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The iBeetle large-scale RNAi screen reveals gene functions for insect development and physiology

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Genetic screens are powerful tools to identify the genes required for a given biological process. However, for technical reasons, comprehensive screens have been restricted to very few model organisms. Therefore, although deep sequencing is revealing the genes of ever more insect species, the functional studies predominantly focus on candidate genes previously identified in *Drosophila*, which is biasing research towards conserved gene functions. RNAi screens in other organisms promise to reduce this bias. Here we present the results of the iBeetle screen, a large-scale, unbiased RNAi screen in the red flour beetle, *Tribolium castaneum*, which identifies gene functions in embryonic and postembryonic development, physiology and cell biology. The utility of *Tribolium* as a screening platform is demonstrated by the identification of genes involved in insect epithelial adhesion. This work transcends the restrictions of the candidate gene approach and opens fields of research not accessible in *Drosophila*.

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The importance of *Drosophila melanogaster* as a model system is in large part due to its amenability to elegant genetic screens, which allow the comprehensive identification of genes required for a given biological process[1,2]. Recently, unbiased genetic screens have also been performed in a few other insects, for example, the hymenopteran *Nasonia vitripennis* and the beetle *Tribolium castaneum*[3–6]. However, technical constraints prohibit saturation screens in these species. Hence, most of what we know about insect gene function remains based on *Drosophila* work. RNA interference (RNAi) has emerged as an alternative tool to knockdown gene function and has thus far been used for genome-wide screens in the nematode *Caenorhabditis elegans*, in *Drosophila* and in cell culture[7–11].

In recent years, reverse genetics based on deep sequencing and RNAi has enabled functional investigations in ever more insect species, broadening the range of biological phenomena that can be analysed. However, the candidate gene approach still prevails: genes are selected based on previous findings in *Drosophila* or other model systems[12–21]. Consequently, the field of insect functional genetics suffers from several limitations. First, the candidate gene approach leads to a bias towards the study of conserved gene functions. Second, it has remained difficult to identify genes required for processes that are not represented in *Drosophila*. Finally, technical limitations and lineage specific gene losses or duplications prohibit the identification of comprehensive gene sets for a particular process in any single species. An unbiased, large-scale RNAi screen in a non-dipteran insect species should overcome many of these limitations.

The red flour beetle, *T. castaneum*, is well suited for this aim. In many respects its biology is more representative for insects than *Drosophila,* as its segmentation proceeds from a posterior growth zone, the larval head is not involuted and its extraembryonic tissues are well developed. Further, its modes of oogenesis and metamorphosis resemble those of other non-dipteran insects[22–28]. Further, *Tribolium* is a representative of the most species-rich animal taxon on earth, the coleopterans (beetles), including many devastating pests[29]. Finally, *Tribolium* research builds on an expanding transgenic toolkit[6,30–32] and a particularly strong systemic RNAi response. Knockdown phenotypes can be induced at all life stages through dsRNA injection into the body cavity, and the RNAi effect spreads throughout the animal and is transferred to the offspring, often phenocopying null mutants[33–36].

Here we present the results of the *iBeetle* screen, where we used RNAi targeting about 5,000 genes to identify novel gene functions during oogenesis, embryogenesis and metamorphosis in an unbiased way. We show that this screen has the power of overcoming the current limitations imposed by the candidate gene approach by the identification of unexpected novel players required for long studied processes. For instance, we describe the first bicaudal phenotype in *Tribolium* elicited by knockdown of the homeobox gene *Tc-homeobrain*; a gene so far not related to anterior–posterior axis formation. Further, we show that the unbiased detection of gene function in *Tribolium* allows opening new fields of research. For instance, many insects have odoriferous glands used for communication and defence but such glands are missing in *Drosophila*. In the screen, a set of genes were identified, which are involved in producing the defensive chemicals of the *Tribolium* odoriferous glands. Importantly, many of these genes were not identified in a recent RNA-seq approach confirming the power of a phenotypic screen. Finally, we show that *Tribolium* is an excellent alternative screening platform, where insect gene functions are efficiently identified. One example is the gene *Tc-Rbm24*, which we found to be required for muscle development in *Tribolium*. *Drosophila* does not have a respective ortholog while the vertebrate ortholog has recently been shown to be involved in muscle formation. As second example, we identified novel genes required for epithelial adhesion in *Tribolium*, the orthologs of which are required in *Drosophila* as well but had not been discovered in *Drosophila* screens.

Results

Design of the iBeetle screen. We developed a procedure that allowed efficient screening of several biological processes. Two screens were performed in parallel by injection of different developmental stages. In the 'pupal injection screen', injected *pig19* transgenic female pupae (somatic muscles marked with EGFP) were scored for late metamorphosis phenotypes and, upon maturation to adults, their offspring embryos were analysed for muscle and cuticle phenotypes as readouts for defects in embryogenesis. This treatment knocked down both maternal and zygotic transcripts in developing embryos. In case of reduced egg production, ovaries of the injected females were analysed for oogenesis defects.

In the 'larval injection screen', penultimate instar larvae (L6) were injected. Female larvae were derived from a cross between *D17Xred* (adult flight muscles marked with EGFP; X-linked DsRed marker allowed sexing of larvae) and *pearl* (white eyed) strains. Muscle phenotypes were scored during the pupal stage, and general morphological defects both at pupal and at adult stages. Ovaries were dissected and analysed whenever egg production was found to be strongly reduced. Finally, adult odoriferous glands were scored for alterations in size or colouring and dissected for closer inspection. Importantly, the larval injection screen allowed the identification of gene functions during metamorphosis without affecting essential functions during embryogenesis that would prevent analysis of later stages. We screened 5,300 genes in the (ongoing) pupal and 4,480 genes in the larval injection screen and present analyses of 3,400 genes included in both screens.

The iBeetle screen was designed as a first pass screen wherein each experiment was performed once, and off-target controls were done only for selected genes. We aimed at minimizing false negative annotations with the trade-off of an increased false positive rate. The genes to be knocked down were selected randomly, except that their annotations were based on RNA sequence data, which may have led to some enrichment of highly expressed genes (see Supplementary Table 1). Using the DEQOR prediction algorithm[37], templates were selected for high RNAi efficiency and a low number of possible off-target sites.

dsRNA fragments with an average length of 479 bp were injected at a concentration of $1\,\mu g\,\mu l^{-1}$. Phenotypes were annotated in an online database according to the EQM system (entity, quality, modifier)[38] and using a controlled vocabulary based on the *Tribolium morphological ontology*[39]. In addition, the penetrance of phenotypes was recorded, and pictures and free text fields were used for further documentation (see Methods section for details). All abovementioned data sets of the larval and pupal injection screen in addition to sequence and orthology information of the entire *Tribolium* gene set are available at http://ibeetle-base.uni-goettingen.de[40]

Tests for Sensitivity and Reproducibility. To assess the sensitivity of our screen, roughly 5% of screened genes were positive controls from a set of 41 different genes (Supplementary Fig. 1). In addition, 48 previously published genes that happened to be within our gene set served as additional blind positive controls. Ninety-three per cent of the selected controls and 95% of the previously published genes were identified during the screen (Fig. 1a,b; Supplementary Table 2), which is similar to the

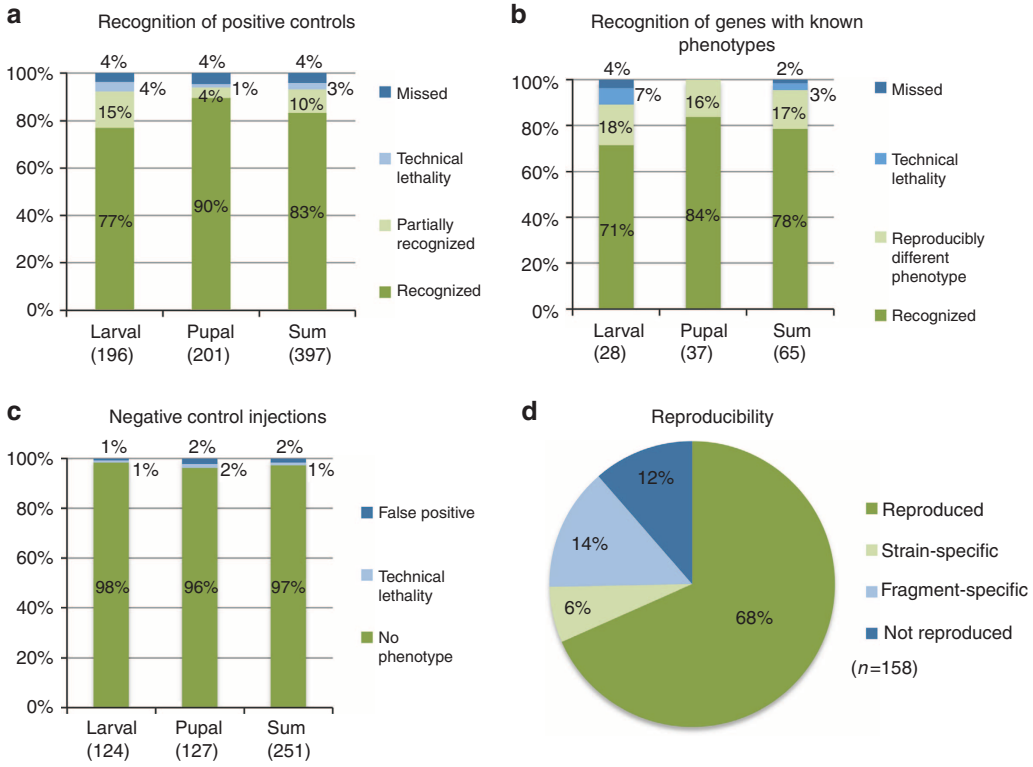

Figure 1 | Sensitivity and reproducibility. (**a**) Recognition rates of 41 different positive controls shown separately for the larval and pupal injection screens (left and middle bars) and for both together (right bar). About 80% of the positive controls were fully recognized while another 10% were 'partially recognized' (that is, not all phenotypic aspects were annotated). Only 4% of the positive controls were missed. 'technical lethality': Expected phenotype not recognized owing to lethality of the animals for example, by injection. (**b**) Recognition rates for dsRNAs targeting 48 genes with published phenotypes, which had by chance been included in the screen. Of all, 78% were recognized with the published phenotype while 17% were annotated with a 'reproducibly different phenotype'; that is, the differing phenotype was reproduced in independent experiments under iBeetle conditions. Hence, these different phenotypes are biologically meaningful and reflect that the timing and the degree of gene knockdown influences the phenotype. See Supplementary Note 1 for discussion of these cases. (**c**) Only 2% of all buffer injections led to false positive annotations. (**d**) A total of 158 dsRNAs were tested in independent injections with non-overlapping fragments. When the phenotype differed from the screening result, we analysed whether it was a false positive ('not reproduced'), or whether the genetic background was the reason for the difference (strain specific). Finally, we tested whether the outcome depended on the dsRNA fragment used (fragment specific), which indicated off-target effects or splice variant specific knockdown.

findings in a genome-wide RNAi screen in *C. elegans*[11]. Interestingly, we found reproducibly different or additional phenotypes for 17% of the published genes, which likely reflects the dependence of the RNAi effect on injection time and dsRNA concentration (see Supplementary Note 1). As expected, negative control injections usually produced no phenotype (Fig. 1c; Supplementary Note 1; Supplementary Fig. 2).

To test for reproducibility, we repeated the screening procedure for 158 genes with high penetrance phenotypes (Fig. 1d; see Supplementary Data 1 for details). Seventy-four per cent of the phenotypes were reproduced (Fig. 1d). Notably, 6% of those phenotypes turned out to depend on the genetic background. This is in line with the emerging view that strain specificity of phenotypes may be more prevalent than appreciated previously[41,42]. Twelve per cent of the re-tested phenotypes turned out to be false positives, while 14% could be reproduced by the original dsRNA fragment but not with a non-overlapping fragment. The latter finding probably reflects off-target effects but in some cases may reflect biologically meaningful differences due to the isoform specific nature of RNAi knockdown in *Tribolium*[43]. Overall, the proportion of off-target effects using systemic RNAi with long dsRNA fragments in *Tribolium* was similar to that observed with short transgenic hairpin constructs in *Drosophila*, where 13% ($n=9$) of the constructs induced unexpected lethality[44], but it was significantly lower than the 28% ($n=18$) or 24% ($n=65$) found with long hairpin constructs[8,45]. Considering 12% of 'not reproduced' phenotypes and a maximum of 14% off-target effects, the maximum false positive rate in the iBeetle screen is 26%. Hence, the phenotypes detected in our first pass screen need to be confirmed by a second assay using non-overlapping fragments.

Reproducibility highly depended on the biological process scored. For lethality or wing blister phenotypes the reproducibility was >95%, while for L1 cuticle phenotypes it was about 60% (see Supplementary Fig. 3 for numbers for different phenotype classes).

To test in how far we missed the strong phenotypes due to incomplete knockdown, we injected dsRNAs targeting 98 genes at concentrations of 1 and 3 $\mu g\,\mu l^{-1}$, and compared the phenotypic quality and strength. We found that phenotype strength was comparable for 86% of these genes.

Essential genes identified in the larval and pupal screens. Of the 3,400 genes tested in both the larval and pupal screens, 56.3% gave any phenotype (Fig. 2a), with 49.6% being lethal for at least one developmental stage (Fig. 2b). In all, 22.9% of the genes displayed a phenotype in both screens, while almost twice as many genes showed a phenotype exclusively in the pupal screen (21.1%) compared with phenotypes restricted to the larval screen

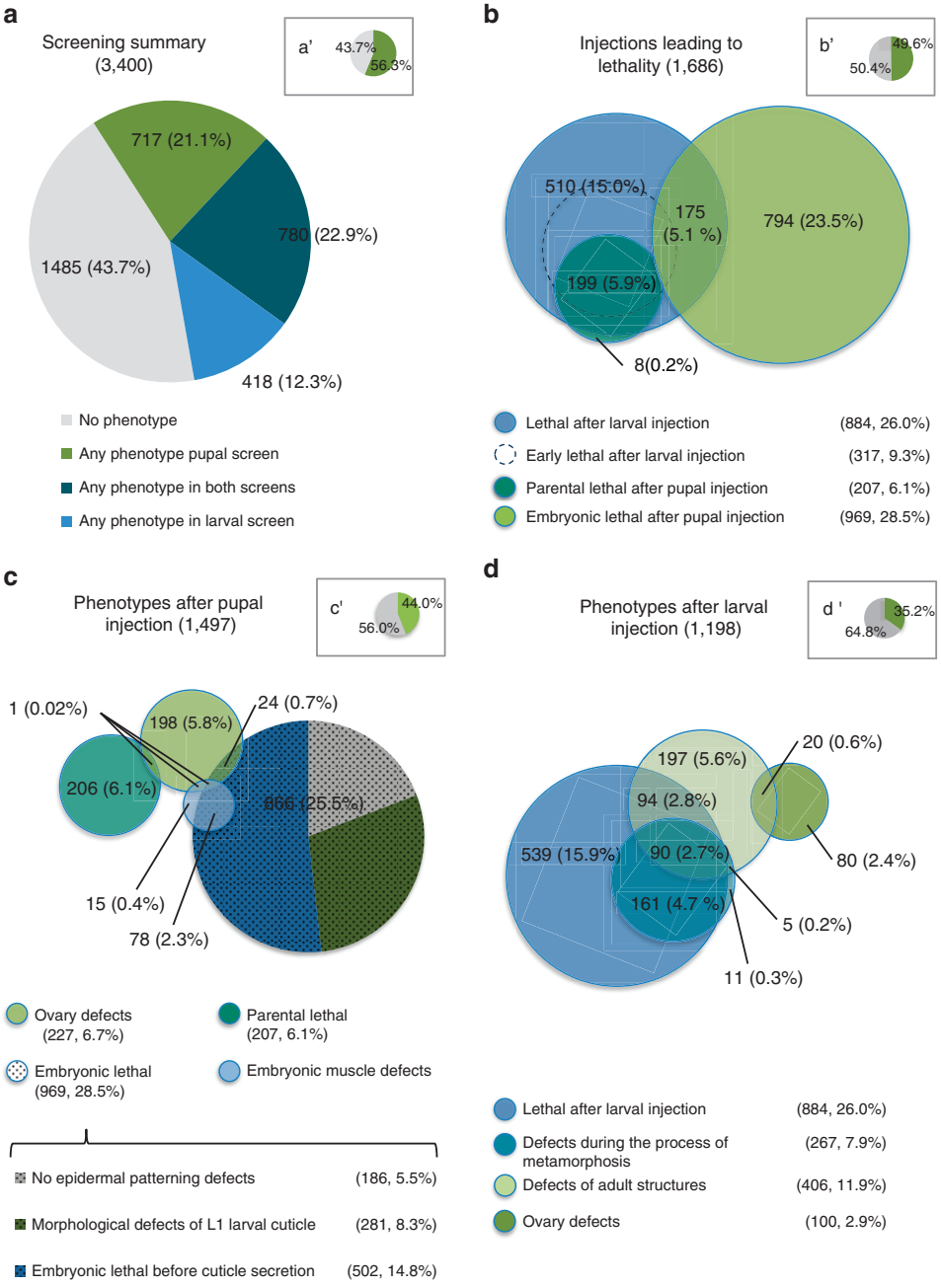

Figure 2 | Essential and lethal genes. (**a**) For more than 56% of the injected genes, phenotypes were observed. The pupal injection screen revealed phenotypes for a larger portion of genes compared with the larval injection screen. (**b**) Death of the injected animals was scored 22 days post injection (larval injection; blue circle) and 11 days post injection (pupal and larval injection; dark green and hatched blue circles). Note that embryonic lethality is based on maternal and zygotic gene knockdown. 'Parental lethal': death of the injected animal. (**c**) Selected phenotypic categories after pupal injection. Embryonic lethal injections are further categorized showing that more than half of the embryonic lethal genes lead to abortion of embryogenesis before cuticle secretion. (**d**) Phenotypic categories after larval injection. 'Defects during the process of metamorphosis': metamorphosis not completed or entered precociously. Insets: relations to the entire data set.

(12.3%; Fig. 2a). Thirteen per cent of the genes showing any phenotype (including lethality) were beetle specific genes (Supplementary Table 1), illustrating the importance of screens in additional model organisms (see Supplementary Table 3 for definition of phenotype classes and Supplementary Data 2 for lists of treatments in the respective classes).

Basically, all genes required for survival to adulthood after pupal injection (5.9% of all genes; Fig. 2b) were also required after larval injection, likely due to housekeeping functions. The set of larval lethal genes appeared to be much larger (26% of all genes; Fig. 2b). However, this difference is in large parts due to low-penetrance lethal phenotypes, which were more likely to reach the threshold in the larval screen, where lethality was checked 22 days post injection instead of 11 days post injection in the pupal screen.

Embryonic lethality was found for 28.5% of all genes following knockdown of maternal and zygotic gene function (Fig. 2b,c). Altogether, 5.5% did not show obvious cuticle defects, while 8.3% displayed cuticle aberrations (Fig. 2c); 14.8% of all genes did not develop a cuticle, leading to a so called 'empty egg phenotype'.

This phenotypic class comprises genes with diverse essential functions, including housekeeping, cuticle formation itself or fertilization; however, some early patterning genes are also known to result in death before cuticle formation in *Tribolium*[46–48]. Further analyses using molecular markers are required to determine which process was affected for a given empty egg phenotype. Interestingly, 5.6% of all genes in the larval injection screen led to alterations of adult morphology without affecting larval survival or fertility, making them interesting candidates for understanding how adult morphologies develop and how they evolved (Fig. 2d).

Comparison of embryonic and postembryonic patterning. For the first time our data allow the systematic comparison of the gene sets required for embryonic and postembryonic development in an insect with typical metamorphosis. Importantly, larval cells are largely re-used to form the adult epidermis in most insects, instead of being replaced by imaginal cells as is the case in *Drosophila*[24]. Nevertheless, the gene sets involved in embryonic and postembryonic patterning turned out to be largely non-overlapping (Fig. 3a). This is true for processes as different as leg or muscle development (Fig. 3b,c). In the case of oogenesis, the respective numbers are probably an overestimation because most genes leading to reduced egg production in the pupal screen were lethal in the larval screen (Fig. 3d). We assume that many of these apparent oogenesis phenotypes reflect incomplete knockdown of genes with basic physiological function because we found that many animals with reduced oogenesis showed a strongly reduced fat body. Subtracting these genes (those outside the dashed line in Fig. 3d), the overlap of genes with defects in both screening parts increases substantially. Together, these data reveal that development during typical insect metamorphosis partially relies on different mechanisms than during embryogenesis.

Essential genes of *Tribolium* and *Drosophila*. The classical genetic screens for embryonic phenotypes in *Drosophila* revealed that about 5,000 genes were lethal when mutated (36% of the *Drosophila* protein coding genes), and for an additional 1,000

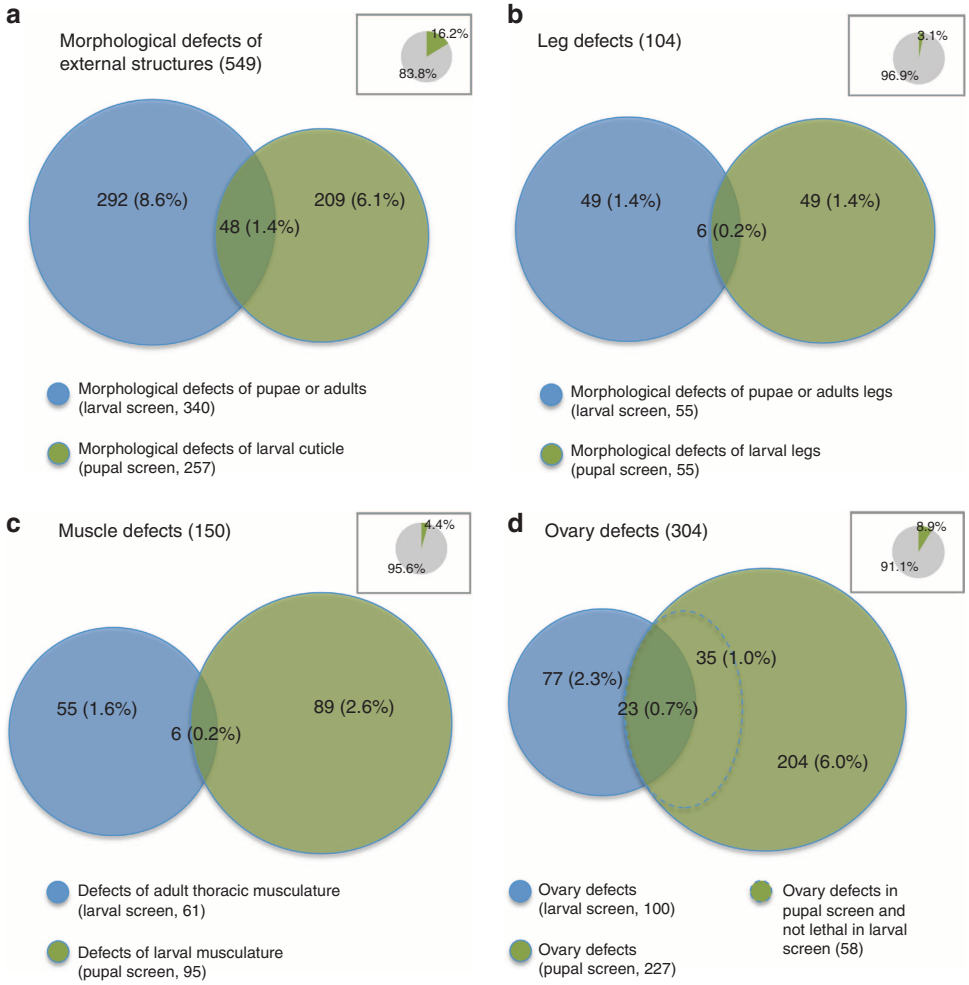

Figure 3 | Comparison of gene sets involved in embryonic versus postembryonic development. (**a**) The gene sets required for cuticle morphology (that is, epidermal patterning) during embryogenesis and typical insect metamorphosis are largely non-overlapping. This indicates that patterning principles may differ to quite some extent between these two stages of major morphological change. (**b**,**c**) This observation also holds true for the subsets affecting leg morphology (**b**) and GFP marked somatic muscles (**c**) indicating that both ectodermal and other patterning processes differ. (**d**) Gene sets required for ovary function. Many genes required for egg production in the pupal injection screen (green circle) were lethal in the larval injection screen. Hence, reduced egg production for these genes was probably due to starvation (green area outside hatched line). When comparing the non-lethal treatments (blue circle and green circle with hatched blue outline) the number of genes with an ovary phenotype in the pupal and larval injection screen are more similar. Insets: relations to the entire data set.

genes non-lethal phenotypes were identified. Hence, mutations in 43% of all loci revealed a phenotype of some kind[49–51]. The respective numbers for *Tribolium* are very similar (37 and 42%; all numbers in this paragraph are corrected assuming 26% false positives; see Methods section for calculations). However, in the iBeetle screen a much larger portion of the lethal genes is embryonic lethal compared with the *Drosophila* genetic screens (58% versus 20%). This is mainly due to a much higher number of embryonic lethal genes that show cuticle defects in *Tribolium* (81% versus 15% in *Drosophila*)[49]. One likely reason for this increased lethality is that in our screen we knocked down both maternal and zygotic gene functions, while in most *Drosophila* genetic screens only the maternal or zygotic contribution was affected[52]. As a consequence, in many cases zygotic functions were not detected because maternal contribution of gene products rescued zygotic mutations throughout *Drosophila* embryogenesis, leading to death after cuticle formation.

Overcoming the candidate gene approach. Most *Drosophila* segmentation genes have already been tested in *Tribolium*, revealing that a different gene set is involved in axis formation[46,53,54]. Overcoming this exhausted candidate gene approach, the iBeetle screen indeed identified novel players. For instance, knockdown of the homeobox gene *Tc-homeobrain* elicited a mirror image duplication of the abdomen similar to the *Drosophila* bicaudal phenotype and was found to be one of the earliest anteriorly expressed zygotic genes (Fig. 4a,e,f). No cuticle phenotype has been described for *Drosophila homeobrain,* and so far no *Tribolium* gene had been found to elicit a bicaudal phenotype. *SoxNeuro* is required for *Drosophila* central nervous system development[55,56], but was not reported to be involved in cuticle patterning. In the iBeetle screen, *Tc-SoxNeuro* knockdown resulted in a dorsalized phenotype, suggesting that *Tc-SoxNeuro* influences early dorsoventral patterning (Fig. 4c). Another example is *Drosophila Dscam,* which is a cell adhesion gene with extensive alternative splicing and which is known to act in neurogenesis and immunity[57,58]. The iBeetle screen identified potential additional essential roles of *Tc-Dscam* in sensory organ formation (Fig. 4d).

New fields of research. Odoriferous stink glands play crucial roles in insect defence and communication but are not present in *Drosophila*[59,60]. In the iBeetle screen, we identified 32 genes with relevant phenotypes, including the absence of the gland contents, altered colour and composition of secretions, or melanosis. Interestingly, only 5 among these 32 genes showed an enrichment of greater than fourfold in odoriferous gland transcriptomes compared with mid-abdominal tissues[60], illustrating that a phenotypic screen can only partially be replaced by transcriptomic approaches (see Supplementary

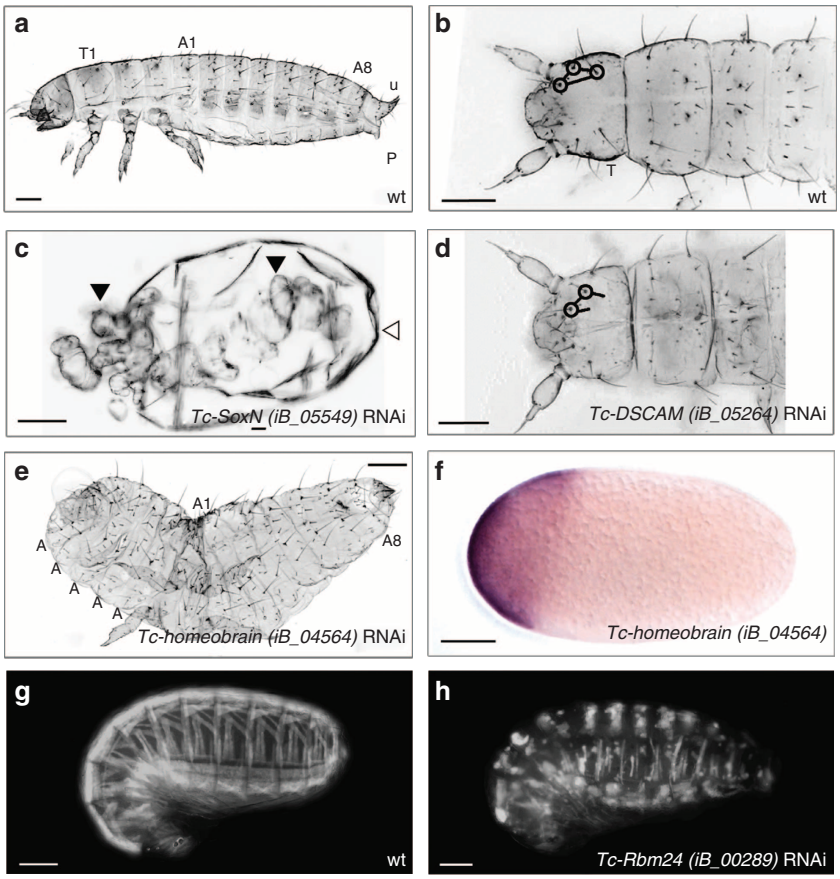

Figure 4 | Embryonic phenotypes. (**a**,**b**) Wild-type L1 cuticles, with head setae marked by circles (**b**). T1: first thoracic segment; A1: first abdominal segment; A8: eighth abdominal segment; U: urogomphi; P: pygopods. (**c**) Unexpectedly, *Tc-SoxN* RNAi led to a strongly dorsalized cuticle phenotype without clear axes (embryo: filled arrowheads; vitelline membrane: open arrowhead). (**d**) *Tc-DSCAM* RNAi induced the deletion of head setae. (**e**) *Tc-homeobrain* RNAi caused a bicaudal phenotype (mirror image abdomina). This function is not known from *Drosophila homeobrain* and in *Tribolium* no bicaudal phenotype has been described before. (**f**) Early anterior zygotic expression of *Tc-homeobrain*. (**g**,**h**) *Tc-Rbm24* RNAi led to detached and shortened body wall muscles (wild type pattern in **g**). A muscle function is conserved in vertebrates while the ortholog was lost in *Drosophila*. Scale bars indicate 100 μm.

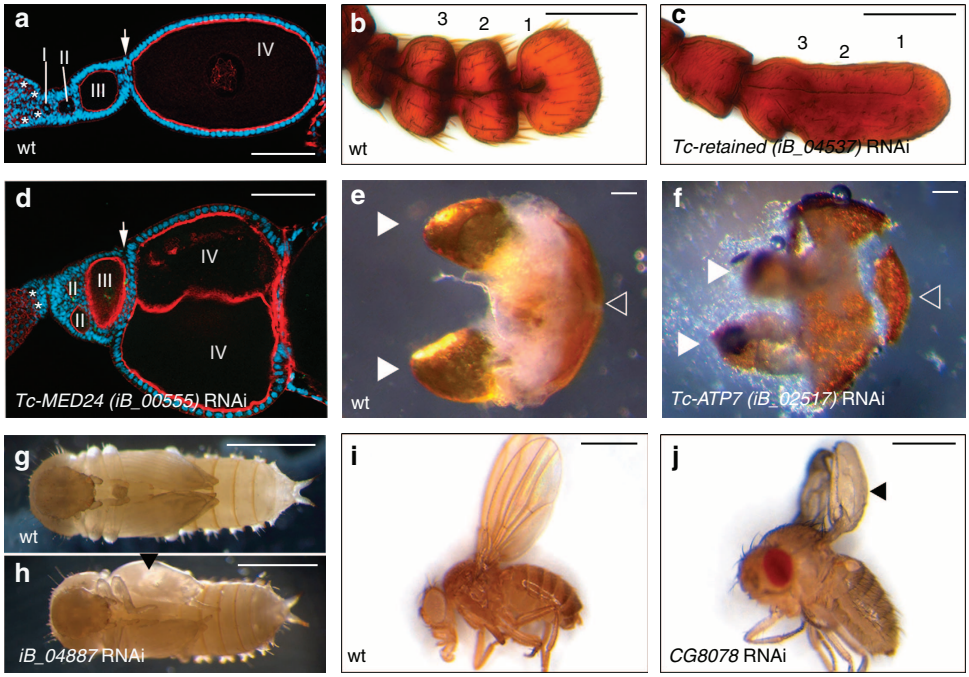

Figure 5 | Postembryonic phenotypes. (**a**) Wild-type ovary stained for F-actin (red) and DNA (blue). Pro-oocytes (asterisks) become encapsulated by somatic follicle cells and separated by stalk cells (arrow). (**d**) Upon *Tc-MED24* RNAi, egg chambers are misarranged, not separated by stalk cells (arrow) and subsequently they fuse (IV). (**b**,**c**) After *Tc-retained*-RNAi the three most distal antennomeres (1–3) of the adult antenna are fused. (**e**,**f**) RNAi against *Tc-ATP7* led to strongly reduced odoriferous gland content and partially melanized secretions (white arrowheads; remnant of posterior abdominal cuticle marked by open arrowhead:). (**g**–**j**) The knockdown of *iB_04887* led to wing blisters in *Tribolium* pupae (arrowhead in **h**). Transgenic RNAi against the *Drosophila* ortholog showed the same phenotype, revealing a novel candidate for integrin mediated adhesion (arrowhead in **j**). Scale bars indicate 100 μm in (**a**–**f**) and 1 mm in (**g**–**j**).

Fig. 4). For instance, the *Tc-copper-transporting-ATPase-I* (*Tc-ATP7*) is neither upregulated nor downregulated (Supplementary Fig. 5) but RNAi mediated knockdown (iB_02517) caused a reduced gland content and melanosis phenotype (Fig. 5f) and a loss of benzoquinones (Supplementary Fig. 6). Another emerging field is the shaping of the adult body during typical holometabolous insect metamorphosis were larval epidermal cells are reused[24]. For instance, *Tc-retained* led to rounded female genitalia and the fusion of distal antennal segments (Fig. 5c). Interestingly, these anatomical features vary in Tenebrionids[61], making this gene a good candidate for morphological evolutionary studies. Finally, one difference between *Tribolium* telotrophic oogenesis and *Drosophila* meroistic oogenesis is that in *Tribolium* germ line stem cells stop proliferating at larval stages while the somatic stem cell lineage remains active throughout life[23]. In *Drosophila*, both lineages remain active and dependent on each other, making it difficult to study the somatic lineage independently. In the iBeetle screen we identified several genes probably required for the somatic lineage, such as *Tc-MED24* whose knockdown led to incomplete separation of egg chambers and a reduced number of follicle cells (Fig. 5d).

***Tribolium* as a screening platform**. Wing blisters indicate the loss of adhesion between the two epithelial sheets of the wing blade. This phenotype has been used in *Drosophila* to identify components of integrin mediated adhesion[62,63]. We found 49 genes associated with wing blisters in our screen. Thirty-four were re-tested with non-overlapping fragments. All were confirmed in the screening strain but one led to lethality in another genetic background. Seventeen of these were previously annotated with GO terms connected to cytoskeletal function or adhesion, but 14 had unrelated functional annotations and 5 did not have any functional annotation at all[64]. We tested 19 genes in *Drosophila* by transgenic RNAi knockdown, using two different wing disc driver lines. Out of seven genes without previous annotations with respect to cell adhesion or cytoskeletal function in *Drosophila*, four uncovered a wing blister or 'crumbled wing' phenotype, indicating an involvement in *Drosophila* epithelial adhesion (See Supplementary Table 4). One example is the *Tribolium* ortholog of CG8078, which is predicted to be involved in tRNA thio-modification and was not implicated in cell adhesion before (Fig. 5h,j). Notably, some wing blister genes known from *Drosophila* were not recovered in the iBeetle screen owing to larval lethality before wing development (see Supplementary Table 5). Therefore, we injected at a later stage (L7) and indeed, two additional genes showed the wing blister phenotype. Hence, a new screen focused on injections at later larval stages is likely to reveal additional wing blister genes. In summary, the *Drosophila* and *Tribolium* screening platforms appear to reveal different subsets of genes involved in a common cellular process, suggesting that the use of *Tribolium* as alternative screening platform may reveal novel players relevant for general insect biology.

We identified many known and novel genes required for muscle development. In *Tc-Rbm24* RNAi embryos, for instance, the muscles form small, round syncytia, which eventually seem to decay (Fig. 4h). The gene codes for an RNA binding protein of the RRM superfamily. Of note, it lacks an ortholog in *Drosophila* but vertebrate orthologs are active in myogenesis[65,66]. Hence, our data show the conservation of myogenic function of *Rbm24* in Bilateria.

Discussion

Here we showed that the iBeetle RNAi screen achieved efficient and sensitive detection of novel gene functions at four developmental stages and in several processes in the red flour

beetle *Tribolium*. The reproducibility varied depending on the phenotypic class. Highly penetrant phenotypes with simple readout (for example, wing blister, lethality) reproduced with more than 95% while the reproduction rate of embryonic developmental defects was significantly lower (about 60%). One reason is that iBeetle was designed as first pass screen without replicates while in cell based RNAi screens replicates and different reagents targeting the same gene function are usually used to minimize the portion of false positive data. Another important reason is that in *Tribolium* moderate knockdown of housekeeping genes during embryogenesis leads to abortion of embryogenesis at different stages leading to diverse cuticle defects. Hence, any off-target effect affecting a housekeeping gene is likely to result in diverse cuticle defects. Hence, it is advisable to focus on phenotypes annotated with high penetrance ($>50\%$) and confirmation of phenotypes using non-overlapping dsRNA fragments is essential.

Our data show that a significant portion of insect gene functions becomes apparent when using additional model systems for several reasons. First, some aspects of insect biology are more representative in *Tribolium* compared with *Drosophila* like for instance metamorphosis based on the re-use of larval epidermal cells. Indeed, we identify novel genes associated with this process, the orthologs of which might have lost their function in *Drosophila* due to its derived mode of metamorphosis. Second, the different characteristics of the screening procedures may lead to detection of different subsets of the gene set involved in a conserved process. For instance, we identified novel genes leading to a wing blister phenotype but not all genes known from *Drosophila* were recovered in our screen. This suggests that neither system appears to have the power of detecting the comprehensive gene set of a given process. Finally, 13% of the genes associated with phenotypes in our screen do not have orthologs in *Drosophila* and therefore need to be studied in other model systems.

In summary, the iBeetle screen helps to overcome several current limitations in insect functional genetics. First, the candidate gene approach prevailing in emerging model organisms cannot reveal novel gene functions. This is superseded by the hypothesis independent identification of genes. Second, new biological processes can now be investigated, the genetic bases of which remained obscure because they are too derived or not present in the fly, or not amenable in the fly due to technical constraints. Third, we show that *Tribolium* is a powerful complementary screening platform for basic processes that are being studied in other organisms, such as epithelial adhesion. Importantly, the dsRNA template library generated in this project facilitates future screens focused on additional topics. Taken together, the iBeetle screen aids in bridging the gap between large-scale gene discovery by next generation sequencing and the small-scale approaches used for uncovering the function of novel genes.

Methods

Selection of genes and dsRNA production. The genes to be knocked down were selected at random. However, we required that the predictions were well supported by cDNA sequence data. Further, we excluded genes close to neighbouring same-strand gene predictions to avoid the double injection of genes, which were erroneously annotated as two genes. As the coverage by RNA-seq was low at project start, the gene set may have been enriched in genes expressed above average. This may explain the higher portion of conserved genes in the set of screened genes (82% in the iBeetle screen versus 59% in the official gene set; see Supplementary Table 1).

The target transcript sequences were analysed using the DEQOR algorithm to identify stretches with highest amount of sequences predicted to be efficiently recognized by the RNAi machinery and the lowest amount of potential off-target sites. Fragments within these stretches were amplified by PCR with gene-specific primers, which were tagged with parts of the T7 (3′ primer) and SP6 (5′ primer) promoter sequences (T7 tag 5′-CTCACTATAGGGAGA-3′; SP6 tag 5′-TGACAC TATAGAAGTG-3′). The sequences are available at the iBeetle-Base (http://ibeetle-base.uni-goettingen.de/) and in Supplementary Data 3. The products from these PCR reactions were used as templates in a second PCR using T7 and SP6-T7 primers to generate templates for bidirectional *in vitro* transcription using T7-RNA polymerase. (Note that due to the different tags on 5′ and 3′ end of the fragments, the iBeetle library can be used for the generation of *in situ* probes as well.) For quality control, all products of the second PCR were checked on a gel and sequenced. All dsRNAs were assessed for purity by gel electrophoresis, the concentration was measured by the Ribogreen Assay (Life Technologies), and adjusted to $1\,\mu g\,\mu l^{-1}$ using injection buffer. Template production was performed by Eupheria Biotech GmbH (Dresden). Overall, 7,200 *in silico* defined templates were channelled into the pipeline leading to the production of 6,147 templates of which 5,670 dsRNAs were produced and sent to the screening centres. The success rate was about 85% for template generation and 92% for dsRNA production, resulting in an overall success rate of about 79%. The average length of the templates was 479 bp.

The screening procedure. Animals for 24 experiments were reared and processed in parallel using the equipment developed by Berghammer *et al.*[67]. For the injections two transgenic lines were used, where EGFP enhancer traps marked larval (*pig19*) or adult muscles (*D17*) in the pupal and larval injection screen, respectively (see Supplementary Fig. 7). An X-linked transgene insertion (*Xred*, expressing DsRed in larval eyes) was used for sexing larvae. dsRNA solution in the volume $1\,\mu g\,\mu l^{-1}$ was injected into 10 animals per experiment using a FemtoJet express device (Eppendorf). It was injected as much as possible without interfering with survival. For injection and morphological inspection, larvae and adult beetles were anaesthetized with ice or carbon dioxide. Pupae were affixed to microscope slides for injection using a double sided sticky tape or rubber based cement (Fixogum). Inspection of pupae and adult morphology and phenotype documentation was performed with epifluorescence stereomicroscopes (Leica M205 FA). The data were documented electronically during analysis using an online interface. The interface allowed the documentation of technical remarks and offered dropdown lists with controlled vocabularies for documentation. In addition, pictures were uploaded for the documentation of the annotations and remained linked to the respective annotation. This allowed displaying the relevant pictures in the search results. For analysis of embryonic muscles, living embryos were dechorionated, embedded in Halocarbon oil (Voltalef 10S) and analysed using upright fluorescence microscopes (Zeiss, Jena). Fourty-one dsRNAs targeting genes with known phenotypes were randomly introduced as positive controls with a frequency of 1 or 2 per 24 injections. The first and last of the 24 injections performed on 1 day were negative control injections (buffer). The throughput of the screen was 21 and 28 genes per week in the pupal and larval injection screen, respectively. The workflow is shown in Supplementary Table 6; the schedules are shown in Supplementary Figs 8 and 9. All phenotypes shown in this paper were reproduced with non-overlapping fragments using the SB strain as a different genetic background (see Supplementary Table 7 for sequences). The data are available at http://ibeetle-base.uni-goettingen.de/[40].

GC–MS of odoriferous stink gland contents. dsRNA was injected in animals at mid-pupa stage (SB strain). Injected pupae as well as uninjected control pupae were kept on whole-grain flour at 32 °C. Ten days after hatching both the prothoracic and the abdominal glands of one beetle were dissected and crushed pairwise in 50 µl methanol (Merck Millipore: SupraSolv). The samples were stored at −20 °C and subjected to gas chromatography mass spectrometry (GC–MS) measurements within 48 h. A volume of 1 µl was loaded per sample by a split injector into a gas chromatograph (Agilent Technologies 6890N Network GC System) connected to a mass spectrometer (Agilent Technologies 5973 Network Mass Selective Detector). For chromatogram analysis the software MSD ChemStation D.02.00.275 (Agilent Technologies) was used.

Comparison of essential genes. The portions of essential (that is, lethal) genes in *Drosophila* that had been published previously vary with the assumed total number of genes at the time. For our comparison, we related the published absolute numbers of essential genes to the number of protein coding genes of the current *Drosophila* genome release (6.02). For *Tribolium* we reduced the numbers observed in the iBeetle screen by the estimated portion of false positives in our data set (26%; see above). See Supplementary Table 8 for numbers and calculations.

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Author contributions

C.S.-E., U.M., J.S., D.S., N.S., N.T. and V.A.D. have contributed the vast bulk of the data: they performed the screening procedure, analysed and documented the data, annotated the phenotypes and contributed data to the NOF analysis. D.G. and T.R. performed the screening procedure, analysed and documented the data, annotated the phenotypes. C.S.-E., N.D.B.K., M.S., M.K. and G.B. developed the screening procedure. M.T., J.D. and B.M. developed the database collaborating with C.S.-E. and G.B. L.G. and M.S. defined the transcripts for template production. M.T. and F.B. defined and produced the iB templates and produced the dsRNAs. I.S., E.K., J.T., S.K. helped with different aspects of the screen. S.L., J.U., J.S., Y.H., K.A.P. and R.S. contributed to the NOF analysis. K.A.P., R.S., M.F., S.R., E.A.W. were involved in the design of the study and the analysis of the data. C.S.-E., M.K., M.S. and G.B. designed the study, led the screen, headed the analysis and wrote the paper

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: M.T. and F.B. are affiliated with Eupheria Biotech GmbH. All other authors declare no competing financial interests.

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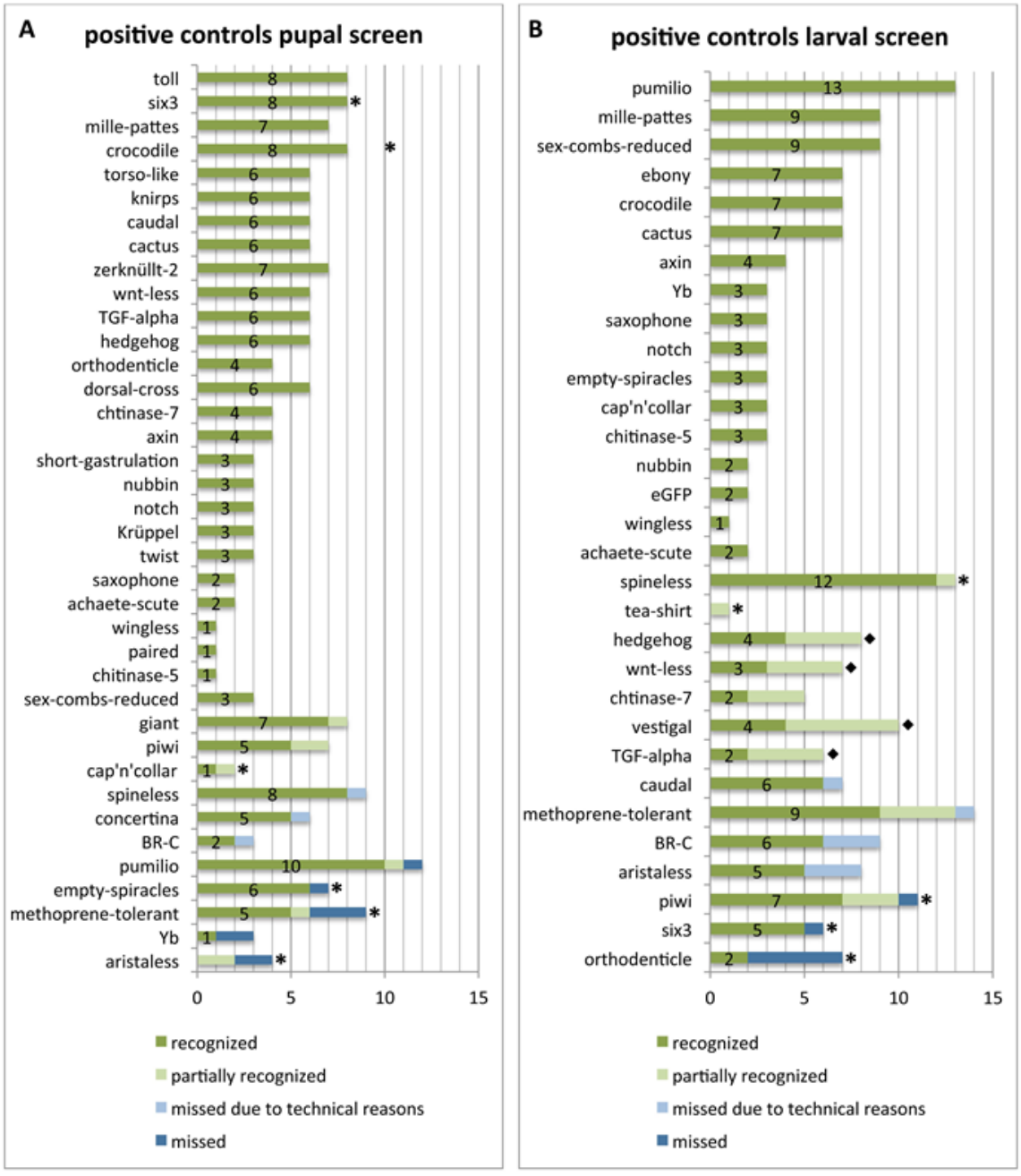
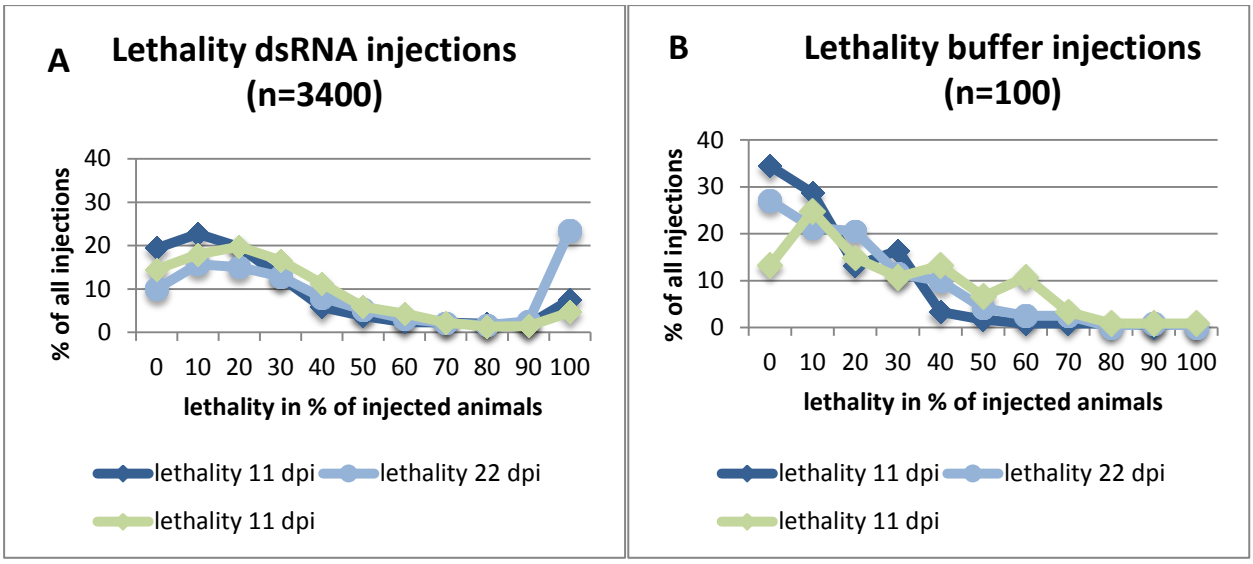


Supplementary Figure 1 | Recognition of positive controls

The length of each bar indicates how often the respective control was used in the screen, and the color code indicates how often it was fully (dark green) or partially identified (light green), and how often it was missed (blue colors).

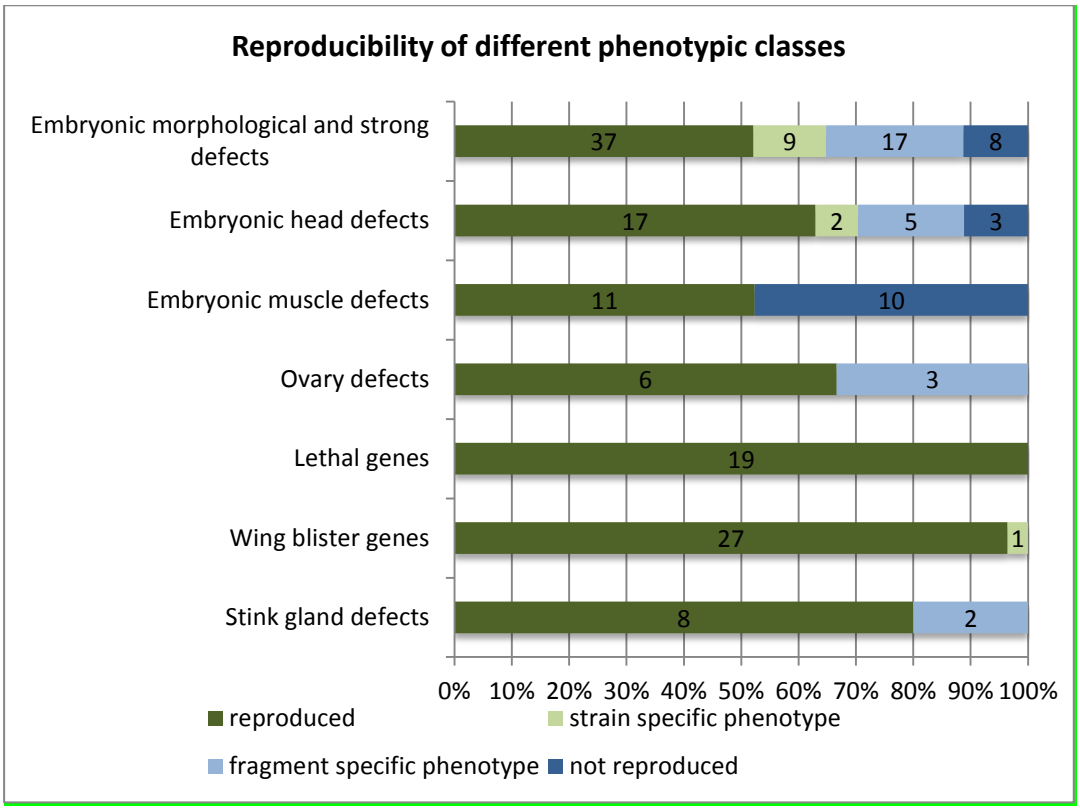
Asterisks indicate controls with a very subtle phenotype. Black diamonds indicate positive controls with complex phenotypes, which were rated as “partially recognized” when three quarter or more of phenotypic aspects were correctly identified.



Supplementary Figure 2 | Lethality of injected animals after dsRNA and buffer injections in the pupal and the larval injection screens

A) Shown is the distribution of lethality rates of the injected animals in RNAi experiments. Lethality was documented at 11 and 22 days post injection (dpi) in the larval injection screen (dark and light blue) and at 11 dpi in the pupal injection screen (green). Most experiments showed a lethality of up to 30%. The distribution dropped up to 80% but increased again from 90% onwards. B) The same distributions shown for buffer injections. Here, the 90 and 100% values were not increased. Taken together, lethality rates below 80% were most likely “technical lethality” while higher lethality rates were probably the consequence of RNAi targeting an essential gene. Hence, we considered “lethality” as a phenotype only when at least 90% of the injected animals had died.

a



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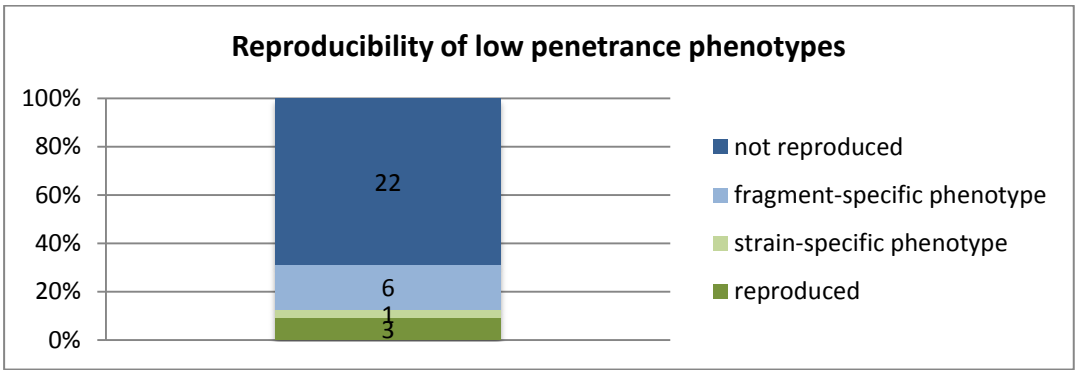
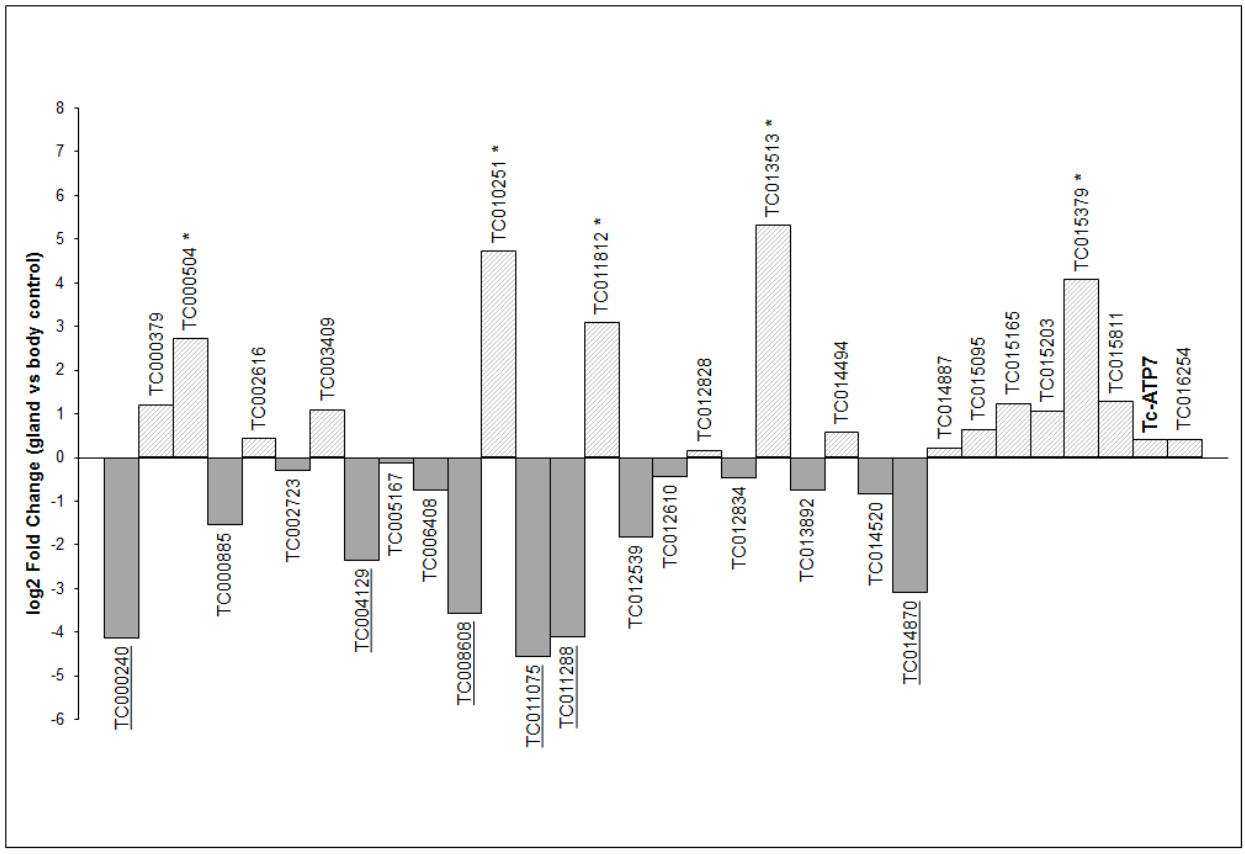


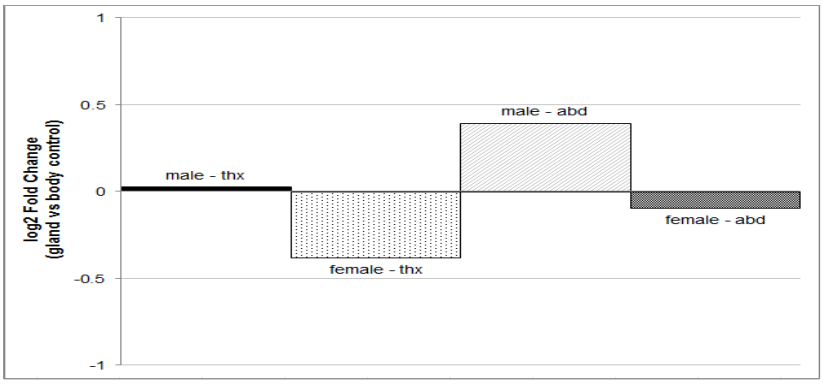
Supplementary Figure 3 | Reproducibility depends on the phenotype class

158 genes matching our criteria for significant phenotypes (see Supplementary Table 3) were tested for reproducibility. Where possible, a non-overlapping fragment was injected into a strain with different genetic background (SB in most cases). If the phenotype was not reproduced, the non-overlapping fragment was injected into the strain used in the screen. This allowed distinguishing whether the non-reproduced phenotypes were due to fragment- or strain-specific differences (i.e. putative off target effects or genetic background effects). a) Phenotypes of different processes with a penetrance > 50% were tested. Some phenotype classes like lethality and wing blistering were reproduced with very high frequency while embryonic phenotypes were more frequently not reproduced (see main text for discussion on likely reason). b) Phenotypes that were annotated with a penetrance <50% were frequently not reproducible.



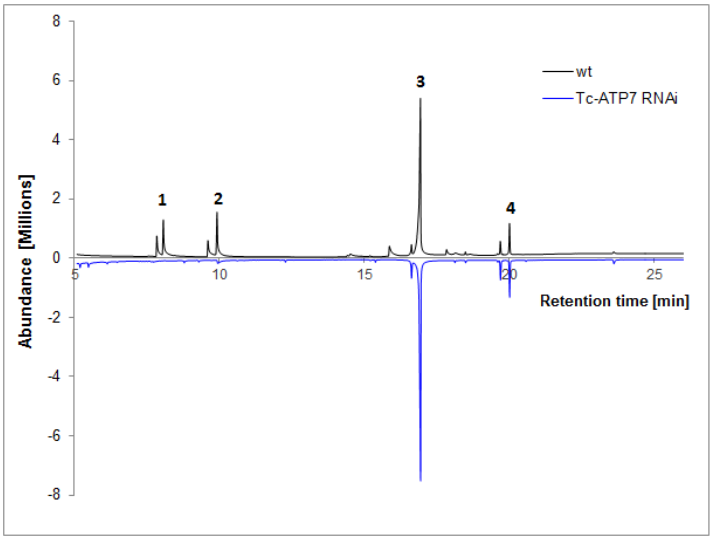
Supplementary Figure 4 | Differential expression of 32 iBeetle-identified gland phenotype-causing genes

We determined for the 32 genes with a confirmed odoriferous gland phenotype, whether the transcripts were enriched in the glands (data taken from Li et al. 2013)[1]. Each bar represents the level of expression of a gene in male abdominal glands relative to its expression in the mid-abdomen body control. Only five genes (marked with an asterisk) showed >4-fold enrichment in the odoriferous glands and would have been chosen as candidates based on a transcriptomics approach, thereby missing genes such as *Tc-ATP7* (bold) that is neither up nor down regulated but revealed a reduced gland content and melanosis phenotype upon knockdown. The iBeetle screen even identified genes causing a gland phenotype upon knock-down whose expression is strongly reduced in the odoriferous glands (underlined TC numbers). Thus, most genes detected in the iBeetle screen would not have been selected in an approach based on differential gene expression. Differences in the expression intensities are given as logarithm 2 fold change, calculated as log2 of the quotient [depth (gland) / depth (control)], where depth is calculated as number of reads multiplied with length of reads (38bp) divided by specific length of gene transcript.



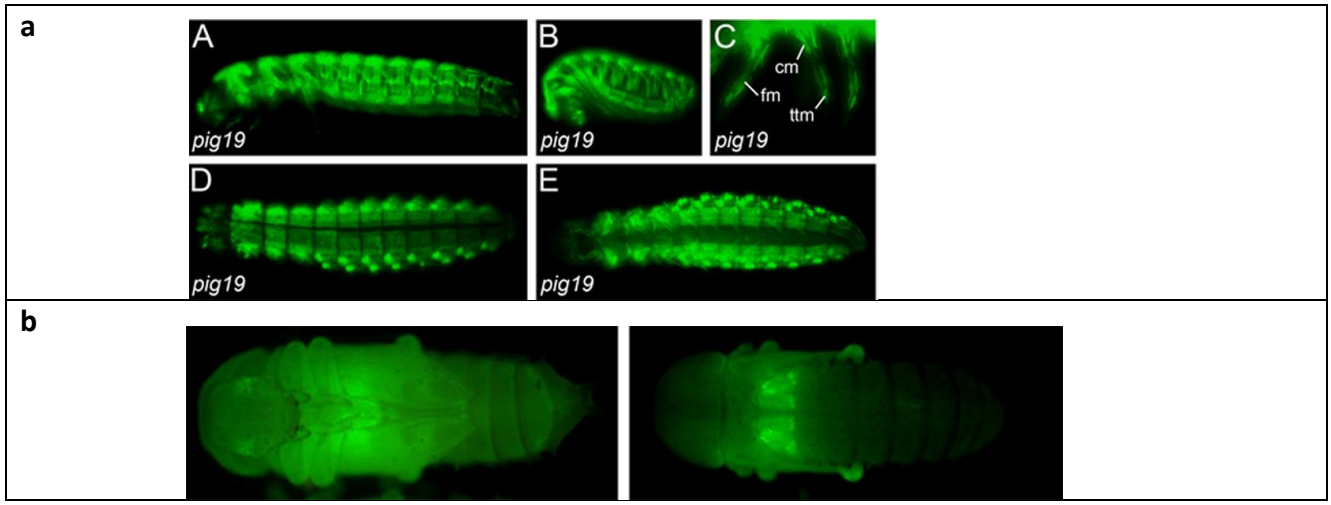
Supplementary Figure 5 | Differential expression of *Tc-ATP7* in different gland tissues

Expression of the copper-transporting ATPase *Tc-ATP7* in male and female thoracic (thx) and abdominal (abd) odoriferous glands is not significantly de- or increased relative to the expression in the mid-abdomen body control.



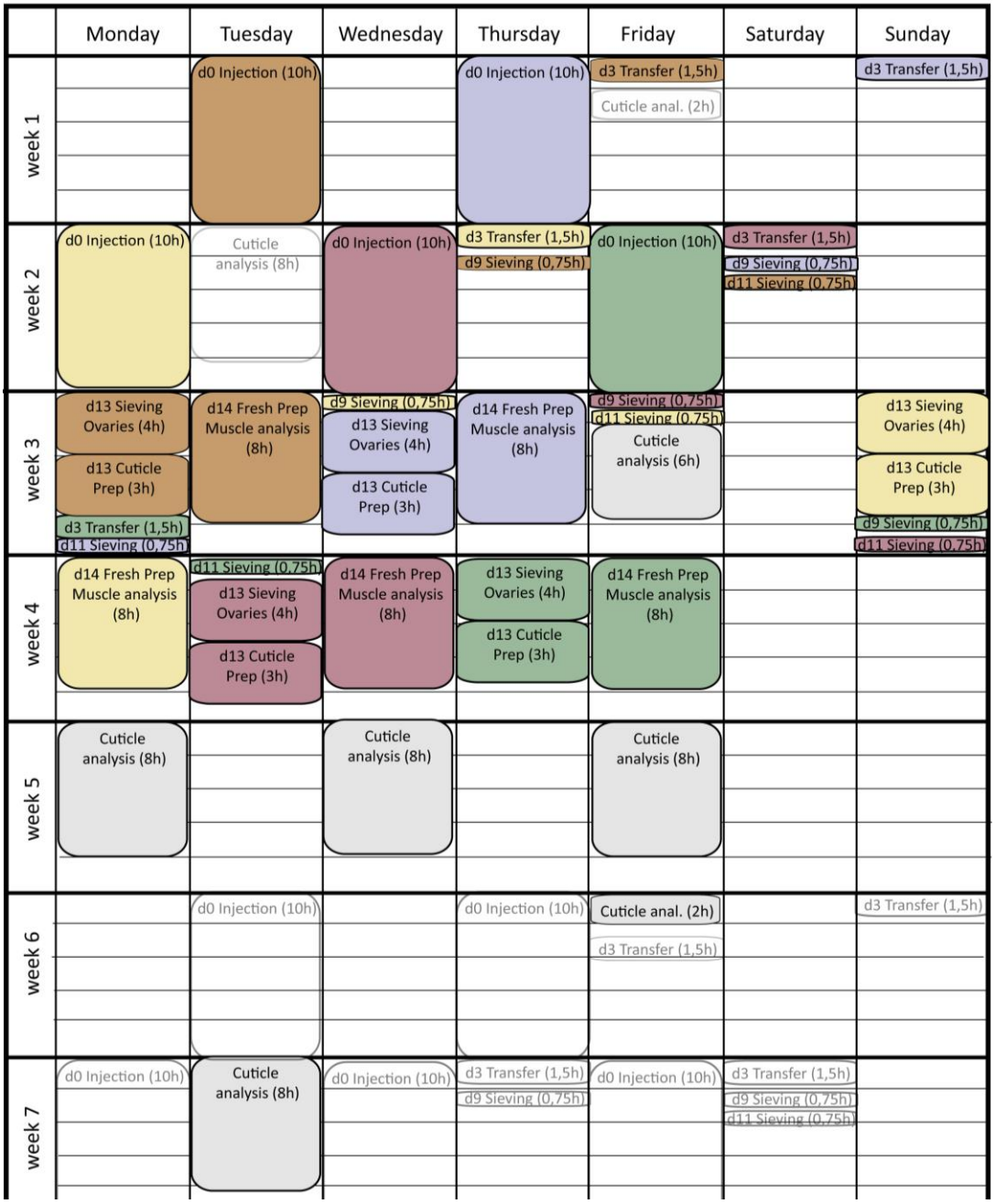
Supplementary Figure 6 | Gas chromatograms of stink gland contents.

The four main volatile substances detected via GC-MS in abdominal glands of wild type beetles (black line) are 1: 2-Methyl-1,4-benzoquinone; 2: 2-Ethyl-1,4-benzoquinone; 3: 1-Pentadecene; 4: 1-Heptadecene. After knockdown of Tc-ATP7 (blue line, for better comparison plotted as negative values) gland secretions lack the benzoquinones (peaks 1 and 2).



Supplementary Figure 7 | Enhancer trap lines used in the screen

a) Females carrying the *pig19* enhancer trap were used for pupal injections. In this enhancer trap line EGFP is expressed in somatic muscles including leg muscles. Upon maturation, injected female pupae were mated with male beetles of the *black* strain (dark cuticle)[2] in order to allow quick assessment of adult survival of injected females without the need for repeated adult sexing.
b) For larval injections we used daughters of a cross between pearl females [3] and males of the D17Xred strain. This line is carrying a Minos transposon [4] insertion (D17) containing the 3xP3-EGFP marker which has captured an adult flight muscle enhancer. In addition, a piggyBac transposon carrying DsRed coding sequence driven by a 6xP3-promoter was inserted on the X-chromosome. Hence, female larvae were identified as larvae expressing the DsRed fluorescent protein in the eyes and the brain. Injected females were crossed to black males.



Supplementary Figure 8 | Schedule of the pupal injection screen

The five repetitions performed in parallel are shown in different colours. Each step of each repetition is shown at the day of processing and the approximate time required for this step is given. After five weeks, the same schedule was repeated (open boxes in week 6 and 7). The cuticle analysis can be performed at any time, because the cuticle preparations are stable over time.

Supplementary Figure 9| Schedule of the larval injection screen

In the larval injection screen, eight repetitions were performed in parallel and the schedule repeated after 6 weeks.

Supplementary Table 1 | Comparison of iBeetle analysis set with official gene set

	Official gene set		iBeetle gene set		Genes with Phenotype in iBeetle		
	N	%	N'	%	N''	%	% of iBeetle genes with phenotype (N''/N')
Genes	16561	100	3400	100	1915	100	56
Conserved	9838	59	2784	82	1659	87	60
Beetle specific	6723	41	616	18	256	13	42
Conserved in *Drosphila*	8334	50	2496	73	1546	81	62
Lost in *Drosophila*	1505	9	288	8	113	6	39

"beetle specific": NCBI blast did reveal orthologs only in beetles;

"lost in *Drosophila"*: NCBI blast revealed orthologs in other insects but not *Drosophila*

Note: the percentage in the rightmost column relates the values in column N'' to respective values in column N', e.g. 39%=113/288.

Supplementary Table 2| iBeetle results for genes with published or known phenotypes

Pupal injection screen

gene	iB-#	TC-#	screening result	reference
achaete-scute	iB_04489	TC008433	loss of larval bristles and setae	Wheeler et al. 2003, this study
axin	iB_04108	TC006314	as expected	Fu et al. 2012
broad-complex	iB_03960	TC005474	no phenotype, potentially due to alternative splicing	this study
cactus	iB_00322	TC002003	as expected	Fonseca et al. 2008; Roth unpublished
CG16778	iB_04153	TC006481	muscle defects, differs from earlier experiments where defects in embryonic head and abdominal development were found	Schoppmeier, unpublished
Delta	iB_03691	TC004114	as expected	Aranda et al. 2007, Schoppmeier unpublished
decapentaplegic	iB_04497	TC008466	lethality of injected animals, differs from earlier experiments where strong embryonic defects, but no lethality were found	Van der Zee et al., 2006; Ober & Jockusch, 2006
dorsocross	iB_05219	TC012346	as expected, dorsal closure defect, appendage and abdominal defects	Panfilio unpublished, positive control in this study
EGF-Receptor	iB_00647	TC003986	as expected, oogenesis defects	Großmann, unpublished
folded gastrulation	iB_04203	TC006722	dorso-ventral patterning	Roth, unpublished
Frizzled-1	iB_02240	TC014055	early embryonic lethality, stronger defect than published	Beermann et al. 2011
glass-bottom-boat	iB_05543	TC014017	as expected, lipid homeostasis and sterility	Namigai & Suzuki 2012, Trauner unpublished
hairy	iB_05339	TC012851	as expected	Aranda et al. 2008
hunchback	iB_05451	TC013553	as expected	Marques-Souza et al. 2008
knirps	iB_03553	TC003413	early embryonic lethality, stronger defect than published	Cerny et al. 2008
lame-duck	iB_06061	TC030749	as expected, muscle defects	Frasch, unpublished
lim1	iB_05727	TC014939	early embryonic lethality, stronger defect than published	Posnien et al. 2011
methoprene-tolerant	iB_03648	TC003908	as expected, subtle leg defects	this study
mirror/irx	iB_03595	TC003634	as expected, oogenesis defects	Schoppmeier, unpublished
odd-skipped	iB_04013	TC005785	as expected	Choe et al. 2006
org-1	iB_05796	TC015327	as expected, muscle defects	Frasch, unpublished
patched	iB_03831	TC004745	as expected	Farzana et al. 2008

pelle	iB_02469	TC015365	as expected, strong embryonic defects	Roth, unpublished
porcupine	iB_03822	TC004714	as expected	Bolognesi et al. 2009
pumilio	iB_03898	TC005073	as expected	Schmitt-Engel et al. 2012
saxophone	iB_02534	TC015948	as expected, reduced fecundity	Roth, unpublished
sex-combs-reduced	iB_00186	TC000917	as expected, slightly stronger than published	Curtis et al. 2001; positive control in this study
sloppy-paired	iB_04421	TC008062	as expected	Choe et al. 2006, Choe et al. 2007
TGF-alpha	iB_03555	TC003429	as expected	Lynch et al. 2010; Roth unpublished
tolloid	iB_01822	TC011197	as expected	Fonseca et al. 2010
Torso	iB_04720	TC009906	as expected	Schoppmeier et al. 2005
torso-like	iB_04423	TC008090	as expected	Schoppmeier et al. 2005
twisted-gastrulation	iB_00592	TC003620	as expected	Fonseca et al. 2010
wingless	iB_05552	TC014084	as expected	Bolognesi et al. 2008
wnt-less	iB_00832	TC005345	as expected	Bolognesi et al. 2008
yb	iB_02707	TC000053	as expected	positive control in this study, Klingler unpublished
zfh2	iB_03646	TC003891	lethality of injected animals, differs from earlier experiments where defects of leg development, but no lethality were found	Prpic-Schäper, unpublished

Larval injection screen

gene	iB-#	TC-#	screening result	reference
achaete-scute	iB_04489	TC008433	as expected, lethality	positive control in this study
bric-a-brac	iB_03591	TC003621	just lethality, published subtle appendage phenotype was missed	Angelini et al. 2009, 2012
broad-complex	iB_03960	TC005474	as expected, slightly stronger	Konopova et al. 2008; Konopova, personal communication
cactus	iB_00322	TC002003	as expected	Fonseca et al. 2008; Roth unpublished
Delta	iB_03691	TC004114	larval lethality, stronger than published	Angelini et al. 2012a+b
decapentaplegic	iB_04497	TC008466	larval lethality, stronger than published	Knorr et al. 2009
ebony	iB_05139	TC011976	as expected	Park et al. 2005

EGF-receptor	iB_00647	TC003986	as expected, lethality	Großmann, unpublished
empty-spiracles	iB_05098	TC011763	wing and labrum defects, pupal lethality, differs from experiments in this study, where some lethality, but no morphological defect was found	positive control in this study
homothorax	iB_04526	TC008629	prepupal lethality, stronger than published	Angelini et al. 2012a+b
knickkopf	iB_04889	TC010653	as expected	Chaudhari et al. 2011
knirps	iB_03553	TC003413	prepupal lethality, differs from experiments in this study, where no phenotype was found, but earlier experiments showed similar defects	positive control in this study, Schmitt-Engel, unpublished
laccase 2	iB_01701	TC010489	larval lethality, stronger than published	Arakane et al. 2005
lim1	iB_05727	TC014939	as expected, slightly stronger	Angelini et al. 2012b
matrix metalloproteinase 1	iB_02266	TC014266	as expected	Knorr et al. 2009
mef2	iB_04920	TC010850	as expected, lethality	Frasch, unpublished
methoprene-tolerant	iB_03648	TC003908	larval lethality, technical artifact	Konopova et al. 2007; positive control in this study
odd-skipped	iB_04013	TC005785	just labial misorientation, weaker defect than published	Angelini et al. 2012
pumilio	iB_03898	TC005073	larval lethality, technical artifact	Schmitt-Engel, unpublished
serrate	iB_04764	TC010113	as expected, slightly stronger	Angelini et al. 2009
sex-combs-reduced	iB_00186	TC000917	as expected	Tomoyasu et al. 2005
sister-of-odd-and-bowl	iB_04014	TC005788	as expected, slightly stronger	Angelini et al. 2009, 2012a,b
Sp8	iB_05083	TC011697	as expected	Beermann et al. 2004
TGF-alpha	iB_03555	TC003429	as expected, morphological defects in pupae	positive control in this study
vestigal	iB_04931	TC010897	as expected	positive control in this study
wingless	iB_05552	TC014084	as expected, morphological defects in pupae and adults	positive control in this study
wnt-less	iB_00832	TC005345	as expected, morphological defects in pupae	positive control in this study
yb	iB_02707	TC000053	as expected, ovariogenesis defects	positive control in this study, Klingler unpublished

Supplementary Table 3 | Definition of phenotypic categories used in large scale comparison

Phenotype	Criteria
no phenotype	not in one of the other categories
lethal after larval injection	> 80 % of the injected animals died within 22 dpi
parental lethal after pupal injection	> 80 % of the injected animals died within 11 dpi
embryonic lethal	> 50 animals in cuticle preparation of clutch 9 dpi, or > 20 animals in cuticle preparation of clutch 9 dpi without any hatched animals, or > 20 animals in fresh preparation of clutch 11 dpi with > 50% embryos died before cuticle secretion/did not develop
morphological defects of larval cuticle	> 50 animals in cuticle preparation of clutch 9 dpi, while > 70 % hatched, preparation with > 50% larvae showing morphological defects but clearly visible polarity and at least 2 distinguishable tagmata; or > 50 animals in cuticle preparation of clutch 9 dpi, while 10 - 70 % hatched, preparation with > 30 % larvae showing morphological defects but clearly visible polarity and at least 2 distinguishable tagmata; or > 20 animals in cuticle preparation of clutch 9 dpi while no animals hatched, preparation with > 30 % larvae showing morphological defects but clearly visible polarity and at least 2 distinguishable tagmata
strong defects of larval cuticle	> 50 animals in cuticle preparation of clutch 9 dpi, while > 70 % hatched, preparation with > 50 % larvae without clear tagmatic division or polarity; or > 50 animals in cuticle preparation of clutch 9 dpi, while 10 - 70 % hatched, preparation with > 30 % larvae without clear tagmatic division or polarity; or > 20 animals in cuticle preparation of clutch 9 dpi while no animals hatched, preparation with > 30 % larvae without clear tagmatic division or polarity
embryonic lethal before cuticle secretion	> 50 animals in cuticle preparation of clutch 9 dpi, preparation with > 50 % egg shells without larval cuticle or >30 % egg shells without larval cuticle in combination with morphological defects (see above); or > 20 animals in cuticle preparation of clutch 9 dpi while no animals hatched, preparation with> 50 % egg shells without larval cuticle or >30 % egg shells without larval cuticle in combination with morphological defects (see above); or > 20 animals in fresh preparation of clutch 11 dpi with > 50% embryos died before cuticle secretion/did not develop
defects in metamorphosis control	> 3 injected animals died during development from prepupal to adult stage
defects of adult structures	> 2 injected animals with morphological defects of pupal or adult structures (except ovary defects)
ovary defects	> 50 % of dissected animals with reduced egg production show morphological defects of ovaries and no reduced fat body 22 dpi or 13 dpi
morphological defects of pupae or adults	> 2 injected animals with morphological defects of external pupal or adult structures

morphological defects of pupal or adult legs	> 2 injected animals with morphological defects of pupal or adult legs
morphological defects of larval legs	> 50 animals in cuticle preparation of clutch 9 dpi, while > 70 % hatched, preparation with > 50% larvae showing leg defects; or > 50 animals in cuticle preparation of clutch 9 dpi, while 10 - 70 % hatched, preparation with > 30 % larvae showing leg defects; or > 20 animals in cuticle preparation of clutch 9 dpi while no animals hatched, preparation with > 30 % larvae showing leg defects
defects of adult thoracic musculature	> 2 injected animals with defects of the developing dorsal thoracic musculature (marked at pupal stage by d17 enhancer trap)
defects of larval musculature	> 20 animals in fresh preparation of clutch 11 dpi with > 30% embryos/larvae showing defects of musculature (marked by pig19 enhancer trap)
odoriferous glands defects	> 1 injected animal showed aberrations of abdominal or thoracic odoriferous glands
blistered wings phenotype	> 4 injected animals show a total or partial separation of dorsal and ventral wing surfaces

Note: We developed criteria for selecting datasets with high likelihood of reproducibility for our dataset wide analysis (shown in Figs. 2 and 3). For each phenotype class, we defined specific cut-off values, which were based on our experience gained during the reproduction experiments (e.g. reproducibility, see Figure 1d and Supplementary Figure 1). The main criterion was the penetrance of a phenotype (>50% for most embryonic phenotypes; >2 animals for most phenotypes observed in the injected animals; >80% penetrance for lethality). This main criterion was complemented with other criteria wherever our experience indicated that we would miss a significant number of phenotypes when only applying penetrance.

Supplementary Table 4 | *Tribolium* wing-blister genes tested in *Drosophila*

iB#	*D.m. ortholog*	Driver:	Bbg-GAL4	Bx-GAL4
			Description of wing phenotype	
Annotations associated with wing blister in Drosophila				
iB_00385	*Ilk (integrin linked kinase)*		blister	crumpled
iB_05522	*Asx (additional sex combs)*		WT	blister, curly
iB_05688	*wb (wing blister)*		blister	blister
iB_00014	*parvin*		blister	blister
Annotations associated with cytoskeleton in Drosophila				
iB_05272	*Mob4*		WT	unable to eclose
iB_00101	*TBCB*		WT	blister
iB_02017	*CG32138*		WT	vein defect
Annotations associated with cell adhesion in Drosophila				
iB_01705	*LanB2 (Laminin B2)*		WT	blister
iB_01221	*Cka (Connector of kinase to AP-1)*		crumpled	wings reduced, blisters unsure
iB_01762	*Pak (PAK-kinase)*	line 1	crumpled	crumpled
		line 2	WT	WT
		line 3	WT	curly, crumpled
iB_00557	*Lar (Leukocyte-antigen-related-like)*		crumpled	curly, shape, low hatchrate
iB_00666	*Sra-1 (specifically Rac1-associated protein 1)*		WT	crumpled
Annotations without cell adhesion or cytoskeleton in Drosophila				
iB_01726	*CG11526*		crumpled, mis-shapen, low hatchrate	wings reduced, blisters unsure
iB_02548	*CG5734*		WT	WT
iB_00300	*plexA (plexin A)*		WT	potential blisters
iB_00499	*SRPK*	line 1	vein defects	vein defects
		line 2	WT	blister
iB_00907	*Eip71CD (Ecdysone-induced protein 28/29 kD)*		WT	curly
iB_00845	*Lrt (Leucine-rich tendon-specific protein)*		WT	WT
iB_04887	*CG8078*		WT	blister, low hatchrate
PS integrins as positive controls (not included in iBeetle screen)				
-	*mew (multiple edematous wings)*		WT	blister
-	*mys (myospheroid)*		unable to hatch	blister, low hatchrate
-	*if (inflated)*		WT	WT

Supplementary Table 5 | *Drosophila* wing blister phenotypes in *Tribolium*

iB-number	name of *Drosophila* ortholog	iBeetle phenotype (Injection of L5/6)	Rescreen phenotype (Injection of L7)
iB_01796	*papillote*	larval lethal	larval lethal
iB_01467	*kopupu/shortstop/kakapo*	larval lethal	larval lethal
iB_03871	*blistered*	larval lethal	blistered wings
iB_05141	*dumpy*	larval lethal	larval lethal
iB_04642	*piopio*	larval lethal	larval lethal
iB_05522	*xenicid/additional sex combs*	blistered wings, deformed genital lobe	-
iB_01537	*rhea/talin*	larval lethal	blistered wings
iB_03691	*delta*	larval lethal	pupal lethality without blistering

Note: The database was screened for genes known to elicit a wing blister phenotype in Drosophila (based on Prout et al. 1997 and Walsh and Brown 1998 [5,6]). In seven out of eight cases, death prior to wing formation had prevented detection of the blister phenotype ("iBeetle phenotype"). We reasoned that injection at the last larval stage (L7) would reduce lethality and, hence, allow the detection of potential wing blister phenotypes. Indeed, two more genes showed the wing blister phenotype after later injection ("Rescreen phenotype"). In total, three out of eight genes (38%) would have been detected in a screen using systemic RNAi in the last larval stage.

Supplementary Table 6 |Workflow of the pupal and larval injection screens

Days after injection	Processing step	Phenotypic aspect screened
Workflow pupal injection screen		
d0	Injection (10 female pupae)	
d3	Inspection of adults	Metamorphosis control
d3-9	1st egg collection	
d9	Inspection of adults	Lethality of injected animals
d9-11	2nd egg collection	
d11	Assessment number of eggs in 2nd collection	Egg productivity
d13	L1 cuticle preparation (based on 1st egg collection) Dissection of ovaries*	Ovary morphology*
d14	Muscle preparation (based on 2nd egg collection)	L1 somatic muscles
d27-32		L1 cuticle morphology#
Workflow larval injection screen		
d0	Injection (10 female larvae)	
d11	Inspection of pupae	Lethality of injected animals Metamorphosis control Pupal morphology Pupal muscles
d16/18	Inspection of adults	Metamorphosis control Adult morphology Melanization
d20-23	Egg collection	
d21-25	Assessment number of eggs Dissection ovaries*	Egg productivity Ovary morphology*
d38-41	Analysis stink glands#	Altered or missing stink glands

*If number of eggs was reduced in second egg collection #Exact timing not critical

Supplementary Table 7 | Sequences of the dsRNA fragments used for the analysis of selected genes

iB-number	iB-fragment	Non-overlapping fragment
iB_05549	AGCACCAGACCCAGAACAACAACAACAACAATACCAAATCGCAAAAC ATGGAGCGGGTCAAACGGCCCATGAACGCCTTCATGGTGTGGTCGCG GGGCCAGCGGCGGAAAATGGCCCAGGAAAACCCCAAAATGCACAATT CGGAAATCTCGAAGCGGCTGGGGGCCGAGTGGAAGCTGTTGAGTGA GGCCGAGAAGCGGCCCTTCATCGACGAGGCCAAGCGGCTGAGGGCC GTGCACATGAAGGAACACCCGGATTACAAGTACCGGCCTAGGAGGAA GACCAAGACGCTCCTGAAGAAGGATAAATATCCCTTGGGAGCGTCCA GCTTGATCCCGACTAGTGACCCGACGCGGACGGCGCCTTCGGCGGTC CAACAGGTGTCCAGCCGGGACATGTACCAGATGCCCAATGGGTACAT GCCCAACGGGTACATGATGCACGACCCCGGGGCCTACCAGCAGCAGT ACACCGGGTCCAACTACGGCCGCTACGACATGTCGCAAATGCAGTACA TGAACGGTTACGGTTACGG	ACCAAGACGCTCCTGAAGAAGGATAAATATCCCTTGGGAGCGTCCA GCTTGATCCCGACTAGTGACCCGACGCGGACGGCGCCTTCGGCGGT CCAACAGGTGTCCAGCCGGGACATGTACCAGATGCCCAATGGGTAC ATGCCCAACGGGTACATGATGCACGACCCCGGGGCCTACCAGCAGC AGTACACCGGGTCCAACTACGGCCGCTACGACATGTCGCAAATGCA GTACATGAACGGTTACGGTTACGGGGCCACCGTGCCCCAGAGTGCA GGCTCCCCCTACGGAATGCAACAGACGTCGTCGCATAGCCCCTCTG GGTCCAGTATAAAATCGGAGCCGGTTTCTCCGAGTTCGGGGCTGCA CACACCGACGCCGGGCGTCAAGCGGGAGTACGGCCAACAGCAGCA GCCCCAGGGGGACCTGCGACAGATGATCTCCATGTACCTGCCCAC
iB_05264	AAGGGCTACTGGGGTTTCACCCGGCGATTACAAAGATTTCAAACCAAA CAATCCCGATATTAAAGTCGAAGACGGCACTCTTACTATTAACAATATC CAGAAAACAAACGAGGGTTATTATTTATGTGAGGCTGTCAATGGGATT GGATCAGGATTATCTGCAGTTATTCAAATCAGTGTTCAAGCTCCCCCAC AGTTTGATATTAAACTCAGGAACCAAACCTCCCGCCGTGGAGACCCTG CCGTCCTCCAATGTGAGGCCAAAGGCGAAAAACCGATTGGTATTTTAT GGAATATCAACAATAAGCGTTTGGAACCAAAAGGCGACAATAGATAC ACGATCCGGGAGGAGATCCTCGCCAATGGTGTTCTTTCCGGCCTCAGT ATCAAACGCACAGAACGCTCCGACTCCGCTCTCTTTACTTGTGTAGCTA CCAACGCCTTCGGCAGTGACGATACCAGCATTAACATGATTGTGCAAG AAGTACCAGAGGTACCATACGGGCTGAA	AAGGGCTACTGGGGTTTCACCCGGCGATTACAAAGATTTCAAACCA AACAATCCCGATATTAAAGTCGAAGACGGCACTCTTACTATTAACAA TATCCAGAAAACAAACGAGGGTTATTATTTATGTGAGGCTGTCAAT GGGATTGGATCAGGATTATCTGCAGTTATTCAAATCAGTGTTCAAG CTCCCCCACAGTTTGATATTAAACTCAGGAACCAAACCTCCCGCCGT GGAGACCCTGCCGTCCTCCAATGTGAGGCCAAAGGCGAAAAACCG ATTGGTATTTTATGGAATATCAACAATAAGCGTTTGGAACCAAAAG GCGACAATAGATACACGATCCGGGAGGAGATCCTCGCCAATGGTG TTCTTTCCGGCCTCAGTATCAAACGCACAGAACGCTCCGACTCCGCT CTCTTTACTTGTGTAGCTACCAACGCCTTCGGCAGTGACGATACCAG CATTAACATGATTGTGCAAGAAGTACCAGAGGTACCATACGGGCTG AA
iB_04564	CGTCTACAGCATCGACCAGATTTTGGGAGTTAACAGCTCGTCCACCTCT GCGAAGAGCATCGAAGGCGAGTCGGATTCCAAAGTCGATTCGGTCAG TGATAGCGAAATGGTTGAGGAAAGCATCGAAGATTTGAACGACACCA GACCGAGAAAAATCCGCAGATCCCGAACGACTTTCACCACCTATCAGC TGCACCAACTGGAGCGAGCTTTCGAAAAAACCCAATACCCCGACGTTT TCACAAGAGAGGAATTGGCTATGCGGCTTGATTTGAGCGAAGCGCGA GTCCAGGTATGGTTCCAAAACCGTCGCGCCAAGTGGAGAAAGCGTGA AAAGG	CGCTTTCCTGTCGCAATACGTGCTCGCAGGCGGCGTCCCCCAACTGA ATCTCCTAAATAGTGGGATTCCGGACGAACGGTCCCGGAGTACGAG TCCGGAAACTCCCAGTCCGTCGCAGTTGTCGCCCTCAGCGCTGGAA GCTTTGAGGTTACGGACACAAGAAATTTTAGTGCCGAGTTCTTCGCC GCAGAAACTGCACGCGAAGTCGTAAATAGATAATATCTAGTGGACA TGTAGTTGTATTGTTAGGGATGTAGGAGATTATTGGCGCTTGATAT GTGGGGGAAATAGACTGAATAGCTGTACCAAGTTTTCGATTTAGTT GTTATTTTTTTTTCATTCTTATGATAGGGAGAATTATTTTTGTATTAA ATTTATTTACAACACTAGTTGCACTAGTTAGTTTAGCGCCAATTTTGT AAATAATTTTGTACATAAAGTGCGTATTTATTTATTGTAGATAAAAA ATCAAGAGGTTTGTTTAAAACATAATAGCATTACAGATAATTATTTT GTGGGGGAAATTAACAATAAGTTGTACCTAATACCTAGTTACGATC GATATTAAACGAAACAATAATTAAAACGGCAGGTGTTTTACTTGTCT TGGT
iB_00289	TCAGAGTGCGAAAATGCTGATGCCCAGTGGACTTCCGGCCAACGAGG CCGATGGTCTTGGACTCGGAGGCCTGGGCACGAACACAAACACCAAC AACGGAGTGCAGCATAAGGACACCACATGGACCAAACTGTTCGTGGG TGGACTGCCGTATCACACCACGGATCAGTCGCTAAGAGAACATTTTTC GGTTTATGGGGAAATCGAAGAAGCGGTGGTCATCACTGATAGACAAA CGGGGAAAAGTCGAGGATATGGATTCGTAATTATGGGAGATAAGTCT TCATCAGATAGAGCATGTAAAGACGCTAACCCCATTATTGACGGGCGA AAAGCGAATGTGAATTTAGCGATTCTGGGAGCGAAACCGAGAGGAAA TGCGCAAACGGGTTTCCCGTTTCAAGGAATCCGGGCTGGTTATCCTGC ACTTCTTCCGGGCCAGTACGGAATGCCTCCTGGTTATGTGTACCAATCT CCATACCTAACTGCTGCTGCTCCTGGAAGTCT	CGCTAACCCCATTATTGACGGGCGAAAAGCGAATGTGAATTTAGCG ATTCTGGGAGCGAAACCGAGAGGAAATGCGCAAACGGGTTTCCCG TTTCAAGGAATCCGGGCTGGTTATCCTGCACTTCTTCCGGGCCAGTA CGGAATGCCTCCTGGTTATGTGTACCAATCTCCATACCTAACTGCTG CTGCTCCTGGAAGTCTT
iB_00555	GCTGATTGC ATTCTCCAA CAAGCGGTA GTCGGTGCC GGGGCCAAT CAGTTGGTT TTGAGTTAC TTGAGACAC TCCTTGAGC GCCCAGTTA GTCTCACAT GCGGCTGTG CTTCAACGC CTCAGCAAA TACAACCAA CTCAGTAAA GTCCACTGC GTGTTTAGC CTACTTGAG TTCCTCGAA GGCATGCTT CCGGGTGTC ACTTGTTGT GGCAAACCG GAAGAAACC GTCCTGGCC ACTGCAATT TTATCAATT GCTTGTTGG CTCCTGACT ATTTTATTG CAATGCAAA GGCACCGCT CATTTGACT CAGAAAGCC TCGTTCTTG CTCACCACG CTCATGAAT GACGATTTT TACGTCTCC ATGATGTGT CTAGCGAGG TACAGCGAT CCTGAATTG TTTACTGAG ACCAACCGC AAATGTATT GAGTTGAGG GCGTCGTTG TCCGATTCT GACGAGTTA TCTAAATGC GTTAAAAAA CTGGAAAAC ATCGATGTT AACATTTTG AGCCTCCCG ATCAATAG	TGCAGAGAGGTGTGGATCTGGCTTTGGCGGCGTTCCATGTCGATAT CAGGGCCTGCACTTTGGAGCTGCTCTCGCATGTTTTGCCCCAAATGT TGTATAACGATCTCCAAGCGGACTCGCTCATGGAGCCGCATTTGATC GCATTGGCTTACTTGACCAGTTATTGCGTGTACACGGCGTTTGATGC GTTTTCGGAAGAACCGGAAGAACCGATGGCTAAAGTTGCGCGGCTT AATGAGGGTGAGGATGGCACGCTGATTGAGCAACTAATTTCGACTC TCAGACAACTGATGACGATTTTTGAGGATGGGATACAAGAGGGTTA CATCACTCAACAGACTTATTTCGCGTTTTATTTGATTAAAAGTCTGGT TGAGGTGAAAGTGAGCACGGCGAGTGCTCTCTTGGCTGCGATTCCG CCGGCTCTTGTGACAGACTTGCTTCGGACTCTGCCTGAACTTTTTAC TTACCCGATTTTGTTGCATCTGCACGATGTGTTTAATACTCACGGTC GGAATAACATGGCAAAGGACTTGTGTATATTGCGAAATTACCACTT GAGGAACGTTTCG
iB_04537	TCATGCAGAAACGGGGTACTCCGATCAATCGACTGCCCATCATGGCCA AATCCGTTCTCGATTTATACGAATTGTACAATTTAGTCATCGCGAGGG GTGGACTTGTTGATGTCATCAATAAGAAACTCTGGCAGGAAATTATCA AAGGGCTGCATCTACCGTCGTCGATAACGTCGGCAGCTTTCACACTCA GGACGCAATACATGAAATACCTGTACCCTTACGAATGCGAGAAACGCC GCCTCAGCACACCGGCCGAGCTCCAGGCGGCCATCGACGGCAACCGT	CGCATGATGGAATACGTCAAGCTCCTCAACAAGGAAATCCGCAGCT CGGCGGCTACTCCGCCACGACAAGGGGACGTGTCGCCCCCTAACGC CACCTCGCCACTCAACCAGCTGGAGCTGTCGCGGATAACGCTCTGG AATATGTACAATAATAATCAACCCCCCGTCGAGCCGCAAAAGGAAG CGTTGAATCTCTCCGACCCGACGCAGCCTTCGGTGAAACGGGAGCC GGAGCATAGAGATTCGCCACCGCCCCCGAAGAAGTTTTCCAAAGAT

	CGCGAAGGCCGCCGCAGCAGCTACGGCCAGTACGACTCCATGCAGCG CTCCCCCAACCCTTCGCAGATGTCTCCTTTGTCCCTCGTC	GATGATGAACAGAAGATCTCGCCC
iB_02517	TCACTCAAAGCCACAGATGCGGTTTTAGTAAAATTGGGGCCCAAGGG CGAAATTTCAAATGAGACACTTGTTCACGTCGATTTGGTGCAACGTGG GGATGTTTTAAAAGTGGTACCGGGGGCCAAAGTACCAGTCGACGGCA AAGTTTTACAGGGCCAATCAATGTGCGACGAGAGCCTCATAACTGGG GAAAGCATGCCGGTACCGAAGAAAATCACAAGTAGTGTGATTGGTGG ATCAATCAATCAGCACGGCCTACTTATAATCGAAGCCACACATACAGG AGAGGCAACAACCCTATCACAAATTGTCAAATTGGTCGAAGAAGCACA AACGTCAAAAGCACCCATCCAACAATTGGCCGATAAAATCGCTGGTTA TTTCGTCCCAACTGTCGTCTTCCTGTCACTTTTGACACTTATTGTCTGGT CTATTATCGGCTCAATCGATATAAACGCACTTCCGGTGAC	CCCCACAGGCGACTACACCTCCGACCAACCAACAATAATCACAGTG TCAGAAGATGACACGATTAAAATCACCGTCTTGGGCATGACGTGCC AAAGCTGTGTCAAAAACATCGAAGAGACCCTGAGTCGTAAACCCGG CATTTACAACATCAAAGTCAGCCTTCAGGAAAAAGCCGCTCTAGTCC ATTATGACACACGCCAACTGACA
iB_04887	GGTGGCAAAGACTCCACAGTTCTGGCGTACGTCATGAAGCTACTTAAC GAAAAGTACGATTATAAGCTGGACTTAGTGCTTTTGTCCATTGATGAG GGTATTACAGGGTACAGGGACGATAGTTTAGATACTGTGAAACAAAA TCGGGACGATTACGGAATGCCTTTGAAAATAATGTCTTATAAGGATTT GTACGGTTGGACAATGGACGAAATTGTGGCTGAGATTGGGAGGAAA AATAACTGTACTTTTTGTGGCGTTTTCAGACGCCAGGCTTTAGACAGA GGGGCGGCTCTTCTAAATGTTGATTATTTAGCAACTGGACATAACGCT GATGATATTGCAGAGACTGTCTTGATGAATATTTTAAGGGGCGATTTG GCACGTCTCAGCCGTTGTACGTCCATTATCACGGACAGTGGTGACGGC ATTCCACGCGTAAAACCCCTCAAATACACCTACGAGAAAGAAATCGTC ATGTACGCCT	TCTCCACTGAGTGTGTCTTCGCCCCCAATGCGTACAGAGGCCATGCC CGGGTGTTGCTCAAAGATTTGGAAAAAATAGACCCTGCTGTTATAA TGAATATTATCCAGTCGGGGGAATCCCTCAAAATCAACGAAAATGC CAATATGCCAACTTTGCAGAAATGCACAAGGTGCGGATATGTGTCG TCGCAGGACGTGTGCAAAGCTTGCGTCCTCTTGGAGGGACTCAATA AAGGATTACCGAAACTAGGAATAGGGAAGTCGAGTAAAGTGAAAC GACATTTACAAGAAAACAGTCCGTGTTGTAAAACGC
iB_05264	AAGGGCTACTGGGGTTTCACCCGGCGATTACAAAGATTTCAAACCAAA CAATCCCGATATTAAAGTCGAAGACGGCACTCTTACTATTAACAATATC CAGAAAACAAACGAGGGTTATTATTTATGTGAGGCTGTCAATGGGATT GGATCAGGATTATCTGCAGTTATTCAAATCAGTGTTCAAGCTCCCCCAC AGTTTGATATTAAACTCAGGAACCAAACCTCCCGCCGTGGAGACCCTG CCGTCCTCCAATGTGAGGCCAAAGGCGAAAAACCGATTGGTATTTTAT GGAATATCAACAATAAGCGTTTGGAACCAAAAGGCGACAATAGATAC ACGATCCGGGAGGAGATCCTCGCCAATGGTGTTCTTTCCGGCCTCAGT ATCAAACGCACAGAACGCTCCGACTCCGCTCTCTTTACTTGTGTAGCTA CCAACGCCTTCGGCAGTGACGATACCAGCATTAACATGATTGTGCAAG AAGTACCAGAGGTACCATACGGGCTGAA	GTTTTGGGTTACCGCAGCTACCACTATTGGAGAAGGGCAACCGTCG AAGAAAGTTACAGTGTCTCCAAGCGCGAGCGTTCCAGCCAAAATCG CCTCGTTTGACGATACCTTCACCACGACGTACAAGGAAGACGTGAC TCTCCCTTGCCTCGCCGTTGGGTTGCCACCACCGGTCATCACATGGA AAATCAAGGGGGTTCAGTTCACCACAAGCGACAAAATCAGGCAACA ACCAGACGGGTCACTGTTTATTCGTGATGTCAGTCGGAATAACGCA GGGGAGTACTCGTGTCACGTTGAGAATGACTATGGACAGGACTCG GTGACTCACCAGTTGATTGTCAATGCTCCTCCACACGCACCACAAAT TGCTCTCACTTCAACTACCACAAATTCGCTCACGTTTAAGTTAAAGCC GCATGAGTCGGATGTTGAGCCGATCCATGGATACACTATTCACTAC AAGCCAGAGTTTGGCGATTGGGAGACGGTCCAGATTGGACCAACT GTCGAAAAGTACACTTTGGAGAAGTTGCTGTGTGG
iB_04564	CGTCTACAGCATCGACCAGATTTTGGGAGTTAACAGCTCGTCCACCTCT GCGAAGAGCATCGAAGGCGAGTCGGATTCCAAAGTCGATTCGGTCAG TGATAGCGAAATGGTTGAGGAAAGCATCGAAGATTTGAACGACACCA GACCGAGAAAAATCCGCAGATCCCGAACGACTTTCACCACCTATCAGC TGCACCAACTGGAGCGAGCTTTCGAAAAAACCCAATACCCCGACGTTT TCACAAGAGAGGAATTGGCTATGCGGCTTGATTTGAGCGAAGCGCGA GTCCAGGTATGGTTCCAAAACCGTCGCGCCAAGTGGAGAAAGCGTGA AAAGG	CGCTTTCCTGTCGCAATACGTGCTCGCAGGCGGCGTCCCCCAACTGA ATCTCCTAAATAGTGGGATTCCGGACGAACGGTCCCGGAGTACGAG TCCGGAAACTCCCAGTCCGTCGCAGTTGTCGCCCTCAGCGCTGGAA GCTTTGAGGTTACGGACACAAGAAATTTTAGTGCCGAGTTCTTCGCC GCAGAAACTGCACGCGAAGTCGTAAATAGATAATATCTAGTGGACA TGTAGTTGTATTGTTAGGGATGTAGGAGATTATTGGCGCTTGATAT GTGGGGGAAATAGACTGAATAGCTGTACCAAGTTTTCGATTTAGTT GTTATTTTTTTTTCATTCTTATGATAGGGAGAATTATTTTTGTATTAA ATTTATTTACAACACTAGTTGCACTAGTTAGTTTAGCGCCAATTTTGT AAATAATTTTGTACATAAAGTGCGTATTTATTTATTGTAGATAAAAA ATCAAGAGGTTTGTTTAAAACATAATAGCATTACAGATAATTATTTT GTGGGGGAAATTAACAATAAGTTGTACCTAATACCTAGTTACGATC GATATTAAACGAAACAATAATTAAAACGGCAGGTGTTTTACTTGTCT TGGT
iB_00289	TCAGAGTGCGAAAATGCTGATGCCCAGTGGACTTCCGGCCAACGAGG CCGATGGTCTTGGACTCGGAGGCCTGGGCACGAACACAAACACCAAC AACGGAGTGCAGCATAAGGACACCACATGGACCAAACTGTTCGTGGG TGGACTGCCGTATCACACCACGGATCAGTCGCTAAGAGAACATTTTTC GGTTTATGGGGAAATCGAAGAAGCGGTGGTCATCACTGATAGACAAA CGGGGAAAAGTCGAGGATATGGATTCGTAATTATGGGAGATAAGTCT TCATCAGATAGAGCATGTAAAGACGCTAACCCCATTATTGACGGGCGA AAAGCGAATGTGAATTTAGCGATTCTGGGAGCGAAACCGAGAGGAAA TGCGCAAACGGGTTTCCCGTTTCAAGGAATCCGGGCTGGTTATCCTGC ACTTCTTCCGGGCCAGTACGGAATGCCTCCTGGTTATGTGTACCAATCT CCATACCTAACTGCTGCTGCTCCTGGAAGTCT	GGAGCACCTTCACCAAAGAGTACATAAACAACGTGGTTAACCCCCT CGGAAACCGGCCCACCAGACCAACGGAAAACCACACTACCACCTCC TCGGAGCGTTCCGCAGCTTCTGATCCTTCTGAAGATCAGAGTGCGA AAATGCTGATGCCCAGTGGACTTCCGGCCAACGAGGCCGATGGTCT TGGACTCGGAGGCCTGGGCACGAACACAAACACCAACAACGGAGT GCAGCATAAGGACACCACATGGACCAAACTGTTCGTGGGTGGACT GCCGTATCACACCACGGATCAGCGCTAACCCCATTATTGACGGGCG AAAAGCGAATGTGAATTTAGCGATTCTGGGAGCGAAACCGAGAGG AAATGCGCAAACGGGTTTCCCGTTTCAAGGAATCCGGGCTGGTTAT CCTGCACTTCTTCCGGGCCAGTACGGAATGCCTCCTGGTTATGTGTA CCAATCTCCATACCTAACTGCTGCTGCTCCTGGAAGTCTT
iB_04887	GGTGGCAAAGACTCCACAGTTCTGGCGTACGTCATGAAGCTACTTAAC GAAAAGTACGATTATAAGCTGGACTTAGTGCTTTTGTCCATTGATGAG GGTATTACAGGGTACAGGGACGATAGTTTAGATACTGTGAAACAAAA TCGGGACGATTACGGAATGCCTTTGAAAATAATGTCTTATAAGGATTT GTACGGTTGGACAATGGACGAAATTGTGGCTGAGATTGGGAGGAAA AATAACTGTACTTTTTGTGGCGTTTTCAGACGCCAGGCTTTAGACAGA GGGGCGGCTCTTCTAAATGTTGATTATTTAGCAACTGGACATAACGCT GATGATATTGCAGAGACTGTCTTGATGAATATTTTAAGGGGCGATTTG GCACGTCTCAGCCGTTGTACGTCCATTATCACGGACAGTGGTGACGGC ATTCCACGCGTAAAACCCCTCAAATACACCTACGAGAAAGAAATCGTC ATGTACGCCT	TCTCCACTGAGTGTGTCTTCGCCCCCAATGCGTACAGAGGCCATGCC CGGGTGTTGCTCAAAGATTTGGAAAAAATAGACCCTGCTGTTATAA TGAATATTATCCAGTCGGGGGAATCCCTCAAAATCAACGAAAATGC CAATATGCCAACTTTGCAGAAATGCACAAGGTGCGGATATGTGTCG TCGCAGGACGTGTGCAAAGCTTGCGTCCTCTTGGAGGGACTCAATA AAGGATTACCGAAACTAGGAATAGGGAAGTCGAGTAAAGTGAAAC GACATTTACAAGAAAACAGTCCGTGTTGTAAAACGC

Supplementary Table 8 | Comparison of number of lethal genes

Drosophila				
	Absolute numbers	**% related to …**		**Reference**
Protein coding genes	13,918	…all genes:		FlyBase; Release 6.02
Lethal loci	5,000	35.9%		Nüsslein-Volhard 1994
Sterile loci	1,000	7.2%		Nüsslein-Volhard 1994
Essential genes (sum lethal plus sterile)	6,000	43.1%		Nüsslein-Volhard 1994
		... lethal genes:		
Embryonic lethal genes	1,000	20.0%		Mullins et al. 1994
		... embryonic lethal genes:		
Embryonic lethal without cuticle phenotype		85.0%		Mullins et al. 1994
…with phenotype		15.0%		

Tribolium				
	Numbers found in the screen	**Numbers corrected for false positive rate of 26%**	**% of corrected numbers related to …**	**Reference**
All analyzed genes	3,400		… all analyzed genes	
Lethal genes	1,686	1,248	36.7%	Fig. 2b, heading
Non-lethal genes with phenotype	229	169	5%	Fig. 2a
			… all lethal genes	
Embryonic lethal genes	969	717	57.5%	
			… embryonic lethal genes	
No cuticle defect	186	138	19.2%	
With cuticle defect & empty egg	783	579	80.8%	

Supplementary Note 1

Detailed information on positive controls

In order to assess reliability and sensitivity of our screen, and to test the alertness of the screeners, we included 41 different positive controls. The screeners did not know which gene was used as positive control in a given repetition, whether this control had a phenotype in the pupal or the larval screen, and due to the high number of different controls, a screener would encounter most controls only once during the screen. However, for technical reasons in the production pipeline, it was not possible to hide the position of positive controls within a repetition. Hence, with some effort, a screener could identify the position of the positive control and scrutinize it more carefully (see details below). As an additional way assessing reliability and sensitivity of the screen, we searched for datasets that by chance had targeted genes with known phenotypes. The identities of these genes were neither known to the screeners nor the PIs such that these datasets represent double blind positive controls (see details below). We observed similar recognition rates as in the added positive controls (see Fig. 1a and b).

It turned out that in our procedure, the phenotypes of some positive controls were reproducibly different from the published phenotypes and novel phenotypic aspects were detected (e.g. due to different dsRNA concentration or different injection stage and timing). Therefore, the entire iBeetle procedure was performed with all positive controls in order to define the phenotypic aspects that had to be recognized in our screen.

In 370 cases (93.2%, n=397) the phenotype was entirely (83.3%) or partially (9.8%) recognized (dark and light green; Supplementary Figure 1). Controls were regarded as “partially recognized” when the majority (but not all) of the aspects of a phenotype were correctly annotated.

Most of the 39 cases that were only partially recognized were derived from five positive control genes. These genes had originally been chosen for controlling the pupal injection screen but turned out to have phenotypes in the larval screen as well (*Tc-vestigial, Tc-hedgehog, Tc-wnt-less/evi* and *Tc-TGF-alpha*; see controls marked with a diamond in Supplementary Figure 1). We scored them as “partially recognized” when at least three out of four aspects were recognized. Four additional cases of partially recognized controls stem from *Tc-metoprene tolerant (Tc-met),* where the lethality of the injected animals was properly annotated but the necrotic head was missed.
With respect to “technical lethality”, in 11 cases (2.8%) the premature death of the injected animals prevented detection of the phenotype (Supplementary Figure 1, light blue). 16 cases were true false negatives (4%; Supplementary Figure 1, dark blue). 62.5% of those cases derived from the analysis of only three positive control genes that elicited subtle morphological defects in the adults, like slightly deformed cuticular structures at the ventral midline of adult beetles after larval *Tc-orthodenticle* RNAi (missed five times), or minor head or leg defects after pupal injections of *Tc-methoprene-tolerant* or *Tc-aristaless* (together five cases).

We analyzed 65 datasets that by chance had targeted genes with previously described phenotypes (

Supplementary Table 2). Hence, this set represents completely blind positive controls. 51 of those (78.5%) were recognized with the previously published phenotype.

In eleven cases (16.9%), we found a phenotype which was reproducibly different from the published one (i.e. reproduced in an independent repetition of the experiment following our screening procedure). Hence, the phenotype differed due to our specific experimental conditions, not due to experimental variation. The relevant parameters responsible for such differing phenotypes could be the different stage and timing of injection, the genetic background of the injected animals and the different dsRNA fragment used for the experiments.

We observed several cases where the RNAi phenotypes were reproducibly different from published data. One case is likely due to strain specific differences: The larval injection of two different fragments against *Tc-empty spiracles* (iB_05098 and a positive control fragment) reproducibly elicited different phenotypes in two different strains. In case of *Tc-odd-skipped* (iB_04013) the difference could be either strain specific or due to different dsRNA concentrations. Larval injection of the iB-fragment in L6 or L7 larvae did not lead to any phenotype in the screen, although defects of mouthparts, antennae and legs had been described previously [7–9]. However, in our hands, neither the iB-fragment nor a non-overlapping fragment, either in the screening strain or in another genetic background, reproduced the published phenotype. In three cases, different dsRNA concentrations are likely to be responsible. For both, iB_03691 (*Tc-delta*) and iB_04526 (*Tc-homothorax*), lethality was observed in the screen. This was reproduced by injection into later stages and by using a non-overlapping fragment in another genetic background. In the publications, the authors mention using diluted dsRNA solutions in order to see the phenotype. Hence, it is likely that the higher concentrations of dsRNA used in the iBeetle screen caused the stronger phenotype. Pupal dsRNA injections targeting *Tc-decapentaplegic* (*Tc-dpp)* always led to lethality or cachexia (starved animals) in our screen and in follow-up experiments with two different fragments in two different strains. This is in contrast to the published results [10,11] but is in line with the recent finding that *Tc-dpp* is also involved in lipid homeostasis [12].

The timing of injection was critical for iB_01701, which targets *Tc-laccase-2*. The expected phenotype was annotated neither in the pupal nor the larval injection screen. Injection into L6 larvae reproducibly led to early lethality instead of the published tanning defect (i.e. pupae with soft and non-pigmented cuticle) while in the pupal screen, no effect was seen. An independent injection of the same fragment into older (L7) larvae did reproduce the published phenotype.

In four cases, either isoform-specific knock down, or off target effects likely led to different phenotypes. *Tc-knirps, Tc-frizzled-1* and *Tc-lim-1* were annotated with the “empty egg phenotype” instead of the published morphological defects [13–15]. These results were reproduced using the iBeetle fragment but non-overlapping fragments produced the published phenotype (*Tc-knirps* and *Tc-frizzled-1)* or wildtype larvae (*Tc-lim-1).* Pupal injection of an iB-fragment against *Tc-broad-complex* (*Tc-BRC,* iB_03960) did not reveal any defect, while injection of another fragment of *Tc-BRC* (used as a positive control) consistently led to various strong defects during embryogenesis. Our follow-up analysis indicates that off target effects are the most likely explanation: In the case of *Tc-BRC*, several splice variants with distinct functions are known for *Drosophila* and *Tribolium* and indeed, the two dsRNA fragments target different isoforms: The positive control fragment (335 bp) lies in the common fourth exon of the *Tc-BRC* isoforms as published by Konopova and Jindra (2008)[16] and leads to a range of embryonic developmental defects. iB_03960 (495 bp) does not overlap with the positive control fragment, but spans the 3' end of common exon 4, common exon 5 (adding up to 321 bp) and part of the alternatively spliced exon 6 of splice isoform Z4 (174 bp). iB_03960 does not lead to any embryonic defects. Due to the fact that both fragments are predicted to target all isoforms, an off target effect of the positive control fragment appears likely. Previous studies using an insertion mutant have shown no or no severe degree of embryonic

lethality. Due to the location of the insertion in the region of alternatively spliced exons it remains unclear what isoforms are actually affected in this mutant [16].

For *Tc-fz1* and *Tc-kni* we checked the most recent annotations of the *Tribolium* and *Drosophila* genomes and the *Tribolium* literature for indication of alternative transcripts. There are no published splice isoforms for *Tc-fz1* and off target analysis did not reveal any putative off target hit using the e-RNAi resource [17]. For *Tc-kni* there are two small 5' introns present in the automatically annotated gene model (TC003413), but not in the published mRNA sequences, which are based on RACE and cDNA sequences[14] (NM_001128495). Anyway, the iBeetle fragment targeting *Tc-knirps* is downstream of this questionable region. Off-target analysis revealed several hits in other genes two of which showed a phenotype in the screen.

One out of 65 phenotypes was overlooked (1.5%) and two were missed for technical reasons (3%). It is a bit surprising that the portion of missed phenotypes of this completely blind set of controls is even lower than the one with the set of not completely blind positive controls. The reason could be that we deliberately included subtle (partly unpublished) aspects of phenotypes as positive controls while the set of published phenotypes may be biased towards strong phenotypes.

Several RNAi phenotypes were missed. Subtle tarsal and antennal defects after *Tc-bric-a-brac* (iB_03591) larval RNAi were not detected in the screen but we were able to elicit this phenotype by repeating the experiment (with the iBeetle fragment). In two cases, technical lethality (e.g. due to injection problems) led to precocious death of the injected animals, which prohibited detection of the later phenotype (*Tc-pumilio* (iB_03898) and *Tc-methoprene-tolerant* (iB_03648)).

Negative controls

The last injection of each injection day was either a positive control or a buffer injection (blind to the screener). We used these buffer injections to assess the rate of false negative annotations: Four out of 155 buffer controls (2.6%) had false negative annotations while in another three cases technical issues led to a false positive annotation of lethality (1.9%) (see Supplementary Figure 2 for distribution of technical lethality in buffer and dsRNA injections).

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