# A morphological novelty evolved by co-option of a reduced gene regulatory network and gene recruitment in a beetle

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**Supplementary Material**
**Figure S1-S7**
**Table S1-S4**

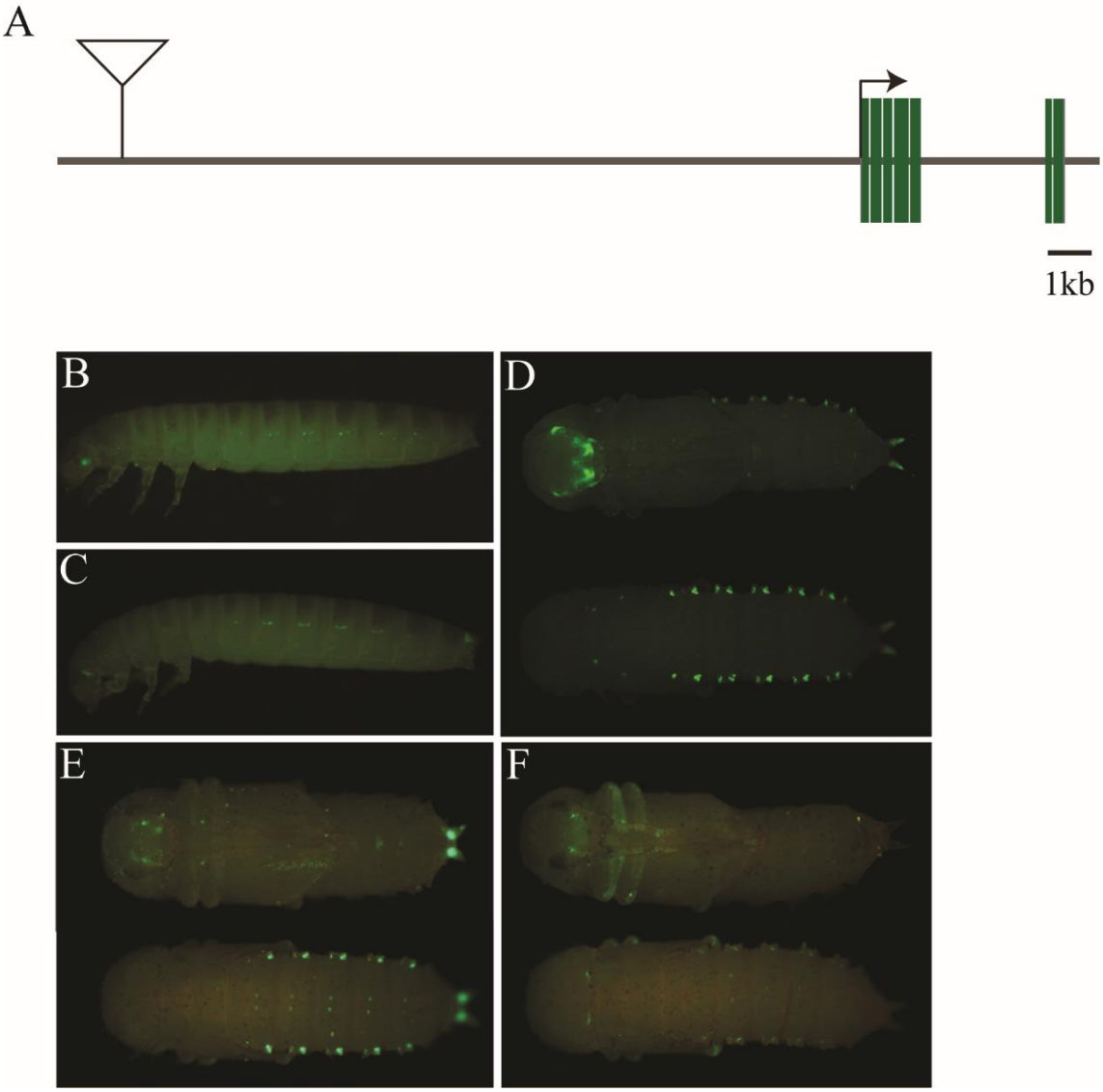


Fig. S1. Characterization of the Gal4 enhancer trap line *GöGal41152*. (A) The insertion site of the construct is upstream of gene *Tc033998* (based on NCBI annotation a sodium-coupled monocarboxylate transporter). dsRNA targeting Tc033998 did not elicit morphological defects in the gin-traps. (B-F) Signal at different developmental stages. (B) First signal is detected immediately after cessation of feeding in late L7. (C) Prepupa (D) 1 day old pupa (E) 2-3 day old pupa (F) 4 day old pupa.

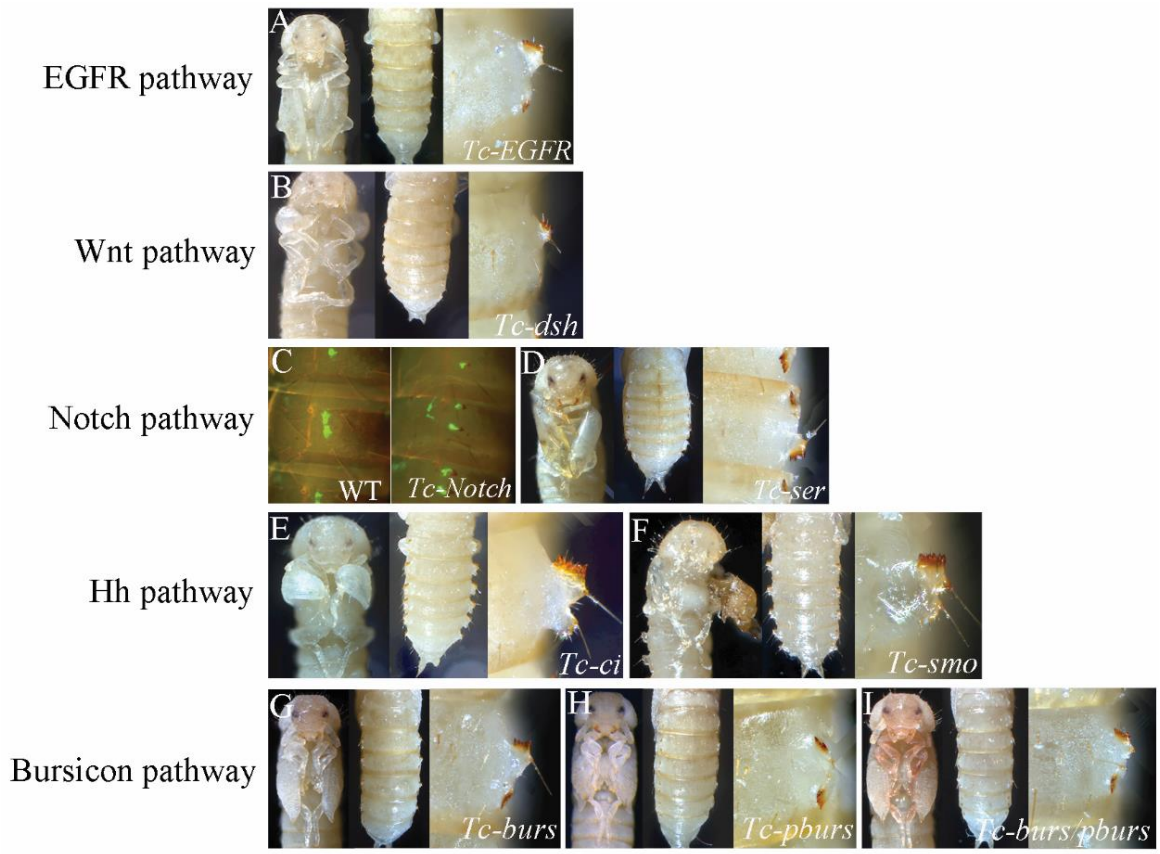


Fig. S2. Effects of five different signaling pathways on gin-trap and wing morphology. (A) *Tc-EGFR* RNAi. (B) *Tc-dsh* RNAi. (C,D) RNAi phenotype of Notch pathway genes: *Tc-Notch* (C) and *Tc-Ser* (D). (E,F) RNAi of *hh* pathway genes: *Tc-ci* (D) and *Tc-smo* (E). (G-I) Genes of the bursicon pathway *Tc-burs* RNAi (G), *pburs* RNAi (H) and *Tc-burs*/*Tc-pburs* double RNAi (I). Each panel shows ventral view of the pupal wing, dorsal view of the pupal abdomen and close-up of the gin-trap on T3. *Notch* RNAi animals did not molt to pupae, hence the images were captured at the pre-pupal stage in line *GöGal41152* (C).


Fig. S3 Wing genes without gin-trap phenotype. (A-I) *Tc-en/inv*, *Tc-dpp*, *Tc-omb*, *Tc-dad*, *Tc-iro*, *Tc-Dll*, *Tc-ASH*, *Tc-srf*, and *Tc-nub*.

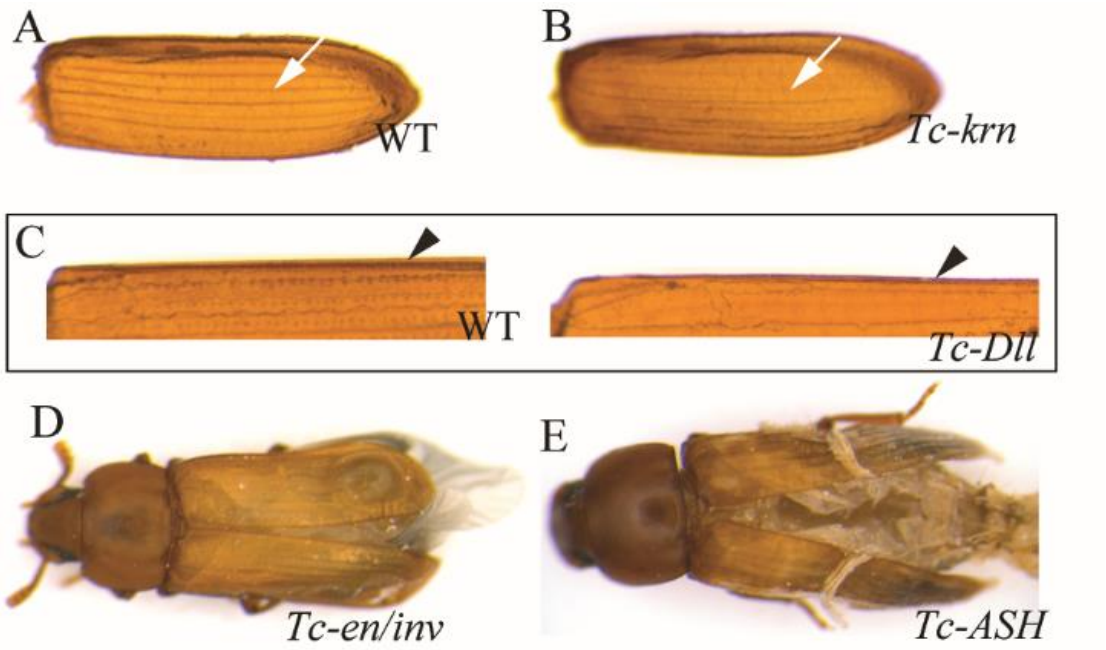


Fig. S4. Effects of RNAi on *Tribolium* adult elytra. (A) Wild type. (B) In *Tc-krn* RNAi the vein pattern of the elytra was affected (compare arrows in A and B). (C) The margin of the elytra was altered after depletion of *Tc-Dll* (arrowheads). (D) *Tc-en/inv* double RNAi led to distal defects in the elytra. (E) *Tc-ASH* RNAi. The development of setae was affected in all epidermal tissues. The wing open phenotype is probably a secondary effect.

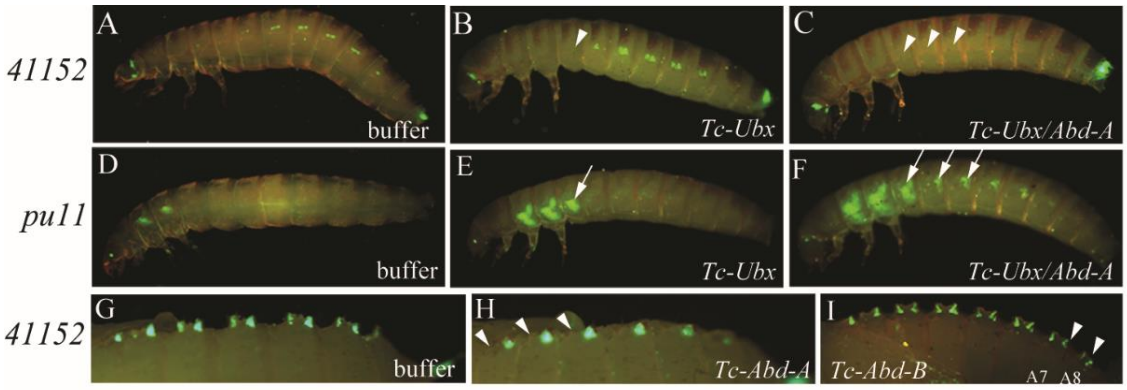


Fig. S5 Effects of Hox genes on gin-trap formation. (A-F) Animals at the prepupal stage injected with buffer (A,D); *Tc-Ubx* dsRNA (B,E) or *Tc-Ubx/Tc-Abd-A* double dsRNA (C,F) in the transgenic lines *GöGal41152* (A-C) or *pu11* (D-F). (G-I) Dorsal view on *GöGal41152* pupae injected with buffer (G), *abdA* (H) and *AbdB* (I). (B,E) *In Tc-Ubx* RNAi the gin-trap marker was lost in A1 (arrowhead in B) but the wing marker appeared at the corresponding position (arrow in E), indicating a transformation from gin-trap to wing identity. (C,F) Double knockdown of *Tc-Ubx* and *Tc-abdA* abolished the gin-trap marker in all abdominal segments (C) while the *pu11* wing marker appeared (F) as reported previously [11,33]. (H) In *Tc-abdA* RNAi pupae the gin-traps on abdominal segments A2 through A6 were more like that in A1, where the anterior part is missing (arrowheads). In strong phenotypes a wing like protrusion formed posterior of gin-traps on A4-A7 (not shown). (I) In *Tc-AbdB* RNAi pupae, the partial gin-trap of A7 (only anterior part is present in wildtype) was complemented by a posterior part and one more pair of intact gin-traps appeared on A8 (arrowheads).

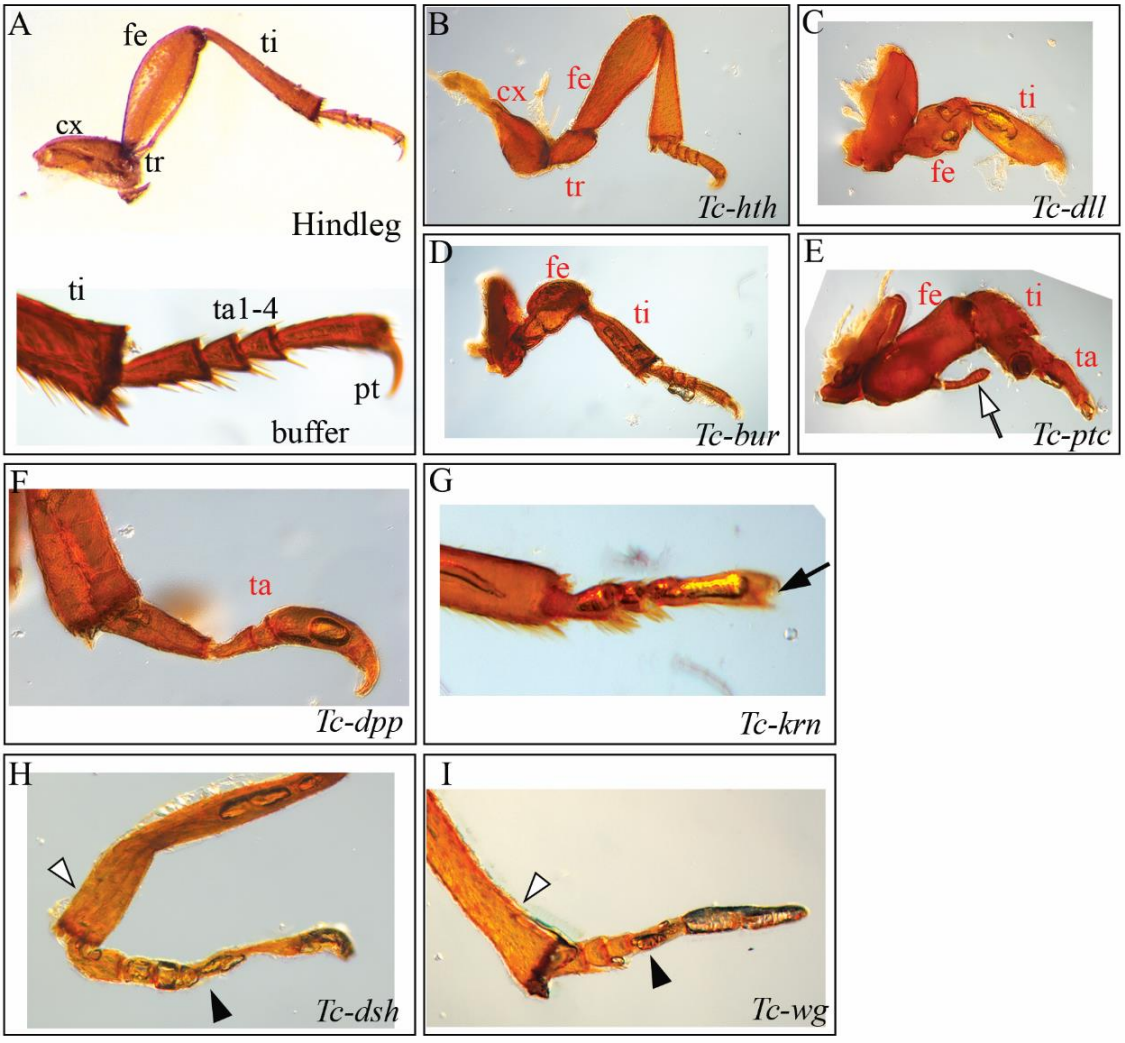


Fig. S6 Effects of wing related genes for adult leg patterning. (A) Hindleg (upper panel) and close-up (lower panel) of tarsus from a buffer injected adult. (B) Depletion of *Tc-hth* induced the transformation of proximal structures (coxa, trochanter, proximal femur) to more distal morphologies. (C) Knocking down of *Tc-Dll* caused the elimination of the segments distal to tibia, and reduction of femur and tibia. (D) The leg was shorter in *Tc-bur* RNAi adults than that in control. (E) Knocking down of *Tc-ptc* led to deformation and fusion of leg segments and ectopic cuticular outgrowth in the femur (open arrow). (F) *Tc-dpp* RNAi resulted in the alteration and partial fusion of tarsomeres. (G) *Tc-krn* caused partial fusion of tarsomeres and elimination of the pretarsus (black arrow). (H and I) Tibia (open arrowhead) and tarsus (black arrowhead) were narrower after knocking down *Tc-dsh* and *Tc-wg*, respectively. Affected structures are marked in red. Abbreviations: cx, coxa; fe, femur; ti, tibia; ta, tarsus; pt, pretarsus.

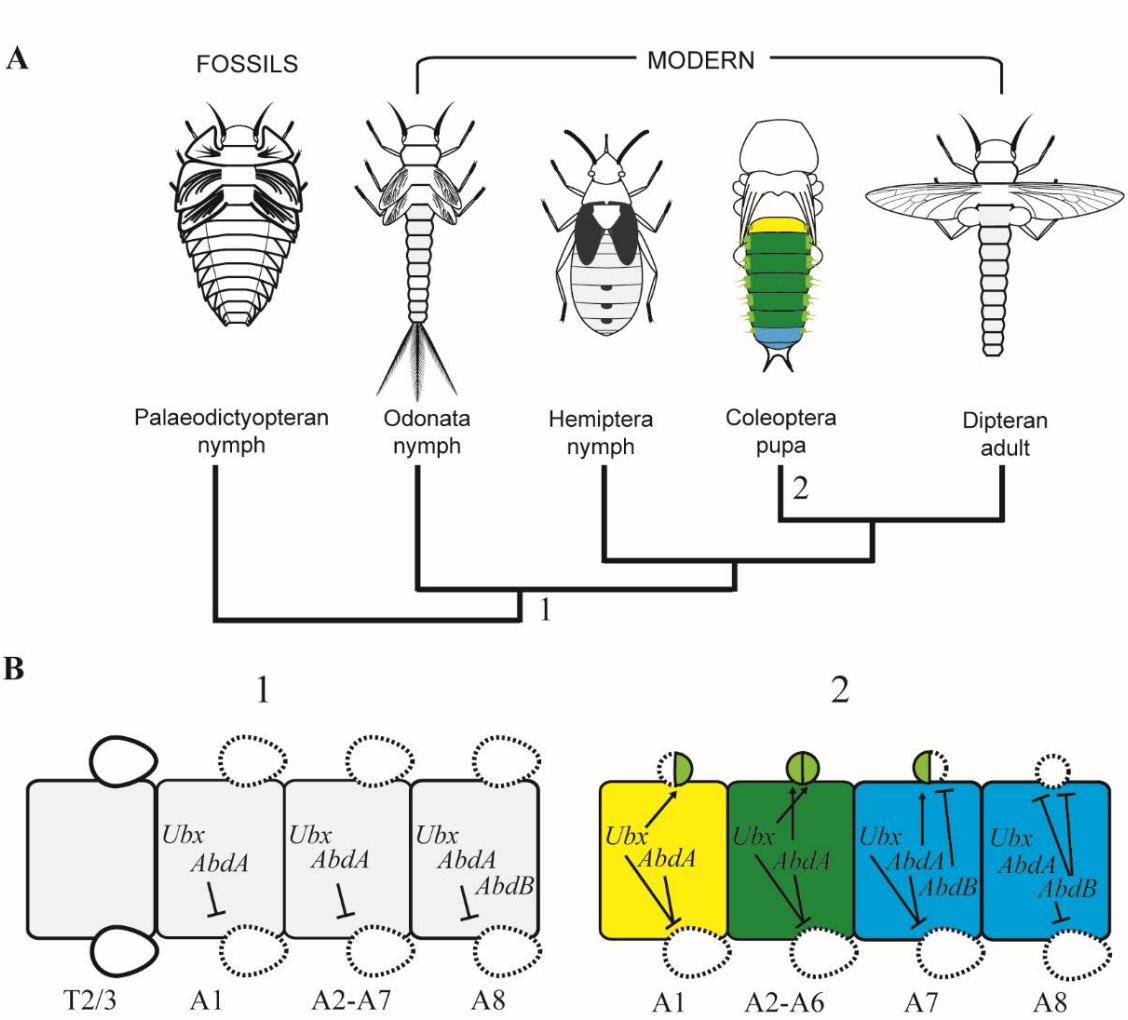


Figure S7 Model for Hox gene function on wings and gin-traps. (A) The fossil record shows early pterygotes with abdominal structures on all thoracic and abdominal segments [57]. Whether these structures are serial homologs to wings remains disputed. B) The wing network probably evolved initially without input from Hox genes because *Antp* is not required for wing formation in extant insects [57]. However, Ubx expressed in T3 modified the hindwings to varying degrees in some insect taxa [11,58–60]. Activation of the wing network in abdominal segments was repressed by abdominal Hox genes (B 1). We propose that the first step for gin-trap evolution was a partial de-repression of the wing GRN leading to an outgrowth based on the co-opted wing GRN. Recruitment of novel genes and pruning and regulatory changes in the GRN led to the gin-trap morphology. For instance, co-option of *Tc-caspar* and evolution of an upstream role of *Tc-ems* were probably required to generate the symmetry of gin-traps in an otherwise AP asymmetric segment. Note the asymmetric requirement of *Tc-abdA* and *Tc-Ubx* for anterior versus posterior part of the gin-traps

**Table S1 Primer sequences used in this study**

| primer name | sequence | use |
|---|---|---|
| Tc-bursF | GTGATCCACGTTTTACAATATC | Cloning of the region used for RNAi |
| Tc-bursR | CCTCAACCCCAGTGCAGG | |
| Tc-pburF | ACAGAGAATATGCAATGGGGAGG | Cloning of the region used for RNAi |
| Tc-pburR | TCGGCTGAAATCGCCACACT | |
| Tc-UbxF | ACTCTTACTTCGAGCAGAGC | Cloning of the region used for RNAi |
| Tc-UbxR | GGTGTATCTGCGTGCCAAC | |
| T7-pJETF | GAATTGTAATACGACTCACTATAGGCGACT CACTATAGGGAGAGC | amplify the template for in vitro transcription to generate dsRNA |
| T7-pJETR | TAATACGACTCACTATAGGAAGAACATCGA TTTTCCATGGCAG | |
| T7 | GAATTGTAATACGACTCACTATAGG | |
| T7-sp6 | TAATACGACTCACTATAGGATTTAGGTGACA CTATAGA | |
| T7-T3 | TAATACGACTCACTATAGGAATTAACCCTCA CTAAAGGG | |
| iPCR5’F1 | GACGCATGATTATCTTTTACGTGAC | 1st round PCR after Bsp143I treatment |
| iPCR5’R1 | TGACACTTACCGCATTGACA | |
| iPCR5’F2 | GCGATGACGAGCTTGTTGGTG | 2nd round PCR after Bsp143I |
| iPCR5’R2 | TCCAAGCGGCGACTGAGATG | treatment |
| iPCR5’Seq | CGCGCTATTTAGAAAGAGAGAG | sequencing |
| | | |
| iPCR3’F1 | CAACATGACTGTTTTTAAAGTACAAA | 1st round PCR after HhaI treatment |
| iPCR3’R1 | GTCAGAAACAACTTTGGCACATATC | |
| iPCR3’F2 | CCTCGATATACAGACCGATAAAAC | 2nd round PCR after HhaI treatment |
| iPCR3’R2 | TGCATTTGCCTTTCGCTTAT | |
| iPCR3’Seq | CGATAAAACACATGCGTCAAT | sequencing |

**Table S2 Summary of iBeetle number, target gene and dsRNA length**

| iBeetle number | Target gene | dsRNA length |
|---|---|---|
| iB_03555 | *Tc-krn* | 510bp |
| iB_04438 | *Tc-rk* | 493bp |
| iB_04697 | *Tc-simj* | 559bp |
| iB_02268 | *Tc-kis* | 470bp |
| iB_04931 | *Tc-vg* | 434bp |
| iB_04737 | *Tc-casp* | 513bp |
| iB_05098 | *Tc-ems* | 481bp |
| iB_05634 | *Tc-mib1* | 476bp |
| iB_05728 | *Tc-Gug* | 495bp |
| iB_06451 | *Tc-Abd-A* | 509bp |
| iB_03099 | *Tc-Abd-B* | 480bp |
| iB_04526 | *Tc-hth* | 530bp |
| iB_05191 | *Tc-tiotsh* | 471bp |
| iB_06279 | *Tc-inv* | 535bp |
| iB_04091 | *Tc033998* | 498bp |
| | *Tc-burs* | 268bp |
| | *Tc-pburs* | 268bp |
| | *Tc-Ubx* | 591bp |
| | *Tc-EGFR* | 974bp |
| | *Tc-ser* | 610bp |
| | *Tc-Notch* | 310bp |
| | *Tc-wg* | 1100bp |
| | *Tc-en* | 885bp |
| | *Tc-hh* | 1148bp |
| | *Tc-smo* | 961bp |
| | *Tc-ci* | 1351bp |
| | *Tc-dpp* | 1129bp |
| | *Tc-omb* | 418bp |
| | *Tc-iro* | 1043bp |
| | *Tc-ASH* | 762bp |
| | *Tc-nub* | 685bp |
| | *Tc-srf* | 872bp |
| | *Tc-dad* | 655bp |
| | *Tc-apA* | 684bp |
| | *Tc-apB* | 798bp |
| | *Tc-dsh* | 525bp |
| | *Tc-sal* | 930bp |

**Table S3 Summary of off-target controls**

| | | | | | gin-trap | | wing | |
|---|---|---|---|---|---|---|---|---|
| iBeetle number | RNAi target | Beetle strain | number of injected larvae | survival to pupal stage | phenotype | penetrance | phenotype | penetrance |
| iB_03555 | *Tc-krn* | *SB* | 10 | 7 | size largely decreased | 100.0% | slightly smooth elytra surface of pupa, rids or vein developed abnormally and number of setea was less in adult elytra | 100.0% |
| iB_03555 | *Tc-krn* | *D17×het* | 10 | 9 | size largely decreased | 100.0% | slightly smooth elytra surface of pupa, rids or vein developed abnormally and number of setea was less in adult elytra | 100.0% |
| iB_03555 NOF | *Tc-krn* | *SB* | 10 | 7 | size largely decreased | 100.0% | slightly smooth elytra surface of pupa, rids or vein developed abnormally and number of setea was less in adult elytra | 100.0% |
| iB_03555 NOF | *Tc-krn* | *D17×het* | 10 | 5 | size largely decreased | 100.0% | slightly smooth elytra surface of pupa, rids or vein developed abnormally and number of setea was less in adult elytra | 100.0% |
| iB_04931 | *Tc-vg* | *SB* | 10 | 4 | absent | 100.0% | size largely decreased or absent | 100.0% |
| iB_04931 | *Tc-vg* | *D17×het* | 10 | 6 | absent | 100.0% | size largely decreased or absent | 100.0% |
| iB_04931 NOF | *Tc-vg* | *SB* | 10 | 3 | absent | 100.0% | size largely decreased or absent | 100.0% |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| iB_04931 NOF | *Tc-vg* | *D17×het* | 10 | 5 | largely decreased or absent | 100.0% | size largely decreased or absent | 100.0% |
| iB_04697 | *Tc-simj* | *SB* | 10 | 6 | largely decreased | 83.3% | smaller and a little deformed | 83.3% |
| iB_04697 | *Tc-simj* | *D17×het* | 10 | 10 | largely decreased | 100.0% | size smaller and distal part more rounded | 70.0% |
| iB_04697 NOF | *Tc-simj* | *SB* | 10 | 2 | largely decreased | 100.0% | size smaller and orientation irregular | 100.0% |
| iB_04697 NOF | *Tc-simj* | *D17×het* | 12 | 9 | largely decreased | 100.0% | size smaller and orientation irregular | 100.0% |
| iB_04737 | *Tc-Casp* | *SB* | 9 | 5 | largely decreased, some showed anterior gin-trap had stronger phenotype than posterior gin-trap | 100.0% | no phenotype | 0.0% |
| iB_04737 | *Tc-Casp* | *D17×het* | 10 | 8 | largely decreased, some showed anterior gin-trap had stronger phenotype than posterior gin-trap | 100.0% | no phenotype | 0.0% |
| iB_04737 NOF | *Tc-Casp* | *SB* | 10 | 4 | anterior gin-trap largely decreased, posterior gin-trap slightly decreased | 100.0% | no phenotype | 0.0% |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| iB_04737 NOF | *Tc-Casp* | *D17×het* | 9 | 3 | anterior gin-trap largely decreased, posterior gin-trap slightly decreased | 66.7% | no phenotype | 0.0% |
| iB_05728 | *Tc-Gug* | *SB* | 10 | 2 | largely decreased | 100.0% | elytra size a little smaller, smooth surface | 100.0% |
| iB_05728 | *Tc-Gug* | *D17×het* | 10 | 1 | largely decreased | 100.0% | elytra size a little smaller, smooth surface | 100.0% |
| iB_05728 NOF | *Tc-Gug* | *SB* | 15 | 5 | largely decreased | 80.0% | elytra size a little smaller, smooth surface | 80.0% |
| iB_05728 NOF | *Tc-Gug* | *D17×het* | 10 | 5 | largely decreased | 100.0% | elytra size a little smaller, smooth surface | 100.0% |
| iB_05634 | *Tc-mib1* | *SB* | 10 | 2 | moderately smaller | 100.0% | mostly absent | 100.0% |
| iB_05634 | *Tc-mib1* | *D17×het* | 8 | 7 | moderately smaller, orientation irregular | 100.0% | largely decreased or absent | 100.0% |
| iB_05634 NOF | *Tc-mib1* | *SB* | 12 | 1 | mildly or moderately smaller and orientation irregular | 100.0% | largely decreased | 100.0% |
| iB_05634 NOF | *Tc-mib1* | *D17×het* | 10 | 2 | slightly smaller, orientation irregular | 100.0% | largely decreased or absent | 100.0% |
| iB_04438 | *Tc-rk* | *SB* | 10 | 2 | slightly smaller, orientation irregular | 100.0% | a little shorter, wrinkled surface | 100.0% |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| iB_04438 | *Tc-rk* | *D17×het* | 10 | 7 | slightly smaller, orientation irregular | 100.0% | a little shorter, orientation irregular | 100.0% |
| iB_04438 NOF | *Tc-rk* | *SB* | 10 | 10 | slightly smaller, orientation irregular | 100.0% | a little shorter, wrinkled surface | 100.0% |
| iB_04438 NOF | *Tc-rk* | *D17×het* | 10 | 6 | slightly smaller, orientation irregular | 100.0% | a little shorter, wrinkled surface | 100.0% |
| iB_05098 | *Tc-ems* | *SB* | 17 | 15 | size decreased, anterior gin-trap largely decreased or absent, posterior gin-trap moderately or largely smaller | 100.0% | elytra a little shorter | 33.3% |
| iB_05098 | *Tc-ems* | *D17×het* | 20 | 5 | anterior gin-trap size largely decreased | 100.0% | elytra a little shorter and surface irregular | 80.0% |
| iB_05098 NOF | *Tc-ems* | *SB* | 10 | 9 | shape irregular, anterior gin-trap smaller and less scleritized jaw | 44.4% | no phenotype | 0.0% |
| iB_05098 NOF | *Tc-ems* | *D17×het* | 12 | 8 | size decreased, anterior gin-trap size largely decreased | 100.0% | no phenotype | 0.0% |
| iB_02268 | *Tc-kis* | *SB* | 18 | 18 | moderately or largely decreased | 100.0% | a little shorter, some showed blistered elytra | 100.0% |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| iB_02268 | *Tc-kis* | *D17×het* | 20 | 13 | moderately decreased | 100.0% | slightly shorter | 100.0% |
| iB_02268 NOF | *Tc-kis* | *SB* | 18 | 17 | moderately or largely decreased | 100.0% | slightly shorter | 100.0% |
| | *Tc-kis* | *D17×het* | 20 | 15 | moderately decreased | 100.0% | slightly shorter | 100.0% |
| iB_06451 | *Tc-Abd-A* | *SB* | 10 | 7 | anterior gin-trap largely decreased, proximal part of gin-traps a little expanded | 100.0% | no phenotype | 0.0% |
| iB_06451 | *Tc-Abd-A* | *D17×het* | 10 | 8 | anterior gin-trap largely decreased, proximal part of gin-traps a little expanded | 87.5% | no phenotype | 0.0% |
| iB_06451 NOF | *Abd-A* | *SB* | 16 | 12 | anterior gin-trap largely decreased, proximal part of gin-traps a little expanded | 100.0% | no phenotype | 0.0% |
| iB_06451 NOF | *Abd-A* | *D17×het* | 10 | 3 | anterior gin-trap largely decreased | 100.0% | no phenotype | 0.0% |

NOF: non-overlapping fragment of dsRNA
penetrance: proportion of pupae with phenotype in all the survived pupae
*D17×het* is the genetic background used in the *iBeetle* screen.

## Table S4 Classification of genes

| **Candidate genes from the known wing gene regulatory network (i.e. phenotype in both *Drosophila* and *Tribolium*)** | | | |
|---|---|---|---|
| | **only wing** | **wing and gin-trap** | **only gin-trap** |
| **pathways** | Dpp | Hh[1]<br>Wnt[2]<br>Notch<br>EGFR | |
| **genes** | *optomotor-blind*<br>*srf*<br>*nubbin*<br>*engrailed/invected*<br>*Distal-less* | *apterous*<br>*vestigial*<br>*teashirt*<br>*homothorax*<br>*spalt*<br>*Grunge/atrophin* | |
| ***Novel genes identified in the iBeetle screen*** | | | |
| | **only wing** | **wing and gin-trap** | **only gin-trap** |
| **pathways** | | | *caspar*[3]<br>*(immunity)* |
| **genes** | | *kismet*<br>*simjang*<br>*ems*[3] | |
| **Gene identified in the iBeetle screen but previously known in wing development in *Tribolium*** | | | |
| | **only wing** | **wing and gin-trap** | **only gin-trap** |
| **pathways** | | Bursicon<br>*(neuropeptide)* | |
| | | | |
| **Notes**<br>[1] only posterior part of gin-trap affected<br>[2] mainly posterior but also anterior part<br>[3] only anterior part of gin-trap affected | | In *Tribolium*, *Tc-iroquois* & *Tc-ASH* RNAi affected bristles on the entire cuticle – therefore not scored as specific wing phenotype. | |
| **Genes/pathways affecting wing patterning:**<br>n=20<br>6 do not affect gin-traps (30%)<br>3 novel in wing patterning (15%) | | **Genes/pathways affecting gin-traps:**<br>n=15<br>1 affects only gin-traps (6.6%) | |
| **Estimation of total number of co-opted genes:**<br>So far, 28% of the genome was screened for metamorphosis phenotypes. In this randomly selected gene set, we found 3 novel genes involved in wing and gin-trap formation and 1 gene active in gin-traps only. Screening the other 72% of the genome we expect at least another 8 genes of the former and 2 genes of the latter category. Together with the genes found by screening wing GRN candidates this would sum up to 25 genes required for gin-trap formation of which 3 would be co-opted (12%). | | | |