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Acute Resistance Exercise Induces Sestrin2 Phosphorylation and p62 Dephosphorylation in Human Skeletal Muscle

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New findings

What is the central question of this study?

- Sestrin and p62 are stress inducible proteins involved in many cellular processes, including suppressing oxidative stress and regulating autophagy. It is unclear how acute and chronic resistance exercise affects these proteins in human skeletal muscle.

What is the main finding and its importance?

- This study shows that 2 h post-exercise, phosphorylation of p62^{Ser403} was downregulated, while there was a mobility shift of Sestrin2, indicative of increased phosphorylation. Both Sestrin2 and p62^{Ser403} are transiently regulated, and may be functionally involved in the adaptive regulatory mechanisms elicited by intense resistance exercise in human skeletal muscle.

Abstract

Sestrins (1, 2, 3) are a family of stress-inducible proteins capable of attenuating oxidative stress, regulating metabolism and stimulating autophagy. Sequestosome1 (p62), is also a stress-inducible multifunctional protein acting as a signalling hub for oxidative stress and selective autophagy. It is unclear whether Sestrin and p62^{Ser403} are regulated acutely or chronically by resistance exercise or training in human skeletal muscle. Therefore, the acute and chronic effects of resistance exercise on Sestrin and p62 in human skeletal muscle were examined through two studies. In Study 1, nine active men (22.1 ± 2.2 years) performed a bout of single-leg strength exercises and muscle biopsies were collected before, 2, 24 and 48 h after exercise. In Study 2, ten active men (21.3 ± 1.9 years) strength trained for 12 weeks (2 days per week) and biopsies were collected pre and post training. Acutely, 2 h post-exercise, phosphorylation of p62^{Ser403} was downregulated, while there was a mobility shift of Sestrin2, indicative of increased phosphorylation. 48 h post-exercise, the protein expression of both Sestrin1 and total p62 increased. Chronic exercise had no impact on the gene or protein expression of Sestrin2/3 or p62, but Sestrin1 protein was upregulated. These findings demonstrated an inverse relationship between Sestrin2 and p62 phosphorylation after a single bout of resistance exercise, indicating they are transiently regulated. Contrarily, 12 weeks of resistance training increased protein expression of Sestrin1, suggesting that despite the strong sequence homology of the Sestrin family, they are differentially regulated in response to acute resistance exercise and chronic resistance training.

Introduction

Physical activity, particularly resistance exercise (RE), is an intense muscle stressor that stimulates adaptive regulation of numerous nutrient and antioxidant-sensitive pathways (Egan & Zierath, 2013). Although mechanisms regulating muscle protein synthesis have been well studied (Dreyer *et al.*, 2006), the complex signalling pathways regulating autophagy in response to RE (Fry *et al.*, 2012) and RE-induced oxidative stress (Çakır-Atabek *et al.*, 2015) remains elusive.

Sestrins are a family of stress-inducible proteins that have multi-functional roles including attenuating oxidative stress, regulating mammalian target of rapamycin complex 1 (mTORC1) and stimulating autophagy (Lee *et al.*, 2013). Mammals have three Sestrin genes *(SESN1/2/3)* that are regulated differently. Whereas Sestrin1 and 2 are regulated by p53, Sestrin3 is regulated by forkhead box O (FOXO) (Parmigiani & Budanov, 2016). Sestrin2

shows intrinsic oxidoreductase activity (Budanov *et al.*, 2004); however, it was later shown that this is not required for its antioxidant functioning (Woo *et al.*, 2009). In circumstances of increased oxidative stress, Sestrin1 and 2 prevent reactive oxygen species (ROS) accumulation by inducing selective autophagic degradation of Kelch-like ECH-associated protein (Keap1), an inhibitor of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2), thereby upregulating Nrf2-dependent antioxidant gene transcription (Bae *et al.*, 2013). Sestrin2 has also been proposed as a leucine sensor (Parmigiani *et al.*, 2014; Wolfson *et al.*, 2016) and *in vitro* analysis has identified it as a phosphoprotein, which in response to leucine deprivation, is phosphorylated and interacts with GTPase-activating protein activity towards Rags 2 (GATOR2) to inhibit mTORC1 activation (Kim *et al.*, 2015; Kimball *et al.*, 2016). Sestrin3, however, is upregulated in the skeletal muscle of type 2 diabetic patients (Nascimento *et al.*, 2013) and maintains insulin sensitivity in overfed mice via protein kinase B (Tao *et al.*, 2015).

p62 is a stress-inducible protein involved in oxidative stress and autophagic clearance of polyubiquitinated proteins (Katsuragi *et al.*, 2015). Autophagy is an evolutionarily conserved process that recycles protein aggregates and malfunctioning organelles. Macroautophagy, microautophagy and chaperone-mediated autophagy are the three main forms of autophagy (Tanida, 2011). p62 plays an important role in selective macroautophagic protein degradation. It binds to ubiquitinated proteins and microtubule-associated protein 1 light chain 3 (LC3), allowing it to recruit these proteins to autophagosomes, which fuse with lysosomes for protein degradation (Lamark *et al.*, 2009). It has been shown that phosphorylation of p62 at Serine 403 (Ser403) plays a critical role in selective macroautophagy, because phosphorylating $p62^{Ser403}$ stabilises the association between p62 and ubiquitinated protein, which enables efficient autophagosome formation (Matsumoto *et al.*, 2011).

Included in the complex functionality of Sestrin2 is its interaction with p62 (Ro *et al.*, 2014). *In vitro*, Sestrin2 associates with p62 and Unc-51-like protein kinase 1 (ULK1), forming a complex that induces ULK1 to phosphorylate $p62^{Ser403}$ (Ro *et al.*, 2014). Phosphorylated $p62^{Ser403}$ enhances its binding affinity to Keap1 (Matsumoto *et al.*, 2011), thereby initiating autophagosome formation around the cargos, which ultimately leads to selective autophagic degradation of Keap1, hence freeing Nrf2 and enabling its translocation to the nucleus to upregulate antioxidant gene expression (Ichimura *et al.*, 2013).

Sestrins are also critical regulators of muscle aging (Budanov *et al.*, 2010). Genetic ablation of *Drosophila* Sestrin (dSesn), induces the early onset of skeletal muscle degeneration and accumulated defective mitochondria (Lee *et al.*, 2010). Resistance training (RT) is one of the most important strategies to prevent muscle wastage (Sanchis-Gomar *et al.*, 2011) however, no studies have assessed the effects of RT on Sestrin in human. To date, there is only evidence of endurance exercise increasing the protein expressions of Sestrin2 and 3 in mouse skeletal muscle which occurs in conjunction with an increase in autophagy (Liu *et al.*, 2015; Lenhare *et al.*, 2017). Whether the three mammalian Sestrin proteins differentially control skeletal muscle function and which plays a more important role on human muscle health is unclear. Similarly, it remains unknown whether Sestrin and p62^{Ser403} are regulated acutely or chronically by RE and RT respectively in human skeletal muscle. Therefore, this study aimed to measure how acute RE affects Sestrin2 and p62^{Ser403} phosphorylation and examined the effects of RE on the protein and mRNA expression of Sestrin paralogs. Separately, the chronic effects of 12 weeks of RT on Sestrin protein and gene expression were also investigated.

Materials and Method

Ethics Approval

All participants were informed of the requirements and potential risks of the studies prior providing their written informed consent. The experimental procedures adhered to the standards set by the latest version of the Declaration of Helsinki and were approved by the Human Research Ethics Committee of The University of Queensland.

Study Design

Subjects in this study were a subset of a larger trial (Roberts *et al.*, 2015). In both studies, all participants had at least 12 months of experience in strength training and were familiar with all exercises used in the studies. In the acute study, nine physically active trained men (22.1 ± 2.2 years old) completed a bout of single-leg strength exercise. 8 repetitions maximum (RM) strength of unilateral knee extension (71 ± 12.0 kg) and unilateral 45° leg press (299 ± 44.8 kg) for both legs was assessed 4-5 days prior to experimental exercise bout. At the same time, familiarisation for the single-leg squats and walking lunge exercise was performed. On the

148 day of the trial, the RE bout included six sets of 45° leg press and knee extensions at
149 8,8,10,12,10 and 10 RM, and three sets of single-leg squats and walking lunges at 12 RM.

150 In the chronic study, ten trained men (21.3 ± 1.9 years old) participated in a 12 week lower
151 body resistance training programme with training twice a week, separated by 72 h. Muscle
152 strength for training load prescription was assessed 10-14 days before the first training
153 session. Bilateral 45° leg press (348 ± 80 kg), knee extension (88 ± 9 kg) and knee flexion
154 (75 ± 11 kg) 1 RMs were determined. For the training session, the loads were set to include
155 fatigue at 8, 10 and 12 RM and weights for walking lunges corresponded to a proportion of
156 each participant's pre-training body mass (PTBM) (79.2 ± 4.4kg). Each training session was
157 approximately 45 min and included six sets of 45° leg press at 8,8,10,12,10,10 RM and three
158 sets of knee extension and flexion at 12 RM. Three sets of walking lunges were also
159 performed with week 1 to 3 having 20% of PTBM, and an additional 5 kg added
160 progressively every 3 weeks. Additionally, three sets of plyometrics exercises comprising of
161 countermovement drop jumps, slow eccentric squat jumps, split lunge jumps and
162 countermovement box jumps were performed at 50% of lunge load. In both studies, after
163 each exercise session, participants completed active recovery by cycling on a stationary
164 bicycle at a low, self-selected intensity for 10 min.

165 To control for post-exercise diet, in the acute study, participants consumed a standardised
166 meal 2 h before the pre-exercise biopsy and consumed 30 g of whey protein before the
167 recovery period. The participants then fasted until the 2 h biopsy, after which they consumed
168 another 30 g of whey protein. Muscles biopsies from the *vastus lateralis* were collected
169 before, 2, 24, and 48 h post-exercise. In the chronic study, biopsies were collected 4-5 days
170 before the first training session and post-training biopsies were collected 6-7 days after the
171 last training session in a fasted-state. All muscle samples were snap frozen in liquid nitrogen
172 and stored at -80°C until further analysis.

173

174 *Western blotting*

175 25 mg of muscle biopsies were homogenised with RIPA lysis buffer (Millipore, Temecula,
176 CA, USA) with added Halt™ protease and phosphatase inhibitor cocktail (Thermo Scientific,
177 MA, USA). After centrifugation, supernatants were collected and total protein concentration
178 was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). Equal
179 amounts of protein were boiled in Laemmli buffer at 95°C for 5 min. 20 μg of protein was

separated by SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories, Inc., CA) using the semidry Trans-Blot Turbo^{TM} device (Bio-Rad). Membranes were incubated with the following primary antibodies, total p62, Sestrin1 and 3 (Abcam, ab56416, ab103121 and ab97792 respectively), Sestrin2 (ProteinTech, 10795-1-AP) and p62^{Ser403} (GeneTex, GTX128171) (all at 1:1,000 dilution, except Sestrin1 which is at 1: 100) overnight and the appropriate anti-rabbit or anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories, PA) linked to horseradish peroxidase (1:10,000) for 1 h at room temperature. The membranes were exposed on a ChemiDoc image device (Bio-Rad) using enhanced chemiluminescence reagent (ECL Select kit; GE Healthcare Ltd., Little Chalfont, UK). Bands were quantified using ImageJ software (NIH, Bethesda, MD). Western blot data was normalised to the housekeeping protein GAPDH (Abcam, ab36840) (1: 10,000).

Sestrin2 Electrophoretic Mobility

To allow for resolution of Sestrin2 into multiple electrophoretic forms as previously demonstrated in (Kimball *et al.*, 2016), samples were electrophoresed through 8% polyacrylamide gels (acrylamide-bis-acrylamide, 19:1). When human embryonic kidney cells (HEK293) were incubated in complete medium, Sestrin2 separated into three bands: α, β and γ. However, when incubated in leucine deficient medium, there was a mobility shift of the protein, resulting in the appearance of a slower migrating δ band (Kimball *et al.*, 2016). To provide evidence that the multiple electrophoretic bands represented different phosphorylated forms of Sestrin2, Kimball et al treated samples with lambda protein phosphatase, which led to a shift in the migration of Sestrin2 into a single band, suggesting additional bands represented multiply phosphorylated forms of the protein. Mass spectrometry analysis of immunoprecipitates of endogenous Sestrin2 further confirmed it as a phosphoprotein as three phosphorylation sites: Thr232, Ser249 and Ser279 were identified (Kimball *et al.*, 2016). In the present study, to measure the intensity of Sestrin2 phosphorylation, the abundance of the slowest migrating δ-form of Sestrin2 was taken as phosphorylation. It is known that phosphorylation results in the protein migrating at a higher, apparent molecular mass (Wegener & Jones, 1984; Peck, 2006). Total Sestrin2 protein was recorded as the expression of all forms of Sestrin2 (δ, γ, β and α-form).

RNA extraction and quantitative real-time PCR

Following the manufacturer's instructions from the AllPrep® DNA/RNA/miRNA Universal Kit (QIAGEN GmbH, Hilden, Germany), total RNA was extracted from 20 mg of muscle biopsies. 1500 ng of input RNA was then used for cDNA synthesis using High-Capacity RNA-to-cDNA™ kit (Life Technologies, Carlsbad, CA). Messenger RNA (mRNA) was measured by RT-PCR on a LightCycler 480 II (Roche Applied Science, Penzberg, Germany) using SYBR Green I Master Mix (Roche Applied Science). Target mRNAs were *SESN1*, *SESN2*, *SESN3* and *p62*. Primers were designed using BLAST software (Ye *et al.*, 2012) with sequences in Table 1. Relative fold changes were determined using the $2^{-\Delta\Delta CT}$ method (Schmittgen & Livak, 2008). To compare the basal expression levels of the different Sestrin paralogs, $2^{-\Delta CT}$ was used. The geometric mean of three reference genes was used for normalisation (Vandesompele *et al.*, 2002). The recently proposed human reference genes (Eisenberg & Levanon, 2013), chromosome 1 open reading frame 43 (*C1orf43*), charged multivesicular body protein 2A (*CHMP2A*) and ER membrane protein complex subunit 7 (*EMC7*) were identified as the least variable and used as reference genes (Table 1).

Statistical analysis

To measure differences across time in the acute study, one way repeated measures ANOVA was performed using SigmaPlot (Systat 218 Software Inc., San Jose). Holm-Sidak post-hocs were used where appropriate to compare post-exercise values to baseline with significance set at $P < 0.05$. For the chronic study, time differences were conducted using a paired Student's *t* test. To compare the basal differences of all three Sestrins, one way ANOVA was used. All values are presented as means ± SEM.

Results

Acute Exercise

Sestrin2 phosphorylation (assessed by the mobility shift of the δ-band) was higher 2 h after exercise ($p<0.001$) (Fig 1A), whereas the phosphorylation of $p62^{Ser403}$ was reduced 2 h after exercise ($P<0.001$) (Fig 1E). No difference was observed in the total protein expression of Sestrin2 (Fig 1B), but its mRNA expression (*SESN2*) increased 2 h post-exercise ($P=0.015$) (Fig 2A). There were no changes in the mRNA expressions of *SESN1*, *SESN3* and *p62* (Fig 2B-D), and protein expression of Sestrin3 (Fig 1D). However, the protein expression of Sestrin1 and total p62 increased 48 h post-exercise ($p=0.025$ and $p=0.031$ respectively) (Fig

1C, F). Basal mRNA expression of *SESN1* was significantly more abundant than *SESN2* ($p<0.001$) or *SESN3* ($p=0.043$). *SESN3* was also more abundant than *SESN2* ($p=0.043$) (Fig 3A).

Chronic Exercise

The phosphorylation states of Sestrin2 and p62 were unchanged following RT (Fig 4A, E). Also no changes in protein and mRNA expressions of Sestrin2, Sestrin3 and total p62 were observed (Fig 4B, D, F). However, Sestrin1 protein was increased with RT ($p=0.026$) (Fig 4C). Similar to the acute study, prior to training, the mRNA expression of *SESN1* was significantly more abundant than *SESN2* ($p<0.001$). *SESN3* was also more abundant than *SESN2* ($P=0.05$) (Fig 3B).

Discussion

The present study confirmed the role of Sestrin2 as a phosphoprotein (Ro *et al.*, 2014; Kimball *et al.*, 2016; Nikonorova *et al.*, 2017) and extended previous findings to show it is responsive to acute RE in human skeletal muscle. There was an electrophoretic mobility shift resulting in increased abundance of a slower migrating δ-band of Sestrin2, indicative of increased phosphorylation acutely following RE. Mirroring the time course of increased Sestrin2 phosphorylation, $p62^{Ser403}$ phosphorylation was transiently downregulated following RE. After 12 weeks of RT, resting total protein abundance and basal phosphorylation of Sestrin2 and p62 were unaltered. However, there was an increased Sestrin1 protein abundance, suggesting that despite the strong sequence homology of the Sestrin family, they are differentially regulated in response to RE and RT.

Effect of Acute Exercise

Following RE, mRNA expression of *SESN2* increased 2 h post-exercise. Exposure of primary human myotubes to reactive oxygen species (H_2O_2) for 6 h also increased mRNA expression of *SESN2* (Nascimento *et al.*, 2013). Although RE is primarily an anabolic stimulus, it has been shown to be a potent inducer of acute oxidative stress (Polotow *et al.*, 2017). Sestrins protect cells from oxidative stress and cellular damage, as their repression upregulated ROS production and induced genetic instability (Kopnin *et al.*, 2007). The upregulation of *SESN2*

post-exercise may be an adaptation to protect skeletal muscle cells from exercise induced-oxidative stress.

There was also an increase in the relative amount of Sestrin2 present in the heavier δ-band at 2 h, returning to pre-exercise levels by 24 h. Sestrin2 has been proposed as a leucine sensor (Parmigiani *et al.*, 2014; Wolfson *et al.*, 2016). In HEK293, increased leucine concentrations in the media resulted in a reduction of the relative amount of the δ-band, which promoted mTORC1 activation, as assessed by an increase in phosphorylation of p70S6K1^{Thr389} (Kimball *et al.*, 2016). In this study, participants consumed 30 g of whey protein before the recovery period. A reduction of the δ-band should be expected, however no inverse relationship between Sestrin2 phosphorylation and mTORC1 activation was observed. Conversely, the intensity of the δ-band increased and p70S6K1^{Thr389} was highly phosphorylated 2 h post-exercise as demonstrated previously (Roberts *et al.*, 2015). The discrepancy could be due to different tissue types and stimuli, as exercise is an intense muscle stressor that affects multiple pathways (Egan & Zierath, 2013). Further, there is limited evidence demonstrating action of leucine on Sestrin function in cells other than HEK293 and mouse fibroblasts (Chantranupong *et al.*, 2014; Parmigiani *et al.*, 2014; Wolfson *et al.*, 2016). Thus, the nature of Sestrin2 leucine sensor properties is still under debate (Lee *et al.*, 2016; Saxton *et al.*, 2016). Therefore, future studies should aim to separate feeding or exercise stimuli with the aim of providing more insight to the possible *in vivo* functioning of Sestrin2 phosphorylation.

In contrast to Sestrin2 phosphorylation, p62^{Ser403} phosphorylation was repressed 2 h post-exercise. Under *in vitro* conditions, the association between Sestrin2, p62 and ULK1 promotes ULK1-mediated p62^{Ser403} phosphorylation, resulting in selective degradation of polyubiquitinated cargos, such as Keap1 (Matsumoto *et al.*, 2011; Ro *et al.*, 2014). Degradation of Keap1 allows Nrf2 to be translocated to the nucleus to upregulate antioxidant gene expression (Ichimura *et al.*, 2013). The acute post-exercise dephosphorylation of p62^{Ser403} observed in the current study could be suggestive of diminished ubiquitin-mediated selective macroautophagic protein degradation. Further, in agreement with the current study, RE has been shown to upregulate total p62, 24 and 48 h following exercise (Ogborn *et al.*, 2015), indicating that acute RE might be suppressing macroautophagy, as p62 accumulates when autophagy is inhibited (Mizushima *et al.*, 2010). Additionally, by measuring the conversion of cytosolic microtubule-associated protein 1 (LC3B-I) to the autophagosomal membrane-associated form, LC3B-II, a marker of enhanced autophagy, it was demonstrated

306 that macroautophagy was depressed in both young and old adults following an acute bout of
307 RE (Glynn *et al.*, 2010; Fry *et al.*, 2012).

308 p62 has been found to be induced at the transcriptional level by ROS under cellular stress
309 (Jain *et al.*, 2010), therefore to evaluate the role of Sestrin2 and p62 in regulating oxidative
310 stress, future studies should explore oxidative stress markers, Keap1 degradation, Nrf2
311 upregulation and antioxidant response. As exercise influences multiple pathways, the present
312 data does not allow for strong mechanistic conclusions regarding the role of Sestrin2
313 phosphorylation in regulating $p62^{Ser403}$ phosphorylation and its implication in redox
314 homeostasis and selective autophagy. Due to limited available muscle tissues, co-
315 immunoprecipitation analyses were not undertaken, making it unclear whether there was a
316 functional association between Sestrin2 and p62. Furthermore, a limitation of the study was a
317 lack of control for feeding at the 24 and 48 h biopsies collections, which could have affected
318 the result observed at these time points. Future studies should investigate the physical
319 association between Sestrin2 and p62 and control for feeding at all time points. The present
320 study demonstrated that following RE, a clear inverse relationship between the
321 phosphorylation status of Sestrin2 and $p62^{Ser403}$ exists, and they are transiently regulated after
322 RE, which may play a role in cellular adaptation in human skeletal muscle.

323

324 *Effect of Chronic Exercise*

325 Skeletal muscle is sensitive to both acute and chronic stresses associated with RE and RT.
326 The mechanisms associated with the acute transient response to RE might be different
327 compared to chronic adaptation which are measured in the rested state, since these responses
328 are influenced by multiple factors including frequency of exercise, recovery period and
329 training history of individuals (Abernethy *et al.*, 1994). Literature on the relationship of
330 Sestrin and exercise is limited. The current understanding is a single bout of aerobic exercise
331 increased Sestrin2 protein in mice (Lenhare *et al.*, 2017), while long-term endurance exercise
332 increased the protein expression of Sestrin2 and 3 and basal level of muscle autophagy (Liu
333 *et al.*, 2015). In this study, long-term RT for 12 weeks did not alter the protein or mRNA
334 expression of Sestrin2, 3 and total p62 or the basal phosphorylation status of Sestrin2 and
335 $p62^{Ser403}$. This could be due to the use of different exercise protocol, since different modes of
336 exercise produce distinct myofibre adaptations, while RT increases strength and muscle fibre
337 cross-sectional area, endurance exercise improves oxidative metabolism by increasing

mitochondrial content and capillary densities (Wilkinson *et al.*, 2008). Interestingly, the protein expression of Sestrin1 increased significantly following RT and also 48 h following RE. However, direct comparison between the acute and chronic effects on Sestrin1 cannot be made, since fasting biopsies were collected in the present chronic study while in the acute study, biopsies were collected in the fed state.

In agreement with a previous study, compared with Sestrin2 and 3, Sestrin1 is more abundantly expressed in skeletal muscle (Peeters *et al.*, 2003). Silencing of Sestrin1 in human embryonic fibroblasts inhibited cell proliferation and accelerated cell senescence due to excess ROS production (Budanov *et al.*, 2004). Moreover, studies from drosophila and mouse models provided a connection between Sestrins and muscle growth, as knockout of Sestrin resulted in muscle degeneration (Lee *et al.*, 2010). Additionally, silencing Sestrin3 in human myotubes increased myostatin expression, which is a negative regulator of muscle growth (Nascimento *et al.*, 2013). Recruited subjects demonstrated an increased in both strength and muscle mass after 12 weeks of training (Roberts *et al.*, 2015). Taken together these observations suggest a potential link between Sestrin1 and the regulation of cell growth, which warrants further investigation to clarify the distinct roles played by each Sestrin family members in human skeletal muscle.

Conclusion

Sestrin and p62 are multifunctional proteins involved in many cellular processes, including suppressing oxidative stress, mTORC1 and autophagy regulation (Katsuragi *et al.*, 2015; Parmigiani & Budanov, 2016). The present analysis demonstrated that while Sestrin family members share considerable sequence homology, each is regulated independently in response to RE. Sestrin3 was not affected by RE, whereas long-term training induced the protein expression of Sestrin1. In response to RE, there was a transient mobility shift of Sestrin2, indicative of increased phosphorylation. Mirroring this response, $p62^{Ser403}$ phosphorylation was downregulated. It appears that both Sestrin2 and $p62^{Ser403}$ are transiently regulated, and may be functionally involved in the adaptive regulatory mechanisms elicited by human skeletal muscle after intense RE.

Conflict of Interest

None declared.

Author Contributions

NZ, JMP, CJM and DCS designed the study; NZ, RFD, VCF, JFM and LAR performed experiments; NZ analysed data; NZ, CJM and DCS drafted manuscript; NZ, RFD, VCF, JFM, LAR, JMP, CJM and DCS critically evaluated and contributed to the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Abernethy PJ, Jürimäe J, Logan PA, Taylor AW & Thayer RE (1994). Acute and chronic response of skeletal muscle to resistance exercise. *Sports Medicine* **17,** 22-38.

Bae SH, Sung SH, Oh SY, Lim JM, Lee SK, Park YN, Lee HE, Kang D & Rhee SG (2013). Sestrins activate Nrf2 by promoting p62-dependent autophagic degradation of Keap1 and prevent oxidative liver damage. *Cell metabolism* **17,** 73-84.

Budanov AV, Lee JH & Karin M (2010). Stressin9 Sestrins take an aging fight. *EMBO molecular medicine* **2,** 388-400.

Budanov AV, Sablina AA, Feinstein E, Koonin EV & Chumakov PM (2004). Regeneration of peroxiredoxins by p53-regulated sestrins, homologs of bacterial AhpD. *Science* **304,** 596-600.

Çakır-Atabek H, Özdemir F & Çolak R (2015). Oxidative stress and antioxidant responses to progressive resistance exercise intensity in trained and untrained males. *Biology of sport* **32,** 321.

Chantranupong L, Wolfson RL, Orozco JM, Saxton RA, Scaria SM, Bar-Peled L, Spooner E, Isasa M, Gygi SP & Sabatini DM (2014). The Sestrins interact with GATOR2 to negatively regulate the amino-acid-sensing pathway upstream of mTORC1. *Cell reports* **9,** 1-8.

Dreyer HC, Fujita S, Cadenas JG, Chinkes DL, Volpi E & Rasmussen BB (2006). Resistance exercise increases AMPK activity and reduces 4E-BP1 phosphorylation and protein synthesis in human skeletal muscle. *The Journal of physiology* **576,** 613-624.

Egan B & Zierath JR (2013). Exercise metabolism and the molecular regulation of skeletal muscle adaptation. *Cell metabolism* **17,** 162-184.

Eisenberg E & Levanon EY (2013). Human housekeeping genes, revisited. *Trends in Genetics* **29,** 569-574.

Fry CS, Drummond MJ, Glynn EL, Dickinson JM, Gundermann DM, Timmerman KL, Walker DK, Volpi E & Rasmussen BB (2012). Skeletal muscle autophagy and protein breakdown following resistance exercise are similar in younger and older adults. *Journals of Gerontology Series A: Biomedical Sciences and Medical Sciences* **68,** 599-607.

Glynn EL, Fry CS, Drummond MJ, Dreyer HC, Dhanani S, Volpi E & Rasmussen BB (2010). Muscle protein breakdown has a minor role in the protein anabolic response to essential amino acid and carbohydrate intake following resistance exercise. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **299,** R533-R540.

Ichimura Y, Waguri S, Sou Y-s, Kageyama S, Hasegawa J, Ishimura R, Saito T, Yang Y, Kouno T & Fukutomi T (2013). Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy. *Molecular cell* **51,** 618-631.

Jain A, Lamark T, Sjøttem E, Larsen KB, Awuh JA, Øvervatn A, McMahon M, Hayes JD & Johansen T (2010). p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. *Journal of Biological Chemistry* **285,** 22576-22591.

Katsuragi Y, Ichimura Y & Komatsu M (2015). p62/SQSTM1 functions as a signaling hub and an autophagy adaptor. *The FEBS journal* **282,** 4672-4678.

Kim H, An S, Ro S-H, Teixeira F, Park GJ, Kim C, Cho C-S, Kim J-S, Jakob U & Lee JH (2015). Janus-faced Sestrin2 controls ROS and mTOR signalling through two separate functional domains. *Nature communications* **6**, 10025.

Kimball SR, Gordon BS, Moyer JE, Dennis MD & Jefferson LS (2016). Leucine induced dephosphorylation of Sestrin2 promotes mTORC1 activation. *Cellular signalling* **28,** 896-906.

Kopnin PB, Agapova LS, Kopnin BP & Chumakov PM (2007). Repression of sestrin family genes contributes to oncogenic Ras-induced reactive oxygen species up-regulation and genetic instability. *Cancer research* **67,** 4671-4678.

Lamark T, Kirkin V, Dikic I & Johansen T (2009). NBR1 and p62 as cargo receptors for selective autophagy of ubiquitinated targets. *Cell cycle* **8,** 1986-1990.

Lee JH, Budanov AV & Karin M (2013). Sestrins orchestrate cellular metabolism to attenuate aging. *Cell metabolism* **18,** 792-801.

Lee JH, Budanov AV, Park EJ, Birse R, Kim TE, Perkins GA, Ocorr K, Ellisman MH, Bodmer R & Bier E (2010). Sestrin as a feedback inhibitor of TOR that prevents age-related pathologies. *Science* **327,** 1223-1228.

Lee JH, Cho U-S & Karin M (2016). Sestrin regulation of TORC1: Is Sestrin a leucine sensor? *Science Signaling* **9,** re5.

Lenhare L, Crisol BM, Silva VR, Katashima CK, Cordeiro AV, Pereira KD, Luchessi AD, da Silva AS, Cintra DE & Moura LP (2017). Physical exercise increases Sestrin 2 protein levels and induces autophagy in the skeletal muscle of old mice. *Experimental Gerontology* **97,** 17-21.

Liu X, Niu Y, Yuan H, Huang J & Fu L (2015). AMPK binds to Sestrins and mediates the effect of exercise to increase insulin-sensitivity through autophagy. *Metabolism* **64,** 658-665.

Matsumoto G, Wada K, Okuno M, Kurosawa M & Nukina N (2011). Serine 403 phosphorylation of p62/SQSTM1 regulates selective autophagic clearance of ubiquitinated proteins. *Molecular cell* **44,** 279-289.

Mizushima N, Yoshimori T & Levine B (2010). Methods in mammalian autophagy research. *Cell* **140,** 313-326.

Nascimento EB, Osler ME & Zierath JR (2013). Sestrin 3 regulation in type 2 diabetic patients and its influence on metabolism and differentiation in skeletal muscle. *American Journal of Physiology-Endocrinology and Metabolism* **305,** E1408-E1414.

Nikonorova IA, Al-Baghdadi RJ, Mirek ET, Wang Y, Goudie MP, Wetstein BB, Dixon JL, Hine C, Mitchell JR & Adams CM (2017). Obesity challenges the hepatoprotective function of the integrated stress response to asparaginase exposure in mice. *Journal of Biological Chemistry* **292,** 6786-6798.

Ogborn DI, McKay BR, Crane JD, Safdar A, Akhtar M, Parise G & Tarnopolsky MA (2015). Effects of age and unaccustomed resistance exercise on mitochondrial transcript and protein abundance in skeletal muscle of men. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **308,** R734-R741.

Parmigiani A & Budanov A (2016). Chapter One-Sensing the Environment Through Sestrins: Implications for Cellular Metabolism. *International review of cell and molecular biology* **327,** 1-42.

Parmigiani A, Nourbakhsh A, Ding B, Wang W, Kim YC, Akopiants K, Guan K-L, Karin M & Budanov AV (2014). Sestrins inhibit mTORC1 kinase activation through the GATOR complex. *Cell reports* **9,** 1281-1291.

Peck SC (2006). Analysis of protein phosphorylation: methods and strategies for studying kinases and substrates. *The plant journal* **45,** 512-522.

Peeters H, Debeer P, Bairoch A, Wilquet V, Huysmans C, Parthoens E, Fryns J-P, Gewillig M, Nakamura Y & Niikawa N (2003). PA26 is a candidate gene for heterotaxia in humans: identification of a novel PA26-related gene family in human and mouse. *Human genetics* **112,** 573-580.

Polotow TG, Souza-Junior TP, Sampaio RC, Okuyama AR, Ganini D, Vardaris CV, Alves RC, McAnulty SR & Barros MP (2017). Effect of 1 Repetition Maximum, 80% Repetition Maximum, and 50% Repetition Maximum Strength Exercise in Trained Individuals on Variations in Plasma Redox Biomarkers. *The Journal of Strength & Conditioning Research* **31,** 2489-2497.

Ro SH, Semple IA, Park H, Park H, Park HW, Kim M, Kim JS & Lee JH (2014). Sestrin2 promotes Unc-51-like kinase 1 mediated phosphorylation of p62/sequestosome-1. *The FEBS journal* **281,** 3816-3827.

Roberts LA, Raastad T, Markworth JF, Figueiredo VC, Egner IM, Shield A, Cameron-Smith D, Coombes JS & Peake JM (2015). Post-exercise cold water immersion attenuates acute anabolic signalling and long-term adaptations in muscle to strength training. *The Journal of physiology* **593,** 4285-4301.

Sanchis-Gomar F, Gómez-Cabrera MC & Viña J (2011). The loss of muscle mass and sarcopenia: non hormonal intervention. *Experimental Gerontology* **46,** 967-969.

Saxton RA, Knockenhauer KE, Schwartz TU & Sabatini DM (2016). The apo-structure of the leucine sensor Sestrin2 is still elusive. *Science Signaling* **9,** ra92.

Schmittgen TD & Livak KJ (2008). Analyzing real-time PCR data by the comparative CT method. *Nature protocols* **3,** 1101-1108.

Tanida I (2011). Autophagosome formation and molecular mechanism of autophagy. *Antioxidants & redox signaling* **14,** 2201-2214.

Tao R, Xiong X, Liangpunsakul S & Dong XC (2015). Sestrin 3 protein enhances hepatic insulin sensitivity by direct activation of the mTORC2-Akt signaling. *Diabetes* **64,** 1211-1223.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology* **3,** research0034-1.

Wegener A & Jones L (1984). Phosphorylation-induced mobility shift in phospholamban in sodium dodecyl sulfate-polyacrylamide gels. Evidence for a protein structure consisting of multiple identical phosphorylatable subunits. *Journal of Biological Chemistry* **259,** 1834-1841.

Wilkinson SB, Phillips SM, Atherton PJ, Patel R, Yarasheski KE, Tarnopolsky MA & Rennie MJ (2008). Differential effects of resistance and endurance exercise in the fed state on signalling molecule phosphorylation and protein synthesis in human muscle. *The Journal of physiology* **586,** 3701-3717.

Wolfson RL, Chantranupong L, Saxton RA, Shen K, Scaria SM, Cantor JR & Sabatini DM (2016). Sestrin2 is a leucine sensor for the mTORC1 pathway. *Science* **351,** 43-48.

Woo HA, Bae SH, Park S & Rhee SG (2009). Sestrin 2 is not a reductase for cysteine sulfinic acid of peroxiredoxins. *Antioxidants & redox signaling* **11,** 739-745.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S & Madden TL (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC bioinformatics* **13,** 134.

Table1. mRNA sequences. Forward and reverse sequences of analysed genes.

Gene	Sequence
CHMP2A (Forward)	CGCTATGTGCGCAAGTTTGT
CHMP2A (Reverse)	GGGGCAACTTCAGCTGTCTG
C1orf43 (Forward)	CTATGGGACAGGGGTCTTTGG
C1orf43 (Reverse)	TTTGGCTGCTGACTGGTGAT

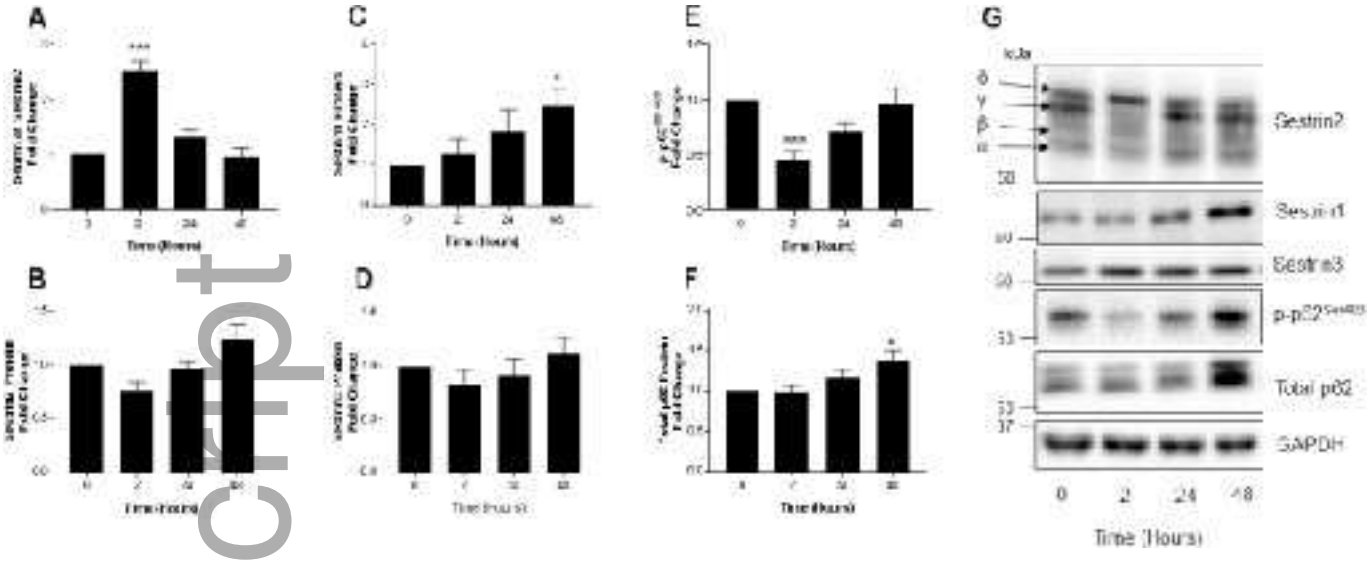
EMC7 (Forward)	GGGCTGGACAGACTTTCTAATG
EMC7 (Reverse)	CTCCATTTCCCGTCTCATGTCAG
SESN1 (Forward)	TTTCGTGTCCAGGACTATTGC
SESN1 (Reverse)	ACTGTCCCACATCTGGATAAAGG
SESN2 (Forward)	CAACCTCTTCTGGAGGCACTT
SESN2 (Reverse)	CCTGCTCAGGAGTCAGGTCA
SESN3 (Forward)	CAGGCAGCAACTTTGGGATTGT
SESN3 (Reverse)	AGACGCCTCTTCATCTTCCCTT
p62 (Forward)	GAATCAGCTTCTGGTCCATCGG
p62 (Reverse)	GCTTCTTTTCCCTCCGTGCT

Figure 1. Effects of Acute Resistance Exercise on Sestrin and p62 protein. The relative abundance of Sestrin2 in δ-form (A); total Sestrin2 protein (B); Sestrin1 protein (C); Sestrin3 protein (D); phosphorylation status of $p62^{Ser403}$ (E); and total p62 protein (F) following acute resistance exercise. Representative western blots (G). Data are expressed as fold change from rest and error bars represent SEM. *P<0.05 and ***P<0.001 *vs.* respective baseline samples.

Figure 2. Effects of Acute Resistance Exercise on mRNA expression. The mRNA expression of *SESN2* (A); *SESN1* (B); *SESN3* (C); and *p62* (D). Data are expressed as fold change from rest and error bars represent SEM. *P<0.05 *vs.* respective baseline samples.

Figure 3. Basal expression of Sestrin paralogs. The basal mRNA expression of *SESN1, 2, 3* in the acute (A) and chronic (B) study. Data are expressed as means ± SEM. *** difference between *SESN1* and *SESN2* P<0.001, # difference between *SESN1* and *SESN3* P<0.05 and Φ difference between *SESN3* and *SESN2* P<0.05.

Figure 4. Effects of Chronic Resistance Exercise on Sestrin and p62 protein. The relative abundance of Sestrin2 in δ-form (A); total Sestrin2 protein (B); Sestrin1 protein (C); Sestrin3 protein (D); phosphorylation status of $p62^{Ser403}$ (E); and total p62 protein (F). Representative western blots (G). Data are expressed as fold change from rest and error bars represent SEM. *P<0.05 *vs.* respective baseline samples.



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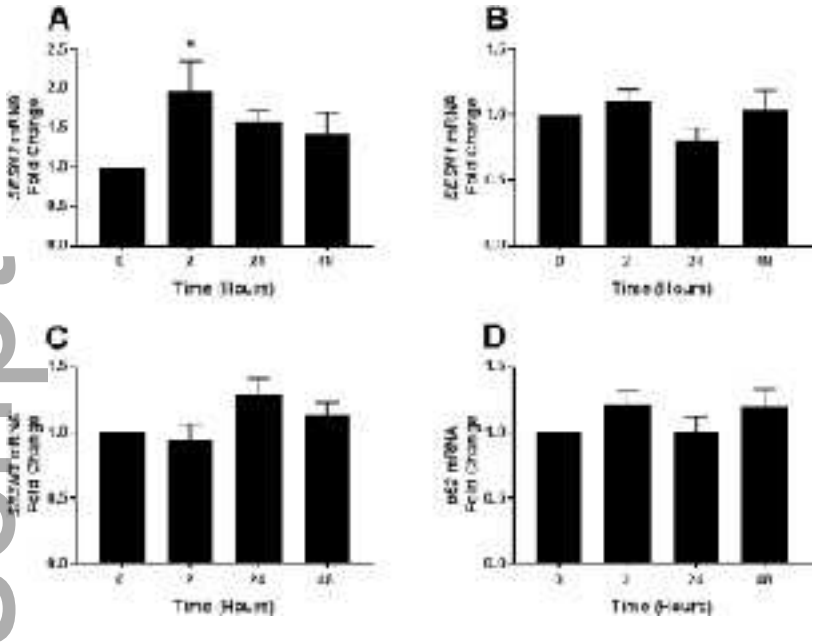

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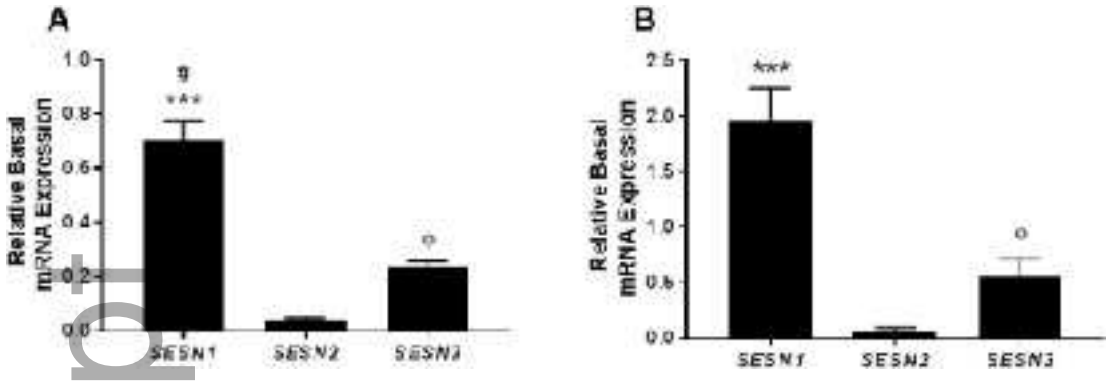

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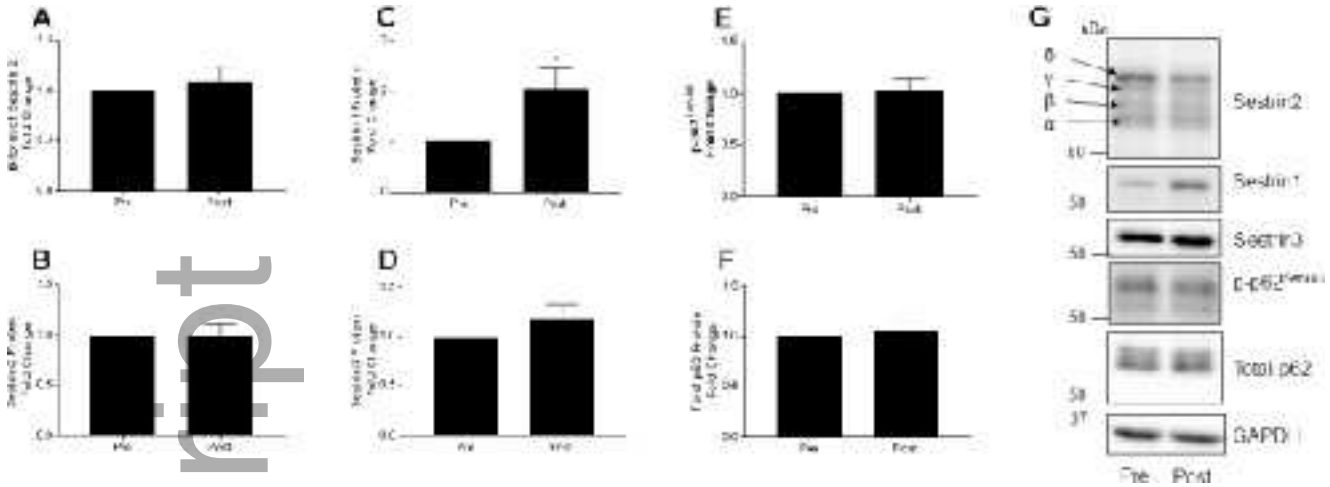

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