Yulink, predicted from evolutionary analysis, is involved in cardiac function

Ming-Wei Kuo†, Hsiu-Hui Tsai†, Sheng-Hung Wang¹, Yi-Yin Chen¹, Alice L. Yu¹,² and John Yu¹,³*

Abstract

Background: The comparative evolutionary genomics analysis was used to study the functions of novel Ka/Ks-predicted human exons in a zebrafish model. The Yulink (MIOS, Entrez Gene: 54,468), a conserved gene from zebrafish to human with WD40 repeats at N-terminus, was identified and found to encode an 875 amino acid in human. The biological function of this Yulink gene in cardiomyocytes remains unexplored. The purpose of this study is to determine the involvement of Yulink in the functions of cardiomyocytes and to investigate its molecular regulatory mechanism.

Methods: Knockdown of Yulink was performed using morpholino or shRNA in zebrafish, mouse HL-1 cardiomyocytes, and human iPSC-derived cardiomyocytes. The expression levels of mRNA and protein were quantified by qPCR and western blots. Other methods including DNA binding, ligand uptake, agonists treatment and Ca²⁺⁺ imaging assays were used to study the molecular regulatory mechanism by Yulink. Statistical data were shown as mean ± SD or mean ± standard error.

Results: The knockdown of yulink with three specific morpholinos in zebrafish resulted in cardiac dysfunctions with pericardial edema, decreased heart beats and cardiac output. The Yulink knockdown in mouse HL-1 cardiomyocytes disrupted Ca²⁺⁺ cycling, reduced DNA binding activity of PPARγ (peroxisome proliferator-activated receptor gamma) and resulted in a reduction of Serca2 (sarcoplasmic reticulum Ca²⁺⁺ ATPase 2) expression. Expression of Serca2 was up-regulated by PPARγ agonists and down-regulated by PPARγ-shRNA knockdown, suggesting that Yulink regulates SERCA2 expression through PPARγ in mouse HL-1 cardiomyocytes. On the other hand, YULINK, PPARγ or SERCA2 over-expression rescued the phenotypes of Yulink KD cells. In addition, knockdown of YULINK in human iPSC-derived cardiomyocytes also disrupted Ca²⁺⁺ cycling via decreased SERCA2 expression.

Conclusions: Overall, our data showed that Yulink is an evolutionarily conserved gene from zebrafish to human. Mechanistically Yulink regulated Serca2 expression in cardiomyocytes, presumably mediated through PPARγ nuclear entry. Deficiency of Yulink in mouse and human cardiomyocytes resulted in irregular Ca²⁺⁺ cycling, which may contribute to arrhythmogenesis.

Keywords: Yulink, SERCA2, PPARγ, Ca²⁺⁺ cycling, Cardiomyocytes
Since 4768 of these predicted new exons had already been recognized as genes or pseudogenes, we used the remaining 8943 potential novel human exons to search for zebrafish orthologs in a zebrafish database (http://www.sanger.ac.uk/Projects/D_rerio/). Previously, we reported that 308 zebrafish orthologs displayed tissue- and/or developmental-specific expression [17]. In this early study, by a reverse screening process involving genetic knockdown (KD), a conserved gene, designated as Yulink, was identified, cloned and functionally characterized.

Here, we demonstrated that yulink promoted cardiac dysfunction in zebrafish hearts, and genetic knockdown resulted in pericardial edema, decreased beating rate and cardiac output. Knockdown of Yulink in mouse and human iPSC-derived cardiomyocytes disrupted Ca\textsuperscript{2+} cycling, reduced the DNA binding activity of PPAR\textgamma, and resulted in a reduction of Serca2 expression. Expression of Serca2 was up-regulated by PPAR\textgamma agonists and down-regulated by PPAR\gamma-shRNA knockdown, suggesting that Yulink regulates SERCA2 expression through PPAR\gamma in mouse HL-1 cardiomyocytes. Therefore, enhancement of nuclear PPAR\gamma activity may provide a mechanistic explanation for the involvement of Yulink in the regulation of Serca2 expression. Finally, knockdown of YULINK in human iPSC-derived cardiomyocytes also disrupted Ca\textsuperscript{2+} cycling via decreased SERCA2 expression. This Yulink was also found in fly as mio (Gene ID: 33399) and required for the maintenance of the meiotic cycle and oocyte identity [12]. Later, it was found as a subunit of GATOR2 complex proteins in HEK-293T cells and inhibition of GATOR2 suppressed mTORC1 signaling and GATOR2 negatively regulated GATOR1 [2]. Therefore, the Yulink is an evolutionarily conserved gene with diverse functions.

Materials and methods
Bioinformatics analysis for Yulink
Protein domains on Yulink were analyzed by SMART server (http://smart.embl-heidelberg.de) using the amino acid sequences. Alignment of protein sequences and phylogenetic tree were performed using the CLUSTALW combined with ETE3 tools on GenomeNet server (https://www.genome.jp) with default parameters.

Animals
Breeding and maintenance of TL strain zebrafish, as well as collection and staging of embryos, were performed in accordance with standard procedures [35] and approved by the Academia Sinica Institutional Animal Care and Utilization Committee. Certain embryos were reared in zebrafish egg water [35], and treated with 0.003% 1-phenyl-2-thiourea to inhibit pigmentation. Developmental times refer to hours (hpf) or days (dpf) post-fertilization.

In situ hybridization
Embryos were fixed in 4% paraformaldehyde buffered with 1 × phosphate-buffered saline at 4 °C overnight, and proceeded to hybridized with DIG-labeled RNA antisense or control sense probes of yulink, embryos were incubated with anti-Dig antibody conjugated to alkaline phosphatase, and developed with NBT-BCIP reagents (Roche, Germany).

Morpholino (MO) knockdown
Zebrafish embryos were obtained by natural mating and microinjected with morpholino (MO) before 4-cell stage. Three different MO antisense oligonucleotides, MO (5'-GGAGACGGTGTTGTTACGTGC-3'), MO-splicing site (5'-AGTGCTTGAGAACATCGTATT-3') and MO-start site (5'-CTGGCTTATAGCCGCCTCGACATGCC-3') were designed, that targeted specifically against the 5' untranslated region (UTR), the splicing site and the start site of the yulink gene, respectively.

In addition, the sequence of the 5 bp mismatch negative control MO (yulink-5mmMO) was as follows: 5'-GGCTGcCAGACTcGCTTcTTAcGTGC-3'. For the experiment, embryos injected with yulink-5mmMO were considered as negative control. Embryos positioned in an agarose injection chamber were injected with MO at 4.6 nl using a Narishige micromanipulator and needle holder (Narishige, Japan). The phenotype was observed using a dissecting microscope (MZ-FLIII, Leica Microsystems, Germany). Images were captured with a digital camera (SPOT, DIAGNOSTIC Instruments, USA). For hemo-dynamic assay, images of yulink KD morphant and WT hearts were dynamically monitored and captured using a dissecting microscope and digital camera at 2 dpf. Heart rate, long and short axis length of ventricle were measured from dynamic heart images. Cardiac output value was enumerated with the following formula: heart rate × (largest ventricle volume — smallest ventricle volume).

qPCR for zebrafish
Total RNA was extracted from adult zebrafish tissues using Tri-reagent (Sigma, St. Louis, MO, USA). Reverse transcription was performed using the Superscript pre-amplification system (Gibco BRL, USA) as described in the manufacturer’s instructions. The cDNA products were amplified by PCR with specific primer sets for yulink or β-actin. The forward primer of zebrafish yulink was 5'-GGAGACGGTGTTGCTGGAGG-3' and the reversed primer, 5'-TGACTGAACCGCCTCCGTG-3'. The β-actin forward primer was 5'-TCACACCTCTCTA
CAACGAGCTGC-3′ and the reversed primer, 5′-GAA GCTGTAGCCTCTCTCGGTCAG-3′.

Specificity of yulink gene MO
For in vivo experiments, the yulink 5′-UTR (46 bp in length) and its partial coding region were amplified by PCR with specific primers (5′-TCTCGAGCTCAAGCT GTTTCAGCTCAAATCTGTA-3′ and 5′-GCAGAA TTCGAAGCTCAAACACTTGCTAGTTT-3′), and the amplicon was ligated into a HindIII-digested pEYFP-N1 plasmid using an In-Fusion HD Cloning Kit (Clontech, USA) to generate pYulink-EYFP. Embryos were injected with pYulink-EYFP plasmid (100 ng/embryo) alone or co-injected with pYulink-EYFP plasmid (100 pg/embryo) and the amplicon was ligated into a HindIII-digested pEYFP-N1 plasmid using an In-Fusion HD Cloning Kit (Clontech, USA) to generate pYulink-EYFP. Embryos were injected with pYulink-EYFP plasmid (100 pg/embryo) alone or co-injected with pYulink-EYFP plasmid (100 pg/embryo) and either yulink-MO or yulink-5mmMO (4.6 ng/embryo). The number of embryos expressing YFP was determined at 1 dpf.

Yulink and PPARγ KD in mouse HL-1 cardiomyocytes
A pGIPZ lentiviral shRNA vector expressing a short hairpin RNA targeting Yulink (V2LMM_11104, mouse Yulink-shRNA) and a non-targeting control shRNA vector (Ctrl vector, RHS4346) were purchased from Open Biosystems (Huntsville, AL, USA). The TRCN0000001657 (PPARγ-shRNA) clone was obtained from the National RNAi Core Facility at the Institute of Molecular Biology (Academia Sinica, Taipei, Taiwan). HL-1 (mouse cardiac muscle) cells were obtained from Dr. W. Claycomb (Louisiana State University Medical Center, New Orleans, LA, USA), and were grown as previously described [32]. Stable KD cell lines were generated by lentivirus applied to HL-1 cells with 8 μg/ml of polybrene (Sigma). RNA was isolated from cells treated with Yulink-shRNA, PPARγ-shRNA or Ctrl vector using the Quick-RNA MiniPrep Kit (Zymo Research, USA), according to the manufacturer’s instructions. The isolated RNA was reverse transcribed with the ReverTra Ace qPCR RT Kit (Toyobo, Japan).

The mRNA expression levels of Yulink, PPARγ, Serca2a and GAPDH were measured by qRT-PCR using a Roche Lightcycler 480 (Roche, Germany). The Serca2a primers were designed with unique sequence at 3′-UTR and not expressed in Serca2b. The final qRT-PCR volume in each well was 20 μl and contained 10 μl of 2× THUNDERBIRD SYBR qPCR Mix (Toyobo, Japan), 10 ng of cDNA, and 50 nM of gene specific primer pairs. The primers used were as follows: Yulink-F: CAGAGTGGGACATCGCTTGTA; Yulink-R: TCAATTCCTGACGCTTCTTCT; PPARγ-F: GAAAAAGCAACGGACACATTACC; PPARγ-R: GGTTTTGAATGGTTGGGCTTG; Serca2a-F: CCTCCAGTCTAATCTCAGGTTC; Serca2a-R: CTGTCTACTGTTGTGATCCTAAA; GAPDH-F: GGTCCTCAGTGTAGCCCAAG; and GAPDH-R: AATTGTTCGGTGCGATCTC. Expression of mRNA was normalized to GAPDH in the same sample.

Western blot
Lysates of HL-1 cells treated with Yulink-shRNA, PPARγ-shRNA or Ctrl vector were isolated using RIPA reagent or NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, USA). Protein content was quantified with the BioRad DC Protein Assay (BioRad Laboratories, USA). For Western blot, protein samples were separated by 4–12% SDS-PAGE and transferred to a PVDF membrane and probed with an appropriate primary antibody at 4 °C overnight. Antibodies were acquired from the following companies: α-Yulink (1G3) from Abnova (Taiwan), α-GAPDH (GTX100118), α-PPARα (GTX28934), α-PPARδ (GTX113250) and α-Histone H1 (GTX114462) from GeneTex (USA), α-SERCA2 (sc-8095) from Santa Cruz Biotechnology (USA), and α-PPARγ (ab27649) from Abcam (UK). Blots were then incubated with HRP-conjugated secondary antibodies (1:5000; GeneTex) for 1 h at room temperature, and proteins were detected using an ECL kit (Millipore, USA). Expression of protein was normalized to GAPDH or Histone H1 in the same sample.

PPARγ DNA binding assays and treatment with PPAR agonists
PPARγ DNA binding activity was measured using a PPARγ transcription factor assay kit (Cayman Chemical, USA). Briefly, 10 μg of extracted nuclear proteins were added to wells containing immobilized dsDNA sequences corresponding to the peroxisome proliferator response element. Bound PPARγ was detected by the addition of specific antibodies against PPARγ. Relative PPARγ DNA binding activity was determined by normalizing the measurements obtained for cells transfected with Yulink-shRNA to those obtained for cells treated with Ctrl vector. Rosiglitazone (Santa Cruz), pioglitazone (Sigma), GW7647 (Sigma), and GW0742 (Sigma) were stored in the dark at −20 °C. Working solutions were prepared by diluting the stock solution in media. Stable cells transfected with Yulink-shRNA or Ctrl vector were treated with or without agonist for 6 h, 12 h, or 2 days. RNA was subsequently isolated from cells using Quick-RNA MiniPrep (Zymo Research, USA), according to the manufacturer’s instructions.

15-Deoxy-Δ12,14-prostaglandin J2 uptake assay
HL-1 cardiomyocytes were incubated in medium containing 1 μM 15-deoxy-Δ12,14-prostaglandin J2-biotin (15d-PGJ2-Biotin) as a PPARγ ligand for 3 h, followed by PBS washing and trypsinization. After fixation with 4%
PFA and staining with streptavidin-Alexa Fluor 647, the signals were analyzed by flow cytometry and immunofluorescence microscope. Approximately 10,000 cells were included in each sample for flow cytometry. The nuclei were stained with Hoechst 33342 in the immunofluorescence staining.

**Differentiation of human iPSC to cardiomyocytes**

Human iPSCs were split at 1:12 ratio using 0.5 mM EDTA in PBS and grown for four days, when cells reached 85% confluency. Medium was changed to cardiomyocyte differentiation medium CDM3, which consisted of RPMI1640 basal medium, 500 μg/ml human albumin, and 213 μg/ml l-ascorbic acid-2 phosphate [4]. Then medium was changed every other day. For day 0–2, medium was supplemented with 6 μM GSK-3 inhibitor CHIR99021. Afterword, medium was changed to CDM3 containing 2 μM Wnt signaling inhibitor Wnt-C59 on day 2–4 [4]. Starting from day 7, contracting cardiomyocytes were observed.

**YULINK KD in human iPSCs derived cardiomyocytes**

A pGIPZ lentiviral shRNAmir vector expressing a short hairpin RNA targeting YULLINK (V3LHS_374795, human YULLINK-shRNA) and a non-targeting control shRNA vector (Ctrl vector, RHS4346) were purchased from Open Biosystems (Huntsville, AL). YULLINK KD cells were generated by lentivirus applied to human iPSCs derived cardiomyocytes with 8 μg/ml of polybrene (Sigma, Germany).

**Ca²⁺ imaging**

The control and Yulink KD cardiomyocytes were seeded in Matrigel-coated 8-well Lab Tek II chambers (Nalge Nuc international, Rochester, NY, USA). Cells were recovered after two days and loaded with 5 μM Rhod-2 AM (Invitrogen) in Tyrode’s solution for 15 min at 37 °C as described by the manufacturer’s protocol. Ca²⁺ imaging was conducted using a Leica SP8 confocal microscope (Wetzlar, Hesse, Germany). Spontaneous Ca²⁺ transients of single beating cardiomyocyte were obtained using a time-lapse line scanning recording mode (512 pixels × 1920 lines) under 40 × objective at room temperature, and the raw data was analyzed using Leica LAS X program. Ca²⁺ signal was normalized to the intracellular basal line (F₀), and the transient amplitude was expressed as F/F₀. In addition, the τ (Tau) is the exponential decay time constant for the speed of calcium uptake, which is commonly used as one method for characterizing the speed of Ca²⁺ recovery. A large τ value indicates a longer recovery time to baseline; the units of τ are in time (seconds). The time constant, Tau, represents the elapsed time required for the calcium amount to decay to 1/e = 36.8% of the original value.

**Over-expression of YULLINK, PPARy and SERCA2 in mouse HL-1 cardiomyocytes**

To generate the over-expression of YULLINK, PPARy or SERCA2 plasmids, the PCR products of their full-length coding regions were generated using pEF1-Flag/His-YULLINK vector or cDNA from human iPSC-derived cardiomyocytes. The specific primer sets were 5’-tagactcagcagatgcgcttatttctgtcctgcagga-3’ and 5’-tcgctgctgcagatgcgcttatttctgtcctgcagga-3’ for YULLINK; 5’-tagactcagcagatgcgcttatttctgtcctgcagga-3’ and 5’-tgctgctgcagatgcgcttatttctgtcctgcagga-3’ for PPARy; 5’-tagactcagcagatgcgcttatttctgtcctgcagga-3’ and 5’-tgctgctgcagatgcgcttatttctgtcctgcagga-3’ for SERCA2. Then these PCR products were inserted into the lentiviral pCDH plasmid containing BFP (System Biosciences, linearized by EcoRI and BamHI double digestion) using an In-Fusion HD Cloning Kit (Clontech). All constructs were checked using Sanger sequencing (Genomics BioSci & Tech). Over-expression of YULLINK and PPARy plasmids were transfected into Yulink KD cells using lipofectamine 3000 transfection reagent (Thermo Fisher Scientific). Over-expression of SERCA2 plasmid was electroporated into Yulink KD cells using Neon electroporation system (ThermoFisher).

**Statistical analysis**

Some data were shown as the mean±SD whereas other values were shown as mean±standard error. A p value or post-hoc p value <0.05 was considered statistically significant.

**Results**

**Structure of Yulink**

Previously, we performed comparative evolutionary genomics analysis to study the functions of 13,711 novel Ka/Ks-predicted human exons using zebrafish (Danio rerio) as a model organism [17, 20–22]. Through a reverse screening process involving genetic knockdown (KD), a novel gene, designated as Yulink (MIOS; Entrez Gene: 54468), which encodes an 875 amino acid protein with WD40 repeats at N-terminus in human and mouse (Fig. 1a). The similarities of protein sequences among Yulink genes of common experimental animals were analyzed by multiple sequence alignment using CLUSTALW (https://www.genome.jp). The classification of these homologs of Yulink was shown with a phylogenetic tree (Fig. 1b). When compared with the protein sequence of human YULINK, the phylogenetic tree indicated that YULINK proteins of rhesus and crab-eating macaque were the most similar homologs,
with 99% of identities in amino acid sequences (Fig. 1b). The most dissimilar homolog was yulink from zebrafish, which exhibited 82% identities (Fig. 1b). The identities for homologs of Yulink from mouse, rat, and rabbit were all approximately 98% (Fig. 1b). These analyses indicated that the Yulink gene is highly conserved in diverse species of animal, implying that the Yulink may have potentially conserved functions from zebrafish to human.

The 3D structure of YULINK protein (Gene ID: 54468) is currently unavailable. A WD40 repeat is traditionally defined as a structural motif with about 40 amino acids composed of four β-strands which is often terminated with Trp-Asp (WD) sequence [37]. In addition,
seven WD40 repeats can further be assembled to form a WD40 domain [37]. Such structural features were conventionally analyzed with computer servers. Currently, to explore these structural features, the following four database servers are available for annotation: (i) Conserved Domain Database (CDD, https://www.ncbi.nlm.nih.gov/cdd), (ii) UniProt (https://www.uniprot.org), (iii) SMART (http://smart.embl-heidelberg.de), and (iv) InterPro (https://www.ebi.ac.uk/interpro) servers.

It was found that with these four computer servers, the region of AA 101 to 297 of YULINK displayed clearly four conserved WD40 repeats (i.e. WD40 #1–#4 in Fig. 1c, yellow). However, two additional candidates for WD40 repeats were also revealed by the Conserved Domain Database (WD40 A and B in Fig. 1c, blue). Furthermore, the UniProt server had annotated one additional WD40 repeat by (WD40 C in Fig. 1c, blue). These analyses thus suggest that YULINK may contain four conventional WD40 repeats and three additional potential candidates.

For further validation, we examined the secondary structure of YULINK in details. Since WD40 repeat is known to possess secondary structure as four β-strands, the distribution of β-strands in YULINK was analyzed with the Jpred4 (http://www.cmpbio.dundee.ac.uk/jpred) (Additional file 1: Fig. S1). As shown, the first half of YULINK contains seven WD40 repeats (#1–#4 and A–C) as predicted by four computer servers to possess the prerequisite features. The distribution of secondary structure as analyzed by Jpred4 server further confirmed that each predicted WD40 repeat displayed three to four tandem β-strands (Additional file 1: Fig. S1); these structural features are reminiscent of the characteristics for typical WD40 repeats. In conclusions, YULINK has four conserved WD40 repeats and three additional potential WD40 repeat candidates.

Specificity of yulink-MO knockdown (KD) in zebrafish morphants

The yulink was expressed in whole zebrafish embryo ubiquitously from 0.5 hpf (zygote stage) to 3 dpf (larval stage) and expressed in heart region starting at 24–30 hpf (Fig. 2a). To investigate the biological function of the yulink gene, zebrafish embryos before the 4-cell stage were injected with the antisense oligonucleotide yulink-MO, which targeted against to 5’-UTR, to knockdown gene function. Yulink KD morphants showed small eyes, a small head, and marked pericardial edema, as compared to the wild type at 3 dpf (Fig. 2b). Increasing the yulink-MO amount from 2.3 to 9.2 ng caused the proportion of the severely affected embryos with pericardial edema from 6 to 54% (Fig. 2b). We had also constructed two other MOs, which targeted against splicing site and translational start site of the yulink gene, respectively, the results are shown in Additional file 1: Fig. S2. The abnormal phenotypes (e.g. small eyes, a small head, marked pericardial edema, etc.) after treatment of these MOs were similar to those in the morphants originally observed in Fig. 2b, at wide range dosage of MOs (1–10 ng/embryo).

In addition, severely affected embryos which showed marked pericardial edema exhibited defects in blood circulation. The yulink KD morphants exhibited slower heart rates (126 beats/min), as compared to mismatch control 5mmMO (187 beats/min, Fig. 2c, Additional file 1: Video). Besides, the yulink KD morphants also exhibited reduced cardiac output averaged 11.7 μl/min, as compared to the 5mmMO averaged 42.7 μl/min (Fig. 2c). Loss of function for serca2a [7] or several ion channel related genes (e.g. the “hyperpolarization activated cyclic nucleotide gated potassium channel 4”, hcn4 [14, 29], and T-type calcium channels, a1G, a1Ha, a1Hb, a1la and a1lb [27]) were reported to reduce the heart rate. Therefore, specific qPCR primers were designed for these genes (Additional file 1: Fig. S3A). The expression levels of serca2a and the ion-channel related genes were found to decrease significantly in yulink KD morphants (orange color) after normalization with the internal control (bactin1) at 3 dpf (Additional file 1: Fig. S3b, c), thus consistent with the observed slower heart rates for the yulink KD morphants.

To further confirm the above findings were not caused by off-target effect, the 5’-UTR of yulink and its partial coding sequences were introduced into the pEYFP-N1 plasmid to generate the pYulink-EYFP plasmid (Fig. 2d). Approximately 58% of the embryos injected with this plasmid (100 pg/embryo) exhibited EYFP fluorescence in vivo (Fig. 2d). In a parallel co-injection with yulink-MO totally suppressed EYFP expression; in contrast, the mismatch control, yulink-5mmMO did not affect EYFP expression (Fig. 2d). To further assess the specificity of the yulink-MO, the ability of synthesized mouse Yulink mRNA to protect against the yulink-MO-induced changes in phenotype was determined, using the β-gal mRNA as an experimental control (Additional file 1: Fig. S4). After injection of yulink-MO into zebrafish embryos, the proportions of morphants with severe (gray color) and moderate (orange color) phenotype changes were, respectively, 65.8% and 18.6%. But, co-injection of the control β-gal mRNA and yulink-MO did not change the proportions of morphants which displayed severe and moderate phenotypes (62.2% and 15.6%); these values were similar to those observed with the injection of yulink-MO alone. However, co-injection with Yulink mRNA reduced the proportions of morphants with severe phenotype significantly to 38.7%. Importantly, the
morphants with moderate or normal phenotypes were found to increase to 30.65% and 30.65%, respectively (Additional file 1: Fig. S4). These results thus implied that morphants caused by injection of *yulink*-MO could be competed (rescued) to become the morphants with less severe (moderate) and WT phenotype with the coinjection of *Yulink* mRNA. It was thus concluded that *Yulink* is important for the heart development and cardiac function in zebrafish; but detailed characterization ... await for future electrophysiological studies.

**Yulink knockdown induces irregular Ca\(^{2+}\) cycling in mouse HL-1 cardiomyocytes**

The *yulink* KD morphants in zebrafish reduced the heart rates and cardiac output suggest that the Yulink may play an important role in cardiomyocytes. To investigate this possibility, mouse HL-1 cardiomyocytes were transduced with lentivirus expressing short hairpin RNA (shRNA) against *Yulink*. Quantitative PCR and western blotting analysis of the transduced cells confirmed a *Yulink* knockdown with efficiency of ~50% (Fig. 3a).

Calcium (Ca\(^{2+}\)) plays a critical role in regulation of excitation–contraction coupling and is essential in the electrical signaling of cardiomyocytes. Abnormal Ca\(^{2+}\) cycling is linked to arrhythmogenesis, which is associated with cardiac disorders and heart failure [10]. Accordingly, we measure Ca\(^{2+}\) cycling from control and *Yulink* KD HL-1 cells using confocal fluorescence microscopy loaded with fluorescent Ca\(^{2+}\) dye Rhod-2 AM. Compared to control, *Yulink* KD demonstrated significant higher...
Ca\textsuperscript{2+} transient irregularities which may relate to triggered arrhythmia-like waveforms (Fig. 3b, red arrows); irregular Ca\textsuperscript{2+} transients were virtually absent in control cells (28.6% for Yulink KD vs. 4.6% for control, Fig. 3c). Moreover, we also observed that the Yulink KD HL-1 cells exhibited defective intracellular Ca\textsuperscript{2+} cycling with a significantly observed reduced amplitude of Ca\textsuperscript{2+} transients (F/F\textsubscript{0} = 1.87 ± 0.31) than in control cells (F/F\textsubscript{0} = 2.89 ± 0.34) (Fig. 3d). In particular, the Yulink KD exhibited defective intracellular Ca\textsuperscript{2+} cycling with a significantly slower Ca\textsuperscript{2+} decay rate (615 ± 51 ms) than control (421 ± 34 ms) (Fig. 3e). These data suggest that knockdown of Yulink contribute to abnormal
intracellular Ca\textsuperscript{2+} release and arrhythmogenic phenotype in mouse HL-1 cardiomyocytes.

**Over-expression of YULINK rescued the phenotypes of Yulink KD HL-1 cells**

In order to study whether the over-expression (OE) of YULINK rescued the phenotypes of Yulink KD HL-1 cells, control (Ctrl) or YULINK plasmid (carrying BFP as indicator of expression marker) were transfected into Yulink KD cells, and then Ca\textsuperscript{2+} cycling were analyzed using the fluorescent Rhod-2 AM dye. In Fig. 4a, the intracellular Ca\textsuperscript{2+} cycling waveforms became normal, when these KD cells were over-expressed with YULINK, as compared to Yulink KD cells. In addition, the results of Ca\textsuperscript{2+} sparks analysis were consistent with the increase of Ca\textsuperscript{2+} transient amplitudes (3.1 ± 0.5 for YULINK-OE, orange bar, vs. 1.9 ± 0.35 for Ctrl, black bar) (Fig. 4a). These cells with YULINK-OE also exhibited a decrease of the percentages of irregular Ca\textsuperscript{2+} transients (8 ± 2% for YULINK-OE, orange bar, vs. 27 ± 3% for Ctrl, black bar) and the reduction of the Ca\textsuperscript{2+} decay rate (430 ± 42 ms for YULINK-OE, orange bar, vs. 605 ± 47 ms for Ctrl, black bar) (Fig. 4a). The level of Ca\textsuperscript{2+} transient amplitudes, the percentages of irregular Ca\textsuperscript{2+} transients, and the Ca\textsuperscript{2+} decay (Tau) rate were all similar between Ctrl (Fig. 3b) and over-expression of Yulink cells. These data indicate that the Yulink-shRNA used in our studies was specific and the observed defects in cells were indeed Yulink-dependent.

**Knockdown of Yulink reduces Serca2 expression in mouse HL-1 cardiomyocytes**

Several studies have suggested that expression and function of Serca2 play a major role in defective intracellular Ca\textsuperscript{2+} cycling [11, 26, 32]. The decreased Serca2 expression may also contribute to mechanical failure in cardiomyocytes [24]. Therefore, it is hypothesized that Yulink may play a role in regulating Serca2 expression. Serca2a, one of the two isoforms for Serca2, was expressed in cardiac muscle, slow-twitch skeletal muscle, and smooth muscle cells, while Serca2b is an ubiquitous isoform expressed in muscle and non-muscle cells [25]. It was also reported that approximately 95% of the Serca2 protein in mouse HL-1 cardiomyocytes are Serca2a [34]. To examine the Serca2a expression in the Yulink KD mouse HL-1 cardiomyocytes, we first performed quantitative PCR using specific Serca2a primers, designed based on a unique sequence at 3′-UTR region which were not expressed by Serca2b. As shown in Fig. 3a, there was significant reduction of Serca2a expression after Yulink KD by qPCR. Similarly, on Western blot analysis, there was also reduction of SERCA2 protein expression (Fig. 3a). Therefore, these results demonstrate that the expression of Yulink is required for Serca2 expression. These data imply that Yulink may be involved in the expression of Serca2 as well as the intracellular Ca\textsuperscript{2+} cycling in mouse HL-1 cardiomyocytes.

**Knockdown of Yulink blocks the entry of PPARγ ligands into cells and decreases PPARγ DNA binding activity in the nuclei**

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors of the nuclear receptor superfamily [13]; one member of this family, PPARγ has been reported to directly bind to a PPARγ response element within the Serca2 gene proximal promoter in pancreatic islet cells [16]. We next evaluate the PPARγ DNA-binding activities in the Yulink KD cardiomyocytes using the immobilized dsDNA corresponding to the peroxisome proliferator response element. As showed in Fig. 5a, the PPARγ DNA-binding amount of Yulink KD HL-1 was significantly decreased to 47%
compared to control cells. Consistent with these findings, we also found that the nuclear PPARγ was decreased in Yulink KD cardiomyocytes by immunofluorescence staining (Fig. 5b). Western blot analysis also showed that the nuclear PPARγ protein level was decreased by 85% in Yulink KD cardiomyocytes, respectively (Fig. 5c). In this assay, the decrease of PPARγ in lysate of Yulink KD cell nuclei also reflected a lower DNA binding activity. The
Yulink KD resulted in diminished PPARγ DNA-binding and nuclear PPARγ protein level in HL-1 cardiomyocytes suggest that Yulink may involve in the nuclear import of PPARγ.

The ligands for PPARγ are known to include fatty acids, arachidonic acid metabolites, and thiazolidinediones, such as rosiglitazone and pioglitazone [28, 31]. In addition, 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) has been reported as an endogenous ligand for PPARγ activation [9, 15]. Therefore, we used the biotinylated 15d-PGJ2 to characterize the ligand uptake in Yulink KD cardiomyocytes by flow cytometry and immunofluorescence (Fig. 5d). The results showed that 89% of the fluorescent 15d-PGJ2-biotin was taken up by control vector-treated cardiomyocytes, but only 2% was found in Yulink KD cardiomyocytes (Fig. 5d). In the immunofluorescence assay, in which 15d-PGJ2-biotin was stained with streptavidin-Alexa Fluor647 (red), and nuclei were stained with Hoechst 33342 (blue), red signals were observed in the cytoplasm and nuclei of control cardiomyocytes, while no red signals were detected in Yulink KD cardiomyocytes (Fig. 5d). These findings indicated that Yulink KD block the ligands of PPARγ into cells and result in a decreased nuclear import of PPARγ.

PPARγ regulates Serca2 expression in mouse HL-1 cardiomyocytes

To examine whether the Yulink regulate Serca2 expression through PPARγ pathway, Yulink KD cardiomyocytes were treated with 50 μM rosiglitazone (a PPARγ agonist) for 6 h. As showed in Fig. 6a, the relative Serca2 mRNA expressions were 1.09 and 2.57 in the presence of 10 and 50 μM rosiglitazone, respectively, compared to the control in the absence of the rosiglitazone. In addition, the SERCA2 protein level could be enhanced to 1.5 folds in control vector-treated cardiomyocytes by 50 μM rosiglitazone treatment for 6 h. Furthermore, in Yulink KD cardiomyocytes, the decreased expression of Serca2 can be
rescued from 0.2 to 0.5 folds (Fig. 6a). In parallel, treatment with another PPARγ agonist pioglitazone (50 μM) for 12 h almost completely rescued Serca2 mRNA expression (Fig. 6b). PPARγ agonists enhanced the SERCA2 expression in the control- and Yulink KD cardiomyocytes, suggesting that the down regulation of Yulink resulted in a decreased SERCA2 expression may through PPARγ pathway.

To confirm the transcription factor PPARγ involved in the Serca2 expression in cardiomyocytes, HL-1 cells were transduced with lentivirus expressing shRNA against PPARγ. Quantitative PCR and western blotting analysis

Fig. 6 Yulink regulated Serca2 expression through the PPARγ pathway. a Treatment with 50 μM rosiglitazone for 6 h rescued and enhanced Serca2 expression at mRNA and protein levels in Yulink KD cardiomyocytes by qPCR (left panel) and Western blot (right panel). Serca2 mRNA levels normalized to values in cells treated with Ctrl vector. (n = 3, **p < 0.01, Student’s t test). Protein levels normalized to internal control, GAPDH. b Treatment with 50 mM pioglitazone (PPARγ agonist) for 12 h increased Serca2 mRNA expression in Yulink KD cardiomyocytes, as shown by qPCR. Data for cardiomyocytes treated with Ctrl vector and Yulink KD cardiomyocytes are shown in blue and red, respectively. Before pioglitazone treatment, Serca2 expression was decreased in Yulink KD cardiomyocytes (n = 3, **p < 0.01, Student’s t test). Pioglitazone increased relative Serca2 expression in Yulink KD cardiomyocytes from 0.25 to 0.82 (n = 3, **p < 0.01, Student’s t test). In Ctrl vector-treated cardiomyocytes, the expressions of Serca2 before and after pioglitazone were 1 and 0.81, respectively (n = 3). c Gene expression and protein levels in PPARγ-shRNA KD cardiomyocytes and cells treated with Ctrl Vector were quantified by qRT-PCR and Western blot, respectively. The mRNA expressions of PPARγ and Serca2 were significantly lower in PPARγ-shRNA KD cardiomyocytes (0.36 and 0.51, respectively) compared to values of 1 in cells treated with Ctrl vector (n = 3, **p < 0.01, Student’s t test). Decreased total cellular PPARγ and SERCA2 protein levels were also observed in PPARγ-shRNA KD cardiomyocytes; values normalized to GAPDH. d KD of Yulink resulted in obvious decreases in nuclear levels of PPARα and PPARβ/δ (left panel, n = 3, **p < 0.01, Student’s t test), but treatment with the PPARα agonist GW7647 (middle panel) or PPAR β/δ agonist GW0742 (right panel) did not statistically increase Serca2 expression in Control vector-treated or Yulink KD cardiomyocytes (n = 3).
of the transduced cells confirmed a PPARγ knockdown efficiency of ~70% (Fig. 6c). The relative mRNA and protein levels of Serca2 were also significantly reduced in these PPARγ KD cells (Fig. 6c). These results confirmed that the regulation of the Serca2 expression by Yulink is mediated via PPARγ in HL-1 cardiomyocytes.

We also observed decreased levels of PPARα and PPARβ/δ in Yulink KD cardiomyocytes (Fig. 6d). Treatment with PPARα and PPARβ/δ agonists, GW7647 and GW0742, however, did not increase Serca2 expression in control or Yulink KD cardiomyocytes (Fig. 6d), suggesting that PPARα or PPARβ/δ does not involved in Serca2 expression. Therefore, these results highlight the involvement of Yulink with PPARγ in regulating Serca2 expression in HL-1 cardiomyocytes.

PPARγ has been shown to bind directly to the PPAR response element in the promoter of the SERCA2 gene of pancreatic islet cells [16]. In this study, KD of PPARγ resulted in significant decreases in the expression of Serca2 mRNA and protein in both normal and Yulink KD cardiomyocytes. Down-regulation of Yulink resulted in a significant reduction in PPARγ DNA binding activity and protein level in the nuclei, demonstrating the role of PPARγ in Yulink-mediated transcriptional regulation of Serca2. Additionally, PPARγ agonists were found to enhance the expression of Serca2 in both normal and Yulink KD cardiomyocytes. But, treatment with PPARα or PPARβ/δ agonists did not protect against the Yulink KD-induced reduction of SERCA2, suggesting the specificity and dependence on PPARγ.

**PPARγ over-expression rescued the phenotypes of Yulink KD cells**

In order to study whether the PPARγ over-expression (OE) rescued the phenotypes of Yulink KD cells, control (Ctrl) or PPARγ plasmid with BFP as expression marker were transfected into Yulink KD cells, and we then analyzed Ca2+ cycling. As compared to Yulink KD control cells, the intracellular Ca2+ cycling waveforms became normal, when these KD cells were over-expressed with PPARγ (Fig. 4a). In addition, the results of Ca2+ sparks analysis were consistent with the increase of Ca2+ transient amplitudes (3.2 ± 0.3 for PPARγ-OE, blue bar, vs. 1.9 ± 0.35 for Ctrl, black bar) (Fig. 4a). The cells with PPARγ-OE also exhibited a decrease of the percentages of irregular Ca2+ transients (10 ± 2% for PPARγ-OE, blue bar, vs. 27 ± 3% for Ctrl, black bar) and the reduction of the Ca2+ decay rate (450 ± 51 ms for PPARγ-OE, blue bar, vs. 605 ± 47 ms for Ctrl, black bar) (Fig. 4a). The level of Ca2+ transient amplitudes, the percentages of irregular Ca2+ transients, and the Ca2+ decay (Tau) rate were all similar between Ctrl (Fig. 3b) and over-expression of PPARγ cells. These data indicate that the PPARγ-OE rescued the phenotypes of Yulink KD cells, suggesting involvement of Yulink with PPARγ in regulating Serca2 expression in HL-1 cardiomyocytes.

**SERCA2 over-expression rescued the phenotypes of Yulink KD cells**

In order to examine whether the SERCA2 over-expression (SERCA2-OE) rescued the phenotypes of Yulink KD cells, control vector (Ctrl) or SERCA2 plasmids were electroporated into Yulink KD cells with Neon electroporation system. As compared to Yulink KD control cells, the intracellular Ca2+ cycling waveforms became normal, when these KD cells were over-expressed with SERCA2 (Fig. 4b). In addition, the results of Ca2+ sparks analysis were consistent with the increase of Ca2+ transient amplitudes (3.2 ± 0.45 for SERCA2-OE, gray bar, vs. 2.02 ± 0.3 for Ctrl, black bar) (Fig. 4b). The cells with SERCA2-OE also exhibited a decrease of the percentages of the irregular Ca2+ transients (11 ± 3% for SERCA2-OE, gray bar, vs. 32 ± 5% for Ctrl, black bar) and the reduction of the Ca2+ decay rate (412 ± 50 ms for SERCA2-OE, gray bar, vs. 635 ± 78 ms for Ctrl, black bar) (Fig. 4b). The level of Ca2+ transient amplitudes, the percentages of irregular Ca2+ transients, and the Ca2+ decay (Tau) rate were all similar between Ctrl (Fig. 3b) and over-expression of SERCA2 cells. These data indicate that the SERCA2-OE rescued the phenotypes of Yulink KD cells, suggesting a specific control for SERCA2 expression by Yulink in regulating calcium cycling in HL-1 cardiomyocytes.

**Yulink KD induces irregular Ca2+ cycling in human cardiomyocytes derived from iPSC**

To investigate whether the YULINK also involves in intracellular Ca2+ cycling in human cardiomyocytes, we analyzed Ca2+ cycling in human iPSC-derived cardiomyocytes using fluorescent Ca2+ dye Rhod-2 AM. Compared to control, the YULINK KD iPSC-derived human cardiomyocytes showed defective intracellular Ca2+ cycling with significant higher arrhythmia-like waveforms (38.4% vs. 12.5% for control) (red arrows in Fig. 7a, b), indicative of the Ca2+ transient irregularities. In addition, smaller Ca2+ transient amplitudes (1.87 ± 0.31 for YULINK KD vs. 2.89 ± 0.34 for control) (Fig. 7c) and slower Ca2+ decay rate (521 ± 30 ms for YULINK KD vs. 351 ± 21 ms for control) (Fig. 7d) were also found. These observations of the Ca2+ transients of human iPSC-derived cardiomyocytes are consistent with the results using mouse HL-1 cardiomyocytes, indicating that irregular Ca2+ cycling is a feature of cardiomyocytes for mouse and human, when Yulink was knocked down.

Furthermore, the contractile function of the control and YULINK KD cardiomyocytes was also examined. The cell contraction rate was counted under a phase-contrast
microscope, it was found that YULINK KD resulted in reduction of spontaneous beating rate, as shown with a reduced beating frequency by 28.3% (Fig. 7e).

To determine the SERAC2 expression in the YULINK KD human cardiomyocytes, western blotting analysis was performed. It was found that there were approximately 90% reduction of SERCA2 expression in the YULINK KD cardiomyocytes (Fig. 7f). These results imply that YULINK regulates SERCA2 expression and intracellular Ca\textsuperscript{2+} cycling in human cardiomyocytes.

**Discussions**

In this study, we have demonstrated that Yulink is highly conserved in zebrafish, mouse, and human. It was expressed in zebrafish embryo ubiquitously from zygote stage to larval stage and expressed in heart region starting from 1 dpf. The yulink KD morphants in zebrafish exhibited pericardial edema, slower heart rate, and reduced cardiac output. Besides, we also observed that down-regulation of Yulink in the mouse and iPSC-derived human cardiomyocytes exhibited defective intracellular Ca\textsuperscript{2+} cycling. Importantly it was observed that YULINK, PPARγ or SERCA2 over-expression specifically rescued the phenotypes of Yulink KD cells. These results thus suggest that Yulink is involved in intracellular Ca\textsuperscript{2+} cycling in cardiomyocytes.

Ca\textsuperscript{2+} cycling is crucial for excitation–contraction coupling of cardiomyocytes and is essential in the electrical signaling of cardiomyocytes. Abnormal Ca\textsuperscript{2+} cycling is linked to arrhythmogenesis, which is associated with cardiac disorders and heart failure [10]. KD of Yulink in mouse and human cardiomyocytes displayed a decrease in SERCA2 expression and exhibited higher Ca\textsuperscript{2+} transient irregularities which are accompanied with the triggered arrhythmia, defective intracellular Ca\textsuperscript{2+} cycling with a reduced Ca\textsuperscript{2+} transient amplitudes, and slower Ca\textsuperscript{2+} decay rate. In addition, YULINK, PPARγ or SERCA2 over-expression restored these phenotypes of mouse Yulink KD cells, indicating that the Yulink-shRNA used in our studies was specific and the observed defects in cells were Yulink-dependent.
When SERCA2 was deleted in mouse heart using the tamoxifen-inducible Cre, the Tau of Ca²⁺ transient was increased by ~90 to 118% in SERCA2-deficient cardiomyocytes [1, 19]. Additionally, it is known that the decreases in SERCA2 expression resulted in a diminished Ca²⁺ content in the sarcoplasmic reticulum, which reduced systolic Ca²⁺ release and impairment of myocardial contractility [10]. Therefore, decrease in Yulink function may play an important role in susceptibility to heart arrhythmia via impairment of cardiac SERCA2 activity.

Our studies indicated that knockdown of Yulink resulted in a decrease of nuclear PPARα and β/δ levels in HL-1 cardiomyocytes. Previously, it was reported that PPARs were major executors for modulating homeostasis of glucose and lipids in heart [3]. Overexpression of PPARα induced several target genes involved in fatty acid utilization and increased fatty acid uptake and oxidation in heart [8]. On the other hand, PPARγ null mice exhibited an increase in glucose transporter expression and glucose uptake [5, 23]. Mice with cardiac-specific deletion of PPARβ/δ were shown to exhibit severe impairments in myocardial fatty acid oxidation gene expression and increased cardiac lipid accumulation [6]. Therefore, the decreased nuclear PPARα and PPARβ/δ levels observed in Yulink KD cardiomyocytes would suggest that Yulink may have other important functions in the maintenance of glucose and lipid homeostasis via regulating PPARα or PPARβ/δ activities.

Some natural ligands can bind PPARγ, like unsaturated fatty acid, 15-Hydroxyeicosatetraenoic acid, 9- and 13-hydroxyoctadecadienoic acid or PGJ2 [18]. It is unknown which ligand is required for cardiomyocytes and entry into cells via Yulink. Transcriptional activity of PPARγ is regulated primarily by ligand binding [33] and 15d-PGJ2 is thought to be the most potent endogenous ligand for PPARγ [9, 36]. The 15d-PGJ2 was detected in exosomes; and exosomes were internalized and accumulated in an endosomal compartment [30]. Here, we used 15d-PGJ2-biotin as PPARγ ligand and KD of Yulink blocked the entry of ligand into cells. Therefore, Yulink KD resulted in a reduced nuclear import of PPARγ, a decreased SERCA2 expression, and abnormal Ca²⁺ cycling in the mouse HL-1 cardiomyocytes. Furthermore, it was reported that PPARγ bound a PPAR response element in the −259 bp proximal region of SERCA2 promoter in pancreatic cells, based on luciferase reporter assay, EMSA, and chromatin immunoprecipitation [16]. In addition, the SERCA2 promoter region of rabbit, rat, mouse and human all displayed the PPARγ binding site [16]. However, there was no direct evidence to demonstrate a binding of PPARγ in cardiomyocytes. We would wait for studies in the future to investigate in details how Yulink modulates uptake of ligand for PPARγ, thus regulating SERCA2 in cells. In addition to PPARγ signaling, other pathways might be impaired when Yulink was knocked down, indicating the need for future studies to validate detailed mechanism.

**Conclusions**

Deficiency of yulink caused cardiac dysfunction in zebrafish, manifested by pericardial edema, decreased beating rate and cardiac output. In addition, down regulation of Yulink in mouse and human iPSC-derived cardiomyocytes resulted in greater Ca²⁺ transient irregularities including defective intracellular Ca²⁺ cycling, reduced Ca²⁺ transient amplitudes, and slower Ca²⁺ decay rate, thereby triggering arrhythmia. Besides, YULINK, PPARγ or SERCA2 over-expression rescued these phenotypes of mouse Yulink KD cells. Mechanistically, deficiency of Yulink reduced expression of cardiac SERCA2 mediated by PPARγ nucleus entry.

Importantly, our results highlight the involvement of Yulink with PPARγ in regulating SERCA2 expression, which may shed light on many debates about the risks and benefits of PPARγ agonists in clinical use. Finally, this Yulink gene was first identified through comparative evolutionary genomics analysis and reverse screening involving genetic knockdown in zebrafish. The strategies of using the initial observations in zebrafish for the identification of biological functions in mouse HL-1 cardiomyocytes and human iPSC-derived cardiomyocytes provide new paradigm for the study of diseases mechanisms of other specific/novel genes.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12929-020-00701-7.

**Additional file 1:** Video.

**Additional file 2: Fig. S1.** Prediction of secondary structure for YULINK.

**Fig. S2.** Similar phenotypes of yulink knockdown were observed in embryos after microinjection with MO that targeted against the splicing site or start site. **Fig. S3.** The expressions of heart rate-related genes were decreased in yulink KD morphants. **Fig. S4.** The phenotypes of the morphants were rescued via over-expression of Yulink.

**Acknowledgements**

We would like to thank the excellent technical support from the Core Facility of the Institute of Cellular and Organismic Biology, Academia Sinica.

**Authors’ contributions**

MWK, HHT, SHW, ALY and JY conceived of the experiments, interpreted results and wrote the manuscript. MWK, HHT and YY C conducted the experiments, and performed the statistical analyses. All authors read and approved the final manuscript.

**Funding**

This work was supported by grants MOST 109-2321-B-182A-005 from the Ministry of Science and Technology of Taiwan, CMRPG3F0971 to CMRPG3F0973, and CMRPG3C0046 from Chang Gung Medical Foundation.
Availability of supporting data
All data generated or analyzed during this study are included in this article.

Ethical approval and consent to participate
Breeding and maintenance of TL strain zebrafish, as well as the collection and staging of embryos, were performed in accordance with standard procedures and approved by the Academia Sinica Institutional Animal Care and Utilization Committee.

Consent for publication
Not applicable.

Competing interests
The author declares that they have no competing interests.

Author details
1 Institute of Stem Cell and Translational Cancer Research, Chang Gung Memorial Hospital at Linkou, Taoyuan 333, Taiwan. 2 Department of Pediatrics, University of California, San Diego, CA, USA. 3 Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan.

Received: 2 July 2020   Accepted: 21 December 2020
Published online: 11 January 2021

References
1. Andersson KB, Birkeland JA, Finsen AV, Louch WE, Sjaastad I, Wang Y, Chen J, Molkenst JD, Chen KR, Sejjersted OM, Christensen G. Moderate heart dysfunction in mice with inducible cardiomyocyte-specific excision of the Serca2a gene. J Mol Cell Cardiol. 2009;47(2):180–7.
2. Bar-Peled L, Chantranupong L, Cherniack AD, Chen WW, Ottina KA, Grabiner BC, Spear ED, Carter SL, Meyerson M, Sabatini DM. A tumor suppressor complex with GAP activity for the Rap GTPases that signal amino acid sufficiency to mTORC1. Science. 2013;340(6136):1100–6.
3. Barger PM, Kelly DP. PPAR signaling in the control of cardiac energy metabolism. Trends Cardiovasc Med. 2000;10(6):238–45.
4. Burridge PW, Matsa E, Shukla P, Lin ZC, Churko JM, Ebert AD, Lan F, Diecke S, Huber B, Mordwinkin NM, Plews JR, Abelez OJ, Cui B, Gold JD, Wu JC. Chemically defined generation of human cardiomyocytes. Nat Methods. 2014;11(8):855–60.
5. Campbell FM, Kozak R, Wagner A, Altarejos JY, Dyck JR, Belke DD, Severson DT, Kelly DP, Lopaschuk GD. A role for peroxisome proliferator-activator receptor alpha (PPARalpha) in the control of cardiac malonyl-CoA levels: reduced fatty acid oxidation rates and increased glucose oxidation rates in the hearts of mice lacking PPARalpha are associated with higher concentrations of malonyl-CoA and reduced expression of malonyl-CoA decarboxylase. J Biol Chem. 2002;277(6):4098–103.
6. Cheng L, Ding G, Qin Q, Huang Y, Lewis W, He N, Evans RM, Schneider MD, Brako FA, Xiao Y, Chen YE, Yang Q. Cardiomyocyte-restricted peroxisome proliferator-activated receptor-delto deletion perturbs myocardial fatty acid oxidation and leads to cardiomyopathy. Nat Med. 2004;10(11):1245–50.
7. Ebert AM, Hume GL, Warren KS, Cook NP, Burns CG, Mohideen MA, Ottina KA. The hyperpolarization-activated channel HCN4 is required for the generation of pacemaker action potentials in the embryonic heart. Proc Natl Acad Sci USA. 2005;102(49):17075–100.
8. Finck BN, Lehman JJ, Leone TC, Welch MJ, Bennett MJ, Kovacs A, Han X, Gross RW, Kozak R, Lopaschuk GD, Kelly DP. The cardiac phenotype induced by PPARalpha overexpression mimics that caused by diabetes mellitus. J Clin Invest. 2002;109(11):121–30.
9. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 15-Deyoxy-delta-12, 14-prostaglandin J2 is a ligand for the adipocyte differentiation factor PPAR gamma. Cell. 1995;83(5):803–12.
10. Frank KE, Bolck B, Erdmann E, Schwinger RH. Sarcoplasmic reticulum Ca2⁺-ATPase modulates cardiac contraction and relaxation. Cardiovasc Res. 2003;57(1):20–7.
11. Hirayama Y, Saitoh H, Atariishi H, Hayakawa H. Electrical and mechanical alternans in canine myocardium in vivo. Dependence on intracellular calcium cycling. Circulation. 1993;88(6):2894–902.
12. Iida T, Lilly MA. missing oocyte encodes a highly conserved nuclear protein required for the maintenance of the meiotic cycle and oocyte identity in Drosophila. Development. 2004;131(5):1029–39.
13. Issmann I, Green S. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature. 1990;347(6334):645–50.
14. Jou CJ, Arrington CB, Barnett S, Shen J, Cho S, Sheng X, McCullagg PC, Bowles NE, Pribble CM, Saarel EV, Pilcher TA, Etheridge SP, Tristani-Firouzi M. A functional assay for sick sinus syndrome genetic variants. Cell Physiol Biochem. 2017;42(5):2011–9.
15. Kleeer SA, Lenhard JM, Willton TM, Patel I, Morris DC, Lehmann JM. A prostanlandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. Cell. 1995;83(5):813–9.
16. Kono T, Ahd G, Moss DR, Gann L, Zarian-Herzberg A, Nishiki Y, Fueger PT, Oghara T, Evans-Molina C. PPAR-gamma activation restores pancreatic islet SERCA2 levels and prevents beta-cell dysfunction under conditions of hyperglycemic and cytokine stress. Mol Endocrinol. 2012;26(2):257–71.
17. Kuo MW, Wang SH, Chang JC, Chang CH, Huang LJ, Lin HH, Yu AL, Li WH, Yu J. A novel puf-A gene predicted from evolutionary analysis is involved in the development of eyes and primordial germ-cells. PLoS ONE. 2009;4(3):e4980.
18. Lee WS, Kim J. Peroxisome proliferator-activated receptors and the heart: lessons from the past and future directions. PPAR Res. 2015;2015:271983.
19. Li L, Louch WE, Niederer SA, Aronsen JM, Christensen G, Sejersted OM, Smith NP. Sodium accumulation in SERCA knockout-induced heart failure. Biophys J. 2012;102(9):2039–48.
20. Nekronen A, Chung WY, Li WH. ETOPE: Evolutionary test of predicted exons. Nucleic Acids Res. 2003a;31(13):3564–7.
21. Nekronen A, Chung WY, Li WH. An evolutionary protein-coding capacity of the human genome. Trends Genet. 2003b;19(6):306–10.
22. Nekronen A, Makova KD, Li WH. The K(A)/K(S) ratio for testing the protein-coding potential of genomic regions: an empirical and simulation study. Genome Res. 2002;12(1):198–202.
23. Panagia M, Gibbons GF, Radka OK, Clarke K. PPAR-alpha activation required for decreased glucose uptake and increased susceptibility to injury during ischemia. Am J Physiol Heart Circ Physiol. 2005;288(6):H2677–2683.
24. Pastore JM, Giroud SD, Laurita KR, Akar FG, Rosenbaum DS. Mechanism linking T-wave alternans to the genesis of cardiac fibrillation. Circulation. 1999;100(10):1385–94.
25. Periasamy M, Kalyanasundaram A. SERCA pump isoforms: their role in cardiac morphogenesis and rhythm in embryonic zebrafish hearts. Proc Natl Acad Sci USA. 2002;102(7):2464–71.
26. Pruvot EJ, Katra RP, Rosenbaum DS, Laurita KR. Role of cardiac cycling versus restitution in the mechanism of repolarization alternans. Circ Res. 2004;94(8):1083–90.
27. Roden DM, Balser JR, George AL Jr, Anderson ME. Cardiac ion channels. Annu Rev Physiol. 2002;64:431–75.
28. Sakamoto J, Kikuma H, Moriyama S, Odaka H, Momose Y, Sugiyama Y, Sawada H. Activation of human peroxisome proliferator-activated receptor (PPAR) subtypes by pioglitazone. Biochem Biophys Res Commun. 1999;207(1):385–94.
29. Senior DP, Lopaschuk GD, Mobideen MA, Siegal G, Yelon D, Fishman MC, Garrity DM. Calcium extrusion is critical for myocardial fatty acid oxidation and leads to cardiomyopathy. Nat Med. 2000;6:1245–50.
30. Subra C, Grand D, Laulagnier K, Stella A, Lambeau G, Paillasse M, De M. Development. 2004;131(5):1029–39.
31. Sweis AH, Pipela CM, Bowles NE, Pilcher TA, Etheridge SP, Tristani-Firouzi M. A functional assay for sick sinus syndrome genetic variants. Cell Physiol Biochem. 2008;20(7):1205–20.
32. Tontonoz P, Spiegelman BM. Fat and beyond: the diverse biology of PPARgamma. Annu Rev Biochem. 2008;77:289–312.
33. Tsai CT, Chiang FT, Tseng CY, Yu CC, Wang YC, Lai LP, Hwang JJ, Lin JL. Mechanical stretch of atrial myocyte monolayer decreases sarcoplasmic reticulum calcium adenosine tetrathionate expression and increases susceptibility to repolarization alternans. J Am Coll Cardiol. 2011;58(20):2106–15.
34. Uliwier C, Balardi CT. The potential of peroxisome proliferator-activated receptor gamma (PPARgamma) ligands in the treatment of hematological malignancies. Mini Rev Med Chem. 2007;7(9):877–87.
34. Vangheluwe P, Louch WE, Ver Heyen M, Sipido K, Raeymaekers L, Wuytack F. Ca\textsuperscript{2+} transport ATPase isoforms SERCA2a and SERCA2b are targeted to the same sites in the murine heart. Cell Calcium. 2003;34(6):457–64.
35. Westerfield M. The Zebrafish book: a guide for the laboratory use of Zebrafish (Danio rerio). Eugene: Univ. of Oregon Press; 1995.
36. Willson TM, Brown PJ, Sternbach DD, Henke BR. The PPARs: from orphan receptors to drug discovery. J Med Chem. 2000;43(4):527–50.
37. Xu C, Min J. Structure and function of WD40 domain proteins. Protein Cell. 2011;2(3):202–14.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.