POPDC1^{S201F} causes muscular dystrophy and arrhythmia by affecting protein trafficking

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Abstract

The Popeye domain containing 1 (*POPDC1*) gene encodes a plasma membrane-localized cAMP-binding protein, which is abundantly expressed in striated muscle. Despite its essential role in regulating structure and function of cardiac and skeletal muscle in animal models, this gene has never been associated with human cardiac and muscular diseases. We describe a novel homozygous missense variant (c.602C>T, p.S201F) in *POPDC1*, identified by whole exome sequencing, in a family-of-four with cardiac arrhythmia and limb-girdle muscular dystrophy (LGMD). This allele was absent in databases and segregated with the phenotype. Further screening in 104 patients with similar phenotype gave a negative result, suggesting this mutation to be family-specific. The POPDC1^{S201F} protein displayed a 50% reduction in cAMP affinity and in skeletal muscle of patients, the mutant POPDC1^{S201F} protein as well as the unaffected POPDC2 displayed impaired membrane trafficking. Forced expression of POPDC1^{S201F} in HL-1 cells increased hyperpolarization and upstroke velocity of the action potential. The homologous mutation (*popdc1*^{S191F}) introduced into zebrafish caused heart and skeletal muscle phenotypes, which resembled those observed in patients. Our study therefore identifies *POPDC1* as a novel disease gene causing 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 co-morbidities and may even precede the onset of the muscle symptoms (4-6). The limb-girdle type muscular dystrophies (LGMD) 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)), or 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 are highly expressed in cardiac and skeletal muscle in an overlapping manner (8, 9). Popdc proteins possess the evolutionary conserved Popeye domain, which functions as a high-affinity 3'-5'-cyclic adenosine monophosphate (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 two-pore-domain potassium channel TREK-1 has been reported (10, 12). In the presence of Popdc proteins, TREK-1 currents are potentiated due to enhanced membrane trafficking (10). The *Popdc1* null mutant in mice 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 novel homozygous recessive mutation in *POPDC1* (c.602C>T, p.S201F) by Whole Exome Sequencing (WES) in a family with LGMD and atrioventricular (AV) block. We demonstrate that POPDC1^{S201F} has strong pathogenic consequences since it affects cAMP binding and subcellular localization of the mutant protein and its interaction partners. Therefore *POPDC1* is a novel 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 town of about 3000 inhabitants located in Calabria (Italy) showed an interesting pseudo-dominant inheritance, compatible with consanguinity and geographic isolation (Figure 1A). The grandfather, currently 81 years old (patient I-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 60. At that time, blood creatine kinase (CK) was elevated (range 750-1300 IU/L). At 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). Electron microscopy analysis revealed the presence of plasma membrane discontinuities and submembraneous vacuoles (Figure 1B-D). Expression of dystrophin, all sarcoglycans, emerin, merosin and caveolin-3 was normal (data not shown). At the end of his 6th decade this patient started to complain of repeated syncope episodes and a second-degree atrioventricular (AV) block was disclosed. A pacemaker was placed one year later at the age of 70. Echocardiography excluded a cardiomyopathy. Muscle testing at age of 72 showed a clear symmetrical weakness of the limbs (neck flexors MRC (Medical Research Council Scale for Muscle Strength) 5; ileopsoas MRC 3.5; quadriceps MRC 2; glutei MRC 1; gastrocnemius MRC 5; foot dorsal flexors MRC 5). The grandfather has two healthy sons and three healthy daughters. One of the daughters has two children showing the same phenotype as the grandfather, although apparently both parents denied consanguinity. The eldest son (patient III-1), currently 25, started to manifest syncopal episodes during exercise at the age of 17, and his CK level was elevated to 1216 IU/L. The electrocardiogram at that age showed a second-degree AV infra-Hisian block with no aberrant intraventricular conduction, and negative response to intravenous flecainide. The Holter monitoring revealed episodes of paroxysmal second degree AV block (Mobitz 2) (Figure 1E, Supplemental Figure 2A-C). Echocardiography was normal. An endocardial double chamber pacemaker was implanted.

The muscle computed tomography (CT) scan was normal (Supplemental Figure 3A). Muscle biopsy was proposed and refused by the patient. The younger brother (patient III-2) started to complain of syncope episode with facial trauma at the age of 12 years. At that age, the CK level was elevated to 7643 IU/L after a prolonged exercise and to 3182 IU/L at rest. During hospitalization, the ECG was normal and the Holter monitoring showed occasional episodes of second degree AV block (Mobitz 1) (Supplemental Figure 2D-F). The Head-up Tilt Test showed sinus bradycardia and escape junctional rhythm in the absence of symptoms. Echocardiography was normal excluding a cardiomyopathy. The endocavitary electrophysiological study documented a normal His bundle – ventricle (HV) interval (30 msec) and an advanced infranodal AV block during atrial pacing at cycle length < 600msec. For these reasons, a ventricular single chamber pacemaker (VVI) endocardial pacemaker was implanted, which after 8 years was upgraded to a double chamber pacemaker. The muscle biopsy was normal (data not shown) and a CT scan at the age of 19 years did not show a skeletal muscle involvement despite of a very high CK level (Supplemental Figure 3B). Currently the patient is periodically checked and did not have further episodes of syncope or other symptoms.

Using WES, we identified in this family a novel homozygous missense mutation (c.602C>T, p.S201F) in *POPDC1.* The S201F variant has not been reported in any of the known databases such as dbSNP (16), 1000 Genomes (17), NHLBI Exome Sequencing Project (18) and ExAC Database (19). This variation was shared in homozygosis in patients III-1 and III-2 (Figure 1F). Sanger sequencing validated the c.602C>T SNP and confirmed that it was present in homozygosis in the two sons and their grandfather (I-1), and in heterozygosis in both non-affected parents (II-5 and II-6) as expected for a pseudodominant inheritance due to inbreeding in the family. Further screening of 104 patients with a similar clinical phenotype gave a negative result, suggesting this mutation to be very rare and family-specific (Supplemental Table 1). The S201F mutation is located within the Popeye domain, which is the most conserved part of POPDC1 and functions as a cAMP-binding domain (10). S201 is a residue of the ultra-conserved DSPE motif, which is thought to be part of the phosphate-binding cassette (Figure 1G) (9, 10, 14). In a 3D-model of the Popeye domain (10), S201 is predicted to be localized to

the nucleotide binding region facing the cyclic AMP molecule and its hydroxyl group might possibly engage via hydrogen bonding either with the 2'-hydroxyl group of the ribose ring or the side chain amine group of K255, which is located in the lid helix αC and potentially controls the allosteric transfer of cAMP-binding (Figure 1H). In the S201F protein model the aromatic side chain of the introduced phenylalanine protrudes into the cAMP-binding pocket thereby likely impairing cyclic nucleotide-binding and exchange.

The S201F mutation affects membrane trafficking of both POPDC1 and POPDC2.

In healthy skeletal muscle fibers, POPDC1 and POPDC2 were prominently expressed in the sarcolemma (Figures 2A,B,E and 3A,B,E). In contrast, in skeletal muscle biopsies of patients I-1 and III-2, a significant reduction in membrane localization of both, POPDC1 and POPDC2 was observed (Figures 2C-E and 3C-E). While plasma membrane labeling was diminished, an increased perinuclear localization of POPDC1 and POPDC2 was observed in the patients' biopsies (Figures 2F-Q and 3C,D). In addition, pronounced expression in cells adjacent to the muscle fiber, which may represent activated satellite cells or myoblasts, was observed and particularly prominent in case of POPDC2 in both patients (Fig. 3C,D). The total level of POPDC1 and POPDC2 in muscle biopsies and in dermal fibroblast was determined by Western blot analysis (Figures 2R,K and 3S; Supplemental Figure 4). The expression level varied slightly between samples, however total protein levels did not correlate with the significant reduction of the plasma membrane levels of POPDC1 and POPDC2 in the patients' 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 patients III-1 and III-2 (Supplemental Figure 1A-C and 3). However, in the grandfather (I-1) fiber size variability, central nuclei, and plasma membrane discontinuities in muscle fibers were observed (Fig. 1E, Supplemental Figure 1D). Transmission electron microscopy of patient III-2 failed to detect membrane discontinuities nor 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 patients I-1 and III-2 in comparison with two 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 biopsies in comparison to controls (Supplemental Figure 5). We also studied dystrophin (DYS), which was co-immunoprecipitated with POPDC1 (Supplemental Figures 6). However, we could not detect any difference in membrane localization between patient and control samples (Supplemental Figures 7). Membrane discontinuities were observed in the muscle biopsy of the grandfather (I-1), a feature, which is typical of dysferlinopathies and related muscle disorders (20, 21). We established association of DYSF with POPDC1 by co-localisation and co-immunoprecipitation (Supplemental Figure 8). However, plasma membrane levels of DYSF were not affected in patients with a homozygous POPDC1^{S201F} mutation (Supplemental Figures 9). These data indicate that POPDC1 does not affect sarcolemmal localization of DYS, 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 POPDC1^{S201F} mutant protein displays a reduction of its affinity for cAMP

The S201 residue is part of the ultra-conserved 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 utilised for the mouse protein (10). We found that the S201F mutant protein has approximately a 50% reduction in affinity for cAMP (Figure 4A). To corroborate this finding we employed a bi-molecular 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 co-transfection of either CFP-tagged wildtype POPDC1 or POPDC1^{S201F} together with YFP-TREK-1. Upon stimulation with isoproterenol (Iso) or forskolin a small but robust decrease of the FRET ratio was observed for wildtype POPDC1 but changes in the case of POPDC1^{S201F}

were much weaker (~50%), confirming in an independent assay the decreased cAMP sensitivity of the POPDC1^{S201F} mutant (Figure 4B; Supplemental Figure 10). No decrease of the FRET ratio was observed when the co-transfected cells were pre-incubated 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 wildtype POPDC1 is based on different cAMP affinities and not due to an impaired interaction between POPDC1^{S201F} and TREK-1, we narrowed down the TREK-1 interaction domain using the mouse protein by deletion and co-immunoprecipitation 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 wildtype POPDC1 co-localize with TREK-1 after co-transfection (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 co-expression 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 co-expression 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 co-expressed with wildtype 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' muscle biopsies, membrane trafficking of TREK-1 in *Xenopus* oocytes was affected in the presence of the S201F mutant. Co-expression of TREK-1 with POPDC1 led to increased current amplitudes for both, wildtype and mutant S201F (Figure 4D,E). Surprisingly, co-expression of the S201F mutant with TREK-1 caused an even more pronounced enhancement of TREK-1 outward currents than wildtype 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 wildtype POPDC1 on TREK-1 currents were basically abolished (Figure 4F). Thus, irrespective of the surface expression, increased cAMP levels neutralized the impact of POPDC1 on TREK-1 conductivity (Figure 4C). Interestingly, perfusion of the oocytes expressing TREK-1 in complex with POPDC1 with 8-Br-cAMP (1 mM) during two-electrode voltage-clamp recordings did not affect TREK-1/POPDC1 current amplitudes (Figure 4G) indicating that the altered conductivity of the TREK-1/POPDC1 complex is not mediated by a pure gating effect (cAMP switch) (9). Most importantly, and consistent with a reduced cAMP sensitivity of the mutant protein, POPDC1^{S201F} retained the ability to enhance TREK-1 currents even in the presence of 8-Br-cAMP (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 CFP-tagged wildtype POPDC1 or POPDC1^{S201F} in HL-1 cells and recorded action potential properties of spontaneously beating cells using patch clamp experiments. Forced expression of wildtype POPDC1 into HL-1 cells resulted in a pronounced afterhyperpolarization (Figure 5A,D) and shortening of the action potential duration (Figure 5A,B), compared to non-transfected 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^{S201F} and TREK-1 cDNA caused a hyperpolarization of the maximal diastolic membrane potential (Figure 5A,D). This effect is in agreement with a more pronounced activation of TREK-1 channels by POPDC1^{S201F} (Figure 4D,E). As cells with POPDC1^{S201F} 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 to wildtype 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 5F,G), in HL-1 cells transfected with POPDC1^{S201F}. In summary, the overexpression experiments in HL-1 cells demonstrate that the mutant can more efficiently than wildtype protein raising potassium conductance in these cardiac myocyte-like cells, leading to more hyperpolarized membrane potentials. These effects might explain the AV-block observed in patients and in the POPDC1^{S191F} 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 15A-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 overall normal with the exception of the presence of a cardiac edema in a large fraction of the injected embryos (70%) (Supplemental Figure 15E,F). Some morphants (20%) displayed a 2:1 AV-block, a phenotype, which has been previously reported for *popdc2* morphants (Supplemental Figure 15G and Supplemental Movies 1 and 2) (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 wildtype POPDC1 was achieved by mRNA injection into fertilized zebrafish eggs. Wildtype POPDC1 did not affect cardiac morphology, however forced expression of POPDC1^{S201F} caused myocardial edema formation indicative of embryonic heart failure (Supplemental Figure 16A,B).

In order to directly test the pathogenicity of the POPDC1^{S201F} mutation, we generated by TALEN-mediated gene editing a genomic mutation of the corresponding serine residue in zebrafish *popdc1* (i.e. *popdc1*^{S191F}). The homozygous *popdc1*^{S191} mutant developed normally during embryogenesis and no significant lethality in comparison to wildtype 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, 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 with approximately only one third of the embryos, which appear 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 phenotpe penetrance is the presenc of three family members, which at least partially are able to functionally substitute for each other (9).

In order to further studying the skeletal muscle phenotype, zebrafish larvae at 5dpf were stained with phalloidin together with vinculin or dystrophin. The trunk skeletal muscle of some homozygous *popdc1*^{s191f} 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 6A-D). Myofiber detachment is a hallmark of muscular dystrophy phenotypes in zebrafish and was previously reported for example for the dystrophin mutant *sapje* (26). Electron microscopy of trunk muscle development in the homozygous *popdc1*^{s191f} mutant demonstrated an almost complete absence of extracellular matrix in the MTJ at 5dpf (Figure 6E,G). In addition to the MTJ phenotype, also a severe reduction in myofibrillar content and disarray was observed (Fig. 6F,H).

We assessed cardiac performance using optical recording, which revealed an overall reduction in heart rate and stroke volume (Figure 6I,J). Between 5dpf and 9dpf a steadily increasing number of homozygotes developed a 2:1 AV-block (Figure 6K). In addition, also more severe forms of AV block such as 3:1 and even longer pauses including electrically silent ventricles were observed in homozygotes (Supplemental Figure 18, 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 which was not observed in wildtype (Figure 6L). Since a majority of mutants developed into adulthood, we asked whether adult *popdc1*^{s191f} 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 wildtype animals were randomly picked and Popdc1 and Popdc2 expression was 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 7A-D). In addition, many mutant fibers displayed an increase in number and signal intensity of immunostained intracellular vesicles particularly in 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. Taken together, the phenotypic spectrum of the homozygous *popdc1*^{S191F} mutant was reminiscent of the phenotypes observed in our patients fully supporting the notion that the *POPDC1*^{S201F} mutation is pathogenic and causes heart and muscle disease.

Discussion

Our work demonstrates that *POPDC1*, which has never before been associated with human inherited diseases, is a novel disease gene of which mutations 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 *POPDC1*^{S201F} mutation was in particular obtained by the knock-in 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 a 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 POPDC1^{S201F} 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 causes 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 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 interaction partners, which may provide additional opportunities to better define the molecular basis for the observed cardiac arrhythmia phenotype.

Significantly, impaired membrane trafficking was reported in many forms of muscular dystrophy, especially those caused by mutations in CAV3, dystrophin and dysferlins (27). However, no discernable effects on the plasma membrane levels of these proteins were observed in our patients, despite the fact that each of them was co-immunoprecipitated with POPDC1. The effects of POPDC1 on CAV3, dystrophin and dysferlins may be subtle and not discernable 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 two 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 localisation. 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 studies.

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 suggests a similar structural remodeling in 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 Popdc 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 places the POPDC1 binding site in the vicinity of the PKA-dependent phosphorylation site of TREK-1. It is therefore possible that POPDC1, when bound to TREK-1, sterically interferes with PKA-dependent phosphorylation and thereby indirectly prevent the inactivation TREK-1 of ("shielding" effect). 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.

POPDC1^{S201F} causes a mild, late onset LDMG. The pathogenesis of the muscle damage is supported by the muscular dystrophy phenotype that we observed in a significant fraction of homozygous *popdc1*^{S191F} zebrafish mutants. 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 extracellular matrix formation as demonstrated by transmission electron microscopy. It can be envisioned that the lack of proper matrix assembly is providing insufficient mechanical linkage leading to fiber detachment when the zebrafish larvae starts to swim. Identical phenotypes were observed for

popdc1 (this study, 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 extracellular matrix accumulation and what role cAMP signaling might have in these processes, however the fact that POPDC1 interacts with dystrophin (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 extracellular matrix (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 dystrophin), 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 novel 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 extracellular matrix 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 novel perspectives to study the genetic basis of cardiac and skeletal muscle disease, and other phenotypes, which are related to the main or corollary functions of *POPDC1*.

Methods

Whole exome sequencing (WES)

Exome sequencing was performed using genomic DNA of the two affected brothers (PTIII-1 and -2) and both parents using NimbleGen Sequence Capture technology (SeqCap EZ Human Exome Library v2.0) according to the manufacturer's instructions. The enriched libraries underwent 90 base pair, paired-end sequencing on a HiSeq2000 next-generation sequencing platform (Illumina, San Diego, CA) at an average of 80X coverage. SOAPaligner (soap2.21) was used to align clean reads to the human reference genome (UCSC hg19, build 37.1) and SNP calling used the SOAPsnp (v.1.03), while BWA and GATK (Genome-Analysis-Tool-Kit) 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 approximately 1000 WES data of normal individuals, were used to filter out previously reported variants. As disease-causing variants have a very high probability to cause amino acid changes, we initially focused on nonsynonymous variants, 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 Exon4-fwd: AAATACTTGTGCCTCAAGAAGTGC, POPDC1 Exon4-rev: CCCCCAATAATTCAT AGCAGTG and the PCR was run in a final volume of 25µl, containing 100ng genomic DNA, 20mM Tris–HCl (pH 8.4), 50mM KCl, 1.5mM MgCl2, 0.2mM dNTPs, 5U Taq DNA Polymerase (Invitrogen) and 0.4µl of each primer. The amplified PCR product was analyzed by direct sequencing on an automatic sequencer (ABIPRISM 3130, Applied Biosystems). DNA libraries 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 75bp + 35bp). Reads were demultiplexed and aligned to 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 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: cardiac arrhythmia, AV block, sudden death, LGMD (see Supplemental Table 1) for mutations in *POPDC1*. Genomic DNA from patients was extracted from peripheral lymphocytes after informed consent by standard methods. PCR primers (Supplemental Table 2) were designed to amplify all seven exons of *POPDC1* as well as their flanking intronic regions. PCRs were run and analyzed using the conditions described above.

Immunohistochemistry

10µm frozen sections of skeletal muscle biopsies of patients PT I-1 and PT III-2 and two control biopsies, or muscles dissected out from adult wildtype and *popdc1*$^{S191F/S191F}$ zebrafish were mounted on Superfrost Plus glass slides (Thermo Scientific) and subjected to immunohistochemistry using the following primary antibodies: POPDC1 (HPA018176, Sigma-Aldrich), POPDC2 (HPA024255, Sigma-Aldrich), dystrophin (D8043, Sigma-Aldrich), CAV3 (610420, BD Transduction Laboratories), dysferlin (NCL-Hamlet, Leica). alpha-sarcoglycan (NCL-a-SARC, Leica), 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) or 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 ~30 muscle fibers was measured using the 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 standard deviation.

Immunocytochemistry

Cos-7 cells (87021302, Sigma-Aldrich) were transfected with constructs encoding HA-tagged POPDC1^{WT} or POPDC1^{S201F} alone or together with a TREK-1-Flag plasmid or myc-tagged mouse Popdc1 together with HA-tagged Dysferlin and stained 24 hrs later with anti-HA (MMS-101R, Covance), anti-Flag (F1804, Sigma-Aldrich), or anti-myc (clone 9c,10, ab32 Abcam) antibodies. As secondary antibodies FITC-conjugated anti-mouse Fab fragments (715-097-003, Jackson ImmunoResearch Laboratories) and AlexaFluor 555-conjugated donkey anti-mouse (A31570, Invitrogen) were used. HL-1 cells (kindly 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 4′,6-diamidin-2-phenylindol (DAPI, Calbiochem). Images were taken using a Zeiss LSM 510 confocal microscope.

Live cell imaging (FRET analysis)

293A cells (R705-07, Thermo-Fischer) were transfected with POPDC1^{WT}-CFP or POPDC1^{S201F}-CFP together with YFP-TREK-1 plasmids at 5:1 ratio. 48 h later, live cell imaging was performed exactly as described previously (10).

Mutagenesis

The S201F point mutation was introduced into POPDC1 cDNA by PCR using following primers (mutant nucleotides are underlined): POPDC1-S201F$_s$: 5'-AACATTTACCCCTGTGCCTTTATAGATTTTCCTGAATTTAGATCAAC-3', POPDC1-S201F$_{as}$: 5'-GTTGATCTAAATTCAGGAAAATCTATAAAGGCACAGGGGTAAATGTT-3'. Correct mutagenesis was confirmed by sequence analysis. For injection into *Xenopus* oocytes and zebrafish eggs, cDNAs were subcloned into pSGEM. For expression in mammalian cell lines, cDNAs were subcloned into the pECFP-N1 vector (Clontech). PCR was used to generate *POPDC1* constructs that encode proteins fused at their carboxy termini to a HA epitope tag.

Tissue lysates

For determining expression levels, proteins were extracted from skeletal muscle using 50 mM Tris pH 8.0, 4 M urea, 10% SDS, 2 mM EDTA pH 8.0. Protein concentrations of lysates were estimated with the help of the DC Protein Assay (Bio-Rad) and equal amounts of proteins were subjected to gel electrophoresis and Western blot analysis. To reduce protein aggregation, lysates were incubated at 37°C for 30 min, or 60°C for 15 min in SDS loading buffer.

cAMP-affinity precipitation

Cos-7 cells (87021302, Sigma-Aldrich) were transfected with POPDC1^{WT} or POPDC1^{S201F}, constructs with HA epitope tags attached to their C-termini using Lipofectamine2000 reagent (Invitrogen), and cells were harvested 24 hours later using a buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA pH 8.0, 1% (v/v) Triton X-100, 0.25% (w/v) gelatin, and protease inhibitors (Roche). Cells lysates were briefly sonicated and cleared twice by centrifugation at >18,000 x g at 4°C for 30 min. 30µl of the lysates were taken as loading controls. Lysates were first incubated with 50 µl ethanolamine (EtOH-NH) agarose (BioLog) for 1 h with rotation at 4°C. After centrifugation at 500 x 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 hrs 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 $MgCl_2$; 10 mM KCl; 500mM NaCl, 0,1% (v/v) Igepal CA-630; 1 mM DTT, 1 mM IBMX) followed by three washes with wash buffer B (10 mM HEPES, pH 7.4; 1.5 mM $MgCl_2$; 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 min 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 h at room temperature and

subsequently incubated with anti-HA (MMS-101R, Covance), anti-FLAG (F1804, Sigma-Aldrich) anti-Actin (sc-1616, Santa Cruz), anti-POPDC1 (sc-49889, Santa Cruz), 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; APrEST72859 (POPDC2), Atlas Antibodies). After several washes, the blots were incubated for 1 h 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.

Co-immunoprecipitation analysis

For co-immunoprecipitation (CoIP) analysis, Cos-7 cells (87021302, Sigma-Aldrich), were transiently transfected with myc-tagged Popdc1 and either Becker dystrophin, M2-microdystrophin (kindly provided by Rolf Stucka, Ludwig-Maximilans-Universität, Munich, Germany), FLAG-tagged Dp71 dystrophin (GenScript), or HA-tagged Dysferlin (DYSF-3HA) provided to Addgene (plasmid # 29767) by Steven Vogel (NIH, Bethesda, U.S.A.) (44), empty vectors were transfected as negative controls. Protein lysates were produced as described above. A sample was taken as input control and the rest of the lysates was subjected to CoIP using the ProFound™ c-Myc Tag IP/co-IP Kit (23620, ThermoFisher Scientific) or the Pierce HA Tag IP/Co-IP Kit (26180, ThermoFisher Scientific) following manufacturer's instructions.

Zebrafish strains and lines

Zebrafish AB and popdc1^{S191F} lines were maintained under standard conditions at 28.5°C according to established protocols (45), which were approved by the Animal Welfare and Ethical Review Board of the Harefield Heart Science Centre and licensed by the Home Office

(PPL 70/7171). Zebrafish were raised and staged in hours post fertilization (hpf) or days post fertilization (dpf). Pigmentation was inhibited with 0.2 mM 1-phenyl-2-thiourea (PTU) in egg water.

Morpholino design and microinjections

Morpholino-modified oligonucleotides (MO) were obtained from Gene-Tools (Philomath, OR). Embryos were injected at the 1-2 cell stage with 0.5 ng to 2 ng of *popdc1*-specific morpholino, co-injection of *p53*-targeting morpholino (5′-GCGCCATTGCTTTGCAAGAATTG-3′) was performed to suppress non-specific morpholino toxicity. Morpholinos targeting *popdc1* were directed against the splice-acceptor site of exon 4 (MO1-*popdc1*: 5'-CTTAATCTGGAATTAA ACAGGAGAA-3') and the splice donor site of exon 4 (MO2-*popdc1*: 5′-TACTTTCCTTTGAG AAGAATGCTGA-3′).

Gene editing

A *popdc1*^{S191F} mutation was generated using the GoldyTALEN modified scaffold (Bedell et al. 2012). The left TALEN (P1E5_S191FL: 5'-AACATTTTACACCAATGCCTT-3') and right TALEN (P1E5_S191FL: 5'-CATCTGAGTTGACCTGAA-3') were assembled via the GoldenGate method. For ease of analysis TALEN recognition sequences flanked a *Ple*I restriction site in exon 5 of *popdc1*, which is destroyed upon successful targeting. TALEN repeat variable di-residues (RVDs) were cloned into pT3TS-driven TALEN scaffold. Resulting mRNA was injected into one-cell stage zebrafish embryos. For the S191F mutagenesis the following oligonucleotide was co-injected together with the TALEN constructs: S191F oligonucleotide: CACCAATGCC**TTC**ATCGACTTCCCAGAATTCAGGTCAA (underlined triplet encodes F191). Larvae were molecularly tested at 2dpf, or raised for germ line mutation analysis. Somatic and germ line TALEN-induced mutations were evaluated by PCR and restriction fragment length polymorphisms. The primer pair: P1TALE5_1434F: 5'-TCAGAAATCCAGACAGCCGTG-3' and P1TALE5_2175R: 5'-AGTTCTCCTCAGCAGCGATC-3' was used to amplify the locus. Subsequently, the PCR fragment was restriction digested with *Ple*I. The mutant allele produced a 741 bp fragment, while the wildtype allele produced two fragments of 365 and 375 bp. For

further analysis, the PCR products were sequenced with the following primer sequences: P1TALE5_1556F: 5'-CAAAACAATAATGGCAGACTG-3' and P1TALE5_2069R: 5'-TCTTTTTT ATTTGTGTGTAGGGAA-3'. Heterozygotes were backcrossed 6 times with AB-line in order to outcross any off-target mutation. Homozygous mutant and wildtype animals were bred, separately.

Forced expression

The human POPDC1^{WT}, and POPDC1^{S201F} cDNAs were subcloned into psGEM vector. To generate mRNA, the clones were linearized with *Xho*I and capped mRNA was synthesized using the T7 mMessage Machine Kit (Ambion).

Whole mount *in situ* hybridization

Whole mount *in situ* hybridization was carried out as described previously (15). A partial clone of *popdc1* cDNA was subcloned into pGEMTeasy vector (Promega). A digoxygenin-labeled antisense probe was generated using T3 polymerase after linearization with *Bam*HI.

Whole mount immunohistochemistry and rhodamine-phalloidin staining

Whole-mount immunostaining was performed as described previously (15). The following primary antibodies were used: monoclonal F59 (DHSB), monoclonal F310 (DHSB), monoclonal anti-sarcomeric alpha-actinin (A7811, Sigma-Aldrich), anti-Dystrophin (D8043, Sigma-Aldrich), anti-Vinculin (FAK100, Millipore) Alexa Fluor 488-coupled donkey anti-mouse (A21202, Invitrogen) was used as secondary antibody. For F-actin staining, zebrafish embryos were incubated with rhodamine-phalloidin (R415, Invitrogen). Stained zebrafish embryos were embedded in 1% low melting agarose and imaged by confocal microscopy (Zeiss, LSM 510).

Live *in vivo* cardiac imaging

The heart rate of zebrafish morphants was recorded using a Leica imaging system equipped with a VGA Miniature GigE CCD Color Camera (DFK 23G618, Scorpion Vision). Video images were recorded at 28°C and processed with ImageJ (43) to obtain heart rate measurements and

perform M-mode analysis. For in detail analysis of the arrhythmic phenotype cardiac performance batches were screened for individuals showing arrhythmic phenotypes without cardiac edema. Cardiac contractility was recorded in tricaine anaesthetized 6 to 9dpf old embryos. For pharmacological characterization 50 individuals were incubated in 10^{-4} M isoproterenol (experimental group) or water (control) for 5 minutes. The percentage of arrhythmic to regular rhythm was counted. The imaging system consisted of a custom-made selective plane illumination microscope (SPIM) equipped with Olympus UMPLFLN20xW 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 (46). 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 cardiotachiogram to calculate mean heart rate. Stroke volume was determined as described (47).

Electron microscopy

5dpf wildtype 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 post-fixed 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 post-stained using uranyl acetate and TEM was performed in 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 transciption kit (Ambion, Austin, Texas, USA). The cRNAs were purified, and photometrically quantified. Human TREK-1c alone, or together with human POPDC1 (wildtype or S201F mutant) was injected into *Xenopus laevis* oocytes at the indicated amounts of cRNA. Oocytes were incubated at 19°C for 24-48 h in ND96 solution containing (in mM) 96 NaCl, KCl, 1 $MgCl_2$, 1.8 $CaCl_2$ and 5 HEPES (pH 7.4-7.5), supplemented with 50 mg/l gentamicin and 275 mg/l sodium pyruvate. For experiments with elevated cAMP levels, 25mM theophylline or 1mM 8-Br-cAMP (Tocris Bioscience) were supplemented to the storage solution, directly following the cRNA injection. Two-microelectrode voltage-clamp measurements were performed with a Turbo Tec-10 C amplifier (npi, Tamm). Data were recorded at a sampling rate of 2 kHz and lowpass filtered with 700 Hz. The oocytes were placed in a small-volume perfusion chamber and superfused with ND96 solution. Oocytes were held at -80 mV and voltage was ramped from -120 to +45 mV within 3.5 s, using a sweep time interval of 4 s. Since current amplitudes vary from one batch of oocytes to the next, currents were normalized to TREK-1c wildtype current amplitudes of the respective batch and recording day.

Patch clamp experiments of HL-1 cells.

HL-1 cells, kindly provided by Dr William C Claycomb, Lousiana State University, USA (22), were transfected as described above. Action potentials were recorded in the whole-cell configuration under current-clamp conditions at room temperature (22°C). For patch clamp experiments, HL-1 cells were superfused with solution containing (in mM) 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 0.33 NaH_2PO_4, 10 glucose, 2 sodium pyruvate and 5 HEPES (pH 7.4 with NaOH) as previously described (48,49). 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 - 4.0 MΩ when filled with a solution containing (in mM): 60 KCl, 65 K^+-glutamate, 5 EGTA, 2 $MgCl_2$, 3 K_2ATP, 0.2 Na_2GTP, and 5 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 s and a sample rate of 200 kHz. Data analysis of action potentials was done using the Fitmaster software (HEKA). For each cell measured, the action potential parameters were averaged by analyzing ten subsequent action potentials, as previously described (48).

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 instructions of the manufacturer. PCR was performed using Taq Gold Polymerase (Applied Biosystems) according to the instructions of the manufacturer. Reaction mixtures were preheated at 96 °C for 5 min followed by 40 cycles at 96 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s. PCR products were visualized by gel-electrophoresis on 2% agarose gels. The following intron-spanning primers were used: GAPDH for: 5'-ACTTCAACAGCAACTCCCACTCT-3', GAPDH rev: 5'-GCTGTAGCCGTATTCATTGTC ATA-3'; TREK-1 for: 5'-G ACGAACTCATCCAGCAAATAGTG-3', TREK-1 rev: 5'-AAAGAAGAA AGAGCTTCCGAGGTC -3'. For both primer pairs the amplification products were confirmed by sequencing. RNA, which was not reverse transcribed was used as negative control.

Statistics

Results are presented as mean ± SEM. Statistical significance was defined as $P < 0.05$. Two groups of measurements in Figures 4A, 6I-L, 7C,D, and Supplemental Fig. 17 were compared by paired Two-tailed Student's *t* test. One-way ANOVA was used to determine the difference between groups of the data presented in Figures 4B-C,E-G, 5B-E,G, and Supplemental Figure 12.

Study approvals

Informed consent for WES analysis and clinical exome analysis for the family was obtained and approval of the local ethics committee in Ferrara, Italy, was given to the project: Neuromics, approved the 30th of May 2013, approval number 05/2013, P. 58-2013, RARER, approved the 20th of June 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 was approved by the Animal Welfare and Ethical Review Board of the Harefield Heart Science Centre and licensed by the UK 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- II/3b/2012). The work with *Xenopus* at the University of Marburg was approved by the Regierungspräsidium Gießen, Germany (V54-19c 20 15 h 02 MR 20/28 Nr.A 4/2013).

Authors contributions

RFRS, JZ, BOB, SS, TS, KLP MF, SR, AF, VON, CG, TM, GT, PS designed and conducted functional experiments. FD, FF, BD and EB clinically characterized the family, AP clinically supervised the project on the families-of four (RARER), CR and EA clinically characterized the patients with cardiac abnormalities screened for mutations. CS, CP, FRDR, RS, FG, MN WL, HJ, XX, ND, performed the DNA studies, patients screening, WES and bioinformatics analysis, and technical validation. JW designing WES studies; TB, AF designing research studies, analyzing data, and writing the manuscript.

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Figures

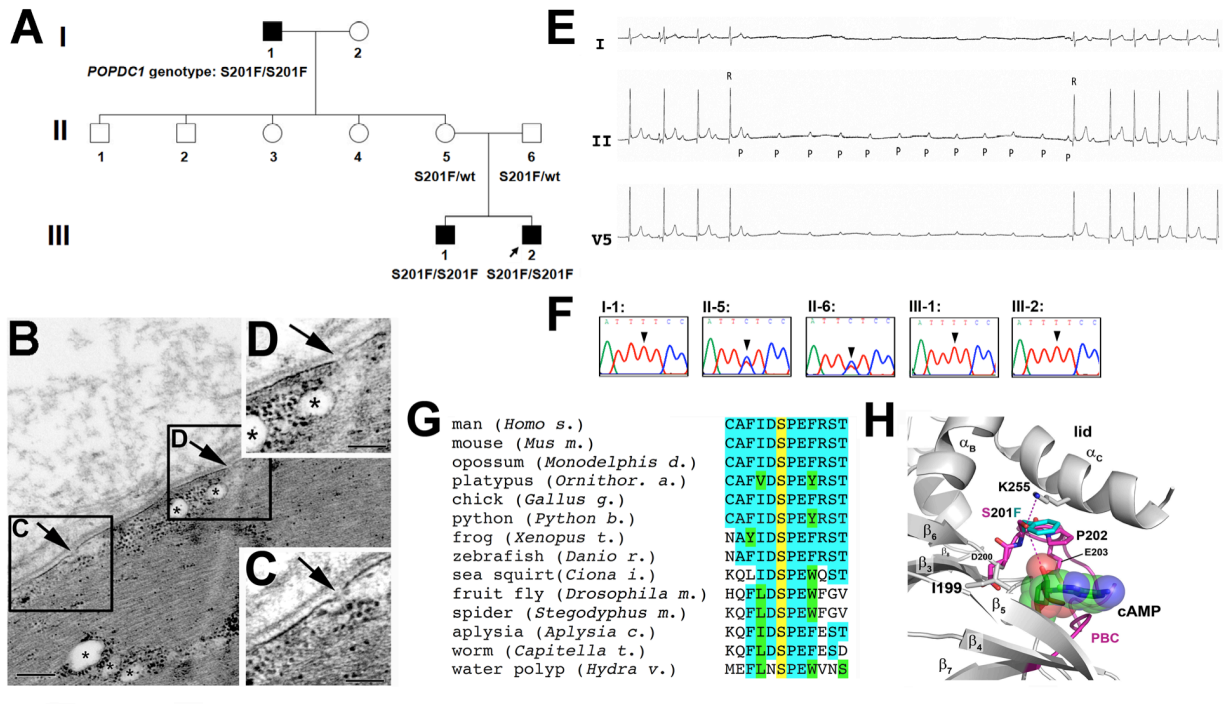


Figure 1. Identification of the *POPDC1*^{S201F} mutation associated with atrioventricular block and limb-girdle muscular dystrophy. (**A**) Pedigree of a family homozygous for the *POPDC1*^{S201F} mutation. The index patient (III-2) and his brother (III-1) are suffering from atrioventricular (AV) block, and the grandfather (I-1) from LGMD and AV block. (**B**) Electron microscopy analysis of skeletal muscle of patient I-1 revealed the presence of plasma membrane discontinuities (arrows) and subsarcolemmal and intermyofibrillar vacuoles (asterisks). (**C**,**D**) are higher magnifications of the boxed areas in (B). Scale bars: (B) 2 µm; (C,D) 1 µm. Data depicted are representative results all derived from a single biopsy. (**E**) Holter ECG of the index patient (III-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 S^{201} (yellow) is ultra-conserved. (**H**) 3D model of the Popeye domain. Only the Phosphate-Binding Cassette (PBC) is shown. Two hydrogen bonds are formed by S^{201}, which are lost in case the F^{201} (turquoise) mutant residue is present, possibly affecting the ligand-binding affinity of the Popeye domain.

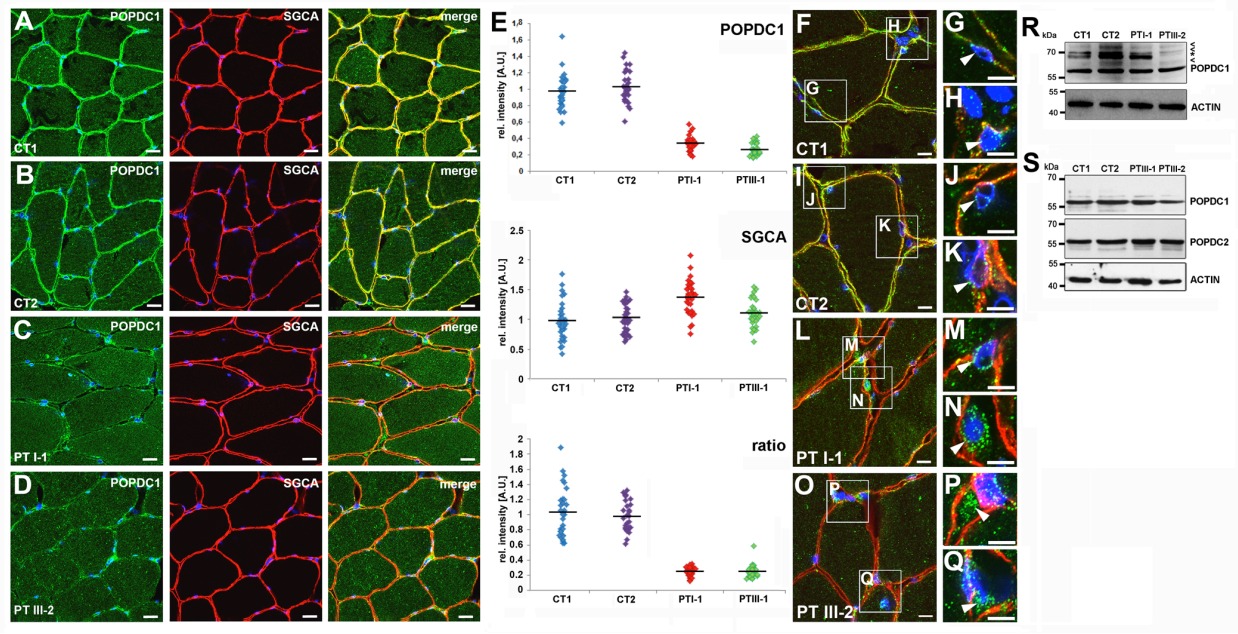


Figure 2. Membrane trafficking of POPDC1 is affected in muscle biopsies. Skeletal muscle biopsies of PTI-1 and PTIII-1 and two controls were immunostained for (**A**-**D**) POPDC1 (green signal) and SCGA (red signal). (**E**) The immunofluorescence signals were quantified in 10 muscle fibers of 3 sections per biopsy. The signals of POPDC1, SGCA and the ratio of both were plotted relative to the means of both controls, which were set as 1. (**F-Q**) Subcellular localisation of POPDC1 in (**F**-**H**) CT1, (**I**-**K**) CT2, (**L**-**N**) PTI-1, and (**O**-**Q**) PTIII-2. Perinuclear localisation 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: (A-D,G,H,J,K,M,N,P,Q) 10 µm; (F,I,L,O) 20µm. (**R,S**) Western blot analysis of (**R**) POPDC1 expression in skeletal muscle biopsies and of (**S**) POPDC1 and POPDC2 in dermal fibroblasts of two controls (CT1 and CT2) and PTI-1 and PTIII-2. Results are representatives of three independent Western blot experiments.

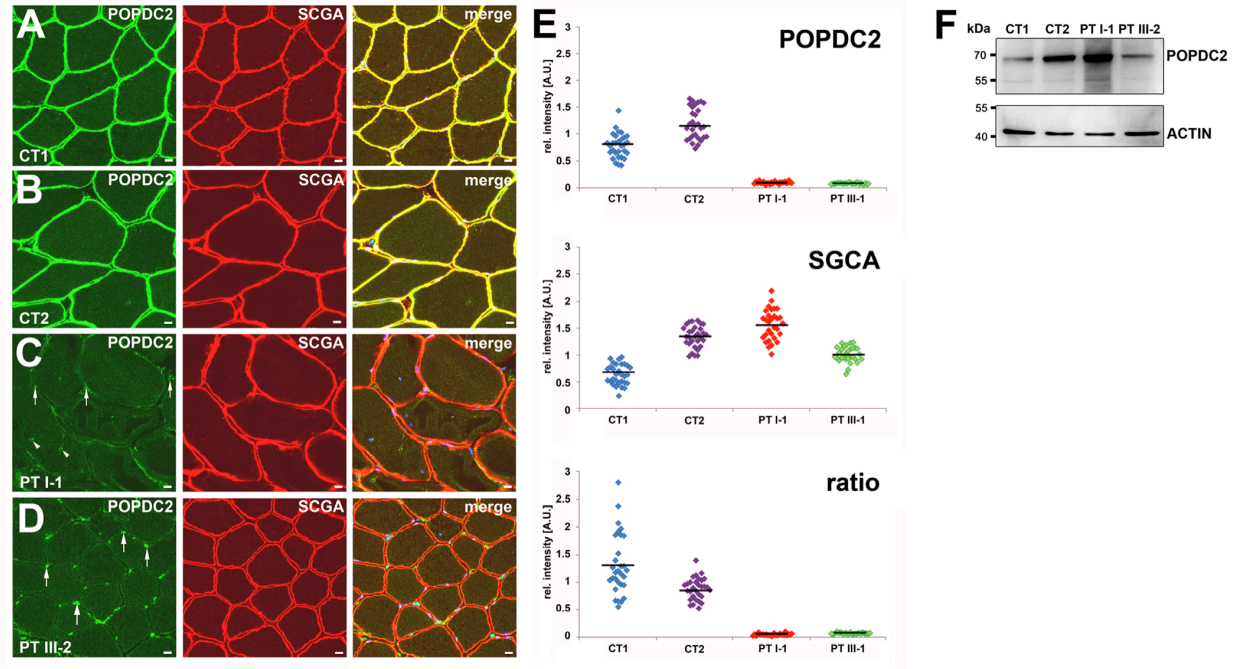


Figure 3. Membrane trafficking of POPDC2 is affected in patients with a homozygous POPDC1^{S201F} mutation. Skeletal muscle biopsies of PTI-1 and PTIII-2 and two controls were immunostained for (**A**-**D**) POPDC2 (green signal) and SCGA (red sigal). (**E**) Quantification of the relative intensities of the plasma membrane staining of POPDC2 and SGCA in 10 fibres each of three sections per biopsy. The signals of POPDC2, SGCA and the ratio of both were plotted relative to the means of both controls, which were set as 1. The normalized intensities revealed a downregulation of the membrane localisation of POPDC2 in the patient material. Scale bars: 10 µm. (**F**) Western blot analysis of POPDC2 expression in the muscle biopsy material of the two controls (CT1 and CT2) and PTI-1 and PTIII-2. Results are representatives of three independent experiments.

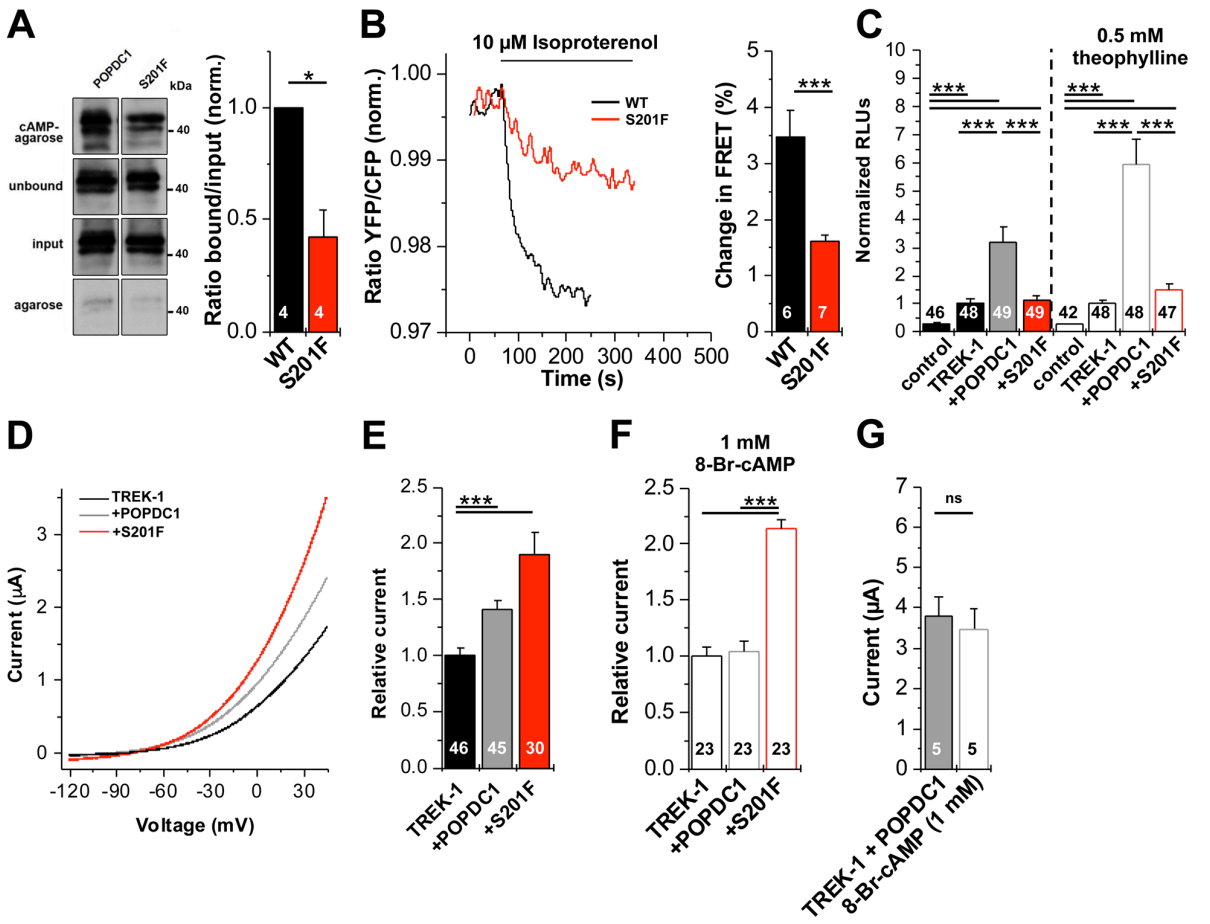


Figure 4. The POPDC1^{S201F} mutant protein displays a reduced cAMP-affinity. (**A**) cAMP affinity precipitation of wildtype or POPDC1^{S201F} protein. Left: precipitated proteins, unbound fraction, input samples as well as protein subjected to control precipitation using EtOH agarose (agarose), were subjected to western blot detection of POPDC1. Right: Quantification of the 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 POPDC1^{S201F}-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 POPDC1^{S201F}. Control, non-injected oocytes. Measurements after incubation with 0.5 mM theophylline are illustrated with open bars. (**D**) Representative two-electrode voltage clamp (TEVC) measurements in oocytes injected with TREK-1 alone (black), or co-injected with POPDC1 (gray) or POPDC1^{S201F} (red). Voltage was ramped from -110 to +35 mV. (**E**) Relative current amplitudes at 0 mV. (**F**) TEVC measurements in oocytes injected with TREK-1, or co-injected with POPDC1 or POPDC1^{S201F} bathed after cRNA injection in a storage solution containing 1 mM 8-Br-cAMP. (**G**) Relative current amplitudes of TREK-1 co-injected with POPDC1, measured in recording solution or directly after 10 minutes of perfusion with 2 mM 8-Br-cAMP. (A-C,E-G) Numbers of experiments are included in the bars. Results are presented as mean ± SEM. Two groups of measurements in Figures 4A were compared by paired Two-tailed Student's *t* test. One-way ANOVA was used for the data presented in Figure 4B,C,E-G. Statistical significance was defined as $P < 0.05$. *, p<0.05; ***, p<0.001. ns, not significant.

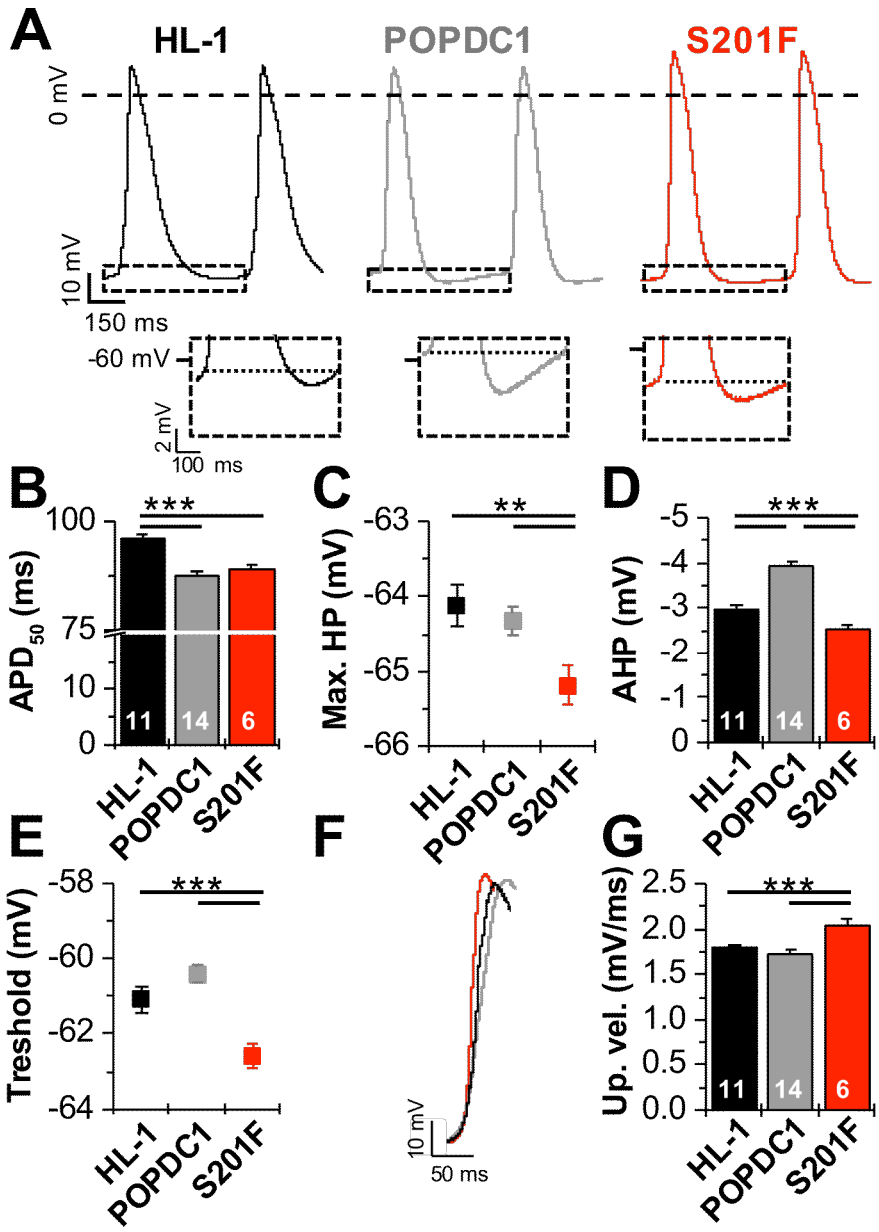


Figure 5. POPDC1^{S201F} hyperpolarizes the membrane potential of spontaneously beating HL-1 cells. (**A**) Representative action potentials recorded for HL-1 cells or HL-1 cells transfected with POPDC1 or POPDC1^{S201F}. The boxes at the bottom illustrate a zoom-in to show the effects on maximal hyperpolarization and afterhyperpolarization. (**B**) Analyses of the action potential duration, analyzed at 50 % of repolarization (APD_{50}). (**C**) Analyses of maximal hyperpolarization (Max. HP), (**D**) afterhyperpolarization (AHP), and (**E**) activation threshold (Threshold) for HL-1 cells or HL-1 cells transfected with POPDC1 or POPDC1^{S201F}. (**F**) Zoom-in showing the upstroke phase of an action potential of HL-1 cells or HL-1 cells transfected with POPDC1 or POPDC1^{S201F}. Traces were aligned to the activation threshold. (**G**) Analyses of the upstroke velocity (Up. Vel.). Results are presented as mean ± SEM. Statistical significance was defined as $P < 0.05$. One-way ANOVA was used to compare multiple variables presented in Figure 5B-E and G. Statistical significance was defined as $P < 0.05$. **, p<0.01; ***, p<0.001.

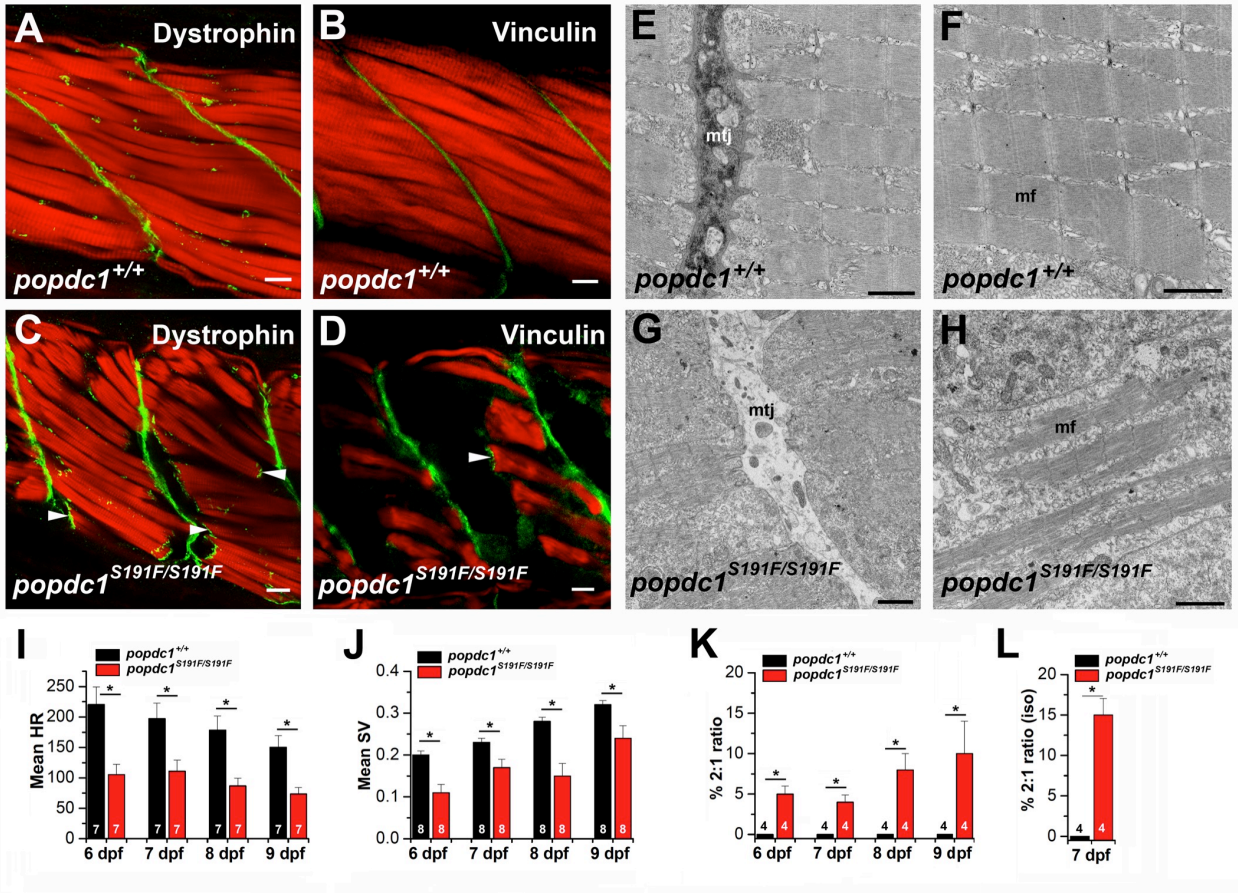


Figure 6: **The popdc1^{S191F} zebrafish mutant displays muscular dystrophy and cardiac arrhythmia.** (**A-D**) Confocal analysis of actin (phalloidin staining, red channel) and (**A**,**C**) Dystrophin (green channel) or (**B,D**) vinculin (green channel) expression in trunk skeletal muscle of (**A,B**) wildtype (*popdc1*$^{+/+}$) and (**C,D**) homozygous mutants (*popdc1*$^{S191F/S191F}$). Lateral view of the tail musculature in larvae at 5 days post-fertilisation (dpf). Homozygous mutants develop lesions in skeletal muscle. Muscle fibers detach from the myotendinous junction (mtj) and retract. Arrowheads in (**C**,**D**) indicate the presence of Dystrophin and Vinculin immunoreactivity at the end of the ruptured fibers, suggesting that the fibers that have lost connection to the mtj are probably physically intact. The results shown are representative of three independent experiments. (**E-H**) Electron microscopy analysis of trunk skeletal muscle of (**E,F)** wildtype and (**G,H**) homozygous *popdc1*^{S191F} 5dpf mutant zebrafish embryos. The mtj in the homozygous mutant is characterised by a lack of electron dense material. The results shown are representative of three independent experiments. (**I-L**) Analysis of heart function in wildtype and homozygous *popdc1*^{S191F} mutant embryos. (**I**) Mean heart rate. (**J**) Mean stroke volume. (**K**) Number of embryos displaying a 2:1 AV block. (**L**) Number of embryos displaying a 2:1 AV block after isoproterenol stimulation. Results are presented as mean ± SEM. Two groups of measurements were compared by paired Two-tailed Student's *t* test. Statistical significance (*) was defined as $P < 0.05$. Scale bar: A-D = 10 µm; E,F = 1 µm, G,H = 2 µm. mf – myofibrils.

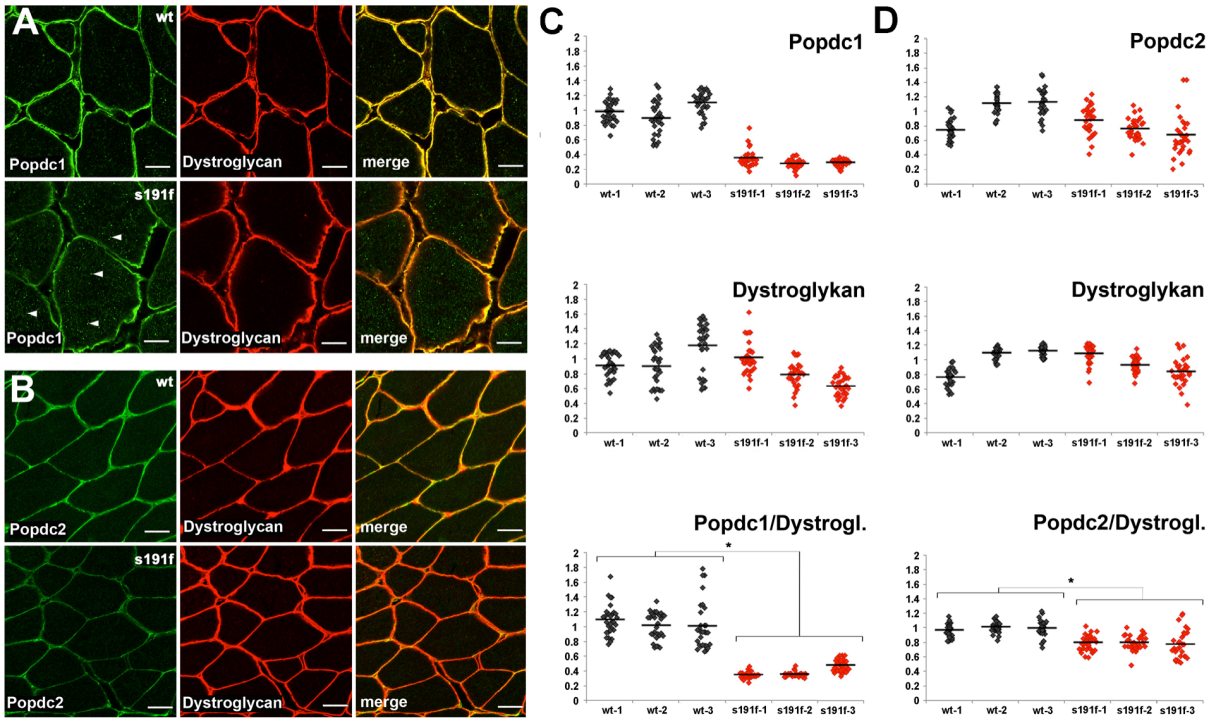


Figure 7 Expression of Popdc1 and Popdc2 in the trunk skeletal muscle of the adult zebrafish. (**A**,**B**) Transversal sections through the trunk skeletal muscles of wildtype (*popdc1*$^{+/+}$) and homozygous *popdc1*^{S191F} mutants (*popdc1*$^{S191F/S191F}$) were stained with antibodies for α-Dystroglycan and **(A)** Popdc1 or (**B**) Popdc2. (**C**,**D**) Quantification of the plasma membrane staining of (**C**) Popdc1 and (**B**) Popdc2 in wildtype and homozygous Popdc1^{S201F} mutant animals, respectively. The regions of interest of 30 fibres were analyzed for each sample. The membrane localization of both, Popdc1^{S191F} and Popdc2 is reduced in the *popdc1*$^{S191F/S191F}$ mutants. In agreement with the reduction in plasma membrane localisation, increased amounts of the Popdc1^{S191F} mutant protein is found in cytoplasmic vesicles (arrowheads in A). For statistical testing, mean values of the Popdc/Dystroglycan ratios of wildtype (n = 3) and mutants (n = 3) were compared by paired Two-tailed Student's *t* test. Statistical significance (*) was defined as $P < 0.05$.