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ORIGINAL ARTICLE

RNAi screening of developmental toolkit genes: a search for novel wing genes in the red flour beetle, *Tribolium castaneum*

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Abstract The amazing array of diversity among insect wings offers a powerful opportunity to study the mechanisms guiding morphological evolution. Studies in *Drosophila* (the fruit fly) have identified dozens of genes important for wing development. These genes are often called candidate genes, serving as an ideal starting point to study wing development in other insects. However, we also need to explore beyond the candidate genes to gain a more comprehensive view of insect wing evolution. As a first step away from the traditional candidate genes, we utilized *Tribolium* (the red flour beetle) as a model and assessed the potential involvement of a group of developmental toolkit genes (embryonic patterning genes) in beetle wing development. We hypothesized that the highly pleiotropic nature of these developmental genes would increase the likelihood of finding novel wing genes in *Tribolium*. Through the RNA interference screening, we found that *Tc-cactus* has a less characterized (but potentially evolutionarily conserved) role in wing development. We also found that the *odd-skipped* family genes are essential for the formation of the thoracic pleural plates, including the recently discovered wing serial homologs in *Tribolium*. In addition, we obtained several novel insights into the function of these developmental genes, such as the involvement of *mille-pattes* and *Tc-odd-paired* in metamorphosis. Despite these findings, no gene we examined was found to have novel wing-related roles unique in *Tribolium*. These results suggest a relatively conserved nature of developmental toolkit genes and highlight the limited degree to which these genes are co-opted during insect wing evolution.

Keywords *Tribolium* · Developmental genes · Wing · Candidate genes · Wing serial homologs

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Introduction

The class Insecta is one of the most diverse metazoan groups, composing nearly 80 % of all known animal species (Grimaldi and Engel 2005). The success of this group can at least partly be credited to their diverged wing structures that provide benefits for their successful radiation, such as mobility, protection, and camouflage (Crowson 1981; Grimaldi and Engel 2005). Insect wings and their derivatives (i.e., dorsal appendages) vary greatly among different insect lineages, and also between the forewing and hindwing in individual species, providing a unique opportunity to study the molecular and genetic mechanisms underlying morphological evolution.

The processes guiding the formation of dorsal appendages are best understood in a dipteran insect, *Drosophila melanogaster*, and the knowledge acquired from these *Drosophila* studies has been utilized as a paradigm to study wing development in other insects (see Brook et al. 1996 to review *Drosophila* wing development. Some examples of studies in other insects are; Tomoyasu et al. 2009 and Tomoyasu et al. 2005 in beetles; Bowsher et al. 2007 and Abouheif and Wray 2002 in ants; Weatherbee et al. 1999, Martin et al. 2012, Macdonald et al. 2010, Shirai et al. 2012, and Stoehr et al. 2013 in butterflies). The genes identified as essential for wing development in *Drosophila* (i.e., wing genes) have been a focus of these studies, as developmental genes are often used in a similar developmental context in different organisms, with slight modifications corresponding to morphological evolution. This type of approach, the

candidate gene approach, has been quite effective in elucidating the conserved as well as diverged genetic mechanisms underlying the diversification of insect wings.

Despite the effectiveness of the candidate gene approach, it is limited by the fact that the pool of genes to choose for analysis can only stem from those identified in *Drosophila*. While the conserved and diverged functions of the wing genes that have been evolutionarily maintained across species can be evaluated by this approach, genes uniquely important for wing development in each species cannot be identified through these candidate genes. In addition, this limitation has a risk of creating a fly-biased view of evolution, in which we treat the fly paradigm as a "starting point" of evolution. To go beyond the candidate gene approach and also escape this fly-centric viewpoint, we need to analyze insects other than *Drosophila* at a similar level of detail as has been achieved in the fly.

With a fully sequenced genome (*Tribolium* Genome Sequencing Consortium 2008), available mutants (Brown et al. 2003; Klingler 2004; Denell 2008; Trauner et al. 2009) and a robust and systemic RNA interference (RNAi) response (Brown et al. 1999; Bucher et al. 2002; Tomoyasu and Denell 2004; Tomoyasu et al. 2008), the red flour beetle, *Tribolium castaneum*, may offer such an opportunity. Coleopterans (beetles) contain vastly diverged wing structures compared to dipterans (flies). Flies, such as *Drosophila*, have typical flight wings on the second thoracic segment (T2) but have strongly reduced dorsal appendages (halteres) on the third thoracic segment (T3). In contrast, the hindwings of beetles are used for flight, while the forewings have been modified and now act as protective armoring structures, called elytra. The acquisition of elytra was an important step in the lineage leading to beetles, which has made Coleoptera one of the most successful animal groups on the planet (Lawrence and Britton 1991; Grimaldi and Engel 2005; Crowson 1981). The candidate gene approach has provided intriguing insights into beetle elytron evolution and development, which include the Hox-free nature of elytra (Tomoyasu et al. 2005) and the multiple co-options of exoskeletalization genes into the conserved wing gene network (Tomoyasu et al. 2009). However, it is also necessary to analyze genes unrelated to the fly candidate genes to gain a more comprehensive view of elytron evolution in beetles.

As a first step to escaping the limitations of the candidate gene approach, we examined the postembryonic function of a group of *Tribolium* genes whose orthologs in *Drosophila* are important for embryonic segmentation and axis establishment, but have not been implicated in wing development. We hypothesized that the highly pleiotropic nature of these developmental genes would increase the likelihood of finding novel wing genes in *Tribolium*. We call this a "semi-candidate gene" approach, as it is still relying on *Drosophila* studies but is independent of genes identified to be important for fly wing development.

We disrupted the function of these genes by RNAi and assessed the effects on the postembryonic development in *Tribolium*. Among the 25 genes we have tested (Table 1), RNAi for *Tc-cactus* (*Tc-cact*) suppressed the proliferation of wing and elytron discs in *Tribolium*, indicating a less characterized (but potentially evolutionarily conserved) role of *Tc-cact* during wing development. We also found that RNAi for the *odd-skipped* family genes disrupted the formation of the pleural plates on the first thoracic segment (T1), which have recently been discovered as wing serial homologs in *Tribolium* (Clark-Hachtel et al. 2013). In addition, we obtained several novel insights into the function of the developmental genes during postembryonic development in *Tribolium*. For instance, *mille-pattes* (*mlpt*) and *Tc-odd-paired* (*Tc-opa*) appear to be important for metamorphosis in *Tribolium*, and the *odd-skipped* family genes show how the redundancy of paralogs shifts over evolutionary time periods. However, despite these findings, no gene we examined was found to have novel wing-related roles in *Tribolium*. Although disappointing, this outcome provides us with two important insights. First, it suggests a relatively

Table 1 List of semi-candidate genes examined in this study

Gene name	Symbol	Protein class
torso	*tor*	Receptor tyrosine kinase
tailless	*tl*	Zn finger TF
nanos	*nos*	Zn finger TF
caudal	*cad*	Homeodomain TF
giant	*gnt*	Leucine zipper
hairy	*h*	HLH TF
mille-pattes	*mlpt*	Short peptides, polycistronic
orthodenticle 1	*otd1*	Homeodomain TF
orthodenticle 2	*otd2*	Homeodomain TF
even-skipped	*eve*	Homeodomain TF
sister of odd and bowl	*sob*	Zn finger TF
odd-skipped	*odd*	Zn finger TF
brother of odd with entrails limited	*bowl*	Zn finger TF
drumstick	*drm*	Zn finger TF
odd-paired	*opa*	Zn finger TF
paired	*prd*	Homeodomain TF
sloppy paired	*slp*	Fork head domain
teneurin-m	*Ten-m* (*odz*)	EGF repeat
deadpan	*dpn*	HLH TF
buttonhead	*btd*	Zn finger TF
Sp1	*Sp1*	Zn finger TF
dorsal	*dl*	rel/NF-kappaB TF
cactus	*cact*	I kappa B TF
aristaless	*al*	Homeodomain TF
bric-a-brac	*bab*	BTB/POZ HTH TF

TF transcription factor

conserved nature of developmental toolkit genes and highlights the limited degree to which these genes are co-opted during insect wing evolution. Second, it reveals the difficulties of a semi-candidate gene approach and demonstrates the necessity of utilizing an approach that is truly independent of candidate genes, such as RNA sequencing and a genome-wide RNAi screen (Bucher and Klingler 2014; also mentioned in Knorr et al. 2013).

Materials and methods

RNA interference in *Tribolium*

Beetles were cultured on whole wheat flour (+5 % yeast) at 30 °C with 70 % humidity. *pu11 nubbin* (*nub*) enhancer trap line (Lorenzen et al. 2003; Tomoyasu et al. 2005; Clark-Hachtel et al. 2013) was used for all RNAi experiments. The plasmids and primers used to make double-stranded RNA (dsRNA) in this study are summarized in Table S1. dsRNA was synthesized using the MEGAscript T7 Transcription Kit (Life Technologies). Detailed dsRNA synthesis and injection procedures were described previously (Linz et al. 2014; Philip and Tomoyasu 2011). At least 20 last instar larvae were injected for each single gene tested (see Table 2 for numbers of larvae injected and their survival rates). The stage of larvae for injection was determined by the size of EYFP positive wing discs in the *pu11 nub* enhancer trap line. We selected larvae that had the initial stage of EYFP expression in the wing disc, which corresponds to 2.5 days after the final larval molt (see Figure S4 of Clark-Hachtel et al. 2013). dsRNA was injected into the dorsal side of the first abdominal segment at a concentration of 1 μg/μl unless otherwise stated. Each larva can hold up to 0.7 μl of dsRNA solution when injected at the stage described above. Detailed information including primer sequences and off-target effect assessment are included in Table S1.

Phylogenetic analysis

Pfam (Finn et al. 2013) was used to identify conserved protein domains. Multiple alignments were created and curated in MEGA 5.2.1 (Tamura et al. 2011). Neighbor-joining and maximum likelihood analyses were performed in MEGA 5.2.1 with bootstrapping using 2000 replicates. Bayesian trees were generated using the GTR+I+ Γ model in MrBayes (Ronquist et al. 2012) and visualized with FigTree 1.4.2 (Rambaut 2014; Hall 2011). The conserved Zn finger domains (amino acid sequences or the corresponding nucleotide sequences) were used to create multiple alignments for the *odd-skipped* family members (see Document S1 for both amino acid and nucleotide alignments).

Image processing and documentation

The *Tribolium* adults were fixed in 95 % ethanol at least overnight. EYFP larval images were taken by submerging larvae in double-distilled water. The images were captured by Zeiss Discovery V12 with AxioCam MRc 5. Zeiss AxioVision Extended Focus module was used to obtain images with increased focus depth. Some pictures were enhanced only for brightness and contrast with Adobe Photoshop CS3.

Results

An RNAi survey for semi-candidate genes in *Tribolium*

The insect body is primarily patterned along two major axes (anterior/posterior (A/P) and dorsal/ventral (D/V)) during embryogenesis. The genes driving these processes are among the most thoroughly examined in *Drosophila* (for a general review, see Carroll et al. 2001; Gilbert 2014). For this study, we chose 25 *Tribolium* genes orthologous to these *Drosophila* embryonic patterning genes (Table 1) and assessed their functions at later postembryonic stages in *Tribolium*. From the four major classes of A/P patterning genes (maternal, gap, pair-rule, and segment polarity genes) (see Sanson 2001 for A/P patterning review), we focused mainly on the first three classes. The last class, the segment polarity genes, has been excluded because these genes are known to be involved in wing development in *Drosophila*. From the D/V patterning genes, we have selected *dorsal* and *cact* (see Lynch and Roth 2011 for D/V patterning review). The paralogs of some appendage genes with no known wing function (e.g., *odd* paralogs) and a unique *Tribolium* gap gene (*mlpt*) were also included (Table 1).

RNAi was performed at the standard 1 μg/μl concentration for each of the 25 genes (Table 2). Lower concentrations were used for genes with high lethality (*mlpt* and *Tc-cact*). A large subset of the 25 genes (15/25) failed to show any noticeable adult abnormalities (Table 2). A small group of genes (3/25) showed RNAi phenotypes previously described or parallel to *Drosophila* loss-of-function phenotypes (Fig. S1), while another set of genes (6/25) showed previously undescribed phenotypes outside of the wing tissues (Table 2, Figs. 1 and 3). Only one of the 25 genes (*Tc-cact*) had a clearly observable effect on wing and elytron development (Table 2 and Fig. 2a–d). The survival rate for genes that showed no phenotype stayed at high levels (>~70 % except *Tc-slp*, which resulted in a lower survival rate due to unknown pleiotropic effects) (Table 2). For the genes whose RNAi have resulted in previously undescribed phenotypes (*mlpt*, *Tc-opa*, and *Tc-cact*), RNAi was performed with two non-overlapping dsRNA fragments that target the same gene to confirm that the observed phenotypes were indeed caused by the reduction of the

Table 2 Summary of RNAi effects

Gene	dsRNA		Larvae injected	Lethality			Survived adults (%)	Abnormality
	Length (bp)	Conc.		Larval (%)	Pupal (%)	Adult (%)		
Tc-tor	601	1 μg/μl	30	13.3	0.0	0.0	86.7	n.o.a.
Tc-tll	686	1 μg/μl	30	10.0	3.3	0.0	86.7	n.o.a.
Tc-nos	520	1 μg/μl	30	16.7	6.7	0.0	76.7	n.o.a.
Tc-cad	510	1 μg/μl	29	24.1	0.0	0.0	75.9	n.o.a.
Tc-gnt	244	1 μg/μl	20	25.0	0.0	15.0	60.0	n.o.a.
Tc-h	430	1 μg/μl	23	8.7	0.0	0.0	91.3	n.o.a.
mlpt	282	1 μg/μl	29	100.0	0.0	0.0	0.0	Figs. 1 and 2
mlpt	282	50 ng/μl	30	100.0	0.0	0.0	0.0	n.o.a.
Tc-otd1	401	1 μg/μl	31	6.5	0.0	0.0	93.5	n.o.a.
Tc-otd2	259	1 μg/μl	30	10.0	0.0	0.0	90.0	n.o.a.
Tc-otd1 + *Tc-otd2*	401/259	1 μg/μl each	22	18.2	0.0	0.0	81.8	n.o.a.
Tc-eve	456	1 μg/μl	31	6.5	0.0	0.0	93.5	n.o.a.
Tc-sob^{unique}	283	1 μg/μl	14	7.1	0.0	0.0	92.9	Figs. 3 and S3
Tc-bow^{lunique}	122	1 μg/μl	15	60.0	6.7	0.0	33.3	Fig. S3
Tc-odd^{cross} + *Tc-bowl*^{unique}	381/122	1 μg/μl each	15	0.0	0.0	0.0	100.0	n.o.a.
Tc-sob^{unique} + *Tc-bowl*^{unique}	283/122	800 ng/μl each	15	46.7	0.0	0.0	53.3	n.o.a.
Tc-sob^{unique} + *Tc-odd*^{cross}	283/381	800 ng/μl each	15	20.0	13.3	0.0	66.7	n.o.a.
Tc-odd^{cross}	381	1 μg/μl	18	0.0	0.0	0.0	100.0	Fig. S3
Tc-drm^{cross}	215	1 μg/μl	31	19.4	0.0	0.0	80.6	Figs. 3 and S3
Tc-sob^{cross}	739	1 μg/μl	10	10.0	0.0	0.0	90.0	Fig. 3 and S3
Tc-bow^{lcross}	437	1 μg/μl	26	27.0	0.0	0.0	73.0	Fig. 3
Tc-odd^{cross} + *Tc-sob*^{unique} + *Tc-bowl*^{unique}	381/283/122	300 ng/μl each	15	40.0	0.0	0.0	60.0	Fig. S3
Tc-opa	634	1 μg/μl	31	16.1	12.9	71.0	0.0	Fig. 1
Tc-prd	323	1 μg/μl	27	25.9	3.7	0.0	70.4	n.o.a.
Tc-slp	604	1 μg/μl	30	36.7	23.3	16.7	23.3	n.o.a.
Tc-Ten-m (*odz*)	812	1 μg/μl	29	31.0	0.0	0.0	69.0	n.o.a.
Tc-dpn	630	1 μg/μl	29	3.4	0.0	0.0	96.6	n.o.a.
Tc-btd	628	1 μg/μl	29	27.6	0.0	0.0	72.4	n.o.a.
Sp8	654	1 μg/μl	30	40.0	6.7	0.0	53.3	Fig. S1
Tc-btd + *Sp8*	628/654	1 μg/μl each	32	6.3	0.0	46.9	46.9	Same as *Sp8* single
Tc-dl	564	1 μg/μl	27	7.4	3.7	3.7	85.2	n.o.a.
Tc-cact	571	1 μg/μl	29	100.0	0.0	0.0	0.0	Figs. 1 and 2
Tc-cact	571	100 ng/μl	20	100.0	0.0	0.0	0.0	n.o.a.
Tc-al	732	1 μg/μl	30	23.3	0.0	0.0	76.7	Fig. S1
Tc-bab	723	1 μg/μl	27	44.4	18.5	0.0	37.0	Fig. S1

n.o.a. no observable abnormality

targeted genes and not by off-target effect (see Table S1 for the non-overlapping dsRNA fragments used in this study).

Evolutionarily conserved postembryonic roles of the embryonic patterning genes

Three genes, *Sp8*, *Tc-bric-a-brac* (*Tc-bab*), and *Tc-aristaless* (*Tc-al*), when knocked down, displayed previously described phenotypes without the indication of novel wing roles in *Tribolium* (Beermann and Schroder 2004; Beermann et al. 2004; Angelini et al. 2009; Angelini et al. 2012). RNAi for *Sp8* (the *Tribolium* ortholog of *Drosophila Sp1*) showed disruptions in leg and antennal segmentation (Fig. S1a-e). *Tc-bab* RNAi induced fusion of tarsal segments in the leg (Fig. S1f-g), and *Tc-al* RNAi showed disruptions in the distal antennae (Fig. S1h-i). These RNAi phenotypes accentuated the

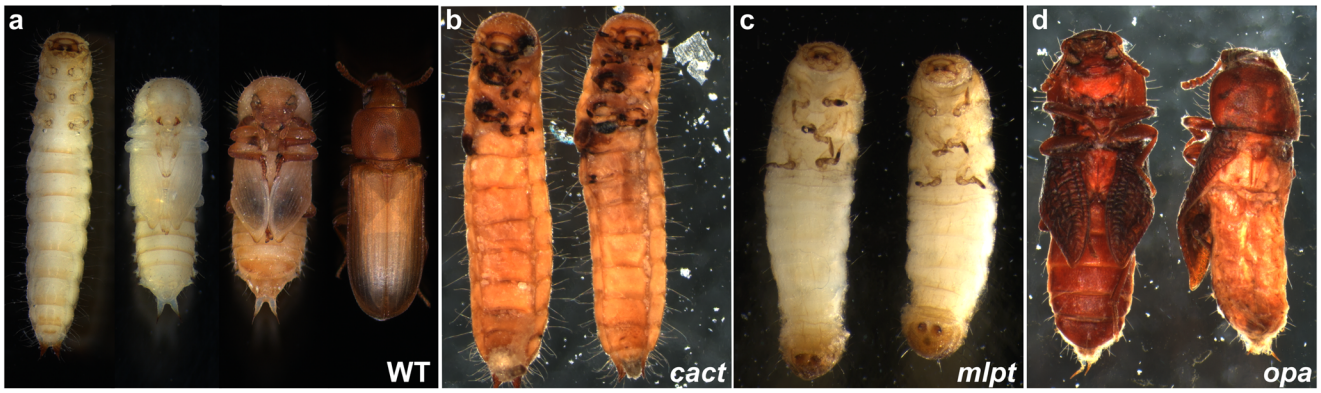

Fig. 1 Metamorphosis defects induced by RNAi for *Tc-cact*, *mlpt*, and *Tc-opa*. **a** wild-type metamorphosis from a prepupa (*left*) to a newly eclosed adult (*right*). **b** *Tc-cact* RNAi. **c** *mlpt* RNAi. **d** *Tc-opa* RNAi. *Tc-cact* and *mlpt* RNAi beetles failed to pupate, while *Tc-opa* RNAi prevented adult eclosion

evolutionarily conserved roles of these genes and also confirmed the effectiveness of our knockdowns.

Tc-cact has an uncharacterized role in wing imaginal disc development in *Tribolium*

cact codes for a IkB family protein that regulates the nuclear entry of Dorsal proteins during the D/V axis formation in the *Drosophila* embryo (Roth et al. 1989; Rushlow et al. 1989; Steward 1989; Moussian and Roth 2005). It has been reported that the D/V axis function of *Tc-cact* and *Tc-dorsal* is conserved in *Tribolium* (Nunes da Fonseca et al. 2008). Our screening has revealed that RNAi for *Tc-cact* caused pupation defects, inducing severe lethality at the last larval stage in *Tribolium* (Table 2 and Fig. 1a–b). The same lethality was observed even with 10 times diluted dsRNA (100 ng/μl) (Table 2). Further examination revealed that the hindwing and elytron imaginal tissues in the *Tc-cact* RNAi larvae failed to proliferate (Fig. 2a–d). In wild-type *Tribolium*, wing tissue proliferation, visualized by the EYFP expression of the *pu11 nub* enhancer trap line, begins at the early last larval stage (Fig. 2a) and continues to the prepupal stage (Fig. 2b) (also see Figure S4 of Clark-Hachtel et al. 2013). In *Tc-cact* RNAi, although we saw an initial sign of proliferation (Fig. 2c), wing imaginal tissues failed to proliferate further. Instead, we observed that the discs maintained their early size until just prior to larval lethality at the time of pupation (Fig. 2d). In contrast, larval RNAi for *Tc-dorsal*, whose protein products are regulated by Cact during the embryonic D/V axis formation, did not cause any noticeable morphological or developmental defects in *Tribolium* (Table 2). These results suggest that *Tc-cact* is essential, while *Tc-dorsal* is dispensable or redundant with other genes, for the development of wing-related tissues in *Tribolium*.

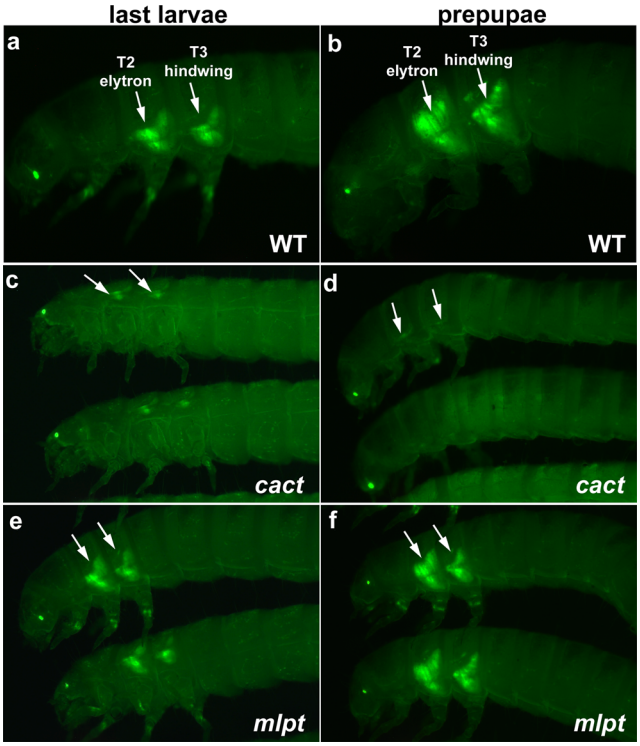

Fig. 2 Wing disc proliferation in *Tc-cact* and *mlpt* RNAi beetles. **a**, **b** *pu11 nub* enhancer trap line showing wing development in the last larval (**a**) and prepupal stages (**b**). Both elytron and hindwing discs are marked by EYFP shown in *green* (*arrows* in **a**–**f**). **c**, **d** *Tc-cact* RNAi larvae. Last instar larvae (**c**) and prepupae (**d**) with reduced wing disc proliferation at both stages. These larvae die just prior to pupation. **e**, **f** *mlpt* RNAi larvae. Last instar larvae (**e**) and prepupae (**f**), showing normal wing disc proliferation even with larval lethality similar to *Tc-cact* RNAi

The *odd-skipped* family genes are critical for the formation of the wing serial homologs in *Tribolium*

The *odd-skipped* gene family was another class of genes included in our screening. In *Drosophila*, this family consists of four genes: *odd*, *sister of odd and bowl* (*sob*), *brother of odd with entrails limited* (*bowl*), and *drumstick* (*drm*) (Hart et al. 1996). Two members of this gene family, *odd* and *bowl*, are involved in embryonic segmentation in *Drosophila* (Nusslein-Volhard and Wieschaus 1980; Wang and Coulter 1996). The *odd-skipped* gene family in *Drosophila* is also important for

appendage segmentation, with three members acting redundantly and the fourth member, *bowl*, having a unique function (Hao et al. 2003; de Celis Ibeas and Bray 2003). The *Tribolium* genome also contains four *odd-skipped* family genes, some of which have been implicated in embryogenesis (Choe et al. 2006), as well as in appendage segmentation (Angelini et al. 2012, 2009).

We first utilized dsRNA molecules for the *odd-skipped* family genes that contain the conserved Zn finger coding regions and are likely to knock down multiple *odd-skipped* paralogs in *Tribolium* (we hereafter refer these potential cross-reacting dsRNA as *Tc-sob*^{cross}, *Tc-drm*^{cross}, *Tc-bowl*^{cross}, and *Tc-odd*^{cross}; see Document S2 for the nucleotide sequence alignments). RNAi with these dsRNA molecules, except *Tc-odd*^{cross}, caused defects in leg joint formation similar to the previously reported phenotype (arrow in Fig. 3a, d, g and Fig. S2a-e; also see Angelini et al. 2012 and Angelini et al. 2009). *odd*^{cross} RNAi did not cause any noticeable abnormalities in adult morphology (Fig. S2e), presumably because *Tc-odd*^{cross} dsRNA failed to knock down multiple *odd-skipped* paralogs despite the high nucleotide sequence conservation in the *odd* Zn finger coding region.

None of these cross-reacting RNAi caused any noticeable abnormalities in elytra or hindwings in *Tribolium*. However, we noticed that these RNAi caused abnormalities in the formation of the pleural plates in the T1 segment in *Tribolium*. This is very intriguing, as we have recently discovered that some of these T1 pleural plates are serially homologous to elytra in T2 and hindwings in T3 in *Tribolium* (i.e., T1 wing serial homologs) (Clark-Hachtel et al. 2013). There are several pleural plates in the *Tribolium* T1 segment, including anterior and posterior trochantin, epimeron, and endopleuron (see Fig. 3 and Fig. S1 of Clark-Hachtel et al. 2013 for detailed annotation of the T1 body wall structure). RNAi with *Tc-sob*^{cross}, *Tc-drm*^{cross}, and *Tc-bowl*^{cross} dsRNA resulted in the reduction of both anterior and posterior trochantin (red and white arrowheads in Fig. 3a–i, arrowheads in Fig. S2) as well as epimeron (arrows in Fig. 3a–i) in *Tribolium*. No pleural

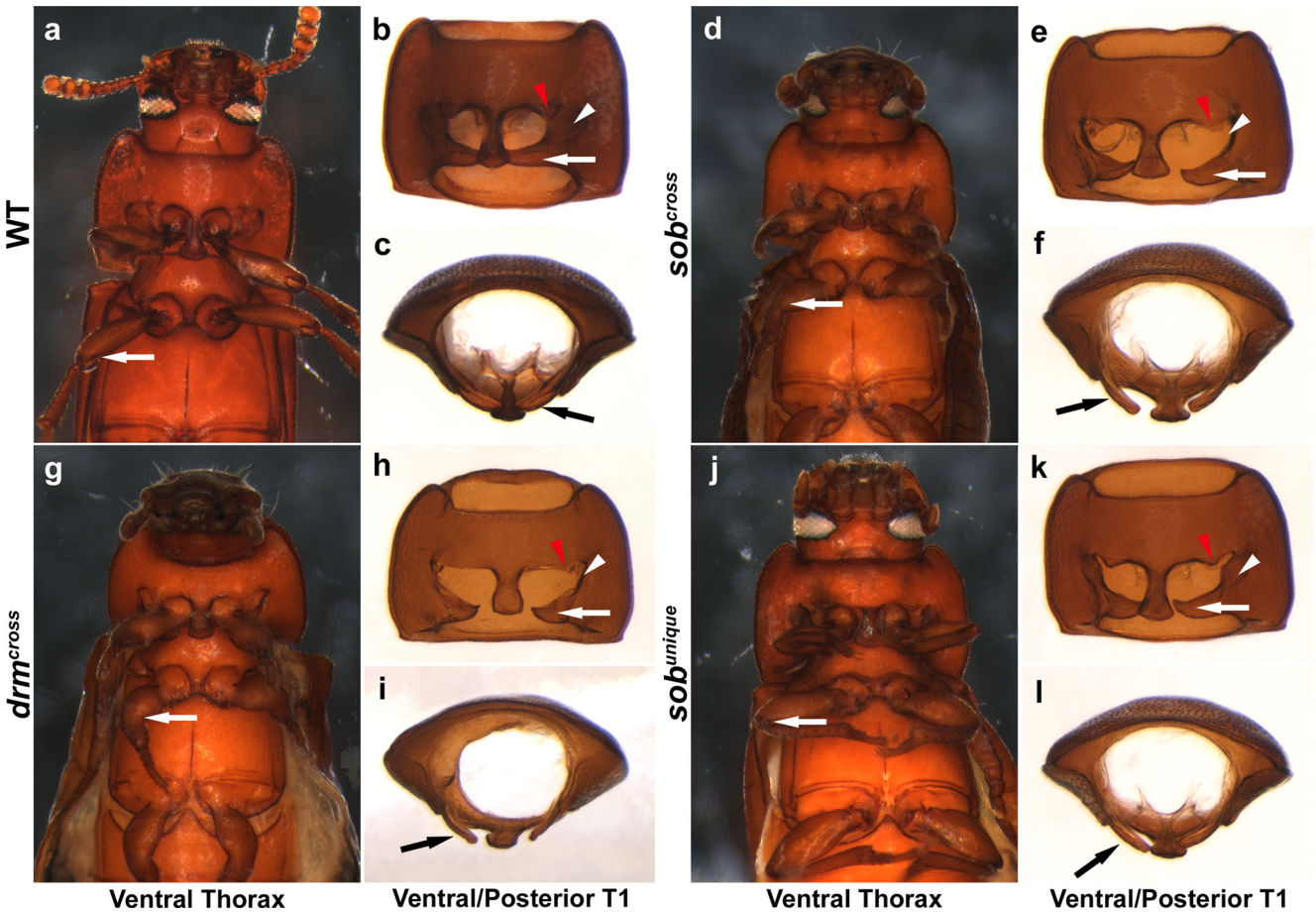

Fig. 3 Defects in the T1 wing serial homologs induced by RNAi for *odd-skipped* family genes. **a**–**c** wild-type. The anterior trochantin (*red arrowhead* in **b**), posterior trochantin (*white arrowhead* in **b**), and epimeron (*white arrow* and *black arrow* in **b** and **c**, respectively) as well as T2 leg (*arrow* in **a**) are indicated. **d**–**f** *Tc-sob*^{cross} RNAi. **g**–**i** *Tc-drm*^{cross} RNAi. **j**–**l** *Tc-sob*^{unique} RNAi. Both anterior and posterior trochantin (*red* and *white arrowheads* in **e**, **h**, **k**) and epimeron (*white arrow* in **e**, **h**, **k** and *black arrow* in **f**, **i**, **l**) are significantly reduced. Leg segmentation was also disrupted (*arrows* in **d**, **g**, **j**)

plates in other thoracic segments were affected (data not shown). These results indicate that, although *odd-skipped* family genes are not important for wing development in T2 and T3, these genes are critical for the formation of the wing serial homologs in T1 in *Tribolium*.

We next sought to investigate the individual function of the *odd-skipped* family members during *Tribolium* adult morphogenesis by utilizing dsRNA fragments unique to *Tc-sob* and *Tc-bowl* (*Tc-sob*^{unique} and *Tc-bowl*^{unique}). We continued to use *Tc-odd*^{cross} fragment for *odd* RNAi, as this fragment did not appear to cross react with other *odd-skipped* paralogs (Fig. S2e). Among RNAi with these dsRNA fragments, only *Tc-sob*^{unique} RNAi caused abnormality in the T1 pleural plates (Fig. 3j–l); both the anterior and posterior trochantin (red and white arrowhead in Fig. 3k) as well as epimeron were reduced (arrow in Fig. 3k–l). However, the abnormalities were less severe compared to those of *Tc-sob*^{cross} RNAi (Fig. 3d–f), suggesting that either the *Tc-sob*^{unique} dsRNA is less efficient to trigger RNAi than *Tc-sob*^{cross} dsRNA or some of the paralogs function partially redundantly with *Tc-sob* in pleural plate formation. In contrast to *Tc-sob*^{unique} RNAi, single RNAi for *Tc-bowl* with *Tc-bowl*^{unique} dsRNA did not induce any noticeable abnormalities (Fig. S2g). *Tc-odd*^{cross} + *Tc-bowl*-^{unique} double RNAi produced weak leg defects; however, the pleural plates were not affected (data not shown). Furthermore, the injection of *Tc-odd*^{cross} and *Tc-bowl*^{unique} dsRNA along with *Tc-sob*^{unique} dsRNA did not enhance (and instead reduced) the *Tc-sob*^{unique} single RNAi phenotypes (Fig. S2h), suggesting that the function of *Tc-odd* and *Tc-bowl* during pleural plate formation is minor (if any) compared to the *Tc-sob* function. The reduction of RNAi phenotypes in the triple RNAi could be due to the reduction of dsRNA concentrations (Table 2) and/or competition among three RNAi treatments (Miller et al. 2012). We could not determine whether *Tc-drm* has a unique role, since we failed to design dsRNA specific to *Tc-drm* due to the short length of the *Tc-drm* gene. Since *Tc-sob* single RNAi phenotype was not enhanced by *Tc-odd* and *Tc-bowl* RNAi, it is possible that *Tc-drm* is the paralog acting in a partially redundant manner with *Tc-sob* in pleural plate formation.

Taken together, our RNAi analysis revealed that the *odd-skipped* family genes are essential for the formation of the T1 pleural plates, with *Tc-sob* (and potentially also *Tc-drm*) having a more significant role than *Tc-odd* and *Tc-bowl*. Since some of these T1 pleural plates are serially homologous to wings in *Tribolium*, our findings indicate that the *odd-skipped* family genes (or at least *Tc-sob*) are novel "wing serial homolog" genes in *Tribolium*.

Two novel metamorphosis genes in *Tribolium*

In addition to the above findings related to wings, RNAi for two more genes, *mlpt* and *Tc-opa*, induced previously uncharacterized defects in *Tribolium* metamorphosis. *mlpt* is a unique polycistronic gene, whose messenger RNA (mRNA) contains multiple short open-reading frames within a single transcript (Savard et al. 2006; Galindo et al. 2007; Kondo et al. 2007). RNAi for *mlpt* at the last larval stage caused complete lethality just prior to pupation (Fig. 1c). Unlike *Tc-cact* RNAi, the proliferation of wing imaginal tissues appears to be unaffected in the *mlpt* RNAi larvae (Fig. 2e–f), suggesting that *mlpt* is involved in metamorphosis but not in the wing tissue proliferation in *Tribolium*.

opa is a pair-rule gene expressed in stripes along the A/P axis of the developing fly embryo (Benedyk et al. 1994). In *Tribolium*, however, *Tc-opa* does not appear to function as a pair-rule gene, as no segmentation defects were observed via RNAi (Choe et al. 2006). *Tc-opa* RNAi at the last larval stage resulted in a defect in adult eclosion in *Tribolium* (Table 2). Approximately 70 % of the *Tc-opa* RNAi beetles failed to eclose, displaying pharate adult lethality, while the remaining 30 % die prior to adult eclosion (Fig. 1d). This result suggests that *Tc-opa* also has an important role in metamorphosis in *Tribolium*.

Although deviating from the primary goal of our screening for novel wing genes, the unique metamorphosis roles of these two genes we identified will contribute to our understanding of the functional evolution of developmental genes, since neither of these genes has been implicated in metamorphosis in *Drosophila*.

Discussion

Drosophila has long been a classic model organism that has pioneered the study for the molecular and genetic mechanisms underlying insect wing development. The genes identified as important for wing development in *Drosophila* (wing genes) have been the main foci when studying wing development in insects other than *Drosophila*. Although this candidate gene approach has been quite successful in elucidating the diverged as well as conserved aspects of insect wing development, it is also limited since the pool of genes for analysis can only stem from those identified in *Drosophila*. As a first step to escaping this limitation, we took a semi-candidate gene approach, in which the selection of the genes still relies on *Drosophila* studies but is independent of *Drosophila* wing genes. Through our screening, we have obtained several novel insights into the function of the developmental toolkit genes in *Tribolium*.

Tc-cact in wing development

RNAi for *Tc-cact* at the last larval stage prevented the growth of wing imaginal discs, indicating that *Tc-cact* has a

previously uncharacterized function in *Tribolium* wing development. *cact* encodes an evolutionarily conserved IkB protein that regulates the nuclear localization of NFkB proteins (Roth et al. 1989; Rushlow et al. 1989; Steward 1989; Moussian and Roth 2005). Dorsal and Dorsal-related immunity factor (DIF) are two NFkB factors that are known to be regulated by Cact in *Drosophila* during the embryonic D/V axis formation (Dorsal) (Roth et al. 1989; Rushlow et al. 1989; Steward 1989) or immune response (Dorsal and DIF) (Ip et al. 1993). Interestingly, RNAi for *Tc-dorsal*, which codes for the dominant binding target of Cact in the embryonic patterning event, did not cause any noticeable wing abnormalities in *Tribolium*. This suggests that either other NFkB factors act redundantly or Cact acts independent of NFkB factors during wing development in *Tribolium*. Recently, a potential role of *cact* in the development of the wing in *Drosophila* has been reported through an RNAi screen (Friedman et al. 2011). This implies that the wing-related function of *cact* might be conserved between flies and beetles. An alternative explanation to the wing imaginal tissue specific role of *Tc-cact* is that *Tc-cact* RNAi might have severely affected larval physiology and growth, causing overall cell proliferation including wing tissues to halt. We favor the former interpretation, as the *Tc-cact* RNAi larvae appear to reach the late prepupal stage with normal body size and shape (Fig. 2d). Nonetheless, further analyses will be required in order to characterize the molecular mechanisms behind the function of *cact* during adult morphogenesis in insects.

Function of *odd-skipped* family genes in the T1 wing serial homologs

We and another group have recently reported that there are tissues serially homologous to wings in some of the wingless segments in beetles (Clark-Hachtel et al. 2013; Ohde et al. 2013). One of the wing serial homologs identified in T1 belongs to a part of the dorsally originated body wall tissue (called carinated margin in Clark-Hachtel et al. 2013 and hypomeron in Ohde et al. 2013). In addition to this dorsal tissue, we found that some of the body wall plates of lateral origin (pleural plates) in T1, trochantin and epimeron, are also wing serial homologs in *Tribolium* (Clark-Hachtel et al. 2013). The merger of the dorsal and lateral wing serial homologs appears to be essential for the complete induction of ectopic wing structures in T1 through homeotic transformation in *Tribolium*.

Pleural plates in insects are thought to have evolved from the most proximal components of legs (such as epicoxa and subcoxa) in ancestral arthropods (Kukalova-Peck 1983, 2008). Since the *odd-skipped* family genes are critical for leg segmentation, the involvement of *odd-skipped* family in the formation of the T1 pleural plates may provide the first genetic evidence supporting the leg origin of insect pleural plates.

The recognition of the two classes of the T1 wing serial homologs being homologous to the two proposed insect wing origins (the paranotal lobe via paranotal hypothesis and the ancestral proximal leg segments via gill/exite hypothesis, respectively) have led us to promote a dual origin hypothesis of insect wings (Clark-Hachtel et al. 2013; Kukalova-Peck 1983, 2008; Niwa et al. 2010; Rasnitsyn 1981). The identification of genes involved in the formation of these T1 wing serial homologs is essential to further test the validity of the dual origin hypothesis. Therefore, although not directly related to the elucidation of the mechanisms involved in the diversification of wings on T2 and T3, our finding regarding the *odd-skipped* gene family in pleural plate formation provides valuable clues to understand the origin and evolution of insect wings.

Independent subfunctionalization among *odd-skipped* gene paralogs between *Drosophila* and *Tribolium*

In addition to our finding regarding the novel function of the *odd-skipped* family genes in the T1 wing serial homologs in *Tribolium*, our RNAi analysis of the *odd-skipped* gene family also provided an interesting look at the evolution of paralogous genes in separate organisms.

In *Drosophila*, *bowl* appears to be an outlier that possesses some non-redundant functions during postembryonic development (Hao et al. 2003). Through the usage of dsRNA fragments that uniquely target some of the *odd-skipped* paralogs, we found that *Tc-sob* (and possibly *Tc-drm*) has a more prominent role than *odd* and *bowl* during pleural plate formation in *Tribolium*. We saw the same tendency even for the evolutionarily conserved functions of this gene family, such as leg and antenna segmentation functions, in *Tribolium* (Fig. 3 and Fig. S2). This suggests that, although the functions of the *odd-skipped* paralogs as a gene family during appendage segmentation are conserved between *Drosophila* and *Tribolium*, the four paralogs have been subfunctionalized and/or neofunctionalized in a lineage-specific manner. Thus, the *odd-skipped* gene family provides us with a unique opportunity to investigate how the functional allocation among paralogous genes changes during evolution. Paralog-specific functional analyses for the *odd-skipped* family in other insects will help further investigate lineage-specific subfunctionalization among family genes.

odd-skipped gene family: lineage-specific duplications or an ancient duplication?

One caveat to the above interpretation regarding *odd-skipped* paralogs is that, although the *Tribolium odd-skipped* family genes have names corresponding to the four *Drosophila* paralogs, these genes might have emerged via linage-specific duplication and the functional assignments may thus

be unique to each lineage. Previous phylogenetic analysis for the *odd-skipped* family genes indeed suggested lineage-specific duplication events (Angelini et al. 2009). This analysis was based on the nucleotide sequence of the genes, which maintains a unique degree of conservation among paralogs in each organism (Angelini et al. 2009).

Our RNAi analysis prompted us to further evaluate the orthology among the insect *odd-skipped* family genes. We first examined the domain architecture of the *odd-skipped* family proteins from three different insect orders (*D. melanogaster*, *T. castaneum*, and *Apis mellifera*). Pfam analysis indicated that *odd-skipped* family proteins contain several Zn fingers unique to this family (Fig. S3). Both the Bowl and Sob proteins from all three species contain four consecutive Zn finger domains, while Odd and Drm proteins contain fewer. Odd has three Zn fingers, except Tc-Odd, which retains a diverged (potentially degenerated) forth Zn finger. Drm is a very short protein, consisting of only two very conserved Zn fingers in all three species. Therefore, the Zn finger configuration in *odd-skipped* family protein is more similar among the same class of the protein than among the proteins in one species. Furthermore, in addition to the Zn finger domains, we also recognized several evolutionarily conserved motifs unique to each class of the proteins (Fig. S3). The unique Zn finger configuration along with the presence of additional motifs unique to each class are strong indications that the emergence of the four different classes of the *odd-skipped* family proteins preceded the radiation of the three holometabolous insect orders examined.

We next attempted to reevaluate the phylogenetic analysis for the *odd-skipped* gene family. The amino acid sequence multiple alignment composed of the two Zn finger domains that are conserved among all four classes of the *odd-skipped* family proteins contains minimal information regarding the divergence of these proteins (95 % identical, data not shown); therefore, we have decided to exclude Drm (the shortest class of the four) from the multiple alignment and focus on the Zn fingers that are conserved among the Odd, Sob, and Bowl classes of proteins. We used the Pfam-defined Zn fingers to compose the multiple alignment (Document S1) and built the phylogenetic trees by using three independent methods; neighbor-joining (Fig. 4), maximum likelihood (Fig. S4), and Bayesian (Fig. S5a). Unlike the previous tree based on the nucleotide sequence (Angelini et al. 2009), all three methods indicated that the duplication of the family preceded the split of *Tribolium*, *Drosophila*, and *Apis*. Interestingly (and rather puzzlingly), however, by using the nucleotide sequences that exactly correspond to the conserved Zn finger domains used for the amino acid-based tree constructions, we were able to recreate the tree obtained by Angelini et al. that suggests lineage-specific duplications (Angelini et al. 2009) (Fig. S5b). It is currently unknown why the nucleotide sequence-based phylogenetic analysis produces such a different outcome compared to that with amino acid sequences.

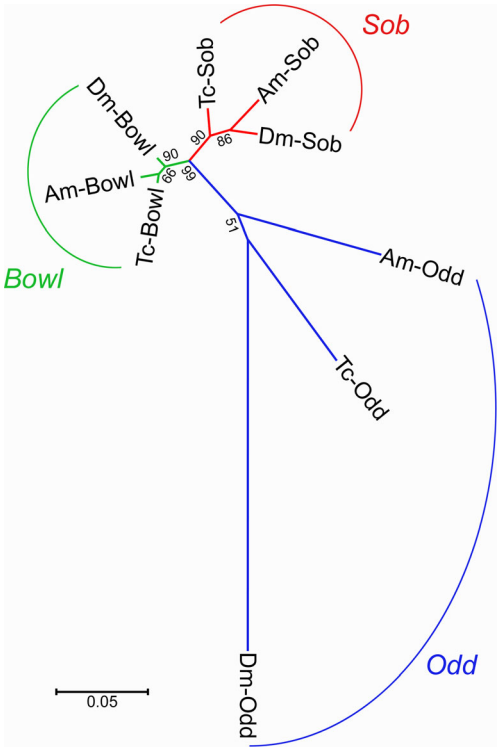

Fig. 4 Phylogenetic analysis of *odd-skipped* family members from three holometabolous insect orders by neighbor joining. The neighbor-joining (NJ) tree is based on the alignment of the conserved Zn finger domains (see Document S1 for the alignment). The individual paralogous members cluster together, suggesting that the duplications that produced these paralogs preceded the emergence of the major holometabolous clades. *Dm Drosophila melanogaster*, *Tc Tribolium castaneum*, and *Am Apis mellifera*

In addition to the domain architecture and phylogenetic analyses, we also examined the genomic structure to explore the origins of this gene family. We noticed that the synteny of the *odd-skipped* family genes is conserved between *Drosophila* and *Tribolium* (Fig. S6). In *Tribolium*, all four *odd-skipped* family genes are clustered within a 200-kb region. The *odd-skipped* family genes are also clustered in *Drosophila*, although *bowl* resides in a slightly more distant position from the other members with an inverted orientation. The conserved microsynteny between *Drosophila* and *Tribolium* further supports the idea that the duplication of the *odd-skipped* gene family preceded the divergence of beetles and flies.

Taken together, our domain architecture analysis, phylogenetic analysis with the amino acid sequences, and genomic architecture analysis are all in agreement that each *odd-skipped* paralog has the one-to-one ortholog in each organism examined, and the emergence of those paralogs preceded the evolution of three holometabolous orders. However, it will require further analysis to understand the discrepancy between the nucleotide-based and amino acid-based phylogenetic trees.

Novel genes in metamorphosis

Although unrelated to wing development, we found that *mlpt* and *Tc-opa* have previously uncharacterized roles in metamorphosis in *Tribolium*. The involvement of *mlpt* in metamorphosis is especially intriguing as the *mlpt* locus is transcribed into a polycistronic mRNA, which codes for a series of evolutionarily conserved small peptides (Savard et al. 2006). The orthologous gene in *Drosophila* has been identified (*polished rice* in Kondo et al. 2007 or *tarsal-less* in Galindo et al. 2007), which is involved in the formation of cuticular structures during embryogenesis (Kondo et al. 2010) and the formation of distal appendage structures (Pueyo and Couso 2008). However, the involvement of the *Drosophila mlpt* ortholog in metamorphosis has not been described. It would be interesting to investigate how the small Mlpt peptides interact with known metamorphosis pathways and if the metamorphosis function of *mlpt* is conserved in *Drosophila* and other insects.

The role of *Tc-opa* in metamorphosis is also noteworthy, as this is the first reported function for this gene in *Tribolium*. In *Drosophila*, *opa* is an important component of the pair-rule patterning mechanism during A/P axis specification (Benedyk et al. 1994). In *Tribolium*, however, Choe et al. did not find a patterning role for the beetle ortholog (Choe et al. 2006). Similar to *mlpt*, determining whether the metamorphic role of *opa* is conserved across other insects is an interesting question to explore in the future.

Evolution of beetle elytra

The evolution of the beetle unique wings, elytra, was an important step in beetles becoming one of the most successful animal groups on the planet (Lawrence and Britton 1991; Grimaldi and Engel 2005; Crowson 1981). Elytra therefore provide an interesting opportunity to study the diversification of insect wings in a context where the structure itself has been evolutionarily integral to the success of the insects bearing those structures. We sought to identify the genes that have been critical to the acquisition of these wing structures unique to beetles. Our semi-candidate gene approach, however, proved to be difficult. Although the *Tribolium* orthologs of *Drosophila* embryonic patterning genes are highly pleiotropic, and we identified several novel insights into the function of these genes, we could not identify any novel wing-related roles of these genes that are unique in *Tribolium*. These data indicate that, instead of screening limited subsets of genes, it will be more beneficial to utilize an unbiased approach to identify novel genes that have been critical to the evolution of beetle elytra. A genome-wide RNAi screening (such as the iBeetle project) (Bucher and Klingler 2014) or wing transcriptome analyses by RNA sequencing will give us a more comprehensive view of genes involved in the formation of beetle elytra and shed light on the evolutionary mechanisms underlying the vast diversity in wing structures seen among insects.

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References

Abouheif E, Wray GA (2002) Evolution of the gene network underlying wing polyphenism in ants. Science 297(5579):249–252. doi:10.1126/science.1071468

Angelini DR, Kikuchi M, Jockusch EL (2009) Genetic patterning in the adult capitate antenna of the beetle Tribolium castaneum. Dev Biol 327(1):240–251. doi:10.1016/j.ydbio.2008.10.047

Angelini DR, Smith FW, Jockusch EL (2012) Extent with modification: leg patterning in the beetle Tribolium castaneum and the evolution of serial homologs. G3 (Bethesda) 2(2):235–248. doi:10.1534/g3.111.001537

Beermann A, Schroder R (2004) Functional stability of the aristaless gene in appendage tip formation during evolution. Dev Genes Evol 214(6):303–308. doi:10.1007/s00427-004-0411-7

Beermann A, Aranda M, Schroder R (2004) The Sp8 zinc-finger transcription factor is involved in allometric growth of the limbs in the beetle Tribolium castaneum. Development 131(4):733–742. doi:10.1242/dev.00974

Benedyk MJ, Mullen JR, DiNardo S (1994) odd-paired: a zinc finger pair-rule protein required for the timely activation of engrailed and wingless in Drosophila embryos. Genes Dev 8(1):105–117

Bowsher JH, Wray GA, Abouheif E (2007) Growth and patterning are evolutionarily dissociated in the vestigial wing discs of workers of the red imported fire ant, Solenopsis invicta. J Exp Zool B Mol Dev Evol 308(6):769–776. doi:10.1002/jez.b.21200

Brook WJ, Diaz-Benjumea FJ, Cohen SM (1996) Organizing spatial pattern in limb development. Annu Rev Cell Dev Biol 12:161–180. doi:10.1146/annurev.cellbio.12.1.161

Brown SJ, Mahaffey JP, Lorenzen MD, Denell RE, Mahaffey JW (1999) Using RNAi to investigate orthologous homeotic gene function during development of distantly related insects. Evol Dev 1(1):11–15

Brown SJ, Denell RE, Beeman RW (2003) Beetling around the genome. Genet Res 82(3):155–161

Bucher G, Klingler M (2014) iBeetle: functional genomics of insect development and metamorphosis. http://ibeetle.uni-goettingen.de

Bucher G, Scholten J, Klingler M (2002) Parental RNAi in Tribolium (Coleoptera). Curr Biol 12(3):R85–R86

Carroll SB, Grenier JK, Weatherbee SD (2001) From DNA to diversity. Science, Blackwell

Choe CP, Miller SC, Brown SJ (2006) A pair-rule gene circuit defines segments sequentially in the short-germ insect Tribolium castaneum. Proc Natl Acad Sci U S A 103(17):6560–6564. doi:10.1073/pnas.0510440103

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 U S A 110(42):16951–16956. doi:10.1073/pnas.1304332110

Crowson RA (1981) The biology of the Coleoptera. Academic, London

de Celis Ibeas JM, Bray SJ (2003) Bowl is required downstream of Notch for elaboration of distal limb patterning. Development 130(24): 5943–5952. doi:10.1242/dev.00833

Denell R (2008) Establishment of tribolium as a genetic model system and its early contributions to evo-devo. Genetics 180(4):1779–1786. doi:10.1534/genetics.104.98673

Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, Heger A, Hetherington K, Holm L, Mistry J, Sonnhammer EL, Tate J, Punta M (2013) Pfam: the protein families database. Nucleic Acids Res. doi:10.1093/nar/gkt1223

Friedman AA, Tucker G, Singh R, Yan D, Vinayagam A, Hu Y, Binari R, Hong P, Sun X, Porto M, Pacifico S, Murali T, Finley RL Jr, Asara JM, Berger B, Perrimon N (2011) Proteomic and functional genomic landscape of receptor tyrosine kinase and ras to extracellular signal-regulated kinase signaling. Sci Signal 4(196):rs10. doi:10.1126/scisignal.2002029

Galindo MI, Pueyo JI, Fouix S, Bishop SA, Couso JP (2007) Peptides encoded by short ORFs control development and define a new eukaryotic gene family. PLoS Biol 5(5):e106. doi:10.1371/journal.pbio.0050106

Gilbert SF (2014) Developmental biology. Tenth Edition edn. Sinauer Associates, Sunderland

Grimaldi D, Engel MS (2005) Evolution of the insects. Cambridge University Press, Cambridge

Hall BG (2011) Phylogenetic trees made easy: a how-to manual, 4th edn. Sinauer Associates, Sunderland

Hao I, Green RB, Dunaevsky O, Lengyel JA, Rauskolb C (2003) The odd-skipped family of zinc finger genes promotes Drosophila leg segmentation. Dev Biol 263(2):282–295

Hart MC, Wang L, Coulter DE (1996) Comparison of the structure and expression of odd-skipped and two related genes that encode a new family of zinc finger proteins in Drosophila. Genetics 144(1):171–182

Ip YT, Reach M, Engstrom Y, Kadalayil L, Cai H, Gonzalez-Crespo S, Tatei K, Levine M (1993) Dif, a dorsal-related gene that mediates an immune response in Drosophila. Cell 75(4):753–763

Kim HS, Murphy T, Xia J, Caragea D, Park Y, Beeman RW, Lorenzen MD, Butcher S, Manak JR, Brown SJ (2010) BeetleBase in 2010: revisions to provide comprehensive genomic information for Tribolium castaneum. Nucleic Acids Res 38(Database issue): D437–D442. doi:10.1093/nar/gkp807

Klingler M (2004) Tribolium. Curr Biol 14(16):R639–R640. doi:10.1016/j.cub.2004.08.004

Knorr E, Bingsohn L, Kanost MR, Vilcinskas A (2013) Tribolium castaneum as a model for high-throughput RNAi screening. Adv Biochem Eng Biotechnol 136:163–178. doi:10.1007/10_2013_208

Kondo T, Hashimoto Y, Kato K, Inagaki S, Hayashi S, Kageyama Y (2007) Small peptide regulators of actin-based cell morphogenesis encoded by a polycistronic mRNA. Nat Cell Biol 9(6):660–665. doi:10.1038/ncb1595

Kondo T, Plaza S, Zanet J, Benrabah E, Valenti P, Hashimoto Y, Kobayashi S, Payre F, Kageyama Y (2010) Small peptides switch the transcriptional activity of Shavenbaby during Drosophila embryogenesis. Science 329(5989):336–339. doi:10.1126/science.1188158

Kukalova-Peck J (1983) Origin of the insect wing and wing articulation from the arthropodan leg. Can J Zool 61(7):1618–1669. doi:10.1139/z83-217

Kukalova-Peck J (2008) Phylogeny of higher taxa in Insecta: finding synapomorphies in the extant fauna and separating them from homoplasies. Evol Biol 35(1):4–51. doi:10.1007/s11692-007-9013-4

Lawrence JF, Britton EB (1991) Coleoptera (beetles). In: The insects of Australia: a textbook for students and research workers, vol 2. Second Edition edn. CSIRO Division of Entomology, Carlton, Australia: Melbourne University Press, pp pp. 543–564

Linz DM, Clark-Hachtel CM, Borras-Castells F, Tomoyasu Y (2014) Larval RNA interference in the red flour beetle, Tribolium castaneum. J Vis Exp (92). doi:10.3791/52059

Lorenzen MD, Berghammer AJ, Brown SJ, Denell RE, Klingler M, Beeman RW (2003) piggyBac-mediated germline transformation in the beetle Tribolium castaneum. Insect Mol Biol 12(5):433–440

Lynch JA, Roth S (2011) The evolution of dorsal-ventral patterning mechanisms in insects. Genes Dev 25(2):107–118. doi:10.1101/gad.2010711

Macdonald WP, Martin A, Reed RD (2010) Butterfly wings shaped by a molecular cookie cutter: evolutionary radiation of lepidopteran wing shapes associated with a derived Cut/wingless wing margin boundary system. Evol Dev 12(3):296–304. doi:10.1111/j.1525-142X.2010.00415.x

Martin A, Papa R, Nadeau NJ, Hill RI, Counterman BA, Halder G, Jiggins CD, Kronforst MR, Long AD, McMillan WO, Reed RD (2012) Diversification of complex butterfly wing patterns by repeated regulatory evolution of a Wnt ligand. Proc Natl Acad Sci U S A 109(31):12632–12637. doi:10.1073/pnas.1204800109

Miller SC, Miyata K, Brown SJ, Tomoyasu Y (2012) Dissecting systemic RNA interference in the red flour beetle Tribolium castaneum: parameters affecting the efficiency of RNAi. PLoS One 7(10): e47431. doi:10.1371/journal.pone.0047431

Moussian B, Roth S (2005) Dorsoventral axis formation in the Drosophila embryo—shaping and transducing a morphogen gradient. Curr Biol 15(21):R887–R899. doi:10.1016/j.cub.2005.10.026

Niwa N, Akimoto-Kato A, Niimi T, Tojo K, Machida R, Hayashi S (2010) Evolutionary origin of the insect wing via integration of two developmental modules. Evol Dev 12(2):168–176. doi:10.1111/j.1525-142X.2010.00402.x

Nunes da Fonseca R, von Levetzow C, Kalscheuer P, Basal A, van der Zee M, Roth S (2008) Self-regulatory circuits in dorsoventral axis formation of the short-germ beetle Tribolium castaneum. Dev Cell 14(4):605–615. doi:10.1016/j.devcel.2008.02.011

Nusslein-Volhard C, Wieschaus E (1980) Mutations affecting segment number and polarity in Drosophila. Nature 287(5785):795–801

Ohde T, Yaginuma T, Niimi T (2013) Insect morphological diversification through the modification of wing serial homologs. Science 340(6131):495–498. doi:10.1126/science.1234219

Philip BN, Tomoyasu Y (2011) Gene knockdown analysis by double-stranded RNA injection molecular methods for evolutionary genetics. In: Orgogozo V, Rockman MV (eds), vol 772. Methods in molecular biology. Humana Press, pp 471–497. doi:10.1007/978-1-61779-228-1_28

Pueyo JI, Couso JP (2008) The 11-aminoacid long Tarsal-less peptides trigger a cell signal in Drosophila leg development. Dev Biol 324(2): 192–201. doi:10.1016/j.ydbio.2008.08.025

Rambaut A (2014) FigTree. http://tree.bio.ed.ac.uk/software/figtree/

Rasnitsyn AP (1981) A modified paranotal theory of insect wing origin. J Morphol 168(3):331–338. doi:10.1002/jmor.1051680309

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Hohna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61(3):539–542. doi:10.1093/sysbio/sys029

Roth S, Stein D, Nusslein-Volhard C (1989) A gradient of nuclear localization of the dorsal protein determines dorsoventral pattern in the Drosophila embryo. Cell 59(6):1189–1202

Rushlow CA, Han K, Manley JL, Levine M (1989) The graded distribution of the dorsal morphogen is initiated by selective nuclear transport in Drosophila. Cell 59(6):1165–1177

Sanson B (2001) Generating patterns from fields of cells. Examples from Drosophila segmentation. EMBO Rep 2(12):1083–1088. doi:10.1093/embo-reports/kve255

Savard J, Marques-Souza H, Aranda M, Tautz D (2006) A segmentation gene in tribolium produces a polycistronic mRNA that codes for multiple conserved peptides. Cell 126(3):559–569. doi:10.1016/j.cell.2006.05.053

Shirai LT, Saenko SV, Keller RA, Jeronimo MA, Brakefield PM, Descimon H, Wahlberg N, Beldade P (2012) Evolutionary history of the recruitment of conserved developmental genes in association to the formation and diversification of a novel trait. BMC Evol Biol 12:21. doi:10.1186/1471-2148-12-21

St Pierre SE, Ponting L, Stefancsik R, McQuilton P (2014) FlyBase 102—advanced approaches to interrogating FlyBase. Nucleic Acids Res 42(Database issue):D780–D788. doi:10.1093/nar/gkt1092

Steward R (1989) Relocalization of the dorsal protein from the cytoplasm to the nucleus correlates with its function. Cell 59(6):1179–1188

Stoehr AM, Walker JF, Monteiro A (2013) Spalt expression and the development of melanic color patterns in pierid butterflies. Evodevo 4(1):6. doi:10.1186/2041-9139-4-6

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739. doi:10.1093/molbev/msr121

Tomoyasu Y, Denell RE (2004) Larval RNAi in Tribolium (Coleoptera) for analyzing adult development. Dev Genes Evol 214(11):575–578. doi:10.1007/s00427-004-0434-0

Tomoyasu Y, Wheeler SR, Denell RE (2005) Ultrabithorax is required for membranous wing identity in the beetle Tribolium castaneum. Nature 433 (7026):643–647. doi:http://www.nature.com/nature/journal/v433/n7026/suppinfo/nature03272_S1.html

Tomoyasu Y, Miller SC, Tomita S, Schoppmeier M, Grossmann D, Bucher G (2008) Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in Tribolium. Genome Biol 9(1):R10. doi:10.1186/gb-2008-9-1-r10

Tomoyasu Y, Arakane Y, Kramer KJ, Denell RE (2009) Repeated co-options of exoskeleton formation during wing-to-elytron evolution in beetles. Curr Biol 19(24):2057–2065. doi:10.1016/j.cub.2009.11.014

Trauner J, Schinko J, Lorenzen MD, Shippy TD, Wimmer EA, Beeman RW, Klingler M, Bucher G, Brown SJ (2009) Large-scale insertional mutagenesis of a coleopteran stored grain pest, the red flour beetle Tribolium castaneum, identifies embryonic lethal mutations and enhancer traps. BMC Biol 7:73. doi:10.1186/1741-7007-7-73

Tribolium Genome Sequencing Consortium (2008) The genome of the model beetle and pest Tribolium castaneum. Nature 452(7190):949–955. doi:10.1038/nature06784

Wang L, Coulter DE (1996) bowel, an odd-skipped homolog, functions in the terminal pathway during Drosophila embryogenesis. EMBO J 15(12):3182–3196

Weatherbee SD, Nijhout HF, Grunert LW, Halder G, Galant R, Selegue J, Carroll S (1999) Ultrabithorax function in butterfly wings and the evolution of insect wing patterns. Curr Biol 9(3):109–115