Screening for Inducible Genetic Variation in Natural Populations of *Drosophila melanogaster*

California Lutheran UNIVERSITY

Neha Soogoor and Madison Katz

CLU Biology Department

ABSTRACT

Transposable elements (TEs) are DNA segments that can transpose within a genome. This study aims to screen for epigenetic cryptic genetic variation (ECGV) in natural populations of *Drosophila melanogaster* which have been induced by P-transposable elements. P-transposable elements are a specific class of transposable elements that have high rates of transposition and are associated with genetic defects like high mutation rates and chromosomal abnormalities. Our screen is based on a model for the production of head defects by the *extra eye* mutation (*ee*), which is incompletely penetrant, variably expressed, and conditionally dominant. The model (Dr. Marcey, personal communication) suggests a gene encoding the repressor STAT, a molecule present in the embryonic eye field establishment, is silenced through RNAi-mediated heterochromatization induced by P-transposable elements. Prior PCR analysis indicated presence or absence of P-elements in 40+ lines of wild type flies collected from various worldwide geographical locations. Lines that harbor P-elements are being crossed to JG1, a highly penetrant *ee* stock, and their F1 progeny intercrossed to establish lines. Lines are being screened for possible cryptic variants that could be revealed as a product of RNAi-induced heterochromatization of genes near P-elements. The results of such crosses are relevant for both genetic and evolutionary biology, as the variation is one of the essential components which natural selection acts upon. This search may uncover what are known as "epialleles" which play a role for variation upon which natural selection can act.

INTRODUCTION

Background

The *extra eye* (*ee*) mutation in *Drosophila melanogaster* is variably expressed, conditionally dominant, and incompletely penetrant. The mutation can result in head deformities that can include missing and/or duplicated bristles and head cuticle, and in its most severe expressions, supernumerary compound eyes and antennae (Figure 1). The insertion of P-transposable element in the 5' exon of the *Cytochrome p450 reductase* (*Cpr*) gene can result in the *ee* mutation. Transcription of *Cpr* gene results in an anti-sense P-element RNA transcript (Figure 2). Genomic P-elements inserted in a normal orientation are present and when transcribed provide for sense P-element transcript. The sense and antisense transcripts can then hybridize, forming double-stranded RNA. The formation of dsRNA results in formation of siRNA via the RNAi pathway, where chromatin remodeling factors are recruited and heterochromatize regions close to P-elements. Heterochromatin can then "spread" and result in silencing of adjacent host genes through a phenomenon called position-effect variegation (Lee, 2015). The heterochromatin spreads to a nearby gene known as *Su(var)2-10,* a suppressor of Position Effect Variegation (Hari et. al., 2001). This heterochromatization results in the *Su(var)2-10* gene silencing. The suppression of the *Su(var)2-10* gene has been associated with the upregulation or increase of JAK-STAT signaling. Overexpression of JAK-STAT results in head deformities in *Drosophila* which have been present in extra eyes.

. **Multiple genetic studies support the Figure 2 model (Marcey, et al., unpublished), including:**

- **Silenced transposable elements have effects on expression of nearby genes, suggesting that there is an evolutionary trade-off between silencing of TEs and increased effect on nearby genes.**
- **Insertion of 3-kilobase P-transposable element resulted in exhibition of P-element induced mutations in *D. melanogaster* progeny, proving evidence that P-elements can successfully mobilize (Spradling and Rubin, 1982).**
- **The study found that new TE insertions in *A. thaliana* resulted in the disruption of crucial genes and many of the TEs were silenced by DNA methylation, indicating that DNA methylation was directly correlated with gene silencing (Hollister and Gaut, 2007).**

Thus, we propose that when lines of *ee* are bred with wild-type lines known to contain P-elements it will result in epigenetic variations such as head abnormalities, eye abnormalities, changes in bristles, and wing abnormalities as proposed by the Marcey lab.

A Test of Inducible Epigenetic Variation

Cryptic genetic variation (CGV) is conditionally expressed as a phenotype when organisms are subjected to environmental or genetic perturbations (e.g. Takahashi, 2013). The existence of CGV could theoretically represent an important store of latent, adaptive alleles, neutral variants, or even deleterious mutations, but CGV is better understood in theory than in nature (e.g. Paaby and Rockman, 2013). Based on the the two-component model for extra eye production (Figure 2), we predicted that crossing *ee* mutant flies with wild-type flies collected from diverse geographical locations (with P-elements at correspondingly diverse genomic loci) could reveal inducible epigenetic variation (IEGV) in natural populations. We expected to recover mutations caused by epigenetic silencing of genes residing at genomic positions near P-elements due to RNAi-induced heterochromatization caused by the *Cpr* anti-sense P-element in the *ee* stock.

In a preliminary screen of only 16 lines, we recovered a new mutation, *crybaby (cby)*, that displays epigenetic hallmarks similar to *ee*. This result supports the *ee* epigenetic model, and suggests that new, inducible, previously cryptic epialleles, revealed by RNAi-mediated heterochromatization, may be a component of heritable phenotypic variation upon which natural selection is brought to bear in wild populations. However, it remained to be seen whether the crybaby mutation was indeed caused by putative induction via the proposed RNAi model (Figure 2), or could be due to the de novo insertion of a P- transposable element into a gene, causing the *cby* disruption. I therefore conducted a series of crosses to resolve this issue.

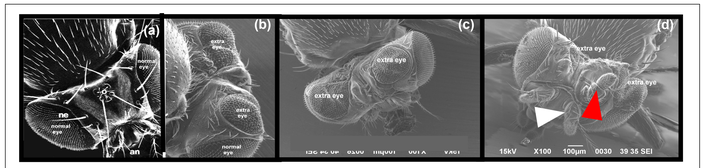

Figure 1. Extreme *extra eye* phenotypes. **(a)** dorsal view of a wild-type head, showing lateral, normal compound eyes, dorsal ocelli (O), antennae (an) and bristles. **(b)** posterior dorsal view of an *ee* fly with missing ocelli and two supernumerary eyes embedded in dorsal cuticle. **(c)** dorsal aspect of an *ee* fly with missing ocelli and large extra eyes on both sides of the head. **(d)** anterior dorsal view of an ee fly, with a duplicated antenna (red arrow), a mirror-image of its normal counterpart (white arrow), as well as a large extra eye fused with its ipsilateral, normal counterpart and a smaller, contralateral extra eye.

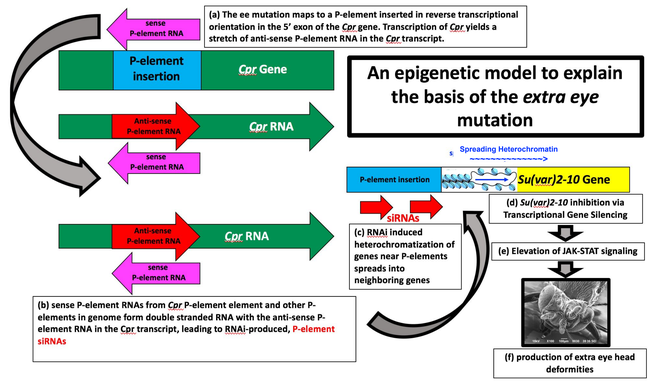

Figure 2. (a) A P-element insertion in the 5' exon of *Cpr* results in an anti-sense P-element RNA within the *Cpr* transcript. **(b)** P-element sense RNAs derived from genomic P-elements produces double stranded RNA with the *Cpr* anti-sense P-element RNA. This yields siRNAs via the RNAi pathway. **(c)** siRNAs are ferried to P-elements in the genome where they recruit chromatin remodeling factors that heterochromatize regions near P-elements. **(d)** Spreading heterochromatization of the genomic region near a *Su(var)2-10*-adjacent P-element leads to *Su(var)2-10* transcriptional gene silencing. **(e)** Inhibition of a *Su(var)2-10* expression leads to an increase of JAK-STAT signaling. **(f)** Overexpression of JAK-STAT signaling leads to head deformities, including extra eyes. The model explains multiple genetic features of the *ee* mutation.

METHODS

In order to examine traits expressed by P-elements in lines of *Drosophila melanogaster,* crosses were performed between *ee* lines of JG1/1245 with wild-type lines found to contain P-elements through PCR analysis. Lines were established at the F2 generation and beyond and screening was conducted once F2 generation was established. Screens were recorded in lab notebooks in which the total number of flies screened and the bottle date was recorded. Crosses were observed for new emerging phenotypes, bristles, eyes, head, limbs, abdomen, and wings were all studied for abnormalities. If mutants were present in cross then they were isolated and collected for virgin males and females. A mutant cross was made between mutant males and females to select upwards for new phenotypes. If mutants were absent and 300 flies were screened, crosses were archived in a 18 C incubator.
Performed crosses included:

Table 1. Crosses between *ee* and wild-type strains of *Drosophila melanogaster.*

Crosses (ee ♀♀x wild-type ♂♂)		
	JG1xDM-15	JG1xDM-185
	JG1xDM-137	1245xKW003
	JG1xDM-126	1245xDM15
	JG1xKW008	JG1xDM-133
	JG1xKW003	JG1x38
	JG1xDM-136	JG1x52
		JG1xDM-198

PREVIOUS RESULTS

A New Mutant Phenotype Uncovered by Crosses to *ee*

We observed a new phenotype, consisting of head deformities most commonly observed as loss of ventral eye tissue and duplication of vibrissae in the F2 generation of crosses of JG1(*ee*) females to KW003 (Brazil) males. The new mutation is named ***crybaby* (*cby*)** because of the teardrop shape of the eyes commonly observed (Figure 3b). That ventral retinal development is primarily impaired by the *cby* mutation is shown by incorporating the *white racing stripe* mutation into a *cby* background. *Racing stripe* is caused by the insertion of a *white*$^{+}$ construct into a *white*$^{-}$ mutant background. The construct expresses the wild-type white gene in a stripe across the midline of the eye (Figure 3c). In a *cby racing stripe* fly, the band of red pigment is observed at the ventral margin of a reduced eye, indicating absence of ventral eye tissue (Figure 3d).

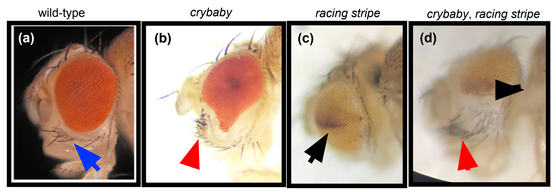

Figure 3. Lateral views of a wild-type and *crybaby* heads. **(a)** a wild-type head showing large compound eye (red) and ventral vibrissae (blue arrow). **(b)** a *crybaby* mutant - note the reduced number of facets in the ventral half of the *crybaby* eye, along with duplications of ventral vibrissae (red arrow). **(c)** the racing stripe phenotype. **(d)** a racing stripe (black arrow) is observed at the ventral margin of a *crybaby* eye – note supernumerary vibrissae (red arrow).

The cryptic *cby* mutation is repeatedly revealed in crosses of WT line #3 (Brazil) to *ee* lines

In order to test the possibility that *cby* was caused by the *de novo* hopping of a P transposable element into a gene (*cby*) in the course of our cross of JG1 (*ee*) to KW003 (Brazil), as opposed to genetic crypsis being revealed by the anti-sense P-element of the *ee* line, we repeated the original cross, and also crossed KW003 (Brazil) males to females from a different *ee* line, 1245 (*ee b pr*). In both additional cases, *crybaby* phenotypes appeared in the F2. An example of a *cby* fly from the F2 of the latter cross is the "*b pr cby*" example of Figure 4f. The probability that the same gene would be targeted by transposable element insertion in 3 separate crosses is extremely rare, leading to the conclusion that crosses of *ee* lines to KW003 (Brazil) reveals a pre-existing, cryptic genetic variant that is expressed in the presence of *ee*.

PREVIOUS RESULTS, continued

The recessive *cby* mutation displays several epigenetic features, and behaves similarly to *ee*

The *crybaby* mutation displays several hallmarks of epigenetic variability in expression and penetrance, as does *extra eye* (Figure 4). *Cby* is variably expressed with mild expressions involving small ventral eye reductions and severe expressions removing all compound ommatidia. The *cby* mutation, like *ee*, is incompletely penetrant and temperature sensitive. Various *cby* sublines, established by outcrossing to balancer chromosome stocks and re-homozygosing *cby*, display different mutant penetrances, and a pronounced elevation of penetrance and expression at 25+ degrees, as compared to development at 18 degrees (Marcey, personal communication).

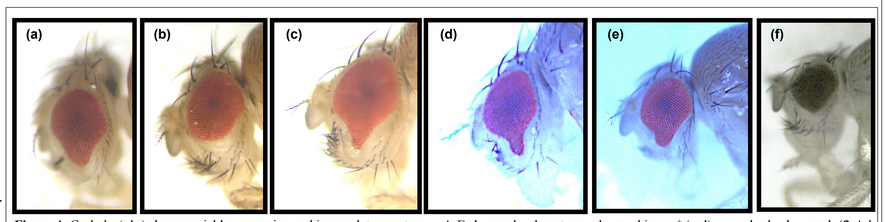

Figure 4. *Crybaby* (*cby*) shows variable expression and incomplete penetrance. A-E shows *cby* phenotypes observed in a w^{+} (red) eye color background. **(f)** A *b pr cby* example obtained in a cross of KW003 to *ee b pr*.

Table 1. Summary table of phenotypes that appeared from the screening of genetic crosses

Crosses	Phenotype
1245xDM-15	*Heartbaby (Hby)*
JG1xDM-133	*Red belly (Rb)*
JG1xJG1xDM-133	*Red belly (Rb)*

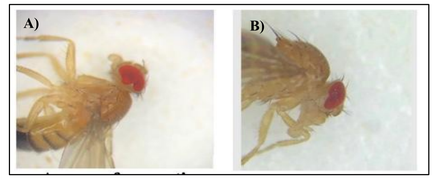

Figure 5. *Heartbaby (Hby)* phenotypic expression from a line established from the 1245 x DM-15 cross. *Hby* phenotype appears variably expressed and incompletely penetrance. A and B show *Hby* phenotypes observed with a WT (w^{+}) eye color. *Hby* expresses an inverted teardrop shape in the ventral region of the eye.

RESULTS

The *Hby* mutations displays phenotypic variation induced by P-elements similar to *Cby*

We observed a phenotype that consisted of eye deformities in the ventral region of the eye similar to the *Cby* mutation and *ee*. The mutation is called **heartbaby *(Hby)*** due to the heart-like appearance of the eye. *Heartbaby* is the result of a cross between *ee* line 1245 and wild type line DM-15 which was isolated from Bahia, Brazil. The new phenotype appears to be variably expressed and present in both males and females. The construct appeared with extensive indention along the middle region of the eye, followed overgrowth of bristles as well as small eye bristle growth that also appeared in *ee* mutations (Figure 5).

New Phenotype Uncovered by Crosses with *ee*

We also observed a new phenotype pertaining to the abdomen region of *Drosophila melanogaster.* The phenotype was obtained from a cross between *ee* line JG1 and wild type strain DM-133. The fruit flies obtained in the F2 generation and beyond possessed red-orange bodies similar to the color of their eyes. The new phenotype was observed in males of the line containing racing stripe eyes (a construct as a result of *ee*). This phenotype was studied further and was also examined to spread from the lower abdomen to the right side of the abdomen (Figure 6). A cross was made to test for penetrance and flies exhibiting such phenotypes were isolated: **JG1 ♀♀ x JG1 x DM-133 (*Rb*)** ♂♂
Cross between the mutants *Rb* and JG1 female virgins resulted in higher penetrance of red belly phenotypes.

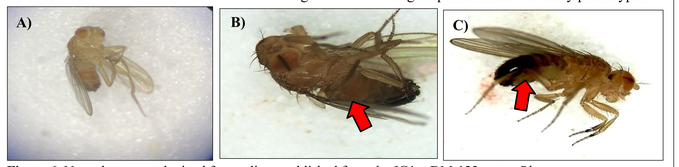

Figure 6. New phenotype obtained from a line established from the JG1 x DM-133 cross. *Rb* phenotype expresses a red-orange stripe that runs along the right portion of lower abdomen. Phenotype is variably expressed and only present in RS males of the line. A has stripe that is darker, meanwhile, B and C express a faint stripe.

DISCUSSION/FUTURE WORK

The varying states of an epigenome that are independent of underlying DNA sequences can be termed as "epigenetics". Epigenetic changes can arise spontaneously and can be inherited. The changes can also regulate chromatin condensation and accessibility, resulting in changes in transcriptional activity (Becker and Weigel, 2012). One consequence of changes in the epigenomes is the release of transposable elements which can lead to gene silencing and result in disruption of the DNA sequence. This gene silencing can result in genetic defects such as head deformities and eye and wing abnormalities. The results described above support our hypothesis that when lines of *ee* are bred with wild-type lines known to contain P-elements it will result in epigenetic variations such as head abnormalities, eye abnormalities, changes in bristles, and wing abnormalities as proposed by the Marcey lab model. Our results indicate that a genetic element found in the *ee* (JG1) line is responsible for the inducing genetic variation in crosses containing transposable elements. This comports with the prediction that these new phenotypes are caused by epigenetic suppression of genomic regions close to P-element caused by heterochromatization of these regions induced due to insertion of P-transposable elements in the *Cpr* gene (see Figure 2). Although these conclusions are still to be tested further and crosses are to be repeated, the results of this study suggest several lines of investigation that will possibly shed light on the molecular nature of the *ee* mutations. For example, given that prior members of the Marcey Lab have already genetically mapped P-element insertions in *cby* and *ee* mutant genotypes, we can use that information to map the *cby* mutation to examine if it co-maps with a known P-element insertion. Another future investigation involves looking for epigenetic molecular markers of heterochromatization, such as di- & tri-methylation of histone 3 lysine 9 (H3K9) (Choi & Lee, 2020). Both of these results would provide support to the proposed *ee* model (Figure 2), highlighting that *ee* is caused by a P-element insertion that is heterochromatizing and therefore, silencing, genes near P-elements. Our hypothesis regarding cryptic epigenetic variation in natural populations of *D.melanogaster*, if confirmed by additional work, has significant implications for both genetics and evolutionary biology, as this type of variation may provide new epialleles that are manifested in the presence of an appropriate antisense transposable element. Such novel, contingently expressed epialleles would provide a collateral source of variation upon which natural selection could act, in addition to DNA sequence changes in coding or regulatory regions of eukaryotic genes.

REFERENCES

- Becker, C. and Weigel, D. (2012). Epigenetic variation: origin and transgenerational inheritance. Current Opinion in Plant Biology. 15: 562-567.
- Hari, K. L., Cook, K. R., and Karpen, G. H. (2001). The Drosophila Su(var)2-10 locus regulates chromosome structure and function and encodes a member of the PIAS protein family. Genetics and Development. 15: 1334-1348.
- Choi J.Y. & Lee Y.C.G. Double-edged sword: The evolutionary consequences of the epigenetic silencing of transposable elements. PLoS Genet. 2020 Jul 16;16(7)
- Hollister, J.D. and Gaut, B.S. (2009). Epigenetic silencing of transposable elements: A trade-off between reduced transposition and deleterious effects on neighboring gene expression. Genome Research. 19: 1419-1428.
- Lee, Y. C. G. (2015). The role of *piRNA*-mediated epigenetic silencing in the population dynamics of transposable elements in *Drosophila melanogaster*. PLoS Genet. 11(6): 1-24.
- Paaby, A.B. and M.V. Rockman (2014). Cryptic Genetic Variation: evolution's hidden substrate. Nature Reviews, Genetics 15: 247-258.
- Spradling, A. C. and Rubin, G. M. (1982). Transposition of Cloned P-elements into *Drosophila* germ line chromosomes. Science. 218(22): 341-347.
- Takahashi, K.H. (2013). Multiple capacitors for natural genetic variation in *Drosophila melanogaster*. Molecular Ecology 22:1356-65.

ACKNOWLEDGEMENTS

We thank Dr. Bryan Swig and Ms. Shannon Andreoli for technical support. Generous support to the Marcey lab has been provided by the Fletcher Jones Foundation and the CLU Biology Department. Thanks to Dr. Marcey for his support, guidance, and knowledge on this project. Lastly, thank you to the Marcey Lab members who helped maintain our stocks, collect virgin flies, make fly food, and perform other routine lab protocols.