Transcription factor neuromancer/TBX20 is required for cardiac function in *Drosophila* with implications for human heart disease

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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 cardiogenic 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

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Fig. 1. Requirement for *nmr/Tbx20* in adult heart function. (A, and B) Double labeling of *nmr-lacZ* (*nmr1*, *H15*) with myofiber marker α -Actinin shows nuclear *nmr* expression in four Tinman-positive cardiomyocytes (arrows in B) and two Seven-up-positive ostia cells (asterisks in B) in each hemisegment. (C) Double labeling for *nmr-lacZ* and muscle-specific transcription factor Dmef2 indicates co-localization of *nmr* and Dmef2 in all myocardial cells including ostiae cells (arrows). (D) Double labeling of *nmr-lacZ* 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*). χ^2 analysis compared to wild type (*w*) and controls (*nmr1*^{614/+} and Df(2L)Exel612/+): *, $P < 0.01$, **, $P < 0.001$, $n = 50-100$. (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 uninduced 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. (I-K) Sections through the inner cardiomyocytes show abnormal arrangement of the spiral myofibers (arrows) in *nmr2* adult specific mutant hearts using either the *24B-Gal4* (J) or *tinC4-Gal4* driver (K).

determine whether cardiac expression persists, we examined *nmr* expression in the adult heart. Double labeling with *nmr*^{H15}-lacZ (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 *A-D*) (34). The two smaller Dmef2-positive nuclei correspond to the ostiae, which form openings in the heart tube controlling movement of the hemolymph into and out of the heart (asterisk in Fig. 1*B* and arrows in Fig. 1*C* and *D*). This observation is consistent with our real time quantitative RT-PCR results in which *nmr1&2* mRNA were both detected in the adult fly heart (see Fig. 4), indicating *nmr* genes are indeed expressed in adult heart.

Pacing-Induced Heart Arrest/Fibrillation in *nmr/Tbx20* Mutants. Since *tinman* is expressed and required in the adult heart, we wondered whether *nmr* also contributes to adult cardiac structure and function. *nmr1* null mutants (*nmr1*^{61d}) are viable and show a mild embryonic heart phenotype, whereas mesodermal *nmr2* knock-down is almost as severe a (cardiac) phenotype as *nmr1*&2 double mutants (12–14). To test the roles of these genes in adult flies, we

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 *nmr*^{I⁶¹⁴} or a larger deficiency covering both *nmr*1&2, 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 *nmr*1 are removed (*nmr*^{I⁶¹⁴}) or when *nmr*2 function is reduced with heart-specific knockdown (tinCΔ4-Gal4 driving UAS-*nmr*2-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 *nmr*2 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 *nmr*2 in the heart only during the adult stage (using the TARGET system (32): *tub*-Gal80-ts;tinCΔ4Gal4 or *tub*-Gal80-ts;24BGal4 crossed to UAS-*nmr*2-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. 2*F*; 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 hearts 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 *C* and *G*) (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. S1*E*), but not in the ventral longitudinal muscle fibers that do not express *tinman* (Fig. 1 *G* and *H* and Fig. S1*E*). No noticeable change in cell death is 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

Fig. 3. Abnormal cardiac function and morphology in *nmr*, *tin* transheterozygotes. (A) Heart arrest/fibrillation rates of transheterozygous combinations of *neuro-mancer/Tbx20* (*Df(2L)Exel6012*) with *tinman* (*tin³⁴⁶*) or *dorsocross/Tbx5/6* (*Df(3L)DocA*), and corresponding controls. Sample sizes are 50–100 flies/data point. χ^2 analysis [with respect to all controls; see Wessells, *et al.* (2004) in ref. 24 for details]; *, $P < 0.01$ (see Table S1 for individual values). (B) Increased heart arrest/fibrillation rate in transheterozygotes of *nmr* and *tinman* or *doc* is rescued by expressing *nmr2* cDNA in the heart. (χ^2 analysis: *, $P < 0.01$; **, $P < 0.001$, see Table S1 for individual values). (C–E) Representative M-mode traces from each genotype. Note the increased incidence of arrhythmia in *nmr*+/-; *tin*+/- flies (arrows in H indicate shortened heart beats, arrowheads in H show heart beats with longer diastolic interval). (F and G) α -Actinin labeling of adult heart. As compared to control (*nmr*+/-; F), *nmr*+/-; *tin*+/- (G) transheterozygous mutants show disruption in the regular myofibril alignment and Z-lines spacings. (H) Quantification of myofiber irregularities. Severity is determined by the average number of segments (5 in total as shown in Fig. 1A) with significant abnormal myofiber structure (as in G). Student *t* test: *, $P < 0.05$.

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 pentology of Fallot (tetralogy of Fallot with ASD). The other exon 4 mutation (601T>C; L197P) was identified in an African American female with pentology of Fallot and in a white female with isolated ASD. None of these variants were identified in 392 ethnic-matched controls (see Tables S3 and S4).

Discussion

Drosophila has been successfully used to study the regulatory genetic networks of inductive signals and transcription factors that determine cardiac specification and morphogenesis, but the genetic control of cardiac physiology is only beginning to be investigated. In this study, we used the *Drosophila* model to identify novel roles for the T-box transcription factor *nmr/Tbx20* and the homeobox gene *tinman* in establishing cardiac function and maintaining the structural integrity of the adult heart.

Fig. 4. Quantitative real-time PCR on candidate genes in *neuromancer*, *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 *tin^{EC40}/+*), *Df(nmr1)/+;tin/+*, showed lower expression than heterozygous *Df(nmr1)/+* 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 *tinCΔ4-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(nmr1)/+* or *tin/+*. (E) 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(nmr1)/+;tin/+* lanes are the normalized averages of four samples and *Df(nmr1)/+* and *tinCΔ4>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-Keules 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 *nmr/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 ab-

Fig. 5. Tbx20 mutations in humans with cardiomyopathy. (A) Patient cohort details. Four types of Tbx20 mutations are identified from patients diagnosed with dilated cardiomyopathy (see *SI Materials and Methods*: Clinical data, for details). (B and C) Pedigrees of the families carrying the TBX20 mutation. (B) In family 3, the 7-year old proband with the W349R mutation died of DCM. The father also died of acute idiopathic DCM (no sample available for genetic studies). (C) In family 4, the 16 year proband and his affected sister were detected with R334Q mutation. One of his unaffected brothers, his father, and some other extended family members are carriers of the mutation. In this family the grandfather and one of the uncles died suddenly of DCM.

normalities. 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 clinical 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. *tin*³⁴⁶/TM3-*ftzlacZ* (2), *tin*^{EC40}/TM3, *Df*(3R)*tin*^{GC14} (1), *Df*(3L)*DocA* (37), *nmr1*⁶¹⁴, *nmr1*²¹⁰ (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 (*twi24B*>; ref. 46), *tin*Δ4-Gal4 (47), *Dmef2*-Gal4 (48), UAS-*tin* (49), UAS-*nmr2*RNAi, UAS-*nmr1*, and UAS-*nmr2* (13).

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