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ANKRD1, the Gene Encoding Cardiac Ankyrin Repeat Protein, Is a Novel Dilated Cardiomyopathy Gene

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Abstract

Objectives—We evaluated ankyrin repeat domain 1 (*ANKRD1*), the gene encoding cardiac ankyrin repeat protein (CARP), as a novel candidate gene for dilated cardiomyopathy (DCM) through mutation analysis of a cohort of familial or idiopathic DCM patients, based on the hypothesis that inherited dysfunction of mechanical stretch-based signaling is present in a subset of DCM patients.

Background—CARP, a transcription coinhibitor, is a member of the titin-N2A mechanosensory complex and translocates to the nucleus in response to stretch. It is up-regulated in cardiac failure and hypertrophy and represses expression of sarcomeric proteins. Its overexpression results in contractile dysfunction.

Methods—In all, 208 DCM patients were screened for mutations/variants in the coding region of *ANKRD1* using polymerase chain reaction, denaturing high-performance liquid chromatography, and direct deoxyribonucleic acid sequencing. In vitro functional analyses of the mutation were performed using yeast 2-hybrid assays and investigating the effect on stretch-mediated gene expression in myoblastoid cell lines using quantitative real-time reverse transcription–polymerase chain reaction.

Results—Three missense heterozygous *ANKRD1* mutations (P105S, V107L, and M184I) were identified in 4 DCM patients. The M184I mutation results in loss of CARP binding with Talin 1 and FHL2, and the P105S mutation in loss of Talin 1 binding. Intracellular localization of mutant

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CARP proteins is not altered. The mutations result in differential stretch-induced gene expression compared with wild-type CARP.

Conclusions—*ANKRD1* is a novel DCM gene, with mutations present in 1.9% of DCM patients. The *ANKRD1* mutations may cause DCM as a result of disruption of the normal cardiac stretch-based signaling.

Keywords

DCM; CARP; *ANKRD1*; mutations

Dilated cardiomyopathy (DCM), a primary disorder of the cardiac muscle characterized by ventricular chamber dilation and diminished cardiac contractility (1), is the most common cause of chronic heart failure (CHF) in the young and the most common indication for cardiac transplantation (2). The underlying etiologies are varied and include genetic, viral (myocarditis), toxins like alcohol, mitochondrial, and metabolic disorders (3–6).

Familial inheritance is seen in ≈30% to 40% of DCM patients (5). Autosomal dominant mode of inheritance is the most common (≈90%), followed by X-linked (5% to 10%), autosomal recessive, and mitochondrial inheritance patterns (<5%) (7). To date, mutations in ≈20 genes have been discovered in patients with DCM (8). Of these genes, the genes encoding Z-band alternatively spliced PDZ-motif protein, titin, lamin A/C, and β-myosin heavy chain may each be responsible for 5% to 10% of familial DCM cases (9–12), with dystrophin thought to contribute in 10% to 15% of boys with DCM (13).

Most of the known DCM-causing mutations are thought to be pathogenic due to resulting deficits in force generation (beta-myosin heavy chain, cardiac troponin T) (14), force transmission (cardiac actin, alpha-tropomyosin, desmin, dystrophin, delta-sarcoglycan, beta-sarcoglycan) (15), or energy production (mitochondrial mutations) (16). Abnormal signaling in response to force (abnormal stretch-based signaling) is another potential mechanism for inherited DCM that merits further investigation.

We hypothesized that inherited dysfunction of mechanical stretch-sensing and stretch-based signaling forms the pathogenic basis for a subset of DCM patients. Telethonin, cysteine- and glycine-rich protein 3 (CSRP3/MLP), and titin, which have been implicated in DCM (12,17,18), have a role in stretch-sensing and stretch-based signaling, in addition to their structural properties, and abnormal mechanotransduction may be 1 of the mechanisms through which mutations in these proteins cause DCM. This hypothesis is further strengthened by the fact that targeted disruption in mice of genes such as β_1-integrin and melusin, which have stretch-sensing functions, results in DCM (19,20).

Based on this hypothesis, we screened ankyrin repeat domain 1 (*ANKRD1*), the gene encoding CARP, a transcription cofactor that translocates to the nucleus in response to mechanical stretch (21) and is up-regulated in both cardiac failure (22,23) and hypertrophy (24). CARP is present in the I-band region of the sarcomere as a member of the titin-N2A mechanosensory unit (21). CARP is induced by mechanical stretch (21,25), α- and β-adrenergic signaling (26,27), and cytokines including transforming growth factor (TGF)-β (28). Studies have shown that CARP acts as a transcription coinhibitor and represses the expression of sarcomeric proteins, including myosin light chain, cardiac troponin T, and myosin heavy chain species (29), and overexpression of CARP in engineered heart tissue causes contractile dysfunction (26).

In this study, we present the results of a comprehensive mutation screening of 208 patients with familial or idiopathic DCM for the presence of nonsynonymous sequence variants in

the coding region of *ANKRD1*, demonstrating that *ANKRD1* is a novel DCM gene and that mutations in *ANKRD1* occur in ≈2% of DCM patients. In addition, our functional analyses show that these mutations lead to impaired protein-protein interactions and altered gene expression in response to mechanical stretch, suggesting that inherited dysfunction of stretch-based signaling is another avenue for the pathogenesis of DCM.

Methods

Study patients

Genomic deoxyribonucleic acid (DNA) from 160 patients from the United Kingdom and 48 patients from Japan with familial or idiopathic DCM were screened for mutations. DCM was diagnosed on the basis of World Health Organization/International Society and Federation of Cardiology Task Force criteria (1), and clinical evaluation of the patients was performed as previously described (18). Genetic studies were performed blinded to clinical information. After written informed consent, blood for DNA extraction was obtained, as regulated by the Institutional Review Boards at St. George's Hospital Medical School, London, United Kingdom, and the Baylor College of Medicine, Houston, Texas, and the Ethics Reviewing Committee of Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan.

Mutation screening

Peripheral blood-derived genomic DNA was used to amplify the 9 coding exons of *ANKRD1* (GenBank Accession no. NM_0143912) by polymerase chain reaction (PCR) using primers derived from the adjoining intronic sequences (PCR primers and reaction conditions available upon request). The PCR amplicons were analyzed using denaturing high-performance liquid chromatography followed by direct sequencing, as previously described. Japanese samples were analyzed by direct sequencing of the PCR products.

Construction of the wild-type and mutant *ANKRD1* vectors

The full-length *ANKRD1* complementary deoxyribonucleic acid (cDNA) was inserted into pEGFP-C1 (providing an N-terminal green fluorescent protein (GFP) tag, Clontech BD Biosciences, Palo Alto, California) and mutations were introduced using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California). The *ANKRD1* cDNA clones were sequenced completely to confirm the presence of the desired mutation, the absence of cloning artifacts, and to ensure that the GFP and *ANKRD1* coding sequences were in-frame. The wild-type and mutant *ANKRD1* cDNAs were subcloned into a pcDNA 3.1 V5-His vector (Invitrogen, Carlsbad, California) to generate a C-terminal V5-His tagged fusion protein. The fidelity of the subcloned fragment was confirmed by direct DNA sequencing. The AdenoX system (Clontech, Palo Alto, California) was used for the generation of replication-incompetent adenoviral vectors carrying the wild-type or mutant V5-His-tagged *ANKRD1* cDNA.

Transfections and immunofluorescent detection of GFP

Cultured C2C12 mouse myoblastoid cells (American Type Culture Collection, Manassas, Virginia) were transfected with the wild-type or mutant GFP tagged *ANKRD1* cDNA constructs using Effectene (Qiagen, Valencia, California). Cells were fixed with cold 2% paraformaldehyde, permeabilized with 0.25% Triton X-100 (Roche Applied Sciences, Indianapolis, Indiana) after 48 h, and incubated with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen) for nuclear staining and visualized by an Olympus epifluorescence microscope.

Yeast 2-hybrid assays

For yeast 2-hybrid mating studies, CARP cDNAs were inserted into the pGBKT7 bait vector (BD Biosciences). We used CARP clones coding for wild-type or mutant CARP as baits to qualitatively compare their interactions. As prey clones (inserted in pGADT7), we used a set of 39 genes: this gene set has been identified by a recent yeast 2-hybrid screen as coding for potential CARP interacting partners (S.H. Witt and S. Labeit, unpublished data, February 2009).

Technically, mating assays were performed as previously described (21). Briefly, the wild-type and mutant CARP cDNA were subcloned into pBKT7 yeast 2-hybrid vectors and the recombinant baits transformed into *Saccharomyces cerevisiae*, strain AH109. The AH109 cells were cotransformed with recombinant library plasmids containing cDNAs of novel CARP-interacting ligands during a 2-hybrid survey of cardiac and skeletal muscle prey libraries for novel CARP-interacting proteins. The transformed cells were incubated for 5 days at 30°C on SD/Leu–/Trp–/His– plates. Subsequent determination of β-galactosidase activities were performed as described previously (21) and ligands with differential binding between the wild-type and mutant CARP proteins identified.

Gene expression assays

Of the 3 disease-associated variants, the P105S and V107I variants were selected for the gene expressions assays. The H9C2 cells (derived from rat embryonic myocardium) were plated in collagen-coated Flexcell stretchable 6-well plates at 80% density and transduced with first-generation adenoviral vectors carrying V5-His-tagged wild-type or mutant *ANKRD1* cDNA, at 100 multiplicity of infection, as per manufacturer's instructions (Clontech). Then, 48 h after transduction, the cells in the stretchable plates were placed in a Flexcell 4000 unit (Hillsborough, North Carolina) in a 37°C incubator with the usual 5% CO_2, and cyclically stretched at a strain rate of 10% and a frequency of 60 Hz for 6 h. The cells were then harvested and ribonucleic acid (RNA) extracted using Trizol (Invitrogen) and purified a second time using RNEasy columns (Qiagen). From each of the RNA samples, 150 ng of total RNA was used as a template in a quantitative real-time PCR reaction, performed in an ABI Prism 7500 Sequence Detection System (Applied Biosystems) with SYBR Green technology. The genes quantitated encode troponins (*TNNT1*, *TNNT2*, *TNNT3*, *TNNC1*), myosin species (*MHY7*, *MLC2*), myogenin (*MYOG*), P53 (*TP53*), calsequestrin (*CASQ2*), early growth response factor (*EGR1*), atrial natriuretic factor (*NPPA*), and TGFβ (*TGFB1*). In addition, a panel of 6 housekeeping genes (beta 2 micro-globulin, GAPDH, Eefig, Hmbs, Cyclophilin, and *ALAS*) was checked, and using Genorm software (Primer Design Ltd., Southampton, United Kingdom), the samples were normalized to their starting template content.

Results

Mutation analysis of *ANKRD1* gene

We identified 3 heterozygous, missense, sequence variants c.313C>T (p.P105S), c. 319G>T (p.V107L), and c.552G>A (p.M184I) in 4 Caucasian patients (Fig. 1): the P105S variant was identified in 2 patients. None of these variants was detected in 180 (360 chromosomes) ethnically-matched healthy normal control subjects and have not been reported in the dbSNP database. No disease-associated mutations were found in Japanese patients.

Proline 105 and Valine 107 are located between the nuclear localization sequence and PEST (amino acids proline [P], glutamic acid [E], serine [S], threonine [T]) sequence, a signal for degradation (Fig. 2A). Methionine 184 is located in the second ankyrin repeat domain, close

to the titin-N2A binding region of CARP (Fig. 2A). CARP is highly conserved across species, including at each of the 3 affected residues (Fig. 2B).

Clinical characteristics of the probands with *ANKRD1* variants

All 4 patients carrying the variants were male. One proband with the P105S variant presented at the age of 15 years with a fractional shortening (FS) of 19% and left ventricular end-diastolic diameter (LVEDD) of 70 mm (Table 1). His father had isolated left ventricular dilation. The second proband with the P105S variant had no family history of DCM and presented at the age of 52 years with an FS of 13% and LVEDD of 72 mm (Table 1). The proband with V107L variant also had no family history and presented at the age of 68 years with an FS of 12% and LVEDD of 61 mm (Table 1).

The proband carrying the M184I variant had a possible autosomal-dominant inheritance. He presented at the age of 33 years with an FS of 10% and LVEDD of 83 mm (Table 1). He had 1 affected sister with isolated left ventricular dilation. The M184I variant was identified in the affected sibling and his unaffected father. Family pedigrees of 3 of the probands are shown in Figure 3.

Intracellular localization of CARP protein

The presence of the substitutions in CARP did not alter its intracellular localization in undifferentiated C2C12 myoblastoid cells in the basal unstretched state or in H9C2 cells after cyclical stretch, with both wild-type and mutant proteins showing intranuclear and cytoplasmic localization (Fig. 4), as previously reported for wild-type CARP (21).

Differential binding of the wild-type and mutant CARP proteins using yeast 2-hybrid assays

The M184I mutation resulted in a loss of binding of CARP with Talin-1 and 4-and-a-half LIM domains 2 (FHL2). Talin-1 is a 270 kD protein located in the β-integrin protein complex and plays an important role in binding the β-integrin subunit with the cytoskeleton. FHL2 is a transcription cofactor and is also located in the titin-N2B and β-integrin complexes. The P105S mutation results in loss of CARP binding with Talin-1. No differential binding with respect to the wild-type protein was identified in the V107L mutation.

Changes in mechanical stretch-induced gene expression after wild-type and mutant CARP expression

Of the 3 DCM-associated *ANKRD1* variants, the P105S and V107L variants were selected for evaluation of mechanical stretch-induced gene expression compared with wild-type CARP. The P105S substitution enhanced the down-regulation of p53 and up-regulation of myogenin seen after transduction with wild-type CARP (Fig. 5), suggesting a gain of function effect. In contrast, the V107L substitution blocked the decreased expression of TGFBR1 and CASQ2 seen in the wild-type expressing cells (Fig. 5). However, this substitution enhanced the down-regulation of EGR1 seen in the wild-type cells and decreased the expression of TNNT1, which was up-regulated in wild-type cells (Fig. 5). The expression of other isoforms of troponin T (TNNT2 and TNNT3) was, however, not changed.

Discussion

ANKRD1 is a novel DCM gene

Our data indicate that *ANKRD1* (encoding cardiac ankyrin repeat protein) is a novel disease gene in DCM, with variants identified in 4 of 208 (1.9%). The 3 nonsynonymous *ANKRD1* variants (P105S, V107L, and M184I) were identified only in the patient cohort, resulted in the substitution of conserved amino acid residues, and altered protein–protein interactions and/or stretch-induced gene expression, suggesting that they are disease causing. The prevalence of *ANKRD1* mutations in our DCM patient cohort is consistent with the published mutation prevalence data for most of the other known DCM-associated genes that vary from 1% to 3% (except *MHC7*, *titin*, *LMNA*, and *LDB3*, which may each account for 5% to 10% of DCM cases).

The role of CARP in cardiac hypertrophy and failure

Since its discovery in 1995, the *ANKRD1* gene and its transcript CARP have elicited significant interest as one of the transcripts found to be persistently up-regulated in cardiac hypertrophy and heart failure, although its exact role in these conditions is not yet clear. CARP is predominantly expressed in cardiac muscle, with lower expression levels in skeletal muscle and endothelial cells. It is one of the earliest markers of cardiac muscle cell lineage and is downstream in the Nkx2.5 pathway that defines the early heart field in the developing embryo (30). The high level of ventricular CARP expression in the fetal heart, down-regulation in the adult ventricle, and significant up-regulation during cardiac hypertrophy (24) indicates that CARP is part of the developmentally regulated fetal gene program (31). CARP has been shown to be a transcription coinhibitor and decreases the expression of myocyte contractile elements including cardiac actin, skeletal actin, and myosin light chain 2V (29). Adenoviral-mediated transduction of C2/C2 cells with CARP decreases overall DNA synthesis, indicating that CARP may play a role in decreasing cellular proliferation. In addition, overexpression of CARP in engineered cardiac tissue results in contractile dysfunction (26).

Significance of altered protein–protein interactions of the P105S and M184I substitutions

Both the P105S and M184I substitutions result in loss of CARP binding with Talin 1. Talin 1 is a key binding partner of the beta-integrin subunit of the integrin-complex (which connects the extracellular matrix with the intracellular cytoskeleton and is a putative cellular mechanosensory unit). Hence, disruption of the CARP-Talin 1 interaction may result in altered mechanical stretch-based signaling. In addition to loss of Talin 1 binding, the M184I substitution also results in loss of CARP interaction with the FHL2 protein. The FHL2 protein is highly expressed in the heart and binds to the N2B domain of titin, which has potential stretch-sensing functions. FHL2 may have dual roles, acting both as an adaptor protein as well as a transcription coactivator and selectively increases the transcriptional activity of the androgen receptor. Recently, an FHL2 variant that significantly decreased FHL2 binding with the titin-N2B segment was reported in a DCM patient (32). Similarly, a DCM-associated mutation has been reported in the titin-N2B region (12). These findings together indicate that interaction between the stretch-based signaling molecules is important in the functional integrity of the cardiomyocyte, and disruption of this interaction may be one of the pathways to DCM.

Implication of the gene-expression changes due to the *ANKRD1* mutations in the pathogenesis of DCM

The P105S variant resulted in down-regulation of p53 and up-regulation of myogenin compared with wild-type CARP, and the V107L variant up-regulated the expression of

TGFβ1 and calsequestrin 2, and down-regulated EGR1 and slow isoform of troponin T compared with wild-type CARP. The significance of these findings and any potential role in the pathogenesis of DCM still needs to be evaluated. The oncogene p53 has been implicated in cardiomyocyte cell cycle control and apoptosis (33). Myogenin, a muscle differentiation factor, also inhibits cell division and may prevent the remodeling (34) that normally occurs in the overloaded heart. TGFβ is a potent stimulator of collagen synthesis by cardiac fibroblasts (35), and elevated levels of TGFβ are seen in patients with idiopathic DCM (36). Calsequestrin serves as the major calcium ion reservoir within the sarcoplasmic reticulum of cardiac myocytes, and its overexpression causes an abnormal sequestration of calcium, leading to dysregulated EC-coupling in the heart (37). EGR1, a transcription factor and one of the immediate early response genes, is induced in alpha-adrenergic–mediated myocardial hypertrophy (38) and regulates the expression of α-myosin heavy chain (39). Isoforms shift in troponin T, a subunit of the troponin complex that regulates actin-myosin cross-bridge formation, has been described in several animal models and various forms of heart failure, with expression of a fetal isoform in the diseased state (40). Hence, we speculate that in response to mechanical stretch, the DCM-associated mutations in CARP may result in altered expression of proteins involved in key cellular pathways such as cell cycle, apoptosis, growth, and cytokine or calcium signaling. Because our experiments were performed in a rat embryonic heart cell line, H9C2, some caution needs to be used in extrapolating these results to DCM patients without confirming them in other model systems more closely representative of the myocardial milieu, such as primary cardiomyocyte cultures.

Inherited dysfunction of stretch-based signaling, another paradigm for the pathogenesis of familial DCM

Most of the proteins encoded by the known DCM-causing genes are structural components of the sarcolemma, cytoskeleton, or sarcomere involved in force transmission or force generation, and hence they form the basis for our earlier hypothesis that the linkage of the sarcolemma, cytoskeleton, and sarcomere would comprise the “final common pathway” of DCM (41). However, with the recent additions to the ever-lengthening list of DCM-causing genes, it is increasingly apparent that DCM is genetically the most heterogeneous of all the primary cardiomyopathies and inherited cardiac disorders. A subset of the DCM-causing genes encode proteins involved in cellular stretch-based signaling, including MLP (42), FHL2 (32), α-crystallin (43), and Tcap (17), and mutations in these genes may result in DCM by interfering with stretch-based signaling. *ANKRD1* belongs to this subset of DCM-causing genes. Hence, we propose that inherited dysfunction of stretch-based signaling is another paradigm for the pathogenesis of familial DCM. Candidate gene screening based on this paradigm may result in the identification of additional novel DCM-causing genes.

Conclusions

In summary, *ANKRD1*, the gene encoding CARP (a transcription cofactor with presumed stretch-based signaling function), is a novel DCM gene, and genetic variants account for 1.9% of cases. DCM-associated variants in *ANKRD1* result in dysfunction of the cellular stretch-based signaling machinery, suggesting that these are disease causing, and provide support to the hypothesis that inherited dysfunction of cardiac stretch-based signaling is present in a subset of DCM patients.

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Abbreviations and Acronyms

ANKRD1	ankyrin repeat domain 1
CARP	cardiac ankyrin repeat protein
cDNA	complementary deoxyribonucleic acid
CHF	chronic heart failure
DCM	dilated cardiomyopathy
DNA	deoxyribonucleic acid
FHL2	4-and-a-half LIM domains 2
FS	fractional shortening
LVEDD	left ventricular end-diastolic diameter
NYHA	New York Heart Association
PCR	polymerase chain reaction
RNA	ribonucleic acid
TGF	transforming growth factor

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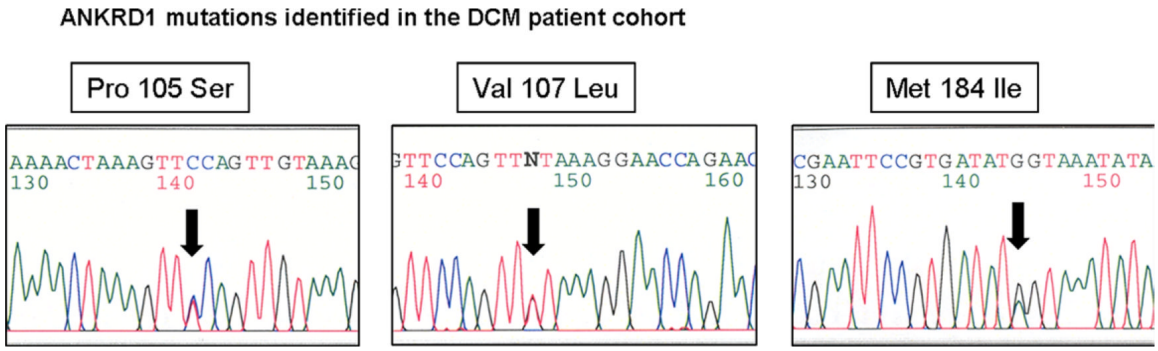

Figure 1. DNA Sequencing of *ANKRD1* Gene

Deoxyribonucleic acid (DNA) sequencing shows the P105S **(left)**, V107L **(middle)**, and M184I **(right)** single nucleotide, heterozygous mutations in the *ANKRD1* gene. DCM = dilated cardiomyopathy.

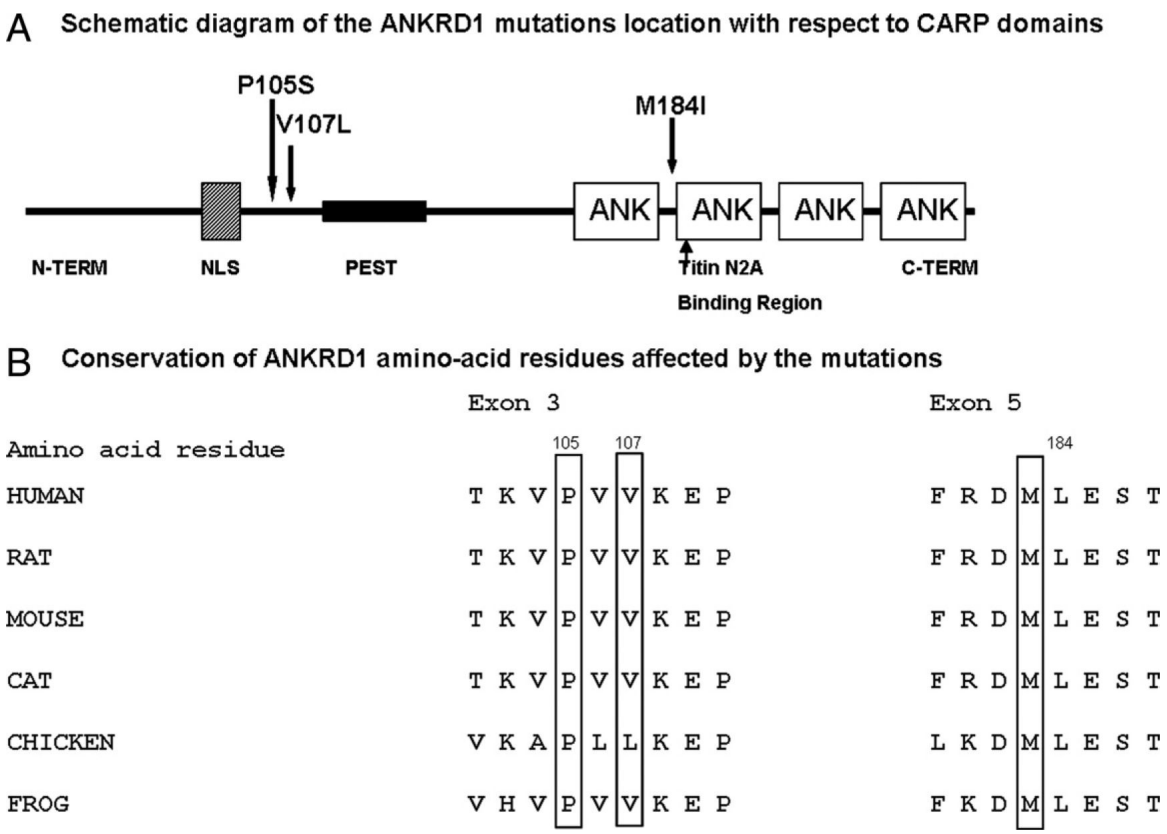

Figure 2. CARP Amino Acid Residues Affected by Mutations

Location and conservation of the cardiac ankyrin repeat protein (CARP [ankyrin repeat domain 1 (*ANKRD1*)]) amino acid residues affected by the mutations. **(A)** The P105S and V107L mutations are in exon 3 and between the nuclear localization region (NLS) and PEST sequence of *ANKRD1*, while the M184I mutation is in exon 5 close to the titin-N2A binding region of the second Ankyrin domain of *ANKRD1*. **(B)** P105, V107, and M184 are evolutionally conserved amino acid residues of *ANKRD1*.

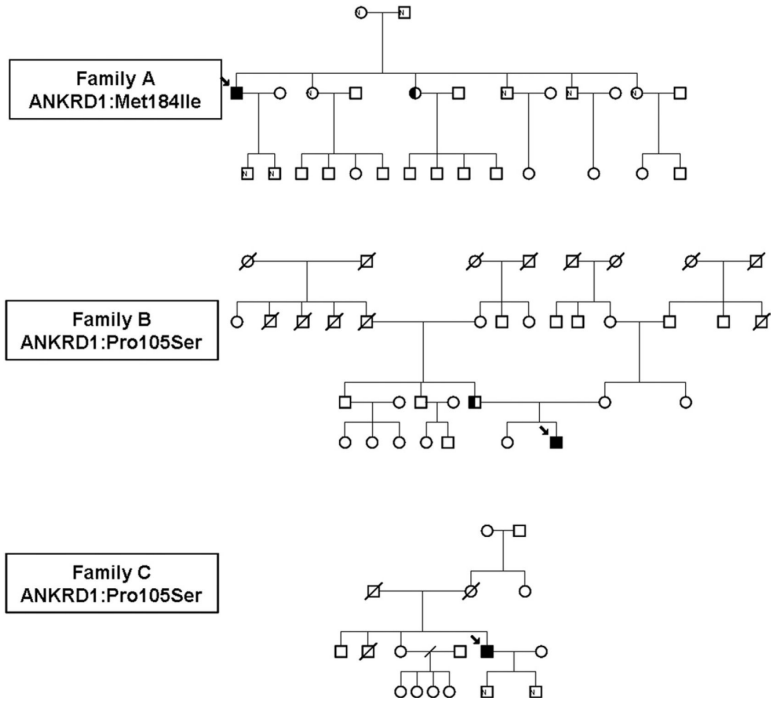

Figure 3. Families With DCM

Pedigree drawings of 3 dilated cardiomyopathy (DCM) families affected by ankyrin repeat domain 1 (*ANKRD1*) mutations. **Squares** indicate male family members; **circles** indicate female family members; **symbols with slash** represent deceased persons; **open symbols** represent unaffected persons; **solid symbols** represent persons affected by DCM; and **half-solid symbols** represent persons with left ventricle enlargement. Probands are identified by **arrows**.

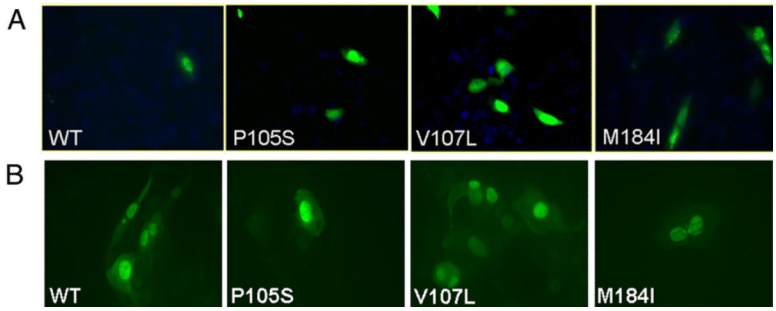

Figure 4. Cytosolic and Nuclear Localization of CARP Protein

Cytosolic and nuclear localization of wild-type (WT), P105S, V107L, and M184I mutant cardiac ankyrin repeat protein (CARP) protein is similar after transfection with GFP-tagged wild-type or mutant *ANKRD1* complementary deoxyribonucleic acid (cDNA) in **(A)** C2C12 cells in basal resting state and **(B)** H9C2 cells after cyclic mechanical stretch.

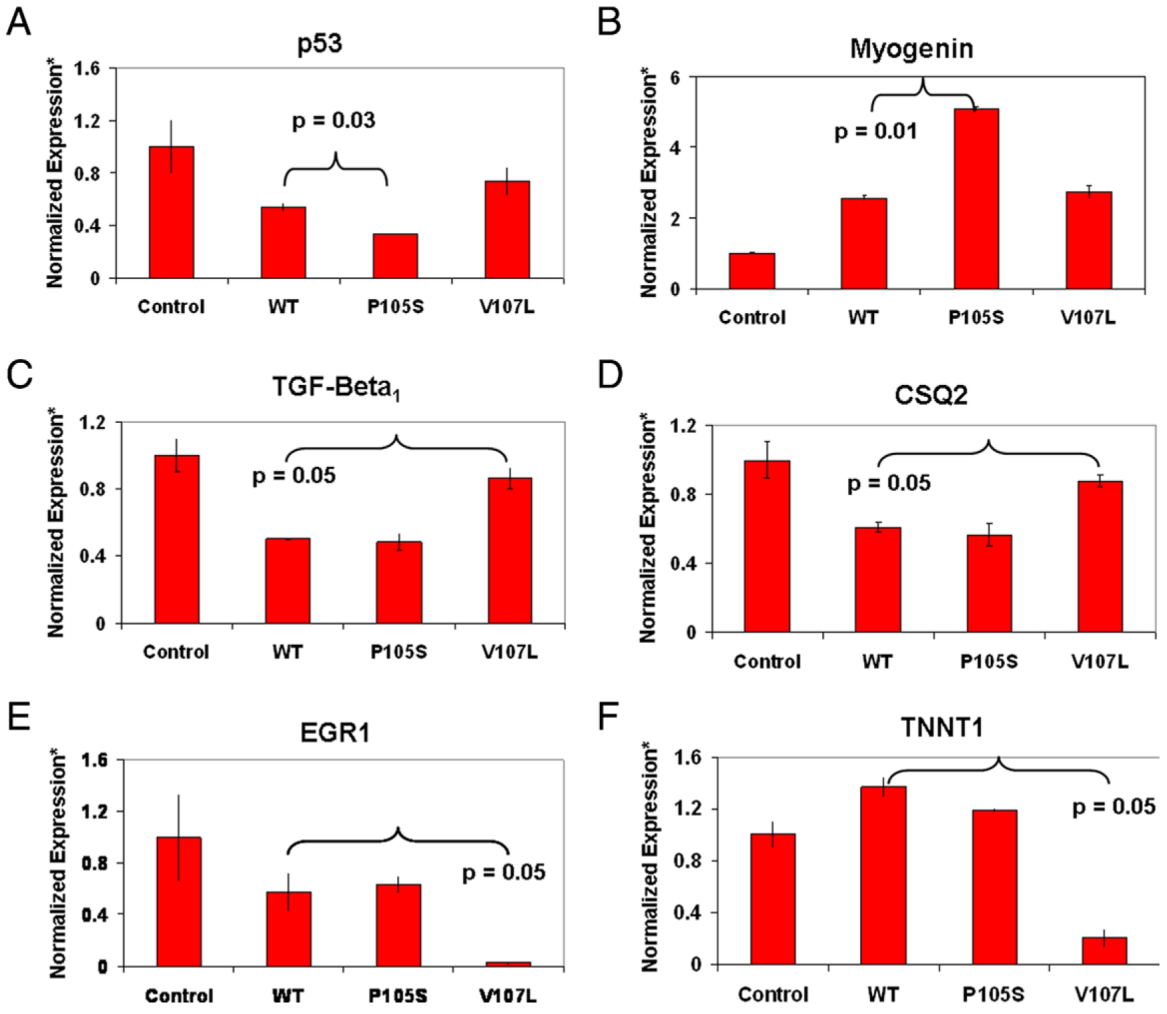

Figure 5. Gene Expression Changes

Gene expression changes in H9C2 cells transduced with wild-type (WT) or mutant *ANKRD1* complementary deoxyribonucleic acid (cDNA) and cyclically stretched for 6 h. **(A)** Expression of p53 is down-regulated (p = 0.03) and **(B)** myogenin expression is up-regulated (p = 0.01) in cells transduced with P105S compared with wild-type *ANKRD1* cDNA. As shown in **(C)** transforming growth factor (TGF)-beta$_1$, (**D**) CASQ2, (**E**) EGR1, and (**F**) TNNT1, TGF-beta$_1$ and CASQ2 are up-regulated (p = 0.05) and EGR1 and TNNT1 are down-regulated (p = 0.05) in cells transduced with V107L compared with wild-type *ANKRD1* cDNA.

Table 1

Clinical Characteristics of DCM Probands With *ANKRD1* Mutations

Mutation	Inheritance	Age (yrs)	Sex	FS (%)	LVEDD (mm)
P105S	Autosomal dominant	15	Male	19	70
P105S	Sporadic	52	Male	13	75
V107L	Sporadic	68	Male	12	61
M184I	Autosomal dominant	33	Male	10	83

ANKRD1 = ankyrin repeat domain 1; FS = fractional shortening LVEDD = left ventricular end-diastolic dimension.