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Role of RhoA in Cardiac Aging with a Focus on Autophagy and Mitophagy

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by

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The thesis of Michelle Tu is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California San Diego

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DEDICATION

This thesis is dedicated to my dad, my mom, and my brother for all their love and sacrifice, and to my friends and all the people I have met throughout my master's for their endless encouragement. I could not have done it without each and every one of you.

From the bottom of my heart,

Thank You.

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ABSTRACT OF THE THESIS

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Aging is commonly associated with the failure to maintain cellular homeostasis, including the maintenance of mitochondrial health. Mitochondrial health, and subsequently cellular homeostasis, can be compromised upon cellular stress and is, therefore, regulated through mitophagy, the mitochondria-specific form of autophagy which is the self-recycling system of the cell. The small GTPase protein, RhoA, participates in various cellular processes

and has been shown to protect the heart against numerous stresses. In this study, we explore whether RhoA protects against aging in the heart and investigate the potential of RhoA in regulating the cellular homeostatic processes, autophagy and mitophagy. While investigating the role of RhoA in cardiac aging, 4 month old (young) cardiac-specific RhoA knockout mice did not show overt signs of cardiac dysfunction, but at 10 months of age (middle-aged) revealed early onsets of age-associated features in the heart such as hypertrophy and contractile dysfunction when compared to wildtype littermates. Although our data in RhoA knockout mice suggest that autophagy is not regulated by RhoA, adenoviral overexpression of RhoA in neonatal rat ventricular myocytes (NRVMs) increased both PINK1 and Parkin mitochondrial protein levels which lead to mitophagy. The observed accumulation of PINK1 was found to result from RhoA-mediated inhibition of PINK1 protein degradation, which was also discovered to involve the localization of active RhoA together with the activation of PKD, a RhoA downstream effector. Our findings suggest RhoA as both a protective signaling molecule against the progression of cardiac aging and a previously unidentified regulator of mitophagy in cardiomyocytes.

INTRODUCTION

Cardiac Aging

Age has long been associated as a major risk factor for many prominent diseases including, but not limited to, neurodegenerative diseases, cancers, as well as cardiovascular diseases [1-3]. With age, heart disease becomes increasingly more prevalent as a cause of death, eventually becoming the leading cause of death in age groups above the age of 65 in the U.S. [4]. Advancements in research and medicine continue to extend our lifespan farther from what it was decades ago, increasing the number of people reaching the age of 65 and above, and hence, further increasing the burden of heart disease in our world today [5]. Because of its rising prevalence, further studies exploring the mechanisms behind age-dependent developments in the heart is of interest for finding better treatment options and lowering the burden of heart disease.

Cardiac aging refers to the age-dependent changes that occur in the heart with time and is known to contribute to the susceptibility of the heart to failure. A commonly observed hallmark of cardiac aging is the development of hypertrophy, the enlargement of cells [6, 7]. When in the face of stress, the heart preserves contractile function through cardiac hypertrophy. However, excessive enlargement of cardiomyocytes can become maladaptive and instead, result in cardiac dysfunction [8, 9]. Hypertrophy has been extensively studied in the heart and observed to largely contribute to diastolic dysfunctioning in numerous cardiac diseases, including the aging heart [8, 10]. Age-related cardiac dysfunction includes slowed left ventricular diastolic filling rate, decreased max heart rate and reduced cardiac contractility [11, 12]. Notably, cardiac contractility is compromised in the aged heart in part due to increased fibrosis. Fibrosis reduces compliance in

the heart and has been observed to begin to do so in those as young as 40 years old [13, 14]. Additionally, aging in the heart is also associated with low levels of chronic inflammation, which has recently been suggested to contribute to the development of heart failure [15, 16].

A major contributing factor of the mentioned features of cardiac aging is the loss of cardiomyocytes due to cell death. Unlike many other cell types, cardiomyocytes lose their ability to undergo cell division at birth, and hence are irreplaceable once lost [17]. Over time, the heart endures and accumulates a lifetime's worth of stress and damage which eventually can promote and lead to cell loss. The heart continuously requires an extensive amount of ATP to constantly deliver nutrients and oxygen to cells throughout the body from birth till death. Thus, cardiomyocytes are rich in mitochondria, a major source of cellular ATP, and not surprisingly, are particularly susceptible to mitochondria-derived reactive oxygen species (ROS)-mediated cell death compared to many other cell types [18]. ROS can wreak havoc in a cell by oxidizing various macromolecules and result in the formation of damaged and misfolded proteins and aggregates [19]. High levels of protein aggregates have been well associated with many age-related diseases both in the brain as well as in the heart [20, 21], leading to the growing number of studies investigating the regulation of cellular maintenance mechanisms, such as autophagy, as potential therapeutic targets for age-associated diseases [22-24].

Additionally, it is not surprising that changes in mitochondrial shape and structure as well as extensive mitochondrial dysfunction are commonly observed in the aged heart due to the particular susceptibility of mitochondrial DNA to ROS-mediated damage [25-27]. In response to stress, the mitochondrial membrane is depolarized and both pro-survival mechanisms such as mitophagy as well as pro-death pathways such as mitochondria-induced cell death are activated

concurrently [28]. When the amount of stress is not excessive, mitochondrial maintenance mechanisms such as mitophagy will be able to clear the damaged mitochondria before mitochondrial cell death pathways are fully activated, successfully protecting the cell from cell death [28]. Of interest in the field of aging is Denham Harman's free radical theory of aging which postulates that the features of aging result from the accumulation of free radical-mediated disruption of cellular homeostasis, and of recent, it is specifically mitochondria-derived ROS that is emphasized to have a large contribution in the development of aging [29].

Autophagy and Mitophagy

Studies investigating the causes of aging have for the most part agreed upon the build up of cellular wastes as a critical culprit of cardiac aging and this includes, but is not limited to, the build up of both misfolded proteins and ROS by-products [30]. Autophagy is the self-recycling system of the cell which maintains cellular homeostasis by degrading damaged cellular components. Cellular components, including misfolded proteins and aggregates, are encased in double-membraned vesicles called autophagosomes that fuse with lysosomes for lysosome-mediated degradation of its components [31]. Mitophagy is a specific form of autophagy that is selective towards the degradation of damaged mitochondria to prevent a cycle of ROS production and cell death by maintaining mitochondrial quality [29, 31]. Interestingly, both autophagy and mitophagy have been found to decline with age [22, 33], and as a result, a large amount of research has been dedicated towards studying the potential of utilizing these pathways as therapeutic targets against age-related diseases.

Mammalian target of rapamycin complex 1 (mTORC1) is a signaling complex that promotes growth, proliferation, and survival in the cell and is well known to negatively regulate autophagy. Many studies have shown that the activation of mTORC1 and its subsequent inhibition of autophagy results in early onsets of cardiac aging [23, 34, 35]. Remarkably, cardiac aging is reversed through treatment with pharmacological mTOR inhibitors such as rapamycin and everolimus, making mTOR a promising target for the maintenance of cardiac health [23, 35]. Caloric restriction has also been established to inhibit mTORC1 signaling leading to the initiation of autophagy. Interestingly, however, it was found that early application of caloric restriction resulted in worsened cardiac health in mice [36]. Additionally, although rapamycin and everolimus induce autophagy and prolong lifespan in mice, these compounds are approved as immunosuppressants which leads to the concern of increased risk of infections in humans that may come with these treatments [37]. As a result, it is increasingly clear that while mTOR is a major regulator of autophagy, better therapeutic targets of mTORC1 signaling are needed and more research must be done before establishing mTORC1 as an ideal target against aging.

Mitophagy is classically induced by the depolarization of the mitochondrial membrane upon mitochondrial damage and is mediated through PINK1, a serine/threonine protein kinase. Depolarization of the mitochondrial membrane inhibits degradation of PINK1, resulting in PINK1 accumulation at the mitochondria to subsequently recruit the E3 ubiquitin ligase, Parkin. Parkin ubiquitinates PINK1 and other mitochondrial proteins to induce the encasement of the damaged mitochondria in a double membrane vesicle called the mitophasome to which a lysosome then fuses with and mediates the degradation of its contents [38]. Features of cardiac aging are found to accelerate in Parkin-deficient mice, while cardiac-specific overexpression of

Parkin is found to delay cardiac aging [39], identifying Parkin as a potential target for the regulation of mitophagy against cardiac aging. Additionally, deletion of PINK1 in mouse models of the age-associated disease, Parkinson's disease, resulted in exacerbated neurodegeneration due to increased mitochondrial dysfunction [40, 41], suggesting a significant role for PINK1 and overall mitochondrial maintenance in age-related diseases. Because PINK1/Parkin mediated mitophagy is conventionally activated upon mitochondrial damage, it is difficult to identify potential target molecules that regulate the PINK1/Parkin pathway without inducing mitochondrial toxicity [42]. Thus, it is important to identify intracellular signaling pathways that modulate mitophagy independent of the classical pathway to discover a safer therapeutic target for age-related heart failure.

RhoA

A small G-protein mediating signaling cascades in its GTP-bound active state has been identified to have significant roles in the cardiovascular system. The small G-protein, RhoA, serves as a proximal downstream effector of numerous GPCRs and has been found to be responsive to different stress signals [43, 44]. Originally, the activation of RhoA was thought to be detrimental in the heart as supraphysiological levels of RhoA expression produced cardiac hypertrophy and fibrosis in mice [45]. Levels of its downstream effector, rho-associated coiled-coil containing protein kinase (ROCK), have also been observed to increase in response to ischemic injury [46] and inhibition of this downstream effector was found to reduce ischemia/reperfusion (I/R) induced infarct size [47].

In the past decade, however, a new light was shed onto the role of RhoA in cardiac pathologies. Low levels of active RhoA expression in the heart did not induce hypertrophy in mice and a study involving the use of cardiac-specific RhoA knockout (KO) mice revealed that pressure-overload induced hypertrophy is not mediated, but rather, is inhibited by RhoA [48]. Additionally, RhoA activation has been demonstrated to confer cardioprotection against not only I/R injury, but also oxidative stress [49, 50], and this protection is inhibited not through the inhibition of ROCK, but through that of another downstream effector, PKD [50, 51].

To further investigate the role of RhoA as a protective molecule against cardiovascular diseases, we explored its potential in protecting the heart against aging, specifically through regulation of autophagy and mitophagy. In our study, cardiac-specific RhoA KO mice ($RhoA^{fl/fl,\ \beta MHC\text{-}cre}$) were compared against their wildtype (WT) littermates at 4 months (young) and 10 months (middle-aged) of age for hypertrophy, fibrosis, inflammation, and cardiac contractile function as well as for the potential of RhoA to regulate autophagy and mitophagy. Our findings suggest that RhoA protects the heart against the progression of cardiac aging plausibly through the activation of mitophagy that is uniquely independent of the conventional mitochondrial membrane depolarization pathway.

MATERIALS AND METHODS

Animals

Procedures involving the use of animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of California, San Diego. Animal experiments were performed on either 4 month or 10 month old wild-type or RhoA knockout mice whose generation has been previously described [49]. Echocardiograms were performed and measured as previously described [52].

NRVM Cell Culture

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1-2 day old Sprague-Dawley rat pups (Harlan, Indianapolis, IN) as previously described by using the Neonatal Cardiomyocyte Isolation System (Worthington, Lakewood, NJ) [53]. NRVMs were plated at a density of 7.0 x 10^6 cells per 15 cm TC treated dishes (from Genesee Scientific Corporation) that had been coated the day before with 1% gelatin. NRVMs were cultured overnight at 37℃ and 5% CO_2 in high glucose Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 15% fetal bovine serum (FBS), 1% L-glutamine, and antibiotics (200 units/mL penicillin and 200 µg/mL streptomycin). Cells were then serum-starved the following morning in high glucose DMEM supplemented with antibiotics (100 units/mL penicillin and 100 ug/mL streptomycin) for 24 hours before cell harvest.

Gateway Cloning Technology (Thermo Fisher Scientific) was used to generate adenoviruses expressing green fluorescent protein (GFP), constitutively active RhoA (L63RhoA), miniSOG-tagged WT PINK1 (WT PINK1), miniSOG-tagged phospho-dead PINK1 (SA PINK1), miniSOG-tagged phospho-mimicking PINK1 (SD PINK1), and PKD as shown in our previous studies [50, 54, 55]. mCherry-tagged Parkin (Parkin) expressing adenovirus was provided by Dr. Asa Gustafsson (UCSD, La Jolla, CA). NRVMs were either infected with or without Parkin, WT PINK1, SA PINK1, and/or SD PINK1 for 4 hours before undergoing a 24 hour infection with GFP, L63RhoA, and/or PKD. NRVMs were also either treated or not treated with 100 μg/mL cycloheximide (CHX) (Cayman Chemical Company) for 10, 15, 20, or 60 minutes or 100 nM of CID 7555673 (CID) (Sigma Aldrich) for 24 hours before harvest.

Active RhoA Pull Down Assay

300 μL of RhoA lysis buffer (500 mM NaCl, 10 mM $MgCl_2$, 50 mM Tris HCl(pH 7.4), 0.1% SDS, and 1% NP-40) supplemented with inhibitors (10 μg/mL leupeptin, 10 μg/mL aprotinin, 200 μmol Na_3VO_4, 1 mM PMSF, and 1 mM PNPP) was used to homogenize powderized adult mouse ventricles over vortex. 700 μL of the RhoA lysis buffer was then added to the homogenates for a final volume of 1 mL before spinning at 14,000 rpm (20,817 g) for 1 minute at 4℃. The supernatant was saved and first frozen in liquid nitrogen for 3 minutes before dethawing and subjected to a micro BCA assay (Thermo Fisher Scientific) for measurement of protein concentrations. Equal amounts of proteins were incubated for 2 hours with agarose-bound rhotekin-RhoA binding domain (Cytoskeleton Inc). After incubation, the beads were subjected to a series of washes and centrifugations before preparing samples in 4x Laemmli

buffer as previously described [51]. Western blotting for RhoA was used to detect activated GTP-bound RhoA.

Whole Heart Homogenates and Whole Cell Lysates

Powderized adult mouse ventricles were homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris (pH 7.4), 1% NP-40 alternative, 1% sodium deoxycholate, 0.1% SDS, 0.2 mM EDTA) supplemented with inhibitors. NRVMs were washed twice with PBS before lysing in RIPA buffer supplemented with inhibitors as mentioned above. Samples were sonicated for 1 minute, vortexed for 30 seconds, and nutated at 4℃ for 10 minutes before spinning down at 14,000 rpm (20,817 g) at 4℃ for 5 minutes. Supernatants were then saved and protein concentrations were measured by using a micro BCA assay (Thermo Fisher Scientific).

Mitochondrial Fractionation

Powderized adult mouse ventricles were homogenized three times in 300 µL of Mito Fractionation D Buffer (20 mM HEPES (pH 7.4), 210 mM Mannitol, 70 mM sucrose, 1 mM EGTA) with 0.025% high purity Digitonin (EDM Millipore) and supplemented with inhibitors (10 µg/mL leupeptin, 10 µg/mL aprotinin, 200 µmol Na_3VO_4, 1 mM PMSF, and 1 mM PNPP), transferring and saving the supernatant after each homogenization. 1100 µL of Mito Fractionation D Buffer with 0.025% high purity Digitonin and supplemented with inhibitors was then added to the saved lysates for a final volume of 2 mL. NRVMs were washed twice with ice-cold PBS before harvesting in 2 mL of Mito Fractionation D Buffer with 0.025% high purity Digitonin and supplemented with inhibitors as mentioned above. Lysates were then sheared five

times through a 3 mL syringe (Becton, Dickinson and Company) with a 25 gauge needle (Becton, Dickinson and Company) before shaking at 4℃ for 20 minutes. Lysates were then spun at 1,000 g at 4℃ for 5 minutes and the resultant supernatants were then centrifuged at 700 g at 4℃ for 10 minutes. The saved supernatant was then spun at 16,000 g at 4℃ for 15 minutes, saving the pellet. The saved pellets were washed in 200 µL of lysis buffer and spun at 16,000 g for 2 minutes. The supernatant was aspirated and pellets were resuspended in 120 µL of RIPA buffer. Resuspended pellets were sonicated for 1 minute, vortexed for 30 seconds, nutated for 5 minutes at 4℃, and spun at 14,000 rpm (20,817 g) for 5 minutes before saving the supernatant as the mitochondrial fraction.

Western Blotting

NuPAGE™ LDS Sample Buffer (4X) (Thermo Fisher Scientific) and 20x DTT (Fisher Bioreagents) were added to cell lysates or mitochondrial fractions, and heated at 100℃ for 15 minutes to create western blot samples. Equal amounts of proteins were loaded into either NuPAGE® Novex® 4-12% or 12% Bis-Tris (Thermo Fisher Scientific) gels and run on ice for 95 minutes at 120 mV and 400 mA. Gels were then transferred to PVDF membranes (Millipore) for 1 hour and 40 minutes at 125 mV before blocking in 5% milk/TBS-Tween for 30 minutes. PVDF membranes were then rinsed 5 times, 10 seconds each, in 0.1% TBS-Tween before primary antibodies were added to probe overnight at 4℃.

The following antibodies used were from Cell Signaling Technologies: GAPDH, COX IV, p-ULK S555, p-ULK S757, Beclin1, LC3B, mTOR, p-mTOR S2481, p-P70S6K T389, p-4EBP1 T37/46, p-4EBP1 S65, p-Akt S473, p-AMPK T172, p-PKD S916, Parkin, and RhoA.

PINK1 antibodies were either from Cayman Chemical Company (for endogenous PINK1) or Novus Biologicals (for miniSOG PINK1). T-ULK1 was from Sigma Aldrich, p62 from Becton, Dickinson and Company, and p-Beclin1 S30 from Signalway Antibody. All antibodies were used in a 1:1000 dilution in 5% BSA/TBS-Tween, except those for p-Beclin1, p62, PINK1 (both Cayman Chemical Company and Novus Biologicals), RhoA, and BAG2 of which were used in 1:500 dilutions.

After overnight probing, PVDF membranes were rocked in 0.1% TBS-Tween for 30 minutes, changing the 0.1% TBS-Tween every 5 minutes, before rocking in either mouse or rabbit peroxidase-conjugated secondary antibodies (Sigma Aldrich) at room temperature in 5% milk/TBS-Tween for 1 hour. 1:3000 secondary antibody dilutions were used to probe for all antibodies, except for RhoA and PINK1 (Novus Biologicals) of which were used in 1:1000 dilutions and Beclin1 and BAG2 of which were used in 1:2000 dilutions. PVDF membranes were once more rocked in 0.1% TBS-Tween for 30 minutes, changing the 0.1% TBS-Tween every 5 minutes before they were imaged by using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific).

Quantitative PCR (qPCR)

Powderized heart tissues were homogenized in 300 μL of Trizol (Ambion), while NRVMs were harvested in 1 mL of Trizol (Ambion). Samples were left to incubate at room temperature for 5 minutes in a final volume of 1 mL of Trizol before adding 200 μL of chloroform (Fisher Scientific) and incubating for another 5 minutes at room temperature, inverting 5 times before and after incubation. Samples were then spun at 14,000 rpm (20,817 g)

at 4℃ for 10 minutes, and the clear supernatant at the top was saved. 500 µL of isopropanol (Fisher Scientific) was added to the saved supernatant and incubated at -20℃ for 20 minutes. After incubation, the samples were spun at 14,000 rpm (20,817 g) at 4℃ for 10 minutes. Pellets were washed with 500 µL of 70% EtOH (Fisher Scientific) and spun again at 14,000 (20,817 g) for 5 minutes. Supernatant was discarded and pellets were left inverted to dry for 30 minutes before dissolving in RNAse-free water (Qiagen), incubating in a 55℃ water bath for 10 minutes.

Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher) was used to synthesize cDNA. TaqMan Universal Mastermix II (Applied Biosystems) together with standard primers from Integrated DNA Technologies, except those for NPPB and MYH7 of which were from TaqMan, were used to perform qPCR through a 7500 Fast Real-Time PCR system (Applied Biosystems). The comparative threshold cycle (Ct) was normalized to GAPDH for data analysis through the comparative C_T method as previously described [56].

Statistical Analysis

The following results were all presented as the average ± the standard error of the mean (SEM). One-tailed, unpaired Student's t-test was used to determine significance between two groups sharing one variable. A p-value less than 0.05 was determined to be statistically significant.

RESULTS

RhoA activity is decreased with age.

To explore the potential of RhoA as a therapeutic target against aging in the heart, RhoA activity levels were first analyzed through an active RhoA pull-down assay conducted in heart samples obtained from WT mice at 5 months (young) and 10 months of age (middle-aged). The active RhoA pull down assay revealed a decrease in levels of active RhoA in the hearts of 10 month old mice (middle-aged) compared to that of 5 month old mice (young) (Figure 1A). Confirming this observation, decreased PKD activity, a downstream effector of RhoA, was also observed in mice at 10 months of age (middle-aged) compared to that of mice at 5 months of age (young) (Figure 1B). These results suggest a decrease in RhoA signaling activity with age.

Cardiac-specific RhoA KO mouse hearts show signs of accelerated cardiac aging.

Cardiac-specific RhoA KO ($RhoA^{fl.fl,\beta MHC\text{-}cre}$) mice have previously been shown to express no basal phenotype in the heart at 2-3 months of age [49]. To assess cardiac contractility, echocardiography was performed in the hearts of 4 month (young) and 10 month (middle-aged) old WT and RhoA KO mice. Supporting previous observations obtained at 2-3 months of age, no significant differences in fractional shortening were observed when comparing WT and KO hearts of 4 month old (young) mice (Figure 2). In contrast, fractional shortening in 10 month old (middle-aged) KO mouse hearts were significantly lower than that of WT mouse hearts (Figure 2), suggesting that cardiac-specific deletion of RhoA results in decreased cardiac contractile function in mice at 10 months of age (middle-aged), but not at 4 months of age (young).

To further investigate the role of RhoA in cardiac aging, mouse hearts were assessed for cardiac hypertrophy, a major feature of cardiac aging [11]. Upon extraction of the heart, heart and body weights were measured to use their ratio as an indicator of cardiac hypertrophy. Both 4 month (young) and 10 month (middle-aged) old KO mice had significantly higher heart to body weight ratios in comparison to that of their corresponding WT littermates (Figure 3A), which suggests an increase in the heart sizes of KO mice. At 4 months of age (young) qPCR analysis revealed an increased, but not statistically significant, expression of hypertrophic genes (NPPB, Acta1, and MYH7) in KO mouse hearts and a robust increase at 10 months (middle-aged) of age when compared to those of WT mice (Figure 3B). These observations suggest a protective role for RhoA against the age-induced development of hypertrophy in the heart.

qPCR analysis was also used to assess fibrotic and inflammatory responses, both of which are also known to increase in the heart with age [13, 57]. Although not significant, Col1a1, Col3a1, and Postn fibrotic gene expressions were also elevated in 4 month old (young) KO mouse hearts relative to that of WT mouse hearts (Figure 4). Similarly observed with hypertrophic gene expression, fibrotic gene expression was significantly increased in KO mice at 10 months of age (middle-aged) compared to that of WT mice at the same age (Figure 4), suggesting that RhoA also has a role in preventing the age-dependent development of fibrosis.

While looking at the effects of cardiac-specific deletion of RhoA on inflammatory gene expression, no obvious differences were found in the mRNA expression of IL-6, CCL2, CCL3, and CXCL2 between 4 month old (young) WT and KO mouse hearts (Figure 5). Again, as discovered with hypertrophic and fibrotic gene expressions, 10 month old (middle-aged) KO mouse hearts revealed significantly higher expression of all four studied inflammatory genes in

relation to those found in WT mouse hearts (Figure 5). Together, these observations suggest that RhoA has a prominent protective effect against the development of inflammation associated with age.

Analysis of mTORC1 signaling in cardiac-specific RhoA KO mouse hearts.

Another hallmark of cardiac aging is an increase in protein aggregates [20]. One way in which protein aggregates are cleared from the cell is through autophagy, the cellular system that recycles misfolded proteins and damaged organelles in the cell [31]. To determine whether RhoA regulates autophagy, the effects of cardiac-specific deletion of RhoA on mTORC1 signaling, a well-established negative regulator of autophagy, were first studied. mTORC1 signaling is regulated by AMPK serving as a negative regulator of mTORC1 and Akt as a positive regulator [58].

WT and cardiac-specific RhoA KO mouse hearts were subjected to western blot analysis for p-AMPK and p-Akt to determine whether AMPK and Akt activity is regulated by RhoA in the heart. Both 4 month (young) and 10 month (middle-aged) old KO mouse hearts showed lower p-AMPK levels when compared to that of WT mouse hearts (Figure 6), suggesting positive regulation of AMPK activity in the heart by RhoA. It was previously shown that constitutively active RhoA in HEK 273E cells results in decreased p-Akt S473 levels [59], and supporting this finding, an increase in p-Akt S473 protein levels was observed in the hearts of 4 month old (young) RhoA KO mice (Figure 6). Interestingly, however, p-Akt S473 levels in 10 month old (middle-aged) KO mice were no different than that observed in WT mice (Figure 6).

Continuing to study the effects of cardiac-specific RhoA deletion on mTORC1 signaling, auto-phosphorylation of mTOR was assessed as an indicator of mTOR activation. KO mouse hearts at 4 months of age (young) had significantly higher mTOR phosphorylation levels than that of WT mouse hearts (Figure 7). In contrast, p-mTOR levels were significantly lower in KO mouse hearts at 10 months (middle-aged) of age compared to that in WT (Figure 7). These results suggest a possibility that the role of RhoA signaling in the regulation of mTOR activation varies with age where RhoA signaling is inhibiting in the young but activating in the middle-aged. Looking at the phosphorylation of mTORC1 downstream substrates as an indicator of mTORC1 activity, a trending increase in p-P70S6K levels was observed in the hearts of KO mice relative to that observed in the hearts of WT mice and were significantly so in the hearts of 10 month old (middle-aged) KO mice (Figure 8). While p-4EBP1 T37/46 levels remained unchanged between WT and KO mouse hearts, p-4EBP1 S65 protein levels were slightly, but not statistically significantly, lower in KO hearts than in WT hearts of both 4 month (young) and 10 month (middle-aged) old mice (Figure 8). These observations suggest that cardiac-specific deletion of RhoA does not have a significant impact in regulating mTORC1 signaling, although further studies will be required.

Analysis of autophagy in cardiac-specific RhoA KO mouse hearts.

To determine whether RhoA regulates autophagy, phosphorylation of ULK1 activity, an autophagy initiating kinase, was analyzed. ULK1 is positively regulated by AMPK-mediated phosphorylation at Ser555, and negatively regulated by mTORC1-mediated phosphorylation at Ser757 [60]. Western blot analysis of whole heart homogenates revealed that levels of both

p-ULK555 and p-ULK757 phosphorylation were not statistically different in the hearts of KO mice at both 4 months (young) and 10 months (middle-aged) of age compared to that of WT mice (Figure 9). Beclin-1 is a downstream target of ULK1 and plays a critical role in the formation of the autophagosome to sequester cellular contents for autophagy. Interestingly, only the hearts of 4 month (young) old KO mice showed a significant increase in the phosphorylation of Beclin1 at Ser30 in comparison to WT mouse hearts (Figure 10). Protein levels of LC3-II and p62 were next measured as markers of autophagy. Although not statistically significant, both LC3-II and p62 protein levels were slightly lower in the KO hearts of 4 month old (young) mice compared to WT mice at the same age, but were no different between WT and KO mouse hearts at 10 months of age (Figure 10). Taken all together, these results suggest that cardiac-specific deletion of RhoA does not play a significant role in the regulation of autophagy in mice at both 4 months (young) and 10 months (middle-aged) of age.

RhoA increases PINK1 protein levels in NRVMs.

In addition to the accumulation of protein aggregates, accumulation of damaged mitochondria is also commonly observed in the aged heart and contributes to the development of cardiac aging. Mitophagy, a mitochondria-specific form of autophagy, plays an important role in maintaining mitochondrial quality and is frequently discussed to have a significant role in the process of aging [38, 61]. Mitophagy is conventionally initiated through the stabilization of PINK1 at the mitochondria [62]. Western blot analysis of whole heart homogenates isolated from WT and cardiac-specific RhoA KO mice at 10 months of age (middle-aged) revealed decreased PINK1 protein levels in RhoA KO mice compared to that in WT mice (Figure 11A), suggesting

that RhoA positively regulates the level of PINK1. This was further supported by a gain-of-function approach in *in vitro* studies utilizing adenoviral-mediated expression of constitutively active RhoA (L63RhoA) in neonatal rat ventricular myocytes (NRVMs). Western blot analysis of whole cell lysates showed increased PINK1 protein levels in L63RhoA expressing NRVMs (Figure 11B). These observations suggest that RhoA may have a regulatory role in mitophagy by inducing PINK1 accumulation at the mitochondria.

RhoA increases PINK1 protein levels by inhibiting PINK1 degradation in NRVMs.

PINK1 proteins undergo constant degradation in the healthy mitochondria, but this is inhibited in response to mitochondrial membrane depolarization upon mitochondrial damage [62]. However, a study using a mitochondrial membrane potential indicator (TMRE) had previously showed that L63RhoA expression in NRVMs does not depolarize the mitochondrial membrane potential [54] and this was also confirmed through microscopic analysis of TMRE fluorescence in NRVMs (Shigeki Miyamoto, unpublished observation). To test whether RhoA increases PINK1 protein levels by increasing PINK1 transcription, qPCR analysis was used to look at PINK1 mRNA levels in WT and cardiac-specific RhoA KO mouse hearts as well as in NRVMs infected with L63RhoA expressing adenovirus. A significant decrease in PINK1 mRNA levels was observed in RhoA KO mouse hearts compared to that of WT mouse hearts at 10 months of age (middle-aged) (Figure 12A), suggesting that RhoA increases PINK1 transcription. Interestingly, however, no significant differences in PINK1 mRNA levels were observed among NRVMs infected with L63RhoA expressing adenovirus for 8, 16, and 24 hours (Figure 12B), suggesting that activation of RhoA does not directly regulate PINK1 transcription.

Considering the possibility that RhoA does not influence the transcription of PINK1, NRVMs were infected with either GFP or L63RhoA expressing adenovirus to determine whether the observed RhoA-mediated accumulation of PINK1 results from the inhibition of PINK1 protein degradation. Protein synthesis was inhibited by treatment with 100 μg/mL of cycloheximide (CHX) for 15 minutes and changes in PINK1 protein levels were examined by western blot analysis of whole cell lysates. Expression of L63RhoA was found to inhibit the decrease in PINK1 protein levels of CHX treated NRVMs (Figure 13). This result suggests the existence of a mitochondrial membrane depolarization independent mechanism for the stabilization of PINK1 in NRVMs that is induced by the RhoA signaling pathway.

PKD is necessary, but not sufficient for RhoA-mediated stabilization of PINK1 and the subsequent increase in Parkin association with mitochondria in NRVMs.

PINK1 accumulation at the mitochondria results in the recruitment of Parkin through a series of phosphorylation events, and in turn, Parkin tags various mitochondrial proteins through ubiquitination for the induction of mitophagy [62]. Mitochondrial fractions isolated from NRVMs were subjected to western blot analysis for not only PINK1, but also Parkin. NRVMs expressing L63RhoA revealed a robust increase in both PINK1 and Parkin protein levels (Figure 14), strongly supporting earlier suggestions of RhoA positively regulating mitophagy. These responses were not induced by wild-type RhoA expression (Figure 14), indicating that activation of the RhoA signaling pathway is involved in the increases in PINK1 and Parkin levels at the mitochondria.

PKD, a downstream effector of RhoA, has previously been shown to have a significant role in RhoA-mediated protection of the heart against I/R injury as well as oxidative stress [49, 50]. To test determine whether RhoA-mediated stabilization of PINK1 and the subsequent recruitment of Parkin to the mitochondria occurs through the activation of PKD, 100 nM of CID 755637, a pharmacological PKD inhibitor, was used in NRVMs expressing L63RhoA. CID 755637 treatment abolished the RhoA-mediated increase in PINK1 protein levels and largely attenuated that of Parkin in the mitochondrial fractions (Figure 14), suggesting the necessity of PKD in RhoA-mediated stabilization of PINK1 and the subsequent recruitment of Parkin to the mitochondria.

To further investigate the role of PKD in RhoA-mediated stabilization of PINK1 at the mitochondria, adenovirus encoding PKD was used to overexpress PKD in NRVMs either with or without L63RhoA expression. Mitochondrial fractions isolated from NRVMs expressing both L63RhoA and PKD showed even higher PINK1 protein levels without significantly affecting Parkin levels when compared to those expressing L63RhoA alone (data not shown). Interestingly, however, overexpression of only PKD failed to induce PINK1 or Parkin accumulation at the mitochondria, indicating that PKD activation is not sufficient to increase either mitochondrial PINK1 or Parkin protein levels (Figure 14), suggesting that PKD plays a critical role in RhoA-mediated PINK1 stabilization and subsequent Parkin recruitment to the mitochondria, but is not sufficient.

The phosphorylation state of the PKD consensus sequence on PINK1 does not affect the degradation rate of PINK1 in NRVMs.

PINK1 was found to contain p-PKD consensus sequences (LQREAS495), and thus we hypothesized that RhoA-mediated PINK1 stabilization results from the phosphorylation of PINK1 at Ser495 by PKD. Phospho-mimicking and non-phosphorylatable mutants of PINK1 were created by mutating Ser495 at the PKD consensus sequence to either aspartic acid to create a phospho-mimicking mutant or to alanine to create a non-phosphorylatable mutant. NRVMs were infected with either the unmutated wild-type (WT), the non-phosphorylatable (SA), or the phospho-mimicking (SD) PINK1 encoding adenovirus and/or treated with 100 μL/mL of cycloheximide (CHX) for 10, 20, or 60 minutes. Whole cell lysates were isolated and subjected to western blot analysis for PINK1. No significant differences in PINK1 protein degradation were observed among NRVMs treated with CHX and infected with WT, SA, or SD PINK1 (Figure 15), suggesting that this putative PKD phosphorylation site on PINK1 is not important for RhoA-mediated PINK1 protein stabilization.

PKD plays an important role in the association of active RhoA with the mitochondria.

While examining the role of PKD in the stabilization of PINK1 induced by RhoA signaling, active RhoA was interestingly discovered to be accumulated at the mitochondria as indicated by the significant increase in RhoA protein levels in the mitochondrial fractions isolated from NRVMs expressing L63RhoA (Figure 16). Additionally, CID 755637 treatment surprisingly inhibited the observed increase in mitochondrial RhoA protein (Figure 15). Conversely, while the expression of L63RhoA together with the overexpression of PKD also

increased the presence of RhoA at the mitochondria, overexpression of PKD alone failed to induce the accumulation of RhoA at the mitochondria (Figure 16). Together, these findings suggest that similar to the observed stabilization of PINK1, the accumulation of active RhoA at the mitochondria is dependent on the activation of PKD, but PKD alone is not sufficient.

Similar to PINK1, RhoA was also found to contain p-PKD consensus sequences, leading to our next hypothesis stating that PKD is involved in the stabilization of PINK1 by mediating the increase in the mitochondrial association of RhoA. To answer whether the accumulation of active RhoA at the mitochondria results from PKD-mediated phosphorylation of active RhoA, non-phosphorylatable and phospho-mimicking mutants similar to those of PINK1 were created with L63RhoA, but have yet to be tested.

Active RhoA increases mitochondrial BAG2 protein levels in NRVMs.

Because our data suggests that PKD is required, but is not sufficient, for RhoA-mediated PINK1 stabilization and that PKD is also important in the association of active RhoA at the mitochondria, we hypothesize that the mitochondrial translocation of active RhoA mediated by PKD plays a key role in the stabilization of PINK1 at the mitochondria. We speculate that this involves an additional molecule that undergoes mitochondrial translocation with and is associated with RhoA. Co-chaperone protein, BAG2, has recently been found to inhibit PINK1 degradation at the mitochondria [63, 64], making it a potential molecule that links together PKD-mediated RhoA mitochondria translocation and subsequent PINK1 stabilization. NRVMs expressing L63RhoA were found to have a significant increase in BAG2 protein levels at the mitochondria (Figure 17), suggesting that RhoA signaling induces the accumulation of BAG2 at

the mitochondria. An immunoprecipitation assay is in the process of being optimized in NRVMs in order to further investigate whether RhoA directly interacts with BAG2 and other mitophagy-related molecules to mediate the induction of mitophagy.

FIGURES

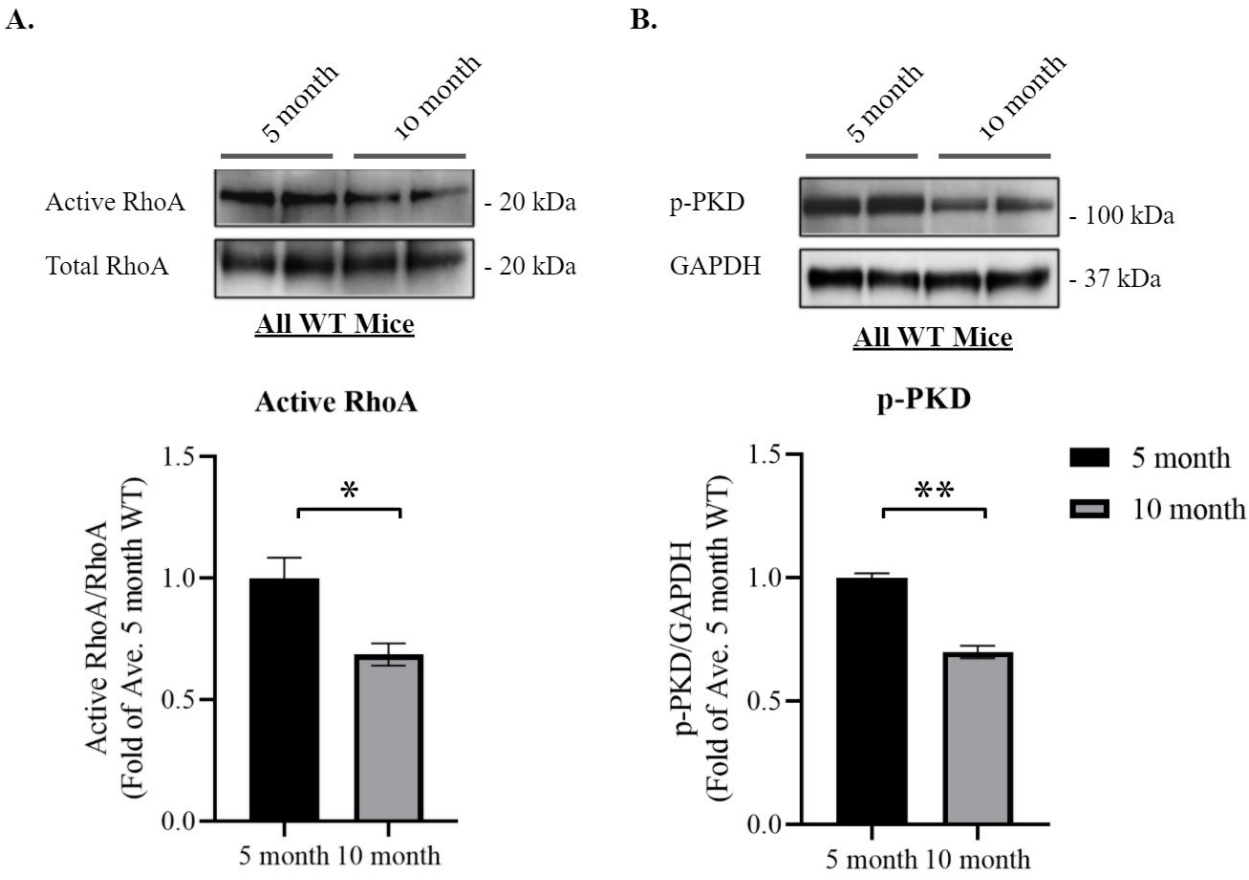

Figure 1. Decreased RhoA activity in WT mice at 10 months of age (middle-aged). Active RhoA pull down assay was used to compare the levels of RhoA activity in 5 month old (young) and 10 month old (middle-aged) mice. Samples were analyzed through western blot analysis for **A.** active RhoA and **B.** p-PKD S916 in whole heart homogenates. *, ** $p < 0.05$, 0.01 vs 5 month (young) WT; n = 2.

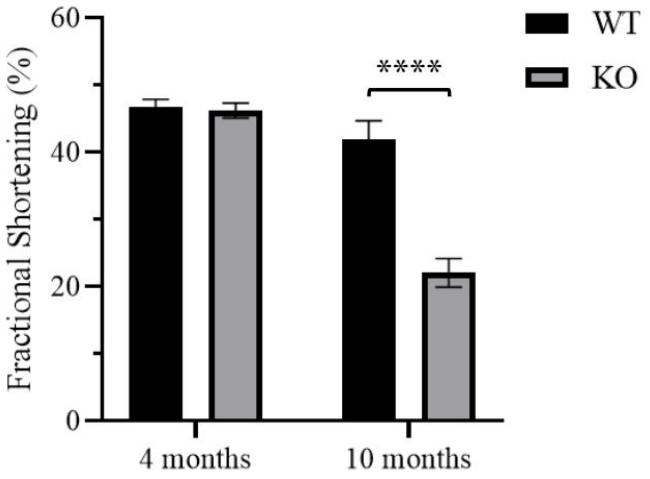

Figure 2. Cardiac contractile function is reduced in middle-aged cardiac-specific RhoA KO mouse hearts. Echocardiography was used to measure the percent fractional shortening of WT and cardiac-specific RhoA KO mouse hearts at 4 months (young) or 10 months (middle-aged) of age. **** $p < 0.0001$ vs. 10 month old (middle-aged) WT; $n > 6$.

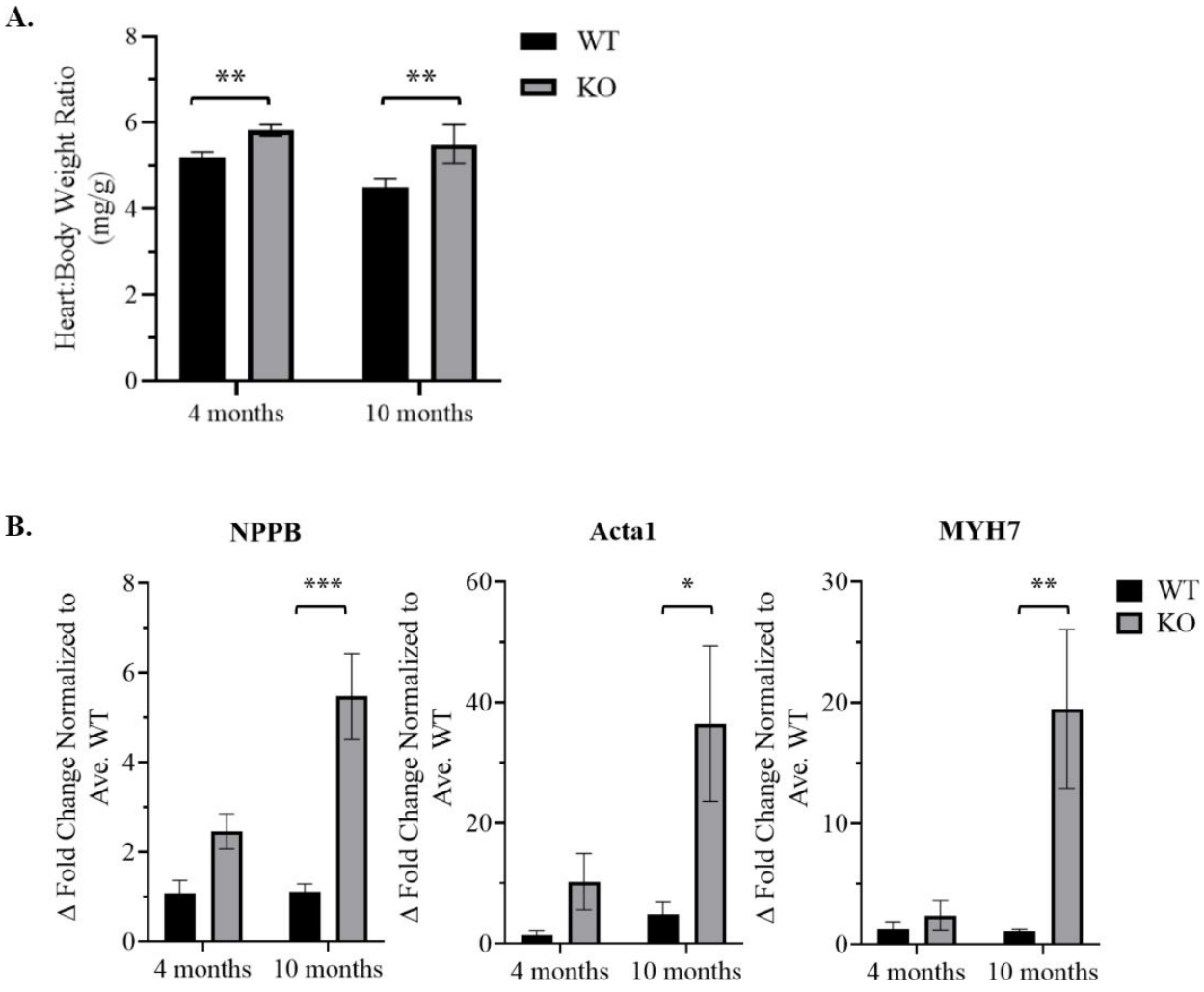

Figure 3. Hypertrophic responses are induced in RhoA KO in an age dependent manner. **A.** Heart weight (mg) to body weight (g) ratio of wild-type (WT) and cardiac-specific RhoA knockout (KO) mice at 4 months (young) and 10 months (middle aged) of age. * $p < 0.05$ vs. WT; $n > 2$. **B.** qPCR analysis of hypertrophic gene (NPPB, Acta1, MYH7) expression in the ventricles of WT and KO mouse hearts. *, **, *** $p < 0.05, 0.01, 0.001$ vs. WT; $n > 2$.

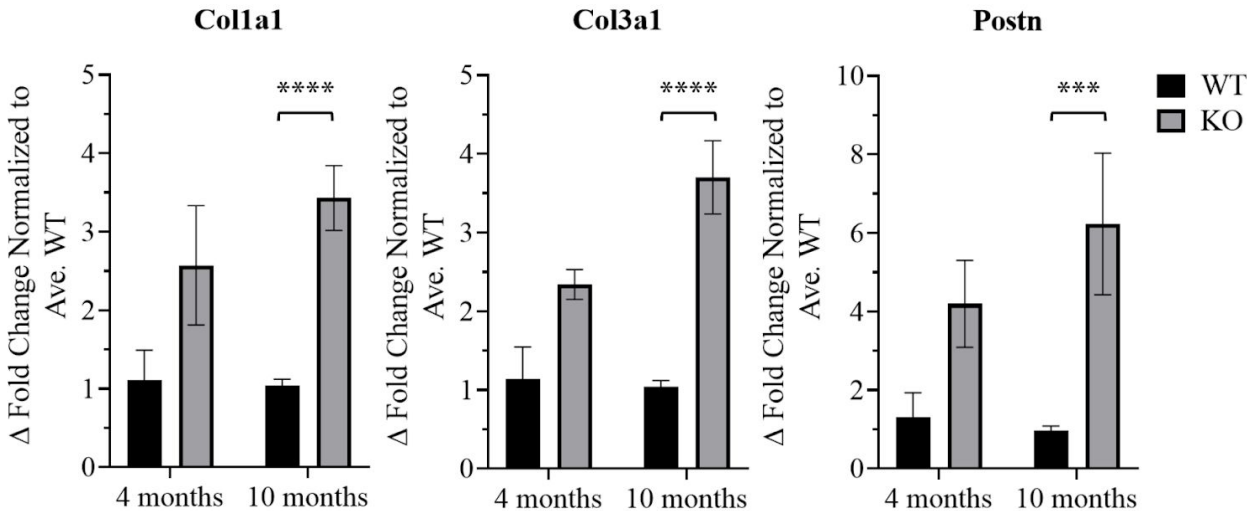

Figure 4. Fibrotic gene expression is increased in 10 month (middle-aged) old cardiac-specific RhoA KO mouse hearts. qPCR analysis of fibrotic gene (Col1a1, Col3a1, and Postn) expression in the ventricles of WT and KO mice at 4 months (young) and 10 months (middle-aged) of age. ***, **** $p < 0.001, 0.0001$ vs WT; $n > 2$.

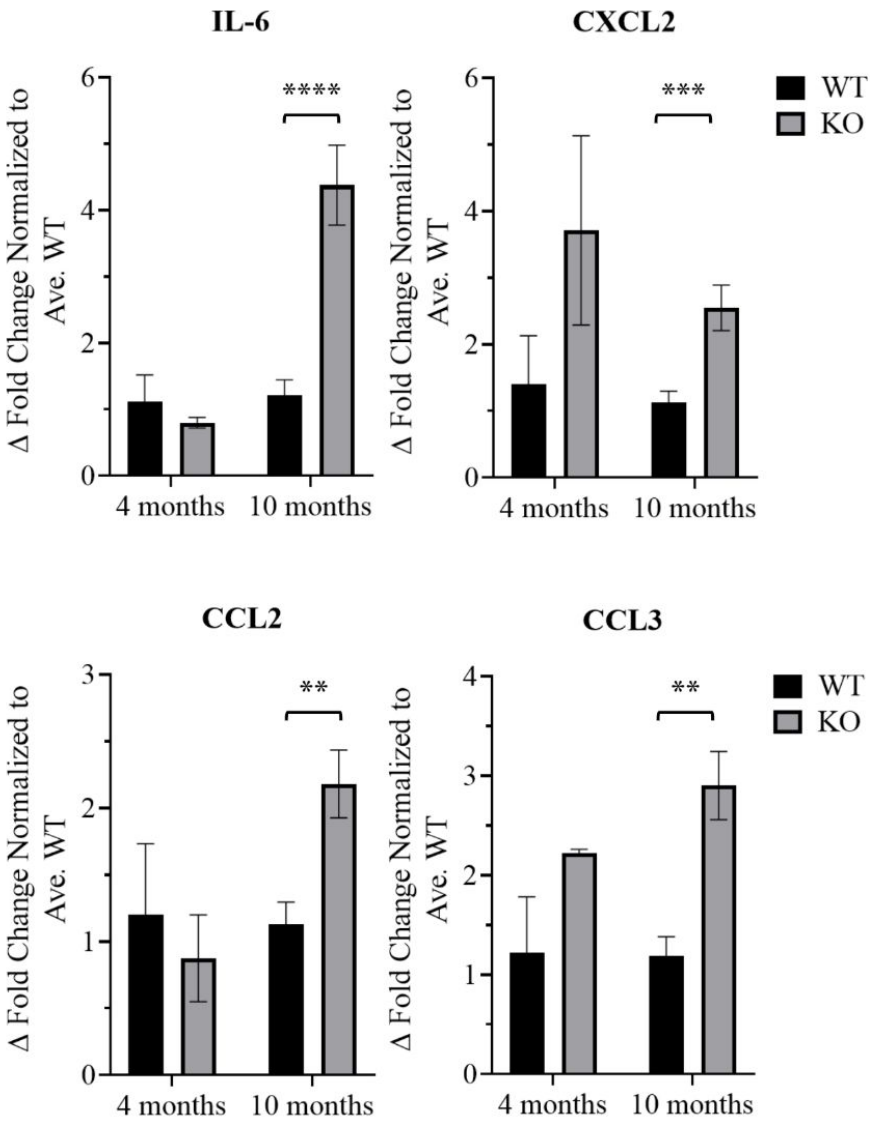

Figure 5. Cardiac-specific RhoA KO mouse hearts show increased inflammatory gene expression in 10 month (middle-aged) old mouse hearts. qPCR analysis of inflammatory gene (IL-6, CXCL2, CCL2, and CCL3) expression in the ventricles of WT and KO mice at 4 months (young) and 10 months (middle-aged) of age. **, ***, **** $p < 0.01, 0.001, 0.0001$ vs WT; $n > 2$.

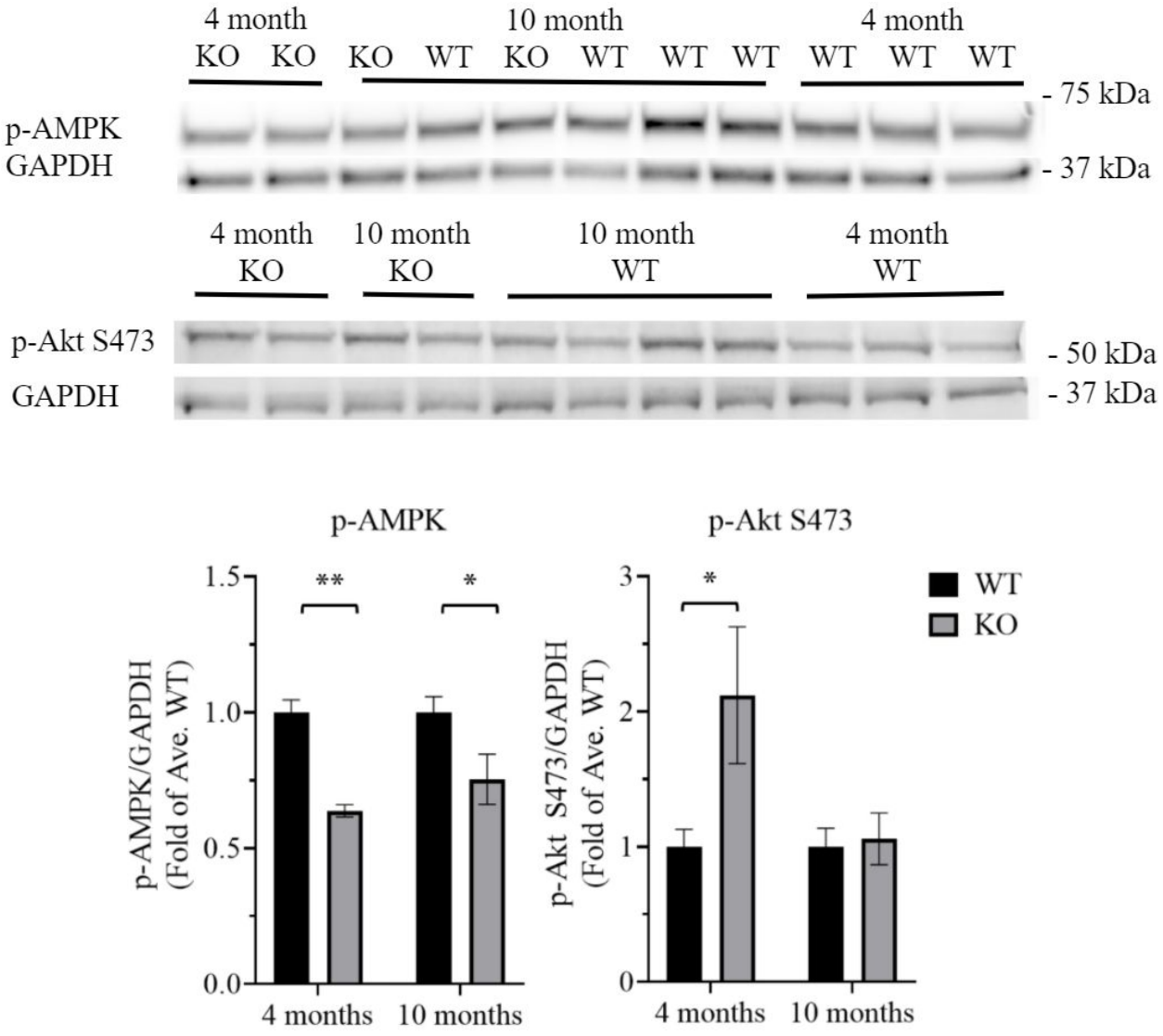

Figure 6. Effects of cardiac-specific deletion of RhoA on upstream kinases of mTORC1. Whole heart homogenate samples were extracted from WT and cardiac-specific RhoA KO heart tissue of mice at either 4 months (young) or 10 months (middle-aged) of age and subjected to western blot analysis for p-AMPK T172 and p-Akt S473. GAPDH was used as the loading control. *, ** $p < 0.05, 0.01$ vs WT; $n > 2$.

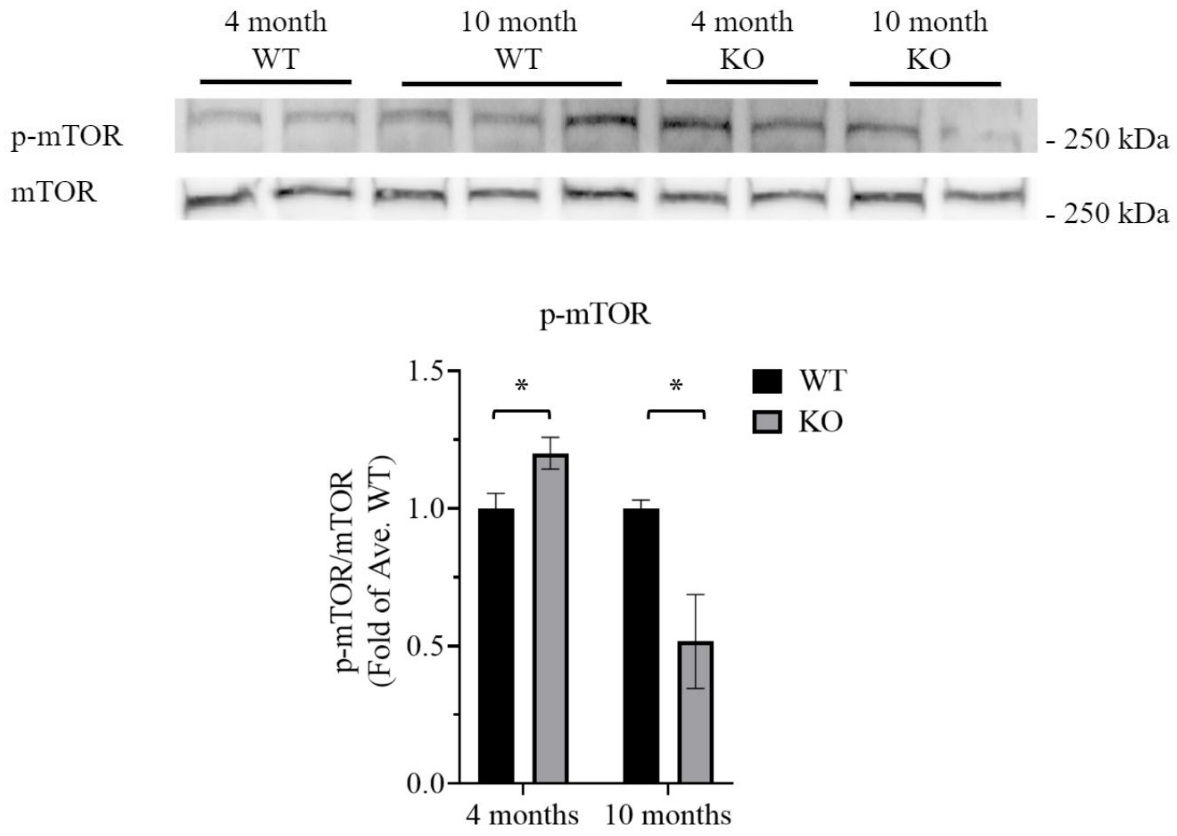

Figure 7. mTOR phosphorylation is increased in 4 month old (young) cardiac-specific RhoA KO mouse hearts and decreased in 10 month old (middle-aged) ones. Whole heart homogenates were extracted from WT and cardiac-specific RhoA KO mice at 4 months (young) or 10 months (middle-aged) of age and subjected to western blot analysis for mTOR and p-mTOR. GAPDH was used as the loading control. * $p < 0.05$ vs WT; $n > 2$.

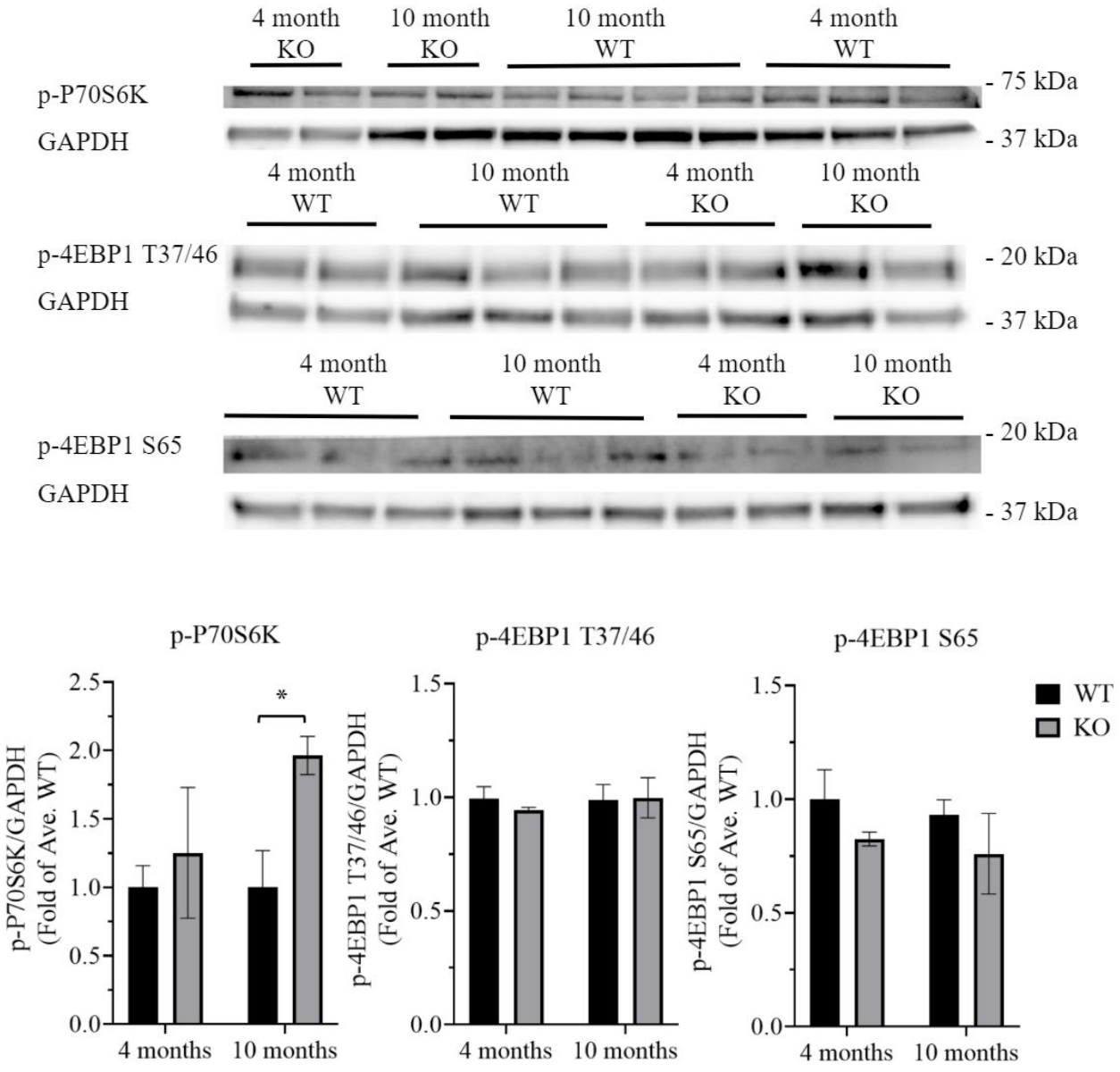

Figure 8. Effects of cardiac-specific deletion of RhoA on mTORC1 downstream signaling. Whole heart homogenates were extracted from 4 month old (young) and 10 month old (middle-aged) WT and cardiac-specific RhoA KO hearts and subjected to western blot analysis of p-P70S6K, p-4EBP1 T37/46, and p-4EBP1 S65. GAPDH was used as the loading control. * $p < 0.05$; $n > 2$.

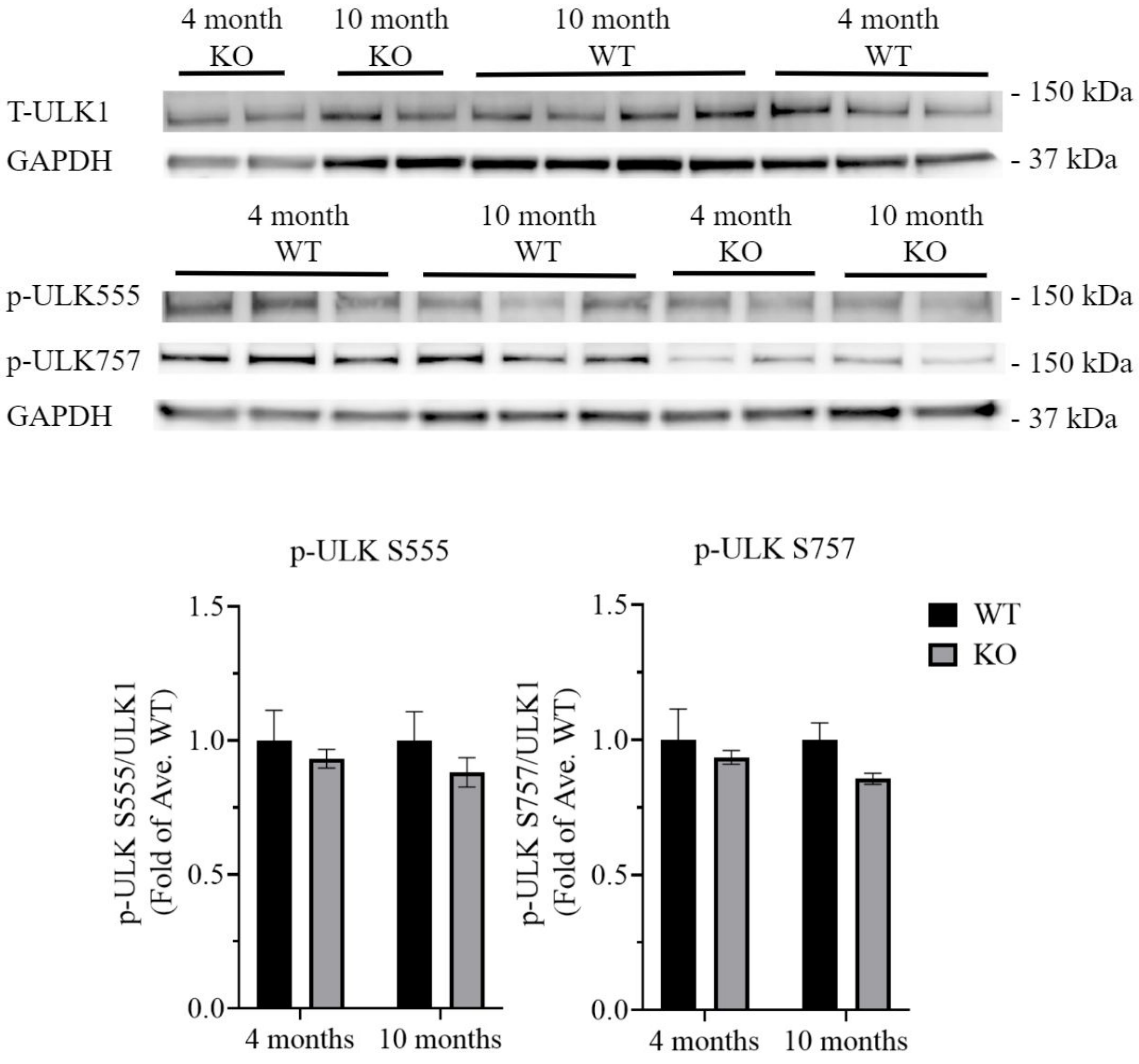

Figure 9. Cardiac-specific RhoA KO mouse hearts show no difference in ULK1 phosphorylation levels. Whole heart homogenates were extracted from WT and cardiac-specific RhoA KO hearts of 4 month old (young) and 10 month old (middle-aged) mice and subjected to the western blot analysis of T-ULK1, p-ULK S555, and p-ULK S757. GAPDH was used as the loading control. ns vs WT; n > 2.

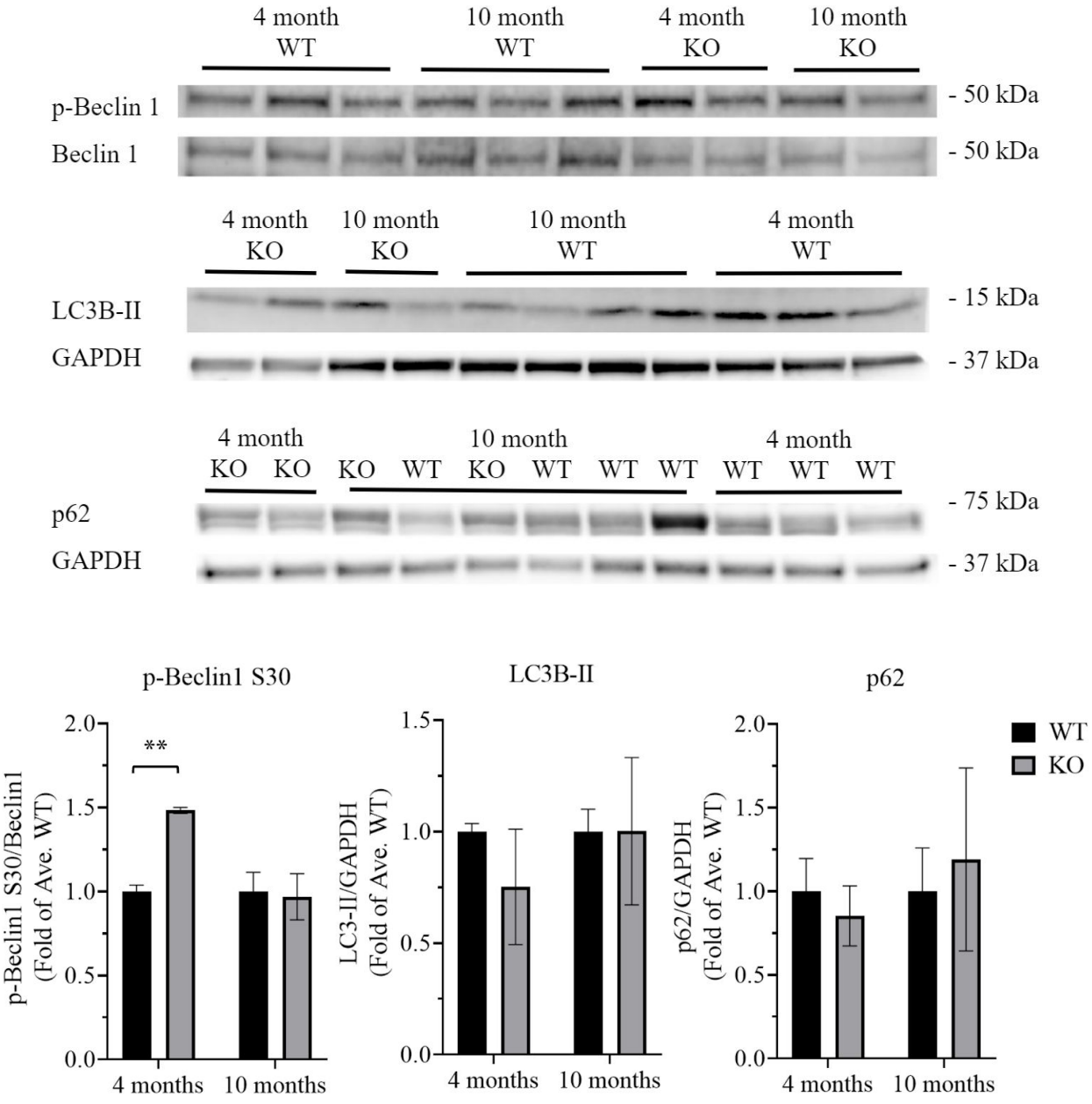

Figure 10. Effects of cardiac-specific deletion of RhoA on autophagic signaling. Whole heart homogenates were extracted from WT and cardiac-specific RhoA KO hearts of mice at 4 months (young) or 10 months (middle-aged) of age and subjected to western blot analysis of Beclin1, p-Beclin1 S30, LC3-II, and p62. GAPDH was used as the loading control. ** $p < 0.01$ vs WT; $n > 2$.

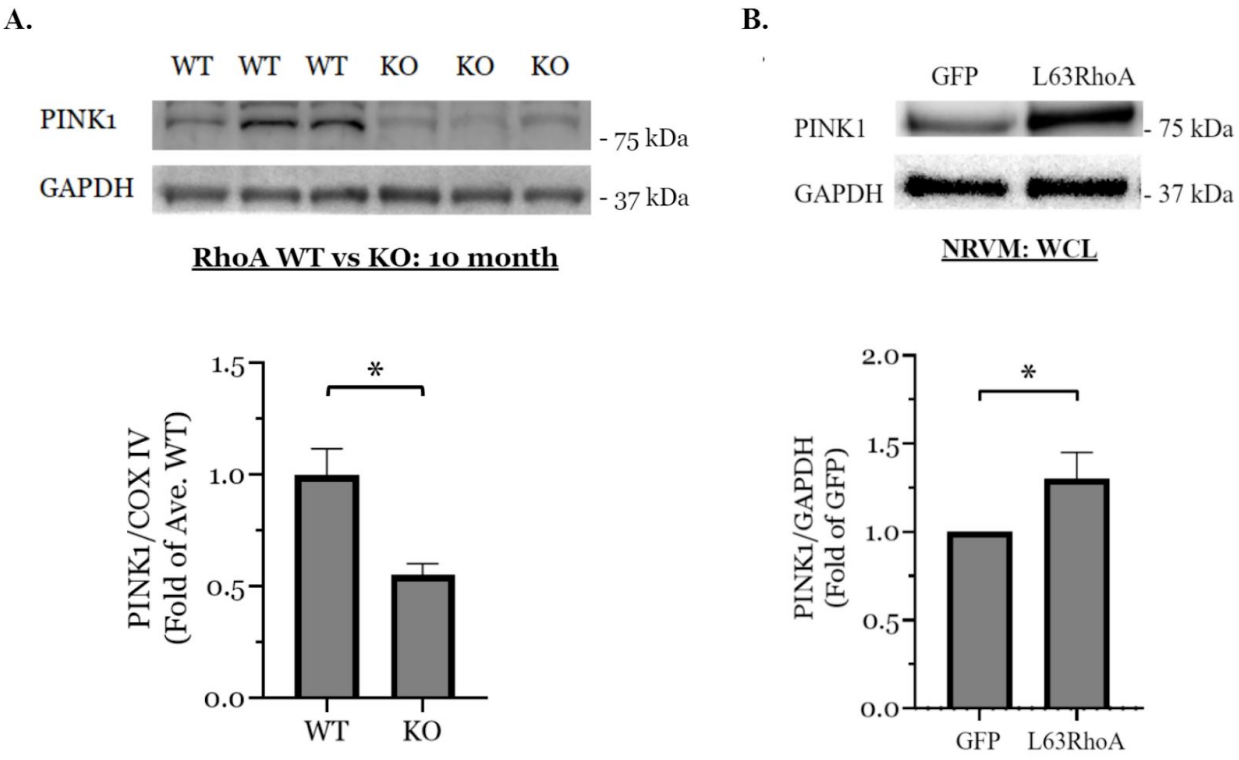

Figure 11. RhoA regulates PINK1 protein levels. A. Whole heart homogenates were extracted from WT and cardiac-specific RhoA KO hearts of mice at 10 months (middle-aged) of age and subjected to western blot analysis for PINK1. COX IV was used as the loading control. * $p < 0.05$ vs WT; n = 3. **B.** 24 hour serum starved NRVMs were infected with either GFP or L63RhoA expressing adenovirus for 24 hours. Whole cell lysates (WCL) were isolated from the NRVMs and subjected to western blot analysis of PINK1 with GAPDH as the loading control. * $p < 0.05$ vs GFP; n =3.

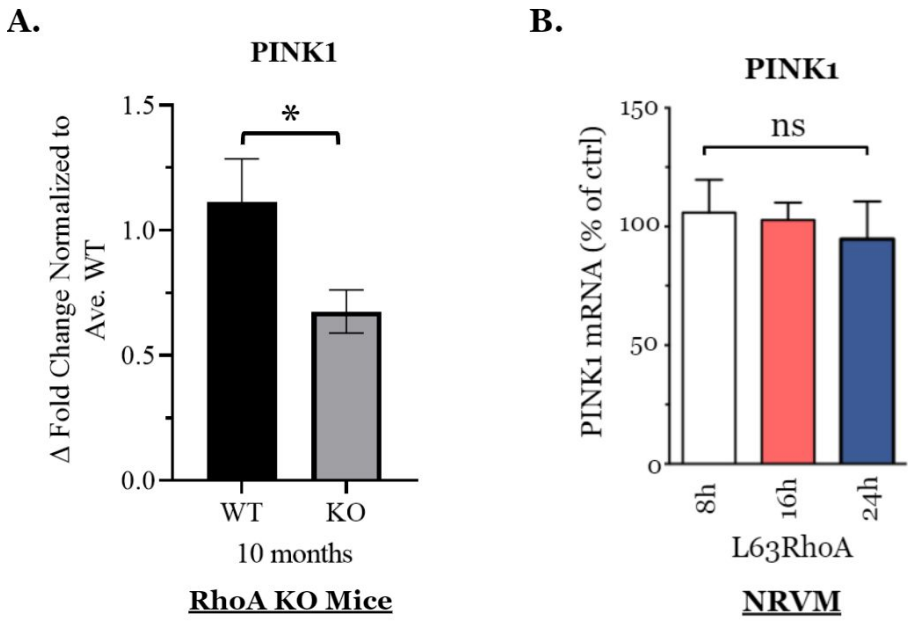

Figure 12. 10 month old (middle-aged) cardiac-specific RhoA KO mouse hearts have decreased PINK1 transcription levels, while L63RhoA expression does not affect PINK1 transcription levels in NRVMs. A. qPCR analysis of PINK1 gene expression in the ventricles of 10 month old (middle-aged) WT and KO mouse hearts. * $p < 0.05$ vs WT; $n > 9$. **B.** 24 hour serum starved NRVMs were subjected to either 8 hour, 16 hour, or 24 hour infection with L63RhoA (constitutively active) expressing adenovirus. RNA was isolated from the NRVMs and qPCR analysis was performed. ns (not significant) vs GFP (control); $n > 7$.

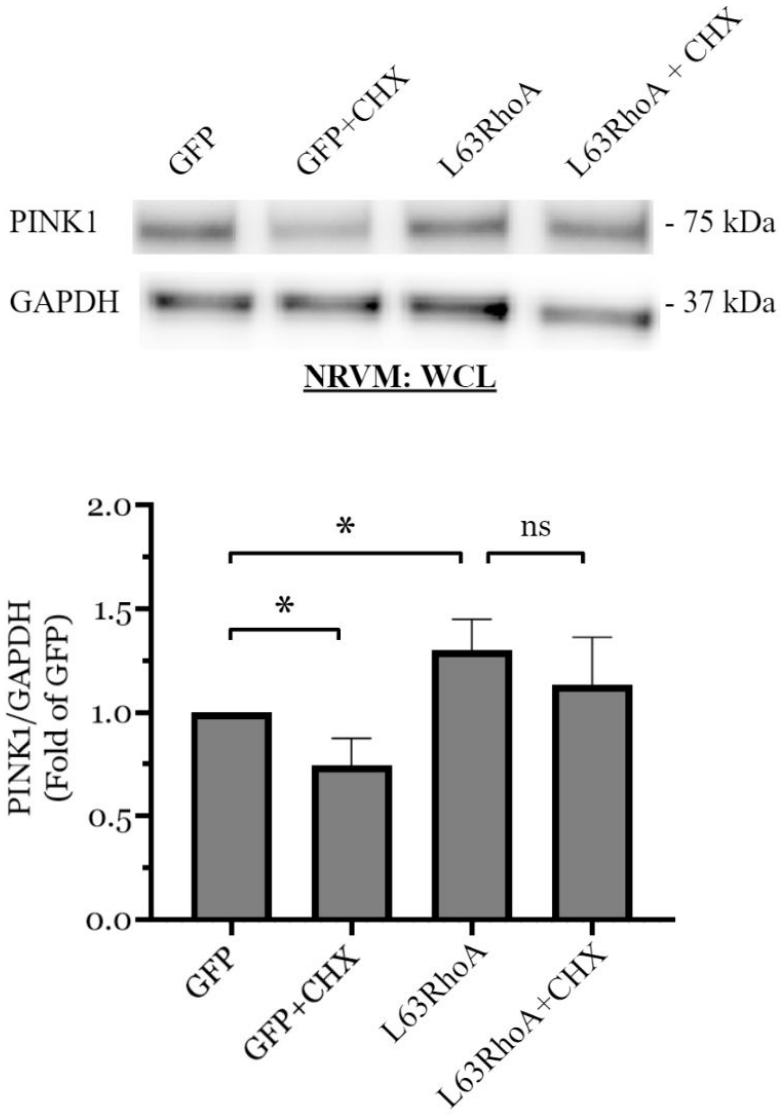

Figure 13. RhoA inhibits PINK1 protein degradation. 24 hour serum starved NRVMs were infected with PINK1 expressing adenovirus for 4 hours. The NRVMs were then washed and infected with either GFP or L63RhoA expressing adenovirus for 24 hours. Cells were treated with 100 µg/mL CHX for 15 min before harvest. Whole cell lysates were isolated from the NRVMs and subjected to western blot analysis of PINK1. * $p < 0.05$ vs GFP. ns vs RhoA; $n > 5$.

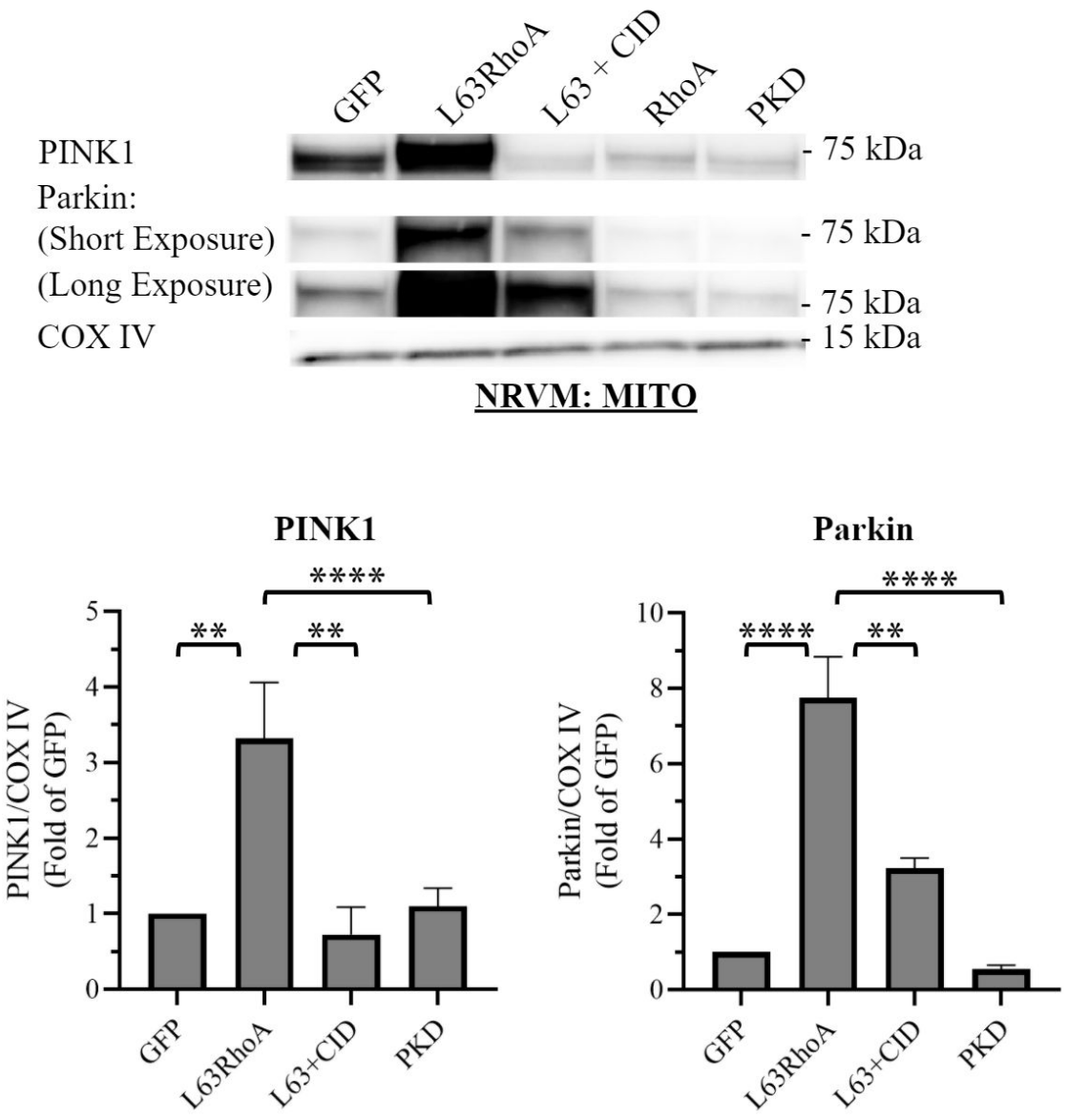

Figure 14. PKD is necessary, but not sufficient, for RhoA-mediated stabilization of PINK1. 24 hour serum starved NRVMs were subjected to 4 hour infection with PINK and PARKIN expressing adenovirus. The NRVMs were then infected with GFP, L63RhoA, WT RhoA, and/or PKD expressing adenovirus and/or treated with 100 nM CID, a pharmacological PKD inhibitor, for 24 hours. Mitochondrial fractions were isolated from the NRVMs and subjected to western blot analysis of PINK1 and Parkin with COX IV used as the loading control. **, **** $p < 0.01$, 0.0001; $n > 4$.

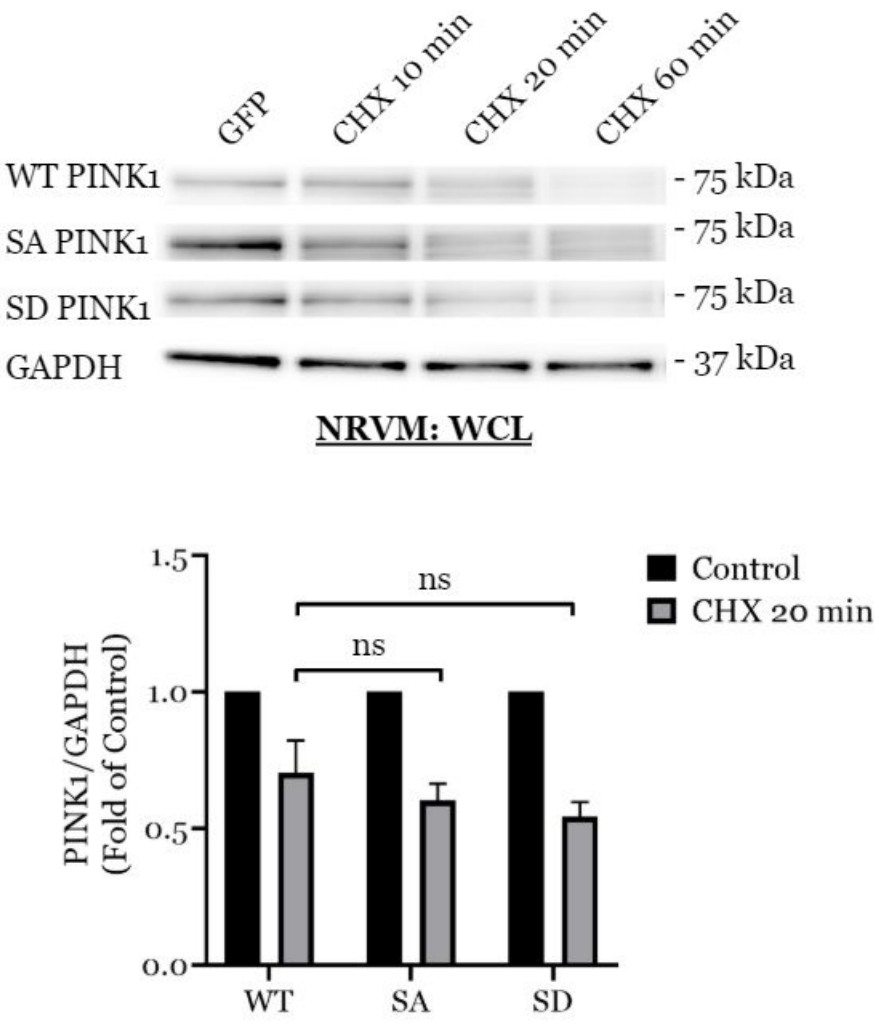

Figure 15. Mutation of the p-PKD consensus sequence on PINK1 does not affect the degradation of PINK1. 24 hour serum starved NRVMs were infected with Parkin expressing adenovirus and either WT PINK, SA PINK1 (non-phosphorylatable mutant), or SD PINK1 (phospho-mimicking mutant) for 4 hours and cultured for 20 hours. Cells were treated with 100 μg/mL CHX for 10 min, 20 min, or 60 min. Whole cell lysates were extracted from the NRVMs and subjected to western blot analysis for PINK1 with GAPDH used as the loading control. ns vs WT PINK1 treated with CHX for 20 min; n = 2.

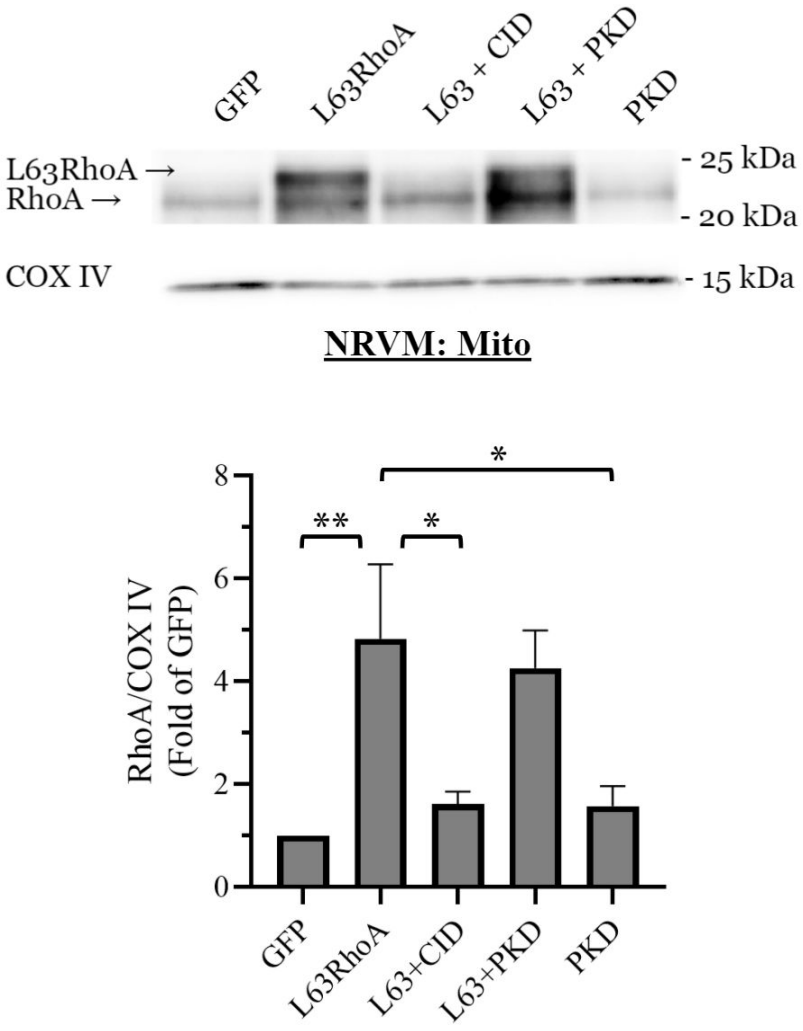

Figure 16. PKD is necessary, but not sufficient, for the mitochondrial accumulation of active RhoA. 24 hour serum starved NRVMs were infected with PINK and PARKIN expressing adenovirus. The NRVMs were then washed and infected with GFP, L63RhoA, WT RhoA, and/or PKD expressing adenovirus and/or treated with 100 nM CID, a pharmacological PKD inhibitor, for 24 hours. Mitochondrial fractions were isolated from the NRVMs and subjected to western blot analysis of RhoA with COX IV used as the loading control. *, ** $p < 0.05, 0.01$; $n > 2$.

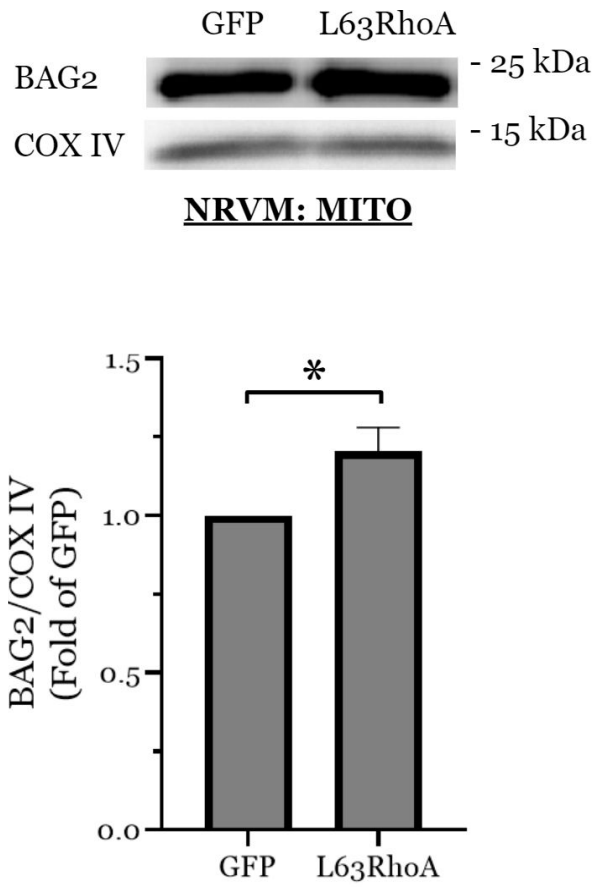

Figure 17. Active RhoA increases mitochondrial BAG2 levels in NRVMs. 24 hour serum starved NRVMs were infected with GFP or L63RhoA expressing adenovirus for 24 hours. Mitochondrial fractions were isolated from the NRVMs and subjected to western blot analysis of BAG2 with COX IV used as the loading control. * $p < 0.05$ vs GFP; $n = 4$.

DISCUSSION

As a low molecular weight G-protein switching between an active and inactive state upon GPCR stimulation, RhoA has interestingly also been shown to protect against various stresses in the heart such as I/R injury, pressure overload, and oxidative stress [48-51]. Age is a major factor in the development of cardiovascular diseases and it has not been determined as to what role RhoA plays in the development of age-dependent cardiac dysfunction. Our study demonstrates that RhoA not only plays a protective role against the progression of aging in the heart, but also regulates mitophagy through its PKD-mediated, mitochondrial membrane depolarization independent stabilization of PINK1, while general autophagy is not affected. Our findings suggest RhoA-mediated mitophagy as a potential mechanism in conferring cardioprotection against aging.

RhoA protects the heart against aging.

Our study revealed that aging in the hearts of cardiac-specific RhoA KO mice already becomes evident at only 10 months of age (middle-aged) (Figures 2-5). Based on survival rate comparisons between mice and humans, mice between the ages of 3-6 months are considered representative of 20-40 year old human adults, while 10-14 month old mice are considered equivalent to middle-aged adults in their 40's [65]. Specifically, a substantial increase in the development of cardiac aging phenotypes such as hypertrophy, fibrosis, inflammation and contractile dysfunction was observed in cardiac-specific RhoA KO mice at just 10 months of age (middle-aged) (Figures 2-5). This early onset of aging in the heart induced by cardiac deletion of

RhoA strongly suggests that RhoA acts as a protective signaling molecule against the progression of age-associated heart failure.

Because cardiac aging is typically not observed until beyond the middle-age years of a healthy human [66], it would be of interest to further extend our studies to 18-24 month old mice, representative of the 59-68 year old human aging population [65]. With such a study, a better causal correlation between the observed cardiac-specific RhoA KO induced changes and the features of cardiac aging can be made. Adeno-associated virus serotype (the most cardiotropic AAV) encoding L63RhoA have been generated in our laboratory to be utilized in a future study as a converse approach to cardiac-specific RhoA deletion to determine whether RhoA activation attenuates cardiac aging.

RhoA is a proximal downstream effector of GPCRs and functions as a signal transducer modulating a wide range of cellular processes by engaging various downstream effectors. ROCK1/2 are currently the most established downstream effectors of RhoA and models inhibiting ROCK have been shown to decrease hypertrophy, fibrosis, and inflammation [67-69], contributing to the view of the RhoA signaling cascade as pathological in the heart. It is important to take note, however, of the fact that ROCK also plays a critical role in regulation of vascular contractility [70] and could be involved in the discrepancies between findings obtained in global inhibition of ROCK and those of cardiac-specific inhibition. Interestingly, a recent study using cardiac-specific ROCK1 knockout mice and cardiac-specific ROCK2 knockout mice suggests that activation of ROCK1 is cardioprotective while that of ROCK2 is deleterious in the heart subjected to pressure-overload [71]. In addition to ROCK, RhoA also modulates a wide range of downstream effectors from kinases including PKD, to transcription regulators such as

MRTF-A and YAP. Thus, the deletion of RhoA should result in the inhibition of all its signaling pathways and produce more complex effects than that from inhibition of just one of its downstream effectors.

Nonetheless, previous studies using cardiac specific RhoA KO mice have demonstrated that RhoA provides protection in the heart against ischemia/reperfusion as well as against pressure-overload [48, 49]. Together with our results, the overall net effect of RhoA signaling is strongly suggested to be protective against the stresses in the heart. While studies have implicated PKD to be involved in RhoA-mediated cardioprotection against ischemia/reperfusion injury, more studies are needed to determine the extent to which PKD activation contributes to the protection against cardiac aging as suggested by this study. The observed decrease in RhoA and PKD activity with age (Figure 1) implicates that the decline in RhoA signaling activity with age contributes to the development of cardiac aging. Our results suggest that stimulation of RhoA/PKD signaling may be a promising therapeutic alternative in preventing the development of age-associated features in the heart that contribute to the aging population's susceptibility to heart failure.

RhoA does not significantly impact mTORC1 signaling and autophagy.

During our investigation as to how RhoA is able to mediate its protective effects against cardiac aging, the increase in p-Akt and decrease in p-AMPK observed in 4 month old (young) cardiac-specific RhoA KO mice (Figure 6) suggest that RhoA may negatively regulate mTORC1 activity which is also supported by the increase in p-mTOR levels (Figure 7). Our findings also coincide with those of another study observing decreased mTORC1 activity levels in

L63RhoA-transfected HEK293 cells as implicated by decreased phosphorylation levels of mTORC1 downstream effectors, P70S6K and 4EBP1 [59]. Interestingly, however, our study found no differences in the phosphorylation levels of these downstream effectors at 4 months of age (young) in RhoA KO mouse hearts (Figure 8), which suggests that the activation of mTORC1 mediated by RhoA deletion is not sufficient to induce changes in the regulation of mTORC1 downstream effectors. This discrepancy could be due to the fact that our model involves chronic deletion of RhoA, possibly resulting in compensatory signaling in response to a sustained increase in mTORC1 activity of which is known to be an underlying mechanism in the age-associated decline of autophagy in the heart [23]. Additionally, these findings obtained by the deletion of RhoA under basal conditions in mice at 4 months of age (young) do not directly suggest the inability of RhoA activation to regulate mTORC1 signaling under stressed conditions. Thus, our future study will examine whether the activation of RhoA mediated by AAV9-RhoA can alter mTORC1 activity in the hearts of elderly mice more accurately representative of the aged human population.

In contrast to these observations made in 4 month old mice (young), those made in 10 month old (middle-aged) WT and RhoA KO mice suggests a different role for RhoA in the regulation of mTORC1 activity. A significant decrease in p-mTOR levels was observed in RhoA KO mice at 10 months of age (middle-aged) (Figure 7), but a significant increase in p-70S6K protein levels and no change in 4EBP1 phosphorylation levels were also observed (Figure 8), suggesting the possible activation of complex compensatory signaling pathways in response to the chronic deletion of RhoA. These observations in 4 month (young) and 10 month old

(middle-aged) RhoA KO mice together suggest that RhoA may not be a critical regulator of mTORC1 activity in young and middle-aged mice.

ULK1 is another mTORC1 substrate, where mTORC1-mediated phosphorylation at S757 leads to the inhibition of its kinase activity, while phosphorylation of ULK1 at S555 mediated by AMPK signifies initiation of autophagy [60]. p-ULK S555 and p-ULK S757 levels in the hearts of RhoA KO mice at both 4 months (young) and 10 months (middle-aged) of age were found not significantly different from those of WT mouse hearts (Figure 9), supporting the idea that RhoA deletion does not significantly influence mTORC1 activity. In line with this, we did not observe significant changes in the level of general autophagy markers, Beclin1, LC3B-II, and p62, in WT and RhoA KO mice at either ages (Figure 10). Interestingly, however, a substantial increase in phosphorylated Beclin1, a downstream effector of ULK1, at Ser 30 was observed in RhoA KO mice at 4 months of age (young), but not at 10 months of age (middle-aged) (Figure 10). Because ULK1 activity assessed by p-ULK1 western blotting is no affected by cardiac-specific deletion of RhoA, the observed increase in p-Beclin1 is suspected to be mediated by other kinases or be the result of a compensatory pathway in response to the chronic loss of RhoA.

When taken all together, our findings implicate that cardiac-specific deletion of RhoA significantly affects neither mTORC1 signaling nor autophagy specifically at 4 months (young) and 10 months (middle-aged) of age and that the early onset of cardiac aging in RhoA KO mice is not due to the impairment of autophagy. Further studies utilizing an inducible overexpression of RhoA or inducible deletion of RhoA in the hearts of mice at various ages including 18-24 month old (old) mice will be required to comprehensively understand the role of RhoA in the regulation of autophagy in the aging heart.

RhoA regulates PINK1-mediated mitophagy through an unconventional pathway.

Regarding the role of RhoA in the regulation of mitophagy, both our in vitro studies using NRVM expressing constitutively active RhoA (L63RhoA) and our in vivo study using 10 month old cardiac-specific RhoA KO mice suggest that RhoA activation leads to PINK1 protein accumulation (Figure 11). One of the most well established pathways for the initiation of mitophagy is through the PINK1/Parkin signaling pathway where PINK1, though is normally constantly degraded in the healthy mitochondria, is stabilized through the depolarization of the mitochondrial membrane potential in the damaged mitochondria [62]. In addition to the accumulation of PINK1, our study further demonstrated Parkin recruitment to mitochondria upon RhoA activation in NRVMs (Figure 14), confirming the subsequent induction of mitophagic events upon the RhoA-mediated accumulation of PINK1 and further supports the suggestion that RhoA signaling positively regulates mitophagy.

While investigating how the expression of active RhoA induces the accumulation of PINK1, it was interesting to see that although the chronic deletion of RhoA in mouse hearts showed a significant decrease in PINK1 transcription, suggesting that RhoA induces PINK1 transcription for PINK1 accumulation, short-term (less than 24 hour) expression of L63RhoA in NRVMs did not affect PINK1 transcription levels (Figure 12). As mentioned earlier, this discrepancy could be the result of compensatory signaling in response to the chronic deletion of RhoA. Our study did find that the observed accumulation of PINK1 in NRVMs may, however, result from RhoA-mediated inhibition of PINK1 degradation as suggested by the smaller difference in PINK1 protein levels between untreated and CHX-treated NRVMs in L63RhoA

expressing NRVMs compared to that of the control (Figure 13). These observations taken all together with the previous finding that RhoA does not induce depolarization of the mitochondrial membrane [54] reveal for the first time that PINK1 protein degradation may also be regulated by RhoA-mediated intracellular signaling for the control of mitophagy. It would be of interest to investigate the application of RhoA signaling as a pharmacological means to positively regulate mitophagy for the treatment of age-associated heart failure by extending our *in vitro* study to an *in vivo* study using AAV9-L63RhoA injected mice at various ages.

Mechanistically, our results suggest that the serine/threonine protein kinase, PKD, is required, but is not sufficient, for RhoA to stabilize PINK1 and induce mitophagy (Figure 14). While our study does show that RhoA potentially mediates its protection against cardiac aging through its regulation of mitophagy as suggested by the decreased PINK1 levels in RhoA KO mice at 10 months of age (middle-aged) (Figure 11A), more studies are needed to directly determine whether it is the RhoA/PKD signaling pathway that plays a significant role in producing the observed protective effects against cardiac aging. PKD has also been previously found to contribute to RhoA-mediated cardioprotection against I/R injury, specifically through the prevention of mitochondrial death pathways [49, 50]. Together, these findings suggest that RhoA/PKD signaling may not only inhibit the mitochondrial cell death pathway, but also regulate mitochondrial quality through mitophagy, preserving the integrity of mitochondria and thereby maintaining cellular homeostasis.

The precise mechanism behind RhoA/PKD stabilization of PINK1 still remains unclear. From our study, it is suggested through the PKD dependent accumulation of active RhoA at the mitochondria (Figure 16) that RhoA may play a more of a direct role in the stabilization of

PINK1 than as just an upstream regulator. Further studies are, however, required to determine if the accumulation of RhoA at the mitochondria is mediated by PKD phosphorylation of RhoA.

Our study suggests the potential involvement of the chaperone protein, BAG2 as increased BAG2 protein levels are observed at the mitochondria in L63RhoA expressing NRVMs (Figure 17). Although very few studies have been conducted on BAG2 in respect to the regulation of mitophagy, it has been recently discovered that BAG2 can interact with PINK1 at the mitochondria to inhibit the ubiquitination of PINK1 and its subsequent proteasome-mediated degradation, resulting in the increase in PINK1 protein levels [63, 64]. Furthermore, BAG2 was shown to protect HEK293 cells against oxidative stress, likely through the observed PINK1-dependent mitochondrial translocation of Parkin for mitophagy [64]. Our finding of the association between the activation of RhoA and the increase in mitochondrial BAG2 protein levels (Figure 17) together with the required involvement of PKD (Figure 14) suggests that the mitochondrial translocation of BAG2 is elicited by RhoA/PKD signaling and may play a key role in the discovered RhoA-mediated unconventional stabilization of PINK1. Our future study aims to examine whether RhoA and BAG2 form a molecular complex that translocates to the mitochondria to regulate PINK1 stability in response to RhoA and PKD activation. More studies are also needed towards delineating the mechanisms by which RhoA regulates PINK1-dependent mitophagy and it would be of interest to further clarify whether it is through this mechanism that RhoA is able to delay the progression of aging in the heart.

Conclusion

Our study contributes to the increasing evidence supporting the protective potential of the small signaling G-protein, RhoA, in the heart. Specifically, we provided for the first time, evidence of the potential of RhoA in serving as an inhibitory signaling molecule against the progression of cardiac aging. From this study, RhoA was also identified as a new and unconventional inducer of mitophagy, bringing to light a novel role for RhoA signaling in respect to maintaining mitochondrial integrity. All together, our study presents the potential of RhoA as a therapeutic target to protect the heart against aging through its unconventional positive regulation of mitophagy.

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