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Two distinct phosphorylation events govern the function of muscle FHOD3

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Abstract

Posttranslational modifications such as phosphorylation are universally acknowledged regulators of protein function. Recently we characterised a striated muscle-specific isoform of the formin FHOD3 that displays distinct subcellular targeting and protein half-life compared to its non-muscle counterpart, which is dependent on phosphorylation by CK2 (formerly casein kinase 2). We now show that the two isoforms of FHOD3 are already expressed in the vertebrate embryonic heart. Analysis of CK2alpha knockout mice showed that phosphorylation by CK2 is required for proper targeting of muscle FHOD3 to the myofibrils also in embryonic cardiomyocytes *in situ*. The localisation of muscle FHOD3 in the sarcomere varies depending on the maturation state, being either broader or restricted to the Z-disc proper in adult heart. Following myofibril disassembly such as in dedifferentiating adult rat cardiomyocytes in culture, the expression of non-muscle FHOD3 is up-regulated, which is reversed once the myofibrils are reassembled. The shift in expression levels of different isoforms is accompanied by an increased co-localisation with p62, which is involved in autophagy, and affects the half-life of FHOD3.

Phosphorylation of three amino acids in the C-terminus of FHOD3 by ROCK1 is sufficient for activation, which results in increased actin filament synthesis in cardiomyocytes and also a broader localisation pattern of FHOD3 in the myofibrils. ROCK1 can directly phosphorylate FHOD3 and FHOD3 seems to be the downstream mediator of the exaggerated actin filament formation phenotype that is induced in cardiomyocytes upon the overexpression of constitutively active ROCK1. We conclude that the expression of the muscle FHOD3 isoform is characteristic for the healthy mature heart and that two distinct phosphorylation events are crucial to regulate its activity in thin filament assembly and maintenance.

Keywords

myofibril; formin; cardiac cytoarchitecture; heart development

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Introduction

The pumping action of the heart is carried out by highly specialised, terminally differentiated cells, the cardiomyocytes. Throughout life the heart has to adapt to changes in demand by changing the size of the individual cardiomyocyte, which results in physiological hypertrophy (induced by developmental growth, exercise or pregnancy), pathological hypertrophy (e.g. induced by hypertension) or atrophy [1]. At the level of the cardiomyocyte this leads to increased assembly or disassembly of the contractile structures, the myofibrils, as well as to changes in their isoform composition especially of myosin and actin [2,3]. However, in addition to physiological adaptation, there is also a continuous replacement of proteins due to maintenance, which has been estimated to result in a half-life between three and four days for some myofibrillar proteins [4]. Currently very little is known, how the maintenance and additional synthesis of actin filaments is achieved in the developing and the mature heart [5,6]. Recently several labs have taken inspiration from the well-characterised events during actin filament formation in migrating cells [7] and could for example show a role for well-known players such as N-WASP during myofibrillogenesis in skeletal myocytes [8]. We have characterised a striated muscle specific splice variant of the formin FHOD3 that appears to be required for myofibril maintenance in cultured cardiomyocytes and was shown to be downregulated in heart disease [9]. This isoform is characterised by the presence of eight additional amino acids at the C-terminal end of the FH2 (formin homology 2) domain, which result in a target site for phosphorylation by CK2 (formerly known as casein kinase 2). CK2 is a constitutively active ubiquitously expressed serine/threonine kinase with a plethora of substrates [10,11]. In addition to its well-established role in cancer [12], embryonic development [13,14] and to its recently described role in proliferation [15,16], CK2 is starting to be also recognised as a regulator of the cytoskeleton and ultimately cell morphology [17]. The phosphorylation by CK2 affects the subcellular targeting as well as the half-life of muscle FHOD3 by preventing its interaction with p62, a protein involved in autophagy, but does not appear to affect the ability of FHOD3 to support actin filament synthesis [9].

FHOD3 is a member of the diaphanous related formins (DRF), which in addition to the formin domains FH1 and FH2 contain an N-terminal GTPase binding domain (GBD), a diaphanous autoinhibitory domain (DID) and a C-terminal diaphanous autoregulation domain (DAD; for reviews on formins see [18–21]. Members of this formin family exist in an autoinhibited state, brought about by the intramolecular interaction between DID and DAD, which has to be relieved by the binding of a small GTPase such as Rac1 or Rho to lead to an active protein that can dimerise and stimulate the synthesis of actin filaments [22]. For FHOD3's closest relative FHOD1 the possibility of a more direct activation was suggested recently, where the phosphorylation of three amino acids in the DAD domain by ROCK1 was sufficient for release of the autoinhibited state [23,24].

Aims of our study were to establish whether the muscle FHOD3 isoform is also expressed during heart development. In addition, we wanted to find out whether its subcellular localisation is indeed regulated by CK2 and how the activity of FHOD3 is controlled in cardiomyocytes. We were able to show that there is a tight correlation between the fully differentiated state of a myofibril and high levels of muscle FHOD3 expression and that the presence of CK2 is needed to target FHOD3 to the myofibrils. Non-muscle FHOD3 is expressed at higher levels in the embryonic atrium and also gets upregulated in dedifferentiating adult rat cardiomyocytes in culture, similar to the situation already described for the diseased heart [9]. In addition, we could demonstrate that the activation of FHOD3 is regulated in a similar way as FHOD1 and that its constitutive activation leads to excessive actin filament synthesis in cultured cardiomyocytes. We propose a link between

the subsarcomeric localisation of FHOD3 and the extent of actin filament synthesis in cardiomyocytes, which is governed by two separate phosphorylation events.

Materials and methods

Preparation of transfection constructs

Cloning of FHOD3-GFP and -HA, the DAD deletion constructs, FHOD3-CT-GFP and GST, as well as the CK2 consensus site (T(D/E)₅XE exon) splice variants and mutants was described previously [9]. FHOD3-3D-GFP and FHOD3-CT-GFP (ROCK3D) were created by standard site directed mutagenesis of S1590, S1596 and T1600 to Aspartic acid using 5'-GAAACGAGACCGGGCCAACCGGAAAGACTTGCGAAGAGACCTGA AG-3'. Since mutation of S1590 failed, 5'-GCCGAGGGAGAGGAAACGAGACCGGGCCAACCGGAAAGA-3' primers were used in a second step. FHOD3-NT-GST was created using 5'-GGAATTCCGCCACCATGGCCACGCTGGCTTGCC-3' forward and 5'-CGGGATCCCGCCCGGCACCCACTGGG-3' reverse primers and FHOD3-GFP as a template by cloning into the EcoRI and BamHI sites of a GST-CI vector. p160-ROCK1-myc and p130-ROCK1-myc were kindly provided by S. Narumiya (Department of Pharmacology, Kyoto University, Japan).

Knockdown by shRNA was achieved by using plasmids described previously that encode for the shRNA under the control of an H1 expression cassette together with GFP under the control of a CMV promoter [9]. The vector just containing the H1 expression cassette and GFP was used as a control.

In vitro phosphorylation assays

FHOD3 CT was subcloned into a pGEX-C1 vector using BglII and SacII restriction sites. The Rho Kinase substrate motif was mutated using the quick change 2 site directed mutagenesis kit (Agilent) following the manufacturers instructions in three consecutive steps with 5'-CGAGGGAGAGGAAACGAGCCCGGGCCA-3', 5'-CGGGCCAACCGGAAAGCTTTGCGAAGAAGACCC-3', and 5'-AACCGGAAAGCTTTGCGAAGAGCCCTGAAGAGC-3' sense and the respective anti-sense primers. FHOD3-CT-GST and CT-3A-GST were expressed in E. coli BL21 Star (Invitrogen). The peptides were purified using glutathion sepharose (Amersham). Equal amounts of CT-GST and CT-3A-GST were incubated with 20 mU recombinant active ROCK-I (Millipore) in 8 mM MOPS, 10 mM MgAc, 0.1 mM ATP and 1 mM EDTA at pH7.0 for 5 minutes at 30°C and then boiled in SDS buffer and blotted with rabbit anti-phosphoserine/threonine (ECM Biosciences), mouse anti-GST (Cell Signaling) and anti-His (Qiagen) antibodies.

Chick embryos

Fertilised hens eggs (mixed flock Henry Stewart, Peterborough, UK) were incubated in a humidified atmosphere at 37°C. Embryos were staged according to Hamburger and Hamilton [25] and RNA was extracted either from whole embryos or from dissected atria and ventricles and used for RT-PCRs. Alternatively, embryos were stored in 4% paraformaldehyde (PFA) in PBS at 4°C until use for in situ hybridisation experiments.

RT-PCR

RNA was extracted with Trizol (Invitrogen) following the manufacturer's instructions. cDNA pools were prepared using M-MLV reverse transcriptase (Promega, Madison, WI, USA).

RT-PCRs for murine samples were performed as described previously [9], for the amplification of T(D/E)₅XE exon containing and excluding isoforms of *G. gallus* FHOD3 we used 5'-AATGATCACAGATACTGATGAAGAAG-3' and 5'-TGATCACAGATTCCGGAAAG-3' forward and 5'-TCCTTCTCAATGACTTCCTATTTG-3' reverse primers, for the amplification of FHOD3 independent of the T(D/E)₅XE exon ("all isoforms") we used an 5'-AGGTTTCACTCATTTTGTATTATG-3' forward and the same reverse primer and for the amplification of *G. gallus* Glyceraldehyde 3-phosphate dehydrogenase (GAP-DH) 5'-CTGAAAATTGTCAGCAATGCATCG-3' forward and 5'-CCAGTGGACGCTGGGATGATGTTC-3' reverse primers.

In situ hybridisation

Fragments of FHOD3 were amplified with 5'-GGGGTACCACAGATACTGATGAAGAAG-3' forward and 5'-GGAATTCTCTCAATGACTTCCTATTTG-3' reverse primers for T(D/E)₅XE specific probes and with 5'-GGGGTACCATCACAGATTCCGGAAAG-3' forward and 5'-GGAATTCTCTCAATGACTTCCTATTTG-3' reverse primers for T(D/E)₅XE excluding probes (DT(D/E)₅XE) and cloned into the KpnI and EcoRI sites of a pGEM-3Z vector (Promega). Digoxigenin (Roche) labelled sense and antisense RNA probes were created by digestion with EcoRI and KpnI, respectively and transcription with SP6 and T7 (both Promega), respectively.

Whole-mount *in situ* hybridisation was carried out as described previously using a probe concentration of 1 µg/ml [26]. For detection embryos were incubated with digoxigenin antibodies conjugated to alkaline phosphatase and subsequently with NBT/BCIP (Roche) for visualisation. Embryos were embedded in a mix of gelatine type A, egg albumin and sucrose and crosslinked by glutaraldehyde [26]. Embryos were cut into 50 µm sections on a VT1000S vibratome (Leica, Wetzlar, Germany), placed on glass microscope slides and mounted in glycerol.

Pictures of embryos and sections were taken on a Leica MZ16 stereo-microscope.

Interaction assays

GST pulldown assays and co-immunoprecipitations were performed as described previously [9]. COS cell lysates for GST pull down assays to assess the FHOD3 autointeraction were prepared in the presence of phosphatase inhibitor (0.5 µM calyculin A, 0.2 mM NaVO₄ and 30 mM sodium pyrophosphate).

Immunofluorescence

Freshly dissected embryos were collected at embryonic day (E) 9.5 and E13.5, washed in PBS and fixed overnight at 4°C with 4% paraformaldehyde in PBS. After fixation, embryos were washed in PBS and stored in 70% ethanol until used. For embedding, embryos were dehydrated by washing them for 5 min in 80% EtOH, 90% EtOH, 96% EtOH, twice in 100% EtOH, and cleared by washing in 1:1 EtOH/xylene and xylene for one hour. Then, embryos were incubated in 1:1 xylene/paraplast (Oxford, St. Louis, USA) for 1 h at 60°C and incubated in 100% paraplast three times at 60°C for 30 min to overnight. Embedded embryos were serially sectioned into 5 µm sections with an 820 microtome (American Optical Company), mounted on Superfrost microscope slides and dried overnight. Sections were deparaffinised by a xylene – ethanol – water series and epitopes were retrieved by boiling in 10 mM citric acid (pH 6.0) for 3x 5 min. MAXblock blocking reagent was used before stainings with the FHOD3 antiserum according to the manufacturers' instructions (Active Motif, Carlsbad, CA, USA). The sections were incubated with the primary antibody

diluted in antibody dilution buffer (1% BSA, 20 mM Tris-Base, 155 mM NaCl, 2 mM EGTA, 2 mM MgCl₂ at pH 7.5) for 1 h at RT and then washed 3 times in PBS, followed by the incubation with the secondary antibody solution for 1 h at RT. Sections were mounted in 0.1 M Tris-HCl/glycerol (3:7) and 50 mg/ml n-propyl-gallate at pH 9.5.

Cells and culture

COS-cells, HeLa cells and Neonatal Rat Cardiomyocytes were prepared and cultured as described previously [9]. Adult Rat Cardiomyocytes were isolated by Langendorff perfusion of hearts and cultured in DMEM with 10% Fetal Calf Serum on collagen coated plastic dishes [27]. Cells were fixed with 4% paraformaldehyde in PBS, permeabilised with 0.2% Triton X-100 and stained as described above for the embryonic mouse sections. Quantification of actin polymerisation activity was performed as reported previously [9].

Confocal microscopy

Confocal microscopy was performed on a LSM510 laser scanning confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) or an SP5 confocal microscope (Leica, Mannheim, Germany) with solid state (diode) laser excitation at 405 nm and emission at 420–480 nm, Ar-laser excitation at 488 nm and emission at 505–530 nm or He-Ne-laser excitation at 563 and 633 nm and emission at 560–615 nm and > 650 nm, respectively using LCI Plan-Neofluar 25x/0.8, Plan-Apochromat 63x/1.4 or Plan-Neofluar 100x/1.3 oil objectives.

Transmission electron microscopy

Embryos were fixed at E10.5 in 4.3% glutaraldehyde (Polysciences)/0.03 mol/L sodium barbital–sodium acetate buffer (pH 7.4)/0.07 mol/L potassium chloride for 24 h at RT. The samples were then rinsed with 1X PBS (3x 15 min), and post-fixed in a solution of 1% osmium tetroxide (Ted Pella)/0.03 mol/L sodium barbital–sodium acetate buffer (pH 7.4)/0.07 mol/L potassium chloride, for 1–2 h at RT. Embryos were then dehydrated through 70, 80, 95, and 100% EtOH washes (15–20 min) and allowed to remain in 100% EtOH for 1 h with 2 changes. A mixture of Araldite 502 and dodecenyl succinic anhydride (DDSA) was prepared by combining equal volumes of Araldite 502 and DDSA. Then, the embryos were placed in a 1:1 ratio of propylene oxide:Araldite 502–DDSA mixture for 30 min, and then treated with a 1:3 ratio of propylene oxide:Araldite 502–DDSA mixture for 60 min. The samples were then immersed in a freshly prepared 1:1 mixture of Araldite 502:DDSA with accelerator (2% benzyl dimethyl amine) and incubated at 60 °C for 18 h for polymerisation. Sections were cut on an LKB Ultratome V. Ribbons of sections showing gray, silver, or slightly gold interference colors were picked up on uncoated 200 mesh Athene Thin Bar copper grids (Ted Pella). Electron micrographs were obtained from sections stained with uranyl acetate followed by lead citrate using either a JEM-1011 or a Philips 300 transmission electron microscope.

Antibodies

The polyclonal rabbit anti-FHOD3 antibody was prepared and characterised as described previously [9]. The monoclonal mouse (mM) anti-sarcomeric alpha-actinin (clone EA-53) and anti-Troponin T (TnT, clone JLT-12) antibodies were obtained from Sigma (St. Louis, MO, USA). The mM anti-GFP (mixture of clones 7.1 and 13.1), anti-c-myc (clone 9E10) and the monoclonal rat anti-HA antibody (clone 3F10) were purchased from Roche Applied Science (Mannheim, Germany). The mM anti p62 was from Abcam (Cambridge, UK). The polyclonal goat anti-GST antibody was obtained from GE-Healthcare (Amersham Place, UK). The mM anti-myomesin clone B4 was a gift from Prof. Jean-Claude Perriard's Lab (Institute of Cell Biology, ETH-Zürich Hönggerberg, Zürich, Switzerland). Horseradish

peroxidase-conjugated anti-mouse Igs were purchased from DAKO (Glostrup, Denmark). Horseradish peroxidase-conjugated anti-rabbit Igs and anti-goat Igs were purchased from Calbiochem (San Diego, CA, USA). Cy2-conjugated anti-rabbit Igs, Cy3-conjugated anti-mouse Igs and Cy5-conjugated anti-rabbit and anti-mouse Igs were all purchased from Jackson Immuno Research (West Grove, PA, USA). Phalloidin (Alexa633- and Alexa546-conjugated, respectively) was bought from Invitrogen.

Results

The expression of FHOD3 isoforms is linked to the establishment of a dense myofibril arrangement

Our initial characterisation of muscle and non-muscle FHOD3 isoforms as distinguished by the presence or absence of the T(D/E)₅XE exon suggested a striated muscle specific expression pattern of T(D/E)₅XE exon-containing isoforms (hence termed muscle FHOD3) [9]. Since this could be a hallmark only of fully differentiated muscle, we wanted to investigate the expression of different FHOD3 isoforms during embryonic heart development. RT-PCR analysis showed that FHOD3 lacking the T(D/E)₅XE exon (Chromosome 2: 85704901:85705524, see [9] for sequence alignment) is already expressed faintly in whole chick embryos of Hamburger Hamilton (HH; [25]) stage 4 and that the first apparent expression of the T(D/E)₅XE exon containing FHOD3 can be detected at HH11 (Supplementary Figure 1A). Using isoform-specific probes *in situ* hybridisation experiments showed a first difference in localisation of both probes at stage HH28 (Figure 1), when expression of the non-muscle isoform is restricted to the atrium, while the muscle isoform is highly enriched in the ventricle. There it is localised in the compact and trabeculated myocardium, as confirmed on cross-sections through the embryos. Interestingly, even in the atrium there is a difference in the expression of both isoforms, the muscle isoform is expressed in almost all atrial regions, while the non-muscle isoform is mainly restricted to the atrial wall. Expression of both isoforms is also seen in the limb buds at this stage. These observations indicate that the muscle isoform, which contains the T(D/E)₅XE exon, is expressed at higher levels in the ventricle, while the non-muscle isoform is expressed higher in the atria. RT-PCR with isoform-specific primer pairs on dissected embryonic atria and ventricles supported this interpretation (Supplementary Figure 1B).

We turned to the mouse as an experimental system to determine whether this developmental shift in FHOD3 isoform expression levels is a general occurrence during vertebrate cardiac development. Analysis of the expression patterns of FHOD3 isoforms in developing mouse hearts by RT-PCR detects expression of both isoforms in whole hearts of E (embryonic day) 14 mouse embryos, although the non-muscle isoform seems to be expressed at higher levels (Figure 2A). That expression ratio changes in favour of muscle FHOD3 during the early postnatal phase of hypertrophic cardiac growth in mouse heart. These observations confirm our hypothesis that the expression of muscle FHOD3 is a hallmark of fully differentiated cardiomyocytes. To analyse the localisation of FHOD3 at a subcellular level we performed immunostainings of mouse heart paraffin sections. Since our FHOD3 antibodies recognise both isoforms and all attempts to produce a muscle isoform specific FHOD3 antibody failed so far, we were unable to study isoform-specific subcellular localisation differences. FHOD3 can be detected in cardiomyocytes as early as E9.5 (Figure 2B) in a diffuse staining pattern with a more intense signal along the myofibrils. As myofibrillogenesis proceeds and the thin filaments get more organised (indicated by the anti-TnT staining) at E13.5, the FHOD3 staining pattern gets restricted and is mostly found around the Z-disc. In the healthy adult heart FHOD3 is exclusively found at the Z-disc, as indicated by its clear alternating pattern with myomesin, a marker of the M-band (Figure 2B). Thus, FHOD3 subcellular localisation seems to be dependent on the maturity of the myofibrils, since while its localisation is restricted to the Z-disc in adult heart, it is found in a much broader distribution around the Z-

disc in the embryonic heart. This localisation pattern nicely mirrors the results obtained by immunostaining or transfection of epitope-tagged FHOD3 in cultured neonatal rat cardiomyocytes (NRC; [9]) and may indicate a higher rate of turnover or myofibrillogenesis activity.

To investigate this further we utilised the model system of adult rat cardiomyocytes (ARC) in high serum culture [27,28]. After isolation by Langendorff perfusion of the heart with collagenase and plating on collagen-coated plastic dishes in medium containing 10% FCS, ARCs undergo a programme of de-differentiation and re-differentiation of their myofibrils and change from a rod-shaped morphology to a more flattened, fried egg-like shape similar to most cells in culture. After two weeks in culture and the re-assembly of their myofibrils they also start again to beat, indicating full functionality of their contractile systems [27,28]. This well characterised disassembly and re-assembly process of the myofibrils allowed us to investigate potential changes in the FHOD3 isoform expression pattern in a purified population of cardiomyocytes and alterations in subcellular targeting pattern.

Analysis of the FHOD3 isoform expression pattern by RT-PCR showed a simultaneous upregulation of the non-muscle and downregulation of the muscle isoform of FHOD3 by day (d) 3 (Fig 3A) and a reversion by d12, when muscle FHOD3 appeared to be the major isoform again. The shift in FHOD3 isoform expression appears to be correlated also with a differential localisation pattern: While displaying nice Z-disc association in freshly isolated ARC, FHOD3 localisation started to become more diffuse 3 days after plating (Fig 3B). Interestingly, around this time FHOD3 started to appear in autophagosome-like vesicles, as judged by co-localisation with p62 (Figure 3C). After day 8 post plating, although still strongly localising to autophagosomal structures in the cell body, we additionally detected targeting of FHOD3 to the newly forming stress-fibre like structures in the regions close to the cell attachments. During the following days more and more FHOD3 was recruited to the actin filaments, coinciding with increased expression levels of the muscle isoform. Two weeks after plating FHOD3 is strongly localised to the actin structures in regions with continuous alpha-actinin staining pattern, indicative of areas of myofibrillogenesis [29]. Additionally, in line with our hypothesis that the changes in targeting are depending on myofibril dynamics, FHOD3 was found in a broad striated pattern similar to that observed in embryonic hearts and NRCs in regions with further advanced sarcomere formation and properly striated alpha-actinin staining (magnified image in Figure 3B, lower panel). Therefore the differential targeting in the sarcomere itself cannot be explained by attributing it to different FHOD3 isoforms, since at this stage muscle FHOD3 seems to be the prevalent isoform. The localisation of FHOD3 to autophagosome-like structures does seem to correlate with isoform identity, though and thus with the ability/inability to interact with p62 [9]. These experiments support our hypothesis that muscle FHOD3 isoform expression and FHOD3 localisation at the Z-disc is a hallmark of mature myofibrils [30] and that challenges to this strictly organised cytoarchitecture lead to an upregulation of the non-muscle isoform and hence increased FHOD3 protein turnover.

Muscle FHOD3 targeting as regulated by CK2 seems required for myofibril maturation in the ventricle

Our results in cultured NRC had indicated that muscle FHOD3 has to be phosphorylated by CK2 to target to the myofibrils [9]. To investigate this experimentally in an *in situ* setting, we made use of the CK2alpha knockout mice [14]. Hearts from CK2alpha knockout mice at E9.5 have less developed myofibrils in comparison to hearts from wild type littermates (Figure 4). Nevertheless, sarcomeric myosin could be detected in a cross-striated pattern, albeit with slightly reduced sarcomere length. However, clear myofibrillar association could never be observed for FHOD3 in CK2alpha knockout hearts, since it localised in a more diffuse fashion (Figure 4B). There is a higher occurrence of FHOD3 speckles in the absence

of CK2alpha (arrows in Figure 4B). This is similar to the localisation that we described for non-muscle FHOD3 [9]. This isoform lacks the CK2 consensus site and is targeted to autophagosomes due to its interaction with p62, both in transient transfection experiments as well as in cardiomyopathy [9]. Unfortunately we were unable to demonstrate the co-localisation of FHOD3 and p62 in the CK2alpha knockout hearts, due to the lack of reactivity of the monoclonal mouse anti p62 on paraffin embedded tissue. Electron micrographs of the CK2alpha knockout hearts also showed more sparse myofibrils with slightly reduced sarcomere length (Figure 5). Interestingly, this phenotype is only apparent in the ventricles, while atrial tissue appears to be spared (Figure 5, bottom row). Since in wildtype embryos CK2alpha is expressed equally in atria and ventricles (Degano et al., manuscript submitted), this suggests that CK2alpha activity and possibly its action on muscle FHOD3 is only required for the assembly and maintenance of myofibrils in the embryonic ventricle.

C-terminal phosphorylation by ROCK1 is sufficient for FHOD3 activation

Members of the subfamily of diaphanous related formins such as FHOD3 are normally found in an autoinhibited form in cells [31]. Active dimers, which can aid the assembly of actin filaments, can only be formed once the autointeraction between the N-terminal DID and the C-terminal DAD domain is prevented. FHOD1, FHOD3's closest paralog, can be activated by ROCK1 [24], which phosphorylates a consensus site in the DAD domain. A similar potential consensus site is conserved in the FHOD3 DAD domain between human and mouse (Supplementary Figure 2A). We therefore wanted to find out, if ROCK1 is also sufficient for the activation of FHOD3. Indeed we found interaction of ROCK1 with FHOD3, which was increased when using a constitutively active form of ROCK1 (Figure 6A). Constructs containing or lacking the T(D/E)₅XE exon bound equally well to constitutively active ROCK1 (Supplementary Figure 2B). To test for the ability of ROCK1 to activate FHOD3 by preventing its autointeraction, pull down assays with GST tagged N-terminal (DID-) and GFP-tagged C-terminal (DAD-) constructs (Fig 6B) were performed. The constructs interacted with each other, but the interaction was strongly reduced in the presence of ROCK1 (Fig 6C) or after mutation of the ROCK1 consensus site to mimic the phosphorylated state (Fig 6D). This confirms that the autointeraction can be inhibited via phosphorylation of the DAD domain by ROCK1. To prove that this activation also has functional consequences, we carried out actin polymerisation assays in HeLa cells transfected with different FHOD3 constructs. Increased actin polymerization activity as indicated by increased phalloidin staining was detected in HeLa cells transfected with the phosphomimetic mutant compared to control or wild type FHOD3 transfected cells (Figure 6E) and the activity of the phosphomimetic mutant (FHOD3-3D) seemed to be comparable to a constitutively active C-terminal truncation (FHOD3-DDAD; Fig 6F). Therefore FHOD3 is regulated in an analogous fashion to FHOD1 and does not appear to require the binding of a small GTPase of the Rho family to its GBD to relieve the auto-inhibited state, as most other members of the DRF family do [22].

While the effect of activated formins on the cytoskeleton of fibroblasts has been studied intensively, very little is known about any effect of constitutively active formins in muscle cells. To investigate the effect of activated FHOD3 on the cytoskeleton of cardiomyocytes, FHOD3 and ROCK1 were co-transfected into NRC. In a cellular environment a dramatic effect on the actin cytoskeleton was already observed for full length ROCK1 and was even more pronounced for constitutively active ROCK1 (Figure 7). While control cells that just expressed HA-tagged muscle FHOD3 displayed nicely cross-striated thin filaments as visualised by phalloidin (Figure 7A, top row), both double-transfections with ROCK1 resulted in much more pronounced actin cables with a lack of striations (Figure 7A, middle and bottom row). This may indicate an excessive amount of actin filament synthesis. Patches

of strong FHOD3 expression coincided with higher F-actin staining (arrows in Figure 7A). The same observations were made for endogenous FHOD3 in ROCK1 overexpressing cardiomyocytes (Figure 7B), as well as for constitutively active FHOD3 constructs (both DAD deletion constructs as well as ROCK1 phosphomimetic mutants, Figure 7C). To provide evidence that FHOD3 is a direct target for ROCK1 phosphorylation, we performed *in vitro* kinase assays (Figure 8A). His-tagged ROCK1 was either incubated with a wildtype construct of the FHOD3 C-terminus or with a construct, where the three putative ROCK1 phosphorylation sites had been mutated to alanines. A robust phosphorylation signal was only detected with the wildtype construct and not with the unphosphorylatable mutant, which indicates that ROCK1 can indeed phosphorylate FHOD3 directly and that the three amino acids, which we had identified by sequence comparison, are the target amino acids.

As shown above, overexpression of constitutively active ROCK1 results in excessive actin filament polymerisation in NRC (Figure 7A, B). Since this could be due to other proteins than FHOD3, we started to investigate double-transfected NRC, which expressed p130-ROCK1, but also had been subjected to FHOD3 knockdown for 5 days (Figure 8B). The FHOD3 knockdown cells showed the same fragmented myofibril phenotype that we had observed before [9], which also included the actin filaments despite expressing constitutively active ROCK1 (bottom row). Control cells transfected with p130-ROCK1 and the negative control knockdown plasmid displayed an exaggerated actin filament phenotype compared to untransfected neighbouring cells, suggesting that FHOD3 is quite likely the downstream effector of this phenotype.

These results indicate that ROCK1 on its own is sufficient to activate FHOD3 by relieving its intramolecular autoinhibition and that this subsequently leads to increased F-actin polymerisation in HeLa cells as well as in cultured cardiomyocytes. This increased F-actin formation is probably directly due to the activated FHOD3.

Taken together our results are the first study on the developmental regulation of expression and differentiation state specific subcellular localisation of a formin family member, FHOD3, in cardiomyocytes and its dependence on CK2 function. In addition we were able to throw light on the mechanism of activation of FHOD3 by ROCK1 and could demonstrate the effects of constitutively active FHOD3 on actin filament synthesis in cardiomyocytes. We conclude that muscle FHOD3 plays an important role in fine-tuning the extent of thin filament assembly in the sarcomere, in particular in ventricular cardiomyocytes.

Discussion

We have previously reported the existence of a muscle specific splice variant of FHOD3 that is important for myofibril maintenance [9]. In the current work we show that this muscle FHOD3 isoform is already expressed in the developing embryo heart and that its presence at Z-discs is tightly correlated with the existence of mature myofibrils. In case of dedifferentiation events such as in adult cardiomyocytes that are adapting to culture conditions or in cardiomyopathy, a shift to the increased expression of the non-muscle FHOD3 is observed, with the consequences of lack of sarcomeric targeting, increased interaction with p62 and shorter half life of FHOD3 [9]. The expression of FHOD3 in mouse embryos from E8 to E10.5 was already studied earlier by *in situ* hybridisation by Xu and colleagues, who detected a cardiac specific expression pattern at these early stages [32]. It is interesting to note that the expression of non-muscle FHOD3 is maintained in the atrium at a time it already has disappeared from the ventricle. A potential explanation could be that atrial cardiomyocytes are known to be smaller and less differentiated and have a higher proliferative activity also later on during development. Therefore they are the only cardiomyocyte population in the heart from which it was possible to establish a cell line, the

HL-1 cells [33]. We could also show here that the atrial myofibrils are not affected in the CK2alpha knockout mice. This suggests that non-muscle FHOD3 is sufficient for the assembly and maintenance of the more loose myofibrillar network in atrial cardiomyocytes, but that the presence of muscle FHOD3 and its proper targeting to sarcomeres is required for the more dense myofibrillar arrangement in ventricular cardiomyocytes. Interestingly, an actin bundling activity in addition to a nucleation activity has been shown for other formin family members such as e.g. FRL 1 and mDia2, but not for mDia1 [34]. We found previously that mDia1 was much less efficient than FHOD3 in the restoration of actin filaments in NRC following latrunculin B treatment [9] and there is a possibility that muscle FHOD3 may also be involved in the lateral integration of thin filaments in the ventricle.

Analysis of the phenotype of the somites in CK2alpha knockout embryos revealed predominantly a defect in proliferation, while the extent of apoptosis was comparable to controls [16]. It has previously been shown that CK2alpha is the principal isoform in the postnatal heart and that its expression is developmentally regulated with lower levels in adult compared to perinatal hearts [35]. Forced expression of CK2alpha in transgenic mice leads to cardiac hypertrophy [36]. Our results suggest that muscle FHOD3 may be an effector molecule in these events, since elevated levels of CK2 activity would lead to lower FHOD3 protein turnover and therefore improved myofibril maintenance and potentially even more myofibrillogenesis.

The timing of the observed change in expression patterns between muscle and non-muscle FHOD3 isoforms fits extremely well with the switch from hyperplastic to hypertrophic heart growth in the early postnatal phase of mouse heart development [37] and suggests that while muscle FHOD3 can be regarded as a marker for mature myocardium, non-muscle FHOD3 can be added to the list of fetal isoforms that are re-expressed in pathological hypertrophy [27,38–40].

Diaphanous related formins (DRFs) are inhibited by the interaction of the C-terminal diaphanous auto-activation domain (DAD) with the N-terminal diaphanous inhibitory domain (DID). This can be either due to autointeraction [22] or, at least in vitro, also due to homo- and heterodimerisation [41]. FHOD1, unlike other diaphanous related formins (DRFs), is activated upon phosphorylation of three residues in the DAD by ROCK1, which releases the inhibitory interaction between DAD and DID [23,24]. Here we could show that the same activation mechanism applies for FHOD3. Our GST-pull down assay and co-immunoprecipitation results confirmed binding of ROCK1 to FHOD3 and we could also demonstrate direct phosphorylation by ROCK1 in an in vitro kinase assay. Furthermore, we could show that ROCK1 activity inhibits the autointeraction and induces assembly of (linear) actin filaments in HeLa cells as well as the formation of thick actin cables in neonatal rat cardiomyocytes. Mutation of the suspected phospho-acceptor residues S1590, S1596 and T1600 (based on the homology to the FHOD1 residues S1131, S1137, T1141 [24]), to aspartic acids mimicked the effect and confirmed the activation of FHOD3 via ROCK1 phosphorylation. ROCK1 is highly expressed in the embryonic heart and was reported to be involved in several steps of heart development [42,43]. Consistent with a predominant early cardiogenic role, its expression is downregulated after birth, but ROCK1 gets re-expressed again during pathological cardiac hypertrophy [40]. Inhibition of ROCK1 disrupts the formation of striated myofibrils in cultured chicken blastoderm [44,45]. Considering the expression pattern of ROCK1 as well as FHOD3's ability to polymerise actin filaments in cardiac myocytes [9], activation of FHOD3 by ROCK1 could be a crucial event during myofibrillogenesis.

In conclusion, we have described the relevance of two different phosphorylation events that determine the localisation and the activity of muscle FHOD3 (for schematic representation

see Figure 9). Phosphorylation of the C-terminal end of the FH2 domain of FHOD3 by CK2 is crucial for its targeting to myofibrils, where FHOD3 is involved in their maintenance and potentially also in novel assembly and lateral integration. For full activation of FHOD3 it seems to be sufficient that three amino acids in its C-terminus are phosphorylated by ROCK1. This regulation by posttranslational modification together with the alterations in the expression levels of different FHOD3 isoforms, CK2 and ROCK1 over time in the heart appear to make an important contribution to hypertrophic growth seen during early postnatal development and in hypertrophic cardiomyopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CK2	casein kinase 2
DID	diaphanous autoinhibitory domain
DAD	diaphanous autoregulation domain
DRF	diaphanous related formins
E	embryonic day
FH	formin homology
HH	Hamburger-Hamilton
P	postnatal day

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Fig. 1.

Distinct expression of FHOD3 isoforms during early development in the chick embryo. *In situ* hybridisation experiments were carried out using probes specific for the muscle isoform (T(D/E)₅XE exon) and probes excluding the T(D/E)₅XE exon (ΔT(D/E)₅XE exon) and thus specific for the non-muscle isoform on chick embryos at HH28 stage, left and middle columns show overviews, the right column shows cross sections taken at the level of the heart. H heart, v ventricle, hnb hind limb bud, flb fore limb bud, lv left ventricle, la left atrium

Fig. 2. FHOD3 isoforms show a dynamic expression pattern and a developmental stage specific subcellular localisation in the developing mouse heart. **A** RT-PCR was carried out on RNA isolated from mouse hearts at E14, P0, P7, P16 and adult (3 months) stages using forward primers in front of or within the mouse T(D/E)₅XE exon (exon 25) or specific for exon 25 lacking transcripts, sarcomeric alpha-actinin was amplified as a loading control. Reactions were run longer as previously published [9] to allow comparison of expression levels between prenatal and postnatal hearts. **B** Confocal micrographs of paraffin sections of embryonic (E9.5, E13.5) and adult heart after staining with antibodies against FHOD3 (all isoforms, shown in green in the merged image) and against troponin T (embryonic hearts) or myomesin (adult heart), shown in red in the merged image

Fig. 3.

Adult rat cardiomyocytes (ARC) display a shift in FHOD3 isoform expression and altered subcellular targeting of FHOD3 during their myofibril degeneration-regeneration phase induced by high serum culture conditions. **A** Schematic drawing of FHOD3, indicating the muscle exon at the C-terminus of the FH2 domain as a yellow triangle. RT-PCR was carried out on RNA isolated from ARC immediately after collagenase digest 0 days (d) or after 3d, 8d and 12d of culture in DMEM with 10% FCS with primer sets amplifying all FHOD3 isoforms (E24–27), specifically the muscle isoform (E25–27) or specifically the non-muscle isoform (DE25). A transient upregulation of the non-muscle FHOD3 is seen at 3 and 8d, with re-expression of muscle FHOD3 by 12d. **B** Confocal micrographs of ARC at 0d, 3d, 8d and 15d in culture, showing the degeneration and regeneration of myofibrils as visualised by staining for sarcomeric alpha-actinin (red signal in merge); confocal sections were taken close to the substrate where maximal cell spreading was seen. At d0 FHOD3 (green signal) is colocalised at the Z-disc, becomes more diffuse during de-differentiation and gets re-localised to the myofibrils only after 15d in culture in the most mature sarcomeres (indicated in the blow-ups). **C** Confocal micrographs of ARC at 0d, 3d, 8d and 15d in culture showing a partial colocalisation of FHOD3 with p62 containing speckles at 3d and 8d (arrowheads), but a total absence of colocalisation at 15d (arrows, also at 3d), when the myofibrils are regenerated and muscle FHOD3 expression levels have returned to normal (data not shown). The confocal section for the 8 days cell was taken from the top of the cell, where most of the degradation seems to take place and not close to the substrate.

Fig. 4. FHOD3 needs CK2 activity for targeting to the myofibrils. Confocal micrographs of paraffin sections of E9.5 wildtype (A) and CK2alpha knockout mice (B), stained for FHOD3 (all isoforms, green signal in the merge) and sarcomeric myosin (red signal in the merge). While FHOD3 shows clear myofibril association in the wildtype hearts (arrows), this targeting seems lost in the CK2alpha knockout hearts and an increased number of speckles is observed (arrowheads)

Fig. 5.

Lack of CK2alpha expression affects myofibrils in the ventricle more dramatically than in the atria. Electron micrographs of E10.5 wildtype (left column) and CK2alpha knockout embryos (right column) taken from the right ventricle (RV), the left ventricle (LV) and the atrium (A). Sparse, misaligned myofibrils with reduced sarcomere length are apparent in both CK2alpha knockout ventricles, while atrial myofibrils appear to be indistinguishable between wildtype and knockout

Fig. 6. Interaction of active ROCK1 with the C-terminus of FHOD3 prevents the autoinhibitory interaction between DID and DAD domain and leads to an activation of FHOD3 as apparent by an increase in F-actin in HeLa cells. **A** Co-immunoprecipitation of myc-tagged full length (p160-ROCK1) and constitutively active (p130-ROCK1) ROCK1 with the C-terminus of FHOD3 (GFP-tagged) shows a stronger interaction with p130-ROCK1. **B** Schematic drawing of the autoinhibitory state and the proposed interaction of C- and N-terminal FHOD3 constructs in the absence or the presence of activation by phosphorylation (indicated by the letter P in the DAD domain). **C** GST pull-down assays of GST-tagged N-terminal FHOD3 constructs with GFP-tagged C-terminal FHOD3 constructs on their own show strong interaction, which is weakened by the presence of constitutively active myc-tagged ROCK1. **D** GST pull-down assays of GST-tagged N-terminal FHOD3 constructs with GFP-tagged C-terminal FHOD3 constructs show an interaction (lane 2) which is abolished in pull-down assays with GFP-tagged C-terminal FHOD3 constructs that mimic phosphorylation (ROCK3D; lane 5) despite equal inputs as shown with the anti GST western blot (5% of input loaded). **E** HeLa cells were transfected with GFP, or different GFP-tagged FHOD3 constructs (full length FHOD3; FHOD3-DAD, which lacks the DAD domain; FHOD3-3D, which phosphomimics the three ROCK1 phosphorylation sites), stained for F-actin with phalloidin and analysed by confocal microscopy. More filamentous actin is obvious in all cases where activated FHOD3 is present (FHOD3-DAD, FHOD3-3D),

which is quantified in **F**. Presented values are the means ($\pm$ SD) between three separate experiments with nine quantified images per construct per experiment

Fig. 7.

Activation of FHOD3 leads to increased F-actin staining in NRC. **A** Confocal micrographs of NRCs that were transiently transfected with HA-tagged full length FHOD3 either on its own or together with wildtype (p160) or constitutively active (p130) myc-tagged ROCK1 constructs. While FHOD3 on its own targets to the myofibrils and clear I-band striations are visualised by Alexa633-phalloidin staining, activation in a cellular environment by ROCK1 leads to increased formation of actin cables (arrows). **B** Endogenous FHOD3 shows a more pronounced co-localisation with actin cables in NRC that were co-transfected with myc-tagged p130 ROCK1 compared to neighbouring control cells. **C** Activation of FHOD3 by truncating the DAD domain or by a phosphomimetic construct of the ROCK1 phosphorylation sites also leads to increased actin cable formation in transiently transfected NRC

Fig. 8.

ROCK1 directly phosphorylates the C-terminus of FHOD3 in vitro and the expression of FHOD3 is necessary to get the actin filament polymerization phenotype that is induced by ROCK1 overexpression. **A** *In vitro* kinase assay using His-tagged ROCK1 on either the wildtype C-terminus of FHOD3 or a mutant construct, where the serines and the threonine were mutated to alanine. A signal with the anti phospho-serine/threonine antibodies could only be detected for the wildtype construct. **B** Neonatal rat cardiomyocytes were double transfected with myc-tagged constitutively active ROCK1 (p130-ROCK1-myc) and either the control (top row) or the FHOD3 knockdown shRNA plasmid also expressing GFP (bottom row). After 5d pronounced actin cables (visualized by Alexa633-phalloidin) induced by ROCK1 can only be seen in the control cardiomyocytes, but not in cardiomyocytes with reduced FHOD3 expression levels, which show only rudimentary myofibrils, as described previously [9]. The cell labeled with an asterisk is a contaminating fibroblast in the cardiomyocyte primary culture. Bar 10 micrometres.

Fig. 9. Scheme depicting the two distinct phosphorylation events that govern muscle FHOD3's localization and activity. Only the muscle isoform of FHOD3 can be phosphorylated by CK2, which is required for its proper targeting to the myofibrils. Phosphorylation by ROCK1 at the C-terminus is alone sufficient for the activation of FHOD3.