Zebrafish VCAP1X2 regulates cardiac contractility and proliferation of cardiomyocytes and epicardial cells

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Sarcomeric signaling complexes are important to sustain proper sarcomere structure and function, however, the mechanisms underlying these processes are not fully elucidated. In a gene trap experiment, we found that vascular cell adhesion protein 1 isoform X2 (VCAP1X2) mutant embryos displayed a dilated cardiomyopathy phenotype, including reduced cardiac contractility, enlarged ventricular chamber and thinned ventricular compact layer. Cardiomyocyte and epicardial cell proliferation was decreased in the mutant heart ventricle, as was the expression of pAKT and pERK. Contractile dysfunction in the mutant was caused by sarcomeric disorganization, including sparse myofilament, blurred Z-disc, and decreased gene expression for sarcomere modulators (smyd1b, mypn and fhl2a), sarcomeric proteins (myh6, myh7, vmhcl and tntt2a) and calcium regulators (yrr2b and slc8a1a). Treatment of PI3K activator restored Z-disc alignment while injection of smyd1b mRNA restored Z-disc alignment, contractile function and cardiomyocyte proliferation in ventricles of VCAP1X2 mutant embryos. Furthermore, injection of VCAP1X2 variant mRNA rescued all phenotypes, so long as two cytosolic tyrosines were left intact. Our results reveal two tyrosine residues located in the VCAP1X2 cytoplasmic domain are essential to regulate cardiac contractility and the proliferation of ventricular cardiomyocytes and epicardial cells through modulating pAKT and pERK expression levels.

The sarcomere is the basic contractile unit of cardiac and skeletal striated muscles1, consisting of central bipolar myosin thick filaments surrounded by parallel actin thin filaments, which are anchored to the Z-disc. Mutations in thin or thick filament components may cause hypertrophic cardiomyopathy or dilated cardiomyopathy (DCM)2,3. In addition to well-known calcium homeostasis and cell signaling events, cardiac contractility is regulated by sarcomere maintenance. The sarcomere is a dynamic structure under constant turnover, and the homeostatic quality control system involves chaperones, the calpain and ubiquitin proteasome system, and autophagy4. In addition to protein quality control, other modulators participate in the maintenance of sarcomere homeostasis in cardiac and skeletal muscle. For instance, Smyd1, a MYND and SET-domain-containing lysine methyltransferase, is specifically expressed in muscle tissues. Smyd1 knockout mice display defects in ventricular cardiomyocyte maturation and formation of the right ventricle5, and the protein localizes to the sarcomere of murine cardiomyocytes where it interacts with myosin at the sarcomeric M-line6. Furthermore, fla/smyd1 mutant zebrafish exhibited disrupted sarcomere assembly in both heart and skeletal muscle, demonstrating the importance of the Smyd1-myosin interaction in the assembly of thick filaments6.

Z-discs contain α-actinin as a core protein7, which interacts with F-actin and titin. These interactions are critical for mechanical function. In addition to its mechanical role, Z-disc proteins are important for cell signaling, gene expression and cell survival8, with mutations in α-actinin, calsarin, telethonin or MLP all causing DCM.

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or hypertrophic cardiomyopathy. The Z-disc is linked to the sarcolemma via desmin intermediate filaments, which connect to costameres. Two major protein complexes, including the dystrophin-glycoprotein complex (DGC) and the integrin-vinculin-talin complex, exist in the costamere. Mutations in several proteins that comprise the DGC may cause cardiomyopathy or muscular dystrophy. The integrin heterodimer transduces signals through focal adhesion kinase or integrin-linked kinase (ILK) to regulate cardiac growth, contractility, and repair. Cardiac deletion of vinculin, β1 integrin or ILK in mice led to dilated cardiomyopathy. Therefore, the costamere-Z-disc axis is important for force and signal transmission between the sarcomere, sarcolemma and extracellular matrix. Further characterization of proteins within this axis will serve to advance our knowledge of mechanotransduction in cardiac muscle.

Recently, the zebrafish has emerged as a model organism for cardiovascular research, partially due to the availability of the zebrafish genome sequence and conserved function of many human and zebrafish genes. Zebrafish heart development and function resemble those of mammals, allowing researchers to use genetic tools for studies on cardiogenesis or cardiac diseases. Intriguingly, zebrafish also have the ability to regenerate heart muscle, suggesting that this model may hold clues to uncovering therapeutic treatments for heart failure.

In this report, we show that the DCM phenotype in the VCAP1X2 mutant is attributed to sarcomere disorganization, decreased pAKT and pERK expression, and reduced gene expression for sarcomere modulators, sarcomeric proteins and calcium regulators. We conducted rescue experiments by injecting embryos with variant mRNAs for either VCAP1X2 or smdy1b (a downstream effector), and probed the underlying signaling mechanisms by treating with PI3K and pMEK inhibitors or activators. Together, our results reveal two tyrosine residues located in the VCAP1X2 cytoplasmic domain are essential to regulate cardiac contractility and the proliferation of ventricular cardiomyocytes and epicardial cells through modulation of pAKT and pERK levels.

Results

VCAP1X2 is expressed in the heart and localized to myocardium plasma membrane. To discover genes involved in cardiac development, we injected Tol2-Gal4-VP16; UAS: EGFP-Tol2 plasmid and produced a gene trap transgenic zebrafish with EGFP expression in the heart (Fig. 1A). The insertion was located in intron 1 of the stch211-74m13.3 gene (ZFIN ID: ZDB-GENE-060503-219) on chromosome 3. Two splice variants (stch211-74m13.3-201, stch11-74m13.3-202) consisting of seven or five exons were produced. The variants encode vascular cell adhesion protein 1 isoform X1 (VCAP1X1) (XP_002661307.2, 551 amino acids) and vascular cell adhesion protein 1 isoform X2 (VCAP1X2) (XP_001564393.1, 360 amino acids). VCAP1X2 protein contains a signal peptide, one intercellular adhesion molecule (ICAM) domain, two immunoglobulin (Ig) superfamily domains, a transmembrane domain, and a cytosolic domain, while VCAP1X1 possesses two extra Ig domains (Supplementary Fig. S1A). Sequence comparison indicated that VCAP1X1 and VCAP1X2 are identical at amino acids 1-303 (Supplementary Fig. S1B). However, strong expression of VCAP1X2, but not VCAP1X1, was identified at 72 and 96 hours post fertilization (hpf) by RT-PCR using specific reverse primers for each isoform, indicating that only VCAP1X2 was expressed during embryonic stages (Supplementary Fig. S1C,D).

RT-PCR analysis indicated splicing of exon 1 and exon 2 was blocked by the insertion, and only exon 1 of VCAP1X2 was synthesized in stch211-74m13.3 gene trap homozygous transgenic fish (Fig. 1B). Normal global embryonic morphology was observed in stch211-74m13.3 gene trap homozygous embryos at 48 hpf and intense EGFP expression was detected in the heart, primordial hindbrain channel and posterior cardiac vein (Fig. 1C).

We then generated a polyclonal antibody using amino acids number 31–308 as an antigen. With this antibody, we detected VCAP1X2 in the heart ventricle at 96 hpf in gene trap homozygous embryos at 48 hpf and intense expression at 48 and 72 hpf (Fig. 1D,E,F,G). VCAP1X2 was co-expressed with myl7 in the myocardium at 24 and 30 hpf (Fig. 1E,H,J), and was also expressed in the primordial hindbrain channel and posterior cardiac vein during different developmental stages (Fig. 1E,H,K,L). VCAP1X2 immunofluorescence signal co-localized with GFP in the primordial hindbrain channel and posterior cardiac vein of Tg(fli1:EGFP) embryos at 48 hpf (Fig. 1E,N–Q).

To further clarify the localization of VCAP1X2 in the heart, we conducted immunofluorescence staining (Fig. 1F). VCAP1X2 was observed at the plasma membrane of EGFP-expressing ventricular myocardium cells in Tg (myl7: EGFP; myl7: H2AFZ mCherry) embryos at 96 hpf (Fig. 1F,A). No VCAP1X2 was detected in GFP-expressing outer epicardial cells of Tg(tcf21:NTR; tcf21:nceEGFP) embryos or in GFP-expressing inner endocardial cells of Tg(fli1:GFP) embryos (Fig. 1F,B,C). Thus, VCAP1X2 is a membrane protein that is exclusively expressed in the myocardium.

VCAP1X2 deficient embryonic heart displays a DCM phenotype. To characterize the role of VCAP1X2 in heart development, pseudodynamic 3D cardiac imaging was applied to zebrafish embryos, and...
Figure 1. The gene trap transgenic zebrafish was characterized as a VCAP1X2 mutant. (A) A diagram of the VCAP1X2 gene trap mutant is shown. Blue boxes indicate exons I-V. White boxes indicate 5′ and 3′ UTRs. Red arrows indicate forward (F) and reverse (R) RT-PCR primers. Yellow triangles indicate 535-bp right Tol2 arm (R) or 517-bp left Tol2 arm (L). UAS, Gal4-responsive upstream activating sequence; E1b, adenoviral E1b minimal promoter element. (B) Splicing of exon 1 and exon 2 was disrupted in VCAP1X2 mutant (mut) embryos at 24 and 48 hpf. (C) Normal morphology of VCAP1X2 mutants and WT embryos at 48 hpf. EGFP expression was detected in the heart (h), posterior cardinal vein (pcv) and primordial hindbrain channel (phbc) of VCAP1X2 mutant (n = 50 for 48 hpf). Scale bar, 25 μm. (D) Expression of EGFP and VCAP1X2 (red) is shown in heart ventricles of Tg(myl7:EGFP; myl7:H2AFZ mCherry) embryos at 96 hpf. No VCAP1X2 expression was detected in the ventricle of homozygous VCAP1X2 mutant heart (n = 15 per condition, N = 3). Scale bar, 50 μm. (E) Expression of VCAP1X2 at shield (a), 5 somite (s) (b), 18 s (c,d), 24 e–g), 30 (h–j), 48 (k,l) and 72 (m) hpf stages. VCAP1X2 was co-expressed with myl7 in the myocardium of embryos at 24 and 30 hpf (g–j). Scale bars, 30 μm. Co-localization of VCAP1X2 and GFP was detected in phbc (n,o) and pcv (p,q) of Tg(fli1:GFP) embryos at 48 hpf (n = 10). Arrows indicate heart, a, atrium; h, heart; v, ventricle. Scale bars, 50 μm. (F) (a) VCAP1X2 (red) was expressed in the plasma membrane of ventricular myocardium cardiomyocytes labeled with EGFP in Tg(myl7:EGFP; myl7:H2AFZ mCherry) transgenic embryos at 96 hpf. (b) VCAP1X2 was not expressed in EGFP-labeled ventricular epicardium cells in Tg(tec21:NTR; tec21:uscEGFP) transgenic embryos at 96 hpf. (c) VCAP1X2 was also not expressed in the EGFP-labeled ventricular endocardium of Tg(fli1:GFP) transgenic embryos at 96 hpf (n = 15 per condition, N = 3). Nuclei were stained by DAPI. Scale bar, 5 μm.
cardiac function was evaluated at 72 hpf (Fig. 2A)\(^8\). Both end-systolic volume ( ESV ) and end-diastolic volume ( EDV ) were substantially increased, while ejection fraction ( EF ) was decreased in VCA\(\text{PIX2}\) mutant compared to Tg ( myl7: EGFP ; myl7: H2AFZ mcherry ) controls (Fig. 2a–c). In parallel, we determined the end-diastolic length and width of VCA\(\text{PIX2}\) mutant hearts by recording a video of the beating heart. Increased end-diastolic length and width, with reduced fractional shortening ( FS ) were observed in VCA\(\text{PIX2}\) mutants relative to controls (Fig. 2B,d–f), further demonstrating that VCA\(\text{PIX2}\) mutant embryos have impaired cardiac function.

Histological analysis revealed a thinner ventricular compact layer ( one-cell layer ) in VCA\(\text{PIX2}\) mutant heart compared to WT ( three-cell layer ) embryos at 120 hpf, and injection of VCA\(\text{PIX2}\) full-length ( FL ) but not LacZ mRNA restored the thickness ( Fig. 2C ). Significantly reduced ventricular cardiomyocyte number and a decreased percentage of BrDU\(^+\) cardiomyocytes were detected in ventricles of VCA\(\text{PIX2}\) mutant compared to control embryos at 72 hpf ( Fig. 2D,c,d ), while similar low levels of apoptosis were detected in ventricles of VCA\(\text{PIX2}\) mutant and control hearts by TUNEL labeling at 72 hpf ( Fig. 2E,F ). Thus, the thinner ventricular compact layer with reduced cardiomyocyte number in VCA\(\text{PIX2}\) mutants may be due to decreased cell proliferation. Together, these results show that VCA\(\text{PIX2}\) deficient embryonic heart exhibits DCM phenotypes, including impaired cardiac function, enlarged ventricular chamber and thinner ventricular compact layer with decreased cardiomyocyte number.

Two tyrosine residues within the VCA\(\text{PIX2}\) cytoplasmic domain are required for heart contractility and cardiomyocyte proliferation. Because phosphorylation of two cytosolic tyrosine residues in platelet endothelial cell adhesion molecule-1 ( PECAM1 ) is known to activate extracellular signal-regulated kinase ( ERK )\(^23\), we wondered if the two cytosolic tyrosine residues ( Y333 and Y341 ) of VCA\(\text{PIX2}\) may also play important functional roles ( Fig. 3A ). We generated five VCA\(\text{PIX2}\) variants, including SP: only signal peptide, \(\Delta N\) Y2F: deleted extracellular ICAM and Ig domains with two cytosolic tyrosines mutated to phenylalanines, Y2F: full-length VCA\(\text{PIX2}\) with cytosolic tyrosine to phenylalanine mutations, \(\Delta N\): deleted extracellular ICAM and Ig domains with two cytosolic tyrosines intact, and FL: full-length VCA\(\text{PIX2}\) ( Fig. 3B ). Initially, we compared heart contractility by measuring FS at 48 hpf in WT or VCA\(\text{PIX2}\) mutant embryos, or mutants injected with SP or FL mRNA. Injection of FL, but not SP mRNA, restored ventricular FS to levels comparable with WT ( \(p < 0.001\), Fig. 3C ). Interestingly, injection of human vascular cell adhesion protein 1 isoform c precursor ( hVCAM1 ) mRNA also rescued decreased FS in injected mutant embryos, despite the fact that VCA\(\text{PIX2}\) only shares 27% amino acid sequence identity with human VCAM1 isoform c ( Supplementary Fig. S2A ). At 72 hpf, FS was restored in VCA\(\text{PIX2}\) mutants injected with \(\Delta N, FL\) or hVCAM1 mRNA, but not in those injected with SP, \(\Delta N\) Y2F, Y2F or LacZ mRNA ( \(p < 0.001\), Fig. 3D ). Furthermore, injection of \(\Delta N, FL\) or hVCAM1, but not SP, \(\Delta N\) Y2F or Y2F mRNA restored ventricular cardiomyocyte number ( \(p < 0.001\), Fig. 3E,F ) at 72 and 96 hpf. Injection of FL or hVCAM1 increased the percentage of ventricular PCNA\(^+\) cardiomyocytes in VCA\(\text{PIX2}\) mutants more robustly ( \(p < 0.001\) ) than \(\Delta N\) ( \(p < 0.05\) ) at 72 hpf ( Fig. 3G ). However, at 96 hpf, only injection of FL, but not \(\Delta N\) or hVCAM1, significantly restored percentage of ventricular PCNA\(^+\) cardiomyocytes in VCA\(\text{PIX2}\) mutants ( \(p < 0.001\), Fig. 3H ).

We then designed a splicing morpholino ( spMO ) that targets the acceptor site of intron 1 and monitored the inhibition of splicing between exon 1 and exon 2 of VCA\(\text{PIX2}\) by RT-PCR ( Supplementary Fig. S3A,B ). VCA\(\text{PIX2}\) immunofluorescence was detected in heart ventricles at 96 hpf in Tg ( myl7: EGFP ; myl7: H2AFZ mcherry ) transgenic fish and 5 mm spMO-injected control embryos but not spMO-injected morphants ( Supplementary Fig. S3C ). A thinner ventricular compact layer was detected in VCA\(\text{PIX2}\) morphant heart, which was rescued by co-injection of VCA\(\text{PIX2}\) but not LacZ mRNA ( Supplementary Fig. S3D ). Co-injection of \(\Delta N\) or FL, but not SP, \(\Delta N\) Y2F or Y2F mRNA also completely restored reduced ventricular FS and ventricular cardiomyocyte number ( \(p < 0.001\), Supplementary Fig. S3E,F ). Together, these results demonstrate that the two cytosolic tyrosine residues of VCA\(\text{PIX2}\) are essential for heart contractility, compact layer thickness and cardiomyocyte proliferation in the ventricle of embryonic heart.

VCA\(\text{PIX2}\) cytosolic tyrosine residues are essential for maintaining pAKT and pERK levels. Cardiomyocyte proliferation is regulated by several signaling pathways, including neuregulin-1/ ErbB2 \(^22\) and insulin like growth factor 2 signaling\(^23\). PI3K–AKT and MAPK–ERK1/2 transduce upstream signals to activate genes involved in different biological processes, including cell proliferation. Since ventricular cardiomyocyte proliferation was reduced in VCA\(\text{PIX2}\) mutants ( Fig. 2 ), we investigated whether pAKT and pERK levels were also affected in the ventricle of VCA\(\text{PIX2}\) mutant embryonic hearts.

Both pAKT and pERK were diminished in VCA\(\text{PIX2}\) mutant heart compared to WT at 96 hpf ( Figs 4A,B and 5A,B ). Injection of \(\Delta N\) or FL mRNA, but not SP, \(\Delta N\) Y2F or Y2F mRNA restored ventricular pAKT and pERK levels ( Figs 4C–G and 5C–G ). Western blots further revealed reductions in pAKT ( 4.2-fold ) and pERK ( 1.8-fold ) levels ( Figs 4C–G and 5C–G ). Injection of FL mRNA further reduced ventricular pAKT and pERK to levels that were comparable to WT at 96 hpf ( Figs 4H and 5H ). Together, these results indicate that the two cytosolic tyrosine residues of VCA\(\text{PIX2}\) are essential for maintaining proper ventricular pAKT and pERK levels as well as cardiomyocyte number in embryonic heart.
Figure 2. VCAP1X2 mutant displayed cardiac dysfunction and impaired cardiomyocyte proliferation. (A) Pseudodynamic reconstructed 3D images and respective bright-field images are shown of end-diastolic phase beating hearts from Tg(myl7:EGFP; myl7:H2AFZ mCherry) (a,b) or VCAP1X2 mutant (c,d) at 72 hpf. Yellow lines indicate the diameter of the ventricular chamber. Green fluorescence marks ventricular myocardium and red indicates ventricular chamber. Scale bar, 50 μm. (B) Parameters related to heart performance were measured from pseudodynamic reconstructed 3D images (a–c, n = 3, N = 2) or CCD images (d–f, n = 20, N = 3) for Tg(myl7:EGFP; myl7:H2AFZ mCherry) or WT and VCAP1X2 mutant at 72 hpf. End-systolic volume (a) and end-diastolic volume (b) were significantly increased in VCAP1X2 mutant compared with Tg(myl7:EGFP; myl7:H2AFZ mCherry) embryos. Ejection fraction (c) was lower in VCAP1X2 mutant than in Tg(myl7:EGFP; myl7:H2AFZ mCherry) embryos. End-diastolic length (d) and width (e) were increased while fractional shortening (f) was decreased in VCAP1X2 mutant compared to WT. Error bars represent standard error. Student’s t-test, **p < 0.01, ***p < 0.001. (C) Paraffin sectioning with hematoxylin and eosin staining revealed a thinner compact layer in the ventricle of VCAP1X2 mutants (b) and LacZ mRNA-injected VCAP1X2 mutants (c) compared to WT (a) or VCAP1X2 mRNA-injected VCAP1X2 mutants (d) (n = 10 per condition). a, atrium, v, ventricle. Scale bar, 50 μm. (D) Immunostaining of BrdU (red) in heart ventricles from Tg(myl7:EGFP; myl7:H2AFZ mCherry) (myl7) (a) or VCAP1X2 mutants (b) at 72 hpf. Significantly decreased cardiomyocyte (CM) number (c) and percentage of BrdU+ CMs (d) in the ventricle was detected in VCAP1X2 mutant compared to Tg(myl7:EGFP; myl7:H2AFZ mCherry) embryos (n = 20 per condition, N = 3). Student’s t-test, ***p < 0.001. Scale bar, 50 μm. Apoptotic cells were analyzed by TUNEL labeling (E) and similar low level of apoptotic cells (F) was detected in VCAP1X2 mutant and Tg(myl7:EGFP; myl7:H2AFZ mCherry) embryos (n = 15, N = 3). Cardiomyocyte nuclei stained by anti-MEF2 antibody. Error bars represent standard error. Scale bar, 50 μm.
Figure 3. Full-length or $\Delta N$ VCAP1X2 or human VCAM1 mRNA rescue decreased fractional shortening and cardiomyocyte proliferation in VCAP1X2 mutants. (A) Amino acid sequence and various domains of VCAP1X2 are shown. (B) A diagram shows various VCAP1X2 variants. (C) Ventricular fractional shortening (FS) was significantly lower in VCAP1X2 mutant (mut) than in WT and the defect was rescued by injection with full-length (FL) VCAP1X2 or human VCAM1 mRNA (hVCAM1) but not with SP VCAP1X2 mRNA at 48 hpf ($n = 20$ per condition, $N = 3$). (D) At 72 hpf, the FS defect was still observed in mutants and was rescued by injection with $\Delta N$, FL VCAP1X2, or hVCAM1 mRNA, but not with LacZ, SP, $\Delta N$ Y2F or Y2F VCAP1X2 mRNA ($n = 20$ per condition, $N = 3$). (E,F) Ventricular cardiomyocyte (CM) number was significantly decreased in VCAP1X2 mutant at 72 hpf (E) and 96 hpf (F) compared to WT. Injection of $\Delta N$ or FL VCAP1X2 or hVCAM1 mRNA but not SP, $\Delta N$ Y2F or Y2F VCAP1X2 mRNA could restore CM number in VCAP1X2 mutant embryos ($n = 20$ per condition, $N = 3$). (G,H) A substantially reduced percentage of ventricular PCNA$^+$ cardiomyocytes was detected in VCAP1X2 mutant at 72 hpf (G) and 96 hpf (H) compared to WT. Injection of $\Delta N$ or FL VCAP1X2 or hVCAM1 mRNA but not SP, $\Delta N$ Y2F or Y2F VCAP1X2 mRNA could restore the decreased percentage of ventricular PCNA$^+$ cardiomyocytes in VCAP1X2 mutant embryos at 72 hpf. However, at 96 hpf, only injection of FL, but not $\Delta N$ or hVCAM1, significantly restored percentage of ventricular PCNA$^+$ cardiomyocytes in VCAP1X2 mutants ($n = 20$ per condition, $N = 3$). Error bars indicate standard error. Quantitative data were analyzed by ANOVA with Bonferroni multiple comparisons. Treatments that are not statistically different ($\alpha = 0.05$) from each other are labeled with the same letter.
VCAP1X2 deficiency and inhibition of pAKT or pERK induced sarcomere disorganization. Since heart contractility was decreased in VCAP1X2 mutants (Fig. 2), we investigated whether sarcomere assembly was affected in the ventricle. Sarcomere organization was examined by transmission electron microscopy, which revealed intact and well-organized structures with clear Z-disc boundaries in WT heart at 96 hpf. By contrast, sparse and irregular myofibrils bordered by blurred Z-discs were observed in VCAP1X2 mutant heart (Fig. 6A). Blurred Z-discs were also detected by α-actinin immunofluorescence in VCAP1X2 mutant heart compared to WT at 96 hpf (Fig. 6B,a,b). Injection of ΔN or FL VCAP1X2 mRNA but not with SP (C), ΔNY2F (D) or Y2F (E) VCAP1X2 mRNA (n = 15 per condition, N = 3). Nuclei were stained with DAPI. Scale bar, 30 μm. v, ventricle. Dashed lines illustrate the ventricular myocardium. Erythrocytes in the ventricular chamber show non-specific staining from secondary antibody. (H) Levels of pAKT or total AKT in embryonic hearts isolated from Tg(myl7:EGFP; myl7:H2AFZ: mCherry) (myl7), VCAP1X2 mutant (mut) or VCAP1X2 mutant injected with Y2F or FL VCAP1X2 mRNA were measured by Western blot with β-actin as loading control. Cropped representative blots for each protein are shown. Uncropped blots are shown in Supplementary Fig. S4. All blotting was performed using the same experimental conditions. (I) Quantification of pAKT/AKT expression ratio among different treatments (N = 3). Error bars indicate standard error. Quantitative data were analyzed by ANOVA with Bonferroni multiple comparisons. Treatments that are not statistically different (α = 0.05) from each other are labeled with the same letter.

Figure 4. Maintenance of pAKT required expression of two tyrosines in the cytoplasmic domain of VCAP1X2. Immunofluorescent staining revealed decreased pAKT expression levels in VCAP1X2 mutants (B) compared to WT (A) at 96 hpf. pAKT expression levels in VCAP1X2 mutants could be restored by injection with ΔN (F) or FL (G) VCAP1X2 mRNA but not with SP (C), ΔNY2F (D) or Y2F (E) VCAP1X2 mRNA (n = 15 per condition, N = 3). Nuclei were stained with DAPI. Scale bar, 30 μm. v, ventricle. Dashed lines illustrate the ventricular myocardium. Erythrocytes in the ventricular chamber show non-specific staining from secondary antibody. (H) Levels of pAKT or total AKT in embryonic hearts isolated from Tg(myl7:EGFP; myl7:H2AFZ mCherry) (myl7), VCAP1X2 mutant (mut) or VCAP1X2 mutant injected with Y2F or FL VCAP1X2 mRNA were measured by Western blot with β-actin as loading control. Cropped representative blots for each protein are shown. Uncropped blots are shown in Supplementary Fig. S4. All blotting was performed using the same experimental conditions. (I) Quantification of pAKT/AKT expression ratio among different treatments (N = 3). Error bars indicate standard error. Quantitative data were analyzed by ANOVA with Bonferroni multiple comparisons. Treatments that are not statistically different (α = 0.05) from each other are labeled with the same letter.
(p < 0.001, Fig. 6C). Again, injection of ΔN, FL or hVCAM1 mRNA, but not SP, ΔN Y2F or Y2F mRNA restored the defect (p < 0.001, Fig. 6C). Similar sarcomere lengths were measured in all groups (Fig. 6D). Therefore, we conclude that two cytosolic tyrosine residues of VCAP1X2 are essential in regulating sarcomere organization.

Since sarcomere disorganization and decreased pAKT and pERK were detected in the ventricles of VCAP1X2 mutant hearts (Figs 4–6), we wondered whether inhibition of pAKT or pERK expression in WT embryos would affect sarcomere organization. We treated WT embryos with an inhibitor of PI3K (LY294002) or pMEK(U0126) at 63 hpf and fixed embryos for α-actinin immunofluorescence at 72 hpf. Blurred shortened Z-discs were detected in heart ventricles of Tg (myl7: EGFP; myl7: H2AFZ mCherry) embryos treated with PI3K or pMEK.
Figure 6. Disorganized sarcomere structure was observed in VCAP1X2 mutant hearts and rescued by VCAP1X2 variant mRNA, hVCAM1 mRNA or a PI3K activator. (A) Transmission electron microscopy shows sparse myofilaments and indistinct Z-discs in ventricular sarcomere of VCAP1X2 mutants at 96 hpf (n = 3 per condition). Arrows indicate Z-discs (Z). Scale bar, 500 nm. (B) Striated Z-discs were detected by α-actinin immunofluorescence in WT heart (a), while a dotted pattern was observed in VCAP1X2 mutants (b) at 96 hpf. ΔN (f) or FL (g), but not SP (c), ΔN Y2F (d) or Y2F (e), restored striated Z-discs (n = 16 per condition, N = 3). Z-disc length and sarcomere length were measured from immunofluorescence images. Z-disc length (C) was reduced in VCAP1X2 mutants and SP, ΔN Y2F or Y2F-injected mutants but not those injected with ΔN, FL, or hVCAM1 mRNA at 96 hpf (n = 160 per condition, N = 3). (D) Slight differences (maximum 8%) were found in sarcomere length, indicating that ventricular sarcomere length was not robustly affected by any treatment at 96 hpf. Data in (C and D) were analyzed by ANOVA with Bonferroni multiple comparisons. Treatments that are not statistically different (α = 0.05) from each other are labeled with the same letter. (E) α-actinin immunofluorescence on sarcomeres of DMSO-treated Tg( myl7:EGFP; myl7:H2AFZ mCherry) embryos (a) or PI3K activator (SC-79)-treated VCAP1X2 mutant heart (e) showed striations, while Tg( myl7:EGFP; myl7:H2AFZ mCherry) embryos treated with inhibitors of PI3K (LY294002) (b) or pMEK (U0126) (c), or DMSO-treated VCAP1X2 mutant hearts (d) showed a dotted pattern. Ventricular myocardium is outlined in right-most panels. Erythrocytes show non-specific staining from secondary antibody. (F) Z-disc length was measured in Tg( myl7:EGFP; myl7:H2AFZ mCherry) embryos and VCAP1X2 mutants treated with PI3K or pMEK inhibitor. Data were analyzed by ANOVA with Bonferroni multiple comparisons (α = 0.05).
The difference between groups a and b was significant \((p < 0.001)\). (G) Similar ventricular sarcomere length was detected in all groups \((n = 200 \text{ per condition}, N = 3)\). ANOVA indicated no significant difference among treatments \((p = 0.96)\). Error bars indicate standard error.

Inhibitor compared to DMSO-treated embryos (Fig. 6E.a–e,F.G), but sarcomere length was similar. We also treated VCAP1X2 mutant embryos with pAKT activator (SC-79) for a similar period prior to α-actinin immunostaining. Complete restoration of Z-disc alignment and length were detected in heart ventricles of SC-79-treated VCAP1X2 mutants compared to DMSO-treated controls (Fig. 6E.d,e,F.G). Together, these results demonstrate that two cytosolic tyrosine residues of VCAP1X2 and proper pAKT and pERK levels are essential for sarcomere organization in the heart ventricle.

Two cytosolic tyrosine residues of VCAP1X2 are essential for epicardium formation and epicardial cell proliferation. A prior study revealed VCAMI-deficient mouse hearts exhibited thinned compact layer, reduction of intraventricular septum and epicardium formation defects\(^{16}\). Because VCAP1X2 mutant embryonic heart showed similar defects of thinner ventricular compact layer and reduced cardiomyocyte number (an effect that could be rescued by hVCAM1 mRNA; Figs 2, 3), we next investigated whether epicardium formation was also affected in VCAP1X2 mutants. Indeed, ventricular epicardium formation was affected in VCAP1X2 mutants, as evidenced by tcf21 RNA staining at 96 hpf (Supplementary Fig. S8A). To further investigate the effect of VCAP1X2 deficiency on epicardium formation, we knocked down VCAP1X2 in Tg(tcf21:nucEGFP) transgenic embryos and labeled proliferative epicardial cells with PCNA antibody at 96 hpf (Supplementary Fig. S8B). A reduced number of ventricular Tcfl \(1^{+}\) epicardial cells was observed in VCAP1X2 morphants and co-injection of \(\Delta N\) or FL, but not SP, \(\Delta N\) Y2F or Y2F mRNA, completely restored Tcfl \(1^{+}\) epicardial cell number compared to un-injected or 5 mm spMO-injected transgenic embryos \((p < 0.001, \text{Supplementary Fig. S8C})\). Similarly, a reduction in the percentage of ventricular Tcfl \(1^{+}\)/PCNA \(^{-}\) cells was completely rescued by co-injection of \(\Delta N\) or FL, but not SP, \(\Delta N\) Y2F or Y2F mRNAs. \((p < 0.001, \text{Supplementary Fig. S8D})\). Thus, the two cytosolic tyrosine residues of VCAP1X2 are also required for proper epicardial cell proliferation and epicardium formation.

**VCAP1X2 deficiency leads to reduced gene expression for proteins involved in sarcomere assembly and calcium homeostasis.** To further investigate the mechanism by which VCAP1X2 regulates heart contractility and epicardium formation, we compared gene expression levels of proteins involved in sarcomere assembly, calcium homeostasis, and epicardium formation. Embryonic hearts of VCAP1X2 mutant and WT zebrafish were evaluated by RT-qPCR at 96 hpf (Fig. 7). Since VCAP1X2 mutant embryonic heart exhibited a DCM phenotype, we selected several zebrafish orthologues of known human DCM-associated genes for comparison\(^{29}\) (Fig. 7A). Expression of genes encoding sarcomere proteins, such as myosin heavy chain 6 (myh6), myosin heavy chain 7 (myh7), ventricle myosin heavy chain like (vmhcl), troponin T (tntt2a), myopalladin (mynp) and four and a half LIM domains 2a (fhl2a), was significantly downregulated in VCAP1X2 mutants compared to WT. Expression levels of two natriuretic peptides (nppa, nppb) were substantially increased in VCAP1X2 mutant heart, similar to other DCM animal models\(^{36}\). We then compared expression levels of the sarcomere modulator, smyd1b, stress response regulators [integrin subunit beta1 binding protein 2 (itgb1bp2)\(^{26}\) and ankyrin repeat domain 1 (ankrd1)\(^{21}\)] and chaperones (hsp90aa1.1, unc45b, B-crystallin/cryab)\(^{28,29}\) in VCAP1X2 mutant and WT hearts (Fig. 7B). Significant downregulation of smyd1b, and upregulation of hsp90aa1.1 and cryab were detected in VCAP1X2 mutants.

Because the contraction and relaxation of cardiac muscle is regulated by cytosolic Ca\(^{2+}\) concentration\(^{30}\), we investigated whether expression of calcium regulators was affected in VCAP1X2 mutant hearts (Fig. 7C). Diminished expression of sarcoendoplasmic reticulum (SER) calcium-release receptor 2 (ryr2b) and sarcolemma solute carrier family 8, member 1a (slc8a1a) were detected in VCAP1X2 mutants. We also compared gene expression for proteins that are essential for epicardium formation, such as GATA4, Tbx5 and BMP4\(^{31,32}\). A trend toward downregulation of gata4 and tbx5a was observed in mutants, with no alteration of bmp4 expression (Fig. 7D). Together, these findings suggest that VCAP1X2 regulates cardiac contractility by modulating expression of smyd1b, mypn or fhl2a for proper sarcomere assembly and Ca\(^{2+}\)-regulatory proteins (ryr2b, slc8a1a) for calcium homeostasis.

**smyd1b mRNA injection restored cardiac dysfunction, sarcomere organization and cardiomyocyte proliferation in VCAP1X2 mutant embryonic heart.** We focused on downregulation of smyd1b as a primary effector in VCAP1X2 mutants, because Smyd1-myosin interaction is essential for thick filament assembly, and zebrafish flia/smyd1 mutants exhibit disrupted sarcomere assembly in both heart and skeletal muscle\(^{37}\). In a rescue experiment, injection of smy1b\(^{\text{ext}}\) or HMT-deficient smy1b\(^{12,49}\) mRNA, but not myosin-interaction-site-absent smy1b\(^{7,8}\) or lacZ mRNA, restored FS, blurred Z-discs and Z-disc length in heart ventricles of VCAP1X2 mutants at 96 hpf (Fig. 8A–D). Furthermore, the reduced cardiomyocyte number and decreased percentage of PCNA \(^{-}\) cardiomyocytes were also restored at 96 hpf in mutant embryos injected with smy1b\(^{\text{ext}}\) but not with smy1b\(^{7,8}\) mRNA \((p < 0.001, \text{Fig. 8E,F})\). Together, these findings suggest that VCAP1X2 regulates cardiac contractility and cardiomyocyte proliferation by modulating expression of smy1b, a myosin-interacting protein that is required for proper sarcomere assembly.

**Discussion**

The costamere-Z-disc interaction is important for transmission of mechanical force and molecular signals between the sarcomere, sarcolemma and extracellular matrix. Identifying proteins that connect the sarcolemma to sarcomeres can further our understanding of mechanical and signal transduction in cardiac muscle. Here, we identified a...
novel cell adhesion molecule, VCAP1X2, which is expressed in the sarcolemma and functions as an important signal transducer from the sarcolemma to the sarcomere and nucleus in cardiac muscle. This protein maintains cardiac contractility as well as proliferation of cardiomyocytes and epicardial cells in zebrafish embryonic heart ventricle.

VCAP1X2 deficient embryonic hearts exhibited DCM phenotypes, including enlarged ventricular chamber, thinner ventricular compact layer with decreased cardiomyocyte number, and impaired cardiac function. Sparse and irregular myofilaments, bordered by blurred Z-discs with decreased length, were observed in VCAP1X2 mutant heart and may be attributed to decreased expression of a sarcomere modulator (smyd1b), proteins associated with Z-discs and I-bands (mypn, fhl2a) and calcium regulators (ryr2b, slc8a1a). Smyd1b is expressed in cardiomyocytes, where it localizes to the M-line of the sarcomere and interacts with myosin. Disrupted myofibrillogenesis was previously reported in cardiomyocytes of zebrafish fla mutants with defective smyd1b. Knockdown of smyd1b induced upregulation of two chaperones (hsp90aa1 and unc45b) and resulted in cardiac sarcomere disorganization. Although unc45b expression was not increased in VCAP1X2 mutant heart, cryabb, which binds to titin in the I-band and protects it from stress, was significantly induced. These upregulated chaperones may be responding to misfolded thick filament myosin during myofibrillogenesis. Furthermore, injection of smyd1bwt but not myosin-interaction-site-absent smyd1b278del mRNA restored FS, Z-disc alignment and Z-disc length in VCAP1X2 mutants, suggesting that Smyd1b-mediated thick filament assembly is regulated by VCAP1X2.

In addition to Smyd1b, Mypn binds α-actinin at the Z-disc and interacts with ANKR1 in the I-band to maintain sarcomere integrity. While ANKR1 is a transcriptional regulator, it also interacts with titin, where it serves as a scaffold to facilitate ERK1/2 phosphorylation of GATA4. Upon GATA4 phosphorylation, the ANKR1/ERK/GATA4 complex translocates to the nucleus to regulate hypertrophic gene expression. GATA4 is known to regulate cardiac muscle-specific gene (α-MHC, β-MHC and MLC) expression and promoted cardiomyocyte proliferation by modulating cardiomyocyte cell cycle activity. In VCAP1X2 mutant embryonic heart, decreased smyd1b expression affected thick filament assembly and sarcomere organization and downregulation of mpykn may disrupt the interaction between Mypn and Ankrd1a to affect Ankrd1a/ERK/GATA4 complex formation and decrease ERK phosphorylation. Reduced GATA4 phosphorylation would affect its transcriptional activity, resulting in decreased expression of thick filament genes (myh6, myh7, vmhcl) and reduced expression of cell cycle genes such as cyclin D2, cyclin A2 and CDK4. As a result, reduced cardiomyocyte proliferation was detected in ventricles of VCAP1X2 mutant hearts, which was restored by injection of smyd1bwt but not smyd1b278del mRNA. In addition, GATA4 was reported to be an essential factor for the production of proepicardium in mice, and decreased GATA4 transcriptional activity may also affect the epicardium formation in VCAP1X2 mutant zebrafish.

Figure 7. Real-time reverse-transcription PCR revealed altered expression of genes related to sarcomere assembly and heart contractility in embryonic hearts from Tg (myl7: EGFP; myl7: H2AFZ mCherry) and VCAP1X2 mutant at 96 hpf. (A) Dilated cardiomyopathy-related genes encoding sarcomeric proteins, including myh6, myh7, vmhcl, tnt2a, mpykn and fh2a, were significantly decreased, while expression of two natriuretic peptides (nppa and nppb) were substantially increased. (B) smyd1b was significantly reduced in VCAP1X2 mutant accompanied by an induction of expression of hsp90aa1.1 and cryabb. (C) Ca2+ handling regulators (ryr2b and slc8a1a) were significantly downregulated in VCAP1X2 mutants. (D) Genes related to epicardium formation were not significantly altered between WT and VCAP1X2 mutants. Error bars indicate standard deviation. Student's t-test, *p < 0.05. ***p < 0.001.
Figure 8. Injection of smydlb<sup>wt</sup> but not myosin-interaction-site-absent smydlb<sup>278del</sup> mRNA restored fractional shortening, sarcomere organization and cardiomyocyte proliferation. Injection of smydlb<sup>wt</sup> or HMT-deficient smydlb<sup>Y247F</sup> mRNA but not smydlb<sup>278del</sup> or LacZ mRNA restored fractional shortening. (A) (n = 24 per condition, N = 4) and Z-disc length (C) (n = 200 per condition, N = 4) at 96 hpf. Data were analyzed by ANOVA with Bonferroni multiple comparisons (α = 0.05). The difference between groups a and b was significant (p < 0.001). Similar ventricular sarcomere lengths (D) were detected in all groups (n = 200 per condition, N = 4). ANOVA indicated no significant difference among treatments (p = 0.49). (B) Striated Z-discs were detected by α-actinin immunofluorescence on sarcomeres in the ventricle of WT (a), smydlb<sup>Y247F</sup> (e) or smydlb<sup>wt</sup> (f) mRNA-injected VCAPIX2 mutant hearts while a dotted pattern on Z-discs was observed in ventricles of VCAPIX2 mutant (b), LacZ (c) or smydlb<sup>278del</sup> mRNA (d)-injected mutant hearts. Enlarged areas are indicated by white dashed rectangles. Nuclei were stained by DAPI. Significantly reduced cardiomyocyte number (E) or percentage of PCNA<sup>+</sup> cardiomyocytes (F) was detected in ventricles of smydlb<sup>278del</sup> mRNA-injected or un-injected (Un-inj) VCAPIX2 mutant hearts compared to smydlb<sup>wt</sup> mRNA-injected or WT embryos at 96 hpf (n = 15, N = 3). Data were analyzed by ANOVA with Bonferroni multiple comparisons. Treatments that are not statistically different (α = 0.05) from each other are labeled with the same letter. Error bars indicate standard error.
Besides the effects on cardiac muscle or cell cycle gene expression by reduced ERK-mediated GATA4 transcriptional activity, PI3K-AKT signaling was also shown to promote expression of genes involved in cardiac structure and Z-disc signaling. Experiments using caPI3K and dnPI3K in transgenic mice indicated that genes encoding several cardiac muscle-related proteins, including Melusin (Igβ1p2), were differentially expressed. Melusin was shown to associate with the cytosolic domain of integrin β1 at the costamere and interact with the p85 regulatory subunit of PI3K. Thinner myofibrils and reduced Z-disc alignment were identified in dnPI3K transgenic heart, and PI3K (p110α) inhibitors prevented the formation of mature myofibers in response to IGF1 stimulation. Therefore, upon IGF1 stimulation, PI3K interacts with insulin receptor substrate 1 and Melusin to activate downstream AKT kinase and regulate expression of several cardiac muscle-associated proteins. Consistent with these studies in mice, we also detected blurred Z-discs with reduced length in heart ventricles of zebrafish embryos treated with inhibitor of PI3K or pMEK. Furthermore, such defects in ventricles of VCAP1X2 mutant hearts could be completely restored by treatment with PI3K activator. The PI3K-AKT pathway interacts with Akt/β-catenin or Hippo-Yap signaling to regulate cardiomyocyte proliferation. PI3K-AKT promoted β-catenin nuclear translocation by inhibiting GSK3β phosphorylation and induced Yap nuclear translocation by dissociation of the S2-Sav-LATS1/2 complex and the inactivation of LATS1/2 via recruiting PDK1 to the plasma membrane. β-catenin and Yap then interact with cofactors to express a variety of cell cycle genes.

Mammalian VCAM1 isoforms contain a highly conserved 19-amino-acid cytoplasmic domain that is not found in VCAP1X2 or zebrafish VCAM1 (Supplementary Fig. S2B). Within the 19-amino-acid sequence, S728 and Y729 are necessary for mouse VCAM1 activation of calcium flux, while S730 and S737 are required for VCAM1 activation of Rac1 during leukocyte transendothelial migration. Similarly, VCAP1X2 possesses a short 30-amino-acid cytoplasmic domain with two tyrosine residues. We demonstrated that these two cytosolic tyrosine residues are essential for VCAP1X2 function. As the VCAP1X2 antibody was not suitable for immunoprecipitation experiments to confirm phosphorylation of the cytosolic tyrosines, we did mutate the residues to phenylalanines to block auto- or trans-phosphorylation.

VCAM1-deficient mouse embryonic hearts displayed thinned compact layer, reduction of intraventricular septum and epicardium formation defects, and the interaction between VCAM1 and integrin αvβ was shown to be important for heart development. Our finding that VCAP1X2 mutant heart exhibits a thin ventricular compact layer and epicardium formation defect is similar to the phenotype in VCAM1-deficient mice. Our data further suggested that decreased pAKT and pERK levels in VCAP1X2 mutant heart caused sarcomere disorganization and reduction in cardiomyocyte and epicardial cell proliferation, which then resulted in morphologic and functional defects. In contrast, zebrafish VCAM1 was shown to interact with Integrin α9 rather than Integrin αv and was essential for lymphatic development. Tyrosine phosphorylation was shown to be crucial during myofibrillogenesis and embryonic heart development in axolotls. Our results further provide a potential molecular mechanism to explain how tyrosine phosphorylation may affect sarcomere structure and function. Like PECAM1, VCAP1X2 may possess a non-conventional ITAM motif with two tyrosine residues in the cytoplasmic domain that may provide a scaffold for adaptor molecule signaling. SH2-containing phosphatase may bind to phosphorylated tyrosine together with Gab1 to recruit PI3K docking or activate Ras-Raf-MEK-ERK. Alternatively, SH2-containing PI3K p85 may directly bind to phosphorylated tyrosine. Activated AKT kinase would then regulate expression of sarcomeric modulators (smyd1b, mypn, fhla2) for proper sarcomere assembly that in turn enhances GATA4 activity by promoting translocation of Ankrd1a/ERK/GATA4 complex to the nucleus and ERK-GATA4 signal transduction, driving cardiomyocyte and epicardial proliferation. In addition to regulating cardiomyocyte and epicardial proliferation, activated AKT also regulates Ca2+ cycling by modulating expression of slc8a1a and ryr2b and activity of the Atp2a2a calcium pump for proper cardiac contractility (Supplementary Fig. S9). A previous study showing that VCAM1 activated PI3K and ERK kinase in human airway smooth muscle cells provides additional support for our finding that VCAP1X2 enhances pAKT and pERK levels.

In conclusion, using a zebrafish gene trap mutant, we demonstrate that VCAP1X2, a cell adhesion molecule, localizes to the myocardium sarcolemma, where it regulates cardiac contractility, and proliferation of cardiomyocytes and epicardial cells by modulating pAKT and pERK expression via two tyrosine residues within the cytoplasmic domain. These results advance our understanding of signal transmission between the sarcolemma and sarcomeres in cardiac muscle cells.

**Methods**

**Zebrafish strains and maintenance.** Adult zebrafish, including wild-type ASAB, Tg (myl7: EGFP; myl7: H2A2FZ mcherry)53, Tg(tcf21:NTR; tcf21:nucEGFP), Tg(tc21:EGFP), Tg(tc21:nucEGFP), Tg(fli1:GFP) and VCAP1X2 mutant, were maintained in 20 L aquaria supplied with filtered fresh water and aeration, or high density, self-circulation systems (Aqua Blue) with a photoperiod of 14 h light/10 h dark cycle. Embryos were raised at 28.5 °C and embryonic stages were determined by morphological criteria defined as described. All animal procedures were approved by the Academia Sinica Institutional Animal Care & Utilization Committee (AS IACUC) (protocol # 12-12-482). All experimental methods were performed in accordance with the approved guideline.

**Generation of VCAP1X2 mutant line.** The Tol2 transposon based SAGVG (splice acceptor-Gal4-VP16;UAS:EGFP) plasmid DNA and Tol2 transposase RNA were injected into ASAB 1–2 cell zygotes. Injected F0 embryos were raised to adulthood and outcrossed with WT to produce F1. F1 Embryos that exhibited intense heart-specific EGFP expression by fluorescence microscopy were raised to adulthood and the F2 generation was produced. Subsequent ligation mediated-PCR showed that the Gal4-VP16; UAS:EGFP vector had been inserted into intron 1 of the VCAP1X2 gene. To determine whether the splicing of VCAP1X2 exon 1 and exon 2 was disrupted, RT-PCR was conducted using forward (5′- GATCTACAACGTGACAGGACA-3′) and
reverse (5′-TGTAATAGTGACTGGAGAG-3′) primers, and total RNA isolated at 24 and 48 hpf. Homozygous VCAP1X2 mutant embryos are able to survive to adulthood. Homozygous populations of adult female and male VCAP1X2 mutants were maintained.

**Morpholino and mRNA injection and RNA synthesis.** Individual MOs (3.3 ng/embryo) were diluted with DEPC dH2O and injected into randomly selected 1–2 cell zygotes using an IM-300 microinjector (NARIISHIGE). For rescue experiments, 33 pg of individual capped VCAP1X2 mRNA variants together with or without VCAP1X2 spMO were injected into randomly selected 1–2 cell zygotes of Tg (myl7: EGFP; myl7: H2AFZ mcherry) transgenic fish or VCAP1X2 mutant. For smydlb rescue experiments, 50 pg of capped smydlb variants, smydlbRNA, or smydlbRNA†+ mRNA were injected by a SP6 mMESSAGE mMACHINE Kit by digestion with NotI. To synthesize RNA probes for RNA in situ hybridization, plasmids were linearized by restriction enzymes and antisense RNA probes were produced as follows (restriction sites and promoters in parentheses): myl7 (NcoI/sp6), tcf21(Ncol/sp6) and VCAP1X2 (Ncol/sp6).

**Analysis of heart function.** 72 hpf Zebrafish larvae were anesthetized with a mixture of tricaine (100 ppm) and isoflurane (100 ppm). Green fluorescent images of Tg(myl7: EGFP; myl7: H2AFZ mcherry) and VCAP1X2 mutant hearts were captured as described. Green fluorometric imaging of the contracting heart was used to measure cardiac function, and the images were constructed and displayed with commercial software (Iamiris, Bioptle). End-diastolic volume (EDV) and end-systolic volume (ESV) correspond to the ventricular volume immediately before a contraction and at the end of the contraction, respectively. Stroke volume (SV), the volume of blood ejected in a single heartbeat, was determined from the equation: \[ SV = EDV - ESV \] and ejection fraction (EF) was defined as \[ EF = SV / EDV \]. The FS of zebrafish embryonic hearts was determined as described. In brief, heart beating was recorded at 37 frames per second for 30 s with a camera (AxioCam HRC) installed on a microscope (Zeiss Imager M1). Videos were converted to frames and the ventricular widths and lengths were measured from single frames showing the maximum ventricular systole (VS) and ventricular diastole (VD) by ImageJ software. FS (%) of the ventricle was calculated as \[ (W_VD - W_{VS}) / W_{VD} \times 100\% \].

**Immunofluorescence staining.** Immunofluorescence staining of zebrafish embryos was performed as described with some modifications. For immunostaining of α-actinin, pERK or pAKT, antigen retrieval was conducted by incubating embryos with 150 mM Tris-HCl (pH 9.0) for 15 min at 70°C. Embryos were then permeabilized by 10 µg/mL protease K for 20 min at 28°C and acetic acid for 20 min at −20°C. Embryonic hearts were then dissected using two 22-gauge needles and transferred to slides. Tissues were blocked with 10% serum for 1 h at room temperature (RT), followed by incubation with primary antibody including α-actinin (1:100, Sigma-Aldrich), pAKT (1:100, Cell Signaling) or pERK (1:100, Cell Signaling) at 4°C overnight. For immunostaining of VCAP1X2 (1:100), embryos were permeabilized in acetone for 7 min at −20°C and 2% Triton-X100 in PBS for 1.5 h at RT, followed by antigen retrieval in 150 mM Tris-HCl (pH 9.0) for 15 min at 70°C. For immunostaining of MEF2 or PCNA, embryos were permeabilized by 2% Triton-X100 in PBS for 1.5 h at RT, followed by antigen retrieval in 150 mM Tris-HCl (pH 9.0) for 15 min at 70°C. Embryonic hearts were then dissected and blocked with 10% serum. Tissues were incubated with primary antibody including MEF2 (1:150, Santa Cruz Biotechnology) or PCNA (1:200, Abcam) at 4°C overnight. After several PBS washes, embryonic hearts were incubated with goat anti-rabbit Alexa Fluor 647 (1:200, Invitrogen) or mouse Alexa Fluor 568 (1:200, Invitrogen) and later were mounted in ProLong Gold Antifade Mountant with DAPI (Invitrogen). To conduct immunostaining of VCAP1X2 on Tg(fli1:GFP) transgenic embryos, fixed Tg(fli1:GFP) embryos were permeabilized with 2% Triton-X100 in PBS for 1.5 h at RT, followed by antigen retrieval in 150 mM Tris-HCl (pH 9.0) for 15 min at 70°C and acetone treatment for 8 min at −20°C. After several PBS washes, embryos were blocked with 10% serum for 1 h at RT and incubated with anti-VCAP1X2 antibody (1:100) at 4°C overnight. After several PBS washes, embryos were incubated with goat anti-rabbit Alexa Fluor 647 (1:200, Invitrogen).

**BrdU incorporation.** BrdU incorporation was conducted as described. 70.5 hpf embryos were incubated with freshly diluted BrdU (final 10 mM, Sigma-Aldrich) in 15% dimethylsulfoxide on ice for 20 min, and then incubated in egg water at 28.5°C for 1 h after washes. Samples were fixed in 4% PFA at 72 hpf at 4°C overnight. Fixed embryos were dehydrated and rehydrated through a methanol/PBST series, followed by permeabilization with 10 µg/mL proteinase K for 30 min and exposure of the BrdU epitope with 2 N HCl for 1 h. Embryos were blocked with 10% serum at 4°C overnight and incubated with BrdU antibody (1:7, BD Biosciences) at 28°C for 2 h. After incubation with goat anti mouse Alexa Fluor 647 (1:200, Invitrogen) for 1 h at RT, embryos were washed with PBS before imaging.

**Apoptosis analysis.** Embryos were fixed at 72 hpf in 4% PFA at 4°C overnight. Fixed embryos were dehydrated and rehydrated through a methanol/PBST series, followed by permeabilization with 10 µg/mL proteinase K for 30 min and fixation in 4% PFA for 20 min at RT. Apoptotic cells were labeled by TdT-mediated dUTP nick-end labeling (TUNEL) assay using in situ cell death detection kit (Roche), according to the manufacturer's
instructions. After washing with PBST, embryos were blocked with 5% serum in PBST for 2 h at RT, and incubated with anti-fluorescein-POD antibody (1:1000, Roche) and anti-MEF2 antibody (1:150, Santa Cruz Biotechnology) overnight at 4°C. After several PBST washes, apoptosis signal was enhanced by TSA Plus Cyanine 5 and Fluorescein System (Perkin Elmer), then embryos were incubated with goat anti-rabbit Alexa Fluor 568 (1:200, Invitrogen) for 3 h at RT. Later, embryonic hearts were dissected using two 22-gauge needles and transferred to slides, where the tissues were mounted in ProLong Gold Antifade Mountant with DAPI (Invitrogen). Both DNaase I-treated positive control and negative control, without DTT enzyme, were included.

**Embryonic heart purification and Western blot analyses.** Embryonic heart purification was performed as described\(^4\). Hearts of 96 hpf Tg(myl7::EGFP; myl7::H2AFZ mcherry) transgenic embryos were isolated by fluid force driven by syringe with a 19-gauge needle and filtered through 100 μm and 40 μm cell strainer (Corning). Isolated hearts were washed with L-15 medium containing 10% charcoal-stripped FBS and examined under a fluorescence microscope for intactness. For Western blot analyses, isolated embryonic hearts were extracted by homogenization in 1X RIPA lysis buffer (Millipore) containing 1X Protease Inhibitor Cocktail tablet (Roche), 1X Phosphatase Inhibitor Cocktail 2 and 3 (Sigma-Aldrich) and 1 mM PMSF (Sigma-Aldrich). Protein concentration was determined by Bradford assay. Total protein was separated by electrophoresis on a 11% SDS–PAGE and transferred to Immobilon-P Transfer Polyvinylidene difluoride (PVDF) Membranes were immunoblotted with total AKT (1:1000), phospho-AKT (1:2000), total ERK (1:2000) or phospho-ERK T202/Y204 (1:2000) antibody from Cell Signaling company, or β-actin (1:10000, Millipore) antibody. Peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (1:5000, Jackson) and peroxidase-conjugated goat anti-mouse IgG (1:5000, Jackson) were used as secondary antibodies. Immuno-reactive signals were detected by Luminata Crescendo Western HRP Substrate (Millipore). Protein concentrations from visualized bands were quantified by Image J.

**Electron Microscopy and sarcomere length quantification.** Transmission electron microscopy analysis was conducted as described\(^5\). For sarcomere length quantification, embryos were incubated with relaxation buffer (20 mM imidazole, 5 mM EGTA, 7 mM MgCl\(_2\), 5 mM creatine phosphate (Sigma-Aldrich), 10 mM ATP (Sigma-Aldrich), 100 mM KCl) for 1.5 h before fixation\(^6\). Z-discs were labelled with anti-α-actinin antibody. Sarcomere length and Z-disc length were measured according to immunofluorescent α-actinin signal by Image J.

**Treatment of inhibitors or activator of PI3K or pMEK.** To inhibit PI3K-AKT or MAPK-ERK1/2 signaling, Tg(myl7::EGFP; myl7::H2AFZ mcherry) embryos were treated with 30 μM PI3K inhibitor (LY294002, Calbiochem) or 40 μM PMEK inhibitor (U0126, Promega) in egg water (final 0.1% DMSO) from 63 to 70.5 hpf, respectively. To activate pAKT, VCAP1X2 mutant embryos were treated with 1 μM SC-79 (Sigma) in egg water (final 0.1% DMSO) from 63 to 70.5 hpf. These embryos were then replaced with relaxation buffer (final 0.1% DMSO) containing the same amount of inhibitors or activators for 1.5 h before fixation at 72 hpf in 4% PFA overnight at 4°C. Control embryos were incubated with egg water or relaxation buffer with 0.1% DMSO.

**Microscopy and statistics.** Bright-field images of embryos were taken using a camera (AxioCam HRC) installed on a microscope (Zeiss Imager M1) in DIC mode. Immunofluorescence stained embryos or hearts were observed with a confocal microscope (Leica TCS-SP5-MP). Ultrastructural sections were examined using an electron microscope (FEI Tecnai G2 TF20 Super Twin, Thermo Fisher Scientific). The total number of embryos analyzed (biological replicates) was reported as the sample number for each experiment. Biological replicates were pooled from at least three separate experiments, conducted independently. For RT-qPCR analysis, each biological replicate was analyzed with three technical replications. Two-tailed Student’s t-test with unequal variance was performed in Microsoft Excel. ANOVA with post hoc multiple comparisons (Bonferroni method) was performed in R\(^7\).

Plasmid construction, morpholino sequence, VCAP1X2 antibody generation and purification, histological analyses, RNA in situ hybridization, reverse transcription polymerase chain reaction (RT-PCR) and quantitative real-time reverse-transcription PCR (RT-qPCR) were performed as described in the Supplementary Information.

**Data availability.** All data analyzed during this study are included in this published article (and its Supplementary Information files).

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**Additional Information**

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