Creating white eye *drosophila* mutants through RNAi

Mercedes Goering, Ioannis Raymond, Ella Smith, Alex Zuffoletti

BioBuilderClub, Renaissance School, Charlottesville, Virginia, USA

Reviewed on 2 May 2020; Accepted on 22 June 2020; Published on 26 October 2020

The white gene in Drosophila determines eye pigment, which is typically red (Hersh 2016). The Drosophila eye contains two pigment types; red and brown (Hersh 2016). When white is nonfunctional, these pigments are never made, so the eye is white (Hersh 2016). This gene can turn nonfunctional using RNAi. RNA interference is a system where a gene is post-transcriptionally silenced. In this experiment, RNAi will be induced through the Dicer pathway, where exogenous double stranded RNA (dsRNA) corresponding to a target gene - in this case the white gene - is introduced into a cell. The first step in this procedure will be to prepare the dsRNA templates. To prepare these templates, the mRNA sequence of the target gene must be determined and synthetically created. This template would then be multiplied via PCR, and combined with RNase-free water, an enzyme mix, and reaction buffer to form a dsRNA bath solution for the fly embryos. Cultured Drosophila embryos can take up exogenous dsRNA without the use of a vector, which allows the RNAi Dicer pathway to easily occur. An F1 generation of the *Drosophila melanogaster* stock will be bred, and the embryos of the F2 generation will be bathed in the dsRNA solution to promote absorption of the dsRNA. The dsRNA is cleaved by the Dicer enzyme into small interfering RNAs, which separate into a passenger and guide strand. The guide strand is loaded into RISC (RNA Induced Silencing Complex), where it binds to the complementary mRNA of the target gene. The mRNA is cleaved by the Piwi domain of the enzyme Argonaute (a component of RISC). Since the mRNA is cleaved and degraded before transcription can occur, the white gene is silenced. The F2 embryos will then be cultured into adult flies and observed phenotypically to determine eye color.

Keywords: *Drosophila*, RNAi, post-transcriptional gene silencing, dsRNA, gel electrophoresis

Mentors: Anna Minutella; Chloe Lohmeyer

Direct correspondence to aminutella@renaissanceschool.org; ChloeLohmeyer2021@u.northwestern.edu

Watch a video introduction by the authors at https://youtu.be/cFwA8JT6CYY

Background

The common fruit fly, *Drosophila melanogaster*, is an ideal model organism for humans due to their large number of homologs to homeotic genes expressed during embryonic development (Hersh 2016). The late-19th and early-20th century biologist Thomas Hunt Morgan was the first to use *Drosophila* in his lab as a model organism at Columbia University. There he observed genetic

recombination through crossover and produced the first genetic maps.

This project will be a proof-of-concept experiment, outlining how RNA interference can be induced in *Drosophila*. However, this project also has important ties to the phenomenon of pleiotropy in which a single gene can have several functions within an organism, and mutational loss of that gene has multiple effects across different physiological systems in the organism (Hersh 2016). This experiment could provide valuable information about the mechanisms of pleiotropy, mechanisms that may function similarly in humans. Therefore, the results of this project may have implications for human health and overall human gene function. Sickle cell anemia, albinism, and certain forms of autism and schizophrenia in humans are caused by pleiotropic effects, illustrating the importance of understanding pleiotropy (Sivakumaran, Agakov, Theodoratou, et al. 2011). If a gene with unknown pleiotropic effects were to be silenced in humans, potentially for a gene therapy treatment, it may affect other parts of the biological system and be potentially harmful.

The *Drosophila* eye contains two pigment types, red and brown (Hersh 2016). Fruit flies typically have red eyes (Hersh 2016). Morgan showed that the gene coding for eye color in *Drosophila* was found on the X chromosome (Hersh 2016). When white is nonfunctional, these pigments are never made, and the eye is white in color (Hersh 2016). The function of the gene appears to be the opposite of its name, since the gene was named for its loss-of-function mutant phenotype (Hersh 2016).

Mutations in the white gene have pleiotropic effects, meaning that white mutants are associated with more than just white eye color (Hersh 2016). They have reduced levels of biogenic amines in the brain, reduced loading of these amine molecules into intracellular vesicles within the brain, and changes in male courtship behavior, anesthesia response, and cyclic GMP transport (Hersh 2016). The white gene was also discovered in 2016 to play a role in the response of *Drosophila* to oxygen deprivation (Xiao and Robertson 2016). One or more of the substances that white acts on — likely a neurotransmitter or a neurotransmitter precursor — is important for recovery from anoxia (Xiao and Robertson 2016). Therefore, when white is not expressed, fruit flies struggle to recover from oxygen deprivation, which can represent a danger of pleiotropy (Xiao and Robertson 2016).

This primary method utilized in this experiment is a gene therapy technique known as RNA interference, or RNAi. Gene therapy is often thought of as the editing of endogenous DNA in the cell. However, RNAi uses the cell's own immune mechanisms to affect protein synthesis without editing the DNA of the cell itself (Lin, Sukasweang, Chuong, et al. 2001). Instead of using tools to edit the cell's DNA, it is possible to augment the immune mechanisms in the cell to work for particular purposes of a lab. RNAi can be used to silence the expression of the white gene, resulting in a decrease in the production of normal eye pigment proteins within *Drosophila*.

It has been observed that when the white gene is deactivated, the pleiotropic effects discussed earlier are observed in *Drosophila*. However, through RNAi the white gene is silenced post-transcriptionally, as the RNAi mechanism does not affect the gene, but instead results in the cleaving of corresponding mRNA. This experiment would determine whether the pleiotropic effects are still observed when the white gene is silenced post-transcriptionally.

When the white gene is silenced through a mutation, then every mRNA corresponding to the gene will be affected by this mutation. The proteins that these mRNA code for are therefore not created, which results in the pleiotropic effects observed with the silencing of the white gene through genetic mutation. In this experiment, we are silencing one particular mRNA associated with the white gene in order to create white-eyed flies. The other mRNA associated with the white gene are unaltered, suggesting that in this experiment the pleiotropic effects typically observed with the silencing of the white gene would not occur. In this way, it can be determined through this experiment whether the pleiotropic effects observed when the gene is mutated are generated simply through altered mRNA that no longer codes for the corresponding proteins, as would seem logical. It could be very informative to silence each individual mRNA coded for by the white gene, and observe how this affects the fly. This would illustrate how the white gene interacts with various systems through the *Drosophila* organism, and could provide information regarding why the pleiotropic effects associated with the silencing of the white gene occur.

Systems level

RNAi has been accomplished in *Drosophila* by injection of dsRNA into embryos in the early syncytial cleavage stage (Kao and Megraw 2004). RNAi has also been demonstrated to be achievable with cultured *Drosophila* cells (Kao and Megraw 2004). *Drosophila* cells in culture take up exogenous dsRNA, so there is no need to use carriers or transfection methods to introduce dsRNA into the *Drosophila* cells to induce RNAi (Kao and Megraw 2004). The RNAi Dicer pathway will be used to silence the white gene in the embryos since they are taking up exogenous dsRNA.

The first step in the procedure will be to prepare templates of RNA to be used for in vitro transcription using PCR. In order to prepare these templates, the mRNA of the target gene must be located, designed, and synthesized for the RNAi to work. To do this, the online program BLOCK-iT™ RNAi Designer by ThermoScientific will be used. The RNA will then be synthesized and shipped to the procedure site. This template will then be multiplied via PCR. An RNA bath solution will then be prepared using the PCR product with RNase-free water, an enzyme mix, and reaction buffer.

An F2 generation of the *D. melanogaster* stock will be bred. The F2 embryos will be bathed in the PCR/dsRNA solution to promote absorption of the dsRNA. These F2 embryos will then be cultured into adult flies and then observed phenotypically for changes in eye color.

In order to validate that the white gene DNA was not directly edited, a gel electrophoresis will be performed. This will be done utilizing a sample of DNA from a fly without RNAi and one with RNAi. The determination of RNAi occurrence will be by phenotypically observing the eyes for white color. Before performing a gel electrophoresis, the DNA of both fly samples will need to be multiplied via PCR (polymerase chain reaction). The PCR samples will be combined with the appropriate amount of buffer and loading dyes before being run through the gel. In order to test if the proteins from the white gene were no longer transcribed, a western blot, or protein gel electrophoresis (that is determined by presence of antibodies) will be used. This will require the PCR product of the previous mentioned samples to be mixed with the western blot buffer and loading dyes then electrophoresed. This would prove that there would be no direct gene editing even though there were changes in the phenotype and production of proteins corresponding with the white gene.

Device level

Cultured *D. melanogaster* embryos have the capability to absorb exogenous dsRNA solution, which can be utilized for RNAi. For this, the cultured embryos would consume the dsRNA since they will not yet have a cell membrane in this early syncytial embryonic stage (Clemens, Worby, Simonson-Leff, et al. 2000).

A cell's natural immune mechanisms to edit post-transcriptionally are known as the Dicer and Drosha pathways (Lin, Sukasweang, Chuong, et al. 2001). Post-transcriptional editing occurs once a gene has been transcribed and the mRNA has been transported out of the nucleus, thus post- transcriptional editing occurs in the cytoplasm and not the nucleus. In the normal cell life cycle, a cell will use Dicer and Drosha pathways to regulate expression of its own endogenous DNA. Therefore, regulate protein abundance via the Drosha pathway or defend against invading viral RNA via the Dicer pathway, thus regulating expression of viral proteins. A deeper understanding of both the Dicer and Drosha pathways is recommended to illustrate the mechanisms utilized for experimental post-transcriptional gene silencing.

The RNAi Dicer pathway would be used to post-transcriptionally silence the white gene. Dicer degrades the invading RNA and turns it into guide strands. Guide strands are 21-23 base pair long strands of RNA (Lin, Sukasweang, Chuong, et al. 2001). These guides are then loaded into the RNA Induced Silencing Complex (RISC) and used to find matching copies of the viral RNA elsewhere in the cell (Lin, Sukasweang, Chuong, et al. 2001). RISC is a complex of proteins the most important of which is a protein called Argonaute. Argonaute is a protein with two lobes which each grab one end of the RNA guide which has been loaded into it. One lobe, called the PAZ domain, holds the RNA by its 3' end, while the other lobe, called the Middle domain, holds the 5' end (Pratt and MacRae 2009). RISC then degrades the sense strand of each guide strand, making it single stranded (Lin, Sukasweang, Chuong, et al. 2001). After each sense guide strand has been degraded, RISC then matches the antisense strand to each sense strand of all other copies of the exogenous RNA present in the cell. These matched strands are called the miRNA duplex. RISC then degrades the miRNA duplex, resulting in the silencing of the target gene expression. Alternatively, the Drosha pathway works similarly, and includes the Dicer enzyme. While Dicer is triggered by exogenous RNA, Drosha is used to regulate endogenous genes. In the nucleus, Drosha copies strands from a transposon gene or gene simply meant to produce RNA for silencing purposes (Lin, Sukasweang, Chuong, et al. 2001). Drosha creates priRNA strands copied from genes on the cells which fold over on themselves into what are called hairpin structures. They are exported from the nucleus where the same events involving Dicer and RISC take place. The hairpin structure is recognized as double stranded RNA, which activates Dicer. They are then diced by Dicer, loaded into RISC, and used as a guide to silence the genes the RNA matches.

Parts level

In *Drosophila*, the target gene for inactivation, the white gene (w), is a transporter-coding gene of the ATP-binding cassette super-family G member 2 (ABCG2) family (FlyBase 2020). The white gene is located on the X chromosome of *drosophila* at base pairs 2,790,599..2,796,466 and is just under 5,600 base pairs in length (FlyBase 2020). In order to have RNA-mediated

gene silencing on the w gene an RNA strand must be created that corresponds to the first 21-25 basepairs of the 5' end of the gene to ensure it is silenced before it has the chance to go through translation and becomes an amino acid chain. The first 27 nucleotides of the *D. melanogaster's* white gene mRNA are as follows:

5' AACUACAAU CCGGCGGAC UUUUACGUA 3'

In the laboratory, the *drosophila* eggs will be bathed in the dsRNA template solution in early embryogenesis to ensure RNAi is induced in the flies during development. The organism will intake the foreign RNA and utilize it in the Dicer pathway.

Safety

This project would not require any materials that are harmful to humans. However, as always, a cautious sterile procedure should be followed. The main safety concern from this project is what would happen if RNAi-induced flies escaped the lab. If it is the case that RNAi induced flies have the same pleiotropic effects observed in flies with a mutated white gene, then if the flies escaped the lab they would be at a significant disadvantage in the wild. *Drosophila* showing these pleiotropic effects would exhibit retinal degradation early on in life and respond more slowly to different environmental stressors such as oxygen deprivation (Ferreiro, Pérez, Marchesano, et al. 2018). Therefore, many of the flies would die off. If RNAi-induced flies have the same pleiotropic effects observed in flies with a mutated white gene, then the only difference between the escaped flies and wild flies would be that the escaped flies have white eyes, which is a naturally occurring mutation. There would also be potential legal concerns if any of the test organisms escaped the lab.

In either case, since RNAi is a mechanism for post-transcriptional gene silencing, the actual genome of the escaped flies would not have any man-made alterations. Therefore, any ensuing generations from the escaped flies would be identical to wild flies, having no sign of human interference.

Discussions

The goal of this project is to knock-down the white gene, which codes for the red eye pigment in *D. melanogaster*. However, there are some potential complications. While the gene knockout may be successful, it may be unknown if the expected pleiotropic effects would occur. There is potential for unknown pleiotropic effects, in which the RNAi affects the expression of other traits on the same gene. This shows the danger of pleiotropy, as there could be intention to silence one trait but instead multiple traits at once. This however would not be an issue if the targeted mRNA only translated to the protein for the target trait. For example, in this project, pleiotropy would not occur if the white gene mRNA only affected eye pigment. However, it has been proven that RNAi gene silencing for this white gene does demonstrate pleiotropic effects like difficulty in anaerobic environments or lower than average amines in the brain. Another potential issue is that the RNAi would prevent other mRNAs than the target from translation, which is similar to off target modification in genomic editing. Although this would be less likely to happen, potential remains. This would result in more genetic traits being silenced, and have similar negative effects to pleiotropy.

A challenge with this project was the effects of the COVID-19 outbreak on our ability to perform laboratory functions.This was originally planned to have been a Lab Report paper, yet it was changed to a Design Brief due to the newly found limitations. It would have been interesting for results to have been produced for this project. The procedure would have been educational on RNAi as well as on basic lab skills.

Acknowledgements

Thank you to BioTreks, Ms. Minutella, and Chloe Lohmeyer for making this paper possible!

This project was accomplished through participation in the BioBuilderClub, an after-school program organized by BioBuilder Educational Foundation. BioBuilderClub engages high school teams around the world to combine engineering approaches and scientific know-how to design/build/test their own project ideas using synthetic biology.

References

Clemens JC, Worby CA, Simonson-Leff N, Muda M, Maehama T, Hemmings BA, and Dixon JE use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. PNAS. 2000;97(12):6499–503.

Ferreiro MJ, Pérez C, Marchesano M, Ruiz S, Caputi A, Aguilera P, Barrio R and Cantera R. *Drosophila melanogaster* White Mutant w1118 undergo retinal degeneration. Frontiers in Neuroscience. 4 January 2018;11. doi: 10.3389/fnins.2017.00732

FlyBase. FlyBase gene report: DmeI\w [Internet]. FlyBase;2020[cited 2020 April]. Available from: https://flybase.org/reports/FBgn0003996

Hersh BM. More than meets the eye: a primer for "timing of locomotor recovery from Anoxia modulated by the white Gene in *Drosophila melanogaster*". Genetics. 2016;204(4): 1369–75.

Kao LR and Megraw TL. RNAi in cultured *Drosophila* cells. Methods Mol Biol. 2004;247:443-57.

Lin SL, Sukasweang S, Chuong CM, Rasheed S and Ying SY. D-RNAi (messenger RNA-antisense DNA interference) as a novel defense system against cancer and viral infections. Curr Cancer Drug Targets. 2001 Nov;1(3):241-7. Erratum in: Curr Cancer Drug Targets. 2003 Jun;3(3):237.

Pratt AJ and MacRae IJ. The RNA-induced silencing complex: a versatile gene-silencing machine. J Biol Chem. 2009 Jul 3;284(27):17897-901.

Sivakumaran S, Agakov F, Theodoratou E, Prendergast JG, Zgaga L, Manolio T, Rudan I, McKeigue P, Wilson JF and Campbell H. Abundant pleiotropy in human complex diseases and traits. Am J Hum Genet. 2011 Nov 11;89(5):607-18. doi: 10.1016/j.ajhg.2011.10.004

Xiao C and Robertson RM. Timing of locomotor recovery from anoxia modulated by the white gene in *Drosophila*. Genetics. 2016;203:787–97.