Dual evolutionary origin of insect wings supported by an investigation of the abdominal wing serial homologs in *Tribolium*

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The origin of insect wings is still a highly debated mystery in biology, despite the importance of this evolutionary innovation. There are currently two prominent, but contrasting wing origin hypotheses (the tergal origin hypothesis and the pleural origin hypothesis). Through studies in the *Tribolium* beetle, we have previously obtained functional evidence supporting a third hypothesis, the dual origin hypothesis. Although this hypothesis can potentially unify the two competing hypotheses, it requires further testing from various fields. Here, we investigated the genetic regulation of the tissues serially homologous to wings in the abdomen, outside of the appendage-bearing segments, in *Tribolium*. We found that the formation of ectopic wings in the abdomen upon homeotic transformation relies not only on the previously identified abdominal wing serial homolog (gin-trap), but also on a secondary tissue in the pleural location. Using an enhancer trap line of *nubbin* (a wing lineage marker), we were able to visualize both of these two tissues (of tergal and pleural nature) contributing to form a complete wing. These results support the idea that the presence of two distinct sets of wing serial homologs per segment represents an ancestral state of the wing serial homologs, and can therefore further support a dual evolutionary origin of insect wings. Our analyses also uncovered detailed Hox regulation of abdominal wing serial homologs, which can be used as a foundation to elucidate the molecular mechanisms that have facilitated the evolution of bona fide insect wings, as well as the diversification of other wing serial homologs.

serial homology | morphological novelty | insect wings | Hox | *Tribolium*

Like the emergence of tetrapod limbs and the evolution of animal eyes, the acquisition of wings in the hexapod taxa represents a profound moment in eukaryotic evolution. The gain of wings allowed insects to enhance their ability to radiate and simultaneously provided a substrate on which they could explore various survival strategies [e.g., using wings for camouflage (1) or converting them into protective shields (2)]. These features of wings, when combined, played a critical role in making insects one of the most successful clades on this planet. The evolutionary origin of insect wings is, however, a longstanding mystery that has been a point of debate for over a century. At present, there are two contrasting hypotheses that explain the acquisition of insect wings (to review the history of the wing origin debate, see refs. 3 and 4). The first hypothesis, called the tergal origin hypothesis (also known as the paranotal hypothesis), proposes that wings originated from an expansion of dorsal body wall (tergum), which allowed insects to first glide and later to fly (5–7). The second hypothesis, called the pleural origin hypothesis (also known as the gill or exite hypothesis), states that wings were derived from ancestral proximal leg segments and the branches (exites) connected to them (7–9). These leg segments are thought to have fused into the body wall, forming the pleural plates in the insect lineage (10). The pleural origin hypothesis proposes that some of the pleural plates, along with the associated exites, migrated dorsally to produce the modern flight structures of insects (8). In addition to these two schools of thought, there is a third idea, which seeks to unify the two competing hypotheses by proposing

contributions of both tergal and pleural components during the evolution of insect wings (4). Although this "dual origin" hypothesis is not new, having been proposed as early as 1916 by Crampton (11) and articulated more clearly by Rasnitsyn (5) in his modified paranotal hypothesis, it has recently been gaining momentum, mainly by receiving support from an evolutionary developmental biology (evo-devo) point of view (4, 12–16). More recently, the dual origin of insect wings was also supported from a paleontological study (17). Currently, all three hypotheses are valid, and the dual origin hypothesis itself can also have variations in regard to the degree of contribution from the two distinct tissues. Therefore, this is a critical moment for the dual origin hypothesis, requiring rigorous testing from various fields (such as evo-devo, taxonomy, neuro- and muscle-anatomy, and paleontology) to be recognized as a third major hypothesis.

In the extant insects, wings and their derivatives (i.e., dorsal appendages) are found only on the second and third thoracic segments (T2 and T3) (18, 19). In *Drosophila*, the *vestigial* gene (*vg*) is often used to trace the tissues that have wing identity, due to its unique expression in the wing-related tissues within the epidermis (20) and its ability to induce wing identity when expressed ectopically (21, 22). Through a functional analysis of *vg* in the red flour beetle (*Tribolium castaneum*), we have previously shown that there are additional *vg*-dependent tissues on the first thoracic segment (T1), a segment typically viewed as wingless. Intriguingly, these two T1 *vg*-dependent tissues, the carinated margin (lateral tergal expansion) and the two pleural plates appear to correspond to the two proposed wing origins (13). Furthermore, induction of an ectopic wing on T1 via manipulation

Significance

Acquisition of morphologically novel structures can facilitate successful radiation during evolution. The emergence of wings in hexapods represents a profound moment in eukaryotic evolution, making insects one of the most successful groups. However, the tissue that gave rise to this novel and evolutionarily crucial structure, and the mechanism that facilitated its evolution, are still under intense debate. By studying various wing-related tissues in beetles, we demonstrated that two distinct lineages of wing-related tissues are present even outside the appendage-bearing segments. This outcome supports a dual evolutionary origin of insect wings, and shows that novelty can emerge through two previously unassociated tissues collaborating to form a new structure.

of the Hox gene *Sex combs reduced* (*Scr*) caused these two groups of tissues to merge to form a complete wing (13). Collectively, these findings led us to support a dual origin of insect wings, whereby a merger of tissues of both pleural and tergal origin led to the evolutionary advent of the insect flight structure. Importantly, our results also revealed how wing serial homologs can be used to provide a glimpse into the transitional state of insect wings, reveal the tissues critical to their formation, and ultimately decipher the evolutionary history of these structures (see also ref. 23 for further discussion on wing serial homologs in evo-devo studies).

Intriguingly, the presence of wing serial homologs is not limited to the appendage-bearing segments. Ohde et al. (24) previously reported that gin-traps, pupal-unique structures, are wing serial homologs in the abdominal segments of another beetle, *Tenebrio molitor*. Gin-traps are formed in the abdomen, and are thought to serve as protective devices for the vulnerable pupae (25–27). The gin-traps in *Tenebrio* were shown to be *vg*-dependent and, by modulation of Hox activity, able to be partially transformed into wings (24). However, the homology between the two (tergal and pleural) T1 wing serial homologs and the abdominal wing serial homologs (gin-traps) has yet to be examined. Spatially, the gin-traps are located at the lateral edge of the terga, suggesting that the gin-traps are homologous to the T1 tergal wing serial homolog (the carinated margin) (Fig. 1 *A–C*). This interpretation raises the possibility of additional wing serial homologs in the pleural region of the abdomen, which have yet to be revealed. Two sets of wing serial homologs contributing to the formation of a complete wing even in the abdomen upon Hox reduction would provide further support for a dual origin of insect wings. Conversely, if the gin-trap cells are sufficient for the formation of a complete wing upon Hox transformation, this outcome would suggest that the presence of the two distinct wing serial homologs in T1 we identified in the *Tribolium* beetle may not represent an ancestral state of wing serial homologs. The presence of a single set of abdominal wing serial homologs, each of which is sufficient

to form a complete ectopic wing, would also contradict with the dual origin of insect wings.

In this study, we examined the relationship among various wing serial homologs and explored the possibility of other groups of wing serial homologs in the abdominal segments of *Tribolium*. We found that the formation of the gin-traps relies on, not only *vg*, but also other wing genes [such as *apterous* (*ap*) and *disheveled* (*dsh*)], similar to the tergal T1 wing serial homolog (the carinated margin). We also found that the tergal *vg*-dependent tissues cover a region larger than just the gin-trap by expanding into the dorsal posterior edge of the tergum. However, we did not detect the secondary, pleural *vg*-dependent tissues in the abdominal segments. Interestingly, our detailed analyses of Hox transformation, along with tracing the wing lineage with a *nubbin* (*nub*) enhancer trap line (*pu11*), have revealed that the formation of abdominal ectopic wings relies not only on the transformation of the cells at the tergal edge (including the gin-traps), but also on the ectopic induction of *vg*-positive and *nub* enhancer trap active cells in the pleural region. Taken together, our study has uncovered two important features regarding the abdominal wing serial homologs in *Tribolium*. First, in contrast to the situation in T1, the induction of pleural wing serial homologs is normally suppressed by Hox in the abdomen of *Tribolium*. This also implies that the gin-traps are serially homologous to the tergal wing serial homologs and only partially serially homologous to bona fide wings. Second, and now parallel to T1, the formation of an ectopic wing (upon Hox transformation) still requires the contribution of two separate groups of tissues (of tergal and pleural origin) even in the abdominal segments. The latter feature suggests that the presence of two distinct sets of wing serial homologs is not unique to T1, and instead represents an ancestral state of the wing serial homologs (i.e., plesiomorphic). This is in contrast to the situation in the wing-bearing segments (T2 and T3), where bona fide wings appear to be composed of two separate lineages of tissues (representing a more derived state; i.e., apomorphic), which can therefore be used to further support a dual origin of insect wings.

Results

Abdominal *vg*-Dependent Tissues in *Tribolium*. We have been using two criteria to identify wing serial homologs in segments outside of the T2 and T3 (typically winged) segments: within the epidermis, (*i*) tissues that have *vg* expression or show functional dependency on *vg*, and (*ii*) tissues that transform into wings upon Hox loss-of-function (4, 13, 23, 24). As mentioned, Ohde et al. (24) previously identified that the gin-traps of *Tenebrio* pupa are wing serial homologs in the abdominal segments. We first performed *vg* RNAi and investigated if there are any additional *vg*-dependent tissues (i.e., possible wing serial homologs) in the abdomen of *Tribolium*. At the pupal stage, the gin-traps are completely missing from the *Tribolium* pupae after *vg* RNAi, confirming the earlier finding by Ohde et al. that the gin-traps are wing serial homologs in the beetle abdomen (arrows in Fig. 1 *A–D*; also see Fig. S1 for a negative control). Unexpectedly, we also observed that, in each abdominal segment, the distinct stripe of posterior pigmentation in the tergum (arrowheads in Fig. 1 *A* and *B*) is strongly reduced or absent from *vg* RNAi pupae (arrowheads in Fig. 1 *C* and *D*). This suggests that, along with the gin-traps, there are additional *vg*-dependent tissues in the dorsal posterior of each abdominal segment. Outside of terga, we did not detect any morphological abnormalities in the abdomen of *vg* RNAi pupae (Fig. 1 *A–D*).

We also analyzed the adult abdomen of *vg* RNAi beetles. Despite our extensive morphological analyses using scanning electron microscopy (SEM), we failed to detect any morphological abnormalities in the *vg* RNAi adult abdomen, either in the tergal or the pleural region (Fig. S2). This result indicates that cells that produce the gin-traps do not contribute to adult morphology in *Tribolium*, and that there appear to be no tissues in the adult abdomen that are functionally dependent on *vg* in *Tribolium*.
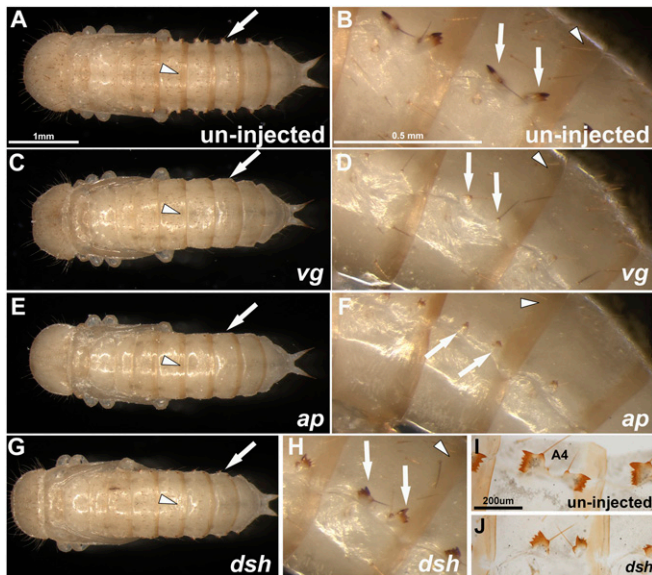

Fig. 1. Pupal RNAi phenotypes for *vg*, *ap*, and *dsh* generated by last larval injection. (*A* and *B*) Uninjected pupa. (*C–J*) Pupae with RNAi for *vg* (*C* and *D*), *ap* genes (*E* and *F*), and *dsh* (*G–J*). Pupae are shown from the dorsal view (*A*, *C*, *E*, *G*) or lateral view (*B*, *D*, *F*, *H*). Arrows indicate gin-traps (both anterior and posterior jaw in *B*, *D*, *F*, and *H*), and arrowheads indicate dorsal posterior stripe of pigmentation. Gin-traps in *vg* and *ap* RNAi are significantly reduced (*C–F*). Gin-traps of *dsh* RNAi are less affected (*G* and *H*), but are smaller, have fewer spiked teeth, and are now disoriented (*I* and *J*). Scale bars in *A* and *B* apply to respective images below the panes. Scale bar in *I* applies to *J*.

We next analyzed the expression of Vg during the pupal stage using an antibody against the *Tribolium* Vg protein (Fig. 2 *A–F*). We detected strong Vg expression in the cells beneath the gin-traps (arrow in Fig. 2 *E* and *F*), demonstrating that *vg* is directly involved in gin-trap formation. Parallel to our *vg* RNAi result, we also detected Vg expression in a strip of cells along the posterior edge of abdominal terga (arrowhead in Fig. 2 *E* and *F*). This Vg expression covers both the posterior compartment [marked by Engrailed (En) expression] and the region in the anterior compartment that is adjacent to the posterior compartment (arrowhead in Fig. 2 *D–F*). The nuclear staining of Tc-Vg antibody was completely removed in *vg* RNAi, confirming the specificity of this antibody (Fig. 2 *G–L*). The posterior compartment (visualized by En) was intact in these *vg* RNAi (Fig. 2*J*), suggesting that, although *vg* is important for the pigmentation of this area, *vg* is dispensable for the survival of the corresponding tissue. The larger Vg expression domain revealed by our staining suggests that the tissues serially homologous to wings may extend beyond the gin-trap cells. In contrast, we did not detect any Vg-positive cells in the ventral side of the pupae (corresponding to pleuron and sternum), except for the sixth abdominal segment (A6) that has a pair of strong Vg-positive tissues in the ventral side of the pupa (Fig. S3). The tergal "L-shaped" Vg expression pattern (that consists of the lateral and posterior tergal edge), along with the missing Vg expression in the ventral side, is consistent with the *vg* expression pattern in late embryos (13). This suggests that the *vg* expression pattern is maintained throughout larval and pupal development in *Tribolium*.

Two important conclusions can be drawn from the above observations. First, the tergal wing serial homologs in the *Tribolium* abdomen might occupy not only the lateral edge of terga (where the gin-traps are formed), but also the posterior edge of the terga, although the *vg*-dependency of these tissues is restricted to the pupal stage. Second, unlike in T1, there appear to be no pleural wing serial homologs in the *Tribolium* abdomen (except for the Vg-positive tissues in A6).

The Overlap of Gene Networks Responsible for the Formation of Wings and Wing Serial Homologs in *Tribolium*. The degree of overlap in gene networks can be used to further assess homology relationships among wings and wing serial homologs. We have previously shown that formation of one of the wing serial homologs in T1, the carinated margin, is dependent on not only *vg*,

but also on several additional members of the wing gene network (*ap* and Wg signaling) in *Tribolium* (13). Moreover, the carinated margin also has *nub* enhancer trap activity, although *nub* itself is dispensable for the formation of the carinated margin (13) (see also Fig. S8C). We performed RNAi for the two *ap* paralogs [*apA* and *apB* (28)] as well as *dsh* [*dsh* codes for a critical intracellular protein necessary for transducing Wg signal (29)], and investigated if the abdominal wing serial homologs also show a similar overlap of the wing gene network. RNAi at the last larval stage for the *ap* genes completely removed the gin-trap structures (arrows in Fig. 1 *E* and *F*). RNAi for *dsh* also disrupted gin-trap formation, although not as strongly as *vg* or *ap* genes (arrows in Fig. 1 *G–J*). In contrast, we did not observe any disruptions in the dorsal posterior of each abdominal tergum as the stripe of pigmentation in pupae remained intact (arrowheads in Fig. 1 *E–H*). We also examined possible *nub* enhancer activity in the *vg*-dependent tissues in pupae using enhanced yellow fluorescent protein (EYFP) expression of the *nub* enhancer trap line (*pu11*), but we failed to detect any EYFP expression in abdominal terga (Fig. 3 *D* and *E*). In addition, RNAi for *nub* did not result in any defects in the gin-traps or other abdominal structures (Fig. S1). These results demonstrated that, similar to the carinated margin, at least the gin-trap portion of the abdominal *vg*-dependent tissues shares a gene network with wings. This outcome raises two possibilities in regard to the homology relationship among the *vg*-dependent tissues: (*i*) because the L-shaped lateral-to-posterior tergal edge is the only *vg*-dependent tissue we detected in the abdominal segments, it is possible that this tergal *vg*-dependent tissue is "entirely" serially homologous to the wings in T2 and T3; or (*ii*) given that the gin-traps and the carinated margin are both positioned similarly at the lateral terga, it is also possible that the abdominal *vg*-dependent tissue is serially homologous to one of the T1 wing serial homologs (the carinated margin), and thus only "partially" serially homologous to wings in T2 and T3. The former possibility argues against the idea that the presence of two distinct sets of wing serial homologs in T1 represents an ancestral state, and therefore contradicts with the dual origin of insect wings (and instead could suggest that bona fide wings are serially homologous to the tergal structures). In contrast, the latter possibility supports the idea that "two sets of wing serial homologs per segment" represents an ancestral state. In this case, bona fide wings are composed of two distinct sets of tissues (of tergal and pleural nature), which supports the dual origin hypothesis. In
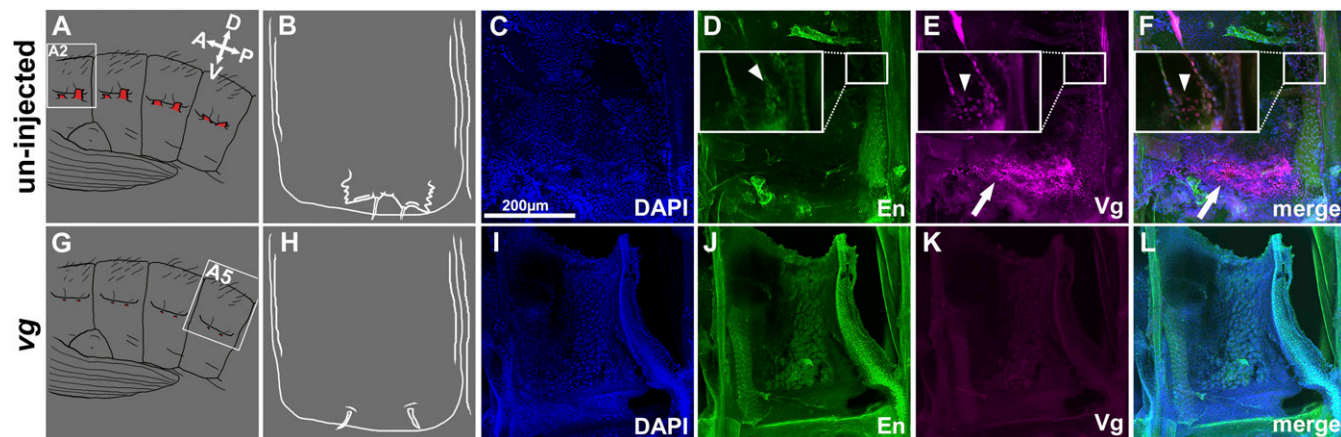

Fig. 2. Vg expression in the pupal epidermis. (*A–F*) Vg antibody staining in an uninjected pupa. Vg is expressed in epidermal cells beneath the gin-trap as well as in the dorsal posterior of the segment (arrow and arrowhead in *E* and *F*). The Vg expression at the dorsal posterior edge of terga includes the posterior compartment marked by En staining (arrowhead in *D* and *F*, Inset), as well as the adjacent cells in the anterior compartment. (*G–L*) Vg antibody staining in *vg* RNAi. Vg expression is absent (*K* and *L*). En expression is unchanged in *vg* RNAi (*J*), suggesting that there is no tissue truncation in this region. The first and second columns are schematic representations of areas imaged in the respective row. The first row is A2, the second row is A5. Scale in *C* applies to all images except for cartoon diagrams.
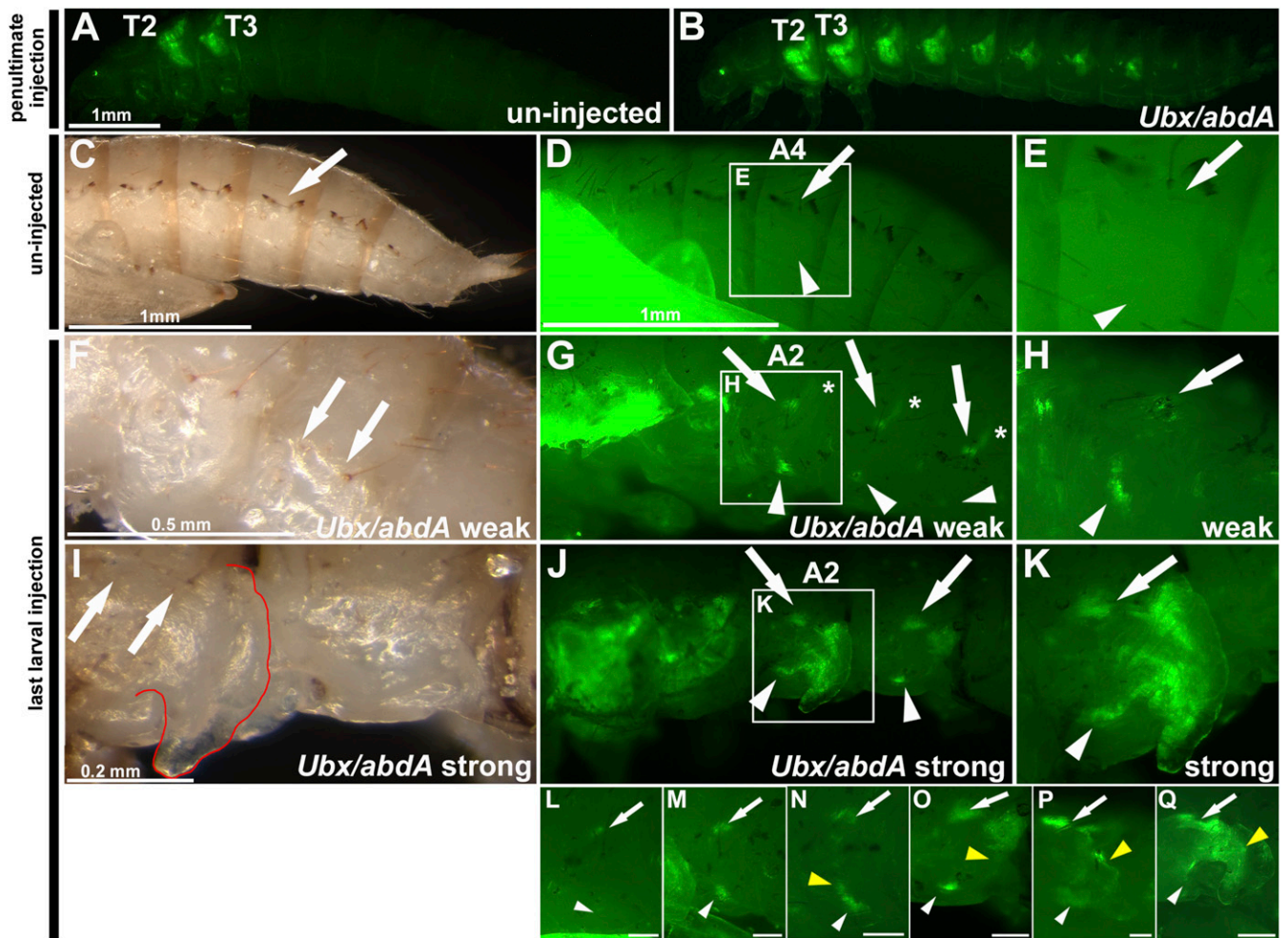
Fig. 3. Transformation of abdominal wing serial homologs by *Ubx/abdA* double RNAi. (*A*) EYFP expression in an uninjected *pu11* larva showing matured wing discs in T2 and T3. (*B*) A last instar larva with ectopic wing discs throughout the abdominal segments resulting from *Ubx/abdA* double RNAi at the penultimate larval stage. (*C–E*) Uninjected *pu11* pupa. Gin-traps are properly formed (arrow in *C*) and no *nub* enhancer trap activity is observed either at the tergal (arrow) or pleural (arrowhead) location in abdominal segments (*D* and *E*). (*F–H*) Weak transformation induced by *Ubx/abdA* double RNAi at the last larval stage. Gin-traps are missing (arrows in *F*). Ectopic *nub* enhancer trap activity is observed at two locations, one at the tergal location (arrow) and the other at the pleural location (arrowhead) (*G* and *H*). *nub* enhancer trap activity also spreads to the dorsal posterior edge of each tergum (asterisks in *G*). (*I–K*) Strong transformation induced by *Ubx/abdA* double RNAi at the last larval stage. Ectopic wings are induced (outlined by red in *I*). Gin-traps are absent (arrows in *I*). Two *nub* enhancer trap-positive groups of cells merge to form an ectopic wing (*J* and *K*). The last larval *Ubx/abdA* double RNAi often shows a phenotypic gradient with stronger transformation in a more anterior segment. In the pupa shown in *J*, A2 exhibits a merger of two groups of *nub* enhancer trap-positive cells and the formation of an ectopic wing, while A3 shows two distinct groups of *nub* enhancer trap-positive cells bridged by additional *nub* enhancer trap-positive cells. (*L–Q*) Transformation transition series after *Ubx/abdA* double RNAi. Weakly transformed abdominal segments (*L*) have subtle *nub* enhancer trap activity at both a tergal location (white arrow) and at the pleural location (white arrowhead). As the strength of the transformation increases (*M–P*) the EYFP expression first becomes more robust (arrow and arrowhead in *M*), and then begins to merge (yellow arrowhead in *N–P*) between the tergal and pleural regions (white arrow and arrowhead in *N–P*). In the most strongly transformed segments (*Q*) ectopic wings are induced (yellow arrowhead in *Q*) connecting tergal and pleural *nub* enhancer trap-active cells (white arrow and arrowhead in *Q*). Segments shown in *M*, *N*, and *Q* are A2 and segments in *L*, *O*, and *P* are A3. Scale bar in *A* applies to *B*, and scale in *D* applies to *G* and *J*. (Scale bars in *L–Q*, 0.1 mm.)

the next section, we assessed these possibilities by analyzing the abdominal *vg*-dependent tissues after varying degrees of homeotic transformation.

Visualizing Cells Contributing to Ectopic Abdominal Wings in *Ubx/abdA* Double RNAi. Serially homologous structures in insects are often evolutionarily modified (or suppressed) by the action of Hox genes (23, 30). These modifications are sometimes so extensive that morphological and functional similarities among serial homologs can be reduced beyond recognition. Hox mutation can strip away the modifications applied to these serially homologous structures, and reveal their developmental "default state." If the abdominal *vg*-dependent tissues identified above are serially homologous to wings, these tissues should transform into wings upon

reduction of abdominal Hox genes [*Ultrabithorax* (*Ubx*) and *abdominalA* (*abdA*)]. This is indeed the case in *Tenebrio*, where double RNAi for *Ubx* and *abdA* transforms the gin-traps into ectopic wings (albeit an incomplete transformation) (24). However, it remains unclear if the tergal *vg*-dependent tissue (including the gin-trap) is sufficient to form a complete wing in each abdominal segment upon reduction of Hox activity [more precisely, into elytra, as the elytron is the developmental default state of wing structures in beetles (31), but described here as a "wing" to avoid confusion]. If sufficient, this will imply that a wing is entirely serially homologous to a tergal structure, supporting the tergal origin of insect wings. On the other hand, if there are additional cells outside of the terga that also contribute to the formation of an ectopic wing, this will support a dual origin. We set out to

identify the cells that contribute to the formation of ectopic abdominal wings by visualizing wing cells with the *pu11 nub* enhancer trap line. *nub* is frequently used as a wing marker, as its expression corresponds to future wing tissues in various insects, including *Tribolium* (28, 32). As mentioned, the *pu11 nub* enhancer trap line does not have any enhancer trap activity in the abdominal epidermis, except in the peripheral nervous system, during the larval and pupal stage (Fig. 3 *D* and *E*; also see arrowhead in Fig. S8*C*), making this line quite useful to identify cells adopting a more bona fide wing identity.

Using *pu11*, Tomoyasu et al. (31) have previously shown that *Ubx/abdA* double RNAi induces what appears to be complete ectopic wings throughout the abdominal segments in *Tribolium* (also see Fig. 3 *A* and *B*). Although impressive, this transformation is less informative for our current study as the transformation is too complete to determine which abdominal cells contribute to the formation of ectopic wings. Therefore, we first evaluated RNAi conditions that result in more intermediate transformations. We found that injection of a high concentration dsRNA (1 µg/µL) for *Ubx* and *abdA* at the penultimate stage is required for the complete transformation, similar to the transformation described by Tomoyasu et al. (31) (Fig. 3 *A* and *B*). Lowering the concentration of double-stranded RNA (dsRNA) can result in a weaker RNAi phenotype (33). We were able to somewhat weaken the *Ubx/abdA* double-RNAi phenotype by injecting a low concentration of dsRNA (500 ng/µL for each gene) at the penultimate stage. These larvae still displayed some induction of wing cells in the abdomen (monitored by EYFP expression of *pu11*); however, most individuals died before pupation, preventing us from analyzing pupal morphologies (Table S1). Some individuals with this condition escaped to the adult stage, but these escapers did not have any detectable abdominal wing cells. As an alternative to lowering the dsRNA concentration, we also tested if delaying the dsRNA injection could result in an intermediate transformation. In contrast to lowering dsRNA concentration, injection of 1 µg/µL *Ubx* and *abdA* dsRNA at the early last larval stage produced a series of transformations (presumably due to slight differences in the timing of injection), and many of the injected larvae survived to the pupal stage (Fig. 3 *F–K*). Therefore, we decided to further study the intermediate transformations obtained by the last larval *Ubx/abdA* double RNAi.

Both Tergal and Pleural Tissues Contribute to Forming Ectopic Abdominal Wings upon Hox Reduction. *Ubx/abdA* double RNAi at the early last larval stage resulted in two noticeable abnormalities in the pupal abdomen: (*i*) a complete absence of the gin-trap structures (Fig. 3 *F* and *I*) and (*ii*) ectopic *nub* enhancer trap activity (i.e., wing identity) (Fig. 3 *C–K*). The absence of the gin-traps occurs even when the ectopic *nub* enhancer trap activity is minimal, suggesting that the function of abdominal Hox genes in the induction of the gin-traps and the suppression of the wing identity can be separable. In contrast to the gin-trap defect, the induction of ectopic wing tissue (*nub* enhancer trap-positive cells) in the abdomen varied from weak to strong in the *Ubx/abdA* double-RNAi beetles injected at the early last larval stage (Fig. 3 *L–Q*). In weakly transformed individuals, cells located underneath the (now absent) gin-trap become *nub* enhancer trap-positive (arrows in Fig. 3 *G*, *H*, and *L–N*), indicating that the gin-trap cells are transforming into wing cells. Intriguingly, we also noticed a second group of *nub* enhancer trap-positive cells at a more lateral (pleural) location (arrowheads in Fig. 3 *G*, *H*, and *L–O*; also see Fig. S4 for annotation of abdominal morphology and location of two regions of wing transformation). Furthermore, in strongly transformed individuals, the two groups of *nub* enhancer trap-positive cells (tergal and pleural) merge and form small but visible ectopic wings (Fig. 3 *I–K* and *O–Q*). The *Ubx/abdA* double-RNAi pupae often show an anterior-to-posterior transformation gradient (with anterior segments being

more strongly transformed), allowing us to observe a merger of the two groups of *nub* enhancer trap-positive cells in anterior abdominal segments and the two separate groups of cells with *nub* enhancer trap activity in more posterior abdominal segments (Fig. 3*J*). This result shows that in the abdomen, similar to T1, induction of ectopic wings requires contribution from both tergal and pleural tissues. We also noticed that the ectopic *nub* enhancer trap activity in the *Ubx/abdA* double-RNAi beetles often expands toward the dorsal posterior edge of terga (asterisks in Fig. 3*G*), suggesting that the abdominal tergal cells that are serially homologous to wings include not only the gin-trap cells but also the more dorsally located cells (corresponding to the L-shaped tergal *vg*-dependent tissue).

The finding of the second group of *nub* enhancer trap-positive cells contributing to the formation of an ectopic abdominal wing is exciting, but also puzzling as we did not detect a *vg*-expressing tissue (i.e., a possible wing serial homolog) in the pleural region of the abdominal segments in *Tribolium*. Immunostaining with the anti-Vg antibody in the *Ubx/abdA* double RNAi has revealed that the tergal Vg-expressing cells are redistributed more posteriorly in each segment, while new Vg expression appears in the pleural region, connecting the tergal and pleural *nub*-positive groups of cells (Fig. 4 *A–F*).

Taken together, our analyses of the intermediate transformation induced by *Ubx/abdA* double RNAi have revealed that there can be two different types of wing serial homologs even in the abdominal segments, similar to those in T1. This implies that the tergal *vg*-dependent tissues (including the gin-trap and the posterior dorsal tergal edge) in abdomen are likely serially homologous to one of the wing serial homologs in T1 (the carinated margin), and thus are partially serially homologous to wings in T2 and T3 (see Fig. 6*A*). However, unlike T1, the induction of the pleural wing serial homologs in the abdomen is usually repressed by Hox (at least based on the current criteria of wing serial homologs having *vg* expression and being capable of transforming into wings) (see Fig. 6*B*). Upon Hox transformation, the pleural wing serial homolog (with *vg* expression) is ectopically induced, and when it merges with the tergal wing serial homolog, an ectopic wing is formed in the abdominal segments (note that the two distinct sets of wing serial homologs do not give rise to two separate complete wings upon Hox transformation, but instead the merger of the two tissues facilitates the formation of one complete wing).

Asymmetric Requirement of *Ubx* and *abdA* in the Abdominal Wing Serial Homologs. Knocking down the function of both *Ubx* and *abdA* appears to be essential for the induction of complete wings in the abdominal segments (31). Immunostaining with FP6.87, which detects both Ubx and AbdA proteins (34), has revealed that the sum of Ubx and AbdA proteins appears to be uniform throughout the abdominal segments at the pupal stage (Fig. S5 *A* and *B*). However, staining with the Tc-Ubx–specific antibody showed a more gradated distribution of Ubx proteins in the abdomen, with a stronger expression in the anterior abdominal segments that gradually decreases in more posterior segments (Fig. S5 *C* and *D*). This implies that the AbdA protein has an opposite gradient of expression, and the sum of the two abdominal Hox proteins (and not each specifically) is important for the suppression of the wing identity in the abdominal segments. If this is the case, we may be able to induce an intermediate transformation by single RNAi for either *Ubx* or *abdA*. Therefore, we next performed single RNAi for each gene and analyzed their phenotypes to further understand the genetic control over the abdominal wing serial homologs.

RNAi for *abdA* at the early last larval stage caused strong abnormalities in the gin-traps, with a complete disruption of the anterior jaw of each gin trap and strong reduction of the posterior jaw (Fig. 5 *A–I* and Fig. S6 *A–D*). In addition, the region posterior
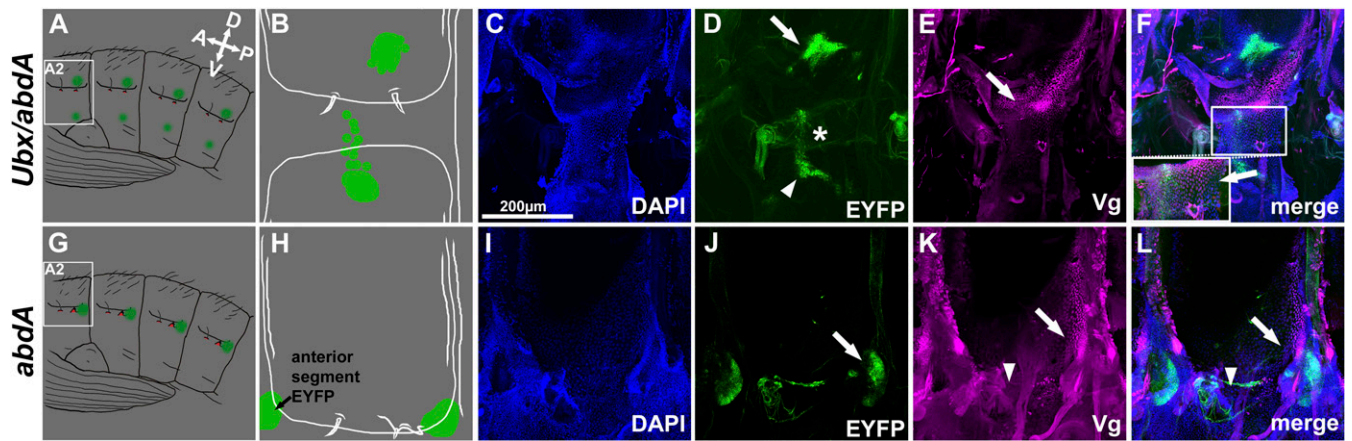
Fig. 4. Vg expression and *nub* enhancer trap activity in Hox RNAi pupal epidermis. (*A–F*) *Ubx/abdA* double RNAi. Gin-traps are missing (*A* and *B*), and *nub* enhancer trap activity appears at two locations, one in the tergal and the other in the pleural region of each segment (arrow and arrowhead in *D*, respectively, also indicated with green in *A* and *B*). *nub* enhancer trap activity also stretches between the two regions (asterisk in *D*). Vg expression is strongest in the gin-trap region (arrow in *E*) and also expands toward the pleural region (arrow in *F*), overlapping with the *nub* enhancer trap activity in the pleuron (*Inset* in *F*). (*G–L*) *abdA* RNAi. Gin-traps are reduced (*G* and *H*). *nub* enhancer trap activity appears posterior to the gin-trap region (arrow in *J*, also depicted with green in *G* and *H*). Cells that express Vg accumulated posteriorly at the lateral tergal region (arrow in *K*), coinciding with the *nub* enhancer trap activity (arrow in *J*). Vg expression near the anterior gin-trap regions is missing (arrowheads in *K* and *L*), suggesting that Vg-expressing cells are redistributed more posteriorly in *abdA* RNAi. The first column and second column are schematic representations of areas imaged in the respective row. Scale bar in *C* applies to all images except for the diagrams.

to the gin-trap in the tergum of each segment forms a bulge (Fig. 5 *G–I*), which contains *nub* enhancer trap-positive cells (arrows in Fig. 5 *J–L*). It is worth emphasizing that the location of strong gin-trap abnormality (anterior) does not match with the location of strong wing transformation (posterior) (Fig. 5 *I* and *L*). This suggests that, in addition to suppressing wing identity, *abdA* is also important for the distribution of wing serial homolog cells toward the anterior at the lateral tergum to form a laterally flattened gin-trap structure. A caveat to this interpretation is that the cells located posterior to the gin-trap that become *nub* enhancer trap-positive upon *abdA* RNAi are distinct from the cells that produce gin-traps. We decided to use another tool to address this possibility. We recently identified a 415-bp region at the *nub* locus that is sufficient to recapitulate the *nub* wing expression when combined with a reporter gene (*Tc-nub1L-mCherry*) (35). Interestingly, this reporter construct drives expression not only in the wings on T2 and T3, but also in other wing serial homologs, including the carinated margin and pleural plates in T1 and the cells underneath the gin-traps in the pupal abdomen (Fig. S7 *A* and *B*). RNAi for *vg* completely removed the mCherry expression (Fig. S7 *C* and *D*), indicating that these mCherry-positive cells are the cells that produce gin-traps. Upon *abdA* RNAi, the mCherry-positive cells colocalize with the *nub* enhancer trap-active cells at the bulge posterior to the gin-trap in each tergum (Fig. S7 *E* and *F*). This result indicates that the cells that are transforming into wings are gin-trap–producing cells that failed to be distributed to a more anterior-lateral region, thus excluding the possibility that the cells ectopically transforming into wings are distinct from the gin-trap cells.

In contrast to the ectopic induction of wing cells at the lateral tergal location, we did not observe any *nub* enhancer trap activity in the dorsal posterior tergal edge or in the pleural region in the *abdA* RNAi pupae (arrow in Fig. 4*J* and Fig. 5*L*). We also examined Vg expression after *abdA* RNAi (Fig. 4 *G–L*) and observed that Vg-expressing cells redistribute, with fewer *vg*-expressing cells in the anterior region under the gin-trap (arrowhead in Fig. 4 *K* and *L*) and more along the dorsal posterior (arrow in Fig. 4 *K* and *L*). Compared with *abdA* RNAi, *Ubx* RNAi resulted in much more subtle abnormalities, with a small bulging protrusion forming on A1 (arrowhead in Fig. 5*M*) and no noticeable

gin-trap defects or induction of wing cells in the remaining posterior abdomen (Fig. 5 *M–R* and Fig. S6 *E* and *F*).

It is possible that the RNAi phenotypes we observed do not reflect the null condition due to incomplete knockdown. To test this possibility, we performed the same RNAi in Hox mutant heterozygous conditions, which should enhance the RNAi phenotypes if knockdown is incomplete (for example, see figure S4 of ref. 28). We used the null allele for each Hox gene, Utx^{M115}/Es (36) and $abdA^{A12}/Ey$ (37). In addition, we also used Df (1–3)/Ey, which contains a deletion spanning nearly the entire Hox complex (*Deformed* to *abdA*) (38). These mutant strains do not exhibit any abnormalities in their gin-traps in a heterozygous condition (Fig. S6 *G* and *H*). We did not see any enhancement in the gin-trap abnormalities in the *abdA* RNAi pupae with Hox deletion heterozygous [Df (1–3)/Ey] (Fig. S6 *G–J*) or *abdA* null heterozygous ($abdA^{A12}/Ey$) (Fig. S6 *M* and *N*), suggesting that the *abdA* RNAi phenotypes we observed are close to the null condition. In contrast, *Ubx* RNAi in Hox deletion heterozygous [Df (1–3)/Ey] (Fig. S6 *K* and *L*) and *Ubx* null heterozygous (Utx^{M115}/Es) (Fig. S6 *O* and *P*) conditions slightly enhanced the *Ubx* RNAi phenotype, as we observed a subtle reduction of the posterior jaw of the gin-traps, suggesting that *Ubx* is also required for gin-trap formation and suppression of wing identity in the gin-trap cells.

Taking these data together, we find that single RNAi for each abdominal Hox gene has revealed an asymmetric requirement of *Ubx* and *abdA* in the development of abdominal wing serial homologs (Fig. 6*B*). *abdA* appears to play a more significant role in the formation of the tergal wing serial homolog (except in A1), by suppressing wing identity and facilitating the distribution of the wing serial homolog cells along the anterior–posterior axis to form a proper gin-trap. However, *Ubx* also plays a role in the tergal wing serial homologs in abdomen, as *nub* enhancer trap activity continues to be suppressed at the dorsal posterior edge of terga in *abdA* single RNAi, and the morphology of the adult tissues that develop from the *nub* enhancer trap-positive cells induced by *abdA* RNAi appear to be more similar to the abdominal dorsal body wall than the wing (Fig. 5 *G–I*). In addition, *Ubx* does play a dominant role in the tergal wing serial homolog in A1, as *Ubx* single RNAi is sufficient to induce partially transformed wing tissue (Fig. 5*M*). In contrast to the asymmetric contribution of
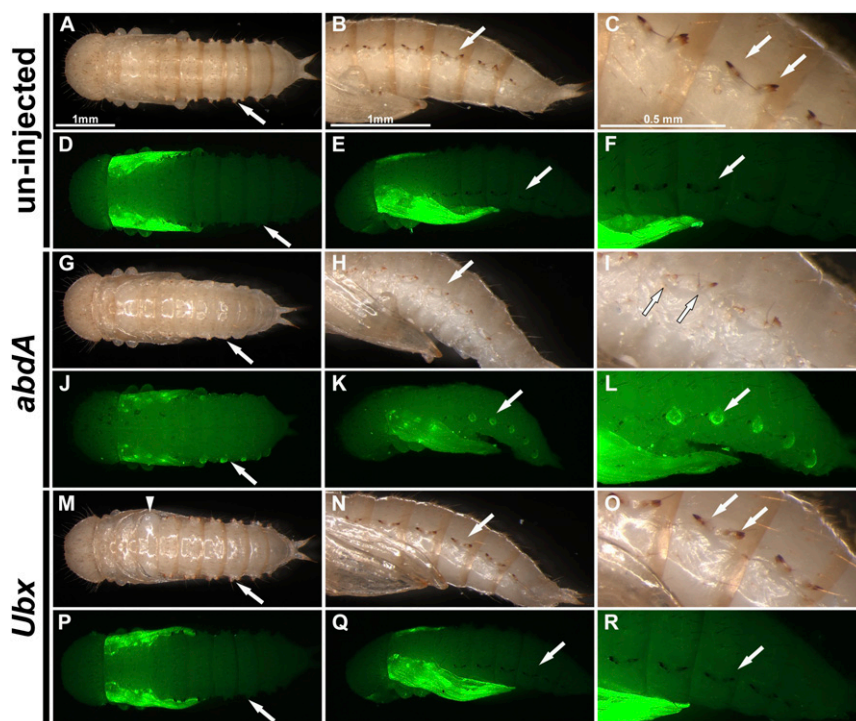
Linz and Tomoyasu

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Fig. 5. Gin-trap formation and transformation in *Ubx* and *abdA* each single RNAi. (*A–F*) Uninjected *pu11* pupa. Gin-traps are formed normally (*A–C*), and the *nub* enhancer trap activity is absent in abdominal segments (*D–F*). (*G–L*) *abdA* single RNAi. Anterior jaw of gin-traps is missing, and the posterior jaw is strongly reduced (arrows in *G–I*). *nub* enhancer trap activity appears in the region posterior to each gin-trap (arrows in *J–L*). (*M–R*) *Ubx* single RNAi. A partial transformation of A1 to a thoracic segment is observed (arrowhead in *M*). Gin-trap formation is unaffected (arrows in *M–O*), and no *nub* enhancer trap activity is observed in abdominal segments (*P–R*), except in A1. Scale bars in *A–C* also apply to other corresponding panels.

Ubx and *abdA* to the tergal wing serial homolog, these two Hox genes appear to be redundant in the suppression of pleural wing serial homologs in the abdominal segments, as we did not see ectopic *nub* enhancer trap activity in the pleural region in either of the single RNAi experiments.

Discussion

A Dual Origin of Insect Wings. The origin of insect wings has been the subject of heated debates for centuries. These debates have culminated into two prominent, yet contrasting hypotheses: the tergal origin hypothesis and the pleural origin hypothesis (4). The dual origin hypothesis is a third hypothesis, which can potentially unify the two competing hypotheses. A dual origin of insect wings was proposed as early as 1916 by Crampton (11), but Rasnitsyn (5) in his modified paranotal hypothesis might have been the first to clearly favor the idea that both tergal and pleural tissues have contributed to the evolution of insect wings. The first evo-devo support for this hypothesis came from expression analyses in several basal insects (12). As mentioned, our previous study in *Tribolium* has identified two wing serial homologs in T1 (one of a tergal and the other of a pleural nature), which has added compelling support for the dual origin hypothesis (13). Since then, this third hypothesis has been gaining momentum, with further support obtained from studies in two additional insects (the milkweed bug, *Oncopeltus fasciatus*, and the German cockroach, *Blattella germanica*) (14, 15), and more recently also from a paleontological study analyzing the thorax of an ancient insect, Palaeodictyoptera (17), and a detailed morphological study in the cricket, *Gryllus bimaculatus* (16). In the present study, we showed that there are two distinct sets of wing serial homologs (of tergal and pleural nature) in each segment even outside of the appendage-bearing segments in *Tribolium*, providing crucial support for a dual origin of insect wings.

On the Tergal Wing Serial Homologs in the Beetle Abdominal Segments. Ohde et al. (24) have previously shown that the gin-trap, a pupal tergal structure, is an abdominal wing serial homolog of beetles. In the present study, we found that the expression of Vg in terga covers a wider domain in *Tribolium*, including not only the gin-trap region but also the dorsal posterior edge of each tergum. Are all of these *vg*-expressing tergal cells serially homologous to wings? Our data suggest that at least some of the cells at the dorsal posterior tergal edge are serially homologous to wings, as these cells could transform into wings (detected by *nub* enhancer trap activity) upon *Ubx/abdA* double RNAi. However, the situation may be more complex, as, unlike RNAi for *vg*, RNAi for two additional wing genes (*ap* and *dsh*) did not affect the pigmentation at the dorsal posterior edge of each tergum (even though the same RNAi disrupted the formation of the gin-traps). A previous report in *Oncopeltus* has revealed that the scutellum, a structure located at the dorsal posterior edge of the thoracic terga, is also *vg*-dependent. This finding, along with our data, may point to a situation where the lateral to posterior edge of terga in insects represents a tissue with one shared identity (as the tergal wing serial homolog). The dependency of these *vg*-expressing tissues on wing gene network components, however, may be more labile, with the cells at the lateral edge of terga having a larger genetic overlap with true wings.

On the Pleural Wing Serial Homologs in the Beetle Abdominal Segments. In addition to the tergal wing serial homologs in the abdomen, we found that, upon *Ubx/abdA* double RNAi, a second group of cells located in the more lateral (pleural) position merge with the tergal cells to form an ectopic wing. This situation parallels our previous reports in *Tribolium* T1, with two separable wing serial homologs in each abdominal segment. This interpretation would imply that the gin-traps, along with the dorsal
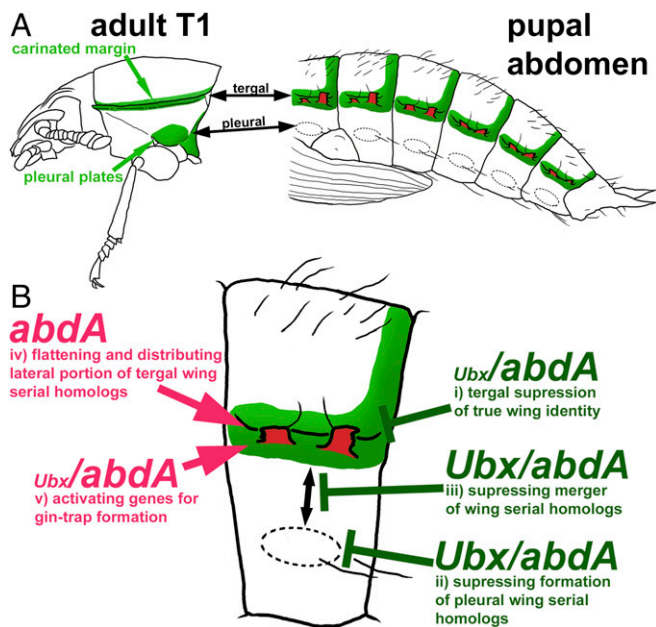
Linz and Tomoyasu

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Fig. 6. Abdominal wing serial homologs and Hox functions. (*A*) Relationship between the T1 and abdominal wing serial homologs. (*B*) Five Hox functions related to abdominal wing serial homologs.

posterior region of each abdominal segment, are serially homologous to the carinated margin in T1 (Fig. 6*A*), and each are thus partially homologous to the bona fide wings on T2 and T3. However, unlike T1, the pleural wing serial homologs in abdominal segments are normally suppressed by the action of Hox.

The suppression of the pleural wing serial homologs in the abdomen is likely connected to the legless nature of the insect abdomen. In most extant insects, formation of leg-related structures (i.e., ventral appendages) in the abdominal segments is suppressed by the action of *Ubx* and *abdA* (39, 40). The expression of *Distalless* (*Dll*), the leg lineage marker gene, is missing in the abdominal segments throughout development in *Drosophila* (39) and in *Tribolium* (41), indicating that the leg serial homologs are never induced in the abdominal segments in these insects (see ref. 23 for more in-depth discussion related to abdominal leg serial homologs). Since insect pleural plates are thought to have evolved from the most proximal segment of ancient legs, it is intriguing to speculate that the formation of some of the pleural plates (including the pleural wing serial homologs) is also suppressed by the same mechanism in the abdominal segments. The leg-derived nature of pleural plates in *Tribolium* has recently been supported from the developmental perspective (42), providing further support to the idea that the formation of pleural wing serial homologs is usually suppressed in the abdomen.

Interestingly, we never observed the formation of ectopic legs in the abdomen by Hox RNAi (even at earlier larval stages) (Fig. S8). This is in contrast to the abdominal pleural wing serial homologs, which can be ectopically induced upon Hox RNAi. Perhaps preexisting abdominal pleural plates can be transformed into wing serial homologs, but these tissues are not large enough (and/or less competent) to transform into the entire leg. Alternatively, there may be a small group of *vg*-expressing cells present in the abdominal segments (i.e., abdominal pleural wing serial homologs), but these cells might be difficult to detect with our current staining method. At least during embryogenesis, we detected only the L-shaped tergal *vg* expression in abdominal segments, while we observed two groups of *vg*-expressing cells in the thoracic segments (13). Further analyses on the development of pleural plates, along with detailed *vg* expression in these tissues, will shed light on the evolutionary and developmental contribution of the pleural tissues to the evolution of insect wings.

In this study, we use the *nub* enhancer trap activity visualized by EYFP in *pu11* as a surrogate for "wing identity." There is a caveat to this interpretation, as *pu11* also has EYFP expression in several tissues other than wings, such as the peripheral nervous system and joints of the leg (Fig. S8 *A–C*). However, the pleural *pu11* enhancer-positive tissue we detected in Hox-transformed abdominal segments is unlikely to be related to these nonwing tissues, as the pleural EYFP-positive cells do not have neuronal morphology and, as mentioned, leg-related structures never appear to be induced in the abdomen even with the strongest transformation upon *Ubx/abdA* RNAi (Fig. S8 *D–F*).

Function of Hox Genes in the Abdominal Wing Serial Homologs. Through the RNAi analysis, we have identified several functions of Hox genes in the abdominal segments during postembryonic development, both related and unrelated to wing serial homologs (Fig. 6*B*). For a function unrelated to wing serial homologs, we noticed that *abdA* is essential for the sclerotization [or more precisely, exoskeletalization (28)] of the A3–A7 abdominal sternites, as *abdA* RNAi has removed exoskeletalized cuticle (additional functions of *abdA* in postembryonic development have also been reported in refs. 37 and 43). For functions related to abdominal wing serial homologs in *Tribolium*, we found that *Ubx* and *abdA* are important for: (*i*) suppressing the true wing identity in the tergal wing serial homolog, (*ii*) suppressing the formation of the pleural wing serial homolog itself, (*iii*) keeping the two wing serial homologs from merging, (*iv*) flattening and distributing the lateral portion of the tergal wing serial homologs toward a more anterior region for the formation of the gin-traps, and (*v*) activating genes essential for gin-trap formation. The first three functions are related to the suppression of bona fide wing identity in the abdomen (and are thus likely conserved throughout Insecta), while the last two functions are for the formation of a structure unique to some of the coleopteran pupa, the gin-trap.

It is currently unknown if *Ubx* and *abdA* are directly involved in some of the gin-trap formation processes [such as function (*v*)], as a misregulation in the suppression of the bona fide wing identity and/or the anterior–posterior distribution of tergal wing serial homolog cells in the abdominal segments [i.e., functions (*i*)–(*iv*)] could indirectly disrupt the formation of the gin-traps. For example, although the anterior jaw of the gin-trap was predominantly affected by *abdA* RNAi (Fig. 5 *G–I* and Fig. S6 *C and D*), this is likely not because *abdA* is important for the formation of the anterior jaw, but because the lateral tergal wing serial homolog cells failed to be distributed more anteriorly in *abdA* RNAi pupae [namely an indirect effect of the disruption in function (*iv*)]. In some cases, however, we observed disruptions in gin-trap formation without the activation of true wing identity (evaluated by the lack of *nub* enhancer trap activity), which may suggest that *Ubx* and *abdA* might also have a direct role in activating genes essential for gin-trap formation.

The role of each Hox gene appears to be asymmetric. Both *Ubx* and *abdA* are essential for the suppression of the formation of the pleural wing serial homolog [function (*ii*)], as the pleural *vg* expression and *nub* enhancer trap activity are induced only in the *Ubx/abdA* double RNAi (Fig. 3 *F–K*). In contrast, *abdA* might be more dominant in suppressing true wing identity in the tergal wing serial homolog [function (*i*)] and in distributing the tergal wing serial homologs toward the anterior region [function (*iv*)], since *abdA* single RNAi was sufficient to disrupt these processes (Figs. 4 *G–L* and 5 *G–L*). However, the transformed *nub* enhancer trap-positive tergal tissue in *abdA* single RNAi (Fig. 5 *J–L*) does not develop into an adult structure with wing morphology, and instead maintains the typical dorsal abdominal cuticle identity, indicating that *Ubx* is also required in suppressing the true wing

Linz and Tomoyasu

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identity in the tergal wing serial homolog. *Ubx* appears to also have a role in gin-trap formation itself; however, the involvement of *Ubx* in this process should be minor as gin-trap disruption can only be observed in *Ubx* RNAi with the *Ubx* mutant heterozygous background (Fig. S6 *K, L, O, and P*). In addition, *Ubx* does have a dominant role in A1, as *Ubx* single RNAi is sufficient to induce partial ectopic wings in A1 (Fig. 5*M*).

Overlaps in Gene Networks Operating Among Wing Serial Homologs. There are two major possibilities to consider when explaining two tissues with a genetic overlap: shared common ancestry and cooption. Our identification of wing serial homologs in the nonwinged segments is based on shared common ancestry, with cooption being a significant caveat to our interpretation. A battery of genes (or a part of a gene network) can be coopted into a novel tissue, which also can result in two tissues with a genetic overlap. The genetic overlap between legs and horns in dung beetles provides a key example of cooption (44). However, in contrast to two tissues that share common ancestry, tissues that have coopted gene networks likely cannot be transformed into each other upon Hox loss-of-function (45) (also see ref. 23 for further discussion on Hox and serial homology). In this study, we showed that the T1 carinated margin and the pupal gin-traps in the abdomen (the tergal wing serial homologs) have a shared dependency on a number of wing gene network components (*vg, ap*, and Wg signal, and *nub* wing enhancer activity) with true wings. The shared dependency of these tissues on the wing genes are likely explained by a shared common ancestry (i.e., these tissues are serially homologous to each other) and not by cooption, since both the carinated margin and the gin-traps can contribute to wing transformation upon Hox RNAi. This also means that the genes shared among these serially homologous tissues were essential before the diversification of these tissues (including the evolution of bona fide wings), and thus are a part of the tergal "core" network. Compared with the tergal wing serial homologs, we found that the pleural wing serial homologs display a smaller genetic overlap with wings (*vg* and *nub* enhancer trap activity upon partial transformation). It is yet to be determined if this limited genetic overlap with wings represents an ancestral state of pleural wing serial homologs or a derived state in the coleopteran lineage.

Two Sets of Wing Serial Homologs per Segment: Ancestral or Derived? We have previously shown that there are two distinct sets of wing serial homologs in T1 of *Tribolium*. As mentioned, this "two wing serial homologs per segment" configuration has also been found in T1 of other insects, such as *O. fasciatus* and *B. germanica* (14, 15). In this study, we showed that the presence of two distinct sets of wing serial homologs is not limited to T1, and instead is also seen even outside of the appendage-bearing segments in *Tribolium*. Considering the pervasive nature of the "two wing serial homologs per segment" configuration in nonwinged segments of various insects, it is likely that this configuration represents an ancestral state of the tissues serially homologous to wings (i.e., plesiomorphic), while wings on T2 and T3 represents a derived state (apomorphic). Nevertheless, caution must be exercised when interpreting the ancestral state of wing serial homologs, as each insect can provide only a snapshot of the evolutionary history of these tissues. The genetic and developmental regulations of the wing serial homologs in the beetle abdomen described in this study (especially the induction of the pleural wing serial homolog upon Hox transformation) can be unique to the beetle lineage. Therefore, it is crucial to investigate the development of wing serial homologs in the abdomen of diverse insects, which will help us determine the evolutionary relationship between abdominal wing serial homologs and bona fide wings. In summary, the "two wing serial homologs per segment" configuration as an ancestral state is one of the key concepts in the dual origin hypothesis, which requires further validation from a wide taxonomy of insects, and perhaps more importantly from species outside of Insecta.

The Spectrum of the Dual Origin Hypothesis. The dual origin hypothesis embraces the strengths of the two original wing origin hypotheses; the complex wing articulation system was derived from the ancestral proximal leg segments (the pleural origin hypothesis), while the large flat tissue was provided from the expansion of terga (the tergal origin hypothesis) (5, 12, 13, 23). Interestingly, there is a significant variation within the dual origin hypothesis in regard to the degree of contributions from tergal and pleural tissues to the evolution of wings, creating "a spectrum" of explanations that can exist within the dual origin hypothesis. For example, the contribution of the pleural tissues can be as little as just a small portion of wing articulation structures, or as large as forming the majority of the wing blade.

In our study, we noticed that a part of the pleural *nub* enhancer trap-positive tissue in the abdomen induced upon Hox RNAi is Vg-negative (Fig. 4 *E* and *F*). Interestingly, in addition to the expression in the wing blades, *pu11* also has strong EYFP expression in the T2 and T3 wing hinge (arrows in Fig. S8*C*), which is outside of the *vg* functional domain. Therefore, it is attractive to speculate that some of the pleural wing serial homolog cells observed in Hox RNAi mainly contribute to the hinge region of the abdominal ectopic wing. Nonetheless, the precise contribution of each wing serial homolog to the formation of a complete wing is still elusive. There are two contexts in which we can study the details of the merger: (*i*) ectopic wings induced by Hox loss-of-function in otherwise wingless segments, and (*ii*) normal wing development in T2 and T3. A lineage tracing experiment will be fruitful to dissect the detailed contribution of each wing serial homolog to the formation of a wing, and can thus help to resolve the spectrum that exists within the dual origin hypothesis. It will also be increasingly important to continue investigating the dual origin hypothesis with a wider taxonomic breadth, which will allow us to identify lineage-specific modifications to the wing serial homologs and to resolve the evolutionary history of the two separate wing serial homologs. These approaches, when combined, will provide critical insight into the mechanisms that facilitated the evolution of insect wings.

Materials and Methods

***Tribolium* Cultures and Injection.** Beetles were cultured on whole-wheat flour (+5% yeast) at 30 °C with 70% humidity. Injections were performed during the early last larval stage (days 1–2) or penultimate larval stage. Detailed genotypes of the beetles used in this study are in *SI Materials and Methods* and Table S1.

dsRNA Synthesis. Detailed cloning and dsRNA synthesis of the genes used in this study (*Tribolium vg, ap* genes, *dsh, nub, Ubx,* and *abdA*) has been previously described (13, 28, 31). Detailed information, including primers used to synthesize the dsRNA templates and the length of the products, are in *SI Materials and Methods* and Table S1.

Tissue Staining and Documentation. *Tribolium* pupae were dissected in PBS, and either were cleared in 90% lactic acid for the pupal cuticle preparation or were fixed in 4% formaldehyde/PBS and used for antibody staining. Adult *Tribolium* abdomen were fixed in 100% ethanol, then air-dried and mounted with carbon conductive tabs for SEM. The images were captured by using Zeiss AxioCam MRc5 with AxioPlan 2 or Zeiss Discovery V12. Confocal images were captured by using Zeiss 710, and SEM images were taken on a Zeiss Supra 35 VP FEG SEM. Detailed tissue dissection and fixation procedures, as well as antibody concentrations, are in *SI Materials and Methods*.

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