A rough guide to *Drosophila* mating schemes (version 3) [1]

This document is one part of a *Drosophila* genetics training package, the entire strategy of which is described in detail elsewhere [82].

Content

[1] Updated versions of this document can be downloaded @ dx.doi.org/10.6084/m9.figshare.106631 and a “lite” version for short term training @ dx.doi.org/10.6084/m9.figshare.156395

Boxes

Figures

Appendix

1. Why work with the fruitfly *Drosophila melanogaster*?

More than a century ago the fruitfly *Drosophila melanogaster* was introduced as the invertebrate model organism that founded the field of classical genetics. It has been argued that *Drosophila*, as an omnipresent follower of human culture, was easy to obtain and maintain in laboratories, and that it was kept in many laboratories as a cheap model for student projects suitable in times of neo-Darwinism (the study of Darwinian evolution with Mendelian genetics) [59]. Several laboratories started using the fly for their main research, but it was the serendipitous discovery of the *white* mutation and recognition of its linkage to the X chromosome in 1910 by T.H. Morgan which kick-started the systematic use of the fly for genetic research, essentially fuelled by Morgan's graduate students Sturtevant and Bridges [2,22,59,94][1]. Building on the sophisticated fly genetics gained during the early decades, research during the second half of the 20th century gradually turned flies into a powerful "boundary object" linking genetics to other biological disciplines [56]. Thus, fly genetics was systematically applied to the study of development, physiology and behaviour, generating new understanding of the principal genetic and molecular mechanisms underpinning biology, many being conserved with higher animals and humans [2,9,12,45,56,62,65,70,102]. Notably, it has been estimated that "...about 75% of known human disease genes have a recognisable match in the genome of fruit flies" [81]. Therefore, besides remaining a powerhouse for unravelling concepts and fundamental understanding of basic biology, *Drosophila* is nowadays often used as a "test tube" to screen for genetic components of disease-relevant processes or pathways, or to unravel their cellular and molecular mechanisms, covering a wide range of disease mechanisms including neurodegeneration and even neurotoxicology [13,50,54,80]. It is therefore not surprising that *Drosophila* is the insect behind six Nobel laureates (Box 1).

Box 1. Nobel prizes for work on *Drosophila* (www.nobelprize.org/nobel_prizes/medicine/laureates/)

1933	Thomas Hunt Morgan - *the role played by chromosomes in heredity*
1946	Hermann Joseph Muller - *the production of mutations by means of X-ray irradiation*
1995	Edward B. Lewis, Christiane Nüsslein-Volhard, Eric F. Wieschaus - *the genetic control of early embryonic development*
2011	Jules A. Hoffmann - *the activation of innate immunity*

Drosophila's enormous success originates from the numerous practical advantages this tiny insect and the community of fly researchers have to offer to the experimenter. The most important advantages are briefly listed below (and tongue-in-cheek here)[2] & [3]:

- Fruit flies are easy and cheap to keep. High numbers of different fly stocks can be kept in a handful of laboratory trays, thus facilitating high-throughput experiments and stock management (section 3).
- A fruit fly generation takes about 10 days (Fig.1), thus fly research progresses rapidly and pedigrees over several generations can be easily planned and monitored.
- The fly genome is of low redundancy, i.e. only one or very few genes code for members of one protein class. In contrast, higher organisms tend to have more paralogous genes encoding closely related proteins that often display functional redundancy and complicate loss-of-function analyses.
- A particular strength of *Drosophila* is the possibility to perform unbiased screens for genes that regulate or mediate biological processes of interest, often referred to as forward genetics (Fig. 2; Box 2). Highly efficient and versatile strategies have been developed that can be adapted to the experimenter's needs [17,42,52,88,91].
- Virtually every gene of *Drosophila* is amenable to targeted manipulations through a wide range of available genetic strategies and tools, ideal to perform reverse genetics (Box 2).

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[1] Dan Lindley (2008) *Drosophila* genetics - The first 25 years @ hstalks.com/?t=BL0341788

[2] for an excellent overview of *Drosophila* genetics see the appendix of the book by Hartwell [48] (http://highered.mcgraw-hill.com/sites/007352526x/student_view0/genetic_portrait_chapters_a-e.html)

[3] Informative lay descriptions of fly research can be found on the Wellcome Trust Blog:
The portrait of a fly (Part 1) - wellcometrust.wordpress.com/2012/11/20/feature-the-portrait-of-a-fly-part-1/
The portrait of a fly (Part 2) - wellcometrust.wordpress.com/2012/11/23/the-portrait-of-a-fly-part-2-fly-on-the-wall/

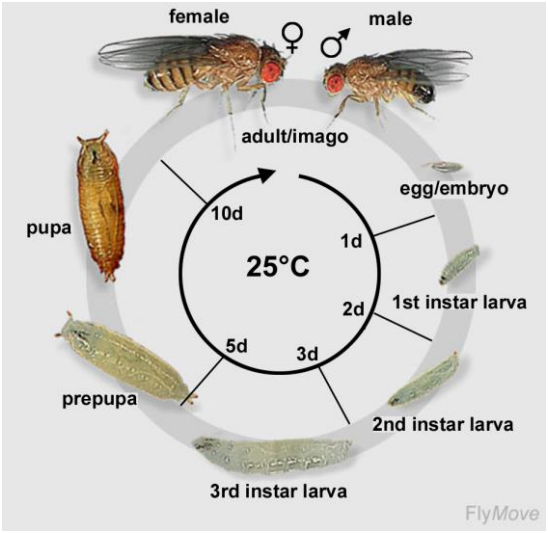

Figure 1. The life cycle of *Drosophila melanogaster*

Fertilised females store sperm in their *receptaculum seminis* for the fertilisation of hundreds of eggs to be laid over several days. At 25°C embryonic development lasts for ~21hr. The hatched larvae (1st instar) take 2 days to molt into 2nd then 3rd instar larvae. 3rd instar larvae continue feeding for one more day (foraging stage) before they leave their food source and migrate away (wandering stage) and eventually pupariate (prepupa then pupa). During the pupal stages, all organs degenerate (histolysis) and restructure into their adult shapes (metamorphosis). 10d after egg-lay, adult flies emerge from the pupal case. After eclosure, males require up to 8 hr to mature sexually, which can be capitalised on for virgin female collection (section 3). The times mentioned here need to be doubled when flies are raised at 18°C [3]. Image modified from FlyMove [101].

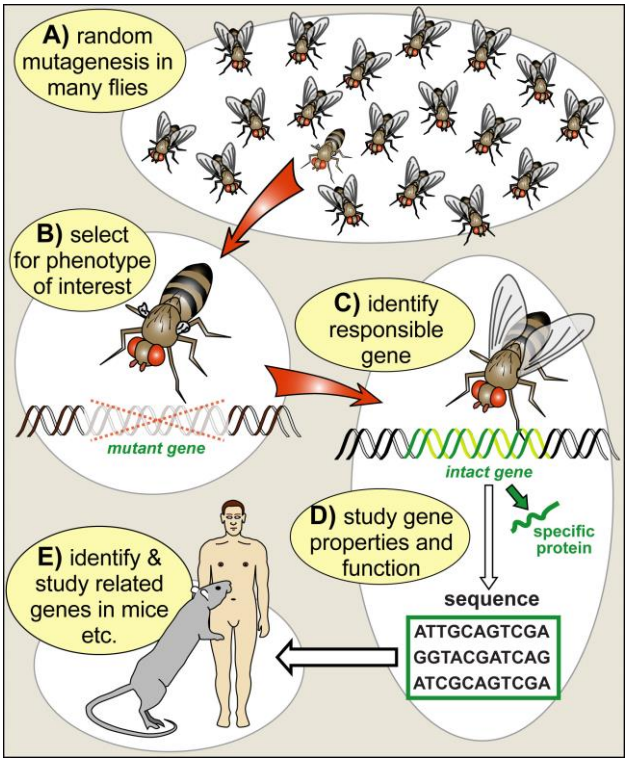

Figure 2 A typical flow diagram of how genetic screens in *Drosophila* contribute to research

A) To induce random mutations, large numbers of flies are treated chemically (e.g. using EMS, ethyl methanesulfonate - **highly carcinogenic!**), manipulated genetically (e.g. through P-element mutagenesis; section 5.1) or exposed to irradiation (e.g. applying X-ray). Other unbiased approaches are screens with large collections of transgenic *RNAi* lines to systematically knock down genes one by one (section 5.2f) or with EP-line collections to systematically over-express genes (section 5.2d). **B)** The essential task is to select those mutant or genetically manipulated animals that display phenotypes representing defects in the biological processes to be investigated. **C)** The responsible gene is either indicated by the specific RNAi- or EP-line inducing the phenotype, or can be identified using classical genetic or molecular strategies to map newly induced mutations to defined genes within the fly genome (Fig. 12B and section 6). **D)** Once the gene is identified, its nature and normal function can be studied. **E)** Vertebrate or human homologues of *Drosophila* genes are usually known (listed under "Orthologs" in FlyBase). Based on knowledge derived from fly research and the empirical assumption that principal mechanisms are often conserved, informed and focussed experiments can be carried out on these genes in vertebrate/mammalian model organisms, or human patients can be screened for mutations in these genes.

- Experimental manipulations and observations of cells and tissues *in vivo* are relatively easy. Thus, organs are of relatively low complexity and size, and can usually be studied live or via straightforward fixation and staining protocols in the whole organism. Only in exceptional cases are these experiments subject to legal requirements or procedures, thus enormously facilitating the fast implementation of experimental ideas. Furthermore, there is a "parallel universe" of complementary *Drosophila* research in cell culture. Firstly, an impressive number of *Drosophila* cell lines is readily available (dgrc.cgb.indiana.edu/cells/Catalog), of which especially S2 cells have achieved considerable recognition beyond the community of fly researchers [29]. Secondly, primary cell cultures (cells directly harvested from the organism) are well established, especially for neurons and haemocytes [78,85], and offer important complementary readouts amenable to the full range of versatile *Drosophila* genetics.

- Finally, more than a century of fly work has produced a huge body of knowledge and a rich resource of genetic tools. From early days of *Drosophila* genetics up to this day, the fly community has maintained a highly collaborative spirit which facilitates research enormously through generous exchange of materials and information [58,59][1]. Well organised databases and stock centres provide easy access to knowledge, fly strains and materials, all of which are well integrated and curated in FlyBase (flybase.org) the central point of reference for fly researchers worldwide [71,90].

Box 2. Concepts for genetic research: forward *versus* reverse genetics & LOF *versus* GOF

Gene manipulations are generally employed to serve two principal strategies: forward and reverse genetics [89]. FORWARD GENETICS is the approach to identify the genes that are responsible for a particular biological process or function. In *Drosophila,* this is usually performed through using unbiased large-scale screens for genetic aberrations that disturb the process/function in question, and the subsequent identification of the genes affected through these aberrations (Fig. 2). REVERSE GENETICS is the approach to unravel the functions behind specific genes of interest, for example when trying to understand molecular mechanisms or functions of genes known to cause human disease (using the fly as a "test tube"). For this, loss- or gain-of-function (LOF, GOF) approaches are employed, using existing mutant alleles and a wide range of transgenic fly lines that are often readily available (Box 3).

GOF approaches attempt to obtain functional information by creating conditions where the gene is excessively or ectopically expressed or its function exaggerated. This can be achieved through classical GOF mutant alleles (section 4.1.2) or through targeted expression of genes, either of their wild type alleles or of constitutive active versions (section 5).

LOF approaches attempt to eliminate a gene's function partially or completely. This can be achieved by employing classical LOF mutant alleles (section 4.1.2), transposable element insertions (existing for virtually all gene loci; section 5.2b-d), knock-down of genes using RNA interference strategies (readily available as transgenic lines for virtually every gene; section 5.2f), the targeted expression of dominant-negative constructs (e.g. catalytically dead versions of enzymes titrating out the function of the endogenous healthy enzyme), or the use of targeted expression of single domain antibodies (Box 3). Furthermore, there are constantly improving strategies for the manipulation of genes *in situ*, i.e. in their chromosomal location, including...

- classical mutagenesis strategies in which mutations are first generated at random and then selected over chromosomal deficiencies uncovering the targeted gene, thus enriching for candidate mutant alleles of this gene (section 6c)
- generation of targeted deletions at the gene locus through recombinase-mediated mobilisation or homologous recombination of local transposable elements (section 5.1)
- targeted manipulations of the gene locus through genomic engineering, using recombinase-based strategies [51], TALEN strategies (transcription activator-like effector nuclease) [61,66] or CRISPR technology (clustered regularly interspaced palindromic repeats) strategies [7,43].

2. The importance of genetic mating schemes

Daily life in a fly laboratory requires performing classical genetic crosses. In these crosses, mutant or genetically modified flies are used (Box 3). These different fly variants are the bread-and-butter of fly research, providing the tools by which genes are manipulated or visualised in action in order to investigate their function. The art of *Drosophila* genetics is to use these tools, not only in isolation but often combined in the same flies. This combinatorial genetic approach significantly enhances the information that can be extracted.

For example, you investigate a certain gene called *Mef2*. You have isolated a candidate mutation in this gene which, when present in two copies in embryos, correlates with aberrant muscle development. You hypothesise that this phenotype is caused by loss of *Mef2* function. A standard approach to prove this hypothesis is to carry out "rescue experiments" by adding back a wild type copy of the gene into the mutant background, analogous to gene therapy. For this, you will need to clone the *Mef2* gene and generate transgenic fly lines for the targeted expression of *Mef2* (section 5.1). To perform the actual experiment, you now need to bring the *Mef2* transgenic construct into *Mef2* mutant individuals. This last step requires classical genetic crosses and the careful design of genetic mating schemes.

[1] All issues of the legendary *Drosophila* Information Service can be browsed here: www.ou.edu/journals/dis/

These mating schemes are a key prerequisite for successful *Drosophila* research. The rules underpinning these schemes are simple. However, they often require thinking ahead for several generations, comparable to planning your moves during a game of chess. To enable you to design such mating schemes, this manual will provide you with the key rules of the game and explain the main parameters that need to be considered.

Box 3. Fly stocks available for *Drosophila* research

1. Flies carrying classical loss- and gain-of-function mutations (including marker mutations) or deficiencies (section 4.1b)
2. Flies with chromosomal rearrangements (duplications, inversions, translocations etc.) [3,46]
3. Flies with balancer chromosomes (section 4.3; Box 6)
4. Flies with transgenic constructs encoding a range of products (section 5.2) including..
   - ..wildtype or mutant versions of genes (including dominant negative constructs) from *Drosophila* or other organisms
   - ..whole chromosomal fragments for rescue, gain-of-function or targeted mutagenesis experiments [98,100]
   - ..reporter genes (encoding ß-Gal, fluorescent proteins, calcium indicators, pH indicators etc.) fused to gene-specific or inducible promoters, or under the control of position-specific activating elements at their chromosomal insertion site (section 5.2a-c)
   - ..exogenous transcription factors (e.g. Gal4, tTA, LexA) with known expression patterns to induce targeted expression of a gene of choice (section 5.2d)
   - ..small interfering RNAs to knock down gene expression (section 5.2f)
   - ..single-domain antibodies against endogenous proteins [63] or designed into anti-GFP nanobodies for the targeted degradation of GFP-tagged proteins [25]
   - ..recombinases (e.g. flippase, ϕC31) or their recombination target sites (e.g. FRT, attP) at specific chromosomal locations; they are jointly used for site-directed insertion of transgenes (section 5.1) or to generate mosaics of mutant cells in the germline or in somatic tissues (section 5.2e)
   - ..genetically encoded toxins (e.g. ricin, tetanus toxin), cell death inducers (e.g. *hid*, *DTS*; Box 8), optogenetic tools (e.g. channel rhodopsin) [53] or other physiological tools (e.g. Kir channels, Shibire^{ts}) for the analysis and/or experimental manipulation of cells

3. Handling flies in the laboratory

3.1. Keeping flies

Before starting the theoretical part, it is necessary to give a brief insight into the practical aspects of fly husbandry and how the genetic crosses are performed. This should allow you to imagine the actual "fly pushing" work required to execute the mating schemes designed on the drawing board.

As indicated in Box 3, many different fly stocks are available for fly work. *Drosophila* research laboratories usually maintain considerable numbers of stocks relevant to their projects (Fig. 3A). But always be aware that stock keeping is work intense since you deal with live animals which need to be cared for like pets! Therefore, you should have a good reason for keeping stocks. For example, they may be unique (in this case also consider to send them on to stock centres or interested colleagues for back-up), or you may want to have them readily available to be able to kick-start practical work on experimental ideas that arise through daily discussion and thought. Always consider that most stocks can be ordered from public or commercial stock centres (FlyBase / Resources / Stock Collections) or by sending requests to colleagues all over the world, most of whom are willing to freely share fly stocks, especially when they are already published in scientific journals. Note that new flies coming into the laboratory should be properly filed (Box 4) and kept in quarantine under observation for a couple of generations in order to exclude diseases or parasites they may carry. Fly stocks are kept in small vials (Fig. 3B) containing food (the main ingredients of which are corn flour, glucose, yeast and agar)[1] and they can easily be transferred to fresh vials for maintenance. These vials are usually stored on trays in rooms or incubators (Fig. 3A) which are temperature-controlled since temperature influences the developmental time of flies (Fig. 1).

[1] fly media recipies: flystocks.bio.indiana.edu/Fly_Work/media-recipes/media-recipes.htm

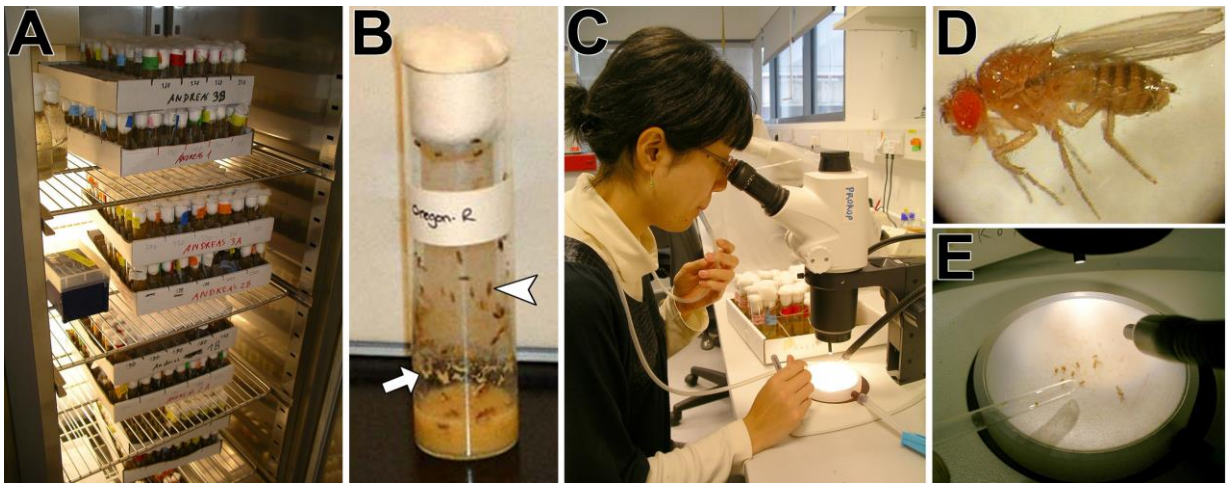

Figure 3. Maintaining and handling flies in the laboratory

A) Fly stocks are stored in large numbers on trays in temperature controlled rooms/incubators[1] (the trays shown here each hold two copies of 50 stocks). **B)** Each fly stock is kept in glass or plastic vials which contain food at the bottom and are closed with foam, cellulose acetate, paper plugs or cotton wool. Larvae live in the food and, at the wandering stage, climb up the walls (white arrow) where they subsequently puparíate (white arrow head). **C-E)** To score for genetic markers and select virgins and males of the desired phenotypes, flies are immobilised on CO_2-dispensing porous pads (E), visualised under a dissecting scope (C, D) and then discarded into a morgue or transferred to fresh vials via a paint brush, forceps or pooter / aspirator[2] (C, E). For further information on how a typical fly laboratory is organised see other sources [3,4,5,92][3].

Box 4. Keeping information about laboratory stocks

Work in a fly laboratory involves constant influx of new fly stocks, although only a small percentage of these will eventually be kept in your stock collection. Follow good practice by making it a rule to instantly document the essential information for each incoming stock in a dedicated folder or data sheet/base before it gets lost and forgotten in daily routine:

1. Keep the full original genetic description and any other information you may find on vials or accompanying notes (e.g. stock centre references or other seemingly meaningless numbers). Note that genetic descriptions you are given by the donor may be incomplete, and your accessory notes may provide unique identifiers for this fly stock when communicating with the donor laboratory.
2. Note down the donor laboratory/person and contact. You will need this information for further enquiries and acknowledgements in future publications.
3. When introducing stocks into your collection, transfer the above information into the accompanying data base/sheet. Make sure there is a proper genetic description, a clearly assigned short hand for daily use, info on the donor and the key reference publication. This information will be most useful when writing up your project and for people succeeding you in the laboratory.

- **Stock keeping** is usually done at 18°C (generation time of about 1 month). It is good practice to keep one young and one two week older vial of each stock. Every fortnight, freshly hatched flies from the month-old vial are flipped into a fresh vial, whilst the two-week-old vial should have produced larvae and serves as back-up. Such a routine allows you to spot any problems on time, such as infections (mites, mould, bacteria, viral infections) [3], the need to add water (if the food is too dry and coming away from the wall) or to reduce humidity (if vials are too moist so that fungus accumulates and/or flies get stuck in the food and at walls).
- **Experiments** with flies tend to take place at room temperature or at certain conventional temperatures, such as 25°C for well-timed experiments or 29°C to speed up development or enhance targeted gene expression with the *Gal4/UAS* system (section 5.2).

[1] Incubators need to be fly-proof: copper is aggressively corroded in the presence of flies and should be replaced by stainless steel or needs at least to be well protected (e.g. thoroughly coated with resin).

[2] for use & construction of pooters see: files.figshare.com/1402420/PootersForDrosophilaGenetics.pdf

[3] detailed stock-keeping instructions: flystocks.bio.indiana.edu/Fly_Work/culturing.htm

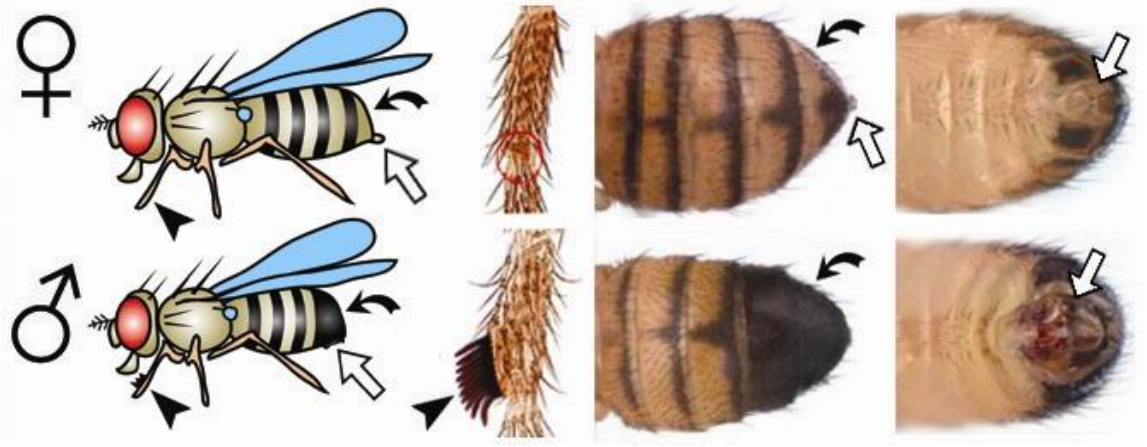

Figure 4. Criteria for gender selection

Images show females (top) and males (bottom): lateral whole body view (1st column), a magnified view of the front legs (2nd column), dorsal view (3rd column) and ventral view (4th column) of the abdomen. Only males display sex combs on the first pair of legs (black arrow heads). Females are slightly larger and display dark separated stripes at the posterior tip of their abdomen, which are merged in males (curved arrows). Anal plates (white arrows) are darker and more complex in males and display a pin-like extension in females. The abdomen and anal plate are still pale in freshly eclosed males and can be mistaken as female indicators at first sight. Photos are modified from [1] and [30]. During a very short period after eclosion, flies display a visible dark greenish spot in their abdomen (*meconium*; not shown) which can be taken as a secure indicator of female virginity even if fertile males are present.

3.2. Performing crosses

To perform crosses, females and males that carry the appropriate genotypes are carefully selected and transferred into one vial for mating (Box 5). Some aspects need consideration:

- **Males** and **females** need to be distinguished using the criteria explained in Figure 4.
- Selected females have to be **virgin**, i.e. selected before they are randomly fertilised by sibling males in their vial of origin. To select virgins, choose vials containing many dark mature pupae from which adult flies are expected to eclose. To start the selection procedure, discard all flies from the vial and thoroughly check that all eclosed flies (including those that transiently stick to the food or walls) have been removed or otherwise eliminated. The key rationale of this procedure is that freshly eclosed males remain sterile for a period of several hours and will not court females. Hence, after clearing vials, all females eclosed within this period will be virgin. This period lasts for 5-8 hrs at 25°C, about double the time at 18°C, and considerably longer at even lower temperatures (we use 11°C to maintain crosses up to two days for subsequent virgin collection). Therefore, a typical routine for virgin collection is to keep vials **overnight** at low temperatures (ideally below 18°C) and harvest virgins first thing in the **morning**. During the day, they are kept at higher temperatures (to enhance yield) and harvested again around **lunchtime** and **early evening**, before moving them back to lower temperature for the night.
- Flies have to be selected for the right **phenotypic markers**. When designing a **mating scheme**, combinations of markers need to be wisely chosen so that the correct genotypes of both sexes can be unequivocally recognised at each step of the scheme (often from parallel crosses). Phenotypic markers will be explained in section 4.2, and the rules how to choose them will become clear from later sections.

In general, more female flies are used in a cross than male flies (unless males are expected to be of low fitness), with two thirds being female as a reasonable approximation. In general, consider that di- and trihybrid crosses (see example in Fig. 6) will have a low yield of the required offspring and that the numbers of flies available for crosses in a complex mating scheme may gradually reduce from generation to generation. Complex mating schemes should therefore be initiated with large volume crosses (also see Box 5).

A frequent problem is that genetic combinations required for your mating scheme may render flies morbid, so that the numbers in which they hatch are far lower than statistically expected (referred to as **semi-** or **sub-lethality**). If you need to perform crosses with such animals and

gender choice is an option, choose males from the morbid stock/genotype and females from the more viable stock/genotype to enhance your chances of establishing the next generation. Furthermore, make sure that you improve the yield of these morbid flies by taking a number of measures:

- Avoid over-population of vials, which tends to negatively select against morbid individuals. For this, transfer parents to new vials when sufficient eggs have been laid (within a time frame of 1 day to 1 week, depending on the fertility of stocks used and numbers of parental flies).
- Morbid flies tend to get stuck and lost in the food. Therefore monitor crosses daily from start of eclosion, even if you want to collect only males. Alternatively you may resort to gender scoring at the larval or pupal stage (Box 5).
- Morbid animals tend to hatch late. For example, males carrying the balancer chromosome *FM7* (Fig. 11) tend to eclose days after their heterozygous female siblings. Therefore, continue scoring for as long as possible, but stop and discard the tube before potential individuals of the grandchild generation start emerging (after ~19 d in a modestly populated tube at 25ºC).
- Make sure that strains are free of bacterial or viral diseases as well as fungal or mite infection [3]. These conditions can pose a threat to the feasibility of mating schemes. The best prophylaxis is careful and regular husbandry of your fly stocks.

Note that these same measures should also be taken when you need to **quantitatively assess** the relative abundance of different phenotypes emerging from a cross. This is required, for example, when carrying out **meiotic mapping** experiments (section 6b), or when you want to perform **geno-/phenotypic counts** of homozygous mutant *versus* heterozygous/balanced animals, in order to determine the degree of lethality as a measure of allelic strength. In these cases you need to take care that morbid animals are not disadvantaged by the stock keeping and harvesting prodecures.

Box 5. How to select flies

Early drosophilists commonly used ether to select flies for gender and phenotypic markers. Nowadays flies are tipped from their vials onto porous pads dispensing CO_2 which acts as a narcotic and is not harmful if exposure is kept to a few minutes (www.youtube.com/watch?v=S7FkmBjrnAs). Using a dissection microscope, flies can be easily inspected and selected on this pad (Fig. 3C-E):

- Small numbers of flies are efficiently selected using a pooter/aspirator, which is a simple rubber tube with a mouth piece at one end and glass pipette at the other (Fig. 3C, E). A pooter is far more efficient than a paint brush, since flies can be simultaneously selected and collected and then directly be blown into vials, even if these already contain flies.
- When scoring large numbers of flies, arrange them into a line across the pad and pull out one phenotypic class at a time. In this way you only have 2 piles of flies on the pad at any one time. Use a funnel to tip the selected flies from the pad into vials. Note that large-scale virgin collection can be automated using stocks carrying inducible lethal factors on the Y chromosome [see *l(2)DTS* in Box 8].
- Morbid fly stocks/crosses do not tolerate overnight storage at lower temperatures (used to guarantee virginity of eclosed individuals in the morning) very well. To maximise yields, individuals can be sexed as larvae or pupae [32], removed from their tubes with a wet paint brush, and transferred into vials separated by gender. After eclosion, flies can be screened for phenotypic markers.
- Especially in complex mating schemes involving multiple markers, a safe way of phenotype and gender selection is to merely separate males from females into distinct vials during your daily routine. Only when enough animals have been collected, perform the marker selection in one single session. This mode is safer and less time-consuming, especially for the inexperienced fly pusher or when various crosses are running in parallel so that keeping an overview becomes a challenge.

Selected flies are added to fresh standard vials properly labelled with gender and genotype (Fig. 3B) and kept at your temperature of choice. Non-selected flies are disposed of in a fly morgue (usually a bottle containing 70% alcohol) and never returned to their vials of origin.

4. How to design a mating scheme

4.1. Genetic rules

In order to design mating schemes for *Drosophila*, the typical rules of classical genetics can be applied. These rules are briefly summarised here and described in greater depth elsewhere [3,46].

4.1.1. Law of segregation

Drosophila is diploid, i.e. has two homologous sets of chromosomes, and all genes exist in two copies (except X-chromosomal genes in males; Fig. 5). By convention, homologous alleles are separated by a slash or horizontal line(s) (Fig. 6, Box 9). According to the first law of Mendel (**law of segregation**), one gene copy is inherited from each parent or, *vice versa*, the two copies of a gene are separated during meiosis and only one copy is passed on to each offspring (Fig. 6). **Non-disjunction** events are rare exceptions in which both copies pass to one gamete.

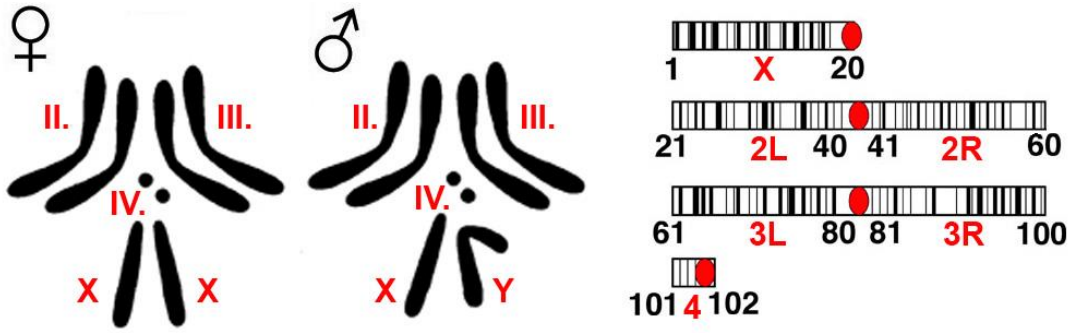

Figure 5. *Drosophila* chromosomes

Cytological images of mitotic *Drosophila* chromosomes. **Left**: Female and male cells contain pairs of heterosomes (X, Y) and three autosomal chromosomes. **Right**: Schematic illustration of *Drosophila* salivary gland polytene chromosomes which display a reproducible banding pattern which can be used for the cytogenetic mapping of gene loci (black numbers; see FlyBase / Tools / Genomic/Map Tools / Chromosome Maps for detailed microscopic images); 2nd and 3rd chromosomes are subdivided into a left (L) and right (R) arm, divided by the centrosome (red dot). Detailed descriptions of *Drosophila* chromosomes can be found elsewhere [49].

4.1.2. Alleles[1]

Genes exist in different alleles. Most **loss-of-function** mutant alleles (**hypo-** or **amorphic/null**) are **recessive**. Their phenotypes are not expressed in heterozygous (-/+) but only in homozygous animals (-/-), i.e. the wildtype allele mostly compensates for the functional loss of one gene copy (see *w*, *vg* or *e* in Fig. 6). Loss-of-function mutant alleles can also be **dominant**. For example, phenotypes are observed in animals heterozygous for *Ultrabithorax* (*Ubx*/+), *Polycomb* (*Pc*/+), or Notch (*N*/+) loss-of-function alleles, i.e. the wildtype allele is insufficient to compensate for loss of one functional gene copy (**haplo-insufficiency**). Dominant alleles can also be **gain-of-function**, usually caused by over-expression of a gene product (**hypermorph** or "dominant negative" **antimorph**) or by ectopic expression or activation of a gene product, potentially conveying novel gene functions (**neomorph**). For example, *BarH1* over-expression in the eye causes kidney-shaped eyes in Bar^{1}/+ individuals (Fig. 6) [60], ectopic *Antp* expression in antennae the antenna-to-leg transformations in $Antp^{73b}$/+ (Fig. 9) [41], and *Krüppel* mis-expression the reduced eyes in If^{1}/+ animals (Fig. 9) [23]. Dominant alleles may display **intermediate** inheritance showing a stepwise increase in phenotype strength from heterozygous to homozygous animals. Thus, the eyes of heterozygous flies (B^{1}/+) are kidney-shaped, whereas they display a stronger slit-shaped phenotype in homo- (*B*/*B*) or hemizygous (*B*/Y) flies (Fig. 6). Animals carrying the loss-of-function mutant allele $abd\text{-}A^{MX1}$ in heterozygosis are viable and show a weak dominant cell proliferation phenotype, whereas homozygous animals are lethal and show a strong cell proliferation phenotype [77]. Note, that the phenotype distribution in pedigrees involving dominant mutant alleles differs from those with recessive mutant alleles (Fig. 6). Also note that the existence of dominant and recessive alleles has impacted on gene names (capitalisation of the first letter), which can be confusing or even misleading (Box 7). As a further matter of complication, a **phenotype** you observe may not always be caused by the gene or mutant allele you believe to study, but a whole range of potential independent factors in the background of your fly stock/cross might modify the strength or quality of the observed phenotype, or be causing the whole phenotype all together. Be aware of this and carry out appropriate control experiments before drawing hasty conclusions (Box 6).

[1] see also http://en.wikipedia.org/wiki/Muller's_morphs

Box 6: Awareness of potential genetic modifiers

Apart from alleles or constructs that you want to study, fly stocks often carry additional features such as marker mutations which have remained as left-overs from gene mapping exercises (section 4.2). These are often not indicated in the genotypic descriptions, but be aware of them since they may have unwanted side effects. For example, the *ebony* marker mutation not only causes dark body colour, but it encodes a multi substrate enzyme that influences circadian rhythm, vision and courtship behaviour [95] (references therein). Furthermore, hidden genetic modifiers throughout the genome can have a significant impact on phenotypes you study [26,27,28,35,97]. A number of factors, singly or in combination, can cause genetic modifications:

- UNKNOWN SECOND-SITE MUTATIONS: Unknown second-site mutations (for example on the balanced chromosome that carries a mutation you want to study) may cause false phenotypes [44]. Note that mutations (especially homozygous-viable or haploinsufficient ones) kept in a stock over a long period, may cause positive selection for second-site mutations which ameliorate the original phenotype. Whilst this effect is unwanted in stock keeping, it is actively capitalised on in enhancer-suppressor screens [88].
- SYMBIONTS AND VIRAL INFECTIONS: For example, the intracellular bacterial symbiont *Wolbachia* is highly abundant in many *Drosophila* colonies and can impact on fly development, behaviour and longevity [72]. It can be detected by PCR or DAPI staining and eliminated using tetracyclin treatment [3]. Far less is known about viral infections, but the finding that about 130 genes including many signalling pathway components were activated upon infection with *Drosophila* C virus (DCV) [38], suggests that such a condition can impact on seemingly unrelated gene functions.
- B-CHROMOSOMES: They are supernumerary chromosomes inherited in a non-Mendelian fashion (like plasmids in bacteria), and they can impact on gene regulation and meiotic processes [8].
- SATELLITE DNA: heterochromatic repetitive elements serve important epigenetic functions; they account for ~30% of the *D. melanogaster* genome, but values may vary in different strains and therefore impose modifying potential [18].
- TRANSPOSABLE ELEMENTS: Transposable elements can insert into the genome in a random manner, impacting on gene function (section 5.1). There are close to 100 different species of transposable elements of euchromatin in *Drosophila* and ~1500 insertions were determined in one single genome [55], indicating their enormous potential as modifiers [104].
- EPIGENETIC INHERITANCE: PIWI-interacting RNAs (piRNAs) act as a kind of “immune system” against transposable elements, but only if they are maternally deposited in the oocyte. Inbred laboratory fly strains are poor in piRNAs and natural strains are rich, and crosses between them lead to the phenomenon of hybrid dysgenesis where laboratory females crossed to natural males cause infertility but not *vice versa* [84,86]. Another form of epigenetic inheritance called genetic imprinting seems absent from *Drosophila* (likely due to lack of gene methylation in fly) [34].
- MITOCHONDRIAL DNA: Mitochondria are producers of energy and reactive oxygen species important for cell physiology. Mitochondria are inherited from the mother, yet 90% of their functional genome is encoded by the cell nucleus and, accordingly, the combination of mitochondrial and somatic genomes can generate unpredictable biological variations, for example of longevity [79].
- Y-CHROMOSOME: The Y-chromosome of *Drosophila* is primarily heterochromatic, yet has regulatory power over autosomal genes which can vary significantly between Y-chromosomes from stocks of different origin [106].

To control for any unknown genetic backgrounds, you must seek independent ways of confirming observed phenotypes, ideally through heteroallelic combinations using mutant stocks from different sources, performing gene knock-down (section 5.2f) and/or rescue experiments (section 2). For highly complex phenotypes (e.g. behaviour, longevity), these strategies will not suffice. In this case, stocks need to be cleaned from infection and isogenised (i.e. the genome around mutant loci has to be replaced by that of a reference stock). Already the classical geneticists, when generating detailed linkage maps of *Drosophila* chromosomes, used standardised inbred stocks, the most famous of which are Canton-S, Berlin-K and Oregon-R [59]. More recently, a fantastic community resource of 192 fully sequenced inbred lines derived from natural stocks was generated by the *Drosophila melanogaster* Genetic Reference Panel (DGRP) [67]. Inbred fly lines are available at the Bloomington Stock Centre (All Browsing Options/Wild-Type Stocks) and can now be used for analysis of population genomics and quantitative traits in genome-wide association studies (GWAS) (e.g.) [69].

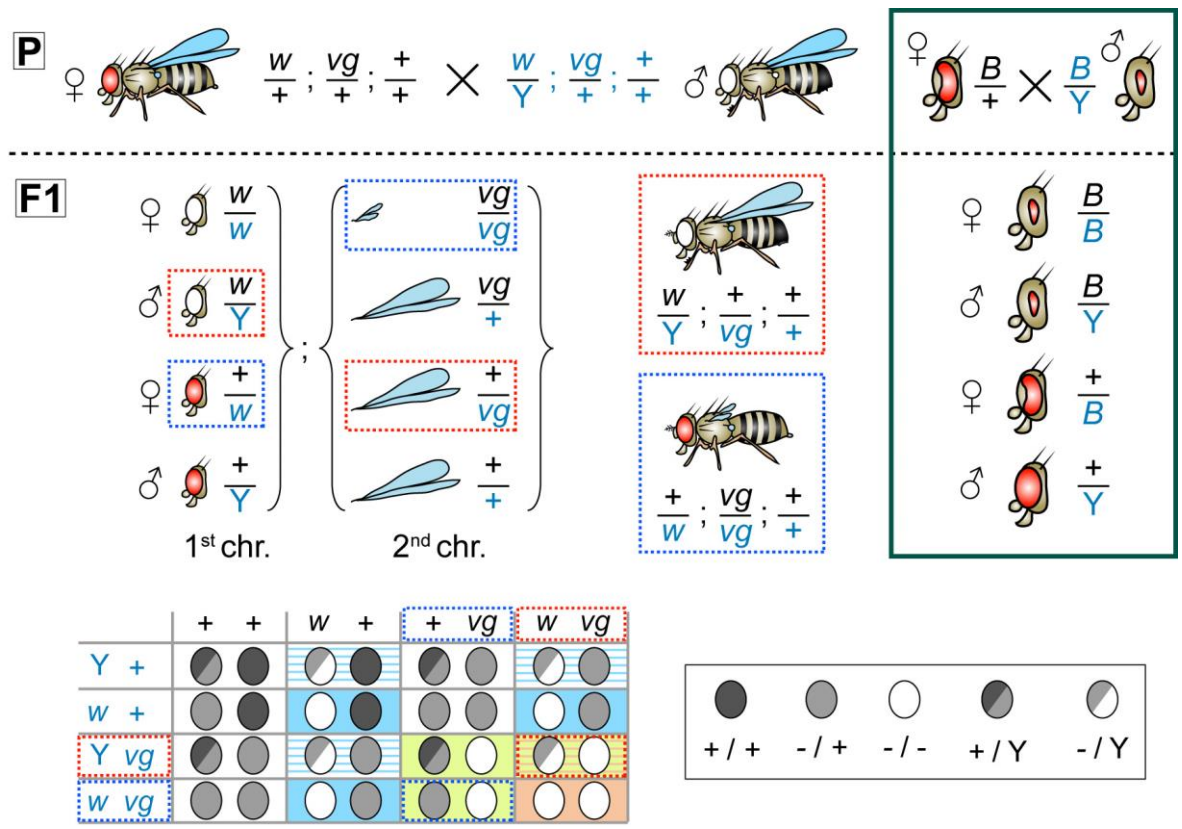

Figure 6. Independent assortment of alleles & comparison of recessive and dominant inheritance

Two examples of crosses between heterozygous parents (P) involving recessive alleles (top left) and a dominant allele (green box top right) are shown. Homologous alleles are separated by a horizontal line; maternal alleles are shown in black, paternal ones in blue. Mutant alleles are *w* (*white*; white eyes), *vg* (*vestigial*; reduced wings), *B* (*Bar*; reduced eyes); phenotypes are indicated by fly diagrams (compare Fig. 9). When comparing inheritance of the eye marker mutations *w* (left) and *B* (right), it becomes apparent that the allele assortments are identical, yet only the heterozygous *B* mutant females show an intermediate eye phenotype.

The left example is a dihybrid cross involving mutant alleles on X and 2nd chromosomes (separated by semicolons). In the first offspring/filial generation (F1) each chromosome has undergone independent assortment of alleles (demarcated by curly brackets) and each of the four possible outcomes per chromosome can be combined with any of the outcomes of the other two chromosomes resulting in 4 x 4 = 16 combinations. In case of two autosomal genes, the phenotypic distribution would be 9:3:3:1 (9 white : 3 blue : 3 yellow : 1 pink coloured fields in the Punnett square), as compared to 3:1 in a monohybrid cross (*vg*/+ X *vg*/+ → only one of 4 animals displays *vg* phenotype). In the above cross, *w* is X-chromosomal which changes the phenotypic distribution to 6:6:2:2 (6 white : 6 plain/hatched blue : 2 yellow : 2 plain/hatched pink in the Punnett square). The Punnett square lists all possible combinations (symbols explained on the right); red and blue stippled boxes in the curly bracket scheme and Punnett square show the same examples of two possible offspring. Note that the Punnett square reflects the numerical outcome of this cross in its full complexity, whereas the curly bracket strategy only qualitatively reflects potential combinations and is easier to interpret for the purpose of mating scheme design (Box 9). The complexity of Punnett squares becomes even more obvious when dealing with trihybrid crosses (Appendix 2).

4.1.3. Independent assortment of chromosomes

Drosophila has one pair of sex chromosomes (heterosomes: X/X or X/Y) and three pairs of autosomes (Fig. 5). Usually, non-homologous chromosomes behave as individual entities during meiosis and are written separated by semicolon in crossing schemes (Fig. 6, Box 9). According to the second law of Mendel (**law of independent assortment**), they assort independently of one another during gamete formation, leading to a high number of possible genotypes (Fig. 6). A good strategy to deal with this complexity during mating scheme design is to define selection criteria for each chromosome independently (curly brackets in Fig. 6; see Box 9). The **4th chromosome** harbours very few genes and its genetics slightly differs from other chromosomes [46]. It plays a negligible role in routine fly work and will therefore not be considered here.

4.1.4. Linkage groups and recombination

Genes located on the same chromosome are considered a **linkage group** that tends to segregate jointly during meiosis. However, when homologous chromosomes are physically paired during meiotic prophase (**synapsis**), the process of **intra-chromosomal recombination (crossing-over)** can lead to exchange of genetic material between homologous chromosomes (Fig. 7; note, that recombination does **not occur on the 4th chromosome**). As a rule of thumb, the recombination frequency increases with distance between gene loci, but non-uniformly across the chromosome arms (**map expansion/compression**). Frequencies are usually high in the middle of chromosome arms and low in regions adjacent to heterochromatin-rich telomeres and centromeres. Recombination frequencies have been used to generate spatial chromosomal maps of gene loci (recombination maps), defining 1% chance of crossing-over between two loci as 1 map unit (or centimorgan, cM) [46][1]. 50% is the maximum detectable crossing-over frequency because crossing-over is happening at the 4-strand stage; only 2 strands are involved in any one event and exchange between sister chromatids produces no observable changes. If two genes are 50 cMs apart then they are equivalent to being unlinked (due to the increase in multiple crossing-over events occurring between them). If the location of two loci is known relative to the cytogenetic map, their position on the recombination map can be roughly estimated and the recombination frequency between them deduced (Fig. 7B and bottom of Box 7).

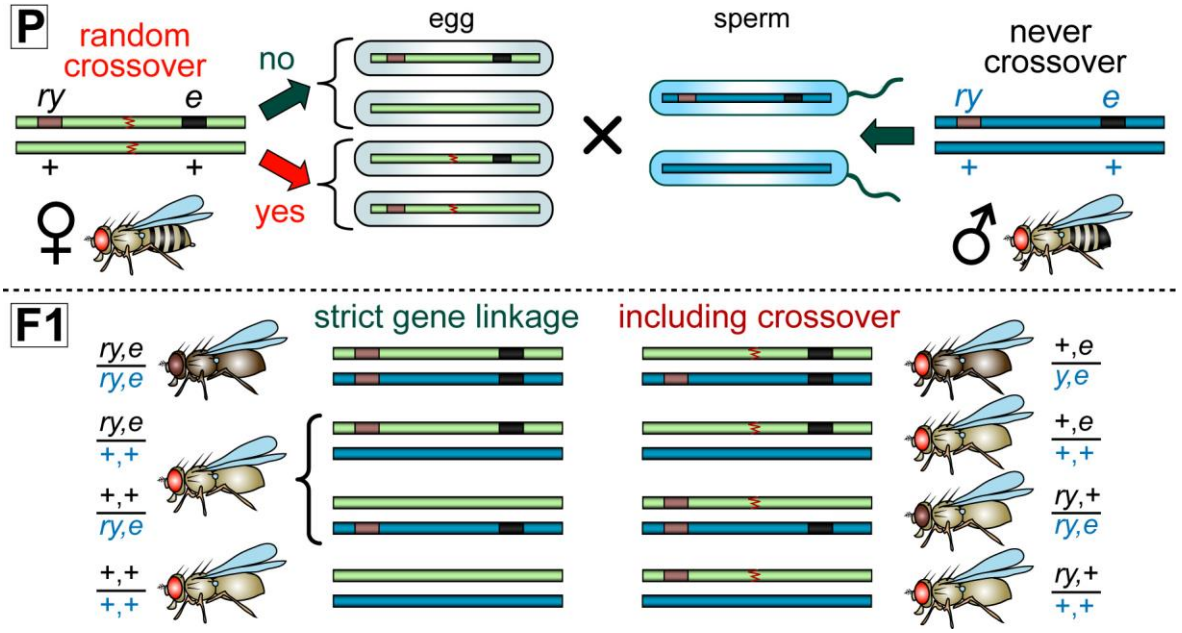

Figure 7. Inheritance of genes on the same chromosome (linked genes)

(**P**) A cross between flies heterozygous for viable recessive mutations of the 3rd chromosomal loci *rosy* (*ry*; brownish eyes, 87D9-87D9) and *ebony* (*e*; black body colour, 93C7-93D1); female chromosomes are shown in beige, male in blue. According to the law of segregation, homologous chromosomes are distributed to different gametes (egg and sperm) during gametogenesis. Male chromosomes do not undergo crossing-over. In females, crossing-overs are possible (red zigzag lines), and recombination between any pair of genes may (yes) or may not (no) occur (at a frequency dependent on their location and distance apart), thus increasing the number of different genotypes. In the first filial generation (**F1**), three potential genotypes and two potential phenotypes would have been expected in the absence of recombination (strict gene linkage); this number is increased to 7 genotypes and 4 phenotypes when including crossing-over.

For mating schemes, recombination can be a threat as well as an intended outcome:

- There are two key remedies to prevent unwanted recombination during mating schemes. The first strategy is to use **balancer chromosomes** (section 4.3). The second strategy is to take advantage of the recombination rule. The **recombination rule** states that there is **no crossing-over in *Drosophila* males** (Fig. 7). The reason for this is not clear but might relate

[1] The first ever linkage map [93]: www.esp.org/foundations/genetics/classical/holdings/s/ahs-13.pdf

to the observation that, the type of genes expressed in male meiosis "is much more similar to mitosis than to female meiosis" [103], albeit reductional divisions occur and haploid gametes are produced.

Box 7. Gene descriptors and locators

- *Drosophila* genes have different descriptors: name, symbol, synonyms, the annotation symbol and the FlyBase ID. As an example, go to the FlyBase home page flybase.org[1]. In the "Quick Search" box click on the "Data Type" tab, select "Data Class / genes" and type "shot" into the text field. This will direct you to the gene page where you will find a full description of the gene *short stop* including various identifiers and locators in the top section and further synonyms in the second last bottom section [e.g. *kakapo/kak*, *groovin/grv*, *kopupu/kop*, *l(2)CA4*]. The naming of genes and chromosomal aberrations follows agreed rules (FlyBase / Documents / Nomenclature), as summarised here:
  - The **names** of *Drosophila* genes (and their associated short forms or **symbols**) reflect the classical (and certainly most human) way to describe a gene or marker mutation. They are most commonly used in daily life and publications and tend to reflect the mutant phenotypes of genes - often in very creative ways (e.g. *faint sausage*, *ether-a-gogo*, *couch potato*). For example, *white* loss-of-function mutations cause white eyes, indicating that *white* gene function is normally required for red eye colour. However, not everybody has followed this tradition when naming genes. Furthermore, mutations of genes which were identified as homologues to known mouse or human genes tend to be named after their mammalian relatives. Note that genes encoding products of similar molecular function may be given names/symbol with identical prefixes (usually indicating the protein class) and unique suffixes (usually referring to a gene's cytogenetic location; e.g. Actin-5C, Actin-42A, Actin-57B). For an entertaining radio feature about fly names listen to www.bbc.co.uk/programmes/b00lyfy1.
  - As illustrated by the *shot* example, genes have often been called differently by independent researchers (**Synonyms & Secondary IDs**), and these names come with their independent symbols. FlyBase usually follows the rule that the first published name for a mutation of a gene (usually not the wildtype locus or protein) becomes official, but a searchable list of all synonymous names is maintained. In any case, FlyBase is your key point of reference and you are advised to use their official naming.
  - The **annotation symbol** (CG number, the **C**omputed **G**ene identifier) originates from the genome sequencing projects and has only been assigned to genetic loci that have been identified as genes. For example, *Cyl/Curly* is a mutation of unknown molecular nature and has therefore no CG number. CG numbers are primarily used if no other name has been given yet.
  - The **FlyBase ID** (FBgn = **F**ly**B**ase **g**e**n**e) is the only unique identifier available for both annotated genes and non-annotated marker mutations. It is often the prime reference during database searches.
- As a general convention, genes/symbols that were FIRST named after recessive mutant alleles (section 4.1.2) start with lower case, those FIRST identified by dominant alleles are capitalised. For example, *abd-A* is lower case due to its original classification as a recessive gene, although subsequent analyses have revealed dominant loss-of-function mutant phenotypes [77]. Capitalisation can be confusing, since identical symbols starting with either upper or lower case represent different gene or marker names (e.g. *syn/syndrome* versus *Syn/Synapsin*). Furthermore, genes named after vertebrate homologues are capitalised, regardless of whether their mutant alleles are dominant or recessive (e.g. *Nrx-IV* /*Neurexin IV* or *Syn/Synapsin*).
- Be aware that short hand for mutant alleles in daily use can differ. For example, "*w;*+;+" or "*w*" or "*w*ˇ" or "*w*ˇ/*w*ˇ" all mean the same thing, i.e. a *white* mutant fly. Whereas the first two versions do not discriminate gender, the fourth option clearly indicates a female.
- Note that genes and their mutant alleles are usually *italicised*, whereas proteins are written in plain and often capitalised (the *shot* gene, the *shot*^{sf20} mutant allele, the Shot protein)
- The genomic location of a gene is given in up to 4 ways: the **chromosome (arm)**, **cytogenetic map** position (both Fig. 5), the **sequence location** within the fully sequenced *Drosophila* genome and, for marker mutations, also the **recombination map** position (e.g. the *shot* gene is on chromosome arm 2R, in cytogenetic map position 50C6-50C9, in sequence location 2R:9,751,742..9,829,615 corresponding to the recombination map position 2-[68]). Use the "Map Conversion Table" (importable in Excel) for determining recombination map positions of other genes (section 4.1.4).

[1] for an easy guide to FlyBase see: http://flybase.org/static_pages/docs/pubs/FlyBase_workshop_2009.pdf

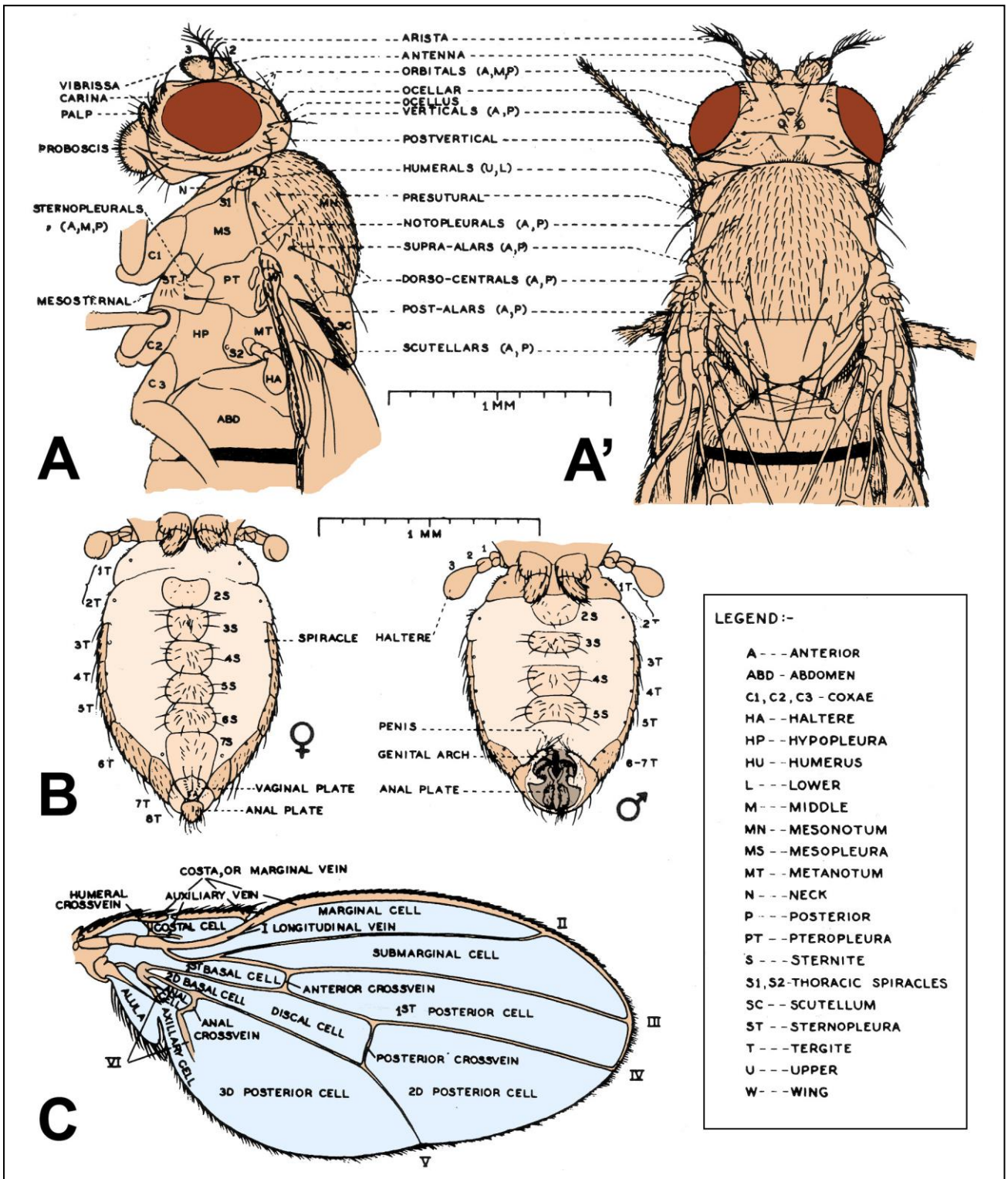

Figure 8. Anatomy of adult *Drosophila*

Lateral (**A**) and dorsal (**A'**) view of the head and thorax region of an imago; body parts and bristles are indicated. **B**) Ventral views of a female (left) and male (right) abdomen; note differences of the anal plate in B which provide easy markers to determine gender (Fig. 4). **C**) Morphology of the wing and its characteristic veins. Image modified from [19].

- In other occasions it can be the intended outcome of a mating scheme to **recombine mutations onto the same chromosome**. For example, in reverse to what is shown in Fig. 7, you may want to combine the *rosy* (*ry*) and *ebony* (*e*) mutations from different fly stocks onto one chromosome in order to perform studies of *ry,e* double-mutant flies. A typical mating scheme for this task is explained in Appendix 1.

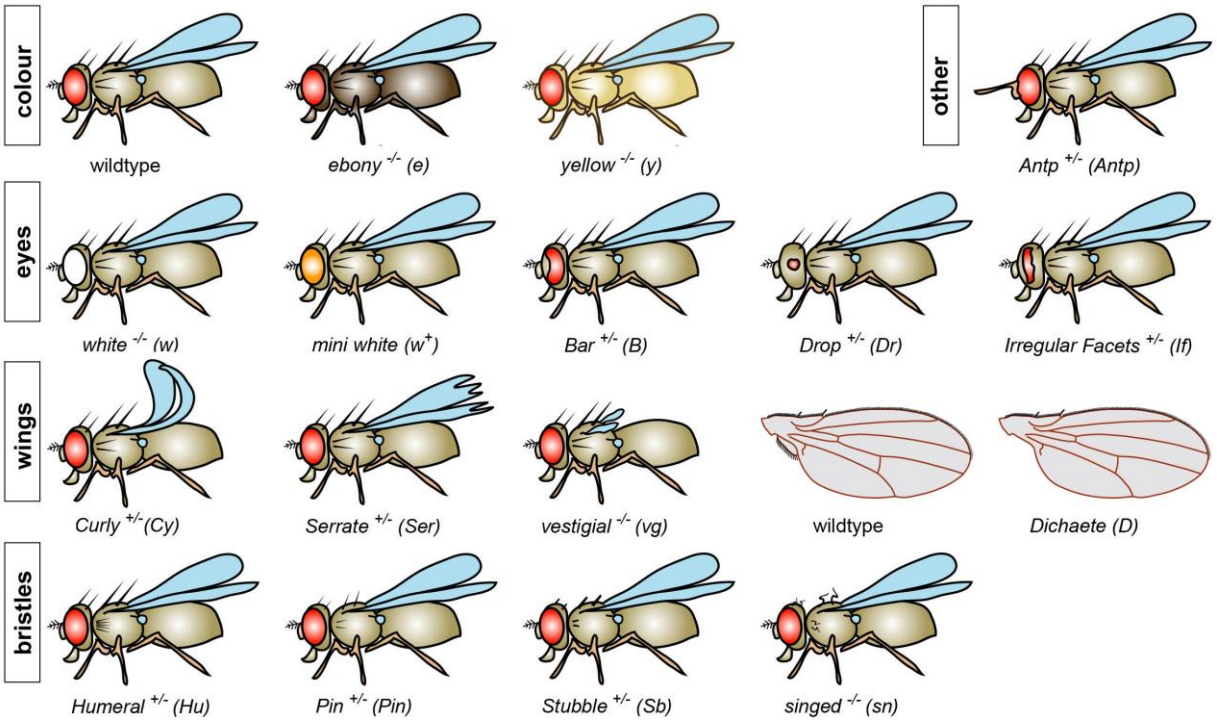

Figure 9. Examples of typical marker mutations used during genetic crosses

Mutations are grouped by body colour (top), eye markers (2nd row), wing markers (3rd row), bristle markers (bottom row), and "other" markers (top right). Explanations in alphabetic order:

- *Antennapedia*^{73b} (dominant; 3rd; antenna-to-leg transformation)
- *Bar*^{1} (dominant; 1st; kidney shaped eyes in heterozygosis, slit-shaped eyes in homo-/hemizygosis)
- *Curly* (dominant; 2nd; curled-up wings; phenotype can be weak at lower temperatures, such as 18ºC)
- *Dichaete* (dominant; 3rd; lack of alula, wings spread out)
- *Drop* (dominant; 3rd; small drop-shaped eyes)
- *ebony* (recessive; 3rd chromosome; dark body colour)
- *Humeral* (dominant; 3rd; *Antennapedia* allele, increased numbers of humeral bristles)
- *Irregular Facets* (dominant; 2nd; slit-shaped eyes)
- *mini-white* (dominant in *white* mutant background, recessive in wildtype background; any chromosome; hypomorphic allele commonly used as marker on transposable elements)
- *Pin* (dominant; 2nd; short pointed bristles)
- *Serrate* (dominant; 3rd; serrated wing tips)
- *singed* (recessive; 1st; curled bristles)
- *Stubble* (dominant; 3rd; short, blunt bristles)
- *vestigial* (recessive; 2nd; reduced wings)
- *white* (recessive: 1st; white eye colour)
- *yellow* (recessive; 1st; yellowish body colour)

Photos of flies carrying marker mutations have been published elsewhere [30,32] [1].

4.2. Marker mutations

The anatomy of the fly is highly reproducible with regard to features such as the sizes and positions of bristles, the sizes and shapes of eyes, wings and halteres, or the patterns of wing veins (Fig. 8). Many mutations have been isolated affecting these anatomical landmarks in characteristic ways [64][2]. On the one hand these mutations can be used to study biological processes underlying body patterning and development (by addressing what the mutant phenotypes reveal about the normal

[1] see also images available on FlyBase or as an App, or download the poster "Learning to Fly".

[2] available on FlyBase at the bottom of "Summary Information" for genes that were listed in the red book

gene function). On the other hand these mutations provide important markers to be used during genetic crosses and, hence, for mating scheme design. A few marker mutations commonly used for fly work are illustrated in Fig. 9.

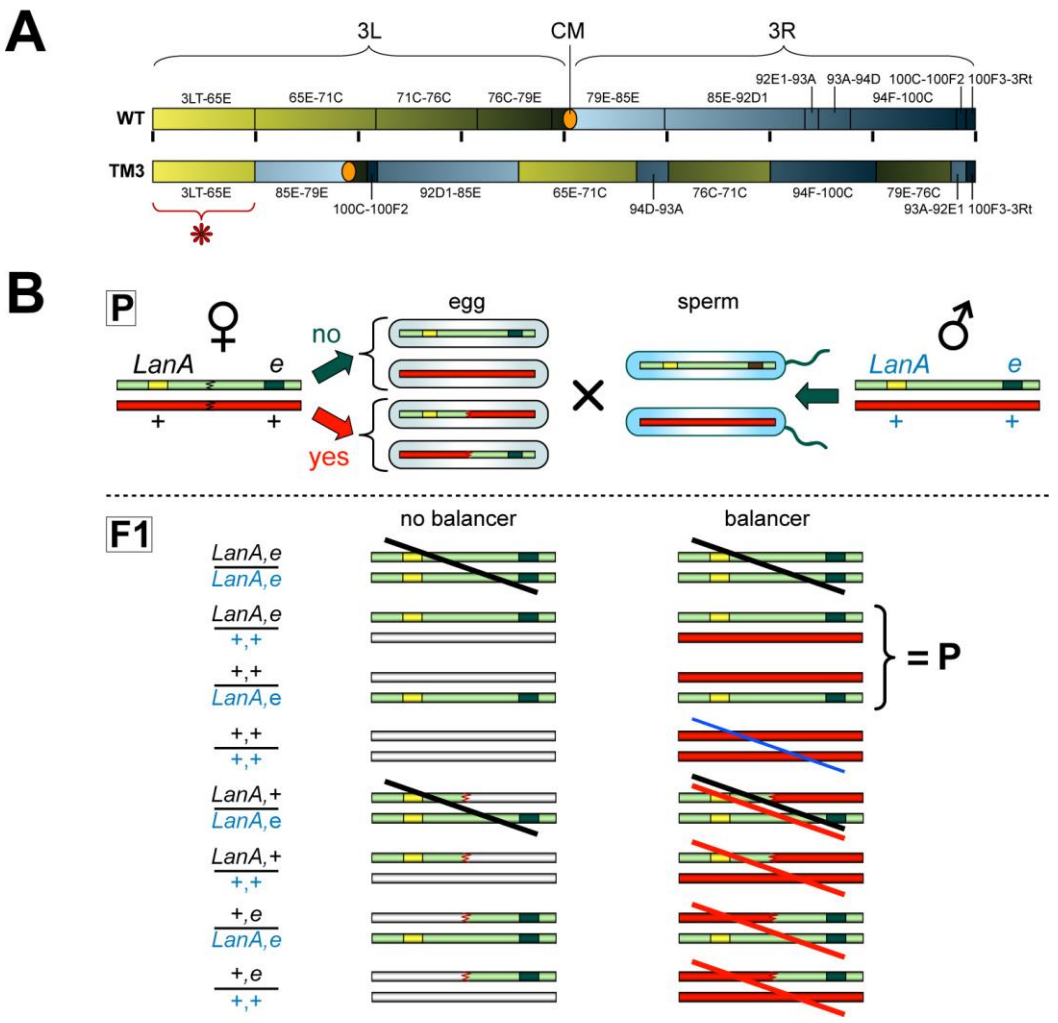

Figure 10. The use of balancers in stock maintenance

A) Chromosome 3 of wildtype (WT; left arm, green gradient; CM, centromere; right arm, turquoise gradient) compared to a TM3 balancer chromosome. Chromosomal rearrangements of TM3 are shown as boxes which correspond to analogous boxes on the wildtype chromosome and display the original colour-code and cytogenetic location in their new orientation (data taken from the Bloomington site: Balancers / Inversion breakpoints present on balancers). The asterisk indicates a non-rearranged region prone to recombination. **B)** A cross of two parents (**P**) heterozygous for *LamininA* (*LanA*; a homozygous embryonic lethal mutation on 3L, 65A8-65A9) and the recessive and viable marker mutation *e* (*ebony*, dark body colour; 3R, 93C7-93D1). Both mutations are on the 3rd chromosome and kept over a balancer. The mutant chromosome is shown in light green, the balancer chromosome in red, parental alleles in blue, maternal in black. The first filial generation (**F1**) is shown on the right. It is compared to a parallel cross (left) where the balancer was replaced by a wildtype chromosome (white). In the parallel cross, only the two combinations containing *LanA* in homozygosis are lethal (black strikethrough). Out of 6 viable combinations, only two are identical to the parents. In the cross with balancers, also the homozygous balancer constellation is eliminated (blue strikethrough) as well as all combinations involving recombination (red strikethrough). Only the combinations identical to the parental genotype are viable, ideal for stock maintenance.

4.3. Balancer chromosomes

Balancer chromosomes are essential for the maintenance of mutant fly stocks as well as for mating scheme design. Balancer chromosomes carry multiple inversions through which the relative positions of genes have been significantly rearranged (Fig. 10A) [3]. Balancer chromosomes segregate normally during meiosis, but they **suppress recombination** with a normal sequence

chromosome and the products of any recombination that does occur are lethal due to duplications and deletions of chromosome fragments (**aneuploidy** of chromosome fragments). The cytological order of breakpoints for each balancer is listed on the Bloomington site (Balancers / Inversion breakpoints present on balancers) and shown as pictograms in THE ATLAS [32], nicely illustrating the weak spots where balancers are prone to recombination (asterisk in Fig. 10A). In addition, most balancer chromosomes are **lethal in homozygosis**. Together these properties are essential for stock maintenance, since they eliminate all genotypes that differ from the parental combination (Fig. 10B). First chromosomal balancers (*FM7, first multiply-inverted 7*) are usually viable in homo- or hemizygosis, but carry recessive mutations such as sn^{X2} and lz^{S} that cause **female sterility** in homozygosis. The principal outcome for stock maintenance is the same (Fig. 11). The third key feature of balancer chromosomes is the **presence of dominant and recessive marker mutations**. Through their dominant marker mutations, balancer chromosomes are easy to follow in mating schemes. For example, by making sure that a recessive mutant allele of interest is always kept over dominantly marked balancers, the presence of this allele can be "negatively traced" over the various generations of a mating scheme - especially since recombination with the balancer chromosomes can be excluded. Examples of balancer chromosomes are listed in Box 8.

Box 8. Examples of balancer chromosomes

Numerous balancer stocks are available from *Drosophila* stock centres (e.g. Bloomington / Balancers):

- **Typical standard balancers** (most marker mutations explained in Fig. 9):
  - ***FM7a*** (*1st multiply-inverted 7a*) - X chromosome - typical markers: *y, w^{a}, sn, B^{1}*
  - ***FM7c*** (*1^{st} multiply-marked 7c*) - X chromosome - typical markers: *y*, *sc*, *w*, *oc*, *ptg*, *B^{1}*
  - ***CyO*** (*Curly derivative of Oster*) - 2^{nd} chromosome - typical markers: *Cy* (*Curly*), *dp* (*dumpy*; bumpy notum), *pr* (*purple*; eye colour), *cn^{2}* (*cinnabar;* eye colour)
  - ***SM6a*** (*2nd multiply-inverted 6a*) - 2^{nd} chromosome - typical markers: *al*, *Cy*, *dp*, *cn*, *sp*
  - ***TM3*** (*3rd multiply-inverted 3)* - 3^{rd} chromosome - typical markers: *Sb, Ubx^{bx-34e},* (*bithorax*; larger halteres) *e, Ser*
  - ***TM6B*** (*3rd multiply-inverted 6B*) - 3^{rd} chromosome - typical markers: *$Antp^{Hu}$, e, Tb* (*Tubby*; physically shortened 3^{rd} instar larvae and pupae)
- **Balancers with extra features** which can make your life easier:
  - most 1^{st} and 2^{nd} chromosomal balancers carry the same dominant markers (*B* and *Cy*, respectively); additional dominant markers, such as *Star*/*S** on *CyO* or *Lobe*/*L^{4}* on *SM1*, can be helpful to distinguish paternal and maternal balancers, e.g. in back-crosses.
  - balancers may carry *l(2)DTS* or transgenic insertions of *hs-hid* (*Wrinkled*) constructs, which cause cellular lethality when elevating the temperature to ~29°C the individuals carrying these balancers are automatically eliminated, thus enriching for animals homozygous for the non-balancer chromosome. Note, that having these features on the Y chromosome can be used to collect virgins at large scale by simply elevating the temperature during development.
  - green/blue balancers carry constructs expressing GFP or β-Gal, ideal to select against balanced animals also in embryos, larvae or pupae - live or in fixed/stained preparations. However, note that some of these balancers were generated through double-insertion of a *Gal4* construct (e.g. *Kr-Gal4*, *twi-Gal4*) and a *UAS-GFP* construct [24,47]; the Gal4 constructs on these balancers will activate any other UAS-constructs kept in the same stock, thus causing potential phenotypes or accumulation of unwanted suppressor mutations over time.
- **Multiple-balancer stocks** carry balancers on more than one chromosome, ideal to cross together and keep mutations / markers on different chromosomes (see also Fig. 15).
- **Translocation balancer stocks** also carry two balancers, but these act as one balancer across different chromosomes; large fragments have been exchanged between these balancers [e.g. *T(2;3)CyO-TM3*] causing lethality in animals that do not inherit both of them.
- **Compound-X / attached-X chromosomes** [e.g. *C(1)DX*] are not true balancers but can be used in similar ways; they consist of two *X* chromosomes fused together so that they do not segregate during meiosis and are jointly passed on to one gamete. Stocks are maintained by *C(1)DX*/*Y* females which inherit the attached-X from their mothers and the Y from their fathers, whereas *C(1)DX*/*X* females carry three X chromosomes and are lethal or sterile. The *X*/*Y* males are the only individuals passing on the non-attached-*X* chromosome - ideal for maintaining dominant female sterile mutations.

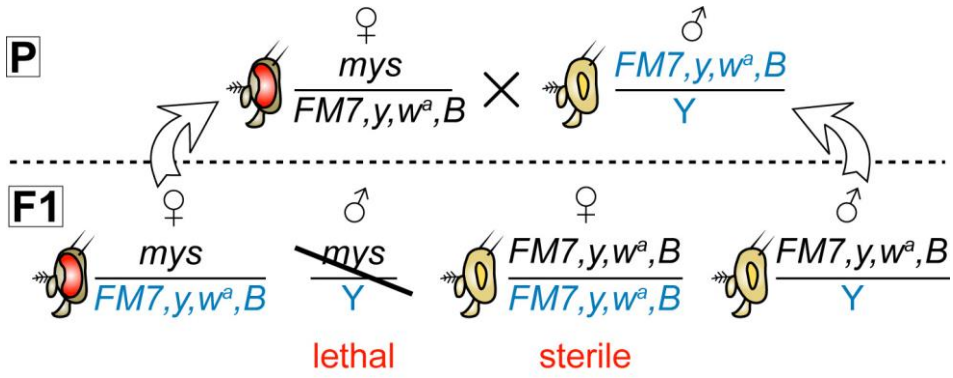

Figure 11. First chromosome balancer, FM7

A stable stock carrying a recessive, homozygous lethal allele of myospheroid (*mys*) balanced over the *FM7* chromosome carrying the following marker mutations: recessive *y* (yellow body colour), recessive w^a (bright orange eyes), dominant Bar^1 (reduced eyes; Fig. 6). In the F1 generation, hemizygous *mys* mutant males die as embryos, females homozygous for FM7 are viable but sterile. Therefore, only the parental genotypes contribute to subsequent generations, thus maintaining the *mys* mutant stock.

Note that the **4th chromosome** does not require balancers since it does not display recombination. Instead the *ci*^{D} mutant allele is used to maintain stocks with lethal/sterile mutations of genes on the 4th chromosome; *ci*^{D} is a recessive lethal, dominant marker mutation caused by a chromosome rearrangement that led to a fusion protein encoded by the *cubitus interruptus* and *pan* genes.

5. Transgenic flies

5.1. Generating transgenic fly lines

Transgenic flies have become a key resource for *Drosophila* genetics with many important applications (see below). Accordingly, transgenic animals are omnipresent in mating schemes, and it is important to understand their principal nature and some of their applications. The generation of transgenic fly lines is based on the use of **transposable elements/transposons**. Transposable elements are virus-like DNA fragments that insert into the genome fairly randomly, where they can be maintained in position over many generations, replicate like endogenous genes and follow Mendelian rules of inheritance. There are ~100 types of natural transposons in *Drosophila melanogaster* and thousands of insertions per individual genome [55]. Transposons encode specialised enzymes called **transposases** which catalyse mobilisation of the transposons into other genomic locations, either through excision/re-integration or through replication (Fig.12A). In *Drosophila*, the most frequently used class of transposon is the **P-element** which will be dealt with primarily in this manual. For the purpose of transgenesis, transposons are **modified genetically**. The transposase gene is removed and replaced by the genes the experimenter wants to introduce into the fly genome, in addition to marker genes and genes/motifs for the selective cloning of the transposable element in bacteria (Fig. 12B), as well as further potential features enhancing the use of these constructs (section 5.2).

To introduce purpose-tailored transposons into the fly genome, they are **injected** into early embryos at the syncytial blastoderm stage. Injection has to take place at the posterior pole where the pole cells will form, which are the precursors of sperm and egg cells (Fig. 12) [6]. If successfully integrated into the genome of some pole cells, the injected transposons will give rise to transgenic offspring. To catalyse genomic insertion of these P-elements, injections are performed in the presence of transposases, through using transgenic fly lines **expressing transposase** in the germline, or co-injecting **helper elements** (Fig. 12C, D). Transgenic transposases are crossed out in the next generation, helper elements don't integrate into the genome and get lost subsequently (Fig. 12D). Through this disappearance of the enzymatic transposase activity, successful P-element insertions are stabilised and can be maintained as stocks. Generating transgenic fly lines through transposon/helper element injection requires technical expertise and specialised equipment, such as micromanipulators and glass needle pullers. It is often more economical to outsource this task to specialised companies (of which there are a number existing worldwide), instead of establishing and maintaining this capacity in individual laboratories.

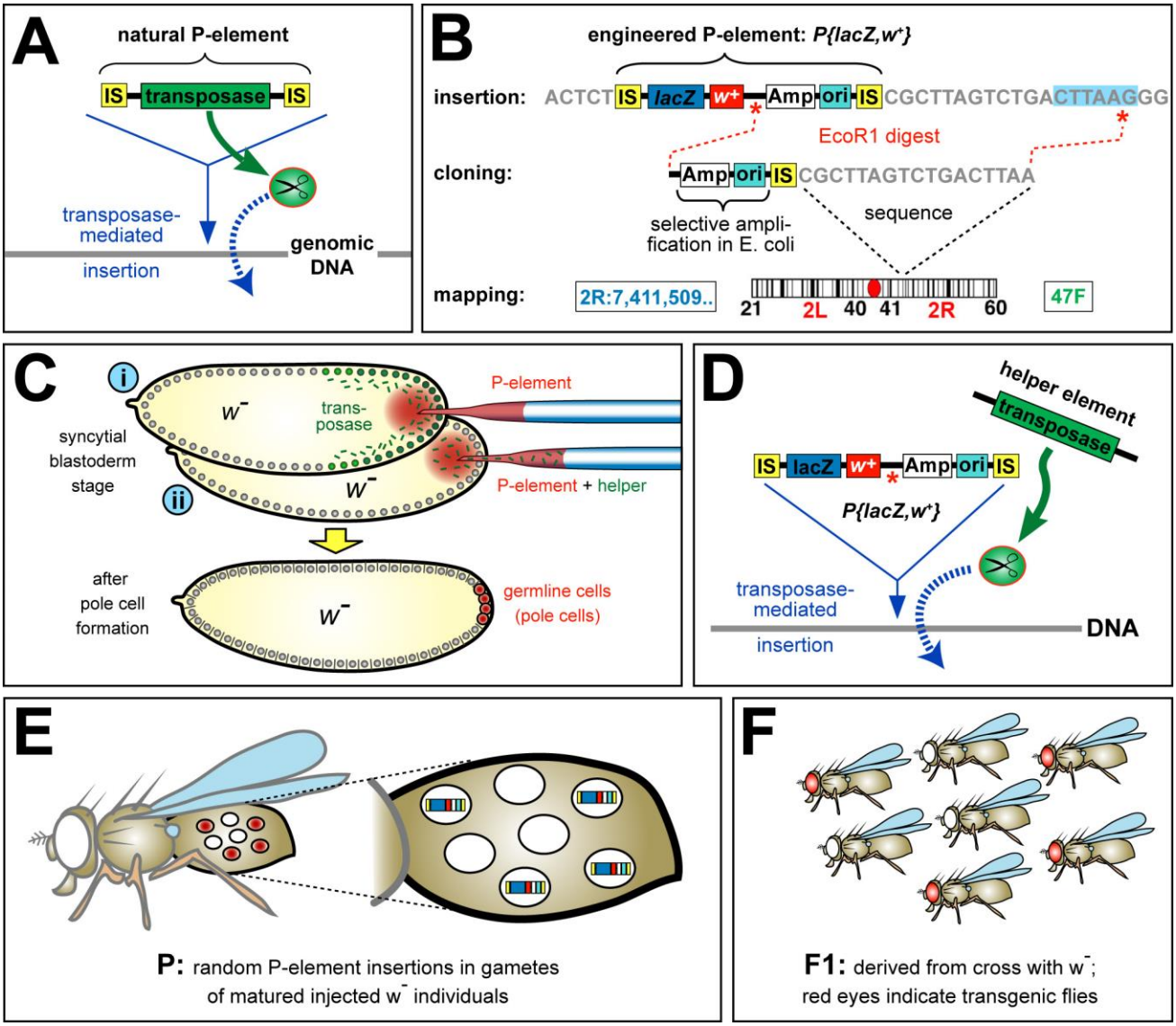

Figure 12. Using P-elements to generate and map transgenic insertions

A) The insertion of natural P-elements into the genome (grey line) requires two key features: firstly, flanking IS motifs (insertion sequences) mediating stem-loop conformation important for the insertion process (blue arrow); secondly, catalytic transposase activity (scissors and dashed blue arrow), and this enzyme is encoded by the P-element itself. **B)** *P{lacZ,w+}* is a classic example of an engineered P-element used for transgenesis where the transposase gene is replaced by: the *lacZ* from *E. coli* (dark blue box) as reporter gene, a *mini-white* gene (red box) as selection marker in F1 (see F), an antibiotic resistance gene (e.g. ampicillin; white box) and an origin of replication (ori; grey box). Once a fly strain with a stable genomic P-element insertion is established, the exact insertion site can be mapped: genomic DNA from these flies is extracted and then digested using defined restriction sites in the P-element (e.g. EcoR1; red asterisk) and random sites for the same enzyme (light blue box) in the nearby genome (grey letters); the obtained restriction fragment contains the C-terminal part of the P-element permitting selective cloning along with the adjacent genomic sequence; the obtained gene sequence can be blasted against the fly genome to map its precise position (box with blue letters) and deduce the cytogenetic map position (box with green letters; see Box 3). **C-F)** Making transgenic flies: P-element solution (red) is injected into the posterior pole of early embryos (C); transposase is either (i) expressed in the embryo or (ii) co-injected with the P-element solution in form of helper elements which lack IS motifs (D) and will therefore not insert but disappear during subsequent cell divisions. P-elements become randomly inserted into the genome of posterior pole cells (D) which will differentiate into egg/sperm cells when the injected individuals mature into *w-* adults (E). When these adults are crossed to other *w-* animals, the transgenic individuals amongst the F1 offspring can be selected by their red eye colour (F), encoded by the *mini-white* gene marker on the inserted P-element (B, D). Note that P-element insertions are still heterozygous in these F1 animals.

Existing P-element insertions can be mobilised to produce excisions and transpositions into new chromosomal locations. For this, the P{Δ2-3} strain (carrying a non-excisable transposase-encoding insertion) is crossed with P-element-carrying flies to induce transposition. In the next generation, P{Δ2-3} is crossed out again to stabilise any newly generated P-element insertions [52]. P-element mobilisation is used for a number of reasons. For example, random P-element insertions into genes can disrupt their functions and provide new mutant alleles for these genes (**P-element mutagenesis**) [52]. In other approaches, reporter genes on P-elements (e.g. lacZ, Gal4 or GFP) are used to interrogate the genome for gene expression patterns (**enhancer/gene/protein trap screens**; details in section 5.2.). Mobilisation of mapped P-element insertions can also be used to induce deletions at their insertion sites. This can occur through a process called **imprecise excision** where the P-element may remove genetic material either side of the insertion site. Deletions can also be generated through **homologous recombination**, a strategy that removes the genomic sequence between two adjacent P-element insertions [68]. For these latter approaches, countless mapped transposable element insertions are readily available for most gene loci, which are carefully listed in FlyBase and the Berkeley *Drosophila* Genome Project (BDGP) [10].

A number of problems with P-elements have been identified and led to improved strategies. For example, P-elements have **size limitations** for the DNA inserts they can successfully insert into the fly genome. Fragment sizes can be significantly increased through the use of BAC (Bacterial Artificial Chromosome) technology, which allows whole genomic loci of greater than 100 kb to be used for transgenesis [98,99,100][1]. Another problem is the so called **position effect**, referring to the fact that identical P-element constructs can have different levels of expression, as a function of their individual genomic insertion sites. This is due to the fact that each genomic locus displays a reproducible base-level of transcriptional activity, caused through factors such as site-specific degrees of chromosomal condensation. One way to deal with this problem is to use specific features that **increase the expression strength** of the transposable elements [75], so that they generate stronger signal even if inserted in less favourite sites. A second strategy is to avoid position effect by using reproducible **site-directed integration** of transposons into specific genomic positions. For example, ΦC31 integrase (as an alternative to the P-transposase) promotes recombination between attP and attB motifs. Consequently, when attB-bearing transposons are injected into ΦC31-expressing fly strains carrying attP sites at defined genomic locations, a high percentage of transposons will insert only at the defined attP site [15]. Note that ΦC31-mediated recombination can also be used to engineer genes or genomic regions within their natural chromosomal location (**genomic engineering**; see Box 2) [51]. Finally, P-elements display a pronounced non-random insertion spectrum (**insertion hot & cold spots**), meaning that certain classes of transposons are biased to insert in certain regions of the genome and avoid others, or that they show preferential insertion in 5' regulatory rather than coding regions of genes. This can be advantageous in some cases, but primarily poses a problem, in particular for unbiased genome-wide genetic screens (Fig. 2). To circumvent this problem, a number of alternative vectors with different or less pronounced preferences are available, such as the lepidopteran *piggyBac* or the Minos transposon [10,52].

5.2 Important classes of P-element lines

There is a great variety of transgenic fly lines (Box 3) and their nomenclature is complex (see FlyBase / Documents / Nomenclature). This nomenclature takes into consideration the respective class of transposon, the molecular components it contains including dominant markers, the insertion site and other unique identifiers. Here we use a "light" version of this nomenclature (Figs. 12 and 13), with **P** indicating P-element as the vector, information **between curly brackets** naming the key transgenic components including ***w*$^{+}$** as the dominant marker, and further information **behind brackets** may indicate the gene locus of insertion. Usually further identifiers in superscript are required to unequivocally describe each individual insertion line but will not be considered here. In the following some important classes of transgenic lines will be explained.

a. Enhancer/reporter construct lines (Fig. 13 A): Enhancers are gene regulatory elements which induce/facilitate the transcriptional activation at gene promoters, in some cases acting over distances of several kilo bases. Usually enhancers act on the promoters of endogenous

[1] P[acman] clones of genes or genomic regions can be found at pacmanfly.org and are distributed at bacpac.chori.org

genes in their region, but they can also activate the promoters on transgenic constructs. Therefore, to identify and characterise enhancers in non-coding regulatory regions of genes, genomic fragments containing these regions can be cloned in front of a P-element promoter (which alone is too weak to initiate gene expression) fused to a reporter gene (e.g. GFP or *lacZ*/ß-Gal from *E. coli*). Transgenic animals carrying these constructs can then be analysed for the spatiotemporal expression pattern of the reporter gene as a readout for enhancer activity. Note, that the technology around these

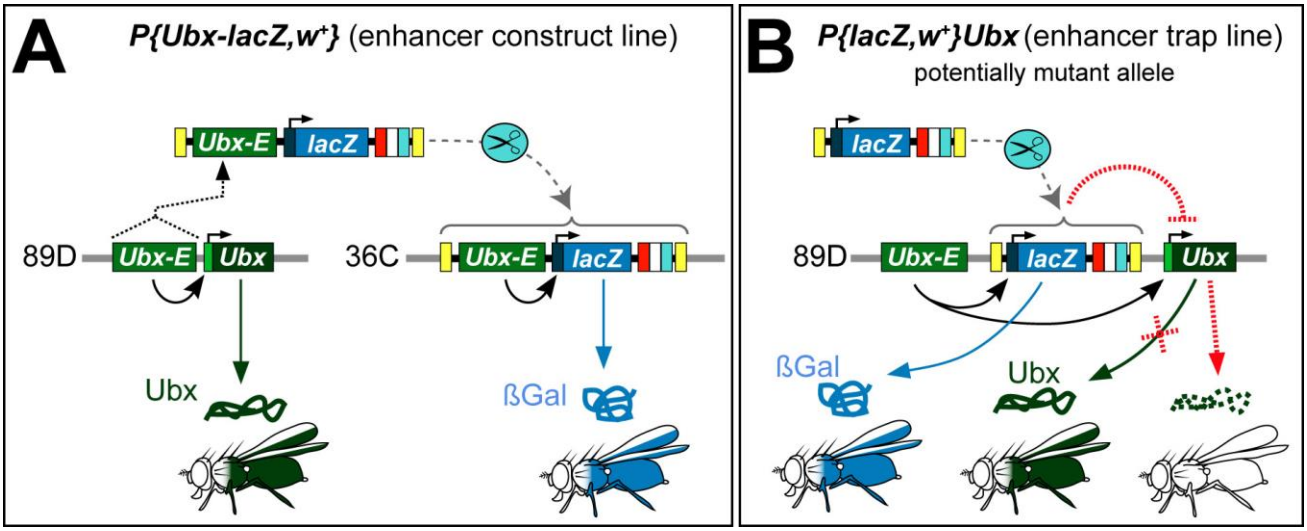

Figure 13. Enhancer trap and enhancer/reporter lines

A) *P{Ubx-lacZ,w+}* illustrating an enhancer/reporter line. An enhancer element that usually activates the promoter of the *Ubx* gene at cytogenetic map position 89D (light green box with right pointing arrow) is cloned (stippled black line) into a P-element, next to a *lacZ* reporter gene with a basal promoter (dark box with right pointing arrow) that alone is insufficient to drive *lacZ* expression. After genomic insertion (scissors; here at cytogenetic map position 36C), *Ubx-E* activates (black arrow) transcription of the basal promoter in a *Ubx*-like pattern translating into a *Ubx*-like ßGal expression pattern in the transgenic flies (blue). **B)** *P{lacZ,w+}Ubx* illustrating an enhancer trap line. A P-element (curly bracket; colour code as in Fig. 12) carrying *lacZ* with a basal promoter is inserted in the *Ubx* gene locus at 89D. The endogenous *Ubx-E* activates expression of the *lacZ* gene on the P-element (blue in fly). Note that the inserted P-element may disrupt (red stippled T) expression or function of the endogenous gene (red stippled X), thus generating a mutant allele (red stippled arrow).

b. Enhancer trap lines (Fig. 13 B): The P-element promoter alone is too weak to initiate gene expression of fused reporter genes. Therefore, transposable elements carrying such a P-element promoter fusion construct will display reporter gene expression only if inserted in a genomic site which lies within the activity range of endogenous enhancers. By generating many random insertions of such P-elements, the genome can therefore be screened for enhancers which are active in specific tissues at certain stages. Such activity often indicates the presence of genes which are expressed and therefore potentially relevant in these tissues. This procedure is referred to as an **enhancer trap screen** [11]. Since P-element insertions frequently affect the function of genes at their insertion site (stippled red T in Fig. 13 B), they can be used for systematic **P-element mutagenesis screens** [52] (see also Fig. 2). Once P-induced insertions have been generated, reporter gene patterns may reveal when and where the gene is active (Fig. 13 B), and efficient cloning strategies can be used to map the insertion and identify the targeted gene (Fig. 12 B).

c. Protein trap lines: A **protein trap screen** is an advanced version of an enhancer trap screen. It uses transposons which carry protein tag-encoding sequences (e.g. *GFP*) flanked by splice acceptor and donor sites. If such a transposon inserts into an intron (within or flanking a gene's coding region), the tag gets spliced into the host gene's natural product. This produces tagged versions of endogenous proteins which are otherwise under their normal regulation (in contrast to GFP-tagged proteins expressed via Gal4/UAS), so that GFP reflects their natural expression and localisation patterns [20,57,87].

d. Gal4/*UAS* lines: Gal4 is a transcription factor from yeast that activates genes downstream of *UAS* (upstream activating sequence) enhancer elements. Gal4 does not exist endogenously in flies and does not act on any endogenous loci in the fly genome. Very many transgenic ***Gal4* fly lines** have been and are still being generated. To illustrate this point, the simple search term "Gal4" produces almost 6000 hits representing individual fly stocks at the Bloomington Stock Centre. Of these, numerous *Gal4* lines are readily available that display Gal4 expression in different tissues or cells at specific developmental stages (Fig. 14 a, b). By simply crossing Gal4-expressing flies to ***UAS* construct lines** (Fig. 14 c, d) or **enhancer-promoter (EP) lines** [83] (Fig. 14 e), the genes downstream of *UAS* enhancers are being activated. *UAS*-linked genes can be of very different nature including reporters, different isoforms of fly genes (or of other species), optogenetic or physiological tools, small interfering RNAs or cytotoxins (Box 3). Once crossed to a Gal4 line, the offspring will display expression of these *UAS*-coupled genes in the chosen Gal4 pattern. This provides an impressively versatile and powerful system for experimentation, the spatiotemporal pattern of which can be further refined through technical improvements such as the use of **Gal80** (a Gal4 repressor), **Split Gal4** or the use of alternative strategies (alone or in combination), such as the **LexA-based binary expression system** [36,40,74]. A further important feature of the Gal4/UAS system is that its **expression strength can be decreased/increased** by keeping Gal4/UAS individuals at lower (e.g. 18ºC)/higher (e.g. 29ºC) temperatures.

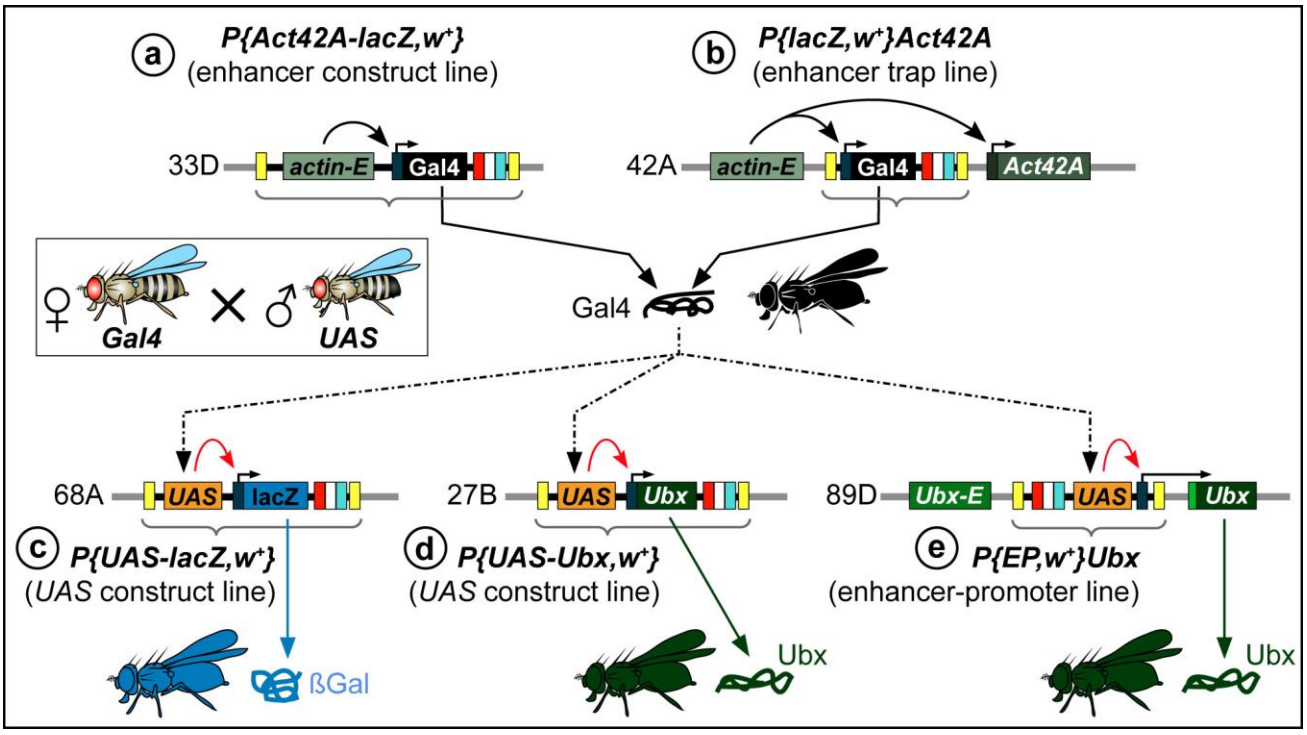

Figure 14. The versatile Gal4/*UAS* system for targeted gene expression

The Gal4/UAS system is a two component system where flies carrying *Gal4*-expressing constructs are crossed to flies carrying *UAS*-constructs (inset). Gal4 (black knotted line) binds and activates *UAS* enhancers (dotted-stippled lines), so that the pattern in which Gal4 is expressed (here ubiquitously in the fly) will determine the expression pattern of any genes downstream of the *UAS* enhancer (here ß-Gal or Ubx). The two components can be freely combined providing a versatile system of targeted gene expression. For example, *Gal4*-expressing constructs can be enhancer construct lines (**a**) or enhancer trap lines (**b**). The shown Gal4 lines are analogous to those in Fig. 12 with some modifications: P-elements carry *Gal4* instead of *lacZ*, the enhancer trap line is inserted into the ubiquitously expressed *Act42A* actin gene at cytogenetic map position 42A, and the enhancer element is the *Act42A* enhancer (*actin-E*) which activates expression of Gal4 ubiquitously in the fly (black). Three examples of *UAS* lines are shown: **c**) *P{UAS-lacZ,w+}* carries a *UAS* enhancer in front of the *lacZ* reporter gene; **d**) *P{UAS-Ubx,w+}* carries the *UAS* enhancer in front of the *Ubx* gene; **e**) *P{EP,w+}Ubx* is an enhancer-promoter (EP) line with a random insertion into the *Ubx* locus at 89D (analogous to enhancer trap line in Fig. 12 A); P-elements of EP lines carry an *UAS* enhancer plus basal promoter which, on Gal4 binding, jointly activate genes that lie downstream of their random insertion sites (here the *Ubx* gene).

e. *FRT* lines: *FRT* (*FLP recognition target*) sites are specifically targeted by the yeast FLP recombinase ("flippase"). The FLP/*FRT* system is widely used in *Drosophila* as an inducible recombination system that has mostly replaced former X-ray based strategies [14,96]. It is used to **excise genetic material** (to activate/inactivate genes or markers) or to cause **somatic recombination** between homologous chromosomes, an event that would normally only occur during meiosis (Fig. 7). Somatic recombination requires specific insertions of FRT-bearing P-elements close to the centromere of both homologous chromosomes. At these FRT sites, FLP will catalyse breakage and exchange of the homologous chromosome arms which can distribute into different cells in subsequent cell divisions. When starting from heterozygous individuals, this method can produce mosaic tissues with homozygous clones of cells surrounded by heterozygous cells [14]. Somatic recombination is used for **MARCM** (Mosaic Analysis with a Repressible Cell Marker) analysis studying the behaviour of single mutant cells or cell groups in normal or mutant tissue [105] (Fig. 15). Another important application is the generation of **germline clones** using Flippase/FRT-mediated recombination at the larval stage. After such animals have developed into female adults, their ovaries contain homozygous mutant germline stem cells which will give rise to oocytes/embryos without maternal gene product (**maternal mutant**; Fig. 16) [31].

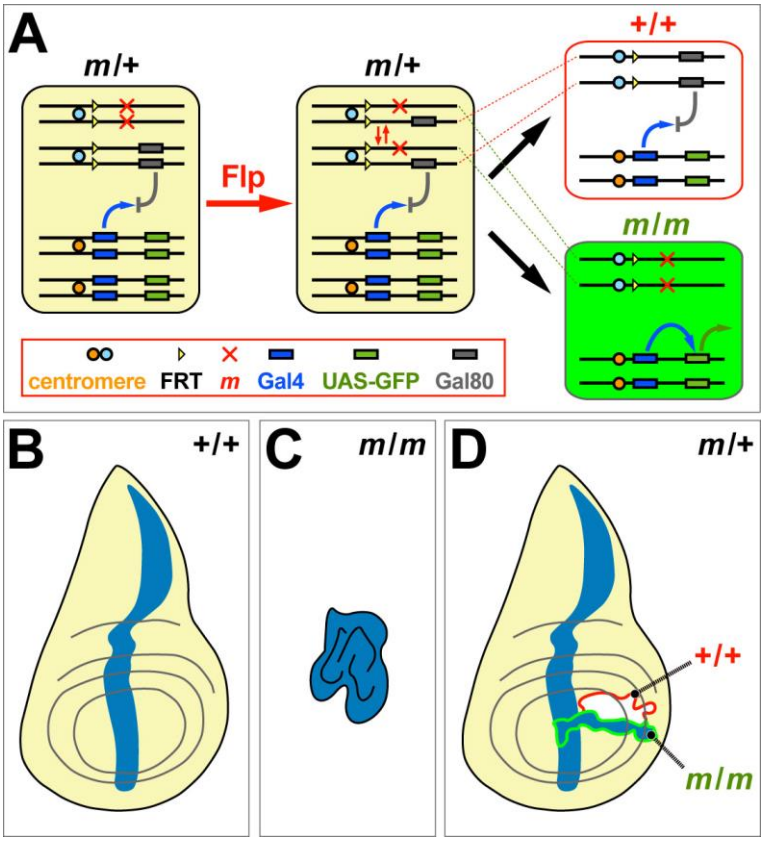

Figure 15. Clonal analysis using MARCM:
A) MARCM scheme: Activation of UAS-GFP through a tissue-specific Gal4 driver (blue arrow) is suppressed (grey T) by the Gal4 inhibitor Gal80 present in heterozygosis on a chromosome with an FRT site; the homologous chromosome carries a mutation of interest and an equivalent FRT site; activation of Flippase (Flp) causes somatic recombination at these FRT sites (red arrows); during subsequent somatic cell divisions the mutant allele may assort into the same daughter cell and become homozygous (*m/m*) creating a parallel twin clone (+/+) that carries both the wildtype allele and Gal80 in homozygosis. Subsequent divisions multiply cells with these genotypes embedded in heterozygous tissue. **B-D)** Illustration for the use of MARCM in research: **B)** Image of a normal wing imaginal disc at the late larval stage displaying blue marker gene expression along the antero-posterior compartment boundary. **C)** In larvae carrying a hypothetical mutation in homozygosis (*m/m*), wing discs express the marker gene throughout and are under-grown and aberrant to a degree that no sensible conclusions can be made about the gene's function. **D)** Small MARCM clones do not disturb the overall morphology of the wing disc and allow the study of mutant cells unequivocally identifiable by their GFP-expression (green outline). In this example, *m/m* mutant cells away from the compartment boundary display ectopic expression of the blue marker gene, suggesting that the wildtype M protein negatively regulates expression of the marker gene.

f. *RNAi* lines: Application of RNA interference strategies in flies has become a powerful alternative to the use of mutant alleles. As one key advantage, fly lines carrying *UAS-RNAi* constructs (available for virtually every gene) [37,73] allow the targeted knock-down of specific genes in a reproducible tissue or set of cells, often at distinct stages of development. Like analyses using mutant FRT-clones (section 5.2e), this approach can therefore overcome problems caused by systemic loss of gene function, such as early lethality (often impeding analyses at postembryonic stages) or complex aberrations of whole tissues that can be

difficult to interpret. However, the use of RNAi lines needs to be well controlled. Demonstration of reduced protein or RNA levels of the targeted gene is not sufficient, since phenotypes can still be due to additional **off-target effects** (i.e. knock-down of independent gene functions). Therefore, it is advised to use more than one independent RNAi line targeting different regions of the gene. Other proof of specificity can come from enhancement of the knock-down phenotype in the presence of one mutant copy of the targeted gene or, *vice versa*, suppression of the knock-down phenotype through co-expression of a rescue construct for the targeted gene (using the degenerate code to protect rescue RNA from knock-down).

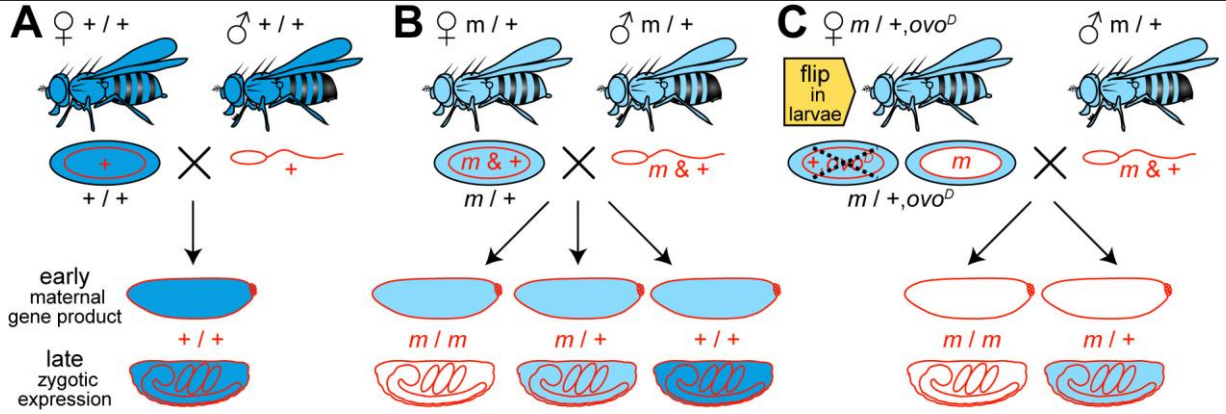

Figure 16. Maternal gene product and germline clones

A) Many genes are expressed in the female germline and their gene products (RNA or protein; blue) are deposited in oocytes (but not in sperm; white), often perduring into embryonic or larval stages or even beyond (depending on the half life of the particular RNA and/or protein); the red "+" indicates the haploid gamete genotypes, the black "+/+" below, describes the somatic phenotype of ovaries. **B)** If mutant alleles of such genes are homozygous lethal, heterozygous parents (*m*/+) have to be used in crosses to generate homozygous mutant F1 individuals (*m/m*); these mutant individuals display maternal gene product (derived from their mother's wildtype copy of the gene) that may mask mutant phenotypes especially at earlier stages of development. **C)** By using Flippase/FRT-mediated recombination at the larval stage (analogously to Fig. 15A), females with homozygous mutant germline stem cells can be generated that will give rise to oocytes without maternal product [31]. If the homologous chromosome carries a dominant, female sterile ovo^D mutant allele (ovo^{D1} or ovo^{D2}) oogenesis is blocked, and only stem cells that lost ovo^D and have become homozygous mutant (*m/m*) can give rise to oocytes. Homozygous mutant embryos developing from these eggs display neither maternal nor zygotic gene function, whereas heterozygous embryos have zygotic expression of the wildtype allele starting at/after embryonic stage 8 (~3.5 hr after fertilisation at 25ºC) [21].

6. Classical strategies for the mapping of mutant alleles or transgenic constructs

You may encounter situations in which the location of a mutant allele or P-element insertion is not known, for example after having conducted a chemical or X-ray mutagenesis (Fig. 2) or when using a P-element line of unknown origin (unfortunately not a rare experience). To map such mutant alleles, a step-wise strategy can be applied to determine the chromosome, the region on the chromosome and, eventually, the actual gene locus. Nowadays, mapping can often be achieved through molecular strategies, such as **plasmid rescue** (Fig. 12 B), **inverse or splinkerette PCR** [76] or **high-throughput genome sequencing** [16]. However, classical genetic strategies remain important and are briefly summarised here.

a. Determining the chromosome: You hold a viable *P{lacZ,w+}* line in the laboratory that serves as an excellent reporter for your tissue of interest, but it is not known on which chromosome the P-element is inserted. To determine the chromosome of insertion you can use a simple two-generation cross using a w^- mutant double-balancer stock (Fig. 17).

b. Meiotic mapping: During meiosis, recombination occurs between homologous chromosomes and the frequency of recombination between two loci on the same chromosome provides a measure of their distance apart (section 4.1.4). To make efficient use of this strategy, **multi marker chromosomes** have been generated that carry four or more marker mutations on the same chromosome (Bloomington / Mapping stocks / Meiotic mapping). Each marker provides

an independent reference point, and they can be assessed jointly in the same set of crosses, thus informing you about the approximate location of your mutation [17,46]. Note that multi-marker chromosomes can also be used to generate recombinant chromosomes where other strategies might fail. For example, recombining a mutation onto a chromosome that already carries two or more mutations, or making recombinant chromosomes with homozygous viable mutations is made far easier with multi-marker chromosomes.

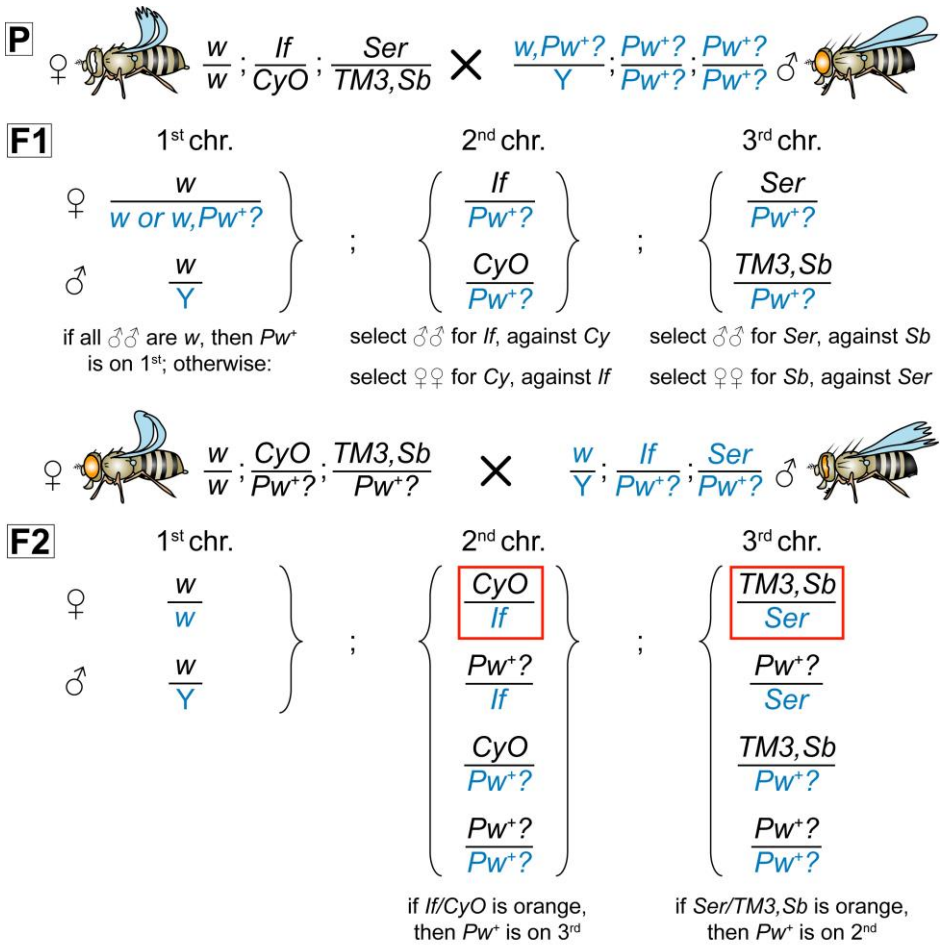

Figure 17. Determining the chromosome of insertion of a P-element

A homozygous viable transgenic fly line carries a *P{lacZ,w+}* insertion on either 1st, 2nd or 3rd chromosome (*Pw+?*). **P**) To determine the chromosome of insertion, males of this line (paternal chromosomes in blue) are crossed to a *white* mutant double-balancer line carrying balancers on both 2nd and 3rd chromosome (note, that the same can be done in two parallel crosses to single balancer stocks carrying balancers on only 2nd and only 3rd; try it out!). **F1**) In the first filial generation potential X chromosome insertions can be determined; if X is excluded, complementary chromosome combinations are selected for a second cross; males can be used for the dominant marker combination (*If* and *Ser*) since recombination is excluded by default in males (section 4.1.4.), whereas recombination in the females is suppressed through using the balancers (*CyO* and *TM3*). **F2**) In the second filial generation, potential 2nd or 3rd chromosomal insertions can be determined (note that helpful stocks for follow-up crosses can be selected at this stage, e.g. *If/CyO;Pw+/Pw+* would facilitate future combinations of the P-element insertion with a mutation on the 2nd chromosome); if *w/w;If/CyO;Ser/TM3,Sb* flies in F2 are still orange, you have a rare event in which your insertion is on the 4th or the Y chromosome.

c. Deletion mapping: Deficiencies are chromosomal aberrations in which genomic regions containing one, few or many genetic loci are deleted. Large collections of balanced deficiencies are available through stock centres (e.g. Bloomington / Deficiencies) and listed in FlyBase. Using improved technology the Bloomington Deficiency Kit now covers 98.4% of the euchromatic genome [33]. These deficiencies provide a rich resource to map genes through classical complementation testing. For this, you cross your mutant to deficiencies of the region determined by meiotic mapping. If your mutation crossed to the deficiency displays its known

phenotype (e.g. lethality) you can infer that the gene of interest is uncovered by this deficiency (**hemizygous constellation**). Note that, when dealing with lethal mutations, only 25% of your offspring are expected to carry the phenotype, so you look for presence/absence of balancer-free animals in F1 (Fig. 6). Absence of the phenotype excludes the group of genes uncovered by the deficiency. By using various deficiencies in the area, the mapping of the gene can be further refined (Fig. 18).

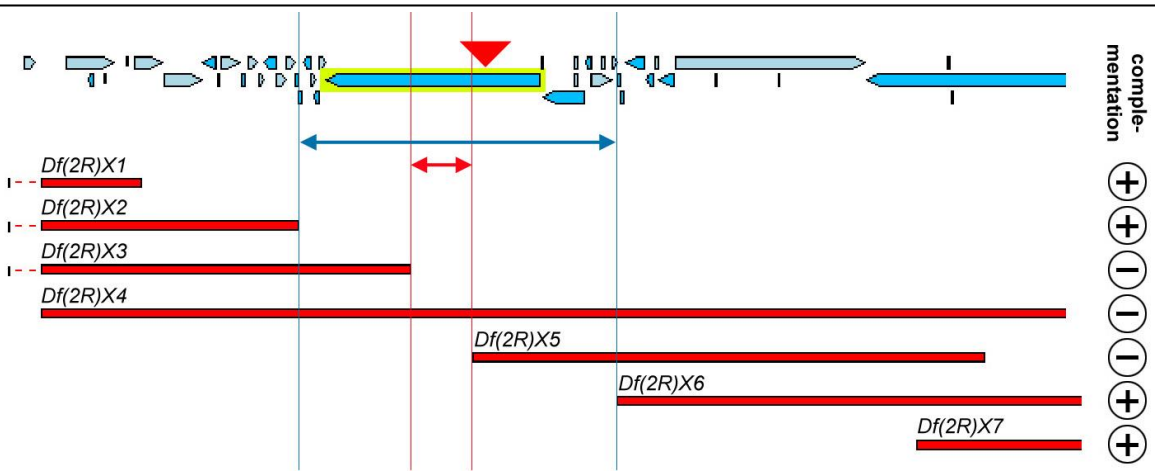

Figure 18. Deletion mapping

A mutation (red triangle) in the yellow highlighted gene locus has been mapped (e.g. through meiotic mapping) to a region of the right arm of chromosome 2 (2R). To refine its mapping, the mutant allele is crossed to deficiencies (Df) that have their breakpoints in this region (red bars indicate the deleted chromosomal region for each deficiency). Closest breakpoints of deficiencies that complement the mutation (+) indicate the region in which the gene is located (blue double-arrow). Closest breakpoints of non-complementing deficiencies (-) may lie within the gene in question and, in this example, clearly identify the mutated gene (red double-arrow).

d. Complementation tests with known loss-of-function mutant alleles: Once the location of your gene has been narrowed down by deletion mapping, you can cross your mutation to available loss-of-function mutations for the genes in this area, basically following the same strategy as for deletion mapping. Presence of the phenotype indicates that the mutations are alleles of the same gene (**hetero-allelic constellation**). Absence of the phenotype suggests that these alleles belong to different genes (**trans-heterozygous constellation**). However, be aware that the nature of a gene may be complex and lead to false interpretations of your complementation analysis:

- Genes may display **transvection**, a phenomenon where different homozygous mutant alleles affecting different areas of the same gene may complement each other [39].
- **Genes can be nested**, i.e. complete genes can be lying within introns of another gene, or they may map to the complementary strand of DNA at the same locus.
- Adjacent genes with separate coding regions might still **share common gene regulatory regions**, and therefore display unusual complementation behaviour.
- Finally, **non-coding RNAs** are encoded by independent loci that may often be considered to represent genes themselves. These loci have important gene regulatory functions and can complicate the analyses of other genes in their vicinity[1].

To circumvent some of these problems, other strategies are available. For example, collections of ***UAS-RNAi* fly lines** (section 5.2f) can be used to systematically knock down the functions of genes in the area of interest. This strategy only works if your mutation has phenotypes characteristic enough to be unequivocally identifiable upon gene knock-down. Furthermore, important clarification can often be obtained from the detailed transcriptional

[1] nice example: http://biobabel.wordpress.com/2012/05/30/a-dual-purpose-rna-and-hox-regulation/

profiles displayed for every gene on FlyBase (at the bottom of the "Expression/Regulation" view in GBrowse).

7. Concluding remarks

You should now have gained the key knowledge and terminology required to design mating schemes for *Drosophila* and to function in a fly laboratory. However, the information given is still basic and requires that you further explore the details behind the various aspects mentioned here. For this, some literature has been provided throughout the text. Should there be mistakes, passages that are hard to understand or information that is missing or wrong, please, be so kind to let me know (Andreas.Prokop@manchester.ac.uk).

Box 9. How to design mating schemes (illustrated in Figs. 6 and 17)

- write 'X' between maternal and paternal genotypes to indicate the crossing step
- genes on the same chromosome may be separated by comma, and also the names of balancer chromosomes may be separated by comma from the list of their markers (e.g. *TM3,Sb,e*)
- genes on homologous/sister chromosomes are separated by a slash or horizontal lines (usually one, sometimes two)
- genes on different chromosomes are separated by a semicolon
- always write chromosomes in their order (1^{st} ; 2^{nd} ; 3^{rd}); to avoid confusion indicate wildtype chromosomes as "+" (e.g. *y/Y ; + ; Sb/+*); note, that the 4^{th} chromosome is mentioned only in the relatively rare occasions that 4^{th} chromosomal loci are involved in the cross
- the first chromosome represents the sex chromosome; always assign a Y chromosome to the male of a cross (see Fig. 6); note that the Y chromosome is sometimes indicated by a horizontal line with a check on its right side (⟝)
- especially as a beginner, stick to a routine order, such as...
  - ...the female genotype is always shown on the left side, male on right
  - ...the maternal chromosomes (inherited from mother) are shown above, paternal chromosomes (grey) below the separating line
- especially as a beginner, always write down all possible combinations resulting from a cross; carefully assign phenotypes to each genotype, define selection criteria and check whether these criteria unequivocally identify the genotype you are after
- to keep this task manageable, use curly brackets for chromosome separation and assess each chromosome individually (Fig. 6). At the end, cross-check whether criteria might clash (for example, a *mini-white* marker on the second chromosome only works as a selection criterion if the first chromosome is homo- or hemizygous for *white*)
- always make sure that you avoid unwanted recombination events by using balancer chromosomes and/or the recombination rules (no crossing-over in males or on the 4th chromosome). If recombination is the task of your cross, make sure you use females during the crossing-over step (usually in F1).
- be aware of fly nomenclature which can be confusing, especially with respect to capitalisation and the indication of whether an allele is recessive, dominant, loss- or gain-of-function (Box 3). Be aware that you understand the nature of the involved alleles, since dominant alleles behave differently to recessive ones in a cross (Fig. 6)
- The nomenclature of transposable elements or chromosomal aberrations can be tedious. To work more efficiently, feel free to use your own unequivocal short hand during the task. For example, "*P{UAS-lacZ,w$^+$}*" and "*P{eve-Gal4,w$^+$}*" could be shortened to "PUw$^+$" and "PGw$^+$".

8. Acknowledgements

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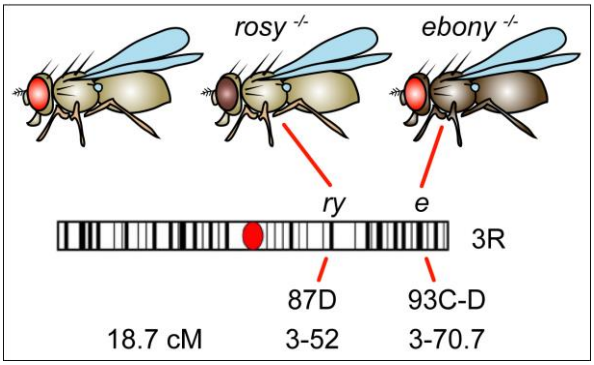
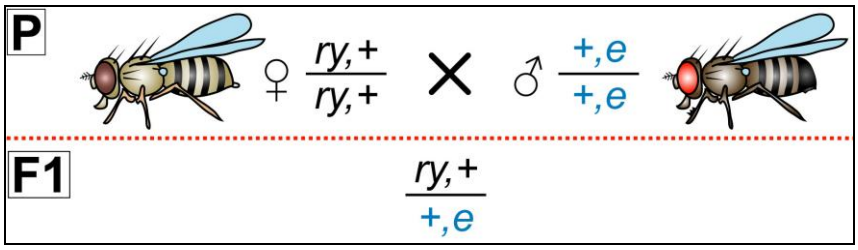
Appendix 1. A recombination scheme

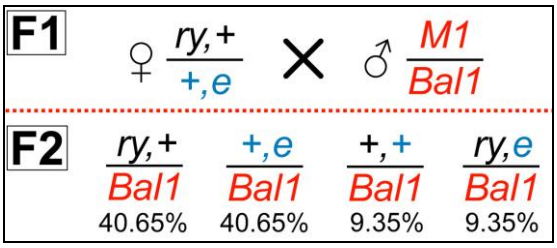
You want to recombine mutant alleles of the viable, recessive, 3rd chromosomal loci *rosy* (*ry*; dark brown eyes) and *ebony* (*e*; black body colour) onto one chromosome. According to FlyBase, *ry* localises to recombination map position 3-52, and *e* to 3-70.7. Hence, they lie 18.7cM apart, indicating that slightly less than 1 in 5 oocytes will carry the desired recombination event.



For this, you start by crossing *ry* females with *e* males or *vice versa* (**P**, parental cross). In the first filial generation (**F1**), all flies are trans-heterozygous (*ry,+/+,e*). Note that the different fly stocks used in this cross will be colour-coded to allow you to easily trace the origin of each chromosome.



According to the recombination rule, you need to take females so that recombination can occur. Note that crossing-over during oogenesis in these females occurs at random, i.e. their eggs which give rise to the second filial generation (**F2**) represent a cocktail of recombination events with a statistical likelihood of 18.7% as calculated above. Note that only half of the tested animals carry the first marker *ry*, out of which only 18.7% display the wanted recombination. Therefore, 9.35% of the single F2 individuals carry a recombinant chromosome with both markers, and 9.35% a recombinant chromosome with wildtype alleles of both markers. The key task is to **identify and isolate these recombination events through a step-wise process**.

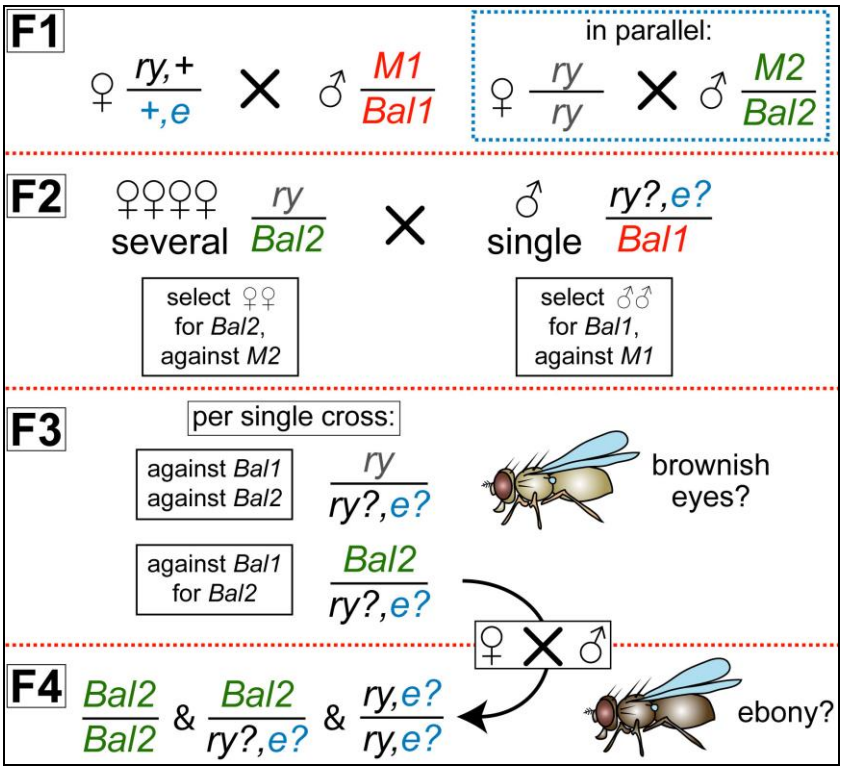


In the first step, recombination events need to be "stabilised" to prevent further recombination. For this, F1 females are crossed to a balancer stock carrying a balancer chromosome (Bal1) over a dominantly marked chromosome (M1; sections 4.2. and 4.3). In the third filial generation (F3), you determine whether one of the markers (here *ry*) is present (remember that, according to the law of segregation, only 50% of balanced F2 individuals carry *ry*). To determine the presence of *ry*, you cross F2 animals back to a *ry* mutant stock. Two important issues need to be considered here.

- Firstly, each individual in F2 is the result of an individual recombination event in its mother's germline. Therefore, **single animals** need to be tested for the presence of *ry*. For practical reasons, use single males since they can fertilise several females and therefore have a higher likelihood to generate enough offspring.

- Secondly, you have to cross back to *ry* mutant flies, but need to be able to distinguish your recombinant chromosome from the *ry* chromosome of the back-cross. For this, cross the *ry* stock to a balancer stock (*Bal2*) that can be distinguished from *Bal1*.

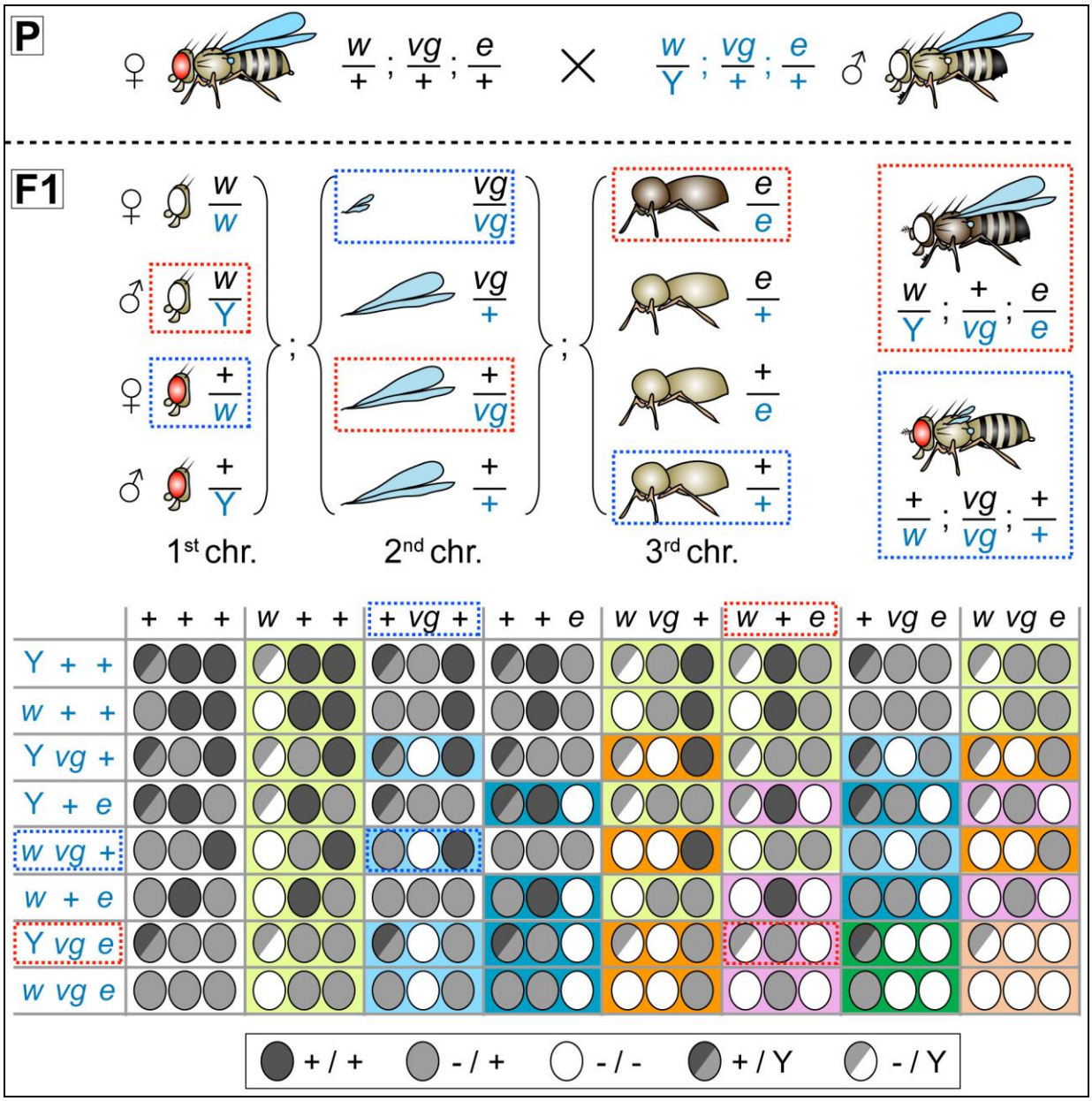
In **F3**, use simple selection to separate out two groups of flies: non-balanced flies allow you to determine whether flies have brownish eyes (i.e. carry *ry* on their potentially recombinant chromosome). If this is the case, flies carrying *Bal2* over the potentially recombinant paternal chromosome (rather than the *ry* chromosome of their mothers) can be used to establish a stable fly stock. The fourth filial generation (**F4**) emerging from these newly established fly stocks will contain non-balanced animals (*ry* and *e* are viable mutations). Stocks in which non-balanced flies have brownish eyes and dark body colour bear the desired recombinant chromosome and will be kept, the rest discarded.



For consideration:

- To have a statistical chance of isolating recombination events, more than 10 single crosses in F2 should be used to match the 9.35% chance of obtaining a recombinant.
- The example of *ry* and *e* represents an unusual case, since they are common marker mutations that are found on several balancer chromosomes (section 4.3.). Using balancers with these markers would allow you to immediately identify the presence of the desired mutations on the potentially recombinant chromosomes. Try it yourself.

Appendix 2. A trihybrid cross



Example of a trihybrid cross between heterozygous parents (P, top) involving recessive alleles on X, 2nd and 3rd chromosomes (separated by semicolons). Homologous alleles are separated by a horizontal line; maternal alleles are shown in black, paternal ones in blue. Mutant alleles are *w* (*white*; white eyes), *vg* (*vestigial*; reduced wings), *e* (*ebony*; dark body colour); phenotypes are indicated by fly diagrams (compare Fig. 9). In the first offspring/filial generation (F1) each chromosome has undergone independent assortment of alleles (demarcated by curly brackets) and each of the four possible outcomes per chromosome can be combined with any of the outcomes of the other two chromosomes resulting in 4 x 4 x 4 = 64 combinations. The Punnett square at the bottom systematically lists all possible combinations (different phenotype classes are colour-coded and display a 18:18:6:6:6:6:2:2 distribution; symbols are explained at the bottom). Red and blue stippled boxes show the same examples of two possible offspring in both the curly bracket scheme and the Punnett square. Note that the Punnett square reflects the numerical outcome of this cross in its full complexity, whereas the curly bracket strategy only qualitatively reflects potential combinations and is easier to interpret for the purpose of mating scheme design (Box 9).