Loss of Rad-GTPase Produces a Novel Adaptive Cardiac Phenotype Resistant to Systolic Decline with Aging

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Short title: Rad-deletion improves heart function

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Abstract

Rationale: Rad GTPase is a regulator of L-type calcium current, with increased calcium current observed in Rad knockout models. While mouse models that result in elevated LTCC have been associated with heart failure, our laboratory and others observe a hypercontractile phenotype with enhanced calcium homeostasis in Rad+. It is currently unclear whether this observation represents an early time point in a decompensatory progression towards heart failure, or whether Rad loss drives a novel phenotype with stable enhanced function.

Objective: We test the hypothesis that Rad+/− drives a stable non-failing hypercontractile phenotype in adult hearts, and we examine compensatory regulation of sarcoplasmic reticulum (SR) loading and protein changes.

Methods and Results: Heart function was measured in vivo with echocardiography. In vivo heart function was significantly improved in adult Rad+/− hearts compared to wildtype. Heart wall dimensions were significantly increased, while heart size was decreased, and cardiac output was not changed. Cardiac function was maintained through 18 months of age with no decompensation. SR releasable Ca²⁺ was increased in isolated Rad+/− ventricular myocytes. Higher Ca²⁺ load was accompanied by SERCA2a protein elevation as determined by immunoblotting and a rightward shift in the thapsigargin inhibitor-response curve.

Conclusions: Rad+/− promotes morphological changes accompanied by a stable increase in contractility with aging, and preserved cardiac output. The Rad+/− phenotype
is marked by enhanced systolic and diastolic function with increased SR uptake, which is consistent with a model that does not progress into heart failure.

Key words: Cardiac hypertrophy; calcium signaling; echocardiography; genetically-modified mice

Abbreviations: SR, Sarcoplasmic reticulum; LV, left ventricle; LTCC, L-type Ca$^{2+}$-channel; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; SERCA, sarco/endoplasmic reticulum calcium ATPase; ANF, atrial natriuretic factor; BNP, brain natriuretic protein; col 3a1, collagen isoform 3a1, RCAN1, regulator of calcineurin 1; NCX, sodium calcium exchanger; RyR, ryanodine receptor; CSQ, calsequestrin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PLB, phospholamban

New & Noteworthy

Deletion of Rad-GTPase results in a novel adaptive cardiac phenotype that includes stable increased cardiac output and preserved function that is maintained in aged mice. Mechanistically, Rad improves Ca$^{2+}$ homeostasis. These studies suggest that Rad-deletion is a potential therapeutic approach for maintaining cardiac function in aging, and perhaps disease.
**Introduction**

Disruptions to calcium cycling contribute to compromised function during heart failure progression. In response to pressure overload, L-type calcium channel (LTCC) single-channel current is increased (32), and sarcolemmal channel expression decreases (6). Impaired calcium homeostasis is further attributed to changes in the expression of calcium-handling proteins in the sarcoplasmic reticulum (SR) membrane, leading to reduction of SR Ca$^{2+}$-load. In particular, recent work has focused on modulation of the sarco/endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA2) and SERCA-inhibitory proteins (16, 21, 30).

Rad is a member of the RGK GTPase family (including Rem, Gem, Kir) that regulates current though the LTCC. Rad deletion or RNAi-mediated knockdown results in an increase in LTCC current (3, 23, 34). Rad protein is down-regulated in the myocardium of failing human hearts (3) suggesting that Rad loss may be an integral signaling component in myocardial adaptation. Our more recent studies have demonstrated that Rad deletion enhances systolic function at the cellular and the whole organ level (23).

Here, we test the specific hypothesis that the absence of Rad-GTPase does not accompany a loss of cardiac function *in vivo*. Rather, we report a novel, beneficial phenotype characterized by stable morphological changes and sustained elevation of function, without decompensation or contractile failure.
Methods

Mouse housing and generation

Global RRAD null mice (Rad+) were generated as previously described (23). We were unable to use littermates as a control because of incomplete record keeping involving the strategy utilized in the generation of the original Rad knockout. Therefore age-matched in-house bred C57/Bl6 mice served as controls. Male mice ages 4 months, 10 month, 18 months, or 21 months were evaluated. The experimental procedures and methods used were approved by the Animal Care and Use Committee of the University of Kentucky, and conformed to the NIH Guide for Care and Use of Laboratory Animals.

Quantitative RT-PCR

Male mice were anesthetized with ketamine and xylazine and hearts were quickly excised, after which the apex of the left ventricle was removed and snap frozen in liquid nitrogen. Frozen tissue was then homogenized, and RNA was isolated using RNAqueous kit (Life Technologies) and quantified using a Nanodrop (ThermoScientific). cDNA was generated from 500ng RNA, which was then amplified via RT-PCR using Taqman probes from Life Technologies: gapdh (Mm99999915_g1), nppa (Mm01255747_g1), nppb (Mm01255770_g1), collagen isoform col3a1 (Mm01254476_m1), and Primetime probes from IDT: ppp3bc, calcineurin (Mm.PT.56a.11865879), and calcineurin responsive rcan1 (Mm.PT.56a.41844729.g).

Threshold values (C_T) for nppa, nppb, col3a1, ppp3bc and rcan1 were normalized by subtraction from gapdh, and wildtype was then subtracted from Rad+ (ΔΔC_T) and fold changes were calculated as 2^{-ΔΔCT}. 
Echocardiography

Echocardiography was conducted using a Vevo2100 high-frequency ultrasound (VisualSonics). Mice were anesthetized with isoflurane. Heart rate was monitored using surface ECG and temperature was controlled at 37°C. A MS550D probe was used to acquire M-mode and B-mode image series along the long, short, and apical axes of the left ventricle. Mitral valve doppler imaging was used to determine the E:A ratio (33), which was taken as an indication for diastolic stiffness. Doppler mitral inflow pattern was acquired, and the two peaks indicating diastolic inflow were identified, followed by a negative outflow during systole. The early peak “E,” was taken as a measure of passive filling, while the later peak, “A” was taken as a measure of inflow due to atrial contraction. The ratio of the former to latter was measured, and an E value higher than A (i.e. E:A ratio is greater than 1) indicates normal passive stiffness. An A value higher than E (i.e. E:A ratio is less than 1) indicates elevated stiffness. Main pulmonary artery (MPA) flow imaging was done as a measure of cardiac output (CO). Pulmonary flow is the product of the main pulmonary area times the velocity time integral of the pulsed wave Doppler of the pulmonary flow. The analysis was done using Vevo2100 software v1.4.

Adult ventricular myocyte isolation

Left ventricular myocytes from Rad⁻/⁻ and wildtype hearts were isolated as previously described (23). Briefly, mice were anesthetized with ketamine and xylazine. Hearts were excised and subjected to retrograde perfusion on a Langendorff apparatus using a modified Tyrode solution. 5-7 mg of Liberase (Roche) was dissolved in perfusion buffer
to digest extracellular matrix, after which the left ventricle was dissected from the rest of the heart and was pulled apart with forceps. Digestion was stopped in 10% fetal bovine serum. Healthy cells were re-suspended in stop buffer and the calcium concentration was gradually increased in a step-wise fashion to 1mM. Prior to calcium elevation, an aliquot of cells was removed and fixed in 4% paraformaldehyde.

Histology
Mice were anesthetized as described above, and hearts were excised and perfused with saline followed by 10% formalin buffered in PBS. These hearts were cut along the short axis to reveal papillary muscles, embedded in paraffin, and cut into 5μm sections. Sections were then deparaffinized, hydrated, and stained with Masson’s Trichrome. Embedding, sectioning and staining was completed with the help of the Department of Molecular Pathology at the University of Texas, Southwest. Stained sections were then photographed at 4x. Images were analyzed using ImageJ for the ratio of blue pixels to red pixels meeting an intensity threshold, and this ratio was used to give a % fibrosis value.

Myocyte size measurements
Fixed myocytes were permeabilized with 1% Triton X-100, and non-selective binding was blocked with incubation with 10% BSA. Cells were then incubated with mouse anti-alpha actinin (Sigma), followed by Alexa-496 conjugated anti-mouse secondary antibody. This resulted in a characteristic striation pattern that was used to visually identify myocytes. Fluorescent images of stained cells were collected using Nikon NIS-
Elements, and area was measured by selecting myocytes and adjusting an intensity threshold until the entire cell was highlighted. Area was calculated automatically with Nikon NIS-Elements.

**Analysis of global calcium transients**

Ventricular myocytes were isolated as described (23), loaded with cell permeable Fura-2AM at room temperature for 5 minutes, washed, and re-suspended in physiological saline solution (PSS) containing 1.8 mM calcium. Myocytes were then field stimulated at 2Hz, and the F_{340/380} ratio was measured as an indicator of changes in cytosolic calcium concentration using Ion Wizard (IonOptix). For SR load evaluation, caffeine-induced calcium release measurements were performed by initially pacing cardiomyocytes for >60 seconds at 2Hz to attain steady state. Caffeine (50 mM) dissolved in PSS was rapidly administered one second after stimulus was halted. For caffeine-induced Ca^{2+} decay constant measurements, bath calcium was reduced to 0 for 20 seconds prior to caffeine puff to eliminate contamination of the calcium signal decay by LTCC current.

Thapsigargan dose-response curves were generated by pre-incubating isolated cells in increasing concentrations of thapsigargan (1.0e-10, 1.0e-9, 1.0e-8) for at least 10 minutes prior to recording.

**Spark Measurement**

Ventricular myocytes were isolated as described above. Cells were loaded with cell-permeable Fluo-4 at room temperature and paced at 3 Hz to steady state, then stopped to measure sparks, on a Live 5 (Zeiss) live cell scanning microscope. Line scans were acquired with a pixel size of 0.13 µm/pixel and a temporal resolution of 1 ms/line. Sparks were detected using Sparkmaster on ImageJ.
Western blotting

Hearts were homogenized by abrasive media (1.0mm zirconium oxide beads) in buffer (20mM Tris-HCl pH7.5, 250mM sodium chloride, 10mM magnesium chloride, 1% Triton X-100, 1mM sodium vanadate, 50mM β-glycerophosphate, 1x protease inhibitor cocktail 1 (Calbiochem, #539131) and 0.5mM DTT using a Next Advance Bullet Blender at 4°C. Samples were heated to 55°C for 15 minutes or 95°C for 5 minutes (for phospholamban blots) prior to running on SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes for 16 hours at low current (0.08mA). Membranes were blocked with casein prior to application of primary antibodies (mouse anti-Phospholamban, Thermo Scientific; rabbit anti-SERCA 2a, Badrilla; rabbit anti-Ryanodine receptor, Santa Cruz Biotechnology; rabbit anti-Calsequestrin, Abcam). Antibodies for Rad GTPase were generated in house. Additional antibody source detail is presented in Table 1. Horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit, Invitrogen; goat anti-mouse, Jackson ImmunoReasearch) were detected with Hyglo chemiluminescent reagent (Denville Scientific) and blots were visualized on a ChemiDoc MP (BioRad). Protein bands were analyzed with Image Lab software (BioRad) and values expressed relative to the wildtype mean.

Statistical Analysis

Significance was determined by Student’s T-test when comparing wildtype and Rad−/− cells or echocardiography-derived parameters. One-way analysis of variance (ANOVA) was used when two or more variables were compared, as in RT-PCR analysis, and two-way ANOVA was used to compare wildtype and Rad−/− echocardiographic
measurements in young and aged mice. Non-linear regression was used to fit thapsigargin dose-response values. p<0.05 was considered statistically significant.
Results

Rad loss results in novel cardiac muscle morphology

Rad protein loss was demonstrated in the Rad\(^{-/-}\) myocardium by immunoblotting (Figure 1A). Echocardiography revealed that Rad\(^{-/-}\) hearts exhibited thicker walls relative to the ventricular lumen inner dimension (h/r ratio, Figure 1B and 1C). Rad\(^{-/-}\) ventricular lumen was decreased at diastole and systole (Figure 1D and E), as was epicardial diameter (Figure 1F and G), suggesting that Rad\(^{-/-}\) hearts are smaller with thicker walls.

Calculated ventricle weight normalized to body weight was unchanged (Figure 1H), which was matched by observed heart weights normalized to body weight (Figure 1I). Normalizing heart weight to tibia length did not reveal a difference either (Figure 1H; HW/TL: WT, 9.65 +/- 0.36; Rad\(^{-/-}\) 9.10 +/- 0.42).

The measured area of isolated ventricular myocytes in Rad\(^{-/-}\) is greater than that of wildtype (Figure 2A and B). These alterations in myocyte dimensions motivated evaluation of the expression of the canonical fetal gene program associated with cardiac hypertrophy. *nppa*, *nbbp*, and *col3a1* are significantly increased in expression in Rad\(^{-/-}\) compared to wildtype hearts (Figure 2C). Additionally, the expression of *rcan1* (Regulator of calcineurin 1) was evaluated to investigate a possible connection between the reported increased diastolic calcium in Rad\(^{-/-}\) (23) and the calcium-responsive calcineurin/NFAT hypertrophic pathway. A similar increase in the expression of this gene was observed, with a corresponding decrease of *ppp3cb* (calcineurin) (Figure 2C). These data suggest that Rad deletion produces structural changes in the shape and size of the heart that are distinct from pathological hypertrophic remodeling.
Rad null mice exhibit greater contractility in vivo

Changes in Rad\textsuperscript{-/-} heart morphology were accompanied by significantly increased LV function (Figure 3A), as expected from previously published data examining the function of individual cells and isolated working hearts (23). Rad\textsuperscript{-/-} hearts displayed improved ejection fraction (Figure 3B) and fractional shortening (Figure 3C). Diastolic function was also measured because previous results showed that diastolic calcium and fibrosis are increased in Rad\textsuperscript{-/-} (3, 23). E:A ratios were significantly above 1 for both wildtype and Rad\textsuperscript{-/-} mice, suggesting that diastolic function is not impaired (Figure 3D). To demonstrate that these differences are not due to changes in intrinsic heart rate reported previously (23), heart rate was titrated with anesthesia to similar levels for all recordings (Figure 3E). Main pulmonary artery (MPA) imaging revealed that cardiac output was no different between Rad\textsuperscript{-/-} and WT (Figure 3F), suggesting that the hypercontractile state observed in Rad\textsuperscript{-/-} hearts is sufficient to maintain stable output despite a smaller LV lumen. Bright, relatively cohesive traces indicate laminar flow in wildtype (Figure 3G, upper left panel). Diffuse, grey shading is observed in the Rad\textsuperscript{-/-}, consistent with turbulence from ejecting blood faster through a fixed diameter MPA (Figure 3G, lower left panel).

Improved function is preserved in aged Rad\textsuperscript{-/-} mice

Enhanced Ca\textsuperscript{2+}-entry acutely increases function, but over-expression of LTCC channels has been found in other models to lead to the development of pathological hypertrophy over a time-frame of about 8 months (28). We therefore allowed a cohort of wildtype
and Rad\(^{-}^{-}\) mice to age into senescence. At the advanced age of 18 months, histological
sections and echocardiography showed no evidence of wall thinning in Rad\(^{-}^{-}\) hearts
(Figure 4A and B). Senescent LV function was significantly greater in Rad\(^{-}^{-}\) compared
to wildtype (Figure 4B-D). Relative wall thickness (h/r ratio) was significantly increased
in Rad\(^{-}^{-}\) with aging but not wildtype (Figure 4E). MPA imaging indicated that cardiac
output was maintained in Rad\(^{-}^{-}\) mice, suggesting a stable phenotype with hyperdynamic
LV function (Figure 4F). Finally, immunoblotting established that Rad protein
expression remained unchanged in senescent wildtype mice (Figure 4G).

Previously published data suggested that fibrosis was increased in Rad\(^{-}^{-}\) mice
compared to wildtype, so we next evaluated whether this is altered in old age. We
confirmed that fibrosis is increased in Rad\(^{-}^{-}\), and we have further found that this
increase is maintained at 18 months (Figure 5A and B). However, E:A ratios do not
show a difference between Rad\(^{-}^{-}\) or wildtype in either young or aged animals,
suggesting that this increase in fibrosis had no detectable effect on myocardial stiffness
(Figure 5C). Further measures of diastology using tissue Doppler (Young mice: e' = -
19.8±0.9 for WT, -20.8±4.0 for Rad\(^{-}^{-}\); Aged mice: e' = -17.2±3.4 for WT, -20.2±5.2 for
Rad\(^{-}^{-}\)) were similarly unchanged.

SR load is increased in Rad\(^{-}^{-}\) ventricular myocytes

Rad\(^{-}^{-}\) myocardium has increased calcium current density (23). Dogma dictates that
chronic increased LTCC activity promotes heart failure. Thus, a Rad\(^{-}^{-}\) phenotype
classified by enhanced heart function despite persistently increased LTCC function
well into old age was unexpected (26, 27). To investigate a potential mechanism by which Rad\(^{+/−}\) hearts avoid heart failure, we examined the SR Ca\(^{2+}\) load. SERCA down-regulation contributes to impaired SR Ca\(^{2+}\)-loading in heart failure (14), and rescue of SERCA expression and SR loading improves heart function (17, 24). To determine the impact of Rad-deletion on SR Ca\(^{2+}\)-load, caffeine-inducible Ca\(^{2+}\)-release was measured from cells after pacing to steady state (Figure 6A). In ventricular myocytes from Rad\(^{+/−}\) hearts, the caffeine–inducible peak was increased (Figure 6B), as was the percentage of Ca\(^{2+}\) available in the SR that was released during each stimulus (Figure 6C). Thus, both SR-load and the extent of SR Ca\(^{2+}\) release were increased in Rad\(^{+/−}\) myocytes.

Interestingly, the decay constant of the caffeine-induced transient was slower in Rad\(^{+/−}\) myocytes (Figure 6D), suggesting that enhanced calcium extrusion from the cell by NCX is not necessarily triggered by elevated Ca\(^{2+}\)-influx. Given that increased SR load is associated with increased leak (4), we next measured spark release as measure of calcium release during diastole. As expected, more sparks were observed in Rad\(^{+/−}\) cells than WT (Figure 6E and 6F).

**SERCA protein is elevated in Rad\(^{+/−}\) hearts**

Immunoblotting Rad\(^{+/−}\) hearts previously showed changes in the phosphorylation state of phospholamban and calcium/calmodulin dependent protein kinase II (CaMKII) (20). This motivated the evaluation of the expression of other calcium handling proteins. Immunoblotting shows that SERCA 2a protein is significantly up-regulated in Rad\(^{+/−}\) hearts (Figure 7A), although the absence of a difference in mRNA suggests that SERCA is not subject to differential transcriptional control in Rad\(^{+/−}\) (Figure 7B). Calsequestrin 2, RyR\(_2\), and phospholamban protein levels are unchanged, and Ca\(_{v}\)1.2
protein is decreased (Figure 7A). In order to determine whether SERCA2 protein levels
are tied to the increased function observed in Rad⁻/⁻ hearts, a concentration-response
curve to the SERCA inhibitor thapsigargin was generated (Figure 7C and 7D). As
previously described (20), thapsigargin reduced contractility by ~50% in wildtype
ventricular myocytes with an IC₅₀ of 6.1e-11. However, the response of Rad⁻/⁻
cardiomyocytes was both attenuated and shifted rightward (IC₅₀ 2.6 e-10), indicating
that more SERCA2 is present. Moreover, a thapsigargin-insensitive component may
contribute to increased sarcomere shortening in Rad⁻/⁻ cells, consistent with increased
SERCA protein increasing SR loading and in conjunction with increased LTCC current,
summing to improved cardiac function.
Discussion

The present study of Rad<sup>-/-</sup> mice characterizes a novel hypercontractile phenotype that is preserved during aging. Rad<sup>-/-</sup> mice exhibit smaller hearts with increased heart wall thickness, and larger cardiomyocytes. However, cardiac remodeling in Rad<sup>-/-</sup> mice is not hypertrophic, as cardiac mass does not differ between wildtype and Rad<sup>-/-</sup> hearts. These hearts remain hypercontractile in aged mice, suggesting that their increased systolic and diastolic function is stable and sustainable. This improved function is associated with increased SR load and up-regulation of SERCA2a.

As our laboratory and others have demonstrated, the loss of Rad increases LTCC current (I<sub>Ca,L</sub>) and calcium transient amplitude, with an associated increase in systolic function (23, 34). We also observe a decrease in CaV1.2 protein, which is the expected result of an increase in LTCC activity due to the loss of a negative current regulator (31). Conversely, all RGK family members block LTCC activity (8-12, 22, 29). Opposite approaches that rely on overexpression models have shown profound block of I<sub>Ca,L</sub>. Over-expression of RGK protein Gem, for example, reduces transient amplitude and function, which supports the findings that RGK proteins like Rad act as negative regulators of function (25). Rad<sup>-/-</sup> mouse hearts mimic several characteristics of young mice overexpressing the α subunit of the LTCC, including increased I<sub>Ca,L</sub> current density, a hyperpolarizing shift in LTCC activation potential, reduced sensitivity to adrenergic stimulation, increased contractility, and heart wall growth (23, 28). However, LTCC-overexpressing mice progress to decompensation after 8 months (26). Importantly,
increased function and preserved calcium handling persist in Rad<sup>−/−</sup> hearts long after this point (Figure 4), and significant mortality was not exhibited in Rad<sup>−/−</sup>, even after 1.5 years. It should also be noted that both the α subunit overexpressing model and Rad<sup>−/−</sup> model appear to be distinct from genetic modifications that target expression of the β subunit of the LTCC directly, such as β2a overexpression or knockdown, which result in severe heart failure (5) or protection from TAC-induced hypertrophy (7), respectively. Rather, Rad deletion seems to produce a sustained stable compensatory phenotype. This appears to occur despite gradual and continuous wall thickening that occurs into old age. These studies assess global Rad loss; therefore, we cannot exclude an effect of Rad loss in non-cardiomyocytes and the potential long-term effect of neural and humoral impacts on cardiac function. However, single cell and isolated working heart measures presented both here and in previously published data (23) mirror in vivo parameters consistent with cardiac specific effects.

SERCA2a has been shown to play a role in the preservation of function under conditions that otherwise would be expected to produce failure (2, 13, 18, 19). SERCA regulatory proteins such as phospholamban and sarcolipin are also associated with improved contractility and mitigation of heart failure(1). We have shown previously that phospholamban is phosphorylated in vivo to a higher degree in Rad<sup>−/−</sup> hearts (23), which would be expected to result in higher SERCA activity. Furthermore, we show here that SERCA protein itself is up-regulated. These data together point to a phenotype whereby increased calcium sequestration via SERCA balances an increase in calcium influx via the LTCC. This increase in uptake may explain the increase of caffeine-
induced SR load in Rad⁻/⁻ myocardium. Increases of SERCA and SR calcium load is observed in models of non-failing hypertrophy (physiological hypertrophy) such as with exercise or pregnancy (15). These changes do not compromise myocardial contractility and are associated with increased SERCA2a expression (35), similar to Rad⁻/⁻. SERCA2a protein, but not mRNA, is also up-regulated in a non-pathological model of hypertrophy induced by AKT overexpression (20). Perhaps most importantly, SERCA2a expression levels have been shown to play a key role in restoring efficient loading of the SR in human heart failure patients, including trials that increase expression of SERCA2a and restore the capacity of the SR to sequester and subsequently release calcium (18, 19). These studies have found that adeno-associated virus-mediated SERCA2a expression is associated with reduced mortality and increased systolic function in patients with heart failure. These studies underscore the clinical relevance of exploring models that increase SERCA2a protein levels. Both immunoblotting and a rightward shift in the IC₅₀ following thapsigargan treatment suggest that SERCA2a protein is up-regulated in the Rad⁻/⁻ myocardium, which provides a potential explanation for the preserved function observed in Rad⁻/⁻ hearts despite increased calcium current. Importantly, maximal thapsigargan concentrations fail to return sarcomere shortening to the same levels observed in wildtype mice, which is consistent with previously published studies by our laboratory and others that elevated L-type calcium channel current density contributes to increased contractile function (20).

The present study is demonstrates stable basal changes in function imposed by Rad-deletion. Our studies complement and expand upon an earlier report of the
dimensional changes of the Rad\(^{-}\) myocardium in response to pressure overload (3). In the earlier study it was proposed that Rad\(^{-}\) hearts show an enhanced hypertrophic response to pathological stimuli; however, measures were limited to cell size from histological sections (subject to error imposed by helical arrangement of myocardial fibers) and qRT-PCR. No echocardiography or other measure of cardiac function was reported. Basal changes in cell size, fetal gene program biomarkers, or heart function were not previously noted. We observe elevated ANF (\textit{nppa}) expression in Rad knockout mice that was not previously seen, although it should be noted that the previous report failed to detect the expected ANF induction in response to thoracic aortic constriction in wildtype mice (3). Consistent with increased Ca\(^{2+}\) homeostasis reflexively decreasing Ca\(_{\text{v}}\)1.2 expression (31), Rad\(^{-}\) exhibits down-regulation of Ca\(_{\text{v}}\)1.2. We also present \textit{in vivo} functional data for Rad\(^{-}\) mice, revealing the unexpected insight that contractility remains elevated in Rad\(^{-}\) mice well into senescence, with a corresponding preservation of cardiac output. In view of these findings, the down-regulation of Rad in long-term human heart failure (3) may be re-contextualized from playing a pathological role to a compensatory one. This is particularly important as novel therapies to promote systolic function must take into account the possibility that prevention of Rad loss in the heart, rather than slowing decompensation, will reduce systolic function and produce further injury. A critically important question is whether SERCA2a up-regulation and enhanced SR-loading caused by Rad-deletion will protect against pathological hypertrophic stimulus, such as pressure overload. Studies are ongoing to determine the answer to this question.
In summary, we demonstrate an \textit{in vivo} characterization of the effects of Rad deletion on the morphology and function of the heart. We demonstrate significant changes in relative ventricular wall thickness without a corresponding change in overall heart size, accompanied by an increase in contractility and preserved cardiac output. These changes are preserved into old age, and do not result in loss of either systolic or diastolic function. This is consistent with an increase in myocyte SR load and increased expression of SERCA2a protein. These data suggest that Rad loss plays a novel role in calcium homeostasis of the heart. Deletion of Rad results in a heart with reduced LV volume compensated by faster ejection. The net outcome is that Rad-deletion safely maintains cardiac output despite increased Ca$^{2+}$ homeostasis.

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\textbf{Disclosures}

None
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Figure 1. Rad deletion alters cardiac morphology

A. Representative immunoblot demonstrating the loss of Rad protein. N= 3 mice for each genotype

B. Representative echocardiographs of WT and Rad^{-/-} at diastole and systole.

C. Relative wall thickness (h/r ratio) of WT and Rad^{-/-} LVs.

D. and E. LVID (LV inner dimension) is reduced in Rad^{-/-} at both diastole (LVIDd, D) and systole (LVIDs, E).

F. and G. Epicardial diameter is similarly reduced in Rad^{-/-} at both diastole (F) and systole (G). G and H. Calculated LV mass normalized to body weight (G), and observed heart weight/body weight measurements are not different in Rad^{-/-} mice compared to WT (H), N=30 mice per genotype for all echocardiographic measures, and 3-4 mice per genotype for heart weight/body weight measures. *p<0.05, **p<0.01, ****p<0.0001 vs. WT.

Figure 2. Rad deletion increases cell size and upregulates fetal and growth markers.

A. Representative isolated ventricular myocytes stained for α-actinin (red) and nucleus (blue).

B. An increase in area is observed in myocytes isolated from Rad^{-/-} hearts. N=3 hearts. *p<0.05 vs. WT
C. Up-regulation of hypertrophic markers *nppa* (ANF), *nppb* (BNP), collagen isoform *col3a1, pp3cb* (calcineurin) and *rcan1* (MCIP) in Rad^{/-} hearts. N=6 hearts. *p<0.05, **p<0.01 vs. WT.

**Figure 3. Rad deletion improves contractile function.**

A. Representative M-mode traces for WT and Rad^{/-} 4 month old mice.

B and C. Ejection fraction (B) and fractional shortening (C) are elevated in Rad^{/-} hearts. N=30 mice per genotype.

D. Diastology is not significantly different between WT and Rad^{/-}. N=9-10 mice.

E. Heart rate of anesthetized mice is maintained at a constant rate for both genotypes.

F. Cardiac output as measured by MPA imaging is not significantly different between WT and Rad^{/-}.

G. Representative MPA cardiac output Doppler traces. ****p<0.0001 vs. WT.

**Figure 4. Aged Rad^{/-} hearts exhibit enhanced function with progressive LV wall thickening.**

A. Representative images of Masson’s trichome-stained sections showing increasing wall thickness in Rad^{/-} from 4 months, 8 months, and 21 months.

B. Representative B-mode images from 4-month and 18-month old mice.
C. and D. Ejection fraction and fractional shortening are elevated in young and aged Rad−/−.

E. Relative wall thickness (h/r) is progressively higher in Rad−/−. N=10-13 4-month, and 3-4 18-month mice. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. WT

F. Cardiac output remains unchanged in WT and Rad−/− mice. N=4-5 mice per genotype.

G. Rad expression is not changed in old age. Data represented as expression in 20-month relative to 8 month old mice. Calsequestrin is shown as a loading control. N= 3 mice per genotype.

Figure 5. Young and aged Rad−/− hearts show increased fibrosis, without an observable effect on myocardial stiffness

A. Representative images from of Masson’s trichome stained sections showing increasing fibrosis in Rad−/− from 4 months and 20 months.

B. Significantly more fibrosis is measured in trichrome stained hearts from Rad−/− compared to WT in both young and aged mice.

C. E:A ratio is unchanged and remains above 1 for both WT and Rad−/− in old age.
*p<0.05,**p<0.01 vs WT. N = 3-6 mice.

Figure 6. SR load is elevated in Rad−/−

A. Representative paced and caffeine-stimulated Ca2+-transients from WT and Rad−/−.
B. and C. The caffeine-induced Ca\(^{2+}\)-release amplitude (B) and the ratio of stimulated
calcium release to total caffeine-induced SR Ca\(^{2+}\)-release (C) was greater in Rad\(^{-/-}\)
compared to WT.

D. The caffeine decay constant (\(\tau\)) is significantly prolonged in Ca\(^{2+}\)-free media.

E. Spark frequency is increased in Rad\(^{-/-}\) compared to WT.

F. Representative line scan showing stimulus (arrowhead) and sparks measured after a
> e-fold decay of the global Ca\(^{2+}\) transient in WT and Rad\(^{-/-}\).

N=3 mice per genotype. *p<0.05, ***p<0.001 vs WT.

**Figure 7.** SERCA is increased in Rad\(^{-/-}\) hearts.

A. SERCA 2a protein is significantly up-regulated, while there is no change in protein
levels of ryanodine receptors (RyR\(_2\)), calsequestrin 2 (CSQ2), or phospholamban (PLB).

Ca\(_{\text{v}}\)1.2 is slightly but significantly down-regulated. Approximate molecular weights of
bands as shown: RyR2 ~ 550 kDa, Ca\(_{\text{v}}\)1.2 ~ 200 kDa, SERCA2a ~ 100 kDa, CASQ2 ~
55 kDa, GAPDH ~ 37 kDa, PLB ~ 10 kDa. N=3-4 mice. *p<0.05.

B. SERCA 2 mRNA is not significantly up-regulated in Rad\(^{-/-}\) hearts.

C and D. Inhibitor-response curves of thapsigargin-induced depression of sarcomere
shortening (C) and concentration-response normalized to maximal function (D) is
shifted rightward in Rad\(^{-/-}\) myocytes. N=3 mice per genotype, 30 cells per concentration.
Manning et al.,
Figure 1
Manning et al.,
Figure 2
Manning et al.,
Figure 3
Manning et al., Figure 4
Manning et al., Figure 5
Caffeine-induced release

**Caffeine Transient Decay constant**

**Spark Density**

**Manning et al., Figure 6**
Figure 7

(A) Expression relative to WT

(B) SERCA2 mRNA

(C) Sarcomere shortening (%) vs. Thapsigargan

(D) % maximum sarcomere shortening vs. Thapsigargan

IC$_{50}$ values:
- SERCA2: $2.6 \times 10^{-10}$
- CaV1.2: $6.1 \times 10^{-11}$