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Genetic strategies to tackle neurological diseases in fruit flies

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Abstract

Drosophila melanogaster is a genetic model organism that has contributed to the discovery of numerous genes whose human homologues are associated with diseases. The development of sophisticated genetic tools to manipulate its genome accelerates the discovery of the genetic basis of undiagnosed human diseases and the elucidation of molecular pathogenic events of known and novel diseases. Here, we discuss various approaches used in flies to assess the function of the fly homologues of disease-associated genes. We highlight how systematic and combinatorial approaches based on recently established methods provide us with integrated tool sets that can be applied to the study of neurodevelopmental and neurodegenerative disorders.

Keywords

Drosophila; RMCE; MiMIC; T2A-GAL4; flippase recombinase

INTRODUCTION

For over a century, the fruit fly, *Drosophila melanogaster*, has been widely used as a model organism to study most aspects of biology [1]. Flies have a short life cycle, produce a large number of offspring, and are inexpensive to maintain. Numerous developmental pathways and physiological processes are conserved between flies and mammals and nearly 75% of human disease genes have functional homologue(s) in flies [2]. In addition, genetic manipulations in the fly have no equal in other multicellular model organisms with a

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Conflict of Interest Statement

Nothing declared.

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complex nervous system. These features provide fly biologists with opportunities to collaborate with human geneticists, discover the genetic basis of new human diseases, unravel pathogenic mechanisms, discover cellular mechanisms and drugs, and establish conserved molecular etiologies underlying numerous neurological diseases.

The goal of this review is to provide examples of techniques used in fly models to gain insight about the pathophysiology of neurological diseases. We discuss recent experimental strategies that build upon Φ C31 recombination-mediated cassette exchange (RMCE) to insert DNA [3]; CRISPR/Cas9 to edit genomes [4,5]; P[acman]/FlyFos genomic libraries to rescue mutants and determine protein localization [6–8]; Minos-mediated integration cassette (MiMIC) to mutate genes and tag proteins [9,10]; and GAL4/UAS approaches [11], including the *Trojan*-GAL4 (T2A-Gal4) method [12], to express transgenes. We highlight how integrating many of these technologies into new approaches has provided an elegant toolset to unravel molecular mechanisms of neurodevelopmental and neurodegenerative diseases by enabling unsurpassed genetic manipulations in flies.

A. Reverse genetic approaches

Reverse genetic approaches to elucidate disease pathogenesis in flies are based on the discovery of disease-associated genes by human geneticists and the availability of reagents related to the fly homologue(s) of the target gene (Figure 1a). The available information in the literature and databases can be screened through MARRVEL [13] and FlyBase (flybase.org). These include mutations induced by chemical mutagens, deletions, or insertions of transposable elements, which can often be excised to create small deletions. The phenotypes associated with the complete loss of the gene of interest provide an important reference point to compare phenotypes associated with missense mutations identified in patients to gauge if they cause loss-of-function (LOF) (amorph or hypomorph), gain-of-function (GOF) (hypermorph or neomorph) or dominant negative (antimorph) phenotype. Alternatively, spatiotemporally controlled expression of RNAi against the gene of interest, using the GAL4/UAS system [11], allows gene knockdown. However, it often does not provide as rigorous a reference point as the null mutant and the need to address the efficacy and specificity of knock-down with each RNAi increases the workload. On the other hand, a large collection of nervous system-specific GAL4 lines [14,15] is available, permitting knockdown of genes in specific neuronal and glial cell (sub)populations. Finally, CRISPR based strategies can be employed to edit genes [16,17]. In all cases, it is highly recommended that the phenotypes associated with mutations that are being studied are rescued using either a UAS-cDNA approach or better, a genomic fragment that encodes the gene (see Sections C and D). Indeed, second site mutations or RNAi off-target effects may cloud the interpretation of phenotypes associated with the gene of interest.

The *Drosophila* community has kept a publicly available database of the majority of fly mutants that were generated. The availability of large collections of LOF alleles from stock centers has been invaluable for many researchers to tackle the pathogenic mechanisms associated with numerous diseases including neurodegenerative and neurodevelopmental disorders. Recent examples include models of Parkinson's disease (PD) [18], amyotrophic lateral sclerosis (ALS) [19], Coffin-Lowry syndrome [20], and lysosomal storage diseases

(LSDs) [21]. Similarly, RNAi mediated knockdown has been successfully utilized to assess the *in vivo* function of disease-associated genes including *parkin* [22], *valosin containing protein (VCP)* [23], amyloid precursor protein (APP) [24], and others [25,26].

B. Forward genetic approaches

Even though reverse genetic approaches can generate valuable information about a gene's function, this research is typically hypothesis-driven. Forward genetic approaches, on the other hand, are unbiased and based on a phenotype of interest. They have led to the discovery of thousands of genes, many of which are relevant to human pathology and disease (Figure 1b). However, in the area of neurobiology there has been a strong bias towards screens that affect neuronal development in flies, and very few high-throughput screens have attempted to isolate mutants that affect neuronal function [27–29] and/or cause the demise of neurons [30,31]. Indeed, most efforts to study genes associated with neurodegeneration have been based on reverse genetic strategies [32,33].

Recently, an unbiased chemical (EMS) mutagenesis screen was performed in flies to isolate essential genes required for neuronal development and maintenance. This screen was designed to isolate lethal mutations on a flippase recognition target (FRT) bearing X-chromosome and to subsequently perform flippase (FLP)-mediated clonal analyses in the eye and thorax/wing. By assessing externally visible eye phenotypes as well as the progressive demise of photoreceptor function based on electroretinograms (ERGs), ~700 mutations were isolated. Unlike other EMS screens, the authors set out to identify the nature of most affected genes by performing duplication mapping [34], whole genome sequencing [35], and systematic transgenic rescue based on BAC clones [36]. This led to the identification of 165 genes associated with neurodevelopmental or neurodegenerative phenotypes [30]. Nearly all isolated mutations were LOF alleles, often providing allelic series of genes whose human homologues were already known to be associated with disease. These alleles have permitted in depth phenotypic characterization of genes associated with Leigh Syndrome (*sicily/NDUFAF6*, *l(1)G0334/PDHA1*, and *ppr/LRPPRC*) [37–39], Charcot-Marie-Tooth Type 2A (CMT2A) (*Marf/MFN2*) [38,40], PD (*Vps35/VPS35* and *Vps26/VPS26*) [41], and Friedreich's Ataxia (*fh/FXN*) [42]. The screen also led to the identification of a novel human gene associated with severe microcephaly (*Ankle2/ANKLE2*) [30], and two other genes associated with acquired microcephaly and neurodegeneration (*Nrd1/NRD1* and *dOgdh/OGDH*) [43]. In summary, the ability to identify the molecular lesions in genes from a single genetic screen in *Drosophila* has driven the discovery of genes that are associated with diseases and follow-up studies have revealed a better understanding of the pathology.

C. Expression of fly or human genes using binary systems

Accumulation of toxic proteins in inclusions or aggregates is a key pathological finding associated with many neurological diseases, including AD, PD, ALS, and prion diseases. These may lead to altered protein function or toxic aggregates that can often be mimicked by protein overexpression. The most commonly used strategy to overexpress genes in the flies is based on the GAL4/UAS-mediated binary expression system [11] using tissue and neuronal sub-type specific drivers to model neurodegenerative and neurodevelopmental

diseases (Figure 2a). By adding the temperature-sensitive GAL80^{ts} to repress GAL4 activity, fine spatiotemporal control of expression can be obtained [44–46]. Similarly, the GeneSwitch (GS) method utilizes a GAL4-progesterone-receptor chimera protein that can be activated by the hormone progesterone to spatiotemporally control the gene expression [44,47]. Alternative binary overexpression systems such as the LexA/LexA operator (LexAop) system [48] and the Q-system (QF/QUAS/QS) [49] are also used for the analysis of gene function and precise spatiotemporal manipulations of gene expression (Figure 2a). These systems can be used along with the GAL4/UAS/GAL80^{ts} system to perform independent labeling and/or manipulation of separate populations of neurons in the same fly [50–53]. Binary overexpression systems also permit screening for modifiers of a gain of function phenotype and unravel other key players that affect protein toxicity [46,54–56].

Another application of the GAL4/UAS approach is to test if the expression of a fly or human cDNA can rescue the phenotypes associated with the loss of a homologous fly gene (Figure 2b). Rescue with the human cDNA indicates functional conservation and permits assessing the consequences of human variant expression in flies. This approach, based on a limited set of roughly 30 genes, is effective for the majority of genes tested (personal communication and [57–59]).

D. Assessing disease variants using genomic rescue constructs

One caveat associated with expression of fly or human cDNAs, especially if the goal is to rescue mutants, is that there are often different isoforms for a single gene, raising the issue as to which isoform should be selected. In addition, when genes are large, full-length fly or human cDNAs are often not available. An alternative strategy to rescue a null mutant phenotype is to use a fly genomic rescue construct that encodes all the transcripts of the gene of interest. Three genomic rescue construct libraries of *Drosophila* are available: two BAC libraries based on P[acman] [7] (bacpacresources.org) and a fosmid library based on FlyFos clones [8] (sourcebioscience.com). In addition, transgenic flies carrying 80 kb P[acman] clones and FlyFos constructs are available [8,36].

A key requirement to analyze the functional consequences of a patient variant is that the protein sequences that display variants in humans are conserved in the fly homologues. For instance, mutations in the voltage-gated Ca²⁺ channel *CACNA1A* cause diverse neurological disorders in humans and two of these mutations with different patient symptoms were recently modeled in flies using a genomic rescue construct [60]. Based on previously identified lethal mutations and neurodegenerative phenotypes caused by mutations in the fly homologue, *cac* [61], the authors tested the rescuing ability of a wild type 80 kb P[acman] clone and clones derived from this BAC in which the human variants were inserted by recombineering [62]. Whereas the wild type P[acman] clone fully rescued the observed phenotypes, the two variants tested showed very different phenotypes in the flies providing a better understanding of the nature of the patient variants in *CACNA1A* [60].

In cases where the gene of interest carries a single exon, the fly exon in the genomic rescue constructs can be replaced by wild type and variant-containing human exon to assess/compare the ability of these constructs to rescue mutants (Figure 2c') [63]. For example, the functional impact of a rare *TM2D3* human variant that was predicted to be non-pathogenic

was identified in association with late onset AD susceptibility [64]. The loss of the fly homologue of *TM2D3*, *almondex* (*amx*), is known to cause embryonic lethality and severe neurogenic defects. The authors showed that a genomic rescue construct containing the human *TM2D3* under the control of endogenous *amx* regulatory elements can functionally substitute for fly *amx*, whereas expression of the AD-associated variant under the same conditions causes a severe LOF. These data strongly suggest that the variant in *TM2D3* observed in AD patients, which is not evolutionarily conserved in flies, plays a role in AD pathogenesis.

E. Introducing disease variants in conserved residues of fly homologues

An alternative way to assess the functional impact of disease-associated variants that may affect the protein function is to introduce the variants in the endogenous fly gene, rather than in a transgene (Figure 2c''). This requires that the human and fly proteins are likely orthologues and that the residues affected in humans are conserved in flies. Mutations or variants can be integrated into the fly homologue of disease-associated genes by homologous recombination (HR) [65] and this strategy was utilized to introduce ALS-associated *SOD1* point mutations in the fly homologue of *SOD1* [16]. This study provided valuable insights about the nature of the different mutations that affect *SOD1*. Currently, the CRISPR/Cas9 technology can be used to precisely modify nucleotides in endogenous genes by injection of single-stranded DNA (ssDNA) [66] (Figure 2c'''). The Cas9 can also be used to perform conditional mutagenesis by ubiquitously expressing the guide RNAs against the gene of interest but restricting the expression of Cas9 with the GAL4/UAS system to specific cells or tissues [17].

F. Integrated approaches to study gene function and disease variants based on MiMIC derived strategies

Some of the basic knowledge required to dissect a gene's function relates to which cells express the gene and where the protein is localized subcellularly. In addition, a tissue specific knockdown strategy that is conditional and reversible can provide invaluable information. These features, as well as the capability to test the rescuing ability of the fly gene or its human homologues and potential pathogenic variants, were recently integrated using MiMIC technology [9] (Figure 3). MiMIC is a transposable element that incorporates two inverted Φ C31 attP target sites flanking a gene-trap cassette: (Splice Acceptor) SA-three stop codons for each possible reading frame-the polyA tail and a transformation marker, *yellow*⁺ (Figure 3a'). Once a MiMIC is in the fly genome, any DNA sequence flanked by attB sites can replace the gene-trap cassette present in MiMIC by Φ C31-mediated RMCE.

The MiMIC cassette can be randomly inserted in almost any genomic location and can be used for different applications depending on the insertion location. Among these, the insertions in introns between two coding exons are the most useful. As shown in Figure 3a, they can be used to integrate an artificial exon: SA-EGFP-SD (Splice Donor), creating a GFP-tagged protein trap (Figure 3a''). The internally tagged proteins are most often functional [10] and permit the determination of the cells that express the protein as well as the subcellular localization of the protein. Importantly, the deGradFP system can be used for a reversible temperature-dependent conditional degradation of GFP-tagged proteins [10,67].

Two alternative strategies based on MiMIC and FLP recombinase can be used for conditional gene inactivation in mitotic or post-mitotic cells such as neurons: Flip-Flop [68] and FlpStop [69] (Figure 3b). Both strategies utilize cassettes that are nested in between attB sites and they can easily be integrated into MiMICs using RMCE. Both cassettes are flanked by two different inverted pairs of FRT sites forming a FLip-Excision (FLEx) switch [70]. This allows inversion of the cassette in the presence of FLP. Flip-Flop contains a protein-trap (PT) module that tags the protein with GFP when inserted in PT orientation and a gene-trap (GT) module that truncates the transcript and marks the mutant cells with mCherry when inserted in GT orientation (Figure 3b'). Hence, starting with a PT and inverting the cassette to a GT allows conditional removal of a gene/protein in developing as well as in non-mitotic cells by simultaneously marking wild type and mutant cells. Similarly, the FlpStop cassette can be inserted in two different orientations: non-disrupting (ND) or disrupting (D) (Figure 3b''). Flippase activity can invert one orientation to the other, providing conditional disruption and rescue, similar to Flip-Flop. The presence of the tdTomato marker in the invertible FLEx switch region of the FlpStop cassette allows detection of cells that undergo the inversion. Hence, FlipFlop and FlpStop permit the removal of proteins at any time and in any tissue and provide powerful additions to the *Drosophila* toolkit.

Another recently developed tool that is also based on intronic MiMIC insertions relies on RMCE-mediated integration of an artificial exon that contains a SA followed by self-cleaving T2A peptide that causes ribosomal skipping, the GAL4 coding sequence, and a polyA signal to create a LOF allele by early transcription termination [12] (Figure 3a'''). Upon translation, the truncated message produces the GAL4 protein that is expressed in the same spatiotemporal expression pattern as the native gene. The GAL4 can then be used to drive a UAS-fly or human cDNA to assess the rescue of mutant phenotype or to express a fluorescent marker like UAS-GFP to assess the cellular pattern of endogenous expression. This strategy was successfully applied to investigate a new neurodevelopmental disorder resulting from *de novo* variants in *EBF3* [71]. The lethality caused by the T2A-GAL4 insertion in the fly *EBF3* orthologue, *knot*, was rescued by expression of human *EBF3* cDNA, showing that human *EBF3* can functionally replace its *Drosophila* orthologue. On the other hand, expression of *de novo* variants observed in patients, abrogated the ability of human *EBF3* to rescue the mutant flies, suggesting that these variants are pathogenic. In another example, the T2A-GAL4 strategy was utilized in combination with the forward genetic approach (discussed in section B), revealing a novel role for NRD1 (Nardilysin) as a mitochondrial co-chaperone for OGDH (2-oxoglutarate dehydrogenase) in flies [43]. The authors showed that loss of the fly homologues, *Nrd1* or *dOgdh* cause progressive neurodegeneration and mutations in both human *NRD1* and *OGDH* are linked to rare neurological conditions. They generated a severe LOF allele using the T2A-GAL4 system and showed that the lethality caused by loss of *dOgdh* can be rescued by expression of wild type fly or human *OGDH*. Expression of the disease associated variant-containing human or fly *OGDH*, on the other hand, did not rescue lethality, suggesting that the disease variant in the patient is a severe LOF mutation.

The above strategies show the power and versatility of the MiMIC system, yet the MiMIC collection contains intronic insertions for only 1,800 genes [10]. Hence, the Gene Disruption Project is creating an additional collection of intronic MiMIC-like elements using a CRISPR

based technology termed CRIMIC (CRISPR-mediated integration of MiMIC-like elements). CRIMIC allows the efficient targeting of MiMIC-like elements in introns of fly homologues of human genes [12,72] and should therefore expedite our understanding of neurological disease pathologies in fly models.

In summary, integrating numerous technologies in a pipeline based on MiMIC allows us to assess the rescue ability of wild type and variants of human genes, to determine the cells expressing the gene of interest, to probe the subcellular localization of wild type and mutant proteins, and enables elegant conditional manipulations *in vivo* at various developmental stages as well as in adults.

CONCLUSION

Drosophila has been a powerful model organism to discover and study human genes. Recently developed techniques further facilitate the study of disease-associated genes and permit assessing the pathogenicity of identified human genetic variants in flies. Integrating these techniques in seamless approaches will continue to drive gene discovery and assessment of pathogenic mechanisms.

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Highlights

- Expression of reference and variant human genes in flies permit functional testing
- Innovative genetic strategies in flies drive discovery of disease associated genes
- Integration of different approaches accelerates discovery of disease mechanisms

FIGURE 1.

Genetic approaches enabling neurological disease research in *Drosophila* **(a)** Reverse genetic approaches in flies are based on the identification of the fly homologue(s) of the disease-associated gene and the assessment of phenotypes caused by genetic manipulations of the gene of interest. Upon examination of the gene's function, modifier screens can be performed to identify additional genes that act as either enhancers or suppressors of determined phenotypes. **(b)** Forward genetic approaches identify mutants with a certain phenotype of interest (such as neuronal dysfunction or neurodegeneration) based on unbiased genetic screens. Upon mapping of the causative mutations to genes whose human homologues are associated with disease, detailed phenotypic characterizations can be performed to provide a better understanding of the gene's function.

FIGURE 2. Methods to model *Drosophila* for assessing disease variants **(a)** Basic binary expression systems used in *Drosophila* **(b)** Upon identification of phenotypes caused by a severe LOF mutation, the effect of overexpression of WT or disease-variant bearing cDNA (of both fly and human) can be tested using binary expression systems: GAL4/UAS, Q-system, and LexA/LexAop. Availability of a wide-range of tissue/developmental stage-specific drivers and conditional use of binary systems allow elegant manipulations on gene expression. **(c)** Disease-associated variants can be inserted into genomic rescue constructs or into the endogenous fly locus when the variant is conserved in the fly homologue. **(c')** If the gene of

interest contains a single exon, the fly exon can be replaced by wild type or disease variant-containing human exon. Introducing these genomic rescue constructs into flies that lack the gene of interest and display certain phenotypes allows testing for rescue with different genomic constructs. (c'') Exogenous fly sequence with the disease variant can be replaced by the endogenous fly locus using ends-out homologous recombination (HR). Cre recombinase induces site-specific recombination between LoxP sites that flank mini white marker (w^+) and removes w^+ , leaving a LoxP within the intron. This method can be used to create knock in disease models in flies. (c''') CRISPR/Cas9 based genome editing can be used to precisely modify nucleotides. RNA-guided Cas9 nuclease induces a double-strand break. Homology directed repair repairs the break by introduction of a donor single-stranded DNA (ssDNA) containing disease-variant.

FIGURE 3.

Integrated gene manipulation approaches using Minos-mediated integration cassette (MiMIC) (a') MiMIC elements contain a gene trap cassette and a *yellow*⁺ selection marker flanked by attP sites. Using recombinase mediated cassette exchange (RMCE) an artificial exon nested in attB sites can be integrated into MiMIC. (a'') Protein traps can be generated using cassettes containing SA-EGFP-SD, which allows the assessment of endogenous localization of the GFP-tagged protein as well as deGradFP strategies for conditional manipulations. Flexible linker sequence is abbreviated as L. (a''') Introduction of an artificial exon that contains a self-cleaving T2A peptide causing ribosomal skipping and a GAL4 sequence creates a truncated protein of interest and a GAL4 protein. GAL4 is expressed in the same spatial and temporal pattern as the fly gene, allowing expression of transgenes including the corresponding UAS-fly or human cDNA as well as UAS-fluorescent protein

(e.g. GFP, mCherry). **(b)** Recently, two flippase-dependent cassettes that can be integrated into MiMIC sites were generated to perform conditional gene inactivation: Flip-Flop and FlpStop. Both cassettes are flanked by two different inverted pairs of FRT sites forming a FLip-Excision (FLEx) switch, thereby making the cassettes invertible in the presence of FLP recombinase. **(b')** FlipFlop cassette contains two modules that are placed in opposite orientations: a protein-trap (PT) module and a gene-trap (GT) module. The PT module contains SA-EGFP-SD and thus tags the protein with GFP. The GT module contains SA-T2AmCherry-polyA and thus truncates the gene/protein. The T2AmCherry in the GT module marks the mutant cells with mCherry. Presence of flippase can invert one module to the other, allowing conditional and reversible regulation of gene disruption. **(b'')** FlpStop cassette is designed to have a non-disrupting (ND) and disrupting (D) mode that are placed in opposite orientations. Flippase activity can invert one orientation to the other providing conditional gene disruption and rescue. Presence of the tdTomato marker in the invertible FLEx switch region allows detection of cells that undergoes inversion.