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

Authors

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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 *TcO33998* (based on NCBI annotation a sodium-coupled monocarboxylate transporter). dsRNA targeting TcO33998 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-DII*, *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 closeup (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 derepression 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

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	amplify the template for in vitro			
	CACTATAGGGAGAGC	transcription to generate dsRNA			
T7-pJETR	TAATACGACTCACTATAGGAAGAACATCGA				
	TTTTCCATGGCAG				
Т7	GAATTGTAATACGACTCACTATAGG				
T7-sp6	TAATACGACTCACTATAGGATTTAGGTGACA				
	CTATAGA				
Т7-Т3	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 Hhal treatment			
iPCR3'R1	GTCAGAAACAACTTTGGCACATATC				
iPCR3'F2	CCTCGATATACAGACCGATAAAAC	2nd round PCR after Hhal treatment			
iPCR3'R2	TGCATTTGCCTTTCGCTTAT				
iPCR3'Seq	CGATAAAACACATGCGTCAAT	sequencing			

Table S1 Primer sequences used in this study

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-В	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
	Тс-сі	1351bp
	Tc-dpp	1129bp
	Tc-omb	418bp
	Tc-iro	1043bp
	Tc-ASH	762bp
	Tc-nub	685bp
	Tc-srf	872bp
	Tc-dad	655bp
	Тс-арА	684bp
	Тс-арВ	798bp
	Tc-dsh	525bp
	Tc-sal	930bp

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

			number of	survival to pupal	gin-trap		wing	
iBeetle number	RNAi	Beetle			phenotype	penetrance	phenotype	penetrance
	target	strain	injected larvae	stage				
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%

Table S3 Summary of off-target controls

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, 100. orientation irregular		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, 100.0% anterior gin-trap largely decreased or absent, posterior gin- trap moderately or largely smaller		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, 44.4% anterior gin-trap smaller and less scleritized jaw		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 100.0% largely decreased, proximal part of gin- traps a little expanded		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

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3 novel in wing patterning (15%)	6 do not affect	gin-traps (30%)		1 affects only gin-t	raps (6.6%)				
	3 novel in wing	patterning (15%)							
Estimation of total number of co-opted genes:	Estimation of t	otal number of co-opted g	enes:						
So far, 28% of the genome was screened for metamorphosis phenotypes. In this randomly	So far, 28% of t	he genome was screened f	or metar	morphosis phenotyp	es. In this randomly				
selected gene set, we found 3 novel genes involved in wing and gin-trap formation and 1 gene	selected gene s	set, we found 3 novel genes	s involve	d in wing and gin-tra	p formation and 1 gene				
active in gin-traps only. Screening the other 72% of the genome we expect at least another 8	active in gin-tra	aps only. Screening the othe	er 72% o	f the genome we ex	pect at least another 8				
genes of the former and 2 genes of the latter category. Together with the genes found by	genes of the fo	rmer and 2 genes of the lat	ter cate	gory. Together with t	he 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%).