Development of functional ectopic compound eyes in scarabaeid beetles by knockdown of orthodenticle

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Complex traits like limbs, brains, or eyes form through coordinated integration of diverse cell fates across developmental space and time, yet understanding how complexity and integration emerge from uniform, undifferentiated precursor tissues remains limited. Here, we use ectopic eye formation as a paradigm to investigate the emergence and integration of novel complex structures following massive ontogenetic perturbation. We show that down-regulation via RNAi of a single head patterning gene—orthodenticle—induces ectopic structures externally resembling compound eyes at the middorsal adult head of both basal and derived scarabaeid beetle species (Onthophagini and Oniticellini). Scanning electron microscopy documents ommatidial organization of these induced structures, while immunohistochemistry reveals the presence of rudimentary ommatidial lenses, crystalline cones, and associated neural-like tissue within them. Further, RNA-sequencing experiments show that after orthodenticle down-regulation, the transcriptional signature of the middorsal head—the location of ectopic eye induction—converges onto that of regular compound eyes, including up-regulation of several retina-specific genes. Finally, a light-aversion behavioral assay to assess functionality reveals that ectopic compound eyes can rescue the ability to respond to visual stimuli when wild-type eyes are surgically removed. Combined, our results show that knockdown of a single gene is sufficient for the middorsal head to acquire the competence to ectopically generate a functional compound eye-like structure. These findings highlight the buffering capacity of developmental systems, allowing massive genetic perturbations to be channeled toward orderly and functional developmental outcomes, and render ectopic eye formation a widely accessible paradigm to study the evolution of complex systems.


Significance

From both evolutionary and biomedical perspectives, ectopic organ development is a promising paradigm to investigate the emergence and integration of novel complex structures outside their normal biological context. However, the best known examples of ectopic organ induction require genetic techniques only available for a few model systems. Here, we report the ectopic induction of complex and functional organs, compound eyes, by down-regulation of a single transcription factor through RNAi, a technique known to work well across a broad taxonomic range. These results provide a remarkable example of the ability of developmental systems to channel massive perturbations toward orderly and functional outcomes and open the use of ectopic eyes as an accessible paradigm of ectopic organ development.


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**Results and Discussion**

**Orthodenticle Down-Regulation Induces Ectopic Structures Externally Resembling Compound Eyes in Onthophagini and Oniticellini.** We first assessed whether the induction of ectopic compound eyes via down-regulation of *orthodenticle* may be restricted to the closely related *Onthophagus* species *O. taurus* and *O. sagittarius* or alternatively constitute a more taxonomically widespread phenomenon. To do so, we cloned *orthodenticle* orthologs in three additional scarabaeid species (*Onthophagus binodis* and *Digitonthophagus gazella*, tribe Onthophagini; and *Liatongus militaris*, tribe O niticellini; Fig. 1 and Dataset S1), followed by larval *otd1 RNAi* in all species. *otd1 RNAi* reduced the size of the metasternal plate and pronotal ornaments, caused head midline defects, eliminated or greatly reduced horns and ornaments located along the posterodorsal

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**Fig. 1.** Beetle *orthodenticle* orthologs and phenotypes observed after *otd1 RNAi*. (A) Gene tree showing relationships between *orthodenticle/OTX* class genes from scarabaeid beetle species and the red flour beetle *Tribolium castaneum*. The * indicates partial gene sequences retrieved from genomic DNA. (B) Table showing distribution of RNAi phenotypes corresponding to the gene indicated in the left row. Each gene was knocked down in the species from which it was cloned; the dendrogram indicates inferred phylogenetic relationships among species. A tick mark indicates the presence of an unambiguous RNAi phenotype; a cross indicates a wild-type–like phenotype. DHM, dorsal head midline defect; EELS, ectopic eye-like structure; EHH, ectopic head horns; VMD, ventral midline defect. (C) Dorsal view of heads at midpupal stage from control (Left) and *otd1* (Right) dsRNA-injected individuals from all five species. Blue half-arrowheads indicate ectopic eyes. (Scale bar, 0.5 mm.) (D) External morphology of ectopic eyes in adults ranges from large, convex structures with evident ommatidial arrangement (Top) to small, inconspicuous pits (Bottom). Right shows a higher magnification view of the boxed areas in Left. Labeling as in C.
head, and induced small ectopic horns in the same area in both tribes (Fig. 1B and Figs. S1–S6). Furthermore, in all species, we observed formation of ectopic compound eyes at the posterodorsal head (8/20 in O. binodis; 52/74 in D. gazella; 7/92 in L. militaris), which first became evident by the midpupal stage as paired pigmented spots flanking the posterodorsal head midline (Fig. 1C). We replicated these results in O. sagittarius (51/139) and O. taurus (where ectopic compound eyes only develop when both Ot-otd1 and Ot-otd2 are knocked down: 5/48). Our data thus show that the ability to develop ectopic compound eyes through larval otd1 down-regulation is not idiosyncratic to the genus Onthophagus.

**SEM Imaging and Immunohistochemistry Reveal Striking Parallels Between Ectopic Compound Eyes and Wild-Type Eye Morphology.** We used scanning electron microscopy to image adult ectopic compound eyes and assess their external morphology. In all species, we observed putative ommatidial lenses organized in hexagonal arrays reminiscent of those found in regular lateral compound eyes. Even though ectopic compound eyes varied widely in their external expression within and between species (the largest and most complex being found in O. sagittarius), ommatidia-like structures were evident in each case (Fig. 1D). Using cryosectioning and immunohistochemical staining, we found that compound eye-like external features of ectopic compound eyes were underlain by corresponding internal features, specifically crystalline cones surrounded by putative photoreceptor and accessory cells, albeit arrayed more irregularly (Fig. 2 A–D). Strikingly, some of the sections showed organized neural-like tissue extending from ectopic eyes onto a deeper flask-shaped structure of nonmuscular identity (Fig. 2E). Taken together, analysis of external and internal morphology supports that ectopic compound eyes share structurally defining traits with regular compound eyes, despite being smaller in size and more irregular in organization.

**The Transcriptional Signature of the Middorsal Head Converges onto That of Regular Compound Eyes Following Orthodenticle Down-Regulation.** Although ectopic compound eyes thus appear morphologically similar to compound eyes, their ability to also function alike would critically depend upon the coordinated ectopic expression of the retinal and photoreceptor gene networks. Thus, we tested whether ectopic compound eyes are transcriptionally closer to regular compound eyes than to the posteromedial head of control animals. Using RNA-sequencing (RNA-seq), we characterized and contrasted the transcriptional repertoire of the posteromedial head (PMH) and wild-type compound eye (EYE) tissues of both control and Dg-otd1RNAi 4-d-old pupae of D. gazella (Fig. 3A). We chose D. gazella due to higher penetrance of the ectopic eye phenotype; furthermore, since ectopic compound eyes in this species appear smaller and simpler than in O. sagittarius, we considered it a conservative choice. Our results showed that the transcriptional profile of EYE tissue for both treatments differs strongly from that of the control PMH tissue. Indeed, three out of four replicates of the Dg-otd1RNAi PMH samples (all of which developed ectopic eyes) clustered more closely with the EYE samples than with control PMH samples (Fig. 3B). Furthermore, a Gene Ontology analysis showed significant enrichment for visual, photoreception, signal transduction, and rhodomere categories of differentially expressed genes in otd1-PMH samples relative to ctrl-PMH samples (Datasets S2–S11). Most of these genes, including orthologs of ninac, arrestin, chaoptin, and slowpoke-binding protein, were also differentially up-regulated in ctrl-EYE relative to ctrl-PMH samples (Fig. 3C. Left). Collectively, these data show that ectopic compound eyes exhibit a transcriptional profile similar to that of wild-type compound eyes.

**Ectopic Compound Eyes Partially Rescue Visual Function in Blindened Beetles.** Combined, our data reveal significant morphological, cellular, neuroanatomical, and transcriptional congruence between ectopic compound eyes induced after otd1 knockdown and wild-type lateral compound eyes. To test whether ectopic compound eyes are sufficiently complex to support functional integration with the rest of the organism, we assessed whether they could rescue visually cued behavioral responses in individuals deprived of regular compound eyes. We designed a behavioral assay where an individual beetle is placed inside a dish in a dark room and allowed to walk; a cold, bright, flickering light is then shone in front of the beetle. Wild-type individuals (B−I−) react without exception by stopping, low-ering their heads, turning around, and quickly moving away from the light source (11/11). By ablating wild-type larval head tissue fated to form compound eyes (14), we generated eyesless beetles (B+I−) that invariably failed to show any reaction to light (0/15). We then...
repeated the ablation in otd1RNAi individuals. We found that most individuals possessing ectopic compound eyes but lacking regular lateral compound eyes (B+/B) eventually reacted to the light stimulus by either lowering their head or turning around or a combination thereof (11/12) (Table S1 and Movie S1). These results indicate that, despite the impact of the eye ablation treatment on overall vigor, ectopic compound eyes can at least partially rescue the behavioral response to light in an otherwise eyeless beetle (P < 0.01) and are thus functionally integrated with the central nervous system of the insect.

**Ectopic Eye Formation Provides a Useful and Widely Accessible Paradigm to Study the Ontogeny and Evolution of Complex Systems.** Targeted overexpression of eyeless/Pax6 is known to induce development of ectopic eye-like structures in fruit flies and frogs, but evidence of full functional integration of ectopic eyes is lacking. In contrast, our results show that in scarabaeid beetles, RNAi-induced depletion of orthodenticle/OTX homologs results in development of functional ectopic compound eyes. As such our results document the formation of a highly complex and functional organ in a novel location following the down-regulation of a single gene, thereby highlighting the flexibility and modularity of developmental systems to generate complex, well-integrated structures even when confronted with major regulatory perturbations and outside their conventional anatomical context. The regulatory mechanisms leading to ectopic compound eye development in beetles remain to be fully investigated, but comparing our RNA-seq dataset to corresponding information on the fly retinal gene network, we find that while the sign of some interactions is conserved, others have reversed. For example, in both *Drosophila* and *Onthophagus*, loss of orthodenticle results in increased wingless expression (Fig. 3C). In contrast, loss of otd expression leads to the loss of hedgehog expression in flies (15) yet to its up-regulation in otd1RNAi beetles (Fig. 3C). Since *hedgehog* is required to induce neurogenesis and synapse formation between brain and photoreceptor neurites (16), we speculate that differences between the fly and beetle retinal networks might explain why fly ectopic eyes do not reach functional integration. A further difference between flies and beetles is that the latter lack ocelli, except for a subset of species in 8 out of ~200 beetle families currently recognized (12). Ocelli are optical sensory structures simpler than compound eyes yet controlled, at least as far as is known in flies, by the same developmental gene network (17). Since ocelli are believed to be part of the basal insect ground plan and thus assumed to have been lost in beetles (12), it is conceivable that ectopic eye development seen after otd1RNAi in this group is mediated by activation of a vestigial and incomplete ocellar-fate program that eventually defaults into a compound eye fate. However, how a vestigial and incomplete developmental program can ultimately yield a complete and functional developmental product as complex as
compound eye remains entirely unclear. A wider taxonomic sampling is clearly needed to determine whether lack of ocelli correlates with the propensity to form dorsal ectopic eyes and which retinal network (beetle or fly) more closely reflects the ancestral arthropod arrangement; fortunately, while targeted gene over-expression techniques are currently limited to a few model systems, RNAi-mediated gene down-regulation works remarkably well in a wide variety of arthropods. Thus, our work opens the possibility of exploring the taxonomic breadth and conservation of ectopic compound eye development induced by orthodenticle knockdown.

More generally, our results show that down-regulation of transcription factors that govern the development of entire body regions does not have to result solely in the morphological disruption of their corresponding domains but may also provide opportunities for developmental systems to settle into alternate stable states including, in at least this case, the formation of a complex and functionally integrated trait in a novel location. Our findings thus provide a remarkable example of the buffering capacity of developmental systems, allowing massive genetic perturbations to be channeled toward orderly and functional developmental outcomes. Further, developmental buffering and guiding toward functional integration as seen here may constitute key factors in promoting both evolutionary as well as technological innovation: On one hand, these developmental features are likely key facilitators of heterotopy, a widespread yet understudied phenomenon whereby the development of an organ or tissue is shifted to a new spatial location (18). On the other hand, the modular, reciprocally self-constraining nature of developmental processes is instrumental to the biomedical goal of growing ex situ organoids for research and transplantation (19). Whether used in basic or applied research, our findings render ectopic eye formation a widely accessible paradigm to study the mechanistic basis of the developmental flexibility that enables the evolution and re- construction of complex systems.

Materials and Methods

Beele Husbandry. Beeltes were obtained from cow pastures around Bloomington, Indiana; Chapel Hill, North Carolina; and Busselton, Australia (O. taurus); Imbil, Australia (O. sagittarius); Kuala, Hawaii (O. sagittarius, D. gazella, L. militaris); and Waimea, Hawaii (O. binodis). Experimental animals were generated and maintained as described elsewhere (8, 20) either at 24 °C (O. taurus, O. binodis) or at 28 °C (O. sagittarius, D. gazella, L. militaris).

Orthodenticle Cloning, Sequencing, and Phylogenetic Analysis. Ot-otd1, Ot-otd2, Os-otd1, and Os-otd2 sequences were retrieved and cloned as previously described from genomic and transcriptomic databases (8, 21, 22). A fragment of Os-otd1 was partially cloned using degenerate PCR from cDNA, a fragment of Ob-otd2 was amplified and sequenced from genomic DNA using cross-specific primers. Dg-otd1, Dg-otd2, and Lm-otd1 sequences were retrieved from transcriptomes assembled de novo from RNA-seq data (see Transcriptome Assembly and Annotation). Multiple sequence alignment (MSA) and phylogenetic analyses were made using Geneious 8.1 (23); the MSA was generated using the “Translation align” tool and the MAFFT method (24), and a gene tree was inferred using RAxML 7.2.8 (25) with the GTR-GAMMA model and default settings.

otd1 RNAi. Targeted gene knockdown was achieved by injection of double-stranded RNA derived from a fragment of each target gene, as detailed previously (8, 26). Template DNA containing ~500-bp orthologous fragments of ottd was amplified by PCR from either cloned plasmids (Ot-otd1, Ot-otd2, Os-otd1, Ob-otd1, Dg-otd1) or from cDNA using species-specific primers flanked by T3 and T7 binding sequences (Lm-otd1). The templates were transcribed in vitro using T3 and T7 RNA-polymerases to generate forward and reverse RNA strands using MEGAscript T7 and T3 Kits (Life Technologies). After DNase I treatment, sRNA was precipitated by lithium chloride or sodium acetate, incubated at ~20 °C for 1 h, spun at 4 °C for 20 min, washed with 80% ethanol, and resuspended in water. After quantification, forward and reverse strands were mixed at a 1:1 ratio by weight and annealed by heating to 80 °C and then incubated at 37 °C for 5 to 35 °C. The concentration of the annealed RNA was measured, confirmed by gel electrophoresis, and stored at ~80 °C until injection. Injections into larvae were carried out as described previously (26). Doses varying from 0.1 to 50 μg of dsRNA were diluted in injection buffer (5 mM KCl, 1 mM NaPO4, pH 6.9) to a total of 3 μL and injected into larvae during the third instar. We injected a total of 334 O. taurus (Ot-otd1, 251; Ot-otd1 ot-otd2, 83), 312 O. sagittarius, 25 O. binodis, 91 D. gazella, and 138 L. militaris. Phenotype scoring is reported as number of dsRNA-injected beetles with ectopic eyes/total number of dsRNA-injected beetles that became pupae. Sham control injections were made exactly as described above, except that larvae were injected with 1 μg nonsense dsRNA from a 167-bp PCR product derived from a pBluescript SK vector. Transcription reactions, DNAs I treatment, transcript annealing, and injections were performed as described above (160 O. taurus, 37 O. sagittarius, 23 O. binodis, 47 D. gazella, 60 L. militaris).

Scanning Electron Microscopy. At least three adult individuals of each species and treatment were fixed in 70% ethanol, then incubated in 100% ethanol. Samples were either critical-point CO2 dehydrated or immersed in hexamethyldisilazane and air-dried, coated with gold-iridium in an argon chamber, and imaged on either a JEOL JSM 5800 LV scanning electron microscope, or on a FEI Quanta 600, at 15 kV.

Sectioning, Immunohistochemistry, and Confocal Imaging. Individuals at mid- to late pupal stage were cut between thorax and abdomen and fixed in PEM buffer (27) and then dehydrated by immersion in 100% methanol overnight. Samples were rehydrated in a decreasing methanol/PBS series and then moved through an increasing sucrose/PBS series until reaching 30% w/v sucrose samples were kept in 30% sucrose (1–2 day) or in 70% w/v sucrose to which compound (Tissue-Tek, Sakura Finetek) and mounted on an aluminum stub at ~20 °C inside a Reichert-Jung Cryocut 1800 cryostat. Samples were sliced to obtain sections between ~18 and 25 μm thick, collected on glass slides, air-dried, and stored at ~20 °C until used. For staining, samples were rehy- drated in PBS and incubated in anti-acetylated α-tubulin antibodies, phal- lolidin, and DAPI as detailed elsewhere (28). Stained samples were imaged under a Leica SP8 laser scanning confocal microscope using LAS software.

RNA Extraction and Sequencing. Whole animals were homogenized in TRIzol using disposable polypropylene RNase-free pellet pestles: For D. gazella, we used three individuals of each sex and late last larval instar, prepupa, first day pupa, and 4–d-old adult stages; for L. militaris, we only sampled a male prepupa and first day pupa. Chloroform was added to the homogenates in 1:1 volume ratio, thoroughly mixed and incubated at room temperature for 2 min, and then spun at 13,600 × g for 20 min at 4 °C, washed with 100% ethanol, air-dried, and resuspended in RNase-free water. Total RNA was quality checked using RNA ScreenTape TapeStation System (Agilent) and quantified with a Quant-it RiboGreen Assay Kit (Thermo Fisher). RNA Stranded RNA-seq libraries were constructed using the TruSeq Stranded mRNA Sample Preparation Kit (Illu- mina) according to the manufacturer’s instructions. Libraries were quantified using a Quant-iT RNA Assay Kit (Thermo Fisher), pooled in equal molar amounts, and single-end sequenced on the NextSeq500 platform (Illumina) using a 75-cycle High Output Kit. Resulting 75-bp read sequences were cleaned using a trimomatic version 0.32 (29) to remove reads from adapter sequences and perform quality trimming. Trimomatic was run with the following parameters: “2:20:5 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:18.”

Transcriptome Assembly and Annotation. The reference transcriptomes for D. gazella and L. militaris were assembled from 75SE reads using Trinity 2.0.6 (30) using “–trimmomatic-55_lib_type R” parameters on a single 16 CPU node with 128 GB of RAM at the Mason computer cluster at Indiana University. Bloom-ington. Candidate coding sequences were predicted using Transdecoder 2.0.1 (transdecoder.github.io), and the assemblies were annotated using the Trino- tate pipeline (31) (https://transrate.github.io/). The annotations were made based on Blast+ (32) searches against the UniProt database and current O. taurus ge- nome annotations (21), HMMPR (33) scans against the Pfam database (34), and predictions for signal peptides (35) and transmembrane domains (36).

Differential Gene Expression Analysis. To compare the transcriptional profile of ectopic eyes to that of control postocomeral head epithelium and regular compound eyes, we dissected both tissues from control and Dg-otd1 dsRNA-injected females of D. gazella (Fig. 3A), and extracted total RNA following a previously described protocol (20), except that RNA was extracted directly out of the TRIzol reagent using Direct-zol RNA MiniPrep Plus spin columns (R2072; Zymo Research). RNA was processed using a Truseq kit and sequenced on a NextSeq500 platform, as described above. We sequenced four biological replicates of each tissue and treatment. The resulting 75 bp single end reads were cleaned using Trimmomatic version 0.32 (29). Read alignment to
The d. gazella reference transcriptome and transcript abundance estimation was performed using the align_and_estimate_abundance.pl and abundance_estimation.pl scripts included in Trinity 20140717 (30), using Bowtie2 (37) as alignment method and RSEM (38) as abundance estimation method. Differential expression analysis was performed by the DESeq2 (39) method (that uses a negative binomial model to perform pairwise Wald tests, adjusting for multiple testing using the Benjamini-Hochberg method) using Trinity v2.4.0's run_DE_analysis.pl and analyze_diff.expm.pl scripts, including GO enrichment analysis by using the examine_GO_enrichment parameter. The default P value cutoff for FDR of 0.001 was used. Tables of differentially expressed genes for each contrast were merged with the transcriptome annotation table described above using customized R (40) scripts. Cluster and correlation analyses were run on a log2-transformed matrix with an added pseudo count of one. A correlation matrix was computed with the Pearson method using R's cor function, an Euclidean distance matrix was computed with the dist function, and hierarchical sample clustering was performed with the hclust function using the complete linkage method. A sample-to-sample correlation heatmap and dendrogram was generated using the heatmap3 function bundled with Trinity v2.4.0. Scripts and data are available as Datasets S2–S11.

**Behavioral Testing of Ectopic Eyes Functionality.** Testing of the ability of regular compound eyes and ectopic eyes to trigger a behavioral response took advantage of the aversive conduct toward sudden, light, strong, light shown by tunneling dung beetles when they are in a tunnel-like environment (41). Assays were conducted in a dark room and used a standard Petri dish (5.2 cm diameter) as arena to allow beetles to turn around and a cold-light dual gooseneck illuminator as light source. The light was blocked using a piece of folded tinfoil as arena to allow beetles to move away from the light source until a ternary choice (that uses a negative biological model to perform pairwise Wald tests, adjusting for multiple testing using the Benjamini-Hochberg method) using Trinity v2.4.0's run_DE_analysis.pl and analyze_diff.expm.pl scripts, including GO enrichment analysis by using the examine_GO_enrichment parameter. The default P value cutoff for FDR of 0.001 was used. Tables of differentially expressed genes for each contrast were merged with the transcriptome annotation table described above using customized R (40) scripts. Cluster and correlation analyses were run on a log2-transformed matrix with an added pseudo count of one. A correlation matrix was computed with the Pearson method using R's cor function, an Euclidean distance matrix was computed with the dist function, and hierarchical sample clustering was performed with the hclust function using the complete linkage method. A sample-to-sample correlation heatmap and dendrogram was generated using the heatmap3 function bundled with Trinity v2.4.0. Scripts and data are available as Datasets S2–S11.

15. Moczek AP (2000) Male horn dimorphism in the scarab beetle, Onthophagus taurus genome annotations v0.5.3. Ag Data Commons, 10.15482/USDA.ADC/1255153.
19. Moczek AP, Emlen DJ (2000) Male horn dimorphism in the scarab beetle, Onthophagus taurus genome annotations v0.5.3. Ag Data Commons, 10.15482/USDA.ADC/1255153.
21. Zattara EE, Hughes DST, Richards S, Kijimoto T, Moczek AP (2016) Orthophasus taurus genome annotations v0.5.3. Ag Data Commons, 10.15482/USDA.ADC/1255153.