VEGF–PLCγ1 pathway controls cardiac contractility in the embryonic heart

Wolfgang Rottbauer,[1,2,4,6] Steffen Just,[1,4] Georgia Wessels,[1] Nicole Trano,[1] Patrick Most,[1] Hugo A. Katus,[1] and Mark C. Fishman[2,3,5]

[1]Department of Medicine III, University of Heidelberg, D-69120 Heidelberg, Germany; [2]Cardiovascular Research Center, Massachusetts General Hospital and Department of Medicine, Harvard Medical School, Boston, Massachusetts 02114, USA; [3]Novartis Institutes for BioMedical Research, Cambridge, Massachusetts 02139, USA

The strength of the heart beat can accommodate in seconds to changes in blood pressure or flow. The mechanism for such homeostatic adaptation is unknown. We sought the cause of poor contractility in the heart of the embryonic zebrafish with the mutation *dead beat*. We find through cloning that this is due to a mutation in the phospholipase C γ1 (*plcγ1*) gene. In mutant embryos, contractile function can be restored by PLCγ1 expression directed selectively to cardiac myocytes. In other situations, PLCγ1 is known to transduce the signal from vascular endothelial growth factor (VEGF), and we show here that abrogation of VEGF also interferes with cardiac contractility. Somewhat unexpectedly, FLT-1 is the responsible VEGF receptor. We show that the same system functions in the rat. Blockage of VEGF–PLCγ1 signaling decreases calcium transients in rat ventricular cardiomyocytes, whereas VEGF imposes a positive inotropic effect on cardiomyocytes by increasing calcium transients. Thus, the muscle of the heart uses the VEGF–PLCγ1 cascade to control the strength of the heart beat. We speculate that this paracrine system may contribute to normal and pathological regulation of cardiac contractility.

[*Keywords*: VEGF; PLCγ1; contractility; heart; zebrafish]

Supplemental material is available at http://www.genesdev.org.

Received March 30, 2005; revised version accepted May 23, 2005.

In vertebrates, the heart and vasculature have great ability to match function to hemodynamic needs. Some of this is due to homeostatic mechanisms extrinsic to the heart. For example, sympathetic nerves and circulating catecholamines enhance contractility in the face of fluid loss. There is, additionally, a powerful system intrinsic to the heart that matches precisely the input to output of the heart. This so-called Frank-Starling relationship, first described in 1918, is manifest even in an isolated heart (Starling 1918). The molecular basis of this intrinsic system is not known. It has been speculated to reflect better alignment of the myofibrillar arrays (Sonnenblick et al. 1973) or regulation of intracellular calcium (Ca^{2+}) (Gordon and Pollack 1980; Van Henningen et al. 1982; Gillebert et al. 1989). Even the embryonic heart manifests a Frank-Starling response well before innervation (Benson et al. 1989).

Phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2), producing two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces a transient increase in intracellular free Ca^{2+}, while DAG directly activates protein kinase C (PKC) (Lamers et al. 1993). To date, 11 mammalian PLC-isozymes have been characterized. Targeted disruption of the *plcγ1* gene in mice leads to severe defects in blood-vessel formation (Liao et al. 2002). *y10* zebrafish mutants, which have recently shown to be at least partially deficient in PLCγ1 have defects in formation of the artery, but not the vein (Lawson et al. 2003).

Vascular endothelial growth factor (VEGF) acts via its receptors FLT-1 and FLK-1 to activate PLCγ1. In the embryo, this activity is essential to establish and maintain the earliest vessels, a process termed vasculogenesis, as well as to direct later sprouting, termed angiogenesis. Disruption of VEGF or its receptors is early embryonic lethal because of this critical role (Neufeld et al. 1999). VEGF is produced by the myocardium, where one role clearly is to enhance vascularization. Indeed, its elimination in the heart prevents vessel formation (Ferrara 2001).

Here, we show that the recessive lethal zebrafish mutation, *dead beat* (*ded*) (Stainier et al. 1996), loses contractility of its ventricle and lacks a lumenized vasculature. By positional cloning, we show that the *ded* mutation is in *zplcγ1*. Cardiac-specific expression of wild-type

[4]These authors contributed equally to this work.
Corresponding authors.
[5]E-MAIL mark.fishman@pharma.novartis.com; FAX (617) 871-3402.
[6]E-MAIL wolfgang.rottbauer@med.uni-heidelberg.de; FAX 0049-6221-564886.
Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.1319405.

zplcγ1 in *ded* mutant cardiomyocytes restores contractility, even in the absence of blood circulation. We demonstrate that VEGF via its receptor FLT-1 acts upstream of PLCγ1 to control cardiac ventricular contractility. Using cultured rat neonatal ventricular cardiomyocytes, we show VEGF–FLT-1–PLCγ1 signals to control contractility in a reversible and rapid manner by modifying cardiomyocyte calcium cycling. These observations indicate that VEGF, its receptor FLT-1, and zPLCγ1 are key components of a pathway that controls the onset and maintenance of cardiac ventricular contractility, independently of its impact on vessel formation.

Results

Zebrafish dead beat (ded) renders the cardiac ventricle noncontractile, and affects key steps in vasculogenesis

We isolated the zebrafish mutation *dead beat* (*ded*^{m582}) in a large-scale ENU-mutagenesis screen for recessive lethal mutations that perturb cardiac function (Stainier et al. 1996). *ded* mutant embryos display progressive, ventricle-specific reduction of cardiac contractility. Aside from pericardial edema, *ded* embryos are not noticeably affected by the lack of normal blood flow during the first week of development (Fig. 1A,B). As in wild-type embryos, the first cardiac contractions in *ded* are peristaltic. By 36 h post-fertilization (hpf), the two chambers contract rhythmically, sequentially, and vigorously. However, shortly after, there is progressive diminution in ventricular contractility of *ded* mutant embryos. In order to quantitate contractility, we applied a fractional shortening assay, which measures systolic contractile function normalized to the diameters of the heart. By 48 hpf, fractional shortening (FS) of the *ded* ventricular chamber is severely reduced from 22.3% ± 5.8% to 1.4% ± 2.2%. By 60 hpf the ventricular chamber becomes completely silent, whereas the atrium of *ded* mutant embryos continues to contract normally (atrial FS of *ded* = 14.8% ± 3.2%; atrial FS of wild-type = 15.9% ± 3%) (Fig. 1C,D; Supplementary Movie 1). This indicates a chamber-specific requirement for *ded* to maintain cardiac ventricular contractility.

ded mutant ventricles are structurally indistinguishable from wild-type hearts. By 72 hpf, an epicardial, myocardial, as well as an endocardial layer are clearly present (Fig. 1E,F). The ventricular myocardium of *ded* thickens properly by addition of myocardial cells, and the number of ventricular cardiomyocytes (342 ± 21 SEM) is indistinguishable from wild type (336 ± 34 SEM), even at 72 hpf, when the *ded* ventricle is completely silent. There is no

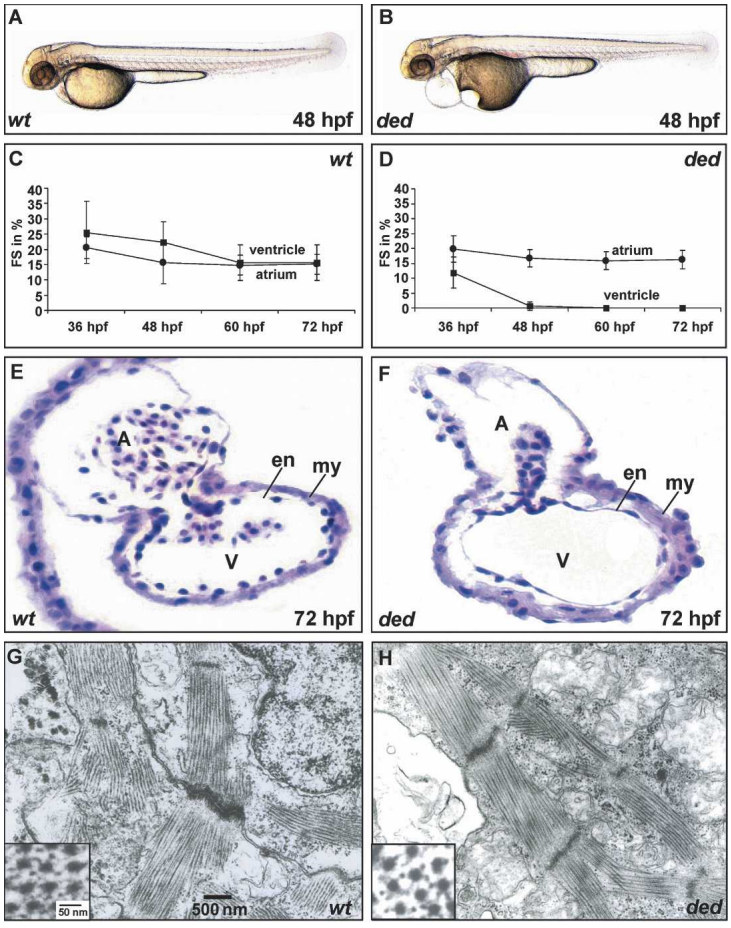

Figure 1. Effects of the *ded*^{m582} mutation on embryonic heart function. (*A*,*B*) *ded* mutants develop pericardial edema due to loss of ventricular contractility, whereas the development of other organ systems proceeds normally. Lateral view of wild-type (wt) and *ded* mutant embryos at 48 hpf. (*C*,*D*) Fractional shortening (FS) of the atrial (filled circles) and ventricular (filled boxes) chamber of wild-type and *ded* mutants at 36, 48, 60, and 72 hpf. Ventricular FS of *ded* mutant ventricles severely decreases over time, whereas atrial contractility is unaffected. (*E*,*F*) *ded* hearts display normal heart morphology. Endocardial and myocardial layers of the heart are unaltered in *ded* mutant hearts after 72 hpf. Transverse sections of a wild-type (*E*) and *ded* mutant (*F*) embryo stained with hematoxylin/eosin. (A) Atrium; (V) ventricle; (en) endocard; (my) myocard. (*G*,*H*) Transmission electron microscopy of wild-type (*G*) and *ded* mutant (*H*) zebrafish embryonic hearts at 72 hpf. Ventricular cardiomyocytes of *ded* mutants show normal cell architecture. *Insets* display transverse sections of myofilaments.

increase in number of apoptotic myocardial cells compared with wild-type embryos using TUNEL-staining (data not shown). Transmission electron microscopy of *ded* ventricular cardiomyocytes reveals normal myocardial cell architecture, including regular arrays of thick and thin myofilaments, Z-discs, and intercalated discs, as well as normal content and appearance of mitochondria (Fig. 1G,H). There is no evident difference in the expression of key-regulatory cardiac transcription factors, such as *znkx2.5* (Chen and Fishman 1996) and *ztbx5* (Garrity et al. 2002), nor for genes encoding thick myofilament components, such as *myosin light chain 2* (*zmlc2*), *ventricular-specific myosin heavy chain* (*zvmhc*) (Yelon et al. 1999) and *atrial myosin heavy chain* (*zamhc*) (Fig. 2A,F; Berdougo et al. 2003).

ded mutant embryos are also defective in embryonic vasculogenesis, lacking a lumenized dorsal aorta and the posterior cardinal vein (Fig. 2G,H).

dead beat *acts cell autonomously in the cardiac myocyte*

To evaluate whether *ded* acts cell autonomously or non-cell autonomously in the ventricular myocardium, we generated fish with hearts mosaic between wild-type and *ded* mutant cardiomyocytes. Labeled wild-type cells integrate normally in *ded*$^{-/-}$ ventricles and contract actively (n = 17) in the *ded* ventricle, even long after *ded* ventricular cardiomyocytes stopped contracting (Supplementary Movie 2). Myocytes from *ded*$^{-/-}$ mutant embryos in ventricles of wild-type embryos do not contract. Rather, they buldge outward during ventricular systole (n = 4) (data not shown). These experiments clearly indicate a ventricle-specific and cell-autonomous role of *ded* in the heart.

The dead beat *(*ded^{m582}*) locus encodes zebrafish phospholipase C γ1 (*zplcγ1*)*

We identified the ENU-induced mutation causing the recessive *ded* mutant phenotype by a positional walk (Fig. 2I). The *ded* mutation maps to the *zplcγ1* gene. By sequence, we identified the *ded*^{m582} mutation to be a guanine to thymine nucleotide transversion (GGA → TGA) in codon 11, predicted to change glycine to a stop codon at amino acid 11, and to cause premature termination of translation of *zplcγ1* (Fig. 2J). Hence, the *ded* mutant

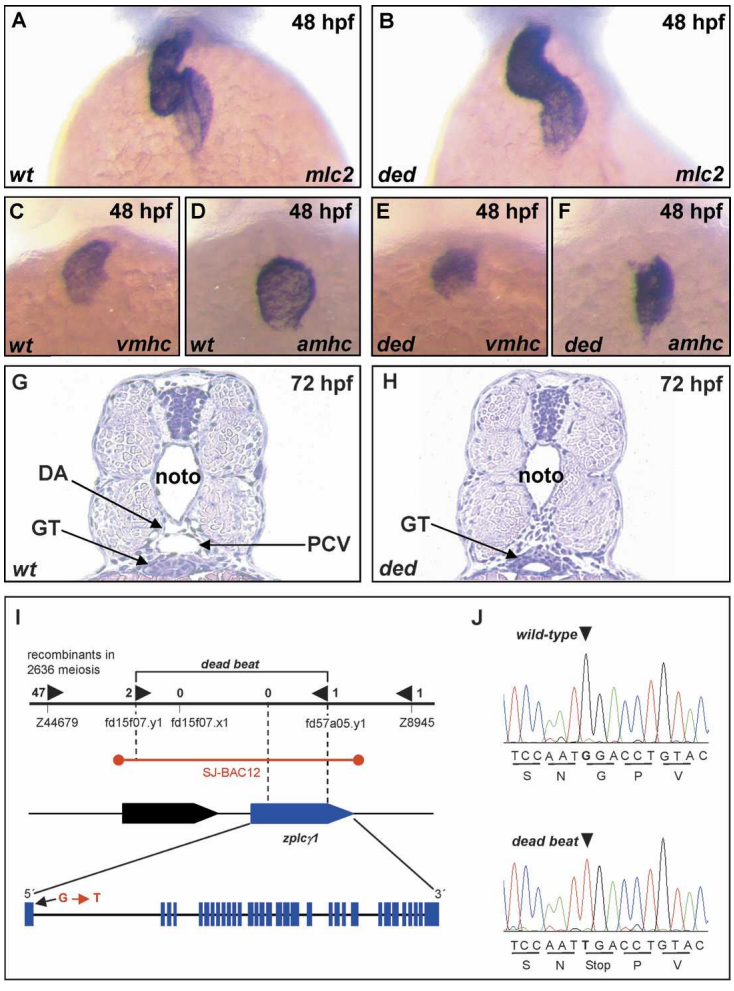

Figure 2. *ded* mutant heart and vascular phenotype due to mutation in the zebrafish *phospholipase C γ1* gene. (*A–F*) Unaltered expression of heart chamber-specific genes in *ded* mutant hearts. At 48 hpf, the expression of *myosin light chain 2* (*mlc2*), *ventricular myosin heavy chain* (*vmhc*), and *atrial myosin heavy chain* (*amhc*) investigated by RNA antisense in situ hybridization, is similar in *ded* and wild-type hearts. (*G,H*) Absence of a lumenized dorsal aorta and posterior cardinal vein in *ded* mutants. Transverse sections of a wild-type (*G*) and *ded* mutant (*H*) embryo at the mid-trunk region, stained with hematoxylin/eosin. (noto) Notochord; (GT) gut tube; (DA) dorsal aorta; (PCV) posterior cardinal vein. (*I*) Integrated genetic and physical map of the zebrafish *ded* region. The *ded* mutation interval is flanked by the microsatellite markers z8945 and z44679. A bacterial artificial chromosome (BAC) clone SJ-BAC12 was isolated covering the *ded* interval and subjected to shotgun sequencing. Sequence analysis of the *ded* mutation interval revealed two zebrafish genes, highly homologous to the *DNA topoisomerase I* and *phospholipase C γ1*. The genomic structure of *zplcγ1* is displayed at the *bottom* of the figure. The *ded* nonsense mutation (G → T) in the first exon of *zplcγ1* is indicated. (*J*) The point mutation (G → T) at cDNA position 31 translates into a premature stop codon (GGA → TGA). The mutated base is marked by an arrowhead.

heart and vascular phenotypes are due to a loss of *zplcγ1* function.

As is the human, *zplcγ1* is encoded by 32 exons. The zebrafish protein encodes 1312 amino acids and has 74% amino acid identity to the human ortholog. As in other species, *zplcγ1* encodes a pleckstrin homology domain (PH domain) in the N terminus of the protein, two catalytic domains (X and Y), and a conserved C2 domain in the C-terminal region. In addition, PLCγ1 includes two SH2 domains, known to recognize the phosphotyrosine-containing sequences in VEGF receptors, one SH3 domain, and one EF-hand domain (Supplementary Fig. S1A; Anderson et al. 1990).

In other species, *plcγ1* is expressed ubiquitously both during embryonic development and in the adult (Gerfen et al. 1988; Liao et al. 2002). We confirm here by RT–PCR expression of *zplcγ1* in the myocardium of both atrium and ventricle during embryonic development and in the adult vertebrate (Supplementary Fig. S1B). By antisense RNA whole-mount in situ staining, we observe *zplcγ1* to be ubiquitously expressed in zebrafish embryos during the first 72 hpf. At 48 hpf, expression of *zplcγ1* RNA is pronounced in the brain, vasculature, and the heart (Fig. 3A–C). By 72 hpf, zPLCγ1 protein is found to be expressed in both the endocardial and myocardial layer of the atrium and the ventricle (Fig. 3D).

Injection of Morpholino-modified antisense oligonucleotides against zebrafish plcγ1 *phenocopies the* ded *heart and vessel phenotypes*

To confirm that the *ded* mutant heart contractility and vascular phenotypes are caused by the absence of zPLCγ1, we injected Morpholino-modified antisense oligonucleotides, either directed against the translational start site (MO1-*zplcγ1*) or the splice donor site of exon 13 (MO2-*zplcγ1*) of *zplcγ1* into one-cell stage wild-type zebrafish embryos. When injected with 4 ng of MO2-*zplcγ1*, 98% of injected embryos reveal the *ded* mutant heart and vascular phenotype (Fig. 3E–G). Blood circulation in lumenized vessels is absent, and ventricular contractility decreases during development, leading to a silent ventricle 60 h after injection. Similar effects and efficacy are observed when injecting 4 ng of MO1-*zplcγ1*.

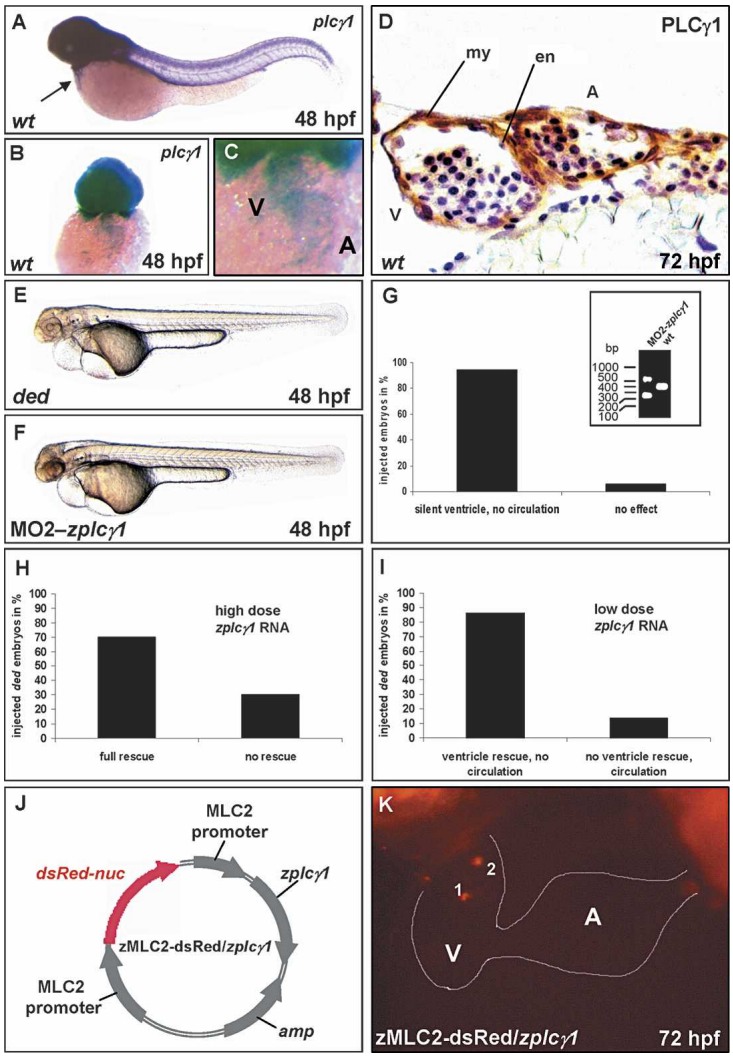

Figure 3. The *ded* ventricular contractility defect can be rescued even in the absence of an intact vasculature. (*A–D*) Ubiquitous *zplcγ1* expression during zebrafish development. (*A–C*) By 72 hpf *zplcγ1*, RNA expression is pronounced in the brain, vasculature, and the heart (arrow). (*D*) Immunostain of wild-type zebrafish embryos using a PLCγ1-specific antibody. PLCγ1 is expressed in the endocardial and myocardial layer of both the atrium and the ventricle. (A) Atrium; (V) ventricle; (en) endocardium; (my) myocardium. (*E–G*) Inhibition of *zplcγ1* function by Morpholino-modified antisense oligonucleotide injection phenocopies the *ded* mutant phenotype. MO2-*zplcγ1*-injected embryos are indistinguishable from *ded* mutant embryos and display severe impairment of ventricular contractility and vasculogenesis. The effectiveness of MO2-*zplcγ1* on mRNA splicing is shown in the *inset* in *G*. Injection of MO2-*zplcγ1* results in abnormal splice products of 602 bp (integration of intron 13) and 250 bp (skipping of exon 13), leading to premature termination of translation of zPLCγ1. (*H*,*I*) Injection of wild-type *zplcγ1* RNA at different dosages rescues the *ded* mutant vascular and heart defects, independently. (*H*) Injection of *zplcγ1* mRNA at a high dose (25 pg) results in the full rescue of the *ded* mutant vascular and heart phenotype in 70% of injected $ded^{-/-}$ embryos. (*I*) If lower amounts (13 pg) of *zplcγ1* mRNA are injected in $ded^{-/-}$ embryos, ~80% of injected embryos reveal normal ventricular contractility after 72 hpf, whereas no vascular rescue was observed. Vice versa, ~15% of rescued $ded^{-/-}$ embryos show complete vascular rescue, whereas ventricular contractility is still absent. (*J*,*K*) Cardiac-specific, mosaic overexpression of wild-type *zplcγ1* rescues the contractile deficit of single $ded^{-/-}$ cardiomyocytes. (*J*) Schematogram of the zMLC2-dsRed/*zplcγ1* vector. (*K*) Injection of zMLC2-dsRed/*zplcγ1* leads to cardiac-specific, mosaic overexpression of *zplcγ1* in two different cardiomyocytes (1, 2). (Lateral view) Head to the *left*. (V) Ventricle; (A) atrium.

RNA analysis confirms the effect of MO2-*zplcγ1* on *zplcγ1* RNA (Fig. 3G, inset).

zplcγ1 *mRNA independently restores heart function and vasculogenesis in* ded *mutant embryos*

To evaluate whether wild-type *zplcγ1* mRNA can restore ventricular contractile function and/or vasculogenesis of homozygous *ded* mutant embryos, we injected *zplcγ1* mRNA into embryos derived from intercrossing *ded* heterozygous zebrafish. After injecting 25 pg of wild-type *plcγ1* mRNA, 70% of *ded* mutant embryos show complete rescue of both the vascular and heart phenotype (Fig. 3H). Blood circulation through a lumenized arterial and venous vasculature is established and the ventricle contracts normally. Cardiac contractility is generally restored by lower amounts than needed to rescue the vasculature. In 86.3% $ded^{-/-}$ embryos injected with 13 pg of *zplcγ1* mRNA, ventricular heart contractility is restored, but no blood circulation is established. In a few cases (13.6%), blood vessels are restored, but the ventricular chamber does not pump (Fig. 3I). Presumably, this reflects a mosaic effect of the injection. These results confirm that the mutation in the *zplcγ1* gene is responsible for the *ded* vascular and heart defects. Furthermore, they demonstrate that the cardiac phenotype is not secondary to the vascular defect.

Cardiac-specific, mosaic overexpression of zebrafish plcγ1 *rescues the* ded *mutant ventricular contractility defect*

In order to examine whether *zplcγ1* is sufficient to rescue ventricular cardiomyocyte contractility in a cell-autonomous fashion, we directed its expression to myocardial cells of the heart using the cardiomyocyte-specific zebrafish myosin-light chain 2 (zMLC2) promoter (Rottbauer et al. 2002). We modified our previous expression construct, such that the fluorescent marker nucDsRed2 is coexpressed (zMLC2-dsRed/*zplcγ1*) (Fig. 3J). We injected the zMLC2-dsRed/*zplcγ1* plasmid into $ded^{-/-}$ embryos, generating mosaic hearts. Cardiomyocytes, which express zebrafish *zplcγ1* and nucDsRed2 under the control of zMLC2 contract vigorously in otherwise silent *ded* ventricles (Fig. 3K; Supplementary Movie 3). This persists beyond 72 hpf, indicating that overexpression of wild-type *zplcγ1* in $ded^{-/-}$ cardiomyocytes can restore the *ded* ventricular contractility defect in single ventricular cells (18 of 30).

VEGF is essential for zebrafish vasculogenesis and cardiac ventricular contractility

PLCγ1 acts downstream of VEGF signaling in many situations (McLaughlin and De Vries 2001; Takahashi et al. 2001; Lawson et al. 2003). In zebrafish, knock-down of *vegf* using Morpholino-modified antisense oligonucleotides (MO-*vegf*) have been noted to cause a vascular phenotype identical to the one observed in *ded* mutant embryos (Nasevicius et al. 2000), a finding we confirm.

In addition, we find that contractility of the ventricle is significantly impaired by Morpholinos directed to *vegf*. Fractional shortening of the ventricular chamber is reduced from wild-type values of 34.7% ± 6.2% to 5.7% ± 3.6% in injected embryos after 48 hpf. By 60 hpf, the ventricle in the Morpholino-injected embryos becomes silent, while the atrium continues to contract (Fig. 4C). Thus, MO-*vegf* injection phenocopies both the vascular and the cardiac phenotypes of *ded* mutant zebrafish embryos (Fig. 4A–D; Supplementary Movie 4), indicating that VEGF is critical to ventricular contractility.

VEGF signals through its receptor VEGFR-1 (FLT-1) to control ventricular contractility

On endothelial cells, where best studied, VEGF is known to exert its effects through at least two receptors, FLK-1, FLT-1, along with the coreceptor Neuropilin-1 (Np-1) (Neufeld et al. 1999; Gu et al. 2003). In zebrafish, only FLK-1 and Np-1 have been identified to date (Thompson et al. 1998; Lee et al. 2002). Therefore, to evaluate which receptor mediates VEGF effects on zebrafish ventricular contractility and vasculogenesis, we first inhibited zebrafish FLK-1 and Np-1 by injections of Morpholinos. However, none of them seems to perturb the heart. Np-1 Morpholino has no obvious effects (data not shown; Lee et al. 2002). As in *flk-1* mutant embryos (Habeck et al. 2002), *flk-1* Morpholino interferes selectively with formation of the intersegmental vessels (in 91.2% of injected embryos, as shown in Fig. 4E–H). Hence, neither FLK-1 nor Np-1 appear to be the relevant receptors for VEGF effects on cardiac contractility or vasculogenesis in the zebrafish.

In mammals and the chick (Yamaguchi et al. 2002), there is an additional VEGF receptor, termed FLT-1 or VEGFR-1. To our knowledge, a FLT-1 receptor has not been identified in zebrafish. In fact, it has been speculated that FLK-1 serves as the only VEGF receptor in that species, similar to the situation in *Drosophila melanogaster* (Habeck et al. 2002). However, since we found no evidence that FLK-1 or the coreceptor Np-1 mediate VEGF effects on cardiac contractility in the zebrafish, we suspected the existence of another, as yet undefined, zebrafish VEGF receptor. We identified in the zebrafish genome sequence an open reading frame with 51% amino acid identity to human FLT-1 (GenBank accession no. AY848694). We located this zebrafish *flt-1* gene on zebrafish linkage group 24 by radiation hybrid mapping. Interestingly, the zebrafish genomic region around the *flt-1* gene (NA54293 from Sanger Zebrafish Assembly 3) is syntenic to a region on human chromosome 13 that includes the *flt-1* gene, further supporting the identification of zebrafish *flt-1* as the ortholog of human *flt-1* (data not shown). Full-length alignment of human and zebrafish FLT-1 protein sequence is provided as Supplemental Material (Supplementary Fig. S3).

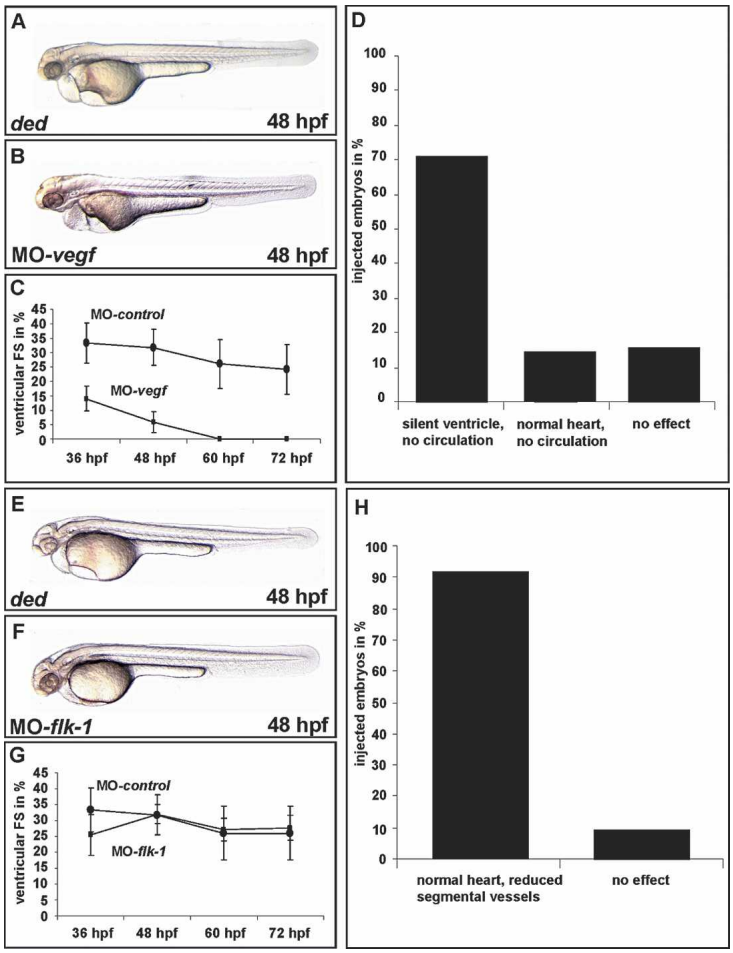

Figure 4. Effects of VEGF on zebrafish cardiac ventricular contractility are not mediated by the receptor FLK-1. (*A–D*) Injection of MO-*vegf* into wild-type embryos phenocopies the vascular and heart contractility defect of *ded* mutant embryos. At 60 hpf, 70% of injected embryos reveal complete absence of circulation, as well as a silent ventricle. About 15% of embryos reveal loss of circulation, but normal ventricular contractility. (*C*) In contrast to mock-injected (MO-*control*) wild-type embryos (filled circles), fractional shortening of the ventricular chamber of MO-*vegf*-injected embryos (filled boxes) is significantly reduced after 36 hpf. By 60 hpf, the ventricle of MO-*vegf*-injected embryos becomes silent (FS = 0%). (*E–H*) Zebrafish embryos injected with MO-*flk-1* (*F*) do not display a vascular and cardiac phenotype similar to *ded* mutants (*E*). (*G*) In comparison with mock-injected (MO-*control*) wild-type embryos (filled circles) injection of MO-*flk-1* (filled boxes) does not lead to a decreased fractional shortening (FS) of the ventricular chamber. (*H*) In ~90% of MO-*flk-1*-injected embryos, intersegmental vessels are absent. None of the embryos displays a heart phenotype similar to *ded* mutant embryos.

To test whether zebrafish *flt-1* transduces VEGF signals, we injected embryos with zebrafish *flt-1* Morpholino (MO1-*flt-1*) (Fig. 5A–C). Eighty percent of injected wild-type embryos phenocopy the vascular phenotype of *ded* mutant embryos (data not shown). In addition, MO1-*flt-1* injection causes progressive reduction of ventricular contractility to a fractional shortening of 4.8% ± 5% by 48 hpf, and finally between 60 and 72 hpf, to a silent ventricle (Fig. 5C; Supplementary Movie 4). This is identical to the cardiac phenotype observed in *ded* mutant and MO-*vegf*-injected zebrafish embryos. As in *ded* mutant embryos, the heart in MO1-*flt-1*-injected embryos retain relatively normal histology of endocardial and myocardial layers (Fig. 5D). Ventricular cardiomyocyte cell numbers are not different between MO1-*flt-1* (325 ± 18 SEM) and embryos injected with MO-*control* (341 ± 21 SEM) at 72 hpf. Injection of a second zebrafish *flt-1* Morpholino (MO2-*flt-1*), which targets a different splice donor site, also phenocopies the *ded* mutation with the same efficacy. RT–PCR analysis demonstrates strong expression of *flt-1* in embryonic and adult cardiomyocytes from zebrafish and rat (Supplementary Fig. S2A). In summary, the data indicate that VEGF signaling, mainly through the VEGF receptor 1 (FLT-1), activates zPLCγ1, and thereby controls ventricular contractility.

VEGF receptors and PLCγ1 are regulators of rat cardiomyocyte contractility

VEGF and PLCγ1 are known to be expressed in cardiomyocytes (Puceat and Vassort 1996; Seko et al. 1999), whereas FLT-1 expression was thought to be mainly on vascular endothelial cells (Shibuya et al. 1994). We therefore evaluated the expression of FLT-1 protein in adult rat hearts by immunostaining. As shown in Figure 5E, FLT-1 is expressed at high levels in the sarcolemma of rat cardiomyocytes. An isotype-matched antibody control does not show any specific immunostaining in the sarcolemma (Supplementary Fig. S2B).

To examine whether VEGF signaling through the receptor tyrosine kinase FLT-1 and the consecutive activation of PLCγ1 affect cardiac myocyte contractility in mammals, we first incubated neonatal rat ventricular cardiomyocytes with the specific VEGF receptor inhibitor PTK787/ZK222584 or the PLC inhibitor U73122 at various concentrations and recorded their spontaneous contractility by video-microscopy (Yule and Williams 1992; Wood et al. 2000). Eighty-four percent of cardiomyocytes treated with PTK787/ZK222584 lose their spontaneous contractile activity within minutes after incubation. Similarly, up to 80% of ventricular rat cardio-

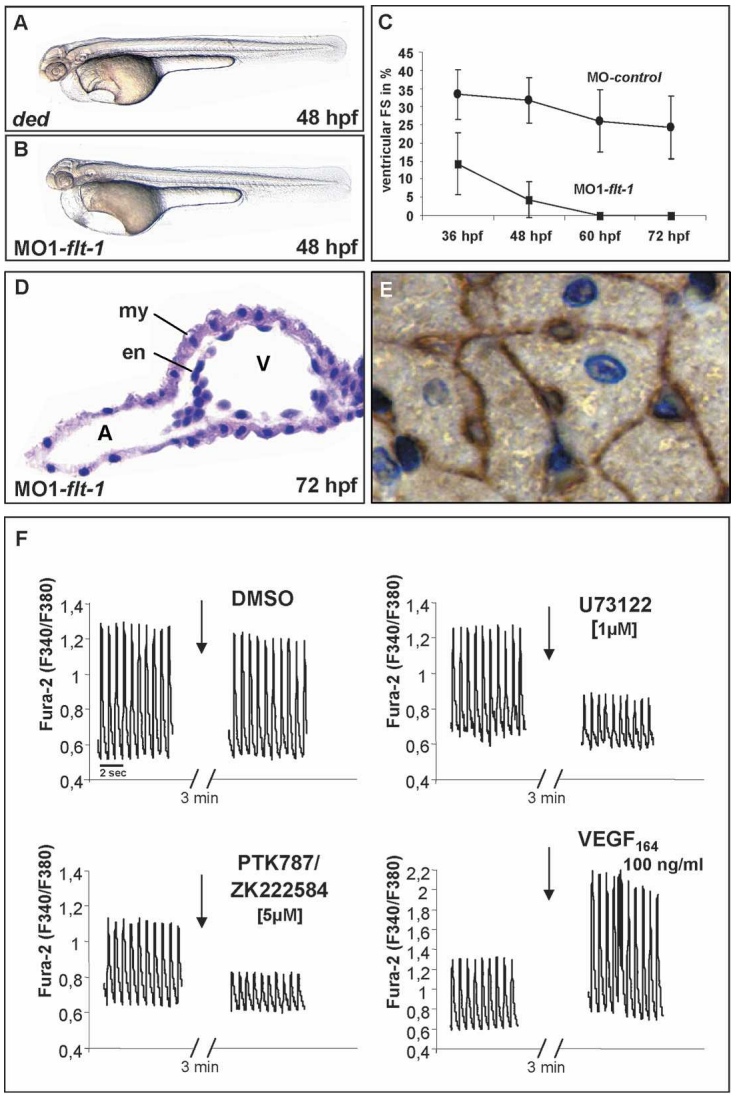

Figure 5. VEGF–FLT-1–PLCγ1 signaling controls cardiomyocyte contractility by modulating calcium cycling. (*A–C*) MO1-*flt-1*-injected embryos (*B*) display a cardiovascular phenotype indistinguishable from *ded* mutant embryos (*A*). (*C*) Injection of MO1-*flt-1* significantly impairs cardiac ventricular contractility. In contrast to MO-*control*-injected embryos (filled circles), fractional shortening of the ventricular chamber of MO1-*flt-1*-injected embryos (filled boxes) is significantly reduced after 36 hpf. By 60 hpf, the ventricle of MO1-*flt-1*-injected embryos becomes silent (FS = 0%). (*D*) Histological sections of MO1-*flt-1*-injected zebrafish embryonic hearts at 72 hpf stained with hematoxylin/eosin. The ventricular and atrial myocardium of MO1-*flt-1*-injected embryos is properly developed by 72 hpf. Both heart chambers display normal myocardial and endocardial layers. (A) Atrium; (V) ventricle; (en) endocard; (my) myocard. (*E*) The VEGF-1 receptor FLT-1 is expressed in the cell membrane of rat ventricular cardiomyocytes. Cell nuclei are counterstained with hematoxylin. (*F*) Ca^{2+} transients of neonatal rat ventricular cardiomyocytes are modified by VEGF–PLCγ1 signaling. Ca^{2+} transients (Fura-2 F340/F380) were measured before and 3 min after treatment with the PLC inhibitor U73122, the VEGF receptor inhibitor PTK787/ZK222584 or $VEGF_{164}$. Cardiomyocytes were field stimulated at a rate of 2 Hz. Inhibition of PLCγ1 function by U73122 (1 µM) or VEGF receptor function by PTK787/ZK222584 (5 µM) leads to significant reduction of the calcium transients, whereas treatment with $VEGF_{164}$ leads to significant increase of calcium cycling.

myocytes treated with the PLC inhibitor U73122 lose their spontaneous contractile activity within minutes after incubation, dependent on the amount of U73122 used (10–50 µM), whereas cardiomyocytes treated with the inactive form of the inhibitor (U73343) continue beating. PTK787/ZK222584 and U73122 treated cardiomyocytes are refractory to electrical pacing. These findings already indicate an essential role for the VEGF–PLCγ1 pathway in mammalian ventricular cardiomyocyte contractility.

To further elucidate the mechanism by which VEGF–FLT-1–PLCγ1 signaling controls cardiomyocyte contractility, we paced neonatal rat ventricular cardiomyocytes at a defined rate (two per second), incubated them with the specific VEGF receptor inhibitor PTK787/ZK222584, the PLC inhibitor U73122 as well as $VEGF_{164}$ at various concentrations, and recorded their calcium transients before and after treatment. As shown in Figure 5F, inhibition of VEGF signaling with either PTK787/ZK222584 (5 µM) or U73122 (1 µM) significantly reduces (~20%–30%) calcium transients in the ventricular cardiomyocytes, whereas treatment with $VEGF_{164}$ (100 ng/mL) almost doubles calcium transients. Treatment of the field-stimulated cardiomyocytes with high concentrations of PTK787/ZK222584 (20 µM) or U73122 (20 µM) completely inhibits calcium cycling within 3 min after incubation (data not shown). These findings indicate that VEGF–PLCγ1 signaling controls mammalian ventricular cardiomyocyte contractility by modulating calcium cycling.

Interestingly, when bathing wild-type zebrafish embryos in either U73122 (1 µM) or PTK787/ZK222584 (5 µM) after 48 h of development, at a time point when both heart chambers are usually vigorously beating and propel blood cells to the aterial and venous vascular system, ventricular cardiomyocytes become silent within 24 h, whereas no effect on the vascular system is observed (Supplementary Movie 5). These findings are compatible with the VEGF–FLT-1–PLCγ1 signaling pathway, playing an essential role in controlling ventricular cardiomyocyte contractility, even in later stages of development.

Discussion

Cardiac contractility is regulated tightly as an essential homeostatic mechanism (Starling 1918; Frank 1959; Schaldach and Hutten 1990). Some of the control is neuro-humoral, but much appears intrinsic to the heart. Here we show that VEGF, in addition to its role in blood-vessel formation, regulates cardiac contractility. Its pathway of action specifically uses the FLT-1 receptor and PLCγ1.

We describe here a mutation in zebrafish *plcγ1*, *dead beat*, which impairs proper arterial and venous vasculogenesis, as well as cardiac ventricular contractility. The mutation completely abolishes *plcγ1* function. PLCγ1 is known to act downstream of VEGF to control arterial vasculogenesis in vertebrates (Liao et al. 2002; Lawson et al. 2003). We find here that PLCγ1 also is essential for vertebrate heart contractility. Antisense-oligonucleotide mediated knock-down of either *vegf*, *flt-1*, or *plcγ1* function in zebrafish leads to a vascular and cardiac phenotype identical to *ded*. Injected embryos lack a lumenized arterial and venous vascular system, and cardiac ventricular contractility declines over time to culminate in a silent heart by 60 hpf. All components of the pathway, namely VEGF, its receptor FLT-1, and PLCγ1, are found to be expressed in vertebrate cardiomyocytes. Inhibition of VEGF signaling reduces calcium transients in neonatal rat ventricular cardiomyocytes, whereas exposure to VEGF leads to increased calcium cycling. Therefore, we postulate that VEGF activates PLCγ1 in ventricular cardiomyocytes, specifically through its receptor FLT-1 to control their contractile force.

Molecular pathways essential for both vasculogenesis and heart development are difficult to dissect genetically in placental animals, because disturbance of either vascular or heart development leads to early embryonic death. Nullizygous *vegf*, *flt-1*, *flk-1*, and *plcγ1* mice die in early embryogenesis due to disturbed vasculogenesis and angiogenesis (Shalaby et al. 1995; Carmeliet et al. 1996; Ferrara 2001; Liao et al. 2002). In contrast to mammals, intact cardiovascular function is not essential for the early development of the zebrafish embryo, because it obtains adequate oxygen by diffusion from its environment (Burggren and Pinder 1991; Stainier and Fishman 1994). Zebrafish embryos, mutant in cardiovascular gene function, survive for days to late larval stages. This permits analysis of the role of zPLCγ1 in the development of cardiac function.

The zPLCγ1 effect is cell-autonomous. Cardiac myocytes express zPLCγ1. Cardiac myocytes without zPLCγ1 do not contract even in a wild-type heart, whereas wild-type cardiac myocytes contract in a $ded^{-/-}$ heart. The contractile deficit of $ded^{-/-}$ ventricular cardiomyocytes can be rescued by cardiac-specific overexpression of *zplcγ1*, even in single ventricular cardiomyocytes that are surrounded by noncontracting cardiac cells.

Although *zplcγ1* is found to be expressed in atrial cardiomyocytes, it seems not to be essential for maintaining atrial cardiomyocyte contractility. The atrium in *ded* mutant embryos continues to contract normally, even long after the ventricle becomes silent. The biology of the myocytes in the two chambers is quite distinct. For example, the α1C L-type calcium channel subunit (C-LTCC) is expressed in both the atrium and the ventricle of the zebrafish heart, but its mutation only affects the ventricle (Rottbauer et al. 2001).

The downstream mediator of PLCγ1 in cardiomyocytes remains to be determined. Activated PLCγ1 enzyme catalyses the hydrolysis of phosphatidylinositol 4,5,-bisphosphate (PIP_2) to the second messenger molecules inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 can cause release of Ca^{2+} from internal stores, and thereby increase cardiomyocyte contractility. DAG activates PKC enzymes. However, the only PKC evaluated in cardiac myocytes to date, PKC-α, appears to inhibit contractility (Braz et al. 2004). Other isoenzymes, such as PKC-δ and PKC-ε might, of course, play different roles (Kang and Walker 2005).

VEGF is known to be produced and secreted by cardiomyocytes upon biomechanical stress (Li et al. 1997; Seko et al. 1999). It clearly is needed for cardiac vascularization, and it makes sense that vessel density might be a response to stress. In zebrafish, we find that a separate role for VEGF in controlling of heart contractility can be demonstrated long before intramyocardial capillaries and coronary arteries are generated (Hu et al. 2000). Therefore, VEGF signaling is not only required for proper arterial and venous vasculogenesis, but also for the maintenance of cardiac ventricular contractility.

In mammals, VEGF is known to exert its effects on endothelial cells through the receptors FLK-1 and FLT-1. FLK-1 has an essential role in vasculogenesis, angiogenesis, and hematopoiesis, and FLK-1 null mice do not establish the framework of a vascular system nor develop blood islands or organized blood vessels (Shalaby et al. 1995). FLK-1-deficient zebrafish embryos, on the other hand (Habeck et al. 2002), manifest more subtle effects on vessel formation, limited to deficiency in angiogenic formation of intersegmental vessels. This suggests the role of another VEGF receptor with regard to vasculogenesis in the zebrafish. As shown here, MO-*flk-1* injections, even at high concentrations, do not affect heart function. We identified and characterized here the zebrafish orthologous gene for the VEGF receptor *flt-1*. The zebrafish orthologous FLT-1 sequence shows high homology to the human FLT-1 protein (~51% amino acid identity) and maps to a zebrafish linkage group in a region syntenic to human chromosome 13, where human *flt-1* is encoded. It is this receptor that appears to mediate VEGF effects on both cardiac contractility and vasculogenesis in the zebrafish.

The heart responds to stretch by increasing contractility. This feature allows it to respond to increased load, for example, as imposed by heightened blood pressure. Clinically, it is this response that underpins much of the compensatory response in the face of early cardiac failure from myocardial ischemia (Starling 1918; Frank 1959). The molecular basis for this phenomenon has been elusive. There is evidence that VEGF secretion increases

with physical stretch of the heart, via glycoprotein gp130 and transforming growth factor (TGF) β (Seko et al. 1999; Funamoto et al. 2000). PLCγ1 itself can be activated through mechanical stress in neonatal rat cardiomyocytes (Seko et al. 1998). Hence, it is conceivable that stretch of the heart, by increasing VEGF, could contribute to enhanced cardiac contractiltity.

Nearly 5 million Americans have heart failure today, and 550,000 new cases are diagnosed each year. The use of medicines that improve contractility by changing Ca^{2+} handling are complicated by concomitant propensity to arrhythmias. No current agents safely enhance cardiac contractility. It will be of interest to examine whether the VEGF–PLCγ1 pathway may offer new opportunities for such intervention.

Materials and methods

Zebrafish strains, compound treatment, and cell transplantation

Care and breeding of zebrafish *Danio rerio* was as described (Westerfield 1995). Zebrafish embryos were incubated at different developmental stages with various concentrations of the VEGF receptor-specific inhibitor PTK787/ZK222584 (Schering) or the PLC inhibitor U73122 (Sigma). Pictures and movies were recorded 24, 48, and 72 h after starting the compound treatment.

Embryos used for transplantation experiments were produced by intercrossing *ded*$^{m582-/+}$ fish. Cell transplantation was performed essentially as described (Rottbauer et al. 2001). By 24, 48, and 72 hpf, embryos were inspected with fluorescence microscopy, and movies of integrated ventricular cells recorded. To inhibit pigmentation, 0.003% 1-phenyl-2-thiourea was added to the embryo medium. To confirm the integration of labeled donor cells into *ded* or wild-type ventricular myocardial layers, whole embryos were stained with antibiotin antibodies, fixed, and sectioned. Labeled donor embryos were genotyped using polymorphic markers, flanking the *ded* gene.

Histology, transmission electron microscopy, RNA antisense in situ hybridization, and immunostaining

Embryos were fixed in 4% paraformaldehyde and embedded in JB-4 (Polysciences, Inc.). Five-micrometer sections were cut, dried, and stained with hematoxylin/eosin. Electron Micrographs were obtained essentially as described (Rottbauer et al. 2001).

Whole-mount RNA in situ hybridization was carried out essentially as described (Jowett and Lettice 1994) using a full-length *zplcγ1* antisense probe, as well as antisense probes for *zflk-1*, *zmlc2*, *zvmhc*, *zamhc*, *ztbx5*, and *znkx2.5*. All RNA probes were digoxigenin labeled.

For immunostaining, adult rat hearts were fixed in 4% formalin and paraffin embedded. FLT-1 immunostaining was performed with the polyclonal anti-VEGF receptor 1 (ab2350, Abcam) and HRP-coupled rabbit IgG (ab6721, Abcam) antibodies. DAB staining was carried out according to the manufacturer's protocol (Sigma) and cell nuclei counterstained with hematoxylin. For immunostaining of Dent's fixed zebrafish embryos, the monoclonal anti-PLCγ1 (Upstate) antibody and the Vectastain ABC kit (VectorLabs) was used. Embryos were embedded in JB-4 (Polysciences, Inc.), 5-μm sections were cut and dried, and cell nuclei were counterstained with hematoxylin.

Genetic mapping, positional cloning, and mutation detection

DNA from 24 *ded*^{m582} mutant and 24 wild-type embryos was pooled and bulked segregation analysis performed as described (Michelmore et al. 1991). The critical genomic interval for *ded* was defined by genotyping 1318 mutant embryos for polymorphic markers in the area. RNA from *ded* mutant and wild-type embryos was isolated using TRIZOL Reagent (Life Technologies) and reverse transcribed. Eight independent clones from mutant and wild-type *zplcγ1* cDNA were sequenced. Genomic DNA from *ded* mutant and wild-type embryos was sequenced around the point mutation.

Injection procedures

Morpholino-modified oligonucleotides were directed against a splice donor site (MO2-*zplcγ1*, 5′-AGAGCGTCCTCCTGA CCTTGATGAG-3′) and the translational start site of *zplcγ1* (MO1-*zplcγ1*, 5′-TCGCAGCCATTTCCCCTGTTTGTTC-3′), against the translational start site of *vegf* (MO-*vegf*, 5′-GTAT CAAATAAACAACCAAGTTCAT-3′) (Nasevicius et al. 2000), against the 5′UTRs of *flk-1* (MO-*flk-1*, 5′-CCGAATGATACTC CGTATGTCAC-3′) and *neuropilin-1* (MO-*np-1*, 5′-GAATCCT GGAGTTCGGAGTGCGGAA-3′), and two different splice donor sites of *zflt-1* (MO1-*flt-1*, 5′-CAGCAGTTCACTCACATCT CCGTTC-3′) and (MO2-*flt-1*, 5′-AAGCAGACTCTCACCATT TACCACC-3′). A standard control oligonucleotide (MO-*control*) (GENETOOLS, LLC) was injected at the same concentration as a negative control.

To drive cardiac-specific expression of *zplcγ1*, the coding sequence was cloned downstream of the zebrafish MLC2 promoter (zMLC2-dsRed- *zplγ1*). Seventy-five picograms of zMLC2-dsRed- *zplγ1* were pressure injected into one-cell stage wild-type and mutant embryos produced by intercrossing *ded*$^{m582-/+}$ fish. *zplcγ1*-expressing cells were identified in vivo by fluorescent video microscopy (EM CCD Camera Proxitronic).

Sense-capped RNA was synthesized using the mMESSAGE mMASCHINE system (Ambion) from pCS2*zplcγ1*. RNA was diluted (26 ng/μL or 50 ng/μL in solution A [0.1% phenol red, 0.2 M KCl]) and microinjected into one-cell stage embryos. Siblings from the same pool were injected using solution A as control.

Cardiomyocyte culture and measurement of Ca^{2+} transients

Preparation and cultivation of neonatal and adult ventricular cardiomyocytes was performed essentially as described (Most et al. 2003). Adult as well as neonatal cardiomyocytes were incubated with different concentrations of the phospholipase C-specific inhibitor (U73122, Sigma) or its inactive form (U73343, Sigma). Futhermore cardiomyocytes were incubated with different concentrations of the VEGF receptor (FLT-1, FLK-1) inhibitor (PTK787/ZK222584, Schering).

Ca^{2+} imaging in field-stimulated neonatal ventricular cardiomyocytes was carried out essentially as described (Most et al. 2003). Isolated cells were incubated in 1 mL HEPES-modified medium 199 (2 mM [Ca^{2+}]) with 2 μM FURA2-AM for 20 min at room temperature. Cells were electrically stimulated with 2 Hz, and measurements were performed with T.I.L.L. Vision software (version 4.01). Ca^{2+} transients were recorded at baseline and throughout the treatment with the VEGF receptor-specific inhibitor PTK787/ZK222584 (Schering), the PLC inhibitor U73122 (Sigma), and $VEGF_{164}$ (Sigma).

Acknowledgments

We thank M.H. Boulos, M. McKee, S. Marquart, and M. Völckers for technical assistance, and A. Doherty for excellent fish care. We are especially grateful to Melanie Peters, who provided us excellent technical assistance and tragically died during the course of this study. We thank N.D. Lawson for providing pCS2zplcg1 and J.G. Burns supplying us zMLC2-dsRed. This work was supported by grants from NIH—5R01HL49579, 5R01DK55383, 1R01HL63206 (M.C.F.), DFG Ro2173/1-1, DFG Ro2173/2-1, and BMBF 01GS0108-TP5, as well as Klaus-Georg and Sigrid Hengstberger Stipendium (W.R.).

References

Anderson, D., Koch, C.A., Grey, L., Ellis, C., Moran, M.F., and Pawson, T. 1990. Binding of SH2 domains of phospholipase C γ 1, GAP, and Src to activated growth factor receptors. *Science* **250:** 979–982.

Benson Jr., D.W., Hughes, S.F., Hu, N., and Clark, E.B. 1989. Effect of heart rate increase on dorsal aortic flow before and after volume loading in the stage 24 chick embryo. *Pediatr. Res.* **26:** 438–441.

Berdougo, E., Coleman, H., Lee, D.H., Stainier, D.Y., and Yelon, D. 2003. Mutation of weak atrium/atrial myosin heavy chain disrupts atrial function and influences ventricular morphogenesis in zebrafish. *Development* **130:** 6121–6129.

Braz, J.C., Gregory, K., Pathak, A., Zhao, W., Sahin, B., Klevitsky, R., Kimball, T.F., Lorenz, J.N., Nairn, A.C., Liggett, S.B., et al. 2004. PKC-α regulates cardiac contractility and propensity toward heart failure. *Nat. Med.* **10:** 248–254.

Burggren, W.W. and Pinder, A.W. 1991. Ontogeny of cardiovascular and respiratory physiology in lower vertebrates. *Annu. Rev. Physiol.* **53:** 107–135.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., et al. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380:** 435–439.

Chen, J.N. and Fishman, M.C. 1996. Zebrafish tinman homolog demarcates the heart field and initiates myocardial differentiation. *Development* **122:** 3809–3816.

Ferrara, N. 2001. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am. J. Physiol. Cell. Physiol.* **280:** C1358–C1366.

Frank, O. 1959. On the dynamics of cardiac muscle. *Am. Heart J.* **58:** 282–317.

Funamoto, M., Fujio, Y., Kunisada, K., Negoro, S., Tone, E., Osugi, T., Hirota, H., Izumi, M., Yoshizaki, K., Walsh, K., et al. 2000. Signal transducer and activator of transcription 3 is required for glycoprotein 130-mediated induction of vascular endothelial growth factor in cardiac myocytes. *J. Biol. Chem.* **275:** 10561–10566.

Garrity, D.M., Childs, S., and Fishman, M.C. 2002. The heartstrings mutation in zebrafish causes heart/fin Tbx5 deficiency syndrome. *Development* **129:** 4635–4645.

Gerfen, C.R., Choi, W.C., Suh, P.G., and Rhee, S.G. 1988. Phospholipase C I and II brain isozymes: Immunohistochemical localization in neuronal systems in rat brain. *Proc. Natl. Acad. Sci.* **85:** 3208–3212.

Gillebert, T.C., Sys, S.U., and Brutsaert, D.L. 1989. Influence of loading patterns on peak length-tension relation and on relaxation in cardiac muscle. *J. Am. Coll. Cardiol.* **13:** 483–490.

Gordon, A.M. and Pollack, G.H. 1980. Effects of calcium on the sarcomere length-tension relation in rat cardiac muscle. Implications for the Frank-Starling mechanism. *Circ. Res.* **47:** 610–619.

Gu, C., Rodriguez, E.R., Reimert, D.V., Shu, T., Fritzsch, B., Richards, L.J., Kolodkin, A.L., and Ginty, D.D. 2003. Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. *Dev. Cell* **5:** 45–57.

Habeck, H., Odenthal, J., Walderich, B., Maischein, H., and Schulte-Merker, S. 2002. Analysis of a zebrafish VEGF receptor mutant reveals specific disruption of angiogenesis. *Curr. Biol.* **12:** 1405–1412.

Hu, N., Sedmera, D., Yost, H.J., and Clark, E.B. 2000. Structure and function of the developing zebrafish heart. *Anat. Rec.* **260:** 148–157.

Jowett, T. and Lettice, L. 1994. Whole-mount in situ hybridizations on zebrafish embryos using a mixture of digoxigenin- and fluorescein-labelled probes. *Trends Genet.* **10:** 73–74.

Kang, M. and Walker, J.W. 2005. Protein kinase C δ and ε mediate positive inotropy in adult ventricular myocytes. *J. Mol. Cell. Cardiol.* **38:** 753–764.

Lamers, J.M., De Jonge, H.W., Panagia, V., and Van Heugten, H.A. 1993. Receptor-mediated signalling pathways acting through hydrolysis of membrane phospholipids in cardiomyocytes. *Cardioscience* **4:** 121–131.

Lawson, N.D., Mugford, J.W., Diamond, B.A., and Weinstein, B.M. 2003. *phospholipase C γ-1* is required downstream of vascular endothelial growth factor during arterial development. *Genes & Dev.* **17:** 1346–1351.

Lee, P., Goishi, K., Davidson, A.J., Mannix, R., Zon, L., and Klagsbrun, M. 2002. Neuropilin-1 is required for vascular development and is a mediator of VEGF-dependent angiogenesis in zebrafish. *Proc. Natl. Acad. Sci.* **99:** 10470–10475.

Li, J., Hampton, T., Morgan, J.P., and Simons, M. 1997. Stretch-induced VEGF expression in the heart. *J. Clin. Invest.* **100:** 18–24.

Liao, H.J., Kume, T., McKay, C., Xu, M.J., Ihle, J.N., and Carpenter, G. 2002. Absence of erythrogenesis and vasculogenesis in Plcg1-deficient mice. *J. Biol. Chem.* **277:** 9335–9341.

McLaughlin, A.P. and De Vries, G.W. 2001. Role of PLCγ and Ca^{2+} in VEGF- and FGF-induced choroidal endothelial cell proliferation. *Am. J. Physiol. Cell. Physiol.* **281:** C1448–C1456.

Michelmore, R.W., Paran, I., and Kesseli, R.V. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci.* **88:** 9828–9832.

Most, P., Boerries, M., Eicher, C., Schweda, C., Ehlermann, P., Pleger, S.T., Loeffler, E., Koch, W.J., Katus, H.A., Schoenenberger, C.A., et al. 2003. Extracellular S100A1 protein inhibits apoptosis in ventricular cardiomyocytes via activation of the extracellular signal-regulated protein kinase 1/2 (ERK1/2). *J. Biol. Chem.* **278:** 48404–48412.

Nasevicius, A., Larson, J., and Ekker, S.C. 2000. Distinct requirements for zebrafish angiogenesis revealed by a VEGF-A morphant. *Yeast* **17:** 294–301.

Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. 1999. Vascular endothelial growth factor (VEGF) and its receptors. *Faseb J.* **13:** 9–22.

Puceat, M. and Vassort, G. 1996. Signalling by protein kinase C isoforms in the heart. *Mol. Cell. Biochem.* **157:** 65–72.

Rottbauer, W., Baker, K., Wo, Z.G., Mohideen, M.A., Cantiello, H.F., and Fishman, M.C. 2001. Growth and function of the embryonic heart depend upon the cardiac-specific L-type calcium channel α1 subunit. *Dev. Cell* **1:** 265–275.

Rottbauer, W., Saurin, A.J., Lickert, H., Shen, X., Burns, C.G., Wo, Z.G., Kemler, R., Kingston, R., Wu, C., and Fishman, M.

2002. Reptin and pontin antagonistically regulate heart growth in zebrafish embryos. *Cell* **111:** 661–672.
Schaldach, M. and Hutten, H. 1990. A physiological approach to different concepts of rate adaptive cardiac pacing. *Med. Prog. Technol.* **16:** 235–246.
Seko, Y., Takahashi, N., Tobe, K., Ueki, K., Kadowaki, T., and Yazaki, Y. 1998. Vascular endothelial growth factor (VEGF) activates Raf-1, mitogen-activated protein (MAP) kinases, and S6 kinase (p90rsk) in cultured rat cardiac myocytes. *J. Cell. Physiol.* **175:** 239–246.
Seko, Y., Takahashi, N., Shibuya, M., and Yazaki, Y. 1999. Pulsatile stretch stimulates vascular endothelial growth factor (VEGF) secretion by cultured rat cardiac myocytes. *Biochem. Biophys. Res. Commun.* **254:** 462–465.
Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.F., Breitman, M.L., and Schuh, A.C. 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* **376:** 62–66.
Shibuya, M., Seetharam, L., Ishii, Y., Sawano, A., Gotoh, N., Matsushime, H., and Yamaguchi, S. 1994. Possible involvement of VEGF–FLT tyrosine kinase receptor system in normal and tumor angiogenesis. *Princess Takamatsu Symp.* **24:** 162–170.
Sonnenblick, E.H., Skelton, C.L., Spotnitz, W.D., and Feldman, D. 1973. Redefinition of the ultrastructural basis of cardiac length-tension relations. *Circ* **48:** 65.
Stainier, D.Y. and Fishman, M.C. 1994. The zebrafish as an model system to study cardiovascular development. *Trends Cardiovasc. Med.* **4:** 207–212.
Stainier, D.Y., Fouquet, B., Chen, J.N., Warren, K.S., Weinstein, B.M., Meiler, S.E., Mohideen, M.A., Neuhauss, S.C., Solnica-Krezel, L., Schier, A.F., et al. 1996. Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. *Development* **123:** 285–292.
Starling, E.H. 1918. *Linacre lecture on the law of the heart*. Longmans, Green and Co., London, UK.
Takahashi, T., Yamaguchi, S., Chida, K., and Shibuya, M. 2001. A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-γ and DNA synthesis in vascular endothelial cells. *EMBO J.* **20:** 2768–2778.
Thompson, M.A., Ransom, D.G., Pratt, S.J., MacLennan, H., Kieran, M.W., Detrich III, H.W., Vail, B., Huber, T.L., Paw, B., Brownlie, A.J., et al. 1998. The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis. *Dev. Biol.* **197:** 248–269.
Van Henningen, R., Rijinsburger, W.H., and ter Keurs, H.E.D.J. 1982. Sarcomere length control in striated muscle. *Am. J. Physiol.* **242:** H411.
Westerfield, M. 1995. *The zebrafish book: A guide for the laboratory use of zebrafish* Danio rerio. Eugene, OR.
Wood, J.M., Bold, G., Buchdunger, E., Cozens, R., Ferrari, S., Frei, J., Hofmann, F., Mestan, J., Mett, H., O'Reilly, T., et al. 2000. PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. *Cancer Res.* **60:** 2178–2189.
Yamaguchi, S., Iwata, K., and Shibuya, M. 2002. Soluble Flt-1 (soluble VEGFR-1), a potent natural antiangiogenic molecule in mammals, is phylogenetically conserved in avians. *Biochem. Biophys. Res. Commun.* **291:** 554–559.
Yelon, D., Horne, S.A., and Stainier, D.Y. 1999. Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish. *Dev. Biol.* **214:** 23–37.
Yule, D.I. and Williams, J.A. 1992. U73122 inhibits Ca2+ oscillations in response to cholecystokinin and carbachol but not to JMV-180 in rat pancreatic acinar cells. *J. Biol. Chem.* **267:** 13830–13835.