FROM SEQUENCE TO PHENOTYPE: REVERSE GENETICS IN *DROSOPHILA MELANOGASTER*

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There has been a long history of innovation and development of tools for gene discovery and genetic analysis in *Drosophila melanogaster*. This includes methods to induce mutations and to screen for those mutations that disrupt specific processes, methods to map mutations genetically and physically, and methods to clone and characterize genes at the molecular level. Modern genetics also requires techniques to do the reverse — to disrupt the functions of specific genes, the sequences of which are already known. This is the process referred to as reverse genetics. During recent years, some valuable new methods for conducting reverse genetics in *Drosophila* have been developed.

Drosophila melanogaster has been an important model organism in genetic studies for more than 90 years. For most of this time, the primary approach has been forward genetics, in which genes are discovered on the basis of mutant phenotypes (see review by Daniel St Johnston on page 176 of this issue[1]). More recently, gene discovery starting with DNA sequence has become increasingly important, as emphasized by the publication of the genome sequence of *Drosophila* in 2000 (REF. 2). Initial analysis of this sequence led to a prediction that there are ~13,600 genes in *Drosophila*. The previous published literature had discussed only ~20% of these genes, and only half of those had been characterized by genetic methods. A significant challenge for the current era of *Drosophila* genetics is therefore to understand the functions of the newly discovered genes. This will require methods of disrupting gene function when only the sequence and position in the genome are known. The term 'reverse genetics' has been used to describe this process.

Methods for reverse genetics fall into two classes. The first class includes what are essentially forward genetics screens — involving, for example, chemical and transposable element mutagenesis — that have been modified to obtain mutations in specific genes of interest. The second class of strategies includes directed approaches, in which the function of the relevant gene is specifically altered. Targeted gene replacement and double-stranded-RNA-mediated gene silencing fall into this category. *Drosophila* researchers have only recently had access to these technologies, but already several innovations have been developed. In this review, we discuss some of the ways in which these reverse genetics strategies have been used in *Drosophila*, with a focus on methods that allow gene disruption without previous knowledge of mutant phenotypes. We consider the strengths and weaknesses of the various technologies, provide examples of how they have been applied and, in the light of these developments, discuss the prospects for attaching phenotypic information to all of the putative genes that have been identified in the *Drosophila* genome.

Chemical mutagenesis

DNA-damaging agents, such as chemical mutagens, have been widely used to induce mutations in forward genetics studies. Chemical mutagenesis offers the advantages of a relatively high mutation rate and broad target range. Since its introduction in 1968 by Lewis and Bacher[3], ethyl methanesulphonate (EMS) has been the most commonly used chemical mutagen in *Drosophila*. EMS is an alkylating agent that produces primarily G/C to A/T transitions[4]. Although there is some effect of

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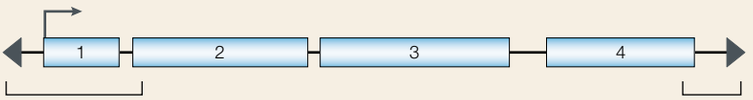
DOI: 10.1038/nrg752

Box 1 | Transposable element vectors: *P* and beyond

A full-length, wild-type *P*-element of 2,907 bp includes a 31-bp inverted repeat (triangles in figure) at each end and a gene for *P*-transposase, which comprises four exons (see figure). To be competent to respond to transposase, a *P*-element construct requires only a few hundred base pairs of *P*-element sequence from each end, including the inverted repeats[71]. Almost any desired sequences can be placed between these ends (brackets in figure). A marker gene, usually the *white* (w^+) or *rosy* (ry^+) eye-colour genes, is included so that integration and excision can be followed phenotypically. Additional sequences can be included to provide other features (FIG. 1). The transposition reaction follows a cut-and-paste mechanism[28,72]: in the excision phase, transposase cleaves within the two inverted repeats to remove a fragment with 17-nucleotide single-stranded 3′ ends[27]; the element then integrates into an 8-bp target site that becomes duplicated at either end of the insertion.

One feature that makes *P*-elements so useful as experimental tools is that standard laboratory stocks are devoid of them, and therefore *P*-element insertions are completely stable. Transposition can be induced at will by expressing transposase. This feature has allowed *P*-elements to become widely used in mutagenesis, gene tagging and transformation. However, the availability of additional transposable element vectors would considerably simplify some applications. For example, genome-wide gene disruption projects would benefit from the use of alternative transposable element vectors, because these might have target-site specificities that are different from those of *P*-elements.

Like *P*-elements, *hobo* elements are absent from many (although not all) laboratory stocks. Indeed, *hobo* has been used in transformation and enhancer trapping, in which it has been shown to have a different insertion specificity from that of *P*[73]. Another approach is to use transposable elements from other species. Although some elements show limited activity outside their endogenous host, several have recently been found to be more promiscuous[74]. Most notable are *mariner* (a *Tc1*-like element derived from *Drosophila mauritiana*), *Hermes* (a *hAT* element from the housefly) and *piggyBac* (cloned from the lepidopteran *Trichoplusia ni*). The use of these elements is not yet widespread, but they have the potential to be developed as new tools with applications in reverse genetics.



sequence context[5], most G/C base pairs are potential targets for EMS mutagenesis, and therefore the probability of inducing a mutation in a specific gene is closely related to the size of the gene. In a typical EMS mutagenesis, a 1-kb region undergoes a sequence alteration in almost 1 in 200 gametes[5].

Chemical mutagenesis has not been used extensively as a method for reverse genetics in *Drosophila*, largely because of the lack of efficient and high-throughput methods for detecting mutations. However, there have been some recent improvements. For example, several methods have been developed for the molecular detection of point mutations, most of which involve PCR amplification[6]. The most sensitive method is direct determination of DNA sequence, but most laboratories will find this to be prohibitively expensive for processing the 10^3–10^4 mutagenized lines that are generated in a typical screen. An alternative method that has recently been shown to be extremely sensitive, while offering the capacity for high throughput, is denaturing high-performance liquid chromatography (DHPLC)[5,7]. In DHPLC, PCR products are generated from heterozygous individuals, the product is heat denatured and the strands are allowed to re-anneal. If there is a sequence difference in the amplified region, a mixture of homoduplexes and mismatched heteroduplexes will be formed. This mixture is then separated on an HPLC column, at a temperature that causes partial denaturation. Because the mismatched regions of heteroduplexes will denature at a lower temperature than homoduplexes, the heteroduplexes will elute at a different time, and can be easily detected in the elution profile. One shortcoming of this procedure is that many deletions and other chromosomal rearrangements will be undetected.

To determine the feasibility of this method in *Drosophila* screens, Bentley *et al.* examined a 672-bp region of the *abnormal wing discs* (*awd*) gene by DHPLC[5]. Among the 4,988 mutagenized chromosomes that were screened, they detected 16 sequence alterations. Twelve of these were within the 459 bp of protein-coding sequence, but only half of these caused an amino-acid substitution. By contrast, a phenotypic screen for EMS-induced loss-of-function mutations in this gene resulted in a much lower recovery of ~1 in 15,000 (REF. 8) (the EMS dose used in this screen was half that used by Bentley *et al.*, which would be predicted to produce ~70% as many mutations[9]). DHPLC therefore seems to be a powerful method for detecting sequence alterations in a gene of interest.

As illustrated by the example above, many sequence alterations that are detected by DHPLC have no effect on the function of the gene. This can be advantageous for some purposes: the ability to recover an assortment of silent, HYPOMORPHIC and null mutations provides a relatively unbiased way to determine which regions of the gene, or amino-acid residues of the gene product, might be important to its function. The primary disadvantage of DHPLC is that it requires a substantial investment in equipment.

P-element insertional mutagenesis

Transposable element insertion is an extremely powerful means of gene disruption. The *P*-element of *Drosophila melanogaster* has been engineered to suit various purposes (BOX 1), including several for reverse genetics studies. The fastest and easiest way to use a *P*-element is simply to order a stock that carries an insertion in the gene of interest. Several systematic *P*-element gene disruption projects have generated thousands of stocks that each harbour a single *P*-element construct inserted at a known location in the genome[10] (TABLE 1). For many of these, the exact sequence at which the element is inserted is known, and it is possible to use the sequence of the gene of interest to search for matches among these flanking sequences. The Berkeley *Drosophila* Genome Project provides an option of searching against the database of flanking sequences on their BLAST search page. When using one of these lines, researchers should bear in mind that these chromosomes might carry other mutations that are not associated with the *P*-element insertion. Therefore, it is preferable to characterize phenotypes in HEMIZYGOUS or COMPOUND HETEROZYGOUS individuals. It is also important to ensure that the phenotype can be reverted by excising the element. The insertional mutagenesis projects have used several different *P*-element constructs, as described below and illustrated in FIG. 1.

HYPOMORPHIC
A partial loss-of-function allele, sometimes called weak or leaky.

HEMIZYGOUS
A diploid genotype that has only one copy of a particular gene, as in X-chromosome genes in a male, or when the homologous chromosome carries a deletion.

COMPOUND HETEROZYGOUS
A diploid genotype in which the two copies of a gene carry different mutations.

ENHANCER-TRAP CONSTRUCT
A transgenic construct used to identify genes that are expressed in specific tissues. When the construct inserts near a tissue-specific enhancer, the weak promoter on the construct comes under the control of the enhancer, resulting in tissue-specific expression of the reporter gene.

Table 1 | **P-element insertion stocks available from stock centres**

Chromosome	X	2	3
Bloomington* Drosophila *Stock Center			
P{LacW}	442	482	167
P{PZ}	0	245	277
P{EP}	166	0	0
P{GT1}	141	160	173
Total[a]	800	957	767
Szeged* P-*insertion Mutant Stock Centre			
P{LacW}	0	1204	2368
P{EP}	410	937	942
Exelixis, Inc. EP flyStation			
P{EP}	410	937	946

[a]Total number of stocks with *P*-elements mapped at least to a cytological region, including constructs not listed, as of January 2002.

Enhancer traps. *P*{PZ} and *P*{LacW} are ENHANCER-TRAP CONSTRUCTS[11]. Each carries a *lacZ* gene fused to the transposase promoter within the 5′ end of the *P*-element. Expression from this promoter can come under the control of regulatory elements in the vicinity of the insertion site. As *P*-elements frequently insert near the promoter of a gene[12], *lacZ* will often be expressed in the same spatial and temporal pattern as the gene into which the element is inserted. *P*{PZ} and *P*{LacW} also carry sequences that are necessary for propagation as circular plasmids in *Escherichia coli*, which allows DNA that flanks the insertion site to be cloned by the PLASMID-RESCUE technique. The primary difference between *P*{PZ} and *P*{LacW} is in the marker gene that is used to monitor transposition and excision: *P*{PZ} has a *rosy* (*ry*) marker, whereas *P*{LacW} has a *WHITE* MINI-GENE (*mini-white*) marker. The advantage of *ry*, which was the first marker to be used in *P*-element transformation[13], is that only a very low level of expression is required to confer a wild-type phenotype. By contrast, the *mini-white* marker gene is sensitive to copy number, DOSAGE COMPENSATION and insertion location. The same *mini-white* gene in different locations might result in yellow, orange or red eyes, corresponding to weak, moderate or complete rescue of the *white* mutant phenotype. Most current *P*-element constructs use the *mini-white* marker, because it allows changes in copy number, flanking sequences or genomic location to be easily detected.

Misexpression screening. The *P*{EP}-element was designed to do the opposite of an enhancer-trap construct. Rather than carrying a gene that can come under the control of nearby regulatory sequences, it uses the Gal4–UAS (upstream activator sequence) system to modify the expression of the gene into which it is inserted[14]. Gal4 is a transcription factor from *Saccharomyces cerevisiae* that activates transcription when it binds to a specific UAS near a promoter. It retains this activity when expressed in *Drosophila* cells[15]. *P*{EP} carries a UAS near one end, so if a *P*{EP}-element is inserted near the 5′ end of a gene, in the proper orientation, the expression of that gene can be induced by expressing *GAL4*. The Bloomington *Drosophila* Stock Center carries almost 100 stocks that express *GAL4* in different tissues or at different developmental stages, allowing controlled misexpression of *P*{EP} target genes in many desired patterns. About 2,300 *P*{EP} insertion lines are available for use in targeted misexpression screens (TABLE 1); many of these have been physically mapped by INVERSE PCR, so that they can be used as reverse genetics tools.

Elements for gene disruption. The aim of the gene disruption projects is to deliver a disrupting insertion into every gene. Although it is relatively straightforward to generate large numbers of *P*-element insertions, it is not uncommon for a given *P*-element insertion to fail to disrupt any gene. This is not surprising, as the *P*-element constructs described above were not specifically designed for gene disruption projects. The continuing *P*-element Gene Disruption Project, being carried out by the laboratories of Hugo Bellen, Gerald Rubin and Allan Spradling, uses two constructs, *P*{GT1} and *P*{SUPor-P}, that are designed to ensure a high frequency of gene disruption. In *P*{GT1}, the *mini-white* gene has its own promoter but lacks a polyadenylation signal sequence. It is therefore expressed at a low level, leading to a pale yellow eye colour. When *P*{GT1} inserts upstream of a functional polyadenylation sequence, increased *mini-white* expression results in orange or red eyes. This construct also carries a *GAL4* gene that lacks a promoter, but is preceded by an artificial splice acceptor sequence; Gal4 will therefore be expressed only if the construct is inserted downstream of an endogenous promoter or splice donor. This 'dual-tagging' method for gene trapping ensures that virtually all inserts that express both *mini-white* and *GAL4* disrupt a gene. In a test of this concept, Lukacsovich *et al.* analysed 27 *P*{GT1} insertions, and found that all of those that passed both tests disrupted a gene[16]. *P*{GT1} insertions should express Gal4 in the exact pattern of the interrupted gene, which also makes this an excellent enhancer-trap construct, and provides additional Gal4 expression lines for misexpression screens.

P{SUPor-P} uses a different strategy for gene disruption. In this construct, the *mini-white* gene is flanked by sequences that have been shown to insulate against position effects on expression[17]. These sequences, derived from the *gypsy* retrotransposon, are believed to block the interaction between enhancers and promoters[18,19]. Therefore, when a *P*{SUPor-P} element inserts into the 5′ regulatory region of a gene, it is more likely to cause a mutant phenotype than an insertion that does not block transcriptional regulators. In practice, a higher fraction of *P*{SUPor-P} inserts seems to be associated with lethality than for other constructs[18].

The *P*-element Gene Disruption Project has produced 581 *P*{GT1} insertions and 1,556 *P*{SUPor-P} insertions so far. Unique lines are deposited in the Bloomington *Drosophila* Stock Center as insertion sites are determined (TABLE 1). It is also possible to search the entire collection, including those not yet available from the stock centre, by genomic location or by target gene (see online links).

PLASMID RESCUE
A method for cloning DNA that flanks a transgenic construct. The construct carries a plasmid backbone and an antibiotic resistance gene. Genomic DNA from a transgenic line is restricted, circularized and transformed into bacteria. After selection for antibiotic resistance, plasmid DNA is recovered and sequenced.

WHITE MINI-GENE
A copy of the *white* gene in which non-essential sequences have been removed. In *mini-white*, either a heterologous promoter is used, or some of the *cis*-regulatory region is removed.

DOSAGE COMPENSATION
The process of compensating for differences in gene dosage between the sexes of organisms that use a chromosomal basis of sex determination. In *Drosophila*, males have one X chromosome, whereas females have two X chromosomes. Dosage compensation results in the increased expression of X-linked genes in males.

INVERSE PCR
A method for cloning DNA that flanks a known sequence. Genomic DNA is digested and ligated into circles, and is then subjected to PCR. Primers correspond to the known sequence, but point out from this sequence. In a circle that contains the known sequence, the unknown flanking sequence will be amplified.

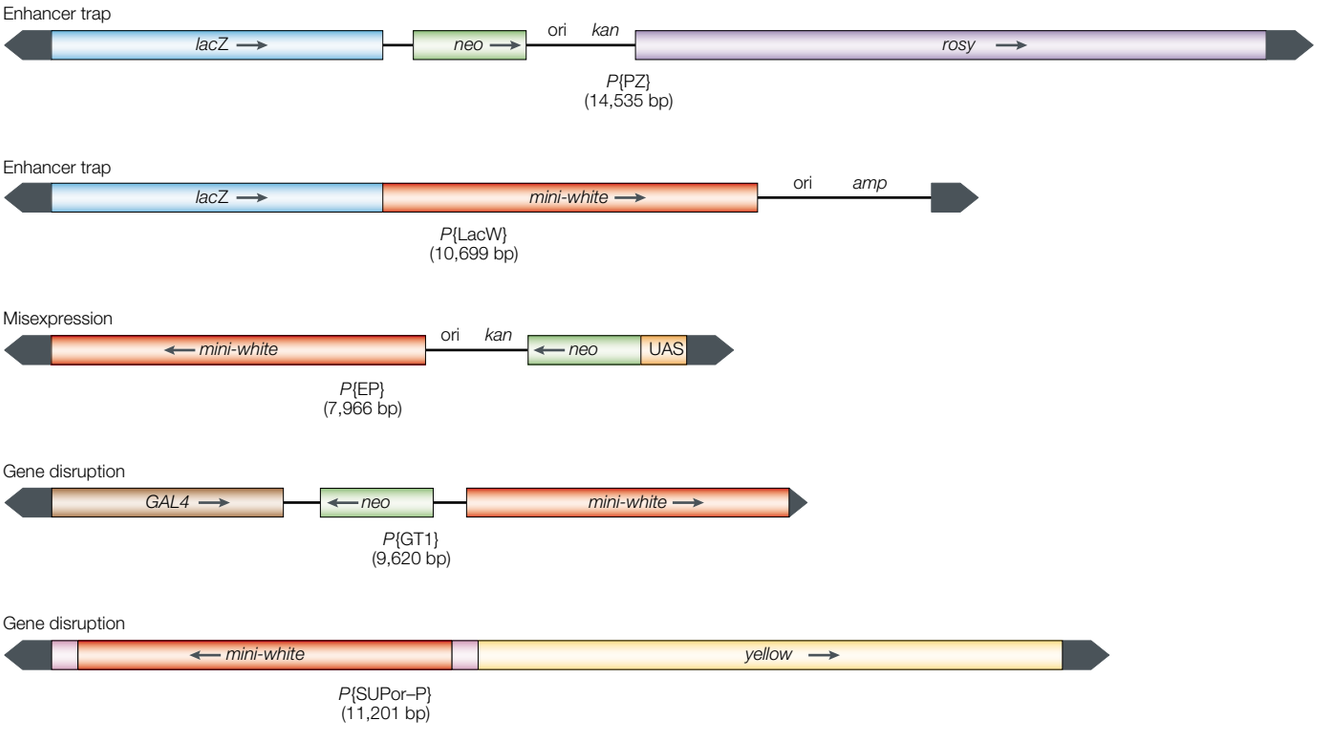


Figure 1 | ***P*-element constructs used for reverse genetics in *Drosophila*.** The *P*-element constructs described in the text are shown in schematic form, with the main features represented. Black arrows represent the *P*-element ends. *P*{PZ} has a *rosy* marker (purple); all other constructs have a *mini-white* marker (red). Three of the constructs also carry a *neo* gene (green), which provides selectable resistance to the antibiotic G418. *P*{PZ} and *P*{LacW} are enhancer-trap constructs that carry a *lacZ* gene (blue) at one end. *P*{EP} has an upstream activating sequence (UAS) (orange) at one end for use in targeted misexpression screens. *P*{PZ}, *P*{LacW} and *P*{EP} each also have a plasmid backbone with an *Escherichia coli* origin of replication (ori) and an antibiotic resistance gene (*kan*, kanamycin; *amp*, ampicillin). *P*{GT1} has a promoter-less *GAL4* gene (brown) at one end. The *mini-white* marker on *P*{GT1} lacks a polyadenylation signal sequence. The *mini-white* gene in *P*{SUPor-P} is flanked by sequences that protect against position effects (pink). This construct also carries a *yellow* gene (yellow). Maps and sequences of each of these elements are available from FlyBase (see online links box).

Local transposition. *P*-element insertion mutations are not yet available for most genes in the *Drosophila* genome. However, many genes are within 10–100 kb of an existing insert, and it is sometimes possible to use these to obtain an insertion into a gene of interest. Zhang and Spradling[20] showed that 'local transposition' of *P*-elements (at intervals of 1–200 kb from the starting site) can occur at a relatively high frequency (~1% of progeny chromosomes). Local transposition screens are easiest when the *P*-element construct carries a *mini-white* marker gene. Because *mini-white* expression is sensitive to both position effects and copy number, it is possible to recover potential transposition events as progeny that have a different level of eye pigmentation than the parental line. These are then screened for insertions into the gene of interest, typically by PCR-based assays. One method that has proved to be useful is to use a set of primers on one strand that are spaced at regular intervals in the gene, coupled with a *P*-element primer[21–23]. An alternative is to use inverse PCR, and to screen a Southern blot using a probe from the target region[24]. This approach has the advantage of being able to detect insertions throughout a relatively large region.

P-element excision mutagenesis

As mentioned above, *P*-element insertions often result in hypomorphic mutations because insertion sites are frequently near or within gene promoters[12]. Although such alleles can be useful in genetic analysis, it is usually desirable to have a complete loss-of-function mutation. Fortunately, there are several strategies in which *P*-element insertions can be used to generate new mutations close to the original insertion. These include methods to make small or large deletions that flank or surround an insertion site, and to bring about targeted replacement of sequences near an insertion (FIG. 2).

Imprecise excision. If a *P*-element is inserted within a few kilobases of a gene of interest, making a null allele can often be accomplished fairly easily using the 'imprecise-excision' (or more correctly, imprecise-repair) method[25,26]. The basis of this method is to induce many independent excisions of the *P*-element and to screen these for any that remove some of the genomic sequence that flanks the insertion site. In a typical imprecise-excision screen, flies that have both the chromosome that bears the *P*-element and a chromosome that expresses high levels of *P*-transposase are

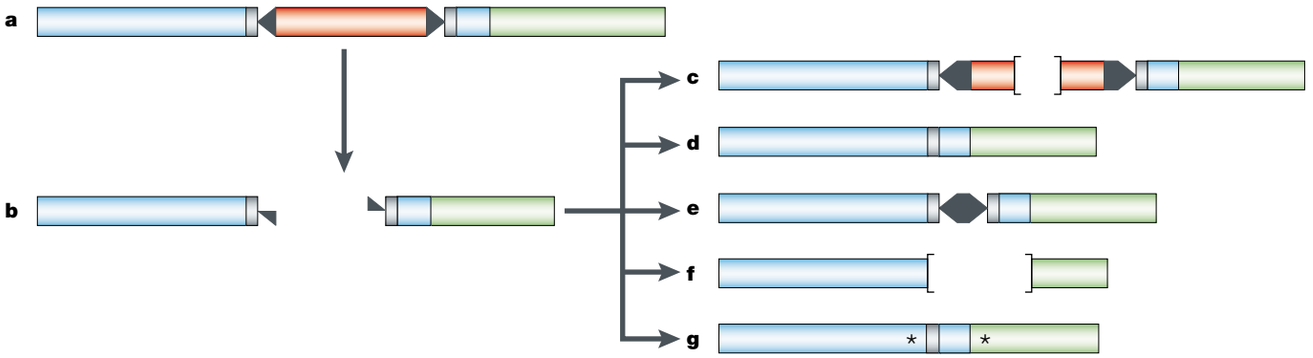


Figure 2 | ***P*-element mobilization mutagenesis. a** | A generic *P*-element construct is inserted into a genomic location near a target gene (green). Grey regions represent the 8-bp target-site duplication, and blue represents the intergenic region. **b** | *P*-transposase catalyses excision of the element, which leaves a double-stranded break with 3′ overhangs derived from the *P*-element inverted repeats (black triangles). Several possible repair products are represented on the right. **c** | Homology-directed repair from the sister chromatid can reproduce the *P*-element, possibly with an internal deletion. **d** | Homology-directed repair from the homologous chromosome can generate a 'precise excision'. **e** | Non-homologous end-joining leaves a footprint that contains sequences from one or both *P* inverted repeats. **f** | Small deletions that extend in one or both directions can be recovered. **g** | Gap repair using an ectopic template can be used to introduce specific sequence changes (asterisks) near the original insertion point.

generated by an appropriate cross. Transposase catalyses the excision reaction, in which the *P*-element is cut out of the donor site. This leaves a double-stranded break (DSB), with the ends that have 17-nucleotide single-stranded 3′ tails derived from the *P*-element inverted repeats[27] (FIG. 2b).

DNA-repair mechanisms can process the DSB by several pathways, which can generate different products (FIG. 2). These pathways fall into two classes: homology directed, in which the ends of the break are used to prime new synthesis from a homologous sequence; and non-homologous end-joining, in which the free ends are ligated to one another. Common end-points of homology-directed repair include restoration of an intact or internally deleted *P*-element (FIG. 2c), or restoration of the original chromosomal sequence without a *P*-element[28] (FIG. 2d). End-joining often leaves a small 'footprint' of 16–18 bp from one or both *P*-element ends[29] (FIG. 2e). For most purposes, these products are not desired, and therefore a strategy should be devised to eliminate them early in the screening process, either phenotypically or by the presence or absence of specific PCR products.

More useful for genetic analysis are the alternative outcomes of the repair process. Before repair, it is possible that the DSB can be enlarged to a gap, and that repair of the gap can result in a deletion that extends in one or both directions from the original insertion site (FIG. 2f). This outcome is often frequent enough (1 in 10 to 1 in 50 excisions that have lost the marker gene) that several such events can be recovered with only a moderate investment of labour. Because *P*-elements frequently insert into sequences between the promoter and protein-coding region, this method can be used to obtain deletions that completely destroy gene function by removing coding sequences (for examples, see REFS 30–32).

Repair with an ectopic template. Homology-directed repair preferentially involves the sister chromatid or the homologous chromosome. Occasionally, however, an ectopic template can be used. This property can be used to convert sequences that are adjacent to an existing *P*-element insertion by using a homologous sequence that is present elsewhere in the genome, such as on another *P*-element[33], or even on an injected plasmid[34]. This makes it possible to introduce site-directed mutations into a gene of interest — provided that a *P*-element is already inserted nearby, preferably within several hundred base pairs of the target for the sequence change. Because it is more straightforward to make a simple loss-of-function mutation by imprecise excision, this method of gene replacement has seen very limited use. Most examples have involved efforts to introduce specific site-directed mutations (reviewed in REF. 35) or to replace one *P*-element construct with a different one at the same location[36–38].

Hybrid element insertion. The methods described above are only practical when an insertion exists within a few kilobases of the gene of interest. However, it is also possible to make larger deletions from more distant *P*-elements. Large deletions are likely to remove more than the gene of interest, so this method has only limited use in reverse genetics. Most of the larger deletions (and some smaller deletions) are probably generated by a different process than the one described above, and so it is advisable to follow different strategies to optimize the probability of recovering such events. Many large deletions are probably the product of a process termed 'hybrid element insertion' (HEI)[39], which, unlike the imprecise repair described above, really can be considered as a form of imprecise excision (FIG. 3). *P*-transposase requires both 5′ and 3′ *P*-element ends for activity,

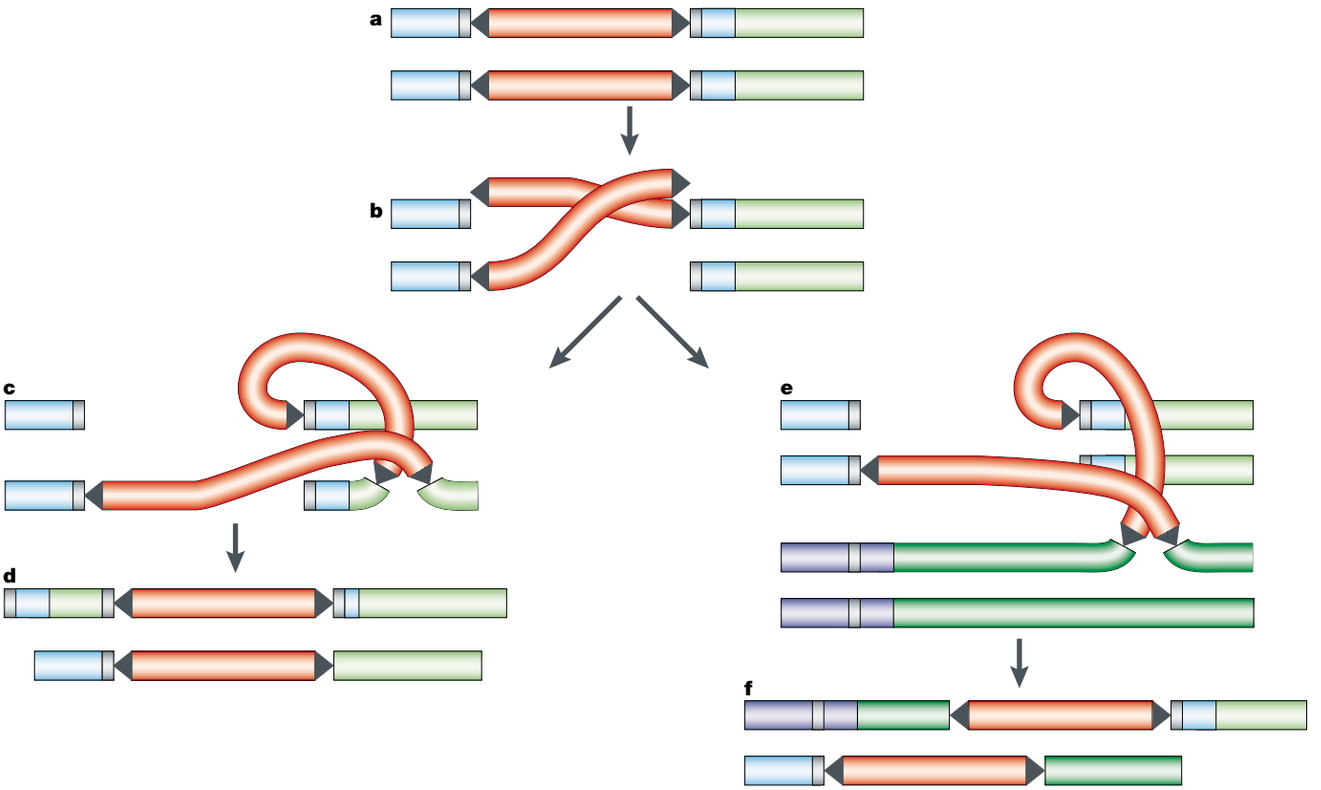


Figure 3 | **Generation of deletions by hybrid element insertion. a** | Sister chromatids carrying a *P*-element insertion near a gene of interest (green) are shown during G2. Grey regions represent the 8-bp target-site duplication, and blue represents the intergenic region. **b** | *P*-transposase cuts the 5′ end of *P* from one chromatid and 3′ end of *P* from the sister. Each end is still continuous with the rest of the chromatid. **c** | The two ends are integrated together into a nearby target location on one of the starting chromatids. **d** | One product (lower) carries a deletion from the starting insertion point into the target gene. The other chromatid (upper) carries a duplication of the same region. **e** | Alternatively, the hybrid element can be integrated into the target gene on the homologous chromosome (dark green). **f** | Integration on the homologue generates products that can carry either a duplication (upper) or deletion (lower). In addition, each product is recombinant for flanking markers on the chromosome.

but these two ends need not be linked *in cis*[27]. When transposition occurs during the G2 phase of the cell cycle, transposase can occasionally cut the 5′ end from one chromatid and the 3′ end from the sister chromatid (FIG. 3b). Integration of these ends as a unit will result in some type of chromosomal rearrangement[39]. If integration occurs near the original insertion (FIG. 3c), or in a comparable region on the homologous chromosome (FIG. 3e), it is possible to obtain a deletion[40] (FIG. 3d,f).

Characteristically, deletions made by HEI begin precisely at the original insertion point and extend unidirectionally. After HEI, a full-length *P*-element should be present adjacent to the deleted region (FIG. 3). If the *P*-element has a *mini-white* marker gene, the new flanking sequence might have different effects on expression than the original insert. Screening for changes in eye colour is, therefore, one method for recovering HEI deletions. An alternative is to screen for 'male recombination' events. An HEI integration on the homologous chromosome generates a crossover[40] (FIG. 3f). Because there is no meiotic crossing over in *D. melanogaster* males, HEI crossovers can be easily detected when they occur in the male germ line, even though the frequency is typically low (0.1–1% of progeny)[41,42]. Recovery of a crossover does not ensure that a deletion has been made; duplications are equally likely, and it is also possible that integration can occur at the same position on the homologous chromosome as the original insertion, resulting in a precise crossover[40]. This property has also been exploited as a method for mapping genes that have been discovered in forward genetics screens[41,42].

Overall, *P*-element mutagenesis is the method of choice for reverse genetics when an insertion is located within or very near the gene of interest. Unfortunately, the success rates of the various *P*-element methods can vary markedly between different insertions, and it is not possible to predict the probability of success with any specific insertion. Nonetheless, most researchers also resort to *P*-element mutagenesis when there is not an insertion nearby, due in part to the lack of alternative methods. Recently, two new methods for the directed disruption of gene function — targeted gene replacement and 'RNA interference' (RNAi) — have proved to be successful in *Drosophila*.

Targeted gene replacement

The aim of targeted gene replacement is to substitute precisely an endogenous gene with an introduced copy. The introduced copy can be engineered to carry a mutation that destroys or alters function in a defined manner, making this an extremely versatile method. Targeted gene replacement is commonly used in fungal and mouse genetics. In these experimental systems, linear DNA is introduced into cells, and the recombination machinery of the cell integrates this DNA into the chromosome, sometimes by homologous recombination with the endogenous gene. A crucial feature that makes this technology feasible is the ability to introduce linear DNA into a large number of cells simultaneously. In yeast, this is done by standard high-efficiency transformation techniques; in mice, DNA is electroporated into cultured embryonic stem cells. Neither of these approaches can be accomplished in *Drosophila* at present. Efforts to culture germ-line stem cells have been unsuccessful, and DNA can only be introduced into the germ line by microinjection into individual embryos.

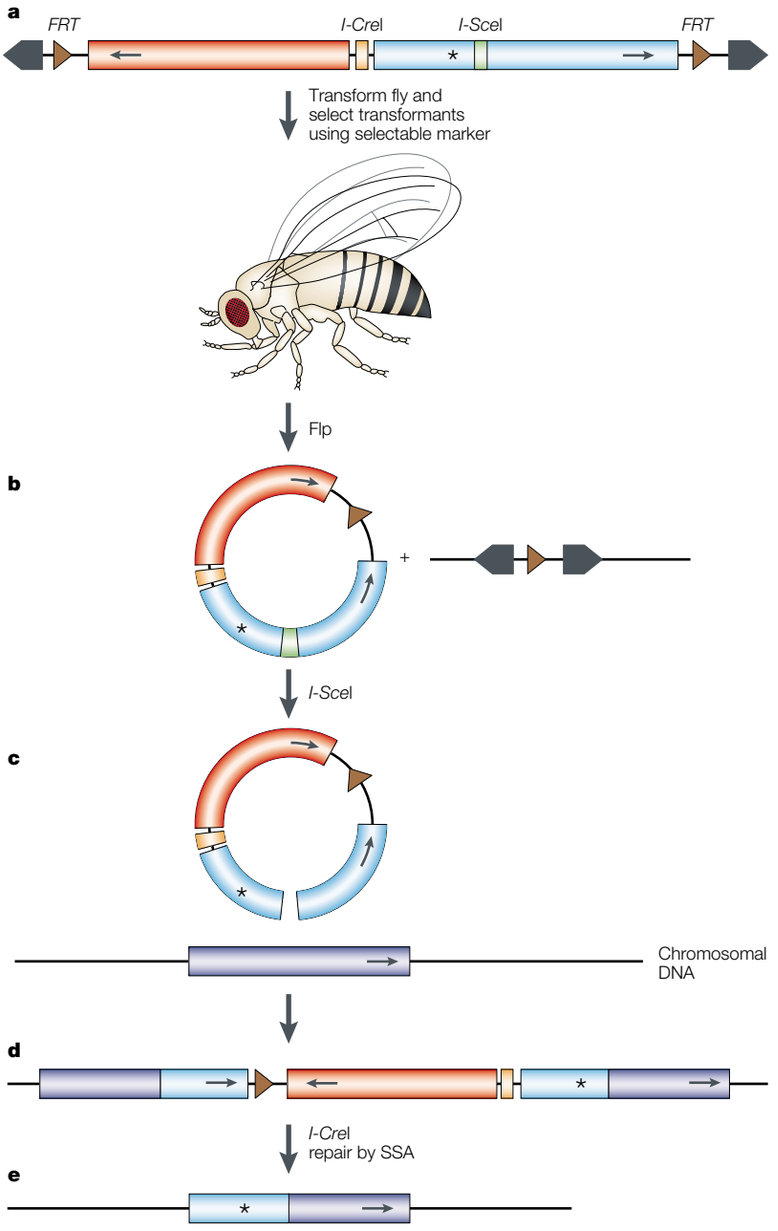


Figure 4 | **Targeted gene replacement in *Drosophila*. a** | The source of the targeting DNA is a donor constructed *in vitro* and introduced into the germ line by *P*-element-mediated transformation. The donor has genomic sequence homologous to the target (blue), two *FRT*s (brown arrowheads), and an *I-Cre*I site (orange). The genomic sequence is altered to carry a desired mutation (asterisk) and an *I-Sce*I site (green). **b** | The targeting DNA is generated *in vivo* by Flp recombinase, which excises the donor as an extra-chromosomal circle, leaving the *P*-element ends and one *FRT* at the chromosomal donor site. **c** | *I-Sce*I endonuclease then makes a double-stranded break in the excised donor. **d** | Recombination with the endogenous target sequence (dark blue) generates a tandem duplication. One copy of the duplication carries the introduced mutation. **e** | Reduction of the duplication to a single copy occurs after generation of a double-stranded break by *I-Cre*I cutting, and repair by the single-stranded annealing (SSA) pathway. The single copy might or might not carry the introduced mutation.

A clever solution to this problem is to generate the linear targeting DNA *in vivo*. To achieve this, Rong and Golic[43] first placed a donor construct (FIG. 4) into the genome by standard *P*-element germ-line transformation. The donor construct has four key features: a segment of DNA that is homologous to the region to be targeted, but carrying an engineered mutation in the gene of interest; a recognition site for *I-Sce*I — a rarely cutting restriction endonuclease for which there are no known sites in the *Drosophila* genome; a *mini-white* marker gene; and two 34-bp recognition sequences for the Flp site-specific recombinase (Flp recombination targets, or *FRT*s). Expression of the Flp enzyme *in vivo* catalyses recombination between the *FRT*s, resulting in excision of a circular DNA that carries the *mini-white* marker gene and the targeting DNA. Expression of the *I-Sce*I enzyme *in vivo* then cleaves the excised circle, thereby producing a linear fragment with ends that are homologous to the region being targeted.

The precise molecular mechanisms of homologous recombination between the donor and target are not completely understood, but several outcomes are possible, so it is important to characterize the products in sufficient detail to ensure that the desired molecular structure is obtained. In the experiments of Rong and Golic, a single DSB is made in the region of homology (FIG. 4c). The expected product is therefore a tandem duplication (FIG. 4d). This has important consequences for the design of the donor construct; if a single mutation is engineered into the donor, then only one copy of the duplicated region will carry the mutation. One strategy to deal with this issue is to introduce mutations on both sides of the insertion. Rong and Golic used this method to disrupt the *pugilist* (*pug*) gene[44]. In their experiment, an internal fragment of *pug* was used in the targeting construct. The resulting duplicated region contains one copy of the gene that lacks the 5′ end and another that lacks the 3′ end. Because efficient targeting is thought to require at least 2 kb of sequence homology, use of only a fragment of the gene in the targeting cannot be done for very small genes, unless flanking sequences are also included. Likewise, making point mutations on both sides of the *I-Sce*I site can also be problematical for very small genes. It is likely that mutations that are too near the *I-Sce*I site can be lost during

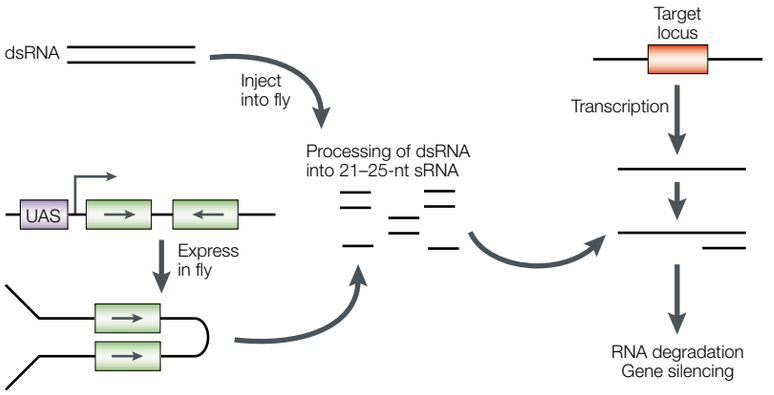


Figure 5 | **RNA interference in *Drosophila*.** Double-stranded RNA (dsRNA) that is homologous to the target gene is delivered by either of two methods: injection of *in vitro*-transcribed RNA into individual embryos (upper left), or expression of an inverted repeat RNA *in vivo* (lower left). The dsRNA is processed into 21–25-nucleotide (nt) small interfering RNAs (siRNA) by the Dicer ribonuclease[75]. The siRNA is used to guide the sequence-specific degradation of mRNA, leading to post-transcriptional silencing of the target locus. UAS, upstream activating sequence.

the targeting. Until this is studied systematically, it is advisable to place mutations at least several hundred base pairs from the *I-Sce*I site.

To circumvent these difficulties, Rong and Golic have devised a more versatile strategy (Y. Rong and K. Golic, personal communication). In yeast, when a DSB is made between two copies of a duplication, a repair pathway termed 'single-stranded annealing' (SSA) leads to recombination between the two copies[45]. As a result, only a single copy of the gene is retained and intervening sequences are lost. SSA or a similar pathway also operates efficiently in *Drosophila*[43]. This is where the *I-Cre*I (another rarely cutting endonuclease) site on the targeting construct comes into play: expression of the *I-Cre*I enzyme generates a DSB between the duplicated regions, resulting in high-frequency reduction to a single copy (FIG. 4e). We have found this step to be extremely efficient (M.D.A. and J.J.S., unpublished data; T. Donaldson and R. Duronio, personal communication).

The ability to reduce the product of targeting to a single copy makes it possible to do knock-ins as well as knockouts, so that specific site-directed mutations (missense mutations, GFP (green fluorescent protein) or epitope tags, and so on) can be made. In the past, this has been accomplished by putting a transgene that carries the desired mutation or a tagged copy of the gene into a genetic background that is deficient in the endogenous gene. However, transgenes are often sensitive to position effects and, with this approach, several independent insertions need to be evaluated[46–48]. In the gene replacement method, the site-directed mutation is made at the endogenous locus, so position effects should not be a factor.

This method for targeted gene replacement is somewhat lengthy, requiring at least six months for the entire procedure. Before doing the targeting crosses, a construct must be designed and built, and transformants must be generated. Different donor inserts of the same construct can generate targeted events at vastly different frequencies (Y. Rong and K. Golic, personal communication; M.D.A. and J. J.S., unpublished data). At present, it is not possible to predict which donor inserts will work best, so it is advisable to establish several lines that carry different inserts. In spite of the relatively long time from initiation to generation of a targeted mutant, we believe that the versatility and high probability of success will make this a widely used method for reverse genetics in *Drosophila*.

RNA interference

The past few years have seen the discovery of a remarkable method of gene regulation termed RNAi[49]. In RNAi, double-stranded RNA (dsRNA) is cleaved *in vivo* into short fragments that guide sequence-specific mRNA degradation or translational repression[50–52] (see animation in REF. 49). RNAi-based phenomena have been observed in many organisms, and are possibly derived from an ancient mechanism to combat viral infections and to tame transposable elements. In animals, RNAi is also used to regulate the expression of specific genes in development[53,54].

Experimentally introduced dsRNA can trigger the RNAi effect in a wide range of experimental organisms, providing a powerful method for disrupting gene function[55]. In *Drosophila*, several methods have been used to deliver dsRNA. The simplest method works for cultured cells: dsRNA added directly to the culture medium silences gene function within 72 h. This can be a powerful method for working out signal-transduction pathways[56], cell-biological phenomena[57,58] and the mechanism of RNAi itself[59].

Although RNAi in cultured cells is rapid and relatively easy, the goal of most reverse genetics studies is to determine phenotypes that are associated with the loss of gene function in the organism. Two methods that use RNAi have been developed to do this in *Drosophila* (FIG. 5). The first relies on the delivery of dsRNA to embryos by microinjection[60]. A main advantage of this method is that both maternally supplied mRNA and zygotically expressed mRNA are degraded. Also, results are obtained within a few days of injection. It is important to keep in mind that injected dsRNA might not produce a phenotype in every embryo, and the severity of the phenotype might vary between embryos. Furthermore, the injection procedure itself can cause aberrant phenotypes or death in some embryos. It is therefore important to report the frequency of any observed phenotype in a population of injected embryos, and to compare this with control injections of non-homologous dsRNA.

Unfortunately, microinjection of dsRNA into *Drosophila* embryos does not efficiently interfere with gene function later in development[60]. An alternative that might work at all stages and in all tissues is to generate the dsRNA *in vivo*. This can be accomplished by expressing an RNA with a long inverted repeat that can fold back on itself to become double stranded[61–63] (FIG. 5). Bacterial hosts do not readily propagate most inverted repeat sequences during cloning, but the stability of these sequences increases markedly if the repeats are interrupted by a spacer of at least 50 bp (REF. 64).

A principal advantage of RNAi by expression is the possibility of interfering with gene function in specific tissues or at specific times during development. This can be done using the Gal4–UAS system described above[15]. Care should be exercised in interpreting results, however, because not all *GAL4*-expressing lines are equally proficient in this method, and insertions of the same UAS–inverted repeat construct into different genomic locations can be associated with different levels of silencing[63]. The effects of *in vivo* RNAi might also be altered by temperature[65]. This method requires a few months for construction of the transgene and generation of transgenic flies, but these transgenic lines are stable and can easily be used for a variety of subsequent experiments.

The future of genome-wide reverse genetics

The completion of several genome-sequencing projects has resulted in the initiation of genome-wide reverse genetics projects in several organisms. Directed approaches have been used in *Saccharomyces cerevisiae* (by targeted replacement of all genes in the genome[66]) and in *Caenorhabditis elegans* (by genome-scale RNAi[67–70]). Unfortunately, for the reasons discussed in this article, neither of these directed approaches is, at present, feasible for large-scale genome projects in *Drosophila*. Nevertheless, both targeted gene replacement and RNAi are relatively immature technologies, and no doubt many improvements, enhancements and extensions to these methods will be developed in coming years.

For the present, then, random mutagenesis approaches seem to have the most promise for *Drosophila*. The *P*-element insertional mutagenesis projects continue to provide an extremely valuable resource for the *Drosophila* community. The first large-scale project, which combined resources from several different screens, succeeded in generating mutations in ~25% of all essential genes[10]. These strains, as well as the enhancer-trap and gene-trap strains, are readily available from stock centres (see TABLE 1 and online links box).

It is unlikely that the goal of producing a *P*-element insertion in every gene will ever be realized. The use of additional transposable element vectors with different target specificities might increase the number of gene interruptions available (BOX 1), but alternative genome-scale reverse genetics approaches will be essential. Random chemical mutagenesis, which for many years was the method of choice for forward genetics screens, could provide an important complement to insertional mutagenesis. A centralized facility could readily generate several thousand stocks, each of which carries a single mutagenized chromosome. These could then be screened by a method such as DHPLC, either systematically or by contract with researchers who seek mutations in specific genes. This strategy is well suited to a large facility because much of the process can be automated.

With the reverse genetics methods now in hand, it should be possible for individual laboratories to conduct thorough genetic analyses of almost any desired sequence in the genome. Given the long history of innovation and development of genetic tools in *Drosophila*, we expect to see additional methods becoming available in the next few years.

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Acknowledgements

We thank Y. Rong and K. Golic for communicating unpublished results. M.D.A. was supported by a postdoctoral fellowship from the American Cancer Society.

Online links

DATABASES

The following terms in this article are linked online to:

LocusLink: http://www.ncbi.nlm.nih.gov/LocusLink/
abnormal wing discs | Dicer | *pugilist* | *rosy* | *white*

FURTHER INFORMATION

Berkeley *Drosophila* Genome Project: http://www.fruitfly.org/
Bloomington *Drosophila* Stock Center: http://fly.bio.indiana.edu/
***Drosophila* Virtual Library:** http://ceolas.org/fly/
Encyclopedia of Life Sciences: http://www.els.net/
Genome, proteome and the quest for a full structure–function description of an organism
European *Drosophila* Genome Project:
http://edgp-dev.ebi.ac.uk/
Exelixis, Inc. EP flyStation: http://cdigraphics.com/flystation2/
FlyBase: http://flybase.bio.indiana.edu/
***P*-element Gene Disruption Project:**
http://flypush.imgen.bcm.tmc.edu/pscreen/
Szeged *P*-insertion Mutant Stock Center:
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