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. Currently, DES mutations were also found in patients with arrhythmogenic right ventricular cardiomyopathy. Currently, the cellular and molecular pathomechanisms of the DES mutations leading to this disease are not exactly known.

Methods and Results—We identified the 2 novel variants DES-p.A120D (c.359C>A) and DES-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 reveal a severe intrinsic filament formation defect causing cytoplasmic aggregates in cell lines and of the isolated recombinant protein. 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—Because of the functional in vivo and in vitro characterization, DES-p.A120D has to be regarded as a rare variant with unknown significance. Presumably, the loss of the desmin-p.A120D (c.359C>A) and severe skeletal and cardiac muscle diseases with heterogeneous phenotypes. Recently, DES mutations were also found in patients with arrhythmogenic right ventricular cardiomyopathy. Currently, the cellular and molecular pathomechanisms of the DES mutations leading to this disease are not exactly known.

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Key Words: arrhythmias, cardiac myopathies, death, sudden, desmin, desmosomes, intermediate filaments

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, 2 coiled-coil dimers form an antiparallel tetramer.1 These tetramers are the essential building blocks of the IF. Eight tetramers anneal in lateral orientation into unit length filaments. In the longitudinal elongation step, these unit length filaments are assembled and radially compacted into IF.2 Since the first reports on DES mutations,3–5 it became obvious that DES mutations cause skeletal myopathies and different forms of cardiomyopathies.6,7

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In the meantime, >60 different DES mutations distributed over the whole sequence are known, which lead to filament formation defects with deposition of cytoplasmic desmin aggregates in the majority of cases.6,8 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 arrhythmogenic right ventricular cardiomyopathy (ARVC).10–15 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 (SCD).16 It is well established that mutations in the genes coding for desmosomal plaque proteins cause ARVC17–19 and rare forms of dilated cardiomyopathy.20 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 seems to interfere particularly with the connection of desmin IF to the ID. Furthermore, we investigate whether 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.

Materials and Methods
Clinical Description of the Patients
In family A, the 34-year-old female index patient (III:24) presented with atrial flutter, variable atrioventricular conduction (Figure I in the online-only Data Supplement), and dilated atria. The average ventricular frequency was 64 bpm, and the atrial frequency was 120 bpm. In the ECG, some polymorphic premature ventricular contractions (PVCs) with a frequency of 45 to 111 bpm were detected (Figure I in the online-only Data Supplement). The cardiological evaluation including 2-dimensional, M-mode, spectral, and color Doppler was performed. These investigations reveal normal left ventricular (LV) systolic function (LV ejection fraction of 67%), a borderline concentration of mitral regurgitation, and which of the desmin mutations impair the connection of the IF system to the cardiac desmosome.

In family B (Figure 1B), the index patient (IV:1) had experienced palpitations from an age of 25 years. Clinical workup showed a borderline ARVC phenotype based on nonsustained ventricular tachycardia of left bundle branch block 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 80s because of syncope. Workup had shown frequent PVCs of left bundle branch block morphology. He died suddenly playing golf at the age of 38 years. The probands uncle (III:3) had experienced syncope during running at the age of 38 years and was diagnosed with a nonischemic cardiomyopathy (ejection fraction, 25%) and nonsustained ventricular tachycardia. His ejection fraction improved to 50% on therapy (angiotensin-converting enzyme inhibitor, β-blocker, digoxin, spironolactone, implantable cardioverter defibrillator), but he developed chronic atrial fibrillation. All 3 individuals (IV:1, III:2, III:3) were tested positive or obligate carriers of the DES-p.H326R (c.977A>G) variant. The probands 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 because they had died >30 years ago. Individual III:5 was diagnosed with a dilated cardiomyopathy and PVCs before experiencing sudden cardiac arrest at the age of 36 years. Individual IV:4 was initially examined at the age of 9 years because of PVCs and nonsustained ventricular tachycardia. Later, her LV was dilated (LV end diastolic diameter, 69 mm) and ejection fraction was at the lowest 25% but improved to 55% with treatment (angiotensin-converting enzyme inhibitor, β-blocker, amiodarone, PVC ablation, implantable cardioverter defibrillator). 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, United Kingdom). The BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA) and an ABI310 Genetic Analyzer (Applied Biosystems, CA) were used for sequencing according to the manufacturer’s instructions. The sequences were analyzed with the Variant Reporter Software v1.0 (Applied Biosystems, CA). The allele frequencies of novel variants were determined in 394 healthy control individuals using the TaqMan SNP Genotyping Assay (Applied Biosystems, CA). 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-Desmin2 and pEYFP-N1-Desmin2 using appropriate primers and the QuickChange Lightning Kit (Agilent Technologies, Santa Clara) according to the manufacturer’s instructions (see Table I in the online-only Data Supplement). The DES coding regions were verified by sequencing using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA). 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) were cultured in DMEM (Invitrogen, Carlsbad) supplemented with 10% FCS, 4.5 g/L glucose, and penicillin/streptomycin. The HL-1 cells (kindly provided by Dr William Claycomb) were cultured in Claycomb medium (Sigma-Aldrich, St. Louis) supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 mmol/L

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norepinephrine, and penicillin/streptomycin. Cardiomyocytes derived from human-induced pluripotent stem cells were generated and cultured as previously described. Lipofectamine 2000 (Invitrogen, Carlsbad) was used to transfect the cells according to the manufacturer’s protocol.

**Immunohistochemistry and Fluorescence Microscopy**

Transfected cells were fixed with methanol (15 minutes, −20°C) and were then permeabilized with 0.1% Triton X-100 (20 minutes, room temperature). After blocking with 1% BSA/PBS, the cells were incubated with 7.5 μg/mL anti-desmin antibodies (R&D Systems, Minneapolis) or 25 μg/mL anti-vimentin antibodies (Sigma-Aldrich, St. Louis) overnight 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 ImmunoResearch, West Grove, PA) for 1 hour at RT. The nuclei were stained with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI, 5 minutes, 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), yellow fluorescence protein, 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 overnight at 4°C (see Table II in the online-only Data Supplement) and afterward with secondary antibodies for 1 hour at room temperature.

**Desmin Expression and Purification**

Bacteria (BL21-Star-DE3) transformed with desmin expression constructs (see Table I in the online-only Data Supplement) were cultured in Luria Broth medium supplemented with ampicillin (100 μg/mL). The desmin expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mmol/L), when the A600 nm reached a value of 0.6 to 0.8. After 4-hour incubation at 37°C, bacteria were harvested by centrifugation and were frozen at −80°C. The inclusion
bodies were isolated as earlier described. Finally, the proteins were dissolved (8 mol/L urea, 20 mmol/L Tris-HCl, 100 mmol/L NaH2PO4, pH 8.0) and supplied to a HiTrap DEAE Sepharose Fast Flow column (GE Healthcare, Chalfont St Giles, United Kingdom) using the ÄKTApurifier system (GE Healthcare, Chalfont St Giles, United Kingdom). Recombinant desmin was eluted by a linear salt gradient (0–0.35 mol/L NaCl), and fractions were pooled and supplied to 5 mL Ni2+-NTA (Qiagen, Hilden, Germany) overnight at 4°C. The column was washed with buffer (8 mol/L urea, 20 mmol/L Tris-HCl, 10 mmol/L imidazole, pH 8.0) until A280nm decreased to a constant value <0.01. Recombinant desmin molecules were eluted with imidazole-containing buffer (8 mol/L urea, 20 mmol/L Tris-HCl, 300 mmol/L imidazole, pH 6.9). The fractions containing >95% recombinant desmin were pooled and stored at −80°C. The desmin concentration was determined by absorption measurement at 280 nm (ε=26,300 (mol/L)−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 mmol/L Tris-HCl, pH 8.5) were incubated with 0.08 mg dithiothreitol (30 minutes, 60°C) and were digested with trypsin or Lys-C (Sigma-Aldrich, St. Louis) according to the manufacturer’s instructions. The lyophilized peptides were dissolved in 0.1% formic acid and identified by peptide mass fingerprinting using electrospray ionization-liquid chromatography/mass spectrometry and were confirmed by electrospray ionization-liquid chromatography/tandem mass spectrometry using a micrOTOF-Q hybrid mass spectrometer (Bruker, Bremen, Germany). A Jupiter 5u C18 (2.0x150 nm, 300 Å) reverse phase column was used for chromatography. The spectrometer was run in multiple reactions monitoring mode (20–25 eV) for tandem mass spectrometry analysis.

Atomic Force Microscopy
After a stepwise dialysis into buffer without urea (5 mmol/L Tris-HCl, 1 mmol/L dithiothreitol, pH 8.4), the filament formation of recombinant desmin was initiated by addition of an equal volume of sodium chloride buffer (200 mmol/L NaCl, 45 mmol/L Tris-HCl, pH 7.0) and subsequent heating to 37°C for 1 hour 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. Topographical AFM imaging was done with a Multimode AFM and Nanoscope IIIa controller (Bruker, Santa Barbara) as previously described.

Results
Genetic Analysis
We identified 2 unreported heterozygous sequence variants p.A120D (c.359C>T) and p.H326R (c.977A>G) in the DES gene (Figure 1C and 1D). Both variants were not present in 394 healthy control individuals. Furthermore, both variants were not present in publicly available databases and in >12,500 alleles from the NHLBI Exome Sequencing Project (http://evs.gs.washington.edu/EVs/).

The index patient (III:24) of family A (Figure 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, TNN3, TAZ, TTR, MYBC3, and LAMP2, which represent the most frequent dilated cardiomyopathy 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 nonpathogenic single-nucleotide polymorphism. In the NHLBI Exome Sequencing Project, the PKP2 variant (c.1577C>T, p.T526M) was found 48× in 12,958 alleles (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 (Figure 1B) has also been screened for mutations in above listed ARVC genes without positive findings.

Filament or Aggregate Formation of the Desmin Variants in Transfected Cells
We investigated the filament formation of the different desmin variants in transfected SW-13, HL-1, H9c2, C2C12, HeLa, and cardiomyocytes derived from human-induced pluripotent stem cells. In these transfection experiments, desmin p.H326R formed in all cell line filaments compared with the WT (Figure 3). In contrast, desmin-p.A120D accumulated into cytoplasmic aggregates independently of the transfected cell line (Figure 3).

We further investigated by immunohistochemistry whether 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 (Figure 4). Desmin and vimentin form heterofilaments when coexpressed within the same cell. Therefore, we used endogenously vimentin expressing HeLa cells to investigate whether the desmin mutants induce a coaggregation with vimentin. In contrast to desmin-p.H326R and desmin-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 AFM
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 WT desmin (Figure 5). In contrast, desmin-p.A120D formed small accumulated fibrils (Figure 5), suggesting severe impairment of the filament elongation step by this mutation.

Investigation of Different Model Mutants at Position A120
Because it is not known whether 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 WT desmin (Figure 6). In contrast, the exchange of A120 against positive or negative amino acids induced desmin aggregation, with the exception of p.A120K (Figure 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). 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 (Figure 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 (Figure 7).

Discussion

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

In recent years, mutations of \( \text{DES} \) associated with an ARVC-related phenotype were found.10–15 In this study, we identified and characterized the 2 novel heterozygous \( \text{DES} \) 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.37 Even lamin of \( \text{Hydra attenuata} \) contains this amino acid.38,39 Interestingly, mutations in the homologous positions of the genes coding for keratins K5,40,41 K10,42 K12,43 K86,44 and lamin A/C45 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.46 Based on yeast 2 hybrid analyses, this protein interaction was claimed to be affected by the desmin mutant p.I451M.46 However, the transgenic murine model of Mavroidis et al47 revealed in contrast that this mutation affects the positioning of desmin to the z-bands but not in the ID. Thus, it remains an open question whether 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 several 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 arrhythmogenic cardiomyopathy patients.34,35 Nevertheless, plakoglobin staining as well as connexin-43 remodeling are controversially discussed in the literature because the absence could not be detected in every patient.46–51 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 \( \text{DES} \)-p.A120D mutation. Based on these experiments, we conclude that remodeling of plakoglobin and connexin-43 does not play a major role for the pathomechanisms caused by this specific \( \text{DES} \) 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 (1) hydrophobic residues (p.A120L and p.A120V), (2) negative (p.A120E), and (3) 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 intramolecular interaction of this part of the desmin coil 1 with its head domain might be affected, which was recently shown for vimentin.\(^{52,53}\)

In summary, although a cosegregation analysis was not possible in family A because of 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.

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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. WT indicates wild type; and YFP, yellow fluorescence protein.

Figure 7. Immunohistological analysis of cardiac tissue heterozygous for the DES-p.A120D mutation. A to C. Representative fluorescence images of a control sample from a human nonfailing control heart showing normal localization of desmin (red) and desmoplakin (green) at the intercalated disks (IDs) and at the z-bands. D to 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.
The amino acid p.H326 is conserved among vertebrate desmins. However, arginine at this position is also found in the human homolog 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, such as biomechanical properties or protein–protein interactions, that is, 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.

Acknowledgments
We thank all participating patients. We are grateful to Birte Bohms for excellent technical assistance. Furthermore, we thank Dr Dawn Lombardo (University of Irvine) for her help in clinical investigations. In addition, we thank Dr Bianca Werner (Heart and Diabetes Centre NRW, Germany) for providing the HeLa cells, Dr Christine Mummery (Leiden University Medical Centre, The Netherlands) for providing the END2 cells, and Dr William Claycomb (New Orleans) for providing the HL-1 cells.

Sources of Funding
Dr Milting was kindly funded by the Erich and Hanna Klessmann Foundation, Güttersloh, Germany, and by FoRUM grant (F649-2009) of the Ruhr-University Bochum. This work was also supported by the grant from the Bundesträger für Bildung und Forschung (BMBF) to T. Šaric (grant No.: 01 GN 0824). J.H. Svendsen was funded by The Danish National Research Foundation, The John and Birthe Meyer Foundation, The Research Foundation of the Heart and Diabetes Center NRW, Ruhr-University Bochum. This work was also supported by AFM of the purified recombinant desmin. Nevertheless, we cannot exclude that other relevant functions of desmin, such as biomechanical properties or protein–protein interactions, that is, 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.

Disclosures
None.

Appendix
From the Erich and Hanna Klessmann Institute for Cardiovascular Research and Development (EHKI), Heart and Diabetes Center NRW, Ruhr-University Bochum, Bad Oeynhausen, Germany (A.B., B.K., T.S., R.C., D.G., U.S., J.G., H.M.); Experimental Biophysics and Applied Nanoscience, Faculty of Physics, Bielefeld Institute for Biophysics and Nanoscience (BINAS), Bielefeld University, Bielefeld, Germany (M.D., V.W., K.T., D.A.); Division of Genetics and Metabolism, Department of Pediatrics, University of California, Irvine (E.D., S.M., J.G., V.K.); Division of Human Genetics, Department of Pediatrics, Cincinnati Children’s Hospital, OH (T.H.); Institute for Neurophysiology, Medical Center, University of Cologne, Cologne, Germany (A.F., T.S.); Physikalisch-Technische Bundesanstalt (PTB), Braunschweig, Germany (H.C.); Department of Cardiology, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark (J.H.S., M.S.O., A.H.C.); and The Danish National Research Foundation Centre for Cardiac Arrhythmia, Copenhagen, Denmark (J.H.S., M.S.O., A.H.C.).

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**CLINICAL PERSPECTIVE**

Mutations in the gene DES might cause skeletal and cardiac myopathies including dilated, hypertrophic, restrictive, and arrhythmogenic cardiomyopathy (arythmogenic right ventricular cardiomyopathy). Currently, the desmin-specific pathomechanisms leading to the different phenotypes are unknown. Here, we present a novel disease-causing heterozygous DES missense mutation (p.A120D) which is clinically associated with dilated atria, arythmias, and sudden cardiac death at young age. In addition, we identified a DES variant of unknown significance in patients with arythmogenic cardiomyo-
pathy (p.H326R). The results of our study indicate that the phenotypes caused by DES mutations could be more diverse and partially overlapping between the different cardiomyopathies which should be considered in genetic counseling. Here, we focus on the molecular characterization of desmin p.A120D which causes a severe filament formation defect in vitro. Furthermore, we provide evidences that this missense mutation also causes strong desmin aggregation in the myocardium of the affected patients and leads to the loss of desmin staining within the intercalated disk. The absence of desmin at the intercalated disk might contribute to the arythmogenic potential of this mutation. In summary, the presented cell culture experiments and the analysis using atomic force microscopy could help to prove the pathogenicity of further desmin variants. These experimental methods could assist to identify the pathogenic impact of DES mutations. This might contribute to risk assessment and genetic counseling of families with desminopathies in the future.
**SUPPLEMENTAL MATERIAL**

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

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<td>4</td>
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<td>SDM of pEYFP-N1-Desmin</td>
<td>XhoI, BamHI</td>
<td>7, 8</td>
</tr>
<tr>
<td>5</td>
<td>pEYFP-N1-Desmin-p.A120E</td>
<td>SDM of pEYFP-N1-Desmin</td>
<td>XhoI, BamHI</td>
<td>9, 10</td>
</tr>
<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>
</tr>
<tr>
<td>7</td>
<td>pEYFP-N1-Desmin-p.A120R</td>
<td>SDM of pEYFP-N1-Desmin</td>
<td>XhoI, BamHI</td>
<td>13, 14</td>
</tr>
<tr>
<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>
</tr>
<tr>
<td>9</td>
<td>pET100D-Desmin</td>
<td>PCR of pCMV6-AC-Desmin(^9) / TOPO-TA</td>
<td>---</td>
<td>17, 18</td>
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<td>10</td>
<td>pET100D-Desmin-p.A120D</td>
<td>SDM of pET100D-Desmin</td>
<td>---</td>
<td>3, 4</td>
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<td>11</td>
<td>pET100D-Desmin-p.H326R</td>
<td>SDM of pET100D-Desmin</td>
<td>---</td>
<td>15, 16</td>
</tr>
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</table>

\(^7\) Origene Technologies, Rockville, USA
Table S2: Overview about the antibodies used for IHC

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Specification</th>
<th>Used concentration (over night, 4°C)</th>
</tr>
</thead>
<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>
</tr>
<tr>
<td>anti Desmoplakin</td>
<td>Acris Antibodies GmbH (Herford, Germany)</td>
<td>AM09122SU-N, monoclonal mouse IgG2b</td>
<td>undiluted</td>
</tr>
<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 S1

S1
Figure S3
The Novel Desmin Mutant p.A120D Impairs Filament Formation, Prevents Intercalated Disk Localization, and Causes Sudden Cardiac Death

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_Circ Cardiovasc Genet._ 2013;6:615-623; originally published online November 7, 2013; doi: 10.1161/CIRCGENETICS.113.000103

_Circulation: Cardiovascular Genetics_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1942-325X. Online ISSN: 1942-3268

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