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Allelic drive: a strategy to correct disease-causing mutations modeled in Drosophila melanogaster

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Allelic drive: a strategy to correct disease-causing mutations modeled in *Drosophila melanogaster*

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

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2020

The Thesis of Brian Valadez is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California San Diego

2020

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ABSTRACT OF THE THESIS

Allelic drive: a strategy to correct disease-causing mutations modeled in *Drosophila melanogaster*

by

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Master of Science in Biology

University of California San Diego, 2020

Professor Ethan Bier, Chair
Professor Justin Meyer, Co-Chair

Allelic drive is a new gene drive technology that allows for the selective cleavage of detrimental alleles at a locus that is separate from the inserted genetic element. This new technology has the potential to drive intact desired alleles in populations, however it is unknown if this strategy can also be used to fix mutations in somatic tissues to restore partial or complete function of mutant alleles. We have defined and created a combination of mutant alleles in the Notch gene of Drosophila Melanogaster amenable to repair using allelic replacement: a Cas9-based strategy, in which selective cleavage of a mutant allele is followed by repair using the wild-type homologous chromosome. In addition, we have identified other phenotypes (shorter lifespan and intestinal stem cell phenotypes) in mutant Drosophila that may be alleviated by a restoration

of gene function. Additionally, we have been able to create a line of Drosophila expressing a guide RNA that can be used in allelic drive to selectively remove a mutated allele. Finally, background experiments have been conducted and data collected in preparation for experiments aimed to measure reduction of known mutant phenotypes in the presence of an allele-specific guide RNA and a Cas9 nuclease. If allelic drive is shown to be successful in restoring Notch gene function, these concepts have the potential to be transferred to more complex animals and even be used to treat human genetic disorders.

INTRODUCTION

In traditional Mendelian genetics, each allele of a heterozygous animal is passed on to its offspring at rates of approximately 50%[1]. The development of gene drive technology has allowed for the creation of transgenic elements that are able to pass themselves on at rates far greater than the traditional 50%[2] . Gene drives are genetic elements that can increase their rate of transmission by either copying themselves to the opposite chromosome or by decreasing the viability of wild-type gametes[2]. A new method of creating gene drives known as Active Genetics was devised in the Bier lab at UC San Diego[1]. Active Genetics uses a CRISPR-Cas9-based genome editing technique to promote transmission of genetic elements at much greater frequencies than the mendelian 50% rate[1]. With the creation of Active Genetics, gene drive technology can be used to spread a gene cassette with potential benefits throughout a population. For example, it has been proposed that a gene drive element could be created in mosquito species, that would prevent transmission of malarial parasites to humans[1]. This shows that Active Genetics is a technology that has the potential to make a positive impact on the world.

In Active Genetic strategies, a genetic cassette is inserted into the genome, encoding a guide RNA (gRNA) and the gene for the Cas9 endonuclease: both of these elements are required for self-replication[1]. The gRNA directs the Cas9 nuclease to cut the homologous chromosome at the site of insertion. The cellular machinery then repairs the double-strand break (DSB) by copying the transgenic cassette at the cut site the insertional cassette, which becomes homozygous[1]. When this process occurs in the germline, these genetic elements are transmitted to the next generation at much higher frequencies than the 50% Mendelian rate (~90%-100%)[1]. This occurs when an individual bearing an active genetic element breeds with a wild type individual, because the active

genetic element will induce cutting of the wild type allele in the germline, resulting in most of the offspring carrying the gene cassette[1].

The concepts of active genetics were then used in 2019 to develop a new technology known as allelic drive[3]. In allelic drive, an active genetic approach is used to alter one or more base pairs at a locus in a separate gene than the inserted genetic element[3]. This could then allow for a desired allele to be propagated, or "driven", at the expense of another (possibly detrimental) allele. Allelic drive differs from active genetics by the introduction of a second gRNA that targets and cuts an undesired allele of a gene at a separate location from the inserted genetic element[3]. The uncut allele can then be used as a template for DNA repair, allowing for that allele to be driven in the population[3]. The development of this technology was demonstrated when a gene drive cassette was created to target the *Notch* gene of *Drosophila melanogaster*[3]. This cassette contained the information to drive itself though a population as well as the gRNA required for Cas9 to cut a specific allele on a different gene[3]. A viable allele of Notch (Ax16) that was selected for this study, which consists of a 1 nucleotide substitution AAG instead of AGG from a wild type sequence[3]. This gRNA used for this study was designed to specifically cut the wild type allele, but not the Ax16 allele[3]. After cleavage of the sensitive allele, the cut resistant allele on the homologous chromosome was used as a template for DNA repair and could be driven in the population[3]. Knowledge of this new allelic drive technology is significant because it opens the door to new possible genetic experiments, such as, repairing mutated alleles in somatic cells using the homologous chromosome as a template. Initial experiments indicated that this process also takes place in somatic cells, albeit less frequently, leading us to believe that other mutations in various genes may also be repaired using this technology.

In humans, the *Notch* signaling pathway consists of an important group of four receptors and five ligands that are critical during in development and cell differentiation[4]. The four *Notch* receptors are activated by Delta and Serrate Ligands expressed in neighboring cells[7]. When bound by a ligand, the *Notch* receptor is cleaved in its transmembrane domain and releases its intracellular domain which migrates to the nucleus to stimulate expression of downstream genes involved in different cell proliferation and differentiation events [5,7]. Many mutations affecting one of the four *Notch* genes inhibit ligand binding, thus interrupting Notch signaling and preventing proper cell differentiation[5]. In humans, *Notch* mutations can lead to a variety of developmental disorders, such as Bicuspid aortic valve disease, Hypoplastic left heart syndrome, Adams-Oliver syndrome, Hajdu-Cheney syndrome, Alagille syndrome, and CADASIL syndrome[4].

Notch's function in development and cell differentiation is highly conserved in a wide variety of species. *Drosophila melanogaster* has only one copy of the gene, which simplifies many studies by avoiding functional redundancy[6]. In *Drosophila*, *Notch* mutations have been shown to cause a variety of visible defects, such as notches in the wing margins[6]. Additionally, *Notch* has also been to play an important role in homeostasis of the intestinal system[7]. In wild-type individuals, *Notch* is important for a process called lateral inhibition operating during differentiation of intestinal stem cells into enterocytes[7]. When Notch signaling is reduced, both daughter cells derived from intestinal stem cells (ISC) remain ISC, instead of one daughter becoming an enteroblast and one remaining an ISC, causing a buildup of intestinal stem cells[7]. Homozygous *Notch* mutations and many allelic combinations of mutations in this gene have been shown to be lethal in *Drosophila*, emphasizing the importance of the function of this gene[8].

It is currently unknown if an approach similar to allelic drive could be used to restore function of a mutated gene in the cells of an adult organism. The purpose of my research is to

design an allelic drive cassette targeted to a mutant allele in the *Notch* gene, and test if it can restore function of the mutated *Notch* gene using the homologous chromosome as a template, in developing or adult *Drosophila*. I will assess the success of this strategy at several levels: restoration of normal life span, differentiation of gut epithelial cells and wild type wing phenotypes. If successful, this would then indicate that this technology has the potential to be used to repair heterozygous mutations in more complex organisms including humans, while also opening the door for such allelic replacement to be used in gene therapy, as a treatment for a wide variety of heterozygous autosomal dominant genetic disorders.

RESULTS

Characterization of Notch- alleles amenable to allelic correction

The purpose of these experiments is to characterize the phenotypes of various Notch allelic combinations in Drosophila melanogaster. We are looking in particular for two alleles that together cause a strong yet viable phenotype, that could be amenable to repair by allelic replacement strategy, leading to a visible phenotypical rescue. Known loss of function alleles of Notch cause a dominant wing phenotype, but do not have any intestinal phenotype. Our goal is to identify an allelic combination that would cause an intestinal phenotype in viable adults. The sequence of the candidate mutations should be such that it can be specifically targeted by a guide RNA (gRNA), while its wild-type counterpart should not be targeted by this gRNA. In order to create a strong loss-of-function allelic combination of Notch with phenotypes potentially amenable to visible correction, we tested various Notch mutant alleles we had created in a previous study, in combination with two weak Notch alleles (N(nd-1) and N(nd-3)), which have the unique property of being viable in males.

Mutant Allele 1	Mutant Allele 2	Viability	Phenotype Strength
N^{-17}	N(nd-3)	Lethal	N/A
N^{-17}	N^{-11}	Lethal	N/A
N^{-17}	N^{-12}	Lethal	N/A
N^{-17}	N^{-37}	Lethal	N/A
N^{-17}	N(nd-1)	Viable	Strong
N^{-32}	N^{-11}	Lethal	N/A
N^{-32}	N^{-12}	Lethal	N/A
N^{-11}	N^{-24}	Lethal	N/A
N^{-12}	N^{-24}	Lethal	N/A
N^{-37}	N^{-24}	Lethal	N/A
N(nd-3)	N(nd-1)	Viable	Mild

Figure (1). Allelic combinations of Notch This table shows various allelic combinations that were created to find a candidate for allelic replacement. The table shows the viability and strength of visible wing phenotype of each created allelic combination. The first mutant allele was provided from the mother of the Drosophila while the second allele was provided from the father.

Only two allelic combinations, N(nd-3)/N(nd-1) and N^{-17}/N(nd-1), were viable in adults. Initial observations showed the N^{-17}/N(nd-1) mutant combination to have a much stronger wing phenotype than either of these mutant alleles alone. The N(nd-1)/N(nd-3) individuals had a wing phenotype similar to what was found in individuals that were homozygous for either mutant allele. For our next experiments, we chose the two mutant alleles N^{-17} and N(nd-1) because they showed the strongest viable wing phenotype, while each allele alone caused a weak or moderate phenotype. This feature is important, as correction of either allele should result in a clear phenotypic rescue. The N^{-17} allele is a homozygous lethal mutation caused by a single nucleotide deletion in the 6th exon of the Notch gene, resulting in a frameshift of the region coding of the extracellular domain of *Notch.* The N(nd-1) mutation is a homozygous-viable 41-nucleotide deletion on the 8th exon of *Notch*, a region encoding for the cytoplasmic domain of the protein[9]. Once this allelic combination was defined, experiments were conducted to document the wing phenotype of the mutations when expressed alone and when expressed together.

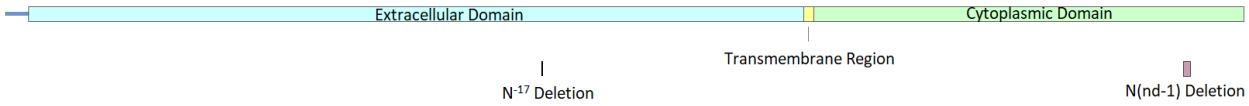

Figure (2). Notch mutations of interest The N^{-17} mutation is a single nucleotide deletion in the extracellular domain of the Drosophila Notch gene. The N(nd-1) mutation is a 41 nucleotide deletion present approximately 4.3 Kb upstream of N^{-17}, in the topological domain of the gene.

Defining wing phenotype

The N(nd-1) homozygote virgin was crossed to a wild type male in order to produce offspring that were heterozygous for the mutation. The same was done with virgins containing the N^{-17} mutation in order to produce heterozygous offspring. The wings of these offspring were compared to the wings of wild type individuals and drosophila that were homozygous for the N(nd-1) mutation. We were able to observe that flies that had only one copy of the mutant N(nd-1) allele had a wing phenotype that was similar to wild type individuals, suggesting that N(nd1) is a weak

mutation. In N(nd-1) homozygous flies, wings present a mild thickening of the 3rd wing vein and a notch is seen on the tips of the wings. Flies that were heterozygous of N^{-17} and wild type forms of Notch had a stronger phenotype than the N(nd-1) homozygote with a large more noticeable notch on the wing tips. Since N(nd-1) had a weak phenotype alone and N^{-17} had a strong phenotype alone, the two mutant flies were crossed to produce offspring that had both mutations. The wings of double mutants had noticeably thicker wing veins, larger notches and tended to be more shriveled than the wings of other observed Drosophila genotypes. Thus, the N^{-17}/N(nd-1) genotype represents an outstanding strong loss-of-function viable combination, with an easily identifiable wing phenotype that could be used to detect rescue by allelic correction. Once these wing phenotypes were defined, experiments were conducted to measure any differences in gut phenotype and lifespan between wild type and N^{-17}/N(nd-1) individuals.

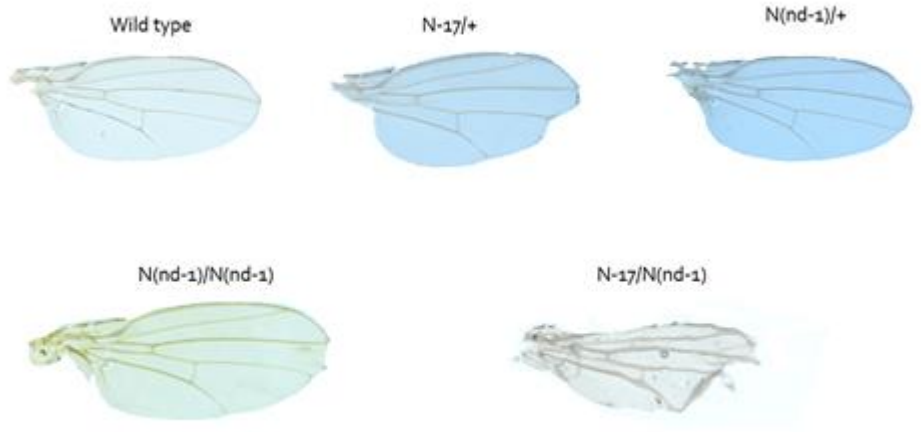

Figure (3). Wing Phenotype of Wild type and Notch Mutant Drosophila Genetic crosses were set up to create five allelic combinations of Notch mutant and wild type alleles. The wings of flies of each genotype were observed and mounted on a microscope slide for imaging.

Lifespan analysis

To measure differences in lifespan, genetic crosses were set up to produce ~30 progenies of the four allelic combinations of *Notch* mutant and wild type alleles in *Drosophila*. The allelic combinations of interest were wild type (+/+), single mutant N^{-17}/+, single mutant N(nd-1)/+, and

double mutant N^{-17}/N(nd-1). Two weeks after the crosses were set up, 30 adult female offspring were collected of each genotype. The N^{-17}/N(nd-1) individuals seemed to have lower viability, and only 23 adult individuals could be collected. Fly viability was followed for 21 days and counts were taken on the number of surviving individuals of each genotype at various time points.

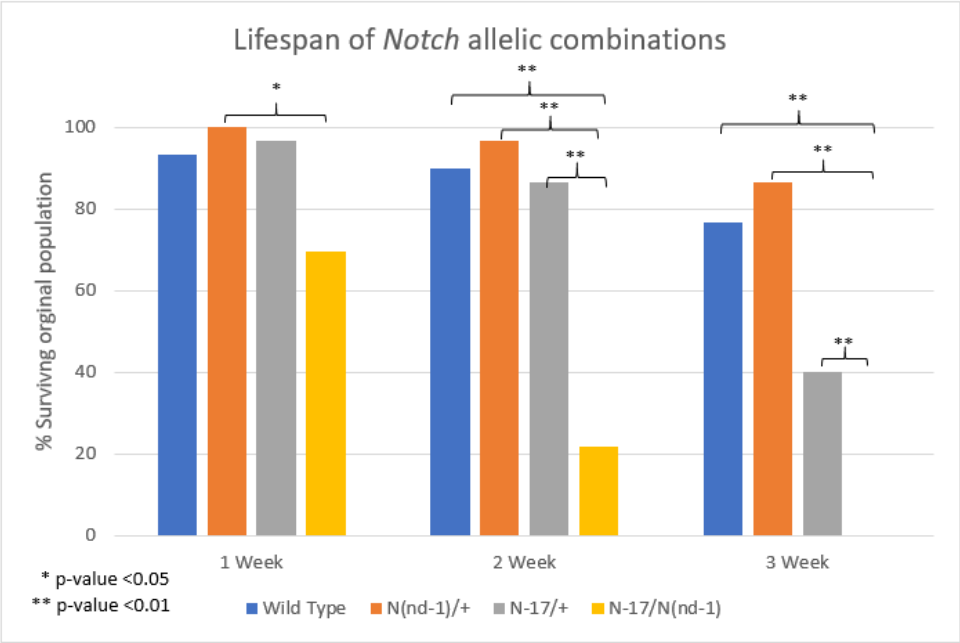

Figure (4a). Death curve Histogram of Drosophila expressing various allelic combinations of Notch. Genetic crosses were set up to create four allelic combinations of Notch mutant and wild type alleles. After 10 days, offspring of these crosses were collected and were observed 21 days. After one week the N^{-17}/N(nd-1) double mutant had significantly fewer surviving individuals when compared to N(nd-1)/+. After two weeks the double mutant had significantly fewer surviving individuals when compared to all other studied allelic combinations.

After two weeks, N^{-17}/N(nd-1) was found to be significantly less viable than all other tested allelic combinations. In contrast, by the end of the study, there was no significant difference in viability between any of the other tested allelic combinations. These results are consistent with the N(nd-1) mutation to be a weak loss-of-function allele and the N^{-17} being a mild loss-of-function allele. This data also supports the idea that N^{-17}/N(nd-1) combination leads to a significantly stronger loss-of-function of phenotype than when the alleles are expressed alone.

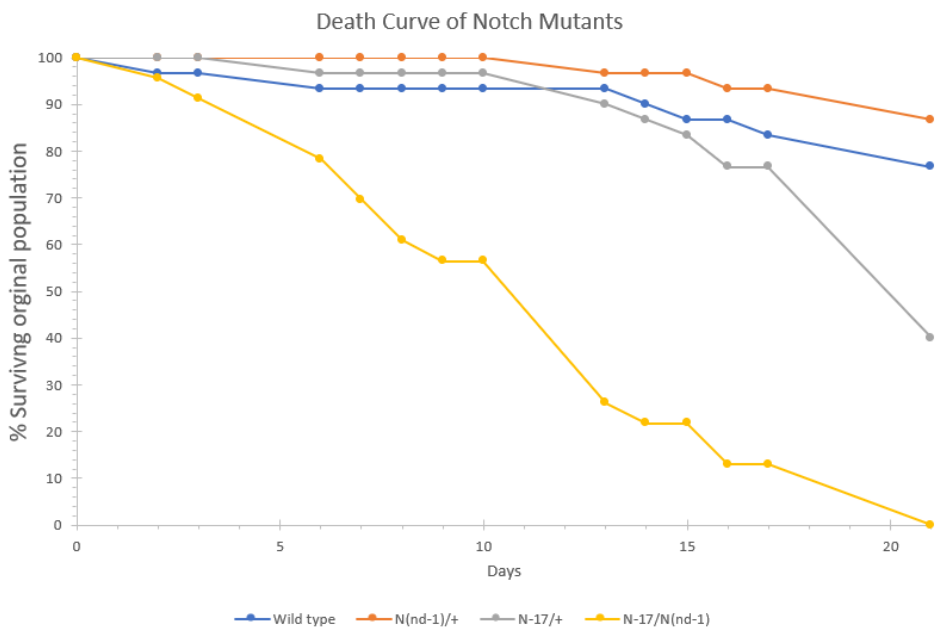

Figure (4b). Death curve of Drosophila expressing various allelic combinations of Notch. 30 adult female flies (of approximately the same age) from each of the four genotypes were collected, however there were only 23 living adults with the N^{-17}/N(nd-1) allelic combination. The flies were then observed for the next 21 days with counts of living flies of each genotype being taken at various time points. This graph shows the percent of remaining individuals of each genotype at various time points.

Intestinal phenotype analysis

In addition to analyzing wing and lifespan phenotypes, we were interested in examining the mid-gut in adult animals of the strongest loss-of-function Notch allelic combination. The gut is an ideal tissue for testing allelic correction, because it is similar in many ways to the human gut10. Both Drosophila and human adult guts have intestinal stem cells (ISC) that can differentiate in to secretory enteroendocrine cells or enterocytes that are important for nutrient absorbtion10. Also, the gut epithelium undergoes constant renewal during adult life, though regular division of intestinal Stem Cells10. In the gut epithelium, the Notch pathway plays a critical role in regulating the asymmetrical division of ISC.7 Such cells can be fluorescently marked using a double transgenic insertion esgGal4; UAS-GFP, in which expression of the GAL4 trans-activator under the control of the escargot regulatory sequences drives expression of GFP. Escargot is a protein expressed in intestinal stem cells that controls differentiation7. In wild type animals, this reporter construct reveals the distribution of ISC: a regular array of single triangular GFP expressing cells,

which are smaller than surrounding enteroblasts and enterocytes. A second marker for Notch function in the midgut is Prospero (Pros), which is specifically expressed in the nucleus of entero-endocrine cells, distributed in a regular array of single Prospero expressing cells.7 Crosses were conducted to create wild type offspring and N^{-17}/N(nd-1) offspring expressing GFP under the control of the esg promoter. Guts from adult females carrying the esgGAL4>UAS-GFP reporter combined with either two wild type alleles of *Notch* or the N^{-17} and N(nd-1) loss of function alleles identified earlier were fixed and stained using an anti-Prospero antibody. A Dapi stain was also added to visualize the nucleus of all intestinal cells by binding to DNA.[7]

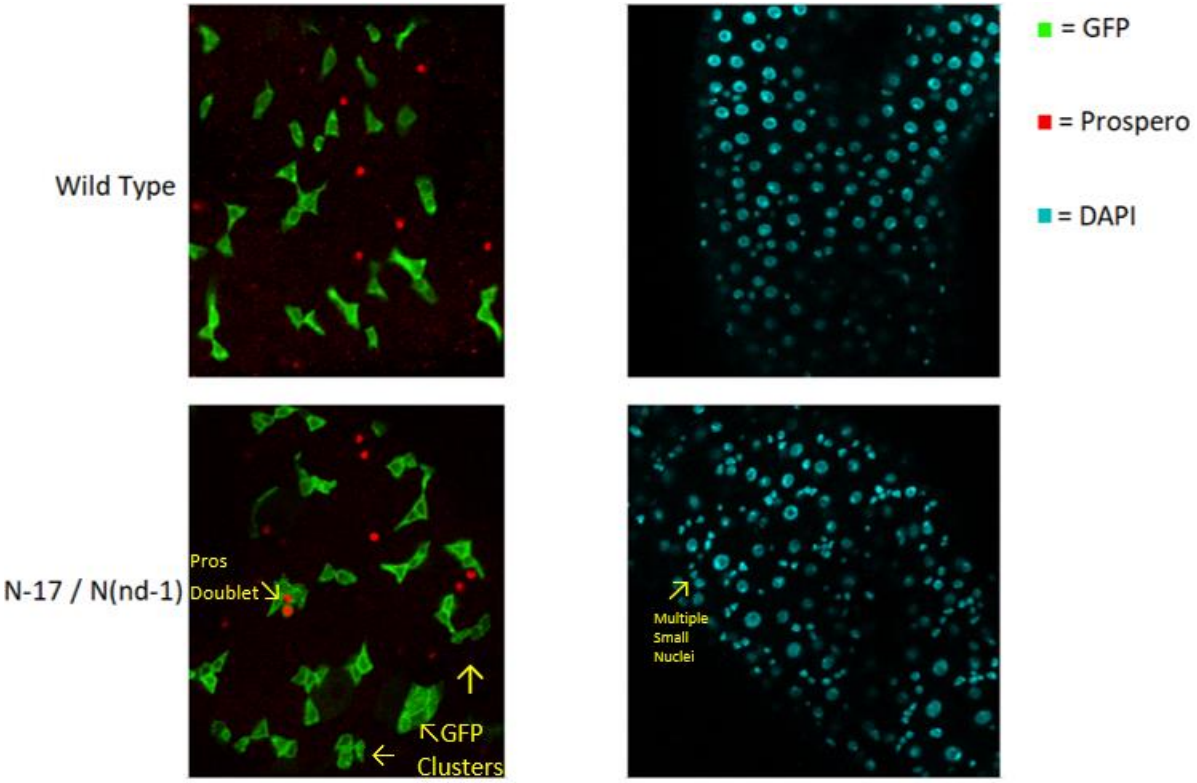

Figure (5). Gut Phenotype of wild type and Notch double mutant Drosophila Intestines from adult Drosophila Melanogaster expressing an esg-GFP protein were collected and stained for GFP, Prospero and DAPI. These images were taken using confocal microscopy. GFP is expressed in stem cells, Prospero is expressed in entero-endocrine cells and differentiating cells, DAPI shows the nucleus of each cell. GFP expression is seen in green, Prospero expression is seen in red and the DAPI stain is seen in blue.

In the gut of N^{-17}/N(nd-1) females, both the GFP and Pros markers showed abnormal distribution compared to wild type *Notch* individuals. ISC (seen by GFP expression) appeared in clusters of two to six cells and were often larger than the ISCs seen in wild type animals. Similarly,

Pros+ cells appeared in doublets, instead of the isolated cell pattern seen in wild type *Notch* guts. Some cells also show both GFP and Pros stains, which is never observed in wild type guts. In addition, the DAPI stain reveals a larger portion of small nuclei, which are characteristic of ISC. Altogether, these phenotypes are consistent with the severe *Notch*- phenotypes observed in wings of N^{-17}/N(nd-1) animals. Wing, lifespan, and gut phenotypes could be used in the future for allelic correction studies in the developing wing, adult gut epithelium, and how ultimately such correction may extend the lifespan in animals carrying a mutation.

Generation of transgenic constructs for allelic correction

Once a desirable allelic combination was identified and its corresponding phenotypes defined, a guide RNA was designed to develop a transgenic construct that could selectively target the N^{-17} mutation for cleavage by Cas9. As the N^{-17} deletion is located 2 base pairs away from an AGG protospacer adjacent motif (PAM) sequence, a gRNA specific for N^{-17} could be designed. The PAM sequence is necessary for the Cas9 nuclease to bind DNA sequence of interest[1]. It is desirable to have our mutation of interest be at or near a PAM site because cleavage occurs three nucleotides upstream from the PAM site. The gRNA also includes a 17-nucleotide sequence that ensures binding to the correct location and minimizes off target cleavage. A Gibson reaction was utilized to introduce our gRNA into a plasmid containing red florescent marker and gRNA for the Drosophila yellow gene. This new construct, named yccN^{-17}, was injected into Drosophila embryos to create transgenic animals expressing a gRNA specific for the N^{-17} allele, that could be identified by the expression of the DsRed florescence marker in adult eyes.

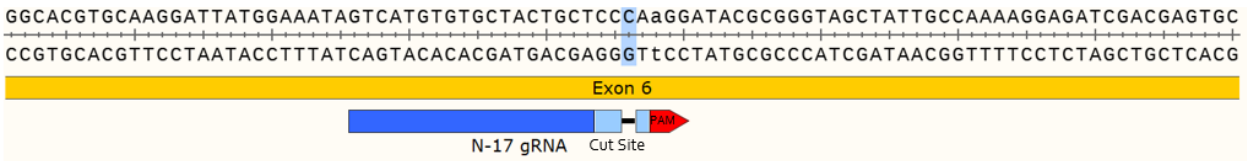

Figure (6). **N^{-17} Guide RNA design** The DNA sequence near the N^{-17} deletion in Notch is shown. The deleted nucleotide is shown highlighted in light blue. Below the sequence, the segments that make up our designed gRNA are labeled.

DISCUSSION

The purpose of our research was to create a Notch mutant combination in Drosophila melanogaster for testing if allelic drive in somatic cells could be used to repair this mutation and alleviate some of the deleterious phenotypes associated with the mutation. Our results show that the N^{-17} and N(nd-1) mutant alleles of Notch, when combined in a trans-heterozygote animal, cause a very severe yet viable loss-of-function phenotype. This is, to our knowledge, the most severe viable allelic combination of Notch. Our N^{-17}/N(nd-1) mutant combination also displays large clumps of esg>GFP+ intestinal stem cells, instead of the normal pattern of single esg>GFP+ cells, suggesting that the reduced function of Notch is preventing proper cell differentiation by favoring the ISC fate over EB fate . Additionally, we have characterized the wing phenotypes of our mutant flies, which could be used as a visible outcome for allelic repair during development. Flies carrying the N^{-17}/N(nd-1) double mutation also have a shortened lifespan. This feature may also be used as a test for allelic repair, which in principle could extend lifespan of these mutant animals. It will be of particular interest to test whether expression of CRISPR/gRNA components can halt the premature death of aging animals, or if allelic correction only improves lifespan when induced at early stages of life. The purpose of creating flies with mutations in the *Notch* gene was to use them as a model for disorders involving Notch signaling deregulation and attempt to alleviate some of the negative outcomes associated with this disorder by using allelic drive technology. We identified the severe N^{-17}/N(nd-1) allelic combination in *Drosophila*, which could be used for a proof of concept study for somatic allelic correction.

With these characterized phenotypes and the guide RNA targeting the N^{-17} allele, we anticipate that experiments designed to test allelic drive for restoring full or partial function of a gene may demonstrate decline of the phenotypes associated with a targeted mutation. We hope

that allelic drive might be able to accomplish this because it has been shown to be able to successfully replace undesired alleles on one chromosome with the other allele of the same locus on the opposite chromosome[3] in germline cells. However, this process is not as well characterized in somatic cells. In principle, the Cas9 endonuclease should be able to selectively cleave the N^{-17} mutant allele and the cell's natural DNA repair machinery should be able to use the intact wild type allele as a template for DNA repair. Nonetheless, it is possible that our allelic replacement treatment will not significantly change the phenotypes that are seen in our N^{-17}/N(nd-1) double mutant. When the mutant allele is cleaved, non-homologous end joining may occur in the cell in attempts to fix the broken DNA strand, which will result in de novo *Notch*- alleles. If this occurs at a high frequency, there may not be enough restoration of function of the *Notch* gene to significantly change the intestinal and lifespan phenotypes that were seen in the N^{-17}/N(nd-1) double mutant.

Future experiments must be conducted in order to test if using allelic replacement strategy via allelic drive will be successful in restoring partial function of the Notch gene. Cross schemes will be conducted to create flies carrying both the N^{-17} and N(nd-1) alleles, combined with the yccN^{-17} insertion expressing the guide RNA targeting the N^{-17} mutation, and a source of Cas9. Different sources of Cas9 could be used, to express Cas9 either constitutively in all cells at various levels (e. g. hsGAL4>UAS-Cas9, actinGAL4>UAS-Cas9), or specifically in intestinal stem cells (e.g. esgGAL4>UAS-Cas9). Also, different Cas9 variants could be tested, such as the Cas9D10A or the Cas9H840A nickases. In animals of these various genotypes, we will first examine wing phenotypes, and sequence the site of the N^{-17} mutation and evaluate whether allelic correction is visible as a shift in favor of the wild type sequence in electrophoretograms. If such phenotypes and sequencing prove promising, a death curve experiment may be conducted to compare the viability

of the yccN^{-17}; N^{-17}/ N(nd-1) genotype in presence or absence of Cas9. This will allow us to assess whether potential allelic correction can ameliorate lifespan. If the experiment is successful, we would expect the death curve to be more similar to the N(nd-1) single mutant than to the N^{-17}/N(nd-1) double mutant. Finally, allelic correction may be tested in the midgut, using the esgGAL4>UAS-GFP ISC marker. Flies from the yccN^{-17}; N-^{17}/ N(nd-1) +/-Cas9 genotypes will be dissected, and their fixed intestines will be stained and imaged. These experiments should help analyze the phenotypical outcomes of allelic replacement in an organ undergoing regular cell divisions. Such phenotypes could be quantified and compared using image analysis. These experiments should help provide a foundation to assess the feasibility and success of allelic replacement as a possible strategy to correct autosomal dominant mutations.

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MATERIALS AND METHODS

Wing phenotype analysis:

11 separate genetic crosses were performed in order to analyze the wing phenotype of *Drosophila melanogaster* carrying different *Notch* mutations. Wild type females were crossed to wild types males to produce wild type offspring that could be used for comparisons. Virgin females carrying a *Notch* mutant allele were crossed to males carrying a different mutant *Notch* in order to generate offspring expressing two different mutant *Notch* alleles. Females expressing the N^{-17} mutant allele and females expressing the N(nd-1) allele were crossed to wild type males to produce heterozygous individuals. The viable offspring of these crosses were allowed to mature to adulthood before their wings were plucked. These wings were then mounted on separate microscope slides in Canada Balsam.

Death Curve:

Four separate genetic crosses were created in order to study the lifespan of *Drosophila melanogaster* carrying the *Notch* alleles of interest. Wild type females were crossed to wild types males to produce wild type offspring that could be used for comparisons. Virgin females containing the N^{-17} mutant allele were crossed to wild type males in order to generate offspring with one copy of the N^{-17} mutant allele and one copy of the wild type allele. Virgin N^{-17} females were also cross males that contained the N(nd-1) mutations in order to produce female offspring that contained both mutant alleles. N(nd-1) homozygous virgin females were also crossed to wild type males in order to create offspring that only contained one copy of the mutant allele. The percentage of surviving individuals of each allelic combination was recorded at various time points over a three-week period. These data points were organized by allelic combination and by what

week the data points were collected. The data from each week was analyzed using a one-way ANOVA with post-hoc Tukey HSD Test using a significance threshold of p=0.05.

Gut Imaging:

Drosophila were generated expressing an Escargot-GFP reporter alongside the N^{-17}/N(nd-1) double mutation. Virgin Females that were homozygous for the N(nd-1) mutation were crossed to males that contained Escargot-GFP to create offspring that were heterozygous for Escargot-GFP containing the N^{-17} mutant allele. The N(nd-1) containing males from this cross were collected and crossed to virgin females containing the N^{-17} mutation. Using the previously characterized wing phenotype we were able to collect offspring of the cross that contained both the N(nd-1) and N^{-17} mutant alleles. Based on concepts of traditional mendelian genetics it was assumed that approximately half of these offspring were also heterozygous for the Escargot-GFP. While this cross was taking place, a separate cross between virgin wild type females and male Escargot-GFP males was taking place to create wild type offspring expressing Escargot-GFP. Once the wild type Escargot-GFP and N^{-17}/N(nd-1) Escargot-GFP *Drosophila* reached adulthood, their intestines were dissected. These intestines were then fixed using a 4% formaldehyde solution before being stained for GFP, Prospero and DAPI in an immunofluorescence experiment. The stained intestines were then mounted on a microscope slide and observed using confocal microscopy.

Construction of the yccN^{-17} insertional element:

A guide RNA (gRNA) was designed to selectively target the N^{-17} allele, and its sequence was inserted into the yccN^{-17} transgene. The guide RNA was comprised of, a 20-nucleotide guiding sequence matching the N^{-17} allele, but not the N+ wild type allele at the same site. Once this gRNA was designed, a Gibson assembly procedure was conducted to create a plasmid containing a red florescent reporter gene and our N^{-17} gRNA in the *Drosophila* Yellow gene. PCR reactions were

conducted to amplify the plasmid as three separate segments. Primers were designed to contain our designed gRNA as well as overhangs that would allow to be inserted into a previously designed plasmid that contained promoters for the *Drosophila Yellow* gene a, DsRed florescence protein, and a gRNA targeting *Yellow*. A PCR reaction was conducted using our designed primers to introduce the gRNA under the control of the *Yellow* gene promoter. A Gibson assembly reaction was used to combine the PCR fragments in to one plasmid that now contains our gRNA. This yccN^{-17} construct was injected in *Drosophila* embryos. The *Yellow* gRNA is used to promote the copying of the yccN^{-17} insertion and the DsRed marker is used for convenient selection of *Drosophila* that are expressing our construct. These flies were used to create a stable genetic line expressing our gRNA.