Hadp1, a newly identified pleckstrin homology domain protein, is required for cardiac contractility in zebrafish

Joshua D. Wythe, Michael J. Jurynec, Lisa D. Urness, Christopher A. Jones, M. Khaled Sabeh, Andreas A. Werdich, Mariko Sato, H. Joseph Yost, David J. Grunwald, Calum A. MacRae and Dean Y. Li

SUMMARY

The vertebrate heart is one of the first organs to form, and its early function and morphogenesis are crucial for continued embryonic development. Here we analyze the effects of loss of Heart adaptor protein 1 (Hadp1), which we show is required for normal function and morphogenesis of the embryonic zebrafish heart. Hadp1 is a pleckstrin homology (PH)-domain-containing protein whose expression is enriched in embryonic cardiomyocytes. Knockdown of hadp1 in zebrafish embryos reduced cardiac contractility and altered late myocyte differentiation. By using optical mapping and submaximal levels of hadp1 knockdown, we observed profound effects on Ca\(^{2+}\) handling and on action potential duration in the absence of morphological defects, suggesting that Hadp1 plays a major role in the regulation of intracellular Ca\(^{2+}\) handling in the heart. Hadp1 interacts with phosphatidylinositol 4-phosphate [PI4P; also known as PtdIns(4)P] derivatives via its PH domain, and its subcellular localization is dependent upon this motif. Pharmacological blockade of the synthesis of PI4P derivatives in vivo phenocopied the loss of hadp1 in zebrafish. Collectively, these results demonstrate that hadp1 is required for normal cardiac function and morphogenesis during embryogenesis, and suggest that hadp1 modulates Ca\(^{2+}\) handling in the heart through its interaction with phosphatidylinositol.

INTRODUCTION

Proper development and function of the heart is dependent upon an intricate network of molecular cues and morphogenetic events that are highly conserved among vertebrates (Harvey, 2002; Srivastava and Olson, 2000). The fact that congenital heart defects are among the most common congenital malformations highlights the exquisite sensitivity of heart formation to alterations in these formative events (Bruneau, 2008; Rosamond et al., 2008).

Studies of early cardiogenesis have identified a number of essential transcriptional mediators, growth factors and myofibrillar components that are required for the initial patterning of the heart (Olson, 2006; Srivastava, 2006; Srivastava and Olson, 2000). The embryonic heart begins to function before its development is complete, and many studies suggest that structure and function are inextricably linked, as they are in the adult heart (Glickman and Yelon, 2002; Hu et al., 2003; Huang et al., 2003a; Sedmera et al., 1999; Ursem et al., 2004). Some aspects of embryonic heart development (such as trabeculation and perhaps endocardial cushion (EC) formation and valve morphogenesis) are sensitive to embryonic function, whereas others (such as looping) do not seem to be dependent on physiological inputs (Auman et al., 2007; Bartman and Hove, 2005; Bartman et al., 2004; Beis et al., 2005; Butcher et al., 2007; Danos and Yost, 1996; Glickman and Yelon, 2002; Hove et al., 2003; Milan et al., 2006; Sehnert et al., 2002; Vermot et al., 2009; Walsh and Stainier, 2001). However, Sultana and colleagues recently demonstrated that early cardiac function is dispensable for valve morphogenesis (Sultana et al., 2008), so the precise extent of the interactions between form and function during cardiogenesis, as well as the factors responsible for these interactions, remain to be determined.

Over the last few decades much has been learned of the factors regulating the structural and functional responses of the adult heart to a broad range of stimuli (Crackower et al., 2002; Frey et al., 2000; Heineke and Molkentin, 2006; Walsh, 2006). The importance of elucidating the mechanisms of early cardiac functional patterning is underscored by the prominent redeployment of the fetal gene expression program early in the onset of hypertrophy in the adult heart (Olson and Schneider, 2003). Indeed, inhibition of reactivation of the fetal gene expression program attenuates the induction of hypertrophy in animal models of pathological cardiac hypertrophy (Backs and Olson, 2006; Barry et al., 2008; Kim et al., 2008; Kong et al., 2006; McKinsey and Olson, 2005; Oka et al., 2007; Xing et al., 2006). There are also emerging data that implicate electrical and Ca\(^{2+}\) transients in the normal function and morphogenetic maturation of the developing heart, although unraveling these signals from their downstream mechanical effectors is challenging (Becker et al., 2011; Chi et al., 2010; Panakova et al., 2010). Here we report the identification and characterization of a previously unknown, evolutionarily conserved gene, heart adaptor protein 1 (Hadp1) in vertebrates. Hadp1 expression is enriched in the vertebrate embryonic heart, and the gene dosage of hadp1 affects cardiac function in the zebrafish embryo. Maximal loss of hadp1 induces morphological defects, such as abnormal looping.
and aberrant atrioventricular (AV) canal (AVC) formation. Using high-resolution physiological measurements, optical voltage mapping and Fura-2-based ratiometric calcium (Ca^{2+}) imaging, we show that cardiac contractility, conduction and Ca^{2+} handling are exquisitely sensitive to hadp1 gene dosage. We demonstrate that HADP1 is a membrane-bound, pleckstrin homology (PH)-domain protein that interacts with phosphatidylinositol derivatives. Finally, we present in vivo pharmacological data implicating PI4-kinase (PI4K) as an upstream regulator of Hadp1 function in the heart. Together, these results indicate that Hadp1 interacts with the PI4K pathway to regulate normal Ca^{2+} dynamics, and thus myocardial contractility and differentiation, within the embryonic zebrafish heart.

**RESULTS**

**Hadp1 is expressed in the vertebrate heart**

To identify genes involved in regulating vertebrate heart development, gene expression changes were analyzed in cardiomyocytes that were isolated from various stages of fetal and postnatal rat hearts (Nechiporuk et al., 2001). An amplicon representing Hadp1 was present at high levels in the fetal cardiomyocyte pool, but was undetectable in postnatal day (P)12 rat cardiomyocytes (supplementary material Fig. S1A,B). Northern analysis showed that expression of Hadp1 is enriched in the embryonic, but not adult, heart in humans and mice (supplementary material Fig. S1B,C). cDNA library screening, northern analysis and rapid amplification of cDNA ends (RACE) determined that alternative splicing occurs at the 5’ end of mammalian Hadp1 to generate two transcripts, Hadp1a and Hadp1b (supplementary material Fig. S1E). Northern analysis and reverse-transcriptase PCR (RT-PCR) determined that expression of both transcripts (Hadp1a and Hadp1b) is enriched at similar levels in the embryonic but not adult heart in rats, mice and humans (supplementary material Fig. S1F,G, and data not shown). Probes designed against the region predicted to be common to both splice variants of Hadp1 also identified two transcripts in zebrafish (supplementary material Fig. S1H). However, 5’ RACE originating in this same region yielded only one transcript in zebrafish embryos (data not shown). Interestingly, RT-PCR determined that hadp1 expression is maintained in the adult zebrafish heart, unlike in mammals (data not shown). Zebrafish hadp1 is the true homolog of murine Hadp1: synteny is conserved and the protein is 55% identical (60% similar) to murine HADP1a (Fig. 1A; supplementary material Fig. S1A,S). Hadp1a contains two WW domains, a PH domain (Mayer et al., 1993; Therrien et al., 1998), two polyproline-rich stretches and an ATP/GTP-binding motif (P-loop) at the N-terminus, and an ATP-binding cassette/structural maintenance of chromosomes family ATPase domain, SBMO1; hereafter referred to as SBMO1 (supplementary material Fig. S5). A splice-blocking morpholino (hadp1SBMO1; hereafter referred to as SBMO1) was designed against an exon-intron boundary sectioning of 72-hpf embryos following whole-mount in situ hybridization (WISH) showed no atrial expression and confirmed that hadp1 was localized to the ventricular myocardium (supplementary material Fig. S4). Examination of various embryonic stages of murine development by WISH revealed expression of Hadp1 throughout all chambers of the heart, unlike its zebrafish ortholog (supplementary material Fig. S5).

Given the high degree of conservation of Hadp1 between multiple species (supplementary material Figs S2, S3), as well as its robust expression in the developing vertebrate heart, we chose to pursue loss-of-function studies of Hadp1 in zebrafish because of the ability to characterize interactions between cardiac morphogenesis, physiology and electrical activity in vivo during the earliest stages of development.

**Hadp1 is required for cardiovascular function in zebrafish**

A splice-blocking morpholino (hadp1SBMO1; hereafter referred to as SBMO1) was designed against an exon-intron boundary...
(common to both predicted transcripts) of hadp1 to disrupt the in vivo function of Hadp1 (Draper et al., 2001). Efficacy of the morpholino was determined by RT-PCR of RNA isolated from MO-injected embryos. SBMO1-injected embryos produced a single, aberrant hadp1 transcript lacking exon 6 (Fig. 2A,B). Loss of the N-terminus of a PH domain [as occurs in the predicted product of the SBMO1 transcript (data not shown)] often abrogates PH-domain folding and recognition of their phosphatidylinositol binding partners (Fukuda and Mikoshiba, 1996; Isakoff et al., 1998; Maroun et al., 1999; Rameh et al., 1997; Salim et al., 1996).

hadp1 morphant embryos appeared morphologically indistinguishable from control MO-injected embryos throughout the first 24 hours of development. At 48 hpf, embryos injected with SBMO1 displayed blood pooling just below the atrium and showed pericardial edema, abnormalities that were not observed in control embryos (Fig. 2C,D). At 55 hpf, SBMO1 morphants displayed bradycardia and reduced cardiac output (as determined by fractional shortening; data not shown) (supplementary material Movies 1, 2).

To determine the extent of altered blood flow in hadp1 morphants, we scored circulation in live 30- to 36-hpf embryos by microangiography. Normally, dye injected into the common cardinal vein (CCV) is drawn into the heart, passes through the aortic arches, into the dorsal aorta (DA) and returns via the posterior cardinal vein (PCV). In 87% of SBMO1 morphants (n=212), however, the dye pooled in the CCV and failed to circulate. Only 13% of control-injected embryos (n=107), and 5% of wild-type embryos (n=134) exhibited a similar phenotype (Fig. 2E-G).

Knockdown of hadp1 in the endothelial-specific reporter line Tg(fli:EGFP)\textsuperscript{33} (Lawson and Weinstein, 2002) demonstrated that loss of hadp1 did not affect vascular patterning (n=30 for each treatment) (Fig. 2H-L). Thus, altered circulation in the morphants was not the consequence of a compromised vascular system.

Because ectopic expression of hadp1 caused severe gastrulation defects, it was impossible to clearly rescue the effects of gene knockdown by widespread overexpression (data not shown). To confirm that the observed physiological defects were a result of hadp1 loss and were not off-target effects, embryos were injected with a second, non-overlapping MO, hadp1SBMO2 (SBMO2). SBMO2-injected embryos produced reduced levels of wild-type hadp1 transcript, along with an alternative transcript (supplementary material Fig. S6A,B) that lacked exon 5 and encoded a predicted premature termination product (data not shown). These animals displayed pericardial edema and circulatory defects at a penetrance similar to that seen in SBMO1 morphants (supplementary material Fig. S6C-I). Thus, elimination of wild-type hadp1 and production of either a truncated Hadp1 protein (SBMO2) or a mutant form of the protein that probably cannot interact with phosphoinositides (SBMO1) resulted in embryos with altered cardiac function. Collectively, these results demonstrate that hadp1 is required for normal cardiovascular function in zebrafish.

**Loss of hadp1 induces dose-dependent bradycardia and contractility defects**

Knockdown of Hadp1 with higher doses (10 ng/embryo) of SBMO1 caused various morphological defects (unlooped hearts, AVC defects, pericardial edema and diminished contractility) (Fig. 2; see below; supplementary material Fig. S6). To uncover the processes most sensitive to loss of hadp1, we developed conditions of partially reduced hadp1 levels. Injection of 1 or 2 ng of SBMO1 did not

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**Fig. 2. Knockdown of hadp1 decreases cardiac output and contractility in zebrafish.**

(A,B) Schematic representation of Hadp1 protein (top) and partial genomic structures (bottom; boxes represent coding exons), with positions of the MO and RT-PCR primers indicated. See Fig. 1 for abbreviations. (B) RT-PCR analysis of control and morphant embryos. \(\beta\)-actin is a loading control. –RT indicates the absence of reverse transcriptase as a negative control. (C,D) Brightfield images of live embryos (anterior is to the left) after injection with 10 ng control MO or 10 ng SBMO1. Arrow and arrowhead indicate pericardial effusion and blood pooling, respectively. (E,F) Microangiography following injection of control MO or SBMO1 reveals that circulation is altered following knockdown of hadp1. Heart (H), common cardinal vein (CCV), dorsal aorta (DA) and the posterior cardinal vein (PCV) are indicated. Circulation was scored by microangiography, or under transmitted light, and the results are quantified in G. (H-K) Confocal micrographs of control MO or SBMO1 morphant fli:EGFP transgenic embryos. Dorsal aorta (DA), posterior cardinal vein (PCV), parachordal vein (PAV), intersegmental arteries (ISA), intersegmental vessel (ISV) and dorsal longitudinal anastomotic vessel (DLAV) are indicated. (L) Quantification of normal vascular patterning.
induce pericardial effusion, blood pooling (data not shown and supplementary material Movies 3-5) or altered cardiac morphogenesis (data not shown). However, these manipulations still affected cardiac performance, as analyzed by heart rate in live embryos, with a dose-dependent relationship between loss of hadp1 and decreased heart rate [control: 186 beats/minute (bpm), \( n = 5 \); 1 ng: 173 bpm, \( n = 6 \), \( P < 0.05 \); 2 ng: 149 bpm, \( n = 5 \), \( P < 0.001 \)] (Fig. 3A).

To objectively monitor ventricular function, we measured myocardial fractional shortening, in the context of reduced doses of SBMO1 (Fig. 3B). Cardiac fractional shortening was significantly reduced in embryos injected with 1 ng (24±3%, \( n = 5 \), \( P < 0.001 \); mean ± s.e.m.) or 2 ng (20±3%, \( n = 7 \), \( P < 0.001 \)) of SBMO1 when compared with control-MO-injected (10 ng) embryos (44±4%, \( n = 4 \)) (Fig. 3C) (supplementary material Movies 3-5). These results demonstrate that reduction of hadp1 expression, with doses of morpholino that do not elicit confounding morphological phenotypes, leads to diminished heart rate and depressed indices of ventricular contractility.

**Loss of hadp1 does not alter heart field specification or cardiomyocyte number**

In an effort to ascertain the underlying cause of altered cardiac function following loss of hadp1, we examined the earliest steps in embryonic cardiac development. Complete loss of hadp1 did not alter cardiomyocyte cell specification, nor did it affect cardiomyocyte migration or fusion into the linear heart tube (Fig. 4A-H). Expression of the cardiac transcription factor nks2.5, as well as the contractile protein gene cmlc2, a definitive cardiomyocyte marker, was indistinguishable between embryos injected with high doses of SBMO1 and embryos injected with control MO. Furthermore, nks2.5 expression was not altered at 30 hpf in morphants, demonstrating that heart tube extension was unaffected by the loss of hadp1 (Fig. 4I,J). These results demonstrate that induction of the cardiac progenitor program, heart tube fusion and heart tube extension are all unaffected by loss of hadp1.

Because hadp1 was identified initially in proliferating cardiomyocytes, we investigated whether loss of hadp1 affected cardiomyocyte proliferation. cmlc2:GFP embryos, which express GFP in atrial and ventricular cardiomyocytes (Huang et al., 2003b), were injected with SBMO1, and GFP-positive cells were counted at 36 and 48 hpf. Although circulation defects were apparent from 30 hpf onwards, the mean number of GFP-positive cells at either time point was indistinguishable between control-injected embryos (36 hpf: 203, \( n = 22 \); 48 hpf: 276, \( n = 6 \)) (Fig. 4K-O) and hadp1 morphants (36 hpf: 196, \( n = 28 \); 48 hpf: 272, \( n = 6 \)), as was the mean number of phosphorylated Histone H3 (H3P)-positive cells in the 48-hpf heart (3.5 vs 4.0, respectively; \( P = 0.63 \)) (Fig. 4P-R). In addition, the endocardium and myocardium of the atrium and ventricle were indistinguishable between control-MO and SBMO1 (\( n = 12 \) for each treatment) embryos, demonstrating that basic morphogenesis occurs normally in the absence of hadp1 (Fig. 4S-V). Examination of 48-hpf morphant hearts by transmission electron microscopy (TEM) revealed no evidence of defects in the sarcomeric ultrastructure of hadp1 morphants (\( n = 6 \) for control MO; \( n = 6 \) for SBMO1; Fig. 4W,X).

**hadp1 is required for AVC myocardial differentiation**

Owing to the absence of defects in early cardiogenesis, we next investigated whether the circulatory abnormalities observed at high doses of hadp1 knockdown resulted from loss of chamber identity, which might lead to perturbation of cardiac rhythm and abnormal contractility. Double in situ hybridization for an atrial-specific marker, atrial myosin heavy chain (amhc), and a ventricle-specific marker, ventricular myosin heavy chain (vmhc), revealed that the expression patterns of both genes were normal in SBMO1 embryos (control MO, \( n = 30 \); SBMO1, \( n = 50 \)) (Fig. 5A,B), although the hearts themselves appeared straighter and sometimes failed to undergo normal looping (data not shown). Immunohistochemical analysis to detect a pan-cardiac myosin (MF20) and an atrial-specific myosin (S46) further confirmed that neither atrial nor ventricular chamber identity is affected following loss of hadp1 (control MO, \( n = 15 \); SBMO1, \( n = 25 \); Fig. 5C,D).

As cardiac development proceeds in zebrafish, several changes occur within the chamber myocardium and in the AV boundary (or AVC) separating the two chambers (from which the endocardial lining will invaginate and develop into the valves of the heart to prevent retrograde blood flow) (Scherz et al., 2008). Prior to ~36 hpf, bmp4 and versican are expressed throughout the anterior-posterior extent of the myocardium, and notch1b throughout the

**Fig. 3. Loss of hadp1 leads to bradycardia and hypococontractility.** (A) Heart rate analysis (bpm) in control and hadp1 morphants at 48 hpf and 21°C. (B) Live, lateral image of a 48-hpf zebrafish heart. The longest line represents the demarcation between the atrium (A) and ventricle (V), whereas the long line in the ventricle represents the end diastolic, or largest area, of the ventricular chamber and the shorter line represents the end systolic, or smallest, ventricular area. The ratio of these two measurements defines ventricular fractional shortening. (C) Analysis of ventricular fractional shortening following loss of hadp1. See supplementary material Movies 3, 4 and 5 for the absence of edema, unlike SBMO1 (10 ng)-injected animals (as shown in Fig. 2 and supplementary material Movies 1 and 2). *P≤0.05; **P≤0.005; ***P≤0.001; ns, not significant.
endocardium. However, by ~37-48 hpf, expression of these genes becomes restricted to the AVC myocardium and endocardium, respectively, prior to EC formation at 72 hpf (Hurlstone et al., 2003; Peal et al., 2009; Vermot et al., 2009; Walsh and Stainier, 2001). In hadp1 morphants \( (n=40) \), but not control embryos \( (n=60) \), the myocardial component of this restriction of gene expression failed to occur: the expression domain of \( \text{bmp4} \) encompassed the entire ventricle as late as 72 hpf (Fig. 5E,F). Interestingly, restriction of the endocardial marker \( \text{notch1b} \) occurred normally at 48 hpf in hadp1 morphants \( (n=24) \), compared with control-MO-injected embryos \( (n=24) \). These data indicate that myocardial and endocardial cross-talk and AVC development was impaired in some \( (\text{Bmp, Wnt}) \) signaling pathways, but not others \( (\text{Notch}) \) (Fig. 5K,L).

Cell morphological changes also differentiate atrial and ventricular myocardium (Auman et al., 2007; Panakova et al., 2010), as well as AVC myocardium and endocardium. Specifically, as the heart develops, myocardium of the AVC becomes more trapezoidal, and endocardial cells within the AVC are identified by their lateral expression of DM-grasp (Beis et al., 2005; Camarata et al., 2010; Scherz et al., 2008). Together, these gene expression and cellular changes are indicative of AVC and chamber myocardial and endocardial differentiation beginning at 48 hpf.
DM-grasp enrichment in the AV cushion myocardium, observed in control-injected embryos ($n=22; 100\%$ at 48 hpf, failed to occur in hadp1 morphants ($n=20; 0\%$) (Fig. 5G,H) (Beis et al., 2005). Similarly, induction of DM-grasp expression within the AVC endocardium failed to occur in morphant embryos (Fig. 5G,H). Furthermore, confocal microscopy on control embryos (Fig. 5I,J) revealed the differentiated, trapezoidal cardiomyocyte morphology and cuboidal endocardial morphology previously reported (Beis et al., 2005). Of note, perhaps as a result of subtle timing differences, we failed to detect the previously reported lateral localization of DM-grasp within the AVC endocardium (Beis et al., 2005) in control (Fig. 5I) or wild-type embryos (data not shown). In contrast to the control embryos, morphant embryos displayed an absence of DM-grasp expression in some AVC cardiomyocytes, abnormal localization of the protein and rectangular cardiomyocyte morphology in the superior portion of the AV canal, and a lack of DM-grasp expression within AVC endocardium. These data suggest that loss of hadp1 leads to abnormal myocardial differentiation as determined by the failure to downregulate $bmp4$ expression in the ventricle, the inability to upregulate $bmp4$ within the AVC myocardium, and the altered myocardial cell morphology in the AVC.

**Loss of hadp1 perturbs Ca$^{2+}$ handling in the heart**

Potential signals that could function to affect both cardiac performance and altered AVC differentiation as observed at maximal hadp1 knockdown include electrical conduction and Ca$^{2+}$ signaling (Chi et al., 2010; Ebert et al., 2005; Panakova et al., 2010). Thus, we examined the electrical properties of the heart as well as cardiomyocyte Ca$^{2+}$ handling in the context of loss of Hadp1. Electrical impulse propagation is crucial for the synchronous contraction of the myocardium. Defects in excitation-contraction...
coupling can perturb contractile function without altering the initial structure or formation of the sarcomeric apparatus (Beauchamp et al., 2006; Costantini et al., 2005; Keating and Sanguinetti, 2001). To determine whether the effects on heart rate and contractility were associated with perturbation of cardiac electrical conduction, we performed optical voltage mapping of the heart at doses of MO that failed to elicit morphological defects (e.g. 2 ng) (Fig. 6A) (Arnaout et al., 2007; Chi et al., 2008b; Milan et al., 2006; Panakova et al., 2010; Sedmera et al., 2003). There was evidence of significant prolongation of action potential duration [APD80 (80% of the duration of an action potential in the heart)] in SBMO1 morphant ventricles (234±11.3 ms vs 324±19.6 ms, P<0.005), but not the atria (129.4±11.1 ms vs 156±16.3, not significant) (Fig. 6B-E; supplementary material Movies 6, 7).

Given the perturbation of electrical function observed in hadp1 morphant hearts, we turned our attention to Ca2+, a major effector of muscle contraction, and thus cardiac contractility. Using ratiometric Ca2+ imaging with Fura-2, we were able to characterize the effects of loss of hadp1 on cardiomyocyte Ca2+ handling in the developing heart (Fig. 7A). There was no significant effect of loss of hadp1 on atrial diastolic Ca2+ concentrations [0.94±0.10 arbitrary ratiometric units (r.u.) vs 0.89±0.11 r.u., not significant] (Fig. 7B,C), but there was a substantial increase in the prolongation of atrial transient amplitude and duration at doses of morpholino that exhibited no morphological effects (e.g. 2 ng) (duration: 157.0±11.6 ms, P<0.05; Ca2+ amplitude: 0.296±0.04 r.u. vs 0.397±0.06 r.u., P<0.05) (Fig. 7A-E). In ventricular cardiomyocytes we also identified a significant increase in the diastolic Ca2+ concentration in hadp1 morphant hearts (0.73±0.09 r.u. vs 0.84±0.07 r.u., P<0.05) (Fig. 7A,B,F-H), and this was accompanied by a substantial increase in the amplitude and duration of the ventricular Ca2+ transient (duration: 233.0±32.4 ms and 309.7±17.3 ms, P<0.05; Ca2+ amplitude: 0.175±0.07 r.u. vs 0.284±0.09 r.u., P<0.05) (Fig. 7A,G,H). These phenomena suggest that loss of hadp1 leads to a reduced rate of extrusion of cytoplasmic Ca2+ during diastole with consequent effects on the duration of the Ca2+ transient and ultimately on the final diastolic Ca2+ concentration (Scoote and Williams, 2004; Ter Keurs and Boyden, 2007).

HADP1 interacts with PI4-kinase derivatives

We sought to identify the biochemical pathway in which HADP1 acts to modulate Ca2+ handling. Because PH-domain-containing proteins and phosphoinositides can affect Ca2+ signaling, we determined whether the predicted PH domain of murine HADP1 (amino acids 164-184) could function as a bona fide phosphoinositide-interacting motif. Using a protein lipid overlay assay (Dowler et al., 2002), we found that the PH domain of HADP1 bound to phosphatidylinositol 4-phosphate [PI4P; also known as PtdIns(4)P], phosphatidylinositol (4,5)-bisphosphate [PI4,5P2; also known as PtdIns(4,5)P2] and phosphatidylinositol (3,4,5)-trisphosphate [PI3,4,5P3; also known as PtdIns(3,4,5)P3] (Fig. 8A). The PH domain also interacted with phosphatidic acid (PA) and cardiolipin. Similar results were obtained using the PH domain fused to maltose binding protein instead of GST (data not shown). Control assays performed in parallel indicated that the PH domain of AKT bound PI3,4,5P3, as previously reported (Franke et al., 1997; Isakoff et al., 1998) (Fig. 8B).

Given the results suggesting that HADP1 can interact with phosphoinositides, we analyzed the subcellular localization of HADP1 in vitro to determine whether it might associate with the...
Hadp1 regulates cardiac contractility

**DISCUSSION**

Owing to the interdependence of cardiac form and function in the developing heart, it is often difficult to discern whether a phenotype results primarily from altered cardiomyocyte morphogenesis, function or both. Studies of several mutants have shown that myocardial functions, specifically contractility and blood flow, are dispensable for initiation of the cardiogenic program and for the progression of early cardiac morphogenesis in zebrafish (Auman et al., 2007; Bartman and Hove, 2005; Bartman et al., 2004; Beis et al., 2005; Chi et al., 2010; Ebert et al., 2005; Hove et al., 2003; Walsh and Stainier, 2001). Although commitment to the cardiac lineage, proliferation, chamber identity, looping, AVC restriction and Ca2+ handling occur normally in some functionally deficient mutants.

PI3,4,5P3, we found that HADP1 continued to be localized to the cell surface in cells depleted of PI3,4,5P3 by starvation (data not shown), suggesting that its localization is not PI3K dependent.

To determine whether the PH domain was required for localization of HADP1, a construct harboring an in-frame deletion of the N-terminus (β1-β3 loop) of the PH domain was transfected into cells. This protein failed to localize to the plasma membrane and instead appeared in a peri-nuclear pool (Fig. S8D). Subcellular fractionation and western blot analysis following transient transfection confirmed these results (Fig. S8E). Higher resolution analysis by Percoll gradient fractionation determined that wild-type HADP1 was normally associated with membrane fractions (determined by the presence or absence of integrin β1, F1 ATPase and calregulin) (Fig. S8F). The ΔPH mutant failed to be enriched in these fractions, but was still detected in the nuclear (unbroken) cell fraction (detected by lamin B reactivity), confirming the immunofluorescence results and demonstrating that the PH domain is required for the plasma membrane localization of HADP1 (Fig. 8G).

From the biochemical and cell culture experiments, we hypothesized that HADP1 interacts with PI4K derivatives in vivo to regulate cardiac contractility. We employed pharmacological inhibition of PI4KIIIα (an enzyme responsible for the plasma membrane pools of PI4P and thus PI4,5P2) (Balla et al., 2008; Balla et al., 2005) activity to test this model. Corroborating our hypothesis, transient transfection of Hadp1a-myc and treatment with the PI4KIIIα inhibitor phenylarsine oxide (PAO) (Godi et al., 2004), followed by subcellular fractionation and western blotting, revealed that PI4KIIIα inhibition abolished membrane localization of HADP1a (Fig. 8H). Furthermore, transient incubation of embryos with PAO beginning after the onset of circulation and cardiac contraction (24 hpf), resulted in diminished cardiac contractility at 48 hpf (supplementary material Fig. S8A). The decrease was dose-dependent and more profound than loss of hadp1, as might be expected given the wide range of cell signaling mediated by PI4K. Notably, PAO treatment also induced a dose-dependent prolongation of atrial and ventricular action potentials in embryonic hearts (supplementary material Fig. S8B,C). In addition, Ca2+ imaging revealed dose-dependent effects of PAO on atrial Ca2+ transient duration and amplitude, and ventricular Ca2+ handling parameters, that mirrored precisely the effects of SBMO1 (Fig. 8I-K). Despite the comparable effects of SBMO1 and PAO, combining submaximal doses of the two failed to produce an additive effect on any Ca2+ handling parameters (Fig. 8I-K).

**Fig. 7.** Ca2+ transient duration is altered following loss of hadp1.

(A-H) Optical mapping and quantification of Ca2+ transients in the embryonic zebrafish heart. (A) Representative images of the mean Ca2+ transient amplitude following optical mapping, using the ratiometric dye Fura-2, in control and hadp1 morphant hearts. The red box denotes an example of the pixel areas that were sampled for quantification of ventricular transients. The far-right scale indicates the transient amplitude intensity. (B) Representative traces of Ca2+ transients from single sites in the atrium and ventricle of control (black) and hadp1 morphants (red). (C-E) Quantification of the atrial diastolic Ca2+ (C), transient amplitude (D) and transient duration (E). (F-H) Quantification of the ventricular diastolic Ca2+ (F), transient amplitude (G) and transient duration (H).

plasma membrane. Myc-epitope-tagged murine Hadp1a was expressed in HEK cells and was constitutively localized to the plasma membrane (Fig. 8C). HADP1b was also constitutively localized to the plasma membrane (supplementary material Fig. S7), demonstrating that the membrane localization was not dependent upon the presence of the N-terminal WW motifs. Although in vitro binding studies indicated that the PH domain of HADP1 can interact with the plasma membrane phosphoinositide...
Hadp1 regulates cardiac contractility

Fig. 8. PI4K activity is necessary for proper subcellular localization of HADP1 and affects Ca\(^{2+}\) transient duration. (A,B) Membrane lipid overlay assay using the purified GST-PH domain of HADP1 (A) and AKT (B). (C,D) HEK293 cells were transiently transfected with myc-epitope-tagged Hadp1 (C) or Hadp1-\(\Delta\)PH (D), and, 36 hours later, were processed for indirect immunofluorescence with an antibody recognizing myc (green) and TOPRO3 (red) to label the nucleus. Arrows indicate cell membrane and peri-nuclear localization of WT and PH-domain mutant (\(\Delta\)PH) HADP1-myc, respectively. (E) In parallel, these cells were subjected to subcellular fractionation and HADP1 was detected by western blotting with anti-myc antibodies. The membrane-enriched protein, integrin \(\beta1\), was probed to confirm separation of fractionations. Input, total cell lysates; cyt, cytoplasmic fraction; mem, soluble membrane fraction. (F,G) Percoll gradient centrifugal density fractionation of HEK293 cells following transient transfection of Hadp1-myc (F) and Hadp1-\(\Delta\)PH-myc (G). I/N, unlysed cells, nuclear-enriched fraction; lanes 1-13 are decreasing density fractions. Myc detects HADP1, integrin \(\beta1\) labels the plasma membrane, lamin B labels the nuclear fraction, transketolase labels the cytoplasm, calregulin labels the endoplasmic reticulum and F1-ATPase labels the mitochondria. (H) Hadp1a-myc transiently transfected HEK293 cells were treated with PAO and subjected to subcellular fractionation, and HADP1 was detected by western blotting with anti-myc antibodies. The membrane-enriched protein integrin \(\beta1\) and the nuclear-enriched protein lamin B were probed to confirm separation of fractionations. (I-K) Optical mapping and quantification of Ca\(^{2+}\) transients in the embryonic zebrafish heart. (I) Representative images of the mean Ca\(^{2+}\) transient amplitude following optical mapping, using the ratiometric dye Fura-2, in control, hadp1 morphant and PAO-treated embryos. The control morphant and hadp1 morphant data from Fig. 7 are included as a reference point for the interaction, and combined effect, of PAO activity on Ca\(^{2+}\) transient activity. The red box denotes an example of the pixel areas that were sampled for quantification of ventricular transients. The far-right scale indicates the transient amplitude intensity. (J,K) Representative traces of Ca\(^{2+}\) transients from single sites in the atrium and ventricle of embryos treated with control (black), SBMO1 (red), 0.5 \(\mu\)M PAO (green), 1.0 \(\mu\)M PAO (purple), SBMO1 + 0.5 \(\mu\)M PAO (blue) and SBMO1 + 1.0 \(\mu\)M PAO (orange).
(Bartman et al., 2004), development of the ECs (Vermot et al., 2009) and the trabeculae (Chi et al., 2008b; Peshkovsky et al., 2011) is abrogated in these mutants, suggesting that proper function is required for some aspects of myocardial differentiation during development, although a recent study might complicate this notion (Sultana et al., 2008). Interestingly, several mutants that specifically affect EC formation [jekyll (ugdh), chys-1, klf2a] show normal cardiac morphogenesis and function through to 36 hpf, yet AVC development and later EC formation is lost, as is later cardiac function (Peal et al., 2009; Vermot et al., 2009; Walsh and Stainier, 2001). These results, as hypothesized by Vermot and colleagues, suggest that the initial signaling events that induce cardiogenesis and AVC formation are genetically hardwired in the myocardium and independent of flow and contraction, but that these epigenetic forces are required for later steps in both endocardial and myocardial differentiation. Our analyses indicate that hadp1 is dispensable for the establishment of the cardiac lineage and for early formation of the heart, but is required for normal cardiac function during embryogenesis (from 36 hpf onwards), for proper AVC formation. However, we found that, at doses of MO that do not affect overall morphogenesis of the heart, loss of hadp1 still exhibited a dose-dependent effect upon both Ca2+ signaling within the heart and cardiac contractility.

As the interplay between form and function is dissected, and as powerful tools emerge for exploring developmental physiology, another layer of functional complexity is beginning to be appreciated in the regulation of cardiac morphogenesis. Evidence suggests that electrical conduction regulates late, but not early, cardiomyocyte morphogenesis as well as differentiation independently of hemodynamic and contractile forces (Chi et al., 2010). We failed to observe morphological defects at doses of MO that clearly affect Ca2+ handling (although hadp1 morphants do not beat asynchronously like the cxnr46 mutants). Using high-resolution video microscopy and optical voltage mapping we have demonstrated depression of cardiac contractility indices and a significant increase in ventricular action potential duration at 48 hpf following loss of hadp1. Concordantly, there was a significant increase in diastolic Ca2+ transient amplitude and transient duration in the ventricle at 48 hpf. Together, these findings suggest a defect in cytoplasmic Ca2+ extrusion in the normal sarcomeric reticulum (SR) release-reuptake cycle. These abnormalities in Ca2+ handling occur at doses of MO that do not affect cardiac morphogenesis, the initial establishment of cardiac chamber identity or AVC formation. Because hadp1 loss also affects cardiac morphogenesis and myocyte differentiation at higher doses of MO, Ca2+ cycling might be a physiological input required for normal myocardial differentiation (Chi et al., 2010; Ebert et al., 2005; Panakova et al., 2010). However, we cannot rule out the possibility that Hadp1 affects intracellular Ca2+ handling independently of its effects on myocyte differentiation and morphogenesis.

There is evidence from others that HADP1 is a cytoplasmic component of the adherens junction (AJ) (Meng et al., 2008; Pulimeno et al., 2010) and is required for AJ stability in human epithelial cells in vitro (Meng et al., 2008). Our immunofluorescence experiments confirm that murine HADP1a and/or HADP1b and zebrafish Hadp1 localize to the plasma membrane in multiple cell lines (data not shown), and our biochemical data establish that HADP1a co-fractionates with components of the plasma membrane. Together, these results implicate Hadp1 in the regulation of cell-cell coupling and the organization of adhesion complexes, including components such as connexins, in cardiomyocytes (Chi et al., 2010; Shaw et al., 2007; Smyth et al., 2010). Despite these inferences, our confocal analysis, TEM and gene expression analyses failed to reveal any myocyte adhesion defects, or the morphological abnormalities predicted to result from disruption of adhesion or AJ stability (Bagatto et al., 2006; Ott et al., 2008; Trinh and Stainier, 2004). Similarly, intercellular coupling (as assessed by in vivo imaging of myocardial action potential propagations) was unaffected, suggesting that AJ structure and coupling functions remain intact (Chi et al., 2010; Smyth et al., 2010). Rather, our results point to an important role for HADP1 in the transduction of junctional signals through interaction with phosphoinositides and subsequent regulation of intracellular Ca2+ handling.

Intracellular Ca2+ signaling is a complex process with multiple independent compartments segregating discrete signals and communicating among themselves (Berridge, 2006). Entry of Ca2+ from outside the cell is tightly regulated both by plasma membrane excitability and by signals from within the intracellular compartments. In the contracting cardiomyocyte, cycling of sarcomeric Ca2+ is regulated on a millisecond timescale by voltage-dependent Ca2+ channels, the consequent ryanodine receptor (RyR) Ca2+ release from the SR to mediate muscle contraction and subsequent active reuptake from the cytoplasm to SR, extracellular compartments or other compartments. An additional mode of internal Ca2+ release results from activation of phospholipase C (PLC) enzymes and their cleavage of PI4,5P2 to generate the second messengers DAG and Ins3,4,5P3. Inositol (1,4,5)-trisphosphate [IP3; also known as Ins(1,4,5)P3] binds and activates the IP3 receptors (IP3Rs) on the SR, which induces Ca2+ release and also sensitizes RyR. Of note, intracellular Ca2+ itself activates PLC (Dupont et al., 2007). Several results have shown that, in addition to acting as a substrate for PI3,4,5P3 and IP3 generation, PI4,5P2 in the plasma membrane directly affects membrane charge and action potentials, and thus regulates intracellular Ca2+ (Balla, 2009; Gamper and Shapiro, 2007; Hilgemann et al., 2001). It has also been shown that PI4P is present within the mammalian plasma membrane (Balla et al., 2008). We have demonstrated that Hadp1 interacts with both PI4,5P2 and PI4P in vitro, and that its localization is dependent upon PI4K activity. Furthermore, loss of Hadp1 stimulates intracellular Ca2+ release and increased transient duration in the heart. From these observations, we hypothesize that Hadp1 normally functions to either inhibit both hydrolysis of PI4,5P2 and IP3-induced Ca2+ release, or to prevent phosphorylation of PI4P to generate PI4,5P2, which could affect IP3-induced Ca2+ release, regulation of reuptake of Ca2+ from the cytoplasm or plasma membrane Ca2+ conductance.

Although our findings confirm the involvement of phosphoinositides in normal embryonic cardiac function, they also suggest an important and, as of yet, undefined role for PI4K and its derivatives in these underlying processes. Indeed, we have recently shown that the patterning of Ca2+ compartments within the cardiomyocyte is itself highly complex, with paradoxical relationships emerging between specific intracellular Ca2+ pools (Panakova et al., 2010). The precise balance of Ca2+ compartments might be important in specifying final cardiomyocyte
determination. These same pathways are also known to play a major role in the regulation of physiological and pathological hypertrophy in adult hearts (Backs and Olson, 2006; Heineke and Molkentin, 2006; Molkentin, 2006; Wilkins and Molkentin, 2004; Zhang et al., 2007). Phosphoinositides and the PH-domain-containing effector molecules that bind them, are also necessary and sufficient for the induction of pathological hypertrophy in mammalian cell culture and animal models (Cantley, 2002; Crackower et al., 2002; Karliner, 2002; Matsui et al., 2003; Matsui et al., 2001; Reiss et al., 1996; Shioi et al., 2000; Shubeita et al., 1990). Recent studies suggest that PI4,5P2 might be involved in the adult hypertrophic response, because ablation of inositol polyphosphate-5-phosphatase f (Inpp5f) results in hypertrophy in adult mice, whereas cardiac-specific overexpression of Inpp5f renders mice resistant to agonist-induced cardiac hypertrophy (Trivedi et al., 2007; Zhu et al., 2009). These findings suggest that the patterning of cardiac Ca2+ signaling is a central determinant of the developmental and adult responses required for adaptation to differing stressors. The work that we have presented establishes a central role for Hadp1 in the fundamental physiological transitions of myocardial development. In future studies it will be important to define the homeostatic mechanisms controlling the relationships between Hadp1-regulated Ca2+ signaling and other Ca2+ pools in healthy and diseased cardiomyocytes in fetal and adult models.

METHODS

Cloning of rat, mouse, human and zebrafish Hadp1

Rat Hadp1 was cloned in a differential display screen (Nechiporuk et al., 2001). The murine homolog was identified in a CDNA library (a gift of Brigid Hogan, Duke University Medical Center, Durham, NC) and was used to generate riboprobes and expression constructs. The rat and mouse sequences were used in a BLAST search of the zebrafish EST database (ZV4; http://www.ncbi.nlm.nih.gov/BLAST/) to identify a potential zebrafish homolog. RT-PCR was performed using primers spanning the ESTs (Forward, 5′-TATAGTAGATTTCAACAGGGAATAATGGCGG-3′; Reverse, 5′-TAGTCTGATCTGACCTGGCGG-TTTGAGCA-3′). The resultant 1.2-kb PCR fragment was extended by 5′ RACE (RLM-RACE kit, Ambion). The full-length open reading frame of zebrafish hadp1 was obtained by RT-PCR (Forward, 5′-AGGGAATGAGGTAGACAGC-3′; Reverse, 5′-CACAAGATTTAACCTCGGACATGG-3′). All verified sequences have been submitted to Genbank (EU380770, EU380771, EU380772).

Embryo culture and zebrafish stock maintenance

AB strain zebrafish Danio rerio were used as wild type. Embryos were generated, cultured and staged as described (Kimmel et al., 1995; Westerfield, 2000). Other lines used were Tg(hs:EGFP)1 (Lawson and Weinstein, 2002), and Tg(cmlc2:GFP) (Huang et al., 2003b). Imaged embryos were grown in 0.002% 1-phenyl-2-thiourea to prevent pigment formation.

Analysis of gene expression

Northern analysis was performed as previously described (Nechiporuk et al., 2001). For WISH, zebrafish embryos were processed as previously described (Park et al., 2005). Following WISH, embryos were dehydrated, embedded, in methacrylate derivative and cut with a Leica 2055 rotary microtome.

Murine embryos were processed as previously described (Park et al., 2003). In situ hybridized embryos were dehydrated, infiltrated with paraffin, embedded and 12-μm sections were counterstained with Nuclear Fast Red (Vector Labs).

Zebrafish hadp1 riboprobe was constructed from a 1.2-kb fragment of the cDNA subcloned into PBS II SK. Murine Hadp1 riboprobe was obtained by subcloning a fragment of the full-length cDNA into pBS II KS. Images were captured on a Leica MZ125 microscope.

MO knockdown of hadp1 translation

Antisense MOs directed against tropomin T (Sehert et al., 2002), the exon 6 (encoding base pairs 428-532)/intron 6 splice donor site of hadp1 (SBMO1: 5′-GATCAACTCTTTACgtctgaag-3′) (intron in capitals, exon in lowercase), the exon 5 (encoding base pairs 332-428)/intron 5 splice donor site of hadp1 (SBMO2: 5′-TTTGTTACTCAGttctgtaa3′) and a mismatch control oligo to SBMO1 (control MO: 5′-GATCAACGTGTTGctctgtaa3′) were obtained from Gene Tools, LLC (Corvalis, OR). MOs were dissolved in 1× Danieau’s Buffer.

To detect hadp1 transcripts, RT-PCR was performed on RNA from pools of 20 control-MO-, SBMO1- or SBMO2-injected embryos. hadp1 was amplified by 25 cycles of PCR using primers in exon 4 (start at bp 262) (hadp1 MO RT For: 5′-ACTACTTACATCCATCCAGTGAGCCAGG-3′) and exon 7 (end at bp 570) (hadp1 MO RT Rev: 5′-AAACATTTTCTCCTTCACACCCCG-3′). Amplicons were confirmed by DNA sequencing. β-actin (forward: 5′-CCTAAGGCGCAACAGGAAA-3′; reverse: 5′-GGTTGTCCCATCTCCTGCTCAA-3′) was amplified from all samples to control for equal cDNA input.

Embryonic cardiovascular physiology

Circulation was scored by eye or under epifluorescence following microinjection of Rhodamine-dextran (10,000 MW; Sigma) into the CCV (Cretkos and Gruenwald, 1999).

Analyses of heart rate, contractility and electrical function were performed at a dose of MO (1 ng and 2 ng) totrivate to avoid the edema and other secondary effects observed at higher doses (10 ng). To measure heart rate, 48 hpf cmlc2-GFP transgenic embryos were equilibrated at 21°C, unless otherwise noted, and the fluorescent hearts were identified using the maximum subsite pixel intensities and 15-second video recordings obtained using a Nikon TE200 microscope and a Hamamatsu ORCA-ER camera. Fast-Fourier transform of the average pixel density over time was performed to determine heart rate. For drug treatment, an inhibitor of PI4K, PAO (Calbiochem) (Godi et al., 2004), was dissolved in DMSO, and dechorionated 24-hpf embryos were soaked in E3 media with 2.5 μM PAO or 0.1% DMSO for 24 hours, and heart rate was scored at 48 hpf.

Ventricular chamber size and contractile function was measured using noninvasive indices derived from videomicroscopy at high spatial and temporal resolution as previously described (Schonberger et al., 2005). For voltage mapping, individual hearts were isolated by microdissection in modified Tyrodes solution (136 mM NaCl, 5.4 mM KCl, 0.3 mM NaH2PO4, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM glucose, 10 mM HEPES, 2% BSA). Cardiac motion
was arrested with the myofibrillar ATPase inhibitor 2, 3-butanedione monoxide (Sigma) at 15–17 mM or with blebbistatin. Hearts were stained with a 7 µM solution of di-4-ANEPPS (Molecular Probes, Eugene, OR) for 10 minutes immediately prior to imaging. The preparations were placed in a custom pacing and imaging chamber and voltage mapping of spontaneous cardiac electrical activity was performed using this chamber and a CCD (CardioCCD-SMQ, RedShirtImaging, Decatur, GA) mounted on a Nikon TE2000 inverted microscope. The hearts were field paced with a square wave at 60 per minute (Grass S48K Stimulator, West Warwick, RI). Samples were illuminated using a 120 W metal-halide Exfo X-Cite 120 with a 480 nm/40 excitation filter and the fluorescence image filtered through a 535 nm/50 emission filter. Data were acquired at a frame rate of 125 Hz. Subsequent analyses were performed using Cardioplex software, including exponential subtraction of baseline photobleaching. Measurements of action potential durations were taken as the average of three or more successive beats for each sample. For all physiological studies, except the inhibitor experiments which used a two-sided t-test, statistical significance was determined by performing one-way analysis of variance (ANOVA) and the Tukey-Kramer post-test, assuming a normal distribution (PRISM4, Graphpad).

For ratiometric Ca$^{2+}$ transient recordings, hearts were loaded for 15 minutes with 50 µM of the Ca$^{2+}$-sensitive dye Fura-2, AM (Invitrogen) and subsequently washed in dye-free NT solution. Hearts were then incubated in NT solution at room temperature for 30–45 minutes to allow complete intracellular hydrolysis of the esterified dye. A high-speed monochromator (Optoscan, Cairn) was used to rapidly switch the excitation wavelength between 340 nm and 380 nm with a bandwidth of 20 nm and at a rate of 500 per second. The excitation light was reflected by a 400-nm cutoff dichroic mirror and fluorescence emission was collected by the camera through a 510/80 nm emission filter.

For the measurement of fluorescence intensities, we used a high-speed 80×80-pixel CCD camera (CardioCCD-SMQ, RedShirtImaging, LLC) with 14-bit resolution. Action potentials were recorded at the maximum frame rate of 2000 per second. For ratiometric Ca$^{2+}$ transient recordings, monochromator and camera were synchronized. Because each wavelength change required a mechanical movement of the grating, the maximum frame rate for Ca$^{2+}$ transient recordings was lower, i.e. 500 frames per second. Each ratio required the recording of four frames, where one frame was used for each transition between wavelengths. Thus, the final ratio rate was 125 per second. Using a 20×0.75NA objective and 380 nm with a bandwidth of 20 nm and at a rate of 500 per second. The excitation light was reflected by a 400-nm cutoff dichroic mirror and fluorescence emission was collected by the camera through a 510/80 nm emission filter.

For the measurement of fluorescence intensities, we used a high-speed 80×80-pixel CCD camera (CardioCCD-SMQ, RedShirtImaging, LLC) with 14-bit resolution. Action potentials were recorded at the maximum frame rate of 2000 per second. For ratiometric Ca$^{2+}$ transient recordings, monochromator and camera were synchronized. Because each wavelength change required a mechanical movement of the grating, the maximum frame rate for Ca$^{2+}$ transient recordings was lower, i.e. 500 frames per second. Each ratio required the recording of four frames, where one frame was used for each transition between wavelengths. Thus, the final ratio rate was 125 per second. Using a 20×0.75NA objective and a 0.5× C-mount adapter, the final magnification was 10×, resulting in a pixel-to-pixel distance of 2.2 µm.

**Immunohistochemistry**

48-hpf embryos were processed for immunohistochemistry as reported elsewhere (Sehnert et al., 2002). Primary antibodies used were MF20 (1:2.5), S46 (1:2.5), Dm-GRASP (Zn8; 1:10) (Developmental Studies Hybridoma Bank), anti-GFP (1:400; Molecular Probes), H3P (1:100; Millipore). Secondary antibodies were goat-anti-mouse-IgG2b-TRITC (Southern Biotechnology), goat-anti-mouse-IgG1b-FITC (Southern Biotechnology), goat-anti-mouse-Alexa-Fluor-546 (Molecular Probes), goat-anti-rabbit-Alexa-Fluor-488 (Molecular Probes) and goat-anti-rabbit-Alexa-Fluor-546 (Molecular Probes) (all at 1:200). After staining, embryos were washed, re-fixed, mounted in 1% low-melt agarose on a 35 mm glass-bottom tissue culture dish (Mat Tek), and images were obtained on a DMRXE laser scanning confocal microscope (Leica). For DM-Grasp and H3P staining, embryos were embedded in agarose and sectioned by vibratome prior to confocal analysis. Tissue culture cells were processed as previously stated (Karnik et al., 2003), using anti-myc (1:200), goat-anti-mouse-Alexa-Fluor-488 (10 mg/ml) and TOPRO-3 (2 µM) (Molecular Probes).

**Scoring of vascular development**

fltl:EGFP embryos were subjected to GFP immunohistochemistry and embedded in agarose as described above. Embryos that contained a DA, PCV, dorsal longitudinal anastomotic vessel, intersegmental vessels, intersegmental arteries and a parachordal vein as determined by confocal microscopy were scored as normal. Embryos lacking any of these structures were counted as abnormal.

**Cardiomyocyte cell counting**

Embryos were dissociated at 36 hpf as previously described (Shu et al., 2003), and cmictc2:GFP-positive cells were counted. 48-hpf intact hearts were imaged by confocal microscopy and cells counted for all planes sampled. For H3P staining in Tg(cmictc2:EGFP) embryos, vibratome sections of the heart were examined by confocal microscopy for double-positive red (H3P) and green (cmictc2:EGFP) cells. Unpaired t-test statistical analysis was performed assuming a normal distribution (PRISM).

**Histology**

TEM was performed as previously described (Juryneč et al., 2008). For standard histology, 48-hpf embryos were fixed in 4% PFA overnight, dehydrated and processed as described (Finkelstein et al., 1999). 5-µm sections were stained with Gill’s hematoxylin (Sigma) and counterstained with an alcoholic eosin Y solution (Shandon).

**In vitro studies**

Early passage HEK293 and HEK293T cells were routinely passaged before they reached 75% confluency. 1.2×10$^5$ cells were seeded onto poly-L-lysine-coated 6-cm dishes and transfected the next day with 3 µg of the appropriate plasmid DNA using FUGENE-6 (Roche). Cells were harvested 36 hours later in 1 ml of hypotonic lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM KCl, 1 mM DTT, 1 mM MgCl$_2$, protease inhibitors), and homogenized in a dounce homogenizer. Homogenates were spun at 400 g for 10 minutes to remove nuclei and the supernatant was subsequently spun at 16,000 g for 30 minutes and saved as the cytosolic fraction. The pellet was resuspended in RIPA and re-spun at 16,000 g, and the supernatant was saved as the soluble membrane fraction. For Percoll gradient fractionation, cells seeded on poly-L-lysine-coated 10-cm dishes were transfected with 10-20 µg of DNA. 24-48 hours later, cells were washed in PBS, then scraped in 5 ml ice-cold 1× PBS/1 mM DTT/protease inhibitors, and the cells were pelleted. The cells were resuspended in 2.5 ml of 250 mM sucrose/50 mM Tris-pH 7.4/5 mM MgCl$_2$/0.5 mM EDTA/1 mM DTT/protease inhibitors and then lysed in a ball-bearing homogenizer. Nuclei, and unlysed cells, were pelleted (400 g for 10 minutes at 4°C; swinging bucket rotor). The pellet was washed and resuspended in 20 mM HEPES pH
Disease Models & Mechanisms

Hadh1 regulates cardiac contractility

7.9/1.5 mM MgCl2, 0.5 M NaCl/0.2 mM EDTA/20% glycerol/1% Triton. The supernatant was then eluted in 750 μl fractions, mixed 1:1 with 2× sample buffer and loaded on a 4-15% gradient SDS-PAGE gel. Western analysis was performed as previously stated (Karnik et al., 2003). Antibodies used were anti-myc (9E10), anti-lamin-B, anti-calregulin, anti-transketolase, anti-F1-ATPase, anti-β-tubulin (Santa Cruz), anti-1-integrin (BD Transduction Labs), and goat-anti-mouse or rabbit HRP (Jackson Labs).

Binding studies
The coding sequence of the PH domain of murine HADH1 (amino acid 164-184) was amplified by PCR (For: 5'-ATTTTATCTCCGATAAAGTGCACAGCTTGG-3'/Rev: 5'-ATGATGATGCGTGCCCACAGTGGCCACATCTTTGG-3'), digested with EcoRI and NotI, and ligated into pGEX-4T1 (Amersham). The Akt-PH-GST plasmid was a gift of Dario Alessi, University of Dundee, UK. Plasmids were transformed into Rosetta 2 (DE3, pLysS-) competent cells (Novagen) and recombinant protein was purified according to standard methods. Protein was dialyzed into PBS/2 mM DTT/20% glycerol and stored at –80°C.

PIP strips (Echelon Biosciences) were blocked with TBS-Tween (TBST)/0.1% ovalbumin (OAB) (Sigma) for 1 hour at room temperature then incubated with 1.0 μg/ml of recombinant GST-fusion protein in TBST/OAB overnight at 4°C. The membrane was then washed in TBST, probed with anti-GST (1:2000; Sigma) at 4°C for 2 hours, washed in TBST, then incubated with goat-anti-mouse HRP (1:10,000), washed and detected by ECL (Amersham).

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COMPETING INTERESTS
The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS
J.D.W., M.J.J., L.D.U., C.A.J., M.K.S., C.A.M. and D.Y.L. conceived of and designed the experiments. J.D.W., M.J.J., L.D.U., C.A.J., M.S., M.K.S., A.A.W. and C.A.M. performed the experiments. J.D.W., M.J.J., L.D.U., C.A.J., M.K.S., MS., A.A.W., H.J.Y., DJ.J., C.A.M. and D.Y.L. analyzed the data. J.D.W., M.J.J., C.A.M., D.J.G. and D.Y.L. wrote the paper, and C.A.J., L.D.U. and H.J.Y. edited the manuscript.

SUPPLEMENTARY MATERIAL
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