Mutations in *NEXN*, a Z-Disc Gene, Are Associated with Hypertrophic Cardiomyopathy

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Hypertrophic cardiomyopathy (HCM), the most common inherited cardiac disorder, is characterized by increased ventricular wall thickness that cannot be explained by underlying conditions, cadiomyocyte hypertrophy and disarray, and increased myocardial fibrosis. In as many as 50% of HCM cases, the genetic cause remains unknown, suggesting that more genes may be involved. Nexilin, encoded by *NEXN*, is a cardiac Z-disc protein recently identified as a crucial protein that functions to protect cardiac Z-discs from forces generated within the sarcomere. We screened *NEXN* in 121 unrelated HCM patients who did not carry any mutation in eight genes commonly mutated in myofilament disease. Two missense mutations, c.391C>G (p.Q131E) and c.835C>T (p.R279C), were identified in exons 5 and 8 of *NEXN*, respectively, in two probands. Each of the two mutations segregated with the HCM phenotype in the family and was absent in 384 control chromosomes. In silico analysis revealed that both of the mutations affect highly conserved amino acid residues, which are predicted to be functionally deleterious. Cellular transfection studies showed that the two mutations resulted in local accumulations of nexilin and that the expressed fragment of actin-binding domain containing p.Q131E completely lost the ability to bind F-actin in C2C12 cells. Coimmunoprecipitation assay indicated that the p.Q131E mutation decreased the binding of full-length *NEXN* to α-actin and abolished the interaction between the fragment of actin-binding domain and α-actin. Therefore, the mutations in *NEXN* that we describe here may further expand the knowledge of Z-disc genes in the pathogenesis of HCM.

Cardiomyopathy is a primary heart-muscle disorder associated with cardiac dysfunction. On the basis of morphological and functional characteristics, it is classified into four categories: hypertrophic, dilated, arrhythmogenic right ventricular, and restrictive.[1] Hypertrophic cardiomyopathy (HCM [MIM 192600]) is characterized by hypertrophy and diastolic dysfunction of cardiac ventricles accompanied by cardiomyocyte hypertrophy, fibrosis, and myofibrillar disarray.[2] Diagnostic criteria for HCM in adults are defined by a maximal left ventricular wall thickness of ≥ 13 mm on echocardiography in the absence of other loading conditions such as hypertension or aortic valve stenosis. Occurring in approximately 1 in 500 individuals, HCM is the most common heritable cardiac disorder and often follows an autosomal-dominant inheritance pattern with incomplete penetrance.[3] HCM can manifest negligible to extreme hypertrophy, minimal to extensive fibrosis and myocyte disarray on microscopy, absent to severe left-ventricular ouflow-tract obstruction, and distinct septal morphologies. The clinical course also varies considerably, from a benign asymptomatic course to that of severe heart failure and sudden cardiac death (SCD). HCM is the most common cause of SCD in young and competitive athletes.[3,4] In recent studies, the annual rate of SCD from HCM varies between 0.1% and 1.7%, a subset of patients having an estimated annual SCD probability between 4% and 5%.[5,6]

HCM is usually caused by mutations in genes that encode components of the cardiac muscle sarcomere. To date, hundreds of mutations implicated in the pathogenesis of HCM have been reported in nine genes encoding sarcomeric filament proteins: β-myosin heavy chain (*MYH7* [MIM 160760]), cardiac myosin-binding protein C (*MYBPC3* [MIM 600958]), cardiac troponin T (*TNNT2* [MIM 191045]), cardiac troponin I (*TNNI3* [MIM 191044]), cardiac troponin C (*TNNC1* [MIM 191040]), cardiac α-actin (*ACTC1* [MIM 102540]), α-tropomyosin (*TPM1* [MIM 191010]), essential myosin light chain (*MYL3* [MIM 160790]), and regulatory myosin light chain (*MYL2* [MIM 160781]). Among these genes, mutations in *MYH7*, *MYBPC3*, *TNNI3,* and *TNNT2* occur most often and account for as many as 50% of reported genotyped HCM cases.[7–10]

With a large portion of HCM cases being genetically unexplained, genes encoding proteins involving potential functional or transcriptional processes of the cardiomyocyte, particularly those encoding the cytoarchitecture proteins localized to the cardiac Z-disc, have been associated with disease pathogenesis. The Z-disc complex is located at either end of the contractile unit of the striated muscle and links titin and actin filaments from opposing sarcomere halves in a lattice connected by α-actinin. The Z-discs provide a backbone for the insertions of actin-based thin filaments and represent a key interface between the

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contractile apparatus and the cytoskeleton. In addition, the complex molecular network of Z-disc proteins is pivotal for reception, transduction, and transmission of mechanical and biochemical signals.[11,12] During recent years, the Z-disc has become the focus of research, and new functions beyond a sole passive mechanical transmitter of force have emerged.[13] Particularly, as a potential myocardial "stretch receptor," the Z-disc would enable the cardiomyocyte to sense increased mechanical load and respond with changes in gene expression, ultimately resulting in cellular hypertrophy.[14,15] Because of the Z-disc's importance in establishing the mechanical coupling and as the stretch-sensor mechanism of the sarcomere, genes encoding these Z-disc proteins were hypothesized to be excellent candidates for involvement in HCM.[16] In 2003, the first Z-disc mutations implicated in HCM were described in muscle LIM protein encoded by *CSRP3* (MIM 600824), which comprises two LIM domains, the first of which directly binds to α-actinin.[17] In 2004, two missense mutations in telethonin, which is a Z-disc protein encoded by *TCAP* (MIM 604488) and represents an important link between titin and other Z-disc-associated proteins, were identified in a cohort of HCM cases.[18] Recent studies have reported that mutations in another five Z-disc genes, *LDB3* (MIM 605906; encoding LIM domain binding 3), *ACTN2* (MIM 102573; encoding α-actinin 2), *VCL* (MIM 193065; encoding vinculin), *MYOZ2* (MIM 605602; encoding myozenin 2), and *ANKRD1* (MIM 609599; encoding ankyrin repeat domain 1), are responsible for HCM.[19–21]

Recently, a cardiac Z-disc protein, nexilin, encoded by *NEXN* (MIM 613121), has been identified as a crucial protein that functions to protect cardiac Z-discs from forces generated within the sarcomere that, when mutated, lead to dilated cardiomyopathy in both humans and zebrafish.[22] Nexilin, consisting of 675 amino acids, was isolated previously as an F-actin-binding protein at cell-matrix adherens junctions.[23] Hassel and colleagues found that nexilin is highly abundant in the heart and skeletal muscle and is located specifically to the Z-disc; loss of nexilin in zebrafish led to perturbed Z-disc stability and heart failure.[22] Moreover, these researchers identified one deletion and two missense mutations in *NEXN* in a large cohort of patients with dilated cardiomyopathy (DCM [MIM 115200]).[22] These mutations account for approximately 1% of the patients studied.

On the basis of the understanding that nexilin plays a unique role in stabilizing and protecting Z-discs from mechanical trauma and that mutations of certain sarcomeric and sarcomeric-associated genes identified in DCM also are known to cause HCM, we hypothesized that mutations in *NEXN* might be involved in the pathogenesis of HCM. Therefore, we performed sequencing analysis of *NEXN* in a cohort of 121 unrelated Han Chinese patients with HCM who previously had not been found to carry mutations in eight common myofilament-associated genes (*MYH7*, *MYBPC3*, *MYL2*, *MYL3*, *TNNT2*, *TNNI3*, *TPM1*, *ACTC1*) responsible for HCM.[7] We selected 192 control subjects from among healthy individuals matched on the basis of gender and ethnic origin. All patients with systemic hypertension, valvular heart disease, and congenital heart disease, including subaortic, valvular, and supravalvular aortic stenosis and coarctation of aorta, were excluded at the beginning of the study. Informed consent was obtained from all participating individuals. This study was approved by the institutional review board of the Cardiovascular Institute, Chinese Academy of Medical Sciences.

Genomic DNA was isolated from peripheral blood leukocytes with a standard salting-out protocol. Primer pairs were designed to amplify all of the coding regions and the intron-exon boundaries of *NEXN* based on the published sequence (GenBank accession number NM_144573.3). PCR amplifications were performed with the use of standard protocols, and PCR products were analyzed by direct sequencing on an Applied Biosystems 3730 Genetic Analyzer with BigDye Terminator chemistry (version 3.1).

We identified two heterozygous missense mutations, c.391C>G (p.Q131E) and c.835C>T (p.R279C), which are located in exons 5 and 8, respectively, in *NEXN* in two probands with familial HCM (Figure 1). Both of the mutations affect highly conserved amino acid residues among 12 interspecies orthologs and are located in the important functional domains of nexilin (Figure 2). In family A, the *NEXN* mutation p.Q131E was detected in individuals II:2, II:3, and III:1, and this mutation segregated with the HCM phenotype in the family members. The proband (II:2) was a 37-year-old female diagnosed with HCM because of typical HCM clinical features. The echocardiogram showed that she had a nonobstructive cardiac hypertrophy with an interventricular septal thickness of 21 mm with normal left ventricular systolic function and an abnormal electrocardiogram (ECG) with T-wave changes. The proband's mother (I:2) had died of SCD at the age of 38 years. Her 34-year-old brother (II:3) and her 16-year-old daughter (III:1), each of whom carry the p.Q131E mutation, also were found to be affected, and the echocardiogram demonstrated that both of them had asymmetrical septal hypertrophy. In addition, II:3 showed an abnormal ECG indicating voltage criteria for left ventricular hypertrophy. The other family members, her father (I:1) and her daughter (III:2), did not demonstrate any clinical evidence of HCM on echocardiography or ECG. Individuals II:4 and III:3 had not had clinical evaluation and genetic testing.

In family B, the proband (III:2), a 45-year-old male carrying the *NEXN* mutation p.R279C, was diagnosed with HCM, with a left ventricular anterior wall thickness of 17 mm. His grandmother (I:2) had died suddenly with suspected heart disease at 40 years of age. His father (II:1) and his younger brother (III:4) were diagnosed subsequently with HCM as a result of clinical screening of the family, and the mutation p.R279C was detected in both

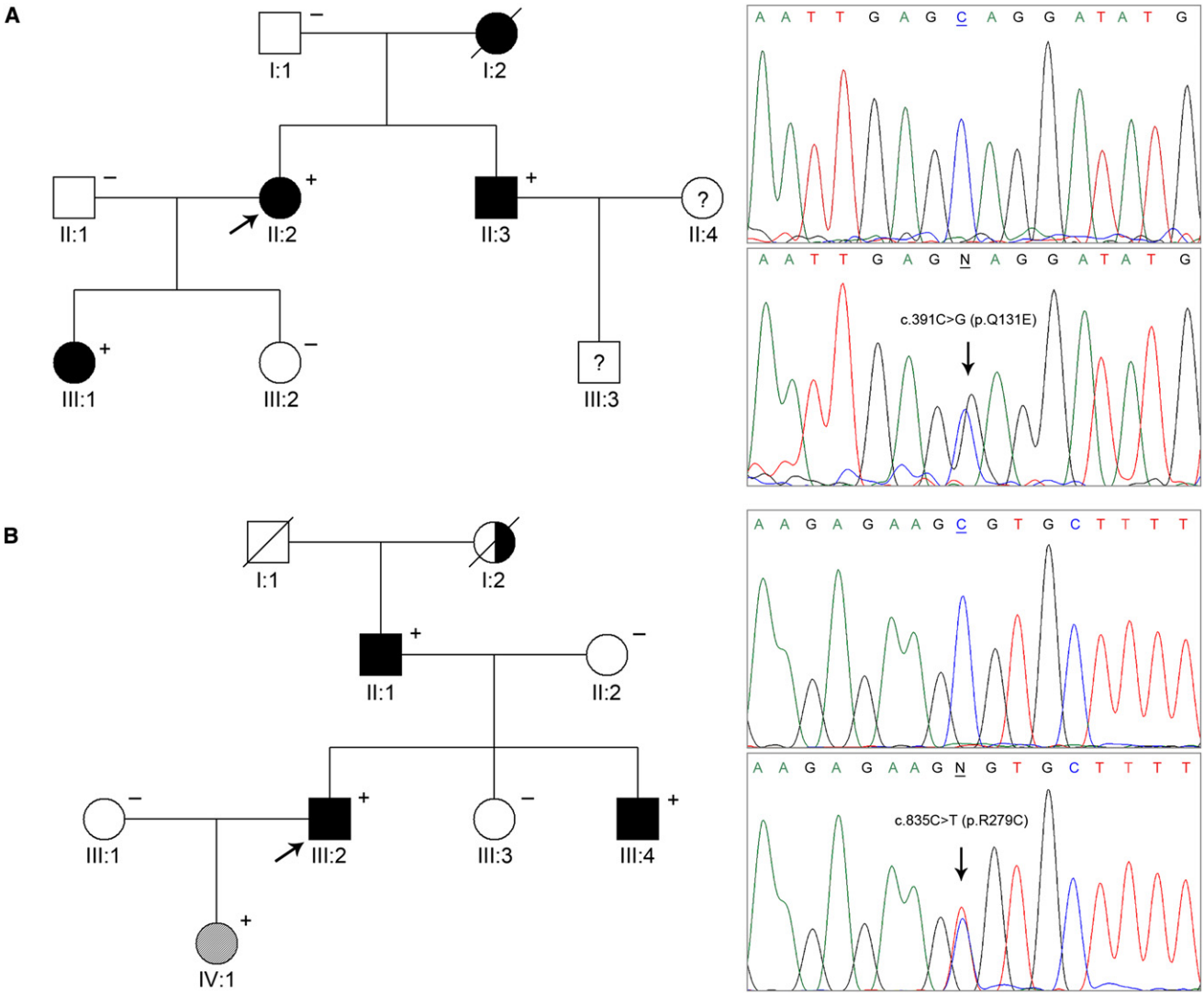

Figure 1. Mutation Analysis of *NEXN* in Familial Hypertrophic Cardiomyopathy
Filled symbols indicate clinically affected individuals; open symbols indicate unaffected individuals; half-filled symbol indicates suspected affected individual; a slash through a circle or square indicates a deceased individual; plus (+) and minus (−) signs indicate the presence or absence of a mutation in *NEXN*, respectively; symbol with "?" indicates an individual who did not have clinical evaluation and genetic testing; squares indicate males; circles indicate females; shaded symbol indicates an individual who was not included in the LOD score calculations. The proband patient in each family is marked with an arrow. The right part of each panel indicates direct sequencing data of *NEXN* mutant sequences compared with a normal control.
(A) Pedigree of Family A. *NEXN* mutation p.Q131E was identified in II-2, II-3, and III-1.
(B) Pedigree of Family B. *NEXN* mutation p.R279C was identified in II-1, III-2, III-4, and IV-1.

of them. The echocardiogram showed that his father had asymmetrical left ventricular hypertrophy with an anterior wall thickness of 22 mm, and the ECG revealed atrial fibrillation and ST-T changes. His younger brother was found to have moderate asymmetrical interventricular septal thickness (16 mm), with an abnormal ECG indicating voltage criteria for left ventricular hypertrophy. The proband's daughter (IV:1) also carried the same mutation and was clinically asymptomatic; however, because she was only 12 years of age at the time of evaluation, she most likely was in the presymptomatic phase of the disease. Clinical findings of the individuals with and without *NEXN* mutations in the two Chinese HCM families are summarized in Table 1 and Table S1 (available online), respectively. Linkage analysis was performed in the two pedigrees with the use of Linkage 5.1. Individual IV:1 in family B is assumed to be below the age of onset and, hence, is not included in the calculation. The LOD score is 0.60 for family A and 0.56 for family B. The combined LOD score of the two pedigrees is 1.16, suggesting that the observed segregation would occur at a random chance of less than 1 in 14. Although the *NEXN* mutations segregate with HCM in these families, the small sample size may limit the strength of the linkage signal.

To exclude the possibility that the two identified sequence variants (p.Q131E and p.R279C) are rare

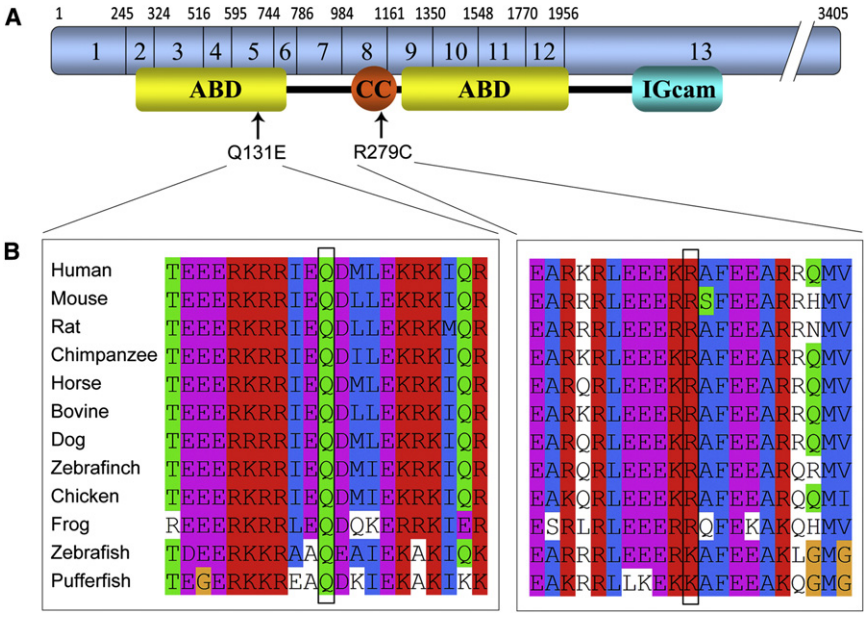

Figure 2. Location and Conservation of the NEXN Protein Amino Acid Residues Affected by the Mutations

NEXN is a 13-exon gene encoding two N-terminally located actin-binding domains (ABD), a coiled-coil domain (CC), and a C-terminal immunoglobulin superfamily class (IGcam).

(A) Schematic structure of *NEXN* mRNA and protein. The p.Q131E mutation is located in exon 5 and in the first actin-binding domain; the p.R279C mutation is located in exon 8 and in the coiled-coil domain.

(B) NEXN peptide sequences surrounding the mutated residues (black box) with multiple interspecies alignments generated by ClustalW. Q131 and R279 amino acid residues of NEXN are highly conserved across species.

polymorphisms without functional significance, we first confirmed that the two variants were not reported in the NCBI dbSNP database. Furthermore, neither of the two mutations could be identified in 192 ethnically matched healthy control subjects. To further confirm that the two mutations (p.Q131E and p.R279C) are likely to be disease causing, we applied two commonly used in silico algorithms, PolyPhen-2 and SIFT, to predict the putative effects of the two mutations on protein function.[24–26] Both of the two algorithms strongly suggested that the two *NEXN* sequence variants might be disease-causing mutations.

In addition to the two mutations, we also identified two SNPs, c.733G/A (p.G245R [rs1166698]) and c.1419A/G (p.R473R [ss252441070]), the frequency of each having no significant difference between HCM cases and controls (Table S2).

HCM has long been considered a disease of the sarcomere, more specifically a disease of the myofilament because of the hundreds of mutations scattered throughout the genes that encode proteins of the myofilament. With the recent discovery of HCM-associated mutations in genes encoding proteins of the Z-disc[17–21] and proteins involved in calcium-induced calcium release,[27,28] the body of knowledge regarding the genetic spectrum of HCM continues to expand. Nexilin, encoded by *NEXN* and recently isolated as a Z-disc protein, plays an important role in stabilizing and protecting Z-discs from mechanical forces.[22] Nexilin is expressed specifically in heart and skeletal muscle and contains two N-terminally located actin-binding domains (ABD), a coiled-coil domain (CC), and a C-terminal immunoglobulin superfamily class domain (IGcam) (Figure 2). Hassel and colleagues identified one deletion mutation (p.G650 del) and two missense mutations (p.P611Y and p.Y652C) in *NEXN* in a large cohort of patients with dilated cardiomyopathy. Notably, the three identified *NEXN* mutations reside in the rather

Table 1. Clinical Features of Affected Patients with *NEXN* Mutations

Family and Subject	Sex	Age (Yrs)	SBP/DBP	LVmax	PW	LVESD	LVEDD	LA	EF (%)	ECG	Mutation
Family A											
II:2	F	37	128/76	21	11	28	46	37	67	T-wave changes	p.Q131E
II:3	M	34	122/82	18	10	29	45	40	62	LVH voltage criteria	p.Q131E
III:1	F	16	108/70	14	8	26	39	41	57	normal	p.Q131E
Family B											
II:1	M	67	135/85	22	12	39	57	46	55	AF, ST-T changes	p.R279C
III:2	M	45	132/80	17	9	28	45	38	65	normal	p.R279C
III:4	M	43	129/75	16	10	30	51	35	71	LVH voltage criteria	p.R279C
IV:1	F	12	95/62	11	7	25	38	34	59	normal	p.R279C

F, female; M, male; SBP/DBP, systolic blood pressure/diastolic blood pressure (mm Hg); LVmax, left ventricular wall maximal thickness; PW, posterior wall thickness (mm); LVESD, left ventricular end–systolic diameter (mm); LVEDD, left ventricular end–diastolic diameter (mm); LA, left atrial anterior-posterior diameter (mm); EF, ejection fraction; ECG, electrocardiogram; AF, atrial fibrillation.

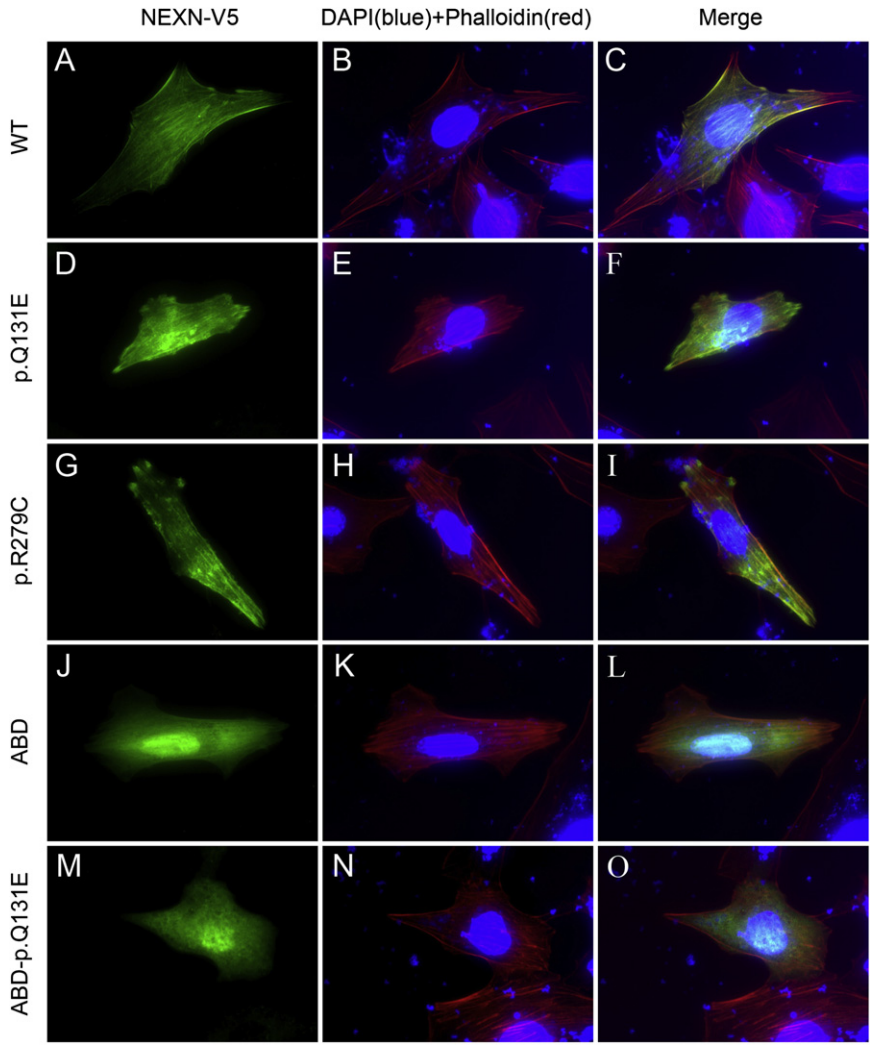

Figure 3. Distribution of V5-Tagged NEXN in C2C12 Cells

C2C12 cells transfected with V5-tagged full-length wild-type (WT) (A–C) or mutants p.Q131E (D–F) and p.R279C (G–I) or with V5-tagged first actin-binding domain (ABD) (J–L) or mutant $NEXN_{ABD}$-p.Q131E (M–O). *NEXN* constructs were fixed 24 hr after the transfection and stained by a V5 antibody followed by a secondary antibody (A, D, G, J, M) and incubated with phalloidin and DAPI (B, E, H, K, N) (Magnification: 400×). Merged images (C, F, I, L, O) are shown. Overexpression of wild-type NEXN was preferentially distributed along F-actin, whereas mutants p.Q131E and p.R279C showed partially abnormal aggregates in the cytoplasm. Expression of the $NEXN_{ABD}$ fragment of amino acids 1–164 in C2C12 cells showed that this fragment tended to be localized in the nucleus and was partially distributed along F-actin, whereas the mutant $NEXN_{ABD}$-p.Q131E fragment was completely dispersed into the nucleus and cytoplasm.

restricted region of amino acids 611–652 located in the IGcam domain of nexilin. Interestingly, the two mutations, p.Q131E and p.R279C, that we identified in the Chinese patients with HCM are located in the first ABD and CC, respectively, each of which is closer to the N terminus of nexilin.

The differences in the location of mutations identified in the two cohort studies may implicate that the molecular mechanisms underlying the *NEXN*-related pathogenesis for DCM and HCM might be different. To determine whether the *NEXN* mutations, p.Q131E and p.R279C, affect the localization of nexilin protein and its binding ability to α-actin, we created *NEXN* expression vectors and performed an immunocytochemistry study. Because the p.Q131E mutation is exactly situated in the first actin-binding domain of nexilin, we also generated fragment clones containing this binding domain to better understand the effects of the mutation on actin binding. The human full-length *NEXN* cDNA and the fragment encoding the first ABD (amino acids 1–164) were cloned into pcDNA3.1/V5 vector. Mutants were generated by site-directed mutagenesis, and all generated constructs were confirmed by direct sequencing. Cultured C2C12 cells were transfected with the wild-type or mutant V5-tagged *NEXN* constructs with the use of Lipofectamine 2000. Cells were stained by rabbit V5 polyclonal antibody followed by secondary donkey FITC-conjugated antibody to rabbit IgG, and Alexa Fluor 633 conjugated to phalloidin was used for F-actin staining. Nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). The results showed that the wild-type NEXN was preferentially distributed along F-actin, whereas the mutants p.Q131E and p.R279C presented local accumulations in the cytoplasm (Figure 3). Expression of the $NEXN_{ABD}$ fragment (amino acids 1–164) in C2C12 cells showed that this fragment tended to aggregate in the nucleus and was partially distributed along F-actin; however, when p.Q131E was introduced, the mutant $NEXN_{ABD}$ fragment was entirely dispersed into nucleus and cytoplasm and completely lost the ability to bind to F-actin (Figure 3). Similar results were observed in differentiated C2C12 cells (Figure S1). Noticeably, intranuclear accumulation of the $NEXN_{ABD}$ fragment points to the possibility that a cytoplasmic localization signal (CLS) exists at the C-terminal part of nexilin. Immunoblot analysis did not show obvious differences in the protein levels of full-length wild-type and mutants or between $NEXN_{ABD}$ and $NEXN_{ABD}$-p.Q131E (Figure S2). To verify the above findings in immunocytochemistry, we then performed a coimmunoprecipitation study. The human α-actin cDNA was cloned into pcDNA3.1/NT-GFP vector and was cotransfected with

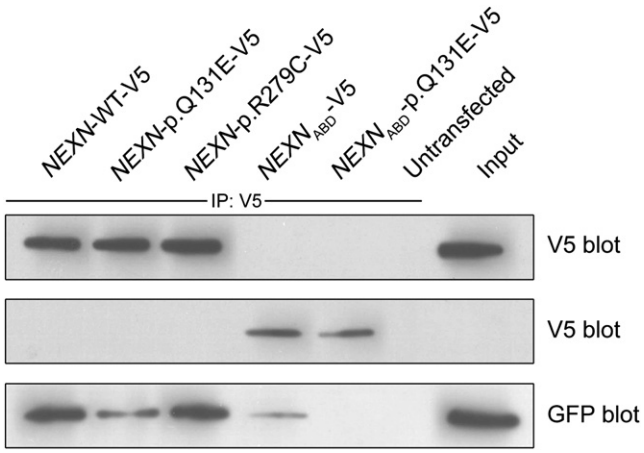

Figure 4. Binding of NEXN to α-Actin
Binding of the full-length NEXN and NEXN fragment to α-actin was analyzed by coimmunoprecipitation and subsequent immunoblotting. Lysates from HEK293 cells cotransfected with NT-GFP-tagged α-actin and V5-tagged *NEXN* constructs (*NEXN*, *NEXN*-p.Q131E, *NEXN*-p.R279C, $NEXN_{ABD}$, or $NEXN_{ABD}$-p.Q131E) were immunoprecipitated with a V5-specific antibody (IP: V5) and subsequently immunoblotted with a HRP-conjugated V5- and GFP-specific antibody. Untransfected cells served as negative controls. Inputs were cell lysates subject to immunoblotting without immunoprecipitation.

NEXN-V5 vectors into human embryonic kidney (HEK) 293 cells. Immunoprecipitation and subsequent immunoblotting were performed, with slight variations, as previously described.[29] Our results showed that the p.Q131E mutation decreased the binding of full-length NEXN to α-actin and abolished the binding of the $NEXN_{ABD}$-p.Q131E mutant fragment to α-actin (Figure 4). In addition, the p.R279C mutation did not change the binding ability of nexilin to α-actin. However, we cannot exclude the possibility that this mutation might be involved in the binding of nexilin to other components of the sarcomere or Z-disc. Taken together, our findings provide the biochemical basis for the possible pathogenesis of HCM associated with *NEXN* mutations.

The Z-disc has received extensive attention recently in cardiac hypertrophy research because mutations in genes encoding several of its constituents have been shown to cause hypertrophic cardiomyopathy.[7,13,16,19,20] Proteins of Z-discs are important in stabilizing sarcomere, integrating mechanical forces, and distributing these within the muscle cell and to the extracellular matrix. In addition, they appear to serve as a docking station for transcription factors, Ca^{2+} signaling proteins, kinases, and phosphatases and as a way station for proteins that regulate transcription by aiding in their controlled translocation between the nucleus and the Z-disc.[13,30] With these roles, a main implication for the Z-disc is its involvement in the cardiomyocyte stretch-sensing and -response systems, which may transduce multiple signaling pathways during stress, translating into hypertrophic responses and remodeling.[15,31] Nexilin has a unique role in stabilizing cardiac Z-discs and is essential for maintaining Z-disc integrity against the extreme forces generated during muscle contraction.[22] As a recently identified member of the Z-disc gene family and because of the potential differences between HCM and DCM in the pathogenesis caused by *NEXN* defects, the function of *NEXN* remains to be further elucidated.

Supplemental Data

Supplemental Data include two tables and two figures and can be found with this article online at http://www.cell.com/AJHG/.

Acknowledgments

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Web Resources

The URLs for data presented herein are as follows:

ClustalW2, http://www.ebi.ac.uk/clustalw2/
dbSNP, http://www.ncbi.nlm.nih.gov/SNP
GenBank, http://www.ncbi.nlm.nih.gov/Genbank/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
SIFT, http://sift.jcvi.org/

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