Chapter 2

Genetic Screens to Identify New Notch Pathway Mutants in Drosophila

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Abstract

Notch signaling controls a wide range of developmental processes, including proliferation, apoptosis, and cell fate specification during both development and adult tissue homeostasis. The functional versatility of the Notch signaling pathway is tightly linked with the complexity of its regulation in different cellular contexts. To unravel the complexity of Notch signaling, it is important to identify the different components of the Notch signaling pathway. A powerful strategy to accomplish this task is based on genetic screens. Given that the developmental context of signaling is important, these screens should be customized to specific cell populations or tissues. Here, I describe how to perform F1 clonal forward genetic screens in Drosophila to identify novel components of the Notch signaling pathway. These screens combine a classical EMS (ethyl methanesulfonate) chemical mutagenesis protocol along with clonal analysis via FRT-mediated mitotic recombination. These F1 clonal screens allow rapid phenotypic screening within clones of mutant cells induced at specific developmental stages and in tissues of interest, bypassing the pleiotropic effects of isolated mutations. More importantly, since EMS mutations have been notoriously difficult to map to specific genes in the past, I briefly discuss mapping methods that allow rapid identification of the causative mutations.

Key words Drosophila, Notch, Forward genetic screen, EMS mutagenesis, Transposable element, RNA interference

1 Introduction

Drosophila melanogaster is a model organism used to elucidate the molecular and cellular mechanisms of intercellular signaling cascades that coordinate the development and physiology of multicellular organisms [1]. There are several key features that underlie the contributions of Drosophila genetics to biomedical research [1–4]. First, most Drosophila genes and proteins involved in signaling pathways are evolutionarily conserved. Second, the complexity of signaling pathways is often easier to unravel in flies than in vertebrates because of the paucity of functional redundancy conferred by paralogs. Third, the wealth of genetic tools and methods available in Drosophila facilitates experimental design.
and permits high cellular resolution [5], allowing assembly of pathways. Finally, *Drosophila* permits the application of large scale, unbiased, forward genetic screens either via spatiotemporally targeted protein overexpression, RNA interference (RNAi), or by generating new mutations that impair intercellular signaling in vivo.

Genetic screens in *Drosophila* can be categorized into three main types depending on whether one relies on (1) gain-of-function, (2) interactions with preexisting mutations, or (3) loss-of-function. Each type of screen has its advantages and disadvantages. Gain-of-function screens can lead to the identification of molecular components whose function may have been masked by functional redundancy [6, 7] using the GAL4/UAS binary expression system [8]. In the past, these screens relied on the availability of transposable element insertions carrying UAS enhancer sequences that are inserted in the proper orientation and location to drive expression of neighboring genes. To enhance the coverage, different transposable elements were engineered [9, 10]. More recently, UAS-transgenic libraries were created, to allow more efficient misexpression screens [11–13].

Modifier screens allow the identification of genetic interactors in a given signaling pathway, and have been productive in the Notch pathway [14–20]. However, the success of genetic modifier screens is very labor intensive and mapping of the mutations can be difficult, unless collections of preexisting mutations are available and mapped.

Loss-of-function screens aim to systematically reduce the function and/or expression of endogenous genes while screening for phenotypes of interest. One approach relies on overexpression of a library of RNAi transgenes that cover the fly genome in a spatiotemporally controlled manner and are expressed in the whole animal or in clones of cells [21]. This strategy was used to identify novel components in the Notch signaling pathway [22, 23]. However, inefficient knockdown of gene and/or off-target effects reduce the accuracy and efficiency of such screens. A similar strategy is to overexpress a set of UAS-micro-RNA transgenes to knockdown their target genes [24–26]. Notch signaling related phenotypes have been used to validate this approach, revealing a complex role of microRNAs in Notch signaling [24]. However, the identification of the miRNA targets remains difficult.

Ethyl methanesulphonate (EMS) is the most widely used mutagen in *Drosophila* because it can be easily administered to flies and causes a high frequency of mutations. Different parameters of EMS mutagenesis have been extensively covered previously [27–29]. In summary, EMS induces mainly point mutations, the vast majority of which are transitions from pyrimidines (G/C) to purines (A/T) and vice versa. However, small deletions, frameshifts, and transversions can also be recovered at low frequency, depending on the conditions of the screen, such as age of flies and dosage of EMS.
EMS mutagenesis is unbiased, covers the whole genome, and is the most efficient method to induce mutations. In standard EMS mutagenesis protocols, the dosage of EMS is 25 mM. This typically induces an average of 1 mutation per 1,000 genes [27, 28]. Importantly, the effective dosage of EMS also varies with respect to the size of the genes, the feeding conditions, the temperature, etc. Hence, one or more pilot runs to define the optimal EMS concentration is advised.

A powerful technique in *Drosophila* genetics is the analysis of loss-of-function mutations in mitotic clones, based on the FLP/FRT system, initially adapted from yeast [30, 31]. When FLP recombinase is expressed under the control of an inducible or tissue specific promoter, it can drive recombination between FRT (FLP Recombination Target) sites (Fig. 1). When FRT sites are located at the same chromosomal position of homologous chromosomes, chromatid exchange can be induced quite efficiently. Upon chromatid segregation during mitosis, some of the progeny cells are homozygous for the induced mutation, while most cells

![Schematic diagram indicating FLP/FRT-mediated recombination gives rise to homozygous mutant clones, otherwise not possible to obtain if the mutation is in an essential gene. In the absence of recombination, chromatids from homologous chromosomes bearing different alleles of a given gene segregate to progeny cells. If “A” is the wild-type allele and “a” is the loss-of-function mutant recessive allele, then the progeny cells are all heterozygous without any phenotype. However, upon mitotic recombination, non-sister chromatids from homologous chromosomes exchange material. After their segregation into progeny cells, homozygous mutant and homozygous wild-type cells arise along with heterozygous ones. The phenotype of the homozygous mutant cells can be examined in comparison to surrounding wild-type tissue. Visible markers such as \( w \) (red or white eyes) and \( yellow \) (brown or yellow bristles) can be used to distinguish between the homozygous mutant cells and wild-type cells.](image)

**Fig. 1** Schematic diagram indicating FLP/FRT-mediated recombination gives rise to homozygous mutant clones, otherwise not possible to obtain if the mutation is in an essential gene. In the absence of recombination, chromatids from homologous chromosomes bearing different alleles of a given gene segregate to progeny cells. If “A” is the wild-type allele and “a” is the loss-of-function mutant recessive allele, then the progeny cells are all heterozygous without any phenotype. However, upon mitotic recombination, non-sister chromatids from homologous chromosomes exchange material. After their segregation into progeny cells, homozygous mutant and homozygous wild-type cells arise along with heterozygous ones. The phenotype of the homozygous mutant cells can be examined in comparison to surrounding wild-type tissue. Visible markers such as \( w \) (red or white eyes) and \( yellow \) (brown or yellow bristles) can be used to distinguish between the homozygous mutant cells and wild-type cells.
are heterozygous. Hence, the loss-of-function phenotype of a mutation can be examined only in subset of cells, bypassing potentially detrimental effects in other tissues or earlier developmental stages. In addition, there are ways to enhance the size of the mutant clones by favoring the growth of mutant tissue over the wild-type neighboring cells [2, 32, 33]. The aforementioned advantages of clonal analysis, namely the efficiency and speed of phenotypic analysis, in combination with the efficacy of EMS mutagenesis have created the unique opportunity to screen rapidly and reliably for novel components of Notch signaling pathway [34–46]. Evidently, the phenotypes of mutants for Notch signaling may range from pattern formation, cell fate acquisition and differentiation to proliferation and apoptosis [47]. For such an endeavor, a collection of males bearing FRT chromosomes is mutagenized and subsequently crossed to females carrying the same FRT chromosome. These females also express FLP under the control of tissue specific promoters, like the regulatory elements of Ultrabithorax (Ubx), which is active in most imaginal discs [36]. Thus, FLP-mediated recombination between FRT chromosomes can create clones of homozygous cells for a randomly induced mutation in the thorax, wings, and eyes. The effect of the mutations can be scored in the F1 generation by visually inspecting the number and morphology of the mechanosensory bristles in these areas, where they develop under the control of Notch signaling pathway [48].

One of the main disadvantages of EMS mutagenesis is the mosaicism in the progeny of mutagenized males, which may negatively affect the yield of recovery of mutations, especially when one performs F1 clonal screens. EMS mutagenizes the post-meiotic sperm DNA that can be repaired in the embryo, upon the first cleavages of division [27, 28]. Inevitably, F1 progeny are therefore often mosaic, i.e., not all somatic cells and, most importantly, not all germ cells carry the induced mutation. Therefore, even if F1 progeny have the desirable phenotype, these animals may not transmit the mutation. Consequently, mosaicism imposes a constraint on the yield of the recovery of new mutations. To avoid the consequences of mosaicism, one needs to expand the chromosome bearing the mutations via backcross and confirm the transmission of the visible phenotype into the subsequent generations.

## 2 Materials

### 2.1 Drosophila Stocks

The list below is specific for an example of screening the right arm of the second chromosome, using FRT42D chromosomes.

1. Isogenized y w; FRT42D.
   or, FRT42D iso—where iso: isogenic, see Note 1. Stock for males for mutagenesis.
2. **y w UbxFLP;FRT42D y**
   for collecting FRT bearing virgins expressing FLP in imaginal discs under the promoter of *Ubx*. The FRT chromosome also carries the body color marker *yellow* (*y*).

3. **y w UbxFLP;FRT42D l(2R) cl y w /CyO**
   for collecting FRT bearing virgins expressing FLP in imaginal discs and favoring the growth of mutant tissue over neighboring cells, which are dying because of homozygosity of a cell lethal (*cl*) mutation. This chromosome also contains the body color marker *yellow* (*y*) and the eye color marker *white* (*w*).

4. **w; noc^loc^ / CyO**
   Stock for balancing and amplification of mutations during backcrossing.

   The above and/or similar stocks can be ordered from Bloomington *Drosophila* Stock Center (BDSC, [http://flystocks.bio.indiana.edu](http://flystocks.bio.indiana.edu)).

### 2.2 Equipment

1. Basic equipment to perform *Drosophila* work (e.g., stereomicroscope, light source, CO₂ anesthetizer, fly food media, etc.).

2. 18 and 25 °C incubators for *Drosophila* culture
   You will be performing the EMS mutagenesis in the fume hood. Keep your supplies at the ready.

3. Empty fly stock culture bottles (e.g., Applied Scientific AS-359) and closures. An old-fashioned glass half-pint milk bottle is ideal.

4. Whatman No. 1 filter paper.

5. One 1 l beaker—for denaturing solution.

6. One 250 ml Erlenmeyer flask—for EMS-sucrose solution.

7. Pipets 5 and/or 10 ml.

8. 10 ml and 1 ml syringes.


10. A container for EMS waste.

11. Handy extra gloves, Kimwipes, diapers, and paper towels.

### 2.3 Chemicals

1. Ethyl methanesulfonyl (EMS) (Sigma #M0880).

2. Sucrose.

3. NaOH.

4. Thioglycolic acid (Sigma #T6750).

5. Inactivating-Denaturing solution: Dissolve 20 g of NaOH in 500 ml of ddH₂O in the 1 l beaker. Add 2.5 ml thioglycolic acid. Thioglycolic acid should be kept at −20 °C to protect from oxidization. The solution should be prepared and used in the fume hood as it can decompose towards hydrogen sulfide.
3 Methods

The following mutagenesis protocol applies to screening of 1,000 genomes after mutagenizing 100 isogenized males (10 genomes per 1 starter mutagenized male, see Note 1). The number of males to be mutagenized can be scaled accordingly. To identify 1–2 novel complementation groups/genes from an F1 clonal screen for visible phenotypes of loss-of-function of Notch signaling, approximately 60,000 flies need to be screened, based on previous experience. In case that larger numbers of genomes need to be screened, it would be better if one follows a “rolling” scheme of mating, which tackles the labor intensive task of genetic screening. Thus, small batches of males (e.g., 100 males) are mutagenized weekly and outcrossed to virgin females of appropriate genotypes, while at the same time previous batches are phenotypically scored and balanced.

3.1 EMS Mutagenesis Protocol

1. Days 1–5: Collect newly eclosed males from the isogenic strain to be mutagenized. Age them for 3–5 days (see Note 2). In parallel, collect approximately three female virgins for every mutagenized male to be crossed. Maintain all flies in vials with yeast and food at 18 °C.

2. Day 6: Place two pieces of Whatman No. 1 filter paper cut to fit on the bottom of clean empty stock bottles. Lightly moisten with approximately 300 μl of water. Place 100 3–5 day old male flies to be mutagenized into each bottle. Incubate overnight at 22–25 °C to starve the males without dehydrating them. Starved flies will efficiently uptake EMS through feeding the next day.

3. Day 7: Prepare the chemical hood for EMS mutagenesis according to safety instructions described in Note 3. Briefly, do not forget to use appropriate protective clothing and double layers of gloves. There must be chemical waste disposal beakers with inactivating solution previously prepared (see Note 3), where all equipment that has been exposed to EMS, such as syringes, fly bottles, and Whatman filter paper, may be discarded and decontaminated. Make sure you decontaminate any spills using Kimwipes previously dipped into inactivating solution. Handle EMS with care in a fume hood, as it evaporates readily.

4. Prepare new clean stock bottles with two pieces of Whatman No. 1 filter paper cut to fit on the bottom.

5. Prepare 100 ml of 1 % sucrose solution in ddH$_2$O in an Erlenmeyer flask.

6. Use a disposable 1 ml syringe with 18 G needle to measure and dispense the EMS into the sucrose solution. Add 0.26 ml
EMS per 100 ml of sucrose solution to achieve a 25 mM final concentration of EMS. Dosage concentration can vary according to pilot tests (see Note 4).

7. Fill the used syringe with inactivating solution and place it in the beaker containing inactivating solution.

8. EMS is oily and forms droplets that sink in the sucrose solution. Disperse the EMS bubbles by repeated cycles of uptake and release through a 10 ml syringe with 18 G needle, applying moderate pressure and taking care not to expel the air in the syringe, so that the solution will not splash. Be sure to keep the needle below the surface of the sucrose solution.

9. Using the same 10 ml syringe, gently uptake and dispense 1.1 ml of EMS solution onto the center of the filter papers at the bottom of each empty stock bottle. Ensure that the EMS solution is applied evenly so that no puddles are formed. Once all bottles have been treated, discard used syringe by filling with inactivating solution then placing within the beaker.

10. Pour an equal volume of inactivating solution into the remaining EMS-sucrose solution. Keep equipment that have been exposed to EMS overnight (~15 h) in the inactivating solution and discard the waste according to the regulations of your facility, after which the glassware is safe to be washed and re-used.

11. Transfer starved males into the EMS treated bottles and leave in the fume hood during the treatment. Males are usually exposed to EMS for an approximate period of 10–12 h. However, one might expose the male flies for 6–15 h. The optimal duration of exposure must be determined empirically in a pilot screen.

12. Day 8: Prepare another 500 ml of inactivating solution. Using a funnel, transfer males onto fresh media to allow them to clean from excess EMS. Pour inactivating solution into the stock bottles used for mutagenesis (leaving the filter paper inside) and leave in the fume hood overnight. Discard appropriately the next day. Let males recover for several hours.

13. During the recovery period, distribute virgin females of the desired genotype into vials with fly food and extra yeast for performing the first cross (F0) (Fig. 2).


15. Day 12: After 4 days remove all males from each cross to prevent the appearance of clusters of identical mutants derived from mutagenized germline stem cells (see Note 5). Transfer the females into fresh food vials with yeast (optional).
16. **Day 18**: At a minimum of 10 days after setting the crosses, start collecting F1 males for phenotypic scoring. Isolate flies of interest and backcross them to ensure genetic transmission as well as amplification of the mutation into a stock (F1) (Fig. 2). 

17. **Day 28**: At a minimum of 10 days after setting the F1 crosses, start collecting F2 males for confirmation of genetic transmission of the pre-observed phenotype. Isolate the flies with the genotype of interest and backcross them to balancer stocks (F2) for amplification of the chromosome and generation of a stock after crossing sibling progeny of the next generation (F3) (Fig. 2).

### 3.2 Formation of Complementation Groups and Mapping of EMS Mutations

After screening, the retrieved EMS alleles must be placed in complementation groups, which most likely correspond to single genes. Overall, the average number of alleles in each complementation group can be used to calculate the saturation of the genetic screen, which corresponds to what degree the specific region of the genome has been probed to retrieve all relevant EMS-induced mutations. The calculation is based on Poisson distribution and is equal to

\[ S = 1 - P(0) = 1 - \left( m^* \left( e^{-m}\right) / 0! \right) \]

where \( S \) stands for the degree of saturation, \( P(0) \) is probability of no-hits, i.e., the probability of the genes that might have not been mutated, \( m \) is the average number of mutations per gene (average number of alleles per complementation group), \( e \) is the natural logarithm, and \( 0! \) is the factorial of 0, which is equal to 1. Calculations of saturation

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**Fig. 2** Schematic diagram of crosses designed in the context of an F1 clonal forward genetic screen performed on the right arm of the second chromosome. In the first generation (F0) crosses are set between mutagenized males and appropriate females. In the next generation (F1), progeny that are scored positive phenotypically are backcrossed for testing for genetic transmission of the mutations and for confirming the phenotypes. F2 progeny are then balanced. To establish a stock, F3 siblings are crossed. All crosses are performed with single males. *Asterisk* represents the EMS-induced mutation. \( cl = l(2)cl \) (cell lethal mutation)
of a genetic screen are only an estimate, since they can be confounded by multiple factors. For example, the rare cases of extragenic (nonallelic) non-complementation may increase the number of alleles in a given complementation group, while intragenic complementation may also undermine the formation of potential complementation groups, splitting them into single allele groups. However, both cases of extragenic non-complementation and intragenic complementation can be particularly informative with respect to pathway structure and protein function respectively. Extragenic non-complementation may reveal novel protein interactors of a given gene, which when mutated together lead to the manifestation of a visible phenotype. Intragenic complementation may become apparent in multi-allelic complementation groups that correspond to large genes with a complex domain structure, such as Notch. In such cases, they may prove to be extremely informative with regard to the interplay among different protein domains [49].

Complementation groups are formed based on a number of genetic schemes which may rely on different characteristics of the alleles. In an F1 clonal screen for Notch pathway mutants, it is expected that the isolated mutations may function pleiotropically in early developmental stages, given the requirement for the Notch signaling pathway in multiple aspects of the development of the nervous system and other organ systems. Thus, the gene of interest is likely to be essential genes, and majority of the alleles may be homozygous lethal, unless they are weak hypomorphs. Consequently, the complementation groups may be formed on the basis of lethality and subsequently, checked individually for the phenotype of interest or even for additional phenotypes in other aspects of Notch signaling. Such analysis can lead to the conclusion whether a given gene affects all or only a subset of Notch signaling-mediated developmental decisions, i.e. whether the affected genes are obligatory or context dependent members of Notch signaling. On that note, one can perform a secondary screen using not visible markers, but in vivo reporters of Notch signaling activity, as described by Housden et al. in Chapter 8.

The most obvious, but also the most tedious, method for establishing complementation groups is to set up crosses among all possible combinations of all isolated alleles in the screen [50]. Alternatively, one can form an initial complementation map based on the non-complementation of different alleles with a set of molecularly defined set of deficiencies that cover the entire fly genome (as performed in [51]; these tools are readily available from the BDSC, http://flystocks.bio.indiana.edu, [52–55]). A variation on this method is to rescue the alleles by a set of molecularly defined chromosomal duplications [56]. Alleles that fail to complement the same deficiency or rescued by the same chromosomal duplication can be further grouped
with respect to the complementation profile amongst themselves as well as against molecularly defined deficiencies in the region of interest [57–59].

Another, more important aspect of EMS screens is the difficulty of mapping of EMS-induced mutations to causative genes. However, recent technical advances have increased the resolution and accuracy of complementation tests and molecular mapping by means of P element based meiotic mapping [60]. P element meiotic mapping is best performed when two alleles of the same complementation group are used, to avoid genetic background effects that will lead to perturbations in the generated genetic map. P element mapping is fast and quite accurate, substituting alternative methods based on SNP maps [61–65] and complementing the power and resolution provided by the engineering and utilization of molecularly defined deletions and duplications [52–54, 56, 66]. Nevertheless, whole genome sequencing has facilitated the identification of molecular lesions in an increasingly effective and affordable fashion [67–69].

Since EMS can cause multiple mutations in the genome in an unbiased fashion, it is advisable to work with multi-allelic complementation groups, consisting of at least two alleles, which provide the opportunity to work with heteroallelic combinations precluding the possibility of analyzing unrelated genes. Accordingly, it is of crucial importance to perform rescue experiments by expressing a wild-type copy of the mutated gene (either as cDNA or in the context of a genomic rescue construct) in the mutant genetic background. Such rescue experiments will confirm that the observed phenotypes are indeed due to the loss-of-function of the gene where the mutations of interest map.

4 Notes

1. It is important to *isogenize* the parental chromosome(s), and therefore the genetic background where the new mutations will be induced, prior to any type of mutagenesis and forward genetic screening experiment. Isogenization ensures that existing polymorphisms or mutations do not interfere with the recovery of newly induced mutations. Isogenization is carried out by the use of balancer chromosomes, which suppress recombination between homologous chromosomes. Multiple isogenized stocks must be established from single balanced males, so that they can be subsequently tested for viability, fertility, and especially the lack of particular phenotypes. Proceed with the healthiest stock. In addition, it is recommended to preserve flies or genomic DNA from the isogenized line immediately after isogenization if one considers gene identification through whole genome sequencing.
2. Forward genetic screens are usually designed so that males are treated with EMS and subsequently crossed to female virgins of the appropriate phenotype. To compensate for loss in fertility and viability of males upon EMS exposure, more males should be treated with EMS. Furthermore, males should be approximately 3–5 days old.

3. EMS is mutagenic, carcinogenic, and teratogenic. Furthermore, EMS evaporates readily, and has a half-life of more than 2 days in water at 25 °C. Contamination by EMS cannot be measured. Thus, EMS should be handled with extreme caution. Any experiments with EMS must be conducted in a closed chemical hood wearing a lab coat, safety glasses, a mask, and double layers of gloves, to avoid accidental tearing. The chemical hood should be lined with a diaper to absorb EMS spills. The highest risk of contamination occurs mainly when the EMS solution is prepared and distributed. In the case of a contaminating spill, one should wipe the spot with Kimwipes, previously dipped in inactivating solution, available in a beaker in the hood. It may seem counterintuitive in terms of accuracy to use a syringe and needle rather than a pipettor for measuring, but you might contaminate your pipettor whereas the syringe is disposable.

4. To avoid inducing multiple lethal hits per chromosome, which may confound subsequent steps of analysis such as for a screen on the X chromosome, a 10 mM EMS concentration should be used [39, 57, 58].

5. Progeny originating from sperm that was in pre-meiotic stages during EMS treatment may lead to the isolation of identical mutations (clonal events). Since spermatogenesis occurs over a period of 5 days at 25 °C, parents must be discarded on the fourth day of treatment.

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