The Novel Desmin Mutant p.A120D Impairs Filament Formation, Prevents Intercalated Disk Localization and Causes Sudden Cardiac Death

**Running title:** Brodehl et al.; Characterization of a novel desmin mutation

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Abstract:

**Background** - The intermediate filament protein desmin is encoded by the gene *DES* and contributes to the mechanical stabilization of the striated muscle sarcomere and cell contacts within the cardiac intercalated disk. *DES* mutations cause severe skeletal and cardiac muscle diseases with heterogeneous phenotypes. Recently, *DES* mutations were also found in patients with arrhythmogenic right ventricular cardiomyopathy (ARVC). Currently, the cellular and molecular pathomechanisms of the *DES* mutations leading to this disease are not exactly known.

**Methods and Results** - We identified the two novel variants *DES*-p.A120D (c.359C>A) and -p.H326R (c.977A>G), which were characterized by cell culture experiments and atomic force microscopy. Family analysis indicated a broad spectrum of cardiomyopathies with a striking frequency of arrhythmias and sudden cardiac deaths. The *in vitro* experiments of desmin-p.A120D evidenced a severe intrinsic filament formation defect causing cytoplasmic aggregates in cell lines and of the isolated recombinant protein, respectively. Model variants of codon 120 indicated that ionic interactions contribute to this filament formation defect. *Ex vivo* analysis of ventricular tissue slices revealed a loss of desmin staining within the intercalated disk and severe cytoplasmic aggregate formation whereas z-band localization was not affected. The functional experiments of desmin-p.H326R did not demonstrate any differences from wild type.

**Conclusions** - Due to the functional *in vivo* and *in vitro* characterization *DES*-p.A120D has to be regarded as a pathogenic mutation, whereas *DES*-p.H326R is a rare variant with unknown significance, respectively. Presumably, the loss of the desmin-p.A120D filament localization at the intercalated disk explains its clinical arrhythmogenic potential.

**Key words:** cardiomyopathy, desmosome, death, sudden, desmosomes arrhythmia, desmin, intermediate filaments, intercalated disk
The intermediate filament (IF) protein desmin is encoded by the gene *DES* and contributes to the mechanical stabilization of the sarcomeres and cell contacts within the cardiac intercalated disk (ID). Desmin is the predominant IF-protein of striated muscles. It belongs to the type III IF-proteins characterized by a uniform assembly mechanism. In the first step of the *in vitro* assembly two coiled-coil dimers form an antiparallel tetramer. These tetramers are the essential building blocks of the IF. Eight tetramers anneal in lateral orientation into unit length filaments (ULFs). In the longitudinal elongation step these ULFs are assembled and radially compacted into IF. Since the first reports on *DES*-mutations it became obvious that *DES*-mutations cause skeletal myopathies and different forms of cardiomyopathies.

In the meantime more than 60 different *DES*-mutations distributed over the whole sequence are known, which lead in the majority of cases to filament formation defects with deposition of cytoplasmic desmin aggregates. However, the pathomechanisms of desmin aggregation leading to skeletal or cardiac myopathies are mechanistically not understood in detail. Moreover, aggregate formation of mutant desmins does not explain *per se* the arrhythmogenic phenotype of some cardiomyopathies.

Recently, different *DES*-mutations were also identified in patients with ARVC. ARVC is an inherited cardiomyopathy clinically characterized by arrhythmias and predominately right ventricular dilatation leading to cardiac syncope, heart failure, or even sudden cardiac death. It is well established that mutations in the genes coding for desmosomal plaque proteins cause ARVC and rare forms of dilated cardiomyopathy (DCM). In the cardiac muscle desmin is found in costamers, the z-disk and -connected via plaque proteins- to the cardiac desmosome within the ID. The molecular processes contributing to the destabilization of the ID through desmin filaments are fragmentarily understood. Especially, it is not known, how and which of
the desmin mutations impair the connection of the IF-system to the cardiac desmosome.

In this study, we report a novel pathogenic DES-mutation (c.359C>A, p.A120D), which appears to interfere particularly with the connection of desmin IF to the ID. Furthermore, we investigate if the DES-variants p.A120D and p.H326R (c.977A>G) affect the IF formation using ectopic expression cell culture systems and atomic force microscopy (AFM). These data reveal that desmin-p.A120D but not desmin-p.H326R inhibits the longitudinal assembly step confirming its pathogenic potential.

Material and Methods

Clinical description of the patients

In family A the 34 years old female index patient (III:24) presented with atrial flutter, variable atrioventricular conduction (Fig. S1) and dilated atria. The average ventricular frequency was 64 beats per minute (bpm) and the atrial frequency was 120 bpm. In the electrocardiogram some polymorphic premature ventricular contractions (PVCs) with a frequency of 45 – 111 bpm were detected (Fig. S1). The cardiological evaluation including 2D, M-mode, spectral and color Doppler was performed. These investigations reveal normal left ventricular systolic function (left ventricular ejection fraction of 67%), a borderline concentric left ventricular hypertrophy. Nevertheless, the left atrium was severely dilated and the right atrium was also dilated. She is a member of a large family with dilated cardiomyopathy (DCM) and several sudden cardiac deaths (SCD) (Fig. 1A). The patient had no signs of a myopathy but received an implantable cardioverter defibrillator (ICD). She had one sister (III:21) and two brothers (III:22, III:23) who died from SCD as teenagers (aged 13, 17 and 13 years, respectively). Her father (II:13) and grandfather (I:3) died due to cardiomyopathy aged 33 and 45 years. Patient III:24 lost three aunts (II:2 aged 34 years, II:4 aged 42 years and II:10 aged 50 years) by SCD. Another four members...
of family A died suddenly at age 13 (III:3, III:4, III:15) and at age 30 years (III:16). One paternal aunt (II:6) of the index patient (III:24) suffering from Ebstein anomaly (EA) was transplanted. All children of II:6 were without cardiac disease. The index patient (III:24) and her aunt (II:6) were heterozygous for the DES mutation p.A120D (c.359C>A). Her son (IV:3) was wild-type and presented no signs of a muscle disease. The mutation was not found in the children of patient II:6. One cousin of the index patient (III:7) was positive for the DES mutation p.A120D. The ECG of patient (III:7) showed normal sinus rhythm with prolonged PR interval, T wave abnormalities and AV block. Furthermore, patient III:7 was examined by echocardiography and magnetic resonance imaging (MRI). The patient revealed normal left ventricular size and normal left ventricular systolic function (left ventricular ejection fraction 64% by Biplane). There were no evidences for left ventricular hypertrophy. In summary, the spectral Doppler showed normal pattern of LV diastolic filling. Normal right ventricular size and systolic function were detected. Of note, the right atrium was severely dilated, similar to his cousin (patient III:24, family A). Nevertheless, because of the young age of this patient and the remarkable similar phenotype compared to the index patient (III:24) it is expected that the clinical symptoms will potentially increase during the next decades. For that reason the patient received a pacemaker/ICD.

In family B (Fig. 1B) the index patient (IV:1) had experienced palpitations from an age of 25 years. Clinical workup showed a borderline ARVC phenotype based on non-sustained ventricular tachycardia of left bundle branch block (LBBB) morphology, a positive signal averaged-ECG, and a suspicious family history. Echocardiography and MRI were normal. Review of her father’s (III:2) medical history revealed that he had been evaluated in the early eighties due to syncope. Workup had shown frequent premature ventricular contractions (PVC) of LBBB morphology. He died suddenly playing golf aged 38 years. The probands uncle (III:3)
had experienced syncope during running at the age of 38 years and was diagnosed with a non-ischemic cardiomyopathy (ejection fraction 25%) and non-sustained ventricular tachycardia. His ejection fraction improved to 50% on therapy (Angiotensin-converting-enzyme inhibitor, beta blocker, digoxin, spironolactone, ICD) but he developed chronic atrial fibrillation. All three individuals (IV:1, III:2, III:3) were tested positive or obligate carriers of the *DES*-p.H326R (c.977A>G) variant. The proband’s brother (IV:2) was phenotype and genotype negative (current age 37 years). No clinical or genetic data were available on individuals II:1 and II:2 as they had died more than 30 years ago. Individual III:5 was diagnosed with a DCM and PVCs before experiencing sudden cardiac arrest aged 36 years. Individual IV:4 was initially examined at the age of nine years due to PVCs and non-sustained ventricular tachycardia. Later, her left ventricle was dilated (left ventricular end diastolic diameter 69 mm) and ejection fraction was at the lowest 25% but improved to 55% with treatment (Angiotensin-converting-enzyme inhibitor, beta blocker, amiodarone, PVC ablation, ICD). Blood was available from individual IV:4 and she did not carry the *DES*-p.H326R variant. None of the patients had signs of peripheral muscular involvement.

**Genetic analysis and mutation detection**

Genomic DNA was isolated and purified from the affected individuals using the illustra blood genomicPrep Mini Spin Kit (GE Healthcare, Chalfont St. Giles, UK). The BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) and an ABI310 Genetic Analyser (Applied Biosystems, CA, USA) were used for sequencing according to the manufacturer’s instructions. The sequences were analyzed with the Variant Reporter Software v1.0 (Applied Biosystems, CA, USA). The allele frequencies of novel variants were determined in 394 healthy control individuals using the TaqMan SNP Genotyping Assay (Applied Biosystems, CA, USA).
The consent of all participants was obtained and the study was approved by the ethics committee.

**Cloning and site-directed mutagenesis**

The sequence variants were inserted into the plasmids pET100D-Desmin and pEYFP-N1-Desmin using appropriate primers and the QuickChange Lightning Kit (Agilent Technologies, Santa Clara, USA) according to the manufacturer’s instructions (see Table S1). The DES coding regions were verified by sequencing using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA). The reference cDNA of human desmin (NM_001927.3) was used for comparison.

**Cell culture**

H9c2-, HeLa-, C2C12- and SW-13-cells (LGC Standards, Middlesex, USA) were cultured in DMEM (Invitrogen, Carlsbad, USA) supplemented with 10% fetal calf serum (FCS), 4.5 g/L Glucose and Penicillin/Streptomycin. The HL-1 cells (kindly provided by W.C. Claycomb) were cultured in Claycomb medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% FCS, 2 mM L-Glutamine, 100 nM Norepinephrine and Penicillin/Streptomycin. Cardiomyocytes derived from human induced pluripotent stem cells (hiPS-CM) were generated and cultured as previously described. Lipofectamin 2000 (Invitrogen, Carlsbad, USA) was used to transfect the cells according to the manufacturer’s protocol.

**Immunohistochemistry and fluorescence microscopy**

Transfected cells were fixed with methanol (15 min, -20°C) and were then permeabilized with 0.1% Triton X 100 (20 min, RT). After blocking with 1% BSA/PBS the cells were incubated with 7.5 μg/mL anti-desmin antibodies (R&D Systems, Minneapolis, USA) or 25 μg/mL anti-vimentin antibodies (Sigma-Aldrich, Saint Louis, USA) over night at 4°C and were gently washed with 1% BSA/PBS. Then the cells were incubated with Cy3-conjugated anti-goat-IgG or
anti-mouse-IgG secondary antibodies (1:400, Jackson Immuno Research, West Grove, PA, USA) for 1 h at RT. The nuclei were stained with 1 μg/mL 4',6-diamidino-2-phenyindole (DAPI, 5 min, RT). The cells were washed with PBS and fluorescence images were recorded with an Eclipse TE2000-U microscope (Nikon, Tokyo, Japan) equipped with a Digital sight DS-2MV CCD-camera (Nikon, Tokyo, Japan), YFP, Cy3 and DAPI filter sets (AHF, Tübingen, Germany) and an oil immersion objective (Plan Apochromat 60x/1.40 Oil; Nikon, Tokyo, Japan).

The paraffin embedded sections (5 μm) were deparaffinized and rehydrated with a standard technique using Xylene and Ethanol. The heart tissue was stained with primary antibodies over night at 4°C (see Table S2) and afterwards with secondary antibodies for one hour at room temperature.

**Desmin expression and purification**

Bacteria (BL21-Star-DE3) transformed with desmin expression constructs (see Table S1) were cultured in LB-medium supplemented with ampicillin (100 μg/mL). The desmin expression was induced with Isopropyl-β-D1-thiogalactopyranoside (IPTG, 1 mM), when the A600 nm reached a value of 0.6-0.8. After 4 h incubation at 37°C, bacteria were harvested by centrifugation and were frozen at -80°C. The inclusion bodies were isolated as earlier described 12. Finally, the proteins were dissolved (8 M Urea, 20 mM Tris-HCl, 100 mM NaH2PO4, pH 8.0) and supplied to a HiTrap DEAE Sepharose Fast Flow column (GE Healthcare, Chalfont St Giles, UK) using the Aktapurifier system (GE Healthcare, Chalfont St Giles, UK). Recombinant desmin was eluted by a linear salt gradient (0-0.35 M NaCl), fractions were pooled and supplied to 5 mL Ni²⁺-NTA (Qiagen, Hilden, Germany) over night at 4°C. The column was washed with buffer (8 M Urea, 20 mM Tris-HCl, 10 mM imidazole, pH 8.0) until A280 nm decreased to a constant value below 0.01. Recombinant desmin molecules were eluted with imidazole containing buffer (8 M
Urea, 20 mM Tris-HCl, 300 mM imidazole, pH 6.9). The fractions containing more than 95% recombinant desmin were pooled and stored at -80°C. The desmin concentration was determined by absorption measurement at 280 nm (ε=26.300 M⁻¹ cm⁻¹, www.expasy.org).

**Proteolysis and mass spectrometry**

The identity of purified desmin variants was proven by mass spectrometry. The desmin variants (20 μg, 100 mM Tris-HCl, pH 8.5) were incubated with 0.08 mg DTT (30 min, 60°C) and were digested with trypsin or Lys-C (Sigma-Aldrich, St. Louis, USA) according to the manufacturer’s instructions. The lyophilized peptides were dissolved in 0.1% formic acid and identified by peptide mass fingerprinting using ESI-LC/MS and were confirmed by ESI-LC/MS-MS using a micrOTOF-Q hybrid mass spectrometer (Bruker, Bremen, Germany). A Jupiter 5u C18 (2.0x150 mm, 300 Å) reverse phase column was used for chromatography. The spectrometer was run in multiple reactions monitoring mode (20-25 eV) for MS-MS analysis.

**Atomic force microscopy**

After a stepwise dialysis into buffer without urea (5 mM Tris-HCl, 1 mM DTT, pH 8.4) the filament formation of recombinant desmin was initiated by addition of an equal volume of sodium chloride buffer (200 mM NaCl, 45 mM Tris-HCl, pH 7.0) and subsequent heating to 37°C for 1 h as previously described. The assembled desmin species (150 μg/mL) were applied to freshly cleaved mica substrates (Plano, Wetzlar, Germany), rinsed with deionized water to remove unbound desmin and dried under a gentle flow of nitrogen. Topographic AFM imaging was done with a Multimode AFM and Nanoscope IIIa controller (Bruker, Santa Barbara, USA) as previously described.
Results

Genetic analysis

We identified two unreported heterozygous sequence variants p.A120D (c.359C>A) and p.H326R (c.977A>G) in the DES gene (Fig. 1C-D). Both variants were not present in 394 healthy control individuals. Furthermore, both variants were not present in publicly available databases and in more than 12,500 alleles from the NHLBI Exome Sequencing Project (ref http://evs.gs.washington.edu/EVS/).

The index patient (III:24) of family A (Fig. 1A) was tested for mutations in ARVC-related genes DSG2, DSC2, JUP, PKP2, DSP, LMNA, TMEM43, PLN and in addition by a gene panel including LDB3/ZASP, TNNT2, SGCD, ACTC1, MYH7, TPM1, TNNI3, TAZ, TTR, MYBC3 and LAMP2, which represent the most frequent DCM genes. We identified in the index patient of family A (III:24) the PKP2-variant (c.1577C>T, p.T526M), which was earlier defined as a non-pathogenic single nucleotide polymorphism 26,27. In the NHLBI Exome Sequencing Project (ESP) the PKP2 variant (c.1577C>T, p.T526M) was found 48x in 12,958 alleles (ref http://evs.gs.washington.edu/EVS/), which is by far above the expected prevalence of the mutation if this was relevant for the disease. The Danish index patient (IV:1) of family B (Fig. 1B) has also been screened for mutations in above listed ARVC-genes without positive findings.

The p.A120D is part of the initial helix motive in coil 1 of the desmin protein and is absolutely conserved in different species (Fig. 2A-B). This mutation is localized in a b-position of the heptade sequence and therefore in close proximity to N116. The other sequence variant p.H326R is localized at the heptade's f-position in coil 2 of the rod domain (Fig. 2B-C).

However, this amino acid is not completely conserved among IF-proteins (Fig. 2C) complicating its pathogenic interpretation.
Filament or aggregate formation of the desmin variants in transfected cells

We investigated the filament formation of the different DES variants in transfected SW-13, HL-1, H9c2, C2C12, HeLa and hiPS-CM. In these transfection experiments desmin p.H326R formed in all cell lines filaments comparable to the wild-type (Fig. 3). In contrast desmin-p.A120D accumulated into cytoplasmic aggregates independently of the transfected cell line (Fig. 3).

We further investigated by immunohistochemistry if the endogenous IFs were impaired by coexpression of the desmin mutants. These analysis revealed that the expression of desmin-p.A120D influences the assembly of endogenous desmin in the muscle cell lines HL-1, H9c2 and C2C12, indicating a dominant inhibiting defect (Fig. 4). Desmin and vimentin form heterofilaments, when coexpressed within the same cell 28. Therefore, we used endogenously vimentin expressing HeLa cells to investigate if the desmin mutants induce a coaggregation with vimentin. In contrast to desmin-p.H326R and -WT the p.A120D mutant induced a partially coaggregation with vimentin in transfected HeLa-cells. Nevertheless, the vimentin network was not strongly affected by the expression of desmin-p.A120D.

In vitro desmin assembly using atomic force microscopy

To get better insights into the putative filament formation defects caused by these new desmin variants, we purified the recombinant desmins by ion exchange and immobilized metal affinity chromatography and analyzed the filament formation in vitro by AFM. In accordance with our cell culture experiments we found that the variant p.H326R formed filaments similar to wild-type desmin (Fig. 5). In contrast, desmin-p.A120D formed small accumulated fibrils (Fig. 5), suggesting severe impairment of the filament elongation step by this mutation.

Investigation of different model mutants at position A120

Since it is not known, if the loss of the methylene group or the gain of the aspartate residue is
causative for aggregation of desmin-p.A120D, we constructed different model variants (p.A120E, p.A120K, p.A120R, p.A120V and p.A120L) to get more insights into the nature of the amino acid residue, which causes the filament formation defect of desmin-p.A120D. In transfected cells, the hydrophobic model mutants (p.A120V and p.A120L) formed filaments similar to wild-type desmin (Fig. 6). In contrast, the exchange of A120 against positive or negative amino acids induced desmin aggregation, with the exception of p.A120K (Fig. 6). In summary, these experiments reveal the essential role of a hydrophobic amino acid at position 120 for the filament formation.

**Immunohistochemistry in cardiac tissue**

Ventricular myocardium of individual II:6 (family A) was available from heart transplantation (Mount Sinai Hospital, Los Angeles, USA). Confirming the *in vitro* results of the cell culture and AFM experiments with the mutant desmin-p.A120D we found a high density of desmin aggregates within the ventricular myocardium, which was undetectable in the ventricles of rejected donor hearts (Fig. 7).

In addition, when the slices of the *DES*-p.A120D heart were costained for desmoplakin colocalization of desmin and desmoplakin could not be observed. Thus, desmin was detectable in the *DES*-p.A120D-heart of the mutation carrier in the z-bands and in prominent protein aggregates but not within the ID (Fig. 7).

Recently, remodeling for plakoglobin (*JUP*)\(^\text{29}\) as well as connexin-43 (*Cx43*)\(^\text{30}\) was described in AC patients. Therefore, we investigated if the localization of both proteins were affected in the patient with the *DES*-p.A120D mutation. These results demonstrate that plakoglobin as well as connexin-43 are localized in the ID similar like in healthy control persons (Fig. S2-S3).
Discussion

At the end of the 1990’s the first mutations in the human DES gene were published. It became obvious that mutations within the DES gene may lead to skeletal and/or cardiac myopathies with a broad spectrum of pathological muscle phenotypes even within the same family. Of note, about 74% of the desminopathy patients develop a cardiac phenotype like conduction disease, arrhythmias or cardiomyopathies. Nevertheless, the molecular pathomechanisms leading to desmin related arrhythmogenic cardiomyopathies are not well understood.

In recent years, mutations of DES associated with an ARVC-related phenotype were found. In this study we identified and characterized the two novel heterozygous DES-variants p.A120D and p.H326R.

The variant p.A120D is localized within the highly conserved IF-consensus motif at the N-terminal segment of coil 1. The amino acid residue A120 is absolutely conserved in different human IF-proteins and among species. Even lamin of Hydra attenuata contains this amino acid. Interestingly, mutations in the homologous positions of the genes coding for keratins K5, K10, K12, K86 and lamin A/C cause severe clinical diseases of eye, skin and muscle, respectively. The desmin variant p.A120D was not detectable in 788 control chromosomes and revealed a severe filament formation defect in cell culture and AFM. These data were confirmed by immunostaining of the failing ventricular myocardium of the affected patient. Interestingly, the desmin-staining within the ID was undetectable in the mutation carrier.

It is known, that desmin is linked to the cardiac desmosome via the plaque protein desmoplakin. Based on yeast two hybrid analyses this protein interaction was claimed to be affected by the desmin mutant p.I451M. However, the transgenic murine model of Mavroides et al. revealed in contrast that this mutation affects the positioning of desmin to the z-bands but not in the
intercalated disk \(^4\). Thus, it remains an open question if a specific (sub-)domain of desmin is linked to the ID via desmoplakin. The lack of desmin within the ID might explain the high prevalence of malignant arrhythmias in the affected family A. Of note, the pedigree of this family reveals a number of SCDs among teenaged family members, which might reflect the arrhythmogenic potential of desmin-p.A120D. Recently, remodeling of plakoglobin and connexin-43 were described in AC patients \(^29,30\). Nevertheless, plakoglobin staining as well as connexin-43 remodeling are controversially discussed in the literature since the absence could not be detected in every patient \(^43–46\). In this study, we demonstrate comparable amounts of plakoglobin and connexin-43 within the ID in heart tissue of a healthy control person and of a patient with the \(DES\)-p.A120D mutation. Based on these experiments we conclude that the remodeling of plakoglobin and connexin-43 does not play a major role for the pathomechanisms caused by this specific \(DES\) mutation.

The mutation p.A120D leads to an exchange of a hydrophobic amino acid side chain against an acidic one. We hypothesized that a hydrophobic amino acid at position 120 is essential for filament formation. For testing this hypothesis we constructed further model mutants with (i) hydrophobic residues (p.A120L and p.A120V), (ii) negative (p.A120E) and (iii) positive charge (p.A120R and p.A120K). Remarkably, ionic side chains at this position disturbed the filament formation with the exception of the lysine residue. Whereas the experiments with hydrophobic amino acid side chains reveal that even a larger mutant amino acid at this position does not disturb filament formation. We conclude from these data that a steric hindrance of the side chain can be excluded as a reason for the p.A120D filament formation defect. We assume that this position within the desmin primary structure, which is in the neighborhood of the recently published mutations p.N116S \(^12\) and p.E114del \(^13\), might be a hotspot for desminopathies.
associated with arrhythmias. It could be speculated that the intra-molecular interaction of this part of the desmin coil 1 with its head domain, might be affected, which was recently shown for vimentin

In summary, although a cosegregation analysis was not possible in family A due to the lack of genomic DNA from suddenly deceased members, we conclude from our experiments that DES-p.A120D is indeed a disease causing mutation with a high potential for SCD.

The amino acid p.H326 is conserved among vertebrate desmins. However, arginine at this position is also found in the human homologue vimentin. In addition this allele was not found in 788 control chromosomes or in the Washington Exome Data. To assess the pathogenic potential of this variant we investigated in vitro the influence of p.H326R on filament formation and performed a cosegregation analysis within the family. When tested in cell culture experiments this recombinant desmin variant did not reveal any filament formation defect, which was also supported by AFM of the purified recombinant desmin. Nevertheless, we cannot exclude that other relevant functions of desmin, like biomechanical properties or protein-protein interactions, i.e. with desmoplakin, might be disturbed by this variant. Of note, the variant did not completely cosegregate within family B. In summary, we regard p.H326R as a rare variant of unknown pathogenic significance.

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**Conflict of Interest Disclosures:** None.

**References**


47. Aziz A, Hess JF, Budamagunta MS, Voss JC, FitzGerald PG. Site-directed spin labeling and


**Figure Legends**

**Figure 1.** Identification and verification of the *DES*-variants. Pedigrees of family A (A) and family B (B). Squares represent males and circles females. Deceased individuals are indicated by slashes. The index patients are marked by a red arrow. Genotypes are shown by present (+) or by absent (-) of the heterozygous *DES* mutations (p.A120D, family A; p.H326R family B).

Electropherograms showing the heterozygous alleles c.359C>A, p.A120D (C) and c.977A>G, p.H326R (D). Converted codons lead to the protein changes p.A120D (C) and p.H326R (D).
Figure 2. Localization of the identified DES-mutations within the desmin sequence. (A)

Schematic domain structure of the desmin protein and the distribution of known disease causing mutations. Some DES mutations affecting the splice sites are not shown (e.g. 49,50). *This sequence variants were also identified in healthy control persons 9. #p.K201RfsX20 and p.R429X were both identified in the same patient 51. **p.A360P and p.N393I were both identified in the same patients 3. ###In the same ARVC patient a pathogenic PKP2 mutation (p.T816RfsX10) was identified 15. ***The mutation p.K240del was originally described as an insertion mutation 52. However, the authors corrected the sequence analysis in 2007. ####Two siblings compound heterozygous for the mutations p.T76fsX21 and p.E108X were described 53. (B, C) Alignment of the desmin/vimentin sequences of Homo sapiens (hs), Mus musculus (mm), Rattus norvegicus (rn), Xenopus laevis (xl) and Dario rerio (dr). The heptad sequence is highlighted in yellow and the positions of the mutations identified in this manuscript are marked in red.

Figure 3. Impairment of filament formation by desmin mutants in transfected cells.

Representative fluorescence images of transfected SW-13, H9c2, HL-1, C2C12 and HeLa cells and hiPS-CM expressing desmin-eYFP constructs (yellow). hiPS-CM were identified by staining for sarcomeric α-actinin using AlexaFluor555-conjugated secondary antibodies (red). Nuclei were stained with DAPI (blue). Scale bars represent 10 μm.

Figure 4. Endogenously expressed IF-proteins in cells transfected with desmin mutants.

Representative fluorescence images of transfected HL-1, H9c2, C2C12 and HeLa cells expressing desmin-eYFP constructs (green), immunostained for desmin (HL-1, H9c2 and C2C12) or vimentin (HeLa) using Cy3-conjugated secondary antibodies (red). The nuclei were stained with DAPI (blue). Scale bars represent 10 μm.
Figure 5. Filament formation of recombinant mutant desmin. Desmin was expressed in E. Coli, purified, prepared on mica, and measured under ambient conditions in AFM tapping mode of operation. Representative AFM topography images of desmin-wt, -p.A120D and p.H326R are shown from left to right in (1 µm x 1 µm) AFM scans (top row) and (5 µm x 5 µm) AFM scans (bottom row). Distinct filament structures could be discerned for desmin-wt and –p.H326R with typical (averaged) dimensions of 500 nm (length), 30 nm (width) and 3-6 nm (height). The apparent width of 30 nm is consistent with a real filament diameter of 8-10 nm that is broadened by artifacts due to a finite AFM tip radius of ~ 20 nm. The reduced height is attributed to surface capillary force effects. In contrast, desmin-p.A120D exhibited complete loss of filament structure, presenting more globular structures with typical size of 85 nm. Representative AFM topography images are shown.

Figure 6. Analysis of model mutants for desmin-p.A120D in different cell lines. Representative fluorescence images of transfected SW-13, H9c2, C2C12 and HeLa cells expressing indicated mutant desmin-eYFP constructs (yellow). Nuclei were stained with DAPI (blue). Scale bars represent 10 µm.

Figure 7. Immunohistological analysis of cardiac tissue heterozygous for the DES-p.A120D mutation. (A-C) Representative fluorescence images of a control sample from a human non-failing control heart, showing normal localization of desmin (red) and desmoplakin (green) at the intercalated discs and at the z-bands. (D-F) Representative paraffin sections of cardiac tissue of patient II:6 demonstrate strong accumulation of desmin (white arrowheads). Of note, the desmin localization is completely lost at the ID. Scale bars represent 10 µm.
**C**

DES c.359C>A (p.A120D)

**D**

DES c.977A>G (p.H326R)
The Novel Desmin Mutant p.A120D Impairs Filament Formation, Prevents Intercalated Disk Localization and Causes Sudden Cardiac Death
Andreas Brodehl, Mareike Dieding, Bärbel Klaue, Eric Dec, Shrestha Madaan, Taosheng Huang, John Gargus, Azra Fatima, Tomo Saric, Hamdin Cakar, Volker Walhorn, Katja Tönsing, Tim Skrzipczyk, Ramona Cebulla, Désirée Gerdes, Uwe Schulz, Jan Gummert, Jesper Hastrup Svendsen, Morten Salling Olesen, Dario Anselmetti, Alex Hørby Christensen, Virginia Kimonis and Hendrik Milting

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**SUPPLEMENTAL MATERIAL**

**Table S1: Designation of constructs and methods of cloning**

<table>
<thead>
<tr>
<th>Construct No.</th>
<th>Construct designation</th>
<th>Source of cDNA/ method of cloning</th>
<th>Restriction sides</th>
<th>Primers</th>
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<tr>
<td>1</td>
<td>pEYFP-N1-Desmin</td>
<td>PCR of pCMV6-AC-Desmin(^7) / TOPO-TA</td>
<td>XhoI, BamHI</td>
<td>1, 2</td>
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<tr>
<td>2</td>
<td>pEYFP-N1-Desmin-p.A120D</td>
<td>SDM of pEYFP-N1-Desmin</td>
<td>XhoI, BamHI</td>
<td>3, 4</td>
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<tr>
<td>3</td>
<td>pEYFP-N1-Desmin-p.A120V</td>
<td>SDM of pEYFP-N1-Desmin</td>
<td>XhoI, BamHI</td>
<td>5, 6</td>
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<tr>
<td>4</td>
<td>pEYFP-N1-Desmin-p.A120L</td>
<td>SDM of pEYFP-N1-Desmin</td>
<td>XhoI, BamHI</td>
<td>7, 8</td>
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<tr>
<td>5</td>
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<td>SDM of pEYFP-N1-Desmin</td>
<td>XhoI, BamHI</td>
<td>9, 10</td>
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<tr>
<td>6</td>
<td>pEYFP-N1-Desmin-p.A120K</td>
<td>SDM of pEYFP-N1-Desmin</td>
<td>XhoI, BamHI</td>
<td>11, 12</td>
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<tr>
<td>7</td>
<td>pEYFP-N1-Desmin-p.A120R</td>
<td>SDM of pEYFP-N1-Desmin</td>
<td>XhoI, BamHI</td>
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<td>8</td>
<td>pEYFP-N1-Desmin-p.H326R</td>
<td>SDM of pEYFP-N1-Desmin</td>
<td>XhoI, BamHI</td>
<td>15, 16</td>
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<td>10</td>
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<td>PCR of pCMV6-AC-Desmin(^9) / TOPO-TA</td>
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<td>3, 4</td>
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<tr>
<td>12</td>
<td>pET100D-Desmin-p.H326R</td>
<td>SDM of pET100D-Desmin</td>
<td>---</td>
<td>15, 16</td>
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</table>

\(^{*}\) Origene Technologies, Rockville, USA
### Table S2: Overview about the antibodies used for IHC

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<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Specification</th>
<th>Used concentration (over night, 4°C)</th>
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<tbody>
<tr>
<td>anti Desmin</td>
<td>R&amp;D Systems Inc. (Minneapolis, MN, USA)</td>
<td>AF3844, polyclonal goat IgG</td>
<td>1:40 (25 ng/µL)</td>
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<tr>
<td>anti Desmoplakin</td>
<td>Acris Antibodies GmbH (Herford, Germany)</td>
<td>AM09122SU-N, monoclonal mouse IgG2b</td>
<td>undiluted</td>
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<tr>
<td>anti Plakoglobin (JUP)</td>
<td>Acris Antibodies GmbH (Herford, Germany)</td>
<td>BM5100, monoclonal mouse IgG2b</td>
<td>1:5 (10 ng/µL)</td>
</tr>
<tr>
<td>anti Connexin-43</td>
<td>Abcam (Cambridge, UK)</td>
<td>AB11370, polyclonal rabbit IgG</td>
<td>1:500 (1.22 ng/µL)</td>
</tr>
</tbody>
</table>
Supplemental Figure Legends:

Figure S1. Electrocardiogram (ECG) of patient III:24 (family A) with DES-p.A120D.
The atrial-ventricular conduction is variable (2:1 and 3:1 conduction). Atrial heart rate is approximately 120 bpm. The ventricular heart rate is approximately 64 bpm. Left axis deviation. Left anterior hemiblock is present with slow progression of R wave and pronounced S in the chest leads. No premature ventricular beats (but polymorphic forms of PVB in 24 h ECG). QRS duration 91 msec. QT/QTc in the normal range.

Figure S2. Immunohistological plakoglobin staining of cardiac tissue heterozygous for the DES-p.A120D mutation.
(A) Representative IHC analysis of paraffin sections of cardiac tissue of patient II:6 demonstrate no reduced expression of plakoglobin at the intercalated disc. (B) Representative fluorescence images of a control sample from a human non-failing control heart. Plakoglobin was stained with FITC-conjugated secondary antibodies (green). The nuclei were stained with DAPI (blue). Scale bars represent 100 µm.

Figure S3. Immunohistological connexin-43 staining of cardiac tissue heterozygous for the DES-p.A120D mutation. (A) Representative IHC analysis of paraffin sections of cardiac tissue of patient II:6 exclude a severe remodeling of connexin-43 at the intercalated disc. (B) Representative fluorescence images of a control sample from a human non-failing control heart. Connexin-43 was stained with Cy3-conjugated secondary antibodies (red). The nuclei were stained with DAPI (blue). Scale bars represent 100 µm.
Figure S2