Functional modeling in zebrafish demonstrates that the atrial-fibrillation-associated gene GREM2 regulates cardiac laterality, cardiomyocyte differentiation and atrial rhythm

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SUMMARY
Atrial fibrillation (AF) is the most common cardiac arrhythmia and carries a significant risk of stroke and heart failure. The molecular etiologies of AF are poorly understood, leaving patients with limited therapeutic options. AF has been recognized as an inherited disease in almost 30% of patient cases. However, few genetic loci have been identified and the mechanisms linking genetic variants to AF susceptibility remain unclear. By sequencing 193 probands with lone AF, we identified a Q76E variant within the coding sequence of the bone morphogenetic protein (BMP) antagonist gremlin-2 (GREM2) that increases its inhibitory activity. Functional modeling in zebrafish revealed that, through regulation of BMP signaling, GREM2 is required for cardiac laterality and atrial differentiation during embryonic development. GREM2 overactivity results in slower cardiac contraction rates in zebrafish, and induction of previously identified AF candidate genes encoding connexin-40, sarcolipin and atrial natriuretic peptide in differentiated mouse embryonic stem cells. By live heart imaging in zebrafish overexpressing wild-type or variant GREM2, we found abnormal contraction velocity specifically in atrial cardiomyocytes. These results implicate, for the first time, regulators of BMP signaling in human AF, providing mechanistic insights into the pathogenesis of the disease and identifying potential new therapeutic targets.

INTRODUCTION
Atrial fibrillation (AF) carries a significant risk of stroke and heart failure and is associated with substantial morbidity and mortality (Feinberg et al., 1995; Stewart et al., 2002). Up to 30% of patients with AF have a family history of the condition, suggesting that the disease has a broad genetic basis (Darbar, 2008; Lubitz et al., 2010; Miyasaka et al., 2006). Cases of ‘lone’ AF, defined by the presence of sustained arrhythmia in the absence of structural heart disease or other identifiable causes in patients younger than 65 years of age, further underscore the contribution of genetic variation to the development of AF (Parvez and Darbar, 2010). Recent studies have identified both common and rare genetic variants contributing to AF susceptibility. Positional cloning and candidate gene approaches have implicated mutations in genes encoding ion channels, gap junctions and signaling molecules in isolated cases and small kindreds (Abraham et al., 2010; Gollob et al., 2006; Hodgson-Zingman et al., 2012). Genome-wide association studies (GWAS) have also recognized AF susceptibility loci (Ellinor et al., 2012) on chromosomes 4q25 near PITX2 (Gudbjartsson et al., 2007; Ritchie et al., 2012), 1q21 in KCNN3 (Ellinor et al., 2010) and 16q22 in ZFHX3 (Gudbjartsson et al., 2009). Even so, most cases of lone AF remain of unknown etiology, are poorly penetrant, and segregate in isolated cases or small families, rendering the identification of causative genes and the design of new therapeutic strategies particularly challenging (Darbar et al., 2012). Moreover, there is a paucity of functional modeling of known variants that could be used to draw putative molecular and cellular pathways contributing to AF symptoms.

In many cases of AF, electrical signals initiate in ectopic atrial locations, often close to the muscle sleeves of the pulmonary veins (Haissaguerre et al., 1998; Levin et al., 2009). Pulmonary veins and pulmonary myocardium develop from pharyngeal mesoderm, a process that depends on transcription factor PITX2 (Liu et al., 2002; Mommersteeg et al., 2007). Recent evidence suggests that aberrant activation of embryonic mechanisms of atrial and pulmonary myocardium development can lead to AF (Mommersteeg et al., 2009) and genetic studies have linked PITX2 to AF patients (Gudbjartsson et al., 2007; Ritchie et al., 2012). Heterozygote Pitx2 knockout mice, which have only 40% lower Pitx2 expression than wild types, are also prone to arrhythmias, indicating that even modest changes in Pitx2 protein levels might promote AF (Kirchhof et al., 2011).

During development, Pitx2 expression is regulated by BMP signaling (Furtado et al., 2008; Monteiro et al., 2008; Schlange et
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TRANSLATIONAL IMPACT

Clinical issue
Atrial fibrillation (AF) is the most prominent heart arrhythmia and carries a significant risk of stroke and heart failure. It affects 2–5 million people in the United States, including young individuals (primarily in an idiopathic form) and older individuals (usually as a complication of various cardiovascular diseases). In most cases of AF, electrical signals begin in aberrant trigger areas, often close to the pulmonary veins. As the molecular causes of AF are not well understood, current treatments are mostly empiric. In addition, treatments can carry significant risks, are frequently ineffective or provide only temporary symptom relief.

Results
By sequencing the gene encoding the bone morphogenetic protein (BMP) antagonist Gremlin-2 (GREM2) in a cohort of idiopathic AF patients, the authors discovered a Q76E variant that increases the capacity of GREM2 to inhibit BMP signaling. The authors went on to analyze this human mutation in a zebrafish model, and assessed its function in live hearts. They made the novel finding that GREM2 is a crucial regulator of the cardiac rhythm gene network that acts upstream of Pitx2 (an important AF-promoting transcription factor). Additional in vivo experiments showed that GREM2 hyperactivity specifically slows conduction velocity in the atrium, without affecting ventricular contraction.

Implications and future directions
These results provide important novel mechanistic insights into the pathogenesis of AF. A major challenge in modern medicine is to understand how the patchwork of genetic variation in each human patient eventually leads to disease. This work provides an experimental template to evaluate the pathologic contribution of genetic variants in AF, and might contribute to finding new AF treatments.

RESULTS
Presence of a GREM2 Q76E variant in two independent probands with lone AF
To identify new variants contributing to AF, we sequenced a cohort consisting of 193 probands with lone AF for GREM2 and identified a single c.226C>G variant in two independent probands (Darbar et al., 2007; Table 1). C.226C>G is a rare variant (rs142343894) with a significantly higher frequency in the AF cohort than found in the general population represented by 1560 subjects in the 1000 Genomes Project (0.5% compared to 0.03%, P=0.034) (Clarke et al., 2012; Mills et al., 2011) (Fig. 1A; supplementary material Table S1). C.226C>G is a missense mutation within exon 2 of the GREM2 gene that results in substitution of glutamine 76 for glutamic acid (Q76E). BMP antagonists such as GREM2 share the same overall protein structure as BMP ligands. Q76 is highly conserved across species and adjacent to C73, the first of the group of six cysteine residues that form the cystine knot motif required for protein folding, dimerization and interaction with BMP receptors (Avsian-Kretchmer and Hsueh, 2004) (Fig. 1B,C).

The Q76E variant increases the inhibitory activity of GREM2
To determine whether the p.Q76E variant alters the ability of GREM2 to antagonize BMP signaling, we transfected expression plasmids carrying wild-type GREM2 or GREM2-Q76E into human embryonic kidney cells. We then exposed cells to recombinant BMP4 and assessed induction of ID2, a prototypical BMP signaling gene target. At maximum expression levels, both wild-type and variant GREM2 efficiently inhibited BMP signaling; however, by titrating the amount of transfected plasmids, we found that at lower concentrations the Q76E variant was at least twofold more potent at antagonizing BMP than wild-type GREM2 (Fig. 1D). These data suggest that the Q76E substitution generates a hypermorphic allele.

Grem2 is required for asymmetric cardiac development and atrial differentiation in zebrafish
Recent studies have implicated GREM2 in placode neurogenesis and craniofacial patterning (Kriebitz et al., 2009; Zuniga et al., 2011). However, its role in cardiac development and cardiac function is unknown. To address this issue, we turned to the zebrafish model that offers excellent genetic and embryological tools for studying cardiac structure and function in vivo. By examining the expression of grem2 and cardiac-specific genes, we found that grem2 was present in the ventral portion of the pharyngeal arch mesoderm, immediately adjacent to the cardiac field, at the time that cardiac progenitor cells migrate from the midline to the left side of the embryo (Fig. 2). Because BMPs are known to modulate proliferation and differentiation of cardiac progenitor cells (Chochron et al., 2007; Schultheiss et al., 1997), the juxtaposition of the Grem2-positive and cardiac territories raised the possibility that Grem2 shapes the gradient of BMP activity across the migratory path of developing cardiomyocytes and thus affects their fate.

To test this possibility, we employed loss-of-function approaches in zebrafish using morpholino antisense oligonucleotides (described in supplementary material Fig. S1). We found that depletion of grem2 led to randomization of the cardiac axis, jogging and looping defects, as well as varied shape of the heart tube (Fig. 3A). Replacement experiments with zebrafish or mouse mRNAs rescued the morpholino defects, suggesting that the phenotype was specific to loss of Grem2 function (supplementary material Figs S2, S3). In zebrafish grem2 morphants, expression of both atrial- and ventricular-specific myosin heavy chains (amhc and vmhc) was reduced, with amhc being more affected. The deficits in cardiac gene expression at 19 hours post-fertilization (hpf) was consistent with a smaller atrium in older embryos (48 hpf), as evaluated by...
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Table 1. Clinical characteristics of AF patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Overall AF (n=1164)</th>
<th>Lone AF probands (n=193)</th>
<th>Gremlin2 c.226C&gt;G AF probands (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis (years)</td>
<td>53±13</td>
<td>43±13</td>
<td>50±18</td>
</tr>
<tr>
<td>Male gender (%)</td>
<td>65</td>
<td>71</td>
<td>50</td>
</tr>
<tr>
<td>Positive family history (%)</td>
<td>17</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Ethnicity: White/Black/Hispanic/Asian (%)</td>
<td>94/6/1&lt;1&lt;1</td>
<td>97/2&lt;1&lt;1&lt;1</td>
<td>100/0/0/0</td>
</tr>
<tr>
<td>Type of AF: paroxysmal/persistent/permanent (%)</td>
<td>58/27/15</td>
<td>80/13/7</td>
<td>50/50/0</td>
</tr>
<tr>
<td>CAD (%)</td>
<td>24</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>57</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>18</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>CHF (%)</td>
<td>19</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Cardiomyopathy (%)</td>
<td>15</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>Ejection fraction (%)</td>
<td>52±13</td>
<td>57±8</td>
<td>60±1</td>
</tr>
<tr>
<td>Left atrial diameter (mm)</td>
<td>44±8</td>
<td>40±6</td>
<td>37±4</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>50±8</td>
<td>48±6</td>
<td>51±6</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>34±10</td>
<td>31±6</td>
<td>32±1</td>
</tr>
</tbody>
</table>

Values are mean ± s.d.

CAD, coronary artery disease; CHF, congestive heart failure; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension.

antibody staining of ventricular and atrial tissues (Fig. 3B). In addition, expression of pitx2, which controls cardiac laterality (Essner et al., 2000; Ryan et al., 1998), was bilaterally upregulated when cardiac progenitors occupy the concentric territory adjacent to the midline, whereas the expression of the nodal antagonist lefty2, which is asymmetrically expressed on the left side when the cardiac tube has jogged to the left (Bisgrove et al., 1999), was absent (Fig. 3B). Grem2 does not appear to be required for asymmetric gene activity during early developmental stages, as illustrated by the normal left side expression of the nodal gene spaw in posterior mesoderm at 16 hpf (supplementary material Fig. S4). These data indicate that Grem2 depletion results in abnormal expression of pitx2 and lefty2, leading to cardiac laterality defects. In addition, atrial development appears to be particularly sensitive to Grem2 depletion.

Grem2 regulates BMP signaling activity in cardiac development

Previous work has shown that precise levels of BMP signaling are necessary for cardiac morphogenesis (Chocron et al., 2007). To test whether Grem2 regulates BMP signaling during zebrafish cardiac development, we analyzed phosphorylated Smad1/5/8 (pSmad) proteins, the main intracellular mediators of activated BMP signaling. We found strong pSmad protein upregulation in the somitic boundaries (Fig. 4A) and the heart of Grem2 morphants. To determine whether attenuation of the high BMP signaling levels in Grem2 morphants could reverse the cardiac deficits, we exposed Grem2-depleted embryos to dorsomorphin (DM), a chemical inhibitor of BMP signaling (Hao et al., 2008) and analyzed cardiac development using cmlc2-egfp and amhc riboprobes. Incubation of Grem2 morphants with DM restored the expression domains of cardiac genes to levels comparable with wild-type embryos, particularly in the atrium (Fig. 4C), whereas DM had no effect on wild-type, DMSO-treated control embryos at concentrations used in these experiments. Furthermore, the differentiation of the atrial bulb itself was restored in 65% of the DM-treated morphants, as assessed by the size of the heart area and the appearance of the morphological characteristics of the atrial bulb (Fig. 4C,D). These data show that aberrantly high BMP signaling in grem2 morphants is responsible, at least in part, for the observed cardiac defects and that Grem2 is a crucial regulator of BMP signaling during cardiac development and essential for atrial patterning and differentiation.

Grem2 overexpression expands atrial cardiomyogenesis

To establish whether Grem2 is sufficient to induce atrial formation in developing embryos, we overexpressed Grem2 by injecting grem2 mRNA into one-cell-stage cmlc2-egfp transgenic zebrafish embryos and evaluated cardiac gene expression. Resultant embryos showed cardiac development not only in its native location, but also contractile activity in ectopic areas in the posterior trunk at 48 hpf (Fig. 5A). At earlier stages (13 hpf), we observed supernumerary expression domains of gata-5, a transcription factor that promotes cardiac specification in mesodermal progenitor cells (Reiter et al., 1999) (Fig. 5B). These results indicate that Grem2 is sufficient to induce ectopic cardiomyogenesis in mesodermal progenitor cells.

To determine the molecular characteristics of the ectopic cardiac territories, we stained grem2-overexpressing embryos with amhc and vmhc riboprobes (Fig. 5C). The expanded ectopic myocardium expressed exclusively atrial mhc (amhc), whereas ventricular mhc (vmhc) expression in the native hearts was significantly reduced as compared with wild type, indicating that high Grem2 activity promotes atrial development and differentiation.

GREM2 Q76E variant is arrhythmogenic

The role of Grem2 in zebrafish atrial development, as well as the association between human GREM2-Q76E and AF, led us to hypothesize that, in addition to its role in cardiac morphogenesis, GREM2 is an upstream regulatory factor of the genetic network controlling cardiac rhythm. To test this possibility, we compared the expression levels of previously identified AF candidate genes.
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(Antzelevitch et al., 2007; Firouzi et al., 2004; Gollob et al., 2006; Gudbjartsson et al., 2007; Hodgson-Zingman et al., 2008; Shanmugam et al., 2011) in differentiated mouse embryonic stem cells stably transfected with empty vector (control), human wild-type GREM2 and variant GREM2-Q76E expressing plasmids (Fig. 6A). The results show that the expression levels of AF candidate genes were significantly increased by wild-type and variant GREM2, supporting the hypothesis that GREM2 regulates genes essential for maintaining cardiac rhythm.

**GREM2 levels are crucial for normal cardiac rhythm**

To determine the functional impact of the GREM2 and GREM2-Q76E on cardiac rhythm, we assessed contraction rates in zebrafish embryos injected with human wild-type GREM2 or GREM2-Q76E mRNAs. We found that contraction rates were significantly slower in embryos overexpressing either form of GREM2 compared with non-injected controls, but the effect was more pronounced in variant than wild-type GREM2-injected embryos (Fig. 6B).

To evaluate whether the observed abnormal contraction rate defect reflects irregular rhythm, abnormal contraction signal propagation, or uncoupling of atrial from ventricular contractions, we developed a method of optical digital cardiography (DCG) for in vivo quantification of heart function in zebrafish. We found that the sequential rhythms of atrium and ventricle were synchronized and thus not perturbed by GREM2 or GREM2-Q76E overexpression (Fig. 6C; supplementary material Movies 1-3). However, quantification of the DCG output showed that although ventricular rhythm remained regular, the atrial rhythm was consistently abnormal in GREM2- and GREM2-Q76E-injected embryos (Fig. 6C; supplementary material Movies 1-3). We concentrated our analysis on morphologically normal hearts; however, it is noteworthy that the severity of the arrhythmias increased in morphologically abnormal hearts (not shown).
Overall, these data support the hypothesis that GREM2 regulates cardiac rhythm in the atrium, and establish that higher than normal GREM2 activity, either by overexpression or gain-of-function mutations such as the Q76E variant, have arrhythmogenic potential. To probe the mechanism of irregular atrial contraction, we investigated whether contraction signal propagation was disrupted by assessing spatiotemporal conduction velocity on two distant test points, upstream (point 1) and downstream (point 2) along the contraction wave of the atrium (Fig. 6D). We found that upstream contraction velocities were similar in all three conditions (control, wild type, variant), whereas downstream contraction velocities were slowed in hearts overexpressing GREM2 or variant GREM2-Q76E (wild type, variant), whereas downstream contraction velocities were similar in all three conditions (control, variant), whereas downstream contraction velocities were slowed in hearts overexpressing GREM2 or variant GREM2-Q76E (wild type, variant), whereas downstream contraction velocities were similar in all three conditions (control, variant). Thus, it appears that high GREM2 activity distorts atrial contraction velocity and wavefront propagation.

**DISCUSSION**

Advanced genetics approaches and sequencing tools have identified a broad spectrum of genetic variation in the coding region of human genes. The current challenge of modern medicine is to determine the contribution of individual pathogenic variants to complex diseases such as familial AF. Our analysis of the GREM2 variant Q76E and the cardiac imaging approaches developed here provide a template for functional modeling of AF-associated variants in zebrafish and differentiating embryonic stem cells. Moreover, because GWAS studies have linked the GREM2 genetic locus to coronary vascular disease (Wellcome Trust Case Control Consortium, 2007), the characterization of the role of GREM2 in cardiac development and function might have broader implications for understanding the contribution of BMP signaling and its specific modulators to cardiovascular health.

Specifically, our results show that Gremlin2, a BMP antagonist that is required for pharyngeal mesoderm patterning, is essential for zebrafish cardiac development. Loss of Gremlin2 leads to overexpression of pitx2 and downregulation of lefty2, two crucial regulators of asymmetric cardiac development (Bisgrove et al., 1999; Essner et al., 2000), randomizing jogging and looping. Moreover, Gremlin2 knockdown increases the levels of activated phosphorylated Smad and BMP signaling within cardiac cells, interfering with both ventricular and atrial differentiation. Atrial differentiation in particular is compromised at high levels of BMP signaling, but favored at low levels.

Our in vitro and in vivo data also support the notion that the GREM2 Q76E mutation generates a hypermorphic allele and a pathogenic genetic variant that increases the risk of AF. Although the allele shows poor penetrance, as often with AF loci, functional analyses in zebrafish revealed that higher than normal GREM2 levels expand atrial differentiation, but reduce cardiac contraction rates and disturb contraction propagation in atrial cardiomyocytes. Because the GREM2 Q76E variant appears to be a stronger BMP inhibitor than wild-type GREM2, it probably has no measurable effect at areas of high GREM2 expression, but might interfere with BMP signaling at border zones of GREM2 and BMP activity. In this setting, overactive GREM2 could promote ectopic differentiation of mesenchymal cells around pulmonary veins to sleeves of atrial muscle, and thus establish foci of ectopic arrhythmogenic activity. Alternatively, GREM2, by inhibiting BMP...
signaling, might function upstream to directly or indirectly downregulate PITX2c expression (Furtado et al., 2008). Thus, increased GREM2 activity could lower PITX2c levels, altering the expression of atrial genes and raising the risk of AF.

Treatment of AF poses significant challenges, with a clear need for new therapeutic targets. The functional modeling of the human GREM2 mutation suggests that defective BMP signaling leads to AF, providing mechanistic insights into the pathogenesis of the disease and a host of novel therapeutic targets. To the best of our knowledge, GREM2 is the first morphogen implicated in cardiac laterality that also regulates cardiac contraction. This raises the intriguing possibility that the pathways establishing asymmetric cardiac development also control the genetic networks of cardiac rhythm, although the precise molecular mechanisms will need to be further investigated in future studies. These findings open new avenues for exploring the causes of AF, ultimately leading to better diagnostic and therapeutic strategies.

MATERIALS AND METHODS

Gene nomenclature

Gene names in the text are according to nomenclature guidelines, with human genes in capitals (e.g. GREM2), mouse first letter capital (Grem2) and zebrafish in small letters (grem2). Gene names are in italic and protein names in roman font.

Study subjects and sample collection

The study protocol was approved by the Vanderbilt University Institutional Review Board and participants took part after informed written consent was obtained. Patients ≥18 years of age with ECG-confirmed AF were enrolled in the Vanderbilt AF Registry, which comprises clinical and genetic databases (Darbar et al., 2007). A detailed medical and drug history was obtained for all patients at enrollment. Clinical characteristics of study subjects are shown in Table 1.

Whole blood was collected for genomic DNA extraction and analysis. The GREM2 coding and flanking regions were amplified by polymerase chain reaction (PCR) using primers 5′-ACCAGATCAAGGAGTGCTG-3′ and 5′-AGAAGTGCTTGCTGAGG-3′, and analyzed using the Reveal Discovery System to identify aberrant conformers, which were then directly sequenced (Abraham et al., 2010). Two unrelated lone AF subjects with GREM2 variants were identified, namely a woman with persistent AF since age 62, and a man with AF since age 37. Both reported vagal triggers, with the former reporting post-prandial and nocturnal onset and the latter reporting nocturnal/obstructive sleep apnea-related onset. As control cohort, subsections of BAM files from 1560 samples containing chromosome 1:240656549-240656551 were downloaded from 1000 Genome Project data (Exome alignment dated 2011-11-14).
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Zebrafish maintenance
Zebrafish, Danio rerio, were maintained as described (Müller et al., 2006). Zebrafish embryos were obtained from crosses between AB and TL lines or the Tg(cmlc2:EGFP)twu34 line.

In situ hybridization and antibody staining
Whole-mount in situ hybridization was performed as described (Müller et al., 2006; Wang et al., 2011). The hybridization mix for the double staining with grem2 and cmlc2 contained two digoxigenin-labeled RNA antisense probes for grem2 (250 ng of grem2 coding region probe and 500 ng of grem2 3' - UTR probe per 250 μl of hybridization mix) and 250 ng of fluorescein-labeled cmlc2 probe. For all other markers, we used 250 ng of digoxigenin-labeled RNA antisense probe per 250 μl hybridization mix.

Whole-mount antibody staining was performed as described (Melville et al., 2011; Sarmah et al., 2010). Mouse monoclonal antibodies MF20 against sarcomeric myosin heavy chain and S46 against atrial myosin heavy chain were obtained from the Developmental Studies Hybridoma Bank, diluted 1:10 in blocking solution, and applied to embryos overnight at 4°C. After five washes in PBS-Triton, embryos were incubated overnight at 4°C with secondary antibodies conjugated to AlexaFluor 488 and AlexaFluor 555 from Molecular Probes, diluted 1:400 in blocking solution. After six washes in PBT-DMSO, samples were transferred to 1:1 glycerol-PBT and stored at 4°C. Samples were incubated overnight at 4°C with anti-pSmad1/5/8 antibody (diluted 1:400 in blocking buffer; Cell Signaling Technology) then washed and incubated with pre-adsorbed alkaline phosphatase-conjugated goat anti-rabbit IgG (diluted 1:1000 in blocking buffer; Jackson ImmunoResearch) for 2 hours at room temperature.

To quantify changes in gene expression levels, signal intensity at the region of interest was measured in the green channel using NIH ImageJ. The background was subtracted and the signal normalized to non-injected, wild-type controls. Statistics were performed using the two-tailed Student's t-test, with P<0.05 being considered significant.

Loss- and gain-of-function experiments and dorsomorphin treatment
We used the following morpholino (MO) antisense oligonucleotides (Gene Tools) to block the splicing (MO1) and translation (MO2) of the zebrafish grem2 transcript: MO1 5'-ACTGCTCATC-CTGGAACACAGAGAG-3' and MO2 5'-ACACAGCCAC-CTTACTGCTCATC-3' (supplementary material Fig. S1). Morpholinos were reconstituted in water. Embryos at 1- to 4-cell stage were injected with 1 nl of morpholino solution into the yolk, close to the blastomeres, with an air injector from WPI (PV820 Pneumatic PicoPump). A concentration of 0.6 mM for MO1 or 0.4 mM for MO2 produced a fully penetrant and expressive phenotype in more than 85% of the injected embryos (morphants) without unspecific toxic effects.

Morphant embryos and non-injected controls were incubated in 2 μM dorsomorphin (Sigma-Aldrich) (Hao et al., 2008) dissolved in DMSO, or in DMSO alone, from 16 hpf until fixation at 48 hpf.

We injected 1 nl zebrafish grem2 mRNA at 10-15 pg/nl into the yolk. Human wild-type and variant GREM2 mRNAs were injected at various concentrations ranging from 2 to 5 pg/nl. Grem2 overexpression caused the expected defect of lack of BMP signaling during early embryogenesis, i.e. dorsalisization of embryos. Only embryos with mild dorsalized phenotypes were processed for molecular characterization at later stages in order to visualize cardiac defects. For all shown experiments, 2.5 pg/nl of human GREM2 mRNA was used because we found it to be the highest concentration that resulted in normal cardiac morphology but abnormal rhythm.
Cardiac morphology and contractility in live embryos

For optical digital cardiography (DCG), live zebrafish Tg(cmlc2:EGFP)twu34 embryos at 48 hpf were embedded into 1.5% agarose and continuous images of the whole heart were taken on a Axio Imager Z1 microscope with a EC Plan-Neofluar 5×/0.15 M27 lens for 30 seconds using Axiovision software (Zeiss). Files were then imported into ImageJ and converted to binary using the ‘threshold’ command. The atrium or ventricle was selected and the two-dimensional surface area was calculated for each frame using the ‘analyze particles’ command. The output values were collected and plotted in Microsoft Excel. Analysis was performed on four embryo hearts for each condition. To determine whether signal propagation was uniform across the atrium, we selected two points on the atrium: one near the location where the contraction wave as it traveled across the atrium. * indicates a possible deterioration of the contraction wave in overexpressing embryos. To quantify the average time that points 1 and 2 spent in systole, we calculated the size of peak areas above the ‘threshold’ command. The atrium or ventricle was selected and the two-dimensional surface area was calculated for each frame using the ‘analyze particles’ command. The output values were collected and plotted in Microsoft Excel. Analysis was performed on four embryo hearts for each condition. To determine whether signal propagation was uniform across the atrium, we selected two points on the atrium: one near the location where the contraction wave begins (point 1), and one far from both the initiation site and the valve that the signal moves towards (point 2). Point 2 was at a 45° angle to the line drawn through the valve.

In order to track points 1 and 2, the two points were marked with a single pixel and the move of the pixel was measured in ~100 successive frames using Imagej to report the coordinates of each selected point. The coordinates were then plotted in Microsoft Excel, assigning as 0 the coordinate at the most expanded location (i.e. furthest distance from the valve) during diastole. The coordinates were then converted to distance values from location 0 and the distances plotted over time as a line chart. Thus, peak values in the middle chart of Fig. 6D represent point locations at the end of systole.

In Grem2 overexpressing hearts, peaks corresponding to distal point 2 appeared broader than the peaks of proximal point 1, indicating that point 2 spent a longer time in systole than point 1 in overexpressing embryos. To quantify the average time that points 1 and 2 spent in systole, we calculated the size of peak areas above the mid-contraction point (Fig. 6D, right panel).

Cloning of human wild-type and variant GREM2

The GREM2 wild-type coding sequence was amplified from human genomic DNA using forward primer 5’-CTAGCGAATTCA-TGTTCGTGAGAAGCTTCCCTGTCTTGTC-3’ and reverse primer 5’-CTAGC TTCATCTGTCCTGCGGAGTCGCT-CAGGTTCCAC-3’. Cloning sites EcoRI and BamHI are underlined. The resulting amplicon was digested with EcoRI and BamHI and cloned into pcDNA3.1/Myc-His(-)A (Invitrogen). The Q76E mutation was introduced with primers 5’-AGTGACTG-GTGCAGACGGAGCCGGTCGGCGAGG-3’ and 5’-CCGTCTGGGCAGCGGTCCTGGCAACAGTCACT-3’. Successful cloning of wild-type GREM2 and GREM2-Q76E was confirmed by restriction enzyme digests and direct sequencing.

Cell culture

Human embryonic kidney 293 (HEK293) cells were transiently transfected with 1, 25, 50 and 100 ng of wild-type GREM2 or GREM2-Q76E plasmids using Lipofectamine 2000 reagent (Invitrogen) and treated with 5 ng/ml BMP4 for 6 hours. RNA was
then extracted and analyzed for *ID2* expression using quantitative RT-PCR. Lipofectamine 2000 (Invitrogen) was used to stably transfec undifferentiated CGR8 mouse embryonic stem cells with 8 μg of linearized wild-type GREM2 and GREM2-C76E plasmids. Positive clones were selected with 200 μg/ml G418 for 10 days and single colonies were picked, expanded, and genotyped. CGR8 cells were cultured and differentiated using the hanging-drop method (Rai et al., 2012).

RNA preparation and qRT-PCR analysis
Total RNA was extracted from mouse embryonic stem cells at day 8 of differentiation using the RNeasy kit (Qiagen) and reverse-transcribed into cDNA (Beck et al., 2008). For quantitative PCR, 20 ng of cDNA was amplified with the iQ SYBR Green Supermix kit on an iCycler (BioRad) using *β-actin* or *GAPDH* as controls. In addition, we normalized qPCR values in Fig. 6 to the relative expression levels of variant and wild-type GREM2. Relative gene expression levels were quantified using the formula $2^{-\Delta\Delta Ct}$ as described (Beck et al., 2008). Primer sequences are included in supplementary material Table S2.

Protein modeling
GREM2 structure was previously modeled on human sclerostin (Rider and Mulloy, 2010). We used the UCSF Chimera system for visualization of the GREM2 structure (Pettersen et al., 2004).

Statistical analysis
Statistical analyses on continuous data were performed using unpaired two-tailed Student’s *t*-test; analysis on categorical data were performed using two-tailed Fisher’s exact test. qPCR results were cultured and differentiated using the hanging-drop method (Rai et al., 2012). Relative gene expression levels were quantified using the formula $2^{-\Delta\Delta Ct}$ as described (Beck et al., 2008). Primer sequences are included in supplementary material Table S2.

ACKNOWLEDGEMENTS
We thank Cory Guthrie and Kirill Zavalin for excellent zebrafish care and Marcia Blair for excellent technical assistance. We are indebted to H. Scott Baldwin and Daniel Levin for critically reading and editing the manuscript.

COMPETING INTERESTS
The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

FUNDING
This work has been supported in part by the zebrafish initiative of the Vanderbilt University Academic Venture Capital Fund; by National Institutes of Health (NIH) grants from the National Institute of Dental and Craniofacial Research (NIDCR) [grant number DE018477 to E.W.K.], the National Heart, Lung, and Blood Institute (NHLBI) [grant numbers HL083958 and HL100398 to A.K.H., HL65962 to D.M.R., and HL09221 to D.D.], an American Heart Association Established Investigator Award [grant number 0940116N to D.D.], and the National Institute of General Medical Sciences (NIGMS) [grant number T32 GM08554]; and by the Cellular, Biochemical and Molecular Sciences Training Program at Vanderbilt (to D.B.M.).

SUPPLEMENTARY MATERIAL
Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl?doi=10.1242/dmm.010488/-/DC1

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Hodgson-Zingman, D. M., Karst, M. L., Zingman, L. V., Heublein, D. M., Darbar, D., Herron, K. J., Ballew, J. D., de Andrade, M., Burnett, J. C., Jr and Olson, T. M.
Gremlin2 in atrial fibrillation


Wellcome Trust Case Control Consortium (2007). Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature 447, 661-678.

**Fig. S1. Design and evaluation of grem2-targeting morpholinos.** (A) Schematic diagram of the positioning of the two anti-grem2 morpholinos (MO1 and MO2) relative to grem2 gene structure. MO1 straddles the exon/intron boundary at the 3’ splice site, blocking splicing of the grem2 transcript. MO2 starts at the beginning of exon 2, the first bases of which contain the initiation of translation ATG codon, blocking synthesis of Grem2 protein. The location of the coding region, stop codon (*) and 3’UTR are indicated. (B) MO-1 morpholino effectively blocks splicing of grem2 as compared to non-injected control embryos (NIC). A set of primers specific for the unspliced (e1f/i1r) and spliced (e1f/e2r) forms of the grem2/prdc transcripts were used to show that MO-1 blocks splicing of the grem2 transcript. The primer pair e2f/e2r2 from the 3’UTR area of the grem2 exon 2 that detects both unspliced and spliced transcripts shows similar levels of grem2 in both samples. The locations of the different primers and MO-1 within the grem2 gene locus are indicated in the left diagram. RNA was extracted from ~30 embryos at 24 hour using the TRIzol reagent and 2 μg of total RNA were reverse transcribed to cDNA for PCR. Primer sequences: e1f: 5’ACTGAAGACTCTCAGCGGCT3’; e2r: 5’TGGCTGACCGTCTG-CCGCAA3’; i1r: 5’GATCCTCTGCTTCATCAGAC3’; e2f: 5’AA CACCTCTGACCGCC-GCAC3’; e2r2: TGCGGCCTGCAGAATGCACA.
**Fig. S2. Quantification of looping and jogging defects in grem2 morphants.** Phenotypic analysis of grem2 morphants generated using various concentrations of MO1 and MO2 as indicated at 24, 36 and 48 hour. The two independently designed morpholinos gave comparable results, confirming that the phenotype is specific to loss of Grem2 function. The data for the graph are included in the Table below.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>hpf</th>
<th>WT (%)</th>
<th>Jogging left, looping right (%)</th>
<th>Straight heart (%)</th>
<th>Jogging right, looping left (%)</th>
<th>N: number of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2 mM</td>
<td>24 h</td>
<td>67</td>
<td>21</td>
<td>10</td>
<td>2</td>
<td>N = 3, n = 152</td>
</tr>
<tr>
<td></td>
<td>36 h</td>
<td>83</td>
<td>10</td>
<td>2</td>
<td>5</td>
<td>N = 3, n = 183</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>68</td>
<td>27</td>
<td>2</td>
<td>3</td>
<td>N = 4, n = 164</td>
</tr>
<tr>
<td>0.4 mM</td>
<td>24 h</td>
<td>28</td>
<td>55</td>
<td>13</td>
<td>4</td>
<td>N = 4, n = 196</td>
</tr>
<tr>
<td></td>
<td>36 h</td>
<td>16</td>
<td>42</td>
<td>5</td>
<td>37</td>
<td>N = 4, n = 129</td>
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<tr>
<td></td>
<td>48 h</td>
<td>25</td>
<td>57</td>
<td>7</td>
<td>9</td>
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<td>12</td>
<td>50</td>
<td>31</td>
<td>7</td>
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<tr>
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<td>45</td>
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<td>75</td>
<td>8</td>
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<tr>
<td>MO 2</td>
<td></td>
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<td>0.4 mM</td>
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<td>12</td>
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<td>26</td>
<td>2</td>
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<td>27</td>
<td>35</td>
<td>14</td>
<td>23</td>
<td>N = 3, n = 127</td>
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<tr>
<td></td>
<td>48 h</td>
<td>24</td>
<td>60</td>
<td>4</td>
<td>13</td>
<td>N = 3, n = 178</td>
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</table>
Quantification of the rescue of the *grem2* morpholino MO1 cardiac defects by co-injection of zebrafish or mouse *grem2* mRNA

<table>
<thead>
<tr>
<th>24 hpf</th>
<th>Concentration</th>
<th>WT (%)</th>
<th>Cardiac defects (%)</th>
<th>N, number of experiments</th>
<th>n, number of embryos</th>
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</thead>
<tbody>
<tr>
<td>MO1</td>
<td>0.4 mM MO</td>
<td>28</td>
<td>72</td>
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<tr>
<td>MO1 +</td>
<td>0.4 mM MO + 15 ng/µl mRNAZ</td>
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<td>50</td>
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<tr>
<td>zebrafish mRNA</td>
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<tr>
<td>MO1 +</td>
<td>0.4 mM MO + 10 ng/µl mRNAM</td>
<td>48</td>
<td>52</td>
<td>N = 3, n = 138</td>
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<tr>
<td>mouse mRNA</td>
<td></td>
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**Fig. S3. Confirmation of *grem2* morpholino specificity by mRNA injection rescue experiments.** To further test the specificity of the morpholino effects, we tested whether zebrafish *grem2* or mouse *Grem2* mRNA rescues the morphant phenotype. The results showed that both zebrafish *grem2* (mRNAZ) and mouse *Grem2* (mRNAm) partially reversed the MO1 morpholino-incurred defects, supporting the notion that the cardiac jogging and looping phenotypes are caused by loss of Grem2 function. The data for the graph are included in the Table below.
Fig. S4. Loss of Grem2 does not affect early asymmetric gene expression of the nodal gene spaw in posterior mesoderm. Spaw expression at 16 hours post-fertilization (h) in grem2 morphants is indistinguishable from wild-types.
Table S1. Phenotypic characteristics of probands and families with GREM2 variants.

<table>
<thead>
<tr>
<th>Pedigree No.</th>
<th>AF</th>
<th>GREM-2</th>
<th>Age at diagnosis/enrollment</th>
<th>AF Triggers</th>
<th>AF risk factors</th>
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<tbody>
<tr>
<td>B-II-1</td>
<td>Affected</td>
<td>Heterozygous</td>
<td>37/52</td>
<td>Vagal-OSA</td>
<td>OSA Obesity (BMI 43)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>variant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-I-2</td>
<td>Unaffected</td>
<td>Heterozygous</td>
<td>**/90</td>
<td>N/A</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td></td>
<td>variant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-II-1</td>
<td>Affected</td>
<td>Heterozygous</td>
<td>62/73</td>
<td>Vagal-post-prandial and nocturnal</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td>variant</td>
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<td></td>
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<td>A-II-3</td>
<td>Unaffected</td>
<td>Heterozygous</td>
<td>**/72</td>
<td>N/A</td>
<td>HTN</td>
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<tr>
<td></td>
<td></td>
<td>variant</td>
<td></td>
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<tr>
<td>A-II-5</td>
<td>Unaffected</td>
<td>Wild type</td>
<td>**/68</td>
<td>N/A</td>
<td>Unknown</td>
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<tr>
<td>A-II-6</td>
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<td>Heterozygous</td>
<td>**/67</td>
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<tr>
<td></td>
<td></td>
<td>variant</td>
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<td></td>
<td></td>
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</tbody>
</table>

BMI=body mass index expressed in kg/m², N/A= not applicable, HTN=hypertension, OSA= obstructive sleep apnea
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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</thead>
<tbody>
<tr>
<td>β−Actin</td>
<td>CTACGAGGGCTATGCTCTCCC</td>
<td>CCGGACTCATCGTACTCCTGC</td>
</tr>
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<td>GAPDH</td>
<td>AAGGTGAAGGTCGGAGTCAAC</td>
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<td>ATAACAGTGGGCAGTTGAACAGCAG</td>
<td>TACCCCAATAACGAATGTGGGAGATG</td>
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<tr>
<td>Kcne2</td>
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<td>GCTTCATGTGGCCTCTGTTCCTCAT</td>
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<tr>
<td>Nppa</td>
<td>GGAGCCTACGAAGATCCAGC</td>
<td>TCCAATCCTGTCAATCCTACCC</td>
</tr>
<tr>
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<td>TCACGAGGAGCCACATAAGG</td>
</tr>
<tr>
<td>ID2</td>
<td>CGACCCGATGAGTCTGCTCTACAAC</td>
<td>GTGTTTCCTCGGTGAAATGGGCTGATAAC</td>
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