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SPOTLIGHT REVIEW

Animal and *in silico* models for the study of sarcomeric cardiomyopathies

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Abstract

Over the past decade, our understanding of cardiomyopathies has improved dramatically, due to improvements in screening and detection of gene defects in the human genome as well as a variety of novel animal models (mouse, zebrafish, and drosophila) and *in silico* computational models. These novel experimental tools have created a platform that is highly complementary to the naturally occurring cardiomyopathies in cats and dogs that had been available for some time. A fully integrative approach, which incorporates all these modalities, is likely required for significant steps forward in understanding the molecular underpinnings and pathogenesis of cardiomyopathies. Finally, novel technologies, including CRISPR/Cas9, which have already been proved to work in zebrafish, are currently being employed to engineer sarcomeric cardiomyopathy in larger animals, including pigs and non-human primates. In the mouse, the increased speed with which these techniques can be employed to engineer precise 'knock-in' models that previously took years to make via multiple rounds of homologous recombination-based gene targeting promises multiple and precise models of human cardiac disease for future study. Such novel genetically engineered animal models recapitulating human sarcomeric protein defects will help bridging the gap to translate therapeutic targets from small animal and *in silico* models to the human patient with sarcomeric cardiomyopathy.

Keywords Cardiomyopathy • Animal models • *In silico* models • Genetics • Sarcomeres

This article is part of the Spotlight Issue on Sarcomeric cardiomyopathies: from bedside to bench and back.

1. Introduction

Cardiomyopathies are defined as myocardial disorders in which the heart muscle is structurally and functionally abnormal in the absence of coronary artery disease, hypertension, valvular disease, and congenital heart disease, sufficient to explain the observed myocardial abnormality.[1] They are grouped into specific morphological and functional phenotypes, including hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy, dilated cardiomyopathy (DCM), and arrhythmogenic right ventricular cardiomyopathy (ARVC).

Animal models of cardiac hypertrophy and heart failure associated with ischaemic heart disease, chronic haemodynamic (volume and/or pressure) overload, and tachyarrhythmias have been available for >40 years, and have proved instrumental in advancing our understanding of pathophysiology and in developing novel therapies of hypertrophy and heart failure.[2–6] In contrast, although some naturally occurring cardiomyopathies in cats and dogs had already been known for some time, animal models of cardiomyopathies have become available only recently with the advent of transgenesis and gene targeting (*Table 1*).

The purpose of this review is to provide an overview of the various naturally occurring and genetically engineered animal models of cardiomyopathy that allow detailed and integrated physiological and molecular studies of cardiomyopathies, as well as the evaluation of therapy. We will end with a novel approach to integrating existing knowledge into an *in silico* model to understand the molecular basis of cardiomyopathies and to predict phenotypes and therapeutic targets.

2. Cat models of cardiomyopathy

HCM is the most common cardiac disease in domestic cats,[7] and is characterized by left ventricular hypertrophy (LVH), particularly of the papillary muscles, systolic anterior motion, and myocardial disarray. It is a progressive disease that starts in the adolescence (generally after 6 months of age) and can result in heart failure, paralysis of the hind legs due to clot embolization originating in the heart, and sudden cardiac death.

HCM is transmitted in an autosomal-dominant trait in the Maine Coon and Ragdoll breeds.[7,8] Two mutations in *MYBPC3* have been identified so far. The first one, identified only in the Main Coon breed, is a c.91G>C

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Table 1 Animal models of sarcomeric cardiomyopathy

Model	Breed	Phenotype	Affected gene	Mutations	Advantages	Disadvantages
Naturally occurring models						
Cat[7–12]	Maine Coon Ragdoll	HCM	*MYPBC3*	c.91G>C [p.Ala31Pro] c.2328C>T [p.Arg820Trp]	- Recapitulates the human phenotype.	- Gene targeting is difficult and expensive.
Dog[13–25]	Port. Waterdogs Great Danes Doberman Boxers	DCM DCM DCM DCM/ARVC	Uncertain Uncertain Uncertain Uncertain	Unknown Unknown Unknown Unknown	- Recapitulates the human phenotype. - Large animals allowing clinical imaging and diagnostics.	- Genes and mutations presently unknown. - Gene targeting is difficult.
Genetically engineered models						
Mouse[26–40]		HCM HCM DCM	*Mybpc3*, *Myh6*, *Tnni3*, *Tnnt2*, *Tpm1* *Mybpc3*, *Myh6*, *Tnnt2* *Mlp/Csrp3*	Transgenesis Knock-in/-out Knock-out	- Transgenesis respects sarcomere stoichiometry (replacement of endogenous protein by exogenous one). - Knock-in mice recapitulate human genetics. - Mammalian heart. - Homozygotes more severe phenotype than heterozygotes.	- Cardiac biology different from large mammals. - Expresses *Myh6*.
Rat[41]		HCM	*Tnnt2*	Transgenesis (truncated protein)	- Mammalian heart. - Reproduce diastolic dysfunction and arrhythmias.	- Cardiac biology different from large mammals.
Rabbit[42]		HCM	*TNNI3*	Transgenesis	- Cardiac physiology close to humans. - Mammalian heart with low heart rate. - Expresses *MYH7* like human heart.	
Zebrafish[43]		DCM	*Mybpc3*	[p.Val762Asp/p.Arg820Gln]	- Embryos develop very fast and are fully transparent: non-invasive *in vivo* imaging. - Forward and reverse genetics toolbox. - Compound heterozygotes severe phenotype.	- Non-mammalian heart.
Drosophila[44,45]		HCM RCM	*mybpc3* *tnnt2*	Wild-type, truncated up[101] *TnT1*	- Mammalian-like early heart development. - Easy gene expression manipulation with the UAS/GAL4 system. - Diastolic dysfunction	- Non-vertebrate heart.

HCM: hypertrophic cardiomyopathy; DCM: dilated cardiomyopathy; ARVC: arrhythmogenic right ventricular cardiomyopathy; RCM: restrictive cardiomyopathy; Gene abbreviations used: *Mybpc3*: cardiac myosin-binding protein C; *Myh6*: α-myosin heavy chain; *Myh7*: β-myosin heavy chain; *Tnni3*: cardiac troponin I; *Tnnt2*: cardiac troponin T; *ilk*: integrin-linked kinase; *mlp/Csrp3*: cysteine and glycine-rich protein; *not3*: CG8426 gene product from the transcript CG8426-RB.

missense mutation in exon 3, which gives rise to the p.Ala31Pro cardiac myosin-binding protein C (cMyBP-C) mutant in the linker region between the C0 and C1 domains of the protein.[9,10] Some rare isolated cases of British Longhair, Ragdoll, or Siberian breeds also carry this mutation.[10,11] The second one, identified only in the Ragdoll breed, is a c.2328C>T transition in exon 26, which results in the p.Arg820Trp cMyBP-C mutant in the C6 domain.[8,10] Both heterozygous and homozygous cats for *MYBPC3* mutations developed LVH (mainly concentric),[12] but some heterozygotes do not exhibit clinical signs of HCM. On the other hand, whereas all homozygotes developed diastolic dysfunction, few heterozygotes developed minor regional myocardial diastolic dysfunction without LVH,[12] suggesting that diastolic dysfunction could be the first feature of the disease, such as observed in heterozygous human patients and mouse model of HCM.[26,47,48] Importantly, the c.91G>C mutation results in a lower amount of cMyBP-C protein in the heart in both heterozygous and homozygous Maine Coon cats,[9] such as seen in human HCM.[49–51] This suggests regulation of mutation expression by protein quality control mechanisms, such as the ubiquitin–protein system, which has been shown to be involved after *MYBPC3* gene transfer in cardiac myocytes and *in vivo* in the *Mybpc3*-targeted knock-in mice.[27,52–56]

Cats with HCM represent therefore a good intermediary model between the many mouse models that have been made and humans to evaluate different causal therapeutic strategies to prevent the development of heart failure and/or sudden cardiac death or to rescue the phenotype in both heterozygotes and homozygotes for *MYBPC3* mutations. Recent evidence that RNA-based therapies, such as exon skipping or *trans*-splicing, can repair *Mybcp3* mRNA,[57,58] and more recently, that *Mybpc3* gene therapy long term prevents the development of the disease phenotype in *Mybpc3*-targeted knock-in mice[59] paved the way to evaluate these strategies in cats.

3. Canine models of cardiomyopathy

Large animal models of inherited cardiomyopathies would be extremely useful for the evaluation of novel pharmacological, gene, cell, and device therapies. To our knowledge, there are no known porcine or canine genetically engineered models of cardiomyopathy. However, several naturally occurring forms of DCM have been described in dogs,[13] and in fact constitute the most common form of heart disease in large- and giant-bred dogs.[14] For example, DCM has been described in Portuguese waterdogs,[15] Great Danes,[16] Doberman Pinschers,[17] and Boxers.[18] The exact genetic basis in each of these breeds remains, however, incompletely understood, with reports showing an association with mutations in genes encoding for sarcomeric,[19] desmosomal,[18] or metabolic proteins.[20–22] Similarly, an autosomal-dominant form of ARVC has been described in Boxers,[23,24] and while no mutations in desmosomal genes (known to be associated with ARVC in humans) were found in these dogs, there was loss of gap junction plaques resembling the phenotype found in humans.[25] These naturally occurring canine models of cardiomyopathy not only provide a model for testing novel therapies, but (as the gene mutations in these breeds appear different from known gene mutations in humans with DCM and ARVC) also provide an interesting target for genetic screening providing novel genes to be tested, in human forms of DCM and ARVC.

4. Genetic approaches for modelling disease in the mammalian heart

Approximately 30 years ago, techniques became available to perform directed genetic experiments on the mouse and rat using genetic engineering approaches in which direct modifications to the DNA coding sequences could be made. Using either gene targeting via homologous recombination or pronuclear injection of fertilized eggs, it became possible to engineer genetically defined changes in the mouse genome.

As first practiced, transgenesis remained a rather blunt instrument and it quickly became apparent that for meaningful questions to be asked and answered, more precise targeting of transgene's (TG's) expression would be needed. Promoter elements that were able to direct high levels of transcription only in cardiomyocytes were developed for the various stages of cardiac development. These promoters, which were derived from the alpha (*Myh6*) and beta (*Myh7*) myosin heavy chain (MHC) genes, were flanked with insulator sequences that shielded their transcriptional activity from the surrounding genetic contexts, making them position independent and copy number dependent in terms of their transcriptional activities. This allowed investigators to perform both developmental stage-specific and dose–response studies and provided the reagents needed to carry out precise TG's expression in the mouse heart[28] and rat heart.[41] Within months of the first reports of these reagents, other laboratories began to use them and, in the ensuing decade, literally hundreds of laboratories took advantage of these promoters to express proteins of their choice in either the fetal or adult cardiomyocyte populations (*Figure 1*). Using binary inducible systems, it became possible to reversibly activate a TG's expression in cardiomyocytes.[60]

While the ability to manipulate the cardiac protein complement with these techniques renders very significant advantages to the mouse as the species of choice for the overwhelming majority of TG's investigations,[29–35] the mouse does have limitations in terms of its relevance to human disease. For example, the basic motor protein that underlies contractile force in the sarcomere, the MHC, differs between the mouse and human ventricle. The mouse heart, which beats at ~600 bpm, expresses the fast cardiac MHC isoform, *Myh6*, whereas the human ventricle, which beats at approximately 1/10 the rate, expresses the 'slow' beta isoform of MHC, *Myh7*. While closely related, the two isoforms do differ in very fundamental ways and our investigations have shown important differences in the sequences that can limit the murine data's applicability to human cardiovascular function. Indeed, even the most ardent defenders of the mouse note that there are significant differences between the mouse models and human disease presentation. These have been best documented for HCM. While those models show some aspects of the human disease (e.g. premature death, cardiac myocyte disarray, interstitial fibrosis, and diastolic dysfunction), LVH, a defining aspect of human HCM, is rarely present.[36] In contrast to regional ventricular systolic dysfunction with maintenance of normal ejection fraction characteristic of human

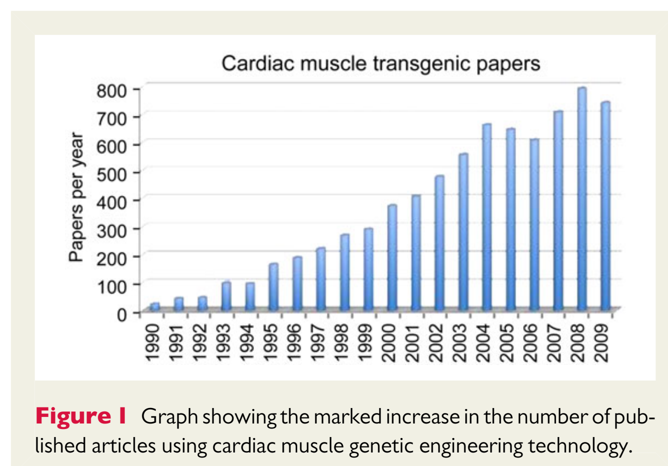

Figure 1 Graph showing the marked increase in the number of published articles using cardiac muscle genetic engineering technology.

disease, the mouse ventricle is globally impaired.[37] In addition to the differences in the motor protein isoform content, the mouse heart is not representative of the human myocardium in other important ways. For example, relative to the mouse, human cardiomyocytes have an increased contribution from the sarcolemmal sodium–calcium exchanger for cytosolic Ca^{2+} removal, with a lesser contribution from the sarcoplasmic reticulum–Ca^{2+}–ATPase (SERCA) pump.[61,62] In contrast, smaller rodents rely more heavily on SERCA2a as the primary mode of Ca^{2+} removal.[61,62]

Because of these types of concerns, it became important to develop a larger animal model to validate some of the findings being made in the mouse models. The rabbit offers an experimental model with significant advantages for cardiovascular research. Compared with the mouse, the larger size and slower heart rate of the rabbit are advantageous for physiological analyses such as echocardiography and cardiac catheterization. Importantly, the rabbit ventricles, like the humans, express *MYH7* and handle Ca^{2+} flux in much the same way as is observed for the human heart.

To establish the potential validity of TG methodology for remodelling a larger four-chambered heart, we explored cardiac-selective expression in TG rabbits. The rabbit *MYH7* promoter was used to express a reporter gene, and TG's expression was quantified in cardiac, skeletal, and smooth muscles as well as in non-muscle tissues. The promoter was able to drive high levels of TG expression in the cardiac compartment and directed slow myocyte-specific expression, showing that TG manipulation of the cardiomyocyte was possible in the rabbit.[42] We then explored the role of the two MHC isoforms in the rabbit ventricle, in order to understand the significance of the small (5–10%) amount of *MYH6* that is present in the human ventricle but is down-regulated in heart failure.[63] To test the effects of persistent *MYH6* expression on the background of *MYH7*, we made TG rabbits that expressed rabbit *MYH6* in the ventricle, so that the endogenous myosin was partially replaced by the transgenically encoded species. We hypothesized that *Myh6*'s unique biochemical properties might offer functional advantages in a failing heart that is normally expressing *Myh7*. This hypothesis cannot be tested in mice or rats because both species express *Myh6* as the predominant isoform.[64] Molecular, histological, and functional analyses showed no significant baseline effects in the TG rabbits, compared with non-TG (NTG) littermates. Cohorts of TG and NTG rabbits were subsequently subjected to rapid ventricular pacing. Although both the TG and NTG rabbits developed DCM, the TG rabbits had a higher shortening fraction, less septal thinning, and more normal haemodynamics than paced NTG rabbits. Thus, in a ventricle whose protein complement closely reflects that of the human, *MYH6* is cardioprotective in experimental tachycardia-induced cardiomyopathy. These results are now being translated into the clinic and, in a small compound screen, a candidate was selected that was able to specifically increase the ATPase activity of the cardiac myosin motor.[65] A phase IIb clinical trial is now underway in which the therapeutic potential of the compound omecamtiv mecarbil® for directly affecting MHC's motor function is being tested.

While cardiomyocyte-specific transgenesis has been widely used, it is less precise than gene targeting. With over 1000 mutations characterized in 10 or more sarcomeric genes, the paucity of gene-targeted models is striking, with only a handful of the mutations being precisely inserted into the relevant gene being reported in the literature. Thus, gene-targeted models for mutations in the MHC,[38] cardiac troponin T (cTNT),[66] cardiac troponin I,[39] and cMyBP-C[26,27] have all been reported. For the sarcomeric proteins, transgenesis has proved to be an effective way of mimicking gene-targeted animals, as the cardiomyocyte has post-translational mechanisms for ensuring that sarcomeric protein stoichiometry is maintained. Thus, when a sarcomeric protein is transgenically overexpressed, the net effect is down-regulation and replacement of the normal protein with the transgenically encoded species.[28] The relative (to transgenic animals) paucity of gene-targeted animals undoubtedly reflects the time (15–30 months or longer) and expense ($20 000–45 000). However, new technologies have recently become operational in a number of laboratories, and one of the authors' laboratory (J.R.) is now producing genetically modified animals using the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 technology and is in the process of bringing TALEN methodology online.[67,68] These have the significant advantage of being more cost effective and more rapid than the usual gene targeting via homologous recombination, in which the desired homologous recombination events occur extremely infrequently (1 in 10^6–10^9 cells).[69] The new gene editing technologies use a series of RNA-guided endonucleases along with other components to greatly increase the efficiency of precisely targeted homologous recombination events,[70] and mice carrying single-site modifications can be generated in ~8 months and for a cost of about $4000. With these technologies now available, we think it likely that the number of cardiomyopathy mouse models carrying precisely targeted, single-site mutations will increase exponentially in the next 5 years.

5. The zebrafish as a model to study cardiomyopathies

In two studies published in 2002, zebrafish (*Danio rerio*) loss-of-function mutants were described in the sarcomeric genes coding for cTnT and titin, both well known for their role in HCM and DCM, respectively.[71,72] Since the first description of these zebrafish models for cardiomyopathies a decade ago, the zebrafish has continued to demonstrate its value in studying the genetics of human cardiomyopathies.[73–75] Zebrafish embryos have a number of advantages that make them particularly well suited to study the genetics of human cardiomyopathies such as large clutches of eggs that are fertilized externally and an extensive genetic toolbox available to the zebrafish researcher.

Sequencing of the zebrafish genome was completed in 2013, which revealed that 82% of the known human disease-related genes have an orthologous gene in the zebrafish genome.[76] Forward genetic screens in zebrafish have been very useful in identifying novel genes required for cardiac development and function,[77] among which was the *integrin-linked kinase* (*ilk*) gene required for maintaining cardiomyocyte integrity in the embryonic heart.[73,78] ILK is located in the Z-disc of cardiomyocytes and when deleted in mouse cardiomyocytes results in DCM, while overexpressing ILK in rat cardiomyocytes can protect against DCM.[79,80] Particularly advantageous is the use of zebrafish to decipher the consequence of genetic variants discovered in the genome of cardiomyopathy patients.[43] In the case of ILK, for example, this was achieved by rescuing the zebrafish *ilk* mutant phenotype by injecting synthetic RNA encoding human *ILK*: while normal *ILK* RNA rescued efficiently, an *ILK* variant (c.785C>T [p.Ala262Val]) derived from a DCM patient failed to rescue the zebrafish *ilk* phenotype.[78] Thus far, reverse genetic approaches to disrupt a particular gene-of-interest have employed transient techniques, such as antisense morpholino oligonucleotides.[81] This technique relies on the specific blockage of protein translation or mRNA splicing, but can create artefacts due to off-target effects.[82] Recently, high-throughput DNA sequencing with chemically induced mutagenesis techniques was initiated, resulting in the

identification of potentially disruptive mutations in 38% of all known zebrafish protein-encoding genes.[83]

A major advantage of zebrafish, compared with other vertebrate models, is that the embryos are transparent allowing high-resolution *in vivo* observations of the heart.[84,85] The transparency of the embryos, combined with genetic engineering approaches where fluorescent proteins are expressed in various cell types of the cardiovascular system, has resulted in improved knowledge of mechanisms that regulate how and when cardiomyocytes differentiate,[86] acquire their typical shape,[87] or how the myocardium is regenerated after damage.[88] The advancement in new microscopy techniques such as light sheet planar illumination microscopy allows high-resolution imaging of the heart during normal or abnormal contraction cycles revealing details about the cellular and sub-cellular level.[89] Automated image analysis techniques in combination with high-speed video imaging has been used to extract functional cardiac parameters such as heart rate, arrhythmia index, and ejection fraction from embryos.[90]

To study gene function in relation to cardiomyopathies, it would be very helpful to study the role of a particular gene only in the heart or even in only one cell type present in the heart, independently from its role in other tissues. Likely, the application of existing technologies such as Cre-recombinase in combination with the recently developed homologous recombination techniques to introduce elements such as lox*P*-sites at specific locations in the genome will fill in this need.[91] The possibility to introduce specific DNA elements by homologous recombination using the TALEN or the CRISPR/Cas9 system opens unexplored territories: it will now be possible to introduce into the zebrafish genome genetic variants that were identified in patients with cardiomyopathies to create patient-specific disease models. These patient-specific models will allow researchers to study the mechanisms by which cardiomyopathies develop and, together with other animal models, will aid in bridging the current gap between patient genotyping and phenotyping. Furthermore, since zebrafish embryos are small (1–2 mm) and take up chemicals from the medium in which they are kept, they are well suited for *in vivo* chemical screens. In combination with newly developed zebrafish disease models, this opens new possibilities for identifying drugs that can restore cardiac function and may be of benefit to specific groups of patients with a well-defined genetic cardiomyopathy. These new developments ensure that zebrafish will remain a valuable model to study the genetics of cardiomyopathies in the future.

6. The *Drosophila* heart as a versatile model system to study cardiomyopathy

Since several years, the *Drosophila* heart has been used as a tool to study various aspects of the heart, such as identification of genes regulating heart development, but also unravelling mechanisms underlying pathophysiology of heart diseases (*Figure 2*). There are several reasons why the *Drosophila* heart is such an interesting tool. The *Drosophila* heart is a linear tube, reminiscent of the primitive vertebrate embryonic heart tube.[92] Although the final heart structure in *Drosophila* is very different compared with that in vertebrates, the basic elements for heart development, function, and ageing are remarkably conserved.[93] Heart development is regulated by an evolutionarily conserved gene regulatory network consisting of functional interconnections between myogenic transcription factors (NK-2, MEF2, GATA, Tbx, and Hand), their downstream target genes expressing contractile proteins, and upstream signalling pathways that direct cardiomyocyte differentiation and cardiac morphogenesis.[92] In addition to conserved heart development between simple model organisms and vertebrates, an important advantage of *Drosophila* is the ability to manipulate gene expression in a highly precise spatial and temporal fashion, by the use of a UAS/GAL4 system.[94,95] In *Drosophila*, the UAS/GAL4 system was successfully utilized to identify genes causing human cardiomyopathies.[95] Moreover, new techniques such as optical coherence tomography allow accurate phenotyping of cardiac diseases (like stretch and arrhythmia) in flies.[95,96] Because of the conserved heart development, the simplicity in structure and availability of powerful genetic tools, the *Drosophila* heart has emerged as a pioneering model system for unravelling the basic genetic and molecular mechanisms of cardiac development, function, and ageing.[93] The *Drosophila* heart has proved to be a valuable asset to elucidate the pathophysiology of human cardiac diseases, including HCM and DCM, channelopathies, congenital heart disease, as well as cardiac tachycardia, such as atrial fibrillation (AF).[44,95,97–99] In addition, the *Drosophila* heart has also been successfully used for drug and genome-wide screening assays, demonstrating the versatility of the *Drosophila* heart as a model system.

For example, the *Drosophila* heart has been used to identify druggable targets and to screen for novel small molecules to treat AF.[100,101] AF is a serious and progressive tachycardia and is a major cause of stroke as well as a precursor for congestive heart failure and cardiomyopathy.[102] AF progression is rooted in structural remodelling, especially sarcomeric protein damage.[101,103] To uncover druggable targets against sarcomeric protein damage, the prepupae of *Drosophila* were subjected to tachypacing by placing them in an electric field.[99,101] Tachypacing of the pupae induced structural damage and contractile dysfunction to the cardiomyocytes.[99] In addition, the *Drosophila* heart was recently utilized to uncover the role of epigenetics in AF by screening various HDAC inhibitors.[101] This screen revealed that HDAC6 is a druggable target, since the specific HDAC6 inhibitor tubacin protected against tachypacing-induced microtubuline network disruption and contractile dysfunction. Taken together, these studies demonstrate the potential of the *Drosophila* heart as a tool for druggable target identification and drug screen assays in heart diseases.

Next to the *Drosophila* as a tool in cardiomyopathies, and druggable target identification, the *Drosophila* heart has been exploited to verify the outcomes of a human genome-wide association study (GWAS) on genes related to heart rate.[46] In this GWAS, 21 loci associated with the heart rate were identified. Experimental down-regulation of gene expression in *Drosophila* confirmed the relevance of 20 genes at 11 loci for heart rate regulation and highlighted a role for the involved signal transduction routes, embryonic cardiac development and the pathophysiology of DCM, congenital heart failure, and/or sudden cardiac death. The *Drosophila* findings provide additional mechanistic insights but also identified new therapeutic targets.

Finally, using cardiac-specific RNAi silencing in *Drosophila*, 7061 evolutionarily conserved genes were knocked down.[45] In this way, a first global roadmap of pathways potentially playing conserved roles in the cardiovascular system was elucidated.[45] One critical pathway identified was the CCR4-Not complex, which was found to play a key role in cardiac function. Silencing of CCR4-Not components in adult *Drosophila* resulted in myofibrillar disarray and DCM. Findings were expanded to mouse studies and humans. In *Not3* knock-out mice, spontaneous impairment of cardiac contractility and increased susceptibility to heart failure was found. In humans, a common *NOT3* SNP was found to correlate with altered cardiac QT intervals, a known cause of potentially lethal

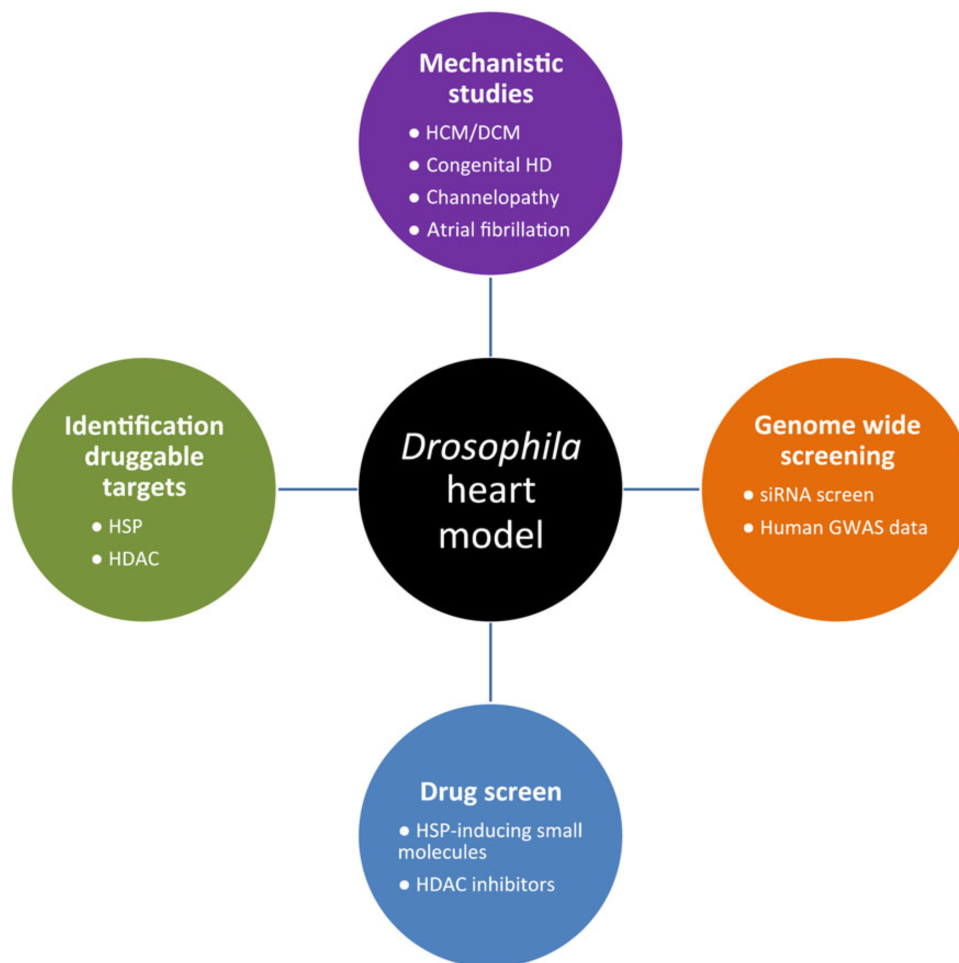

Figure 2 Examples of studies utilizing the *Drosophila* heart model as a tool.

ventricular tachyarrhythmias.[45] Thus, verification and application of genome-wide screens in *Drosophila* can identify candidate genes that translate into conserved mammalian genes involved in heart function.

7. Computational approaches in sarcomeric cardiomyopathies

Among the many model systems for studying complex genetic cardiomyopathies, computational approaches are relatively new. The use of computation has arisen in response to both a wide range of basic unanswered biological questions and rapidly growing genetic datasets. To understand the current use and promise of these varied methodologies, it is important to first define the precise role of each approach and where the resultant data fit in an integrative system to fully define the molecular pathogenesis of sarcomeric cardiomyopathies. As was noted in the recent ACC/AHA guidelines for the management of HCM (the most common genetic form of cardiomyopathy), the *identity* of individual sarcomeric mutations cannot be used in the clinical management of patients.[104] For a disorder that was first identified as having a genetic cause in 1990, this is a sobering state of affairs and represents a crucial roadblock to the development of genotype-driven management.[105] Although the issues that contribute to this limitation are multifactorial and beyond the scope of the current discussion (for review see ref.[106]), there is clearly a pressing need for novel approaches that directly address the primary mechanisms that define the molecular basis of this 'disease of the cardiac sarcomere'. Given that >1400 mutations have been linked to sarcomeric cardiomyopathies to date, computational approaches are a compelling answer to this growing challenge.

The development of computational model systems has focused on several central unmet needs in the study of sarcomeric cardiomyopathies. In particular, the growing availability of genetic testing (leading to a rapidly growing number of putative disease mutations) and the fact that many sarcomeric mutations arise *de novo* combine to vastly complicate the most basic question of whether a single amino acid substitution in a protein component of the cardiac sarcomere is sufficient to cause disease in an affected individual. For the practicing cardiologist, this is the '*raison d'être*' for submitting patient samples for testing. The sheer number of proteins affected, the many possible mutations and the limitations in identifying comprehensive, accessible and reproducible *in vitro* measures of protein function has led to the development of a host of algorithmic approaches for predicting allele 'pathogenicity'.[107] For sarcomeric cardiomyopathies, the two most commonly utilized are PolyPhen-2 and 'Sorting Tolerant from Intolerant' (SIFT).[108,109] PolyPhen-2 is a robust algorithm that mines known protein databases for existing information regarding local structure, known, or predicted

molecular function (e.g. binding domains, salt bridge formation, and hydrophobicity) and, combined with overall amino acid conservation maps, develops a template to determine the potential effects of amino acid substitutions. For proteins with known 3D structural information, it is possible to incorporate secondary and tertiary structures to assess allelic effects on intramolecular interactions, thus adding to the fidelity of the predictions. SIFT serves a similar role and utilizes a comprehensive approach that focuses on amino acid conservation compiled from known protein structure and a robust use of homology mapping to extend predictions beyond what is known for a given protein. Both algorithms have been optimized for use with large datasets and are also extensively used in industry and basic research to predict the structural and potentially functional effects of individual substitutions (and more recently indels) in sarcomeric proteins.

The application of the database-driven algorithms to predict primary pathogenicity is a reasonable approach; from the mechanistic standpoint, however, sarcomeric proteins present a unique challenge in that they are components of a complex multisubunit 'machine'.[106] Moreover, the basic function of this machine is highly dynamic and fully dependent on allosteric interactions, Ca^{2+} regulation, and load and post-translational modifications, conditions that cannot be easily incorporated into models based on static structures of single proteins. Thus, from the computational standpoint, it is necessary to develop more comprehensive, biologically complex models that can incorporate structural and functional information across multiple levels of experimental resolution with a goal of developing a coupled system that can be validated from the single molecule to the whole organ level.[110] While multiscale modelling has yet to be applied to sarcomeric mutations, its promise was recently demonstrated by Sheikh *et al.*,[111] where computational modelling of skinned fibre data suggested a novel two-tiered role for myosin light chain 2v phosphorylation in regulating cardiac muscle contraction and potentially modulating preclinical remodelling in a murine DCM model. As illustrated by this study, it is important to note that a major role for computational modelling going forward is to shed light on potential molecular mechanisms that would not be detected via standard, low-resolution experiments and provide unique, testable hypotheses for discovery and validation.

Given the complexity of the cardiac sarcomere, a crucial component of the multiscaled approach is the ability to define atomic-level changes in protein dynamics, both intra- and intermolecular. Alterations in protein dynamics likely represent an important proximal manifestation of sarcomeric mutations at the molecular level and can be addressed computationally via molecular dynamics (MD) simulations. MD is based on an atom-level determination of the time dependence of molecular interactions.[112] It is well suited to the study of dynamic biological systems like the cardiac thin filament because of its scalability and the ability to map atomic motions over predicted trajectories for a given unit of time, resulting in a high-resolution 'window' into both the local and distant effects of contractile protein mutations. The first application of MD to sarcomeric cardiomyopathies was performed by Ertz-Berger *et al.*,[113] and focused on the effects of known single amino acid substitutions at Residue 92 in cTnT. Results from this and a subsequent study revealed mutation-specific alterations in local protein flexibility and, importantly, for the first time established that thin filament mutations within alpha-helical regions can lead to a propagation of structural and presumably functional effects at a distance from the mutated residue.[114] Similar MD results were observed by Li *et al.*[115] for the tropomyosin Asp175Asn and Glu180Gly mutants, in that both were found to have effects on local and global flexibility. The findings regarding propagation effects are particularly important. First, the understanding that changes in structure and dynamics at the primary mutational site may not be the sole effect of a given mutation broadens our functional interpretation of mutational effects. Secondly, independent mutations at significant distances from each other may propagate structural effects to the same functional domain, thus providing a novel approach to define groups of mutations. The latter observation provides a framework for the design of therapeutic small molecules that can be used for an array of mutations.

There are many exciting new computational approaches on the horizon. In particular, as shown by Manning *et al.*,[116] the development of a full all-atom model of the cardiac thin filament, coupled with advances in computing power, will allow for an ever more detailed understanding of the most proximal molecular causes of disease. As computational powers grow (for example, via the inclusion of graphic processing units or GPUs), it becomes conceivable that a fully atomistic model of the thin filament in explicit solvent will be available. That coupled with a more detailed understanding of chemistry in the thick filament will serve as an input to higher-level multiscale models that will provide an fully integrative, iterative, and eventually predictive approach to understanding these common genetic cardiomyopathies.

8. Concluding remarks and future perspectives

Over the past decade, our understanding of cardiomyopathies has improved dramatically, which is not only due to improvements in screening and detection of gene defects in the human genome, but also to the availability of a variety of novel animal and *in silico* computational models. These novel experimental tools have created a platform that is highly complementary to the naturally occurring cardiomyopathies in cats and dogs that had been available for some time. It is likely that a fully integrative approach, which incorporates all these modalities, is required for major steps forward into understanding the molecular underpinnings and pathogenesis of cardiomyopathies. Finally, the recent development of new technologies, such as CRISPR/Cas9, which has already been proved to work in zebrafish and in mice, is currently being employed to engineer sarcomeric cardiomyopathy in larger animals, including pigs and non-human primates. In the mouse, the increased speed with which these techniques can be employed to engineer precise 'knock-in' models that previously took years to make via multiple rounds of homologous recombination-based gene targeting promises multiple and precise models of human cardiac disease for future study. Such novel genetically engineered animal models recapitulating human sarcomeric protein defects will help bridging the gap to translate therapeutic targets from smaller animal and *in silico* models to the human patient with sarcomeric cardiomyopathy.

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