Online Supplements

Zebrafish care, breeding, injection procedures and functional analysis

Care and breeding of zebrafish (*Danio rerio*) was carried out as previously described(1). Morpholino-modified antisense oligonucleotides were designed against the translational start site of zebrafish ORAI1 [NM_205600] (MO-ORAI1^{ATG} 5'-AGTGCTCGCTCCGACTCATCTTCAT-3') and the splice donor site of zebrafish ORAI exon 1 (MO-ORAI1 5'-AAACAGCGCGGAGACTCACCATTGC-3'). 2-4ng of either MO-ORAI1^{ATG}, MO-ORAI1 or standard control morpholino (Genetools, LLC) was injected into one-cell-stage embryos. Functional assessment of cardiac contractility was carried out essentially as described previously(2). Statistical significance was evaluated with a 1-sided unpaired Student t test. Differences were considered significant if the probability value was $p<0.05$ and highly significant if the probability value was $p<0.01$.

Myocardial infarction and transaortic constriction

Mice surgical procedures have been described previously(3). Briefly, myocardial infarction was produced by ligating the left anterior descending (LAD) branch of the coronary artery using a 8-0 suture (Ethicon).

Immunohistochemistry

Animals were sacrificed at time points described in the text; the abdominal aorta was cannulated and perfused with phosphate buffered heparin. Following perfusion the heart was arrested in diastole by the injection of CdCl2 through the aortic cannula. Hearts were then flushed with formalin for 15 minutes, excised and fixed in formalin for 24 hours at room temperature. For paraffin processing, heart samples were placed in an automated tissue processor. Samples were then embedded and sectioned at a thickness of 4µm. Slides were deparaffinized and rehydrated using xylene and decreasing amounts of alcohol ending with a wash in deionized water. Antigen retrieval was performed using 10mM citrate, pH 6.0, for 15 minutes at 50% power in an 1100 W microwave. Slides were allowed to cool to room temperature and washed several times in distilled water. Next endogenous peroxidase activity was quenched using 3% H2O2 in DI water for 20 minutes at room temperature. Slides were then washed three times with DI water, twice with 1X TN (150mM NaCl, 100mM Tris, pH 7.5) and blocked with TNB (1X TN and blocking reagent supplied by Perkin Elmer) for 1 hour. Primary antibodies were applied overnight at 4° C in TNB. The following day the slides were washed three times in 1 x TN and secondary antibodies were applied for 2 hours at room temperature in TNB. Orai1 signal required detection using a Tyramide Signal Amplification (TSA) kit (Perkin Elmer) following manufacturers recommendations. Slides were washed and then incubated with Streptavidin horseradish peroxidase (HRP) 1:100 in TNB for 1 hour, washed and developed with tyramide substrate 1:50 for 10 minutes. Once slides were developed in tyramide substrate they were washed and coverslipped using VectaShield mounting media. In order to visualize nuclei, slides were treated with To-pro3 Iodide (Topro), Molecular Probes, at 1:10,000 for 20 minutes in 1X TN, washed and cover slipped. Micrographs were acquired using a Leica TCS-SP2 confocal laser scanning microscope. All stainings were accompanied by a section not given primary antibody as a negative control, when inspected these slides had no staining indicating specific staining for that primary antibody and not non-specific signal from the secondary antibody or subsequent Tyramide amplification.

NRCMs grown on glass coverslips were treated with Orai1 or scrambled siRNA as described above. After 48 h cells were fixed, permeabilized and labeled with antibodies specific to Tropomyosin followed by the corresponding Cy3-conjugated,

secondary antibodies (Jackson ImmunoResearch Lab) (1/100). Confocal images (CLSM) were obtained using a 63× water objective on a Leica TCS SP laser scanning confocal microscope. Digitized confocal images were processed by Adobe Photoshop.

Western Blot Analysis

Whole cell lysates isolated from neonatal cardiac myocytes from 2-3 old rats were prepared in 1X SDS sample buffer. Lysates were sonicated briefly then boiled for 5 minutes and loaded or stored at -80°C. The amount of protein was estimated using Bradford protein quantitation assay and equal amount of protein were loaded into an Invitrogen 4-12% Tris-Glycine mini-gel and run at 150V for 1.5 hours on an invitrogen electrophoresis apparatus. Separated proteins were transferred to a PVDF membrane pre-incubated in methanol then blocked for 1 hour with 5% dry milk in TBS-T (50 mmol/liter Tris-HCl (pH 7.6)/150 mM NaCl/ 0.1% Tween 20). After transfer the membrane was probed with primary antibodies overnight at 4$_o$C with gentle agitation in blocking buffer. Primary antibodies consisted of GAPDH (Invitrogen), Orai1 (Santa Cruz). The calsarcin antibody was a kind gift of Dr. Frey (Kiel, Germany).The next day blots were washed with TBS-T three times, probed with fluorescent or alkaline phosphatase-conjugated secondary antibodies 1:2000 in blocking solution (Jackson Labs) for 2 hours at room temperature. Following three washes with TBS-T blots were scanned

Isolation and primary culture of neonatal rat ventricular cardiomyocytes

Ventricular cardiomyocytes from 1 to 2 day old rat neonatal hearts (NRCMs) were prepared by trypsin digestion as described previously. After 24 h the medium was replaced by a 0.5% serum-containing DMEM and NRCM were transfected with indicated siRNAs. 48 h after transfection cells were stimulated with phenylephrine for up 24 h. Cells were washed with PBS and subjected to lysis buffer (PBS pH 7.4, SDS 2%, 2 mM EGTA/EDTA) containing a mixture of 1% v/v phosphatase inhibitors.

small RNA interference

Custom-designed synthetic Orai1 small interfering RNA (siRNA) and scrambled siRNA as a negative control were purchased from Applied Biosystems. NRCMs were transfected with Orai1 and control siRNA oligonucleotides (25 nM) by using HiPerfect transfection reagent according to the manufacturer's instructions (QIAGEN).

RNA isolation, reverse transcription and quantitative real-time PCR
Real-time RT-PCR

Total RNA was isolated from frozen heart or cultured cells by using Quick-RNA™ MiniPrep (Zymo Research) and reverse-transcribed into complementary DNA (cDNA) by using iScript cDNA Synthesis Kit (BioRad).. Real-time PCR was performed on all samples in triplicate using QuantiTect™ SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions. All primer sequences are shown in Online Table I of the Online Data Supplement.

Whole mount in-situ hybridization, immunofluorescent imaging and transmission electron microscopy

Whole mount in-situ hybridization and immunofluorescent staining of zebrafish embryos was carried out essentially as described previously(4). Immunofluorescent stained embryos were imaged on an Nikon C1si spectral confocal microscope. The

following antibodies were used: MF20 (Developmental Studies Hybridoma Bank, developed by Fishman, D.A.) – 1:20; anti-Vinculin (Sigma) – 1:50.
Transmission electron microscopy analysis was carried out essentially as described previously(5).
The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Supplemental Table I. Primer sequences for real-time PCR

Species	Gene	Forward	Reverse
rat	Orai1	5' -3'	5'- -3'
	β-actin	5'-GAAGATCAAGATCATTGCTCCTCCT-3'	5'-GAAGGTGGACAGTGAGGCCA-3'
mice	Orai1	5'- -3'	5'- -3'
	β-actin	5'-CATGAAGATCAAGATCATTGCTCCT-3'	5'-GCTGATCCACATCTGCTGGAA-3'

Online Figure 1.

(A). ORAI1 expression in the heart decreased after birth and is significantly reduced 2 months after birth. **(B)** Morpholino-modified antisense oligonucleotides efficiently knock-down zORAI1. MO-ORAI1 effectiveness on zORAI1 knock-down was demonstrated by analyzing mRNA of MO-ORAI1 injected embryos by RT-PCR. *orai1* is encoded by two exons separated by an intron spanning over 9kb genomic sequence. MO-ORAI1 is predicted to block the splicing of exon1-intron1 leading to integration of intron1. The primers used to examine correct splicing of ORAI1 amplify almost full-length *zorai1* (left lane). Integration of intron1, occurring after injection of MO-ORAI1, results in a transcript far longer than 9kb, unable to amplify with the used PCR conditions and thereby evident by the absence of correctly spliced *zorai1* (right

lane). *β-actin* was used as a RT-PCR control. . **(C)…… (D)** ORAI1 is an evolutionary highly conserved protein**.** Amino acid sequence alignment (CLUSTALW and BOXSHADE) shows high conservation of ORAI1 protein from human, to rat and zebrafish. Black boxes indicate identical amino acids; grey boxes indicate similar residues; white boxes indicate different amino acids

Online Figure 2.

(**A-B**) Western Blot of cardiac myocytes after knockdown of ORAI1 **(A)** and densidometric analysis **(B)**. (**C-D**) Knockdown of ORAI1 in CM inhibits hypertrophic growth after Phenylephrine (10μM for 24h). Representive Immunostaining with an antibody against tropomyosin reveals organization of the cytoskeleton in the control myocytes. Note the impaired formation of cytoskeletal fibers and reduced incorporation of tropomyosin in the siORAI1 myocytes **(C)**. **(D)** Quantification of the cell surface area after treatment with vehicle or Phenylephrine for 24hours.

Supplemental Movie 1

Loss of zORAI1 leads to heart failure in zebrafish. Control injected and MO-ORAI1 injected zebrafish hearts are shown at 48hpf. Head to the left, tail to the right

Supplemental Movie 2

Contractility decreases at 72hpf upon ORAI1 knock-down. Control injected and two MO-ORAI1 injected zebrafish hearts are shown at 72hpf. Head to the left, tail to the right

References:

1. Westerfield, M. 1995. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), 3rd Edition. *Eugene, OR, University of Oregon Press,385 (Book)*.
2. Rottbauer, W., Just, S., Wessels, G., Trano, N., Most, P., Katus, H.A., and Fishman, M.C. 2005. VEGF-PLCgamma1 pathway controls cardiac contractility in the embryonic heart. *Genes Dev* 19:1624-1634.
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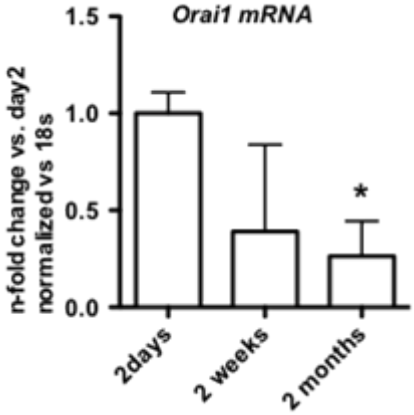
A



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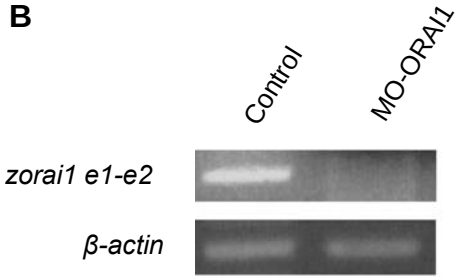

C

Western for MO-ORAI1^{ATG}

D

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        MSLNEHSMQALSWRKLYLSRAKLKASSRTSALLSGFAMVAMVEVQLDTDHDYPPGLLIVF
drORAI1 MSRSEHSLQALSWRKLYLSRAKLKASSRTSALLSGFAMVAMVEVQLDTNHDYPPGLLIAF

hsORAI1 SACTTVLVAVHLFALMISTCILPNIEAVSNVHNLNSVKESPHERMHRHIELAWAFSTVIG
        SACTTVLVAVHLFALMISTCILPNIEAVSNVHNLNSVKESPHERMHRHIELAWAFSTVIG
drORAI1 SACTTVLVAVHLFALMVSTCILPNIEAVSNVHNLNSVKESPHERMHRHIELAWAFSTVIG

hsORAI1 TLLFLAEVVLLCWVKFLPLKKQPGQPRPTSKPPAGGAAANVS-TSGITPGQAAAAIASTTI
        TLLFLAEVVLLCWVKFLPLKRQAGQPSPTKPPAESVIVANHSDSSGITPGEAAAAIASTAI
drORAI1 TLLFLAEVVLLCWVKFLPIK-----PKDQK-----------NGTVSAGVAAAITSTSI

hsORAI1 MVPFGLIFIVFAVHFYRSLVSHKTDRQFQELNELAEFARLQDQLDHRGDHP--LTPGSHY
        MVPCGLVFIVFAVHFYRSLVSHKTDRQFQELNELAEFARLQDQLDHRGDHS--LTPGTHY
drORAI1 MVPFGLVFIVFAVHFYRSLVSHKTDRQFQELEELED---LQNELDHREEVSTLQSPGSLY

hsORAI1 A
        A
drORAI1 P
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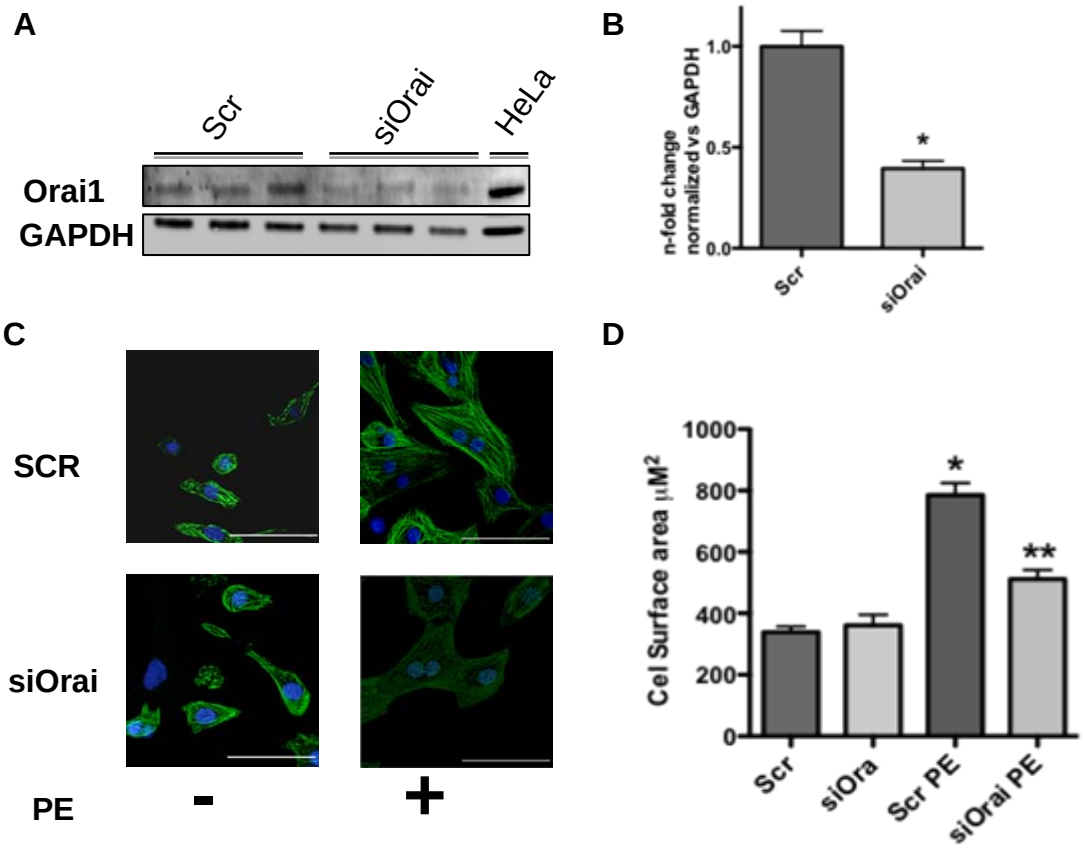
A
Scr
siOrai
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GAPDH
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n-fold change normalized vs GAPDH
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C
SCR
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