GENETICS

The authors note that the following acknowledgment was omitted from the article: “T.A. was supported by the Sanford Fellowship of the Sanford Children’s Health Research Center at Burnham Institute for Medical Research.”

www.pnas.org/cgi/doi/10.1073/pnas.0907125106

RETRACTION

MEDICAL SCIENCES

The authors wish to note the following: “During efforts to extend this work, we have been unable to replicate the data shown in Fig. 1C of the paper. This calls into question a conclusion of the paper. The authors therefore regretfully retract the paper.”

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www.pnas.org/cgi/doi/10.1073/pnas.0906977106
neuromancer/Tbx20 (nmr) genes are cardiac T-box transcription factors that are evolutionarily conserved from flies to humans. Along with other known congenital heart disease genes, including tinman/Nkx2–5, dorsocross/Tbx5/6, and pannier/Gata4/6, they are important for specification and morphogenesis of the embryonic heart. The Drosophila heart has proven to be an excellent model to study genes involved in establishing and maintaining the structural integrity of the adult heart, as well as genes involved in maintaining physiological function. Using this model, we have identified nmr as a gene required in adult fly hearts for the maintenance of both normal myofibrillar architecture and cardiac physiology. Moreover, we have discovered synergistic interactions between nmr and other cardiac transcription factors, including tinman/Nkx2–5, in regulating cardiac performance, rhythmicity, and cardiomyocyte structure, reminiscent of similar interactions in mice. This suggests a remarkably conserved role for this network of cardiac transcription factors in the genetic control of the adult heart. In addition, nmr-tinman interactions also influence the expression of potential downstream effectors, such as ion channels.

Interestingly, genetic screening of patients with dilated cardiomyopathy and congenital heart disease has revealed Tbx20 variants in three sporadic and two familial cases that were not found in controls. These findings suggest that the fly heart might serve as an identifier of candidate genes involved in human heart disease.

Arrhythmia | Cardiac disease | T-box genes | Tinman

The Drosophila homeobox transcription factor (Tinman) was the first key determinant of heart development identified in the animal kingdom (1–3). Its vertebrate homolog, Nkx2–5, was subsequently identified in vertebrates. Since then, the identified components that make up the transcriptional network controlling heart formation have become increasingly complex. The factors that guide cardiac specification and differentiation in the fly appear to be highly conserved across species and now include a number of homeodomain, GATA, MADS-box, and T-box factors (3–6). Although much progress has been made in determining the roles of these factors during cardiac development, their roles in adult heart function remain to be elucidated.

The T-box transcription factor Tbx20 is a member of a family of ancient T-box genes related to the Tbx1 subfamily (7). Studies of Tbx20 in vertebrate models indicate a critical role in the induction and support of a core cardiac transcription factor network (8–11). For example, in Tbx20 knock-out mice, the expression of other cardiac transcription factors is downregulated or delayed, similar to observations in flies with mutations in the Tbx20-related neuromancer genes (nmr1&2; also known as H15 and midline, respectively) (12–14). Mutations in Tbx5, Nkx2–5, and Gata4 cause a range of congenital heart diseases (CHDs) including atrial-septal and conduction defects (15–19). Several findings suggest that Tbx20 is also likely to be involved in CHD. Tbx20 directly interacts with these cardiac transcription factors (10, 20, 21), but its role in adult heart function is not known because Tbx20 knock out mice are embryonic lethal and conditional heart-specific manipulations have not been done as of yet. Thus, the importance of Tbx20 interactions for adult heart structure and function in vivo needs to be further clarified.

Drosophila models are increasingly popular for studying a number of postnatal human diseases including cancer, diabetes, or neurodegenerative diseases (22, 23). Recently, Drosophila has also become a unique and well-suited genetic model for studying potential heart disease genes (6, 22, 24–26), including the cardio- genic NKX, GATA and Tbx transcription factors. For example, tinman/Nkx2–5 function in both flies and mice has been shown to be required not only for embryonic cardiogenesis (1, 2, 27, 28) but also for adult heart morphology and function (29, 30).

In this study, we use image-based cardiac contraction analysis and electrical pacing-induced cardiac stress tests (26, 31) to examine the role of nmr/Tbx20 in the adult Drosophila hearts. Using the TARGET system (32) to spatially and temporally manipulate gene expression in the adult fly heart, we find that this cardiac transcription factor is also required in the adult for maintaining heart function. In addition, we identify a genetic interaction between nmr and tinman and show that this interaction is required for cardiac morphology, performance, and heartbeat rhythmicity. These studies demonstrate that Drosophila has potential as a postnatal model system for exploring the genetics underlying heart disease. As a first step in evaluating this potential, human subjects with structural congenital heart disease, as well as heart muscle dysfunction were examined. In 96 human subjects with clinical evidence of dilated cardiomyopathy (DCM), as characterized by left ventricular dilation and systolic dysfunction, DNA analysis identified three different variants of Tbx20 in individuals with sporadic and familial DCM cases, suggesting Tbx20 gene may be involved in the development of cardiomyopathy. In addition, Tbx20 variants were identified in four children with atrial septal defect (ASD) with or without additional congenital heart defects. This underlines the potential utility of genetic screening in Drosophila as a discovery tool for candidates in human cardiovascular disease.

Results

Expression of nmr in Adult Cardiomyocytes. nmr genes are prominently expressed in the embryonic myocardium (12–14). To
movement of the hemolymph into and out of the heart (asterisk to the ostiae, which form openings in the heart tube controlling mRNA were both detected in the adult fly heart (see Fig. 4), indicating with our real time quantitative RT-PCR results in which (13), and Tinman-positive nuclei of the contractile myocardium (Fig. 1). Since whether tinman is expressed and required in the adult heart, we determined whether cardiac expression persists, we examined nmr expression in the adult heart. Double labeling with nmrH115-IacZ (13), and α-Actinin (33), a Z-line marker of myofibers, shows that nmr is expressed in the adult heart and co-labels with Dmef2 and Tinman-positive nuclei of the contractile myocardium (Fig. 1). The two smaller Dmef2-positive nuclei correspond to the ostiae cells (asterisks in B) in each hemisegment. (C) Double labeling for nmr-IacZ and muscle-specific transcription factor Dmef2 indicates co-localization of nmr and Dmef2 in all myocardial cells including ostia cells (arrows). (D) Double labeling of nmr-IacZ with Tinman cardiomyocyte nuclei. Notice that Tinman is absent in the ostia (arrows), (E) Loss of nmr1 or cardiac knock-down of nmr2 results in a dramatic increase in heart arrest/fibrillation rate (percentage of flies whose hearts failed when paced, see Materials and Methods) χ² analysis compared to wild type (w) and controls (nmrY16/+ and Df(2L)Exel6012); ∗, P < 0.01, **, P < 0.001, n = 50–100. (F) TARGET-mediated knock-down of nmr2 in the adult myocardium after eclosion results in increased arrest/fibrillation rate upon pacing stress (29 °C) compared to unduced controls (18 °C). See Table S1 for details of statistical analysis. (G–K) α-Actinin labeling of the adult heart (one segment), (G and H) Confocal sections through the outer longitudinal heart-associated muscle layer indicate no difference between nmr2 knockdown hearts and controls. (K) Sections through the inner cardiomyocytes show abnormal arrangement of the spiral myofibers (arrows) in nmr2 adult specific mutant hearts using either the 248-Gal4 (U) or tinCΔ4-Gal4 driver (K). measured cardiac performance in nmr mutants using an electrical pacing protocol, which stresses the heart and induces fibrillation or arrest, especially in aged or compromised hearts (31). Although we have previously used the term “heart failure” to describe this cardiac dysfunction (24, 31), this term does not imply the “pump failure” used to describe mammalian heart function but rather an alteration or cessation of normal cardiac pumping as a result of the pacing induced stress. First, we examined flies heterozygous for nmr1614 or a larger deficiency covering both nmr1 and nmr2, Df(2L)Exel6012. No significant increase in heart arrest/fibrillation following pacing was observed in these flies compared to wild type (Fig. 1E and supporting information (SI) Table S1). However, when both copies of nmr1 are removed (nmr1614 or when nmr2 function is reduced with heart-specific knockdown (tinCΔ4-Gal4 driving UAS-nmr2-RNAi) (13), there is a dramatic increase in the rate of pacing-induced heart arrest/fibrillation (Fig. 1E and Table S1), which further increases with age (Fig. S1 A and D). The down-regulation of nmr2 in the heart also results in a severely truncated lifespan (Fig. S1B), similar to what is observed for flies with a cardiac-specific loss of tinman expression (30). To distinguish between an embryonic versus an adult requirement for nmr we knocked down nmr2 in the heart only during the adult stage (using the TARGET system (32): tub-Gal80-tub-Gal4 or tub-Gal80-ts24BGal4 crossed to UAS-nmr2-RNAi, see Materials and Methods). In these flies, we also observed an increased rate in pacing-induced heart arrest/fibrillation compared to control flies (Fig. 1F, Fig. S1D, and Table S1). Although we cannot categorically rule out that some RNAi off-target effects may contribute to observed phenotype, these data strongly suggest that nmr function is indeed required for normal cardiac performance in adult flies. This conclusion is further substantiated by the effects on cardiac function and morphology as a result of genetic interactions between nmr deficiencies and tinman (see Fig. 3).

Arrhythmias and Abnormal Myofibrillar Structure in nmr/Tbx20 Mutants. We conducted image-based contraction analysis in nmr mutant flies. High-speed movies were taken from dissected and exposed hearts and contractions were displayed as M-modes (see Materials and Methods) showing the dynamics of heart wall movements over time (26). Heart specific knock-down of nmr2 causes a considerably irregular beating pattern and an overall increase in the heartbeat length compared to the controls (Fig. 2 A–C). These arrhythmias are due to the increased variability of diastolic (but not the systolic) interval lengths of the heart period (Fig. 2 A–F). The severity of arrhythmia is quantified by the standard deviation of the mean heart period for each fly (“arrhythmia index,” Fig. 2F; see ref. 26). Thus, nmr mutant hearts not only beat more slowly but are also arrhythmic. Depleting nmr levels in the adult heart possibly accelerates the cardiac aging process, since both the incidence of arrhythmias and length of the heart period normally increase with age (26).

Given the heart function defects observed for nmr mutants, we were also interested in examining these flies for cellular defects. During metamorphosis, the fly heart undergoes a remodeling process producing a regular spiral or transverse muscle fiber pattern within the tinman-expressing myocardial cells (see Fig. 1 A–D). In addition, a new set of longitudinal fibers that express Dmef2 but not tinman forms ventral to the heart (Fig. 1 G and C) (34, 35). We examined nmr2 knockdown hearts using α-Actinin antibodies, which localize to Z-lines of Drosophila muscle fibers (33). Using this marker we find that the regularity of the myofibrillar alignment and the spacing between Z-lines is disrupted within the Tinman-positive myocardium, where nmr2 expression is diminished (Fig. 1 I-K and Fig. S1E), but not in the ventral longitudinal muscle fibers that do not express tinman (Fig. 1 G and H and Fig. S1E). No noticeable change in cell death was observed; however, death in 1–2 cells per heart could possibly contribute to the observed myofibrillar disarray. Changes in cardiomyocyte size can also be estimated from measurements of diastolic diameter (DD) and systolic diameter.
Genetic Interaction Between nmr/Tbx20 with tinman/Nkx2–5 in Regulating Adult Heart Function. One of the enduring problems in the study of cardiac disease loci is the relative contribution of the genetic background or polygenic interactions that may ameliorate or aggravate the phenotypic expression of genetic abnormalities. This is the case for known cardiac disease loci, such as Nkx2–5, Tbx5, and Gata4, which cause a phenotype as heterozygotes (15–18, 36). Therefore, we examined whether the fly’s T-box and Nkx homologs interact genetically to control adult heart physiology. We combined the heterozygous nmr1&2 deficiency Df(2L)Exel6012 with heterozygotes for either tinman or Df(3L)DocA (which span all three Drosophila Tbx5/6 related genes) (14, 37). The pacing-induced heart arrest/fibrillation rate for Df(2L)Exel6012/+;tinman+ or Df(2L)Exel6012/+;Df(3L)DocA/+ is dramatically elevated compared to the single heterozygous controls (Fig. 3A and Table S1). This phenotype could be rescued by expressing wild-type nmr2 cDNA specifically in the heart (Fig. 3B and Table S1), indicating that the observed phenotype is indeed due to the genetic interaction of tinman or the doc genes with the nmr locus. An interaction of Df(2L)Exel6012/+ with tinman+/− was also observed with respect to the development of arrhythmias (Fig. 3C–E, Fig. S2 A–E), the level of cardiac expression of potential downstream effector genes (Fig. 4), and the alignment of myofilaments within the cardiac myocytes (Fig. 3 F–H). The genes whose expression is most affected as a result of tinman-nmr interactions are the nmr genes themselves, a potassium channel, an ATPase (SERCA), and dystrophin, all of which are likely to be involved in heart morphogenesis or function (Fig. 4). Indeed a dystrophin deficiency has previously been shown to cause myofibrillar misalignment in the fly heart (38). Whether the observed changes in gene regulation are direct, indirect, or the consequence of a compensatory mechanism remains to be examined. Nevertheless, our data suggest that cardiogenic transcription factors interact strongly in establishing and maintaining adult heart structure and function.

Cardiac performance exhibits a progressive deterioration with age, which is manifest by an increase in pacing-induced heart arrest/fibrillation rate and in arrhythmias (24, 26). Since tinman and nmr significantly influence adult heart function, we wondered whether reduced cardiac performance in old flies could be rescued by overexpression of these factors. When nmr2 is overexpressed beginning at the 5th week of adult life (Fig. S2F; see Materials and Methods), pacing-induced arrest/fibrillation rate is significantly reduced compared to non-overexpressors (Fig. S2G). Similar effects were observed with tinman cDNA overexpression in aging fly hearts (Fig. S2H). This suggests that maintaining high levels of tinman or nmr expression in older hearts halts or slows the age-dependent cardiac performance decline.

TBX20 Mutations in Humans with Dilated Cardiomyopathy. Since most, if not all, transcription factors with a demonstrated role in the fly heart also function in vertebrates, we wondered whether TBX20 sequence variants could be associated with cardiomyopathies in human patients. For this purpose, unrelated probands with clinically apparent dilated cardiomyopathy (DCM), who presented with echocardiographic evidence of left ventricular (LV) dilation, systolic dysfunction, and clinical evidence of heart failure and cardiac rhythm disturbance were enrolled for this study. All subjects and controls had blood drawn, DNA was isolated, and lymphoblastoid cell lines were developed. Patient (n = 96) and control (n = 392) DNA samples were screened for non-synonymous variants (mutations) in TBX20 and NKK2–5 genes by direct DNA sequencing (for primers see Table S2). This revealed three missense mutations (L196V, R334Q and W349R) in the TBX20 gene in Caucasians, two of which occurred with incomplete penetrance in familial cases (see Fig. 5 and Fig. S3). None of these mutations were identified in 392 ethnic-matched controls (nearly 800 chromosomes; see Table S1). Two of the variants had alterations in conserved residues in the (SD) of the heart tube (Fig. S1 F and G). We found a moderate decrease in DD and a mild increase in SD for nmr2 adult-specific knockdown mutants. These two changes result in a significant decrease in fractional shortening compared to controls, indicating a change in contractility in nmr knockdown hearts (Fig. S1H). The observed abnormalities in myofibrillar structure in response to decreased nmr expression likely contribute to these functional defects.
DNA-binding T-box of TBX20 (Fig. S3). Notably, variants in the familial cases of DCM demonstrated reduced penetrance, which is a common finding in inherited forms of cardiomyopathy. In Family 4, for instance, only half of the individuals with the R334Q variant had clinically apparent DCM at the time of the evaluation. All non-synonymous variants found in patients and ethnic-matched controls are listed in Tables S3 and S4. No potentially disease-causing mutations in NKX2-5 were identified in the examined patient cohort and no affected subject lacked the variant in family 4. These findings imply that candidate gene functions found in the Drosophila model are potentially relevant for human cardiac disease. However, definitive causality cannot be claimed at this point, in part because of the sporadic or incompletely penetrant nature of the cases identified. Although this is a common observation in human cardiovascular diseases such as cardiomyopathies and arrhythmia disorders (40, 41), it limits our ability to conclude a cause-and-effect relationship at this time. To demonstrate a causal relationship between gene mutation and disease, it will be important to conduct linkage analysis in large families with identified and functional studies of the identified variants. Nevertheless, our observations of a number of patients (but not controls) with non-synonymous variations in conserved regions of the coding region of TBX20 make this gene a promising candidate to potentially be involved in the human disease.

**Role of TBX20 in the Genetics of Congenital Heart Disease in Humans.** Kirk, et al. previously reported that, in addition to DCM, mutations in TBX20 are also associated with ASD and mitral valve disease (41). Consequently, we screened children with CHD including ASDs and ventricular septal defects (VSDs), as well as more complex CHD associated with these septal defects. In addition, mitral valve disease was evaluated. We screened 96 children with ASD and 96 with VSD associated disease. In four children, sporadic TBX20 variants were identified. A TBX20 variant in exon 4 resulting in a 597C>G alteration, which changes a histidine to an aspartic acid (H186D), was found in two unrelated children. In one child, an African American female, an atrioventricular canal with secundum and primum ASDs, as well as a cleft mitral valve with moderate mitral regurgitation was noted. The second child, a Hispanic male, had pentalogy of Fallot (tetralogy of Fallot with ASD). The other exon 4 mutation (601T>C; L197P) was identified in an African American female, an atrioventricular canal with secundum and primum ASDs, as well as an aortic coarctation, and a ventricular septal defect. In three children, sporadic TBX20 variants were identified. A TBX20 variant in exon 1 resulting in a 134A>G alteration, which changes a glutamic acid to a histidine (H45H), was found in an African American female with pentalogy of Fallot and in a white female with isolated ASD. None of these variants were present in an African American male with isolated ASD. The other exon 4 mutation (601T>C; L197P) was identified in an African American female, an atrioventricular canal with secundum and primum ASDs, as well as an aortic coarctation, and a ventricular septal defect. In three children, sporadic TBX20 variants were identified. 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In contrast, the transheterozygotes showed higher level of dys expression in the nmr2 knockdown flies is significantly reduced, compared to wild type (w1118) as well as to Df(nmr)/+; tin+/+, showed lower expression than heterozygous Df(nmr)/+ or tin+/+ alone, indicating that nmr1 itself, nmr2, Ca-P60A, and dys were positively regulated by Nmr and Tinman. The expression level of nmr2 is also significantly reduced in the progeny of a cross between the heart-specific driver tinCl4a-Gal4 and UAS-nmr2 RNAi line. nmr2 expression in the nmr2 knockdown flies is significantly reduced, compared to wild type (w1118) as well as to Df(nmr)/+ or tin+/+. (D) In contrast, the transheterozygotes showed higher level of elk expression indicating that elk may be negatively regulated by Nmr and Tinman. The normalized (to w1118) average of independently prepared samples plus standard error is shown. We used 20 fly hearts for each sample (RNA extraction). Two samples were prepared for each genotype, thus tin+/+ and Df(nmr)/+; tin+/+ lanes are the normalized averages of four samples and Df(nmr)/+ and tinCl4a-homo-nmr2 RNAi the normalized averages of two samples each. For statistical analysis we used non-repeated measures of ANOVA, then the differences were calculated between all combinations of each experimental group by Student-Newman-Keuls test (parametric) shown in the following triangles; ***, P < 0.01; *, P < 0.05.

**Fig. 4.** Quantitative real-time PCR on candidate genes in neuromancher, tinman mutants. Relative expression of nmr1, nmr2, Calcium ATPase at 60A (Ca-P60A; a SERCA homolog), dystrophin (dys), and eag-like K+ channel (elk) in 1-week-old adult hearts were standardized by rp49 expression. Wild-type control (w1118) was set as one (indicated by thicker horizontal line). (A-D) The transheterozygotes of nmr1&2 deficiency (Df[2L];Exel6012) and tinman null mutants (tin346 or tin346+), Df(nmr)/+;tin+/+, showed lower expression than heterozygous Df(nmr)/+ or tin+/+ alone, indicating that nmr1 itself, nmr2, Ca-P60A, and dys were positively regulated by Nmr and Tinman. The expression level of nmr2 is also significantly reduced in the progeny of a cross between the heart-specific driver tinCl4a-Gal4 and UAS-nmr2 RNAi line. nmr2 expression in the nmr2 knockdown flies is significantly reduced, compared to wild type (w1118) as well as to Df(nmr)/+ or tin+/+. (D) In contrast, the transheterozygotes showed higher level of elk expression indicating that elk may be negatively regulated by Nmr and Tinman. The normalized (to w1118) average of independently prepared samples plus standard error is shown. We used 20 fly hearts for each sample (RNA extraction). Two samples were prepared for each genotype, thus tin+/+ and Df(nmr)/+; tin+/+ lanes are the normalized averages of four samples and Df(nmr)/+ and tinCl4a-homo-nmr2 RNAi the normalized averages of two samples each. For statistical analysis we used non-repeated measures of ANOVA, then the differences were calculated between all combinations of each experimental group by Student-Newman-Keuls test (parametric) shown in the following triangles; ***, P < 0.01; *, P < 0.05.

Tbx20 has been widely studied in several model organisms, and the outcomes suggest a crucial developmental role of Tbx20 in cardiac specification and morphogenesis. Although a potential function of Tbx20 in the adult heart is likely (10), it has not been directly demonstrated. By using conditional and cardiac-specific manipulation, we found that nmr1/Tbx20 continues to have a regulatory role in the adult fly heart, and its depletion dramatically interferes with cardiac performance and disrupts contractile myofibrillar patterning. Since the cardiogenic gene Nkx2–5 is also required at later stages for proper cardiac function in vertebrates (29), as tinman and nmr are in flies (this study; ref. 30), it is likely that Tbx20 also has an essential and conserved role in maintaining function and structural integrity in adult vertebrate hearts. Interestingly, tinman and nmr show a strong double heterozygous interaction in our Drosophila heart model that leads to severe arrhythmias, increased stress sensitivity, and muscle structure abnormalities. In mice, Tbx20 and Nkx2–5 double heterozygotes also exhibit a synergistic interaction, which seem to be primarily confined to atrial defects (10). It is thus possible that the fly heart is sensitive to genetic interactions that are particularly relevant to the proper function of the mammalian atrium. These data imply that the fly heart can be used a discovery tool to identify synergistic genetic interaction that play a role in the mammalian heart in health and disease.

In an attempt to gage the predictive potential of the fly heart model in human heart function and dysfunction, we studied patients with DCM and performed genetic screening for Tbx20 mutations in these patients. While mutations in Nkx2–5 have been well-documented to be associated with congenital heart disease in humans (16, 42), heart defects potentially related to human Tbx20 mutations have only recently been identified (this study; ref. 39). Here, we demonstrate three distinct Tbx20 variants occurring in one sporadic and two familial cases of DCM. Clinically, the affected patients had classic features of DCM with associated rhythm disturbance, including, in some subjects, sudden death, heart failure, and arrhythmias. In the two families with Tbx20 mutations, all subjects hosting the variants were afflicted with DCM. Additionally, multiple affected individuals had arrhythmias with rapid ventricular rhythm, while in others, atrioventricular (AV) block with bradycardia was notable. AV block was also noted in the sporadic case. The variants seen in these patients, which were not found in controls, disrupt conserved regions of the gene, including the T-box. These findings raise the possibility of an association of these variants with the disease. A demonstration, however, that the identified Tbx20 mutations are causal in the development of the various observed heart disease phenotypes has to be achieved by functional studies. The identification of linkage to the Tbx20 locus in large multigenerational families with heart disease would also strongly support a causal association. As predicted by the “final common pathway” hypothesis, the cytoskeleton and contractile apparatus interface, and its relationship to channel function, is likely to be affected by the Tbx20 mutations (43). The finding of DCM, as well as the reduced penetrance that is seen in our...
study, is consistent with the findings reported by Kirk, et al. in human and murine studies of cellular phenotype in TBX20 mutants (39). Furthermore, studies of children with CHD identified two additional mutations in four individuals with various forms of ASD. These findings are also consistent with the report by Kirk, et al. (39). Our findings in the fly model, which led to our subsequent evaluation of humans with structural and myocardial disease, resulted in the suggestion of TBX20 as a promising candidate for a heart disease-causing gene that warrants further investigation and validation. These findings support the contention that the fly heart can serve as a promising and sensitive genetic predictor of gene candidates potentially involved in human heart disease.

Materials and Methods

Drosophila Stocks. tinC(1)TM3;ftzUacZ(2), tinC(9)CG4;TM3, Df(3R)twi(G14 (1), Df(3L)DoaC(37), nmrR1(4), nmrR1(13), Df(2L)Exel6012 (38), also indicated as Df(nmr), UAS-Gal4 system was used as in (45). Gal4 and UAS lines used: twi-Gal4 (twi>), ref. 44), 24B-Gal4 (24B>; ref. 45), twi-Gal4,24B-Gal4 (twi>24B>; ref. 46), twi-C4-Gal4 (47), DmeR-Gal4 (48), UAS-tn(49), UAS-nmr2RNAI, UAS-nmr1, and UAS-nmr2(13).


Electrical Pacing. The electrical pacing was conducted as previously described (24, 31). Briefly, 50–100 flies were paced with a square wave stimulator at 40 V and 6 Hz for 30 s and scored for heart arrest/fibrillation rate.

Image Analysis and M-mode Traces on Semintact Preparations. Semintact heart preparations for image analysis were as in Ocorr, et al. (26). M-modes from movies were generated using Simple PCI software and Matlab-based image analysis (26).

Quantitative Real-Time PCR. RNA was extracted from 20 1-week old hearts for each genotype and subjected to qRT-PCR as described in SI Text.

Patient Evaluation. Patients were evaluated by chest radiography, electrocardiography, echocardiography, and MRI. Left ventricular size and function were evaluated by M-mode and two-dimensional Doppler and color Doppler echocardiographic images. Further details are described in SI Text.

ACKNOWLEDGMENTS. We thank the Bloomington stock center and Developmental Studies Hybridoma Bank for sending fly-stocks and antibodies. L.Q. and J. L. were supported by a predoctoral fellowship from the American Heart Association. This work was funded by grants from National Heart, Lung, and Blood Institute of the National Institutes of Health to J.A.T. and R.B.