophilum* (mayfly). (G–N) *Pedetontus unimaculatus* (jumping bristletail). (A–C) Expression of *ap* in whole embryo (A), thoracic limb (B), and abdominal hemisegments (C) at middle stage. Intense signal is detected at dorsal ectoderm (Ecto) of body segments and mesoderm (Meso) of limb base of gnathal and thoracic segments. Mesodermal signal in abdominal segments is weaker than thoracic segments. (D–F) Lateral view of late embryo (D), thoracic (E), and abdominal segments (F) showing *ap* expression at developing terga. Orientation: anterior is left (A, D), dorsal is up (B, C, E, F). (G–J) Expression of *ap* in thoracic (G and H) and abdominal segments (I and J). (G and H) Lateral view of meso-, and metathoracic segments (G) and cross sectional view of mesothoracic hemisegment (H) showing intense *ap* expression at terga and weak expression in mesoderm of coxopodites. (I and J) Cross-sectional view of *ap* expression in abdominal segments at early (I) and late (J) stages. (K–M) High-magnification view of expression of *wg* (K), *vg* (L), and *ap* (M) near the tergum-coxopodite boundary (dotted line) of the leg. (N) Schematic diagram of the expression domains of the three genes showing intense signals of *wg* and *vg* at the tergal margin specified by *ap* activity. Orientation: dorsal is up, anterior is left (G), midline of segment is left (H–N). Asterisk indicates nonspecific signals in gnathal limbs. A3 and A9, third and ninth abdominal segments; Mn, mandibular segment; Stn, sternum; Sty, stylus; T, tergum; T1 and T3, first and third thoracic segments; Vs, ventral sac. Scale bars: 50 μm.

drift of the dorsal limb branch location. We speculate that changes in the enhancer sequences that regulate expression of *wg* and *vg* genes in response to dorsal–ventral positional cues is one explanation for the drift of dorsal appendage positions in the lateral body region during insect evolution (Fig. 5).

Margin outgrowth activity in the body wall of basal apterygote insect

In *Pedetontus* embryos, the wing-related genes are expressed not only in the dorsal limb branch (stylus) but also in the dorsal body wall (tergum) (Figs. 3 and 4, G–N). Particularly, the region of paranotal margin shares remarkable similarity to the dorso-ventral compartment boundary in the *Drosophila* wing pouch in terms of gene expression patterns and cell arrangement (Figs. 3, U and V, and 4, K–N) (Fristrom and Fristrom 1993; Kim et al. 1996; Ng et al. 1996). These results suggest that the development of paranotal lobe is regulated by *ap, wg*, and *vg* which act as a module for margin outgrowth, and favors the paranotal hypothesis of wing origin. In addition to apterygote bristletails, the ventral extension of the lateral lobe of tergum is described in analogous positions in many extant and extinct arthropods, such as trilobites and crustaceans (Kukalová-Peck 1978). It was recently demonstrated that, in cladoceran crustacean *Daphnia*, the flat sheet-like growth of the dorsal shield uses a strikingly similar signaling mechanism to that of insect wings (Y. Shiga in Tokyo University of Pharmacy and Life Sciences, personal communication), suggesting that the module for margin outgrowth was independently deployed for body wall expansion in basal arthropods before the appearance of the insect wing.

Re-evaluation of the crustacean gill theory

Gill of crustacean limbs and related respiratory organs (book lung, book gill) of chelicerates have been suggested to be

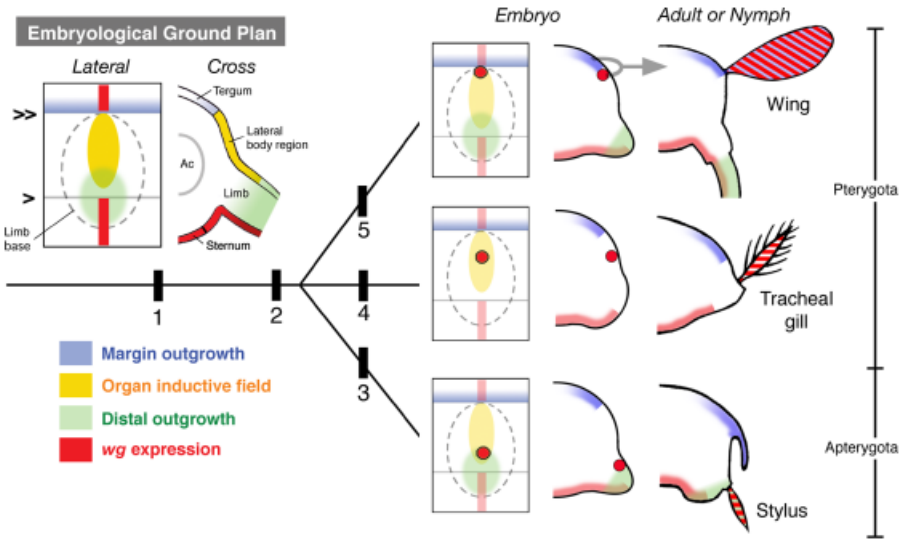

Fig. 5. Embryological ground plan of insect segments and a combinatorial model for evolution of the insect wing. Left. Lateral and cross-sectional view of a generalized insect segment showing *wg* expression (red) and subdivision into three regions; tergum, sternum, and lateral body region. The boundary of tergum-lateral body wall is the region for margin outgrowth (blue) specified by the module involving *ap, wg*, and *vg*. The lateral region lacks *wg* expression and provides a relatively open field for reactivated *wg* (red circles) to induce dorsal limb branch (organ inductive field, yellow). Right. Proposed scenario of insect wing evolution (solid lines) with key events: (1) Establishment of segmental ground plan with *wg*-free lateral region, (2) Acquisition of the *wg–vg* module for dorsal limb branch induction, (3) Stylus formation, (4) Tracheal gill formation, (5) Wing formation. Position of the inductive *wg–vg* expression varied within lateral region (evolutionary drift). A successful combination of the two modules for dorsal limb branch induction and margin outgrowth near the tergum lateral-body wall boundary facilitated rapid emergence of the sheet-like wing outgrowth.

evolutionary related to insect wing based on the expression of *ap* and another wing-related genes *nubbin* in those respiratory appendages (Averof and Cohen 1997; Damen et al. 2002). If *ap* expression indeed represents deep homology among those movable limb branches and insect wing, two predictions can be made. First, *ap* expression in basal arthropods should reflect at least some aspect of essential function of *ap* in wing formation. In the case of insect wing the key function of *ap* is to specify dorsal compartment so that the dorsal–ventral compartment boundary function as the wing margin organizer. In gills or related organs in crustaceans and chelicerates, *ap* is expressed broadly and does not show any sign of compartmentalization (Averof and Cohen 1997; Damen et al. 2002), suggesting that *ap* cannot play any compartment-related role in those organs. Indeed, those gills have no margin-like morphological feature. Second prediction is that movable limb branches of intermediate species between crustaceans and winged insects should inherit *ap* expression. In the present study of dorsal limb branches of basal insects, we found no sign of *ap* expression in tracheal gill of mayfly or stylus of bristletail (Fig. 4, C, D, I, J), thus *ap* cannot be used as a marker for tracing related limb branches in close relative of winged insects. At present, it is equally possible that *ap* activities in the crustacean or cheliceratan limbs were gained in each lineages, and has no relation to insect wing evolution. Indeed, the compartmentalized expression of *ap* homolog *Lmx1* in vertebrate limb buds (Riddle et al. 1995) suggests that *ap* has independently adopted the role of compartment-related function in distant lineages. Whereas available evidence suggests that dorsal limb branches of basal insects are related to branched limbs of crustaceans, whether the wing-related characters have already existed in crustacean-like ancestors remains an open question.

Combinatorial model of insect wing evolution

Our analysis in basal insects indicates that the *wg*-negative lateral body region serves as the field for organ induction where dorsal limb branches are induced by *wg* and *vg* activity. We further show that, in *Pedetontus*, the growing tergal margin expresses a set of genes required for the margin outgrowth in the wing primordium of *Drosophila* (Fig. 4, K–N). These results suggest that, in apterygotes, two developmental modules involving *wg* and *vg* are present in the lateral body region; one induces the dorsal appendage and the other is coupled to the border of *ap* expression and specifies margin outgrowth. We propose a developmental model for wing evolution in which the inductive *wg–vg* signal drifted its position within the lateral body region, allowing the primordia of the movable dorsal limb branch to become incorporated morphologically into the tergum, where pre-existing margin specifying activity of *ap–wg–vg* is integrated to promote a sheet-like outgrowth. The modules for organ induction and margin outgrowth, both involving *wg* and *vg*, are controlled by common positional signals at the tergum-lateral body boundary, and this combination synergistically catalyzes the rapid emergence of a sheet-like wing (Fig. 5). In this combinatorial model, the thoracic tergal edge (paranotal lobe) is considered to be a key source of wing margin formation. And, as the insect's lateral body wall is derived from the dorsal part of the coxopodite (Snodgrass 1935; Matsuda 1970), our model also supports the hypotheses that the insect wing correlates to an organ derived

from a proximo-dorsal part of the arthropod leg, such as crustacean epipods (Averof and Cohen 1997) or putative epicoxal exites (Kukalová-Peck 1983). Thus, our model builds on the paranotal theory (Crampton 1916; Snodgrass 1935; Hamilton 1971) and limb branch theory (Wigglesworth 1973, 1976; Kukalová-Peck 1983; Averof and Cohen 1997) for the origin of wings. Shared modules for induction of the stylus, tracheal gill and wing, however, does not necessarily indicate serial homologies or stepwise modifications among these organs, as there are proven examples of non-homologous structures that share developmental modules (Bolker and Raff 1996). Rather, we propose that the wing is one of a number of organ types that have developed from the dorsal side of the appendicular territory in arthropods, in which a common genetic ground plan for organ induction is conserved.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Sequence analysis of Wg and Vg of *Pedetontus unimaculatus* (bristletail) and *Ephoron eophilum* (mayfly). (**A**) Multiple alignment of *Wg*/*Wnt1* orthologs from arthropods and vertebrates. Asterisks indicate identical amino acids including conserved cysteine residues (shaded). Arrows indicate positions of PCR primers used to isolate *wg* homologs. (**B, C**) Gene-tree depicting the sequence relationship of *Pedetontus*-Wg (Pu-Wg) and *Ephoron*-Wg (Ee-Wg) to *Drosophila* Wnt family members (B), and with Wg/Wnt1 orthologs from arthropods and vertebrates (C). Trees show clear assignment of the Pu-Wg and Ee-Wg to the Wg/Wnt1 groups of insects. Phylogenetic relationships were deduced by the neighbor-joining method and support values (>50) for branches were determined by performing 1000 bootstrap repetitions. Scale bars indicate the number of amino acid substitutions per site. Accession numbers of sequences are as follows: Q9NIF7 (*Thermobia wg*; *Td-wg*), Q9GRA6 (*Gryllus wg*; *Gb-wg*), Q9TX64 (*Tribolium wg*; *Tc-wg*), P49340 (*Bombyx wg*; *Bm-wg*), P09615 (*Drosophila wg*; *Dm-wg*), Q8T396 (Spider *wg*; *Cs-wg*), P24257 (Zebrafish *Wnt1*; *Dr-Wnt1*), P10108 (*Xenopus Wnt1*; *Xl-Wnt1*), Q3UR96 (Mouse *Wnt1*; *Mm-Wnt1*), P28465 (*Dm-Wnt2*), P40589 (*Dm-Wnt4*), P28466 (*Dm-Wnt5*), Q9VM26 (*Dm-Wnt6*), Q9VFX1 (*Dm-Wnt8*/*D*), Q9VM25 (*Dm-Wnt10*).

Fig. S2. Sequences of conserved domains of Vg and Ap of *Pedetontus unimaculatus* (bristletail) and *Ephoron eophilum* (mayfly). (**A**) Multiple alignment of conserved regions of the scalloped interaction domain (SID) (Halder and Carroll 2001) in Vg orthologs from insects and vertebrates. (**B**) Multiple alignment of conserved LIM domains and homeodomain (Cohen et al. 1992) in Ap orthologs from arthropods. The alignments show that each domain is also highly conserved in *Pedetontus* and *Ephoron*. Amino acid identities identical to *Pedetontus* and/or *Ephoron* proteins are shaded. Asterisks indicate conserved amino acids among all species. Accession numbers of sequences are as follows: Q17G05 (Mosquito *vg*), Q26366 (*Drosophila vg*), Q5RJA2 (Zebrafish *Vgl2*), Q7T0X1 (*Xenopus Vgl2*), Q8BGW8 (Mouse *Vgl2*), Z98880-5 (Human *Vgl2*), X65158 (*Drosophila ap*), Y09914 (*Artemia ap*), AJ420132 (*Cupiennius ap-1*).

Supplementary References

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