

RESEARCH ARTICLE | -Omic Approaches to Understanding Muscle Biology

Arrdc2 and Arrdc3 elicit divergent changes in gene expression in skeletal muscle following anabolic and catabolic stimuli

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Gordon BS, Rossetti ML, Eroshkin AM. Arrdc2 and Arrdc3 elicit divergent changes in gene expression in skeletal muscle following anabolic and catabolic stimuli. *Physiol Genomics* 51: 208–217, 2019. First published April 19, 2019; doi:10.1152/physiolgenomics.00007.2019.—Skeletal muscle is a highly plastic organ regulating various processes in the body. As such, loss of skeletal muscle underlies the increased morbidity and mortality risk that is associated with numerous conditions. However, no therapies are available to combat the loss of muscle mass during atrophic conditions, which is due in part to the incomplete understanding of the molecular networks altered by anabolic and catabolic stimuli. Thus, the current objective was to identify novel gene networks modulated by such stimuli. For this, total RNA from the tibialis anterior muscle of mice that were fasted overnight or fasted overnight and refed the next morning was subjected to microarray analysis. The refeeding stimulus altered the expression of genes associated with signal transduction. Specifically, expression of alpha arrestin domain containing 2 (Arrdc2) and alpha arrestin domain containing 3 (Arrdc3) was significantly lowered 70–85% by refeeding. Subsequent analysis showed that expression of these genes was also lowered 50–75% by mechanical overload, with the combination of nutrients and mechanical overload acting synergistically to lower Arrdc2 and Arrdc3 expression. On the converse, stimuli that suppress growth such as testosterone depletion or acute aerobic exercise increased Arrdc2 and Arrdc3 expression in skeletal muscle. While Arrdc2 and Arrdc3 exhibited divergent changes in expression following anabolic or catabolic stimuli, no other member of the Arrdc family of genes exhibited the consistent change in expression across the analyzed conditions. Thus, Arrdc2 and Arrdc3 are a novel set of genes that may be implicated in the regulation of skeletal muscle mass.

atrophy; autophagy; growth; protein degradation

INTRODUCTION

Skeletal muscle regulates various processes in the body including glucose homeostasis, thermoregulation, and physical function (4, 41, 43). Accordingly, the loss of skeletal muscle mass that occurs during catabolic conditions such as cancer, aging, chronic obstructive pulmonary disease, heart failure, and chronic kidney disease increases the risk of morbidity and mortality (2, 42, 48, 51, 53). In addition to these consequences for the individual, the loss of muscle mass also represents an

important public health problem as it imposes a large financial burden on the healthcare system (3), and options to treat muscle atrophy are limited.

Skeletal muscle mass is regulated by various external stimuli. For instance, nutrient consumption and mechanical overload impart a growth stimulus on the skeletal muscle (16, 26), while starvation or testosterone deprivation impart an atrophy stimulus on skeletal muscle (17, 52). While this is known, the molecular networks altered by these various anabolic/catabolic stimuli are not completely defined, consequently limiting the development of therapies to treat or prevent muscle atrophy. In an attempt to increase our understanding of the molecular networks altered by these stimuli, microarray analysis was used to compare the global gene expression patterns from skeletal muscle of rodents in the fasted metabolic state versus those in the refed metabolic state. As nutrient consumption represents a potent anabolic stimulus in skeletal muscle (21, 26), the purpose of this hypothesis-generating approach was to identify novel genes and/or gene programs that would provide the basis for future study related to the mechanisms regulating skeletal muscle plasticity. Accordingly, gene programs associated with signal transduction were among those altered when comparing the fasted and refed conditions. Within these functional categories, we identified expression of the tumor suppressor gene, alpha arrestin domain containing 3 (Arrdc3) (10, 47, 60) as one of the nutrient responsive genes. In addition, this analysis also identified alpha arrestin domain containing 2 (Arrdc2) as a nutrient responsive gene. Subsequent analysis showed that expression of Arrdc2 and Arrdc3 was consistently altered in skeletal muscle by other anabolic/catabolic stimuli. Interestingly, no other member of the Arrdc family of genes exhibited these consistent changes in expression. In all, these findings expand our knowledge of the skeletal muscle gene networks that are sensitive to various anabolic and catabolic stimuli and suggest a possible role for changes in the expression of Arrdc2 and/or Arrdc3 in the regulation of muscle mass.

METHODS

Animals. Male C57BL/6NHsd mice (Envigo, Indianapolis, IN) were used to generate samples for microarray analysis and the castration study, while male B6129F1 mice (Taconic, Hudson, NY) were used to generate samples for the synergistic ablation and acute aerobic exercise studies. These mice were purchased, housed, and treated as described previously (18, 20, 52). For all other experiments, male C57BL/6NHsd mice were obtained from Envigo, housed individually in a temperature- (25°C) and light (12 h/12 h light-dark)-controlled

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environment within the vivarium at Florida State University. Mice were provided 5001 rodent chow (LabDiet, St. Louis, MO) and water ad libitum. The Institutional Animal Care and Use Committee of The University of Central Florida, Pennsylvania State University College of Medicine, and Florida State University approved the animal facilities, and all procedures as pertinent to each experiment.

Fasting/refeeding for microarray analysis sample generation. This model has been previously described by our group to elicit an anabolic response in skeletal muscle (19, 21, 49, 52), and this model was used to generate samples needed to define alterations to global gene expression by microarray analysis. Mice were deprived of food beginning at 1700. The next morning (~0800–1000), mice were randomized to remain fasted until euthanized or to have access to irradiated PicoLab 5053 rodent chow (LabDiet) food pellets for 30 min (i.e., refeed). After 30 min, food was removed and mice were euthanized 4.5 h later.

Fasting/refeeding and muscle contractions. Mechanical overload was induced via unilateral, high frequency, electrically induced muscle contractions as previously described (19, 52). To assess the effect of combining nutrient intake and mechanical overload on Arrdc2 and Arrdc3 expression, we first subjected mice to an overnight fast. The following morning, mice were randomized to remain fasted or refeed with 5001 rodent chow (LabDiet) in a manner as described above. Immediately following completion of the refeeding, or for those mice that remained fasted, 3% isoflurane was used as an anesthetic before the left thigh was shaved, and two bipolar electrodes were inserted subcutaneously and placed on either side of the sciatic nerve. The electrodes were used to stimulate the nerve using a constant current stimulator (Aurora Scientific, Ontario, Canada) causing all muscles of the lower limb to contract. Following contractions, all mice received a subcutaneous injection of warm saline (500 μ l) before returning to their cages where they had free access to water, but not food. Mice were allowed to recover for 4 h following the contractions before removal of the tibialis anterior (TA) muscles and euthanization under isoflurane anesthesia. Muscles were snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Rapamycin injections and muscle contractions. Rapamycin was purchased from LC laboratories (Woburn, MA). It was dissolved in DMSO (VWR; cat. #97063-136) yielding a 5 $\mu\text{g}/\mu\text{l}$ stock solution. The necessary amount of the stock was then diluted into 200 μl of phosphate-buffered saline (PBS) to yield a 3 $\mu\text{g}/\text{g}$ of body weight solution. To assess the role of the mechanistic target of rapamycin in complex 1 (mTORC1) on the mechanically induced change in Arrdc2 and Arrdc3 expression, mice were initially fasted overnight. The next morning, mice were injected with the rapamycin solution or the vehicle only via the intraperitoneal cavity 1 h before an acute bout of muscle contractions by the protocol described above with the exception that all mice remained fasted before the contractions. This rapamycin pretreatment timeframe was based on preliminary experiments that showed mTORC1 activity was completely blocked within 1 h following rapamycin administration. The rapamycin dose used was 2 \times more concentrated than the dose needed to prevent muscle growth following synergistic ablation (16), or the activation of mTORC1 following acute muscle contractions (54).

RNA extraction, microarray, and microarray data analysis. Muscle samples were homogenized in 500–600 μl of Zymo Tri Reagent

(Irvine, CA), and RNA was isolated with a Zymo RNA Miniprep kit with on column DNase treatment (Irvine, CA). RNA quantity was determined spectrophotometrically by 260-to-280 nm ratio. Microarray analysis was performed as previously described (20). Total RNA ($n = 3/\text{condition}$) from the TA was subjected to microarray analysis at the Analytical Genomics Core within the Sanford Burnham Prebys Medical Discovery Institute (Orlando, FL). The quality of total RNA was assessed by the Agilent Bioanalyzer Nano Chip (Agilent Technologies, Santa Clara, CA). Labeled, single-stranded cDNA (ss-cDNA) was generated using 300 ng of total RNA according to the Whole Transcript Sense Target Labeling Assay protocol using Affymetrix's GeneChip WT PLUS Reagent kit (Santa Clara, CA; cat. #902281). Then, 5.5 μg of ss-cDNA was fragmented and labeled using Affymetrix WT Terminal according to the manufacturer's protocol. The labeled ss-cDNA was then hybridized onto GeneChip Mouse Gene 2.0 ST Arrays (Affymetrix, Santa Clara, CA). Arrays were scanned using Affymetrix GeneChip Command Console Software and GeneChip Scanner 3000 7G. All procedures were conducted according to the manufacturer's instructions.

Microarray data analysis was performed at the Bioinformatics Core at the Sanford Burnham Prebys Medical Discovery Institute (La Jolla, CA) as previously described (20). Raw data files from Mouse Gene ST Arrays were imported into Partek's Genomic Software for microarray expression analysis (Partek, Chesterfield, MO). The robust microarray average method was used in Partek for data processing (5, 23, 24, 59). ANOVA analysis was used to define differentially expressed genes (DEGs) using a flexible P value (fold change ≥ 1.5 and P value without false discovery rate < 0.05). Only those genes with a RefSeq were included in the subsequent analysis. The list of DEGs was analyzed for functional gene categories using DAVID analysis software (freely accessible at <https://david.abcc.ncifcrf.gov>). Gene expression data have been made available at the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo>) for this study (GSE125233).

cDNA synthesis and quantitative RT-PCR. cDNA was synthesized using a Superscript VILO cDNA synthesis kit (Invitrogen) or a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA) from 1–2 μg RNA. Quantitative (q)RT-PCR was performed using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) on a QuantStudio5 (Thermo Fisher Scientific) or a Bio-Rad CFX Connect (Hercules, CA) qRT-PCR thermal cycler. The conditions for the qRT-PCR included an initial 2 min at 50°C and 2 min at 95°C , followed by 40 cycles which included a 15 s denature step at 95°C , a 15 s annealing step at 55°C , and a 1 min extension step at 72°C within each cycle. A melt curve analysis was performed for each primer pair to ensure that a single product was efficiently amplified. Relative expression levels of each gene were normalized by using the delta delta Ct method with GAPDH as the control gene (19, 52). Primer sequences are shown in Table 1.

Western blot analysis. Western blotting was conducted as previously described (52). Whole muscle protein was extracted from the TA by glass on glass homogenization in 10 volumes of buffer (10 $\mu\text{l}/\text{mg}$) consisting of 50 mM HEPES (pH 7.4), 0.1% Triton-X 100, 4 mM EGTA, 10 mM EDTA, 15 mM $\text{Na}_4\text{P}_2\text{O}_7$, 100 mM β -glycerophosphate, 25 mM NaF, 5 mM Na_3VO_4 , and 10 $\mu\text{l}/\text{ml}$ protease inhibitor cocktail (#P8340; Sigma Aldrich, St. Louis, MO). The

Table 1. Primer sequences for RT-PCR using SYBR Green

Gene Symbol	Forward (5'-3')	Reverse (5'-3')	Amplicon Size, bp
Arrdc1	TCGGGAGTCTCAAACAATAGGTG	GGTCAAAGGCAAGCATGAAG	161
Arrdc2	CAATGACTATGTGAGCAATCAGACC	ACCTTCACAGATCTCTTCTCATCC	183
Arrdc3	GACTTGCTTCTGATCGTGAC	GAAAGCTGGGCAGAGAGATG	167
Arrdc4	AAGACGGATTGCCCTCATTT	AGTGCTAAGACCGCTGCATT	191
Arrdc6 (a.k.a. TXNIP)	TGGTGCTGACGAAGATGAAG	ATGATCCGAGCAGCGTTTAG	154
GAPDH	GTTGTCTCTCGGACTTCA	TGCTGTAGCCGTATTCATTG	124

Table 2. Functional gene categories altered by nutrient consumption

Functional Category	Genes, <i>n</i>	<i>P</i> Value
G protein-coupled receptor	19	0.00000072
Transducer	19	0.0000012
Olfaction	14	0.000014
Receptor	20	0.000056
Sensory transduction	14	0.000057
Cell membrane	22	0.00086
Transmembrane helix	27	0.047
Transmembrane	27	0.048
Monooxygenase	3	0.053
Heme	3	0.082

muscle extract was centrifuged at 10,000 *g* for 10 min, and the protein content of the supernatant fraction was quantified by the Bradford method. Proteins in the supernatant were fractionated on 4–20% Bio-Rad (Hercules, CA) Criterion precast gels and transferred to PVDF membranes. Membranes were stained with Ponceau-S to confirm effective transfer and equal protein loading. Membranes were incubated with antibodies overnight at 4°C. Antibodies against phospho-70 kDa ribosomal protein S6 kinase 1 (p70S6K1) (Thr389) (cat. #9205), phospho-eukaryotic initiation factor 4E binding protein (4E-BP1) (Ser65) (cat. #9451), and microtubule-associated protein 1B light chain (LC3B) (cat. #2775) were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against total p70S6K1 and

total 4E-BP1 were custom made by Bethyl Laboratories (Montgomery, TX) and kindly provided by Dr. Scot Kimball (Pennsylvania State University College of Medicine, Hershey, PA). Antibodies against regulated in development and DNA damage 1 (REDD1, cat. #10638-1-AP) were obtained from ProteinTech (Chicago, IL). Following incubation with secondary antibodies (cat. #A120-101P; Bethyl Laboratories, Montgomery, TX), the antigen-antibody complex was visualized by enhanced chemiluminescence using Clarity Reagent (Bio-Rad, Hercules, CA) and a ChemoDoc Touch imaging system (Bio-Rad). The pixel density from all blots were quantified using Image J software (National Institutes of Health, Bethesda, MD). All antibodies used herein have been previously validated in experiments that included nutrient stimulation (i.e., mTORC1 activation and LC3B gel migration) or comparing wild-type cells to knockout cells (i.e., REDD1+/+ vs. REDD1−/−) (18, 19, 21).

Statistical analysis. Individual data points for each experiment are presented with error bars illustrating the means \pm SE. Analysis of the microarray was described above. The following methods were used to analyze nonarray data. Student's *t*-test was used to compare the means of two variables. Two-way ANOVA was used to evaluate the means of the groups when two factors were present. Fisher's least significant difference was used post hoc to determine specific differences between groups if an interaction was observed. Analysis of select relationships was performed using Pearson product moment correlation. All analysis was performed using GraphPad Prism Software (La Jolla, CA). Significance was set at $P \leq 0.05$ for all analyses.

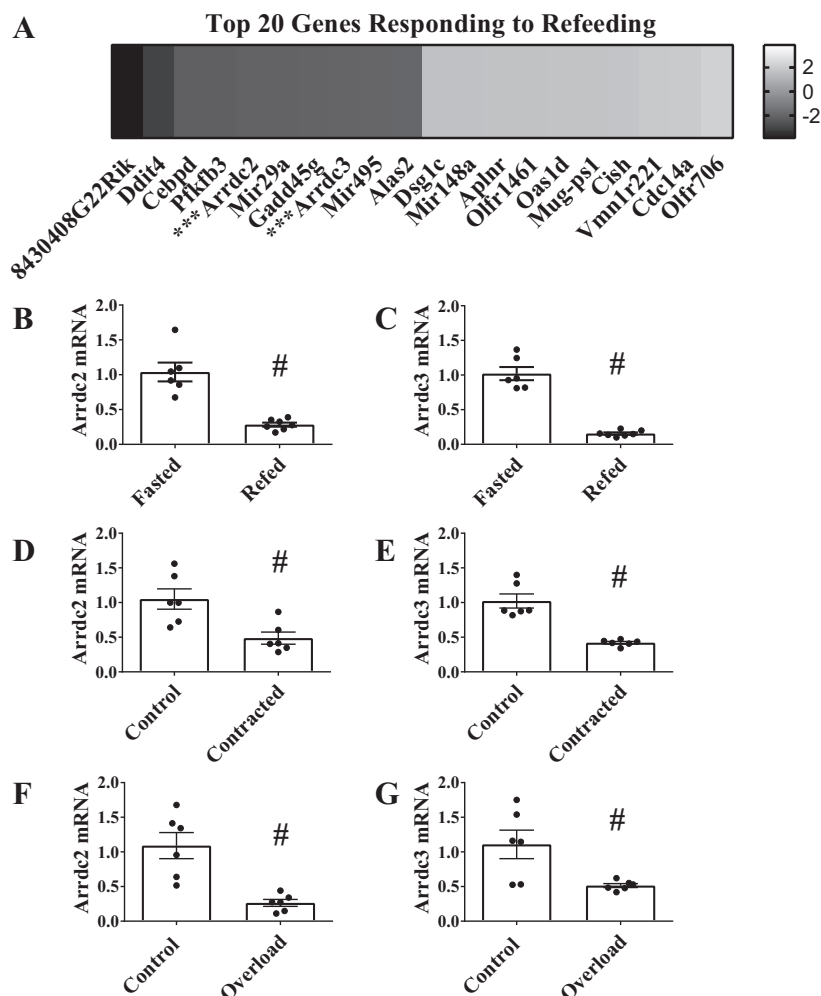


Fig. 1. Arrdc2 and Arrdc3 expression following nutrient consumption and mechanical overload. A: heat map illustrating the top 20 genes whose expression in skeletal muscle was altered by refeeding. B, C: male mice were fasted overnight. The next morning, mice were randomized to remain fasted or refeed. Total RNA from the tibialis anterior (TA) muscle was subjected to microarray analysis. The mRNA expression of Arrdc2 or Arrdc3 in the TA was determined by quantitative (q)RT-PCR. D, E: male mice were fasted overnight. The next morning, mice were subjected to a bout of unilateral, high force muscle contractions. The mRNA expression of Arrdc2 or Arrdc3 in the TA was determined by qRT-PCR. F, G: the plantaris muscle of male mice were subjected to 3 days of mechanical overload via synergistic ablation. The mRNA expression of Arrdc2 or Arrdc3 in the plantaris was determined by qRT-PCR. # Significant difference compared with control group. $n = 6-7$ /group. Significance at $P < 0.05$.

RESULTS

Nutrient consumption altered the expression of genes associated with cell signal transduction, specifically, *Arrdc2* and *Arrdc3*. Nutrient consumption imparts an anabolic stimulus in the skeletal muscle (12, 26). To identify novel gene networks altered in the skeletal muscle by this anabolic stimulus, we subjected total RNA from the TA muscle of mice in the fasted or refed metabolic state to microarray analysis. The validity of the microarray analysis was confirmed visually by observing changes in the expression of genes known to be altered by refeeding, which included a reduction in *Ddit4* expression (a.k.a. REDD1) (21). Upon validation, DAVID analysis showed enrichment of various genes involved with signal transduction (Table 2). We then compared the genes from the identified functional categories with the top 10 genes whose expression was increased and the top 10 genes whose expression was reduced following the refeeding stimulus (Fig. 1A), which identified the tumor suppressor gene *Arrdc3* (10, 47, 60), as a gene whose expression was significantly lower in the muscle following nutrient consumption. Further investigation found another member of the alpha arrestin family of genes, *Arrdc2*, was also among those genes whose expression was lower in the muscle following refeeding. qRT-PCR validation confirmed the microarray (Fig. 1, B and C). Attempts at measuring *Arrdc2* and *Arrdc3* protein content were unsuccessful

due to the lack of a suitable antibody based upon positive control experiments.

Expression of *Arrdc2* and *Arrdc3* is lower in the skeletal muscle by mechanical overload. Mechanical overload exerts an anabolic stimulus on the skeletal muscle that is independent of nutrients (18, 40). To determine whether the changes in *Arrdc2* and *Arrdc3* expression in the muscle were also lower following mechanical overload, mice were fasted overnight before the TA muscle was subjected to a single bout of unilateral, high force eccentric contractions. Compared with the noncontracted control muscle, the contracted muscle had significantly lower levels of *Arrdc2* and *Arrdc3* mRNA (Fig. 1, D and E). To determine whether this was exclusive to the TA muscle or the model of overload, we measured *Arrdc2* and *Arrdc3* expression in the plantaris of mice subjected to 3 days of mechanical overload via synergistic ablation. Consistent with the previous observation, expression of *Arrdc2* and *Arrdc3* was lower in the plantaris muscle following this alternative model of mechanical overload (Fig. 1, F and G).

Combination of nutrient consumption and mechanical overload acts synergistically to lower *Arrdc2* and *Arrdc3* expression. Nutrients and mechanical load activate growth pathways in a synergistic manner (19, 40). Because both nutrient consumption and mechanical overload independently lowered expression of *Arrdc2* and *Arrdc3*, we sought to deter-

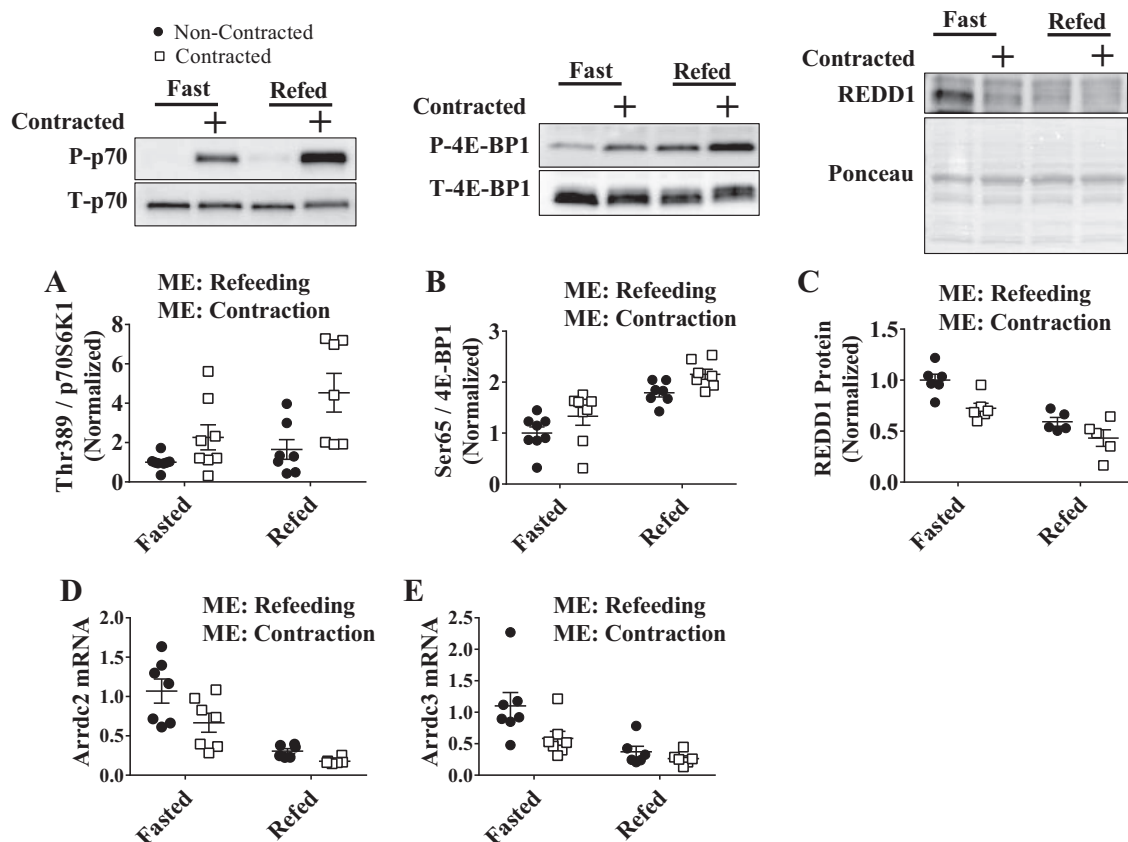


Fig. 2. *Arrdc2* and *Arrdc3* expression following the combination of nutrient consumption and mechanical overload. Male mice were fasted overnight. The next morning, mice were randomized to remain fasted or were refed for 30 min. All mice were subjected to a bout of unilateral, high-force muscle contractions. The ratio of phosphorylated to total protein for p70S6K1 (Thr389) (A) and 4E-BP1 (Ser65) (B) in the TA was determined by Western blot analysis. C: the protein content of REDD1 in the TA was determined by Western blot analysis. The mRNA expression of *Arrdc2* (D) or *Arrdc3* (E) in the TA muscle was determined by qRT-PCR. ME, main Effect. $n = 6-8/\text{group}$. Significance at $P < 0.05$.

mine whether their combination had a synergistic effect. For this, mice were initially fasted overnight. The next morning, half of the mice were randomized to remain fasted while the other half were allowed to refeed for 30 min. All mice were then subjected to a bout of unilateral high-force eccentric contractions. The efficacy of fasting/refeeding and muscle contractions was confirmed by significant main effects of refeeding or contractions to activate mTORC1 [increased phosphorylation of p70S6K1 (Thr389) and 4E-BP1 (Ser65)], and significant main effects of refeeding or contractions to lower expression of regulated in development and DNA damage 1 (REDD1), a nutrient and mechanically sensitive upstream regulator of mTORC1 (Fig. 2, A–C) (8, 19). While refeeding or mechanical overload was each sufficient to lower Arrdc2 and Arrdc3 expression, the combination of the two anabolic stimuli had a synergistic effect (Fig. 2, D and E). Thus, nutrients and mechanical load act synergistically to lower expression of Arrdc2 and Arrdc3.

Lower Arrdc2 and Arrdc3 expression following mechanical overload is independent of mTORC1 activation. Activation of mTORC1 is one of the most important regulators of muscle growth following mechanical overload (16, 32). As such, we sought to determine if activation of this pathway was contributing to the lower expression of Arrdc2 and Arrdc3 following

mechanical overload. For this, mice were administered rapamycin 1 h before a single bout of unilateral high force muscle contractions. Though rapamycin effectively blocked the contraction-mediated activation of mTORC1 as assessed by phosphorylation of p70S6K1 (Thr389) and 4E-BP1 (Ser65) (Fig. 3, A and B), expression of the upstream regulator, REDD1, was lower in the muscle following contractions independent of rapamycin (Fig. 3C). Similar to REDD1, expression of Arrdc2 and Arrdc3 was lower in the muscle following the mechanical stimulus independent of rapamycin (Fig. 3, D and E). Thus, the lower expression of Arrdc2 and Arrdc3 following mechanical overload occurs upstream of, or in parallel with, mTORC1.

Changes in Arrdc2 and Arrdc3 expression following nutrient and/or mechanical load are related to changes in the LC3 II/I ratio marker of autophagy activation. Nutrients and mechanical load shift muscle protein balance toward net protein accretion by increasing muscle protein synthesis and decreasing muscle protein breakdown (40, 57). Autophagy is a degradative pathway consisting of the nonselective, bulk removal of muscle proteins through lysosomal proteases (45, 50). Previous work has shown that Arrdc3 is localized to the lysosome (33), and alpha arrestins (including Arrdc3) have been implicated in lysosomal trafficking (1, 9), suggesting a possible link between changes in the expression of Arrdc3 (and Arrdc2) with changes

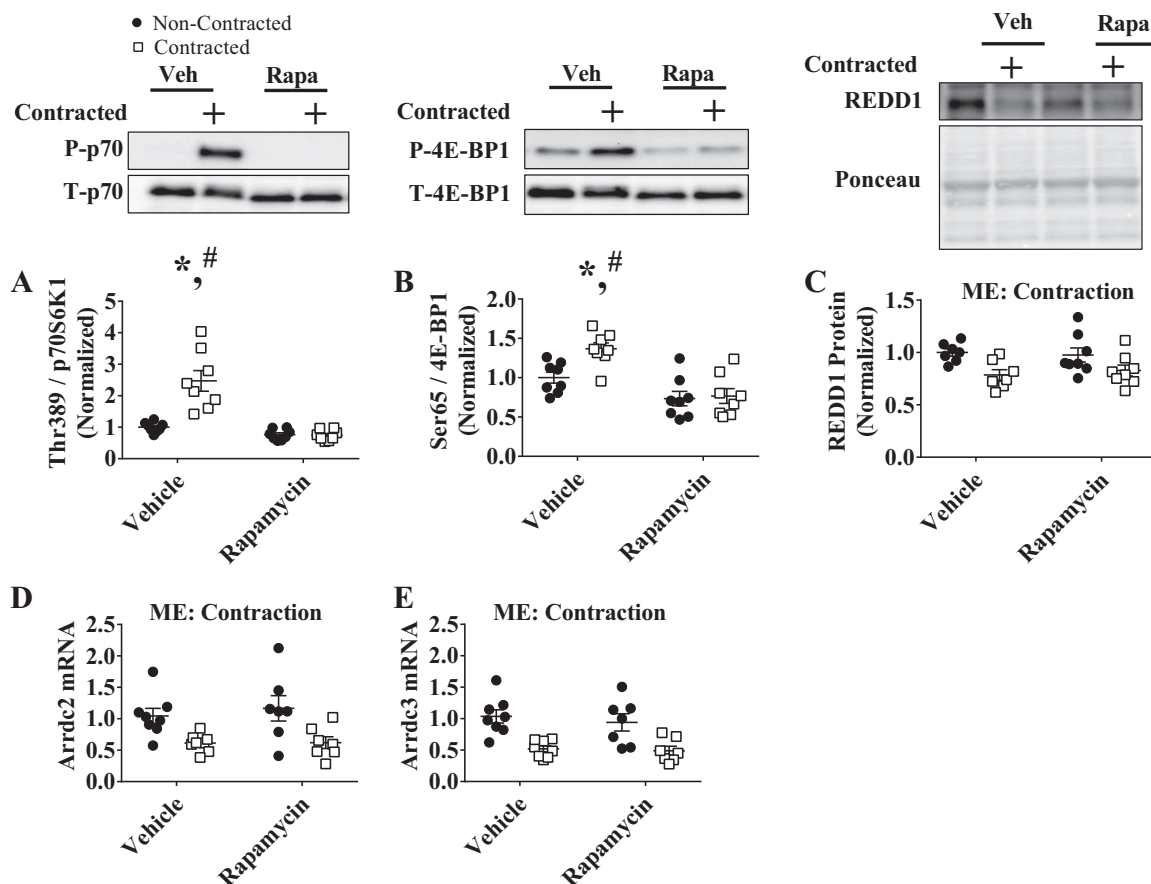


Fig. 3. Arrdc2 and Arrdc3 expression following the mechanical overload in the presence of rapamycin. Male mice were fasted overnight. The next morning, mice were randomized to receive an intraperitoneal injection of vehicle (Veh) or rapamycin (Rapa) before a bout of unilateral, high-force muscle contractions. The ratio of phosphorylated to total protein for p70S6K1 (Thr389) (A) and 4E-BP1 (Ser65) (B) in the TA was determined by Western blot analysis. C: the protein content of REDD1 in that TA was determined by Western blot analysis. The mRNA expression of Arrdc2 (D) or Arrdc3 (E) in the TA muscle was determined by qRT-PCR. *Significant difference compared with noncontracted muscle. #Significant difference compared with contracted muscle of rapamycin-treated mice. ME, main effect. $n = 7-8/\text{group}$. Significance at $P < 0.05$.

in autophagy activation. Autophagy activation can be monitored by following changes in the ratio of the lipidated (II) to the nonlipidated (I) form of LC3 via Western blot, with an increase/decrease in this ratio indicating autophagy activation/suppression, respectively (25). Interestingly, direct relationships were observed between Arrdc2 or Arrdc3 expression and the LC3 II/I ratio in the muscles from mice subjected to fasting/refeeding and muscle contractions (Fig. 4, A–C). Of note, exclusion of the potential outlier in Fig. 4C did not substantially alter the r value (0.49–0.46), the P value, or the overall conclusion. Though p62 is a complementary marker of autophagy activation, acute high force contractions did not alter this autophagy marker (52).

Though mTORC1 signaling contributes to the regulation of autophagy activation (28), mTORC1-independent regulators have also been described (39). Consistent with the latter, rapamycin treatment did not prevent the contraction-mediated decrease in the LC3 II/I ratio (Fig. 4D). Interestingly, the expression of Arrdc2 and Arrdc3 was still related to the LC3 II/I ratio at this postcontraction time point (Fig. 4, E and F). Attempts to measure other markers of protein breakdown (i.e., ubiquitin content and LC3B mRNA) failed to show consistent changes and, therefore, were not related to changes in Arrdc2 or Arrdc3 expression (data not shown). Thus, these data show that changes in the expression of Arrdc2 and Arrdc3 following anabolic stimuli are related to changes in the LC3 II/I ratio marker of autophagy activation.

Expression of Arrdc2 and Arrdc3 is higher in skeletal muscle following catabolic stimuli other than fasting. Previous work showed that Arrdc3 expression was higher in the skeletal muscle of mice following a 14 h fast when compared with

values in mice that were freely fed (35). Additionally, previous work showed that Arrdc2 expression was higher in the atrophied muscle of mice treated with dexamethasone (14), suggesting that the expression of these genes is not only lower in the muscle following anabolic stimuli, but that levels are higher in the muscle following catabolic stimuli. As such, we sought to determine whether expression of Arrdc2 and Arrdc3 were enhanced in the muscle by growth suppressive stimuli other than fasting and dexamethasone. In males, a decrease in the production or bioavailability of androgens (i.e., testosterone) leads to muscle atrophy (12, 52, 55). As such, we tested whether Arrdc2 and Arrdc3 expression was higher in the muscle following testosterone depletion induced by castration. The expression of both Arrdc2 and Arrdc3 was elevated in the atrophied TA of castrated mice compared with the values in the TA of mice with intact testosterone production (Fig. 5, A and B). Our group has previously shown that the LC3 II/I ratio was higher in those TA muscles of castrated mice in which Arrdc2 and Arrdc3 expression was also elevated (52). Since changes in Arrdc2 and Arrdc3 were related to changes in the LC3 II/I ratio following anabolic stimuli, we sought to determine whether these relationships were maintained following castration. Indeed, direct relationships were still observed between Arrdc2 or Arrdc3 and the LC3 II/I ratio (Fig. 5, C and D).

Though long-term aerobic exercise training is beneficial for overall muscle health (29, 44), the metabolic stress imposed on the muscle by an acute bout of exercise imparts a temporary suppression on growth processes and a temporary induction of catabolic processes. For instance expression of REDD1 and activity of 5' AMP-activated protein kinase are temporarily increased, while signaling through mTORC1 is temporarily

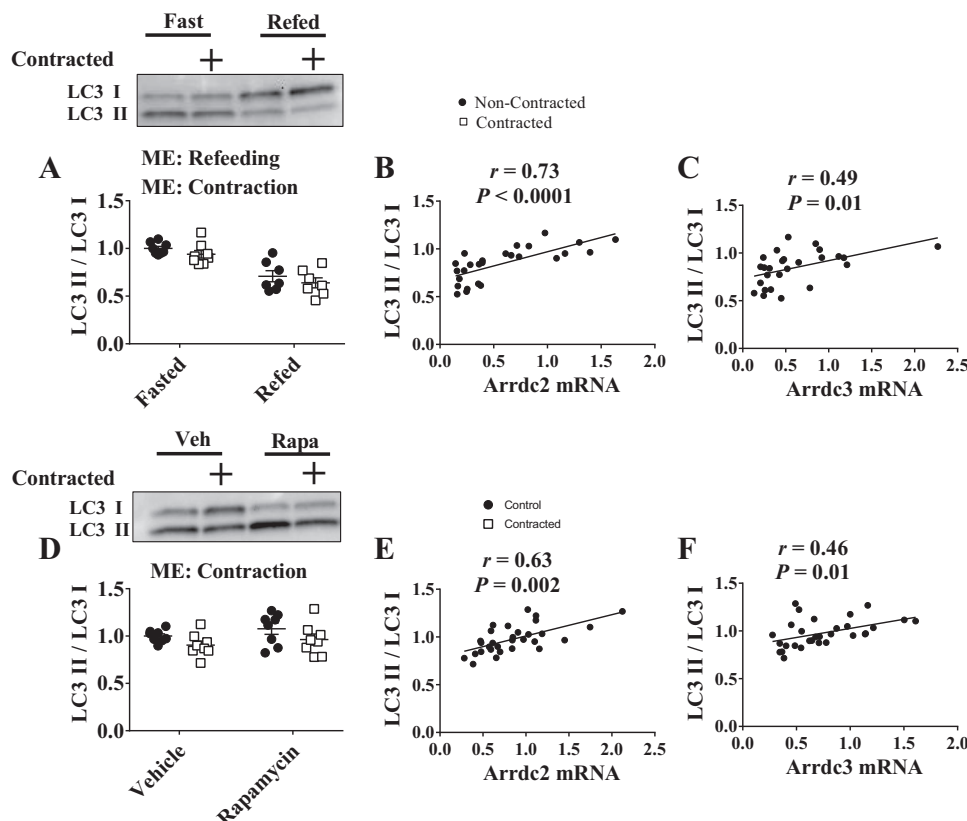


Fig. 4. Relationships between expression of Arrdc2 or Arrdc3 with the LC3 II/I marker of autophagy. The ratio of LC3 II to LC3 I in the TA muscle of mice subjected to fasting/refeeding and unilateral muscle contractions (A) or vehicle/rapamycin treatment (D) before unilateral muscle contractions was determined by Western blot analysis. B, C and E, F: select relationships between Arrdc2 or Arrdc3 and the LC3 II/I ratio were determined by Pearson product moment correlation. ME, main effect. $n = 6-8$ /group. Significance at $P < 0.05$.

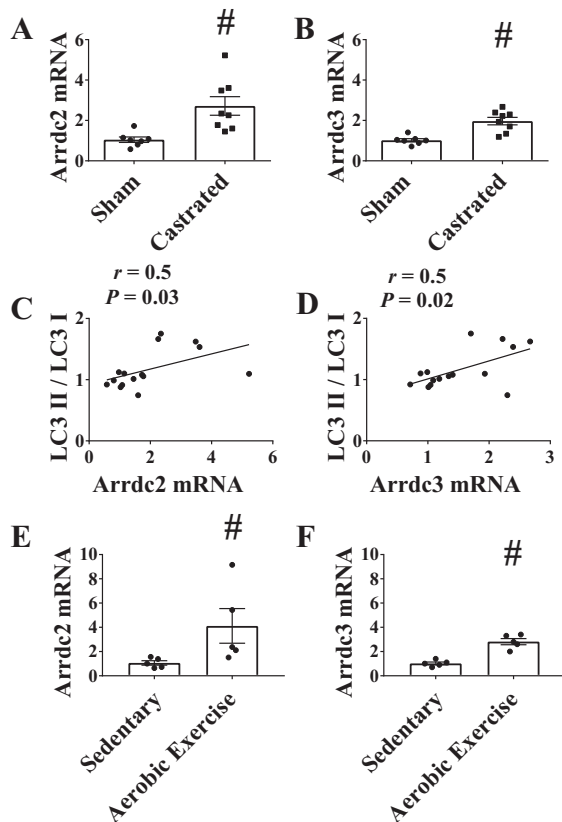


Fig. 5. Arrdc2 and Arrdc3 expression following catabolic stimuli. A, B: male mice were subjected to a sham or castration surgery and allowed to recover for 8 wk. The mRNA expression of Arrdc2 or Arrdc3 was determined in the TA by qRT-PCR. C, D: the relationship between Arrdc2 or Arrdc3 with the LC3 II/I ratio in the TA muscle of sham and castrated mice was determined by Pearson product moment correlation. E, F: male mice were randomized to remain sedentary or undergo an acute bout of aerobic exercise. Mice were euthanized 1 h postexercise. The mRNA expression of Arrdc2 or Arrdc3 in the plantaris of exercised and sedentary mice was determined by qRT-PCR. #Significant difference compared with corresponding control group. $n = 5-8/\text{group}$. Significance at $P < 0.05$.

decreased, by the metabolic stress imposed by an acute bout of aerobic exercise (11, 20, 36, 56). However, these perturbations are highly transient as they return to sedentary values rather quickly following cessation of the exercise stress, which is why long-term aerobic exercise training does not induce muscle atrophy. Accordingly, the metabolic stress imposed by an acute bout of aerobic exercise resulted in higher expression levels of both Arrdc2 and Arrdc3 in the plantaris muscle relative to the nonexercised values (Fig. 5, E and F). Arrdc3 levels were no longer higher in the muscle 3 h following cessation of the exercise stimulus, illustrating the highly transient nature of this induction (data not shown). A lack of cDNA precluded measurement of Arrdc2 at this later postexercise time point. Collectively, these data show that Arrdc2 and Arrdc3 expression is higher in the muscle following growth suppressive stimuli.

Other members of the Arrdc family of genes do not exhibit consistent changes in expression following anabolic and catabolic stimuli. There are six known members of the Arrdc family of genes termed Arrdc1–6 (Arrdc6 a.k.a. thioredoxin interacting protein; TXNIP) (38). Following the observed changes in Arrdc2 and Arrdc3, we sought to determine whether

other members of this gene family show similar and/or consistent changes in expression following the various stimuli. Interestingly, no consistent changes in expression of the other members of the Arrdc family of genes were observed across the analyzed conditions (Table 3). Of note, even though all members of Arrdc family of genes were lowered by refeeding, Arrdc2 and Arrdc3 expression was substantially lower than that which was observed by all other members. Furthermore, no Arrdc5 was detected in our muscle samples. These data show that anabolic/catabolic stimuli selectively and consistently alter expression of Arrdc2 and Arrdc3 in skeletal muscle.

DISCUSSION

Anabolic and catabolic stimuli alter various molecular networks to induce changes in muscle mass. Using a hypothesis-generating experimental approach similar to previous work (7), we show herein that genes involved in the regulation of cell signaling/signal transduction were among those sensitive to anabolic and catabolic stimuli. Of those genes identified, we found that anabolic and catabolic stimuli induced consistent changes in the expression of the tumor suppressor gene, Arrdc3. Follow-up analysis showed that Arrdc2 exhibited similar changes in expression following such stimuli, but interestingly, other members of the Arrdc family of genes did not. Moreover, the changes in Arrdc2 and Arrdc3 were consistently related to the LC3 II/I ratio marker of autophagy, suggesting a possible link between changes in these genes and the activity of this degradative process. In all, these data support a potential role for Arrdc2 and/or Arrdc3 in the regulation of muscle mass.

Initial reports described Arrdc3 as a growth regulatory gene. For example, Arrdc3 mRNA expression was significantly lower in cancerous breast tissue compared with noncancerous breast tissue (10). Furthermore, manipulation of the expression of Arrdc3 was sufficient to regulate growth as overexpression of Arrdc3 suppressed, while knockdown enhanced, cell growth (10, 33, 47, 60). As such, the presently reported changes in Arrdc3 expression following anabolic or catabolic stimuli are consistent with such a growth regulatory function. Though the contribution of Arrdc3 to muscle growth/atrophy remains unknown, previous evidence showed that Arrdc3 regulates signaling pathways that are known to alter muscle mass. For instance, beta adrenergic receptor agonists (e.g., clenbuterol) induce skeletal muscle growth (30), and Arrdc3 has been shown to suppress beta-adrenergic receptor stimulation by facilitating beta receptor degradation (35). Though the role of the beta-adrenergic receptor in regulating skeletal muscle mass under nonpharmacologically stimulated conditions is ill defined, previous work suggests this receptor functions to regulate muscle mass through changes in protein breakdown. For

Table 3. Change in expression of Arrdc1, Arrdc4, and Arrdc6 (TXNIP) following the various anabolic and catabolic stimuli

Stimulus	Arrdc1	Arrdc4	Arrdc6 (TXNIP)
Refeeding	↓	↓	↓
High-force contractions	↔	↑	↑
Synergistic ablation	↑	↑	↓
Castration	↔	↔	↔
Acute aerobic exercise	↔	↔	↔

example, acute epinephrine infusion into humans reduced skeletal muscle protein breakdown (13), while epinephrine depletion in rodents increased muscle protein breakdown, including autophagy (22). As such, a decrease in Arrdc3 following an anabolic stimulus may contribute to such a change in muscle protein balance by allowing endogenous levels of epinephrine to impart a greater stimulus on the beta-adrenergic receptor. Such a hypothesis would also be consistent with the relationships observed between changes in Arrdc3 expression and the corresponding changes in the LC3 II/I marker of autophagy-mediated protein breakdown, though a direct proof of concept is still needed. This hypothesis is further supported by the observation that mice lacking the Arrdc3 gene exhibited enhanced sensitivity of the beta-adrenergic receptor in adipose tissue to epinephrine stimulation (35). Alternatively, Arrdc3 was shown to limit renal cell growth by suppressing the HIPPO signaling pathway via increased degradation of the yes associated protein (YAP) transcription factor (60). YAP expression is increased in the skeletal muscle by mechanical overload, and overexpression of YAP was sufficient to induce muscle fiber growth in an mTORC1-independent manner (15). Thus, the decrease in Arrdc3 expression following anabolic stimuli may also contribute to mTORC1-independent growth by stabilizing the YAP protein. Overall, the observed changes in the expression of Arrdc3 are consistent with a growth regulatory function of this gene in skeletal muscle.

Despite a paucity of information regarding the function of Arrdc2, previous work also supports a possible growth regulatory role for Arrdc2 in skeletal muscle. Arrdc2 was one of the most highly upregulated genes in the atrophied skeletal muscle following 3 or 14 days of dexamethasone administration (14), which is a synthetic corticosteroid hormone that induces atrophy (6). Presently, the data herein show concordant changes in Arrdc2 expression following the growth/atrophy stimuli, supporting a growth regulatory role for Arrdc2. On the contrary, it is possible that changes in Arrdc2 expression simply represent alterations in glucocorticoid receptor activation rather than having a direct role in growth regulation. For instance, changes in glucocorticoid levels/receptor activation underlie, or have been proposed to occur with, each of the conditions analyzed herein (i.e., nutrient consumption, mechanical overload, aerobic exercise, testosterone depletion) (20, 27, 37, 46, 58). Thus, the change in Arrdc2 expression may simply provide a readout of changes in glucocorticoid receptor activation without actually contributing to growth regulation. Additional work is needed to define the role of Arrdc2 in skeletal muscle, particularly following anabolic/catabolic stimuli.

Although Arrdc2 and Arrdc3 were the only members of the Arrdc family of genes to exhibit consistent changes in expression following anabolic/catabolic stimuli, it is possible that other members of this gene family also regulate changes in muscle metabolism. For instance, expression of Arrdc4 and Arrdc6 (a.k.a. TXNIP) has been shown to inhibit glucose uptake (34). Thus, the decrease in expression of both genes following the refeeding stimulus (i.e., Table 3) would be consistent with enhanced glucose uptake following nutrient consumption. Although that is possible, Arrdc4 and TXNIP expression was increased by mechanical overload even though glucose uptake is enhanced by this stimulus (31). While perplexing, this suggests the possibility that regulation of glucose

uptake by Arrdc4 and/or TXNIP is either time and/or context dependent.

In conclusion, we identify Arrdc2 and Arrdc3 as novel genes exhibiting a consistent change in expression following a variety of anabolic and catabolic stimuli. Though little is known of Arrdc2 function, the previously described role of Arrdc3 as a tumor suppressor implies a potentially novel mode of regulating muscle growth through various signaling pathways including the beta-adrenergic receptor and HIPPO signaling. Additional mechanistic work to understand whether these genes contribute to changes in muscle size will advance our overall knowledge of the networks underlying skeletal muscle plasticity, which is required to generate therapies to counteract atrophy during diseased states.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

B.S.G. conceived and designed research; B.S.G. and M.L.R. performed experiments; B.S.G., M.L.R., and A.M.E. analyzed data; B.S.G. interpreted results of experiments; B.S.G. prepared figures; B.S.G. drafted manuscript; B.S.G. and M.L.R. edited and revised manuscript; B.S.G., M.L.R., and A.M.E. approved final version of manuscript.

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