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Probing the polygenic basis of cardiomyopathies in *Drosophila*

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Abstract

In trying to understand the causes for congenital heart disease and cardiomyopathies, it is difficult to study polygenic interactions that contribute to the severity of the disease, which is in part due to genetic complexity and generation time of higher organisms that hinder efficient screening for modifiers of primary causes of heart disease. The adult *Drosophila* heart has recently been established as a model to probe genetic interactions that lead to cardiac dysfunction in this genetically simple and short-lived organism. This has made it possible to systematically and efficiently screen for polygenic modulators of heart dysfunction inflicted by known heart disease genes. As heart development and fundamental aspects of cardiac physiology show remarkable evolutionary conservation, it has become possible to uncover new heart disease candidates by using *Drosophila* genetic tools in combination with sensitive heart function assays. Here, we review the discovery of several new genes, genetic pathways, and interactions that will help understand human heart disease. For example, interactions between cardiogenic transcription factors, discovered in *Drosophila*, are also critical for adult heart function in flies and mammals. These include interactions between *tinman/Nkx2-5* and *neuromancer/Tbx20*, which led to the discovery of possibly disease-causing familial variants in human *TBX20*. A new genetic pathway from *tinman/Nkx2-5* to *Cdc42*, involving the microRNA *miR-1*, was recently discovered in flies and subsequently validated to function similarly in mouse heart. Thus, the fly heart has proven to be a useful discovery tool for screening genetic interactions that are otherwise difficult to conduct.

Keywords: *Drosophila* • cardiac • ageing

Introduction

In a quest to elucidate the genetic basis of heart disease, we and others have used *Drosophila* as a model system to identify and define the first set of 'cardiogenic' genes that specify the heart within a developing embryo. The insights gained from *Drosophila* have served as a prototype to elucidate the fundamentally conserved mechanisms of cardiac development in higher organisms. The discovery of the homeobox transcription factor Tinman in *Drosophila* and related factors in vertebrates marked the beginning of our understanding of the genetic basis of heart development in the

animal kingdom [1–4]. Since then, we and others have unravelled much of the complex interplay between inductive and transcription factors networks that lead to the specification of the heart (reviewed [5]). In turn, these findings lead to the elucidation of the (conserved) regulatory cardiogenic network in vertebrate models and paved the way for a molecular understanding of human heart disease [6–9].

The recent advent of quantitative heart function assays in *Drosophila* (Figs 1 and 2) [10–13] has allowed cardiomyopathy models

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to be established in this genetically tractable system (e.g. [14, 15]). Using such models, we have discovered that the cardiogenic network of transcription factors, encoded by homeobox, GATA and T-box genes (e.g. *tinman/Nkx2-5*, *pannier/Gata4* and *neuromancer/Tbx20*, respectively), not only specifies the heart during development but also plays a critical role in establishing and maintaining heart function in the adult fly [16–18]. Importantly, there is increasing evidence that homologues of these transcription factors also regulate cardiac function in humans (e.g. [17–23]). It is thus critical that we understand the functions and interactions of these genes in the mature, in addition to the developing, heart. Recent studies suggest that the discovery of heart genes in *Drosophila* may identify new candidates and mechanisms to understand human heart disease [24], and in particular may reveal polygenic modulators of the core cardiogenic network of transcription factors [17, 18]. As polygenic contributions to heart disease are difficult to study in mammalian systems, new approaches to study adult *Drosophila* heart physiology (Fig. 2) show great promise as an important paradigm for identifying fundamental mechanisms of heart disease. Here, we review examples of such polygenic interactions.

Recent studies suggest that the evolutionary conservation of factors controlling cardiac function may extend beyond cardiogenic transcription factors such as *tinman*, *pannier* and *neuromancer*, to include structural components as well. We will first discuss fly models of arrhythmias involving KCNQ and HERG potassium channels ([10,11]; K. Ocorr, unpublished data), as well as cardiomyopathy models involving myosin heavy chain (MHC) and Dystrophin [14, 15], and then review recent evidence for polygenic interactions involving cardiogenic transcription factors in the adult heart [17, 18].

Arrhythmia and cardiomyopathy models in *Drosophila*

In humans, efficient repolarization of the cardiac action potential is dependent on fast activating and inactivating potassium (K) channels. A number of arrhythmogenic disorders have been shown to directly involve such K channels. These include KCNQ1, the α -subunit of a K channel responsible for the slow repolarizing current (I_{KS}), and the Human Ether-a-go-go Related Gene (HERG), which encodes a channel underlying the rapid phase of cardiac repolarization (I_{KR}) (for reviews, [25, 26]). Mutations in these K channels often lead to reduced cardiac repolarization, as manifest in prolonged QT intervals with an elevated risk of Torsades de Pointes ventricular arrhythmias, which are believed to be a cause of sudden cardiac death. Remarkably, null mutations in the single *Drosophila* KCNQ gene also lead to arrhythmias in flies, which become much more pronounced with increasing age [11,27]. Reminiscent of the Framingham study showing an age-dependent increase in atrial fibrillation in humans [28], wildtype flies also develop an increased incidence of arrhythmias with age [11] and they become more susceptible to pacing-induced cardiac dysfunction ('heart failure'; [12]). One reason for this age-dependent deterioration of heart function may be that cardiac KCNQ expression is decreased in ageing fly hearts, which may result in a



Fig. 1 *Drosophila* carrying a transgene for a *tinman* heart enhancer driving GFP (green) expression, illustrating the location of the heart tube along the dorsal midline of the abdomen. (Micrograph is courtesy of Dr. Geo Vogler).

suboptimal repolarization capacity of the cardiomyocytes. This hypothesis was corroborated by the observation that forced expression of KCNQ in ageing hearts markedly reduced the frequency of arrhythmias [11]. However, overexpression of KCNQ in young fly hearts had the opposite effect, resulting in a higher prevalence of arrhythmias [27]. These findings suggested that a robust and regular (myogenic) beating pattern in unperturbed hearts depends on the well-balanced expression of appropriate ion channels. As KCNQ and HERG K channels play a major role in cardiac action potential repolarization of humans [25, 26], but apparently not of adult rodents [29], the *Drosophila* model may be a useful alternative for studying the roles of these K channels to cardiac repolarization and rhythmicity.

Many mutations have been identified in the muscle myosin heavy chain (MHC) gene that cause hypertrophic or dilated, as well as restrictive cardiomyopathy in humans (reviewed in [30]). Interestingly, MHC mutations that cause dilated or restrictive cardiomyopathy phenotypes have also been identified in *Drosophila* [14]. For example, a mutation that decreases the ATPase activity of MHC results in a slower and more arrhythmic heartbeat than that of wildtype flies, and the mutant hearts are markedly dilated and reduced in their contractile capacity. A complementary mutation that increases the ATPase activity of MHC results in a more constricted, but equally compromised contractility as the dilated mutant. This suggests that there are remarkable similarities in fundamental aspects of cardiomyopathies between flies and humans.

As for MHC, mutations of Dystrophin (Dys) are known to cause dilated cardiomyopathy, in addition to their role in causing muscular

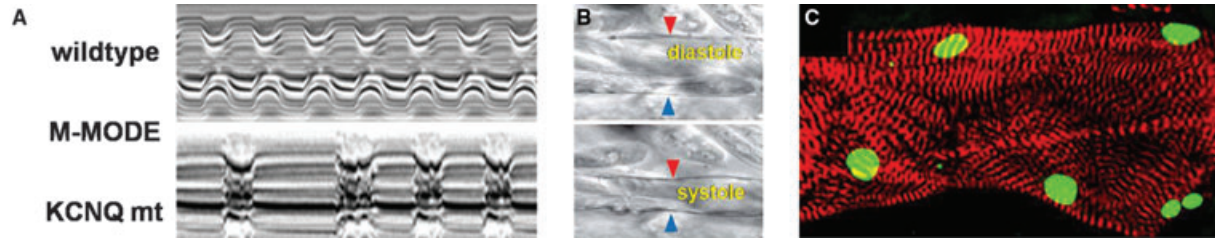


Fig. 2 (A) M-mode traces prepared from high-speed movies of dissected flies. One pixel-wide region with both edges of the heart tube is defined in a single movie frame. The same regions are then electronically cut from all of the movie frames and aligned horizontally to produce the M-mode trace. Note the prolonged arrhythmic contractions in KCNQ mutants compared with the regular heartbeat in wildtype flies ([11]; M-mode images are courtesy of Dr. Karen Ocorr). **(B)** Portion of the fly heart tube in diastole (top) and systole (bottom). Arrowheads indicate the heart walls. **(C)** Portion of the fly heart tube stained for Neuromancer/H15 (fly Tbx20) enhancer trap Lac-Z reporter (green) in cardiomyocyte nuclei, and for the Z-line marker α -actinin (red).

dystrophy [30, 31]. Similarly, Dys-deficient *Drosophila* also exhibit a dilated heart phenotype, as well as disruption of myofibrillar integrity [15]. This suggests that Dys may function similarly in flies and mammals, emphasizing not only that they share fundamental aspects of normal heart function but also that dysfunctional homologous molecular components can lead to equivalent pathological cardiac phenotypes.

Although the *Drosophila* cardiomyopathy models are unlikely to replicate exactly human heart diseases, there is much to be learnt by investigating the basic mechanisms and genetic interactions related to cardiomyopathies. In particular, genetic modifiers that can alter heart disease phenotypes are difficult to identify and study in mammalian systems. Thus, polygenic interactions that are critical for the severity of the disease manifestation can be screened for systematically using *Drosophila* genetics.

Congenital heart disease genes *tinman/NKX2-5*, *neuromancer/TBX20* and *pannier/GATA4* also function in maintaining adult heart function

The genes *tinman/Nkx2-5* (*tin*), *neuromancer/Tbx20* (*nmr*) and *pannier/GATA4/6* (*pnr*) encode evolutionarily conserved cardiac transcription factors important for the specification and morphogenesis of the embryonic heart. Mutations in *NKX2-5* and *GATA4* have been implicated in causing human cardiac disease. The expression of each of these factors spans a large developmental window of cardiogenesis and continues in the mature heart. Consequently, the specific function of each of these key factors may be different during the progression of cardiogenesis compared with their role in maintaining cardiac function or during cardiomyocyte maturation. Therefore, we took advantage of the *Drosophila* heart model to examine the function of these genes in maintaining heart physiology and myofibrillar architecture in the adult, which is difficult to address in higher organisms because of the lethality associated with defective vertebrate heart function.

We first tested genetically whether *tin* function is required at a later stage in the adult heart. Based on the known arrangement of the different enhancer elements, we generated genomic *tin* constructs that support normal patterns of *tin* expression in the early mesoderm, but do not support expression in the forming cardiomyocytes at later stages of embryonic development or in the adult. The cardiac-only loss of function of *tin* caused hypoplasia, impaired heart function and shortened life span [32]. While characterizing the role of *pnr* in adult fly hearts, we discovered a cardiac autonomous requirement also of *pnr* in regulating heart physiology, where adult-specific disruption of *pnr* function, mediated by a dominant-negative form of *pnr*, caused elevated arrhythmias and defective cardiac performance in response to electrical pacing [16], similar to the phenotype observed in *pnr* heterozygotes (Fig. 3). To explore the possible mechanism, we performed genetic epistasis analyses and found that *nmr* is a potential downstream mediator of *pnr* in controlling cardiac performance and rhythm regularity.

In further work, we found that *nmr* function is indeed required to maintain normal heart function in the adult fly because reducing *nmr* function in the adult heart leads to an irregular beating pattern, increased pacing-induced heart failure and an overall decrease in heart rate [17]. Structurally, we found that the myocardium of *nmr* mutant hearts had irregular myofibre alignment and disrupted spacing between Z-lines. Interestingly, we also observed a double heterozygous interaction between *nmr* and *tin* (*nmr*^{+/-};*tin*^{+/-}, Fig. 3), resulting in elevated pacing-induced heart failure rate, increased arrhythmias, altered cardiac expression of potential downstream effector genes and misalignment of myofibres within the cardiac myocytes [17]. In addition, genetic rescue experiments suggested that maintaining high levels of *tin* or *nmr* expression in older hearts slowed the age-dependent decline of cardiac performance in wild type flies. Given that the *tin* homologue, *NKX2-5*, is also implicated in human cardiac disease [33], we performed genetic screening for *TBX20* variants in patients with dilated and other cardiomyopathies. Interestingly, we identified four non-synonymous variants altering the protein coding sequence in two sporadic and two familial cases, suggesting that candidate genes identified in the *Drosophila* cardiomyopathy models may be directly relevant for human cardiac disease [17]. Additional vari-

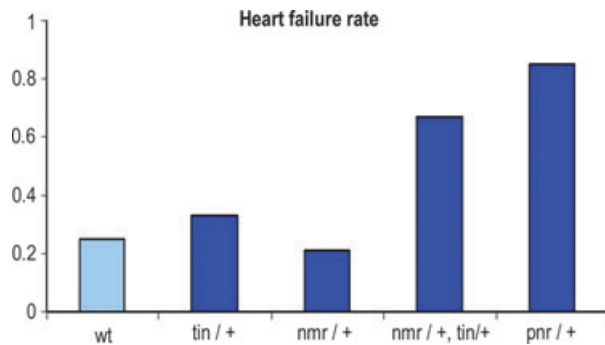


Fig. 3 Synergistic genetic interactions between *tinman/Nkx2-5* and *neuromancer/Tbx20*. Flies of the indicated genotypes were subjected to electrical pacing and assayed for normal heart function [12]. Shown is the fraction of flies with heart dysfunction ('heart failure'). Flies heterozygous for *tinman* (*tin/+*) or a deficiency for *neuromancer 1 and 2* (*nmr/+*) have a low incidence of heart failure, similar to the wildtype (*wt*) flies. In contrast, the double heterozygotes (*nmr/+;tin/+*) have a dramatically increased heart failure rate, indicative of their strong genetic interaction for maintaining normal heart function (see [17]). Flies heterozygous for *pannier* (fly counterpart of *Gata4*) alone also show a high heart failure rate (*pnr/+*; see [16]), reminiscent of the effects of GATA4 variants present in some patients with congenital heart disease [19].

ants of TBX20 were found concurrently in patients with other cardiac pathologies, and the results were reported in an independent publication [21].

A new cardiac pathway from *tinman* to *Cdc42* via *miR-1*: the *Drosophila* heart model as a tool to systematically probe for new heart disease gene candidates

Based on the conserved cardiogenic transcription factor network during embryonic heart development and the newly discovered role of key factors (e.g. *tin*, *nmr*, *pnr*) in adult heart function, it is possible that the genetic mechanisms that establish and maintain adult cardiac performance may also be conserved. To explore this idea, we conducted a screen for mutations that aggravate the cardiac stress response of flies heterozygous for *tin*, similar to the *nmr-tin* double heterozygotes (Fig. 3). Our aim was to uncover novel genes having critical cardiac functions that act in concert with, or downstream of, known cardiac determinants. Among the interactions with *tin*, we found the small Rho-GTPase encoded by *Cdc42* to be an important regulator of embryonic morphogenesis (G. Vogler; J. Liu and R.B., unpublished data), as well as adult function of the heart [18]. Dominant-negative *Cdc42* expression in the adult heart was sufficient to cause functional and structural abnormalities, such as increased arrhythmias and myofibrillar disorganization. Our genetic and functional evidence suggests that *Cdc42* may act through the Pak (a serine/threonine protein kinase) pathway to regulate actin filament assembly and thus myofibrillar structure in the fly heart. While analysing embryonic heart development we also found that *Cdc42* null mutants were

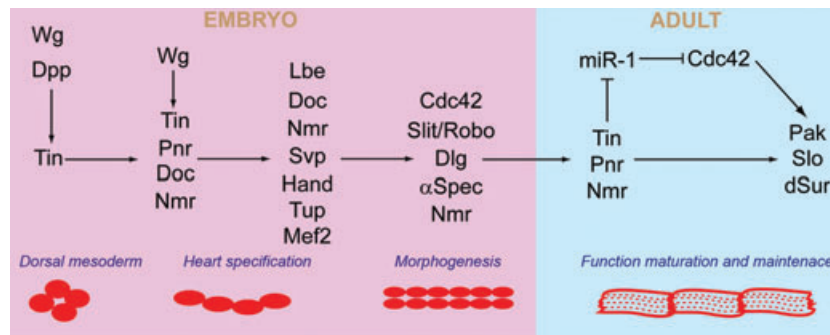


Fig. 4 Summary of regulatory interactions involved in *Drosophila* heart development (left, EMBRYO) and cardiomyocyte maturation function (right, ADULT). Inductive ectodermal signals (Wingless, Wg/Wnt and Decapentaplegic, Dpp/Bmp) to the underlying mesoderm specify the prospective heart field in the presence of Tinman/Nkx2-5 in the dorsal part of fly embryo. Cardiac progenitor specification is established by expression of the cardiogenic transcription factors Tinman (Tin/Nkx2-5), Pannier (Pnr/GATA), and the T-Box genes Dorsocross (Doc) and Neuromancer (Nmr). Further cardiac differentiation and lineage determination involve Ladybird (Lbe), Dorsocross (Doc), Neuromancer (Nmr), Sevenup (Svp), Hand, Tailup (Tup) and Mef2 (see Ref. [5], for details). After cardioblasts are specified, they migrate towards dorsal midline and acquire an apical-basal cellular polarity – a process that requires the heart-specific expression and/or polarized localization of the Robo receptors and their ligand Slit, as well as other polarity genes (e.g. Discs large – Dlg and α -Spectrin – α Spec) and transcription factor Nmr [5, 17, 34]. Finally, the bilateral rows of cardioblasts fuse together to form the lumen of the heart, ready to beat in late embryonic stages. In the adult fly, the key cardiogenic transcription factors Tin, Pnr and Nmr are re-utilized to establish and maintain the proper contractility and rhythm of the heart [16, 17, 32]. The Rho GTPase *Cdc42* genetically interacts with Tin via microRNA miR-1 [18]. The downstream mediators of this interaction probably involve the Pak-kinase and the potassium channels Slowpoke (Slo) and dSur, which by themselves are required to maintain normal heart function and myofibrillar structure of the cardiomyocytes [18, and unpublished data]. Vertebrate homologues of some of the mentioned genes: *wg-Wnt*; *Dpp-BMP*; *tin-Nkx2-5*; *pnr-Gata4/5/6*; *doc-Tbx5/6*; *nmr-Tbx20*; *lbe-Lbx1/2/3*; *tup-Isl1*; *Mef2-Mef2a-d*; *dHand-Hand1/2*; *dSur-Sur1/2*.

impaired in myocardial tube morphogenesis, similar to that seen in mutants of the Robo-Slit pathway [34], and including defective heart lumen formation (G. Vogler; J. Liu and R.B., unpublished data).

In further analysing the interaction of *tin* with *Cdc42*, we found that double heterozygous mutants exhibited irregular heart beating and increased diastolic intervals, slowing the heart rate. To elucidate a possible mechanism by which *Cdc42* and *tin* might interact to maintain heart function, we investigated the role of two K channels, encoded by *dSUR* and *slowpoke (slo)*, which were down-regulated in *tin-Cdc42* double heterozygous hearts. Based on further genetic interaction experiments between *Cdc42* and these K channel genes, they are likely the downstream mediators of the *tin-Cdc42* pathway [18]. Specifically, a *dSUR* enhancer, known to be regulated by Tinman, is further induced in combination with *Cdc42*, acting as a likely co-activator in a cell culture assay. This finding is in accord with the postulated synergistic interaction between *tin* and *Cdc42*.

To determine whether the discoveries made in the fly can translate to mammalian organisms, we examined compound *Cdc42*, *Nkx2-5* heterozygous mice. As in the fly model, cardiac contraction defects were observed in the murine *Nkx2-5;Cdc42* double heterozygotes, as manifested by significantly decreased fractional shortening, ejection fraction, stroke volume and cardiac output [18]. Furthermore, the combined reduction of *Cdc42* and *Nkx2-5* function resulted in prolonged atrial depolarization, manifested by increased P duration, and prolonged QRS complex, QT and QTc intervals, again suggesting that *Cdc42* interacts strongly with *Nkx2-5* in the mouse, as it does the fly heart. Therefore, this genetic interaction is indispensable for proper cardiac contraction, electrical conduction and rhythm across species (illustrated in Fig. 4).

We further explored the mechanism by which *tin/Nkx2-5* genetically interacts with *Cdc42*, and identified *tin/Nkx2-5* consensus binding sites in the enhancer region of the microRNA *miR-1* in both flies

and mice [18]. In addition, we found a *miR-1* consensus target site in the 3'-UTR of *Cdc42* in the mouse (but curiously, not in the fly), suggesting a double-negative regulatory pathway, which was further validated by extensive luciferase assays, qPCR, and western blotting. On the basis of these findings, we proposed a novel conserved pathway that is required for the establishment and/or maintenance of normal heart function: *Cdc42* is a direct target of *miR-1*, which in turn is negatively regulated by *tin/Nkx2-5*. This model was also supported in *Drosophila*, despite the lack of *miR-1* sites in fly *Cdc42*, as we found that cardiac overexpression of *miR-1* induced functional defects similar to those observed in *Cdc42* mutant hearts, suggesting that *miR-1* regulation of *Cdc42* involves a non-canonical or indirect mechanism.

We conclude that the *Drosophila* heart (Fig. 1) serves as an efficient discovery system to uncover new potential cardiac disease genes, and to elucidate polygenic interactions, pathways and mechanism that are difficult to pursue in the intact mammalian heart (see Fig. 4, a summary diagram involved in heart development and establishment and maintenance of heart function).

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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