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Summary

Mutations in the store-operated Ca^{2+} entry pore protein ORAI1 have been reported to cause myopathies in human patients but the mechanism involved is not known. Cardiomyocytes express ORAI1 but its role in heart function is also unknown. Using reverse genetics in zebrafish, we demonstrated that inactivation of the highly conserved zebrafish orthologue of ORAI1 resulted in severe heart failure, reduced ventricular systolic function, bradycardia and skeletal muscle weakness. Electron microscopy of Orai1-deficient myocytes revealed progressive skeletal muscle instability with loss of myofiber integrity and ultrastructural abnormalities of the z-disc in both skeletal and cardiac muscle. Isolated Orai1-deficient cardiomyocytes showed loss of the calcineurin-associated protein calsarcin from the z-discs. Furthermore, we found mechanosignal transduction was affected in Orai1-depleted hearts, indicating an essential role for ORAI1 in establishing the cardiac signaling transduction machinery at the z-disc. Our findings identify ORAI1 as an important regulator of cardiac and skeletal muscle function and provide evidence linking ORAI1-mediated calcium signaling to sarcomere integrity and cardiomyocyte function.

Key words: ORAI1, CRAC, Heart failure, Myopathy, Ca^{2+} signaling

Introduction

Calcium signaling plays a fundamental role in muscle cells, including regulation of growth and differentiation, metabolism and gene expression. In addition, altered calcium signaling plays an important role in the pathophysiology of cardiac diseases such as myocardial infarction and pathological hypertrophy. Ca^{2+}-release-activated Ca^{2+} entry (CRAC) is a established mechanism for nonexcitable cells to replenish intracellular calcium stores and for subsequent Ca^{2+}-dependent signaling cascades (Feske, 2007). Calcium-release-activated calcium channel protein 1 (ORAI1), the calcium-selective pore that mediates CRAC current (Prakriya et al., 2006; Vig et al., 2006), was shown to be expressed in isolated cardiac myocytes and is an important regulator involved in hypertrophic growth in neonatal cardiomyocytes (Voelkers et al., 2010). ORAI1 mutations or deficiencies cause myopathy (McCarl et al., 2009). ORAI1-deficient mice die of unknown cause shortly after birth, and the role of ORAI1 in intact hearts is unknown (Gwack et al., 2008; Vig et al., 2008). To examine the role of ORAI1 in the intact heart in vivo, we used reverse genetics in zebrafish, an intriguing model organism for evaluating effects of gene loss on cardiovascular function (Hassel et al., 2009; Nasevicius and Ekker, 2000; Rottbauer et al., 2005; Sehnert et al., 2002), because the absence of blood flow for several days does not affect development of other organs (Shin and Fishman, 2002). We provide evidence that Ca^{2+} entry through ORAI1 is necessary to maintain sarcomere integrity and establish proper z-disc function, in part through regulation of the localization of the calcineurin-associated protein calsarcin. Our results have identified a mechanism by which excitable cells spatially control Ca^{2+}-dependent signaling events, independently from excitation contraction coupling.

Results and Discussion

ORAI1 is expressed in the mouse heart in vivo

Immunohistochemistry of mice hearts at different stages showed ubiquitous ORAI1 expression in embryonic tissues, including the heart (Fig. 1A) (Gwack et al., 2008; Vig et al., 2008). *Orai1* mRNA expression was largely decreased at 2 months compared with 2 days after birth (supplementary material Fig. S1A). In adult hearts, ORAI1 localized to the plasma membrane of cardiomyocytes and was highly expressed in the vasculature (Fig. 1B). In addition, ORAI1 was detected using a proximity ligation assay in adult hearts (Fig. 1C) as well as in isolated adult cardiomyocytes (supplementary material Fig. S2C), suggesting that ORAI1 is expressed throughout development and in adult cardiomyocytes.

Identification and expression of the zebrafish ORAI1 orthologue

To evaluate the role of ORAI1 in cardiac function in zebrafish, we identified the zebrafish orthologue (zOrai1 for zebrafish Orai1), which shows high evolutionary conservation to the mouse (77%) and human (76%) proteins (supplementary material Fig. S1B). Using whole-mount in situ hybridization,

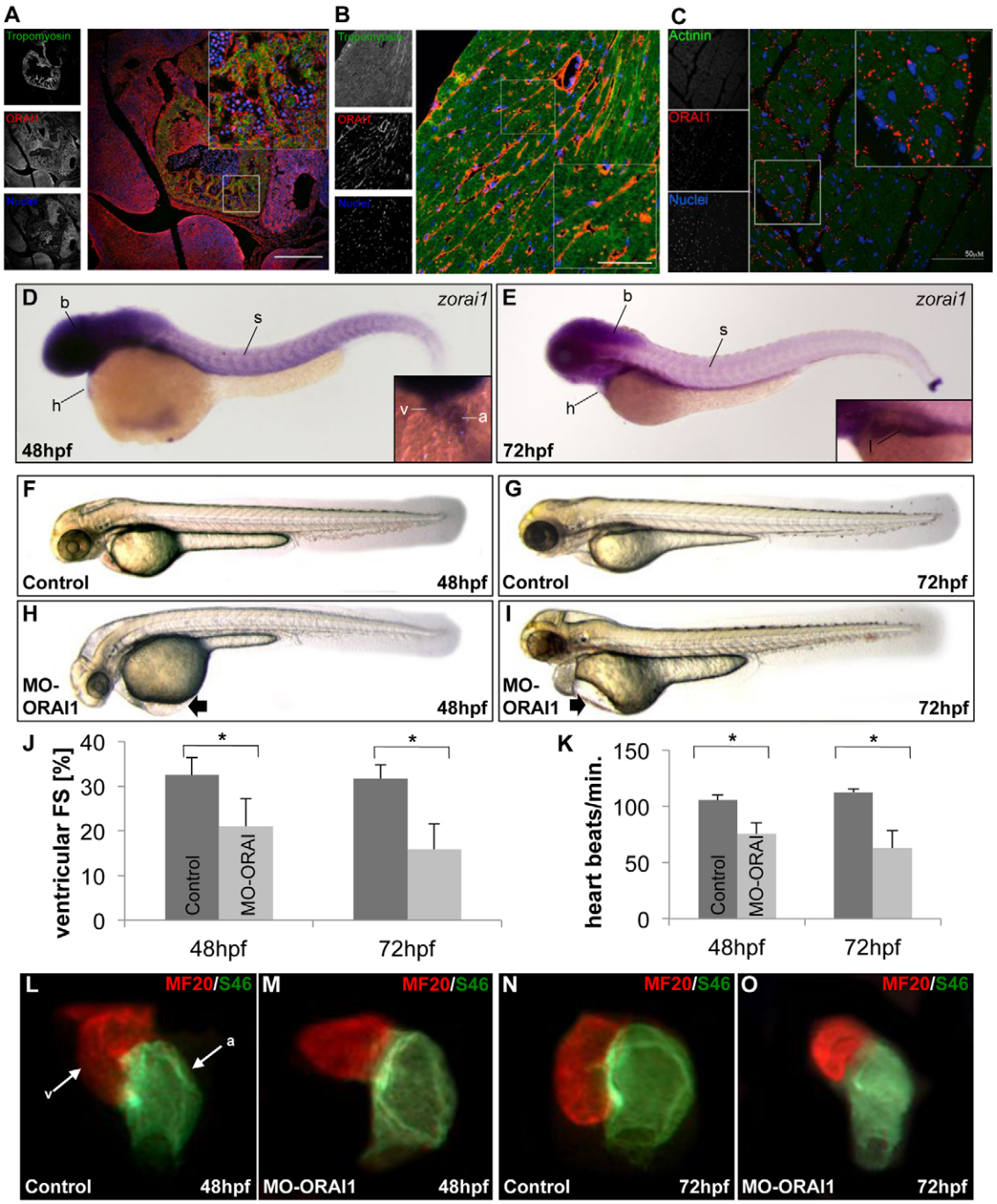

Fig. 1. *Orai1* expression in mice and zebrafish and consequences of ORAI1 knockdown. (**A–C**) Representative immunostaining with antibodies against tropomyosin (green), ORAI1 (red) and nuclei (Sytox Blue) show ORAI1 expression in mouse cardiomyocytes at 11 days postcoitum (A) and 2 months after birth (B). (C) Proximity ligation assay confirmed ORAI1 expression in the adult mouse heart in vivo. Note that one red dot represents one ORAI1 protein complex. (**D**,**E**) In situ hybridization of *orai1* (*zorai1*) in zebrafish at 48 (D) and 72 (E) hpf shows high expression in brain (b), heart (h; and inset in D), somites (s) and liver (l; inset in E). (**F–I**) MO-ORAI1-injected embryos display characteristics of cardiac dysfunction at 48 hpf that had progressed at 72 hpf (H,I) and was not seen in controls (F,G): diminished blood flow and pericardial edema and blood congestion at the sinus venosus (arrow). (**J**,**K**) Ventricular fractional shortening (FS; J) and heart rate (K) in control and MO-ORAI1-injected embryos. Values are means ± s.d.; $*P<0.05$, $n\geq10$. (**L–O**) Immunostaining for atrium- and ventricle-specific myosin heavy chains (MF20 and S46, respectively) show that these are expressed normally in ORAI1-deficient (M,O) embryos; (L,N) controls.

we detected *zorai1* transcripts ubiquitously in various tissues, including heart, throughout development (Gwack et al., 2008; Vig et al., 2008). At 24 hours postfertilization (hpf), *zorai1* was expressed ubiquitously (not shown). By 48 hpf, *zorai1* expression was more restricted, with pronounced expression in brain, liver, somites and heart (Fig. 1D). This pattern was maintained at 72 hpf, with diminishing expression in somites (Fig. 1E).

Orai1 is required for proper heart and skeletal muscle function

Next, we inactivated Orai1 by injecting morpholino-modified antisense oligonucleotides targeting the splice donor site of exon 1 (MO-ORAI1) or the translational start site (MO-ORAI1^{ATG}) into one-cell zebrafish embryos (supplementary material Fig. S1C). Identical phenotypes were obtained with both morpholino oligonucleotides, whereas embryos injected with a control oligonucleotide developed normally. MO-ORAI1-injected embryos were indistinguishable from controls on day 1 of development. By 48 hpf, Orai1-deficient embryos developed severe heart failure, decreased blood circulation and blood congestion at the inflow tract (Fig. 1F–I; supplementary material Movie 1), and most failed to hatch, indicating reduced skeletal muscle force generation. Ventricular contractility, determined by ventricular fractional shortening, was reduced at 48 hpf and further reduced at 72 hpf (Fig. 1J; supplementary material Movie 2). Orai1-deficient embryos developed severe bradycardia by 48 hpf overlapping with onset of reduced contractility (Fig. 1K). Maturation of chamber myocardium proceeded normally with proper growth of ventricular myocardium, and atrial and ventricular cardiomyocytes expressing myosin heavy chains in the regular chamber-specific pattern (Fig. 1L–O; supplementary material Fig. S2B). Furthermore, Orai1-depleted hearts properly expressed cardiac marker genes (supplementary material Fig. S2A). Hence, ORAI1 deficiency caused severe heart failure, bradycardia and skeletal muscle weakness without affecting early cardiogenesis and cardiomyocyte differentiation.

In mice, ORAI1-deficiency causes muscle weakness (Gwack et al., 2008; Vig et al., 2008) and perinatal death, probably from myopathy. Mice lacking STIM1, the calcium sensor of the endoplasmic and sarcoplasmic reticulum required to activate ORAI1, also develop skeletal myopathy and severe immunodeficiency, suggesting that CRAC signaling is essential for normal muscle function (Stiber et al., 2008). All humans with ORAI1 mutations have skeletal myopathy and usually die from severe immunodeficiency and lethal infections by 1 year of age (Feske et al., 2006; McCarl et al., 2009). No assessment of cardiac performance in such patients has been reported so far. We demonstrate for the first time that loss of ORAI1 causes heart failure, without affecting initial myocyte differentiation.

Loss of zebrafish Orai1 leads to loss of skeletal and cardiac muscle integrity

Many skeletal myopathies cause structural and ultrastructural abnormalities in myocytes. We determined whether skeletal muscle weakness in Orai1-deficient embryos reflected altered muscle organization. Immunofluorescence analysis of MO-ORAI1-injected embryos revealed frequent disruption of myofilaments and detachment of myofibrils from myosepta at 48 hpf, with increased severity at 72 hpf, leading to myofiber-free spaces in somites, similar to that seen in mutants deficient for dystrophin–glycoprotein-complex-associated proteins or integrin-linked kinase (Cheng et al., 2006; Guyon et al., 2005; Postel et al., 2008) (Fig. 2A–D). Additionally, at 72 hpf, myofiber density within the somites appeared to be reduced.

Detachment of myofibers is often accompanied by loss of myoseptal integrity (Etard et al., 2010; Parsons et al., 2002; Postel et al., 2008). To evaluate whether loss of Orai1 function affects myoseptal integrity, we performed immunostaining against vinculin, an adaptor protein linking muscle fibres, through integrins, to the extracellular matrix (Critchley, 2000; Postel et al., 2008). At 48 hpf, vinculin level was reduced in myosepta of Orai1-deficient embryos, and vinculin localization at the somite boundaries was disturbed and present ectopically in somites (Fig. 2E,F), indicative for loss of myoseptal integrity (Etard et al., 2010; Postel et al., 2008). At 72 hpf, a stage with advanced myofiber disruption, vinculin levels further declined and mislocalization increased in Orai1-deficient skeletal muscle. Myosepta frequently appeared as tattered, blurry stripes rather than distinct, thin lines separating adjacent somites, as in controls. Thus, loss of Orai1 in zebrafish caused progressive disruption of myofibers and loss of myoseptal integrity, suggesting that Orai1 function is not required for initial muscle assembly but essential for maintaining and straightening of muscle structure. The decreasing myofiber density in somites at 72 hpf suggests that reduced Orai1 disturbs muscle fiber growth and thereby adaptation of muscle force to higher demands during development.

To elucidate the mechanism that effects muscle integrity, we analyzed the ultrastructure of skeletal muscle cells in MO-ORAI1-injected embryos. At 72 hpf, myofibrils in controls were densely packed with precisely aligned and highly organized thick and thin filaments delimited by well-defined z-discs (Fig. 2I). By contrast, even though organized sarcomeric units were generally present, the myofibrils appeared thin and instead of being densely packed, individual myofibrils were separated by cytoplasm (Fig. 2J). Frequently, myofibrils changed from being well organized to disorganized within one to two sarcomeric units, with progressively dispersing thin and thick filaments (Fig. 2J). Additionally, instead of the well-contrasted and distinct z-discs seen in control embryos, the z-lines in MO-ORAI1-injected skeletal muscle cells appeared faint.

To our knowledge, ORAI1-deficient myocytes have not been thoroughly analyzed. Consistent with loss of ORAI1, ultrastructural evaluation of STIM1-deficient skeletal muscle similarly showed muscle atrophy (Stiber et al., 2008). Additionally, we found that Orai1 inactivation caused abnormalities of myofibril organization and of the z-line in skeletal and cardiac muscle in zebrafish, characteristics not described for STIM1-deficient mice.

We speculated that defects similar to those in skeletal muscle cells might explain the decreased contractility in Orai1-deficient hearts. Cardiomyocytes from MO-ORAI1-injected embryos similarly had fewer, thinner myofibrils than control cardiomyocyte (Fig. 2K,L). The z-discs between sarcomeres in Orai1-deficient cardiomyocytes were stretched, blurry and often seemingly absent. Thus, Orai1 deficiency induces similar defects in skeletal and cardiac myocytes.

The fact that correctly assembled, although thinner and less densely packed, sarcomeres were present in both skeletal and cardiac tissues suggests that Orai1 loss does not impair initial sarcomerogenesis but disrupts myofibril growth, possibly limiting adaptation of muscle force to increasing demands during development. Our data on vinculin, as well as the aggravation of myofiber disruption, further suggests that ORAI1-deficiency affects sarcomere stability in part by altering the integrity of adhesion complexes linking muscle fibers through adaptor proteins to the extracellular matrix. Because z-discs are crucial for sarcomere stability, the abnormalities in Orai1-deficient skeletal and cardiac z-discs might contribute to the instability and disaggregation of myofibrils (Hassel et al., 2009).

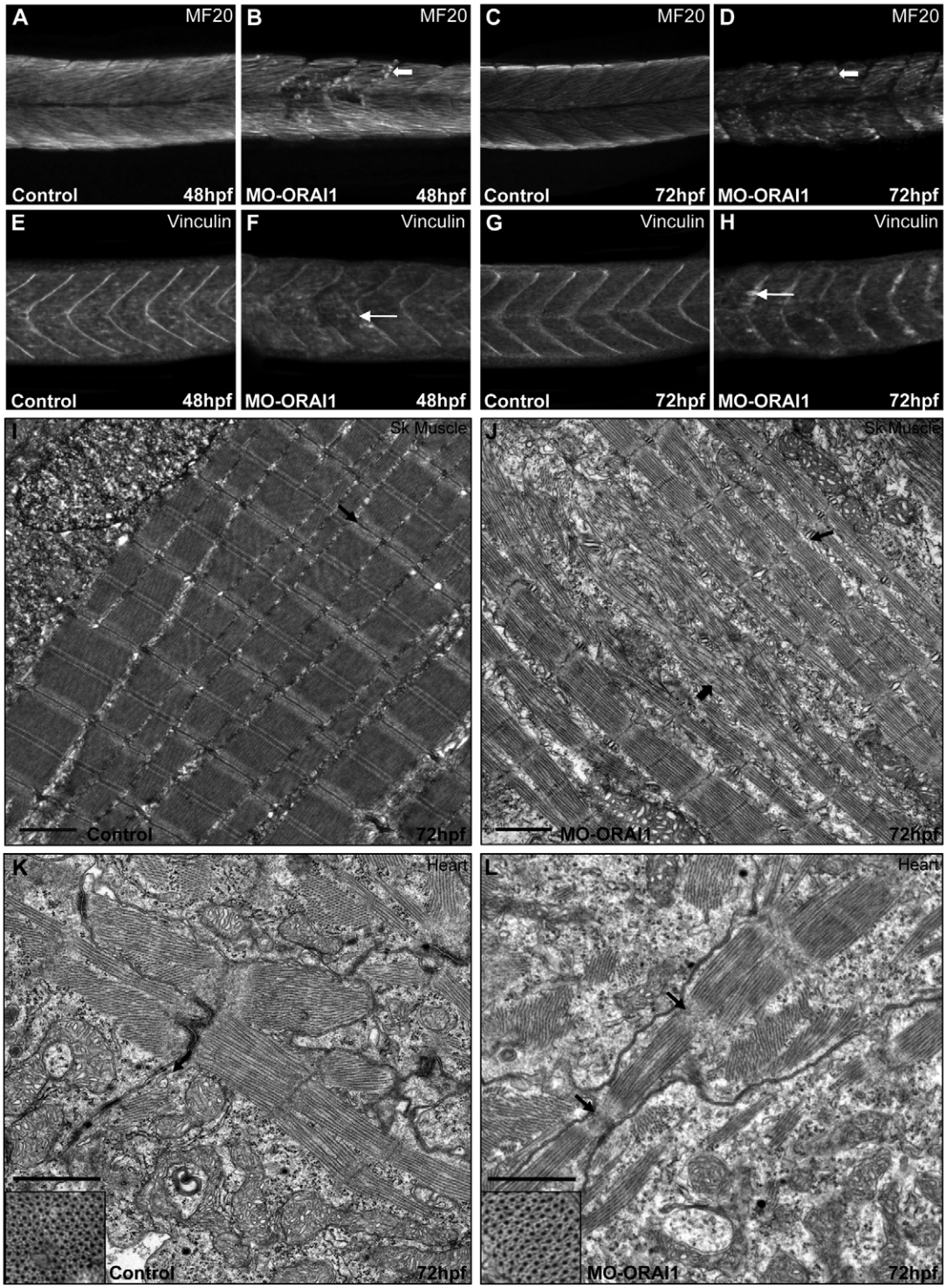

Fig. 2. Consequences of ORAI1-deficiency on skeletal and cardiac muscle. (**A–D**) Immunostaining with anti-myosin (MF20) reveals progressive myofilament disorganization and frequent detachment of myofibrils from the myosepta (bold arrow) in Orai1-deficient zebrafish embryos at 48 (B) and 72 (D) hpf, compared with controls (A,C). (**E–H**) Immunofluorescent staining for vinculin reveals disturbed somite boundaries and diminished expression and mislocalization (arrow) in Orai1-deficient embryos at 48 (F) and 72 (H) hpf compared with controls (E,G). (**I–L**) Transmission electron micrographs of MO-ORAI1-injected embryos at 72 hpf reveal fewer and thinner myofibrils in skeletal muscle cells (J) than in control muscle (I). Myofibrils often collapsed gradually (bold arrow in J). Z-discs often appeared to be missing (arrow). Ultrastructural analysis in Orai1-deficient cardiomyocytes (L) and control cells (K) similarly revealed fewer and thinner sarcomeres flanked by stretched and barely noticeably z-lines (arrows in L). Cross sections through cardiac sarcomeres displayed normal primary organization of thick and thin filaments in hexagonal lattices (insets in K and L). Scale bars: 1 μm.

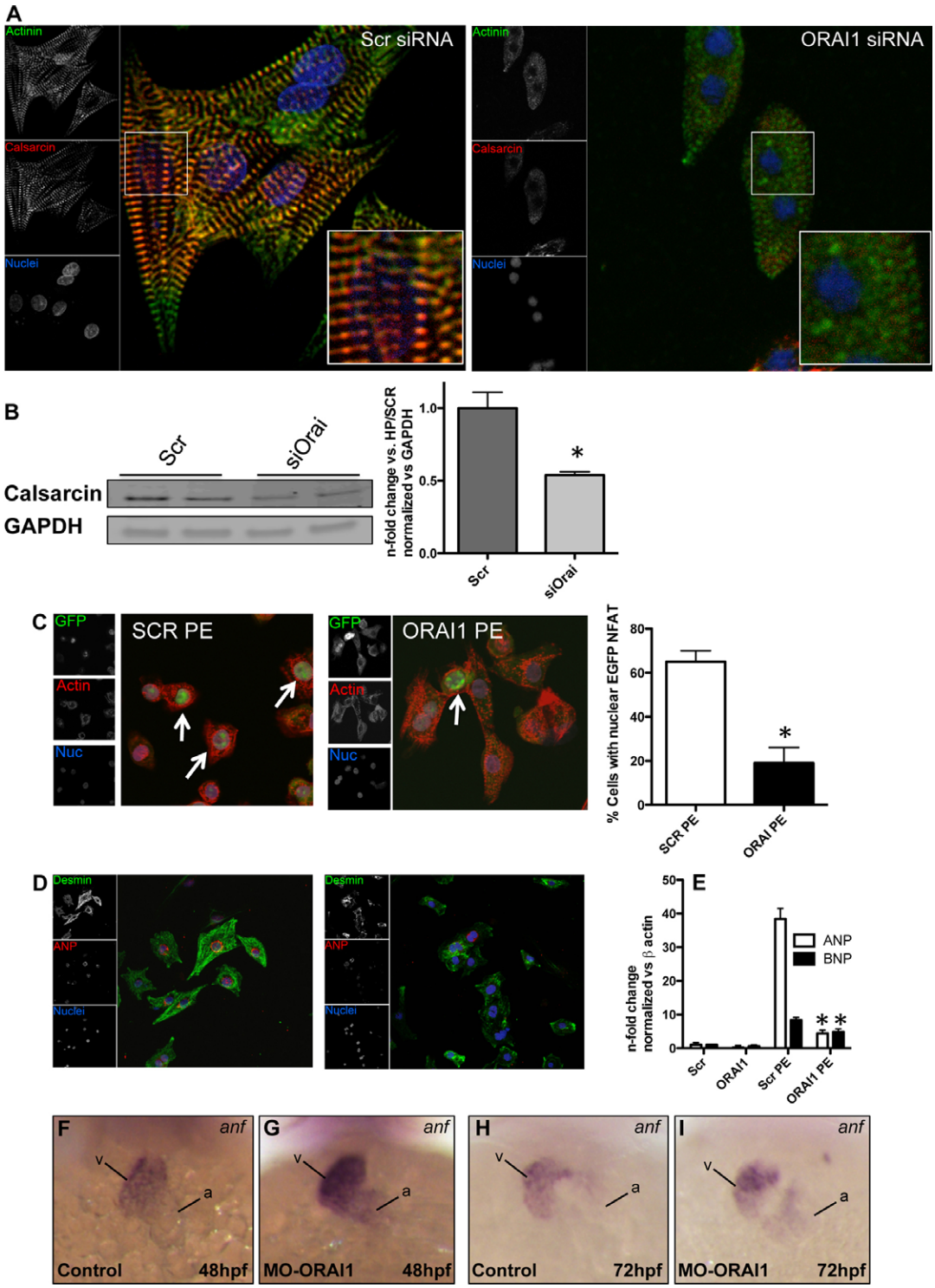

Fig. 3. Loss of ORAI1 signaling in cardiomyocytes leads to impaired calsarcin localization and defective stretch sensing. (**A**) Immunostaining of mouse cardiomyocytes with anti-actinin (green) and anti-calsarcin shows z-disc localization of calsarcin in control cardiomyocytes and severe sarcomeric disorganization and growth defects in ORAI1-deficient cardiomyocytes with absence of calsarcin at the z-disc. Insets show higher magnification. (**B**) Representative western blot and quantification of calsarcin expression after ORAI1 knockdown in cardiomyocytes. (**C**) Nuclear localization of NFAT was analyzed using a EGFP–NFAT adenovirus. After treatment with phenylephrine (PE) more than 60% of the cells showed nuclear localization of NFAT, whereas ORAI1 KD prevented the nuclear localization of NFAT ($n=2$ experiments; $^*P<0.05$). White arrows indicate cells with nuclear localization of NFAT. (**D**) Immunostaining against ANP shows fewer ANP-positive nuclei after ORAI1 KD. Cells were treated for 25 hours with phenylephrine. (**E**) RT-PCR shows that the induction of the 'fetal gene program' (ANP and BNP) is blunted after ORAI KD. Cells were treated for 24 hours with phenylephrine ($n=2$ experiments; $^*P<0.05$). (**F–I**) RNA levels of the stretch-responsive gene *anf* are elevated in MO-ORAI1-injected zebrafish embryos. Compared with the expression of *anf* in control hearts at 48 (F) and 72 (H) hpf, *anf* expression in ORAI1-deficient hearts was upregulated at 48 (G) and 72 (I) hpf.

Loss of ORAI1 impairs expression and localization of calcium-dependent signaling components in cardiomyocytes

Phenylephrine-induced hypertrophic growth of neonatal rat ventricular cardiomyocytes (NRCM) represents an excellent model to study myofibril addition and sarcomere growth in vitro. To test our hypothesis that ORAI1 is necessary for sarcomeric growth, we decreased ORAI1 expression in neonatal rat ventricular cardiomyocytes with ORAI1-specific short interfering RNA (siRNA). At 48 hours after transfection, ORAI1 expression was reduced by 61% (supplementary material Fig. S3A,B). Treatment with phenylephrine increased cell surface area 2.3-fold at 24 hours together with a remarkable organization and growth of the sarcomeric structure (supplementary material Fig. S3C,D). However, si*Orai1*-treated cells failed to organize sarcomeres and hypertrophic growth was considerably attenuated (1.4-fold increase of the cell surface area over baseline).

Next, we analyzed the z-disc structure of neonatal cardiomyocytes by immunostaining for actinin and calsarcin. ORAI1 is implicated in regulating calcium–calcineurin–NFAT signaling. Calcineurin binds to calsarcins, a family of striated muscle-specific proteins located at the sarcomeric z-disc, and calsarcin-deficiency results in z-disc remodeling (Frank et al., 2007). In si*Orai1*-transfected cardiomyocytes, sarcomeres were thinner and less organized than in controls (Fig. 3A). Interestingly, calsarcin failed to localize to z-discs, and global calsarcin expression was decreased (Fig. 3A,B). These findings support our in vivo data in that the Ca^{2+} entry through the ORAI1 channel is essential for proper sarcomeric growth and function in cardiomyocytes, and furthermore indicate that ORAI1 is required for localization of the calcineurin–NFAT signaling component calsarcin to the cardiac z-disc. This finding is supported by the notion that analysis of the nuclear localization of NFAT using an EGFP–NFAT adenovirus is prevented by ORAI1 knockdown (KD; Fig. 3C). In addition the induction of the 'fetal gene program' is blunted in ORAI1 KD cardiomyocytes (Fig. 3D,E).

Calsarcin depletion was previously shown to result in the activation of calcineurin–NFAT-mediated hypertrophic gene transcription (Frey et al., 2004). Because calsarcin inhibits calcineurin signaling, global downregulation of calsarcin might represent a compensatory attempt to restore defective calcineurin signaling after ORAI1 knockdown in vivo. Importantly, calsarcin interacts with important components of the z-disc, including telethonin (also known as T-Cap), Cypher (ZASP) and myotilin, all of which can cause cardiomyopathy or muscular dystrophy when dysfunctional, highlighting the importance of calsarcin–calcineurin–NFAT regulation by ORAI1 (Frank et al., 2006; Hayashi et al., 2004; Moreira et al., 2000; Olive et al., 2005; Selcen and Engel, 2005; Vatta et al., 2003). Further analysis in adult cardiomyocytes lacking ORAI1 are needed to determine the molecular mechanism in more detail, but are hindered by the perinatal death of ORAI1-deficient mice (Gwack et al., 2008).

ORAI1-deficient cardiomyocytes develop disturbed stretch sensing

Besides cross-linking thin filaments and stabilizing sarcomeres by transmission of force generated during contraction, z-discs are nodal points for mechanotransduction (i.e. stretch sensing) (Frank et al., 2006). Given the ultrastructural abnormalities and mislocalization of calsarcin (an important Ca^{2+}-signaling component) in ORAI1-deficient z-discs, we hypothesized that ORAI1 is required to establish a functioning, properly signal-transducing z-disc. Therefore, we assessed expression of the stretch-responsive gene encoding atrial natriuretic factor (*anf*) by whole-mount RNA in situ hybridization (Bendig et al., 2006). At 48 hpf *anf* was markedly upregulated, and still slightly increased at 72hpf (Fig. 3F–I), indicative of cardiac dysfunction and impaired stretch sensing in zebrafish (Bendig et al., 2006). Calcineurin interacts directly with the mechanosignaling transduction protein muscle-LIM-protein (MLP), and calsarcin and calcineurin are displaced from the z-disc upon MLP depletion (Heineke et al., 2005). Animals lacking calcineurin or calsarcin express higher levels of *Anf*, endorsing our data in zebrafish (Frey et al., 2004; Schaeffer et al., 2009).

ORAI1 expression is increased in response to cardiac damage

Our results show that ORAI1 is necessary for muscle growth and physiological adaptation to increasing performance requirements. To determine whether ORAI1 signaling contributes to pathological aspects of hypertrophy, we analyzed ORAI1 expression in mice after myocardial infarction or transaortic constriction (TAC) to induce pressure overload-mediated

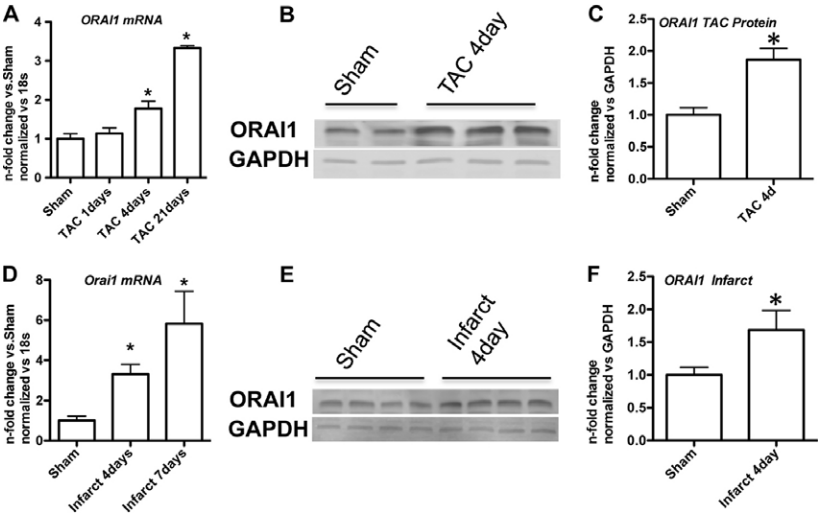

Fig. 4. Upregulation of ORAI1 expression after pathological challenge in mice. (**A–C**) *Orai1* mRNA level (A) and protein expression (B,C) have increased 4 days after TAC. (**D–F**) *Orai1* mRNA (D) and protein (E,F) levels are increased 4 days after myocardial infarction (*$P<0.05$).

hypertrophy. Sham-operated mice served as controls. After TAC, ORAI1 protein and mRNA increased significantly ($P<0.001$; Fig. 4A–C) and the fetal gene program was reactivated (data not shown). ORAI1 expression also increased after myocardial infarction (Fig. 4D–F). Thus, ORAI1 might contribute to the pathology of hypertrophy, even in humans, although the molecular mechanism is unknown.

In summary, we confirmed that loss of Orai1 causes skeletal muscle weakness in zebrafish (Gwack et al., 2008; McCarl et al., 2009). Structural and ultrastructural analysis revealed that the weakness is due to myofibrillar disruption and progressive mechanical instability in skeletal myocytes, both distinctive features of muscular dystrophy in humans and zebrafish (Emery, 2002; Postel et al., 2008). Furthermore, we show, for the first time, that Orai1 deficiency in zebrafish causes heart failure. Sarcomeres in Orai1-deficient hearts exhibit lateral growth and ultrastructural abnormalities at the cardiac z-disc with decreased expression and displacement of calsarcin from the z-disc. Loss of Orai1 also caused defective signal transduction at the cardiac z-disc. Our findings link ORAI1-mediated calcium signaling to sarcomere physiology, in part by affecting z-disc composition and function mediated by the calcineurin-calsarcin-NFAT signaling pathway. Thus, the molecular machinery mediating SOCE might represent a valuable pharmacological target to modulate Ca^{2+}-dependent signaling to improve the outcome of pathological cardiac hypertrophy.

Materials and Methods

Zebrafish care and breeding, injection procedures and functional analysis

Care and breeding of zebrafish (*Danio rerio*) was carried out as previously described (Westerfield, 1995). Morpholino-modified antisense oligonucleotides were designed against the translational start site of zebrafish ORAI1 [NM_205600] (MO-ORAI1ATG 5′-AGTGCTCGCTCCGACTCATCTTCAT-3′) and the splice donor site of zebrafish ORAI1 exon 1 (MO-ORAI1 5′-AAACAGCGCGGAGACTC-ACCATTGC-3′). Functional assessment of cardiac contractility was carried out essentially as described previously (Rottbauer et al., 2005).

Myocardial infarction and transaortic constriction

Surgical procedures on mice have been described previously (Muraski et al., 2007). Briefly, myocardial infarction was produced by ligating the left anterior descending coronary artery with an 8-0 suture (Ethicon). For trans-aortic constriction (TAC), the aorta was ligated between the innominate artery and the left common carotid artery by using a 7-0 polypropylene suture (Ethicon) with an overlying 27-gauge needle to produce a discrete stenosis. All animal experiments were performed according to the relevant regulatory standards.

Whole-mount in situ hybridization, immunohistochemistry, histology and transmission electron microscopy

Whole-mount in situ hybridization and immunofluorescence staining of zebrafish embryos was carried out essentially as described previously (Hassel et al., 2009). The following antibodies were used: MF20 (1:20; Developmental Studies Hybridoma Bank, developed by D. A. Fishman) and anti-vinculin (1:50; Sigma). 72 hpf control and MO-ORAI1-injected embryos were used for histological analysis of the heart. Histological sections were prepared as described previously (Rottbauer et al., 2001). Transmission electron microscopy analysis was carried out as described by Kurrasch et al. (Kurrasch et al., 2009).

Immunohistochemistry of mouse sections have been described before (Muraski et al., 2007). In brief, hearts were flushed with formalin for 15 minutes, excised, and fixed in formalin for 24 hours at room temperature. Sections were cut and deparaffinized using standard procedures. Primary antibodies were applied overnight at 4℃ in nitro blue tetrazolium. The *Orai1* signal was detected with a Tyramide Signal Amplification kit (Perkin Elmer), used according to the manufacturer's recommendations. To reveal nuclei, slides were treated with To-pro3 iodide (Molecular Probes; 1:10,000) for 20 minutes in 1× TN (Tris-based neutral buffer), washed, and a coverslip place on top. Micrographs were acquired with a Leica TCS-SP2 confocal laser scanning microscope. Neonatal rat ventricular cardiomyocytes (NRCMs) grown on glass coverslips were treated with *Orai1* or scrambled siRNA as described before (Voelkers et al., 2010). After 48 hours cells were fixed, permeabilized and stained using standard procedures. A proximity ligation assay to detect ORAI1 was used. Blocking, staining, hybridization, ligation, amplification and detection steps were performed according to manufacturer's instructions (OLINK).

Western blot analysis

Whole-cell lysates of hearts and NRCM lysates were prepared in 1× SDS sample buffer and immunoblotted. Primary antibodies used were against GAPDH (Invitrogen) and Orai1 (Santa Cruz Biotechnology). Calsarcin antibody was a gift from Norbert Frey (Kiel, Germany). The day after application of the antibodies, blots were washed three times with Tris-buffered saline plus 0.1% Tween-20 (TBS-T), probed with fluorescent or alkaline phosphatase–conjugated secondary antibodies (1:2000 in blocking solution; Jackson Immunoresearch Laboratories) for 2 hours at room temperature, washed three times with TBS-T, and scanned.

Isolation and primary culture of neonatal and adult ventricular cardiomyocytes

Ventricular cardiomyocytes from 1- to 2-day-old rat neonatal hearts (NRCMs) were prepared by trypsin digestion as described previously (Voelkers et al., 2010). Adult ventricular cardiomyocytes were isolated using standard procedures as previously described (Most et al., 2006). In a subset of experiments NRCMs transfected with the siRNAs were transfected on the same day with an EGFP–NFAT adenovirus and the cellular localization of EGFP–NFAT were analyzed 48 hours after transfection and 24 hours after treatment with phenylephrine.

RNA interference

Custom-designed synthetic *orai1* siRNA and scrambled siRNA as a negative control were 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

Total RNA was isolated from frozen heart or cultured cells with Quick-RNA MiniPrep (Zymo Research) and reverse-transcribed into cDNA with an iScriptcDNA Synthesis kit (Bio-Rad). Real-time PCR was performed on all samples in triplicate with a QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions. All primer sequences are shown supplementary material Table S1.

For RT-PCR analysis, total RNA from hearts derived from control and MO-orai1-injected embryos at 72 hpf was extracted using Trizol reagent (Invitrogen). RNA was subsequently treated with DNase and reverse transcribed using a random hexamer primer and SuperScript II reverse transcriptase (Invitrogen). Gene-specific primer sequences are available upon request. Amplification involved 35–40 cycles. *Elongation factor I alpha* (*elfa*) was used as endogenous control (McCurley and Callard, 2008).

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