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2 **Two sets of wing homologs in the crustacean,**
3 ***Parhyale hawaiensis***

4 Courtney M. Clark-Hachtel and Yoshinori Tomoyasu
5 Department of Biology, Miami University, Oxford, Ohio, USA
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7 **The origin of insect wings is a biological mystery that has fascinated scientists for centuries.**
8 **Through extensive investigations performed across various fields, two possible wing origin**
9 **tissues have been identified; a lateral outgrowth of the dorsal body wall (tergum) and**
10 **ancestral proximal leg structures^{1,2}. With each idea offering both strengths and weaknesses,**
11 **these two schools of thought have been in an intellectual battle for decades without**
12 **reaching a consensus³. Identification of tissues homologous to insect wings from lineages**
13 **outside of Insecta will provide pivotal information to resolve this conundrum. Here,**
14 **through expression analyses and CRISPR/Cas9-based genome-editing in the crustacean,**
15 ***Parhyale hawaiensis*, we show that a wing-like gene regulatory network (GRN) operates**
16 **both in the crustacean terga and in the proximal leg segments, suggesting that (i) the**
17 **evolution of a wing-like GRN precedes the emergence of insect wings, and (ii) that both of**
18 **these tissues are equally likely to be crustacean wing homologs. Interestingly, the presence**
19 **of two sets of wing homologs parallels previous findings in some wingless segments of**
20 **insects, where wing serial homologs are maintained as two separate tissues⁴⁻⁷. This**
21 **similarity provides crucial support for the idea that the wingless segments of insects indeed**
22 **reflect an ancestral state for the tissues that gave rise to the insect wing, while the true**
23 **insect wing represents a derived state that depends upon the contribution of two distinct**
24 **tissues. These outcomes point toward a dual origin of insect wings, and thus provide a**
25 **crucial opportunity to unify the two historically competing hypotheses on the origin of this**
26 **evolutionarily monumental structure.**

27 The identification of serially homologous structures can be a powerful approach to reveal
28 the life history of complex structures, as serially homologous structures can undergo varying
29 degrees of evolutionary change in different body parts (such as in different segments of
30 insects)^{8,9}. Recent molecular attempts to identify wing serial homologs in some wingless
31 segments of insects have shed light on the insect wing origin debate^{4-7,10}, some of which have

32 suggested that wings have a dual origin and are formed from a combination of the two previously
33 proposed origin tissues⁴⁻⁷. However, this approach is inherently limited to the lineages where
34 wings have already evolved, preventing us from obtaining a comprehensive evolutionary history
35 of this structure. Identifying homologous tissues between different taxa can circumvent this
36 limitation by helping us reconstruct the tissues that were likely present in the common ancestor
37 of these groups, thus providing crucial information on how novel structures arise. As a member
38 of a sister group to insects, the crustacean, *Parhyale hawaiiensis*, provides an excellent
39 opportunity to broaden the search for wing homologs. *Parhyale* are a well-established crustacean
40 model for evo-devo studies¹¹⁻¹³ and their dorso-ventral (DV) body plan remains largely similar to
41 that of insects. Both of the tissues that correspond to the two proposed wing origins, the dorsal
42 terga and the proximal leg segments (Fig. 1a), are present in *Parhyale*, allowing us to evaluate
43 the evolutionary relationship of these tissues to the insect wing and other wing serial homologs.

44 We first investigated the possible wing homologs in *Parhyale* via expression and
45 functional analysis of *vestigial* (*vg*). The *vg* gene in insects is considered a critical wing gene
46 because of its unique function in the ectoderm to orchestrate wing development^{14,15} and its
47 potential to induce ectopic wings when overexpressed in certain contexts^{16,17}. We and others have
48 previously demonstrated that *vg* is quite powerful at identifying tissues serially homologous to
49 wings in the wingless segments of insects^{3,4,7,10,18,19}. In *Parhyale*, we found that the *vg* ortholog
50 (*Ph-vg*, Extended Data Fig. 1) is expressed in the edge of the terga, as well as in parts of the
51 proximal leg, including the edge of the coxal plate (cp) and part of the basis (Fig. 1d, SI movie
52 1). In addition, knocking out (KO) *Ph-vg* via CRISPR/Cas9 genome editing (Extended Data Fig.
53 2) resulted in deletion of the tergal edge, the entire cp, and the expansion of the basis (Fig. 1b
54 and e, SI movie 2 and 3), validating the functionality of *Ph-vg* in these tissues. The penetrance of
55 these *Ph-vg* KO phenotypes was very high, with the majority of G0 somatic cells displaying all
56 relevant phenotypes (Extended Data Table 1 and 2). Intriguingly, although the gills of
57 crustaceans have been previously proposed as crustacean wing homologs²⁰, the gills of *Ph-vg* KO
58 individuals remain intact (Fig. 1c and f, SI movie 2 and 3), suggesting that the gill of *Parhyale*
59 may not be related to insect wings (however, the homology among crustacean gills requires
60 further evaluation). Together, these findings suggest that both the terga (a dorsal tissue) and the
61 proximal leg segments (homologous to insect pleural plates²¹⁻²³ (Bruce et al. accompanying ms))
62 are the possible wing homologs in this crustacean.

63 To further investigate the genetic overlap between the two *vg*-dependent tissues in
64 *Parhyale* and insect wings, we analyzed two additional genes in *Parhyale*, *nubbin* (*Ph-nub*) and
65 *apterous* (*Ph-ap*) (Extended Data Fig. 1). In insects, *nub* is strongly expressed in future wing
66 tissues and loss of its function disrupts the development of wing-related tissues^{5,24-26}. *ap* is
67 expressed in the dorsal compartment of the wing as well as in the tergum in *Drosophila*²⁷⁻²⁹ and
68 other insects^{26,30}. In the *Drosophila* wing disc, *ap* acts as the dorsal selector to establish the DV
69 organizer²⁷. Both *nub* and *ap* have been used previously to identify wing homologs in
70 crustaceans, leading to the identification of the gill as a potential wing homolog²⁰. In *Parhyale*,
71 *Ph-nub* is expressed strongly in the gill and weakly but broadly in the cp (Fig. 1g, SI movie 4).
72 *nub* is also expressed in a ring pattern in each segment of the leg, which appears to match the *nub*
73 leg segmental expression of various arthropods^{31,32} (Fig. 1g, SI movie 4). Upon knock-out, *Ph-*
74 *nub* mutants consistently show loss of gills (Fig. 1j, Extended Data Fig. 3, Extended Data Table
75 1 and 2, SI movie 5). *Ph-nub* KO also causes reduction or curling of the cp, as well as leg
76 miniaturization (Fig. 1h and i, SI movie 5). Interestingly, although the presence of *Ph-nub*
77 expression in terga is somewhat ambiguous, we detected occasional mild tergal defects in *Ph-*
78 *nub* KO individuals (Extended Data Table 2, Extended Data Fig. 4). Therefore, in *Parhyale*, the
79 *nub* gene is essential for the proper formation of terga, as well as various leg components,
80 including the gill, cp, and distal leg segments.

81 We previously identified the two classes of *ap* genes in arthropods, termed *apA* and *apB*,
82 with *apA* being a dominant paralog during wing development^{26,33}. The *ap* gene that has
83 previously been tested in another crustacean appears to belong to the *apB* class^{20,26} (Extended
84 Data Fig. 1). We identified two *ap* genes in *Parhyale*, corresponding to the two classes (*Ph-apA*
85 and *Ph-apB*) (Extended Data Fig. 1). Our expression analysis revealed that *Ph-apA* is the
86 relevant *ap* paralog for wing homolog identification, as *Ph-apB* is only expressed in the brain of
87 *Parhyale* (Extended Data Fig. 5). In contrast, *Ph-apA* is expressed diffusely throughout the terga,
88 cp, and basis (Fig. 1k, SI movie 6) and acutely where the cp and gill join the coxa (Fig. 1k'). *apA*
89 KO (Extended Data Fig. 2 and 3, Extended Data Table 1) causes curling in the edge of the terga,
90 the cp, and in the expansion of the basis (Fig. 1l and m, SI movie 7). In addition, *apA* KO
91 individuals are often missing the entire gill, even though *Ph-apA* expression is limited to the base
92 of the gill (Fig. 1n, Extended Data Table 2, SI movie 7). Full deletion or severe reduction of all
93 relevant tissues (the tergum, cp, and gill) was also observed with *apA* KO, but we were only able

94 to recover one such individual (Extended Data Fig. 4). This low penetrance of severe KO
95 phenotype might be due to high lethality when the majority of the somatic cells are *apA* KO.
96 Taken together, our expression and functional analyses for the three “wing” genes in *Parhyale*
97 have revealed that, although the expression and functional domains for each of these genes do
98 not overlap completely, all three genes are critical for the formation of both terga and
99 components of the proximal leg (Fig. 2a). Therefore, both of these two tissues are equally likely
100 to be the wing homologs of crustaceans.

101 The debate on the origin of insect wings is like a pendulum that has been swinging back
102 and forth between the two possible origin tissues for more than 200 years³. Previous molecular
103 evidence of a wing GRN operating in crustacean gills²⁰ strongly swayed this pendulum in the
104 direction of a proximal leg origin of insect wing. Our identification of the wing-like GRN
105 operating in both the terga and the proximal leg segments prior to the evolution of insect wings
106 returns the swinging pendulum back to a neutral position, where either origin tissue can be
107 implicated. As mentioned, there is a third direction for the pendulum to swing, namely toward a
108 dual origin of insect wings³. Although not new, this idea has only recently been gaining
109 momentum^{4-7,34,35}. Below, we argue that the data presented here provide critical support for the
110 dual origin model, and in combination with previous observations of wing serial homologs in
111 wingless segments of insects, push the pendulum further in this new direction. First, the
112 functional dependency of the two tissues on the wing-like GRN is not due to a common cell
113 lineage of these tissues, as cell lineage tracing in the developing *Parhyale* embryo has identified
114 that the tergum and the leg (including the most proximal components such as coxa) have
115 different identities even early in development³⁶. These data establish that the separation of these
116 two lineages of tissues is deep in evolutionary time. Second, the presence of two separate sets of
117 tissues per segment in *Parhyale*, both of which rely on a similar wing-like GRN, is reminiscent
118 of the situation observed in the wingless segments of some insects where wing serial homologs
119 are maintained as two separate tissues of tergal and pleural (i.e. ancestral proximal leg) nature⁴⁻
120 ^{7,19} (Fig 2). This similarity provides crucial support for the idea that the wingless segments of
121 insects indeed reflect a plesiomorphic (ancestral) state for wing serial homologs, while the *bona*
122 *fide* insect wing represents an apomorphic (derived) state that depends upon the contribution of
123 two distinct tissues (*i.e.* a dual origin)¹⁹.

124 It is intriguing to speculate how a similar GRN has come to operate in the two distinct
125 tissues (the terga and the proximal leg segments). Co-option of the GRN from one tissue to the
126 other is a strong possibility that has recently been proposed³⁷. Another possibility is shared
127 ancestry between the terga and the proximal leg segments, as suggested in the accompanying
128 paper (Bruce et al. accompanying ms). In either case, our data indicate that the wing-like GRN
129 was already operating in both the terga and the proximal leg segments in the common ancestor of
130 hexapods and crustaceans prior to the evolution of *bona fide* wings. It is also worth mentioning
131 that the gill in *Parhyale* might not be homologous to insect wings, despite some genetic overlaps
132 observed between these two structures. A previous study has demonstrated that the gill GRN has
133 a larger overlap with the insect “respiratory GRN”³⁸. Furthermore, here we showed that the gill
134 lacks dependency on *Ph-vg*, a critical wing gene in insects. Therefore, the crustacean gill might
135 be more homologous to the insect respiratory system than to the wing.

136 The evolutionary route from the two sets of wing homologs to the *bona fide* insect wing
137 is still a mystery for future studies. The accompanying paper highlights one of the critical steps
138 in the evolution of insect wings, *i.e.* the evolution of pleural plates (Bruce et al. accompanying
139 ms). Bruce et al., provides compelling evidence supporting the idea that the most proximal part
140 of the *Parhyale* leg (coxa) is equivalent to the insect subcoxa, a structure that has evolved into
141 pleural plates in modern insects²¹⁻²³. Considering our finding that similar wing-like GRNs are
142 operating in both the terga and the proximal leg, the merger of the proximal leg into the body
143 wall to form the pleural plates of insects could have been a key step in bringing these highly
144 similar developmental modules closer together. Subsequently, this event may have caused a
145 “cross-wiring” of the two similar GRNs operating in these two tissues, resulting in one fused
146 tissue now functionally dependent on the merged GRN (*i.e.* an ancestral wing GRN) (Fig. 2b).

147 Although the evidence for a dual origin of insect wings is mounting^{3-7,34,35}, this third
148 hypothesis requires rigorous further testing from various fields. Recently established genetic
149 techniques (such as the CRISPR/Cas9 genome editing used in this study) will allow us to delve
150 deeper into the molecular basis underlying the evolution of insect wings. Meanwhile, the
151 pendulum of the wing origin debate continues to attract more researchers to the unveiling of the
152 origin and history of this evolutionarily monumental structure.

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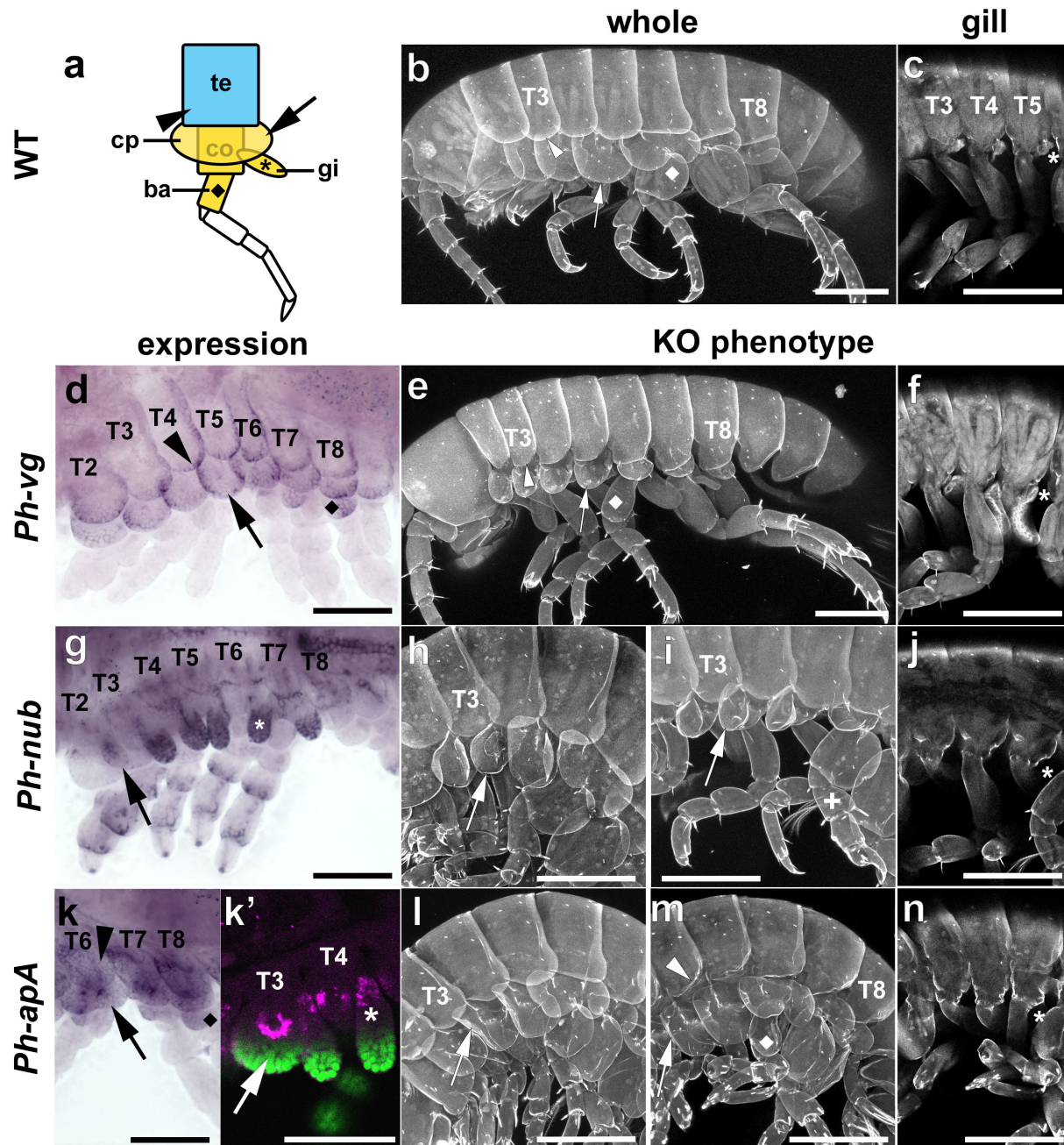
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244 **Author Contributions** C. CH. and Y.T. conceived the experiments. C. CH. performed the experiments. C. CH and Y.T.
245 analysed the data and wrote the manuscript.

246

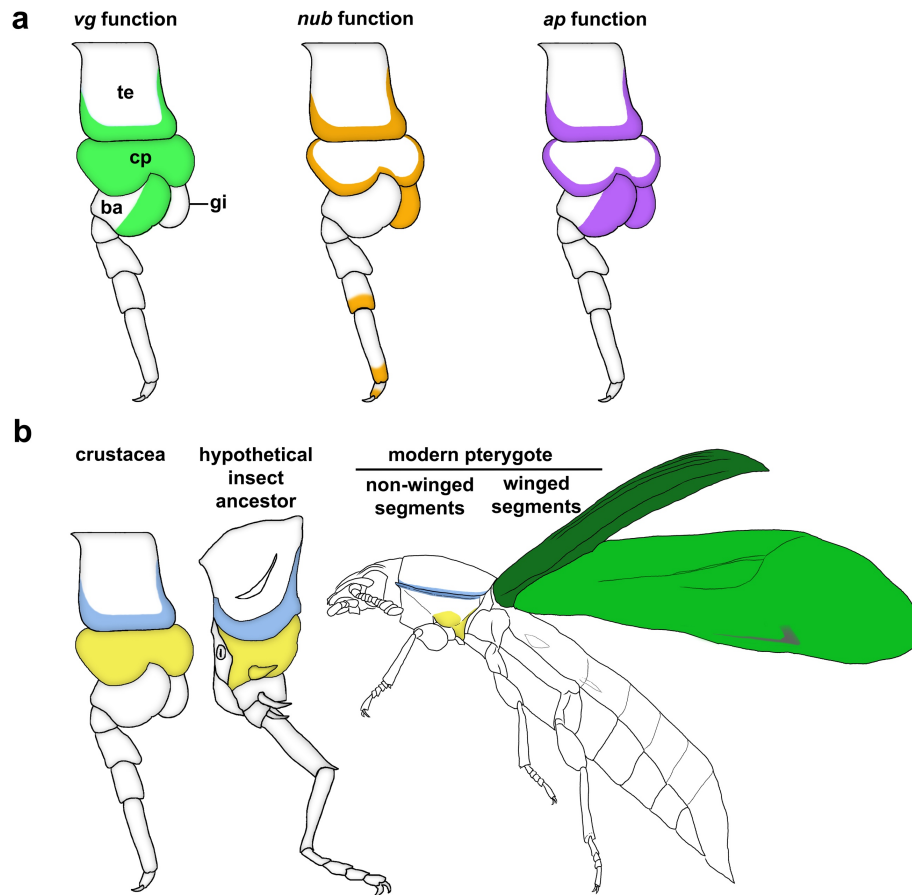
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250 **Figure 1 | Expression and functional analyses of *vg*, *nub* and *ap* in *Parhyale*.** **a**, Schematic of the
 251 dorsal-ventral body organization of *Parhyale*. te=tergum (arrowhead), co=coxa, cp=coxal plate (arrow),
 252 gi=gill (*), ba=basis (◆). Blue and yellow correspond to tergal and proximal leg tissues, respectively. **b**,
 253 **c**, WT whole (**b**) and optical section (**c**). **d-n**, *in situ* hybridization and CRISPR/Cas9 KO analyses for *vg*
 254 (**d-f**), *nub* (**g-j**), and *ap* (**k-n**). **k'**, optical section at the attachment of gill and coxal plate to coxa. *Ph-apA*:
 255 purple, DAPI: green. + in **i** indicates stunted leg. Scale bars 200µm, except in **d**, **g**, **k** and **k'** 100µm.



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258 **Figure 2 | The proposed wing homologs of *Parhyale* and the evolutionary relationship among wing**
 259 **homologs. a**, the functional domains of the three genes examined in this study. te=tergum, cp=coxal
 260 plate, gi=gill, ba=basis. **b**, the evolutionary relationship among wing homologs. The tergal edge (blue)
 261 and proximal leg segment (cp, yellow) are two possible crustacean wing homologs, in which two similar
 262 wing-like GRNs operate (left in **b**). These crustacean tissues correspond to the two proposed wing origin
 263 tissues; the crustacean tergum corresponds to the insect tergum, and the crustacean proximal leg segments
 264 to the insect pleural plates (middle in **b**). A similar situation can be found in the wingless segments of
 265 pterygote insects (right in **b**, tergal and pleural tissues are colored in blue and yellow, respectively). A
 266 wing-like GRN operates in both of these tissues, and they merge to form a complete wing upon homeotic
 267 transformation^{4,6}, indicating that both tissues are wing serial homologs in the wingless segments of
 268 pterygotes (right in **b**). Through these observations, we propose that, prior to the evolution of insect
 269 wings, the apterygote ancestor of hexapods possessed two distinct tissues (of tergal and pleural nature),
 270 both of which had similar wing-like GRNs operating in them (middle in **b**). The evolution of pleural
 271 plates from the most proximal leg segment (*i.e.* subcoxa) has juxtaposed these two distinct tissues that
 272 rely on a similar GRN, which might have resulted in one functional unit of tissues (green) with a “cross-
 273 wired” GRN (ancestral wing GRN). In contrast, in the wingless segments, Hox genes have evolved to
 274 prevent this merger, maintaining the tissues serially homologous to wings as two separate sets (one of
 275 tergal and the other of pleural nature).