The importance of a halotyrosine dehalogenase for Drosophila fertility

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Running title: A dehalogenase mediates Drosophila fertility

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ABSTRACT

The ability of iodotyrosine deiodinase to salvage iodide from iodotyrosine has long been recognized as critical for iodide homeostasis and proper thyroid function in vertebrates. The significance of its additional ability to dehalogenate bromo- and chlorotyrosine is less apparent and none of these functions could have been anticipated in invertebrates until recently. Drosophila, as most arthropods, contains a deiodinase homolog encoded by CG6279, now named condet (cdt), with a similar catalytic specificity. However, its physiological role cannot be equivalent because Drosophila lacks a thyroid and its associated hormones and no requirement for iodide or halotyrosines has been reported for this species. We have now applied CRISPR/Cas9 technology to generate Drosophila strains in which the cdt gene has been either deleted or mutated to identify its biological function. As previously shown in larvae, expression of cdt is primarily limited to the fat body and we now report that loss of cdt function does not enhance sensitivity of larvae to the toxic effects of iodotyrosine. In adult flies by contrast, expression is known to occur in testes and is detected at very high levels in this study. The importance of cdt is most evident by the decrease in fertility observed when either males or females carry a deletion or mutation of cdt. Therefore, dehalogenation of a halotyrosine appears essential for efficient reproduction in Drosophila and likely contributes to a new pathway for controlling viability in arthropods.

The flavoprotein iodotyrosine deiodinase (IYD) was discovered first in humans while studying the biochemical basis of thyroid disease (1, 2). This enzyme is necessary for maintaining iodide homeostasis and acts by salvaging iodide from iodotyrosines (I-Tyr and I2-Tyr, Scheme 1) that are formed during thyroxine (tetraiodothyronine, thyroid hormone) biosynthesis. This process was originally presumed unique to the phylum Chordata because only these organisms are known to generate thyroxine and hence require iodide. In mammals, IYD is primarily expressed in the thyroid gland where this hormone is produced but it is also expressed at lower levels in the digestive tract, kidney and liver (3) where it may also dehalogenate chloro- and bromotyrosine (Cl-Tyr, Br-Tyr) (Scheme 1) (4, 5). These additional halotyrosines are generated during inflammation by activation of myeloperoxidase (6, 7). Human IYD obtained by heterologous expression demonstrates a nearly equal ability to bind and dehalogenate Br-Tyr and I-Tyr (8). Cl-Tyr also binds with similar affinity but is dehalogenated at a lower rate (20-fold) (8). Whether these additional activities of IYD are necessary or incidental in humans has yet to be determined.

The historical link between IYD and thyroid function might have suggested a possible coevolution. However, genes encoding IYD homologs now appear independent of thyroxine biosynthesis and are common to most animals as well as certain eubacteria and archaea (9, 10). This distribution suggests that IYD may play a variety of different roles in biology and the prevalence of its substrates, halotyrosines, may be underestimated (11, 12). To date, over 10
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homologs of IYD from diverse phyla have been expressed and purified and all of these promote deiodination of $I_2$-Tyr (9). Honey bee was selected as the first test of an IYD from arthropods based on its relatively low molecular weight and minimal Cys content. After its deiodinase activity was confirmed (9), attention has turned to Drosophila melanogaster for learning the biological role of IYD in an organism that is not known to require iodide (13, 14). Drosophila offers an excellent and well-established model organism but was not initially considered due to complications from alternative splicing of the gene locus (Figure S1). The IYD gene was originally listed in flybase as CG6279 and has since been named cdt, in honor of Dr. Jean François Condet who discovered that ingestion of iodide could reduce goiter (15). Isoform A of cdt encodes a 484 amino acid N-terminal sequence of unknown structure and function that extends from the canonical IYD domains. This region is common to all Drosophila species sequenced to date but is not shared by other Diptera (16). Isoform B contains only the three domains associated with most metazoan IYDs: an N-terminal membrane anchor, an intermediate region and a C-terminal active site structure (Figure S1) (10, 17).

Heterologous expression of the combined intermediate and active site domains encoded by cdt produces a stable and soluble enzyme (15). Its profile of activity is very similar to that of human IYD. For example, both have high affinities for I-Tyr, Br-Tyr and Cl-Tyr with $K_d$ values ranging from 0.1 - 0.6 μM (15, 18). Turnover efficiencies for I-Tyr and Br-Tyr are also nearly equivalent (ca. 7 - 9 x 10$^3$ M$^{-1}$s$^{-1}$) (8, 15). Dehalogenation of Cl-Tyr is less efficient by ca. 3-fold and 20-fold for Drosophila and human IYD, respectively. No IYDs have yet demonstrated an ability to dehalogenate fluorotyrosine (10, 19) and thus only I-Tyr, Br-Tyr, Cl-Tyr and their derivatives are possible substrates in vivo. An iodide requirement for Drosophila has yet to be reported but feeding studies with $[^{131}]$-iodide have established that iodide can accumulate in the protein fraction of cuticle and may also generate I-Tyr and $I_2$-Tyr (20). Other halotyrosines have also been identified in the scleroproteins of numerous invertebrates (21, 22) and likely result from a peroxidase activity associated with cuticle sclerotization (23). A need to recover bromide or chloride from Br-Tyr and Cl-Tyr would be even more surprising than a corresponding salvage of iodide. Both bromide and chloride are required by Drosophila but are readily available in the environment in contrast to iodide (24-26).

Existing precedence was not sufficient for speculating on the role(s) of IYD in invertebrates and its expression pattern in Drosophila only adds to its intrigue. Levels of cdt mRNA increase from larvae to adult where it is detected most abundantly in fat body and testis, respectively (27, 28). CRISPR/Cas9 (29) has now been used to create Drosophila mutant strains that either have a deletion at this gene locus (cdt$^\Delta$) or contain a point mutation E154Q (cdt$^Q$) with low catalytic activity (15) as an initial effort to learn the biological role of IYD in an organism not known to require iodide. As described below, a lack of the dehalogenase during the larval stage did not sensitize Drosophila to the toxic effects of I-Tyr. Thus, its function in juveniles remains to be established. However, high levels of cdt expression have now been confirmed in testes using in situ hybridization and loss of IYD or its dehalogenase activity reduces fertility. Surprisingly, these effects are observed when either the adult male or female lack sufficient dehalogenase activity.

Results and discussion

Generation of Drosophila strains with limited IYD activity in vivo

The biological function of an enzyme can often be defined on the basis of the phenotypes generated by mutants with compromised activity. CRISPR/Cas9 technology has revolutionized such loss-of-activity studies through its ability to remove or alter genes as desired relatively easily in most organisms. To delete the dehalogenase gene cdt (chromosome 3L, bases 11093371 to 11096245) from Drosophila (30), its sequence was analyzed for potential CRISPR sites using the “CRISPR Optimal Target Finder” algorithm (29). From this, a pair of CRISPR target sites that flanked over 90% of the cdt gene was selected (Figure S1B). These sites also minimized the chance of potential off-target reactions that were estimated to be fewer than seven and all contain at least 3 mismatches to the designed gRNAs. A single-stranded oligonucleotide (ODN1, Figure S2) was created
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The third candidate (lane 6, Figure S4) contained no additional nucleotide insertions, deletions or mutations from 278 bases upstream to 241 bases downstream of the targeted mutation. This strain was used for studies on the effects of the E154Q mutation (cdtQ).

A possible metabolic role for IYD in Drosophila

Expression of cdt in the larval fat body suggested a possible protective role for dehalogenation. Feeding studies had previously determined that I-Tyr was toxic to Drosophila larvae (32). This toxicity is apparently derived from the ability of I-Tyr to inhibit tyrosine hydroxylase and ultimately deplete dopamine levels. If tyrosyl residues within proteins became iodinated in Drosophila as they are in vertebrates, then subsequent hydrolysis of these proteins would release I-Tyr. Sensitivity towards I-Tyr might consequently increase if the dehalogenation activity of IYD was compromised. Thus, feeding studies were repeated with the cdtQ and cdtQ strains as well as two controls established by the parent vasa-cas9 and the standard wild-type (yellow, white) strains.

Addition of 100 µM I-Tyr to standard solid Bloomington media had little effect on the ability of Drosophila larvae to develop into adults (Figure 1). With an increase of I-Tyr to 150 µM, a decrease in survival was observed for the strain expressing the active site mutant of IYD (cdtQ). However, no decrease in viability was noted for the deletion mutant (cdtQ) or the control flies. Once the I-Tyr supplement was increased to 200 µM, survival of larvae to adults decreased in all strains. The cdtQ mutant strain again appeared to be the most sensitive to I-Tyr. The origin of this effect is not yet clear since a complete lack of the catalytic activity (cdtQ) does not induce a similar sensitivity to I-Tyr. All strains suffered almost complete lethality when I-Tyr concentration was increased to 300 µM. We also confirmed that these results were not a consequence of rejecting the food containing I-Tyr by adding a dye to the media and tracing the food intake. These tests showed that larvae continued to eat the media supplemented with I-Tyr (Figure S5).

Feeding studies were also repeated with alternative supplements of Br-Tyr and Cl-Tyr. Neither halotyrosine demonstrated the high toxicity of I-Tyr. Significant lethality was not
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By contrast, cdt expression was almost undetectable in bag of marbles (bam) mutant testes (RPKM=0.12). Because the bam mutation arrests male germ cell differentiation at the mitotic spermatogonial stage (36, 37), lack of cdt in bam testes suggests that cdt is not transcribed in mitotic male germ cells (38). By contrast, meiosis is initiated in aly mutant testes but its germ cells are arrested at the G2 phase as primary spermatocytes (39). The robust expression of cdt in aly mutant and wild-type testes indicates that it is tightly associated with the meiotic program of the male germ cells.

Antisense RNA probes were designed to detect the N-terminal extension unique to isoform A of cdt and the active site domain common to both isoforms A and B, respectively. Consistent with the RNA-seq results, in situ hybridization in wild-type testes detected enriched cdt mRNA in spermatocytes but not in the spermatogonial cells (marked with * in Figure 3A-B). The region and intensity of staining were also similar for both antisense probes. As controls, the corresponding sense probes produced no detectable signals in wild-type testes (Figure 3C-D). A similar expression pattern in the meiotic male germ line has not been reported in humans since no IYD has been detected in gonads or other reproductive organs.3

Drosophila fertility is promoted by cdt

Fertility assays were first performed for male flies using both the cdt\textsuperscript{A} and cdt\textsuperscript{Q} alleles, due to the high expression of cdt in adult testes. A trans-heterozygous strain carrying the cdt\textsuperscript{A} allele over a deficiency chromosome (Df) encompassing the cdt locus was also used to avoid any complication due to a second mutation on the same chromosome that may occur if homozygotes are used. A similar trans-heterozygous strain was also generated using cdt\textsuperscript{Q} (cdt\textsuperscript{Q}/Df). Corresponding heterozygotes of cdt\textsuperscript{A}/Df represented a wild-type control. Each of these fly strains was mated with wild-type females (vasa-cas9 strain, cdt\textsuperscript{Q}/cdt\textsuperscript{Q}) for 24 h and adult progeny were counted on successive days of egg laying (Figure 4A). Males with one copy of wild-type IYD (cdt\textsuperscript{Q}/Df) yielded an average of 45 adults from eggs laid during the first day. The number of subsequent progeny slowly decreased to an average of 25 adults on the fifth day (Figure 4B).

evident for Br-Tyr until its concentrations was increased to 1.5 mM (Figure 2). Under these conditions, cdt\textsuperscript{A} inexplicably suffered least lethality and variability was observed between the two control strains. Again, the presence of Br-Tyr did not discourage consumption of the media (Figure S5). Only the wild-type control (y,w) strain exhibited a measurable lethality when supplemented with an equivalent concentration of Cl-Tyr. As expected, the products of dehalogenation including Tyr, iodide and bromide were not detrimental to Drosophila when added to the media at high concentrations (Figures S6). The insensitivity to excess iodide is consistent with previous studies concluding that iodide did not inhibit a peroxidase-dependent production of melanin (20). Additionally, no adverse effect on survival of Drosophila had previously been observed after exposure to thyroxine or a combination of iodide and tyrosine (33).

In contrast to larvae, adult Drosophila demonstrated no sensitivity to halotyrosines. Survival of adults was unchanged even in the presence of 2 mM I-Tyr (Figure S7). Equivalent concentrations of Br-Tyr and Cl-Tyr also had no obvious effect. Again, ingestion of a dye included in the food demonstrated that the presence of halotyrosines did not affect the overall eating pattern (Figure S8). These data along with those from the larvae studies indicate the dehalogenase activity is not essential for detoxifying potential accumulation of halotyrosines that might form during generation or degradation of cuticle. IYD is also not likely required for iodide salvage under standard growth conditions as it is in vertebrates since Drosophila larvae and adults well tolerate deletion of IYD in contrast to humans (34). Attention turned next to the necessity of IYD in fertility since its expression is even higher in testes than in the larval fat body (27, 28).

Expression of cdt in testes

Both RNA-seq and affymetrix-based microarray methods revealed high levels of cdt expression in wild-type testes compared to levels in ovaries and male carcass samples (adult males with testes removed) (27, 28). More specifically, wild-type testes generated 303 reads per kilobase of transcript per million mapped reads (RPKM) for cdt followed by always early (aly) mutant testes with RPKM= 107 for cdt (35).
Males with the deletion of IYD (cdt^3/Df) generated almost the same average progeny (38) on the first day as that of the cdt^+/Df strain but progeny declined significantly over the following days to an average of only 1 adult progeny on day 5. This represents a 96% loss of fecundity on the last day of observation. In addition, the cdt^Q/Df males had an intermediate effect with a 72% loss of fecundity on day 5. Thus, the ability of Drosophila to dehalogenate halotyrosine impacts the fertility of adult males.

Complementary studies mating adult female flies with genotypes of cdt^3/Df, cdt^Q/Df or cdt^+/Df with wild-type males (vasa-cas9 strain, cdt^+/cdt^+) under the same protocol generated similar results (Figure 4C). Again, slight differences in the average progeny were observed on the first day but differences became more obvious when progenies from subsequent days were counted, suggesting an overall decrease in fertility for females with a cdt mutation. This result may be surprising based on the negligible expression of cdt in ovaries (RPKM = 0.25) (35). However, weak expression has been observed in female spermatheca (27), the organ that stores sperm prior to egg fertilization. Thus, cdt expression has the potential to effect sperm in both male and female flies and accordingly, loss of cdt activity in either sex has a significant effect on fertility. The impact is further enhanced by the lack of cdt in both sexes. Mating homozygous strains either lacking IYD (cdt^3) or containing its mutant form (cdt^Q) generated even fewer progeny than that observed when only one partner contained a deficiency in cdt (Figure 5).

In all examples, dehalogenation plays an unanticipated role in Drosophila reproduction. Human IYD is not directly associated with fertility and thus the biological function of Drosophila and human IYD are very different despite the similarities in their specificities of halotyrosine dehalogenation (15, 18). Expression of IYD in other insects such as a mosquito (Anopheles gambiae) is not principally observed in testes and is instead distributed widely through most organs at low levels (41). Still, this may be sufficient to affect reproduction in Diptera since even low levels of IYD in female Drosophila are sufficient to maintain fertility when mated with males lacking cdt.

Drosophila IYD has the potential to dehalogenate I-Tyr, Br-Tyr and Cl-Tyr in vivo but neither its ability to detoxify I-Tyr nor salvage halides likely represents its primary function as suggested by the feeding studies above. Instead, the contribution of IYD to reproduction lends support to an intriguing proposal that halotyrosines may represent a progenitor of thyroxine by functioning as a hormone (42). In both examples, signaling would be dependent on an organism’s ability to both halogenate and dehalogenate the parent compounds. Thyroxine controls more than just metabolic rate in mammals and is essential for proper neonatal development (43). Thyroxine is also crucial for signaling the metamorphosis of amphibians as illustrated most famously by conversion of tadpoles to frogs (44, 45). Limited examples have similarly demonstrated that iodinated tyrosines, thyroxine and thyroxine-like compounds may effect metamorphosis of invertebrates. For example, thyroxine acquired through diet appears to accelerate development of sea hare, sea urchin and sand dollar (46, 47). Most interestingly, I2-Tyr appears to be generated endogenously in jelly fish for regulating strobilation of its polyp into free-swimming medusa (48).

While a role for halotyrosine and cdt in sperm maturation is possible, an additional hypothesis would be necessary to explain the decrease of fertility observed when cdt mutations are only present in female Drosophila (Figure 4C). A more appealing alternative would have a common basis in both sexes such as maintaining the viability of sperm during development in testes and storage in spermatheca. For example, sperm are highly sensitive to reactive oxygen species and at least in the spermatheca, their longevity appears to depend on controlling metabolism and preventing accumulation of these species (49, 50). Such an activity is consistent with the time-dependent loss of fertility observed for cdt mutants and provides a testable hypothesis for future investigations on the details of cdt and halotyrosines in Drosophila. The link between cdt and fertility should also inspire broad investigations into the general requirement of halotyrosines and IYD homologs throughout the phylum Arthropoda.

MATERIALS AND METHODS

General
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Standard procedures were used for all cloning protocols. Oligonucleotides (ODNs) were purchased from IDT (Coralville, IA). *Drosophila* were raised using standard Bloomington medium at 25 °C unless specified. 3-Bromo-L-tyrosine (95% pure) was purchased from AEchem Scientific Corporation (Naperville, IL). 3-Fluoro-L-tyrosine (97% pure) was purchased from Astatech Inc (Bristol, PA). 3-Iodo-L-tyrosine (97% pure) was purchased from AcrO Organics (Waltham, MA). Solutions of 3-halotyrosines and Tyr were prepared in 0.1 N HCl and concentrations were determined using published extinction coefficients (21). Aqueous sodium hydroxide was used to neutralize the media after addition of the acidic halotyrosine solutions.

**CRISPR/Cas9-based deletion and mutation of the Drosophila gene cdt**

Sense and antisense ODNs encoding the gRNAs (Figure S1) were cloned separately into the pCFD3-U6:3-gRNA vector using published protocols (51, 52). The resulting pair of gRNA encoding vectors (250 ng/µl each) for targeted gene deletion and a single-stranded ODN template (100 ng/µl) were co-injected into *Drosophila* embryos expressing Cas9 in the germline (BDSC #51323, *vasa-cas9* (29)) by Bestgene Inc (Chino Hills, CA). To generate the E154Q mutant of cdt, a single gRNA encoding vector (500 ng/µl, Figure S1) and a single-stranded ODN repair template (100 ng/µl) that coded for E154Q were similarly co-injected in *Drosophila* embryos (*vasa-Cas9*) by Bestgene Inc. The resulting larvae were mated with a MKRS/TM6B balancer and males from the third generation (F3) were screened by PCR (See supporting information for details). Strains with the desired homozygous genotypes (*cdt*Δ or *cdt*Q) were confirmed by DNA sequencing. A control strain containing the wild-type gene (*cdt*Δ) was generated with equivalent crosses between adults from the MKRS/TM6B balancer strain and those from the parent *vasa-cas9* strain. The *Drosophila* strain yellow, white (*yw*) (53), commonly used as a reference in laboratory experiments, was also employed as a control for all feeding experiments.

**Larval feeding**

Mature egg laying adults of the desired genotype (homozygous) were transferred to vials containing apple juice agar medium (40% Giant brand apple juice, 1.5% agar and 0.05% Tegosept) and allowed to lay eggs. Adults were removed after 24 h and L1 larvae were collected between 24 to 30 h after egg laying. Fifty L1 larvae were transferred to each vial containing the standard feeding media, FD&C blue 1 dye (0.007% w/v) and the indicated supplement. Food consumption by the larvae was detected through ingestion of the dye as described above and using larvae raised solely on the standard Bloomington media containing halotyrosine and dye (Figure S6). Vials were maintained at 25 °C and the number of larvae surviving to adulthood was quantified.

**Adult feeding**

One day old male and female adult flies (25 each) were transferred to a vial containing a tissue (KimWipe, Kimberly Clark) soaked in 3 ml of an apple juice-halotyrosine feeding mixture (75% v/v apple juice, 2 mM halotyrosine, 0.05% w/v Tegosept and 0.05% w/v FD&C blue 1 dye). Consumption of the liquid mixture by adult flies was confirmed through ingestion of the dye (Figure S8). Adult *Drosophila* were maintained on apple juice-halotyrosine mixtures at 25 °C for 5 days and the number of surviving adults was quantified.

**In situ hybridization to identify cdt expression**

Expression of wild type cdt in testes (*yw* strain, whole mount) was detected by RNA *in situ* hybridization using standard procedures (54, 55) Briefly, primers 5'-CATGAAAGTTTCCGTTAGAA GAG-3' (forward) and 5'- CATCTGCACTTGC TGGCTATT-3' (reverse) were used to amplify a 405 bp region of cdt isoform A from a *Drosophila* testes cDNA library (35) using Taq DNA polymerase (Thermo Scientific). Similarly, primers 5'-ATTGTGGAACAGGAGG AGCTG-3' (forward) and 5'-ATTCTTTCTCGC CAAGTCGGG-3' (reverse) were used to amplify a 393 bp region of cdt that is common to both isoforms A and B. The PCR products with 5'-A overhangs were then individually ligated into the linearized pGEM-T Easy plasmid (Promega) with 3'-T overhangs using T4 DNA ligase (Promega, Madison, WI). Plasmids were purified from single colonies and the desired inserts were subsequently subcloned into the pBluescript II vector using SacII and PstI restriction sites.
Transformants containing inserts were identified via blue-white colony screening and the orientation of the inserts was determined by DNA sequencing (Genewiz, South Plainfield, NJ). Based on the orientation of the DNA inserts, anti-sense and sense hybridization probes were synthesized using T3 and T7 RNA polymerases (Roche Life Science, Indianapolis, IN) after linearizing the vector with PstI or SacII, respectively. A DIG label was incorporated in the probes through DIG labeled UTPs (DIG RNA labeling mix, Roche Life Science). All subsequent procedures including hydrolysis of RNA probes, testes preparation and in situ hybridization were performed as described previously (54, 55). Testes were imaged with a Zeiss HXP120C Apotome microscope.

**Fecundity after a limited mating period**

The balanced deficiency (Df) fly stock for cdt carrying a deletion on chromosome 3L (11070526 to 11247145) was obtained from the Bloomington Drosophila stock center (BDSC # 27578) (56). Homozygous virgin females of genotype cdt∆ and cdtQ derived from the vasa-cas9 strain and a wild-type control (cdt+) were individually mated with Df/TM6B males. The first generation without the humeral phenotype provided the cdt∆/Df, cdtQ/Df and cdt+/Df strains. Newly eclosed virgin males and females of each strain were maintained separately for 2-3 days and 3-4 days respectively prior to mating. Concurrently, newly eclosed virgin males and females of the cdt+/cdt− genotype were maintained equivalently and then combined individually with the specified strains for 24 h at 25 °C. Males were then removed, and the females were transferred to new vials every 24 h for 5 consecutive days to lay eggs. The progeny emerging from each vial was quantified from day 10 to day 18 as described previously (57). Vials were excluded from analysis if any of the adults died during this period.

The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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This article contains Figs. S1 – S8 and supporting information.

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2 The abbreviations used are: aly, always early; bam, bag of marbles; Br-Tyr, 3-bromo-L-tyrosine; Cl-Tyr, 3-chloro-L-tyrosine; IYD, iodo-tyrosine deiodinase; I-Tyr, 3-iodo-L-tyrosine; I2-Tyr, diiodotyrosine; RPKM, reads per kilobase of transcript per million mapped reads; Drosophila containing wildtype iodotyrosine deiodinase (cdt+), the E154Q variant (cdtQ) and its deletion (cdt∆).
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References

1. Hartmann, N. (1950) Über den Abbau von Dijotyrosin im Gewebe, Z. Physiol. Chem. 285, 1-17
2. Roche, J., Michel, O., Michel, R., Gorbman, A., and Lissitzky, S. (1953) Sur la deshalogénation enzymatique des i odorotyrosines par le corps thyroïde et sur son rôle physiologique. II, Biochim. Biophys. Acta 12, 570-576
3. Uhlén, M., Fagerberg, L., Hallström, B. M., Lindskog, P., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., and et a. (2015) Tissue-based map of the human proteome, Science 347, 1260419
4. Mani, A. R., Ippolito, S., Moreno, J. C., Visser, T. J., and Moore, K. P. (2007) The metabolism and dechlorination of chlorotyrosine in vivo, J. Biol. Chem. 282, 29114-29121
5. Mani, A. R., Moreno, J. C., Visser, T. J., and Moore, K. P. (2016) The metabolism and debromination of bromotyrosine in vivo, Free Rad. Biol. Med. 90, 243-251
6. Wu, W., Samoszuk, M. K., Comhair, S. A., Thomasen, M. J., Farver, C. F., Dweik, R. A., Kavuru, M. S., Erzurum, S. C., and Hazen, S. L. (2000) Eosinophils generate brominating oxidants in allergen-induced asthma, J. Clin. Invest. 105, 1455-1463
7. Buss, I. H., Senthilmohan, R., Darlow, B. A., Mogridge, N., Kettle, A. J., and Winterbourn, C. C. (2003) 3-Chlorotyrosine as a marker of protein damage by myeloperoxidase in tracheal aspirates from preterm infants: association with adverse respiratory outcome, Pediatr. Res. 53, 455-462
8. Bobyk, K. D., Ballou, D. P., and Rokita, S. E. (2015) Rapid kinetics of dehalogenation promoted by iodotyrosine deiodinase from human thyroid, Biochemistry 54, 4487-4494
9. Phatarphekar, A., Buss, J. M., and Rokita, S. E. (2014) Iodotyrosine deiodinase: a unique flavoprotein present in organisms of diverse phyla, Mol. BioSyst. 10, 86-92
10. Sun, Z., Su, Q., and Rokita, S. E. (2017) The distribution and mechanism of iodotyrosine deiodinase defied expectations, Arch. Biochem. Biophys. 632, 77-87
11. Agarwal, V., El Gamal, A. A., Yamanaka, K., Poth, D., Kersten, R. D., Schorn, M., Allen, E. E., and Moore, B. S. (2014) Biosynthesis of polybrominated aromatic organic compounds by marine bacteria, Nat. Chem. Biol. 10, 640-647
12. Wang, L., Zhou, X., Fredimoses, M., Lia, S., and Liu, Y. (2014) Naturally occurring organoiodines, RSC Advances 4, 57350-57376
13. Eales, J. G. (1997) Iodine metabolism and thyroid-related functions in organisms lacking thyroid follicles: are thyroid hormones also vitamins? Proc. Soc. Exp. Biol. Med. 214, 302-317
14. Heyland, A., and Moroz, L. L. (2005) Cross-kingdom hormonal signaling: an insight from thyroid hormone functions in marine larvae, J. Exp. Biol. 208, 4355-4361
15. Phatarphekar, A., and Rokita, S. E. (2016) Functional analysis of iodotyrosine deiodinase from Drosophila melanogaster, Protein. Sci. 25, 2187-2195
16. Akiva, E., Copp, J. N., Tokuriki, N., and Babbit, P. C. (2017) Evolutionary and molecular foundations of multiple contemporary functions of the nitroreductase superfamily, Proc. Nat. Acad. Sci. (USA) 114, E9549–E9558
17. Friedman, J. E., Watson, J. A., Jr., Lam, D. W.-H., and Rokita, S. E. (2006) Iodotyrosine deiodinase is the first mammalian member of the NADH oxidase / flavin reductase superfamily, J. Biol. Chem. 281, 2812-2819
18. Hu, J., Chuenchor, W., and Rokita, S. E. (2015) A switch between one- and two-electron chemistry of the human flavoprotein iodotyrosine deiodinase is controlled by substrate, J. Biol. Chem. 290, 590-600
19. McTamney, P. M., and Rokita, S. E. (2009) A mammalian reductive deiodinase has broad power to dehalogenate chlorinated and brominated substrates, J. Am. Chem. Soc. 131, 14212–14213
20. Wheeler, B. M. (1950) Halogen metabolism of Drosophila-Gibbersosa. 1. Iodine metabolism studied by means of I-131, J. Exp. Zoology 115, 83-107
21. Hunt, S. (1984) Halogenated tyrosine derivatives in invertebrate scleroproteins: Isolation and identification, Methods Enzymol. 107, 413-438
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22. Valverde-R., C., Orozco, A., Becerra, A., Jeziorski, M. C., Villalobos, P., and Solis-S., J. C. (2004) Halometabolites and cellular dehalogenase systems: an evolutionary perspective, *Int. Rev. Cytol.* 234, 143-199

23. Hopkins, T. L., and Kramer, K. J. (1992) Insect cuticle sclerotization, *Annu. Rev. Entomol.* 37, 273-302

24. Johnson, K. S., Coale, K. H., and Jannasch, J. W. (1992) Analytical chemistry in oceanography, *Anal. Chem.* 64, 1065-1075

25. Piper, M. D. W., Blanc, E., Leitão-Gonçalves, R., Yang, M., He, X., Linford, N. J., Hoddinott, M. P., Hopfen, C., Soutoukis, G. A., Niemeyer, C., Kerr, F., Pletcher, S. D., C., R., and Partridge, L. (2007) A holidic medium for *Drosophila melanogaster*, *Nat. Methods* 11, 100-107

26. McCall, A. S., Cummings, C. F., Bhave, G., Vanacore, R., Page-McCaw, A., and Hudson, B. G. (2014) Bromine is an essential trace element for assembly of collagen IV scaffolds in tissue development and architecture, *Cell* 157, 1380-1392

27. Chintapalli, V. R., Wang, J., and Dow, J. A. T. (2007) Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease, *Nat. Genet.* 39, 715-720

28. modENCODE-Consortium, Roy, S., Ernst, J., Kharchenko, P. V., Kheradpour, P., Negre, N., Eaton, M. L., Landolin, J. M., Bristow, C. A., Ma, L., Lin, M. F. et al. (2010) Identification of functional elements and regulatory circuits by Drosophila modENCODE, *Science* 330, 1787-1797

29. Gratz, S. J., Ukken, F. P., Rubinstein, C. D., Thiede, G., Donohue, L. K., Cummings, A. M., and O’Connor-Giles, K. M. (2014) Highly specific and efficient CRISPR/Cas9-catalyzed homology-directed repair in *Drosophila*, *Genetics* 196, 961-971

30. Attrill, H., Falls, K., Goodman, J. L., Millburn, G. H., Antonazzo, G., Rey, A. J., and Marygold, S. J. (2016) FlyBase: establishing a gene group resource for *Drosophila melanogaster*, *Nucleic Acids Res.* 44, D786-D792

31. Ingavat, N., Kavran, J. M., Sun, Z., and Rokita, S. E. (2017) Active site binding is not sufficient for reductive deiodination by iodotyrosine deiodinase, *Biochemistry* 56, 1130-1139

32. Neckameyer, W. S. (1996) Multiple roles for dopamine in *Drosophila* development, *Develop. Biol.* 176, 209-219

33. Flatt, T., Moroz, L. L., Tatar, M., and Heyland, A. (2006) Comparing thyroid and insect hormone signaling, *Int. Comp. Biol.* 46, 777-794

34. Moreno, J. C., Klootwijk, W., van Toor, H., Pinto, G., D’Alessandro, M., Lèger, A., Goudie, D., Polak, M., Grütters, A., and Visser, T. J. (2008) Mutations in the iodotyrosine deiodinase gene and hypothyroidism, *N. Engl. J. Med.* 358, 1811-1818

35. Gan, Q., Chepelev, I., Wei, G., Tarayrah, L., Cui, K., Zhao, K., and Chen, X. (2010) Dynamic regulation of alternative splicing and chromatin structure in Drosophila gonads revealed by RNA-seq., *Cell Res.* 20, 763-771

36. Eun, S. H., Stoiber, P. M., Wright, H. J., McMurdie, K. E., Choi, C. H., Gan, Q., Lim, C., and Chen, X. (2013) MicroRNAs downregulate bag of marbles to ensure proper terminal differentiation in the *Drosophila* male germline, *Development* 140, 23-30

37. Chen, D., Wu, C., Zhao, S., Geng, Q., Gao, Y., Li, X., Zhang, Y., and Wang, Z. (2014) Three RNA binding proteins form a complex to promote differentiation of germline stem cell lineage in *Drosophila*, *PLoS Genet* 10, e1004797

38. McKearin, D. M., and Spradling, A. C (1990) Bag-of-marbles: a *Drosophila* gene required to initiate both male and female gametogenesis. *Genes Dev.* 4, 2242-2251

39. White-Cooper, H., Leroy, D., MacQueen, A., and Fuller, M. T (2000) Transcription of meiotic cell cycle and terminal differentiation genes depends on a conserved chromatin associated protein, whose nuclear localisation is regulated. *Development* 127, 5463-5473

40. Sitnik, J. L., Gligorov, D., Maeda, R. K., Karch, F., and Wolfner, M. F. (2016) The female post-mating response requires genes expressed in the secondary cells of the male accessory gland in *Drosophila melanogaster*, *Genetics* 202, 1029-1041

41. Baker, D. A., Nolan, T., Fischer, B., Pinder, A., Cristanti, A., and Russell, S. (2011) A comprehensive gene expression atlas of sex- and tissue-specificity in the malaria vector, *Anopheles gambiae*, *BMC Genomics* 12, 296
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42. Crockford, S. J. (2009) Evolutionary roots of iodine and thyroid hormones in cell-cell signaling, Integr. Comp. Biol. 49, 155-166
43. Forhead, A. J., and Fowden, A. L. (2014) Thyroid hormones in fetal growth and prepartum maturation, J. Endocrin. 221, R87-R103
44. Tata, J. R. (1999) Amphibian metamorphosis as a model for studying the developmental actions of thyroid hormone, Biochimie 81, 359-366
45. Dickhoff, W. W., and Darling, D. S. (1983) Evolution of thyroid function and its control in lower vertebrates, Amer. Zool. 23, 697-707
46. Heyland, A., Price, D. A., Bodnarova-Buganova, M., and Moroz, L. L. (2006) Thyroid hormone metabolism and peroxidase function in two non-chordate animals, J. Exp. Zoology 306B, 551-566
47. Heyland, A., Reitzel, A. M., and Hodin, J. (2004) Thyroid hormones determine development mode in sand dollars, Evol. Develop. 6, 382-392
48. Silverstone, M., Galton, V. A., and Ingbar, S. H. (1978) Observations concerning the metabolism of iodine by polyps of Aurelia aurita, Gen. Comp. Endocrinol. 34, 132-140
49. Heifetz, Y., and Rivlin, P. K. (2010) Beyond the mouse model: using Drosophila as a model for sperm interaction with the female reproductive tract, Theriogenology 73, 723-739
50. Ribou, A.-C., and Reinhardt, K. (2012) Reduced metabolic rate and oxygen radicals production in stored insect sperm, Proc. R. Soc. B 279, 2196-2203
51. Port, F., Chen, H.-M., Lee, T., and Bullock, S. L. (2014) Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in Drosophila, Proc. Nat. Acad. Sci. (USA) 111, E2967-E2976
52. Port, F. Cloning gRNA expression vectors with pCFD3. http://www.crisprflydesign.org/
53. Ryuda, M., Tsuzuki, S., Tanimura, T., Tojo, S., and Hayakawa, Y. (2008) A gene involved in the food preferences of larval Drosophila melanogaster, J. Insect Physiol. 54, 1440-1445
54. Schulz, C. (2007) In situ hybridization to Drosophila testes, CSH protocols. doi:10.1101/pdb.prot4764
55. Morris, C. A., and Benson, E. (2009) Determination of gene expression patterns using in situ hybridization to Drosophila testes, Nat. Protocol 4, 1807-1819
56. Bloomington Drosophila Stock Center: Deficiency Kit information. http://flystocks.bio.indiana.edu/Browse/df/dfkit-info.htm
57. Xie, J., Wooten, M., Tran, V., Chen, B.-C., Pozmanter, C., Simbolon, C., Betzig, E., and Chen, X. (2015) Histone H3 threonine phosphorylation regulates asymmetric histone inheritance in the Drosophila male germline, Cell 163, 920-933
**Figure 1.** Sensitivity of *Drosophila* larvae to I-Tyr. The survival of larvae in three or more independent cohorts (50 each) from the first instar to adults was compared under increasing concentrations of I-Tyr for the indicated homozygous genotypes based on the parent strain *vasa-cas9*. The wild-type parent (*cdt+*) and a yellow, white strain (*yw*) act as controls. The midline indicates the average of the measurements. (*) indicates a p-value <0.01 and (**) indicates a p-value <0.05. Statistical significance was determined using the Student’s t-test for a two tailed distribution of unequal variance.

**Figure 2.** Sensitivity of *Drosophila* larvae to Br-Tyr and Cl-Tyr. The survival of larvae in three independent cohorts (50) from the first instar to adults was compared in the presence of equal concentrations of Br-Tyr and Cl-Tyr (1.5 mM), respectively. The homozygous genotypes are based on the parent strain *vasa-cas9*. The wild-type parent (*cdt+*) and a yellow, white strain (*yw*) act as controls. The midline indicates the average of the measurements. (*) indicates a p-value <0.01 and (**) indicates a p-value <0.05. Statistical significance was determined using the Student’s t-test for a two tailed distribution of unequal variance.
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**Figure 3.** *In situ* hybridization of Drosophila testes with Digoxigenin (DIG)-labeled RNA probes. Antisense probes targeting (A) a unique region of *cdt* isoform A, and (B) a common region of *cdt* isoforms A and B, show intense staining in the region of the testes housing meiotic germ cells. Corresponding sense RNA probes (C and D) were used as controls. Scale bars: 50 µm. The region of spermatogonial cells is indicated by a bracketed with an asterisk.

**Figure 4.** Fertility tests showed decreased progenies over time after an initial mating. (A) Single males (2-3 day old) and several females (3-4 days old) were placed together in vials for 24 h. Males were then removed and females were transferred to new vials each day. The adult progeny from each vial was then counted. (B) Mutant and control males crossed with wild-type (cdt+/cdt) females. (C) Mutant and control females crossed with wild-type (cdt+/cdt) males. The box plots are defined by the 25th and 75th percentiles. The midline indicates the average and the bar indicates standard deviation. (*) indicates a p-value <0.01 and (***)) indicates a p-value <0.001 with respect to the control data of (cdt+/cdt) x (cdt+/Df). Statistical significance was determined using the Student’s t-test for a two tailed distribution of unequal variance.
Figure 5. Fertility tests indicate a significant decrease of progeny over time after an initial mating of males and females with the same deletion or mutation of IYD. Procedures described in Figure 4 were repeated for the designated genotypes. The box plots are defined by the 25th and 75th percentiles. The midline indicates the average and the bar indicates standard deviation. (***) indicates a p-value <0.001 with respect to the control data of (cdt+/cdt+) x (cdt+/cdt+). Statistical significance was determined using the Student’s t-test for a two tailed distribution of unequal variance.
