Functional analysis of candidate genes in 2q13 deletion syndrome implicates FBLN7 and TMEM87B deficiency in congenital heart defects and FBLN7 in craniofacial malformations

Mark W. Russell1,∗, Maide O. Raeker1, Sarah B. Geisler1, Peedikayil E. Thomas2, Tracy A. Simmons1, John A. Bernat1, Thor Thorsson1 and Jeffrey W. Innis1,2,∗

1Departments of Pediatrics and 2Human Genetics, University of Michigan Ann Arbor, MI, USA

Recurrent 2q13 deletion syndrome is associated with incompletely penetrant severe cardiac defects and craniofacial anomalies. We used an atypical, overlapping 1.34 Mb 2q13 deletion in a patient with pathogenically similar congenital heart defects (CHD) to narrow the putative critical region for CHD to 474 kb containing six genes. To determine which of these genes is responsible for severe cardiac and craniofacial defects noted in the patients with the deletions, we used zebrafish morpholino knockdown to test the function of each orthologue during zebrafish development. Morpholino-antisense-mediated depletion of fibulin-7B, a zebrafish orthologue of fibulin-7 (FBLN7), resulted in cardiac hypoplasia, deficient craniofacial cartilage deposition and impaired branchial arch development. TMEM87B depletion likewise resulted in cardiac hypoplasia but with preserved branchial arch development. Depletion of both fibulin-7B and TMEM87B resulted in more severe defects of cardiac development, suggesting that their concurrent loss may enhance the risk of a severe cardiac defect. We postulate that heterozygous loss of FBLN7 and TMEM87B account for some of the clinical features, including cardiac defects and craniofacial abnormalities associated with 2q13 deletion syndrome.

INTRODUCTION

Congenital heart defects (CHD) occur in nearly 6 out of every 1000 live births making them the most common type of birth defect. Advances in surgical and medical care for patients with heart defects has dramatically improved survival for most types of CHD but has come at a cost to the health care system. Heart defects account for approximately one-third of admissions and approximately half of hospital costs ($1.4 billion) for birth defect-related conditions (http://www.hcup-us.ahrq.gov/reports/statbriefs/sb24.pdf, last accessed on 12 April 2014). Furthermore, there remains a very high morbidity and mortality for the most severe types of heart defects with a 5-year survival of ~70% for single-ventricle types of heart lesions (1). Identification of the genes responsible for heart defects may promote development of new treatment strategies to improve long-term outcomes in patients with the most severe abnormalities.

Advances in molecular diagnostics have allowed the characterization of small recurrent chromosomal deletions or duplications associated with severe types of human heart defects that can be used to localize genes for CHD (2–4). This approach led to the identification of CHD genes associated with the DiGeorge and Williams syndromes, TBX1 and ELN, respectively (5,6), and was recently used to identify novel genes associated with heterotaxy (7). Additional loci have been proposed based on the study of rare copy number variants (CNVs) which occur more commonly in patients with CHD than in the normal population (2,8). Characterization of atypical chromosomal microdeletions, which often lie within or overlap with recurrent deletions, has also promoted the elucidation of the roles of specific genes in congenital defects or cognitive impairment.

Recurrent 1.6–1.7 Mb deletions of chromosome 2q13 (roughly chr2:111442130–113065779; hg19) are associated with developmental delay, autism spectrum disorder, attention
deficit hyperactivity disorder, craniofacial malformation, CHD and other features. Two out of three subjects reported by Rudd et al. (9) had cardiac phenotypes including heterotaxy, total anomalous pulmonary venous return, ventricular septal defect (VSD), patent ductus arteriosus (PDA) and coarctation. At least three additional patients described in Cooper et al. (10) and Soemedi et al. (2) also had heart defects. Yu et al. described four additional unrelated deletion cases and one with duplication. In their review of the literature involving 15 microdeletion and 4 microduplication cases they showed that of patients with 2q13 genomic imbalance 93% have cognitive or developmental problems and 63% displayed craniofacial dysmorphisms. One of the four new deletion cases had a VSD. Based on comparison with the Database of Genomic Variants the microdeletion is overrepresented in affected patient populations compared with normal controls and the authors proposed that it is clinically relevant. Examination of the ISCA and DECIPHER databases disclosed 35 total reported cases of recurrent 2q13 deletion. Of the 29 with any phenotypic description, two had heart defects. However, phenotypic descriptions for patients in those databases are incomplete and cardiac evaluations may not have been performed in many cases. Perhaps a better measure of disease risk is from the Signature database (10) in which 2 of 12 subjects with a deletion of 2q13 had a significant cardiac defect. Although phenotypic descriptions are more standardized and consistent for that database, cardiac evaluations are not routinely performed so even that database may underestimate cardiac defect risk of a given variation. Therefore, based on the recorded four cardiac defects in 41 individuals with 2q13 deletions described in the three databases (ISCA, DECIPHER, and Signature Genomics), we would conservatively estimate that $\approx 10\%$ of patients with 2q13 deletions demonstrate congenital cardiac defects.

In this study, we describe two new patients with overlapping genomic deletions, one with the recurrent 2q13 deletion syndrome and one with an atypical deletion and similar, severe congenital cardiac defects. We use zebrafish gene knockdown to analyze the functional contribution of each critical region (CR) gene and identify FBLN7 and TMEM87B as candidate genes for vertebrate heart and craniofacial development.

RESULTS
Characterization of 2q13 deletions in patients with severe congenital cardiac defects

Two patients with severe congenital cardiac defects were identified here at the University of Michigan (UM) with overlapping deletions of chromosome 2q13 using Array-Comparative Genomic Hybridization (Array-CGH) (Fig. 1). Both deletions were confirmed by fluorescence in situ hybridization (FISH) and by quantitative PCR (see Materials and Methods; data not shown). Additional known pathogenic deletions or duplications were absent in both patients. Patient 1 was a newborn female with an unbalanced, complete atrioventricular septal defect with double outlet right ventricle, hypoplastic left ventricle, pulmonary atresia, several small secundum defects and a large PDA. In addition, Patient 1 also had right sided cleft lip and palate, poorly developed posterior ear helices with absent lobules and overfolded superior helices, single umbilical artery, hypertonicity and reduced flexibility at the elbows, wrists and knees. There was no known family history of congenital heart disease. Patient 2 is a male who was evaluated as 1 month old, who had pulmonary valve atresia with tetralogy of Fallot, hypoplastic main pulmonary artery and confluent branch pulmonary arteries filled by a large PDA. In addition, Patient 2 also had hypoplasias, only four metatarsals and distal elements on the right foot, the bilateral absence of the middle phalanges of the toes, and chylothorax. In common to both patients were pulmonary atresia and an abnormality of septation of the ventricular outflow tract, suggesting a shared pathogenetic mechanism with possibly a common genetic etiology. Previous studies have noted the presence of a recurring deletion, matching that in Patient 1, on chromosome 2q13 (Fig. 1) spanning $\approx 1.62$ Mb including 10 genes (2,9,11). The deletion in Patient 1 was maternally inherited; although phenotypically normal, the mother was lost to follow-up before an echocardiogram could be obtained. The atypical deletion in Patient 2 was not inherited from the mother; the father could not be examined molecularly. Available maternal and paternal family history did not disclose congenital heart disease.

The region of overlap or CR for these two deletions, contains six genes: fibulin-7 (FBLN7), anaphase protein C1 (ANAPC1), transmembrane protein 87B (TMEM87B), c-mer proto-oncogene tyrosine kinase (MERTK) and two zinc finger protein genes (ZC3H8 and ZC3H6), none of which have a known role in cardiac development.

TMEM87B antisense morpholino-mediated depletion leads to cardiac defects

To explore the genes potentially responsible for the severe cardiac defects noted in the patients, we used the developing zebrafish embryo model system to examine the effect of knockdown of these six genes in early cardiac development. In silico techniques were used to identify all zebrafish orthologues of MERTK, TMEM87B, FBLN7, ANAPC1, ZC3H6 and ZC3H8. Antisense morpholinos were designed to target the translation start site and an internal splice site of each gene. Antisense morpholino injection experiments targeting the translation start sites of each of the zebrafish orthologues of MERTK, ANAPC1, ZC3H6 and ZC3H8, individually, in a minimum of 50 embryos with each morpholino, did not result in a detectable cardiac or craniofacial phenotype (Table 1 and Supplementary Material, Table S1). To verify that the absence of a phenotype was not due to inadequate transcript suppression, each gene was also targeted using an antisense morpholino recognizing a conserved splice site. Downregulation of transcript expression or aberrant splicing was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) (Supplementary Material, Fig. S1 and Table S2). In all cases, cardiac and craniofacial development was similar to uninjected and control morpholino-injected embryos (Supplementary Material, Fig. S2).

Very little is known regarding TMEM87B. It has been demonstrated to physically interact with human papillomavirus type 18 E6 oncogene in yeast two hybrid studies (12). TMEM87Btranscripts have been identified in multiple adult and fetal tissues including the brain and the heart. We identified the orthologue of TMEM87B on zebrafish chromosome 13. Using in silico techniques, we determined that the predicted TMEM87B protein is highly homologous to the human TMEM87B protein at the carboxy terminus including a conserved seven-pass transmembrane domain. The amino terminus
is less well conserved. The putative human translation start site was identified in sequenced cDNAs from zebrafish expressed sequence tags in the Zebrafish Information Network (ZFIN) database (Fig. 2); this putative start site was targeted by an antisense morpholino (see below). Zebrafish TMEM87B was expressed at all-time points studied from 3 to 72 hpf by RT-PCR (data not shown). By RNA in situ hybridization, there was a dynamic pattern of localization during development; there was diffuse expression at 24 hpf, including the developing somites, with expression becoming increasingly restricted to the head and the branchial arches by 72 hpf (Fig. 2).

To determine the dependence of vertebrate development on TMEM87B, an antisense morpholino, targeting the putative translation start site was injected into 1–4 cell zebrafish embryos both alone and in combination with a p53 antisense

Figure 1. Array-CGH results of patients with CHD. Region of chromosome 2q13 showing the recurrent 1.62 Mb deletion (Patient 1; hg19, chr2:111442130–113065779) and the overlapping, novel 1.35 Mb deletion (Patient 2; hg19, chr2:112592087–113937615). For Patient 1, the nearest proximal and distal probes not affected by the deletion were 73 and 145 kb, respectively, distant from the boundaries of the deletion. For Patient 2, the nearest proximal and distal probes not affected by the deletion were 115 and 45 kb, respectively, distant from the boundaries of the deletion. Patient deletions (long red bars) are shown relative to the genes involved. Six genes are deleted (whole or part) in common among those with the recurrent deletion and the novel 1.34 Mb overlapping deletion in the critical region (CR) of 474 Kb.

Table 1. Screening of candidate genes within the 2q13 CR by antisense morpholino knockdown of the zebrafish orthologues in developing zebrafish embryos

<table>
<thead>
<tr>
<th>Genes</th>
<th>MO Amt</th>
<th>MO Target</th>
<th>Small head</th>
<th>Curved tail</th>
<th>Bradycardia</th>
<th>Heart hypo V</th>
<th>Dilated V</th>
<th>Cardiac unlooped</th>
<th>Looping inverted</th>
<th>Normal heart</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBLN7A</td>
<td>MO 0.2</td>
<td>Splice</td>
<td>1</td>
<td>30</td>
<td>3</td>
<td>21</td>
<td>8</td>
<td>9</td>
<td>22</td>
<td>19</td>
<td>55</td>
</tr>
<tr>
<td>FBLN7B</td>
<td>MO1 0.2</td>
<td>Start</td>
<td>5</td>
<td>7</td>
<td>3</td>
<td>8</td>
<td>20</td>
<td>22</td>
<td>3</td>
<td>19</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>MO2 0.2</td>
<td>Start</td>
<td>23</td>
<td>26</td>
<td>25</td>
<td>26</td>
<td>10</td>
<td>31</td>
<td>4</td>
<td>0</td>
<td>38</td>
</tr>
<tr>
<td>TMEM87B</td>
<td>MO 0.2</td>
<td>Splice</td>
<td>23</td>
<td>26</td>
<td>25</td>
<td>26</td>
<td>10</td>
<td>31</td>
<td>4</td>
<td>0</td>
<td>38</td>
</tr>
<tr>
<td>TMEM87B + p53</td>
<td>MO 0.2 + 0.27</td>
<td>Start</td>
<td>4</td>
<td>42</td>
<td>12</td>
<td>42</td>
<td>12</td>
<td>8</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MERTK</td>
<td>MO 0.4</td>
<td>Start</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>49</td>
<td>55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANAPC1</td>
<td>MO 0.4</td>
<td>Start</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>53</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>ZC3H6</td>
<td>MO 0.4</td>
<td>Start</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>49</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>ZC3H8</td>
<td>MO 0.4</td>
<td>Start</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>49</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>MO 0.4</td>
<td>NA1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>49</td>
<td>50</td>
</tr>
</tbody>
</table>

Note that few FBLN7B (MO2) and TMEM87B morphant embryos demonstrated normal cardiac structure and function. The FBLN7B developmental abnormalities were noted with two different morpholinos (MO1 and MO2) and the TMEM87B morphant phenotype was not significantly altered by p53 morpholino co-injection. MO amount refers to concentration of injected antisense morpholino in mM. Hypo V refers to hypoplastic ventricle. Total refers to number of injected embryos for each morpholino or combination. FBLN7A morpholino is MO2.
Figure 2. Zebrafish TMEM87B. (Top panel) Alignment of the proposed human and zebrafish (zf) TMEM87B amino acid sequences showing initiator methionine. The proposed amino acid sequence is derived from cDNA. Identical amino acid residues are hidden (.), gaps in the alignment are noted (−) and identical or conserved residues are enclosed in boxes. Alignment was performed using Clustal-W. (Bottom panel) Expression of TMEM87B in developing zebrafish embryos. RNA in situ hybridization demonstrates TMEM87B expression in 24 (A.1, A.2), 48 (B.1, B.2) and 72 (C.1, C.2) hpf zebrafish embryos. Sense strand RNA controls for the same time points are shown (A.3, B.3, C.3). (A) At 24 hpf, note the expression in the developing somites of the tail (A.1: arrowhead), hindbrain (A.2: dark arrowhead) and rhombomeres (A.2: white arrowhead). By 48 hpf, there is expression noted in the branchial arches (B.2: arrowhead) which becomes more prominent as the arches continue to develop by 72 hpf (C.1, C.2: arrowheads). Scale bars are 200 μm.
morpholino to inhibit non-specific p53-mediated, morpholino-induced apoptosis (13). Depletion of TMEM87B was associated with defects of the heart (Fig. 3) which persisted despite co-injection of the p53 antisense morpholino. Cardiac looping was noted to be normal in most cases with an absence of looping in a small subset. There was clear division into atrial and ventricular domains by 48 hpf but there was an impaired restriction of the atrioventricular canal with increased reversal of blood flow and resulting cardiac dilatation in the more mildly affected embryos (data not shown). However, marked cardiac hypoplasia was the most common cardiac phenotype and this became more pronounced during development leading to a stretched appearance to the heart with significant pericardial edema by 72 hpf in 84% of the embryos injected with 0.2 mM concentration of the morpholino (Table 1 and Fig. 3).

Due to uncertainty regarding the translation initiation site of FBLN7A, antisense morpholinos were designed to target two internal splice sites of the FBLN7A transcript. Only one (MO2) of the two morpholinos resulted in aberrant splicing (Supplementary Material, Fig. S3). A 0.1 mM morpholino injection did not result in detectable phenotypic abnormalities. However, at a dose of 0.4 mM, mild abnormalities in heart development and craniofacial structure were noted in a subset of embryos. In addition, curling of the tail occurred in more than half of the injected embryos (Table 2).

FBLN7B depletion likewise affected both cardiac and craniofacial development but with a more penetrant phenotype. FBLN7B expression was targeted using start site and splice site morpholinos (MO1 and MO2, respectively) injected individually. FBLN7B MO2 injection resulted in diminished expression of the wild-type FBLN7 transcript and two aberrantly spliced products (one lacking exon 2 and one lacking exons 2 and 3 (Supplementary Material, Fig. S4). Injection of either morpholino resulted in developmental abnormalities that were confined to craniofacial structures, the branchial arches and their derivatives, and the outflow region of the heart (Fig. 5). By 4 days postfertilization, most morphant embryos (nearly 100% for MO2 and over 60% for MO1) developed pericardial edema and had structural abnormalities that included the absence of cardiac looping and cardiac hypoplasia. The observed FBLN7B morphant phenotype was very similar to that previously noted in zebrafish upon exposure to PCB126, which disrupts branchial arch development (15). In both instances, the embryos had hypoplasia of the outflow tract of the heart, leading to a ‘stretched’ heart appearance by 96 hpf and underdevelopment of the lower jaw.

Since depletion of FBLN7B and TMEM87B, individually, resulted in significant cardiovascular defects, depletion of the genes in combination was performed to determine if there was

Fibulin-7 antisense morpholino-mediated depletion leads to cardiac and craniofacial defects

In silico analysis revealed 3 FBLN7 orthologues in zebrafish, located on chromosomes 13 (FBLN7A), 18 (FBLN7B) and 25 (FBLN7C) (Fig. 4). The zebrafish underwent a genome duplication event after their divergence from other vertebrates so having two orthologues of a single human gene is relatively common (14). In the case of FBLN7, it appears, based on sequence similarity that FBLN7B and FBLN7C were formed during the initial duplication and then FBLN7A may have arisen from FBLN7B after a second gene duplication event. Expression of FBLN7A and FBLN7B was examined in developing zebrafish embryos. Both were found to be expressed in the craniofacial region and branchial arches by 72 hpf (Fig. 4) and therefore were determined to be potential candidates for mediating cardiac and craniofacial development.
Figure 4. Zebrafish fibulin-7. (Top panel) Alignment of the proposed human and zebrafish (zf) FBLN7 amino acid sequences. The proposed amino acid sequence is derived from cDNA sequences and in silico analysis. Three zebrafish FBLN7 genes were identified, FBLN7A (chromosome 13), FBLN7B (chromosome 18), and FBLN7C (chromosome 25). Alignment was performed using the Clustal-W algorithm. Amino acid residues identical to the human FBLN7 are hidden (.) and residues identical to or conserved with the human are boxed. Phylogenetic analysis demonstrates that FBLN7A is most closely related to the human FBLN7 followed by FBLN7B. (Bottom panel) Similar expression patterns noted for FBLN7A and FBLN7B. Both are restricted in expression to the branchial arches including the notochord and the mandibular arches (arrows). Sense strand FBLN7A and FBLN7B RNA controls showed no staining (not displayed). Scale bars are 200 μm.
an additive effect of FBLN7A, FBLN7B and TMEM87B depletion on cardiac development. Injection of FBLN7A and FBLN7B morpholinos, each at 0.1 mM concentration, did not result in significantly more severe or more frequent cardiac abnormalities than were noted with FBLN7B depletion alone. However, co-injection of FBLN7A, FBLN7B and TMEM87B yielded a higher incidence of cardiac defects than injection of FBLN7A + FBLN7B or TMEM87B (Table 2). The frequency of observed cardiac defects in the embryos depleted of FBLN7B and TMEM87B suggests that combined haploinsufficiency of both genes, as occurs in patients with del 2q13, may increase the risk of congenital heart disease beyond what would be noted with the haploinsufficiency of either gene individually.

### Depletion of FBLN7B or TMEM87B does not affect the number of aortic arch vessels

Flil-EGFP zebrafish (16), which express enhanced green fluorescent protein (EGFP) under the direction of the flil promoter in Table 2.

<table>
<thead>
<tr>
<th>Genes</th>
<th>MO Amt</th>
<th>MO target</th>
<th>Craniofacial</th>
<th>Curved tail</th>
<th>Edema</th>
<th>Heart hypo V</th>
<th>Dilated V</th>
<th>Cardiac unlooped</th>
<th>Looping inverted</th>
<th>Normal heart</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBLN7A</td>
<td>MO 0.1</td>
<td>Splice 1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>49</td>
<td>50</td>
</tr>
<tr>
<td>FBLN7A</td>
<td>MO 0.4</td>
<td>Splice 1</td>
<td>10</td>
<td>29</td>
<td>5</td>
<td>1</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>39</td>
<td>50</td>
</tr>
<tr>
<td>FBLN7B</td>
<td>MO 0.1</td>
<td>Splice 29</td>
<td>2</td>
<td>9</td>
<td>9</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>37</td>
<td>50</td>
</tr>
<tr>
<td>FBLN7A FBLN7B</td>
<td>MO 0.1 ea</td>
<td>Splice 36</td>
<td>2</td>
<td>9</td>
<td>5</td>
<td>11</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>34</td>
<td>50</td>
</tr>
<tr>
<td>TMEM87B</td>
<td>MO 0.1</td>
<td>Start 0</td>
<td>0</td>
<td>0</td>
<td>12</td>
<td>8</td>
<td>3</td>
<td>5</td>
<td>0</td>
<td>35</td>
<td>50</td>
</tr>
<tr>
<td>FBLN7A FBLN7B TMEM87B</td>
<td>MO 0.1 ea</td>
<td>Mix 42</td>
<td>1</td>
<td>35</td>
<td>21</td>
<td>9</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>50</td>
</tr>
</tbody>
</table>

Cardiac defects occurred in fewer than 30% of embryos injected with morpholinos individually targeting FBLN7A, FBLN7B (0.1 mM dose) or TMEM87B (0.1 mM dose). However, concurrent targeting of all three genes (0.1 mM dosing) resulted in cardiac abnormalities in 80% of the injected embryos. Craniofacial abnormalities were mild and were specific to those embryos in which FBLN7A and/or FBLN7B were targeted using antisense morpholino-mediated depletion.

**Figure 5.** Fibulin-7B morphant. (A, A’) Control embryo at 96 hpf. (B, B’) fibulin-7B morphant embryo at 96 hpf [0.4 mM splice-site morpholino (MO2)]. Note that fibulin-7B knockdown (B, B’) results in cardiac hypoplasia and pericardial edema (white arrowheads) and underdevelopment of the lower jaw (black arrowheads). (C) At 72 hpf, MO2 morphant embryos (C.2–C.4) demonstrated a high incidence (over 98% of 0.4 mM MO2-injected embryos) of cardiac defects including reversed looping (C.2) or hypoplastic ‘stretched’ ventricle (C.3, C.4) compared with control (C.1).
neural crest-derived vascular smooth muscle cells, were used to assess the effects of FBLN7B or TMEM87B depletion on patterning of the aortic arch vessels and the development of the brachial arches (Fig. 6). Depletion of either FBLN7B or TMEM87B resulted in normal patterning of the aortic arches although the arches were underdeveloped in those embryos with marked cardiac hypoplasia. As growth and development of the vessels will be dependent on flow (17) and blood flow is severely compromised in the embryos with cardiac hypoplasia, it is not surprising that those vessels are small in caliber. While patterning of the arch vessels was maintained, the branchial arches in the FBLN7B morphant embryos were not well developed in marked contrast to the control morphant and the TMEM87B morphant embryos.

Fibulin-7B depletion leads to reduced cartilage deposition in the pharyngeal arches and impaired branchial arch development

Alcian blue was used to stain the cartilaginous structures of control and FBLN7B morphant (0.2 mm) embryos. Antisense morpholino-mediated depletion of FBLN7B resulted in reduced cartilage deposition in the pharyngeal region. While patterning of cartilage deposition was intact, there was a marked reduction in staining with almost no detectable staining of the ceratobranchial structures by 120 hpf in the morphant embryos (Fig. 7). Not surprisingly, the reduction in cartilage deposition was most evident in the embryos with the most severe cardiac manifestations (Fig. 7A, A'). That there was not a global delay in development, as noted by similar embryo size and tail development, suggests that the observed abnormalities of craniofacial development are specific.

Assessment of patient FBLN7 CNVs and FBLN7/TMEM87B DNA sequences of the non-deleted alleles

Given the zebrafish knockdown results, it is conceivable that penetrance for CHDs in patients with heterozygous 2q13 deletions is influenced by mutations or CNVs residing within the non-deleted FBLN7 or TMEM87B genes. To explore this hypothesis we evaluated both patients with quantitative PCR directed to a known FBLN7 CNV loss encompassing exon 1. 

Figure 6. Effects of TMEM87B or FBLN7B depletion on branchial arch vessel patterning. (A) A.1 and A.2 show two views of a control embryo at 96 hpf demonstrating intact development of aortic arches 3, 4, 5 and 6 (arrowheads). (B) B.1 and B.2 show two views of an embryo subjected to TMEM87B depletion yet showing preserved patterning of the aortic arches (arrowheads in B.2). However, the arch vessels are markedly hypoplastic as might be anticipated due to cardiac insufficiency (note the marked hypoplasia and stretched appearance of the heart). (C) C.1 and C.2 show two views of an embryo subjected to FBLN7B depletion yet showing preserved patterning of the aortic arches (arrowheads in C.2).
as well as to **FBLN7** downstream exons. Both tests revealed only heterozygous gene dosage loss in both patients (data not shown). We then proceeded to sequence exon and exon/intron boundaries of the non-deleted **FBLN7** and **TMEM87B** genes in both patients. No pathogenic sequence variants were found. Therefore, the occurrence of CHDs in these patients is not associated with a detectable loss of function mutation in either gene on the other chromosome.

**DISCUSSION**

In this study, characterization of a patient with a novel deletion of chromosome 2q13 restricted the candidate interval for the gene(s) potentially responsible for at least the cardiac defects in the patients with 2q13 deletion syndrome to six genes, none of which had been previously demonstrated to have a role in heart development. Sequential depletion of each gene in developing zebrafish embryos suggested that haploinsufficiency of **FBLN7** and/or **TMEM87B** may be responsible for the observed cardiac phenotypes, an assertion supported by identification of tetralogy of Fallot in another patient lacking only four genes in the region, including **FBLN7** and **TMEM87B** (2). The results are instructive in identifying two candidate genes for CHD in humans and support a potentially important role for the extracellular matrix and cell–extracellular environment interactions in cardiac development. Loss of **FBLN7** also appears to affect craniofacial development, consistent with its expression in the branchial arches.

**The extracellular matrix (ECM) is critical in heart development**

A significant body of work has demonstrated the importance of the ECM in the endothelial–mesenchymal transition that shapes the atrioventricular canal or inlet portion of the heart (18). Less attention has been paid to its role in the patterning of the conotruncus or outlet portion of the heart. It has long been recognized that neural crest cell migration from the dorsal neural tube, through the branchial arches to the arterial pole of the heart is critical for the normal patterning of the asymmetric aortic arches, for the division of the aorta and pulmonary artery and for the septation of the ventricles (19). Ablation of the neural crest or interference with the migration process results in marked abnormalities of conotruncal development (20). Migration depends on the interaction of integrins on the cell surface with components of the surrounding extracellular matrix (21). Other elements of the ECM which have important roles in the migration process include fibronectin, versican, chondroitin sulfate and heparin sulfate (22). Versican is a secreted chondroitin sulfate proteoglycan that is cleaved by metalloproteases including ADAMTS-1 and -9, an essential step in conus and truncal development (23–25). Thus, disruption of the ECM components can interfere with heart development.

**Fibulins are an important part of the ECM**

Fibulins are ECM glycoproteins that have important roles in elastic fiber assembly and tissue remodeling (26). They interact with other matrix components including elastic fibers and the...
Fibulin-7

Very little is known about fibulin-7, a relatively recently described member of the fibulin gene family. It was initially cloned and characterized from tooth germ mRNA. It is a glycoprotein that is proposed to interact with cells via integrin-and heparan sulfate-binding and has been demonstrated to bind to heparin, fibronectin, dentin, sialophosphoprotein and fibulin-1 (31). Given its role in the differentiation of odontoblasts (31), it has been proposed to contribute to the craniofacial abnormalities (including widely spaced teeth (9), micrognathia [ISCA database] and cleft palate [this study, Patient 1]) noted in several patients with deletions or duplications of this region. Our data on reduced cartilage deposition in the branchial arches would support a role for fibulin-7 in craniofacial development.

Yet fibulin-7’s potential role in the cardiac manifestations of 2q13 del has not been evaluated. Its ability to bind to fibulin-1 (31) in vitro suggests an important functional interaction between the two that may contribute to its role in cardiac morphogenesis. Given the noted abnormality in cardiac cushion development (both conotruncal and atrioventricular) in the study patients, it is noteworthy that fibulins have a central role in epithelial–mesenchymal transformation, which is required for cardiac development. Loss of function of Fibulin-1 in mice is associated with failure of neural crest-derived cardiac development leading to double outlet right ventricle, overriding aorta, atrial septal defects and VSDs (24). Dependence on the phenotypic expression on the genetic background of the knockout strain suggests that there are important functional FBLN1 interactions that may either amplify or compensate for its loss during cardiac development.

Potential role of TMEM87B in heart development

As noted above, even less is known about TMEM87B. It appears to be expressed in a broad range of tissues, including the heart and brain, and has been detected in developing embryos. It has a conserved transmembrane motif that suggests that it is inserted into and spans the cell membrane, potentially allowing it to interact with the ECM or soluble extracellular signals to effect changes in cardiac myocyte differentiation, migration or organization. While it may not interact with the fibulins or any other components of the ECM, our results, combined with its structural features, would suggest that it mediates interactions between the cell and its environment in a way that affects development.

Based on the current study, we cannot exclude the possibility that the cardiac, craniofacial and cognitive defects noted in patients with 2q13 del are due to contiguous gene losses involving not only FBLN7B and TMEM87B, but also other genes within or in close proximity to the CR defined here. Examination of genes in the composite deletion interval (chr2:111442130-113937615; hg19) defined in our paper by the recurrent and atypical deletion, disclosed no known gene involved in cardiac malformation; the same conclusion was derived even after extension by another 500 kb on each side. Further evaluation of composite deletion interval genes for expression in the heart through the human GNF Expression Atlas 2 using U133A and GNF1H chips (UCSC Genome Browser) and at E14.5 in the EMouse Atlas (http://www.emouseatlas.org/emage/) disclosed several with some heart expression whose role is unknown (Supplementary Material, Table S3). Whether expression of any of these genes is affected with the recurrent or atypical deletions and subsequently has additional effects on cardiac or craniofacial morphogenesis or function is unknown at this time. However, our work in the zebrafish model suggests that both fibulin-7 and TMEM87B may have novel and important roles during development and that their combined loss may increase the likelihood of congenital cardiac defects. The recent identification of a patient with tetralogy of Fallot (a conotruncal defect) and a smaller deletion, encompassing only MERTK, TMEM87B, FBLN7 and ZC3H8 (2), further supports this assertion. In addition, the contribution of loss of function mutations in single genes within this interval to the occurrence of any of the phenotypes of 2q13 genomic imbalance is unknown. It is conceivable that the increased risk for clinical problems occurs because of the combined loss of TMEM87B and FBLN7, and perhaps others, and may not be observed with only single gene losses.

In summary, the chromosome 2q13 deletion syndrome is associated with an increased risk of congenital cardiac defects. Using functional analysis in developing zebrafish, we have identified two genes within the region, TMEM87B and FBLN7 that, individually or in combination, have important roles in human cardiac and craniofacial development.

MATERIALS AND METHODS

Patient samples

Clinical genetic evaluation (JWI) and testing in the Michigan Medical Genetics Laboratories at the University of Michigan (UM) demonstrated heterozygous 2q13 deletions by Array-CGH. Deletions were confirmed by FISH in the UM Cytogenetics Human Molecular Genetics, 2014, Vol. 23, No. 16 4281
laboratory in Patient 1 with BAC probe RP11-55409 and in Patient 2 with BAC probe RP11-67L14. Quantitative PCR was also used as described below to demonstrate the deletions.

**Array-CGH**

A custom-designed EMArray Cytos6000 Array-CGH platform (37) a sex-mismatched, pooled normal DNA control comparison sample, and routine Michigan Medical Genetics Laboratories protocols at UM were used in the identification of the 2q13 chromosomal deletions as described (38). The array data were imported into, analyzed and plotted by the Agilent CGH Analytics version 3.5 or DNA Analytics version 4.0.81 software (Agilent Technologies). For reporting of copy number changes, nucleotides are numbered according to the UCSC hg19 assembly.

**Quantitative PCR**

We performed Q-PCR for a FBLN7 CNV overlapping exon 1 (DGV 74219; coordinate hg19, chr2:112896227) using an Applied Biosystems Pre-Designed TaqMan Copy Number Assay available from Life Technologies (Probe # Hs00576266_cn) and for a region between FBLN7 exons 4 and 5 (coordinate hg19, chr2:112934974; Probe # Hs05819029_cn). Genomic DNA (gDNA) samples were purified using a Qiagen M-48 Bio-Robot. The patient, control and calibrator samples were diluted to 10 ng/μl (+/-1.0 ng/μl) with DNase/RNase free water. The real-time PCR assays consisted of a control water sample, a control sample (pooled gDNA of 10 male patients with normal Array-CGH results), a control sample (pooled gDNA of 10 female patients with normal Array-CGH results), and the gDNA of each human research subject being tested. Each patient, control, calibrator and water sample was set up in a PCR plate in four replicates, and each replicate consisted of 6 μl of DNase/RNase free water, 10 μl of Applied Biosystems 2× Genotyping master Mix, 1 μl of Applied Biosystems TaqMan Probe (Hs05819029_cn or Hs00576266_cn), 1 μl of Applied Biosystems TaqMan Reference assay, and 2 μl of DNA or water (total volume for each replicate was 20 μl). The reference assay using RNAseP was performed in duplicate with the copy number assay in real-time PCR to detect and measure the copy number. The reaction plate was amplified using sequence specific primers and the TaqMan probe on an Applied Biosystems Step One Plus Real-Time PCR System. For the Copy Number assay, FAM was used as the Reporter and NFQ-B as the Quencher. For the RNAseP assay, VIC was used as the Reporter and TAMRA as the Quencher. All PCR reactions were carried out using Accuprime pfx (Life Technologies) and BioRad T100 Thermocycler. Primer sequences are in Supplementary Material, Table S1. Controls were No RT sample (for RT) and No template (for PCR). Two microliters of each product were separated by electrophoresis on a 2% agarose gel, and visualized on GelDoc XR+ (BioRad). Volumetric analysis was performed using Image lab software (BioRad). To examine for splicing defects, PCR product bands were excised from the gel, purified by Gel extraction kit (Qiagen), ligated into pCR®-Blunt Vector using ZeroBlunt PCR cloning kit (Life Technologies, USA), and transformed into ME DH5alpha. DNA was prepared from selected colonies and sequenced at the University of Michigan Sequencing Facility using Applied Biosystems DNA Sequencers (Model 3730 XL).

**Zebrafish maintenance and breeding**

Wild-type adult, cmlc2-EGFP (39), and fli1-EGFP (16) transgenic zebrafish were maintained as previously described (40). Morpholino oligonucleotide microinjection

Morpholino (MO) antisense oligonucleotides targeting splice sites and/or translation start sites of the orthologous zebrafish FBLN7A, FBLN7B, TMEM87B, MERTK, ANAPC1A, ANAPC1B, ZC3H6, and ZC3H8 genes were designed by Gene Tools, LLC (Philomath, OR) (see Supplementary Material, Table S1 for sequences targeted and MO sequences for each gene). Antisense morpholinos were injected at 0.1 ng (0.15 ng/nl), 0.2 ng (0.4 ng/nl) or 0.4 ng (0.2 ng/nl) into the 1–4 cell wild-type or cmlc2-EGFP or fli1-EGFP embryos. A standard control (Gene Tools, LLC) MO (5′-CTCTTTACCTCAGTTCAATTATA-3′) was used at 0.4 mM concentration for control injections. A Leica MZ16F dissecting microscope equipped with a DFC340 FX camera was used to visualize the embryos and record the observed phenotypes. To confirm that the phenotypes observed with FBLN7B and with TMEM87B deletion were specific, morpholino injections were repeated in the presence of a p53 antisense morpholino (13) which inhibits non-specific morpholino-mediated apoptosis.

**Confirmation of splice-site morpholino effectiveness using RT-PCR**

Total RNA was isolated from embryos at 72 hpf using Qiagen RNeasy Mini Kit (Life Technologies). Five hundred nanograms of RNA was used to transcribe cDNA using iScript cDNA synthesis kit (BioRad Laboratories, Hercules, CA). Primers were designed to amplify each target via PCR along with housekeeping gene ElfA as a control. All PCR reactions were carried out using Accuprime pfx (Life Technologies) and BioRad T100 Thermocycler. Primer sequences are in Supplementary Material, Table S2. Controls were No RT sample (for RT) and No template (for PCR). Two microliters of each product were separated by electrophoresis on a 2% agarose gel, and visualized on GelDoc XR+ (BioRad). Volumetric analysis was performed using Image lab software (BioRad). To examine for splicing defects, PCR product bands were excised from the gel, purified by Gel extraction kit (Qiagen), ligated into pCR®-Blunt Vector using ZeroBlunt PCR cloning kit (Life Technologies, USA), and transformed into ME DH5alpha. DNA was prepared from selected colonies and sequenced at the University of Michigan Sequencing Facility using Applied Biosystems DNA Sequencers (Model 3730 XL).

**RNA in situ hybridization**

Digoxigenin-labeled antisense RNA probes were synthesized from cDNA clones of zebrafish FBLN7A, FBLN7B and TMEM87B by *in vitro* transcription using T3 and T7 poly-merases and DIG RNA labeling kit (Boehringer-Mannheim). Embryos were fixed in 4% paraformaldehyde (PFA) for 5 h at room temperature and subjected to *in situ* hybridization with the above RNA probes. Stained embryos were visualized under an Olympus BX-51 light microscope.

**Alcian blue staining**

Embryos were fixed at 120 hpf with 4% PFA overnight at 4°C and washed with phosphate buffered saline and then dehydrated with 50% ethanol. Dehydrated embryos were stained with 0.02% Alcian blue staining.
sequences were aligned to the reference genome (hg19). Primer selection was designed using Primer3 to amplify all exons and exon/intron boundaries of each human gene (hg19). Primer selection was adjusted to avoid known single nucleotide polymorphisms (SNPs) (dbSNP135) to minimize the possibility of allele dropout. Where necessary, PCR conditions were adjusted to generate single band PCR products, which were sequenced bidirectionally. Where necessary, PCR conditions were adjusted to generate single band PCR products, which were sequenced bidirectionally. Sequence chromatograms were visually examined and sequences were aligned to the reference genome (hg19).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

**ACKNOWLEDGEMENTS**

The authors would like to thank the families for their participation in this research. We thank Katie Rudd for helpful discussions.

Conflict of Interest Statement. None declared.

**FUNDING**

This work was supported by American Heart Association grant #13GRNT15810006 (M.W.R. and J.W.I.), Braylon’s Gift of Hope Fund (M.W.R.), the Aaron Stern Professorship in Pediatric Cardiology (M.W.R.) and the Morton S. and Henrietta K. Sellner Hope Fund (M.W.R.), the Aaron Stern Professorship in Pediatric Cardiology (M.W.R. and J.W.I.), Braylon’s Gift of Hope Fund (M.W.R.), and the Morton S. and Henrietta K. Sellner Hope Fund (M.W.R.), the Aaron Stern Professorship in Pediatric Cardiology (M.W.R.), and the Morton S. and Henrietta K. Sellner Hope Fund (M.W.R.).

**REFERENCES**


