Spring 4-24-2023

Localization of CTG-Repeat-Containing Transgenes in Drosophila melanogaster Myotonic Dystrophy Models

Andrea Waltrip

LOCALIZATION OF CTG-REPEAT-CONTAINING TRANSGENES IN *DROSOPHILA MELANOGASTER* MYOTONIC DYSTROPHY MODELS

by

Andrea

Waltrip

Thesis

Submitted in partial fulfillment of the requirements for Honors in Biology at

the University of Mary Washington

Fredericksburg, Virginia

April 23, 2023

This Thesis by Andrea Waltrip is accepted in its present form as satisfying the thesis requirement for Honors in Biology.

Date: April 24, 2023

Approved:

signature

Theresa Grana

Chairman of Honors Committee

Date: April 24, 2023

signature

Ginny Morriss

Honors Advisor

Date: April 24, 2023

signature

Deborah O'Dell

Honors Committee Member

Andrea Waltrip was born in Virginia on April 26, 2001. She received an Associate Degree in Arts and Sciences with high honors from Southside Virginia Community College. She will receive a Bachelor Degree in Biomedical Sciences with Magna cum Laude from the University of Mary Washington in the Spring of 2023. She will attend the Basic Biomedical Sciences Program at the University of Texas at Southwestern, where she intends to receive her Doctor of Philosophy.

Acknowledgements

I would like to thank Dr. Ginny Morriss, Dr. Ruben Artero, Dr. Daniel Babcock, Delaney Baratka, Shyanne Michael, Noah Smith, Emma Gardner, Madeline Brunt, Victoria Ashton, Larry Sizemore, Charlotte Russell, the Department of Biology, and the Jepson Scholars Program for supporting me throughout my project.

Abstract

Myotonic Dystrophy Type 1, DM1, is a multi-systemic muscle wasting disorder that results from expression of expanded CTG repeats in the *DMPK* gene in humans. Three transgenic *Drosophila melanogaster* lines have been created containing 60, 250, or 480 CTG repeats to model DM1. The transgenic repeats are expressed using the GAL4/UAS system. Expression of long-repeat transgenes ($(CTG)_{250}$ and $i(CTG)_{480}$) produces phenotypes consistent with DM1, relative to control lines ($(CTG)_{60}$). The precise chromosomal location of insertion of the transgenes has not been reported. We used classical genetic approaches to localize CTG-repeat transgene insertion to a specific chromosome. We used GAL4 drivers with known locations to drive expression of repeats and assessed eye color phenotypes of the F_2 generation. Results from the genetic analysis suggest that the $(CTG)_{250}$ and $i(CTG)_{480}$ transgene are likely localized to chromosome 2 and the $(CTG)_{60}$ transgene is likely localized to chromosome 3. Knowing the location of the transgenes can allow for more practical mating schemes to study DM1 disease mechanisms, as well as provides crucial information for understanding transgene expression. The climbing velocity and flight capability tests were used to look at the effect of different drivers on muscle performance. The *Mef2*-GAL4 driver had the largest statistical difference in the climbing velocity test and the *Hsp70*-GAL4 driver had the largest statistical differences in the flight capability. These drivers should be chosen to improve the physiological differences seen in DM1 flies in future studies utilizing these tests. With the CTG repeat transgenes localized to a respective chromosome, researchers can better utilize these models to investigate disease mechanisms and potential treatments for DM1.

Table of Contents

Section 1: Introduction

Myotonic Dystrophy Type 1, DM1, is a muscle wasting disorder that results from expansion of CTG repeats in the 3' Untranslated Region, UTR, of the *dystrophia myotonica protein kinase*, *DMPK*, gene on the 19th chromosome in humans (Bargiela *et al.* 2015). DM1 is the most common form of muscular dystrophy with adult onset and occurs once in every 8000 adults (Bargiela *et al.* 2015). Classical DM1 symptoms include myotonia, cardiac conduction issues, cataracts, weak and wasting muscle, and shorter life expectancy (Bird 1999). There are currently no treatments that can substantially alter the outcome of the disease, only medications and devices that can lessen each individual symptom the patient has (Bird 1999). At least fifty repeats are needed to have a phenotype consistent with DM1; longer repeats create a more severe phenotype and lowers the age of onset.

Drosophila melanogaster, fruit flies, are used as model organisms to study DM1 as *Drosophila* have been shown to model human muscle diseases closely (Kreipke *et al.* 2017). Multiple transgenic DM1 *Drosophila* lines have been created that contain different numbers of CTG repeats with 60 uninterrupted CTG repeats ($(CTG)_{60}$), 250 uninterrupted CTG repeats ($(CTG)_{250}$), and 480 repeats consisting of synthetic CTG repeats interrupted every 20 units by the CTCGA sequence ($i(CTG)_{480}$) (Garcia-Lopez *et al.* 2008; Cerro-Herreros *et al.* 2017). The locations of these transgenes are unknown. The locations of the transgenes are crucial information when using these lines to research DM1, because researchers can use the location to make more efficient mating schemes to test genetic modulation of the disease and could lead to more wide spread use of the model. The CTG repeat transgenes are expressed using the GAL4/UAS system, in which the yeast GAL4 gene is integrated into the fruit fly genome where the GAL4 can affect its target, the

upstream activating sequence (UAS), which is also integrated into the genome of another fly line (Osterwalder *et al.* 2001).

Classical genetics was used to narrow down the location of the inserted transgenes chromosomally. Genetic crosses were used to drive expression of repeats to assess phenotypic ratios of eye color, climbing velocity, and flight capability. Phenotypic ratios of F_2 progeny from crosses using the CTG repeats and GAL4 drivers on different chromosomes indicate chromosomal location. The climbing velocity and flight capability tests were used to look at the effect of different drivers on muscle performance. Drivers that showed statistical differences in muscle performances can be chosen to increase differences in DM1 and control flies muscle performances in future studies. In future studies, molecular approaches will be used to identify into which part of the chromosome the CTG-repeat transgenes have been inserted.

Key terms: Parental generation are the initial cross. F_1 progeny are the offspring of the parental cross. F_2 progeny are the offspring produced when the F_1 progeny are crossed.

Section 2: Literature-based Rationale

Myotonic Dystrophy Type 1, DM1, is caused by the expansion of CTG repeats in the 3' Untranslated Region, UTR, of the *dystrophia myotonica protein kinase*, *DMPK*, gene (Bargiela *et al.* 2015). DM1 is autosomal dominant and has multisystem features, including myotonic myopathy, cataract, and cardiac conduction disease (Bargiela *et al.* 2015; Thornton 2014). The number of CTG repeats in the gene varies in the general population, with the repeats ranging from 5 to 37 (Thornton 2014). Individuals with at least 50 CTG repeats are diagnosed with DM1, but some cases can have upwards of

3,000 CTG repeats in the *DMPK* gene (Thornton 2014). The CTG repeats are unstable in the somatic and germline cells and further expansion is favored (Thornton 2014). The DM1 expansion is more unstable in non-dividing cells like the skeletal muscle, brain, and heart, making these organs carry the brunt of the symptoms (Thornton 2014).

Typically, patients with 50-70 repeats have mild symptoms after age 60, while patients with 70-90 repeats have mild symptoms beginning after age 40; however, there are exceptions depending on the severity of expansion over the patient's lifetime. Congenital DM1, on the other hand, is associated with more than 1,000 repeats and has severe symptoms with fetal-onset with an 18% neonatal mortality rate. About 5% of families that have DM1 will have sequence interruptions in the CTG repeats (i(CTG)). These interruptions have been shown to stabilize the repeats and minimize further expansion. There are currently no treatments that change the outcome of the disease (Thornton 2014).

The main cause of the symptoms in DM1 patients is sequestration of the Muscleblind-like (MBNL) proteins to the nucleus by the expanded repeats from expressed DMPK mRNA, preventing the normal function of MBNL proteins in alternative mRNA splicing, allowing mis-spliced RNAs to be translated into proteins (Sznajder *et al.* 2016). The muscleblind (mbl) protein in *Drosophila melanogaster*, fruit flies, has a similar function to the MBNL protein in humans (Thornton 2014). Most of the mechanisms underlying DM1 are unknown, so having a simple model may help to untangle the causes of the disease (Morriss *et al.* 2018).

The Artero research group created three transgenic *Drosophila* models expressing 60 pure repeats, denoted $(CTG)_{60}$, 250 pure repeats, denoted $(CTG)_{250}$, and 480 interrupted

CTG repeats (Garcia-Lopez *et al.* 2008; Cerro-Herreros *et al.* 2017). The 480 CTG repeats are interrupted every 20 trinucleotides, this is denoted by $i(CTG)_{480}$ (Garcia-Lopez *et al.* 2008). The $i(CTG)_{480}$ flies are largely flightless and have a reduction in indirect flight muscle mass when driven by the *Mhc*-GAL4 driver (Bargiela *et al.* 2015; Garcia-Lopez *et al.* 2008). The $(CTG)_{250}$ flies showed decreased climbing velocity, flight ability, and muscle size when driven by the *Mhc*-GAL4 driver (Cerro-Herreros *et al.* 2017). The $(CTG)_{60}$ line was created as a control, and these flies have no detectable flight defect or appreciable effect on muscle size when driven by the *Mhc*-GAL4 driver (Garcia-Lopez *et al.* 2008). Fruit flies expressing the $i(CTG)_{480}$ and $(CTG)_{250}$, but not the $(CTG)_{60}$ transgene showed sequestration of muscleblind proteins (Garcia-Lopez *et al.* 2008; Cerro-Herreros *et al.* 2017).

The CTG repeats are expressed via the GAL4/UAS system (Garcia-Lopez *et al.* 2008). In yeast, GAL4 encodes a protein that regulates genes by binding to sites called Upstream Activating Sequences (UAS), this system allows GAL4 to selectively drive expression of RNA or proteins whose mRNA is fused to the UAS element (Duffy 2002). Brand and Perrimon adapted the UAS/GAL4 system for *Drosophila* to achieve targeted transgene expression (Duffy 2002; Brand *et al.* 1993). In the absence of GAL4 drivers, the UAS genes will not be expressed assisting in the study of lethal and semi-lethal genes (Duffy 2002). The expression pattern is determined by the chosen GAL4 driver, regulatory DNA is used to create ubiquitous expression, tissue specific expression, or late onset of expression through environment temperature changes (Garcia-Lopez *et al.* 2008; Duffy 2002).

The UAS genes and GAL4 drivers were created by molecular cloning into plasmid

vectors (Duffy 2002). Specifically, the pUAST is the vector which allows a coding region to be placed under GAL4 control (Duffy 2002). The transgene is not placed randomly at any point on the genome; instead, the transgene is placed in a P transposable element (Duffy 2002, Liao *et al.* 2000). P transposable elements were discovered in *Drosophila* as the cause of a genetic disease called hybrid dysgenesis (Majumdar 2015). P elements are 14 base pair palindromic patterns which allow access to the chromatin (Liao *et al.* 2000). P elements, as a means to edit and transfer genes, changed the field of *Drosophila* genetics (Majumdar 2015). There are many P elements in the *Drosophila* genome and each of the three UAS-CTG repeat transgenes could have been inserted into any of them (Majumdar 2015). Neither the exact location nor the chromosomal location of the three CTG repeats has ever been reported. Determining the chromosomal location of the CTG transgenes is the objective of this project and determining the transgenes exact location is the ultimate goal.

Section 3: Experimental Design and Analysis

All CTG repeat transgene were injected into *white*, w^-, flies. The pUAST plasmid contains the *mini- white* gene; this means that without a transgene the fly will have white eyes and with a transgene the fly will have pigmented eyes. If the fly has a transgene on two chromosomes, then the fly will have red or dark orange eyes; if the fly has one transgene then the fly will have light orange or yellow eyes. Mating schemes were planned and conducted with at least eight replicate crosses and were tested with reciprocal crosses. Calculations from mating schemes indicate when the F_2 generation has no white eyed flies for 300 or more flies, the CTG repeat and the driver can be assumed to be on the same chromosome (see Table 1). The eye color ratios were

calculated and chi-square analysis was used to analyze the results. After 300 or more flies in a genetic cross were phenotyped, the chromosomal location was determined. The drivers used for the crosses were *Mhc*-GAL4 driver, *Hsp70*-GAL4 driver, as well as *Mef2*-GAL4 driver, located on the X chromosome, second chromosome, and third chromosome respectively. The *1151*-GAL4 driver is an X chromosome driver which was used for crosses, because the *Mhc*-GAL4 driver created such a severe phenotype for the i$(CTG)_{480}$ flies that many of the F_1 flies did not survive pupation. Phenotyping was conducted through climbing velocity tests and flight capability tests. The climbing velocity test was used to show if there is a decline in the physiological function of the climbing muscles when using different drivers. This test was done by placing flies in clear vials in front of a white background, after being tapped down the flies will try to climb up the vial instinctively (Nichols *et al.* 2012). The procedure was recorded on video for precise measurement of velocity. The velocity of each fly was calculated by dividing the millimeters of the predetermined point of the vial by the number of seconds the fly took to reach the point. If the fly did not reach the point after 15seconds, the distance the fly traveled was measured in millimeters instead. Following the climbing velocity test, the same flies were subjected to the flight capability test. The flight capability test was used to show if there is a decrease in flight muscle performance when using different drivers. This test was done by placing flies in vials, after dropping the vial down a tube to eject the flies out of the vials into a large tube with a sticky sheet of plastic on the inside, the flies will try to fly instinctively (Babcock and Ganetzky 2014). If the flies can fly, the flies will be trapped on the sticky plastic, the better the flight muscles in the flies the closer to the top of the tube the landing height of the flies will

be. If the flies have defective flight muscles, the flies will have a landing height closer to the bottom of the tube or will land in the oil placed at the bottom of the tube. The plastic sheet is then removed from the tube, stretched out and placed on a white background for photographs for precise measurements. The distance each fly flew was calculated by digitally measuring how far up in centimeters the fly was from the bottom of the tube. The two-way ANOVA test and the Tukey Honest Significant Differences test were used for statistical analysis of the climbing velocity and the flight capability tests.

Section 4: Results

After completing genetic crosses, the *Hsp70*-GAL4 driver on the second chromosome did not produce white eyed F_2 flies for the $(CTG)_{250}$ and $i(CTG)_{480}$ fly lines, out of 1701 flies and 840 flies respectively. The *Mef2*-GAL4 driver on the third chromosome did not produce white eyed F_2 flies for the $(CTG)_{60}$ fly line, out of 1122 flies. All other crosses had white eyed F_2 flies (see Table 2). The results were statistically analyzed using a Chi-square analysis for the eye-phenotype, this resulted in significant P values for only the CTG repeats crosses with the driver line where no white eyed F_2 flies were found (see Table 3). This indicates with statistical significance that the driver is located on the same chromosome as the CTG repeat supporting the calculated ratio of white eyed flies from mating schemes (see Table 1).

The climbing velocity test was measured by seconds to a set point in the vial unless the fly did not reach the point after 15seconds in which case distance was measured in millimeters from starting point. The flight capability test was measured in centimeters of landing height from the ground. The two-way ANOVA was used to analyze the climbing velocity tests and flight tests for statistical significance. The two-way ANOVA analysis of

the climbing velocity tests resulted in the number of CTG repeats being a significant contributing factor to climbing velocity for the *Mef2*-GAL4 driver flies (Pr(>F) = 2.06e-10) (see Table 5). The Tukey Honest Significant Differences analysis of the climbing velocity tests resulted in the $(CTG)_{60}$ flies, which are used for the control, climbing with significantly higher velocity compared to the severe phenotypic $(CTG)_{250}$ and $i(CTG)_{480}$ flies for both sexes when using the *Mef2*-GAL4 driver (see Table 6). The ANOVA and Tukey results are seen in Figure 1C by the higher velocity of the interquartile range of the $(CTG)_{60}$ flies compared to the $(CTG)_{250}$ and $i(CTG)_{480}$ flies. The two-way ANOVA analysis of the flight capability tests resulted in the number of CTG repeats being a significant contributing factor for the *Mhc*-GAL4, *Hsp70*-GAL4, and *Mef2*-GAL4 drivers (Pr(>F) = 0.00149, <2e-16, 0.00892 respectively) (see Table 8). The Tukey Honest Significant Differences analysis of the flight capability tests resulted in the $i(CTG)_{480}$ flies having significantly less capability to fly compared to the $(CTG)_{60}$ and $(CTG)_{250}$ flies for both sexes when using the *Hsp70*-GAL4 driver (see Table 9). The ANOVA and Tukey results are seen in Figure 2B by the lower distance of the interquartile range of the $i(CTG)_{480}$ flies compared to the $(CTG)_{60}$ and $(CTG)_{250}$ flies. The Tukey analysis of the flight capability tests also showed that the female $(CTG)_{250}$ and $i(CTG)_{480}$ flies flew significantly different when using both the *Mhc*-GAL4 and the *Mef2*-GAL4 drivers (see Table 9); this can be seen to a lesser extent in Figure 2A and 2C.

Section 5: Discussion

The main objective of the study was to localize the three CTG repeat transgenes to a chromosome using genetic crosses, this was accomplished. Through looking at the number of white eyed F_2 flies in crosses with a CTG repeat line and a driver line on a

known chromosome. It was found that the $(CTG)_{60}$ when crossed with *Mef2*-GAL4 driver on the third chromosome there were no white eyed F_2 flies. It was found that the $(CTG)_{250}$ and $i(CTG)_{480}$ when crossed with the *Hsp70*-GAL4 driver on the second chromosome there were no white eyed F_2 flies. This localizes the $(CTG)_{60}$ to the third chromosome and localizes the $(CTG)_{250}$ and $i(CTG)_{480}$ to the second chromosome. Fluorescent *in situ* hybridization (FISH) of polytene chromosome preparations will be used in a future study to narrow down the transgenes' locations on the chromosomes. The polytene chromosomes used for FISH will be isolated from salivary glands. Probes will be generated using long-range PCR followed by nick-translation fluorescent labeling and FISH. After the location of the transgenes are verified and narrowed down further, PCR of the region, to which the transgenes have been farther localized, will be completed. Then the samples will be sent out for sequencing to determine the exact location of the transgenes. The climbing velocity and flight capability tests were used to test for differences in muscle performance across the different drivers. The *Mef2*-GAL4 driver showed the largest change in the climbing performance (see Figure 1). The *Hsp70*-GAL4 driver showed the largest change in flight performance (see Figure 2). This knowledge will be used for future studies that use these tests to improve the physiological differences seen in DM1 flies. Now that the CTG repeat transgenes have been localized to a respective chromosome, researchers can develop well thought-out mating schemes making the process more efficient. The fly models can now be better utilized to investigate disease mechanisms and potential treatments for DM1.

Section 6: Literature Cited

Babcock DT, Ganetzky B. An Improved Method for Accurate and Rapid Measurement of Flight Performance in *Drosophila*. Journal of Visualized Experiments : JoVE. 2014;(84):51223. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4089396/. doi:10.3791/51223

Bargiela A, Cerro-Herreros E, Fernandez-Costa J, Vilchez J, Llamusi B, Artero R. Increased autophagy and apoptosis contribute to muscle atrophy in a myotonic dystrophy type 1 *Drosophila* model. The Company of Biologist. 2015;8:679-690 doi:10.1242/dmm.018127

Brand A, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 1993;118(2):401–415. doi:10.1242/dev.118.2.401

Bird, Thomas D. "Myotonic Dystrophy Type 1." GeneReviews, edited by Margaret P. Adam *et al.*, University of Washington, Seattle, 1993, http://www.ncbi.nlm.nih.gov/books/NBK1165/.

Cerro-Herreros E, Chakraborty M, Pérez-Alonso M, Artero R, Llamusí B. Expanded CCUG repeat RNA expression in *Drosophila* heart and muscle trigger Myotonic Dystrophy type 1-like phenotypes and activate autophagocytosis genes. Scientific Reports. 2017;7(1):2843. doi:10.1038/s41598-017-02829-3

Duffy JB. GAL4 system in *Drosophila*: A fly geneticist's swiss army knife. genesis. 2002;34(1–2):1–15. https://onlinelibrary.wiley.com/doi/abs/10.1002/gene.10150. doi:10.1002/gene.10150

Garcia-Lopez A, Monferrer L, Garcia-Alcover I, Vicente-Crespo M, Alvarez-Abril M, Artero R. Genetic and chemical modifiers of a CUG toxicity model in *Drosophila*. PLoS ONE. 2008;3(2):e1595. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2220037/. doi:10.1371/journal.pone.0001595

Kreipke RE, Kwon YV, Shcherbata HR, Ruohola-Baker H. *Drosophila melanogaster* as a model of muscle degeneration disorders. In: Pick L, editor. Current Topics in Developmental Biology. Vol. 121. Academic Press; 2017. p. 83–109. https://www.sciencedirect.com/science/article/pii/S0070215316301442. doi:10.1016/bs.ctdb.2016.07.003

Liao G, Rehm E, Rubin G. Insertion site preferences of the P transposable element in *Drosophila melanogaster*. Proceedings of the National Academy of Sciences. 2000;97(7):3347–3351. https://www.pnas.org/doi/10.1073/pnas.97.7.3347. doi:10.1073/pnas.97.7.3347

Majumdar S, Rio DC. P transposable elements in *Drosophila* and other eukaryotic organisms. Microbiology spectrum. 2015;3(2):MDNA3-0004–2014. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4399808/. doi:10.1128/microbiolspec.MDNA3-0004-2014

Morriss GR, Rajapakshe K, Huang S, Coarfa C, Cooper TA. Mechanisms of skeletal muscle wasting in a mouse model for myotonic dystrophy type 1. Human Molecular Genetics. 2018;27(16):2789–2804. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6077786/. doi:10.1093/hmg/ddy192

Nichols CD, Becnel J, Pandey UB. Methods to Assay *Drosophila* Behavior. JoVE (Journal of Visualized Experiments). 2012;(61):e3795. https://www.jove.com/v/3795/methods-to-assay-*drosophila*-behavior. doi:10.3791/3795

Osterwalder T, Yoon KS, White BH, Keshishian H. A conditional tissue-specific transgene expression system using inducible GAL4. Proceedings of the National Academy of Sciences. 2001;98(22):12596–12601. https://www.pnas.org/doi/10.1073/pnas.221303298. doi:10.1073/pnas.22130329

Sznajder, Łukasz J., et al. "Mechanistic Determinants of MBNL Activity." Nucleic Acids Research, vol. 44, no. 21, Dec. 2016, pp. 10326–42, https://doi.org/10.1093/nar/gkw915.

Thornton C. Myotonic Dystrophy. Neurologic clinics. 2014;32(3):705–719. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4105852/. doi:10.1016/j.ncl.2014.04.011

Appendix

Standard labeling if statistical significance dictates * = a P value < 0.05, ** = a P value < 0.01, and *** = a P value < 0.001.

Table 1: Listed below are the expected ratios of number of white eyed F_2 Flies, when the driver and repeat transgene are not on the same chromosome.

Expected Ratios of White Eyed F_2 Flies When Driver and Repeat are Not on the Same Chromosome

Mating Schemes	Predicted Ratios of White Eyed F_2 Flies
$(CTG)_{60}$ x *Mhc*-GAL4	1/16
$(CTG)_{60}$ x *Hsp70*-GAL4	1/16
$(CTG)_{60}$ x *Mef2*-GAL4	1/16
$(CTG)_{250}$ x *Mhc*-GAL4	1/16
$(CTG)_{250}$ x *Hsp70*-GAL4	1/16
$(CTG)_{250}$ x *Mef2*-GAL4	1/16
Female $i(CTG)_{480}$ x Male *1151*-GAL4 or *Mhc*-GAL4	1/128
Male $i(CTG)_{480}$ x Female *1151*-GAL4 or *Mhc*-GAL4	1/128
$i(CTG)_{480}$ x *Hsp70*-GAL4	1/16
$i(CTG)_{480}$ x *Mef2*-GAL4	1/16

Table 2: The number of white eyed F_2 flies from each cross is shown. Transgenic flies will have white eyes if no transgenes are present in the genome. This will occur in the F_2 generation of a cross between parental flies carrying two transgenes, if the transgenes are on different chromosomes. The number in parentheses are the total number of F_2 flies produced. The *1151*-GAL4 driver is an X chromosome driver which was used for crosses, because the *Mhc*-GAL4 driver created such a severe phenotype for the $i(CTG)_{480}$ flies that many of the F_1 flies did not survive pupation.

Number of White Eyed F_2 Flies in Each Cross

Driver	$(CTG)_{60}$	$(CTG)_{250}$	$i(CTG)_{480}$
X Chromosome:*Mhc*-GAL4 or *1151*-GAL4	42 (436)	17 (129)	5 (699)
2nd Chromosome: *Hsp70*-GAL4	237 (3554)	0 (1701)	0 (840)
3rd Chromosome: *Mef2*-GAL4	0 (1122)	25 (366)	15 (333)

Table 3: Chi-square and P values for likelihood the CTG repeat is on the same chromosome. If the driver is on the same chromosome as the CTG repeat transgene, then the P value will be significant.

Chi-square Analysis of Eye Color Data

Chromosome	$(CTG)_{60}$	$(CTG)_{250}$	$i(CTG)_{480}$
X	χ^2=3.28, p>0.05	χ^2=0.91, p>0.5	χ^2=0.039, p>0.8
2nd	χ^2=1.07, p>0.5	χ^2=113.31, p<0.01**	χ^2=56, p<0.01**
3rd	χ^2=74.81, p<0.01**	χ^2=0.21, p>0.5	χ^2=1.73, p>0.05

Table 4: Listed below are the mean, standard error of the mean, and the group size of each cross for the climbing velocity data. The group size includes only flies with pigmented eyes. The $(CTG)_{250}$ x *Hsp70*-GAL4 cross was not tested for climbing velocity

Mean, SEM, and N for Climbing Velocity Data

		Mhc-GAL4	*Hsp70*-GAL4	*Mef2*-GAL4
$(CTG)_{60}$				
Females	Mean	12.753	17.262	12.844
	SEM	1.191	2.729	2.207
	N	79	30	16
$(CTG)_{60}$				
Males	Mean	19.437	20.37	13.275
	SEM	1.757	5.603	0.902
	N	118	30	18
$(CTG)_{250}$				
Females	Mean	11.842	N/A	2.844
	SEM	2.443	N/A	1.474
	N	50	0	17
$(CTG)_{250}$				
Males	Mean	25.05	N/A	7.658
	SEM	3.09	N/A	1.311
	N	60	0	17
$i(CTG)_{480}$				
Females	Mean	10.383	14.472	5.738
	SEM	2.181	2.437	0.991
	N	10	43	21
$i(CTG)_{480}$				
Males	Mean	22.246	34.25	3.386
	SEM	3.913	8.083	0.874
	N	17	60	30

Table 5: Two-way ANOVA test of climbing velocity test data. The purpose was to find a driver where the repeat had a significant effect on performance. The *Mef2*-GAL4 is the only driver where this is the case. The F value is the difference between the means of the groups, the larger this number is the more significant the difference.

Two-way ANOVA Analysis of Climbing Data

Driver		F value	Pr (>F)
Mhc-GAL4	Sex	21.2	5.93e-06***
	Repeat	0.858	0.425
	Sex: Repeat	1.205	0.301
Hsp70-GAL4	Sex	5.584	0.019*
	Repeat	0.122	0.727
	Sex: Repeat	1.16	0.283
Mef2-Gal4	Sex	0.096	0.7571
	Repeat	27.347	2.06e-10***
	Sex: Repeat	4.149	0.0182*

Table 6: The significant P values from Tukey Honest Significant Differences of the climbing velocity test data for different driver lines. The Sex: Repeat column indicates the groups being compared. Lack of significant P values for the *Hsp70*-GAL4 driver may be due to lack of $(CTG)_{250}$ cross. The $(CTG)_{60}$ flies climb significantly better thanthe severe phenotypic $(CTG)_{250}$ and $i(CTG)_{480}$ flies for both sexes when using the *Mef2*-GAL4 driver.

Tukey Honest Significant Differences Analysis of Climbing Velocity Data

Driver	Sex: Repeats	P value
Mhc-GAL4	M:$(CTG)_{250}$-F:$(CTG)_{250}$	0.00184**
	F:$(CTG)_{60}$-M:$(CTG)_{250}$	9.95e-4***
Hsp70-GAL4	No significance	--
Mef2-GAL4	F:$(CTG)_{60}$-F:$(CTG)_{250}$	1.74e-5***
	M:$(CTG)_{60}$-F:$(CTG)_{250}$	3.3e-6***
	M:$(CTG)_{60}$-M:$(CTG)_{250}$	0.0424*
	F:$(CTG)_{60}$-F:$i(CTG)_{480}$	0.003**
	F:$(CTG)_{60}$-M:$i(CTG)_{480}$	4.5e-6***
	M:$(CTG)_{60}$-F:$i(CTG)_{480}$	7.94e4***
	M:$(CTG)_{60}$-M:$i(CTG)_{480}$	5.0e-7***

Table 7: Listed below are the mean, standard error of the mean, and the group size of each cross for the flight capability data. The group size includes only flies with pigmented eyes.

Mean, SEM, and N for Flight Capability Data

		Mhc-GAL4	*Hsp70*-GAL4	*Mef2*-GAL4
$(CTG)_{60}$ Females	Mean	53.019	52.626	49.101
	SEM	9.94	2.028	2.29
	N	13	307	218
$(CTG)_{60}$ Males	Mean	65.848	49.559	60.219
	SEM	6.294	2.368	1.793
	N	36	225	302
$(CTG)_{250}$ Females	Mean	33.166	50.653	61.344
	SEM	6.505	2.212	4.555
	N	37	249	62
$(CTG)_{250}$ Males	Mean	55.945	44.226	64.759
	SEM	8.959	3.041	3.572
	N	37	169	60
$i(CTG)_{480}$ Females	Mean	68.716	7.294	44.893
	SEM	7.781	1.624	3.224
	N	15	171	120
$i(CTG)_{480}$ Males	Mean	74.954	6.554	59.577
	SEM	3.496	1.58	2.426
	N	30	175	144

Table 8: Two-way ANOVA test of flight capability test data. The purpose was to find a driver where the repeat had a significant effect on performance. The F value is the difference between the means of the groups, the larger this number is the more significant the difference. All of the drivers had the repeats significantly impact the flight capability with the *Hsp70*-GAL4 showing the repeats having most significant contribution.

Two-way ANOVA Analysis of Flight Data

Driver		F value	Pr (>F)
Mhc-GAL4	Sex	12.468	5.49e-4***
	Repeats	6.793	0.001496**
	Sex: Repeats	0.664	0.516
Hsp70-GAL4	Sex	9.928	0.00166**
	Repeats	210.149	<2e-16***
	Sex: Repeats	0.696	0.49
Mef2-GAL4	Sex	25.287	5.96e-07***
	Repeats	4.744	0.00892**
	Sex: Repeats	1.272	0.28

Table 9: The significant P values from Tukey Honest Significant Differences of the flight capability test data for different driver lines. The Sex: Repeat column indicates the groups being compared. The $i(CTG)_{480}$ flies flew significantly different from the $(CTG)_{60}$ and $(CTG)_{250}$ flies for both sexes when using the *Hsp70*-GAL4 driver.

Tukey Honest Significant Differences Analysis of Flight Capability Data

Driver	Sex: Repeats	P value
Mhc-GAL4	M:$(CTG)_{60}$-F:$(CTG)_{250}$	0.0022**
	F:$(CTG)_{250}$-F:$i(CTG)_{480}$	0.019*
	F:$(CTG)_{250}$-M:$i(CTG)_{480}$	8.2e-4***
Hsp70-GAL4	F:$(CTG)_{250}$-F:$i(CTG)_{480}$	0.00***
	F:$(CTG)_{250}$-M:$i(CTG)_{480}$	0.00***
	M:$(CTG)_{250}$-F:$i(CTG)_{480}$	0.00***
	M:$(CTG)_{250}$-M:$i(CTG)_{480}$	0.00***
	F:$(CTG)_{60}$-F:$i(CTG)_{480}$	0.00***
	F:$(CTG)_{60}$-M:$i(CTG)_{480}$	0.00***
	M:$(CTG)_{60}$-F:$i(CTG)_{480}$	0.00***
	M:$(CTG)_{60}$-M:$i(CTG)_{480}$	0.00***
Mef2-GAL4	F:$(CTG)_{250}$-F:$i(CTG)_{480}$	0.014*
	F:$(CTG)_{60}$-M:$(CTG)_{250}$	0.011*
	M:$(CTG)_{250}$-F:$i(CTG_{480}$	0.0014**
	M:$(CTG)_{60}$-F:$(CTG)_{60}$	0.0015**
	F:$(CTG)_{60}$-M:$i(CTG)_{480}$	0.03*
	M:$(CTG)_{60}$-F:$i(CTG)_{480}$	1.6e-4***
	M:$i(CTG)_{480}$-F:$i(CTG)_{480}$	0.0032**

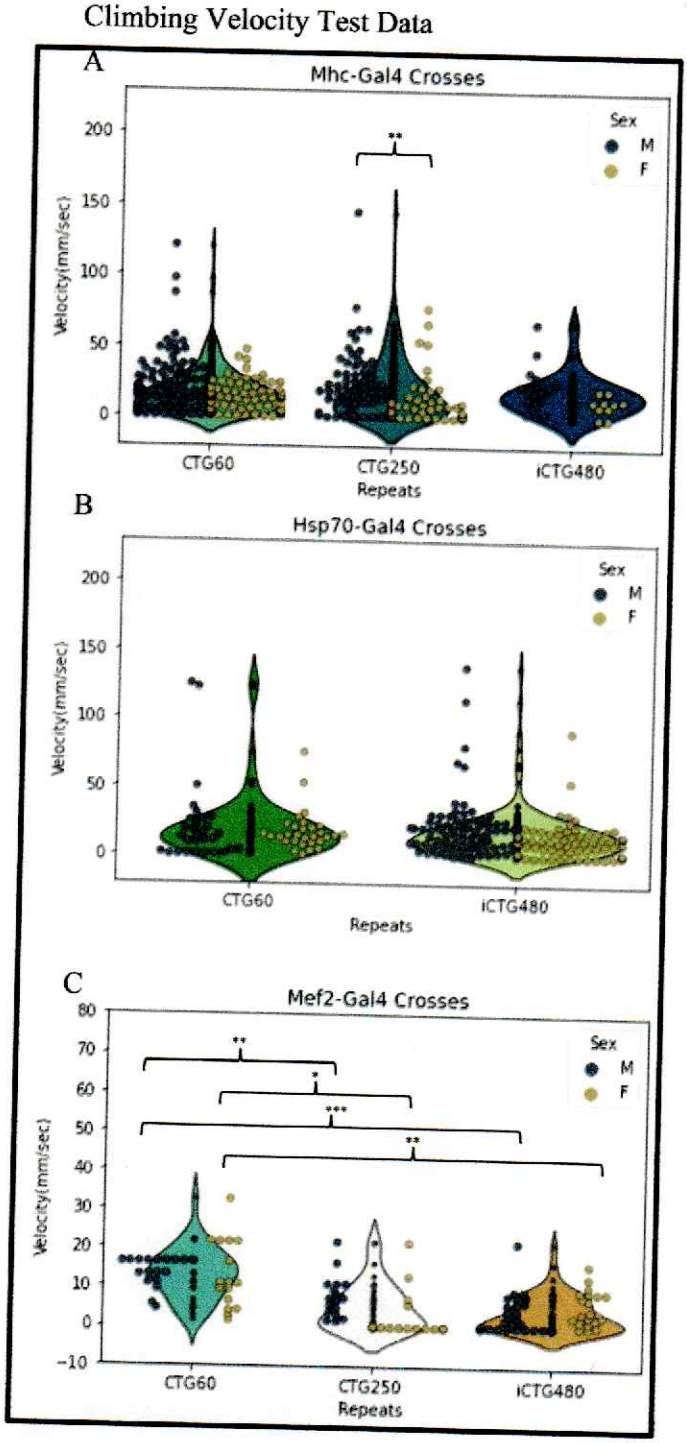

Figure 1: Violin plots showing the climbing velocity test data. Males and females are separated in each CTG repeat category. The important significant Tukey Pr(>F) values are shown.

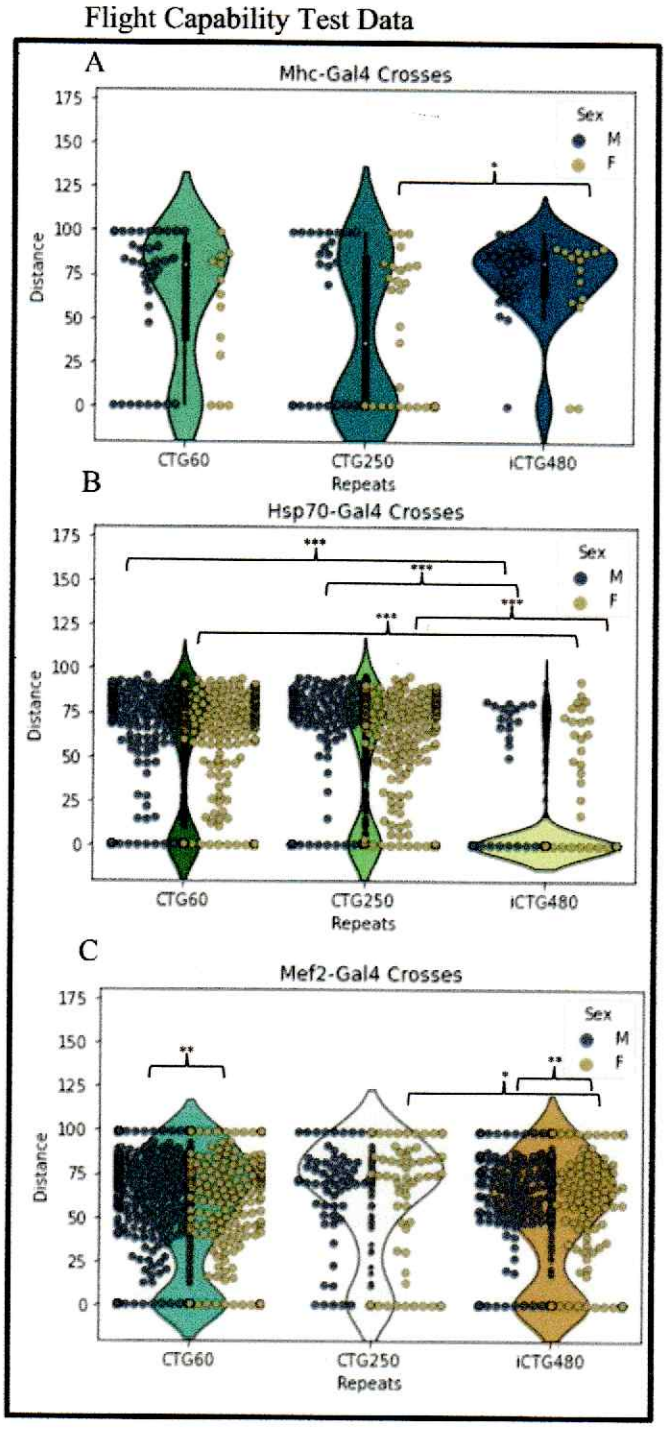

Figure 2: Violin plots showing the flight capability test data. Males and females are separated in each CTG repeat category. The important significant Tukey Pr(>F) values are shown.