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RalGDS-Dependent Cardiomyocyte Autophagy is Required for Load-Induced Ventricular Hypertrophy

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Abstract

Recent work has demonstrated that autophagy, a phylogenetically conserved, lysosome-mediated pathway of protein degradation, is a key participant in pathological cardiac remodeling. One common feature of cell growth and autophagy is membrane biogenesis and processing. The exocyst, an octomeric protein complex involved in vesicle trafficking, is implicated in numerous cellular processes, yet its role in cardiomyocyte plasticity is unknown. Here, we set out to explore the role of small G protein-dependent control of exocyst function and membrane trafficking in stress-induced cardiomyocyte remodeling and autophagy. First, we tested in cultured neonatal cardiomyocytes (NRCMs) two isoforms of Ral (RalA, RalB) whose actions are mediated by the exocyst. In these experiments, mTOR inhibition in response to starvation or Torin1 was preserved despite RalA or RalB knockdown; however, activation of autophagy was suppressed only in NRCMs depleted of RalB, implicating RalB as being required for mTOR-dependent cardiomyocyte autophagy. To define further the role of RalB in cardiomyocyte autophagy, we analyzed hearts from mice lacking RalGDS (*Ralgds*$^{-/-}$), a guanine exchange factor (GEF) for the Ral family of small GTPases. RalGDS-null hearts were similar to wild-type (WT) littermates in terms of ventricular structure, contractile performance, and gene expression. However, *Ralgds*$^{-/-}$ hearts manifested a blunted growth response ($p<0.05$) to TAC-mediated pressure-overload stress. Ventricular chamber size and contractile performance were preserved in response to TAC in *Ralgds*$^{-/-}$ mice, and load-induced cardiomyocyte autophagy was suppressed. Interestingly, TAC-induced activation of the fetal gene program was similar in both genotypes despite the relative lack of hypertrophic growth in mutant hearts. Together, these data implicate RalGDS-mediated induction of autophagy and exocyst function as a critical feature of load-induced cardiac hypertrophy.

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Conflicts of Interest Disclosures None

Keywords

autophagy; cardiac hypertrophy; exocyst; small GTPase

1. Introduction

In response to pathological stress such as neurohormonal activation, hypertension, or myocardial injury, the heart is capable of robust changes in cardiac mass [1]. From a teleological perspective, the early phases of cardiac hypertrophy may be a compensatory response to increased workload, serving to normalize wall stress and thereby minimize oxygen consumption. In the long run, however, pathological hypertrophy is a milestone in the pathogenesis of heart failure [2]. Numerous signaling pathways have been implicated in the regulation of cardiac hypertrophy [3]. More recently, autophagy has emerged as critical process involved in cardiac hypertrophy [4].

Autophagy, or more precisely, macroautophagy, is an evolutionarily conserved, near-ubiquitous mechanism for the degradation of long-lived proteins and clearance of organelles [5]. Autophagy is involved in numerous disease processes, including neurodegeneration, cancer, and infectious disease [6]. Our group and others have demonstrated a role for autophagy in a variety of forms of cardiovascular disease, as well. A major question that remains largely unanswered about autophagy in the heart is whether autophagy is an adaptive or maladaptive response to stress [7, 8]. Our findings point to a maladaptive role of robust activation of autophagy in the setting of severe pressure overload stress [9]. Conversely, inactivation of Atg5, a gene required for autophagy, points to an adaptive role for autophagy [10]. These seemingly contradicting results imply that autophagic activity exists on a continuum, where too little or too much autophagy in response to stress is maladaptive, but a minimum amount of constitutive autophagic activity is required for cell survival.

The dichotomous roles of cardiac hypertrophy and cardiomyocyte autophagy led us to consider a common feature of the two processes: membrane biogenesis and processing. A plausible nexus for these two processes lies in the exocyst, an octomeric protein complex involved in vesicle trafficking. This complex serves a necessary role in the targeting of Golgi-derived vesicles to the basolateral membrane of polarized epithelial cells and to the growth cones of differentiating PC12 cells [11–13]. Recent work has demonstrated a role for the exocyst in autophagosome assembly as well; the small G protein, RalB, and an Exo84-dependent subcomplex of the exocyst were demonstrated to be critical for nutrient starvation and pathogen-induced autophagosome formation [14]. While many small GTPases have critical effects on cardiac plasticity [15], the role of small G protein-dependent membrane trafficking in stress-induced cardiomyocyte remodeling and autophagy remains largely unexplored.

Whereas previous studies have suggested a role for the Ral family of small GTPases in cardiac hypertrophy [16], underlying mechanisms remain unknown. Here, we set out to test the role of these enzymes and their associated guanine exchange factor (GEF), RalDGS (Ral GDP dissociation stimulator), in the control of membrane trafficking in stress-induced cardiomyocyte remodeling and autophagy.

2. Materials and methods

2.1. Animal models and echocardiography

Male C57/BL6 mice (8–10 weeks old) were subjected to thoracic aortic constriction (TAC) [17] for 3 weeks as previously described [18]. Control animals underwent sham operations. The Animal Care and Use Committee of the University of Texas Southwestern Medical Center approved all animal care and procedures. Echocardiograms were performed on conscious, gently restrained mice using a Vevo 2100 system with a MS400C scanhead. LVEDD and LVESD were measured from M-mode recordings. Fractional shortening (FS) was calculated as (LVEDD - LVESD)/LVEDD and expressed as a percentage. All measurements were made at the level of the papillary muscles.

2.2. Primary culture of neonatal cardiomyocytes, siRNA transfection and adenovirus infection

Neonatal rat cardiomyocytes were isolated and cultured as described previously [19]. After 24 hours, NRCMs were transfected with Silencer Select Pre-designed siRNA constructs (Ambion) using Lipofectamine RNAiMax (Invtirogen) in Optimem (Gibco) for 4 hours, and then switched to basal media: DMEM supplemented with 3% fetal bovine serum (FBS), BrdU, and antibiotics. After 48 hours, cells were infected with adenovirus (MOI 10) for 1 hour in Optimem and then switched to basal media. Torin1 and Bafilomycin A1 (LC Laboratories) were used at a concentration of 50 nM.

2.3. Real-time RT-PCR

Total RNA was harvested from NRVMs or mouse LV using TRIzol (Invitrogen) according to the manufacturer's protocol. cDNA was prepared from RNA using a high capacity cDNA reverse transcription kit (Applied Biosystems). Real-time PCR was performed using SYBR green on an ABI 7000 Prism Sequence Detection System (Applied Biosystems). To confirm amplification specificity, the PCR products were subjected to melting curve analysis. Negative controls containing water instead of cDNA were run concomitantly. Data for each transcript were normalized to reactions performed 18S rRNA primers, and fold change was determined using the comparative threshold method [20].

2.4. Immunoblot analysis

Tissues were either homogenized immediately or quick frozen in liquid nitrogen and stored at −80 °C for later use. To harvest protein, tissues were homogenized at 4°C in M-PER® mammalian protein extraction reagent (Thermo Scientific) supplemented with protease inhibitors (Roche), and phosphatase inhibitors (Roche). Whole cell lysates from cultured neonatal myocytes were prepared by directly harvesting cells in M-PER®. Homogenates were passed over glass wool to remove DNA. Proteins were separated by SDS/PAGE, transferred to a supported nitrocellulose membrane, and immunoblotted. The following antibodies were used: rabbit anti-LC3 (described previously [9]); mouse anti-p62 (Abnova); RalA (BD Biosciences); mouse anti-RalB (provided by Larry Feig, Tufts University); mouse anti-S6 Ribosomal Protein (Cell Signaling); rabbit anti-Phospho-S6 Ribosomal Protein (Ser235/236) (Cell Signaling); mouse anti-mTOR (Cell Signaling); rabbit anti-Phospho-mTOR (Ser2448) (Cell Signaling); mouse monoclonal anti-gapdh (Fitgerald Industries Int.); rabbit anti-α-tubulin (Abcam); mouse anti-α-tubulin (Sigma). Blots were scanned, and bands were quantified using an Odyssey Licor (version 3.0) imaging system. Immunoblots from a representative experiment are shown. The graphs shown indicate mean ± SD for 3 experiments.

2.5. Histology

All tissues were fixed in 4% paraformaldehyde overnight at 4°C, rinsed, and transferred to PBS followed by paraffin embedding. Terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) staining for apoptotic cells was performed by labeling with fluorescein, and the sections were counterstained with propidium iodide.

2.6. Myocyte cross-sectional Area

Images of tissues stained with wheat germ agglutinin were paraffin fixed (Vector Laboratories), and images were acquired on a confocal microscope (TCS SP5; Leica) with Leica LAS AF software. The following lenses were used: HC PL APO 20×/0.70, HCX PL APO 40×/1.25–0.75 oil, and HCX PL APO 63×/1.40–0.60 oil. All images were taken at room temperature and processed in ImageJ for CSA analysis. Occasionally, images were linearly rescaled to optimize brightness and contrast uniformly without altering, masking, or eliminating data.

2.8. RalB activity assay

Assays were performed according to the recommended protocol for the Active Ras Pull-Down and Detection Kit (Thermo Scientific). Briefly, 1 mg protein from flash frozen heart lysates was subjected to affinity purification with GST-RalBP1-RBD, a domain with affinity for the activated GTP-RalB. This purified aliquot was then subjected to immunoblot analysis as above.

2.7. Statistics

Data are presented as mean ± SD. The unpaired Student's t test was used for comparison between two groups, and ANOVA with Bonferroni correction was used for comparison among multiple groups. Values of $p < 0.05$ were considered significant.

3. Results

3. 1. RalB is necessary for cardiomyocyte autophagy

To test whether Ral small GTPases are required for cardiomyocyte autophagy, we selectively depleted NRCMs of RalA or RalB using RNAi. The siRNA constructs were specific for their respective isoform and were able to selectively deplete their target protein 48 hours after transfection and without a compensatory increase in the abundance of the other isoform (Fig. 1 A). To evaluate autophagy, we incubated NRCMs in Earle's Balanced Salt Solution (EBSS) for 2 hours, thereby simulating starvation, a powerful inducer of autophagy [5]. Nutrient deprivation led to increased levels of the faster migrating, lipidated form LC3-II, indicating an accumulation of autophagosomes (Fig. 1 B). Concomitant treatment with the lysosomal inhibitor Bafilomycin A1 led to a further increase in LC3-II, demonstrating that the increase in LC3-II reflects induction of autophagic flux and is not simply a block in degradation of autophagosomes. When RalB levels were depleted by RNAi, the expected increase in LC3-II in response to starvation was abrogated. However, the autophagic response to starvation in NRCMs lacking RalA remained intact (Fig. 1 B). Degradation of the autophagy substrate p62 was observed in response to starvation in NRCMs, as well. When RalB was depleted, however, p62 degradation was attenuated. In fact, treatment with the lysosomal inhibitor failed to increase p62 in these cardiomyocytes, consistent with diminution of autophagic flux in RalB-depleted NRCMs. These results suggest that RalB is necessary for cardiomyocyte autophagy in response to starvation.

Autophagic activation is governed by tonic inhibition by mTOR, and the autophagic response to starvation, or more specifically amino acid withdrawal, is mediated by release of

mTOR-dependent repression [21]. To test whether the inhibition of starvation-induced autophagy in the setting of RalB knockdown occurred because of persistent mTOR activation, we examined phosphorylation of mTOR and its substrate s6 (ribosomal protein s6). NRCMs exposed to EBSS showed diminished phosphorylation of mTOR at serine 2448 and marked loss of phosphorylation of s6 at serines 235 and 236 as well (Fig. 1 C). This response was similar when cells were depleted of either RalA or RalB, demonstrating that autophagy inhibition observed with loss of RalB was not due to a block in the mTOR response to starvation.

As another method for probing autophagic activation, we evaluated LC3 accumulation on autophagosomes as formation of GFP-tagged LC3 puncta (Fig. 1 D). NRCMs were infected with adenovirus harboring a GFP-LC3 transgene and subsequently exposed (2h) to EBSS. This short-term starvation stress triggered accumulation of GFP-LC3 puncta, indicative of increases in autophagosome abundance. The number of GFP-positive puncta per cell was significantly elevated in NRCMs exposed to EBSS (Fig. 1 D). RNAi-dependent depletion of RalA did not alter the autophagic response in NRCMs, as GFP-positive puncta were similarly increased in response to EBSS exposure (Supp. Fig. 1 A). By contrast, NRCMs depleted of RalB did not manifest a starvation-induced increase in GFP-positive puncta (Fig. 1 D). These results provide additional evidence that RalB is required for cardiomyocyte autophagy.

As an alternative method for inducing autophagy, we turned to pharmacological suppression of mTOR. Similar to the effects of nutrient deprivation, direct mTOR inhibition by exposure to Torin1 [22] (2h) provoked increases in LC3-II and declines in p62 (Fig. 1 E). With lysosomal inhibition, LC3-II was further increased and p62 degradation blocked, consistent with increases in autophagosome biogenesis induced by Torin1. In NRCMs depleted of RalB, however, autophagy flux was suppressed as indicated by a failure to increase LC3-II and attenuated degradation of p62 (Fig. 1 E). Exposure to Torin1 (2h) similarly induced localization of GFP-LC3 to distinct puncta in control NRCMs. This response was abrogated in NRCMs depleted of RalB (Fig. 1 F), but remained intact in NRCMs lacking RalA (Supp. Fig. 1 B). Together, these results lend further support to a model wherein RalB is necessary for cardiomyocyte autophagy triggered by nutrient deprivation or mTOR suppression.

3. 2. RalGDS is necessary for autophagy

GTPases function as molecular switches that cycle between an inactive GDP-bound state and an active state that binds GTP. GTP is hydrolyzed to GDP through the intrinsic GTPase activity of these proteins, rendering them inactive. GEFs (guanine nucleotide exchange factors) facilitate GTP loading and thereby activate the small G protein. To determine whether the GEF for the Ral family GTPases, RalGDS, is necessary for autophagy, we depleted NRCMs of RalGDS by means of RNAi knockdown using two sequence-independent siRNA constructs. After 48 hours, we quantified RalGDS mRNA by RT-PCR, because no commercially available anti-RalGDS antibody is available, confirming efficient knockdown of RalGDS (Fig 2 A).

To determine whether RalGDS is required for starvation-induced autophagy, we exposed NRCMs to EBSS (2h), in the presence/absence of lysosomal inhibition by Bafilomycin A1. As expected, NRCMs treated with control siRNA were competent for autophagic flux, responding to 2 hours of starvation with an increase in LC3-II accumulation and a further increase when Bafilomycin A1 was added (Fig. 2 B). In contrast, NRCMs treated with siRNA for RalGDS exhibited blunted autophagic flux. Only modest increases in LC3-II accumulation were observed when the NRCMs were additionally challenged with lysosomal inhibition (Fig 2 B), consistent with blunted flux. Similar results were obtained when we assayed for GFP-LC3 puncta formation; NRCMs depleted of RalGDS manifested

significantly diminished numbers of GFP-LC3 puncta in response to 2 hours of starvation, as compared with cells exposed to scrambled RNAi (Fig. 2 C).

To determine whether the attenuated autophagic flux response in RalGDS-depleted cells was a consequence of alterations in the mTOR pathway, we probed for phosphorylation of mTOR and its substrate s6. NRCMs starved for 2 hours manifested diminished phosphorylation of mTOR (serine 2448) and marked loss of phosphorylation of s6 (serines 235 and 236) despite depletion of RalGDS (Fig. 2 D). Together, these results demonstrate that nutrient depletion-dependent inhibition of mTOR remains intact despite depletion of RalGDS, situating this mechanism downstream of mTOR. They further suggest that the dependence of autophagic flux on RalGDS occurs through its action on RalB.

As a further test of a RalGDS requirement for cardiomyocyte autophagy, we examined whether this protein is necessary for autophagy induced by pharmacological blockade of mTOR. NRCMs were treated with Torin1 for 2 hours with or without Bafilomycin A1. In NRCMs depleted of RalGDS, autophagic flux triggered by Torin1 was inhibited as evidenced by lack of an increase in LC3-II abundance in response to Torin1 or an additional increment of LC3-II accumulation when lysosomal degradation was inhibited (Fig. 2 E).

Similar results were obtained when we evaluated accumulation of GFP-LC3 puncta in RalGDS-depleted cells subjected to mTOR inhibition (Fig. 2 F). NRCMs manifested increased abundances of GFP puncta in response to Torin1 treatment. However, NRCMs depleted of RalGDS did not reveal similar Torin1-induced increases, either in the absence or presence of lysosome inhibition with Bafilomycin A1. Collectively, these results indicate that RalGDS is required for cardiomyocyte autophagy.

3. 3. RalGDS$^{-/-}$ hearts manifested a blunted growth response and preserved function in response to pressure-overload stress

We have demonstrated previously that activation of cardiomyocyte autophagy is required for load-induced hypertrophic growth of the heart [9, 23]. Now, having established the necessity for RalB and its GEF, RalGDS, in NRCM autophagy *in vitro*, we set out to test the role of RalGDS-dependent cardiomyocyte autophagy in pathological cardiac remodeling *in vivo*. To accomplish this, male mice lacking RalGDS (KO) and their wild-type (WT) littermates, aged 8–10 weeks, were subjected to pressure overload stress by thoracic aortic constriction (TAC), a model which in our hands induces cardiac hypertrophic growth that reaches steady state at 3 weeks [18]. Hearts from KO mice manifested a hypertrophic growth response which was significantly attenuated compared with WT mice subjected to TAC (Fig. 3 A). Heart weight normalized to body weight (HW/BW) was as follows: WT Sham: 5.0 ± 0.5 mg/g, n=12; WT TAC: 6.9 ± 1.2, n=15; KO Sham: 4.7 ± 0.5, n=11, KO TAC: 5.6 ± 0.5, n=12; $p<0.05$) (Fig. 3 B). Heart weight normalized to tibia length (HW/TL) was: WT Sham: 8.5 ± 1.3 mg/mm, n=12; WT TAC: 11.4 ± 1.5, n=15; KO Sham: 8.0 ± 0.8, n=11, KO TAC: 9.2 ± 1.0, n=12; $p<0.05$). (Fig. 3 C). Measures comparing normalized left-ventricular weights confirmed that the KO hearts exhibited a diminished hypertrophic response to pressure overload stress (Supp Fig. 2 A & B). These data, consistent with our *in vitro* findings in NRCMs, point to a specific requirement of RalGDS in load-induced ventricular hypertrophy.

To assess cardiac function, echocardiograms were performed 3 weeks following surgery (Fig. 4 A). Ventricular systolic performance, assessed as % fractional shortening (%FS) on M-mode recordings, was decreased mildly, albeit significantly, in WT mice exposed to TAC (Fig. 4 B). By contrast, %FS was essentially unchanged in KO mice exposed to Sham operation or TAC (WT Sham: 71 ± 5 %, n=3; WT TAC: 53 ± 8, n=5; KO Sham: 65 ± 13, n=7, KO TAC: 61 ± 9, n=6). The decline in %FS in WT mice derived from significant

increases in both left ventricular internal diameter in diastole (LVIDd; WT Sham: 2.8 ± 0.2 mm, n=3; WT TAC: 3.4 ± 0.5, n=5; $p<0.05$) (Fig. 4 C) and left ventricular internal diameter in systole (LVIDs; WT Sham: 0.8 ± 0.2 mm, n=3; WT TAC: 1.6 ± 0.5, n=5; $p<0.05$) (Fig. 4 D). These increases, however, were not observed in KO mice in terms of LVIDd (KO Sham: 3.1 ± 0.8, n=7, KO TAC: 3.1 ± 0.4, n=7; $p<0.05$) (Fig. 4 C) or LVIDs (KO sham: 1.2 ± 0.7, n=7, KO TAC: 1.2 ± 0.4, n=6; $p<0.05$) Fig. 4 D). Thus, RalGDS-depleted hearts manifested a blunted growth response to pressure overload, in terms of both hypertrophic growth and development of systolic dysfunction.

Quantification of the mean cross-sectional area of 80–100 cardiomyocytes in transverse sections of ventricular septa stained for wheat germ agglutinin in each of 3 mice per group revealed significant increases in WT hearts exposed to TAC (Figs. 5 A, 5 B). By contrast, the cross-sectional areas of cardiomyocytes from KO mice were not significantly increased (CSA; WT Sham: 505 ± 20 μm^2; WT TAC: 733 ± 27; KO Sham: 484 ± 14, KO TAC: 557 ± 15; $p<0.05$). Interestingly, TAC-induced activation of the fetal gene program was similar in both genotypes despite the relative lack of hypertrophic growth in KO hearts (Fig. 5 C).

3. 4. Ralgds$^{-/-}$ mice exhibit diminished load-induced cardiomyocyte autophagy

We have found that RalB (Fig. 1) and its GEF, RalGDS (Fig. 2), are required for autophagy in NRCMs exposed to nutrient depletion and pharmacological mTOR inhibition. To determine whether the autophagic response triggered by pressure overload manifests a similar requirement for RalGDS, we assessed LC3-II levels in WT and KO hearts subjected to TAC or Sham operation. Here, we found that hearts from KO mice subjected to TAC exhibited diminished load-induced cardiomyocyte autophagy; heart lysates from WT mice manifested increased LC3-II abundance with TAC surgery, whereas LC3-II accumulation was absent in TAC-exposed hearts from KO mice (Fig. 5 D). As expected, cardiomyocytes from WT and KO mice did not display significant apoptosis in response to pressure-overload stress as quantified by TUNEL-positive nuclei (Supp. Fig. 3 A & B).

We detected a trend for increased p62 abundance in response to TAC, which was absent in KO hearts (Fig. 5 D). While p62 is sometimes used as a readout for autophagic degradation, there is no clear correlation between increases in LC3-II and decreases in p62 [24]. Indeed, increased abundance of p62 may serve as a marker of stress in the cardiomyocyte, as it has been reported in the setting of pressure overload stress [10] and a model of cardiac proteinopathy [25]. Interestingly, and in contrast with our findings in NRCMs, KO mice exhibited an increased LC3-II/LC3-I ratio in response to starvation for 24 hours (Supp. Fig. 4), pointing to significant differences in the triggers of autophagy in these two models.

Expression of *Rala*, *Ralb* and *Ralgds* transcripts were not significantly altered in hearts subjected to pressure-overload stress (Supp. Fig. 5 A). Also, an assay utilizing a GST-fusion protein of the Ral-binding domain of RalBP1 (GST-RalBP1) to purify the active GTP-bound form of RalB (GTP-RalB), did not detect a significant increase in GTP-RalB in hearts subjected pressure-overload stress (Supp. Fig. 5 B). Thus, mechanisms governing RalB activity in response to pressure-overload stress remain unclear.

4. Discussion

Prior to this report, the role of RalGDS/Ral signaling in cardiomyocyte remodeling and autophagy was unexplored. Here, we report that the small GTPase RalB involved in membrane trafficking is specifically required for cardiomyocyte autophagy and hypertrophic growth. Its GEF, RalGDS, is similarly required. Both of these mechanisms are situated downstream of mTOR-dependent suppression of autophagosome biogenesis and stress-induced activation of the fetal gene program. Finally, we show that TAC-induced

hypertrophy is attenuated in RalGDS-deficient hearts, and yet ventricular size and performance are maintained despite the presence of elevated afterload. Together, these novel data unveil a requirement for RalGDS in the autophagic response, and hypertrophic growth, elicited by pressure-overload stress. In so doing, they point to the conservation of mechanisms involving RalB signaling through the exocyst to engage autophagy, a response required in cardiomyocyte remodeling (Fig. 6).

4. 1. RalGDS is an activator of cardiomyocyte autophagy

The small GTPase RalB and an Exo84-dependent subcomplex of the exocyst are critical for nutrient starvation and pathogen-induced autophagosome formation in human epithelial cells [14]. Here, we report that RalB is a necessary regulatory switch to promote autophagosome biogenesis in the cardiomyocyte triggered by nutrient deprivation or mTOR suppression. NRCMs lacking RalB are capable of sensing nutrient depletion, which provokes mTOR inhibition, but they are unable to mount the necessary autophagy response.

Previous studies have suggested a role for the Ral family of small GTPases in cardiac hypertrophy [16]. Cardiotrophin-1 (CT-1) activated RalGDS mRNA expression and induced Ral activation in an *in vitro* model of cardiac hypertrophy; similarly Ral activity was elevated in hypertrophied hearts in a rat aortic banding model [16]. However, mechanisms whereby RalGDS influences cardiac hypertrophy have remained unknown.

RalA has recently been shown to promote mTORC1 activation, potentially through PLD1 and phosphatidic acid-dependent mTORC1/2 assembly [26–28]. In the cardiomyocyte, we observed that autophagic activity under basal conditions and in response to starvation is increased when RalA is depleted within the cell; yet, mTOR activity remains unaffected. It is plausible that in the setting of RalA depletion, RalGDS is increasingly available to activate RalB and thus facilitate autophagy. This suggests a model where RalA and RalB represent an antagonistic switch for the autophagy response.

Our work goes on to demonstrate that RalGDS, a GEF for the Ral family of small GTPases, is itself a bona fide requirement for cardiomyocyte autophagy. Again, our results suggest that RalGDS is a proximal regulator of autophagosome biogenesis, as mTOR inhibition in response to starvation remains intact despite RalGDS depletion. It is likely that RalGDS serves as a GEF that targets, and activates, RalB in its autophagy-promoting role.

An assay for RalB activity was unable to detect a significant increase in active, GTP-bound RalB in hearts subjected to pressure-overload stress. We speculate that subcellular domains of RalB exist within cardiomyocytes which are differentially regulated in response to afterload stress and which escape detection by this assay.

4. 2. RalGDS is required for cardiac hypertrophy in response to pressure-overload stress

Our findings demonstrate that RalGDS is a critical requirement for load-induced cardiac hypertrophy. Previous findings had shown that over-expression of RalGDS in NRCMs was sufficient to activate the promoters of two fetal genes, beta myosin heavy chain and alpha skeletal muscle actin [29]. Here, we have extended the role of RalGDS to an *in vivo* model of cardiac hypertrophy in response to pressure-overload stress, observing that RalGDS-null hearts manifested a blunted hypertrophic response to pressure stress.

4. 3. Cardiomyocyte autophagy is required for load-induced hypertrophy

In preclinical studies [18, 30] and correlative studies in patients with heart disease [31], attenuation of load-induced hypertrophy is well tolerated; suppression of pathological hypertrophy is well tolerated, and ventricular dilation and decompensation do not occur.

Consistent with this now-established theme, RalGDS-null hearts manifest a blunted hypertrophic response to pressure stress and yet ventricular size and function were preserved even in the setting of markedly elevated afterload. Interestingly, TAC-induced activation of the fetal gene program was preserved in the KO hearts. This suggests that the autophagy-dependent myocyte growth pathway is independent of the previously described calcineurin-dependent transcriptional pathway for cardiac hypertrophy [32].

We have reported that activation of autophagy is required in the remodeling responses of the cardiac myocyte [9, 23]. Here, we uncovered an association between lack of hypertrophy and lack of autophagy, consistent with this model, in the RalGDS-null heart. However, further work will be required to determine whether this association reflects a causal link.

4. 4. Summary and perspective

Together, studies reported here implicate RalGDS-mediated induction of autophagy as a critical feature of load-induced cardiac hypertrophy. As such, these findings contribute to a growing literature pointing to stress-induced cardiomyocyte autophagy as a critical feature of the pathological remodeling response. Given this, we posit that the autophagic process may have relevance as a therapeutic target of clinical importance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

[1]. Hill JA, Olson EN. Cardiac plasticity. The New England journal of medicine. 2008; 358:1370–80. [PubMed: 18367740]

[2]. Levy D, Garrison RJ, Savage DD, Kannel WB, Castelli WP. Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. N Engl J Med. 1990; 322:1561–6. [PubMed: 2139921]

[3]. Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. Nature reviews Molecular cell biology. 2006; 7:589–600.

[4]. Rothermel BA, Hill JA. Autophagy in load-induced heart disease. Circulation Research. 2008; 103:1363–9. [PubMed: 19059838]

[5]. Klionsky DJ. Autophagy: from phenomenology to molecular understanding in less than a decade. Nature reviews Molecular cell biology. 2007; 8:931–7.

[6]. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature. 2008; 451:1069–75. [PubMed: 18305538]

[7]. Gustafsson AB, Gottlieb RA. Recycle or die: the role of autophagy in cardioprotection. Journal of Molecular and Cellular Cardiology. 2008; 44:654–61. [PubMed: 18353358]

[8]. Nemchenko A, Chiong M, Turer A, Lavandero S, Hill JA. Autophagy as a therapeutic target in cardiovascular disease. J Mol Cell Cardiol. 2011; 51:584–93. [PubMed: 21723289]

[9]. Zhu H, Tannous P, Johnstone JL, Kong Y, Shelton JM, Richardson JA, et al. Cardiac autophagy is a maladaptive response to hemodynamic stress. The Journal of clinical investigation. 2007; 117:1782–93. [PubMed: 17607355]

[10]. Nakai A, Yamaguchi O, Takeda T, Higuchi Y, Hikoso S, Taniike M, et al. The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. Nature Medicine. 2007; 13:619–24.

[11]. Hsu SC, Hazuka CD, Foletti DL, Scheller RH. Targeting vesicles to specific sites on the plasma membrane: the role of the sec6/8 complex. Trends in cell biology. 1999; 9:150–3. [PubMed: 10203793]

[12]. Guo W, Sacher M, Barrowman J, Ferro-Novick S, Novick P. Protein complexes in transport vesicle targeting. Trends in cell biology. 2000; 10:251–5. [PubMed: 10802541]

[13]. Mostov KE, Verges M, Altschuler Y. Membrane traffic in polarized epithelial cells. Current opinion in cell biology. 2000; 12:483–90. [PubMed: 10873817]

[14]. Bodemann BO, Orvedahl A, Cheng T, Ram RR, Ou YH, Formstecher E, et al. RalB and the exocyst mediate the cellular starvation response by direct activation of autophagosome assembly. Cell. 2011; 144:253–67. [PubMed: 21241894]

[15]. Lezoualc'h F, Métrich M, Hmitou I, Duquesnes N, Morel E. Small GTP-binding proteins and their regulators in cardiac hypertrophy. Journal of Molecular and Cellular Cardiology. 2008; 44:623–32. [PubMed: 18339399]

[16]. Kawai M, Kawashima S, Sakoda T, Toh R, Kikuchi A, Yamauchi-Takihara K, et al. Ral GDP dissociation stimulator and Ral GTPase are involved in myocardial hypertrophy. Hypertension. 2003; 41:956–62. [PubMed: 12642511]

[17]. Rockman HA, Ross RS, Harris AN, Knowlton KU, Steinhelper ME, Field LJ, et al. Segregation of atrial-specific and inducible expression of an atrial natriuretic factor transgene in an in vivo murine model of cardiac hypertrophy. Proc Natl Acad Sci U S A. 1991; 88:8277–81. [PubMed: 1832775]

[18]. Hill JA, Karimi M, Kutschke W, Davisson RL, Zimmerman K, Wang Z, et al. Cardiac hypertrophy is not a required compensatory response to short-term pressure overload. Circulation. 2000; 101:2863–9. [PubMed: 10859294]

[19]. Ni YG, Berenji K, Wang N, Oh M, Sachan N, Dey A, et al. Foxo transcription factors blunt cardiac hypertrophy by inhibiting calcineurin signaling. Circulation. 2006; 114:1159–68. [PubMed: 16952979]

[20]. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25:402–8. [PubMed: 11846609]

[21]. Noda T, Ohsumi Y. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J Biol Chem. 1998; 273:3963–6. [PubMed: 9461583]

[22]. Thoreen CC, Kang SA, Chang JW, Liu Q, Zhang J, Gao Y, et al. An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. J Biol Chem. 2009; 284:8023–32. [PubMed: 19150980]

[23]. Cao DJ, Wang ZV, Battiprolu PK, Jiang N, Morales CR, Kong Y, et al. Histone deacetylase (HDAC) inhibitors attenuate cardiac hypertrophy by suppressing autophagy. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108:4123–8. [PubMed: 21367693]

[24]. Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, et al. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy. 2012; 8:445–544. [PubMed: 22966490]

[25]. Zheng Q, Su H, Ranek MJ, Wang X. Autophagy and p62 in Cardiac Proteinopathy / Novelty and Significance. Circulation Research. 2011; 109:296–308. [PubMed: 21659648]

[26]. Maehama T, Tanaka M, Nishina H, Murakami M, Kanaho Y, Hanada K. RalA functions as an indispensable signal mediator for the nutrient-sensing system. J Biol Chem. 2008; 283:35053–9. [PubMed: 18948269]

[27]. Toschi A, Lee E, Xu L, Garcia A, Gadir N, Foster DA. Regulation of mTORC1 and mTORC2 complex assembly by phosphatidic acid: competition with rapamycin. Mol Cell Biol. 2009; 29:1411–20. [PubMed: 19114562]

[28]. Voss M, Weernink PA, Haupenthal S, Moller U, Cool RH, Bauer B, et al. Phospholipase D stimulation by receptor tyrosine kinases mediated by protein kinase C and a Ras/Ral signaling cascade. J Biol Chem. 1999; 274:34691–8. [PubMed: 10574935]

[29]. Kawai M, Kawashima S, Sakoda T, Toh R, Kikuchi A, Yamauchi-Takihara K, et al. Ral GDP dissociation stimulator and Ral GTPase are involved in myocardial hypertrophy. Hypertension. 2003; 41:956–62. [PubMed: 12642511]

[30]. Frey N, Katus HA, Olson EN, Hill JA. Hypertrophy of the heart: a new therapeutic target? Circulation. 2004; 109:1580–9. [PubMed: 15066961]

[31]. Mathew J, Sleight P, Lonn E, Johnstone D, Pogue J, Yi Q, et al. Reduction of cardiovascular risk by regression of electrocardiographic markers of left ventricular hypertrophy by the angiotensin-converting enzyme inhibitor ramipril. Circulation. 2001; 104:1615–21. [PubMed: 11581138]

[32]. Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, et al. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell. 1998; 93:215–28. [PubMed: 9568714]

HIGHLIGHTS

- The small GTPase RalB is required for cardiomyocyte autophagy and hypertrophic growth.
- Its GEF, RalGDS, is similarly required.
- Both are downstream of mTOR-dependent suppression of autophagosome biogenesis.
- Both are downstream of stress-induced fetal gene program activation.
- TAC-induced hypertrophy is attenuated in RalGDS-deficient hearts.

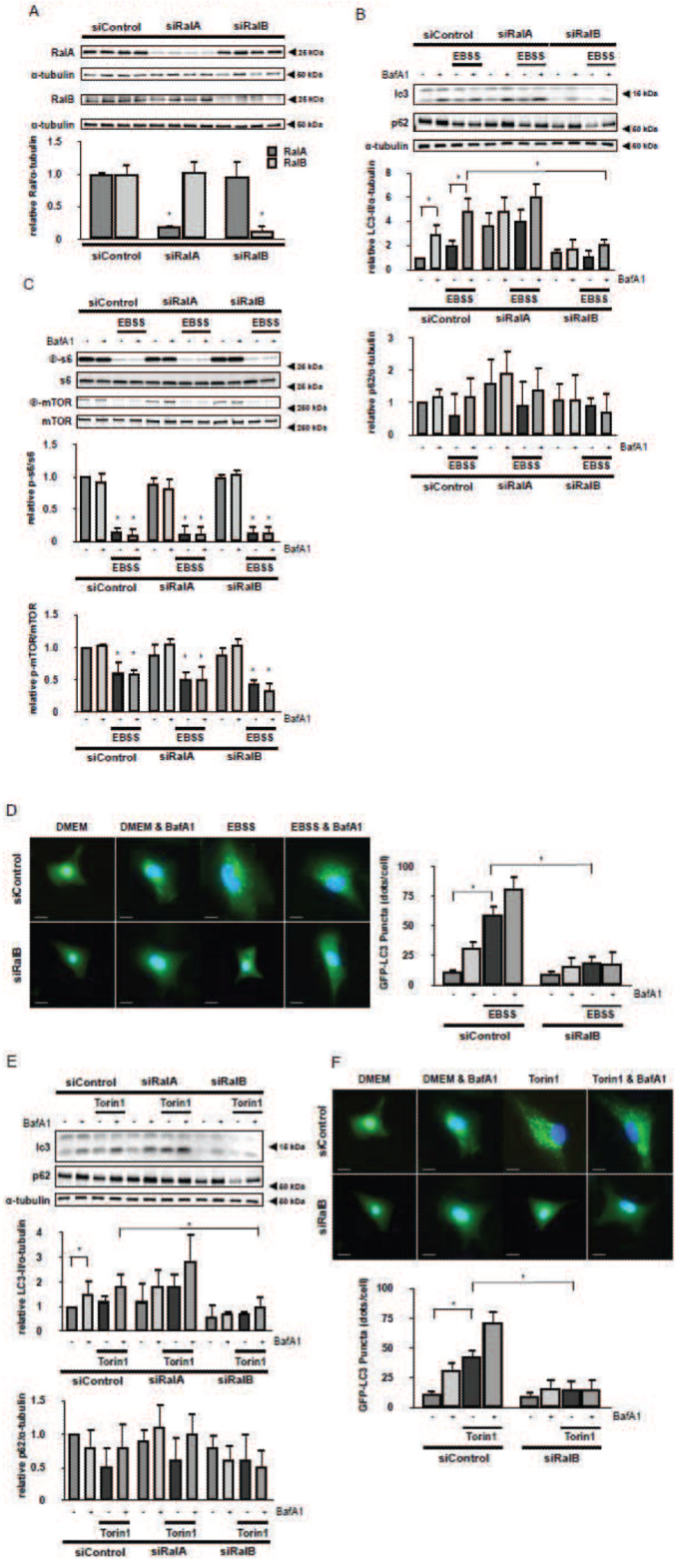

Figure 1. RalB is required for autophagy

(A) Immunoblot detection of NRCM lysates after 48 hour exposure to siRNA-mediated knockdown showing isoform-specific protein knockdown (*p<0.05 compared to siControl).

(B) Immunoblot detection of NRCM lysates after 2-hour starvation in Earle's Balanced Salt Solution (EBSS) with or without Bafilomycin A1 showing decreased LC3-II formation with siRalB (average of 3 independent experiments; *$p<0.05$). (C) Immunoblot detection of NRCM lysates after 2-hour starvation in EBSS with or without Bafilomycin A1 showing mTOR inhibition (average of 3 independent experiments; *$p<0.05$). (D) Representative images of cardiomyocytes expressing GFP-LC3 and exposed to 2-hour starvation in EBSS with or without BafA1 showing decreased GFP-LC3 puncta with siRalB (n=80 cells per treatment, *$p<0.05$). (E) Immunoblot detection of NRCM lysates after 2-hour treatment with Torin1 with or without Bafilomycin A1 showing decreased LC3-II formation with siRalB (average of 3 independent experiments; *$p<0.05$). (F) Representative images of cardiomyocytes expressing GFP-LC3 and exposed to 2-hour treatment with Torin1 with or without BafA1 showing decreased GFP-LC3 puncta with siRalB (n=80 cells per treatment, *$p<0.05$).

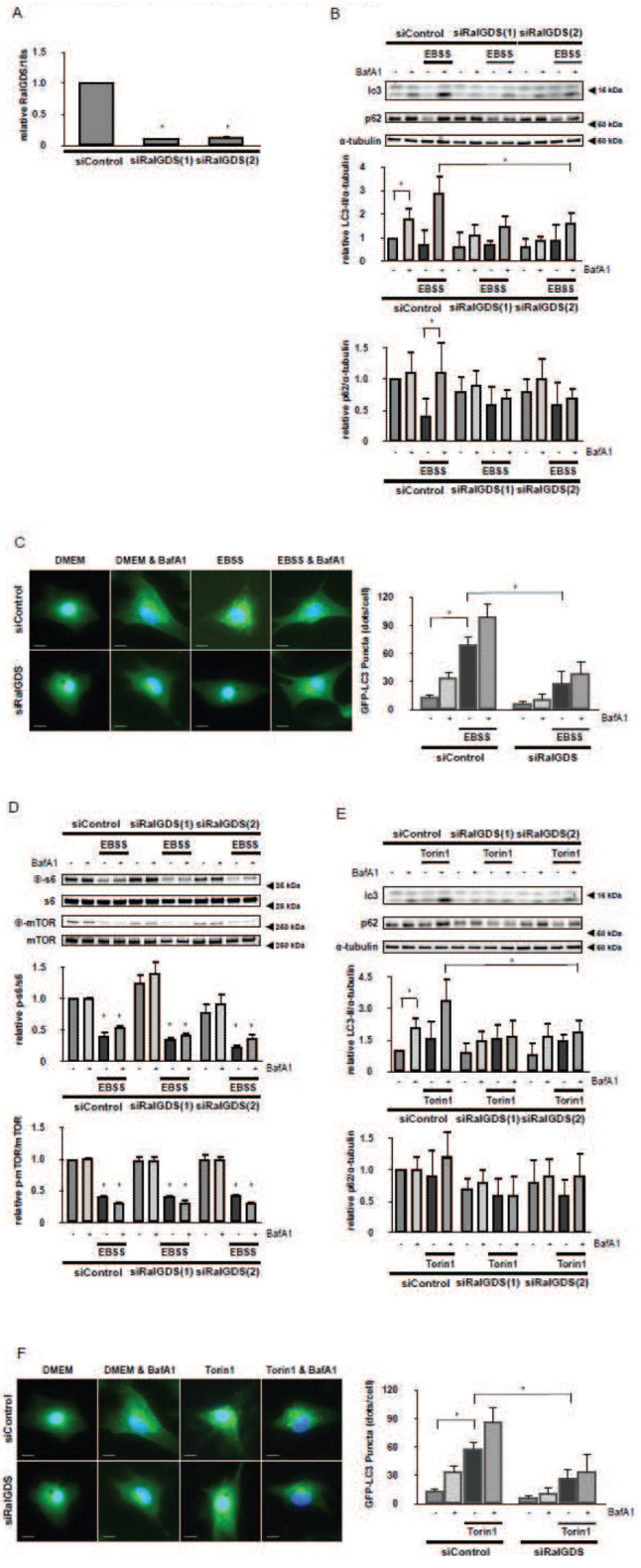

Figure 2. RalGDS is required for autophagy

(A) Relative RalGDS mRNA levels after 48 hour knockdown with 2 independent siRNA constructs (*p<0.05 compared to siControl). (B) Immunoblot detection of NRCM lysates after 2-hour starvation in EBSS with or without Bafilomycin A1 showing decreased LC3-II formation with siRalGDS (average of 3 independent experiments; *p<0.05). (C) Immunoblot detection of NRCM lysates after 2-hour starvation in EBSS with or without Bafilomycin A1 showing mTOR inhibition (average of 3 independent experiments; *p<0.05). (D) Representative images of cardiomyocytes expressing GFP-LC3 and exposed to 2-hour starvation in EBSS with or without BafA1 showing decreased GFP-LC3 puncta with siRalGDS (n=80 cells per treatment, *p<0.05). (E) Immunoblot detection of NRCM lysates after 2-hour treatment with Torin1 with or without Bafilomycin A1 showing decreased LC3-II formation with siRalGDS (average of 3 independent experiments; *p<0.05). (F) Representative images of cardiomyocytes expressing GFP-LC3 and exposed to 2-hour treatment with Torin1 with or without BafA1 showing decreased GFP-LC3 puncta with siRalGDS (n=80 cells per treatment, *p<0.05).

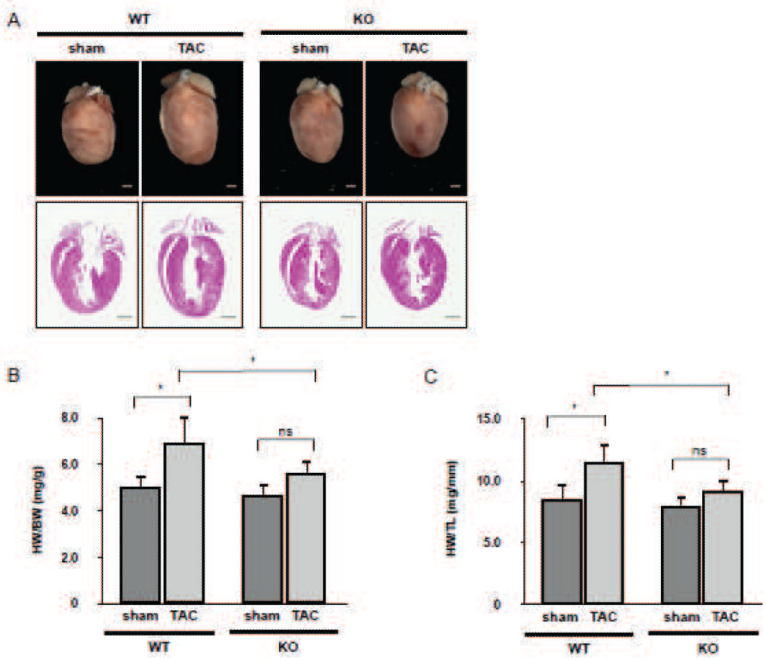

Figure 3. RalGDS$^{-/-}$ hearts manifest a blunted growth response to pressure-overload stress

Male mice lacking RalGDS (*Ralgds*$^{-/-}$) and their wild-type (WT) littermates, aged 8 to 10 weeks, were subjected to pressure-overload stress by TAC or sham operation. After 3 weeks, hearts from *Ralgds*$^{-/-}$ mice manifested a blunted growth response as seen by representative (A) formalin-fixed whole hearts and H&E histology sections. The blunted growth response was quantified and normalized by (B) heart weight to body weight ratios and (C) heart weight to tibia length ratios (*p<0.05).

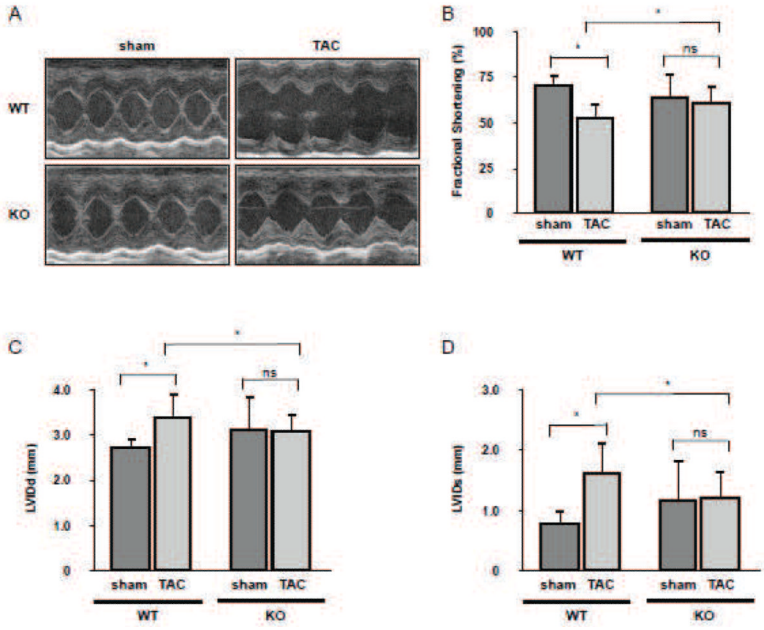

Figure 4. RalGDS$^{-/-}$ hearts exhibit preserved contractile function in response to pressure-overload stress

(A) Representative M-mode tracings. (B) Fractional shortening (C) Left ventricular internal diameter in diastole (mm). (D) Left ventricular internal diameter in systole (mm). (*p<0.05).

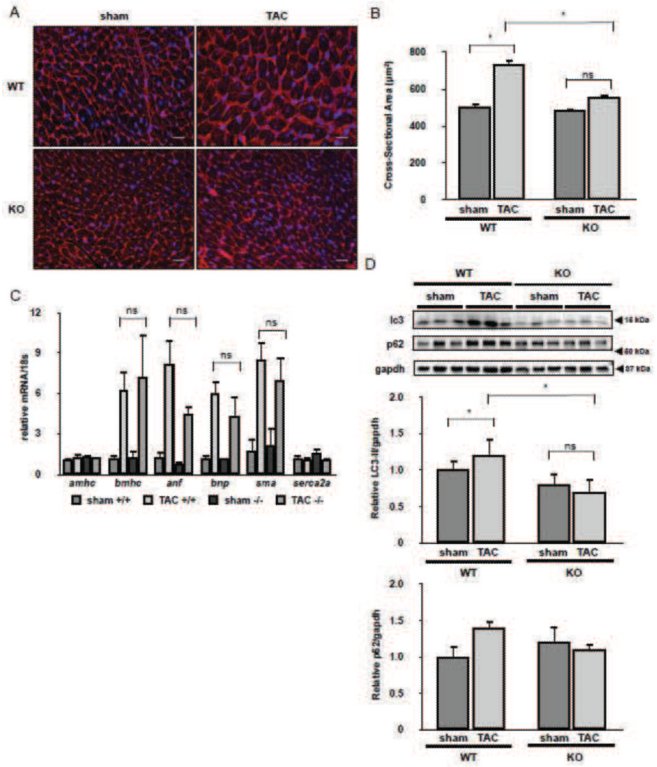

Figure 5. Blunted cardiomyocyte growth and autophagy of RalGDS$^{-/-}$ hearts, yet preserved activation of fetal gene program

(A) Representative sections of DAPI-labeled (blue) and wheat germ agglutinin-stained (red) hearts. (B) Quantification of mean cross-sectional area of 80–100 cardiomyocytes in the ventricular septum from each of 3 mice per group (*p<0.05). (C) Mean relative mRNA expression in relation to 18s rRNA and normalized to sham +/+ levels (*amhc* alpha myosin heavy chain, *bmhc* beta myosin heavy chain, *anf* atrial natriuretic factor, *bnp* brain natriuretic peptide, *sma* smooth muscle actin, *serca2a* sarcoplasmic reticulum Ca(2+) ATPase). (D) Immunoblot detection of left ventricular lysates following one week of TAC or sham surgery and quantification of relative levels of LC3 and p62 expressed as fold induction over WT sham surgery (*p<0.05).

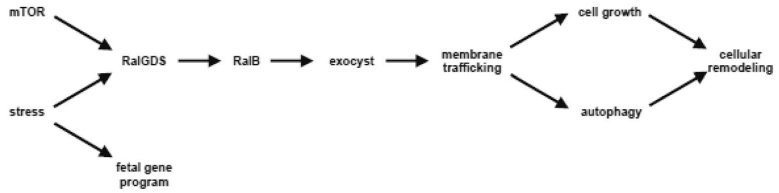

Figure 6. Working model of RalGDS-dependent cardiomyocyte remodeling

Findings reported here point to a model where growth cues activate a RalGDS-RalB signaling axis which governs exocyst-dependent membrane trafficking required for both cellular growth and autophagy.