Know thy fly

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The generation and analysis of mutants is central to studies of gene function in model organisms. Methods for random mutagenesis in *Drosophila melanogaster* have been available for many years, but an alternative approach – targeted mutagenesis using homologous recombination – has only recently been developed. This approach has the advantage of specificity, because genes of interest can be altered. One might expect with a gene-targeting approach that the frequency of background mutations would be minimal. Unfortunately, we have found that this is not the case. Although the possibility of background mutations arising during homologous-recombination-based gene targeting has been raised in the literature, it is not routinely taken into account when using this technique. Our experience suggests that it can be a considerable problem but that it has a relatively simple solution.

A new generation of mutagenesis in *Drosophila*

The Human Genome Project has contributed to the identification of a large number of novel genes of unknown function, many of which are associated with particular human diseases. One approach to improve our understanding of the function of specific genes and the pathogenic pathway(s) by which their mutation leads to disease is through the use of animal models. Targeted gene mutagenesis has enabled analysis of the phenotypic consequences of specific gene mutations; however, such analysis is based on the assumption that the model animals have a uniform genetic background. Recently, some of the concerns associated with inbred strains of mice and their variable genetic backgrounds were raised in this journal [1]. *Drosophila* is another organism that can provide useful insights into the function of orthologous genes, and methods have been established recently to carry out targeted mutagenesis in *Drosophila*. Our experience is that although gene targeting in *Drosophila* is effective, it can also result in a significant number of background mutations that can either lead to a phenotype on their own or contribute to the observed phenotypes.

In the past, mutagenesis of *Drosophila* involved a random whole-genome approach, in which a mutagen such as radiation or DNA-damaging chemicals was applied to

the organism or in which random insertion of transposons was induced. It is well established that the multiple 'hits' generated by these approaches provide background mutations that can interfere with subsequent phenotypic analyses. Targeted approaches for mutagenesis have been available in the mouse and in yeast for many years. The power of this type of approach lies in the ability to introduce a desired mutation(s) into any gene of interest, with no reliance on phenotype for identification of the resultant mutant alleles and no reliance on existing genetic lesions in the region of interest. Recently, techniques for the targeted mutagenesis of genes by homologous recombination (HR) have been developed for *Drosophila*. Despite this method having the considerable advantage of specificity, it does not seem to have been widely embraced by the *Drosophila* research community. It is possible that because HR approaches in *Drosophila* are time consuming and involve multiple steps with various genetic rearrangements occurring at unpredictable frequencies (Figure 1), and because researchers are familiar with more established techniques, HR might be the least favoured method for mutagenesis. However, the benefits of the specificity of HR-generated mutations can outweigh the investment required for their generation.

Successful gene targeting using HR in *Drosophila* was first reported in 2000 by Rong and Golic [2], and we have found 29 subsequent reports [3–31]. These reports include examples of 'ends-in' and 'ends-out' targeting approaches, the details of which are outlined in Figure 1. We have carried out ends-in targeted mutagenesis for the *Drosophila* gene *Wwox* (also known as *CG7221*) and found nontargeted mutation(s), in addition to the desired mutations, that have significant effects on phenotypic outcomes. In this Opinion article, we detail our findings, including the difficulty that we experienced in uncovering the problem of background mutations, to illustrate the nature of this issue.

Background nontargeted mutations generated by HR: some unpleasant surprises

We carried out ends-in targeting to introduce two premature stop codons and a frameshift mutation at the start of the endogenous *Drosophila* gene *Wwox*. We found that the resultant mutant flies, which were homozygous for *Wwox*^L, were viable and fertile but showed an increased

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Figure 1. Ends-in and ends-out approaches to targeted mutagenesis in *Drosophila melanogaster*. **(a)** Ends-in gene-targeting scheme. The relevant features of the targeting vector pTV2 [25] containing the mutated genomic region of interest are indicated: FLP recombinase target (FRT) sites (green), white (w^+) selectable marker (white) and I-CreI recognition site, two exons of a hypothetical gene of interest (red) with the red asterisk representing the introduced mutation(s). The whole construct is contained within P element ends (black) for transformation. There are two steps involved in this process. Step 1 involves induction of gene expression for FLP recombinase and the endonuclease I-SceI under the control of a heat shock (*hs*) promoter. This results in excision and circularization of the genomic region between the two FRT sites, followed by cleavage at the I-SceI site to facilitate integration into the endogenous target locus (blue). This can produce a tandem duplication of the target locus, with one copy carrying the introduced mutation(s). Step 2 involves reduction to a single allele and is carried out by heat-shock-induced gene expression of I-CreI (*hs::I-CreI*), which cleaves at its recognition sequence. This chromosomal break is enlarged by endogenous exonucleases and repaired by a single-strand annealing mechanism, leaving a single copy of the target locus. PCR screening with allele-specific primers can then be used to identify the recovered mutant alleles. **(b)** Ends-out gene-targeting scheme. The ends-out donor construct contains many of the elements of ends-in donor construct. The w^+ selectable marker (white) is inserted into the target locus such that it disrupts the gene of interest (red). The targeting region is flanked at either end by I-SceI sites and directionally oriented FRT sites. The whole construct is contained within P-element ends (black) for transformation. There is only one step in this process. Induction of gene expression for FLP recombinase and the endonuclease I-SceI in transgenic flies carrying the targeting construct results in excision and circularization of the genomic region between the two FRT sites, followed by cleavage at the two I-SceI sites. This results in a linear DNA molecule with breaks at the end of the region of homology between the targeting construct and the endogenous genomic region. The linear targeting molecule recombines with the target locus (blue), resulting in a cassette exchange and the generation of a mutant allele.

sensitivity to ionizing radiation [29] (Figure 2a). Genetic tools that remove large regions of the genome (i.e. deficiency strains) were unavailable in the region where the *Wwox* gene resides, so the phenotype could not be verified using *trans*-heterozygosity with a deficiency allele. However, sensitivity to ionizing radiation was observed in multiple homozygous strains generated from independent reduction events (step 2 of ends-in targeting; Figure 1), and rescue was achieved by introduction of a transgene encoding either *Drosophila Wwox* or human WWOX [29].

Sensitivity to ionizing radiation was subsequently also observed for an independent allele of *Wwox* ($Wwox^{f04545}$) that resulted from insertion of a *piggyBac* transposon into the second intron of *Wwox* [32]. However, sensitivity to ionizing radiation was not observed in flies that were *trans*-heterozygous for both $Wwox^1$ and $Wwox^{f04545}$ (Figure 2a), suggesting, to our surprise, that in homozygous strains for each of these independent alleles of *Wwox* the phenotype of

sensitivity to ionizing radiation is independent of the mutations in *Drosophila Wwox*. To confirm this, we carried out four rounds of backcrossing of each of these alleles of *Wwox* to the parental ('wild type') strain used for the targeted mutagenesis (i.e. w^{1118} , a strain that has a small deletion in the *white* eye colour gene but which is otherwise wild type). In both cases, we found that the resultant homozygous mutant strains showed a significant decrease in sensitivity to ionizing radiation after backcrossing to w^{1118} (Figure 2a). Therefore, the sensitivity to ionizing radiation must be independent of mutations induced at the *Wwox* locus in these cases. It has been reported that 0.3–0.5% of *piggyBac* insertion strains contain unlinked background mutations [32], and these could account for the radiation sensitivity that we have observed in $Wwox^{f04545}$ mutants. Our data, therefore, show that background mutations can arise during a targeted mutagenesis approach.

Figure 2. Background mutations following HR mutagenesis at the gene *Wwox*. (a) *Wwox*¹ mutants are no longer sensitive to ionizing radiation after backcrossing to wild-type flies (*w*¹¹¹⁸). Radiation sensitivity was determined as percentage survival to adulthood after treatment of wandering third instar larvae (~5 days of development) with 30 Gy of ionizing radiation, as previously described [28]. Homozygous strains of HR-generated *Wwox*¹ and piggyback-transposon-generated *Wwox*^{f04545} mutants show sensitivity to ionizing radiation compared with *w*¹¹¹⁸. Sensitivity to ionizing radiation was significantly decreased in homozygous strains of *Wwox*¹ and *Wwox*^{f04545} after backcrossing to *w*¹¹¹⁸ and was not observed in flies that were heterozygous for these two alleles of *Wwox* (*Wwox*¹ and *Wwox*^{f04545}). The graph shows the average percentage and the standard deviation for at least three separate experiments for each genotype, where n = 100 for each experiment. (b) Protein profiles of HR-generated *Wwox*¹ mutants before and after backcrossing to wild-type flies (*w*¹¹¹⁸). Protein homogenate from *w*¹¹¹⁸ and *Wwox*¹ homozygous mutant embryos (at 2–4 h of development), before and after backcrossing to *w*¹¹¹⁸, was subjected to DIGE analysis, as described in Gong *et al.* [33]. The green curve shows the frequency distribution of the log ratios of proteins from *Wwox*¹ mutants to proteins from *w*¹¹¹⁸. The number of individual protein spots on a DIGE gel showing a twofold or greater increase (blue) or decrease (red) in abundance in the *Wwox*¹ mutants compared with *w*¹¹¹⁸ was determined. Each spot on the graph represents an individual protein spot on a DIGE gel. A significant decrease in the proteins that changed in abundance was observed in *Wwox*¹ mutants after backcrossing to *w*¹¹¹⁸ (the wild-type strain used for the mutagenesis).

Proteomic analysis of HR-generated alleles of *Wwox*

The observed difference in phenotype – i.e. sensitivity to ionizing radiation – between HR-generated homozygous *Wwox*¹ mutants before and after backcrossing to *w*¹¹¹⁸ suggests that there are difference(s) at the genomic level. Therefore, we used 2D difference gel electrophoresis (DIGE) analysis [33] to compare the protein profiles of homozygous *Wwox*¹ mutants before and after backcrossing to *w*¹¹¹⁸. Several changes that were observed in the proteins produced by homozygous *Wwox*¹ mutants compared with *w*¹¹¹⁸ – the parental strain used for the mutagenesis – are no longer present after backcrossing to the same parental strain (Figure 2b); therefore, we conclude that these

changes were introduced, either directly or indirectly, as a consequence of the targeting procedures.

Changes in the genetic background following HR

The possibility of generating background nontargeted mutations has previously been raised in the literature on HR. However, it has most often been mentioned only briefly in the ‘Materials and Methods’ section or included as ‘Online Supplementary Material’ and, therefore, is not often highlighted. Background nontargeted mutations affecting viability have been reported following HR mutagenesis of *pug*, *NLaz* [25] and *Nap1* [19], whereas background nontargeted mutations are proposed to contribute

to variability in meiotic phenotypes as a consequence of mutations in *Ercc1* [5]. We have also identified background nontargeted mutations resulting in semi-lethality and sterility of females following HR mutagenesis of *Rgk1* and *Rgk2*, respectively (P. Smibert and R. Saint, unpublished).

The presence of background nontargeted mutations could also explain differences in stress tolerance tests observed between strains that are homozygous for a mutation in the gene *desaturase-2* [14]. This was reported as an unlikely explanation, because the independently reduced strains (i.e. independent strains following step 2 of ends-in targeting; Figure 1) were assumed to be genetically identical. Claims of genetic identity, other than at the target locus, have also been made for wild-type flies and for mutant flies generated by HR procedures to induce mutations in the *Or83b* [26] and *OdsH* [9] loci. Similarly, the assumption that a particular chromosome remains isogenic after HR procedures has been reported in studies of independent targeted mutations of three members of the *metallothionein* gene family [3]. As a result of these claims in the literature, it is possible that the contribution of background nontargeted mutations is also not fully appreciated by others. Here, our aim is to highlight the significant impact that background nontargeted mutations can have on phenotypic outcomes.

Minimizing the impact of background nontargeted mutations

The importance of proper control of genetic background following HR has been discussed recently following conflicting reports of stress tolerance assays of strains that are homozygous for mutation in *desaturase-2* [14,34]. The segregation and reassortment over time of alleles obtained from various strains used for the mutagenesis has been suggested as an explanation for the differences. A proposed strategy to control for changes in genetic background is the replacement of chromosomes, other than that on which the gene of interest resides, with those from a wild-type strain [35]. However, we propose multiple rounds of backcrossing to the parental strain as a preferred approach for the elimination of potential background nontargeted mutations, because this will ensure that alleles on all chromosomes will be replaced by assortment and recombination with those from the parental strain used for the mutagenesis.

Backcrossing has been reported in five of 29 published studies of *Drosophila* with mutations generated by HR. Multiple rounds of backcrossing to either the parental strain or another wild-type strain is routinely carried out before conducting behavioural studies. Backcrossing was performed before male courtship studies of flies with HR-generated mutations in *fruitless* [6,28]. In addition, ten rounds of backcrossing were carried out for flies with mutations generated in *Or43b* [16], which encodes an odorant receptor, and an unspecified number of rounds were carried out for flies with mutations in *p53* [20]. In the case of the cation channel PKD2, a more thorough approach was undertaken: the chromosomes other than those carrying the targeted mutation were first replaced and then ten rounds of backcrossing were carried out [15]. However, in the remaining 25 reports of mutations

generated by HR, there is no mention of backcrossing to eliminate potential background nontargeted mutations.

We found that four rounds of backcrossing of *Wwox*¹ mutants to *w*¹¹¹⁸ was sufficient to minimize the effect of background nontargeted mutations on radiation sensitivity. This is a relatively time-consuming process (one *Drosophila* generation is a little less than 2 weeks), requiring at least four generations of backcrossing, in addition to the crosses required for the generation of homozygous strains. Together, this process takes in excess of 3 months. For ends-out targeting approaches, these backcrossing steps are more straightforward, because the mutations carry a visible eye colour marker, whereas mutations generated by an ends-in approach need to be followed by allele-specific PCR. However, there is no substitute for using good basic genetics after HR mutagenesis (similar to any other form of mutagenesis).

Verifying HR-generated alleles

The possibility of background nontargeted mutations following HR mutagenesis (as for all types of mutagenesis) necessitates verification that observed phenotypes are specifically attributable to loss of function of the gene of interest. Independently derived alleles of the gene of interest that show the same phenotype, both as independent homozygous strains or in a *trans*-heterozygous combination, provide strong support for this attribution, as discussed by Rong *et al.* [25]. However, our experience is that independently reduced HR-generated alleles (i.e. after step 2 of ends-in mutagenesis; Figure 1) following a single integration event cannot be treated as truly independent. This finding suggests that nontargeted mutations can be introduced during step 1 of ends-in targeting (Figure 1), and these can then be carried through step 2 to all of the mutant alleles. In addition, verification of HR-generated mutant phenotypes can also be carried out by rescue experiments following either introduction of a construct bearing the corresponding genomic region or ectopic expression of the corresponding cDNA [25]. However, these rescue experiments often involve crossing strategies that can effectively rescue the phenotypic effects of background mutations by the introduction of wild-type alleles on other chromosomes, as we observed in our studies with the *Wwox* gene. In each of these cases, backcrossing of HR-derived alleles before commencing any phenotypic and/or rescue analyses would considerably decrease any confounding contribution of background nontargeted mutations.

What is the origin of background nontargeted mutations after HR?

It is reasonable to propose that nontargeted mutations can arise during HR mutagenesis procedures, because the enzymes that are used (Δ 2-3 transposase, FLP recombinase and the endonucleases I-SceI and I-CreI) could cleave at nonspecific sites, especially because they are ectopically expressed. Alternatively, it is also possible that the background nontargeted mutations could have been introduced directly, or indirectly, from the various strains used for the mutagenesis. Irrespective of the origin of background nontargeted mutations resulting from HR mutagenesis procedures, their effects will be minimized following

multiple rounds of backcrossing to an appropriate wild-type strain.

What is the extent of the problem?

There is not enough information in the published reports of HR-generated mutations to know whether any of the previously reported phenotypes might need to be revised because background mutations contribute to the phenotype. Furthermore, we do not wish to analyse other studies that might have been impaired by such mutations. Our aim is specifically to raise awareness of the potential contribution of background nontargeted mutations following HR mutagenesis.

Concluding remarks

In summary, we have found nontargeted background mutations following HR mutagenesis in *Drosophila*. Although there is nothing new about the appearance of background nontargeted mutations affecting phenotypic outcomes following various mutagenic procedures in *Drosophila*, and indeed in all species, background nontargeted mutations are rarely reported and are not often given sufficient focus of attention. The possibility of such mutations is acknowledged in some reports in the literature, but steps to minimize the impact of these mutations are taken only infrequently. We propose that phenotypic analysis of alleles generated by HR-based gene targeting ought to be carried out only after efforts have been made to control for genetic background, by repeated backcrossing to the parental strain from which the mutants were derived.

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