Suppression of Rap1 Impairs Cardiac Myofibrils and Conduction System in Zebrafish

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Abstract
Numerous studies have revealed that Rap1 (Ras-proximate-1 or Ras-related protein 1), a small GTPase protein, plays a crucial role in mediating cAMP signaling in isolated cardiac tissues and cell lines. However, the involvement of Rap1 in the cardiac development in vivo is largely unknown. By injecting anti-sense morpholino oligonucleotides to knock down Rap1a and Rap1b in zebrafish embryos, and in combination with time-lapsed imaging, in situ hybridization, immunohistochemistry and transmission electron microscope techniques, we seek to understand the role of Rap1 in cardiac development and functions. At an optimized low dose of mixed rap1a and rap1b morpholino oligonucleotides, the heart developed essentially normally until cardiac contraction occurred. Morphants hearts showed the myocardium defect phenotypes, most likely due to disrupted myofibril assembly and alignment. In vivo heart electrocardiography revealed prolonged P-R interval and QRS duration, consistent with an adherens junction defect and reduced Connexons in cardiac myocytes of morphants. We conclude that a proper level of Rap1 is crucial for heart morphogenesis and function, and suggest that Rap1 and/or their downstream factor genes are potential candidates for genetic screening for human heart diseases.

Introduction
In the heart, cardiomyocytes are responsible for pumping blood into the circulation, a process called the cardiac contraction, which is cAMP-dependent. An important cAMP sensor, Epac (exchange proteins directly activated by cAMP), has been extensively studied for its role and regulation in cardiovascular physiology and pathophysiology [1–3]. It is now clear that upon cAMP binding, the GEF (guanine-nucleotide-exchange factor) domain of Epac is exposed, allowing Epac to activate small Ras-like GTPase proteins, such as Rap1 (Ras-proximate-1 or Ras-related protein 1). In addition, Epac regulates the activity of various cellular compartments of cardiac myocytes and influences calcium homeostasis and excitation-contraction coupling, and thus, is potentially involved in many cardiovascular disorders, such as cardiac hypertrophy and remodeling [4,5]. Recent experiments have demonstrated that Rap1 and its effectors mediate functions of Epac, perhaps by directly targeting or modulating the affinity of integrins at cell membranes [6,7].

Rap1 is a small cytosolic protein that acts like a cellular switch for signal transductions. Rap1 carries an effector domain similar to that of Ras, and functions to inhibit Ras signaling. As an evolutionarily conserved protein, Rap1 has been found in many animal species to play distinct roles in the worm (C. elegans), fly (Drosophila), zebrafish (Danio rerio), frog (Xenopus) and mouse. [8–13]. Previous studies have declared that Rap1 induces calcium release in cardiac excitation-contraction [14], a proposed function in cAMP metabolism and homeostasis in adrenergic signaling [15]. Originally thought to be a transformation suppressor with the ability to remodel the Ras-transformed phenotype, recent studies showed that Rap1 has many functions in cell attachment, migration and cell junction, and coupling of extracellular stimulation to intracellular signaling through second messengers. A vital role of Rap1 in the cardiovasculature was revealed, reflecting its multiple roles at different stages of heart development/remodeling and cardiac functions [16].

Rap1a knock out mouse are unexpectedly viable and fertile [17], presumably due to functional compensation by Rap1b. A study in frog and fish showed that a loss of both Rap1a and Rap1b functions can lead to severe convergence extension defect during gastrulation, and Rap1 serves as a component of the non-canonical Wnt pathway [10]. Moreover, in endothelial junction formation, knocking down rap1b alone causes specific cerebral hemorrhage [19], suggesting that Rap1a and Rap1b may be functionally redundant and yet possess individually unique functions. The fact that Rap1a and Rap1b have distinct roles in the cardiovasculature, from in vitro studies, emphasizes a critical role of Rap1 in heart functions however, its role in heart development remains blurry.

The coordinated cardiac conduction relies on gap junction (GJ) mediated electrical excitation [16,20]. It is known that a connexon...
of each adjacent cardiomyocytes pairs to form a GJ, and a loss of the most abundant Connexin 43 (Cx43) leads to cardiac arrhythmias. On the other hand, cAMP-treated cardiac myocytes show increased Cx43 expression and the neoformation of functional GJs [21]. Interestingly, Somekawa et al. showed that cAMP treatment increases GJs through activating Epac-Rap1 signaling and adherens junctions [22]; whereas the activation of Rap1 by Epac results in impeding ERK5, and thus in the decreasing of myocyte growth [23]. This suggests a role of Rap1 in myocardium function, thus likely to change cardiac development (the GJ remodeling).

We set to analyze if and how Rap1a and Rap1b regulate heart development and functions in vivo. The simplicity of the fish heart, with a single atrium and ventricle, and the ease of using time-lapsed recording tools can delineate complex functions of Rap1 during fish heart development, and distinguish its role in heart functions from developmental process. We carefully determined a combined dose of anti-sense morpholino oligonucleotides (MOs) against rap1a and rap1b, and produced specific heart, trunk and medial fin-fold defects. We found that loss of both Rap1a and Rap1b generates persistent myocardium defect phenotype as well as the first degree A-V block. At optimal MO injection, early heart development appeared to be normal until cardiac dilation progressively occurred from 20–24 hpf onward and some cardiac cells failed to move into the heart tube during heart morphogenesis. The 20 hpf is the stage when embryonic cardiac muscle cells gain their contractile/relaxation ability, and consuming/requiring more energy. Therefore, we believe that Rap1 plays an essential role in development/remodeling of cardiac functions, resembles the in vivo observation using mammalian cultured cardiac cells.

Materials and Methods

Zebrafish Maintenance and Transgenic Lines

Wild type Tubingen strain and all transgenic fish lines were raised under standard conditions [24]. mp137e is a transgenic line obtained from Toł2 mediated enhancer trap screening at the Peking University, named Egitata2e:eGFP/pha5, Tg(flk1:mCherry)pha6 is a home-made transgenic line using a flk1:mCherry plasmid (a gift from Shuo Lin’s lab at UCLA).

Ethics statement: All animal experiments were approved by Institutional Animal Care and Use Committee (IACUC) of Peking University. The reference from IACUC of Peking University, named is a transgenic line (a gift from Shuo Lin’s lab at UCLA).

RT-PCR and Gene Cloning

Total RNA was extracted from 50 wild type or morpholino injected embryos using the Trizol RNA isolation kit (Invitrogen) raised under standard conditions [24]. Specific primer sets for mRNA synthesis. Specific primer sets for rap1a and rap1b cloning are listed below. The long primers were used to synthesize mRNA in mis-expression experiments and the short primers for in situ anti-sense RNA probes.

**rap1a Long sense:** 5’- GCCCATCGATATCGGCAATATA-TAAGCTTGTG-3’

**rap1a Long anti-sense:** 5’- CCAACTCGAGTTACAGCAGGACA-GGACGAGTTTGA-3’

**rap1a Short sense:** 5’- ATGCCTGAAATACAAGTGTA-TAGTC-3’

**rap1a Short anti-sense:** 5’- TTACACAGGCAATAGGTGTA-TAGTC-3’

**rap1b Long sense:** 5’- GCCCATCGATATCGGCAATATA-TAAGCTTGTG-3’

**rap1b Long anti-sense:** 5’- ATTA CTCGAG TTAGAG-CAACTTGGAAGGTG-3’

**rap1b Short sense:** 5’- ATGCCTGAAATACAAGTGTA-TAGTC-3’

**rap1b Short anti-sense:** 5’- TTACACAGGCAATAGGTGTA-TAGTC-3’

**rap1 MO**

**rap1a splicing-blocking MO:** 5’- CCTCTTACCTCATTACATTAAAA-3’

**rap1b splicing-blocking MO:** 5’- CAATAGAATGTAGCGA-GACTTGCC-3’

**Standard Control MO:** 5’- CCTCTTACCTCATTACATTAAAA-3’

**p53 MO:** 5’- GCGGATTTGTTCGCAAATATTGG-3’

**p53 MO** (n-mismatch): 5’- TGCAGATATAGTCTTCCCCAAA-3’

One-cell stage embryos were injected with 1 nl of various concentrations of individual (5 ng/ul) or mixed MOs (2.5 ng/ul each) dissolved in ddH2O from a Femtojet (Eppendorf). rap1MOs were co-injected with p53 MO to prevent nonspecific cell death and/or artificial defect.

mRNA Rescue Experiment

The cDNAs of rap1a and rap1b were cloned into pCS2+ and used for mRNA synthesis. Specific primer sets for rap1a and rap1b cloning are listed below.

**rap1a long S:** (5’- GCCCATCGATATCGGCAATATA-TAAGCTTGTG-3’);

**rap1a long A:** (5’- CCAACTCGAGTTACAGCAGGACA-GGACGAGTTTGA-3’);

**rap1a Short S:** (5’- GCCCATCGATATCGGCAATATA-TAAGCTTGTG-3’);

**rap1a Short A:** (5’- CCTCTTACCTCATTACATTAAAA-3’);

**rap1b Long S:** (5’- ATTA CTCGAG TTAGAG-CAACTTGGAAGGTG-3’);

**rap1b Long A:** (5’- CCTCTTACCTCATTACATTAAAA-3’);

**rap1b Short S:** (5’- ATTA CTCGAG TTAGAG-CAACTTGGAAGGTG-3’);

**rap1b Short A:** (5’- ATTA CTCGAG TTAGAG-CAACTTGGAAGGTG-3’);

**mRNA was synthesized** using MESSAGE MACHINE SP6 system (Ambion), and purified with RNeasy Mini (QIAGEN). Fertilized eggs were injected with 1 nl of mixed rap1a, rap1b and p53 MOs and quickly were subjected to another injection of rap1a and rap1b mRNAs (1nl/egg). In control group, 1nl of ddH2O was used in the second injection.

Whole-Mount in situ Hybridization and Immunostaining

In situ probes were synthesized with a DIG RNA labeling Kit (Roche), and purified with RNeasy Mini Kit (QIAGEN). Prehybridization and hybridization were performed at 65°C for 4 hours to overnight. The embryos were washed in 66% formamide/2×SSCT at 65°C for 30 min, in 2×SSCT at 65°C for 15 min, and twice in 0.2×SSCT at 65°C for 30 min. Then the embryos were incubated in blocking solution with 1/4000 volume of anti-digoxigenin-AP Fabfragments (Roche) at 4°C overnight. Embryos were washed in coloration buffer at room temperature for 25 min three times. The signals were detected by using BCIP/NBT Color Development Substrate (Promega). The reaction was stopped by washing with PBS.
Whole-mount in situ hybridization for cmlc2, cmhc, rapla and raplb was performed as described above. Whole-mount immunofluorescence experiment was performed as described [25], using primary monoclonal antibodies against sarcomeric myosin heavy chain (MF20/S14 and Connexin 43 (Cx43) of gap-junction component ANTI-C-JA1, Sigma). Briefly, we fixed the young fish, and performed whole-mount antibody staining procedure. We then isolated the heart tube from each 3 dpf fish and pressed it on a glass slide for observation.

Histology and Transmission Electron Microscopy

Whole embryos and isolated hearts at 3 dpf were fixed with 2.5% glutaraldehyde and 2.0% paraformaldehyde and post fixed in 1% osmium tetroxide. After washing, they were embedded in the Spur resin, and sectioned. The sections were stained with uranyl acetate and lead citrate and observed under a Philips CM100 transmission electron microscope at an accelerating voltage of 100 kV.

Imaging and Statistics

Embryos were examined with Zeiss Observer A1 and Leica S8APO, and photographed with a Zeiss Axiocam camera. 3-D images were obtained with confocal Zeiss LSM 510. Figures were processed with Adobe Photoshop CS4 software (Adobe).

To provide clear contrast of each part of the heart shown in Fig. 1E, we merged three photos of bright (DIC) and green fluorescent (highlighting the atrium) fields; and red field (the green fluorescence, highlighting the ventricle, was converted into red color via Photoshop).

TEM figures were further analyzed by counting numbers of thin and thick filaments in a 100 nm × 100 nm of cardiomyocyte myofibrils region. From Cx43 antibody staining experiment, a 200 μm × 200 μm of cardiomyocyte region (in confocil photo) was analyzed by ImageJ (National Institutes of Health) with manually set Cx43 signal threshold, and the result is represented as averaged value ± S.D.

Statistic figures (Fig. 1D, 3D, and 3E) were generated by GraphPad Prism 5.01 (GraphPad Software, Inc.). Columns and error bars represented mean ± S.D. Statistic symbols: ns for *p > 0.05; * for 0.01 < p < 0.05; ** for 0.001 < p < 0.01; *** for p < 0.001.

Electrocardiograph (ECG) Recording

The working platform was encased in a grounded metal cage. A pair of silver wires were suspended within pre-pulled borosilicate micropipettes (1.5 mm O.D., 2–3 MΩ in water; VitalSense Scientific Instruments Co., Ltd) filled with cell outside buffer (140 mM NaCl, 4 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 10 mM Glucose, 10 mM Hepes, pH 7.4). Zebrafish embryos at 3 dpf were sedated in 0.63 mM tricaine (Ethyl 3-aminobenzoate methanesulfonic acid salt, 98%; Sigma) in E3 solution (containing 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, and 0.33 mM MgSO4) and moved into M199 medium (Invitrogen) with 10% FBS and 20mM BDM (2, 3-butanedionemonoxime; Sigma). To exclude motion contamination, two micropipettes were used to disclose pericardial sac and expose heart to BDM. When the hearts stop beating, the positive and reference electrodes were introduced in atrium and ventricle respectively. The ECG signal was amplified (AM3000H-220-50, ADInstruments), digitized (ML870, ADInstruments), and recorded in 1-s sweeps with digital filtering at 0.5–10 Hz (Chart 5, ADInstruments).

Results

Loss of rap1 Functions Led to Specific Defects, Including the Heart Malformation, in Zebrafish

To investigate the role of rap1 in zebrafish development, we used morpholino anti-sense (MO) oligonucleotides that were previously used to block the translational start site of rapla [19] and a splice donor site of raplb [19]. Because of the ubiquitous expression of both rap1 genes at the embryonic stages (Fig. 1A-B), we reasoned that the dose of MO was crucial to distinguish artifacts from real loss-of-function phenotypes. During MO dose dependency tests, we observed variable phenotypes ranging from mild to severe. In low dose (<3 ng/ml of each) of morpholino injection, embryos and larvae survived normally. In high dose (>8 ng/ml of each), almost all injected embryos died or possessed severe abnormalities after 3dpf (data not shown). At 5 ng/ml, raplaMO injection led to fairly specific heart, trunk muscle and caudal fin defects, while the intracranial hemorrhage phenotype was specific to raplbMO injected animals (Table1, data not shown). Because the specificity of raplbMO could easily confirmed by RT-PCR (data not shown; see also ref. 19), to ensure the specificity of raplaMO, we used a mismatch raplaMO to test if the consistent phenotypes observed in rapla morphants was entirely due to raplaMO. Similar to the un.injected control (182/185), the mismatch raplaMO injection resulted in 118/122 normal fish at 3 dpf, indicating the specific loss of function phenotypes of rapla morphants.

Because Rapla and Raplb may have redundant functions (Table1), to fully evaluate Rapl functions and avoid obvious early developmental defects, we injected embryos with MO oligonucleotides each at 2.5 ng/ml, and observed constant defects, i.e., over 90% of double rap1 knock-down morphants showed heart, trunk and caudal fin-fold phenotypes but intracranial hemorrhage remains a specific phenotype caused by raplbMO (Fig. 1C, and Table1). This suggested additively specific effects of both morpholino oligonucleotides (Table1 and Fig. 1). Thus, in the following, we used rapIMO to refer to co-injection with MO oligonucleotides against both rapla and raplb each at 2.5 ng/ml. In rapIMO embryos, the heart was misshaped and the cardioeolum expanded compared with those of wild-type fish (Fig. 1E). We also observed that rapIMO embryos had curved trunks, as well as blisters at the tip of the caudal fin (Fig. 1C, data not shown).

By injecting rapla and raplb mRNA into embryos pre-injected with rapIMO, we found that rapl mRNAs can rescue the heart deficiency in a dose dependent manner. There was no obvious rescue or side effect when small amount mRNAs was used (<0.1 ng/ml). While in a relatively low dose (0.25 ng/ml) of mRNAs, over 30% of rapIMO embryos had been partially rescued (Fig. 1D). Interestingly, when we further increased the dose of mRNA to 0.5 ng/ml, we observed no higher ratio of rescue but more distorted embryos (data not shown), suggesting that Rapl level should be properly maintained in vivo.

Using transgenic embryos carrying cardiomyocyte-specific GFP (transgenic fish line mp157e, Fig. 2A-B and 2A’–B’), we observed malformed hearts in rapIMO embryos (Fig. 2). Although the initial heart development (18–22 hpf) was normal, rapIMO embryos showed cardiac defect from 24 hpf onward (Fig. 2B and 2B’), but cmhc probe still hardly distinguished the morphant heart from the control heart (Fig. S1C, C’). The characteristic phenotypes of rapIMO embryos included abnormal cardiac looping and incomplete heart chamber formation in transgenic mp157e carrying flk1mCherry (Fig. 2C–D and 2C’–D). However, nearby blood vessel cells expressing mCherry in rapIMO embryos appeared to be normal, indicating the specificity of cardiac defects caused by loss-
of Rap1 function. In older larvae (after 5dpf), rap1MO embryos showed persistent ventricular hypoplasia, heart dilation, and pericardial edema (data not shown), suggesting a correlation between rap1 and cardiomyocyte function of the heart.

Table 1. Phenotype summary of the rap1MO fish.

| MO oligo mix | Injected | Percentage of Embryos with Phenotype |
|--------------|----------|-------------------------------------|
| (5 ng/ml, each or mixed rap1MO) (pooled from 3 experiments) | Heart | Somite | Tail | Intracranial hemorrhage(ICH) |
| rap1a+p53 | 683 | 85 | 89 | 78 | 3 |
| rap1b+p53 | 540 | 31 | 36 | 10 | 67 |
| rap1a+rap1b+p53 | 526 | 93 | 96 | 90 | 60 |

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Early heart development, starting from around 10-somite stage, was essentially normal, judged by normal cardiac expression of nkx2.5, gata4 and tbx5 in rap1MO embryos until 21 hpf (Fig. S1, data not shown) when the cardiac myocytes started to contract. rap1 morphants mainly had smaller and shorter heart tubes (68%, n = 81). At 48 hpf, some rap1MO embryos failed to form their heart ‘S’ loops (46%, n = 144), and the defect could be captured from confocal images of both ventral and lateral views (Fig. S2). Expression of cmlc and vmhc indicated the essential normal shapes of morphant hearts (Fig. S3A, 3A’, 3D and 3D’). At 72 hpf, the ventricular defect was more obvious, showing the pericardium edema (48%, n = 25) (Fig. S3B, B’). These results collectively indicated that rap1 persistently and predominantly functioned to regulate ventricular development of the heart.

Rap1MO Animals had Disrupted Cardiac Structures

Starting from 24 hpf, some GFP’ (cardiac) cells failed to strictly follow their destined paths to the developing heart or to incorporate into the cardiac tissue when Rap1 function was lost (Fig. 2B’–D’, white arrows) and cardiac myocytes of heart tubes appeared to be loosely packed in rap1MO embryos (Fig. 2C–D, C’-D’). This is suggestive of heart structure and/or function defects.

Compared to normal hearts, in which myofibrils were organized into hexagonal lattices with regularly arranged thick and thin filaments (Fig. 3A, 3A’), the units of myofibrils in rap1MO hearts had significantly fewer thin filaments but no difference in thick filaments compared to those of wild type fish heart (Fig. 3D). It was unclear if abnormal primary assembly of myofibrils was due to a lack of thin filaments; however, at least the absence of Z-discs may contribute to the formation of detached sarcomeric units (data not shown).

In normal heart, there were plenty of adherens junctions (AJs) along the membrane of adjacent cardiac myocytes; while in rap1MO hearts, there were only few or none AJs (data not shown). The close-up images of AJs in rap1MO hearts showed low electron density (Fig. 3B and 3B’) indicated that much less myofibrils were present, with obvious larger space between cell membranes of adjacent myocytes.

We then used antibody against Cx43 to stain normal and morphant hearts at 3 dpf. The antibody highlighted 2 adjacent connexons that form GJ channels between two bordering cells (Fig. 3C, 3C’), and Cx43 was the predominant connexins in working cardiomyocytes. The number of connexon pairs, highlighted by the presence of Cx43 in ventricular cardiomyocytes, was significantly reduced in rap1MO hearts (Fig. 3E). Therefore, fewer GJs in ventricular cardiomyocytes, due to a suppression of rap1, may lead to heart dysfunction.

Loss of rap1 Function led to Atrio-ventricular Block (A–V Block) in Zebrafish

Heart beating is the driving force to enable circulation of hematopoietic cells, and the blood flow promotes ventricular cells to enlarge and elongate in a process of heart development/remodeling. Upon imaging both control and morphant fish larvae in the cardiac regions, we found that in wild-type embryos, the heartbeats on average were 85/min at 36 hpf and 120/min at 72 hpf, while the averaged rap1MO heartbeats were 68 and 103, respectively. In addition to an obvious reduction in heart rate over time, ejection fraction and contraction volumes also decreased (data not shown).

In addition, the GJ deficiency (Cx dependent) could be correlated to the conduction system of rap1/MO animal hearts. To determine if there was any conduction problem, we measured the conduction of heart in 3dpf fish larvae in our own way, and in 10 control fish, we obtained fairly reproducible electrocardiography (ECG) readings. ECG of hearts at 3 dpf was recorded in

Figure 2. Time-lapsed observation revealed abnormal heart development in Rap1 knock-down zebrafish. Transgenic fish mp157e without any injection (A) and injected rap1MO (A’) were indistinquishable in early heart development. By 24 hpf, rap1MO injected mp157e embryos, crossed in flk1:mCherry background, started to show abnormality in heart development and remodeling (B to B’), including the failure of heart tube extension, abnormal cardiac looping and incomplete heart chamber formation. In higher magnification views, the morphants show that a small portion of GFP labeled cardiac cells (arrows in B’ to D’) did not migrate properly to be eventually packed in the heart tube. Dorsal view, head to left in A to C and A’ to C’. Lateral view, head to left in B to D and B’ to D’. Scale bar, 100 μm in A to C, and A’ to C’; 40 μm in D and D’.

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sedated zebrafish with their hearts exposed to 20 mM BDM to avoid any motion interferences. The P and R waves were easily recognized by ECG recording of atrium or ventricular alone (data not shown). In control larvae, the P-R intervals were averaged at 0.664s (Fig. 4, A and Table 2); while in rap1MO fish (n = 12), the P-R intervals were averaged at 1.278s (Fig. 4, B–D and Table 2). The duration of QRS complex in rap1MO fish was also significantly longer than that of the wild type. Therefore, rap1 deficiency in zebrafish hearts results in characteristic ECG changes such as first-degree atrio-ventricular block and the prolongation of the QRS duration, further suggesting that suppressing rap1 may lead to abnormal heart function.

Discussion

In the present study, we provided in vivo evidence to reveal that Rap1 participates in cardiac development and function. We showed that by simultaneously knocking down two ubiquitously expressed Ras family members, namely zebrafish Rap1a and Rap1b, rap1MO larva exert abnormal cardiac AJs and show reproducible A-V block pattern and prolonged QRS duration, as well as slower heart beating activity and reduced ejection and contraction volumes (our unpublished observations). Comparison of ultra structures of normal and morphant hearts revealed that myofibril assembly and alignment were disrupted when rap1 was suppressed, and the small number of thin filaments in cardiomyocytes may also contribute to slower heart beating rate observed in the morphants. These defects ultimately led to the heart failure.

It is known that the cardiac impulse conduction is in part governed by myocardium cell-cell coupling. GJs, in the ventricles of the heart and mainly composed of Cx43, enable low resistance communication between adjacent cells, thereby increasing the cell-cell coupling. Our finding that Cx43 level in the rap1MO heart was significantly lower than that of the normal heart (Fig. 3C, C’) is thus intriguing, because it is suggestive of a correlation between Rap1 and GJ, a fact that was previously demonstrated in cultured cells, in which the Epac-Rap1 signal accelerates AJ formation, leading to enhanced GJ formation [22]. Because previous studies also showed the Rap1 dependency of AJ positioning, and demonstrated that GJ assembly is dependent of and differentially regulated by AJ adhesion molecules [12,26], the presence of poorly

Figure 3. Ultra-structural changes of cell junctions and sarcomeres in Rap1 knock-down zebrafish heart. Transmission electronic scan revealed that the myofibril units, regularly organized into hexagonal lattices with thick (green arrow) and thin (yellow arrows) filaments (A) with adherens junctions (AJs) along the membrane of adjacent cardiac myocytes (B). In rap1MO heart, thick filaments (green arrow) number was not markedly changed but thin filaments (yellow arrows) number was significantly and statistically reduced (A’ and D), and less myofibrils attached to AJs (arrows in B and B’), with larger space between the membrane of adjacent cardiac myocytes (B’). Antibody against Connexin 43 stained a significantly and statistically less GJ signal (arrowheads in C and C’) in rap1MO cardiomyocytes at 3 dpf, compared to a normal heart (C, C’, and E). Scale bar, 50 nm in A and A’; 350 nm in B and B’; 50 μm in C and C’. Columns and error bars in D and E showed mean ± S.D. n = 9 in filaments experiment (D) for each group, and n = 10 in C43 antibody staining experiment (E) for each group. Unpaired two-tailed t-test was used to test the significance between two columns in each group in D. *** in statistic graph represent p < 0.001 in the t-test.

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formed AJs in rap1 morphant heart strongly suggests that the abnormal cell-cell coupling in cardiomyocytes, i.e., the A-V block and prolonged QRS duration in morphants, is ultimately due to an insufficient amount of Rap1. However, similar atrio-ventricular block measurements were evident in Cx40^{−/−} mouse hearts [27], rather than in Cx43^{−/−} mouse (Cx43^{−/−} is embryonic lethal) [28]. Interestingly, a longer QRS duration was both evident in Cx40^{−/−} and Cx43^{+/−} hearts [27,28], suggesting that Rap1 may target a Cx-complex composed of connexins(s) in addition to Cx43.

Numerous studies reveal that Rap1 is vital for effective cAMP signal transduction, which is downstream of Epac. However, whether Rap1 is involved in heart development is unknown. In previous studies, knocking down Rap1 functions either leads to severe defects in axial development (rap1a/rap1b MOs), making evaluation of heart development impossible, or the heart development is largely normal (rap1b MO). Because both rap1a and rap1b are ubiquitously expressed (Fig. 1A–B), it is expected that a complete lack of Rap1 during development is deleterious. In order to reveal the role of Rap1 in zebrafish heart development, we determined the dosage of rap1 MOs, and found that Rap1 regulates overall development in a dose dependent manner. At a low level of anti-sense oligonucleotides, heart and somite defects were not observed by either rap1a MO or rap1b MO (2.5 ng/ml) alone, but only by their combination. The defects were certainly enhanced when both rap1a MO and rap1b MOs (each at 2.5 ng/ml) were used (Table 1). On the other hand, the fin-fold defect was specific to rap1a MO, while the intracranial hemorrhage was specific to rap1b MO, thus suggesting that the two zebrafish Rap1s are not entirely redundant in their functions.

### Table 2. Summary of ECG features.

| ECG                | WT (beats/min) | MO (beats/min) | p value |
|--------------------|----------------|----------------|---------|
| Heart rate         | 120±4          | 103±5          | 0.012   |
| P-R interval (s)   | 0.664±0.047    | 1.278±0.142    | 0.000   |
| QRS duration (s)   | 0.305±0.028    | 0.449±0.034    | 0.000   |
| Q-T interval (s)   | 0.768±0.063    | 0.716±0.078    | 0.147   |

Note: Data was shown as mean ± S.D. P value was calculated by unpaired two-tailed t test.

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Figure 4. Rap1 knock-down zebrafish have prolonged P-R intervals, resembling the first-degree atrio-ventricular block. Zebrafish heart electrocardiography (ECG) was measured in wild type larvae (A), and rap1MO hearts at 3 dpf (B to D). 

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In *rap1* morphants, the heart defect was first observable at 20–22 hpf, when initial cardiomyocyte contractions started during normal linear heart tube formation [29], and became more obvious when a small fraction of cardiac cells failed to integrate into the developing heart chambers (Fig. 2C–D, 2B’–D’). These observations led us to believe that the knock down of Rap1 function in zebrafish, at our experimental condition, has a limited effect on normal cardiac development at early stages (before 20 hpf). However, it was still surprising that massive heart failure eventually happened in the morphant. Because of a dramatic cardiocardiac morphogenesis between 20 and 30 hpf, and a rash emergence of uncoordinated cardiac cells in the morphant hearts, it was reasonable to conclude that cardiomyocytes required Rap1 during heart remodeling process. It was possible that the lower than normal Rap1 level in morphant, initially compensated by during heart remodeling process. It was possible that the lower than normal Rap1 level in morphant, initially compensated by

**Supporting Information**

**Figure S1** The expression of *nkx2.5* in developing hearts. The cardiac primordial marker gene, *nkx2.5*, was expressed essentially normal between 18–21 hpf (A–C), compared to that of wild type heart (D) Dorsal view, anterior to the left. Scale bar, 200 μm. (TIF)

**Figure S2** Morphant zebrafish has consistent defects in cardiac looping. Compared to control *mp157e* embryos (A, B), *rap1*MO injected *mp157e* embryos, crossed in *flk1:mCherry* background, showed faulty heart tube ‘S’ loop and thin cavity, in both ventral and lateral views (C,D). Shown are ventral view (A, C) and lateral view (B, D), with anterior to the left. (TIF)

**Figure S3** The cardiac looping defect was also evident by in situ hybridization. At 24 hpf, both control and *rap1*MO animals showed alike *vmhc* expression pattern in their hearts. Unlike the wild type hearts (A, C, D), the heart tube failed to extend left (C, C’) at 24 hpf or failed to form ‘S’ loop (A, A’, D and D’) in *rap1*MO animals at 48 hpf. At 72 hpf, ventricular defects, including pericardium edema and abnormal chamber differentiation, was observed in *rap1*MO (B, B’). Ventral views were shown in A-A’, B-B’ and D-D’, anterior to the left; dorsal view are shown in C-C’, anterior to the bottom. Scale bar, 150 μm in A, A’, D and D’; 120 μm in B and B’; 80 μm in C and C’. (TIF)

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### Author Contributions

Conceived and designed the experiments: WD CX DL. Performed the experiments: WD ZY FY JW YZ. Analyzed the data: DL WD ZY FY JW YZ CX BX XT. Contributed reagents/materials/analysis tools: BW. Wrote the paper: WD XT DL. Designed and conducted EGG: FY XT. Proofread the manuscript: DL WD ZY FY JW YZ CX BX XT.

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