THE ART AND DESIGN OF GENETIC SCREENS: *DROSOPHILA MELANOGASTER*

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The success of *Drosophila melanogaster* as a model organism is largely due to the power of forward genetic screens to identify the genes that are involved in a biological process. Traditional screens, such as the Nobel-prize-winning screen for embryonic-patterning mutants, can only identify the earliest phenotype of a mutation. This review describes the ingenious approaches that have been devised to circumvent this problem: modifier screens, for example, have been invaluable for elucidating signal-transduction pathways, whereas clonal screens now make it possible to screen for almost any phenotype in any cell at any stage of development.

POLYTENE CHROMOSOME
A giant chromosome that is formed by many rounds of replication of the DNA. The replicated DNA molecules tightly align side-by-side in parallel register, which creates a non-mitotic chromosome that is visible by light microscopy.

PROTOSTOME–DEUTEROSTOME
The two principal divisions of animal phyla, based on how the mouth forms in the embryo.

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The fruitfly *Drosophila melanogaster* has been one of the favourite model organisms of geneticists, since Thomas Hunt Morgan decided to use it to investigate the chromosomal theory of inheritance at the beginning of the last century[1]. Morgan chose *Drosophila* because it is easy and cheap to rear in the laboratory, has a ten-day generation time and produces many progeny. However, he soon discovered that it has several other advantages for genetic analyses. For example, there is no meiotic recombination in males, and there are only four chromosomes, which can be directly visualized in the giant POLYTENE CHROMOSOMES of the larval salivary gland. Furthermore, its exoskeleton provides a wealth of external features, such as bristles, wing veins and compound eyes, which can be affected by mutations, and for which the resulting mutant phenotypes can be scored simply by looking down the stereomicroscope. This early start has been built on by succeeding generations of drosophilists, who have developed an ever-increasing repertoire of techniques that make *Drosophila* one of the most tractable multicellular organisms for genetic analysis[2]. In fact, *Drosophila* has only one main drawback, which is that the stocks have to be continuously maintained in the laboratory because it is not possible to freeze them (and successfully revive them afterwards).

An unfortunate feature of genetic model organisms is that the easier they are to work with, the worse they are as models for the animal that most funding agencies find most interesting, namely ourselves. In this respect, however, *Drosophila* provides a very happy compromise. A surprisingly large number of developmental processes seem to be conserved between flies and vertebrates, even though they diverged at the PROTOSTOME–DEUTEROSTOME split ~700 million years ago. To cite two of the more famous examples: the dorsoventral (D/V) axes of the *Drosophila* and vertebrate embryo are patterned by opposing gradients of Decapentaplegic (Dpp; BMP4 (bone morphogenetic protein 4) in vertebrates) and Short gastrulation (Sog; CHRD (chordin) in vertebrates), even though the orientation of the axes is reversed; whereas Hedgehog (Hh) and its vertebrate counterpart, sonic hedgehog (SHH), have remarkably similar roles in limb patterning in both systems[3,4]. The sequencing of the *Drosophila* genome has now revealed the true extent of these similarities[5]. *Drosophila* has only ~15,000 genes, which is fewer than has *Caenorhabditis elegans*, but twice as many of these have clear homologues in humans (*E*-value $<10^{-50}$)[6]. Furthermore, 197 out of 287 known human disease genes have *Drosophila* homologues, and even those that do not can produce very similar symptoms when expressed in flies[7,8]. So,

Box 1 | **Mutagenesis in *Drosophila***

Ethyl methane sulphonate

This is the most commonly used mutagen in *Drosophila* because it is easy to administer and causes the highest frequency of mutations. It mainly induces single-base changes (point mutations), which disrupt gene function by causing missense or nonsense mutations, and the frequency at which a gene can be mutated therefore depends on the size of the coding regions and the number of crucial amino acids that it contains. Using the standard mutagenesis protocol with 25 mM ethyl methane sulphonate (EMS), the mutation rate for the average gene is ~1 in 1,000 (REF. 93). This varies enormously, however, and mutations in very large genes, such as *dumpy* (*dp*) are recovered at more than 20 times this rate. A disadvantage of EMS in the past has been that it was very difficult and laborious to map point mutations to specific genes. This problem has been solved largely by the development of single nucleotide polymorphism (SNP) maps that allow the rapid meiotic mapping of mutations to regions of less than 50 kb, and SNP maps that are specifically designed for mapping mutants from *FRT* (Flp recombinase target) screens (see main text) are now available[94,95]. A second drawback of EMS is that the progeny of mutagenized males are often mosaic (that is, some of the cells carry the mutation, whereas others do not), and mutants identified in F_1 screens will therefore not be transmitted to the next generation, unless the germ cells are also mutant. This mosaicism arises because an EMS-induced base change in one strand of the spermatid DNA segregates from the unmutated strand during the first zygotic division, if the mismatch has not been repaired yet. To overcome this problem, F_1 screens are often carried out using X-ray irradiation as the mutagen. This is about an order of magnitude less efficient than EMS, but induces mainly double-stranded DNA breaks that do not cause mosaicism. Because many X-ray-induced mutations are chromosomal rearrangements or deletions, they can often be detected cytologically in larval polytene chromosomes, which allows mutations to be mapped rapidly to a region and then identified on Southern blots.

***P*-transposable elements**

Another popular strategy is to screen for mutations caused by *P*-element insertions, because the mutated gene can be rapidly and easily identified using the *P*-element as a tag. *P*-elements are very inefficient mutagens, however, so the most common approach is to screen the large collection of *P*-element insertions that are available from the Berkeley *Drosophila* Genome Project, rather than generating new insertions by mobilizing the *P*-element oneself. The existing collection contains insertions in about one-quarter of the essential genes[89]. Because most genes are predicted to be cold spots for *P*-element insertion, saturation screens cannot be carried out, but this type of screen does provide a very efficient way of identifying some of the genes that are involved in a process.

there are now more reasons than ever for taking advantage of the powerful genetics of *Drosophila* to investigate the basic biological questions that are common to flies and humans. One of the most important tools that *Drosophila* provides is the ability to carry out large-scale genetic screens for mutations that affect a given process or, to coin a term I recently heard from a mouse geneticist, "forward functional genomics". The advantage of this approach is that it provides an unbiased way to identify the genes that function in a particular process, whereas the mutants themselves are a very valuable resource for dissecting the function of the gene.

Traditional genetic screens

Although the early drosophilists isolated many visible mutations, these were all spontaneous alleles from natural populations, and genetic screens only became possible once better ways to generate mutations were developed. The most efficient method to do this is to feed flies ethyl methane sulphonate (EMS), which induces point mutations, following the protocol described by Lewis and Bacher in 1968 (REF. 9) (BOX 1).

Until 1980, most drosophilists were still preoccupied with understanding the nature of the gene, and most mutagenesis studies were designed to discover new alleles of existing genes or to find out how many genes there were in a particular region of the genome. This all changed when Christiane Nüsslein-Volhard and Eric Wieschaus published their Nobel-prize-winning *Nature* paper on mutations that affect the patterning of the embryo[10] (FIG. 1a). This work was revolutionary, because it was the first mutagenesis in any multicellular organism that attempted to find most or all of the mutations that affect a given process, and because it was one of the first screens for phenotypes in the embryo rather than the adult, which allowed them to identify null or strong mutations in most of the essential patterning genes that are used throughout development[11] (FIG. 2a,b). As Peter Lawrence pointed out, half of the talks at the *Drosophila* meeting in Crete ten years later were about genes that were identified in this screen, which gives some idea of the impact of the paper[12]. Two features of *Drosophila* development had a profound effect on the success of the screen. First, because *Drosophila* has an exoskeleton, the larval cuticle provides an exquisite readout of the patterning of the embryo. Second, *Drosophila* embryogenesis has evolved to occur as rapidly as possible, and the mother therefore loads the egg with most of the products of genes that do not need to be transcribed in a precise pattern in the embryo[13]. This means that, in contrast to other organisms, very few mutations block embryonic development at early stages, and most mutants in housekeeping genes complete embryogenesis and secrete a normal cuticle. The screen was therefore very efficient at identifying the transcription factors and signalling molecules that generate positional information in the embryo.

No genetic screen can find everything, and it is worth considering what sort of genes could not be identified in the famous Heidelberg screen. The analysis of the zygotic genes that pattern the anteroposterior (A/P) and D/V axes of the larvae revealed that the genes at the top of the hierarchy (the gap genes, and the D/V genes, such as *dpp*, *zerknullt* (*zen*), *twist* (*twi*) and *snail* (*sna*)) are already expressed in discrete domains, even though they are among the first genes to be transcribed in the embryo. This indicated that they must be regulated by maternal determinants that are deposited in the egg. To find these factors, the Nüsslein-Volhard lab, and Eric Wieschaus and Trudi Schüpbach, carried out saturation mutageneses for maternal-effect mutations, which identified many of the genes that are involved in generating the four maternal signals that define the two main axes of the embryo[14,15] (FIG. 2c).

A second class of genes that were missed in the screen comprises those that have specific roles in the patterning of internal structures, such as the nervous system, because it is obviously impossible to identify mutants that have no effect on the structure that is being screened. Indeed, several groups have subsequently used a very similar approach to carry out large-scale screens for mutants that affect the organization of the central nervous system, which led to the discovery of genes that

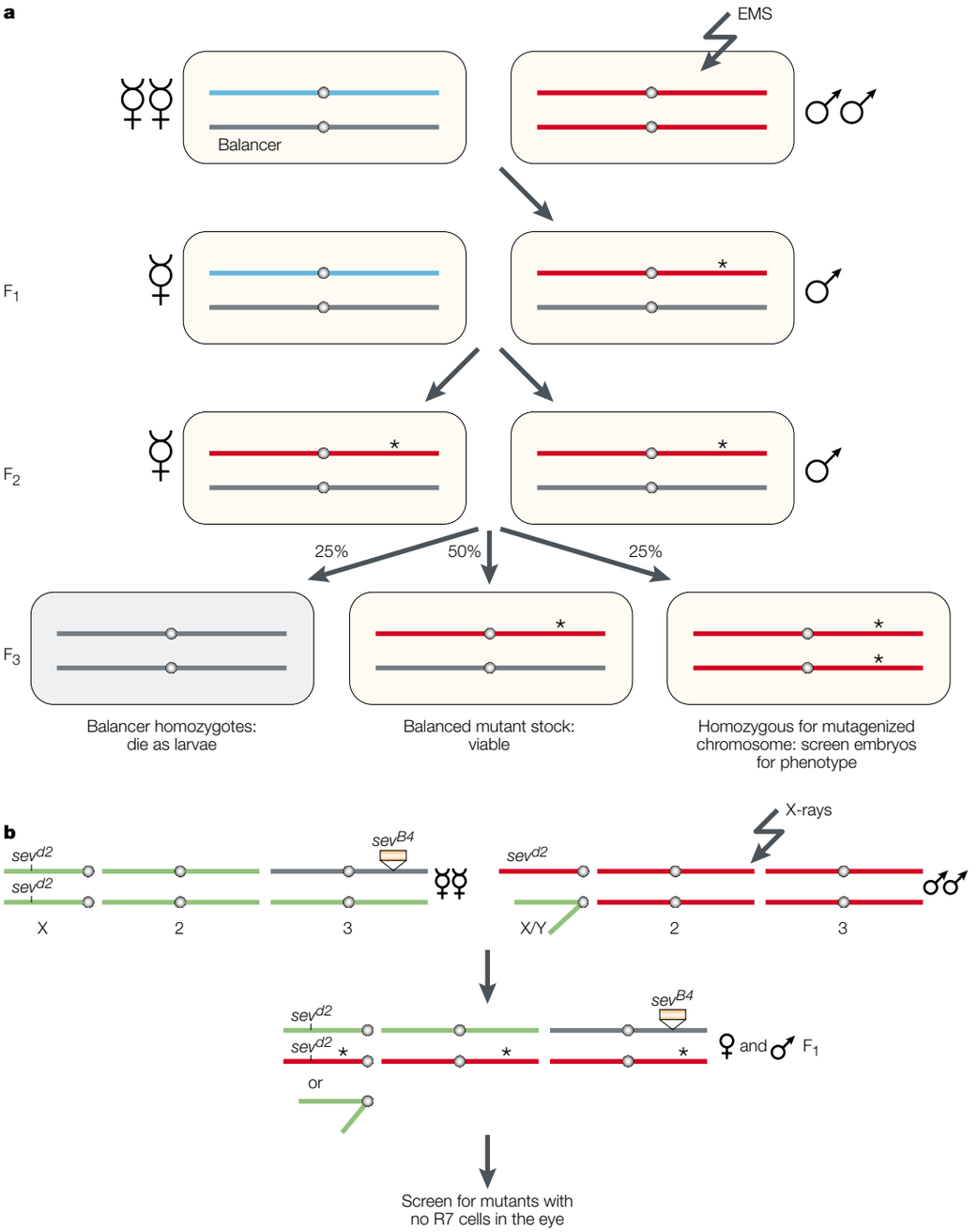


Figure 1 | **Outline of the crossing schemes used in the Heidelberg screen and the first screen for enhancers of *sevenless*.** **a** | A typical crossing scheme for a screen for mutants on an autosome that produce a zygotic phenotype, based on those used in the Heidelberg screens for mutants that affect the pattern of the larval cuticle. Male flies are fed ethyl methane sulphonate (EMS) to induce mutations and are crossed *en masse* to virgin females (indicated by the symbol ☿) that carry a BALANCER for the chromosome to be screened (grey). As the mutations are induced in mature spermatids, each F_1 male inherits a mutagenized chromosome (red) carrying a different spectrum of mutations (asterisk). Single F_1 males that carry a mutagenized chromosome *in trans* to the balancer are then backcrossed to balancer stock to generate F_2 males and females that carry the same mutagenized chromosome. When these are crossed to each other, 25% of the F_3 progeny will be homozygous for the mutagenized chromosome. As only one chromosome is screened at a time, the other chromosomes are not shown. In the Heidelberg screens, the lines were first screened for the absence of flies that were homozygous for the mutagenized chromosome, which indicated that it carried a zygotic lethal mutation, and cuticle preparations were then done on the embryos from these crosses to see if 25% showed a phenotype. In the screens for MATERNAL-EFFECT MUTATIONS (see main text), the eggs laid by homozygous females from non-lethal lines were screened for phenotypes. **b** | The crossing scheme for the original screen for enhancers of *sev* (REF. 32). Males that are hemizygous for a null allele of *sev* (sev^{d2}) were mutagenized with X-rays and crossed to sev^{d2} homozygous females that carry a temperature-sensitive allele of *sev* (sev^{B4}) as a transgene inserted on the third chromosome balancer, TM3. The F_1 TM3 flies were then screened for a reduction in the number of R7 photoreceptor cells in the eye.

BALANCER CHROMOSOME
A chromosome with one or more inverted segments that suppress recombination. They are used as genetic tools because they allow lethal mutations to be maintained without selection.

MATERNAL-EFFECT MUTATION
Homozygous-viable mutation that causes little or no phenotype in the mutant mothers, but leads to the development of abnormal offspring.

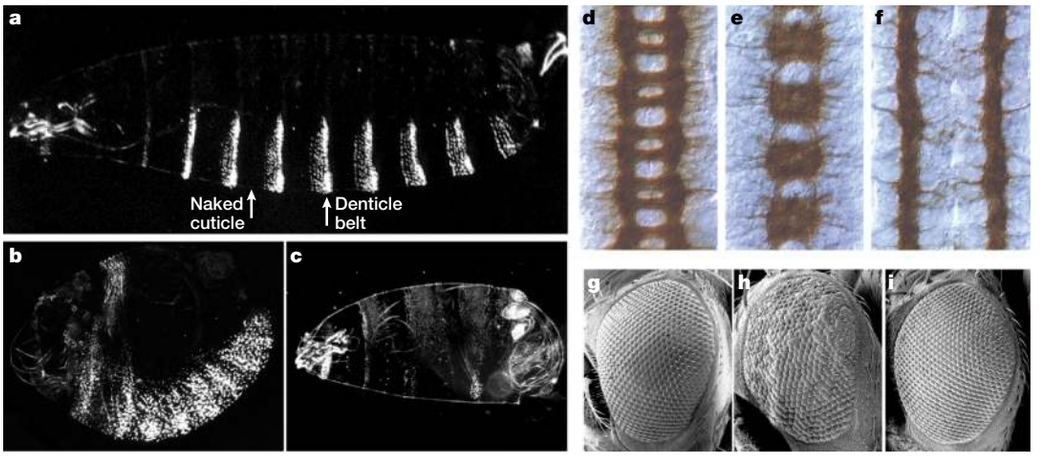


Figure 2 | **Examples of mutant phenotypes from standard F_3 screens, and from a screen for enhancers and suppressors of a rough-eye phenotype. a–c** | Cuticle preparations of first instar larvae. **a** | Wild type. The alternating denticle belts and naked cuticle along the anteroposterior axis (left–right) are indicated. **b** | A *wingless* mutant embryo, showing the loss of naked cuticle between the denticle belts in the ventral region of each segment. **c** | An embryo laid by a female that is homozygous for a null mutation in *staufen* (*stau*), which disrupts the localization of the anterior and posterior determinants. The embryo has a reduced head and no abdomen, but forms a normal thorax and telson (the posterior-most region of a *Drosophila* embryo). **d–f** | The central nervous system of the embryo stained with a marker for longitudinal and commissural axons. **d** | In the wild-type embryonic nervous system, each segment contains two commissures that cross between the longitudinal axonal tracts. **e** | In *roundabout* mutants, IPSILATERAL AXONS are no longer prevented from crossing the midline, and axons that should only cross once, recross multiple times. **f** | In embryos that are mutant for *commissureless*, which encodes a product that is required for axon guidance, most axons fail to cross the midline, and the commissures fail to form. **g–i** | Scanning electron micrographs of adult eyes. **g** | Wild type. **h** | A rough eye produced by overexpression of the Egfr (Epidermal growth factor receptor) inhibitor, Argos (GMR–*argos*/+). **i** | A mutation in *sprouty* (*sty*) acts as a dominant suppressor of the GMR–Argos rough-eye phenotype. (Panel **b** courtesy of Bénédicte Sanson, University of Cambridge, UK; panels **d–f** courtesy of Guy Tear, MRC Centre for Developmental Neurobiology, Guy's Campus, London, UK, and reproduced with permission from REF. 100 © (1998) Elsevier Science; panels **g–i** courtesy of Matthew Freeman, MRC–LMB, Cambridge, UK, and **h**, **i** reproduced with permission from REF. 44 © (1999) Elsevier Science.)

control axon pathfinding, such as *roundabout* (*robo*) and *commissureless* (*comm*)[16–19] (FIG. 2d–f). As our ability to visualize the details of patterning has advanced, more sophisticated screens have become possible. For example, a recent screen used a Gal4 driver (BOX 2) to label the dendrites of peripheral neurons with green fluorescent protein (GFP), and this has allowed the discovery of new genes that control dendritic morphology[20–22].

Although the embryonic lethal screen identified mutants in most of the signalling molecules that are involved in patterning the embryo, such as Wingless (Wg), Dpp, Hh, Spitz (Spi; an epidermal growth factor-like molecule) and Delta (Dl), they were much less successful at finding the other components of the signal-transduction pathways through which these ligands act. For example, the only components of the Wg pathway that give a clear SEGMENT-POLARITY phenotype in homozygous mutant embryos are *wg* itself, *arrow* (*arr*), *armadillo* (*arm*; also known as β-catenin) and *pangolin* (*pan*; also known as *TCF/LEF1*), which interact to form the transcriptional activator at the end of the pathway[23–26]. Many of the remaining components, such as the proteins that are required for the processing and secretion of Wg, the Frizzled (Fz) receptors, Dishevelled (Dsh) and those of the Adenomatous polyposis coli (Apc) complex, were not identified, because their maternal contribution is sufficient for early Wg signalling. Wingless signalling is used over and over again in development, however, and mutants in these genes could not be found in the screens for maternal-effect mutations, because they need to be expressed zygotically to mediate later Wg signalling events and are therefore lethal. This problem applies to many essential proteins that are supplied maternally to the embryo, as homozygous mutants in these genes only show a zygotic phenotype when the maternal contribution runs out, and this often happens gradually, making the phenotype hard to analyse.

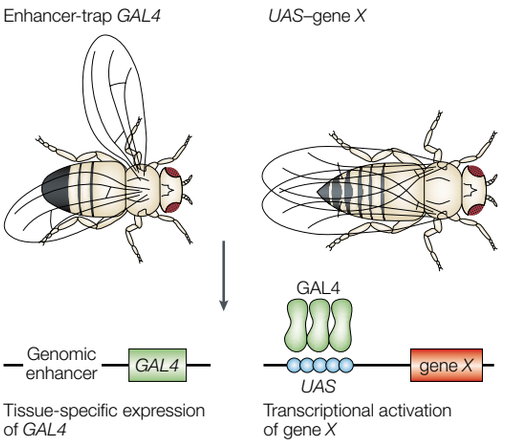
The discussion above illustrates a general limitation of genetic screens for homozygous lethal phenotypes, which is that only the first essential function of a gene can be analysed. Indeed, Eric Wieschaus and others have carried out an elegant series of experiments that have taken this to its logical extreme. They looked at embryos that were homozygous for a series of large deletions (also called deficiencies) that remove hundreds of genes at the same time. Because most genes are not required zygotically for early development, the first defects in the embryo reveal the phenotype of the gene within the deficiency that has the earliest essential function. By using a series of deletions that span most of the genome, they could therefore identify most or all of the regions that contain these early-acting genes, and this led to the surprising conclusion that development to the cellular blastoderm stage requires only seven zygotic genes[27,28]. As deficiencies are equivalent to null mutations in all of the genes that are deleted, this type of deficiency screen provides a rapid way to scan the genome for zygotically acting genes that are

IPSILATERAL AXON
An axon that does not cross the midline.

SEGMENT-POLARITY GENE
A gene that is required for anteroposterior patterning within each segment, such as *wingless*, *engrailed* and *hedgehog*.

Box 2 | **The *GAL4–UAS* system for directed gene expression**

The yeast transcriptional activator Gal4 can be used to regulate gene expression in *Drosophila* by inserting the upstream activating sequence (*UAS*) to which it binds next to a gene of interest (gene *X*)[96]. The *GAL4* gene has been inserted at random positions in the *Drosophila* genome to generate 'enhancer-trap' lines that express *GAL4* under the control of nearby genomic enhancers, and there is now a large collection of lines that express *GAL4* in a huge variety of cell-type and tissue-specific patterns[97]. Therefore, the expression of gene *X* can be driven in any of these patterns by crossing the appropriate *GAL4* enhancer-trap line to flies that carry the *UAS*–gene *X* transgene. This system has been adapted to carry out genetic screens for genes that give phenotypes when misexpressed in a particular tissue (modular misexpression screens)[79].



involved in any early developmental process, but it can also be used to look for dominant effects in deficiency heterozygotes, which is a quick means of assessing the number of potential target genes in an enhancer or suppressor screen (see below)[29]. To facilitate deficiency screens, the Bloomington stock center has assembled a collection of deficiencies that provide maximal coverage of the genome in the minimum number of stocks, which is often referred to as the 'deficiency kit'.

Enhancer and suppressor screens

One way to find the missing components of a developmental pathway that cannot be found in traditional screens is to carry out a screen for dominant enhancers or suppressors. Loss-of-function mutations in almost all genes are recessive, which indicates that 50% of the wild-type level of a protein is sufficient for normal development. When a particular process is already partially disrupted by another mutation, however, this amount might no longer suffice, and mutations in the genes that are involved in the pathway can therefore be identified as dominant enhancers or suppressors in this sensitized genetic background. Some of the most successful screens of this type were carried out to find the components in the signal-transduction pathway downstream of Sevenless (Sev), which is a receptor tyrosine kinase (RTK) that controls cell-fate choice between one of the eight photoreceptors in the eye, the R7 cell, and non-neuronal cone cells[30]. A weak allele of *sev* provides just enough signalling activity for most of the R7 cells to form, but these cells are transformed to cone cells if there is any further reduction in the efficiency of signal transduction. Under these conditions, halving the dose of some of the downstream components in the pathway causes most of the R7 cells to become cone cells, without affecting signalling through the other RTKs in the fly. This sensitized genetic background therefore allowed Simon *et al.*[30] to identify mutants in components of the RTK signalling pathway as dominant enhancers of a visible eye phenotype. This approach has several important advantages over traditional screens (FIG. 1b). First, the progeny of mutagenized flies can be screened directly (an F_1 screen), because the mutations do not need to be made homozygous, which means that an order of magnitude more flies can be screened than in an F_3 screen. Second, lethal mutations in essential genes that function at many stages of development can be isolated, because the sensitized background only affects the eye, which is not required for viability. Third, the whole genome can be screened at once, because there is no need to use balancers to make particular chromosomes homozygous. At the time this screen was carried out, biochemical experiments had identified several RTK substrates, but the pathway through which they signal to the nucleus was still unclear. The mutants from the screen led to the demonstration that Sev, and other *Drosophila* RTKs, such as the Epidermal growth factor receptor (Egfr) and Torso (Tor), signal through Son of Sevenless (Sos), Downstream of receptor kinase (Drk; an SH3/SH2 adaptor protein) and Corkscrew (Csw; a protein tyrosine phosphatase), to activate Ras[31–34]. Two other types of screen for modifiers of *sev* identified partially overlapping sets of genes, and illustrate the different ways that this type of screen can be designed. Rogge *et al.*[35] used a stronger *sev* hypomorphic mutation and screened for dominant suppressors that increase the number of R7 cells. This led to the identification of the opposite types of mutant to the enhancer screen, such as a gain-of-function mutant in *Sos*. By contrast, a screen for dominant suppressors of a constitutively active form of the Sev receptor identified a loss-of-function allele of *drk*[36].

Although these screens were remarkably effective, they did not find the genes that function downstream of *ras*, presumably because these act too far downstream of *sev* for a reduction in their levels to have a significant effect in the sensitized backgrounds. Because *ras* is an essential gene that is required throughout development, none of the alleles gave a viable phenotype that was suitable for modifier screens; however, this problem could be circumvented by making a transgenic construct in which the *sev* enhancer drives the expression of an

OMMATIDIA
The compound eye of *Drosophila* is formed from 800 ommatidia, each of which contains eight photoreceptor cells, surrounded by four cone cells that secrete the lens, and seven pigment cells.

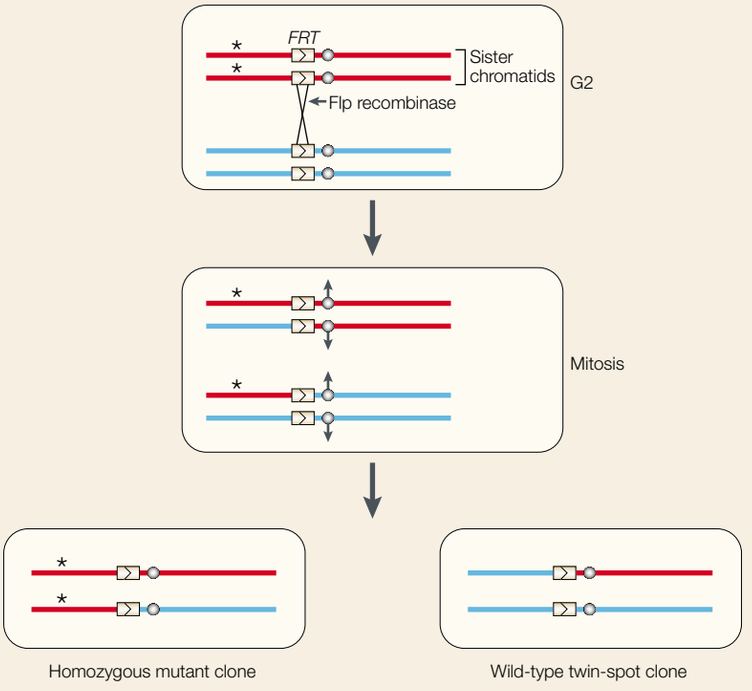
activated form of Ras in the eye. The eye-specific activation of the RTK pathway by this transgene transforms cone cells into R7 cells to produce a rough-eye phenotype (in which the surface of the eye loses its smooth appearance; see FIG. 2g,h). A genetic screen for dominant enhancers and suppressors of this phenotype resulted in the identification of mutants in the downstream components, such as Raf (a serine/threonine kinase), mitogen-activated protein (MAP) kinase kinase, MAP kinase (encoded by *rolled* (*rl*)), and Phyllopod (Phyl; a nuclear protein)[37]. A similar approach was taken by Dickson *et al.*[38], who screened for modifiers of an activated form of Raf and identified many of the same genes. This is not the end of the story, however, because new screens for modifiers of other components of the pathway continue to identify new genes that are important in this process[39–42].

The screens for mutants in the Sev pathway illustrate several important features for designing successful modifier screens. Although I have mentioned some of the interacting genes that proved to have a direct role in the pathway, many mutants were also isolated from these screens that modify the eye phenotype for less obvious reasons, and it is therefore crucial to have simple assays to sort out the different classes of interactors. For example, some mutants might modify the phenotype by interfering with unrelated processes that occur in the same cells, whereas others might do so because they alter the expression of the allele or construct used to generate the sensitized background. This latter class can be screened out because they should also suppress constructs that are under the same transcriptional regulation but produce the opposite phenotype, whereas bona fide components of the pathway should have the converse effect. Second, not all components of a pathway will necessarily be dosage sensitive. Only one allele of Raf was isolated in all of these screens, whereas hundreds of mutations were recovered in *rl* (MAP kinase), which indicates that the levels of the former are probably not crucial for the efficiency of signal transduction in the eye. Third, each screen isolated a different spectrum of mutations, and it might be necessary to carry out screens in various different sensitized backgrounds to find most of the components in a pathway. Finally, many of the mutations turned out to be homozygous viable and have no phenotype when removed from the sensitized background. Suppression or enhancement of the phenotype of interest is therefore no guarantee that a gene has an essential role in the process.

Many other enhancer and suppressor screens have been carried out in *Drosophila*, and they continue to provide a very effective means of finding new components in almost any process[29,43–45]. Some idea of their impact comes from the hundreds of genes listed in FlyBase, whose names indicate that they were identified as either enhancers or suppressors, even though they represent only a subset of the genes found in this way (for example, *Enhancer of split* (*E(spl)*), *Suppressor of Hairless* (*Su(H)*) and *Suppressor of deltex* (*Su(dx)*) in the Notch pathway). The eye is by far the most popular place in which to conduct such screens, because it is not essential for viability or fertility, and is easy to score. Furthermore, it is particularly suited to modifier screens because it is composed of ~800 regularly packed OMMATIDIA, and numerous defects in cell-fate determination and differentiation give a rough-eye phenotype, the severity of which reflects the number of ommatidia affected. It is even possible to carry out screens for modifiers of genes the functions of which have not been characterized in the eye, if the eye-specific expression of a wild-type or mutant construct of the gene produces a rough-eye phenotype[46].

Box 3 | **Using the Flp/*FRT* system to generate mitotic clones**

Flp recombinase mediates site-specific recombination between *FRT* (Flp recombinase target) sites during replication of the yeast 2μ plasmid, and works very efficiently when expressed in *Drosophila*[49]. Flp-mediated recombination can be used to generate mitotic clones by creating flies with transgenic *FRT* sites at identical positions on homologous chromosomes[98]. If the site-specific recombination between homologues occurs after DNA replication, and the daughter chromatids segregate appropriately, the region of the chromosome arm that lies distal to the *FRT* site will be made homozygous, with each daughter cell inheriting two copies of this region from one of the parental chromosomes (see figure). This site-specific recombination event can be used to make a mutagenized chromosome arm (red) homozygous in clones of cells, which can then be screened for a phenotype. The principal advantages of this approach are: first, F_1 screens can be carried out for recessive loss-of-function phenotypes, as there is no longer a need to go through two additional generations to make the mutagenized chromosomes homozygous, as is the case in a traditional genetic screen; and second, by controlling where and when the recombination occurs, only the cells of interest are made homozygous. The tissue-specific phenotypes of mutations in essential genes can therefore be identified, regardless of their other functions in development. One disadvantage is that Flp/*FRT* screens cannot detect mutations that lie proximal to the *FRT* site. However, *FRT* insertions have been recovered close to the centromere on all of the main chromosome arms, and it is now possible to screen ~95% of the euchromatin, although this requires carrying out separate screens for each of the five arms[56,99]. See animation of mitotic recombination online.



IMAGINAL DISC
Sac-like infolding of the epithelium in the larva. They give rise to most of the external structures of the adult. Imaginal disc cells are set aside in the embryo and continue to divide until pupation, when they differentiate.

Clonal screens

Another extremely powerful way to get around the problem of only being able to analyse the first phenotype of a mutation is to carry out screens in mitotic clones, in which only the cells of interest are homozygous for the mutagenized chromosome, whereas the rest of the organism is heterozygous. The old-fashioned way of generating clones was to use X-rays to induce mitotic recombination between homologous chromosomes, and this technique was used successfully to carry out genetic screens for X-linked mutations that give phenotypes in germ-line clones[47,48]. However, a much more efficient way to generate clones is to use the Flp recombinase from the yeast 2μ plasmid, which mediates efficient site-specific recombination between its target sites, called *FRT* sites, when these are integrated into the *Drosophila* genome[49]. The Flp recombinase works surprisingly well when the transgenic *FRT* sites are present at identical positions on homologous chromosomes, and this is probably because homologues are paired in mitotic cells in *Drosophila*, in contrast to most other organisms. This site-specific recombination event can, therefore, be used to make a mutagenized chromosome arm homozygous in clones of cells, which can then be screened for a phenotype (BOX 3).

By expressing Flp in larvae under the control of a heat-inducible promoter, any embryonic phenotype can be bypassed by specifically inducing clones in the IMAGINAL DISCS. The first Flp/*FRT* screens used this approach to identify mutants that affect the growth and patterning of the discs on the basis of their visible adult phenotypes, and show the immense power of this technique. Xu *et al.*[50] searched for mutations that produce tumorous outgrowths in the discs, and identified the tumour-suppressor gene *warts* (*wts*; also known as *lats*), and a negative regulator of the Dpp and Wg signalling pathways, *supernumerary limbs* (*slmb*; also known as *slimb*). Jiang and Struhl also identified *slmb*, and isolated an allele of Protein kinase A, which was found to be a negative regulator of the Hh pathway[51,52] (FIG. 3a). Similar screens for mutants that produce bubbles in the wing identified 14 loci that are required for adhesion between the dorsal and ventral compartments, and led to the identification of novel components of the integrin adhesion complexes that hold the two surfaces together[53–55] (FIG. 3b).

Germ-line clones. An ingenious refinement to the Flp/*FRT* system, called the dominant female sterile (DFS) technique, has been developed by Chou and Perrimon to select for mutant clones in the germ line[56,57]. The DFS method uses the dominant ovo^D mutation to kill the non-recombinant germ cells, so that females lay only eggs that derive from homozygous mutant germ-line clones. This approach provides a powerful way to screen for the maternal-effect phenotypes of lethal genes (BOX 4) and has allowed the identification of many of the essential genes that were missed in previous screens because their products are required for embryogenesis but are supplied maternally[58]. For example, these screens have led to the discovery of several new genes that are involved in signal-transduction pathways in the embryo, such as *tout velu* (*ttv*), which is required for the diffusion of Hh protein and encodes the homologue of the human disease gene exostoses (*EXT*), and *sugarless* (*sgl*) and *sulfateless* (*sfl*), which are both enzymes involved in the synthesis of heparan sulphate glycosoaminoglycans, which are required for Wg and fibroblast growth factor (Fgf) signalling[59–62].

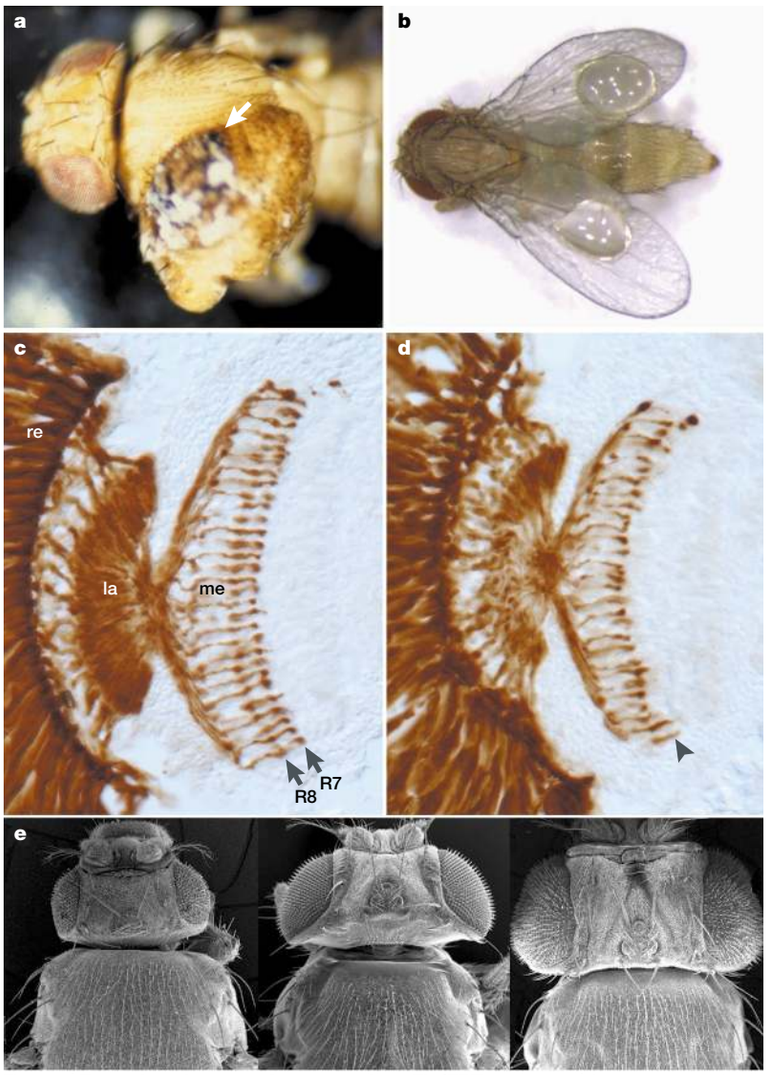


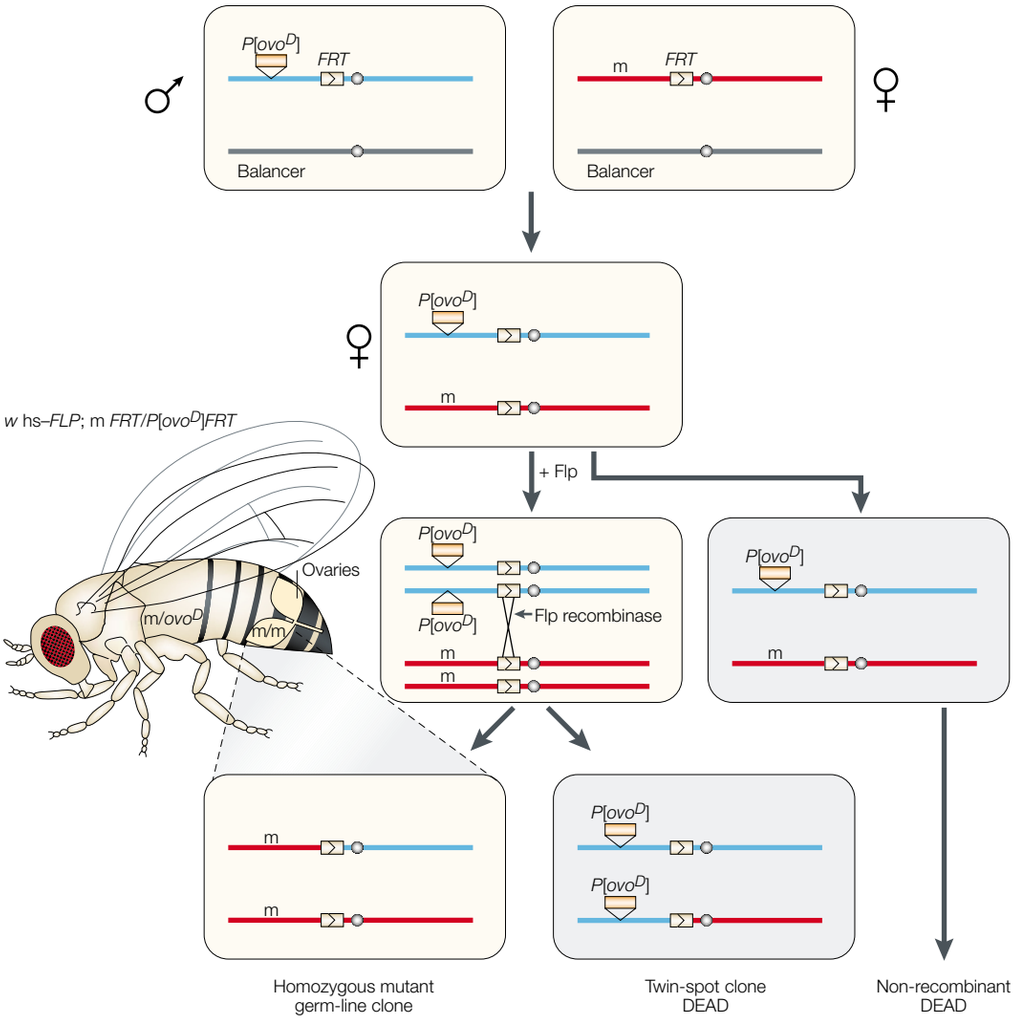
Figure 3 | **Examples of mutant phenotypes from Flp/*FRT* screens. a** | A NOTUM containing a homozygous *lats/warts* mutant clone, which has overgrown to form a large tumerous outgrowth (arrow). **b** | An adult fly with bubbles in both wings produced by clones of a mutant in *piopio* (*pio*), which disrupts adhesion between the dorsal and ventral surfaces of the wing. **c**,**d** | Section through an adult head showing the projections of retinal axons into the lamina (la) and medulla (me) of the optic lobe. 're' marks the position of the retina. **c** | Wild type. The R7 and R8 axons project to two distinct layers in the medulla. **d** | The R7 and R8 axons terminate in the same region of the medulla in *Leukocyte-antigen-related-like* (*Lar*) mutant clones, generated using *eye–FLP* with the *Minute* technique. In panels **a**–**d**, anterior is to the left. **e** | Scanning electron micrographs of the head and thorax of a wild-type fly (centre), and flies from the 'pinhead' screen with either a smaller (left) or larger (right) than normal head. (Panel **a** courtesy of Tain Xu, Yale University, USA, and reproduced with permission from REF. 50 © (1995) The Company of Biologists, Ltd; panel **b** courtesy of Nick Brown and Christian Boekel, Wellcome/CRC Institute, Cambridge, UK; panels **c**, **d** courtesy of Barry Dickson, Institute for Molecular Pathology, Vienna, and reproduced with permission from REF. 72 © (2001) Elsevier Science; panel **e** courtesy of Ernst Hafen, University of Zürich, Switzerland.)

Because the *ovo*^{D} selection means that all of the progeny of mutant females show the phenotype, it is not possible to recover the mutant chromosomes from the females, and these screens must, therefore, be carried out in the F_2 generation, so that the mutant can be isolated from the sibling males. More recently, Luschnig *et al.*[63] have adapted this approach to carry out F_1 screens for lethal mutations with maternal-effect phenotypes, by marking the non-mutagenized *FRT* chromosome with GFP. The embryos from the homozygous mutant germ-line clones can therefore be selected by the absence of maternal GFP and screened for phenotypes, and the mutations can be recovered from the eggs that are derived from non-recombinant germ cells, which express GFP.

Flp/*FRT* screens can be targeted to particular tissues by putting the Flp recombinase under the control of a tissue-specific promoter, which removes any risk of losing mutants because of the effects of clones in other tissues. Duffy *et al.* have generated upstream activating sequence (*UAS*)–*FLP* lines that allow the

Box 4 | **The dominant female sterile technique for selecting homozygous germ-line clones**

Dominant female sterile (DFS) mutations in the *ovo* gene (*ovo*^{D} mutants) cause female germ cells to die during early oogenesis, and therefore completely block egg production. These mutants can be used to eliminate non-recombinant cells in Flp/*FRT* screens for mutations that give phenotypes in germ-line clones, by placing them on the *FRT* chromosome arm that is to be selected against. In the absence of recombination, the *ovo*^{D} transgene kills all of the germ cells, so the only egg chambers to survive are those that have lost *ovo*^{D} and are homozygous for the other *FRT* chromosome arm (see figure). By mutagenizing this chromosome, genetic screens can be carried out for lethal mutations that give maternal-effect phenotypes, in which 100% of the eggs that are laid derive from homozygous mutant germ-line clones, and this provides a very powerful way to find mutants in maternally supplied components that are essential for embryonic development[58]. Although the *ovo* gene is on the X chromosome, Chou and Perrimon have generated a set of transgenic lines that carry the mutant form of the gene and a proximal *FRT* site on each of the four major autosomal chromosome arms[55]. This makes it possible to use the DFS technique to do germ-line clone screens of most of the genome. hs, heat shock; m, mutation; *w, white* gene.



NOTUM
The dorsal or upper surface of the thoracic segment of any insect.

FOLLICLE STEM CELL
Each ovariole (chambers in the ovary through which the egg passes during development) contains 2–3 follicle stem cells, which produce the somatic follicle cells that surround the chambers. The follicle cells then differentiate into several cell types, including the border cells, which migrate from the anterior of the egg chamber towards the oocyte, where they contribute to the formation of the micropyle. At the end of oogenesis, the follicle cells secrete the eggshell and undergo apoptosis.

targeted expression of Flp with any Gal4 driver, and have shown that a driver that is expressed in the FOLLICLE STEM CELLS can make up to 30% of the follicle cells homozygous for a particular chromosome arm (that is, 60% of the cells have undergone recombination)[64]. This has made it possible to carry out clonal screens for mutants that affect several aspects of follicle cell behaviour. For example, Liu and Montell have screened for mutants that disrupt the migration of the border follicle cells towards the anterior of the oocyte and found two new transcription factors that regulate this movement, whereas Pai *et al.* identified a new dorsalizing mutation that proved to be an allele of *Cbl*, which targets the activated Egf receptor for degradation[65–68].

The most sophisticated Flp/*FRT* screens combine tissue-specific expression of the recombinase with a selection for the homozygous mutant clones, and, perhaps not surprisingly, these have been devised for carrying out screens in the eye (BOX 4). Flp has been expressed specifically in the developing eye disc either by using an *eyeless* (*ey*)–*GAL4* driver and *UAS–FLP*, or by fusing the recombinase directly to the *ey* regulatory region (*ey–FLP*). In addition, several different tricks have been used to select against the non-recombinant cells and the twin-spot clones (the reciprocal product of the recombination event, which inherits two copies of the non-mutagenized chromosome arm)[69,70]. Newsome *et al.*[69] used *FRT* chromosomes that carry *Minute* (*M*) mutations,

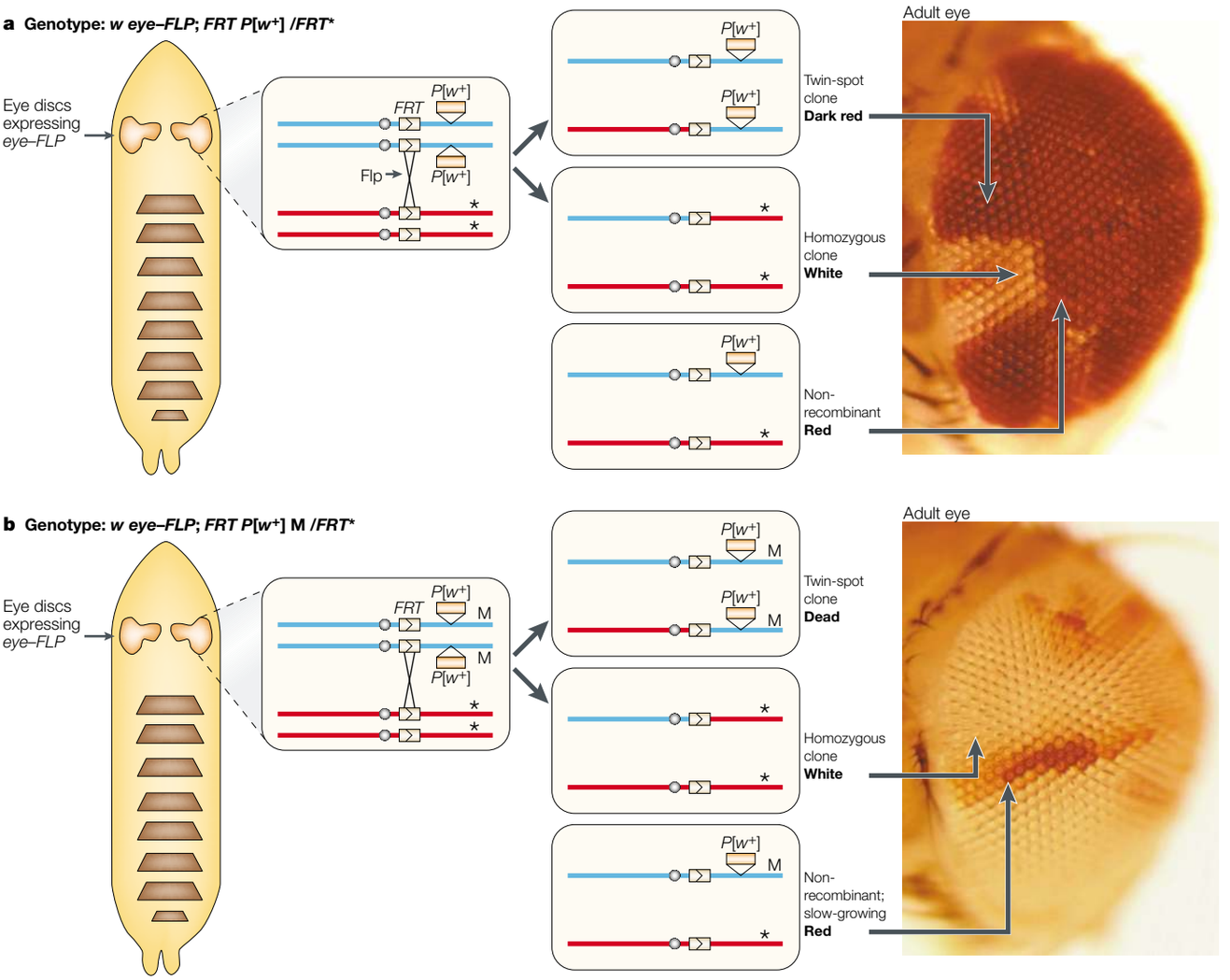


Figure 4 | **The *eye–FLP* technique for targeting clones to the eye.** By placing the *FLP* recombinase gene under the control of the *eyeless* enhancer (which drives expression specifically in the eye–antennal imaginal disc), Flp/*FRT*-mediated recombination can be targeted to this disc to generate homozygous mutant clones in the eye in flies that are otherwise heterozygous. **a** | The non-mutant chromosome (the asterisk indicates a mutation) is marked by a *mini-white* transgene, but there is no selection against the twin-spot clones or non-recombinant cells, and both the mutant clones (white) and the twin-spot clones (darker red, because they carry two copies of *white*$^+$) are relatively small. **b** | The effects of incorporating a *Minute* mutation (*M*) onto the non-mutant *FRT* chromosome. The mutant clones now occupy almost all of the eye, because they outcompete the slow-growing non-recombinant cells (which are *M*/+), whereas the twin-spot clones die. (Photographs courtesy of Barry Dickson, Institute of Molecular Pathology, Vienna, and reproduced with permission from REF. 69 © (2000) The Company of Biologists, Ltd.)

which are mutants in essential components of the translation apparatus, such as ribosomal proteins, that are recessive lethal and cause a dominant inhibition of cell growth[71]. The twin-spot cells that are homozygous for the *M* mutation therefore die, whereas the non-recombinant cells grow more slowly and are largely eliminated through competition with the faster growing M^+ clones (FIG. 4). Stowers and Schwarz went even further, and completely eliminated the non-recombinant and twin-spot cells by creating an *FRT* with a dominant eye-specific cell-lethal construct, in which another eye-specific promoter, GMR (glass multiple reporter), drives the expression of *head involution defective* (*hid*; also known as *wrinkled*) — a gene that triggers apoptosis[70]. It turns out, however, that *ey–FLP* is so efficient that nearly all of the cells undergo recombination, so there is no need to eliminate the non-recombinant cells. The twin-spot cells can simply be removed by placing a recessive cell-lethal mutation on the other *FRT* chromosome[69]. These reagents make it possible to generate flies in which almost all eye cells are mutant, but the rest of the animal is wild type, and allow simple F_1 or F_2 screens for the eye-specific phenotypes of all genes.

One of the first screens to take advantage of the *ey–FLP* approach used a *lacZ* line that was expressed in the photoreceptor cells to screen for defects in their axonal projections into the optic lobe of the brain. This approach identified 210 mutant lines that specifically disrupt axon pathfinding, without affecting photoreceptor cell differentiation[69]. Although most of these mutants remain to be characterized, two of the complementation groups correspond to the mutants in the receptor tyrosine phosphatases, PTP69 and *Leukocyte-antigen-related-like* (*Lar*; also known as *Dlar*)[72] (FIG. 3c–d). Similar mutants have also been recovered in an elegant F_1 screen, which used a behavioural assay to select mutant flies that do not detect motion[73,74]. Finally, there are some types of screen that can be conceived only as clonal screens, and particularly ingenious examples are screens for mutants that cause cells to divide more rapidly than normal, without disrupting patterning or causing a tumorous overgrowth phenotype. These take advantage of the fact that a mutant clone and a twin-spot clone are generated at the same cell division, and therefore normally grow to be about the same size. Mutant and twin-spot clones in the eye that were marked by the presence of either no copies or two copies of the *MINI-WHITE* GENE were generated using *ey–FLP*, and these were screened for cases in which the eye was normally proportioned, but the mutant clone was much larger than the twin spot. Mutants from these screens fall into more than 20 complementation groups, and include alleles of the *Drosophila* homologues of the human tuberous sclerosis complex 1 and 2 genes, *Pten* and *archipelago* (*ago*), which regulates cyclin E degradation and is the homologue of the human tumour-suppressor gene *HCDC4* (REFS 75–77). The Hafen lab is carrying out a related 'pinhead' screen for mutants that disrupt size regulation, by using the *ey–FLP* system with a selection against the twin spots to generate flies in which all of the regions of the head that derive from the eye–antennal imaginal disc are mutant, whereas the rest of the body is wild type, and by screening for flies with small heads (FIG. 3e) (S. Oldham, H. Stocker and E. Hafen, personal communication). Because flies naturally come in different sizes depending on their nutritional state, it is much easier to spot a mutant that affects size control when only the head is affected, and the rest of the body acts as an internal control.

The efficiency of the Flp/*FRT* system for generating clones has made *Drosophila* the only multicellular organism in which it is now possible to carry out screens in almost any cell at any stage of development, without having to worry about the pleiotropic effects of mutations. Only a few examples of mutants that have been isolated in such clonal screens have been published so far, but they already illustrate the enormous potential of this approach, and the range of such screens can be further enhanced by combining them with sophisticated ways of visualizing particular attributes of a cell. For example, mutations that affect the expression or localization of a specific protein can be screened for, by generating the appropriate transgenic GFP reporter constructs[78]. Given the versatility of this technique, it seems very likely that many more Flp/*FRT* screens will be carried out in future to address a host of biological questions.

Misexpression screens

All of the screens described so far can be carried out on a sufficiently large scale that, in theory, it should be possible to identify all of the genes in which loss-of-function mutations give the phenotype of interest. Not all genes can be found in this way, however, and one class that might be missed comprises genes that have redundant functions. A useful complement to these screening strategies is to use the Gal4 system (BOX 2) to carry out a targeted misexpression screen. Rørth *et al.* have generated a *P*-element vector called the EP element that carries *UAS* sites at one end, so that any gene that it inserts next to can be activated by Gal4 (REF. 79). This makes it possible to carry out a gain-of-function screen by simply crossing a Gal4 driver that is expressed in the appropriate tissue to a large number of EP insertion lines, and screening for phenotypes caused by the mis- or overexpression of the adjacent genes[80]. The effectiveness of this type of 'modular misexpression screen' in identifying functions that would be missed in loss-of-function screens is exemplified by a screen for genes that impair border-cell migration when overexpressed in either the border cells or the germ cells through which the border cells migrate[81,82]. Migration was disrupted by 2 out of 8,500 EP insertions tested, and these led to the demonstration that EGF and PDGF/VEGF (platelet-derived growth factor/vascular endothelial growth factor) homologues function as redundant signalling molecules that direct migration

MINI-WHITE GENE
A truncated version of the *white* gene that is commonly used as the selectable marker in transformation constructs. One copy of the transgene usually produces yellow or orange eyes in a *white* mutant background, whereas two copies give more complete rescue and produce darker eye colours. This allows more than one transgene to be followed at a time, and flies that are either heterozygous or homozygous for a particular insertion to be distinguished.

Box 5 | **What type of screen to do?**

Although a successful screen can generate enough mutants to keep a lab busy for many years, doing the screen and characterizing the mutants from it involve a huge amount of work, and it is therefore very important to design an efficient and effective screening strategy. The most appropriate solution will be different in each case, but there are several factors to bear in mind.

- **F_1 screens for viable phenotypes are much less work than F_3 screens, but it is always more work to map the mutations and to organize them into complementation groups than to do the screen itself.**
- **Because characterizing the mutants requires so much effort, it is important to minimize the background of mutations that do not affect the process of interest. For this reason, it is sometimes better to do a more laborious but more specific screen than a faster screen with a higher background, and the best way to do this is to screen for a phenotype that is directly related to the process of interest. A good example of this approach is the screen for retinal axon-guidance mutants by Newsome *et al.*[69], which is very labour intensive because it requires the staining of dissected heads, but unambiguously identifies the mutants that affect axon guidance, thereby reducing the frustration of mapping irrelevant mutations. A disadvantage of this approach, however, is that not as many flies can be screened, and many mutations might not fall into complementation groups. In other cases, it is more efficient to carry out a less specific but easier screen, such as an F_1 suppressor or enhancer screen, provided that simple re-screening assays are available that distinguish the interesting mutants from the noise at an early stage.**
- **It is often quite difficult to predict how well a particular screening strategy will work and what types of background mutation it will isolate, and it is therefore a good idea to do a small pilot screen first.**
- **It is very important to ISOGENIZE the starting stocks beforehand, to ensure that there are no lethal mutations present in the background. Otherwise, there is a risk of isolating the same pre-existing mutation over and over again, or of identifying spurious lethal complementation groups, because two independent mutations carry the same background lethal mutation.**

towards the oocyte. Other screens of this type have led to the identification of the cell-cycle inhibitor Tribbles (Trbl), a large number of genes that disrupt axon guidance or synapse formation when expressed in motor neurons and a similar number that perturb the development of the adult sensory organs when expressed in the sensory organ precursor cells[83–85]. Although this type of screen can never be a saturating screen, because *P*-elements do not insert into every gene, large collections of EP insertions are available in the public stock centres.

Prospects

I hope that this review has given a flavour of the types of genetic screen that are possible in *Drosophila*, and will provide ideas for new screens (BOX 5). It is worth considering, however, whether the time will come when forward genetic screens are no longer used. One reason that this might occur is if there are faster ways of knocking out gene function, and RNA INTERFERENCE (RNAi) now provides an alternative strategy for doing this[86]. Because the genome sequence can be used to design double-stranded RNAs against every predicted gene, it should be possible to carry out genome-wide screens by RNAi, as is now being done in *C. elegans*[87,88]. One disadvantage of this approach at the moment is that it cannot be carried out by simply feeding flies double-stranded RNA (as in worms), and it has to be injected into the embryo. This means that RNAi screens will have the same limitations as traditional screens for homozygous mutations, in that they will only be able to detect the first zygotic function of each gene. Although the problem could be overcome by generating transgenic constructs that express double-stranded RNA for each transcript, this would be very laborious. In addition, there are several types of experiment that can be done with a mutant that are not possible with RNAi. For example, a common way to dissect the functions of a protein is to determine whether transgenic constructs that express truncated or altered forms of the protein rescue a null mutation, and this is difficult to do using RNAi, because this exogenous RNA will also target the transgenic gene products.

A second reason why forward genetic screens might become obsolete is that there is only a finite number of genes in the genome and, at some point in the future, there might be mutations in all of them. At the moment this seems a long way off, because there are reported mutations in only a few thousand of the ~15,000 predicted genes, but the Berkeley *Drosophila* Genome Project is rapidly increasing this coverage by screening through tens of thousands of *P*-element insertions[89]. Although this approach alone will not saturate the genome, it might be possible to generate a null mutation in every gene by using a combination of transposable elements. Nevertheless, I believe that there will still be good reasons for doing genetic screens, even when the 'gene knockout kit' is available. As screens have become more sophisticated, they have used more and more complex genetic backgrounds, which often contain several different transgenes; it is much more work to cross ~15,000 mutations individually into this background than to generate new mutations in the appropriate stocks. More importantly, forward genetic screens can generate a variety of alleles of a gene, ranging from amorphs (null mutations) to weak hypomorphs (partial loss-of-function mutations), and the latter are often invaluable for elucidating its functions. For example, most mutations that are identified in behavioural screens turn out to be hypomorphic mutations in essential genes, and these phenotypes would probably not be detected using null mutations[90]. Many proteins carry out more than one function in a cell, and this is often revealed by having a series of alleles of differing strengths. To take another example from our own work, the key functions of *par-1* and *mago nashi* (*mago*) would not have been noticed without having hypomorphic mutations, as the null mutants block oogenesis before these later functions become apparent[91,92]. So, forward genetic screens are likely to be an important tool in *Drosophila* for many years to come, particularly if people continue to be so inventive in coming up with better ways to do them.

ISOGENESIS
A way of homogenizing the genetic background of a line that is used for mutagenesis. In an isogenic stock, the two homologous chromosomes of each pair are identical, which ensures that no recessive lethal allele is present.

RNA INTERFERENCE
(RNAi). A process by which double-stranded RNA specifically silences the expression of homologous genes through degradation of their cognate mRNA.

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