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The Popeye domain containing 2 (*popdc2*) gene in zebrafish is required for heart and skeletal muscle development

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Abstract

The Popeye domain containing (Popdc) genes encode a family of transmembrane proteins with an evolutionary conserved Popeye domain. These genes are abundantly expressed in striated muscle tissue, however their function is not well understood. In this study we have investigated the role of the *popdc2* gene in zebrafish. *Popdc2* transcripts were detected in the embryonic myocardium and transiently in the craniofacial and tail musculature. Morpholino oligonucleotide-mediated knockdown of *popdc2* resulted in aberrant development of skeletal muscle and heart. Muscle segments in the trunk were irregularly shaped and craniofacial muscles were severely reduced or even missing. In the heart, pericardial edema was prevalent in the morphants and heart chambers were elongated and looping was abnormal. These pathologies in muscle and heart were alleviated after reducing the morpholino concentration. However the heart still was abnormal displaying cardiac arrhythmia at later stages of development. Optical recordings of cardiac contractility revealed irregular ventricular contractions with a 2:1, or 3:1 atrial/ventricular conduction ratio, which caused a significant reduction in heart frequency. Recordings of calcium transients with high spatiotemporal resolution using a transgenic calcium indicator line (*Tg(cmlc2:gCaMP)*^{s878}) and SPIM microscopy confirmed the presence of a severe arrhythmia phenotype. Our results identify *popdc2* as a gene important for striated muscle differentiation and cardiac morphogenesis. In addition it is required for the development of the cardiac conduction system.

Keywords

Arrhythmia; Calcium transient; Heart; Skeletal muscle; Craniofacial muscle; Development; Popeye domain containing genes

Introduction

Electrical activity is autonomously generated in the vertebrate heart. The sinus node acts as the primary cardiac pacemaker and electrical activity is rapidly propagated through the atria. The electrical impulse is delayed at the atrioventricular (AV) junction due to the presence of slow conducting AV node cells. This arrangement allows the atria to contract before the ventricle. A fast-conducting network of specialized cells in the His-Purkinje system ensures that the ventricle is electrically excited from the apex to the base allowing efficient ejection of blood (Christoffels et al., 2010). Several transcription factors have been recognized that do play an important role in specifying and shaping the transcriptional programs of the cardiac conduction system (CCS) (Blaschke et al., 2007; Hoogaars et al., 2007; Moskowitz et al., 2007). Likewise, signaling molecules involved in CCS development including neuregulin and endothelin have been identified (Mikawa and Hurtado, 2007). However, many aspects of CCS development are currently not well understood.

The zebrafish has recently emerged as a novel system to study cardiac electrophysiology and the development and physiology of the cardiac conduction system (Milan and Macrae, 2008). In zebrafish the cardiac conduction system lacks the anatomical boundaries that are found in the mammalian heart. Nonetheless, a distinct cardiac pacemaker has been functionally defined in the embryonic zebrafish heart (Arrenberg et al., 2010). Impulse delay at the atrioventricular canal (AVC) allowing sequential contraction of atrial and ventricular chambers is first observed 36–40 h post fertilization (hpf) and is correlated with a cell shape change of AVC myocytes (Chi et al., 2008; Milan et al., 2006a). A fast conducting ventricular conduction tissue develops at 100 hpf and its functionality is correlated with the development of ventricular trabeculation (Chi et al., 2008). Despite significant anatomical differences, the electrophysiology and the beating rates of zebrafish and human hearts were found to be surprisingly similar (Kopp et al., 2005; Leong et al., 2010). Many of the ionic currents involved in the generation and shaping of the cardiac action potential are identical, although some differences are also present (Milan et al., 2006b; Nemtsas et al., 2010). Several zebrafish mutants have been identified that faithfully model human conduction diseases such as long or short QT syndromes, which are difficult to model in the mouse due to the high heart rate and differences in potassium currents being active during repolarization (Arnaout et al., 2007; Hassel et al., 2008; Langheinrich et al., 2003; Milan et al., 2003; Salama and London, 2007). Thus, the zebrafish appears to be a powerful system to study cardiac electrophysiology and recent pharmacological and mutagenesis screens in the zebrafish model resulted in the identification of several novel genes involved in the developmental or functional control of the cardiac conduction system (Chi et al., 2008; Milan et al., 2009).

The Popeye domain containing (Popdc) gene family encodes a unique class of membrane proteins harboring three transmembrane domains and an evolutionary conserved intracellular Popeye domain (Andrée et al., 2003; Brand, 2005; Knight et al., 2003; Osler et al., 2006). In vertebrates, the Popdc gene family consists of three members, *Popdc1* (also known as *Bves*), *Popdc2*, and *Popdc3* (Brand, 2005; Osler et al., 2006). *Popdc2* is uniquely present in vertebrates, whereas *Popdc1* and *Popdc3* orthologs are also found in lower chordates and insects (Brand, 2005; Lin et al., 2007). All three genes in vertebrates display an overlapping and evolutionary conserved expression pattern, being highly abundant in cardiac and skeletal muscle, suggesting an important function in these tissues (Andrée et al., 2000; Breher et al., 2004; Froese and Brand, 2008; Hitz et al., 2002; Parnes et al., 2007; Torlopp et al., 2006). Popdc genes are also expressed in other cell types such as smooth muscle cells, neurons, and several types of epithelial cells, which indicate a more widespread role for this gene family (Brand, 2005; Osler et al., 2006). In *Drosophila*, antisense-mediated knockdown of the *Popdc1* homolog causes abnormal epithelial movement and

failure of pole cell migration (Lin et al., 2007). Similarly, morpholino-mediated knockdown of *Popdc1* in *Xenopus* causes an arrest in gastrulation (Ripley et al., 2006). In contrast, the null mutant of *Popdc1* in mice is viable. However, skeletal muscle tissue in this mutant displays impaired regeneration after experimental wounding (Andrée et al., 2002). *Popdc1* has been proposed to act in cell adhesion since it is rapidly recruited to sites of cell–cell contact formation (Wada et al., 2001). Moreover, *Popdc1* has been reported to control membrane localization of ZO-1 and thereby modulate tight junction integrity in epithelial cells (Osler et al., 2005). Recently, protein partners for *Popdc1* have been identified, the guanine nucleotide exchange factor T (GEFT), which acts as a GEF for Rho-family GTPases (Smith et al., 2008), and the SNARE proteins VAMP2/VAMP3, which control vesicular transport and β 1-integrin recycling (Hager et al., 2010). A null mutation of *Popdc2* has recently been engineered in mice (Froese and Brand, 2008) and interestingly, a cardiac arrhythmia phenotype was present in null mutants (Froese et al., 2012).

Here we demonstrate that during zebrafish development *popdc2* is expressed in skeletal muscle and heart. Global loss of *popdc2* affected skeletal muscle development resulting in abnormal facial and trunk muscle formation. The alignment of muscle fibers as well as establishment of myotendinous junctions was defective in the morphants. An even more severe defect was seen in craniofacial muscle development. Here many muscles were malformed, or reduced in size. In the heart looping was defective and the chambers were misshaped. After reducing the morpholino concentration, muscle and heart development appeared morphological normal, however the heart became arrhythmic at later stages of development, showing irregular action potential durations, and an AV block. Thus, *popdc2* in zebrafish is essential for muscle and heart development and is required for cardiac conduction system development.

Material and methods

Zebrafish strains and lines

Zebrafish were raised under standard conditions at 28.5 °C and staged as previously described (Westerfield, 2003). The following transgenic lines were used: *Tg(cmlc2:gCaMP)^{s878}* (Arnaout et al., 2007), *Tg(cmlc2:eGFP)* (Huang et al., 2003), *Tg(flk1:eGFP)^{s843}* (Jin et al., 2005), *Tg(cmlc2:eGFP-ras)^{s883}* (D'Amico et al., 2007), *Tg(gata1:dsRed)^{sd2}* (Traver et al., 2003), *Tg(acta1:GFP)* (Higashijima et al., 1997).

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 morpholino. Morpholino sequences were directed against the splice-acceptor site of exon 2 of *popdc2* (MO1-*popdc2*: 5'-CTTAATCTGGAATTAAACAGGAGAA-3') and the splicedonor site of exon 1 of *popdc2* (MO2-*popdc2*: 5'-GTTCAATTGTTTCTCACCTGCCAGA-3'). Embryos were injected at the 1–2 cell stage with 0.5–1 ng MO1-*popdc2* for analysis of the cardiac phenotype and 1–2 ng MO1-*popdc2* for analyzing the tail and craniofacial muscle defects. In case of the injection of MO1/MO2 morpholinos 1 ng of each morpholino was used. For control purposes a standard control morpholino: 5'-CCTCTTACCTCAGTTACAATTTATA-3' was used at the respective concentration.

Phenotype rescue

The ability of murine *Popdc2* mRNA to rescue the morphant phenotype was tested by subcloning the coding sequence of *Popdc2* into the *Stu* I restriction site of pCS2+. The plasmid was linearized by enzymatic digestion with *Sac* II and capped mRNA was

synthesized using the SP6 mMessage Machine Kit (Ambion). Rescue was achieved using 100 pg cRNA and 1 ng of MO1-*popdc2*.

RNA isolation, cDNA synthesis, and RT-PCR analysis

RNA of adult tissues and embryos was isolated with Trizol Reagent (Invitrogen) and purified according to manufacturer's instructions. cDNA was synthesized from DNase-treated total RNA using AMV reverse transcriptase (Promega). The following primer pairs were used for RT-PCR analysis to study the extent of interference with splicing after morpholino injection (Figs. 3B,C): E_{fw} (5'-CTGCTGATGCTGCAGTGTTT-3') and E_{rev} (5'-GGCACGTCCTCTTTGGTATC-3'); I_{fw} (5'-TGCAGCTGAAATCTGAATGG-3') and I_{rev} (5'-GTTTAAAGCAGGCCACCTGA-3'). The following primer pairs were used for RT-PCR analysis of gene expression (Figs. 2U,V): *zf-popdc2*-fw (5'-GACGGGGAACAGAAGCACAGACA-3') and *zf-popdc2*-rev (5'-ACCGCCCATATAGCCCAGAAAAA-3'); β -actin-fw (5'-CTTGCGGTATCCACGAGAC-3') and β -actin-rev (5'-GCGCCATACAGAGCAGAA-3').

Rhodamine-phalloidin staining

Zebrafish embryos were fixed overnight at 4 °C with 4% paraformaldehyde in phosphate buffered saline (PBS). After extensive washing in PBS, the embryos were equilibrated for 30 min in 0.2% Triton X-100/PBS. Subsequently the embryos were incubated with rhodamine-phalloidin (Molecular Probes; 1:150 dilution in 0.2% Triton X-100/PBS) for 20 min. Subsequently, the embryos were washed several times in 0.2% Triton X-100/PBS and embedded in 1.5% LMW agarose for confocal microscopy. Confocal stacks were obtained with a Leica TCS-SP2 AOBS.

Whole mount in situ hybridization

Whole mount in situ hybridization was carried out as described previously (Jowett and Lettice, 1994). The following digoxigenin-labeled antisense probes were used: *cmlc2* (Yelon et al., 1999), *vmhc* (Yelon et al., 1999), *myod* (Weinberg et al., 1996), *notch1b* (Walsh and Stainier, 2001), and *bmp4* (Chen et al., 1997). A full-length *popdc2* cDNA clone was identified in the IMAGE library (IMAGE clone: 2602337; NCBI accession number: 153906) and was fully sequenced. The nucleotide sequence has been deposited in GenBank (accession no.: AAQ57588). An antisense probe was generated using T3 polymerase after linearization with *Bam*HI.

Whole mount immunostaining

For whole-mount immunostaining, embryos were fixed in 4% paraformaldehyde or in case of FAK with Dent's fixative (Dent et al., 1989) at 4 °C overnight. Blocking was performed using PBS with 5% goat serum, 0.2% Triton X-100 and 1% DMSO. Subsequently the embryos were incubated with the primary antibody at 4 °C over night followed by intense washing steps in PBS with 1.5% goat serum, 0.2% Triton X-100 and 1% DMSO. The following primary antibodies were used: monoclonal anti-vinculin (Sigma, 1:400), polyclonal anti-FAK (Santa Cruz Biotechnology, 1:200), monoclonal beta-catenin (BD Transduction Laboratories, 1:200), polyclonal beta-catenin (Cell Signaling, 1:200), monoclonal F59 (DHSB, 1:20), monoclonal F310 (DHSB, 1:20), monoclonal anti-Alcam (Zn8, DHSB, 1:250), monoclonal anti-sarcomeric α -actinin (Sigma, clone EA-53, 1:500), and monoclonal anti-tropomyosin (Sigma, clone TM311, 1:200). The secondary antibody was conjugated with Alexa Fluor 488 or 566 (Invitrogen, 1:400) and incubation was performed for 2–3 h at room temperature.

Bone and cartilage staining

4 dpf and 5 dpf zebrafish embryos were fixed in 3.5% formaldehyde/0.1 M sodium phosphate buffer for 1 h and stored in 70% methanol. The protocol for simultaneous bone and cartilage staining was previously described in Spoorendonk et al. (2008) and adapted from Walker and Kimmel (2007). Embryos were rinsed in 50% ethanol and cartilage staining was performed using 0.2 mg/ml alcian blue 8 GX (Sigma) in 70% ethanol/80 mM MgCl₂. After several wash steps in 0.02% Triton X-100, embryos were bleached in 1% H₂O₂/1% KOH for 30 min and rinsed in a saturated sodium tetraborate solution. The embryos were digested for 1 h in 1 mg/ml trypsin (Sigma) in 60% saturated sodium tetraborate. The bones were stained with 0.04 mg/ml alizarin red S (Sigma) in 1% KOH, destained with an increasing glycerol series and stored at 4 °C in 70% glycerol.

Electron microscopy

3 dpf zebrafish embryos were analyzed by electron microscopy using a Zeiss EM-10 following standard procedures according to (Schoft et al., 2003).

In vivo whole mount confocal imaging

Zebrafish embryos (*Tg(cmlc2:eGFP-ras)*^{s883} (Jin et al., 2005) and *Tg(flk1:eGFP)*^{s843} (D'Amico et al., 2007) were embedded in 2% low melting agarose supplemented with tricaine and imaged by confocal microscopy (Zeiss, LSM 710).

High-speed video imaging

Cardiac contractility was recorded in tricaine anesthetized 5–7 dpf old embryos. The imaging system consisted of an inverted microscope (Zeiss Axiovert 25) equipped with a digital high-speed video camera (Basler Ahrensburg, Germany). Video images were recorded at 28 °C (20,000 images, 50 fps) and heart rates were measured as described previously (Schwerte and Fritsche, 2003). 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. In this 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 cardiogram, which was analyzed by power spectrum analysis to evaluate frequency domains.

Isolated calcium transient recordings by selective plane illumination microscopy (SPIM)

For electro-mechanical isolation, 5 and 6 dpf *Tg(cmlc2:gCaMP)*^{s878} embryos were transferred to 40 mM butanedione monoxime (BDM) in E3 for 1–2 min and then embedded in 1.5% low melting agarose in E3 supplemented with 20 mM BDM and subjected to SPIM microscopy to detect calcium transients as described (Arnaout et al., 2007). The obtained video sequences were analyzed with Matlab (The Mathworks, Natick, MA). Six areas within the video sequence were selected and fluorescence intensity in these areas was plotted over time in a semi-logarithmic manner.

Results

Expression of *popdc2* during skeletal muscle and heart development

We identified a *popdc2* homolog in zebrafish through database screening (Fig. S1). Expression analysis by RT-PCR revealed that *popdc2* mRNA was detectable at 4 hpf (Fig. 1A). Expression level peaked at 24 hpf, and gradually decreased afterwards. Expression analysis using whole mount in situ hybridization revealed that *popdc2* was not detectable during early stages of somitogenesis, but when skeletal muscle differentiation commenced at

14–16 somites, *popdc2* expression became detectable (Figs. 1B–D). Expression displayed an anteroposterior gradient, since newly formed somites did not express *popdc2*, however, expression was detected in somites that were more mature. Myocardial expression was first observed at 22 hpf in the forming heart tube (Fig. 1E). At 24 hpf, expression in the heart was confined to differentiated cardiac muscle cells (Figs. 1F,H,J). At the same time *popdc2* was expressed in the entire myotome (Figs. 1F,G,I). At 48 and 72 hpf, expression in the trunk muscle cells disappeared, but was present in muscle cells of the pectoral fin bud and facial muscles. At this time in development, prominent expression in both, atrial and ventricular chambers was observed in the heart (Figs. 1K–R). In the adult, *popdc2* was present in the heart and faintly expressed in skeletal muscle (Fig. 1S).

Knockdown of *popdc2* affects muscle and heart development

In order to study the function of *popdc2*, we interfered with its expression using two different morpholino oligonucleotides, which were targeted to the splice donor (MO2) and acceptor sequences (MO1) of intron 1, respectively (Fig. S2A). Both morpholinos, which were used singly (Fig. S2B,C), or in combination (Fig. S2D) suppressed expression of the *popdc2* transcript with similar efficiency. In each case aberrantly spliced *popdc2* transcripts retaining intron 1 sequences were detected by PCR using intron-specific primer (Fig. S2B–D). Injection of MO1, MO2 or a combination of thereof evoked similar phenotypes in heart and skeletal muscle development (Fig. S2E–H). A majority (n=73/92) of MO1-*popdc2* morphants displayed aberrant tail morphology at 24 hpf. Identical phenotypes at similar frequency were seen in case of MO2 or MO1/MO2 injected embryos. After onset of blood circulation the morphants developed a pericardial edema (Fig. S2E–H), which was often accompanied by prominent blood accumulation at the venous pole at 48 hpf. At later stages, the edema became more extensive, resulting in stretching of the heart, and ultimately the heart was ruptured due to the massive inflation of the pericardial sac (data not shown).

Trunk and craniofacial skeletal muscle development is impaired in *popdc2* morphants

In order to more closely evaluate the skeletal muscle phenotype, embryos receiving MO1-*popdc2*, a combination of MO1 and MO2, or control morpholinos, respectively, were stained using rhodamine-labeled phalloidin (Figs. 2A–F). In control embryos, the myotomal segments were chevron-shaped and evenly spaced. The myofibrillar content, which at 24 hpf was initially sparse increased in density by 72 hpf (Figs. 2A,D). In contrast, in the MO1-*popdc2* morphants the myotomal segment borders were u-shaped and the segmental borders were ill defined and irregularly spaced (Figs. 2B,E). Myofibrils were often unevenly distributed and areas lacking myofibrils were observed. Somitogenesis was normal as revealed by MyoD staining (Fig. S3), and therefore aberrant segmental borders in trunk muscle tissue were independent of paraxial mesoderm segmentation. A combined injection of MO1- and MO2-morpholinos exaggerated the phenotype (Figs. 2C,F). Knockdown of *popdc2* did not affect muscle-fiber type specification as shown by the normal distribution of both fast and slow muscle fibers in the *popdc2* morphants (Figs. 3A,D,G,J). However, the overall muscle mass was noticeably reduced in the *popdc2* morphant. Both muscle fiber types were morphologically abnormal. While the multinucleated fast muscle fibers in control embryos were relatively even in size and were stacked neatly in parallel rows, morphant fast muscle displayed varying cell shapes and was highly disorganized (Figs. 3B,E). Likewise slow muscle fibers in the *popdc2* morphant also displayed malalignment and tissue gaps in the otherwise tight arrangement (Figs. 3H,K). By 3 dpf, both fiber types became increasingly disorganized in the morphant embryos with occurrence of ruptured fast muscle fibers, and many slow muscle fibers with a wavy appearance and the presence of widening gaps (Figs. 3C,F,I,L). The myofibrillar apparatus displayed severe myofiber disarray as revealed by α -actinin staining (Fig. S4). The development of the myotendinous junctions was studied using FAK and vinculin as marker proteins (Henry et al., 2005). Early

histogenesis of trunk skeletal muscle at 17 and 18 hpf appeared to be unaffected by the loss of *popdc2* (Fig. S5). However by 24 hpf the myotendinous junction was much thinner and discontinuous suggesting a failure to build up or maintain proper myotomal boundaries (Fig. 4). We analyzed whether the severe trunk muscle defect was rescued by mouse *Popdc2* cRNA (Fig. 5). *Popdc2* cRNA injected zebrafish (n=60/60) displayed a clearly structured tail indistinguishable from control embryos (Fig. 5). In contrast, MO1-*popdc2* injected embryos (n=59/62) showed a disorganized tail at 24 hpf and developed pericardial edema, which started to become visible at 48 hpf (Fig. 5). Embryos co-injected with *Popdc2* cRNA and MO1-*popdc2* displayed a rescue of the trunk muscle phenotype at 24 hpf in 22 out of 82 (27%) embryos and normal heart development at 48 hpf. In addition 8 embryos retained the pericardial edema but a rescue of the tail phenotype was seen (weak phenotype, Fig. 5). We conclude that the observed defects in skeletal muscle development can be partially rescued by the addition of mouse *Popdc2* to counteract the knockdown by the *popdc2* morpholino.

At 3 dpf, the morphants were often lying motionless on the substrate, or displayed abnormal circular swimming behavior after stimulation. While the observed morphogenetic defects in trunk skeletal muscle development are likely to be causal for the observed muscle dysfunction, we cannot rule out that this phenotype is secondary and caused for example by cardiac insufficiency.

Craniofacial muscle development was also severely affected by the loss of *popdc2*. Confocal analysis of the heads of 3 dpf old Tg(*acta1*:GFP) embryos revealed a loss of craniofacial muscles, or a massive reduction in size and those muscles were dysmorphic (Fig. 6). Similar results were obtained after whole mount staining of the head of morphants using MF20 antibody (Fig. S6). The impaired development of craniofacial muscle was not accompanied by aberrant craniofacial bone and cartilage formation, which was unaffected in the *popdc2* morphant (Fig. S7). Similarly the formation of pharyngeal arch tissue was indistinguishable between WT and *popdc2* morphants (data not shown) suggesting that the cranial phenotype was confined to the skeletal muscle compartment.

Knockdown of *popdc2* induces a cardiac conduction phenotype

At a morpholino concentration of 2 ng/embryo, the heart was dysmorphic and showed aberrant looping and a lack of trabeculation (Figs. 7A,B). Myocyte shape appeared to be unaffected, however the development of the myofibrillar apparatus in the *popdc2* morphants was less advanced when compared to the control morphant (Figs. 7C–F). The heart displayed arrhythmia, which became more severe as development progressed and most embryos had a silent ventricle at 3–4 dpf. Due to the development of a severe pericardial edema the heart was stretched and therefore it is unclear whether the arrhythmia phenotype was primary or due to the morphological aberrations. In order to study the conduction phenotype further, the morpholino concentration was reduced to 1 ng/embryo. At this concentration, pericardial edema was not present between 24–72 hpf and skeletal muscle development appeared undisturbed. At 72 hpf some morphants displayed cardiac arrhythmia. At 4 dpf, a mild pericardial edema developed in many morphants (n=38/103) and a minor fraction of them displayed ventricular pauses (n=8/103). At 5 dpf, the total number of animals with cardiac defects increased (n=63/103) as well as the cardiac phenotype became more severe: i.e. pericardial edema (n=16/103), and cardiac arrhythmia (n=41/103). Cardiac arrhythmia was characterized by ventricular pauses with a 2:1 and 3:1 AV block pattern (Fig. 8A; Movies 1 and 2 in the supplementary material). At 5 dpf the 2:1 AV block occurred with high frequency (30 ± 6 /min (n=4)), and a few 3:1 ventricular pauses (3 ± 1 (n=4)), were also observed. Due to these ventricular pauses, the resulting heart frequency (182 ± 4) was significantly different from controls (235 ± 18 ; p 0.05; Fig. 8B) and similar observations were made at 6 and 7 dpf. At 7 dpf, morphant hearts also displayed extensive pauses of atrial and ventricular contraction. A mean of 1.2 ± 0.4 pauses per min

with a duration of 9.8 ± 1.8 s was seen (Movies 3 and 4 in the supplementary material), which resulted in a further drop of the mean heart frequency, which was 165 ± 15 in the *popdc2* morphants vs. 201 ± 8 ($p = 0.05$) in controls.

The loss of *popdc2* affects both atrial and ventricular conduction

In order to further study the cardiac conduction phenotype in the *popdc2* morphants, cardiac Ca^{2+} cycling was studied in the *Tg(cmlc2:gCaMP)^{s878}* line, which expresses a voltage-sensitive fluorescent Ca^{2+} sensor in myocytes (Arnaout et al., 2007). In order to image Ca^{2+} -oscillations in these hearts with high spatial and temporal resolution, selective plane illumination microscopy (SPIM) was utilized (Huiskens and Stainier, 2009). Cardiac contraction was uncoupled from electrical excitation using butanedione monoxime (BDM) and in vivo images were obtained at 5 and 6 dpf. In wild-type hearts ($n=4$), repetitive fluorescent waves representing systolic Ca^{2+} -release were visible, spreading from the atrium via the AV-junction to the ventricle (Fig. 9A and Movie 5 in the supplementary material). In MO1-injected embryos ($n=6$), several different aberrant patterns of Ca^{2+} -waves were visible (Figs. 9B–D and Movies 6, 7, and 8 in the supplementary material). In two of the imaged morpholino-treated embryos (Figs. 9B, C), intervals between individual Ca^{2+} -peaks were irregular in the atrium and this might have its impact on electrical excitation in the ventricle (Fig. 10). Several of the intervals between atrial Ca^{2+} -peaks were very short and the electrical impulses possibly reached the AV-junction at a time when the ventricle was still in its refractory state and therefore unable to respond. In three other cases, the atria displayed normal rhythmicity and no interval variations were seen, however, an AV block with different severity was observed (data not shown). Another phenotype that we observed was a sinoatrial block (Fig. 9D).

Confocal analysis of cardiac myocyte morphology using the *Tg(cmlc2:eGFP-ras)^{s883}* line revealed normal atrial and ventricular chamber dimensions and trabeculation (Fig. S8A–D). Moreover, cardiac myocyte morphology in the atrium and in the AV canal showed no obvious alterations (Fig. S8E–H). This was also the case for the endocardium and valve leaflets, which were analyzed in morphants with a *Tg(flk1:eGFP)^{s843}* background (Fig. S8I–L). Taken together no evidence was found for a morphological correlate explaining the observed cardiac conduction abnormalities. The expression of *vmhc* and *cmlc2* was also studied and both genes were normally expressed and no difference was seen between *popdc2* morphants and control embryos (Fig. S9A–D). Since in the *popdc2* morphants an AV-block was observed, the expression of *bmp4* and *notch1b*, two established myocardial and endocardial AV canal marker genes were studied. The expression of both genes was not affected by the loss of *popdc2* (Fig. S9E–H). Histology and ultrastructure of 72 hpf *popdc2* morphants and control embryos also revealed no difference (Fig. S10). In order to make sure that at the time the hearts of *popdc2* morphants became arrhythmic, the morpholinos were biological active, RT-PCR analysis using RNA isolated from embryos at 4 and 5 dpf and exon and intron-specific primers was performed (Fig. S11). At both time points the morpholinos efficiently suppressed *popdc2* expression and the transcripts that were present retained intron 1. We therefore conclude that the observed cardiac conduction phenotype was present at a time the *popdc2* morpholinos still had their full biological activity.

Discussion

This study represents the first functional analysis of the *popdc2* gene. We report that the zebrafish *popdc2* gene is required for skeletal and cardiac muscle development. During trunk muscle development the morphants displayed signs of a myopathy, which was characterized by a loss of individual myofibers, aberrant muscle segmentation, and myofibrillar disarray. A similar phenotype has also been reported in a number of other genes acting in different cellular pathways during muscle development (Dowling et al., 2008;

Nixon et al., 2005; Raeker et al., 2010; Zoeller et al., 2008). Interestingly, in case of craniofacial muscle development, we observed a malformation and size reduction of individual muscles. It will be interesting to find out whether the phenotypes in the head and trunk muscles are based on the same molecular mechanism. In order to gain further insight in the underlying pathology, it will be important to study migration, fusion and differentiation of muscle precursor cells by time-lapse analysis in vivo. Interestingly, a muscle phenotype was also observed in case of the related family member *Popdc1* in mice (Andrée et al., 2002). The *Popdc1* null mutant displayed an impaired ability of skeletal muscle regeneration in the adult. In this regard it is also noteworthy that two recently identified *Popdc1*-interacting proteins, GEFT and VAMP2/VAMP3 have both been implicated in skeletal muscle regeneration (Bryan et al., 2005; Hager et al., 2010; Smith et al., 2008; Tajika et al., 2007). Thus, apparently members of the *Popdc* gene family have essential functions in the development and regeneration of skeletal muscle.

Many *popdc2* morphants displayed a cardiac conduction defect characterized by 2:1 or 3:1 AV block. In addition, variability in action potential duration in the atrium was observed and in rare cases a sinoatrial block was seen. At later stages of development most of the injected embryos had a silent heart phenotype leading ultimately to the death of the animals. The cardiac electrical phenotype was not observed during early heart development and was first seen in some embryos at 3 dpf. The electrical phenotype became more robustly visible at 4–5 dpf. Recently, it has been proposed that in zebrafish the development of the cardiac conduction system consists of three developmental steps (Chi et al., 2008). In the first step, primary pacemaker development is accomplished at 24 hpf. The atrioventricular delay of electrical conduction develops at 36 to 48 hpf, and at 96 hpf the fast ventricular conduction system becomes fully functional. Cardiac electrical activity is however subject to further steps of maturation such that the QRS interval time is continuously decreasing during larval development (Yu et al., 2010). Maturation of the cardiac electrical system is probably accomplished by anatomical changes and improved electrical coupling between myocytes but may also relate to changes in expression level, membrane localization and complex formation of ion channels and regulatory proteins, which are involved in action potential generation. Whether the defects seen here in the *popdc2* morphants are caused by aberrant conduction tissue development or are due to an essential function of *popdc2* in action potential generation needs to be further studied. The AV block, which is observed in many *popdc2* morphants resembles the phenotype of the *breakdance* mutant (Kopp et al., 2005), which is defective in the *Kcnh2* potassium channel involved in cardiac repolarization (Arnaout et al., 2007; Langheinrich et al., 2003). The variability in the peak intervals in the *popdc2* morphant can be explained on the basis of a defect in cardiac repolarization. The mouse *Popdc* proteins are localized to the plasma membrane in cardiac myocytes and an interaction with TREK-1, a member of the tandem pore family of potassium channels has been observed (Froese et al., 2012). Therefore it will be important to define whether also in case of the zebrafish protein an interaction with ion channels can be demonstrated. Significantly the *Popdc1* and *Popdc2* null mutants in mice display cardiac arrhythmia (Froese et al., 2012), suggesting an evolutionary conserved function in cardiac electrical activity. Novel molecular insight may also come from studying the electrophysiological properties of cardiac myocytes isolated from *popdc2* morphants. It will also be important to study the phenotype of *popdc1* and *popdc3* morphants in zebrafish. It is likely that cardiac and skeletal muscle phenotypes are also present in these morphants given that in all vertebrates studied so far, the *Popdc* genes are expressed in muscle and heart (Andrée et al., 2000; Breher et al., 2004; Froese and Brand, 2008; Hitz et al., 2002; Parnes et al., 2007; Torlopp et al., 2006).

Gene defects in ion channel genes causing cardiac conduction defects such as long QT syndrome in humans have been successfully modeled in zebrafish through mutagenesis or

morpholino-mediated suppression of the corresponding homologs (Morita et al., 2008). The phenotypes of those mutants resembled to a large extent the conduction defects observed in patients and this is probably due to the fact that man and zebrafish have similar heart rates and utilize similar sets of ion channels for action potential generation (Milan and Macrae, 2008). Therefore the here observed conduction defects in zebrafish could be taken as an indication for a possible association of *POPDC2* mutations with cardiac conduction defects in patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Expression of *popdc2* during zebrafish development. (A) RT-PCR analysis of *popdc2* expression during development. (B–R) Whole mount in situ hybridization analysis using a full-length *popdc2* probe of zebrafish embryos at (B,C) 16-somites stage, (D,E) 22 hpf, (F–H) 24 hpf, (K–M) 48 hpf, and (P–Q) 72 hpf. Stained embryos are viewed from (B,D,F,G,K,L,P,Q) left lateral, (C,E) dorsal, and (H,M) ventrofrontal. Panel (I,J,N,R) depicts transversal and (O) sagittal sections through stained embryos. (S) RT-PCR analysis of *popdc2* expression in adult tissues. Abbreviations: a—atrium; cc—cardiac conus; dpw1–5—dorsal pharyngeal wall muscles 1–5; eom—extraocular muscles; fm—facial muscles; GI tract—gastro-intestinal tract; h—heart; ht—tubular heart; m—myotome; pf—pectoral fin bud; s—somites; skm—skeletal muscle; tv1–5—transversus ventralis muscles; v—ventricle. Scale bar=100 μ m.

Fig. 2.

Aberrant skeletal muscle development in *popdc2* morphants. Skeletal muscle tissue formation in the trunk of (A–C) 24 hpf and (D–F) 72 hpf embryos, which were (A,D) wild type (WT), or injected with (B,E) MO1-*popdc2*, and (C,F) MO1/MO2-*popdc2* morpholinos, respectively. Skeletal muscle development was studied by confocal microscopy using rhodaminephalloidin staining. (A'–F') depict the morphology of single muscle segments at higher magnification, which are labeled in (A–F) with a white square. The trunk musculature is highly aberrant in the morphants. Segment borders are not well developed and variable in size (square brackets in B). Moreover, muscle fibers are missing in some areas (arrows in E, F) or are detached (arrowheads in C). Scale bar=100 μ m.

Fig. 3.

Fast and slow muscle formation in *popdc2* morphants. Confocal imaging of transverse sections (A,D,G,J) through the 27 hpf trunk and lateral flatmounts of (B,E,H,K) 1 dpf and (C,F,I,L) 3 dpf embryos, which were injected with (A–C,G–I) MO-control (MO-CTR) or (D–F,J–L) MO1/MO2-*popdc2* morpholinos, respectively and stained (A–F) with F310 antibody to visualize fast muscle fibers, and (G–L) with F59 antibody to detect slow muscle fibers. Sections stained for slow muscle fibers (G,J) were also stained with phalloidin detecting F-actin to visualize the fast muscle compartment (green). Asterisks in (K,L) pointing to gaps in the fiber arrangement. Arrows in (F) indicate ruptured fast muscle fibers and in (L) point to slow muscle fibers with a wavy morphology. Scale bars=50 μ m.

Fig. 4.

Myotendinous junctions are altered in *popdc2* morphants. Confocal imaging of the trunk musculature of 24 hpf embryos in a lateral view, anterior to the left. (A,B) FAK and (C,D) vinculin expression in (A,C) MO-control and (B,D) MO1-*popdc2* injected embryos. In the *popdc2* morphants the shape of the myotomal boundaries is disturbed and the expression domains of FAK and vinculin are discontinuous (arrow heads). (A'–D') Beta-catenin staining. (A''–D'') Merge of panel (A–D) and (A'–D'). Scale bar=50 μ m.

Fig. 5.

Murine *Popdc2* is able to rescue the morphant phenotype. (A–H) Lateral view of the (A,E) uninjected wild-type embryos (WT) as controls and embryos injected with either (B,F) 100 pg *Popdc2* cRNA, (C,G) 1 ng of MO1-*popdc2*, or (D,H) 100 pg *Popdc2* cRNA and MO1-*popdc2* morpholino, which were photographed at (A,C,E,G) 24 hpf, or (B,D,F,H) 48 hpf. (I–L) Lateral confocal stacks of the zebrafish trunk labeled with phalloidin rhodamine at 48 hpf. (I) control, (J) *Popdc2* cRNA, (K) MO1-*popdc2*, (L) *Popdc2* cRNA and MO1-*popdc2* morpholino. (M) Graphic depiction of the percentage of embryos with wildtype appearance (WT) (blue), or weak (pink), and severe (red) pathological phenotypes. Significant difference ($p < 0.05$) is marked with an asterisk. Scale bar=100 μ m.

Fig. 6.

The craniofacial skeletal muscles in *popdc2* morphants exhibit an abnormal morphology. Confocal analysis of heads of 3 dpf Tg(acta:GFP) embryos, which were injected with (A,C) MO-control (MO-CTR) or MO1/MO2-*popdc2* morpholinos, respectively. In the morphants craniofacial muscles are malformed and in many cases significantly reduced in size. Abbreviations: ah, adductor hyoideus; am, adductor mandibulae; ao, adductor operculi; do, dilator operculi; hh, hyohyoideus; ih, interhyoideus; ima, intermandibularis anterior; imp, intermandibularis posterior; io, inferior oblique; ir, interior rectus, lap, levator arcus palatini; mr, medial rectus; sh, sternohyoideus; so, superior oblique; tv 1–5, transversus ventralis 1–5. Scale bar=50 μ m.

Fig. 7.

Cardiac dysmorphogenesis in the *popdc2* morphant. Zebrafish embryos were injected with 2 ng/embryo of (A,C,E) control morpholino (MO-CTR) or (B,D,F) MO1/MO2 *popdc2* morpholinos. (A,B) Confocal analysis of 3 dpf transgenic Tg(*cmhc2*:GFP) hearts. The heart of the *popdc2* morphants is dysmorphic and lacks trabeculation. (C,D) Anti-Alcam antibody staining reveals normal cell shape in the *popdc2* morphants. (E,F) Anti-tropomyosin antibody staining shows a reduction in myofibrillar content in cardiac myocytes of the *popdc2* morphants. Abbreviations: A, atrium; V, ventricle. Scale bar=10 μ m.

Fig. 8.

Popdc2 morphants display cardiac conduction defects. (A) Luminance periodogram of hearts injected with control morpholino (CTR), or *MO1-popdc2* morpholino at 5 dpf. Black bars indicate 3:1 and light gray bars 2:1 atrio/ventricular rhythm. (B) Beating frequencies of *popdc2* morphants and control animals. The number of animals that were analyzed at each time point is given at the base of each column. The difference in beating frequency of control and *popdc2* morphants reached statistical significance ($p < 0.05$) at 5 dpf and 7 dpf.

Fig. 9.

The hearts of *popdc2* morphants display a variety of cardiac conduction defects. SPIM videos of the heart of zebrafish embryos injected with (A) control (CTR) and (B–D) *MO1-popdc2* morpholino in the *Tg(cmlc2:gCaMP)s878* background. The individual movies (see SI Movies 5–8) were processed to determine the fluorescence intensity of selected regions of the heart over time. Each selected region has a corresponding number plotted below. (A) In a control (CTR), fluorescence intensity varies with time in atrial and ventricular regions as the depolarization wave propagates through the heart. (B) Heart displaying a single incidence (asterisks) of a 4:1 AV block. (C) Heart displaying alternating phases of normal conduction and 2:1 AV block (asterisks). Both hearts depicted in (B) and (C) also display irregular atrial conduction waves. (D) Example of a 6 dpf heart with sinoatrial block (asterisk).

Fig. 10.

Variability in the peak interval between control and MO1-*popdc2* morphants. The intervals between individual peak maxima in (A) atrium and (B) ventricle of control and *popdc2* morphants were measured during a ten second period and depicted as a box and whisker plot. The data are derived from the tracings depicted in Figs. 4A–D. In the case of MO1-A and MO1-B the peak intervals show variability in the atria and ventricle suggesting that an electrophysiological defect is present in both chambers of the *popdc2* morphants.