Highly Efficient Knockout of a Squid Pigmentation Gene

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Summary

Seminal studies using squid as a model led to breakthroughs in neurobiology. The squid giant axon and synapse, for example, laid the foundation for our current understanding of the action potential (1), ionic gradients across cells (2), voltage-dependent ion channels (3), molecular motors (4–7), and synaptic transmission (8–11). Despite their anatomical advantages, the use of squid as a model receded over the past several decades as investigators turned to genetically tractable systems. Recently, however, two key advances have made it possible to develop techniques for the genetic manipulation of squid. The first is the CRISPR-Cas9 system for targeted gene disruption, a largely species-agnostic method (12, 13). The second is the sequencing of genomes for several cephalopod species (14–16). If made genetically tractable, squid and other cephalopods offer a wealth of biological novelties that could spur discovery.

Within invertebrates, not only do they possess by far the largest brains, they also express the most sophisticated behaviorals (17). In this paper, we demonstrate efficient gene knockout in the squid *Doryteuthis pealeii* using CRISPR-Cas9. Ommochromes, the pigments found in squid retinas and chromatophores, are derivatives of tryptophan, and the first commited step in their synthesis is normally catalyzed by Tryptophan 2,3 Dioxygenase (TDO; (18–20)). Knocking out TDO in squid embryos efficiently eliminated pigmentation. By precisely timing CRISPR-Cas9 delivery during early development, the degree of pigmentation could be finely controlled. Genotyping revealed knockout efficiencies routinely greater than 90%. This study represents a critical advancement towards making squid genetically tractable.

Results

Selection of *D. pealeii* as a model

D. pealeii was selected as a model because of several favorable characteristics. It is readily available, its embryos are transparent and their development has been well characterized (21). In addition, oocytes can be fertilized *in vitro* and the period between fertilization and the first cell division is relatively long, enabling reagents to be delivered early (22). Finally, transcriptome sequences are available (23, 24) and, although unpublished, the genome has been sequenced and was available at the onset of this study. Despite these attributes, *D. pealeii* requires >6 months to reach sexual maturity (25) and its life cycle has not been closed. Therefore, our goal was to determine whether we could produce G0 knockouts.

Selection of *tdo* for knockout

As a target for knockout, we wanted a non-essential gene with a clear phenotype during embryonic development. In cephalopods, the pigments in the eyes and chromatophores are ommochromes, a derivative of tryptophan (Figure 1A) (18–20). Ommochromes also pigment the eyes of *Drosophila* where their genetics and biochemistry has been studied in detail (26). In

invertebrates, Tryptophan, 2,3 Dioxygenase (TDO) catalyzes the first committed step in ommochrome biosynthesis, converting Tryptophan to N-Formylkyneurenine ((27) Figure 1B). We hypothesized that its disruption would reduce or eliminate ommochrome synthesis in squid as well. To verify that TDO was an appropriate choice, we determined whether a TDO-selective inhibitor (680C91) impeded pigmentation in developing embryos. The development of *D. pealeii* has been divided into 30 stages and eye pigmentation appears at ~stage 25, while chromatophore pigmentation starts at stage 26 (21). We added 680C91 to developing embryos at stage 20, and it clearly blocked pigmentation in both the eyes and chromatophores, with animals developing normally otherwise (Figure 1C). These data supported our choice of TDO.

A single TDO gene was annotated in the *D. pealeii* genome based on similarity to homologs from other species. The gene is large and highly fragmented, consisting of 13 exons spanning over 120 KB (Figure 1D). A phylogenetic comparison of this sequence with diverse TDOs, and indolamine-2,3-dioxygenases (a different enzyme that catalyzes the same reaction), supported its identity as TDO (Figure S1). We next analyzed the expression profile of *D. pealeii* TDO. *In situ* hybridization of stage 22 embryos showed punctae on the arms and mantle, consistent with chromatophore expression (Figure 1E). Expression was robust within the eyes as well. Comparative expression calculated from RNAseq data yielded a similar picture for adult specimens, with highest expression in the retina and the chromatophore layer of the skin (Figure S1B). Taken together, these data suggested that disrupting *tdo* would lead to a loss of pigmentation in the chromatophores and eyes.

Microinjection in *D. pealeii* embryos

To knockout *tdo*, we adopted a standard approach using CRISPR gRNAs and Cas9 nuclease. Single gRNAs were designed to exons 1 and 2 of *tdo* (Figure 1D) and synthesized chemically with protecting groups. These were mixed with recombinant *Streptococcus pyogenes* Cas9 protein to form ribonuclear complexes. Our intention was to inject these components into early

stage embryos, however there were several obstacles to overcome (Figure 2). First, the chorion surrounding *D. pealeii* embryos is thick and tough, resisting even beveled, quartz injection needles. To overcome this issue, we made partial cuts through the chorion above the site of injection using “micro-scissors” fashioned from #5 forceps (see methods; Figure 2 B&C). This was a delicate process: if the cuts were too large, the embryo and yolk would extrude through them, arresting development. For stability, embryos were nested blastodisc side up in agarose and beveled quartz needles were passed through the cuts. A second challenge became apparent following the preliminary injections of dyes. Unlike zebrafish embryos, which form their blastodisc via microfilament-guided streams directing cytoplasm up through the yolk cell (28), squid embryos form their blastodiscs via microtubule driven cortical streaming (29, 30). Therefore, injections had to be made directly into the blastodisc, a relatively shallow target with a depth of 20-40μm. Figure 2D shows a small bolus of DiI (1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate) injected through a cut in the chorion. This panel shows an embryo injected after 4th cleavage to demonstrate the precision of our injections. Figure 2E summarizes the features relevant to injections. After injections, embryos were cultured until hatching and monitored for pigmentation.

To maximize the potential knockout efficiency, we performed the majority of our injections before the first cleavage. Embryos were fertilized *in vitro*. It took approximately 3.5 hours post fertilization (hpf) to reach the onset of the first cell division, defining our window for injections. As *D. pealeii* oocytes are transparent, we could monitor the events leading to the first cell division to time injections precisely. Following fertilization, the zygote membrane pulls away from the chorion surface and the blastodisc begins to form (29, 30). The egg nucleus resides within the thin layer of cortical cytoplasm eccentric to the micropyle in the unfertilized egg and has yet to complete meiosis. With fertilization, meiosis is completed before egg pronuclear migration and fusion. At room temperature (20-21^{o}C), the first polar body forms 20 minutes post fertilization, while the second polar body is released 1.5 hpf. Egg pronuclear migration toward the sperm nucleus,

98 located beneath the micropyle, begins shortly thereafter and terminates in nuclear contact 3 hpf.
99 The realtive proximity of the pronuclei is an excellent indicator of the time post-fertilization (Figures
100 2E and 4 “I” panels).

101 **Knockout phenotypes**

102 The injection of two sgRNAs and Cas9 resulted in a reduction of chromatophore and eye
103 pigmentation, however the extent depended on the precise time of injection (Figure 3). Panel 3A
104 shows an example of a control embryo at hatching and one that was injected 2 hpf (30 minutes
105 post 2nd polar body formation). For embryos injected at this stage, pigmentation is completely
106 absent from the chromatophores and is minimal in the eyes which are light red (n=9). The large
107 black spot near the posterior of the mantle is the ink sack. Ink pigment contains melanins, not
108 ommochromes, and is unaffected by TDO disruption. Panel 3B shows more examples of embryos
109 injected during early pronuclear migration (2.5 hpf). Most, but not all, of the chromatophores are
110 missing pigmentation and the eyes are light red (n= 14, 8 white, 4 nearly white, 2 with darker eyes
111 and a few pigmented chromatophores). In panel 3C, injections were after nuclear contact (3 hpf)
112 but before first cleavage (3.5 hpf). These produced mosaic patterns of chromatophore
113 pigmentation (n=21, all with mosaic pigmentation) and a range of eye pigmentation from pink to
114 dark brown, often in the same embryo. Injection into a single blastomere following first cleavage
115 (3.75 hpf) often produced embryos where chromatophores were missing over half of the mantle
116 (Panels 3D&E). Thus, stronger phenotypes were produced with earlier injections; however, the
117 blastodisc is narrower at earlier stages and a more difficult target. In addition, embryo survival
118 was poorer at earlier times of injection (~40-50% loss for embryos injected between 1.5 – 2.0 hpf).
119 These data showed that *tdo* sgRNAs produced appropriate and consistent reductions of
120 pigmentation.

121

Knockout genotypes

To corroborate the phenotypes produced by the sgRNA injections, we examined genetic disruptions of *tdo*. At hatching, DNA was extracted from whole embryos and amplicons bracketing the targets for sgRNA1 and sgRNA2 were amplified by PCR. Individual amplicons were sequenced using MiSeq, yielding on average ~ 50,000 paired-end reads that covered the entire amplicon. Figure 4A shows an example of a hatchling that was injected before first cleavage with both sgRNAs. Most pigmentation in the chromatophores and the eyes is missing. For amplicons covering the targets of each sgRNA, we then calculated the frequency that each base was either missing (blue) or had an insertion at that position (red). For each amplicon, there is a peak at the position specified by the CRISPR gRNA for Cas9 cleavage (see triangle). Deletions were more common than insertions and the shape of the plot indicates that multiple deletion events occured.

Assuming that indels at each guide are independent, we estimate that the total *tdo* disruption in this individual was ~90%. Figure 4B shows the same analysis for a control embryo where no indels are present at the specified sites (for sgRNA1 there is a small peak of deletions at ~nt10 present in all control and the experimental samples that was due to a PCR error over highly repetitive intronic sequence). We performed a similar analysis on 20 CRISPR-Cas9 injected individuals and 3 controls (Figure 4C). The cumulative disruption of *tdo* ranged from ~30-95%. sgRNA1 performed marginally better than sgRNA2, but they both were effective. Figure 4D presents a breakdown of the individual indel events from a representative specimen. In this case 46 indels were identified, the most frequent representing less than 20% of the total. Other specimens had a large range of events (from 8-78) and in few cases did a single event occur at a frequency of more than 20% (Figure S2A&B). Across samples, there was a large range of indel sizes: for sgRNA 1, 95% of the deletions were less than 43 bp and 95% of the insertions were less than 8 nt (median deletion = 9nt, median insertion = 2 bp) and for sgRNA 2, 95% of the deletions were less than 25bp and 95% of the insertions were less than 7bp (median deletion = 7

147 nt, median insertion = 3 bp; Figure S2 A&B). These data indicate that CRISPR-Cas9 injections
148 produced multiple indels at the targeted locations.

149 **Discussion**

150 This study demonstrates that squid genes can be efficiently disrupted using the CRISPR-
151 Cas9 system. We routinely disrupted *tdo* at efficiencies >90 %, resulting in an almost complete
152 lack of pigmentation. Given that both sgRNAs worked well, we expect similar efficiencies at other
153 targets. Similarly efficient G0 knockouts have proven to be useful research tools for other
154 organisms, including butterflies, amphipods and even zebrafish (31–35). We expect that they will
155 be similarly useful with squid. *D. pealeii* is not culturable in captivity at this point. We routinely
156 raise embryos through hatching, but mortality is high thereafter. Accordingly, this species will not
157 be used to establish genetic lines, but it still offers great utility, particularly for developmental
158 studies. Life-cycle culture is possible for other squid species, including *Euprymna scolopes*, a
159 model with a published genome that is commonly used to investigate bacterial-animal symbioses
160 (16, 36, 37). We expect that our methods, with modifications, will be transferable.

161 Interestingly, injecting before the first cleavage produced high knockout efficiencies; however,
162 it also produced large numbers of indels, and single events did not dominate (Figure 4D and
163 Figure S2). The average number of distinct indels was 38 ± 13 (SD) per specimen and the average
164 frequency of the most frequent event was 15 ± 8% (SD). In no case did we observe an event at
165 a frequency greater than 30%. This indicates that indels were not formed before the first cleavage,
166 and that they were being created continuously across subsequent cell divisions. While delivering
167 CRISPR-Cas9 prior to the first cleavage ensures that it is present in subsequent blastomeres, it
168 is unclear why the precise timing of reagent delivery before the first division is important.
169 Considering that Cas9 protein was injected and its activity was still delayed, the injection of Cas9
170 mRNA would probably be less effective.

Our methods for gene knockout should be readily adopted by other research groups. *Loliginid* squid are available worldwide and our methods do not require specialized equipment. When the *D. pealeii* genome is released, CRISPR sgRNAs can be designed to avoid off-target edits. The ability to knockout genes in squid will enable us to ask new questions. Some examples include: how does the cephalopod brain encode complex behaviors in comparison to the vertebrate brain? What is the mechanistic basis of high-level mRNA recoding in cephalopods and how is it deployed to respond to the environment (23, 24, 38)? How is camouflage produced structurally and controlled by the brain? And what controls development of the unique cephalopod body plan? This study provides a way forward to investigate these questions, and many others.

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Author contributions

KC developed the microinjection techniques, and injected, cultured and imaged the embryos in this study. KK researched squid pigmentation pathways and performed preliminary TDO inhibitor studies. KC performed the reported TDO inhibitor studies. CA identified the tdo locus with input from TDO trancriptome sequences provided by KK and JR. CA performed all gene expression studies. JR and NA prepared genotyping samples. NA developed injection needles. JD performed all computational work on genotyping. KC designed approaches for microinjection and embryo culture. JR and CA designed CRISPR-Cas9 knockouts and JD designed bioinformatic scripts for analyses. JR wrote the manuscript in collaboration with KC, CA, and JD, along with editing support from KK, and NA.

Declaration of interests

The authors declare no competing interests

Figure Legends

Figure 1. TDO activity is required for pigmentation in chromatophores and retinas in developing *D. pealeii*. A) A recently hatched *D. pealeii* with relevant anatomical structures labelled (dorsal view). B) A schematic for ommochrome biosynthesis from tryptophan. KF = kyneurenine formamidase, KMO = kynurenine 3-monooxygenase, POS = phenoxazinone synthase. C) Stage 27 embryos that were treated with either DMSO (left) or 3.15 μM TDO inhibitor 680C91 (right) starting at stage 20. D) The TDO locus spans 123Kb in the D. pealeii genome, with 13 exons. Intron size is to scale. E) Fluorescent *in situ* hybridization for TDO in stage 22 *D. pealeii* embryos demonstrates expression in the developing eyes and chromatophores on the mantle, above the eyes, and on the arm primordia. Anterior (top) and posterior (bottom) views of the in situs (left) are shown along images of stage matched live embryos (right). * indicates arms,

t = tentacle, pff = posterior funnel fold, ap = anal papilla, gp = gill primordia, st = statocyst. Scale bar = 250 μm. See Figure S1 for more details on TDO intentification and expression.

Figure 2. Microinjection method for squid embryos. A) Side view whole embryo. The tough outer chorion (white arrow) is visible along with the blastodisc (at the animal pole, white arrow) and yolk in this telolecithal embryo. The polar bodies (black arrow) are also visible. B) Close up view of micro-scissors used to clip small cuts in the chorion. C) Animal pole view showing the chorion cuts (white arrows) and polar bodies on the surface of the zygote. D) Reference view, DiI injected embryo (black arrowhead). This embryo was injected at the 8-cell stage and has progressed through 4th cleavage. The chorion cuts (white arrows) and polar bodies (black arrow) are shown. The polar bodies reside near or in the anterior mid-line. E) Diagram of an embryo nested in agarose in which a beveled quartz injection needle has been passed through the chorion cut and into the blastodisc layer. The egg (black arrowhead) and sperm (blue arrowhead) pronuclei are represented in this panel. Inset, depicts the relative position of the polar bodies in an embryo ~2.5 hpf. Scale bars = 250 μm.

Figure 3. Timing of CRISPR-Cas9 injection affects pigmentation. Ai) Embryo blastodisc 2.0 hpf; Bi) 2.5 hpf; Ci) 3.0 hpf; and Di) 3.75 hpf. The blastodisc (a thin lens of cytoplasm) increases in thickness throughout this series of images. The egg pronucleus (black arrowhead) approaches the sperm pronucleus (blue arrowhead) in Ai, Bi, and Ci. Di) One of the nuclei of first cleavage, along with the meroblastic cleavage furrow (white arrow) are visible in this panel. Aii) The embryo on the left is a control hatchling; note the black and reddish brown chromatophores evenly placed across its mantle, head and tentacles. In contrast, the embryo on the right was injected with 2 CRISPR sgRNAs αTDO and Cas9 at 2 hpf and has very few pigmented chromatophores, in addition to light pink to light red eyes. Close inspection reveals that there are 2 diminutive pigmented cells positioned medial to each eye. Bii) CRISPR-Cas9 αTDO embryos injected at 2.5 hpf. These embryos are missing nearly all their pigment and exhibit a range of eye color from faint

pink to darker red. Cii). These embryos were injected post nuclear contact, 3.0 hpf and exhibit a range of mosaic patterns, from embryos missing some pigmented chromatophores to others missing large patches or regions of pigmented chromatophores. A range of eye pigmentation from deep brown to light red is also typical in this group. Dii) CRISPR-Cas9 TDO embryo injected post first cleavage, ~3.75 hpf, into one cell only. This embryo is missing pigmentation on half its body, the side that would form from the injected cell. E. Control embryo with normal chromatophore pigmentation and patterning. All views are ventral. Ai, Bi, Ci, and Di are all at the same magnification, scale bar in Ai = 250μm. Aii, Bii, Cii, Dii, and E, Scale bar = 500 μm.

Figure 4. Efficient *tdo* gene disruption using CRISPR-Cas9. Hatchlings are shown for an individual that was injected with Cas9 and two sgRNAs targeting tdo (A) vs a control embryo (B). Pictures reveal a loss of pigmentation in the chromatophores of the experimental animal. Genomic DNA was extracted from these animals and amplicons surrounding the target sites for sgRNA 1 and sgRNA2 were generated by PCR and then sequenced with MiSeq. Histograms show the frequency that each position in the amplicon is deleted (blue) or contains an insertion (red). Triangles indicated the Cas9 cut site as specified by the CRISPR sgRNA. C) In similar experiments on 3 control animals and 20 CRISPR-Cas9 injected animals, the percent *tdo* gene disruption was determined for each sgRNA alone and together. D) A representative histogram of the frequency of specific indels (46 in total) are presented for a CRISPR-Cas9 injected individual. See Figure S2 for more details on indel characterizations.

Star Methods

Resource availability

Lead contact: Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Joshua Rosenthal (jrosenthal@mbl.edu).

Materials availability:

This study did not generate unique reagents.

Data and code availability

The scripts (Cumulative.py, indels.py, Event_frequency.py) generated during this study are available at Github (https://github.com/pipedq/CRISPR_G0_Genotyping). The sequence for the *D. pealeii* TDO locus is available at Genbank (MT648678). RNAseq reads are deposited as Bioproject PRJNA641326. Reads from the amplicon sequencing are available on request.

Experimental Model and Subject Details

Adult specimens of *D. pealeii* were obtained from the Vineyard Sound by otter trawl between July and October 2019 by the Marine Resources Center of the Marine Biological Laboratories, Woods Hole, MA. Embryos were obtained via *in vitro* fertilization and maintained at 18 °C in well aerated 0.22μM filtered seawater (FSW).

Statement on the ethical treatment of animals

Although the use of cephalopods for research is not currently regulated in the USA, the Marine Biological Laboratory has implemented strict internal policies to ensure their ethical and humane treatment. All specimens of *Doryteuthis pealeii* used in this study conformed to the Marine Biological Laboratory's "Policy for the use of cephalopods for research and teaching."

METHOD DETAILS

Identifying the TDO gene in *D. pealeii*

The human TDO and IDO protein sequence was used as bait to search the proteomes of *D. pealeii*, *Octopus bimaculoides*, *Euprymna scolopes*, *Architeuthis dux*, *Crassostrea gigas*, *Capitella teleta*, *Drosophila melanogaster*, *Tribolium castaneum*, *Mus musculus*, and *Homo sapiens* with BLASTP. The identified sequences were aligned using MUSCLE (39), and an approximately-maximum likelihood tree was built with FastTree2 (40) and illustrated with FigTree (http://tree.bio.ed.ac.uk/software/figtree). The TDO sequence was mapped using BLAST onto the *D. pealeii* genome assembly (in preparation, provided by C Albertin, a member of the genome project) to determine the structure of the locus. The sequence of the *tdo* locus is deposited in Genbank (MT648678).

Specimen collection and oocyte preparation

Oocytes and spermatophores were removed by dissection from adult *D. pealeii* and fertilized *in vitro* at room temperature as previously described (22). Upon sacrifice and dissection, mature oocytes were collected from the oviduct of the gravid female and placed into well aerated FSW in a medium sized (4.5 inches) glass fingerbowl. Eggs were washed 2x with FSW by gentle swirling and decanting. Next, several spermatophores (5-6) were collected from the distal tip of the penis of the male, placed into a small (2 inch) glass Syracuse dish half filled with aerated FSW, and compressed to induce the release of sperm. Concentrated drops of eggs, collected with a fire polished large bore glass Pasteur pipette are next added to the Syracuse dish with the FSW sperm mixture. After 20 minutes, the egg/sperm mixture is washed back into FSW in a medium finger bowl and washed several times with FSW to remove excess sperm. Following fertilization, embryos were cultured in 60-mm plastic Petri dishes lined with 0.2% agarose (Type II-A, Sigma),

and filled with FSW supplemented with 1:100 dilution of Penicillin-Streptomycin 100x (Gibco, 15-140-122) at 18ºC. Agarose was prepared by microwaving in FSW until melted, allowed to cool and then poured to line (non-plasma treated) Petri dishes. Dishes were stored at 4ºC in closed plastic containers until needed. Freshly fertilized embryos 10-20 embryos were plated per dish in well aerated FSW-PS.

Oocyte injection

After second polar body formation (1.5 hpf), embryos laying naturally on their sides were held gently in place against the agarose cushion with a standard pair of watchmaker's forceps. Using the modified "scissors" made from forceps (see below), a partial clip was made to the apex of the chorion to create two small cuts (~8-10µm long cuts) angled away from each other. It is important that these cuts are partial, and the two blades of the scissors do not fully close. When done properly, each blade should make a small incision in the chorion. Partial cuts heal after injection allowing the chorion to elevate normally. If the cut is too great and the blades brought too close together, a large "V" shaped incision results and the gel-like embryo/yolk extrudes through the small opening, destroying the embryo.

Once multiple embryos are clipped, they are positioned blastodisc side up within the soft agarose base using a polished Pasteur pipette formed into the shape of a "hockey stick" (~1mm in diameter) over a flame. In this position, the micropyle, chorion cuts, polar bodies (marking the outer membrane surface of the zygote) and deeper yolk are easily visible. Embryos were injected by passing a beveled quartz needle (2-3 µm tip with a 15º angled bevel) through one of the chorion cuts and up to but not through the zygotic membrane. Using diffracted light, it is possible to see the outer membrane of the embryo begin to indent from the pressure of the needle and with that, a gentle tap to the long end of the micromanipulator control knob will "pop" the needle tip into the

342 blastodisc, but not into the yolk. Driving the needle into the blastodisc with the micromanipulator
343 can easily result in overshooting the blastodisc and wounding the yolk. Significant trauma to the
344 yolk layer results in death.

345 The microinjection set-up consisted of a Xenoworks Digital Pressure Injector (Sutter
346 Instruments, Novato CA), mounted on a Discovery V8 steroscope (Zeiss) using an MMO-202ND
347 manual 3-axis manipulator (Narishige). Quartz micropipettes were pulled on a P-2000G Pipette
348 Puller (Sutter Instruments) and beveled using a BV-10 Micropipetted Beveller (Sutter
349 Instruments). The program used to pull the pipettes consisted of one line with the following
350 settings: heat 750, filament 4, velocity 60, delay 140, pull 175. After pulling, they were bevelled
351 for 30 seconds at a 20º angle. Settings on the injector were Pressure 74, Width 0.21, and Positive
352 Pressure Flow 10. Using these settings, we estimated that 0.221 pL were injected per oocyte by
353 measuring the volume under these settings injected into a drop of oil.

354

355 CRISPR sgRNAs and Cas9 protein

356 Chemically modified CRISPR sgRNAs were synthesized by Synthego (Menlo Park, CA) as was
357 recombinant 2X NLS Cas9 protein (Cas9 2NLS Nuclease). The gene-specific sequence for
358 sgRNA1 was CAUCCAAUCAGUGCCGAAGC and for sgRNA2 was
359 UGGCAGCUGAGGUUCGUGUU. The solution that was injected into oocytes consisted of 34 μM
360 sgRNA (17 μM each), 7μM Cas9 protein and 1.7X PBS. Given the volume that was injected, we
361 estimate that 3.76 amol of each sgRNA and 1.54 amol of Cas9 protein were injected per oocyte.

362

363 Post-injection care of embryos

Following injections, embryos were observed daily and moved using clean, large bore polished glass pipettes to fresh culture dishes with MFSW-PS every two days. Some embryos were cultured within their chorions while others were mechanically dechorionated using fine forceps during early organogenesis (after 5 or 6 dpf) to facilitate photography. Dechorionated and chorionated embryos were co-cultured in the same dishes. Dechorionated embryos were not distinguishable developmentally from their siblings. Embryos were cultured for at least 18 days or until control embryos began to hatch from their chorions. For imaging, embryos were anesthetized in 6% Ethanol in FSW and imaged with a Nikon CoolPix 995 camera mounted on a Zeiss Stemi 2000-C Trinocular scope. Embryos were staged according to (21).

Forceps-scissor fabrication

Using a new set of forceps (Inox; #5), the final ~1 mm of each tip is bent 15 to 30 degrees using the fine pliers of a forceps repair kit. The tips should both bend inward. The tips should engage as miniature scissors. A high magnification image of the scissors used in this study are presented in Figure 2.

TDO Inhibitor Treatment

Embryos were treated with 3.15 μM TDO inhibitor 680C91 (Sigma-Aldrich) in MFSW-PS from early organogenesis stage 20 (21), through late organogenesis (stage 27). Dilutions were prepared from a 42 mM stock solution dissolved in dimethyl sulfoxide (DMSO, 10 mg/ml). Dishes and treatment solutions were refreshed every other day. Control embryos were cultured in the presence of DMSO without the inhibitor.

In situ hybridizations

In situ hybridization was carried out as in Shigeno et al. with several modifications (41). A 751 bp sequence of the *D. pealeii* TDO gene (nt 417-1167) was amplified by PCR (using primers GAGCAATCGCGTCAAGTACA and GGCGTTGTCTTCAGGGTAGA) and cloned into pGEM T-Easy. Digoxigenin-labeled nucleotides (Roche 11277073910) were incorporated into antisense riboprobes generated with SP6 reverse transcriptase (New England Biolabs M0207L) according to the manufacturer's instructions. Embryos were anesthetized in 2% ethanol in FSW and fixed overnight at 4°C in 4% paraformaldehyde (Electron Microscopy Sciences 15714) in filtered sea water. Fixed embryos were washed for five minutes two times and for 30 minutes once in DEPC-PBS (phosphate buffered saline treated with diethyl pyrocarbonate) and stored in hybridization solution (50% formamide, 5xSSC, 1% SDS, 250g yeast RNA, and 0.1g heparin sulfate per 500mL) at -20°C until use. Tissue was prehybridized for at least 1 hour at 72°C and incubated overnight with 30μL antisense RNA probe reaction at 72°C. Tissue was washed once quickly, four times for 30 minutes, and once for 1h in preheated Solution X (50% formamide, 1% SDS, 2xSSC). Embryos were then washed three times for 15 minutes in TBST (25mL 1M Tris-HCl, 8g NaCl, 0.2g KCl per 1L with 1% Tween) and blocked overnight at 4°C in 10% Roche blocking buffer in TBST. Riboprobes were detected with an anti-DIG antibody coupled to horseradish peroxidase (Sigma 11207733910) diluted to 1:250 in 10% Roche blocking buffer in TBST and incubated at room temperature for 2 hours. Following antibody incubation, embryos were washed three times for 15 minutes and three times for 30 minutes with TBST, followed by two 5 minute washes in TNT (0.1M Tris HCl pH 7.5, 0.15M NaCl, and 0.05%. Tween). Embryos were washed in 50μL amplification diluent and incubated for 1 hour in the dark with Cy5 tyramide (Perkin Elmer) diluted 1:50 in amplification diluent. Embryos were washed three times for 15 minutes in TBST, and twice in Solution X preheated to 72°C and stored in TBST until imaging. Embryos were mounted in Fluoromount-G with Dapi (Southern Biotech) and imaged on an LSM-710 confocal microscope (Zeiss).

411

412 TDO expression in the transcriptome

413 RNAseq reads generated from the *D. pealeii* genome project (Bioproject PRJNA641326) were
414 mapped onto the genome with Star 2.7.0 (42). Transcripts per million (TPM) for selected tissues
415 were plotted using Rstudio.

416

417 Genotyping CRISPR-Cas9 embryos

418 In order to genotype embryos, genomic DNA was isolated from whole individuals according
419 to a method originally used for Ciona, which uses 25 uL of a Ciona DNA Extraction buffer (made
420 of 1% Triton X, 100 mM NaCl, 20 mM Tris-HCL pH 7.8 and 1mM EDTA), 25 uL H20 and 2.5 uL
421 Proteinase K to digest the ground up embryo, all incubated at 55 °C for 2 hours (42). Samples
422 then underwent an extraction with phenol: chloroform (1:1) followed by a further purification using
423 the Monarch DNA kit (NEB). Nested PCR amplifications surrounding sgRNA-targeted sequences
424 in TDO were the performed. For sgRNA1 we used primer pair
425 GCCTCAAAACAACCCATATTATTGAGG + GAGTTGTAGCGCATCTGAGCAC followed by
426 primer pair TAAATACTTGTGTTCATAGGGTACAC + GGTAAACCCGCTCTGAGTTATTTCCC
427 which resulted in a 215 bp amplicon. For sgRNA2 we used primer pair
428 GCGTGCTATTCTGCATTAGCAC + CGTTAAACCAGTTCTGCCCTCAAG followed by primer
429 pair CCCTAACCATAACCTTAACGTCTC + GCATTCTGTACGATGACACTAAGC which resulted
430 in a 390 bp amplicon. In some cases, we added partial Illumina tags to the nested reactions
431 (Forward ACACTCTTTCCCTACACGACGCTCTTCCGATCT and reverse
432 GACTGGAGTTCAGACGTGTGCTCTTCCGATCT) but in other cases they were added by
433 Genewiz during library preparation. PCR products were then gel extracted and quantified prior to

sequencing on the MiSeq platform (paired end 250 nt) using the Amplicon EZ service at Genewiz (South Plainfield, NJ).

Reads were aligned to the amplicon reference sequence using Bowtie2 with the local configuration (43). Because we were expecting misalignment to the reference sequence due to indels, we reduced the gap open and extend penalty from 5 and 3 respectively (default setting) to 3 and 1. Indel analysis was performed by processing the aligned reads using the mpileup function in the SAMtools package filtering out base-calls with Phred Quality (Q) <40 (44). A summary of alignment and filtering results are shown in Table S1. From the mpileup files that were generated, the Indel.py script was used to determine the percentage of deletions per position (number of reads that were missing a position over the total number of reads covering the position). The percentage of insertions per position were calculated in the same way, and both percentages were used to graph the InDel histograms presented in Fig 5A&B. To calculate the total disruption per animal, the script Cumulative.py examined a region spanning -1nt to +1nt from the expected Cas9 cut site designated by the CRISPR sgRNA (total number of reads that contain a deletion and/or insertion event and divided by the total number of reads). Finally, to identify specific indels, the Event_frequency.py script (GitHub) was used to determine the number of reads at a position that have a distinct deletion (e.g. -1A, -2AG etc…) or insertion (e.g. +1A, +1G etc…) event and analysis was limited a region spanning -5nt to +5nt from the expected Cas9 cut site designated by the CRISPR sgRNA. Events that occurred at a frequency higher than 0.1% were used for analysis.

Quantification and statistical analyses

To quantify the overall frequency of specific deletions or insertions for all animals, the script Cumulative_Event_frecuency.py was used. The scripts takes in indel frequency data of all

animals and create a histogram that records how frequent the deletion or insertion of X number of bases in all reads is in all reads in all the animals for each CRISPR sgRNA. The script also encodes for equations needed for obtaining the parameters of descriptive statistics (mean, median, quartiles, e.g) used to characterize the frequency distribution.

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