Chapter 3

Mosaic Analysis in the *Drosophila melanogaster* Ovary

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Abstract

Drosophila melanogaster oogenesis is a versatile model system used to address many important questions of cell and developmental biology such as stem cell regulation, cell determination, cell polarization, cell–cell signaling, cell–cell adhesion, and cell-cycle regulation. The ovary is composed of germline and somatic cells of different origins and functions. Mosaic analysis using the powerful genetic tools available in *Drosophila melanogaster* allows deciphering the contribution of each cell type in the different processes leading to the formation of a mature egg. Germ cells and follicle cells are produced by actively dividing stem cells, which permit the use of recombinases, such as FLP, to generate genetic mosaics using mitotic recombination. This chapter summarizes the different methods used to create genetic mosaics in the germline and in somatic cells of adult ovaries. We briefly introduce the morphology and development of the adult female ovary. We then describe in practical terms how to generate mosaics with examples of cross schemes and recombining strains. We also explain how to identify the appropriate progeny and how to prepare clonal tissues for phenotypic analysis.

Key words *Drosophila*, Mitotic recombination, FLP recombinase, Clonal analysis, Germline, Oogenesis

1 Introduction

1.1 The Adult Ovary

The *Drosophila melanogaster* ovary is composed of 16–20 ovarioles, each of which contains a chain of progressively more and more mature egg chambers [1] (Fig. 1). New egg chambers are generated at the anterior of the ovariole in a region called the germarium, which can be divided into four regions according to the developmental stage of the cyst. Oogenesis begins in region 1, when a Germline Stem Cell (GSC) divides asymmetrically to produce a posterior cystoblast (CB), and a new GSC, which remains attached to neighboring somatic cells at the anterior. The cystoblast then undergoes precisely four rounds of mitosis with incomplete cytokinesis to form a cyst of 16 germline cells, which are interconnected by stable cytoplasmic bridges called ring canals. These 16 cells are thus all sister cells and share the same cytoplasm. However, only one cell will become the oocyte and complete meiosis, while the 15 other cells become nurse cells, endoreplicate

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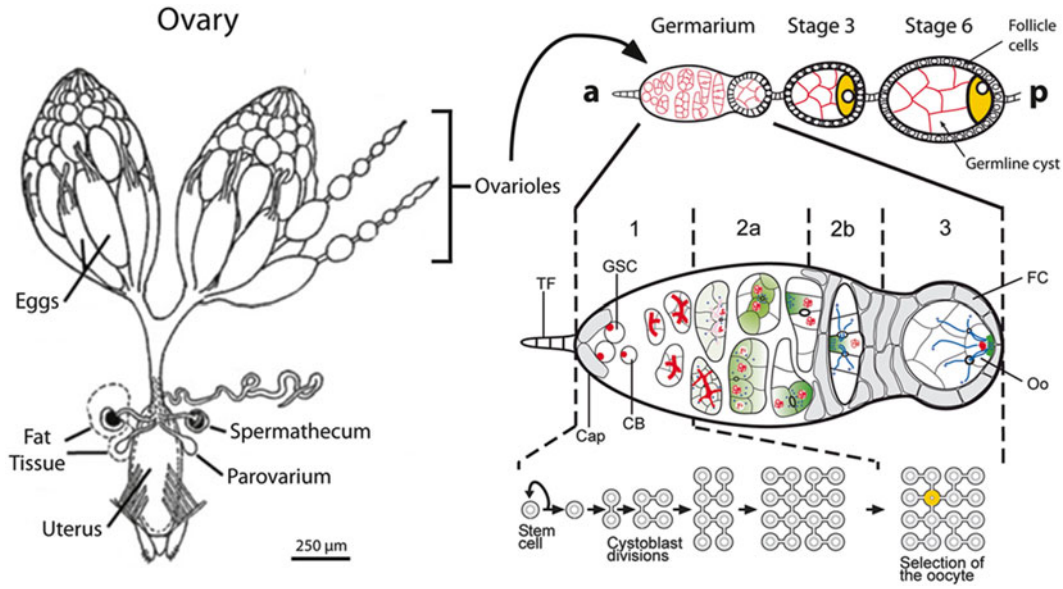

Fig. 1 ***Drosophila*** **Oogenesis**. *On the left*—depiction of the female reproductive tract redrawn after [55], Scale Bar is ~250 μm; *on the top right*—structure of an ovariole; *on the bottom right*—organization of the germline development in the germarium. Each ovariole is made of a chain of progressively more mature egg chambers toward the posterior (p). An egg chamber comprises 16 germline cells surrounded by a monolayer of follicle cells. The egg chambers are produced in a specialized structure, called the germarium, at the anterior (a) of the ovariole. The germarium is divided into four morphological regions along the anterior–posterior axis. The germline stem cells reside at the anterior tip of the germarium (*left*) and divide to produce cystoblasts, which divide four more times in region 1 to produce 16 cell germline cysts that are connected by ring canals. The stem cells and cystoblasts contain a spectrosome (*red circles*), which develops into a branched structure called the fusome, which orients each division of the cyst. In early region 2a, the synaptonemal complex (*red lines*) forms along the chromosomes of the two cells with four ring canals (pro-oocytes) as they enter meiosis. The synaptonemal complex then appears transiently in the two cells with three ring canals, before becoming restricted to the pro-oocytes in late region 2a. By region 2b, the oocyte has been selected (*yellow*), and is the only cell to remain in meiosis. In region 2a, cytoplasmic proteins, mRNAs and mitochondria (*green*), and the centrosomes (*blue circles*) progressively accumulate at the anterior of the oocyte. In region 2b, the minus-ends of the microtubules are focused in the oocyte, and the plus-ends extend through the ring canals into the nurse cells. The follicle cells (*gray*) also start to migrate and surround the germline cells. As the cyst moves down to region 3, the oocyte adheres strongly to the posterior follicle cells and repolarizes along its anterior–posterior axis, with the microtubule minus-ends and specific cytoplasmic components now localized at the posterior cortex

their DNA and provide the oocyte with organelles and nutrients. During the four divisions, a cytoplasmic structure called the fusome anchors one pole of each mitotic spindle and therefore ensures that cells follow an invariant pattern of divisions [2]. This leads to the formation of a symmetric cyst with two cells with four ring canals, two with three, four with two, and eight with one. This invariant pattern of divisions is important, as the oocyte always differentiates from one of the two cells with four ring canals, which are therefore called the pro-oocytes.

Three types of somatic cells can be distinguished in region 1. Cap Cells (CCs) are directly in contact with GSCs and send signals, which regulate GSC behavior. CCs define a niche for GSCs. More anteriorly, Terminal Filament Cells (TFCs) attach each ovariole to the surrounding muscular sheath. More posteriorly, Escort Cells (ECs) unsheathe each GSC and dividing germline cysts. Importantly, CCs, ECs, and TFCs do not divide under normal conditions in the adult ovary; it is thus impossible to perform mosaic analysis using mitotic recombination in these cell types in the adult. Once the 16-cells cyst has formed, it enters region 2a of the germarium. At this stage, all the cells of one cyst appear similar, but by the time it reaches region 2b, one cell will have differentiated as an oocyte. This differentiation can be followed with several types of markers [3]: (1) oocyte-specific proteins, such as Bicaudal-D (BicD), Orb, Barentsz (Btz), and Cup, and mRNAs, such as *oskar*, *BicD* and *orb*, which first concentrate in the two pro-oocytes, and come to lie on either side of the largest ring canal which connects them [4–9]; (2) the centrosomes of each cell of the germline cyst appear to be inactivated after the last mitotic division, and migrate along the fusome into the pro-oocytes, and then into the oocyte [10–12]; (3) the oocyte is the only cell of the cyst to remain in meiosis, and this can be followed by the formation of the synaptonemal complex as the chromosomes pair during the pachytene stage [13, 14]. By region 2b of the germarium, all these markers are restricted to only one cell of the cyst showing that the oocyte is already clearly selected. These components remain associated with the fusome remnants and therefore accumulate at the anterior of the oocyte to form a Balbiani body [15] .

Follicle Stem Cells (FSCs) are located at the border between region 2a and 2b. FSCs are somatic cells producing follicle cells, which migrate and surround each germline cyst to form an egg chamber. As the cyst moves down to region 3 (also called stage 1), it rounds up to form a sphere with the oocyte always lying at the posterior pole. Follicle cells form a monolayered epithelium encasing the 16 germline cells. Main body follicle cells surround most of the egg chamber, while terminal cells cover the anterior and posterior poles of each egg chamber. Two additional populations of somatic cells, called polar and stalk cells, have a common ancestor. Each pair of polar cells patterns the follicular epithelium at each pole, while stalk cells connect two adjacent egg chambers. FCs divide until stage 6 of oogenesis reaching around 900–1000 cells. Interestingly, FC divisions are also incomplete and groups of FCs remain linked by ring canals. Some level of synchrony can thus be detected among small groups of FCs and small molecules can diffuse from one cell to another. This can complicate the analysis of mosaic experiments in the follicular epithelium. After stage 6, FCs remain interconnected but stop dividing, endoreplicate their DNA and become polyploid. During the final stages of oogenesis, there

is a huge increase in the oocyte volume due to yolk uptake through the follicular cells and transfer of nurse cell cytoplasm into the oocyte. FCs undergo important morphogenetic movements to accommodate the volume increase and to form the micropyle and the dorsal appendages.

1.2 Mitotic Recombination

1.2.1 Mosaic Mutant Analysis: Background and Theory

To investigate the function of a specific gene, geneticists use mutations disrupting the function of this gene. However, developmental biologists want to know in which cell(s) this gene is required. In other words, in which cell(s) does the mutation cause the observed phenotype? Geneticists have designed methods allowing them to generate a single individual/tissue/organ with cells homozygous and cells heterozygous for this mutation, and cells with two wild type copies of the gene. Thus, within the same individual, all the cells do not have the same genotype and this individual/tissue/organ is thus called a genetic mosaic. This method has many advantages. When studying a mutation inducing lethality in the homozygous state, it allows the generation of homozygous mutant cells only in specific organs, while the rest of the fly is heterozygous for the mutation and thus viable. It allows the determination of whether the defects caused by a mutation are restricted to the genetically mutant cells (cell autonomous) or if the mutation affects neighboring cells (cell non-autonomous). It is equally important to mark the different genotypes to identify the homozygous, heterozygous, or wild type cells. Mosaic analysis is thus combined with dominant markers, which are genetic and heritable by the progeny. It is thus possible to perform lineage tracing, i.e., to determine the number and fate of cells that descend from one original cell by successive mitotic divisions, for the establishment of fate and specification maps.

One widespread method to generate homozygous mutant cells in an otherwise heterozygous individual is to induce mitotic recombination at the G2 stage (each chromosome is then made of two sister chromatids) in mitotically active cells (Fig. 2). After recombination between non-sister chromatids and depending on the orientation of chromosomes on the metaphase plate, there is 50 % chance that both mutant alleles will segregate into the same cell (homozygous mutant), while the two wild type copies of the gene under study will segregate into the sister cell (aka "twin spot"). In the absence of recombination, only heterozygous cells are produced. In the following divisions, the homozygous mutant cell will only generate homozygous mutant cells. This group of mutant cells derived from a single mutant cell is called a mutant "clone." Likewise, the twin-spot wild-type cell will generate a "clone" of wild type cells. The twin-spot clone is used as an internal control for the mutant clone. Heterozygous cells surround both wild type and mutant clones. The number of cells within a clone depends on the mitotic rate and the timing of clone induction.

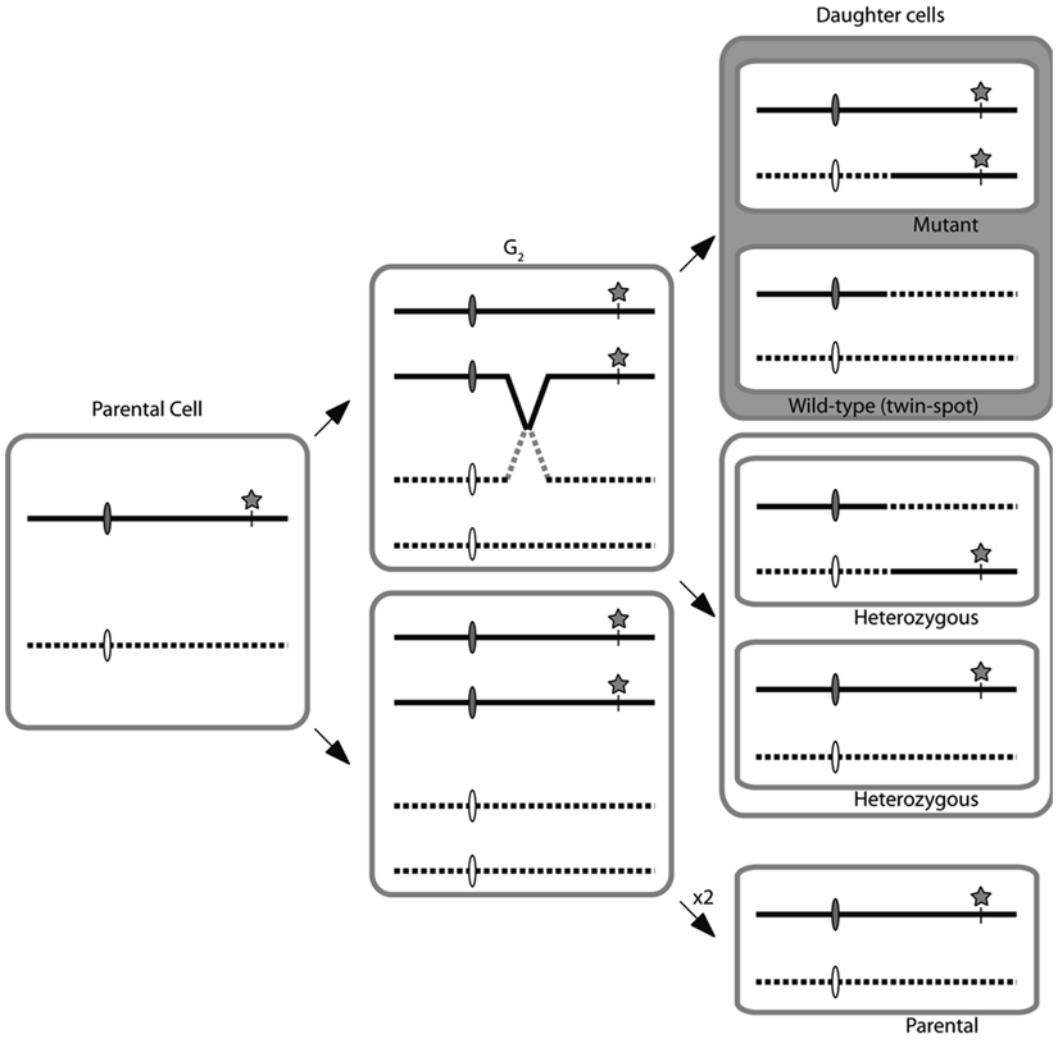

Fig. 2 Mitotic recombination. In the absence of mitotic recombination, the daughter cells are identical to the parent cell (*bottom way*). Each daughter cell has an arm of each of two homologous chromosomes: one carrying the mutation (*star*) and the other the wild-type allele. If recombination occurs, there is recombination between homologous chromosomes before mitosis. After the mitotic division, the mother cell has a 50 % chance of giving a daughter cell homozygous for the mutation and a daughter cell homozygous for the wild-type allele (*upper way*). The remaining 50 % produces two daughter cells both heterozygous for the mutation (*middle way*)

1.2.2 Recombination Methods

Ionizing radiation was the first method used to induce mitotic recombination between two chromatids. Adult flies, larvae or embryos heterozygous for a given mutation are placed in an X-ray machine, and subjected to a calibrated dose of radiation. Recombination events occur randomly along chromosomes. This method has the advantage to be simple and can be used with any genotype. However, radiation is unhealthy for the fly, and lethality is common.

X-rays can also induce genetic rearrangements. It can then be difficult to distinguish phenotypes caused by the mutation of interest or by the radiation exposure.

Genome manipulation has been transformed by the use of site-specific DNA recombinases such as Cre or FLP to perform site-specific recombination (SSR) [16–20]. Cre and FLP belong to the same family of recombinases, and recognize sequence-specific loxP or FRT sites, respectively. The more efficient Cre/loxP system is widely used in mammals, while the less efficient, but less toxic, FLP/FRT system is commonly used in *Drosophila*. FRT and loxP sites do not exist in the host genome and are uniquely recognized by the enzyme that can mediate recombination between two sites. This recombination event can induce chromatid exchange between two homologous chromosomes, but can also induce the deletion of an endogenous gene, the activation of a transgene or a reporter gene for cell-lineage analysis [16–20]. The expression of Cre or FLP can be targeted to specific groups of cells by the use of specific promoters driving the transcription of the gene encoding the enzyme.

The FLP/FRT method relies on the use of the site-specific recombination enzyme, FLP recombinase, from *Saccharomyces cerevisiae*. This enzyme specifically targets FRT sites (FLP recombination target site) and mediates site-specific recombination reactions between two identical FRT sites [21, 22]. The minimal FRT site is 34 bp and comprises 13 bp inverted repeats on either side of an asymmetric sequence or "spacer." FLP binds a FRT site, dimerizes with another FRT-bound FLP, making a synaptic complex between the two FRT sites, and catalyzes a recombination event between them [23]. The asymmetry of the spacer imposes directionality on the FRT sites, and enzyme mediated recombination only occurs between FRT sites in a specific orientation. The position and relative orientation (i.e., same or opposite direction) of the two FRT sites determine the outcome (i.e., insertion, excision, inversion, or reciprocal translocation) of the FLP recombinase-mediated recombination reaction [24]. FLP can be expressed in specific groups of cells with the use of specific promoters. However, a limited number of promoters have been well characterized over extended periods of time and in the entire organism [25]. These promoters are often expressed in several groups of cells and not in single cells, and their expression can vary in time, or in contrast they can remain constitutively active after specific time points. Promoters are available that lead to constitutive expression in certain tissues (e.g., *actin*-FLP, *tubulin*-FLP) and near complete mosaicism, whereas others are inducible (e.g., *heat-shock*-FLP). Since most of the FRT insertion stocks were equipped with the transgene encoding the *hs*-FLP, planning for a FLP/FRT experiment is especially user-friendly. With *hs*-FLP, the level of expression is dependent on the severity and duration of the heat shock [26] (*see* **Note 1**). Brief heat shocks at lower temperatures result in

lower levels of expression whereas long heat shocks at higher temperatures cause higher expression levels. The *hs*-FLP is the most widely used FLP to induce clones both in germline cells and in somatic cells.

The major advantages of the FLP/FRT mitotic recombination system are: (1) the recombination rates are much higher than achieved by X-ray irradiation; (2) the site of FLP-mediated recombination is precisely determined by the location of the FRTs; (3) the system does not cause significant cell death or developmental delay. The main disadvantages of this system include: (1) Cells must divide after the clone induction to follow the mutant progeny; (2) it requires to first recombine a mutation onto the appropriate FRT chromosome; (3) mutations on the fourth chromosome and those proximal to the FRT sites on the other chromosomes cannot be analyzed (recombining a mutation and an FRT site is not easily possible on the fourth chromosome, because no meiotic recombination occurs on this chromosome).

In the Cre/Lox system, Cre comes from bacteriophage P1 and catalyzes recombination between two loxP sites. Cre is in the same family of site-specific recombinases as FLP, therefore its function and mechanism of action is analogous [23]. Likewise, loxP sites have the same inverted repeat and spacer structure as FRT sites. However, Cre appears to be toxic to *Drosophila* cells in some cases, but these effects can be mitigated by reducing the expression levels [27]. Over the years, the Cre/Lox system has not been commonly used to induce mosaics of whole chromosome arms. LoxP sites have not been inserted close to each centromere. This system is mostly used in a "FLPout" configuration to remove transient markers or selection cassettes (*see* Subheading 1.2.7).

1.2.3 Production of Germline Clones of Maternal-Effect Genes

The first stages of embryonic development do not rely on the zygotic genome, but on mRNAs deposited by the mother in the egg cytoplasm during oogenesis. Thus, an embryo can be genetically homozygous for a given mutation, but the consequences of this mutation can be masked during the early stages of embryonic development by the presence of wild type mRNAs of the same gene deposited by the mother during oogenesis. To remove this "maternal contribution," the wild type copies of the gene need to be removed in the germline of the mother during the formation of the egg. This can be achieved by inducing mitotic recombination in the mother's germline stem cells which are the progenitors of all germ cells [28]. Mitotic recombination in germline stem cells can be induced using X-rays, but are more commonly done using the FLP/FRT system. FRT sites have been introduced close to centromeres of chromosome X, 2L, 2R, 3L, and 3R. FLP-mediated recombination between two corresponding FRT-sites will then produce an exchange of an entire chromosome arm [16, 20, 29]. If a mutation is localized distally (away from the centromere) on

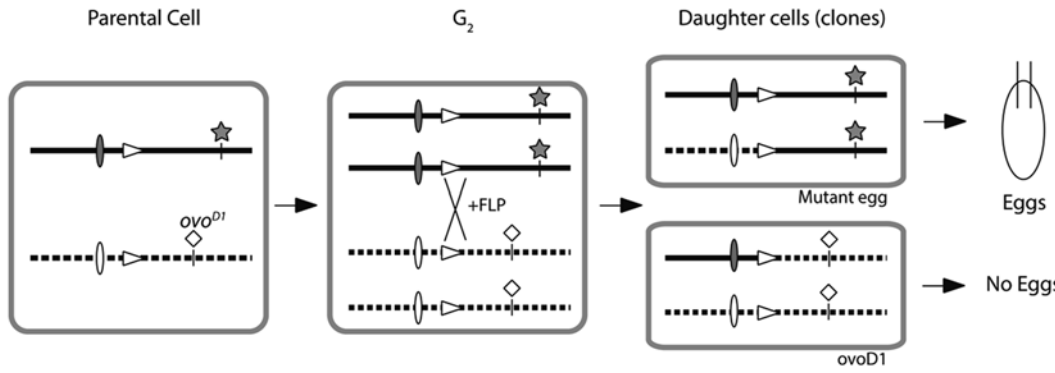

Fig. 3 Generating females with homozygous mutant germ cells. Before mitotic recombination, a parental cell has an arm of each of two homologous chromosomes: one carrying the mutation (*star*) and the other carrying the DFS *ovoD1* mutation. Activation of the FLP recombinase induces recombination between the two FRT sites present on homologous chromosomes before mitosis. After the mitotic division, the mother cell has a 50 % chance of giving a daughter cell homozygous for the mutation and lacking the DFS *ovoD1* mutation, and thus oogenesis can proceed with a homozygous mutant germline. The other daughter cell will be homozygous for the DFS o*voD1* mutation

this chromosome arm, there is a 50 % chance that the same cell will inherit both mutant copies. If this event happens in a germline stem cell, all the daughter cells produced by this stem cell will be homozygous for the mutation of interest, and the egg will be devoid of wild type mRNAs.

One important issue with this method is the ability to distinguish the homozygous cells from the heterozygous and wild-type twin spot cells. In order to identify each chromatid (mutant or wild type), a dominant marker is placed on the wild type chromatid arm. Thus, homozygous mutant cells can be recognized by the absence of the dominant marker. This method can even be improved by adding a selectable dominant marker such that only the homozygous mutant cells will develop. Indeed, in regular conditions homozygous mutant cells can be counter-selected when surrounded by wild type cells. However, this can be reversed if all heterozygous or wild type cells expressed a selectable marker slowing down their growth (Minute mutations) or arresting their development. In the case of germ cells, the *ovoD1* dominant mutation arrests oogenesis at stage 6 and females are thus sterile (Fig. 3) [30]. The only germ cells, which will develop past stage 6 are thus the ones which have recombined out any copy of *ovoD1*, meaning the germ cells homozygous for the mutation of interest [31]. The only eggs laid by these females are those produced by homozygous mutant germline stem cells. The combination of the FRT system with the *ovoD1* mutation allows a positive selection of eggs with no maternal contribution. The original *ovoD1* mutation is located on the X-chromosome, but transgenes expressing the mutation have

been inserted on each chromosome arm. This method is thus applicable to all genes on the X, 2 and 3 chromosomes [29, 31].

Importantly, when planning an experiment using the *ovoD1* mutation, one should keep in mind that the perdurance of the OvoD1 protein can last for a few cell divisions [30]. The OvoD1 protein may be stable in the cytoplasm of a germline stem cell even though this GSC has segregated away all copies of the *ovoD1* transgene. The antimorphic activity of *ovoD1* can thus be detected in a few daughter cells before being diluted out by cell division. This can create artifacts such as an abnormal number of germ cells in each egg chamber [32].

An alternative method to generate mosaic ovaries with mutant germ cells is to transplant pole cells from a donor X into a host Y. Pole cells are the future germline stem cells. They can be removed from the posterior of an embryo with a glass pipet and injected at the posterior pole of a different embryo. They will be incorporated into the future ovaries and form germline stem cells, which will produce germ cells of genotype X. Usually the donor X is homozygous mutant for a gene of interest and the host is a mutant embryo with no germ cells, such as *germ cell-less* or *grandchildless* mutants. In the resulting adult female, all germ cells will be of genotype X, while all somatic cells, including follicle cells, will be of genotype Y. However, this method is technically very challenging, and the transplantation procedure can introduce artifacts not caused by the mutation.

1.2.4 Germline Clones of Genes Required for Oogenesis

The induction of germline clones mutant for a specific gene during oogenesis follows the same principle as removing the maternal contribution using the FLP/FRT system as described above. In both cases, mitotic recombination is induced in mitotically active germline stem cells (Fig. 4). GSCs then produce homozygous mutant cysts until they differentiate. However, it is highly advised not to use the *ovoD1* transgene as a dominant marker. Firstly, *ovoD1* stops oogenesis at stage 6, and it is thus impossible to distinguish mutant from wild type clones before this stage. Secondly, the perdurance of the OvoD1 protein can induce phenotypes independently of the mutation. Instead, one can use a ubiquitous and neutral GFP or β-gal marker to label heterozygous and wild type cells [13, 33]. The homozygous mutant cells are thus identified by the absence of these markers.

1.2.5 Production of Clones in a Cyst

As cyst cells undergo four rounds of mitosis, it is possible to induce mitotic recombination during these divisions. The germline cyst can thus also be mosaic and all 16 cells will not be genetically identical. The number of cells sharing the same genotype depends on when the recombination has been induced. For example, if recombination happens during the first division, there is a 50 % chance that half of cyst cells will be identical, and 8 cells is the maximum

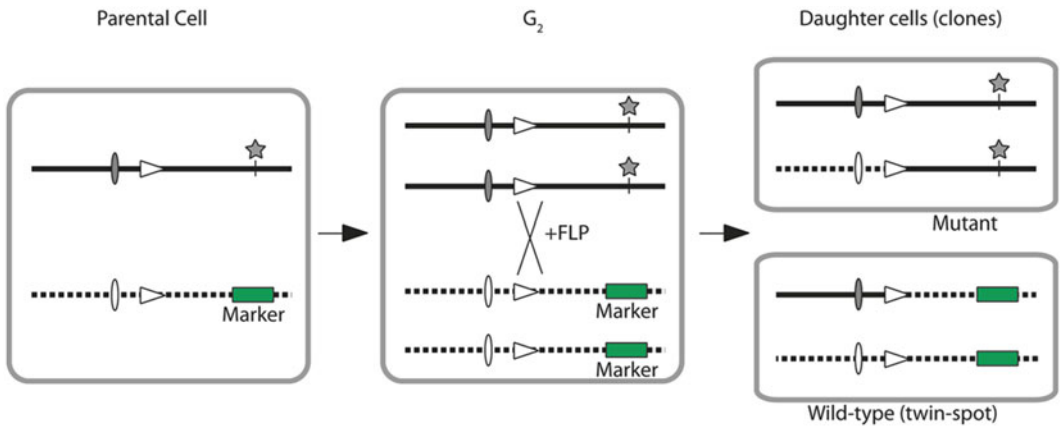

Fig. 4 Generating mutant clones using FLP-mediated mitotic recombination and a visible dominant marker. Before replication, both homologous chromosomes have one chromatid each: one carrying the mutation (*star*); and the other carrying the wild-type allele associated with a dominant marker gene such as a GFP. Activation of the FLP recombinase in G2 induces recombination between the two FRT sites present at the centromere of each homologue. After mitotic division, the mother cell has a 50 % chance of giving a daughter cell homozygous for the mutation and lacking the marker. The other daughter cell will be homozygous for the wild-type allele (giving rise to the "twin-spot") and will express two copies of the genetic marker. Cells, which have not recombined, will be heterozygous and identical to the parental cell

number of cells in intra-cyst clones. These experiments can be useful to decipher the relative contribution of the oocyte compared to nurse cells to a given process. However, these cells share the same cytoplasm through ring canals, which makes the analysis more challenging. GFP or β-gal can diffuse from one cell to another, and the genotype of each cell may not correspond to the visible marker. Secondly, mutant and wild type proteins can also pass through the ring canals, it is thus difficult to conclude on the cell autonomy or non-autonomy of a gene.

To circumvent the problem of diffusion of GFP or β-gal between germ cells, it is possible to directly label the chromatids with strictly cell-autonomous markers (Fig. 5). These germline mosaics are made with a transgene containing direct repeats of the *lac* operator (*lacO*) as a strictly cell autonomous genetic marker [34–36]. The presence of the transgene is indicated by the binding of a nuclear GFP-tagged Lac repressor protein (GFP-LacI) that binds to the *lacO* transgene and yields a discrete focus of nuclear fluorescence. In ovaries from females heterozygous for the lacO transgene, a single focus is present in the oocyte nucleus, whereas in the nurse cell nuclei, which are highly polyploid with partially dispersed chromatids multiple foci are visible. To create germline mosaics, one must construct a chromosome containing the *lacO* transgene in cis to the mutant allele, so that homozygous mutant cells would be homozygous for the transgene as well. This configuration allows the recognition of a homozygous mutant oocyte

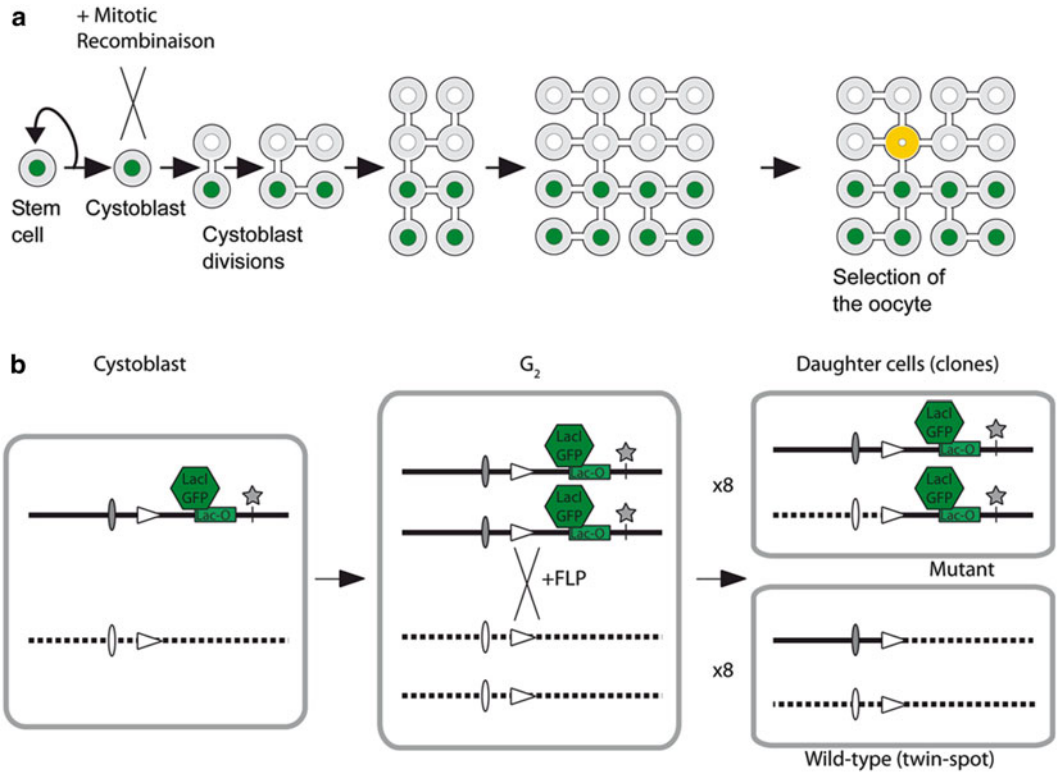

Fig. 5 Generation of mosaics in the germline cyst. (**a**) Mitotic recombination during the first division of a heterozygous cystoblast results in a mosaic cyst made of eight wild-type cells (*white*) and eight mutant cells (*green*), while recombination in the germline stem cell divisions generates clonal germline cysts (not shown). In such mosaics, the oocyte nucleus can be either wild type or mutant. (**b**) Generation of mosaics with the lacO/GFP-LacI system. Before mitotic recombination, a parental cell has an arm of each of two homologous chromosomes: one carrying the mutation (*star*) in *cis* to the *lacO* transgene, as well as the GFP-LacI transgene (not shown), and the other arm carries the wild-type allele. All cells exhibit nuclear GFP-LacI fluorescence, and discrete fluorescent foci are visible in the nuclei of cells with the *lacO* transgene. Heterozygous females exhibit a single focus of GFP in the oocyte nucleus and multiple foci in the polyploid nurse cells. Activation of the FLP recombinase induces recombination between the two FRT sites presents on homologous chromosomes before mitosis. After the mitotic division, the mother cell has a 50 % chance of giving rise to a daughter cell homozygous for the mutation and exhibiting two GFP foci. The other daughter cell is homozygous for the wild-type allele and is lacking the GFP focus

directly by the presence of two clear foci of GFP fluorescence within the oocyte nucleus [37]. However, when using this method, it must be kept in mind that proteins can diffuse through cytoplasmic bridges, consequently the absence of a phenotype in a mutant cell could be due to a contribution from the neighboring wild-type cells.

1.2.6 Production of Clones in Ovarian Somatic Cells

The FLP/FRT system can be equally used to induce homozygous mutant clones in somatic cells. The main limitation is that these cells have to be mitotically active to induce mitotic recombination.

In the adult ovary, the terminal filament cells, cap cells and escort cells are not dividing. To generate mutant clones in these cell lineages, one has to induce recombination during the previous larval stages. However, these different cell types often share some common progenitors, so it can be difficult to induce mutant clones in only one cell type [38]. Follicle stem cells divide throughout adult life and follicle cells also divide until stage 6. Clonal analysis is thus easier in these cell types. However, as in the case of germ cells, cytokinesis remains incomplete in most follicle cells. Follicle cells thus remain connected by small ring canals, through which markers and proteins can diffuse [39]. This should be taken into account when analyzing the cell autonomy of a given phenotype [40]. Furthermore, different types of follicle cells also share some common ancestors and it can be difficult to induce clones in only one cell type. For example, stalk cells and polar cells have a common progenitor very early on in the germarium.

Here, mutant cells are identified by the absence of markers. One should be careful that the absence of markers is not due to the absence of the whole cell. Mechanical damage during dissection can induce holes in the epithelium, which look like mitotic clones because of the absence of dominant markers [41]. Similar types of damage can also be caused by pipetting up and down the ovaries before immunostaining. We thus strongly advise not to use this method of dissection.

1.2.7 Clonal Misexpression: Flip-Out and MARCM Techniques

The advent of the Gal4/UAS system has introduced the ability to misexpress or overexpress genes in specific groups of cells [38]. Despite an ever-increasing number of promoters driving Gal4 expression, clonal expression in a very restricted number of cells is difficult to achieve. It is now possible to combine the FLP/FRT system with the Gal4/UAS system to create genetic mosaics in germline and somatic cells.

The Flip-out technique allows the induction of constitutive expression of one or several transgenes in single cells or clones of cells [42]. The expression of the transgene is switched on by the excision of a "FLPout" cassette, inserted between the transgene and its promoter. The removal of the FLPout cassette, containing the transcriptional termination site, leads to the fusion of the promoter to the coding sequence of the transgene and consequently to its expression (Fig. 6). For example, the ubiquitous *actin5c* (Act5C) or *tubulin1α* (tub1α) promoters are commonly used (*see* ref. 25 for a list of useful promoters to study oogenesis). The FLPout cassette, flanked on both sides by an FRT sequence, contains a marker gene (e.g., yellow, CD2) and a transcriptional termination site (polyA signal). Since these two FRT sites are orientated in the same direction, FLP-mediated recombination will lead to the excision of the FLPout cassette. This "FLPout" event is induced and adjusted through the timing and levels of FLP expression.

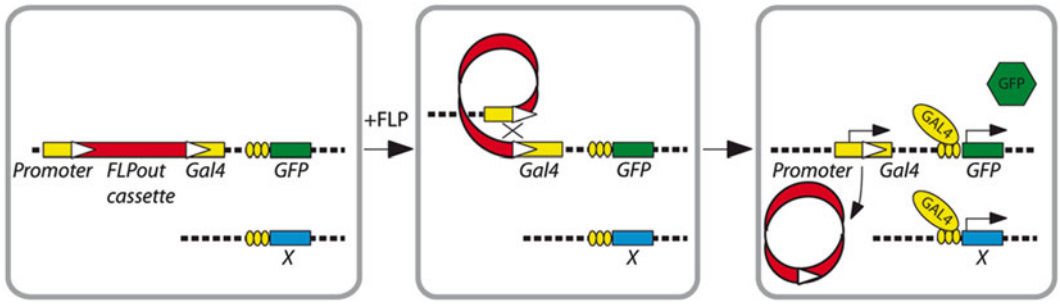

Fig. 6 The FLPout-Gal4 technique. Before recombination, a parental cell has a chromosome carrying one FLPout cassette. Activation of the FLP recombinase induces recombination between two flanking FRT sites, leading to the release of the cassette and thus to the expression of the Gal4 transgene under the exogenous promoter. GAL4 will in turn activate UAS-regulated transgenes, including UAS-driven marker genes (such as GFP). This technique also allows the expression of multiple UAS-transgenes at the same time

Eventually, since the single FRT site obtained after the excision fuses the promoter to the coding sequence of the transgene, the induced transgene expression is transmitted to the progeny of the cells that underwent the FLPout event. As the founder FLPout cell, the FLPout progeny can be identified by the absence of the FLP-out-cassette markers. In most experiments, FLP is expressed under the heat-shock-inducible promoter hsp70 [42, 43]. The timing and strength of the heat shock modulate "FLPout clone formation". Whereas early clone induction generally results in larger clones, the strength of the heat shock influences the number of cells that undergo a FLPout event. The FLPout system has been combined with the Gal4/UAS system. This "FLPout-Gal4 system" combination allows the ubiquitous expression of the Gal4 transcription factor after the FLPout event [44, 45]. Clones expressing Gal4 will in turn activate any UAS-transgene present. Therefore, these Gal4-expressing clones can be positively marked, for example, by the presence of a UAS-GFP or UAS-lacZ construct.

With the regular FLP/FRT system, homozygous clones are identified by the lack of a dominant marker. As previously described, these clones may sometimes be difficult to distinguish from a complete absence of mutant cells. One way to circumvent this problem is to mark positively the homozygous mutant cells. It is possible using a method called MARCM (mosaic analysis with a repressible cell marker), which relies on the combination of the FLP/FRT system, the activator Gal4/UAS system and its repressor Gal80 (Fig. 7). Before recombination, Gal80 inhibits the activity of the Gal4 transcription factor, whose respective transgenes are located on each arm of the same chromosome. A FRT site is located proximal to the ubiquitously expressed *tubP*-Gal80 transgene. On the homologous FRT chromosome, a mutation under study is located

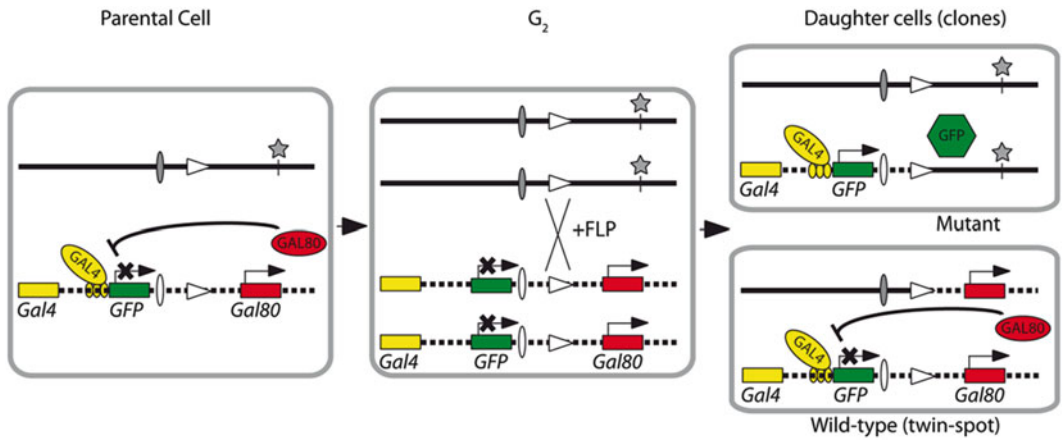

Fig. 7 Mitotic recombination with the MARCM system. Before mitotic recombination, a parental cell has one chromosome carrying a mutation (*star*) and the homologous chromosome carrying the wild type allele and a transgene expressing the Gal80 repressor. For convenience the Gal4 gene, which is driven by a constitutive promoter, and the UAS marker gene are drawn on the same chromosome; however, they can also be located on different chromosomes. Activation of the FLP recombinase induces recombination between the two FRT sites present on homologous chromosomes before mitosis. After the mitotic division, the mother cell has a 50 % chance of giving rise to a daughter cell homozygous for the mutation and lacking the Gal80 repressor transgene. In the absence of GAL80, GAL4 can drive the expression of a UAS-marker transgene (e.g., GFP) in the homozygous mutant cell. The other daughter cell is homozygous for the wild-type allele (giving rise to the "twin-spot") and is expressing two copies of the Gal80 repressor transgene

on the same FRT chromosome arm. After FLP/FRT induced recombination, both copies of the Gal80 transgenes have 50 % chance to segregate into one of the daughter cells. Consequently, in the twin cell, the mutation is homozygous, the Gal4 activity is recovered, and genes downstream of UAS sequences become expressed (e.g., UAS-marker transgene UAS-GFP or UAS-lacZ). This technique also allows the expression of a UAS-transgene in a marked cell clone that is accompanied by a wild-type sister clone for comparison. Importantly, this system offers the possibility to drive, in addition to the marker, other UAS-transgenes in the mutant clone.

This technique is mostly used in somatic cells. It has not been used in germline cells to our knowledge. It is theoretically possible to use it in germline cells with some modifications: (1) UAS sequences have to be UASp which work in germ cells [46]; (2) the Gal4 activator should **not** be a chimaera with VP16, as it is often the case with germline drivers such as nanos-Gal4::VP16. Indeed, the repressor Gal80 binds the activating part of Gal4, but does not recognize VP16.

1.2.8 Screens

The power and efficiency of the FLP/FRT system has allowed using this technique not only to study the function of single genes but also to interrogate functionally the entire genome for novel

genes involved in the formation of a mature egg. Genetic screens have been performed using the FLP/FRT system either in the germline or in somatic cells. Flies carrying FRT chromosomes are directly mutagenized and random mutations are induced on FRT chromosomes. Homozygous cells are then generated either in the germline or in somatic cells. The study of ovarian development was previously limited to female sterile mutations. Instead, these mosaic screens have allowed the identification of many lethal genes, which were required to complete oogenesis. The most commonly used mutagen with FRT chromosomes is the alkylating agent EMS, which makes mostly point mutations in the genome. Random insertions of P-elements are impossible to use because the FRT insertion are themselves P-based transgenes; and the mobilization of P-elements in the genome would also trigger the mobilization of FRT insertions. Instead, existing collections of P-element insertions have been recombined onto FRT chromosome corresponding to their insertion site. It is however possible to use PiggyBac mutagenesis directly on FRT chromosome as the two transposons do not use the same transposase [47].

In germ cells, genetic screens have been performed with the OvoD1 system to score for late phenotypes (after stage 6) and with GFP for genes involved in the early steps of oogenesis [48]. Indeed, recent studies have shown that key developmental decisions, such as the selection, polarization, or localization of the oocyte (the future egg cell) are made during the very early steps of oogenesis, before the arrest caused by the *ovoD1* mutation (reviewed in ref. 3). To uncover novel genes involved during these early stages, a mosaic genetic screen for mutations causing an early arrest of oogenesis have been performed using the FLP/FRT system [49, 50]. The morphology of arrested egg chambers was sufficient to discriminate several phenotypic classes without any staining procedures [50].

2 Materials

2.1 Fly Stocks: FLP, FRT, and Balancer Chromosomes

The Bloomington Stock Center maintains a wide variety of FLP and FRT chromosomes with many combinations of markers and mutations (http://flystocks.bio.indiana.edu/). Many more are available from the laboratories that generated the initial mutants. The list in Table 1 is a starting point for available FRT chromosomes carrying neo, GFP, RFP markers and also *ovoD1* mutations (*see* Table 1). Table 2 is a starting point for FLP lines (*see* Table 2). The Bloomington Stock Center also keeps useful balancer chromosomes: CyO, y+ for the second chromosome, TM6B, Tb or TM6B, y+ for the third chromosome, and T(2;3) SM6a; TM6B, Tb (a translocation between SM6a and TM6B, which segregates the second and third chromosomes together).

Table 1
FRT lines to induce somatic and/or germline clones

Name	Marker	Map	OvoD	GFP	RFP	Arm-Z
X						
101	w^+	14A-B	+	+		
18A	ry^+neo^+	18A		+		+
9-2	w^+	18E	+ ovoD2	+		
19A	ry^+neo^+	19A	+ ovoD1-18	+	+	
2L						
40A	ry^+neo^+	40A	+ ovoD1-18	+	+	+
2R						
G13	w^+	42B	+ ovoD1-18	+		
42D	ry^+neo^+	42D			+	+
43D	ry^+neo^+	43D				+
3L						
2A	w^+	79D-F	+ ovoD1-18	+	+	
80B	ry^+neo^+	80B			+	+
3R						
82B	ry^+neo^+	82B	+ ovoD1-18	+	+	+

2.2 Reagents, Buffers and Equipment

1. Phosphate buffered saline (1× PBS) or Schneider Medium.
2. PBT: 1× PBS with 0.2 % Triton™ X-100. Store at 4 °C for up to 1 week.
3. Fix Solution: 1× PBS with 4 % paraformaldehyde (PFA).
4. Methanol 100 %.
5. Block Solution: PBT and 10 % BSA (*see* **Note 2**). Store at 4 °C.
6. The Developmental Hybridoma Studies Bank (DSHB) produces and sells many monoclonal antibodies relevant for clonal analysis (Myc, GFP, beta-Galactosidase) at a low price (http://dshb.biology.uiowa.edu). Similar antibodies are also commercially available.
7. Halocarbon oil 10S (Voltalef, VWR).
8. Standard fly food.
9. G418 (Geneticin/neomycin; Invitogen, cat. no. (11811).

Table 2
FLP lines to induce somatic and/or germline clones

Name	Chromosome	Efficiency
{hsFLP1}	X	+ [26]
{hsFLP12}	X	+++ [43]
{hsFLP22}	X	+++ [43]
{hsFLP38}	2	++ [16]
{hsFLP86E}	3	++ [16]
{betaTub85D-FLP}1	X	ND
{ovo-FLP.R}	X	ND
{ovo-FLP.R}	2	ND
{ovo-FLP.R}	3	ND
{UAS-FLP.Exel}1	X	ND
{UAS-FLP.Exel}3	3	ND
{3XUAS-FLPG5.PEST}	X	ND
{3XUAS-FLPG5.PEST}	2	ND
{20XUAS-FLPG5.PEST}	X	ND
{20XUAS-FLPG5.PEST}	2	ND
{20XUAS-FLPG5.PEST}	3	ND
{UASp-FLP.G}1	X	ND
{UASp-FLP.G}3	3	ND
{UAS-FLP.MB}	X	ND
{UAS-FLP1.D}	2	ND
{UAS-FLP1.D}	3	ND

10. 25 °C fly culture incubator.
11. Temperature-adjustable water bath tank (or 37 °C bacteria incubator).
12. Dissecting microscope.
13. Dissecting tools: three-well dissecting dishes, fine forceps, tungsten needles.
14. Table top rotator for 1.5 ml tubes.
15. Microscope glass slides (22 × 40 mm, 0.17 mm thickness) and coverslips (18 × 18 mm, 0.17 mm thickness).
16. Mounting Medium: Glycerol, Cityfluor, or VECTASHIELD.
17. Epifluorescence or confocal microscope to analyze ovaries.

3 Methods

The methods described here are used in female ovaries, though they can also be used for male testis. Before starting a mosaic analysis, we suggest you to read some general advices on how to set up your crosses (*see* **Note 3**). The control samples should be treated exactly in the same conditions as those of the experimental samples. The first step is to generate flies bearing the mutation of interest recombined onto the appropriate FRT chromosome. Germline clones can then be induced using the FRT-OvoD1 system to study late oogenesis stage or the FRT-GFP system to study early stages as well as follicle cells. The most common method to induce expression of the FLP is to use heat-shock inducible promoter. For advice on heat-shock duration and timing *see* **Note 1**. It is also possible to perform double mutant mosaic analysis (*see* **Note 4**), mosaic analysis of mutations located proximal to FRT insertions or located on chromosome 4 (*see* **Note 5**). Unfortunately, it is also possible that you do not get clones in some case (*see* **Note 6**).

3.1 Construct a Chromosome with Both an FRT Site and a Mutation (m^-)

There are two types of FRT insertions with different selection markers (*see* Table 1). The first category is a FRT insertion with a mini-*white* (*w*) marker. The FRT insertion can then be followed by red pigmentation of the eye. Both the FRT insertion and the mutation of interest need to be in a *w* background to follow the FRT insertion. The second category of FRT insertions contains a Neomycin-resistant cassette and a *rosy* (*ry*) transgene. The FRT insertion can be followed by the ry^+ cassette if all flies are in an *ry* background. The ry^+ cassette can be difficult to score so it is advised to use the accompanying Neomycin-resistant cassette. If Neomycin/G418 is added to the fly food, only the larvae containing the *neo*-cassette will survive. FRT-bearing larvae are thus positively selected. The *neomycin* gene is under the control of a heat-shock promoter, therefore 37 °C heat-shocks can improve significantly the survival rate.

A third method to follow the FRT insertions is to use PCR primers to amplify directly the FRT sequences. This method works for both categories of FRT sites (*see* **Note 7**).

3.1.1 Recombining a Mutation onto FRT on an Autosome

As an example, the following steps describe how to proceed with a mutation (m−) located on the right arm of the second chromosome (*see* **Note 7**).

1. Set up the cross on neomycin/G418-containing fly food using 8–10 virgin heterozygous females and 3–5 males with the appropriate balancer at 25 °C:

   ♀ *w;* m^-*/P[*ry^+*;hs-neo;FRT]42D* × ♂ *w; Sco/CyO*

2. Transfer parental flies daily into a new vial with freshly prepared G418-containing food.

3. Perform the heat shock treatment on the offspring at 1–3 days of age, at 37 °C for 1 h, to drive the expression of *hs-neo*.
4. After hatching, select single *w* males and cross them individually to a balancer stock (such as *w*; *Sco/CyO*) to establish independent recombinant lines. The number of independent stocks needed to be established depends on the genetic distance between your mutation of interest and the FRT insertion. Usually, when the mutation is on the other half of the chromosome arm, 30 stocks are sufficient. One can go up to 200 lines when the mutation is very close to the FRT site. Each of these lines can then be tested for the presence of the mutation, either by crossing it to another allele of the same gene or a deficiency uncovering the region. The obtained genotype is: *w;* m⁻*,P[ry+;hs-neo;FRT]42D/CyO*.

3.1.2 Recombining a Mutation onto FRT on X Chromosome

As an example, the following steps describe how to proceed with a mutation (m–) located on the long left arm of the X chromosome (*see* **Note 7**).

1. Set up a cross on neomycin/G418-containing media using 8–10 virgin heterozygous females and 3–5 males with the appropriate balancer at 25 °C:

   ♀ m⁻*/P[ry⁺;hs-neo;FRT]18A* × ♂ *Y/FM7*

2. Transfer parental flies daily into a new vial with freshly prepared G418-containing food.
3. Perform the heat shock treatment on the offspring at 1–3 days of age, at 37 °C for 1 h, to drive the expression of *hs-neo*.
4. After hatching, select single *FM7* females and cross them individually to a balancer stock male (such as *Y/FM7*) to establish independent recombinant lines:

   ♀ m⁻*, P[ry⁺;hs-neo;FRT]18A/FM7* × ♂*Y/FM7*

   The obtained genotype is: *w;* m⁻*, P[ry⁺;hs-neo;FRT]18A/FM7*

   - Cross the resulting m⁻*, P[ry⁺;hs-neo;FRT]18A/Y male* with m⁻*/FM7* females:

     ♀ m⁻*/FM7* × ♂ m⁻*, P[ry⁺;hs-neo;FRT]18A/Y*

   - If in the progeny m⁻/m⁻*, P[ry+;hs-neo;FRT]18A* females exhibit the expected m⁻ homozygous phenotype, then the above corresponding recombinant line contains both the FRT and m⁻ on the X chromosome.

3.2 Generating Germline Clones with the FRT/OvoD1 System

The following steps describe, as an example, how to proceed with a mutation (m⁻) located on the left arm of the second chromosome (*see* **Note 8**).

1. Produce by standard crosses female flies that are heterozygous for the mutation under study and the ovo^{D1} chromosome by

crossing ovo^{D1} males to females that have the mutation balanced and present distal to an FRT site on that chromosome arm. The ovo^{D1} can only be brought in by males.

2. Set up the cross using 8–10 virgin heterozygous females and 3–5 males at 25 °C.

♀ *w;* m⁻, *FRT-40A/CyO*× ♂ *hs*-FLP/Y; ovo^{D1}, *FRT-40A/CyO*

3. Perform the heat shock treatment on these offspring at 1–3 days of age, to drive the expression of the FLP recombinase. Females of the following genotype should be dissected:

♀ *hs*-FLP/+; m⁻, *FRT-40A*/ovo^{D1}, *FRT-40A*

3.3 Generating Germline Clones with the FRT/GFP System

The following steps describe, as an example, how to proceed with a mutation (m⁻) located on the left arm of the second chromosome.

1. Produce by standard crosses female flies that are heterozygous for the mutation under study and the GFP-FRT chromosome by crossing GFP-FRT males to females that have the mutation balanced and on an FRT chromosome arm. In this case, the cross can be done in both directions, by bringing in the mutation either by males or females. When using a lethal mutation on the X chromosome, the mutation can only be introduced by females.
2. Set up the cross using 8–10 virgin heterozygous females and 3–5 males at 25 °C.

♀ *w;* m⁻, *FRT-40A/CyO*× ♂ *hs*-FLP/Y; *ubi*-GFP, *FRT-40A/CyO*

3. Perform the heat shock treatment on these offspring at 1–3 days of age, to drive the expression of the FLP recombinase. Females of the following genotype should be dissected:

♀ *hs*-FLP/+; m⁻, *FRT-40A*/*ubi*-GFP, *FRT-40A*

3.4 Generating Mutant Somatic Clones with the FRT/GFP System

The procedure to generate somatic clones with the FRT/GFP system is exactly the same as for germline clones (*see* Subheading 3.3). The main parameter to be adjusted is the timing of the heat-shock. It is possible to hit the FSCs when heat-shocking the third instar larvae using the same procedure as for germline clones. However, FSCs are still actively dividing at the adult stage, so heat-shocks can also be performed on adult female. It is also possible to generate small clones by inducing recombination while the follicle cells are still dividing before stage 6. These clones are however transient and the flies should be dissected only a few days after recombination. *See* Subheading 1.2.3 for specific considerations when analyzing mosaics in somatic cells.

3.5 Overexpressing a Candidate Gene (X) in GFP-Marked Mutant (m^-) Clones

As mentioned above, the MARCM technique has been used only in somatic cells to our knowledge. Here, we describe its use in follicular cells (*see* **Note 9**).

1. Produce by standard crosses flies with the following genotype:

   ♀ *y, w, hs*-FLP/+; *actin*-GAL4, *UAS*-EGFP/ *UAS-geneX; FRT82B, tub*-GAL80/*FRT82B*

2. Perform the heat shock either on larvae or adults, depending on your developmental stage of interest.

3.6 Antibody Staining of Ovaries

1. Dissect the ovaries in 1× PBS or Schneider Medium with tungsten needles (*see* **Note 10**).
2. Fix the ovaries in Fix Solution with rocking for 20 min.

2′. Optional: Wash 3× in methanol, the ovaries can then be stored at −20 °C, if required.

3. Wash 10 min in 1× PBS.
4. Permeabilize for 30 min in 1× PBT.

4′. Optional: Incubate the ovaries in Blocking Solution for 1 h.

5. Wash 3× 10 min with 1× PBS.
6. Add 200–500 µl of the primary antibody in 1× PBS. Incubate overnight, with rocking at room temperature (usually 20 h).
7. Aspirate the primary antibody and recycle it.
8. Wash 4× 30 min with 1× PBS with rocking.
9. Add the secondary antibody in 1× PBS (usually at 1/200 final concentration).
10. Incubate for 2–4 h with rocking at room temperature.
11. Aspirate the secondary solution and wash 3× 10 min in 1× PBS.
12. Proceed to staining for Phalloidin, DAPI (1/500) or Hoechst (1/500) during the wash.
13. Mount in one of several types of Mounting Medium: Glycerol, Cityfluor, or VectaShield.

3.7 Live Imaging of Ovaries

1. Dissect the ovaries in a drop of halocarbon oil (*see* **Note 10**).
2. Remove the muscular sheath around each ovariole.
3. Pull a line of parallel germaria thus making them stick to coverslips in the oil.
4. Put two late stage egg chambers on each side of this line, just making it easier to find the germarium and to position the coverslip on top of the microscope stage.

4 Notes

1. The efficiency of recombination depends on each FRT insertion and the levels of expression of the FLP [26]. In general, there is not much choice for the FRT insertions available for a given mutation. However, the efficiency of recombination does vary for different FRT insertions. The levels of *hs*-FLP can also vary greatly from one line to another (*see* Table 2). Very efficient FLP could be due to multiple insertions of the same *hs*-FLP transgene on the same chromosome. For each FLP line, it is possible to control the levels of FLP with two parameters: (1) temperature and (2) duration of each shock. The maximal and usual temperature is 37 °C, but FLP can be induced already at 29 °C. An example of a strong heat-shock regime to induce germline clones with the FRT-OvoD1 system is 2 h of heat-shock at 37 °C once per day for 3 consecutive days. This strong regime is used during genetic screens when lots of clones need to be induced in each individual.

   It is important to keep track of the number and duration of heat-shocks for each vial. This can be written directly on the vial or on the plug (date of crosses/date of first heat-shock/numbers of heat-shock).

   It has been worked out that third instar larvae (when they start crawling up the vial) is the best stage of development to induce germline clones. Since the heat-shock promoter is ubiquitous, clones will be induced in any dividing cells of third instar larvae. However, at this time, the future GSCs are actively dividing, while cells in imaginal discs have mostly stopped dividing. Therefore, although this procedure enriches for the number of clones in germ cells, it is not restricted to germ cells.

   If a mutation is detrimental to GSCs viability, it is advised to limit recombination to the adult stage. Indeed, GSCs are still dividing at the adult stage (every 24 h on average) and mitotic recombination is still possible. The probability of inducing mosaics will be however lower than during the larval stage. On the other hand, if the mutation under study induces a rapid loss of GSCs, mutant GSCs can have already disappeared at the adult stage if recombination is induced in third instar larvae.

   In follicle cells, inducing clones at the adult stage is more efficient than in germ cells, and can be used routinely. It is also possible to use UAS-FLP transgenes and Gal4 drivers that are expressed in FSCs or in a subpopulations of follicle cells such as border cells [52, 53].

   Heat shocks can be given in either a 37 °C water bath or an air incubator. Water baths are slightly more efficient in the transduction of heat. However, when large number of vials

needs to be heat-shocked simultaneously (during a genetic screen for example), an air incubator is a good alternative. Fly food tends to dry a bit more in an air incubator, so it is a good idea to add some water.

2. The species from which the serum is derived is not important when using highly specific primary and secondary antibodies. We routinely add sodium azide (0.02 %) to ensure that there will be no microbial growth.
3. Even a light regime of heat-shocks can be detrimental to a fly's health, and more larvae or adult flies will die than in non-heat-shocked vials. One should be careful to set up crosses with more adults than regular crosses in order to obtain more larvae per vial for heat-shocks. Larvae are also more resistant than adult flies to long heat-shocks. Although the focus of this chapter is on ovaries, and it is important to keep males in the same vials as the females, because the presence of males stimulate oogenesis.
4. It is possible to induce clones homozygous for two different mutations. The easiest case is when both mutations can be recombined onto the same chromosome arm. A single marked FRT chromosome can then be used as in regular clonal analysis.

   A second case is when the two mutations are on different chromosomes. One can either use two FRT chromosomes with the same marker such as GFP. The homozygous double mutant cells will be identified by the complete lack of GFP, but single mutant cells for any of the two mutations cannot be distinguished. One can also used two different markers, one FRT-GFP and one FRT-LacZ or RFP. Alternatively, one can also use antibody staining against one of the mutated genes if the corresponding protein is completely absent [54]. Double homozygous mutant cells can be identified by the lack of both markers and the protein.

   A third case is when both mutations are on the same chromosome but on different chromosome arms. Special FRT chromosomes need to be used carrying two FRT insertions on both sides of centromeres. Such chromosomes can be found at the Bloomington Stock Center. For an example, *see* ref. 47.
5. The regular FLP/FRT system cannot be used to analyze mosaics for genes located between an FRT insertion and a centromere or for genes located on the fourth chromosome. One alternative is to construct a transgene that is able to rescue completely the mutation of interest, and to recombine this transgene onto a FRT chromosome. Homozygous mutant cells will be induced by the absence of the rescue cassette in a background completely homozygous for the mutation of interest.

6. It is possible that no clones will be identified in the recombining females, even when following the protocol carefully. If this occurs, it is suggested to check whether: (1) There is a FRT insertion recombined with the mutation of interest. This can be done by PCR or by inducing clones in the eye disc with eyeless-FLP; (2) The dominant marker is present and expressed as it should be on the marker chromosome. One can dissect the FRT-marker stock without crosses or heat-shocks to test for the presence of the marker; (3) The mutation is cell-lethal. Homozygous clones may be induced, but could disappear as mutant cells either die or are expelled from the tissue. The presence of "twin-spot" cells is an indication that recombination events have taken place, but that homozygous cells have disappeared. You can then adjust the clone induction procedure and dissect quickly after the clone induction (*see* **Note 1**).
7. As controls during the positive selection, take flies from the corresponding *hs-neo* :FRT (the parents of the original cross) line into one vial with G418, and follow the same procedure. DO NOT use FRT 101, 18E (also called 9-2), 42B (also called G13), or 2A FRT strains as positive controls, since these FRTs do not contain a *neo* resistance gene. In practice, the *hs-neo* can be leaky in many strains. Heat-shocks are thus not always required for survival. Transfer the recombined adults to a new vial before the next generation begins to hatch. When the above recombinant lines hatch, check the corresponding controls. If flies emerged from the negative control vial, you cannot be confident that the selection worked efficiently. Different choices are then available:
   - Induce clones with *ey*-FLP to validate the presence of the FRT chromosome. It is easy to check if clones have been induced in the eye without any dissection or staining procedure.
   - Screen for the presence of the FRT by PCR. This second option can be a good choice if the mutation of interest is more than ~20 map units from the FRT site because it is likely that many of the flies recovered are good recombinants. (Map unit positions of genes can be found in the gene reports in Flybase). Single-fly DNA preps for PCR protocol [51] works well to amplify FRT sequences. FRT primers are:

   Sense sequence: TGAAGTTCCTATACTTTCTAGAGAATA GGAACTTC

   Antisense sequence: GAAGTTCCTATTCTCTAGAAAGTA TAGGAACTTCA
8. As a control, dissect females with the same genotypes from vials, which have NOT been heat-shocked. It will confirm that OvoD1 is working correctly and that the genotypes of the flies in the cross are correct.

9. As for the FLP-FRT lines, the FLPout and FLPout-Gal4 lines may show leaky expression, and control experiments without FLP induction are strongly advised for misexpression studies. This method eliminates possible artifacts due to dissection (*see* Subheading 1.2.4) when whole cells detached from the epithelium. However, one should still be careful of communication between follicle cells by small ring canals, either for the interpretation of loss-of-function experiments, but also for diffusion of markers and/or overexpressed proteins.
10. Before dissection, put the females for one night on fly food supplemented with yeast to get healthy, enlarged ovaries.

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