Drosophila as a model system for genetic and genomic research

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ABSTRACT

The sequencing of the *Drosophila* genome allowed the identification of most coding sequences, highlighting the necessity for a functional assignation of the identified genes. The information extracted from the sequence directly classified a considerable fraction of genes into known molecular categories, although there is still a large proportion of them that, due to poor sequence conservation, are not included into any informative class. Furthermore, in many instances the molecular nature of a protein is not particularly revealing about its functional requirements and network of interactions. In this manner, complementary genomic approaches to gene identification by sequence conservation are fundamental both in *Drosophila* and other organisms to assign particular functions to annotated genes. The approach more successful in the *Drosophila* field is the undertaking of genetic screenings to identify sets of interacting genes and genes controlling particular cellular processes. Classic genetic screens comprise all those based on a "phenotypic" paradigm, where the generation of large collections of mutant chromosomes is followed by their mapping. This approach has been recently expanded to include "genomic" tools, such as the use of microarrays and interference RNA, as well as reverse-genetics techniques, seeding the way to a "functional" annotation of the *Drosophila* genome.

INTRODUCTION

For almost a century, *Drosophila* has been a favourite creature in genetic research, and the knowledge of the fundamental principles of genetic organization in eukaryotic organisms stems from the early work carried out by the school of Thomas Hunt Morgan during the early years of the XX century (1). The success of the fruit fly in relation to other model organisms is due in part to several characteristics of *Drosophila*, including relatively short generation time, large number of progeny and low genome complexity. These features, combined to the rich adult and embryonic morphologies, complex physiology, stereotyped behaviour, and the variety of ecological adaptations of *Drosophilids*, have contributed to expand the use of *Drosophila* from basic genetic research, mostly gene organization and transmission, to the analysis of the mechanistic basis of Development and Genome evolution. Furthermore, the conservation in the coding sequences between *Drosophila* and other organisms, including humans, has trigger an enormous expansion in the application of flies into basic biomedical research. Apart from the intrinsic characteristics of the *Drosophila* biology, other important aspects necessary to understand the success and power of *Drosophila* as a model organism are the traditions of sharing genetic strains and information among the fly community, as well as the effort of many individuals to systematize and make accessible in a comprehensive manner the wealth of information available to *Drosophila* researches. In this manner, of paramount importance to the *Drosophila* community today are the databases generated by the Flybase Consortium, Flymine and the Berkley Drosophila Genome Project (see Table 1). Flybase is a database of genetic, molecular and bibliographic data for *Drosophila*, whereas the related BDGP aims to finish the sequence of the *Drosophila* genome and to maintain biological annotations of this sequence. Additional information and reposition of research materials, including fly strains, cell lines, cDNA clones and antibodies exists in several databases funded by public grants (see Table 1 for a summary). With the help from these initiatives, *Drosophila* has become today a versatile and practical model organism with applications in many fields of Biological research.

The primary reason to choose *Drosophila* for the study of almost any aspect of eukaryotic biology is still the immense possibilities offered by the sophisticated genetic techniques available. Thus, not only a wealth of genetic information already exists for a

large fraction of the *Drosophila* genome, but also several methods are at hand to generate mutations in genes of interest, to identify genes on the basis of genetic interactions and to construct transgenic flies for studies of gene regulation and function. Moreover, the sequencing and annotation of the *Drosophila* genome has opened the door for the application of high-throughput techniques, leading to the determination of gene expression profiles during development and in a variety of experimental conditions, and to construct a preliminary global map of protein-protein interactions. High throughput approaches allow extending the range, speed and type of genetic screens, from whole-organism screens to others based on cell-cultures. Sequence information and genome annotation also permits the development of reverse-genetics techniques, mainly through the use of interference RNA, Tilling techniques, and, to a lesser extent, gene targeting by homologous recombination. In this manner, studies in *Drosophila* are providing key insights into related processes occurring in other species, such as embryonic development, neural organization, growth and tissue morphogenesis, as well as helping to clarify the function of genes involved in genetic diseases in humans. Certainly, it appears that the goal of determining how the approximately 14.000 *Drosophila* genes generate a functional multicellular organism is within reach in the next decades.

Name	Web Site	Description
FlyBase	http://flybase.org	Drosophila Genetic and molecular data
FlyMine	http://www.flymine.org/	Drosophila and Anopheles genomics
Berkeley Drosophila Genome Project	http://www.fruitfly.org/	Drosophila genome anotation
Drosophila Genomics Resource Center	http://dgrc.cgb.indiana.edu/	cDNA clones, cells lines, vectors and microarrays
Homophila	http://superfly.ucsd.edu/homophila/	Drosophila genes related to human genetic deseases
Bloomington Drosophila Stock Center	http://flystocks.bio.indiana.edu/bloomhome.html	Drosophila Stock Center
Harvard Drosophila Stock Collection	http://drosophila.med.harvard.edu/	Exelixis collection of Drosophila stocks
DrosDel Project	http://www.drosdel.org.uk/	Isogenic deficiency kit for Drosophila melanogaster
Japanese National Institute of Genetics	http://www.shigen.nig.ac.jp/fly/nigfly/	Stock center from the NIG, Japan
BDGP/Baylor Gene Disruption Project	http://flypush.imgen.bcm.tmc.edu/pscreen/	Gateway to the Gene Disruption Project
FlyView	http://flyview.uni-muenster.de/html/About.html	Gene expression patterns
Drosophila Heterocromatin Project	http://www.dhgp.org/index.html	Drosophila heterochromatin gene project
Drosophila RNAi Screening Center	http://flyrnai.org/RNAi_index.html	High throughput RNAi screens
Developmental Studies Hybridoma Bank	http://www.uiowa.edu/~dshbwww/	Storage and distribution of monoclonal antibodies

TABLE 1

Summary of Drosophila resources available on the WWW. For a complete list see Flybase (http://flybase.org/allied-data/resources.html).

In this review, we will summarize the different methods used in the *Drosophila* field, and their applications to the identification and characterisation of genes contributing to a variety of biological processes. We will include the use and recent modifications of conventional loss-of-function screens, the contribution and applications of transposable elements, and the recent impact of post-genomic technologies, such as the identification of expression profiles using microarray techniques and genetic screens based in interference RNA in cultured cells.

1. - THE TRADITION OF *DROSOPHILA* AS A MODEL ORGANISM FOR GENETIC STUDIES

The first reason for the success of *Drosophila* as an experimental organism was the high frequency with which mutants flies turned up in breeding experiments carried out in the Morgan laboratory in the early 1910. This was mainly a consequence of the scaling up in the number of cultures maintained in the laboratory, in a process described as autocatalytic: "the more crosses were done, the more mutants turned up" (2). By the late 1910 the number of mutants available, and the development of conceptual approaches to use them in neo-mendelian experiments, leaded to the construction of the first genetic maps, using the frequency of meiotic recombination as a measure of physical distances between mutants (1). Early *Drosophila* workers from the Morgan group were mainly concerned with genetic transmission and mapping, and in many ways their work established most of the parameters, principles and techniques used today in *Drosophila* genetics. In the nine decades of *Drosophila* use in the laboratory since then, novel applications and technical advances have moved hand by hand, speeding the conversion of *Drosophila* from a pure genetic workhorse to a versatile

model in developmental and cellular biology. The technical advances include the use of ionizing radiation and chemical agents to induce mutants, the identification and posterior domestication of transposable elements, the development of transformation techniques using P-elements, DNA cloning by genomic walk, the construction of cDNA libraries, the adoption of methods to study *in situ* gene expression, the use of techniques to construct mosaic animals and the design of direct ways to modify gene expression in the organism. The availability of these techniques, combined with the detailed histological description of embryonic and imaginal development immensely promoted the use of *Drosophila* in Developmental Biology studies. In this way, the technical achievements and the understanding of the basic rules governing *Drosophila* development set the perfect stage to use the information provided by the sequence, accelerating even more the rate of discovery in new areas of Biology important not only for the fly, but also for general biology and biomedicine.

2. - THE *DROSOPHILA* GENOME

The sequencing of the *Drosophila* genome was the result of the collaboration between the Berkeley *Drosophila* Genome Project (BDGP), led by Gerry Rubin, and the genomics company Celera. The method to sequence the genome followed by the BDGP and the related European *Drosophila* Genome Project (EDGP) was a 'clone-by-clone' strategy, using the genome coverage generated in Cosmid, YAC, P1 and BAC clones. This approach resulted in sequencing approximately the 20% of the euchromatic DNA by 1999. These projects were greatly accelerated in 1998, when Celera applied its 'shotgun' sequencing strategy to *Drosophila*. In brief, shotgun sequencing relies on breaking the DNA into random pieces that are sequenced and assembled by computational methods. The combined approaches of the public consortiums and Celera resulted in the publication of what was named "Release 1" of the annotated sequence by 2000 (3). Gene annotation relied mainly in gene-prediction programs, and was helped by the BDGP sequence data of full-length cDNAs and expressed-sequence tags (ESTs). Since the first release of the sequence, the annotation has been improved by the use of additional functional and comparative data, and by large-scale sequencing of cDNAs, resulting in the public availability of subsequent Releases, from 1 to 5 by August 2006 (4).

Sequence information is extremely useful on its own, because it speeds up research in the field and broads the range of experimental approaches available for researches. Additionally, the annotation of the genome acquires particular relevance when the information content is compared among different species. This applies to species belonging to the same family, the *Drosophilids*, and to species phylogenetically distant, such as *C. elegans* and *A. gambiae* (5). Comparing related species helps in many ways to genome annotation, allows the identification of conserved regions beyond the protein-coding DNA, and leads to the analysis of evolutionary rates. The sequencing of the *Drosophila melanogaster* genome was followed by a coordinated project to sequence several other *Drosophilids*. So far, ten different *Drosophila* species have been sequenced, and it is expected that the core manuscripts describing the basic assemblies, annotation sets and overall comparative descriptions will be submitted by September 2006 (see Flybase). A comparative analysis of the predicted proteins from the genomes of *Drosophila melanogaster*, *C. elegans*, and *S. cerevisiae* undertaken in the context of cellular and developmental processes, showed that the non-redundant protein sets of flies and worms are similar in size and are only twice than that of yeast (5). This analysis assigned 7419 fly proteins to either protein families or domain families. Furthermore, about half of the fly protein sequences show similarity to mammalian proteins. In the context of the uses of *Drosophila* as a model system for biological research, several conclusions are particularly revealing. First, it was noted that approximately 47% of *Drosophila* coding units have single orthologs counterparts in other species (6). This number increases to 85% when only genes with defined roles in development are considered, suggesting that these functions are ancient and under considerable evolutive constrain (6). The degree of genomic conservation is even more impressive in the case of signalling pathways. For example, almost all members of the *decapentaplegic* signalling pathway (Dpp is member of the BMP/TGFβ superfamily of ligands) are represented by individual orthologs genes in *Anopheles*, and only the negative regulator *brinker* and the ligand *screw* from *Drosophila* appear to be absent in *Anopheles* (6). A second large category of close similarity includes a total of 579 orthologs restricted to *Anopheles* and *Drosophila*. These genes do not share domains with genes in other organisms, and they likely determine insect-specific characters, such as odour and taste reception, cuticle formation, and insect-specific defence mechanisms. Interestingly, only about 100 of these have been functionally characterised in

Drosophila, suggesting that mutants in the insect-specific set of genes cause either very subtle phenotypes, affect traits under the control of multiple redundant inputs, or are involved in processes unlikely to cause easily-scored phenotypes. Perhaps not very surprisingly, only a handful of the genes unique to *Drosophila* (84 of 2570) have functional annotations mapped to Gene Ontology (GO) terms (6). In this manner the class of genes predicted from the sequence includes the larger fraction of uncharacterized genes. It has been argued that 'predicted' genes have a higher rate of protein evolution than genes selected for experimental study on the bases of mutant phenotypes (6, 7).

Of great importance is the fact that sequence searches using 289 human genetic diseases genes show that 61% have orthologs in *Drosophila*, favouring the use of the available genetic techniques of the fly in biomedical research (6, 8). Furthermore, there is a large correspondence of *Drosophila* proteins and their human counterparts considering those involved in the regulation of gene expression, cell signalling pathways, basic cell biology processes such as cell adhesion and migration, cell cycle and cell death, cytosqueleton dynamics, protein transport across cell membranes, as well as DNA, RNA and bioenergetic metabolisms, reinforcing the validity of the fly as a convenient experimental model for basic research (6). Finally, gene duplication affects only a very small fraction of *Drosophila* genes, and in most cases *Drosophila* contains single representatives of genes forming complex families in humans (3). This has the advantage of reducing the possibility of functional redundancy between orthologues, facilitating the characterization of gene functions. The availability of the *Drosophila* genome has deeply affected the way experiments are conducted in the field, stimulating the development of new technologies. First, sequence information saves time with regard to the mapping of mutations and cloning of genes, guiding and speeding conventional genetic analyses. As a consequence, the number of genetic screens carried out in flies has increased in the last years, from 8 published papers in 1999 to 22 in 2005 (authors search in PubMed). Second, the identification of all transcriptional units allows the development of new functional genomics approaches, such as the construction of complete *Drosophila* microarrays and interference RNA libraries.

3. - GENE SEARCH IN *DROSOPHILA*

The functional analysis of any gene mainly consists in the meticulous study of the consequences of its loss on the morphology, physiology or behaviour of the animal. The entry point for these studies is a mutation affecting a particular tract of interest. Searches for mutations are carried out by "genetic screens", involving the isolation and phenotypic classification of a large number of mutants, their mapping to chromosomal regions and, ideally, to the affected genes. In the best of cases this approach provides a powerful mean to analyze complex biological processes, and relies in the ability to manipulate the genome to obtain mutations. In general genetic screens are restricted to either loss-of-function alleles, generated by chemical mutagenesis, or, alternatively, to gain-of-function alleles, through the application of the GAL4/UAS system (9, 10). Complementary to conventional "phenotypic screens", large-scale gene searches also use the criterion that the domain of gene expression is indicative of specific functional requirements. The object of the experiment is to identify sets of genes with similar temporal or spatial expression patterns. In either case, these approaches do not necessarily require prior knowledge of the genome, although they are much more efficient when the sequence information is available. In what follows, we will discuss the more frequent applications of *Drosophila* genetics, from forward genetic screens to the more recent development of reverse genetics techniques.

3.1 Gene identification through mutagenesis screens

The variety of genetic methods developed to identify genes and to link them to their biological functions have in common their dependence on the ability to detect phenotypes and the subsequent mapping of mutations to single genes. These approaches have the limitation of being time-consuming, particularly when chemical mutagenic agents are employed. The main advance in this area have been the extension of the phenotypic traits scored, from altered external morphologies to a wide range of biological aspects, including internal tissues by using tissue-specific reporters and methods of generating mosaics, fly behaviour and immunity.

3.1.1   Loss of function screens

Since the more reliable information about the function of a gene is the phenotype of its absence, it is not surprising that a large proportion of genetic screens aim to identify loss-of-function mutations. The requisite for these experiments to be successful are: 1) to select a trait easy to score, 2) to design a clever protocol of crosses, including the establishment of stable stocks, that minimize fly handling, 3) to construct isogenic chromosomes to facilitate the posterior mapping of the mutants and 4) to scale up the screen to reach genome saturation. Loss-of-function screens generally involve the generation of a large collection of randomly induced mutations in balanced stocks. This step is the most time-consuming and a burden for the laboratory resources, and therefore the screening of the stocks for the desirable phenotypes must be done simultaneously to the initiation of the novel mutant stocks. The end result of this approach is a collection of mutations induced on the same genetic background that display a phenotype in the tissue of interest. In the more favourable cases, the grouping of the mutants in phenotypic classes allows the dissection of the process under study into a number of genetic steps, and the classification of the genes into sets with similar requirements. This information is extremely useful about the way genes interact to control a particular biological process, and it does not require prior knowledge about their identity or molecular nature.

The screens conducted in the late 1970s and 80s to identify genes affecting embryonic development exemplified this approach (11, 12). This work aimed to identify mutations in genes controlling embryonic segmentation by screening for phenotypes in the embryo cuticle. The cuticle is secreted by the epidermis, and contains patterned elements arranged in a segment-specific manner. Because each cell secretes cuticle depending of its position in the segment, cuticle differentiation and cell identity are directly related, and the screens isolated a large fraction of the genes involved in generating positional information in the embryo. For example, many of the genes subsequently classified as members of the Hedgehog, Dpp/BMP, Notch and Wnt signalling pathways were identified in these screenings, as well as a large number of transcription factors involved in the subdivision of the epidermis into segments and in smaller regions within segments (13). The grouping of the mutants into coherent phenotypic classes such as "gap", "pair-rule" and "segment polarity" also allowed, before the molecular nature of the affected genes was identified, to establish the mechanistic basis of segmentation (11). These screens used EMS as a mutagen, and

were optimized to identify genes required in the zygote with no maternal contribution. Subsequent screens were designed to identify maternal-effect mutations, resulting in the identification of the genes that establish the dorsal-ventral and anterior-posterior axes during oogenesis (14-16). The characterization of the genes identified in these screens took almost a decade for a large fraction of the ever-growing *Drosophila* research community.

Other loss-of-function screens used collections of P-element insertions, chromosomal deficiencies or newly induced sets of mutants, and were focussed to dissect processes as diverse as gravitaxis (17), synaptic transmission (18) and synaptogenesis (19), the auditory response (20), dendritic morphogenesis (21, 22), larval optic lobe development (23), behavioural response to touch (24), muscle development (25), meiosis (26), DNA replication (27), heart function (28) and metastasis (29).

3.1.2 Loss of function screens in adult structures

The adult fly was the main phenotypic realm for *Drosophila* researches up until the embryo took a more prominent place in the 1980s. However, early work was engaged either with spontaneous mutations arising from natural populations or with the isolation of novel alleles either in known genes or in particular chromosomal regions (7, 30, 31). The handicap of screens for adult characters is the recessive lethality of most mutations affecting genes with basic cellular and developmental roles. Some early efforts to identify genes involved in adult development coupled mutagenesis to clonal analysis, to look for cell markers in the adult cuticle (32). However, before adult screens could be carried out efficiently, it was necessary to develop methods to induce mosaics of treated chromosomes with high efficiency. Such methods were only available after the adoption of the yeast system of recombination, based on the site-specific recombination induced in FRT sites by the enzyme FLP recombinase (33). Site-specific recombination was used to make treated chromosomal arms homozygous, but only in clones of cells that were screened for a phenotype. The expression of the FLP enzyme can be driven either by a heat-shock promoter, or more efficiently, by directing its expression in a particular tissue of interest, using a combination of a tissue-specific Gal4 line and FLP cloned under control of UAS sequences (34). These screens

involve only one generation (F1) of flies, and by using the appropriate crosses permit to construct healthy flies heterozygous for newly induced mutants, but formed by homozygous cells in the tissue of interest (35). Screens in mosaic animals have been carried out to identify genes required for the adhesion between the dorsal and ventral wing surfaces (36), tumour suppressors (37), synaptic transmission mutants (18), axon guidance in the eye (38) and cell migration defects (39), among others.

3.1.3   Gain-of-function screens

Since it is estimated that two-thirds of *Drosophila* genes are not required for viability (40), a large fraction of the genome is not accessible to conventional loss-of-function screens. An alternative gene identification method relies on the phenotypic consequences of the ectopic and/or increased expression of genes in a particular tissue of interest. It has been observed that this manipulation of gene expression results in phenotypes that are informative about the normal function of the gene. For example, during vein formation the ectopic expression of dominant-negative forms of proteins belonging to the Dpp, Notch and EGFR pathways results in phenotypes very similar to those of the corresponding loss-of-function alleles, whereas ectopic expression of activated forms of the same genes cause opposite phenotypes (41). In addition, the gain-of-function approach has the potential to uncover genes that, due to functional redundancy, are not easily found in loss-of-function screens.

To allow systematic miss-expression screens in *Drosophila*, Rørth (1996) developed a modular system combining P-element insertional mutagenesis with GAL4 regulated gene expression (9). The system is designed to allow conditional expression of genes that are randomly tagged by the insertion of a P-element (10). The P-element carries GAL4 binding sites (UAS sequences) and a basal promoter oriented to direct expression of genomic sequences adjacent to the P-element insertion site (Fig. 1). When combined with a source of GAL4, the P-element directs expression of any gene that lie next to its insertion site. The basis of identifying genes by the consequences of their over-expression is that mutant phenotypes result from the expression of a gene in a place where it is not normally present (ectopic expression) and/or by its expression at higher than normal levels (gain-of-expression). However, the main limitation of over-expression screens is that P elements insert non-random, but with some sites being

highly preferred (hot-spots) and others very rarely targeted by the P element (cold spots) (50). The P-element bias implies that only a fraction of genes will be targeted with a reasonable frequency to allow their identification. The second restriction of over-expression screens is related to the uncertainty about unspecific effects caused by ectopic gene expression on a particular developmental system. In general, gain-of-function screens using P-UAS elements consist in the analysis of the phenotypes resulting from the combination of a previously established collection of mapped P-UAS strains and a Gal4 line expressed in the tissue of interest. Alternatively, large numbers of P-UAS lines can be generated by transposition, and crossed to a Gal4 line of interest to select and map only those P-UAS which in combination with the Gal4 result in a mutant phenotype (51). The use of different P-UAS constructs suitable for gene targeting (42, 52), and the construction of Gal4 lines expressed in restricted patterns, has allowed the undertaking of gain-of-function screens directed to identify genes affecting, among others, imaginal development (52), sensory organ formation (53), thorax formation (54), synaptogenesis and motor axon guidance (55), the development of the central nervous system (56), wing and eye disc growth and development (57, 58), vein patterning (51), male germ-line stem cell maintenance (59), muscle patterning (60), and the formation of the antennal lobes (61).
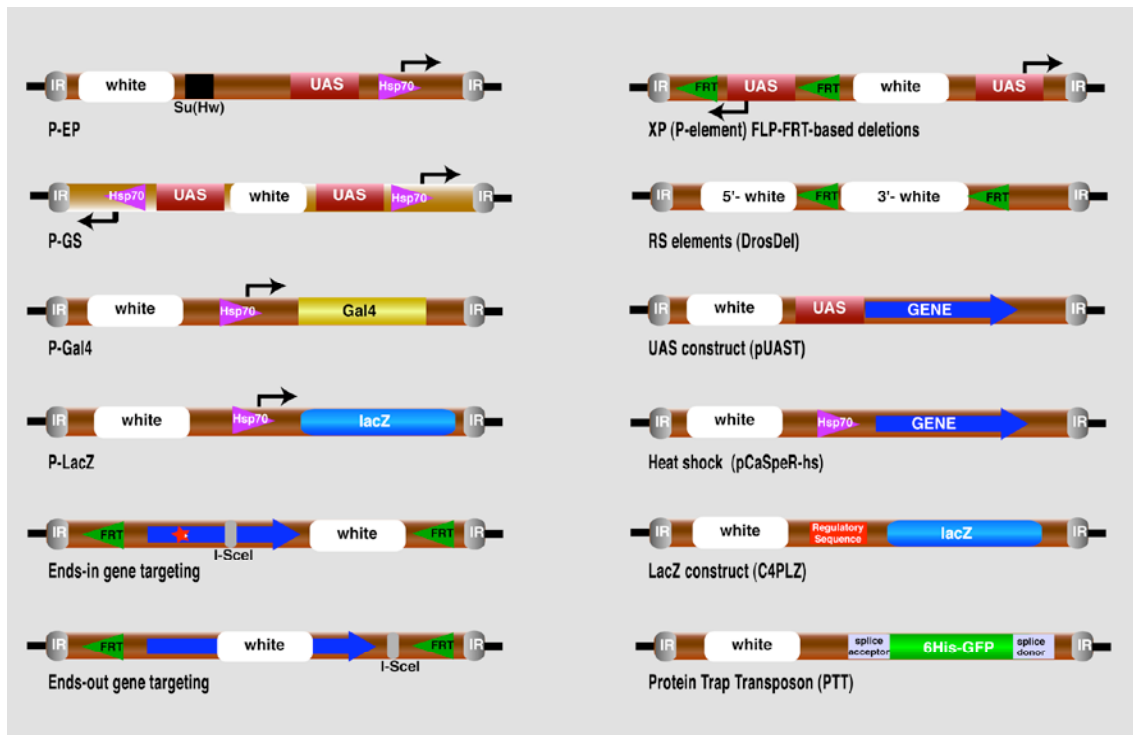
FIGURE 1

Summary of P-element based vectors commonly used in Drosophila genetics. P-EP (10), P-GS (42), P-Gal4 (9), P-lacZ (43, 44), Gene targeting vectors (45), XP, (46), RS (47), UAS (9), Hs (48) and Protein trap (49).

3.1.4 Searching for modifiers: loss and gain of function screens in sensitized genotypes

All of the above mentioned genetic screens were carried out in wild-type genetic backgrounds. Additionally, screens can be conducted in particular mutant backgrounds of interest, named "sensitized background". In these cases, the screen aims to identify genes belonging to pre-determined sets of interacting genes, such as elements of a signalling pathway or a transcriptional network. The rationale for these screens was defined in a seminal paper in which regulatory genes of the *bithorax* and *achaete-scute* gene complexes were identified (62). In modifying screens, it is expected that the background genotype restricts and determines the set of mutants susceptible to be identified. The underlying idea is that the gene-dose of elements belonging to a given pathway became critical for the phenotypic outcome after particular perturbations in the

pathway. When this happens, mutants that in heterozygosis don't show a phenotype are able to increase ("enhancers") or reduce ("suppressors") the phenotype of a particular genetic condition. This allows conducting the experiment in one generation, facilitating the screening of large numbers of treated chromosomes. In general, all screens of the modifier type consist in crossing into a defined genetic background, chromosomal deficiencies, P-UAS elements or newly induced mutants.

There are many examples of successful screens aiming to identify members of known signalling pathways, such as those targeting the Sevenless and Epidermal growth factor receptor (63-70), Notch (71-75), Ecdysone (76), Dpp (77-79), JAK/STAT (80, 81), Hedgehog (82, 83), TNF (84) and wingless (85-88) pathways. Similar screens have also been conducted to isolate interactors of different transcription factors, such as Deformed (89-91), Seven in absentia (92), Cut (93), fushi tarazu (94), Kruppel (95, 96) and Brahma (97), as well as cell-survival factors (98), tumour suppressor proteins (99), cell-cycle regulators (100-104), and interactors of non-muscle myosin-II (105) and Presenilin (106)

An important advantage of modifiers screenings is that they can be easily adapted to searchers for mutants affecting the phenotype caused by the miss-expression of engineered proteins (107), including non-*Drosophila* proteins (108). This has allowed the design of modifier screens in a *Drosophila* model of tauopathy (109) and other neurodegenerative diseases (110-112), as well as other human genetic diseases such as Multiple Endocrine Neoplasia Type 2 (113), cardiomyopathies (114) and Adenomatous Polyposis Coli (APC)(115), among others.

3.1.5 Public resources for loss- and gain-of-function searches

Most screens are carried out by individual groups, which in some cases generate the collection of mutants or P-element lines to be searched for either specific phenotypes or interacting genes. However, a number of resources are already available to conduct these experiments with established public collections of P-elements or genetic deletions (Table 1). In this manner, any screen can be carried out without the necessity of first generating novel variants. Some of the resources for gene mapping and modifying

screens are the collection of large chromosomal deletions with a genome-wide coverage maintained by the Bloomington stock center and the high-resolution and molecularly-mapped deficiencies deleting only few genes per fly strain from the DrosDel Project (47) and the Exelexis collection (116). Similarly, a BDGP project is in progress to target all the *Drosophila* genome with P-elements, which currently includes about 70% of the annotated genes (117, 118). Even if not all insertions result in a loss of function, because P-elements are generally located in introns or other non-coding regions, the use of imprecise excision should made possible to generate loss-of-function alleles in any gene targeted by a P element. Additional resources, such as large collections of P-UAS inserts are available at the Bloomington stock center and National Genetics Institute in Japan (10, 42). Finally, a large-scale effort is in progress to generate transgene-mediated RNAi for the complete *Drosophila* gene content (http://www.shigen.nig.ac.jp/fly/nigfly/). Such lines could be used to obtain loss-of-function conditions in genes not amenable to conventional mutagenesis, as well as facilitate genome-wide screens.

3.2. - Genetic identification through expression pattern analysis

Cell-fate changes that occur during development are usually accompanied by changes in gene expression. Thus, exhaustive knowledge of the spatial and temporal gene expression patterns is necessary to understand the regulatory networks directing development (119). Large-scale searches for genes based in expression patterns were first conducted using engineered P-elements carrying a reporter gene and a basal promoter (Fig. 1).

The more widely reporters are the *lacZ* gene and, more recently, GFP, which allows visualization of gene expression in living animals. Screens aiming to identify expression patterns are named "enhancer trap" screens, because in these cases the target of the screen is genomic DNA able to direct in restricted spatial patterns the expression of the reporter (43, 120, 121). Recent adaptations of enhancer trap screenings to adult territories include the "yellow+" and "GFP" methods, where the mobilised P-element encodes GAL4 (122, 123). In the yellow+ method, the Gal4 protein directs the expression of a *yellow* transgene, which rescues the pigmentation defect caused by the *yellow* mutation only in specific subsets of the adult pattern (122). Enhancer trap

screens revealed the enormous richness of regulatory information contained in the genome, and contributed to the identification of many genes with restricted expression patterns to particular tissues. A repository of gene expression patterns during embryogenesis documented with extensive digital images, controlled vocabulary annotations and microarray profiles, is been generated by the BDGP, aiming to determine systematically the expression of all annotated genes using in situ hybridization (124). The generation of modified P-elements with splicing sites flanking the coding region of GFP (Fig. 1) permits coupling the determination of the expression pattern with the identification of the protein sub-cellular localization. This gene-targeting technique might prove extremely powerful when applied in sufficient large scale to identify the cellular compartments where the proteins are localised, as well as to study changes in subcellular distribution of proteins under different experimental conditions.

4. - Genetic identification using high-throughput techniques

Genetic screens in all their many formats continue to be a very active field of research in the *Drosophila* model, and the complete sequencing of the genome certainly has reinforced this approach. In addition, the availability of a high-quality genomic annotation makes possible the application of other high-throughput techniques, which have been quickly adopted by the fly community. In particular, two aspects that we will consider are microarray techniques and the use of interference RNA-based screens carried out in cell cultures.

4.1 The use of microarrays in gene search

Before the development of microarrays for *Drosophila*, the only way to identify large sets of genes expressed in a tissue of interest was to use laborious techniques of subtractive hybridization. This approach was used to identify genes that are expressed at the beginning of gastrulation (125), mesoderm (126, 127), and in purified follicle cells (128). The complete sequencing of the genome and the development of microarrays have enabled global gene expression analysis, which combined with cell sorting and genetic manipulations has immense power to analyse gene networks. Microarrays rely on the hybridisation of a labelled sample, prepared from mRNA

extracted from the tissues of interest, to nucleic acids fixed to a slide, and allow thousands of hybridisation assays in one experiment (129). The reactions can be competitive, where the relative hybridisation of two samples labelled with different fluorescent molecules is measured, or non-competitive reactions with a single labelled sample. Currently, *Drosophila* researchers use three main array platforms; spotted cDNA amplicons, genomic amplicons and oligonucleotides. cDNA arrays are limited by the coverage of the available cDNA collections, where oligonucleotides and genomic amplicon arrays rely mostly on genome annotations, being capable of a complete coverage of transcripts (see (129) for a comprehensible review).

Microarray studies are being used in *Drosophila* to analyse many complex biological processes that can be grouped into several categories including cell- or organ-type expression profiling, transcriptional responses to environmental challenges, the analysis of gene expression during developmental transitions, the identification of genes affecting particular molecular processes, and the detection of changes in gene expression caused by genetic manipulations. In this last case, the object of the experiment is to compare the wild type versus a mutant or versus the over-expression of a gene of interest using the Gal4/UAS system. Microarray studies can be conducted on whole organisms, on particular tissues easy to dissect or even on GFP-labelled cells isolated using automated cell sorters. In addition, they can also be applied to cell cultures, allowing the description of global expression patterns in cells treated with particular interference RNAs (see below). A bibliographic search in PubMed using the terms "microarray" and "Drosophila" reveals the extent and scope that microarray-based approaches are having in the field. Thus, cell-type expression profiling has been carried out in migrating cells during oogenesis (130), germ stem cells (131, 132), miogenesis (133, 134), Malpighian tubules (135, 136), glial cells (137), neural midline cells (138), wing disc and pupal cells (139-141), and larval haemocytes (142). Similarly, the number of developmental processes analysed by expression profiling comprises circadian rhythms (143-145), transdetermination in imaginal discs (146), aging (147-150), cellularisation (151), gastrulation (152), mating (153), metamorphosis (154, 155), dorso-ventral patterning in the embryo (156), segment identity (157), sex-specific expression (158) and different aspects of the immune defense (159-163).

The analysis of molecular processes using microarrays includes the identification of microRNA targets (164), non sense-mediated mRNA decay (165), splicing (166, 167) and DNA replication (168). The transcriptional responses to environmental challenges is another favourite field of research and includes the study of heat-shock induced transcripts (169-171), response to cold hardening treatment (172), dietary changes (173-176), genetic and environmental stress (177, 178), ionizing radiation (179), sleep-deprived and sleeping flies (180), defence responses to toxins (181, 182) or to other chemicals such as Methotrexate (183) and DDT (184-186). The area more promising includes the identification of changes in gene expression caused by genetic manipulations. So far, microarrays have been applied to polyglutamine toxicity (187), mutants with acute or chronic alterations in neuronal activity (188), response to hypercontraction-induced myopathy (189), identification of targets of of the transcription factors Eyeless (190), Ultrabithorax (191), Retinoblastoma factor (192), dFOXO (193), the male-specific lethal complex (194). }, dMyc (195), transcriptional repressor complexes containing Histone deacetylases (196), activating transcription factor-2 (197), Hrp59 (198), the trithorax protein Ash2 (199), proneural proteins (200) and dMed6 (201). Other experiments aim to identify posttranscriptional regulation by Pumilio (202, Ras-induced overproliferation in the hemocytes {Asha, 2003 #443), EGFR (203), TGFβ (204), JNK or AP-1 dependent transcription in neurons (205), guanylyl cyclase targets (206). the tumor supresor brat (207) and steroid hormone targets (208). Finally, microarrays are increasingly been used for evolution and population genetics studies (209-212).

Microarrays generate awesome datasets that are a serious challenge to analyse. In general, the microarray experiment is followed by the annotation of the identified genes using GO standards, and because only in a small fraction of cases their function is known, the challenge is to determine them using conventional genetics or interference RNA approaches. Excellent examples of this approach are the analysis of myogenesis by transcriptional profiling, in situ hybridization and functional studies of selected candidates (133), and the study of the transcriptional network downstream of the Dorsal transcription factor, combining microarray assays, in situ hybridization and bioinformatics methods (156).

4.2. - Genetic screens in cell cultures using reporters and RNAi techniques

Drosophila cells in culture take up double-strand RNA (dsRNA) using bathing protocols, and in general a 72 hours treatment cause a specific and dramatic depletion of the targeted gene product (213, 214). The sources of dsRNAs are long RNA molecules, which are processed in the cell into small interference-RNA without the necessity of expensive production by chemical synthesis. The long dsRNA can be synthesized from PCR products obtained either from existing cDNA collections or from genomic DNA, followed by an in vitro transcription reaction with RNA polymerases. Experiments using dsRNA involve the design and synthesis of a dsRNA library, the incubation of cells with the dsRNAs and the detection of fluorescent signals revealing the expression of a reporter or a cellular antigen. The screens conducted in cells allow the detection of cell phenotypes not easily scorable in the organism, such as cell lethal factors or specialized gene functions monitored through the use of reporter-based assays.

The use of dsRNA makes feasible the analysis of gene families, by looking for the phenotypes of all genes, either singly or in combination, that contain a common protein domain. This approach was used to study the involvement of kinases and phosphatases in the Hedgehog signalling pathway (215), and the function of histone deacetylase proteins (196). dsRNA screens have also be applied to the entire genome, searching for novel components of known signalling pathways, such as Wingless (216), JAK/Stat (217), TNF (218) and Hedgehog (215, 219). In addition, other published screens aimed to identify genes affecting a variety of cellular processes, including cell morphology (220), cytokinesis (221), cell proliferation (222), the response to micobacterial infection (223), and components of innate immunity (224).

The strengths of dsRNA screens are their versatility, based in the diversity of cell lines available and functional assay designs, and their speed, because the experiment can be coupled to automated detection and acquisition of images. The possibility of undertaking the experiments in specialized centres offering dsRNA facilities, such as the *Drosophila* RNAi Screening Center at Harvard Medical School (http://flyrnai.org/RNAi_index.html), will certainly contribute to make the technique available to small laboratories. In some cases, it might still be required to use dsRNA in vivo on a large scale. Large-scale experiments using dsRNA have been conducted in

Drosophila embryos (225), although the necessity of injection by hand limits the applicability of this approach. However, the generation of a repository of RNA interference strains with a genome-wide coverage at the National Institute of Genetics (http://www.shigen.nig.ac.jp/fly/nigfly/) will facilitate the application of dsRNA for large-scale functional genomics in the organism.

5. - **Gene targeting**

Phenotypic screening of random mutations or dsRNA is very powerful for implicating genes in processes where their involvement was not suspected. In general, the association of mutations to genes requires a considerable investment of time and effort, with standard procedures involving complementation analysis to chromosomal deficiencies, mapping by meiotic recombination to visible markers, and, more recently, to single-nucleotide polymorphisms (226, 227). In other cases, the workflow progress from a gene of interest to the generation of mutants, using reverse genetic techniques (228). In those cases where the gene is targeted by a P element, the more frequent way to generate loss of function alleles are imprecise excision of the P-element and male recombination (229, 230). In both cases, the end results are deletions of DNA flanking the P-element insertion site. Additionally, when the P-element carries UAS sequences capable of directing the expression of the gene in presence of Gal4, an easy manner to recover loss-of-function alleles is searching for revertants of the miss-expression phenotype in a conventional chemical mutagenesis experiment (EMS or ENU) (231). The generation of large sets of strains carrying modified transposable elements containing FLP recombinase target sites (FRT; Fig. 1) has promoted the generation of small and molecularly mapped deficiencies by recombination between adjacent P elements or PiggyBac elements (47, 116). Thus, because the P or PiggyBac element insertion sites are mapped to the sequence, the extent of the deficiency is predetermined and restricted to the DNA comprised between the two P or PiggyBac elements used (47, 116).

Other techniques based exclusively on sequence information intended to generate mutants in genes of interest are homologous recombination and tilling mutagenesis. Homologous recombination consists in the insertion of linear DNA molecules within the corresponding chromosomal region through recombination of

paired sequences. The main advantage is that specific changes can be engineered to modify the structure of the target gene, eliminating coding regions, introducing particular base changes that cause amino acid substitutions, or targeting a GFP molecule in frame to study subcellular localization. The donor DNA linear molecule contains an *I-SceI* endonuclease recognition site, and it is cloned into a P-element flanked by FRT sites (45). In transgenic flies containing this P-element and a source of FLP site-specific recombinase and I-SceI, the expression of FLP and I-*Sce*I excises the donor sequence from its chromosomal location and generates a double-strand break within the sequence that is homologous to the target gene, permitting homologous recombination between the donor and the corresponding chromosomal target locus (45). This technique, however, is not applicable on a genomic scale, and it is also time-consuming and technically demanding. Tilling mutagenesis is based on the large-scale detection of point mutations by Cel-I-mediated heteroduplex cleavage (232). This approach allows screening a large collection of newly induced mutants for DNA alterations in a particular gene of interest (232). Alternatively, PCR fragment-length polymorphisms coupled to mutagens inducing small deficiencies, such as hexamethylphosphoramide, has been applied as a reverse genetic technique for efficient mutation-detection (233). Because in both cases the identification of mutants relies exclusively in their molecular mapping to a region of interest, the experiments do not require any previous knowledge of the consequences of the inactivation of a gene.

CONCLUSION

Drosophila genes are conserved and present in a single copy in the genome, making this organism a very useful model in establishing the functions of mammalian orthologs. The described techniques, coupled to genome-wide efforts to generate interference RNA, P-UAS, P-Gal4, gene trap, chromosomal deficiencies and gene disruption lines, in combination to EST projects, global spatial-temporal expression analyses by RNA in-situ hybridization and large-scale protein interaction maps using the Yeast-2-Hybrid system (234), are making possible to address a wide range of biological questions in the *Drosophila* model. The task of global functional annotation is also greatly facilitated by the diverse initiatives to organize, curate and make available to the research community the immense amount of information related to the functional analysis of gene functions.

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