HIGH SCHOOL EDITION
Journal of Student Research
Volume 12 Issue 4 (2023)

Modeling Retinoblastoma in Adult *Drosophila* by Reducing *Rbf* Gene Expression

Pria Chandarana[1] and Cathy Messenger[#]

[1]Los Gatos High School, USA
[#]Advisor

ABSTRACT

Retinoblastoma is an eye cancer that begins in the retina due to genetic mutations in the retinal cells, typically of the *Rb1* tumor suppressor gene, which encodes for the Rb protein. Rb inhibits the E2F transcription factor, which drives the expression of genes needed for entry into S-phase of the cell cycle. Loss of function of Rb dysregulates E2F, causing uninhibited cell-cycle proliferation and tumor formation. Current treatments for retinoblastoma are highly invasive such as surgery to remove the eye or chemotherapy impacting patients' lives. If an animal model of retinoblastoma is constructed, less invasive therapies can be tested. *Drosophila melanogaster* have a short life cycle, are cost-effective, and have an *Rb1* homologue called *Rbf1*, making them an efficient model. An RNAi system was used to knock down the expression of Rbf1 protein via the gsGAL4-UAS gene switch system of gene regulation. A fly line in which the gsGAL4 transcription factor is expressed under the eye-specific *elav* promoter was crossed with a line in which an siRNA targeting Rbf1 mRNA is expressed under the control of an upstream activating sequence (UAS). When the offspring of this cross were fed RU486, the normally cytoplasmic gsGAL4 entered the nucleus of the retinal cells in which it was expressed and bound to the UAS, driving the expression of the siRNA, which was expected to knock down levels of Rbf1 protein in these flies. RU486-treated flies exhibited a change in eye morphology, suggestive of tumor formation, as well as differential Kaplan-Meier survival curves.

Introduction

"[Retinoblastoma, or cancer of the eye] can have devastating consequences for the children affected by it. If treated too late, it can lead to the loss of the eye, invasion of the brain and death" (Bowman, 2018).

Retinoblastoma is a type of eye cancer that begins in the retina due to genetic mutations in the retina's photoreceptor cells (Dimaras et al., 2017). Current treatments for retinoblastoma, such as chemotherapy or surgery to remove the eye entirely (American Cancer Society 2018), are toxic or highly invasive and therefore negatively impact patients' quality of life (Binotto et al., 2020). There are many side effects to chemotherapy: hair loss, permanent damage to organs such as the kidney or heart, nausea, and fatigue (American Cancer Society 2020). If an animal model is developed that can provide large pools of affected organisms for each generation, then rapid screening of multiple potential less invasive treatments and statistically significant pre-clinical trials could be conducted.

Drosophila melanogaster have a fast life cycle and are cost-effective to maintain. In addition, they can be grown in large quantities, making them an ideal model for testing different treatments. The complete life cycle of *D. melanogaster* through the 4 stages of egg or embryo, larva, pupae, to adult, takes about 10 days (Fernández-Moreno et al., 2016). Drosophila have 4 chromosomes in total; the sex chromosomes (also called chromosome 1), and three autosomes (non-sex chromosomes) which are referred to as chromosomes 2, 3, and 4 (Larsson et al., 2001). The small number of chromosomes makes genetic manipulation easier, another advantage to using *D. melanogaster* in generating a model organism for retinoblastoma.

Retinoblastoma begins as an overgrowth of cells due to excessive proliferation. Normally, cells enter their cycle's replicative phase (S-phase) when signaled to do so by activating a protein called E2F (He et al., 2000). E2F is a transcription factor, a protein responsible for controlling the expression of genes, and it drives the expression of genes encoding proteins needed to initiate entry into the S-phase and the process of cell division (Adams, 2008). The RB1 gene encodes the retinoblastoma (Rb) protein which binds to and inhibits E2F (Ahlander et al., 2009) until a phosphate group is added to Rb due to a pathway activated when a growth signal is received by the cell (Dick et al., 2013). The Rb tumor suppressor normally inhibits E2F to restrict cell cycle proliferation, thereby preventing tumor formation. Thus, Rb is referred to as one of the tumor suppressor proteins. Retinoblastoma occurs when a mutation in Rb (in the homozygous state) reduces or eliminates the E2F-inhibitory activity of Rb (Cruz et al., 2017). If there is reduced Rb activity, E2F can promote the cell cycle far more than it should, thereby causing uninhibited cell proliferation, resulting in a retinoblastoma tumor that eventually takes over the eye's retina.

In *Drosophila,* Rbf1 is the homolog for the Rb protein in humans. The main goal of this project, to create a *Drosophila* retinoblastoma model, requires performing a targeted reduction of expression of the Rbf1 gene only in the eye and only in adults. The silencing mechanism RNA interference (RNAi) is an essential tool used to do this, in which a short, double-stranded RNA, called an siRNA, is generated to interfere with the translation or degradation of a target mRNA (Xu et al., 2019). Specifically, the expression of an siRNA targeting Rbf1 mRNA (within the organism's cells) will cause the degradation of the mRNA, resulting in reduced levels of protein production. A construct in which the siRNA targeting Rbf1 mRNA is transcribed under the control of an upstream activation sequence (UAS) which was introduced into fruit flies. The expression of the GAL4 activator protein binds to the UAS used to drive the expression of the siRNA (Rodríguez et al., 2013). Since this should only occur in the eyes of adult flies, a modified version of this activation system called the geneswitch GAL4-UAS system was utilized, in which the GAL4 induces expression of a transgene only when the drug RU486 is introduced (Murugia et al., 2019). The gsGAL4 binds to the UAS and drives expression of the associated gene requires a drug called RU486 to do so. This allows expression of the Rbf1-targeting siRNA to be induced by gsGAL4 only in adults that have been fed RU486.

Furthermore, in addition to the geneswitch, expression of gsGAL4 was also placed under the control of the eye-specific *elav* promoter. Therefore, when RU486 was administered to the flies, gsGAL4 would only be expressed in the eye (Roman et al., 2001). In other words,the combination of the *elav* promoter and geneswitch system - used to to regulate gsGAL4 expression and function - allows the gsGAL4 to activate the expression of the Rbf1-targeting RNAi expression (hereafter called Rbf1-RNAi), and thus results in the inhibition of Rbf1 protein expression only in the adult eye, and not in other areas of the fly body. Therefore, the degradation of the Rbf1 mRNA and the resulting reduction of Rbf1 protein may cause retinoblastoma tumors to occur only in the adult *Drosophila* eye. This study hypothesizes that using RNAi to knock down levels of Rbf mRNA in adult *D. melanogaster* will result in reduced Rbf protein activity, inducing retinoblastoma in fruit flies. Once the adult model is established with reduced levels of Rbf1 and the fly is confirmed to develop retinoblastoma tumors, less invasive treatments for retinoblastoma can begin to be tested.

Once this double homozygous line is generated, flies from the line can be treated with RU486 to induce retinoblastoma, and less invasive therapies can be tested. These include less invasive drug therapies or using the same RNAi mechanism to silence different tumor microenvironment genes important for cancer metabolism.

Materials and Methods

Fly Lines and Generation of Flies Homozygous for Chromosome 2 Containing TRiP-UAS-Rbf RNAi

Two fly lines were obtained from the Bloomington Drosophila Stock Center. One with a UAS-driven RNAi cassette targeting Rbf1 (TRiP-UAS-Rbf1 RNAi) inserted into chromosome 2 (catalog # 41863) and the other with the inducible geneswitch GAL4 system expressed under the control of the eye-specific *elav* promoter (elav-gsGAL4) inserted into chromosome 3 (catalog # 43642). These flies were maintained in vials containing Carolina Blue Instant Media at 23℃ in an incubator. While the elav-gsGAL4 fly line was already homozygous, the TRiP-UAS-Rbf1 RNAi fly line included heterozygotes containing a floating Curly-wing (CyO) balancer chromosome 2. Consequently, the flies in the vial of the TRiP-UAS-Rbf RNAi line were a mix of homozygotes for the chromosome 2 containing TRiP-UAS-Rbf RNAi and heterozygotes for the chromosome 2 containing TRiP-UAS-Rbf RNAi and a chromosome 2 containing the recessive lethal CyO gene for dominant curly wings and inversions that prevent meiotic homologous recombination. To generate homozygous TRiP-UAS-Rbf RNAi flies, five males and five females from the TRiP-UAS-Rbf RNAi fly line were placed into a new vial with Carolina Blue Instant Media, deionized water, and Fleischmann's Dry Active Yeast, and allowed to mate (Figure 1). Since the offspring could not be homozygous for the balancer (as that leads to lethality), the offspring should either be curly-winged heterozygotes with the chromosome 2 carrying TRiP-UAS-Rbf RNAi or homozygous for the TRiP-UAS-Rbf RNAi containing chromosome 2. The parents were removed from the vial once pupae were observed. When the adult offspring emerged from the pupae, male flies were segregated from female flies, and, for both sexes, only flies without curly wings were retained. Males were collected and females were further observed to identify virgin females based on the presence of a black mark on the abdomen.

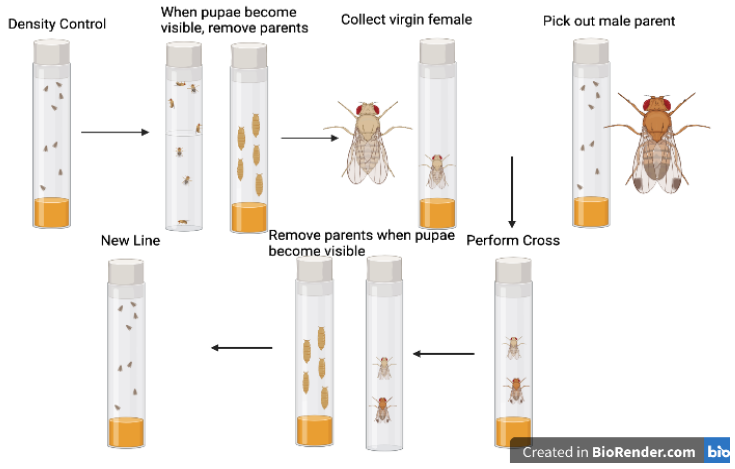

Figure 1. Procedure for crossing flies to generate a new fly line.

Generation of Flies Heterozygous for Both Chromosome 2 Containing the TRiP-UAS-Rbf RNAi Cassette and Chromosome 3 Containing the elav-gsGAL4 Cassette

Virgin TRiP-UAS-Rbf RNAi homozygous females were collected and placed in a new vial with Carolina Blue Instant Media, deionized water, and Felischmann's Dry Active Yeast. A homozygous elav-gsGAL4 male from the original stock was then transferred into each such vial for mating. When pupae were observed, the parents were removed. Fruit flies that emerged from the pupae were heterozygous for TRiP-UAS-Rbf RNAi on chromosome 2, and for the elav-gsGAL4 on chromosome 3.

Production of RU486 Containing Media and Dosing of Flies

In order to produce a concentrated stock solution of 20 mg/ml of RU486 in ethanol, the solution had to be vortexed for 3-4 minutes, followed by incubation for 10 minutes, to fully dissolve the RU486. Despite this procedure, due to RU486's insolubility in water, when this stock solution was used to generate a 500 µM (0.21 mg/ml) working solution in water, this working solution formed a gelatinous substance. Fortunately, when this working solution was added to the Carolina Media powder, the powder absorbed the solution. Thus, when the TRiP-UAS-Rbf RNAi and elav-gsGAL4 double heterozygous flies were placed in tubes with this media, they consumed RU486 at the 500 µM concentration that was previously reported to be the optimal dosage (Garschall et al., 2017).

Statistical Analysis of Results – Kaplan Meier Survival Curve

A Kaplan Meier Survival curve was generated for the flies treated with RU486 (or for controls). Each death was designated as an "event" and denoted by a drop in the curve from the original 100% survival. While all surviving flies at the end of the experiment were designated as "censored events." The curves were generated using online software found at Statists Kingdom (https://www.statskingdom.com/kaplan-meier.html).

This website automatically performed a log-rank test, which is a form of a chi-square test that compares trends or Kaplan-Meier Survival curves between the experimental groups and control groups. The null hypothesis for the test is that the experimental and control groups exhibit no difference in overall survival.

Results

Flies homozygous for chromosome 2 with an integrated TRiP-UAS-Rbf1-RNAi cassette, and lacking the floating CyO-containing balancer chromosome were generated by crosses of the fly line obtained from Bloomington Drosophila Stock Center. Then these homozygous flies were crossed with flies homozygous for chromosome 3 with an integrated elav-gsGAL4 cassette to generate double heterozygous flies. The offspring generated from the experimental cross (n=7) were fed RU486 after emerging from the pupae. This treatment was expected to relocate gsGAL4, a version of the GAL4 transcription factor fused to the estrogen receptor and expressed only in the eye due to the use of the eye-specific *elav* promoter, into the nucleus, from where it would drive the expression of an siRNA targeting the *D. melanogaster* Rbf1 mRNA in the retina (Figure 2). This was, in turn, expected to reduce levels of Rbf1 protein.

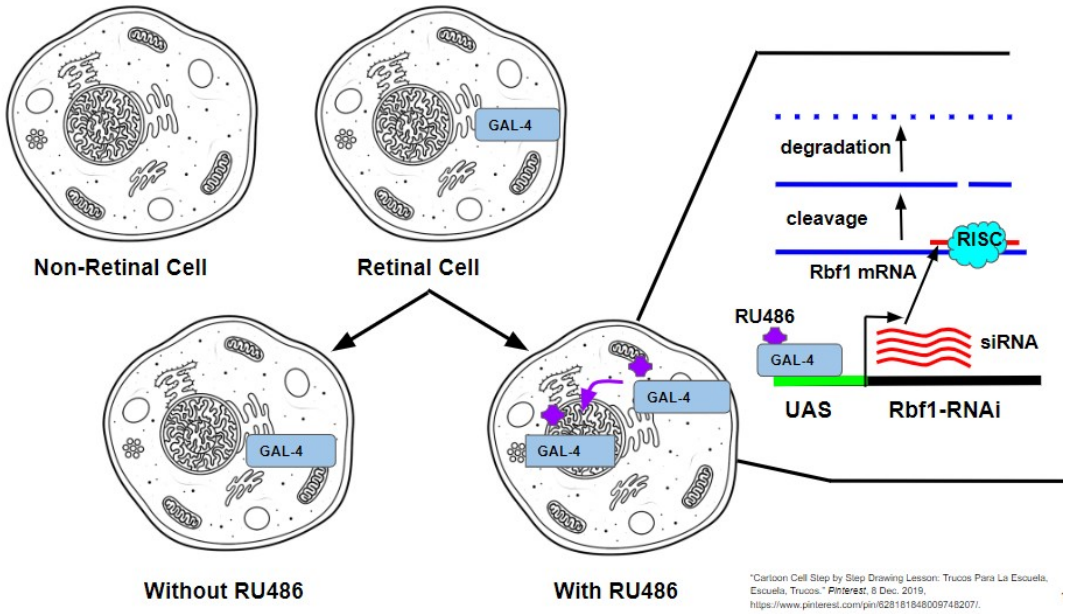

Figure 2. Experimental design involving the use of RU486 to drive expression of siRNA targeting Rbf1 mRNA and thereby knock down Rbf1 protein in D. melanogaster retinal cells.

The gsGal4 protein is a fusion of the Gal4 transcription factor with the estrogen receptor that is retained in the cytoplasm until bound by estrogen or an analog. As the mRNA encoding gsGal4 is transcribed under the control of the *elav* promoter, gsGal4 is expected to be expressed only in the eye. Treatment with RU486, an estrogen analog, therefore allows gsGal4 to enter the nucleus and drive the expression of the siRNA (red line) targeting Rbf1 mRNA (blue line) and leading to cleavage of the mRNA by the RNA induced silencing complex (RISC). This would prevent expression of Rbf1 protein, and therefore allow uncontrolled activity of the E2F proto-oncogenic transcription factor. This was expected to cause retinoblastoma.

Five out of the seven flies in the experimental group developed eye deformities such as indentations of the eye or tumor-like extensions to the tissue of the eye (Figure 3).

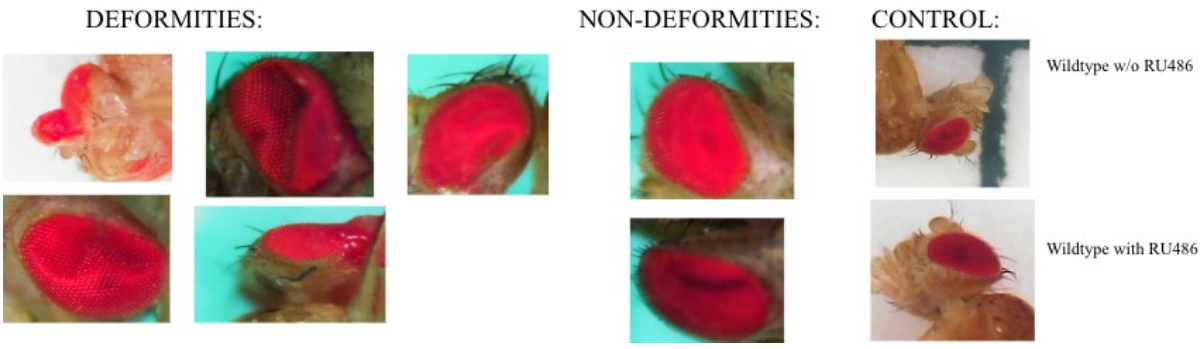

Figure 3. Image of eye morphologies of TRiP-UAS-Rbf1-RNAi and elav-gsGAL4 double heterozygous flies fed RU486-containing media, or, as controls, of wildtype flies fed RU486-containing media (Wildtype with RU486) or normal media (Wildtype w/o RU486).

This experimental group was then compared to a control group of wildtype flies fed RU486, and to another control group with wildtype flies fed normal media. The number of flies that died was tracked in each

group, and a Kaplan Meier survival curve was generated on the 5th day (Figure 4). There was a statistically significant difference in survival for the experimental groups versus the control groups, with a p-value from a log-rank test of the survival curves of 0.001, which was less than the 0.05 cutoff needed to reject the null hypothesis that there was no difference between the overall survival of the groups.

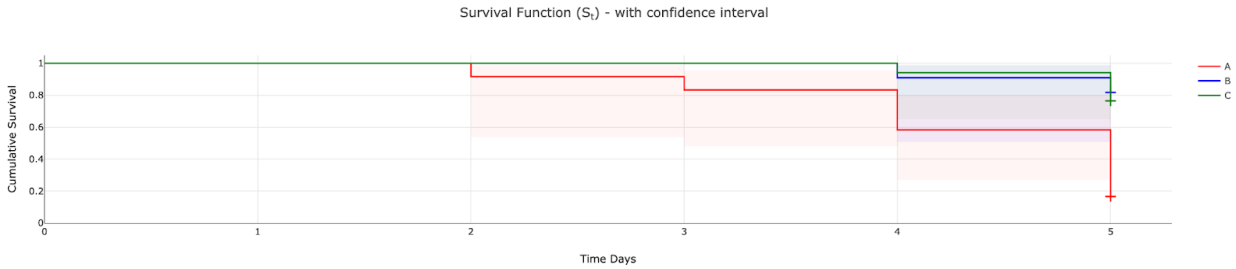

Figure 4. Kaplan Meier Survival Curve with one experimental group (A), wildtype control (B), and wildtype control fed RU486 (C). Log Rank test p-value = 0.001.

Discussion

Feeding RU486 to the *elav*-gsGAL4, TRiP-UAS-Rbf1RNAi, double heterozygous flies, resulting in the knock-down of the Rbf1 gene, specifically in the adult eyes, gave rise to eye deformities in 5 out of 7 experimental flies. Additionally, when the survival of the flies was compared to the untreated or RU486-fed wildtype control flies, there was a statistically significant difference (by log-rank test, p = 0.001) in the Kaplan-Meier survival curves. Thus, driving expression of Rbf1-targeting RNAi specifically in the adult fly eye appears to lead to the formation of retinal tumors, which may contribute to lethality. Therefore, XYZ seems to recapitulate the retinoblastoma tumors of patients with mutations in *RB1*. However, larger trials with more double heterozygous flies need to be conducted to be sure.

Furthermore, studies in flies that are double homozygous for *elav*-gsGAL4 and TRiP-USA-Rbf1RNAi might yield an even clearer phenotype and could be utilized as a model to test various therapies for retinoblastoma. However, his adult model must first be established. With the help of balancer chromosomes, a fly line that is homozygous for both the TRiP-USA-Rbf-RNAi cassette in chromosome 2 and the elav-gs-GAL4 in chromosome 3 can be produced. *Balancer chromosomes* are genetically engineered chromosomes containing inversions that prevent recombination with the homologous chromosome (Bohnekamp et al., 2016). Additionally, since balancer chromosomes contain genes that drive a dominant phenotype in heterozygotes but are homozygous lethal, crosses between heterozygous flies will generate only other heterozygotes with the dominant phenotype or homozygotes for the other chromosome (in this case, the elav-gsGAL4 and Rbf1-RNAi chromosomes) and lacking the balancer phenotype (Bloomington Drosophila Stock Center 2021). Thus, to create the double homozygous fly retinoblastoma model, a series of crosses needs to be performed to get Rbf1-RNAi/Rbf1-RNAi on chromosome 2 and elav-gsGAL4/elav-gsGAL4 on chromosome 3. A Pm/Cy; D/Sb double balancer line from Carolina Biological Supply, in which the balancer version of chromosome 2 is marked with either the Plum (Pm) or Curly Wing (Cy) phenotypically dominant but recessive lethal allele and the balancer version of chromosome 3 is marked with either the Dichaete (D) or Stubby Bristles (Sb) phenotypically dominant but recessive lethal allele, can be obtained. A double balancer male fly (with any combination of chromosome 2 and chromosome 3 balancer dominant phenotypes) can be bred with a homozygous *elav*-gsGAL4 virgin female, and offspring exhibiting the two balancer phenotypes and heterozygous for the *elav*-gsGAL4 cassette on chromosome 3 can be bred to each other to obtain chromosome 2 balancer heterozygotes and chromosome 3 balancer and *elav*-gsGAL4 heterozygotes. These can then be bred to each other to obtain

chromosome 2 balancer heterozygotes that are homozygous for the elav:gsGAL4 cassette on chromosome 3, based on lack of the chromosome 3 balancer phenotype. Similarly, male double balancer fly can be bred with a homozygous TRiP-UAS-Rbf1-RNAi virgin female, and offspring exhibiting both balancer phenotypes and heterozygous for the TRIP-UAS-Rbf1-RNAi cassette on chromosome 2 can be bred to each other to obtain chromosome 2 balancer and TRIP-UAS-Rbf1-RNAi heterozygotes and chromosome 3 balancer heterozygotes. These can then be bred to each other to obtain chromosome 3 balancer heterozygotes that are homozygous for the TRIP-UAS-Rbf1-RNAi cassette on chromosome 2, based on lack of the chromosome 2 balancer phenotype. Finally, the chromosome 2 balancer heterozygotes that are homozygous for the elav:gsGAL4 cassette on chromosome 3 can be bred to the chromosome 3 balancer heterozygotes that are homozygous for the TRIP-UAS-Rbf1-RNAi cassette on chromosome 2 to obtain offspring that exhibit both balancer phenotypes and therefore are heterozygous both for the chromosome 2 balancer and chromosome 2 with the TRIP-UAS-Rbf1-RNAi cassette and for the chromosome 3 balancer and chromosome 3 with the *elav*-gsGAL4 cassette. Crossing these to each other and selecting for offspring without either balancer phenotype will guarantee the generation of the double homozygous fly line. Once this animal model is fully established, the homozygous model will be fed RU486 to ensure they develop retinoblastoma. Less invasive therapies can then be tested via this model of retinoblastoma such as alternative drug therapies or silencing different genes. One example of a potential avenue for therapy is to starve the cancer cells of glucose.

Typically, cells use aerobic cellular respiration to obtain energy from glucose or other organic molecules, but cancer cells grow so densely that they can cut off their supply of oxygen, thus, making them dependent solely on glycolysis and fermentation (The Harvard Gazette, 2022). As a result, cancer cells need substantial quantities of glucose to support themselves (Cancer Research UK 2020). In many cases, even after the tumor has driven angiogenesis to restore blood flow and therefore oxygen to the hypoxic region the tumor, the cancer cells still utilize glycolysis and fermentation as their major energy derivation process; this use of aerobic fermentation and increased need for glucose is known as the Warburg effect (Luengo et al., 2021). Cells import glucose through their membranes using multiple glucose transporters (Lizák et al., 2019). One of these glucose transporters is known as GLUT-1, and expressing it helps cancer cells grow (Simmons, 2017). Thus, knocking down or inhibiting GLUT-1 expression in retinoblastoma cells might be a way to kill the retinal tumor, and this can be tested in the fruitfly homozygous Rbf1 mRNA knockdown model. Drugs and other therapies can also be tested on this animal model to help improve the quality of life of cancer patients.

Conclusion

Driving expression of RNAi targeting Rbf1 mRNA, the fruitfly homolog to human RB1 mRNA, solely in the adult fly eye appears to result in the formation of retinal tumors that reduce survival, but more trials need to be conducted to be sure. If this holds up, a double homozygous fly line can be generated that can be used as a preclinical model to test various less invasive therapies for retinoblastoma.

Acknowledgments

I would like to thank Dr. John Santiago and Dr. Jennifer Sanders at Brown University for their mentorship and support. I would also like to thank Mrs.Messenger for all of her guidance and support.

References

1. Ahlander, J., & Bosco, G. (2009). The rb/e2f pathway and regulation of RNA processing. *Biochemical and Biophysical Research Communications*, *384*(3), 280-283. https://doi.org/10.1016/j.bbrc.2009.04.107
2. Binotto, M., Reinert, T., Werutsky, G., Zaffaroni, F., & Schwartsmann, G. (2020). Health-related quality of life before and during chemotherapy in patients with early-stage breast cancer. *Ecancermedicalscience*, *14*. https://doi.org/10.3332/ecancer.2020.1007
3. *BioRender* [Computer software]. (n.d.). https://www.biorender.com/
4. Del valle rodríguez, A., Didiano, D., & Desplan, C. (2011). Power tools for gene expression and clonal analysis in drosophila. *Nature Methods*, *9*(1), 47-55. https://doi.org/10.1038/nmeth.1800
5. Dick, F. A., & Rubin, S. M. (2013). Molecular mechanisms underlying RB protein function. *Nature Reviews Molecular Cell Biology*, *14*(5), 297-306. https://doi.org/10.1038/nrm3567
6. Dimaras, H., Corson, T. W., Cobrinik, D., White, A., Zhao, J., Munier, F. L., Abramson, D. H., Shields, C. L., Chantada, G. L., Njuguna, F., & Gallie, B. L. (2015). Retinoblastoma. *Nature Reviews Disease Primers*, *1*(1). https://doi.org/10.1038/nrdp.2015.21
7. Fadaka, A., Ajiboye, B., Ojo, O., Adewale, O., Olayide, I., & Emuowhochere, R. (2017). Biology of glucose metabolization in cancer cells. *Journal of Oncological Sciences*, *3*(2), 45-51. https://doi.org/10.1016/j.jons.2017.06.002
8. Fernández-moreno, M. A., Farr, C. L., Kaguni, L. S., & Garesse, R. (2007). Drosophila melanogaster as a model system to study mitochondrial biology. *Methods in Molecular Biology*, 33-49. https://doi.org/10.1007/978-1-59745-365-3_3
9. Garschall, K., Dellago, H., Gáliková, M., Schosserer, M., Flatt, T., & Grillari, J. (2017). Ubiquitous overexpression of the DNA repair factor dPrp19 reduces DNA damage and extends drosophila life span. *Npj Aging and Mechanisms of Disease*, *3*(1). https://doi.org/10.1038/s41514-017-0005-z
10. *Gene: Dmel\w*. (n.d.). https://flybase.org/reports/FBgn0003996.html
11. *The Harvard Gazette*. (n.d.). https://news.harvard.edu/gazette/story/2022/03/closer-look-at-cancer-cells-ability-to-rewire-thrive-survive/
12. He, S., Cook, B. L., Deverman, B. E., Weihe, U., Zhang, F., Prachand, V., Zheng, J., & Weintraub, S. J. (2000). E2F is required to prevent inappropriate s-phase entry of mammalian cells. *Molecular and Cellular Biology*, *20*(1), 363-371. https://doi.org/10.1128/mcb.20.1.363-371.2000
13. *An Introduction to Balancers*. (n.d.). Bloomington Indiana Drosophila Screening Center. Retrieved 2021, from https://bdsc.indiana.edu/stocks/balancers/balancer_intro.html#:~:text=Balancer%20chromosomes%20keep%20homozygous%20lethal,deleterious%20mutations%20and%20inversion%20breakpoints.
14. *Kaplan Meier Survival Analysis*. (n.d.). https://www.statskingdom.com/kaplan-meier.html
15. Larsson, J., Chen, J. D., Rasheva, V., Rasmuson-lestander, Å., & Pirrotta, V. (2001). Painting of fourth, a chromosome-specific protein in Drosophila. *Proceedings of the National Academy of Sciences*, *98*(11), 6273-6278. https://doi.org/10.1073/pnas.111581298
16. Lizák, B., Szarka, A., Kim, Y., Choi, K.-S., Németh, C. E., Marcolongo, P., Benedetti, A., Bánhegyi, G., & Margittai, É. (2019). Glucose transport and transporters in the endomembranes. *International Journal of Molecular Sciences*, *20*(23), 5898. https://doi.org/10.3390/ijms20235898
17. Malagón, J. N., Ahuja, A., Sivapatham, G., Hung, J., Lee, J., Muñoz-gómez, S. A., Atallah, J., Singh, R. S., & Larsen, E. (2014). Evolution of Drosophila sex comb length illustrates the inextricable interplay between selection and variation. *Proceedings of the National Academy of Sciences*, *111*(39). https://doi.org/10.1073/pnas.1322342111

18. Mathews, K. W., Cavegn, M., & Zwicky, M. (2017). Sexual dimorphism of body size is controlled by dosage of the X-Chromosomal gene Myc and by the sex-determining gene tra in Drosophila. *Genetics*, *205*(3), 1215-1228. https://doi.org/10.1534/genetics.116.192260
19. *National Cancer Institute*. (n.d.). https://www.cancer.gov/research/key-initiatives/ras/ras-central/blog/2021/vander-heiden-warburg-effect
20. *Natural Library of Medicine*. (n.d.). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5998401/#:~:text=Although%20retinoblastoma%20is%20relatively%20uncommon,does%20not%20affect%20everyone%20equally.
21. *Nature Education*. (2014). https://www.nature.com/scitable/topicpage/the-complexity-of-gene-expression-protein-interaction-34575/
22. *Retinoblastoma*. (n.d.). https://www.mayoclinic.org/diseases-conditions/retinoblastoma/symptoms-causes/syc-20351008
23. Robles-murguia, M., Hunt, L. C., Finkelstein, D., Fan, Y., & Demontis, F. (2019). Tissue-specific alteration of gene expression and function by ru486 and the geneswitch system. *Npj Aging and Mechanisms of Disease*, *5*(1). https://doi.org/10.1038/s41514-019-0036-8
24. Roman, G., Endo, K., Zong, L., & Davis, R. L. (2001). P{Switch}, a system for spatial and temporal control of gene expression in Drosophila melanogaster. *Proceedings of the National Academy of Sciences*, *98*(22), 12602-12607. https://doi.org/10.1073/pnas.221303998
25. *ScienceDirect*. (n.d.). https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/glut1
26. *ScienceDirect*. (n.d.). https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/balancer-chromosome
27. Sladek, T. L. (1997). E2F transcription factor action, regulation and possible role in human cancer. *Cell Proliferation*, *30*(3-4), 97-105. https://doi.org/10.1046/j.1365-2184.1997.00085.x
28. *Treating Retinoblastoma*. (n.d.). https://www.cancer.org/cancer/retinoblastoma/treating.html
29. Trivedi, D. (2021). Using crispr-cas9-based genome engineering tools in drosophila melanogaster. *Progress in Molecular Biology and Translational Science*, 85-121. https://doi.org/10.1016/bs.pmbts.2021.01.006
30. Vélez-cruz, R., & Johnson, D. (2017). The retinoblastoma (RB) tumor suppressor: Pushing back against genome instability on multiple fronts. *International Journal of Molecular Sciences*, *18*(8), 1776. https://doi.org/10.3390/ijms18081776
31. Xu, W., Jiang, X., & Huang, L. (2019). RNA interference technology. *Comprehensive Biotechnology*, 560-575. https://doi.org/10.1016/B978-0-444-64046-8.00282-2
32. *How to Draw a Cartoon Cell - How to Draw Cartoons*. (n.d.). https://www.pinterest.com/pin/cartoon-cell-step-by-step-drawing-lesson--628181848009748207/