Published in final edited form as:

J Mol Cell Cardiol. 2013 July ; 60: 121–128. doi:10.1016/j.yjmcc.2013.04.014.

Functional integrity of the t-tubular system in cardiomyocytes depends on p21-activated kinase 1

Jaime DeSantiago^{1,2}, Dan J Bare^{1,2}, Yunbo Ke^{1,3}, Katherine A. Sheehan^{1,3}, R. John Solaro^{1,3}, and Kathrin Banach^{1,2,*}

¹Center for Cardiovascular Research, University of Illinois at Chicago, 840 S. Wood Street, Chicago, IL 60612, USA

²Dept. of Medicine, Section of Cardiology, University of Illinois at Chicago, 840 S. Wood Street, Chicago, IL 60612, USA

³Dept. of Physiology and Biophysics, University of Illinois at Chicago, 840 S. Wood Street, Chicago, IL 60612, USA

Abstract

p21-activated kinase (Pak1), a serine-threonine protein kinase, regulates cytoskeletal dynamics and cell motility. Recent experiments further demonstrate that loss of Pak1 results in exaggerated hypertrophic growth in response to pathophysiological stimuli. Calcium (Ca) signaling plays an important role in the regulation of transcription factors involved in hypertrophic remodeling. Here we aimed to determine the role of Pak1 in cardiac excitation-contraction coupling (ECC).

Ca transients were recorded in isolated, ventricular myocytes (VMs) from WT and Pak1^{-/-} mice. Pak1^{-/-} Ca transients had a decreased amplitude, prolonged rise time and delayed recovery time. Di-8-ANNEPS staining revealed a decreased t-tubular density in Pak1^{-/-} VMs that coincided with decreased cell capacitance and increased dis-synchrony of Ca induced Ca release (CICR) at individual release units. These changes were not observed in atrial myocytes of Pak1^{-/-} mice where the t-tubular system is only sparsely developed. Experiments in cultured rabbit VMs supported a role of Pak1 in the maintenance of the t-tubular structure. T-tubular density in rabbit VMs significantly decreased within 24h of culture. This was accompanied by a decrease of the Ca transient amplitude and a prolongation of its rise time. However, overexpression of constitutively active Pak1 in VMs attenuated the structural remodeling as well as changes in ECC.

The results provide significant support for a prominent role of Pak1 activity not only in the functional regulation of ECC but for the structural maintenance of the t-tubular system whose remodeling is an integral feature of hypertrophic remodeling.

Introduction

The p21-activated kinase1 (Pak1) is a serine/threonine kinase that was shown to attenuate cardiac hypertrophic growth. It belongs to group I Paks (1–3), which are regulated by auto-

© 2013 Elsevier Ltd. All rights reserved.

*Corresponding Author: Kathrin Banach, Ph.D., Section of Cardiology, Department of Medicine, University of Illinois at Chicago, 840 South Wood Street (MC 715), Chicago, Illinois 60612-7323, Phone: 312 996 5018, kbanach@uic.edu.

Disclosures: None

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

inhibition that can be relieved by GTP bound Rac and Cdc42, members of Rho-related GTPases [1, 2]. Pak1 is abundant in the cardiac muscle [3, 4] and predominantly localizes to the Z-disc, cell and nuclear membranes as well as the intercalated discs [5–7]. In cardiomyocytes Pak1 regulates cellular excitation and contractility. Through the regulation of troponin I and myosin binding protein C phosphorylation, Pak1 enhances myofilament Ca sensitivity [7, 8], while via activation of the phosphatase PP2A [7] it reduces $I_{Ca,L}$, $I_{K,r}$, and connexin 43 phosphorylation thereby attenuating β -adrenergic stimulation and reducing intercellular coupling [5, 6]. Recent work demonstrated that loss of Pak1 signaling resulted in exaggerated cardiac hypertrophic growth in response to isoproterenol (Iso), angiotensin II (AngII) or trans-aortic banding [9, 10]. The negative regulation of Erk- [10] as well as JNK-dependent NFATc3 phosphorylation [9] were proposed as mechanisms by which Pak1 attenuates hypertrophic signaling under patho-physiological conditions.

Over-expression of constitutively active Pak1 was shown to prolong Ca transient decay constant (τ_{Ca}) and decrease the amplitude and width of Ca sparks [8]. In addition, Pak1 as an activator of PP2A promoted anti-adrenergic signaling by attenuating the Iso-induced increase in $I_{Ca,L}$ and phospholamban (PLN) phosphorylation [11–13]. PP2A inhibition itself resulted in increased Ca influx, enhanced spontaneous Ca release from ryanodine receptors (RyR), PLN activity, and decreased myofilament Ca sensitivity [12, 14]. These experiments indicate that modulation of Pak1 activity can have a significant impact on excitation-contraction coupling (ECC).

However, beyond the PP2A dependent regulation of β -adrenergic signaling, other mechanisms by which Pak1 influences cardiac ECC have not yet been identified. Since changes in $[Ca]_i$ play a significant role in the activation of transcription factors that promote hypertrophic remodeling, we tested the hypothesis that lack of Pak1 signaling results in changes of cardiac ECC, which could further contribute to hypertrophic remodeling. We used isolated ventricular myocytes from WT and Pak1^{-/-} mice [10, 15] and determined excitation induced action potentials and Ca transients. Our experimental results demonstrate that attenuation of Pak1 signaling induces a significant change in ECC that can in part be explained by sub-cellular remodeling of VMs. Sub-cellular remodeling is a characteristic feature of hypertrophic remodeling making Pak1 an attractive pharmacological target in the treatment of cardiac hypertrophy.

Methods

Ventricular myocytes

Ventricular myocytes were isolated from 3–6 month old WT and Pak1 knockout mice (Pak1^{-/-}) as well as from WT rabbit hearts by Langendorff perfusion as previously described [16, 17]. Left and right atria were dissected from the Langendorff perfused heart, cut into strips and further incubated in digestion buffer (mg/L): 0.1 Liberase TM (Roche), 0.14 trypsin (Gibco/Invitrogen), 1 Protease type XIV (Sigma) for 20 minutes at 37°C. Cell dissociation was enhanced by gentle cell dispersion with a Pasteur pipette. The digestion was stopped by addition of bovine calf serum (Hyclone) before Ca in the solution was reintroduced in a step-wise manner [18].

All animal procedures were performed with the approval of the IACUC of the University of Illinois at Chicago and Rush University and in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals. Cells were plated on laminin (Sigma Aldrich: 1mg/ml) coated glass coverslips in standard tyrode solution (in mmol/L: NaCl 130, KCl 5.4, CaCl₂ 1, MgCl₂ 1.5, NaHCO₃ 10, Glucose 10, HEPES 25; pH 7.4).

Intracellular Ca^{2+} measurements

To visualize changes of the intracellular Ca^{2+} concentration ($[\text{Ca}]_i$), VMs were incubated (15 min) at room temperature with fluo-4acetoxymethyl ester (5 $\mu\text{mol/L}$; fluo-4/AM; Invitrogen). After washout, 15 min were allowed for de-esterification of the dye. $[\text{Ca}^{2+}]_i$ measurements were performed as previously described [19]. Whole-cell Ca^{2+} -transients were obtained from confocal line scan images through single VMs (1200 lps; 0.2 $\mu\text{m}/\text{pixel}$) or by 2D confocal imaging (VTeye; VisiTech International, Sunderland, UK) at sampling frequencies of 120 to 360 Hz. The resulting images were analyzed using Metamorph (Molecular Devices) and ImageJ. Ca^{2+} -transients are presented as the change in background-subtracted fluorescence normalized to the diastolic fluorescence (F_0) at the beginning of the recording ($\Delta F/F_0$). VMs were stimulated at a frequency of 0.5 Hz for the duration of the experiment.

SDS-PAGE and Immunoblotting

Isolated VMs were lysed directly with the addition of hot 1-X Laemmli sample buffer without β -mercaptoethanol (β -ME) or bromophenol blue dye and heated to 95°C for 5 min. Sample protein determinations were made with a BCA protein assay kit (Pierce) and then β -ME and dye were added to the final concentrations for 1-X sample buffer and heated as before. Cell lysates were separated using pre-cast 4–20 % Novex tris-glycine gels (Invitrogen) and following standard electrophoresis protocols for SDS-PAGE and immunoblotting as previously described [16, 20]. Typically 25–45 μg of protein were loaded per well. Primary antibodies used for western blotting were directed against the sarcoplasmic reticulum Ca ATPase (Serca2a: Cell Signaling, # 4388), a-subunit of the L-type Ca channel ($\text{Ca}_v1.2$ $\alpha 1\text{c}$: Millipore, # AB10515), p21-activated kinase 1 (PAK1: Invitrogen, # 71-9300), brain natriuretic peptide (pro-BNP: Millipore, AB1549), α -skeletal muscle actin (Novus Biologicals; NB100-91648), glyceraldehyde 3-phosphate dehydrogenase (GAPDH: Cell Signaling, #5174) and α -actinin (Sigma A7811). Species-specific horseradish peroxidase-conjugated secondary antibodies were used and visualization was accomplished using Western Lighting chemi-luminescence reagents (PerkinElmer) and Kodak BioMax film.

Patch clamp recordings

VMs were used for patch clamp studies of the L-type Ca current ($I_{\text{Ca,L}}$). Experiments were performed in the ruptured patch configuration with microelectrodes filled with pipette solution containing (mmol/L): CsCl 125, TEA-Cl 20, EGTA 10, HEPES 10, phosphocreatine 5, Mg_2ATP 5, GTP 0.3; pH = 7.2. Pipette resistance ranged from 1 M Ω to 3 M Ω . To isolate $I_{\text{Ca,L}}$, VMs were voltage clamped to a holding potential of –50 mV and voltage pulses of 300 ms duration were applied from –50 to +50 mV in 10 mV increments [16]. Cell capacitance was measured during application of –10 mV voltage pulses from a holding potential of –50 mV in the voltage-clamp mode. Action potentials (APs) were recorded in the whole cell configuration in the current clamp mode during stimulation of VMs at 0.5 Hz. The pipette solution contained (mmol/L): K-aspartate 100, KCl 40, MgCl_2 1, MgATP 5, Hepes 10, NaCl 7, EGTA 5, CaCl_2 0.5, pH 7.2.

Di-8-ANNEPS staining

Freshly isolated atrial and ventricular myocytes were maintained in Kraft-Bruehe (KB)-solution containing in mmol/L: Hepes 5, MgSO_4 5, glucose 10, KCL 90, K_2HPO_4 30, sodium pyruvate 5, EGTA-Tris 0.5, taurine 20, creatine 5, sodium butyrate 5 and NaOH 1, pH 7.3. Di-8-ANNEPS(di-8-butyl-amino-naphthyl-ethylene-pyridinium-propyl-sulfonate) was added at a concentration of 5 $\mu\text{mol/L}$ and cells were incubated for 10 min. Using a laser scanning confocal microscope in the 2D scanning mode z-stacks were obtained

at 1–2 μm intervals with a pixel width $< 0.2 \mu\text{m}$. T-tubular density was determined in Image J as previously described [21, 22]. Binary fluorescent images were generated using the median average fluorescence as the threshold. An area within the cell was outlined excluding the sarcolemmal membrane and the nucleus, and the percentage area occupied by positive staining was determined and averaged for each cell over 6 consecutive images.

Viral transfection of isolated rabbit VMs

After isolation rabbit VMs were plated on laminin coated coverslips and transfected with a recombinant adenovirus expressing constitutively active Pak1 (AdPak1) at a multiplicity of infection of 100 [23]. Cells were maintained in Claycomb medium (51800C; SAFC Biosciences) supplemented with penicillin/streptomycin (50 U/ml; 50 $\mu\text{g}/\text{ml}$) for up to 48 h.

Statistics

Data sets were statistically evaluated using un-paired *t* test. All averaged data are presented as means $\pm$ S.E.M.. The number of experiments (*n*) refers to the number of cells examined and is indicated in the text. For each experimental group cells from at least 2 different cell isolations/animals were used.

Results

To determine if loss of Pak1 results in changes in cellular Ca handling, we recorded Ca transients in field stimulated (0.5 Hz), isolated VMs that were loaded with the Ca sensitive dye Fluo4/AM (Fig. 1AB). In comparison to WT, VMs isolated from Pak1^{-/-} mice exhibited a decreased Ca transient amplitude (Fig. 1C) ($\Delta F/F_0$: WT: 1.78 ± 0.2 , *n* = 10; Pak1^{-/-}: 1.26 ± 0.2 , *n* = 7; *p* < 0.05) that coincided with a prolonged Ca transient rise time (Fig. 1E; time to peak: WT: 79 ± 5 ms, *n* = 10; Pak1^{-/-}: 119 ± 8 ms, *n* = 7; *p* < 0.05) and recovery time at 90% decay (Fig. 1D) (RT_{90%}: WT: 0.91 ± 0.11 s, *n* = 10; Pak1^{-/-}: 1.33 ± 0.08 s, *n* = 7; *p* < 0.05).

The Ca transient amplitude depends on Ca influx across the plasma membrane and the release of Ca from the sarcoplasmic reticulum through CICR from RyR [24]. Protein levels of Ca_v1.2 (Fig. 2G) and voltage clamp recordings of I_{Ca,L} from isolated VMs did not exhibit a significant difference between WT and Pak1^{-/-} myocytes (Fig. 2ABC)(current density (I_{Ca,L}) at -10 mV: WT: -4.1 ± 0.16 pA/pF, *n* = 11; Pak1^{-/-}: -4.65 ± 0.52 pA/pF, *n* = 10). Also no significant difference in the time constant of inactivation ($\tau_{Ca,L}$) was determined ($\tau_{Ca,L}$ at -10 mV: WT: 45.7 ± 2.68 ms, *n* = 11; Pak1^{-/-}: 38.4 ± 3.65 ms, *n* = 10). The caffeine (10 mM) transient amplitude recorded as a measure of SR Ca load [24] was not statistically different between the two cell types (Fig. 2D) (caff ($\Delta F/F_0$): WT: 5.5 ± 0.3 , *n* = 10; Pak1^{-/-}: 4.8 ± 0.3 , *n* = 7) consistent with comparable expression levels of SERCA (Fig. 2G). The results indicate that neither changes in Ca influx nor SR load contributed significantly to the reduction in Ca transient amplitude.

To further evaluate the Ca sensitivity of RyRs we analyzed the conduction velocity of spontaneous Ca waves in WT and Pak1^{-/-} VMs. The Ca waves in WT and Pak1^{-/-} VMs were comparable in amplitude (Fig. 2E) and velocity (Fig. 2F; WT: $127 \pm 15 \mu\text{m}/\text{s}$, *n* = 30; Pak1^{-/-}: $126 \pm 12 \mu\text{m}/\text{s}$, *n* = 22). Nevertheless the fractional release (Fig. 3A) as well as the ECC gain determined for I_{Ca,L} at -10 mV voltage pulse (Fig. 3B) are reduced in Pak1^{-/-} VMs supporting that the functional interaction between Ca influx and Ca release is attenuated.

Changes in the AP of Pak1^{-/-} ventricular myocytes

Changes in the cardiac action potential (AP) can influence the excitation-induced Ca transient by reducing the driving force for Ca entry through $I_{Ca,L}$. To identify potential modifications in the electrophysiological properties we recorded APs in isolated WT and Pak1^{-/-} VMs. The resting membrane potential (Fig. 3C: WT: -77 ± 1 mV, $n = 9$; Pak1^{-/-}: -74 ± 2 mV, $n = 8$) and the AP amplitude (WT: 102 ± 5 mV, $n = 9$; Pak1^{-/-}: 104 ± 3 mV, $n = 8$; not shown) were not significantly different between WT and Pak1^{-/-} VMs; however, in Pak1^{-/-} VMs the repolarization of the AP was slowed resulting in an overall prolongation of the APD at 50% (APD₅₀: WT: 14 ± 1 ms, $n = 9$; Pak1^{-/-}: 21 ± 1 ms, $n = 8$; $p < 0.05$) (Fig. 3D) and 90% (APD₉₀: WT: 40 ± 5 ms, $n = 9$; Pak1^{-/-}: 78 ± 3 ms, $n = 8$; $p < 0.05$; not shown) repolarization, respectively. Early repolarization in VMs depends on the activation of the transient outward potassium current (I_{to}). To identify the role of I_{to} in the changes of the Ca transient amplitude and rise time we superfused WT ($n = 10$) and Pak1^{-/-} ($n = 14$) VMs with the I_{to} inhibitor 4-aminopyridine (100 μ mol/L). Ca transient rise time was prolonged in both cell types (Fig. 3E); however, it remained delayed in Pak1^{-/-} VMs indicating that changes in I_{to} are not likely to be the main reason for the observed changes in ECC. Nevertheless, a decrease in early repolarization could contribute to the changes in the Ca transient amplitude.

Decreased t-tubular density in Pak1^{-/-} ventricular myocytes

The patch clamp recordings further revealed that cell capacitance of Pak1^{-/-} VMs was significantly reduced (Fig. 4A; C_m : WT: 206 ± 13 pF, $n = 9$; Pak1^{-/-}: 160 ± 21 pF, $n = 8$; $p < 0.05$) despite the fact that the cell volume (defined as: cell length * cell width * cell height; Fig. 4B) of WT and Pak1^{-/-} VMs remained unchanged (WT: 64.3 ± 5.6 pL, $n = 10$; Pak1^{-/-}: 58 ± 4.2 pL, $n = 12$). In VMs the membrane invaginations that form the t-tubular system contribute up to 40% of total membrane area [25]. To determine if changes in cell capacitance are due to alterations of the t-tubular membrane, freshly isolated VMs were stained with di-8-ANNEPS (5 μ mol/L). WT myocytes exhibited a t-tubular density of 35.3 ± 1.64 % ($n = 28$) whereas in Pak1^{-/-} VMs t-tubular density was significantly decreased to 25.8 ± 1.59 % ($p < 0.01$). A decrease in the t-tubular density is often linked to cellular remodeling in cardiac diseases like hypertrophy. However, western blot analysis of cell lysates obtained from WT and Pak1^{-/-} isolated VMs did not show changes in the expression of cardiac hypertrophic marker proteins such as pro-BNP, α skeletal actin (Fig. 4D) or SERCA (Fig. 2G).

T-tubules provide the means by which AP-induced Ca influx triggers CICR in the depth of the VM allowing for a homogeneous rise in $[Ca]_i$ [26]. To determine if the reduction in the t-tubular density in Pak1^{-/-} myocytes has functional consequences for ECC, we obtained rapid line scans (1200 lps; 0.2 μ m/pixel) along the longitudinal axis of the VMs (Fig. 5AB) [27]. The rising phase of the stimulation induced Ca transient was analyzed for areas (only areas $> 2 \mu$ m were quantified) along the line of scan, where CICR did not reach F_{50} 20 ms after the stimulus (Fig. 5C). In Pak1^{-/-} VMs the number of areas with delayed release were significantly increased. In addition an increased standard deviation (SD) of the rise time was determined (time to 50% (TT50); WT: 7.77 ± 2.1 , $n = 6$; Pak1^{-/-}: 17.8 ± 3.0 , $n = 6$; $p < 0.05$; Fig. 5D). Both parameters indicate an increased number of sites where the functional coupling between Ca influx and Ca release is delayed. To verify that the delay is not based on modifications within the individual couplons, we analyzed the change in $[Ca]_i$ at individual early release sites. When the Ca transient amplitude and rise time was analyzed at fast release sites of WT and Pak1^{-/-} VMs only, neither a difference in the amplitude, time to peak (Fig. 5E) nor in the ECC gain at -10 mV (Fig. 5F) could be determined. The results support that the modification in Pak1^{-/-} VMs is not due to changes in the individual couplons but rather due the decrease in the number of fast release sites.

Changes of the Ca transient kinetics depend on t-tubular remodeling

The density of the t-tubular system in ventricular myocytes is species-dependent [28] and changes during pathophysiological remodeling [29]. To identify if enhanced Pak1 activity is directly linked to the maintenance of the t-tubular system, we used freshly isolated rabbit VMs that in contrast to mouse VMs, exhibit a spontaneous decrease in their t-tubular density when cultured for 24h to 48 h [28]. Isolated rabbit VMs were used either on the day of isolation or after 24 h in culture. Stimulation induced Ca transients were recorded and the t-tubular density of the cells was quantified by di-8-ANNEPS staining. After 24h of culture rabbit VMs exhibited significantly decreased Ca transient amplitudes (Fig. 6A: $\Delta F/F_0$: Ctrl: 2.5 ± 0.3 , $n = 7$; Ctrl_{24h}: 1.7 ± 0.2 , $n = 13$; $p < 0.05$) and the transients exhibited a prolonged rise time (Fig. 6B: time to peak: Ctrl: 0.23 ± 0.01 s, $n = 7$; Ctrl_{24h}: 0.26 ± 0.01 s, $n = 13$; $p < 0.05$). These changes in ECC coincided with a decrease in t-tubular density (Fig. 6C) (t-tubules: Ctrl: 20.73 ± 1.49 %, $n = 21$; Ctrl_{24h}: 8.52 ± 0.75 %, $n = 17$; Ctrl_{48h}: 5.13 ± 0.84 %, $n = 10$; $p < 0.05$ vs. Ctrl) and a dramatic culture dependent down-regulation of Pak1 as evaluated by western blotting (Fig. 6D, top). However, when VMs were transfected with AdPak1 at the day of isolation Pak1 expression was maintained (Fig. 6D) and after 24 h in culture Ca transient kinetics ($\Delta F/F_0$: AdPak1_{24h}: 2.3 ± 0.3 , $n = 10$; TTP: 0.22 ± 0.01 s, $n = 10$) and t-tubular density (t-tubules: AdPak1_{24h}: 13.54 ± 1.55 %, $n = 15$; $p < 0.05$ vs. Ctrl_{24h}; AdPak1_{48h}: 10.57 ± 0.98 %, $n = 16$; $p < 0.05$ vs. Ctrl_{48h}) were better maintained than those from untransfected or AdLacZ transfected VMs.

To determine if the changes in ECC of Pak1^{-/-} VMs are the consequence of the structural remodeling of the t-tubular system, we analyzed Ca transients in isolated atrial myocytes of WT and Pak1^{-/-} mice. Atrial myocytes isolated from the right atria of WT or Pak1^{-/-} mice did not exhibit an extensive t-tubular system (Fig. 7AB) (t-tubular density: WT: 2.46 ± 0.69 %, $n = 9$; Pak1^{-/-}: 1.92 ± 0.72 %, $n = 9$) and Ca transient amplitude (Fig. 7CD) ($\Delta F/F_0$: WT: 3.2 ± 0.4 , $n = 15$; Pak1^{-/-}: 3.17 ± 0.3 , $n = 14$) and rise time (WT: 102 ± 8 ms, $n = 15$; Pak1^{-/-}: 101 ± 3 ms, $n = 14$) were not significantly different. The results support the hypothesis that changes in VMs Ca transient rise time and amplitude closely linked to the remodeling of the t-tubular system in Pak1^{-/-} mice.

Discussion

Pak1 has been shown to negatively regulate the changes in ECC promoted by β -adrenergic stimulation. In this study we further demonstrate that loss of Pak1 signaling results in changes in cardiac ECC that are in part based on the sub-cellular remodeling of the ventricular myocytes t-tubular structure. The decrease in t-tubular density is associated with a decreased amplitude and a delayed rise time of the Ca transient based on a spatial and temporal dissociation of Ca influx through $I_{Ca,L}$ and CICR from RyR.

In the heart the basis for every excitation cycle is the AP that leads to the voltage-dependent activation of Ca influx ($I_{Ca,L}$), and the subsequent CICR from the RyRs localized in the SR [24, 30]. Ca influx through $I_{Ca,L}$ during phase 0 and phase 1 of the cardiac AP forms the predominant trigger for CICR. In Pak1^{-/-} VMs Ca-current density, AP upstroke velocity and maximum diastolic depolarization are comparable to WT myocytes. This indicates that $I_{Ca,L}$ activation should remain unchanged at the onset of the AP where with the fast depolarization only a short delay in $I_{Ca,L}$ activation is expected [31].

We determined a decreased early repolarization of the AP in Pak1^{-/-} VMs. In mouse VMs, an attenuated early repolarization could occur due to a reduction in the transient-outward potassium-current (I_{to}) [32], as well as a decrease of the Ca dependent inactivation of $I_{Ca,L}$ [33]. Under both circumstances an overall prolongation of APD would be expected as seen in the Pak1^{-/-} VMs. Both mechanisms would decrease $I_{Ca,L}$ during the plateau phase of the

AP due to a decreased driving force for Ca [34] but prolong the overall Ca influx. Block of I_{to} in our hands did not eliminate the differences in the Ca transient upstroke velocity supporting the hypothesis that it is not the predominant reason for the reduced Ca transient amplitude in $Pak1^{-/-}$ VMs.

In mouse VMs the Ca transient amplitude predominantly depends on SR Ca-release [35]. The decreased Ca transient amplitude in $Pak1^{-/-}$ VMs could reflect an attenuated SR load due to either increased leak of Ca from the SR through RyR, or a decreased uptake of Ca into the SR. Our experiments show that SR Ca load is comparable between WT and $Pak1^{-/-}$ VMs and no significant change in PLN phosphorylation was determined in lysates from isolated VMs (not shown). The data exclude a decrease in SR load as the limiting factor for the Ca transient amplitude and the comparable propagation velocity of Ca waves support that RyR open probability remains unaffected. Other than the Ca-influx and overall Ca release, the Ca transient amplitude in VMs further depends on the time course and synchrony of Ca release throughout the cell, which is enabled by an extensive t-tubular network. T-tubules are membrane invaginations, which contain a high density of membrane proteins involved in cardiac ECC [29]. Of the total VM current 80% of $I_{Ca,L}$, 63% of I_{NCX} , 76% of $I_{K,s}$ and 30% of I_{to} were reported to be localized to the t-tubules of rat VMs [21]. T-tubules closely align with the SR at the z-line, allowing a close spatial proximity between $I_{Ca,L}$ and RyRs. The formation of these Ca release units facilitates the fast and synchronous AP-triggered CICR throughout the depth of the cell [30, 36]. The early-triggered Ca release events in WT and $Pak1^{-/-}$ VMs should represent the release from intact couplons [36, 37]. We did not identify differences in the Ca release kinetic or ECC gain during these early release events that could be indicative of changes in number of L-type or RyR channels or their interaction within one couplon. In VMs during the pathophysiological remodeling of t-tubules the close spatial interaction between $I_{Ca,L}$ and RyR is partially lost and a decreased synchrony of CICR from individual release units is the consequence [27, 38]. In $Pak1^{-/-}$ VMs we determined a decreased t-tubular density, which coincided with a decreased capacitance of the cells [39]. Together with the increased delay of Ca release observed during the up-stroke of the Ca transient we therefore propose that the subcellular remodeling in $Pak1^{-/-}$ VMs forms the basis for the overall delayed rise time and decreased amplitude of the Ca transient. As shown here in the mouse preparation, atrial myocytes of some species are void of an extensive t-tubular network [40, 41]. In these cases the rise of the Ca transient is delayed in the center of the myocyte where Ca release from RYR depends in part on Ca diffusion [41]. Atrial myocytes of $Pak1^{-/-}$ VMs did not exhibit any changes in the Ca transient rise time and amplitude compared to WT cells, which further supports our hypothesis that Pak1 maintains ECC by stabilization of the t-tubular network.

Nevertheless, besides the changes in the Ca transient, we observed changes in the AP of $Pak1^{-/-}$ VMs. As previously discussed, the attenuated early repolarization could be the result of decreased I_{to} expression; alternatively, an enhanced depolarizing current could mask the early repolarization. While $I_{Ca,L}$ density remains unchanged in $Pak1^{-/-}$ cells, during an AP the amount of Ca entering the cell critically depends on the Ca-dependent inactivation of the channel. Modifications of $[Ca]_i$ in the vicinity of the Ca channel (Cav1.2) such as changes in NCX expression and block of SR Ca release, dynamically change the channels inactivation kinetics [33, 42]. A difference in Ca dependent inactivation of $I_{Ca,L}$ was also described between channels localized in the t-tubules and those in the sarcolemmal membrane. The latter display a slower inactivation kinetic [43]. We did not determine differences in $\tau_{Ca,L}$, however the currents were recorded under buffering conditions with 10 mM EGTA included in the pipette solution [44]. We propose that the attenuated early repolarization could be mediated by a delayed Ca-dependent inactivation of Cav1.2 due to a prolonged rise in $[Ca]_i$ at the Ca release units or an increased contribution of sarcolemmal Ca channels to the total $I_{Ca,L}$.

Changes in the t-tubular network are associated with pathophysiological remodeling of the cardiac tissue. Decreased t-tubular densities have been reported in association with human heart failure and ischemic heart disease [45, 46], as well as cardiac hypertrophy, failure and infarct [39, 47, 48] in mouse. We, and others have shown that Pak1^{-/-} VMs do not exhibit signs of hypertrophic remodeling as demonstrated by the lack of expression of hypertrophic markers, the maintenance of cell surface area, and normal contractile performance [9, 10]. That this remodeling can occur without changes in contractile function of the heart was demonstrated in a model of cardiac hypertrophy [47]. The concept of t-tubular remodeling as a direct consequence of the loss of Pak1 is supported by the ability of Pak1 signaling to antagonize t-tubular remodeling in isolated rabbit VMs that down-regulate Pak1 in culture.

Mechanisms of t-tubular remodeling are poorly understood. In vivo, exercise or blockade of the phosphodiesterase 5 maintain and restore a hypertrophy-induced decrease in t-tubular density [49]. Deletion of PI3K α and γ isoforms on the other hand resulted in a loss of this structure [50]. Bin1 has been identified as a protein critical in the formation of the membrane invaginations [51] and is required for the directed transport of I_{Ca,L} to the t-tubules [52]. In vitro data support the idea that t-tubular maintenance depends on cytoskeletal integrity, in particular the stabilization of actin filaments [53, 54]. Pak1 is a critical regulator of the cytoskeleton by controlling actin, tubulin, or desmin assembly [2, 55, 56] and the mechanism by which Pak1 signaling maintains the t-tubular structure will be the subject of future studies.

Conclusion

We demonstrate that Pak1 signaling under physiological conditions in vivo as well as in vitro plays a critical role in the maintenance of the t-tubular structure of ventricular myocytes. An absence of Pak1 activity leads to changes in cardiac ECC that are predominantly based on the loss of t-tubules that result in a reduction of functional Ca release units that consist of L-type Ca channels and RyRs. The changes in ECC can not only attenuate cardiac contractility but also potentially modulate the Ca dependent regulation of kinases, phosphatases and transcription factors. Prior experiments demonstrate, that maintenance and restoration of t-tubules can attenuate the progression of heart disease [49, 57, 58], which would make Pak1 a promising therapeutic target in the prevention of hypertrophy induced sub-cellular remodeling.

Acknowledgments

The work was supported by grants from the National Institutes of Health HL089617 and HL089617-03S1 to KB, HL64035 to RJS.

References

1. Clerk A, Sugden PH. Activation of p21-activated protein kinase alpha (alpha PAK) by hyperosmotic shock in neonatal ventricular myocytes. FEBS letters. 1997; 403:23–5. [PubMed: 9038353]
2. Edwards DC, Sanders LC, Bokoch GM, Gill GN. Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. Nature Cell Biology. 1999; 1:253–9.
3. Jaffer ZM, Chernoff J. p21-activated kinases: three more join the Pak. The international journal of biochemistry & cell biology. 2002; 34:713–7.
4. Szczepanowska J. Involvement of Rac/Cdc42/PAK pathway in cytoskeletal rearrangements. Acta Biochim Pol. 2009; 56:225–34. [PubMed: 19513348]
5. Ai X, Jiang A, Ke Y, Solaro RJ, Pogwizd SM. Enhanced activation of p21-activated kinase 1 in heart failure contributes to dephosphorylation of connexin 43. Cardiovascular Research. 2011; 92:106–14. [PubMed: 21727092]

6. Ke Y, Lei M, Collins TP, Rakovic S, Mattick PA, Yamasaki M, et al. Regulation of L-type calcium channel and delayed rectifier potassium channel activity by p21-activated kinase-1 in guinea pig sinoatrial node pacemaker cells. *Circ Res.* 2007; 100:1317–27. [PubMed: 17413045]
7. Ke Y, Wang L, Pyle WG, de Tombe PP, Solaro RJ. Intracellular localization and functional effects of P21-activated kinase-1 (Pak1) in cardiac myocytes. *Circ Res.* 2004; 94:194–200. [PubMed: 14670848]
8. Sheehan KA, Ke Y, Wolska BM, Solaro RJ. Expression of active p21-activated kinase-1 induces Ca²⁺ flux modification with altered regulatory protein phosphorylation in cardiac myocytes. *Am J Physiol Cell Physiol.* 2009; 296:C47–58. [PubMed: 18923061]
9. Liu W, Zi M, Naumann R, Ulm S, Jin J, Taglieri DM, et al. Pak1 as a Novel Therapeutic Target for Antihypertrophic Treatment in the Heart. *Circulation.* 2011; 124:2702–15. [PubMed: 22082674]
10. Taglieri DM, Monasky MM, Knezevic I, Sheehan KA, Lei M, Wang X, et al. Ablation of p21-activated kinase-1 in mice promotes isoproterenol-induced cardiac hypertrophy in association with activation of Erk1/2 and inhibition of protein phosphatase 2A. *J Mol Cell Cardiol.* 2011; 51:988–96. [PubMed: 21971074]
11. El-Armouche A, Bednorz A, Pamminger T, Ditz D, Didie M, Dobrev D, et al. Role of calcineurin and protein phosphatase-2A in the regulation of phosphatase inhibitor-1 in cardiac myocytes. *Biochem Biophys Res Commun.* 2006; 346:700–6. [PubMed: 16774736]
12. Terentyev D, Belevych AE, Terentyeva R, Martin MM, Malana GE, Kuhn DE, et al. miR-1 overexpression enhances Ca²⁺ release and promotes cardiac arrhythmogenesis by targeting PP2A regulatory subunit B56alpha and causing CaMKII-dependent hyperphosphorylation of RyR2. *Circ Res.* 2009; 104:514–21. [PubMed: 19131648]
13. Ke Y, Sheehan KA, Egom EE, Lei M, Solaro RJ. Novel bradykinin signaling in adult rat cardiac myocytes through activation of p21-activated kinase. *Am J Physiol Heart Circ Physiol.* 2010; 298:H1283–9. [PubMed: 20154261]
14. duBell WH, Gigena MS, Guatimosim S, Long X, Lederer WJ, Rogers TB. Effects of PP1/PP2A inhibitor calyculin A on the E-C coupling cascade in murine ventricular myocytes. *Am J Physiol Heart Circ Physiol.* 2002; 282:H38–48. [PubMed: 11748045]
15. Monasky MM, Taglieri DM, Patel BG, Chernoff J, Wolska BM, Ke Y, et al. P21-Activated Kinase Improves Cardiac Contractility during Ischemia-Reperfusion concomitant with changes in Troponin-T and Myosin Light Chain 2 Phosphorylation. *Am J Physiol Heart Circ Physiol.* 2011; 302:H224–30. [PubMed: 22037191]
16. Desantiago J, Bare DJ, Semenov I, Minshall RD, Geenen DL, Wolska BM, et al. Excitation-contraction coupling in ventricular myocytes is enhanced by paracrine signaling from mesenchymal stem cells. *J Mol Cell Cardiol.* 2012; 52:1249–56. [PubMed: 22465692]
17. Maxwell JT, Domeier TL, Blatter LA. Dantrolene prevents arrhythmogenic Ca²⁺ release in heart failure. *Am J Physiol Heart Circ Physiol.* 2012; 302:H953–63. [PubMed: 22180651]
18. Louch WE, Sheehan KA, Wolska BM. Methods in cardiomyocyte isolation, culture, and gene transfer. *Journal of molecular and cellular cardiology.* 2011; 51:288–98. [PubMed: 21723873]
19. Kapur N, Banach K. Inositol-1,4,5-trisphosphate-mediated spontaneous activity in mouse embryonic stem cell-derived cardiomyocytes. *J Physiol.* 2007; 581:1113–27. [PubMed: 17379641]
20. Ramos-Franco J, Bare D, Caenepeel S, Nani A, Fill M, Mignery G. Single-channel function of recombinant type 2 inositol 1,4, 5-trisphosphate receptor. *Biophys J.* 2000; 79:1388. [PubMed: 10969001]
21. Pasek M, Brette F, Nelson A, Pearce C, Qaiser A, Christe G, et al. Quantification of t-tubule area and protein distribution in rat cardiac ventricular myocytes. *Progress in biophysics and molecular biology.* 2008; 96:244–57. [PubMed: 17881039]
22. Heinzel FR, Bito V, Volders PG, Antoons G, Mubagwa K, Sipido KR. Spatial and temporal inhomogeneities during Ca²⁺ release from the sarcoplasmic reticulum in pig ventricular myocytes. *Circ Res.* 2002; 91:1023–30. [PubMed: 12456488]
23. Ke Y, Lei M, Solaro RJ. Regulation of cardiac excitation and contraction by p21 activated kinase-1. *Prog Biophys Mol Biol.* 2008; 98:238–50. [PubMed: 19351515]
24. Bers DM. Calcium cycling and signaling in cardiac myocytes. *Annu Rev Physiol.* 2008; 70:23–49. [PubMed: 17988210]

25. Brette F, Orchard C. T-tubule function in mammalian cardiac myocytes. *Circ Res.* 2003; 92:1182. [PubMed: 12805236]
26. Brette F, Despa S, Bers DM, Orchard CH. Spatiotemporal characteristics of SR Ca²⁺ uptake and release in detubulated rat ventricular myocytes. *J Mol Cell Cardiol.* 2005; 39:804–12. [PubMed: 16198369]
27. Heinzel FR, Bito V, Biesmans L, Wu M, Detre E, von Wegner F, et al. Remodeling of T-tubules and reduced synchrony of Ca²⁺ release in myocytes from chronically ischemic myocardium. *Circ Res.* 2008; 102:338–46. [PubMed: 18079411]
28. Pavlovic D, McLatchie LM, Shattock MJ. The rate of loss of T-tubules in cultured adult ventricular myocytes is species dependent. *Experimental physiology.* 2010; 95:518–27. [PubMed: 20061354]
29. Ibrahim M, Gorelik J, Yacoub MH, Terracciano CM. The structure and function of cardiac t-tubules in health and disease. *Proc Biol Sci.* 2011
30. Fearnley CJ, Roderick HL, Bootman MD. Calcium signaling in cardiac myocytes. *Cold Spring Harb Perspect Biol.* 2011; 3:a004242. [PubMed: 21875987]
31. Bers DM, Lederer WJ, Berlin JR. Intracellular Ca transients in rat cardiac myocytes: role of Na-Ca exchange in excitation-contraction coupling. *Am J Physiol.* 1990; 258:C944–54. [PubMed: 2333986]
32. Sun X, Wang HS. Role of the transient outward current (I_{to}) in shaping canine ventricular action potential—a dynamic clamp study. *The Journal of physiology.* 2005; 564:411–9. [PubMed: 15649977]
33. Takamatsu H, Nagao T, Ichijo H, Adachi-Akahane S. L-type Ca²⁺ channels serve as a sensor of the SR Ca²⁺ for tuning the efficacy of Ca²⁺-induced Ca²⁺ release in rat ventricular myocytes. *J Physiol.* 2003; 552:415–24. [PubMed: 14561825]
34. Clark RB, Bouchard RA, Giles WR. Action potential duration modulates calcium influx, Na(+)-Ca²⁺ exchange, and intracellular calcium release in rat ventricular myocytes. *Annals of the New York Academy of Sciences.* 1996; 779:417–29. [PubMed: 8659858]
35. Li L, Chu G, Kranias EG, Bers DM. Cardiac myocyte calcium transport in phospholamban knockout mouse: relaxation and endogenous CaMKII effects. *Am J Physiol.* 1998; 274:H1335–47. [PubMed: 9575939]
36. Heinzel FR, MacQuaide N, Biesmans L, Sipido K. Dyssynchrony of Ca²⁺ release from the sarcoplasmic reticulum as subcellular mechanism of cardiac contractile dysfunction. *J Mol Cell Cardiol.* 2011; 50:390–400. [PubMed: 21075114]
37. Xu M, Zhou P, Xu SM, Liu Y, Feng X, Bai SH, et al. Intermolecular failure of L-type Ca²⁺ channel and ryanodine receptor signaling in hypertrophy. *PLoS biology.* 2007; 5:e21. [PubMed: 17214508]
38. Gomez AM, Valdivia HH, Cheng H, Lederer MR, Santana LF, Cannell MB, et al. Defective excitation-contraction coupling in experimental cardiac hypertrophy and heart failure. *Science.* 1997; 276:800–6. [PubMed: 9115206]
39. Horiuchi-Hirose M, Kashihara T, Nakada T, Kurebayashi N, Shimojo H, Shibazaki T, et al. Decrease in the density of t-tubular L-type Ca²⁺ channel currents in failing ventricular myocytes. *Am J Physiol Heart Circ Physiol.* 2010; 300:H978–88. [PubMed: 21193586]
40. Li X, Zima AV, Sheikh F, Blatter LA, Chen J. Endothelin-1-induced arrhythmogenic Ca²⁺ signaling is abolished in atrial myocytes of inositol-1,4,5-trisphosphate(IP3)-receptor type 2-deficient mice. *Circ Res.* 2005; 96:1274–81. [PubMed: 15933266]
41. Sheehan KA, Blatter LA. Regulation of junctional and non-junctional sarcoplasmic reticulum calcium release in excitation-contraction coupling in cat atrial myocytes. *J Physiol.* 2003; 546:119–32. [PubMed: 12509483]
42. Pott C, Goldhaber JJ, Philipson KD. Homozygous overexpression of the Na⁺-Ca²⁺ exchanger in mice: evidence for increased transsarcolemmal Ca²⁺ fluxes. *Annals of the New York Academy of Sciences.* 2007; 1099:310–4. [PubMed: 17446472]
43. Brette F, Salle L, Orchard CH. Differential modulation of L-type Ca²⁺ current by SR Ca²⁺ release at the T-tubules and surface membrane of rat ventricular myocytes. *Circ Res.* 2004; 95:e1. [PubMed: 15192026]

44. Ozdemir S, Bito V, Holemans P, Vinet L, Mercadier JJ, Varro A, et al. Pharmacological inhibition of na/ca exchange results in increased cellular Ca²⁺ load attributable to the predominance of forward mode block. *Circ Res.* 2008; 102:1398–405. [PubMed: 18451338]
45. Crossman DJ, Ruygrok PN, Soeller C, Cannell MB. Changes in the organization of excitation-contraction coupling structures in failing human heart. *PLoS ONE.* 2011; 6:e17901. [PubMed: 21408028]
46. Lyon AR, MacLeod KT, Zhang Y, Garcia E, Kanda GK, Lab MJ, et al. Loss of T-tubules and other changes to surface topography in ventricular myocytes from failing human and rat heart. *Proceedings of the National Academy of Sciences of the United States of America.* 2009; 106:6854–9. [PubMed: 19342485]
47. Wei S, Guo A, Chen B, Kutschke W, Xie YP, Zimmerman K, et al. T-tubule remodeling during transition from hypertrophy to heart failure. *Circ Res.* 2010; 107:520–31. [PubMed: 20576937]
48. Biesmans L, Macquaide N, Heinzel FR, Bito V, Smith GL, Sipido KR. Subcellular heterogeneity of ryanodine receptor properties in ventricular myocytes with low T-tubule density. *PLoS ONE.* 2011; 6:e25100. [PubMed: 22022376]
49. Xie YP, Chen B, Sanders P, Guo A, Li Y, Zimmerman K, et al. Sildenafil Prevents and Reverses Transverse-Tubule Remodeling and Ca²⁺ Handling Dysfunction in Right Ventricle Failure Induced by Pulmonary Artery Hypertension. *Hypertension.* 2011
50. Wu CY, Jia Z, Wang W, Ballou LM, Jiang YP, Chen B, et al. PI3Ks maintain the structural integrity of T-tubules in cardiac myocytes. *PLoS ONE.* 2011; 6:e24404. [PubMed: 21912691]
51. Lee E, Marcucci M, Daniell L, Pypaert M, Weisz OA, Ochoa GC, et al. Amphiphysin 2 (Bin1) and T-tubule biogenesis in muscle. *Science.* 2002; 297:1193–6. [PubMed: 12183633]
52. Hong TT, Smyth JW, Gao D, Chu KY, Vogan JM, Fong TS, et al. BIN1 localizes the L-type calcium channel to cardiac T-tubules. *PLoS Biol.* 2010; 8:e1000312. [PubMed: 20169111]
53. Leach RN, Desai JC, Orchard CH. Effect of cytoskeleton disruptors on L-type Ca channel distribution in rat ventricular myocytes. *Cell Calcium.* 2005; 38:515–26. [PubMed: 16137761]
54. Hammer K, Ruppenthal S, Viero C, Scholz A, Edelmann L, Kaestner L, et al. Remodelling of Ca²⁺ handling organelles in adult rat ventricular myocytes during long term culture. *J Mol Cell Cardiol.* 2010; 49:427–37. [PubMed: 20540947]
55. Delorme-Walker VD, Peterson JR, Chernoff J, Waterman CM, Danuser G, Dermardirossian C, et al. Pak1 regulates focal adhesion strength, myosin IIA distribution, and actin dynamics to optimize cell migration. *J Cell Biol.* 2011; 193:1289–303. [PubMed: 21708980]
56. Allen JD, Jaffer ZM, Park SJ, Burgin S, Hofmann C, Sells MA, et al. p21-activated kinase regulates mast cell degranulation via effects on calcium mobilization and cytoskeletal dynamics. *Blood.* 2009; 113:2695–705. [PubMed: 19124833]
57. Sachse FB, Torres NS, Savio-Galimberti E, Aiba T, Kass DA, Tomaselli GF, et al. Subcellular Structures and Function of Myocytes Impaired During Heart Failure Are Restored by Cardiac Resynchronization Therapy. *Circ Res.* 2012; 110:588–97. [PubMed: 22253411]
58. Stolen TO, Hoydal MA, Kemi OJ, Catalucci D, Ceci M, Aasum E, et al. Interval training normalizes cardiomyocyte function, diastolic Ca²⁺ control, and SR Ca²⁺ release synchronicity in a mouse model of diabetic cardiomyopathy. *Circ Res.* 2009; 105:527–36. [PubMed: 19679837]

Highlights

- Loss of Pak1 activity modulates cardiac excitation-contraction coupling
- Ventricular myocytes from Pak1^{-/-} mice have a reduced t-tubular density without signs of hypertrophic remodeling
- Pak1 deficiency attenuates functional coupling of Ca influx and Ca-induced Ca release
- Pak1 over-expression can attenuate t-tubular remodeling in culture

Figure 1. Decreased Ca transient amplitude in *Pak1*^{-/-} VMs
 F/F_0 plots from field stimulated WT (A) and *Pak1*^{-/-} (B) VMs. Ca transients in *Pak1*^{-/-} VMs had a decreased amplitude (C), prolonged recovery time to 90% decay (RT_{90%}) (D), and (E) Ca transient rise time, expressed as 'time to peak' (*, p < 0.05).

Figure 2. Ca influx and SR Ca load remain unchanged in *Pak1*^{-/-} VMs

(A) Current voltage relationship of $I_{Ca,L}$ in WT and *Pak1*^{-/-} VMs obtained in the whole cell patch configuration from a holding potential of -50 mV (-40 to +40 mV), in 10 mV increments. (B) Representative current traces from WT and *Pak1*^{-/-} VMs during a voltage pulse to 10 mV. (C) $I_{Ca,L}$ at -10 mV and (D) the amplitude of the caffeine (10 mmol/L) induced Ca transient were not significantly different in WT and *Pak1*^{-/-} VMs. (E) The propagation velocity of spontaneous Ca waves and their amplitude (F) was comparable in WT and *Pak1*^{-/-} VMs. (G) Western blotting analysis did not reveal differences in the protein expression of SERCA2a or $Ca_v1.2$ in 4 and 6 month old *Pak1*^{-/-} VMs. α -actinin is shown as a loading control.

Figure 3. The AP in *Pak1*^{-/-} VMs exhibits a delayed repolarization

(A) The fractional release obtained from the ratio of the twitch vs. the caffeine induced transient amplitude, and (B) ECC gain obtained as the ratio of the twitch transient amplitude vs. $I_{Ca,L}$ at a voltage pulse to -10 mV were reduced in *Pak1*^{-/-} VMs (*, $p < 0.05$). (C) Representative stimulation induced APs recorded from WT (black) and *Pak1*^{-/-} (grey) VMs. The APD at (C) 50% repolarization was significantly prolonged in *Pak1*^{-/-} VMs. (E) Suppression of I_{to} by 4-AP ($100 \mu\text{mol/L}$) increased the time to peak of the Ca transient in WT and *Pak1*^{-/-} VMs but maintained the difference between cell types (*, $p < 0.05$).

Figure 4. Pak1^{-/-} VMs exhibit changes in t-tubular density independent of hypertrophic remodeling

(A) Patch clamp recordings revealed a significantly reduced cell capacitance in Pak1^{-/-} VMs that did not correlate with a change in (B) VMs cell volume. Di-8-ANNEPS staining revealed a significant decrease in (C) t-tubular density within the WT and Pak1^{-/-} VMs. (D) Western blotting analysis did not reveal an increased expression of the hypertrophic marker proteins BNP and α-sk actin in 4 and 6 month old Pak1^{-/-} VMs. GAPDH and α-actinin are shown as loading controls. (E) Representative confocal images from a WT (top) and Pak1^{-/-} VM (bottom).

Figure 5. Pak1^{-/-} VMs exhibit an increased asynchrony in stimulation induced Ca release
 Line scans and F/F_0 plots from (A) WT and (B) Pak1^{-/-} VMs obtained at a scanning frequency of 1200 lps. F/F_0 plots were obtained immediately before (black) and 20 ms after (red) stimulation of the Ca transient; time points are indicated by arrows. Pak1^{-/-} VMs displayed an increased number of regions (C) where $[\text{Ca}]_i$ did not reach half maximal fluorescence (F50) 20 ms after the stimulus. Further an increased standard deviation of the time to peak (SD TT50; D) was recorded. Individual analysis of 'early' Ca release sites did not reveal differences in the time to peak (E) or ECC gain at -10 mV when only the sites of fast Ca rise were analyzed(*, $p < 0.05$).

Figure 6. Pak1 overexpression prevents t-tubular remodeling in rabbit VMs

Culture of adult ventricular myocytes after 24 h (black bars) results in a decrease of (A) the Ca transient amplitude and (B) a prolongation of the Ca transient rise time (time to peak) compared to Ctrl VMs at the time of isolation (white bars). In VMs transfected with constitutively active Pak1 (gray bars) culture induced changes in the Ca transient were attenuated. (C) T-tubular density in rabbit VMs non-transfected, or transfected with AdLacZ or AdPak1 after 0, 24, and 48 h of culture. T-tubular remodeling was attenuated in AdPak transfected cells (*, $p < 0.05$). (D) Western blot analysis of endogenous Pak1 (top panel) after 24h in culture indicated a loss of detectable Pak1 expression whereas adPak1 overexpression in VMs occurred at 24h and 48 h (bottom panel) of culture; α -actinin or GAPDH are shown as loading controls, respectively. Representative original confocal images of di-8-ANNEPS stained rabbit VMs that were (E) non-transfected or (F) transfected with AdPak1.

Figure 7. Changes in Pak1^{-/-} ECC are based on t-tubular remodeling

Representative original confocal images of di-8-ANNEPs stained atrial myocytes isolated from right atria of (A) WT and (B) Pak1^{-/-} mice. The staining did not reveal an organized t-tubular system. In contrast to VMs (C) Ca transient amplitude and (D) rise time (time to peak) were unchanged in WT and Pak1^{-/-} atrial myocytes.