Cardiac-Specific Deletion of Orai3 Leads to Severe Dilated Cardiomyopathy and Heart Failure in Mice

Jesse Gammons, BS; Mohamed Trebak, PhD; Salvatore Mancarella, PhD

BACKGROUND: Orai3 is a mammalian-specific member of the Orai family (Orai1–3) and a component of the store-operated Ca\textsuperscript{2+} entry channels. There is little understanding of the role of Orai channels in cardiomyocytes, and its role in cardiac function remains unexplored. Thus, we developed mice lacking Orai1 and Orai3 to address their role in cardiac homeostasis.

METHODS AND RESULTS: We generated constitutive and inducible cardiomyocyte-specific Orai3 knockout (Orai3 cKO) mice. Constitutive Orai3- loss led to ventricular dysfunction progressing to dilated cardiomyopathy and heart failure. Orai3 cKO mice subjected to pressure overload developed a fulminant dilated cardiomyopathy with rapid heart failure onset, characterized by interstitial fibrosis and apoptosis. Ultrastructural analysis of Orai3-deficient cardiomyocytes showed abnormal M- and Z-line morphology. The greater density of condensed mitochondria in Orai3-deficient cardiomyocytes was associated with the upregulation of DRP1 (dynamin-related protein 1). Cardiomyocytes isolated from Orai3 cKO mice exhibited profoundly altered myocardial Ca\textsuperscript{2+} cycling and changes in the expression of critical proteins involved in the Ca\textsuperscript{2+} clearance mechanisms. Upregulation of TRPC6 (transient receptor potential canonical type 6) channels was associated with upregulation of the RCAN1 (regulator of calcineurin 1), indicating the activation of the calcineurin signaling pathway in Orai3 cKO mice. A more dramatic cardiac phenotype emerged when Orai3 was removed in adult mice using a tamoxifen-inducible Orai3 cKO mouse. The removal of Orai1 from adult cardiomyocytes did not change the phenotype of tamoxifen-inducible Orai3 cKO mice.

CONCLUSIONS: Our results identify a critical role for Orai3 in the heart. We provide evidence that Orai3-mediated Ca\textsuperscript{2+} signaling is required for maintaining sarcomere integrity and proper mitochondrial function in adult mammalian cardiomyocytes.

Key Words: calcium signaling ■ dilated cardiomyopathy ■ ion channels ■ Orai3

Dilated cardiomyopathy (DCM) is a severe condition, usually idiopathic, resulting in heart enlargement, progressive systolic dysfunction and arrhythmia.\textsuperscript{1,2} DCM has a poor prognosis leading to heart failure (HF) and death, cardiac transplantation remains the ultimate treatment for patients with end-stage HF. The molecular mechanisms that trigger the development of DCM remain poorly understood. Genetic abnormalities in the sarcomere, Z-disc, Ca\textsuperscript{2+} regulatory and cytoskeletal proteins complex may be causal for DCM.\textsuperscript{3,4}

Experimental evidence suggests that Orai1 (Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} modulator 1), is involved in maintaining cardiac pump function and sarcomere structure in invertebrates and lower vertebrates.\textsuperscript{5} Loss of Orai1 in zebrafish leads to progressive disruption of myofibers resulting in skeletal muscle weakness and HF.\textsuperscript{6} According to the same study, Orai1-loss was found to induce disruption of the calcineurin signaling transduction machinery at the Z-disc level.\textsuperscript{6} Orai channels are activated by the STIM1 (stromal interaction molecule 1), a single transmembrane protein primarily

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cardiac muscle. Our study provides evidence that constitutive and conditionally cardiac myocyte-specific genetic deletion of Orai3 induces dilated cardiomyopathy and heart failure in adult mice.

Cardiomyocytes from hypertrophic rat hearts exhibit a voltage-independent, inward current activated by arachidonic acid, a known activator of the Orai1/Orai3 channel complex. Moreover, a recent study reported that acute inhibition of Orai3 using siRNA accelerates isoproterenol-induced HF. Taken together, these data suggest that Orai3, and maybe Orai1, play a critical role in preserving the structural integrity and function of the mammalian heart. However, the role of these channels in cardiac physiology and pathology remains elusive.

For a detailed investigation of the physiological role of cardiac Orai channels, we used a Cre-LoxP system to generate conditional and inducible Orai3 cardiomyocyte-specific knockout mice (Orai3cKO). We show that deletion of Orai3, specifically in adult mouse cardiomyocytes, induces DCM characterized by progressive loss of ventricular function leading to HF and premature death. We found that cardiomyocytes from Orai3cKO mice exhibited abnormal cytoskeletal structure associated with mitochondrial dysfunction and dysregulation of critical elements of the excitation contraction-coupling (EC-coupling). These results suggest an indispensable role for Orai3 in maintaining cardiac structure and function. We conclude that

has remained elusive, and some controversy has been generated as to whether Orai1 is expressed in adult mammalian cardiomyocytes. For instance, patients lacking functional STIM1 and Orai1 develop a severe form of combined immunodeficiency associated with skeletal muscle myopathy, but these patients show no obvious cardiac muscle-related phenotypes. In lymphocytes, as well as in neonatal rat cardiomyocytes (NRCM), STIM1 and Orai1-mediated Ca^2+ signaling to the calcineurin/nuclear factor of activated T-cells (calcineurin/NFAT) pathway. This highly Ca^2+-dependent signaling pathway enables tightly controlled regulation of gene expression through different stimuli that can promote pathological remodeling of the adult heart. Therefore, STIM/Orai has been proposed as an upstream source of Ca^2+ that activate the calcineurin/NFAT, and it has been postulated that inhibition of Orai1 channel activity or suppression of STIM1 expression in the heart is protective against pathological remodeling. However, transgenic mice with cardiac-restricted Orai1 deletion did not show any cardiac structural abnormalities, and did not protect against angiotensin-II-induced pathological cardiac remodeling.

Orai2 and Orai3 are homologs of Orai1. In contrast to the Orai1 gene, which is present in invertebrates and vertebrates alike, the Orai3 gene is exclusive to mammals, and has been relatively under-investigated. Perhaps the exclusive existence of Orai3 in mammals could explain why Orai1 loss of function in Zebrafish and Drosophila has deleterious effects not seen in human patients carrying STIM1/Orai1 mutations.

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localized in the endo/sarcoplasmic reticulum. The STIM1/Orai complex mediates the store-operated Ca^2+ entry (SOCE). Petersen et al., recently reported that cardiac-specific suppression of Orai1 or STIM1 in Drosophila causes abolition of SOCE, highly disorganized myofibers, and DCM, further supporting the role of these proteins in healthy cardiomyocytes. Despite mounting evidence suggests a role of Orai in the myocardium, the function of Orai in adult heart

Nonstandard Abbreviations and Acronyms

| Abbreviation | Description |
|--------------|-------------|
| α-Myh        | alpha-myosin heavy chain |
| a.u.         | arbitrary units |
| cKO          | cardiomyocyte-specific knockout |
| DCM          | dilated cardiomyopathy |
| DRP1         | dynamin-related protein 1 |
| EC           | excitation-contraction |
| NRCM         | neonatal rat cardiomyocytes |
| RCAN-1       | regulator of calcineurin 1 |
| STIM1        | stromal interaction molecule 1 |
| TAC          | transverse aortic constriction |
| TRPC6        | transient receptor potential canonical type 6 |
| TUNEL        | terminal deoxynucleotidyl transferase dUTP nick end labeling apoptosis |

What Is New?
- We report that Orai3 is the most abundant Orai isoform expressed in healthy mouse cardiomyocytes.
- We provide evidence that constitutive and conditional cardiomyocyte-specific genetic deletion of Orai3 induces dilated cardiomyopathy and heart failure in adult mice.
- Orai3 is required for cytoskeletal and energy homeostasis; we have positioned Orai3 as a key protein in cardiac function and structure.

What Are the Clinical Implications?
- Because dilated cardiomyopathy is a significant problem that contributes to a wide array of cardiovascular diseases, our identification of Orai3 as a new regulator of cardiac function points to previously unknown mechanisms that may emerge as therapeutic targets of heart disease.
- Our findings point to the Orai3 channel as a novel Ca^2+ source required to maintain cardiac signaling necessary for cardiac structure and could be a future target in patients with idiopathic dilated cardiomyopathy.

CLINICAL PERSPECTIVE

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Orai3cKO mice resemble the morphological and clinical cardiac features as those observed in acquired adult DCM in humans and may be used as a model for this condition.

METHODS

Data Availability Disclosure Statement
The data, analytic methods, and study materials will be made available on request to other researchers for purposes of reproducing the results or replicating the procedures reported here.

Mouse Models Generation
All animal procedures were approved by the Institutional Animal Care and Use Committees at the University of Tennessee Health Science Center and comply with US regulations on animal experimentation. Mice were housed in a facility maintained at 22°C with an alternating 12-hour light/dark cycle and had free access to food and water ad libitum. Orai1 flox/flox and Orai3 flox/flox mice were crossed with transgenic mice FVB-Tg(Myh6-cre)2182Mds/J, expressing Cre-recombinase under the cardiomyocyte-specific α-Myh6 (α-myosin-heavy chain promoter) (Jackson Laboratory, cat. 011038). Mice were backcrossed for 6 generations with C57BL/6N mice to harbor cardiac-specific Orai1- or Orai3- deficient (Orai1 cKO and Orai3cKO) mice. Tamoxifen-inducible, cardiomyocyte-specific, Orai1 cKO and Orai3 cKO mice were obtained by crossing Orai1 flox/flox and Orai3 flox/flox with tamoxifen-inducible cardiomyocyte-specific, Orai1 cKO and Orai3 cKO mice were obtained by crossing Orai1 flox/flox and Orai3 flox/flox with tamoxifen-inducible Cre recombinase under the α-Myh cardiac-specific promoter: (C57BL)-Tg(Myh6-cre/Esr1*)1Jmk/J, (Jackson laboratory, cat. 5657).

Cardiac Phenotyping
Mice from both groups were anesthetized with isoflurane and placed on the warming pad of a recording stage of a Vevo 2100 ultrasound machine. The anterior chest was previously shaved, ultrasound coupling gel was applied, electrodes were connected to each limb, and an ECG was simultaneously recorded while body temperature was monitored. Two-dimensional (short axis-guided) M-mode measurements at the level of the papillary muscles were taken using an 18 to 32 MHz MS400 transducer as previously described. Images were also recorded in the parasternal long-axis. For analysis purposes, ≥3 beats were averaged; measurements within the same heart rate interval (500±50 bpm) were used for analysis. Measurements were performed before and after left anterior descending coronary artery ligation. Pressure-volume loop measurements were taken with the ADV 500 PV system (Transonic Scisense).

Histological and Morphometric Analysis
The heart was arrested in diastole by nominally Ca2+-free Tyrode solution containing 2,3-butanediene monoxime, (10 mmol/L), and was fixed in 4% paraformaldehyde for 20 minutes under pressure (diastolic arterial pressure). The heart was excised, weighed, and stored for 24 to 48 hours in 4% paraformaldehyde. Picrosirius Red staining was performed on 5-µm-thick paraffin-embedded tissue sections. Wheat-germ agglutinin (WGA) staining was performed using FITC-conjugated lectin (Sigma Aldrich L4895). Heart sections were incubated with WGA solubilized in PBS for 20 minutes at room temperature. After washing 3 times with PBS, the sections were mounted with Prolong Gold containing DAPI (Invitrogen, cat. P36931) and kept at 4°C in dark. Terminal deoxynucleotidyl transferase dUTP nick end labeling apoptosis (TUNEL) staining was performed using Invitrogen Click-IT Plus TUNEL assay (ThermoFisher, cat. C10618). Images were obtained for all histological experiments using a Zeiss LSM 710 confocal laser scanning microscope. Fibrosis, apoptosis, and cell area were measured using ImageJ.

Myocyte Isolation From Neonatal and Adult Hearts
NRCM were isolated from Sprague-Dawley rats (1–3 day-old) as described earlier. Briefly, neonatal rat hearts were removed, atria were trimmed off, the remaining ventricles were minced and the myocytes were dissociated with a Ca2+-bicarbonate free Hanks buffer (pH 7.5) containing penicillin (50 000 U/L)/streptomycin (50 mg/L), gentamicin (50 mg/L), heparin (5000 USP units/mL), DNase I (2 mg/mL), and trypsin (1.5 mg/mL) at 25°C with gentle mixing. The dissociated NRCMs were pre-plated for 2 hours to reduce non-myocyte contamination, the medium was then replaced with DMEM ( Gibco, Carlsbad, CA, USA) supplemented with 5% FBS (HyClone Laboratories, Logan, UT, USA), Bromodeoxyuridine / 5-bromo-2′-deoxyuridine (BrDU) (0.1 mmol/L; Sigma Chemical, St. Louis, MO, USA), vitamin B12 (1.5 mmol/L; Sigma), and antibiotics. After the first 24 hours, the medium was replaced again with DMEM supplemented with: insulin (10 µg/mL; Sigma), transferrin (10 µg/mL; Sigma), vitamin B12 (1.5 mmol/L; Sigma), and antibiotics. NRCM were grown on fibronectin-coated coverslips. Cells were transfected with G-GECO1-Orai3 and STIM1-mCherry using lipofectamine 3000 (Life Technologies). Live-cell imaging was conducted using the IX83 Olympus microscope attached to a Zyla 5.5 sCMOS camera.
Adult mouse cardiomyocytes were isolated using the langendorff retro-perfusion method and digested using collagenase type II ( Worthington, Lakewood, NJ) as described earlier. Mouse myocytes loaded with...
different fluorophores as indicated in the text (ie, fura-2, JC-1), according to the manufacturer. Cells were observed on a Nikon Eclipse TE300 or IX83 Olympus microscope. Cells were used within 6 hours from the isolation.

**Transmission Electron Microscopic Analysis**

Cardiac muscle samples were obtained and fixed in 2.5% glutaraldehyde and 2% paraformaldehyde for 2 hours at 4°C. After washing in cold cacodylate buffer, the specimens were post-fixed in 1% osmium tetroxide, stained in aqueous uranyl acetate, and then dehydrated through a graded ethanol series and embedded in epoxy resin. Ultra-thin sections (600–800 Å) from the resin blocks were stained using lead citrate and examined by transmission electron microscopy (TAM), (JEOL 2000EX).

**Cell Shortening and Intracellular Ca2+ Measurements**

Morphological and mechanical properties of the myocytes and intracellular Ca2+ activity were measured using the methods described earlier.\(^31,32\) Briefly, after a period of Ca2+ re-adaptation, isolated cardiomyocytes were stored in Tyrode solution (0.5 mmol/L CaCl2) and used within 6 hours from the isolation. Cell shortening was measured in naïve cells placed in a perfusion chamber mounted on the stage of an inverted microscope (Eclipse T300, Nikon). The cells were left to attach on a coverslip pre-coated with laminin and were bathed with modified Tyrode solution, which contained (in mmol/L) 137 NaCl, 10 glucose, 5.4 KCl, 1.3 MgSO\(_4\), 2.0 CaCl2, and 10 HEPES (pH 7.4). Cells were electrically paced, video motion edge detector software (Ionoptix, Milton, MA) was used to analyze and calculate the myocyte shortening fraction and the maximal rates of the cell shortening and re-lengthening. Cell length was measured in 15 to 25 myocytes/heart. For intracellular Ca2+ measurements, the myocytes were incubated with fura-2 for 30 minutes at room temperature (22°C–24°C). Excess of fura-2 was washed out and the cells were re-suspended in physiological buffer with 0.5 mmol/L CaCl2. Cells were excited at 360/380, fluorescence emission was detected at 510 (interpolated method) with a dual-excitation fluorescence multiplier system (IonOptics). Steady-state contractions or Ca2+ transients were averaged for each cell and used. SOCE was measured with the following method: fura2-loaded cardiomyocytes were electrically stimulated to confirm the presence of Ca2+ transients, then in the absence of electrical stimulation, cells underwent store depletion by adding thapsigargin (5 µmol/L) and caffeine (10 mmol/L) to the extracellular, Ca2+ free buffer. This procedure is commonly used to induce STIM1 oligomerization and activation of SOCE. When intracellular Ca2+ reaches baseline, extracellular Ca2+ was added and the cytosolic fluorescence signal was recorded.

**Western Blots**

Total proteins were extracted from mutants and control hearts by bead homogenization in RIPA Lysis Buffer (ThermoFisher cat. #89900) in the presence of a mixture of protease inhibitors (Halt Protease Inhibitor Cocktail cat. #78438). The homogenates were centrifuged at 13 000 rpm (20 784g) for 10 minutes at 4°C to obtain clarified lysates. Cell lysates were separated by precast PAGE gels (Genscript) and transferred to polyvinylidene difluoride membranes (PVDF). In this study, the following primary antibodies were used for Western blot and immunohistochemical detection: mouse anti-Orai1 (Santa Cruz, cat. sc-377281), rabbit anti-Orai2 (Proteintech, cat. 26766), rabbit anti-Orai3 (ProSci, cat. 4117), rabbit anti-TRPC6 (transient receptor potential canonical type 6) (Abcam, cat. ab62641), rabbit anti-tubulin (Cell Signaling, cat. 2146), rabbit anti-GAPDH (Cell Signaling, cat. 5174), rabbit anti-DRP1 (dynamin-related protein 1) (Invitrogen, cat. pa5-20176), mouse anti-SERCA2a (sarco/endoplasmic reticulum Ca2+-ATPase) (Santa Cruz, cat. sc-376235), rabbit anti-NCX1 (Abcam, cat. ab177952), rat anti-ERTR7 (fibroblast marker, Santa Cruz, cat. sc-73355), phalloidin (Invitrogen, cat. R415). Protein loading was normalized to a reversible total protein stain (Li-Cor, cat. 926-11010), GAPDH or beta-tubulin. Primary antibodies were detected by appropriate secondary antibodies, and membranes were imaged using blot imager LI-COR Odyssey system according to manufacturer protocols.

**Data Analysis**

All data were expressed as mean±SEM. Comparisons between the control group and Orai1KO were performed with the Student t-test on raw data before normalization. Comparisons between >2 groups were conducted on raw data with ANOVA followed by Bonferroni correction, which was calculated with GraphPad Prism, version 9 (GraphPad Software; San Diego, CA, USA). Cardiac phenotype studies were performed blind with experimenters not knowing the genotype of the mouse. In cases when the 2 control groups were combined, statistics were performed on the pooled raw data of both control groups and compared with Orai1KO mice with the Student t-test. Experiments using isolated cardiomyocyte (n) were obtained from several heart (N) preparations.
RESULTS

Orai Isoform Expression in Cardiac Tissue and Cardiomyocytes

We first evaluated the expression pattern of all 3 Orai isoforms (Orai1–3) in the mouse heart. Protein immunoblots of crude cardiac tissue lysate produced a band at ≈55 kDa corresponding to Orai1; this is higher than the predicted size because of protein glycosylation in its native environment. Orai2 and Orai3 were detected near the expected molecular size at ≈28 and ≈37 kDa, respectively. In contrast, lysates enriched with freshly isolated cardiomyocytes from adult mice displayed the presence of Orai3 protein, while Orai1 and Orai2 proteins were not detectable (Figure 1A and 1B).

The spatial distribution of Orai1 and Orai3 in adult mouse heart appeared to be cell-specific. Orai1 immunoreactivity was mainly associated with fibroblasts and co-stained with the fibroblast marker ER-TR7. Cardiomyocytes stained poorly with Orai1 antibody (Figure 1C, a through d). In contrast, anti-Orai3 staining colocalized mainly with phalloidin staining, which suggested the presence of Orai3 in cardiomyocytes with a weak signal from the interstitial cells (Figure 1C, e through h). It has been reported that gene expression of STIM and Orai in the heart is dynamic and...
undergoes pronounced changes during development.\textsuperscript{17,34} We show that while STIM1 and Orai1 were abundantly expressed in the NRCM, Orai3 protein level was barely detectable at this stage. In contrast, in the adult myocardium, we observed a dramatic downregulation of STIM1 and Orai1 and a rise of Orai3 expression (Figure S1A and S1B). These findings suggest a distinct spatial distribution of Orai isoforms in the mouse cardiac tissue and that Orai3 is the predominant isoform expressed in the post-natal heart.

**Orai3\textsuperscript{cko} Mice Develop DCM and HF**

To address the physiological role of Orai3 channels in the myocardium, we generated Orai3\textsuperscript{cko} mice. Mutant mice were viable and born at the expected Mendelian ratios, indicating the absence of embryonic lethality. Orai3\textsuperscript{cko} mice were fertile and were indistinguishable from control mice expressing the Orai3 wild type (wt) allele and cre-recombinase (Orai3\textsuperscript{wt/wt-α-Myh-\textsuperscript{cre}}). Examination of 5-week-old Orai3\textsuperscript{cko} mice confirmed the lack of Orai3 protein from cardiac tissue homogenates (Figure 1D and 1E), further suggesting that most of the Orai3 present in the heart is expressed in cardiomyocytes. The Orai3\textsuperscript{cko} mice had a reduced lifespan, with death occurring between 6 and 10 months of age, as quantified by Kaplan–Meier survival curve analysis (Figure 1F).

Echocardiographic analysis of adult (12–16 weeks) Orai3\textsuperscript{cko} mice revealed dilation of the left ventricular (LV) chamber as suggested by increased end-diastolic LV internal dimension associated with thinning of the LV anterior wall and interventricular septum (Figure 1G through 1J). Reduced cardiac function was observed in the Orai3-null mice, shown as decreased fractional shortening percentage (Figure 1K). The heart weight to body weight ratio was increased in the Orai3\textsuperscript{cko} mice when compared with the control group (Figure 1L). Overall, Orai3\textsuperscript{cko} mice showed the anatomic features of eccentric hypertrophy characterized by dilation of the ventricular chambers and compromised cardiac function. Our data provide evidence that Orai3 protein expression in the adult myocardium is required for maintaining cardiac function and structure.

**Orai3 Expression Protects the Heart From Pressure Overload-Induced HF**

STIM1 and Orai1 have been implicated in stress-induced maladaptive cardiac remodeling.\textsuperscript{25,34} In contrast, the role of Orai3 in the heart remains unknown. To test the hypothesis that Orai3 is involved in pathological cardiac remodeling in mammalians, we subjected Orai3\textsuperscript{cko} and control mice to transverse aortic constriction (TAC) to induce LV pressure-overload and HF. Because of the severity of the cardiac phenotype of Orai3\textsuperscript{cko} older mice, we used young (1–2 months) mice that exhibited a mild cardiac phenotype as assessed by echocardiography.

Cardiac function was evaluated by 2D echocardiography at various times post-surgery. Two weeks post-TAC, Orai3\textsuperscript{cko} mice showed a significant increase of LV internal dimension accompanied by a mild reduction in cardiac contraction measured as fractional shortening percentage. The LV chamber further dilated and cardiac function progressively degraded at the 4-week time point. In contrast, the control group exhibited a typical compensatory response with thickening of the free ventricular walls and mild LV chamber dilation (Figure 2A through 2C). Post-mortem analysis confirmed cardiac chamber dilation and cardiac hypertrophy in Orai3\textsuperscript{cko} mice (Figure 2D and 2E). Most Orai3\textsuperscript{cko} mice with TAC died prematurely at the 7-week timepoint because of HF and pulmonary edema with occasional left atrial fibrin clots.

Wheat-germ agglutinin staining for cell size assessment confirmed the presence of cell hypertrophy in post-TAC control cardiac tissue. In contrast, Orai3\textsuperscript{cko} showed a decrease of cross-sectional area (Figure 2F and 2G) associated with greater fibrosis and collagen deposition than the relatively healthy hearts from the control group (Figure 2H and 2I). Cardiac apoptosis is an essential mechanism of pathological stimuli-induced cell death and a significant factor underlying pressure overload-induced cardiac dysfunction and cardiovascular disease.\textsuperscript{35,36} Therefore, we tested the hypothesis that loss of Orai3 induces cardiac structural and functional deterioration by increasing cardiomyocyte susceptibility to apoptosis. TUNEL analysis performed on cardiac section from mice subjected to TAC showed a higher incidence of apoptotic cells in Orai3\textsuperscript{cko} hearts than control hearts. These results indicate that Orai3 is required for the maintenance of normal heart function by preventing cardiomyocyte cell death in response to cardiac stress.

**Orai3-Loss Causes Aberrant Myocyte Cytoarchitecture and Altered Mitochondrial Morphology**

Orai1 has been linked to the maintenance of sarcomere integrity in skeletal and cardiac muscle of invertebrates and lower vertebrates.\textsuperscript{5,37} However, these findings were not corroborated in mammalians. Based on our data and in silico analyses of Orai isoforms distribution pattern, we reasoned that Orai3 compensates for the lack of Orai1 in mouse cardiomyocytes. Morphologic and ultrastructural examination of ventricular myocardium showed that sarcomeres from the Orai3\textsuperscript{cko} heart were disorganized compared with those from matched controls. The ultrastructure of the Z-line in left ventricular cardiomyocytes Orai3\textsuperscript{cko} exhibited a lower density of myofibrils and defect in the demarcation of
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Z-line and M-line (Figure 3A, a and b). Orai3-deficient cardiomyocytes displayed wider Z-lines (461.1±121.6 versus 102.9±12.1 nm; \( P < 0.001 \); Orai3-deficient versus control, respectively). The M-line structure was mostly lost in areas with severe disruption of Z-line architecture. Intercalated discs of control samples were clearly observed connecting adjacent myocytes end-to-end. However, in Orai3<sup>CKO</sup> intercalated disc structures were variably affected, highly convoluted, and gaps of the intercalated discs were significantly widened in some cases. Overall, the number of gap junctions in Orai3<sup>CKO</sup> hearts was significantly reduced (Figure 3B, a and b).

Cytoskeletal disarray increases susceptibility to apoptosis and is associated with mitochondrial dysfunction.\(^ {38,39} \) We then investigated whether the cytoarchitecture aberration observed in Orai3<sup>CKO</sup> mice was associated with altered mitochondrial morphology and distribution. We performed ultrastructural and morphometric analysis on a subset of myocardial samples (Orai3<sup>CKO</sup> versus control). A mean of 805 isolated mitochondria (defined as organelles enclosed by a double-contoured membrane) was analyzed from each sample (range, 500–1230; SD±116.18). As shown in Figure 3B (c and d), mitochondria from Orai3<sup>CKO</sup> heart are increased in number and appear smaller as compared with control. The absolute number of mitochondria per area was increased in Orai3<sup>CKO</sup>, while the mean cross-sectional area of individual mitochondria was reduced in Orai3<sup>CKO</sup> compared with controls (Figure 3C). During apoptosis, mitochondrial membrane potential decreases, inducing the releases of mitochondrial apoptogenic factors.\(^ {23} \) To test whether loss of mitochondrial function...
Figure 3. Sarcomere and mitochondrial dysfunction in Orai3<sup>cKO</sup> mice.
A, a and b showing representative transmission electron microscopy pictures of the sarcomere structure of cardiac muscle from 1-month-old male mice. Scale bar=500 nm. High magnification images (inset, c-d) depicting normal Z-line (arrowhead) and M-band (red parenthesis) in the control and disorganized Z-line and shallow M-band in Orai3<sup>cKO</sup> sections. Scale bar=10 nm. B, Cross-sections through intercalated disc showing large mixed-type junction (area composita) surrounded by two dense desmosome structures (a and b, black arrows). Representative ultrastructural images of left ventricle from control and Orai3<sup>cKO</sup> hearts showing normal (control) and altered (Orai3<sup>cKO</sup>) mitochondrial morphology and density (c and d). C, Graphs summarizing mitochondria number, mitochondrial size, mean cross-sectional area, and optical. Data are expressed as mean±SEM t-test, *P<0.01 for Orai3<sup>cKO</sup> vs. control. D and E, Representative photograph and analysis of cardiomyocytes loaded with JC-1 to measure mitochondrial membrane potential, emissions at 525/585 are visualized in green and red fluorescence, respectively. Scale bar=50 µm. Cells isolated from 3 mice each group, t-test: *P<0.01. F, Immunoblot and analysis showing that the expression of DRP1 (dynamin-related protein 1) was increased in Orai3<sup>cKO</sup> hearts when compared with the control group. Loading was normalized using total protein (Figure S5). DRP1 indicates dynamin-related protein 1; LV, left ventricle; Orai3<sup>cKO</sup>; Orai3 cardiomyocyte-specific knockout; and TEM, transmission electron microscopy. t-test: *P<0.01.
membrane potential ($\Delta \Psi m$) was associated with the increased apoptosis observed in the Orai3$^{\text{cKO}}$, we measured $\Delta \Psi m$ in JC-1 loaded viable control and Orai3$^{\text{cKO}}$ isolated cardiomyocytes. JC-1 is a positively charged carbocyanine dye that accumulates in the inner mitochondrial membrane and fluoresces in green at low and in red at high membrane potentials. JC-1–stained control cells displayed healthy mitochondria with high red to green ratio, whereas in many Orai3-deficient cardiomyocytes exhibited predominantly green fluorescence, which indicated dysfunctional depolarized mitochondria (Figure 3D and 3E). Mitochondrial fragmentation usually precedes apoptosis in a phenomenon mediated by DRP1, a fission-related protein. Accordingly, we observed that the expression level of DRP1 was elevated in Orai3$^{\text{cKO}}$ mice (Figure 3). In conclusion, these data suggest that Orai3 is required for promoting proper cardiomyocyte cytoarchitecture and maintain optimal mitochondria shape and function.

**Orai3 is Essential for Maintaining Normal EC-Coupling**

The potential effect of Orai3-loss on the intracellular Ca$^{2+}$ cycling was measured in individual myocytes isolated from hearts of 3- to 4-month-old mice. Cardiomyocytes from the Orai3$^{\text{cKO}}$ mice exhibited higher resting Ca$^{2+}$ levels during pacing protocols (1Hz) that significantly dropped when pacing was ceased (Figure 4A). Electrically evoked Ca$^{2+}$ transients showed reduced peak amplitude in Orai3-deficient cells (Figure 4B). The decay time of the Ca$^{2+}$ transient was significantly prolonged at 50% and 90% (Figure 4C). We found that endo/sarcoplasmic reticulum Ca$^{2+}$ loading, measured as caffeine-induced Ca$^{2+}$ release, was decreased in Orai3-deficient cardiomyocytes (Figure 4D).

Orai3 is a member of the store-operated Ca$^{2+}$ channels (SOCC), hence, we evaluated its response to intracellular store emptying in isolated adult cardiomyocytes. Cells were subjected to Ca$^{2+}$ free buffer and exposed to thapsigargin, an SERCA inhibitor, to deplete internal Ca$^{2+}$ stores. Ca$^{2+}$ was then returned to the media and entry into the cell was monitored by a change in the fura-2 fluorescence absorbance ratio. About 50% of adult control myocytes showed a subtle but significant Ca$^{2+}$ entry. In contrast, all Orai3$^{\text{cKO}}$ cells analyzed did not show Ca$^{2+}$ entry when Ca$^{2+}$ was reintroduced to the extracellular solution (Figure S2).

Since Ca$^{2+}$ clearance was affected in the Orai3-deficient cells we further analyzed the expression of essential proteins involved in the Ca$^{2+}$ cycle. We found increased NCX (sodium Ca$^{2+}$ exchanger) expression (Figure 4E). This was in keeping with the prolonged Ca$^{2+}$ decay time and more in general with the HF phenotype of Orai3$^{\text{cKO}}$ mice. Some members of the TRPC channels family have often been suspected of acting as (SOCC) or being affected by them. We found elevated levels of TRPC6 protein in the Orai3$^{\text{cKO}}$ heart lysates (Figure 4F). A sustained increase in intracellular Ca$^{2+}$ level can trigger downstream gene transcription believed to play a causative role in adverse cardiac remodeling and the pathogenesis of HF. Accordingly, we found that the RCAN1 (regulator of calcineurin-1) expression was increased in Orai3$^{\text{cKO}}$ mice (Figure 5G), suggesting calcineurin signaling activation, this was probably attributable to the Ca$^{2+}$ overload from the defective Ca$^{2+}$ cycle and the TRPC6 upregulation in response to Orai3 deletion.

We previously reported that STIM1-tagged with a genetic Ca$^{2+}$ sensor, the GCaMP6, associates with Ca$^{2+}$ concentration microdomains in cardiomyocytes. To determine whether Orai3 mediate Ca$^{2+}$ influx, NRCMs were co-transfected with mCherry-tagged STIM1 and Orai3 tagged with GECO1.2, the Ca$^{2+}$ sensor, attached in its cytosolic portion of the channel. This highly informative model allows the visualization of STIM1, Orai3, and Ca$^{2+}$-entry detected by the GECO1.2 domain in live cardiomyocytes (Figure 5A). When overexpressed, the spatial distribution of STIM1-mCherry suggested the formation of clusters as we and others have reported earlier, and shown in Figure 5B and 5C. STIM1/Orai3 form complexes when expressed together, however, Orai3-GECO1.2 aggregates in cells not expressing STIM1-mCherry, probably indicating Orai3 interacting with endogenous STIM1. STIM1/Orai3 complex is independent of the electrically evoked global Ca$^{2+}$ transients.

**Rapid Orai3 Downregulation in the Adult Myocardium Causes DCM and HF**

Our findings that Orai3 is predominantly expressed in the adult myocardium prompted us to evaluate the impact of Orai3 deletion on the cardiac function of adult mice. We generated a tamoxifen-inducible cardiomyocyte-specific Orai3 knockout (TM-Orai3$^{\text{cKO}}$) mouse by crossing mice carrying IoxP-flanked alleles of Orai3 with the cardiac-specific α-Myh promoter directing expression of a tamoxifen-inducible Cre recombinase (MerCreMer) in adult cardiomyocytes. To rule out any contribution from Orai1, which has been reported to be expressed in cardiomyocytes during pathological conditions, the same strategy was used to generate TM-inducible
Figure 4. Intracellular Ca\textsuperscript{2+} recordings and regulation of essential proteins for the excitation-contraction-coupling.

A, Ca\textsuperscript{2+} transients from fura-2 loaded adult mouse ventricular myocyte. Cells were initially stimulated at 1 Hz; the stimulator was turned off as indicated by the arrow. B, Mean Ca\textsuperscript{2+} transient amplitude in Orai3\textsuperscript{cKO} and control myocytes. C, Decay kinetics Ca\textsuperscript{2+} transient in Orai3\textsuperscript{cKO} and control myocytes using an exponential smoothed curve fitting function at 10% decay, 50% decay, and 90% decay. D, SR load determined by caffeine-induced Ca\textsuperscript{2+} release. Statistical analysis, t-test, *P<0.05 vs control (n, 28-24 myocytes per group).

E and G, Immunoblot image and quantification analysis on TRPC6 and RCAN-1 immunoblots. Both TRPC6 and RCAN-1 expression was higher in Orai3\textsuperscript{cKO} than control. All values were normalized to β-tubulin or total protein to correct for minor loading variations. Statistical analysis. T-test, (P<0.05). EC indicates excitation-contraction; NCX, sodium-calcium exchanger; Orai3\textsuperscript{cKO}, Orai3 cardiomyocyte-specific knockout; RCAN-1, regulator of calcineurin 1; SERCA, sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase; SR, endo/sarcoplasmic reticulum; and TRPC6, transient receptor potential canonical type 6.
Orai1/Orai3 cardiomyocyte-specific double knockout mice. Two weeks after tamoxifen injection, expression of Orai3 protein in TM-Orai3cKO mouse hearts was almost totally abolished compared with controls (Figure 6A and 6B). TM-injected TM-Orai3cKO mice died prematurely. In contrast, the survival rate in the controls was not significantly affected (Figure 6C). Serial echocardiography revealed that 15 days after TM injection, mice expressing MerCreMer (used as a control group) exhibited depressed cardiac function. However, such detrimental effect was greater in the TM-Orai3cKO and TM-inducible Orai1/Orai3 cardiomyocyte-specific double knockout mice (Figure 6D). The LV internal dimension and LV volume increased significantly in the MerCreMer mice and was significantly higher in TM-Orai3cKO and TM-inducible Orai1/Orai3 cardiomyocyte-specific double knockout mice. Thirty days post-TM injection, cardiac dysfunction induced by MerCreMer was reduced, and heart function recovered to levels comparable with those of wild-type mice. However, most of the TM-Orai3cKO and TM-inducible Orai1/Orai3 cardiomyocyte-specific double knockout mice had died at this point, and those that survived showed significantly depressed cardiac function and exaggerated LV dilation (Figure 6D and 6E). Pressure-volume loop measurements in both control and TM-Orai3cKO mice 30 days after TM injection are illustrated in Figure 6F, and hemodynamic parameters are reported in Figure 6G. Compared with
control mice, TM-Orai3<sup>cko</sup> mice showed a significant LV volume increase, the slope of the end-diastolic pressure-volume relationship, which describes the diastolic function and passive properties of the ventricle, was elevated in TM-Orai3<sup>cko</sup> mice, suggesting an increase in diastolic myocardial stiffness in TM-Orai3<sup>cko</sup> mice. Collectively, these data show impaired cardiac function in the adult myocardium subsequent to genetic Orai3 deletion. Quantitative analysis of apoptosis-positive nuclei in cardiac myocytes revealed increased apoptotic cell death in the TM-Orai3<sup>cko</sup> hearts. At the cellular level, cardiomyocytes were elongated and thinner (Figure S4). Overall, the cardiac phenotype of TM-Orai3<sup>cko</sup> was consistent with the DCM observed in the constitutive Orai3<sup>cko</sup> mice.
Figure 6. Deletion of Orai3 in adult cardiomyocytes results in dilated cardiomyopathy.

A and B, Representative and summarized Western blot analysis of Orai3 protein in control and TM-Orai3cKO, 2 weeks post-tamoxifen injection, n=5 mice each group, t-test, *P<0.01. C, Kaplan–Meier survival curve shows that Orai3 loss reduces survival of TM-Orai3cKO mice. D, Echocardiographic analyses of TM-Orai3cKO mice and ctrl mice at 10- and 30-days post-tamoxifen injection. LV, LVID, and LV vol measurements are shown. Two-way ANOVA followed by Bonferroni test was used to compare the mean differences between groups, P<0.05. E, Frames extracted from echocardiographic videos performed 30 days post-tamoxifen injection showing trans-thoracic B-mode and M-mode from controls and TM-Orai3cKO mice in both long- and short-axes view. F and G, Representative left ventricular pressure-volume loops performed 30 days post-tamoxifen from TM-Orai3cKO and control group illustrate the emerging systolic dysfunction (ie, rightward shift in the end-systolic pressure-volume relationship and the upward shift of end-diastolic pressure-volume relationship) in TM-Orai3cKO mice; n=5 mice each group, t-test, *P<0.05. ESPVR indicates end-systolic pressure volume relationship; FS, fractional shortening; LV, left ventricle; LVID, left ventricular internal diameter; MHC, myosin heavy chain. Orai3 ff, Orai3 flox/flox; Orai1  Orai3 ff; Orai1 and Orai3 double floxed; TM, tamoxifen-inducible; TM-Orai3cKO, tamoxifen-inducible cardiomyocyte-specific Orai3 knockcoulout; and WT, wild-type.

DISCUSSION
The role of local Ca\textsuperscript{2+} signaling that governs cardiac homeostasis and remodeling has been extensively studied, but remains one of the least understood processes. In our present study, we used several experimental models and found that Orai3 Ca\textsuperscript{2+} channel is critical for maintaining the proper function and structure of the mouse heart. First, we established that among all 3 Orai isoforms, Orai3 is the most abundant in the healthy adult mouse myocardium. In contrast, Orai1 and STIM1 are more abundant in neonatal cardiomyocytes and are downregulated in adult cardiomyocytes. This implies the presence of a maturation-specific regulation network that was not recognized previously.

Second, we showed genetic disruption of Orai3 results in severe myofilament disarray that impacted both sarcomere structure and mitochondrial maturation. Third, we reported that the STIM1/Orai3 mediated Ca\textsuperscript{2+} entry is independent of, and coexists with, the EC-coupling Ca\textsuperscript{2+} machinery. Fourth, loss of Orai3 causes abnormalities of intracellular Ca\textsuperscript{2+} handling resulting in calcineurin signaling cascade activation with detrimental effects on cell survival, cell structure, mitochondrial morphology.

The new information acquired in this study provides potential guidance to improve cellular structural stability and metabolic demands. For example, sarcomere disarray is a typical phenotype observed in various clinical settings, including hypertrophic and DCMs. Furthermore, muscle cytoarchitecture is organized to promote efficient communication between mitochondria, endo/sarcoplasmic reticulum, and sarcomeres, cytoarchitectural perturbations could promote energetic dysfunction.\textsuperscript{42} This indicates that processes which regulate sarcomere organization are likely to be key mechanisms whose manipulation can improve functional integrity of the sarcomere and energy transfer.

We found that Orai3 is the main isoform expressed in the adult mouse cardiomyocyte, while Orai1 and Orai2 proteins were undetectable. In contrast, NRCMs express high levels of Orai1 and STIM1 with low levels of Orai2 and Orai3 protein. RNA-Seq transcriptomic of developmental changes in Orai3 expression across 4 developmental stages in rats agree with our results.\textsuperscript{43} Furthermore, many laboratories showed undetectable Orai1 and Orai2 proteins expression in the adult mouse myocardium.\textsuperscript{12,13} Our findings are in line with the absence of a cardiac phenotype in patients with loss-of-function mutations in Orai1 and with a recently described cardiac-specific Orai1 knockout mouse, which lacks a cardiac phenotype under normal conditions and shows no protection against pathological cardiac hypertrophy.\textsuperscript{21} However, it is still possible that low levels of Orai1 protein is expressed in the adult mouse cardiomyocytes that may interact with Orai3 to form heterodimers that may or may not require STIM1 activation and are sensitive to arachidonic acid.\textsuperscript{44,45} Previous studies in non-excitatory cells showed that Orai3 channel activity is significantly weaker than Orai1 and that Orai3 can heterodimerize with Orai1 to dampen the activity of store-operated Ca\textsuperscript{2+} entry.\textsuperscript{46} Therefore, it can be argued that the weaker activity of Orai3 channels is better suitable to coexist with the EC-coupling in cardiomyocytes. Further studies are needed to shed light on the biological reason for these findings.

Orai3 downregulation achieved by pharmacological treatment or siRNA results in accelerated HF.\textsuperscript{24} Here, we used novel constitutive and inducible Orai3cKO animal models as our key strategy. These models generated new information that significantly updated our previous understanding of the role of Orai3 in cardiomyocytes. For example, we demonstrate that Orai3 is necessary for adult cardiac homeostasis and its deletion causes DCM similarly to invertebrates lacking Orai1, further supporting the idea that Orai3 likely substitutes for Orai1 in the mammalian adult heart.

Our data suggest an increase of TRPC6 expression in Orai3cKO mice. This can be a compensatory response to the loss of Orai3. Indeed, Orai channels coexist with TRPC channels, and they affect each other’s function and expression.\textsuperscript{37} Furthermore, TRPC6, 3 and 1 have been linked to cardiac remodeling through activation of calcineurin.\textsuperscript{48} In our
case, TRPC6 upregulation could provide enough Ca\textsuperscript{2+} entry to activate calcineurin and its downstream target, RCAN-1 (regulator of calcineurin 1). Similar to our results, in zebrafish, Orai1-loss also resulted in calcineurin over-activation.\textsuperscript{37} However, intracellular Ca\textsuperscript{2+} overload because of alteration of SERCA and NCX could contribute to RCAN-1 activation. Further investigation will have to clarify these modalities in cardiomyocytes.

Orai3 genetic deletion from cardiomyocytes results in mitochondrial dysfunction and elevated apoptosis in the Orai3-null myocardium. Indeed, constitutive rise in intracellular Ca\textsuperscript{2+} activates calcineurin, which induces dephosphorylation and translocation of DRP1, triggering exaggerated mitochondrial fission, mitochondrial fragmentation, and loss of ΔΨ\textsubscript{m}, known hallmark of early apoptosis,\textsuperscript{49} and are consistent with our findings. Interestingly, disorders in the mitochondrial organization and the presence of abnormally small and fragmented mitochondria have been observed in end-stage human DCM, myocardial hibernation, and ventricular-associated congenital heart disease.\textsuperscript{50}

We show, for the first time, that in NRCM, STIM1/Orai3 microdomain can trigger Ca\textsuperscript{2+} entry that is distinct from the global intracellular Ca\textsuperscript{2+} transient observed during the EC-coupling. Such microdomains coexist with the global Ca\textsuperscript{2+} transients in electrically paced NRCMs. Remarkably, despite a dramatic rise in local Ca\textsuperscript{2+} in correspondence with STIM/Orai3 domains, the signal remained spatially constrained without affecting the global Ca\textsuperscript{2+} transient. These findings concur with previous observations that in cardiac and skeletal muscle, STIM1 clusters independently of the endo/sarcoplasmic reticulum Ca\textsuperscript{2+} load.\textsuperscript{12,25,51} We show an unprecedented phenomenon of STIM1/Orai3 channel activation, where only a few of the STIM/Orai clusters trigger Ca\textsuperscript{2+}-entry in NRCMs. Although we show that Orai3 is activated by intracellular Ca\textsuperscript{2+} store depletion, the mechanisms leading to Orai3-mediated Ca\textsuperscript{2+} entry in cardiomyocytes need to be further elucidated.

Altogether, these data suggest that Orai3-signaling is involved in the regulation gene transcription program that maintain sarcomere integrity and plays an important role in cellular energy balance as well as in stress response to pressure-induced cardiac stress. Future studies should be conducted to explore the potential for Orai3 as a therapeutic target for patients with HF.

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**Disclosures**

None.

**Supplementary Material**

Video S1

Figures S1–S5
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SUPPLEMENTAL MATERIAL
Figure S1. Age-dependent STIM1, Orai1, and Orai3 expression.

A) Western blot showing STIM1, Orai1, and Orai3 in hearts from adult (three months old) and neonatal (2-3 days old) rats. B) Quantitative analysis of Western blot data, protein expression, normalized to total protein. Values represent means ± S.E.M, (N = 5 adult rats, and 5 independent NRCM isolation), (P<0.05). neonatal rat cardiomyocytes – NRCM; stromal interavtion molecule 1 - STIM1.
A-B) Representative Ca^{2+} traces from fura-2-loaded adult cardiomyocytes treated with thapsigargin in zero extracellular Ca^{2+}. Buffer with Ca^{2+} was later added and store operated Ca^{2+}-entry was captured by fura-2 in ctrl cells, but it was absent in cardiomyocytes isolated from Oria3^{cKO} mice. All data were collected in multiple myocytes from three to six mice, P<0.05.
Figure S3. STIM1 and Orai3-GECO expression in NRCM.

A) Micrographs of NRCM transiently transfected with STIM1-mCherry (a), Orai3-GECO (b), and merged (c). Note that 2 cells express Orai3-GECO, only one express STIM1-mCherry. Arrowheads indicate selected Orai3 puncta, probably interacting with endogenous STIM. D-F) magnified insets. (Scale bar, 20 µm). B) NRCM expressing STIM1-mCherry (a), (b-e) consecutive frames showing global Ca\(^{2+}\) transient and Ca\(^{2+}\) rise at the level of the STIM/Orai3 microdomain. genetically encoded calcium indicators for optical imaging - GECO; neonatal rat cardiomyocytes – NRCM; stromal interavtion molecule 1 - STIM1.
Figure S4. Enhanced apoptosis and elongated myocytes in Tam-Orai3\textsuperscript{cko} hearts.

A) Confocal microscopy of control and Tam-Orai3\textsuperscript{cko} heart sections stained with DAPI and TUNEL show TUNEL-positive cells (red) Tam-Orai3\textsuperscript{cko}. Scale bar = 100\,\textmu m B) TUNEL-positive cells quantification showing a higher incidence of apoptotic events in Tam-Orai3\textsuperscript{cko} sections. C) Cell length of single cardiomyocytes isolated from controls and Tam-Orai3\textsuperscript{cko} and ctrl hearts. D-E) Quantification and analysis of cell area and cell length from controls and Tam-Orai3\textsuperscript{cko} hearts. Scale bar = 100\,\textmu m 4',6-diamidino-2-phenylindole – DAPI; Tamoxifen – Tam; Terminal deoxynucleotidyl transferase dUTP nick end labeling – TUNEL.
Figure S5. The full membranes used for DRP-1, NCX, and SERCA detection stained for total protein and used for normalization.

dynamin-related protein 1 - DRP-1; sodium-calcium exchanger – NCX; sarco/endoplasmic reticulum Ca2+-ATPase – SERCA.
Supplemental Video Legend:

Video S1. NRCM expressing STIM1-mCherry and Orai3-GECO (only Orai3-GECO was visible in the green channels). The cell was paced at 0.5Hz and a microdomain can be observed at around 10 seconds from the beginning. Frames from this video were extracted and showed in Figure 5 and Figure S3B. Best viewed with Windows Media Player.