FUNCTIONAL CHARACTERIZATION OF A NOVEL MUTATION IN NKX2-5 ASSOCIATED WITH CONGENITAL HEART DISEASE AND ADULT-ONSET CARDIOMYOPATHY.

Costa: NKX2-5 mutation in familial DCM.

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Abstract

Background: The transcription factor NKX2-5 is crucial for heart development and mutations in this gene have been implicated in diverse congenital heart diseases (CHD) and conduction defects (CD) in mouse models and humans. Whether NKX2-5 mutations have a role in adult-onset heart disease is unknown.

Methods and Results: Mutation screening was performed in 220 probands with adult-onset dilated cardiomyopathy (DCM). Six NKX2-5 coding sequence variants were identified, including 3 non-synonymous variants. A novel heterozygous mutation, I184M, located within the NKX2-5 homeodomain (HD), was identified in one family. A subset of family members had CHD, but there was an unexpectedly high prevalence of DCM. Functional analysis of I184M in vitro demonstrated a striking increase in protein expression when transfected into COS-7 cells or HL-1 cardiomyocytes, due to reduced degradation by the ubiquitin-proteasome system (UPS). In functional assays, DNA binding activity of I184M was reduced, resulting in impaired activation of target genes, despite increased expression levels of mutant protein.

Conclusions: Certain NKX2-5 HD mutations show abnormal protein degradation via the UPS and partially impaired transcriptional activity. We propose that this class of mutation can impair heart development and mature heart function, and contribute to NKX2-5-related cardiomyopathies with graded severity.

Key words: dilated cardiomyopathy, transcription factors, gene mutations, ubiquitin-proteasome system, NKX2-5.
Introduction

Dilated cardiomyopathy (DCM) is a major cause of morbidity and mortality. Significant progress towards new management strategies for DCM is critically dependent on a better understanding of disease pathophysiology. Over the past decade, it has been recognized that inherited genetic factors are causative of DCM in a substantial proportion of cases, and more than 40 chromosomal loci and disease genes have been identified to date\(^1\). Functional characterization of these genetic variants indicates that diverse cardiomyocyte structural and signaling defects can result in the DCM phenotype\(^2\).

The homeobox transcription factor Nkx2-5 is expressed in cardiac precursor cells from *Drosophila* to humans, and is essential for heart development and determination of myocardial cell fate\(^3,4\). Absence of Nkx2-5 in mice results in impaired cardiac growth and chamber formation, deranged gene regulatory network and early embryonic lethality\(^5,6\). Heterozygous and conditional deletion at postnatal stages has shown that Nkx2-5 activity is also essential for maintenance of the cardiac conduction system\(^7,9\). Mutations in the human *NKX2*-5 gene have been associated with a diverse range of CHD and conduction system defect (CD) phenotypes, including atrial and ventricular septal defects, atrioventricular conduction block, tetralogy of Fallot, hypoplastic left heart, transposition of the great arteries, dextracardia and valvular malformations\(^10\text{-}14\), and overexpression of NKX2-5 underpins conduction defects in myotonic dystrophy, a toxic RNA disease\(^15\).

In addition to its role in early cardiac morphogenesis, studies in *Drosophila* and mice suggest that Nkx2-5 and its transcriptional co-factors are critical determinants of later ventricular myocyte maturation and adult heart function\(^7,16\text{-}18\). In families with CHD-causing *NKX2*-5 mutations, left ventricular contractile dysfunction has been observed in a few cases\(^10,14\). DCM may in fact occur as a late clinical manifestation of a predominant CHD phenotype; however, the relative importance of *NKX2*-5 mutations as a direct cause of DCM has not been
determined. DCM can be a primary manifestation of heterozygous mutations in \textit{TBX20}, an Nkx2-5 transcriptional cofactor, in humans and mice\textsuperscript{17,19}. Here we report the results of a mutation screening study in a large cohort of individuals with adult-onset familial DCM. Detailed functional analysis of a novel \textit{Nkx2-5} mutation, I184M, showed increased protein stability, in association with reduced transcriptional activation. Our findings highlight a previously unexplored role of defective ubiquitin-mediated protein degradation in NKX2-5-related cardiac pathologies.

\textbf{Methods}

An expanded Methods section is available in the online Data Supplement.

\textbf{Study subjects}

Caucasian probands with a family history of DCM were recruited from St Vincent’s Hospital (n=146) and Harvard Medical School (n=74). A supplementary group of unrelated individuals with left ventricular non-compaction (LVNC, n=24) was recruited from Royal Prince Alfred Hospital and the National Australian Childhood Cardiomyopathy Study. One hundred healthy Caucasian volunteers comprised a control group. All participants gave informed written consent and study protocols were approved by the relevant institutional Human Research Ethics Committees.

\textbf{Mutation screening}

Protein-coding sequences of the \textit{NKX2-5} gene were amplified by PCR from genomic DNA using primers derived from intron sequences. Amplimers were sequenced using the Big Dye terminator and were analysed on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems).

\textbf{Molecular modeling}

A homology model of human Nkx2-5 HD was generated using X-ray crystallography data
derived from Nkx2-5 HD complexed to DNA\textsuperscript{20} and Insight II software (Accelyrs).

**Cell Transfections**

COS-7 and HL-1 cells were transfected using Lipofectamine/Plus (Invitrogen) or electroporation (NEON system, Invitrogen) with reporter plasmids and plasmids containing untagged and FLAG, HA, Myc-tagged point-mutations at murine amino acid sites equivalent to human Nkx2-5 HD mutations or reported functional variants\textsuperscript{10,13,14,21,22}. Results were expressed as fold-activity after normalization for Renilla activity.

**Electrophoretic mobility shift assay (EMSA) and GST pull-down analyses**

GST-cleaved Nkx2-5HD and \textit{in vitro} translated \( {^{35}}S \)-labelled Nkx2-5 proteins (TNT Quick Coupled System, Promega) were produced and EMSA was performed as described\textsuperscript{23}. For pull-down analysis, GST-fusion protein was incubated with \textit{in vitro}-translated \( {^{35}}S \)-Nkx2-5 protein, subjected to SDS-PAGE and visualized by autoradiography.

**Immunoprecipitation, \textit{in vitro} ubiquitylation and Western blotting**

Lysates prepared from transfected cells were subjected to co-immunoprecipitation and western blotting according to manufacturer protocols. \textit{In vitro} ubiquitylation studies were performed using Ubiquitin Protein Conjugation Kit (Calbiochem) and \textit{in vitro} translated \( {^{35}}S \)-radiolabeled Nkx2-5 proteins (Promega).

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

**Results**

Evaluation of the \textit{NKX2-5} gene in 220 family probands with adult-onset DCM identified 6 coding sequence variants, including 3 non-synonymous variants (Table 1). One proband had an \textit{R25C} change that has been associated with CHD and results in a modest deficit in Nkx2-5
dimerisation\textsuperscript{10,12,24}. Although we did not detect R25C in 100 control subjects, it has been reported in other Caucasian and Afro-American control populations\textsuperscript{12}. A second proband had an A119S change reported previously in an individual with thyroid ectopy but also in relatives with normal thyroid function and showed reduced DNA binding affinity\textsuperscript{25}. These data suggest that both R25C and A119S are likely to be rare, disease-modifying polymorphisms.

A novel I184M variant was found in one proband with DCM and CD (Family DO, Figure 1A, B) and in all other affected family members (Figure 1C). It was absent in unaffected relatives, 100 healthy individuals and 146 CHD patients evaluated by our group\textsuperscript{11}. This variant was also not seen in the Exome Sequencing Project or 1000 Genomes databases.

Coding sequence variants in the 15 most common adult-onset familial DCM genes, \textit{BAG3, Cypher/ZASP, DES, DMD, LMNA, MYBPC3, MYH6, MYH7, RBM20, SCN5A, TNNC1, TNNI3, TNNT2, TPMI, TTN}, were excluded in the family proband, DO-II-4. Significantly, mice expressing an \textit{Nkx2-5} transgene with an isoleucine to proline substitution at amino acid 183, which is equivalent to codon 184 in humans, develop rapidly progressive DCM and CD during early adult life\textsuperscript{16}. The proband DO-II-4 presented recurrent syncope and complete heart block at 46 years of age. Echocardiography showed moderate to severe DCM with apical left ventricular thickening indicative of left ventricular noncompaction (LVNC) (Table 2). A patent foramen ovale was identified, but there was no significant CHD. His youngest son, DO-III-7, had a right bundle branch block with a normal echocardiogram. Individuals DO-II-3 and DO-III-4 had atrial septal defects diagnosed in childhood that had required surgical repair. Neither of these individuals had experienced cardiac symptoms but clinical screening at the time of study recruitment showed DCM and CD. Individual DO-III-1 had tricuspid atresia diagnosed shortly after birth with progressive heart failure and death by 3 years of age.
LVNC may occur in association with CHD and has been observed in some humans with Nkx2-5 mutations and in mice with a ventricle-restricted knockout of Nkx2-5. LVNC was present in 3 proband in which Nkx2-5 mutations were not identified. To further evaluate possible associations between Nkx2-5 and LVNC, a cohort of 24 individuals with LVNC was evaluated however no additional mutations were found.

**Predicted structural consequences of I184M Nkx2-5**

The human Nkx2-5 HD contains 60 amino acids and is comprised of three α-helices. I184 is located in the third helix at a position in which isoleucine is highly conserved from humans to flies (Figure 2A). The third helix lies in close proximity to the major groove of DNA and the region N-terminal to helix 1 is positioned along the minor groove (Figure 2B). Loss of the β-methyl group at I184 reduces hydrophobic contacts with neighbouring amino acids and ribose rings in the DNA backbone, whereas methionine replaces a relatively compact side-chain with a linear one and introduces a sulphur atom that creates electrostatic clashes with glutamine at position 187 and increases hydrogen-bonding potential with asparagine at position 188 (Figure 2C-E). These changes alter the orientation of neighbouring side-chains in the HD helix as well as their interactions with bases in the DNA backbone and would be expected to reduce Nkx2-5 affinity for its target sites.

**I184M Nkx2-5 retains interaction with other transcription factors but has impaired transcriptional activity**

Our modelling study suggested that I184M mutation alters functional properties of the Nkx2-5 protein. Using EMSA, we evaluated relative binding affinity of WT and mutant Nkx2-5 proteins for the Nppa promoter, a well-known target of Nkx2-5. Nkx2-5 protein was produced by in vitro transcription/translation of full length Nkx2-5 cDNA, and bacterial expression of the Nkx2-5 HD. Specificity was confirmed by retardation of migration of the
WT DNA/protein complex upon addition of Nkx2-5 antibody (Figure 3A, lanes 2-4). Using full-length Nkx2-5 protein, the I184M mutation showed no detectable DNA binding (Figure 3A, lanes 5-7). Bacterially-expressed WT Nkx2-5HD bound to the Nppa promoter as a monomer at low protein concentrations and progressively as a dimer with increasing concentration (Figure 3B, lanes 2-6). I184M Nkx2-5HD showed only weak DNA binding to Nppa promoter and only as a monomer (Figure 3B, lanes 7-11). Comparison of monomer band intensities suggested that I184M-Nkx2-5 binding is at least 10-fold less than WT Nkx2-5 under these conditions.

We have also analysed the ability of I184M Nkx2-5 to activate its primary target promoters using luminescence assays in COS-7 cells. I184M variant showed 2-3-fold reduced transcriptional activity on the Nppa promoter (Figure 4A). This reduction was similar in assays using both Nppa and Gja5 promoters and in which Nkx2-5 acts in synergy with cofactors Gata4 and Tbx20 (Figure 4B-C). We further compared the transcriptional activity of I184M with two other variants, I184P and Y191C, which also have markedly reduced DNA binding affinity. I184P (also affecting I184), although not found in humans to date, was previously designed as a possible dominant negative Nkx2-5 protein based on the activity of proline substitutions occurring in this same amino acid in other HD proteins, and when over-expressed in hearts in transgenic mice it lead to rapid onset-DCM.\(^{16,24}\). Y191C was identified in a family with CD and atrial septal defects (ASDs)\(^ {10}\) and interrupts the unique tyrosine that specifies the NK-2 class of HD protein and is critical for their atypical DNA-binding specificity [REFS]. Transcriptional activity of I184P and Y191C variants was more strongly compromised than that of I184M (Figure 4B,C). While I184M Nkx2-5 was unable to compete with WT Nkx2-5 in synergy with Gata4 and Tbx20 over a range of doses, both I184P and Y191C showed dominant negative activity, with Y191C completely abrogating transcriptional activation, even at the lowest dose (Figure 4D). These results suggest that the
I184M mutant retains partial transcriptional activity and, unlike I184P, is not a strong dominant-negative protein *in vitro*\textsuperscript{16,24,27}. Overexpression of Nkx2-5 I184M mutant in the murine atrial cardiomyocyte cell line HL-1\textsuperscript{28} showed impaired expression of endogenous *Nppa* relative to the WT protein (Figure 4E). In this context *Gata4* and *Gja1* were unaffected. These data nonetheless confirm the decreased functionality of the I184M mutant.

Protein-protein interactions between Nkx2-5 HD and its co-factors enhance transcriptional activation *in vitro* and likely form a critical layer of the cardiac gene regulatory network. Using a GST-fusion protein pull-down assay, no difference was found in the capacity of Nkx2-5 I184M to interact with *in vitro*-translated co-factors *Gata4*, *Tbx5* or *Tbx20*, nor with Nkx2-5HD, reflecting Nkx2-5 homodimerisation (Figure 4F), suggesting that the previously observed impairment in transcriptional activity is mostly due to the inability of the mutant protein to bind DNA.

**I184M Nkx2-5 protein localization and stability**

The N-terminal region of the Nkx2-5 HD contains a nuclear localization signal (NLS) and several *NKX2-5* mutations in this domain show aberrant nuclear localization, directly impacting on protein function\textsuperscript{21}. We confirmed that the cellular distribution of Nkx2-5 I184M mutant protein was comparable to that of WT proteins, showing diffuse nuclear localization. However, we noted that immunofluorescence detection of Nkx2-5 I184M gave a more intense signal than WT Nkx2-5 (Figure 5A). Although levels of WT and I184M mutant transcripts were similar (Suppl. Figure 1A,B), there was a \textgreater 3-fold increase in I184M protein expression by western blotting (Figure 5B). As a result of this finding, we reassessed transcriptional activity of I184M following normalization of protein levels, and confirmed transcriptional impairment on the *Nppa* and 3xHA promoters (Suppl. Figure 1C). Transiently expressed Nkx2-5 WT, I184M and I184P in HL-1 cardiomyocytes also showed increased
mutant protein levels, most pronounced for I184P (Figure 5C). Steady state protein levels are a measure of the balance of newly synthesized protein versus degradation. Inhibition of protein biosynthesis using cycloheximide (CHX), an inhibitor of protein translation, showed that robust levels of both Nkx2-5 I184M and I184P persisted beyond 12 hrs when Nkx2-5 WT protein had substantially diminished, indicating increased protein stability of the mutants (Figure 5D).

Elevated protein levels were also observed for several other Nkx2-5 HD mutations located between HD residues 171 to 190. These mutations target helices 2 and 3, which comprise the “helix-turn-helix” backbone of the HD fold. Higher protein levels were not seen for the Y191C mutation, which lies just to the edge of the helix-turn-helix region, and, as noted, interrupts the unique tyrosine found in this position in NK2 class HD proteins that is critical for their unique DNA binding specificity (Figure 5E). No changes in protein levels were observed for two other mutations that lie outside of helices 2 and 3: S164A, that destroys a casein kinase II phosphorylation site involved in transactivation, and R142C, located within the NLS.

The ubiquitin-proteasome system (UPS) is the major pathway for degradation of intracellular proteins. To evaluate Nkx2-5 mutant protein turnover, we transiently transfected COS-7 cells with WT or I184M Nkx2-5 vectors followed by treatment with the specific proteasome inhibitor Lactacystin. WT Nkx2-5 showed a 3.1-fold increase in protein levels over controls upon UPS inhibition (p<0.01), while no significant changes were observed for the Nkx2-5 I184M mutant (Figure 6A and Suppl. Figure 2A). Cyclin D1 is known to undergo proteasomal degradation, and treatment with Lactacystin led to its accumulation, thus confirming the active disruption of proteasomal activity (Figure 6A). The same accumulation patterns were seen when MG132, another commonly used UPS inhibitor, was administered (Suppl. Figure 2B). Co-transfection of FLAG-tagged Nkx2-5 and HA-tagged ubiquitin
vectors and immunoprecipitation with FLAG antibody allowed the detection of high molecular weight ubiquitin-linked Nkx2-5 intermediates by western blotting with HA antibody (Figure 6B). Nkx2-5/ubiquitin complexes were more prominent in the presence of MG132 (Figure 6B, lanes 4-5), consistent with accumulation of intermediates marked for degradation. The I184M mutant was also ubiquitylated (Figure 6C). *In vitro*-translated I184M, I184P, and Y191C mutant proteins were also ubiquitylated *in vitro* (Figure 6D). Collectively, our findings suggest that Nkx2-5 levels are regulated by UPS-mediated degradation, and that mutations at I184 show defective degradation. The defective accumulation of I184M occurs downstream of ubiquitylation.

**NKXkx2-5 transcriptional potential and turnover**
For a subset of transcription factors, transcriptional activation is intimately linked to degradation. In such cases, ubiquitin enzymes and proteasomal components are found within the nuclear transcriptional complex\textsuperscript{30,31}, and increased transcriptional activity leads to increased turnover. In transfected cells, Nkx2-5 achieves maximum activity after deletion of the region C-terminal to the HD\textsuperscript{32}, which contains several conserved domains that regulate transcriptional activity\textsuperscript{3}. To explore a potential relationship between increased Nkx2-5 transcriptional activity and turnover, we assessed protein levels of Nkx2-5 that were WT or I184P mutant in the HD, in the context of the full length protein or a deletion of the repressive C-terminal region (Nkx2-5ΔC)\textsuperscript{32}(Figure 7A). Overall, the C-terminally truncated Nkx2-5 appeared more stable than Nkx2-5 WT, results that appear contrary to those previously published for other transcription factors. However, both Nkx2-5 I184P and Nkx2-5ΔC I184P were significantly more stable than their non-mutant counterparts, with Nkx2-5ΔC I184P more stable than Nkx2-5 I184P. While the mechanisms remain unclear, these data suggest a complex relationship between activity and turnover in the context of I184P mutation.

It is likely that many, if not all members of the HD superfamily, are regulated by the UPS. To determine whether there is a common link between mutations in helices 2 and 3 of the HD and disturbed UPS-mediated degradation in divergent HD proteins, we analysed the effects of substituting proline for isoleucine/valine at position 184 in the NK2-class HD protein Bapx1, whose HD sequence is highly related to that of Nkx2-5, in the more distantly related LIM-HD protein Islet1, and the paired HD protein Pax7 (Figure 7B). Transfection of WT or mutant proteins into COS-7 cells showed increased stability only for members of the NK2 class - Nkx2-5 and Bapx1 - but not for Islet 1 or Pax7, despite all proteins being substrates for ubiquitylation \textit{in vitro} (Figure 7C, D). Thus, the increased stability seen for I184P and other helix2-helix3 HD mutations in Nkx2-5 does not reflect a universal feature of the
ancient HD fold.

Discussion

Here we report the identification and functional analysis of a novel NKX2-5 mutation, I184M, causative for CHD and CD, but also associated with an unusually high prevalence of familial adult-onset DCM. The I184M Nkx2-5 protein had significantly reduced DNA binding and transcriptional activity, and it accumulated in the nuclear compartment of transfected cells due to defective UPS-mediated degradation. Nkx2-5 accumulation was also seen for several other human helix 2 and 3 NKX2-5 HD mutations, and our studies on the Nkx2-5ΔC mutation support a link between activation and degradation of NK2-class HD transcription factors. Differences in the degree of protein accumulation between I184M and I184P, and in their transcriptional and dominant negative effects, suggest a graded severity in these features that likely affect phenotypic outcome. We hypothesise that certain HD mutations leading to accumulation of protein can be causative for adult-onset DCM with graded severity.

Nkx2-5 HD mutations reduce DNA binding and transcriptional activation

Most CHD-causing mutations in NKX2-5 HD alter DNA binding affinity. Reduced affinity has been attributed to structural changes at specific residues that change interactions with the major groove of DNA$^{21,24}$. Although I184 does not interact directly with DNA strands, molecular modelling predicted that the I184M substitution alters topographical and electrostatic characteristics of this site and could secondarily result in reduced DNA binding affinity. The murine variant I183P, equivalent to the human 184 position (I184P), retains its ability to homodimerize but is nonetheless unable to bind DNA$^{16,27}$. The I184M variant found in family DO showed ~10-fold reduced monomeric DNA binding. It is important to stress that the I184P mutation is not found in humans, but was generated as a potential dominant
negative protein based on proline substitutions occurring at an equivalent position in other HD proteins. Its overexpression in a mouse transgenic model led to rapid-onset DCM\textsuperscript{16}. Collectively, our data point to helices 2 and 3, and position 184 in particular, as a focus for DCM-causative mutations with a graded severity of functional effects, depending on both the position and nature of the amino acid substitution.

**Transcriptional activation defects in the pathogenesis of DCM**

During embryonic development, Nkx2-5 is essential for activation and maintenance of the cardiac regulatory network. Nkx2-5 mutants have cardiomyocyte specification and maturation deeply compromised, resulting in death\textsuperscript{33}. Nkx2-5 is also essential for post-natal maturation and homeostasis of cardiomyocytes and the conduction system\textsuperscript{7-9,16,17,19,34}. Transcriptional defects could result in primary developmental defects of the myocardial wall and/or altered growth and survival responses to the mechanical stresses of repetitive contraction and hemodynamic load throughout adult life. Indeed, in individuals with CHD-causing \textit{NKX2-5} mutations, altered intracardiac blood flow could contribute to the development of contractile dysfunction with aging. However, it is notable that the Family DO proband developed DCM in the absence of CHD and in other family members occurred after repair of structural malformations in childhood. A link between transcription factor mutations and adult cardiac functional deficit has been highlighted by the discovery of mutations in the transcriptional co-activators EYA\textsuperscript{35} and T-box factor TBX\textsubscript{20}\textsuperscript{19} in families with DCM. Taken together, our data suggest that mutations in NKX2-5 and other transcription factors should also be considered to have a major role in the pathogenesis of adult-onset familial DCM.

**Nkx2-5 HD mutations alter UPS-mediated degradation**
The UPS has been implicated in the quality control and degradation of a number of cardiac proteins\textsuperscript{29}. We determined that Nkx2-5 is also a target for ubiquitylation and suggest that high levels of I184M Nkx2-5 protein may result from interference with UPS-mediated degradation downstream of the ubiquitylation event, as WT and I184M Nkx2-5 proteins are ubiquitylated to a similar extent. It is clear that factors other than ubiquitylation, such as protein misfolding, alterations in the pattern of ubiquitylated residues, impaired recognition of mutant proteins by the 19S proteasome or reduced proteolytic activity of the 20S proteasome, must be operative for I184M and other Nkx2-5 HD mutants. Altered UPS activity may contribute to cardiac dysfunction due to a variety of causes\textsuperscript{29,36-40}. For example, truncated cardiac myosin binding protein C mutants that cause familial hypertrophic cardiomyopathy are preferentially targeted by the UPS and rapidly degraded\textsuperscript{38}, while extensively misfolded proteins associated with DCM-causing mutations in cardiac actin are resistant to proteasomal degradation\textsuperscript{39}. Mutant proteins can directly promote cytoplasmic aggregate formation and/or have non-specific effects due to progressive overloading of proteasomal proteolytic capacity with subsequent accumulation of downstream protein targets\textsuperscript{41}. Protein aggregation is thought to underlie contractile dysfunction in desmin\textsuperscript{36} and αβ-crystallin transgenic mice\textsuperscript{37} and has been observed in human myopathic hearts\textsuperscript{40}. A mutant form of the cardiac TF TBX1 bearing a poly-alanine stretch elongation found in a patient with tetralogy of Fallot, showed nuclear and cytoplasmic aggregates in transfected cells\textsuperscript{42}. It is not clear, however, whether NKX2-5 mutations inhibit the UPS via protein aggregation.

**Interdependence of UPS defects and transcriptional activation**

Activation and degradation of transcription factors are closely linked\textsuperscript{30,31} and the sequences that specify activation domains overlap with those that signal proteolysis\textsuperscript{43}. Mandatory degradation of active transcriptional complexes allows transcriptional control over time in a binary fashion. Hence, while the mechanism underlying I184M pathogenesis is currently not
fully elucidated, functional characteristics of Nkx2-5 within transcriptional complexes may provide a critical clue as to why UPS-mediated Nkx2-5 degradation is disrupted in certain HD mutants. This notion is supported by our findings that the truncation mutant Nkx2-5ΔC, normally more active, when combined with the I184P mutation, showed additionally elevated stability. Disrupted Nkx2-5 UPS-mediated turnover may contribute to, or be reflective of, Nkx2-5-related cardiomyopathies. Nevertheless, it distinguishes a sub-class of Nkx2-5 mutations and our data on familial I184M mutation combined with the rapid-onset DCM seen in I184P transgenic mice\textsuperscript{16} collectively suggest that this sub-class may be causative for DCM with graded severity.

**Complex mechanism underlies development of cardiac dysfunctions**

Our work demonstrates that there are two different aspects associated with Nkx2-5 mutations at I184 residues that may play interdependent roles in cardiac dysfunction: (1) isoleucine changes to methionine or proline at residue 184 lead to profound differences in Nkx2-5 transcription activity; and (2) a consistent increase in protein levels, observed in both mutations at I184 and others located in helices 2 and 3 of the HD. Levels of Nkx2-5 protein may influence the dominant negative activity of mutations, the expression of target genes that can be accessed by mutant protein via cofactor interactions, or the expression of off-targets that may contribute to pathology.

Another important aspect of human \textit{NKX2-5} mutations is that patients bearing these changes usually develop progressive conduction defects\textsuperscript{10,11,13}, reproduced in mouse models. Complete post-natal ablation of Nkx2-5 leads to progressive loss of cells of the cardiac conduction system concomitant with the development of cardiac arrhythmias\textsuperscript{8}. However, it is important to understand how these observations in a full ablation model actually correlate to conduction defects in patients with heterozygous \textit{NKX2-5} point mutations. Impairment of transcriptional activation of target genes, such as \textit{Nppa}, protein accumulation and UPS
dysfunction, and differential cofactor interactions, might all play important roles in these cases. Ultimately, it remains to be seen how molecular disturbances detected *in vitro* might reflect on physiological function in whole organisms, where multiple levels of network regulatory control exist.
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Disclosures
None.
References


### Table 1:
**NXK2-5** coding sequence variants identified in 220 probands with familial DCM

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<td>I184M</td>
<td>Novel</td>
</tr>
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Table 2:
Clinical phenotype of affected individuals in Family DO

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<th>CHD</th>
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<td>CHB, PPM</td>
<td>PFO</td>
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<td>ASD</td>
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<td>III-7</td>
<td>(15)</td>
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AF, atrial fibrillation; ASD, atrial septal defect; CD, conduction-system disease; CHB, complete heart block; CHD, congenital heart disease; DCM, dilated cardiomyopathy; NA, not applicable; PFO, patent foramen ovale; PPM, permanent pacemaker; RBBB, right bundle branch block.

* Age at DCM diagnosis (age at study) expressed as years.
Figure Legends

Figure 1

Novel I184M NKX2-5 mutation with high association with DCM. (A) Pedigree showing DO family members. The family proband is indicated by an arrow. Phenotypes are indicated as: conduction-system abnormalities (black, right half, upper quadrant), dilated cardiomyopathy (red, left half, upper quadrant), congenital heart disease (ASD or PFO, black, right half lower quadrant; Tricuspid atresia, black, left half lower quadrant), unknown (gray symbols). (B) Sequence electropherograms showing wild type sequence (upper trace) and a C to G substitution that alters the amino acid 184 from Isoleucine to Methionine (lower trace) identified in the Family DO proband, (DO-II-4). (C) The presence of the variant G allele in affected family members was confirmed by loss of a BgIII restriction enzyme site. CD - conduction defect; DCM - dilated cardiomyopathy; CHD - congenital heart disease; ASD - atrial septal defects; PFO - patent foramen ovale.

Figure 2

Structural consequences of aminoacid variation in position I184 in the NKX2-5 HD. (A) Alignment of NKX2-5 HD sequence in various species showed complete evolutionary conservation of isoleucine (I) at amino acid 184 (arrow). Homologous amino acids are highlighted in blue. Positions of previously reported human HD mutations (*) are shown. (B) Conformational model of human NKX2-5, shows helix 3 (arrowhead) in close contact with the major groove of DNA. (C) I184 is located into a pocket formed by Q187 and N188. The underlying interacting DNA binding strand (pink ribbon) and deoxyribonucleic acid are shown. Atoms indicated are: carbon (green, except at position 184, where WT is shown as orange and mutant as cyan), nitrogen (blue), oxygen (red), sulphur (yellow), phosphorus (dark pink) and hydrogen (white). (D) One possible conformation of 184M has the sulphur atom in close proximity to the oxygen atom of the Q187 side chain, resulting in disruption of
the underlying helical structure. (E) An alternative 184M conformation allows a hydrogen bond to be formed between the sulphur atom and one of the amide hydrogens of N188, altering position of the N188 side chain and disrupting the structure of the helix. HD – homeodomain

**Figure 3**

I184M Nkx2-5 mutation causes decreased DNA affinity. (A) EMSA assay using $[^{35}S]$-labelled Nkx2-5 showed specific interaction of WT Nkx2-5 with the NKE site present in the *Nppa* promoter (lane 2). Binding was inhibited by competition with self-oligonucleotide (lane 3) or anti-Nkx2-5 antibody (lane 4). No interaction was observed when the I184M variant was used (lanes 5-7). (B) EMSA performed with increasing amounts of GST-purified Nkx2-5 HD showed that WT Nkx2-5HD (arrowheads, lanes 2-6) displayed at least 10-fold greater DNA binding affinity than Nkx2-5 I184M (lanes 7-11).

**Figure 4**

I184M mutation leads to partial transcriptional impairment. (A-C) I184M Nkx2-5 has decreased activation of the cardiac promoters *Nppa* and *Gja5* in transfected COS-7 cells, while I184P and Y191C variants showed no detectable activation. (D) In the presence of WT Nkx2-5, increasing doses of I184M Nkx2-5 allowed transcriptional activation in transfected COS-7 cells but did not restore normal levels of *Nppa* activation, while I184P and Y191C caused strong dominant effects by further depressing promoter function. (E) Quantification of Gata4, Nppa and Gja1 transcripts in HL-1 cells following transient overexpression of WT or I184M Nkx2-5 mutant protein. qPCR showed a 3-fold increase in Nppa transcript level when WT protein was transfected, while addition of I184M decreased basal activation. (F) GST-pull down showed that I184M Nkx2-5 had normal self-dimerization and interaction with
Gata4, Tbx5 and Tbx20. Input represents 20% of the *in vitro* translated protein used in each pull-down assay.

**Figure 5**

I184M Nkx2-5 is localized to the nucleus but displays increased levels when compared to WT Nkx2-5. (A) Normal nuclear localization of both WT and I184M Nkx2-5 in COS-7 cells was evident by immunofluorescence microscopy, but mutant protein displayed more intense signal. (B-C) Levels of WT and I184M Nkx2-5 in transfected cells by western blot in both COS-7 and cardiac HL-1 cells showed much higher expression of I184M and I184P when compared to WT Nkx2-5. (D) Increased stability of I184 mutants was confirmed by CHX treatment. WT protein was dramatically reduced after 24 hours, while mutant protein expression was sustained. α-tubulin was used as a loading control in all experiments. (E) Increased protein expression was also observed for a cluster of mutations located in helices 2 and 3 of the Nkx2-5 HD, as per marked asterisks in yellow strand.

**Figure 6**

Nkx2-5 protein levels are regulated by the UPS. (A) Protein levels in transfected COS-7 cells were evaluated by western blot before and after treatment with the specific proteasome inhibitor Lactacystin. WT Nkx2-5 levels were increased by 3.1 fold (**, P<0.01, Students t-test, Prism Software) upon UPS inhibition. I184M Nkx2-5 levels were unchanged (1.1 fold), indicating that the mutant had decreased proteasome degradation before the drug is added. Effective inhibition of the UPS was confirmed increased cyclin D1 levels; α-tubulin was used as a loading control. (B,C) Immunoprecipitation to detect poly-ubiquitylated Nkx2-5 conjugates. FLAG antibody was used to pull-down tagged Nkx2-5 protein, while western blotting has performed using anti-HA antibodies that detected tagged ubiquitin. Similar amounts of input Nkx2-5 were confirmed using an anti-Nkx2-5 antibody (lower panel). (D)
*In vitro* ubiquitylation kinetics assay for WT, I184M, I184P and Y191C Nkx2-5 variants confirmed that these mutations did not impair ubiquitylation.

**Figure 7**

Comparison of expression levels among Nkx2-5 protein variants and other HD proteins. (A) Western blots showing protein levels of I184 mutant were still elevated upon deletion of the C-terminal domain (ΔC) as compared to the full length (FL) WT or I184P protein. (B) Alignment of HD proteins showing molecular divergence among family members and presence of isoleucine or valine at position 184. (C) High protein levels associated with variation at position 184 were observed only for Nkx2-5 and closely related Bapx1, but not for the more distantly related Pax7 and Islet1, even though all proteins were ubiquitylated *in vitro* (D).
Figure 1

A

B

C

Figure 1
**Figure 2**

A. Schematic representation of the NKX2-5 transcription factor with annotated helices (helix 1, helix 2, and helix 3) and the highlighted homeodomain (HD) region spanning residues 137 to 201.

B. Cartoon diagram illustrating the structure of the NKX2-5 protein with the homeodomain region emphasized.

C. Comparative structural analysis showing the interaction of NKX2-5 with its DNA-binding site.

- **i.** Wild-type NKX2-5 interaction with DNA, demonstrating the binding sites Q187 and N188.
- **ii.** Mutant NKX2-5 (Q187I) showing altered binding at DNA site.
- **iii.** Additional mutant (I184M) with modified interactions at DNA site.

The images highlight the importance of specific amino acid residues (Q187, N188, and I184) in DNA binding and the structural changes that occur with mutations.
Figure 3

A

Nkx2.5 IVT
Ab competitor

1 2 3 4 5 6 7

WT I184M
- - + - - + - -

B

Nkx2.5HD (ng)

1 2 3 4 5 6 7 8 9 10 11

50 100 150 250 500 500

WT I184M
- - 50 100 150 250 500 7 8 9 10 11
Figure 4
Figure 5

A. DAPI, Nkx2-5, and Merged images showing WT and mutant Nkx2-5.

B. Western Blot (IB) of Nkx2-5 and α-tubulin showing WT and I184M bands.

C. Western Blot (IB) of Nkx2-5-HA showing WT, I184M, and I184P bands.

D. Western Blot (IB) of Nkx2-5 and α-tubulin with CHX treatment for 0, 1, 4, and 24 hours.

E. Diagram of HD region with amino acid mutations indicated.

Figure 5
Figure 6

A

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B

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C

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Figure 7

A

Myc-Nkx2-5FL
Myc-Nkx2-5ΔC

B

Nkx2-5
Bapx1
Pax7
Isl1

Nkx2-5 Bapx1 Pax7 Isl1

IB:

HD proteins
β-Gal

C

Nkx2-5 FL
Nkx2-5 ΔC
Bapx1
Pax7
Isl1

IB:

β-Gal

D

Nkx2-5FL Nkx2-5ΔC

IB:

Nkx2-5
α-tubulin

homology to Nkx2-5

25.7% 69.5% 18.2%
23.5% 37.3% 24%
15.4% 23.7% 13.2%

Figure 7
Supplementary Figure 1

A. Bar graph showing FOLD expression of WT and I184M samples.

B. Gel image showing Nkx2-5 expression over different cycles.

C. Bar graph showing FOLD expression for ANF and 3xHA in WT and I184M samples.
Supplementary Figure 2

A

Increase upon UPS inhibition - lactacystin

![Bar chart showing pixel average](image)

B

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