Using Recombination to Separate Genetic Components of the *extra eye* Mutation in *Drosophila melanogaster*

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CLU Festival of Scholars
Spring, 2021

ABSTRACT

The *extra eye* (*ee*) mutation in *Drosophila melanogaster* produces head deformities which range from missing and/or duplicated bristles and head cuticle to supernumerary compound eyes and antennae. It is incompletely penetrant and conditionally dominant. Marcey (personal communication) has developed an epigenetic, two-component model to explain the exotic genetic behavior of *ee*. The first component centers around a transposable P-element inserted in the 5′ exon of the *Cytochrome p450 reductase* (*Cpr*) gene in a reverse orientation with respect to the transcriptional polarity of *Cpr*. This reverse-transcriptional orientation causes for an RNAi-based tightly packaging of DNA, heterochromatization, at the site of the anti-sense P-element and all other P-elements in the genome. The second component of the model predicts that there is a P-element that exists near the *Su(var)2-10* gene, which is important for normal eye development. The RNAi-induced heterochromatization of this P-element subsequently suppresses the expression of *Su(var)2-10*, causing for the observed *extra eye* phenotypes. In order to test the model's validity, the present study works to isolate these two components in *ee* lines by splitting the chromosome on which both components reside, the 2nd chromosome, through recombinational means. It was expected that the resultant recombinationally-generated descendants with split *extra eye* chromosomes would not produce any phenotypes characteristic of the *extra eye* mutation in any capacity. To conduct this project, *ee* flies were mated to flies with a heavily mutated 2nd chromosome, a mapping chromosome, so as to track where recombination events took place. Resultant progeny were assayed for desirable recombination events. Such offspring were mated with balancer stocks to preserve the split chromosome and then preserved into a line of flies for studying potential *extra eye* phenotypes. This project is important as it provides further insight into the mechanisms of animal development, especially in understanding the role of transposable elements in gene expression.

INTRODUCTION

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, 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.**

In this study, our focus is to test the validity of the *ee* model by conducting a series of genetic crosses with *ee* lines and test stocks aimed at utilizing recombination to separate the *Su(var)2-10* mutation and the *Cpr* P-element insertion in descendants. These descendants, with only components of the *extra eye* mutation, will be screened for *extra eye* phenotypes. Based on the model for *ee* presented in **Figure 2**, we hypothesize that these recombinationally generated descendants with only components of the *extra eye* mutation will not be able to cause RNAi-induced heterochromatization of P-elements, which will result in no expression of the *extra eye* phenotype. The experiments are expected to yield both data and *Drosophila* stocks that speak to the hypothesis stated above. Generated stocks will be scored for *extra eye* phenotype presence and severity. We expect there to be no *extra eye* phenotypes in the resultant stocks as the components of the *extra eye* mutation as presented in the **Figure 2** model have been separated and will not be wholly present.

Discovering the mechanisms of development and processes in *Drosophila melanogaster* has provided key insights into animal development in general, and human development in particular. For this reason, this model organism continues to play an important role in biomedical research. This investigation is expected to be relevant to understanding the possible roles of transposable elements in causing changes in gene expression due to RNAi-induced DNA heterochromatization.

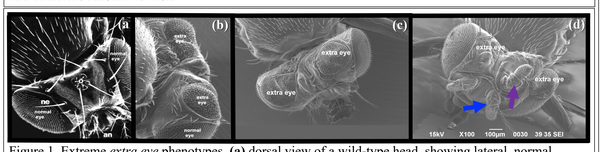

Figure 1. Extreme *extra eye* phenotypes. **(a)** dorsal view of a wild-type head, showing lateral, normal compound eyes, dorsal ocelli (O), antennae (an) and bristles. **(b)** posterior dorsal view of an *ee* fly with missing ocelli and two supernumerary eyes embedded in dorsal cuticle. **(c)** dorsal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

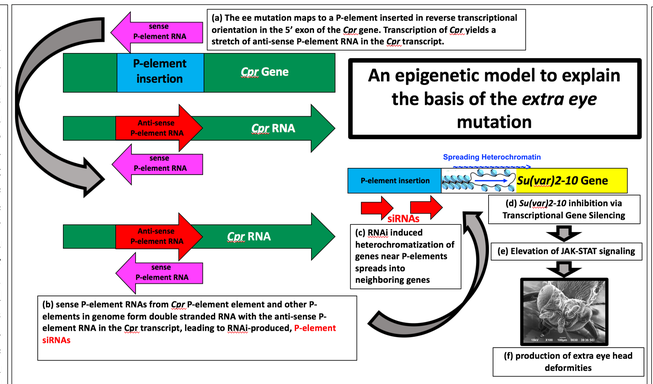

Figure 2. **(a)** A P-element insertion in the 5' exon of *Cpr* results in an anti-sense P-element RNA within the *Cpr* transcript. **(b)** P-element sense RNAs derived from genomic P-elements produces double stranded RNA with the *Cpr* anti-sense P-element RNA. This yields siRNAs via the RNAi pathway. **(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.

RESULTS

Phase 1 of the experiment ran relatively smoothly. A sufficient number of JG1 and 156 flies were gathered and mated. In scoring the F_2 offspring, we found that the *dumpy* gene was unable to be distinguished between its mutant a wildtype expression. While suboptimal, this did not impede the progress of the experiment. In total, we collected many flies that expressed phenotypes associated with an ideally-split chromosome (scoring was simplified to look for the presence of the *purple* gene without the *black* gene or vice versa); however, we were only able to advance twelve males to Phase 2. We were only able to advance twelve males with ideally-split chromosomes to Phase 2 because we experienced a shortage of females with balanced 2nd chromosomes.

Phase 2 of the experiment ran rather poorly. Many of the twelve males that we advanced from Phase 1 died before they could mate with their daughters. Some males who did interact with their daughters did not mate with them. Unfortunately, this resulted in only two flies being able to advance to Phase 3. These lines, A and J as labeled in the experiment, have ideally-split chromosomes that are depicted in **Figure 4**. Line A possesses the mapping genes *aristaless*, *black*, *plexus*, and *speck*, indicating that it possesses the purported transposable element located near *Su(var) 2-10*. Line J possesses the mapping genes *aristaless*, *purple*, *curved*, *plexus*, and *speck*, indicating that it possesses the purported transposable element located in a reverse transcriptional orientation to that of *Cpr*.

Phase 3 is still in progress. We have been able to make twelve crosses beginning Phase 3 from Line A. We have been able to make three crosses beginning Phase 3 from Line J. Of the twelve crosses from Line A in Phase 3, only one has progressed to the second check, most failed the first check, and we are still waiting for many offspring to hatch. None of the offspring from the crosses from Line J in Phase 3 have developed. We aim to have one of these crosses be solidified into a true-breeding line of flies for future study of *extra eye* expression and rendering.

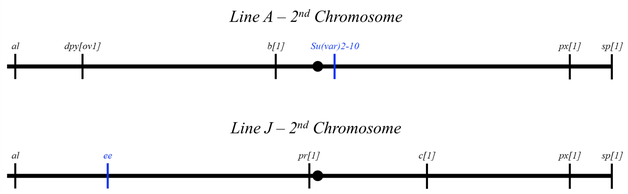

Figure 4. Schematic illustrations of the ideally-split chromosomes that are currently in Phase 3 of the project. **Line A** illustrates that the recombination event that took place left the male fly that began this line with the transposable element purported to be near the *Su(var)2-10* gene, shown in blue. The line also possesses the *aristaless*, *black*, *plexus*, and *speck* mutations from the mapping chromosome. While we could not determine the presence or absence of the *dumpy* mutation, we have placed it in the illustration as its presence more likely than the alternative. **Line J** illustrates that the recombination event that took place left the male fly that began this line with the transposable element purported to be in a reverse-transcriptional orientation within the *Cpr* gene, shown in blue. The line also possesses the *aristaless*, *purple*, *curved*, *plexus*, and *speck* mutations from the mapping chromosome. These two lines contain the two genetic components of the *extra eye* mutation as modeled in Figure 2.

METHODS

This study encompasses a multitude of highly specific crosses and backcrosses in order to accurately isolate the components of the *extra eye* mutation from each other in lines of flies. Therefore, great care was taken in properly executing these crosses to ensure the highest degree of certainty towards the genetic makeup of the generated flies. The "Cross Scheme" is depicted in **Figure 3**. The extra eye line that will be used as the basis for this experiment will be the stock of flies known as JG1. It possesses the white (w^{1118}) mutation on its first chromosome, the *ee* mutation on its second chromosome, and is doubly balanced on its third chromosome with Racing Stripe (*RS*) and Stubble Bristles (*TM3*). This stock was chosen out of two possible lines, the other being 1245. JG1 was chosen because its second chromosome only caries the *ee* mutation whereas the second chromosome of 1245 possesses both the *black* (*b*) and purple (*pr*) mutations in addition to *extra eye*. Because the *ee* and *Su(var)2-10* P-element insertions cannot be tracked on any consistent, reliable, or practical basis, this experiment required a mapping chromosome. The mapping line, 156, only possesses mutations on its second chromosome, spread out across the entire length of the chromosome, making it ideal for this study.

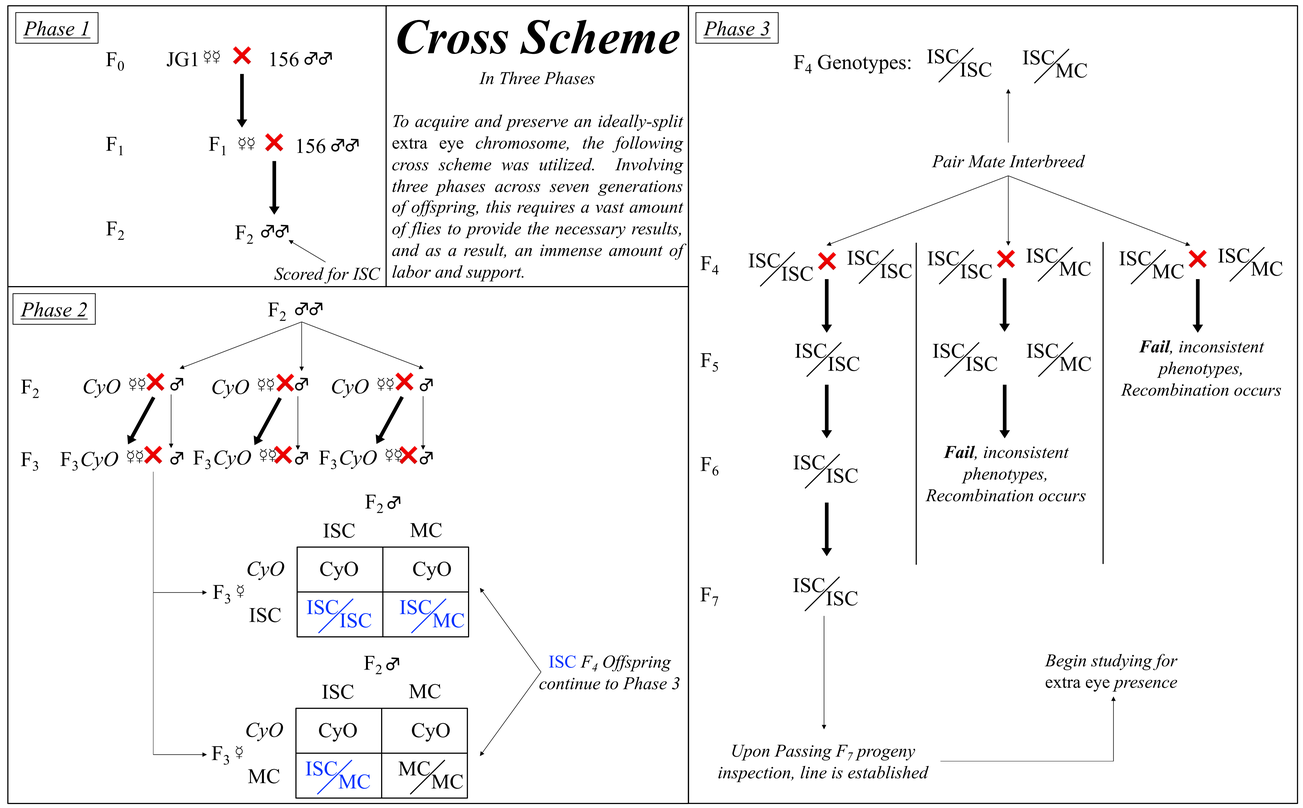

Figure 3. Cross Scheme in Three Phases. **Phase 1** *extra eye* line JG1 virgin females are mated with mapping line 156 males. F_1 virgin female offspring are backcrossed to 156 males. This is where the recombination to split the *extra eye* chromosome occurs. F_2 males are scored for the presence of an ideally-split chromosome (depicted here as ISC). **Phase 2** F_2 males possessing ideally-split chromosomes are individually mated to balanced (depicted here as *CyO*) virgin females. F_3 virgin female offspring possessing the balancer chromosome are then backcrossed to their father (the scored F_2 male who sired them). F_4 offspring are then scored for the phenotype associated with the ideally-split chromosome. **Phase 3** Selected F_4 offspring are interbred with each other in a series of pair matings. Resultant F_5 offspring are scored for inconsistencies in phenotypes. Crosses who fail are discarded. Crosses who pass are then interbred. Resultant F_6 offspring are scored for inconsistencies in phenotypes. Crosses who fail are discarded. Crosses who pass are then interbred. Resultant F_7 offspring are scored for inconsistencies in phenotypes. Crosses who fail are discarded. Crosses who pass are then established lines containing the ideally-split chromosome. Resultant lines will then be studied for the presence or absence of *extra eye*.

DISCUSSION

Though the final results of this project are still ongoing, we can report that, up until now, the project has been a success. With the COVID-19 pandemic hindering much of this study's progression, we made every effort to continue as normal; however, we were never able to get enough flies for crossing or offspring for scoring to make the success of the project assured. Despite this, we can assess certain aspects of the study and its success. As expected, Phase 1 yielded recombinant phenotypes associated with those of ideally-split chromosomes, indicating that recombination between the *extra eye* chromosome and the mapping chromosome took place. It should be noted that while scoring for *extra eye* phenotypes in the offspring of Phase 1 was not the priority, no *extra eye* phenotypes were observed. Phase 2 further behaved as expected in terms of offspring phenotypes. The balancer chromosomes successfully prevented recombination from occurring in the females so that they may mate with their fathers. Phase 2 did also not see any *extra eye* phenotypes. And though Phase 3 is still ongoing, it shows promise that there could be crosses that are between homozygous individuals for the ideally-split chromosome. Of the flies that have been scored, no *extra eye* phenotypes have been observed. Looking ahead, should Phase 3 elicit one or two lines of ideally-split flies, then these lines of flies will have only one component of the **Figure 2** model, depending on the actual results with chromosomes that are those depicted in **Figure 4**. These lines can then be further studied by the Marcey Lab for not only *extra eye* phenotypes but also how they respond when crossed to flies that have the *extra eye* mutation and to flies with transposable elements, thus improving our understanding of the role that transposable elements play in gene expression.

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ACKNOWLEDGEMENTS

We thank Dr. Bryan Swig and Ms. Shannon Andreoli for technical support. We thank Dr. Marcey for mentorship, guidance, and support in all phases of the project. Generous support to the Marcey lab has been provided by the Fletcher Jones Foundation and the CLU Biology Department. We thank the Marcey Lab members for their continued support. We also thank the flies for the generous donation of their lives.