Origin and diversification of wings: Insights from a neopteran insect

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Edited by Sean B. Carroll, Howard Hughes Medical Institute and University of Wisconsin-Madison, Madison, WI, and approved November 3, 2015 (received for review May 18, 2015)

Winged insects underwent an unparalleled evolutionary radiation, but mechanisms underlying the origin and diversification of wings in basal insects are sparsely known compared with more derived holometabolous insects. In the neopteran species *Oncopeltus fasciatus*, we manipulated wing specification genes and used RNA-seq to obtain both functional and genomic perspectives. Combined with previous studies, our results suggest the following key steps in wing origin and diversification. First, a set of dorsally derived outgrowths evolved along a number of body segments including the first thoracic segment (T1). Homeotic genes were subsequently co-opted to suppress growth of some dorsal flaps in the thorax and abdomen. In T1 this suppression was accomplished by *Sex combs reduced*, that when experimentally removed, results in an ectopic T1 flap similar to prothoracic winglets present in fossil hemipteroids and other early insects. Global gene-expression differences in ectopic T1 vs. T2/T3 wings suggest that the transition from flaps to wings required ventrally originating cells, homologous with those in ancestral arthropod gill flaps/epipods, to migrate dorsally and fuse with the dorsal flap tissue thereby bringing new functional gene networks; these presumably enabled the T2/T3 wing's increased size and functionality. Third, "fused" wings became both the wing blade and surrounding regions of the dorsal thorax cuticle, providing tissue for subsequent modifications including wing folding and the fit of folded wings. Finally, *Ultrabithorax* was co-opted to uncouple the morphology of T2 and T3 wings and to act as a general modifier of hindwings, which in turn governed the subsequent diversification of lineage-specific wing forms.

wing origins | *Sex combs reduced* | *Ultrabithorax* | RNA-seq | *vestigial*

Some 350 million years ago, the development of insect wings was a seminal event in the evolution of insect body design (1, 2). The ability to fly was critical to insects becoming the most diverse and abundant animal group, and the origin of such novelty has been a focus of intense scientific inquiry for more than a century (3, 4). More recently, through studies of genetic model systems such as *Drosophila*, the mechanisms of wing morphogenesis have been elucidated (5–12). Still lacking however is a comprehensive understanding of transitional steps connecting the morphology of structures observed in the fossil record with that of the modern-day insects, including wing origins and subsequent diversification.

The initial stages of insect wing evolution are missing from the fossil record and it is therefore necessary to use indirect evidence from fossils that postdate the origin and initial radiation of pterygotes (2). Larvae of many of those taxa featured dorsally positioned outgrowths on each of the thoracic and abdominal segments (2, 13), apparently serial homologs (i.e., similar structures likely arising from a common set of developmental mechanisms). Diverse lineages independently lost those dorsal appendages on the abdomen while undergoing parallel modifications of wing-like structures on thoracic (T1–T3) segments. Specifically, the T1 winglets were always much smaller in fossils and apparently lacked hinge articulation whereas T2 (fore-) and T3 (hind-) wings were fully operational in adults, featuring muscles, venation, and size that rendered them capable of flapping flight (14). T1 winglets were subsequently repressed in multiple lineages (15–18) whereas T2 and T3 wings acquired morphology similar to modern day Paleoptera (mayflies and dragonflies) and other extinct paleopterous orders. The transition from Paleoptera, which rest with wings extended from the body, to Neoptera, which rest with wings folded flat against the body, required changes in the hinge mechanism, with many orders also evolving a precise mechanical fit between wing margins and the adjacent body wall of the dorsal thorax. Finally, the radiation of Neoptera encompassed a further divergence between the fore- and hindwings in terms of their shape, size, and texture (5, 19, 20). Together, this set of transitions accounts for major features of the diversity and lineage-specific wing morphologies among fossil and extant taxa.

To gain insight into genetic mechanisms governing these transitional steps, we used a direct-developing neopteran species, the milkweed bug (*Oncopeltus fasciatus*; Hemimetabola, Hemiptera). The phylogenetic placement of *Oncopeltus*, basal to the more derived holometabola (e.g., flies, beetles, bees, butterflies, and so forth that have a pupal stage), is important because holometabolous appendage development occurs from imaginal discs, which are collections of cells that form and commit to appendage

Significance

De-repressing appendage growth induces development of ectopic wings on the dorsal prothorax (T1) of the neopteran insect *Oncopeltus*. These T1 wings, albeit fully developed, are small and of primarily dorsal origin. Transcriptome data indicate that incorporation of ventrally originating tissue was a key evolutionary innovation for generating large and useful T2 and T3 wings. Complimentary functional experiments reveal that wings and an adjacent thoracic plate are not developmentally distinct structures, and are coregulated to create tight wing folding that arose during the transition from paleopteran to neopteran insects. Finally, *Ultrabithorax* regulates the divergence of fore- and hindwing morphology, a culminating but also ancient feature of insect wing diversity. These innovations account for major features of insect wing origin and diversification.

Author contributions: V.M., J.H.M., and A.P. designed research; V.M., H.W.F., J.P.D., and N.M. performed research; H.W.F., J.P.D., and J.L. contributed new reagents/analytic tools; V.M., J.H.M., and A.P. analyzed data; and V.M., J.H.M., and A.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA), www.ncbi.nlm.nih.gov/sra (accession no. SRP066252).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1509517112/-/DCSupplemental.

identity early in larval development. *Oncopeltus* and other hemimetabolous insects lack imaginal discs and acquire adult morphology gradually through a series of nymphal stages, similar to early fossil insects. Hence, examination of wing development in a hemimetabolous insect can help resolve the ancestral versus derived status of developmental traits present in holometabola and in general may provide new evolutionary perspectives.

Oncopeltus features brightly colored forewings with a stiff proximal region and a more flexible membranous apex (hemelytra), entirely membranous hind wings, and a well-developed dorsal T2 structure (scutellum) that fits precisely against trailing edges of the folded wings. To examine wing developmental mechanisms underlying these features, we combined a candidate gene approach in which we depleted (via RNA interference, RNAi) the expression of *Oncopeltus* orthologs of wing specification genes, and a global approach (RNA-seq) (21) that characterized all expressed genes in wild-type T2 and T3 wings, ectopic T1 wings, and wild-type T1 body wall. The results provide independent and expanded insights into the origins and fate of T1 wings, the transition from paleoptera to neoptera, and the eventual diversification of T2 and T3 wing morphology.

Results

Insights About Wing Origins and Functionalization from T1 Ectopic Wings. Depletion via RNAi of the homeotic gene *Sex combs reduced* (*Scr*), which regulates T1 segmental identity (8), results in the formation of a small ectopic wing on T1 (16). Similar small T1 wings are present in diverse early insect fossils (2). Therefore it is possible that repression of *Scr* releases an ancient wing developmental program. Alternatively, the small T1 ectopic wing (T1 EW) may be a partial conversion to T2 segmental identity; that is, a lower-penetrance phenotype but otherwise analogous to what occurs in T3 when *Ultrabithorax* (*Ubx*) is repressed (see below).

To begin to resolve these alternatives, we characterized the transcriptome (RNA-seq) (21) of the developing wing pads of fifth-instar nymphs on all three thoracic segments (including T1 EW in *Scr* RNAi individuals) and wild-type T1 thoracic body wall (Fig. 1). In the 500 genes most up-regulated in each of the three wing types compared with the T1 body wall, genes involved in imaginal disc morphogenesis were among the most enriched Gene Ontology (GO) categories [Functional group analyses, Database for Annotation, Visualization and Integrated Discovery (DAVID) (22), analyzing Biological Processes level 5: development] (Table S1). Among those was a set of known wing specification genes [*vestigial* (*vg*), *scalloped* (*sd*), *wingless* (*wg*), and *apterous* (*ap*)] (Fig. 2*A*) whose expression did not differ among the three wing types ($P = 0.22$). However, the wing disc and general appendage specification gene *Distal-less* (*Dll*) had reduced expression in T1 EW (more than fourfold lower than T2 wing), as did a number of genes detected only in T2/T3 (Fig. S1 and Table S2).

Most conspicuously absent from T1 EW was *trachealess* (*trh*) (Fig. 1), an arthropod ventral appendage gene (23) expressed in the epipods of *Artemia* (24). During *Drosophila* embryogenesis *trh* is expressed in a cell population that migrates from the ventral ectoderm dorsally to become either glands (corpora cardiaca, prothoracic gland) in anterior segments, or tracheae in segments T2 through A8 (25, 26). Also absent from T1 EW (Table S2) was a wing-specific isoform of *salimus* (*sls*), a gene involved in muscle development and attachment (27–29) that in *Drosophila* is coexpressed with *trh* in ventral-origin cells. Another tracheal morphogenesis gene, *forked* (*f*) (30), involved in formation of mechanosensory wing bristles (31), was among the wing-specific genes missing from T1 EW. Additional *trh*-regulated genes (*vvl*, *spalt*, and *Stat92E*) (26) and the ventral-origin wing specification gene *nubbin* (*nub*) (32) were detectable but reduced in T1 EW compared with T2 wing ($P = 0.0001$; mean difference > twofold) (Fig. 2*B*). The transcriptome data are therefore robust in showing that T1 EW is developmentally a wing but lacks or has markedly reduced expression of ventral-origin genes such as *Dll*-, *nub*-, *trh*-, and *trh*-regulated genes that are consistently up-regulated in T2 and T3 wings.

A previous study demonstrated fusion of dorsal and ventral cell populations during the development of wings in basal insects (mayflies: Paleoptera, Ephemeroptera) (33) and hence a strong up-regulation of both dorsal and ventral-origin genes may be a fundamental feature of normal insect wing morphogenesis, with ventral-origin genes specifically absent in T1 wings that were an evolutionary dead end. Alternatively, it is possible that the timing of ectoptic wing development on T1 in *Oncopeltus* differs from that of T2/T3 wings and therefore the differences in size and gene expression might reflect delayed development of the T1 EW. Hence, we used the transcriptome data to determine if there is evidence for delayed development in T1 EW, and if there is broad support for a reduced ventral contribution to the T1 EW. First, to address developmental timing, we examined expression of *lama*, a gene that preserves the pluripotency of disc cells and is not expressed after tissue determination (34). *lama* was more highly expressed in T2 and T3 wings whereas it was nearly undetectable in T1 EW and the T1 body wall (Fig. 1). Absence of *lama* from T1 EW indicates completion of development rather than delay. In addition, functional studies in *Periplaneta americana* (cockroach, another neopteran) show that the depletion of *Scr* also generates small prothoracic wings (17) despite the difference that RNAi can be applied consecutively over five to six nymphal stages (versus only two stages in *Oncopeltus*). Furthermore, a mutation in another cockroach (*Blatella germanica*) creates small T1 wings (35). Hence, the small size of T1 ectopic wings in neoptera is highly unlikely to be an artifact resulting from insufficient developmental time to fully grow these appendages. The uniqueness of T1 EW is further supported by the hundreds of genes down-regulated at least fourfold in T2 wings (and generally similar in T3 wings) but not down-regulated in T1 EW (green points in Fig. 1). These independent lines of evidence fully support the notion that the ectopic T1 wing is both a unique and fully differentiated structure rather than being an incompletely developed forewing.

To determine if there is broad transcriptome-wide evidence for underrepresentation of ventral-origin genes in the T1 EW, we determined the expression level of all genes with *Drosophila* best-hit homologs having GO Biological Processes annotations containing "dorsal closure" or "ventral furrow" (but not both), based on the assumption that these genes are expressed more strongly at opposite ends of the dorso-ventral body axis. T2 wings had reduced expression of dorsal closure genes ($n = 149$) compared with T1 EW, but greater expression of ventral furrow genes ($n = 17$; $P = 0.03$ for the mean T2-T1 EW difference). The expression

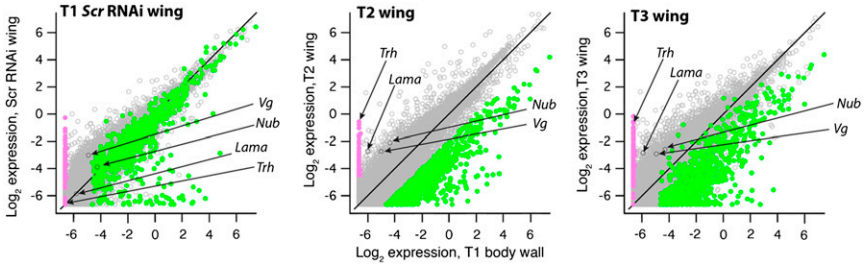

Fig. 1. Gene expression [mean $\log_2$ (normalized read count + 0.01)] in the three wing libraries compared with the same gene in T1 body wall. Genes undetectable or nearly so in T1 body wall but up-regulated >fourfold in T2 wings are colored pink. Genes down-regulated >fourfold in T2 wings are shown in green.

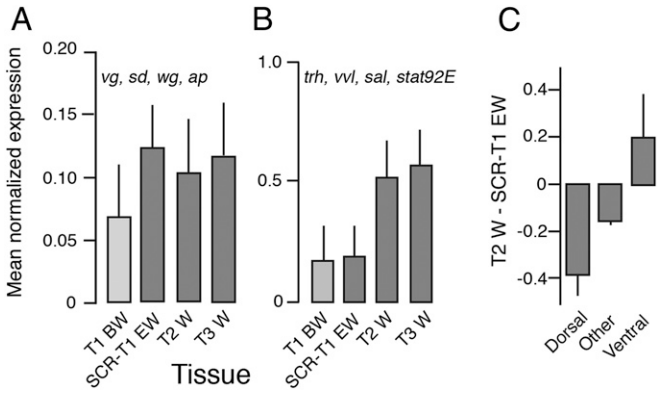

Fig. 2. Least-square means (+ SE) of normalized read counts of (*A*) a set of wing specification genes (*vg*, *sd*, *wg*, and *ap*) equally expressed in all wing types, and (*B*) the ventral origin genes *nub* and tracheal genes (*trh*, *vvl*, *sal*, and *stat92E*) that are missing in the T1 *Scr* RNAi ectopic wing. These means are from a model that included "gene" and "tissue" as independent variables. (*C*) Transcriptome-wide mean expression difference between T2 wing and T1 *Scr* RNAi ectopic wing for genes involved in development of dorsal closure, ventral furrow, or neither.

difference of all other genes (n = 12,257) was intermediate (Fig. 2*C*). This result provides broad support for reduced expression of ventral-origin genes in the T1 ectopic wing.

Effects of Wing Specification Genes on Neopteran Thoracic Segments. In adult *Oncopeltus* from nymphs treated with RNAi against key wing genes (*vg*, *sd*, and *nub*), the main effect in T1 was localized in the posterior dorsal pronotum (T1) (Fig. S2). No visible effects were observed on the ventral T1 plates, including the regions corresponding to sternum and epimeron (Fig. S2 *E*–*H* and *E*′–*H*′). Although these two regions are completely missing in *Tribolium vg* RNAi adults (18), the only change observed on the ventral prothorax in *Oncopeltus* is restricted to an area surrounding the leg base (compare Fig. S3*B* with Fig. S3*E*). Hence, the functions of wing specification orthologs in *Oncopeltus* T1 are mainly localized to the dorsal pronotum and have an effect primarily on its overall width (Fig. S4).

Adult T2 morphology in *Oncopeltus* is comprised of semimembranous wings (hemelytra) that fit tightly against a large, triangularly shaped scutellum on the dorsal side (Fig. 3 *A*, *B*, *F*, and *J*). Tucked beneath the hemelytra is a pair of membranous T3 hindwings, with distinct shapes and pigmentation (Fig. 3*B*). Both pairs of wings in *vg* RNAi adults had alterations in their shape and size, especially the hindwings, which became greatly reduced (Fig. 3*C*). The *sd* RNAi wings displayed similar changes, but to a lesser degree (Fig. 3*D*), consistent with studies in *Drosophila* where the wings of *sd* mutants do not result in a complete loss of wings (36). The depletion of *nub* also caused a reduction in the fore- and hindwing size, and a change in forewing shape (Fig. 3*E*).

These genes also affected the scutellum on T2. No such effects were previously observed in beetles (18, 37), possibly because radical reconfiguration of the thorax during the pupal state in holometabolous insects may override any such effects. In *Oncopeltus vg* RNAi adults, the scutellum lost its tip, and its distinct triangular morphology was changed to a shield-like shape (Fig. 3*G*). *sd* RNAi also affected the posterior half of the scutellum, but to a lesser degree (Fig. 3*H*). In *nub* RNAi adults, the scutellum was not affected but there was a curving of the forewing clavus [the location of *nub* expression in *Drosophila* wings (38)], preventing the wings from lying flat (Fig. 3*M*).

To determine which part of the developing wing pad gives rise to thoracic structures, we performed heat lesions to the central region and lateral extensions (Fig. S5). The former resulted in a misshapen scutellum and unaffected wings whereas the latter had the opposite affect: the wings were malformed and the scutellum remained unchanged. This result suggests that the central region of the developing wing pad gives rise to the scutellum and the lateral extensions form the wing blade. When combined with the RT-PCR results that show *vg* is expressed in both the central region and lateral extensions, whereas *nub* is expressed in only the extensions (Fig. S6), these observations corroborate the above RNAi phenotypes, where *vg* affected both the wing blade and scutellum but *nub* affected only the wing blade (Fig. 3 *C* and *G* vs. Fig. 3 *E* and *I*).

What Developmental Mechanisms Create a Fully Formed and Functional Wing, Including Fit with Thoracic Plates? The present insights from *Oncopeltus* show, to our knowledge for the first time, that *vg*, which can be thought of as a master wing regulator, also has a function in the T2 notum, namely its central region: the scutellum. Hence, the scutellum may also represent part of the wing program based on the interpretation from previous studies where *vg*-dependent tissues are considered to be wing serial homologs (18, 37).

A possible caveat to results depicted in Fig. 3*G* is that the RNAi was performed at fourth and fifth nymphal stages when wing pads already contained a well-developed scutellum primordium. In other words, the obtained phenotype may be a hypomorph and only partially representative of the full function of *vg* in the dorsal T2 notum. Ideally, one would perform such experiments at the initial stages of development of the structures under study. Such an opportunity exists in *Oncopeltus*, where the depletion of *Scr* at late nymphal stages leads to the development of both ectopic scutellum and wings on the prothorax (Fig. 4*B*) (16), thus allowing us to examine the functions of *vg* while these structures are developing de novo. First, we performed the double *Scr*/*vg* RNAi. If *vg* has a key function in wing development with a secondary role in the scutellum, then the T1 wings should not develop but scutellum will, and only its posterior region will be modified. Instead, however, neither structure developed in *Scr*/*vg* RNAi individuals (Fig. 4 *C* and *C*′). This result reveals that in addition to its function in wings, *vg* also acts as a key regulator for initialization of the entire

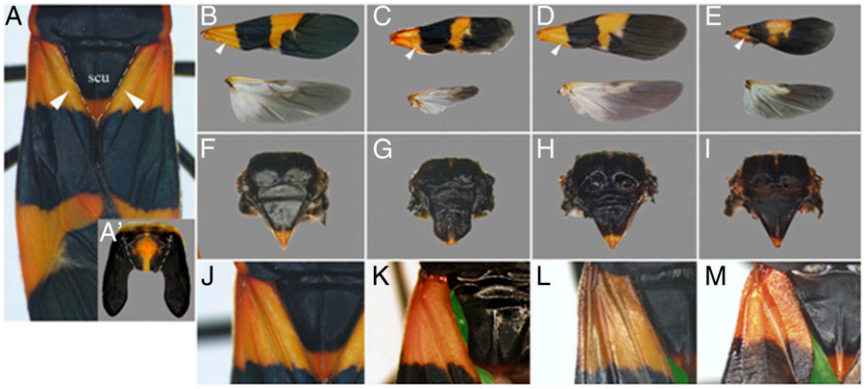

Fig. 3. The functions of wing genes on the mesothorax (T2) of *Oncopeltus*. (*A*) Dorsal morphology of the adult T2 segment; arrowheads point to the clavus of the forewings. (*A*′) The dissected dorsal T2 plate of a fifth nymph. The dotted line denotes the proximal-most boundary of wing primordia. (*B*) Wild-type fore- and hindwings. (*C*–*E*) The effects of *vg* RNAi, *sd* RNAi, and *nub* RNAi on adult wing morphology, respectively. (*F*) Dissected dorsal T2 notum of wild-type adult. (*G*–*I*) The effect of *vg* RNAi, *sd* RNAi, and *nub* RNAi, respectively. (*J*–*M*) The alignment of the scutellum and clavus of the forewing in wild-type (*J*), *vg* RNAi (*K*), *sd* RNAi (*L*), and *nub* RNAi (*M*) adults, respectively. In *K*–*M*, this alignment is disturbed causing scutellum and clavus to lose their close contact to one another (the created open space is artificially colored in green). scu, scutellum.

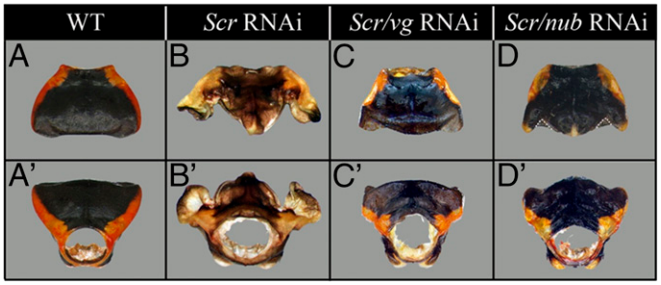

Fig. 4. Effects of *Scr*, *Scr/vg*, and *Scr/nub* RNAi on the T1 morphology. (*A*–*D*) Dorsal and (*A′*–*D′*) frontal view of wild-type T1 plate (*A* and *A′*), *Scr* RNAi T1 plate (*B* and *B′*), *Scr/vg* RNAi T1 plate (*C* and *C′*), and *Scr/nub* RNAi T1 plate (*D* and *D′*). To better distinguish the shape of the ectopic scutellum, its outer edges are outlined with a dotted white line in *D*.

scutellum development. In other words, *vg* functions as both the wing and scutellum master gene. Second, we performed the double *Scr/nub* RNAi (Fig. 4 *D* and *D′*) to determine the degree to which the independent development of the wings and scutellum observed in *nub* RNAi can be applied to the ectopic T1 structures. Because in T2 *nub* affects only the wings and not the scutellum (Fig. 3 *E* and *I*), we should observe similar effects in T1. Consistent with its conserved role in wing blade formation, the depletion of *nub* reduces the development of ectopic T1 wings (although not to the same extent as observed in *vg* knockdowns). In addition, although T1 scutellum was initialized and featured a diagnostic brightly colored posterior tip (Fig. 4 *D* and *D′*), it was much smaller and incompletely developed. In summary, these double-RNAi results indicate that initialization of both the wings and scutellum are dependent on *vg* function, whereas the increase in size of these structures requires *nub* function.

Coordinated development of the scutellum and wing blade likely affects the wing hinge and wing-folding mechanics, the latter of which became highly specialized following the origin in neoptera of the ability to fold the wings flat against the body. To begin to address this we examined the role of *tiptop* (*tio*), the *Oncopeltus* ortholog of *tea-shirt* (*tsh*), which plays a role in hinge development and wing posture in *Drosophila* (39, 40). In *tio* RNAi adults we found that the wings failed to fold flat against the body (Fig. S7*A* vs. Fig. S7*E*), consistent with previous observations in *tsh* mutants in *Drosophila* and similar to the ancestral wing posture still present in Paleoptera. This improper folding of the wings is the result of changes to the shape of the scutellum (Fig. S7*F*) as well as the development of the hinge (Fig. S7*G*), but not changes in the wing itself (Fig. S7*D* vs. Fig. S7*H*). This result points to *tio* as a gene that may overcome the otherwise coordinated development of wing blade + scutellum to form the hinge, thereby enabling the transition from paleoptera to neoptera.

***Ultrabithorax* Function in T2/T3 Wing Divergence and Evolutionary Diversification.** Divergence of T2 and T3 wing shape and texture, along with changes in wing-associated thoracic structures, and diversification of wings and thoraces among insect species, required changes to the default T2 morphology (6). In *Drosophila*, loss of *Ubx* converts the T3 segment to a T2 identity, including transformation of halteres to fully developed forewings (19, 20). Similarly, *Ubx* inhibition in *Tribolium* transforms the membranous hindwings to forewing-like sclerotized elytra (5). *Oncopeltus Ubx* RNAi adults had hindwings possessing all key features of forewing morphology, including their distinct shape, texture, and pigmentation (Fig. 5 *A′* and *B′*); this includes the transformation of the proximal anal lobe present in wild-type T3 wing, which assumed a clavus-like T2 morphology (blue arrowheads in Fig. 5 *B* and *B′*). Therefore, although the default state of the wing may vary, *Ubx* functions the same way in each case by always modifying an existing T2 wing to create a divergent hindwing (halteres in *Drosophila* or membranous wings in both *Tribolium* and *Oncopeltus*). In addition to wings, the *Ubx* RNAi T3 dorsal notum also showed significant alterations and developed an ectopic scutellum (Figs. 5 *C* and *D* and *C′* and *D′*). The extent of the change is best visible in dissected plates (Fig. 5 *E* and *E′*), enabling a full view of their structure. On the ventral side, the oval-shaped T3 sternum (Fig. 5*F*) assumed the V-shaped morphology of the T2 sternum (Fig. 5*F′*). These results show that in a hemimetabolous species *Ubx* also specifies a unique T3 segment morphology by altering the default T2 segment program. Similar to holometabola, these effects involve both the wings and thoracic plates (5, 8). In terms of its dorsal function, *Ubx* "makes" hindwings by altering the default forewing program while also repressing the formation of a T2 scutellum. These functions of *Ubx* most likely arose early in pterygote evolution because divergences in T2/T3 wings occur in certain Paleoptera (Ephemeroptera) as well as Neoptera.

Discussion

Uniqueness of the T1 Wing and Its Lack of Ventral Genes. Previous work in beetles revealed that the prothorax (T1) contains *vg*-dependent tissue, leading to the proposal that the affected T1 regions represent wing serial homologs (18, 37). Our present results show that, in addition to *vg*, several other genes involved in wing development also have a function in the wingless prothorax of *Oncopeltus*. By showing that *Oncopeltus* T1 EW is a unique type of wing and a characteristically T1 structure, rather than a conversion to T2 segmental identity, these results suggest, to our knowledge

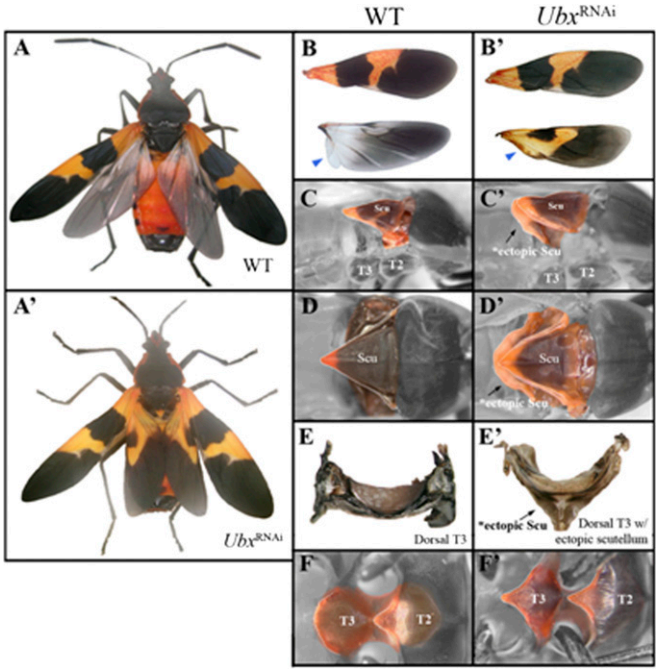

Fig. 5. The role of *Ubx* in the *Oncopeltus* metathorax. (*A* and *A′*) Dorsal views of the wild-type and *Ubx* RNAi adults. (*B*) Wild-type fore- and hindwing display distinct morphologies with regards to their shape, color, and size. (*B′*) Although forewings look the same, hindwings are transformed into forewings in *Ubx* RNAi adults. (*C*) A side view of the dorsal plates in the wild-type showing that the T2 segment features a well-developed scutellum. (*C′*) A side view of *Ubx* RNAi adult indicates a presence of an ectopic scutellum on T3 (black arrow). (*D*) Close-up view focused on wild-type scutellum. (*D′*) In *Ubx* RNAi adults, a second ectopic scutellum forms beneath the T2 scutellum (black arrow). (*E*) Dissected T3 plate of wild-type adult. (*E′*) Dissected T3 plate of an *Ubx* RNAi adult. (*F*) A close-up view of T2 and T3 ventral plates in wild-type. The ventral T2 has a triangular shape, whereas the T3 is oval. (*F′*) In *Ubx* RNAi adults, the ventral T3 is transformed into a ventral T2 sternum. Abbreviations: Scu, scutellum; T2, mesothoracic segment; T3, metathorax thoracic segment.

for the first time, the presence of a T1 wing serial homolog in a contemporary hemimetabolous insect. In contrast to the situation observed in *Tribolium*, where T1 EW is derived from both dorsal and ventral tissues and is indicative of a conversion of T1 to T2 segmental identity, these wings in *Oncopeltus* are of strictly dorsal morphological origin and have gene-expression profiles indicating either complete absence or marked reduction of ventral-origin cells.

Implications of the T1 Ectopic Wing for Insect Wing Origins. Small T1 wings are present in a phylogenetically diverse set of fossil insects, but no insects with T1 wings persist in fossils more recent than about 250 Mya (1). Our results in *Oncopeltus*, combined with recent developmental insights from *Drosophila*, provide a hypothesis to explain why T1 wings were likely limited to small size, reduced functionality, and were lost independently in multiple insect orders. Ventrally originating cells in *Drosophila* embryos migrate dorsally and undergo an epithelial–mesenchymal transition to form the corpora cardiaca and prothoracic glands in the head and T1 segments (25). Serially homologous cells in segments T2 through A8 also migrate dorsally and undergo epithelial–mesenchymal transition to form the main tracheal trunks while also playing a role in T2/T3 wing development. Stunting of the T1 wing may therefore demonstrate the limited potential of notal primarily cuticular cells to form a large and useful wing without the functions contributed by ventral-origin mesenchyme. The gene *ventral veins lacking* (*vvl*) acts downstream of *trh*, suggesting that the ventral component of wing vein formation (critical for wing stiffening) also derives from ventral cells that may have been diverted to gland formation in the T1 segment.

Tracheal placodes and leg primordia arise from a common pool of cells in *Drosophila* (23) with differences in their fate controlled by the activation state of the wingless signaling pathway. Tracheal cells and spiracles on T2 and T3 arise from locations lateral to the leg bases (23) possibly homologous with the association between gills and appendages in crustaceans, a hypothesis strengthened by the finding that homologs of tracheal inducer genes (*trh*, *vvl*) are specifically expressed in the gills of crustaceans (24, 41). Notably, those genes were absent from the small T1 ectopic wings of *Oncopeltus*. Furthermore, *nub* affected leg-adjacent morphology of the *Oncopeltus* T1 ventral thorax and was expressed only in the blade portion of the T2 wing but not the adjacent scutellum, similar to the way *nub* is expressed only in the posterior compartment of crustacean gills (23). Starting with fossil evidence (42) and continuing with developmental data (18, 23, 25, 32, 33) and present insights from *Oncopeltus*, genes expressed in crustacean gills appear to mark a cell population that evolved into both tracheae and wings in insects. In wings, the fusion of both dorsal and ventral contributions appears to be required for formation of fully developed and functional wings. Hence, after many decades of debate between proponents of the paranotal lobe vs. wings-from-gills hypotheses for insect wing origins, the answer is becoming increasingly apparent: each side was half correct.

Overview of the Origin and Divergence of Insect Wings. The origin and divergence of wings involved a multistep process outlined in Fig. 6. First, we propose that dorsally derived outgrowths evolved along a number of body segments, most notably T1 and some abdominal segments. Fossils containing these features (including larvae) gave rise to the paranotal theory of wing origins (1, 43). Dorsal origins

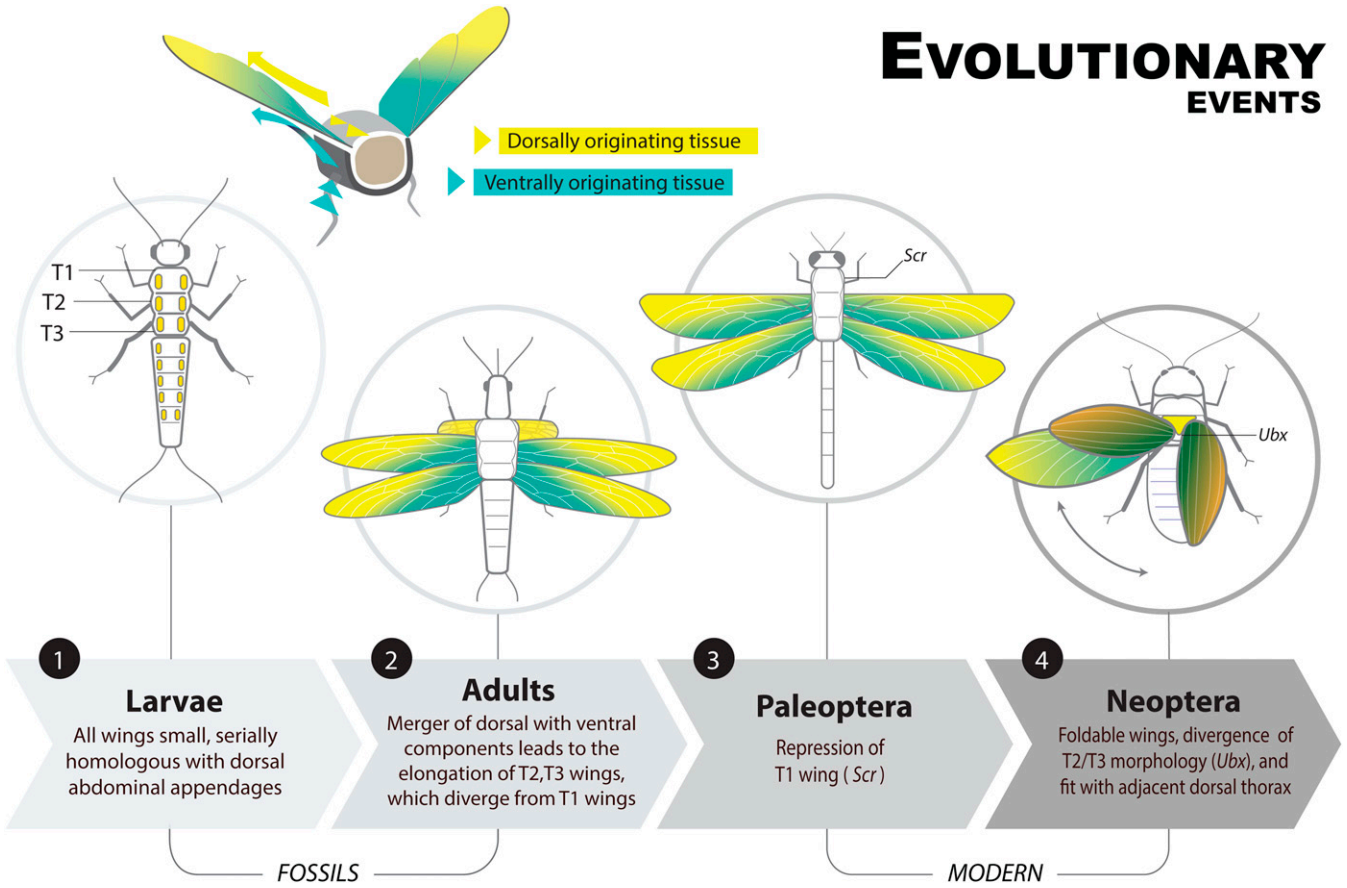

Fig. 6. Major events in the divergence and segmental diversification of insect wings. Note the presence of a T1 winglet in fossil insects, which persisted until after T2 and T3 wings elongated. Additionally, the divergence of T2/T3 wing morphology occurs also in some Paleoptera (Ephemeroptera), which if mediated by *Ubx* would place this feature at a more basal location than depicted here. Illustration by Daorong Fang.

are evident in the position and the expression of primarily dorsally derived genes in the ectopic T1 wings of *Oncopeltus*. Next, *Hox* genes were co-opted to suppress a number of these dorsal flaps (7). Either before or after this step, ventrally derived cells possibly homologous to ancestral arthropod epipods migrated dorsally and fused with the cells that give rise to the dorsal flaps (32). This fusion brought gene networks that likely enabled an increase in size of the outgrowths (shown empirically here by the effect of *nub* on the size of the T1 EW) as well as features necessary for articulation. Elongation of these dorsal flaps also featured regionalization, with a discrete lateral blade (the wing) connected by a hinge to a central region (the scutellum); these structures coevolved and became independently regulated, allowing formation of a hinge region without affecting wing blade development, which ultimately permitted wing folding. Finally, *Ubx* was co-opted to uncouple the wing morphologies of T2 and T3, facilitating the divergence of wing forms within species and diversification among species. These results represent a broad outline of key mechanisms underlying the origin and diversification of insect wings, which brought about the first flying animals and triggered the largest evolutionary radiation of multicellular life.

Materials and Methods

Gene Cloning, dsRNA Synthesis, and RNAi. Injections and dsRNA synthesis were performed as previously described (16). Detailed information including primer sequences and RT-PCR controls, are in *SI Material and Methods* and Fig. S8.

Library Preparation, Transcript Assembly, and Blast Annotation. Bar coded libraries ($n = 12$, one for each combination of treatment and replicate; NCBI SRA accession SRP066252) were prepared using the TruSeq RNA Sample Preparation Kit (Illumina). Contigs from the assembly were searched using BLASTx against the following sets of proteins: Uniprot, *Drosophila melanogaster*, *Tribolium casteneum*, and *Daphnia pulex*. Detailed information including RNA isolation, high-throughput sequencing, read processing, assembly and functional group analysis are in *SI Material and Methods*.

ACKNOWLEDGMENTS. We thank Thomas R. Lemonds, Dr. Mark VanBerkum, and two anonymous reviewers for helpful comments that greatly improved this manuscript; Venessa Verstraete for help with imaging; and Daorong Fang for her work on the illustration in Fig. 6. This work was partially supported by National Institutes of Health Grant GM071927 (to A.P.), Wayne State University Graduate Enhancement Funds (to V.M. and J.L.), funds from the Huck Institutes for Life Sciences and Grants NSF IOS-0950416 and IOS-1354667 (to J.H.M.), and National Science Foundation Grant PGRR- 0922742.

1. Grimaldi D, Engel MS (2005) Insects take to the skies. *Evolution of the Insects* (Cambridge Univ Press, New York), pp 155–187.
2. Wootton RJ, Kukalová-Peck J (2000) Flight adaptations in Palaeozoic Palaeoptera (Insecta). *Biol Rev Camb Philos Soc* 75(1):129–167.
3. Dudley R (2000) *The Biomechanics of Insect Flight: Form, Function, and Evolution* (Princeton Univ Press, Princeton, NJ).
4. Jockusch EL, Nagy LM (1997) Insect evolution: How did insect wings originate? *Curr Biol* 7(6):R358–R361.
5. Tomoyasu Y, Wheeler SR, Denell RE (2005) *Ultrabithorax* is required for membranous wing identity in the beetle *Tribolium castaneum*. *Nature* 433(7026):643–647.
6. Lewis EB (1978) A gene complex controlling segmentation in *Drosophila*. *Nature* 276(5688):565–570.
7. Carroll SB, Weatherbee SD, Langeland JA (1995) Homeotic genes and the regulation and evolution of insect wing number. *Nature* 375(6526):58–61.
8. Struhl G (1982) Genes controlling segmental specification in the *Drosophila* thorax. *Proc Natl Acad Sci USA* 79(23):7380–7384.
9. Angelini DR, Kaufman TC (2005) Insect appendages and comparative ontogenetics. *Dev Biol* 286(1):57–77.
10. Jockusch EL, Ober KA (2004) Hypothesis testing in evolutionary developmental biology: A case study from insect wings. *J Hered* 95(5):382–396.
11. Shubin N, Tabin C, Carroll S (1997) Fossils, genes and the evolution of animal limbs. *Nature* 388(6643):639–648.
12. O'Donnell BC, Jockusch EL (2010) The expression of *wingless* and *Engrailed* in developing embryos of the mayfly *Ephoron leukon* (Ephemeroptera: Polymitarcyidae). *Dev Genes Evol* 220(1-2):11–24.
13. Staniczek A, Bechly G, Godunko R (2011) Coxoplectoptera, a new fossil order of Palaeoptera (Arthropoda: Insecta), with comments on the phylogeny of the stem group of mayflies (Ephemeroptera). *Insect Syst Evol* 42(2):101–138.
14. Wootton RJ, Kukalova-Peck J, Newman DJS, Muzon J (1998) Smart engineering in the mid-carboniferous: How well could Palaeozoic dragonflies Fly? *Science* 282(5389):749–751.
15. Rogers BT, Peterson MD, Kaufman TC (1997) Evolution of the insect body plan as revealed by the *Sex combs reduced* expression pattern. *Development* 124(1):149–157.
16. Chesebro J, Hrycaj S, Mahfooz N, Popadić A (2009) Diverging functions of *Scr* between embryonic and post-embryonic development in a hemimetabolous insect, *Oncopeltus fasciatus*. *Dev Biol* 329(1):142–151.
17. Hrycaj S, Chesebro J, Popadić A (2010) Functional analysis of *Scr* during embryonic and post-embryonic development in the cockroach, *Periplaneta americana*. *Dev Biol* 341(1):324–334.
18. Clark-Hachtel CM, Linz DM, Tomoyasu Y (2013) Insights into insect wing origin provided by functional analysis of *vestigial* in the red flour beetle, *Tribolium castaneum*. *Proc Natl Acad Sci USA* 110(42):16951–16956.
19. Pavlopoulos A, Akam M (2011) Hox gene *Ultrabithorax* regulates distinct sets of target genes at successive stages of *Drosophila* haltere morphogenesis. *Proc Natl Acad Sci USA* 108(7):2855–2860.
20. Bender W, et al. (1983) Molecular genetics of the Bithorax complex in *Drosophila melanogaster*. *Science* 221(4605):23–29.
21. Wang Z, Gerstein M, Snyder M (2009) RNA-seq: A revolutionary tool for transcriptomics. *Nat Rev Genet* 10(1):57–63.
22. Huang W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4(1):44–57.
23. Franch-Marro X, Martín N, Averof M, Casanova J (2006) Association of tracheal placodes with leg primordia in *Drosophila* and implications for the origin of insect tracheal systems. *Development* 133(5):785–790.
24. Mitchell B, Crews ST (2002) Expression of the *Artemia trachealess* gene in the salt gland and epipod. *Evol Dev* 4(5):344–353.
25. Sánchez-Higueras C, Sotillos S, Castelli-Gair Hombría J (2014) Common origin of insect trachea and endocrine organs from a segmentally repeated precursor. *Curr Biol* 24(1):76–81.
26. Chung S, Chavez C, Andrew DJ (2011) *Trachealess* (*Trh*) regulates all tracheal genes during *Drosophila* embryogenesis. *Dev Biol* 360(1):160–172.
27. Furlong EEM, Andersen EC, Null B, White KP, Scott MP (2001) Patterns of gene expression during *Drosophila* mesoderm development. *Science* 293(5535):1629–1633.
28. Hakeda S, Endo S, Saigo K (2000) Requirements of *Kettin*, a giant muscle protein highly conserved in overall structure in evolution, for normal muscle function, viability, and flight activity of *Drosophila*. *J Cell Biol* 148(1):101–114.
29. Zhang Y, Featherstone D, Davis W, Rushton E, Broadie K (2000) *Drosophila D-titin* is required for myoblast fusion and skeletal muscle striation. *J Cell Sci* 113(Pt 17):3103–3115.
30. Okenve-Ramos P, Llimargas M (2014) *Fascin* links Btl/FGFR signalling to the actin cytoskeleton during *Drosophila* tracheal morphogenesis. *Development* 141(4):929–939.
31. Guild GM, Connelly PS, Ruggiero L, Vranich KA, Tilney LG (2005) Actin filament bundles in *Drosophila* wing hairs: Hairs and bristles use different strategies for assembly. *Mol Biol Cell* 16(8):3620–3631.
32. Averof M, Cohen SM (1997) Evolutionary origin of insect wings from ancestral gills. *Nature* 385(6617):627–630.
33. Niwa N, et al. (2010) Evolutionary origin of the insect wing via integration of two developmental modules. *Evol Dev* 12(2):168–176.
34. Berger C, et al. (2012) FACS purification and transcriptome analysis of *Drosophila* neural stem cells reveals a role for *Klumpfuss* in self-renewal. *Cell Reports* 2(2):407–418.
35. Ross MH (1964) Pronotal wings in *Blattella germanica* (L.) and their possible evolutionary significance. *Am Midl Nat* 71(1):161–180.
36. Campbell S, et al. (1992) The *scalloped* gene encodes a novel, evolutionarily conserved transcription factor required for sensory organ differentiation in *Drosophila*. *Genes Dev* 6(3):367–379.
37. Ohde T, Yaginuma T, Niimi T (2013) Insect morphological diversification through the modification of wing serial homologs. *Science* 340(6131):495–498.
38. Zirin JD, Mann RS (2004) Differing strategies for the establishment and maintenance of *teashirt* and *homothorax* repression in the *Drosophila* wing. *Development* 131(22):5683–5693.
39. Soanes KH, Bell JB (1999) Rediscovery and further characterization of the *aeroplane* (*ae*) wing posture mutation in *Drosophila melanogaster*. *Genome* 42(3):403–411.
40. Soanes KH, et al. (2001) Identification of a regulatory allele of *teashirt* (*tsh*) in *Drosophila melanogaster* that affects wing hinge development. An adult-specific *tsh* enhancer in *Drosophila*. *Mech Dev* 105(1-2):145–151.
41. Chavez M, et al. (1999) *APH-1*, a POU homeobox gene expressed in the salt gland of the crustacean *Artemia franciscana*. *Mech Dev* 87(1-2):207–212.
42. Kukalova-Peck J (1978) Origin and evolution of insect wings and their relation to metamorphosis, as documented by the fossil record. *J Morphol* 156(1):53–125.
43. Rasnitsyn AP (1981) A modified paranotal theory of insect wing origin. *J Morphol* 168(3):331–338.
44. Li H, Popadić A (2004) Analysis of *nubbin* expression patterns in insects. *Evol Dev* 6(5):310–324.
45. Hrycaj S, Mihajlovic M, Mahfooz N, Couso JP, Popadić A (2008) RNAi analysis of *nubbin* embryonic functions in a hemimetabolous insect, *Oncopeltus fasciatus*. *Evol Dev* 10(6):705–716.
46. Mahfooz N, Turchyn N, Mihajlovic M, Hrycaj S, Popadić A (2007) *Ubx* regulates differential enlargement and diversification of insect hind legs. *PLoS One* 2(9):e866.
47. Grabherr MG, et al. (2011) Full-length transcriptome assembly from RNA-seq data without a reference genome. *Nat Biotechnol* 29(7):644–652.
48. Ewen-Campen B, et al. (2011) The maternal and early embryonic transcriptome of the milkweed bug *Oncopeltus fasciatus*. *BMC Genomics* 12:61.
49. Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25(14):1754–1760.
50. Li B, Dewey CN (2011) RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12:323.
51. Robinson MD, Oshlack A (2010) A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol* 11(3):R25.