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Masamitsu Yamaguchi *Editor*

Drosophila Models for Human Diseases

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Preface

Fifteen years ago when I gave a talk on "*Drosophila* models for human diseases" to medical doctors, they were not particularly interested in this subject. They said that even for mouse models it was hard to interpret the results, so that flies must be even more difficult. When I gave the equivalent talk to *Drosophila* communities at a similar time, they were also not very interested in the subject of "*Drosophila* models for human diseases." They were more interested in basic biological and/or developmental processes and evolutionary genetics. However, now the situation has dramatically changed. Facilities keeping transgenic and knockout mice are all full in many universities and institutes all over the world and medical scientists are starting to look for animal models that can substitute for, or complement, mouse models. The Drosophilists have also realized the great potential in using *Drosophila* models for medical science. *Drosophila* is now attractive to scientists in various fields as a useful and highly tractable model organism for studying human diseases. Most biological pathways and physical and neurological properties are highly conserved between humans and *Drosophila* and nearly 75% of human disease-causing genes have a functional homologue in *Drosophila*. We can therefore *Recycle* the knowledge and data accumulated with *Drosophila* for studies of human diseases. The costs involved in experimentation with *Drosophila* are relatively low (*Reasonable price*) compared to mouse and other rodent models. The life cycle and life-span of *Drosophila* are much shorter than that of the mouse so that researchers can perform experiments more *Rapidly*. Many genetic and experimental tools have been developed in *Drosophila* to examine gene function, genetic interactions, and environmental influences. Moreover, statistical analyses with large number of offspring in *Drosophila* can make the data obtained *Reliable*. The *Drosophila* model can thus provide a *4R platform* for studies of human diseases.

This book provides information about various *Drosophila* models for human diseases including Alzheimer's disease, Parkinson's disease, repeat expansion disease, amyotrophic lateral sclerosis, Charcot-Marie-Tooth disease, muscular dystrophy, cancer, leukemia, diseases of replication/repair genes, diabetes, and so on. This knowledge is useful for scientists and graduate students in the field of Applied Biology, Pharmaceutical Science, and Medicine. In the first chapter, specific and commonly used *Drosophila* techniques will be provided so that readers can easily understand the subsequent sections. In addition, for many of the following chapters, the authors provide a section dealing with protocols commonly used in their laboratories related to each subject. This is useful for beginners who want to start using *Drosophila* as a model for their studies on human disease. The last chapter introduces a unique approach by designers to develop a screening kit for medicine using the *Drosophila* model. This kind of multidisciplinary approach opens new possibilities for the studies of human diseases using *Drosophila* models.

Finally, I would like to acknowledge the international grants that were helpful to establish international networks to produce this book. The JSPS Core-to-Core Program, Asia-Africa Science Platforms, the JSPS Program for Advancing Strategic International Networks to Accelerate the Circulation of Talented Researchers (Grant No. S2802), the JSPS Japan-UK collaborative research, the JSPS Japan-Vietnam collaborative research, and the JSPS Japan-Korea collaborative research. I would also like to acknowledge invaluable help from Project Co-ordinator, Kripa Guruprasad and Project Manager, Kandrakota Maadhuri

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Drosophila as a Model Organism

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Masamitsu Yamaguchi and Hideki Yoshida

Abstract

Drosophila melanogaster has been widely used in classical and modern genetics for more than 100 years. The history of the *Drosophila* model in the study of various aspects of life sciences will be summarized in this chapter. Furthermore, commonly used techniques and tools with *Drosophila* models will be briefly described, with a special emphasis on the advantages of *Drosophila* models in the study of various human diseases.

Keywords

Drosophila · History · Biology · Chromosome · Genome · GAL4-UAS

1.1 History of Studies with *Drosophila*

Drosophila melanogaster is one of the most commonly used experimental organisms and was first studied experimentally by Dr. Castle (Castle 1906) and used by Dr. Morgan for genetic experiments from 1909 (Sturtevant 1959). During the following 30 years, a number of the main principles of classical genetics were established by studying *Drosophila*, which advanced our understanding of genes, chromosomes, and the inheritance of genetic information (Ashburner and Bergman 2017). Mutagenesis techniques using radiation and chemicals were also developed with *Escherichia coli*, yeast, and *Drosophila*, allowing scientists to clarify gene functions by studying the phenotypes induced by mutations.

After 1970, various molecular, developmental, and biological techniques began to be applied to *Drosophila*, such as gene cloning, hybridization, P-element-based transformation, and clonal analyses. These techniques allowed scientists to perform analytical rather than descriptive studies on the development and behavior of *Drosophila*. In 1994, the Nobel Prize in Physiology or Medicine was awarded to Dr. Lewis for his studies with *Drosophila* to elucidate gene structures, as well as Drs. Weischaus and Nusslein-Volhard for their pioneering work on embryogenesis and the identification of a large number of genes involved in all aspects of *Drosophila* development, including segmentation. Most of the mammalian homologues of these genes were then found to be essential for mammalian development. In addition, many tumor suppressor genes were initially identified in *Drosophila*, and their human homologues were subsequently detected and proven to play important roles in oncogenesis.

The genome project of *Drosophila melanogaster* (*D. melanogaster*) was completed in 2000.

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A few years later, the human genome project was also finished, and comparisons of both genome sequences revealed high homologies between the *Drosophila* and human genomes, thereby confirming the importance of *Drosophila* as a model to study human diseases (Adams et al. 2000; Myers et al. 2000). Nearly 75% of human disease-related genes have been estimated to have functional orthologues in *Drosophila* (Pandy and Nichols 2011; Yamamoto et al. 2014). Overall identity at the nucleotide or amino acid sequence between *Drosophila* and mammals is approximately 40% between homologues. Regarding the conserved functional domains of proteins, identity may be more than 80%. The completion of the fly genome also promoted the study of transcription, protein binding to specific DNA sequences, and genetic variations at the molecular level. Based on genome information, we may perform RNA-sequence analyses or microarrays for expression profiling, targeted to all known or predicted coding regions or against the entire *Drosophila* genome including noncoding regions. We may also perform the genome-wide mapping of binding sites for chromatin-associated proteins at a high resolution using DNA adenine methyltransferase identification (DamID) (Sun et al. 2003; Bianchi-Frias et al. 2004), chromatin immunoprecipitation assays (ChIP assays), or a ChIP-sequence analysis (MacAlpine et al. 2004; Birch-Machin et al. 2005). Genome-wide surveys for polymorphisms using high-throughput PCR strategies are now also available (Glinka et al. 2003). Thus, *Drosophila* is always at the forefront of modern biology, in which genes, gene engineering, and other new findings are often achieved first in *Drosophila* and then generalized to other organisms including humans. It is important to note that the Nobel Prize in Physiology or Medicine in 2017 was awarded to Drs. Hall, Rosbash, and Young for their discovery of the molecular mechanisms controlling the circadian rhythm in *Drosophila*.

1.2 Biology of *Drosophila*

Drosophila has a relatively rapid life cycle. A single fertile mating pair may produce hundreds of genetically identical offspring in approximately 10 days at 25 °C. This is markedly faster than the commonly used rodent models. *Drosophila* may also be regarded as a model organism defined by its developmental stage: the embryo, larva, pupa, and adult (Pandy and Nichols 2011).

After fertilization, embryos undergo highly synchronized nuclear division cycles. These cycles, which are composed of only G and S phases, proceed very rapidly, requiring approximately 10 min for one cycle to form a multinuclear syncytial blastoderm. After nine nuclear division cycles, most nuclei move to the surface of the embryo and undergo four successive nuclear divisions at the surface of the embryo. These nuclei then simultaneously cellularize to form a cellular blastoderm. After cellularization, they undergo segmentation processes. Early embryos store large amounts of DNA replication enzymes that are enzymatically and cytologically characterized by the embryo (Yamaguchi et al. 1991). The embryo may be used in studies on fundamental developmental biology by examining pattern formation, cell fate determination, organogenesis, central/peripheral neuronal development, and axon pathfinding.

The larva, particularly the wandering third instar larva, is commonly used to study developmental and physiological processes as well as less complex behaviors such as foraging. A group of cells called imaginal discs produce the future adult external structures of *Drosophila* and are contained within the larva. They are primarily composed of an undifferentiated epithelium. In the late third instar larval stage and subsequent pupal stage, imaginal discs undergo morphological changes that produce adult external structures such as the antenna, compound eye, wings, and

legs. Imaginal disc cells undergo a typical G1, S, G2, and M phase cycle. In terms of cell cycle studies, eye imaginal discs in the third instar larvae are particularly useful. Cells in the anterior region to the morphogenetic furrow undergo random cell division, and those at the morphogenetic furrow are arrested at the G1 phase and then synchronously undergo the S, G2, and M phases to double the cell number. Then all cells fall into the G1/G0 and undergo differentiation. Thus, the eye imaginal disc provides a naturally occurring synchronized cell system that is very useful for characterizing the genes involved in the regulation of the cell cycle and DNA replication (Yamaguchi et al. 1999). The differentiation processes of eight (R1–R8) photoreceptor cells have also been studied in detail. Clarification of the mechanisms responsible for the developmental processes of imaginal discs has provided significant insights into *Drosophila* and human biologies. Furthermore, learning and memory assays are possible with larvae.

In the pupal stage, *Drosophila* undergoes metamorphosis, and during metamorphosis, imaginal discs undergo cell proliferation, differentiation, and organogenesis to produce various adult external structures, while most larval tissues undergo autophagy and cell death (Aguila et al. 2007). Cells undergo these processes in response to the hormone 20-hydroxyecdysone (ecdysone), which initiates larval-prepupal and prepupal-pupal transitions (Baehrecke 1996). Consequently, *Drosophila* undergoes morphological changes with the tight regulation of various biological pathways. During metamorphosis, the metabolic rate of *Drosophila* follows a U-shaped curve in which energy consumption is high during the first stages, declines toward the mid-pupal stage, and increases again toward the last phases of the larval-adult transformation (Merkey et al. 2011). Further details on the metabolic changes that occur during the development of *Drosophila* are described in Chap. 14.

The *Drosophila* adult provides a complex model organism that is somewhat similar to mammals in many aspects. The adult fly has organs that are functionally similar to the mammalian heart, lung, kidney, gut, and reproductive tract. The adult fly brain contains more than 100,000 neurons that form discrete circuits and neuropils, which mediate complex behaviors including wake and sleep circadian rhythms, learning and memory, feeding, aggression, courtship, and grooming. More significantly, the responses of *Drosophila* to various drugs that act on the central nervous system are similar to the effects observed in mammals (Rothenfluh and Heberlein 2002; Satta et al. 2003; Wolf and Heberlein 2003; Nichols 2006; Andretic et al. 2008). Therefore, *Drosophila* provides a useful model for screening therapeutic drugs for various human neuropathies.

1.3 Chromosomes of *Drosophila*

1.3.1 Overview

D. melanogaster has four sets of chromosomes, the X and Y sex chromosomes, two autosomal chromosomes 2 and 3, and the very small chromosome 4 (Metz 1914; Deng et al. 2007). Female flies carry two X chromosomes and males carry a single X and Y chromosome. Females and males carry two sets of the autosomal second, third, and fourth chromosomes. The X chromosome is acrocentric and may be divided into two arms by the centromere, a large left arm and a markedly smaller right arm. The Y chromosome is also acrocentric with a slightly longer long arm and shorter arm. In contrast, chromosomes 2 and 3 are metacentric with the centromere located in nearly the center of two left and right arms, named 2L, 2R, 3L, and 3R, respectively. The fourth chromosome is also acrocentric, carrying a small left arm and larger right arm.

Drosophila chromosomes may be functionally and structurally divided into heterochromatic and euchromatic regions. Heterochromatin is designated as the darkly staining regions in karyotyping. The heterochromatic region is also known to be late replicating in the S phase of the cell cycle and is enriched with highly repetitive nucleotide sequences and transposable elements (Dimitri 1997). The X, second, and third chromosomal regions adjacent to the centromeres are

darkly staining and are designated as pericentric heterochromatin. The Y and fourth chromosomes are also darkly staining and entirely heterochromatic, although the fourth chromosome has a small euchromatic right arm. The gene densities of the heterochromatic regions of the genome are lower than those of euchromatic regions. The Y chromosome is not necessary for the viability of *Drosophila*; however, XO males lacking the Y chromosome are sterile. Furthermore, XXY flies are female, indicating that the Y chromosome plays no role in sex determination in *Drosophila*. Sex is determined by the balance between the X chromosome and autosome in *Drosophila*: X:A = 1 is female and X:A = 0.5 is male.

1.3.2 Polytene Chromosomes

In *Drosophila*, after differentiation, most cells undergo endoreplication in which the S and G phases are repeated without any M phase. The most typical endoreplicating tissue in *Drosophila* is the larval salivary glands. In the case of the third instar larval salivary gland, the ploidy level reaches 1024 (Rodman 1967; Hammond and Laird 1985). The levels of polyploidy are mainly reached by the euchromatic regions of the genome because the heterochromatic regions are under-replicated. Furthermore, homologous chromosomes undergo somatic pairing in the polytene chromosome. Thus, the combination of polyploidy and pairing may produce 1024 DNA strands for each euchromatic chromosome arm. All chromosome arms corresponding to X, 2L, 2R, 3L, 3R, and the small 4 expand from a central region called the heterochromatic chromocenter (Fig. 1.1). The heterochromatic chromocenter is composed of pericentric heterochromatin and, in the case of males, the Y chromosome. Polytene chromosomes are sufficiently large to be easily observed using a standard light microscope. Each of the euchromatic arms shows a unique banding pattern caused by the differential condensation of chromatin to form darkly stained bands and less stained interbands (Fig. 1.1).

In *Drosophila*, the band pattern of salivary gland polytene chromosomes is highly reproducible among individuals (Bridges 1935). Each large arm is cytologically divided into 20 roughly equal numbered segments (X = 1–20; 2L = 21–40; 2R = 41–60; 3L = 61–80; 3R = 81–100; 4 = 101–102). Each of these num-

Fig. 1.1 In situ hybridization of the *white* gene on salivary gland polytene chromosomes. The arrowhead indicates the *white* gene locus (3B6)

bered segments is further divided into six roughly equal lettered segments, A to F, and the bands in each lettered segment are numbered. Therefore, each band has a unique address, and its position is easily discernible from the address. Moreover, the positions of genes may now be mapped on these addresses.

Various research tools have been developed to mark functional regions on polytene chromosomes. Anti-phosphorylated RNA polymerase II is used to mark the transcriptionally active domain of polytene chromosomes, and anti-heterochromatin protein 1 (HP-1) marks heterochromatic and heterochromatin-like regions on chromosomes (Kato et al. 2007, 2008). By immunostaining chromatin-binding proteins with specific antibodies in combination with various markers, it is possible to identify the protein of interest binding to the relevant functional region of chromosomes, such as euchromatic or heterochromatic and transcriptionally active or inactive regions.

1.3.3 Balancer Chromosomes

Balancer chromosomes are an extremely valuable tool in studies on *Drosophila*. They contain extensive inversions through the entire chromosome that prevent the recovery of chromosome exchange events, thereby isolating and maintaining the sequences in the balancer and balanced chromosome. They do not prevent crossing over but inhibit the recovery of exchanged chromatids (Kaufman 2017). Balancer chromosomes are used to stably maintain lethal and sterile mutations in the *Drosophila* stock without the selection process. Balancer chromosomes are also useful for effectively screening for mutations by maintaining the linear integrity of a mutagenized homologue (Kaufman 2017). These processes are very difficult to perform in other model organisms, such as the mouse without balancers.

Balancer chromosomes carry a recessive lethal mutation that is not related to the lesion being balanced and, thus, may efficiently balance lethal and sterile mutations. Balancer chromosomes also carry dominant visible mutations, and scientists may easily follow flies carrying the balancer in crossing schemes. Many balancer chromosomes also carry a set of recessive visible mutations that are useful for designing screens and distinguishing complex genotypes. Transgenic flies carrying a set of new and useful visible markers to the balancer have been developed as follows. Transgenes expressing visible markers, such as LacZ, GFP, or other fluorophores, in various spatial and temporal patterns have been inserted into different balancers as new and useful dominant markers. These transgenic flies may be used to easily distinguish the marked balancer flies from non-balancer flies at various developmental stages (Kaufman 2017). A list of balancers may be found at the BDSC site (http://flystocks.bio.indiana.edu/Browse/balancers/balancer_main.htm).

1.3.4 *Drosophila* Genome

The genome size of *D. melanogaster* is approximately 180 Mb, with 2/3 (120 Mb) representing the euchromatic region and 1/3 (60 Mb) the heterochromatic region. After the first report of the *D. melanogaster* genome by the consortium of the Berkley Drosophila Genome Project and Celera Genomics (Adams et al. 2000; Myers et al. 2000), the annotation of the genome has been revised several times by incorporating data from genome-wide RNA sequencing analyses and those on heterochromatin (modENCODE et al. 2010; Graveley et al. 2011; Boley et al. 2014; Brown et al. 2014; Chen et al. 2014; Kaufman 2017). Based on the current release, the total sequence length is 143,726,002 bp with a total gap length mainly in heterochromatin, including major and minor scaffolds of 1,152,978 bp (Kaufman 2017). The sequence is assembled into 1870 scaffolds with the majority of the sequence, 137.6 Mbp, residing on the seven chromosome arms (X, Y, 2L, 2R, 3L, 3R, and 4) and the entire mitochondrial genome. The sequence includes contiguous portions of the pericentric heterochromatin of X, 2, 3, and 4. Some may be mapped to the highly repetitive rRNA-encoding genes in the nucleolus organizer of X and Y (He et al. 2012).

Annotation of the genome currently identifies 17,726 genes, 13,907 of which are protein coding that encode 21,953 unique polypeptides. The remaining 3821 identified loci are genes encoding various types of noncoding RNA, 147 for rRNA, 313 for tRNA, 31 for snRNA, 288 for snoRNA, 256 for miRNA, 2470 for lncRNA, and 315 for pseudogenes (Kaufman 2017). The importance of many of these genes is now being recognized, particularly in relation to human diseases. Further details on noncoding RNA related to human diseases will be described in Chap. 8.

1.4 Strategies and Techniques to Study Human Diseases Using *Drosophila*

There are two main strategies to study human diseases using the *Drosophila* model: forward and reverse genetics.

1.4.1 Studies with Forward Genetics

In forward genetics, mutations are induced at random, and flies are screened for a phenotype of interest. Mutations may be generated by ethyl methanesulfonate (EMS) or the insertion of transposons, such as P-element and piggyback (Venken and Bellen 2014). Mutants may also be isolated by screen RNAi libraries or chromosome deficiency kits that cover 95% of the euchromatic region of the *Drosophila* genome (Ida et al. 2009; Cook et al. 2012). These strategies are useful for identifying uncharacterized mutations in already known disease-related genes as well as genes that have not yet been linked to disease. Therefore, this represents a useful strategy for identifying previously unknown genes and clarifying various biological events.

1.4.2 GAL4-UAS Targeted Expression System

A commonly used approach to express or knockdown specific genes in *Drosophila* is the so-called GAL4-UAS targeted expression system (Brand and Perrimon 1993). GAL4 is a yeast transcription factor that is used to control the spatial and temporal expression of target genes, which consequently directs gene activity at a specific developmental stage and specific cells and tissues. In one parental strain, promoter regions for a particular gene are designed to drive the expression of GAL4 in some tissues. In another strain, the GAL4-binding upstream-activating sequence (UAS) is placed in front of the transgene. When these two strains are genetically crossed, their progenies express the transgene in specific tissues driven by the GAL4-UAS system. In combination with RNA interference (RNAi), it is also possible to knockdown specific genes by expressing double-stranded RNAs targeted to specific mRNAs using the GAL4-UAS system. A useful resource for this purpose is the collection of UAS-RNAi responder strains of the Vienna Drosophila Stock Center (VDRC) (http://stockcenter.vdrc.at/control/main). These RNAi knockdown strains cover nearly 90% of all *Drosophila* protein-coding genes and are available to the research community from VDRC (Dietzl et al. 2007). The basic GAL4-UAS targeted expression system has been modified to further refine cell and tissue specificities as well as temporal expression specificities (Roman et al. 2001: McGuire et al. 2004).

1.4.3 Studies with Reverse Genetics

In reverse genetics, mutations are generated in *Drosophila* homologues of human genes to characterize their phenotypes in vivo. There are several approaches to knockdown or knockout genes in *Drosophila*. One strategy is targeted gene disruption using clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) (Beumer and Carroll 2014), transposon-mediated mutagenesis, and the excision of pre-existing transposable elements. The other is gene silencing with RNAi combined with the GAL4-UAS system or CRISPR (Mohr 2014). In addition to these loss-of-function studies, a wild-type or mutant form of a human disease-causing gene

may be introduced and overexpressed in *Drosophila* in order to examine its effects in specific tissues and organs (Feany and Bender 2000).

Drosophila is also useful for studying the pathogenesis of rare variants that are linked to human diseases (Ugur et al. 2016). There are US-based initiatives to identify human disease-causing genes by deep sequencing of the whole exomes or genomes of patients and their families, which are coordinated by the Centers for Mendelian Genomics (http://www.mendelian.org/) and Undiagnosed Diseases Network (UDN; http://undisgnosed.hms.harvard.edu/). Similar strategies have been performed in other countries, such as the UK (http://www.uk10k.org/) and China (Guangzhou Drosophila Resource Center and the Center for Genomic Sciences in the University of Hong Kong). These strategies are sometimes not sufficient for identifying the disease-causing gene if only a few individuals are assessed. In these cases, the *Drosophila* orthologue may be knocked out or down in order to examine phenotypes. If the observed phenotype is rescued by the expression of wild-type UAS-human-cDNA, but not by its human variant, disease causality may be confirmed (Bellen and Yamamoto 2015; Wangler et al. 2015).

1.5 Advantages of *Drosophila* in the Study of Human Diseases

Several model organisms are intensively studied in life sciences, such as the mouse, zebrafish, *Xenopus*, *Arabidopsis*, *Drosophila*, *Caenorhabditis elegans*, yeast, and *E. coli*. These are specific species that are extensively examined in research laboratories. Studies using these model organisms will advance our understanding of cellular functions, development, and human diseases. The knowledge obtained from these model organisms may also be applied to other organisms, which will result in the generalization of findings.

The following characteristics of *Drosophila* demonstrate that it is a good model organism. It is small, easy to handle, and inexpensive to maintain and manipulate in the laboratory. *Drosophila* has a short life span and produces a large number of offspring, which facilitates statistical analyses of the data obtained. *Drosophila* development is external, and, thus, it is very easy to follow using various microscopes. Many mutants and transgenic fly lines may be obtained from stock centers such as the Bloomington Drosophila Stock Center (https://bdsc.indiana.edu/), Kyoto Stock Center (http://www.dgrc.kit.ac.jp/), and VDRC. A plethora of information from previous experiments and discoveries is available. Sequencing of the genome is nearly complete, as described above. Homologues for nearly 75% of human disease-related genes have been identified (Pandy and Nichols 2011; Yamamoto et al. 2014). *Drosophila* shows complex behaviors including social activity. In addition, there are fewer ethical concerns because the insect is outside animal laws in many countries. In combination with genome-wide genetic screening, genome-wide analyses with deep sequencers, such as RNA-seq and ChIP-seq, and metabolomics analyses, *Drosophila* is now commonly used as a model to study human diseases with the aim of identifying novel biomarkers or therapeutic targets for human diseases together with the screening of candidate substances for their treatment (Pandy and Nichols 2011). *Drosophila* is now used in the study of various human diseases related to the central and peripheral nervous systems such as neurodegeneration, Alzheimer's disease (Chap. 3), Parkinson's disease (Chap. 4), triplet repeat expansion disease (Chap. 5), sleep disorders (Pandy and Nichols 2011), seizure disorders (Pandy and Nichols 2011), cognitive and psychosis disorders (Pandy and Nichols 2011), amyotrophic lateral sclerosis (Chap. 6), and Charcot-Marie-Tooth disease (Chap. 7). It is also used as a cancer model including tumor formation and metastasis (Chaps. 10 and 11). *Drosophila* may also be employed in the study of cardiovascular diseases (Pandy and Nichols 2011; Ugur et al. 2016). Although the fly heart has only one cardiac chamber, it may still be used to study some steps of heart development and its

defects. *Drosophila* also provides a model for inflammation/infectious diseases, metabolic disorders, and diabetes (Chaps. 13 and 14).

There are some limitations to *Drosophila* models. *Drosophila* does not possess hemoglobin (Adams et al. 2000; Myers et al. 2000), and, thus, *Drosophila* models cannot be generated for human diseases related to hemoglobin. Smaller organisms, such as yeast and *E. coli*, which have shorter generation times, smaller genomes, and produce more offspring than *Drosophila*, are preferred for the study of cell autonomous functions, such as DNA replication and repair. Therefore, an ideal study of human diseases will be a parallel analysis with relevant models. For example, cell autonomous effects will be studied in yeast, while multicellular or inductive events mediated by genes will be examined using *Drosophila.* A more accurate disease model needs to be established in the mouse. In any case, the benefits of *Drosophil*a may be summarized as follows. A number of genes related to human diseases have already been discovered and various useful techniques developed. Powerful tools for studying developmental/neurological disorders and cancer are now available. Therefore, *Drosophila* is a very effective model with more simplicity than mammalian models and greater complexity than yeast and bacterial models.

1.6 Commonly Used Websites for *Drosophila* Studies

Commonly used online databases for *Drosophila* studies are now available to support experimental design, the identification of relevant fly stocks, research tools, reagents such as antibodies, and related human diseases (Table 1.1). These databases are particularly useful for beginners. More

Table 1.1 A list of websites providing information about *Drosophila* or human diseases

Website	URL
FlyBase	http://flybase.org
modENCODE	http://modencode.sciencemag.org/drosophila/introduction
Drosophila Genomics Resource Center	https://dgrc.bio.indiana.edu/Home
Berkeley Drosophila Genome Project	http://www.fruitfly.org/
Drosophila Genomics and Genetic Resources	http://www.dgrc.kit.ac.jp/
Bloomington Drosophila Stock Center	https://bdsc.indiana.edu/
Vienna Drosophila Resource Center	http://stockcenter.vdrc.at/control/main
NIG-FLY	https://shigen.nig.ac.jp/fly/nigfly/
The Exelixis Collection at Harvard Medical School	https://drosophila.med.harvard.edu/
DRSC/TRiP Functional Genomics Resources	https://fgr.hms.harvard.edu/fly-in-vivo-rnai
FlyORF	http://flyorf.ch/index.php/orf-collection
FlyExpress 7	http://www.flyexpress.net/
FlyBook	http://www.genetics.org/content/flybook
FlyMove	http://flymove.uni-muenster.de/
Fly-FISH	http://fly-fish.ccbr.utoronto.ca/
Flygut	http://flygut.epfl.ch/
FlyMine	http://www.flymine.org/
Gene Disruption Project	http://flypush.imgen.bcm.tmc.edu/pscreen/index.php
The Interactive Fly	http://www.sdbonline.org/sites/fly/aimain/1aahome.htm
Textpresso for Fly	http://www.textpresso.org/fly/
BruinFly	http://www.bruinfly.ucla.edu/index.php
Virtual Fly Brain	https://www.virtualflybrain.org/site/vfb_site/home.htm
Fruit Fly Brain Observatory	http://fruitflybrain.org/
DroID – The Drosophila Interactions Database	http://flygut.epfl.ch/
J-FLY	http://jfly.iam.u-tokyo.ac.jp/index.html
Neuromuscular Disease Center	http://neuromuscular.wustl.edu/

specialized bioinformatics resources for *Drosophila* scientists are described in Chap. 15.

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Adult Intestine Aging Model

2

Koji Takeda, Takashi Okumura, Kiichiro Taniguchi, and Takashi Adachi-Yamada

Abstract

The *Drosophila* adult has an intestine composed of a series of differentiated cells and tissue stem cells, all of which are similar to the mammalian intestinal cells. The aged adult intestine shows apparent characteristics such as multilayering of absorptive cells, misexpression of cell type-specific genes, and hyperproliferation of stem cells. Recent studies have revealed various gene networks responsible for progression of these aged phenotypes. The molecular mechanism for senescence of the *Drosophila* adult midgut and its relation with the corresponding mechanism in mammals are overviewed. In addition, a basic method for observing aged phenotypes of the midgut is described.

Keywords

Drosophila · Midgut · ISCs · Senescence · Dl · JNK · Upd · Integrin · AstA · Dh31

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2.1 Maintenance of *Drosophila* Adult Midgut

The abdomen of a wild-type adult *Drosophila melanogaster*, which has a length of 1 mm, has a folded gastrointestinal tract with a length of approximately 8–9 mm. It is divided to three parts, namely, foregut, midgut, and hindgut, which morphologically and functionally correspond to the mammalian esophagus, small intestine, and large intestine, respectively (Fig. 2.1A). The main functions of the midgut are food digestion and nutrient absorption as well as defense and immune responses to microbes, all of which are similar to those of the mammalian small intestine. The midgut is a regenerative organ with multipotent tissue stem cells in its monolayer epithelium, which also resembles that of the mammalian small intestine.

The intestinal stem cells (ISCs) in the *Drosophila* adult midgut were first described in 2006 (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006). They show a scattered distribution among polyploid enterocytes (ECs) for nutrient absorption, and they are juxtaposed to the progenitor cell enteroblast (EB) at high frequency (Fig. 2.1B). In most cases, one of the ISC daughter cells differentiates to the EB, while the other daughter cell becomes an ISC again. The diploid EB grows to the EC via two or more cycles of endoreplication without cell division. Furthermore, enteroendocrine cells (EEs), which produce various peptide hormones, often arise

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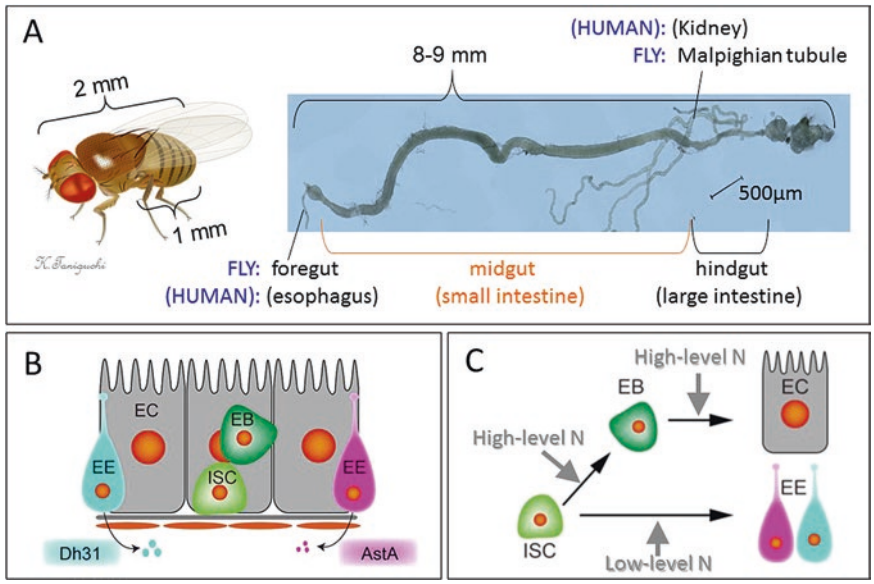

Fig. 2.1 Schematic representation of (**A**) whole intestine of adult *Drosophila*, (**B**) structure of midgut epithelium, and (**C**) cellular lineage of midgut
(**A**) The intestine (folded in the abdomen) of an adult fly is composed of the three parts: foregut, midgut, and hindgut, which are analogous to the human esophagus, small intestine, and large intestine, respectively. The two Malpighian tubules branched from the joint between the midgut and hindgut are analogous to the human kidney
(**B**) The monolayer epithelium of the midgut contains four types of cells: ISCs (yellow-green), EBs (green), ECs (gray), and EEs (described in text in detail). EEs are further classified to the two subtypes: class I (producing AstA, magenta) and class II (producing Dh31 and Tachykinin, cyan). The gray belt beneath the ECs represents the basement membrane. Brown ellipses beneath the basement membrane represent visceral muscles surrounding the midgut epithelium
(**C**) When the ISCs are divided to two daughter cells, one becomes an ISC again, but the other becomes an EB in most cases or an EE in minor cases. The EBs grow into the ECs. Differentiation from ISCs to EB/EC requires high levels of N, while that to EE requires low levels of it

directly from the daughter cells of ISCs (Fig. 2.1B). EEs are classified as two subtypes, namely, class I and class II, according to the hormones they produce.

The main signaling molecule required for differentiation from ISC to EB/EC is Notch (N), which is the transmembrane protein for juxtacrine signaling by the ligand Delta (Dl) expressed in the ISCs. When Notch is activated in EBs, they terminate cytokinesis and grow to mature ECs. In contrast, when Notch is inactivated in ISCs, the cells continue to proliferate, and ISCs are maintained. Artificial and continuous inactivation of the N signal in ISCs generates ISC tumors in most cases and EE tumors in some cases (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006). In both cases, accordingly, cells also lose their scattered distribution and contact together with E-cadherin. Therefore, one of the Notch functions other than the trigger for EB/EC differentiation is considered to be degradation of E-cadherin in the contact plain between the ISC and EB (Maeda et al. 2008). In development of normal EEs, low-level activation of N signal is observed (Perdigoto et al. 2011). Subtype differentiation of EEs is also regulated by the N signal (Beehler-Evans and Micchelli 2015). Although the effect of N activation on proliferation of the progenitor cells is converse in the cases of *Drosophila* and mammals, the fact that N inactivation is required for differentiating EEs is common to both. Maintenance of the ISCs and differentiation to all of the other types of cells require a kind of master gene for encoding the transcription factor GATAe (Buchon et al. 2013; Okumura et al. 2016).

2.2 Molecular Mechanism of Midgut Senescence

Senescence phenotypes of the *Drosophila* midgut were first documented in 2008 (Choi et al. 2008). It was reported that ISCs proliferate at higher rate, and with more-frequent Dl expression, in the midgut 30 days after eclosion. Similar increase of cells was found also in the EB and EE, while frequency of EC was decreased. Later analyses clarified that the more-frequent Dl expression is not due to simple proliferation of ISCs; instead, it was considered to be caused by ectopic expression of Dl in EC-like polyploid cells (Biteau et al. 2008). That ectopic gene expression in EC-like polyploid cells was not only observed for Dl but also for an EB marker, *Suppressor of hairless* (*Su(H)*), and an ISC/EB marker, *escargot* (*esg*) (Choi et al. 2008). All of these phenotypes became more severe with aging and were accelerated by Paraquat treatment, indicating that they are the reactive oxygen species (ROS)-dependent senescence phenotypes.

One important factor responsible for initiating and propagating these senescence phenotypes is c-Jun N-terminal kinase (JNK) (Biteau et al. 2008). JNK activity is elevated with aging throughout the midgut. When JNK is inactivated in the aged midgut, overproliferation phenotype of ISCs is suppressed. However, JNK might be a modulator to express exaggerated phenotypes by other cellular signal(s) promoting ISC proliferation because JNK is reported to act as a factor in manifesting all-or-none responses (Bagowski et al. 2003). The extracellular diffusible signaling factors responsible for propagating senescence phenotypes are considered to be PDGF- and VEGF-related factor 2 (PVF2), Leptin/IL-6-like ligands named "Unpaired" (Upds), and epidermal growth factor family members (EGFs). PVF2 is normally expressed in ISCs and EBs. Overexpression of PVF2 and its receptor PVR in ISCs and EBs induced premature senescence phenotypes, while inactivation of PVF2 blocked appearance of the senescence phenotypes (Choi et al. 2008).

Upds in the midgut were identified as factors required for regenerative ISC division and proper differentiation in response to EC apoptosis, enteric infection, and JNK activation in ECs (Jiang et al. 2009; Buchon et al. 2009). In ECs under these conditions, expression of Upd3 is the highest among all of the three Upd paralogs (Upd, Upd2, and Upd3), although all of their expression are induced. Similar responses are found in normally aged midgut (Li et al. 2016). Upds are required for age-dependent elevation of ISC proliferation rate and non-cell autonomous propagation of stress signaling to outer cells through downstream JAK/STAT signaling.

Three family members of the *Drosophila* EGF, namely, Vein (Vn), Spitz (Spi), and Keren (Krn), also act in a similar process for ISC overproliferation (Jiang and Edgar 2009; Jiang et al. 2011). Among three ligands, Vn shows the strongest induction level. Like Upds, they are expressed in response to EC apoptosis, enteric infection, and JNK activation in ECs. However, unlike Upds, their expression is not only observed in ECs but also in the visceral muscles (VM) surrounding the midgut epithelium. When the EGF receptor was knocked down, normal and regenerative ISC growth was completely blocked (Jiang et al. 2011). Similar responses are found also in normally aged midgut. These EGFs are required for age-dependent elevation of ISC proliferation rate and non-cell autonomous propagation of stress signaling to outer cells through downstream Ras/MAPK signaling.

Canonical Wnts, which are matricrine extracellular ligands, are known to act as a trigger for cell proliferation and differentiation in a wide variety of organs and organisms. The mammalian homologs Wnt3, 6, and 9B play a pivotal role in proliferation of intestinal stem cells, and *adenomatous polyposis coli* (*APC*), namely, a gene for repressing the Wnt signal, is the most representative tumor suppressor gene for colon cancer. Although Wingless (Wg) in *Drosophila* is the ortholog of canonical Wnts in mammals, its normal expression in VMs surrounding the midgut epithelium does not act as a stronger mitogen for normal ISC proliferation than expected from the above mammalian knowledge (Lin et al. 2008; Xu et al. 2011) (Fang et al. 2016). However, in the case of regenerative ISC proliferation, Wg

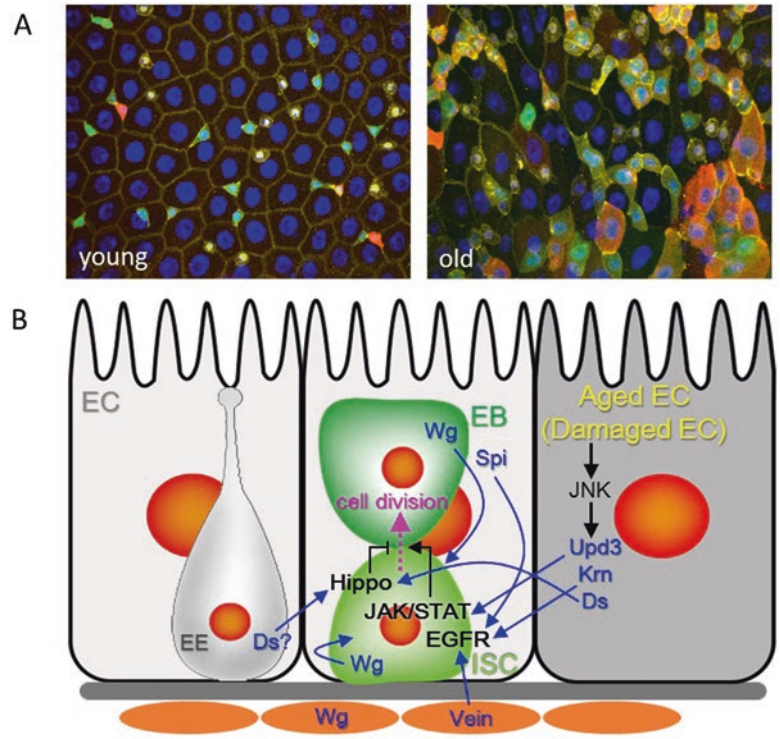

Fig. 2.2 Molecular mechanisms for initiating and propagating senescence phenotypes in adult posterior midgut (**A**) Confocal images of representative midgut epithelia taken from young (left) and old (right) adults. Various cell types show a well-organized array and specific gene expression in the young adult, while they show ambiguous cell differentiation through ectopic gene expression in the old adult. Color: *esg* expression (green, ISCs and EBs), *Su(H)* expression (red, EBs), *prospero* expression (white, EEs), Armadillo (yellow, plasma membrane), and DAPI (blue, nuclei)
(**B**) Various kinds cellular of signaling for senescence phenotypes. Extracellular signaling ligands and the direction of their actions are expressed in blue. In the aged or damaged ECs, activity of JNK is elevated, which overproduces secretory ligands Upds for the JAK/STAT pathway and Krn for the EGFR pathway. At the same time, the circular muscle cells overexpress another EGFR ligand Vein (Vn), and the ISCs and EBs overexpress Wg and the third ligand Spi for the EGFR pathway. The transmembrane ligand Ds, namely, a hippo signaling trigger, is normally expressed in the ECs and EEs. All of these extracellular ligands are considered to cooperatively activate proliferation of ISCs by increasing Dl expression in ISCs

expression in ISC and EB is elevated to contribute as a trigger of proliferation (Cordero et al. 2012) (Fig. 2.2). Mathematical models suggested that Wg expression in ISCs/EBs/EEs or VMs induces a similar effect on ISC proliferation and differentiation (Kuwamura et al. 2010, 2012).

Another mitogenic signal in the aged midgut is an anti-Hippo signal. Hippo is a cytoplasmic serine/threonine kinase, and its activation represses cell proliferation and induces apoptosis. When Hippo is inactivated, cells show overproliferation in various organs including the midgut. At that time, one of the Hippo-repressible target genes, i.e., *expanded* (*ex*), is known to be expressed. In the aged midgut or a midgut with bacterial infection or Paraquat treatment, expression of *ex* is widely and probably non-cell autonomously induced, indicating that the Hippo activity is repressed under these conditions. This Hippo inactivation elicits regenerative proliferation of ISCs through induction of Upds (Shaw et al. 2010; Karpowicz et al. 2010). The extracellular ligands involved in this Hippo inactivation were not elucidated. However, lack of Dachsous (Ds), namely, an atypical cadherin family transmembrane protein (which is a Hippo signaling

extracellular ligand acting in normal appendage development), is a predictable way to inactivate Hippo signal in this case. The site of Ds expression in the normal midgut is reported to be ECs and EEs (Karpowicz et al. 2010).

2.3 Regulation of Midgut Senescence by Integrin

A membrane protein superfamily, called Integrin, of which alpha and beta subunits form a heterodimer, is localized to the basal side of the epithelial cells. In vertebrates, it accumulates in the focal adhesion area or exists as a component of hemidesmosome, both of which are structures found in the basal side of the epithelial cells. However, in invertebrates, including *Drosophila*, the hemidesmosome is not known to be present. In any cases, Integrin is known to be responsible for firm attachment of epithelial cells to the basement membrane. Therefore, geriatric diseases such as bullous pemphigoid (in which Integrin in the skin does not function properly) show a detachment of the epidermis from the basement membrane and dermis to form blisters on the skin.

In regard to *Drosophila*, five genes for the alpha subunit and two genes for the beta subunit have been documented. One of the beta subunits, namely, Myospheroid (Mys), is widely expressed in most of the epithelial tissues and shows severe phenotypes in its mutants. For example, in the adult midgut, *mys* does not only anchor ISCs to the basement membrane but also maintains and proliferates ISCs probably due to its requirement for niche formation (Goulas et al. 2012; Lin et al. 2013; Patel et al. 2015). Thus, the mutants do not easily show the epithelial detachment phenotype that is covered by the proliferation inhibitory phenotype found in ISCs. In contrast, another beta subunit βν-integrin shows a midgut-specific expression pattern throughout the development of *Drosophila*, and its null mutant is viable and fertile. When the midgut of this null mutant is continuously observed after eclosion, premature senescence phenotypes, such as ectopic expression of ISC marker Dl, increase of mitotic index, and multilayering of ECs, were clearly found. All of these phenotypes are considered to be caused by mild detachment of ECs from the basement membrane. Thus, the two distinct beta subunits encoded by the *Drosophila* genome show contrasting influences on proliferation of ISCs, and that fact explains the evolutionary conservation of both types of beta subunits among various *Drosophila* species (Okumura et al. 2014).

These senescence phenotypes were strengthened and propagated with aging through a positive feedback loop between JNK activation and Upd expression (Okumura et al. 2014) (Fig. 2.3). The mechanism of that aging process seems to be common to normal bacterial infection-induced senescence progression (Jiang et al. 2009; Buchon et al. 2009; Li et al. 2016). Therefore, lacking the cell-to-substrate contact could be a primary cause for senescence phenotypes in epithelial tissues of various organs, such as the skin and intestine.

2.4 Regulation of Longevity and Organ Senescence by Midgut Hormones

One of the interesting themes regarding senescence mechanism to be explored is the recently discovered hormonal regulation of longevity and organ senescence by intestinal hormones (Takeda et al. 2018). It has long been known that several kinds of peptide hormones are commonly produced in the brain and intestine in both vertebrates and invertebrates (Fujita et al. 2012; Endo et al. 1990; Bloom and Polak 1980; De Loof and Schoofs 1990). However, definitive functions of the gut-producing fraction of these brain-gut hormones have not been clarified because it is not possible to distinguish whether the functional molecule circulated in hemolymph is derived from the brain or gut.

It was reported that in the posterior midgut (PMG) of a *Drosophila* adult, the two subtypes of enteroendocrine cells exist by producing peptide hormones. Class I EEs produce allatostatin A (AstA), and class II EEs produce a calcitonin-like peptide, namely, diuretic hormone 31 (Dh31) and tachykinin (Beehler-Evans and Micchelli 2015).

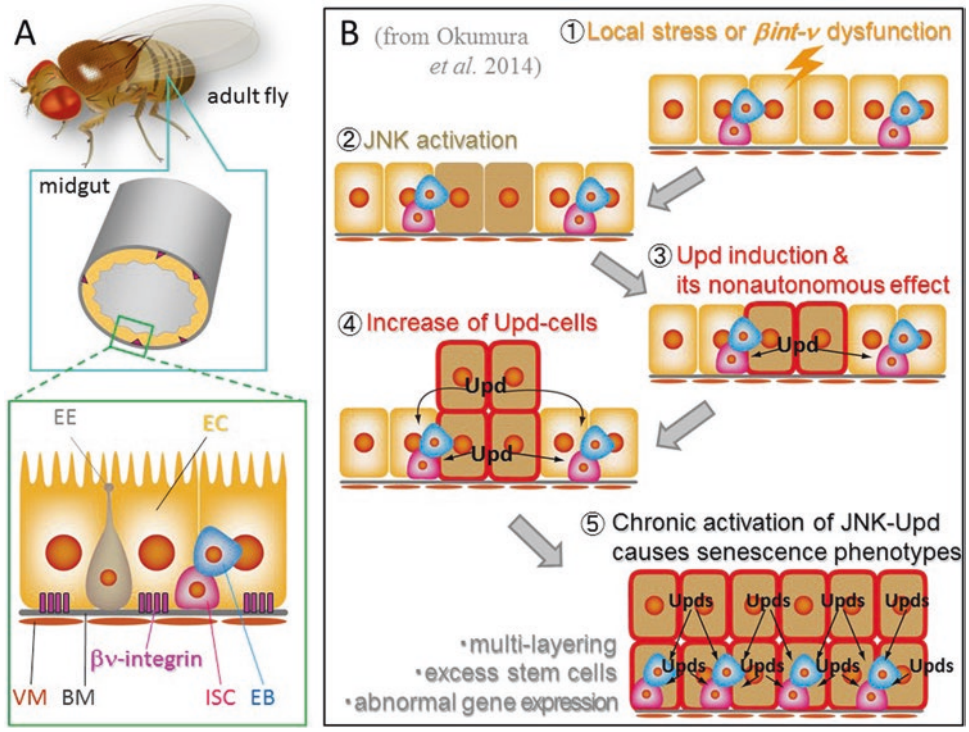

Fig. 2.3 Mechanisms for premature senescence of midgut by loss of βν-integrin. (Reproduced from Okumura et al. 2014)
(**A**) βν-integrin (magenta) is localized to the basement membrane of the adult midgut epithelium. The gray belt beneath the ECs represents the basement membrane (BM). Brown ellipses beneath the basement membrane represent visceral muscles (VM) surrounding the midgut epithelium.
(**B**) Mechanism for propagation of senescence phenotypes caused by impaired βν-integrin function. ① Local stress or dysfunction of βν-integrin causes a slight damage to the epithelium. ② JNK is activated in the damaged cells. ③ Expression of *upd* genes is induced in response to JNK activation, and secreted Upd stimulates surrounding ISCs. ④ JNK-Upd-active ECs are proliferated and stratified. ⑤ JNK-Upd circuits are propagated with abnormally ectopic gene expressions

Although these two subtypes of EEs are reported to be generated alternatively from ISCs, the frequencies of each subtype are not always 50%, except at the middle part of the PMG. Actually, the frequency of AstA cells is highest at the posterior end of the PMG and decreases gradually toward the anterior region. Conversely, the frequency of Dh31 cells is highest at the anterior end and decreases gradually toward the posterior region. Thus, the densities of both EE subtypes create a double gradient with opposite direction.

This remarkable distribution of EEs mentioned above led us to predict that the hormones produced by each EE subtype elicit contrasting responses in various physiological phenotypes (Takeda et al. 2018). During studies in this vision, an obviously contrasting effect of AstA/Dh31on adult longevities was discovered. Gut-preferential knockdown of AstA led to shortening of longevity, while that of Dh31 led to extension of longevity by 30% in both cases compared with a control. Consistently, these changes in longevity simultaneously correlate with the above-described gut senescence phenotypes such as ectopic expression of the ISC marker Dl and increase of mitotic index. That is, AstA knockdown induced premature senescence phenotypes, while Dh31 knockdown induced delayed senescence phenotypes (Figs. 2.4 and 2.5). However, since knockdown of receptors of AstA/Dh31 has no effect on adult-longevity regulation, gut senescence is not thought to affect adult longevity.

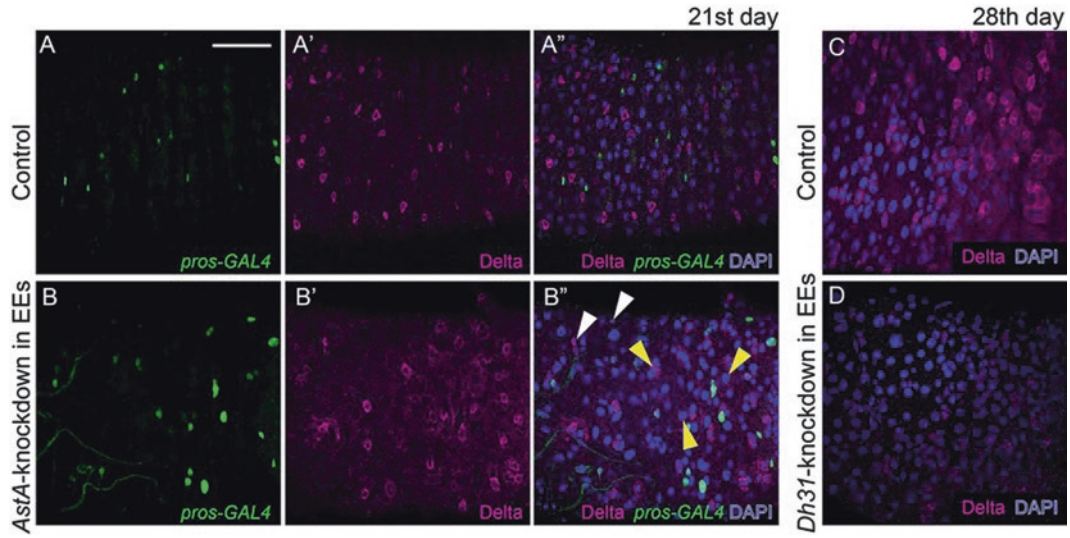

Fig. 2.4 Midgut-preferential RNAi of AstA or Dh31 induces premature or delayed senescence phenotypes, respectively. (Reproduced from Takeda et al. 2018) (**A**-**A**″) Control midgut on 21st day after eclosion. (**B**-**B**″) Midgut of *AstA* knockdown in EEs on 21st day after eclosion. Premature senescence phenotypes can be observed. White arrowheads denote normal Dl expression in ISCs. Yellow arrowheads denote abnormally ectopic expression of Dl in polyploid EC-like cells. (**C**) Control midgut on 28th day after eclosion. Naturally occurring senescence can be observed. (**D**) Midgut of *Dh31* knockdown EEs on 28th day after eclosion. Appearance of senescence phenotypes is delayed. Color: *pros-GAL4* expression in EEs (green), Dl protein (magenta), DAPI (blue, nuclei)

Together with these results, it appears that the target of longevity regulation by AstA/Dh31 is not the gut but multiple organs (including the gut) that collectively affect longevity. However, senescence of the male accessory gland, an internal reproductive organ, inversely correlates with that of the gut and longevity regulation when Dh31 is knocked down. This inverse correlation suggests that senescence progression by Dh31 action in various organs is not mediated by hemolymph-circulating factors such as insulin (Takeda et al. 2018). Thus, the relationship between longevity regulation and organ senescence is complicated, and further studies are needed to reveal its underlying mechanism.

The target cell types involved in regulation of gut senescence by AstA/Dh31 were elucidated through cell type-specific knockdown of their receptors. Consequently, the target cell is EB in the case of AstA, while it is EB, ISC, and EC in the case of Dh31. These target cell types display a slight difference from the case of accelerated senescence phenotypes shown after bacterial infection. In the case of bacterial infection, senescence response starts from injured ECs that produce and release Upds as diffusible ligands for further propagation of senescence (Jiang et al. 2009). However, in the case of AstA/Dh31 action, the target cells are more naïve or undifferentiated cells (ISC/EBs) described above. Furthermore, in the case of bacterial infection, the diffusible Upd ligands are Upd, Upd2, and Upd3 (Buchon et al. 2009), while in the case of knockdown of AstA knockdown, it is only Upd3. On the other hand, other factors contributing to senescence propagation, such as JNK and anti-Hippo signaling marker gene *ex*, are commonly used. Therefore, molecular mechanisms for initiating and expanding senescence are somewhat common and specific in various aspects in the two cases.

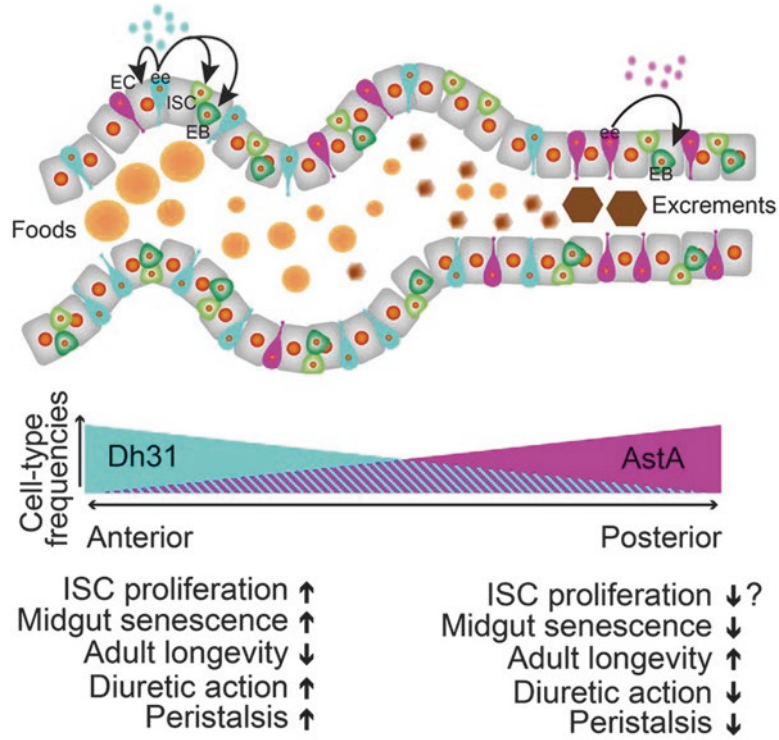

Fig. 2.5 Schematic representation of posterior midgut epithelium with gradients and paracrine actions of two peptide hormones, AstA and Dh31. (Reproduced from Takeda et al. 2018)
In the adult posterior midgut, two subtypes of EEs distribute with complementary gradients, in which AstA-producing cells (magenta) and Dh31-producing cells (cyan) scatter with posterior and anterior peaks, respectively. Under normal feeding conditions, in accordance with stimulation by intestinal contents, AstA acts on EBs for appropriate ISC proliferation rate simultaneously with inactivation of diuretic action and peristalsis. In contrast, Dh31 acts on ISC, EB, and EC for increasing ISC proliferation rate simultaneously with activation of diuretic action and peristalsis. These hormonal actions may also affect adult longevity and difference in senescence progression between organs

2.5 Concluding Remarks

The *Drosophila* midgut shows apparent senescence phenotypes, which are considered to be an excellent model for studying the mechanism for progression of organ senescence. It is interesting that overproduction of stem cells and various differentiated cells as a senescence phenotype uses multiple intercellular signaling pathways. That fact might mean that nonautonomous synchronization in senescence levels is important for intestinal functions such as barrier and immune responses to microbe infection. Similar senescence phenotypes in overproduction of specific cell types can also be found in Paneth cells and goblet cells in the mammalian small intestine (Nalapareddy et al. 2017). Elucidating the molecular mechanisms in the *Drosophila* midgut may be a precedent for understanding these common senescence mechanisms.

Another interesting observation in this field is the similarity between cellular response to aging and that to bacterial infection and massive apoptosis. This similarity leads us to predict that frequencies in pathological bacterial infection and apoptosis are increased under an aged condition of individuals. In fact, impairment of cell-substrate contact by Integrin dysfunction can be understood as damage to the barrier against infection, suggesting that the resultant premature senescence shows phenotypes related to bacterial infection. Under an aged condition of individu-

als, increase of epithelial permeability and expression of a human Upd, i.e., homolog IL-6 (Man et al. 2015), in addition to change of microbiota (Mitsuoka 1996) were also observed in the human intestine.

On the other hand, the requirement of JNK activation for progression of the midgut senescence phenotype is difficult to understand in view of the fact that JNK activation elongates adult life span (Wang et al. 2003, 2005). Inactivation of JNK delays senescence progression of the midgut but reduces longevity of individuals. Thus, it seems that the same signaling molecule JNK plays a role in both increasing and decreasing life span. The ultimate factor to resolve this discrepancy is awaited. In addition, recently found roles of some hormones produced in the midgut for its senescence regulation and unexpected longevity control are interesting issues to be resolved. Relation between organ senescence and longevity control will be one of the foci in the near future. Furthermore, a new finding in regard to inverse response in senescence progression between the midgut and accessory gland is intriguing in its physiological significance.

2.6 Commonly Used Protocol

Methods for preparing confocal images of senescence phenotypes in the adult midgut are described below.

Equipment

PYREX® 9 Depression Glass Spot Plates (#722085 Corning, #13748B Fisher)
Sharp and fine forceps (e.g., #5 Dumont)
Micro-spring scissors (e.g., #15002–08 FST)
Dissection stereomicroscope
Microscope slides and 22/22 mm coverslips
Micropipettes and blue/yellow tips
Microcentrifuge tubes (e.g., #131-815C WATSON)

Solution and Reagents

PBS (phosphate-buffered saline) (e.g., #T900 Takara-Bio)
Fixative: 4% formaldehyde in PBS (1:3 dilution of commercial 16% methanol-free stock solution)
Washing solution: PBT (PBS with 0.1% (v/v) Triton X-100)
Primary antibody: Mouse anti-Dl 1:200 dilution (#C594.9B DSHB)
Secondary antibodies: e.g., goat anti-Mouse IgG Alexa Fluor 555 probes 1: 200 dilution (#A21424 Invitrogen), Cy3 AffiniPure Goat Anti-Chicken IgY (IgG) probes 1:200 dilution (#103–165-155 Jackson Immuno Research)
Mountant: 80% nonfluorescent glycerol
Fast-dry transparent nail polish (without fluorescence)

Useful *Drosophila* Strains

esg-GAL4 on 2nd: e.g., *P{w[+mW.hs] = GawB} NP6267* (#113889 Kyoto Stock Center)
UAS-GFP on 2nd: e.g., *P{UAS-GFP.S65 T} Myo31DF^{T2}* (#106363 Kyoto Stock Center)
UAS-GFP on 3rd: e.g., *P{UAS-GFP.S65 T}eg^{T10}* (#106364 Kyoto Stock Center)
Expression of *UAS-GFP* driven by *esg-GAL4* is found specifically in ISCs and EBs in young adult midgut, while it expands ectopically to the polyploid EC-like cells in old fly midgut (green in Fig. 2.2).
pros-GAL4 on 3rd: e.g., *prospero^{V1}-GAL4* (Balakireva et al. 1998)
Expression of *UAS-GFP* driven by *pros-GAL4* is found specifically in EEs (green in Fig. 2.4).
Su(H)-GAL4 on X: e.g., *Su(H) + GBE-GAL4* (Zeng et al. 2010)
Expression of *UAS-GFP* driven by *Su(H) + GBE-GAL4* is found specifically in EBs (green in Fig. 2.2).
puc-lacZ on 3rd: e.g., *P{ry[+t7.2] = A92} puc[E69]* (#109029 Kyoto Stock Center)
JNK activity is a good indicator of senescence levels in the adult midgut, which can be monitored by expression of JNK inactivator kinase Puckered (Puc) (Martin-Blanco et al. 1998; Adachi-Yamada et al. 1999a).
UAS-dominant negative JNK on X: e.g., *P{UAS-bsk.DN}2* (#6407 BDSC)

Senescence progression can be blocked by JNK inactivation with forced expression of dominant-negative form of JNK (Adachi-Yamada et al. 1999b).

upd3-Redstinger: This strain can be used to trace *upd3* expression in the aged midgut through fluorescence of Redstinger, a modified version of red fluorescent protein (RFP) produced by coral *Discosoma* (Takeda et al. 2018). This strain will be delivered by Kyoto Stock Center in the near future.

Dissection of Adult Midgut

See Fig. 2.6 illustrating methods for dissecting male body (A–G) and female body (H–J). It is reported that the senescence mechanisms of the midgut of male and females slightly differ (Regan et al. 2013).

(A) Remove the wings, legs, and head from the body of an aged *Drosophila* adult.
(B) Put the ventral side up and insert the blade of micro-spring scissors to the cut end between the head and thorax.
(C) Cut the ventral epidermis along the midline (magenta broken line) until immediately in front of the external genitalium.
(D) Soak the cut body in PBS in a holed glass plate, and open the epidermis from the midline cut ends by two forceps.
(E) Pinch the epidermis and external genitalium by each forceps.
(F) Isolate the internal organs by pulling away of epidermis and external genitalium.
(G) Remove the internal reproductive organ complex composed of testes, seminal vesicles, accessory glands, and ejaculatory duct. Isolate the midgut by removing the hindgut, Malpighian tubules, and external genitalium.
(H) In the case of female body, see text of C.
(I) See text of F.
(J) Remove the internal reproductive organ complex composed of ovaries, oviduct, spermathecae, bursa copulatrix, and external genitalium. After that, isolate the midgut as described in G.

Fixation and Staining

1. Put the midgut in a new microcentrifuge tube by forceps.
2. Add 200 μl of fixative and mix by tapping gently but firmly (about 30 times). Note that this is the most important step for good staining.
3. Leave the tube to stand for 30 min, but mix it sometimes by gentle tapping as above.
4. Remove as much of the fixative as possible and add 200 μl of PBT followed by gentle tapping as above. To avoid histological damage, be careful not to suck the midgut into the yellow tip. For this reason, resuspend the sunk midgut by pipetting, and gently push the yellow tip to the bottom of microcentrifuge tube. Then, suck up the solution through the narrow gap between the tube bottom and yellow tip.
5. Immediately remove the PBT and add 200 μl of PBT again followed by gentle tapping. Repeat this step twice.
6. Leave the tube to stand for 10 min to diffuse residual fixative.
7. Similarly exchange PBT and leave the tube to stand for 10 min again.
8. Remove as much PBT as possible, and add 200 μl of primary antibody solution followed by gentle tapping as above.
9. Leave the tube to stand for 1 h at 37 ° C or overnight at 4 ° C. The experiment can be paused at this step at 4 ° C if the period is less than 1 week.
10. Remove as much of the primary antibody solution as possible, and add 200 μl of PBT followed by gentle tapping as above.
11. Rinse the midgut as explained in steps 5–7.
12. Remove as much PBT as possible, and add 200 μl of appropriate secondary antibody solution followed by gentle tapping as above. At the same time, various kinds of counter staining can be optionally carried out by mixing with fluorescent dyes such as DAPI (nuclei staining) and fluorescent phalloidin (F-actin staining).
13. Leave the tube to stand for 1 h at room temperature or overnight at 4 ° C. The experi-

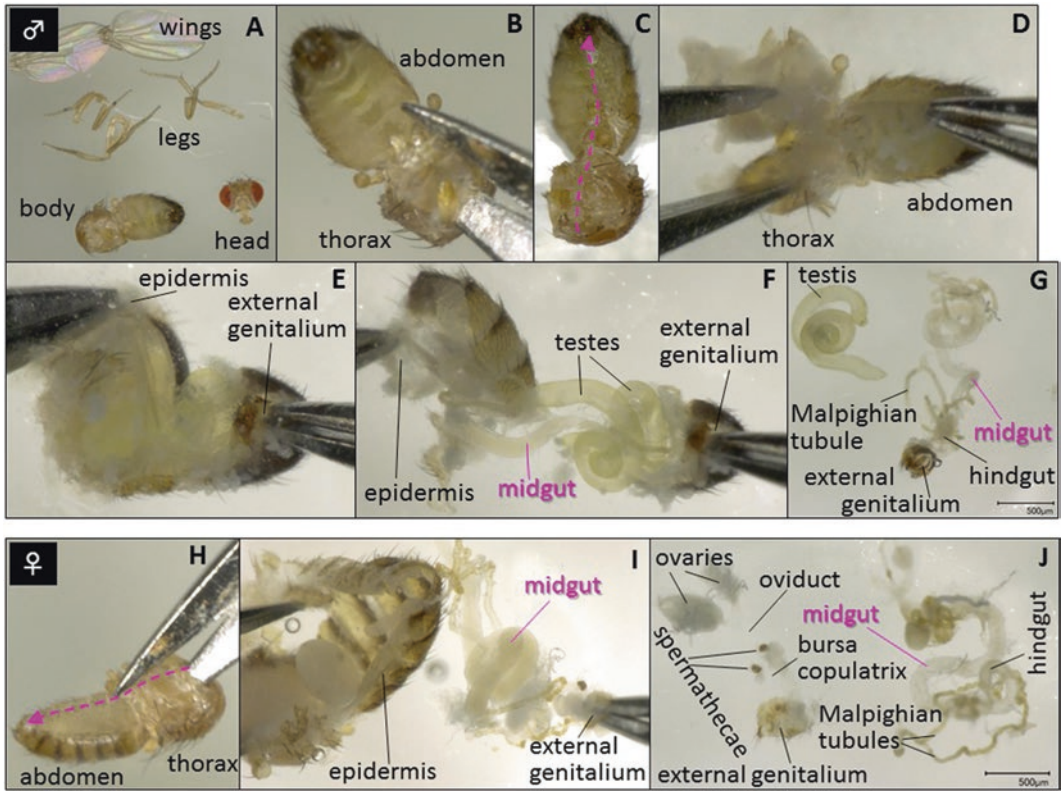

Fig. 2.6 Dissection for *Drosophila* adult midgut (**A**–**G**) Dissection of male body. (**H**–**J**) Dissection of female body. (**A**) Ventral view of male body (bottom) from which the wings, legs, and head are removed. (**B**) A micro-spring scissors blade inserted into the cut end between the head and thorax. (**C**) Direction for cutting of the ventral epidermis along the midline (magenta broken line). (**D**) Opening of the epidermis from the midline cut ends by two forceps in PBS. (**E**) Pinching of the epidermis and external genitalium by each forceps. (**F**) Isolation of male internal organs by pulling away epidermis and external genitalium. (**G**) An organ complex composed of the midgut, hindgut, Malpighian tubules, and genitalium is shown on the right. The removed testis is shown at the left top. Accessory glands and other reproductive organs are not shown in this photo. (**H**) Blade of micro-spring scissors inserted into the cut end between the head and thorax of the female body, from which the wings, legs, and head are removed (lateral view). (**I**) Isolation of female internal organs by pulling away epidermis and external genitalium as shown in F (for male). (**J**) An organ complex composed of midgut, hindgut, and Malpighian tubules is shown on the right. The removed reproductive organ complex composed of ovaries, oviduct, spermathecae, bursa copulatrix, and external genitalium is shown on the left

ment can be paused at this step at 4 ° C if the period is less than 1 week.

14. Remove as much of the secondary antibody solution as possible, and add 200 μl of PBT followed by gentle tapping as above.
15. Rinse the midgut as explained in steps 5–7.
16. Cut the yellow tip to expand its hole, and gently suck up the midgut into the yellow tip. Then, transfer the midgut on to a microscope slide, and carefully put a drop of 80% non-fluorescent glycerol onto the midgut as a mountant, and mix it into the residual PBT on the slide.
17. Put the cover slip gently onto the midgut so as not to leave air bubbles, and close the four sides of the cover slip by transparent nail polish.

Observation

It is recommended to use a laser confocal microscope for finer observation and obtaining sec-

tional images of the midgut. However, a standard fluorescent microscope can also be used if expansion of *Dl* and *esg* expression is only judged as senescence phenotypes.

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Alzheimer's Disease Model System Using *Drosophila*

3

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Abstract

Alzheimer's disease (AD) is the most epidemic neuronal dysfunctions among elderly people. It is accompanied by neuronal disorders along with learning and memory defects, as well as massive neurodegeneration phenotype. The presence of intracellular neurofibrillary tangles (NFTs) and extracellular amyloid plaques, called senile plaques (SPs), and brain atrophy are typically observed in the brains of AD patients. It has been over 20 years since the discovery that small peptide, called beta-amyloid (Aβ), has pivotal role for the disease formation. Since then, a variety of drugs have been developed to cure AD; however, there is currently no effective drug for the disorder. This therapeutic void reflects lacks of ideal model system, which can evaluate the progression of AD in a short period. Recently, large numbers of AD model system have been established using *Drosophila melanogaster* by overproducing Aβ molecules in the brain. These systems successfully reflect some of the symptoms along with AD. In this review, we would like to point out "pros and cons" of *Drosophila* AD models.

Keywords

Alzheimer's disease · Beta-amyloid · *Drosophila* · Chemical biology

3.1 Introduction

Recent Japanese research project reported that about 462 million people are thought to be affected by dementia in Japan (List of Statistical Surveys conducted by Ministry of Health 2012). Since more than half of dementia is expected to be categorized into Alzheimer's disease (AD), therapeutic strategies against AD are the most urgent issue to solve in the highly elderly society like Japan. However, despite all the researchers' effort around the world, there are no therapeutic drugs, which can suppress progression of AD. This therapeutic void reflects lacks of ideal animal model system for the AD drug development. Growing numbers of studies suggest that *Drosophila melanogaster* provide us an ideal model system to analyze human disease formation. In this chapter, we would like to explain the usefulness and the limit of *Drosophila* system as a model for the study of AD.

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3.2 What Is Alzheimer's Disease?

In 1906, Dr. Alois Alzheimer in Germany reported historically an important symptom of a 50-year-old woman, who seemed to have suffered from Alzheimer's disease (Terry and Davies 1980). Dr. Alzheimer reported prominent features of the disorder: there were senile plaques (SPs), neurofibrillary tangles (NFTs), as well as severe brain atrophy (Terry and Davies 1980). From the molecular studies of AD within these 30 years, many types of causative factors, such as amyloid precursor protein (APP), presenilin1 and 2 (PSEN1, 2), have been isolated (Benilova et al. 2012). Among them, APP is thought to be one of the key molecules for AD formation (Fig. 3.1a).

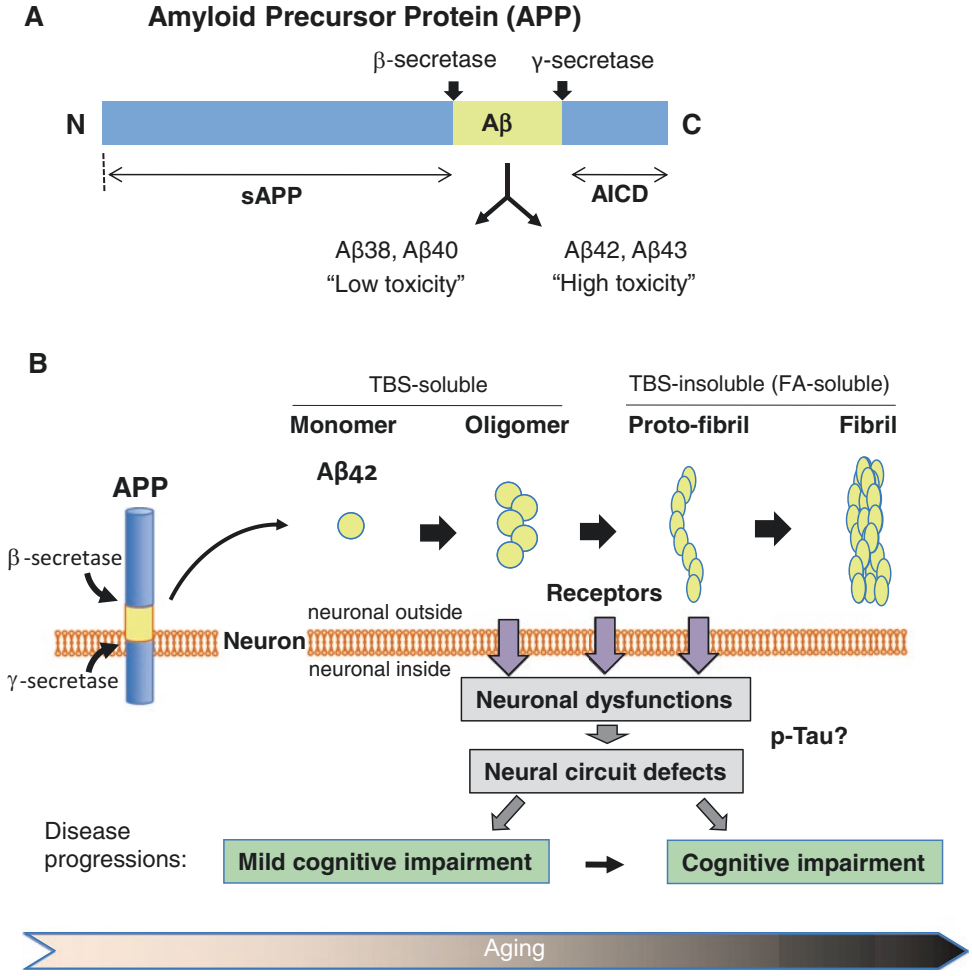


Fig. 3.1 Amyloid precursor protein and amyloid hypothesis. (**a**) Structure of amyloid precursor protein (APP) and their modification sAPP = secreted form of the beta-amyloid precursor protein, AICD = APP intracellular domain. Combinatorial action between ß-secretase and γ-secretase produces Aß small peptides: low-toxic Aßs (Aß38, Aß40) or high-toxic Aßs (Aß42, Aß43). (**b**) Model of Aß hypothesis. Aß42 is produced from APP by the enzymatic combination of ß-secretase and γ-secretase. Single Aß42, a monomer form of Aß42, is assembled and makes a complex, called oligomer. The oligomer is going to make a fibril formation through semi-condensed fibril, called proto-fibril. During these processes, it has been thought that the aggregated form of Aß binds to the cell surface receptors and produces neuronal dysfunction. Phosphorylated Tau (p-Tau) is thought to be involved in these processes. These events are thought to be causative element for the mild cognitive impairment (MCI) and the following cognitive impairment. MCI is a disorder that has been associated with risk for Alzheimer's disease

Many studies indicate that truncated peptide, called Aβ, is produced from the APP by the sequential proteolysis with the enzymatic combination: β-secretase (cysteine proteases and β-site APP-cleaving enzyme (BACE)) and γ-secretase (a multimeric protein complex composed of presenilin, nicastrin, Aph-1, and Pen-2) activity (Fig. 3.1b) (Benilova et al. 2012; Cescato et al. 2000). Aß42 is a very aggregative molecule: in vitro analysis showed that monomeric Aß42 (molecular mass ~4 kDa) accumulated into fibril formation (molecular mass ~20 kDa) through assemblies of several monomers, called oligomer, and proto-fibrils (Fig. 3.1b) (Benilova et al. 2012). Biochemical analysis has shown that the monomer and oligomer form of Aß can be dissolved by the mild condition (TBS-soluble), while proto-fibril and fibril form of Aß cannot (TBS-insoluble) (Fig. 3.1b). It has been shown that the TBS-insoluble fraction of Aß can be dissolved by folic acid and is called as FA-soluble form (Fig. 3.1b). The aggregation of Aß peptide in the brain is thought to be a pathological hallmark of AD. Aß, especially Aß42, is an aggregative molecule and seems to produce neuronal toxicity through membrane neuronal receptors, such as Prion receptor (PrP), metabotropic glutamate receptors (mGluRs), or N-methyl-D-aspartate (NMDA) receptor (Fig. 3.1b) (DeArmond 1993; Lee et al. 2004; Snyder et al. 2005). The neuronal dysfunctions are thought to induce cognitive defects, such as mild cognitive impairment (MCI) and cognitive impairment during aging (Fig. 3.1b).

N-terminal region of Aβ has been shown to be modified after the production of this peptide (Mori et al. 1992). Most prominent figure of the N-terminal modification is pyroglutamation: the second or tenth amino acid of N-terminal residues of Aß42 is truncated by the enzymatic activity, and remained glutamate at N-terminal region of Aß is circulized (Saido et al. 1995). This process is called pyroglutamation and mediated by the enzyme, called glutaminyl cyclase (QC) (Schiling et al. 2004). It has been shown that about half of Aß42 in SPs are pyroglutamated form (pGlu-Aß) at the later stage of AD formation (Mori et al. 1992). The biochemical analysis revealed that the properties of pGlu-Aß42 are more aggregative than normal Aß42, suggesting that pGlu-Aß42 is a more toxic component than Aß42 itself (Schiling et al. 2006). To support this idea, it has been reported that inhibition of pGlu-Aß formation, by QC knockout background, reduced accumulation of Aß42. From this result, it has been proposed that pGlu-Aß is acting as a "seed" for Aß accumulation (Jawhar et al. 2010).

Thus, people believe that pGlu-Aß42 is a major contributor for the late-onset neurodegeneration during AD. Given that pGu-Aß42 production seems to have an important role for the AD formation, the inhibitor of QC is expected to be a therapeutic drug for AD. Recent study reported that PQ912, an inhibitor for QC, is under the phase II trial on the way to AD drug development (Hoffman et al. 2017).

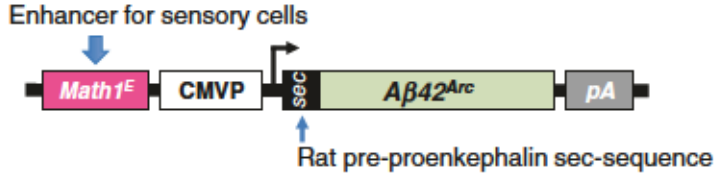
3.3 Mouse AD Model Systems

Since APP was identified as a causative factor for AD from familial AD patients and the first mouse model has been reported by Games et al. (Games et al. 1995), many types of mouse AD models were established: most of them are expressing APP in their brain (Fig. 3.2a) (Ittner and Gotz 2011). Thus, those mice are called as "APP-mouse." In the APP-mouse, there are SPs in the cortex of the brain; however, NFTs are barely observed in the brain of the APP-mouse (Games et al. 1995). To solve the problem, human Tau and Psen1 were introduced into the APP-mouse background (e.g., 3xTg; Fig. 3.2a) (Oddo et al. 2003). In this combination type, we can observe NFTs and synaptic dysfunctions within several months after the birth. However, it calls for caution about this system, in which many of factors are overproduced. In 2014, Takaomi Saido's group of RIKEN Brain Science Institute, Japan, reported about a new type of AD mouse, in which APP-mouse was changed to human-type sequence and knocked into the genomic region of APP-mouse (Fig. 3.2a) (Saito et al. 2014). This knock-in AD mouse model (KI-mouse) showed relatively low expression levels of APP and prominent correlation with human pathologies (Saito et al. 2014).

A Mouse AD models (APP-mouse)

Charactors	Representative models
APP over-expression	*Tg2576, APP23, PDAPP, J20*
APP knock-in	*APP^{NL-F}-KI*
Combination type	3xTg (APP, human Tau, and Psen1)

B *Math1^{E}-Aβ42^{Arc}* mouse



C ABR recording data

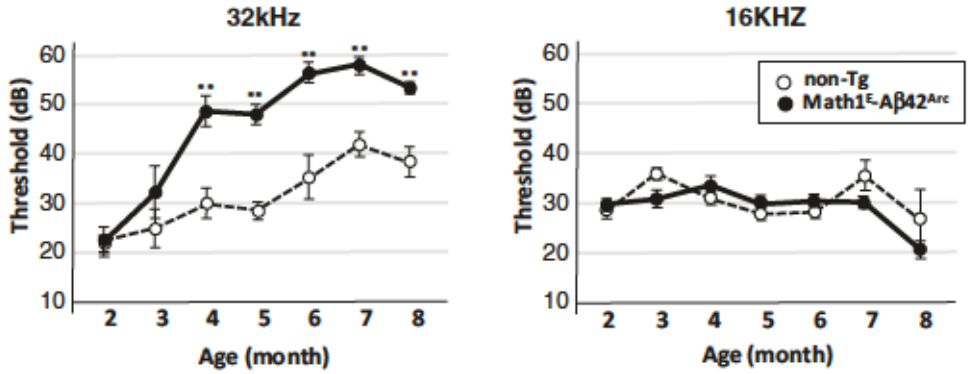


Fig. 3.2 Mouse AD models. (**a**) The ordinary mouse AD models. Representative three types of mouse AD models are shown. APP-overexpressing or knock-in types are expressing human APP under neuron-specific promoters or endogenous APP promoter, respectively. Combination type, such as 3xTg, is that three genes, human APP, MAPT (human Tau), and human Psen1, those of which contain familial AD mutations, are expressing under the neuron-specific promoter. (**b**) Construct of Math1^{E}-Aß42^{Arc} mouse. Math1^{E} = a minimal enhancer of Math1 gene, CMVP = cytomegalovirus promoter, pA = polyadenylation signal. (**c**) Representative ABR threshold data. Non-Tg = siblings of Tg (Math1^{E}-Aß42^{Arc}). Threshold shift was observed. These data are modified from the reference (Omata et al. 2016)

AD is thought to be a typical neurodegenerative disorder, which leads to severe brain atrophy; however, the molecular mechanism of the neurodegeneration in AD patients remains to be elucidated (Haass and Selkoe 2007). This discrepancy is due to the lack of ideal model system to analyze neurodegeneration in AD. Although APP-mouse and KI-mouse showed cognitive dysfunctions and synaptic-loss phenotypes, neurodegeneration is barely observed in those systems (Games et al. 1995; Saito et al. 2014). Given that overproduction of pGlu-Aß triggered neurodegeneration in mouse brain, it has been implicated that pGlu-Aß might have important roles for the late-onset neurodegeneration in AD (Wirths et al. 2009).

The most prominent properties of APP-mouse and KI-mouse are that we can examine the learning and memory defect (Games et al. 1995; Saito

et al. 2014). The defects of learning and memory in those models can be detected by the behavior assay, such as Morris water maze test or Y-maze test (Webster et al. 2014). The behavior analysis, however, is easily affected by the environmental condition; therefore, it is difficult to perform quantitative analysis. Also, the process of behavior assay system is time-consuming; Morris water maze test requires more than a week to obtain the data (Chen et al. 2000). Furthermore, it takes almost a year to show the prominent cognitive defects in APP-mouse and KI-mouse (Games et al. 1995; Saito et al. 2014). It is no doubt that APP-mouse and KI-mouse models are very important tool for the study of pathologies of AD; however, we need a new mouse system to evaluate the effect of therapeutic drugs against AD. To compensate for the APP-mouse and KI-mouse models, recently we produced a new transgenic mouse as a tool for the AD drug evaluation (Fig. 3.2b) (Omata et al. 2016). Previously, it has been shown that the sensory system, such as olfactory, photoreception, or audioreception, is defected in the patient of AD (Wilson et al. 2007; Sivak 2013; Zheng et al. 2017). Furthermore, sensory cells and neurons contain similar characters; both types of cells have synaptic regulation and similar machineries of neurotransmission (Travis and Paukin 2014). We reasoned that expressing Aß42 in sensory cells also causes synaptic dysfunctions in sensory cells (Omata et al. 2016). We introduced Aß42 with familial AD mutation (E22G; Arctic) under the control of minimal enhancer of Math1 ($Math1^{E}$), which can drive gene expression at sensory cells and established a new transgenic mouse (Tg), $Math1^{E}$-$Aß42^{Arc}$ (Fig. 3.2b). Since auditory ability can be quantified by the electrophysiological method, such as auditory brainstem response (ABR), we have monitored the effect of auditory response in $Math1^{E}$-$Aß42^{Arc}$ by analyzing the threshold of ABR (Fig. 3.2c). We found that $Math1^{E}$-$Aß42^{Arc}$ caused auditory defects at 4 months after the birth (Omata et al. 2016). Interestingly, hearing ability against high-frequency sound stimulation (>32 kHz) was only defective in the mouse (Fig. 3.2c). This is an interesting data, given that the sense of high-frequency sound stimulation seems to be easily declined at old age and that aging is the biggest risk factor for AD (Liberman 2017). Like the central nervous system (CNS) in the APP-mouse, this system showed synergistic interaction between Aß42 and human Tau (Omata et al. 2016; Chabrier et al. 2014). Co-expression of Aß42 with human Tau showed severe auditory defect even at 2 months after the birth. It supported that the new system might reflect, if not all, human pathology of AD and that the $Math1^{E}$-$Aß42^{Arc}$ system is an ideal system for the evaluation of the toxic effect of Aß quantitatively. Furthermore, the $Math1^{E}$-$Aß42^{Arc}$ system also showed degeneration phenotype at the auditory hair cells after 6 months after the birth. This also supports the idea that the new mouse model can compensate for the known AD mouse model, since degeneration phenotype is not detected in known AD mouse models. Furthermore, the toxicity of Aß can be easily monitored by the electrophysiological way (ABR) in this new AD model, so one can evaluate the effect of drugs against toxicity of Aß quantitatively with time course. Although $Math1^{E}$-$Aß42^{Arc}$ system can't estimate the effect of drugs in terms of learning and memory defects in AD, this new mouse system might be very powerful by the combinatorial using APP-mouse.

3.4 The Present Conditions of the AD Drug Development and Problems

Since Aß was recognized as a causative factor for AD, people have been trying to develop therapeutic drugs for AD, those of which inhibit the production or toxic effects of Aß (Fig. 3.1b). Many laboratories have been trying to produce the inhibitors of the APP-cleavage enzymes, such as γ-secretase inhibitors or ß-secretase inhibitors (BACE inhibitors) (Lanz et al. 2003; Ghosh et al. 2012). To inhibit the toxic effect of Aß42 itself, monoclonal antibody against Aß also has been developed. In 2011, for example, Bachmeier et al. published that the data about anti-Aß inhibited the formation of SPs (Bachmeier et al. 2011).

Aß is easily aggregated in in vitro experiments; therefore, people have been trying to identify the inhibitors to block in vitro aggregation of Aß (Esler et al. 1997). 3-Amino-1-propanesulfonic acid (3-APS) is one of the inhibitors to perturb in vitro aggregation of Aß42 (Alsen et al. 2006). In spite of all the effort for the development of therapeutic drugs against AD, however, most of the approaches above were in fail. The trial for γ-secretase inhibitor or BACE inhibitor, monoclonal antibody against Aß, and 3-APS were all withdrawal in phase III trials (De Strooper 2014; Merck pulls plug on phase 2/3 BACE inhibitor trial 2017; Vandenberghe et al. 2016; Aisen et al. 2011). One possible reason for the failures is that Aß is not a correct target for the therapeutic drugs for AD. However, given that the earlier treatment of anti-Aß seems to suppress the cognitive defects of AD, it seems to be too early to make a conclusion (Sevigny et al. 2016).

Another possibility for the failures is that the known mechanism of AD formation is not enough and there might be unidentified new processes underlying AD. Despite the detailed analysis of the production of Aß or aggregation processes of Aß, little is known about how toxicity of Aß is produced in AD patients, supporting the idea that there might be unknown mechanism of Aß activity (Benilova et al. 2012). Thus, to solve the problem about the lack of AD drugs, one possible approach is to perform in vivo screening and identify the candidate compounds, which can inhibit the toxic effect of Aß. This chemical-biological approach might compensate for the known approach so far in the field of AD. Once AD drug candidate was identified, we can isolate the binding partner of this chemical compound by biochemical analysis. Functional analysis of this binding protein might provide us a molecular mechanism of AD formation. Therefore, this chemical-biological approach might contribute not only for the therapeutic field but also basic science in the AD formation. One of the most important points in performing in vivo drug screening is how to choose model organisms. As we will discuss below, *Drosophila melanogaster* provides us a powerful tool to study AD and develop the therapeutic AD drugs in vivo.

3.5 *Drosophila* Model System for the Study of AD

3.5.1 *Drosophila* Provides Many Types of AD Model Systems

Based on the genomic analysis, it has been estimated that more than 70% of human disease-related genes are conserved in *Drosophila* (Runbin et al. 2000). Notably, many kinds of human neurodegenerative disorders, such as Huntington's, ALS, or Parkinson's disease, have been shown to be analyzed by fly system (Jackson et al. 1998; Arquie et al. 2008; Feany and Bender 2000). As mouse AD model systems, fly systems also have been applied to reveal the molecular mechanism of AD. Recent advancement of genome-wide association study analysis (GWAS analysis) revealed that many types of risk factors for AD are identified and *Drosophila* homologue of those risk factors showed genetic interaction with fly model of AD (Waring and Rosenberg 2008; Shulman et al. 2011). These results strongly suggest that fly model system is useful for the study of AD.

There is a homologous molecule with APP, called APP-like (APPL) in the genome of *Drosophila* (Wasco et al. 1992). Furthermore, it has been shown that there seems to be γ-secretase activity in fly (Fossgreen et al. 1998). There does not seem to be significant sequence similarity around Aß region in APPL, suggesting that toxic Aß is not produced in fly. Chakraborty et al., however, have shown that expressing human APP with human BACE in fly nervous system (APP/BACE) produced Aß and shorten the life span (Chalraborty et al. 2011). They have shown that memory loss is induced by the APP/BACE fly system. Human Tau, when it is expressed with APP/BACE, caused severe neurodegeneration in fly, suggesting the "APP-fly" showed related symptoms of AD (Torroja et al. 1999).

In *Drosophila*, many types of induction system have been established (Phelps and Brand 1998). Among them, UAS/Gal4 system is the most common used as an induction system (Fig. 3.3a) (Phelps and Brand 1998). Gal4 is a transcription factor in yeast and recognizes a

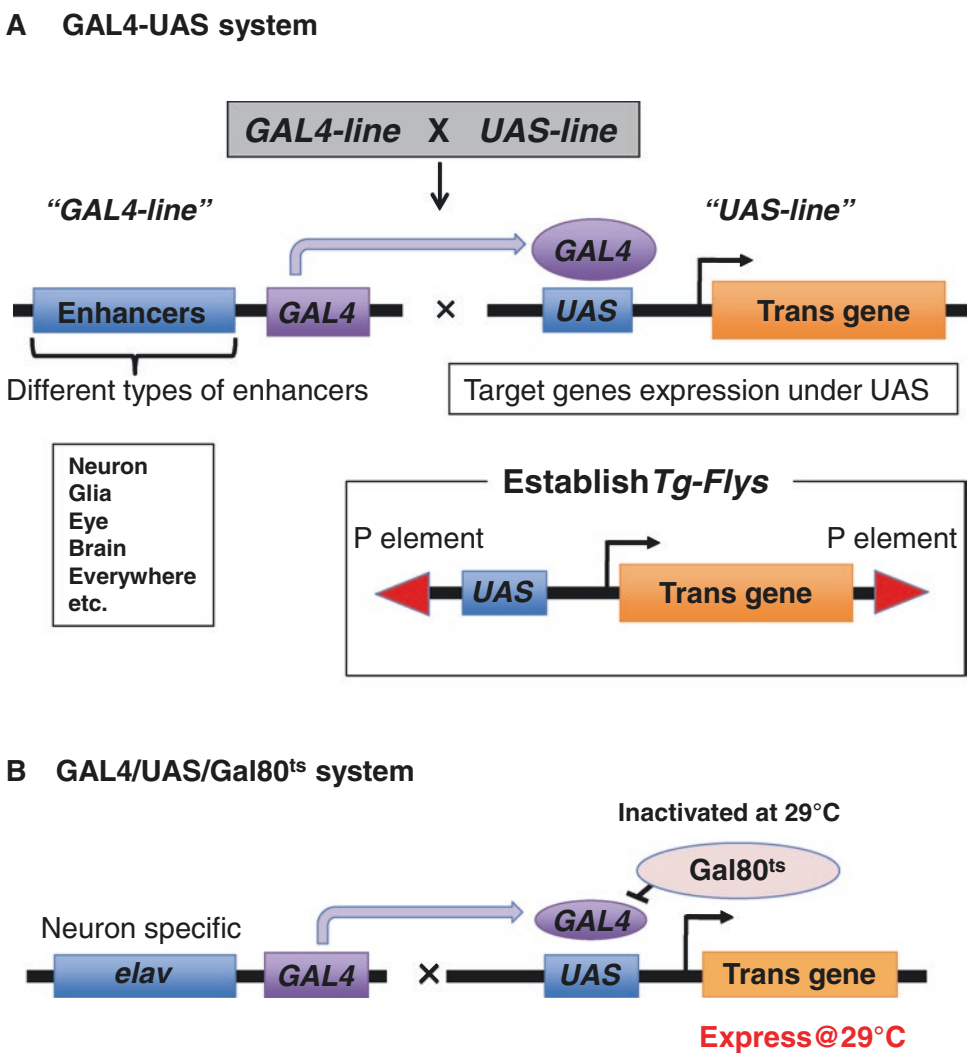


Fig. 3.3 Gene expression system of *Drosophila*. (**a**) Gal4/UAS system. There have been several Gal4-expressing lines, those of which are expressing Gal4 under the local enhancer. Target genes under *UAS* sequence can be expressed by these *Gal4* lines. (**b**) Gal4/UAS/Gal80ts system. Gal80ts is a Gal4 inhibitor with a temperature-sensitive mutation. Gal80ts is expressing everywhere by the ubiquitous promoter, such as actin. The Gal4 inhibitor activity of Gal80ts is inactivated by the temperature shift over 29 °C. Therefore, constitutively expressing Gal80ts, such as actin-Gal80ts line, blocks the Gal4/UAS system until feeding temperature was shift over 29 °C

specific sequence, called upstream activation sequence (UAS), at the promoter region of the target genes (Guarente 1988). Therefore, any genes can be overexpressed under UAS, and many types of Gal4 insertion lines, those of which are expressing Gal4 at the tissue specific manner, have been established. To elucidate the effect of drugs against AD model system, most of the individuals should be synchronized. Therefore, we need inducible gene expression system. Gal80ts is a temperature-sensitive mutant of Gal4 inhibitor and has been shown to be inactivated at 29 °C (Johnston 1987). Thus, UAS/Gal4/gal80ts system allows us to induce gene expression of fly only by the shift of temperature from 18 to 29 °C (Fig. 3.3b) (Suster et al. 2004).

To reveal the in vivo effect of Aß on the surface of neural cells, Aß with secretion peptide is

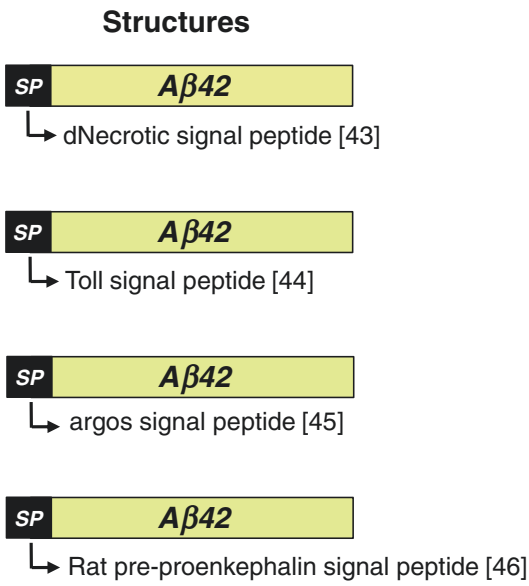


Fig. 3.4 Aß secretion system in *Drosophila*
Representative four types of *Drosophila* AD models are shown. All constructs contain the secretion signal, which allow each Aß to secret to the outside of cells. Each construct is expressed in neurons by Gal4/UAS system. dNecrotic: *Drosophila* necrotic protein

invented; there are several types of Aß secretion systems in *Drosophila*. *Drosophila* necrotic protein (dNecrotic), *Toll*, *Argos*, or Rat pre-proenkephalin-derived signal peptides are used for this purpose (Fig. 3.4) (Crowther et al. 2005; Casas-Tinto et al. 2011a; Allan et al. 2014; Iijima et al. 2004; Omata et al. 2014). The fusion Aß42 when they are expressing CNS by UAS/Gal4 system caused Aß42 secretion and aggregation. The fusion molecule reduced longevity and induced progressive locomotion defects (Crowther et al. 2005; Casas-Tinto et al. 2011a; Allan et al. 2014; Iijima et al. 2004; Omata et al. 2014). Although this secretion form Aß is a very powerful tool to reveal the toxic effect of Aß in vivo, recent study calls for caution about this approach: the signal peptide from dNecrotic showed additional Gln in the N-terminus, and this additional form caused toxic effect (Speretta et al. 2012).

3.5.2 Assay System for the Evaluation of Toxic Effect of Aß in Neuron Using *Drosophila*

To elucidate the Aß-induced toxic effect on the neuronal cells in *Drosophila*, many types of assay system have been performed. For the study of Aß aggregation, for example, besides biochemical analysis, thioflavin staining is used to detect aggregated form of Aß in the brain (Burns et al. 1967; Palutke et al. 1987). Thioflavin S and thioflavin T (anionic and cationic fluorochromes, respectively) have been used for histological staining and biophysical studies of AD (Palutke et al. 1987). It has been shown that thioflavins bind to beta-sheet-rich architectures, such as beta-amyloid aggregation (Burns et al. 1967). It displays enhanced fluorescence and a specific emission spectrum shift when it makes a complex with protein aggregation with beta-sheet. Thus, thioflavin S is used for the critical methods for the detection of Aß, those of which are called as thioflavin assay. In *Drosophila*, thioflavins are aggregated when the Aß is expressed (Palutke et al. 1987). Thus, aggregated form of Aß might be a causative factor for the toxicity of Aß in *Drosophila* as well as mammalian cells. In the mammalian case, it has been thought that the stability of Aß might contribute for the toxicity of Aß, and neprilysin, a metabolic enzyme for Aß, is shown to have an important role for the Aß degradation (Acerra et al. 2014). In *Drosophila*, there seems to be a homologous molecule of neprilysin in the genome (Iwata et al. 2000). Loss-of-function mutation increased the stability of Aß, while overexpression of this molecule decreased the stability of Aß, suggesting that neprilysin is also acting as a metabolic enzyme for Aß in *Drosophila* (Thomas et al. 2005).

Synaptic dysfunction leads to the defect of neuronal activity. This might be a causative factor for the defect of behavior, such as sleep, locomotion, and/or learning-memory defects in AD

(Iijima-Ando et al. 2008). As is the case of mammalian system, *Drosophila* also showed sleep, locomotion, and learning-memory defects when Aß is expressed in the nervous system (Kent and Mistlberger 2017; Gerstner et al. 2017). Memory defect is detectable by olfactory-dependent learning-memory analysis (Tabuchi et al. 2015). This is robust system; however, it requires large number of flies (about 100 flies per assay). Therefore, it does not seem to be an ideal system for the drug discovery. *Drosophila* showed negative geotactic property, and Aß42 when it is expressed in the nervous system inhibited the negative geotactic (Davis 2005). Recent study, however, revealed that it is not the sense of negative geotactic, but the speed of their working might be a major target of the defect in Aß expression system (Watanabe and Anderson 1976).

Electrophysiological analysis of *Drosophila* supports the idea that Aß42 suppresses the activity of synapse (Rhodenizer et al. 2008). Adult synaptic activity can be monitored by the electrophysiological analysis using giant fiber system (GFS) (Fig. 3.5a). Giant fiber (GF), which consisted of a pair of command interneurons that convey information from sensory centers in the brain to motor neurons in the thoracic ganglion that control the mesothoracic legs and wings, mediates escape behavior from the changing intensity of light. Brain stimulation caused the peaks of response when the recoding needles are inserted at the terminal muscle (TTMn and DLMn, respectively) (Fig. 3.5b) (Zhao et al. 2010). Huang et al. expressed Aß42 in the GF neurons and found that the synaptic activity seemed to be reduced (Mejia et al. 2013). They have shown that a vesicle recycling at the presynaptic junctions of GF was severely disrupted (Huang et al. 2013). This is a very interesting data, since the defect of recycling endosome is thought to be one of the typical symptoms along with AD (Nixon 2017).

As we have discussed earlier, the molecular mechanism behind the degeneration phenotype in AD remains to be clarified. This is also the case in *Drosophila*. Even in the fly model, whether Aß42 can cause neurodegeneration is a big controversial issue. *Drosophila* compound eyes consisted of about 800 ommatidia, and each ommatidium contains 8 photoreceptor neurons that are arranged like crystal structures (Fig. 3.6a–c) (Colley 2012). Therefore, this system has been used as a model for the human neurodegenerative disorders, such as Huntington's, ALS, or Parkinson's (Jackson et al. 1998; Arquie et al. 2008; Feany and Bender 2000). Fly compound eye system also have been applied for a model of neurodegeneration caused by Aß42. However, in most of the case, Aß42 expression in the eye caused morphological deficit (Casas-Tinto et al. 2011b; Cao et al. 2008). These suggest that some differentiation defects might contribute for the neurodegeneration phenotype in the eye of AD fly models.

3.5.3 *Drosophila* System as a Tool for the Discovery of Therapeutic Drugs for AD

An insect has an open blood vascular system; therefore drugs are easily delivered to the target organ including the brain (Wang et al. 2013). There are several approaches that have been thought to administer drugs to flies (Bhan and Nichols 2011). Injection of drugs or dropping drugs directly onto the exposed nervous system of flies has been performed. However, the problem as a drug screening is that it is time-consuming; therefore, administration of drugs to the food or the filter paper with sucrose/drug mixture seems to be one of the best ways to administer drugs to flies (Xiang et al. 2011). Usually drug screening is performed by high-throughput screening (HTS) from a large library (~100,000 compounds) within a month, whereas most of the fly system for the drug discovery might be the order 100–500 small compounds per month. Although the fly system can handle such small numbers of chemical compounds, one must keep in mind that the quality of hits from the screening is the key of success and in vivo screening using *Drosophila* system might provide us a chance to get the high-quality hit.

There are several drug screenings that already have been performed using fly AD model. Liu

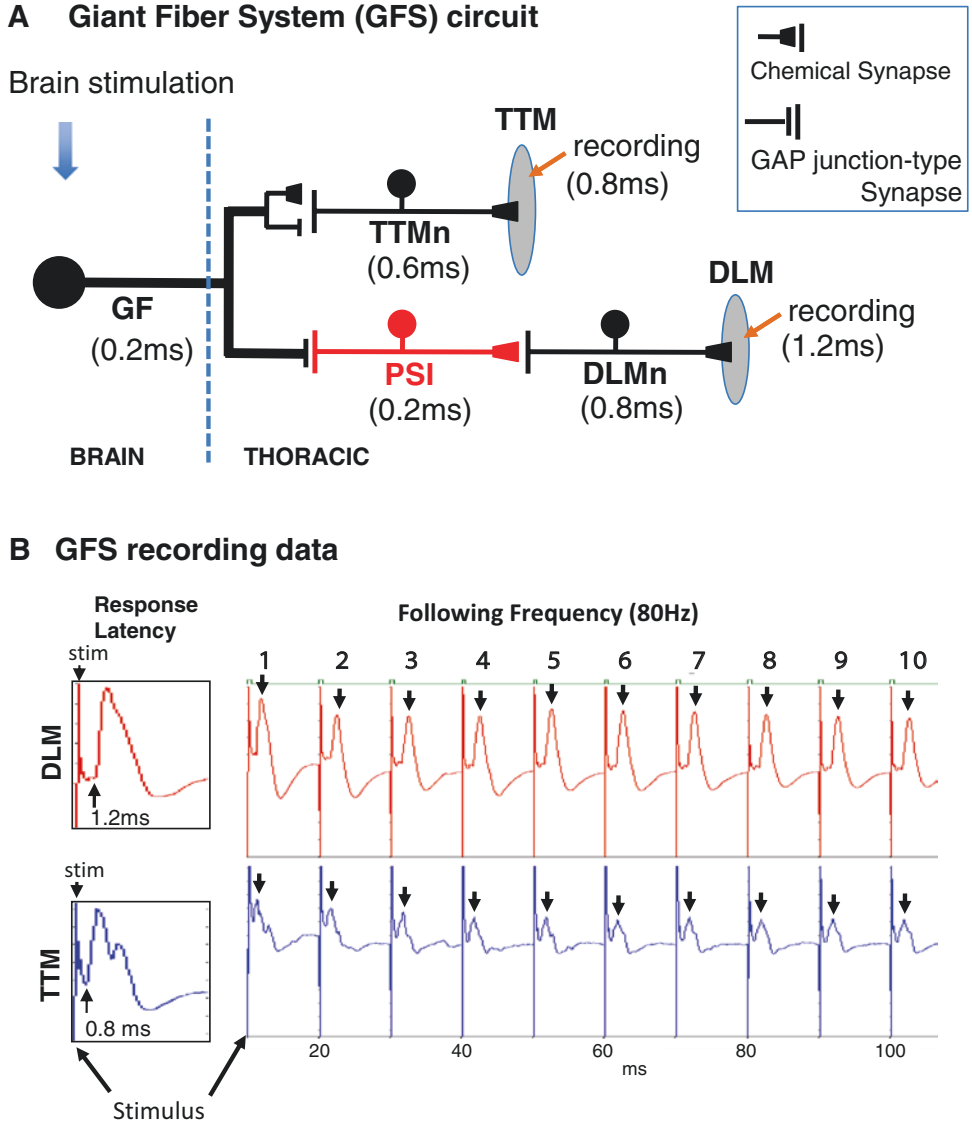


Fig. 3.5 Giant fiber system in *Drosophila*. (**a**) Representative illustration of one lateral half of the giant fiber (GF) circuit (Allen et al. 2006). Dark circle represents cell bodies and dendrites. The GF cell body and dendrite are localized in the brain, and each extends a single axon into the thoracic neuromere, where it makes a mixed electrical (GAP junction) and chemical synapse (see the inset) onto the tergo trochanteral motorneuron (TTMn). TTMn innervates the jump muscle (TTM). The GF also makes an electrical synapse onto the peripheral synapsing interneuron (PSI). PSI makes a cholinergic synapse onto the dorsal longitudinal motorneurons (DLMn) that innervate the flight muscle (DLM). It has been shown that the cholinergic synapse between PSI and DLMn is the most sensitive synapse in GFS when Aß42 was expressed (Zhao et al. 2010). (**b**) The representative recording after the brain stimulation. Ten times of stimulation (80 Hz) and response were recorded by the electrode inserted at TTM and DLM, respectively. The response at DLM is very sensitive, easily affected by Aß42 (Zhao et al. 2010)

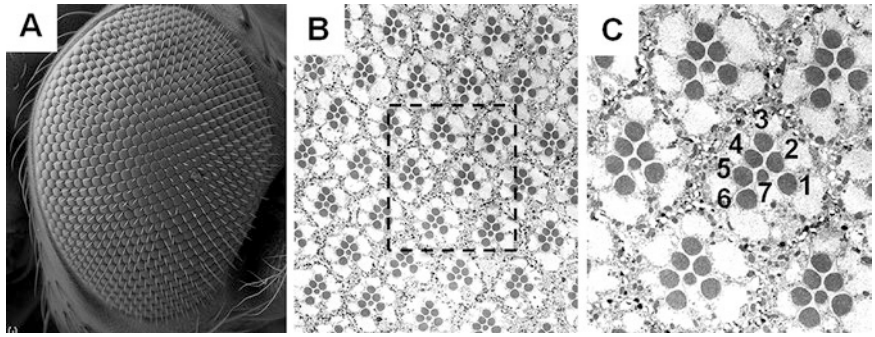


Fig. 3.6 *Drosophila* compound eye. (**a**) Scanning electron microscopic figure of *Drosophila* compound eye. Compound eye consisted of about 800 ommatidia. (**b**) Section of the compound eye. Each ommatidium contains rhabdomeres (a dark structure consisted of multiple microvilli in the center of ommatidia). (**c**) The magnified figure of (B) (dotted square). Each ommatidium is polarized, and rhabdomeres in the ommatidia are arranged in an orderly manner. Although there is supposed to be eight photoreceptor cells in each ommatidium, only seven rhabdomeres can be observed in relation to the position

et al. has reported that application of traditional medical plans to the fly AD system leads to identify five plant extracts, which can suppress the survival defects caused by Aß (Kiu et al. 2015). In this experiment, they have shown that those medical plants reduced Aß42-induced ROS levels. Another example was reported by Wang et al. (Wang et al. 2012). They showed that inhibitors for the epidermal growth factor receptor (EGFR) suppressed learning and memory deficit induced by Aß42. To identify a novel compound, which can suppress Aß42-inducing learning-memory deficit, they performed a drug screening using 2000 synthetic chemical compounds with structures that are presumably targeted to protein kinase activities. They identified 45 compounds out of 2000 components. Notably, they have shown that some of the candidate compounds suppressed learning-memory defects in APP-mouse. This suggests that chemical screening using fly system could identify the compound, which is available for the mammalian system.

3.6 Perspective

Drosophila system seems to reflect many aspects of AD pathologies; therefore, we will be able to identify chemical compounds, which can suppress the toxic effect of Aß42. However, our goal is to develop therapeutic drugs for human. Thus, we need to evaluate the effect of drugs using mouse model, before we administer the candidate compound identified from fly screening to human. The combinatorial using *Drosophila* system and recently established mouse AD model might be a powerful tool to develop the therapeutic drugs to the AD patient (Fig. 3.7).

3.7 Commonly Used Protocol

3.7.1 Feeding Drugs on *Drosophila*

Several approaches are developed to feed drugs to *Drosophila*. One of the common methods is to mix the drugs into the fly food. Briefly, the food was prepared with distilled water containing 2% (wt/vol) yeast, 7% (vol/vol) corn syrup liquids, and 1.5% (wt/vol) agarose and autoclaved. The food was mixed as a liquid with drugs at 60 °C. The drugs were dissolved in DMSO and mixed into the melted fly food at appropriate concentrations (final 1% DMSO). For larval feeding, parental crosses were placed for 1 day in the vials with standard *Drosophila* food, containing the respective drug at appropriate concentration (prepared as above). Larvae were allowed to feed and develop in the vials at 25 °C. For the drug feeding at adult stage, the flies were transferred to vials (10 flies per vial) containing fly food and tested compounds. Every 2–3 days, the flies were trans-

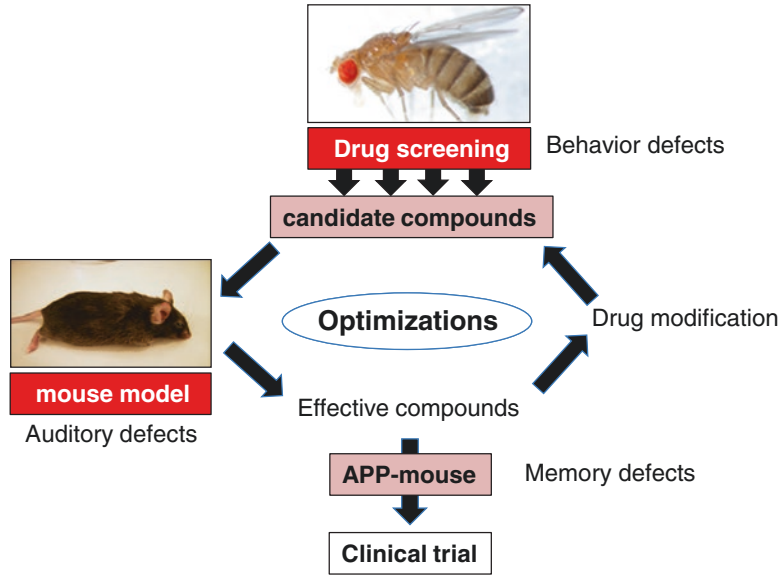


Fig. 3.7 Combinatorial using fly and mouse AD models for the therapeutic AD drug development. Using *Drosophila* model, candidate compounds, which suppress the behavior defects caused by Aß42, can be identified out of chemical libraries. The effects of the candidate compounds are easily assessed by the new type of mouse AD model (*Math1*^{E}-*Aß42*^{Arc} system). Chemical modifications of these compounds and the evaluation of the effect of the compounds using *Drosophila* and mouse models are repeatedly applied and optimize the effect of the AD drugs (optimization). Finally, the AD drug can be administered to human after the evaluation of the drug effect on the learning and memory defect caused by APP-mouse

ferred to fresh vials. As I have mentioned below, we have investigated the amount of drugs, which was taken by flies, and noticed that each individuals were taking different amount of drugs in the ordinary method. Thus, we have been modifying ordinary feeding method. Briefly, we prepare 200 μM drug solution at the final concentration of 5–100 μM/(1% DMSO, 0.1 M sucrose). This solution is applied to the 3MM paper (1 cm × 1 cm) at the bottom of an empty vial. Flies are transferred into these vials and incubated at 25 °C. Next day, flies were transferred to vials containing fly food and tested compounds.

3.7.2 Estimation of Drug Uptaking in *Drosophila*

When we look at the effect of drug using *Drosophila*, one of the most important issues is the uptake of the drugs. We have to monitor how much flies are taking foods including drugs. To estimate the amount of drugs that flies are taking, several approaches have been developed. I would like to show some of those assay systems. The first approach is using radioisotope, which was originally performed by Carvalho et al. (2005). We have modified the method (Xiang et al. 2011). Briefly, virgin male and female flies were collected (20 animals/vial × 3). Flies were transferred to a medium containing 5% EtOH or drugs in 5% EtOH, supplemented with 6.5 kBq/ml [32P]dCTP and allowed to feed for 24 h. Flies were then transferred to empty vials to groom for 30 min to ensure removal of cuticular radioactive deposits. Flies were anesthetized on ice and assayed in 3 ml scintillation cocktail (Aquasol-2, Packard) for 4 min/sample using an LSC-5100 scintillation counter. The second approach is using Brilliant Blue FCF (Blue no.1). 0.5 mg/mL of Blue no.1 is added into the fly food, and flies are allowed to feed for 24 h. Flies were anesthetized on ice and homogenized in PBS, and we assay the concentration of the no.1 dye using spectrophotometer (580 nm).

To make more quantitative estimation system for the actual ingestion in individual *Drosophila*, Ja et al. developed the capillary feeder (CAFE) assay, which allows flies consuming liquid food from a graduated glass microcapillary (Ja et al. 2007). This capillary-based method is applied for monitoring real-time ingestion for periods ranging from minutes to the entire life. Recently, Qi et al. modified the CAFE assay and developed more sophisticated method for the evaluation of drug ingestion, called manual feeding (MAFE) assay (Qi et al. 2017). Briefly, 4–6-day-old male flies were starved in vials containing 2% agar only, before the assay. Individual flies were gently aspirated into the large end of a 200 μL micropipette tip and lodged in the micropipette tip so that its head is at the opening as the thorax becomes wedged against the side. Subsequently, 3 μL liquid food (added with 5% Blue dye no.1) is filled in a fine graduated capillary (VWR, #53432-604). And this equipped liquid food was delivered to the proboscis of flies. The tip of the capillary could be retrieved a bit away from the flies to allow the full extension of proboscis. Once the flies stopped feeding and retrieved proboscis, the food stimulation was repeated until the flies became unresponsive to a series of ten food stimuli. Flies that exhibited prolonged water consumption or no proboscis extension reflex (PER) to liquid food were excluded from the calculation of average meal size. This MAFE assay system has temporal resolution to analyze drug uptaking and detect a fast-acting mechanism that senses the drug solution.

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Parkinson's Disease Model

4

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Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disease worldwide. It is known that there are many factors, either genetic or environmental factors, involved in PD, but the mechanism of PD is still not fully understood. Several animal models have been established to study the mechanisms of PD. Among these models, *Drosophila melanogaster* has been utilized as a valuable model to get insight into important features of PD. *Drosophila melanogaster* possesses a well-developed dopaminergic (DA) neuron system which is known to play an important role in PD pathogenesis. The well understanding of DA neurons from early larval through adult stage makes *Drosophila* as a powerful model for investigating the progressive neurodegeneration in PD. Besides, the short life cycle of *Drosophila melanogaster* serves an advantage in studying epidemiological features of PD. Most of PD symptoms can be mimicked in *Drosophila* model such as progressive impairment in locomotion, DA neuron degeneration, and some other non-motor symptoms. The *Drosophila* models of PD, therefore, show a great potential in application for PD genetic and drug screening.

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Keywords

Drosophila melanogaster · Parkinson's disease · PD-like symptoms · Drug screening · Genetic screening

4.1 Introduction

Parkinson's disease (PD) which is characterized by progressive impairment in locomotive ability such as tremor, rigidity, and bradykinesia was first described in 1817 by Dr. James Parkinson. PD impacts 1% of the population over 60 years old and is considered as the second most common neurodegenerative disorder after Alzheimer's disease. Previous studies have shown that PD resulted from the loss of DA neurons in substantia nigra and Lewy body formation in brains (Nussbaum and Polymeropoulos 1997; Forno 1996; Thomas and Beal 2007). Many genes and their variants have been demonstrated to be involved in PD such as *α-synuclein* (*PARK1/SNCA*); *leucine-rich repeat kinase 2* (*PARK8/LRRK2*); *parkin RBR E3 ubiquitin protein ligase* (*PARK2/PARKIN*); *Parkinson protein 7* (*PARK7/DJ-1*); *PTEN-induced putative kinase 1* (*PARK6/PINK1*); *glucosidase*, *beta*, *acid* (*GBA*); and *ubiquitin carboxyl-terminal esterase L1* (*PARK5/UCH-L1*) (Polymeropoulos et al. 1997; Seidel et al. 2010; Paisán-Ruíz et al. 2004; Zimprich et al. 2004; Di Fonzo et al. 2005; Kitada et al. 1998; Hoenicka et al. 2002; Bonifati et al. 2003;

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Annesi et al. 2005; Valente et al. 2004; Hedrich et al. 2006; Aharon-Peretz et al. 2004; Sidransky et al. 2009; Leroy et al. 1998; Liu et al. 2002). Besides, several environmental factors are discovered as causes of PD or to be associated with PD including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA), rotenone, and paraquat. In addition, exposure to pesticides or heavy metal, well water consumption, and poor working conditions have been implicated as factors increasing the risk of PD (Pezzoli and Cereda 2013; Montgomery 1995). A variable range of genetic and environmental interaction is also thought to result in PD (Ross and Smith 2007). However, mechanism which causes PD is still unclear. In order to understand PD, some toxin-based models and gene-based models were established. Among those models, *Drosophila melanogaster* have successfully provided valuable insights into the PD (Tieu 2011; Lim and Ng 2009; Dawson et al. 2010; Jagmag et al. 2016).

Drosophila melanogaster has been recognized as a powerful organism for modeling human neurodegenerative diseases including PD. Firstly, many PD-related genes are found to have homologues in *Drosophila*. Secondly, in *Drosophila melanogaster*, most of DA neurons are generated at embryogenesis, matured and gathered into clusters during first larval stage. In adult flies, nine DA neuron clusters can be distinctively recognized by the position of cell body, dendrite, and the number of DA neuron in each cluster. The feature of *Drosophila* is appropriate for applying *Drosophila* PD models in studying the progressive degeneration of neurons (Blanco et al. 2011; Budnik and White 1988). Together with strong points of shortness in life span, large number of population, and easiness in maintenance, the use of *Drosophila* model for PD study has various advantages in genetic analysis in vivo, generation-population analysis.

4.2 Parkinson's Disease and Models for Studying Parkinson's Disease

4.2.1 Parkinson's Disease

Parkinson's disease (PD), a disorder of the basal ganglia, is recognized as one of the most common neurologic disorders, affecting approximately 1% of individuals older than 60 years old. There are two major neuropathologic findings in PD: the loss of pigmented dopaminergic neurons in the substantia nigra and the presence of Lewy bodies. Most cases of idiopathic Parkinson's disease (IPD) are believed to be due to a combination of genetic and environmental factors. The prevalence of PD is about 0.3% of the whole population in industrialized countries. PD is more common in the elderly, and prevalence rises from 1% in those over 60 years of age to 4% of the population over 80. Although 5–10% of cases, classified as young onset, begin between the ages of 20 and 50, the mean age of onset is around 60 years. Some studies have proposed that it is more common in men than women, but others failed to detect any differences between the two sexes. The incidence of PD is between 8 and 18 per 100,000 person-years (Nussbaum and Polymeropoulos 1997; Thomas and Beal 2007; de Lau and Breteler 2006).

In the brain, dopamine plays an important role in controlling muscle activity. When the levels between dopamine and acetylcholine are equal, damping effect occurred in which the basal ganglia will transmit signals to spinal cord to control muscle activity. However, in the PD patients, it is found that dopamine is not produced. Consequently, levels of dopamine and acetylcholine are imbalance, and damping effect has not occurred. Therefore, muscle could not be controlled and resulted in muscle tension and/or tremor (Mayes-Burnett 2016).

Misfolded proteins are known to involve in Parkinson's disease. Misfolded α-synuclein (SNCA), ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1), parkin, PTEN-induced putative kinase 1 (PINK1), leucine-rich repeat kinase 2 (LRRK2 or dardarin), and DJ-1 caused overloading the ubiquitin (proteasomal) and lysosomal degradation pathways, thereby resulted in neurodegeneration and PD (Tan et al. 2009; Lee and Hsu 2017).

On the other hand, genetic factors related to oxidative stress, mitochondrial dysfunction, accumulation of α-synuclein, or defects in the ubiquitin-proteasome system are also known to involve in PD (Shadrina et al. 2010). Mutations in specific genes have been conclusively shown to cause PD. In most cases, people with these mutations will develop PD. For example, defects in parkin, UCH-L1, and α-synuclein proteins lead to an error in the protein degradation pathway and caused neurodegeneration. Mutant proteins, such as parkin and UCH-L1, which belong to the ubiquitin-proteasome system, may no longer exert their ubiquitin ligase activity, thus damaging the ability of the cellular machinery to detect and degrade misfolded proteins. PINK1, parkin, and DJ-1 play important roles in maintaining the normal function of mitochondria; therefore mutations in these proteins can result in mitochondrial dysfunction (Ebrahimi-Fakhari et al. 2012; Moon and Paek 2015) (Table 4.1).

Some environmental factors including insecticide, MPTP containing herbicide, rotenone, and paraquat are demonstrated as causes of PD. Besides, air pollution, aging, and working environment are also involved to the high risk of PD (Pezzoli and Cereda 2013; Montgomery 1995). The complex interaction between environmental and genetic factors is also thought to result in PD, but the interlink between these factors still remains unknown.

Table 4.1 Parkinson's disease-related proteins

Protein	Organ/functional system
α-Synuclein	Mitochondria, ubiquitin-proteasome system
Parkin	Mitochondria, ubiquitin-proteasome system
UCH-L1	Ubiquitin proteasome system
PINK1	Mitochondria
DJ-1	Mitochondria, ubiquitin-proteasome system
LRRK2	Mitochondria
HtrA2	Mitochondria
GBA	Lysosome
POLG	Mitochondria

Although many genes, proteins, and environmental factors are known to be involved in PD, the mechanism of this disease is still unclear, leading to many limitations in studying and finding PD drugs. In order to find out a therapy for PD, recently, there are many researches focus on mechanism of PD which is based on Lewy body, oxidative stress, mitochondria, and ubiquitin-proteasome system.

4.2.2 Models for Studying Parkinson's Disease

To study on Parkinson's disease, many models have been established and utilized. The models of PD can be divided into two different approaches: toxin-based models (such as 6-OHDA, MPTP, rotenone, and paraquat) and gene-based models (such as α-synuclein, LRRK2, Parkin, DJ-1, PINK1) (Tieu 2011; Lim and Ng 2009; Dawson et al. 2010; Jagmag et al. 2016; Dauer and Przedborski 2003; Hisahara and Shimohama 2011). Many cellular and animal models of PD have been developed to investigate the mechanism of PD and develop new therapeutic strategies. An ideal model of PD should display pathophysiologic features and symptoms of PD; however, the current models are not able to recapitulate all PD features. Each model has both advantages and disadvantages, and the selection of the most suitable model depends on particular purposes of the research study (Fig. 4.1).

4.2.2.1 Cellular Models

Cellular models have been used for studying PD mechanism, drug screening, and developing new therapeutic strategies. In addition to the strengths of cell-based model including easy access of cells in culture and allowing high-throughput screening, PD cellular models can display fea-

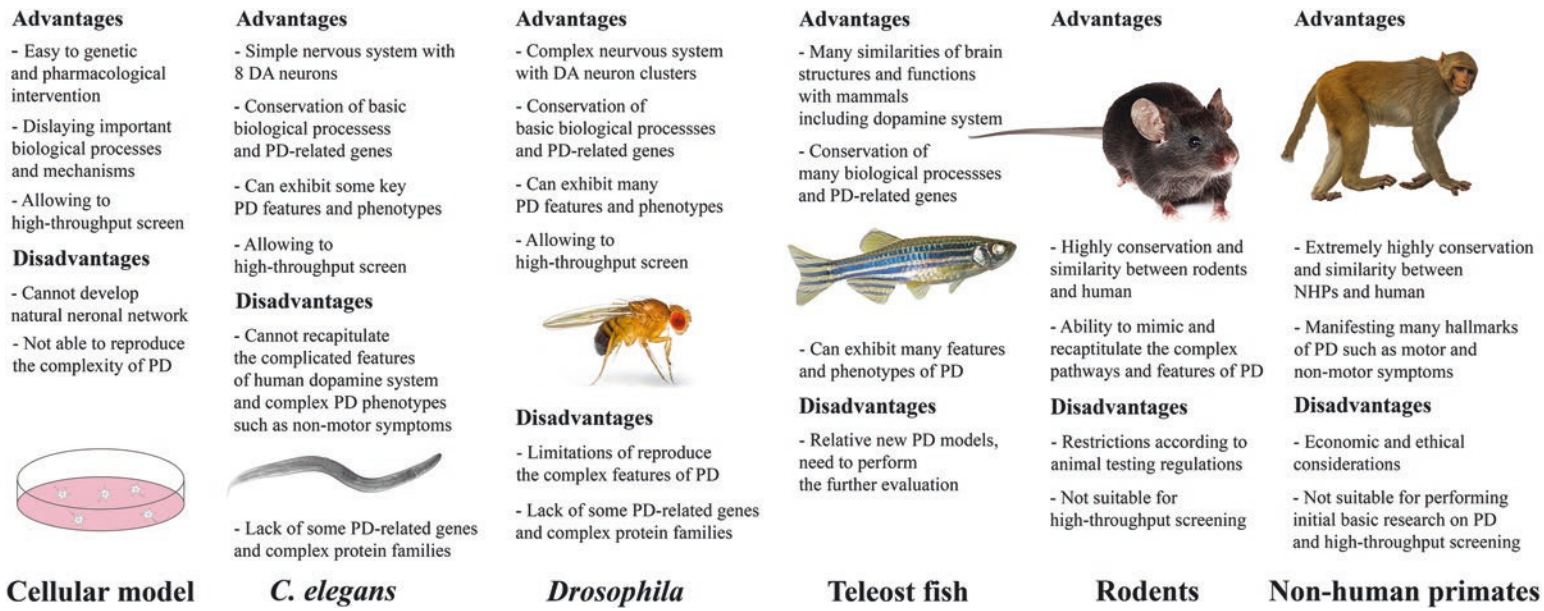

Fig. 4.1 Animal models in studying Parkinson's disease: advantages and disadvantages

tures of PD (such as DA neuron degeneration and protein aggregates containing α-synuclein) and important biological processes (such as apoptosis, oxidative stress, mitochondrial impairment, altered proteolysis, and dysfunctional mitophagy) (Alberio et al. 2012; Falkenburger and Schulz 2006; Falkenburger et al. 2016). However, the weaknesses of cellular models are that culture cells do not develop natural neuronal network and lack the interaction of different cell types and cellular microenvironment; therefore they are not able to reproduce the complexity of PD (Falkenburger and Schulz 2006).

Human neuroblastoma cell line SH-SY5Y and rat pheochromocytoma cell line PC12 are cell lines widely used for modeling PD. They possess the machinery to produce and release catecholamines and can develop neuron-like features. Numerous studies have used these cell lines as PD models to not only screen causative factors that can cause PD as well as compounds that can treat PD but also study the molecular and cellular mechanism related to PD (Xicoy et al. 2017; Malagelada and Greene 2008). Besides that, immortalized lund human mesencephalic (LUHMES) cells can be used for modeling PD because of their ability to differentiate and develop to dopaminergic-like neurons (Zhang et al. 2014). Another approach to model PD is using patient-specific cell lines (Schule et al. 2009). Cybrid (cytoplasmic hybrid) cell lines are created by fusion of mtDNA-lacking cell and donated platelets containing mtDNA from PD patients. The PD cybrid cell lines can represent the impairment in mitochondrial functions and have been used to investigate the relationship between mtDNA gene mutation and mitochondrial dysfunction and PD pathogenesis (Trimmer and Bennett 2009). Recently, the development of human-induced pluripotent stem cells (iPSCs) has supported the studies of human diseases including PD. The abilities to derive iPSCs from PD patients and differentiate these iPSCs into DA neurons exhibiting PD phenotypes enable them to become a promising model to study mechanism and drug discovery (Martinez-Morales and Liste 2012; Byers et al. 2012).

4.2.2.2 Animal Models

There are numerous animal models that have been developed from invertebrates such as nematode roundworm and fruit fly to vertebrates including fish, rodent, and nonhuman primates. The uses of these models have been significantly contributed to our knowledge of PD pathogenesis and potential treatment.

4.2.2.2.1 Nematode Roundworm: *Caenorhabditis elegans*

C. elegans possesses many advantages of modeling PD for studying the complex interaction of genetic and environmental factors and drug screening. This simple organism shares many conserved molecular and cellular pathways to human such as protein degradation machinery, oxidative stress, and signal transduction (Harrington et al. 2010). Specially, *C. elegans* has simple nervous system with exactly 302 neurons including 8 dopaminergic neurons and the conserved dopaminergic pathways (Harrington et al. 2010; Sulston et al. 1975). Although the simple dopaminergic system is useful to study the effects of factors on morphology and number of DA neurons, it cannot recapitulate the complex features of human dopamine neurons.

The *C. elegans* genome encodes many homologues of PD-related genes such as Parkin, PINK1, DJ-1, UCH-L1, and LRRK2, so this organism can be used for studying the functions of these genes involved in PD. For example, the study on Lrk-1, a homolog of LRRK2 in *C. elegans*, demonstrated the role of this protein in regulating cellular responses to mitochondrial dysfunction (Saha et al. 2009). Although there is an absence of the *C. elegans* α-synuclein homolog, transgenic roundworm model which overexpresses human α-synuclein has been developed. The *C. elegans* model established by Lakso et al. showed that the overexpression of α-synuclein in DA neurons led to neurodegeneration (Lakso et al. 2003). Remarkably, a whole genome microarray analysis on α-synuclein-overexpressing *C. elegans* was performed to identify gene expression changes. That supported confirmation of known molecular functions and suggestion of

new pathways related to PD and contributed to understand the role of α-synuclein in PD pathogenesis (Vartiainen et al. 2006). Neurotoxins such as 6-OHDA and MPP+ are also used to develop *C. elegans* models of PD (Li and Le 2013). In addition to study PD mechanism, *C. elegans* is also a suitable model for drug discovery (Chen et al. 2015).

4.2.2.2.2 Fruit Fly: *Drosophila melanogaster*

The completion of the genome sequence showed that 77% of human disease genes are conserved in *Drosophila (*Adams et al. 2000*)*. Notably, many homologues of PD-related genes were identified in fruit fly such as dardarin/LRRK2, parkin, PINK1, Omi/HtrA2, DJ-1, UCH-L1, GIGYF2, PLA2G6, and GBA with exception of α-synuclein, ATP13A2, and FBXO7 (Whitworth 2011). *Drosophila* possesses more complex dopaminergic neuron system containing DA neuron clusters. In larval stage, there are 21 DA neurons grouped into 7 DA neuron clusters per hemisphere: DM1a, DM1b, DM2, DL1a, DL1b, DL2a, and DL2b (Blanco et al. 2011). In adult stage, DA neurons are classified into nine distinct clusters: PAM, PAL, PPM1, PPM2, PPM3, PPL1, PPL2ab, PPL2c, and VUM (Fig. 4.2) (Nassel and Elekes 1992; Mao and Davis 2009). The locations of DA neuron clusters have been identified; the effects of environmental or genetic factors on the number, morphology, or locations of DA neurons can be examined. Moreover, beside the similarity in some main functions of nervous system between human and fly, the basic biological processes such as cell death regulation are also conserved in *Drosophila (*Jennings 2011; Vernooy et al. 2000*)*. Considering these strengths, *Drosophila* is a powerful tool for study of PD.

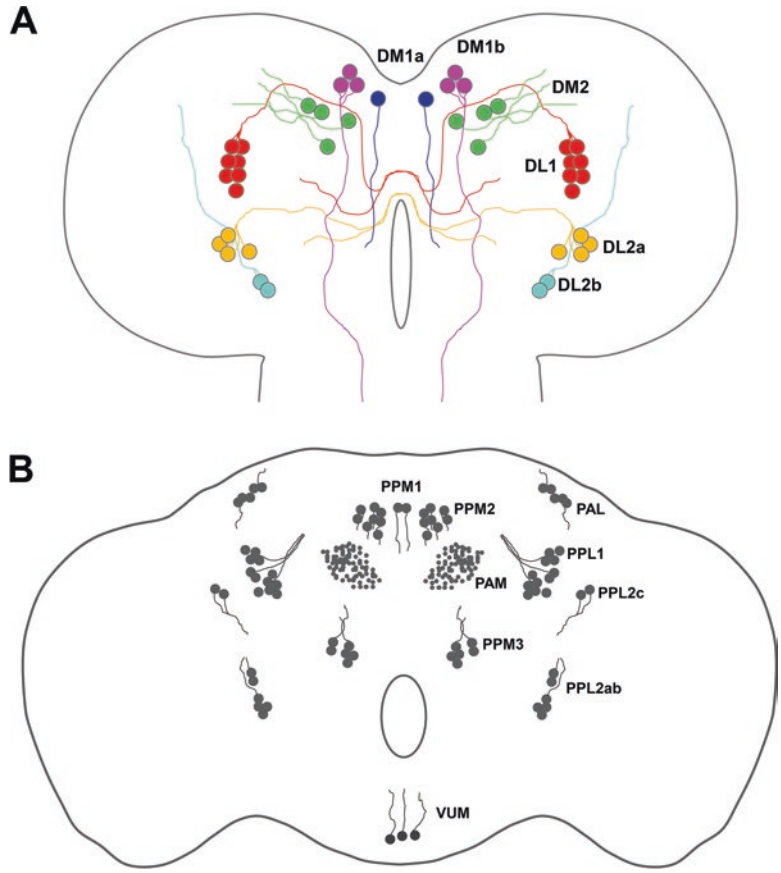

Fig. 4.2 Schematic representation of DA neuron systems in the *Drosophila* larval and adult stages. (**a**) Illustration of six DA neuron clusters DM1a, DM1b, DM2, DL1, DL2a, and DL2b in *Drosophila* larval central brain. The illustration was redrawn based on the study of Blanco et al. (Blanco et al. 2011). (**b**) A schematic representation of seven DA neuron clusters PAL, PAM, PPM1, PPM2, PPM3, PPL1, and PPL2 in *Drosophila* adult central brain. It was redrawn based on the study of Nassel et al. and Mao et al. (Nassel and Elekes 1992; Mao and Davis 2009)

4.2.2.2.3 Teleost Fish: Zebrafish and Medaka Fish

Teleost fish including zebrafish and medaka fish has been widely used as model for studying developmental biology and recently emerged as a new vertebrate model of PD (Matsui and Takahashi 2017). These fish have several strengths such as transparency high fecundity, their rapid development, and ease of maintenance and handling (Xi et al. 2011; Matsui et al. 2012). Notably, teleost fish is a vertebrate, so these fish possess many similarities of brain structures and functions with mammals including dopamine system. DA neuron clusters (A8–A10) in the diencephalon-midbrain are closely related to PD (German et al. 1989). Although teleost midbrain does not contain DA neurons, they are located in the paraventricular organs, the periventricular nucleus of the posterior tuberculum, and the posterior tuberal nucleus. Within these DA neurons, some neurons in the periventricular nucleus of the posterior tuberculum may be equivalent to mammalian A9 and A10 neurons because of their projection pattern (Matsui 2017).

There are several zebrafish and medaka fish models of PD induced by genetic (parkin, PINK1, DJ-1, LRRK2, ATP13A2, and GBA) or toxin factors (MPTP and 6-OHDA). Several fish models exhibited some features of PD including reduction of locomotive ability (swimming movement) and loss of DA neurons (Xi et al. 2011; Matsui et al. 2012). These models have been used for studying the contributions of lysosome dysfunction and mitochondrial dysfunction to PD (Matsui and Takahashi 2017). Recently, Zhang et al. developed zebrafish model combining PINK1 deficiency and rotenone for drug screening (Zhang et al. 2017). However, these teleost fish are relative new PD models; therefore, the further evaluation of these organisms as PD models needs to perform.

4.2.2.2.4 Mammals: Rodent and Nonhuman Primate

The highly conservation and similarity between mammals including rodent and nonhuman primates (NHP) and human make these organisms as good PD models. Rodent and NHP models are expected to exhibit complex features of PD and closely match to human pathology. Similar to abovementioned models, PD rodent models can be classified into environmental models, induced by several neurotoxins such as MPTP and 6-OHDA and genetic models with knock-in or knockout of PD-related genes. These models have provided insight into pathways involved in PD and contributed to therapeutic development (Vingill et al. 2017). The well-established NHP model of PD is induced by MPTP and manifests many hallmarks of PD including DA cell loss and motor and non-motor symptoms such as cognitive impairment and sleep/wake disturbances (Porras et al. 2012). Recently, another approach to model PD NHP model is using AAV1/2 vector to overexpress α-synuclein; however, this methodology is relatively new and needs extended study (Koprich et al. 2016). NHP model has been used as a preclinical model of PD and plays an important role in developing treatment therapies for PD (Blesa et al. 2017). However, the use of these mammalian models is limited by economic and ethical considerations, and these models are not suitable for performing initial research on PD because of their complexities.

4.3 *Drosophila* Model in Studying Parkinson's Disease

4.3.1 *Drosophila* Models of Parkinson's Disease

Many *Drosophila* models of Parkinson's disease based on pathogenic molecular mechanisms have been developed, either by gene transfer or by induction with poison. Fly models have been reported to exhibit strong PD-like phenotypes characterized by locomotion defects and DA neuron degeneration as well as defects associated with mitochondrial dysfunction, oxidative stress, and protein aggregation (Whitworth 2011). Many *Drosophila* models of PD induced by genetic factors including α-synuclein, LRRK2, Parkin, DJ-1, and PINK1 and environmental factors such as rotenone and paraquat have been developed, and studies on these models provided some profound insights into PD pathogenesis (Whitworth 2011; Navarro et al. 2014) (Table 4.2). For instance, research on *Drosophila* has clarified the functions

Table 4.2 *Drosophila* models of Parkinson's diseases

Toxin-based models of PD						
Toxin			PD-like phenotypes			Relevant biological processes
			Locomotive defects	LB-like aggregations	Loss of DA neurons	
Rotenone			Yes (Coulom and Birman 2004)	No (Coulom and Birman 2004)	Yes (Coulom and Birman 2004)	Mitochondrial oxidative stress (Hosamani et al. 2010), and the mitochondrial fusion/fission machinery (Hwang et al. 2014)
Paraquat			Yes (Ameel et al. 2007)	No data	Yes (Ameel et al. 2007)	Oxidative stress, mitochondrial dysfunction (Shukla et al. 2016; Hosamani 2013), and DNA damage (Mehdi and Qamar 2013)
Genetic-based models of PD						
Gene	*Drosophila* homolog (identity)	Genetic intervention	PD-like phenotypes			Relevant biological processes
			Locomotive defects	LB-like aggregations	Loss of DA neurons	
SNCA	No	Expression of human WT/ A30P/ A53T	Yes (Feany and Bender 2000)	Yes (Feany and Bender 2000)	Yes (Feany and Bender 2000)	Lipid metabolism, energy production, membrane transport (Scherzer et al. 2003), and oxidative stress (Botella et al. 2008; Trinh et al. 2008)
		Expression of S129D	No data	Yes (Chen and Feany 2005)	Yes (Chen and Feany 2005)	
		Expression of WT 1–120 construct	No data	Yes (Periquet et al. 2007)	Yes (Periquet et al. 2007)	
LRRK2	dLRRK (26%) (Whitworth 2011)	Expression of human WT/ G2019S	Yes (Liu et al. 2008)	No data	Yes (Liu et al. 2008)	Oxidative stress, protein translation (Imai et al. 2008), energy demand (Hindle et al. 2013), vesicular transport (Dodson et al. 2012, 2014; Arranz et al. 2015; Linhart et al. 2014), and cytoskeleton regulation (Lee et al. 2010)
		Expression of human R1441C	Yes (Islam et al. 2016)	No data	Yes (Islam et al. 2016)	
		dLRRK null mutant	Yes (Lee et al. 2007)	No data	No (Lee et al. 2007; Wang et al. 2008)	
Parkin	Parkin (42%) (Whitworth 2011)	Expression of human Q311X/T240R	Yes (Sang et al. 2007)	No data	Yes (Sang et al. 2007)	Mitochondrial dysfunction, apoptosis (Greene et al. 2003), mitochondrial fusion/ fission machinery (Deng et al. 2008), oxidative stress, innate immune responses (Greene et al. 2005; Whitworth et al. 2005), and ER stress (Celardo et al. 2016)
		Parkin null mutant	Yes (Whitworth et al. 2005)	No data	Yes (Whitworth et al. 2005)	

(continued)

Table 4.2 (continued)

DJ-1	DJ-1α (56%) (Whitworth 2011)	DJ-1α null mutant	No data	No data	No (Meulener et al. 2005)	Oxidative stress, apoptosis (Yang et al. 2005; Hwang et al. 2013), and mitochondrial dysfunction (Hao et al. 2010)
		Knockdown of DJ-1α by Ddc-Gal4, TH-Gal4, and Elav-Gal4	No data	No data	Yes (Yang et al. 2005)	
	DJ-1β (52%) (Whitworth 2011)	DJ-1β null mutant	Yes (Park et al. 2005; Lavara-Culebras and Paricio 2007)	No data	No (Park et al. 2005; Lavara-Culebras and Paricio 2007)	
PINK1	PINK1 (32%) (Whitworth 2011)	PINK1 null mutant	Yes (Park et al. 2006)	No data	Yes (Park et al. 2006)	Mitochondrial dysfunction (Park et al. 2006) and mitochondrial fusion/fission machinery (Yang et al. 2008)
		Knockdown of PINK1 by Da-Gal4 or TH-Gal4	Yes (Yang et al. 2006)	No data	Yes (Yang et al. 2006)	
GBA	dGBA1a (32%) dGBA1b (31%) (Whitworth 2011)	Double heterozygous dGBA1a and dGBA1b mutant	Yes (Maor et al. 2013)	No (Maor et al. 2016)	Yes (Maor et al. 2016)	ER stress (Maor et al. 2016; Suzuki et al. 2013)
		Expression of human N370S/L444P	Yes (Maor et al. 2013)	No data	Yes (Maor et al. 2016)	
UCH-L1	dUCH (45%) (Whitworth 2011)	Knockdown of dUCH by TH-Gal4	Yes	No data	Yes	Oxidative stress

of PINK1 and parkin which are associated with familial forms of PD. Many studies on fly model were performed and showed that parkin acts as a downstream of PINK1, and this pathway regulates mitochondrial integrity and mitochondrial fission/fusion dynamics (Guo 2010). Besides that, *Drosophila* has been also considered as a model for high-throughput screening of candidate compounds that can prevent this disease and developing therapeutic strategies (Whitworth 2011; Whitworth et al. 2006). Fly with PD symptoms caused by oxidative stress can be used for rapid screening of potential therapeutic antioxidant drugs in treating PD such as melatonin with the paraquat model and polyphenols with the α-synuclein model (Medina-Leendertz et al. 2014; Takahashi et al. 2015).

4.3.2 Parkinson's Disease Symptoms and PD-Like Phenotypes in *Drosophila* Models

The basic symptoms of Parkinson's disease are difficulty in walking, slow movement, stiff and trembling limbs, balance disorders, and facial paralysis. Symptoms appear gradually and not marked, and it is difficult to recognize and often may be confused with other diseases. Causes are attributed to lack of dopamine, a chemical that plays an important role in nerve signal transmission, due to degeneration/loss of dopaminergic neurons. Besides, the presence of Lewy body was also reported as one of the PD symptoms although it is not clear to be a cause or a result of PD

(Nussbaum and Polymeropoulos 1997; Mayes-Burnett 2016; Fahn and Sulzer 2004).

4.3.2.1 PD-Like Phenotype of Movement

In fly model, the progressive impairment in locomotive ability of PD has been characterized through crawling ability in larval stage and climbing ability in adult stage. Many studies on *Drosophila* PD models showed the similarity of locomotor behaviors including decline in climbing ability of *Drosophila* overexpressing human wild-type and PD-related mutant forms of alpha-synuclein, reduction in crawling ability of parkin mutant third instar larvae and locomotor dysfunction, and early mortality in *Drosophila* overexpressing human wild-type and PD-associated mutant forms of LRRK2 (Feany and Bender 2000; Liu et al. 2008; Sang et al. 2007).

The assay to quantify the locomotor ability of *Drosophila* larvae (crawling assay) was first described by Min and Condron in 2005 (Min and Condron 2005). In this assay, larvae in the third instar stage were randomly picked up from PD fly models and placed on agar plate to examine crawling ability. Larval movement was recorded, and then the recorded videos were analyzed to track larval movement and draw motion paths. The average velocity was also calculated, statistically analyzed, and graphed. The PD model larvae displayed a tremor-like behavior which was tracked as tight wavy line when moving horizontally on agar plates. Additionally, these larvae accomplished a shorter moving path compared to normal flies. The mean velocity of PD larvae was also reduced in comparison with the normal flies (Fig. 4.3).

Locomotor ability of adult flies can be estimated by startle-induced negative geotaxis assay which was first described in 1992 by Le Bourg and Lints as climbing activity (Le Bourg and Lints 1992). Flies were transferred from food vials to climbing cylinders and then were tapped to the bottom, and the movement of flies was recorded. The data then were statistically analyzed. The PD model flies showed the decline in climbing ability in comparison with normal flies (Fig. 4.4).

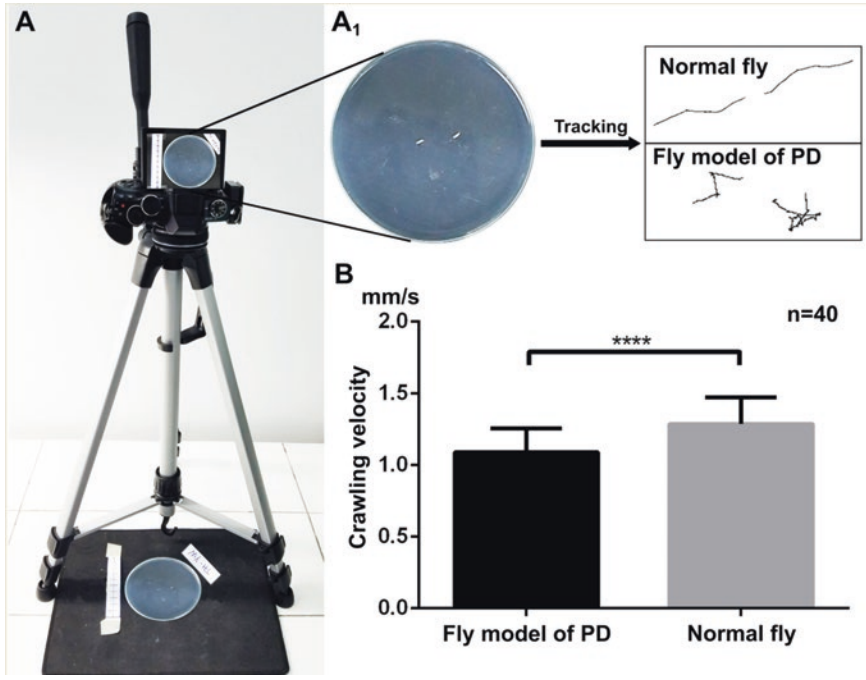

Fig. 4.3 PD-like phenotype of movement in larvae can be scored by crawling assay. (**a**) Larval movement and draw motion path. (**b**) Crawling velocity

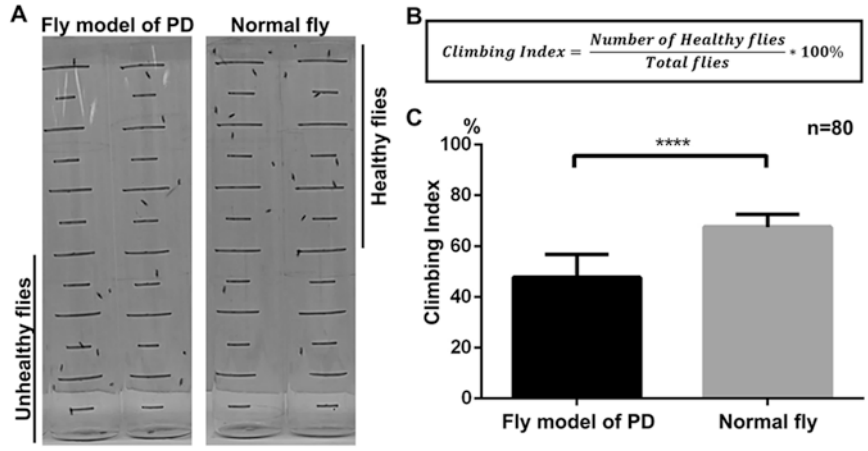

Fig. 4.4 Climbing assay: an acquisition of PD-like phenotype of movement in adult fly. (**a**) Visualization of climbing assay. (**b**) Formula of climbing index. (**c**) A representation of climbing index

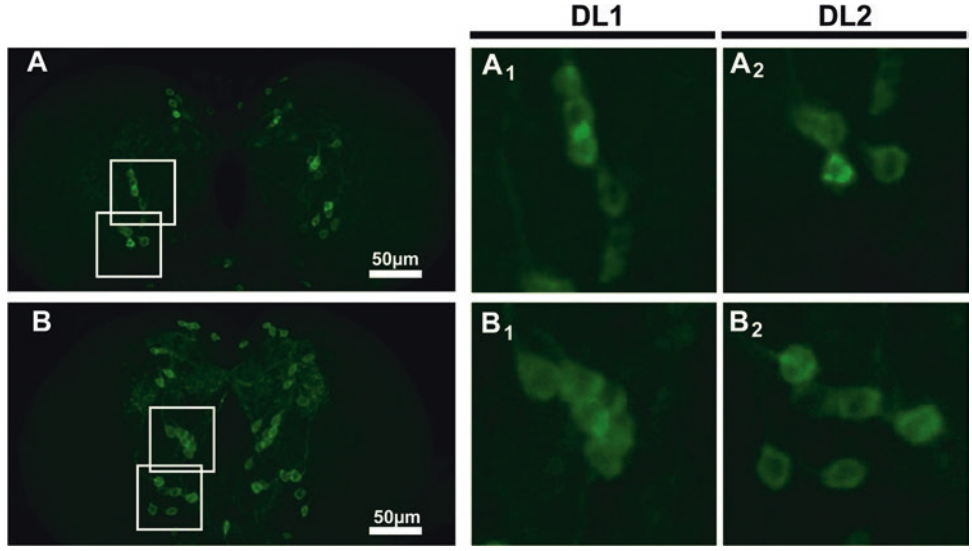

Fig. 4.5 The loss of DL1 and DL2 dopaminergic neurons in larval brain with knockdown of dUCH, a homolog of UCH-L1 in *Drosophila*. DA neuron clusters in the third instar larval central brain were stained with anti-TH. (**a**) PD model larval brain. (**b**) Normal larval brain. The boxed area marks DL1 and DL2 clusters were magnified in A_1 and B_1 and A_2 and B_2. Number of DA neurons in DL1 and DL2 clusters in PD model larval brain was less than those in normal flies

4.3.2.2 PD-Like Phenotype of DA Degeneration

Since *Drosophila* possess a complex dopaminergic neuron system containing DA neuron clusters, fly models can emulate PD symptom of DA loss/degeneration. DA neuron in fly can be visualized by immunostaining with anti-tyrosine hydroxylase (anti-TH), an enzyme that plays a key role in dopamine synthesis pathway. Number of DA in each DA cluster can be examined at both larval and adult stage (Figs. 4.5 and 4.6).

4.3.2.3 PD-Like Phenotype of Aging-Dependent Progression

Parkinson's disease is not only characterized by the degeneration but also by the progressive loss

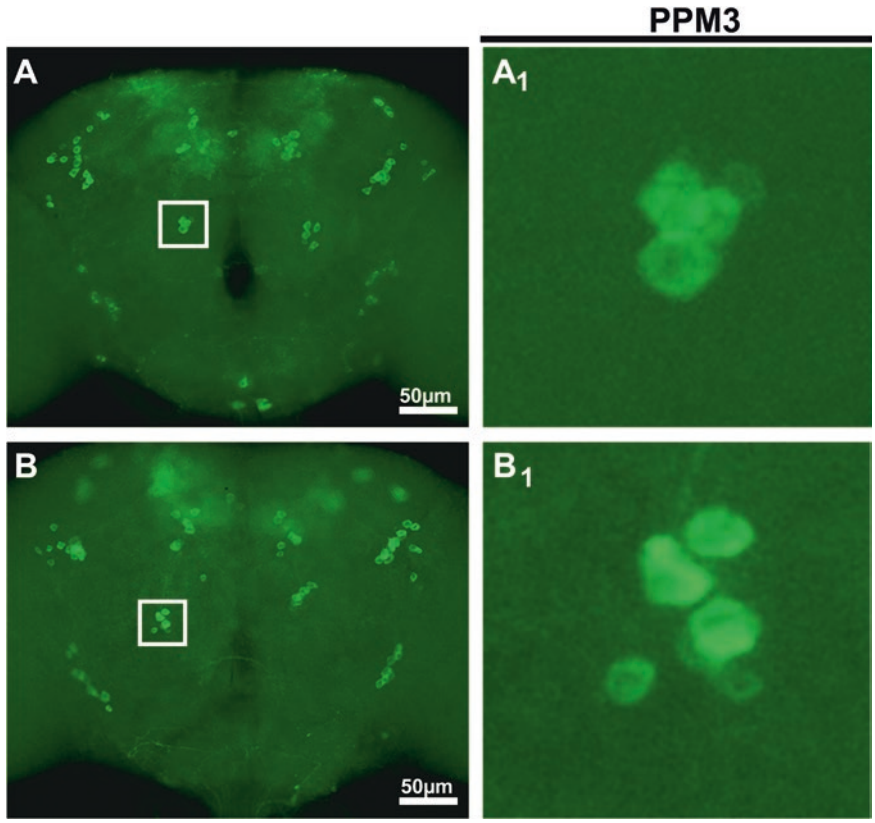

Fig. 4.6 The susceptibility of PPM3 dopaminergic neurons in adult brain of dUCH knockdown fly. DA neuron clusters in fly brain were stained with anti-TH. (**a**) PD model fly brain. (**b**) Normal fly brain. The boxed area marks PPM3 cluster was magnified in A_1 and B_1

of DA neurons in the course of aging. The short life span of *Drosophila* makes it convenient to perform aging-dependent analysis in relatively short time periods. Thereby, *Drosophila* models of PD own a strong point in observing the aging-dependent PD characteristic. The observation of DA neurons in fly brain can be performed from 1-day-old to 40-day-old adult fly brains to see if PD model brains exhibit gradual reduction in the number of DA neurons.

Besides, in the epidemiological point of view, the percentage of individuals with PD in the population increases throughout aging. The most advantage of *Drosophila* models in studying PD is the easiness to handle numerous samples at one time, by which *Drosophila* models can provide reliable data for statistical analysis without bias. Together with a strong point of life span shortness, *Drosophila* serves as a good model for calculating the percentage of flies which showed aberrant DA neuronal phenotype in PD model fly population from 1 to 40 days old. The prevalence can be count in correlation with aging (Fig. 4.7).

4.3.2.4 PD-Like Phenotype of Dopamine Shortage

Reduction of neurotransmitter dopamine was found in PD patients and was declared as PD clinical symptoms (Jankovic 2008). Dopamine is mainly produced in DA neurons through catecholamine biosynthesis pathway. Dopamine in fly brains can be quantified by high-performance liquid chromatography (HPLC). Adult fly heads are collected and homogenized in homogenization buffer containing 0.1 M perchloric acid and 3% trichloroacetic acid. Supernatants of the homogenates are used for performing HPLC. Studies on *Drosophila* model have dem-

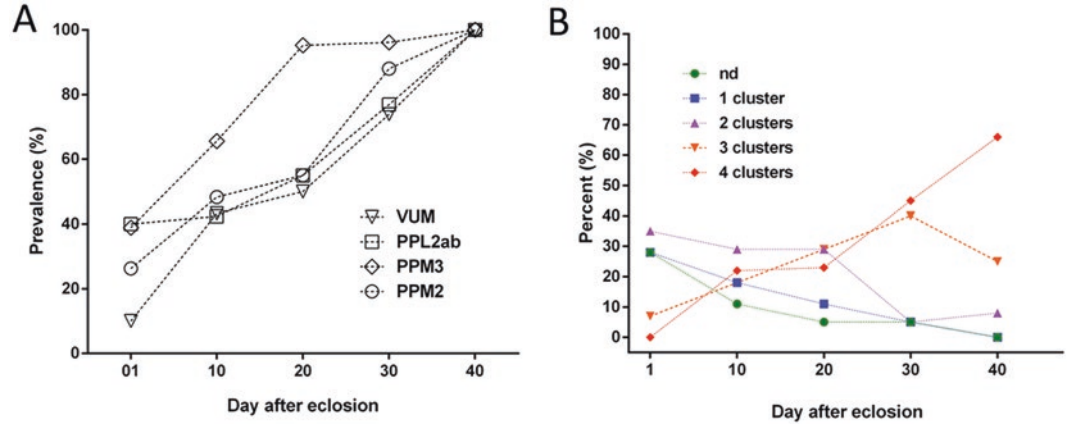

Fig. 4.7 The progressive loss of DA neurons in the course of aging in dUCH knockdown fly brain. (**a**) Prevalence was increased in correlation with aging in VUM, PPL2ab, PPM3, and PPM2 clusters. The prevalence in PPM2 and VUM increased in regular manner from 1 to 40 days old, while the prevalence in PPM3 increased rapidly from 1 to 20 days old and then went to stationary phase from 20 to 40 days old, and the prevalence in PPL2ab slowly increased from 1 to 40 days old. (**b**) The percentage of dUCH knockdown flies with no damage on DA neuron system was 28.6% at 1 day old and decreased regularly from 1 to 40 days old. The similar phenomenon occurred in one and two DA cluster-damaged flies with 28.6% and 35.7%, respectively, at 1 day old, and they also decreased regularly from 1 to 40 days old. In contrast, three and four DA cluster-damaged flies with 7.1% and 0%, respectively, at 1 day old experienced rapid increases from 1 to 30 days old

onstrated the *Drosophila* locomotor activity involved in dopamine level (Riemensperger et al. 2013). In addition, some other *Drosophila* life activities such as olfactory conditioning, sleep and arousal regulation, and memory and learning process also relate to dopamine production (Selcho et al. 2009; Ueno et al. 2012; Berry et al. 2012). Those mentioned activities are known as non-motor features of PD.

4.3.2.5 Lewy Body-Like Aggregation in *Drosophila*

Lewy body (LB), a fibrillar aggregation in brain, has been considered as a histological hallmark of Parkinson's disease (PD). Since neuronal loss is found in predilection sites of LBs, LB formation has been considered as a marker for neurodegeneration. The main component of LB is known to be α-synuclein (α-syn), which is the first protein in which mutants A30P and A53T were found to cause PD. Ectopic expression of human α-syn either wild-type or PD-linked mutants (A53T and A30P) in *Drosophila* mimics some aspects of PD such as locomotion dysfunction, LB accumulation, and neurodegeneration (Feany and Bender 2000; Chen and Feany 2005; Periquet et al. 2007). Furthermore, role of molecular chaperones and protein degradation systems in protecting against α-syn misfolding has been investigated by using α-syn *Drosophila* models (Mizuno et al. 2011).

4.3.2.6 Non-motor PD Phenotypes in *Drosophila*

In addition to impairment in locomotion, Parkinson's disease is also known as a multisystem disorder with non-motor features. Throughout the course of PD, reflecting the neurodegeneration, various clinical symptoms have been observed in PD patients. The symptoms are involved not only in the dopaminergic degeneration but also in damaging of other brainstem areas such as serotonergic, noradrenergic, and cholinergic frontal brainstem (Perez-Lloret and Barrantes 2016). Non-motor symptoms in PD occurred throughout the course of the disease either in early or late stage. In later development of PD, several non-motor symptoms including sleep, smell, and mood problems have been observed. Some symptoms such as sleep and autonomic disturbances occurred diversely in early and later PD stages. Other non-motor features are also found in de novo, untreated PD

patients such as cognitive impairment and autonomic dysfunction (Perez-Lloret and Barrantes 2016; Goldman and Postuma 2014). In *Drosophila*, modeling of PINK1 and parkin loss-of-function mimic a range of non-motor PD features. Abnormalities in learning and memory were recorded in both Pink1 and Parkin *Drosophila* models of PD. Besides, weakness of circadian rhythm was also observed (Julienne et al. 2017). The *Drosophila* model of PD therefore showed its advantage in studying PD with non-motor phenotypes.

4.4 *Drosophila* Model of Parkinson's Disease and Applications

4.4.1 The Contributions of *Drosophila* to Study PD

After Feany and Bender established the first *Drosophila* model of PD by expressing normal and mutant forms of human α-synuclein in 2000 (Feany and Bender 2000), numerous *Drosophila* models have been developed induced by both environmental and genetic factors for studying PD. Research on *Drosophila* has provided several important insights into PD pathogenesis. One of the outstanding contributions of fly model is elucidating the endogenous functions of PINK1 and parkin from studies on *Drosophila* homologues of these genes. The studies on fly model have provided the strong evidence that PINK1 and parkin function in regulating mitochondrial integrity. Flies with null mutants in parkin manifest locomotive impairment, mitochondrial defects, and DA neuron degeneration (Greene et al. 2003; Whitworth et al. 2005; Pesah et al. 2004). Subsequent studies showed that PINK1 mutants resulted in phenotypes similar to parkin mutants including mitochondrial dysfunction. Furthermore, overexpression of parkin can suppress the phenotypes induced by PINK1 mutant, whereas PINK1 overexpression cannot rescue parkin mutant phenotypes. The data indicated that Parkin functions downstream of PINK1 in a common pathway for maintaining mitochondrial integrity (Park et al. 2006; Yang et al. 2006; Clark et al. 2006). Notably, these findings on *Drosophila* model are consistent with human and mice. Cells from PD patient with parkin or PINK1 mutants and human cell with knockdown of PINK1 showed defects in mitochondrial morphology and functions (Muftuoglu et al. 2004; Grunewald et al. 2010; Gegg et al. 2009; Exner et al. 2007). The observations in mouse models indicated that knockout of PINK1 or parkin also caused impairments in mitochondrial respiration but not morphology (Palacino et al. 2004; Gautier et al. 2008). Moreover, aberrant mitochondrial morphology in PINK1 knockdown cell was rescued by expression of parkin (Exner et al. 2007).

The further investigations on *Drosophila* showed that PINK1 and parkin play important roles in mitochondrial dynamics and mitophagy. Several studies indicated that PINK1 and parkin interact with regulators of fusion/fission machinery. The phenotypes of parkin or PINK1 mutants such as defects in locomotive abilities and mitochondrial morphology were suppressed by overexpression of fission factor drp1 (dynamin-related protein 1) or reduction of fusion factors mfn (mitofusin) and opa1 (optic atrophy 1) (Deng et al. 2008; Yang et al. 2008; Poole et al. 2008; Park et al. 2009). The data show that PINK1/parkin pathway promotes mitochondrial fission and/or inhibits fusion. Subsequently, Parkin was demonstrated to induce the ubiquitination of Mfn in fly models (Poole et al. 2010; Ziviani et al. 2010) and mammalian cells (Tanaka et al. 2010). Moreover, PINK1/parkin pathway also promotes mitophagy. A study in fly model using proteomic approach showed that parkin null mutants slowed the mitochondrial protein turnover and PINK1 mutants resulted in selective impairment in mitochondrial respiratory chain subunit turnover. The study on *Drosophila* model of PD provides the evidence of the function of PINK1/parkin pathway in mitophagy (Vincow et al. 2013).

In addition to studying functions of PINK1 and parkin, *Drosophila* model also provided key insights into the relationship between other genetic and environmental factors and biological processes, as well as the interaction of these factors. For example, *Drosophila* models of PD

induced by toxins showed that rotenone toxicity is related to mitochondrial oxidative stress (Hosamani et al. 2010) and the mitochondrial fusion/fission machinery (Hwang et al. 2014). In PD fly models induced by genetic factors, several studies indicated that dLRRK/LRRK2 is involved in processes including oxidative stress, protein translation (Imai et al. 2008), energy demand (Hindle et al. 2013), vesicular transport (Dodson et al. 2012, 2014; Arranz et al. 2015; Linhart et al. 2014), and cytoskeleton regulation (Lee et al. 2010). Another PD-related gene, dDJ-1/DJ-1, was reported to play roles in oxidative stress response, apoptosis (Yang et al. 2005; Hwang et al. 2013), and mitochondrial function (Hao et al. 2010). Moreover, the sensitivity of dDJ-1 mutant flies to oxidative stress-inducing toxin exposure suggested that dDJ-1 play a role in the protection from environment oxidative stress and provided a link between genetic and environmental factors in PD pathogenesis (Meulener et al. 2005). In other studies, dDJ-1 knockout flies exhibited mitochondrial defects, and upregulation of dDJ-1 can rescue muscle defects caused by PINK1, but not parkin, mutants. The results obtained in this study suggested complex interaction between DJ-1 and PINK1/parkin pathway (Hao et al. 2010).

Previous studies implicated mitochondrial dysfunction, oxidative stress, altered proteolysis, and inflammation in the pathogenesis of PD (Shadrina et al. 2010; Dexter and Jenner 2013; Klemann et al. 2017). The complex interaction between environmental and genetic factors is considered to result in PD; however, the roles of these factors as well as the interactions between them leading to this disease have not yet been elucidated in detail. The findings in *Drosophila* model contribute to our knowledge about PD pathogenesis.

4.4.2 The Applications of *Drosophila* to Genetic and Drug Screening

Drosophila possesses many useful features such as short life cycle, available genetic tools for manipulation of genome, and the conservation of basic biological processes and PD-related genes. Besides that, many key neuropathologic and clinical features of PD are reproduced in fly model. Therefore, *Drosophila* is also considered as a powerful tool for genetic and drug screening. The genetic screens allow genomic-wide analysis of genetic interactions to identify genes that can enhance or suppress the phenotypes caused by a mutant gene of interest (Fig. 4.8). For instance, *Drosophila* was used in a genome-wide screening project for modifiers parkin and PINK1 mutant phenotypes. In the study, flies with knockdown of parkin or PINK1 and PINK1 null mutant were crossed with deficiency lines, and analysis of wing phenotype, longevity, and fertility was performed. By analyzing cytological regions interacting with parkin and/or PINK1, five candidate genes were identified including opa1, drp1, dbr, Pi3K21B, and β4GalNAcTA (Fernandes and Rao 2011). Another study identified acon (aconitase) as a dominant suppressor of PINK1 by performing a genetic modifier screening in PINK1 mutant fly model (Esposito et al. 2013).

In the field of compound screening, there are two distinct approaches. The first approach is screening toxins that can induce abnormal phenotypes in wild-type flies. The second approach is testing drug that can rescue aberrant phenotypes induced by mutation, RNAi, transgenesis, or chemical (Giacomotto and Ségalat 2010). Drug screening on *Drosophila* model helps to discover potential therapeutic compounds for PD (Fig. 4.9). For example, dDJ-1β mutant fly was used for performing modifier compound screen. This study identified candidate chemicals such as dexrazoxane, tocopherol, sodium phenylbutyrate, dalfampridine, methylene blue, and minocycline that are able to improve climbing ability. Furthermore, these positive candidate compounds also attenuate H2O2-induced cytotoxicity of DJ-1 mutant human cells (Sanz et al. 2017). In another study, *Drosophila* expressing human mutant LRRK2 (G2019S) was utilized to validate seven phenolic compounds which show kinase inhibitor activity. The results showed that piceatannol, thymoquinone, and esculetin reduced oxidative stress and the loss of DA neurons and locomotor defects caused by expressing

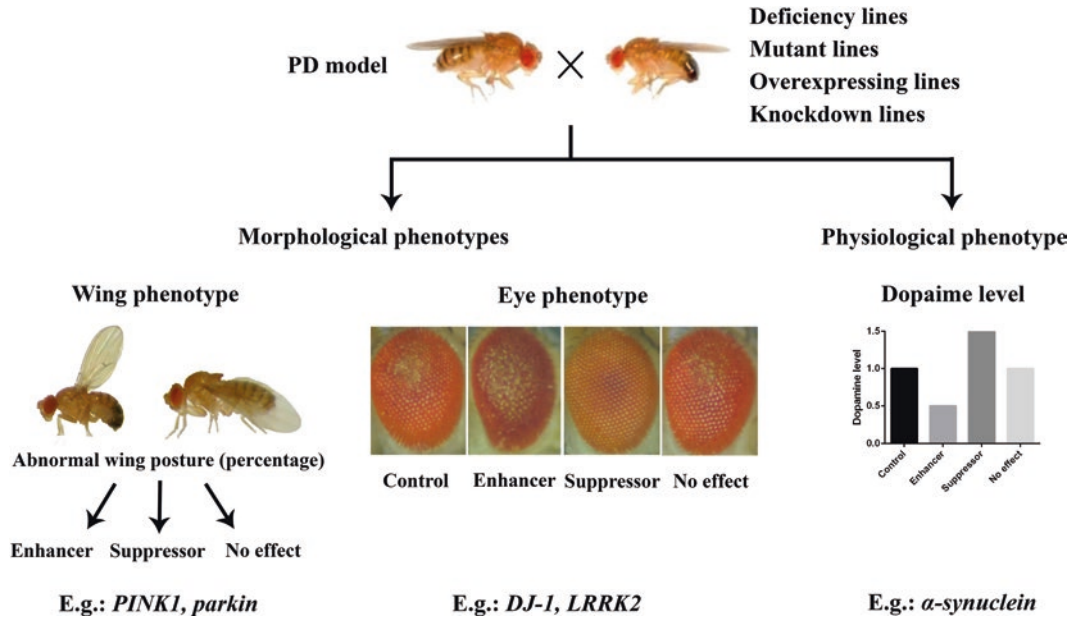

Fig. 4.8 Application of PD-like *Drosophila* model in genetic screening. PINK1, pink (Fernandes and Rao 2011), DJ-1 (Yang et al. 2005), LRRK2 (Venderova et al. 2009), and α-synuclein (Butler et al. 2012)

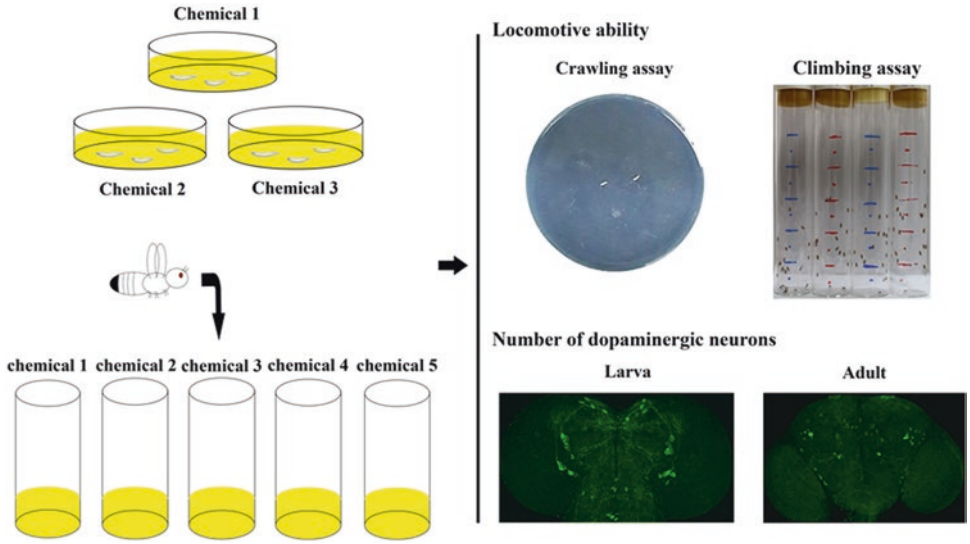

Fig. 4.9 Application of PD-like *Drosophila* model in drug screening

G2019S (Angeles et al. 2016). The other examples of drug screening dVMAT mutant fly were used for screening 1000 known drugs to evaluate the effects of these drugs on locomotor deficits (Lawal et al. 2014). In addition to identifying potential therapeutic compounds, these studies also support the use of *Drosophila* for PD drug discovery.

4.5 Conclusion and Perspective

After Alzheimer disease, PD is the second most common neurodegenerative disease. PD is more commonly associated with motor dysfunction and DA neurodegeneration and is known to show a range of non-motor features. Although many studies demonstrated links of PD to several genetic and environmental factors, mechanism of PD still remains as an interest to investigate. The more PD mechanism is understood, the more advantages in PD therapy and prevention are gained. Currently, it seems to have no potent therapy to cure PD; the application of medicine has just help to control PD symptoms. Therefore, many cellular and animal models of PD have been developed to study PD and discover drug for PD. Among those models, *Drosophila* has been successfully used to mimic PD phenotypes. The *Drosophila* model of PD well displays PD symptoms either motor symptoms, DA neurodegeneration or non-motor symptoms. Owning quite a lot of advantages such as short life span, genetic similarity with PD-related genes, and easiness in maintenance with large populations, *Drosophila* model of PD so far has had a great contribution in PD study. It enables us to further work that may help to understand PD mechanisms, thus identifying new targets for PD treatments.

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Repeat Expansion Disease Models

5

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Abstract

Repeat expansion disorders are a group of inherited neuromuscular diseases, which are caused by expansion mutations of repeat sequences in the disease-causing genes. Repeat expansion disorders include a class of diseases caused by repeat expansions in the coding region of the genes, producing mutant proteins with amino acid repeats, mostly the polyglutamine (polyQ) diseases, and another class of diseases caused by repeat expansions in the noncoding regions, producing aberrant RNA with expanded repeats, which are called noncoding repeat expansion diseases. A variety of *Drosophila* disease models have been established for both types of diseases, and they have made significant contributions toward elucidating the molecular mechanisms of and developing therapies for these neuromuscular diseases.

Keywords

Repeat expansion diseases · *Drosophila* · Polyglutamine diseases · Noncoding repeat expansion diseases · Neurodegenerative diseases · Spinocerebellar ataxia · Amyotrophic lateral sclerosis · RNA foci · Repeat-associated non-ATG translation

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5.1 Introduction

In 1991, expansion mutations of repeat sequences in the genome were discovered to cause human hereditary diseases, namely, a CGG trinucleotide repeat expansion in the fragile X mental retardation 1 (*FMR1*) gene causing fragile X syndrome (FXS) and a CAG trinucleotide repeat expansion in the androgen receptor (*AR*) gene in spinal-bulbar muscular atrophy (SBMA) (La Spada et al. 1991; Verkerk et al. 1991). Since these initial findings, more than 23 expansion mutations of 3 or more nucleotide repeats were found to cause various inherited neurological and neuromuscular diseases (Table 5.1) (La Spada and Taylor 2010). These repeat expansion disorders are largely classified into two groups depending on the location of the repeat sequences in the genome, i.e., the coding region or the noncoding region.

In the former group, expanded CAG repeats produce proteins containing an expanded polyglutamine (polyQ) tract, triggering neurodegeneration via toxic gain-of-function mechanisms in Huntington's disease (HD); spinocerebellar ataxia (SCA) types 1, 2, 3, 6, 7, and 17; dentatorubral-pallidoluysian atrophy; and SBMA, which are collectively called the polyQ diseases (Katsuno et al. 2014; Takeuchi and Nagai 2017). Expansions of the polyQ tract are thought to trigger misfolding and aggregation of these causative proteins, eventually causing

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Table 5.1 Clinical and molecular characteristics of repeat expansion disorders in which repeat sequences are located in the coding region of a gene

Disease	Repeat	Gene	Repeat length		Main clinical features
			Normal	Disease	
HD	CAG	*HTT*	6–35	36–180	Chorea, psychiatric disturbance, dementia
SCA1	CAG	*ATXN1*	6–39	39–83	Ataxia, bulbar palsy
SCA2	CAG	*ATXN2*	14–32	32–200	Ataxia, bulbar palsy, parkinsonism
SCA3	CAG	*ATXN3*	12–41	55–84	Ataxia, spasticity, parkinsonism
SCA6	CAG	*CACNA1A*	4–19	20–33	Ataxia, nystagmus
SCA7	CAG	*ATXN7*	4–35	37–306	Ataxia, retinal degeneration
SCA17	CAG	*TBP*	25–44	46–63	Ataxia, dementia, parkinsonism
DRPLA	CAG	*ATN1*	6–36	49–88	Ataxia, myoclonic epilepsy, choreoathetosis, dementia
SBMA	CAG	*AR*	9–36	38–65	Muscle weakness, bulbar palsy
OPMD	GCN	*PABPN1*	6–10	12–17	Ptosis, bulbar palsy

HD, Huntington's disease; SCA, spinocerebellar ataxia; DRPLA, dentatorubral-pallidoluysian atrophy; SBMA, spinal and bulbar muscular atrophy; OPMD, oculopharyngeal muscular dystrophy; *HTT, huntingtin*; *ATXN, ataxin*, *CACNA1A, calcium channel voltage-gated channel subunit alpha1 A*; *TBP, TATA box-binding protein*; *ATN1, atrophin 1*; *AR, androgen receptor*; *PABPN1, poly(A) binding protein, nuclear 1*

neurodegeneration. Expansion mutations of GCN repeats encoding a polyalanine (polyA) tract have also been reported in oculopharyngeal muscular dystrophy (OPMD) and other diseases, which can lead to both gain-of-function and loss-of-function pathogenic mechanisms (Messaed and Rouleau 2009).

In the latter group, the repeat sequences are located in the noncoding region, such as the 5′-UTR, 3′-UTR, or introns in the genome in FXS and fragile X tremor ataxia syndrome (FXTAS); fragile XE syndrome (FRAXE); myotonic dystrophy (DM) types 1 and 2; Friedreich ataxia; SCA8, 10, 12, 31, 36, and 37; C9orf72-linked amyotrophic lateral sclerosis and frontotemporal dementia (C9-ALS/FTD); and Huntington's disease-like 2 (Table 5.2) (Orr and Zoghbi 2007; Rohilla and Gagnon 2017; Seixas et al. 2017). Since these repeat sequences do not directly encode amino acid sequences in proteins, their pathogenic mechanisms are much more complicated. At least three molecular mechanisms underlying the pathogenesis of these noncoding repeat expansion diseases have been proposed (Nelson et al. 2013; Rohilla and Gagnon 2017). First, loss-of-function of the mutant genes due to silencing of or reduction in gene expression by the repeat expansion mutation has been suggested in FXS, FRAXE, and Friedreich ataxia (Pieretti et al. 1991; Bidichandani et al. 1998). Second, gain-of-function due to aberrant RNAs containing expanded repeats transcribed from the mutant gene have been suggested in most of these diseases, including DM1 and 2, FXTAS, SCA8, 10, 31, and 36, and C9-ALS/FTD. These expanded repeat-containing RNAs were shown to be accumulated as RNA foci in affected tissues and to recruit their corresponding RNA-binding proteins (RBPs), resulting in their loss-of-function (Miller et al. 2000; Mankodi et al. 2001; Jin et al. 2007). Furthermore, a third mechanism has emerged from recent studies, in which expanded repeat RNAs were surprisingly shown to be translated into aberrant repeat polypeptides despite the lack of an initiation codon, via unconventional translation, so-called repeat-associated non-ATG (RAN) translation (Zu et al. 2011; Mori et al. 2013; Ash et al. 2013; Pearson 2011). Subsequent studies demonstrated that these repeat polypeptides produced by RAN translation cause toxicity via gain-of-function mechanisms (Kwon et al. 2014; Mizielinska et al. 2014). However, the molecular mechanisms of RAN translation still remain to be understood, and research toward elucidation of the pathogenic mechanisms of these disorders is still ongoing.

In this chapter, we will introduce a number of studies using a variety of fly models to elucidate

Table 5.2 Clinical and molecular characteristics of repeat expansion disorders in which repeat sequences are located in the noncoding region of a gene

Disease	Repeat	Gene	Repeat location	Repeat length		Main clinical features
				Normal	Disease	
FXS	CGG	*FMR1*	5′-UTR	6–55	> 200	Mental retardation, autism
FXTAS	CGG	*FMR1*	5′-UTR	6–55	55–200	Ataxia, kinetic tremor, parkinsonism
FRAXE	GCC	*FMR2*	5′-UTR	4–39	200–900	Mental retardation
DM1	CTG	*DMPK*	3′-UTR	5–37	50–10,000	Muscle weakness, myotonia, cataract, heart defect
DM2	CCTG	*ZNF9*	Intron	10–26	75–11,000	Muscle weakness, myotonia, cataract
FRDA	GAA	*FXN*	Intron	8–33	> 90	Ataxia, bulbar palsy, scoliosis, cardiomyopathy
SCA8	CTG	*ATXN8*	3′-UTR	15–50	71–1300	Ataxia, bulbar palsy
SCA10	ATTCT	*ATXN10*	Intron	10–29	280–4500	Ataxia, bulbar palsy
SCA12	CAG	*PPP2R2B*	5′-UTR	7–45	51–78	Tremor, ataxia
SCA31	TGGAA	*BEAN/TK2*	Intron	0	45–760	Ataxia
SCA36	GGCCTG	*NOP56*	Intron	3–14	> 650	Ataxia, bulbar palsy, hearing loss
SCA37	ATTTC	*DAB1*	Intron	0	31–75	Ataxia
C9-ALS/FTD	GGGGCC	*C9ORF72*	Intron	2–25	25–1600	Motor neuron disease, frontotemporal dementia
HDL2	CTG	*JPH3*	3′-UTR	6–28	41–58	Chorea, dystonia

FXS, fragile X syndrome; FXTAS, fragile X tremor ataxia syndrome; FRAXE, fragile XE syndrome; DM, myotonic dystrophy; FDRA, Friedreich ataxia; C9-ALS/FTD, C9ORF72-related amyotrophic lateral sclerosis and frontotemporal dementia; HDL2, Huntington's disease-like 2; *FMR1, fragile X mental retardation 1*; *FMR2, fragile X mental retardation 2*; *DMPK, DM1 protein kinase*; *ZNF9, zinc finger 9*; *FXN, frataxin*; *PPP2R2B, protein phosphatase 2 regulatory subunit B, beta isoform*; *BEAN, brain expressed, associated with Nedd4*; *TK2, thymidine kinase 2*; *NOP56, nucleolar protein 56*; *DAB1, Disabled-1*; *C9ORF72, chromosome 9 open reading frame 72*; *JPH3, junctophilin 3*; UTR, untranslated region

the pathogenic mechanisms of these repeat expansion disorders. We will also discuss the advantages of fly models as human disease models for studying pathogenic mechanisms and investigating potential therapies for these disorders.

5.2 Fly Models of Repeat Expansion Disorders

5.2.1 Huntington's Disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by loss of neurons mainly in the striatum and cortex, leading to progressive motor impairments, cognitive decline, and psychiatric symptoms. HD is caused by an abnormal expansion of CAG repeats encoding the polyQ tract in exon 1 of the huntingtin (Htt) gene. In the polyQ diseases, such as HD, there is a threshold length of polyQ repeats for clinical manifestation of approximately 35 to 40 repeats in general, and longer repeats are associated with earlier age of onset and severity of disease. The abnormal aggregation of mutant proteins into nuclear inclusions (NIs) is also commonly observed in the brains of patients with the polyQ diseases (DiFiglia et al. 1997; Becher et al. 1998; Paulson et al. 1997).

Jackson et al. (1998) first generated fly models of HD, each expressing the exon 1 fragment of the Htt protein with tracts of either 2, 75, or 120 glutamine residues (Httex1-Q2, Q75, or Q120, respectively) in photoreceptor neurons of the eye. Expression of Httex1-Q2 had no effect on the fly eyes, whereas expression of Httex1-Q75 or Q120 caused repeat length- and age-dependent degeneration of photoreceptor neurons. Although the mutant Htt protein accumulated in the cytoplasm and nucleus of the photoreceptor neurons just after eclosion, nuclear accumulation of mutant Htt was observed in aged HD flies, suggesting that accumulation of the mutant Htt protein in the nucleus plays a crucial role in neurodegeneration. Gunawardena et al. (2003) also established HD fly models expressing the exon 1 fragment of the Htt protein with a 93 polyQ tract and showed that overexpression of this mutant Htt causes axonal transport defects accompanied by accumulation of the pathogenic Htt protein. Lee et al. (2004) established other HD fly models expressing the longer 548 amino acids fragment of the Htt protein with a 128 polyQ tract and also reported the disruption of axonal transport and accumulation of aggregates at synapses, indicating that cytoplasmic accumulation of the pathogenic Htt protein leads to neuronal dysfunction. Interestingly, they did not find axonal transport defects in flies expressing an expanded polyQ tract alone, which show only nuclear aggregates. On the other hand, new HD fly models expressing the full-length Htt protein containing a 128 polyQ tract have been established (Romero et al. 2008), and these flies showed behavioral, neurodegenerative, and electrophysiological phenotypes. They found that increased neurotransmission rather than axonal transport defects is at the root of the neurodegeneration caused by full-length mutant Htt during the early stages of pathogenesis (Romero et al. 2008). The results of these studies indicate that pathogenic outcomes can be affected by the protein context of the polyQ proteins.

5.2.2 Spinocerebellar Ataxia Type 1

Spinocerebellar ataxia type 1 (SCA1) is a dominantly inherited ataxia characterized by progressive cerebellar ataxia, dysarthria, dysphagia, and variable neurological symptoms and is caused by an abnormal expansion of the CAG trinucleotide repeat in the coding region of the ataxin-1 gene.

Fernandez-Funez et al. (2000) created a fly model of SCA1 by introducing transgenes encoding the full-length human ataxin-1 with a normal (SCA1-Q30) or expanded (SCA1-Q82) length polyQ repeats. Expression of SCA1-Q82 caused progressive neurodegeneration, as expected, and notably, flies expressing SCA1-Q30 at a high level also showed neurodegenerative phenotypes, indicating that even wild-type ataxin-1 can cause neurodegeneration. Genetic modifier screening using the SCA1 fly models identified several modifiers involved in protein folding/degrada-

tion, RNA processing, transcriptional regulation, and cellular detoxification. These findings shed light on a previously unrecognized new pathogenic mechanism of SCA1: the normal function of ataxin-1 could contribute to SCA1 pathogenesis. Subsequent studies also clarified modifiers involved in the signal transduction pathways by genetic interaction analyses using SCA1 fly models, in combination with mammalian-based genetic and proteomic analyses (Chen et al. 2003; Tsuda et al. 2005; Lam et al. 2006; Park et al. 2013).

In addition, genetic interaction between ataxin-1 and ataxin-2 was demonstrated using the SCA1 fly model (Al-Ramahi et al. 2007). The authors showed that wild-type *Drosophila* ataxin-2 is a major genetic modifier of the phenotypes of SCA1-82Q flies. They also showed that nuclear accumulation of ataxin-2 contributes to mutant ataxin-1-induced toxicity. Altogether, these findings suggest common mechanisms of neurodegeneration in different types of ataxia.

5.2.3 Spinocerebellar Ataxia Type 3

Spinocerebellar ataxia type 3 (SCA3), also known as Machado-Joseph disease (MJD), is the most common dominantly inherited ataxia and is characterized by progressive cerebellar ataxia and variable neurological symptoms. SCA3 is caused by an abnormal expansion of the CAG trinucleotide repeat in the coding region of the ataxin-3 gene.

The first genetically engineered fly models that were established for human neurodegenerative diseases were the SCA3 models (Warrick et al. 1998). These SCA3 fly models express the C-terminal region of the ataxin-3 protein containing normal (MJDtr-Q27) or pathogenic (MJDtr-Q78) length polyQ repeats. Expression of MJDtr-Q78 in the eye led to late-onset cell degeneration and NI formation (Fig. 5.1), similarly to the characteristics observed in SCA3 patients, whereas the expression of MJDtr-Q27 had no effect (Warrick et al. 1998). In a subsequent study, the same group demonstrated that HSP70, a major stress-induced molecular chaperone, suppresses polyQ-induced neurodegeneration in the SCA3 fly model (Warrick et al. 1999). They also showed that the full-length ataxin-3-Q27, which is a polyubiquitin-binding protein with ubiquitin protease activity, suppresses neurodegeneration and delays NI formation in MJDtr-Q78 flies, depending on its ubiquitin-associated activities and proteasome function (Warrick et al. 2005). These results indicate that the physiological function of the host protein plays a crucial role in SCA3 pathogenesis, as well as indicates the potential therapeutic role of ataxin-3 activity for the polyQ diseases. Moreover, Bilen and Bonini (2007) performed a genetic modifier screen using the SCA3 model fly and identified a set of genes that affects protein misfolding. Importantly, some modifiers of the

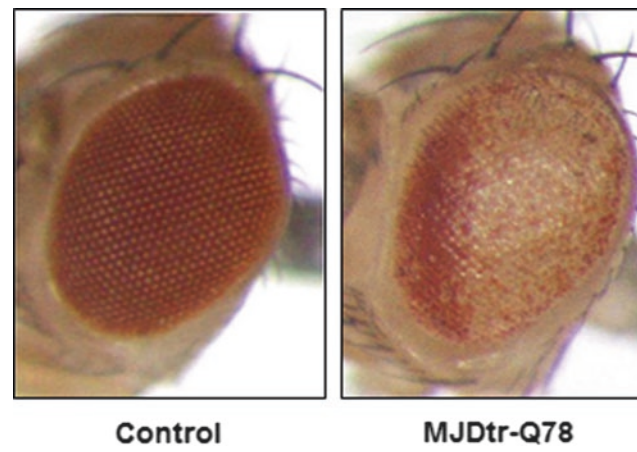

Fig. 5.1 The fly model of SCA3 Expression of MJDtr-Q78 in the eye causes severe eye degeneration as compared to control. Fly genotypes are *gmr-Gal4*/+ (left) and *gmr-Gal4*/+; *UAS-MJDtr-Q78S*/+ (right)

SCA3 flies also modulated toxicity of tau, which is involved in Alzheimer's disease and frontotemporal dementia, demonstrating common mechanisms of neurodegeneration between distinct neurotoxic proteins. We also showed that the loss of p62/sequestosome 1, which is involved in selective autophagy, delays the degradation of MJDtr-Q78 protein oligomers and exacerbates eye degeneration, indicating that p62 plays a protective role against polyQ-induced neurodegeneration in the SCA3 fly model (Saitoh et al. 2015). Taken together, these results suggest that chaperone activity and the protein-folding pathway play important roles in the pathogenesis of SCA3.

It is widely accepted that mutant ataxin-3 proteins containing an expanded polyQ tract cause neurodegeneration. However, Li et al. (2008) provided evidence for a pathogenic role of CAG repeat RNA in polyQ disease pathogenesis using SCA3 fly models. They performed modifier screening for polyQ-induced neurodegeneration and unexpectedly found that muscleblind, a gene implicated in the RNA toxicity of CUG expansion diseases, enhanced eye degeneration in SCA3 flies. Furthermore, they tested the possible role of RNA toxicity by expressing the CAG repeat in the untranslated region, and found that mRNA expression of an untranslated CAG repeat of pathogenic length induced progressive neuronal dysfunction. These results demonstrate the role of RNA toxicity in the pathogenesis of SCA3.

5.2.4 Spinal and Bulbar Muscular Atrophy

Spinal and bulbar muscular atrophy (SBMA), also known as Kennedy disease, is an adult-onset neurodegenerative disorder with an X-linked recessive inheritance. The disease mainly affects motor neurons and is characterized by slowly progressive limb and bulbar muscle weakness and atrophy and gynecomastia. As described in the Introduction section, SBMA is caused by an abnormal expansion of the CAG repeat encoding a polyQ tract in exon 1 of the *AR* gene (La Spada et al. 1991).

A fly model of SBMA was generated by introducing a transgene encoding the AR protein with a tract of 52 polyQ (AR-Q52) into flies (Takeyama et al. 2002). Although no obvious phenotype was observed in the photoreceptor neurons of the eyes of these flies, administration of androgen or its antagonists led to marked neurodegeneration accompanied with nuclear translocation of the mutant AR. These findings suggest that ligand binding to polyQ-expanded AR leads to its structural alteration and subsequent nuclear translocation, which eventually leads to neurodegeneration in male SBMA patients (Takeyama et al. 2002). Regarding involvement of native AR functions in the pathogenesis of SMBA, Nedelsky et al. (2010) showed that not only the nuclear translocation of AR but also the DNA-binding activity of AR and recruitment of transcriptional coregulators is necessary for its toxicity. These findings indicate that the native functions of AR play a crucial role in the pathogenesis of SBMA.

5.2.5 Oculopharyngeal Muscular Dystrophy

Oculopharyngeal muscular dystrophy (OPMD) is an adult-onset muscular disorder generally with autosomal dominant traits and is characterized by progressive swallowing difficulties, ptosis, and proximal limb weakness. OPMD is caused by a short expansion of the GCG trinucleotide repeat in the coding region of the nuclear poly(A)-binding protein 1 (*PABPN1*) gene, which encodes a protein that is involved in the polyadenylation of mRNAs and poly(A) site selection (Brais et al. 1998). Whereas the normal *PABPN1* allele has a $(GCN)_{10}$ repeat encoding a 10 polyalanine (polyA) stretch, OPMD patients carry expanded alleles with $(GCG)_{12–17}$ repeats, encoding expanded polyA tracts in the N-terminal domain of PABPN1 (Brais et al. 1998).

Chartier et al. (2006) established a fly model of OPMD expressing mutant PABPN1 with a 17 polyA tract in muscle and demonstrated progressive muscle degeneration and nuclear inclusions composed of mutant PABPN1 in these flies, which are reminiscent of the characteristics of human OPMD patients. Notably, in this OPMD fly model, the polyA tract was not sufficient to

cause muscle degeneration, and the RNA-binding domain (RRM) of PABPN1 was also required. This suggests that OPMD does not only result from polyA toxicity but also from an intrinsic property of mutant PABPN1 that is dependent on the RRM. The authors also identified several suppressors of the muscular phenotype such as the molecular chaperone HSP70 and the anti-apoptotic protein p35 using the OPMD fly model, demonstrating the protective role of molecular chaperones and involvement of apoptosis in mutant PABPN1-induced muscle degeneration.

Recently, Chartier et al. (2015) found that mRNAs encoding mitochondrial proteins are downregulated starting at the earliest stages of progression in fly and mouse models of OPMD. Since the downregulation of these mRNAs correlates with their shortened poly(A) tails, the authors propose that impaired nuclear polyadenylation is an early defect in OPMD.

5.2.6 Spinocerebellar Ataxia Type 8

Spinocerebellar ataxia type 8 (SCA8) is an adult-onset slowly progressive ataxia with autosomal dominant inheritance, which is associated with an expansion of the CTG repeat in the noncoding region of the ataxin-8 opposite strand gene, and possibly the complementary CAG repeat in the ataxin-8 gene. This was the first example of an expansion mutation of a noncoding trinucleotide repeat in SCA, in contrast to most other repeat expansion mutations occurring in the coding regions in other SCAs (Koob et al. 1999). As the CTG trinucleotide repeat is believed to be located in the noncoding region, toxic gain-of-function mechanisms of repeat RNA are thought to be involved in the pathogenesis of SCA8.

To investigate this possibility, Mutsuddi et al. (2004) generated fly models for SCA8 by expressing 9 (normal) or 112 (expanded) CTG repeats. Both flies expressing normal and expanded CTG repeats in the eye showed late-onset and progressive eye degeneration. Using these SCA8 fly models, they performed a genetic modifier screen and identified four RBPs that are expressed in neurons.

Later, bidirectional expression of CUG and antisense CAG repeat transcripts were reported in an SCA8 mouse model, as well as in SCA8 patients (Moseley et al. 2006). Most surprisingly, the CAG repeat sequence located in the noncoding region was discovered to be translated into repeat polypeptides in the absence of an initiation ATG codon (Zu et al. 2011) in cell and mouse models of SCA8, as well as in SCA8 patients. This unconventional translation was named repeat-associated non-ATG (RAN) translation. These results suggest that toxic gain-of-function mechanisms at both the protein and RNA levels may contribute to the pathogenesis of SCA8.

5.2.7 Spinocerebellar Ataxia Type 31

Spinocerebellar ataxia type 31 (SCA31) is a late-onset autosomal dominant cerebellar ataxia, which is caused by a complex penta-nucleotide (TGGAA)n repeat insertion in the overlapping intron of the brain expressed, associated with Nedd4 gene and the thymidine kinase 2 gene in the antisense strand (Sato et al. 2009). In the brains of SCA31 patients, RNA foci containing UGGAA repeats were observed (Niimi et al. 2013), supporting a toxic gain-of-function mechanism caused by UGGAA repeat RNA in the pathogenesis of SCA31.

To gain insight into the pathogenic mechanisms of SCA31, we generated SCA31 model flies expressing expanded UGGAA repeats (UGGAAexp) and showed that the expression of UGGAAexp causes neurodegeneration accompanied by the accumulation of UGGAAexp RNA foci and pentapeptide repeat proteins produced by repeat-associated translation, as observed in SCA31 patient brains (Ishiguro et al. 2017). Moreover, the ALS-associated RBPs, TAR DNA-binding protein (TDP-43), fused in sarcoma (FUS), and heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2/B1) bind to UGGAAexp RNA, alter the structure of UGGAAexp RNA, and suppress UGGAAexp-mediated toxicity. These results demonstrate that these RBPs function as RNA chaperones and

regulate repeat-associated translation, suggesting that defects of RNA metabolism associated with RBPs contribute to the pathogenesis of SCA31.

5.2.8 Fragile X Tremor Ataxia Syndrome

Fragile X tremor/ataxia syndrome (FXTAS) is a late-onset neurodegenerative disease characterized by kinetic tremor, gait ataxia, parkinsonism, and dementia. FXTAS is caused by a premutation expansion of CGG repeats (55–200) in the 5′-UTR of the *FMR1* gene, which is found in FXS carriers and belongs to the *FMR1*-related disorders, including FXS and *FMR1*-related primary ovarian insufficiency.

To investigate whether premutation alleles of *FMR1* lead to neurodegeneration *in vivo*, Jin et al. (2003) established FXTAS fly models expressing 60 or 90 CGG repeats. They showed that expression of premutation CGG repeats alone is sufficient to cause neurodegeneration in a dose- and repeat length-dependent manner, suggesting RNA-mediated neurodegeneration in these fly models. In their following study, the authors screened for CGG repeat RNA-binding proteins from mouse brain lysates and identified Pur α and hnRNPA2/B1 as RBPs binding to CGG repeat RNA. They further showed that Pur α suppresses neurodegeneration caused by CGG repeat RNA in the FXTAS fly models, indicating that Pur α plays an important role in the pathogenesis of FXTAS (Jin et al. 2007). Sofola et al. (2007b) also identified RBPs such as hnRNP A2/B1 and CUG-binding protein 1 (CUGBP1) that bind to the CGG repeat and suppresses its toxicity in the FXTAS fly models. These results suggest sequestration of RBPs by CGG repeat RNA as one of the pathogenic mechanisms of FXTAS.

They also reported that co-expression of CGG repeat RNA together with CCG repeat RNA, whose expansion in the *FMR2* gene causes another type of X-linked mental retardation, FRAXE, decreases their independent toxicities with each other, by reducing their transcript levels through the RNAi pathway (Sofola et al. 2007a). Furthermore, Sellier et al. (2013) found that the double-stranded RNA-binding protein DGCR8 binds to CGG repeats and is sequestered in CGG RNA aggregates together with its partner, DROSHA, resulting in a reduction in microRNA processing. These results suggest that alteration of the microRNA-processing machinery is involved in the pathogenic mechanisms in FXTAS.

Intriguingly, Todd et al. (2013) demonstrated that CGG repeats work as a template for RAN translation to produce polyglycine-containing proteins, which accumulate in ubiquitin-positive inclusions in the FXTAS fly models and FXTAS patient brains. Moreover, CGG repeat toxicity is suppressed by eliminating RAN translation and is enhanced by increased polyglycine production via ATG-initiated translation, indicating that RAN translation, which produces aberrant polypeptides, is involved in the neurodegeneration in FXTAS.

5.2.9 Myotonic Dystrophy Type 1

Myotonic dystrophy type 1 (DM1) is an autosomal dominant muscular dystrophy characterized by myotonia and muscular dystrophy, together with multisystem impairments, including cataracts, hypogonadism, endocrine dysfunction, heart defects, and cognitive decline. DM1 is the most common muscular dystrophy affected in adulthood, but it also appears as a congenital form. DM1 is caused by an abnormal expansion of CTG repeats in the 3′-UTR of the dystrophia myotonica protein kinase gene (Mahadevan et al. 1992; Brook et al. 1992). In DM1 patients, expanded CUG repeat-containing RNA accumulates as RNA foci in the nucleus of affected tissues and recruit two major RBPs, muscleblind like splicing regulator 1 (MBNL1) and CUGBP1, which bind to the CUG repeat RNA, resulting in their misregulation and alteration of RNA metabolism (Philips et al. 1998; Miller et al. 2000; Timchenko 2013).

To provide further insight into the pathogenic mechanisms of DM1, Houseley et al. (2005) generated DM1 fly models expressing expanded (162), intermediate (48, 56), or normal (11) CTG

repeats in the 3′-UTR of a *GFP* reporter gene. In muscle cells, expanded CUG repeats formed RNA foci and colocalized with muscleblind, which is the *Drosophila* ortholog of human MBNL1, whereas normal and intermediate CTG repeats did not. However, no pathological phenotype, such as locomotor impairment, shortened life span, or muscular pathology, was detectable in this fly model. Further investigation was conducted by creating a more severe fly disease model with a larger number (480) of interrupted CUG (iCUG) repeats (de Haro et al. 2006). Expressions of this expanded iCUG repeat caused eye and muscle degeneration and the accumulation of expanded iCUG transcripts in nuclear RNA foci. Moreover, expression of MBNL1 was found to suppress expanded iCUG-induced toxicity, whereas expression of CUGBP1 worsened the iCUG-induced toxicity in these DM1 fly models (de Haro et al. 2006). Using this DM1 fly model, de Haro et al. (2013) further identified smaug, which is the *Drosophila* ortholog of human Smaug1/Samd4A, a translational repressor, as a suppressor of iCUG repeat-induced toxicity. Smaug was found to physically and genetically interact with CUGBP1 and suppresses iCUG-induced myopathy via restoration of the translational activity of CUGBP1 (de Haro et al. 2013).

5.2.10 C9orf72-Linked Amyotrophic Lateral Sclerosis and Frontotemporal Dementia

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease predominantly affecting upper and lower motor neurons, resulting in muscle weakness and atrophy, bulbar dysfunction, and eventual respiratory impairment. Frontotemporal dementia (FTD) is a neurodegenerative dementia characterized by cognitive impairment together with behavioral and personality changes. Since the discovery of TDP-43 as a key molecule aggregated in the pathological inclusions of both diseases, these intractable neurodegenerative diseases have been considered to belong to the same disease spectrum with overlapping genetic and neuropathological features (ALS/FTD) (Ling et al. 2013). In 2011, an abnormal expansion of a GGGGCC repeat in the first intron of the *C9orf72* gene was identified as the most common genetic mutation of ALS/FTD (C9-ALS/FTD) (Renton et al. 2011; DeJesus-Hernandez et al. 2011). Three hypotheses in the pathogenesis of C9-ALS/FTD have been proposed so far, as follows: loss-of-function of the C9ORF72 protein, toxic gain-of-function of expanded GGGGCC repeat RNAs, and toxic gain-of-function of dipeptide repeat (DPR) proteins generated from expanded repeat RNAs by RAN translation (Ling et al. 2013). However, an FTD patient homozygous for the *C9orf72* GGGGCC repeat expansion mutation was reported to demonstrate clinical and pathological features that fit within the range of those of heterozygous patients (Fratta et al. 2013). This fact, together with the lack of *C9orf72*-coding mutations in ALS patients, excludes the possibility of a loss-of-function mechanism in C9-ALS/FTD (Harms et al. 2013). Moreover, knockout mice for the *C9orf72* gene demonstrate immunological defects, but no or mild neurological dysfunction (Koppers et al. 2015; Atanasio et al. 2016; Jiang et al. 2016; Sudria-Lopez et al. 2016). Thus, loss of *C9orf72* function may not play a key role in the pathogenesis of C9-ALS/FTD.

Although *Drosophila* do not have an ortholog of the *C9orf72* gene, fly models were employed to explore the toxic gain-of-function mechanisms in the pathogenesis of C9-ALS/FTD. The first C9-ALS/FTD fly model was established by expressing expanded 30 GGGGCC repeats with a CTCGAG interruption (iGGGGCC). Flies expressing iGGGGCC repeats in the eye caused eye degeneration, and those in motor neurons demonstrated motor dysfunction with aging (Xu et al. 2013). To distinguish the toxic gain-of-function mechanisms between expanded repeat RNAs themselves and DPR proteins produced by RAN translation, Mizielinska et al. (2014) generated three C9-ALS/FTD fly models, as follows: (1) flies expressing expanded pure GGGGCC repeats that produce both expanded RNAs and DPR proteins, (2) RNA-only flies expressing

stop codon-interrupted expanded GGGGCC repeats that only produce expanded RNAs, and (3) DPR protein-only flies expressing non-GGGGCC RNAs with alternative codons that only produce DPR proteins. They found that flies expressing pure GGGGCC repeats showed neurodegenerative phenotypes, such as rough eye and decreased life span, whereas RNA-only flies showed no apparent phenotype, despite RNA foci formation in both pure GGGGCC repeat and interrupted repeat RNA-only flies. These findings suggest that expanded GGGGCC repeats cause neurotoxicity through the DPR proteins, and RNA foci may not be a direct cause of neurodegeneration in these fly models. The authors further investigated whether expression of the DPR protein alone is sufficient to induce toxicity using DPR protein-only flies. They found that only poly-GR and poly-PR proteins cause eye degeneration, whereas poly-GA and poly-PA proteins do not, indicating that arginine-containing DPR proteins are the major cause of neurodegeneration in C9-ALS/FTD fly models (Mizielinska et al. 2014). Tran et al. (2015) reported a new C9-ALS/FTD fly model expressing 160 GGGGCC repeats flanked by human intronic and exonic sequences. Spliced intronic 160 GGGGCC repeat RNA formed RNA foci in the nucleus of neurons but resulted in low levels of DPRs and no neurodegeneration. These results also indicate that the accumulation of RNA foci is not sufficient to drive neurodegeneration, and the sequences flanking the GGGGCC repeats may modulate RAN translation.

Toward elucidation of the molecular mechanisms underlying the pathogenesis of C9-ALS/FTD, several groups have performed genetic modifier screening using C9-ALS/FTD fly models. Zhang et al. (2015) identified Ran GTPase-activating protein (RanGAP), which is a key regulator of nucleocytoplasmic transport, and showed a genetic interaction between GGGGCC repeats and the nucleocytoplasmic transport machinery. Freibaum et al. (2015) performed genetic modifier screening using flies expressing GGGGCC repeats and GFP in frame to monitor RAN translation and identified 18 genes involved in the nuclear pore complex and nucleocytoplasmic transport. Boeynaems et al. (2016) also discovered genes encoding components of the nuclear pore complex, importins, exportins, Ran-GTP regulators, and arginine methylases as modifiers of C9-ALS/FTD flies. These findings provide evidence that nucleocytoplasmic transport contributes to the pathogenesis of C9-ALS/FTD.

5.3 Perspectives

As introduced above, a number of studies on repeat expansion disorders have been performed using fly models and have contributed toward elucidating the molecular mechanisms of these diseases. In particular, by taking advantage of fly models in rapid and efficient genetic analyses, various modifier genes have been identified by genetic screening, providing insight into the pathogenic mechanisms of these disorders.

The other remarkable advantage of fly models is their short generation cycle, which is useful for research on intergenerational repeat instability. Repeat instabilities are commonly observed in most of the repeat expansion disorders, and further elongation of expanded repeats in the next generation often results in earlier onset and more severe disease phenotypes, which is called anticipation (Mirkin 2007; Orr and Zoghbi 2007). Such elongation of expanded repeats is thought to occur during meiosis in germline cells, whereas repeat instability during mitosis is also known to cause somatic mosaicism (Pearson et al. 2005; Kovtun and McMurray 2008). Jung and Bonini (2007) used a fly model of SCA3 expressing an expanded CAG repeat to clarify the mechanisms underlying repeat instability. They found that repeat instability was enhanced by transcription and was modulated by Rad2/XPG, which is involved in DNA repair mechanisms. Furthermore, repeat instability was increased in SCA3 flies by the loss of CREB-binding protein, which is a histone acetyltransferase, and treatment with trichostatin A, a histone deacetylase (HDAC) inhibitor suppressed this repeat instability. These results clearly indicate the usefulness of fly models to study the mechanisms of repeat

instability, which is thought to underlie the fundamental etiology of repeat expansion disorders.

In addition, several studies have shown the usefulness of fly models for the identification of potential drug targets. Using HD fly models, Steffan et al. (2001) first identified HDAC inhibitors, which increase the acetylation levels of histones, as therapeutic candidates for HD. They showed that the administration of sodium butyrate and suberoylanilide hydroxamic acid (SAHA) to HD flies by feeding suppressed neurodegeneration. Based on these findings, the therapeutic potential of HDAC inhibitors for HD were further explored in mouse models, and the therapeutic effects of SAHA were indeed replicated in a HD mouse model (Hockly et al. 2003). Several molecules targeting the misfolding and aggregation of polyQ proteins, such as polyglutamine binding peptide 1 (QBP1), Congo red, and methylene blue, have also been analyzed using fly models for their therapeutic potential (Nagai et al. 2003; Apostol et al. 2003; Sontag et al. 2012).

Although fly models have significantly contributed to extend our knowledge of repeat expansion disorders as mentioned above, we need to recognize the limitations of fly models in studying human diseases, due to the many differences between flies and humans, such as in their development, physiology, metabolism, nervous system, etc. Nevertheless, considering their rapid generation cycle, cost-effectiveness, and advantages in genetic analyses, fly models are powerful tools for studying human diseases (McGurk et al. 2015; Koon and Chan 2017).

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Amyotrophic Lateral Sclerosis Model

6

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that affects upper and lower motor neurons in the brain and the spinal cord. Due to the progressive neurodegeneration, ALS leads to paralysis and death caused by respiratory failure 2–5 years after the onset of symptoms. There is no effective cure available. Most ALS cases are sporadic, without family history, whereas 10% of the cases are familial. Identification of variants in more than 30 different loci has provided insight into the pathogenic molecular mechanisms mediating disease pathogenesis. Studies of a *Drosophila melanogaster* model for each of the ALS genes can contribute to uncovering pathophysiological mechanism of ALS and finding targets of the disease-modifying therapy. In this review, we focus on three ALS-causing genes: *TAR DNA-binding protein* (*TDP-43*), *fused in sarcoma/translocated in liposarcoma* (*FUS/TLS*), and *chromosome 9 open reading frame 72* (*C9orf72*).

Keywords

Amyotrophic lateral sclerosis · Neurodegeneration · Motor neuron disease · TAR DNA-binding protein 43 · Fused in sarcoma/translocated in liposarcoma · Chromosome 9 open reading frame 72

6.1 Introduction

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a fatal neurodegenerative disease that is characterized by progressive neurodegeneration of upper and lower motor neurons. The upper motor neurons originate in the motor cortex and send signal to the lower motor neurons via synapses in the brainstem or spinal cord. The lower motor neurons send signals to skeletal muscles via neuromuscular junctions (NMJ) (Fig. 6.1). ALS typically starts focally, either an upper limb or a lower limb or the bulbar region, and spread to other regions over time. Patients lead to paralysis and death 2–5 years after disease onset (Kanouchi et al. 2012). The pathological hallmark of ALS is abnormal protein inclusions in neurons called Bunina bodies (BBs) and skein-like inclusions

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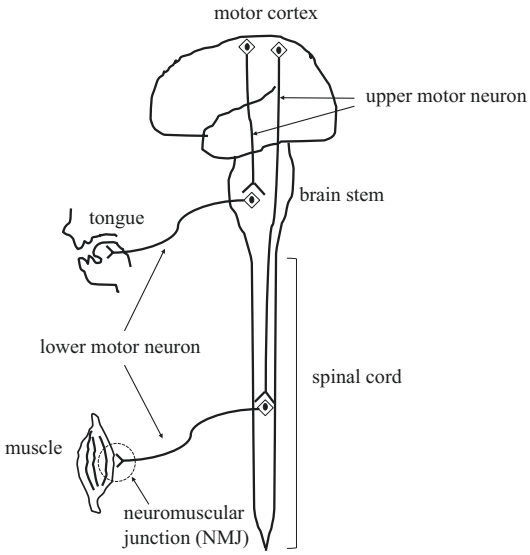

Fig. 6.1 Components of the nervous system Upper motor neurons make direct or indirect connections with lower motor neurons, which innervate skeletal muscles and trigger their contraction

(Piao et al. 2003). The mean prevalence of ALS is about 3.4–5.4 cases per 100,000 people all over the world (Chiò et al. 2013).

About 10% of ALS patients have family history (familial ALS) (Mitchell and Borasio 2007). So far, more than 30 causative genes have been identified (Al-Chalabi et al. 2017; White and Sreedharan 2016). These genes play important roles in the pathogenic mechanism of not only familial ALS but also sporadic ALS. Although no effective therapy has been established yet, targeting the causative genes and modifier genes of familial ALS may be useful for developing an effective treatment for ALS.

Table 6.1 summarizes familial ALS-causing genes, their *Drosophila* homologues, and the papers of *Drosophila* ALS model published as before. The phenotypes of *Drosophila* ALS model in this review are briefly shown in Table 6.2. Superoxide dismutase (*SOD1*), the firstly identified as familial ALS-causing gene in

Table 6.1 ALS *Drosophila* models

Human gene (ALS locus)	Drosophila gene	References
SOD1 (ALS1)	Sod1	Bahadorani et al. (2013), Islam et al. (2012), Kumimoto et al. (2013), and Watson et al. (2008)
Alsin (ALS2)	Als2	Takayama et al. (2014)
FUS (ALS6)	cabeza	Baldwin et al. (2016), Chen et al. (2011), Daigle et al. (2013), Frickenhaus et al. (2015), Jäckel et al. (2015), Lanson et al. (2011), Machamer et al. (2014), Miguel et al. (2012), Sasayama et al. (2012), Shahidullah et al. (2013), Wang et al. (2011), and Xia et al. (2012)
VAPB (ALS8)	Vap33	Chai et al. (2008), Chen et al. (2010), Deivasigamani et al. (2014), Forrest et al. (2013), Han et al. (2012), Moustaqim-Barrette et al. (2014), Ratnaparkhi et al. (2008), Sanhueza et al. (2014), Tsuda et al. (2008), and Yang et al. (2012)
TARDBP (ALS10)	TBPH	Diaper et al. (2013a, b), Elden et al. (2010), Estes et al. (2011), Estes et al. (2013), Feiguin et al. (2009), Fiesel et al. (2010), Gregory et al. (2012), Hanson et al. (2010), Hazelett et al. (2012), Ihara et al. (2013), Kim et al. (2012, 2014), Li et al. (2010), Lin et al. (2011), Lu et al. (2009), Miguel et al. (2011), Ritson et al. (2010), Sreedharan et al. (2015), Voigt et al. (2010), and Zhan et al. (2013)
VCP (ALS14)	TER94	Kim et al. (2013a, b), Ritson et al. (2010), and Wang et al. (2016)
UBQLN2 (ALS15)	Ubqn	Jantrapirom et al. (2018)
C9orf72 (ALSFTLD1)		Burguete et al. (2015), Celona et al. (2017), Freibaum et al. (2015), Kramer et al. (2016), Lee et al. (2016), Mizielinska et al. (2014), Mizielinska et al. (2017), Simone et al. (2017), Tran et al. (2015), Wen et al. (2014), Xu et al. (2013), and Zhang et al. (2015)
hnRNPA2	Hrb87F/hrp36	Kim et al. (2013a, b)

Table 6.2 Summary of phenotypes of *Drosophila* ALS models

	Aberrant eye morphology	Crawling defect	Climbing defect	Eclosion	Bouton numbers	Active zone	Dendritic branching	Branch length	Aggregates
hTDP-43 WT	+	+	+	↓	↓	↑	↑ or ↓	nd	+
hTDP-43 mutation	+	+	+	↓	↓	↑	↑ or nc	nd	+
TBPH WT	+	+	+	↓	↑ or ↓	nd	↑ or nc	nd	+
TBPH mutation	+	+	+	↓	↓	nd	↓ or nc	nd	+
TBPH KO or KD	+	+	+	↓	↑ or ↓	nd	↓	↓	–
hFUS WT	+	+	+	↓	↓	↓	nd	nd	–
hFUS mutation	+	+	+	↓	↓ or nc	↓	nd	nd	–
Cabeza WT	+	+	+	↓	↓	nd	nd	↓	–
Cabeza KD or KD	+	+	+	↓ or nc	↓	nd	nd	↓	–
GGGGCC repeat expansion	+	+	+	↓	↓	↓	nd	nd	+

KO knockout, *KD* knockdown, *nc* no change, *nd* not determined

1993, encodes a protein protecting cells against oxidative stress by catalyzing the conversion of superoxide anions into oxygen and hydrogen peroxide (Fridovich 1986; Rosen et al. 1993). The SOD1 fly ALS model showed progressive motor dysfunction, mitochondrial change, coupled with electrophysiological defects and aggregation (Bahadorani et al. 2013; Watson et al. 2008).

Based on the findings that some causative genes for ALS were also associated with another disorder, frontotemporal lobar degeneration (FTLD), these two disorders are thought to form continuum of a broad neurodegenerative disorder (Murphy et al. 2007) (Fig. 6.2a). FTLD is a clinically diverse dementia syndrome, characterized by behavioral change and language dysfunction (Cairns et al. 2007). Each of ALS and FTLD presents as extremes of a spectrum of overlapping clinical symptoms (Fig. 6.2a).

In this review, we focus on three ALS-causing genes mainly related to both ALS and FTLD: *TAR DNA-binding protein-43* (*TDP-43*), *fused in sarcoma/translocated in liposarcoma* (*FUS/TLS*), and *chromosome 9 open reading frame 72* (*C9orf72*).

6.2 *Drosophila* Models of ALS

6.2.1 TAR DNA-Binding Protein 43 (TDP-43)

6.2.1.1 *TDP-43*-Related ALS

Mutations in TAR DNA-binding protein 43 gene (*TARDBP*, *TDP-43*) account for 4% of familial ALS and inherited in autosomal dominant manner (Fig. 6.2b) (Picher-Martel et al. 2016). It is of note that TDP-43 pathology is observed in most ALS patients, both sporadic and familial ALS. The TDP-43 has been first identified as the major component of the ubiquitin-positive neuronal inclusion bodies observed in patients with ALS and FTLD (Arai et al. 2006; Neumann et al. 2006). Subsequently, mutations of *TDP-43* were identified both in ALS and FTLD families (Gitcho et al. 2008; Kabashi et al. 2008; Sreedharan et al. 2008; Van Deerlin et al. 2008). TDP-43 is a highly conserved 43 kDa RNA/DNA-binding protein and contains two RNA recognition motifs, RRM1 and RRM2, and glycine-rich domain relating to binding single-stranded DNA, RNA, and proteins (Buratti and Baralle

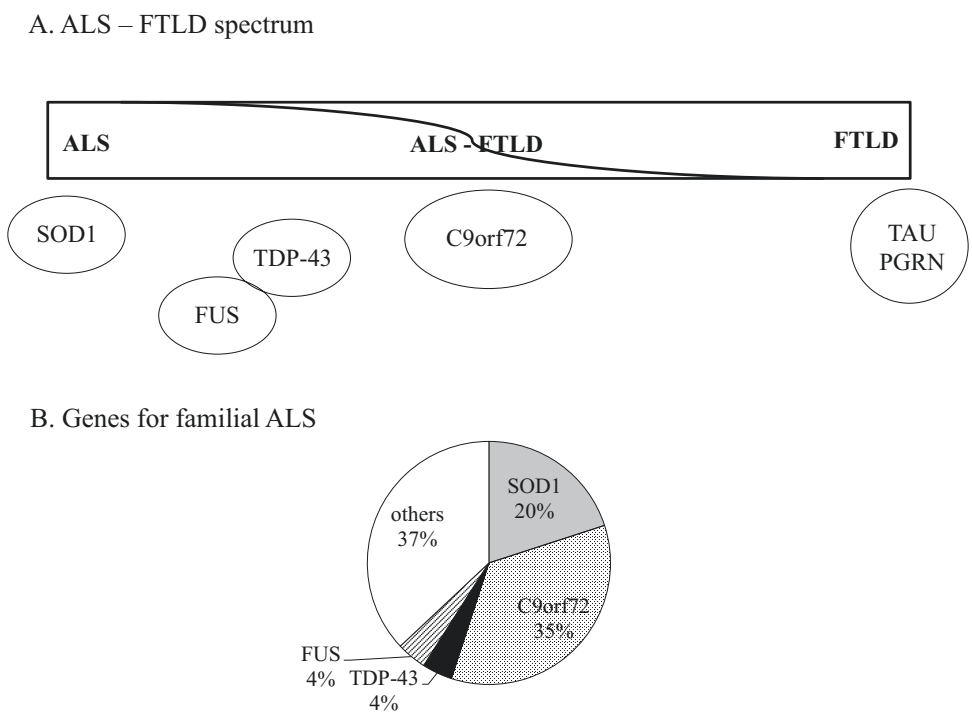

Fig. 6.2 (**a**) ALS-FTLD spectrum. (Modified from Neuron 2013; 79: 416–438). ALS and FTLD may share common neurodegenerative pathways and may be part of a spectrum. (**b**) Genes for familial ALS. (Modified from Acta Neuropathol Commun 2016; 4: 70)

2001; Mackenzie et al. 2010; Ou et al. 1995). Of more than 40 mutations of *TDP-43* identified so far, the most frequent ones G298S, A315T, M337V, G348C, and A382T are localized in glycine-rich domain (Corcia et al. 2012). Because of limited number of patients of each mutation, it is hard to clarify genotype-phenotype correlation of *TDP-43*-related ALS. However, of the five common mutations, G298S and A315T showed rapid and slow progression, respectively, and the remaining mutations showed intermediate progression (Corcia et al. 2012).

TDP-43 is predominantly localized in the nucleus and has multiple functions including RNA processing, splicing regulation, and transcriptional regulation (Ratti and Buratti 2016). In autopsied tissues of ALS patients, TDP-43 abnormally redistributes from nucleus to cytosol and forms cytosolic inclusions in motor neurons. The pathological mechanisms underlying TDP-43-driven neurodegeneration in ALS have not been completely clarified. Presence of cytosolic inclusions suggests gain-of-function hypothesis that aggregated TDP-43 is toxic, whereas loss of nuclear localization of TDP-43 suggests loss-of-function hypothesis because TDP-43 physiologically functions in the nucleus (Vanden Broeck et al. 2014) (Fig. 6.3).

The *Drosophila* models for TDP-43-associated ALS based on either gain-of-function or loss-of-function have been reported since 2009 (Feiguin et al. 2009; Lu et al. 2009).

6.2.1.2 Gain-of-Function Model

Based on gain-of-function hypothesis, overexpression models using GAL4-UAS system have been reported. Transgenic *Drosophila* expressing wild-type (WT) human TDP-43 (hTDP-43) leads to aberrant eye morphology, climbing and crawling defect, reduced life span, eclosion defect, increased larval turning time, the reduced synaptic bouton number, the decreased or increased

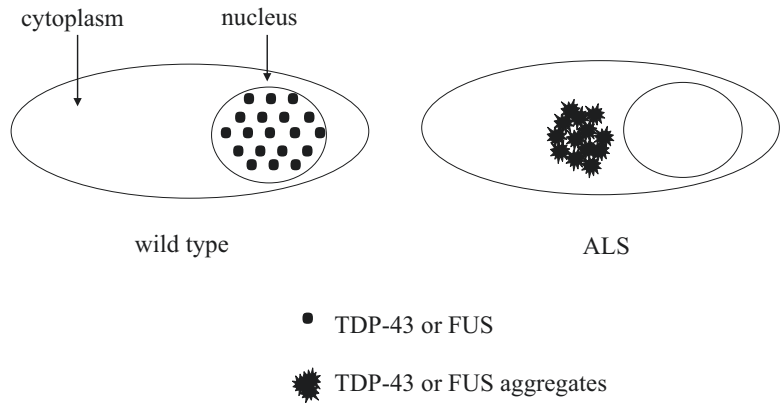

Fig. 6.3 Intracellular localization of TDP-43 or FUS associated with ALS pathology TDP-43 or FUS is normally found in the nucleus, but in ALS, TDP-43 or FUS abnormally redistributes from nucleus to cytosol and forms cytosolic inclusions

dendritic branching, the increased active zone at NMJs, day and night sleep fragmentation, and cytoplasmic and axonal aggregates (Estes et al. 2011; Estes et al. 2013; Hanson et al. 2010; Ihara et al. 2013; Li et al. 2010; Lu et al. 2009; Voigt et al. 2010).

Overexpression of ALS mutant hTDP-43 also leads to progressive degeneration and functional deficits in flies. Mutant hTDP-43-induced phenotypes cannot be distinguished from WT hTDP-43 induced ones. Mutant hTDP-43-induced phenotypes reported include aberrant eye morphology (D169G, G298S, A315T, M337V, N345K), life span reduction and eclosion defect (G287S, A315T, G348C, A382T, N390D), climbing and crawling defect (A315T), increased larval turning time, the decreased bouton numbers, the increased or no change of dendritic branching, the increased active zone at NMJs (D169G, G298S, A315T, N345K), day and night sleep fragmentation (D169G, G298S, A315T, Q331K, N345K), and cytoplasmic and axonal aggregates (D169G, G298S, A315T, N345K) (Estes et al. 2011; Estes et al. 2013; Ritson et al. 2010; Sreedharan et al. 2015; Voigt et al. 2010).

TBPH is the well-conserved *Drosophila* homologue of h*TDP-43*. Overexpression of WT *TBPH* induced aberrant eye morphology, eclosion defect, climbing and crawling defect, learning deficiency, the vesicle transport dysfunction, the increased or no change of dendritic branching at NMJs, and TBPH aggregates (Baldwin et al. 2016; Diaper et al. 2013a, b; Estes et al. 2011; Lin et al. 2011; Lu et al. 2009; Magrane et al. 2014). The different reports as for dendritic branching might be due to the difference of genetic backgrounds and/or position of the *UAS-TBPH* inserted among lines used.

Overexpression of mutant TBPH (A315T, Q367X) was also reported to lead to aberrant eye morphology, axonal aggregates, crawling defect, eclosion defect, decreased bouton numbers, and decreased or no change in dendritic branching at NMJs (Estes et al. 2011; Lu et al. 2009).

6.2.1.3 Loss-of-Function Model

Based on loss-of-function hypothesis, knocking out or knockdown of endogenous *TBPH* fly models has been reported. They showed eclosion defect, climbing defect, crawling defect, the decreased or increased synaptic boutons, the reduced dendritic branching, the dysfunction of synaptic transmission, and axonal transport (Baldwin et al. 2016; Diaper et al. 2013a; Diaper et al. 2013b; Feiguin et al. 2009; Lin et al. 2011; Lu et al. 2009; Magrane et al. 2014). The controversial reports of the synaptic bouton phenotype might result from the difference of genetic backgrounds and/or deletion alleles among lines used.

6.2.1.4 Modifiers of *TDP-43*-Associated Phenotypes

Genetic interaction analysis by mating flies allows efficient identification of modifier genes for disease. Here we introduce the genes identified as modifiers of TDP-43-associated phenotypes in flies.

6.2.1.4.1 Stress Granule-Related Genes

Yeast plasmid overexpression screening and cellular experiments suggest that stress granule is related to TDP-43 aggregation (Colombrita et al. 2009; Kim et al. 2014). Of genes encoding stress granule components and related genes, *Ataxin-2* (*ATXN2*), *PEK*, *Rox8*, *Gadd34*, and *PABP* were reported as modifier of TDP-43 toxicity in flies (Elden et al. 2010; Kim et al. 2014). *ATXN2* is a causative gene of spinocerebellar degeneration type2. ATXN2 and PABP are major components of stress granule. PEK, Rox8, and Gadd34 modulate phosphorylation levels of eIF2α, which correlate with amount of stress granule.

6.2.1.4.2 Modifier Genes Identified from Comprehensive Screening

RNA-seq analysis of knockdown and overexpression of *TBPH* expression showed that most upregulated gene was *Map 205*, *which* encodes a PAM2 motif containing neuronal microtubule binding protein (Rolls et al. 2007). TBPH directly bound to *Map 250* mRNA and neuron-specific knockdown of *Map 205* suppressed the late pupal lethality in *TBPH* knockdown flies (Vanden Broeck et al. 2013).

Through genetic screening using a subset of the Bloomington deficiency kit library, *wallenda* (*wnd*), which encodes a conserved mitogen-activated protein kinase kinase kinase (MAPKKK) homologous, was identified as a modifier of TDP-43 elicited neurotoxicity. Reducing *wnd* gene dosage or overexpression of *highwire*, which encodes ubiquitin E3 ligase regulating wnd through proteasomal clearance, partially rescued TDP-43-associated lethality. JNK and p38 are phosphorylated by wnd and play important and potentially opposing roles in TDP-43-induced neurodegeneration. Overexpression or null allele of fly *JNK*, *Bsk*, increased or decreased life span of TDP-43 fly, respectively. However, overexpression or null allele of p38 decreased or increased life span of TDP-43 fly, respectively (Zhan et al. 2015).

From RNAi screening in Hela cells, *ITPR1* was identified as a strong modifier of TDP-43 localization. *ITPR1* encodes an endoplasmic reticulum (ER)-resident calcium channel (Cardenas et al. 2010). Mutation in *Drosophila ITPR* improved the life span and climbing defects in neuron-specific WT hTDP-43 flies, suggesting that ITPR-mediated Ca^{2+} signaling contributes to TDP-43-induced neurotoxicity (Colombrita et al. 2009; Liu-Yesucevitz et al. 2010).

6.2.1.4.3 Genes Encoding TDP-43 Binding Proteins

Human heterogeneous nuclear ribonucleoproteins (hnRNPs) A2B1 and A1 are well known as TDP-43 partners. The knockdown of *Hrp38*, *Drosophila* ortholog of human hnRNP A/B family, enhanced the locomotive deficit, life span reduction, and the neuropil degeneration caused by *TBPH* knockdown (Romano et al. 2014).

By using gene suppression screening of mating flies, knockdown of some *hnRNPs* (*Hrb87F*, *Glo*, *Heph*, *Bl*, and *Sm*) enhanced the locomotion defect induced by *TBPH* knockdown, whereas knockdown of other *hnRNPs* (*Hrb27c*, *CG42458*, *Glo* and *Syp*) rescued overexpression of *TBPH* toxicity (Appocher et al. 2017).

6.2.1.4.4 ALS-Related Gene

Valosin-containing protein (*VCP*) encodes a highly conserved AAA (ATPase associated with a variety of cellular activities) family of proteins (Meyer et al. 2012; Ritson et al. 2010). *VCP* is a causative gene of inclusion body myopathy with early-onset Paget disease and frontotemporal dementia (IBMPFD) (Watts et al. 2004) and also reported as one of familial ALS genes (Johnson et al. 2010). During screening of modifier of IBMPFD fly models by using *Drosophila* deficiency kit, R152H mutant of *ter94*, *Drosophila VCP*, was found to decrease nuclear localization of TDP-43, suggesting that *VCP* is a modifier of *TDP-43* (Ritson et al. 2010). We found that overexpression of *ter94* suppressed the phenotypes of *TBPH* knockdown in the eyes and neurons (Kushimura et al. 2018). The VCP-Ufd1-Npl4 complex regulates proteasomal processing. *Npl4* was reported to be genetically interacted with *TBPH* (Byrne et al. 2017).

6.2.1.4.5 Chromatin Modeling and RNA Export-Related Genes

EMS-based genetic screening of mating flies revealed that loss of *sgg/GSK3*, *hat-trick*, or *xmas-2* suppressed TDP-43 toxicity in motor axon and NMJ morphology. These genes encode proteins relating chromatin modeling and RNA export (Sreedharan et al. 2015).

6.2.1.4.6 Genes Encoding NMJ Proteins

It was reported that TBPH maintains NMJ growth and microtubule organization through the function of *futsch,* which encodes microtubule binding protein (Godena et al. 2011). Recent study demonstrated that *futsch* mRNA may be a target of TDP-43 in *Drosophila*. Overexpression of *futsch* mitigated the locomotive dysfunction and life span reduction induced by WT and MT hTDP-43 overexpression (Coyne et al. 2014).

Cacophony, which encodes the type II voltage-gated calcium channel necessary for NMJs (Kawasaki et al. 2004), was identified as a potential modifier of *TBPH* toxicity. Loss of *TBPH* caused a reduction in *cacophony* expression and that genetically restoring *cacophony* in motor neurons in loss of *TBPH* was sufficient to rescue the locomotion defects (Chang et al. 2014; Lembke et al. 2017).

6.2.1.4.7 Mitochondrial Dynamic Regulatory Genes

Fragmented mitochondrial morphology has been observed both in ALS and TDP-43 flies (Altanbyek et al. 2016; Sasaki et al. 2007). Recent study focusing on mitochondrial dynamics, fission and fusion, identified *Marf*, *Opa1*, and *Drp1* as modifiers of TDP-43 phenotype in flies (Altanbyek et al. 2016).

6.2.1.4.8 Candidate Genes from Drug Screening

In drug screening of 1200 FDA-approved compounds, PPARγ agonist pioglitazone could rescue WT and mutant hTDP-43-neurotoxicity of eclosion defect and larval locomotive dysfunction in glia and motor neurons (Joardar et al. 2015). In the nervous system, activation of the nuclear hormone receptor PPARγ has been shown to have anti-inflammatory and neuroprotective effects (Kapadia et al. 2008).

6.2.2 *Fused in Sarcoma/Translocated in Liposarcoma* (FUS/TLS)

6.2.2.1 *FUS*-Related ALS

Mutations in *fused in sarcoma/translocated in liposarcoma* (FUS/ TLS, FUS) represent around 4% of familial ALS and inherited mostly in autosomal dominant manner (Fig. 6.2b) (Picher-Martel et al. 2016). Patients with *FUS*-related ALS tend to have earlier onset and more rapid progression compared with *SOD1*- and *TDP-43*-related ALS (Yan et al. 2010).

Similar to TDP-43, FUS is an ubiquitously expressed and highly conserved multifunctional protein whose activities included RNA processing, RNA/DNA binding, splicing, and transcriptional regulation (Tan and Manley. 2010; Wang et al. 2008; Yang et al. 1998). In addition, FUS is normally found in the nucleus, but in ALS, it localizes in the cytoplasm as inclusions (Fig. 6.3). It is of note that TDP-43 is absent in FUS pathology (Kwiatkowski et al. 2009; Vance et al. 2009), suggesting disease pathways of FUS and TDP-43 are independent of each other.

Although the pathological mechanisms underlying FUS-driven neurodegeneration in ALS have not been clarified, disease-associated FUS mutations may cause neurotoxicity by a gain of function or a loss of function similar to TDP-43 (Fig. 6.3).

The *Drosophila* models for FUS-associated ALS based on gain of function or loss of function have been reported since 2011 (Chen et al. 2011; Lanson Jr. et al. 2011; Wang et al. 2011).

6.2.2.2 Gain-of-Function Model

Based on gain-of-function hypothesis, overexpression models using GAL4-UAS system have been reported. Transgenic *Drosophila* expressing WT human FUS (hFUS) leads to aberrant eye morphology, reduced life span, eclosion defect or normal eclosion, climbing and crawling defect,

the reduced synaptic bouton number, the decreased active zone at NMJs, axonal degeneration, enlargement of the motoneurons, and wing defect (Baldwin et al. 2016; Chen et al. 2011; Jäckel et al. 2015; Lanson et al. 2011; Miguel et al. 2012; Xia et al. 2012).

Overexpression of ALS mutant hFUS also leads to progressive degeneration and functional deficits in flies. Mutant h*FUS*-induced phenotypes reported include aberrant eye morphology (R518K, R521C, R521G, R521H, R524S, P525L), eclosion defect (R518K, R521C, R521H, R521G), climbing and crawling defect (R518K, R521C, R521G, R521H, R524S, P525L), reduced synaptic bouton number (R521G, R524S, P525L) or no change (R521C, R521H), and the decreased active zone (R518K, R521C, R521H, P525L) at NMJs, (Baldwin et al. 2016; Chen et al. 2011; Jäckel et al. 2015; Lanson Jr. et al. 2011; Machamer et al. 2014; Xia et al. 2012). The climbing defect by R521C-flies is more severe than hFUS WT (Lanson Jr. et al. 2011). *Cabeza* (*caz*) is the well-conserved *Drosophila* homologue of hFUS. The overexpression of WT *caz* induced progressive toxicity in multiple tissues: aberrant eye morphology, wing defect, eclosion defect, climbing and crawling defect, and the reduced bouton number at NMJs (Baldwin et al. 2016; Jäckel et al. 2015; Xia et al. 2012).

6.2.2.3 Loss-of-Function Model

Knocking out endogenous *caz* caused aberrant eye morphology, decreased viability, life span reduction, crawling and climbing defect, the reduced bouton numbers, and synaptic branches (Baldwin et al. 2016; Frickenhaus et al. 2015; Sasayama et al. 2012; Shimamura et al. 2014; Wang et al. 2011; Xia et al. 2012).

6.2.2.4 Modifiers of *FUS*-Associated Phenotypes

Genetic interaction analysis by mating flies allows efficient identification of modifier genes for disease. Here we introduce the genes identified as modifiers of FUS-associated phenotype in flies.

6.2.2.4.1 ALS-Related Genes

From screening of ALS-causing genes other than *FUS*, we found that *ter94*, the *Drosophila* ortholog of human *VCP*, suppressed the *caz* knockdown phenotype. The decreased level of *caz* in the nucleus and the resultant motor disturbance induced by *caz* knockdown could be rescued by overexpressed *ter94* despite lacking any change of caz protein in the CNS, probably via the nucleocytoplasmic transport of ter94/VCP (Azuma et al. 2014).

6.2.2.4.2 Modifier Genes Identified from Comprehends Screening

Through genetic screening using a Drosophila Genetic Resource Center deficiency kit library, we showed that genetic link between *caz* and EGFR signaling pathway genes. Mutation in EGFR pathway-related genes, such as *rhomboid-1*, *rhomboid-3*, and *mirror*, suppressed the rough eye phenotype induced by *caz* knockdown. *Caz* negatively regulates the EGFR signaling pathway required for determination of cone cell fate in *Drosophila* (Shimamura et al. 2014).

6.2.2.4.3 Genes Encoding FUS Binding Protein

By affinity purification, Pur-α was identified as a protein binding to FUS C-terminal region, where FUS mutations mainly localize (Di Salvio et al. 2015). It is of interest that Pur-α was also identified as transcript of GGGGCC repeat of *C9orf72* (see below). The overexpression of Pur-α significantly exacerbated the aberrant eye morphology caused by FUS mutations (R521G, R522G, R524S, and P525L), whereas the downregulation of Pur-α significantly improved those climbing defect (Di Salvio et al. 2015).

6.2.2.4.4 Cancer-Related Genes

Because some ALS-causing genes are also involved in cancer, we focus on cancer-related genes as modifiers of ALS (Yamaguchi et al. 2016). We will soon report a genetic link between *caz* and *Hippo*, the *Drosophila* ortholog of human *Mammalian sterile 20-like kinase* (*MST*) *1* and *2*. Loss-of-function mutations of *hpo* rescued *caz*

knockdown-induced phenotypes in eyes and neurons (Azuma et al. in submitting). The Hippo pathway plays a role as a tumor suppressor in mammals. Other tumor suppressor *folliculin* (*FLCN*) was reported to be a positive regulator of TDP-43 translocation (Xia et al. 2016).

Nucleophosmin-human myeloid leukemia factor1 (*NPM-hMLF1*) fusion protein could suppress the aberrant eye morphology induced by WT hFUS. *NPM-hMLF1* may bind to hFUS to hold it in nucleus to protect from degradation by proteasome (Yamamoto et al. in submitting).

6.2.2.4.5 Molecular Chaperon

From candidate approach, *HSPA1L*, human ortholog of *Drosophila HSP70*, decreased the FUS aggregates and suppressed eye degeneration and life span reduction induced by WT-hFUS flies (Miguel et al. 2012).

The imbalance of mitochondrial dynamics is one of the key pathogenic mechanisms in ALS. Downregulating HSP60, the mitochondrial chaperoning, reduced mitochondrially localized FUS and partially rescued mitochondrial defects and neurodegenerative phenotypes caused by WT-hFUS and hFUS P525L-flies in the eye and motoneurons (Deng et al. 2015).

6.2.2.4.6 Mitochondrial Dynamic Regulatory Genes

Similar to TDP-43, the mitochondrial morphology of WT-hFUS-expressing flies was highly fragmented and those were rescued by co-expression of mitochondrial dynamic regulatory genes such as *Marf*, *Opa1*, and the dominant negative mutant form of *Drp1* (Altanbyek et al. 2016).

6.2.2.4.7 Gene Related to Methylation of FUS

The methylation of h*FUS* by *protein arginine methyltransferase 1* (*PRMT1*) reduced its ability to bind to transportin playing a role in nuclear import in cell culture (Dormann et al. 2012; Du et al. 2011). Knockdown of *Drosophila transportin* caused cytoplasmic retention of WT-hFUS and enhanced eye phenotypes (Jäckel et al. 2015). The genetic ablation of *DART1*, *Drosophila* homologue of human *PRMT1*, exacerbates the external eye degeneration in WT-hFUS and hFUS R521C-flies (Scaramuzzino et al. 2013).

6.2.3 Chromosome 9 Open Reading Frame 72 (C9orf72)

6.2.3.1 *C9orf72*-Related ALS

C9orf72 account for 35% of familial ALS in Caucasians (Fig. 6.2b) (Picher-Martel et al. 2016), whereas extremely low frequency in Asian or Oceanian populations (Ishiura and Tsuji 2015). Hexanucleotide repeat expansion (HRE), (GGGGCC)*n*, in the noncoding region of *C9orf72*, was linked to ALS and FTLD (Dejesus-Hernandez et al. 2011; Renton et al. 2011). In healthy individuals, the sequence GGGGCC was present as 2–23 repeats, but in affected individuals, it was expanded to hundreds or thousands of repeats (Taylor et al. 2016). Three potential mechanisms have been proposed about *C9orf72*-mediated ALS and FTLD: RNA-mediated toxicity through generation of RNA foci and sequestration of RNA-binding proteins from their normal targets, expression of dipeptide repeat proteins by repeat associated non-ATG (RAN) translation which occurs in the absence of the initiation codon ATG, and haploinsufficiency (Dejesus-Hernandez et al. 2011; Gendron et al. 2014; Mori et al. 2013; Renton et al. 2011).

Drosophila lacks *C9orf72* homologue. Fly models for *C9orf72*-associated ALS have been reported by overexpression of repeat sequence since 2013 (Xu et al. 2013).

6.2.3.2 Phenotype

Based on RNA-mediated toxicity hypothesis, studies using transgenic *Drosophila* expressing GGGGCC repeat expansion were reported. Thirty to fifty repeats of GGGGCC-induced phenotypes are aberrant eye morphology, crawling and climbing defects, the decreased numbers of synaptic bouton and active zone at NMJs, and eclosion defect compared to control 3–6 repeats (Celona et al. 2017; Freibaum et al. 2015; Kramer et al. 2016; Xu et al. 2013; Zhang et al. 2015).

Fly models based on RAN hypothesis were also reported. In RAN translation, sense and antisense transcripts of GGGGCC repeat expansions can be translated to five dipeptide repeat proteins (DRPs) (antisense, poly-PA (proline-alanine); poly-PR (proline-arginine); sense, poly-GR (glycine-arginine); poly-GA (glycine-alanine); and sense and antisense, poly-GP (glycine-proline)) (Gendron et al. 2014; Mori et al. 2013). Antibody against each DRP showed that inclusions of patients with *C9orf72* mutation contained poly-GA, and to a lesser extent, poly-GP and poly-GR, supporting this hypothesis (Mori et al. 2013). Expression of each DRP with non-GGGGCC repeat sequence but alternative codon constructs in flies showed that expression of poly-GR or poly-PR DPR proteins in GGGGCC repeat expansions resulted in severe eye degeneration and pupal lethality, whereas poly-GA, poly-GP, or poly-PA had no effect (Freibaum et al. 2015; Lee et al. 2016; Mizielinska et al. 2014; Wen et al. 2014). Cellular experiments showed that overexpression of poly-GR or poly-PR resulted in nuclear localization of these DPR and enlarged nuclei, suggesting DPR-induced nucleolar dysfunction (Mizielinska et al. 2014). Neuronal expression of poly-GR in flies showed significantly enlarged nuclei (Mizielinska et al. 2017).

Recent report using transgenic flies expressing GGGGCC-160 repeats suggested that nuclear RNA foci are nontoxic, whereas the levels of DPR proteins are a major source of toxicity. GGGGCC-160 repeats induced RNA foci but had little toxicity in flies. In contrast, GGGGCC-36 repeats, producing >100-fold more DPR protein than GGGGCC-160, were highly toxic (Tran et al. 2015).

6.2.3.3 Modifiers of *C9orf72*-Associated Phenotypes

Genetic interaction analysis by mating flies allows efficient identification of modifier genes for disease. Here we introduce the genes identified as modifiers of *C9orf72*-associated phenotype in flies.

Pur-α was identified as the RNA-binding protein of GGGGCC repeats. Overexpression of Pur-α suppressed GGGGCC repeat-mediated neurodegeneration in *Drosophila* eyes. In addition, Pur-α inclusions colocalizing with ubiquitin were present in flies expressing GGGGCC-30 repeats flies, but not in control flies expressing GGGGCC-3 repeats flies (Xu et al. 2013).

The candidate-based screening previously shown to bind to GGGGCC identified RanGAP, *Drosophila* ortholog of human RanGAP1, was a modifier of flies expressing GGGGCC-30 repeats. RanGAP is a key regulator of nucleocytoplasmic transport. Gain-of-function allele of *RanGAP* suppressed neurodegeneration in photoreceptor neurons and locomotive dysfunction of that lines (Zhang et al. 2015).

By biotinylated RNA pulldown of mouse Neuro-2a cell nuclear extract, the zinc finger protein *Zfp106* was identified as a GGGGCC RNA repeat-binding protein. Overexpression of *Zfp106* suppressed the locomotive dysfunction and eclosion defects in flies expressing GGGGCC-30 repeats, indicating that *Zfp106* is a potent suppressor of neurodegeneration in a *C9orf72*-mediated *Drosophila* model (Celona et al. 2017).

Through genetic screening using Bloomington Drosophila Stock Center deficiency kit, 18 genetic modifiers of *Drosophila* expressing 58 GGGGCC repeats were identified. These modifiers encoded components of the nuclear pore complex (NPC). The strongest suppressor was *Ref1*, and the strongest enhancer was *Nup50* (Freibaum et al. 2015).

Yeast plasmid genetic screening revealed that mutation of *Spt4* reduced the transcription of long CAG trinucleotide repeats associated with polyglutamine disease (Liu et al. 2012). Based on the hypothesis that *Spt4* inhibition also reduces transcript of GGGGCC repeats, effect of *Spt4* was tested in *C9orf72* fly. Knockdown of *Drosophila Spt4* partially suppressed the eye degeneration and life span reduction, supporting the hypothesis (Kramer et al. 2016).

6.3 Protocol

This protocol is widely used for study of the immunostaining of *Drosophila* larval central nervous system (modified from *Drosophila* Protocols (2000) Cold Spring Harbor Laboratory Press, Edited by William Sullivan, Michael Ashburner, R. Scott Hawley. Cold Spring Harbor Laboratory Press).

6.3.1 Dissection Techniques

Supplies and Equipment

- Dissecting microscope.
- Dissecting tools: Forceps. Dumont #5 (Ted Pella 505-NM; or Fine Science Tools 11,252–30) or Dumont #55 (Fine Science Tools 11,255–20) forceps are recommended; the #55 forceps have lighter and finer shanks than the #5 forceps.
- Sylmar dissection dish. This is an indispensable for dissections, as the soft base prevents the dissecting tools from getting damaged.
- Slides and coverslips.
- Confocal laser scanning microscope for observation.

Solutions and Reagents

- 4% paraformaldehyde/phosphate-buffered saline (PBS)
- 1 × PBS
- PBS containing 0.3% Triton X-100
- PBS containing 0.15% Triton X-100
- Primary antibody, secondary antibody
- Alexa 488-conjugated phalloidin
- 4,6-diamidino-2-phenylindole dihydrochloride
- Vectashield

6.3.2 Immunostaining and Mounting of the Brains

Note that all steps should be performed at room temperature, except for the incubation with the primary antibody, which is usually performed at 4 °C.

1. Larval Central Nervous System (CNS) Dissection

   Location and identification of imaginal discs in the larva (Fig. 6.4a). The larval CNS consists of the two brain hemispheres and the compound ventral ganglion (Fig. 6.4b).

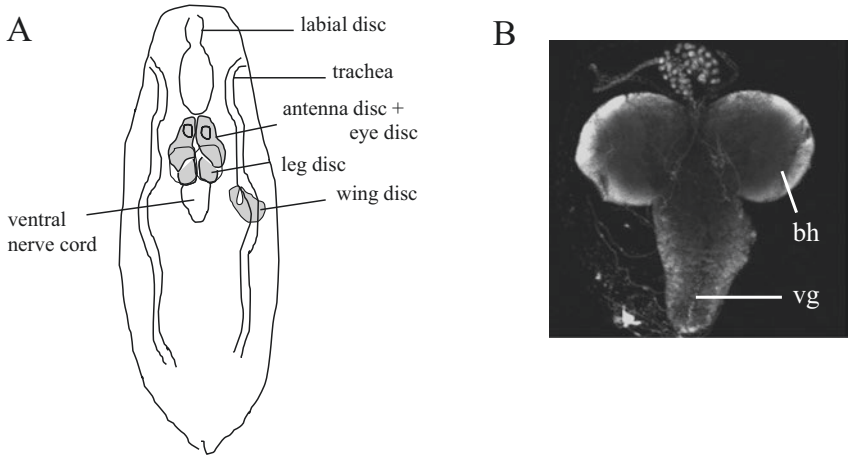

Fig. 6.4 (**a**) Location and identification of imaginal discs in the larvae. (Modified from *Drosophila* Protocols (2000) Cold Spring Harbor Laboratory Press, Edited by William Sullivan, Michael Ashburner, R. Scott Hawley. Cold Spring Harbor Laboratory Press). (**b**) Larval central nervous system. bh, brain hemisphere; vg, ventral ganglion

Perform the following dissection with the larva immersed in dissection buffer (1 × PBS).

To remove the central nervous system (CNS) from third instar larvae, use one pair of forceps to gently hold the larva at approximately one third of its length from the interior end. With a second set of forceps, grab a firm hold at the base of the mouth hooks and then pull the mouth parts away from the rest of the body.

Typically, the brain with attached eye-antennal imaginal discs and salivary glands, as well as other tissues, will be removed as a single mass. Fix the CNS tissues in 4% paraformaldehyde/phosphate-buffered saline (PBS) for 15 min at 25 °C.

2. Wash four times for 15 min with PBS containing 0.3% Triton X-100.
3. Incubate with Alexa 488-conjugated phalloidin (1 unit/200 μl) in PBS containing 0.3% Triton X-100 for 20 min at 25 °C.
4. Wash four times for 15 min with PBS containing 0.3% Triton X-100.
5. Incubate with blocking buffer (PBS containing 0.15% Triton X-100 and 10% normal goat serum) for 30 min at 25 °C.
6. Incubate with diluted primary antibodies in PBS containing 0.15% Triton X-100 and 10% normal goat serum for 20 h at 4 °C.
7. Wash four times for 15 min with PBS containing 0.3% Triton X-100.
8. Incubate in the dark with secondary antibody solution for 3 h at 25 °C.
9. Wash four times for 15 min with PBS containing 0.3% Triton X-100.
10. Incubate with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (0.5 μg/ml)/PBS/0.1% Triton X-100 for 10 min at 25 °C.
11. Wash with PBS containing 0.15% Triton X-100.
12. Mount in Vectashield (Vector Laboratories Inc.). Remove all extraneous tissues, leaving CNS.
13. View under a confocal laser scanning microscope (OLYMPUS FLUOVIEW FV10i).

6.4 Conclusion

There has been remarkable progress toward defining the new disease-causing genes and molecular biology of ALS. Genetic interaction analysis by mating flies has allowed efficient identification of novel modifier genes and elucidation of the pathomechanisms of ALS.

Based on these findings, further approaches including drug screening using ALS flies should be necessary to develop effective cure of ALS.

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Drosophila Charcot-Marie-Tooth Disease Models

7

Masamitsu Yamaguchi and Hiroshi Takashima

Abstract

Charcot-Marie-Tooth disease (CMT) was initially described in 1886. It is characterized by defects in the peripheral nervous system, including sensory and motor neurons. Although more than 80 CMT-causing genes have been identified to date, an effective therapy has not yet been developed for this disease. Since *Drosophila* does not have axons surrounded by myelin sheaths or Schwann cells, the establishment of a demyelinating CMT model is not appropriate. In this chapter, after overviewing CMT, examples of *Drosophila* CMT models with axonal neuropathy and other animal CMT models are described.

Keywords

Drosophila · Charcot-Marie-Tooth disease · Mitochondria · Neuromuscular junction

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7.1 Introduction

In 1886, Drs. Jean Charcot, Pierre Marie, and Howard Henry Tooth described Charcot-Marie-Tooth disease (CMT), an inherited group of peripheral neuropathies (Charcot and Marie 1886; Tooth 1886). Although CMT is the most common hereditary motor and sensory neuropathy with a prevalence of approximately 1:2500 persons (Skre 1974), it is still regarded as a rare disease. CMT shows onset in childhood in many cases, and CMT patients generally exhibit a combination of slowly progressive symptoms of sensory defects and distal muscle debility; however, the severity and progression of symptoms markedly vary (Saifi et al. 2003). Patients show foot deformities caused by defects in the foot muscles. Nerve conduction velocity (NCV) studies are commonly performed to classify the clinical forms of CMT. CMT type 1 (CMT1) is characterized by a decreased NCV (≦38 m/s), while CMT type 2 (CMT2) shows normal NCV (>38 m/s) or slightly reduced muscle action potentials. Intermediate CMT shows 30–45 m/s. CMT1 patients exhibit demyelination and re-myelination (onion bulb formation) processes that may be detected using nerve biopsy. In contrast, CMT2 patients show a reduced number of axons and the absence or fewer Schwan cells with no evidence of demyelination (Schroder 2006; Barisic et al. 2008).

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CMT shows not only clinical but also genetic heterogeneity. Although CMT frequently exhibits autosomal dominant inherited neuropathy, it also shows autosomal recessive (AR) and X-linked inheritance, and the disease frequently appears sporadically. Recent studies with a deep sequencer revealed that more than 80 genes are associated with CMT (Pareyson et al. 2017), and many are listed in Table 7.1.

7.2 CMT Classification

According to NCV data, inheritance patterns, and CMT-causing genes, CMT is mainly classified into four genetic types (CMT1, CMT2, CMT4, and CMTX). More than 90% of genetically defined CMT patients were found to have a mutation in only one of the following four genes: *peripheral myelin protein 22 kDa* (*PMP22*), *gap junction beta 1* (*GJβ1*), *myelin protein zero* (*MPZ*), and *mitofusin 2* (*MFN2*) (Murphy et al. 2012; Saporta et al. 2011).

7.2.1 CMT1

CMT1 (abnormal myelin, autosomal dominant) has been further classified into five subtypes: CMT1A, 1B, 1C, 1D, and 1F. CMT1A is the most common type of CMT, accounting for approximately 55% of familial CMT caused by the duplication of a 1.4-Mb region on chromosome 17p11.2 containing the *PMP22* gene (Lupski et al. 1991). CMT1B is the fourth most common type of CMT caused by point mutations in the *MPZ* gene on chromosome 1q22–23 (Hayasaka et al. 1993; Saporta et al. 2011). MPZ has been identified as the major component of peripheral myelin, consisting of at least 50% of this protein (Greenfield et al. 1973). CMT1C is caused by mutations in the *LITAF* gene on chromosome 16p13.3–12 (Street et al. 2003), while CMT1D is caused by mutations in the *early growth response 2* (*EGR2*) gene, which is localized on chromosome 10q21.1–22.1 (Warner et al. 1998). Both of these subtypes are rare causes of CMT, accounting for less than 1% of CMT cases. EGR2 is the transcription factor responsible for the regulation of differentiation and myelin gene expression in Schwann cells. CMT1F is caused by mutations of the *NEFL* gene, which firstly discovered the cause of CMT2E. Neurofilament light chain (NEFL) works for organization of neurofilaments.

7.2.2 CMT2

CMT2 (axonopathy, autosomal dominant) and AR-CMT2 (axonopathy, autosomal recessive) comprises around 20% of genetically defined CMT (Murphy et al. 2012; Saporta et al. 2011). CMT2A is associated with mutations in the *MFN2* gene on chromosome 1p35-p36, which are estimated to comprise 10–30% of CMT2 (Zuchner et al. 2004; Murphy et al. 2012; Saporta et al. 2011). In addition to motor symptoms, some CMT2A have been reported to cause optic atrophy (Zuchner and Vance 2006). MFN2 is involved in mitochondrial dynamics and axonal transport. CMT2B characterized by mild to moderate sensory loss is caused by mutations in the *RAB7* gene, which is located at chromosome 3q13-q22 (Auer-Grumbach et al. 2000; De Jonghe et al. 1997; Verhoeven et al. 2003). CMT2C is caused by mutations in the *TRPV4* gene on chromosome 12q23–24 (Chen et al. 2010; Deng et al. 2010; Landoure et al. 2010). CMT2C is associated with motor rather than sensory axonal neuropathy. CMT2D is caused by mutations in the *GARS* (*glycyl-tRNA synthetase*) gene (Antonellis et al. 2003), and mutations in this gene also cause distal spinal muscular atrophy type V (dSMA-V). dSMA-V is a neuromuscular disorder that is similar to CMT2D, but is distinguished from CMT by the lack of sensory loss (Antonellis et al. 2003). Aminoacyl-tRNA synthetases (ARS) ensure the accurate transfer of information in the genetic code. In addition to *GARS*, *AARS* (*alanyl-tRNA synthetase*), *KARS* (*lysyl-tRNA synthetase*), *MARS* (*methionyl-tRNA synthetase*), *HARS* (*histidyl-tRNA synthetase*), and *YARS* (*tyrosyl-tRNA synthetase*) are associated with CMT2. CMT2E has been linked to mutations in the *NEFL* (*neurofilament light*

Table 7.1 The List of CMT-causing genes and their *Drosophila* homologues

Gene symbol	Inheritance	Classification	Phenotype	Phenotype MIM number	*Drosophila* homologues (score)	*Drosophila* models
AARS	AD	CMT	Charcot-Marie-Tooth disease, axonal, type 2N	613287	—	
COX6A1	AR	CMT	Charcot-Marie-Tooth disease, recessive, axonal, or mixed	–	*CG17280* (*levy*) (11 of 11), *CG30093* (8 of 11), *CG14077* (6 of 11)	
DHTKD1	AD	CMT	Charcot-Marie-Tooth disease, axonal, type 2Q	615025	*CG1544* (11 of 11)	
DYNC1H1	AD	CMT	Charcot-Marie-Tooth disease, axonal, type 20	614228	*CG7507* (*dynein heavy chain 64C*) (11 of 11)	
EGR2	AD, AR	CMT	Charcot-Marie-Tooth disease, type 1D	607678	*CG7847* (*stripe*) (5 of 11)	
FGD4	AR	CMT	Charcot-Marie-Tooth disease, type 4H	609311	*CG8606* (*RhoGEF4*) (3 of 11)	
FIG4	AR	CMT	Charcot-Marie-Tooth disease, type 4J	611228	*CG17840* (*dFIG 4*) (10 of 11)	Bharadwaj et al. (2016) and Kyotani et al. (2016)
GAN	AR	CMT	Giant axonal neuropathy-1	256850	—	
GARS	AD	CMT	Charcot-Marie-Tooth disease, type 2D	601472	*CG6778* (*GlyRS, gars*) (11 of 11)	Ermanoska et al. (2014) and Niehues et al. (2016)
GDAP1	AD	CMT	Charcot-Marie-Tooth disease, axonal, type 2K	607831	*CG4623* (*Gdap1*) (10 of 11)	López et al. (2015)
	AR	CMT	Charcot-Marie-Tooth disease, axonal, with vocal cord paresis	607706		
	AR	CMT	Charcot-Marie-Tooth disease, recessive intermediate, A	608340		
	AR	CMT	Charcot-Marie-Tooth disease, type 4A	214400		
GJB1	XR	CMT	Charcot-Marie-Tooth neuropathy, X-linked dominant, 1	302800	—	

(continued)

Table 7.1 (continued)

Gene symbol	Inheritance	Classification	Phenotype	Phenotype MIM number	*Drosophila* homologues (score)	*Drosophila* models
GNB4	AD	CMT	Charcot-Marie-Tooth disease, dominant intermediate F	615185	*CG10545* (*Gβ13F*) (8 of 11)	
HARS	AD	CMT	CMT2 (peripheral neuropathy, sensory predominant)	–	*CG6335* (*HisRS*) (11 of 11)	
HK1	AR	CMT	Neuropathy, hereditary motor, and sensory, Russe type	605285	*CG3001* (*Hex-A*) (7 of 11)	
HSPB1	AD, AR	CMT	Charcot-Marie-Tooth disease, axonal, type 2F	606595	*CG4167* (*heat shock protein family B (small) member 1*)(3 of 11)	
HSPB8	AD	CMT	Charcot-Marie-Tooth disease, axonal, type 2L	608673	—	
INF2	AD	CMT	Charcot-Marie-Tooth disease, dominant intermediate E	614455	*CG33556* (*formin 3*)(6 of 11)	
KARS	AD, AR	CMT	Charcot-Marie-Tooth disease, recessive intermediate, B	613641	—	
KIF1B	AD	CMT	Charcot-Marie-Tooth disease, type 2A1	118210	*CG8566* (*uncoordinated 104*) (9 of 11)	
LITAF	AD	CMT	Charcot-Marie-Tooth disease, type 1C	601098	*CG13510, CG13559, CG32280* (3 of 11)	
LMNA	AR	CMT	Charcot-Marie-Tooth disease, type 2B1	605588	*CG6944* (*Lam*) (8 of 11), *CG10119* (*LamC*) (7 of 11)	
LRSAM1	AD, AR	CMT	Charcot-Marie-Tooth disease, axonal, type 2P	614436	—	
MARS	AD	CMT	CMT2(peripheral neuropathy, sensory predominant)	–	*CG15100* (*methionyl-tRNA synthetase*) (10 of 11)	
MED25	AR	CMT	Charcot-Marie-Tooth disease, type 2B2	605589	*CG12254* (*MED25*) (9 of 11)	

(continued)

Table 7.1 (continued)

Gene symbol	Inheritance	Classification	Phenotype	Phenotype MIM number	*Drosophila* homologues (score)	*Drosophila* models
MFN2	AD, AR	CMT	Charcot-Marie-Tooth disease, type 2A2	609260	*CG3869* (*Marf*) (9 of 11)	Eschenbacher et al. (2012)
		CMT	Hereditary motor and sensory neuropathy VI	601152		
MPZ	AD	CMT	Charcot-Marie-Tooth disease, dominant intermediate D	607791	—	
		CMT	Charcot-Marie-Tooth disease, type 1B	118200		
		CMT	Charcot-Marie-Tooth disease, type 2I	607677		
		CMT	Charcot-Marie-Tooth disease, type 2J	607736		
		CMT	Dejerine-Sottas disease	145900		
MTMR2	AR	CMT	Charcot-Marie-Tooth disease, type 4B1	601382	*CG9115* (*mtm*) (11 of 11)	
MTMR5	AR	CMT	Charcot-Marie-Tooth disease, type 4B3	615284	—	
NDRG1	AR	CMT	Charcot-Marie-Tooth disease, type 4D	601455	*CG15669* (*MESK2*) (NDRG3 8 of 11, NDRG1 6 of 11)	
NEFL	AD, AR	CMT	Charcot-Marie-Tooth disease, type 1F	607734	—	
		CMT	Charcot-Marie-Tooth disease, type 2E	607684		
PDK3	XD	CMT	Charcot-Marie-Tooth disease, X-linked dominant, 6	300905	*CG8808* (*Pdk*) (11 of 11)	
PMP22	AD	CMT	Charcot-Marie-Tooth disease, type 1A	118220	—	
		CMT	Charcot-Marie-Tooth disease, type 1E	118300		
		CMT	Dejerine-Sottas disease	145900		
		CMT	Neuropathy, recurrent, with pressure palsies	162500		

(continued)

Table 7.1 (continued)

Gene symbol	Inheritance	Classification	Phenotype	Phenotype MIM number	*Drosophila* homologues (score)	*Drosophila* models
PRPS1	XR	CMT	Charcot-Marie-Tooth disease, X-linked recessive, 5	311070	*CG6767* (*PRPS2* 10 of 11, *PRPS1* 9 of 11)	
PRX	AR	CMT	Charcot-Marie-Tooth disease, type 4F	614895	—	
		CMT	Dejerine-Sottas disease, autosomal recessive	145900		
RAB7A	AD	CMT	Charcot-Marie-Tooth disease, type 2B	600882	*CG5915* (*Rab7*) (9 of 11)	
SBF1	AR	CMT	Charcot-Marie-Tooth disease, type 4B3	615284	*CG6939* (*Sbf*) (*SBF1* 10 of 11, *SBF2* 10 of 11)	
SBF2	AR	CMT	Charcot-Marie-Tooth disease, type 4B2	604563		
SH3TC2	AR	CMT	Charcot-Marie-Tooth disease, type 4C	601596	—	
SURF1	AR	CMT	Charcot-Marie-Tooth disease, type 4	–	*CG9943* (*Surf1*) (10 of 11)	
TRIM2	AR	CMT	AR-CMT2	–	*CG15105* (*tn*) (*TRIM2* 4 of 11), *CG10719* (*brat*) (*TRIM3* 5 of 11, *TRIM2* 4 of 11)	
YARS	AD	CMT	Charcot-Marie-Tooth disease, dominant intermediate C	608323	*CG4561* (*Tyrosyl-tRNA synthetase*) (12 of 12)	Storkebaum et al. (2009)
DNM2	AD	CMT	Charcot-Marie-Tooth disease, axonal, type 2M	606482	—	
		CMT	Charcot-Marie-Tooth disease, dominant intermediate B	606482	—	
TRPV4	AD	CMT	Hereditary motor and sensory neuropathy, type Ic	606071	*CG5842* (*nanchung*) (4 of 12), *CG4536* (*inactive*) (4 of 12)	

(continued)

Table 7.1 (continued)

Gene symbol	Inheritance	Classification	Phenotype	Phenotype MIM number	*Drosophila* homologues (score)	*Drosophila* models
FBLN5	AD	CMT	Cutis laxa, autosomal dominant 2	614434	—	
		CMT	Cutis laxa, autosomal recessive, type IA	219100	—	
VCP	AD	CMT	Charcot-Marie-Tooth disease, type 2		*CG2331* (*TER94*) (11 of 12)	
PLEKHG5	AR	CMT	Charcot-Marie-Tooth disease, recessive intermediate C	615376	*CG42674* (5 of 12)	
ARHGEF10	AD	CMT	Slowed nerve conduction velocity, AD	608236	*CG43658* (7 of 12)	
HOXD10	AD	CMT	Charcot-Marie-Tooth disease, foot deformity of vertical talus, congenital	192950	—	
MME	AR	CMT	Charcot-Marie-Tooth disease, axonal, type 2T	617017	*CG5905* (*neprilysin 1*) (8 of 12)	
SPG11	AR	CMT	Charcot-Marie-Tooth disease, axonal, type 2X	616668	*CG13531* (8 of 12)	
PNKP	AR	CMT	Charcot-Marie-Tooth, axonal, recessive	–	*CG9601* (10 of 12)	
MORC2	AD	CMT	Charcot-Marie-Tooth disease, axonal, type 2Z	616688	—	

Abbreviations: AD autosomal dominant, *AR* autosomal recessive, *XD* X-linked dominant, *XR* X-linked recessive

chain) gene (Mersiyanova et al. 2000). Neurofilaments are major components of the axonal cytoskeleton. However, CMT2E patients exhibit both axonal and/or demyelinating phenotypes (Mersiyanova et al. 2000; Yoshihara et al. 2002). CMT2F is caused by mutations in the *HSPB1* (*HSP27*) gene, which is a member of the heat shock protein superfamily of genes. CMT2K is caused by mutations in the *GDAP1* gene located on chromosome 8q13–21 (Crimella et al. 2010). GDAP1 (ganglioside-induced differentiation-associated protein 1) is involved in the fission of mitochondria. CMT2L is caused by mutations in *HSPB8* (*HSP22*) located on chromosome 12q24, which is also a member of the heat shock protein superfamily of genes. More recently, other CMT2 genes encoding cytoplasmic dynein 1 heavy chain 1 (DYNC1H1), E3 ubiquitin-protein ligase LRSAM1 (LRSAM1), DNA-binding protein SMUBP-2 (IGHMBP2), DnaJ homologue subfamily B member 2 (DNAJB2), and MORC family CW-type zinc finger protein 2 (MORC2) have been found and designated as CMT2O, CMT2P, CMT2S, CMT2T, and CMT2Z, respectively (Siskind et al. 2013; Ekins et al. 2015).

Many types of AR-CMT2 has been reported, AR-CMTA (B1) caused by Lamin A/C, AR-CMT2B by MED25, AR-CMT2F by HSPB1, AR-CMT2K by GDAP1, AR-CMT2P by LRSAM1, AR-CMT2R by tripartite motif-containing protein 2 (TRIM2), AR-CMT2S by IGHMBP2, and AR-CMT2T by membrane metalloendopeptidase (MME). Some of the disease-causing genes cause both autosomal recessive and autosomal dominant CMT.

CMT3 is also called Dejerine-Sottas neuropathy which indicated early-onset severe hypomyelinating or demyelinating CMT. Original family has been reported as seems to be autosomal recessive inheritance. However, molecular diagnosis indicates the patients with CMT3 are attributed to the same mutations of genes that are responsible for autosomal dominant CMT1A (*PMP22*), CMT1B (*MPZ*), and CMT1D (*EGR2*) as or autosomal recessive CMT4 (*PRX*). Therefore, currently CMT3 is no longer used for the designation based on the CMT-causing genes.

7.2.3 CMT4

CMT4 (myelinopathy, autosomal recessive) includes demyelinating or hypomyelinating forms of autosomal recessive CMT that is divided into subtypes based on the causing gene. CMT4 is divided into subtypes based on the causative genes. CMT4A is caused by mutations in the *GDAP1* gene on 8q13–21, showing the demyelinating or axonal phenotype (Baxter et al. 2002). CMT4B1 is associated with mutations in MTMR2 localized at chromosome 11q22 (Bolino et al. 2001). MTMR2 is a member of the myotubularin family of phosphoinositide-3-phosphatases, which dephosphorylate phosphatidylinositide-3,5-phosphate (PIP_2), an important signaling molecule that is crucially involved in the biogenesis and maintenance of myelin and is also suggested to play a role in membrane trafficking. CMT4B2 is associated with mutations in the *SBF2* gene. SBF2, also known as myotubularin-related 13 (*MTMR13*), is located at chromosome 11p15 (Azzedine et al. 2003). CMT4C is caused by mutations in *SH3TC2* located on chromosome 5q23-q33 (Senderek et al. 2003), and CMT4E is associated with recessive mutations in the inhibitory domain of EGR2 (Warner et al. 1998). CMT4F is caused by mutations in the *periaxin* (*PRX*) gene on chromosome 19q13 (Boerkoel et al. 2001; Guilbot et al. 2001). PRX is a cytoskeletal component of Schwann cells and is necessary for the formation of cytoplasmic compartments, Cajal bands, in Schwann cells. CMT4H and CMT4J are caused by mutations in *FGD4* and *FIG4*, respectively (Chow et al. 2007).

7.2.4 CMTX

CMTX shows axonopathy with secondary myelin changes that are X-linked. CMTX includes all forms of X-linked CMT. CMTX1 is characterized by motor and sensory neuropathies exclusively in males. CMTX1-carrier females only exhibit mild or no symptoms. CMTX is caused by mutations in the *GJβ1* gene on chromosome Xq13.1, encoding the connexin-32 protein (Bergoffen et al. 1993). CMTX1 is the second most common form of CMT, accounting for 90% of CMTX and at least 10% of all CMT patients (Murphy et al. 2012; Saporta et al. 2011). CMTX2 patients have intellectual disabilities, while CMTX3 patients show spasticity and pyramidal tract signs. CMTX4, also known as Cowchock syndrome, is associated with mutations in the *AIFM1* gene encoding apoptosis-inducing factor 1. CMTX5 is caused by mutations in the *PRPS1* gene encoding ribose-phosphate pyrophosphokinase 1, while CMTX6 is associated with mutations in the *PDK3* gene encoding pyruvate dehydrogenase kinase isoform 3.

7.3 Murine CMT Models

The first genetically modified murine models for CMT were generated approximately 20 years ago (Martini et al. 1995; Huxley et al. 1996; Sereda et al. 1996; Magyar et al. 1996). A number of mouse and rat CMT models that mimic human CMT have since been developed. The most extensively studied rodent CMT models are

those with the altered expression of *PMP22*, *GJβ1*, and *MPZ*, which correspond to models for the most common subtypes of human CMT1A, CMTX1, and CMT1B, respectively (Fledrich et al. 2012a, b). Some examples of murine CMT models are shown below.

7.3.1 Murine CMT1 Model

The low copy number type of *PMP22* transgenic mice and rats exhibit a mild dysmyelination phenotype and apparently no alterations in their axons when they are young; however, they show progressive demyelination and axonal loss with aging, which mimics the pathogenesis of CMT1A (Robertson et al. 2002; Grandis et al. 2004; Fledrich et al. 2012a, b). MPZ is the most abundant myelin protein in peripheral nerves, and human studies reported that the deletion of MPZ serine 63 resulted in the demyelinating mild late onset of CMT1B (Hayasaka et al. 1993; Kulkens et al. 1993). Transgenic mice carrying the MPZ serine 63 deletion exhibit distantly pronounced demyelination, decreased NCV, and atrophied muscle, resembling CMT1B. EGR2-deficient mice, representing CMT1D, die at birth and exhibit severe peripheral dysmyelination, while the differentiation of Schwann cells is arrested in a pre-myelinating state (Wrabetz et al. 2006).

7.3.2 Murine CMT2 Model

Transgenic mice expressing the mutant NEFL P222S in adult neurons exhibit an aberrant hindlimb posture, motor defects, and the loss of muscle innervation, recapitulating the key feature of CMT2E (Dequen et al. 2010). In a mouse model of human CMT2F, the expression of the mutant form of HSPB1 resulted in a decrease in the acetylation level of alpha-tubulin and induced severe defects in axonal transport (d'Ydewalle et al. 2011). The prevention of the deacetylation of alpha-tubulin by inhibiting histone deacetylase 6 (HDAC6) suppressed impaired axonal transport and rescued the CMT phenotype of the mouse model, demonstrating the first promising animal model for the development of therapy for axonal CMT (d'Ydewalle et al. 2011). Mice carrying mutations in the *LRSAM1* gene encoding an E3 ubiquitin ligase represent the CMT2P model. This mouse model showed mild neuropathy with aging but was sensitive to neurotoxins causing axonal degeneration (Bogdanik et al. 2013).

7.3.3 Murine CMT4 and CMTX Models

Periaxin (PRX)-deficient mice develop progressive demyelination with the lack of Cajal bands and a decreased intermodal length (Court et al. 2004). This mouse model shows a broad sensory phenotype, making them a useful tool for studying CMT4F (Gillespie et al. 2000). Mice with truncated MTMR2, a phosphatidylinositide-3-phosphatase of PIP_2, show distantly pronounced myelin out-folding that is similar to human CMT4B1 patients (Bonneick et al. 2005). Mice lacking FIG, a 5-phosphatase of PIP_2, display the pale tremor phenotype accompanied by extensive neurodegeneration and peripheral neuropathy (Chow et al. 2007). These findings demonstrate the importance of PIP2 homeostasis for myelination (Vaccari et al. 2012).

Mice lacking *GJβ1* or transgenically expressing the mutant type of *GJβ1* (R142W) exhibit the late onset of demyelinating neuropathy that mainly affects motor neurons, resembling that in human CMTX1 patients (Anzini et al. 1997; Scherer et al. 1998).

7.4 Zebrafish CMT Models

The zebrafish is sometimes useful as an alternative model organism to murine models (Kozol et al. 2016). The small size and optical transparency of the zebrafish are suitable for the in vivo visualization of cells during early development (McLean and Fetcho 2011). Moreover, the zebrafish nervous system is less complex than that of mammals and, thus, may simplify functional studies on neural circuits (Goulding 2009).

Although knockout mice for MFN2 resulted in embryonic lethality (Chen et al. 2003; Strickland et al. 2014), MFN2 mutant zebrafish developed normally but showed progressive motor dysfunction, as observed in some human CMT2A patients (Chapman et al. 2013). Examinations of mitochondrial transport in the neurons of MFN2 knockout zebrafish revealed a defect in the retrograde transport of mitochondria. These phenotypes are very similar to those of human CMT2A patients, suggesting that this zebrafish model is very useful in the search for potential drugs to cure defects in mitochondrial dynamics and axonal transport. Another gene, called *solute carrier family 25 member 46* (*SLC25A46*), is also involved in mitochondrial dynamics and some neurodegenerative diseases including CMT, optic atrophy, and cerebellar degeneration (Abrams et al. 2015). The disruption of *SLC25A46* in zebrafish showed a phenotype with reduced mitochondrial fission, altered mitochondrial distribution in motor neurons, and a defect in the maintenance of neuronal processes; however, swimming deficits were mild (Kozol et al. 2016).

7.5 *Drosophila* CMT Models

Drosophila does not have axons surrounded by myelin sheaths or Schwann cells. Therefore, *Drosophila* is not suitable for developing the demyelinating type of CMT; however, several models have recently been developed for the axonal type of CMT.

7.5.1 *Drosophila* Mitochondrial CMT Models

Mitochondria undergo dynamic fission and fusion processes, the tight regulation of which is necessary for mitochondrial function, with defects causing various diseases (Itoh et al. 2013). The mechanism controlling mitochondrial dynamics is highly conserved among eukaryotes (Sanchis-Gomar et al. 2014). The process of fusion is controlled by three GTPases: mitofusin 1 and 2 (MFN1 and MFN2), located at the outer membrane of mitochondria, and OPA1, located at the inner membrane. The fission of mitochondria is controlled by DRP1 and FIS1, which regulate the formation of a contractile ring to divide mitochondria. Mitochondrial dynamics are necessary for not only the morphology and structure of the mitochondrial network but also its function. The high conservation of these proteins among eukaryotes also supports the importance of mitochondrial dynamics for its proper function. Mitochondrial dynamics, such as the balance between fusion and fission, are required for mitochondrial biogenesis (Gomes and Scorrano 2013). Mitochondrial dynamics also play important roles in quality control involving the destruction of damaged mitochondria by autophagy (mitophagy) (Verstreken et al. 2005; Pla-Martin et al. 2013). In addition, the precise subcellular localization and transport of mitochondria are necessary for its function. The axonal transport of mitochondria is necessary for supplying energy at synapses, and the localization of mitochondria at the vicinity of the endoplasmic reticulum is suitable for regulating calcium homeostasis (Verstreken et al. 2005). Therefore, mutations in genes involved in mitochondrial dynamics and function are related to neuropathologies in both the central and peripheral nervous systems.

MFN2 and *GDAP1* have both been identified as causative genes for CMT and are involved in mitochondrial dynamics. MFN2 is required for mitochondrial fusion, while

GDAP1 participates in mitochondrial fission (Pedrola et al. 2005; Niemann et al. 2005). Therefore, the function of MFN2 appears to be antagonistic to GDAP1. Although the GDAP1 homologue has not yet been found in yeast, the expression of human GDAP1 in yeast may complement a defect in Fis1 that is involved in mitochondrial fission in yeast (Estela et al. 2011). *GDAP1* is associated with autosomal dominant CMT2K and AR CMT4A, while *MFN2* is autosomal dominant CMT2A in most cases, as described above. Mutations in both the *MFN2* and *GDAP1* genes show a similar type of pathology and appear to interact with each other. However, it currently remains unclear why

mutations in *GDAP1* and *MFN2* result in similar phenotypes, despite their functions being antagonistic to each other (Vital et al. 2012).

Analyses with genomic databases suggested that *Drosophila CG4623* (*dGdap1*) is an ancestor gene of mammalian *GDAP1* and *GDAP1L1*, which appear to have originated by gene duplication (López et al. 2015). GDAP1 belongs to the family of glutathione *S*-transferases. The overexpression and knockdown of *dGdap1* in eye imaginal discs by the *GMR-GAL4* driver induced the loss of some photoreceptor neurons in an age-dependent manner, suggesting that a proper level of dGdap1 is required for the survival of photoreceptor neurons. The expression of human GDAP1 rescued the neurodegeneration phenotype induced by the knockdown of *dGdap1*, indicating that human GDAP1 complements the function of its *Drosophila* counterpart. The overexpression and knockdown of *dGdap1* also induced an aberrant axon morphology. dGdap1 is expressed in muscle, and the muscle-specific overexpression of *dGdap1* by *myosin heavy chain* (*Mhc*)-*GAL4* induced the degeneration of myofibrils accompanied by the fragmentation of mitochondria (López et al. 2015). Similarly, the muscle-specific knockdown of *dGdap1* induced the degeneration of myofibrils accompanied by extensive mitochondrial fusion. Therefore, muscular degeneration appears to be tissue autonomous and not dependent on innervation. Mitochondrial dysfunction may increase oxidative stress. In young flies with altered levels of dGdap1, no significant differences were noted in the production of reactive oxygen species (ROS). However, in aged flies, decreases and increases in ROS levels were observed in *dGdap1*-overexpressing and *dGdap1*-knockdown flies, respectively (López et al. 2015). Since marked changes were not detected in young flies, in which mitochondrial defects had occurred, the generation of oxidative stress does not appear to be the primary cause of neuromuscular degeneration but rather a long-term effect of mitochondrial dysfunction.

Although CMT-linked mutations in human mitofusin 2 (hMfn2) are predominantly within the GTPase domain, two rare mutations in hMfn2 heptad repeat 1 (HR1), hMfn2 M393I and R400Q, were poorly characterized. These mutations in the hMfn2 HR1 domain have been characterized in a *Drosophila* model (Eschenbacher et al. 2012). Wild-type hMfn2 and the two mutants were expressed in *Drosophila* eyes or heart tubes, which are deficient in endogenous *Drosophila* Mfn (dMfn). The two mutants induced similar *Drosophila* compound eye phenotypes. In contrast, hMfn2 R400Q induced more severe cardiomyocyte mitochondrial fragmentation and cardiac phenotypes than hMfn2 M393I. These detailed analyses using a *Drosophila* model indicated the organ-specific and differential effects of the two hMfn HR1 mutations (Eschenbacher et al. 2012).

Currently, recessive mutations in the *cytochrome c oxidase assembly factor 7* (*COA7*) was identified in four unrelated patients among a Japanese case series of 1396 CMT patients or other inherited peripheral neuropathies including complex forms of CMT (Higuchi et al. 2018). COA7 has a role in assembling mitochondrial respiratory chain (MRC) complexes that function in oxidative phosphorylation. *Drosophila* contains a single homologue (dCOA7) to human COA7. The identity and the similarity of the amino acid sequences of dCOA7 and human COA7 are 48% and 70%, respectively. With respect to conservation of specific COA7 domains, SEL1-like domain, which is believed to be involved in protein-protein interactions, is highly conserved between human COA7 and dCOA7 and showed 44% identity. The similarity of the human and *Drosophila* SEL1-like domain is as high as 78%. The *dCOA7* knockdown models have been developed (Higuchi et al. 2018). Pan-neuron-specific *dCOA7* knockdown by the *Elav-GAL4* driver caused a shorter life span than that of control flies and also reduced mobility evaluated by climbing assays in adults. Analyses of the morphology of motor neuron presynaptic terminals at NMJs in muscle 4 of the third instar larvae of *dCOA7* knockdown flies revealed that the total length of the synaptic branches of motor neurons in knockdown flies was shorter than that of control flies. The knockdown of *dCOA7* in eye imaginal discs by the *GMR-GAL4* driver induced

morphologically aberrant rough eyes with fused ommatidia and a lack of bristles in adults. These results suggest that loss-of-function *COA7* mutation is responsible for the phenotype of the presented patients (Higuchi et al. 2018).

7.5.2 *Drosophila* CMT Models Targeting tRNA Synthetases

Previous studies reported that dominant mutations in six distinct aminoacyl-tRNA synthetases induced axonal and intermediate CMT, such as glycyl-tRNA synthetase (GARS) (Antonellis et al. 2003), tyrosyl-tRNA synthetase (YARS) (Jordanova et al. 2006), alanyl-tRNA synthetase (AARS) (Latour et al. 2010), histidyl-tRNA synthetase (HARS) (Vester et al. 2013), lysyl-tRNA synthetase (KARS) (McLaughlin et al. 2010), and methionyl-tRNA synthetase (MARS) (Gonzalez et al. 2013). Aminoacyl-tRNA synthetases catalyze the aminoacylation of tRNA with their cognate amino acids, and this is an essential process in translation. All CMT-related aminoacyl-tRNA synthetases form homodimers for their activation.

Drosophila CMT models targeted to *YARS* recapitulated the characteristics of CMT, including progressive locomotive defects, terminal axonal degeneration, and electrophysiological defects (Storkebaum et al. 2009). Biochemical and genetic complementation assays using *Drosophila* CMT models revealed that the loss of enzyme activity was not a common feature of the CMT-like phenotype. Transgenic flies carrying human wild-type GARS or three CMT-mutant GARS carrying the missense mutations E71G, G240R, and G526R have been established using a landing system to target the transgene to specific chromosomal sites (Niehues et al. 2016). The effects of these mutations have been characterized to show that the enzyme carrying the E71G mutation is enzymatically active, whereas that carrying the G240R or G526R mutation is inactive. The ubiquitous expression of mutant GARS induced lethality with the phenotypic strength of the mutations ranging from G240R > G526R > E71G, whereas no effect was observed with wild-type GARS. The ubiquitous expression of mutant GARS in adults using the GAL80 system reduced life spans with the phenotypic strength of the mutations ranging from G240R > G526R > E71G, whereas no effects were observed with wild-type GARS. Moreover, the expression of mutant GARS in motor neurons by the *OK371-GAL4* driver resulted in defects in locomotion evaluated by climbing assays, whereas no effects were noted with wild-type GARS (Niehues et al. 2016). Therefore, mutant GARS are intrinsically toxic to motor neurons. Consistent with these findings, the motor neuron-specific expression of mutant GARS G240R and G526R reduced synapse sizes in all muscles analyzed, whereas that of GARS E71G only reduced synapse sizes in distal muscles. Thus, reductions in synapse sizes were more prominent in distal muscles than in proximal muscles.

In motor neurons of third instar larvae, wild-type GARS and YARS proteins localize in the cytoplasm, with the homogenous staining of cell bodies, axons, and neuromuscular junctions (NMJs). No significant differences were observed in the subcellular localization of mutant GARS and YARS proteins, indicating that the subcellular localization of these proteins is not the cause of defects in motor and sensory neurons in *Drosophila* CMT models (Niehues et al. 2016).

The effects of mutant GARS expression on global protein synthesis rates in larval motor neurons were evaluated. A previous study reported that the expression of the mutant GARS proteins G240R and G526R reduced the levels of newly synthesized proteins, whereas those of wild-type GARS and the mutant GARS E71G did not alter the translation rate (Niehues et al. 2016). Thus, the expression of two out of the three CMT-associated mutant GARS proteins compromises global protein synthesis in motor neurons in vivo. Translational slowdown is not due to altered glycyl-tRNA aminoacylation and cannot be rescued by the overexpression of *Drosophila* GARS, indicating some gain-of-toxic function mechanism. The expression of CMT-mutant YARS also impairs translation, suggesting a common pathogenic mechanism for CMT related to various *aminoacyl-tRNA synthetase* genes. More detailed

studies revealed that the inhibition of global protein synthesis in motor and sensory neurons was sufficient to induce CMT-like phenotypes (Niehues et al. 2016).

Another group also established a model for GARS-associated neuropathy by expressing two mutant forms of GARS (G240R, P234KY) in *Drosophila* (Ermanoska et al. 2014). The phenotypes of these flies recapitulated several characteristics of CMT and were similar to the phenotypes observed with the *Drosophila* model of YARS-associated neuropathy. In addition, genetic modifiers of mutant YARS that were identified by a retinal degeneration screen also modified the phenotype of mutant GARS, suggesting a shared mechanism for peripheral neuropathies induced by aminoacyl-tRNA synthetases (Ermanoska et al. 2014).

7.5.3 *Drosophila* CMT Models Targeting FIG4

Factor-induced gene 4 (FIG4) carries a sac phosphatase domain consisting of seven conserved motifs that define phosphoinositide phosphatase activity. FIG4 forms a complex with Vac 14 and Fab1, a 5′-kinase of PI(3)P (Gary et al. 2002). This protein complex mediates the conversion of PI(3)P to PI(3,5)P2, which is associated with the intracellular vesicles of early and late endosomes (Sbrissa et al. 2007; Huotari and Helenius 2011). Mutations in the *FIG4* gene are associated with CMT 4 J (Chow et al. 2007), as described above, Yunis-Varon syndrome (YVS) (Campeau et al. 2013), and epilepsy with polymicrogyria (Baulac et al. 2014). YVS is an AR disorder with cleidocranial dysplasia, digital anomalies, and severe neurological involvement. Homozygous *FIG4*-null mice exhibit neurodegeneration and enlarged vacuoles in neurons (Chow et al. 2007). In addition, the FIG gene has been identified as a genetic susceptibility factor for amyotrophic lateral sclerosis (ALS) (Chow et al. 2009).

In *Drosophila*, there is a single orthologue for human FIG4, designated as dFIG4. GAL4-UAS targeted expression combined with RNAi was employed to knockdown *dFIG4* in various tissues (Fig. 7.1). Pan-neuron-specific *dFIG4* knockdown by the Elav-GAL4 driver caused a shorter life span than that of control flies and also reduced mobility measured by climbing assays in adults (Kyotani et al. 2016). Mobility defects appeared to be enhanced with aging. Analyses of the morphology of motor neuron presynaptic terminals at NMJs in muscle 4 of the third instar larvae of *dFIG4* knockdown flies revealed that the total length of the synaptic branches of motor neurons in knockdown flies was shorter than that of control flies (Fig. 7.2). The knockdown of *dFIG4* in eye imaginal discs by the GMR-GAL4 driver induced morphologically aberrant rough eyes with fused ommatidia and a lack of bristles in adults. Although all eight photoreceptor cells (R1-R8) appeared to differentiate normally, the number of cone cells per ommatidium in pupal retinae was decreased in *dFIG4* knockdown flies. In addition, extra secondary and tertiary pigment cells surrounding cone cells were produced. Therefore, the knockdown of *dFIG4* by the *GMR-GAL4* driver disrupts the differentiation of pupal ommatidial cell types, particularly cone cells and pigment cells. In the *Drosophila* eye, axonal projections from photoreceptor cell neurons innervate the centers of the brain to generate visual connections. Differentiating photoreceptor cell neurons form an axonal bundle that targets different layers of the brain. Photoreceptor R1–R6 innervates laminae, and R7 and R8 extend into the medulla of the brain. The innervation of photoreceptor neurons was aberrant in dFIG 4 knockdown flies driven by pan-neuron-specific *Elav-GAL4*. These phenotypes are summarized in Fig. 7.3.

dFIG4 null mutants were produced by another group. *dFIG4* null mutants are viable but exhibit markedly enlarged lysosomes in muscle cells and neurons, accompanied by an age-related decline in flight ability (Bharadwaj et al. 2016). Transgenic flies expressing *dFIG4* missense mutations that correspond to human CMT mutations may partially rescue the lysosome expansion phenotype. The *dFIG 4* mutations predicted to inactivate phosphatase activity may rescue the lysosome expansion phenotype, and mutations in Fab1 also cause the lysosome expansion pheno-

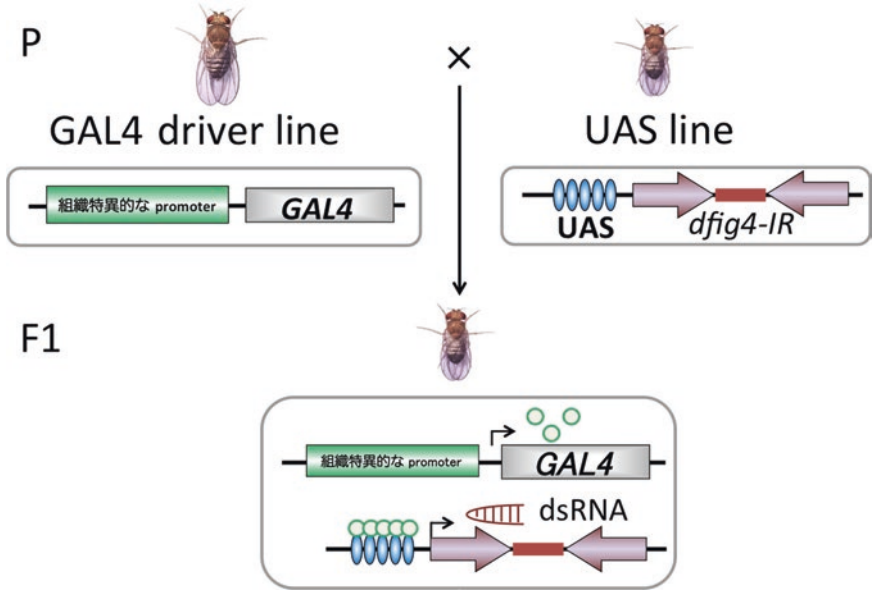

Fig. 7.1 Gal4-UAS targeted expression system to knock-down *dFIG4*. GAL4 driver strain was crossed with RNAi lines targeted to *dFIG4* gene. In the progeny, double-stranded RNA is expressed tissue, specifically to knock-down *dFIG4*, and the phenotype was examined

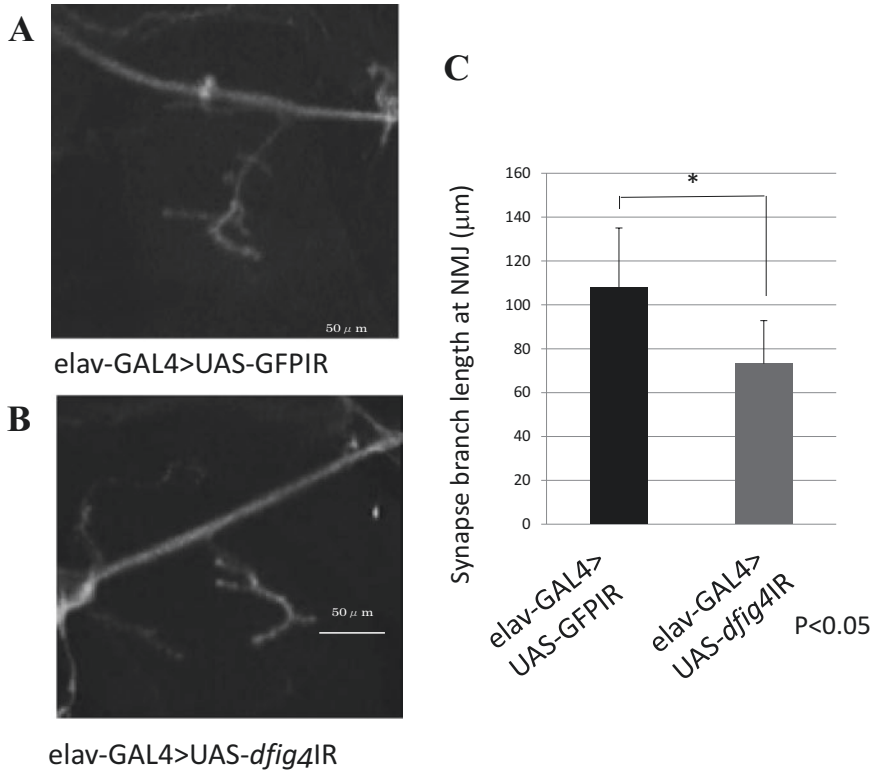

Fig. 7.2 Confocal images of anti-HRP staining of muscle 4 synapse in third instar larvae. (**a**) elav-GAL4 > UAS-GFPIR (*w*; UAS-GFP-IR/+; *elav*-GAL4/+). (**b**) elav-GAL4 > UAS-*dfig4*IR (*w*; UAS-*dFIG4*-IR$_{516–523}$/+; *elav*-GAL4/+). Image was taken by a confocal laser scanning microscopy (Olympus Fluoview FV10i). (**c**) Total branch length of the NMJ from muscle 4 for each of the indicated genotypes

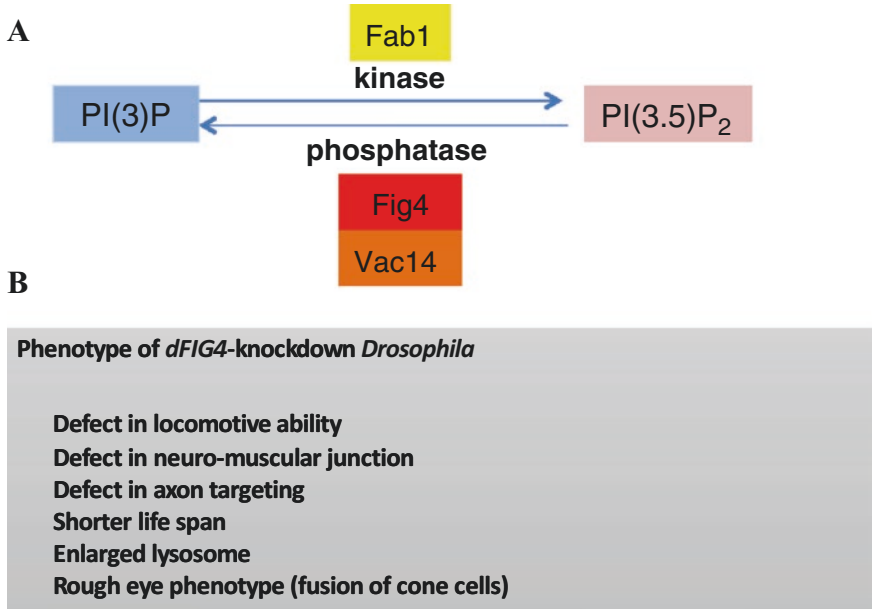

Fig. 7.3 Summary of phenotype of *dFIG4* knockdown flies. (**a**) Biochemical role of FIG 4. (**b**) Phenotype of *dFIG4*-knockdown flies

type (Bharadwaj et al. 2016). These findings suggest that dFIG4 has a phosphatase-independent biosynthetic function that is essential for lysosomal membrane homeostasis. Rab7 mediates endolysosomal trafficking through interactions with two different protein complexes, the HOPS complex and retromer complex. Lysosomal phenotypes in *dFIG4* mutants are reported to be suppressed by the depletion of Rab7 or the HOPS complex, demonstrating that dFIG 4 functions after endosome-to-lysosome fusion. Furthermore, the disruption of the retromer complex, implicated in recycling from lysosomes to Golgi, does not lead to similar phenotypes as *dFIG4* mutants. These findings from *dFIG4* null mutants suggest that lysosomal defects are not due to the compromised retromer-mediated recycling of endolysosomal membranes. Thus, dFIG4 plays a critical non-catalytic role in maintaining lysosomal membrane homeostasis, and this function is disrupted by mutations causing CMT4J and YVS. These findings are important for explaining the pathogenesis of CMT4J and YVS.

7.6 Perspectives

More than 35 *Drosophila* homologues of CMT-causing human genes have been identified to date (Table 7.1). Many of them belong to causative genes for the axonal type of CMT because *Drosophila* do not have axons surrounded by myelin sheaths and Schwann cells. These genes have a number of functions, such as endosomal sorting, cell signaling, mitochondrial maintenance, ER and Golgi functions, the formation of nuclear envelopes, mRNA processing, proteasome and protein degradation, the regulation of ion channels, axonal transport, and synaptic transmission. In addition to *dFIG4*, we are currently investigating several other CMT-causing genes in *Drosophila* by knocking them down specifically in pan-neuron or eye discs. They exhibit very similar phenotypes, such as defects in locomotive ability and NMJs as well as a rough eye phenotype. Therefore, we predict that these CMT-causing genes relate to common genes or common pathways in cells (Fig. 7.4). The identification of common genes and path-

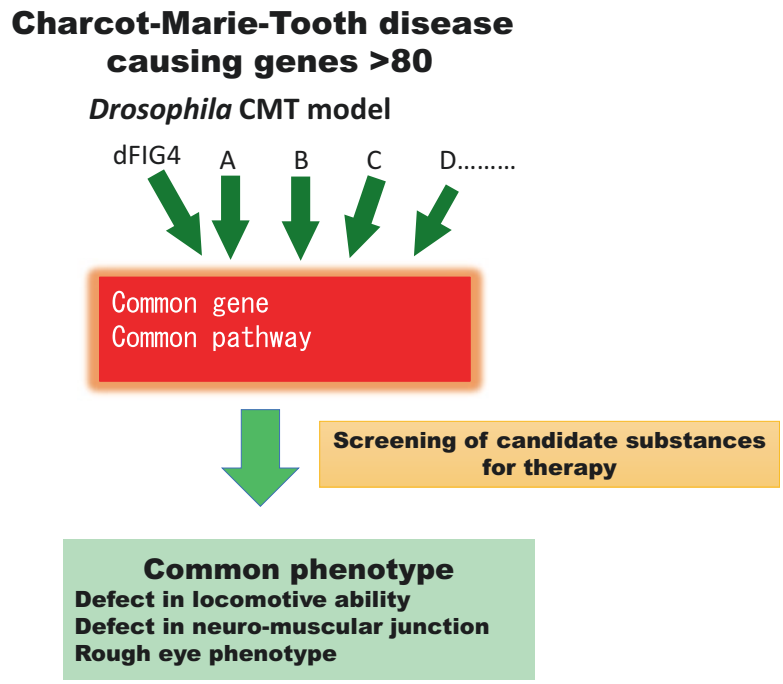

Fig. 7.4 Identification of common genes and pathways for CMT-causing genes

ways by genetic screening with *Drosophila* may provide insights for the development of novel therapies for CMT by targeting these common genes and pathways.

7.7 Commonly Used Protocol

Methods for the visualization of *Drosophila* NMJ by a super-resolution microscope (N-SIM, Nikon) are described below. In structured illumination microscopy (SIM), the cellular ultrastructure is elucidated by analyzing the moiré pattern produced when illuminating the specimen with a known high-frequency patterned illumination. N-SIM shows super resolution of up to 115 nm in multiple colors. The typical image of NMJ in muscle 4 of third instar larva is shown in Fig. 7.5.

Dissection

Pick up 4 third instar larvae using forceps and wash with *Drosophila* ringer. Dissect them in HL3 saline. Fix the dissected larvae on a 5.0-cm plastic petri dish using small pins. A movie showing the dissection of NMJs is available at this site (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2762896/) (Brent et al. 2009).

Fixation and Staining

Remove HL3 saline from the sample, and then add one drop of 4% paraformaldehyde in PBS to the sample.

Incubate at 25 °C for 30 min. Cover the dish with a lid to avoid evaporation.

After fixation, transfer the samples into 1.5-ml Eppendorf tubes.

Wash samples three times for 10 min each with PBS containing 0.3% Triton X-100.

After removing the washing solution, add 400 μl of PBS containing 0.15% Triton X-100 and 10% normal goat serum (NGS), and then incubate at 25 °C for 30 min.

Add primary antibodies in PBS containing 0.15% Triton X-100 and 10% NGS, and then incubate at 4 °C for 16 h.

Wash samples five times for 10 min each with PBS containing 0.3% Triton X-100.

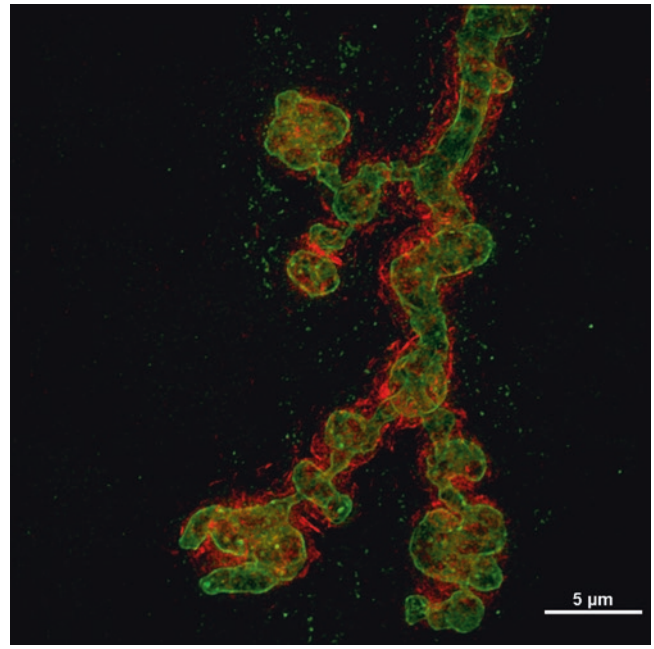

Fig. 7.5 Visualization of NMJ in muscle 4 of third instar larva. Image shows NMJ that was double-stained with anti-HRP (green) and anti-Dlg (red). Image was taken by a super-resolution microscope (N-SIM, Nikon)

Add secondary antibodies in PBS containing 0.15% Triton X-100 and 10% NGS, and then incubate at 25 °C for 3 h.

Wash samples three times for 10 min each with PBS containing 0.3% Triton X-100.

Transfer samples onto a slide glass, and cut out the head and tail regions using a sharp knife.

Add the mounting solution, ProLong Diamond (Invitrogen), and then gently overlay the cover glass.

Observation

Inspect samples with N-SIM. The images obtained were processed with MetaMorph software (Molecular Devices).

Primary Antibodies

Rabbit anti-GFP IgG (1:200, Medical & Biological Laboratories [MBL], 598), mouse anti-discs large (Dlg) (1:500, Developmental Studies Hybridoma Bank [DSHB], 4F3), and mouse anti-Bruchpilot (Brp) IgG (1:200, DSHB, nc82)

Secondary Antibodies

Alexa 594(1:400), 488(1:400), or 594 (1:400)-conjugated anti-mouse IgG, or anti-rabbit IgG. FITC-conjugated goat anti-HRP IgG (1:400)

HL3 (hemolymph-like solutions)

70 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose, and 5 mM HEPES or BES, pH 7.2

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8 *Drosophila* as a Model to Gain Insight into the Role of lncRNAs in Neurological Disorders

Luca Lo Piccolo

Abstract

It is now clear that the majority of transcription in humans results in the production of long non-protein-coding RNAs (lncRNAs) with a variable length spanning from 200 bp up to several kilobases. To date, we have a limited understanding of the lncRNA function, but a huge number of evidences have suggested that lncRNAs represent an outstanding asset for cells. In particular, temporal and spatial expression of lncRNAs appears to be important for proper neurological functioning. Stunningly, abnormal lncRNA function has been found as being critical for the onset of neurological disorders. This chapter focus on the lncRNAs with a role in diseases affecting the central nervous system with particular regard for the lncRNAs causing those neurodegenerative diseases that exhibit dementia and/or motor dysfunctions. A specific section will be dedicated to the human neuronal lncRNAs that have been modelled in *Drosophila*. Finally, even if only few examples have been reported so far, an overview of the *Drosophila* lncRNAs with neurological functions will be also included in this chapter.

Keywords

lncRNAs · Neurological disorders · Dementia · Motor system disorders · *Drosophila* · RNA processing · hnRNPs · Toxic aggregates

8.1 Biology of lncRNAs

The classic view of the central dogma of biology stating that "DNA makes RNA and RNA makes protein" was changed upon the past decade, when the advances in genome-wide analysis have revealed that up to 90% of the human genome is transcribed, but only 1–2% of RNA is effectively translated into proteins. Stunningly, the remaining transcripts are non-protein-coding (ncRNAs). This phenomenon is now known as "pervasive transcription" and explains how almost all loci produce a plethora of interlaced and overlapping transcripts in both sense and antisense orientation.

At the beginning, because no obvious functions, the non-coding portion of a genome has been defined as useless or sometimes "selfish DNA", because it was believed that it was not contributing to an organism's fitness. However, further evidences have clearly shown that when

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the complexity of organism increases, the amount of ncRNAs within their genome also increases. Therefore, this evidence has suggested that the development of complex organisms do not exclusively depend on their protein-coding genes and that the ncRNAs could provide an additional critical level of regulation. Indeed, in the last decade, researchers have collected many evidences to highlight that a large number of ncRNAs is in fact involved in crucial biological processes for normal development, physiology and diseases (Esteller 2011; Kaikkonen et al. 2011; Roberts et al. 2014; Sana et al. 2012; Zhou et al. 2010).

Non-coding transcripts are divided into housekeeping and regulatory, where the first one are constitutively expressed and include ribosomal (rRNAs), transfer (tRNAs), small nuclear (snRNAs) and small nucleolar (snoRNAs) RNAs (Fig. 8.1).

To date, there is no clear taxonomy between regulatory ncRNAs, and different authors in fact apply diverse classification system and/or nomenclature to organize the subclasses. However, largely shared distinction is made with respect to the size of transcripts. The regulatory ncRNAs are generally divided into small and long, establishing up to 200 nucleotides (nt) the size limit in this classification. Those less than 200 nt include micro, small interfering and Piwi-associated RNAs, while those longer than 200 are known as long non-coding RNAs (lncRNAs) (Fig. 8.1).

Previous reports had suggested to consider the vast majority of the lncRNAs similar to mRNA, since they are transcribed by RNA pol II and processed via 5′ end-capping, 3′ end-polyadenylation and finally, through alternative splicing. However, recent studies have highlighted new features of lncRNAs that have led to further distinguish them from mRNAs (Quinn and Chang 2016).

A function-based classification of lncRNAs entails a four distinct groups of lncRNAs, reviewed in 2012 (Ip and Nakagawa 2012) (Fig. 8.2). Accordingly, lncRNAs can be involved in transcription (Fig. 8.2, I) and post-transcriptional regulation (Fig. 8.2, II). Moreover, lncRNAs show the ability to establish a huge number of diverse interaction with other cellular components to generate multiple levels of regulation (Fig. 8.2, III). The largest group includes lncRNAs that associate with chromatin modifiers and take part in epigenetic regulation of gene expression. Strictly, 20% of lncRNAs are co-immunoprecipitated with

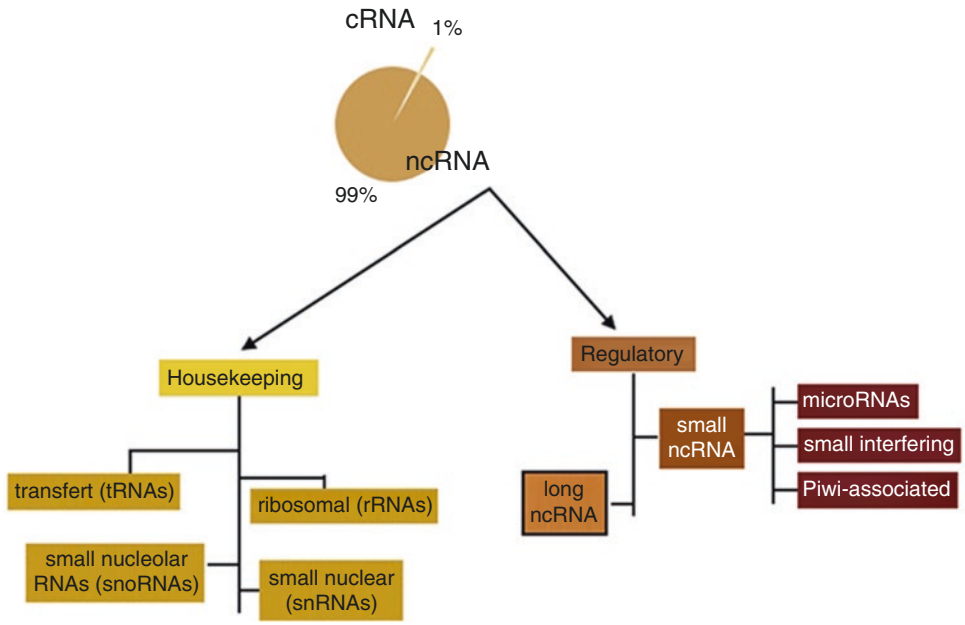

Fig. 8.1 Diversity of non-coding RNAs. cRNA, coding RNA; ncRNA, non-coding RNA

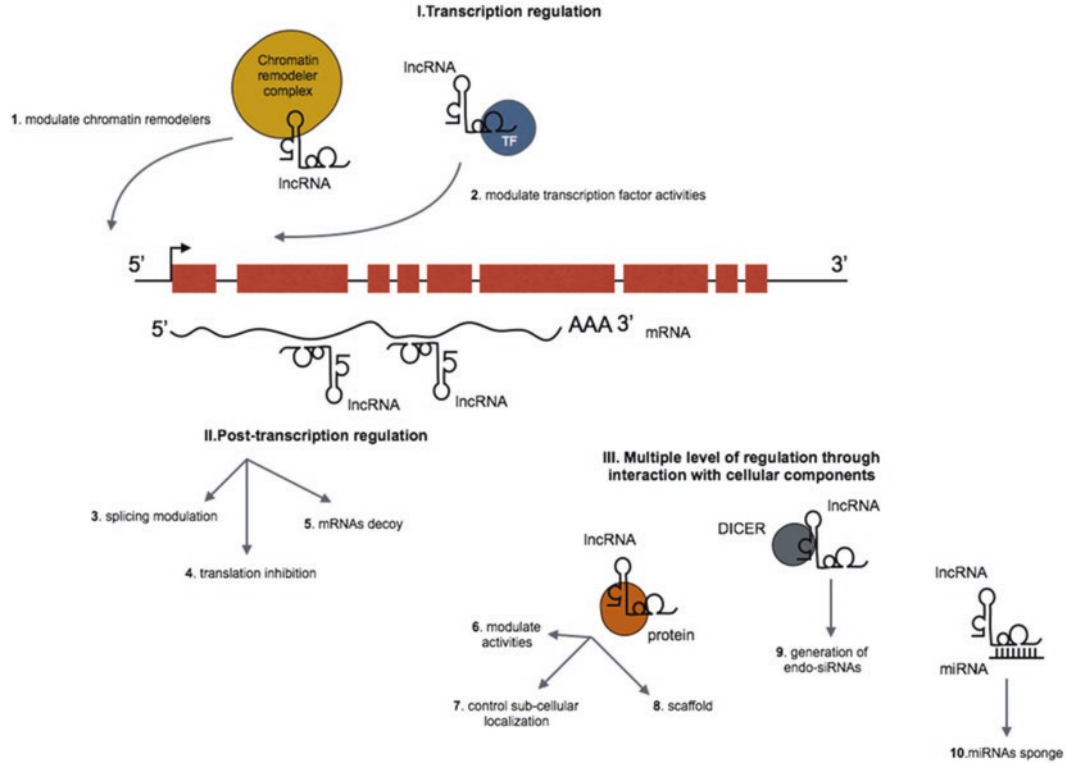

Fig. 8.2 Function-based classification of lncRNAs. TF, transcription factor. (Adapted from Ip and Nakagawa (2012))

histone methyltransferase complex PCR2 (Davidovich and Cech 2015; Khalil et al. 2009; Marchese and Huarte 2014) allowing that the chromatin modification may be one of the representative functions of lncRNAs (Fig. 8.2).

The lncRNAs which act as precursors of the small RNAs to take part in gene suppression via RNA silencing mechanisms belong to the second group (Fig. 8.2, II).

Recent findings remarked the importance of lncRNAs as components of nuclear bodies (NBs), thus allowing their classification into the third group (Fig. 8.2, III-8 "scaffold"). Extensive reviews on these classes are available since 2012 (Cheng et al. 2016; Chujo et al. 2016; Ip and Nakagawa 2012).

Other ncRNAs are tentatively categorized into a fourth group, but this classification suffers for the lacking information on the large diversity of lncRNA's functions.

A position-based classification was recently summarized by Riva and colleagues (Riva et al. 2016), and it might represent a convenient depiction for educational purposes. Accordingly, lncRNAs can be broadly classified into two large categories, herein illustrated in Fig. 8.3.

To make out an extensive report of the wide variety of biological functions involving lncRNAs is quite hard given they control nearly every level of gene regulation—pretranscriptional, transcriptional and posttranscriptional—through DNA–RNA, RNA–RNA or protein–RNA interactions. Indeed, lncRNAs may consist of multiple binding modules and are therefore capable of connection to any cellular components.

Since the lncRNAs intervene in the co-transcriptional recruitment of protein factors to specific loci, they have an intrinsic cis-regulatory capacity; thus, it's largely accepted that the best-defined molecular function of lncRNAs is the *cis*-acting epigenetic gene regulation (Sun and Kraus 2013). However, some new reports are now inviting to consider that the primary lncRNA's mode of gene regulation is *in trans* because some

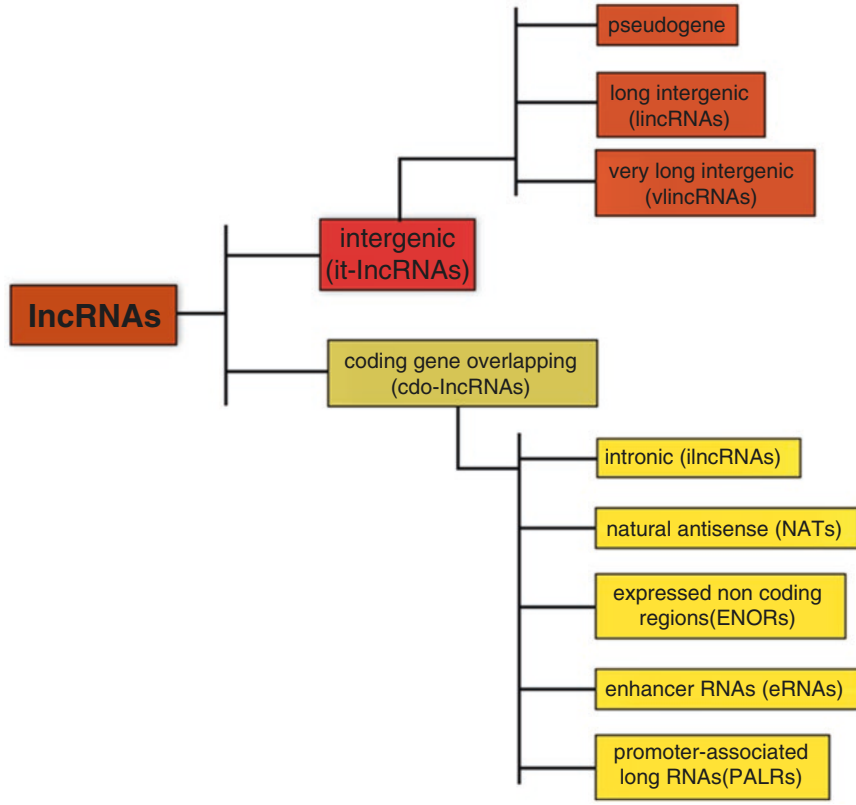

Fig. 8.3 Position-based classification of lncRNAs. (Adapted from Riva et al. (2016))

emerging evidences have shown that knockdown of several lncRNAs do not affect neighbouring genomic loci (Guttman et al. 2011). Regardless their controversial primary mode of action, *cis* and/or *trans*, the impact of lncRNAs in biological systems is just beginning to be elucidated. To date, most characterized lncRNAs have been implicated in development and differentiation or found to be involved in pathways associated with cell proliferation and cell death.

Despite the huge number of long non-translated transcripts, the functional significance of very few lncRNAs has been brought to light so far, especially those exhibiting differential expression in tumours, but almost the lncRNAs remain just annotated, with unknown functions.

A large body of evidence has revealed that lncRNAs play essential roles in all stages of carcinogenesis and metastasis (Weidle et al. 2017). On the other hand, different lncRNAs are deregulated in cancer, and many evidences have revealed that they are highly tumour- and lineage-specific, often associated with somatic copy number alterations, promoter hyper-methylation and/or cancer-associated SNPs (Bartonicek et al. 2016; Cerk et al. 2016; Weng et al. 2017). Strictly, lncRNAs can act either as tumour suppressors or as oncogenes to mediate several cancer-associated processes, such as epigenetic regulation and DNA damage or cell cycle control and miRNAs silencing. Interestingly, lncRNAs and PI3K have been shown to be interconnected in several different cancer subtypes with the ability to enhance aberrant cell proliferation, epithelial-to-mesenchymal transition, migration and invasion and also cancer cell metabolism. A recent review has highlighted the lncRNAs and PI3K cross-talk in cancer (Benetatos et al. 2017).

The involvement of lncRNAs in cancer makes them as a critical class of effectors or regulators. For instance, aberrantly expressed lncRNAs can be able to interact with protein and coding partners to cause deregulation of normal cellular pro-

cesses up to drive the cell towards a malignant state. Because of these crucial interactions, lncRNAs are actually considered as ideal targets for cancer therapy and biomarkers.

Numerous recent reviews focus on the role of lncRNAs in carcinogenesis and metastasis (Chen et al. 2017; Li et al. 2017; Rao et al. 2017; Weidle et al. 2017), and their involvement in these processes will not be discussed in this chapter.

8.2 Databases for lncRNAs: Emerging Tools with Great Expectations

As described up here, we have formed the impression that the lncRNAs have a critical cellular role and that they take part in intricate networks to support the cell fate, whereby new approaches to support the molecular and cellular research on lncRNAs are required to contribute in understanding of lncRNA function and mechanism of action. In fact, there is a large gap between the real number of lncRNAs that intervene into the cell and the number of those that we have figured out how, where and when function.

According to the current release of NONCODE Ev4 ID (http://www.noncode.org/analysis.php), the estimated number of the human lncRNA genes is 90.062, which is significantly higher than that of coding genes, actually estimated in 19.815 as reported on the current version of GENCODE (http://www.gencodegenes.org/stats/current.html).

Multiple high-quality resources of annotations are needed to identify and characterize lncRNAs in genomic studies. An increasing number of databases dedicated to lncRNAs are becoming available, whereby we are witnessing a rapid accumulation of large-scale data sets and novel computing tools. This is a good time for the research on lncRNAs. For instance, in the last 5 years, the number of publications about the role of lncRNAs in the human brain was exponentially growing. The merit is certainly to be attributed to intense research in the field of diseases that has led researchers to study not only the protein-coding transcripts but also to explore the unknown and unexpected functions of non-coding protein transcripts. In this context, the use of new databases will eventually enable the generation of new hypotheses about the roles of lncRNAs in different disease phenotypes (Table 8.1).

Existing genomics data could be re-annotated in terms of non-coding genes or transcripts to provide an understanding of their putative clinical relevance. In this respect, a recent analysis of the Cancer Genome Atlas (TCGA) data identified potentially clinically relevant non-coding transcripts. The expression of specific lncRNAs seems to be linked to patient survival, copy number alteration or histological subgrouping in glioblastoma as well as in lung, ovarian and prostate cancers.

The use of large data set could help to guide the research on lncRNAs and might provide unexpected connection with old and new cellular networks.

Recently, diverse resources dedicated to lncRNAs have been developed, which differ in data coverage and quality. A comprehensive compendium of human lncRNAs is offered by Lncipedia (http://www.lncipedia.org) which provides information on human lncRNA transcript sequence and structures.

An extensive and comparative analysis on the lncRNA database was recently proposed by Fritah and colleagues (Fritah et al. 2014) to offer a paradigmatic approach of how to query these databases to address putative lncRNA functions in human diseases.

DIANA-LncBase is the only database that specifies the incorporation of lncRNA annotations originating from the literature, from computational predictions and from primary data repositories. This database includes lncRNA annotations that are supported by experimental evidences (Paraskevopoulou et al. 2013).

An interesting example of lncRNA database is offered by ChIPBase v2.0 which is an open source for studying the transcription factor binding sites and motifs and for decoding the transcriptional regulatory networks of lncRNAs, miRNAs, other ncRNAs and protein-coding genes from ChIP-seq data. This database currently contains ~10,200 curated peak data sets

Table 8.1 Database for lncRNAs

Database	Description	Website	References
lncRNABase	Designed for decoding miRNA-lncRNA (lncRNAs, pseudogenes, circRNAs) and miRNA-ceRNA interaction networks from 108 CLIP-Seq data sets. This database also provides information about the interaction networks of lncRNAs, miRNAs, ceRNAs, mRNAs and RNA-binding proteins accordingly to data from 14 cancer types with >6000 tumour samples	http://starbase.sysu.edu.cn/mirLncRNA.php	Li et al. (2014)
NRED	The ncRNA expression database integrates annotated expression data from various sources, mainly from human and mouse models	http://nred.matticklab.com/cgi-bin/ncrnadb.pl	Dinger et al. (2009)
DmeLncDB	The *Drosophila melanogaster* lncRNA database provides integrated information for each lncRNA record such as loci, presence and number of exons, coding potential and secondary structures	http://dmelncdb.bime.ntu.edu.tw	
COME	The coding potential calculation tool based on multiple features is a robust coding potential calculation tool for lncRNA identification and characterization	https://github.com/lulab/COME.	Hu et al. (2017)
LNCediting	A database for functional effects of RNA editing in lncRNAs	http://bioinfo.life.hust.edu.cn/LNCediting/	Gong et al. (2017)
CHIPBase	Database for decoding the transcriptional regulation of lncRNAs and microRNA	http://rna.sysu.edu.cn/chipbase/	Yang et al. (2013)
DIANA-LncBAse	Experimentally verified and computationally predicted microRNA targets on lncRNAs	http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=lncBase/index	Paraskevopoulou et al. (2013)
LNCipedia	A database for annotated human lncRNA transcript sequences and structures	https://lncipedia.org	Volders et al. (2013)
lncRNAdb	Database providing comprehensive annotations of eukaryotic lncRNAs	http://www.lncrnadb.org	Amaral et al. (2011)
lncRNADisease	Experimentally supported lncRNA-disease associations	http://cmbi.bjmu.edu.cn/lncrnadisease	Chen et al. (2013))
LNCMap	The connectivity map of lncRNAs provides to establish the correlations among diseases, physiological processes and the action of small molecule therapeutics, by attempting to describe all biological states in terms of lncRNA signatures	http://www.bio-bigdata.com/LNCmap/	Yang et al. (2017)
lncRNome	Comprehensive database of lncRNA in humans	http://genome.igib.res.in/lncRNome/	Bhartiya et al. (2013)
Noncode	Noncode is an integrated knowledge database dedicated to non-coding RNAs (excluding tRNAs and rRNAs) with particular regard to lncRNAs	http://www.noncode.org	Zhao et al. (2016)

derived from ChIP-seq methods in 10 species (Zhou et al. 2017).

The current version of DmeLncDB (http://www.dmelncdb.bime.ntu.edu.tw) with 3014 is actually the most exhaustive database of lncRNAs of *Drosophila melanogaster*. In DmeLncDB, the integrated information for each lncRNA record includes loci, exons, directionality, cross-species conservation score, chromatin signatures, coding potential, predicted secondary structure, expression profiles in different developmental stages and the list of co-expressed coding genes. In summary, DmeLncDB is expected to serve as an important resource for lncRNA studies not only related to *Drosophil*a.

A new lncRNA database was recently released with the purpose to use lncRNA signatures to find the correlations among diseases, physiological processes and the action of small molecule therapeutics (Yang et al. 2017). By re-annotating the microarray data from the Connectivity Map database, the new LncRNA Connectivity Map (LNCmap) has obtained 237 lncRNA signatures of 5916 instances which correspond to 1262 small molecular drugs. This database could significantly improve our understanding of the biological roles of lncRNAs and provide a unique resource to reveal the connections among drugs, lncRNAs and diseases.

8.3 LncRNAs in the Central Nervous System

LncRNAs are predicted to function, probably preferentially, in the nervous system where they may play roles in mediating neuronal development, behaviour and cognitive functions. Indeed, annotation via the GENCODE suggests that about 40% of differentially expressed lncRNAs are specific to the brain (Derrien et al. 2012), and moreover, within the brain, the expression of lncRNAs seems to be particularly region-specific and highly dynamic during neural differentiation (Ramos et al. 2013). For instance, the important work of Mercer and colleagues has shown that 849 lncRNAs on 1328 examined are expressed in the adult mouse brain in association with specific neuroanatomical regions (Mercer et al. 2008). Intriguingly, as some authors comment, the ductile and elaborate functions of lncRNAs seem to be in accord with the diversity and complex nature of the central nervous system (CNS). These proprieties make the lncRNAs ideal candidates to explain the rapid evolution of human CNS and in the same time represent a promising breakthrough to gain insight into the molecular mechanisms of CNS development and neuropsychiatric diseases. Finally, a conspicuous body of work has revealed that the lncRNAs have functions in neuronal differentiation and maintenance (Pollard et al. 2006; Wu et al. 2013).

8.3.1 LncRNAs and Neuronal Development

The differentiation programme of progenitor neuronal cells seems to be under control of lncRNAs, and recently, a review has been proposed to summarize lncRNAs with function in neuronal development (Clark and Blackshaw 2014). Some interesting examples are the lncRNAs *AK055040*, *AK091713* and *AK124684* that are required for neuronal induction of ES cell (Ng et al. 2012) or the long intergenic ncRNA (lincRNA) *Cyrano* which are maybe involved in *miR-7* decoy transcript, and its loss of function in mice results in small eyes and brains due to a reduction in neural specification (Ulitsky et al. 2011). To date, other six lincRNAs are important for neuronal cell fate specification. Among them, the most recently identified is the lncRNA *TUNA* which regulates pluripotency by recruiting RNA-binding proteins (RBPs) to Sox2, Nanog and Fgf4 promoters (Lin et al. 2014).

The number of lncRNAs involved in CNS development is expected to grow in the next years through the improvement of techniques that will allow the identification and then the characterization of new lncRNAs. Indeed, in order to identify lncRNAs, a direct detection of the transcribed RNA is necessary, but actually, conventional gene expression microarrays are only designed to reveal the expression of protein-coding mRNAs; thus, the use of new advanced techniques like tiling arrays, serial analysis of gene expression (SAGE), cap analysis of gene expression (CAGE) and high-throughput RNA sequencing (RNA-seq) is now providing new resources. To comprehensively identify lncRNAs, a very challenge due to their low level expression and/or their more cell type specificity than protein-coding genes. An interesting attachment to a recent review clearly describes the methodologies for lncRNA identification and analyses (Fatica and Bozzoni 2014).

A new approach by using an ab initio transcriptome reconstruction using eight purified cell populations from mouse cortex has allowed Dong

and colleagues to the identification of more than 5000 lncRNAs. Specifically, they have performed a motif searches in ENCODE DNase I digital footprint data and Mouse ENCODE promoters in order to infer the occupancy of transcription factors (TFs). By integrating TF binding and cell-type-specific transcriptomic data, they have constructed a novel framework that seems to be useful for the systematic identification of lncRNAs with high potential activity in the brain and in particular with critical role in neuronal cell fate determination (Dong et al. 2015). Based on this integrative analysis, they have identified lncRNAs that are regulated during oligodendrocyte precursor cell (OPC) differentiation from neural stem cells (NSCs) and that are likely to be involved in oligodendrogenesis. The approach of Dong and colleagues has finally allowed to find a novel role for the lncRNA *lnc-OPC* in OPC fate determination.

Among the last identified lncRNAs with important roles in neural development and neural cell fate determination, the *lncOL1*and *FMR4* are maybe two very interesting examples. Indeed, lncOL1 overexpression promotes precocious oligodendrocyte differentiation in the developing brain, whereas genetic inactivation of *lncOL1* causes defects in CNS myelination and remyelination upon an injury (He et al. 2017). Functional analyses have further illustrated that *lncOL1* can interact with Suz12 which is a component of polycomb repressive complex 2 (PRC2) to promote the maturation of oligodendrocytes. Finally, the lncRNA *FMR4* has been described as chromatin-associated lncRNA with the ability to alter in trans the chromatin state and the expression of several hundred genes (Peschansky et al. 2016).

8.3.2 LncRNAs and Neurodegenerative Diseases

Our appreciation that lncRNAs have a broad spectrum of functions in the normal brain development to orchestrate synaptic plasticity and take part in cognitive and memory process has increased together with the discovery of their impact in aged brain and CNS disorders. An increasing number of studies have shown that lncRNAs are associated with several neurodegenerative disorders (Table 8.2). To date, an accurate report has been proposed by Wan and colleagues (Wan et al. 2017) to provide a list of detailed information on lncRNAs and their expression level in diseases like Alzheimer and Parkinson. Moreover, Lourenco and colleagues have identified a number of lncRNAs that are dysregulated upon a depletion of the aggregation-prone proteins TDP43 or FUS, which in turn are known to be involved in proteinopathies or neurodegenerative diseases such as FTLD and ALS (Lourenco et al. 2015). It is important to note that only few reports have described the molecular mechanism through a lncRNA intervention in the neurodegenerative disease. More often instead, a global overview of the expression level of the lncRNAs in pathological conditions in comparison with normal phenotype has been provided. Finally, whether the modulation of lncRNA expression is a cause or a consequence of disease yet remains hard to address.

Bearing in mind that how the lncRNAs impact the diseases has not fully elucidated so far, the following paragraphs are an attempt to report and describe the diverse mechanisms by which some lncRNAs involve neurodegenerative diseases. In particular, with a view to simplifying herein, the neurodegenerative diseases have been in short classified in those showing dementia and in those exhibiting locomotive dysfunctions.

8.3.2.1 LncRNAs in Neurodegenerative Diseases with Dementia

The Alzheimer's disease (AD) is one of the most common neurodegenerative diseases with more than 80% of dementia cases in people aged older than 65 years (Anand et al. 2014). AD is characterized by apraxia, agnosia and other devastating symptoms due to the progressive decline in mental and behavioural functions. The neuropathological hallmark of AD is the deposit of extracellular senile plaques and intracellular neurofibrillary tangles composed of amyloid beta

Table 8.2 Human lncRNAs that have been discussed because of their role in neurological and neurodegenerative diseases

lncRNA	Classification	Function	Disease associated	References
AK055040	Promoter-associated	Located upstream of CACN2D1; interacts with SUZ12; neuronal development	Neurological disorders	Ng et al. (2012)
AK091713	Overlapping	Contains mir125B and LET7A and the nuclear-encoded mitochondrial protein BLID within its introns; neuronal development	Neurological disorders	Ng et al. (2012)
AK124684	Intergenic	Interacts with REST; neuronal development	Neurological disorders	Ng et al. (2012)
TUNA	Intergenic	Regulates pluripotency by recruiting Sox2, Nanog and Fgf4; neuronal cell fate specification	HD	Lin et al. (2014)
Lnc-OPC	Promoter-associated	Regulates oligodendrocyte precursor cell differentiation; neuronal cell fate specification	Intellectual disability	Dong et al. (2015)
lncOL1	Chromatin-associated	Abnormal expression can cause alteration in oligodendrocyte differentiation	Neurological disorders	He et al. (2017)
FMR4	NAT	Chromatin-associated with the ability to alter the expression of several genes involved in neuronal development; neuronal development	Intellectual disability and autism spectrum disorder (ASD)	Peschansky et al. (2016)
BACE1-AS	NAT	Positive regulator of BACE1 expression; it can induce overproduction of Aβ42	AD	Faghihi et al. (2008)
LRP1-AS	NAT	Negatively controls the LRP1 gene expression by modulating HMGB2 activity	AD	Yamanaka et al. (2015)
17A	NAT	Regulates the pre-mRNA processing of GPR51/GABBR2	AD	Massone et al. (2011)
NDM29	NAT	Aberrant expression can induce overproduction of Aβ42	AD	Massone et al. (2012)
51A	NAT	Controls the alternative splicing of SORL1	AD	Ciarlo et al. (2013)
Expansion repeats in promoter and/or intron of C9orf72	Promoter-associated	C9orf72 is a protein-coding gene that plays an important role in the regulation of endosomal trafficking and has been shown to interact with Rab proteins that are involved in autophagy and endocytic transport	ALS/FTD	Moens et al. (2017)
NEAT1	Intergenic, arcRNA	Regulates splicing processes by modulating the activity of several hnRNPs into the paraspeckles	ALS/FTD, HD	Johnson (2012), Lagier-Tourenne et al. (2012) and Sunwoo et al. (2017)
MALAT1	Intergenic arcRNA	Regulates splicing processes by modulating the activity of several hnRNPs into the nuclear speckles	ALS/FTD	Lagier-Tourenne et al. (2012) and Liu et al. (2017)
FRG1–3	NAT	N.D.	PD	Soreq et al. (2014)
AS Uchl1	NAT	Drives the Uchl1 translation through a combined SINEB2 repeat	PD	Carrieri et al. (2015)

(continued)

Table 8.2 (continued)

lncRNA	Classification	Function	Disease associated	References
naPINK1	NAT	Increases the stability of a splice variant PINK1 antisense (svPINK1)	PD	Scheele et al. (2007)
HOTAIR	NAT	Mediates the trimethylation of histone H3 at lysine 27 and the demethylation of histone H3 dimethyl Lys4 by recruiting the polycomb repressive complex 2 and the lysine-specific demethylase 1/co-repressor of RE1-silencing transcription factor (coREST)/REST complex to the target gene promoters, which leads to gene silencing	PD	Liu et al. (2016)
HAR1	Intergenic	Target of REST	HD	Johnson et al. (2010)
TUG1	Intergenic	Necessary for retinal development	HD	Johnson (2012)
MEG3	Intergenic	Binds the PCR2 epigenetic silencing complex	HD	Johnson (2012)
ATXN8OS	NAT	Brain-specific function	SCA8	Daughters et al. (2009)
Lnc-SCA7	Retro-pseudogene	Post-transcriptionally regulates the expression of ATXN7	SCA7	Tan et al. (2014)

(Aβ) protein and hyperphosphorylated tau protein. In the last decade, a conspicuous number of reports have revealed the molecular pathomechanism of AD to show that amyloid precursor protein (APP) is sequentially cleaved by β-site APP cleaving enzyme-1 (BACE1) and γ-secretase during Aβ biosynthesis, with γ-secretase initiating the "amyloid cascade". It is known that the Aβ peptides can aggregate into soluble oligomers which become able to induce mitochondrial dysfunction and oxidative damage. Finally, the soluble oligomers allow to start a cascade of detrimental events that induce synaptic dysfunction and apoptosis.

Although alterations in the expressions of non-coding RNAs have been studied in AD, most research focused on the involvement of microRNAs, and comprehensive expression profiling of lncRNAs in AD has been lacking. Almost the information on the role of lncRNAs in AD pathogenesis come from large data set analysis such as re-annotated microarrays of post-mortem human patient's tissues and high-throughput screening of altered gene expression profile. Few studies on transgenic mice also have achieved to unrevealing the involvement of lncRNAs in this devastating disease. Recently, a microarray analysis has been performed to collect the expression profile of lncRNAs dysregulated in a triple transgenic model of AD (3xTg-AD), and a total of 4622 lncRNAs have been analysed (Lee et al. 2015). In this study, one of the most highly upregulated lncRNAs has shown a 395 bp core sequence that overlaps with multiple chromosomal regions. Interestingly, the work of Lee and colleagues represents the first study that comprehensively has identified dysregulated lncRNAs in 3xTg-AD mice, and in the future, it might likely facilitate the development of therapeutics targeting lncRNAs in AD.

Despite a remarkable number of lncRNAs found dysregulated in AD (AD-associated lncRNAs), only few of them have been clearly characterized (Wu et al. 2013; Zhou and Xu 2015). Strictly, beta-secretase 1 RNA antisense (*BACE1-AS*) and low-density lipoprotein receptor-related protein RNA antisense (*LRP1-AS*) are the best studied, and both are natural antisense lncRNAs (NATs). *BACE1-AS* is highly expressed in AD patients, and it has been shown to be capable of upregulating *BACE1* mRNA (Faghihi et al. 2008). Other NATs have been shown involved in neurodiseases through the ability to form the duplex complex with the

sense of coding mRNA with a detrimental effect on its stability and a resulting mRNA translation inhibition, but *BACE1-AS* interestingly can function by increasing *BACE1* mRNA stability to allow the overproduction of Aβ42.

Among lncRNAs, the NATs have emerged as a large class of regulatory lncRNAs especially with role in neurodegenerative diseases, and they have been found in more than 70% of all transcriptional units and 20% of human genes. Since the functional knockdown of NATs has positive or negative influences on the expression of neighbouring protein-coding genes, it has been recently proposed that they can have a critical role in regulation of gene expression. Intriguingly, another lncRNAs AD-associated is also NAT. In fact, a recent work of Yamanaka and colleagues has shown that *LRP1-AS* lncRNA can negatively control the *LRP1* gene expression through a modulation of non-histone chromatin modifier HMGB2 activity (Yamanaka et al. 2015). *LRP1* is a member of the low-density lipoprotein receptor family, with a role in a variety of physiological processes including the cellular transport of cholesterol, endocytosis of ligands, and transcytosis across the blood-brain barrier. Recently, *LRP1* has been implicated in the systemic clearance of AD amyloid-beta (Aβ), and the level of *LRP1* expression is critical for AD progression. The study of Yamanaka and colleagues has shown that in the brain of AD patients, LRP1 is lowly expressed, while LRP1-AS is highly abundant. Due to the augmentation of *LRP1-AS* in AD brains, it has been proposed that *LRP1-AS* can play a central role to downregulate *LRP1* transcription, whereby it might be critical in AD pathomechanism (Yamanaka et al. 2015). Further investigations are required to understand the functional implication of LRP1-AS in the pathological processes underlying AD. However, it appears intriguing that two different lncRNAs involve in AD as NATs, and it seems worth looking at whether this is a common lncRNA's mode of action in degenerative diseases.

More recently, the use of a genome-wide screening has led to the identification of other lncRNAs with an aberrant expression in post-mortem human AD brains (Zhou and Xu 2015). A large part of these new lncRNAs have been classified as intergenic, and the overall expression profile has been considered as a specific AD-associated signature. In particular, among the new identified AD-associated lncRNAs, three have been characterized in further experiments such as the *17A*, *NDM29* and *51A* lncRNAs (Ciarlo et al. 2013; Massone et al. 2011, 2012). Interestingly, all of them have been found upregulated in human AD brains with the ability to finally induce unbalance in Aβ42/Aβ40 ratio to drive the accumulation of Aβ oligomers.

Briefly, the lncRNA *17A* is generated by antisense orientation in the third intron of the human G protein-coupled receptor 51 (*GPR51*, also known as *GABBR2*) gene, and it regulates the pre-mRNA processing of *GPR51*/*GABBR2*. The lncRNA *17A* allows the generation of splicing isoform B of *GABABR2*, which finally is not functional, as confirmed in human neuroblastoma cells. Some evidences have shown that an upregulation of the lncRNA 17A can cause the formation of a defective GABA signalling so that it has been proposed that the lncRNA 17A might induce the secretion of the Aβ. Similarly, the antisense lncRNA *51A*, deriving from the first intron of *SORL1* gene (a well-recognized risk factor for AD), by controlling the alternative splicing of *SORL1* can promote the Aβ formation.

As remarked in a recent review (Riva et al. 2016), it seems interestingly to note that both *17A* and *NDM29* lncRNA expression can be induced by inflammatory stimuli, which represent a pathogenic mechanism in AD. It will be important to extend our understanding of how the inflammation takes a part in the lncRNA-AD network because, due to the effects of lncRNAs on Aβ as above mentioned, one could hypothesize to indirectly modulate the Aβ synthesis by using anti-inflammatory drugs.

Frontotemporal dementia (FTD) is the second important cause of dementia after AD in elderly population. From clinical perspective, FTD is a heterogeneous neurodegenerative disorder, including behavioural variant FTD (bvFTD), semantic dementia, progressive non-fluent aphasia (PNFA), FTD-parkinsonism, and FTD-motor neuron disease. At least 8 causative genes have

been identified in patients with FTD, so far. Among them, the microtubule-associated protein tau (MAPT), progranulin (GRN) and chromosome 9 open-reading frame 72 (C9orf72) have been identified as responsible for almost all the familial cases and for about 20% of all cases (Lashley et al. 2015). Interestingly, the *C9orf72* gene on chromosome 9 carries a hexanucleotide repeat region in its promoter or in the intron 1 (depending on the transcript variant). A massive expansion of this repeat region triggers this syndrome (Chan 2014).

In the last years, neuropathological and genetic data have suggested that FTD might be a disease continuum with amyotrophic lateral sclerosis (ALS). For instance, alteration of RNA metabolism due to cytoplasmic inclusions of TDP43 and FUS RNA binding protein (RBPs), the aberrant function of lncRNAs such as *NEAT-1* and *MALAT1* in subcellular compartmentalization of RBPs and the hexanucleotide expansion in *C9orf72* represent both a common hallmark and fundamental causative events, respectively. For the reason above, the lncRNAs involved in FTD will be discussed in detail in the next paragraph with regard to ALS.

8.3.2.2 LncRNAs in Motor System Disorders

In this chapter those lncRNAs with role in disorder of the central nervous system characterized by abnormal and involuntary movements will be taken into consideration. Although a distinct classification is normally applied for motor system disorders and motor neuron disorders, hence leading to the diseases like Parkinson (PD) and amyotrophic lateral sclerosis (ALS) to different groups, here just for convenience, they will be discussed together, under a general classification of motor system disorders (MSDs).

There are few examples of lncRNAs underlying such a degeneration process, and again, like previously described for AD, they mainly act as antisense of neighbouring genes (NATs). However, new findings suggest that the impact of lncRNAs in motor system disorders could be more miscellaneous, including indirect transcriptional regulation or epigenetic control of fundamental genes in the central nervous system.

PD is one of the most common movement dysfunctions and belongs to a group of conditions called MSDs, characterized by the loss of dopaminergic (DA) neurons in the midbrain and the presence of intra-neuronal cytoplasmic inclusions, called Lewy bodies. Lewy bodies are composed of neurofilaments and ubiquitin, and α-synuclein is the most abundant protein included. The characteristic motor symptom of PD is particularly akinesia, which should be caused by intense dopamine depletion in the striatum. Despite the increasing number of studies about the onset of parkinsonian degeneration, the exact phenomena causing the degeneration process is yet to be fully defined (Majidinia et al. 2016; Wu et al. 2011). The oxidative stress may play an important role in the degeneration of substantia nigra, and defects in protein trafficking machineries have been also proposed as one of the critical dysfunctions to drive the death of DA neurons. According to the recent findings, the α-synuclein, leucine-rich repeat kinase 2 (LRRK-2), parkin (PRKN/PARK2), UCHL1/PARK5, phosphatase and tensin homologue (PTEN)-induced kinase1 (PINK1/PARK6) and oncogene DJ-1 have been classified as major causative proteins to be participated in the initiation/development of PD. Furthermore, some other genes, such as α-synuclein polymorphism, glucocerebrosidase (GBA), microtubule-associated protein and tau/saitohin (MAPT/STH), have been also included in the list because they have been associated with the risk of PD development/progression.

The first study on PD-associated lncRNAs has been released in 2014 by Soreq and colleagues (Soreq et al. 2014). This study has led to the identification of at least five specifically PD-induced lncRNAs such as the spliceosome component *U1* and the muscular dystrophy-associated *RP11-462G22.1* (*lnc-FRG1-3*). However, none of these lncRNAs PD-associated have been characterized, so far. Similarly to lncRNAs AD-associated, the most extensively studied lncRNAs in PD are also NATs.

In 2015, Carrieri and colleagues have conducted a study on the previously identified *AS*

Uchl1, an antisense lncRNA to the mouse ubiquitin carboxy-terminal hydrolase L1 (*Uchl1*) gene (*AS Uchl1*) (Carrieri et al. 2015). Notably, the locus *UCHL1/PARK5* is one of the 28 PD causative genes, mutated in rare cases of early-onset familial PD, and moreover, the loss of UCHL1 activity has been reported in many neurodegenerative diseases. The innovative study of Carrieri and colleagues has revealed that Nurr1, a major transcription factor involved in dopaminergic cells' differentiation and maintenance, can control the expression of *AS Uchl1* which in turn is able to drive the Uchl1 translation through a combined SINEB2 repeat. The *AS Uchl1* can finally induce an augmentation of *Uchl1* expression. The authors of this study have also provided evidences of a strong downregulation of *AS Uchl1* in neurochemical models of PD in vitro and in vivo, and they have remarked the impact of *AS Uchl1* in PD pathomechanism.

The human-specific lncRNA transcribed from the splice variant *PINK1* antisense (*svPINK1*) is another characterized AS lncRNA involved in PD (*naPINK1*) (Scheele et al. 2007). PINK1 is a serine-threonine kinase that has been directly linked to a recessive form of familial parkinsonism. Recently a molecular mechanism to link PINK1 to PD has been reported (DasBanerjee et al. 2017). It has been shown that mitochondrial protein kinase A (PKA) and PINK1 can interact in mitochondria and that they can regulate dendrite remodelling, mitochondrial morphology, content and trafficking in dendrites. Finally, PINK1 has shown the ability to activate a neuroprotective signalling pathway to maintain dendrite connectivity. In this context, it has been found that the lncRNA naPINK1 can increase the stability of svPINK1, but how this event can involve the PD progression needs to be clarified.

Recently, Liu and colleagues have found that lncRNA homeobox (HOX) transcript antisense RNA (*HOTAIR*) is upregulated in the midbrain of mice treated with MPTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) which is a drug notoriously used to induce PD. Interestingly, they have shown that *HOTAIR* can promote the onset of PD through the regulation of *LPPK2* (leucine-rich repeat kinase 2) (Liu et al. 2016).

As mentioned above, the lncRNAs PD-associated similarly to those AD-associated mainly involve the direct binding of target mRNA because they act as NATs. However new discoveries have shown that lncRNAs can also involve neurodegenerative disease with different mechanism of action. In fact, the well-known lncRNA metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) MALAT1 has been recently shown to play also a critical role on the onset of PD (Liu et al. 2017). *MALAT1* is a spliced lncRNA which is highly conversed in mammals and widely expressed in human tissues, and its mis-expression has been reported in various types of cancers (Gutschner et al. 2013). Previous studies have proven that *MALAT1* is an endogenous regulator of breast cancer progression because it can downregulate *miR-124* to activate the CDK4/E2F1 signalling pathway. The new intriguing hypothesis of Liu and colleagues is that *MALAT1* can sequester *miR-124* through a sponging effect to further regulate the apoptosis of DA neurons. Therefore, the new mode of action proposed is that *MALAT1* can function in PD because it can directly bind *miR-124* to negatively control *miR-124* expression. Finally, the authors have speculated that the downregulation of *MALAT1* might lead to an inhibition of DA neuron apoptosis in MPTP-induced PD mice (Liu et al. 2017).

Regarding Huntington's disease (HD), to date, only a few studies have addressed the implications of lncRNA dysregulation. By genome-wide date screening, Johnson and colleagues have provided two independent studies where a subset of lncRNAs have been identified as aberrantly expressed in HD (Johnson 2012; Johnson et al. 2010).

The Huntington's disease is an inherited disorder that results in death of brain cells, characterized by a general lack of coordination as initial symptom until severest movement dysfunctions make person unable to talk. Mental abilities generally decline into dementia. At a molecular level, HD is caused by trinucleotide expansion in the first exon of the ubiquitously expressed gene encoding huntingtin (Htt). The mutant form of this protein (mutHtt) is neurotoxic, and it leads to a relocation of the RE1-silencing transcription

factor (REST). Since REST is known to be involved in repressing thousands of targets, it has been found that the HD's brains have widespread changes in gene expression profile.

In the first work, Johnson and colleagues by the screening of genome-wide data for novel non-coding targets of REST have identified the human accelerated region 1 (*HAR1*). It has been shown that the direct targeting of REST to *HAR1* depends on specific DNA regulatory motifs, and it results in a potent transcriptional repression (Johnson et al. 2010). However, the lack of knowledge about *HAR1* transcript functions has led to limited understanding of its mechanistic relevance to the neurodegeneration in HD.

A second high-throughput screening approach by interrogating published microarray gene expression data from HD patient caudate nuclei has allowed Johnson to discover seven new lncRNAs dysregulated in HD with specific genomic binding sites for REST. In particular, *TUG1* (necessary for retinal development) and *NEAT1* (a structural component of nuclear paraspeckles) have been found upregulated, while the brain-specific tumour-suppressor *MEG3* has been found downregulated. However, how these new identified HD-associated lncRNAs can be involved in the onset of HD or HD's progression has not yet understood. Since many lncRNAs are able to regulate gene expression through formation of epigenetic ribonucleoprotein complexes, as in the case of *TUG1* and *MEG3*, the authors have proposed that alteration of epigenetic gene regulation dependent on mis-functional lncRNAs might have a strong impact to neurodegeneration (Johnson 2012).

Upon to now, the chapters highlighted a link between lncRNAs' altered expression and the initiation and/or progression of neurodegenerative diseases. However, to make a general statement on the role of lncRNAs is arduous, and, on the other hand, to firmly establish whether the mis-expressed lncRNAs in neurodegenerative diseases are harmful or protective on neuronal survival is complicated. Recently, an intriguing report shed light on a new perspective on how the lncRNAs might be involved in neurodegeneration. In particular, Sunwoo and colleagues by transfecting neuro2A cells with the NEAT1 short isoform vector followed up H_2O_2-induced injury have been able to determine the biological effects of NEAT1 on neuronal survival. Since they have found that NEAT1-transfected cells can increase their viability under oxidative stress, the authors have proposed that NEAT1could have a protective role so that the augmentation of NEAT1 transcript in HD patients might be due to a cellular mechanism of neuronal protection (Sunwoo et al. 2017) more than have a negative impact on the onset of disease. Interestingly, this is not an isolated case. Indeed Francelle and colleagues have also reported that the downregulation of the mouse lncRNA transcribed from the opposite strand of abhydrolase domain containing 11 (*Abhd11os*) in the striatum of R6/2 mice could be neuroprotective against mutant huntingtin in vivo (Francelle et al. 2015). These examples have revealed that lncRNAs can induce complicated networks. Therefore, in order to discern the specific impact of lncRNAs on diseases, it is desirable to use the genome-wide data to select a pool of ideal lncRNA candidates, which should be further examined by in vivo and in vitro approaches and with the support of animal models.

Next part of this chapter concerns those lncRNAs which are involved in neurodegenerative disorders as a result of mutations. Apparently, these lncRNAs do not have a physiological role in MSDs, but they became risk or causative factors as a consequence of mutations. Likely, the clearest examples of these class of lncRNAs are the *ATXN8OS* and *C9ORF72* that are able to cause the spinocerebellar ataxia type 8 (SCA8) and the amyotrophic lateral sclerosis (ALS), respectively, after nucleotide expansions.

The spinocerebellar ataxias (SCAs) are a clinically heterogeneous group of disorders, rare and slowly progressing neurological diseases that affect the cerebellum and its related pathways. Currently, SCAs have been distinguished in accord with the order of gene description (SCA1-SCA25). The pathophysiology of these diseases still remains understood. Although missense mutations have been recently found, the feature

of SCAs is the expansions of repeated trinucleotides. The expansion of CAG repeats has been associated with SCA1, SCA2, SCA3, SCA6, SCA7, SCA17 and DRPLA, while the expansions of CTG repeated has been found in SCA8. Moreover, a pentanucleotide repeat expansion (ATTCT) is related to SCA10 (Manto 2005).

It must be emphasized that in diseases associated with nucleotide expansions such as SCAs, it should be taken into consideration not only the detrimental effects of the untranslated mutant transcripts but also the eventually toxic proteins that could be encoded by the mutant expanded nucleotides. In particular, a CTG triplet expansion can occur in the lncRNA *ATXN8OS*, which is a brain-specific transcript, partially overlapping with the neighbouring protein-coding gene *KLHL1*. In SCA8 patients, both a CUG expansion RNAs (CUG^{exp}) as a result of the nucleotide expansion on the *ATXN8OS* gene and a polyglutamine (polyQ) expansion protein as a result of the *ATXN8* CAG^{exp} translation of antisense *ATXN8* CAG^{exp} transcripts have been found. However, the mechanism underlying the SCA8 is actually confusing and still remains to be elucidated. Daughters and colleagues have proposed that the triplet expansion in the lncRNA *ATXN8OS* can generate a RNA gain-of-function because it is able to accumulate on RNA foci and it also shows the capability to sequester important splicing factors (Daughters et al. 2009).

Recently, the expanded UGGAA ($UGGAA^{exp}$) repeat, responsible for SCA31, has been modelled in *Drosophila*, where it can almost recapitulate the neuropathological conditions observed in SCA31 patient brains such as neurodegeneration accompanied by accumulation of $UGGAA^{exp}$RNA foci and translation of repeat-associated pentapeptide repeat (PPR) proteins (Ishiguro et al. 2017). The elegant work of Ishiguro and colleagues have revealed that TDP43, FUS and hnRNPA2B1 can bind the pentanucleotide repeat to induce alteration in the RNA structure and they can be able to suppress the toxicity induced by $UGGAA^{exp}$ in *Drosophila*. According to the model proposed, these RNA-binding proteins (RBPs) might work as RNA chaperones to guide the proper $UGGAA^{exp}$ folding and regulation of PPR translation. Since non-toxic short UGGAA repeat RNAs can suppress the aggregation of mutated RBPs, the authors have revealed a new functional cross talk between RNA and RBPs with the ability to finely regulate their own quality and balance.

As mentioned above, the hexanucleotide GGGGCC expansion (G_4C_2) in the protein-coding gene *C9ORF72* represents the most common genetic cause of both ALS and FTD (Paul and Gitler 2014).

In the healthy population, the repeat region has been reported to be variable up to around 30 repeats, while more than 400 have been typically observed in patients. No evidence of risk factor of the smallest hexanucleotide nor other mutation types have been reported yet, and the function of the *C9orf72* protein is not well understood. To date, three mechanisms have been proposed to explain how *C9orf72* hexanucleotide expansions lead to ALS/FTD (C9ALS/FTD), either by loss-of-function or by two different gain-of-function mechanisms. A complete review has been recently proposed by Moens and colleagues (Moens et al. 2017). Although the loss of function of *C9orf72* transcript in models has provided evidence to support its role in critical neuronal functions, a mutation potentially capable of causing a heterozygous loss of function has been found only in a single sporadic ALS patient. Therefore, it is plausible that a gain-of-function rather than a loss-of-function mechanism could be the primary cause of this disease. Interestingly, the *C9orf72* repeat-expanded is transcribed in both sense and antisense directions, and it has been shown that it can form both nuclear and, more rarely, cytoplasmic sense and antisense RNA foci. Since many RBPs have been found engaged in *C9orf72* RNA foci, it has been proposed that the hexanucleotide expansion might sequester important RBPs such as TDP43 and FUS to cause a detrimental alteration of RNA metabolism. An ideal mechanism underlying the RBPs sequestration might be a sponging effect.

In addition to the formation of RNA foci, it has been observed that both sense and antisense *C9orf72* hexanucleotide expansion can contain repeat-associated, non-ATG-initiated (RAN)

translation sequence that can generate five, potentially toxic, repetitive dipeptide proteins (DPRs). It has been shown that these dipeptides are able to form p62-positive, TDP43-negative inclusions, abundant in the neocortex, hippocampus and cerebellum (Ash et al. 2013; Mackenzie et al. 2013; Mori et al. 2013; Zu et al. 2013). However, whether the toxicity is primarily mediated by *C9orf72* sponging or DPRs still remains an open question and a debate in the field.

The overview of the lncRNAs involved in neurological disorders up to here reported has brought out that two classes of lncRNAs are mainly critical: those acting as NATS and those that affect the CNS functions after the accumulation of mutations (nucleotide expansion). The last part of this chapter will take into consideration two different lncRNA modes of action that have been described so far in the pathomechanism of neurodegenerative diseases. In fact, it will herein introduce the examples of lncRNAs that work as scaffold or in synergistic network with miRNAs.

Some lncRNAs have shown the ability to organize factors like RBPs to shape the cell nucleus. For example, *NEAT1* and *MALAT1* have been described as being essential for the formation and maintenance of the nuclear bodies (NBs), paraspeckles and nuclear speckles, respectively. For these critical abilities, *NEAT1* and *MALAT1* have been recently defined architectural lncRNAs (arcRNAs) (Chujo et al. 2016). The NBs are the sites of the biogenesis, maturation, storage and sequestration of specific RNAs, proteins and ribonucleoprotein complexes (Stanek and Fox 2017).

A number of evidences have highlighted the important roles of TDP43 and FUS in controlling the expression profile of many RNAs with particular regard to arcRNAs. An exhaustive review has been proposed by Lourenco and colleagues (Lourenco et al. 2015). Some reports have been made from striatum samples of FTD/ALS mice models when both TDP43 and FUS loss of function or depletion have allowed to increase the expression of *NEAT1* and *MALAT1* (Lagier-Tourenne et al. 2012; Polymenidou et al. 2011). Moreover, similar results have been obtained from human post-mortem samples, when the augmented formation of *NEAT1* RNA foci has been found being a consequence of altered TPD43 subcellular localization in the early stage of ALS pathogenesis (Nishimoto et al. 2013; Tollervey et al. 2011). In the light of these evidences, TDP43 and FUS might control arcRNAs by distinct mechanisms. In fact, these two RBPs can modulate the RNA transcription and/or stability; they can trigger the formation of the functional RNA structure to allow the lncRNAs to properly work; and they can work as chaperon in a similar manner described in the abovementioned work of Ishiguro and colleagues. Since, in ALS and FTD neurodegenerative diseases, TDP43 and/or FUS are aberrantly mis-localized in the cytoplasm into insoluble inclusions, it might be plausible that their loss of function can lead to a wide RNA dysfunction and to the alteration of lncRNA activities. In this point of view, the aberrant arcRNA transcription in ALS/FTD might simply be a consequence of impaired RBP's activities.

To make this scenario more complicated, there are two important reports which define a fine-tuned cross talk of arcRNA-RBPs. Firstly, the compromised paraspeckle formation through both loss and gain of FUS function has been reported to impair the protective responses in neurons (Shelkovnikova et al. 2014). On the other hand, a new knockdown *Drosophila* model of the arcRNA hsrω has recently revealed that the subcellular localization of *Drosophila* FUS (dFUS) is affected by the alteration of the lncRNA *hsrω* transcript (Lo Piccolo and Yamaguchi 2017). These evidences might suggest that the aberrant cytoplasmic RBP localization typically found in ALS/FTD not only can disrupt the physiological role of lncRNAs but itself can be a consequence of altered arcRNA transcript regulation.

A cross talk between lncRNA and miRNA has been found fundamental for the pathogenesis of SCA7 (Tan et al. 2014). Indeed, the lncRNA *lnc-SCA7* which is defined as retro-pseudogene (*ATXN7L3B*) can post-transcriptionally regulate the expression of ataxin type 7 gene (*ATXN7*).

The SCA7 neurodegenerative disease is caused by an in-frame CAG tri-nucleotide repeat expansion in the first coding exon of *ATXN7*. It

has been reported that the translation of the mutated *ATXN7* allele can cause diverse detrimental consequences such as the polyglutamine (polyQ) tract expansion, the formation of protein aggregates and the decreased ATXN7 protein activity. Since *ATXN7* is an ubiquitously expressed housekeeping gene, it has been hard to understand why the expanded CAG mutations lead to the degeneration of only retinal and cerebellar neurons. The work of Tan and colleagues has provided a new suggestion to explain the tissue-specific neurodegeneration caused by alteration of the housekeeping *ATXN7* gene. The authors have found that along with ATXN7 the miR-124 is target of mammalian SAGA (Spt/Ada/Gcn5 acetylase)-like multi-subunit complex (STAGA). It has been shown that mutations in *ATXN7* allow the formation of cytoplasmic ATXN7 aggregates with a reduced activity of STAGA, whereby the mutant ATXN7 by altering the activity of STAGA complex can decrease the expression of *miR-124*. Moreover, the *lnc-SCA7* has shown being a post-transcriptional regulator of *ATXN7* with the ability to modulate ATXN7 abundance. The authors have reported that *miR-124* can mediate the interaction between *lnc-SCA7* and Atxn7, whereby one of the targets of STAGA complex can affect the activity of the complex which controls its transcription. In this context, the alteration of *miR-124* abundance by modulating the interaction between ATXN7 and the *lnc-SCA7* can cause an augmentation of the level of ATXN7 which in turn may cause a reduction of STAGA activity. The complicated network *lnc-SCA7-miR-124*-ATXN7 has demonstrated the critical role in CNS of the lncRNAs and miRNAs cross talk, and it has revealed that alteration in these tangled networks might cause neurodiseases.

Cumulatively, all the observations so far discussed have proven the complexity and heterogeneity of lncRNAs involved in neurodegenerative diseases and in CNS functions. It is clear that the understanding of lncRNA's biology represents a new challenge for researchers and a new frontier in neuroscience. The use of animal model, computer design and genome-wide data all together can represent a powerful tool to explore the function of these extraordinary molecules.

8.4 Investigating lncRNAs in Animal Models

LncRNAs are found in many organisms across different taxa, including not only human and mice but also *Xenopus tropicalis*, *Drosophila melanogaster*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, *Medicago truncatula* and *Zea mays* (Au et al. 2011). Commonly used animal models vary in complexity and evolutionary divergence from human. The past use of *C. elegans* and *D. melanogaster* has aided in understanding the molecular mechanisms of apoptosis, whose abnormal functionality drives cancer development and progression. Less evolutionary divergent animal models include rat and mouse which have largely contributed to better model the complexity of tumour growth and metastasis due to their physiological similarity to humans.

In addition to human, numerous lncRNAs have been found in all of these animal models (Feyder and Goff 2016), despite their primary sequence is weakly conserved across the species. Against this background, it is considered that higher-order structure rather than primary sequence may be evolutionary conserved.

In particular, *Drosophila* and *C. elegans* are preferentially used to model ageing because of their shorter life span in comparison to mouse and rat, and in this point of view, they are proving to be valuable resource in understanding the role of lncRNAs in neurodegenerative diseases.

8.4.1 The Role of *Drosophila* in Studying Human lncRNAs Involved in Neurodegenerative Diseases

Drosophila was introduced into scientific research over 100 years ago and quickly has become an invaluable tool that empowers our dis-

coveries and understanding of a wide range of biological processes, such as embryogenesis, neural development, synaptic plasticity and even complex behaviours such as decision-making and learning and memory (Bellen et al. 2010; Spindler and Hartenstein 2010). Sequencing of the *Drosophila* and the human genomes have revealed a high similarity between the fly and humans (Adams et al. 2000). Outstandingly, about 75% of the genes implicated in human genetic disorders have at least one homologue in *Drosophila* (Reiter et al. 2001). According to the Ensembl database, the proportion of lncRNAs with respect to the whole annotated gene number is similar between human and *Drosophila* because it accounts in both species in the average of 13%.

Important success have been achieved so far by the use of *Drosophila* as model to gain insight into the molecular mechanism of human neurodegenerative diseases such as the rescuing of disease-like phenotypes in fly models of fragile X syndrome (FXS) (Chang et al. 2008), prolonged survival of DA neurons in fly models of PD (Auluck et al. 2005; Faust et al. 2009) and lifespan extension in fly models of AD (Rajendran et al. 2008).

Despite a limited pool of human lncRNAs have been modelled in *Drosophila* so far, the use of fly in exploring the functions of lncRNAs is proving to be a fruitful approach particularly as a model to study RNA toxicity of repeat expansion-associated neurodegenerative and neuromuscular diseases (Koon and Chan 2017; Rogoyski et al. 2017). Since the theme of this chapter is the lncRNAs, it will be considered herein only the non-coding expansion disorders that typically involve large expansion from 100 up to 1000 copies and reside in the non-coding regions of genes, while the expansions affecting exon coding region will not be addressed.

The group of human non-coding expansion disorders that have been modelled in *Drosophila* so far includes the fragile X-associated tremor/ataxia syndrome (FXTAS), Friedreich's ataxia, the C9-ALS/FTD, the myotonic dystrophy type 1 and type 2 (DM1 and DM2) and the SCA8. Recently, the role of *Drosophila* in understanding the concept of RNA toxicity associated to repeat expansion in neurodegenerative diseases was reviewed, and an exhaustive list of published transgenic *Drosophila* lines for modelling repeat expansion-associated diseases (READs) has been provided (Koon and Chan 2017).

The idea that RNA can itself act as major cause of toxicity on neurodegenerative and neuromuscular diseases came from a study on DM1 in the early 1990s, but one of the earliest *Drosophila* studies that has shed light on the emerging RNA toxicity was pioneered by Rebay and colleagues in 2004 when by overexpressing the human non-coding transcript SCA8 locus in the fly eye, they have been able to identify several SCA8 genetic modifiers such as the *Drosophila* RNA-binding protein genes, staufen, muscleblind (Mbl), split ends and CG3249 (Mutsuddi et al. 2004).

An important contribution in understanding the mechanism of FXTAS has been made by *Drosophila*, where the overexpression of the CGG repeat expansion in specific eye compound has showed severe neurodegenerative phenotype. Through this modelling, RNA expanded-interacting proteins have been found and studied for their ability to enhance or rescue the FXTAS-associated phenotype. It has been reported that overexpression of Purα as well as the overexpression of RNA-binding proteins hnRNP A2/B1 and CUGBP1 can suppress rCGG-mediated neurodegeneration in a dose-dependent manner (Jin et al. 2007; Sofola et al. 2007). Moreover, other studies have been shown that CGG expanded repeat expression in fly can decrease the expression of Rm62 post-transcriptionally, leading to the nuclear accumulation of *Hsp70* transcript and additional mRNAs involved in stress and immune responses. These evidences have suggested that the abnormal nuclear accumulation of these mRNAs, likely as a result of impaired nuclear export, could contribute to FXTAS pathogenesis (Qurashi et al. 2011). An intriguing study has correlated the role of selective miRNAs with the CGG repeats because some of them, including *miR-277*, have been found altered specifically in *Drosophila* brains expressing the repeat associated to FXTAS. All together these studies have

provided important findings to show that sequestration of specific CGG repeat-binding proteins can lead to aberrant expression of selective miRNAs with the ability to modulate the pathogenesis of FXTAS post-transcriptionally (Tan et al. 2012).

Drosophila is an excellent model not only to explore the molecular mechanism of neurodiseases but also prove to be useful for unbiased drug screening. For instance, a chemical screen has revealed some small molecules with the ability to suppress abnormal phenotype induced by expression of CGG repeats. The study of Qurashi and colleagues has showed that specific inhibition of phospholipase A(2) activity could mitigate the neuronal deficits caused by FXS CGG repeats, including lethality and locomotion deficits (Qurashi et al. 2012).

As mentioned above, the expansion of G_4C_2 hexanucleotide over 400 repeats has been typically observed in ALS/FTLD patients where it can induce neurodegeneration through a complex mechanism that involves both RNA toxicity of the repeats and the abnormal capability of the dipeptide repeats (DPRs) derived from G_4C_2 expansion to sequester critical RNA-binding proteins into toxic aggregates. The use of *Drosophila* has played a pivotal role in understanding the pathogenesis of neurodegenerative diseases associated with G_4C_2 hexanucleotide repeat expansion (Mizielinska et al. 2014; Stepto et al. 2014; Wen et al. 2014; Xu et al. 2013). More recently, by employing transgenic flies it has been possible to clarify the RNA toxicity associated to G4C2 hexanucleotide expansion. In transgenic flies expressing $(G_4C_2)_n$, a total of 19 new genetic interactors have been found associated with the expanded repeats (Freibaum et al. 2015; Zhang et al. 2015). Interestingly, some of these proteins are components of nuclear pore complex, and others take part in machinery that coordinates the exports of nuclear RNAs and the import of nuclear proteins. These study have revealed a novel mechanism of neurodegeneration caused by the $(G_4C_2)_n$ repeats to compromise the nucleocytoplasmic transport through the nuclear pore. Finally, in fly, it has been observed that DPRs are able to impair the assembly, dynamics and functions of membrane-less organelles, thus unrevealing the wide spectrum of cellular dysfunctions induced by the $(G_4C_2)_n$ repeats (K. H. Lee et al. 2016).

All these studies in *Drosophila* have contributed to clarify that RNA toxicity play a significant role in the pathologies of many READs. More importantly, these studies have also demonstrated the fruit fly as an excellent model for studying human READs and RNA toxicity.

8.4.2 Neural Functions of *Drosophila* lncRNAs

To date, similar to other animal model and humans, only a very small portion of known *Drosophila* lncRNAs have been thoroughly characterized. In silico and expression analyses have revealed that similar to vertebrates even in *Drosophila*, lncRNAs are highly abundant in the nervous system. Moreover, the examination of genomic loci has shown that lncRNAs are located in proximity of development-related protein-coding genes mediating nervous system development, sensory organ and ventral cord development (Inagaki et al. 2005; Li and Liu 2015; Young et al. 2012).

An overview of the so far known *Drosophila* lncRNAs with functions in locomotion and complex neurological processes will be described below.

8.4.2.1 The Heat Shock lncRNA *Omega*

Among the *Drosophila* species, the 93D locus is highly conserved and carries the very long noncoding hrsω RNA. The locus is also called heat shock RNA omega (*hsrω*) because it is one of the most active genes after heat exposure, although it is constitutively expressed at relatively high levels in different cell types and it is also uniquely responsive to amides like benzamide, colchicine, etc. (Lakhotia and Sharma 1996). The 93D locus produces three transcripts using alternative polyadenylation sites and splicing. The longer transcript from 93D locus is nucleus-limited (*hsrω-n*), and it covers the entire length of the gene. The

hsrω-n spans 10–20 kb and contains two exons followed by a long stretch of a 280 nucleotide tandem repeat unit, and it is also polyadenylated. Curiously, the intron between the two exons is not spliced out (Mutsuddi and Lakhotia 1995). Significantly, the 93D locus is not coding for any protein but is essential under conditions of stress as well as for normal development (Lakhotia et al. 2001). For many years, the lncRNA *hsrω* has been studied for its ability to restore the correct nuclear distribution of key regulator factors such as several hnRNPs, HP1 and RNA pol II after thermal stress. Several studies have shed on light the fundamental role of *hsrω* in cellular reprogramming event and organismal survivor (Lakhotia et al. 2012). Indeed, the lncRNA *hsrω* has the ability to form the ω-speckles, a class of distinct nuclear bodies which are built by the remodelling activity of the chromatin remodeler ISWI and engages various hnRNPs (Onorati et al. 2011; Prasanth et al. 2000). It is believed that the omega speckles are dynamic storage sites for the various RNA-processing and related proteins from which the different proteins are released as required by the state of nuclear activities at any given moment. The lncRNA *hsrω* was also defined as novel regulator of apoptosis because it is able to regulate the level of *Drosophila* inhibitor of apoptosis (DIAP1) (Mallik and Lakhotia 2009a).

The first evidence that the lncRNA hsrω may play a critical role in neurodegenerative diseases has been provided as results of genetic interaction with human expanded polyQ proteins. Indeed, it has been shown that the mutation P292 can enhance the SCA1 eye degenerative phenotype (Fernandez-Funez et al. 2000), while the loss of *hsrω-n* lncRNA is able to suppress the eye-specific degeneration mediated by GMR-GAL4-driven expression of the 127Q or MJDtr-Q78 or ataxin1 82Q or httex1p Q93 transgene (Mallik and Lakhotia 2009b).

It should be noted that a high-throughput RNA sequence from the central nervous system of third instar larvae has shown that the lncRNA *hsrω* directly or indirectly affects several genes with important functions in neuronal development, oxidative stress response and synaptic transmission, thus emphasizing the critical role of *hsrω* in neurons (Lo Piccolo et al. 2017).

Additional studies carried out by taking advantage of GAL4 system have recently found that the pan neuronal- and motor neuronal-specific knockdown of *hsrω* affects locomotive abilities of flies and impairs the structures of neuron muscular junctions (NMJs) (Lo Piccolo and Yamaguchi 2017). Interestingly, it has been shown that *hsrω* has the ability to control the hnRNP Cabeza (the human orthologue of FUS) at different levels because RNAi of *hsrω* can reduce the mRNA abundance of *Cabeza* (*dFUS*) and is also able to induce an abnormal cytoplasmic localization of the nuclear dFUS (Lo Piccolo and Yamaguchi 2017). In this context, it is worth recalling that the mis-regulation of dFUS is a condition leading to neurodegenerative phenotype in flies (Sasayama et al. 2012), and the augmented cytoplasmic distribution of human FUS as mentioned above is a prerequisite for the formation of pathological aggregates. These evidences have highlighted the critical role of the lncRNA *hsrω* in aberrant neuronal activities and have revealed that alteration of its function can drive pathological conditions.

8.4.2.2 The Novel lncRNA *CRG*

The Drosophila Ca2+/calmodulin-dependent protein kinase (Caki) is the human orthologue of CASK and is critical for locomotor behaviour. The *Drosophila* CASK (dCASK) has been found highly abundant during embryogenesis, larval and pupal life, almost exclusively in the central nervous system, while in adult flies, CASK immunoreactivity has been detected in the head, lamina, neuropil of the medulla and the central brain (Martin and Ollo 1996). According to the latest released annotation of *Drosophila* genome, the *CASK* transcript accounts for about 40 kb long and the relative encoded protein exists in two isoforms of which the longer contains the N-terminal CaM-kinase-like and L27 domains. Further examinations have revealed that the lack of these two domains is associated with locomotor dysfunctions that include initiation and motor maintenance defects (Slawson et al. 2011).

By making use of in silico and molecular approaches, Li and colleagues have found a new lncRNA (*CR44887*) with a 2672 nt length, non-spliced, polyadenylated, with a very low coding potential, that interestingly is located downstream of *CASK* gene, with an overlapping region between *CRG* 5′ end and *CASK* 3' UTR region (Li et al. 2012). In situ hybridization has shown that this new lncRNA is neuro-specific because its expression has been found concentrated in the central brain and in the regions between the central brain and the optic lobes. By taking advantage of diverse neuronal specific drivers, Li and colleagues have been able to demonstrate that this new lncRNA is critical to locomotor functions in fly and that it can interact with the dCASK.

As mentioned above, one of the main abilities of the lncRNAs is to regulate the transcription of neighbouring protein-coding genes. In their work, Li and colleagues have found that the nullisomy of the new identified lncRNA can negatively affect the abundance of the *CASK* transcript and protein. In turn, the overexpression of *CR44887* can rescue the normal walking speed in a *CR44887* null mutant background. Therefore, because of its ability to regulate CASK, the new lncRNA has been classified as *CASK* regulatory gene (*CRG*) (Li et al. 2012). The study of the molecular mechanism underlying the control of CASK expression has revealed that the lncRNA *CRG* has the ability to enhance the association between the transcription initiation complex and the *CASK* promoter regions. Indeed, it has been demonstrated that the occupancy of Pol (II) on the *CASK* promoter can decrease in the *CRG* null mutant, while the *CRG* overexpression is involved in the restoration of the normal Pol(II) interaction with the CASK promoter.

More than 35 different mutations in the human *CASK* gene have been associated with 2 mental disorders such as the *CASK*-related intellectual disability and the FG syndrome 4, whereby in human, CASK plays a critical role in the central nervous system. It will be interesting to see whether human neuronal lncRNAs might be involved in CASK-associated diseases. From this perspective, the *Drosophila* model of *CRG-CASK* cross talk could represent an important resource.

8.4.2.3 The lncRNA *yar*

During a study aimed to characterize the non-*gypsy* binding regions and the role of Su(Hw) insulator, Soshnev and colleagues have discovered that the intergenic region between the *yellow* and *achete* genes contains a previously uncharacterized gene (called *yar*, for *y-ac intergenic RNA*) which exhibits a low coding potential, a multiple alternatively spliced and unconventional polyadenylation signal sequence AAATACA (Soshnev et al. 2008). Because the incapacity to encode proteins and due to the transcript length over 200 nt, the *yar* gene has been defined as lncRNA.

The initial studies of Soshnev and colleagues have shed on light that the non-*gypsy* region 1A-2 is a composite insulator containing both enhancer blocking and facilitator elements and that it is required for the transcriptional activation of the lncRNA *yar* (Soshnev et al. 2008).

The lncRNA yar is part of a neural gene cluster. In fact, the upstream yellow gene involves in male sexual behaviour (Drapeau et al. 2003) while the downstream achete gene is associated with the development of the central and peripheral nervous systems (Gibert and Simpson 2003; Negre and Simpson 2009).

Further examinations have revealed that the lncRNA *yar* accumulate in the cytoplasm, and unlike other lncRNAs, it is not required for the transcriptional regulation of the neighbouring genes. Indeed, the nullisomy of *yar* has shown a normal bristle number and cuticle pigmentation consistently with the normal expression of *yellow* and *achete* genes (Soshnev et al. 2011). Finally, flies lacking *yar* RNAs have been found viable.

Because of the genomic localization of the lncRNA *yar*, additional studies have been carried out to definitively explore a potential involvement of *yar* in neurological functions. Indeed, Soshenev and colleagues have further evaluated the general locomotor geotactic ability and the sleep behaviour in yar mutants and found that the lncRNA yar is not required for the general locomotion, but it is critical for sleep maintenance

and homeostasis. In particular, the *yar* null mutant has shown a short night-time sleep period within a normal circadian sleep-wake cycle and a reduced level of sleep rebound after sleep deprivation (Soshnev et al. 2011).

Considering the cytoplasmic localization of *yar* and its incapacity to affect transcription of the neighbouring genes, a possible link with miRNA has been proposed by Soshenev and colleagues as a mechanism to control set of *yar* RNA targets. Indeed, about 33 miRNAs have been uncovered to match with *yar* exons corresponding to 19 confirmed miRNAs. However, the mechanism of *yar*-miRNA remains to be characterized.

8.5 Conclusions

LncRNAs are emerging as important regulators of critical neurological functions. However, only few lncRNAs have been characterized so far, and globally, our understanding of lncRNA activity remains limited. Improved molecular techniques and upgraded databases are providing new tools to explore the lncRNA abilities.

Aberrant lncRNA activity has been associated with devastating neurodegenerative diseases such as AD, PD and ALS. In this regard, the group of lncRNAs so far identified and characterized has shown the ability to work as NATs. Moreover, a fine-regulated cross talk between lncRNAs and miRNAs is emerging as another outstanding asset for cell, and alterations of this network have been recently found in diverse neurodiseases.

Drosophila is proving very useful to understand the human lncRNAs with particular regards for the nucleotide expansion repeats associated with neurodegeneration. As the evolutionary conservation of critical network, future characterizations of *Drosophila* lncRNAs might contribute to expand the knowledge of the human lncRNAs and might help to depict how lncRNAs are involved in CNS functions.

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Muscular Dystrophy Model

9

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Abstract

Muscular dystrophy (MD) is a group of muscle weakness disease involving in inherited genetic conditions. MD is caused by mutations or alteration in the genes responsible for the structure and functioning of muscles. There are many different types of MD which have a wide range from mild symptoms to severe disability. Some types involve the muscles used for breathing which eventually affect life expectancy. This chapter provides an overview of the MD types, its gene mutations, and the *Drosophila* MD models. Specifically, the Duchenne muscular dystrophy (DMD), the most common form of MD, will be thoroughly discussed including Dystrophin genes, their isoforms, possible mechanisms, and signaling pathways of pathogenesis.

Keywords

Muscular dystrophy · Muscular atrophy · Dystrophin-glycoprotein complex · Neuromuscular junction · Expertise: molecular cell biology · *Drosophila* as a Duchenne muscular dystrophy (DMD) model

9.1 Introduction

Muscular dystrophy (MD) is a group of degenerative diseases of muscle characterized by progressive loss of muscle fiber all over the body. Patients normally have muscle weakness with or without additional organ abnormalities such as cardiomyopathy. The severity depends upon the types of MD. Some types can lead to rapid progression and short life span. Most patients with severe forms of MD die from respiratory or cardiac failure. On the other hand, some MD types result in mild muscle weakness with normal cognitive ability and average life expectancy. Almost all of MD patients have normal brain function. Only some forms like Duchenne muscular dystrophy (DMD) and congenital MD (CMD) have cognitive impairment. There is no specific treatment for MD because the knowledge concerning molecular mechanisms of pathophysiology of MD disease is not complete yet and requires tremendous investigation. Therefore, extensive research in both cell-based and animal models need to be done. Studying in a mouse model has

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been revealed to be successful for exploring basic human biology as well as discovering effectiveness of certain agents in curing specific diseases. One of the benefits of using rodents as a model is because the regulation of many genes and the physiological functions of some proteins are conservative in rodents and humans. However, in some cases, the control of gene expression and certain cellular signal communication are totally different. Specially, for MD disease, one protein can be encoded by several different genes. Therefore, knocking down a particular gene may not be enough to eliminate the generation of a functional protein since other genes can compensate the loss of protein function. This complex gene control in rodents makes it difficult to perform gene manipulation to understand the consequences of a single gene defect. Luckily, *Drosophila* gene organization is much less complex, and many individual proteins are encoded by an individual gene. *Drosophila melanogaster* has proven to be an insightful and powerful model system for studying human diseases including muscular dystrophy (Table 9.1). *Drosophila* is an excellent model for understanding the central nervous system phenotypes of muscular dystrophies, as well as for identifying the complex array of regulatory and downstream signaling molecules of the dystrophin-glycoprotein complex (DGC). The ability to easily manipulate genes in *Drosophila* like RNAi knockdown, UAS-GAL4, and P-element systems together with the fly's short life cycle makes *Drosophila* a powerful genetic tool for studying muscular dystrophy diseases. Moreover, an ethical approval for *Drosophila*, compared to mammals, is much easier. Many *Drosophila* models for muscular dystrophy have been reported. Since there are several types of MD and many genes associated with particular types have been established, the content in this chapter will discuss mainly about certain types of MD in humans, particularly Duchenne muscular dystrophy models and currently available *Drosophila* MD models relevant to each type of MD.

9.2 Types of Muscular Dystrophy

9.2.1 Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is a common X-linked recessive, fatal genetic disorder characterized by progressive muscle wasting. The disease was named after the French neurologist Guillaume-Benjamin-Amand Duchenne who described a DMD boy in 1861 (Parent 2005). The onset of the disease could initially be seen at the age of 1–3 years with delayed walking, climbing stairs, difficulty in running, and frequent falls (Ryder et al. 2017). His muscle weakness rapidly progressed within 8–14 years and eventually required a wheelchair. The average survival rate (40.95 years) of DMD patients born between 1970 and 1994 was dramatically increased compared to those patients born between 1955 and 1969 (25.77 years) (Ryder et al. 2017). The prevalence of DMD per 100,000 males was 10.9, 1.9, 2.2, and 6.1 for France, the USA, the UK, and Canada, respectively (Ryder et al. 2017). DMD is caused by the mutation of DMD gene which is one of the largest genes in the human genome, spanning 2.3 Mb. In vertebrate, there are three dystrophin homologs which are dystrophin, utrophin, and dystrophin-related protein 2 (DRP2). This gene has three upstream promoters that control the expression of full-length dystrophin, Dp427, and four internal promoters which regulate the expression of the short dystrophin isoforms, Dp260, Dp140, Dp116, and Dp71 (Pilgram et al. 2010). The expression of Dp427, Dp260, Dp140, Dp116, and Dp71 is specifically localized in the skeleton muscle, the retina, the brain and the kidney, the peripheral nervous system, and throughout the mammalian brain, respectively (Pilgram et al. 2010).

In the skeletal muscle, dystrophin is a part of a large protein complex called dystrophin-glycoprotein complex (DGC) (Fig. 9.1) (Pilgram et al. 2010). The N-terminal actin-binding domain of dystrophin binds to F-actin. The cysteine-rich region of dystrophin binds to β-dystroglycan, and the C-terminus of dystrophin is associated with

Table 9.1 Summary of muscular dystrophy (MD) diseases

MD	Human genes (protein)	Causes/symptoms	Fly genes (protein)	Models	Phenotypes
DMD	*DYS* (DYS)	Decrease or absence of dystrophin protein	*DLP2*	DLP2 knock out (P-element)	Defect on synaptic homeostasis, increased presynaptic neurotransmitter release
BMD	Isoforms: *DP427*, *Dp260*, *Dp140*, *Dp116*, and *DP71*	Onset 1–3 years	*Dp117*	RNAi of Dp117	Disorganized actin-myosin filaments and the cellular hallmarks of necrosis
		Progressive muscle weakness			
		Short life expectancy	*Dp186*	Dp186 knock out (P-element)	Increased neurotransmitter release at presynaptic areas of motoneuron
CMD or WWS or LGMD	Multiple genes *POMT1*	Muscle weakness with congenital ocular and brain malformation	*dPOMT1* or *rt*	dPOMT1 mutant	Synaptic defect
					Climbing and flying defect
					Reduction of synaptic DGluRIIB
				Knocking down *rt*	Twisted abdomen phenotype
	POMT2		*dPOMT2* or *tw*	Knocking down *tw*	Twisted abdomen phenotype
				dPOMT2 mutant showed	ultrastructural defect of muscle
DM	DM1 type:	CTG repeats in *DMPK*		Overexpression of CTG repeats in larval muscle	Degeneration and vacuolization of the indirect flight muscle
	DMPK	Symptoms related to repeat number			Loss of the structure of muscle fibers
		Prolonged muscle contraction			Hypercontraction of larval muscles
		Respiratory failure			
	DM2 type:	CCTG repeats in *CNBP*		Overexpression of the CCTG 106 and 700 repeats in the muscle and the eye	Eye defect
	CNBP or *ZNF9*	Symptoms not related to the number of repeats			No muscle atrophy but has ribonuclear foci formation in the muscle
		Grip myotonia		Overexpression of the CCTG 1100 repeats in the muscle and the cardiomyocyte	
		No respiratory failure			Severe muscle degeneration and cardiac dysfunction
					Ribonuclear foci formation in the muscle
FSHMD	*DUX4* (DUX4)	Overexpression of DUX4-fl in the muscle		Overexpression of human DUX4-fl in germline by UASp-	Eye defect
	FRG1				Impaired muscle function for flight
	FRG2	Onset ~ age 20		DUX4	
		Slow progression		Overexpression of human FRG1in thoracic muscle	
		Weakness of the facial muscles and the dorsiflexors of the foot			
		Winging scapula			

(continued)

Table 9.1 (continued)

MD	Human genes (protein)	Causes/symptoms	Fly genes (protein)	Models	Phenotypes
OPMD	*PABPN1*	GCG repeats in *PABPN1*	*PABP2* (polyalanine in various proteins)	Overexpression of human PABPN1-17-alanine repeats in muscle tissue	Adult: two wing position phenotypes (wings up and wings down). Indirect flight muscle (IFM) fibers are irregular, thin, disorganized myofibrils with broken Z band and no M bands
		Adult onset			
		Ptosis and dysphagia			Third instar larvae: thin muscle fibers and irregular pattern of nuclei
		Proximal muscle weakness			
DD	Many genes and proteins	Distal muscle weakness	*FLN90*	Filamin null mutant	Absent SSR
				Filamin knockdown	Reduced expression of GluRIIA but not GluRIIB
	FLNC (filamin C)				
EDMD	*EMD* (emerin) *LMNA* (laminA/C)	Early contractures	*Lamin C*	Expression of truncated Lamin C	Mispositioned nuclei
		Slow progression muscle weakness			Nuclear organization and muscle defect
		Cardiac muscle and conduction defect		Null mutation of Lamin C	Twist leg phenotype
					Tendon-cell defects
	SYNE1 (nesprin1)		Msp-300 or *Drosophila* nesprin-1protein	Deletion of KASH domain in Msp-300	Larval locomotion defect
	SYNE2 (nesprin2)				Decreased neurotransmitter release and GluRIIA receptor at the NMJ
	TMEM43 (luma)				
SBMA	*AR*	CAG repeats (polyQ) at *AR*		Overexpression of polyQ in human *AR* in the fly eye	Eye defect
		Adult onset			
		Muscle cramp progressive muscle weakness			
		Bulbar signs			
SMA	*SMA1*, *SMA2* (SMA1, SMA2)	Mutation of *SMA*	dSMA	smn^{73Ao} mutant	Loss of body wall contraction
		Degeneration of motor neurons in anterior horn cells in the spinal cord		Hypomorphic *Smn* mutant (Smn^{E33})	Disorganization and an increased number of enlarged boutons
		Variation in age onset and symptoms			Reduction in the EPSCs

AR, androgen receptor gene; DMD, Duchenne MD; BMD, Becker MD; LGMD, limb-girdle MD; CMD, congenital MD; DM, myotonic MD; FSHMD, facioscapulohumeral MD; OPMD, oculopharyngeal MD; DD, distal MD; EDMD, Emery-Dreifuss MD; SBMA, spinobulbar muscular atrophy; SMA, spinal muscular atrophy; SSR, subsynaptic reticulum; WWS, Walker-Warburg syndrome; PABPN1, poly(A)-binding protein 1; PABP2, poly(A)- binding protein 2; EPSCs, excitatory postsynaptic currents

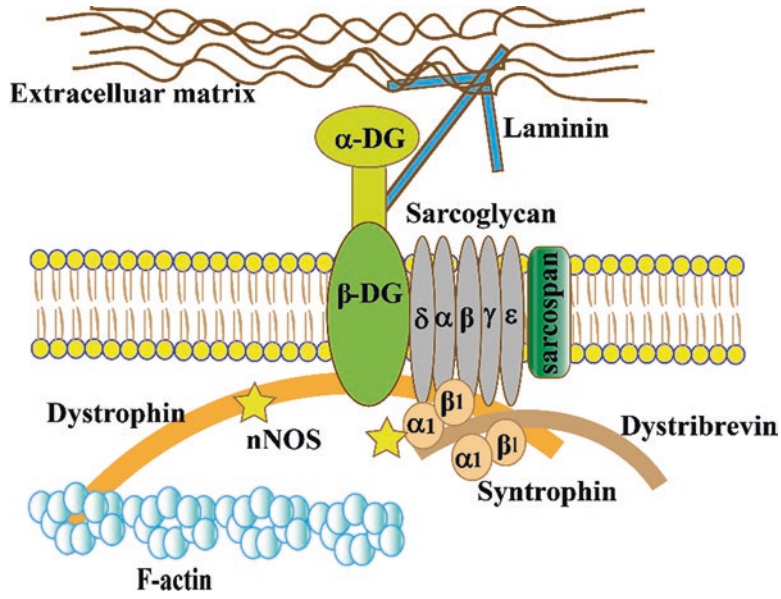


Fig. 9.1 Schematic picture of dystrophin in the dystrophin-glycoprotein complex (DGC) at sarcolemma of mammalian muscle. F-actin binds to the N-terminal region of dystrophin which is linked to β-dystroglycan and α-dystrobrevin2. DG, dystroglycan

α-dystrobrevin2. β-dystroglycan is linked to the extracellular α-dystroglycan which eventually binds to laminin α2 along the sarcolemma. In addition, β-dystroglycan associates with δ-sarcoglycan by which the sarcoglycan-sarcospan complex is stabilized at the sarcolemma. The DGC binds to four syntrophins (α1 and β1subunits) where sodium channels are localized at its PDZ domain. Moreover, syntrophin recruits signaling molecules, such as nNOS to the spectrin-like repeats of dystrophin.

9.2.1.1 *Drosophila* Models for DMD

Unlike mouse DMD models such as mdx model, *Drosophila* DMD models have no redundancy of the dystrophin homolog, utrophin, which compensates the lack of dystrophin gene and leads to muscle regeneration which makes mouse models more complicated to study the authentic effect of dystrophin functions. *Drosophila* has one dystrophin (*DYS*) gene localized on the third chromosome. However, *Drosophila* produces many dystrophin isoforms homologous to human dystrophin proteins. Dp427, Dp260, Dp140, Dp116, and DP71 isoforms in humans are similar to DLP1, DLP2, DLP3, Dp205, and Dp186 in the fly, respectively. Each isoform of dystrophin contains unique sequences at the N-terminal region. However, all dystrophin isoforms contain conserved sequence at the C-terminus.

Knocking down dystrophin DLP2 isoform in muscle was first established using RNA interference specific for the N-terminal region of DLP2 (RNAi-DysNH2). The DLP2 knockdown flies did not show any appearance muscle defect (Fig. 9.2b, e, h). However, reducing all dystrophin isoforms by RNAi knockdown at the conserved C-terminal part of dystrophin gene (RNAi-DysCO2H) in muscle (24B-Gal4 or DMEF2-Gal4) exhibited severe muscle degeneration at 3rd instar larval stage. The muscles were ruptured or absent, or the fibers were detached from their attachment sites at tendon cells (Fig. 9.2n, q, o, r). The muscle in the embryonic stage did not show any defects (Fig. 9.2a–c, 9.2j–l) which is similar to what occurs in patients whose muscle is normal at birth. Knocking down dystrophin in tendon cells using Stripe-Gal4 did not affect muscle integrity (Fig. 9.2f, i). Interestingly, only Dp117 knockdown at muscle fibers displayed disorganized array of fibers (Fig. 9.2m, p) (van der Plas et al. 2007). Dp117 is a muscle-specific dystrophin isoform (van der Plas et al. 2007) which is a homolog of utrophin found in humans. In general, utrophin is homologous to dystrophin and it is named from ubiquitous dystrophin.

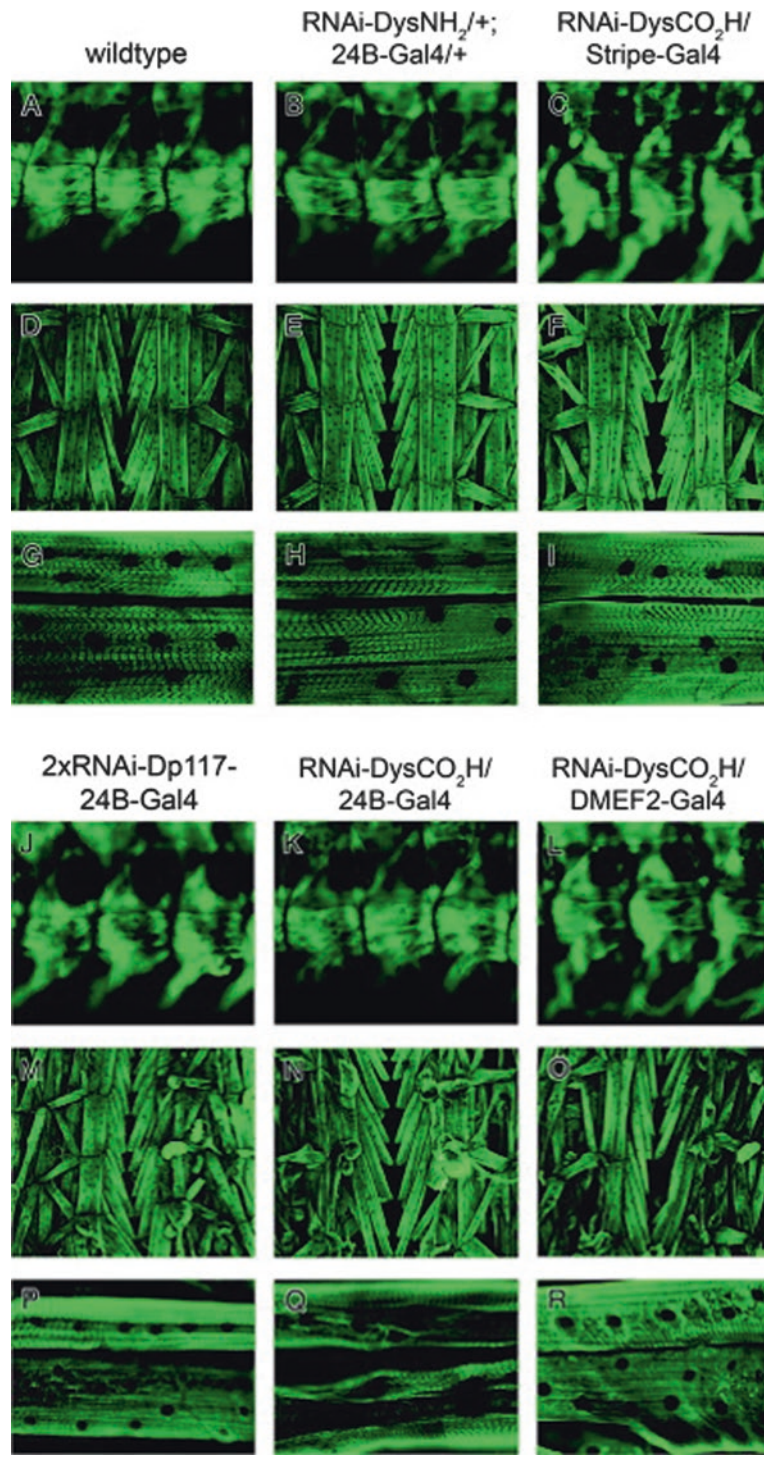


Fig. 9.2 Myosin filament of embryonic (**a**–**c**, **j**–**l**) and larval body wall (**d**–**i**, **m**–**r**) stained with an anti-muscle myosin antibody. Muscle degeneration only occurs in larvae when either all dystrophin isoform (**n**,**q**) or only the Dp117 isoform (**m**, **p**) expression levels are reduced in muscle (24B-Gal4 and DMEF2-Gal4). Reduction of dystrophin in tendon cell (Stripe-Gal4) (**c**, **f**, and **i**). (Reprinted with permission)

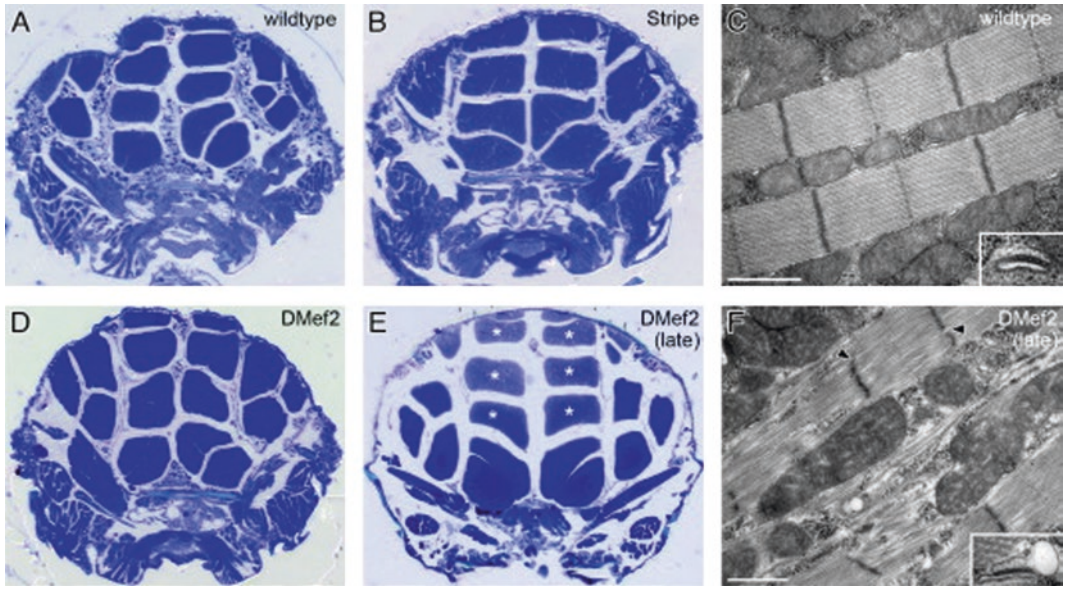


Fig. 9.3 Transverse sections of thoracic muscles of wild type (**a**, **c**), RNAi-DysCO2H/Stripe-Gal4 (**b**), and RNAi-DysCO2H/DMef2-Gal4 (**d**, **e**, and **f**). Animals were collected as uneclosed pharate adults at 72 h APF (**a**–**d**) and late stage (**e**, * shows less well-stained muscle fibers). Transmission electron microscopy sections of muscle fiber of wild-type muscle fiber (**c**) and RNAi-DysCO2H/DMef2-Gal4 (**f**). Z-lines are also disrupted or shortened (**f**, arrowheads). Dyads are shown in inset. (Reprinted with permission)

Utrophin and dystrophin have complementing roles in normal functional or developmental pathways in muscle (Deconinck et al. 1997). Even though there is no report of utrophin mutation in humans, utrophin is upregulated in DMD patients and the Mdx mouse model (Deconinck et al. 1997; Kleopa et al. 2006). Reduction of Dp117 affects not only the muscle integrity but also the life span of flies which is dependent on the degree of decreased Dp117 expression. Knocking down Dp117 homozygous alleles (two copies) showed early death of larvae, while knocking down only one copy of the gene resulted in death at the white pupae stage (van der Plas et al. 2007).

New muscle fibers develop and mature during pupation to form the adult musculature. The thoracic muscle fibers in RNAi-DysCO2H/DMef2-Gal4 *Drosophila* were not impaired in the early pupae (72 h after pupa formation) (Fig. 9.3d) but obviously degenerated in the late pupae (partial eclosion) (Fig. 9.3e). This indicates that the depletion of dystrophin is not required in the process of myogenesis. Electron microscopy of late pupae with dystrophin depletion showed rupture and disorganization of myofilaments (Fig. 9.3f). Moreover, the sarcoplasmic reticulum (SR) was swollen (inset of Fig. 9.3f). Knocking down the level of dystrophin in tendon cells did not exhibit muscle aberration in adult flies. The RNAi-mediated knockdown of dystrophin in the muscle showed progressive climbing defect and severe muscle degeneration in adult flies (Shcherbata et al. 2007). Besides *Drosophila* muscular dystrophy model, flies have been successfully used to study the tissue-specific functions of dystrophin in other types of muscles. For example, knocking down the level of the long *DLPs dys* isoforms and a short form, *Dp117*, caused heart defects with age-dependent disruption of cardiac myofibrillar architecture, chamber dilation, and diastolic dysfunction similar to the dilated cardiomyopathy seen in DMD patients (Taghli-Lamallem et al. 2008).

It is very interesting to see that the lack of dystrophin in *Drosophila* that causes delayed degen-

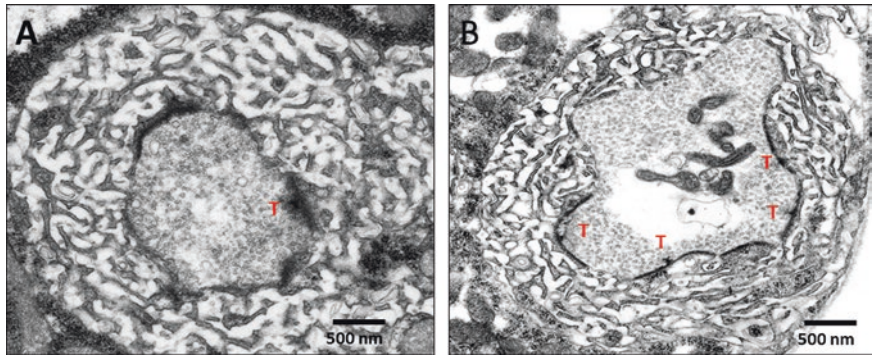


Fig. 9.4 Transmission electron microscopy of one bouton of the 3rd instar larval body wall of wild type (**a**) and dystrophin mutant (**b**). Letter T stands for T-bar in a bouton

eration of muscle is reminiscent of the process found in DMD patients where there is no noticeable symptom of muscle weakness observed in infants but the symptoms rapidly progress when the muscle is used. Therefore, *Drosophila* model is considered to be an excellent model to explore the mechanism of disease development as well as to discover effective therapeutic agents to treat DMD in humans.

In addition to the knowledge obtained from studies related to structural defect found in dystrophin-depleted muscle, dystrophin has been discovered to play essential roles in other biological processes. The null mutant of DLP2 (dysE6) isoform using P-element excision mutagenesis has been established and can be used to study synaptic transmission at the neuromuscular junction (NMJ) (van der Plas et al. 2006). *Drosophila* lacking DLP2 isoform did not show any muscle defect but showed alteration of synaptic transmission assayed by electrophysiological analysis (protocol below). This technique is performed by linking the motor nerve (normally associated with motoneuron in neuropile) with the stimulating electrode that stimulates the action potential [excitatory junction potential (EJP)] and neurotransmitter release at the neuromuscular junction (NMJ), and the electrical impulse is detected by a recording probe inserted in the muscle of a larva. The basal level of neurotransmitter release at the NMJ is measured as spontaneous miniature excitatory junction potential (mEJP) amplitudes. The neurotransmitter release or quantal content (QC) per NMJ was calculated by dividing the mean EJP amplitudes with the mean mEJP amplitude. The dysE6 or DLP2 null mutant showed an increase in EJP, but there was no change in mEJP. Therefore, the QC of dysE6 mutant was drastically increased. An increase in neurotransmitter release was found to be associated with an increase in neurotransmitter release site or T-bars as observed by electron microscopy (Fig. 9.4) but not the number of bouton in NMJ or the distribution of the postsynaptically localized glutamate receptor subunits (DGluRIIA and DGluRIIB) (van der Plas et al. 2006).

It is interesting to observe that when dystrophin was expressed postsynaptically (muscle site) of dys^{E6} mutant, neurotransmitter release was rescued to that of the wild-type level. However, dystrophin expression at the presynaptic site (in motoneuron) did not rescue the over-released level of neurotransmitter release in the dys^{E6} mutant. This observation indicates that dystrophin is specifically required at the muscle site for maintaining synaptic homeostasis. Synaptic homeostasis between muscle and motoneuron is regulated by anterograde and retrograde signaling (Berke et al. 2013). In general, the type II BMP receptor wishful thinking (Wit) in the motoneuron is activated by the glass bottom boat (Gbb) ligand released from the muscle. The retrograde bone morphogenetic protein (BMP) signaling is important for increasing neurotransmitter release in the dys^{E6} mutant. The

absence of Wit receptor prevented an increase in neurotransmitter release in dystrophin mutants, suggesting that the dystrophin-modulated retrograde signal goes through the Wit signaling pathway (van der Plas et al. 2006).

In addition to its known functions of dystrophin in maintaining muscle integrity, dystrophin has been discovered to play a crucial role in the central nervous system (CNS), specifically in the synaptic transmission. Based on the observation that dystrophin defect can cause not only muscle weakness but also mental retardation in many DMD patients (D'Angelo et al. 2011), it is reasonable to think that certain types of dystrophin may be expressed and function in the brain. To support this idea, Dp140 depletion was shown to be linked with the cognitive performances in DMD patients with mental defect (D'Angelo et al. 2011). Consistently, in *Drosophila*, the short isoform or Dp186 is mainly expressed in the brain and plays an important role in synaptic homeostasis in the CNS. Dp186 null mutant was established by P-element excision mutagenesis (van der Plas et al. 2006). Flies that lack Dp186 for two copies (homozygous) were viable and had normal larval crawling movement. The adult mutant had no observable muscle defect with normal climbing and flight. However, the loss of Dp186 increased presynaptic neurotransmitter release which could be fully rescued to wild-type levels by expressing Dp186 in the motoneuron. This observation was similar to that seen in DLP2 mutant at the NMJ (van der Plas et al. 2006).

9.2.2 Becker MD (BMD)

BMD is an X-linked recessive inherited disorder characterized by slowly progressive muscle weakness of the legs and pelvis. BMD is caused by dystrophin gene mutations typically in-frame deletions in the DYS gene. These mutations lead to partial loss or mild reduction of dystrophin protein in the muscle which is different from an event occurred in most DMD patients where dystrophin protein is completely deleted or severely reduced. The incidence of BMD accounts for approximately 1.5–6 in 100,000 male births. The symptom development resembles that of DMD but is in lesser extent and usually occurs during late childhood or early adulthood. Early symptoms include cramps after exercising and problems while walking, running, and climbing stairs. Most of BMD patients have normal cognitive function. Only some cases have learning defect which is typically minor. BMD can occur as a result of a new mutation. Therefore, not all mothers of BMD patients carry in-frame deletions in the DYS gene. BMD can also occur through mosaicism where only some cell lines are affected. There are various gene defects at different parts of dystrophin gene that can give rise to BMD.

9.2.2.1 *Drosophila* Models for BMD

Since BMD is mainly caused by the reduction of some amount of dystrophin protein rather than complete deletion of the molecule, therefore, it is considered to be the mild form of DMD. *Drosophila* model for studying BMD can be simply achieved by performing DYS knockdown to reduce the level of dystrophin protein for studying various aspects related to BMD. For instance, Taghli-Lamallem et al. showed that dystrophin-RNAi-mediated knockdown in the mesoderm shortened *Drosophila* life span. The loss of dystrophin function led to an age-dependent disruption of the myofibrillar organization within the myocardium and eventually altered cardiac performance (Taghli-Lamallem et al. 2008).

9.2.3 Congenital MD (CMD)

CMD is defined as muscle weakness at birth with delayed motor development and associated with eyes and brain abnormalities. At least 30 CMD subtypes have been classified. This chapter will mention only the congenital muscular dystrophy caused by defect in the glycosylation of α-dystroglycan. This disease is due to the mutation of genes that are involved in the glycosylation of α-dystroglycan. They include *POMT1*, *POMT2*, *POMGnT*, *FKTN*, *FKRP*, and *LARGE*. Many subtypes of CMD are classified

including Fukuyama congenital muscular dystrophy (FCMD), muscle-eye-brain disease (MEB), and Walker-Warburg syndrome (WWS). WWS is an autosomal recessive disease with muscle weakness with congenital ocular and brain malformation (Vajsar and Schachter 2006). Normally, patients with WWS rarely survive to birth. Even if they survive, the life expectancy is about 2–3 years. The disease is caused by mutation of genes encoding protein O-mannosyltransferase 1 and 2 (*POMT1* and *POMT2*). Even though WWS has similar POMT1 deficiency as LGMD2K, there is a slight difference in the cause of mental retardation (MR). In WWS, MR is severe and likely caused by structural abnormalities from cell migration defects, while MR in LGMD2K can be mild and is not related to structural defect of the brain. Moreover, the age onset is the key to distinguish CMD, which occurs at birth, from LGMD which occurs in late childhood or adulthood (Sparks et al. 1993). Protein O-mannosyltransferase transfers mannose to the Ser/Thr residues of α-dystroglycan via forming a heterodimer with POMT2 which contributes to the stabilization of sarcolemma by binding to laminin (Muntoni et al. 2004). Mutations of *POMT1* gene lead to a defect of protein O-mannosylation (Akasaka-Manya et al. 2004). Coexistence of POMT1 and POMT2 is required for POMT activity (Manya et al. 2004). Due to the similarity of CMD and LGMD, the *Drosophila* models for CMD and LGMD are mentioned in the LGMD topic.

9.2.4 Limb-Girdle MD (LGMD)

LGMD is named because muscle weakness is limited to the limb musculature including the shoulder, upper arms, pelvic girdle, and upper thighs. Proximal muscle wasting is greater than that of distal muscle. LGMD can be classified into two major types: LGMD1 (dominant inheritance) and LGMD2 (recessive inheritance) (Pegoraro and Hoffman 1993). Each type of LGMD can be categorized into different subtypes depending upon mutations in certain proteins. Many protein defects including sarcoglycanopathy, calpainopathy, dysferlinopathy, glycosylation defects, or dystroglycanopathy can contribute to LGMD. For instance, a mutation in POMT1 protein results in a defect in mannosylation of α-dystroglycan complex. This defect eventually leads to LGMD2K in which clinical features including slow progression, mild muscle hypertrophy, mental retardation, and joint contractures at the ankles can be observed (Rocha and Hoffman 2010). Therefore, there has been an attempt to utilize *Drosophila* to explore more about this particular subtype of LGMD2. LGMD2 is often associated with mental retardation which can be mild and is not associated with structural defect in the brain. This observation provokes neuroscientist to hypothesize that the loss of function of POMT1 protein may be the cause of neuronal structure or function aberrance.

9.2.4.1 *Drosophila* Models for WWS and LGMD2K

Drosophila model for WWS has been established by mutation of *Drosophila* orthologs of *POMT1* and *POMT2* which are called *rotated abdomen* (*rt*) and *twisted* (*tw*), respectively (Ichimiya et al. 2004; Ueyama et al. 2010), or *dPOMT1* and *dPOMT2*, respectively (Wairkar et al. 2008). Both dPOMT proteins colocalize in the endoplasmic reticulum compartment within *Drosophila* cells (Haines et al. 2007). They play a role in myogenesis, muscle architecture, and cell adhesion. Knocking down *rt* or *tw* showed a "twisted abdomen phenotype," in which the abdomen is twisted 30–60° (Ichimiya et al. 2004).

dPOMT1 is expressed in the embryonic mesoderm and midgut but not in the ectoderm. The dPOMT1 mutants were created using P-element insertion alleles in the first exon of the gene resulting in null or strong hypomorphic mutations (Martín-Blanco and García-Bellido 1996). The dPOMT1 mutant had defects in embryonic muscle development and a clockwise helical rotation of the body (Martín-Blanco and García-Bellido 1996). The larval body wall of dPOMT1

mutants showed deficient or thin muscles at abdominal segment. Shortened life span occurred in ubiquitous expression of RNAi for *dPOMT1* gene but not in neuron or glial cell expression. The adult dPOMT1 mutant flies showed reduced climbing and flying ability which reflect the defect at leg and flight muscles, respectively. The flight muscles in the thorax develop from myoblast in the wing imaginal disc. The number of myoblast did not decrease in the mutant wing imaginal disc. The mutant showed excessive apoptosis of myoblasts (Ueyama et al. 2010). The climbing abilities of mutants rapidly decreased with age similar to WWS patients who have difficulty in walking with age (Ueyama et al. 2010). The dPOMT2 mutant was also created from P-element insertion technique. It had defects similar to those of dPOMT1 mutants (Haines et al. 2007; Ueyama et al. 2010). The dPOMT2 mutant showed ultrastructural defect of muscle, sarcomeric disarray, irregular Z-lines, filament disorganization, swollen sarcoplasmic reticulum, accumulation of glycogen granules, enlargement of mitochondria, and duplication of basement membranes (Ueyama et al. 2010). The larvae of both mutants showed muscle attachment and muscle contraction phenotypes identical to those associated with reduced Dg function (Haines et al. 2007) which reflects a requirement of O-linked mannose on *Drosophila* Dg. Genetic interaction study also convinced that Dg interacts with rt and/or tw (Wairkar et al. 2008). Similar to human, co-expression of wild-type rt and tw is required of POMT activity in *Drosophila* cells (Ueyama et al. 2010). Expressing only rt or tw is not sufficient to produce POMT activity.

Besides muscular defect, dPOMT1 mutant exhibited the synaptic abnormalities. The mutant showed the reduction of synaptic DGluRIIB but normal level of DGluRIIA glutamate receptor subunit which might be related to the severe impairment of neurotransmitter release (Wairkar et al. 2008). This phenotype was similar to that of the dystroglycan (Dg) deficiency mutant. The heterozygous or one copy defect of dPOMT1 or Dg mutant did not show any neurotransmitter defect. Interestingly, when combined one copy of dPOMT1 to Dg mutant, the decrease of neurotransmitter release was clearly seen. This indicates that dPOMT1 and Dg mutant has genetic interaction (Wairkar et al. 2008).

9.2.5 Myotonic MD (DM)

Myotonic MD is abbreviated as DM due to its Greek synonym "dystrophia myotonica." DM is a common adult onset muscular dystrophy characterized by prolonged muscle contraction (myotonia) or difficulty of muscle to relax after contraction followed by progressive muscle wasting and weakness. DM affects more than 1 in 8000 people worldwide. The clinical features including disease onset, signs and symptoms, and severity vary among individual patients. The age of onset starts from new born until late adulthood (> 40 years). DM patients may have mild symptoms such as mild myotonia, hypotonia, facial weakness, cataract, or severe conditions such as heart conduction defect and respiratory failure. DM patients usually have CNS defect involvement, for example, attention deficit, learning disability, social interactions or communication problems, apathy, hypersomnia, and difficulty on concentration and word function (Gourdon and Meola 2017). Currently, the mechanism underlining DM disease involves abnormal RNA splicing which causes an RNA expansion in the noncoding region of gene. RNA repeats are formed as ribonuclear foci which are the hallmark of DM pathogenesis. These foci are predominately present in the nucleus and affect a subset of proteins which alter the activity of RNA-binding proteins (RNA-binding protein 1, CUGBP1 and the muscleblind-like proteins, MBNL1) that regulate splicing. Aberrant splicing of mRNA of insulin receptor, chloride channel CLCN1, cardiac troponin T, RYR1, and MTMR1 causes insulin resistance, myotonia, cardiac abnormalities, muscle weakness, and CNS effect, respectively (Turner and Hilton-Jones 2014). DM is classified in two types: DM type 1 and DM type 2.

9.2.5.1 DM type1 (DM1)

The majority of DM patients is DM1. DM1 is an autosomal dominant neuromuscular disorder associated with a CTG expansion in the 3′ region of the myotonic dystrophy protein kinase gene (DMPK) which is located on the long arm of chromosome 19. Normal individuals have CTG repeats between 5 and 37 repeats, whereas patients have the repeat in a range of 50–4000 CTG repeats (Turner and Hilton-Jones 2014). The symptoms of the disease vary and depend on the amount of the repeats. An increase in numbers of the repeats causes an increase in severity and early onset. DM patients are classified into five categories: congenital, childhood onset, juvenile, adult onset, and late onset/asymptomatic. Congenital DM1 in which symptoms occur at birth usually has more than 1000 CTG repeats (Gourdon and Meola 2017), whereas the late onset (> 40 year) has 50–100 CTG repeats. Congenital DM1 is a severe form with massive generalized weakness, hypotonia, respiratory failure, and learning disability. The late onset can be presented with only mild myotonia and cataract (Gourdon and Meola 2017).

9.2.5.2 DM Type 2 (DM2)

DM2 is caused by an expansion of CCTG repeats in the nucleic acid-binding protein gene (*CNBP*), previously known as zinc finger 9 gene, *ZNF9*, on chromosome 3. DM2 does not have a congenital or early childhood form (Meola and Cardani 2015). The onset of DM2 begins at the age of 20–60. In many patients, the first symptom is grip myotonia. Myotonia is often less obvious and milder in DM2 than in DM1. The hypertrophy of calf muscle is commonly found in DM2 patients. The symptoms can be presented with only mild weakness of hip extension, thigh flexion, and finger flexion. Cataract, cardiac, and CNS involvement are also found in DM2 but are in lesser extend compared to DM1. In contrast with DM1, there is no report of respiratory failure in DM2.

9.2.5.3 *Drosophila* Models for DM1

Incorporating the large human CTG repeats into the fly is difficult because of instability and failure to amplify by PCR. Currently, the largest expression of 162 CUG repeats was performed by Housely et al. (Houseley et al. 2005). This CUG repeat caused accumulation of nuclear foci containing mbl without muscle degeneration (Houseley et al. 2005), meaning that the presence of these ribonuclear foci is not sufficient to cause toxicity in *Drosophila*. The fly might be refractory to CUG-induced toxicity since 162 CUG repeats are in the range of pathogenic in DM1 patients.

The first fly model of DM was generated by expressing a noncoding RNA containing an expanded, interrupted CUG repeat (iCUG)480 in *Drosophila* (de Haro et al. 2006). The synthetic, interrupted CTG repeat minigenes were used to interrupt every 20 CTG units by the sequence CTCGA (Philips et al. 1998). When iCUG480 was overexpressed in the muscle, it colocalized with muscleblind (mbl) protein, the *Drosophila* orthologue of human MBNLs, in nuclear foci and caused progressive muscle degeneration, similar to what has been observed in the muscle of patients with this disease. In short, the pathognomonic hallmark of DM1, ribonuclear foci, is conserved in *Drosophila* muscles.

The expression of variation of interrupted CTG repeats (240, 600, and 960 interrupted CTG repeats) in third instar larvae muscle exhibited muscle hypercontraction, reduced fiber size or myoblast fusion defects, and caused splitting of muscle fibers (Picchio et al. 2013). The muscle splitting phenotype in third instar larva muscle is more sensitive to create DM phenotype than visible ribonuclear foci (Picchio et al. 2013), since the iCTG240 repeats in muscle showed splitting of muscle fiber and displayed motility defect without any visible ribonuclear foci.

The mbl protein is a key step in the pathogenesis of the DM disease (de Haro et al. 2006; Picchio et al. 2013). Reduction of endogenous mbl by driven mblRNAi in larval muscle showed muscle degeneration, whereas over the expression of human MBNL1 in *Drosophila* muscle suppressed muscle degenerative phenotypes (de Haro et al. 2006). Generally, mbl protein plays a role in regulating RNA splicing. One target of mbl protein is the *Drosophila* sarcoendoplasmic reticulum calcium ATPase (dSERCA) which is a calcium pump involved in muscle contraction.

When mbl protein is deficient in DM flies, mis-splicing of dSERCA can occur where the exon 8 or 11 of dSERCA is spliced out, leading to the production of altered dSERCA isoforms that lack the transmembrane domain and exhibit ectopic the expression of dSERCA in the nuclei. The expression of the membrane dSERCA isoform is sufficient to rescue a DM1-induced hypercontraction phenotype. This means that the decrease of dSERCA transmembrane isoform is responsible for hypercontraction phenotype in a *Drosophila* model (Picchio et al. 2013).

Besides muscle degeneration, cardiac alteration is also seen in DM1 *Drosophila* model. Overexpression of 250CUG repeats in *Drosophila* heart using GMH5-Gal4 resulted in increased lengthening of the heart period with prolonged systolic and diastolic intervals, reduction in a percentage of fractional shortening, and increased arrhythmia index (Cerro-Herreros et al. 2017). However, the expression of short (20CUG) repeats only showed a slight increase in the systolic interval duration (Chakraborty et al. 2015). Similar to humans, the amount or length of CUG repeats is related to the severity of disease symptoms.

Several transcripts of muscle proteins are known to be mis-spliced in DM muscle. They include troponin T and α-actinin (Machuca-Tzili et al. 2006; Garcia-Lopez et al. 2008). Genetic and chemical modifier screens of CUG-mediated toxicity (Garcia-Lopez et al. 2008) by using iCUG480 expression in the adult eye (sevenless-GAL4) which causes rough eye phenotype were performed to identify possible genes. The *viking* (*vkg*) gene, alpha 2-chain type IV collagen involved in cell adhesion, enhanced CUG toxicity, while *cnc* (bZIP transcription factor), *foi* (zinc ion transporter), and *coro* (F-actin-binding protein coronin) were suppressors (Garcia-Lopez et al. 2008). The pro-apoptotic *spin* and apoptosis inhibitor *th* also interact with CUG-mediated toxicity. Several chemicals have been identified as suppressors of a CUG-induced lethality. They include nonsteroidal anti-inflammatory agents (ketoprofen, indomethacin), dopamine receptors and monoamine uptake inhibitors (nefopam hydrochloride, metoclopramide), muscarinic inhibitor (orphenadrine), and aldosterone antagonist (spironolactone).

9.2.5.4 *Drosophila* Models for DM2

The transgenic flies expressing noncoding CCUG 106 repeats showed only disrupted eye structure with no muscle atrophy (Yenigun et al. 2017). Consistently, the expression of 700 CCUG repeats caused retinal and eye disruption without muscle atrophy (Yu et al. 2015). Even though the flies with CCUG 106 and 700 repeats did not show muscle atrophy, the ribonuclear foci formation and changes in alternative splicing could be seen in these flies similar to DM2 patient's muscle. Overexpression of human MBNL1 protein in the eye using GMR-GAL4 rescued the retinal degeneration of flies with CCUG repeats (Yu et al. 2015; Yenigun et al. 2017).

Recently, the expression of 1100 CCUG repeats of noncoding RNA in the muscle and the cardiomyocyte of the flies exhibited severe muscle degeneration and cardiac dysfunction (Cerro-Herreros et al. 2017). These flies had increased the expression of autophagy-related genes; Atg4, Atg7, Atg8a, Atg9, and Atg12. Similar to DM1, *Drosophila* DM2 showed RNA mis-splicing and repeat aggregation in ribonuclear foci along with the mbl protein (Cerro-Herreros et al. 2017).

9.2.6 Facioscapulohumeral MD (FSHD)

FSHMD is a weakness of the facial muscles and the stabilizers of the scapula (winging scapula) or the dorsiflexors of the foot. It is one of the most common adult onset muscular dystrophy. The prevalence of FSHD is about 1:15,000–1:20,000 in adults (Lemmers et al. 1993; Statland and Tawil 2014). This disease is an autosomal dominant which is caused by inappropriate expression of *DUX4* (double homeobox 4) gene in muscle cells. In general, *DUX4* is in a repressed state due to tightly wound chromatin. Once chromatin is opened, the expression of *DUX4* results in FSHMD which is considered as a

gain-of-function disease. *DUX4* lies in the macrosatellite repeat D4Z4 on chromosome 4q35. This gene encodes at least two isoforms; a nonpathogenic short form (DUX4-S) and a full-length form (DUX4 full length or DUX4-fl). Normally, DUX4-fl is expressed at low level in human testis, pluripotent stem cells, and some somatic cells (Snider et al. 2010) where it functions during germ line and embryonic development. However, epigenetic changes in the chromosome 4q35 lead to relaxation of repression of chromatin followed by an increase in the pathogenic alternative splicing isoform of the *DUX4* gene or DUX4-fl. Recently, there are other two candidate genes for FSHD: *FRG1* (FSHD region gene 1) and *FRG2* (FSHD region gene 2). These genes are proximally located on chromosome 4q35 and direct targets of DUX4-fl (Thijssen et al. 2014; Ferri et al. 2015).

FSHD is classified into two types based on the underlying genetic lesions: the common form (95%) FSHD1 and the lessor extend (5%) FSHD2. FSHD1 is caused by the shortening of the D4Z4 allele which leads to chromatin relaxation at the D4Z4 locus and DUX4 promoter. FSHD2 is caused by mutations in the chromatin modifier *SMCHD1* leading to chromatin relaxation at D4Z4 without having a D4Z4 contraction. Chromatin relaxation at D4Z4 eventually causes DUX4 expression. Both FSHD1 and FSHD2 have similar symptoms: progressive muscle weakness involving the face, scapular stabilizers, upper arm, peroneal muscles, and hip girdle and asymmetrical muscle weakness. Muscle weakness occurs by the age of 20 with slow progression, and 20% of the cases finally require a wheelchair. However, the life expectancy is not affected.

9.2.6.1 *Drosophila* Models for FSHD

In contrast with other *Drosophila* MD models, FSHD models are difficult to establish due to the potent cytotoxicity of DUX4-fl in somatic cells. For example, the expression of DUX4-fl in vertebrate somatic cells led to apoptotic cell death in vertebrate system (Wuebbles et al. 2010). In 2016, Jones TI et al. successfully produced transgenic *Drosophila* lines for investigating the involvement of DUX4 and FRG1 genes in FSHD by expressing DUX4-fl or FRG1 under the control of the GAL4-upstream activation sequence (UAS) (Jones et al. 2016). The expression of DUX4-fl using the pUAST somatic expression vector did not generate any transgenic lines due to lethality or sterility. However, two transgenic flies were successfully created using the UASp germline expression vector: UASp-DUX4# 26 and UASp-DUX4# 55. These flies were crossed with *nanos-GAL4:V16* for testis- and ovary-specific expression. All progenies were alive. Progeny female flies were fertile and had normal ovaries. However, progeny male flies were sterile in contrast to humans where the testes normally express DUX4-fl. Ubiquitous expression of UASp-DUX4 (*tubP-GAL4* and *Act5C-GAL4*) and specific expression in adult thoracic muscle (*DJ667-GAL4*) caused lethality. Expression UASp-DUX4 in the eye (*IGMR-GAL4)* caused eye phenotype.

FRG1 is highly conserved among invertebrates and humans (50% amino acid identity and 66% similarity) (Grewal et al. 1998). The *Drosophila FRG1* (*DmFRG1*) overexpression in thoracic muscle (*DJ667-GAL4*) was accumulated in the nucleus and particularly in the nucleolus with the pattern similar to that of vertebrate species (Hanel et al. 2011). Overexpression of *FRG1* in thoracic muscle caused impaired muscle function for flight. These flies could walk and jump for takeoff but could not maintain the flight. Their dorsal longitudinal muscles were misshapen, fused together, disorganized, and degenerated.

9.2.7 Oculopharyngeal MD (OPMD)

OPMD is characterized by weakness of subset of muscle at the eye lid and pharynx (Trollet et al. 1993). It is an adult or late-onset progressive muscle disorder with eyelid drooping (ptosis), swallowing difficulties (dysphagia), and proximal limb weakness. The mean age of onset of ptosis is usually 48 years, and the onset of dysphagia is 50 years followed by proximal leg weakness which starts before age 60. OPMD does not affect

life span, but severe dysphagia can lead to potential life-threatening aspiration pneumonia and poor nutrition (Trollet et al. 1993). The estimated prevalence is 1 in 100,000 in Europe (Abu-Baker and Rouleau 2007). OPMD can be autosomal dominant and autosomal recessive MD and is caused by a trinucleotide repeat expansion of polyalanine tract at the N-terminal part in (poly(A))-binding protein 1 (PABPN1). The OPMD locus was mapped to chromosome 14q. PABPN1 is important for nuclear polyadenylation, and a poly (A) tail is added to an RNA at the end of transcription. On mRNAs, the poly(A) tail protects the mRNA molecule from enzymatic degradation in the cytoplasm and aids in transcription termination (Weill et al. 2012). The PABPN1 mutation contains a GCG trinucleotide repeat at the 5′ end of the coding region. Normally, PABPN1 gene has (GCG) 6 repeats, but OPMD patients have (GCG) 8–13 repeats at the N-terminus. The mutated PABPN1 aggregates and forms filamentous intranuclear inclusion in muscle nuclei which can cause cell death. This inclusion is a pathologic hallmark of OPMD.

9.2.7.1 *Drosophila* Models for OPMD

The poly (A)-binding protein 2 (PABP2) is a *Drosophila* PABPN1 homolog. PABP2 has similar function as PABPN1 in nuclear polyadenylation. However, PABP2 does not have a polyalanine tract at the N-terminus. The *Drosophila* OPMD model was established by expressing the 17 alanine repeats of mammalian PABPN1 in *Drosophila* using UAS/GAL4 system (Chartier et al. 2006). The expression of human PABPN1-17-alanine repeats in ubiquitous (daughterless-GAL4) was too toxic to the flies shown as death at the embryo or pupal stage. Expression those repeats with muscle-specific driver (Mhc-GAL4) induced abnormal wing position and age-dependent muscle degeneration caused by apoptosis. Muscles showed dense nuclear inclusions, disorganized myofibrils, lack of mitochondria, and many vacuoles. All those flies were flightless. Indirect flight muscles (IFMs) composed of dorso-longitudinal muscles (DLM) and dorsoventral muscles (DVM) which are involved in flight and wing position became thin and irregular after day 6 of eclose from pupae. This fits for adult onset muscle degeneration with rimmed vacuoles and nuclear inclusions that are very similar to those seen in OPMD patients.

Using *Drosophila* as OPMD models generates knowledge about the essential domain of PABPN1 which gives rise to the disease phenotype. Generally, PABPN1 has many domains from the N-terminus to the C-terminus as an alanine tract, a coiled-coil domain, an RNP-type RNA-binding domain (RPM), and an arginine-rich C-terminal domain. When the RPM domain was deleted or mutated by point mutations and expressed in the muscle of flies, those flies had no longer OPMD phenotype even 17 alanine repeats were presented. This indicates that RPM is an important domain responsible for OPMD-like phenotype (Chartier et al. 2006). Chartier et al. also demonstrated that alanine tract or 17 alanine repeats is not a direct cause of OPMD phenotype, but the ability of PABPN1 to bind to RNA within RPM domain is likely to be the cause. It has been shown that RPM domain is essential for specific binding of PABPN1 to poly A (Kühn et al. 2003).

The mRNA regulation does not depend only on polyadenylation but also on degradation involved with deadenylation. Chartier et al. further showed that downregulation of mRNA in deadenylation can improve muscle function of OPMD flies (Chartier et al. 2015). The CCR4-NOT deadenylation complex and Smaug, the deadenylation regulators, are the complex that reduces the amount of specific mRNAs encoding mitochondrial proteins. Active deadenylation complex leads to their destabilization and mitochondrial dysfunction. In contrast, if this complex is downregulated, it can partially improve muscle function. Consistently, sternocleidomastoid muscle biopsy from OPMD patients also had deregulation and downregulation of mitochondrial proteins which is similar to the observation in *Drosophila* where downregulated mRNAs encode for 53% of orthologous mitochondrial proteins (Chartier et al. 2015).

9.2.8 Distal MD (DD)

Distal muscle dystrophy (DD) is a rare disease characterized by muscle weakness at distal part of muscles including muscle of the hands, forearms, lower legs, and feet (Udd 2014; Dimachkie and Barohn 2014). DD is caused by mutations of many genes that affect protein necessary to the function of muscles. However, the cause of DD is difficult to identify. Many proteins involved in DD are desmin, αB-crystallin, myotilin, Z-disc alternatively spliced PDZ domain-containing protein (ZASP), caveolin, dysferlin, nebulin, myosin, telethonind, and filamin C. DD does not shorten the life span and not affect the brain and intellectual functions. Due to the slow progression of this disease, patients may not recognize their symptoms until very late age. There are at least eight types of DD which are distal myopathy with vocal cord and pharyngeal weakness, Finnish (tibial) distal myopathy, Gowers-Laing distal myopathy, hereditary inclusion-body myositis type 1, Miyoshi distal myopathy (LGMD2B), Nonaka distal myopathy, Welander's distal myopathy, and ZASP-related myopathy.

The two mutations of the DD were identified at p.Ala193Thr and p.Met251Thr of the calponin homology domains (CH2 domain) of the N-terminal actin-binding domain of filamin C protein from *FLNC* gene (Duff et al. 2011).

9.2.8.1 *Drosophila* Models for DD

The FLN90 is an isoform of the *Drosophila* ortholog filamin presented at synaptic boutons. It is a part of glutamate receptor clusters and plays a role in development of postsynaptic membrane folds called subsynaptic reticulum (SSR) of the larval NMJ (Lee and Schwarz 2016). Lee and Schwarz demonstrated that the filamin null mutant loss SSR formation at the bouton. The forming of SSR requires the exocyst complex to be recruited to the synapse which occurs by the small GTPase Ral. Knocking down filamin in muscles reduces type-A glutamate receptor at the postsynapse but show normal distribution of type-B glutamate receptor (Lee and Schwarz 2016). FLN90 is required for localization of the kinase dPak (*Drosophila* p21-activated kinase) and downstream GTPase Ral. The *Drosophila* Ral shows important role in exocyst which regulates autophagy process (Tracy et al. 2016).

9.2.9 Emery-Dreifuss MD (EDMD)

This disease is named after Alan Eglin H. Emery and Fritz E. Dreifuss who described this muscular disease. EDMD is a rare genetic muscular dystrophy disease characterized by early contractures and slow progression muscle weakness usually at the shoulder and lower leg muscles (Helbling-Leclerc et al. 2002). Patients also present with cardiac muscle and conduction defect. The onset of the disease is around childhood to teenager. Several gene mutations are involved in EDMD, including mutations of *EMD* (Emerin gene), *LMNA* (lamin A/C gene), *SYNE1* (Spectrin Repeat Containing Nuclear Envelope Protein 1 gene), *SYNE2* (Spectrin Repeat Containing Nuclear Envelope Protein 2 gene), and *TMEM43* (Transmembrane protein 43 gene) genes. These genes are associated with proteins emerin, lamin A/C, nesprin-1, nesprin-2, and luma, respectively, which are proteins that have a mechanical link between the nucleoskeleton and cytoskeleton. There are three major types of EDMD – EDMD1, EDMD2, and EDMD3 – which are X-linked, autosomal dominant, and autosomal recessive inheritance, respectively. EDMD1 is caused by mutations in the *EMD* gene on the X chromosome that codes for the nuclear envelope protein emerin which is a ubiquitous inner nuclear membrane protein. The cause of EDMD2/EDMD3 is due to mutations in *LMNA* gene located on chromosome 1. *LMNA* gene encodes at least four different types of mRNA: lamin A, lamin Adel10, lamin C, and lamin C2. Lamin A/C proteins are components of the nuclear envelope and are located in the lamina, a structure associated with the nucleoplasmic surface of the inner nuclear membrane. Mutations of A-type lamins cause a muscular dystrophy.

Beside laminA/C, nesprin-1 (nuclear envelope spectrin repeat protein 1) is a core protein complex of the linker of nucleoskeleton and cytoskeleton (LINC) which connects nuclei to cytoskeleton by its C-terminal region called

KASH domain (Klarsicht, ANC-1, Syne Homology). Mutation of nesprin is involved in laminopaties and cardiomyopathies (Rajgor and Shanahan 2013).

9.2.9.1 *Drosophila* Models for EDMD

Lamin C gene in *Drosophila* is an ortholog of human A-type *Lamin* gene. Transgenic flies for EDMD model were demonstrated by expressing a mutant form of Lamin C. Larval muscle-specific the expression of truncated lamin C (lacking first 42 amino acids or loss the N-terminal head domain) exhibited nuclear organization and muscle defect. Moreover, those flies had abnormality at leg imaginal disc and leg morphogenesis called twist leg phenotype. The contraction of larval body wall muscles is essential for leg elongation. Thus, the lack of larval muscle function resulted in malformed leg (Dialynas et al. 2010). The twist leg phenotype in Lamin C mutant is similar to that of ecdysone mutant. Ecdysone is a major steroid hormone in insect and plays a role in transition such as molting. Ecdysone regulates many genes including the orphan nuclear receptor β*Ftz-F1* which is involved in a muscular response to the prepupal ecdysone pulses. The lack of βFtz-F1 expression in Lamin C mutant at the pupal stage of development led to limited muscle contractions necessary for leg extension. In addition, the expression of truncated lamin C caused alterations in gene expression due to nuclear periphery which causes a transcriptionally repressive environment and gene repression. Since the mispositioned nuclei is one appearance of EDMD, the position of nuclei in *Drosophila* that had disruptions in genes linked to EDMD was evaluated and found that genes including *Otefin* (*Drosophila* emerin), *bocksbeutel* (*Drosophila* emerin), *klaroid* (*Drosophila* SUN), and *klarsicht* (*Drosophila* nesprin) were necessary for the initial separation of nuclei into distinct clusters and proper nuclei position (Collins et al. 2017).

Lamin C null mutants in *Drosophila* was first established by P-element technique. The mutant flies showed abnormality in musculature formation during pupal metamorphic stages. This musculature abnormality is a result of tendon-cell defects. (Uchino et al. 2013). The null mutant showed severe fragmentation and lobulation of muscle cell nuclei at the late larval stages. Restoration of Lamin C into the null mutant tendon cells but not skeletal muscle cells efficiently rescued the phenotype. This indicates that lamin C is required in tendon cells for normal formation of muscle nuclei (Uchino et al. 2013).

MSP-300 corresponds to the N-terminal two-thirds of the *Drosophila* nesprin ortholog. It is located at the sarcomeric Z-line of both skeletal and cardiac muscle (The Nesprins Are Giant Actin-Binding Proteins 2002). It plays a role in glutamate receptor density at the *Drosophila* neuromuscular junctions (Morel et al. 2014). Morel et al. showed that deletion of KASH domain in Msp-300 (Msp-300$^{\Delta KASH}$ mutant) impairs locomotion of *Drosophila* larvae. The Msp-300$^{\Delta KASH}$ mutant showed the decrease of neurotransmitter which was related to low density of GluRIIA receptor at the NMJ.

9.2.10 Spinobulbar Muscular Atrophy (SBMA)

Spinobulbar muscular atrophy (SBMA) is a rare and late-onset neuromuscular disease. It is an X-linked recessive, adult onset neurodegenerative disease which is caused by the degeneration of motor neurons in the brain stem and spinal cord. Patients present with muscle cramp progressive muscle weakness. Bulbar signs occur due to the defect of nerve from the brain stem that supplies muscles involved in swallowing, speech, and other functions of the throat. The endocrine symptoms may present with breast enlargement, erectile dysfunction, infertility, and testicular atrophy. SBMA is a sex-linked recessive inheritance and associated with the mutation of androgen receptor (AR) gene. SBMA is caused by the expansion of a CAG repeat in the first exon of androgen receptor gene (trinucleotide repeats). The CAG repeat encodes a polyglutamine (polyQ) tract in androgen receptor protein (Beitel et al. 2013). Normal individual has 8–34 polyQ stretches while SBMA patients have more than 40 glutamine residues. Even if SBMA is not a fatal disease, patients finally end up with a wheelchair.

9.2.10.1 *Drosophila* Models for SBMA

A SBMA model in *Drosophila* was first established by the expression of various human AR mutants in the fly eyes (Takeyama et al. 2002). Human AR structure contains at least five domains: the N-terminal regulatory domain (A/B domain), DNA-binding domain (DBD), hinge region, ligand-binding domain (LBD), and the C-terminal domain. Polyglutamine (polyQ of Q52, Q92, Q112, or Q212) stretches in many regions of AR were created and expressed in the fly eye. The flies clearly showed eye defect when mutated human AR (the polyQ expanded at the N-terminal A/B domain in full-length AR) was driven to the fly eye (by GMR-GAL4) in the presence of dihydroxytestosterone (DHT) (Takeyama et al. 2002). Overexpression of fragment of the polyQ expanded at the N-terminal A/B (no LBD or full-length AR) fused with the nuclear localization signal (NLS) did show eye phenotype without DHT. Moreover, trapping the polyQ-expanded human AR mutants in the cytosol by using a nuclear export signal (NES) prevented eye defect or neurodegeneration. This indicates that the nuclear translocation of mutated AR was required for eye defect, but not ligands binding mechanism. However, when androgen ligand, DHT, bound at LBD, the mutated AR was able to translocate into the nucleus and caused toxicity to photoreceptor neurons.

Serine phosphorylation sites for the MAP kinase Erk2 in human AR are also important for inducing toxic aggregate forming in the cells (Funderburk et al. 2009). Wild-type androgen receptor which has polyQ 22 stretches in the N-terminus did not show any eye defect when overexpressed using eye driver (GMR-GAL4). However, when two serine phosphorylation residuals (serine 424 and 514) at the N-terminus were mutated to alanine and overexpressed in the eye and the brain, the eye and locomotion defect were clearly seen in the presence of DHT, respectively (Funderburk et al. 2009).

Studying SBMA in *Drosophil*a models provides more insight into the pathophysiologic process that (1) the full binding of androgen to LBD in the polyQ expanded at the N-terminus in the hAR mutants leads to structural alteration with nuclear translocation and results in the onset of SBMA in male patients and that (2) mutations of serine phosphorylation residual at the N-terminus of AR cause toxicity in the cell.

9.2.11 Spinal Muscular Atrophy (SMA)

SMA is caused by the degeneration of motor neurons in anterior horn cells in the spinal cord connecting the brain and spinal cord to the body's muscles. It is a group of autosomal recessive disorders associated with the mutation of survival motor neuron (SMN) genes on chromosome 5q11.2–5q13.3 (Brzustowicz et al. 1990). There are two SMN genes – SMN1 and SMN2 – that produce SMN proteins. The SMN proteins are expressed in most tissues and associated with nuclear riboproteins (nRNPs, small and heterogeneous) and other RNA-binding proteins. It is presented in a complex that functions in the formation and transport of spliceosomal snRNPs which plays a role in mRNA biogenesis. The incidence of SMA is 1:11,000 live births (Kolb and Kissel 2015). SMA is mostly presented with muscle weakness and normal cognitive function. The clinical severity of each SMA type correlates with SMN2 copy number which is able to compensate for the loss of SMN1 gene (Kolb and Kissel 2015). The severity of the disease depends on copy number of SMN2. SMA is classified into five types according to the onset of the disease and age-related symptoms and how much physical mobility a person has (Kolb and Kissel 2015). For instance, SMA Type 0 occurs in infant with less than 1 month and has one copy of SMN2 gene. This type is the most severe form of the disease and is characterized by decreased fetal movement, areflexia, facial diplegia, atrial septal defects, and joint contractures. Patients usually cannot survive beyond 1 month due to the respiratory failure. In contrast to SMA type IV which has the onset of disease after 21 years, it has 4–8 copies of SMN2 gene. Patients are usually able to reach all the major motor function including independent walking. These patients are usually ambulatory until age 60 .

9.2.11.1 *Drosophila* Models for SMA

SMN gene is highly conserved across species. *Drosophila* has a single copy of *SMN* gene (d*SMN*) which encodes a highly conserved homolog of SMN (Chan et al. 2003). Null mutations in single-copy SMN gene are lethal in every organism (Monani 2005). The point mutation in the coding region of *dSMN* gene, the smn^{73Ao} mutant, has been established to study SMN function in the flies (Chan et al. 2003). This point mutation is similar to SMA patient who had a single G insertion at the end of exon 1 which results in a missense mutation (Skordis et al. 2001). The smn^{73Ao} mutant that is homozygous is lethal at the late larval stages. The mutant never reaches the adult fly life stage. This larval mutant showed a decrease in contraction rate as observed by the loss of mobility of locomotory body wall contraction. The mutant larvae showed disorganization and an increased number of enlarged boutons. The mutant showed decreased efficiency at the NMJ as reduction in the amplitude of excitatory postsynaptic currents (EPSCs). This might be caused by reduction of large glutamate receptor (GluR) clusters in the postsynaptic muscle site. The mutant phenotype can be almost completely rescued only when the SMN protein is expressed in both neuronal and muscle site of the NMJ suggesting that the SMN protein is required both pre- and postsynaptically. Rescue experiment had shown that bringing back the N-terminus-deleted part (deletion of up to 63 amino acids from total 226 amino acid) partially rescued the phenotype, while deletion in the carboxy-terminus of SMN protein (misses all amino acids after 157) did not show any rescue. This suggests that the carboxy-terminus of SMN protein is required for rescue of the neuromuscular phenotype (Chan et al. 2003). Chia-Hao Chang et al. performed a genetic approach to screen for genes that affect *Smn*-dependent processes using the Exelixis collection of transposon-induced mutations. They indicated that SMN influences retrograde BMP activity through Wit receptor (type II BMP receptor). Overexpression of Wit in neurons in a heterozygous smn^{73Ao} mutant resulted in phenotype rescue by reducing the NMJ bouton numbers. The downstream effector of BMP pathway, *Mothers against dpp* (*Mad*) or *Drosophila* homolog of *R-Smad*, confirmed the role of SMN in BMP activity. The hypomorphic Mad mutant clearly enhanced SMN-dependent NMJ defect, but the loss of *Daughters against dpp* (*Dad*) which is a *Mad* antagonist decreased NMJ defect of *Smn* mutation. Elevating the BMP activity through a complete loss of *Dad* function suppressed the effect of *Smn* mutation on the NMJ. This suggests the potential therapeutic target for SMA as an increase BMP signaling may decrease Smn-associated NMJ defect (Chang et al. 2008).

Since the smn^{73Ao} mutant was homozygous lethal, Rajendra et al. established the hypomorphic *Smn* mutant called Smn^{E33} as a model for SMA in adult fly. A *P* element insertion located 94 bp upstream of the transcription start site of *Smn* gene was used to generate Smn^{E} mutant by imprecise excision of the *P* element and screened for neuromuscular phenotype in adult flies. From 170 independent excisions, the Smn^{E33} mutant was identified. This mutant displayed severe atrophy of indirect flight muscles (IFMs), but it stayed alive until adult with the lack of its ability to jump or fly (Rajendra et al. 2007). This fly showed disorganization in the IFMs in both dorsal longitudinal muscles (DLMs) and dorsoventral muscles (DVMs). It is characterized by irregular with numerous bulges and constrictions throughout the muscles. The motoneuron branching in DLM of Smn^{E33}mutant showed smaller routing and defect in secondary branching and arborization. The failure of motoneuron innervation of DLMs resulted in a decrease in the expression of actin filament (Rajendra et al. 2007).

Electrophysiology at the NMJ (Intracellular Recording in the *Drosophila* Larval Muscle)

The *Drosophila* NMJ has been extensively used as a model to study the molecular mechanisms underlying synaptic transmission. Performing electrophysiology at the NMJ provides the information of neurotransmitter release from the presynaptic nerve terminal to activate glutamate receptor at motor endplate on the muscle. In *Drosophila*, the postsynaptic membrane is called

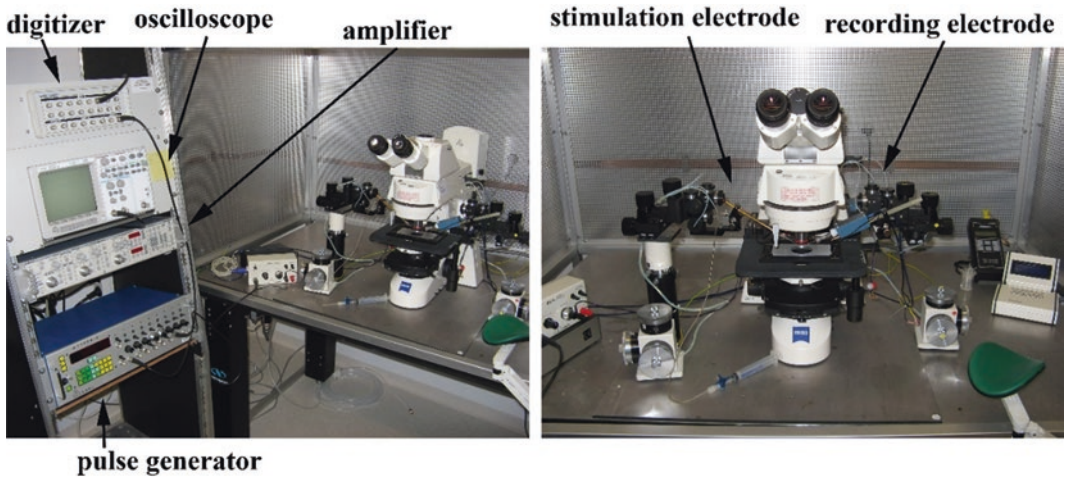


Fig. 9.5 Electrophysiology setting at electrophysiology unit of Prof. Dr. Jasprien Noordermeer Laboratory, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, the Netherlands: Photo from Chap. 1 of thesis dissertation "A *Drosophila* Model for Duchenne Muscular Dystrophy" of Mariska C. van der Plas (24 Jan 2008)

the subsynaptic reticulum (SSR). The glutamate receptors are located opposite the active zones. When glutamate binds to its receptor, the receptor allows Ca^{2+} to enter the muscle, which causes the muscle membrane to depolarize. The absence of an action potential in the *Drosophila* muscle is one of the reasons why it is a desired model for NMJ study. In mice, the action potential has to be prevented by blocking Na^{+} channels in order to be able to measure the endplate potential. *Drosophila* third instar larval NMJs can be measured at room temperature, maintaining their electrical properties for many hours (Fig. 9.5).

Materials

1. 3 M KCl
2. HL3 (hemolymph-like saline) solution without Ca^{2+} (70 mM NaCl, 5 mM KCl, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose, 5 mM HEPES, pH 7.2)
3. HL3 solution with 0.6 mM $CaCl_2$
4. Sylgard
5. Dissecting apparatus: insect pins (#0), ophthalmologists scissor, and *Drosophila* forcep
6. Recording microelectrode (Sutter capillary glass)
7. Stimulation or suction microelectrode (Sutter capillary glass)

Equipment

1. Stereomicroscope and a dark-field illuminator on vibration-free table
2. Micropipette puller P-97 (Sutter Instrument, CA, USA)
3. GeneClamp 500B amplifier (Axon Instruments, Union City, CA), low-pass filtered at 10 kHz, high-pass filtered at 0.5 Hz
4. Digitizer DigiData 1322A and pClamp9 software (Axon Instruments)
5. Pulse generator (Master-8; AMPI)

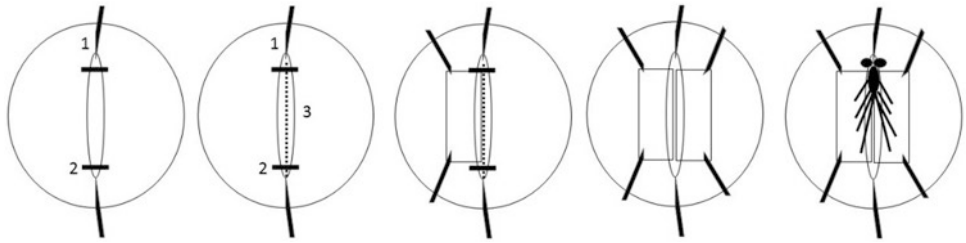
Methods

1. Prepare a recording microelectrode.
   - Recording microelectrode is prepared from Sutter capillary glass (the borosilicate glass, inside diameter ~ 0.7 mm) by Sutter glass pipette puller.
   - Fill in 3 M KCl in recording microelectrode and avoid air bubble by using

1 mL syringe with long lumbar puncture needle.
- Insert recording electrode to manipulator connecting an amplifier.

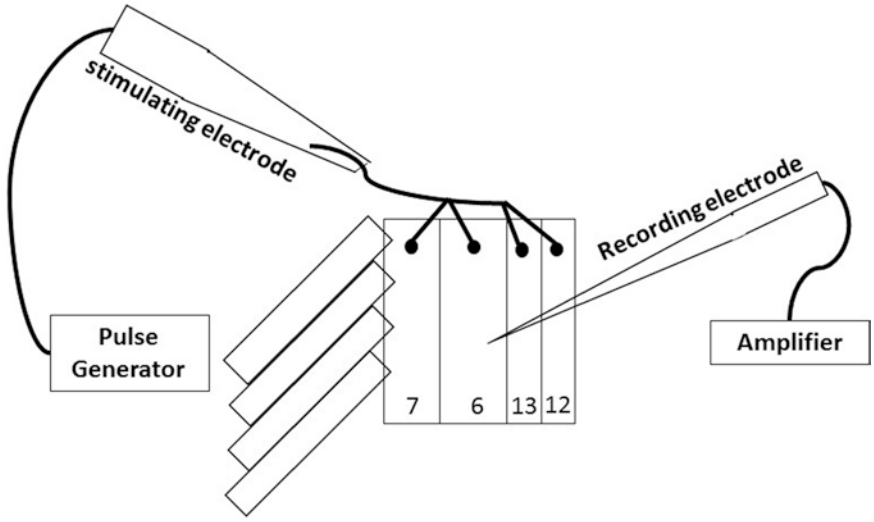
2. Prepare a stimulation microelectrode or suction electrode.
   - Stimulation electrode is prepared by Sutter glass pipette puller.
   - Under dissecting stereomicroscope, use forceps to break the tip of stimulation electrode making a small hole for suction the nerve.
   - Connect stimulation electrode with a manipulator connecting the pulse generator.

3. Dissection of 3rd instar larva body wall (figure).
   - Wash 3rd instar larva with phosphate buffer saline and place on sylgard-coated dish.
   - Facing up of dorsal side of larva.
   - Place the pin at the head near mouth hook, stretch a larva with pin, and place the pin at posterior spiracles.
   - Add HL3 solution without $CaCl_2$.
   - Make a horizontal incision by scissor near both pins (1 and 2).
   - Make a vertical incision along the body (dash line of 3).
   - Place the pin at each edge of larval body wall.
   - Remove the gut and residual tissue until the brain and motorneuron branches are exposed.

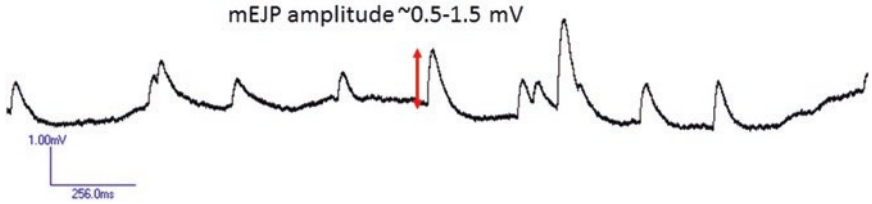


   - Cut motonerve at the distal end of the neuropile and remove the brain.
   - Wash the sample twice with HL3 with 0.6 mM of $CaCl_2$.
   - Place sylgard dish with dissected larva under stereomicroscope electrophysiology plate form.

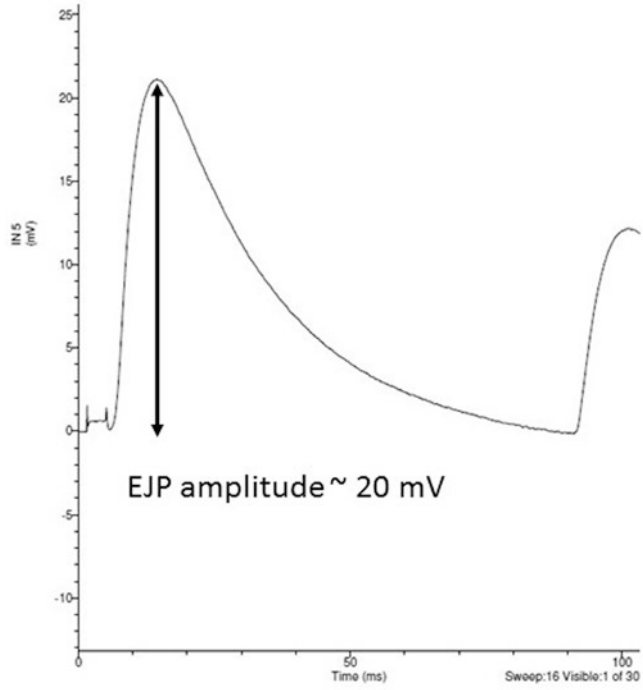
4. Electrophysiology recording.
   - Gently insert recording electrode in the muscle cell at abdominal muscle A2–A4 of muscle 6.
   - Slightly adjust the depth of recording electrode and observe the synaptic signals until the resting membrane potential is below −50 mV (on average, the membrane potential was –60 mV in all samples).



- Electrical input resistance of all muscle fibers recorded should be above 4 MΩ.
- Record a spontaneous small depolarizations of the muscle membrane, called miniature excitatory junction potentials or mEJPs, continuously for 1 min.



- Take up the motonerve of recorded muscle via suction electrode.
- Stimulate the nerve by a pulse generator at 0.3 Hz stimulation to get a large depolarizations of the muscle membrane called (evoked) excitatory junction potentials or (e) EJPs.



- Record EJP continuously for 30 stimulation.

5. Analysis for neurotransmitter release or quantal content.

mEJPs represent the activation of glutamate receptors as a result of the spontaneous release of a single neurotransmitter vesicle or quanta from the motoneuron. The amplitude of the mEJPs gives information about the amount of neurotransmitter in a vesicle and the amount of glutamate receptors able to respond.

The mean mEJP amplitude and frequency are analyzed by using the peak detection feature of Mini-Analysis 6.0 (Synaptosoft).

EJPs are fired when an action potential in the motoneuron triggers the release of many neurotransmitter containing vesicles. EJP amplitudes are analyzed using Clampfit 9.0, and amplitudes are normalized to a membrane potential of −60 mV.

Since the measurements are done in current clamp, it means that the amount of current flowing through the recording electrode is constant. We have to add a correction to the calculation to compensate for nonlinear summation. This correction is only necessary in current clamp mode.

$$\text{Formula}: EJP'' = EJP' / \left(1 \quad f^{*}\left(EJP' / Vr \quad Vm\right)\right)$$
$$= EJP' / \left(1 - 0.4^{*}\left(EJP' / 50\right)\right)$$

where EJP″ represents the average EJP amplitude corrected for nonlinear summation

EJP' represents the average EJP amplitude normalized to a Vm of −60 mV

$$F = 0.4$$

Vr represents the reversal potential = −10 mV

Vm represents the membrane potential = −60 mV

NMJ quantal content (QC) or amount of vesicles released can be calculated by dividing the mean EJP amplitude (calculated from 30 events) corrected for nonlinear summation (B. A. Stewart, personal communication) by the mean mEJP amplitude (calculated from 100 events).

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Drosophila As a Cancer Model

10

Masato Enomoto, Carmen Siow, and Tatsushi Igaki

Abstract

Over the last few decades, *Drosophila* cancer models have made great contributions to our understanding toward fundamental cancer processes. Particularly, the development of genetic mosaic technique in *Drosophila* has enabled us to recapitulate basic aspects of human cancers, including clonal evolution, tumor microenvironment, cancer cachexia, and anticancer drug resistance. The mosaic technique has also led to the discovery of important tumor-suppressor pathways such as the Hippo pathway and the elucidation of the mechanisms underlying tumor growth and metastasis via regulation of cell polarity, cell-cell cooperation, and cell competition. Recent approaches toward identification of novel therapeutics using fly cancer models have further proved *Drosophila* as a robust system with great potentials for cancer research as well as anti-cancer therapy.

Keywords

Cancer · Cell competition · Cell-cell cooperation · Clonal evolution · Tumor heterogeneity · Tumor microenvironment · Anticancer therapy

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10.1 Introduction

Cancer research in *Drosophila* has a long established history. Notably, the first cancer-causing mutation was discovered in *Drosophila*. In 1967, a genetic screen performed by Gateff and Schneiderman reported a "recessive mutant" that caused affected cells to behave like "malignant tumors" (Gateff and Schneiderman 1967). This mutation, known as the *lethal giant larvae* (*lgl*), was described to expedite aggressive growth and eventually kill the host animal. Although the existence of "recessive oncogenes" had long been predicted by Boveri (Boveri 1929), Gateff and Schneiderman's discovery was the first example of an inactivating mutation demonstrated in a living organism, even before the term "tumor suppressor" was described (Gateff 1978). Before the discovery of *lgl*, skepticism remains in regard to the functional homology between fly and human cancers. Until the last few decades, comparative cancer research in lower organisms including *Drosophila* sparked interest in manipulating simple models to understand fundamental cancer processes. Considering substantial conservation of basic cellular pathways between flies and humans, the use of *Drosophila* model opens up many possibilities to address difficult propositions in cancer biology in vivo, especially those involved in tumor progression, metastasis, and oncogenic cell-cell interactions.

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In recent years, vast array of *Drosophila* genetic tools have been made available. Among them, one of the most important inventions is the genetic mosaic technique (Xu and Rubin 1993), with further modifications made especially the establishment of mosaic analysis with a repressible cell marker (MARCM) system (Lee and Luo 1999). This technique induces somatic clones of mutant cells in living tissues, allowing coexistence of cells with different genotypes in a single organism. This elegant approach provides a huge advantage in cancer studies, considering the etiology of cancer and how it progresses. While single genetic lesion is able to transform cells into tumors, malignant cancer is developed by subclones with sequential acquisitions of multiple oncogenic mutations. Hence, by establishing clonal cell populations in *Drosophila*, we could unveil conserved tumor suppressor genes and analyze cancer progression in vivo. Certainly this classical view of how tumor develops depicts a cell-autonomous process in which mutant cells become malignant after acquiring mutations sequentially. However more recently, through genetic mosaic analyses the aspect of non-cell autonomy in cancer progression has drawn growing interest. Tumor tissues contain interacting heterogenous subclones of mutant cells surrounded by wild-type or other mutant cells, which generate oncogenic cell-cell cooperation and competition (Egeblad et al. 2010; Enomoto et al. 2015b). In this chapter, we review concepts of cancer biology revolving cell-cell communication, tumor heterogeneity, and its microenvironment which emerged from *Drosophila* genetics and describe how the fly model can be implemented in understanding human cancer progression.

10.2 Discovery of Tumor Suppressors in *Drosophila*

10.2.1 "Hyperplastic" Tumor Suppressors

Drosophila tumor suppressor genes were subsequently characterized since the discovery of *lgl*, in which these mutants showed tumorous phenotype. Although *lgl* was identified as a mutant that forms neoplastic tumors, the molecular role of Lgl remained an enigma in cancer progression for a long time. It was until the last two decades, Lgl was shown to act in a similar genetic pathway alongside *scribble* (*scrib*) and *discs large* (*dlg*), which were initially isolated as the classical *Drosophila* mutants. Lgl, Scrib and Dlg cooperatively maintain epithelial apicobasal polarity, and their mutants show disorganization of tissue architecture and subsequently develop multilayered metastatic tumors (Bilder et al. 2000). Although a single loss-of-function mutation illustrated by these tumor suppressors is sufficient to cause tumor formation, there is a huge limitation in studying the effects of mutations in cancer that would otherwise show lethality in the whole organism. In 1993, Xu and Rubin developed the genetic mosaic technique that enables coexistence of oncogenic mutant clones and wild-type clones in a single organism, generated by FLP (flippase)/FRT (FLP recognition target)-based mitotic recombination in vivo. Since then, crucial modifications for further refinement of the mosaic technique are made, which include the MARCM (mosaic analysis with repressible marker) technique (Protocol I). This technique recapitulates development of human cancers in which homozygous somatic clones of oncogenic mutant cells are generated from single cells in heterozygous mutant tissue. It was through genetic mosaic screens that "hyperplastic tumor suppressor genes" were discovered. The first of these hyperplastic mutants identified, unlike "neoplastic" mutants such as *lgl*, *scrib*, and *dlg*, is *warts* (*wts*)/*large tumor suppressor (lats)*. Mutant clones carrying homozygous mutations in *wts*/*lats* overgrow when generated in normal tissues (Fig. 10.1a) (Xu et al. 1995; Justice et al. 1995). In contrast to neoplastic tumors that exhibit multilayered and undifferentiated tissues, hyperplastic tumors normally maintain the characteristics of an epithelial monolayer in the larva and eventually differentiate into adult tissue. Successively through genetic screens, a series of hyperplastic tumor suppressor genes were identified, which include *salvador* (*sav*)/*shar-pei*, *hippo* (*hpo*), *mob as tumor suppressor* (*mats*),

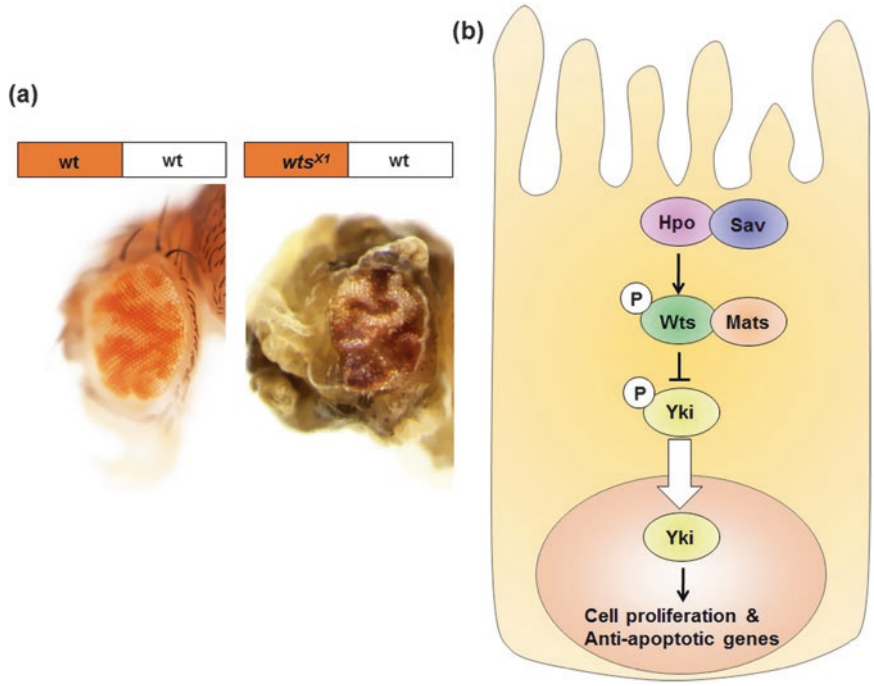

Fig. 10.1 Epithelial overgrowth by hyperplastic tumor-suppressor mutation
(**a**) Adult eye of eyFLP-MARCM-induced wt//wt and wts^{X1}//wt mosaics. Adult eye with wt//wt clones shows normal eye morphology. Adult eye with wts^{X1}//wt mosaics shows folded-eye phenotype, a characteristic of overgrown epithelial tissue. (**b**) The core components of *Drosophila* Hippo signaling. Hippo (Hpo)/Salvador (Sav) phosphorylates and activates Warts (Wts), which, together with Mats, inactivates Yorkie (Yki) via phosphorylation-dependent cytoplasmic retention

Tsc1, and *archipelago* (*ago*) (Gao and Pan 2001; Potter et al. 2001; Moberg et al. 2001; Jia et al. 2003; Tapon et al. 2002; Kango-Singh et al. 2002; Harvey et al. 2003; Udan et al. 2003; Lai et al. 2005; Wu et al. 2003; Pantalacci et al. 2003). Particularly, *sav*, *hpo*, and *mats* turned out to be components of a common pathway, named the Hippo pathway. The Hippo pathway is a kinase cascade that negatively regulates cell proliferation and survival by sequestering the transcriptional coactivator *yorkie* (*yki*; a Yap/Taz homolog) in the cytoplasm (Fig. 10.1b) (Huang et al. 2005). More recently, it has been shown that several cues including mechanical stress, cell polarity, and cell adhesion control Hippo pathway activity. Although most Hippo pathway components were identified in fly, delineation efforts found highly conserved orthologs in mammals. Intriguingly, mutations in core components of the Hippo pathway are hardly observed in human cancers, although nuclear localization of Yap is observed in hepatocellular carcinomas and non-small-cell lung cancers (Harvey et al. 2013). In addition, gene amplification of Yap has been reported in liver cancers (Overholtzer et al. 2006; Zender et al. 2006), suggesting that dysregulation of the Hippo pathway activity also contributes to human cancers. (Harvey et al. 2013; Pan 2010).

10.2.2 "Neoplastic" Tumor Suppressors

Genetic screen using mosaic technique not only identified hyperplastic tumor suppressors but also neoplastic tumor suppressor genes other than the mentioned, i.e., *scrib/lgl/dlg*. One example is the syntaxin *avalanche* (*avl*) mutant, a component of the intracellular vesicle trafficking machinery. Clones of *avl* mutant cells undergo

slow growth and normally disappear from tissues, but become neoplastic when surrounding wild-type cells are removed, resembling the phenotype of *scrib* or *dlg* mutants (Lu and Bilder 2005). Loss of *avl* impairs endocytic trafficking and induces accumulation of the apical protein Crumbs (Crb) in early endosomes (Lu and Bilder 2005). Crb accumulation disrupts apicobasal polarity and subsequently causes neoplastic tumor growth. Likewise, some other mutants of the endocytic machinery also show similar phenotype (see "Cellular cooperation"). It is also shown that neoplastic mutants for *Drosophila* Polycomb group (PcG) components develop into aggressive tumors that lose normal epithelial architecture (Classen et al. 2009). Importantly, Unpaired (Upd, an IL-6 homolog) is a transcriptional target for PcG complex. Therefore, mutations in some core PRC1 (polycomb repressive complex 1) components drive tumor growth through Upd-mediated JAK-STAT signaling. In addition, PcG transcriptionally represses components of the Notch pathway, which explains massive growth of mutant *polyhomeotic* (*ph*), a PcG component (Martinez et al. 2009). Thus, PcG proteins exert tumor-suppressive activity by controlling multiple signaling pathways.

10.3 Cancer Progression by Cell-Cell Communication in *Drosophila*

10.3.1 Cellular Cooperation in Tumorigenesis

Tumors, due to its heterogeneous populations of mutant and wild-type cells, develop through a repertoire of cell-cell interactions. Such interactions which include oncogenic cell-cell cooperation between mutant and wild-type cells have been widely demonstrated in the fly model attributing the major signaling pathways like the Hippo and JNK (c-Jun N-terminal kinase) pathways. Interestingly, through genetic mosaic screens in *Drosophila*, many genes were identified to cause non-cell autonomous tumor growth or progression. An example is the *vps25* (*vacuolar protein-sorting-associated protein 25*), a component of the ESCRT (endosomal sorting complexes required for transport) machinery which controls endocytic trafficking of transmembrane proteins. Deregulation of such sorting system affects signaling pathways triggered by the transmembrane proteins, resulting in tumorigenesis (Mattissek and Teis 2014). Mechanistically, *vps25* mutant cells promote endosomal accumulation of Notch, which leads to elevation of Notch signaling activity. This upregulates Notch signaling target Upd and thereby induces overproliferation of surrounding cells via activation of JAK-STAT signaling (Herz et al. 2006; Thompson et al. 2005; Vaccari and Bilder 2005). As a consequence, *vps25* mutant cells undergo apoptotic cell death and are eventually excluded from imaginal epithelia. Alongside *vps25*, *erupted* (*ept*; a *tsg101* homolog), a component of the endocytic pathway, also cause nonautonomous tissue overgrowth via similar mechanisms through Upd-JAK-STAT signaling (Moberg et al. 2005). However, when the entire tissue contains only *vps25* or *ept* mutant cells, these tumors behave as neoplasms, suggesting that these non-autonomous phenotypes are dependent on cell-cell interaction. Another similar mutant of the endocytic machinery is *rab5*, which also displays non-cell autonomous overgrowth via JAK-STAT signaling, but with distinct mechanisms. *rab5* mutant cells activate Hippo effector Yki via cooperation with Eiger/TNF (Tumor necrosis factor)-JNK and EGFR-Ras signaling, thereby inducing Upd and resulting in non-autonomous overgrowth (Takino et al. 2014). Cellular cooperation is also illustrated by classical oncoprotein Src, a non-receptor tyrosine kinase suggested to be linked with multiple human solid cancers when its activity/expression is increased (Yeatman 2004). It is demonstrated in *Drosophila* imaginal epithelia that clones with activation of Src64B (a *c-Src* homolog) are outcompeted by surrounding wild-type cells (Enomoto and Igaki 2013). Simultaneously, Src64B-activated cells assist surrounding tissue overgrowth by propagating Yki activity to their neighboring cells via JNK activation (Fig. 10.2) (Enomoto and Igaki 2013). Cellular cooperation revolving the Hippo and

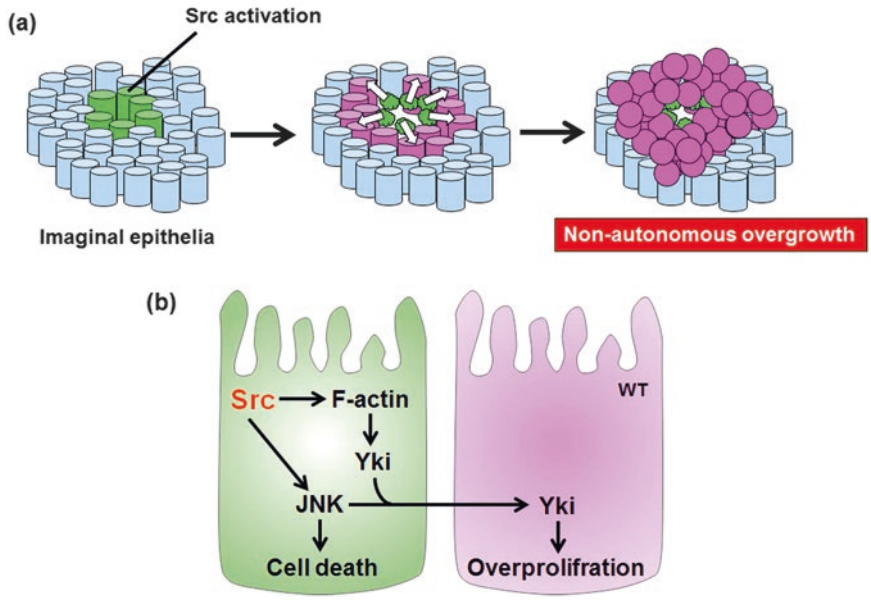

Fig. 10.2 Oncogenic cell-cell cooperation between Src-activating cells and surrounding wild-type cells (**a**) General scheme illustrating a subclone of imaginal epithelia acquires Src activation and progressively stimulates growth of neighboring wild-type cells non-autonomously. (**b**) Src-activating cells induce F-actin accumulation and thus result in the activation of Hippo effector Yki. JNK signaling is also activated by elevated Src activity and thereby concurrently aids in the propagation of Yki to surrounding wild-type cells, causing overgrowth of their neighbors

JNK pathways described above are few of the many identified mechanisms through genetic analysis in *Drosophila*. Nonetheless, these examples demonstrated in flies suggest a phenomenon in which oncogenic mutant cells generate tumor microenvironment by cooperating with normal cells.

10.3.2 Cell Competition in Tumorigenesis

Apart from cellular cooperation, cell competition is among the diverse behaviors of distinct tumor subpopulations, which is demonstrated through *Drosophila* mosaic analyses. Competitive behavior occurs when elimination of viable cells is compelled by their neighbors. This phenomenon is classically found in the interaction between *Minute*/+ cells (heterozygous ribosomal protein mutants) and wild-type cells in *Drosophila* wing imaginal disc (Morata and Ripoll 1975). As described previously, tissues mutant for apico-basal polarity genes such as neoplastic *scrib* or *dlg* aggressively develop into invasive tumors (Fig. 10.3a), but when surrounded by normal cells, the mutants are eliminated from imaginal epithelia (Fig. 10.3b) (Brumby and Richardson 2003; Pagliarini and Xu 2003). How can polarity-deficient cells, with such intrinsic potential to overgrow aggressively, be disqualified? Collective findings associated JNK signaling for being responsible in the elimination of *scrib* or *dlg* cells through various cellular effectors. Eiger, a TNF homolog, is shown to endocytically induce JNK activation in *scrib* or *dlg* cells thus promoting elimination of these mutant cells (Fig. 10.3c) (Igaki et al. 2009). Apart from Eiger, a recent study revealed that the Slit-Robo2 system, a conserved neural axon guidance component important in cell repulsion and migration, acts downstream of JNK signaling to extrude *scrib* mutant cells via dysregulation of E-cadherin (Fig. 10.3c) (Vaughen and Igaki 2016). Interestingly, JNK signaling is also activated in neighboring cells of *scrib* mutants and thereby

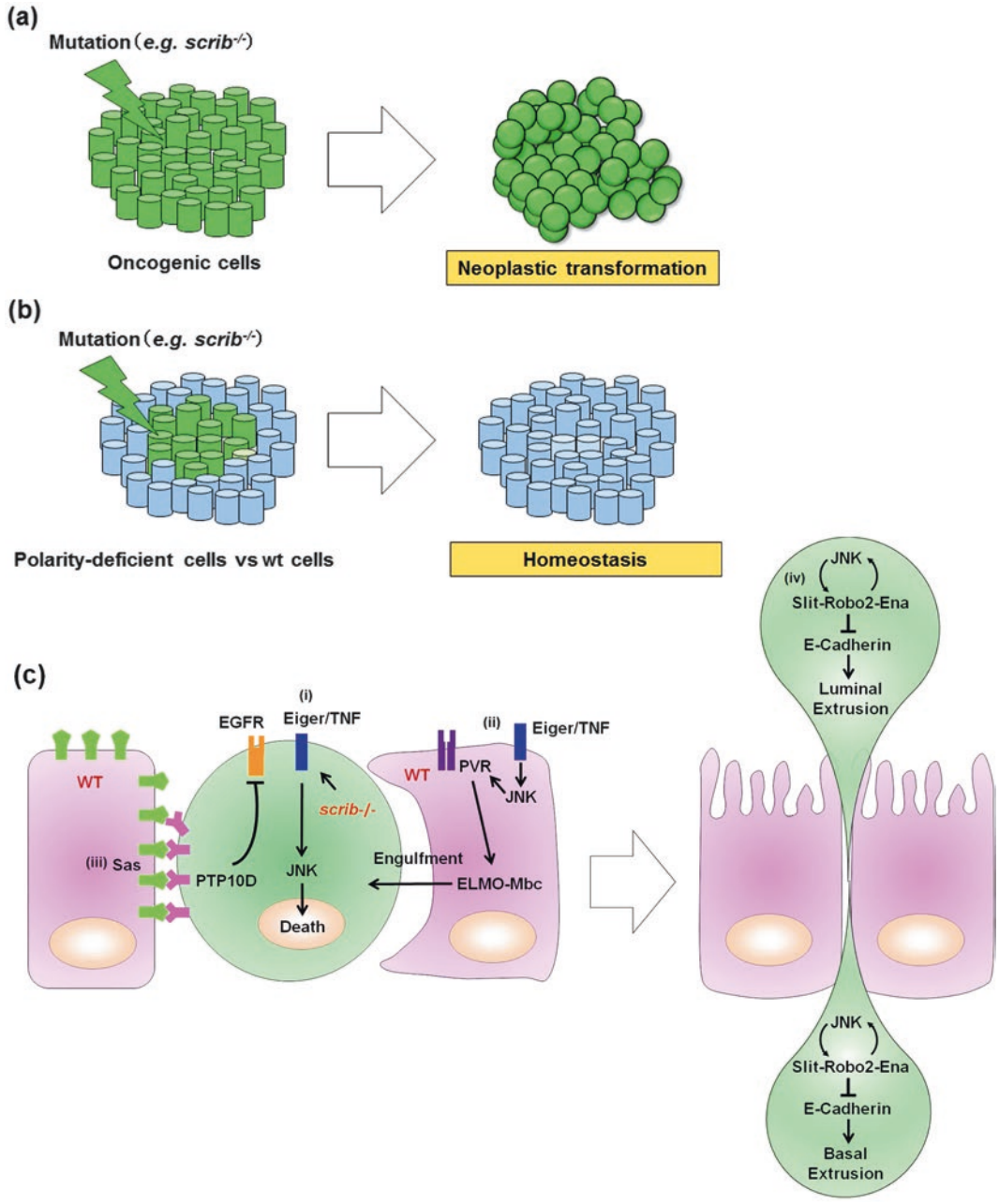

Fig. 10.3 Tumor-suppressive cell competition that eliminates polarity-deficient cells
(**a**) When a tissue entirely consists of oncogenic polarity-deficient cells, e.g., *scrib*$^{-/-}$, the tissues transform over time into neoplastic tumors. (**b**) When polarity-deficient cells are surrounded by wild-type cells, cell competition occurs to achieve tissue homeostasis, hence eliminating mutant clones from the tissue. (**c**) Polarity-deficient cells like *scrib*$^{-/-}$ clones are eliminated by cell competition when surrounded by wild-type cells through three mechanisms: (*i*) *scrib*$^{-/-}$ mutants activate Eiger/TNF to promote JNK-mediated cell death and (*iv*) JNK-Slit-Robo2-Ena-mediated cell extrusion (both luminally and basally) via downregulation of E-cadherin, (*ii*) Eiger/TNF-JNK signaling is also activated in surrounding wild-type cells, which upregulate PVR, resulting in ELMO-/Mbc-mediated engulfment of neighboring mutant cells, and (*iii*) Sas-PTP10D trans-activation in *scrib*$^{-/-}$ cells inhibits EGFR-Ras activity, which allows elevated JNK signaling in *scrib*$^{-/-}$ cells to be used for cell elimination. PVR, PDGF/VEGF receptor; ELMO, engulfment and cell motility; Mbc, myoblast city (Ced-5/DOCK180 homolog)

induces engulfment of *scrib* cells through ELMO-Mbc/Dock180 signaling (Fig. 10.3c) (Ohsawa et al. 2011). A *Drosophila* genetic screen also identified Sas (stranded at second), a cell-surface ligand protein that normally localizes at the apical surface of epithelial cells, and its receptor PTP10D, an apical receptor tyrosine phosphatase, which acts as the ligand-receptor system to drive cell competition. In wild-type "winner" cells, Sas relocalizes to the lateral surface of the cell at the interface between *scrib* and wild-type cells. This allows direct *trans*-interaction of Sas with its receptor PTP10D, which is also laterally relocalized in *scrib* mutants, thereby inhibiting EGFR (epidermal growth factor receptor)-Ras signaling in *scrib* cells and enabling their elimination via JNK signaling. If Sas-PTP10D *trans*-interaction is absent, JNK signaling cooperates with EGFR-Ras activation to cause Yki activation, thereby leading to overgrowth of *scrib* mutants (Fig. 10.3c) (Yamamoto et al. 2017). Thus, normal epithelium by itself possesses multiple intrinsic tumor suppression mechanisms that recognize and eliminate pre-malignant cells from the tissue. Another example of cell competition that is involved in tumor regulation is reported in cells expressing EGFR and microRNA (miRNA) *miR-8*. Such mutant cells undergo cytokinesis failure through downregulation of *peanut* (a Septin7 homolog) and develop into polyploid neoplastic tumors, which outcompete surrounding normal cells by engulfment cell competition (Eichenlaub et al. 2016).

In contrast to neoplastic *scrib* or *dlg*, hyperplastic Hippo component mutants or proto-oncogene Myc-overexpressing cells are instead the "winners," which expand within tissues by outcompeting surrounding normal cells (de la Cova et al. 2004; Moreno and Basler 2004; Tyler et al. 2007). In Myc-induced cell competition, Toll-related receptor signaling leads to expression of an apoptotic gene *hid* through Relish (Rel; a NF-κB homolog) activation, which causes cell death of wild-type cells (Meyer et al. 2014). Another report showed an alternative mechanism in which the death of wild-type cells is caused by *azot* (an EF-hand protein) accumulation (Merino et al. 2015). These two mechanisms may cooperatively drive Myc-induced cell competition. Interestingly, Yki activation, which is caused by Hippo pathway inactivation, stimulates *myc* expression (Neto-Silva et al. 2010; Ziosi et al. 2010). In addition, growth-related Wg/Wnt and JAK-STAT signaling also contribute to tumor cell expansion by eliminating neighboring cells (Rodrigues et al. 2012; Vincent et al. 2011). Furthermore, *apc* mutant cells in the adult fly midgut activate JNK and Yki to induce cell competition and tumor growth (Suijkerbuijk et al. 2016). Thus, cell competition, albeit an intrinsic tumor suppression mechanism for some polarity or endocytic mutants, can drive tumorigenesis by outcompeting neighboring cells when mutated for hyperplastic tumor suppressors or proto-oncogenes.

10.4 *Drosophila* Model of Clonal Evolution

Cancer is developed through sequential oncogenic mutations such as activation of oncogenes and inactivation of tumor suppressor genes. Such sequential alterations trigger tumorigenesis via Darwinian selection of advantaged subclones, termed "clonal evolution" (Greaves and Maley 2012; Vogelstein et al. 2013; Cairns 1975; Nowell 1976). Indeed, evidence from *Drosophila* genetic studies demonstrates that cancers progress by clonal evolution wherein mutant clones become malignant after further mutations of more genes. Reiterating our previous example of polarity-deficient mutants, they are naturally eliminated from tissues when confronted with normal cells. However, interestingly, a polarity mutation (e.g., *scrib*) confers metastatic behavior to Ras-induced benign tumors (Protocol II) (Igaki et al. 2006; Pagliarini and Xu 2003; Brumby and Richardson 2003). In malignant mutant clones of $scrib^{-/-}$ + Ras^{V12} cells, their metastatic behavior is caused by not only ectopic Ras activation but cooperatively with Eiger-JNK signaling that activates Yki (Doggett et al. 2011; Enomoto et al. 2015a). It was also shown that in $scrib^{-/-}$ + Ras^{V12} mutants, JNK signaling induces

Mmp1 (matrix metalloproteinase 1), a collagenase that disrupts extracellular matrix during cell invasion (Srivastava et al. 2007). More evidence obtained from fly studies corroborates the importance of JNK signaling in tumor development, in which mutants that stimulate JNK activity cause metastasis of Ras^{V12} clones (Khoo et al. 2013; Ma et al. 2013; Ma et al. 2017). Apart from JNK activation, mutations that abrogate lysosomal function also cause tumor growth and metastasis of Ras^{V12} clones in flies (Chi et al. 2010).

Clonal evolution demonstrated by fly genetics reflects the potent influence on human cancers and could represent the molecular link to identify therapeutic targets. Gliomas, the malignant type of mammalian brain tumors, frequently harbor mutations that activate both EGFR and PI3K signaling. In *Drosophila* glia and glial precursors, it was shown that co-activation of EGFR and PI3K deregulates cell cycle regulators, including *strings* (*stg*; a cdc25 homolog) and *cyclinD/Cdk4*, thereby driving neoplasia by promoting cell cycle progression (Read et al. 2009). Likewise, multistep development of colon cancer is illustrated in the adult midgut of *Drosophila* with accumulation of genetic mutations such as *apc* (*adenomatous polyposis coli*) and *Ras*, which causes tumorigenesis (Martorell et al. 2014). An exception is the type 2 diabetes, which is specifically contingent to metabolic changes for malignant transformation, rather than merely adapting sequential mutations (Giovannucci et al. 2010). For instance, clones of mutants for the src inhibitor *csk* (*C-terminal src kinase*) do not overgrow even with Ras^{V12} activation, but when flies bearing $csk^{-/-}$ + Ras^{V12} clones are fed high dietary sucrose, metastatic tumors formed resemble the metabolic defects seen in type 2 diabetes (Hirabayashi et al. 2013; Na et al. 2013). Following this report, a subsequent fly study demonstrated that high-sugar diet activates Slk (salt-inducible kinase) that upregulates Yki-induced Wg signaling, causing insulin receptor expression that persistently retains insulin signaling sensitivity in $csk^{-/-}$ + Ras^{V12} clones (Hirabayashi and Cagan 2015). Thus, aggressive transformation of oncogenic cells may not entirely depend on Darwinian sequential mutations of tumor subclones but may also be driven by extrinsic environment, e.g., in nutrient-rich conditions such as in the case of obesity.

10.5 *Drosophila* Model of Tumor Heterogeneity

While cancers are developed by clonal expansion of oncogenic cells, growing evidence revealed that cancer tissues exhibit heterogeneity of distinct tumor subpopulations (Marusyk et al. 2012). Such diverse tumor populations could mutually cooperate or compete during cancer progression. *Drosophila* genetic mosaic technique provides a model for tumor heterogeneity, as it can induce multiple cell populations with distinct oncogenic mutations within an epithelium. It has been shown that heterogeneity of Ras-activating (Ras^{V12}) clones and *scrib* mutant clones mosaically induced in the eye imaginal epithelium causes Ras^{V12} clones to develop into metastatic tumors. In this process, *scrib* clones propagate JNK signaling to surrounding Ras^{V12} cells, causing these neighbors to secrete Upd and induce metastatic behavior via activation of JAK-STAT signaling (Wu et al. 2010). Similar interclonal oncogenic cooperation can also be triggered by mitochondrial dysfunction. Mutations that disrupt mitochondrial respiratory function were identified as inducers of non-cell autonomous tissue overgrowth in conjunction with Ras^{V12}. $Ras^{V12}/mito^{-/-}$ (Ras activation with mitochondrial dysfunction) clones produce reactive oxygen species (ROS) that activates JNK signaling, which cooperates with Ras^{V12} to activate Yki, and thereby upregulate Upd and Wg. Thus, heterogeneity of $Ras^{V12}/mito^{-/-}$ and Ras^{V12} clones mosaically induced in the imaginal epithelium, which mimics human cancers with frequent mitochondrial dysfunction, causes Ras^{V12} clones to develop into metastatic tumors (Fig. 10.4) (Ohsawa et al. 2012). Further study showed that $Ras^{V12}/mito^{-/-}$ cells undergo cell cycle arrest through p53-dependent cellular senescence (Fig. 10.4) (Nakamura et al. 2014). Senescent cells highly express secreted growth factors and inflammatory cytokines, a conserved phenome-

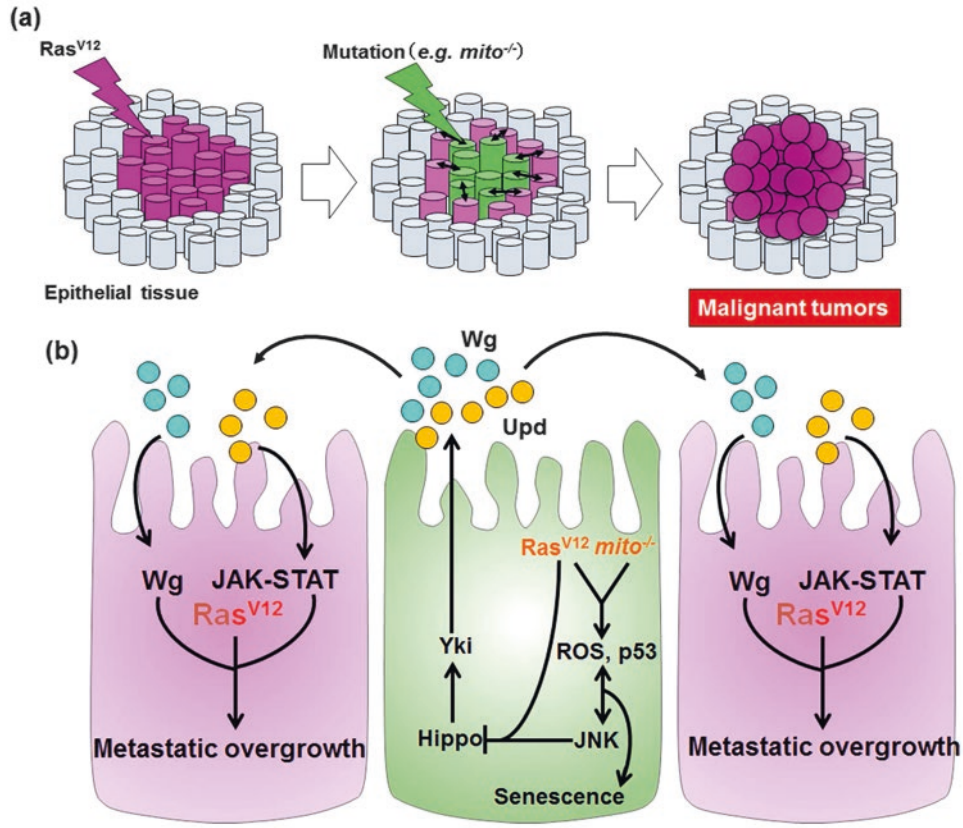

Fig. 10.4 Tumor heterogeneity that drives tumor progression via cell-cell communication
(**a**) General scheme illustrating *Ras*^{V12} cells achieve malignancy and invasiveness non-autonomously when a subset of the *Ras*^{V12} cells acquire a second mutation, e.g., *mito*$^{-/-}$ (mitochondrial dysfunction). (**b**) *Ras*^{V12}/*mito*$^{-/-}$ cells activate JNK signaling by processes such as ROS generation and p53 activation, resulting in cellular senescence. *Ras*^{V12}/*mito*$^{-/-}$ cells induce non-cell autonomous overgrowth of neighboring *Ras*^{V12} cells through JNK-mediated secretion of Upd and Wg ligands. This causes neighboring *Ras*^{V12} cells to activate JAK-STAT and Wg pathways and hence induce metastatic overgrowth

non called senescence-associated secretory phenotype (SASP) (Coppé et al. 2010), inadvertently causing non-cell autonomous proliferation of malignant tumors. Ras-activating clones were also shown to stimulate exocytosis of the ligand Eiger, which in turn promote JNK signaling in neighboring cells. As a response, JNK signaling in wild-type cells induces the expression of ligand Upd to activate JAK-STAT signaling in *Ras*^{V12} clones non-cell autonomously (Chabu and Xu 2014). Interestingly, it has been shown that Eiger-JNK-mediated activation of Upd-JAK-STAT signaling in *scrib*$^{-/-}$ + *Ras*^{V12} clones trigger non-autonomous autophagy in their surrounding cells, which promote *scrib*$^{-/-}$ + *Ras*^{V12} tumor growth by providing amino acids to tumors (Katheder et al. 2017). A similar phenomenon was found in mammals where pancreatic ductal adenocarcinoma triggered nonautonomous autophagy in pancreatic stellate cells, thereby generating alanine and fueling tumor growth (Sousa et al. 2016). Thus, these studies in *Drosophila* may open new avenues for understanding and manipulating cancers driven by tumor heterogeneity.

10.6 Cancer Cachexia in *Drosophila*

Local perturbation of tissue or organ by tumors cannot entirely explain how and why cancers effectuate mortality of the host. Growing evidence emphasizes that cancer lethality can also be driven by distant tumor-host interactions, such as cachexia (Tisdale 2002; Fearon et al. 2012). Cancer cachexia refers to a complex metabolic state that promotes irreversible, progressive tissue wasting in cancer patients. *Drosophila* has recently emerged as a model to study mechanisms underlying cancer cachexia by allograft method, which is performed by transplantation of tumors into an adult host (Rossi and Gonzalez 2015). The allograft method has vast advantages when it comes to characterizing an overgrowth tissue as malignant, whereby limitless time of tumor expansion is allowed, and visualization of secondary tumor growth in metastatic tissues and long-range interactions like cachexia can be attained. In the context of cachexia, two independent fly studies using the allograft method reported that cancer cachexia systematically drives peripheral organ wasting by reinforcing insulin resistance in distant tissues. Tumors such as $scrib^{-/-}$ + Ras^{V12} or Yki-activated clones secrete ImpL2 (insulin growth factor-binding protein; IGFBP), an antagonist of insulin signaling, which inhibits insulin signaling in peripheral tissues including the ovaries, fat bodies, and muscles in host animals bearing tumors (Figueroa-Clarevega and Bilder 2015; Kwon et al. 2015). These studies demonstrated in *Drosophila* conveniently illustrate multiple aspects of cancers, which can be dissected by genetics.

10.7 Tumor Microenvironment in *Drosophila*

In epithelial tissues with tumors, tumor cells not only communicate among themselves but also with other cell types, such as immune cells and mesenchymal cells (Fig. 10.5). Different types of interacting cells around the tumor together with the extracellular matrix (ECM) components make up the tumor microenvironment that is crucial for cancer initiation, progression, and even metastasis. In concert, distinct cellular players around the tumor influence cancer growth by providing a conducive microenvironment to communicate via various means, including cell-cell junction molecules, receptors, hormones, and other soluble factors (Bissell and Hines 2011). In *Drosophila*, upon basement membrane disruption by malignant $scrib^{-/-}$ + Ras^{V12} tumors, hemocytes (*Drosophila* hematopoietic cells) are recruited to the tumor-bearing damaged tissue, along with JNK activation and expression of Upd (Pastor-Pareja et al. 2008). This indicates that flies possess a similar system to tumor-associated macrophage (TAM) recruited to human cancers, forming a tumor microenvironment. However, the mechanism of how hemocytes drive tumor progression remains controversial. It was shown that hemocytes induce JNK activation in malignant tumors in the imaginal epithelium by secreting Eiger (Cordero et al. 2010). In contrast, it was reported that JNK is activated in epithelial malignant tumors when tumor tissues are transplanted into flies lacking hemocytes (Muzzopappa et al. 2017).

Apart from the hemocytes, in vivo cell-cell interaction between epithelial and mesenchymal cells was also shown to generate tumor-prone microenvironment to induce *Drosophila* neoplasm. In the larval wing imaginal disc, epithelial cells in the notum associate with myoblast that will form myofibrils (flight muscles) in dorsal thorax. It was shown that two miRNAs, *miR-10* and *miR-375*, transform EGFR-activated epithelial cells into neoplastic tumors via epithelial-mesenchymal interaction in the wing disc. Mechanistically, these miRNAs target a PcG gene *pipsqueak* (*psq*), thereby increasing the level of secreted proteoglycan perlecan. High level of perlecan enhances EGFR-induced decapentaplegic (Dpp/BMP) signaling to expand the size of mesenchymal populations, thus promoting tumor growth (Herranz et al. 2014). This shows that an oncogenic positive feedback loop between epithelial tumors and mesenchymal cells is present in *Drosophila*.

It was also shown in *Drosophila* that intrinsic tissue structures also influence the onset of epi-

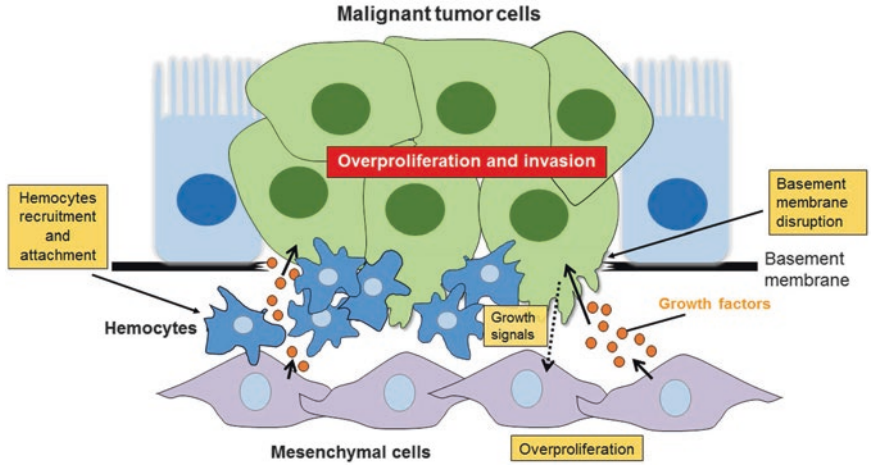

Fig. 10.5 Tumor microenvironment generated by epithelial tumors, mesenchymal cells, and hemocytes in *Drosophila*
As epithelial cells acquire invasive behavior due to mutations, e.g., $scrib^{-/-}$ + Ras^{V12} and subsequently disrupt the basement membrane, hemocytes are recruited to the damaged sites as a result of an immune response. Mutations that cause deregulation of cellular processes, e.g., increased perlecan in EFGR-activating epithelial mutants, can mediate the secretion of growth factors by surrounding mesenchymal cells to jointly promote proliferation of tumors. Epithelial tumors can also cooperatively induce proliferation of mesenchymal cells via growth-related signaling, e.g., TGF-β/Dpp signaling

thelial tumors. At certain "tumor hotspots" in the wing imaginal epithelium, *scrib* or *lgl* mutant cells display robust basal structures but lack apical enrichment of microtubules (cytoskeletal structure important for epithelial structure maintenance), thereby forcing pro-tumor cells to delaminate from apical side. Apical delamination is shown to be caused by deregulated RhoGEF2 activity and increased JAK/STAT activation to promote tumorigenesis. Conversely, "tumor coldspots" are characterized by their loosely organized ECM laminae with straight basal epithelial layer, which prevents *scrib* or *lgl* cells in these "coldspots" from delamination, and thus are eliminated by cell competition (Tamori et al. 2016). These findings suggest that structural integrity or endogenous nature of the epithelial tissue within tumor microenvironment plays a crucial role in cancer development. In summary, multiple interactions either within (i.e., communication between mutant vs. wild-type cells) or around a tumor (i.e., tumor microenvironment) involve distinct cellular players and specific epithelial architecture, so as to act in concert to initiate cancer progression in *Drosophila* model.

10.8 Anti-cancer Therapy in *Drosophila*

Drawing from the findings obtained from *Drosophila* cancer models described above, advancement in genetic manipulation tools has produced significant insights into mechanisms and characteristics of cancers and how these relate to mammalian systems. To date, huge efforts have been made to exploit the fly model further for the discovery of anti-cancer therapies and also drug screening strategies. Main advantages of using *Drosophila* system for drug discovery are its high-throughput and cost-effective whole organism-based screening of therapeutic agents, simple genetic manipulation and huge selection of tools, established annotations of the fly genome, and striking conservation between flies and humans (Gao et al. 2014). An important aspect in cancer research is the development of effective anti-cancer drugs that not only specifically target cancer processes but also cause minimal toxic side effects to non-tumor cells. However, drug discovery approaches are shifting from single-target anticancer drugs to multi-target drugs, termed polypharmacology, wherein

more effective drugs modulating multiple targets could offer higher efficacy in treating complex diseases such as cancer. Recent research trials combining fly genetics and chemical biology gained popularity as a screening platform in developing new generation of chemical compounds with systems polypharmacology. An example is a study of a germline mutation in *Ret* receptor-type tyrosine kinase that is commonly found in multiple endocrine neoplasia type 2 (MEN2) patients having medullary thyroid carcinoma. Flies overexpressing oncogenic $dRet^{M1007T}$ (mimicking gain-of-function *Ret* mutation for MEN2B) in the eyes exhibit "rough-eye" phenotype due to tumor overgrowth (Read et al. 2005). Using this fly MEN2 model, a kinase inhibitor drug vandetanib (ZD6474) was found to effectively suppress the "rough-eye" phenotype in a dose-dependent manner (Vidal et al. 2005). Notably, the effect of vandetanib has already been assessed in clinical trials and approved as the first-line drug for the treatment of medullary thyroid carcinoma (Thornton et al. 2012). In a follow-up study using the same fly model, AD57 was identified as the optimal polypharmacological drug that exerts maximal therapeutic effect and low toxicity profile for suppressing thyroid tumors (Dar et al. 2012).

Apart from the MEN2 model, two research groups explored the fly system to generate multigenic models of colorectal cancer whereby key features of its progression involve sequential mutations in *apc*, *Ras*, *p53*, *TGF-β*, and *pten* genes (Bangi et al. 2016; Martorell et al. 2014). In *Drosophila* hindgut, multigenic combination of *apc/ras/p53/pten* recapitulates characteristics of colon cancer such as hyperproliferation, disrupted tissue architecture, cell migration, and evasion of cell death. Interestingly, this multigenic fly model was reported to exhibit resistance toward PI3K/TOR inhibitor BEZ235, a drug currently in clinical trials for the treatment of advanced solid tumors. Although this compound is deemed less effective when used independently, they found that combinational treatment of the fly colorectal cancer model with BEZ235 together with SC79 (an Akt activator) or bortezomib (a TORC1 activator) promotes sensitivity to PI3K pathway inhibition and thus was able to successfully suppress tumor expansion and dissemination (Bangi et al. 2016). This combinational therapy was further proven in the study to be effective in allografts of cultured human tumor cells and mouse models of colorectal cancer (Bangi et al. 2016).

Using fly genetic-chemical screening approach, more anticancer agents for human cancers were identified or designed, which include a combination of trametinib and statin for lung cancer (Levine and Cagan 2016) and a glutamine analogue acivicin for solid tumors (Levine and Cagan 2016; Levinson and Cagan 2016; Willoughby et al. 2013). A large-scale drug screen using the adult fly revealed an unexpected side effect caused by drug administration. It was found that 14 out of 88 FDA-approved chemotherapy drugs have dual properties in suppressing tumor growth of Raf-activating intestinal stem cells (ISCs) and additionally cause overgrowth of wild-type ISCs when the tumors are transplanted into the abdomen of wild-type hosts (Markstein et al. 2014). This side effect was reported to be caused by Upd3 (an isoform of Upd) induction in enterocytes (ECs) as a consequence of drug administration (Kwon et al. 2015). Interestingly, ECs undergoing environmental stresses (e.g., detachment from the basement membrane) activate JNK and Yki and thus subsequently upregulate Upd3 that promotes ISC tumor growth (Patel et al. 2015). This suggests that tumor recurrence could be caused by detachment of ECs from visceral muscle as a result of side effect from drugs. Thus, recent findings through systematic anticancer drug screens in *Drosophila* have opened new avenues toward drug discovery and cancer modeling to be extrapolated to human cancers and hence further supported the fly system as a robust whole-animal approach against cancer (Table 10.1).

10.9 Future Perspectives

Drosophila cancer models derived from various genetic techniques have made important contributions to our understanding toward fundamental

Table 10.1 Drugs discovered or designed by *Drosophila* cancer models

Drugs	Type	Molecular targets/pathway/ mechanism	Target human cancer	Fly model	References
Vandetanib ZD6474	Kinase inhibitor	Vascular endothelial growth factor receptor, EGFR, and Ret kinase	Medullary thyroid carcinoma	MEN2 model with $dRet^{M1007T}$mutation	Vidal et al. (2005)
AD57	Kinase inhibitor	Ret, Raf, Src, Tor, and S6K kinases	Medullary thyroid carcinoma	MEN2 model with $dRet^{M1007T}$mutation	Dar et al. (2012)
BEZ235 + SC79 or Bortezomib	Kinase inhibitor (BEZ235), transcriptional activator (SC79 and bortezomib)	PI3K/TOR, Akt, and TORC1	Colorectal cancer	Colorectal cancer model with combinatorial *apc*, *Ras*, *p53*, and *pten* mutation	Bangi et al. (2016)
Trametinib + fluvastatin	Kinase inhibitor (trametinib), reductase inhibitor (fluvastatin)	MEK, HMG-CoA reductase, and Ras and PI3K pathway	Non-small cell lung cancer	Lung cancer model with $Ras1^{G12V}$ and $Ras1^{G12V}$ $PTEN^{-/-}$- mutation	Levine and Cagan (2016)
Acivicin	Glutamine analogue	Gamma-glutamyltransferase	Solid tumor	Ras and Notch model with Ras^{V12} and N^{intra} model	Willoughby et al. (2013)

cancer processes in humans. Drawing from *Drosophila*'s many historical contributions, some of the important tumor suppressor genes were discovered in flies. For instance, the Hippo pathway originally discovered in flies was later shown to be conserved in vertebrates, and its dysregulation has been implicated in human cancer development. Notably, the mechanistic link between loss of cell polarity and JNK, JAK-STAT, Hippo, and other intracellular signaling pathways extensively studied in flies is proven to be intimately involved with human tumors of epithelial origin. In addition, the use of *Drosophila* mosaic techniques uncovered a conceptual insight of tumor social biology whereby oncogenic cells establish various forms of interactions with distinct cellular players non-cell autonomously. Recent modifications to the genetic mosaic tools including the coupled-MARCM technique combining two independent expression systems (Gal4/UAS and QF/QUAS) (Potter and Luo 2011) enable researchers to study cell-cell and inter-organ communication in vivo during cancer initiation, progression, and metastasis. Nonetheless, topics covered in this chapter represent only a subset of many excellent findings unveiled from the fly field, thereby a broader grasp of more aspects in fly cancer research is deeply encouraged. Lastly, fresh perspective toward identification of novel therapeutics using fly cancer models shows promising discovery for the past few years and thus further proves *Drosophila* as a robust system with great potentials in cancer research and even anti-cancer therapy.

10.10 Commonly Used Protocol: Studying Tumor Progression In Vivo

In this section, we describe a detailed protocol to analyze invasion of oncogenic subpopulations in living tissues of *Drosophila* larva using genetic mosaic technique through eyFLP mosaic analysis with repressible cell marker (MARCM). To explain the practicality of genetic mosaic tools, the principles underlying the MARCM technique will be discussed in particular. Induction of mosaics specifically in the eye-antennal imaginal disc (EAD) of *Drosophila* larva allows for visualization and quantification of tumor invasiveness in the ventral nerve cord (VNC) complex situated posterior to the EAD structure. Here, we also illustrate the procedures on how to dissect the cephalic complex and then further process them for staining and imaging of positively labeled transgenic clones. Lastly, we define a simple quantification method to count tumor invasiveness by visualization of reporter expression spreading into the VNC. In summary, the techniques described are widely used as a robust experimental system in fly cancer studies especially in the context of tumor development, progression, and metastasis. Moreover, inducing mosaics as patches of oncogenic clones surrounded by wild-type cells enables for the study of social tumor biology including cell-cell competition and cooperation and even non-cell autonomous aspect of cancer.

Mosaic analysis developed in *Drosophila* is used extensively in the study of tumor growth and progression. Recent years have seen significant improvement and modifications to the existing genetic tools, among these include the MARCM technique (Wu and Luo 2007). MARCM induces genetic mosaics wherein coexisting cells of distinct genotypes can be produced and marked with fluorescence in a living organism. This technique combines the traditional FLP/FRT (flippase/flippase recognition target) system with Gal4/UAS (upstream activator sequence), and a FLP-out mechanism to control inducible transgene expression governed by the UAS system (detailed principle underlying the MARCM technique and mitotic recombination steps is shown in Protocol I(a)). As a result, wild-type and heterozygous cells are unmarked, but homozygous mutant clones are positively labeled with fluorescence and thus easily tracked using imaging tools. The ability to generate clones through mitotic recombination gives unparalleled benefits in cancer studies especially in modeling tumor development and progression in vivo. By inducing mosaic clones in a tissue, we can understand many aspects of social tumor biology as oncogenic cells are positioned in a population of

cells surrounded by wild-type cells (or by oncogenic cells with other mutations) and immune cells, alongside the extracellular matrix (ECM) exposed to various soluble factors in a tumor microenvironment (Bissell and Hines 2011). Substantial awareness in cancer's etiology is raised as more evidence showed that cancers are developed by sequentially acquiring oncogenic mutations and by establishing intimate interactions among cellular players (refer to Protocol II for an example of Ras^{V12} + $scrib^{-/-}$ mosaic analysis). Given the feasibility of this technique in modeling multiple mutations in a tissue, MARCM is used to study various cancer processes like tumor metastasis (Pagliarini and Xu 2003). Here, we describe a protocol for mosaics induced by MARCM in the EAD of *Drosophila* larva to study tumor growth and metastasis with detailed procedures and explanation.

10.11 Materials

1. *Equipment*

Forceps (e.g., Dumont No. 5), microscissors, nail polish, petri dish with fine pin attached, stereomicroscope, fluorescence confocal microscope, and microscope slides and 18×18 mm coverslips.

2. *Solutions and Reagents*

Phosphate-buffered saline (PBS), PBS-T (PBS with 0.1% Triton X-100), 4% paraformaldehyde (PFA), and DAPI and mounting medium (e.g., SlowFade® Gold Antifade Mountant with DAPI (Thermo Fisher Scientific. Co.)).

3. *Methods*

3.1. Mosaic Clone Generation

To generate genetic mosaics specifically in the larval EAD, choose an appropriate eyFLP-MARCM tester line carrying *Gal4*, a reporter gene, e.g., *GFP*, and *Gal80* to mark mitotic clones. Transgene of interest should be designed under the control of a *UAS*, which should in most cases be inserted on a different chromosome arm from the *FRT* site. Refer to Protocol I(a) for an illustrative design of MARCM experiment.

(i) Collect virgin females from MARCM tester lines, and cross them with *UAS*-transgene or mutant males with FRT site.

(ii) Incubate crosses at 25 °C for 5–7 days after egg deposition (AED) to obtain third instar larvae. Temperature is important for optimal activity of the Gal4/UAS system.

Note: Development of larvae can be delayed or completely voided due the expression of inserted transgenes.

3.2. Dissection of Larvae

(i) Collect wandering third instar larvae of desired phenotype (e.g., fluorescently labeled EAD or morphology indicating the presence of transgene copy) using forceps, and transfer them into a petri dish filled with PBS.

Note: Larvae carrying random fluorescent spots throughout the body may exist due to non-specific expression of *FLP*-driven UAS transgenes (mostly in gonads). Exclude these larvae from the analysis.

(ii) Under a stereomicroscope, cut the posterior end of the larval body near the genitalia using a microscissors to expose the inner parts of the body.

(iii) Using forceps, gently hold the middle part of the larval body, and flip the larva inside out to expose the inner body parts. (This can be done by using a narrow pin head attached to the bottom of a petri dish. Push the head unto the attached pin by rolling the cuticle over the pin head to flip the larva inside out.)

(iv) Carefully remove unwanted tissues including the salivary glands, fat body, and intestines. This preparation leaves the cephalic complex attached to the mouth hooks on the cuticle for ease of further manipulation procedures.

(v) Transfer the EAD-brain complex into microcentrifuge tubes filled with PBS, and keep them on ice until further steps.

3.3. Fixing, Staining, and Mounting

(i) For fixing the tissues, remove PBS solution from the microcentrifuge tubes containing the cephalic complex, and add 4% PFA to fix samples. Incubate mixture on ice for 5 min and in room temperature for 20 min.

(ii) Before staining procedures, wash the tissues thoroughly with sufficient PBS-T (about 0.5–1 mL) 3 times for 20 min.

(iii) For DAPI staining, replace PBS-T with appropriate DAPI solution (e.g., 1–2 drops of SlowFade® Gold Antifade Mountant with DAPI), and incubate overnight at 4 °C

Note: Incubation times are not definitive and can be modified according to specific needs and optimization.

(iv) For mounting, place the cephalic complexes attached to the cuticle on a microscope slide with sufficient mounting medium for final step of dissection.

(v) Separate EAD-brain complex from the cuticle by pinching the end of the mouth hook with a pair of forceps. For analysis of invasion, EAD pairs can be further separated from the brain-VNC complex for ease of imaging.

(vi) Place a coverslip over tissue preparations without formation of bubbles. Finish the mount by sealing the edges with conventional nail polish. Store slides in 4 °C or less until imaging.

3.4. Confocal Imaging

(i) Acquire images using a confocal fluorescence microscope with 10X or 20X dry objective lens and appropriate laser excitation/absorption wavelengths (e.g., conventional GFP fluorescence profile is about 480/510 nm). For example of EAD mosaic images, see Protocol I(b).

(ii) Z-stacks can be imaged to ensure the visualized clone sizes are a general representation of the whole tissue section.

Note: During image acquisition, use the same parameters for all genotypes to enable comparable quantification and visualization of clones overgrowth or undergrowth. Settings to note include image resolution, pinhole size, scan speed/averaging, gain/offset, and also pixel saturation.

3.5. Analyses of Invasion

(i) Noninvasive cell clones can be seen in the EAD/optic lobe region of the brain, but cannot be observed in other tissues such as VNC. If fluorescently labeled mutant cells are seen in the VNC for most of the living larvae in a given population, these mutant cells have acquired invasiveness, and thus the mutant flies die before adulthood (see Protocol II). A simple scoring system can be used to quantify the percentage of invasiveness.

(ii) Score the number of positively labeled VNC per genotype for all larvae collected from vials. Calculate statistical significance using appropriate tests, e.g., Mann-Whitney test.

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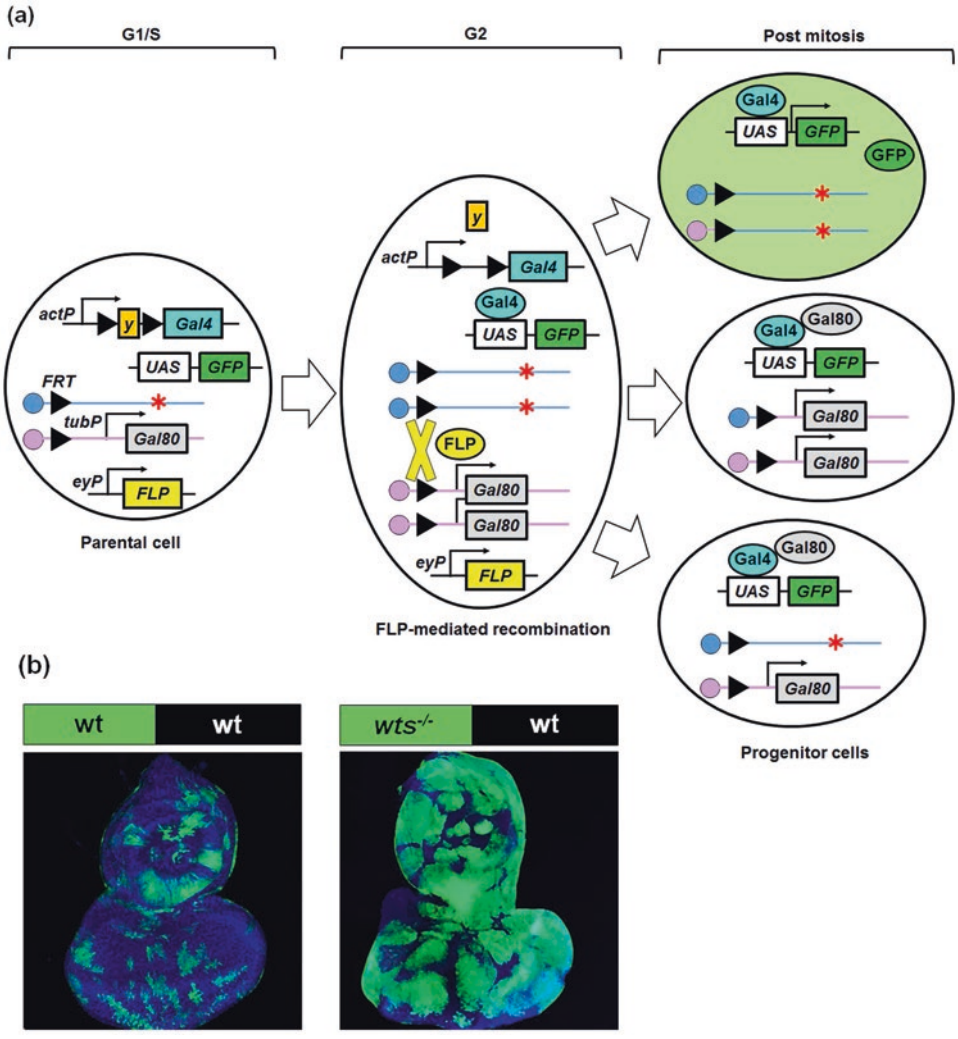
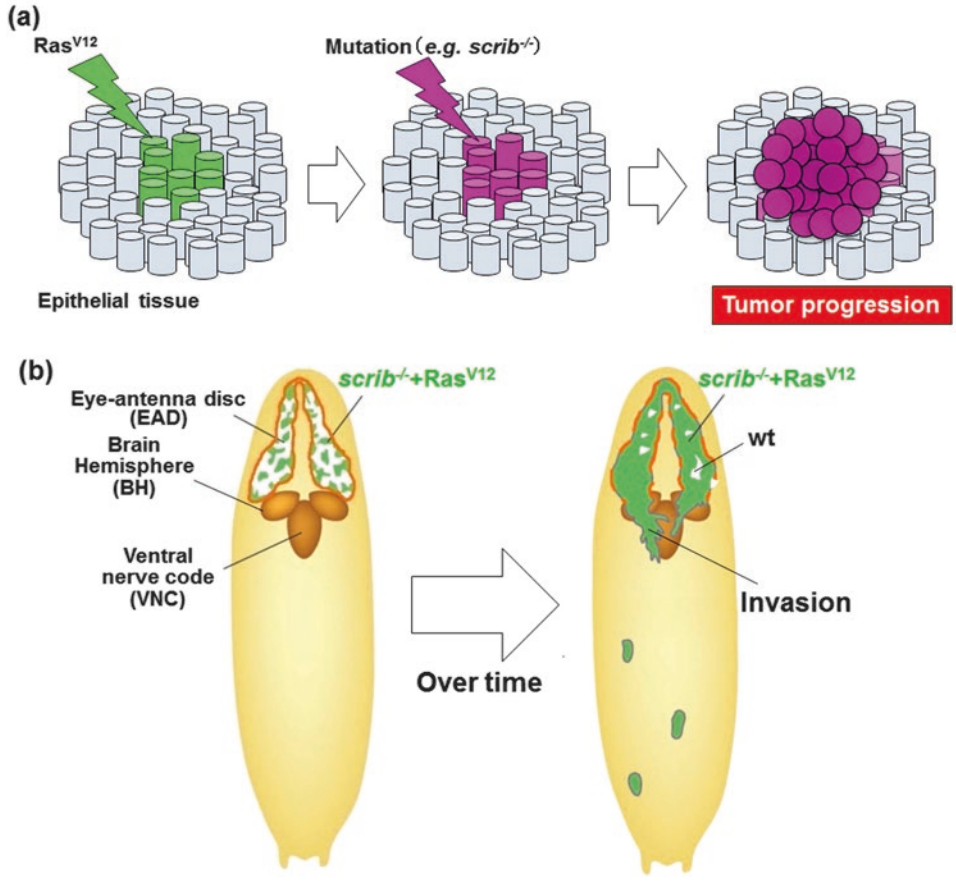
Commonly Used Protocol

Protocol I MARCM technique used in *Drosophila* cancer models. (*a*) The MARCM technique allows expression of target transgenes coupled with GFP specifically in clones. The parental cell contains chromosomes with homologous *FRT* sequence located at the same position, heterozygous *Gal80* controlled by ubiquitous promoter *tubP* located at a distal site from *FRT*, heterozygous mutation (denoted as *) distal to *FRT* but in trans to *Gal80* chromosome, and *FLP* sequence controlled by *eyP* specific to the eye-antennal imaginal discs (EAD). The Gal4/UAS system is governed by ubiquitous promoter *actP* with a *y* spacer tagged downstream with a *UAS-GFP* marker sequence. The *y* spacer includes a transcriptional stop codon so that prior to activation of the FLP recombinase (and subsequent FLP-out), the gene downstream (*UAS-GFP*) of the spacer is not transcribed. After DNA replication, FLP expressed specifically in the EAD mediates mitotic recombination at FRT sites (arrows) and concurrently allows FLP-out of the *y* spacer. Three types of distinct progeny (as mosaics in an EAD tissue) can be produced after mitosis and cell division, in which cells with one/two copies of *Gal80* are unlabeled as wild type, while cells without Gal80 is homozygous for the mutation and are labeled with GFP fluorescence. Fluorescently labeled transgenic cells are a result of the loss of GAL80 repression on GAL4, thus allowing GAL4 to drive expression of any other *UAS* transgenes. (**b**) An example of a confocal image showing wt//wt or $wts^{-/-}$//wt EAD mosaics generated by MARCM. Cell nuclei are stained with DAPI (blue). DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein; *actP*, *actin promoter*; *tubP*, *tubulin promoter*; *eyP*, *eyeless promoter*; FLP, flippase; FRT, flippase recognition target; wt, wild type.





Protocol II The process of malignant transformation and *Drosophila* model of tumor progression. (*a*) General scheme illustrating autonomous malignant transformation of Ras^{V12} mutant subclones in a tissue after acquiring sequential mutation, e.g., $scrib^{-/-}$. (**b**) Schematic drawing showing an EAD mosaic in a $scrib^{-/-}$ + Ras^{V12} mutant larva generated by eyFLP-MARCM technique. Green spots depicted are GFP-labeled patches of $scrib^{-/-}$ + Ras^{V12} mutant clones surrounded by unlabeled wild-type cells in the EAD attached to the brain-VNC complex. Over time, $scrib^{-/-}$ + Ras^{V12} mutant cells acquire malignant behavior and invade to adjacent ventral nerve code (VNC). Overgrowth of $scrib^{-/-}$ + Ras^{V12} clones outcompetes wt subclones, as shown by an increase in GFP-labeled mutants and a decrease in unlabeled wt cells.

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From *Drosophila* Blood Cells to Human Leukemia

11

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Abstract

The hematopoietic system plays a critical role in establishing the proper response against invading pathogens or in removing cancerous cells. Furthermore, deregulations of the hematopoietic differentiation program are at the origin of numerous diseases including leukemia. Importantly, many aspects of blood cell development have been conserved from human to *Drosophila*. Hence, *Drosophila* has emerged as a potent genetic model to study blood cell development and leukemia in vivo. In this chapter, we give a brief overview of the *Drosophila* hematopoietic system, and we provide a protocol for the dissection and the immunostaining of the larval lymph gland, the most studied hematopoietic organ in *Drosophila*. We then focus on the various paradigms that have been used in fly to investigate how conserved genes implicated in leukemogenesis control blood cell development. Specific examples of *Drosophila* models for leukemia are presented, with particular attention to the most translational ones. Finally, we discuss some limitations and potential improvements of *Drosophila* models for studying blood cell cancer.

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11.1 Introduction

Cells of the hematopoietic system are essential for maintaining the homeostasis of the organism, notably by participating in the immune response, removing apoptotic or cancerous cells, and producing various cytokines or clotting factors (Provan and Gribben 2010). Nonetheless, these cells have both protective and pathogenic functions in antimicrobial defense, autoimmune diseases, inflammatory reaction, metabolic disorders, or tumorigenesis. Hence, their development and function have to be tightly regulated. Accordingly mutations affecting blood cell development can lead to various hemopathies including leukemia. This heterogeneous class of malignancies affecting the hematopoietic lineages represents ±3% of all classes of cancers. It is characterized by the presence in the bone marrow and in peripheral tissues of misdifferentiated blood cells with proliferative and/or survival advantage that eventually outnumber normal blood cells, leading to deadly illnesses. The emergence of a leukemic clone is usually associated with the stepwise accumulation of a limited number of genetic mutations in hematopoietic stem or progenitor cells (Ferrando and Lopez-Otin 2017).

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The identification of the mutated genes and the characterization of their mode(s) of action remain an important issue to decipher the mechanisms of blood cell transformation and develop new therapies.

The development of animal models, in particular mouse, has been instrumental in characterizing how hematopoietic cell fate and function are controlled in vivo under normal and pathological conditions (Kohnken et al. 2017). In addition, it has become clear that researches in *Drosophila melanogaster* can also provide relevant information to gain insights into these processes. Indeed works from several labs over the last 20 years have revealed that the molecular pathways controlling blood cell production and function are highly conserved from human to *Drosophila*. Notably, several key transcription factors and signaling pathways implicated in normal and malignant blood cell development in human control hematopoiesis in fly too. Hence, thanks to the outstanding genetic toolbox available in *Drosophila* and to the development of more and more sophisticated markers and assays to characterize *Drosophila* blood cell status and functions, this organism can serve as a valuable model to investigate various aspects of blood cell biology relevant to cancer. Here, we will focus on the use of *Drosophila* to study leukemogenesis. First, we provide a rapid survey of the development of the *Drosophila* hematopoietic system, together with a protocol to assess blood cell status in the lymph gland, a well-described larval hematopoietic organ. Then, we present the three main approaches that have been developed to gain insights into leukemogenesis using *Drosophila*: (1) the study of the so-called melanotic tumors, which can arise from leukemic-like processes, (2) the expression in non-hematopoietic cell types of oncogenic variants of genes participating in blood cell transformation in human, and (3) the study of these oncogenic variants or their homologues in the *Drosophila* hematopoietic system. We present specific examples showing how these various strategies have shed light on blood cell transformation and/or helped tackle the mechanisms of action of specific proteins implicated in leukemia in humans. Finally, we present some possible directions to improve the use of *Drosophila* in leukemia research.

11.2 *Drosophila* Hematopoiesis

As the development and regulation of the *Drosophila* hematopoietic system have been covered extensively in several recent reviews (Gold and Bruckner 2015; Letourneau et al. 2016; El Chamy et al. 2017; Yu et al. 2017), we only provide here a description of its salient features, and we refer interested readers to the aforementioned reviews for further details.

11.2.1 Development of the *Drosophila* Hematopoietic System

Reminiscent of the situation in vertebrates, *Drosophila* hematopoiesis takes place in successive waves (Holz et al. 2003). First, in the early embryo, a pool of pluripotent blood cell progenitors (called prohemocytes) is specified in the head mesoderm and gives rise to peripheral blood cells, which populate the body cavity (hemocoel) of the larva (Makhijani et al. 2011). A second population of prohemocytes arises later during embryonic development from the lateral/cardiac mesoderm, which generates a specialized larval hematopoietic organ called the lymph gland (Mandal et al. 2004; Jung et al. 2005). Under normal conditions, blood cells produced in the lymph gland are released into the hemolymph only at the end of larval life (Honti et al. 2010; Grigorian et al. 2011). In the adult fly, blood cells generated during these two distinct waves of hematopoiesis are present, with limited blood cell proliferation or differentiation (Holz et al. 2003; Honti et al. 2014; Ghosh et al. 2015). Overall, there are ± 700 hemocytes in late embryos (Tepass et al. 1994), while third instar larvae contain ±8000 peripheral hemocytes (Lanot et al. 2001, Petraki et al. 2015) and 4000–8000 lymph gland hemocytes (Krzemien et al. 2010). The number of blood cells in the adult is difficult to assess but is estimated to ±2000 cells

(Lanot et al. 2001), declining with age (Mackenzie et al. 2011; Horn et al. 2014).

It is worth reminding that beside the larval and adult heart tube, which is open at both ends, *Drosophila* has no proper vascular network (Hartenstein and Mandal 2006), and blood cells travel freely within this open circulatory system. If most of the peripheral larval hemocytes and adult hemocytes are sessile and form patches of cells under the epidermal wall (Braun et al. 1998; Elrod-Erickson et al. 2000; Markus et al. 2009; Makhijani et al. 2011), significant turnaround has been observed between sessile and circulating hemocytes in the larva (Makhijani et al. 2011). In addition, hemocytes are also associated with other tissues such as the eye imaginal discs (Fogarty et al. 2016), the heart (Elrod-Erickson et al. 2000; Ghosh et al. 2015), the gut (Zaidman-Remy et al. 2012; Ayyaz et al. 2015; Chakrabarti et al. 2016), or the ovaries (Brandt and Schneider 2007; Van De Bor et al. 2015).

11.2.2 *Drosophila* Blood Cell Lineages

As most metazoans, *Drosophila* lacks equivalents of the lymphoid lineages, and its mature blood cells, collectively called hemocytes, can be subdivided into three specialized cell types functionally related to vertebrate myeloid cells: the plasmatocytes, the crystal cells, and the lamellocytes (Parsons and Foley 2016). Plasmatocytes are professional phagocytes and represent the vast majority of the differentiated blood cells (>90%); they are functionally similar to mammalian monocytes, macrophages, and neutrophils (Wood and Martin 2017). They recognize and engulf small pathogens as well as apoptotic cells, and they are a major source of extracellular matrix components, thus playing important functions in the innate cellular immune response but also in tissue remodeling and homeostasis. Plasmatocytes are highly motile cells and constitute a popular model to study the conserved mechanisms regulating cell migration in vivo and by extension to gain insights into metastatic processes (Fauvarque and Williams 2011, Wood and Martin 2017). While plasmatocytes are usually considered as a single entity, populations expressing different subsets of markers have been identified (Jung et al. 2005; Honti et al. 2014). Moreover, two plasmatocyte subpopulations with distinct functions in the adult immune response have been identified (Clark et al. 2011). A better assessment of plasmatocyte heterogeneity is thus certainly needed. Crystal cells are involved in melanization, an insect-specific defense response related to clotting (Whitten and Coates 2017). They are named according to the presence of large paracrystalline inclusions in their cytoplasm, which contain some of the enzymes required for melanin production. Upon wounding, melanization limits fluid loss and participates in the fight against infection notably by trapping microbes and producing microbicidal reactive oxygen species. Finally, lamellocytes are large flat cells (30–60 μm) that are absent in healthy larvae but whose production can be massively induced in response to some stresses and immune challenges such as the infection by parasitoid wasp eggs (Lanot et al. 2001; Eslin et al. 2009) but also in several cancer-related conditions (see below). Together with the plasmatocytes, the lamellocytes adhere to the wasp egg and form a multilayered capsule, which eventually melanizes and kills the intruder. In contrast with plasmatocytes and crystal cells, which are observed in the embryo, the larva, and the adult, lamellocytes are only produced during the larval stages (Honti et al. 2014).

The lineage relationship between the three mature blood cell types and the presence of genuine hematopoietic stem cells are still a matter of debate, which is out of the scope of this chapter. In short, the prevailing view is that blood cell progenitors present in the early embryo and in the lymph gland are transient populations, which do not persist in the larva or in the pupa, respectively (Grigorian et al. 2011; Makhijani et al. 2011; Dey et al. 2016). It is not clear whether the "undifferentiated" blood cells described in the adult are long-lasting, multipotent, and capable of self-renewing (Ghosh et al. 2015). Moreover, *Drosophila* prohemocytes can give rise to plasmatocytes, crystal cells, and lamellocytes

(Krzemien et al. 2010), but larval peripheral plasmatocytes can also proliferate (Makhijani et al. 2011; Anderl et al. 2016) and transdifferentiate into crystal cells (Leitao and Sucena 2015) or lamellocytes (Markus et al. 2009; Avet-Rochex et al. 2010; Stofanko et al. 2010; Anderl et al. 2016). Thus, it seems that the production of the different blood cell types can be achieved by various routes.

11.2.3 Control of *Drosophila* Hematopoiesis

Hematopoietic progenitor maintenance, hemocyte differentiation, and the overall homeostasis of the hematopoietic system are finely tuned by intrinsic factors and by environmental stimuli. These features have been particularly well studied in the larvae. For instance, in the lymph gland, high levels of reactive oxygen species (ROS) (Owusu-Ansah and Banerjee 2009), activation of the wingless signaling pathway (Sinenko et al. 2009), and expression of the EBF transcription factor Collier (Benmimoun et al. 2015; Oyallon et al. 2016) are required in prohemocytes to promote their maintenance. In addition, prohemocyte fate is controlled by local signals from the neighboring heart tube (Morin-Poulard et al. 2016), posterior signaling center (Krzemien et al. 2007; Mandal et al. 2007), and differentiated hemocytes (Mondal et al. 2011; Zhang and Cadigan 2017), as well as by systemic signals released in response to nutrient levels (Benmimoun et al. 2012; Shim et al. 2012) and olfactory stimulations (Shim et al. 2013). Similarly, in peripheral hemocytes, local cues from the peripheral nervous system attract plasmatocytes to subepidermal hematopoietic pockets and promote their survival, their proliferation, and their differentiation into crystal cells (Makhijani et al. 2011, 2017).

The larval hematopoietic system is highly responsive to immune challenges and stresses. The infection of the larva by parasitoid wasp eggs causes lymph gland expansion and premature dispersal, as well as differentiation of lamellocytes from lymph gland progenitors and from peripheral plamatocytes at the expense of crystal cell development (Sorrentino et al. 2002; Crozatier et al. 2004; Markus et al. 2009; Ferguson and Martinez-Agosto 2014; Anderl et al. 2016). Moreover, bacterial infection was recently found to promote blood cell progenitor differentiation in the lymph gland (Khadilkar et al. 2017) and to induce some proliferation in adult hemocytes (Ghosh et al. 2015). Finally, mechanical stress (Petraki et al. 2015), oxygen levels (Mukherjee et al. 2011), nutrition (Benmimoun et al. 2012; Shim et al. 2012), and odors (Shim et al. 2013) can greatly influence larval blood cell homeostasis. By contrast, the development of the embryonic blood cells seems rather stereotypical (Bataille et al. 2005), and it remains to be shown whether it can be influenced by external factors.

11.2.4 Protocol: Immunostaining in the Larval Lymph Gland

The larval lymph gland is currently the most popular system to study hematopoiesis in *Drosophila* (Letourneau et al. 2016). One of its advantages is the presence of a large pool of blood cell progenitors and of all their differentiated progenies within a confined organ from which they are normally not released in circulation until metamorphosis. Therefore, the lymph gland is well suited to study the control of progenitor blood cell fate and to gain insight into the gene networks regulating blood cell homeostasis. In addition, thanks to the effort of many teams, a large set of well-characterized markers and genetic tools are now available to study lymph gland homeostasis and specifically label or manipulate the different cell types present in this complex organ (Evans et al. 2014). Below, we give a brief presentation of the lymph gland organization and a generic protocol that we use to prepare larval lymph glands for immunostaining.

In the larva, the lymph gland is lining the anterior part of the dorsal vessel/cardiac tube, just behind the ring gland and the brain. It is composed of a large pair of anterior lobes followed by 2–4 pairs of posterior lobes. Each lobe is surrounded by a layer of extracellular matrix and separated from its posterior neighbor by a pericardial cell. The lymph gland anterior lobes are specified in the embryo and grow considerably during the larval stages (Jung et al. 2005), with a shift from blood cell progenitor proliferation toward differentiation in the late second larval instar stage (Krzemien et al. 2010). The ontogeny of the posterior lobes is not well characterized, but they are detectable in late first instar larvae. They constitute a large pool of blood cell progenitors that enter differentiation later than those present in the anterior lobes. In mid-third instar larvae (96h after egg laying), the posterior lobes mostly comprise undifferentiated blood cells, while the anterior lobes contain blood cell progenitors in their inner/medullary zone and differentiated hemocytes in their outer/cortical zone (Fig. 11.1). In addition, a small group of ±30 cells located at the posterior tip of each anterior lobe form the so-called posterior signaling center (PSC). The PSC expresses various signaling molecules that regulate blood cell fate, and it exerts a prominent role in the response to infection (Letourneau et al. 2016).

Even in third instar larvae, the lymph glands are small and fragile organs that are tedious to dissect as compared to other tissues such as the imaginal discs. While the initial steps of the protocol described below are relatively straightforward, some practice is necessary to mount properly the lymph glands before observation, especially to keep the posterior lobes intact or for the observation of first/second instar larva lymph glands. Moreover blood cell number and proliferation/differentiation status evolve significantly during larval life, are sensitive to various external stimuli, and show interindividual variations. It is thus essential to work under well-controlled breeding conditions and to analyze a sufficient (minimum ten) number of stage-matched samples to make sure of the significance of any phenotype.

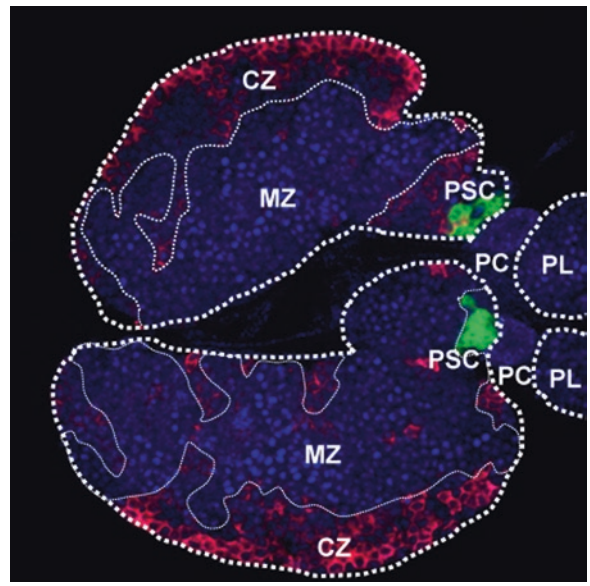

Fig. 11.1 The *Drosophila* larval lymph gland. Confocal image showing the expression of the plasmatocyte marker P1/NimC1 (red), the prohemocyte marker *dome-meso-lacZ* (blue), and the posterior signaling center (PSC) marker *col-GAL4,UAS-GFP* (green). In the anterior lobes, a line demarcates the medullary zone (MZ) from the cortical zone (CZ). PC, pericardial cells. PL, posterior lobes

11.2.4.1 Materials

11.2.4.1.1 Equipment

- Glass dissection dishes (Electron Microscopy Science).
- 6- or 12-well tissue culture plate (Corning).
- Forceps (Fine Science Tools, Dumont #5).
- 1 ml syringes (TERUMO).
- Needles (TERUMO, 0.9*38 mm).
- Microscope slides and 18x18 mm coverslips.
- Stereomicroscope (for dissection).
- Fluorescent microscope (for analysis).
- Transfer pipette (Sterilin).
- 1.5 ml centrifuge tube (Eppendorf).

11.2.4.1.2 Solutions and Reagents

- Sterile phosphate-buffered saline (PBS) (e.g., Dulbecco).
- Bovine serum albumin (BSA).
- 4% formaldehyde (made from 16% stock, Electron Microscopy Sciences) in 1xPBS
- Wash solution: 1xPBS 0.1% Triton X-100 (PBST).
- Permeabilization solution: 1xPBS 0.3% Triton X-100.
- Blocking solution: 1xPBST 1% BSA.
- Primary antibodies and fluorescent-dye conjugated secondary antibodies (e.g., Alexa Fluor).
- DNA staining solutions (e.g., DAPI 5 mg/ml in H_2O, or TO-PRO-3 1mM in DMSO).
- Mounting medium (e.g., Vectashield H-1000, Vector Laboratories).
- Glycerol.
- Optional: Methanol.

11.2.4.2 Methods

11.2.4.2.1 Larvae Collection

1. Set up the appropriate fly cross(es), and transfer the adults to fresh vials every 12 h. Make sure to avoid overcrowding of the larvae.
2. Delicately transfer third instar wandering larvae (or other appropriately staged larvae) from the vials to a dissecting dish or a 6-well plate containing 1xPBS with a pair of forceps or a paintbrush.
3. Using a transfer pipette, wash the larvae with 1xPBS.

11.2.4.2.2 Larvae Dissection

1. Transfer a single larva in a clean dissection dish containing 1xPBS under a stereomicroscope.
2. Orient the anterior part of the larva to the left (for a right-handed person). Using the left hand, clamp the posterior part of the larva (second or third segment from the end) with a pair of forceps. With the right hand, rip the posterior part of the larva with a second pair of forceps.
3. With both pair of forceps, invert the larvae by pushing the mouth hook through the body. Using the left hand, hold the larva anterior part while placing with your right hand a single tine of the forceps in the mouth hooks. Then roll the larvae inside out by gradually pushing the larva on the forceps tine with your left hand. Extend completely the larva so that the internal organs are fully apparent on the outside and the cuticle stretched on the inside. Remove carefully the stretched inverted larva from the forceps tine.
4. Hold the larval carcass with the left-hand forceps, and using the right-hand forceps, unwind and remove the gut, the proventriculus, and as much as possible of the fat body without damaging the dorsal vessel/lymph gland region (which is lined by two dorsal patches of fat body).
5. Transfer the carcass with the brain, the lymph gland, and the heart/aorta in a 1.5 ml microcentrifuge tube containing 1 ml of 1xPBS on ice.
6. Repeat steps 1–5 to prepare as many larvae as needed. *Note: steps 1–6 need to be performed as quickly as possible to limit perturbation of lymph gland homeostasis*. Do not exceed 30 min in total before proceeding to step 7/ fixation.
7. Replace buffer with 1 ml of freshly prepared fixative solution (4% formaldehyde in 1xPBS), and incubate 30 min on a rocking platform at room temperature.
8. Wash the fixed larvae with 1 ml of 1xPBST (1xPBS 0.1% Triton) for 10 min. Repeat twice.

9. Fixed larvae can be stored at 4 °C overnight, but we usually proceed to the immunostaining straight away. For long-term storage, wash the larvae once in 0.5xPBS- 50% methanol and twice in 100% methanol before storing them in 100% methanol at -20 °C. In this case, serial rehydration steps in PBS of the samples will be needed before use. Note however that some epitopes are sensitive to methanol and may not be detected similarly as on freshly prepared tissues.

11.2.4.2.3 Immunostaining

Unless specified, all washes and incubations are performed at room temperature on a rocking platform.

1. Discard 1xPBST, and permeabilize the tissues by adding 1 ml of 1xPBS, 0.3% Triton for 30 min.
2. Discard permeabilization buffer, and wash twice with 1 ml of 1xPBST.
3. Replace wash buffer with 1 ml blocking solution (1xPBST, 1% BSA) for 30 min.
4. Discard blocking solution, and replace with 1xPBST containing the primary antibody diluted at the appropriate concentration. Flip the tube to mix well, and incubate overnight at 4 °C. Note: a minimal volume of 50 µl of diluted primary antibody can be used for 10–15 larvae.
5. Remove primary antibody, and wash with 1 ml of 1xPBST for 10 min; repeat twice.
6. Discard wash buffer, and add 500 µL of 1xPBST containing the secondary antibody diluted at the appropriate concentration (usually 1:1000). Mix well, and incubate for 4 h in the dark. Overnight incubation at 4 °C is also possible.
7. Discard secondary antibody, and wash with 1 ml of 1xPBST for 10 min in the dark; repeat twice.
8. Replace wash buffer with 1 ml of 1xPBST + DNA stain (TO-PRO-3 or DAPI, 1:1000), and incubate 20 min in the dark.
9. Discard DNA stain, and wash rapidly twice with 1 ml of 1xPBST and twice with 1 ml of 1xPBS, and then transfer to 1xPBS 5% glycerol (the presence of glycerol helps to prevent drying out during the final dissection).
10. Store at 4 °C in the dark, or begin the mounting procedure.

11.2.4.2.4 Mounting Lymph Glands for Microscopy

1. Place 10µl of mounting medium on a microscope slide.
2. Using fine forceps, transfer the carcasses next to the drop of mounting medium.
3. Under a stereomicroscope and using syringe needles, carefully separate the lymph glands from the other tissues. The lymph glands are normally still attached to the ring gland and the brain on their anterior side and to the dorsal vessel on their posterior. Separate the ring gland from the brain. You can then use the ring gland or the dorsal vessel to drift the dissected lymph gland into the mounting medium. Align as much as possible the lymph gland lobes along their anterior-posterior axis, anterior to the left and posterior to the right. Proceed similarly with the other larvae. Up to 16 lymph glands can be prepared in 10 µl of mounting medium, but beginners may prefer to split their samples between different slides. Discard the larval carcasses and remaining tissues from the slide.
4. Place the 18 × 18mm coverslip to the left of the lymph glands/drop of mounting medium, and lay it down slowly on the mounting medium to keep the lymph glands well positioned. Note: other sizes of coverslip can be used, in which case the amount of mounting medium needs to be adjusted.
5. Store the slides at 4 °C in the dark until imaging (usually by confocal microscopy).

11.3 *Drosophila* and Leukemogenesis

The presence of hereditary tumors in *Drosophila* was reported one century ago (Stark 1919; Wilson 1924), and the participation of blood cells in these tumors was described more than 60 years ago (Oftedal 1953; Rizki 1960). As we shall see

below, since then, many studies have used *Drosophila* first to identify genes whose deregulation causes these tumors and then to characterize the mode of action of conserved genes mutated in human leukemia or to develop specific models for human leukemogenic proteins.

11.3.1 Melanotic Tumor or *Drosophila* "Leukemia"

Historically, the first strategy to identify and characterize genes regulating blood cell development and potentially involved in leukemia has been to study melanotic tumor formation (Sang and Burnet 1963; Sparrow 1974; Gateff 1978; Gateff 1994; Watson et al. 1994). These melanotic masses, which are easily observable through the cuticle of the larvae (or the adult), are mostly composed of blood cells that have aggregated together or around another tissue and have melanized (Rizki and Rizki 1979, Minakhina and Steward 2006). Their presence is generally associated with increased blood cell numbers, enlarged or precociously ruptured lymph glands, and lamellocyte differentiation. As such, they might represent a model of leukemogenesis in *Drosophila*, and, indeed, several genetic screens using this phenotype as a readout have been performed to unveil new genes controlling blood cell homeostasis. These screens initially relied on classical mutagenic events such as P-element-mediated insertion or EMS mutagenesis (Hanratty and Ryerse 1981; Watson et al. 1991; Torok et al. 1993; Luo et al. 1997; Wu et al. 2001). More recently the UAS/GAL4 system and the advent of genome-wide UAS-RNAi libraries made it possible to target gain or loss of function specifically in the hematopoietic compartment (Zettervall et al. 2004; Stofanko et al. 2008; Avet-Rochex et al. 2010; McNerney et al. 2013). Importantly, enhancers and suppressors of melanotic tumor genes can then be sought in modifier screens (Luo et al. 1995; Shi et al. 2006; Anderson et al. 2017), leading to further characterization of the pathways regulated by these genes (see below). To date, more than 150 genes have been identified that are associated with melanotic tumor formation. These include loss of function mutations in ribosomal proteins (Watson et al. 1992), which could be related to human ribosomopathies that are associated with predisposition to leukemia (Danilova and Gazda 2015), or activating mutation in the Toll/NF-κB pathway (Qiu et al. 1998) that is also constitutively activated and promotes cell survival in a number of hematological malignancies (Gasparini et al. 2014).

However, one important caveat of the "melanotic tumors" is that they can arise from two distinct origins: on the one hand, they can form as a consequence of immune response to damaged tissues, and on the other hand, they can be caused by cell-autonomous deregulation of the hematopoietic program (Wu et al. 2001; Minakhina and Steward 2006; Avet-Rochex et al. 2010; Zang et al. 2015). Hence, their presence often reflects the spurious activation of the lamellocytes rather than a leukemic-like process, and in-depth follow-up studies are necessary to delineate whether the function of these "melanotic tumor genes" is more relevant to tissue homeostasis, immunity, or blood cell cancer.

11.3.2 From Melanotic Tumor to Human Leukemia: The JAK/STAT Pathway

The most notorious example of "melanotic tumor gene" whose study turned out to be highly significant to human leukemia is certainly *hopscotch* (*hop*). *Hop* encodes the *Drosophila* homologue of the JAK kinase, a key component of the eponym JAK/STAT pathway, which mediates signaling from various cytokines (such as Unpaired1, 2, and 3 in *Drosophila* or erythropoietin and granulocyte colony-stimulating factor in human) and their cognate receptors (Amoyel et al. 2014).

The mutation *tumorous-lethal,* an allele of *hop* ($hop^{Tum\text{-}l}$), was identified more than 40 years ago as a recessive lethal temperature-sensitive mutation associated with melanotic tumor formation (Corwin and Hanratty 1976; Hanratty and Ryerse 1981). Importantly, the hypertrophic lymph

glands of $hop^{Tum\text{-}l}$ larvae are neoplastic and can give rise to lethal tumors upon serial transplantations into recipient adult flies (Hanratty and Ryerse 1981, Luo et al. 1995) *(Note: while secondary transplantation is standard in mouse to assess blood cell oncogenic transformation, this experiment is unfortunately seldom used in Drosophila)*. Molecular analyses showed that $hop^{Tum\text{-}l}$ encodes a hyperactive Hop kinase due to the G341E substitution in JAK homology domain 4 (JH4) (Harrison et al. 1995; Luo et al. 1995). Overexpression of $Hop^{Tum\text{-}l}$ in the lymph gland is sufficient to induce lymph gland hypertrophy, lamellocyte differentiation, and melanotic tumor formation (Luo et al. 1995). Likewise, the Hop^{T42} allele, which causes similar phenotypes, also gives rise to a hyperactivated form of Hop due to the E695K substitution in the JH2 domain (Luo et al. 1997). The primary effect of these mutations is to activate in a ligand-independent manner the JAK/STAT pathway by phosphorylating transcription factors of the STAT family, thereby inducing their dimerization and nuclear translocation. Consistent with this idea, decreasing the dosage of the *Drosophila* STAT factor STAT92E in $hop^{Tum\text{-}l}$ larvae is sufficient to reduce melanotic tumor incidence (Hou et al. 1996; Yan et al. 1996; Luo et al. 1997; Shi et al. 2006), whereas the overexpression of an active STAT92E induces melanotic tumor formation (Ekas et al. 2010). These findings thus pointed toward an oncogenic role of the JAK/STAT pathway in leukemia. Strikingly, since 2005, it has been demonstrated that a functionally equivalent activating point mutation in JAK2 (V617F, in the JH2 domain) is one of the most common initiating events in various human myeloproliferative neoplasms (MPN), such as polycythemia vera, essential thrombocytosis, or primary myelofibrosis (Jones et al. 2005; Kralovics et al. 2005; Levine et al. 2005). This mutation accounts for ±70% of MPN, and other point mutations or translocations leading to JAK2 constitutive activation have been identified at lesser frequencies in other hematopoietic malignancies (Kantarcioglu et al. 2015; Vainchenker and Kralovics 2017). Hence, understanding how JAK activation promotes leukemia has become a major issue.

Further studies in *Drosophila* blood cells have brought to light a number of modulators of the JAK/STAT pathway potentially implicated in leukemia. In particular, different genetic screens for second-site modifiers of $hop^{Tum\text{-}l}$-induced melanotic tumor formation have been performed. For instance, using a set of genetic deficiencies uncovering ± 70% of the *Drosophila* autosomes, Shi et al. identified more than 30 genes acting as dominant modifiers of $hop^{Tum\text{-}l}$ -induced melanotic tumor formation in the adult (Shi et al. 2006). Notably, they showed that JAK overactivation promotes proliferation and tumorigenesis by counteracting heterochromatin gene silencing. This effect seems to involve a noncanonical mechanism whereby the unphosphorylated STAT92E is targeted to the heterochromatin by the linker histone H1 and maintains heterochromatin protein 1 (HP1) localization and heterochromatin stabilization (Shi et al. 2006, Xu et al. 2014). A similar link between JAK/STAT and heterochromatin gene silencing has been observed in human: unphosphorylated STAT5 was found to bind HP1α and stabilize heterochromatin (Hu et al. 2013), while JAK2 activation was shown to displace HP1α from the heterochromatin, potentially by directly phosphorylating histone H3 (Dawson et al. 2009). Moreover, JAK2 promotes the survival of primary mediastinal B cell lymphoma and Hodgkin lymphoma cells by promoting heterochromatin formation in cooperation with the histone demethylase JMJD2C (Rui et al. 2010). It is thus possible that heterochromatin alteration is implicated in MPN development, and the role of unphosphorylated STAT in leukemia certainly deserves further investigations.

Thanks to another deficiency screen for modifiers of $hop^{Tum\text{-}l}$, Anderson et al. recently found that the Hippo signaling pathway is activated by Hop and contributes to melanotic tumor development by inducing blood cell proliferation in peripheral larval hemocytes (Anderson et al. 2017). On the other hand, Terriente-Felix et al. showed that JAK-induced hypertrophy of the lymph gland was mediated by the p38 MAPK pathway (Terriente-Felix et al. 2017). It will thus be interesting to test whether these two pathways are activated in MPN and contribute to blood cell neoplasia.

Another modifiers of JAK/STAT overactivation in *Drosophila* blood cells is *abnormal wing disc* (*awd*) (Zinyk et al. 1993), the homologue of the tumor suppressor Nm23. Awd regulates the endocytosis of several receptors including the JAK/STAT pathway receptor Domeless (Dome) (Nallamothu et al. 2008), and some evidence suggests that Nm23 is implicated in leukemia in human (Lilly et al. 2015). Interestingly, Dome is required for Hop-induced lymph gland hypertrophy (Terriente-Felix et al. 2017), which is consistent with ex vivo experiments showing that JAK2V617F requires a cytokine receptor scaffold for its transforming and signaling activities (Lu et al. 2005). In addition, Hop induces a feed-forward loop by activating the expression of Dome ligand Upd3, which also contributes to lymph gland hypertrophy (Terriente-Felix et al. 2017). Along the same line, JAK2 V617F-induced MPN in a mouse model seems to depend on the expression of thrombopoietin and its receptor MPL (which is also subject to activating mutations in some MPN) (Sangkhae et al. 2014). Hence, cytokine receptors may participate in JAK/STAT-induced blood cell proliferation by several mechanisms.

Beside these in vivo studies, it is worth mentioning that *Drosophila* blood cell lines (such as Kc167 or S2 cells) are particularly well suited for genome-wide RNAi screens. Using this approach, two studies identified more than 100 genes regulating JAK/STAT-dependent transactivation of a reporter gene (Baeg et al. 2005; Muller et al. 2005), including *BRDWD3* and *Ptp61F*, which also genetically interacted with $hop^{Tum\text{-}l}$ in vivo (Muller et al. 2005). Besides, transcriptomic profiling in Kc167 cells and in larval tissue led to the identification of JAK/STAT target genes, some of which, like *G protein a 73B*, *chinmo*, or *eukaryotic initiation factor 1A*, contribute to $hop^{Tum\text{-}l}$-induced hematopoietic tumor formation (Myrick and Dearolf 2000; Bina et al. 2010; Flaherty et al. 2010; Bausek and Zeidler 2014). Whether homologues of these genes are implicated in JAK/STAT signaling and leukemia in human certainly warrants further investigation. All together these data illustrate how a variety of approaches in *Drosophila* can highlight multiple levels of regulation and of action of the JAK/STAT pathway relevant to blood cell transformation.

11.3.3 Study of Leukemogenic Proteins in *Drosophila* Non-Hematopoietic Tissues

The development of transgenic or knock-in animal models expressing a human leukemogenic protein has been instrumental to decipher how these proteins interfere with the normal functions of the cells. Of course, mouse remains the prevalent model for such studies (Kohnken et al. 2017), but *Drosophila* offers a cost- and time-effective surrogate to assess in vivo the function(s) and mode(s) of action of human proteins involved in leukemia. While targeting their expression in hematopoietic cells may seem most suitable (see below), "ectopic" expression in unrelated tissue can also present some advantages (e.g., tissue accessibility, previous knowledge of the system, available tools, etc.), and this approach has been used in a few cases in *Drosophila*.

Actually, the first human leukemogenic protein studied in *Drosophila* was BCR-ABL, the product of the notorious Philadelphia chromosome, which is responsible for almost all cases of chronic myeloid leukemia (CML) and some cases of acute lymphoid leukemia (ALL) (Mughal et al. 2016). *BCR-ABL* is generated by a balanced translocation between *c-Abelson (Abl)* on chromosome 9 and the *breakpoint cluster region* (*bcr*) on chromosome 22. Depending on the location of the breakpoint within *bcr*, two main fusion proteins are generated: p210 in most CML and p185 in most ALL. In both proteins, the dimerization domain coded by *bcr* induces the constitutive activation of the tyrosine kinase ABL. To gain insight into the respective mode of action of these two isoforms, Fogerty et al. generated transgenic flies expressing p210 or p185 human/fly chimeras (Fogerty et al. 1999): BCR and the N-terminal ABL were derived from human, whereas the divergent C-terminal tail of ABL was from *Drosophila.* Both p210 and p185 rescued the lethality of *dAbl* mutant flies and activated ABL signaling pathway. Yet, their overex-

pression generated distinct phenotypes and ectopically activated some pathways not employed by ABL (Fogerty et al. 1999; Stevens et al. 2008). Further work using this model may thus help to identify components of the BCR-ABL signaling cascades and the differences underlying the distinct clinical features of p210- and p185-associated leukemia.

A similar strategy was employed to study two different leukemogenic fusions involving *mixed lineage leukemia* (*MLL,* also known as *KMT2A* for *lysine-specific methyltransferase 2A*), which is translocated in 5–10% of patients with acute myeloid or lymphoid leukemia (AML/ALL) (Slany 2016, Yokoyama 2017). *Mll* is the homologue of *Drosophila trithorax*. It is the target of more than 100 different chromosomal rearrangements that result in the expression of a fusion protein between MLL, deprived of its PHD and SET domains, and the C-terminus of its partner. The two most common translocation products are MLL-AF9 in AML and MLL-AF4 in ALL. AF4 and AF9 interact with each other and also directly recruit other transcriptional coactivator complexes. Using various drivers (including some blood cell drivers), it was shown that the expression of MLL-AF4 or MLL-AF9 but not MLL causes larval to pupal lethality (Muyrers-Chen et al. 2004). In addition, these two fusions had different effects on proliferation and chromosome condensation in larval brains and displayed largely nonoverlapping binding patterns on polytene chromosomes. These findings thus suggested that the C-terminal partners of the MLL fusion proteins may modify differentially MLL activity notably by regulating its targeting to distinct set of genes. Consistent with this idea, recent ChIP-seq experiments in human leukemia cell lines showed that MLL-AF4 and MLL-AF9 have distinct binding site repertoires (Prange et al. 2017).

Finally, a recent study has developed a transgenic fly model for the transactivator Tax-1 (Shirinian et al. 2015). Tax-1 is encoded by the human T cell leukemia virus type 1 (HTLV-1), a retrovirus that causes an aggressive adult T cell leukemia/lymphoma in ± 5% of infected individuals (Bangham and Ratner 2015). Tax-1 is essential for HTLV-1 oncogenic properties, and several lines of evidence indicate that the binding of IKK kinases by Tax-1 and the ensuing activation of the NF-κB pathway are critical for T cell transformation. The expression of Tax-1 in the *Drosophila* eye or in the plasmatocytes, respectively, caused a rough eye phenotype and an increase in larval blood cell number (Shirinian et al. 2015). In contrast, the expression of Tax-2, which is encoded by the genetically related but non-oncogenic retrovirus HTLV-2, did not alter eye development or hemocyte number. Moreover, further experiments demonstrated that the deleterious function of Tax-1 in the *Drosophila* eye was mediated by activation of the NF-κB pathway. These findings thus established that *Drosophila* could be used as a genetic model to investigate the mode of action of Tax-1 in cell transformation.

The above three examples illustrate how works in non-hematopoietic tissues of *Drosophila* can help to describe the activity of human leukemogenic proteins. In each case, the expression of the oncogene gave rise to a robust phenotype (rough eye for BCR-ABL and Tax-1, larval/pupal lethality for MLL-AF9 and MLL-AF4) that could be used as readouts in a modifier screen. Hence, further experiments exploiting the genetic tools available in *Drosophila* could undoubtedly bring interesting insights into the mode(s) of action of these factors in vivo.

11.3.4 Study of Leukemogenic Proteins in *Drosophila* Hematopoietic Cells

A seemingly more relevant approach is to study the function of human leukemogenic protein or that of their *Drosophila* counterparts, directly in the fly hematopoietic system.

The best example here is provided by studies of the transcription factor RUNX1 and of its oncogenic derivative RUNX1-ETO. *RUNX1* is a key regulator of several steps of blood cell development in vertebrates (de Bruijn and Dzierzak 2017). Recurrent point mutations or translocations affecting *RUNX1* are among the most frequent genetic abnormalities in human leukemia (Sood et al. 2017). For instance, the prototypical

t(8;21) translocation, which accounts for ±10% of all cases of AML, gives rise to a fusion protein between RUNX1 DNA-binding domain and the transcriptional corepressor ETO (also known as RUNX1T1). The resulting chimera, RUNX1-ETO, chiefly acts by interfering directly with the regulation of RUNX1 target genes. Understanding how deregulation of *RUNX1* activity leads to hematological malignancies is thus a field of intense investigation, and several RUNX1-ETO animal models have been developed in mouse, zebrafish, and fly. Of note, in *Drosophila*, the RUNX1 homologue Lozenge (Lz) is expressed in the crystal cell lineage and is absolutely required for the development of this blood cell type (Waltzer et al. 2010). In addition, Lz is well known for its function in the eye where it regulates photoreceptor and cone cell fate (Canon and Banerjee 2000). Besides an early study where RUNX1-ETO was expressed in the *Drosophila* eye and found to interfere with Lz function by acting as a constitutive transcriptional repressor of two Lz target genes (Wildonger and Mann 2005), two concurrent studies investigated RUNX1-ETO impact on the development of *Drosophila* hematopoietic system (Osman et al. 2009, Sinenko et al. 2010).

In one case, RUNX1-ETO expression was induced in the majority of the circulating larval blood cells using the *hml-GAL4* driver (Sinenko et al. 2010). Reminiscent of the phenotypes observed in mouse models, RUNX1-ETO expression caused a sharp increase in the number of circulating hemocytes along with an expansion of the immature blood cell population. This "leukemic" phenotype required RUNX1 DNA-binding activity and its interaction with its cofactor CBFß as well as different domains of ETO known to interact with transcriptional corepressors. Further analyses revealed that elevated ROS levels were crucial for RUNX1-ETO-induced expansion of the immature blood cells in *Drosophila*, suggesting that a similar mechanism might participate in t(8;21)$^+$ AML. Actually increased ROS levels are observed in many cases of leukemia (Udensi and Tchounwou 2014). In addition, RUNX1-ETO expression was associated with the development of melanotic tumors (Sinenko et al. 2010). By screening a panel of 231 deficiencies and 1500 automosal insertional mutations, 10 suppressors and 12 enhancers of RUNX1-ETO-induced melanotic tumor formation/blood cell number increase were identified. It will be thus of particular interest to test whether the homologues of these genes also interfere with or promote RUNX1-ETO oncogenic activity in human.

In a second case, RUNX1-ETO was expressed in the Lz$^+$ blood cell lineage using the *lz-GAL4* driver to mimic more closely the t(8;21) situation (Osman et al. 2009). As in mammals, it was found that RUNX1-ETO interferes with the function of the endogenous RUNX protein Lz. This led to the accumulation of a high number of Lz$^+$ cells that failed to differentiate in crystal cells. However, RUNX1-ETO did not solely behave as a repressor on Lz target genes in the hematopoietic system. Indeed, experiments in human t(8;21)$^+$ AML cell have since then revealed that RUNX1-ETO binding can lead both to activation and repression of its target genes (Ptasinska et al. 2012). In addition, *lz*-driven expression of RUNX1-ETO caused pupal lethality. Using an in vivo RNAi-based screen strategy, more than 2000 genes were individually knocked down in RUNX1-ETO-expressing cells to identify genes that are cell-autonomously required for its activity. Among the nine suppressors of RUNX1-ETO-induced lethality, the protease calpain B and the AAA+ ATPase RUVBL1/Pontin were studied in more detail. Interestingly both are required for Lz$^+$ blood cell number increase and differentiation blockade caused by RUNX1-ETO, while their knockdown or mutation does not affect Lz$^+$ blood cell development in a wild-type situation (Osman et al. 2009; Breig et al. 2014). It appears that calpain B is required for RUNX1-ETO stability in Lz$^+$ cells (Osman et al. 2009). Strikingly, in human, calpain inhibition also causes RUNX1-ETO degradation and specifically impairs the viability and clonogenic growth of t(8;21)$^+$ AML cells, which are known to depend on RUNX1-ETO expression. Therefore, the regulation of RUNX1-ETO by calpains seems conserved, and calpain inhibitors might be used as therapeutic agents in leukemia. Similarly, works in human cell lines showed that

Pontin expression is activated by RUNX1-ETO and cooperates with this oncogene to sustain leukemic blood cell proliferation and survival (Breig et al. 2014). As RUNX1-ETO-expressing cells appear sensitized to Pontin knockdown, recently developed chemical inhibitors of its ATPase activity might be useful therapeutic agents in t(8;21)$^+$ AML.

Incidentally, other studies in *Drosophila* blood cells have revealed a mechanism of regulation of RUNX activity relevant to human AML. Myeloid leukemia factor (MLF) was found to be required for Lz-induced transactivation in a genome-wide RNAi screen in Kc167 cells (Gobert et al. 2010; Bras et al. 2012). In human, *MLF1* was identified as the target of a rare translocation in patients with myelodysplastic syndrome and AML (Yoneda-Kato et al. 1996) and more recently as a tumor suppressor in infant T cell acute lymphoblastic leukemia (Mansur et al. 2015). Yet the function and mode of action of this conserved family of protein remain largely unknown (Gobert et al. 2012). Analyzing *mlf* function in *Drosophila* revealed that it regulates Lz$^+$ cell number by stabilizing Lz (Bras et al. 2012). Interestingly, the expression of human MLF1 can rescue *mlf* mutant defects, indicating that MLF function is conserved through evolution (Martin-Lanneree et al. 2006; Bras et al. 2012). Moreover, MLF1 appears to be required for RUNX1-ETO stable expression and the growth of t(8;21)$^+$ AML cells (Bras et al. 2012). At the molecular level, MLF acts at least in part as a component of a conserved Hsp70/DnaJ chaperon complex to promote RUNX protein stability (Dyer et al. 2017, Miller et al. 2017). Whether this chaperon complex is also involved in RUNX-dependent AML remains to be assessed. Still it is interesting to note that haploinsufficient mutations in *RUNX1* are associated with AML, indicating that a tight regulation of its level is critical to prevent leukemogenesis (Sood et al. 2017). In fly, reducing Lz level impairs Lz-mediated repression of *Notch* and causes a myelodysplastic-like phenotype due to sustained overactivation of the Notch pathway (Miller et al. 2017). Given the importance of activated Notch signaling in hematological malignancies (Gu et al. 2016), a similar functional relationship between RUNX1, MLF1, and Notch might be at stake in human blood cell transformation.

A slightly different strategy was used to dissect leukemia-associated isocitrate dehydrogenase (IDH) mutants in *Drosophila*. In that case, rather than using the human oncoproteins, the authors used a transgenic approach to drive the expression of their *Drosophila* counterpart carrying homologous mutations. In human, somatic mutations in conserved arginine residues within the active sites of IDH1 and IDH2 are very frequent in AML and in gliomas (Gagne et al. 2017). While IDH normally converts isocitrate into alpha-ketoglutarate (αKD), the mutated forms produce D2-hydroxyglutarate (D2-HG), an oncometabolite that accumulates at high levels in cancer tissues and inhibits the activity of αKD-dependent enzymes, some of which are implicated in chromatin compaction, DNA methylation, collagen modification, or response to hypoxia. To develop a genetically tractable model for IDH-associated leukemia, Reitman et al. generated transgenic lines expressing mutated forms of the single *Idh Drosophila* gene (Reitman et al. 2015). The overexpression of these IDH mutants in circulating larval hemocytes using the *hml-GAL4* driver caused an increase in blood cell number, lamellocyte differentiation, and melanotic tumor formation but also a rise in undifferentiated blood cells, similar to what is observed in mouse models of IDH-mutated leukemia. Interestingly, the severity of the phenotypes was correlated with the level of D2-HG production. Moreover, using a UAS-based co-expression strategy, enhancers and suppressors of IDH mutant-associated phenotypes (melanotic tumor formation or wing expansion defects) were sought. This screen suggested that the phenotypes of IDH mutants are caused by increased levels of ROS, as observed in the case of RUNX1-ETO (Sinenko et al. 2010). In contrast to wild-type IDH that reduces NADP+ to NADPH to produce αKG, mutant IDH consumes NADPH to produce D2-HG (Gagne et al. 2017). Therefore a decreased level of NADPH (an important source

of reducing power) in IDH1/2-mutated cells could interfere with ROS detoxification. It would thus be interesting to further study the contribution of ROS to IDH-associated leukemia in humans.

Finally a recent report investigated the consequences of expressing the human NUP98-HOXA9 (NA9) fusion protein in the *Drosophila* lymph gland (Baril et al. 2017). Several HOX transcription factors and their cofactors MEIS and PBX are deregulated in AML (Alharbi et al. 2013). Similarly, recurrent chromosomal translocations between *NUP98*, which encodes a nucleoporin, and several partner genes, including some *HOX*, occur in AML (Gough et al. 2011). Among them, the rare and high-risk t(7;11)(p15;p15) translocation generates a fusion between the N-terminal of NUP98 and the DNA-binding and heterodimerization domains of HoxA9. In *Drosophila*, *hml*-driven expression of NA9 in differentiating larval hemocytes caused a massive increase in circulating blood cells as well as lymph gland hyperplasia, reminiscent of the myeloproliferative disease induced by NA9 in mouse models (Baril et al. 2017). Also, as in mammals, NA9 activity required interaction with DNA and with PBX mediated by HoxA9 moiety. Interestingly, NA9 expression in differentiating hemocytes not only increased the number of differentiated cells at the expanse of the blood cell progenitors but also altered PSC size and morphology, indicating that NA9 affects lymph gland niche/microenvironment nonautonomously. These phenotypes were due to defective PVR (PDGF/VEGF related) signaling, a receptor tyrosine kinase (RTK) related to several mammalian RTK involved in leukemia (Gough et al. 2011). In mammals, remodeling of the bone marrow niche by AML cells is thought to promote leukemia at the expanse of normal hematopoiesis (Shafat et al. 2017). It is tempting to speculate that NA9 could hijack the hematopoietic stem cell niche by interfering with RTK signaling. Importantly too, these findings also highlight that *Drosophila* can be used to investigate cross-regulatory interactions between leukemic cells and their microenvironment.

11.4 Conclusion

The various examples above show that works in *Drosophila* can contribute to our understanding of leukemia pathogenesis. *Drosophila* seems particularly well suited to decipher the mechanisms of action of proteins involved in human leukemia that have clear homologues in fly. The use of genetic interaction screen in *Drosophila* is an extremely powerful means to identify regulators or mediators of these leukemogenic proteins. In addition, the relative simplicity of the fly hematopoietic system offers a good opportunity to study its different components and the interaction between them at the cellular, developmental, and molecular levels. Yet, the lack of clearly identified hematopoietic stem cells, the limited functional homologies between mammalian and *Drosophila* blood cell types, or the more restricted set of genes known to control *Drosophila* hematopoiesis also hamper the development of leukemia models in this system or at least restrain their heuristic value. Still, there are means to take further advantage of *Drosophila* hematopoietic system to develop more refined models of leukemia. Leukemia arises as a consequence of a limited number of mutations. While most models in fly have focused on the expression of a single oncogenic factor, it is possible to generate *Drosophila* avatars where the expression or function of multiple genes is modified in specific blood cell lineages and/or in a temporal series. This could bring important information as to the mechanisms of oncogenic cooperation that drives blood cell transformation. Besides somatic mutations, germline mutations, for instance, in *RUNX1* or *GATA2*, are associated with predisposition to develop leukemia. The CRISPR/Cas9 technology could be used to generate relevant knock-in as well as allelic series to study these familial cases of leukemia. In addition, a wider use of cell sorting or cell transplantation assays could help define the characteristics of *Drosophila* leukemic blood cells. A better knowledge of the *Drosophila* adult hematopoietic system could also favor the analysis of leukemia models past the larval stage. Indeed, it would be particularly interesting to follow the evolution of "leukemic clones" over a

longer time period and during aging. In addition, the interactions between "leukemic blood cells" and their surrounding cellular environment can easily be studied in fly, and a great deal of knowledge could be drawn from such analyses. Furthermore, *Drosophila* provides a simple model to investigate exogenous factors (nutrition, microbiota, infection, etc.) susceptible to influence leukemia development. It could also easily be used to perform chemical screens for compounds interfering with leukemogenic proteins function, which would open avenues for new therapeutic development. Finally, and perhaps most importantly, a genuine effort is still needed to transfer more effectively the knowledge obtained from *Drosophila* models toward human leukemia. Indeed, except for a few cases, the discoveries made in fly have not been tested thereafter in human leukemic cells or in mammalian models. Nonetheless, several features of leukemia have been recapitulated in *Drosophila*, and new insights into leukemic proteins mode(s) of action have been gained thanks to this model. In light of these promising results, we should endeavor to take a step further by translating these findings to human and eventually to clinic.

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Diseases Associated with Mutation of Replication and Repair Proteins

12

Sue Cotterill

Abstract

Alterations in proteins that function in DNA replication and repair have been implicated in the development of human diseases including cancer, premature ageing, skeletal disorders, mental retardation, microcephaly, and neurodegeneration. *Drosophila* has orthologues of most human replication and repair proteins and high conservation of the relevant cellular pathways, thus providing a versatile system in which to study how these pathways are corrupted leading to the diseased state. In this chapter I will briefly review the diseases associated with defects in replication and repair proteins and discuss how past and future studies on the *Drosophila* orthologues of such proteins can contribute to the dissection of the mechanisms involved in disease development.

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Keywords

DNA replication · DNA repair · Disease · *Drosophila*

12.1 Introduction

Although specific cells such as immune cells are programmed to generate large changes in their chromosomal DNA and small errors fixed into the DNA form the basis of evolution, in general, cells expend a lot of energy maintaining the correct sequence of the DNA contained in the chromosomes. Tightly co-ordinated complexes of proteins are responsible for the accurate copying of DNA during DNA replication and for maintaining DNA sequence in response to constant bombardment from a wide range of physical and chemical insults. Changes in the catalytic activities of these proteins, or of the way they interact with each other, have deleterious effects for the cell and lead to cellular malfunctioning and cell death or disease.

This chapter will discuss how work in *Drosophila* has and will impact the diseases that arise from the presence of mutant replication and repair proteins. It will focus mainly on the problems caused by mutations in proteins that are

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directly involved in the catalysis of the processes. In addition to these, there is large network of proteins that are involved in signalling and coordinating these processes, and mutations in many of these are also known to contribute to human disease. These will not be discussed in any detail here, but information about these has been well reviewed (Awasthi et al. 2016; Blackford and Jackson 2017; Yazinski and Zou 2016; Hustedt and Durocher 2016; Scanlon and Glazer 2015; DNA replication controls 2017).

12.2 DNA Replication Pathways

DNA replication is a well-studied cellular process, and there are a number of good recent reviews of both the mechanism of replication (Fragkos et al. 2015; Dewar and Walter 2017) and DNA replication in *Drosophila* (Hua and Orr-Weaver 2017). Replication is initiated at origins of replication. The nature of replication origins differs between *Saccharomyces cerevisiae*, which initiates replication in regions containing a specific DNA sequence (the ARS sequence), and other eukaryotes where origins are less localised and are not defined by one specific DNA sequence. For eukaryotes apart from *S. cerevisiae*, origins and their characteristics are most likely defined by a combination of certain chromosomal characteristics. Despite this proteins that carry out DNA replication are well conserved from yeast to humans.

Replication is initiated by formation of the preRC complex consisting of the ORC complex (ORC 1-6), cdc6, cdt1, and the MCM complex (MCM2-7). This is activated by a series of phosphorylation events to form the pre-initiation complex (preIC), which is accompanied by the loading of another set of proteins including Treslin, Topbp1, DNA polymerase epsilon, the GINS complex, cdc45, recQ4 mcm10, AND1/Ctf4, and DNA polymerase alpha. Further phosphorylation events allow loading of elongation proteins, initiation of replication, and full-scale synthesis of the leading and lagging strands. Termination of replication is a poorly understood process but probably involves dissolution of the replisome complex via ubiquitination of the MCM complex and resolution of topological issues. The full repertoire of proteins involved in the later step is unclear but almost certainly include topoisomerases and helicases.

The proteins involved in DNA replication, their roles, and their known *Drosophila* orthologues are summarised in Table 12.1. *Drosophila* proteins corresponding to most of the major replication proteins have been characterised. *Drosophila* ORCA and MCMbp proteins have not been characterised. The *Drosophila* genome does contain proteins with homology to both of these proteins; however whether they are true orthologues has yet to be established. *Drosophila* Treslin has also not been reported, and in this case, no protein with significant sequence homology is present in the *Drosophila* genome.

Table 12.1 also shows diseases known to be associated with replication proteins. Most replication proteins are not well represented in terms of specific diseases. This is probably because they are so intrinsic to cell function that most changes are likely to cause lethality, and only subtle changes allow life to progress long enough for disease to develop.

12.3 DNA Repair Pathways

There are a wide variety of agents that can damage DNA including both intrinsic factors (such as replication errors and products generated during normal cellular metabolism) and extrinsic factors (such as ionising radiation and DNA damaging chemicals in the environment). There are, therefore, a wide range of different pathways to deal with the damage, each pathway showing specificity for a subset of different lesions. Some pathways deal with chemical alterations occurring directly on nucleotide bases, e.g. direct reversals deal with specific UV and chemically induced base changes (Yi and He 2013), nucleotide excision repair (NER) largely deals with bulky lesions such as those generated by UV damage (Schärer 2013), and base excision repair (BER) (Krokan and Bjørås 2013) deals with small non-helix-distorting base lesions such as those arising from

Table 12.1 Replication proteins

Human protein	Replication function (1–3)	Disease related to mutation	*Drosophila* orthologue (4)
ORC 1-6	Component of preRC, central to defining origins replication	Meier-Gorlin syndrome (5)	Yes – all
Cdc6	Component of preRC	Meier-Gorlin syndrome (5)	Yes
Cdt1	Component of preRC	Meier-Gorlin syndrome (5)	Yes
MCM 1-6	Component of preRC forms core of CMG helicase needed for initiation and elongation	Meier-Gorlin syndrome and MCM4 disease (5) (6)	Yes
Geminin	Inhibitor cdt1 mcm loading	Meier-Gorlin syndrome (7)	Yes
Cdc45	Component of preIC, component of CMG helicase	Meier-Gorlin syndrome (8)	Yes
GINS complex (the Go Ichi Ni San proteins)	Component of preIC, component of CMG helicase	No specific disorders reported	Yes
Mcm10	Component of preIC	No specific disorders reported	Yes
Treslin	Component of preIC	No specific disorders reported	Not reported
RecQ4	Component of preIC	RTS, BGS, and RAPADILINO (9)	Yes
AND-1 (ctf4)	Component of preIC and aids interaction CMG helicase and DNA polymerase alpha	No specific disorders reported	Yes
TopBp1	Component of preIC	Breast cancer (10)	Yes
Polymerase alpha	Synthesises initiation primer and Okazaki fragment primers	X-linked N syndrome (11)	Yes
Polymerase delta	Bulk lagging strand synthesis	Colon and endometrial cancer (12)	Yes
Polymerase epsilon	Bulk leading-strand synthesis	Colon and endometrial cancer (12)	Yes
RPA (RPA1-3)	ssDNA-binding protein	No specific disorders reported	Yes
RFC (RFC1-5)	Clamp loader for bulk synthesis polymerases	No specific disorders reported	Yes
PCNA	DNA clamp for bulk synthesis polymerases	Cockayne-related disorder[a] (13)	Yes
Mcmbp	Unclear but may modulate MCM complex activity	No specific disorders reported	Not reported
ORCA (LRWD1)	Control of initiation of late-firing origins	No specific disorders reported	Not reported
DNA2	Okazaki fragment processing	Seckel syndrome (14)	Yes [b]
FEN1	Okazaki fragment processing	No specific disorders reported	Yes
Ligase 1	Okazaki fragment joining	No specific disorders reported	Yes
Topoisomerases	Relieving torsional stress generated by unwinding and synthesis	No specific disorders reported	Yes

1. Burgers and Kunkel (2017) 2. Dewar and Walter (2017) 3. Fragkos et al. (2015) 4. Hua and Orr-Weaver (2017) 5. Bicknell et al. (2011) 6. Vetro et al. (2017) 7. Burrage et al. (2015) 8. Fenwick et al. (2016) 9. Larizza et al. (2010) 10. Karppinen et al. (2006) 11. Starokadomskyy et al. (2016) 12. Rayner et al. (2016) 13. Baple et al. (2014) 14. Shaheen et al. (2014)

[a]Probably through its role in repair

[b]Probably through repair function

oxidative and alkylative damage. One pathway, mismatch repair (Kunkel and Erie 2015), deals predominantly with the incorrect base incorporation during DNA replication. Additional separate pathways exist to repair double-stranded breaks (DSBs) in DNA (Jasin and Rothstein 2013). Lesions involving strand breakage are particularly harmful, as they have the potential to cause loss of information and generate translocation between chromosomes. Interstrand cross-links are also potentially highly mutagenic. These have their own specific repair pathway (Hashimoto et al. 2016) which includes some unique proteins but in addition also co-opts proteins from other repair pathways. Finally there are particular mechanisms which deal with problems in ongoing replication, some of which are 'error prone' such as translesion synthesis, and others which are more accurate such as strand switch bypass (Branzei and Szakal 2016). Many of these processes have been well discussed in recent reviews and won't be discussed in detail here, but the proteins that are thought to catalyse these processes, together with their biochemical function and diseases associated with them, are shown in Table 12.2. This table also shows the *Drosophila* orthologues for these proteins. For repair the *Drosophila* repertoire is less complete than for replication (reviewed (Sekelsky et al. 2000; Sekelsky 2017)), but most of the pathways still seem to exist in *Drosophila* to some extent. Specific details are included in the relevant sections below.

Repair proteins are more likely to cause disease than replication proteins. This could be due to the fact that there is some degree of redundancy between pathways. It could also be related to the fact that most proteins involved in repair pathways are needed by the cell under more limited conditions, and so alterations in these proteins can be tolerated sufficiently well for an individual to survive for a significant amount of time.

In addition to the catalytic proteins, there are an especially large number of proteins involved in control of DNA repair, particularly in signalling pathways. Some of these are well known and well studied, for instance, p53, and ATM, ATR; however detailed discussions of these are outside the scope of this article.

12.4 Diseases of Replication and Repair Proteins

Although mutations in replication and repair proteins are most commonly associated with susceptibility to cancer, they can also cause a large number of other disorders which show a surprising variety of symptoms. For many of these disorders, there is a high degree of overlap in symptoms. This is probably because mutations in replication and repair proteins interfere with the same cellular processes; therefore the types of changes that result, and their consequences, are similar. However this does call into question whether all of these disorders are truly distinct diseases, and in fact in many cases proteins that function in the same pathway are recognised as causing the same disease. There are also a few examples where mutations in a protein can cause different symptoms depending on the position of the mutation (discussed later). This may be because a protein functions in multiple repair pathways, or may have both replication and repair function, so that mutations in different regions of the protein might have a differential effect on outcome. For some multi-pathway proteins, the pathways affected by individual mutations are known, and for others it has yet to be determined.

12.4.1 Cancers

Mutations in many repair and replication proteins show a general cancer predisposition. In most cases this general predisposition can manifest in multiple different types of cancers in multiple tissues. For proteins that have specific disease associations, the cancer predisposition may be part of the symptoms of the disease, but it can also be independent of the disease and related to particular and different mutations in the protein. In a few cases, mutations in specific replication and repair proteins can be shown to be causative for particular cancers.

12.4.1.1 Breast Cancer

Proteins involved in the homologous recombination pathway of DSB repair have been shown to be causative for the development of breast and ovarian cancers (Scott 2004). The best known of these are the BRCA1 and BRCA2 (O'Donovan and Livingston 2010) proteins; however breast cancer is also associated with mutations in other proteins in the same pathway, specifically in abraxas (Solyom et al. 2012) and rap80 (Nikkilä et al. 2009). A similar link has also been proposed for the replication repair protein Topbp1 (Karppinen et al. 2006).

Drosophila has a somewhat stripped down pathway for homologous recombination (see Table 12.2). Although it retains most of the proteins involved in the basic catalytic process, there are some notable missing components such as orthologues of BRCA1 complex proteins, including BRCA1 itself. In humans several rad51 homologues are involved in the homology search; *Drosophila* has retained a Rad51 orthologue but lacks rad52, which helps rad51 in the search process. The TR complex is also incomplete; the BLM and topo III components are present, but RM1 and RM2 are missing. *Drosophila* also does not appear to possess orthologues of the E3 ubiquitin ligases (RNF8 and RNF168, which transduce the DNA double-stranded break response signal by ubiquitinating DSB sites on H2a and H2aX proteins (Mattiroli et al. 2012).

12.4.1.2 Colon Cancer

Various types of colon cancers are associated with alterations in the mismatch repair pathway. Mismatch repair is required to deal with errors generated during DNA replication and so is likely to be more important in tissues that have a high cellular turn over. These would include the lining of the gut and endometrium, and in fact endometrial cancers are also associated with mutation of mismatch repair proteins.

The best-studied type of colon cancer involving mutations in MMR proteins is called Lynch syndrome (HNPCC) (Lynch et al. 2015). This is associated with mutations in the mlh1, msh2, msh6, and pms2 proteins and is characterised by lack of polyp formation prior to cancer onset. Apart from colon and endometrial cancers, individuals with mutations in mismatch repair proteins can also get cancers of the stomach, liver, gall bladder urinary tract, brain, and skin. There is also a particular subtype Muir Torre (lynch II) that is associated with sebaceous skin tumours.

Mismatch repair has been seen to occur in *Drosophila* even though it is missing half of the components – there are no *Drosophila* orthologues of msh3, mlh3, pms1, msh1, msh4/5 (Sekelsky 2017). It is likely that the roles of the missing proteins have been taken on by other proteins in the pathway. Although details of how this occurs are unclear, the observation that many of the proteins involved in MMR in humans act as hetero-dimers suggests that this could be a plausible scenario (Kunkel and Erie 2015).

A milder type of familial adenomatous polyposis, called autosomal recessive familial adenomatous polyposis, has been associated with mutations in the Mutyh glycosylase protein (Lipton and Tomlinson 2006) – a BER protein that is missing in *Drosophila*. This is distinct from Lynch syndrome due to the presence of large numbers of polyps before cancer development.

More recently, germline mutations in the replicative DNA polymerases delta and epsilon have also been shown to be responsible for some types of colon and endometrial cancers (Rayner et al. 2016). These can be distinguished from other types of colon cancer due to a lack of microsatellite instability (MSI). Somatic mutations in DNA polymerase epsilon are also associated with very high mutation rate in tumours. The earliest polymerase mutations that were shown to be causative for cancer development were seen in the exonuclease region of the polymerase, which is concerned with maintaining fidelity. However loss of exonuclease activity cannot be the only mechanism involved as some mutants retain partial activity. In addition loss of exonuclease activity alone cannot explain the hypermutation phenotype.

Mutations in the other replicative polymerase – polymerase alpha – have not been associated with colon cancer but instead are seen to cause X-linked N syndrome. This has some colon

Table 12.2 Repair proteins

Pathway	Proteins	Disease association (details in text – note not including cancer predisposition)	*Drosophila* orthologues reported/missing
NER (Global)	ERCC1, XPA, XPB(ERCC3), XPC, XPD/ERCC 2, DDB2/XPE, ERCC4/XPF, ERCC 5/XPG, POLH/XPV (1)	Xeroderma pigmentosum, (2) trichothiodystrophy (3)	All present
NER (TCR) transcription-coupled specific	As above except not XPC and plus ERCC6 and ERCC8 (1)	Cockaynes, cerebro-oculo-facio-skeletal syndrome (4), De Sanctis syndrome (5)	Missing ERCC6, ERCC8
Interstrand cross-link repair	FANCA, FANCB, FANCC, FANCD1 (BRCA2), FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ (BRIP1), FANCL, FANCM, FANCN (palb2), FANCO (RAD51c), FANCP (SLX4), FANCQ (ERCC4), FANCR (RAD51), FANCS (BRCA1), FANCT (UBE2T) FAN1 (6)	Fanconi anaemia (7, 8)	Reported FANCD1(BRCA2), FANCD2, FANCI, FANCL, FANCM, FANCO (RAD51c- spnD), FANCP (SLX4), FANCQ (ERCC4), FANCR (RAD51 spnA)
Base excision repair/SSBR	Glycosylases (>11 specific for different lesions, APE1, (HAP1/apex), APE2 DNA polymerase beta, ligase 1/Lig3, PARP, XRCC1 SSBR does not use glycosylases but needs aprataxin (APTX) and TDP1 (9)	Mutyh glycosylase Familial adrenomatous (10)	Fewer glycosylases No mutyh 1 unique ung No pol beta 1APE and missing aprataxin
Mismatch repair	MSH2, MSH3,MSH6, MLH1,MLH3, PMS1,PMS2, EXO1 MSH1 (mitochondrial) MSH4/5 (meiotic) (11)	Lynch syndrome, Muir Torre (lynch II) (12)	Missing msh3, mlh3 and pms1, msh1, msh4/5
Homologous recombination	MRN, Ctip, BRCA1 complex, (BRCA1, BARD, abraxas, BRCC36, rap80, BRE, NBA1), BRCA2, RNF8, RNF168, ubc13, MDC1, RAD51b/c/d, rad52, XRCC2/3, rad54, DNA ligase 1, DNA2, EXO1, TR complex (BLM, topo IIIα, RMI1, RMI2) (13)	Breast cancer, lymphomas,(14) ataxia-related disorders, (15) Nijmegen breakage syndrome (NBS), and related disorders, (16, 17) Subtypes Seckel syndrome (18) Riddle syndrome (19) Blooms syndrome (20) *Nephronophthisis-related ciliopathies* (21)	Missing BRCA1 complex, rnf8, rnf168 rad51b, rad52, RM1,RM2
	Mcm8/9 associated with HR after ICL repair	Premature ovarian failure (22)	Missing Mcm9

(continued)

Table 12.2 (continued)

Pathway	Proteins	Disease association (details in text – note not including cancer predisposition)	*Drosophila* orthologues reported/missing
Nhej	MRN, 53BP1, KU, DNAPKCs, WRN, pol beta, XRCC4, DNA ligase 4, XLF Polmu, pol lambda	SCID related (23, 24) Ataxia related, NBS related (as above) Lig4 disease (25) Werner syndrome (26) *Nephronophthisis-related ciliopathies*	Missing 53 bp1, dnapkcs, pol beta, Pol lambda or pol mu and WRN split
alt nhej	MRN, 53 bp1, FEN1, pnpk, XRCC1, DNA ligase 3 Pol theta (13)	*Early infantile epileptic encephalopathy* (27)	
Vdj joining	Rag1/2, KU, DNAPKCs, Artemis		Missing Artemis, DNAPKcs,
Translesion synthesis	Pol eta, iota, kappa, zeta REV1, PrimPol (synthesis across and restart after lesion) RAD6, RAD18 (28)	PrimPol high myopia	Missing pol kappa, PrimPol, rad18
Strand switch bypass	RAD51, EXO1, TR complex, RAD6, RAD18, MMS2, Ubc13, HLTF, SHPRH ZRANB3, SMARCAL1 (28)	SMArcal1 – SCHIMKE IMMUNOOSSEOUS DYSPLASIA – although likely to be via transcription not replication role (29)	Missing RM1, RM2, ZRANB3, *Drosophila* smarcal1 (marcal1) does not have the same properties
Direct reversal	Photolyase, alkyltransferases, alkb dioxygenases (30)	No specific reported	Representatives of all types

1. Schärer (2013) 2. DiGiovanna and Kraemer (2012) 3. Hashimoto and Egly (2009) 4. Natale (2011) 5. Rahbar and Naraghi (2015) 6. Hashimoto et al. (2016) 7. Walden and Deans (2014) 8. Zhou et al. (2012) 9. Krokan and Bjørås (2013) 10. Lipton and Tomlinson (2006) 11. Kunkel and Erie (2015) 12. Lynch et al. (2015) 13. Jasin and Rothstein (2013) 14. Scott (2004) 15. Taylor et al. (2004) 16. Chrzanowska et al. (2012) 17. Waltes et al. (2009) 18. Qvist et al. (2011) 19. Mattiroli et al. (2012) 20. Ellis et al. (1995) 21. Chaki et al. (2012) 22. Bouali et al. (2017) 23. Mathieu et al. (2015) 24. Dvorak and Cowan (2010) 25. Chistiakov et al. (2009) 26. Yu et al. (1996) 27. Shen et al. (2010) 28. Branzei and Szakal (2016) 29. Yi and He (2013)

association causing enterocolitis; however the other symptoms are more diverse including recurrent infections and sterile inflammation in various organs, diffuse skin hyperpigmentation, lack of sweating, and corneal inflammation and scarring (Starokadomskyy et al. 2016).

12.4.2 Immune Deficiency Diseases

As well as being cancer associated, the NHEJ repair proteins XLF (NHEJ1), DNA-PKcS, and artemis have been associated with minor subgroups of the immunodeficiency disorder SCID (Dvorak and Cowan 2010; Mathieu et al. 2015). The general symptoms of SCID are frequent and often severe respiratory infections, poor growth, eczema-like rashes, chronic diarrhoea, and recurrent oral thrush. However the symptoms vary depending on the exact protein and mutation, for instance, in DNA-PKS – SCID, there is often no growth delay, but microcephaly is seen.

Lig4 disease (Chistiakov et al. 2009), although not classified as SCID, shows similar features of immunodeficiency coupled with developmental delay, odd facial features, microcephaly, radiosensitivity, and skin and blood abnormalities.

Most of the above NHEJ proteins are particularly associated with the use of the NHEJ pathway in the vdj recombination pathway in immune cells, and it is likely that the immune symptoms are caused by problems in this pathway rather

than general repair of DNA damage. Consistent with the roles of these proteins, *Drosophila* has orthologues of XLF and lig4 – both of which are involved in DNA repair-based NHEJ – but lacks orthologues of PKcS and artemis which only act in vdj recombination.(Sekelsky 2017).

12.4.3 Ataxia-Related Disorders

Ataxia telangiectasia-like disorder (ATLD) is caused by the loss of the DSBR protein Mre11 (Taylor et al. 2004). Patients lack telangiectasia and do not have widespread immune deficiencies, but cancer predisposition has hypersensitivity to ionising radiation, cerebellar degeneration, progressive cerebellar ataxia, slurred speech, and abnormal eye movements. Mre11 is involved in both HR and NHEJ (Jasin and Rothstein 2013), and so it is not clear which is the affected pathway; however three other proteins involved in the NHEJ pathway of DSBR have also been shown to cause related ataxia-associated disorders: Pnkp (polynucleotide kinase 3′-phosphatase) (ataxia-oculomotor apraxia), XRCC1 (spinocerebellar ataxia), and XRCC4, the latter giving both ataxia microcephaly and short stature. (Bras et al. 2015; Guo et al. 2015; Hoch et al. 2017).

Mutations in PNKP can also cause one form of early infantile epileptic encephalopathy (Shen et al. 2010). This is not ataxia related but, like mutations in XRCC4, can cause microcephaly, seizures, and severe mental and physical developmental delay.

Consistent with the importance of NHEJ in dsb repair, *Drosophila* is fully competent to carry out the process, and retains orthologues for most of the proteins involved, and all of those that have been associated with ataxias (Sekelsky 2017). A surprising exception to the conservation of NHEJ proteins in *Drosophila* is the lack of 53 bp1, which in mammals seems to be important for the recognition of the dsb and the choice of repair pathway. However, one way 53 bp1 has been suggested to influence pathway choice is via competition with BRCA1, and since *Drosophila* lacks BRCA1, perhaps the lack of 53 bp1 is more tolerable (Malewicz 2016).

12.4.4 Triplet Disorders

Malfunctioning of the replication elongation proteins, conserved in all species including *Drosophila*, has been suggested to be involved in variation of repeat numbers in triplet disorders. However at this point, no particular gene has been specifically tied into the process. An earlier suggestion for a specific involvement of FEN1 did not stand up to further scrutiny (Otto et al. 2001). In addition, the involvement of replication proteins in triplet disorders is in generation of the repeats, rather than the actual disease itself. For these reasons, and also because triplet disorders are discussed in more detail in another chapter in this book, they will not be discussed further here.

12.4.5 Pleiotropic Disorders

Mutations in many replication and repair proteins are associated with diseases that cause a wide range of symptoms with variable penetrance. In addition, there is often a large amount of overlap between the diseases caused by different proteins and pathways. Table 12.3 shows a comparison of the symptoms between these pleiotropic disorders.

12.4.5.1 Diseases Caused by Mutations in NER Pathway Proteins

The global NER pathway in *Drosophila* is completely conserved, although *Drosophila* appears to totally lack the proteins associated with the transcription-coupled branch of the pathway (Sekelsky 2017). In addition there is no evidence that *Drosophila* carries out transcription-coupled NER (de Cock et al. 1992). Therefore it is unlikely that the actions of missing TcNER proteins are substituted by other proteins, as seems to be the case for missing repair proteins in other repair pathways in *Drosophila*. Mutations in NER proteins cause several disorders with overlapping symptoms:

Xeroderma Pigmentosum (XP) is perhaps the most well known of the diseases caused by defec-

Table 12.3 Diseases with pleiotropic symptoms

Disease	Odd facial features	Short stature	Micro-cephaly	Neurological problems	Development abnormalities	Photosensitivity	Eye problems	Skin changes	Tumours	Skeletal abnormalities	infertility	other
XP						×	×	Pigment	The skin			Premature skin ageing
CSA/CSB		×	×	×	Delay	×	×			×		Cold hands and feet, hearing and vision loss
De Sanctis		×	×	×		×	×		skin		×	Premature skin ageing
Trichothiodystrophy		Rare		Rare	Delay (rare)	Rare	×	Scaly				Brittle hair, no sweating, occasional recurrent infections
FA		×	×	×	Urogenital/ kidneys				The head, neck, skin GI, and genital tract, AML	×	×	Bone marrow failure
CIN					Delay							Progressive kidney disease, abnormal liver function, adrenal insufficiency, NK cell deficiency, and mild recurrent respiratory infections
MCM8 disease												Premature ovarian failure
Seckel syndrome	×	×	×		Delay							Haematological abnormalities
MGS	×	×	×							×		Breathing difficulties
FGD		×	×	×	×			×	×	×		Recurrent infection

Table 12.3 (continued)

Disease	Odd facial features	Short stature	Micro-cephaly	Neurological problems	Development abnormalities	Photosensitivity	Eye problems	Skin changes	Tumours	Skeletal abnormalities	infertility	other
NBS	×	×	×	×					Lymph		×	Radiation sensitivity, respiratory tract problems, immunodeficiency
NBS like	×	×	×	×					Lymph		×	Radiation sensitivity, respiratory tract problems
SIHIWES	×	×	×	×	Cardiac/ urogenital					×		Hearing impairment
NPHP-RC				×			×					Kidney problems
BLM	×	×		Mild		×		Pigment	×		Mild	Increased risk diabetes, mild immune problems
WRN									×			Early-onset ageing and age-related diseases
RTS/BGS/RAP	×	×		×	GI/genital			Pigment	Osteosarcoma, basal/ squamous cell carcinoma	×	×	Early-onset ageing, sparse hair eyebrows and eyelashes, GI problems

References as in Table 12.2

tive NER. This is associated with mutations of ERCC1, XPA, XPB(ERCC3), XPC, XPD/ERCC 2, DDB2/XPE, ERCC4/XPF, ERCC 5/XPG, POLH/XPV. Symptoms of XP are largely confined to the skin and eyes, with the exact severity and penetrance of the symptoms somewhat dependent on the exact protein mutated and the position of the mutation (DiGiovanna and Kraemer 2012).

Cockaynes Syndrome (CSA/B) is also associated with mutations in proteins that function in NER, specifically in this case with the proteins that are involved in the transcription-coupled branch of the pathway ERCC6 and ERCC8, although mutations in PCNA also cause a related disorder. The symptoms of CS are more severe than for XP and not just confined to the skin and eyes. In addition to photosensitivity (sunburn/skin blistering), CS causes changes in development, skeletal defects, neurological defects and hearing and vision loss. CSA is associated with changes in ERCC8. CSB is the more severe form of the disease associated with mutation of ERCC6 and also known as cerebro-occular-facio-skeletal syndrome. This manifests much earlier than CSA, sometimes prenatally, and the symptoms are much more pronounced. Notably, unlike XP, CS is not associated with increased incidence of skin cancer (Natale 2011).

De Sanctis Syndrome is also associated with mutations in the ERCC6 protein – but at different sites to those seen in CSB. Symptoms are similar to those of XP but also include neurological, fertility and skeletal complications (Rahbar and Naraghi 2015).

Trichothiodystrophy is not always associated with altered NER; however 80% of individuals diagnosed with this condition also show mutations in the NER pathway proteins ERCC2 and ERCC33. It is milder than the other NER-associated diseases, with the main symptoms being cloudy lens, brittle hair, scaly skin and lack of sweating. Rarely patients also show some of the more serious symptoms associated with CS, but as for CS, there is no increased incidence of skin cancer (Hashimoto and Egly 2009).

12.4.5.2 Fanconi Anaemia (FA)

Mutations of the proteins responsible for the repair of interstrand cross-links cause Fanconi anaemia (Walden and Deans 2014). This includes mutations both in proteins specific to ICL repair and those that have dual function in ICL and other repair pathways and mutations causing FA have been seen in 20 different proteins (see Table 12.2). As for other repair-/replication-related pleiotropic disorders, the exact manifestation of the symptoms and disease classification is dependent on the protein and the position of the mutation. FA is characterised by skeletal and developmental defects, infertility and bone marrow failure, together with an increased risk for cancer particularly AML.

A related disease, chromic interstitial nephritis (CIN), is caused by mutations in the nuclease FAN1 (fancd2/fanci-associated nuclease gene 1) (Zhou et al. 2012). This lacks many of the symptoms of FA, perhaps because its actions are thought to be somewhat independent of the main FA pathway proteins but manifests as progressive chronic kidney disease, mild and recurrent respiratory tract infections, abnormal liver function, growth retardation with additional defects of adrenal insufficiency and NK cell deficiency.

Although MCM8 and 9 are associated with HR during ICL repair (Nishimura et al. 2012), they have not been associated with FA; however mutations in MCM8 have been seen to cause premature ovarian failure (Bouali et al. 2017).

As for other repair pathways, *Drosophila* appears to be competent to carry out ICL repair but apparently possesses a more minimal collection of FA-related proteins, showing orthologues for only 9 of the 19 human proteins regarded as FA group proteins and lacking both Fan1 and mcm9 (Sekelsky 2017).

12.4.5.3 Seckel Syndrome

For this disorder and the related disorder autosomal dominant OCS, most of the mutations are

seen in the ATR protein (Alderton et al. 2004) – a protein involved in signalling the presence of DNA damage. However a small percentage of cases of Seckel syndrome seem to be associated with mutations of the DNA2 protein which functions both in repair (Shaheen et al. 2014) and in the maturation of Okazaki fragments during replication and the Ctip protein (Qvist et al. 2011) which acts in DSB repair to promote strand resection. Orthologues of both of these proteins are present in *Drosophila*. Seckel syndrome is associated with developmental disorders similar to other repair/replication diseases, but although it causes haematological abnormalities is not associated with cancer predisposition.

12.4.5.4 Meier-Gorlin Syndrome (MGS)

Unlike most of the other diseases discussed so far, MGS appears to be caused by defective DNA replication rather than repair. Despite this there is a large overlap with the previously discussed repair-related disorders in terms of the skeletal and development abnormalities that are observed. Unlike most of these however MGS is not cancer related.

MGS is associated with alterations in well-conserved proteins that are involved in the initiation of DNA replication and many in the original preRC complex, specifically ORC1, ORC4, ORC6, cdt1, cdc6, geminin and MCM5 (Bicknell et al. 2011; Burrage et al. 2015; Vetro et al. 2017). More recently mutation of CDC45 a preIC component and part of the replicative CMG helicase has also been detected in an MGS patient. (Fenwick et al. 2016).

The MCM4 protein is also part of the preRC and forms part of a complex with MCM5. However, mutations in MCM4 have not so far been identified as associated with MGS but have been linked with a mild form of another disorder – familial glucocorticoid deficiency (FGD) (Meimaridou et al. 2013). Although there is some overlap in symptoms with MGS, the predominant mechanism of these changes seems to be via glucocorticoid and NK cell deficiency and adrenal insufficiency. Individuals have mild susceptibility to cross-linking agents and are slightly cancer prone. This disorder is more commonly associated with changes in non-replication-related genes, and only a small fraction of cases are associated with MCM4 deficiency. The mechanism by which mcm4 causes this disorder and whether other members of the MCM complex can cause similar disorders is not clear.

12.4.5.5 NBS (Nijmegen Breakage Syndrome)-Related Syndromes

This group of disorders is caused by mutations in proteins that are involved in dsb repair, and the proteins involved are almost all conserved in *Drosophila*. The proteins are mostly associated with the HR repair pathway, although proteins that form part of the MRN complex are involved in both HR and NHEJ. NBS is associated with mutation of NBN (Chrzanowska et al. 2012), which forms part of the MRN complex with mre11 and rad50. Mutations in another component of the complex, rad50, cause, NBS like syndrome (Waltes et al. 2009).

The symptoms of the diseases associated with deficiencies in these proteins are very similar and also show a high degree of overlap with other diseases caused by repair and replication proteins (Table 12.3). However there is some variation between them, for instance, NBS is associated with immunodeficiency, whereas NBS-like syndrome is not.

Another disorder that shows similar features is Riddle syndrome, which is associated with mutation of Rnf168 an E3 ubiquitin ligases ring finger protein involved in HR but which is lacking in *Drosophila* (Stewart et al. 2009) (http://www.ensembl).

MRE11 mutations have not been linked to NBS-related syndromes but in addition to ATLD are responsible for nephronophthisis-related ciliopathies (NPHP-RC), a group of recessive diseases that affect the kidney, retina and brain (Chaki et al. 2012).

12.4.6 Monogenic Syndromes

Some replication/repair disorders have so far only been linked to mutation in one individual

protein. This does not rule out the possibility that, with more study, other genes may be linked to these disorders. In addition the symptoms of these monogenic syndromes often show high overlap with other replication and repair-related disorders suggesting some commonality in their mechanism of generation. Some monogenic disorders have been mentioned in previous sections of the chapter, for instance, MCM4- and MCM8-related diseases. Others have not been well studied, for instance, the high myopia associated with PrimPol mutations. In contrast monogenic diseases associated with mutations in RecQ helicases have received significant attention. Humans have five RecQ helicases, but only three of them wrn, blm and Recq4 have well-characterised disease association (Croteau et al. 2014). *Drosophila* has three intact RecQ helicases including BLM and RecQ4 and a fourth – the WRN orthologue which is represented only by a homologous C-terminal exonuclease domain (Sekelsky 2017; Saunders et al. 2008).

12.4.6.1 Blooms Syndrome

This is caused by mutations in RecQ family helicase Blooms (BLM) which appears to function in several repair pathways as part of the TR complex (Ellis et al. 1995). This shows symptoms a wide range of symptoms similar to those caused by deficiencies in other repair proteins but particularly characterised by a butterfly-shaped patch red skin on the nose and cheeks.

12.4.6.2 Werner's Syndrome

Mutations in another RecQ family helicase, Werner's helicase, which functions in DSBR, are responsible for this syndrome (Yu et al. 1996), which manifests quite distinctly from most of the other diseases discussed in this chapter. The predominant symptom of this disorder is early-onset ageing (symptoms appearing in sufferers in their 20s). These individuals also suffer from a number of age-related diseases, thin arms and legs and abnormal fat deposition in trunk and cancer predisposition.

12.4.6.3 Rothmund-Thomson/ Baller-Gerold/RAPADILINO Syndromes

A third RecQ helicase, RecQ4 (RecQL4), shows mutations in the coding region in more than 80% of sufferers from these three disorders (Larizza et al. 2010). They are usually treated as three separate disorders but are more likely variations of the same disorder. RecQ4 has both replication and repair roles associated with the N and C terminus of the protein, respectively. The position of disease-associated mutations suggests that it is likely to be the loss of the repair function which is causative; however the exact pathway affected has yet to be determined as RecQ4 has been suggested to act in multiple pathways. All of the diseases show a range of overlapping developmental, skeletal and skin abnormalities. They also all show infertility, early-onset ageing and cancer predisposition. BGS in addition shows unusual head shape, prematurely fused skull bones and mental retardation. RAP shows the common features plus loss of kneecaps, cleft palate and café au lait spots. As for other disorders, the severity and penetrance of the disease vary with the position of the mutation along the genome.

12.4.7 Diseases Caused by Defects in Mitochondrial Replication and Repair

Mitochondrial DNA replication is carried out mainly by specific mitochondrial proteins; however some nuclear replication proteins have also been shown to be important. Nuclear proteins are also important for mitochondrial DNA repair due to the apparent lack of dedicated mitochondrial repair pathways. It is likely that the redundancy of the mitochondrial genome makes DNA repair capability less important for mitochondria, and in fact these organelles possess mechanisms which promote the breakdown of genomes which are too badly damaged (Alexeyev et al. 2013). The mitochondrial-specific replication proteins in

most eukaryotes including *Drosophila* are a DNA polymerase (pol gamma) and a helicase (twinkle), and both of these show disease association (Table 12.4). These diseases mainly manifest with mtDNA depletion leading to a reduced or suboptimal mitochondrial function.

12.4.7.1 Mutations in DNA Polymerase Gamma

Polymerase gamma is the dedicated DNA polymerase associated with mitochondrial replication and consists of two subunits. Mutations in this protein lead to a several recognised disorders all of which show a subset of a range of symptoms (Copeland and Longley 2014; Copeland 2012) including slow growth, progressive spastic quadriparesis, progressive cerebral degeneration leading to mental deterioration and seizures, cortical blindness, deafness, liver failure, ataxias and progressive weakness of the extraocular eye muscles which causes strabismus and ptosis. These diseases are generally distinguished from each other by the range of symptoms shown, the severity of the disease and the age of onset of symptoms (Table 12.4). However there is still significant overlap between symptoms, and as each disorder is associated with mutations in different regions of the pol gamma gene, sequence is often used as definitive. Visual defects seem to be strongly associated with pol gamma-related syndromes. In this regard it may be relevant that mutations in two proteins that are thought to have both nuclear and mitochondrial functions also cause vision-related problems. Mutations in PrimPol, a protein carrying both polymerase and primase activities, are thought to be causative for high myopia, and mutations in DNA 2 are associated with progressive external ophthalmoplegia.

In addition to the core symptoms, many women with PEO from *POLG* mutations go through early menopause and suffer from high gonadotropin and low oestrogen concentrations, indicative of premature ovarian failure. Another observation that may be significant is that cosegregation of parkinsonism with mutations in *POLG* gene has been described in two individuals with adPEO.

12.4.7.2 Mutations in Twinkle

Twinkle is the mitochondrial helicase, and mutations in this protein have been seen to cause mainly adPEO but also epileptic encephalopathy with mtDNA depletion or infantile-onset spinocerebellar ataxia (Copeland and Longley 2014; Copeland 2012).

Twinkle mutation is also associated with a couple of less serious disorders: Perrault syndrome, a condition characterised by hearing loss in affected males and females and abnormalities of the ovaries in affected females, and progressive external ophthalmoplegia, a disorder that weakens the muscles that control eye movement and causes the eyelids to droop (ptosis).

Table 12.4 Diseases associated with mitochondrial replication malfunction

Disease	Abbreviation	Seen with mutations in
Alpers-Huttenlocher syndrome	AHS	Pol gamma
Childhood myocerebrohepatopathy spectrum	MCHS	Pol gamma
Myoclonic epilepsy myopathy sensory ataxia	MEMSA	Pol gamma
Mitochondrial recessive ataxia syndrome[a]	MIRAS	Pol gamma, twinkle
Sensory ataxia neuropathy dysarthria and ophthalmoplegia[a]	SANDO	Pol gamma, twinkle
Autosomal recessive progressive external ophthalmoplegia	arPEO	Pol gamma
Autosomal dominant progressive external ophthalmoplegia	adPEO	Pol gamma, twinkle
Epileptic encephalopathy with mtDNA depletion (very similar AHS)[a]		Twinkle
Infantile-onset spinocerebellar ataxia	IOSCA	Twinkle
Perrault syndrome		Twinkle
Progressive external ophthalmoplegia		Twinkle

All information from Copeland (2012) and Copeland and Longley (2014)
[a]Together make up ataxia neuropathy spectrum (ANS)

12.5 *Drosophila* Associations with Disease Research

To understand the diseased state of a pathway, it is important to understand the normal functioning of that pathway, and studies in *Drosophila* have made significant contributions to dissection of the basic mechanisms involved in both DNA replication and DNA repair. Early *Drosophila* embryos contain large amounts of replication proteins injected by the mother to enable the very rapid early cycles with average cell cycle times of just under 9 min (Farrell and O'Farrell 2014). This allowed the early biochemical isolation and characterisation of a number of core replication proteins, e.g. pol alpha primase, PCNA and RPA (Hua and Orr-Weaver 2017). Characterisation of *Drosophila* mutants that showed replication defects also contributed to the identification of central replication factors, for instance, metazoan cdt1 was first identified as the *Drosophila* mutant *double parked* (Whittaker et al. 2000). Work in *Drosophila* also made early significant contributions to the understanding of DNA repair. The discovery of several of key DNA repair pathways, including synthesis-dependent strand annealing, and DNA polymerase theta-mediated end joining was made in *Drosophila* (Boyd et al. 1990). In addition a number of repair proteins were also first recognised as *Drosophila* mutants that were defective in response to various types of challenge, for instance, the *mus*, *mut* and *mei* groups of mutants (Baker et al. 1976; Boyd et al. 1976, 1981).

More recent studies, both genetic and biochemical, have also made major contributions to our understanding of both of these pathways. The study of *Drosophila* mutants (both natural and engineered) has been particularly important in determining how DNA replication and DNA repair are controlled in an intact organism or specific organs, during development (Hua and Orr-Weaver 2017).

Although the utility of *Drosophila* as a model for human disease is well established (Chen and Crowther 2012; Millburn et al. 2016; Pandey and Nichols 2011; Sonoshita and Cagan 2017), it is at present underused to develop models for diseases specifically related to the DNA repair and DNA replication pathways. Specific *Drosophila* models have been established to study cancer and triplet diseases and are discussed in some detail in other sections of this book; however these have tended to focus on proteins outside of the repair and replication pathways. In addition mitochondrial diseases have also been modelled in *Drosophila* (Foriel et al. 2015), but again these largely do not focus on replication and repair but are directed towards proteins involved in oxidative phosphorylation. Although the mechanisms of some replication repair diseases are well understood, there remains a significant number, particularly those which cause pleiotropic symptoms, for which mechanistic details remain to be clarified. Therefore the potential of *Drosophila* as a model to study diseases related to defective replication and repair proteins still remain to be exploited.

Drosophila has a number of advantages as a model for replication and repair diseases. As can be seen from Tables 12.1 and 12.2, many disease-related proteins have well-conserved *Drosophila* orthologues, allowing meaningful construction of disease-related mutations. In addition although defects in replication and repair proteins can produce a complex array of different symptoms, assays relevant for the assessment of most of these have already been developed in flies. *Drosophila* has additional advantages in the study of the effects of mutations on complete organisms. Often disease-causing mutations in replication and repair proteins occur at multiple locations along a gene, requiring the manufacture of multiple differently mutated proteins to allow a systematic analysis of the differential effects of individual mutations. In most other organisms, the costs of generating large numbers of mutations would be prohibitive. Some of the diseases develop later in the life of an individual, and short life cycle of the fly facilitates the study of such disorders. In addition many of the diseases are recessive, resulting in low availability of a range of patients to study. There are also often a limited number of individuals carrying identical mutations making it hard to determine whether differences in penetrance of symptoms observed are

due to the particular mutation or the background of the individual carrying the mutation. In this regard it is possible to generate many flies carrying identical mutations and also to study the penetrance of symptoms in different backgrounds. Finally the ability to specifically express mutant proteins in particular tissues aids the study of mutations resulting in early lethality. Studies in *Drosophila* therefore have the potential to make a significant contribution to the study of diseases causes by mutations in replication and repair proteins, and hopefully ongoing work in a number of labs will allow this to be recognised over the next few years.

12.6 Nearest Neighbour Analysis of Protein Interactions (Crevel et al. 2001)

Many of the activities involved in DNA replication and DNA repair are carried out by large protein complexes; it is therefore of value to determine the nature of the complexes and the way that the proteins interact with each other. This protocol describes a way in which it is possible to map networks of proteins by determining those proteins that make direct contact with each other. It involves the use of DSP – a cleavable cross-linking agent with a short spacer arm to link proteins that show direct interaction with each other. The efficiency of the cross-linker is such that under the right conditions it is possible to predominantly produce linkage of only two proteins rather than higher-order structures, allowing identification of direct protein neighbours.

These assays can be carried out with either soluble extracts or with proteins that are chromatin associated.

Materials

Buffer A – 15 mM HEPES pH 7.9, 15 mM NaCl, 60 mM KCl, 2 mM EDTA, 0.34 M sucrose and complete™ protease inhibitors (Roche).

Buffer B – phosphate-buffered saline (PBS), 1% Triton X-100 plus complete™ protease inhibitors (Roche).

DSP (dithiobis (succinimidyl propionate)/ Lomant's reagent) – Sigma.

DMSO – dimethyl sulphoxide.

0.1% SDS.

1% Triton X-100.

PBS (phosphate buffered saline) – 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH of 7.4.

RIPA buffer.

Cross-linking of Soluble Extracts

Homogenise *Drosophila* embryos (1 g) or S2 cells (50×10^6 cells) in buffer A using a loose pestle. Centrifuge the homogenate centrifuged for 5 min at 5000 g at 4 °C in a benchtop centrifuge. Collect the middle layer and use directly or flash freeze in liquid nitrogen for later usage.

To the clear supernatant, add DSP to a final concentration of 0.5 mg/ml (freshly prepared in DMSO at 10 mg/ml). After an appropriate length of time on ice, stop the reaction by the addition of Tris pH 8 (final concentration 25 mM). 30 min is a convenient amount of time, but the timing may need to be titrated depending on the extract.

Denature the cross-linked material by adjusting to 1% SDS and incubating for 5 min at 100 °C. Centrifuge the extract for 30 min at 100,000 r.p.m. (Beckman TL100.3 rotor) at 20 °C, and adjust to 0.1% SDS and 1% Triton X-100 by the addition of 9 vol buffer B. Prior to immunoprecipitation filter the extract through a 0.22 μm filter.

Cross-linking of Chromatin Extracts

Homogenise *Drosophila* embryos (1 g) or *Drosophila* S2 cells (50×10^6 cells) in buffer A with a loose pestle. If using embryos filter the homogenate through Miracloth, then centrifuge for 20 min at 4000 r.p.m. (SS34, Sorvall) and wash once with the same buffer. If using cells leave out the filtration through Miracloth resuspend the nuclei-enriched pellet in 2 vol buffer A.

Add DSP to a final concentration of 0.5 mg/ml and incubate carried on ice. In this case 20 mins is a convenient amount of time, but again some titration may be necessary. Stop the reaction by the addition of Tris pH 8 to 25 mM and pellet the nuclei by centrifugation for 10 min at 7000 r.p.m.

(SS34, Sorvall). To remove the nuclear membrane and nucleoplasm, resuspend the pellet in 2 vol Buffer A, adjusted to 1% Triton X-100 and centrifuged for 10 min at 7000 r.p.m. (SS34, Sorvall). Repeat this step twice.

Resuspend the final pellet in 0.1 vol PBS and denature by the addition of SDS to 1%. Reduce the viscosity of the homogenate by sonication and remove particulate material by centrifugation for 30 min at 100000 r.p.m. (TL100.3). Adjust the clear supernatant to 0.1% SDS and 1% Triton X-100 in PBS and filter through a 0.22 μm filter prior to immunoprecipitation.

Immunoprecipitations

Incubate appropriate antibody beads with extracts overnight at 4 °C. Wash beads ten times with 20 vol RIPA buffer (adjusted to 650 mM NaCl). Elute immunoprecipitated proteins using 2% SDS in PBS and analyse by western blotting or PAGE.

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13 *Drosophila* Models to Investigate Insulin Action and Mechanisms Underlying Human Diabetes Mellitus

Yoshihiro H. Inoue, Hiroka Katsube, and Yukiko Hinami

Abstract

Diabetes is a group of metabolic diseases in which the patient shows elevated levels of blood sugar. In healthy condition, there is the regulatory system that maintains constant glucose levels in blood. It is accomplished by two hormones, insulin and glucagon acting antagonistically. Insulin is produced in β cells in pancreas and secreted to blood. It specifically binds to its receptors on plasma membrane and activates the intracellular signaling pathways. At the end, glucose in blood are taken into the cells. The diabetes is classified into two types. In type 1 diabetes (T1D), patients' pancreas fails to produce sufficient insulin. Hence, in type 2 diabetes (T2D), the target cells of insulin fail to respond to the hormone. The metabolic syndrome (MS) is characterized as a prediabetes showing lowered responsiveness to insulin. *Drosophila* has been expected to be a usefulness model animal for the diabetes researches. The regulatory system maintaining homeostasis of circulating sugar in hemolymph is highly conserved between *Drosophila* and mammals. Here, we summarize findings to date on insulin production and its acting mechanism essential for glucose homeostasis both in mammals and *Drosophila*. Subsequently, we introduce several *Drosophila* models for T1D, T2D, and MS. As a consequence of unique genetic approaches, new genes involved in fly's diabetes have been identified. We compare their cellular functions with those of mammalian counterparts. At least three antidiabetic drugs showed similar effects on *Drosophila*. We discuss whether these *Drosophila* models are available for further comparative studies to comprehend the metabolic diseases.

Keywords

Drosophila · Glucose homeostasis · Insulin-like peptides · Diabetes · Type 1 diabetes models · Type 2 diabetes models · Metabolic syndrome models

13.1 Introduction

Diabetes mellitus is a group of metabolic diseases in which the patient shows increased levels of blood sugar. The number of people with diabetes has risen four times for the past three decades. The global prevalence of diabetes among adults over 18 years has almost doubled for the periods. In 2015, an estimated 1.6 million deaths were directly caused by diabetes in the world. The WHO estimated that diabetes will be the seventh

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leading cause of death in 2030 (WHO 2016). The metabolic diseases have been considered to be basically caused by disturbance of sugar homeostasis in our bodies. Normally, there is a sophisticated regulatory system that maintains constant levels of glucose in blood. It is accomplished by two hormones acting, insulin and glucagon acting antagonistically (Aronoff et al. 2004). Both hormones are secreted from different specialized cells in Langerhans islet of pancreas. Insulin produced in pancreatic β cells are secreted to blood, and it specifically binds to its receptors on plasma membrane of target cells. The hormone activates the intracellular signaling pathway. At the end, the signal transmitted to a glucose transporter. Then, glucose existing in blood are taken through the transporter into the cells.

Diabetes are basically caused by a perturbation of somewhere in the process. The inefficient glucose uptake resulted in tissue dysfunction and/or infertility in adults. A development of various tissues and organs is also affected in fetal and neonatal diabetes. The hyperglycemia is caused by either insulin production or its action (David and Mervyn 2009). Therefore, diabetes is classified into two types. In type 1 diabetes (T1D), patients' pancreas fails to produce sufficient insulin. On the other hand, in type 2 diabetes (T2D), the target cells of insulin fail to respond to the hormone. T1D which were previously known as insulin-dependent, juvenile, or childhood-onset is characterized by deficient insulin production. Recently, lowered responsiveness to insulin (insulin resistance) has also caught a great deal of attention as a hallmark of a lifestyle diseases called metabolic syndrome. It is quite important for us to understand onset and pathogenesis of diabetes. Identification of novel genes involved in the processes provides great opportunity for development of therapeutic targets. Furthermore, understanding of basic mechanisms of the insulin production, secretion, and its action contributes to the advances of cell and development biology.

For the purpose, simple animal models allow us to proceed the biological studies faster and more conveniently. As described in other chapters of this book, *Drosophila* has been used as a quite useful animal model for medical studies on various human diseases. For the diabetes research, it has great advantages. The regulatory system that maintains homeostasis of circulating sugar in hemolymph is highly conserved between *Drosophila* and mammals (Baker and Thummel 2007; Haselton and Fridell 2010). In addition to the most sophisticated techniques of genetic analyses, a large amount of information on developmental biology and physiology is available in *Drosophila*. In this chapter, we, firstly, would like to summarize findings to date on insulin production and its acting mechanism essential for glucose homeostasis, remarkably conserved in mammals and *Drosophila*. Subsequently, we introduce several *Drosophila* models for T1D, T2D, and the metabolic syndrome considered as an initial stage of T2D, which have been so far established. And we describe new genes involved in fly's diabetes, identified using the diabetes models. Finally, we try to evaluate whether they are useful as animal models for diabetes studies. Furthermore, we discuss whether these *Drosophila* models are available for large-scale screens to develop new antidiabetic medicines (Pandey and Nichols 2011).

13.2 Glucose Homeostasis in Mammals and *Drosophila*

Before introducing *Drosophila* diabetes models and discussing their availability as human diseases models, we firstly compare regulatory systems to maintain glucose homeostasis in between mammals and *Drosophila* (Fig. 13.1). In mammals, insulin is a unique hormone that plays an indispensable role that controls glucose hemostasis in mammals (Wilcox 2005). The hormone is produced and secreted from β cells existing in the islet of Langerhans in pancreas. Another hormone, glucagon which acts antagonistically to insulin, is also secreted from α cells of the islet. A balance between these two hormones maintains a constant of circulating glucose in blood. The insulin gene encodes the insulin precursor, called preproinsulin, composed of signal peptides, A-chain, C-peptides, and B-chain in this order.

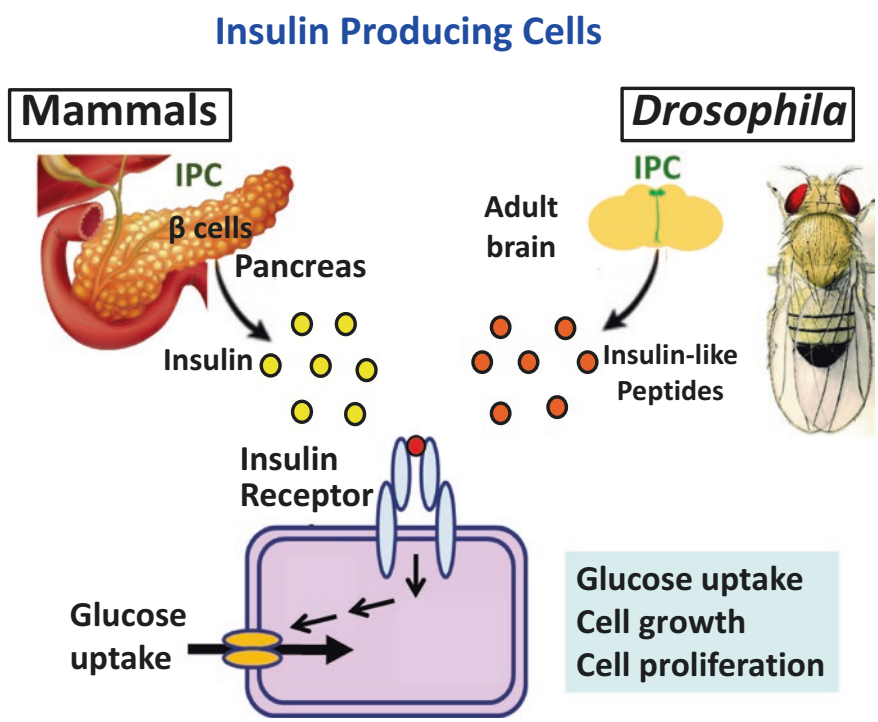

Fig. 13.1 Production, secretion, and signaling of insulin-like peptides and their effects to stimulate glucose uptake in *Drosophila*

After the signal peptide of the preproinsulin is cleaved, the polypeptide folds into the endoplasmic reticulum (ER), forming proinsulin. After the protein folding, the proinsulin is transported to the trans-Golgi network where immature granules containing proinsulin are formed. The polypeptide undergoes maturation into active insulin. After cleavage of the C-peptide located in the central portion of proinsulin, separated the B- and A-chains linked together again by two disulfide bonds, consists of matured insulin. Upon sensing increase of blood glucose and stimuli that promote secretion, secretary vesicles containing matured insulin releases from β cell by exocytosis that are mediated by SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) (Jewell et al. 2011). Insulin in blood binds to insulin receptor of target cells at high affinity, while it can also bind to IGF-1R (insulin-like growth factor 1 receptor) and IGF-2R (insulin-like growth factor 2 receptor) at a lower affinity (Jones and Clemmons 1995; Nakae et al. 2001). Both IGF-1 and IGF-2 play a role as growth factors that promote cell proliferation as well as cell growth, rather than hormones stimulating glucose uptake. These overlapped signaling is referred as the insulin-insulin-like growth factor signaling (IIS). The glucose transporters Glut1 and Glut3 in target tissue cells are involved in sensing of circulating glucose level (Joost and Thorens 2001; Rutter et al. 2015). According to the sensing, the insulin is subsequently released from pancreatic β cells, and it also stimulates glycolysis. This eventually results in stimulation of ATP synthesis in mitochondria, leading to inactivation of ATP-gated potassium channels (K_{ATP}), which allows plasma membranes of the β cell to depolarizing. Then, fusion of the plasma membranes and the insulin-containing vesicles and subsequently insulin secretion takes place (Kreneisz et al. 2010). Insulin secreted from the pancreatic β cells binds

to the extracellular α subunit of the InR, activating tyrosine kinase domain intrinsic to its β subunit (Lee and Pilch 1994). Binding of insulin to the α subunit induces a conformational change, resulting in the autophosphorylation of several tyrosine residues in the β subunit (Van Obberghen et al. 2001). These phosphor-amino acid residues are recognized by phosphotyrosine-binding domains of adaptor proteins such as the insulin receptor substrate family (IRS1~4) (Saltiel and Kahn 2001; Lizcano and Alessi 2002). The phosphotyrosine residues on IRS proteins are recognized by the SH2 domain of the p85 regulatory subunit of PI_3-kinase. The catalytic subunit of the kinase, p110, then phosphorylates PIP_2 converting to the formation of PIP_3. A downstream effector of PIP_3 is another kinase, AKT, which is recruited to the plasma membrane. Activation of the AKT requires the protein kinase 3-phosphoinositide-dependent protein kinase-1 (PDK1). Once AKT is activated, it catalyzes phosphorylation and inactivation of glycogen synthase kinase 3 (GSK3). A major substrate of GSK3 is glycogen synthase, an enzyme that catalyzes the critical step in glycogen synthesis. Phosphorylation of glycogen synthase by GSK3 inhibits glycogen synthesis; therefore, the inactivation of GSK3 by AKT promotes energy storage as glycogen. These protein kinases are responsible for mediating many of the ultimate metabolic actions of insulin, including translocation of GLUT4 in mammals, activation of glycogen synthesis, and suppression of gluconeogenesis by inhibiting transcription of the gene encoding phosphoenolpyruvate carboxykinase (Brady et al. 1998; Lochhead et al. 2001).

Whereas, in *Drosophila*, sugars in diet are taken from the digestive duct and transported into the fat body (FB), which acts as a homologous tissue of both the liver and adipose tissue in insects, respectively. They are converted to trehalose in FB. The disaccharide is once converted to glycogen and stored in the FB. And it is released into the hemolymph when needed, while glucose is only contained less than one-hundredth of trehalose (Nation 2002; Ugrankar et al. 2015). The sugar levels in hemocytes are antagonistically controlled by *Drosophila* insulin-like peptides (DILPs) and the glucagon-like peptide, adipokinetic hormone (AKH) (Ikeya et al. 2002; Rulifson et al. 2002; Kim 2004; Lee and Park 2004). The DILPs that belong to the insulin-relaxin superfamily are composed of eight members involved in Dilp1~8 (Fig. 13.2). (Wu and Brown 2006; Colombani et al. 2015). But no orthologues of genes encoded by mammalian IGF peptides have been found in *Drosophila* genome. Dilp8 is divergent from other members to some extent and bind to relaxin-type membrane receptor, Lgr3 (Colombani et al. 2015; Vallejo et al. 2015). The DILPs except Dilp8 binds to a single receptor, termed the insulin receptor homolog (InR) on the plasma membrane of their target cells (Nishida et al. 1986; Fernandez et al. 1995).

The Dilp binding leads to activation of tyrosine kinase domain furnished in the receptor and recruiting of docking protein, Chico/IRS orthologue, and Lnk (Fig. 13.2). Though the docking protein binds the p60 regulatory subunit of PI_3K (Pi3K92E), the InR recruits the protein together with the catalytic subunit (Pi3K21B) to the plasma membrane. The active PI_3K converts PIP_2 to PIP_3 in the plasma membrane. A formation of the PIP_3 is negatively regulated by the PTEN protein, a phosphatase that dephosphorylates PIP_3 and coverts it to PIP_2. The signaling molecule, PIP_3, recruits two kinases, PDK1 (Pdk1) and AKT (dAkt1), to the plasma membrane, enabling the PDK1 to phosphorylate AKT. Akt1 is the core kinase component of the insulin/insulin-like growth factor signaling (IIS) pathway. It functions downstream of Pi3K92E and is activated by phosphatidylinositol binding and phosphorylation. It mediates versatile signaling pathways essential for cell growth and survival. The AKT phosphorylates several substrates including to the transcription factor, Foxo (forkhead box protein O), a critical transcription factor for metabolism and stress responses. It activates another kinase, glucose synthase kinase 3 (GSK-3/Sgg). And it results increased glucose uptake and fatty acid synthesis at the end. A con-

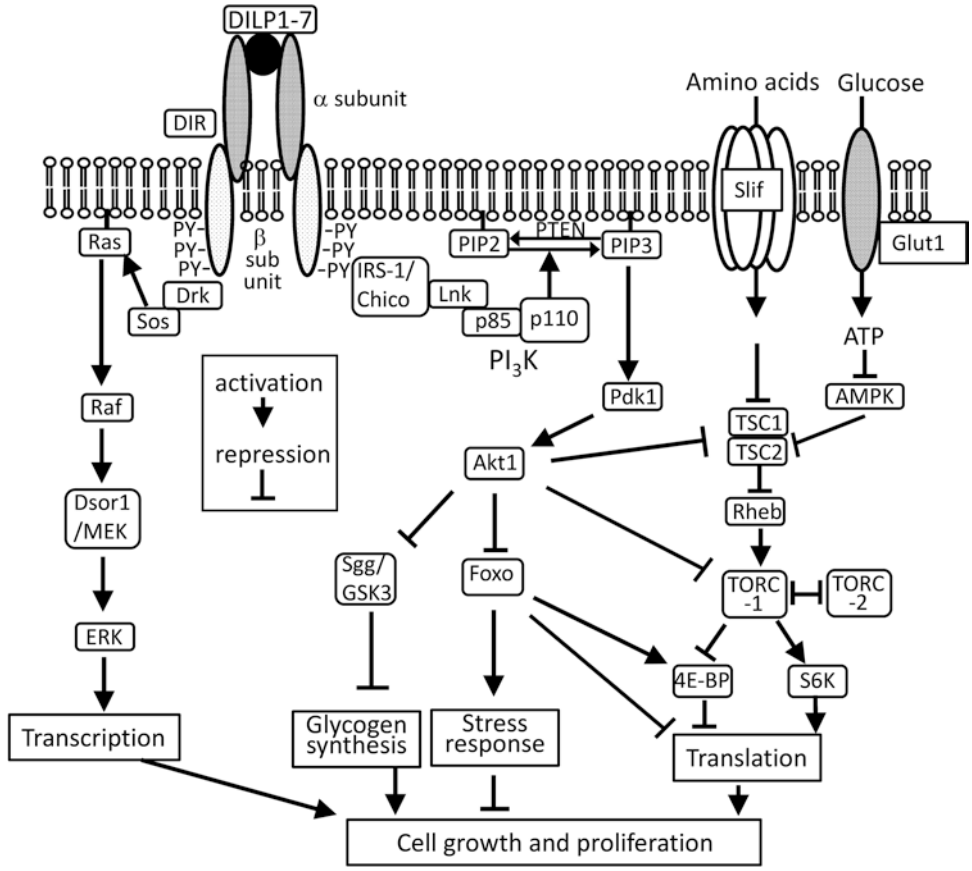

Fig. 13.2 Signal transduction pathway of DILPs and a transport of glucose and amino acids in *Drosophila*. The insulin-like peptides (Dilp1-7) (black sphere) bind to the extracellular α subunit of their specific receptor, DIR, on the plasma membrane. The binding activates the tyrosine kinase in the β subunit and signaling pathways locating downstream. The binding leads to activation of tyrosine kinase domain furnished in the receptor and recruiting of docking protein, Chico/IRS orthologue, and Lnk. Though the docking protein binds the p60 regulatory subunit of PI_3K, the DIR recruits the protein together with the catalytic subunit (p110) to the plasma membrane. The active PI_3K converts PIP2 to PIP3 in the plasma membrane. A formation of the PIP3 is negatively regulated by the PTEN protein, a phosphatase that dephosphorylates PIP3 and coverts it to PIP2. The signaling molecule, PIP3, recruits two kinases, Pdk1 and Akt1, to the plasma membrane, enabling the PDK1 to phosphorylate AKT. Akt1 is the core kinase component of the insulin/insulin-like growth factor signaling (IIS) pathway. It functions downstream of PI_3K and is activated by phosphatidylinositol binding and phosphorylation. It mediates versatile signaling pathways essential for cell growth and survival. The Akt1 phosphorylates several substrates including to the transcription factor, Foxo, a critical transcription factor for metabolism and stress responses. The kinase phosphorylates and inhibits the Tsc1/Tsc2 complex, which is an inhibitor of the Tor signaling pathway, an essential regulator of growth and metabolism. The Akt1 also activates another kinase, Sgg/GSK-3 essential for inhibition of glycogen synthesis. A concentration of glucose in the hemolymph is sensed by GLUT1 and the glucose uptake occurs through the glucose transporter, associated with the insulin signal transduction

served mechanism to sense the glucose concentration in hemolymph is also present in *Drosophila* (Rulifson et al. 2002; Kim 2004; Kreneisz et al. 2010). In conclusion, as insulin peptides and the IIS pathway are well conserved between *Drosophila* and mice, *Drosophila* can be used as one of animal models for studies on insulin production, secretion, and glucose homeostasis.

13.3 Insulin-Like Peptides that Control Growth and Proliferation as well as Sugar Metabolism in *Drosophila*

A series of genetic studies on mutants for DILPs and signaling factors of the IIS pathway have revealed that they play essential roles in cell growth and proliferation of various tissues during *Drosophila* development (Fig. 13.4) (Brogiolo et al. 2001; Hsu and Drummond-Barbosa 2009; Kannan and Fridell 2013). There are some differences in action of insulin-like peptides between *Drosophila* Dilps and mammalian insulin. In mammals, insulin and IGFs basically divide respective role, insulin for glucose uptake and IGFs for cell proliferation and growth. On the other hand, in *Drosophila*, DILP1~7 have both growth factor function and metabolic function to maintain sugar homeostasis (Fig. 13.1). Furthermore, these functions are different from each other in between larval stage and adult stage. DILPs regulate growth of all tissues in both somatic and germline cells, whereas their effects in adults are predominantly restricted to metabolite homeostasis, stress response, fecundity, and longevity (Broughton et al. 2005; Gronke et al. 2010). Insulin is known to play an essential role in glucose uptake in *Drosophila* cells (Ceddia et al. 2003), and seven genes encoding insulin-like peptides (ILPs) have been identified in the *Drosophila* genome. These peptides are synthesized in clusters of medial neurosecretory cells in the *Drosophila* brain (Rulifson et al. 2002). The InR and its downstream signaling cascade are well conserved in *Drosophila* (Fernandez et al. 1995; Bohni et al. 1999). It has been reported that InR and its signaling cascade can stimulate both cell proliferation and growth in cultured *Drosophila* cells as well as in larval imaginal cells (Chen et al. 1996; Brogiolo et al. 2001).

In addition to proliferation and growth of somatic cells, Dilps also play a critical role in cell growth, proliferation, and maintenance of germline cells during gametogenesis. Division of germline stem cells is a critical step that determines the numbers of germ cells. Tissue-extrinsic signals that reflect the nutrient condition of the organisms influences stem cell proliferation and their maintenance in both females and males (Hsu and Drummond-Barbosa 2005; Hsu et al. 2009; Ueishi et al. 2009). Hypomorphic *InR* mutant females exhibit infertility, and the number of cysts produced from female GSCs decreased. Furthermore, growth of nurse cells was also inhibited in the mutant ovaries (LaFever and Drummond-Barbosa 2005). This result suggests that the IIS signaling is required for female GSC division and cell growth of their progenies within the egg chambers (Hsu and Drummond-Barbosa 2009). *Drosophila* oogenesis is dependent on environmental nutrient conditions (Fuller and Spradling 2007). Therefore, it is reasonable to assume that both cell number and growth in egg chambers are directly regulated by hormonal control via DILPs. Furthermore, maintenance of the stem cells depends on local signals provided by niches, in which the stem cells reside (Fuller and Spradling 2007). In addition to common regulatory factors required for maintaining stem cells, Drummond-Barbosa's group showed that insulin signaling integrates the effects of nutrient and age on germline stem cell (GSC) maintenance. This is mainly regulated by Notch signaling mediated by interaction between GSC and niche cells, called cap cells, sending the signal that maintain GSC, mediated by E-cadherin (Inaba et al. 2010). The authors also reported that the loss of GSC and niche cell occurs with age and that the age-dependent impairment can be suppressed by increased levels of Dilp2. These results indicate that the Dilp signal plays an important role in the regulation of stem cell niches and, thereby, of stem cell numbers.

Wheares, Spermatogenesis in *Drosophila* commences with cell division of GSCs to produce male germline cells at the tip of the testis. The study of spermatogenesis in *Drosophila* can aid in understanding the regulatory mechanisms underlying cell proliferation and growth during development. In young adult *Drosophila* males, 5

to 8 GSCs are usually present at the tip of the testis. To maintain their multipotential stem cell characteristics, GSCs receive signals from the adjoining hub cells. Both a ligand encoded by the unpaired gene and the JAK-STAT signaling cascade are involved in this signal transfer (Yamashita 2008); (Tulina and Matunis 2001). The proximal cell of the two daughter cells derived from asymmetric division of a stem cell exclusively receives the unpaired signal and becomes a self-renewed GSC. For self-renewal and differentiation of GSC daughters, it is crucial to set up cell division axis perpendicular to a cluster of the hub cells (Yamashita et al. 2003). The distal daughter cell leaves the niche and differentiates as a spermatogonium, which then undergoes cell division four times to produce a 16-spermatocyte cyst. Ueishi and colleagues found that inhibition of insulin production and insulin signaling mutations resulted in decreased numbers of germline cells in *Drosophila* testes. GSC numbers were maintained in young mutant males, with a gradual decrease in abundance of GSCs with age. Furthermore, in mutants, a lower frequency of GSC division was seen. Insulin signaling was found to promote cell cycle progression of the male GSCs at the G2/M phase. The spermatocytes differentiated from a progeny of GSC enter a growth phase during which they increase remarkably in volume by up to 25-fold. This is the largest extent of cell growth that proliferative cells can accomplish. Although the extracellular signal and the signaling cascade that maintain GSC numbers have been partially identified (Fuller and Spradling 2007); Inoue et al. 2012), the signals and regulatory factors that allow the spermatocytes to increase up to such a remarkable extent before meiotic initiation had been identified.

The *Drosophila* premeiotic spermatocytes have achieved most distinctive cell growth up to 25 times after premeiotic DNA replication. Ueishi and colleagues reported that a loss of DILPs by specific apoptosis induction to insulin-producing cells interfered growth of spermatocytes, suggesting that the spermatocyte cell growth is required for DILPs (Ueishi et al. 2009). They further showed that an accumulation of active Akt form phosphorylated by its upstream factor, PDK1, in the growing spermatocytes. A diameter of spermatocytes from mutant males for InR or IRS orthologue encoded by *chico* decreased in size. We further showed that the expression of constitutive active form of PI_3-kinase catalytic subunit significantly stimulated the spermatocyte growth (Ogata and Inoue unpublished). These genetic data strongly suggest that the ILPs and its signaling cascade through PI_3-kinase to Akt play a role in induction of the remarkable cell growth in *Drosophila*. As mammalian insulin can also activate the Ras-MAP kinase cascade after the insulin receptor (as a review, (Avruch 1998)), we further showed that $Ras85D^{v12}$, a constitutively activated mutation for *Ras85D* (Kim et al., 2006) also induced approximately 10 % increase of cell diameter in length (Ogata, Azuma and Inoue, unpublished). These genetic data suggest that both PI_3K-Akt cascade and Ras-MAP kinase cascades acting downstream of InR are essential for induction of the premeiotic spermatocyte growth. The *InR* mutations also interfered cell growth within egg chambers in ovaries without affecting cyst morphology and cell numbers (LaFever and Drummond-Barbosa 2005). Taking together, these findings indicate that the IIS plays a critical role in both oogenesis and spermatogenesis in *Drosophila*.

As other elucidated roles of the Dilps in adult stage, they are involved in resistance to various stresses such as starvation stress and oxidative stress and lifespan (as a review, (Owusu-Ansah and Perrimon 2014)). The peptides are also involved in regulations of adult vision, behavior, and their appetite. The specific neurons producing Dilp7 stimulates the adult intestine as to promote their appetite (Miguel-Aliaga et al. 2008; Cognigni et al. 2011). The Dilp7 regulates adult female behavior to decide egg-laying (Sousa-Nunes et al. 2011; Bai et al. 2012). This is the reasonable regulation that allows female flies to coordinate their food uptake with promotion of fecundity.

13.4 Human Diabetes: Type 1, Type 2, and Metabolic Syndrome

Human diabetes is a group of metabolic diseases defined by the increased levels of blood sugar, termed hyperglycemia. They are generally classified into either since the pancreas fails to produce sufficient insulin (T1D) or since the target cells of insulin fail to respond to the hormone (T2D) (Katsarou et al. 2017; DeFronzo et al. 2015). T1D account for an estimated less than 10% of all diabetes cases. This type disease had been previously considered to be induced by an autoimmune condition in which the immune system is activated to destroy the IPC in the pancreas (Hanafusa and Imagawa 2007). Alternatively, another class of T1D that the β cell loss arises from unknown causes, without autoantibodies, has been reported. It has been suspected whether inflammation, various cell stresses, and insulin resistance that take place in the insulin-producing cells would lead to the cell death (Eizirik et al. 2009; Bluestone et al. 2010; Atkinson et al. 2011; Katsarou et al. 2017). Particularly, it has been attracted interest that the endoplasmic reticulum (ER) stress is involved in the β cell loss, which might result from continuously enhanced insulin production in the insulin resistance condition. Before the cell death has seriously occurred in cells where ER stress has accumulated, reduction and/or loss of cell activity has been commonly observed in many cases (Sreenan et al. 1999; Ferrannini 2010). Because of that, the patients with this form of diabetes failed to produce or secrete sufficient insulin.

On the other hand, T2D is defined as a long-term metabolic disorder that is characterized by high blood sugar (hyperglaycemia), insulin resistance, and relative lack of insulin response. The insulin resistance is commonly observed from earlier stage of T2D. This class accounts for about 90% of cases of diabetes, with the other 10% due primarily to T1D 1 and gestational diabetes. One of the most characteristic properties of T2D is the impaired response of target organs to insulin, called insulin resistance (Weyer et al. 1999; Kahn et al. 2014). The primary causes of insulin resistance have not yet been clarified in most of the cases. As the primary causes of the resistance, one can simply speculate that impairment of insulin production and secretion, such as downregulation of a IIS factor, takes place. It has been shown that mutations in the insulin gene and InR gene were responsible for sever hyperglycemia syndromes associated with insulin resistance, such as type A insulin resistance and the Rabson-Mendenhall syndrome (Liu et al. 2015; Meur et al. 2010; Jiang et al. 2011). Some mutations decrease the amount of insulin receptors localized on the cell surface. Other mutations impair the functions of the insulin receptor (refer https://www.ncbi.nlm.nih.gov/gene/3643). Patients carrying mutations at both alleles display more severe phenotype than are patients heterozygous for the mutation. And furthermore, if a non-cell autonomous factor, which prevent insulin from binding to its receptor, is expressed, the IIS is certainly inhibited. To compensate elevated glucose levels due to the resistance, organisms try to raise insulin secretion. This counteracts the insulin sensitivity of the target tissues, leading to rather worsen the symptom (Kasuga 2006; Kahn et al. 2014). Due to inadequate compensation, the glucose intolerance arises from the combination of insulin resistance and deficiency of functional insulin.

Metabolic syndrome (MS) is a collection of risk factors that includes glucose dysregulation, central obesity, dyslipidemia, and hypertension. There are multiple definitions that have been described regarding the criteria of the syndrome. This clustering of risk factors is obviously linked to an increased risk of developing T2D. The MS is also characterized by insulin resistance. Prediabetes, which is a combination of insulin resistance and excess body fat, is considered an underlying cause of MS. Therefore, similar biomedical and genetic studies to those having been performed to clarify the T2D pathogenesis can be applied to studies on onset and development of the MS. For the purpose, diabetes models representing the characteristics of T1D, T2D, and MS, have been individually established in *Drosophila*.

13.5 *Drosophila* Models for Type 1 Diabetes

For understanding mechanisms that the insulin-producing cells (IPC) are lost by the cause other than autoimmune condition as observed in T1D patients, experimental animals that allow us to induce cell death in the insulin-producing cells might provide a valuable suggestion. Here, we would like to describe some *Drosophila* models reproducing the IPC loss and phenotype of human T1D (Figs. 13.3a and 13.4a). Immunostaining experiments with anti-Dilps antibodies revealed that three major Dilps (Dilp2, 3 and 5) among seven Dilp members are synthesized, stored, and secreated from specialized neuronal cells, named insulin-producing cells in brains (Ikeya et al. 2002; Broughton et al. 2005). Although the IPCs correspond to only 14 median neurosecretory cells in the *Drosophila* central nervous system, these cells can play an equivalent role as IPCs to the β cells in mammals. Thus, if cell death exclusively occurs in these 14 of IPC cells, this can reproduce T1D-like phenotypes in the *Drosophila* (Fig 13.4a). To generate *Drosophila* lacking of IPC, genetic ablation of IPC was achieved by ectopic induction of apoptosis exclusively in the cells using Gal4/UAS system. As mentioned in previous chapter, the Gal4/UAS system allows us to carry out ectopic induction of any genes located under the UAS sequences (see Chapter 1, (Phelps and Brand 1998)). Using the system, Rulifson et al. induced apoptosis specifically in larval IPC neurons by ectopic expression of pro-apoptotic gene, *reaper* (Rulifson et al. 2002) (See Figs. 13.3a and 13.4b, c). The genetic ablation of the larval IPC resulted in the elevated circulating sugar levels in the larval hemolymph. And the hyperglycemia phenotype resulted in developmental delay and growth retardation at larval stage. Eventually smaller adult flies emerged (Rulifson et al. 2002). These phenotypes are reminiscent of hallmarks of T1D, a loss of pancreatic β cells, and undernutrition. Another experiment that carried out the ablation of IPCs using a different procedure provided the same phenotypes in both larvae and adults. A genetic ablation of IPCs was performed in adults and compared their phenotypes with those seen in larvae (Haselton et al. 2010). For ablation of IPC cells at adult stage, a modified Gal4/UAS system called the GeneSwitch was used. This is based on a GAL4-progesterone receptor chimera that is hormone inducible, which is specifically activated after binding of the activator RU486, which is fed to flies (Osterwalder et al. 2001). Normal adults contain circulating sugars in hemolymph at a lower concentration during and

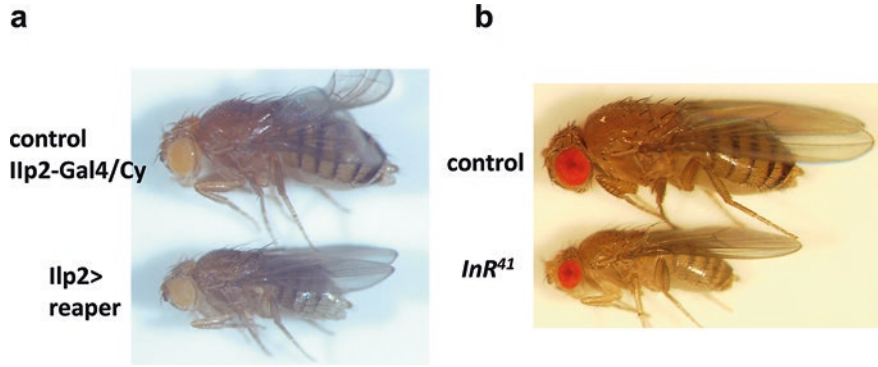

Fig. 13.3 A *Drosophila* type 1 model having genetic ablation of the insulin-producing cells (IPCs) and a model fly showing insulin resistance which is a hallmark of type 2 diabetes. (**a**) (upper) A control fly (*InR /TM3, Sb*). carrying a balancer chromosome carrying Cy (*ilp2-Gal4/Cy*). (lower) A fly derived from larvae expressing pre-apoptotic gene, *reaper*, exclusively in IPCs by Gal4/UAS system (*ilp2>reaper*). (**b**) (upper) A control fly *(InR41/TM3, Sb)*. (lower) A fly homozygous for a hypomorphic *InR* mutation (InR^{41}), displaying insulin resistance due to a reduced expression of *InR* gene. Note that both models show significantly smaller than their sibling controls, indicating that a growth retardation has occurred during larval and pupal stages in both cases

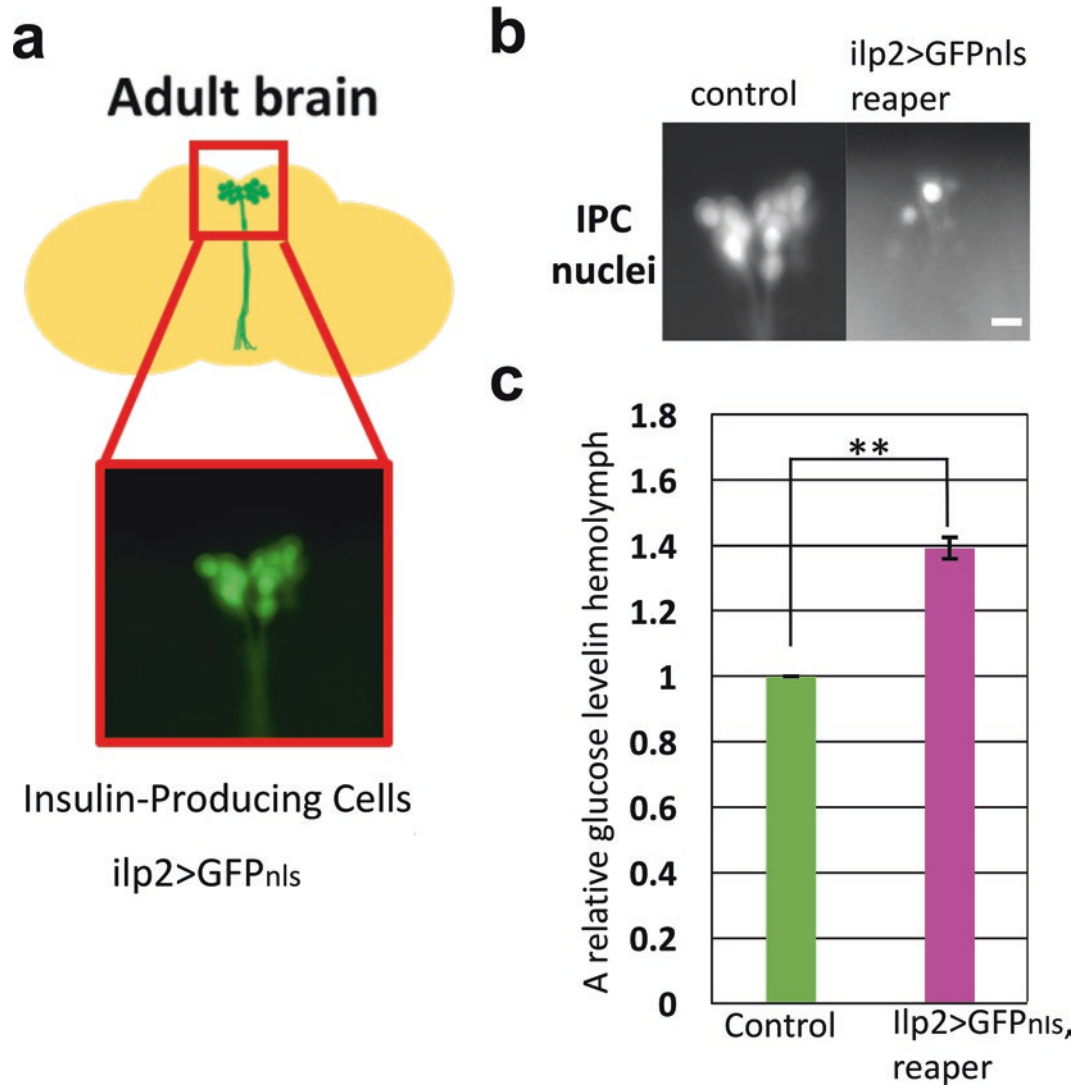

Fig. 13.4 A visualization of IPC in adult brains and genetic ablation of the cells by ectopic induction of apoptosis. (**a**) A illustration of insulin-producing cells in an adult brain. The producing cells can be visualized by the IPC-specific expression of GFP having nuclear localization signal sequences using Gal4/UAS system (ilp2>GFPnls). (**b**) Observation of adult IPC. Note that a distinctive decrease of the cells was observed in the adult brain expressing the preapoptotic gene, reaper (middle). (**c**) Quantification of circulating glucose levels in larval hemolymph. A significant elevation of the levels is seen in the IPC-ablated flies, compared with those of control flies

after fasting (glucose clearance). Once the flies resumed feeding, the sugar level immediately increased. On the other hand, adults lacking IPC by the genetic ablation at adult stage displayed higher sugar levels and slower glucose clearance. Interestingly, the flies lacking of the IPCs displayed extended adult lifespan as an effect of calorie restriction (Haselton et al. 2010). The glucose intolerance seen in the flies was rescued by injection of mammalian insulin, indicating that the flies did not show the strong insulin resistance. The adult stage-specific ablation of the

insulin-producing neurons modulates glucose homeostasis and extends lifespan without insulin resistance. Regarding to the hyperglycemia phenotype, the effects of the IPC ablation are consistent between larval stage and adult stage. Its influence is largely restricted to metabolic homeostasis, resistance to stress, fecundity, and lifespan rather than growth aspects in adults (Owusu-Ansah and Perrimon 2014). Both glucose feeding and fasting experiments more easily manipulate in adults rather than in larvae. It is more difficult to create fasting condition in larvae. Similar to glucose clearance in mammals, wild-type adults displayed the rapid response to decline glucose levels in hemolymph after fasting. Whereas, the IPC-ablated flies showed higher sugar levels than control adults did (Haselton et al. 2010). These evidences suggest that one can obtain more reliable results regarding on regulation of glucose homeostasis by experimental systems using adults rather than larvae.

Among known eight Dilps in *Drosophila*, Dilps1-7 play positive roles essential for cell growth and cell prolifeation. They are also required for glucose homeostasis or roles related to the issues (Brogiolo et al. 2001; Ikeya et al. 2002; Rulifson et al. 2002; Broughton et al. 2005; Gronke et al. 2010; Yang et al. 2008; Veenstra et al. 2008; Okamoto et al. 2009; O'Brien et al. 2011; Bai et al. 2012), In contrast, the Dilp8 has a role in regulation of adaptive development in a response to tissue damages (Colombani et al. 2012; Garelli et al. 2012). Genetic interaction exists among genes required for sugar homeostasis. There is a functional redundancy rescued by another member(s) of DILPs. Thus, a deletion of each *Dilp* gene has no phenotypes in every case. Therefore, their functions have been speculated by mainly expression patter and dominant phenotypes by overexpression experiments (Owusu-Ansah and Perrimon 2014). Three Dilps, Dilp2, 3, and 5 expressing in the IPCs, play a central role in glucose homeostasis. Dilp2 presents highest sequence homology with human insulin. These three Dilps play a central role in glucose homeostasis as well as cell growth and proliferation. Dilp1 is also produced in the IPCs and involved in body size determination, while Dilp4 expressing in larval midgut is involved in larval growth. Dilp6 and Dilp7 expressing in other tissues than the IPCs are more related to adult activity such as growth after diet feeding and egg-laying behavior, respectively (Brogiolo et al. 2001; Yang et al. 2008; Okamoto et al. 2009; Bai et al. 2012; Yang et al. 2008). Homozygous mutants for five *Dilps1-5* genes showed a sever growth defects and developmental delay, quite similar to those seen in the IPC ablation (Zhang et al. 2009). Some of homozygous flies deficient for five Dilps genes (*Dilp1-5*) can survive until adult stage, although many of them died during development. The survivors showed the small fly phenotype, indicating a strong growth retardation. Phenotypes of homozygous mutants for five *Dilps* (*Dilp1-5*) genes were mostly overlapped with those generated from the IPC ablation as described above. Diabetes symptoms, growth defects, and development delay have also been observed in the larvae deficient for major *Dilps* genes (Zhang et al. 2009). The authors also reported that the animals contained increased sugar levels in hemocytes; instead they had reduced triacylglycerides (TG) which is a major fat stored in their bodies in amount and reduced heat production. This is a reflection of lowered metabolic activity. These phenotypes are all reminiscent of T1D hallmarks.

There is a bit difference in phenotypes induced by the insulin depletion between *Drosophila* models and mouse mutants. The knockout mouse deficient for the insulin genes in both alleles results in lethal at neonatal stage (Duvillie et al. 1997). Mouse has two insulin genes, *Ins1* and *Ins2*. Double homozygous mutant pups displayed severe growth retardation. They did not show any glycosuria at birth. But soon after suckling, they developed diabetes with ketoacidosis and died within 48 h. The insulin deficiency did not preclude pancreas organogenesis and the appearance of the various cell types of the endocrine pancreas. Although some of homozygous flies deficient for five Dilps genes (*Dilp1-5*) were viable, many of them died during development. The survivors showed the small fly phenotype indicating a strong growth retardation. In conclusion, both

Drosophila T1D models generated from genetic ablation of the IPCs and those homozygous for mutations of major *Dilp* genes appear to be suitable for genetic studies on T1D.

13.6 *Drosophila* Models for Human Type 2 Diabetes

As we described previous sections, one of the most characteristic properties of T2D is the insulin resistance. This symptom has been considered as earlier stage of the T2D pathogenesis. To gain insight into mechanism by which insulin resistance occurs and progress into T2D, simple model organisms, particularly *Drosophila*, have been considered to be more suitable for the purpose, because they are capable of performing precise genetic analyses.(Fig. 13.3b).

High-sugar diet models: A simple *Drosophila* model for studies on diet-induced T2D was initially established (Musselman et al. 2011). The authors fed wild-type larvae on high-sugar diet (HSD) containing seven times higher sucrose than control diet. The larvae raised on HSD had increased levels of both circulating glucose and trehalose in the larval hemolymph. The larval development of the larvae delayed significantly, and the fat accumulation increased, as observed in T1D fly models. Injection of mammalian insulin to the larvae failed to restore the impaired insulin response only partially as speculated by levels of phosphorylated Akt in IIS. This suggests that insulin resistance occurred as seen in T2D patients. Expression of Dilp2 peptide has risen in the larvae adapting to a prolonged higher level of glucose. This phenotype corresponds to a human hyperinsulinemia ((DeFronzo et al. 2015)). And furthermore, microarray analysis to identify genes which transcription has changed demonstrated that target genes of the Foxo transcription factor were upregulated in the models. This is consistent with observations in gluconeogenic livers of insulin-resistant mice (Michael et al. 2000) and T2D patients (O'Brien et al. 2011). The insulin resistance seems to occur by evolutionarily conserved mechanisms, because the transcriptional effects of high-sugar feeding was observed commonly in mouse, human, and *Drosophila* insulin resistance. From studies using another model of the HSD-feeding larvae established independently, same conclusions were obtained. Continuous feeding of HSD for a longer period resulted in hyperglycemia, growth retardation, hyperinsulin induction, and excess accumulation of fat (Pasco and Leopold 2012). Similarly, adults raised on HSD also displayed a diet-dependent weight gain, metabolic dysfunction, elevated Dilp mRNA, and decreased activity of insulin signaling as far as examined in fat body cells. These phenotypes indicate that insulin resistance, which is a hallmark of human T2D, can be reproduce in *Drosophila* larvae and adults raised on HSD (Morris et al. 2012). Another HSD-induced adult model also displayed hyperglycemia, insulin resistance, increased fat accumulation, and shortened lifespan (Na et al. 2013). The HSD-induced models have an advantage that more sever hyperglycemia phenotype can be observed rather than in flies possessing IPC ablation (Rulifson et al. 2002; Song et al. 2010).

The insulin resistance eventually resulted in induction of the target genes of the stress JNK cascade (Musselman et al. 2011). This result suggested that there is a genetic interaction between the insulin signaling pathway and the stress MAP kinase pathway. Pasco and Leopold further obtained interesting genetic results that the HSD-triggered insulin resistance was suppressed by ectopic overexpression of a *Drosophila* orthologue of lipocalin, Nlaz. Expression of the *Nlaz* gene increased in larvae raised on HSD (Pasco and Leopold 2012). It had previously been known that lipocalin 2, a small extracellular protein, can modulate of diabetes phenotype in mouse (Gavi et al. 2007; Kim et al. 2012). In *Drosophila* larvae, *Nlaz* mutants or restricted depletion of the gene in fat body can rescue metabolic disorders seen in HSD-induced larval models. These genetic data from mouse and *Drosophila* models suggest that there is a therapeutic potential to rescue diabetes type 2 patients. These genetic evidences derived from *Drosophila* diabetes models can contribute to mammalian studies to verify mechanism as well as to develop therapeutic protocol.

Other *Drosophila* T2D models generated by genetic modifications have also been established. For example, heterozygotes for an *InR* mutation exhibited reduced the receptor activity, and the insulin signaling must be impaired in the mutant flies (Fig. 13.4b) (Tatar et al. 2001). As a result, the DILP secretion was enhanced. The FB-specific depletion of InR reproduced the hyperinsulin production and insulin resistance (Park et al. 2014a). These fly phenotypes are reminiscent of hyperinsulinemia in live-specific gene disruption of InR gene in mouse (Michael et al. 2000).

To understand the mechanisms underlying T2D pathogenesis, it is essential to identify new molecular markers for gene diagnosis and targets for therapeutic intervention of T2D. To achieve the purpose, genome-wide association studies (GWAS) have been carried out in T2D patients. Over 90 disease-associated SNP loci associated with human T2D have been identified (Renstrom et al. 2009; Dimas et al. 2014). However, it is not certain whether these genetic loci are really involved in pathogenesis of insulin resistance and H2D. If this is the case, for understanding molecular mechanism underlying the T2D pathogenesis, it is important to clarify the role of individual genes in the disease. *Drosophila* is one of the most suitable animals for the genetic analyses. For example, one of GWAS candidate loci for T2D encodes a transcription factor, GLIS3 (Yang et al. 2009). Its *Drosophila* orthologue is *lmd* (*lame duck*). Depletion of the gene in *Drosophila* IPC by induction of dsRNA against its mRNA was carried out (Park et al. 2014a). The depletion resulted in hyperglycemia phenotype. And it resulted in a significant decrease of *Dilp2* mRNA, indicating that the lmd protein is required for transcription of the *Dilp* gene. It is consistent with results from the mammalian studies that the GLIS3 is required for the insulin gene expression. The genetic results proposed that the human *GLIS3* locus is associated with susceptibility for T2D (Dupuis et al. 2010; Nogueira et al. 2013). Similar genetic approaches seem to be quite effective to elucidate gene function and examine whether these genes are required for T2D pathogenesis. In addition, the approaches did not only allow us to clarify gene functions of individual genes but also to discover the gene network in which the gene is involved in.

Drosophila models to investigate metabolic syndrome: Metabolic syndrome is one of lifestyle diseases relevant to T2D. Currently, it has drawn worldwide attention. As patients suffering from it also display insulin resistance, the disease is placed as a pre-stage of type 2 diabetes. The syndrome is associated with the risk of developing type 2 diabetes and cardiovascular disease. Not only insulin resistance but also several overlapping aspects have been pointed out between metabolic syndrome and prediabetes. Insulin signals also regulate energy storage in fat body. Therefore, disruption of IIS eventually results in lipid metabolic disorder, that is, the metabolic syndrome. One of conserved factors crucial for metabolism of lipids and glucose is AKH in *Drosophila*. This peptide is secreted from specialized cells called corpora cardiaca (CC) of the ring gland. AKH stimulates lipolysis in fat body. Subsequently, TAG breakdown to free fatty acid (FFA). Glycogen breakdown is also stimulated, as AKH activate glycogen phosphorylase. Accordingly, trehalose is released from fat body to hemolymph. Protein kinase A is involved in the process. FFA moves to oenocytes which is a counterpart of adipocytes in mammals to produce energy. Thus, by lowering the IIS pathway, not only hyperglycemia but lipid accumulation in fat body is also promoted. The accumulation eventually results in insulin resistance. Next, regarding insulin resistance, it is possible to speculate the following hypothesis about the process toward onset of the insulin resistance. The lipid accumulation in fat body activates TORC1 in TOR pathway (Gutierrez et al. 2007). The TORC1 can activate S6 kinase. The protein kinase phosphorylates and inhibits a InR substrate, IRS-1, encoded in *chico* gene in *Drosophila*. Once the adaptor protein is phosphorylated, this form of the protein cannot transduce the IIS signal substantially. At the end, a responsiveness to insulin becomes lowered.

A continuous feeding of high-fat diet containing saturated fatty acids (HFD) bring about increased glucose levels in adult flies (Birse et al.

2010). Fat deposition was observed in both adipose tissues, fat body, and other tissues such as the midgut in the flies raised on the HFD. This is reminiscent of a common symptom seen in metabolic syndrome and T2D patients. Conversely, flies fed on HSD indeed increased fat deposition in the fat body and displayed severely lowered responsiveness to insulin, insulin resistance (Musselman et al. 2011). These are evidences indicating that there is a close relationship between fat and glucose metabolism. In the flies, it was also reported that insulin signaling was substantially suppressed in fat body. Instead, the stress-responsible pathway mediated by JNK was upregulated. The JNK phosphorylates and inhibits a *Drosophila* lipocalin orthologue, NLaz which we described at previous section. The downregulation of NLaz impaired responsiveness to insulin in peripheral tissues. Furthermore, reduced insulin signaling resulted in induction of expression of Foxo target genes (Musselman et al. 2011). It leads to insulin resistance.

For a long time, it has not been clarified how downregulation of IIS and upregulation stress signaling pathway in fat body interact with each other to develop insulin resistance and metabolic syndrome. It has been recently argued that the inflammation is associated with obesity and onset of metabolic syndrome in human and model organisms (Hoffmann et al. 2013). *Drosophila* fat body plays a multiple role in maintenance of metabolic homeostasis, stress responses, and production of antimicrobial peptides against infectious microbes (Arrese and Soulages 2010). Therefore, these cells can simultaneously respond to multiple intracellular signals, dependent on the situation. However, it is possible to interpret that the diapause of metabolic signaling was recognized as a sort of inflammation and that it may have resulted in activation of JNK cascade in the fat body. Thus, it is worth to consider the following hypothesis. In a response to inflammation derived from fat accumulation in the fat body, one of inflammation cytokines, TNFα, was possibly induced. It activates the stress JNK pathway. It can phosphorylate IRS-1, which is a substrate of the InR. The phosphorylation interferes the insulin signaling (Boucher et al. 2014). Eventually, it leads to appearance of insulin resistance and hyperglycemia one after another in adult flies fed on HSD or HFD for longer period (Hoffmann et al. 2013).

In conclusion, high-sugar diet or mutations and depletion of IIS factors can reproduce insulin resistance in the fly models. HSD possibly induces inflammation factor, TNFα expression. It resulted in insulin resistance. These MS and T2D models are useful to reveal mechanism underlying T2D pathogenesis.

13.7 Genetic Identification of New Genes Required for Glucose Homeostasis and Diabetes Pathogenesis Using *Drosophila*

It has been considered that complex genetic interaction between genetic loci controlling T2D susceptibility is involved in pathogenesis of human T2D. To identify the susceptibility loci for the disease, human genome-wide association studies (GWAS) have been performed, as described in previous section. However, it is usually difficult to assign specific causative genes, although it can identify a genomic region existing SNP marker linked most closely to susceptible genetic trails associated with the disease. One needs further to identify the causative gene within the region and to evaluate whether these candidate genes are really responsible for the disease. For the purpose, *Drosophila* provides excellent experimental system. Here, we introduce several susceptibility genes identified from different genetic approaches.

For instance, Pendse et al. selected the HHEX gene encoding Hox-class transcription factor, which gene polymorphisms seem to be associated with human T2D (van Vliet-Ostaptchouk et al. 2008). They carried out fat body-specific depletion for its *Drosophila* orthologue, *dHHEX*. The depletion resulted in elevated glucose levels in adult hemolymph and reduced insulin sensitivity (Pendse et al. 2013). Another research group has also carried out IPC-specific depletion of *Drosophila* orthologues for several human candi-

date genes. These genetic analyses revealed that *Imd* (a *Drosophila* orthologue of human *GLIS3* gene) and *CG9650* (a *Drosophila* orthologue of human *BCL11A* gene) are required for Ilp2 production or secretion (Yang et al. 2009; Park et al. 2014b) It is highly likely that these two human orthologue genes are also responsible for T2D.

Large-scale *Drosophila* RNAi screens called glucome screening to find out genes, whose depletion resulted in hyperglycemia of flies have been performed. Fat body-specific and muscle-specific depletion screens of ~1000 genes yielded ~160 candidate genes for hyperglycemia genes (Ugrankar et al. 2015). As one of the candidate genes involved in regulation of glucose metabolism, *CSNK1α1* gene encoding the alpha subunit of Casein kinase 1 were identified. The authors further demonstrated that heterozygous and homozygous mutants for the murine orthologue in their adipose tissue developed diabetes, indicating that the kinase plays a conserved role in glucose metabolism in both *Drosophila* and mouse.

Another type of genetic screens also contributed identification of novel modifiers for insulin signaling in *Drosophila*. Colombani et al. accomplished overexpression screen to identify growth modifiers by *Gal4*-dependent altering expression of each gene. They demonstrated that downregulation of *slimfast* gene specifically in fat body caused a global growth defect due to local repression of PI3 kinase signaling. The gene encodes amino acid transporter (Colombani et al. 2003). Teleman et al. also carried out gain-of-function screen for genes affecting tissue growth. Consequently, they identified a new modulator gene, *melted*, which encodes a PH domain protein that interacts with Tsc1 and FOXO. It can recruit FOXO to the plasma membrane in an insulin-regulated manner (Teleman et al. 2005). To identify new components that express and regulates the production of the Dilps in IPC, Cao et al. isolated mRNA from IPC collected by laser microdissection and performed transcriptome analysis of the mRNAs (Cao et al. 2014). Among those mRNAs expressing abundantly in IPCs, *unc-104* encoding a kinesin 3 family was essential for insulin secretion. Rab protein, which is a key regulator of intracellular vesicle transport, was also required for insulin production or secretion. These two proteins are required for a transport of the vesicles containing Dilps. Other genetic studies have also uncovered several regulators essential for insulin production and/or secretion in *Drosophila*. They include a small GTPase, Steppke, characterized as guanine nucleotide exchange factor (GEF) for ARF, a key regulator of both retrograde and anterograde traffic at the Golgi complex ARF at the Golgi complex. This finding suggests that ARF, a key and its GEF are essential for insulin signaling in larval stage. Another genetic screen to identify regulators of insulin sensitivity revealed that MAPK involves maintenance of glucose in hemolymph at appropriate levels through transcriptional control of InR gene (Zhang et al. 2011).

In addition, it has been uncovered that several miRNAs are involved in insulin production in IPC or its responsiveness in peripheral tissues. miR-278 mutant flies had elevated glucose levels and displayed increased insulin production, indicating that they are insulin resistant. The miRNA contributes to regulate energy homeostasis by regulating insulin responsiveness (Teleman et al. 2006). miR-14 regulates insulin production in IPCs through its target, *sugarbabe* mRNA encoding a predicted transcription factor regulating insulin gene expression (Varghese et al. 2010). Through the negative regulation of insulin gene expression, the miRNA regulates overall glucose metabolism. More recently, Ueda et al. reported that *Drosophila* miR-305, showing high homology with seed sequences of miR-239 in *C. elegans*, is involved in aging (Ueda et al. 2018). They showed that the lifespan of adults overexpressing miR-305 was significantly shorter. Conversely, a reduction in miR-305 expression led to a longer lifespan than that in control flies. miR-305 overexpression accelerated the impairment of locomotor activity and promoted the age-dependent accumulation of poly-ubiquitinated protein aggregates in the muscle, as flies aged. Thus, they concluded that the ectopic expression of miR-305 has a deleterious effect on aging in *Drosophila*. miR-239 in *C. elegans* can activate the IIS pathways (de

Lencastre et al. 2010). It has been reported that the inhibition of these pathways results in lifespan extension (Friedman and Johnson 1988; Clancy et al. 2001; Tatar et al. 2001; Holzenberger et al. 2003; Altintas et al. 2016). RNA-seq analysis to identify target genes of miR-305 demonstrated that the *tobi* mRNA, a target gene for insulin in the brain, and mRNAs for Dilp2 and Dilp5 increased following *miR-305* overexpression. Conversely, mRNAs for other insulin-like peptides, Dilp6 and Dilp8, were somehow downregulated to the controls following *miR-305* overexpression. Dilp6 inhibits *dilp2* expression. *Dilp6* overexpression extended lifespan in flies (Okamoto et al. 2009; Bai et al. 2012). Thus, Ueda et al. speculate that *miR-305* targets and negatively regulates the *dilp6* mRNA, which results in the increased expression of *dilp2*. It has been reported that a reduction in the sugar metabolism eventually leads to an extension in lifespan in *Drosophila* (Huang et al. 2015; Altintas et al. 2016). Another report also demonstrated that the ectopic expression of Foxo, which is negatively regulated by the IIS signaling pathway, resulted in lifespan extension (Demontis and Perrimon 2010). Assuming that *dilp6* is one of the targets of *miR-305*, its downregulation results in an unexpected activation of the IIS pathway. Thus, oxidative phosphorylation in the mitochondria is certainly stimulated, and as a consequence, reactive oxygen species (ROS) production is enhanced. These results are consistent with the observations showing that an increased sensitivity against oxidative stress and the induction of the oxidative stress marker are observed in adults overexpressing *miR-305*. (Ueda et al. 2018).

13.8 Development of Antidiabetic Drugs Using *Drosophila* Models

As pointed out throughout the book, *Drosophila* is one of the most suitable model animals in deciphering mechanisms of many human diseases as well as in identification of new genes involved in onset and development of the diseases. Many of cell biological, physiological, and neurological properties are conserved between mammals and *Drosophila*. More than 75% of human genes responsible for diseases are conserved and working in the fly. These approaches lead to identify new genes and pathways that could be future targets of drug design. Furthermore, it also has an advantage in the discovery of therapeutic agents (Pandey and Nichols 2011). Drug development process currently commences high-throughput screens for small chemicals or natural resources based on *in vitro* assays using cultured cells or on biochemical assays such as target-to-chemical binding assays. The second steps are usually processes that consume time and costs, as a large number of rodent models are utilized. Nevertheless, the majority of candidates are usually removed out of the selection in this process. If one would incorporate another selection using *Drosophila* models after initial large selections into the therapeutic discovery process, it allows us to proceed the screens more rapidly at lower expenses for the drug discovery.

In reality, it has been demonstrated that several types of known antidiabetic drugs or related substances have similar effects to *Drosophila* individuals. Among them, we introduce examples of three chemical compounds here. Firstly, sulfonylureas have been extensively used for treatment of T2D (Sola et al. 2015). The drugs act by stimulating insulin release from the β cells in the pancreas. Sulfonylureas bind to the specific receptor, blocking the inflow of potassium ion (K^+) through the ATP-dependent channel. The plasma membrane becomes depolarized. This opens voltage-gated Ca^{2+} channels. The rise in intracellular Ca^{2+} leads to increased fusion of vesicles containing proinsulin with the cell membrane and therefore increased secretion of insulin. *Drosophila* corpora cardiaca (CC) cells also express the sulfonylurea receptor and ATP-sensitive K^+ channels regulating release of AKH corresponding to glucagon in mammals. Kim and Rulifson demonstrated that homeostasis of circulating glucose

was significantly impaired by exposure to sulfonylureas (Kim and Rulifson 2004).

Metformin is another antidiabetic drug prescribed most commonly. The drug reduces glucose production in the liver and increases its uptake in muscle and adipose tissues (Bailey and Turner 1996). Metformin inhibits complex I in mitochondria. These effects result in increase of the AMP/ATP ratio in the cells, activating of AMP-activated protein kinase (AMPK) and triggering a cascade that inhibits gluconeogenic gene expression and energy-consuming processes. As it also interferes lipogenesis, it results in inhibition of protein kinase C due to reduction of diacylglycerol, which is an activator of the kinase. As a result, it eventually leads to release the InR from negative regulation by protein kinase C. Thus, it restores insulin resistance. Diet and administration of metformin ameliorated high-fat diet-induced hyperglycemia and obesity phenotypes in *Drosophila* adults. Kim et al. showed that the metformin's effect was entirely dependent on an endosomal Na+/H+ exchanger, a possible molecular target of the drug in both *Drosophila* and *C. elegans* (Kim et al. 2016).

Epigallocatechin gallate (EGCG) rich in green tea extract can extend lifespan of *Drosophila* adults through induction of endogenous antioxidant enzymes (Li et al. 2007). It also affects glucose metabolism and increases fitness. These effects went along an increased expression of Spargel, a *Drosophila* orthologue of mammalian PGC1α essential transcription factor for expression of the genes involved in energy metabolism (Wagner et al. 2015). The EGCG downregulates Dilp5 and phosphoenolpyruvate carboxykinase, major regulators of glucose metabolism, as well as the *Drosophila* homolog of leptin, Upd2. The decrease in glucose metabolism in connection with an upregulated expression of Spargel rather contributes to the lifespan expression in EGCG-fed flies. Taking together, these observations strongly suggest that *Drosophila* is a useful experimental model that allows us to evaluate known antidiabetic drugs and perform low- to high-throughput screens to discover novel antidiabetic drugs.

13.9 Conclusion Remarks and Outlook

Drosophila has a great potential as an experimental model system to study on sugar homeostasis including production and acting mechanism of insulin-like peptides. Previous *Drosophila* studies uncovered that the IIS pathways activated by the peptides control sugar metabolism, organism growth, reproduction, and longevity. The IIS pathways showed many conserved features with the mammalian pathways. Furthermore, a genetic disruption of Dilps or IIS factors resulted in diabetes-like phenotypes. On the basis of these results, it is possible to conclude that *Drosophila* is a suitable animal model for diabetes studies.

Certainly, major circulating sugar of insects is a trehalose, which has a less sugar toxicity than glucose has (Benaroudj et al. 2001). Although physiological harmful influences and developmental defects were observed in many *Drosophila* diabetes models, we should consider more carefully whether findings from *Drosophila* directly can apply to human. Nonetheless, *Drosophila* has great advantages for researchers to set up express genetic approaches that allow them to obtain results more quickly. The *in vivo* characterization using the organism confirmed that candidate human diabetes susceptibility genes suggested from GWAS are required for glucose homeostasis and involved in pathogenesis of diabetes (Pendse et al. 2013; Park et al. 2014b). Although *Drosophila* models were used to validate predicted functions of the human genes in these cases, other distinctive genetic studies by partial genomic RNAi screening called the glucome screening have been also performed to find out new genes required for glucose homeostasis. As consequences of these large screens and accumulation of individual studies, *Drosophila* researchers have so far identified new modulators including some microRNAs (Teleman et al. 2005, 2006; Ueda et al. 2018; Varghese et al. 2010). They are essential modulators for the IIS and TOR pathways. As some of them are novel factors that have been uncovered in mammals, yet they must be good examples verifying that the

Drosophila findings could contribute to mammalian studies in this field.

In addition, the involvement of the IIS and relevant regulatory factors are different from developmental stages and cell types. In larval stage, many imaginal cells are undergoing mitotic divisions, and some of larval cells continue to grow. The Dilps and the IIS play a more important role in stimulation cell growth and proliferation at the stage. Hence, they act on maintaining sugar homeostasis in adults in which fewer proliferative cells except germline cells are contained. However, in gametogenesis, the peptides are important for induction of germline stem cell division and cell growth of premeiotic cells before meiotic division. The Dilps and IIS pathways are critical key factors for cell proliferation and growth in *Drosophila*. Therefore, it is important to investigate their diverged functions and regulations in each tissues and development stage, in addition to understand integrated regulatory system to maintain metabolic homeostasis in whole organisms. Particularly, in mammals, influence of hyperglycemia condition to gametogenesis and fetal development have not been well characterized yet (Nandi et al. 2010). *Drosophila* provide a good experimental model system to investigate the issues.

It has been recently reported that *Drosophila* can respond to some antidiabetic drugs, as human and mammalian models do. In contrast to therapeutic medication for neuro-diseases, studies and trials on the drug discovery using *Drosophila* diabetes models are still developing stages. However, the evidences encourage us to push forward the trials. It is possible to set up experiments that can evaluate more easily effects of candidate antidiabetic drugs by examining alternation of simple phenotype, such as body size change, which diabetes model flies display. Researchers will be able to perform the experiments at a large scale but at considerably lower costs within a limited time. In facts, such antidiabetic drug screens using *Drosophila* models are beginning in earnest in some collaborative research projects and several pharmaceutical companies. Once antidiabetic drugs will have been identified, subsequently trials to look for target proteins or genes for the drugs possibly are carried out using *Drosophila* genetic techniques. These studies make it possible to elucidate modes of drug action. It is reasonable to expect that these efforts result in discovery of new regulatory system that has been uncovered in mammals. It is also promising that comprehensive RNAi screens to isolate *Drosophila* larvae or adults showing diabetes phenotype can find new genes essential for regulation of sugar metabolism.

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14 Metabolomics: State-of-the-Art Technologies and Applications on *Drosophila melanogaster*

Phan Nguyen Thuy An and Eiichiro Fukusaki

Abstract

Metabolomics is one of the latest "omics" technology concerned with the high-throughput identification and quantification of metabolites, the final products of cellular processes. The revealed data provide an instantaneous snapshot of an organism's metabolic pathways, which can be used to explain its phenotype or physiology. On the other hand, *Drosophila* has shown its power in studying metabolism and related diseases. At this stage, we have the state-of-the-art knowledge in place: a potential candidate to study cellular metabolism (*Drosophila melanogaster*) and a powerful methodology for metabolic network decipherer (metabolomics). Yet missing is advanced metabolomics technologies like isotope-assisted metabolomics optimized for *Drosophila*. In this chapter, we will discuss on the current status and future perspectives in technologies and applications of *Drosophila* metabolomics.

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Drosophila · Metabolomics · Metabolism · State of the art

14.1 Introduction

14.1.1 Metabolomics

Metabolomics is a rapidly emerging field of the high-throughput identification and quantification of the small molecule metabolites (Putri et al. 2013) (Fig. 14.1). The complete set of metabolites within an organism, cells, or tissues is called metabolome. As metabolites are the substrates and products of metabolism, the changes in the metabolome will reflect the effects of genetic, pathophysiological, developmental, and/or environmental factors (Fukusaki 2014). The dramatic breakthrough in the field of metabolomics within the past decade offers valuable insights in the correlation of metabolism with phenotype. Since metabolites are the final products of cellular processes, metabolomics is the puzzle piece fitting in the current central dogma. In the concept of system biology, metabolomics together with genomics, transcriptomics, and proteomics can give a more complete picture of the living organisms, cells, or tissues physiology (Nielsen 2017).

Same as other "omics" fields, the ideal condition for metabolomics is studying the whole metabolome in biological samples under certain

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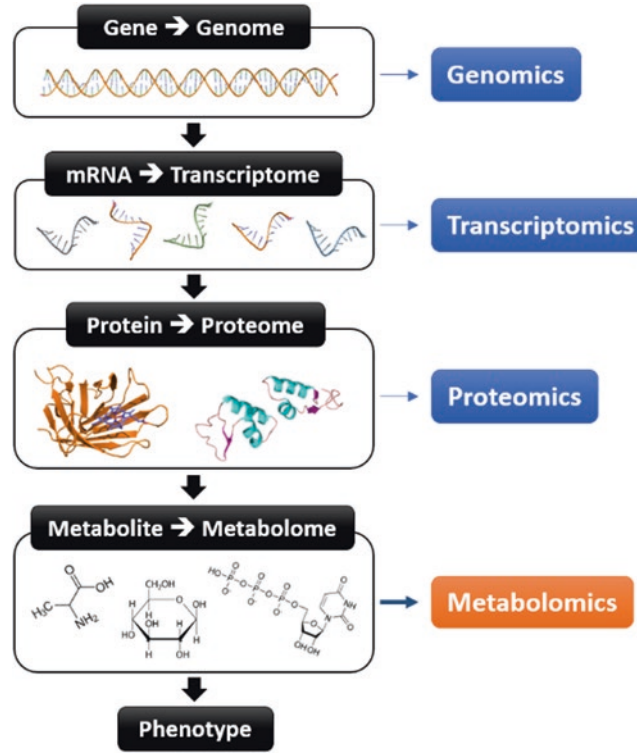

Fig. 14.1 The central dogma of biology referring the "omics" cascade

conditions. However, many metabolites are still unknown, and not any analytical platforms can cover all metabolites. Each metabolite has different characteristics that make the identification and quantification of all detected metabolites very complicated. Thus, the following approaches are now commonly used in the metabolomics field (Fukusaki 2014; Fiehn 2002):

(1) *Metabolic profiling* is an approach focusing on predefined biochemical pathways or specific classes of compounds. The strategy will be developed based on biochemical questions or hypothesis that motivates the research. With the development of analytical platforms, targeted metabolomics becomes one of the most powerful and rapid strategies for metabolic profiling.
(2) *Metabolic fingerprinting* is used to find the differences among the samples caused by their biological relevance. In this strategy, it is not necessary to give the detailed metabolite information. First, the nontargeted metabolomics method is useful to screen as many metabolites as possible without preference. Then, only the metabolites that show significant differences will undergo further investigations. This method provides a broader coverage, which has great potential to give insights into fundamental biological processes.
(3) *Metabolic footprinting (exometabolomics)* focuses on extracellular metabolites in cell culture media before and after culturing to provide a reflection of metabolite excretion or uptake by cells (Silva and Northen 2015).

14.1.2 *Drosophila* as a Model to Study Metabolism

Drosophila has been well established as one of the most tractable multicellular organisms for researches in areas of developmental biology, cell biology, and neurobiology (St Johnston 2002). Thanks to the development of robust analytical methods to evaluate cellular metabolism and the extent knowledge on which organ systems

have functional analogues to vertebrate counterparts, *Drosophila* applications have been expanded to study metabolism in the past decade (Hoffmann et al. 2013; Rajan and Perrimon 2013; Graham and Pick 2017; Herranz and Cohen 2017).

Drosophila organs responsible for absorptions and storage of nutrients share similar structures and functions to those of mammals including the midgut (works like the intestine and stomach) (Pitsouli and Perrimon 2008), the fat body (stores nutrients and functions as a nutrient sensor) (Rajan and Perrimon 2013), the oenocytes (take part in cycle and storage energy) (Gutierrez et al. 2007), and the Malpighian tubules (perform basic functions as kidneys) (Dow and Davies 2006). The energy homeostasis, including carbohydrate and lipid metabolisms, is highly conserved between *Drosophila* and mammalians (Mattila and Hietakangas 2017; Bharucha 2009). Thus, *Drosophila* model has great contributions to widen the knowledge on Type 1/Type 2 diabetes, obesity, metabolic syndrome, and insulin resistance diseases (Hoffmann et al. 2013; Rajan and Perrimon 2013; Graham and Pick 2017).

Besides, the cardiovascular system and tracheal system work independently in *Drosophila*, allowing the investigation on various aspects of metabolic dysfunction and cardiac dysfunction without compromising viability (Rajan and Perrimon 2013). Flies' cardiovascular system is an open circulatory system essential for the circulation of nutrients and immune cells, while the tracheal system is responsible for oxygen delivery (Choma et al. 2011). As cardiac dysfunction is usually a consequence of metabolic disorder, *Drosophila* has recently drawn a lot of attentions as a powerful paradigm to provide insights into high-fat- and sugar-induced cardiovascular disease (Na et al. 2013; Birse et al. 2010).

The advantages of *Drosophila* model in studying metabolism are attributable to the conservation in the signaling pathways controlling cell growth, proliferation, and death. Many reports have shown that the insulin, TOR, and JNK signaling pathways not only controlled normal cellular metabolism but also related to tumor formation and aging process (Herranz and Cohen 2017; Owusu-Ansah and Perrimon 2014; Newgard and Pessin 2014). Otherwise, the cellular metabolites such as sugar and free amino acids and metabolites in purine/pyrimidine metabolism could also regulate the cell signaling (Ben-Sahra and Manning 2017; Pavlova and Thompson 2016). As a result, the cellular metabolism has recently been recognized as a hallmark of cancer and aging (Pavlova and Thompson 2016; López-Otín et al. 2013). Therefore, *Drosophila melanogaster* is emerging as a valuable model to study multiple aspects of the connection between cellular metabolism and signaling pathways.

14.2 General Workflow for *Drosophila* Metabolomics Studies

As a high-throughput approach, metabolomics study required the integration of analytical chemistry, biology, mathematics, and informatics to interpret the data and unravel the biological insights. The Metabolomics Society conceived the Metabolomics Standards Initiative (MSI) focusing on community-agreed reporting standards, which allow data from different research institutes to be shared, integrated, and interpreted (Fiehn et al. 2007). In metabolomics field, MSI has been wildly used as the basic requirement for publication on peer-reviewed journals. In this chapter, the metabolomics approaches for *Drosophila* will be introduced based on the general workflow of metabolomics matched with MSI standard:

- **Experimental design**: As with any scientific studies, the design of a metabolomics experiment is the most curial step and depends on the scientific question under consideration. Though the whole *Drosophila* metabolome still remains unclear, many metabolic pathways are remarkably well conserved between *Drosophila* and mammals (Graham and Pick 2017; Alfa and Kim 2016). Researchers can choose the metabolomics approach (metabolic profiling, metabolic fingerprinting, or

metabolic footprinting) that suites their research objectives, sample types, and working conditions. Several applications of metabolomics established on *Drosophila* model can be used as references (Table 14.1), though not any methods have been reported for *Drosophila* metabolic footprinting.

- ***Drosophila* growing conditions**: Various food types are now utilizing to rear *Drosophila* including "homemade" food or commercial instant food. As the diet will directly affect the metabolism of an organism, the consistent diet throughout research is crucial to avoid experimental errors. Active yeast (*S. cerevisiae* or *S. carlsbergensis*) has been routinely supplemented to *Drosophila* food to mediate attraction, oviposition, and development (Becher et al. 2012). However, the use of yeast as supplement is not recommended in metabolomics study to prevent the alteration in fly's metabolism caused by the host-microbe interaction. Besides, the growing temperature throughout the study should also be controlled strictly. Many methods to control gene expression levels in *Drosophila* require switching temperature from permissive condition (18–22 °C) to restrictive condition (28–30 °C) such as temperature-conditional mutations, GAL4/GAL80ts/UAS system, and FLP/FRT system (Theodosiou and Xu 1998; Duffy 2002). Previous studies show that changes in rearing temperature had a dramatic effect on the metabolic profiles of *Drosophila* even in non-stressed conditions (22–29 °C) (Hariharan et al. 2014; An et al. 2017a; An 2017). Thus, flies in control and experimental test should be reared in exactly same temperature.
- **Sample preparation**: Several procedures for sample collection, quenching, extraction, and storage methods have been developed for every developmental stages of *Drosophila* (Table 14.1). While collecting sample, anesthesia/euthanasia methods (Colinet and Renault 2012; Overmyer et al. 2015), genetic backgrounds (An et al. 2017a), and genders (An 2017) are important factors to consider to prevent unexpected sample variations. Samples are immediately quenched in liquid N_2 and sometimes subsequently lyophilized to inhibit enzyme activities, avoid metabolite turnover, and capture the instant snapshot of the metabolic profile. The selection of extract solvent depends on the metabolite of interest (e.g., hydrophobic, hydrophilic) and the analytical platforms. It is recommended not to leave the samples sitting at room temperature long time before storage or analysis (Stringer et al. 2015). During sample preparation, the measurement of the pooled quality control/quality assurance (QC/QA) samples is highly recommended throughout the analysis to evaluate the stability and reproducibility of the analytical system (Dunn et al. 2012).
- **Metabolome analysis**: To date, the two most commonly used analytical platforms for metabolomics studies are nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy (MS) (Alonso et al. 2015). Each method has its own features, and both have been applied for *Drosophila*; the choice of analytical technique will be depending on characteristic of sample and target pathways (Emwas et al. 2013; Wang et al. 2015). Basically, NMR is a nondestructive method with simple sample preparation and high analytical reproducibility but relatively low sensitivity compared to MS. NMR is accepted as the gold standard to elucidate metabolite structural. Otherwise, MS-based metabolomics (mainly gas chromatography (GC) and liquid chromatography (LC) coupled with MS) has the advantages of high sensitivity, high selectivity, high throughput, and deep coverage. However, it usually requires more than one analytical method to wide range of molecules, requiring more optimization steps (extraction, derivatization, ionization, etc.). As a destructive method, MS-based metabolomics has more complicated data acquisition and metabolite identification.
- **Data acquisition**: Data acquisition (baseline correction, noise filtering, peak detection, peak alignment, normalization, and scaling) is used to archive accurate identification and quantification of detected metabolites (Alonso et al. 2015). Data acquired from metabolomics

Table 14.1 Metabolomics on studies using *Drosophila**

Application field		Analytical platform	Sample type	Important metabolites/pathways	References
Cold tolerance		GC-MS LC-MS NMR	Adult fly	Increase in gluconeogenesis, amino acid synthesis, and cryoprotective polyol synthesis	Hariharan et al. (2014), Teets et al. (2012), MacMillan et al. (2016), Koštál et al. (2012), Colinet et al. (2012), Colinet et al. (2013), Olsson et al. (2016), Kostal et al. (2011) and Colinet et al. (2016)
Heat stress		NMR	Adult fly	Alterations in the levels of free amino acids, maltose, galactoside, and 3-hydroxykynurenine	Malmendal et al. (2006), Pedersen et al. (2008) and Sarup et al. (2016)
Hypoxia tolerance		NMR	Adult fly	Flexibility in energy metabolism supports hypoxia tolerance in *Drosophila* flight muscle and in correlation with aging	Feala et al. (2007) and Coquin et al. (2008)
Oxidative stress		LC-MS	Adult fly	The metabolic response, especially glutamine level, to oxidative stress (superoxide, paraquat, and allopurinol)	Knee et al. (2013) and Al Bratty et al. (2011)
Infection	*Staphylococcus aureus* infection	NMR	Adult fly	Sepsis survivors had a metabolic signature characterized with decreased glucose, tyrosine, beta-alanine, and succinate	Bakalov et al. (2016)
	Listeria monocytogenes infection	GC-MS LC-MS	Adult fly	Lose both energy stores, triglycerides and glycogen, and show decreases in intermediate metabolites for beta-oxidation and glycolysis	Chambers et al. (2012)
Insecticide	Insecticidal activity (Bowman-Birk inhibitors)	GC-MS	Adult fly	Increase in F6P and decrease in citric acid/isocitric acid levels	Li et al. (2010)
Fundamental research	Effects of CO_2 anesthesia	GC-MS	Adult fly	The most important metabolic changes were the accumulation of succinate and G6P	Colinet and Renault (2012)
	Metabolic profile throughout life cycle	GC-MS	All stages	Over all metabolic transitions during *Drosophila* developmental stages	An et al. (2017a), An (2017), Tennessen et al. (2014a) and An et al. (2014)
	Tissue-specific metabolomes	LC-MS	Adult fly	A baseline tissue map of *Drosophila* for polar metabolites and for a range of lipids	Chintapalli et al. (2013)
	The influences of light cycles and temperatures	NMR	Adult fly	Alterations in the levels of free amino acids	Gogna et al. (2015)

(continued)

Table 14.1 (continued)

Application field		Analytical platform	Sample type	Important metabolites/pathways	References
	Rosy, *y* mutation	MS	Adult fly	Metabolomics profiling of *Drosophila*	Kamleh et al. (2008) and Bratty et al. (2012)
	Effect of storage temperature of quiescent larvae	GC-FID LC-MS	Larvae	Over all metabolic transitions	Tennessen et al. (2011)
	Different among wild-type strains	MS	Adult fly	Differences in genotype caused the differences in metabolome	Kamleh et al. (2009) and Reed et al. (2014)
	Effect of genetic background and growing	GC-MS	Pupae	Purine and pyrimidine metabolisms were altered between CS and OR	An (2017)
	temperature	LC-MS		Alterations in the metabolic profile in nonstress temperatures (22–29 °C)	
	Estrogen-related receptor	GC-MS	Larvae	Abnormally high levels of circulating sugar and diminished concentrations of ATP and triacylglycerides	Tennessen et al. (2011)
	Transient receptor potential TRPA1	GC-MS	Adult fly	Downregulation of intermediates in the methionine salvation pathway, in contrast to the synchronized upregulation of a range of free fatty acids	Lee et al. (2016)
	Effects of diet and development	FTMS	Larvae, pupae	The overall changes of lipidome	Carvalho et al. (2012)
	dG9a function in starvation tolerance	GC-MS LC-MS	Adult fly	dG9a-controlled energy reservoirs including amino acid, trehalose, glycogen, and triacylglycerol levels	An et al. (2017b)
Aging	Effect of diet, age, sex, and genotype on aging	LC-MS	Adult fly	Pathways involving sugar and glycerophospholipid metabolism, neurotransmitters, amino acids, and the carnitine shuttle were affected	Hoffman et al. (2014), Laye et al. (2015), Sarup et al. (2012) and Parkhitko et al. (2016)
Diseases	Charcot-Marie-Tooth disease (gene GDAP1)	NMR	Adult fly	Alterations in the levels of free amino acids and carbohydrate metabolism	López del Amo et al. (2017)
	Obesity	GC-MS LC-MS	Larvae	CoA is required to support fatty acid esterification and to protect against the toxicity of high sugar diets.	Palanker Musselman et al. (2016) and Heinrichsen et al. (2014)
	m-Aconitase deficiency	LC-MS	Adult fly	Reduced triacylglyceride and increased acetyl-CoA	Cheng et al. (2013)
	Alzheimer's disease	NMR	Adult fly	Metabolomic changes may lead to the age-relatedtoxicity of the amyloid beta (Aβ) peptide	Ott et al. (2016)

(continued)

Table 14.1 (continued)

Application field		Analytical platform	Sample type	Important metabolites/pathways	References
	Parkinson-like model (paraquat exposure)	GC-MS	Adult fly	Alteration in 24 metabolites, including amino acids, carbohydrates, as well as fatty acids	Shukla et al. (2016)
	Drug efficacy test	LC-MS	Adult fly	The alterations in these metabolites were associated with perturbations in amino acid and fatty acid metabolism, in response to insomnia through immune and nervous system	Yang et al. (2012)

**Adapted and updated from An PNT* (An 2017)

study could be from different batches or analytical platforms. Hence, appropriate methods for merging or comparing data should be applied to avoid experimental errors. In MSI standard, it is recommended to publish the raw data together with the paper or to other open sources such as MetaboLights (Haug et al. 2013).

- **Metabolite identification**: This is one of the major challenges of high-throughput metabolomics analysis. For the "known" metabolites, whose identities are already cataloged in accessible databases, the identification can be very accurate and efficient. The available metabolite library from different analytical techniques is growing continuously (Table 14.2). Since the differences in spectra comparing to structural isomers can be very small or not present at all, it is necessary to compare the library search results with a reference spectrum of the standard and desirably by chromatographic retention of the standard (Dettmer et al. 2007). In opposition, the identification of "unknown" metabolites is quite challenging due to the lack of commercial standard compounds. In MSI, the identification of metabolites is classified into four levels:
  Level 1 – Identified metabolites: metabolite identification is verified by analyzing the authentic chemical standard in the same condition with experimental data acquired.
  Level 2 – Putatively annotated compounds: metabolite identification is acquired by comparing with in-house database or online database.
  Level 3 – Putatively characterized compound classes: the metabolite cannot be identified but can be classified based on the functional groups.
  Level 4 – Unknown metabolites: the use of "identified" or "annotated" metabolites is very much different; it is important to clarify the level of metabolite identification in the publication.
- **Statistical analysis**: In metabolomics, chemometric methods including multiple univariate analysis (UVA) and multivariate analysis (MVA) are critical part to deal with big dataset (Madsen et al. 2010). MVA using non-supervised methods (e.g., HCA, PCA, and SOMs) can be used first to achieve a general view of the dataset. Then, MVA using supervised methods (e.g., PLS, PLS-DA, and OPLS-DA) will be utilized to explore the differences in the metabolic profiles among samples and reveal the important metabolites. While utilizing MVA supervised methods, cross-validation test is always required to prove the model is not overfitting or overprediction (Eriksson et al. 2003). UVA (e.g., student's *t*-test and ANOVA) will be used simultaneously for the validation of candidate metabolite credentials. Details of these

Table 14.2 Available spectral database*

Database	Spectral data	Website	Information	References
HMDB	MS/NMR	http://www.hmdb.ca	114,100 metabolite entries including both water-soluble and lipid-soluble metabolites	Wishart et al. (2013)
LMSD	MS	http://www.lipidmaps.org	37,500 lipid structures with MS/MS spectra	Sud et al. (2007)
METLIN	MS	http://metlin.scripps.edu	961,829 molecules (lipids, steroids, plant and bacterial metabolites, small peptides, carbohydrates, exogenous drugs/metabolites, central carbon metabolites, and toxicants). Over 14,000 metabolites have been individually analyzed, and another 200,000 has in silico MS/MS data	Tautenhahn et al. (2012)
IsoMETLIN	MS	https://isometlin.scripps.edu	All computed isotopologues (>1 million) derived from METLIN on the basis of *m/z* values and specified isotopes of interest (^{13}C or ^{15}N)	Cho et al. (2014)
NIST	MS/NMR	http://chemdata.nist.gov/	Reference mass spectra for GC/MS, LC-MS/MS, NMR, and gas phase retention indices for GC	Simón-Manso et al. (2013) and Babushok et al. (2007)
PRIME	MS/NMR	http://prime.psc.riken.jp/	Standard spectrum of standard compounds generated by GC/MS, LC-MS, CE/MS, and NMR	Akiyama et al. (2008) and Sakurai et al. (2013)
TOCCATA COLMAR	NMR	http://spin.ccic.ohio-state.edu	Multiple spectral NMR datasets: ^{1}H- and ^{13}C-NMR, 2D ^{13}C–^{13}C TOCSY (n = 463), 2D ^{1}H–^{1}H TOCSY and ^{13}C–^{1}H HSQC-TOCSY (n = 475), and 2D ^{13}C–^{1}H HSQC (n = 555)	Robinette et al. (2008), Bingol et al. (2015), Bingol et al. (2014) and Bingol et al. (2012)
MassBank	MS	http://www.massbank.jp	Shared public repository of mass spectral data with 41,092 spectra (LC-MS, GC-MS …)	Horai et al. (2010)
Golm Metabolome	GC-MS	http://gmd.mpimp-golm.mpg.de	2019 metabolites with GC-MS spectra and retention time indices	Hummel et al. (2008)
BMRB	NMR	http://www.bmrb.wisc.edu	9841 biomolecules with ^{1}H, ^{13}C, or ^{15}N spectra	Ulrich et al. (2008)
Madison	NMR/MS	http://mmcd.nmrfam.wisc.edu	794 compounds with NMR spectra (e.g., ^{1}H, ^{13}C, ^{1}H–^{1}H, ^{1}H–^{13}C) and calculated masses for different monoisotopic compositions ($^{12}C^{14}N$, $^{13}C^{14}N$, $^{12}C^{15}N$, $^{13}C^{15}N$)	Cui et al. (2008)
NMRShiftDB	NMR	http://nmrshiftdb.nmr.uni-koeln.de	42,840 structures and 50,897 measured spectra	Steinbeck et al. (2003)
Birmingham Metabolite Library	NMR	http://www.bml-nmr.org	208 metabolites and 3328 1D- and 2D-NMR spectra	Ludwig et al. (2012)

**Adapted and updated from An PNT* (An 2017)

analyses must be reported to show the objective and unbiased data analysis of one study.

- **Data interpretation**: Up to now, there is still a large knowledge gap exists in the translation from changes in the metabolite concentrations to the actual physiological interpretation in an organism. Many informative metabolic databases are available for *Drosophila* such as KEGG (Kanehisa et al. 2012), MetaboAnalyst (Xia et al. 2015), MetaCyc (Caspi et al. 2008), Reactome (Fabregat et al. 2016), and WikiPathways (Kelder et al. 2012). As mentioned above, many metabolic pathways are well conserved between *Drosophila* and mammals (Graham and Pick 2017; Alfa and Kim 2016); the database for other organisms can be used as cross-references. From these databases, we can map the metabolites of interest to the metabolic pathways, find related genes/proteins, search for active cellular processes (DNA repair, cell cycle, or programmed cell death), and compare the metabolic pathways of interest among different organisms.
- **Additional validations for the changes in *Drosophila* metabolic profiles**:
  (1) *Using quantitative metabolomics*: In general, metabolite abundance in metabolomics is measured in relative or absolute quantification (Lei et al. 2011). In relative quantification, the signal intensity of metabolites in the samples and in standard solutions (for calibration curves) will be normalized to the signal intensity of an internal standard or another relative metabolite. In absolute quantification, external standards or internal isotopically labeled standards are utilized.
  (2) *Using assays to study the metabolism* (Tennessen et al. 2014b): Since not any analytical methods can cover all the metabolites, many assays are now available to study the metabolism such as measurements of total proteins, triglycerides, cholesterol, glucose, trehalose, and glycogen. This information can support the hypothesis raised by metabolomics studies.
  (3) *Testing gene, mRNA, and/or protein levels*: As an advantage of high-throughput study, a hypothesis generated from metabolomics is usually not limited at one metabolite but metabolic pathways. Therefore, additional information on the related gene, mRNA, and/or protein abundances will provide more evidences if these pathways are suppressed or upregulated.

14.3 Isotope-Assisted Metabolomics: State of the Art and Potentials for *Drosophila* Studies

Up to now, the available database on metabolic networks of *Drosophila* like KEGG was developed based on genome data. If researchers are interested in the information on the turnover of a metabolite and/or its functions in the metabolic networks, additional analyses should be done. For that purpose, isotope-assisted metabolomics approaches, including metabolite turnover analysis and isotope-assisted absolute quantification, are the state-of-the-art methodologies (You et al. 2014).

14.3.1 Metabolite Turnover Analysis for Metabolic Network Exploration

Metabolite turnover analysis (stable isotope tracer analysis) is a method allowing researcher to trace the fate of a metabolite in metabolic networks (Fig. 14.2) (You et al. 2014; Chokkathukalam et al. 2014). The cells or animals will be exposed to a fully labeled isotope substance of interest, and then samples are collected over time. The changes of labeling in downstream metabolites can be detected by both MS and NMR. Once a single isotope exists, the mass of a molecule will increase one atomic mass unit (amu). Notably, MS/MS-based platform is a very powerful tool to examine the exact location of the stable isotope in the molecule. As a result,

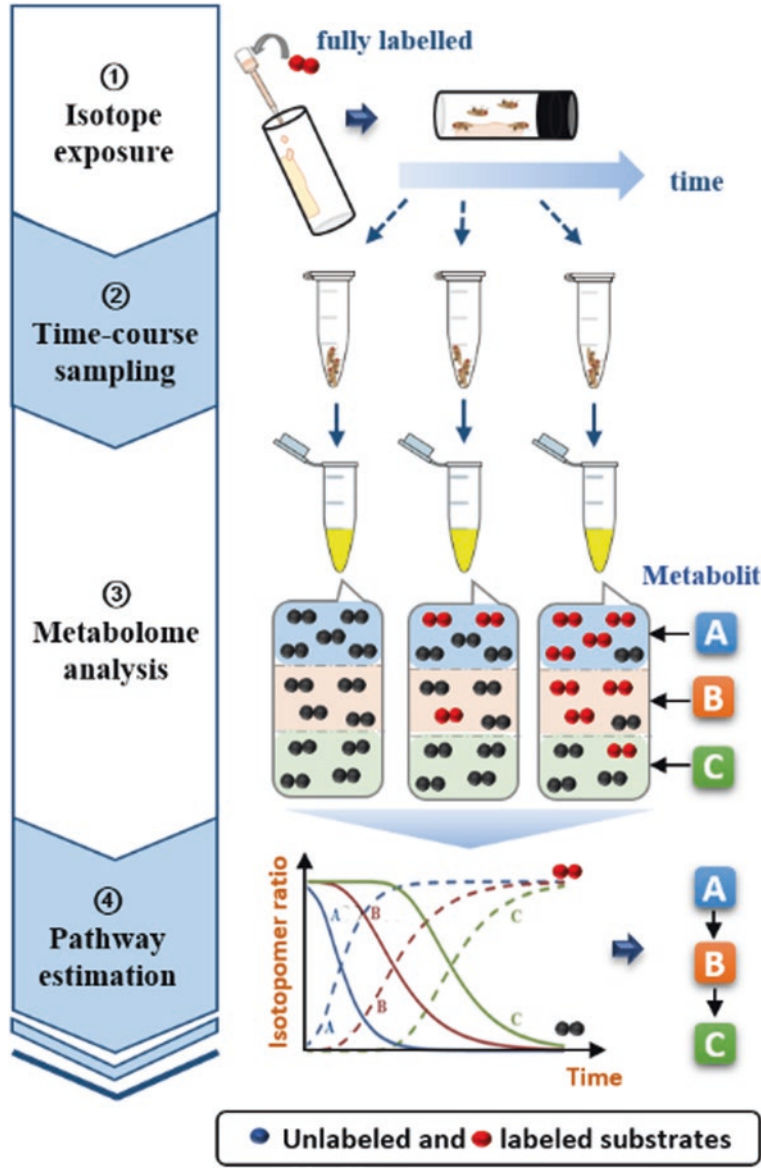

Fig. 14.2 Workflow of metabolite turnover analysis

an overview of which pathways are involved in the turnover of the target metabolite will be revealed. The stable isotopes of hydrogen (^{2}H; deuterium), carbon (^{13}C), and nitrogen (^{15}N) are commonly used in metabolomics fields.

The most important point to set up this experiment is the culture/medium components should be well defined, which makes it easy to modify only the target metabolite to stable isotope. That is the reason why the main application field of this method is on microorganism and cells, while only a few studies applied on *Drosophila* (Coquin et al. 2008; Nicolay et al. 2013). One remarkable case study example is from Dyson group in 2013; the authors applied metabolite turnover analysis on *Drosophila* larvae (Nicolay et al. 2013). By tracing the fate of glutamine, they found that inactivation of the retinoblastoma tumor suppressor (RBF1) increased the flux of glutamine toward glutathione synthesis, apparently reducing oxidative stress. Recently, a "holidic medium" for *Drosophila melanogaster* has been developed

by Piper et al. in 2014 (Piper et al. 2014). Even though it has not been applied for metabolomics study, holidic medium could be very useful if researchers want to strictly control and manually modify the diet.

14.3.2 Isotope Ratio-Based Approach for Absolute Quantification of Metabolites

Isotope-assisted absolute quantification in metabolomics is an advanced method allowing the accurate measurement of all detected metabolite abundances (Bennett et al. 2008). The main obstacle to quantify all metabolites is each metabolite's signal intensity which can be influenced by many factors such as its concentration, its structure, and its matrix effects. Using only one internal standard is not ideal for normalization of all metabolites with different characteristics. To overcome these challenges, one of the best solutions is using isotope internal standard mixtures which have similar profile to sample's metabolome (Chokkathukalam et al. 2014). The isotope compounds are able to account for sample processing variations and matrix effects encountered during analysis because they behave identically to their unlabeled equivalents in sample extracts. Fully labeled ^{13}C-metabolites are the most commonly used; they are however quite expensive. The isotope internal standard mixtures are usually generated by growing the cells with fully labeled substrates to yield exclusively labeled intracellular metabolites (Bennett et al. 2008). The internal standard mixtures will be added and extracted together with samples and standard solutions. After analysis, absolute quantitation of the metabolite levels is calculated using $^{12}C/^{13}C$ ratio-based calibration curves.

Even though isotope-assisted absolute quantification is the most accurate method to measure metabolite levels, it was applied on *Drosophila* only when a small number of metabolites needed to be quantified (Kostal et al. 2011). Hence, if a proper method to yield exclusively labeled intracellular metabolites is developed for *Drosophila*, it will be advantageous to expand applications of metabolomics on *Drosophila* model.

14.4 Central Carbon Metabolism of *Drosophila* Throughout the Life Cycle

The full genome of *Drosophila melanogaster* was successfully sequenced in 2000 (Adams et al. 2000; Fortini et al. 2000). The mRNA expression levels throughout the life cycle (Graveley et al. 2011) and in different organs of adult flies (Brown et al. 2014) were published in 2011 and 2014, respectively. Many projects to explore *Drosophila* proteome are now ongoing with 21,973 protein entries which can be accessed on UniProt (http://www.uniprot.org). Thus, *Drosophila* metabolome information is a viable counterpart to gain deeper understanding on *Drosophila* and expand its applications as a model organism. We recently reported *Drosophila* metabolic profiles at different developmental stages using the combination of GC-MS and ion-paired LC-MS/MS (An et al. 2017a; An 2017; An et al. 2014). Here, we will discuss the changes metabolic profile throughout *Drosophila* life cycle focusing on the central metabolic pathways (amino acids, sugars, and organic acids, as well as intermediates of central metabolism, such as sugar phosphates and cofactors).

During *Drosophila* embryogenesis (Fig. 14.3), free amino acids (FAAs) were proposed to be essential during *Drosophila* embryogenesis, and different amino acids appear to play distinct roles in different developmental stages of the embryo. High levels of aspartic acid, methionine, and glutamic acid were detected at the first 4 h after egg laying, which includes the rapid nuclear division cycles of *Drosophila* embryos. As aspartic acid, methionine and glutamic acid related to purine and pyrimidine synthesis (Bender 2012), high levels of these amino acids might be a crucial for supplying substrates and energy for DNA replication during the of early *Drosophila* embryogenesis. The changes in purine and pyrimidine metabolism also matched with this hypothesis. On the other hand, essential amino acids

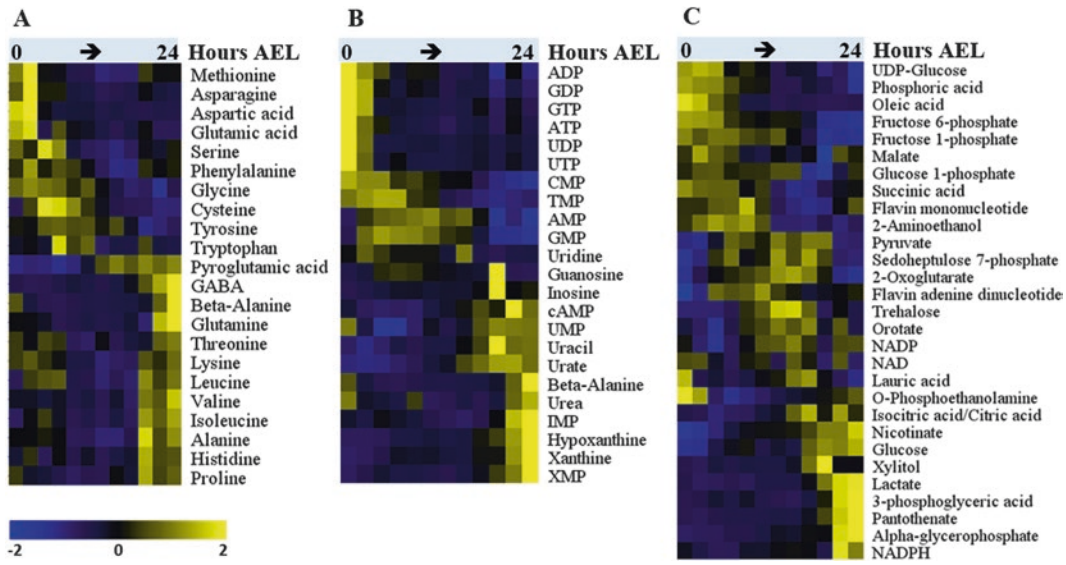

Fig. 14.3 Metabolic profiles of Canton S during embryogenesis. (**a**) Free amino acids. (**b**) purine and pyrimidine metabolism. and (**c**) glycolysis, TCA cycle, fatty acids, and cofactors. Embryos of Canton S were incubated at 25 °C in which samples were collected every 2 h from 0 to 24 h AEL (h after egg laying). The color scale is plotted at the bottom left of the figure

(leucine, isoleucine, threonine, valine, lysine, and histidine) increased significantly at the end of embryogenesis. The only source for essential amino acids in *Drosophila* embryo was from protein degradation, and insects do not carry out gluconeogenesis from lipid substrates (Rockstein 2012). Thus, the embryo must be endowed with an abundance of maternally supplied products, and these amino acids possibly provide another pathway to control energy production during embryogenesis.

Metabolites in sugars metabolism changed drastically during the development of embryos. The level of UDP-glucose decreased, while the level of glucose increased throughout embryogenesis. Interestingly, trehalose was found to be accumulated in an abundant level during gastrulation. Previous transcriptome study reported that *Tret 1–1* and *Tret 1–2* (encoding for trehalose transporters) were highly expressed during gastrulation, while *Treh* (encoding for trehalase that converts trehalose into glucose) was expressed throughout embryogenesis (Fisher et al. 2012). Therefore, trehalose was proposed as the energy source for glycolysis to supply glucose for the cells during embryogenesis. Unlike in larval stage that glucose in the fat body is utilized to generate trehalose (Elbein et al. 2003; Chen and Haddad 2004), trehalose used in embryogenesis must be generated from other sources (properly from the yolk) because the level of glucose is quite low in the early stage. Consistent with this hypothesis, a study from Tennessen et al. showed that the level of triacylglycerol and glycogen decreased during *Drosophila* embryogenesis (Tennessen et al. 2014a).

In larval stage, first, second, and third instar larvae had distinct metabolic profiles (Fig. 14.4). High-abundance metabolites in amino acid, purine, and pyrimidine metabolism were detected in first instar larvae than other larval stages. At the early stage of larval development, these metabolites likely came from their diet since the flies started to uptake food and obtain amino acids from their food by ingesting protein (Nation 2008). The major events during larval stage are rapid growth and proliferation of imaginal discs as well as endoreplication of other tissues. Therefore, high levels of metabolites in purine and pyrimidine metabolism, materials for cell division and cell growth, were detected. Second- and third-instar larvae stages are the extensive

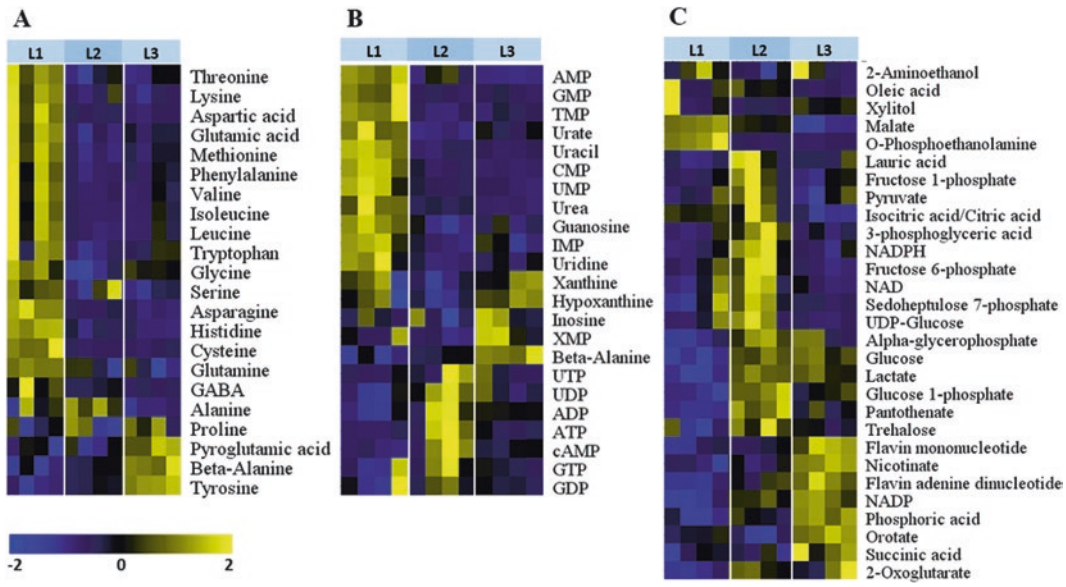

Fig. 14.4 Metabolic profiles of Canton S during larval stage. (**a**) Free amino acids. (**b**) purine and pyrimidine metabolism. and (**c**) glycolysis, TCA cycle, fatty acids, and cofactors. The color scale is plotted at the bottom left of the figure. L1, first instar larvae; L2, second instar larvae; L3, third instar larvae

feeding stage; larvae uptake nutrients not only to fuel developmental reorganization but also to survive during metamorphosis and early adult stage (Church and Robertson 1966). Thus, metabolites related to energy metabolism including some FAAs, sugars, and TCA intermediates were detected in high abundances during the late stage of larval development.

The metabolic profiles of *Drosophila* during metamorphosis were grouped into two main groups including 0–6 h AWP (prepupal period marked by pupariation) and 12–90 h AWP (main pupal period) (Fig. 14.5). Throughout metamorphic processes, the adult progenitor cells such as imaginal discs undergo cell proliferation, differentiation, and organogenesis to give rise to the adult structures, while most larval tissues undergo autophagy and cell death (Aguila et al. 2007). The most significant change was the increase of FAA levels, which was matched with the changes of urea abundance. Further investigation found that the total protein amount in flies decreased throughout metamorphosis. These results suggested that the cells broke down proteins intensively to recycle the amino acids. As flies cannot uptake food from pupal stage until 8 h after eclosion (Chiang and Tactic 1963), these FAAs were likely used as material to construct proteins or to produce energy via gluconeogenesis. Moreover, insects always have to maintain a high hemolymph level (2.9–23.4 mg/ml) comparing to most of vertebrates (0.5 mg/ml) (Gilbert and Schneiderman 1961; Wigglesworth 2012). FAAs were also reported as the most abundant metabolites in ten different tissues of adult flies (Chintapalli et al. 2013). Therefore, the regulation of FAA levels not only maintains the normal development of *Drosophila* pupae but also has to keep a high abundant level of FAAs in the body of adult flies at the end of development. Moreover, the metabolites in purine, pyrimidine, and energy metabolisms (fatty acids, sugars, and TCA intermediates) also changed drastically during the main pupal stage. Autophagy and apoptosis are the important processes for the degradation and turnover components in the cells of an organism (Mariño et al. 2014). These results suggested that in the nutrient-limited environment, the pupae used the materials from cell histolysis for the differentiation of the tissues as well as to generate energy for cellular activities.

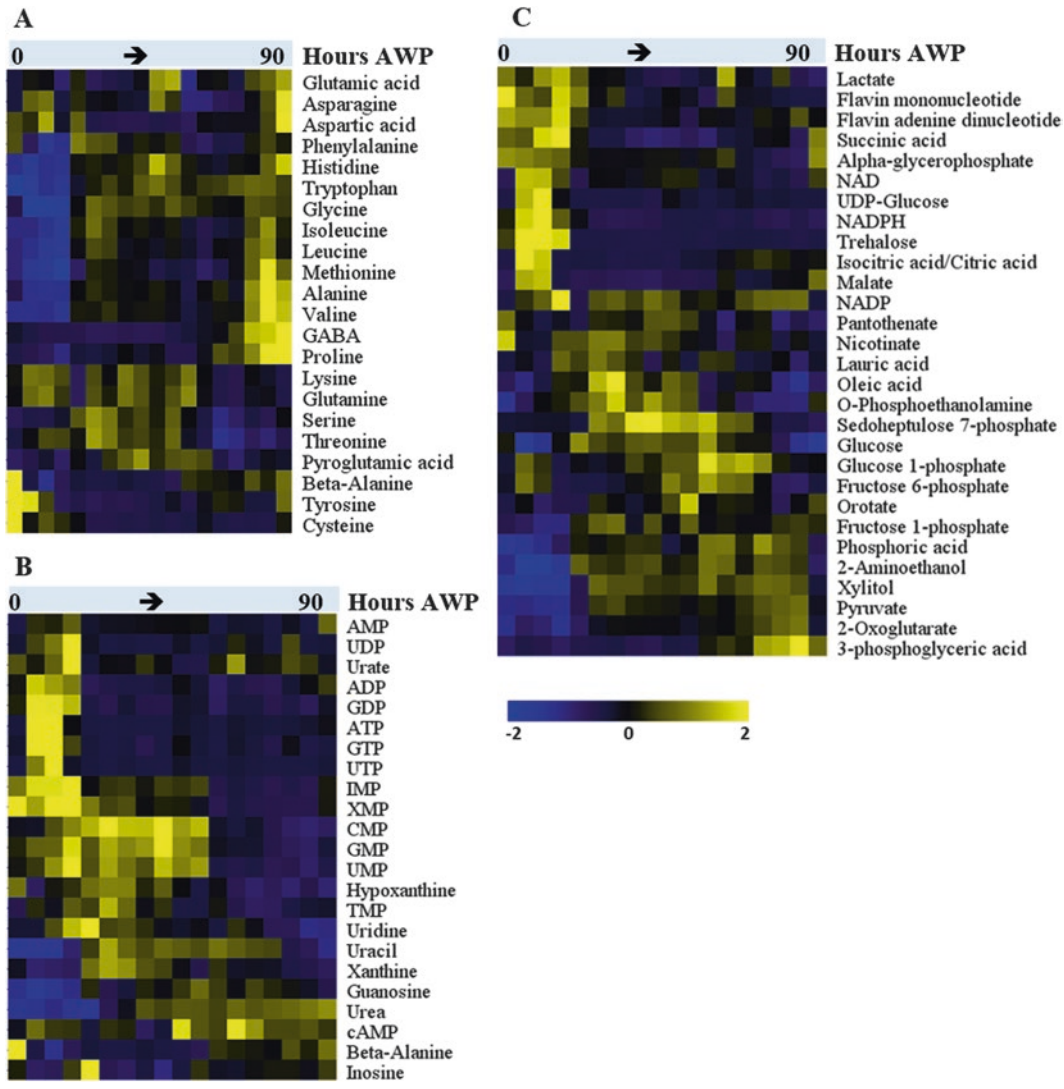

Fig. 14.5 Metabolic profiles of Canton S during metamorphosis. (**a**) Free amino acids. (**b**) purine and pyrimidine metabolism. and (**c**) glycolysis, TCA cycle, fatty acids, and cofactors. At this stage, the time course sampling started when the animals reached white pupal stage. The samples were collected from 0 to 90 h AWP (h after white pupae). The color scale is plotted at the bottom left of the figure

In adult stage, male and female flies appeared to have distinct metabolic profiles especially in purine and pyrimidine metabolism (Fig. 14.6). These data were supported by a previous study showing that female flies required higher DNA biosynthesis and RNA transcription to produce eggs from germ cells (Rong et al. 2014). Moreover, the levels of UDP-glucose, glucose 1-phosphate, fructose 1-phosphate, and fructose 6-phosphate were significantly higher in female flies. Since adult flies can uptake nutrients directly from food, these results indicated that female *Drosophila* had higher energy demand. This finding matched with previous data suggested that female flies had relatively bigger fat body and more storage lipid to maintain the reservoirs the reproductive organs and eggs (Scheitz et al. 2013; Parisi et al. 2011). A transcriptome study in male and female *Drosophila* found that the major differences in gene expression were

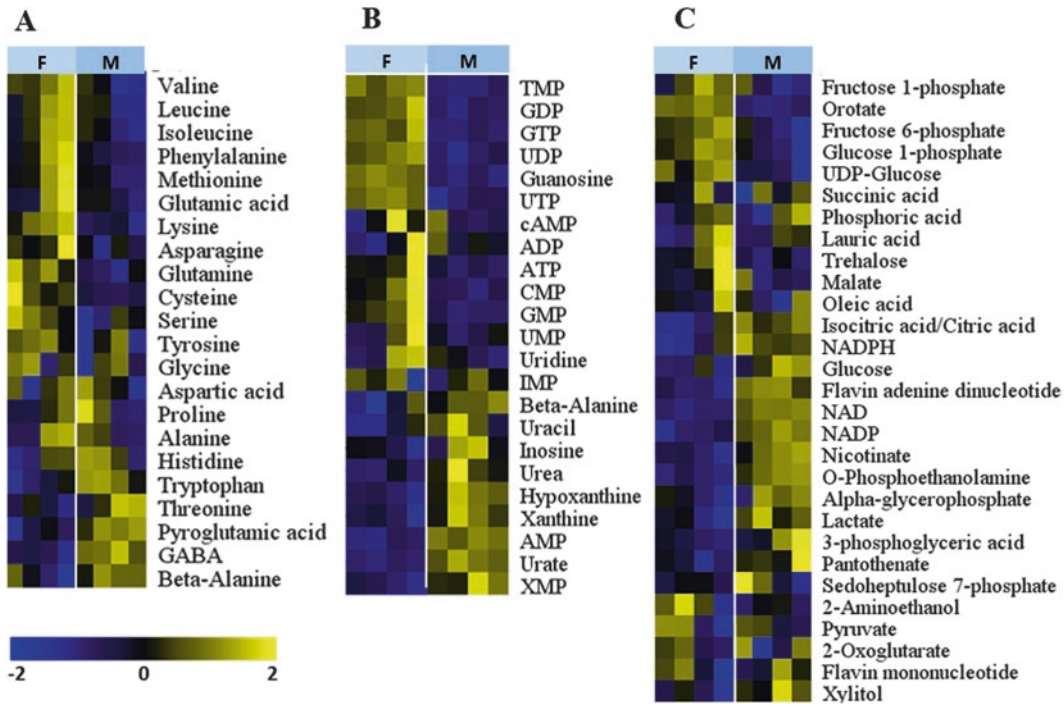

Fig. 14.6 Metabolic profiles of Canton S during metamorphosis. (**a**) Free amino acids. (**b**) purine and pyrimidine metabolism. and (**c**) glycolysis, TCA cycle, fatty acids, and cofactors. For adult stage, the 5-day-old virgin files of each gender were collected after emerging. The color scale is plotted at the bottom left of the figure. *F*, female, *M*, male

attributable to the germ cells (Parisi et al. 2004). Therefore, the differences in the metabolic profiles discovered in this study were likely due to the reproductive systems. Even though the full mechanism was not clearly understood, the metabolism was sex biased in *Drosophila*.

14.5 Future Perspectives of *Drosophila* Metabolomics

In general, *Drosophila* metabolomics is still immature comparing to other application fields of metabolomics. However, it is helping us to gain more insights into *Drosophila* metabolism than any other technologies could have done. Current workflow in *Drosophila* metabolomics is mainly adopted from other organisms. The lack of optimum methods specifically for *Drosophila* is one of the biggest bottlenecks interfering the use of advanced metabolomics technologies such as turnover analysis or absolute quantification. As mention above, *Drosophila* "holidic medium" proposed by Piper et al. could be the key to solve this problem. Besides, expanding the sample types can so widen the applications of *Drosophila* metabolomics. Most of the reports used whole body extract, and only a few studies focus on specific tissues. The current metabolomics technologies can access to the metabolic profile of not only large tissues but also cell lines (in cell culture) (Muschet et al. 2016), single cell (Emara et al. 2017), and even subcellular organelles (Chen et al. 2016; Dietz 2017). Obviously, *Drosophila* cell culture has been using as a quick screening system for basic researches in molecular and cellular biology (Baum and Cherbas 2008). Moreover, the use of *Drosophila* imaginal discs has been providing new insights into a number of discoveries in developmental and cellular biology (Beira and Paro 2016). Therefore, if we can take advantage of metabolomics technologies

and apply to various *Drosophila* experimental systems, *Drosophila* metabolomics will give huge impact on achieving new insights into the cell phenotype and physiology.

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15 Humanized Flies and Resources for Cross-Species Study

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Abstract

The completion of whole-genome sequences has greatly broadened our understanding of genes and genomes. The availability of model organism databases facilitates the sharing of information. However, it is still challenging to predict the pathogenicity of missense mutations, and it is more difficult to evaluate the functional impact of noncoding variants. What is more, it is a primary question to understand what variants interact to express phenotypes. Powerful genetic tools and resources available in *Drosophila* now make it much easier to replace endogenous genes with exogenous DNA. This allows us to directly investigate and compare the functions of orthologs, variants, and fragments in a single genetic background, the value of which should be widely appreciated. To take one example, we are currently studying so-called ultra-conserved elements, which have been conserved over hundreds of millions of years of vertebrate evolution. Many highly conserved elements are in noncoding regions and are thought to play a pivotal role in gene regulation. We generated transgenic fly lines carrying human ultra-conserved elements for enhancer reporter assay and indeed observed the reporter expression in one or more tissues of embryos and larvae in all elements tested. Currently, transgenic human-ORF lines expressing human genes under the control of GAL4/UAS system are also been developed, which will greatly facilitate the cross-species in *Drosophila*. In this chapter, I introduce useful tools and resources available in *Drosophila* to nonspecialists, encouraging their further use in many applications.

Keywords

Cross-species study · Humanized fly · Fly resources · Ultra-conserved element

15.1 Introduction

Fruit fly (*Drosophila melanogaster*) has long been a favorite model organism for many researchers since T. H. Morgan found the first white-eyed mutant fly in 1910. This small (a few mm) invertebrate can easily be breed and maintained in a vial of 25 mm in diameter x 95 mm in height, containing about 8 ml of fly medium. It takes about 10 days from egg to adult at room

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temperature, 1 day for egg to hatch, 4–5 days for the larva to molt twice (into second and third instar larva) and to pupate, and another 4–5 days before the adult fly emerges. Adult fly lives for several weeks to a few months, and each female lays hundreds of eggs during her lifetime. Researchers can breed multiple of generation of flies and examine a mutant phenotype in homozygous condition in a few of months.

Drosophila and humans diverged from a common ancestor about 700 million years ago (Douzery et al. 2004) but still share many cellular and molecular components. Thanks to the deep conservation, findings in *Drosophila* have had profound implications. Genetic analyses in *Drosophila* have led to the discovery of many basic principles of signal transduction, such as *Notch*, *Hedgehog*, *Wnt*, and *Hippo* pathways, to name a few. Our understanding of anterior-posterior axis formation has also emerged from studies in *Drosophila*. The availability of genome sequences and advance in genetic tools for manipulation have now paved a way for more direct functional analyses of human genomes in vivo. Accordingly, the types and number of databases and resources for studying human genes and variants are rapidly expanding. It is now feasible to routinely replace *Drosophila* genes with human orthologs and to investigate their functions. This is not limited to humans, but that the functions of orthologous genes from diverse species are now analyzed and directly compared in a single model organism like *Drosophila*. In this chapter, I introduce some useful genetic tools and resources available in *Drosophila* to nonspecialists, encouraging their further use in many applications in a wide variety of fields.

15.2 Two Kinds of Simplicities that Make *Drosophila* an Excellent Model Organism

Besides the ease of maintenance in a laboratory and the short generation time, there are two kinds of simplicity that make *Drosophila* an excellent model organism. One is the genomic simplicity. Fruit fly has a genome of about 200 Mb distributed mainly on the X and two autosomes (the second and third chromosomes), while the fourth chromosome is much smaller (about 2% of the genome). Its genome has not experienced a whole-genome duplication event and contains fewer paralogs than those of vertebrates. It contains 13,931 protein-coding genes, 2508 long noncoding RNA genes, 115 rRNA genes, 312 tRNA genes, 289 snoRNA genes, and 259 miRNA genes (FB2017_06 Release Notes). When compared to the human genome containing about 21,000 protein-coding and 99,000 RNA genes on their 23 chromosomes, the fly thus has a simple and compact genome. Nevertheless, the two species share more than 60% of genes (orthologs). This simplicity is particularly useful for cross-species study and accelerates translational research.

Drosophila eggs are about 500 μm in length and 180 μm in diameter. Embryogenesis is completed within 22–24 h at room temperature and easy to observe. Embryonic phenotypes are thus quickly recognized and well scored. *Drosophila* is a holometabolous insect, and the majority of adult organs are derived from the so-called imaginal discs, which are each composed of two epithelial layers, a squamous peripodial membrane and a columnar epithelium (disc proper). The latter gives rise to adult external structures, for example, wing and notum from the wing disc. The anatomical simplicity of embryos and larvae has made it much easier to study cell-cell interactions, which are critical for organ development and relevant to disease progression.

15.3 Tools and Resources for Studies in *Drosophila*

15.3.1 Special Chromosomes that Make *Drosophila* Unique

A series of balancer chromosomes makes *Drosophila* very unique. They carry multiple inversions along their chromosomes for crossover suppression. They also carry a dominant

visible marker (or markers) and a recessive lethal (or lethals) to prevent loss of the chromosome of interest. They are created on the X (first multiple series, such as FM6), second (e.g., SM5 and *CyO*), and third chromosomes (e.g., TM3 and TM6). These special chromosomes allow a selected chromosome to be preserved intact in the heterozygous condition (without recombination) or to be made homozygous in a few generations (in as little as 1 month after an initial cross). Taking these advantages, many kinds of mutations have been generated and studied for their effects and functions.

The availability of the balancer chromosomes facilitates the generation of many deficiency chromosomes by X-ray irradiation or chemical and transposon mutagenesis; a collection of such deficiencies has been widely used as null alleles, for rapid mapping of new mutations and phenotypic traits, and for enhancer and suppressor screening. To further improve the performance, the yeast FLP1/FRT site-specific recombination system was introduced to create new collections of deficiencies (Ryder et al. 2004; Parks et al. 2004). They are relatively small with molecularly defined breakpoints and common genetic backgrounds, thus allowing fine-scale mapping and more valid comparison of measurements. In total, the deficiencies, which are publically available in the stock centers, cover more than 98% of the euchromatic genome. Collections of large duplications can also be amenable to high-throughput modifier screening.

The value of quantitative phenotypic measurements is increased, and the data sharing is more effective when commonly used wild-type strains are included as controls in the analysis. The *Drosophila* community shares "reference" strains such as Canton-S, Oregon-R, and other highly inbred strains via Bloomington Drosophila Stock Center and Kyoto Stock Center (DGRC). However, there is no *super fruit fly*, and all laboratory flies are *mutants* in a sense. For instance, one of the most popular "wild-type" strain Canton-S is found to have reduced male fertility (Kanamori et al. 2014), although causative mutation or mutations remain unidentified. Therefore, multiple reference strains are desirable in all model organisms and available to the *Drosophila* community.

At the same time, naturally occurring genetic variation is a valuable resource for studying how genes and environments produce a phenotype. Along this line, *Drosophila melanogaster* Genetic Reference Panel (DGRP) consisting of more than 200 inbred strains derived from a natural population is constructed, sequenced, and phenotyped for traits such as starvation resistance, pigmentation, and aggressive behavior (Mackay et al. 2012; Dembeck et al. 2015; Shorter et al. 2015). Gene expression data (Huang et al. 2015) are also available at http://dgrp.gnets.ncsu.edu and can be used for analysis of expression quantitative trait loci (eQTLs).The more we know about the genetic architecture of complex traits in experimental populations, the better we will be able to predict complex phenotypes such as disease risk. A better understanding of the relative impact of functional variants, age, interactions, and environments on trait phenotypes is a key to making personalized medicine.

15.3.2 Tools and Applications That Are Embedded in *Drosophila*

One of the most important genetic tools in *Drosophila* genetics is binary (driver/responder) transgene induction systems, where the driver activates expression of the downstream responder transgene in a specially and temporally restricted manner. The most common ones are budding yeast transcription activator-based GAL4/UAS (Brand and Perrimon 1993), bacterial repressor-based LexA/lexAop (Lai and Lee 2006), and red bread mold transcription activator-based QF/QUAS systems (Potter et al. 2010). They are activated independently and, importantly, can be repressed; GAL80 represses GAL4 and QS does QF. Moreover, the DNA-binding domain and transcriptional activation domain of the driver genes are separable and independently targeted with different promoters, in which these two elements must be expressed together in the same cell to become transcriptionally active (Luan et al. 2006). By combining these techniques, we

can refine the expression patterns of transgenes and induce expression of distinct transgenes in different patterns in the same fly.

In the binary systems, the driver lines are generated independently from the responder. One way to generate is to fuse the driver gene to the known regulatory elements; for example, heat-inducible *Heat-shock-protein-70Bb* (*Hsp70*), pan-neuronal *neuronal Synaptobrevin* (*nSyb*), germ cell-specific *nanos* (*nos*), and ubiquitous *α-Tub84B* (*α-Tubulin at 84B*) drivers were generated. However, because of the limited number of promoters and enhancers that have been identified and characterized, two alternative approaches have been proposed and used successfully to generate many thousands of driver lines. The first one is so-called enhancer trapping, in which the driver (e.g., GAL4) gene is fused to a minimal promoter and then randomly inserted in the genome. It is expressed in patterns specified by adjacent regulatory sequences (Brand and Perrimon 1993). Currently, about 4300 such lines (NP lines, Hayashi et al. 2002) are available from Kyoto Stock Center. In the second method, the driver gene is directly fused to a short genomic fragment as a potential regulatory sequence, together with a minimal promoter, and then inserted into a specified genomic location (Pfeiffer et al. 2008). These driver lines generally drive expression in fewer cells than the enhancer trap lines do and available from Bloomington Drosophila Stock Center (Jenett et al. 2012) and Vienna Drosophila Resource Center (Kvon et al. 2014).

Genetic mosaic analysis is also a powerful tool for dissecting complex gene functions and indeed widely used in *Drosophila* research, especially in two-dimensional epithelial tissues like eye and wing discs proper. It is especially useful for embryonic lethal genes. In *Drosophila*, mitotic recombination occurs spontaneously, although at very low frequency, in both females and males, which could generate two different homozygous cells (one for each allele). Currently, FLP recombinase is most often used to induce mitotic recombination. Indeed, it is highly efficient. A representative application example is the mosaic analysis with a repressible cell marker system (MARCM; Lee and Luo 1999). This system also depends on the GAL4/UAS system and the GAL80 repressor to label positive cells of interest and to trace cell lineages at a single-cell resolution. There are many strategies and applications of mosaic analysis (Germani et al. 2018), and they can be used for studying cell-cell interactions.

PhiC31 integrase and FLP and Cre recombinases are also used to capture and use the special and temporal expression pattern of genes. *Minos*-mediated integration cassette (MiMIC) is a *Minos* transposon-based gene-trap vector, which consists of a gene-trap cassette and the *yellow*$^{+}$ marker flanked by two inverted *attP* sites and two *Minos* inverted repeats (Venken et al. 2011; Nagarkar-Jaiswal et al. 2015). The gene-trap cassette contains a splice acceptor site followed by stop codons for all three reading frames and a polyadenylation signal. Therefore, insertions into coding introns very likely disrupt the genes. The sequence between the two *attP* sites can be replaced through recombinase-mediated cassette exchange (RMCE) with a plasmid containing two inverted *attB* sites. Exchange with a correction cassette lacking the splice acceptor site and stop codons is supposed to revert the disruptive mutation; exchange with a mutagenic GAL4 cassette results in gene trapping; and exchange with a cassette containing a reporter gene (e.g., GFP) surrounded by a pair of a splice acceptor and a donor site makes a protein trap line.

Like many other animals and plants, the CRISPR/Cas9 system has been successfully introduced into *Drosophila* (Bassett et al. 2013; Gratz et al. 2013; Kondo and Ueda 2013; Yu et al. 2013) and widely used for genome editing. The method has been significantly improved, and many applications have been developed. For highly efficient and specific conditional gene disruption, it is combined with the GAL4/UAS system (Port and Bullock 2016); together with guide RNAs, catalytically inactive Cas9 (dCas9) is targeted to loci of interest for transcriptional repression and activation (Lin et al. 2015; Ghosh et al. 2016); CRISPR/Cas9-based homologous recombination is used for the plant-based auxin-inducible degradation system (Bence et al. 2017);

and many more. The new genome-editing technology will further stimulate cross-species study.

Facilitated by these tools, large-scale fly resources have been made systematically to induce loss-of-function and gain-of-function mutations. For targeted gene silencing, UAS-hairpin RNAi (RNA interference) libraries have been made available in *Drosophila* (e.g., Hu et al. 2017). These hairpin RNAs are expressed under the control of various GAL4 drivers, which causes cell-autonomous sequence-specific degradation of RNA molecules. The libraries have gone through two major changes: site-specific integration mediated by phiC31 integrase instead of P element-based random integration and short hairpin RNAs (shRNAs) instead of long hairpin RNAs. Both changes are expected to improve the effectiveness and the latter to reduce off-target effects. Such large collections of transgenic flies are available in the three stock centers (the following figures refer only to protein-coding genes): 13,185 lines in Bloomington Drosophila Stock Center, 31,393 lines in Vienna Drosophila Resource Center (Dietzl et al. 2007), 17,405 lines in National Institute of Genetics, Japan, and, when combined together, 13,354 genes (95.9%) are targeted (as of Feb 1, 2018).

Overexpression or ectopic expression is an alternative efficient means for studying gene function and for identifying genes associated with a given process or phenotype. In *Drosophila*, this sort of misexpression can be achieved with UAS enhancer-promoter insertion lines, which induce expression of the downstream genes when GAL4 is present (Rorth 1996; Crisp and Merriam 1997; Toba et al. 1999; Beinert et al. 2004; Bellen et al. 2004; Thibault et al. 2004; Staudt et al. 2005). EP, EPgy2, PBAC{WH}, and other libraries are available at Bloomington Drosophila Stock Center (in total, 6181 lines as of Feb 5, 2018), while GS and LA libraries are obtained from Kyoto Stock Center (DGRC) (in total, 7824 lines as of Feb 5, 2018). Unfortunately, because of the nontargeted nature of the insertions, transcripts induced by binding of GAL4 protein to each element are not necessarily known. Alternatively, in vivo UAS-ORF (open reading frame) lines are a valuable resource for assessing protein localization and function. As of Feb 7, 2018, 3211 fly ORF lines for 2817 genes (excluding miRNA genes) are available at FlyORF at University of Zurich, Switzerland.

15.3.3 *Drosophila* Stock Centers

The abovementioned strains are deposited in and distributed from the public fly stock centers such as Bloomington Drosophila Stock Center (BDSC, USA), Vienna Drosophila Resource Center (VDRC, Austria), National Institute of Genetics (Japan), and Kyoto Stock Center (Japan). Our Kyoto Stock Center houses about 30,000 stocks including balancers, deficiencies, mutants, NP, GS, and protein trap lines. Upon shipment, we charge handling and postage fees, but not stock-maintenance fee. The other activities of the center, such as stock maintenance, inspection, acquisition, disposal, and database maintenance, are financially supported by the Japan Agency for Medical Research and Development (The National BioResource Project) and Kyoto Institute of Technology. On the other hand, about 75% of expenses at BDSC is covered by user fees. Like other data and biological material repositories, sustainability is always an issue. The current fee structure may be revised in the future.

Another important issue at the fly stock centers is that fly strains have to be maintained constantly as living cultures; they cannot be cryobiologically preserved. This raises concerns that strains may be accidentally lost or their properties be changed genetically or epigenetically during maintenance. In particular, preservation of the reference strains and original strains used in transgenic experiments without deterioration is urgently needed. Cryopreservation methods, once developed for embryos (Steponkus et al. 1990; Mazur et al. 1992) and ovaries (Brüschweiler and Gehring 1973), do not work well for large stock collections due to the need of time-consuming individual optimization. Collaborated with Satoru Kobayashi's group at the University of Tsukuba, Japan, and Daisuke Tanaka at National Agriculture and Food

Research Organization, Japan, we recently succeeded to cryopreserve primordial germ cells from early embryos of a few of strains. Because of low strain-to-strain variability of the optimum time of cell collection, this method is promising for application in the stock centers.

15.3.4 Bioinformatics Resources

Everyday drosophilists visit the FlyBase database (http://flybase.org) for retrieval of data on gene expression, phenotype and function, genome browsing, stock search, finding orthologs, and so on. While this is a sophisticated database in which we can do many things, it is not easy to use for non-experts because of numerous technical terms and FlyBase-specific data structure. This is particularly so for phenotypic data. Indeed, the FlyBase uses original structured controlled vocabularies for anatomy and development and annotation. The same happens when drosophilists use other organism and disease databases. The National Library of Medicine's controlled vocabulary thesaurus, MeSH, is very helpful, but does not cover all the fly vocabulary. We will come back on this issue later in Sect. 15.5.

Cross-species study requires researchers to gather and process information from several different databases. However, it is time-consuming and confusing to go back and forth due to the scattering of information. It is, therefore, desirable to have websites that integrate multiple information systems and that provide a concise summary of the information in easy-to-understand terms for nonspecialists. For example, MARRVEL (Wang et al. 2017; http://marrvel.org) and InterMine warehouse system (Smith et al. 2012; Kalderimis et al. 2014; http://intermine.org) facilitate integrative analysis of diverse data from humans and model organisms.

On the other hand, obvious anatomical differences apparently limit the direct extrapolation of findings in fruit fly to human and other model organisms. A method is developed to find non-obvious equivalences between mutant phenotypes in different species (McGary et al. 2010; Woods et al. 2013). Here, orthologous phenotypes (phenologs) are defined as those related by the orthology of the associated genes in two organisms. For example, a high incidence of male progeny in *Caenorhabditis elegans* and human breast cancer is in a phenolog relationship because of a significant overlapping of the two sets of causative or linked genes (McGary et al. 2010). Thus, the phenolog identifies novel candidate genes for human diseases. So far, *Drosophila* phenologs have not been developed; the development is eagerly awaited.

15.4 Cross-Species Study

The model organisms including *Drosophila* have made the great contribution to molecular and developmental biology. They reduce the complexity of the system under study and greatly enhance our understanding of the basic principles. The advantages of *Drosophila* can also be directly applied to cross-species study of complex biological processes and traits including human diseases. The high conservation between *Drosophila* and human makes such studies feasible.

Functional conservation of fly and vertebrate genes was first demonstrated by misexpression studies of the mouse and human *Hox* genes in *Drosophila,* where their gain-of-functional phenotypes are nearly identical to those caused by the orthologous fly genes (Malicki et al. 1990; McGinnis et al. 1990). One of the most striking examples is the finding that the *Drosophila eyeless* gene and its mouse ortholog *Pax6* induce ectopic eye formation in *Drosophila* (Halder et al. 1995). Subsequently, the conservation has also been shown by rescue of *Drosophila* mutants by vertebrate orthologs (e.g., Wang et al. 2002; Yamamoto et al. 2014). In yeast, since the early successful humanization (Kataoka et al. 1985; Lee and Nurse 1987), hundreds of yeast genes are humanized, and many are shown to rescue yeast loss-of-function mutations. Indeed, 48% (200/414) of essential genes tested were successfully rescued by the human orthologs (Kachroo et al. 2015). Although there is no systematic study of replaceability of *Drosophila* mutants

with the human orthologs, the success rate should be higher than this. So far, the results are promising (Wangler et al. 2017). The functional replaceability indeed encourages the use of *Drosophila* as an in vivo model to evaluate the effects of human variants on disease risk.

15.4.1 *Drosophila* as an In Vivo Animal Model of Human Diseases

It is currently estimated that all human beings are born with ~100 de novo mutations (Kong et al. 2012; Rahbari et al. 2016) and carry 100 or more loss-of-function or damaging mutations in the genome (Lohmueller et al. 2008; MacArthur and Tyler-Smith 2010; The 1000 Genomes Project Consortium 2012; Tennessen et al. 2012; MacArthur et al. 2012). Furthermore, each person differs from the reference sequence at 10,000 or more non-synonymous sites, of which 2500 or more occur at conserved sites (The 1000 Genomes Project Consortium 2012). They could potentially contribute to disease risk. A step toward personalized health care is to make personalized strains of model organisms, each expressing a unique human variant gene and then to evaluate its effects on phenotypes. Needless to say, *Drosophila* is suitable because of its simplicity, efficiency, and tools mentioned above. Indeed, humanized, personalized flies are now actively developed for this purpose.

Undiagnosed Disease Network (UDN) in the United States aims to improve the level of diagnosis and care for patients with undiagnosed diseases, to facilitate research into the etiology of undiagnosed diseases, and to create an integrated and collaborative research community to identify improved options for optimal patient management. To this end, UDN brings together seven clinical sites, a coordinating center, and core facilities (Cores). Cores include two DNA sequencing cores, a metabolomics core, a central biorepository, and a model-organism screening center. The screening center at the Baylor College of Medicine and the University of Oregon mainly focuses on *Drosophila* and zebra fish to predict the pathogenicity of missense variants identified by whole-exome sequencing. Thus, the screening center is developing new fly resources. Indeed, the group at the Baylor College of Medicine works together with Lawrence Berkeley National Laboratory and Kyoto Institute of Technology (Kyoto Stock Center) and is rigorously developing UAS-human ORF fly strains for rescue experiments.

Human variants that are gain-of-function can be simply tested for phenotypes of ectopic expression of variant genes in the model organisms. On the other hand, loss-of-function variants demand more effort. We must first study the phenotypes of loss-of-function mutations of the *Drosophila* ortholog and then attempt to rescue the phenotypes by expressing human genes in the damaged tissue. Experimental evidence that the wild-type human gene can rescue but that the variant cannot supports the pathogenicity of the mutation. However, driving the expression by a tissue-specific or a ubiquitous driver line may not be suitable, because it does not fully recapitulate the spatial and temporal expression pattern of the endogenous gene. In this sense, the MiMIC insertion lines and RMCE technology mentioned above are useful because they fully capture the endogenous expression pattern and allow us to conduct better-controlled rescue experiments. Indeed, recent applications to cross-species complementation predicted human variant pathogenicity (e.g., Chao et al. 2017; Yoon et al. 2017).

15.4.2 Use of *Drosophila* for Functional Analysis of Noncoding Regulatory Elements

Of course, protein-coding regions are not enough; noncoding regions are also essential parts of the genome. Indeed, a large portion of the human genome is inferred to be functionally active (The ENCODE Project Consortium 2012). However, identification and characterization of regulatory regions remain a challenge. For instance, genome sequence analyses of tumor cells have identified hundreds of potential oncogenic mutations in

protein-coding regions (e.g., Vogelstein et al. 2013) but very few of point mutations in noncoding regions (Rheinbay et al. 2017). The majority of disease and trait-associated variants identified by genome-wide association studies (GWAS) are in noncoding regions, but the functional evaluation of putative regulatory variants is very difficult without prior knowledge of regulatory elements or chromatin structure (e.g., Musunuru et al. 2010; Maurano et al. 2012). Thus, there is a large gap between identifying noncoding trait-associated variants and knowing their functional mechanisms.

An important indicator to identify regulatory elements in noncoding regions is the sequence conservation. The so-called ultra-conserved elements in the human and rodent genomes are extreme examples of conservation (Bejerano et al. 2004). There are thousands of such elements in vertebrate genomes (Visel et al. 2008). Most ultra-conserved elements are noncoding and thought to play an important role in the regulation of gene expression, although some ultra-conserved noncoding elements are known to be dispensable for viability in mice (Ahituv et al. 2007). Consistent with this expectation, the enhancer activity of these elements was detected in in vivo mouse reporter assay (Pennacchio et al. 2006). However, the detection rate falls below 50%, which is most likely due to the transient, single time-point expression assays. Stable transgenic lines should increase the sensitivity and accuracy of identification of expressing cells with the aid of multiple observations of the same line in multiple developmental stages. For this reason, we generated transgenic fly lines carrying human ultra-conserved elements for enhancer reporter assay. Three examples are shown in Fig. 15.1, in which element 113 drove broad expression in the ventral nerve cord of larvae and elements 381 and 1871 in subsets of cells in the central brain and ventral nerve cord. In the mouse assay, the reporter gene expression was observed in the dorsal root ganglion in element 113 and eye in element 381, but not detected in element 1871 (Pennacchio et al. 2006). So far, we observed the reporter expression in one or more tissues of embryos and larvae in all elements tested (unpublished data), implying the usefulness of stable transgenic lines. For detection of enhancer and other regulatory activity, *Drosophila* could be a useful material.

15.5 Future Challenges Facing Cross-Species Study

The increasing availability of the state-of-the-art technology and resources of *Drosophila*, aided by web-based data mining tools, advances our understanding of human and other genomes. However, there are still reasons that prevent researchers from using other model organisms. To increase the use of fruit fly in other species communities and to facilitate cross-species study, we must face the following five challenges.

The first challenge is to deal with community-specific dialects; different communities have dif-

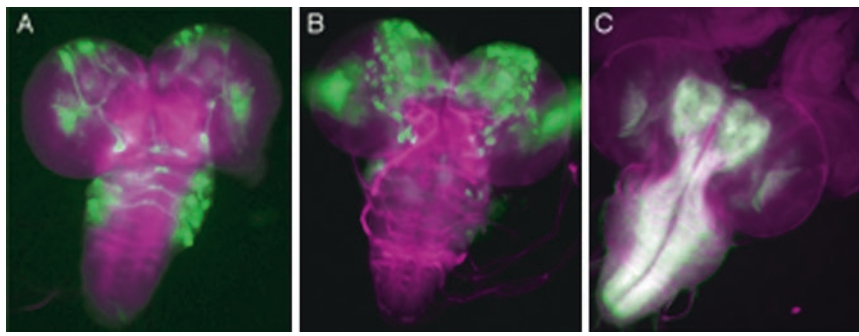

Fig. 15.1 *Drosophila* transgenic enhancer assay effectively detects enhancer activity of noncoding ultra-conserved elements from human. (**a**) element 381; (**b**) element 1871; and (**c**) element113

ferent languages. This is especially so in phenotypic description. Gene ontology (GO) terms, molecular function, cellular component, and biological process, are based on sequence and expression data; therefore, they are relatively easily standardized across species. On the other hand, phenotypes are described by the genetic, anatomical, and developmental nomenclature, which often makes species-specific controlled vocabularies. As mentioned above, like many other databases, FlyBase uses original vocabularies for phenotypes. For instance, while FlyBase uses "male sterility," Medical Subjects Headings (MeSH) recommends "male infertility" for reduced fertility in males. This prevents from or at least delays mutual understanding between researchers using different organisms. Phenotypes are increasingly important for cross-species study. Easy sharing of information will be a key to lower the barriers between model-organism communities.

The second challenge is to deal with context dependency of mutational effects; damaging or deleterious mutations in one species may not be so in another. For example, the chimpanzee genome is reported to have 16 missense variants that cause human diseases (The Chimpanzee Sequencing and Analysis Consortium 2005). Such pathogenic variants are referred to compensated pathogenic deviation (CPD) because their potential harmful effects are thought to be compensated by some other compensatory mutation or mutations (Kondrashov et al. 2002). CPDs are indeed prevalent in vertebrate genomes (estimated at ~10%; Kondrashov et al. 2002; Jordan et al. 2015). This phenomenon cannot be ignored in a cross-species study.

The third challenge is that functions of noncoding regions, particularly of the human genome, are poorly understood and functional effects of noncoding variants are difficult to identify. However, the development of efficient, fast, and reliable identification method is essential because noncoding variants comprise the majority of single-nucleotide polymorphisms (SNPs) associated with disease and other human traits. Although precise comparison with the expression pattern of endogenous genes would be required, fruit fly can potentially be used for the enhancer and other regulatory function assay as shown in Fig. 15.1.

DNA sequencing data have been rapidly accumulating in non-model organisms, leading to the identification of many CPDs as mentioned above. These CPDs are used to identify compensatory variants (e.g., Plotnikova et al. 2007), which may, in turn, deepen our understanding of the molecular nature of the pathogenic effects and provide therapeutic targets. Ortholog and paralog relationships and their functional conservation provide a better means to understand the evolution of gene regulatory networks. At any rate, the potential use of the data from non-model organisms should be explored further.

Besides these challenges, there is a physical barrier for non-drosophilists to start their fly experiments. Despite relatively low cost and small space demands, they must have basic facilities such as cooking equipment and space, breeding incubators, and sterilization equipment. Providing experimental space and facilities for temporary users would greatly expand the use of *Drosophila*. The stock centers have potentials for such activity. Currently, collaborated with National Institute of Genetics, Kyoto Institute of Technology proposes to establish a screening center for cross-species study. Any such center could play a pivotal role in advancing this field in a short period of time.

15.6 Final Remarks

The scope of translational research in model organisms is becoming wider, and the priority for funding such research is clearly increasing. However, it is not the only way for *Drosophila* to contribute to science. Too much focusing of future research activities on humans is not my intention either. To give one example, the evolutionary, developmental study would be expanded to a much longer time scale by using *Drosophila*. As an excellent model organism, *Drosophila* continues to contribute to many fields of biology through cross-species study including and beyond humans.

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Designs for Flies + of Mice and Men: Design Approaches to *Drosophila melanogaster*

16

Julia Cassim, Frank Kolkman, and Marcel Helmer

Abstract

Designs for Flies is an award-winning design-led interdisciplinary project between KYOTO Design Lab (D-Lab), the Department of Applied Biology at the Kyoto Institute of Technology (KIT) and Charcot-Marie-Tooth (CMT), Japan. Within the framework of speculative design yet using an inclusive methodology, Frank Kolkman, a young Dutch designer, took Professor Masamitsu Yamaguchi's climbing assay experiment with *Drosophila* in his genetic mapping for CMT as the point of departure. Kolkman sought to address two questions raised during his initial research: "Could alternative strategies be used to generate interest from pharmaceutical companies for obscure, complicated or 'unmarketable' diseases in drug research?" and "Could transgenic *Drosophila* be used for the wild-card testing of drug compounds directly by patients at home in the search for a possible cure?" The chapter will describe its genesis, design process and the challenges and potential of interdisciplinary projects of this nature along with the impact of the resulting concept, which incorporated service, system, product and interaction design. It won the Services and Systems category of the Dutch Design Awards (DDW) in 2016, and Kolkman was named DDW's Young Designer of the Year in October 2017. It was followed by *Of Flies, Mice and Men: drosophila and the interconnected landscape of genes*, a *Drosophila*-related science communication project by Marcel Helmer, Kolkman's successor as D-Lab Design Associate for which the design brief was based on issues raised by the first project. This is also described to highlight the differing issues, design approaches and results of this science/design collaboration.

Keywords

Speculative design · Inclusive design · Communication design · Design process · Interdisciplinary collaboration · Science communication · *Drosophila melanogaster*

16.1 Introduction

In May 2014, KYOTO Design Lab (D-Lab) was established at the Kyoto Institute of Technology (KIT) funded under the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) Global Centre of Excellence (COE) programme (http://www.jsps.go.jp/english/e-globalcoe/). The overall aim of the

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COE programme is to raise the international competitiveness of Japanese universities. In KYOTO D-Lab's case, this is to be an incubator for new educational approaches to design and architecture with an emphasis on "Innovation by Design", alongside international collaboration with leading schools worldwide. Julia Cassim was appointed Project Professor for Design charged with developing initiatives to support this vision.

Three linked programmes were established – an Open Workshop, a Design Associate and a Designer-in-Residence programme – each with separate aims. The Open Workshop programme is run in collaboration with universities abroad or established design practitioners with expertise in the selected theme. These 5-day intensive workshops are open to all, advertised via the D-Lab website, and attract students from different departments in KIT and other universities as well as practicing designers and participants from different sectors. As a network-building vehicle, the programme aims to demonstrate new interdisciplinary models of design education – ones that explore themes that are more reflective of the social realities with which young designers and the design industry as a whole must now deal.

The two projects described in this chapter resulted from this Open Workshop programme in conjunction with the Design Associate programme. For this, a young designer is invited to spend a period of up to 6 months at D-Lab working in collaboration with an existing research team. The areas chosen are those where design applications can be readily identified and in which KIT excels, namely, materials science, fibroscience and the life sciences. Three overarching themes were chosen under which projects would be advanced – *Making and Materials*, *Designing Social Interactions* and *Critical Curation and Interpretation* – again to reflect KIT research strengths in these areas. The latter theme was chosen since the university is home to the KIT Museum and Archive, which offers opportunities for new approaches in curation, communication design and interpretation. The programme's overall aim is to show how design, when applied to scientific research, can be the springboard for innovation.

Designs for Flies and *Of Flies, Mice and Men* came under the *Designing Social Interactions* theme. They were a collaboration between Professor Masamitsu Yamaguchi's research team in Applied Biology and the Centre for Advanced Insect Science (CAIR), Professor Toshiyuki Takano of KIT's *Drosophila* Genetic Resource Center (DGRC) (Fig. 16.4), Drs Yumiko Azuma and Yukie Kushimura of the Kyoto Prefectural Medical University Hospital and Keiko Ota and Takahiro Hosoi of Charcot-Marie-Tooth Japan (Fig. 16.1).

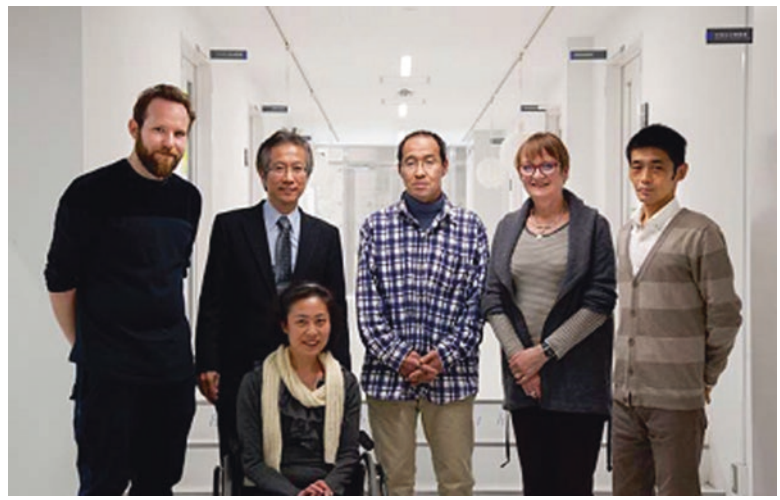

Fig. 16.1 The project team: from left, Kolkman, Yamaguchi, Hosoi, Cassim, Yoshida, Ota (front). (Photo: Juuke Schoorl)

Fig. 16.2 Climbing assay experiment. (Photo: Juuke Schoorl)

The two projects were intended to serve as model templates to demonstrate how cross-disciplinary collaborations of this kind can be realised to encourage other such initiatives in higher education. Before describing their design process and results, it is perhaps wise in a book devoted to science to give a contextual description of the complex landscape in which contemporary designers operate in a post-digital age and to discuss the methodologies underpinning the different design approaches used (Fig. 16.2).

16.1.1 The New Landscape of Design in a Digital Age

In 1931, when the Finnish architect and designer Alvar Aalto (1898–1976) was commissioned to design a tuberculosis sanatorium at Paimio, his approach was holistic. He intended it to be a "medical instrument" that would actively contribute to the healing process of the patients. Accordingly, he designed not only the building but also its interior furniture, including the bent-wood Paimio Chair, an original version of which now sits in the Museum of Modern Art (MOMA) in New York. The chair consists of a single thin sheet of laminated birch plywood that ends at its top and base in an elegant scroll. Aalto chose the optimum angle for the back that would allow the tuberculosis patients, who would be seated on it for long periods, to breathe easily. The chair embodies the sought-after qualities that distinguish a design classic from its mundane contemporaries – simplicity, beauty and truth to form, innovation in materials' use and importantly that it could be mass-produced and was therefore affordable. In addition, the inclusive thinking that underpinned its design meant that it was fit for purpose, one reason behind the design's longevity. The simple linear transactional relationship between Aalto the designer and Artek the firm that he founded and which still produces the chair was similarly clear-cut as was the process by which the design was advanced into production. It was one that persisted until the advent of the digital age when products and the way in which they were designed and manufactured underwent seismic change.

16.1.2 From Independent Product to Interface

In this new digital age, many products, which incorporated digital technology, lost their independent status and identity, becoming instead the primary interface to delivery of a broader service, system or network. As such they became incomplete in their own right since their success or failure was bound not by the functional values, aesthetic appeal or implicit use that distinguished modernist design icons of the past. Instead their useful life was determined by a swiftly evolving technology that would be obsolete a few years hence. Their development required the expertise

of a multi-skilled team, in which designers were often in a minority – one which could deliver all elements of the product, service and system to which this primary interface was umbilically attached. Accordingly, design as a profession expanded to incorporate new predominantly multidisciplinary genres. It is no surprise that the biggest sector now is digital design which incorporates web, communication and service design. Between 2010 and 2015, this grew by 109.7% in the UK, while product and industrial design activity shrunk by 18.8% (UK Design Council 2015).

In tandem with this, the design process itself had to be redesigned to accommodate this new multidisciplinary digital reality. In its best inclusive form, it can be described as akin to a boat race with all participants in communication at any given time yet where leadership shifts according to the expertise needed at the particular point of design development. This differs from the conventional linear relay race, which is sequential in its delivery of a design result. In complex projects, this can result in the intention of the original design brief being lost because of miscommunication between the different disciplines, each of which has their own terminology and process for advancing ideas. Problematic processes such as these can be likened to the party game of Chinese whispers with results that are inevitably not fit for purpose and where the results can be catastrophic in the area of healthcare and medical device design where patient safety is placed at risk by poor design. (NHS 2003)

The second aspect of change has been the necessity for individual designers wishing to address complex subjects to become multi-skilled as is the case with Kolkman and Helmer. Both were required to have a scientific grasp of the subject and strong conceptual ideation ability alongside physical prototyping, programming, research skills and the skill set they brought from the disciplines in which they had originally been trained – Kolkman as a product designer, while Helmer comes from a communication and interaction design background. These factors influenced the way in which the final design result was embodied, overlaid as each was by the particular conceptual emphasis of the Design Interactions programme at the Royal College of Art (RCA) of which they were contemporaries and graduates.

16.1.3 Speculative Design, Inclusive Design: What's in a Name?

16.1.3.1 Speculative Design, Inclusive Design: What's in a Name?

> Few people think about it or are aware of it. But there is nothing made by human beings that does not involve a design decision somewhere. — Bill Moggridge, IDEO founder (Kelley and Kelley 2013)

Design can be said to be a broad church in which different movements rise and draw their adherents only to be subsumed into new poles of interest or superseded by new terminology. Broadly speaking, this project embraced two schools of thought – Inclusive Design and Speculative Design. The major thrust of Inclusive Design, known also as Universal Design or Design for All, depending on geographical context, is simple – to ensure that the products, services, environments and communications that we design do not actively exclude the diverse range of people who use or depend on them in ergonomic, cognitive, sensory, economic, social or digital terms. This is achieved by understanding the context of use and potential application for the proposed idea and the nature of design exclusion, through dialogue and interaction with potential users and the tailoring of the process in which the design is advanced (Cassim 2014).

In the *Designs for Flies* case, Inclusive Design was used as an organisational framework to interrogate the issues and viewpoints of all stakeholders involved in the subject, particularly where they related to people living with CMT – the only missing group were drug companies themselves. Their position nevertheless was considered when Kolkman inbuilt the crucial commercial quid pro quo incentive into the concept that would be necessary to secure their interest. Helmer's project, which targeted a more general audience and built on the findings of the first, similarly took into

consideration the difficulties expressed by the scientific team in communicating the importance of *Drosophila*-related research to diverse audiences rather than a specialist one alone.

Both designers used their training in speculative design as a conceptual tool of enquiry and expression. Speculative design is a design approach articulated by Anthony Dunne and Fiona Raby who founded the influential Design Interactions programme at the RCA: "When people think of design, most believe it is about problem solving ….There are other possibilities for design: one is to use design as a means of speculating how things could be – speculative design. …. This form of design thrives on imagination and aims to create spaces for discussion and debate about alternative ways of being, and to inspire and encourage people's imaginations to flow freely. Design speculations can act as a catalyst for collectively redefining our relationship to reality" (Dunne and Raby 2013).

As a school of thought, it is referred to by other names, which are again context specific – critical design, conceptual design, design for debate, adversarial design, design fictions or discursive design and so on. Irrespective of the terminology used, the aims are similar: "all remove the constraints from the commercial sector that define normative design processes; use models and prototypes at the heart of the enquiry; and use fiction to present alternative systems and world" (Auger 2013). Central to the approach, "speculative design is not only to encourage contemplation on the technological future but can also provide a system for analysing, critiquing and re-thinking contemporary technology" (Auger, ibid).

Thus, design is used not for its conventional business or problem-solving purpose but rather as a means to question the status quo; to stimulate debate, particularly around ethical issues that arise from the use and potential abuse of new technologies; to speculate about their future use; or to posit new possibilities. This latter aspect separates designers of this type from those who do the same in other disciplines. A designer's medium of expression is not a set of text-based theoretical proposals but manifests itself as concrete material or graphic entities or potentially valid products backed by a convincing hinterland of service and systems ideas as is the case with *Design for Flies*. The starting point of enquiry for Kolkman's project fits Auger's definition, but the result was rooted in reality backed by an inclusive process that took into consideration the needs and aspirations of the design partners living with CMT who were involved from the project start.

Helmer's background is graphic and interaction design rather than product design which, nevertheless, was employed to create a physical language of gears but for a different purpose, not for a functional object but rather as a metaphorical language of association that was readily understandable and could provide the core basis of reference to understand the comparative relationships – a "perceptual bridge" as Auger has called it – the means by which the designer engages their audience. As such it did not fall within the category of speculative design but rather of communication design.

Both projects were supported throughout by the expertise of Professor Yamaguchi's team which provided the key scientific knowledge that acted as an initial ideation platform for both Kolkman and Helmer, alongside actual demonstration of the scientific processes they employed in their *Drosophila*-related experiments (Fig. 16.3). In addition, Professor Toshiyuki Takano, head of KIT's *Drosophila* Genetic Resource Center (DGRC) showed how *Drosophila* were bred as well as the logistical technicalities of how they were dispatched safely to research institutions in Japan and abroad (Fig. 16.4). In Helmer's case, it was crucial that the scientific data communicated was accurate and verifiable; thus consultation extended throughout the 6-month project period, while with Kolkman, it was limited primarily to the initial concept ideation process and to proof of concept verification.

16.1.4 Defining the Project Brief

The project was initiated by Cassim in consultation with Yamaguchi, in each case prior to selection of the designers. Yamaguchi organised an annual Bio-Art class for his 2nd year students

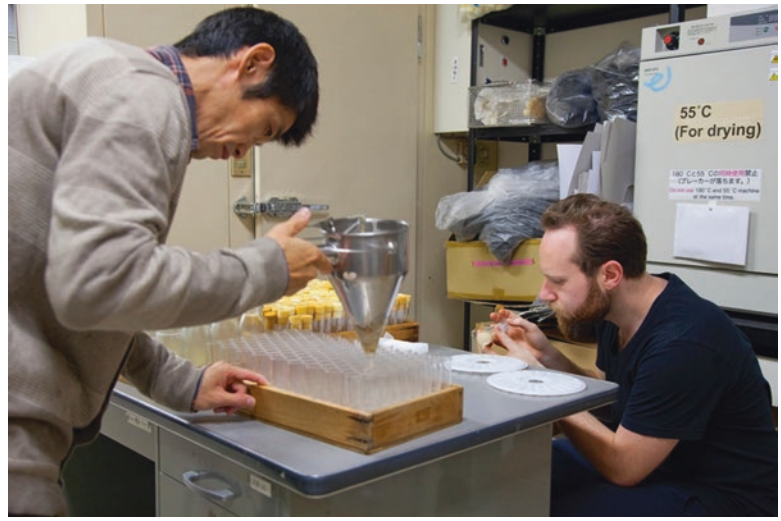

Fig. 16.3 Background research by Kolkman with Associate Professor Hideki Yoshida on the *Drosophila* breeding process. (Photo: Juuke Schoorl)

and interested ones from KIT's design school based on the use of genetically modified *E. coli* ink as a tool for artistic expression. (http://2014.igem.org/Team:KIT-Kyoto/Project) Thus, he was no stranger to the idea of creative collaborations of this kind. After discussion on potential research areas that would lend themselves to design applications, Charcot-Marie-Tooth (CMT) disease, a rare hereditary disease of the peripheral nervous system, was chosen. Yamaguchi's team was in the process of creating a genetic map for this complex and incurable condition, which became the basis for the initial design brief. This was kept open-ended but stated some of the key issues that would have to be considered in relation to the final design result, which had been discussed by Cassim with Yamaguchi and his team prior to Kolkman's arrival.

The design brief for *Designs for Flies* posed the following design question: "Can a physical model that visualises and demonstrates this genetic map and CMT's cause and effect be created that will effectively and simply communicate this complexity?" The aim would be to create greater understanding among three constituencies – clinical researchers, drug companies and the general public – and thereby contribute to a wider awareness on the part of drug companies to the importance of investing in CMT research. Could it also enhance understanding of the emotional difficulties caused by those living with CMT among the wider public? In the event, Kolkman chose to interpret the brief not as physical model of the genetic map but as a speculative response to the issues raised inspired conceptually by Yamaguchi's climbing assay experiment (Fig. 16.2). Yet it was embodied in a product and interlinked system and service that could feasibly be implemented. Irrespective of whether this would happen, *Designs for Flies* serves also as a powerfully persuasive communicative medium for the issues raised.

This is particularly the case as they related to the drug companies and the emotional impact of the condition on those living with CMT and their sense of powerlessness and exclusion, which they had expressed to Kolkman.

In contrast, the design brief for *Of Flies, Mice and Men* derived from the first project and was communication design specific. It was inspired by a chance remark by one of the CMT participants who expressed surprise that a "dirty" insignificant fly could be a powerful research tool into a possible cure for her condition. This issue was linked to the overall low status of *Drosophila* within research and its apparent hierarchical pecking order of human, mouse and fly. This seemed to ignore the vital role *Drosophila* can play in establishing first principle research concepts because of its fast reproductive rate; short

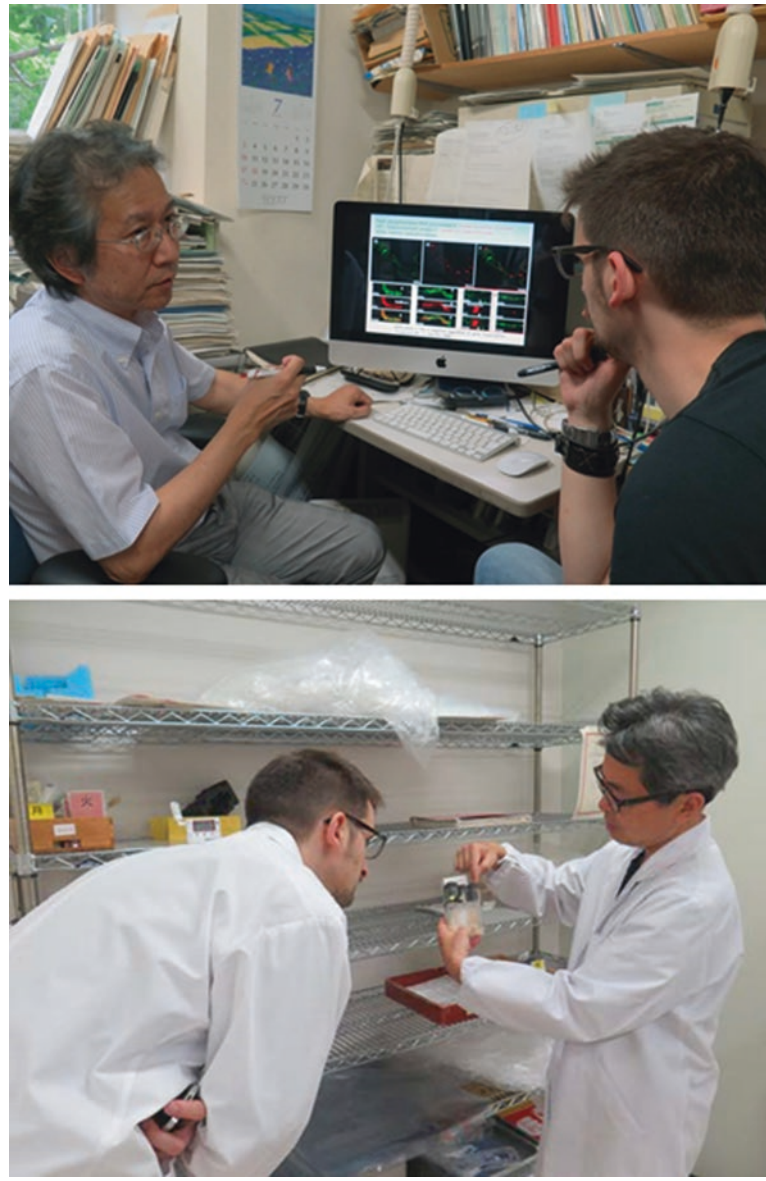

Fig. 16.4 Helmer in the initial contextual briefing phase with Professor Masamitsu Yamaguchi (above left) and Professor Toshiyuki Takano of KIT's Drosophila Genetic Resource Center (below right). (Photos: Julia Cassim)

life cycle; the relative ease by which it could be used for genetic research in different contexts, particularly in the Third World; and importantly the little-known fact among the general population that 75% of human disease-causing genes have a functional homologue in *Drosophila*. There was an evident correlation between this and the way designers come up with "quick and dirty" concepts using an iterative prototyping process, where physical models are serially developed, trialled and refined before the final concept is decided. The question was how could this be communicated? Helmer's approach to the brief is discussed in detail later in this chapter.

16.1.5 Selection of the Designers

Kolkman had been a participant in a 4-day KYOTO D-Lab Open Workshop organised by Cassim in December 2014 and led by Anthony Dunne and James Auger of the RCA's Design

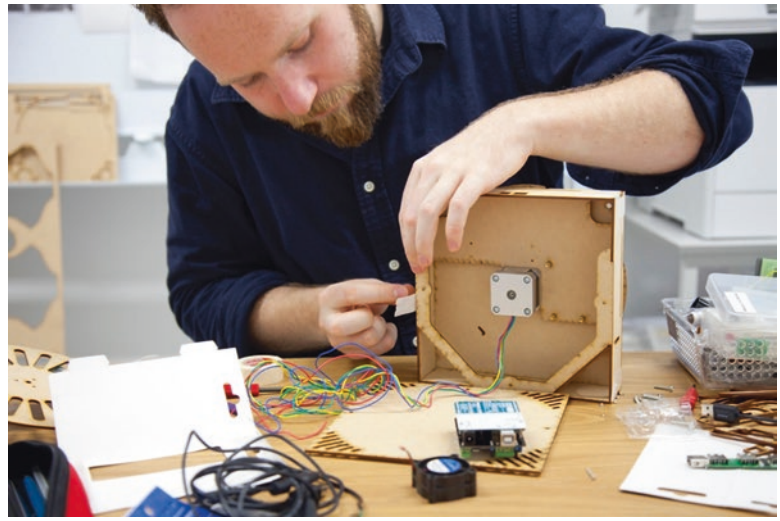

Fig. 16.5 Kolkman at work constructing the prototype. (Photo: Juuke Schoorl)

Interactions programme. The theme had centred on robots in healthcare and the contrasting cultural attitudes to their use in Japan and the UK (KYOTO Design Lab 2014). *Open Surgery (a DIY surgical robot)*, the initial concept he rough-prototyped, then became the basis for his graduation work which went on to win prizes at Ars Electronica and the Beazley Design of the Year Awards in London (http://www.opensurgery.net/). Kolkman's impressive skill set and interest in the subject area made him the obvious candidate to undertake the project (Fig. 16.5). As a communication designer, Helmer was selected based on Kolkman's recommendation and his pre-existing understanding and interest in *Drosophila* dating back to his biology classes in high school.

In the following two sections, the projects are described in detail as case studies:

16.2 Project One: Designs for Flies – User-Participatory Domestic Drug Screening Kit

16.2.1 Contextual Background

Drug discovery is a lengthy, expensive process involving complex interplays between industry, academia, investors, jurisdiction and marketing. Where no significant commercial benefits can be expected from the treatment of particular disorders because they are considered to be rare or too complex, the one hope of therapeutic advancement comes from philanthropic funding. This leaves patients with these conditions feeling neglected and powerless to influence or change the status quo.

Designs for Flies looks at creating alternative strategies for drug research to generate interest from pharmaceutical companies for rare, complicated or "unmarketable" diseases. What if patients living with these conditions could become active participants in their own pharmacological research? What if they could do this in their own homes and thereby be instrumental in finding potential cures? The project proposes a personalised drug screening strategy for patients living with Charcot-Marie-Tooth (CMT) disease, an obscure neurodegenerative disorder that has largely been ignored by pharmaceutical companies for reasons of cost and complexity. Building upon Professor Masamitsu Yamaguchi's use of *Drosophila melanogaster* as a research methodology to build a genetic map for CMT, the project promotes the use of *Drosophila* as a candidate for random drug screenings. After feeding personalised, transgenic fruit flies with randomly selected chemical compounds, a specially designed device monitors their behaviour to discern any therapeutic effects and is supervised by patients at home.

16.2.2 The Design of the Drug Screening Device

16.2.2.1 Physical Design of the Device

The portable device consists of a turntable with two overhead webcams. On the turntable, a sealed plastic cassette can be fitted, containing both wild-type and transgenic flies in alternating rows. The function of the device is to record and analyse the differences in the movements of the flies in the test group (transgenic flies) versus the control group (wild-type flies). During a short period of rotation, the turntable forces the flies to the outside perimeter of the cassette. When the turntable comes to a standstill, the flies will instinctively walk towards the inside of the disk. At this time, their movements are monitored by the webcams, with image analysis software plotting the location of the flies in every frame. From this, their speed, distance and endurance can be deducted (Fig. 16.6 and 16.7).

Under normal circumstances, movements of the flies in the test group will be measurably slower according to the characteristics of CMT. If, however, a single fly in the test group displays speeds and agility more akin to its control group counterpart that has been fed with the same compound, the particular chemical compound used to feed it may be displaying some therapeutic effects. This would trigger further investigation and could potentially provide a starting point for medication to be developed.

When left alone, the device will operate in automatic mode, performing a spin cycle every 15 min. However, there is also a button at the front of the device that can be pressed to perform an additional manual test cycle. The manual mode interrupts the automatic test schedule and is intended for additional visual inspection with the naked eye. It can however only be induced after enough time has passed since the last test cycle.

During a test cycle, only a single pair of flies is monitored by the webcams. However, with every consecutive cycle, the disk moves forward one step to another pair. Therefore, every pair will produce the same amount of data, while all flies will be subject to the same conditions throughout (Fig. 16.7).

After a week, the cassette needs to be replaced by a new one, including new flies and different chemical compounds. The data collected over the course of the previous week will then be emailed to the donor in the form of a visual report. It will also be sent to a server to be processed and compared to data retrieved from other devices, thereby making it accessible as an open source to donors and researchers worldwide (Fig. 16.8).

16.2.2.2 How the Device and Underlying System and Service Work

- After filling out a subscription form and paying the initial membership fees, a person living with CMT collects a saliva sample and sends it to the lab.

Fig. 16.6 Keiko Ota of CMT Japan operates the final prototype. (Photo: Juuke Schoorl)

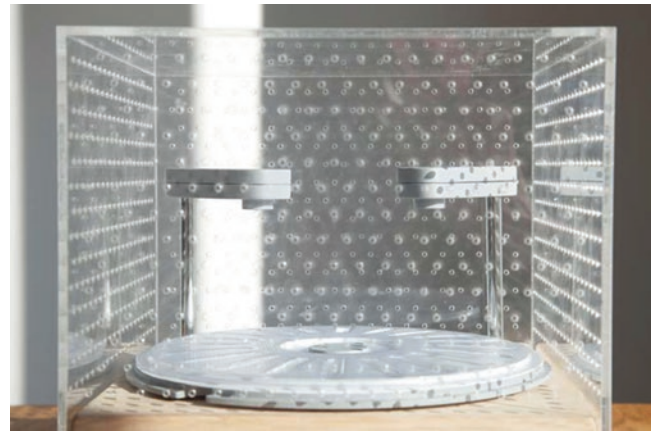

Fig. 16.7 Close-up of turntable and overhead webcams. (Photo: Juuke Schoorl)

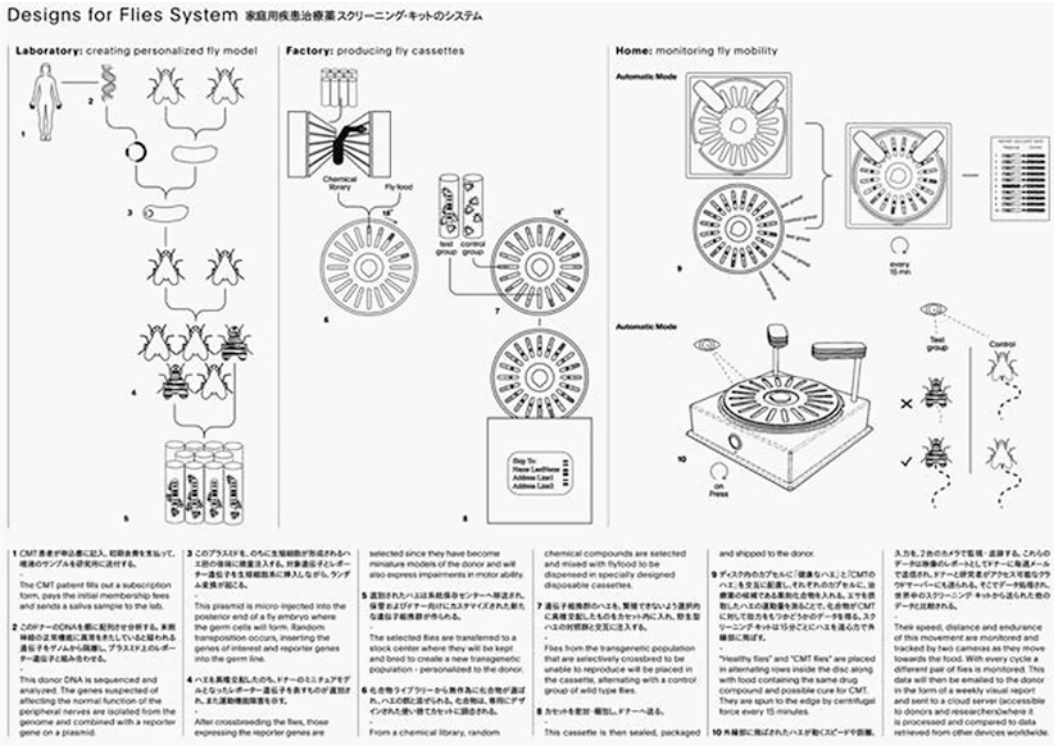

Fig. 16.8 Designs for Flies system and service process diagram

- In the lab, this donor's DNA is then sequenced and analysed. The genes suspected of affecting the normal function of the peripheral nerves are isolated from the genome and are combined with a reporter gene on a plasmid.
- Next, this plasmid is micro-injected into the posterior end of a fly embryo where the germ cells will form. Random transposition occurs, inserting the genes of interest and reporter genes into the germ line.
- From a chemical library, random chemical compounds are selected and mixed with fly food to be dispensed in specially designed disposable cassettes.

- A number of flies from the population – selectively crossbred to be unable to reproduce – will also be placed in the cassette, alternating with a control group of wild-type flies.
- This cassette is then sealed, packaged and shipped to the donor.
- After receiving a package weekly via mail, the donor must place the new cassette on the device and dispose of the old one. The resulting data can then be collected and compared with all participating donors (Fig. 16.8 and 16.9).
- After crossbreeding the flies, the flies expressing the reporter genes are selected, as these will also carry the genes of interest. They have become miniature models of the donor as they are likely to express impairments in motor ability.
- The selected flies can then be transferred to a stock centre where they will be kept and bred to create a new transgenic population – personalised to the donor.

16.2.2.3 Why It Works

The potential of this service as a legitimate strategy for drug research lies in the fact that it brings together the interests of three constituencies:

- The commercial requirement of pharmaceutical companies to generate a profit
- The desire of patients and family members to participate in efforts to develop a possible cure and thereby have some emotional and practical control over their condition
- The needs of researchers to understand the causes of CMT

The sale of unproven chemical compounds instead of finished medicine may provide pharmaceutical companies with a viable business model, offering high reward against relatively low risk. This business model in turn ensures continuity of the research for the researchers and patients while also providing the patients with a psychological sense of control. The additional data produced might also help scientists better understand the origins and function of the disease. Furthermore, if and when a cure is found, the collective nature of this strategy would limit the disruptive potential of patents, ensuring that treatments could be made available at reasonable prices. Although presented as such, this strategy is not limited to CMT but could be applied to many different genetic disorders.

16.3 Project Two: *Of Flies, Mice and Men – The Interconnected Landscape of Genes*

16.3.1 Contextual Background

This science education design project centred on finding a new way of communicating the quali-

Fig. 16.9 Prototype of packaging. (Photo: Juuke Schoorl)

ties of *Drosophila*, their close relationship to humans and their value as scientific tools within medical research and biology. The intended audience was determined to be the spectrum of visitors to a science museum, which would include schoolchildren, researchers and general visitors with or without an interest in the subject. These have been termed "streakers, strollers and students" by George MacDonald of the Canadian Museum of Civilisation and Museum, Victoria (Pontin 2013)[i], while Veron and Lavasseur (Veron and Levasseur 1989), who tracked visitors to a natural history museum, classified them more poetically in four categories as:

- Ants – move methodically from object to objects
- Butterflies – move back and forth, alight on some displays
- Grasshoppers – choose specific objects and hop from one to the other
- Fish – glide in and out of exhibitions with few stops

The challenge for the project, therefore, was to ensure that information was delivered in such a way that these different parties could engage with it at their particular level of interest or pre-existing knowledge or conversely lack of it and that it was not pitched at those with deep understanding of the subject alone.

It was clear from discussions with the scientific team that despite the obvious advantages of cost, time and comparative ease of breeding *Drosophila* for genetic research or as models of human diseases, the fruit fly is still viewed, particularly by drug companies and funding bodies as the poor cousin to the transgenic mouse. Funding applications involving *Drosophila* may be overlooked in favour of those involving transgenic mice or require validation by the latter method even where solid results are acquired. Mice have an extensive cultural history to draw upon in children's literature, animation and so on that humanise them and give them emotional significance beyond their status of the rodent. In contrast, fruit flies, despite their complexity as a species, are seen merely as the inevitable companion of rotting food, fungal material or plants and in no way connected to humans despite their close genetic relationship. The project sought to address how these misconceptions could be addressed and perhaps reversed.

16.3.2 Physical Design of the Triptych and Mechanical Object

Helmer created two automata to illustrate different aspects of their close relationship with man and mouse (Fig. 16.10 and 16.11). The large mechanical triptych that is activated by motors introduces us to the similarities of flies, mice and men (Fig. 16.10). It uses the single gear as a metaphor and physical representation of a gene

Fig. 16.10 Overall view (above) and detail (left) of triptych. (Photo: Tomomi Takano)

Fig. 16.11 Full view of mechanical object. (Photo: Tomomi Takano)

and is marked with its specific number. For each element, the number of gears in relation to its complementary pieces represents the numbers of encoding genes within the respective organism. The design and layout show both their genetic connectedness and how the three species differ from each other. Charcot-Marie-Tooth (CMT) was used as the base model.

The smaller mechanical object demonstrates a fly's unique abilities and its short life of 30 days (Fig. 16.11). In comparison to the transgenic mouse, scientists are able to breed *Drosophila* quickly and in multiple versions. This allows them to rapidly establish research principles and pathways that can be explored later in depth, much as a designer develops their concept through iterative prototyping. The "faster rotation" of a fly's life allows us to look at the fly we see now and also at succeeding generations – an especially important consideration when researching hereditary diseases.

16.3.3 The Use of Metaphors as a Visual Language

There was a need to create a metaphorical language to express the interlinked relationship between the three species, which may not be apparent to a non-scientific audience that lacked any prior knowledge of the subject yet, which served as a cogent and accurate expression to a scientific one. The metaphor had to be a point of reference to which newcomers to the subject could refer at any stage of exploration of the automatas' accompanying text and serve as a conceptual anchor and "perceptual bridge" (Auger 2013)[ii] to the bigger picture. It had to simultaneously accommodate all features of *Drosophila melanogaster*'s specific qualities while striking the right balance between depth and easy engagement for a wide-ranging audience. The fact that *Drosophila melanogaster* share such a high percentage of the human genome was unlikely to be common knowledge to a general audience. Thus, it was important to underscore the idea that two organisms may be quite different in their physical appearance and evolutionary history, but many of their common regulatory functions are based on similar biochemical properties and processes. In Helmer's initial conversations with Professors Yamaguchi and Yoshida, the latter had explained that he used the following analogy to describe the role of *Drosophila* to his students:

> If the human genome is like a computer, the fly is like a radio. It is simpler but uses the same or similar building blocks and principles. If we can understand and test the simple radio, if becomes easier to understand the computer.

16.4 Emotional Associations

This remark was the initial inspiration for the overall mechanical language of gears and levers that Helmer adopted in both automata (Fig. 16.12). Its aim was to create a more objective yet engaging comparison between humans, mice and flies and detach the topic from bio-horror and personal impressions of insects as villainous carriers of dirt and disease – one propagated in particular by vintage public health posters and science fiction cinema.

A more conventional science educational approach would have been to stress the wonder and complexity of small organisms. Modern microscopy and photography are able to give formerly invisible processes a face. This, however, may not be always beneficial for communication purposes. Flies and insects at their own scale summon up emotional responses linked to disgust, disease and dirt in many peoples' minds or conversely can highlight their apparent insignificance. Scaling them up might be fascinating to some, but others could recoil from seeing detailed aspects of what would then be viewed as alien beings so different to our own anatomy, an approach that ties in with the visual strategies of bio-horror movies. In addition, zooming in to a molecular level creates a language that is detached from our own perception of the world and does not permit a global understanding of the big picture. We had to achieve a view that was detached from the biology and was more engaging than models of atoms and molecules yet which communicated the details of the reactions and dependencies of each element.

16.5 Complexity and Prior Knowledge

It would have been both challenging and interesting to look into a specific experiment linked to KIT's current research or published papers. This would have the advantage of creating a link to tangible developments and potential applications for state-of-the art *Drosophila* research. Conversely, this would focus on a particular aspect alone and require complex contextual explanation of many terms and processes. For an audience with prior knowledge of the subject, such as students, this would be an interesting approach particularly where the visual language employed could potentially be used as a communication method for an ongoing series of specific experiments. However, the desired audience was not just students or those with an understanding of the basics of molecular biology but a more diverse one. The project was interlinked with *Designs for Flies* and thus had to address issues arising from it and the constituencies involved.

Fig. 16.12 Helmer assembling the triptych. (Photo: Julia Cassim)

16.6 Data Visualisation: Numbers to Images to Narratives

The existing vocabulary we found when looking into the scientific abilities of the fly was a language of numbers. Numbers are precise and carry little emotional value – an important parameter in the world of science – but we wanted people outside this world to learn and understand. The use of graphs and visualisations is not unique to the world of design. Translating numbers into abstract shapes is just a first step. While this may increase the visual impact and comparability, it does not necessarily form a lasting memory. The numbers had to be connected to more than abstract shapes and linked to objects or known experiences with which the audience could form a narrative connection, hence the use of the gears and levers. An important aim was to reduce the actual amount of numbers or text context to understand the general principles expressed overall as visual metaphors that could be intuitively understood. So instead of starting the story at the dramatic finish, it had to be built from the ground up to let the audience decide how far they wanted to walk along that road. Based on this approach, the layout of the initial object was constructed. The process starts by sketching the relationships, understanding the numbers and comparing them and their actual relationship and what the realistic scales were to convert them to make them understandable, interesting and detailed.

The general triangle shape was the first, followed by the number of gears (Fig. 16.10). To include more structural aspects and differences, it was necessary to look into the actual possibilities of gears themselves. What are the different mechanical systems used in watches, for example? How can we translate these different technical elements to represent elements of biochemical processes to allow comparison to each other? Design decisions such as colour and material are not entirely aesthetic but include possibilities of meaning and information. Which gears would be coloured and what would be their potential meaning, or could they be a tool to differentiate specific aspects that needed to be highlighted? The two automata (Fig. 16.10 and 16.11) were exhibited at KYOTO D-Lab's gallery in Tokyo accompanied by their technical blueprints, illustrated explanations of their key elements and a bilingual video and evinced strong interest from visitors.

16.7 Challenges and Benefits of Interdisciplinary Collaboration

In the 3 years of the KYOTO D-Lab Design Associates programme, Cassim has supervised six interdisciplinary projects under the *Making and Materials* and *Designing Social Interactions* themes. All have involved collaborations between individual researchers or existing teams in the materials, fibro- and life sciences. In each case, the process has reversed the conventional order and been design-initiated and led rather than science or technology led, or alternatively one where design was included as an afterthought. In such latter scenarios, design is introduced, not from the outset, but at the stage of development where an application is sought for a newly developed technology or is used to clad an existing device in a set of aesthetic clothes in preparation for its market release as a product or service – the "lipstick-on-a-pig" approach. Both follow the commonplace and highly limited view of what designers do and ignore their inherent conceptual strengths and ability to synthesise and visualise complex information in a tangible language that is unreliant on textual means.

Other reasons can be where the disciplines involved and the processes for advancing ideas are close, but the emphasis is different, resulting in a procedural tug of war. Engineering, for example, is more linear and focussed on function, while design is more discursive, particularly at the front-end concept development stage, and function may not be the overriding concern. In contrast, for disciplines such as biology and design which are quite separate, there is no procedural tug of war to advance ideas since the methodology, aims, terminology and expertise being applied are dissimilar. Daisy Ginsburg

defined the essentially different ideation process of the two worlds: "In art and design, I use the "experiment" as an open-ended process to open up and reveal potential ideas; in science, the "experiment" is a tool to generate data to test a hypothesis. Repeating an experiment and achieving the same results is key to the scientific method, whereas the experimental process in art often seeks out the exceptional or unique" (Ginsburg et al. 2013).

In addition, for interdisciplinary projects of this type, an overall curatorial vision and logistical framework is required under which they are advanced. In equal measure, they should be based on the strong mutual interest and benefits to all parties involved – what may be termed the *quid pro quo* factor. Design is an applied activity and hence needs subjects to address, while for materials scientists, the advantages of working with designers to develop applications for new materials are clear. However, the benefits may be less obvious to those working in the life sciences. Yamaguchi explained the particular role that a design perspective can play:

> In bio-science, a large variety of model organisms have been developed. Bio- science research has been further sub-divided and advanced rapidly in recent years. This has made the significance of each model organism and its research results less comprehensible. Sometimes researchers have tended not to see the forest for the trees. When science meets and integrates with design, it becomes more familiar and easier to understand. When science researchers are put in touch with a designer's creativity, they are able to have a bird's-eye overview, which allows them to reassess the potential of their own research and connect with society differently. (KYOTO D-Lab 2016)

16.8 Impact of *Designs for Flies*

On October 28, 2017, Frank Kolkman was selected as the 2017 Young Dutch Designer of the Year by the prestigious Dutch Design Awards (DDW). This followed his success in the previous year when *Designs for Flies* won the Services and Systems category of the DDW. The 2017 international jury citation read: "Frank Kolkman's critical, provocative way of thinking merges science and design in complex and bold processes that yield an abundance of visually convincing designs. Reviewing his portfolio, the judges appreciate the fact that in some instances, his work advances a solution, while in others, it challenges or highlights an issue. And with this, his designs are infectiously radical and wittily presented. In a category that, this year, overflows with talented submissions, when it comes to innovation and research, Kolkman is in a class of his own. A highly talented designer dedicated to his work. The jury believes that Kolkman is ready to take the next step, and looks forward to the tangible results of his noteworthy, speculative and inspiring ideas"(DDW 2017).

16.9 Conclusion

Interdisciplinary projects of this kind are challenging on a range of levels but the rewards for all parties can far outweigh the logistical complexities and other headaches involved and are both tangible and intangible.

The particular benefits to scientists have been described earlier by Professor Yamaguchi and in more general terms on the design side by Anthony Dunne:

> Designers should not define futures for everyone else but working with experts, including ethicists, political scientists, economists and so on, generate futures that act as catalysts for public debate and discussion about the kinds of futures people really want. Design can give experts permission to let their imaginations flow freely, give material expression to the insights generated, ground those imaginings in everyday situations, and provide platforms for further collaborative speculation. (Dunne and Raby 2013)

Amen to that!

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Videos of Projects

Designs for Flies

http://www.d-lab.kit.ac.jp/projects/2016/designsfor flies/

https://www.youtube.com/watch?time_continue=10&v=FjNCBftnXTw

Of Flies Mice and Men

http://www.d-lab.kit.ac.jp/projects/2017/drosophila-video/

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