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Identification of Deletions in the Hdc Gene of Drosophila Melanogaster Generated Through Transposon-Excision Mutagenesis

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Thesis Title Page

Identification of deletions in the *Hdc* gene of *Drosophila melanogaster* generated through transposon-excision mutagenesis

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Abstract

Histamine is a biogenic amine that functions as a neurotransmitter in a number of vertebrate and invertebrate systems and is synthesized from its precursor histidine by the enzyme histidine decarboxylase (HDC). In *Drosophila*, histamine has been shown to have function in photoreceptors, mechanoreceptor cells, as well as centrally located neurons. Mutations of the *Hdc* gene, such as Hdc^{JK910}, exhibit defects in histamine synthesis and display altered behaviors such as blindness, inability to groom, impaired thermal tolerance, and altered sleep rhythms. However, all *Hdc* mutants obtained thus far demonstrate some transcriptional activity.

In order to remove *Hdc* expression completely, part of the *Hdc* gene was removed via *Minos* transposon-excision mutagenesis using the $Mi\{ET1\}Hdc^{MB07212}$ fly, which bears a *Minos* transposon within the *Hdc* gene (a hypo-morphic allele). *Minos* excision mutagenesis of *Hdc* was achieved by mating flies from the $Hdc^{MB07212}$ strain with another fly carrying the *Minos*-specific transposase gene – to induce imprecise excision in the progeny's genome to cause a deletion. The *Mi*{ET1} transposon also contains a gene encoding the green fluorescent protein (GFP) under the control of an eye-specific promoter, the loss of which can be used to visually identify a potential *Minos* excision. Once loss of GFP (GFP^-) individuals were identified, breeding lines were established and flies from each line examined using histamine immunostaining to determine the presence or absence of histamine in the ventral nerve cord of larvae.

Progeny obtained fell into the following categories: (1) flies with wild-type levels of histamine, indicating rescue of *Hdc* expression due to a precise *Minos* excision from *Hdc*; (2) flies with trace levels of histamine, indicating an excision event that disrupted GFP expression from the *Mi*{ET1} transposon but did not rescue *Hdc* expression; (3) flies having no histamine,

indicating an imprecise excision with an associated loss of expression from the *Hdc* gene. Molecular lesions associated with each class of flies were characterized using a PCR approach. Results indicate that of the 98 GFP^- strains examined thus far each fell into one of the 3 expected categories, with 29 exhibiting elimination of *Hdc* expression.

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Chapter 1: Introduction

Introduction

Histamine functions as a neurotransmitter in fruit flies and is produced from its precursor, histidine, by the enzyme histidine decarboxylase. The gene responsible for the histidine decarboxylase enzyme in the fruit fly is the *Hdc* gene. Mutant flies defective for histamine synthesis ability display traits such as blindness, inability to groom, and impaired temperature tolerance. Thus far, all of the mutations that disrupt the *Hdc* gene have been caused by point mutations that were induced chemically over 30 years ago. A new set of mutations that remove (or delete) the *Hdc* gene will be useful in the examination of the effects that removal of *Hdc* has on the viability of the fly, as has been traditionally done when characterizing genes as essential to an organism's viability. Additionally, deletions of the *Hdc* gene will be useful in studying the regulation of *Hdc* gene function in the fruitfly, *Drosophila melanogaster*.

Purpose

The purpose of this study was to eliminate *Hdc* gene function by generating a deletion of the gene using transposon-excision mutagenesis. A *Minos* transposon has been reported to be present within the *Hdc* gene, and has been used to generate deletions of other genes through transposon mutagenesis.

Scope

Putative deletion mutants were generated by crossing flies with the *Minos* transposon to flies with a heat-inducible transposase gene. Those mutants were then analyzed for histamine in nervous system tissue using histamine immunofluorescence staining, with a result of no histamine indicating a disruption of the gene. Secondly, examination of the DNA structure surrounding the *Minos* insertion using a polymerase chain reaction (PCR) approach in flies that were shown to have a disrupted *Hdc* gene function has revealed removal of the region containing the *Minos* transposon.

Assumptions

It has been reported that only 1.5 – 3.6% of *Minos* excision events result with imprecise excisions, so it was estimated that 90 – 100 mutant lines would need to be generated using transposon-excision mutagenesis in order to identify a deletion within the *Hdc* gene. Negative results for both histamine staining and PCR would be considered evidence of an *Hdc* deletion mutation. As a result of this work, approximately 29 deletion mutants of the *Hdc* gene have been identified and are available for further molecular analysis.

Research Question

The research question that drove this project was "Is it possible to isolate a homozygous fly with a deletion containing only the *Hdc* region, or will elimination of *Hdc* activity cause lethality in the fly?"

Significance

There are existing mutants that exhibit *Hdc* deficiency, but all of them still have some residual expression. Deletions of most, if not all, of the *Hdc* gene should enable gene regulation studies to move forward, as there has been some evidence generated by the Burg lab (personal communication, M. Burg) that multiple copies of the *Hdc* promoter can suppress endogenous *Hdc* expression. If available, deletions in the promoter control region of the gene may aid in this scientific question.

Definitions

Transposons are sometimes referred to as "jumping genes" because they can excise and then re-insert into various regions within a genome. If a transposon excises imprecisely, it takes adjacent genetic material along with it and causes a deletion.

Chapter 2: Literature Review

Neurotransmitters are the foundation of neural function and play an important role in effecting mechanical function, sensory function, and cognition (reviewed in: Ludwig and Pittman 2003). While numerous neurotransmitter substances have been studied in humans, histamine's role as a neurotransmitter is less understood (Haas et al., 2008). Currently, lack of histamine has been shown to have some role in neurological function for example in some seizure disorders (Kenji et al., 1992) and has been implicated in one rare form of Tourette's syndrome (Baldan et al., 2015). Overall, there are still many questions that exist regarding histamine's role in the vertebrate nervous system and what implications it may have on human health. We have focused on examining the role of histamine in the fruitfly *Drosophila melanogaster* as it represents a robust and accessible model system.

Histamine is a biogenic amine that has been shown to function as a neurotransmitter in a number of invertebrate systems (Stuart 1999). Histamine can be produced from histidine, by the enzyme histidine decarboxylase (HDC) (Sarthy 1991). In *Drosophila*, histamine is the transmitter used by photoreceptors in the compound eye (Nässel et al., 1988; Pollack and Hofbauer, 1991; Sarthy 1991; Burg et al., 1993), and is also used by mechanoreceptors of the macrochaeta sensilla (Buchner et al., 1993; Melzig et al., 1996). Mutant flies have been derived and studied, such as the Hdc^{JK910} mutant, which exhibit defects in histamine synthesis ability (Burg et al., 1993), and display traits such as blindness, inability to groom (Buchner et al., 1993; Melzig et al., 1996), impaired temperature tolerance (Hong et al., 2006), disrupted circadian rhythm, and trouble with courtship (Oh et al., 2013). However, all of the *Hdc* mutants identified by sequence analysis (Birdsey et al, 2006) have some detectable transcriptional activity (Boozer

et al., 2008), leaving to question what complete elimination of *Hdc* expression (a transcriptional null mutation) could cause in the fly. According to the online fly community resource, Flybase (dos Santos et al., 2015), there are no small deletions available that remove only the *Hdc* gene. Thus, an approach to generating small deletions in the *Hdc* gene needed to be employed in order to obtain an *Hdc* deletion mutation.

One potential approach to generating mutations is chemical mutagenesis. The ability of chemicals like ethyl methanesulfonate (EMS) to reliably induce mutagenesis made it a favorite technique used by geneticists for some time. EMS induces transition mutations predominantly, changing G-C nucleotide base pairs to A-T (Pastink et al., 1991). All of the originally isolated *Hdc* mutations were generated using this method (Pak 1975). The challenge of this approach is the random nature of the mutations generated (Blumenstiel et al., 2009), as well as the effort required to identify the mutation location. A second, more convenient method is the exploitation of transposons, sometimes called "jumping genes." Transposons exist in the genomes of many species, and during times of stress can provide the host with useful local genetic variation to aid in survival (McClintock 1984). When utilized by geneticists, transposon insertion usually interferes with gene function and results in a mutant phenotype, and therefore is extremely useful in analyzing gene regulation and function (Cooley et al., 1988; Bellen et al., 1989; Bier et al., 1989). Once identified in a position of a particular gene, the subsequent removal (or excision) of the transposon can lead to a deletion (or removal) of the gene through imprecise excision of the transposon (Loukeris et al., 1995a; Vanrobays et al., 2010).

One recent large-scale effort in *Drosophila* genetics research using transposon mutagenesis techniques is the *Drosophila* Gene Disruption Project (Bellen et al., 2011); its goal was to use transposon insertions to disrupt every *Drosophila* gene identified. Transposable elements differ in their target site specificity (Bellen et al., 2004; Thibault et al., 2004), the P-element for example inserts near promoters on actively transcribed gene "hotspots" (Spradling et al., 1995; Liao et al., 2000; Tower et al., 1993), whereas the piggyBac transposon shows very little target site specificity (Horn et al., 2003). Both the P-element and piggyBac transposons frequently disrupted the gene in which they were inserted, but it was the P-element's proclivity to cause deletions through imprecise excision that made it the most used in transposon mutagenesis studies during the early years of the *Drosophila* Gene Disruption Project (Lin et al., 2014). But in 1991, a new transposon belonging to the Tc1/mariner superfamily (*Minos*) was isolated from *Drosophila hydei* (Franz and Savakis 1991). Tc1/mariner transposons do not require any host-specific factors for transcription and were potentially active in all organisms (Lampe et al., 1996; Vos et al., 1996). For instance, *Minos* became the first transposon to successfully transform a species (the medfly, *Ceratitis capitata*) outside of the element's original host genus (Loukeris et al., 1995b). Unlike the piggyBac transposon, *Minos* has the ability to sometimes cause deletions through imprecise excision (Pastink et al., 1991). It was soon predicted that *Minos* elements would enable genome-wide mutagenesis (Venken et al., 2011), and today more than 95% of *Drosophila melanogaster* genes now contain at least one transposon insert (Bellen et al., 2011). The existence of a line with the *Minos* element in the *Hdc* gene (5 base pairs 5' to the second intron) provided an opportunity to use transposon-excision mutagenesis using *Minos* as a suitable method for generating a deletion in the *Hdc* gene of *Drosophila melanogaster*.

According to the online fly community resource, Flybase (dos Santos et al., 2015), one of the sites of *Minos* transposon insertion was in the Histidine decarboxylase (*Hdc*) gene – 4 base pairs 5' to the second intron (see Fig. 1). Because there is a *Minos* transposon (MB07212) within the *Hdc* gene, it was possible to follow procedures previously described to induce an imprecise excision of the *Minos* transposon (Metaxkais et al., 2005) from the *Hdc* locus. Transposase expression in remobilization experiments can be controlled using heat shock and show rates of transposition between 30 – 50% (Metaxkais et al., 2005). Excision of the *Minos* element does not always lead to a deletion of adjacent genomic DNA, yet 1.5 – 3.6% of excision events reported thus far have resulted in such an imprecise excision, leading to a deletion of a neighboring gene (Metaxkais et al., 2005). Based on these findings, it was estimated 90 – 100 mutant lines would need to be generated using transposon excision mutagenesis in order to identify a deletion within the *Hdc* gene.

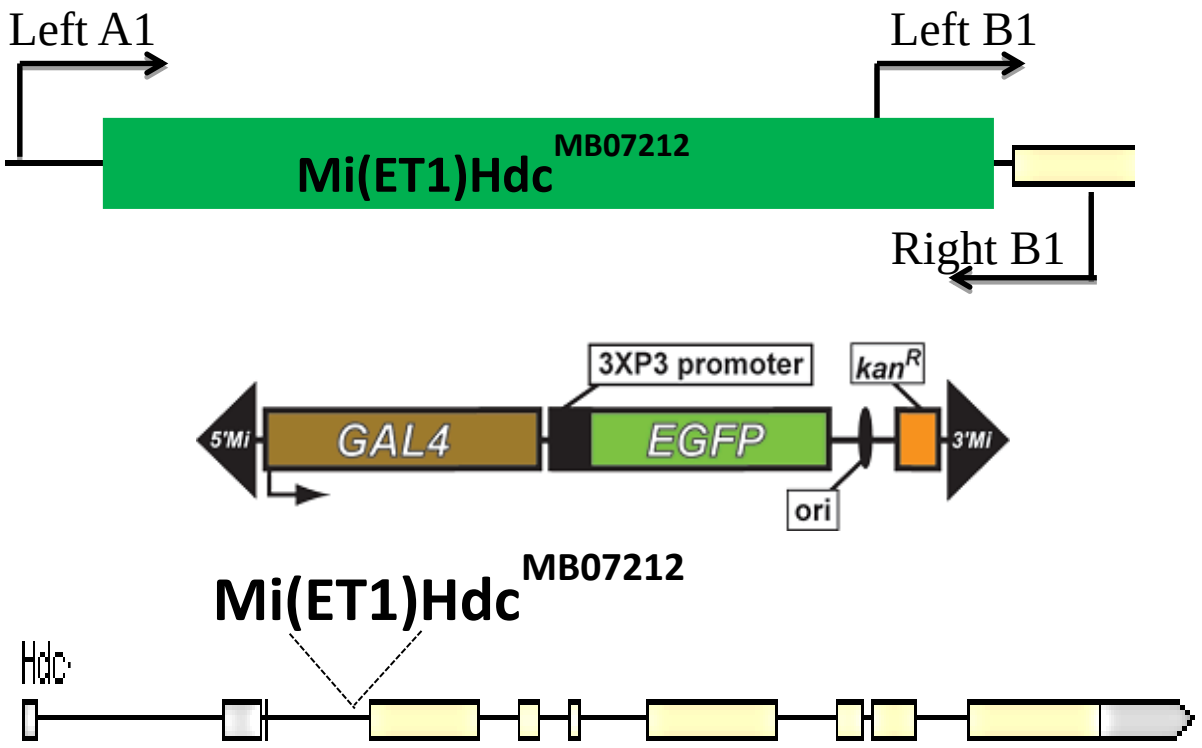
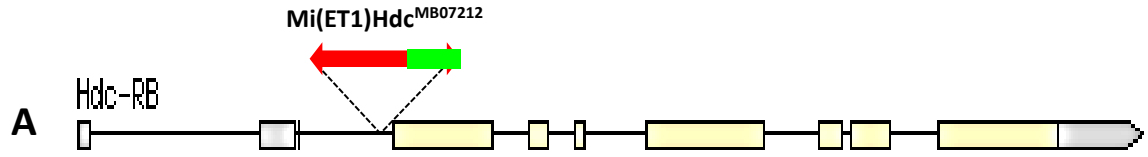


Figure 1: Location of the *Minos* transposon within the 9.4 kilo base pair *Hdc* gene (lower image). Location of the GFP gene within the *Minos* element (middle image). Location of PCR primers used for detection of the 7.5kb *Minos* element (upper image). In the *Minos*^{MB07212} fly, the *Minos* element is 4 base pairs upstream from the second intron within *Hdc*. The expected PCR product size of the first primer pair (Left A1 and Right B1) with a precise *Minos* excision is 407 bp (base pairs), and the expected PCR product size of the second primer pair (Left B1 and Right B1) without a *Minos* excision is 517bp. Image adapted from Flybase (dos Santos et al., 2015).

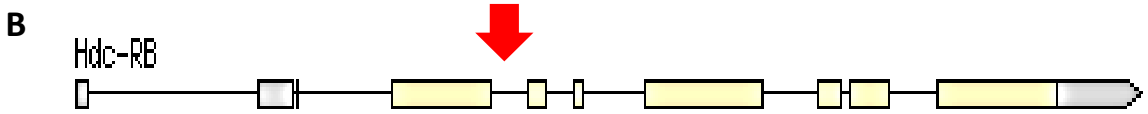
Chapter 3: Research Methods

Inducing imprecise excision of the *Minos*^{MB07212} transposon.

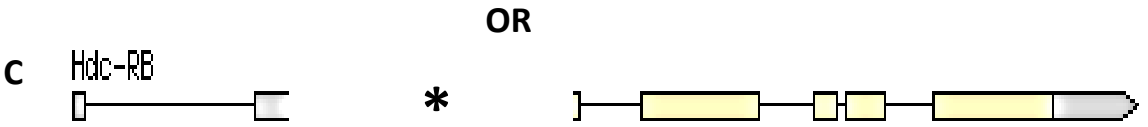
Because there is a 7.5 kilo-base pair *Minos* transposon (MB07212) in the middle of the *Hdc* gene (4 base pairs 5' to the second intron, see also Fig. 2), it is possible to follow procedures previously described to induce an imprecise excision of the *Minos* transposon and thereby disrupt *Hdc* expression (Metaxkais et al., 2005). The existence of a GFP gene under an eye-expressing promoter within *Minos* (Horn et al., 2000), allows the loss of the transposon to be detected due to the change in GFP expression in the compound eye (see Fig. 4; images taken using an Amscope stereomicroscope and the Nightsea fluorescent attachment). To induce a *Minos* transposon excision event and isolate any resulting deletion from such an excision event, the breeding scheme outlined in Figure 3 was followed. While an excision of the *Minos* element may not always lead to a deletion of adjacent genomic DNA (see Figure 2, outcome 2), 1.5 – 3.6% of cases reported thus far in similar experiments have resulted in such an imprecise excision (Metaxkais et al., 2005), even leading to a deletion of a neighboring gene. The details of this cross scheme (Figure 3) should result in a fly that has lost the GFP (green eyes) marker (detected by microscopic examination using a fluorescent excitation and detection system – Nightsea, Inc.), indicating that at least the portion of the *Minos* element containing GFP had been excised.



The *Mi(ET1)Hdc*^{MB07212} transposon location in the *Hdc* gene. Flies carrying this transposon exhibit green fluorescent eyes. Excision of this transposon, using the *Minos* transposase, will result in progeny flies without green fluorescent eyes. The excision of the Minos transposon *Mi(ET1)Hdc*^{MB07212} can occur either precisely (B) or imprecisely (C).



Result 1: Precise excision: elimination of fluorescent green eye results, but with no deletion of the *Hdc* gene, fly gains *Hdc* function and contains histamine in the brain.



Result 2: Imprecise excision; deletion of the *Hdc* gene created (see gap,*), and fly loses *Hdc* function and, as a consequence, has no detectable histamine in the brain.

Figure 2: Transposon-mediated excision method used to generate a deletion in the *Hdc* gene. (A) Location of the *Mi(ET1)Hdc*^{MB07212} transposon in the *Hdc* gene as reported in (Flybase). Once initiated, transposon excision can either be (B) precise or (C) imprecise, which potentially causes a deletion mutation in the *Hdc* gene. (Figure adapted from Flybase; dos Santos et al., 2015)

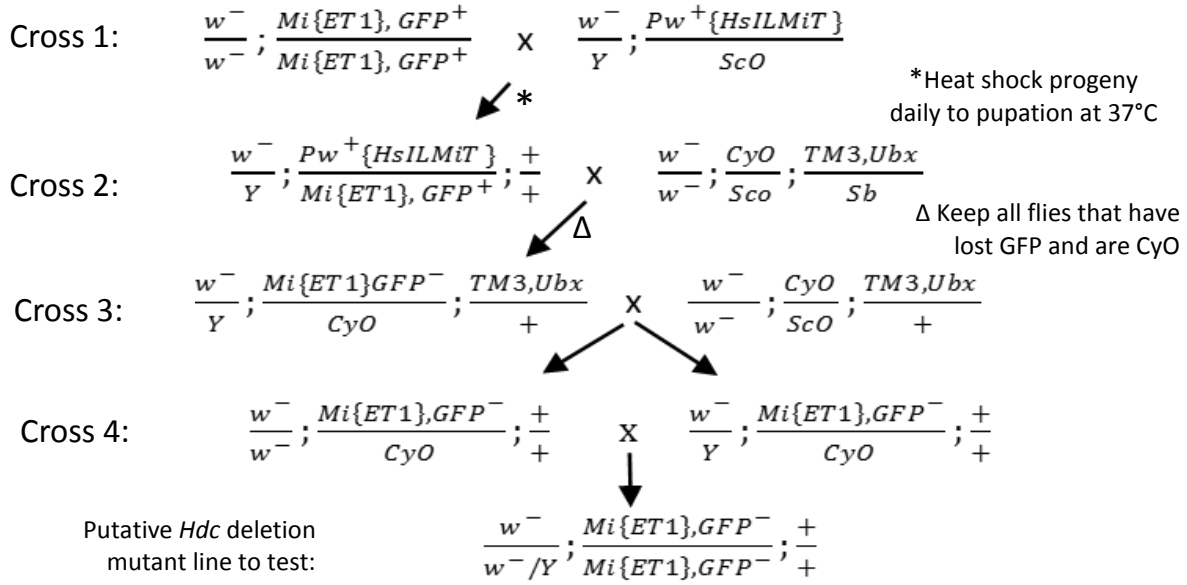


Figure 3: Mating scheme used to generate *Hdc* deletion mutants taking advantage of imprecise excision of a *Minos* element located in the *Hdc* gene. Males used in cross 2 were single-pair mated to females to generate GFP^- flies, representing excision of the Mi{ET1},GFP^+ transposon. Flies obtained for further study were progeny from Cross 4 that were derived into stably mating stocks.

Histamine Staining

Larval brains from mutant flies were subjected to histamine immunofluorescence staining analysis (see Appendix for procedure) once putative *Hdc* deletion alleles (Hdc^{Δ}) were identified and stable breeding stocks established by the selection of GFP^{-} individuals (Fig. 3, Cross 3 and 4; see also Fig. 4). By failing to detect histamine in the nervous system, histamine immunofluorescence staining enabled identification of true breeding GFP^{-} lines disrupted in *Hdc* function (Fig 5). Wild-type (normal) flies were stained as a positive control during these experiments to ensure that any negative staining result could be interpreted properly (Fig. 5).

Analysis of the genomic region including the Mi(ET1)Hdc^{MB07212} insertion in GFP$^-$ flies that are histamine deficient

Once a fly line had been established to have a GFP$^-$ and histamine deficient phenotype, 5 – 10 flies from each line identified (Fig. 3) were collected and their genomic DNA isolated using standard preparation techniques (Qiagen, Inc; see Appendix for specific procedural details). The *Hdc* gene region was amplified using Polymerase Chain Reaction (PCR) carried out via a standard reaction design for the FastStart High Fidelity Enzyme Blend (Roche, Inc.), adjusting the temperature of annealing depending on the primers used. PCR generated DNA samples were prepared for electrophoretic analysis to determine if the expected fragment was detected. The PCR enzyme used was able to amplify fragments up to 5000 base pairs, and the *Minos* transposon is 7819 bp long, so if the *Minos* element was present the expected result would be no amplicon present in the gel. As most of the excision events to be detected were likely to be precise, identification of failed PCR amplification reactions in the midst of successful ones enabled the identification of putative deletions (Vanrobays et al., 2010; Metaxkais et al., 2005). Amplification was repeated using the same DNA preparation on those lines that failed to produce an amplicon.

Complementation tests using *Hdc*^{JK910} and known lethal deletions in the *Hdc* region

In addition to the 98 stable breeding GFP$^-$ lines that were generated in this experiment and were tested (See Fig. 3), 6 additional breeding lines remained heterozygous, suggesting the presence of a recessive lethal mutation being present as a result of the excision experiment. These 6 mutant lines were thought to harbor a lethal mutation on the second chromosome and, if mapped to the *Hdc* gene, could demonstrate that an *Hdc* deletion can be lethal. These fly strains which did possess a lethal mutation on Chromosome 2 were crossed to *Hdc*^{JK910} flies as well as flies with known deletions uncovering the *Hdc* gene to determine whether the lethal mutation was affecting the *Hdc* gene and that it was in fact due to the absence of the *Hdc* gene.

Chapter 4: Results

Establishment of putative *Hdc* deficiency strains

The goal of these experiments was to generate 100 mutant flies, identified by the loss of GFP expression in the eye. Once a GFP^- fly was identified (Fig. 4), an additional cross was carried out to create a strain of flies in which homozygous deficiency-bearing flies could be identified at the larval stage as well as to preserve the potential Hdc^{Δ} allele (Fig. 3, Cross 4). Of the 33,800 flies screened, 119 GFP^- flies (0.35%) were identified – and of the 169 Cross 2's carried out (Fig. 3), 81 of them (47.9%) produced at least one GFP^- fly.

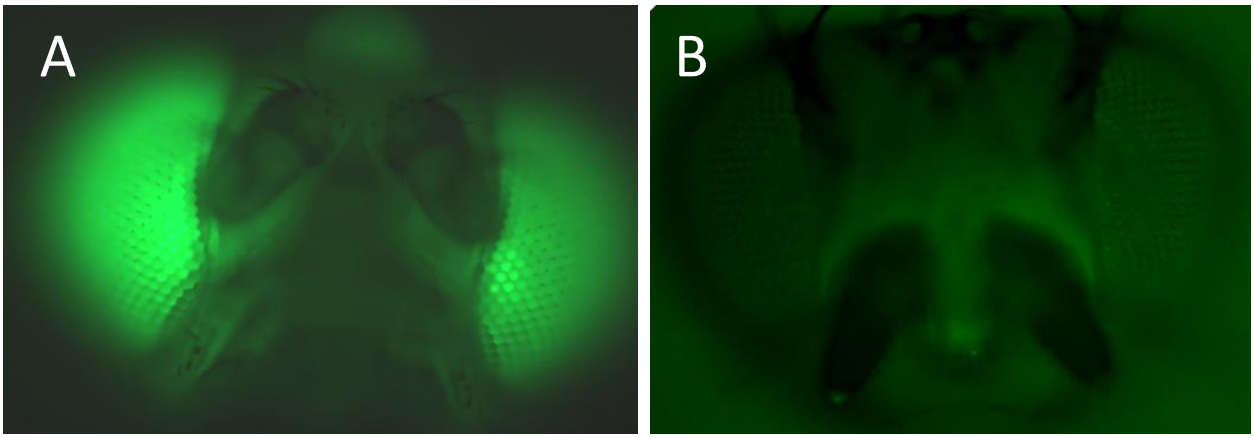


Figure 4: Illustration of the GFP^+ (A) or GFP^- (B) phenotypes used to identify putative Mi{ET1} excision events that could yield deletions of the *Hdc* gene. Males from cross #2 (Fig. 3) were examined for the presence (A) or the absence (B) of the GFP phenotype. (A) The GFP^+ (or 'green eye') phenotype allows for easy selection of a fly with GFP using an Amscope stereomicroscope at 10x magnification and the Nightsea fluorescent attachment. (B) Mi{ET1},GFP^- fly indicating an excision had occurred, through the loss of GFP in the eye. In this project, approximately 33,800 flies were screened, yielding about 169 GFP- lines to study, of which 29 resulted in a complete disruption of *Hdc*. (photo courtesy of Aaron Ripley)

Histamine staining to identify disruptions of the *Hdc* gene

In addition to the 29 lines that were GFP^- and did not stain positive for histamine, 56 GFP^- fly lines did demonstrate strong histamine immunofluorescence detection. These lines likely represent precise excisions of the *Minos* element, eliminating the effect that the *Minos* element had on *Hdc* gene function. As the original *Minos* insert fly line is a hypo-morphic allele, a precise *Minos* excision returns the fly to a wild type phenotype. There were also 13 GFP^- flies that stained weakly for histamine, likely resulting from an internal deletion of the *Minos* element, disrupting GFP expression. We conclude that putative small deletions from within the transposon showed no difference in histamine staining, as the *Minos* insert line itself also stained weakly for histamine.

Some of the 29 strains that demonstrated no detectable histamine immunoreactivity were also crossed with the Hdc^{JK910} mutant strain and their progeny examined for the presence of histamine to genetically confirm that a deletion of *Hdc* occurred in that fly line (Fig. 5F). This was done to ensure the HA^- phenotype was actually caused by a disruption of the *Hdc* gene, presumably through transposon-mediated excision.

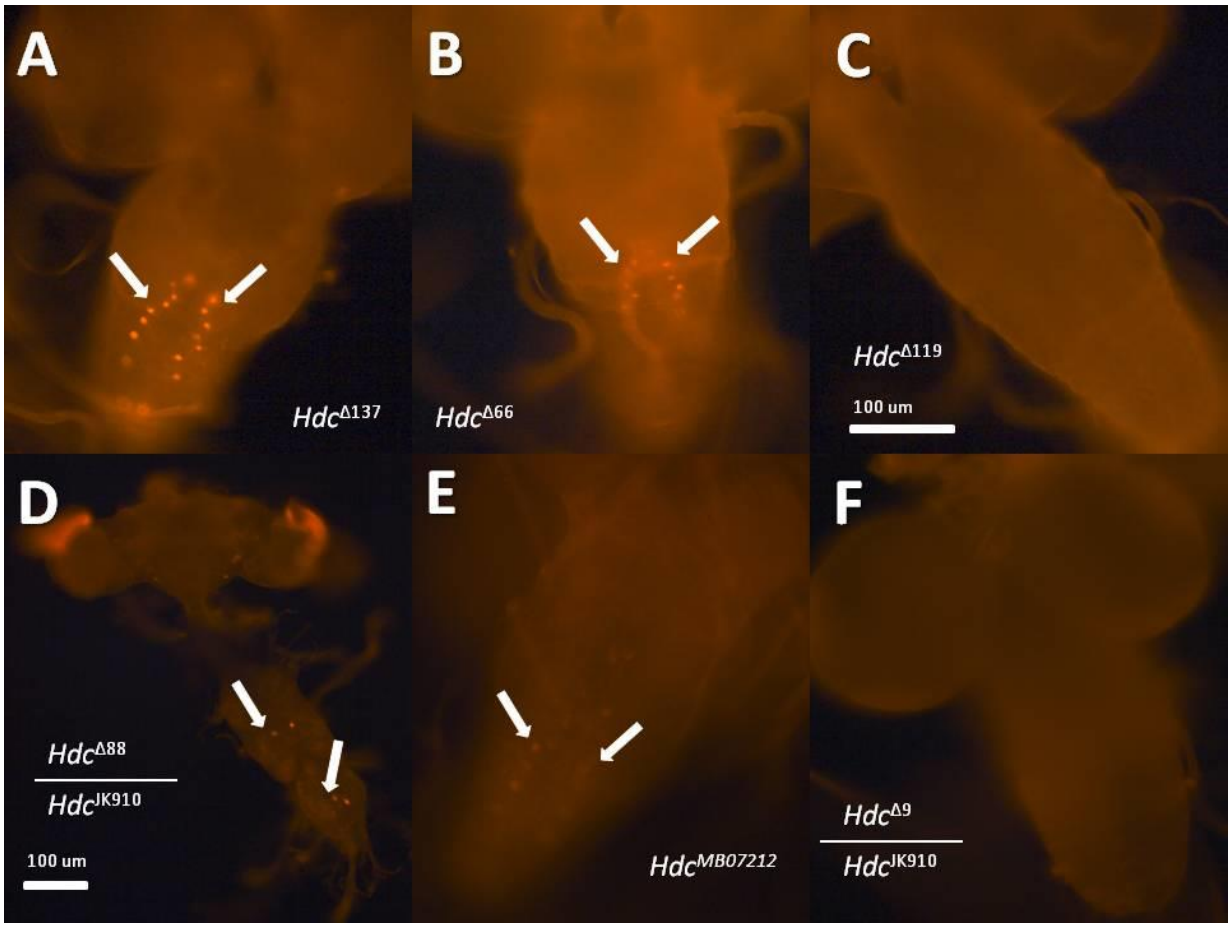


Figure 5: Histamine immuno-localization used to detect mutant flies with a potential deletion in the *Hdc* gene. Histamine immuno-localization of GFP^- flies demonstrate whether an excision of the Minos element had disrupted the *Hdc* gene in the GFP^- flies obtained. When *Hdc* is not disrupted (A), histamine is detected using a histamine antibody (A, arrows). Note that centrally located histaminergic neurons (arrows) are easily visible in the ventral nerve cord of larvae. A weak histamine signal (B, arrows) is observed in some flies that likely have a disrupted transposon remaining at the inserted site, similar to phenotype of the original Minos-bearing insertion used (E). In a third type of fly obtained, no histamine was detected, presumably due to a deletion of all or part of the *Hdc* gene (C). Histamine deficient mutant crossed to Hdc^{JK910} (F), and adult dissection of lethal mutant crossed to Hdc^{JK910} (D) – both crosses to test if the location of the disruption lies within the *Hdc* gene.

Polymerase Chain Reaction analysis of *Hdc* region

When all 29 of the lines staining negative for histamine were analyzed using a PCR approach, 12 failed to produce an amplicon as expected – the remaining 17 did produce a PCR amplicon, perhaps alluding to deletions as small as 5 base pairs. Out of the 20 retained lines staining positive for histamine, 5 were selected for analysis and all 5 produced an amplicon of the expected size. The flies staining weakly for histamine were presumed to have a partial removal of the *Minos* transposon (GFP gene), and when an alternate a primer set was used on 2 such mutants to determine if the 3' end of *Minos* remained, amplicons of the expected size were observed.

Mutant Stock#	HA staining	PCR Amplicon?	Mutant Stock#	HA Staining	PCR Amplicon?
4	weak	*No*	85	weak	*Yes*
4*	weak	*Yes*	85*	weak	*Yes*
69	positive	*Yes*	87	positive	*Yes*
108	positive	*Yes*	137	positive	*Yes*
147	positive	*Yes*			
7	negative	*Yes*	9	negative	*Yes*
13	negative	*Yes*	18	negative	*Yes*
19	negative	*Yes*	22	negative	*Yes*
26	negative	*Yes*	31	negative	*Yes*
32	negative	*Yes*	34	negative	*Yes*
35	negative	*Yes*	43	negative	*Yes*
45	negative	*Yes*	47	negative	*Yes*
48	negative	*Yes*	49	negative	*Yes*
100	negative	*Yes*			
51	negative	*No*	101	negative	*No*
114	negative	*No*	118	negative	*No*
119	negative	*No*	120	negative	*No*
121	negative	*No*	138	negative	*No*
140	negative	*No*	141	negative	*No*
146	negative	*No*	77	negative	*No*

Table 1: Summary table for HA staining and PCR results for the GFP^- lines initially isolated. PCR primers used: Left A1 and Right B1 (See Appendix). (*) signifies the use of the primer Left B1 instead of Left A1. For complete histamine staining and PCR results of all lines examined, see Appendix.

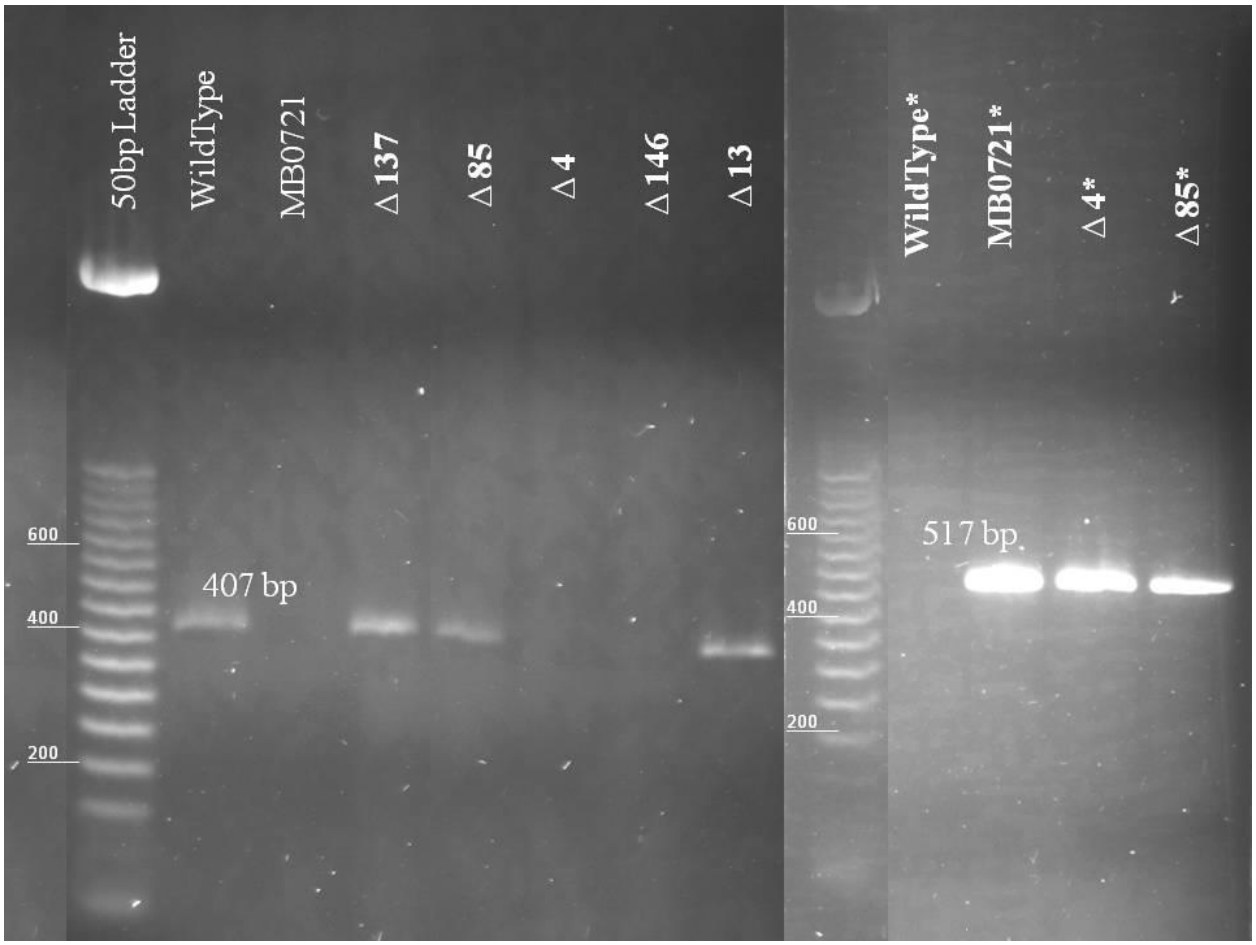


Figure 6: Gel Electrophoresis representative results from each type of mutant found. The first 7 lanes used the first primer set (Left A1 and Right B1), the final 4 lanes used the second primer set (Left B1 and Right B1; see Fig. 1 for primer locations). *Hdc*$^{\Delta 137}$ (lane 3) represents the 5 HA^{+} mutants tested (of 20 retained); all of the HA^{+} mutants produced the expected sized amplicon, indicating no significant disruption in the Hdc gene area tested. These, I postulate, are the flies that have reverted to a wild-type phenotype due to a precise excision of the *Minos* element. *Hdc*$^{\Delta 85}$ and *Hdc*$^{\Delta 4}$ (lanes 4 and 5, then 10 and 11) were the only 2 weak staining histamine mutants tested via PCR, and while they both produced the expected amplicon using the second primer set, *Hdc*$^{\Delta 85}$ also produced an amplicon with the first primer set. Finally *Hdc*$^{\Delta 146}$ and *Hdc*$^{\Delta 13}$ (lanes 6 and 7) represent the 29 HA^{-} mutants.

Complementation tests using *Hdc^{JK910}* and known lethal deletions in the *Hdc* region

Each of the 6 mutant lines thought to harbor a lethal mutation on the second chromosome were crossed to both *Hdc*^{JK910} and flies with known deletions uncovering the *Hdc* gene (Figure 7). These crosses were performed to determine whether the lethal that had been isolated genetically actually mapped to the *Hdc* genomic region. The positive staining result of the mutants crossed to *Hdc*^{JK910} (Fig. 5D) demonstrated that the lethal mutation did not disrupt *Hdc* and must therefore be outside the *Hdc* gene. Chi Square test results of the crosses to flies with known deficiencies uncovering *Hdc* showed mutants *Hdc$^{\Delta 88}$* and *Hdc$^{\Delta 107}$* with a p-value < 0.05 (See Appendix), suggesting that the deficiencies that uncover *Hdc* do not uncover these lethal mutations, therefore the lethal mutation is not within the *Hdc* gene.

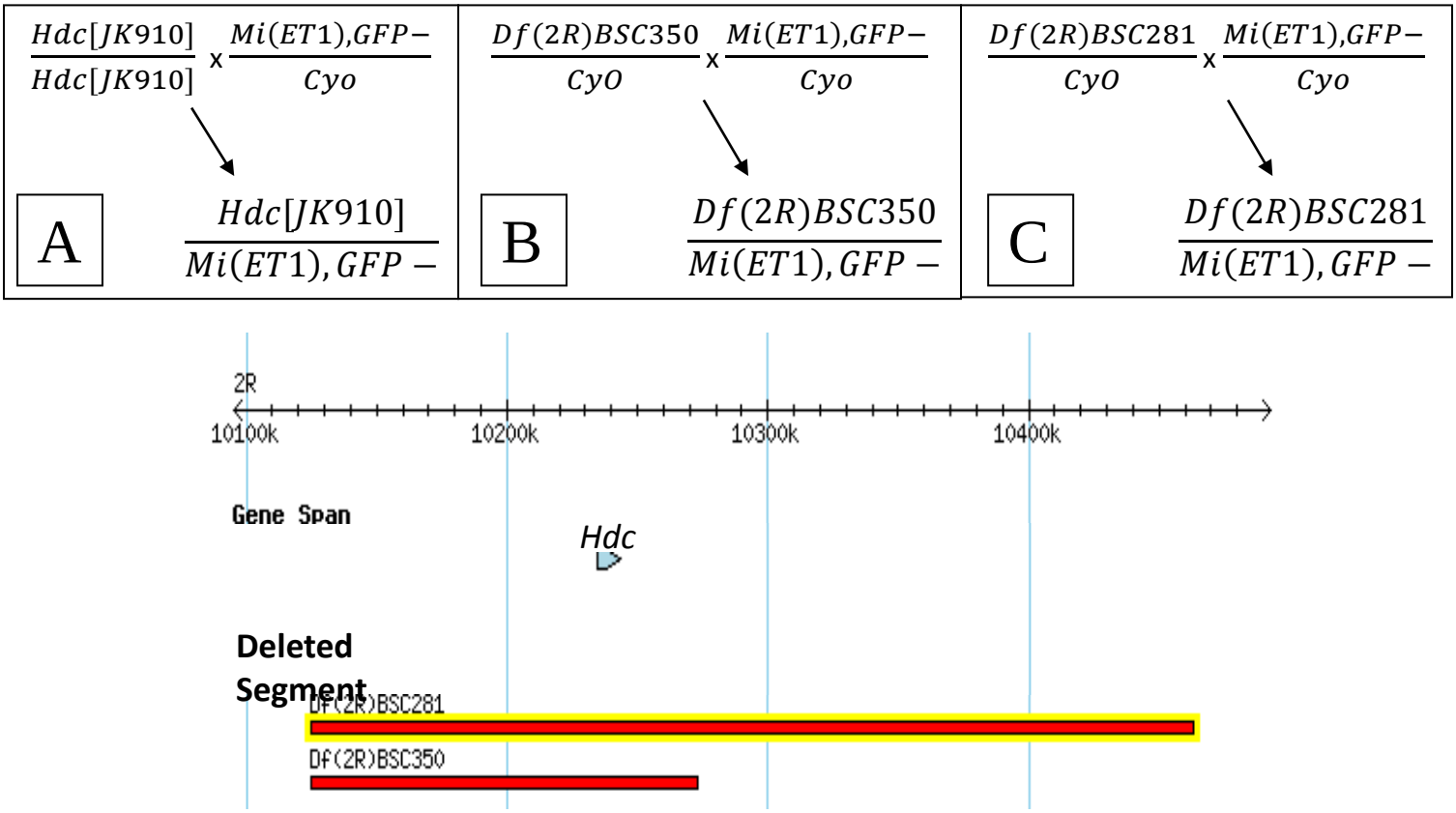


Figure 7: *Hdc*^{JK910} complementation crosses to lethal mutants (A), and mutant lethal crosses to flies with known deficiencies that have been previously shown to uncover the *Hdc* gene (B and C). The location of the known deficiencies relative to the *Hdc* gene are also included (lower image; adapted from Flybase).

Chapter 5: Discussion

Previous reports indicate 1.5 – 3.6% of *Minos* excision events result in an imprecise excision that leads to a deletion of a neighboring gene (Metaxkais et al., 2005). The goal of this study was the identification of at least one mutant with a deletion in the *Hdc* gene. Through *Minos* transposon excision mutagenesis, 98 mutant lines were generated and identified by the loss of GFP expression, then examined via PCR and immunohistochemistry staining. Results obtained indicate that each of the 98 GFP^- strains examined fell into one of the three expected categories (positive staining, weak staining, and no staining), with 29 of the strains demonstrating an elimination of histamine synthesis ability as a result of no histamine being detected in their larval brain structures. When these flies were analyzed using a PCR approach, only 12 of the 29 strains failed to produce an amplicon as expected, suggesting that these 12 lines do have a physical elimination of at least enough of the *Hdc* locus to eliminate gene expression. The remaining 17 strains that showed no histamine staining did produce an amplicon and therefore could represent small deletions as small as 5 base pairs. As *Minos* is 4 base pairs 5' to the second intron in *Hdc*, a deletion that small and difficult to discern on a gel could be large enough to disrupt *Hdc* gene function. Future work will analyze the molecular nature of these lines to confirm the presence of the disruptions. Crosses will be conducted between these mutants and Hdc^{JK910} to confirm the HA^- phenotype is due to *Hdc* disruption, and genome sequencing will be performed to discern to precise extent of the putative deletion.

In addition to the identification of new *Hdc* mutations, 56 GFP$^-$ strains stained positive for histamine, suggesting that a precise excision of the *Minos* transposon caused this result. This is supported by the fact that each (5 of the 20 retained) of the positively staining mutants analyzed with PCR showed an amplicon, suggesting no presence of the *Minos* element. The PCR enzyme used amplifies fragments up to 5000 base pairs, and the *Minos* transposon is 7819 bp long. Therefore if the *Minos* element was present, the expected result would be no amplicon present in the gel. Sequence analysis of these amplicons can show whether this is the case, and will be done in the near future.

A final group of expected histamine-staining categories are the 13 strains that weakly stained for histamine. It was presumed that flies staining weakly for histamine may represent a partial removal of the *Minos* transposon that disrupts the GFP gene within the *Minos* element (see Fig. 1), but retains some *Minos* element that still disrupts the *Hdc* gene. When a PCR analysis was performed on 2 of the weak staining flies using the alternate left primer (Left B1) inside the 3' portion of the *Minos* transposon, an amplicon was shown in the gel. This indicates that the right-most portion of the *Minos* transposon failed to completely excise. Additionally, although both weak staining mutants showed a PCR amplicon using the alternate primer set on the 3' end of the *Minos* transposon, they did not show the same result with the original primer set on either end of *Minos*. Future PCR work could include analyzing the 5' end of the *Minos* transposon and repeating those tests on the remaining 11 weak HA staining strains.

The final group of generated variant flies are those that failed to produce a homozygous breeding stock, and are being kept as heterozygotes (the assumption being that they harbor a recessive lethal mutation on the second chromosome). These flies were first crossed to the Hdc^{JK910} mutant to determine if a lethal deletion occurred within the *Hdc* gene. The positive histamine staining result (see Fig. 5D) demonstrated that the lethal mutation did not disrupt *Hdc* and must be outside the *Hdc* gene. The recessive lethal strains identified were next crossed to flies bearing deficiencies that included the region containing the *Hdc* gene, under the presumption that a lethal mutation could likely manifest in that region. The presence of straight-winged progeny indicated that any lethal mutation would have to be elsewhere in the second chromosome but not in the *Hdc* region itself. Chi Square results (see Appendix) for mutants $Hdc^{\Delta 88}$ and $Hdc^{\Delta 107}$ actually showed a higher than expected number of straight-winged flies when crossed to known deficiencies, further demonstrating the lethal mutation is not within *Hdc*. Future work will cross these mutants with each other to determine if just one gene or multiple genes have been disrupted.

The original experimental design for this project was to first conduct PCR on all new GFP^- mutants and then perform histamine staining on only those strains that did not produce a PCR amplicon – the expectation being those strains would be deletions and therefore show a negative histamine staining result. When the time came, however, the PCR reaction was not working satisfactorily and did not show anything – and did not show any amplicons even for wild type flies until later when new enzyme and primers were obtained. Because of this difficulty in reliably performing PCR, the decision was made to instead perform histamine staining on all GFP^- mutant strains and then analyze select mutants for PCR analysis later. Although this

method took more time, more information was gained through this sequence of analysis – such as the group of weak HA staining flies and the 17 HA^- strains that did show a PCR amplicon.

Overall, it is clear from the histamine staining and PCR results that new alleles of *Hdc* have been generated that are likely caused by deletions. The molecular extent of the 12 HA^- strains that did not produce a PCR amplicon needs to be explored via PCR using oligonucleotide primers that are 500 bp either upstream or downstream of the original insert site. Once an amplicon is generated, DNA sequencing of the resulting fragment can be utilized to determine the precise extent of the deletions for these histamine deficient mutant flies. These new *Hdc* mutants generated could be useful in various behavioral studies as well as gene regulation studies of the *Hdc* promoter, assuming deletions are large enough to extend into the promoter region. While this research focused on the expression of histamine in the central brain of larvae, recent work in the lab has suggested the presence of histamine in the gut of *Drosophila* and other structures. Histamine in the gut as well as in mechanoreceptors could be examined in future studies using these *Minos* excision alleles of *Hdc*. It should lastly be noted that flies can acquire histamine from their food instead of producing it from histidine using the enzyme histidine decarboxylase (Melzig et al., 1996), so future tests focused on the photoreceptors and gut of the generated mutants should utilize food shown to be devoid of histamine.

Appendix

Histamine Staining Protocol

1. Dissect flies in 4% carbodiamide fixative at 4°C
2. Incubate in the carbodiamide fixative at 4°C for 2.5-3 hours.
3. Wash flies twice with Drosophila Ringer's solution
4. Incubate dissections in 5% normal goat serum (NGS) for 30 minutes at room temperature
5. Remove excess serum and add primary rabbit histamine antibody (1:500 dilution)
6. Incubate overnight at 4°C in a humid chamber
7. Allow dissections to warm to room temperature for 30 minutes
8. Wash twice for 20 minutes each in TBS + 1% NGS + 0.3% Triton X-100
9. Incubate for 60 minutes in secondary IgG anti-rabbit antibody conjugated Alexa Fluor 555 (1:1000 dilution)
10. Wash once for 20 minutes in TBS + 1% NGS + 0.3% Triton X-100
11. Wash for 20 minutes in PBS
12. Plate and coverslip with support in PBS

DNA Isolation Protocol

1. Turn on water bath, set to 65°C, grab ice.

2. Dispense 300µL cell lysis solution into 1.5mL micro centrifuge tubes. Put on ice.

3. Isolate 5-10 flies from each stock.

4. Place flies directly into lysis solution. Keep on ice. Homogenize solution using pestle (keep pestle clean with 70% ethanol).

5. Incubate in 65°C water for 10-15 minutes. Cool to room temperature by placing on ice.

6. Add 100µL protein precipitation solution and vortex vigorously for 20 seconds at high speed. Keep sample on ice for 5 minutes.

7. Centrifuge at 13,000-16,000x for 6 minutes.

8. Pipette 300µL isopropyl alcohol into clean 1.5mL micro centrifuge tubes. Add the supernatant from previous step.

9. Mix with 50 inversions.

10. Centrifuge again for 1 minute at 13,000-16,000x.

11. Discard the supernatant, leave the pellet undisturbed.

12. Add 300µL of 70% ethanol to pellet and invert several times to wash.

13. Centrifuge at 13,000-16,000x for 1 minute.

14. Carefully discard the supernatant onto absorbent paper.

15. Let air dry for 10-15 minutes to evaporate the ethanol.

16. Add 50µL of DNA hydration solution.

17. Incubate at 65°C for 1 hour.

18. Incubate at room temperature overnight on a gentle shake plate.

19. Centrifuge for 5 minutes at 13,000-16,000x and transfer to smaller storage tubes.

20. Store samples at -20°C until PCR.

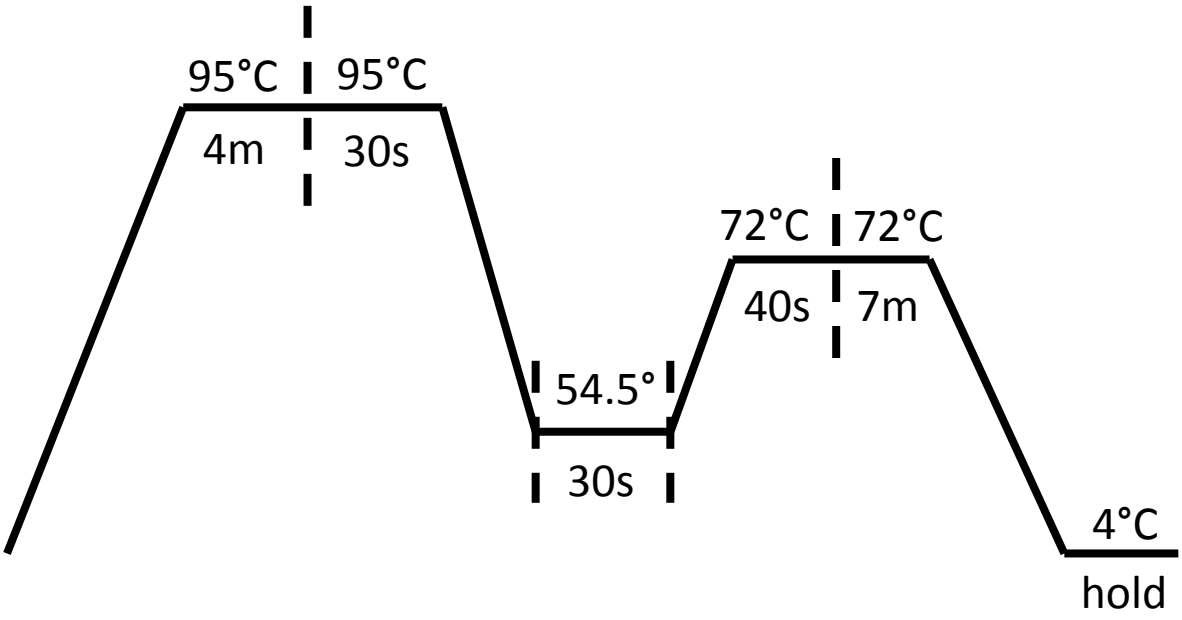
PCR Protocol

1. For generation of the "Master Mix," add the following for each sample you plan to run:

- 10.8µL of PCR H_2O	(108µL if running 10 samples)
- 2.5µL of 10x Buffer	(25µL if running 10 samples)
- 0.5µL of dNTP Mix	(5µL if running 10 samples)
- 5µL of Primer "Left A1"	(50µL if running 10 samples)
- 5µL of Primer "Right B1"	(50µL if running 10 samples)
- 0.2µL of FastStart High Fidelity Enzyme	(2µL if running 10 samples)

2. Place 24µL of "Master Mix" and 1µL of isolated DNA into each PCR tube

3. Place samples into the Thermocycler for 30 cycles of PCR



Standard thermocycler settings as described in FastStart High Fidelity Enzyme Blend (Roche, Inc.) data sheet. Primers:

(Left A1) 5'-CACACACGTGGTTAACATAATCTAC-3',

(Right B1) 5'-CGATTGCCAGTGGGTTATG-3',

(Left B1) 5'-GGATCTCATGCTGGAGTTCTTC-3'

Chi Square Test: *Hdc* mutants crossed to known lethal deletion strains (see Fig. 7 C and D)

Lethal Mutant Stock #	Wing Phenotype	Observed	Expected	p-value	Total
$Hdc^{\Delta 88}$	*straight*	31	27.6		83
	curly	52	55.3		
				0.43766	
$Hdc^{\Delta 89}$	*straight*	21	24.6		74
	curly	53	49.3		
				0.36589	
$Hdc^{\Delta 90}$	*straight*	19	22.3		67
	curly	48	44.6		
				0.38766	
$Hdc^{\Delta 107}$	*straight*	28	31		93
	curly	65	62		
				0.50931	
$Hdc^{\Delta 110}$	*straight*	29	24		72
	curly	43	48		
				0.2113	
$Hdc^{\Delta 124}$	*straight*	25	24		72
	curly	47	48		
				0.80259	

Chi Square Test: Putative *Hdc* lethal mutants crossed to Hdc^{JK910} (See Fig 7A)

Lethal Mutant Stock #	Wing Phenotype	Observed	Expected	p-value	Total
$Hdc^{\Delta 88}$	*straight*	34	22		66
	curly	32	44		
				0.001727951	
$Hdc^{\Delta 89}$	*straight*	35	27		81
	curly	46	54		
				0.059346439	
$Hdc^{\Delta 90}$	*straight*	33	25.3		76
	curly	43	50.6		
				0.062104863	
$Hdc^{\Delta 107}$	*straight*	40	30.6		92
	curly	52	61.3		
				0.038999953	
$Hdc^{\Delta 110}$	*straight*	23	21		63
	curly	40	42		
				0.592980098	
$Hdc^{\Delta 124}$	*straight*	36	30		90
	curly	54	60		
				0.179712495	

List of GFP^{-} Mutants

Mutant	Histamine Staining	PCR Amplicon?
Hdc$^{\Delta 69}$	Positive	Yes
Hdc$^{\Delta 87}$	Positive	Yes
Hdc$^{\Delta 108}$	Positive	Yes
Hdc$^{\Delta 137}$	Positive	Yes
Hdc$^{\Delta 147}$	Positive	Yes
Hdc$^{\Delta 5}$	Positive	
Hdc$^{\Delta 24}$	Positive	
Hdc$^{\Delta 53}$	Positive	
Hdc$^{\Delta 83}$	Positive	
Hdc$^{\Delta 133}$	Positive	
Hdc$^{\Delta 50}$	Positive	
Hdc$^{\Delta 76}$	Positive	
Hdc$^{\Delta 86}$	Positive	
Hdc$^{\Delta 52}$	Positive	
Hdc$^{\Delta 145}$	Positive	
Hdc$^{\Delta 80}$	Positive	
Hdc$^{\Delta 97}$	Positive	
Hdc$^{\Delta 92}$	Positive	
Hdc$^{\Delta 117}$	Positive	
Hdc$^{\Delta 116}$	Positive	
Hdc$^{\Delta 7}$	Positive	
Hdc$^{\Delta 9}$	Positive	
Hdc$^{\Delta 19}$	Positive	
Hdc$^{\Delta 39}$	Positive	
Hdc$^{\Delta 40}$	Positive	
Hdc$^{\Delta 41}$	Positive	
Hdc$^{\Delta 44}$	Positive	
Hdc$^{\Delta 46}$	Positive	

Mutant	Histamine Staining	PCR Amplicon?
Hdc$^{\Delta 47}$	Positive	
Hdc$^{\Delta 48}$	Positive	
Hdc$^{\Delta 53}$	Positive	
Hdc$^{\Delta 56}$	Positive	
Hdc$^{\Delta 58}$	Positive	
Hdc$^{\Delta 59}$	Positive	
Hdc$^{\Delta 60}$	Positive	
Hdc$^{\Delta 61}$	Positive	
Hdc$^{\Delta 62}$	Positive	
Hdc$^{\Delta 64}$	Positive	
Hdc$^{\Delta 65}$	Positive	
Hdc$^{\Delta 73}$	Positive	
Hdc$^{\Delta 84}$	Positive	
Hdc$^{\Delta 94}$	Positive	
Hdc$^{\Delta 99}$	Positive	
Hdc$^{\Delta 102}$	Positive	
Hdc$^{\Delta 114}$	Positive	
Hdc$^{\Delta 115}$	Positive	
Hdc$^{\Delta 119}$	Positive	
Hdc$^{\Delta 122}$	Positive	
Hdc$^{\Delta 123}$	Positive	
Hdc$^{\Delta 126}$	Positive	
Hdc$^{\Delta 131}$	Positive	
Hdc$^{\Delta 135}$	Positive	
Hdc$^{\Delta 139}$	Positive	
Hdc$^{\Delta 142}$	Positive	
Hdc$^{\Delta 143}$	Positive	
Hdc$^{\Delta 144}$	Positive	

Mutant	Histamine Staining	PCR Amplicon?
Hdc$^{\Delta 7}$	Negative	Yes
Hdc$^{\Delta 9}$	Negative	Yes
Hdc$^{\Delta 13}$	Negative	Yes
Hdc$^{\Delta 18}$	Negative	Yes
Hdc$^{\Delta 19}$	Negative	Yes
Hdc$^{\Delta 22}$	Negative	Yes
Hdc$^{\Delta 26}$	Negative	Yes
Hdc$^{\Delta 31}$	Negative	Yes
Hdc$^{\Delta 32}$	Negative	Yes
Hdc$^{\Delta 34}$	Negative	Yes
Hdc$^{\Delta 35}$	Negative	Yes
Hdc$^{\Delta 43}$	Negative	Yes
Hdc$^{\Delta 45}$	Negative	Yes
Hdc$^{\Delta 47}$	Negative	Yes
Hdc$^{\Delta 48}$	Negative	Yes
Hdc$^{\Delta 49}$	Negative	Yes
Hdc$^{\Delta 100}$	Negative	Yes
Hdc$^{\Delta 51}$	Negative	No
Hdc$^{\Delta 77}$	Negative	No
Hdc$^{\Delta 101}$	Negative	No
Hdc$^{\Delta 114}$	Negative	No
Hdc$^{\Delta 118}$	Negative	No
Hdc$^{\Delta 119}$	Negative	No
Hdc$^{\Delta 120}$	Negative	No
Hdc$^{\Delta 121}$	Negative	No
Hdc$^{\Delta 138}$	Negative	No
Hdc$^{\Delta 140}$	Negative	No
Hdc$^{\Delta 141}$	Negative	No
Hdc$^{\Delta 146}$	Negative	No

Mutant	Histamine Staining	PCR Amplicon?
Hdc$^{\Delta 4}$	Weak	No *(Yes with alternate primers)*
Hdc$^{\Delta 85}$	Weak	Yes *(Yes with alternate primers)*
Hdc$^{\Delta 6}$	Weak	
Hdc$^{\Delta 16}$	Weak	
Hdc$^{\Delta 66}$	Weak	
Hdc$^{\Delta 67}$	Weak	
Hdc$^{\Delta 113}$	Weak	
Hdc$^{\Delta 125}$	Weak	
Hdc$^{\Delta 127}$	Weak	
Hdc$^{\Delta 129}$	Weak	
Hdc$^{\Delta 132}$	Weak	
Hdc$^{\Delta 134}$	Weak	
Hdc$^{\Delta 136}$	Weak	
Hdc$^{\Delta 88}$	N/A (kept as heterozygotes)	
Hdc$^{\Delta 89}$	N/A (kept as heterozygotes)	
Hdc$^{\Delta 90}$	N/A (kept as heterozygotes)	
Hdc$^{\Delta 107}$	N/A (kept as heterozygotes)	
Hdc$^{\Delta 110}$	N/A (kept as heterozygotes)	
Hdc$^{\Delta 124}$	N/A (kept as heterozygotes)	

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