Molecular Insights into Arrhythmogenic Right Ventricular Cardiomyopathy Caused by Plakophilin-2 Missense Mutations

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Background—Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an inherited cardiac disorder mainly caused by dominant mutations in several components of the cardiac desmosome including plakophilin-2 (PKP2), the most prevalent disease gene. Little is known about the underlying genetic and molecular mechanisms of missense mutations located in the armadillo (ARM) domains of PKP2, as well as their consequences on human cardiac pathology.

Methods and Results—We focused on in vivo and in vitro studies of the PKP2 founder mutation c.2386T>C (p.C796R), and demonstrated in cardiac tissue from 2 related mutation carriers a patchy expression pattern ranging from unchanged to totally absent immunoreactive signals of PKP2 and other desmosomal proteins. In vitro expression analysis of mutant PKP2 in cardiac derived HL-1 cells revealed unstable proteins that fail to interact with desmplakin and are targeted by degradation involving calpain proteases. Bacterial expression, crystallization, and structural modeling of mutated proteins impacting different ARM domains and helices of PKP2 confirmed their instability and degradation, resulting in the same remaining protein fragment that was crystallized and used to model the entire ARM domain of PKP2.

Conclusions—The p.C796R and other ARVC-related PKP2 mutations indicate loss of function effects by intrinsic instability and calpain proteases mediated degradation in in vitro model systems, suggesting haploinsufficiency as the most likely cause for the genesis of dominant ARVC due to mutations in PKP2. (Circ Cardiovasc Genet. 2012;5:400-411.)

Key Words: arrhythmogenic right ventricular cardiomyopathy • desmosome • gene expression • plakophilin-2 • structural modeling

A rrhythmogenic right ventricular cardiomyopathy (ARVC) is an inherited myocardial disorder characterized by fibro-fatty replacement of myocardium and a high frequency of ventricular arrhythmias and sudden cardiac death.1,2 To date, several disease genes have been identified and most of them encode proteins of the cardiac desmosome.3 Dominant mutations in plakophilin-2 (PKP2) were found in approximately 25% to 50% of patients with ARVC, suggesting that PKP2 is the major disease gene.4-6

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Plakophilin-2 belongs to the p120ctn subfamily of armadillo (ARM)-related proteins that are characterized by a series of repeated motifs of about 42 amino acids called ARM repeats.5,9 PKP2 binds to the cytoplasmic desmosomal cadherins and hereby is involved in regulating adhesive activity and signaling. It also provides binding sites for desmplakin, which maintains the link to the intermediate filaments, such as desmin.10

Mutations in PKP2 often are associated with a translational frameshift, leading to truncated proteins that, in case of their instability, may lead to haploinsufficiency. Some of the identified mutations cause amino acid replacements in the translated protein, raising the question of whether those changes have a neutral or a disease-causing effect by acting as "poison proteins" through a dominant negative effect. The underlying genetic mechanisms, potential consequences,
and control mechanisms in the disease process, however, remain to be defined.

The involvement of protein quality control mechanisms is essential for regulation of normal protein turnover as well as for the identification and disposal of damaged or misfolded proteins.11 Degradation of aberrant proteins is mainly through the ubiquitin-proteasome system (UPS) or by lysosome-mediated autophagy. Moreover, other cellular proteolytic mechanisms such as calpains, which are calcium-dependent cysteine proteinases, have been shown to be important for many pathological conditions due to excessive proteolysis of their substrates.12,13

Few studies of human cardiac tissue have shown that the amount of detectable PKP2 protein varied in samples of ARVC patients, but especially cardiac specimens of subjects bearing PKP2 frameshift mutations have demonstrated diminished protein levels of PKP2 in immunoblot and immunohistochemical analyses.14,15 Moreover, expression levels of other desmosomal proteins, in particular plakoglobin, have been suggested to be reduced in ARVC-related cardiac tissue.16–19 In addition, cellular expression of 2 PKP2 nonsense mutations has produced unstable PKP2 proteins;20,21 however, the process of protein degradation remains unclear. To date there are limited data about molecular and genetic mechanisms of missense mutations in the C-terminal armadillo domains, as well as their consequences in the context of human cardiac disease.

The objective of this study was to determine the underlying genetic mechanism of ARVC-related missense mutations in PKP2 and their effects on protein structure, stability, and intercellular junctional pathology. We investigated cardiac tissue from patients carrying the heterozygous c.2386T>C (p.C796R) missense mutation in PKP2 and found a patchy expression pattern ranging from unchanged to totally absent levels of PKP2 expression and other junctional proteins. In vitro expression analysis and protein modeling of different mutant PKP2 proteins consistently revealed their instability following proteolytic degradation by calpain proteases. Taken together, our data suggest that haploinsufficiency may play a role in the genesis of ARVC-related mutations in PKP2.

**Methods**

**Clinical Evaluation and Mutation Analysis**

Participating individuals have been clinically evaluated for ARVC according to the task force criteria (TFC).22 After written informed consent under an institutional review board approved protocol, the cardiac biopsies of subject III-1 were taken from the right ventricular (RV) septum. The index case was screened for mutations in PKP2, DSC2, DSG2, DSP, JUP, and DES, as well as for the TMEM43 S358L mutation by Sanger sequencing. Family members underwent targeted analysis of the C796R mutation.

**Construction of Plasmids**

The human PKP2b cDNA (NM_001005242), including an N-terminal Myc-tag, was ligated into pTRE2pur-HA (Clontech) to generate the inducible expression plasmids. Mutant constructs have been generated by site directed mutagenesis according to the particular human mutation (see online-only Data Supplement). For bacterial expression and crystallization, the PKP2a isoform (NM_00100524, the dominant isoform in the heart) was used instead of PKP2b. The PKP2a isoform is 44 amino acids shorter than PKP2b. Thus, the numbering of the amino acids are changed accordingly (eg, the C796R mutation of PKP2b corresponds to C752R of PKP2a, the same for all other variants).

**Cell Culture and Transfection**

HEK293 cells stably expressing the tetracycline reverse transcription activator element23 were maintained in DMEM, and supplemented with 10% fetal bovine serum at 37°C in a 5% CO2 incubator. HL-1 cells were maintained according to Claycomb24 and cotransfected with the rtTA construct. Both cell lines were transfected using PEI (CELLINKTEC) in accordance with the manufacturer’s recommendations. Protein expression was induced with 100 nmol/L doxycycline (SIGMA), and cells were analyzed 72 hours after transfection. Cells were treated with proteasome inhibitors: MG132 (MERCK); calpain inhibitors: ALLN (Peptides International), ZLL (MERCK); lysosome inhibitor: bafilomycin; autophagy inhibitor: 3-MA; and endosome inhibitor: BFA (all SIGMA), respectively.

**Histology, Immunofluorescence, and Immunoblot Analyses**

Sections from paraffin-embedded heart tissue of proband II-1 were stained with Masson’s trichrome. De-paraffinized and rehydrated paraffin-embedded sections were antigen unmasked with sodium citrate buffer and blocked with 5% goat serum for immunofluorescence-analysis. Cells on glass coverslips and frozen tissue sections were fixed in 4% paraformaldehyde, and permeabilized with phosphate buffered saline 1% TritonX-100 blocked in 5% bovine serum albumine and incubated with primary antibodies (see online-only Data Supplement) overnight at 4°C. Secondary antibodies, donkey anti-rabbit, mouse, and rat IgG were conjugated with Alexa Fluor 488 or 555. Samples were mounted with ProLong Gold antiadhere containing DAPI (Invitrogen) and visualized using laser scanning confocal microscopy (SP5, Leica). The immunoblot was performed as previously described25 (see online-only Data Supplement).

**Bacterial Expression, Protein Purification, and Mass Spectrometric Analysis**

The ARM domain of wild-type and mutant human PKP2a (amino acids 346–817) were overexpressed as N-terminal His₆-tagged proteins in E. coli Rosetta (DE3; Merck). Proteins were purified by Ni²⁺-affinity and, in case for wild-type and C752R variant, additionally by size-exclusion chromatography. For mass spectrometric analysis, purified proteins from excised gel bands were converted to peptides by in-gel digestion with trypsin (see online-only Data Supplement).

**Protein Crystallization, Structure Determination, and Prediction**

The C752R variant was crystallized using the hanging drop vapor diffusion method at 20°C by mixing equal volumes of protein (5 mg/ml in 20 mmol/L Hepes–NaOH pH 7.5, 0.1 mol/L NaCl, and 3 mmol/L DTT) and reservoir solution (22% (wt/vol) PEG 3350 and 75 μmol/L malonic acid pH 7.0). Before flash-freezing in liquid nitrogen, the crystal was transferred in a cryoprotectant consisting of reservoir solution supplemented with 25% glycerol. The structure was solved by molecular replacement using the ARM domain of human PKP1 (PDB ID 1xm9)26 as search model (see online-only Data Supplement). The coordinates and structure factors have been deposited with the Protein Data Bank (www.pdb.org, PDB ID code 3TT9). Structure prediction of the entire ARM domain of wild-type PKP2a was performed with the I-Tasser server27–29 using the structure of the stable ARM domain fragment of the PKP2a C752R variant and the ARM domain of human PKP1 as template models.

**Statistical Analyses**

Data are summarized as median and compared using the Mann-Whitney U test. Values of P<0.05 were considered significant.
Results
Regional Changes in Expression Patterns of Desmosomal Proteins in Myocardial Tissue of PKP2 p.C796R Mutation Carriers

A family with a history of arrhythmias was clinically and genetically evaluated (Figure 1A; see online-only Data Supplement Table I); the 20-year-old index patient (IV-1) was initially seen because of palpitations due to nonsustained left bundle branch block-type tachycardia, and subsequently diagnosed with ARVC based on TFC, as well as genotyped for the heterozygous missense mutation c.2386T>C (p.C796R) in the PKP2 gene, a previously described Dutch founder mutation.4–6 Genotyping of her 47-year-old mother (III-1), who fulfilled TFC, and her 76-year-old grandfather (II-1) revealed that both carry this PKP2 mutation. A second cousin died suddenly while exercising at the age of 32 (III-3), and a reported autopsy suggested ARVC. The grandfather (II-1) died unexpectedly after gastric bleeding; records of the ECG and echocardiogram did not show diagnostic signs of ARVC. Postmortem analysis of the heart revealed that the RV was normal in size with extensive fibrosis, scar tissue, and inflammatory changes, but without remarkable fatty tissue (see online-only Data Supplement Table I).

To further determine changes in the expression level and pattern of desmosomnal proteins in RV tissue, we investigated fresh-frozen endomyocardial biopsy samples taken from the septum of individual III-1, and postmortem RV tissue of individual II-1 by immunoblot and immunofluorescence analysis.

We detected equal amounts of PKP2 protein in biopsy tissue of patient III-1 in comparison with control tissue by using a C-terminal antibody against PKP2. The amounts of all other desmosomal proteins also appeared to be widely unchanged (Figure 1B). Immunostaining confirmed this observation; the signal level and localization of PKP2 at the intercalated disc was unaltered (Figure 2A). Other desmosomal proteins, including plakoglobin (JUP), did not show significant expression differences, neither in immunoblot nor in their immunostaining patterns (Figure 2B; see online-only Data Supplement Figure IA).

Next, we analyzed paraffin embedded autopsy tissue of patient II-1. The tissue was taken from different areas of the RV and matched to histological findings. In contrast to the unchanged expression of PKP2, JUP, and desmoplakin (DSP) in areas of histological normal myocardium (eg, area 1), we detected severely reduced signal levels of those proteins in sections next to massive fibrosis (eg, area 2), suggesting a regional process of expression alterations adjacent to advanced tissue remodelling (Figures 2C and 2D; see online-only Supplement Figure IB). Signal levels of the desmosomal cadherins desmoglein-2 and desmocollin-2 also were diminished in certain areas of the RV (data not shown).

Figure 1. Inheritance pattern and immunoblot analysis of the plakophilin-2 mutation p.C796R. A, Pedigree of an arrhythmogenic right ventricular cardiomyopathy family demonstrating disease status and heterozygous mutation carriers (+). Squares represent males, circles females, slash denotes deceased. B, Immunoblot analysis of a right ventricular (RV)-cardiac biopsy of subject III-1 compared with control RV tissue from a rejected donor heart. Samples were assayed for desmosomal proteins; cardiac troponin T and Glyceraldehyde-3-phosphate dehydrogenase served as loading controls. No significant expression differences could be observed.

Mutant C796R PKP2 Protein Remains Unstable, Dissociates From the Junctional Plaque, and Fails to Interact With Desmoplakin

To study the effect of the p.C796R mutation on protein stability and cellular localization in vitro, we transfected cardiac derived HL-1 cells with constructs expressing HA-PKP2 wild-type (WT) or HA-PKP2 C796R. Although comparable with the abundance of PKP2 WT mRNA (data not shown), the expression levels of the HA-PKP2 C796R protein were significantly reduced (30.5±2.4% of HA-PKP2

AB

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WT; P<0.05), suggesting that the mutant protein is intrinsically unstable and undergoes degradation (Figure 3A through C). Immunocytochemical analysis revealed a diffuse cytoplasmic expression pattern with an accumulation around the nucleus in cells expressing the PKP2 missense mutation, whereas the WT protein showed the expected localization at the cell membrane (Figure 3D). However, the expression of the mutant protein did not disrupt the localization of other endogenous junctional components (eg, JUP; Figure 3E).

The C796R mutation is predicted to have a significant effect on protein damage (see online-only Data Supplement Table II), whereas a cysteine to methionine exchange at the same position did not show any expression differences compared with WT, suggesting that the specific residue arginine is responsible for protein instability (Figure 3A through D).

Previous studies have shown that PKP2 is associated with the intermediate linker protein DSP, providing the link to the desmosomal cadherins. To further determine the interaction of the mutant HA-PKP2 C796R with FLAG-DSP, we coexpressed both proteins in HELAM2 cells lacking endogenous PKP2 and DSP. In contrast to the proper membrane localization of PKP2 and DSP after cotransfection of both WT proteins, mutant PKP2 C796R protein was not able to interact with FLAG-DSP to enable assembly at the junctional plaque.
Pathogenic PKP2 Missense Mutations Lead to Unstable Proteins, Whereas Undetermined Variants Remain Stable

To determine whether other missense and frameshift mutations in armadillo domains of PKP2 have the same effect as shown for the p.C796R mutation, we selected 2 presumably pathogenic missense mutations c.1844C>T (p.S615F) and c.1960A>C (p.K654Q), the frameshift mutation c.2076_2077delAA (p.C693fsX741), and 2 variants of unknown significance (VUS), causing protein changes p.V587I and p.I531S to perform expression studies in HELAM2 cells. First, we calculated the probability of those variants to have effects on protein structure and function by using common prediction algorithms, such as PolyPhen-2 and SIFT. In silico analysis revealed that PKP2 missense variants p.C796R, p.S615F, p.C693fsX741, and p.K654Q were classified consistently as pathogenic, whereas the variants p.V587I and p.I531S showed less evidence to be disease associated (see online-only Data Supplement Table II).

Expression studies in HELAM2 cells of variants predicted to be pathogenic demonstrated significantly decreased amounts of expressed mutant HA-PKP2 proteins (C796R: 14.5±3.0%, C693fsX741: 14.8±2.3%, S615F: 18.4±1.1%, K654Q: 13.6±2.7%), in comparison with the HA-PKP2 WT (100%) and a clear cytoplasmic localization. In contrast, both VUS were stably expressed (I531S: 74.5±13.9%, V587I: 103.4±3.2%) and localized at the cell membrane. N-Cadherin and JUP are not altered. n=3. Scale bar, 7.5 μm.
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Bacterial Expression Analysis, Crystallization, and Modeling of PKP2a ARM Repeats Confirm Destabilization and Degradation Due to Pathogenic Mutations

Plakophilin-2 contains an N-terminal head and a C-terminal ARM domain with 9 ARM repeats (Figure 5A). To elucidate the molecular basis of PKP2 disease-related mutations and presumed polymorphisms occurring in the ARM domain, we therefore subjected both WT PKP2a (dominant isoform in the heart) and variants of the ARM domain to crystallization (attempts obtaining sufficient amounts of soluble full-length protein failed). While the WT PKP2a ARM domain, as well as the ARM domain variants I487S and V543I, could be stably expressed in bacteria and purified, the PKP2a ARM domain mutations C752R, S571F, and C649fsX697 resulted in unstable proteins that, despite addition of protease inhibitors, degraded during purification until a stable fragment was formed (Figure 5B; see online-only Data Supplement Figure III). The purified stable ARM domain fragments were analyzed via mass spectrometry and shown to include amino acid residues of the first 5 ARM repeats (see online-only Data Supplement Figure IV), leaving about half of the protein.

Protein of the entire ARM domain of PKP2a resisted crystallization. The purified stable ARM domain fragment of the PKP2a C752 variant was crystallized successfully. The structure was solved by molecular replacement using the atomic coordinates of the homologous human PKP122 ARM-domain structure (see online-only Data Supplement Table III for structure determination and refinement statistics). The overall structure of the stable ARM domain fragment of the PKP2a C752R variant is represented in Figure 6A. The final model includes amino-acid residues 346 to 576, plus 2 additional amino acids (Gly-Ser) at the N-terminus, resulting from the His-tag sequence. The structure is similar to other known ARM-domain proteins (see online-only Data Supplement Figure V A–C), and comprises 5 ARM repeats that each display the typical ARM fold, with most repeats consisting of 3 helices (H1, H2, and H3) that form a compact helical bundle.8,9,28,29

To understand the destabilization and degradation behavior of the disease-related PKP2 variants with mutation sites outside the crystallized fragment, we modeled the structure of the entire ARM domain of human PKP2a. The prediction was performed using our crystal structure of the stable ARM-domain fragment of the PKP2a C752 variant and the structure of the human PKP1 ARM domain as template models. The predicted model of the entire ARM domain of PKP2a bears 9 ARM repeats (R1–R9), with each repeat consisting of 3 α-helices (H1, H2, and H3), except for repeats R1 and R6 that lack the corresponding H1 helix (Figure 6B; see online-only Data Supplement Figure VI).

The disease-causing C752 residue is located in the H3 helix of repeat ARM8 (Figure 5A; see online-only Data
Supplement Figure VI). Its side chain forms a hydrogen bond to the main chain carbonyl oxygen atom of T748 contributing, next to the hydrogen bond linking the backbone amide of C752 to the backbone carbonyl oxygen atom of “T748” to the intrahelical stabilization of H3 (see online-only Data Supplement Figure VD). In the disease-related C752R variant, the long and bulky arginine side chain would clash with the side chain of I781 and the main chain carbonyl oxygen atom of A792 within helices H2 and H3 of ARM9, respectively (Figure 6C). This and the missing additional intrahelical stabilization mentioned above lead to destabilization and degradation of the C752R variant protein until the stable fragment is formed (Figure 5B; see online-only Data Supplement Figure IV).

The S571 residue, on the other hand, is located in helix H3 of arm5 (Figure 5A; see online-only Data Supplement Figure VI). Its side chain is hydrogen-bonded to the side chain of Y642 in arm6-H2 and to the main chain carbonyl oxygen atom of L567 in ARM5-H3, contributing to inter- and intrahelical stabilization, respectively (see online-only Data Supplement Figure VE). In the disease-related S571F variant, the long and bulky phenylalanine side chain interferes with these interactions (Figure 6D), resulting in a destabilization and degradation of the S571F variant protein, until the stable fragment consisting of ARM repeats 1 to 5 is formed (Figure 5B; see online-only Data Supplement Figure IV).

For the V543I and I487S variants, no such disturbing interactions are observed on mutation, explaining the WT-like behavior (Figure 4; see online-only Data Supplement Figure III). The frameshift mutation C649fsX697 starts right after helix H2 of ARM6 (see online-only Data Supplement Figure VI). Thus, no inter-ARM–repeat stabilizations are possible beyond that, explaining the observed stable protein fragment for the purified C649fsX697 variant protein (see online-only Data Supplement Figures III and IV).

**Decreased Levels of Mutant Plakophilin-2 Proteins Are Due to Proteolytic Degradation Involving Calpain Proteases**

Degradation of most proteins in eukaryotic cells involves proteolytic pathways, such as UPS, activated calpain proteases, lysosomal degradation through autophagy, or the Golgi-endosome pathways, by removing proteins that are either abnormal or no longer needed.11 To prevent further degradation of mutated PKP2 proteins, we used several inhibitors of the calpain and UPS, as well as those of lysosome pathways. Pretreatment of transfected HELAM2 cells with the potent calpain inhibitor ALLN (100 nmol/L for 8 hours) abolished degradation of the mutant HA-PKP2 C796R protein significantly, demonstrated by an increased density (DMSO: 17.6±5.8% versus ALLN: 61.3±9.6% of PKP2 WT protein; P<0.05) in immunoblot analysis (Figure 7A and B). In addition, the population of cells expressing the mutant C796R protein increased from 25.1±1.8% to 78.5±8.6% (P<0.05) by ALLN treatment, which corresponds to the numbers of cells expressing the WT protein (Figure 7C and D). ALLN also affects the lysosome; however, specific lysosome inhibitors, such as Bafilomycin and 3-MA (5 mmol/L for 8 hours), did not significantly prevent degradation of the mutant protein, whereas the Golgi-endosome inhibitor Brefeldin A (10 μmol/L for 8 hours) did not significantly prevent degradation...
Finally, we confirmed the effect of calpain inhibitors observed in HELAM2 cells in cardiac derived HL-1 cells. ALLN and ZLL treatment increased the level of mutant C796R protein significantly (DMSO: 100±0.7%; ALLN: 361.4±17.3 P<0.05; and ZLL: 218.4±1.8%; P<0.05), but less efficiently compared with HELAM2 cells (Figure 7G and H). Interestingly, mutant protein aggregates colocalize with calpain (Figure 7I), suggesting that aggregates are targets for calpain proteases. Given that calpains are calcium-dependent cysteine proteases, we assumed that calcium addition (5 nmol/L Ca²⁺, 6 hours) would activate endogenous calpain proteases, leading to pronounced degradation. Indeed, we found remarkable further decreased levels of the mutant C796R protein after activation of endogenous calpain proteases via calcium addition (13.2±2.4%), compared with mutant protein levels detected in experiments without supplemented calcium (37.1±1.3%, P<0.05; Figure 7J and K).

Discussion

In the majority of mutations in PKP2 resulting in truncated proteins, about 10% are missense mutations and their effect on protein structure and impact on intercellular junction assembly remains to be analyzed. In this study we focused on the C796R missense mutation located in the armadillo domain of PKP2. We found unchanged PKP2 expression in an endomyocardial biopsy of a C796R carrier, whereas RV autopsy tissue of another mutation carrier showed a patchy expression pattern, demonstrating regional differences in immunoreactive PKP2. In vitro expression studies demonstrated that the replacement of Cys with an Arg at position 796 leads to protein instability and degradation involving calpain proteases. Moreover, this particular exchange dramatically affects protein folding, resulting in destabilization of ARM domains, leaving a stable fragment containing the first 5 of the 9 ARM repeats. Additional expression studies and structure modeling of ARM domain variants of PKP2 confirmed also that other pathogenic mutations affect protein structure and folding in the same way, resulting in protein instability and degradation.

Data about in vivo expression patterns of desmosomal proteins in cardiac tissue of PKP2 mutation carriers are very limited. To understand the consequences of the mutated PKP2 protein in vivo, we examined expression levels and localization of desmosomal proteins in cardiac tissue of 2 related subjects (II/1, III/1; Figure 1) carrying the p.C796R mutation. Surprisingly, we found almost unchanged signals of immunoreactive PKP2 and all other desmosomal proteins in fresh frozen RV biopsy tissue, whereas the autopsy tissue revealed regional expression differences, showing normal PKP2 expression in areas of histologically unaffected myocardium and reduced signals of immunoreactive PKP2, DSP, and JUP in areas adjacent to characteristic hallmarks of tissue degeneration and fibro-fatty replacement. Although reduced quality of autopsy tissue, as well as end-stage disease remodeling, might limit the interpretation of the normal expression, N-Cadherin indicates at least sufficient tissue quality (Figure 2). Our findings suggest a regional process of disturbed cell adhesion and tissue repair, which might be triggered by localized mechanical stress, inflammation, or other factors. Given this patchy pattern of expression.

Figure 6. Structural analysis of the armadillo (ARM)-repeat domain of human plakophilin-2 (PKP2)a. A. Crystal structure of the stable fragment of the ARM domain of the PKP2a C752R variant. The N- and C-terminus are marked with N or C, respectively. Each ARM repeat is colored differently. The overall fold of the predicted model of the entire ARM domain of human PKP2a. The H3 helices of each ARM repeat are highlighted. The local environments of the disease related mutations C752R in ARM8 (C) and S571F in ARM5 (D) are shown in stereo representations, demonstrating steric clashes that lead to protein destabilization and degradation. The coloring of the helices is as in panel B.

(Figure 7A and B). None of the inhibitors showed significant effects on WT protein stabilization.

Assuming the involvement of calpain proteases in protein degradation, we next determined the half-life of expressed mutant C796R and WT PKP2 proteins by pulse-chase analysis with and without ALLN pretreatment. The C796R mutant protein was degraded significantly faster (half-life t½ of 0.62±0.23 hours) compared with WT (t½=3.75±0.45 hours). Pretreatment with ALLN (100 nmol/L) led to a prolonged half-life of the mutant protein to 2.35±0.55 hours (Figure 7E and F).
Figure 7. Inhibition of the degradation process by calpain proteases in plakophilin-2 (PKP2) C796R. A, Immunoblot analysis of HELAM2 cells expressing wild-type (WT) and C796R PKP2 proteins in absence (DMSO) or after 8 hours treatment with calpain inhibitors ALLN, ZLL, or the endosome inhibitor BrefeldinA (BFA). Alpha-tubulin serves as loading control. Note that the calpain inhibitors ALLN and ZLL are able to significantly stabilize the mutant PKP2 protein, also shown in (B) by quantitative densitometric analysis of HA-PKP2/α-tubulin ratios, presented as dot plots. *P<0.05. n=3. C, Representative confocal immunofluorescence (IF) images of HELAM2 cells expressing the WT or C796R, respectively, in absence (DMSO) or after 8 hours treatment with the calpain inhibitor ALLN. Note the marked increase of mutant HA-PKP2 positive cells after ALLN treatment. D, Quantitative HA-PKP2/cell number ratios are presented as dot plots. *P<0.05, n=3. Scale bar, 50 μm. E, Representative autoradiograph of HELAM2 cells expressing Myc-PKP2 WT and Myc-PKP2 C796R proteins pulse-labeled with [35S]methionine/cysteine and chased for indicated times. Anti-myc immunoprecipitation was subjected to autoradiography. F, Plots of the decay curve illustrating the time-course of the degradation process relative to WT. The half-life of C796R PKP2 protein prolonged after ALLN treatment. The blots were normalized to 100% at time 0. n=3. G, Immunoblot analysis of HL-1 cells expressing the C796R PKP2 protein in the absence (DMSO) or after 8 hours treatment with MG132, ALLN, and ZLL. Alpha-tubulin served as loading control. Note that the ALLN and ZLL are able to increase significantly the mutant PKP2 protein compared with DMSO control. H Quantitative densitometric scan of HA-PKP2/α-tubulin ratios are presented as dot plots. *P<0.05. n=3. I, Representative IF images of HL-1 cells expressing HA-PKP2 C796R colocalized with calpain proteases in merged image (arrowheads). n=3. Scale bar, 5 μm. J, Immunoblot analysis of HL-1 cells expressing HA-PKP2 WT or C796R proteins with (+) and without (-) calcium treatment for activation of endogenous calpain proteases. Note the significant reduction of mutant PKP2 protein after calcium compared with the WT protein. Alpha-tubulin served as loading control. K, Quantitative densitometric scan of HA-PKP2/α-tubulin ratios are presented as dot plots. *P<0.05, n=3.
in our cases, the dilemma of targeting an affected area by taking endomyocardial biopsies appears obvious, especially in diagnostic settings.

Previous reports have shown that in particular expression levels of JUP seem to be reduced in cardiac tissue from ARVC patients carrying mutations in desmosomal proteins. However, our findings of unchanged expression levels are consistent in qualitative and quantitative immunofluorescence analyses of the endomyocardial biopsy, as well as in certain areas of the RV autopsy tissue (Figures 1C and 2), but our investigations are limited to a specific missense mutation in PKP2 and tissue of 2 carriers, which will not allow us to generalize those findings. Interestingly, and in contrast to Asimaki et al, our cardiac tissue samples came from 2 mutation carriers who showed only minor signs of disease, which might suggest that changes in the expression of desmosomal proteins develop with disease progression. Further investigations of cardiac tissue samples of PKP2 mutation carriers are needed to establish this more firmly.

The unchanged expression level of PKP2 in the cardiac biopsy sample also raised the question about the suggested null-allele hypothesis, implicated by our in vitro data set. Assuming that the mutant PKP2 protein remains unstable and is processed by a degradation pathway, the involvement of compensating feed-back mechanisms, possibly at the transcriptional level, results in up-regulation of the wild-type allele, and could explain the approximately similar levels of protein observed in vivo.

In contrast to mutations in other desmosomal components that have been shown to produce stable proteins, in vitro studies in cardiac derived HL-1 cells indicate that mutations in ARM domains of PKP2 result in unstable proteins that undergo degradation (Figure 3; see online-only Data Supplement Figure III). Given that previous observations of N-terminally located PKP2 mutations (Q59L, Q62K, R79X) expressed in different cells have shown a similar mode of action, we proposed that a simple cell culture model may help to decide whether a PKP2 variant is likely benign or pathogenic based on their in vitro expression pattern in HELAM2 cells. Interestingly, we found that variants predicted to be pathogenic were unstable and diffusely expressed in cytoplasm, whereas the 2 VUS (V587I, I531S) remained stable and localized at the cell membrane. Thus, the cellular expression pattern of undetermined variants in PKP2 may help in the decision process of variant classification in the future.

Moreover, our observations in cellular expression systems could be further confirmed by bacterial expression experiments of WT and mutant PKP2a armadillo domains, followed by mass spectrometric analysis of resulting stable protein fragments. While the ARM domain variants I487S and V543I could be expressed stably in bacteria, all 3 pathogenic mutations C752R, S571F, and C649fsX697 resulted in instable proteins that degraded during purification, leaving the same stable fragment of the first 5 ARM repeats (Figure 5; see online-only Data Supplement Figure III). Although the positions of the mutations impact different ARM domains and helices, the process of degradation resulted in the same protein fragment, which could be crystallized in case of the C752R mutation and used to model the entire ARM domain of PKP2a (Figure 6; see online-only Data Supplement Figure V). Detailed structural analyses could explain the destabilization...
process of each ARM domain variant at molecular level. Further structural modeling of other protein variants may aid in predicting the consequences of the mutation on protein folding and stability. Eukaryotic cells are equipped with protein degradation systems by removing proteins that are either abnormal or no longer needed. To understand how unstable mutant proteins are further degraded, we blocked the main proteolytic pathways in cell culture. Neither specific inhibitors for UPS nor for the lysosomal degradation through autophagy resulted in stabilization of the mutant protein. However, treatment with specific calpain inhibitors ALLN and ZLL partially rescued the expression of mutant C796R protein in both HELAM2 and HL-1 cells, respectively (Figure 7). Moreover, increased calcium levels leading to activation of endogenous calpains resulted in faster degradation of mutant proteins, supporting the possibility that calpains might be involved in the degradation process. Within the cell, the calpain system covers a broad range of physiological functions, including proteolysis of proteins involved in cell cycle, apoptosis, cytoskeleton organization, and signal transduction.12,13,31 To our knowledge there is no direct link between calpains and desmosomal proteins, indicating their specific involvement. But, considering that calpains are well known to play a role in several signaling pathways by modulating gene expression and regulating proteolysis in muscle tissue, it is reasonable to speculate that the life-long process of degradation mutant proteins itself may participate in the pathology of ARVC. Moreover, given an imbalance of calpain activation and regulation in muscle tissue, there might also be further implications on cardiac function considering that calpains are the key initiators of myofibrillar degradation. Further work will be required to sort out the importance of the calpain system in the degradation process of mutant PKP2 proteins.

In summary, our study shows that PKP2 missense mutations lead to protein instability and degradation in our in vitro model systems, suggesting haploinsufficiency as the most likely cause for the genesis of dominant ARVC due to mutations in PKP2.

Acknowledgments

We thank Janett Tischer, Tracy Dornblut, Ingrid Berger, Silke Kurths, and Iska Liebner for excellent technical assistance. We are also thankful to Yvette Roske, who assisted in diffraction data collection and processing, and acknowledge Uwe Müller and the beamline support by the staff of the Helmholtz-Zentrum Berlin.

Sources of Funding

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Disclosures

None.

References

CLINICAL PERSPECTIVE

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a primary cardiomyopathy with a genetic etiology and autosomal dominant inheritance, with incomplete penetrance that occurs most often. The disease is characterized clinically by a high incidence of ventricular arrhythmias and sudden cardiac death, and pathologically by degeneration of myocytes with replacement of fibro-fatty tissue. About half of the clinically diagnosed patients carry mutations in genes encoding components of the cardiac desmosome, including plakophilin-2 (PKP2), which is the most common disease gene for ARVC. Interestingly, the majority of PKP2 mutations are associated with translational frameshifts, leading to truncated proteins. However, there are also deleterious missense mutations that have been described to cause the disease. The present study focuses on genetic and molecular mechanisms of PKP2 missense and frameshift mutations, which we found lead in vitro to protein instability and degradation, suggesting haploinsufficiency as the main pathogenetic mechanism. Furthermore, we provide evidence that calpain proteases might be involved in the degradation of mutant proteins. We also established a cellular expression system to distinguish between variants of unknown significance (VUS) and pathogenic mutations; hereby selected VUS introduced into the PKP2 protein and expressed in vitro remain stable, whereas pathogenic mutations undergo protein degradation. This observation could be confirmed by bacterial expression experiments and structural modeling. Assuming a common mechanism of haploinsufficiency for all PKP2 mutations, cellular expression of mutant recombinant PKP2 proteins can provide further evidence for the pathogenicity of PKP2 mutations and might help in the decision process of variant classification in the future.
Molecular Insights into Arrhythmogenic Right Ventricular Cardiomyopathy Caused by Plakophilin-2 Missense Mutations
Florian Kirchner, Anja Schuetz, Leif-Hendrik Boldt, Kristina Martens, Gunnar Dittmar, Wilhelm Haverkamp, Ludwig Thierfelder, Udo Heinemann and Brenda Gerull

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Plasmids and site-directed mutagenesis
Full-length DSP-FLAG plasmid was a gift from K.J. Green. Plakophilin-2 constructs carrying several different mutations were generated by PCR mutagenesis using Pfu polymerase (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. The mutations were confirmed by direct DNA sequencing.

Primary antibodies
The primary antibodies used in this study were mouse monoclonal antibodies against plakophilin-2, desmoplakin, desmoglein2 (all Progen, Heidelberg, Germany), plakoglobin, N-cadherin (both BD Bioscience Clontech; Franklin Lakes, NJ), desmocollin2 (Invitrogen), α-tubulin (SIGMA) and cardiac troponin T (DSHB; Iowa City, IA); rabbit polyclonal antibodies against N-cadherin, plakoglobin, FLAG (all Santa Cruz; Santa Cruz, CA), calpain1+2 (abcam; Cambridge, UK), lacZ (Rockland; Gilbertsville, PA) rat monoclonal antibody against HA (ROCHE; Basel, Switzerland).

Immunoblot analysis
Cells were lysed in RIPA buffer (SIGMA), including protease inhibitor cocktail (ROCHE). Proteins were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes, blocked with 5% non-fat dry milk powder in TBST. Membranes were probed with primary antibodies and were revealed using an ECL system (Amersham Bioscience). The band intensities were quantified using NIH image software.

Bacterial expression and protein purification
DNA encoding the ARM domain of wild-type and mutant human PKP2 isoform a (amino acids 346-817) was subcloned into the pQLinkH vector. The genes encoding the N-terminal His-tagged proteins were overexpressed in E. coli Rosetta (DE3) (Merck). Cells were grown at 37°C in Terrific Broth to an OD600 of about 2, induced with 0.5 mM isopropyl-1-thio-D-galactopyranoside, and incubated overnight at 20°C. For purification of the ARM domain of wild-type PKP2 and the C752R variant, cell paste was resuspended in lysis buffer (50 mM sodium/potassium phosphate buffer,
pH 7.5, 0.5 M NaCl, 5% glycerol, 1 mM DTT, 0.1% (w/v) CHAPS, 0.1 mM phenylmethylsulfonyl fluoride, Complete EDTA-free Protease Inhibitor mixture tablet (Roche), and benzonase (Merck). Cells were lysed by sonication. The clarified lysate was applied on a 5 ml HisTrap FF crude column (GE Healthcare), charged with Ni²⁺. The column was washed with 50 mL of 20 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl and 50 mM imidazole. The protein was eluted with the same buffer containing 250 mM imidazole. The protein was further purified to homogeneity using size-exclusion chromatography on a Superdex 200 prep grade column (26 × 60, GE Healthcare) equilibrated with 20 mM HEPES–NaOH pH 7.5, and 0.2 M NaCl. Other variants were purified on small scale. Cells from 50 mL cell culture were lysed by lysozyme, and clarified lysates were applied onto a 1 mL His GraviTrap column (GE Healthcare) equilibrated with 20 mM Tris pH 8.0, 0.2 M NaCl, and 5 mM imidazole. Before elution with the same buffer containing 250 mM imidazole, columns were washed with 10 column volumes of a buffer containing 20 mM imidazole.

Pulse chase assay
HELAM2 cells were pulse-labeled for 2h in methionine-free DMEM (Invitrogen) Supplementaled with [35S]methionine/cysteine (3.7 Ci/mL, Perkin-Elmer, Waltham, MA) and then chased in DMEM with 1 mM methionine. Where indicated, calpain inhibitors were included both in pulse and chase media. Anti-myc immunoprecipitation was done in Tris/HCl pH 9 containing 1% (v/v) Triton X-100, 0.5% SDS, 0.25% sodium deoxycholate, 1 mM EDTA, and protease inhibitors for 2h at 4°C. Immunocomplexes were collected with anti-myc agarose (SIGMA), and bound proteins were analyzed by SDS-PAGE followed by autoradiography and quantification (NIH software).

X-ray data collection, structure determination and refinement
X-ray diffraction data were collected at beamline BL14.1 of the Helmholtz-Zentrum Berlin für Materialien und Energie and Freie Universität Berlin at BESSY. Data reduction was performed using XDS and XSCALE. Data collection statistics are reported in Supplement Table 2. The structure was solved by molecular replacement using the program PHASER and the ARM domain of human PKP1 (PDB ID 1xm9) as search model. The structure was refined using REFMAC. The graphics program
COOT was used for model building and visualization\textsuperscript{9}. Figures were created with PyMOL Molecular Graphics System (Version 1.3, Schrödinger, LLC).

**Mass spectrometric analysis**
Purified proteins from the excised gel bands were converted to peptides by in-gel digestion with trypsin (Promega). The resulting peptides were desalted on an stage-tip micro column\textsuperscript{10} and separated on an in-house prepared (15 cm length, 75 μm inner diameter, Reprosil, Dr. Maisch) reverse phase column using an 1h gradient (EksigentnanoUltra). The separated peptides were directly sprayed into the mass spectrometer using a Proxeon ion-source, attached to a LTQ-Orbitrap (Thermo Scientific). The recorded spectra were analyzed using the Max-Quant software package\textsuperscript{11} or the Mascot software package (Matrix Science, Cambridge).
<table>
<thead>
<tr>
<th>ARVC Task Force Criteria 2010</th>
<th>Individual IV-1</th>
<th>Individual III-1</th>
<th>Individual II-1</th>
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<tr>
<td>Structural Alterations Imaging (MRI/Echo)</td>
<td>cardiac MRI: localized RV aneurysms with diastolic bulging, dilation of the RV with decreased RV systolic function, no LV abnormalities</td>
<td>cardiac MRI: mild segmental RV dilatation and hypokinesis, no diastolic bulging (no TFC)</td>
<td>Echo: Normal LV and RV function, concentric LV hypertrophy, enlarged LA, mild MVR</td>
</tr>
<tr>
<td>Tissue characterization on RV-biopsy and Autopsy</td>
<td>N/A</td>
<td>RV biopsies (septum): no pathological abnormalities found, no evidence of fibrofatty replacement or interstitial/perivascular inflammatory cell infiltrates</td>
<td>Clinical Autopsy Report: LV: moderate muscular hypertrophy, minimal fibrosis, small scars; RV: normal RV size, diffuse perivascular and interstitial fibrosis with transition to scar tissue, regional infiltrates of eosinophilic leukocytes and plasma cells; RA: massive fatty tissue leading to a separation of small islands of remaining cardiomyocytes, in areas of those islands massive fibrosis and scar tissue</td>
</tr>
<tr>
<td>Repolarization Abnormalities</td>
<td>Inverted T-waves in V1-V5</td>
<td>Inverted T-waves in V1-V3</td>
<td>No inverted T-waves</td>
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<tr>
<td>Depolarization Abnormalities</td>
<td>SAECG (N/A), no epsilon wave, QRS: 115ms</td>
<td>SAECG (N/A), no epsilon wave, QRS: 100ms</td>
<td>SAECG (N/A), no epsilon wave, QRS: 98 ms</td>
</tr>
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<td>Arrhythmias</td>
<td>non-sustained and sustained monomorphic ventricular tachycardia of LBBB morphology with superior axis</td>
<td>paroxysmal common type atrial flutter, 24hour Holter: more than 2000 monomorphic ventricular extrasystoles of LBBB morphology</td>
<td>atrial fibrillation, 24hour Holter: very few extrasystoles (morphology not reported)</td>
</tr>
<tr>
<td>Family History/Genetics</td>
<td>mutation carrier</td>
<td>ARVC confirmed in a first degree relative, mutation carrier</td>
<td>ARVC confirmed in a first degree relative, mutation carrier</td>
</tr>
<tr>
<td>Age of Onset</td>
<td>20 years</td>
<td>47 years</td>
<td>N/A</td>
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<td>Symptoms</td>
<td>palpitations, several exercise induced syncope</td>
<td>two exercised induced syncope</td>
<td>None</td>
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<tr>
<td>Score: Major/Minor</td>
<td>4/0</td>
<td>2/1</td>
<td>1/0</td>
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Supplemental Table 1: Clinical Characteristics of ARVC family members.
**Supplemental Table 2**

<table>
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<tr>
<th>Nucleotide Change</th>
<th>Coding Effect</th>
<th>Reported ARVC Cases</th>
<th>Highest reported Prevalence in Controls</th>
<th>ARVC Genetic Database* Classification</th>
<th>Polyphen-2 prediction</th>
<th>SIFT prediction</th>
<th>Cellular expression pattern</th>
</tr>
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<tbody>
<tr>
<td>c.2386C&gt;T</td>
<td>C796R</td>
<td>several (Dutch founder)</td>
<td>0</td>
<td>pathogenic</td>
<td>probably damaging</td>
<td>highly conserved</td>
<td>affects protein function</td>
</tr>
<tr>
<td></td>
<td>C796M</td>
<td>none</td>
<td></td>
<td>pathogenic</td>
<td>probably damaging</td>
<td>highly conserved</td>
<td>cytoplasmatic distribution</td>
</tr>
<tr>
<td>c.1844C&gt;T</td>
<td>S615F</td>
<td>3</td>
<td>0</td>
<td>pathogenic</td>
<td>probably damaging</td>
<td>highly conserved</td>
<td>affects protein function</td>
</tr>
<tr>
<td>c.1960A&gt;C</td>
<td>K654Q</td>
<td>1</td>
<td>0</td>
<td>pathogenic</td>
<td>possibly damaging</td>
<td>highly conserved</td>
<td>affects protein function</td>
</tr>
<tr>
<td>c.2076_2077del AA</td>
<td>C693fsX741</td>
<td>1</td>
<td>0</td>
<td>pathogenic</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>c.1759G&gt;A</td>
<td>V587I</td>
<td>6</td>
<td>3/600</td>
<td>unknown</td>
<td>possibly damaging</td>
<td>Ile in zebrafish, conserved among mammals not conserved</td>
<td>tolerated</td>
</tr>
<tr>
<td>c.1592T&gt;G</td>
<td>I531S</td>
<td>several</td>
<td>15/500</td>
<td>no known pathogenicity</td>
<td>possibly damaging</td>
<td>affects protein function</td>
<td>cell membrane</td>
</tr>
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* [http://www.arvcdatabase.info/](http://www.arvcdatabase.info/)
**Supplemental Table 3.** Data collection and refinement statistics. Numbers in parentheses are for the highest resolution shell.

<table>
<thead>
<tr>
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<td><strong>Data collection</strong></td>
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<td>Wavelength [Å]</td>
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<td>Resolution [Å]</td>
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<td>Space group</td>
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<tr>
<td>Unique reflections</td>
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<td>R\text{meas} [%]</td>
<td>7.1 (58.4)</td>
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<tr>
<td>Completeness [%]</td>
<td>99.4 (98.3)</td>
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<tr>
<td>\langle I \rangle/\sigma(I)</td>
<td>17.9 (2.9)</td>
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<tr>
<td>Wilson B factor [Å²]</td>
<td>19.9</td>
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<tr>
<td><strong>Refinement</strong></td>
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<tr>
<td>Resolution range [Å]</td>
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<tr>
<td>R\text{cryst}</td>
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<tr>
<td>R\text{free} (test set of 5%)</td>
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<td>No. of non-H atoms protein</td>
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<td>glycerol</td>
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<tr>
<td>water</td>
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<tr>
<td>Average isotropic B factor [Å²]</td>
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<tr>
<td>main chain</td>
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<td>side chain</td>
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<td>Rmsd for bond lengths [Å]</td>
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<td>Rmsd for bond angles [°]</td>
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<td>Most favorable [%]</td>
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<tr>
<td>Outliers [%]</td>
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Supplemental figure 1

A

RV control sample

DSP

NCAD

Merged

RV biopsy C796R (III/1)

area 2

area 1

RV autopsy C796R (II/1)

B

DSP

NCAD

Merged

RV control sample

area 1

area 2

RV autopsy C796R (II/1)
Supplemental figure 3

A

head domain

arm-repeat domain

PKP2a

PKP2a_arm

B

delAA  V543I  I487S

u e i s  u e i s  u e i s

kDa

15  20  25  30  35  40  45  50  55  60  65  70  75

U = uninduced  e = expression after IPTG induction
i = insoluble (protein sequestered into inclusion bodies)  s = soluble and affinity beads purified protein
Supplemental figure 4

(A) PKP2a_wt
MKHHHHHHHG AAGTSLYKKA GENLYFQGSN ADMEMTLERA VSMLEAHML
PSRISAAATF IQHECFQKSE ARKRVNQLRG ILKLLQQLKV QNEDVQRAVC
GALRNLFED NDNKLEVAEL NGVPRLQQLV QKTRDLETKK QITGLLWNLS
SNDKLKNLMI TEALLTLTEN IIIPFSGWPE GDYPKANGLL DFDIFYNVTG
CLRNMSSAGA DGRKMARRCD GLIDSLVHYV RGTDIAYQPD DKATENCVCI
LHNLSSQLEA ELPEKYSQNI YIQNRNIQTD NNKSIGCFGS RSRKVKEQYQ
DVPMPKEKSNU PKGVEWLWHS IVIRMYLSTI AKSVRNYTQE ASLGLALQNLT
AGSGPMPTSV AQTVQKESG LQHTRKLMLHV GDPSVKKTATI SLLRNLSRN
SLQNEIAKET LPDLVSIIID LPSTDLDLLIE TTASACYTIN NIIQNSYQNA
RDLLNTGGIQ KIMAIAGDA YASNKASKAA SVLLYSLWAH TELHAYKKA
Q

(B) PKP2a_C752R
MKHHHHHHHG AAGTSLYKKA GENLYFQGSN ADMEMTLERA VSMLEAHML
PSRISAAATF IQHECFQKSE ARKRVNQLRG ILKLLQQLKV QNEDVQRAVC
GALRNLFED NDNKLEVAEL NGVPRLQQLV QKTRDLETKK QITGLLWNLS
SNDKLKNLMI TEALLTLTEN IIIPFSGWPE GDYPKANGLL DFDIFYNVTG
CLRNMSSAGA DGRKMARRCD GLIDSLVHYV RGTDIAYQPD DKATENCVCI
LHNLSSQLEA ELPEKYSQNI YIQNRNIQTD NNKSIGCFGS RSRKVKEQYQ
DVPMPKEKSNU PKGVEWLWHS IVIRMYLSTI AKSVRNYTQE ASLGLALQNLT
AGSGPMPTSV AQTVQKESG LQHTRKLMLHV GDPSVKKTATI SLLRNLSRN
SLQNEIAKET LPDLVSIIIPD LPSTDLDLLIE TTASACYTIN NIIQNSYQNA
RDLLNTGGIQ KIMAIAGDA YASNKASKAA SVLLYSLWAH TELHAYKKA
Q

(C) PKP2a_S571F
MKHHHHHHHG AAGTSLYKKA GENLYFQGSN ADMEMTLERA VSMLEAHML
PSRISAAATF IQHECFQKSE ARKRVNQLRG ILKLLQQLKV QNEDVQRAVC
GALRNLFED NDNKLEVAEL NGVPRLQQLV QKTRDLETKK QITGLLWNLS
SNDKLKNLMI TEALLTLTEN IIIPFSGWPE GDYPKANGLL DFDIFYNVTG
CLRNMSSAGA DGRKMARRCD GLIDSLVHYV RGTDIAYQPD DKATENCVCI
LHNLFYQLEA ELPEKYSQNI YIQNRNIQTD NNKSIGCFGS RSRKVKEQYQ
DVPMPKEKSNU PKGVEWLWHS IVIRMYLSTI AKSVRNYTQE ASLGLALQNLT
AGSGPMPTSV AQTVQKESG LQHTRKLMLHV GDPSVKKTATI SLLRNLSRN
SLQNEIAKET LPDLVSIIIPD LPSTDLDLLIE TTASACYTIN NIIQNSYQNA
RDLLNTGGIQ KIMAIAGDA YASNKASKAA SVLLYSLWAH TELHAYKKA
Q
Supplemental figure 4

(D) PKP2a_delAA

MKHHHHHHHG AAGTSLYKKA GENLYFQGSN ADMEMTLERA VSMLEADHML
PSRISAAATF IQHECFQKSE ARKRVNQLRQ ILKLLQLLLKV QNEQDVQAVC
GALRNLVFED NDNKLEYAEL NGVPRLLQVQ KQTRDLETKK QITGGLLNLS
SDNKLNLMIM TEALLTLTEN IIPFSGWPE GDYPKANGLL DFDIFYNVTVG
CLRNMSSAGA DGRKAMRRCD GLIDSLVHYV RGTIADYQPD DKATENCVCI

(E) PKP2a_I487S

MKHHHHHHHG AAGTSLYKKA GENLYFQGSN ADMEMTLERA VSMLEADHML
PSRISAAATF IQHECFQKSE ARKRVNQLRQ ILKLLQLLLKV QNEQDVQAVC
GALRNLVFED NDNKLEYAEL NGVPRLLQVQ KQTRDLETKK QITGGLLNLS
SDNKLNLMIM TEALLTLTEN IIPFSGWPE GDYPKANGLL DFDIFYNVTVG
CLRNMSSAGA DGRKAMRRCD GLIDSLVHYV RGTIADYQPD DKATENCVCI

(F) PKP2a_V543I

MKHHHHHHHG AAGTSLYKKA GENLYFQGSN ADMEMTLERA VSMLEADHML
PSRISAAATF IQHECFQKSE ARKRVNQLRQ ILKLLQLLLKV QNEQDVQAVC
GALRNLVFED NDNKLEYAEL NGVPRLLQVQ KQTRDLETKK QITGGLLNLS
SDNKLNLMIM TEALLTLTEN IIPFSGWPE GDYPKANGLL DFDIFYNVTVG
CLRNMSSAGA DGRKAMRRCD GLIDSLIHYV RGTIADYQPD DKATENCVCI

Q
Supplemental figure 7

A

<table>
<thead>
<tr>
<th></th>
<th>PKP2 WT</th>
<th>PKP2 C796R</th>
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<tbody>
<tr>
<td>DMSO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG132</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bafilomycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-MA</td>
<td></td>
<td></td>
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</tbody>
</table>

HA-PKP2

α-tubulin

B

HA-PKP2/α-tubulin expression

0% 20% 40% 60% 80% 100% 120% 140%

DMSO MG132 Bafilomycin 3-MA DMSO MG132 Bafilomycin 3-MA

PKP2 WT PKP2 C796R
Supplemental Figure Legends

**Supplemental Figure 1:** Confocal immunofluorescence images of desmoplakin (DSP) expression in cardiac tissue heterozygous for the p.C796R mutation.

(A) Representative images of cryosections of an endomyocardial biopsy from subject III/1 show normal expression of DSP at the intercalated discs of cardiomyocytes compared to control tissue. The adherens junction protein N-cadherin is also equally detectable in both samples. Scale bar, 25µm.

(B) Paraffin sections of RV cardiac autopsy tissue demonstrate absent signals of DSP in area 1 at the intercalated discs of subject II/1, whereas N-cadherin is normally expressed in subject II/1 and the control RV sample. In contrast, in other areas (e.g. area 2) of the same section the DSP expression levels show no differences to the control RV sample. Scale bar, 25µm.

**Supplemental Figure 2:** Co-expression of mutant PKP2 with desmoplakin (DSP) in HELAM2 cells.

(A) Representative confocal immunofluorescence of exogenously expressed GFP-FLAG counterstained with endogenous JUP in HELA cells lacking desmosomes. Note the intercellular location of DSP-GFP.

(B) Immunolocalisation of HELA cells co-transfected with DSP-FLAG and HA-PKP2 wild-type, and C796R, respectively. Note that the HA-PKP2 wild-type protein co-localized with the DSP-FLAG at the cell membrane, whereas the co-transfection with the mutant HA-PKP2 shows a completely absent DSP-FLAG from the cell-cell contact and co-localized with mutant PKP2 in the cytoplasm. n=3. Scale bar, 10µm.
Supplemental Figure 3: Bacterial expression of various PKP2a variants.

(A) The asterisks mark the position of the respective mutation (I487S, V543I, C693fsX741=delAA). Numbers indicate the amino acid position.

(B) Expression and purification of human PKP2a ARM-domain variants. Proteins were expressed as N-terminal His7-tag fusions and purified using affinity beads. The resulting proteins differ in their stability due to bearing certain mutations.

Supplemental Figure 4: Mass spectrometric analysis of purified arm domains of human PKP2a wild-type and variants thereof.

The entire arm domain was expressed as an N-terminal His7-tag fusion protein and purified using affinity beads. The resulting proteins differ in their stability due to bearing certain mutations. Mass spectrometric analyses were performed to identify what part of the protein remains as a stable fragment. Peptide fragments identified via mass spectrometry are highlighted in red.

Supplemental Figure 5: Structural analysis of human PKP2a.

(A-C) Superimpositions of selected ARM-repeat protein crystal structures. The crystal structure of the ARM-repeat domain of human PKP2a C752R variant (purple; this work) is superimposed on the structures of (A) human PKP2 full length model (orange; this work), (B) human PKP1 (light blue, 1xm7), and (C) human p120 catenin (green, 3l6y12). The orientation is identical in all panels. The related human ARM-domain proteins PKP1 and p120 catenin are the closest structural homologues of PKP2a as revealed by the SSM program13. The Cα atoms in the structures of PKP2a and PKP1 superimpose with a root-mean-square deviation (rmsd) of 1.11 Å (over 207 Cα atoms). The p120 catenin superimposes onto PKP2a with an rmsd of 1.28 Å (over 209 Cα atoms). While secondary structure elements superimpose well, structural changes are observed in the loop regions connecting H2 and H3 helices in arm 4, and in loop regions connecting the fifth and the sixth arm repeat, regions that are least conserved within the p120ctn family.
(Supplement Figure 6). (D) Stereo view of the C752 interactions within the ARM8 repeat. (E) Stereo view of the S571 interactions within the ARM5 repeat.

**Supplemental Figure 6:** Structure-based sequence alignment of human PKP1 (lower line) and human PKP2a (upper line) ARM-repeat domains (modified after\textsuperscript{7}).

The sequences of the ARM-repeat domains are aligned according to the H1–H3 helices of each repeat to highlight the sequence conservation (residues shaded gray). The repeat numbers and the corresponding amino acid ranges are shown on the left. The secondary structure elements shown above the alignment correspond to human PKP2a (this work). Italicized residues are disordered in the PKP1 structure. Consensus positions are assigned specific residue identities if the residue is present in at least two-thirds of the repeats or if two amino acids are present in at least four repeats each. Consensus positions with predominantly large or small hydrophobic residues are shown as filled or open boxes, respectively, and those with general hydrophobic residues as half-filled boxes, basic residues with “+”. For comparison, the consensus ARM-repeat motif derived from the β-catenin structure is indicated at the bottom. Conserved residues that mediate contacts between the insert and repeats 5–7 are underlined. Analyzed mutations are labeled with * (I487S, S571F, V543I, C752R) or || (C693fsX741 = delAA).

**Supplemental Figure 7:** Inhibition of several cellular degradation processes.

(A) Immunoblotting analysis of transfected HL-1 cells with HA-PKP2 WT and C796R protein in the absence (DMSO) or after 8h treatment with proteasome inhibitor MG132 (5µM), lysosome inhibitor bafilomycin (50 nM) and autophagy inhibitor 3-MA (5 mM). α-Tubulin served as loading control. Note that inhibitors have no significant effect of protein stability. (B) Quantitative densitometric scan of HA-PKP2/α-tubulin ratios are presented as mean ± SEM. n=3.
Supplemental References


