More than Meets the Eye: A Primer for "Timing of Locomotor Recovery from Anoxia Modulated by the *white* Gene in *Drosophila melanogaster*"

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SUMMARY A single gene might have several functions within an organism, and so mutational loss of that gene has multiple effects across different physiological systems in the organism. Though the *white* gene in *Drosophila melanogaster* was identified originally for its effect on fly eye color, an article by Xiao and Robertson in the June 2016 issue of *GENETICS* describes a function for the *white* gene in the response of *Drosophila* to oxygen deprivation. This Primer article provides background information on the *white* gene, the phenomenon of pleiotropy, and the molecular and genetic approaches used in the study to demonstrate a new behavioral function for the *white* gene.

KEYWORDS education; Drosophila; pleiotropy; behavior

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THE *white* gene was the first *Drosophila melanogaster* mutant discovered by Thomas Hunt Morgan in 1910, following an exhaustive search for variant forms of the fly (Morgan 1910). So important was this fly to Morgan's efforts to understand the nature of heredity that Morgan's wife, Lilian (herself an accomplished fly geneticist who identified the first attached-X and ring-X chromosome variants), is reported to have exclaimed "Oh, I do hope the white-eyed fly is still alive" from her hospital bed after having just delivered her own baby (Green 1996). Morgan showed that the inheritance pattern of the white-eyed phenotype correlated with transmission of the X chromosome (Morgan 1910). His

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student, Calvin Bridges, combined cytological and genetic analysis of nondisjunction of the X chromosome to demonstrate experimentally the chromosome theory of heredity (Bridges 1916).

Given the historical importance of the *white* mutant to modern genetics, the identification of a new gene function over a 100 years after the gene's discovery and initial characterization may seem odd, especially when that gene function is related to behavior, rather than directly to eye color. However, the *white* gene serves as an excellent example of the common phenomenon of pleiotropy, when a single gene affects more than one trait such that mutations in that single gene have multiple, complex effects on phenotype. The article by Xiao and Robertson (2016) provides an interesting exploration of pleiotropy and the genetic basis of behavior, applying molecular genetic tools commonly used across many different experimental questions in the *Drosophila* model system.

Molecular Nature of the *white* Gene

The *Drosophila* eye contains two pigment types, the red drosopterins and the brown ommochromes. When *white* (the gene) is nonfunctional, these pigments are never made, and the eye is white in color. Note that gene names in *Drosophila* are italicized; they can be in lower case if they confer a recessive mutant phenotype or capitalized if they confer a dominant mutant phenotype. This observation of the *white* mutant phenotype leads to the counterintuitive outcome, so common in genetic analysis, where we conclude that the *white* gene is critical for the production of red and brown pigment. The function of the gene appears to be the opposite of its name, since the gene was named based on its loss-of-function mutant phenotype.

At the molecular level, the *white* gene and the *brown* and *scarlet* genes, which also contribute to eye pigmentation, encode members of the ABC transporter family (O'Hare *et al.* 1984). ABC transporters bind ATP to move a wide variety of substrates across the plasma membrane and intracellular membranes. These substrates include nucleotides, various amino acids, fatty acids, peptides, and metal ions. Therefore, ABC transporters are involved in processes as diverse as cholesterol metabolism, insecticide resistance, and cardiac function (Vasiliou *et al.* 2009; Dermauw and Van Leeuwen 2014). In the case of the *Drosophila* eye, the White protein (protein names in *Drosophila* are not italicized, but are either capitalized or in all capital letters) is responsible for transporting pigment precursors into cells rather than being involved directly in the biochemical reactions that synthesize the red and brown pigments. When the precursors are not present in the cells, the biosynthetic enzymes have nothing to act upon and the pigments are not produced, leading to the loss of normal eye color.

However, because pigment precursors are not the only compounds that these ABC transporters act upon, the organismal effects of mutations in the *white* gene extend beyond eye color. *white* mutants have reduced levels of the biogenic amines (histamine, serotonin, and dopamine) in the brain, and reduced loading of these amine molecules into intracellular vesicles within the brain (Borycz *et al.* 2008). In addition, alterations in *white* activity levels are associated with changes in male courtship behavior (Anaka *et al.* 2008), anesthesia response (Campbell and Nash 2001), and cyclic GMP transport (Evans *et al.* 2008). Furthermore, serotonin in *Drosophila* is involved in regulating larval locomotor behavior, sleep, and other circadian behaviors, and aspects of memory, so any effects of *white* on serotonin level or distribution may also carry through to affect those neural functions as well. The article by Xiao and Robertson (2016) is not the first to establish that *white* has a neural function outside of the eye, but it does suggest that one or more of the substrates the White ABC transporter acts on—likely a neurotransmitter or a neurotransmitter precursor in this case—is important for consistent locomotor recovery from anoxia. It also adds to the data establishing *white* as a locus exhibiting a high degree of pleiotropy.

The Challenge of Pleiotropy

Although pleiotropy of the sort observed for the *white* gene is a common genetic phenomenon, its definition and application can be complicated and confusing. Pleiotropic effects can be spurious if the traits affected are actually influenced by mutations at multiple genes. Therefore, a critical step in assessing pleiotropy is first to determine conclusively that the various phenotypic effects are actually due to mutation at a single gene and not to additional mutations that an individual is harboring. The full genetic complement of a strain is often referred to as the "genetic background" for the strain. Any two strains may include both recognized and unrecognized genetic differences, and so it is important to be certain that the phenotypic difference of interest (in this case, locomotor recovery) is not due to one of these unrecognized genetic differences in the genetic background. To rule out this confounding issue in genetic model systems like *Drosophila*, individuals bearing the mutation of interest can be crossed to a "clean" or standardized genetic background, often for several generations, to reduce the possibility of linked mutations. Following the crosses, the multiple traits are assessed to determine whether they were maintained together through the crossing procedure. If not, then we can conclude that more than one mutation is responsible and that our potentially pleiotropic gene is better understood as multiple genes that each independently affect phenotype. For example, these same investigators found that wild-type flies preferred the outer edge (or boundary) of a small enclosed space, but that flies from a *white* mutant strain would more often stray away from the edge; however, when they performed crosses to the clean genetic background, they determined that the altered boundary preference was not genetically linked to the *white* gene (Xiao and Robertson 2015) and that the *white* mutant strain also must carry an

additional mutation associated with the boundary preference behavior. In the current paper, however, the response to oxygen deprivation did travel with the white-eye phenotype across several generations of backcrosses, demonstrating that this trait was a pleiotropic consequence of mutation in the *white* gene (Xiao and Robertson 2016).

Even once it is established that a single gene affects multiple traits, it may not be possible to have a single, clear interpretation of what that tells us about gene function. In some instances, the variants at one gene that are proximally responsible for a particular trait may in turn cause downstream effects on another trait (or set of traits). This form of cascading effects is sometimes referred to as mediated pleiotropy (Solovieff *et al.* 2013). An example of this form of pleiotropy is observed in frizzle-feathered chickens, which have feathers that curl outward, away from the body, instead of lying flat. However, these chickens also typically have enlarged hearts, spleens, and gizzards, as well as increased heart rate and oxygen consumption, and decreased fertility. At the molecular level, a mutation in the KRT75 α-keratin gene alters the development of the rachis, or feather shaft, causing it to improperly curl outward (Ng *et al.* 2012). The metabolic and physiological defects of the birds can be attributed to a reduced ability to regulate body temperature due to the abnormal feathering. A single molecular change leads to a relatively simple change in the feather architecture, which in turn triggers a cascading set of physiological changes contributing to the overall altered phenotype of the frizzle-feathered birds. Because of the wide range of effects on phenotype, the frizzle mutation is a clear example of pleiotropy at the level of the organism. However, the mutation has a direct effect on only one trait–feather architecture–and all of the additional physiological effects are indirect and due to the feather shape. That is, the KRT75 α-keratin gene has a single function, to generate the shape of the feather shaft, and since the mutation only alters that single function, this scenario is distinct from other ways in which a gene can affect multiple traits.

Even when a single gene directly affects multiple traits, the molecular basis for that pleiotropy can be varied. For example, for a single gene that influences multiple traits, we may observe that any mutation in that gene inevitably alters all of those traits; the traits are inseparable by mutation. By contrast, we may observe that, for some pleiotropic genes, different mutations may affect only individual traits or different suites of traits. That is, the multiple traits are instead separable by mutation (as long as those mutations all affect the same gene, unlike the spurious interpretation of pleiotropy caused by the presence of multiple mutations in different genes, described above). The contrast between mediated and direct pleiotropic effects may seem like a semantic concern. However, pleiotropy has important practical implications for understanding how genes function as well as how constraints on their function can affect the process of evolution.

For example, the *Pitx1* gene, which governs aspects of skeletal development in vertebrates, demonstrates both the inseparable direct form pleiotropy that affects multiple traits by mutation of the protein coding region and the separation of its influences on individual traits by mutation of gene regulatory regions. *Pitx1* encodes a transcription factor that is required for proper development of the hindlimbs, craniofacial bones, and pituitary gland in mice. Mutations that disrupt the protein-coding region of *Pitx1* in mice lead to lethality shortly after birth due to this host of defects. The Pitx1 transcription factor can directly bind to regulatory elements of multiple target genes in the developing hindlimbs and pituitary gland in mice, affecting the expression of those target genes. Thus, disruption of the *Pitx1* gene leads to alterations of expression at multiple targets, directly causing severe developmental abnormalities across different physiological systems (Lanctot *et al.* 1999; Szeto *et al.* 1999). The multiple effects are more direct than the cascading effects observed for the chicken frizzle-feather mutation, and any variant of the protein-coding portion of the *Pitx1* gene in mice will result in this outcome. However, in the threespine stickleback fish, *Pitx1* variants are associated with perfectly viable individuals. Populations of threespine sticklebacks that live in marine environments typically have prominent, armored pelvic fins (or spines), whereas several populations of freshwater sticklebacks have, in parallel, lost this hefty pelvic apparatus. The loss of the pelvic spines is genetically linked to the *Pitx1* gene, but the protein coding sequence of *Pitx1* does not contain any differences between marine and freshwater populations. Rather, in marine populations, the *Pitx1* gene is expressed in the developing pelvic region, thymus, and sensory structures, but the freshwater populations have lost expression in just the pelvic region (Shapiro *et al.* 2004). The lethal pleiotropic consequences of disrupting the protein-coding sequence of the *Pitx1* gene have been avoided by mutations in the gene's regulatory regions that disrupt *Pitx1* function only in the pelvic region by decreasing expression of the gene in the developing pelvic tissues (Chan *et al.* 2010). Whether evolution can act in this way depends on whether the pleiotropic effects of a gene are due to separable functions that can be affected by mutation independently or are due to cascading effects downstream of a single function. In the case of *Pitx1*, evolution can, and has, separated its functions into individually modifiable components.

Xiao and Robertson (2016) were able not only to add another affected trait to the pleiotropic effects associated with the *white* gene, but were also able to separate those effects from one another using clever double-mutant analysis that recapitulated the white-eye trait but did not affect locomotor recovery. In addition, their tissue-specific disruption of *white* gene function demonstrated that the effects also were separable between tissues.

Tissue-Specific Expression and RNA Interference (RNAi)

To disrupt expression of the *white* gene in specific tissues, Xiao and Robertson (2016) use the GAL4/UAS binary gene expression system, which is a common and widely used part

of the *Drosophila* molecular toolkit (reviewed in Duffy 2002). The two parts of the system are the GAL4 transcription factor, derived from baker's yeast, *Saccharomyces cerevisiae*, and the Upstream Activating Sequence (UAS) response element to which GAL4 binds to activate transcription. The GAL4 gene previously has been inserted into various locations in the *Drosophila* genome, and its expression is determined by the regulatory elements near that insertion site. The UAS regulatory DNA sequence also can be attached to a target gene of interest to control activation of that target gene. When a fly bears both a GAL4 "driver" of expression and a UAS "responder," the target gene will be activated only in the specific tissues where GAL4 is present. Thus, the regulatory elements directing GAL4 expression to a specific tissue can, in turn, be used to control expression of virtually any target gene in that tissue. Xiao and Robertson (2016) use GAL4 lines that are expressed in the central nervous system, glial cells, or in subsets of serotonin-producing neurons to manipulate expression of the *white* gene. However, their goal is not to express the *white* gene at higher levels in particular cells, but rather to reduce the activity of the *white* gene product in those cells. They achieve this outcome by using the UAS to express short inverted repeat sequences matching the *white* gene. These repeat sequences will form self-complementary double-stranded RNA hairpins that are processed by the Dicer enzyme and incorporated into the multi-protein, RNA-induced silencing complex (RISC). The active RISC, guided by the incorporated RNA sequence, will target *white* mRNA for degradation, preventing production of the ABC transporter protein encoded by *white*. This process of RNAi eliminates gene function by reducing the pool of mRNA of the target gene without altering the DNA sequence of that gene. Thus, RNAi is typically said to "knock-down" gene activity, in contrast to the "knock-out" of gene activity that occurs with a targeted deletion of a gene's DNA sequence. However, combining RNAi with the GAL4/UAS system allows a quick and flexible means to reduce the function of virtually any *Drosophila* gene in a tissue-specific manner.

Understanding the Experimental Details

Establishing a behavioral phenotype

When attempting to study the genetic basis of a behavior, it is critical to have a clear, well-defined assessment of that behavior. Genetic influences on the behavior might be subtle, and without a well-designed assay, changes in the behavior in different genetic backgrounds may be missed. The behavioral trait that Xiao and Robertson (2016) examine is locomotor recovery from 30 sec of anoxia (caused by flooding the fly exposure chamber with nitrogen), which means that they must have a clear definition of what it means for a fly to be moving appropriately. They first observed unexposed flies in a small circular arena to establish a threshold value for continuous walking, which they set at 0.3 cm-path length per second; because sporadic events during recovery might cause a fly to surpass this threshold value, Xiao and Robertson also set the criterion that a fly must display 10 instances of above-threshold movement within 60 sec to be classified as recovered. With these criteria established, they collected data on movement levels for wild-type (Canton-S) and *white* mutant (w^{1118}) flies (Figure 1, A–B in Xiao and Robertson 2016). When anoxic exposure is applied (arrow), the movement levels (black lines) drop; as time progresses (*x*-axis), the flies begin to move again. Even before the application of statistical analysis, it is obvious that recovery time in wild-type flies is less variable than in the *white* mutant, and that variability is in the direction of increased recovery time for the mutant. The statistical analysis (Figure 1C) confirms these observations, using a box-and-whisker plot to represent the data instead of a single mean value with error bars. In this plot, the box represents the values from the 25th to the 75th percentile, and the line within the box represents the median (or middle) value; the whiskers, which need not be equally sized, here indicate the full range of the data, clearly showing the increased variability in recovery time in the mutant strain.

Introgression: eliminating the trivial

Once the difference in locomotor recovery time has been established between the wild-type and *white* mutant strains, the next major hurdle is to show that the observed difference is actually due to the mutation in *white* and not due to another mutation elsewhere in the genetic background of the w^{1118} strain. Xiao and Robertson perform three sets of crosses (Figure 2, A–C in the original article), which progressively narrow in on *white* as the causal genetic locus for the phenotypic difference. When reading complex *Drosophila* genotypes (relevant for this figure and several others in the article), heterozygous individuals are indicated with the two homologous chromosomes bearing different alleles separated by a "/" (as in w^{+}/w^{1118} designating a female heterozygous for a wild-type and mutant *white* allele). Hemizygous males are indicated with a *Y* as one of the chromosomes, typically below the "/." Homozygous individuals are usually shown with only a single allele designator (*e.g.*, w^{1118}), even though they actually bear two copies of that homologous chromosome. When describing flies carrying mutant alleles on several different chromosomes, a ";" is used to separate the chromosomes. Thus, a fly of genotype w^{+}/Y; *cn*, *bw* (Figure 3A) is a male carrying a wild-type *white* allele on the X chromosome and homozygous for the *cinnabar* and *brown* mutations on chromosome II. Flies have four chromosome pairs, and any chromosomes not shown (*e.g.*, chromosomes III and IV are not listed in the previous example) are assumed to bear wild-type alleles at all loci.

In the first experiment (Figure 2A), researchers observed the male offspring of a single generation of crosses, which have the same sets of second and third chromosomes, but differ at their X chromosome (the location of the *white* gene). The second experiment involved four generations of crosses, repeatedly crossing wild-type males to homozygous (parental generation) or heterozygous (all subsequent generations)

mutant females. This process is referred to as introgression, and completely replaces the second and third chromosomes of the w^{1118} mutant strain with chromosomes from the wild-type strain. In introducing the wild-type chromosomes into the mutant strain, the introgression extends the results of the first experiment, virtually eliminating the second and third chromosomes as potential sources of the locomotor recovery difference. The third experiment involves a similar scheme of crosses carried out for 10 generations. Because of chromosome recombination during these 10 generations, this scheme will also swap portions of the X chromosome, further narrowing the genetic region that could be responsible for the phenotypic difference. In all cases, the longer, variable recovery time was associated with the chromosome carrying the w^{1118} mutant allele and the shorter, consistent recovery time was associated with the chromosome carrying the wild-type (or w^+ allele).

Together, these experiments demonstrate that differences in the locomotor recovery time trait are associated with the w^{1118} mutation, and are unlikely to be due to mutations elsewhere in the genetic background of the mutant strain. Thus, this trait is a true instance of pleiotropy at the *white* locus.

Dosage and position effect: complicating the story

With the effect of *white* mutations on recovery time established, a logical next step is to determine whether this effect is dominant or recessive. Because the *white* gene is located on the X chromosome, typically one could only assess this question in heterozygous females. Males are normally hemizygous for the *white* gene (and any gene located on the X chromosome), meaning they only carry a single copy of the locus. Heterozygous females (Figure 2E in the original paper) exhibit the longer, variable recovery, which would suggest that the mutant locomotor phenotype is dominant and that a single copy of the wild-type *white* gene is insufficient to rescue (or fix) that phenotype. However, Xiao and Robertson generated a male with the w^{1118} mutant allele, but also carrying a normal version of the *white* gene on the Y chromosome (the Y chromosome does not carry any copy of the *white* gene, so this experiment uses a modified chromosome in which part of the X chromosome has been translocated to the Y chromosome). Therefore, these altered males are actually heterozygous, with one wild-type and one mutant copy of *white*, and they exhibit the short recovery time phenotype (Figure 2D), which is somewhat surprising, given the opposite result observed in females. These conflicting results may suggest that this behavioral phenotype is extremely sensitive to variations in the dosage of *white* (that is, how many functional copies of the gene are present and how they are being expressed).

The authors further explore this phenomenon by manipulating gene dosage in flies by varying the number of copies they carry of the mini-*white* (mw^+) construct, a commonly used marker for making transgenic flies, and which eliminates much of the upstream, downstream, and intronic noncoding DNA associated with the normal *white* gene [for more detailed information on how transgenic flies are generated and identified, as well as other *Drosophila* molecular techniques, such as RNAi and the GAL4/UAS system, consult the *GENETICS Drosophila* organismal primer (Hales *et al.* 2015)]. Because the mini-*white* DNA is missing the proper regulatory elements, its expression is influenced by the regulatory sequences in the area of the genome in which the transgene inserts (Hazelrigg *et al.* 1984), a phenomenon known as "position effect" (that is, the physical position of the gene in its chromosomal location affects its expression, and therefore function). To ensure greater reliability of their transgene results, the authors tested several insertion locations for the mw^+ transgene in reaching their conclusions. When they examined flies with one, two, or four copies of mw^+, all genotypes displayed the slower, variable recovery phenotype (Figure 4 in Xiao and Robertson). This result has two important consequences. First, because the presence of this common transgenic marker alone does not rescue the locomotor phenotype, the authors can safely interpret the results of any experiments they perform using transgenic flies that bear the mw^+ construct. Second, the regulatory information of the normal *white* locus is critical for its proper function in this behavioral context, again suggesting that the locomotor recovery phenotype is extremely sensitive to the proper location and amount of *white* gene function.

Molecular tricks: dissecting function and location of action

Given the apparent complexities in dosage and gene regulation for the effect of *white* on this behavior, Xiao and Robertson proceed to examine two specific aspects of the molecular genetic basis for locomotor recovery: how it relates to other known functions of *white* and in which tissues *white* function is important for proper anoxia response.

To test the relationship between eye pigmentation and locomotor recovery, Xiao and Robertson (2016) generated phenotypically white-eyed flies using two mutations other than *white*. The *brown* mutation prevents synthesis of the red pteridine pigment and the *cinnabar* mutation prevents synthesis of the brown ommochrome pigment, resulting in white eye color independent of the *white* gene. And despite the fact that the Brown protein is also an ABC transporter, these flies displayed normal locomotor recovery (Figure 3, A–B in Xiao and Robertson), indicating that eye color and anoxia response are separable and that the substrates Brown protein transports are not critical for anoxia response. Furthermore, disrupting *white* gene function throughout the nervous system using the elav-GAL4 pan-neuronal driver had no effect on eye color but did alter locomotor recovery (Figure 3, C–D).

That result indicates that the *white* gene acts somewhere in the nervous system to facilitate locomotor recovery, but Xiao and Robertson (2016) are able to further pinpoint location of action using additional GAL4 driver lines that express in subsets of nervous system tissue (Figure 5 in Xiao and Robertson 2016). In Figure 5D, we are looking head-on at the *Drosophila* brain, with the central brain in the middle and the

large optic lobes toward each side. The bright green cells are labeled with green fluorescent protein (GFP) that is driven by the GAL4/UAS system, indicating the specific cells where several of the GAL4 drivers disrupted *white* gene function. A GAL4 driver to disrupt *white* function in glial cells did not disrupt locomotor recovery, indicating that *white* function is not necessary in that tissue. However, RNAi of *white* specifically in dopaminergic and serotonergic neurons did disrupt recovery time. Further, RNAi directed by any of three GAL4 drivers, which were each were active in subsets of serotonin-containing neurons, also disrupted recovery time. Thus, a well-coordinated, consistent recovery from anoxia requires proper function of the *white* gene in serotonin neurons, suggesting perhaps that the reduction of serotonin (both overall and in vesicles in the brain) in *white* mutants may cause the slower, variable recovery.

Suggestions for Classroom Use

Although pleiotropy is an important topic in genetics, it is often discussed in classrooms at a superficial level, with little regard to the complex ways it may or may not elucidate gene function. Xiao and Robertson (2016) provide an example of a well-known, familiar gene in *white*, but demonstrate a new behavioral function for this gene. They also demonstrate that some of the functions of this gene are separable from one another, and so provide a detailed account of pleiotropy at the *white* locus. In addition, the response to anoxia analyzed in Xiao and Robertson (2016) is a relatively simple behavioral phenotype to understand, and so the experiments also provide an introduction to the genetic analysis of behavior. Reading and discussing Xiao and Robertson (2016) may serve as either an introduction to, or supplement for, topics related to pleiotropy, behavioral genetics, and molecular tools for analysis of complex phenotypes. This Primer article outlines some of the conceptual complexities of pleiotropy to accompany the specific example of *white* in Xiao and Robertson (2016). In addition, this Primer describes briefly the techniques used in Xiao and Robertson (2016) for behavioral analysis and tissue-specific disruption of gene function. It is recommended that students read the introduction to Xiao and Robertson (2016) and the introductory portions of this Primer before attempting the results and discussion sections. Whereas seasoned readers will focus on the figures of an article, students with limited exposure to journal articles may spend a considerable amount of time reading the text and relatively little time examining the figures. To facilitate student interaction with the figures, several faculty at Allegheny College have adopted variations on the following figure analysis exercise. For each figure in the article, students prepare written responses to 5 questions: (1) What is the specific experimental question addressed by this particular figure; (2) what method or methods were used to obtain the data; (3) what is the justification for the experimental design (or "why did they do it *this way*?"); (4) what is the meaning of the data for the specific experimental question described in your response to question #1; and (5) what is the meaning of the data for the overall argument ("big question") of the entire article? This activity can be modified for different levels of experience with the scientific literature. Graduate students or advanced undergraduates might be asked to prepare responses prior to class to allow discussion of the entire paper in a single class session. Undergraduate students without prior exposure to the literature might work in small groups to prepare responses for only a single figure, with each group then sharing their responses with the rest of the class. Other articles that explore the functions of the *white* gene or that use similar molecular tools to analyze additional complex behaviors could be used for further discussions extending from the original article.

Questions for Discussion

1. In the figures with data on time to recovery (TR; *e.g.*, Figure 1, C and D), the data are rendered in box-and-whiskers plots. What information does this depiction give the reader that a similar graph showing mean value ± SD would not? Why do you think the authors chose to use this approach to displaying the data?
2. In interpreting a box-and-whiskers plot, if the median line is closer to the top of the box than to the middle, what does that indicate about the variation within the data? If the median line is closer to the bottom than to the middle, what would that indicate?
3. Figure 1D introduces six new strains of flies: Hikone-AS, Amherst-3, Florida-9, w^1, w^a, and w^{cf}. Why was the recovery time of these strains compared to one another? Why was the comparison between Canton-S (CS) and w^{1118} not sufficient?
4. Using yarn of two different colors (or red and black licorice!) to represent chromosomes, simulate the genetic crosses the experimenters performed to generate the F1 and F4 flies in Figure 2, A and B. What process does this simulation miss that might affect the overall genetic background of the fly strains?
5. What process might account for the discrepancy in phenotype observed in Figure 2, D and E, where heterozygous males are similar to the wild-type phenotype, whereas heterozygous females are more similar to the homozygous *white* mutant? How might this process that occurs differently in males and females result in the difference?
6. The authors examined the effects of the mini-*white* (mw^+) insertion and determined that the insertion could not substitute for the normal w^+ allele and the number of mw^+ copies, ranging from 1 to 4, had no effect on recovery time (Figure 4). Since the authors had already tested dosage effects in Figure 2, why did they perform this set of experiments? What do the authors mean in distinguishing between "site-specific" and "random" locations for mw^+ in these experiments?
7. How do the authors reach the conclusion that the *white* gene is not expressed in glial cells? Describe how the

authors might be using the terms "expression" and "function" in this context. Are there further experiments you might want to perform before being certain that White protein is not present in glial cells?

8. How did the authors use the UAS-GAL4 system to identify serotonergic neurons in the *Drosophila* brain (Figure 6)? What is the role of GFP in this system?
9. Each enhancer in Figure 5 represents a subset of the ~100 serotonergic neurons in the *Drosophila* brain, but knockdown of *white* by RNAi using any of the enhancers increases time to recovery. Propose an explanation for this result.
10. Describe another specific example of pleiotropy, where a mutation in a single gene has more than a single effect on phenotype. How does the scenario you have described qualify as pleiotropy?
11. Does the pleiotropy of the *white* gene more closely resemble that of the *KRT75* frizzle-feather gene or the *Pitx1* gene? Explain how you reached that conclusion.

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