CRISPR/Cas9 induced mutations of the white gene of haplo-X and diplo-X *Drosophila melanogaster.*

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Genome editing is the process of changing the DNA structure of a gene by deleting or replacing nucleotides, or by replacing an old gene with a new DNA sequence (Cox *et al*., 2015). The CRISPR/Cas9 bacterial system has revolutionized the genome editing process (Haimovich *et al*., 2015; Govindan and Ramalingam, 2016). This genome editing system consists of a CRISPR guide RNA that locates the gene of interest and a Cas9 endonuclease that cleaves the targeted DNA to form a double-strand break. Mistakes in DNA repair of this breakage often lead to base deletions or insertions. This technique can be used in any organism where the sequence of a gene of interest is known. For example, CRISPR/Cas9 has been used to eliminate the HIV virus in human cells (Kaminski *et al*., 2016), knock out genes involved in aging (Harel *et al*., 2015), correct disease-causing mutations (Wu *et al*., 2013; Yin *et al*., 2014; Nelson *et al*., 2016), inactivate pig retroviruses in human cells (Yang *et al*., 2015), drive genes and populations of *Drosophila* and mosquitoes to extinction (Gantz and Bier, 2015; Hammond *et al*., 2016), and modify the gene that causes mushrooms to brown (Waltz, 2016).

Experimental Plan

In this study two *Drosophila melanogaster* stocks were used to induce mutations in the white gene using the CRISPR/Cas9 system; mutations caused a change of brown or red eyes to white eyes. One stock (GRN-0006) has the CRISPR RNA guide sequence for the white gene, while the second stock (CAS-0001) contains the Cas9 endonuclease. These two stocks, received from the Genetic Strains Research Center, National Institute of Genetics, Japan, were first crossed as follows in Cross Scheme One. In these crosses, y^1 or y^2 = yellow body color, y^+ = grey body color, *f* = forked bristles, cho^2 v^1 = brown eyes, CyO/+ = curly wings, CyO/CyO flies die as early embryos, C(1)DX = two X chromosomes joined at their centromeres, attP40{U6.2-w-ex6-1, y^+} = CRISPR, and attP40{nos-Cas9} = Cas9 (Lindsley and Zimm, 1992; Kondo and Ueda, 2013). Note that the G2 males in this cross contain the full CRISPR/Cas9 system, with the white-gene guide RNA and Cas9 together on the second chromosomes.

Cross Scheme One

G1 GRN-0006 (y^2 cho^2 v^1; attP40{U6.2-w-ex6-1, y^+})/CyO) CRISPR females

×

CAS-0001 (y^2 cho^2 v^1 / Y; attP40{nos-Cas9})/CyO) Cas9 males

↓

G2 GRN-0006 (y^2 cho^2 v^1 / Y; attP40{U6.2-w-ex6-1, y^+})/CAS-0001 (attP40{nos-Cas9}) males (not CyO)

×

C(1)DX, *y f* / Y females

↓

Score G3 males for brown or white eyes (new mutations at the white locus).

As controls, GRN-0006/CyO females were mated to wild-type (Canton-S) males, and CAS-0001/CyO males were mated to wild-type (Canton-S) females. Then G2 males from each cross that did not have curly wings were mated with C(1)DX, *y f* / Y females, and G3 males were scored for red or white eye colors. Neither control cross should exhibit mutations of the white gene, showing that CRISPR and Cas9 must be together in the same fly to induce mutations.

To test the efficiency of the CRISPR/Cas9 system, we also determined if mutations could be induced at both white genes in wild-type females with attached-X chromosomes, using the following Cross Scheme Two.

Cross Scheme Two

G1 C(1)DX, *y f* / Y females

×

GRN-0006 (y^2 cho^2 v^1; attP40{U6.2-w-ex6-1, y^+})/CyO males

↓

G2 C(1)DX, *y f* / Y; GRN-0006 (y^2 cho^2 v^1; attP40{U6.2-w-ex6-1, y^+})/ + females (not Cy)

×

CAS-0001 (y^2 cho^2 v^1 / Y; attP40{nos-Cas9})/CyO males

↓

G3 C(1)DX, *y f* / Y; GRN-0006 (attP40{U6.2-w-ex6-1, y^+})/CAS-0001 (attP40{nos-Cas9}) females (y+ and not Cy)

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×

Sibling males

↓

The G4 females were scored for white eyes, caused by mutations in both of the white loci of the C(1)DX, *y f* / Y G3 female gametes and for red eyed females, which will have either no mutations at the white loci, or mutations in just one of the two white loci in G3 female gametes (since white mutations are recessive). As a positive control, G4 white-eyed males should be recovered as expected, because some sibling G3 males from this cross will contain both CRISPR and Cas9.

Anticipated Results

Based on results of preliminary crosses of GRN-0006 and CAS-0001stocks, we expect up to 90 percent of G3 males in Cross Scheme One to have white eyes due to CRISPR/Cas9 induced mutations in the white gene (Kondo and Ueda, 2013). Although we do not know the percentage of the G4 C(1)DX, *y f* / Y females in the Cross Scheme Two that will have white eyes, they should occur at a lower frequency than in males of Cross Scheme One, which only have one copy of the X-linked white gene. We also expect no mutants will be recovered in the two control crosses, as CRISPR and Cas9 together are required to induce mutations in the targeted gene.

Results and Discussion

Controls: In 20 crosses where chromosome two contained CRISPR only (CRISPR/+) or Cas9 only (Cas9/+), no white-eyed mutant males were observed among 639 red-eyed males (Table 1).

Table 1. Number of white-eyed mutant males and red-eye, non-mutant, males containing either CRISPR (CRISPR/+) or Cas9 (Cas9/+), but not both.

Vial number	Red eyed males	White eyed males	Total # of males
1	54	0	54
2	25	0	25
3	27	0	27
4	27	0	27
5	30	0	30
6	22	0	22
7	34	0	34
8	31	0	31
9	33	0	33
10	19	0	19
11	50	0	50
12	35	0	35
13	54	0	54
14	43	0	43
15	21	0	21
16	21	0	21
17	39	0	39
18	20	0	20
19	38	0	38
20	16	0	16
Totals	639	0	639

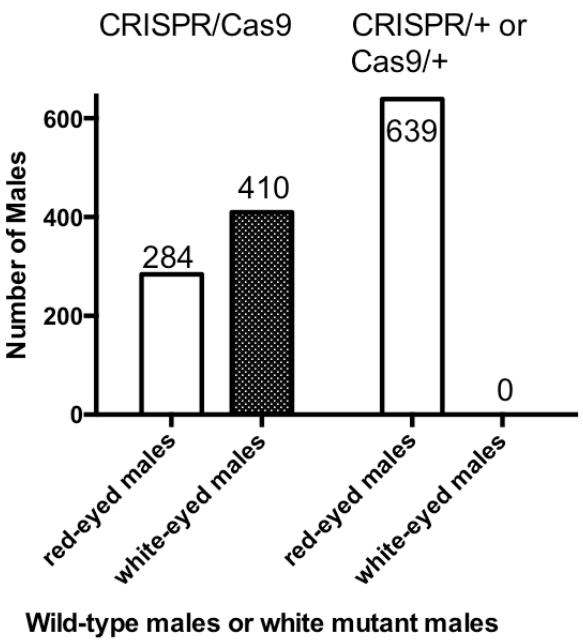

Figure 1. Recovery of white mutations in males

Mutations in males: In 28 crosses of Cross Scheme One, there were 410 white eyed males and 284 red eyed males, for a frequency of 59.1% white-eyed mutant males (410/694 = 59.1%; see Table 2 and Figure 1). White-eyed mutant males were observed in each of the 28 crosses. A total of 24 white-eyed mutant males were mated with C(1)DX, *y f* /Y females, and all bred true as white mutants in subsequent generations. The percent of white-eyed mutants in the CRISPR/Cas9 crosses is significantly different from the lack of mutants recovered in the CRISPR/+ or Cas9/+ crosses ($P < 0.001$ for 410/649 *vs* 0/639).

Mutations in both white genes in C(1)DX, *y f* / Y females: As expected white-eyed mutant males were recovered showing that the CRISPR/Cas9 system was functioning in Cross Scheme Two. Furthermore, in 30 crosses of Cross Scheme Two, 83 C(1)DX, *y f* / Y females had white eyes and 404 had red eyes, for a frequency of 17% females with both white genes mutated by the CRISPR/Cas9 system (see Table 3). Six of the G4 females with mutations in both white genes were crossed and the white mutation bred true into the G5 generation.

Mosaic eyes (spots of red and white pigment) were also observed in G4 females (these females were also *y* and *f*, confirming that they were C(1)DX, *y f* / Y) and in males of cross scheme two (Figure 2). Some of these G4 mosaic flies were also *Cy*, suggesting that they did not carry CRISPR and Cas9 in their somatic cells. It could be that these mosaics were caused by maternal deposition of CRISPR and/or Cas9 into the embryo (Lin and Potter, 2016). Two G4 mosaic flies also gave rise to white-eyed G5 progeny, showing that the white-eyed mutation in mosaics can include germ-line tissues.

Table 2. Number of white-eyed mutant males and red-eye, non-mutant, males from Cross Scheme One CRISPR/Cas9 males.

Vial number	Red eyed males	White eyed males	Total # of males
1	8	20	28
2	11	6	17
3	12	16	28
4	1	21	22
5	10	29	39
6	8	11	19
7	6	27	33
8	8	14	22
9	7	19	26
10	7	4	11
11	12	9	21
12	20	29	49
13	15	31	46
14	30	20	50
15	4	33	37
16	16	19	35
17	18	4	22
18	13	15	28
19	8	17	25
20	24	16	40
21	9	3	12
22	6	9	15
23	7	5	12
24	5	2	7
25	4	16	20
26	4	1	5
27	3	7	10
28	8	7	15
Totals:	284	410	694

Figure 2. Mosaic eyes of a G4 female of cross scheme two.

The results of this study clearly show that the CRISPR/Cas9 genome editing system induces a high percentage of mutations at the targeted white locus in haplo-X males (59.1%) and in diplo-X females (17%). One can compare the frequency of CRISPR/Cas9 induced white mutations in males (59.1%) with the observed spontaneous frequency of white mutations in males (10/668,631 = 0.002%) (Woodruff *et al*., 1983). The female results also mean that both copies of the targeted genes in autosomes could be mutated by this system. The percentage of diplo-X females with mutations in both white genes (17%), however, is signifiantly lower than would be expected if the CRISPR/Cas9 system in these females was the product of the frequency in haplo-X males ($0.591 \times 0.591 = 35\%$; $p < 0.0001$).

A class discussion of the results of this study could include: 1) The CRISPR/Cas9 system can also be used to insert a new DNA sequence into a gene of choice. For example, insertion of a mutant *Sonic hedgehog*

Table 3. Number of white-eye mutant, C(1)DX, *y f* / Y, females and red-eye non-mutant, C(1)DX, *y f* / Y, females from Cross Scheme Two.

Vial Number	Red eyed females	White eyed females	Total # of females
1	16	0	16
2	15	1	16
3	7	0	7
4	12	0	12
5	18	4	22
6	7	0	7
7	4	1	5
8	5	1	6
9	12	2	14
10	5	0	5
11	7	0	7
12	12	1	13
13	5	0	5
14	17	6	23
15	18	0	18
16	8	3	11
17	10	1	11
18	14	4	18
19	18	11	29
20	25	9	34
21	11	18	29
22	14	14	28
23	27	2	29
24	11	0	11
25	16	1	17
26	19	2	21
27	24	0	24
28	20	0	20
29	8	0	8
30	19	2	21
Total	404	83	487

gene from a python into a mouse caused the mice to develop little nubs of legs, suggesting that the ancestors of snakes may have lost their legs by a similar mechanism (Kvon *et al*., 2016). 2) Has the CRISPR/Cas9 system been used in humans? Yes. For example, this system has been used to inactivate HIV in somatic cells and to inactivate a gene in triploid zygotes (Liang *et al*., 2015).

References: Cox, D.B.T., R.J. Platt, and F. Zhang 2015, Nature Medicine 21: 121-131; Gantz, V.M., and E. Bier 2015, Science 348: 442-444; Govindan, G., and S. Ramalingam 2016, J. Cell. Physiol. 231: 2380-2392; Haimovich, A.D., P. Muir, and F.J. Isaacs 2015, Genetics 16: 501-516; Hammond, A., *et al.*, 2016, Nature Biotechnology 34: 78-83; Harel, L., *et al.*, 2015, Cell 160: 1013-1026; Kaminski, R., *et al.*, 2016, Sci. Rep. 6: 22555; doi: 10.1038/srep22555; Kondo, S., and R. Ueda 2013, Genetics 195: 715-721; Kvon, *et al*., 2016, Cell 167: 633-642; Lang, *et al*., 2015, Protein and Cell 6: 363-372; Lin, C., and C.J. Potter 2016, G3 6: 3785-3691; Lindsley, D.L., and G.G. Zimm 1992, *The Genome of* Drosophila melanogaster. Academic Press, New York; Nelson, C.E., *et al*., 2016, Science 351: 403-407; Waltz, E., 2016, Nature 532: 293; Woodruff, R.C., B.E. Slatko, and J.N. Thompson, jr. 1983, *The Genetics and Biology of* Drosophila, Vol. 3c, pp. 37-124 (Ashburner, M., H.L. Carson, and J.N. Thompson, jr., eds.), Academic Press, New York; Wu, Y., *et al.*, 2013, Cell Stem Cell 13: 659-662; Yang, L., *et al.*, 2015, Science 350: 1101-1104; Yin, H., *et al.*, 2014, Nature Biotechnology 32: 551-553.