



# **NKX2-5: An Update on this Hypermutable Homeodomain Protein and its Role in Human Congenital Heart Disease (CHD)**

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**NKX2-5: An Update on this Hypermutable Homeodomain Protein and its Role in Human Congenital Heart Disease (CHD)**

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Key Words:	heart development, congenital heart disease, cardiac malformations, transcription factors, NKX2-5, mutations

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**NKX2-5: An Update on this Hypermutable Homeodomain Protein  
and its Role in Human Congenital Heart Disease (CHD)**

Stella Marie Reamon-Buettner and Juergen Borlak\*

Molecular Medicine and Medical Biotechnology, Fraunhofer Institute of Toxicology and  
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## ABSTRACT

Congenital heart disease (CHD) is among the most prevalent and fatal of all birth defects.

Deciphering its causes, however, is complicated as many patients affected by CHD have no family history of the disease. There is also widespread heterogeneity of cardiac malformations

among affected individuals. Nonetheless, there have been tremendous efforts towards a better understanding of the molecular and cellular events leading to CHD. Notably, certain cardiac-

specific transcription factors have been implicated in mammalian heart development and

disruption of their activity has been demonstrated in CHD. The homeodomain transcription

factor NKX2-5 is an important member of this group. Indeed, more than 40 heterozygous

NKX2-5 germline mutations have been observed in individuals with CHD and these are

spread along the coding region, with many shown to impact protein function. Thus, NKX2-5

appears to be hypermutable (?) highly mutable, yet the overall detection frequency in sporadic

CHD is about 2% and NKX2-5 mutations are one-time detections with single-positives or

private to families. Furthermore, there is lack of genotype-phenotype correlation, in which the

same cardiac malformations have been exhibited in different NKX2-5 mutations or the same

NKX2-5 mutation associated with diverse malformations. Here, we summarize published

NKX2-5 germline mutations and explore different avenues in disease pathogenesis to support

the notion of a multifactorial cause of CHD where possibly several genes and associated

pathways are involved.

**Keywords:** heart development, congenital heart disease, cardiac malformations, transcription factors, NKX2-5, mutations

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Dr. Mark Paalman  
The Managing Editor  
Human Mutation

Dear Dr. Paalman:

Many thanks for the encouraging news on our manuscript " NKX2-5: An Update on this Hypermutable Homeodomain Protein and its Role in Human Congenital Heart Disease (CHD)" Human Mutation Paper No. humu-2010-0256. Attached is the revised manuscript, as well as the response to the managing editor's comments.

We look forward to seeing our work published in Human Mutation.

Sincerely yours,  
JUERGEN BORLAK

**RESPONSE TO MANAGING EDITOR'S COMMENTS**

1) The mutation nomenclature appears to follow the format indicated in the Author Instructions (see the website <http://www.hgvs.org/mutnomen/> and also the nomenclature checklist at <http://www.hgvs.org/mutnomen/checklist.html>). Things to watch for:

a-Mention the GenBank reference sequence and version number for the gene(s) studied (i.e., include the decimal point following the accession number in the sequence record) in the Materials and Methods and as a footnote to the relevant tables and figure.

b-Clearly indicate in the Methods text and tables that the DNA mutation numbering system you use is based on cDNA sequence. Explanatory text is required in legends and text, for example:  
"Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines ([www.hgvs.org/mutnomen](http://www.hgvs.org/mutnomen)). The initiation codon is codon 1."

**RESPONSE :**

*The footnote below is incorporated in Table 1, Supp.Tables S2 and S3.*

*"Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence NM\_0004387.3, according to journal guidelines ([www.hgvs.org/mutnomen](http://www.hgvs.org/mutnomen)). The initiation codon is codon 1. "*

c-Authors are advised to check sequence variant descriptions using the Mutalyzer program (<http://www.LOVD.nl/mutalyzer/>) - using batch mode, all variants for a gene can be analyzed at once. See the article Wildeman et al. Hum Mutat 29, 6-13, 2008 <http://www3.interscience.wiley.com/cgi-bin/fulltext/116842325/PDFSTART>

**RESPONSE :**

*The batch mode of Mutalyzer was used to check the list of NKX2-5 mutations.*

d-All authors should verify that the mutations reported (especially novel ones) have been or will be submitted to an existing locus-specific database for the genes involved. Visit the HGVS-LSDB list to search for databases: <http://www.hgvs.org/dblist/glsdb.html>

**RESPONSE :**

*Review paper, and no novel mutations being reported.*

2) On resubmission:

a-Please double check the author names and affiliations carefully. These are often a source of typographical errors.

**RESPONSE :**

*Authors' names and affiliations checked.*

b-Title Page, Abstract (180-200 words max), Key Words, Main Text, References, and Figure Legends should be combined into one file for the manuscript and submitted as a \*.doc file.

**RESPONSE :**

*The submitted\*.doc. file (NKX2-5 humu10-0256\_text.doc) contains the specified components; Abstract is 200 words.*

c-Figures for main article must be submitted as separate files with high resolution (at least 200 dpi) as \*.tif or \*.eps format only.  
--For color figures in print: submit two files for each color figure: one in CMYK color space and one in RGB color space (with the true color you wish to have published). If you cannot provide CMYK color e-files for the print version, please let me know.

**RESPONSE :**

*Figure3 is 200 dpi, the rest are 300 dpi; both CMYK and RGB files for Figure 3 and Figure 4.*

d-Tables must be submitted individually as separate \*.doc files (with their titles and legends included). Please use the MS Word table format if possible. Excel (\*.xls) files should not be submitted.  
--Do not use custom paper sizes - only use Letter or A4.

**RESPONSE :**

*Table 1. Functional Consequences of Identified NKX2-5 Mutations in CHD: Functional Assays or Bioinformatic Analysis is contained in \*.doc.file NKX2-5 humu10-0256\_Table 1.doc.*

*Table 2. Detection Frequency of NKX2-5 Germline Mutations in Congenital Heart Disease is contained in \*.doc.file NKX2-5 humu10-0256\_Table 2.doc.*

e-Any Supporting Tables or Figures should be named and cited from the text as follows: 'Supp. Table S1' and 'Supp. Figure S1' (see below).  
--If possible, do not use custom paper sizes - Letter or A4 are preferred.

**RESPONSE :**

*As instructed Supplementary Materials are named in the text accordingly.*

f-Supporting Figures and Tables should be prepared in a single MS Word \*.doc file labeled 'Supp\_Mat', with Figures preceding Tables. Each table/figure should be accompanied with its legend.

**RESPONSE :**

*The single file NKX2-5 humu10-0256\_Supp\_Mat.doc contains the following:*  
**Supp. Figure S1.** Geographical distribution of germline NKX2-5 mutations in CHD along the coding sequence of the gene; **Supp. Table S1.** Studies on NKX2-5 Mutations Associated with Congenital Heart Disease (CHD); **Supp. Table S2.** Summary of Published Germline NKX2-5 Mutations in Patients with Congenital Heart Defects; **Supp. Table S3.** Functional Significance of Published Germline NKX2-5 Mutations in Patients with Congenital Heart Disease

3) Please check to see that the references follow the journal's standard format and are cited properly. All authors must be listed, for example. See our online Author Instructions <http://www3.interscience.wiley.com/cgi-bin/jabout/38515/ForAuthors.html>

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*References are according to journal's format and updated to the latest status of publication.*  
4) Figures 3 and 4 require color in the print version in order to be fully understood. We will offer you one free color print page. I think that both figures should fit on one color page. If any additional color is needed or desired, it would cost \$500 per page.

**RESPONSE :**

*We are amenable to this suggestion, and many thanks for your offer of one free color print page.*

5) Human Mutation can accommodate researchers funded by agencies requiring open access publication. More information on Wiley-Blackwell's policy is available at: <http://www.interscience.wiley.com/openaccess>

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**RESPONSE :**

*We proofread the revised manuscript carefully, and no substantial changes carried out except for some minor style editing (e.g. abstract), and updating of citations and references (e.g. Rauch et al., 2010, De Luca et. al 2010).*

b-It is essential that you submit a copyright transfer agreement (CTA) upon submission of your revised manuscript. This will avoid delay in publication of your article upon acceptance. If possible, the CTA must be signed by the corresponding author and should be signed by all contributing authors if practical. All authors must be made aware of the CTA and the rights it conveys to them.  
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and must be filled out completely, including the article title and manuscript number. Please fax it to this number: (201) 748-6091.

**RESPONSE :**

*A signed CTA has been prepared and will be faxed to the above indicated number, as soon as the revised manuscript has been uploaded.*

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Contract grant sponsors: Ministry of Science and Culture, Lower Saxony, Germany; Contract  
grant number: 25A.5-76251-99-3/00

## ABSTRACT

Congenital heart disease (CHD) is among the most prevalent and fatal of all birth defects. Deciphering its causes, however, is complicated as many patients affected by CHD have no family history of the disease. There is also widespread heterogeneity of cardiac malformations within affected individuals. Nonetheless, there have been tremendous efforts towards a better understanding of the molecular and cellular events leading to CHD. Notably, certain cardiac-specific transcription factors have been implicated in mammalian heart development and disruption of their activity in CHD; foremost is the homeodomain transcription factor *NKX2-5*. Indeed, more than 40 heterozygous *NKX2-5* germline mutations are spread along the coding region, many impact protein function. Thus, *NKX2-5* appears to be hypermutable, yet the overall detection frequency in sporadic CHD is about 2% and *NKX2-5* mutations are one-time detections with single-positives or private to families. Furthermore, there is lack of genotype-phenotype correlation, in which the same cardiac malformations have been exhibited in different *NKX2-5* mutations or the same *NKX2-5* mutation associated with diverse malformations. Here, we summarize published *NKX2-5* germline mutations and explore different avenues in disease pathogenesis to support the notion of a multifactorial cause of CHD where possibly several genes and associated pathways are involved.

**Keywords:** heart development, congenital heart disease, cardiac malformations, transcription factors, *NKX2-5*, mutations

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**Introduction**

Cardiac malformations in congenital heart disease (CHD) result from errors during heart development, a process which is very complex and completed in a relatively short period of time. Essentially within eight weeks of embryogenesis, the human heart undergoes several stages of development involving cardiac crescent formation, linear heart tube formation, cardiac looping, chamber formation and maturation, septation and valve formation. At the molecular and cellular levels, heart development entails precise orchestration of genetic events, as well as the interplay of master transcriptional regulators, signaling pathways and chromatin remodeling rendering the understanding of CHD a challenging task. But great efforts are underway towards the unravelling of mechanisms leading to CHD, as conveyed in recent reviews (Bruneau, 2008;Nemer, 2008;Joziassse et al., 2008;Bajolle et al., 2009;Leong et al., 2009;Combs and Yutzey, 2009). Notably, the pivotal role of certain transcription factors in heart development has been demonstrated, and gene targeting of cardiac-specific transcription factor genes in animal models especially in mice has provided valuable insights into heart anomalies (Nemer, 2008).

Over the years, family-based studies identified mutations in several genes controlling heart development, especially transcription factor genes (see reviews, Clark et al., 2006; Bruneau, 2008;Benson, 2010). Among these transcription factor genes with mutations associated with human CHD include *NKX2-5* (MIM# [600584](#)), *GATA4* (MIM# [600576](#)) and *TBX5* (MIM# [601620](#)) and these mutations are often observed in patients with atrial, ventricular and atrioventricular septation defects. Although these family studies have been useful in uncovering genes linked to CHD, population-based studies show so far, rare detection of mutations in unrelated patients with CHD (e.g. Rajagopal et al., 2007;Posch et al., 2008); therefore, pointing to a multifactorial cause of disease. Furthermore, mutations exhibit variable expression, in which different cardiac malformations occur even from the same mutation, suggesting modifying factors and epigenetic effects contributing to disease

manifestation. Indeed, a recent study which examined the effect of genetic background variation on the incidence of specific heart defects in heterozygous knock-out *Nkx2-5*<sup>+/-</sup> mice and in crosses to two different strains revealed that such genetic modifiers to play an important role in buffering cardiac developmental pathways against a major perturbation, while directing the manifestation of disease in a few (Winston et al., 2010).

In this paper, we will review efforts in identifying disease-associated mutations in the transcription factor gene *NKX2-5*, and the clinical significance of such mutations in the pathogenesis of CHD. We summarize published germline *NKX2-5* mutations and corresponding CHD phenotypes, their detection frequency, geographical location, as well as functional consequence as determined by assays or bioinformatic prediction. Finally, we explore different avenues in CHD pathogenesis, some of which challenge the well-accepted view of a single mutation as a direct cause of disease.

## ***NKX2-5* and its Role in Cardiac Development and Disease**

*Nkx2-5* belongs to the NK-2 family of homeodomain-containing transcription factors, which are conserved in many organisms from flies to humans (Bartlett et al., 2010). Through the 60-amino-acid homeodomain (HD), *Nkx2-5* interacts with DNA through a helix-turn-helix DNA-binding motif of three alpha helices, with helix 3 providing binding specificity. Important for *Nkx2-5* function are also other conserved regions namely, the transactivation (TN) domain, and the NK2-specific domain (NK2-SD). *Nkx2-5* is a transcriptional regulator during early embryonic heart development, and its absence in mice models results in embryonic lethality and arrested heart development at the looping stage (see reviews, Harvey et al., 2002; Akazawa and Komuro, 2005; Bartlett et al., 2010). *Nkx2-5* is also essential for the formation, maturation and maintenance of the conduction system (Pashmforoush et al., 2004; Jay et al., 2004). *Nkx2-5* regulates the number of cells in the cardiac conduction system in a dose-dependent manner (Jay et al., 2004). While null *Nkx2-5* mice mutants appear to lack

the primordium of the atrioventricular node, heterozygotes have half the number of cells. Anatomic hypoplasia of the conduction system may explain some of the defects in Nkx2-5 loss-of-function mutants. Notably, Nkx2-5 is expressed in both the first heart field (FHF) and the second heart field (SHF), the two distinct sources of cardiac progenitor cells contributing to different parts of the heart (Buckingham et al., 2005). This expression in progenitor cells indicates a higher role for Nkx2-5 in the hierarchy of transcriptional regulators of heart development.

Recent studies show further roles for Nkx2-5 in heart development and perinatal hearts. In mice, Nkx2-5 repression of Bmp2/Smad1 signaling regulates SHF proliferation and outflow tract (OFT) morphology (Prall et al., 2007). In the cardiac fields of Nkx2-5 mutants, genes controlling cardiac specification and maintenance of the progenitor state were upregulated, leading initially to progenitor overspecification, but subsequently to failed SHF proliferation and outflow tract (OFT) truncation. Nkx2-5-null embryos exhibited decrease in SHF proliferation, resulting in a smaller outflow tract and right ventricle. In vivo Cre-lox P-based lineage tracing in mice showed that Nkx2-5-expressing cardiac progenitors contributed to the proepicardium (Zhou et al., 2008). Indeed, Nkx2-5 knockout resulted in abnormal proepicardial development, and decreased expression of the transcription factor Wilm's Tumor 1 (Wt1), which in the heart its expression is confined to the proepicardium and epicardium. Furthermore, inhibiting the function of Nkx2-5 homologs (nkx2.5 and nkx2.7) in zebrafish showed their involvement in the regulation of heart tube extension as well as ventricular and atrial cell number (Targoff et al., 2008). The nkx-deficient heart tube failed to elongate normally: its ventricular portion was atypically short and wide, and its atrial portion was disorganized and sprawling. This atrial phenotype was associated with a surplus of atrial cardiomyocytes; ventricular cell number decreased in nkx-deficient embryos later in development, when cardiac chambers are emerging.

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Lastly, it has also been demonstrated using tamoxifen-inducible *Nkx2-5* knockout mice that *Nkx2-5* function is critical not only during cardiac development, but also in perinatal hearts, by regulating expression of several important gene products involved in conduction and contraction (Briggs et al., 2008). The perinatal loss of *Nkx2-5* resulted in rapid conduction and contraction defects within 4 days after the deletion of *Nkx2-5* alleles. Furthermore, using tamoxifen-inducible Cre transgene (Cre-ER), floxed-*Nkx2-5* alleles were deleted beginning at 2 weeks of age (Takeda et al., 2009). The loss of *Nkx2-5* beginning at 2 weeks of age also resulted in conduction and contraction defects, but disease progression was slower than in perinatal loss of *Nkx2-5*.

## **An Update of *NKX2-5* Germline Mutations**

The human *NKX2-5* gene maps to chromosome 5q34 and consists of two exons that encode a 324-amino-acid protein. Since the first report implicating *NKX2-5* mutations in human CHD (Schott et al., 1998), the coding region of *NKX2-5* has been consistently searched for additional disease-associated sequence alterations. There are at least 28 studies where germline *NKX2-5* mutations have been analyzed or identified in CHD (Supp. Table S1). *NKX2-5* mutations were identified in different countries, but majority were found in studies conducted in the United States and affect different regions of the gene (Supp. Figure S1, Supp. Table S2). Some mutations may appear though to cluster geographically, such as the three deletions from Japan, and the two substitutions from Germany (Supp. Figure S1).

At least 41 different *NKX2-5* germline mutations, most of which lead to amino acid change (nonsynonymous mutations) have been reported (Figure 1, Supp. Table S2). Of these 41 mutations, 33 are single nucleotide substitutions, 6 deletions and 2 insertions. The *NKX2-5* mutations are spread along the gene, and except for one at a splice site, all are located in the coding region. Mutations affect conserved regions, where 2 are in the TN domain, 14 in the homeodomain (HD), and 2 are in the NK2-SD domain. Only 5 of 41 (12%) have been

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reported more than once in unrelated individuals, with c.73C>T (p.R25C) being frequently detected. However, p.R25C was also found in unaffected relatives and controls; hence, its pathogenicity remains unclear (Goldmuntz et al., 2001; Akcaboy et al., 2008; Esposito et al., 2009; Gioli-Pereira et al., 2010).

Furthermore, *NKX2-5* mutations have been identified in both familial and sporadic cases of CHD, in which 25 of 41 are familial, 14 are sporadic, and 2 are sporadic/familial cases (Figure 1, Supp. Table S2). Patients are heterozygous for the *NKX2-5* mutations, and frequent CHD malformations in patients with *NKX2-5* mutations are atrial septal defect (ASD) with or without atrioventricular block (AV) block. Specifically, 29 of 41 mutations were identified in patients with ASD II, 26 in AV block, and 25 in combined ASD II and AV block. Other CHD phenotypes were also found including Tetralogy of Fallot (TOF) in 7 mutations, as well as ventricular septal defect (VSD) in 9 mutations. A quick characterization of the 41 *NKX2-5* mutations identified in patients with CHD is depicted in Figure 2.

**Functional Significance of *NKX2-5* Mutations**

Of the 40 mutations identified in the coding region of *NKX2-5*, 25 are missense, 7 nonsense, 7 frameshift and 1 leading to an amino acid deletion. Most of these would affect protein function as predicted by bioinformatic methods or by functional assays (Table 1). So far, only 4 mutations have been predicted to have no deleterious effects on protein function. Specifically, 17 mutations would lead to a truncated protein in which 11 affect both the homeodomain and NK2-SD. Notably, certain mutations that are not outright nonsense in the homeodomain would result in the breakage of the secondary structure (Figure 3) and /or affect highly conserved residues (Figure 4). Moreover, 16 mutations exhibited impaired function in all or any of the assays particularly DNA binding, transactivation activity, protein-protein interaction and nuclear localization (Table 1, Supp. Table S3). Essentially, *NKX2-5* mutations

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3 affecting the homeodomain resulted in reduced or loss of DNA binding, transactivation  
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5 activities, and protein-protein interactions particularly with GATA4 and TBX5 (Kasahara et  
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7 al., 2000;Kasahara and Benson, 2004;Zhu et al., 2000;Monzen et al., 2002;Inga et al.,  
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9 2005;Esposito et al., 2009).

## 16 **Lack of Genotype-Phenotype Correlation**

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18 Despite *NKX2-5* hypermutability and the pathogenic potentials of *NKX2-5* germline  
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20 mutations, there is lack of genotype-phenotype correlation of *NKX2-5* mutations in CHD (see  
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22 also review, Benson, 2010). Notably, the detection frequency of *NKX2-5* mutations in  
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24 sporadic cases of CHD is about 2% and in several studies none was found (Table 2). Most of  
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26 the identified *NKX2-5* mutations are unique and diverse cardiac malformations have been  
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28 associated with *NKX2-5* mutations (Figure 1). Furthermore, same CHD phenotypes have been  
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30 exhibited by patients with different *NKX2-5* mutations or same *NKX2-5* mutation gave  
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32 different CHD phenotypes in regards to severity even within families (see for instance, Figure  
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34 1 and Supp. Table S2). Indeed, more than half of identified *NKX2-5* mutations are familial  
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36 cases, and families have their private mutations.

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38 Even as new studies on *NKX2-5* mutations keep coming in, the picture remains  
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40 basically unchanged. After genetic analysis of *NKX2-5* mutations in 230 patients with TOF, a  
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42 detection frequency of 0.9% was obtained (Rauch et al., 2010). Two patients with non-  
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44 syndromic TOF harbored an unknown variant each (i.e. p.C270Y and p.V315L), both located  
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46 at the carboxy terminal and which did not show an effect on the transcriptional reporter assay  
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48 used. The most frequent variant p.R25C was also detected in two patients. In another study,  
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50 no *NKX2-5* mutations were found in 96 patients with idiopathic atrial fibrillation, a condition  
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52 of arrhythmia but no visible structural defects (Boldt et al., 2009). After sequencing *NKX2-5*  
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54 in 34 patients with stroke and patent foramen ovale (PFO), no pathogenic mutations were  
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56 obtained (Belvis et al., 2009). All the while, *NKX2-5* mutations are not exclusive to CHD.  
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Indeed, *NKX2-5* mutations (p.A119S, p.R25C, p.R161P) were found in thyroid dysgenesis, in patients with or without cardiac anomalies (Dentice et al., 2006), suggesting a broader role for *NKX2-5* in organogenesis and pathophysiology.

There are several factors that may contribute to this lack of genotype-phenotype correlation. For one, genetic background can modify the phenotypic expression of single gene mutations in mice and humans (Rajagopal et al., 2007). Indeed, modifier genes have been shown to affect *NKX2-5* mutations in the pathogenesis of CHD (Kaiser et al., 2005; Winston et al., 2010). There is also the possible influence of synonymous (silent) mutations and dbSNPs affecting protein expression and function (Sauna et al., 2007). Such silent variants and dbSNPs eventually serve as risk factors for CHD. Furthermore, buffering that can result from compensation by a normally functioning second allele or a duplicated gene or a pathway that maintains residual function may also explain reduced penetrance of mutations in sporadic cases of CHD (see review, Bentham and Bhattacharya, 2008). These authors proposed that the genetic architecture of sporadic CHD likely includes accumulation of rare nonsynonymous variants in cardiac developmental genes leading to mutational loading of cardiac developmental networks, copy number variation in cardiac developmental genes, and common variants that may not be obviously linked to cardiac development but may alter genetic buffering pathways. Moreover, as one review commented (Weismann and Gelb, 2007), germline mutations may not be compatible with survival of the developing embryo because many of the genes that control cardiac development also play critical roles in the development of other organ systems and in any case, would not likely cause CHD only. Summing up, a single germline *NKX2-5* mutation as the direct cause of disease or a simple Mendelian inheritance alone may not sufficiently explain disease pathogenesis. Needless to say, none of the patients studied so far are homozygous for *NKX2-5* variant alleles.

## Molecular Basis of CHD Pathogenesis

The landscape for CHD pathogenesis is complex. First of all, majority of CHD cases are sporadic in which patients have no family history of the disease. Indeed, the contribution of family history of any CHD among first-degree relatives to the overall prevalence of CHD is small, approximately 2-4% (Oyen et al., 2009). There is clinical or phenotype heterogeneity within affected individuals, as well as within and between families. Disease association is difficult owing to lack of clear genotype-phenotype correlation of mutations. For instance, patients harboring the same *NKX2-5* mutation have different malformations; or mutations in different genes give the same disease phenotype. Specifically, common cardiac malformations associated with *NKX2-5* mutations are AV block, ASD, and VSD. But the same structural anomalies are also observed in mutations in other transcription factor genes (see reviews, Clark et al., 2006; Nemer, 2008). ASD is associated with mutations involving *GATA4*, *TBX5* and *ZIC3*; AV block in *NKX2-5* and *TBX5*; or VSD in *GATA4* and *TBX5*. Thus, CHD is considered as a multifactorial disease, likely resulting from gene-environment interactions which eventually determine disease penetrance and expressivity. Among the environmental risk factors for CHD are chemicals, drugs, infections, and maternal diseases (reviewed in Jenkins et al., 2007).

At the molecular and cellular levels, heart development can practically go wrong in many directions leading to diverse cardiac malformations observed in CHD. Notably, heart development requires spatial and timely interactions of different transcription factors to regulate downstream targets, and breakdown of transcriptional interactions can lead to various CHD phenotypes (see reviews, Nemer, 2008; Bruneau, 2008). For example, *Nkx2-5* through the homeodomain interacts physically and synergistically with *Gata4* and *Tbx5*, such that abrogation of their interaction leads to CHD. Indeed, *NKX2-5* mutations identified in patients affecting the homeodomain have been demonstrated to result in reduced interaction with *Gata4* and *Tbx5* (Kasahara and Benson, 2004). Recently, it has been shown that *Nkx2-5* can

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directly interact with Mef2c, and the physical, functional, and genetic interactions between Nkx2-5 and Mef2c are necessary for ventricle formation (Vincentz et al., 2008). While individual mutants for both Nkx2-5 and Mef2c had identifiable ventricles, double mutants displayed ventricular hypoplasia, a more severe cardiac phenotype. Besides transcription factors, Nkx2-5 can also interact with a histone methyltransferase to regulate target genes. A recent study linked Nkx2-5 to Wolf-Hirschhorn syndrome (MIM #194190) where cardiac septal defects are among the anomalies, through an association with Whsc1, an H3K36me3-specific histone methyltransferase in mouse embryonic hearts (Nimura et al., 2009). The effects of *Whsc1* haploinsufficiency increased in heterozygous *Nkx2-5* mutant hearts, and reporter assays showed that Whsc1 negatively modulated the transcriptional activity of Nkx2-5.

A complex network of signaling molecules and their signal transduction pathways participates in the precise orchestration of the many morphogenetic events in heart development especially in the specification, proliferation and differentiation of cardiac progenitor cells (reviewed in Wagner and Siddiqui, 2007a;Wagner and Siddiqui, 2007b;Niessen and Karsan, 2008;Nemir and Pedrazzini, 2008;Rochais et al., 2009). Shown to regulate cardiogenesis include bone morphogenetic proteins (BMP), fibroblast growth factors (FGF), Wnt, Hedgehog, Nodal, retinoic acid, neuregulin, vascular endothelial growth factor (VEGF) and Notch signaling pathways. Errors in these signaling pathways can impair heart development. For instance, mutations in the Notch signaling pathway (McBride et al., 2008) or in the Nodal signaling pathway (Roessler, 2008) have been identified in patients with various cardiac anomalies.

Furthermore, there are now studies showing that defects in chromatin remodeling can lead to CHD. Chromatin remodeling allows transcriptional machinery access to gene regulatory elements and is essential in heart development. Silencing of *Smarca*d3, which encodes Baf60c, a subunit of the Brg1-associated-factor (BAF) chromatin remodeling

complex, caused in ES-cell-derived mouse embryos severe cardiac malformations including hypoplastic atrium, hypoplastic right ventricle, single ventricle, abnormal outflow tract and severely impaired trabecular formation (Lickert et al., 2004). Furthermore, Baf180, a specific subunit of the PBAF form of the BAF complex is required for cardiac and placental development (Wang et al., 2004). Ablation of Baf180 in mouse embryos resulted in severe hypoplastic ventricle, ventricular septal defect and trophoblast placental defects. Recently, it has been shown that myocardial trabeculation requires Brg1, a chromatin remodeling protein, (Stankunas et al., 2008a). Trabeculation defects occurred in the absence of endocardial Brg1 which resulted from the derepression of a secreted matrix metalloproteinase, ADAMTS1, which normally only increases in expression later in development to prevent excessive trabeculation.

Thus, there is justification for proposing the concept of one heart disease-several mechanisms-several genes to explain the heterogeneity of certain cardiac malformations, which is not by chance but by the heterogeneity of mechanisms (Bajolle et al., 2009). According to this concept, a certain malformation may originate from different embryology mechanisms, which in turn controlled by multiple genes. Given as example is common arterial trunk (CAT), which may result from a participation defect of progenitors from the second cardiac field and/or a migration defect of the neural crest cells and/or a rotation defect of the myocardium and/or a formation abnormality of the endocardial cushions. Further example is atrioventricular valve formation which is regulated by different genetic pathways (Joziassse et al., 2008). Disturbances in each subset of a particular pathway, i.e. ligand receptor, transcription factor or extracellular matrix proteins can all result in similar cardiac defects.

Recently, a study showed that interactions between alleles of three *Pbx* genes, which encode homeodomain transcription factors, are sufficient to determine the phenotypic presentation of CHD in mice (Stankunas et al., 2008b). A major role is served by *Pbx1*, whose

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3 inactivation results in persistent truncus arteriosus (PTA). Reduction or absence of *Pbx2* or  
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5 *Pbx3* leads to *Pbx1* haploinsufficiency and specific malformations that resemble Tetralogy of  
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7 Fallot, overriding aorta with ventricular septal defect, and bicuspid aortic valves. Disruption  
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9 of *Meis1*, which encodes a *Pbx* DNA-binding partner, results in cardiac anomalies that  
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11 resemble those caused by *Pbx* mutations. Altogether, these findings among others challenge  
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13 the well-accepted paradigm in autosomal dominant monogenic disease that 1 mutation in a  
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15 single gene as the direct cause of disease, and have major implication in clinical evaluation,  
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17 diagnosis, and management of families with a genetic cardiovascular disease (see for instance,  
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27 **Learning from the Leipzig Collection of Malformed Hearts**

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30 Model organisms especially mice provide insights into the molecular mechanisms  
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32 leading to CHD. But results can be species-specific and only a minority of mutations in these  
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34 genes have been related to CHD in humans (see Bruneau, 2008). Furthermore, genetic studies  
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36 on CHD are usually based on blood samples and such analysis may not reveal complete  
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38 genetic alterations leading to the disease. To gain insights into the molecular causes of CHD,  
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40 we conducted in the past genetic analysis of *NKX2-5* and other cardiac-specific transcription  
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42 factor genes, most recently on *HAND1* (MIM# [602406](#)), in a rare collection of  
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44 morphologically, well-characterized explanted, malformed hearts stored in formalin for many  
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46 years (Reamon Buettner and Borlak, 2004; Reamon-Buettner et al., 2004; 2008; 2009). This  
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48 research identified somatic mutations of cardiac-specific transcription factor genes as a  
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50 possible disease-causing mechanism as discussed below.

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53 Direct sequencing of different cardiac-specific transcription factor genes in 'affected'  
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55 tissues of malformed hearts revealed frequent disease-associated mutations which were  
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57 mainly absent in 'unaffected' tissues in the same patient's heart. This finding indicates somatic  
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59 mosaicism and somatic origin of mutations. Many patients displayed multiple deleterious  
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mutations not only in one but several transcription factor genes. While for some investigators in the field, it would be easiest to explain the findings from the Leipzig heart collection as artifacts on the basis of degraded DNA owing to many years of storage in formalin solution (Draus et al., 2009), the study on such a rare collection of malformed hearts may lead to important insights on heart development and CHD. Here are some of the pros and cons of the scientific discussion.

### ***1. Somatic mutations as a molecular rationale for CHD***

Somatic mutations may result from environmental insults and epigenetic instability to provide a molecular rationale for CHD. Although somatic mutations are well known in cancer, they can also occur in non-cancer diseases. For example, somatic mutations in the gene *TEK* encoding the endothelial cell tyrosine kinase receptor TIE2 were identified in lesions from patients with sporadic venous malformations (Limaye et al., 2009). The somatic mutations included a frequent p.L914F substitution and several double mutations in *cis*, all of which resulted in ligand-independent TIE2 hyperphosphorylation in vitro. Somatic mutations were also identified in *GJA5* gene encoding cardiac gap-junction protein connexin 40 in cardiac tissues of patients with atrial fibrillation (Gollob et al., 2006). Atrial fibrillation is the most common type of cardiac arrhythmia and a leading cause of cardiovascular morbidity, particularly stroke.

### ***2. Multiple mutations in complex cardiac malformations***

The presence of multiple mutations in patients with complex heart malformations may be a reflection of disease severity. Indeed, there are now studies to show that the presence of multiple disease-causing mutations in the same gene or in different genes can be an indication of disease severity (Kelly and Semsarian, 2009). In familial hypertrophic cardiomyopathy (FHC) which is characterized by genetic and clinical heterogeneity, patients with two FHC-causing mutations typically had more severe phenotype (i.e. earlier onset of disease, greater left ventricular hypertrophy and a higher incidence of sudden cardiac death events) compared

with carriers of single mutation (Tsoutsman et al., 2008a;Tsoutsman et al., 2008b). Furthermore, in familial transposition of great arteries (TGA) some patients harbored more than one mutation in laterality genes which presumably influenced cardiac phenotype (De Luca et al., 2010).

**3. A structural malformation may result from combined effects of deleterious mutations in several transcription factor genes**

Certain malformations may have resulted from combined effects of deleterious mutations in several transcription factor genes. In the hypoplastic hearts group, a frameshift mutation p.A126fs (HAND1) was frequently detected in hypoplastic ventricles (Reamon-Buettner et. al., 2008). But further analysis led to the detection of additional loss-of-function mutations affecting the 2nd zinc finger of GATA4 and homeodomain of NKX2-5 (unpublished results). For example, in the hypoplastic left ventricle of patient A82, we detected in HAND1 p.A126fs; in GATA4 p.R266X; and in NKX2-5 homozygous for p.L116P, p.T178M, p.K192T.

**4. Indications of transient hypermutability/ genetic instability in cardiac tissues of CHD patients**

Hypermutability varied among the different transcription factor genes, but mutations were non-random and closely-spaced. The multiple mutations observed in the affected tissues were possibly the result of various haplotypes. Specifically, depending on the stretch of DNA being analyzed, the closely-spaced sequence alterations could involve those in the coding or non-coding regions, and when in the coding region, mutations could be synonymous or non-synonymous. For example, in two patients affected by VSD, we found 11 non-random, closely-spaced mutations in NKX2-5 within 362-bp spanning intronic and coding sequences of exon 2. In this window, the separation of an NKX2-5 mutation to the next ranged from 3 bp to 121 bp, but majority was less than 30 bp. Moreover, after cloning of a PCR fragment 'heterozygous' for the multiple mutations in an analyzed transcription factor gene, more than



the two expected haplotypes or alleles were found. In some cases, all the variants occurred in *cis*. The occurrence of closely-spaced multiple mutations in human genes has been suggested to be possible signatures of transient hypermutability, a mutational mechanism with the potential to generate multiple synchronous mutations, resulting from a perturbation of one of the many transactions that maintain genetic stability (Chen et al., 2009). Specifically, transient hypermutability may result from (1) the deregulated expression of, or conformational change in, either a replicative DNA polymerase or another protein involved in the maintenance of replication fidelity; (2) the disruption of the balance of the nucleotide pool; or (3) the recruitment of error-prone DNA polymerases in DNA replication or repair (reviewed in Chen et al., 2009).

## Future Directions

Understanding the molecular process leading to CHD should provide important insights into the etiology and treatment of patients. This is a difficult task as CHD is complex entailing defects in multiple genes and different pathways. A good grasp of CHD would thus require a whole-genome functional approach, in which for a specific structural defect a combination of different genes and associated pathways involving transcriptional machinery, signal transduction, and chromatin remodeling will be simultaneously interrogated. But with high-throughput technologies in different 'omics' available, as well as accumulating evidence from animal models, this may be no longer a monumental undertaking. Eventually, there is the task ahead to translate discoveries to human CHD, although in future genetic studies corresponding molecular events may have been identified by then to explain genetic heterogeneity, reduced penetrance and variable expressivity owing to complex inheritance of the disease. Nevertheless, there is also a need to better understand environmental factors that possibly cause CHD, such as intrauterine stress shortly after conception owing to environmental contaminants. Indeed, there is evidence that in utero DNA damage from



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environmental pollution is associated with somatic gene mutation in newborns (Perera et al., 2002), and that there are indications for increased risk of CHD in polluted areas (Brook et al., 2004). Additional research areas should be addressed:

1. To gain deeper knowledge of how epigenetic regulation influence molecular mechanisms of heart development and its disruptions in CHD. Epigenetics describes heritable changes in gene expression that are not coded in the DNA sequence, but an interplay of DNA methylation, histone modifications and expression of non-coding RNAs, in the regulation of gene expression patterns. Epigenetic mechanisms are essential for development and differentiation, but they can become misdirected leading to birth malformations and various human diseases. Indeed, efforts are now underway in this direction, where for instance, deletion of miRNAs in mice during cardiogenesis has been shown to result in double-outlet right ventricle with a concurrent ventricular septal defect (Saxena and Tabin, 2010).

2. To decipher mechanisms of chromatin remodeling and DNA repair in heart development and CHD. Essentially, DNA is packaged, through histone and non-histone proteins, into a condensed structure termed chromatin. The nucleosome is the basic repeating unit of chromatin, consisting of about 147-bp of DNA wrapped approximately two times around a histone core comprising two copies of each of the histone proteins H2A, H2B, H3 and H4. Through chromatin remodeling, histone-DNA is disrupted resulting in a 'relaxed' chromatin, which then allows transcriptional machinery contact DNA to turn genes 'on' or 'off'. But more important, chromatin remodeling also facilitates DNA-damage-response proteins access DNA lesions and eventually initiate repair to maintain genome integrity (for reviews, Osley and Shen, 2006;Osley et al., 2007). Chromatin remodeling factors may involve those modifying enzymes that catalyze post-translational modifications of residues on both histone core and tails; or those enzymes that use ATP hydrolysis to disrupt histone-DNA leading to structurally

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3 altered nucleosomes, or to sliding or eviction of nucleosomes. Although there are now studies  
4 showing an essential role of chromatin remodeling with respect to transcriptional regulation in  
5 heart development and CHD (Lickert et al., 2004; Wang et al., 2004), none has been found so  
6 far on DNA repair. The role of DNA repair would be of utmost importance during early heart  
7 development, notably in embryonic cells and committed precursors of the mesoderm.  
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17 3. To identify molecular causes of hypermutability of genes and subsequent contribution to  
18 CHD, and explore whether activation-induced cytidine deaminase (AID) causes somatic  
19 hypermutability of cardiogenic genes in CHD. AID is a master regulator of diversification of  
20 antibody genes by initiating the processes of somatic hypermutation (SHM), gene conversion  
21 (IGC), and class-switch recombination (CSR) in B-lymphocytes (see reviews, Teng and  
22 Papavasiliou, 2007; Liu and Schatz, 2009). Essentially, AID induces somatic hypermutability  
23 of immunoglobulin (*Ig*) genes by cytosine deamination resulting in the generation of a uracil  
24 and thus converting a C:G basepair into a U:G mismatch, which is then processed into a  
25 mutation by different mechanisms. For instance, DNA replication over the uracil leads to C to  
26 T or G to A transition mutations. Alternatively, removal of the uracil by uracil-DNA  
27 glycosylase (UNG) generates an abasic site in the DNA, which when replicated across yields  
28 transition and transversion mutations at the C:G basepair. The U:G mismatch can also be  
29 recognized by the mismatched repair factors allowing the generation of either mutations at  
30 A:T basepairs by error prone patch repair or a nicked DNA recombination substrate.  
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32 However, there are now evidences for AID to deaminate not only *Ig* genes, but many more  
33 genes including numerous oncogenes implicated in B-cell tumorigenesis (Liu and Schatz,  
34 2009). Indeed, AID can be potentially functional in a variety mammalian of non-B cells  
35 triggering genome-wide hypermutation and consequently to genetic instability and neoplasm  
36 (Perez-Duran et al., 2007).  
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For Peer Review

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**Legend to Figures**

**Figure 1.** Summary of germline *NKX2-5* mutations in CHD, detection frequency in sporadic or familial cases, and associated cardiac malformations.

**Figure 2.** Facts about germline *NKX2-5* mutations.

**Figure 3.** Predictions of the effect of *NKX2-5* mutations on the secondary structure of the protein. *NKX2-5* interacts with DNA through a helix-turn-helix DNA-binding motif of three alpha helices within the homeodomain (HD). The amino acids in the alpha helices are underlined and those affected by *NKX2-5* mutations are indicated by asterisks. Some *NKX2-5* mutations can lead to breakage of the structure (arrows), and thus a truncated protein. For predictions, amino acids in the Swiss Model for *NKX2-5* (P52952) (<http://swissmodel.expasy.org>) were mutated using tools contained in Swiss-PdbViewer (<http://spdbv.vital-it.ch/>), and the resulting structure viewed through Protein Explorer (<http://proteinexplorer.org>).

**Figure 4.** *NKX2-5* mutations affect conserved regions of the homeodomain. Protein modelling and identification of conserved regions were undertaken using Consurf (<http://consurf.tau.ac.il/>) and Chimera (<http://www.cgl.ucsf.edu/chimera/>).



**Table 1.** Functional Consequences of Identified *NKX2-5* Mutations in CHD: Functional Assays or Bioinformatic Analysis

Functional Consequence	Mutations***
5 truncated protein before HD	c.215_221del AGCTGGG, p.E72fs c.223_224delCG, p.P77fs c.262delG, p.A88fs c.312delG, p.D105fs c.325G>T, p.E109X
6 truncated protein within HD	c.445C>T, p.Q149X c.498_499 insC, p.E167fs c.508C>T, p.Q170X c.533C>T, p.T178M c.561G>C, p.Q187H c.564C>A, p.N188K
2 truncated protein after HD	c.592C>T, p.Q198X c.605_606delTG, p.L202fs
4 truncated protein after NK2-SD	c.701_702insTCCCT, p.A235fs c.768T>A, p.Y256X c.777C>A, p.Y259X c.792C>A, p.C264X
16 defective in functional assays* (DNA binding/transactivation activity/protein-protein interaction/ nuclear localization)	c.73C>T, p.R25C c.244T>A, p.C82S c.424C>T, p.R142C c.445C>T, p.Q149X c.508C>T, p.Q170X c.512T>C, p.L171P c.533C>T, p.T178M c.561G>C, p.Q187H c.564C>A, p.N188K c.565C>G, p.R189G c.569G>A, p.R190H c.572A>G, p.Y191C c.592C>T, p.Q198X c.656C>T, p.A219V c.777C>A, p.Y259X c.334 +1G>T
8 predicted to be damaging**	c.44A>T, p.K15I c.61G>C, p.E21Q c.65A>C, p.Q22P c.65A>G, p.Q22R c.479A>C, p.Q160P c.554G>T, p.W185L c.823C>A, p.P275T c.967G>A, p.A323T
4 predicted to be tolerated**	c.124G>C, p.A42P c.188C>T, p.A63V c.380C>A, p.A127E c.839C>T, p.P280L

\* For the functional studies, see (Kasahara et al., 2000; Kasahara and Benson, 2004; Zhu et al., 2000; Monzen et al., 2002; Inga et al., 2005; Esposito et al., 2009).

\*\* predicted by PolyPhen (<http://genetics.bwh.harvard.edu/pph/>); SIFT (<http://sift.jcvi.org/>)

\*\*\* Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence NM\_0004387.3, according to journal guidelines ([www.hgvs.org/mutnomen](http://www.hgvs.org/mutnomen)). The initiation codon is codon 1.



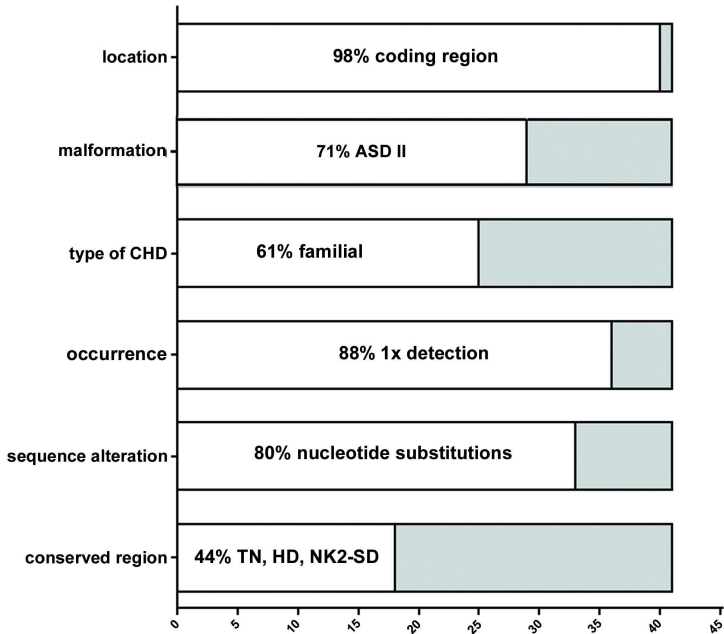
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**Table 2.** Detection Frequency of *NKX2-5* Germline Mutations in Congenital Heart Disease

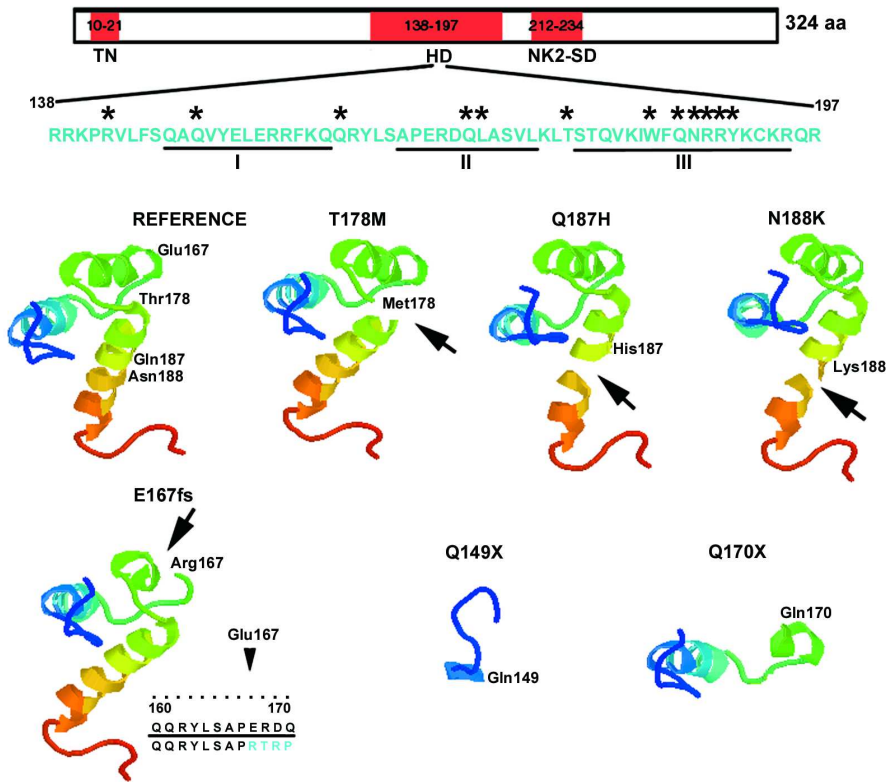
Mutation- Positive Patients	Patients Analyzed	Frequency (%)	Study	Country Study/Patients
1	109	0.9	(Ikeda et al., 2002)	Japan
18	608	3.0	(McElhinney et al., 2003)	USA
2	146	1.4	(Elliott et al., 2003)	Australia, USA
0	227	0	(Hobbs et al., 2005)	USA
3	16	18.8	(Hirayama-Yamada et al., 2005)	Japan
0	205	0	(Posch et al., 2008)	Germany
1	72	1.4	(Akcaboy et al., 2008)	Turkey
2	159	1.3	(Gioli-Pereira et al., 2010)	Brazil
0	230	0	(Zhang et al., 2009a)	China
0	62	0	(Zhang et al., 2009b)	China
6	125	4.8	(Esposito et al., 2009)	Italy
0	104	0	(Hamanoue et al., 2009)	Indonesia
33	2063	2.0	Total	

			Malformations							
			Mutation +	CHD	AV block	ASD II	TOF	VSD	Others	
c.28-63	TN		c.44A>T, p.K151I	n=1	S		X			
		c.61G>C, p.E21Q	2	S/F		X	X			
		c.65A>C, p.Q22P	1	S			X			
		c.65A>G, p.Q22R	1	S		X				
		c.73C>T, p.R25C	17	S/F			X	X		
		c.124G>C, p.A42P	1	S					X	
		c.188C>T, p.A63V	1	F					X	
		c.215_221delAGCTGGG, p.E72fs	5	F	X	X				
		c.223_224delCG, p.77fs	4	F	X	X				
		c.244T>A, p.C82S	1	S					X	
		c.262delG, p.A88fs	4	F	X		X			
	c.312delG, p.D105fs	2	F	X		X				
	c.325G>T, p.E109X	5	F	X		X		X	X	
	c.380C>A, p.A127E	1	F			X				
	c.424C>T, p.R142C	13	F	X		X	X	X	X	
	c.445C>T, p.Q149X	13	F	X		X		X		
	c.479A>C, p.Q160P	4	F	X		X				
	c.498_499insC, p.E167fs	1	S	X		X				
	c.508C>T, p.Q170X	4	F	X		X				
	c.512T>C, p.L171P	9	F	X				X		
c.412-591	HD		c.533C>T, p.T178M	17	F	X		X		X
		c.554G>T, p.W185L	3	F	X		X		X	
		c.561G>C, p.Q187H	6	F	X		X			X
		c.564C>A, p.N188K	5	F	X		X			X
		c.565C>G, p.R189G	5	F	X		X			X
		c.568C>T, p.R190C	1	F	X		X			
		c.569G>A, p.R190H	3	F	X		X			
		c.572A>G, p.Y191C	1	F	X		X		X	
		c.592C>T, p.Q198X	4	F	X		X			
		c.605_606delTG, p.L202fs	3	F	X		X		X	
c.634-702	NK2-SD		c.646C>T, p.R216C	1	S			X		
		c.656C>T, p.A219V	1	S			X			
		c.701_702insTCCCT, p.A235fs	1	S		X				
		c.768T>A, p.Y256X	5	F	X		X			
		c.777C>A, p.Y259X	7	F	X		X		X	
		c.792C>A, p.C264X	1	F	X		X			
		c.823C>A, p.P275T	1	S						X
		c.839C>T, p.P280L	1	S						X
		c.871_873delAAC, p.N291del	1	S						X
		c.967G>A, p.A323T	1	S				X		

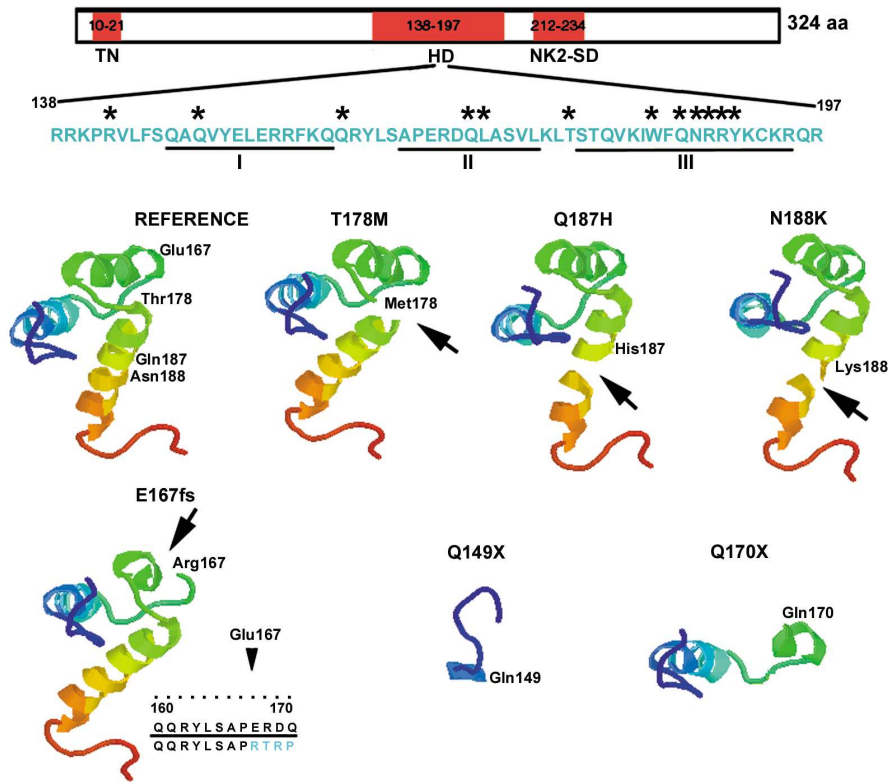
Summary of germline NKX2-5 mutations in CHD, detection frequency in sporadic or familial cases, and associated cardiac malformations.  
215x189mm (300 x 300 DPI)



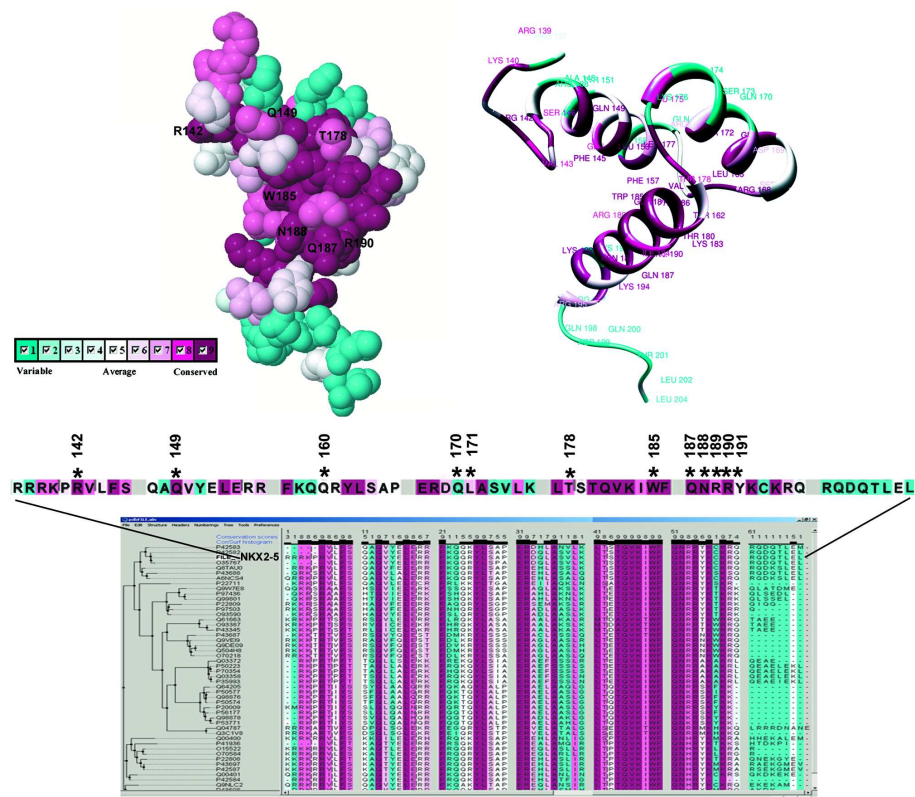
Facts about germline NKX2-5 mutations.  
227x188mm (300 x 300 DPI)



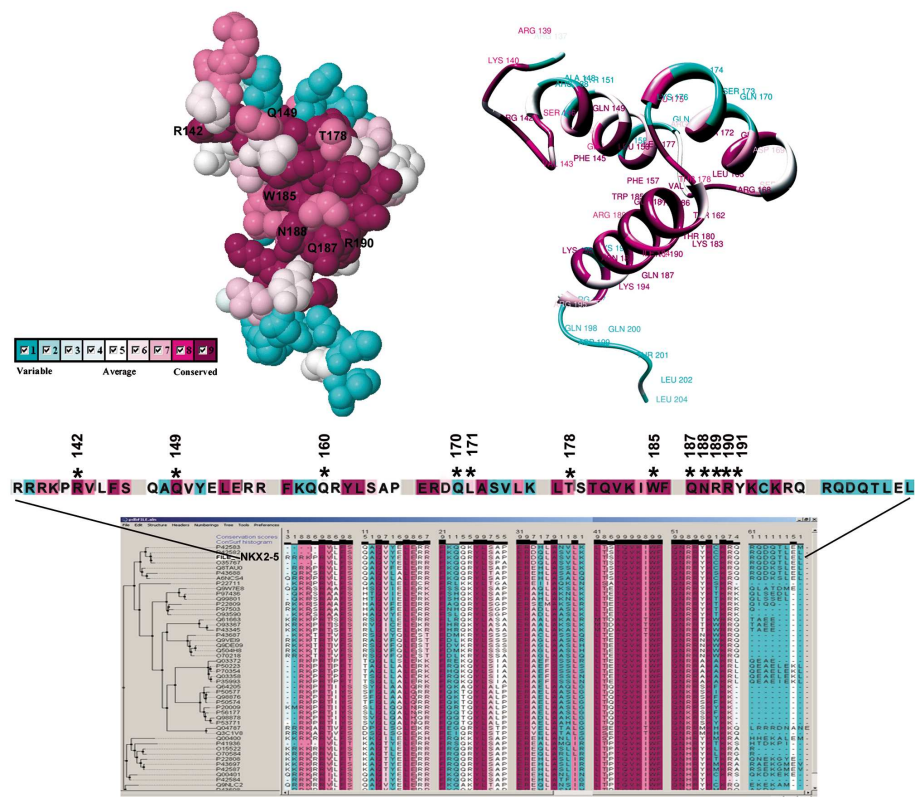
Predictions of the effect of NKX2-5 mutations on the secondary structure of the protein. NKX2-5 interacts with DNA through a helix-turn-helix DNA-binding motif of three alpha helices within the homeodomain (HD). The amino acids in the alpha helices are underlined and those affected by NKX2-5 mutations are indicated by asterisks. Some NKX2-5 mutations can lead to breakage of the structure (arrows), and thus a truncated protein. For predictions, amino acids in the Swiss Model for NKX2-5 (P52952) (<http://swissmodel.expasy.org>) were mutated using tools contained in Swiss-PdbViewer (<http://spdbv.vital-it.ch/>), and the resulting structure viewed through Protein Explorer (<http://proteinexplorer.org>).  
227x188mm (200 x 200 DPI)



Predictions of the effect of NKX2-5 mutations on the secondary structure of the protein. NKX2-5 interacts with DNA through a helix-turn-helix DNA-binding motif of three alpha helices within the homeodomain (HD). The amino acids in the alpha helices are underlined and those affected by NKX2-5 mutations are indicated by asterisks. Some NKX2-5 mutations can lead to breakage of the structure (arrows), and thus a truncated protein. For predictions, amino acids in the Swiss Model for NKX2-5 (P52952) (<http://swissmodel.expasy.org>) were mutated using tools contained in Swiss-PdbViewer (<http://spdbv.vital-it.ch/>), and the resulting structure viewed through Protein Explorer (<http://proteinexplorer.org>).  
227x188mm (200 x 200 DPI)



NKX2-5 mutations affect conserved regions of the homeodomain. Protein modelling and identification of conserved regions were undertaken using Consurf (<http://consurf.tau.ac.il/>) and Chimera (<http://www.cgl.ucsf.edu/chimera/>).  
187x155mm (300 x 300 DPI)

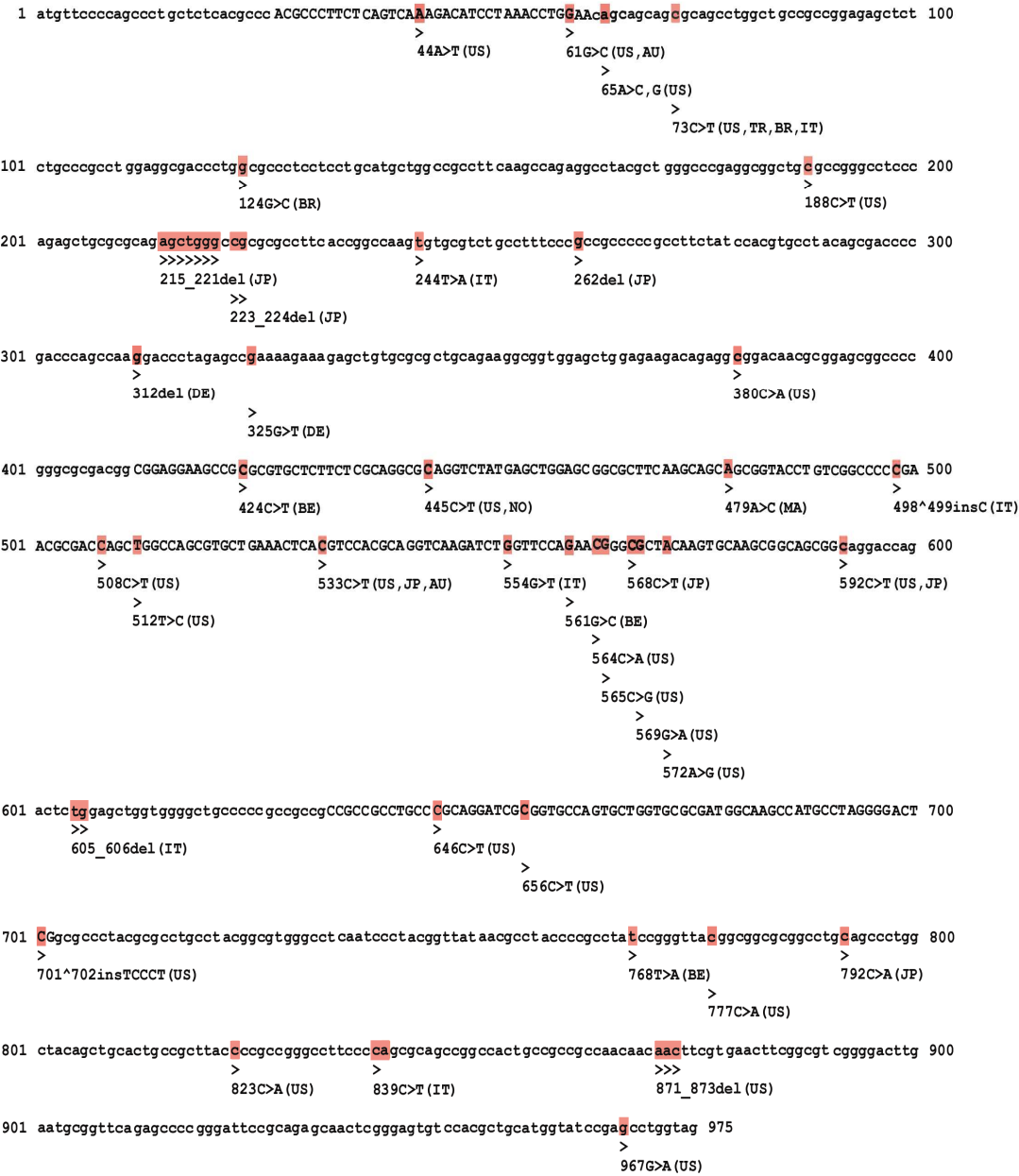


NKX2-5 mutations affect conserved regions of the homeodomain. Protein modelling and identification of conserved regions were undertaken using Consurf (<http://consurf.tau.ac.il/>) and Chimera (<http://www.cgl.ucsf.edu/chimera/>).

187x155mm (300 x 300 DPI)



Supp. Figure S1. Geographical distribution of germline *NKX2-5* mutations in CHD along the coding sequence of the gene.





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**Supp. Table S1.** Studies on *NKX2-5* Mutations Associated with Congenital Heart Disease (CHD)

Ref. No.	Authors/Year	NKX2-5 Germline Mutation	
		present	absent
1	Schott et al., 1998	x	
2	Benson et al., 1999	x	
3	Hosoda et al., 1999	x	
4	Goldmuntz et al., 2001	x	
5	Watanabe et al., 2002	x	
6	Ikeda et al., 2002	x	
7	Gutierrez-Roelens et al., 2002	x	
8	McElhinney et al., 2003	x	
9	Elliott et al., 2003	x	
10	Kasahara and Benson, 2004	x	
11	Sarkozy et al., 2005	x	
12	Hobbs et al., 2005		x
13	Hirayama-Yamada et al., 2005	x	
14	Gutierrez-Roelens et al., 2006	x	
15	Rifai et al., 2006	x	
16	Konig et al., 2006	x	
17	Majumdar et al., 2006		x
18	Pabst et al., 2008	x	
19	Akcaboy et al., 2008	x	
20	Posch et al., 2008		x
21	Khetyar et al., 2008		x
22	Bjornstad and Leren, 2009	x	
23	Esposito et al., 2009	x	
24	Draus, Jr. et al., 2009	x	
25	Hamanoue et al., 2009		x
26	Zhang et al., 2009a		x
27	Zhang et al., 2009b		x
28	Gioli-Pereira et al., 2010	x	

**Supp. Table S2.** Summary of Published Germline *NKX2-5* Mutations in Patients with Congenital Heart Defects

No.	Mutation	Amino acid change	Gene region	Phenotypes of mutation-positive patients	Occurrence of CHD	No. of mutation-positive CHD patients	Authors	Country where study conducted
1	c.44A>T	p.K15I	TN domain	ASD II	sporadic	n = 1	McElhinney et al., 2003)	USA
2.	c.61G>C	p.E21Q	TN domain	TOF	sporadic	n = 1 ( 2 unaffected family members positive)	(Goldmuntz et al., 2001) (McElhinney et al., 2003)	USA
	c.61G>C			ASD II	familial	n = 1 (2 affected family members negative)	(Elliott et al., 2003)	Australia
3.	c.65A>C	p.Q22P	5' coding region	TOF	sporadic	n = 1	(McElhinney et al., 2003)	USA
4.	c.65A>G	p.Q22R	5' coding region	ASD II	sporadic (?)	n = 1	(Draus, Jr. et al., 2009)	USA
5.	c.73C>T	p.R25C	5' coding region	TOF, VSD conoventricular	sporadic	n = 1 (unaffected mother and 2 brothers negative)	(Benson et al., 1999)	USA
	c.73C>T			TOF	sporadic/familial	n = 3 (also detected in an affected father of a positive patient, and in 2 of 43 control)	(Goldmuntz et al., 2001)	USA
	c.73C>T			TOF, TA, IAA,HLHS	sporadic/familial	n = 7 (includes 4 TOFs Benson, Goldmuntz) 4 TOF (father of 1 TOF had VSD and also positive) 1 TA, 1 IAA and 1 HLHS	(McElhinney et al., 2003)	USA
	c.73C>T			TOF	sporadic	n = 1 (also detected in the unaffected father of	(Akcaboy et al., 2008)	Turkey

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						patient, and 2 unrelated healthy control		
	c.73C>T			TOF +PDA+right sided-aortic arch	familial	n = 1 (unaffected brother positive, sister with VSD negative)	(Gioli-Pereira et al., 2010)	Brazil
	c.73C>T			accessory atrioventricular connection	sporadic, familial	n=4 (unaffected parents positive)	(Esposito et al., 2009)	Italy
6.	(c.124G>C)**	p.A42P	5' coding region	Ebstein's anomaly	sporadic	n = 1 (unaffected father positive)	(Gioli-Pereira et al., 2010)	Brazil
7.	c.188C>T	p.A63V	5' coding region	L-TGA	sporadic	n = 1	(McElhinney et al., 2003)	USA
8.	c.215_221del AGCTGGG	p.E72fs	5' coding region	AV block, ASD (3 sinus venosus type)	familial	n = 5 (4 AV block + ASD; 1 AF)	(Watanabe et al., 2002)	Japan
9.	c.223_224delCG	p. R75fs (p.P77fs)**	5' coding region	AV block, ASD II	familial	n = 4 (3 AV block +ASD II, 1 AV block)	(Watanabe et al., 2002)	Japan
10.	c.244T>A	p.C82S	5' coding region	accessory atrioventricular connection	sporadic	n=1	(Esposito et al., 2009)	Italy
11.	c.262delG	p.A88fs	5' coding region	AV block, ASD II	familial	n = 4 (3 AV block +ASD II 1 AV block without cardiac malformation)	(Hirayama-Yamada et al., 2005)	Japan
12.	c.312delG	p.K104fs (p.D105fs)**	5' coding region	AV block, ASD II	familial	n = 2 (both AV block + ASD II)	(Konig et al., 2006)	Germany

13.	c.325G>T	p.E109X	5' coding region	AV block, associated with ASD II, PAS, PFO, VSD	familial	n = 5 (all with AV block +ASD II, plus other defects: 2 PFO, 1 PAS, 1 VSD)	(Pabst et al., 2008)	Germany
14.	c.380C>A	p.A127E	5' coding region	ASD II	familial	n = 1 (grandfather with bileaflet aortic valve and also positive)	(McElhinney et al., 2003)	USA
15.	c.424C>T	p.R142C	HD	AV block, ASD II, PS, VSD, TOF	familial	n = 13 (positive members principally had AV block and ASD II)	(Gutierrez-Roelens et al., 2002)	Belgium
16.	c.445C>T	p.Q149X	HD	AV block, ASD II, VSD	familial	n = 6 (5 AV block, 4 ASD, 1 VSD cono-ventricular associated with TOF, and 2 VSD muscular )	(Benson et al., 1999)	USA
	c.445C>T	p.Q149X	HD	AV block, ASD II	familial	n = 7 (AV block+ ASD II)	(Bjornstad and Leren, 2009)	Norway
17.	(c.479A>C)**	p.Q160P	HD	AV block, ASD II (ASD within the oval fossa)	familial	n = 4 (all with AV block+ ASD II)	(Rifai et al., 2006)	Morocco
18.	c.498_499insC	p.E167fs	HD	AV block, ASD II	sporadic	n = 1 (parents unaffected)	(Sarkozy et al., 2005)	Italy
19.	c.508C>T	p.Q170X	HD	AV block, ASD II	familial	n = 4 (4 AV block, 2 ASD II)	(Schott et al., 1998)	USA
20.	c.512T>C	p.L171P	HD	AV block, ASD II, VSD	familial	n = 9 (all with AV block, and	(Kasahara and Benson, 2004)	USA

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7 with ASD, 1 VSD)

21.	c.533C>T	p.T178M	HD	AV block, ASD II	familial	n = 12 (2 families; 12 AV block, 11 ASD II)	(Schott et al., 1998)	USA
	c.533C>T			AV block, ASD II, atrial fibrillation (AF)	familial	n = 2 1 AV block+ASD II, 1 ASD II+AF)	(Hirayama- Yamada et al., 2005)	Japan
	c.533C>T			ASD II, HLHS	familial	n = 3 (1 phenotypically normal family member also positive; 1 ASD II +HLHS, 2 ASD II)	(Elliott et al., 2003)	Australia
22.	c.554G>T	p.W185L	HD	AV block, ASD II, VSD	familial	n = 3 (1 AV block +ASD II, 2 ASD II+ muscular VSD (swiss-cheese VSD)	(Sarkozy et al., 2005)	Italy
23.	c.561G>C	p.Q187H	HD	AV block, ASD II, anomalous systemic venous return	familial	n = 6 (2 positive members had anomalous venous return)	(Gutierrez- Roelens et al., 2002)	Belgium
24.	c.564C>A	p.N188K	HD	AV block, ASD II, abnormal tricuspid valve, reduced LV function	familial	n = 5 ( 5 AV block, 5 ASD II, 3 abnormal TV, 1 reduced LV function )	(Benson et al., 1999)	USA
25.	c.565C>G	p.R189G	HD	AV block, ASD II abnormal tricuspid valve, reduced LV function	familial	n = 5 (5 AV block ), 4 ASD, 1 abnormal TV, 3 reduced LV function	(Benson et al., 1999)	USA

26.	c.568C>T	p.R190C	HD	AV block, ASD II (cribriform type)	familial	n = 1	(Hirayama- Yamada et al., 2005)	Japan
27.	c.569G>A	p.R190H	HD	AV block, ASD II	familial	n = 3 (3 AV block +ASD I, 2 with VSD)	(Kasahara and Benson, 2004)	USA
28.	c.572A>G	p.Y191C	HD	AV block, ASD II, VSD conoventricular	familial	n = 1 (parents and siblings negative)	(Benson et al., 1999)	USA
29.	c.592C>T	p.Q198X	3' coding region	AV block, ASD II	familial	n = 3	(Schott et al., 1998)	USA
	c.592C>T			AV block, ASD II	familial	n = 1	(Hosoda et al., 1999)	Japan
30.	c.605_606delTG	p.L202fs	3' coding region	AV block, ASD II VSD	familial	n = 3 (2 AV block +ASD; 1 AV block+ASD+VSD)	(Sarkozy et al., 2005)	Italy
31.	c.646C>T	p.R216C	NK domain	TOF	sporadic	n = 1	(Goldmuntz et al., 2001) (McElhinney et al., 2003)	USA
32.	c.656C>T	p.A219V	NK domain	TOF	sporadic	n = 1 (unaffected mother positive)	(Goldmuntz et al., 2001) (McElhinney et al., 2003)	USA
33.	InsTCCCT701 (c.701_702insTCCCT)**	D235AFSter (p.A235fs)**	3' coding region	AV block, ASD II	sporadic	n = 1	(McElhinney et al., 2003)	USA
34.	c.768T>A	p.Y256X	3' coding region	AV block, ASD II, AF, ventricular arrhythmias	familial	n = 5 (5 AV block, 1 ASD II + mitral valve prolapse, 1 ASD II)	(Gutierrez- Roelens et al., 2006)	Belgium

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35.	c.777C>A	p.Y259X	3' coding region	AV block, ASD II VSD conoventricular, VSD muscular	familial	n = 7 (7 AV Block, 6 ASD, 2 VSD conoventricular, and 1 VSD muscular	(Benson et al., 1999)	USA
36.	(c.792C>A)**	p.C264X	3' coding region	AV block, ASD II	familial	n = 1	(Ikeda et al., 2002)	Japan
37.	c.823C>A	p.P275T	3' coding region	CoA	sporadic	n = 1	(McElhinney et al., 2003)	USA
38.	c.839C>T	p.P280L	3' coding region	accessory atrioventricular connection	sporadic	n = 1	(Esposito et al., 2009)	Italy
39.	delAAC871 (c.871_873delAAC)**	del291N (p.N291del)**	3' coding region	DORV	sporadic	n = 1	(McElhinney et al., 2003)	USA
40.	c.967G>A	p.A323T	3' coding region	TOF	sporadic	n = 1	(McElhinney et al., 2003)	USA
41.	Int1 DSG+1T (c.334+1G>T)		intronic, splice donor site	AV block	sporadic	n = 1	(Benson et al., 1999)	USA

\*\* supplied based on description in the paper or written in accordance with recommended mutation nomenclature. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence NM\_0004387.3, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

Watanabe et al 2002: c.223\_224delCG (p.P77fs because p.P77 is the first aa to be affected by the frameshift, p.A76 is silent change)

Koenig et al, 2006: c.312delG (p.D105fs because p.D105 is the first aa to be affected by the frameshift)

McElhinney et al, 2003: c.701\_702insTCCCT (p.A235fs because p.S235 is the first aa to be affected by the frameshift, p.S234 is silent change)

Benson et al. 1999: Int1 DSG+1T (intron1, splice donor site (+1) G>T), c.334 +1G>T

**Supp. Table S3.** Functional Significance of Published Germline *NKX2-5* Mutations in Patients with Congenital Heart Disease

No.	Mutation	Authors	Gene region	Function (as predicted by its location or bioinformatic analysis and functional assays)	Bioinformatic methods/assays
1	c.44A>T, p.K15I	(McElhinney et al., 2003)	TN domain	damaging	SIFT/Polyphen
2.	c.61G>C, p.E21Q	(Goldmuntz et al., 2001)/(McElhinney et al., 2003) (Elliott et al., 2003)	TN domain	alters a conserved amino acid within the TN domain, damaging	SIFT/Polyphen
3.	c.65A>C p.Q22P	(McElhinney et al., 2003)	5' coding region	damaging	SIFT/Polyphen
4.	c.65A>G p.Q22R	(Draus, Jr. et al., 2009)	5' coding region	damaging	SIFT/Polyphen
5.	c.73C>T p.R25C	(Benson et al., 1999) (Goldmuntz et al., 2001),(McElhinney et al., 2003) (Akcaboy et al., 2008) (Gioli-Pereira et al., 2010) (Esposito et al., 2009)	5' coding region	(Kasahara et al., 2000) reduced DNA binding affinity as dimer, WT + R25C increase in transactivation than in WT alone  (Esposito et al., 2009) EMSA analysis no significant reduction in DNA binding significant reduction in transactivation activity at lower concentration of protein (100 ng), but masked at higher concentration	functional assays
6.	(c.124G>C)** p.A42P	(Gioli-Pereira et al., 2010)	5' coding region	tolerated	SIFT/Polyphen
7.	c.188C>T p.A63V	(McElhinney et al., 2003)	5' coding region	tolerated	SIFT/Polyphen



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4	8.	c.215_221del	(Watanabe et al.,	5' coding	truncated protein affecting HD, NK-SD	
5		AGCTGGG	2002)	region		
6		p.E72fs				
7						
8	9.	c.223_224delCG	(Watanabe et al.,	5' coding	truncated protein affecting HD, NK-SD	
9		p.R75fs	2002)	region		
10		(p.P77fs)**				
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12	10.	c.244T>A	(Esposito et al., 2009)	5' coding	EMSA analysis no significant reduction in DNA binding	functional assay
13		p.C82S		region	significant reduction in transactivation activity at lower	
14					concentration of protein (100 ng), but masked at higher	
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18					Note: according to authors not conclusive!	
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20	11.	c.262delG	(Hirayama-Yamada	5' coding	truncated protein affecting HD, NK-SD	
21		p.A88fs	et al., 2005)	region		
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24	12.	c.312delG	(Konig et al., 2006)	5' coding	truncated protein affecting HD, NK-SD	
25		p.K104fs		region		
26		(p.D105fs)**				
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29	13.	c.325G>T	(Pabst et al., 2008)	5' coding	truncated protein affecting HD, NK-SD	
30		p.E109X		region		
31						
32	14.	c.380C>A	(McElhinney et al.,	5' coding	tolerated	SIFT/Polyphen
33		p.A127E	2003)	region		
34						
35	15.	c.424C>T	(Gutierrez-Roelens et	HD	(Kasahara and Benson, 2004)	functional assay,
36		p.R142C	al., 2006)		reduced DNA binding, reduced transactivation, reduced	ConSurf
37					interaction with NKX2-5, GATA4 and TBX5	
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39	16.	c.445C>T	(Benson et al., 1999)	HD	(Kasahara et al., 2000)	functional assay
40		p.Q149X	(Bjornstad and Leren,		no DNA binding and transactivation	
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17.	(c.479A>C)** p.Q160P	2009) (Rifai et al., 2006)	HD	damaging	SIFT/Polyphen
18.	c.498_499 insC p.E167fs	(Sarkozy et al., 2005)	HD	truncated protein affecting HD, NK-SD	
19.	c.508C>T p.Q170X	(Schott et al., 1998)	HD	(Kasahara et al., 2000) no DNA binding, no transactivation, inhibited transcriptional activity of NKX2-5 WT (WT + Q170X less transactivation than in WT alone) (Zhu et al., 2000) loss of transactivation, WT + Q170X less transactivation than in WT alone, no DNA binding, diffused localization in nucleus and cytoplasm, inhibited synergistic NKX2-5-GATA4 interaction (Monzen et al., 2002) reduced expression and transactivation of cardiac-specific genes, inhibited cardiomyocyte differentiation, promoted stress-induced cell death of cardiomyocytes	functional assay/ in vitro cardiomyocyte differentiation system
20.	c.512T>C p.L171P	(Kasahara and Benson, 2004)	HD	(Kasahara and Benson, 2004) anomalous nuclear localization, reduced DNA-protein binding reduced transactivation, less efficient interaction with GATA4 and TBX5	functional assay
21.	c.533C>T p.T178M	(Schott et al., 1998) (Hirayama-Yamada et al., 2005) (Elliott et al., 2003)	HD	(Kasahara et al., 2000), markedly reduced DNA binding and transactivation  (Zhu et al., 2000) reduced transactivation, inhibited transcriptional activity of NKX2-5 WT (WT + T178M less transactivation than in WT alone, reduced DNA binding), inhibited NKX2-5-GATA4 synergistic interaction	functional assay/ in vitro cardiomyocyte differentiation system

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(Monzen et al., 2002)  
reduced expression and transactivation of cardiac-specific genes,  
inhibited cardiomyocyte differentiation, promoted stress-induced  
cell death of cardiomyocytes

(Kasahara and Benson, 2004), anomalous nuclear localization, less  
repressor activity than GAL4-NKX2-5\_WT (repressor)

(Inga et al., 2005)  
loss-of-function mutant in yeast assay

22.	c.554G>T p.W185L	(Sarkozy et al., 2005)	HD	damaging	SIFT/Polyphen
23.	c.561G>C p.Q187H	(Gutierrez-Roelens et al., 2002)	HD	(Kasahara and Benson, 2004) reduced DNA binding, reduced transactivation, reduced interaction with TBX5	functional assay
24.	c.564C>A p.N188K	(Benson et al., 1999)	HD	(Kasahara et al., 2000) markedly reduced DNA binding and transactivation (Kasahara and Benson, 2004) less repressor activity than GAL4-NKX2-5_WT (repressor)	functional assay
25.	c.565C>G p.R189G	(Benson et al., 1999)	HD	(Kasahara et al., 2000) markedly reduced DNA binding and transactivation, inhibited transcriptional activity of NKX2-5 WT (WT + R189G much less transactivation than in WT alone) (Kasahara and Benson, 2004) anomalous nuclear localization	functional assay
26.	c.568C>T p.R190C	(Hirayama-Yamada et al., 2005)	HD	damaging	SIFT/Polyphen
27.	c.569G>A p.R190H	(Kasahara and Benson, 2004)	HD	(Kasahara and Benson, 2004) compartmentalized instead of diffused nuclear localization as in wt, reduced DNA binding, reduced transactivation, reduced interaction with NKX2-5, GATA4 and TBX5	functional assay

28.	c.572A>G p.Y191C	(Benson et al., 1999)	HD	(Kasahara et al., 2000),(Kasahara and Benson, 2004) markedly reduced DNA binding and transactivation, inhibited transcriptional activity of NKX2-5 WT (WT + Y191C less transactivation than in WT alone)	functional assay
29.	c.592C>T p.Q198X	(Schott et al., 1998) (Hosoda et al., 1999)	3' coding region	(Kasahara et al., 2000) reduced DNA binding affinity as dimer (Zhu et al., 2000) reduced transactivation, WT + Q198X much increased transactivation than in WT alone, enhanced NKX2-5-GATA4 synergistic interaction, overexpression in cultured cardiomyocytes induced apoptosis (Monzen et al., 2002) lower cardiomyocyte differentiation efficiency, promoted stress- induced cell death of cardiomyocytes	functional assay / in vitro cardiomyocyte differentiation system
30.	c.605_606delTG p.L202fs	(Sarkozy et al., 2005)	3' coding region	truncated protein leading to deletion of NK-SD	
31.	c.646C>T p.R216C	(Goldmuntz et al., 2001),(McElhinney et al., 2003)	NK2-SD domain	damaging	SIFT/Polyphen
32.	c.656C>T p.A219V	(Goldmuntz et al., 2001),(McElhinney et al., 2003)	NK2-SD domain	(Inga et al., 2005) reduced-function mutant in yeast assay	functional assay
33.	InsTCCCT701 (c.701_702insTCCCT) ** D235AFSter (p.A235fs)**	(McElhinney et al., 2003)	3' coding region	truncated protein	

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34.	c.768T>A p.Y256X	(Gutierrez-Roelens et al., 2006)	3' coding region	truncated protein with 68 aa lacking, predicted to affect ability of NKX2-5 to form dimers	
35.	c.777C>A p.Y259X	(Benson et al., 1999)	3' coding region	(Kasahara et al., 2000) reduced DNA binding affinity as dimer, reduced transactivation, inhibited transcriptional activity of NKX2-5 WT (WT + Y259X much less transactivation than in WT alone)	functional assay
36.	(c.792C>A)** p.C264X	(Ikeda et al., 2002)	3' coding region	truncated protein	
37.	c.823C>A p.P275T	(McElhinney et al., 2003)	3' coding region	damaging	SIFT/Polyphen
38.	c.839C>T p.P280L	(Esposito et al., 2009)	3' coding region	tolerated	SIFT/Polyphen
39.	delAAC871 (c.871_873del AAC)** del291N (p.N291del)**	(McElhinney et al., 2003)	3' coding region	deleted amino acid	
40.	c.967G>A p.A323T	(McElhinney et al., 2003)	3' coding region	damaging/tolerated	SIFT/Polyphen
41.	Int1 DSG+1T (c.334 +1G>T)	(Benson et al., 1999)	intronic, splice donor site	(Kasahara et al., 2000) abolished intron splicing, poor accumulation of mutant protein in the cell, no DNA binding, and transactivation	functional assay

\*\*supplied based on description in the paper or written in accordance with recommended mutation nomenclature. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence NM\_0004387.3, according to journal guidelines ([www.hgvs.org/mutomen](http://www.hgvs.org/mutomen)). The initiation codon is codon 1.