The Popeye domain–containing 1 (POPDC1) gene encodes a plasma membrane–localized cAMP-binding protein that is abundantly expressed in striated muscle. In animal models, POPDC1 is an essential regulator of structure and function of cardiac and skeletal muscle; however, POPDC1 mutations have not been associated with human cardiac and muscular diseases. Here, we have described a homozygous missense variant (c.602C>T, p.S201F) in POPDC1, identified by whole-exome sequencing, in a family of 4 with cardiac arrhythmia and limb-girdle muscular dystrophy (LGMD). This allele was absent in known databases and segregated with the pathological phenotype in this family. We did not find the allele in a further screen of 104 patients with a similar phenotype, suggesting this mutation to be family specific. Compared with WT protein, POPDC1S201F displayed a 50% reduction in cAMP affinity, and in skeletal muscle from patients, both POPDC1 S201F and WT POPDC2 displayed impaired membrane trafficking. Forced expression of POPDC1 S201F in a murine cardiac muscle cell line (HL-1) increased hyperpolarization and upstroke velocity of the action potential. In zebrafish, expression of the homologous mutation (popdc1S202F) caused heart and skeletal muscle phenotypes that resembled those observed in patients. Our study therefore identifies POPDC1 as a disease gene causing a very rare autosomal recessive cardiac arrhythmia and LGMD, expanding the genetic causes of this heterogeneous group of inherited rare diseases.

Introduction
The liaison between muscular dystrophy and heart dysfunction is well known in medical genetics. More than 90 muscular dystrophy phenotypes have been identified, of which the majority also display cardiac manifestations (1–3). Both dilated cardiomyopathies and cardiac arrhythmia phenotypes are often found as comorbidities and may even precede the onset of the muscle symptoms (4–6). The limb-girdle muscular dystrophies (LGMDs) are inherited diseases with onset after birth and are characterized by progressive weakness and muscle atrophy predominantly affecting the hips, shoulders, and proximal extremity muscles (3). There are both autosomal dominant (LGMD1) and autosomal recessive (LGMD2) subtypes known. Today, a total of 31 different LGMD loci have been identified (7). A variety of mutations are known in the corresponding genes, which belong to several different cellular pathways, including sarcolemmal glycoproteins (dystroglycan and sarcoglycans), scaffolding proteins (caveolin-3 [CAV3]), and proteins involved in membrane repair and vesicle trafficking (dysferlin [DYSF] and anoctamin-5 [ANO5]) (7).

POPDC1, which is also known as BVES, is a member of the Popeye domain–containing (Popdc) gene family, encoding transmembrane proteins, which is highly expressed in cardiac and skeletal muscle in an overlapping manner (8,9). POPDC proteins possess the evolutionarily conserved Popeye domain, which functions as a high-affinity cAMP-binding site (8,10). POPDC proteins are localized primarily at the plasma membrane and in t-tubules (10), although they have also been found at the nuclear envelope of striated muscle cells (11). An interaction of POPDC proteins with CAV3 and the 2-pore domain potassium channel TREK-1 has been reported (10,12). In the presence of POPDC proteins, TREK-1 cur-
The town of about 3,000 inhabitants located in Calabria (Italy) showed an interesting pseudodominant inheritance, compatible with consanguinity and geographic isolation (Figure 1A). The grandfather, 81 years old at the time of writing (PTI-1), started to complain of lower limb-girdle weakness around the age of 40 and lost the ability to walk without aids around the age of 60. At that time, blood creatine kinase (CK) was elevated (range, 750–1300 IU/l). At the age of 60, a muscle biopsy was performed in his left deltoid muscle showing muscular dystrophy changes, with diameter variability, increased central nuclei, and the presence of a few necrotic and regenerating fibers (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI79562DS1). Transmission electron microscopy (TEM) analysis revealed the presence of plasma membrane discontinuities and submembranous vacuoles (Figure 1, B–D). Expression of dystrophin (DYS), all sarcoglycans, emerin, merosin, and CAV3 was normal (data not shown). At age 59, this patient started to complain of repeated syncope episodes, and a second-degree AV block was disclosed. A pacemaker was placed 1 year later at the age of 60. Echocardiography excluded a cardiomyopathy. Muscle testing at the age of 72 showed a clear symmetrical weakness of the limbs (neck flexors Medical Research Council Scale for Muscle Strength [MRC] 5; ileopsoas MRC 3.5; quadriceps MRC 2; glutei MRC 1; gastrocnemius MRC 5; foot rents are potentiated due to enhanced membrane trafficking (10). The Popdc1 null mutant displays a retardation of muscle regeneration (13). Moreover, an impaired recovery from cardiac ischemia and an increase in infarct size have been described in the Popdc1 null mutant (12). Both Popdc1 and Popdc2 null mutants display a stress-induced sinus node bradycardia, which develops in an age-dependent manner (10, 14). In zebrafish, popdc2 morphants develop embryonic heart failure, atrioventricular (AV) block, and muscular dystrophy (15). Thus, both heart and skeletal muscle pathologies have been associated in model organisms with the loss of either Popdc1 or Popdc2. However, so far, Popdc genes have never been associated with human hereditary diseases.

We report here the identification of a homozygous recessive mutation in POPDC1 (c.602C>T, p.S201F) by whole-exome sequencing (WES) in a family with LGMD and AV block. We demonstrate that POPDC1S201F has strong pathogenic consequences, since it affects CAMP binding and subcellular localization of the mutant protein and its interaction partners. Therefore POPDC1 is a disease-causing gene associated with LGMD and cardiac arrhythmia.

**Results**

POPDC1 is a disease-causing gene associated with cardiac disturbances and LGMD. A family originating from a small Albanian enclave of about 3,000 inhabitants located in Calabria (Italy) showed an interesting pseudodominant inheritance, compatible with consanguinity and geographic isolation (Figure 1A). The grandfather, 81 years old at the time of writing (PTI-1), started to complain of lower limb-girdle weakness around the age of 40 and lost the ability to walk without aids around the age of 60. At that time, blood creatine kinase (CK) was elevated (range, 750–1300 IU/l). At the age of 60, a muscle biopsy was performed in his left deltoid muscle showing muscular dystrophy changes, with diameter variability, increased central nuclei, and the presence of a few necrotic and regenerating fibers (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI79562DS1). Transmission electron microscopy (TEM) analysis revealed the presence of plasma membrane discontinuities and submembranous vacuoles (Figure 1, B–D). Expression of dystrophin (DYS), all sarcoglycans, emerin, merosin, and CAV3 was normal (data not shown). At age 59, this patient started to complain of repeated syncope episodes, and a second-degree AV block was disclosed. A pacemaker was placed 1 year later at the age of 60. Echocardiography excluded a cardiomyopathy. Muscle testing at the age of 72 showed a clear symmetrical weakness of the limbs (neck flexors Medical Research Council Scale for Muscle Strength [MRC] 5; ileopsoas MRC 3.5; quadriceps MRC 2; glutei MRC 1; gastrocnemius MRC 5; foot

**Figure 1. Identification of the POPDC1S201F mutation associated with AV block and LGMD.** (A) Pedigree of a family homozygous for the POPDC1S201F mutation. The index patient (PTIII-2) and his brother (PTIII-1) suffered from AV block and the grandfather (PTI-1) from LGMD and AV block. (B) TEM analysis of skeletal muscle of PTI-1 revealed the presence of plasma membrane discontinuities (arrows) and subsarcolemmal and intermyofibrillar vacuoles (asterisks). C and D show higher magnifications of the boxed areas in B. Scale bars: 2 μm (B); 1 μm (C and D). Data depicted are representative of results derived from a single biopsy. (E) Holter ECG of the index patient (PTIII-2) displaying an episode of a paroxysmal AV block. (F) Electropherogram of the Sanger sequencing of genomic DNA isolated from family members. (G) Sequence alignment of part of the Popeye domain demonstrating sequence conservation. Many residues are identical (turquoise) or similar (green); however, S201 (yellow) is ultraconserved. (H) 3D model of the Popeye domain. Only the phosphate-binding cassette (PBC) is shown. Two hydrogen bonds are formed by S201, but these are lost when the F201 (turquoise) mutant residue is present, possibly affecting the ligand-binding affinity of the Popeye domain.
Figure 2. Membrane trafficking of POPDC1 is affected in muscle biopsies. Skeletal muscle biopsies of PTI-1 and PTIII-1 and 2 controls were immunostained for (A–D) POPDC1 (green signal) and SGCA (red signal). (E) The immunofluorescence signals were quantified in 10 muscle fibers of 3 sections per biopsy. The signals of POPDC1 and SGCA and the ratio of both were plotted relative to the means of both controls, which were set at 1. (F–Q) Subcellular localization of POPDC1 in (F–H) CT1, (I–K) CT2, (L–N) PTI-1, and (O–Q) PTIII-2. Perinuclear localization of POPDC1 is only weakly present in control samples, whereas in patients’ muscle biopsies, a significant perinuclear accumulation of the mutant protein was seen. Scale bars: 10 μm (A–D, G, H, J, K, M, N, P, and Q); 20 μm (F, I, L, and O). (R and S) Western blot analysis of (R) POPDC1 expression in skeletal muscle biopsies and of (S) POPDC1 and POPDC2 in dermal fibroblasts of 2 controls (CT1 and CT2) and PTI-1 and PTIII-2. The main POPDC1 isoform in R is labeled by POPDC1. Additional isoforms are indicated by arrows, while bands, which are considered to be unspecific and remain present after peptide competition (Supplemental Figure 4, A and B), are indicated by an asterisk. Results are representative of 3 independent experiments.
The S201F mutation affects membrane trafficking of both POPDC1 and POPDC2. In healthy skeletal muscle fibers, POPDC1 and POPDC2 are found in the plasma membrane. However, in patients with a homozygous POPDC1S201F mutation, the membrane localization of POPDC2 is downregulated compared to controls. The relative intensity of POPDC2 and SCGA was quantified in muscle biopsy samples from patients and controls. Western blot analysis of POPDC2 expression in the muscle biopsy material of controls and patients showed a reduction in expression in patients with the mutation. These findings suggest that the S201F mutation affects the trafficking and localization of POPDC2 in muscle cells.

Figure 3. Membrane trafficking of POPDC2 is affected in patients with a homozygous POPDC1S201F mutation. Skeletal muscle biopsies of PTI-1 and PTIII-2 and 2 controls were immunostained for (A–D) POPDC2 (green signal) and SCGA (red signal). (E) Quantification of the relative intensities of the plasma membrane staining of POPDC2 and SCGA in 10 fibers each from 3 sections per biopsy. The signals of POPDC2 and SCGA and the ratio of both were plotted relative to the means of both controls, which were set at 1. The normalized intensities revealed a downregulation of the membrane localization of POPDC2 in the patient material. Scale bars: 10 μm. (F) Western blot analysis of POPDC2 expression in the muscle biopsy material of the 2 controls (CT1 and CT2) and PTI-1 and PTIII-2. Results are representatives of 3 independent experiments.
The Journal of Clinical Investigation

Research Article

muscle fibers, suggesting that the S201F mutation predominantly causes a reduction in plasma membrane trafficking rather than affecting protein expression levels.

The histological analysis and CT scan demonstrated absence of severe fibrosis and fat replacement in PTIII-1 and PTIII-2 (Supplemental Figure 1, A–C, and Supplemental Figure 3). However, in the grandfather (PTI-1), fiber size variability, central nuclei, and plasma membrane discontinuities in muscle fibers were observed (Figure 1E and Supplemental Figure 1D). TEM of PTIII-2 failed to detect membrane discontinuities or any other abnormality of the caveolae, basal membrane, and nuclear pores (data not shown), which is in accordance with the late onset of muscle pathology in this family. We studied the expression of proteins involved in membrane stability and repair in PTI-1 and PTIII-2 in comparison with 2 controls (Supplemental Figures 5–9). We previously reported protein-protein interaction of POPDC1 with CAV3 (12). However, the membrane presence of CAV3 was not altered in the patients’ muscle fibers, suggesting that the S201F mutation predominantly causes a reduction in plasma membrane trafficking rather than affecting protein expression levels.

Figure 4. The POPDC1S201F mutant protein displays a reduced cAMP affinity. (A) cAMP affinity precipitation of WT or POPDC1S201F protein. Left panel: precipitated proteins, unbound fraction, and input samples as well as protein subjected to control precipitation using ethanolamine-agarose (agarose) were subjected to Western blot detection of POPDC1. Right panel: quantification of cAMP affinity, calculating the ratios between input and cAMP agarose–bound protein fractions. (B) Left: example of a FRET measurement of 293A cells transfected with YFP–TREK-1 together with POPDC1–CFP or POPDC1S201F–CFP. Right: relative change in FRET signal as a measure of cAMP affinity. (C) Chemiluminescence assay in Xenopus oocytes analyzing the surface expression of extracellularly HA-tagged TREK-1 in the absence or presence of POPDC1 or POPDC1S201F. Control, noninjected oocytes. Measurements after incubation with 0.5 mM theophylline are illustrated with white bars. (D) Representative 2-electrode voltage clamp measurements in oocytes injected with TREK-1 alone (black) or coinjected with Popdc1 (gray) or POPDC1S201F (red). Voltage was ramped from −110 to +35 mV. (E) Relative current amplitudes at 0 mV. (F) 2-Electrode voltage clamp measurements in oocytes injected with TREK-1 or coinjected with POPDC1 or POPDC1S201F bathed after cRNA injection in a storage solution containing 1 mM 8-Br-cAMP. (G) Relative current amplitudes of TREK-1 coinjected with POPDC1, measured in recording solution or directly after 10 minutes of perfusion with 2 mM 8-Br-cAMP. (A–C and E–G) Numbers of experiments are included in the bars. Results are presented as mean ± SEM. Two groups of measurements in A were compared by paired 2-tailed Student’s t test. One-way ANOVA was used for the data presented in B, C, and E–G. *P < 0.05; ***P < 0.001.
muscle biopsies in comparison with controls (Supplemental Figure 5). We also studied DYS, which was communoprecipitated with POPDC1 (Supplemental Figure 6). However, we could not detect any difference in membrane localization between patient and control samples (Supplemental Figure 7). Membrane discontinuities were observed in the muscle biopsy of the grandfather (PTI-1), a feature that is typical of dysferlinopathies and related muscle disorders (20, 21). We established association of DYSF with POPDC1 by colocalization and communoprecipitation (Supplemental Figure 8). However, plasma membrane levels of DYSF were not affected in patients with a homozygous POPDC1S201F mutation (Supplemental Figure 9). These data indicate that POPDC1 does not affect sarcoplasmic localization of DYSF, DYSF, and CAV3. Possibly, these protein-protein interactions only become essential under particular physiological conditions, such as in response to membrane wounding or during muscle regeneration (13).

The POPDC1S201F mutant protein displays a reduction of its affinity for cAMP. The S201 residue is part of the ultraconserved DSPE motif, which is essential for cAMP binding (10). We therefore assessed whether the change of serine to phenylalanine might cause a reduction in cAMP affinity of POPDC1 with the help of a ligand precipitation assay, previously utilized for the mouse protein (10). We found that the S201F mutant protein has an approximately 50% reduction in affinity for cAMP (Figure 4A). To corroborate this finding, we employed a bimolecular fluorescence resonance energy transfer (FRET) sensor based on the interaction of POPDC1 and TREK-1, analogous to what was previously described for mouse POPDC1 (10). FRET was observed after cotransfection of either CFP-tagged WT POPDC1 or POPDC1S201F together with YFP–TREK-1. Upon stimulation with isoproterenol (Iso) or forskolin, a small but robust decrease of the FRET ratio was observed for WT POPDC1, but changes in the case of POPDC1S201F were much weaker (~50%), confirming in an independent assay the decreased cAMP sensitivity of the POPDC1S201F mutant (Figure 4B and Supplemental Figure 10). No decrease of the FRET ratio was observed when the cotransfected cells were preincubated with propranolol prior to Iso stimulation, demonstrating that the Iso-induced change in FRET ratio represented a β-adrenergic receptor-dependent event (Supplemental Figure 10).

To demonstrate that the different response to rising cAMP levels of mutant and WT POPDC1 is based on different cAMP affinities and not due to an impaired interaction between POPDC1S201F and TREK-1, we narrowed down the TREK-1 interaction domain using the mouse protein by deletion and communoprecipitation analysis (Supplemental Figure 11). The interaction with POPDC1 was lost when 219 amino acids were deleted from the carboxy terminus. These data suggest that the TREK-1 interaction domain of POPDC1 is located between residues 139 and 186 and therefore is positioned at some distance from the phosphate-binding cassette (residues 198–218). Consequently, it is unlikely that the S201F mutation directly affects the interaction of POPDC1 with TREK-1. This conclusion is also supported by the observation that both mutant and WT POPDC1 colocalize with TREK-1 after cotransfection (Supplemental Figure 12).

Enhanced surface expression of TREK-1 by POPDC1 is lost by the S201F mutation. Enhanced TREK-1 outward currents are observed after coexpression of mouse POPDC1 and TREK-1 in Xenopus oocytes (10). For mouse POPDC2, we were able to establish that the increased outward current of TREK-1 is most likely caused by an enhanced surface expression of the channel (10). Here, we show that the coexpression of TREK-1 with human POPDC1 also caused strongly increased surface expression of the channel (Figure 4C). Interestingly, the S201F mutation impaired the ability of POPDC1 to enhance the surface expression of TREK-1 (Figure 4C). Moreover, raising intracellular cAMP levels by the phosphodiesterase inhibitor theophylline resulted in an almost 6-fold increase in surface expression of TREK-1 when coexpressed with WT POPDC1. However, in the presence of the S201F mutant, the surface expression of TREK-1 was not affected (Figure 4C). Thus, similar to the impaired plasma membrane localization of POPDC2 in the patients’ mus-
cle biopsies, membrane trafficking of TREK-1 in Xenopus oocytes was affected in the presence of the S201F mutant. Coexpression of TREK-1 with POPDC1 led to increased current amplitudes for both WT and mutant S201F (Figure 4, D and E). Surprisingly, coexpression of the S201F mutant with TREK-1 caused an even more pronounced enhancement of TREK-1 outward currents than WT POPDC1. While POPDC1 increased TREK-1 currents by 40%, the S201F mutant caused an increase of the TREK-1 current by 90%. Thus, despite the inability of augmenting TREK-1 surface expression, the S201F mutant was able to enhance TREK-1 outward currents, which was presumably caused by an increased conductivity of the channels in complex with POPDC1 S201F. In the presence of the membrane-permeable cAMP analog 8-Br-cAMP, the effects of WT POPDC1 on TREK-1 currents were basically abolished (Figure 4F). Thus, despite the inability of POPDC1 S201F to affect the surface expression of TREK-1 (Figure 4C), the mutant protein caused increased TREK-1 outward currents. These seemingly opposing effects of the S201F mutant protein to cause impaired channel trafficking while enhancing gating may explain to some extent the cardiac arrhythmia phenotype in the patients.

Forced expression of POPDC1 S201F hyperpolarizes the membrane potential of spontaneously beating HL-1 cells. Since POPDC1 is predominantly expressed in striated muscle cells, we used HL-1 cells, which are spontaneously beating sinoatrial node-like myocytes (22), to probe for changes in cellular electrophysiology by forced expression of the POPDC1 S201F mutant. Both POPDC1 and POPDC2 are endogenously expressed in HL-1 cells and are membrane localized (Supplemental Figure 13). We transfected

![Image](http://www.jci.org)
CFP-tagged WT POPDC1 or POPDC1<sup>S201F</sup> in HL-1 cells and recorded action potential properties of spontaneously beating cells using patch-clamp experiments. Forced expression of WT POPDC1 into HL-1 cells resulted in a pronounced afterhyperpolarization (Figure 5, A and D) and shortening of the action potential duration (Figure 5, A and B) compared with nontransfected HL-1 cells. These results are well in agreement with the overexpression of a TREK-1–activating protein in HL-1 cells, as these cells contain endogenous TREK-1 channels (Supplemental Figure 14). Most importantly, transfecting the same amount of POPDC1<sup>S201F</sup> and TREK-1 cDNA caused a hyperpolarization of the maximal diastolic membrane potential (Figure 5, A and D). This effect is in agreement with a more pronounced activation of TREK-1 channels by POPDC1<sup>S201F</sup> (Figure 4, D and E). As cells with POPDC1<sup>S201F</sup> were more hyperpolarized (Figure 5C) and the activation threshold was also shifted to more negative membrane potentials (Figure 5E), there was a reduced afterhyperpolarization compared with WT POPDC1 (Figure 5D). The more hyperpolarized maximal diastolic membrane potential might lead to an increased availability of sodium channels in these cells (23, 24). This assumption is in agreement with the observation of a more hyperpolarized activation threshold (Figure 5E) and the increased upstroke velocity (Figure 5,F and G) in HL-1 cells transfected with POPDC1<sup>S201F</sup>. In summary, the overexpression experiments in HL-1 cells demonstrate that the mutant is more efficient than WT protein at raising potassium conductance in these cells.
cardiac myocyte-like cells, leading to increased hyperpolarized membrane potentials. These effects might explain the AV block observed in patients and in the POPDC1S191F zebrafish mutant (see below), as hyperpolarization of cells in the AV node will further delay the propagation of excitation to the ventricles.

Loss of popdc1 in zebrafish causes an impairment of heart and skeletal muscle development. Zebrafish popdc1 is expressed in both skeletal muscle and heart (Supplemental Figure 15, A–C). To assess popdc1 function in zebrafish, a morpholino-based knockdown approach targeting the splice junctions of exon 4 was utilized. Morpholino injection caused a significant reduction of popdc1 mRNA expression (Supplemental Figure 15D). Macroscopically, the morphants appeared normal overall, with the exception of the presence of a cardiac edema in a large fraction of the injected embryos (70%) (Supplemental Figure 15, E and F). Some morphants (20%) displayed a 2:1 AV block, a phenotype that has been previously reported for popdc2 morphants (Supplemental Figure 15G, Supplemental Videos 1 and 2, and ref. 15). Upon closer inspection, skeletal muscle was also affected in 80% of the morphants and was characterized by myofibrillar misalignment and fiber detachment (Supplemental Figure 15H). The myotendinous junction (MTJ) forms the attachment site for muscle fibers, and was found to be malformed in the morphants and possibly the cause for the fiber detachment (25). Forced expression of mutant and WT POPDC1 was achieved by mRNA injection into fertilized zebrafish eggs. WT POPDC1 did not affect cardiac morphology; however, forced expression of POPDC1S191F caused myocardial edema formation indicative of embryonic heart failure (Supplemental Figure 16, A and B).

In order to directly test the pathogenicity of the POPDC1S191F mutation, we generated by TALEN-mediated gene editing a genomic mutation of the corresponding serine residue in zebrafish popdc1 (i.e., popdc1S191F). The homozygous popdc1S191F mutant developed normally during embryogenesis, and no significant lethality in comparison with WT embryos was observed (data not shown). However, a significant fraction of homozygotes developed cardiac and skeletal muscle phenotypes (Supplemental Figure 17). Mutants displayed cardiac edema and aberrant formation of the trunk musculature, and, in the most severely affected embryos, an edema involving the entire larval body was observed. The mutant phenotype displayed a reduced penetrance of approximately only one-third of the embryos, which appeared to be affected at 5 days post fertilization (dpf). Thus, a majority of homozygotes developed into adulthood. One of the reasons for the reduction in phenotype penetrance was the presence of 3 family members that at least partially were able to functionally substitute for each other (9).

In order to further study the skeletal muscle phenotype, zebrafish larvae at 5 dpf were stained with phalloidin together with vinculin or DYS. The trunk skeletal muscle of some homozygous popdc1S191F mutants displayed myofibrillar misalignment, aberrant formation of the MTJ, and myofiber detachment, probably secondary to the impaired MTJ formation, with various degrees of severity (Figure 6, A–D). Myofiber detachment is a hallmark of muscular dystrophy phenotypes in zebrafish and was previously reported, for example, for the DYS mutant sapje (26). TEM of trunk muscle development in the homozygous popdc1S191F mutant demonstrated an almost complete absence of extracellular matrix (ECM) in the MTJ at 5 dpf (Figure 6, E and G). In addition to the MTJ phenotype, a severe reduction in myofibrillar content and disarray was also observed (Figure 6, F and H).

We assessed cardiac performance using optical recording, which revealed an overall reduction in heart rate and stroke volume (Figure 6, I and J). Between 5 dpf and 9 dpf, a steadily increasing number of homozygotes developed a 2:1 AV block (Figure 6K). In addition, more severe forms of AV block such as 3:1 and even longer pauses including electrically silent ventricles were also observed in homozygotes (Supplemental Figure 18 and Supplemental Video 3). The arrhythmia phenotype displayed a reduced penetrance, with approximately 5%-10% of the embryos being affected. Interestingly, Iso induced an approximately 3-fold increase in the number of homozygous mutant embryos developing cardiac arrhythmia, an effect that was not observed in WT embryos (Figure 6L). Since a majority of mutants developed into adulthood, we asked whether adult popdc1S191F mutants also displayed any pathology. To this end, we confined our analysis to skeletal muscle and studied membrane localization of Popdc1 and Popdc2. Adult homozygous mutants and WT animals were randomly picked, and Popdc1 and Popdc2 expression were assessed immunohistochemically in the trunk musculature. Similar to what we observed in patients’ biopsies, membrane localization of Popdc1 and Popdc2 was significantly diminished in the mutant muscle (Figure 7, A–D). In addition, many mutant fibers displayed an increase in number and signal intensity of immunostained intracellular vesicles, particularly in the case of Popdc1 (Figure 7A), suggesting that the serine 191 to phenylalanine mutation in zebrafish resulted in impaired membrane trafficking of the mutant Popdc1 protein and Popdc2. Therefore, the phenotypic spectrum of the homozygous popdc1S191F mutant was reminiscent of the phenotypes observed in our patients, fully supporting the notion that the POPDC1S191F mutation is pathogenic and causes heart and muscle disease.

Discussion

Our work demonstrates that mutations in POPDC1 are responsible for heart and muscle pathologies. Indeed, WES identified the disease-segregating homozygous missense mutation S201F in POPDC1 in a family with LGMD and AV block. Further screening of POPDC1 mutations in 104 patients with heart and/or myopathic phenotypes resulted in a negative outcome for the presence of homozygous or compound heterozygous mutations, as expected for a very rare, family-specific recessive allele.

Evidence for the pathogenic nature of the POPDC1S201F mutation was in particular obtained by the knockin mutation in zebrafish, which displayed several heart and muscle pathologies that were reminiscent of the symptoms found in the patients. Furthermore, the in vitro assays provided strong evidence for an essential role of cAMP binding to execute POPDC1’s biological functions. cAMP-affinity precipitation and FRET analysis revealed that the switch from serine to phenylalanine caused an approximately 50% reduction in cAMP binding. Further evidence for reduced cAMP sensitivity and the resulting electrophysiological consequences was obtained from experiments in Xenopus oocytes and HL-1 cells. The fact that Iso stimulation caused an increase in the number of zebrafish mutants displaying cardiac arrhythmia suggests that the cAMP-binding ability of POPDC1 has an important physiological function.
in the heart. The sarcolemmal localization of the mutant protein was reduced in the patients’ muscle biopsies and in the trunk musculature of the zebrafish mutant. These data suggest that cAMP binding of POPDC1 is somehow involved in modulating its own membrane transport. Moreover, membrane trafficking of TREK-1 was impaired in the presence of mutant POPDC1S201F protein. Similarly, POPDC2 displayed a trafficking defect in muscle tissue, suggesting that cAMP binding of POPDC1 not only modulates its own membrane trafficking, but also that of associated proteins. Mutations in POPDC1, which cause a lack or a reduction in cAMP affinity, may actually result in a gain of function, increasing hyperpolarization and upstroke velocity of the cardiac action potential. Both parameter changes could be interpreted as effects of POPDC1 on TREK-1 current. However, it is unlikely that TREK-1 alone is sufficient to fully explain the clinical phenotypes observed in the patients. Nonetheless, this protein-protein interaction served throughout our study as a surrogate to assess POPDC protein family function. Further work will be required to identify additional POPDC protein interaction partners; this may provide additional opportunities to better define the molecular basis for the observed cardiac arrhythmia phenotype.

Markedly, impaired membrane trafficking was reported in many forms of muscular dystrophy, especially those caused by mutations in CAV3, DYS, and DYSF (27). However, no discernible effects on the plasma membrane levels of these proteins were observed in our patients, despite the fact that each of them was coimmunoprecipitated with POPDC1. The effects of POPDC1 on CAV3, DYS, and DYSF may be subtle and not discernible by immunohistochemistry. We may speculate that these interactions might be more important in response to plasma membrane rupture or during muscle regeneration (13). Therefore, the physiological and pathophysiological roles for these protein-protein interactions have yet to be defined.

The Xenopus oocyte experiments demonstrated a dual impact of POPDC1 on TREK-1, with both processes being modulated by cAMP. We observed a cAMP-dependent increase in membrane localization of TREK-1 as well as a decrease in channel gating. We recently proposed 2 alternative models to describe how POPDC1 might modulate ion channel function: the switch and the cargo model (9). The cargo model proposes that POPDC1 might be involved in membrane trafficking. Our data give further support to this model, since cAMP binding to POPDC1 causes an increase in membrane localization. It is noteworthy that an interaction of POPDC1 with the vesicle-associated membrane protein VAMP3 has recently been reported (28). However, the significance of this interaction with regard to the regulation of membrane localization in skeletal muscle is presently unclear and deserves further study.

The switch model predicts that cAMP binding induces a conformational change in the POPDC1 protein, affecting the biological properties of interacting proteins. Ligand-induced conformational changes have been described for other cAMP effector proteins (29), and the POPDC1/TREK-1 FRET data suggest a similar structural remodeling in the case of POPDC1. However, the switch model is not supported by the fact that the TREK-1 current in oocytes is not acutely affected by cAMP, suggesting a more complex regulation. Furthermore, in the presence of S201F, membrane transport of TREK-1 is impaired; however, an increase in current can be measured. These paradoxical results could be explained by the fact that cAMP not only binds POPDC1, but also activates other effector proteins. For example, it is well established that protein kinase A interferes with TREK-1 gating through phosphorylation (30, 31). Our unpublished mapping data place the POPDC1-binding site in the vicinity of the PKA-dependent phosphorylation site of TREK-1. It is therefore possible that binding of POPDC1 to TREK-1 results in steric interference with PKA-dependent phosphorylation, thereby indirectly shielding TREK-1 from PKA-mediated inactivation. Experiments with a TREK-1 mutant, which lacks the PKA-dependent phosphorylation site, or pharmacological studies using PKA inhibitors will be informative in this context (32). Interestingly, POPDC1 is also subject to β-adrenergic–dependent phosphorylation (33, 34). Thus, the regulation of POPDC1 involving cAMP binding, subcellular localization, and phosphorylation of itself and of interacting proteins deserves further investigations.

POPDC1S201F causes a mild, late-onset LGMD. The pathogenesis of the muscle damage is supported by the muscular dystrophy phenotype that we observed in a significant fraction of homozygous popdc1S201F zebras. Detachment of muscle fibers from the MTJ was evident, as reported in many other zebrafish mutants with dystrophic phenotypes (25, 26, 35–37). The myofiber detachment is probably based on the failure of proper ECM formation, as demonstrated by TEM. It can be envisioned that the lack of proper matrix assembly provides insufficient mechanical linkage, leading to fiber detachment when the zebrafish larvae start to swim. Identical phenotypes were observed for popdc1 (this study and Supplemental Figure 14) and popdc2 morphants (15), suggesting that both genes are probably acting together in the same pathway. Presently, it is unclear how POPDC proteins are linked to MTJ formation and ECM accumulation and what role cAMP signaling might have in these processes; however, the fact that POPDC1 interacts with DYS (and DYSF and CAV-3) suggests that it might be involved in laminin-DAG linkage formation (37, 38). In skeletal muscle, laminins connect the muscle cell to the ECM by binding either dystroglycan or integrins at the cell membrane. Epistasis experiments will be required to find out whether POPDC1 is interacting with dystroglycan (possibly via DYS), integrins, or both. Alternatively, POPDC1 may be involved in matrix deposition, as POPDC1 has recently been implicated in autocrine matrix deposition (39).

In conclusion, we have discovered a disease gene, POPDC1, causing a very rare LGMD with recessive inheritance and mild disease progression in skeletal muscle. However, the heart is strongly affected by this mutation, producing a severe form of AV block. Aberrant membrane trafficking and ECM abnormalities probably form the molecular basis for the observed heart and skeletal muscle phenotypes present in this family. The functional interaction of POPDC1 with a variety of membrane and membrane-associated proteins opens up perspectives for studying the genetic basis of cardiac and skeletal muscle disease and other phenotypes that are related to the main or corollary functions of POPDC1.

Methods
WES. Exome sequencing was performed using genomic DNA of the 2 affected brothers (PTIII-1 and PTIII-2) and both parents using NimbleGen Sequence Capture technology (SeqCap EZ Human Exome Library v2.0) according to the manufacturer’s instructions. The enriched librar-
ies underwent 90 base pair, paired-end sequencing on a HiSeq2000 next-generation sequencing platform (Illumina) at an average of 80× coverage. SOAPAligner (soap2.21) was used to align clean reads to the human reference genome (UCSC hg19, build 37.1), and SNP calling used SOAPsnp (v.1.03), while BWA and the Genome Analysis Tool Kit (GATK) were used for the detection of indels. After filtering PCR duplicates, some public databases, including dbSNP135 (with frequency greater than 0.5%), 1000 Genome variants database, HapMap exomes, and an in-house database, which contains WES data from approximately 1,000 normal individuals, were used to filter out previously reported variants. As disease-causing variants have a very high probability of causing amino acid changes, we initially focused on non-synonymous variants and splice-site and indel mutations. For potential disease-causing variants, the consequences of the amino acid changes were predicted using Polyphen2 (40), SIFT (41), and Pmut (42). In order to confirm that the variation was not the product of sequencing errors, PCR amplification and Sanger sequencing were performed on the variant. Primer pairs to amplify the sequence surrounding the mutation were synthesized (POPDC1 exon 4–forward, AAATACCTTGCCCCTCAAGAAGTGC; POPDC1 exon 4–reverse, CCCCCAAATTCTCATAGCAGTGT), and the PCR was run in a final volume of 25 μL containing 100 ng genomic DNA, 20 μM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTPs, 5 μM Taq DNA Polymerase (Invitrogen), and 0.4 μL of each primer. The amplified PCR product was analyzed by direct sequencing on an automatic sequencer (ABI Prism 3130, Applied Biosystems). DNA library construction and target capture were performed following the manufacturer’s protocol before sequencing on the SOLiD 5500xl (Life Technologies) using a paired-end protocol (read length 75 bp + 35 bp). Reads were demultiplexed and aligned to a human reference genome (hg19). Duplicate reads and those with low mapping quality score (<8) were removed. Variant calling was carried out using diBayes (SNPs) and small indel modules as well as GATK v1.5-20 and Samtools v0.1.18. Alignment and coverage was carried out using diBayes (SNPs) and small indel modules as well as GATK v1.5-20 and Samtools v0.1.18. Alignment and coverage metrics were calculated using Picard v1.40, BedTools v2.12, and in-house Perl scripts. GATK CallableLoci Walker was used to identify target genomic regions covered sufficiently for variant calling (minimum depth > 4 with base quality > 20 and mapping quality > 10).

POPDC1 mutation screening in patients. We screened an additional 104 unrelated patients with different clinical features, including cardiac arrhythmia, AV block, sudden death, and LGMD (see Supplemental Table 1), for mutations in POPDC1. Genomic DNA from patients was extracted from peripheral lymphocytes by standard methods. PCR primers (Supplemental Table 2) were designed to amplify all 7 exons of POPDC1 as well as their flanking intronic regions. PCs were run and analyzed under the conditions described above.

Immunohistochemistry. The 10-μm frozen sections of skeletal muscle biopsies of patients PT1-1 and PTII-2 and 2 control biopsies or muscles dissected out from adult WT and popdc1−/− zebrafish were mounted on Superfrost Plus glass slides (Thermo Fisher Scientific) and subjected to immunohistochemistry using the following primary antibodies: POPDC1 (HPA018176, Sigma-Aldrich), POPDC2 (HPA024255, Sigma-Aldrich), DYS (D8043, Sigma-Aldrich), CAV3 (610420, BD Transduction Laboratories), DYSF (NCL-Hamlet, Leica), α-sarcoglycan (NCL-α-SARC, Leica), and dystroglycan (MANDAG2/7D11, DSHB). For the detection of primary antibodies, the following secondary antibodies were employed: Alexa Fluor 488–conjugated donkey anti-rabbit (A-21206, Invitrogen) and Alexa Fluor 555–conjugated donkey anti-mouse (A31570, Invitrogen). For counterstaining, DAPI (Calbiochem) was employed. The fluorescent signal was analyzed by confocal microscopy. Membrane signal intensities of approximately 30 muscle fibers were measured using Icy software and corrected for background. To calculate relative signal intensity levels, individual measurements from mutant fibers and control fibers were taken as a percentage of mean of control samples. Error bars are to 1 SD.

Immunocytochemistry. Cos-7 cells (87021302, Sigma-Aldrich) were transfected with constructs encoding HA-tagged POPDC1WT or POPDC1S201F alone or together with a TREK-1–Flag plasmid or myc-tagged mouse POPDC1 together with HA-tagged DYSF and stained 24 hours later with anti-HA (MMS-101R, Covance), anti-Flag (F1804, Sigma-Aldrich), or anti-myc (clone 9c10, ab32 Abcam) antibodies. As secondary antibodies, FITC-conjugated anti-mouse Fab fragments (715-097-003, Jackson ImmunoResearch Laboratories) and Alexa Fluor 555–conjugated donkey anti-mouse (A31570, Invitrogen) were used. HL-1 cells (provided by Emmanuel Dupont, Imperial College London) were stained using anti-POPDC1 (HPA018176, Sigma-Aldrich) and anti-POPDC2 (HPA024255, Sigma-Aldrich) as primary and Alexa Fluor 594–conjugated donkey anti-rabbit (A-21207, Invitrogen) as secondary antibodies. Nuclei were stained with DAPI (Calbiochem). Images were taken using a Zeiss LSM 510 confocal microscope.

Live cell imaging (FRET analysis). 293A cells (R705-07, Thermo Fisher Scientific) were transfected with POPDC1WT-CFP or POPDC1S201F-CFP together with YFP–TREK-1 plasmids at a 5:1 ratio. Forty-eight hours later, live cell imaging was performed exactly as described previously (10).

Mutagenesis. The S201F point mutation was introduced into POPDC1 cDNA by PCR using the following primers: POPDC1-S201F s, 5′-AACAGTATTACCCTGGCTTATTTAGATGTTCTGAGATTTTAGATACCAC-3′; POPDC1-S201F as, 5′-GGTGATCTAAATTCGAGGATAGTTCTGAGATTTTAGATACCAC-3′; POPDC1-S201F & controls. Error bars are to 1 SD. Mutant fibers and control fibers were taken as a percentage of mean of control samples. Error bars are to 1 SD. Mutant fibers and control fibers were taken as a percentage of mean of control samples. Error bars are to 1 SD.

Immunocytochemistry. Cos-7 cells (87021302, Sigma-Aldrich) were transfected with constructs encoding HA-tagged POPDC1WT or POPDC1S201F constructs with HA epitope tags attached to their C termini using Lipofectamine 2000 reagent (Invitrogen), and cells were harvested 24 hours later using a buffering containing 50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, pH 8.0, 1% (w/v) Triton X-100, 0.25% (w/v) gelatin, and protease inhibitors (Roche). Cell lysates were briefly sonicated and cleared twice by centrifugation at approximately 18,000 g at 4°C for 30 minutes. Then 30 μL of the lysates were taken as loading controls. Lysates were first incubated with 50 μL ethanolamine (EtOH-NH) agarose (BioLog) for 1 hour with rotation at 4°C. After centrifugation at 500 g at 4°C for 5 minutes, the supernatant was supplemented with 1 mM IBMX (3-isobutyl-1-methylxanthine; Sigma-Aldrich) and...
added to 4.8 mg cAMP agarose (A0144, Sigma-Aldrich). After incubation for 2 hours with rotation at 4°C, both EtOH-NH and cAMP agaroses were washed with wash buffer A (10 mM HEPES, pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 500 mM NaCl, 0.1% [v/v], Igepal CA-630, 1 mM DTT, 1 mM IBMX) followed by 3 washes with wash buffer B (10 mM HEPES, pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 0.1% [v/v], Igepal CA-630, 1 mM DTT). Agarose-bound proteins were eluted in SDS sample buffer by boiling at 96°C for 5 minutes and subjected to gel electrophoresis and Western blot.

**Gel electrophoresis and Western blot.** Proteins were size separated and transferred onto nitrocellulose membrane (BioTrace NT, Pall). The membrane was washed with TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% [v/v] Tween-20) and blocked in 5% (w/v) low-fat milk in TBST for 1 hour at room temperature and subsequently incubated with anti-HA (MMS-101R, Covance), anti-FLAG (F1804, Sigma-Aldrich), anti-actin (sc-1616, Santa Cruz Biotechnology Inc.), anti-pOPDC1 (sc-49889, Santa Cruz Biotechnology Inc.), or anti-pOPDC2 (HPA024255, Sigma-Aldrich) overnight at 4°C. For peptide competition assays, membranes were incubated with popDC1 or popDC2 antibodies together with a 10-fold excess of the respective neutralizing peptide (sc-49889 P [popDC1], Santa Cruz Biotechnology Inc., APtEST72859 [POPD2], Atlas Antibodies). After several washes, the blots were incubated for 1 hour at room temperature with horseradish peroxidase–coupled anti-mouse (PI-2000, Vector Laboratories), anti-goat (PI-9500, Vector Laboratories), or anti-rabbit antibodies (PI-1000, Vector Laboratories). After washing, signals were detected using an enhanced chemiluminescence protein detection method (Millipore). Exposed films were digitalized and band intensities were determined using ImageJ (43). Ratios between input and cAMP agarose-bound protein fractions were calculated and values were statistically analyzed using GraphPad Prism.

**Coimmunoprecipitation analysis.** For coimmunoprecipitation (Co-IP) analysis, Cos-7 cells (87021302, Sigma-Aldrich) were transiently transfected with pCMV-Tag and the respective plasmid using FuGENE 6 (Promega) according to the manufacturer’s recommendations. Whole-mount immunostaining was performed as described previously (45). The following primary antibodies were used: monoclonal anti-βcatenin (BD, 610155), monoclonal anti-vinculin (FAK100, Millipore), Alexa Fluor 350–coupled donkey anti-mouse (A21205, Invitrogen) was used as secondary antibody. For F-actin staining, zebrafish embryos were incubated with rhodamine-phalloidin (R415, Invitrogen). Stained embryos were washed in PBS for 30 minutes and imaged by confocal microscopy (Zeiss, LSM 510).
10^{-3} M Iso (experimental group) or water (control) for 5 minutes. The percentage of animals displaying cardiac arrhythmia was determined. The imaging system consisted of a custom-made selective plane illumination microscope (SPIM) equipped with an Olympus UPLFLN 20 x W objective and a digital high-speed video camera, Hamamatsu Orca Flash 4.0, in transmitted light mode. Images were recorded at 28°C (2,000 images, 100 fps) and imported into Fiji (47). Luminance periodograms of pixels being passed by the ventricle or atrium were filtered by an equi-ripple low-pass filter with a pass frequency of 0.1 Hz and a stop frequency of 10 Hz (Lab View 2013, National Instruments). In these filtered periodograms, peaks were detected and peak-to-peak distances (reflecting beat-to-beat distances) were converted to a beat-to-beat frequency for each interval. These frequencies were plotted against time to a cardiactochiagram to calculate mean heart rate. Stroke volume was determined as described (48).

**TEM.** Five-dpf WT or popdc1 S191F /S191F zebrafish larvae were sacrificed using MS-222 and fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C overnight and then postfixed in 1% (w/v) osmium tetroxide in 0.1 M sodium cacodylate buffer at room temperature for 1 hour. After 2 buffer washes, samples were dehydrated using a graded ethanol series of 50%, 70%, 90%, and 100% (v/v). Larvae were then immersed in propylene oxide and infiltrated with 1:1 propylene oxide/araldite resin (Agar Scientific) overnight and then in pure resin for 2 hours. After several changes of resin, samples were embedded and the resin was polymerized at 60°C for 24 hours. After polymerization, 100-nm-thick sections were cut with an ultramicrotome and mounted on 300 mesh copper TEM grids. Thereafter, samples were poststained using uranyl acetate and TEM was performed with a JEOL 1200 EX electron microscope.

**Measurement of TREK-1 current.** *Xenopus laevis* was maintained and oocytes isolated under standard conditions according to established protocols. Capped cRNA transcripts were synthesized in vitro using the mMessage mMachine T7 transcription kit (Ambion). The cRNAs were purified and photometrically quantified. Human TREK-1c alone or together with human POPDC1 (WT or S201F mutant) was injected into *Xenopus laevis* oocytes at the indicated amounts of cRNA. Oocytes were incubated at 19°C for 24 to 48 hours in ND96 solution containing 96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 5 mM HEPES (pH 7.4–7.5) supplemented with 50 mg/l gentamicin and 10 mM glucose, 2 mM sodium pyruvate, and 5 mM HEPES (pH 7.4 with NaOH) as previously described (49, 50). Patch-clamp experiments were performed in the whole-cell configuration using pipettes pulled from borosilicate glass capillaries. The pipettes had a tip resistance of 3.0 to 4.0 MΩ when filled with a solution containing 60 mM KCl, 65 K⁺-glutamate, 5 mM EGTA, 2 mM MgCl₂, 3 mM K₂ATP, 0.2 mM NaGTP, and 5 mM HEPES (pH 7.2 with KOH). Data acquisition and command potentials were controlled with a commercial software program (Patchmaster, HEKA) with a sweep time interval of 1 second and a sample rate of 200 kHz. Data analysis of action potentials was done using Fitmaster software (HEKA). For each cell measured, the action potential parameters were averaged by analyzing 10 subsequent action potentials, as previously described (49).

**RT-PCR.** Total RNA was isolated from HL-1 cells using High Pure RNA Isolation Kit (Roche) containing DNaseI. Reverse transcription (RT) was performed with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. PCR was performed using Taq Gold Polymerase (Applied Biosystems) according to the manufacturer’s instructions. Reaction mixtures were preheated at 96°C for 5 minutes, followed by 40 cycles at 96°C for 30 seconds, 58°C for 30 seconds, and 72°C for 45 seconds. PCR products were visualized by gel electrophoresis on 2% agarose gels. The following intron-spanning primers were used: GAPDH forward, 5′-ACTTCAGACCGAATCCCCTCATC-3′; GAPDH reverse, 5′-GCTTGACCGGTATTCATTGTCATA-3′; TREK-1 forward, 5′-AGCAGGACTTACGCAAGGACCT-3′; TREK-1 reverse, 5′-AAAGGAAAGAGCCTCCAGGAGT-3′. For both primer pairs, the amplification products were confirmed by sequencing. RNA that was not reverse transcribed was used as negative control.

**Statistics.** Results are presented as mean ± SEM. Statistical significance was defined as P < 0.05.

**Study approval.** Informed consent for WES analysis and clinical exome analysis for the family was obtained. Approval of the Ethical Committee University Hospital Ferrara was given to the project as follows: Neuromics, approved May 30, 2013, approval number 05/2013, P. 58-2013; RARER, approved June 20, 2013, approval number 06/2013, P. 95-2013 and routine molecular diagnosis approval number 26/7/2012, P. 7/2012. The zebrafish work at Imperial College London was approved by the Animal Welfare and Ethical Review Board of the Harefield Heart Science Centre and licensed by the United Kingdom Home Office (PPL 70/7171). The zebrafish work at the University of Innsbruck was approved by the Austrian Bundesministerium für Bildung, Wissenschaft, und Kultur (animal ethics permission GZ 66.008/0004-I/3b/2012). The work with *Xenopus* at the University of Marburg was approved by the Regierungspräsidium Gießen, Germany (V54-19c 2015 hours 02 MR 20/28 Nr.A 4/2013).

**Author contributions**

RFRS, BOB, SS, TS, KLP, SR, A Froese, VON, CG, TM, GT, and PS designed and conducted functional experiments. FD, FF, BD, and EB clinically characterized the family. AP clinically supervised the project on the families of 4 (RARER). CR and EA clinically characterized the patients with cardiac abnormalities screened for mutations. CS, CP, FRDR, RS, FG, MN, WL, HJ, and XX performed the DNA studies, patient screening, and technical validation. JZ and MF performed the WES and bioinformatics analysis. JW designed and analyzed the electrophysiological.
studies and helped write the manuscript. TB and A Ferlini designed research studies, analyzed data, and wrote the manuscript.

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