Crybaby Induction by Genetic Elements in the *Extra Eye* Strain of *Drosophila melanogaster*

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ABSTRACT

This project is based on a model for the production of head defects by *extra eye* (*ee*), which is an incompletely penetrant, variably expressed, and conditionally dominant mutation in *Drosophila melanogaster*. The mutation maps to a P-transposable element in the *Cpr* gene, inserted in a reverse transcriptional orientation to *Cpr*. The model posits P-transposable element, RNAi-mediated transcriptional suppression of genes near P-element insertions that are implicated in embryonic eye field establishment. Based on this model, screens were conducted by crossing *ee* to wild-derived lines in an attempt to generate inducible, epigenetic variants that would be revealed by the *ee-Cpr* anti-sense P-element. In one screen, a variant was recovered, named *crybaby* (*cby*), based on the tear-drop shape of the eye caused by ventral eye development impairment. The experiments reported here represent an important control to test whether the *cby* phenotype is inducible by any P-element line, or whether this putative "epiallele" is induced exclusively by genetic components in the *ee* genotype, as predicted by the *ee* model. My results examine whether *cby* was induced by 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. I predicted that the *cby* phenotype would only appear in my cross with JG1 (*ee* line) and would not be inducible by other P-element lines (e.g. KW006). I also predicted that the *cby* phenotype would not be induced in crosses of KW003 to a stock that lacks P-elements (e.g. Canton-S). As expected, based on the original screen, the *cby* phenotype appeared in a line established from a cross of JG1 and KW003. No *cby* mutant phenotypes were noted in the lines established from the KW006 and Canton-S crosses to KW003.

INTRODUCTION

Background

The variably expressed *extra eye* (*ee*) mutation in *Drosophila melanogaster* produces 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). In addition to variable expression, the mutation possesses several other exotic features: *ee* is both incompletely penetrant and conditionally dominant. The *ee* mutation is likely caused by a transposable P-element element insertion into a 5' exon of the *Cytochrome p450 reductase* (*Cpr*) gene. Sequencing of this P-element and flanking genomic DNA shows a reverse orientation of the P-element with respect to the transcriptional polarity of *Cpr*, which results in the presence of anti-sense P-element RNA within the *Cpr* transcript – see Figure 2 (Marcey, unpublished).

Marcey (personal communication) has developed a P-element induced, RNAi-based, epigenetic model to explain the exotic genetic behavior of *ee* that proposes a down regulation of a negative regulator of the JAK-STAT signaling pathway (Figure 2). JAK-STAT signaling is a potent inducer of dorsal eye field development, and overexpression of JAK in developing heads can produce extra eyes similar to the ones elicited by *ee* (Harrison, et al., 1995). *Su(var)2-10* is the *Drosophila* ortholog of mammalian Protein Inhibitor of Activated STAT (PIAS). *Su(var)2-10* inhibits JAK-activated STAT92E in early eye development; the *Su(var)*/STAT92E ratio is important in determining correct eye size (Betz, et al., 2001). The *ee* mutation is posited to down regulate *Su(var)2-10* expression by the P-element insertion into *Cpr* and subsequent RNAi-induced heterochromatization of the *Su(var)2-10* genomic region due to a nearby P-element insertion. **Multiple genetic studies support the Figure 2 model (Marcey, et al., unpublished), including:**

- **P-elements from stocks unrelated to *ee* and on multiple chromosomes are potent modifiers (enhancers) of *ee* penetrance;**
- **A component of the *ee* mutation behaves genetically as a suppressor of PEV, and acts synergistically *with Su(var)2-10* mutations in restoring eye pigmentation of white-mottled[4] PEV;**
- **Mutant alleles of *Su(var)2-10* induce significant conditional dominance of *ee* when combined with the *ee* chromosome;**
- **Mutants that decrease heterochromatization, *pleiohomeotic* (*pho*), *Su(var)3-9*, and *brahma* (*brm*), exhibit a significant suppression of *ee* penetrance, indicating that the level of heterochromatization influences *ee* penetrance, putatively through effects on *Su(var)2-10* expression.**

Thus, we hypothesize that the *ee* mutation up-regulates the JAK-STAT signaling by *Su(var)2-10* inhibition, producing head deformities and supernumerary eye fields during head development.

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.

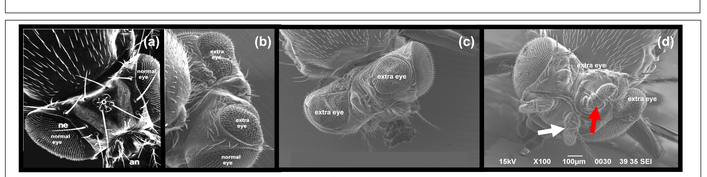

Figure 1. Extreme *extra eye* phenotypes. **(a)** dorsal view of a wild-type head, showing lateral, normal compound eyes, dorsal ocelli (O), antennae (an) and bristles. **(b)** posterior dorsal view of an *ee* fly with missing ocelli and two supernumerary eyes embedded in dorsal cuticle. **(c)** dorsal 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.

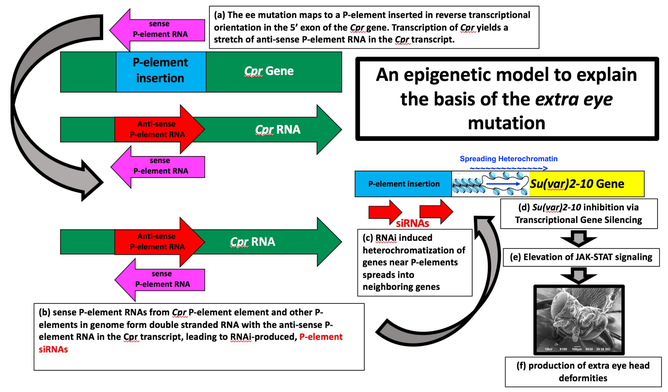

Figure 2. (a) A P-element insertion in the 5' exon of *Cpr* results in an anti-sense P-element RNA within the *Cpr* transcript. **(b)** P-element sense RNAs derived from genomic P-elements produces double stranded RNA with the *Cpr* anti-sense P-element RNA. This yields siRNAs via the RNAi pathway. **(c)** 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 whether *cby* was induced by 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, the following crosses were performed. All crosses were incubated at 25° C to ensure high reproductive rates of the flies.

Shown below is the crossing scheme that I replicated from the original cross made to establish the *cby* phenotype. It is derived from progeny of *ee* (**JG1**) virgin females mated to wild-type male flies from diverse worldwide locations, specifically Brazil (**KW003**). The F_1 progeny of these crosses were interbred to establish lines that were screened for the *cby* mutant phenotypes.

(JG1) *w* / *w* ; *ee b pr* / *ee b pr* ; TM3 / RS1 ♀♀ ✕ (KW003) + / Y; + / +; + / + ♂♂

The second cross I performed was a control crosses to examine if cby is inducible by another P-element line, or whether this putative "epiallele" is induced exclusively by a genetic element in the JG1 (*ee*) line. I crossed virgin females from KW006, a line established from wild collected flies in the Democratic Republic of Congo, and which has been shown to contain P-elements (Marcey, personal communication), to KW003. The F_1 progeny of these crosses were interbred to establish lines that were then screened for the *cby* mutant phenotype. (KW006) + / + ; + / +; + / + ♀♀ ✕ (KW003) + / Y; + / +; + / + ♂♂

Finally, a control cross was performed to examine if *cby* would be induced in the absence of P-elements. Virgin females from a non-P-element containing, long-established, wild type line (Canton-S - CS #1) were crossed with KW003 males. The F_1 progeny of these crosses were interbred to establish lines that were then screened for the *cby* mutant phenotype.

(CS #1) + / + ; + / +; + / + ♀♀ ✕ (KW003) + / Y; + / +; + / + ♂♂

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).

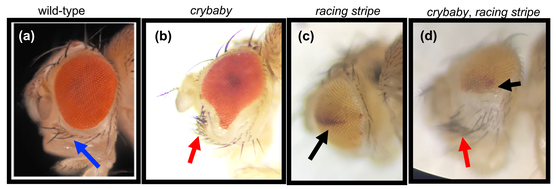

Figure 3. Lateral views of a wild-type and *crybaby* heads. **(a)** a wild-type head showing large compound eye (red) and ventral vibrissae (blue arrow). **(b)** a *crybaby* mutant - note the reduced number of facets in the ventral half of the *crybaby* eye, along with duplications of ventral vibrissae (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* (see Discussion).

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).

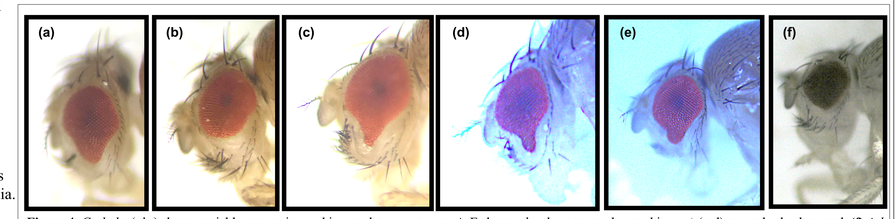

Figure 4. *Crybaby* (*cby*) shows variable expression and incomplete penetrance. A-E shows *cby* phenotypes observed in a *w*$^+$ (red) eye color background. **(f)** A *b pr cby* example obtained in a cross of KW003 to *ee b pr*.

RESULTS

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

F_2 generation	WT phenotype	Cby phenotype	Total progeny counted
JG1♀x KW003♂	278	38	316
KW006♀x KW003♂	300	0	300
CS #1 ♀ x KW003♂	325**	0	325

**2 flies showed a *non-cby* defect

After screening the established lines from the 3 crosses mentioned above, the phenotypic results from the progeny are summarized at left (Table 1). As expected, based on previous experiments (see Background), the *cby* phenotype appeared in a line established from a cross of JG1 and KW003 (see Figure 6). There were 38 mutant *cby* phenotypes that appeared in a total progeny of 316 flies. Roughly every 1/10 flies expressed the mutant *cby* phenotype in this cross. No *cby* mutant phenotypes were noted in the lines established from the KW006 and Canton-S crosses to KW003.

The *cby* phenotypes uncovered in the JG1 x KW003 line feature head disruptions that typically include ventral eye reductions. Mild expressions of *cby* display small ventral eye reductions (Figure 6A, 6B, 6D), resulting in a tear drop shape, whereas more severe expression results in an almost complete loss of ventral eye (Figure 6C, 6E, 6F). Often, duplications and thickening of vibrissae (bristles bordering the ventral eye margin) accompany ventral eye reductions (Figure 6E and 6F). These perturbations suggest that *cby* causes severe disruptions in signaling mechanisms in the developing head. *cby* displays several hallmarks of epigenetic variability: incomplete penetrance and variable expression. These features also characterize the *ee* mutation.

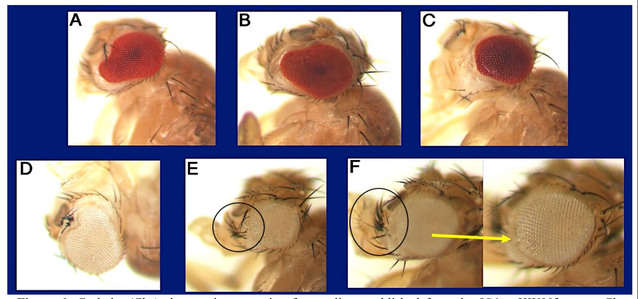

Figure 6. *Crybaby (Cby)* phenotypic expression from a line established from the JG1 x KW003 cross. *Cby* phenotype appears variably expressed and incomplete penetrance. The top row (A-C) shows *cby* phenotypes observed with a WT (w$^+$) eye color. The bottom row (D-F) shows varying expressions of the *cby* phenotype with a white mutant eye color. A, B, & D highlight the extreme teardrop eye shape due to loss of ventral eye tissue. Notice the thickening and duplication of vibrissae in E and F (circled).

DISCUSSION/FUTURE WORK

Cryptic genetic variation (CGV) may be an important component of adaptive, deleterious, or neutral variation that is contingent upon environmental or genetic circumstances to be expressed. CGV therefore qualifies as a "hidden substrate" of evolution (Paaby and Rockman, 2014). Cryptic epigenetic variation (CEGV) is a subset of CGV that may be revealed upon changes in the packaging state of genes or their regulatory components as opposed to DNA sequence variation that is contingently expressed. To date, only limited examples of CEGV in nature have been reported (e.g. Weigle and Colot, 2012; Pecinka, et al., 2013).

The results described above support my hypothesis that *cby* would be exclusively induced in crosses of KW003 to JG1, the *ee* containing line: 1) The *cby* phenotype was observed in the F_2 and beyond in the JG1 x KW003 cross, confirming previous results (Marcey, personal communication); 2) No *cby* phenotypes were observed in crosses of KW006 (a P-element containing line) or CS (a wild-type line lacking P-elements) to KW003. My results indicate that a genetic element found in the *ee* (JG1) line is responsible for the induction of *cby* and comports with the prediction that *cby* is caused by epigenetic suppression of genomic regions near P-element inserts that is rooted in RNAi mediated transcriptional gene silencing caused by the antisense P-element in the *Cpr* gene in *extra eye* lines (see Figure 2). Although these latter conclusions have yet to be rigorously tested, the results of this study suggest several lines of investigation that will possibly shed light on the molecular nature of the *ee* and *cby* 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 concerning cryptic epigenetic variation in natural populations, 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.

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