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Drosophila, Genetic Screens, and Cardiac Function

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Abstract

The fruit fly, *Drosophila melanogaster*, has been used to study genetics, development, and signaling for nearly a century but only over the past few decades has this tremendous resource been the focus of cardiovascular research. Fly genetics offers sophisticated transgenic systems, molecularly-defined genomic deficiencies, genome-wide transgenic RNAi lines, and numerous curated mutants to perform genetic screens. As a genetically-tractable model, the fly facilitates gene discovery and can complement mammalian models of disease. The circulatory system in the fly is comprised of well-defined sets of cardiomyocytes and methodological advances have permitted accurate characterization of cardiac morphology and function. Thus, fly genetics and genomics offers new approaches for gene discovery of adult cardiac phenotypes to identify evolutionarily conserved molecular signals that drive cardiovascular disease.

Keywords

Drosophila; Cardiomyopathy; Genomics

Introduction

> "…the small and totally harmless fruit fly, *Drosophila*. This animal has been extremely cooperative in our hands - and has revealed to us some of its innermost secrets and tricks for developing from a single celled egg to a complex living being of great beauty and harmony."
>
> Christiane Nüsslein-Volhard, Nobel Banquet Speech, 10 Dec 1995

Advantages of *Drosophila* as a Model System

Over the past century, the study of *Drosophila melanogaster* has yielded insight into fundamental concepts that underlie basic biology. The initial work by Morgan, Sturtevant, Bridges, and Muller formed that basis for modern genetics and built a framework that facilitated investigations into the concepts of the gene, mutagenesis, chromosomal structure,

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the inheritance of complex traits, evolutionary development of organisms, and population genetics.[1]

Fruit fly genetics offers a set of resources currently unavailable in other models systems that are uniquely advantageous for gene discovery. First, the fly has a short life cycle of ~ 10 days from mating of adult flies to deposition of fertilized eggs, embryo development to first instar larvae that proceed to second and third instar larvae, pupal formation and subsequent eclosion to produce the next generation of adults.[2, 3] Adults achieve reproductive maturity within a few hours of emerging from the pupal case and have a lifespan of 60 to 100 days under standard laboratory conditions. Therefore, genetic crosses and the establishment of inbred lines requires considerably less time compared to mammalian models. Second, fly genetics has the unique advantage of balancers chromosomes that contain multiple inversions and suppress meiotic recombination with a corresponding non-rearranged chromosome.[4] Recessive, often times lethal, mutations can be maintained in the presence of balancer chromosomes as stable stocks and followed in subsequent genetic crosses. Third, the presence of specific mutations, transgenes, and balancers can be followed by easily observed physical traits. For example, the presence of a transgene is usually accompanied by a mini-white gene cassette that results in an eye color change from white to red.[5] Lastly, the *Drosophila melanogaster* genome is significantly smaller that mammalian genomes thereby decreasing the time and increasing the efficiency of screening. The genome is ~5% the size of the human genome, comprised of five chromosomes (X, Y, 2L/R, 3L/R, and 4) that encode ~125 million base pairs of DNA comprised of ~13,000 predicted gene products (Figure 1)[6–8]. Although smaller than that of the mouse and human, the fly genome efficiently encodes genes that have multiple spliced isoforms, use different promoter start sites, and genes are sometimes contained within the intronic sequences of other genes. Thus, the compact fly genome encodes similar gene products that are present in higher vertebrates. In fact, analyses of *Drosophila* and human genomes have shown ~80% of human diseases in which the disease-related gene has been identified have an orthologue in *Drosophila*.[9–11]

Genetic Engineering in *Drosophila*

Several accomplishments in fly transgenics over the past several years have further advanced the use of the *Drosophila* as a model system. Ectopic transgene expression in the fly is usually achieved using the bipartite Gal4-upstream activating sequence (UAS) system derived from gene expression for galactose metabolism in yeast (Figure 2A).[5, 12] Transgenic flies that are designated GAL4 expression flies harbor promoters of interest and control the tissue-specific expression of the yeast Gal4 transcription factor or designated UAS-target gene lines harbor transgenes of interest downstream of a specific UAS sequence. Typically, different Gal4-driver lines are crossed with UAS-target gene lines and the effects of tissue-specific gene expression are examined in the progeny. The Gal4-UAS system provides a number of distinct advantages including: promoter expression patterns when using UAS-beta-galactosidase (UAS-lacZ) or UAS-Green Fluorescent Protein (GFP) as a tissue marker; ectopic expression in a variety of tissues; and the tissue-specific effects of specific gene knockdown using UAS-RNAi lines (http://www.flyrnai.org/DRSC-OVR.html). [13, 14] For example, tinC-GAL4 can be used to drive cardiac-specific transgenes to express recombinant cDNA to examine ectopic protein expression or RNAi to examine specific gene knockdown in the heart.[15] Compared to strategies to generate cardiac specific transgene expression in the mouse, transgenic flies can be engineered in a timeframe of 6–8 weeks. Further refinements in transgenic expression in the fly include PhiC31 site-specific integrase that uses attB and attP sites to achieve targeted integration of transgenes into the fly genome.[16, 17] This approach controls for positional effects due to transgene location.

Gal4-driver lines also can be engineered to include the ubiquitous expression of a temperature-sensitive Gal80 ($Gal80^{ts}$) that reversibly suppresses Gal4 activity at 18°C and permits Gal4 binding to UAS at 29°C thereby permitting temporal control in the context of tissue-specific transgenes expression (Figure 2B).[18, 19] Other systems based on hormone or tetracycline-sensitive Gal4 based systems have been described that allow for inducible transgene expression.[12, 20]

Strategies to achieve homologous recombination for gene knockout ("ends-out" targeting) or replacement ("ends-in" targeting) in the fly based on FLP site-specific recombinase, and its target site, FRT, have been developed by Golic and colleagues (Figure 3A and B).[21–28] The "ends-out" approach is based on using a transgenic fly harboring a mini-white cassette that is flanked by genomic targeting sequence along with unique I-SceI recognition sites within two FRT sites (Figure 3A). The genomic targeting construct undergoes mobilization and linearization for in vivo homologous recombination in progeny that are bred in to a fly line that harbors hsp70-FLP and hsp70-SceI. Progeny are then screened for potential homologous recombination and target gene disruption by insertion of the mini-white cassette into the endogenous gene locus. The 'ends-in" approach is similar but results in a tandem gene duplication with the targeted construct near the endogenous gene locus and subsequent reduction to produce the targeted replacement of the gene of interest (Figure 3B). One of the main differences between gene targeting strategies in the mouse and fly is that homologous recombination using the mouse is monitored in embryonic stem cells prior to injecting mouse blastocysts while homologous recombination using the fly occurs *in vivo* and the progeny produced from fly crosses are screened.[21] The timeframe for generating transgenic flies using the "ends-in" or "ends-out" strategies is on the order of a few months depending on the culture conditions. Additional strategies based on recombination-mediated genetic engineering, referred to as recombineering, have been developed to create large genomic DNA insertions based on recombination with bacterial artificial chromosomes.[17, 29] These strategies can be combined with site-directed mutagenesis to perform gene product structure-function studies.

Drosophila Genetic Resources

To promote advancement of discovery, the community of *Drosophila* researchers has complied and shared mutants and reagents leading to the creation and maintenance of stock collections including Bloomington, Vienna, and Kyoto (http://flystocks.bio.indiana.edu/, http://stockcenter.vdrc.at/control/main, and http://www.dgrc.kit.ac.jp/en/index.html). Information pertaining to the Bloomington Stock collection was initially curated and described in the "Red Book" ("Genetic Variations of *Drosophila melanogaster*" (1967) and later "The Genome of *Drosophila melanogaster*" (1992)) that contributed significantly to the Flybase consortium (http://flybase.org).[30, 31] As of 2010, the Bloomington Stock center maintained 30,810 stocks and distributed 196,930 subcultures to the scientific community (http://flystocks.bio.indiana.edu). Additional resources include a searchable database of high-throughput in situ hybridization studies that contains >100,000 images of expression from >4000 genes (FlyExpress.net). This platform provides a resource to examine the spatiotemporal expression patterns of genes expressed during *Drosophila* embryogenesis.

Recently, the *Drosophila* model organism ENCyclopedia Of DNA Elements (modENCODE) project has produced large data sets of transcript profiles, histone modifications, transcription factors, and replication programs in cell lines, isolated tissues, and whole organisms across several developmental stages.[32] This powerful resource is designed to provide insight into potential new functions for genes, a better understanding of developmental- and tissue-specific gene regulation, and integration of functional changes in the transcriptome with the genome.

Efforts to systematically examine the effects of gene knockdown have led to the creation of large collections of transgenic flies that harbor specific RNAi under the control of UAS have been generated by Vienna Drosophila RNAi Center (http://stockcenter.vdrc.at/control/main) and the Transgenic RNAi Project (TRiP) at Harvard Medical School http://www.flyrnai.org/TRiP-HOME.html).[13, 14] In combination with tissue specific promoters that drive Gal4 expression, these UAS-RNAi lines have been used to conduct large scale genome-wide screens of gene function in a variety of contexts. For example, RNAi screens have been performed to examine muscle development, cardiac function, and extracellular signal-regulated kinase (ERK) signaling (see below).[15, 33, 34]

Collectively, the resources available in the field of *Drosophila* genetics and genomics have advanced the identification of genes involved in a variety of basic biological processes including signal transduction, cell differentiation, and organ development. While other models, including mouse and large animals, are well suited to investigate the pathophysiology underlying human disease, *Drosophila* research provides a genetically-tractable model system to identify new genes and signaling pathways. Therefore, applying the unique set of resources available in the fields of fly genetics and genomics has the potential to further the understanding of human cardiovascular disease.

General Screening Strategies in *Drosophila*

The major strength of the fruit fly as a model of cardiovascular disease relies on the remarkable, diverse resources that facilitate large-scale genetic screens, otherwise currently unattainable in mouse models. The strategies to identify genes that impact fly cardiac function can be separated into two general concepts: (1) genes that directly cause cardiac abnormalities or (2) genes that suppress or enhance abnormal cardiac phenotypes, so called suppressor or enhancer screens. In the first case, wild-type fly lines are mutagenized and then screened for the occurrence of new measurable phenotypes. This powerful approach has been the cornerstone of genetic screens in the fly and has led to new insights into signaling pathways that are conserved among multiple species, including mammals. Mutagenesis screens based on development of the fly eye or wing vein morphology have identified key components of receptor tyrosine kinase signaling pathways including epidermal growth factor receptor (EGFR), Ras, Raf, Sos, Grb2, and PTP11N.[35–45] Additionally, fly screens have identified components in pathways involving Notch, Wnt, bone morphogenetic protein (BMP), Smad, Salvador/Warts/Hippo and G protein-coupled receptor (GPCR) signaling in a variety of contexts.[46–55]

In a suppressor or enhancer screen, sensitized lines that express particular phenotypes are mutagenized or bred into another mutagenized strain to identify suppressors and enhancers of a particular phenotype or phenotypic trait. The literature in the fly genetics field provides a wealth of information pertaining to mutagenesis screens and describes the identification of key components in many signaling pathways. Mutagenesis approaches include: chemical, irradiation, P-element, and genomic deficiencies. For example, genetic screens performed in the context of Ras or Raf mutations have identified dominant suppressors and enhancers of the Ras-Raf-ERK pathway.[38]

Chemical/Irradiation Mutagenesis

The wealth of resources that are available in the fly genetics and genomics community provides multiple approaches to examine gene function in relationship to a particular measurable trait. Traditionally, gene function has been elucidated by examining nulls (complete loss of function allele), hypomorphs (partial loss of function allele), hypermorphs (gain of function allele), or antimorphs (antagonist of wild-type allele) that are generated by chemical or radiation-induced mutagenesis.[2, 3] When combined with a high-throughput

phenotyping strategy, chemical mutagenesis can generate a number of interesting mutants that can be mapped using several strategies to identify candidate genes.[56]

P-element Mutagenesis

Mutagenesis strategies based on P-element inserts are unique to fly genetics. P-elements are foreign pieces of engineered DNA that insert throughout the fly genome and potentially disrupt gene function by physically altering gene structure. Large collections of mutants have been generated in which the exact positions of the P-element insertions in the genome have been mapped and sequenced to studying gene function.[57–59] In some cases, the P-elements have been engineered to encode GFP (i.e., P(GawP)) or beta-galactosidase (i.e., P(lacW or P(PZ)) to examine the expression patterns of genes. P(EP) and P(XP) P-elements harbor UAS sequences designed to potentially amplify gene transcription near the P-element insertion.

Molecularly-defined Genomic Deficiencies

More recently, large collections of molecularly-defined genomic deficiencies have been engineered by two groups, Exelixis and DrosDel.[60–63] The deficiencies were created using FLP recombinase strategies. Collections of flies were generated that harbored P-elements containing FRT sites that are recognized by FLP recombinase. Deficiencies were created using FLP recombinase mediated excision of genomic DNA flanked by two neighboring *in trans* P-elements (i.e., Piggybac and P(RS3)/P(XP) P-elements) (Figure 1).[60, 62, 63] These deficiency mutants, maintained in a haploinsufficient state in the context of balancer chromosomes and in isogenic backgrounds, are powerful resources to examine the phenotypic effects of gene deletion. These large collections also provide a unique approach to identify and map candidate genes that affects particular traits. Using these methods, the combination of the Exelixis and DrosDel P-element collections can be used to construct ~500,000 theoretical molecularly-defined genomic deficiencies (http://www.drosdel.org.uk/fdd/fdd_info.php).[60–63]

The *Drosophila* Circulatory System

The Embryonic Heart (Dorsal Vessel)

While the fly circulatory system is simple compared to higher vertebrates, investigations of the *Drosophila* heart have yielded new insights into mammalian cardiac development and function. The fly circulatory system develops from an orchestrated set of fundamental signals that occur in the mesoderm.[64–67] Secreted ligands including decapentaplegia (dpp) of the bone morphogenetic protein family regulate embryonic heart development and trigger cell fate specification in the developing mesoderm. A set of cardiac precursor cells that expresses specific transcription factors including tin, the fly othologue of Nkx2.5 and GATA family members arise in the embryo, migrate along the mesoderm, and form the recognizable single layered dorsal vessel at stage 16 of embryonic development.[68] The dorsal vessel is divided into the heart proper and the anterior and posterior aorta (Figure 4A). Many of the temporal and tissue specific signals required for dorsal vessel formations are evolutionarily conserved among mammals.[69] For example, genetic studies have identified *tin* as a critical component in heart development and mutations that remove tin cause a complete absence of heart cells.[68] Mutations in human Nkx2.5 are associated with congenital heart disease including atrial septal defects and arrhythmias.[70]

Each side of the embryonic dorsal vessel is arranged in sets of four tin positive cardioblasts that form the contractile cells of the heart and are separated by pairs of Svp-expressing ostial cells (Figure 4). Pericardial cells are non-contractile cells that surround the heart and have

been implicated in detoxification of the hemolymph.[71, 72] Additionally, the dorsal vessel is surrounded by an extracellular matrix composed of pericardin, a type IV collagen-like protein.[71]

The Larval and Adult Heart

As the larva develops from the embryo, the dorsal vessel maintains its general morphology. Then, during the formation of the pupal stage, when the larva transitions into the adult, the fly circulatory system undergoes tremendous morphological change.[73, 74] During morphogenesis, the heart proper of the dorsal vessel involutes and the posterior aorta in the first abdominal segment becomes the main pumping chamber of the adult heart.[73–75] The adult heart consists of a single layer of cardiomyocytes that have circumferentially oriented myofibers and is closely juxtaposed to the ventral longitudinal muscle (also referred to as the “dorsal diaphragm”) and sets of suspensory muscles arising from the dorsal cuticle (called alary muscles) (Figure 4B). The ventral longitudinal muscle belongs to a distinct non-cardiac muscle type that does not express cardiomyocyte-specific *tin* and arises from lymph cells via trans-differentiation during the pupal stage of development.[76] The larval and adult hearts continue to possess ostial cells derived from the Svp-expressing cells of the embryonic dorsal vessel.[77] The ostial cells function as valves that facilitate the entry of hemolymph into the circulatory system. Additionally, pericardial cells are also maintained in close proximity to the larval and adult hearts.[72]

The embryonic and larval dorsal vessel lack innervation and generate a completely myogenic cardiac impulse. However, pairs of transverse glutamergic nerves innervate the lateral edges of the cardiac chamber and abdominal segments of circulatory system.[78] The adult fly heart has anterior (rostral) and posterior (caudal) pacemakers that generate measurable retrograde and anterograde propagating calcium transients.[79] Consistent with these observations, earlier work by Wasserthal using a linear optosensor chip and an IR-light beam demonstrated anterograde and retrograde pulses of hemolymph.[80] During anterograde beating, the hemolymph enters the heart through all inflow ostia and is propelled through the narrow aorta located in the thorax. During retrograde beating, the hemolymph enters the heart through the first ostia and moves toward the rostral end. Thus, hemolymph circulates through sets of valves along the caudal circulatory system into the lumen of the tube and is propelled in a rostral or caudal direction.

Heart rate in the fly heart responds to agonist stimulation suggesting a level of neurohormonal control.[81–83] For example, the fly heart responds to several bioactive amines including octopamine, serotonin, norepinephrine, and dopamine as well as peptide hormones, including crustacean cardioaccelerator peptide (CCP).[81]

The dissected fly heart also facilitates the examination of myocardial calcium handling using cardiac-specific genetically encoded calcium-dependent fluorescent proteins (Figure 5A).[79] Recently, flies harboring GCaMP2 under the control of the cardiac-specific *tinC* driver have been used to characterize myocardial calcium transients in adult flies.[79] The calcium transients in adult flies share several properties observed in with mammalian hearts. The propagating calcium transients in the fly heart are dependent on extracellular sodium, inhibited by chelation of extracellular calcium or pharmacological inhibition of L-type calcium channels, and are sensitive to thapsigargin-mediated depletion of intracellular calcium stores. Moreover, a troponin-I mutant that has impaired cardiac function and an enlarged heart chamber demonstrated abnormalities in myocardial calcium handling including a decreased response to caffeine-augmented calcium release, consistent with some observations in human cardiomyopathies.[79]

It should be noted that unlike mammals, oxygen transport occurs through diffusion from spiracles that invaginate from the cuticle into the interior of the adult fly.[72] Thus, the flow of hemolymph serves to transport immune cells, nutrients, and molecules that are necessary to maintain homeostasis. The fly is emerging as a model to examine metabolomics, lipidomics, and mitochondrial function.[84–87] The major fuel sources in Drosophila flight muscle are glycogen, free glucose, and trehalose, a disaccharide composed of two glucose molecules. A model of tissue hypoxia tolerance has been used to examine changes in metabolic profiles using NMR-based measurements and principle component analyses.[88, 89] An age-dependent reduction in the hypoxia tolerance was attributed to a decrease in ATP production during reoxygenation due to reduced recovery of mitochondrial respiratory pathways.[88]

Similar to protein and carbohydrate metabolism, flies and mammals demonstrate a conservation among genes that encodes proteins that are involved in lipid metabolism. Mass-spectrometry based analyses of ~400 different lipids from Drosophila showed similarities between fly and mammalian lipidomes.[90] Additionally, fly mutants have been isolated that encode genes involved in the metabolism of ceramides, sphingolipids, cholesterol esters, fatty acids, glycerophospholipids, triacylglycerides, and lipid droplets.[85, 90–99] The unique abilities to combine fly genetics and metabolic profiling in different tissues suggests a unique approach to examine myocardial metabolomics in a genetically-tractable model system.

Evaluating Cardiac Structure and Function in *Drosophila*

Genetic Screens Using the Embryonic and Larval Heart

A number of approaches have been developed to characterize the fly circulatory system. In general, these approaches are based on an examination of cardiac morphology or function. Initial screens of fly mesodermal and heart development, conducted by Bodmer and Frasch, were based on the morphologic examination of the embryonic dorsal vessel.[64, 66–68] These seminal studies identified a number of transcription factors that were critical for cardiac development and are highly conserved throughout mammals, including humans. Skeath and coworkers have examined the cell lineage of all heart cells in the fly embryo and identified the ETS-transcription factor, *pointed*, and the GATA transcription factor, *pannier*, as important components from a screen of ~2000 3rd chromosome P-element lines.[100] The identification of *tin* and subsequent understanding of the regulatory networks including *dMef*, *Hand*, *Svp*, *ladybird*, *eve*, *pannier*, and *pointed* have contributed significantly to the understanding of cardiac development in humans.[66–69]

An interesting screen was conducted to examine modifiers of heart cell fate using a P-element mediated gain-of-function strategy by Bidet and coworkers.[101] Fly lines that harbor single P-element insertions encoding a UAS promoter were bred with the pan-mesodermal driver 24B–Gal4 fly line and dorsal vessel cells were examined for abnormalities in heart morphology. Using this strategy, the effects of over-expression of genes near each P-element insertion was correlated with alterations in dorsal vessel cell number and suggested that alteration in the levels of *rhomboid*, the transcription co-factor *yan*, and the Rho-GTPase *Rac2* influenced cardiac development.[101]

Additional genetic screens based on the examination of the embryonic heart have led to new insights into signaling pathways that control cardiac development. For example, a P-element screen identified mutants in which the pericardial cells dissociated from the cardioblasts during dorsal vessel development.[102, 103] The mutations were subsequently mapped to 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the mevalonate pathway, and the G-protein gamma subunit 1 that requires geranylgeranylation for proper function. These

findings, in conjunction with an examination of septate junction proteins, suggest that G-protein function is necessary to maintain cardiac integrity during fly heart development.

Genetic Screens Based on Heart Rate and Rhythm in Pupae and Intact Adults

During the past few years, attention has been directed towards the adult fly heart as a model of human cardiac disease.[104–107] Several approaches have been developed to characterize the pupal and adult cardiac function and structure (Figure 5B and C). Pasternostro and co-workers initially described an age-associated decline in cardiac function in adult Drosophila.[108] Wessells and Bodmer developed methods to measure heart rate frequency in early pupae that retain the translucent properties of larvae or in adults using high-speed image capture of cardiac chamber movement under bright field microscopy.[109] These strategies have also been used to monitor adult heart rate responses to externally applied electrical pacing to characterize the effects of aging on cardiac parameters in adult flies. For example, an age-related decline in cardiac function, manifest as changes in basal heart rate and altered heart rate response to external pacing, were minimized in flies that had alterations in Insulin-like growth factor (IGF) receptor signaling. Furthermore, cardiac-specific expression of dPTEN, a phosphatase that inhibits insulin-receptor dependent PI3K signaling, rescued the age-dependent changes in cardiac function attributed to insulin-IGF receptor signaling.[109] The cardiac-specific expression of the forkhead transcription factor dFOXO or reductions in dTOR rescued or attenuated age-dependent changes in cardiac function attributed to insulin-IGF receptor signaling.[110]

Furthermore, eukaryotic translation initiation factor 4E (Eif4e) binding protein (Eif4e–BP) acts downstream on dTOR and dFOXO and can modulate the age-associated changes in cardiac function attributed to IGF signaling abnormalities.[111] In flies, the FOXO/Eif4e–BP pathway has been shown to control protein homeostatsis (called “proteostasis”) by regulating autophagy.[112] In transgenic mice, the cardiac-specific ablation of TOR resulted in a dilated cardiomyopathy that was characterized by apoptosis, autophagy, altered mitochondrial structure, and accumulation of Eif4e–BP.[113]

Genetic Screens Based on Heart Rate, Rhythm, and Function in Dissected Heart Preparations

Adult fly cardiac function can be studied using methods based on the dissection of the surrounding tissues from the fly heart thus providing a specimen that is attached to the dorsal cuticle and can be maintained by perfusion in artificial hemolymph.[114–116] This approach provides a platform for multiple phenotyping strategies. Bright field or fluorescence microscopy with high-speed image capture is used to evaluate heart rate, rhythm, and cardiac function by measuring the movement of the lateral walls of the in the second and third abdominal segments. Using these methods, potential mechanisms that contribute to age-related changes in cardiac function have been examined. Examples include: mutations in KCNQ potassium channels mutations have been associated with cardiac arrhythmias mimic the effects of aging in the fly and investigations of sestrin as a feedback inhibitor of TOR that prevents age-related pathologies.[115, 117] Recently, Bodmer and Penninger performed a global in vivo RNAi screen using the Vienna *Drosophila* RNAi collection to identify genes related to cardiac dysfunction.[15] Transgenic flies harboring UAS-RNAi were crossed to the cardiac-specific tinCdelta-4-Gal4 driver lines and the progeny were examined. Developmental and adult lethality at 25°C and temperature-induced stress at 29°C in adults were examined as the primary phenotypic screen. After multiple rounds of screening, 7116 (89.2%) viable adult transformants, 365 (4.6%) lethal transformants, and 490 (6.2%) transformants that were considered to have an adult heart defect were identified. From these studies, a global network of heart function was generated and the silencing of the CCR-NOT pathway was found to cause a dilated cardiomyopathy.

Genetic Screens Based on Cardiac Function in Awake, Adult Flies

To examine cardiac chamber size and function in awake, adult *Drosophila* an approach has been developed based optical coherent tomography (OCT).[18, 106, 118, 119] OCT uses 1310 nm near-infrared light to provide non-invasive, non-destructive images that are analogous to transthoracic echocardiography in humans and mice (Figure 5B). Therefore, OCT imaging facilitates relatively fast analyses of cardiac function in adult flies without the cardiovascular effects associated with prolonged exposure to anesthesia or damage to surrounding tissues during dissection. Moreover, due to the non-invasive properties of OCT, the effects of temporal, cardiac-specific transgene expression can be examined serially in individual flies.[18] Genetic screens based on OCT imaging of heart chamber size and function from stocks of molecularly-defined genomic deficiencies from the Exelixis and DrosDel collections have led to the identification of genes that cause cardiomyopathies in the fly model. An examination of deficiencies of the 3rd chromosome identified mutants in rhomboid-3 that had enlarged cardiac chambers.[119] Rhomboid-3 is a member of an evolutionarily conserved family of seven-transmembrane serine proteases that hydrolyze membrane bound Spitz, the fly orthologue of epidermal growth factor (EGF).[120, 121] In fact, the post-developmental inhibition of EGFR by means of cardiac-specific expression of dominant-negative EGFR results in progressive deterioration of cardiac function suggesting that proper EGFR signaling is required to maintain adult cardiac function.[119] Importantly, these findings suggest that the fly may model cardiovascular-specific aspects of EGFR signaling and provide insights into the mechanisms that contribute to chemotherapy-induced cardiomyopathies.

The screen of genomic deficiencies has also identified a new gene, *weary* (*wry*), that is important for the maintenance of normal heart function in the adult fly.[118] Wry encodes a protein that has a similar structure to members of the Notch family but lacks a DSL (Delta-Serrate-Lag) domain commonly found in other *Drosophila* Notch ligands.[118] Cell aggregation assays and gamma-secretase inhibitors demonstrate that Wry can mediate cellular adhesion with Notch expressing cells and transactivate Notch to promote signaling and nuclear transcription. Interestingly, deficiencies in wry produced by genomic deficiencies, P-element insertions, or cardiac-specific RNAi knockdown results in a dilated cardiomyopathic phenotype. Mutations in human Notch signaling and mutant mouse models of Notch signaling have been implicated in congenital heart disease, however the involvement of Notch signaling in adult mammalian cardiac disease remains unclear. Notch ligands appear important to maintain cardiac function in adult flies and gene orthologs involved in Notch signaling may be important in pathogenesis of mammalian dilated cardiomyopathy. Moreover, Notch signaling components may represent therapeutic targets for the treatment of dilated cardiomyopathies.

Comparison of *Drosophila* to other model systems and limitations of the fly model of cardiovascular disease

It is clear that *Drosophila* is an excellent discovery engine that continues to provide a tremendous resource to identify new genes in cardiovascular biology. Despite the advantages of the fly as a genetically-tractable system, there are obvious limitations compared to models that use Zebrafish, mice, or large animals (Table I). For example, the fly lacks genetic redundancy of more complex organisms, thereby providing the possibility of more efficient evaluation of candidate genes since fewer genes need to be targeted and less time may be required for analyses. However, the lack of genetic redundancy may represent a limitation since specific gene regulatory mechanisms that are present in mammalian systems may not be present in the fly.

The translation of fly cardiac physiology to mammals is difficult due to a single chamber open circulatory system compared to Zebrafish and mammals. Ischemia-reperfusion studies cannot be conducted in the fly because the fly does not have a coronary circulation and relies on oxygen transport by diffusion. The cardiac conduction system of the fly is distinctly different from the two chambered heart of the Zebrafish or the four chambered heart of mammals. The presence of two pacemakers, anterograde and retrograde pulses, and irregularities in adult heart rate can make assessment of arrhythmia difficult in intact flies. Importantly, the wealth of resources available to *Drosophila* research and the evolutionary conservation of signaling mechanisms make the fly a valuable model to identify genes that cause or contribute to human cardiovascular diseases

Conclusions

The rich history and tremendous resources available in fly genetics continues to offer new avenues to identify signaling pathways that are important in many biological processes. In conjunction with recent advances in cardiac phenotyping, the fly provides a genetically-tractable approach to understand the complex signals that lead to cardiac dysfunction. Thus, continued studies based on fly genetic and genomics has the potential to advance our understanding of human cardiovascular diseases.

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Non-standard Abbreviations and Acronyms

BMP	Bone morphogenetic protein
dFOXO	*Drosophila* O subclass of the forkhead family of transcription factors
dTOR	*Drosophila* target of rapamycin
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular signal-regulated kinases
FLP	recombinase that recognizes FRT site
FLP	recombinase that recognizes FRT site
GAL4	transcriptional activator from yeast
Gal80^{ts}	temperature-sensitive protein that interact with GAL4
GCaMP2	circularly permutated enhanced green fluorescent protein fused with M13 helix of myosin light chain kinase at the N terminus and calmodulin at the C terminus
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
KCNQ	potassium channel gene
modENCODE	model organism ENCyclopedia Of DNA Elements
OCT	optical coherence tomography

RNAi	small interference RNA
tinC	DNA sequence within the tinman gene used to drive cardiac-specific transgene expression
TRiP	Transgenic RNAi Project at Harvard Medical School
UAS	Upstream Activating Sequence from yeast

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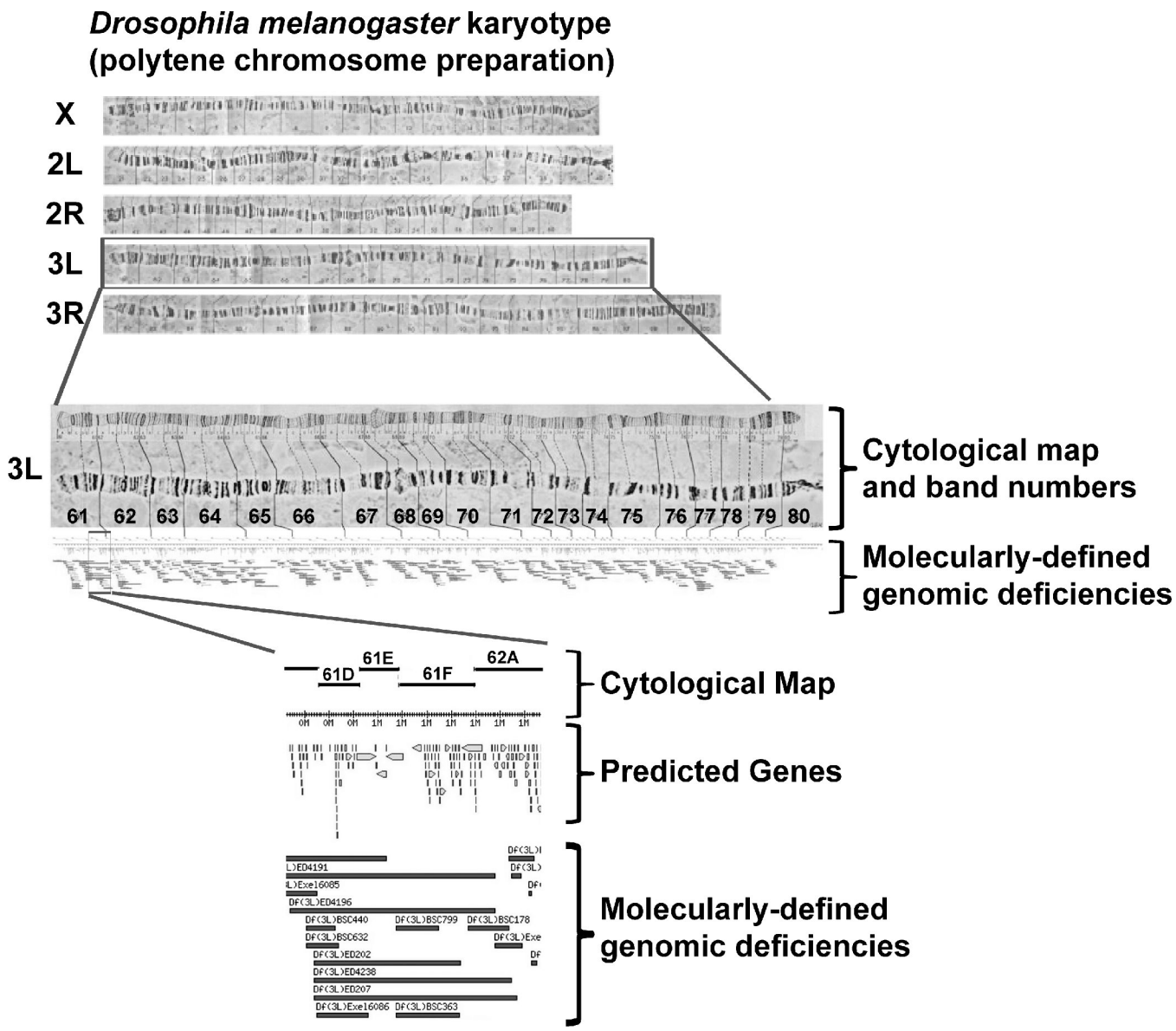

Figure 1. The *Drosophila melanogaster* genome

(A) The karyotyping of *D. melanogaster* prepared from the salivary gland showing chromosomes X, 2L/R, and 3L/R. The Y and fourth chromosomes are not depicted. **(B)** An enlarged view of the 3L chromosome with cytological map showing bands 61 through 80 (above) and molecularly-defined deficiencies (below). **(C)** An enlarged view of the 61D through 62A cytological map showing predicted genes encoded within the region and corresponding molecularly-defined genomic deficiencies from the Exelixis and DrosDel collections.[60–63] Figures adapted from http://flystocks.bio.indiana.edu.

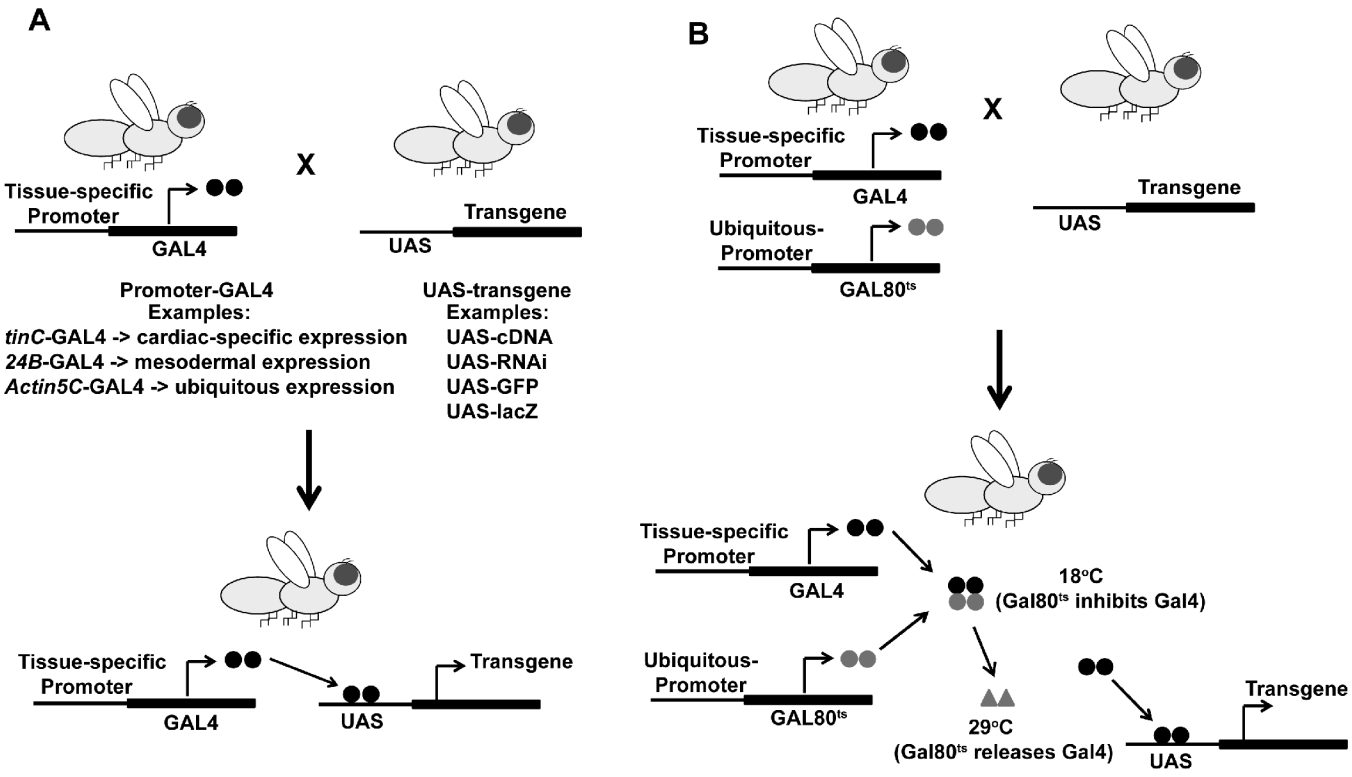

Figure 2. Transgenic-Expression Systems in *Drosophila*

(A) The Gal4-UAS bipartite transgene expression approach relies on breeding transgenic flies that harbor either a tissue-specific promoter that drives Gal4 production or UAS-transgene constructs. The progeny possess one copy of the Gal4 and UAS constructs and express the transgene of interest under the control of the promoter of interest. This system allows versatility by using different promoters to control transgene expression. **(B)** The ubiquitous expression of a temperature-sensitive Gal80 ($Gal80^{ts}$) is incorporated into the Gal4-UAS system to add temporal control of transgene expression using a shift from a restrictive to permissive temperature. $Gal80^{ts}$ reversibly suppresses Gal4 activity at 18°C and permits Gal4 binding to UAS at 29°C.

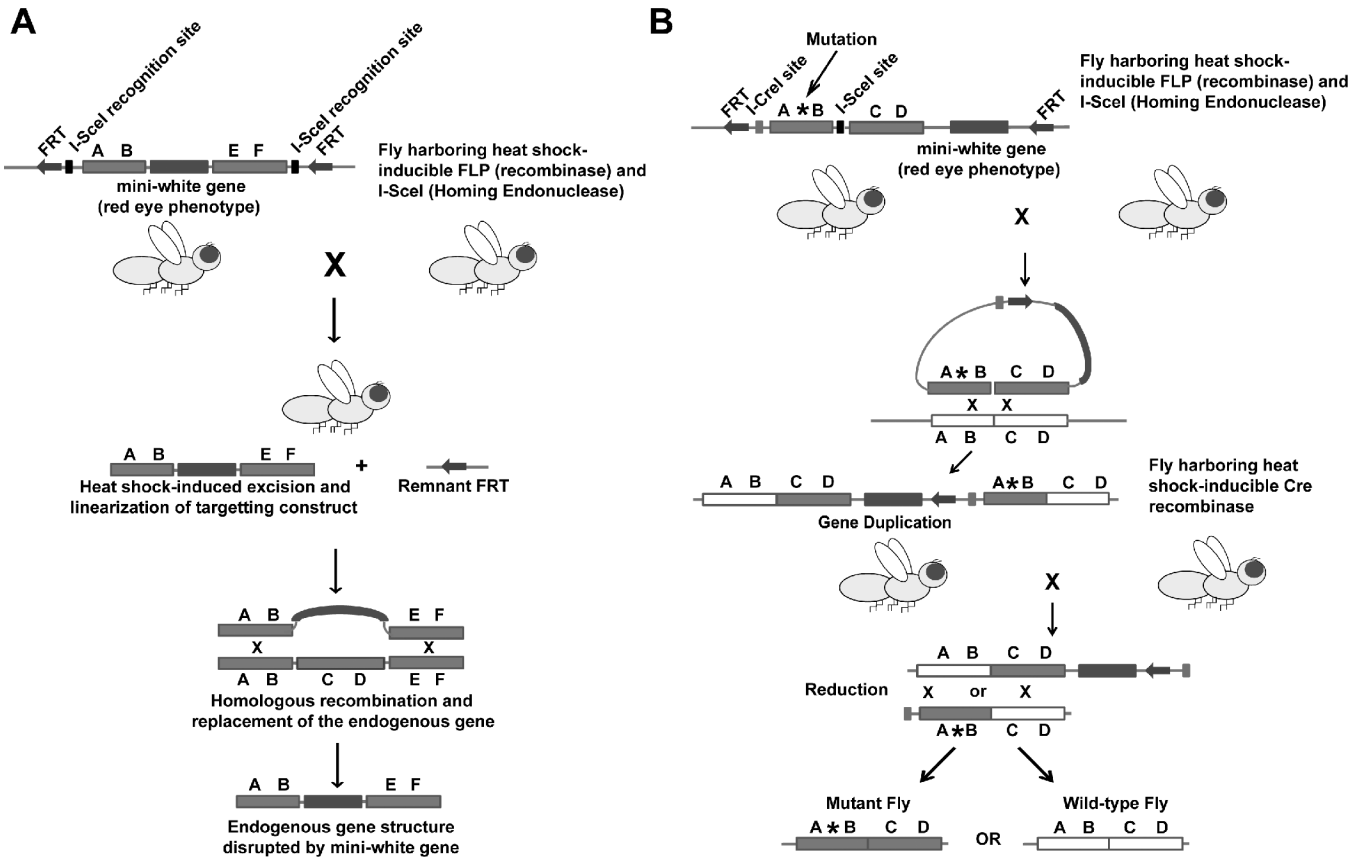

Figure 3. Targeted gene knockout and gene replacement in *Drosophila*

(A) The “ends-out” approach for gene knockout. A fly harboring a transgenic construct containing the mini-white gene within the genomic sequence of a gene targeted for replacement is bred with a fly that harbors an inducible FLP recombinase and I-SceI homing enzyme. In a subset of progeny, the targeting construct is excised by the FLP recombinase, linearized by I-SceI, and undergoes homologous recombination with the endogenous gene. The endogenous gene is disrupted by replaced with the mini-white gene. **(B)** The “ends-out” approach for gene replacement. A fly harboring a transgenic construct that contains an engineered mutation (asterisk) and the mini-white gene within the genomic sequence of a gene targeted for replacement is bred with a fly that harbors an inducible FLP recombinase and I-SceI homing enzyme. In a subset of the first generation, the targeting construct is excised by FLP recombinase, linearized by I-SceI, and undergoes homologous recombination with the endogenous gene locus to produce tandem gene duplication. Then, the fly containing the gene duplication is bred with a fly that harbors an inducible Cre recombinase that recognizes the I-CreI site that is engineered in the targeting construct. In a subset of the progeny, the tandem genes undergo homologous recombination and reduction of gene copy number thereby producing flies that harbor either a wild-type gene or the mutant gene. The figure is based on Maggert, K.A. *et al*[122]

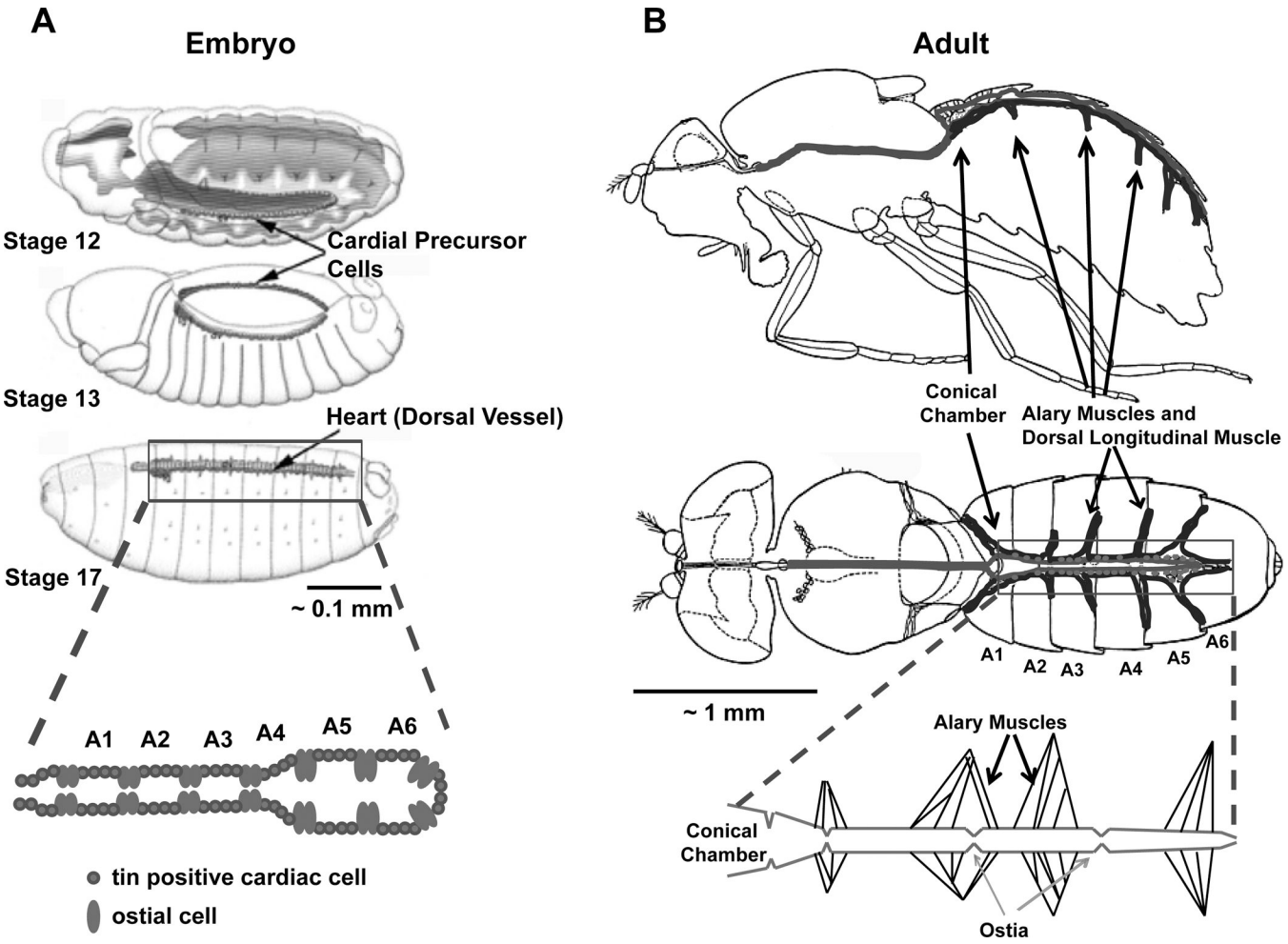

Figure 4. The embryonic and adult *Drosophila* circulatory system

(A) The developing embryonic circulatory system arises from cardial precursor cells that migrate to form the dorsal vessel at Stage 16. Stages 12, 13, and 17 are shown. The figure is adapted from Fly Embryo RNAi Project (http://flyembryo.nhlbi.nih.gov). **(B)** The adult fly circulatory system consists of an open system with the main conical chamber, heart, located along the dorsal aspect of the A1 abdominal segment. Suspensory muscles including the Alary and Ventral Longitudinal Muscle also referred to as the Dorsal Diaphragm. Pericardial cells are closely juxtaposed along the length of the abdominal portion of the circulatory system. The figure is adapted from Miller.[72] A 1 mm scale bars are shown for comparison.

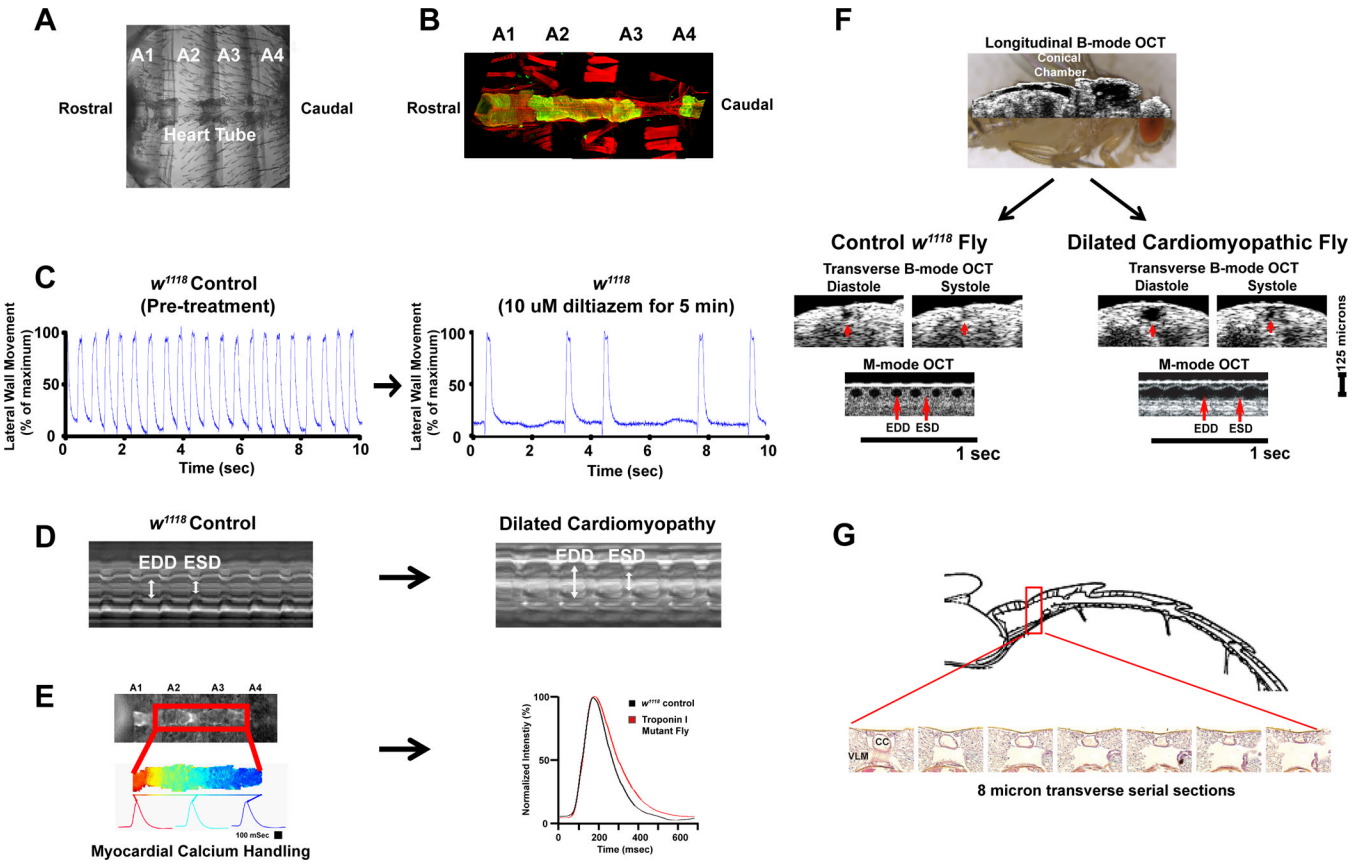

Figure 5. Strategies to assess adult *Drosophila* cardiac morphology and function

(A) A dissected adult fly heart perfused in artificial hemolymph is shown. Abdominal segments are denoted as A1, A2, A3, and A4. This specimen preparation can be used to assess heart rate and rhythm, myocardial calcium handling, cardiac morphology, and cardiac function. **(B)** An example of cardiac morphology in a tinC-GFP transgenic fly depicting the cardiac tube (green) and actin stained with phalloidin (red) using confocal microscopy with Z-stack reconstruction as previously described.[119] **(C)** Examples of heart rate and rhythm obtained using a Leica 165FC steromicroscope equipped with an Andor iXon high-speed camera. Heart rate was measured by examining movement of the lateral wall as described by Wessells and Bodmer.[105, 109] An example of the effects of Diltiazem, a calcium-channel blocker, is shown. **(D)** Representative m-mode of cardiac function was obtained using high-speed brightfield imaging similar to methods as described.[123] M-modes showing w^{1118} (control) and a dilated cardiomyopathic mutant are shown for comparison. **(E)** An example of myocardial calcium transients measured in adult transgenic flies that had cardiac-specific expression of GCaMP2 in a w1118 background (control) or in the presence of hdp2, a mutation in Troponin-I, that has a dilated cardiomyopathy phenotype are shown.[79] **(F)** Longitudinal B-mode OCT image during diastole superimposed on a live adult fly (left) and transverse B-mode OCT during diastole and systole with representative M-mode OCT showing cardiac chamber size and function in w^{1118} (control) and a mutant that has dilated cardiomyopathy as described by Wolf et. al.[106] **(G)** Example of transverse sectioning and hemotylin/eosin staining of a fixed adult fly. The cardiac chamber (CC) and surrounding tissues including the ventral longitudinal muscle (VML) are shown. The image is adapted from Yu et. al.[119]

Table I
Comparison of Model Systems of Cardiovascular Diseases

The strengths and limitations of fly, zebra fish, mouse, and large animal models of cardiovascular diseases are shown.

Model	Advantages	Disadvantages

Fly	- Short life cycle
	- Balancer chromosomes
	- Easily observed physical traits
	- Low maintenance costs
	- Well-annotated databases
	  Flybase.org
	  Bloomington
	- Gal4/UAS bipartite transgenic system permits transgene expression under tissue and temporal control
	- Extensive Genetic/Genomic Resources
	  Chemically mutated stocks
	  P-element mutants
	  Molecularly-defined genomic deficiencies
	  Transgenic RNAi lines
	- Amenable to high-throughput suppressor/enhancer and drug screens
	- Lacks genetic redundancy of more complex organisms (more efficient evaluation of candidate genes with respect to number of gene knockdowns and time of analyses)
		- Single chamber open circulatory system
		- No coronary circulation (oxygen transport by diffusion)
		- Two pacemakers and irregularities in adult heart rate can make assessment of arrhythmia difficult in intact flies
		- Lacks genetic redundancy of more complex organisms (the fly may lack specific gene regulatory mechanisms that are present in mammalian systems)

Zebrafish	- Well-developed resources for lineage tracing, transgenic expression, and knockdown with morpholinos - Two chambered heart with distinct atrium and ventricle - Cardiac conduction system has similarities to mammals - Amenable to large scale drug studies - Heart has regenerative capacity	- Imaging the cardiac chamber can be challenging given the highly trabeculated ventricle - Costs associated with maintaining stock - Mapping new mutants can be difficult

Mouse	- Four chambered heart is similar to humans
	- Conduction system is similar to humans
	- Amenable to measurement of cardiac physiology
	  Intra-cardiac hemodynamic measurements
	  Intra-cardiac electrophysiology testing
	  Trans-aortic constriction pressure overload
	  Coronary ligation/Ischemia-reperfusion studies
	- Well-developed transgenic systems (overexpression/gene knock-out/gene replacement by homologous recombination)
		- Costs associated with maintaining mouse colonies
		- Breeding time to develop complex genetic crosses
		- Difficult to perform high-throughput forward genetic screens

Model	Advantages	Disadvantages
	- Quantitative trait loci (QTL) mapping - Congenic strains	
Large Animal (pig/rabbit/sheep/dog)	- Four chambered heart is similar to humans - Conduction system is similar to humans - Amenable to measurement of cardiac physiology Intra-cardiac hemodynamic measurements Intra-cardiac electrophysiology testing Trans-aortic constriction pressure overload Coronary ligation/Ischemia-reperfusion studies - Well suited for developing surgical techniques/ delivery of Therapeutics	- Difficult to perform genetic loss of function studies