A Gain-of-Function TBX5 Mutation Is Associated With Atypical Holt–Oram Syndrome and Paroxysmal Atrial Fibrillation


Abstract—Holt–Oram syndrome (HOS) is a heart/hand syndrome clinically characterized by upper limb and cardiac malformations. Mutations in T-box transcription factor 5 (TBX5) underlie this syndrome. Here, we describe a large atypical HOS family in which affected patients have mild skeletal deformations and paroxysmal atrial fibrillation, but few have congenital heart disease. Sequencing of TBX5 revealed a novel mutation, c.373G>A, resulting in the missense mutation p.Gly125Arg, in all investigated affected family members, cosegregating with the disease. We demonstrate that the mutation results in normal Nkx2-5 interaction, is correctly targeted to the nucleus, has significantly enhanced DNA binding and activation of both the Nppa(Anf) and Cx40 promoter, and significantly augments expression of Nppa, Cx40, Kcnj2, and Tbx3 in comparison with wild-type TBX5. Thus, contrary to previously published HOS mutations, the p.G125R TBX5 mutation results in a gain-of-function. We speculate that the gain-of-function mechanism underlies the mild skeletal phenotype and paroxysmal atrial fibrillation and suggest a possible role of TBX5 in the development of (paroxysmal) atrial fibrillation based on a gain-of-function either through a direct stimulation of target genes via TBX5 or indirectly via TBX5-stimulated Tbx3. These findings may warrant a renewed look at the phenotypes of families and individuals hitherto not classified as HOS or as atypical but presenting with paroxysmal atrial fibrillation, because these may possibly be the result of additional TBX5 gain-of-function mutations. (Circ Res. 2008;102:0-0.)

Key Words: atrial fibrillation ■ congenital heart defects ■ transcription factor ■ TBX5 ■ Holt–Oram syndrome

Congenital heart defects are among the most common congenital defects in children, occurring in 1% to 2% of live births and in ~5% of stillbirths. Congenital heart defects can either appear as a spontaneous defect or as part of a syndrome. One such syndrome is the Holt–Oram Syndrome (HOS) (Online Mendelian Inheritance in Man no. 142900), appearing in 1 of 100 000 live births and segregating in an autosomal dominant fashion. It is characterized by bilateral forelimb deformities and congenital heart defects. Clinically, there are 3 variations of HOS: affected individuals may have only skeletal anomalies (27.4%), only cardiac defects (3.9%), or both (68.7%). The limb and heart malformations can vary from mild to severe, even within families, and no correlation exists between the severity of the cardiac and skeletal abnormalities of the patient. The congenital heart malformations are generally secundum atrial septal defects (ASD II) or ventricular septal defects, but others, such as mitral valve defects and cardiac conduction defects, most notably atrioventricular block, have also been reported. Single-gene mutations were identified in the T-box transcription factor 5 (TBX5) in multiple HOS patients. TBX5 is a member of the T-box transcription factor family that regulates a wide variety of developmental processes in vertebrates and invertebrates, including specification of the mesoderm and development of the heart, vasculature, and limbs. Mutations in several other T-box genes have been associated with malformations such as septal defects and dilated cardiomyopathy (TBX20), cleft palate (TBX22), and syndromes such as DiGeorge syndrome (TBX1) and Ulnar–Mammary syndrome (TBX3). TBX5 is expressed, among others, in the embryonic heart and forelimbs. In the heart, it regulates transcription of downstream genes such as the atrial natriuretic factor (NPPA) and fibroblast growth factor 10 (FGF10) by the binding to T-box binding elements (TBEs), often in combination with the NKX2-5 transcription factor. Seventy percent of the identified TBX5 mutations lead to a premature stop codon and, in these patients HOS, is presumably caused by haploinsufficiency. Although the HOS-
associated mutations are distributed across all exons of TBX5, the majority are found within the T-box DNA binding domain. However, there is no evidence that either the type of mutation or the location of a mutation is predictive for the severity of the heart or limb malformations in HOS patients.

Here, we describe a family with an atypical form of HOS, previously reported by Van Bever et al in 1996. Besides some characteristics of HOS, 6 of 9 affected alive patients also experience paroxysmal atrial fibrillation (AF). We identified a novel TBX5 mutation (p.G125R) that cosegregates with the disease and, in contrast to previously reported TBX5 mutations, causes a gain-of-function. We speculate that this novel TBX5 mutation results in the atypical HOS seen in this family.

Patients and Methods

Clinical Details
The clinical findings in this family have been previously described by van Bever et al. Additional family members were investigated, new symptoms developed in several persons, and (affected) family members were born (see Results). The study was performed according to a protocol approved by the local ethics committees. Informed consent was obtained from all patients.

Linkage Analysis, Mutation Screen, and Confirmation
TBX5 haplotype analysis in the family was carried out using microsatellite markers D12S129, D12S821, D12S354 and D12S369 flanking the gene (Table II in the online data supplement, available at http://circres.ahajournals.org). Mutation screening is detailed in the online data supplement; in short, whole exons were PCR amplified using intronic primers (supplemental Table I) from peripheral blood genomic DNA and sequenced.

Plasmid Constructs and Reporter Gene Assays
Expression plasmids (FLAG-TBX5, full-length mouse FLAG-Nkx2-5) and reporter plasmids (pGL3-Nppa-luciferase, pGL3-Cx40-luciferase) and introduction of mutations in these are detailed in the online data supplement. Neonatal rat heart myocytes, immortalized with a temperature-sensitive SV40 T antigen (H10 cells) and an atypical HOS seen in this family.

RNA Isolation, CDNA Synthesis, and Quantitative PCR
H10 and H9C2 cells were used for quantitative PCR as detailed in the online data supplement. For calculation of PCR efficiency, the program LinReg was used. Measurements per RNA sample were in triplicate and were averaged. The experiments were repeated at least 3 times. Results are expressed as means ± SEM, as indicated in the legends. To remove variations from multisession experiments attributable to day-to-day differences in transfection efficiency and expression levels, a factor correction was applied. The corrected data set was used for statistical analysis. Comparisons between 2 groups was performed using Student’s t tests. P < 0.05 was considered statistically significant.

Results

Clinical Details
The family presented with an atypical form of HOS (Figure 1); the major clinical details are summarized in the Table. The skeletal findings include: (sub)luxation of the radial head in all examined affected patients (Figure 2A), carpal synostosis in the majority, and scoliosis and scapular dysplasia in some.

In Vitro Yeast Two-Hybrid X-Gal Assay and Binding Assays
All procedures concerning the yeast-2-hybrid system were carried out as described previously. In short bait (pcDNA3.1-mouse NXX2-5 carrying the GAL4 DBD fusion construct) and prey (pcDNA3.1-human TBX5wt/Gly125Arg carrying the GAL4 AD fusion construct) were cotransformed into Saccharomyces cerevisiae AH109 (Clontech), and the ability to grow on minimal media was examined using β-galactosidase filter assays. Binding assays were set up as described previously. Basically, equivalent amounts of myelin basic protein (MBP)/MBP fusion protein were loaded onto PBS-Triton columns equilibrated with amylose resin (New England Biolabs). After washing, the purified glutathione S-transferase (GST)/GST fusion was passed over. After washing and elution, the eluate was used for SDS-PAGE and Western blot analysis. Further details are found in the online data supplement.
Because TBX5 mutations are associated with HOS, we performed linkage analysis with 4 microsatellite markers surrounding the TBX5 gene in 6 affected family members and 1 nonaffected relative related by marriage (1 branch, II-5 and further, and his brother II-10, the other haplotypes are implied).

Linkage to the TBX5 gene was found (with a multipoint logarithm of the odds score of 0.98), because all investigated affected members share the same 4-2-4-1 haplotype (Figure 1). Inclusion of the G125R mutation increases the logarithm of the odds score to 1.51. Subsequent sequencing of TBX5 revealed a heterozygous single–base pair substitution at nucleotide 373.

Figure 1. Pedigree of the family with atypical HOS and paroxysmal AF, including linkage with microsatellite markers surrounding TBX5 and the resultant 4-2-4-1 haplotype cosegregating with the phenotype. Carriers of the p.G125R TBX5 mutation are noted with +; individuals with wild-type TBX5 are noted with −.

### Table. Summary of Clinical Features of the Atypical HOS Family

<table>
<thead>
<tr>
<th>Family</th>
<th>Member</th>
<th>CHD</th>
<th>Echo Parameters</th>
<th>ECG</th>
<th>Arhythmia, Age of Onset (Years)</th>
<th>Radial Luxation</th>
<th>Carpal Synostosis</th>
<th>Scoliosis</th>
<th>Other Anomalies</th>
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<tr>
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<td>(-)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>(+)</td>
<td>?</td>
<td>?</td>
<td>Scapular Dysplasia</td>
</tr>
<tr>
<td>I0.2</td>
<td>(-)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>(+)</td>
<td>(+)</td>
<td>?</td>
<td>(−)</td>
<td>Scapular Dysplasia</td>
</tr>
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<td></td>
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<td>40</td>
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<td>0.12</td>
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<td>Normal</td>
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<td>54</td>
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<td>sinus rhythm</td>
<td>+</td>
<td></td>
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</table>

*indicates according to pediatric values; #, abnormal septal motion; +, present; (+), anamnestically present; −, absent; (−), anamnestically absent; ?, unknown; ASDII, atrial septal defect secundum type; VSD, ventricular septal defect; pAF, paroxysmal atrial fibrillation (age at diagnosis between brackets); AT, atrial tachycardia; IRBBB, incomplete right bundle branch block; LVED, left ventricular end diastolic dimension (mm); LVSD, left ventricular systolic dimension (mm); SF, shortening fraction (%); LA, left atrial dimension (mm).
(Figure 4A), resulting in a G>C substitution shared by all the affected patients (c.373G>C; DNA was unavailable of I-2, II-2, II-4, and II-12). This nucleotide change results in the substitution of an evolutionarily conserved glycine for an arginine, p.G125R (Figure 4A and 4B; reference sequence NM_000192.3). Family members without skeletal findings, heart anomalies, or the risk haplotype do not carry the TBX5 mutation. The p.G125R mutation is absent from 600 chromosomes of control individuals. According to a TBX5 homology model, the introduction of such a positively charged arginine at position 125 could lead to a favorable charge–charge interaction with the negatively charged DNA, because the mutant arginine side chain is in close proximity to the DNA (7Å) (Figure 4C).

No mutations were found in either connexin (Cx)40 or TBX3, nor were Cx40 haplotypes associated with AF. See the online data supplement for details.

G125R TBX5 Protein Displays Enhanced DNA Binding and Normal Nkx2-5 Interaction

The primary function of TBX5 proteins is the regulation of gene transcription by binding to DNA target sequences, often in cooperation with protein partners. One of the best studied examples of this is the interaction with the Nkx2-5 transcription factor. Initially, we assessed the DNA binding of the G125R TBX5 mutant protein in HEK-293 cells transfected with FLAG-tagged TBX5 expression plasmids. In addition, G80R, a well-described TBX5 mutation was included, as was G125E, to study the effect of a negatively charged side chain in comparison with the positively charged arginine at position 125. All of the wild-type and mutant proteins were successfully expressed (data not shown). Subsequently, using the nuclear extracts of the transfected HEK-293 cells, EMSAs were performed using a probe corresponding to the TBE site at position –250 in the Nppa promoter, previously shown to be required for T-box factor–mediated Nppa gene regulation in vivo. This element also contains a NKE, required for Nkx2-5 binding. In Figure 5, we show the effect of TBX5 missense mutations on Nppa-DNA binding activity. Both wild-type TBX5 and the G125R and G125E mutants bind to the probe, whereas G80R mutant is unable to bind (Figure 5A). To investigate the DNA binding strength, we varied the amounts of wild-type and mutant G125 proteins. As can be appreciated from Figure 5B, mutant TBX5 G125R protein shows a strong and significantly enhanced DNA binding, up to 6 times, in comparison with wild-type TBX5 or G125E TBX5 at all protein amounts. G125E shows a slightly attenuated DNA binding, which is in line with the effect of a negative charge on this position, although this was not significant (P=0.07).

The interaction between the TBX5 mutants and Nkx2-5 and the result on DNA was investigated using EMSAs (Figure 5A), an in vitro yeast 2-hybrid X-gal assay (Figure 6A) and a pull-down assay (Figure 6B). There was no difference in the strength of the interaction of wild-type or mutant TBX5 with Nkx2-5; this is further explained in the online data supplement.

G125R TBX5 (Over)stimulates the Nppa and the Cx40 Promoter

Subsequently, we investigated the effect of the G125R and G80R TBX5 mutants on transcriptional activation of the
Nppa and Cx40 (Figure 7A). In accordance with previously published work, wild-type TBX5 gives higher relative expression in the presence of Nkx2-5, whereas the G80R TBX5 mutation displays significantly lower Nppa activation with or without Nkx2-5 compared with wild-type TBX5. Strikingly, the G125R TBX5 mutant displays a significantly higher activation of the Nppa promoter in the presence of Nkx2-5 in contrast to wild-type Tbx5 (mean difference, 3 [95% confidence interval 0.7 to 5.3]). In absence of Nkx2-5 (0.70 [0.13 to 1.53]), a similar trend can be identified although this difference is not significant (P=0.09) (Figure 7A). This effect is dose-independent, because the transactivation remains equal for both wild-type and G125R TBX5 regardless of the dosage of TBX5 (data not shown). Similar experiments were performed on the Cx40 promoter (Figure 7B), again the G125R TBX5 mutation shows a significantly higher activation of the promoter than wild-type TBX5 (0.21 [0.04 to 0.39]), whereas the G80R mutant does not show any activation.

**Figure 3.** A, Twelve-lead ECG of patient IV-1 showing AF. B, ECG of patient III-7 with recorded supraventricular, presumably atrial, tachycardia.

**Trafficking of G125R TBX5 to the Nucleus Is Normal**

To investigate whether the G125R mutant is correctly targeted to the nucleus, we transfected COS7 cells with both G125R mutant and wild-type TBX5. Using either an anti-FLAG antibody (Figure 6C) or a TBX5–yellow fluorescent fusion protein (data not shown). The G125R TBX5 protein is located in the nucleus, similar to wild-type TBX5 protein, indicating that the mutant is correctly transported to the nucleus.

**TBX5 G125R Stimulates Gene Expression**

To assay the effect of the mutant G125R protein on gene transcription, we transfected neonatal rat heart myocytes, immortalized with a temperature-sensitive SV40 T antigen (H10 cells), with both the wild-type and mutant TBX5 protein. Endogenous levels of TBX5 in H10 are negligible (data not shown). Subsequently, using quantitative PCR, the transcription levels of various genes reported to act down-
stream of TBX5 (Tbx3, Cx40, Nppa, Myh6) or genes involved in or associated with AF (Kcnj2, Cx40, Cx43, Hcn4, Kcne5, Kcnq1, Kcne1, Kcne3, Kcne1) were measured. Consistent with the promoter analysis, transcripts of both Nppa and Cx40 were significantly upregulated in H10 cells when transfected with the G125R mutant protein in comparison with wild-type TBX5 and vector control (Figure 7C). Interestingly, besides Cx40, transcripts of Tbx3 and Kcnj2 were also significantly upregulated on transfection with G125R TBX5 in comparison with vector control or wild-type TBX5 (Figure 7C). In contrast, although the expression levels of Cx43, Hcn4, Scn5a, and Kcnh2 were detectable by quantitative PCR, these genes did not demonstrate a significant up- or downregulation in the presence of TBX5 G125R, in comparison with wild-type TBX5 (data not shown). Unfortunately, although transcript levels of Myh6 are well known to respond to TBX5 levels, endogenous levels of this gene in H10 cells are not detectable by quantitative PCR, so the effect of G125R TBX5 on transcript levels could not be determined. Similarly, the transcripts levels of Kcne5, Kcne5, Kcne1, Kcne3, and Kcnq1 were undetectable in H10 cells with or without wild-type or G125R TBX5. This was corroborated in a second cell line (supplemental Figure I) derived from embryonic heart tissue (H9C2 cells)17; this is further detailed in the online data supplement.

Discussion

We have identified a novel missense mutation in TBX5; the p.G125R mutation is present in a large family with an atypical form of HOS, in which the majority of affected patients have both mild skeletal deformations and AF and few have congenital heart disease. The mutation results in normal Nkx2-5 interaction, enhanced DNA binding, and activation of the Nppa (Anf) and Cx40 promoter, and upregulation of various genes.

Van Bever et al in 199615 already described the initial phenotype of the family and compared it with symptoms found in similar entities, such as HOS and heart-hand syndrome type II. It was concluded that the phenotype did not satisfy all HOS characteristics, because thumb, pectoral, or clavicle anomalies were found in none of the family members. Renewed careful examination, however, showed slight
thenar muscular atrophy and limited opposition of the thumbs. These symptoms, however, are much less severe than expected in patients with HOS. Furthermore, only 13% of the affected individuals have a congenital heart malformation compared with 75% in the regular HOS population. However, among the most striking features is the occurrence of paroxysmal AF in the majority of the affected members, especially because most developed it at an unusually young age and in the absence of congenital heart disease. AF has occasionally been described in patients with HOS,²³ although principally in the setting of congenital heart disease and as a result of hemodynamic effects. However, the question remains whether the detection of a TBX5 mutation justifies the diagnosis of HOS. To date, no TBX5 mutations have been found in individuals with atypical phenotypes of HOS who did not meet the strict phenotypic criteria for HOS.²⁴ We suggest that the specific phenotype in this family with atypical signs of HOS relates to an underlying pathological mechanism different from other HOS patients, i.e., a gain-of-function (see below). Likewise, the phenotypes of other families or individuals hitherto not classified as HOS or as atypical may similarly be the result of a gain-of-function mutation.

TBX5 functions by DNA binding and activating target genes such as Nppa and Cx40, with or without a synergistic interaction with Nkx2-5.¹²,²⁵ It has been established that the N-terminal part of the protein, including the T-box, is essential for DNA binding, and that removal of the C-terminal 281 amino acids significantly enhanced DNA binding affinity.¹¹ In contrast to all currently known HOS TBX5 (missense) mutations, the G125R mutation exhibits normal Nkx2-5 interaction and transactivation, has a significantly increased DNA binding affinity, displays increased transcriptional activity on the Nppa and Cx40 promoters, and significantly stimulates transcription of various endogenous target genes in comparison with wild-type TBX5. The location of the G125R mutation in the tertiary structure of the T-box supports an increase in DNA binding activity, because it is juxtaposed in close proximity to the bound DNA. A glycine-to-arginine substitution at this position could generate an extra favorable charge–charge interaction with the DNA, resulting in enhanced DNA binding. Although the R279X TBX5 mutation is also reported to have an increased DNA binding, this was not quantified, nor was it shown that this mutant protein is actually present in the cell. Such a large

Figure 5. A, The effect of missense mutations of TBX5 on DNA binding activity in the presence and absence of Nkx2-5. Note that p.G125R retains DNA binding ability and Nkx2-5 interaction. The bottom blot shows a Western blot indicating that equal amounts of all proteins used in the EMSA were loaded. B, Blot demonstrating the binding strength between DNA and various amounts of wild-type, p.G125E, and p.G125R TBX5 protein; nonspecific bands are marked with NS. Bottom, Quantification of DNA binding strength according to optical density using densitometric quantification of DNA (n=3, SD). *Statistically significant differences (Student’s t tests, P<0.05) between G125R and wild-type or G125E TBX5.
deletion could lead in vivo to nonsense-mediated decay and consequently haploinsufficiency, as was also noted by the authors. For TBX1 mutations, however, 3 gain-of-function mutations were recently reported. The authors concluded that this was likely the result of enhanced stabilization of the protein dimer DNA complex. In contrast, to the atypical HOS phenotype caused by the TBX5 gain-of-function mutation reported in this study, the phenotype of the gain-of-function TBX1 mutation patients was the same as those with loss-of-function or deletions. An extended discussion on this topic can be found in the online data supplement.

The initial formation of limbs is initiated by the expression of FGF10 in the limb field mesenchyme and this expression is directly regulated by TBX5. The subsequent patterning of the digits, wrist bones, and sternum occurs via Cx40-containing gap junctions, again regulated by TBX5. Because both FGF10 and Cx40 are highly sensitive to TBX5 dosage, it is not surprising that the skeletal phenotypes of the family reported here reflects the underlying mutation, namely a mild skeletal phenotype as a result of a gain-of-function mutation, in which the core functionality of TBX5 is retained, in contrast with the more severe phenotypes caused by the common loss-of-function TBX5 mutations. We also investigated whether the prevalent paroxysmal AF in this family could be the consequence of a change in expression levels of genes that are known to be associated with AF. Several studies indicate that genetic variations in the Cx43 and Cx40 may predispose to arrhythmia vulnerability in humans. There was no association between promoter Cx40 polymorphisms and AF, but G125R TBX5 leads to a significant enhanced transcription of endogenous Cx40. However, these results are difficult to interpret because G125R TBX5 also enhances expression of TBX3 (see below), which is known to decrease Cx40 levels. Recently, a familial KCNJ2

Figure 6. A, In vitro yeast 2-hybrid X-gal assay with p.G125R and wild-type TBX5 as prey and Nkx2-5 as bait, quantified with β-galactosidase filter assay; mean values±SD of 4 measurements on independent transformations (Student’s t tests, P<0.05). B, Binding assays of wild-type and mutant TBX5 MBP/MBP fusion proteins. C, Trafficking of both wild-type and p.G125R TBX5 in COS7 cells. Transfected cells overexpressing TBX5 are immunostained with anti-FLAG for TBX5 and the nucleus is stained with 4',6-diamidino-2-phenylindole.
mutation cosegregating with AF was found. Interestingly, the G125R mutant significantly enhanced the transcription of KCNJ2 in comparison with wild-type TBX5. Furthermore, because overexpression of KCNJ2 in mice induces AF, and acquired AF in human is associated with increased expression of the $I_{K1}$ channel, increased expression of KCNJ2 under the influence of G125R TBX5 could potentially contribute to or underlie the observed AF.

Recent publications demonstrated that TBX3 is highly sensitive to TBX5 dosage and that it controls the sinoatrial node gene program. Strikingly, ectopic expression of TBX3 in mice induces pacemaker genes and leads to ectopic pacemaker activity, which is a precursor for AF. Thus, because we found significantly enhanced transcription of TBX3 in the presence of G125R TBX5, this could suggest that induced TBX3 expression may contribute to or underlie the observed AF.

In conclusion, contrary to previously published HOS mutations, we identified a gain-of-function TBX5 mutant that associates with an atypical, mild form of HOS and paroxysmal AF. A possible role of TBX5 in the development of paroxysmal AF based on a gain-of-function is suggested either through a direct mechanism via TBX5 or indirectly via TBX5 stimulated TBX3, although other mechanisms cannot be ruled out. These findings might warrant a renewed look at the phenotypes of families and individuals hitherto not classified as HOS or as atypical presenting with paroxysmal AF, because these may possibly be the result of additional TBX5 gain-of-function mutations.

**Limitations of the Study**

We identified a cosegregating TBX5 missense mutation in a family with atypical HOS and paroxysmal AF, and we argue that this mutation underlies both phenotypes. Because we did not have myocytes of the proband or family members, and we are not able to get them, we lack a human molecular correlate and thus cannot proof in vivo that this mutation leads to enhanced transcription of the various identified (AF) genes. One way around this is to test the functional consequence of the gain-of-function in a Tbx5 knock-in mouse model. Although feasible, it is likely that (because of the small size) these mice might not develop AF, limiting its usefulness, although they may exhibit electrophysiological changes in atrial conduction and excitability. We are currently exploring this possibility.

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Disclosures

None.

References

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Material and Methods

Linkage analysis, Mutation screen

Genomic DNA of family members was extracted from peripheral blood according to standard procedures. PCR amplification, labelling with Cy5dCTP (Amersham) and electrophoresis using automated laser fluorescence (ALF, Pharmacia Biotech) of these markers was performed according to standard methods. TBX5 was PCR amplified using TBX5 specific primers (table 2), located in flanking intronic sequences. PCR products were analyzed by direct sequencing, using the BigdyeTerminator v3.1 Kit on an ABI 3700 Genetic Analyzer (Applied Biosystems). Mutation analysis of NKX2-5, CX40 and TBX3 was also performed in family members III-7, III-9, IV-1 and IV-3. Primers were located in flanking intronic sequences (see table 2).

Plasmid constructs

The full-length human FLAG-TBX5 and full-length mouse FLAG-Nkx2-5 expression plasmids and the pGL3-Nppa-luciferase and pGL3-CX40-luciferase reporter plasmids have been described previously\(^1\),\(^2\). The missense mutations p.Gly125Arg, p.Gly125Glu (control) and p.Gly80Arg\(^3\) were introduced into the wild-type FLAG-TBX5 construct by PCR-based site-directed mutagenesis (QuickChange II Site-Directed Mutagenesis Kit, Stratagene) and verified by sequencing.

Reporter gene assays

Neonatal rat heart myocytes, immortalized with a temperature-sensitive SV40 T antigen (H10 cells\(^4\)) and the embryonic heart tissue cell line(H9C2 cells\(^5\)) were cultured in a 12-well plate at 33°C under standard conditions for 48h after transfection. One day before transfection, cells were plated at a density of 2×10\(^5\) cells/well. Escort V (SIGMA) was used for transfection of H10 cells according to the manufacturer’s instructions. Cells were transfected,
per well, with 700ng of luciferase reporter plasmids and 100ng of each expression plasmid. The total amount of DNA used for transfection was kept constant at 700ng by addition of empty pcDNA3 vector. In every experiment 1-2.5ng of Renilla luciferase plasmid, phRL vector (Promega) was co-transfected as an internal control. Transfected cells were washed once the following day with medium before being refreshed with new medium. 48 hours after transfection, cells were lysed and luciferase activity was measured with a luminometer (Glomax 20/20, Promega) by using the Renilla reporter assay system (Promega).

**Electromobility shift assays and Western blot analyses**

Electromobility shift assays (EMSAs) are used to detect the interaction of DNA binding proteins with their DNA recognition sequences. Purified proteins or crude cell extracts are incubated with a radiolabelled DNA probe. The complexes are separated from the free probe by migration through a nondenaturing polyacrylamide gel, with the complexes migrating more slowly. Nuclear extracts were prepared from HEK cells cultured under standard conditions for 48h after transfection, in 10cm dishes (1×10⁶ cells/dish) and transfected with 40μg of expression constructs. EMSA was performed as described previously. Oligonucleotide used was: TN (corresponding to the TBE-NKE site at position −250 in the Nppa promoter, 5′-TCTGCTCTTCACACCTTTGAAGGGGGGTCTCTTTG). Western blot analysis of nuclear extracts was performed according to standard procedures. Primary antibody used was anti-FLAG rabbit IgG (1:700; ABR) and the secondary antibody was anti-rabbit IgG-HRP (1:5000; Amersham). The complexes were visualized using the ECL detection kit (Amersham).

**In vitro Yeast two-hybrid X-gal assay**

To obtain full-length TBX5 cDNA, we screened a gt11 human fetal heart cDNA library (Clontech). We generated the p.G125R TBX5 mutant by PCR and subcloned these into a
pcDNA3 plasmid (Invitrogen). Bait (pcDNA3.1-mouse NKX2-5 carrying the GAL4 DBD fusion construct) and prey (pcDNA3.1-human TBX5wt/Gly125Arg carrying the GAL4 AD fusion construct) were co-transformed into Saccharomyces cerevisiae AH109 (Clontech), and then the ability to grow on minimal media was examined. The strength of protein interaction was quantitated using a β-galactosidase filter assay. To measure the interaction quantitatively, β-galactosidase liquid assays were carried out using four independent colonies. Chlorophenol red-β-D-galactopyranoside (CPRG) was used as substrate.

**In vitro binding assays**

Basically, equivalent amounts of MBP/MBP-fusion protein from cleared BL21 DE3 cell lysates, were loaded onto PBS-triton equilibrated with 300μl amylose resin (New England Biolabs) columns. The MBP/MBP-fusion loaded resin was subsequently washed with 1ml PBS-triton. A total volume of 1ml PBS-triton containing approximately 100μg of purified GST/GST-fusion was then passed over the MBP/MBP-fusion loaded amylose resin. The column was subsequently washed with 4ml PBS-triton before elution of the MBP/MBP-fusion with 0.5ml PBS-triton containing 20mM maltose. 10μl of eluate was used for SDS-PAGE and Western blot analysis.

**Immunocytochemistry**

COS7 cells were seeded on cover slips (18mm, Fisher Emergo) in a standard 12-well plate at a density of 1×10^5 cells and cultured under standard conditions for 24-48h after transfection. Cells were then fixed in 2% paraformaldehyde, washed in PBS, permeabilized in 0.3% Triton X-100, washed in PBS and incubated with the primary antibody anti-FLAG rabbit IgG (1:1000; ABR) in PBS/1% BSA at 4°C overnight. The next day the secondary antibody, Alexa488-conjugated goat anti-rabbit IgG (Molecular Probes), was added and incubated at room temperature for 1h. Slides were mounted using anti-fading vectashield with DAPI and
cells were viewed under a Zeiss Axiophot fluorescence microscope equipped with a Leica DFC 320 camera.

**RNA isolation, cDNA synthesis and qPCR**

H10 cells were plated at a density of $4 \times 10^5$ cells per well on a 6-well plate. Escort V (SIGMA) was used for transfection according to the manufacturer’s instructions. Cells were transfected, per well, with 1.25 µg of each expression plasmid in the presence of DMEM 1x (GIBCO). Transfected cells were washed the following day once with medium before refreshing them. 48h after transfection, total RNA was prepared using the Nucleospin RNA II kit (Clontech) following the manufacturer's recommendations. Isolated RNA was reverse transcribed and amplified using oligo(dT) primer and the Superscript II RT-PCR kit (Invitrogen) following the manufacturer's protocol.

For qPCR, briefly, a master mix was aliquoted to a 384-well plate (ROCHE). Each well contained 0.3-5.0 µl (depending on the gene of interest) cDNA, 5 µl of 2x SybrGreen I mix (ROCHE), 0.5 µl of primer mix (10 pmol/µl of each primer, see table 2) and supplemented with H2O up to 10 µl. Real-time PCR protocol was as follows: preincubation 95°C 5 min, followed by 45 cycles of: 95°C 10 sec, 58 or 60°C (depending on the gene of interest) for 20 sec and 72°C for 20 sec. Readings were carried out on an ROCHE LC 480 Lightcycler System. The average/mean PCR efficiency was subsequently used for calculating the average mRNA copy number. HPRT expression levels were used to correct for variations in RNA input. qPCR levels were normalized on vector only.

**Statistical analysis**

Results are expressed as mean±SD or SEM as indicated in the legends. To remove variations from multi-session experiments due to day-to-day differences in transfection efficiency and expression levels, a factor correction (calculated as previously described⁶) was applied. The
corrected data set was used for statistical analysis. Comparisons amongst two groups was performed using Student's t tests. $P<0.05$ was considered statistically significance.

Results

TBX3 and CX40 genetic screen

Polymorphisms in the promoter region of $CX40$, an atrial specific gap junction protein, are implicated in atrial fibrillation. Haplotype analysis did not reveal linkage between the SNPs in the $CX40$ promoter region and atrial fibrillation (data not shown), nor were mutations present in the coding region of $CX40$. As overexpression of $TBX3$ has recently been shown to lead to ectopic pacemaker activity (see discussion), and because it lies in very close proximity to $TBX5$ and is present in the linking chromosomal region, it was also screened. No mutations were identified.

TBX5, NKX2-5 Interaction

Co-transfection of Nkx2-5 expression plasmids allowed us to investigate the interaction between TBX5 mutants and Nkx2-5 and the result on DNA binding. With the addition of Nkx2-5, complexes of TBX5-Nkx2-5 can be seen in the wild-type as well as the G125R and G125E mutants (figure 5A), but these complexes are absent in the presence of the G80R mutant. Moreover, using an in vitro yeast two-hybrid X-gal assay, we determined that there was no difference in the strength of the interaction of wild-type TBX5 or G125R TBX5 with Nkx2-5 (figure 6A), indicating that the mutation does not disturb protein-protein interaction. This was further confirmed by use of a pull down assay, as both wild-type and G125R TBX5 protein can be pulled down using Nkx2-5 protein (figure 6B), indicating that the TBX5 G125R mutant binds DNA and associates with Nkx2-5 in a manner comparable to wild-type TBX5 protein.
TBX5 G125R also stimulates gene expression in H9C2 cells

we performed similar experiments in a second cell line derived from embryonic heart tissue (H9C2 cells). Correspondingly to the response of G125R TBX5 in H10 cells, transcripts of both Nppa and Cx40 were significantly up-regulated in H9C2 cells transfected with G125R mutant protein in comparison to wild-type TBX5 and vector control (supplemental figure 1). Although Tbx3 levels showed a comparable trend, this was not statistically significant between wildtype and mutant TBX5, but Tbx3 was significantly up-regulated for both groups in comparison to vector only. Unfortunately, endogenous levels of Kcnj2 were not detectable by qPCR in these cells.

Discussion
TBX1 vs TBX5 gain-of-function phenotypical discussion

In contrast, to the a-typical HOS phenotype caused by the TBX5 gain-of-function mutation reported in this study, the phenotype of the gain-of-function TBX1 mutation patients was the same as those with loss-of-function or deletions. This was in line with the fact that mice that over- or under-express Tbx1 have identical phenotypes, supporting the idea that both loss-of-function and gain-of-function mutations lead to the same phenotype for TBX1 mutations. In contrast, the gain-of-function TBX5 mutant reported here results in a phenotype distinctly different from loss-of-function TBX5 mutations, indicating a difference in dosage sensitivity in the heart and limbs for TBX5. Although Tbx5 overexpression has been studied in mouse models with a cardiac-specific promoter, the level of over-expression is relatively high, i.e. much higher than what would be expected in case of a gain of function mutation that we describe. The general result is a thinned and hypoproliferative ventricular myocardium, which is roughly reminiscent of what is published about Tbx5 knockout mice, but less dramatic. However, unpublished data from our lab, using a similar overexpression model,
suggest that heart development is arrested and the chambers fail to form, but, unlike the Tbx5 knock-out, activation of $Nppa$ and $Cx40$ is seen. Taken together, even high levels of over-expression of this very dose-sensitive transcription factor leads to a response distinct from deficiency.

**Online Supplemental Legends**

**Online Figure 1.** Quantitative expression of $Nppa$, $Cx40$, and $Tbx3$ upon transfection of H9C2 cells with vector only, wild-type Tbx5 or G125R Tbx5. Values are normalized on vector only construct, expressed as average ± SEM, representing three independent transfections, each done in triplicate, n=9. * indicates statistically significant differences between the wild-type or G125R Tbx5 and vector only, and # between G125R Tbx5 and wild-type Tbx5 (Student's t tests, P<0.05).

**Online Table 1.** Summary of the primer pairs used for amplification of human Tbx5 and rat qPCR primers, included are amplicon size and PCR touch down protocol annealing temperatures.

**Online Table 2.** The names, location and heterozygosity of microsatellites and genes used in this study. Basepairs are according to build 36.2 of the human genome, centiMorgans according to the Marshfield genetic map.

**References**


### Online Table 1. Primer pairs for amplification of human TBX5 and rat qPCR primers

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#### Gene

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*primers work on rat cDNA*

### Online Table 2. Location of microsatellites and genes

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*primers work on rat cDNA*