

The role of skeletal muscle mTOR in the regulation of mechanical load-induced growth

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Non-technical summary Chronic mechanical loading (CML) of skeletal muscle induces growth and this effect can be blocked by the drug rapamycin. Rapamycin is considered to be a highly specific inhibitor of the mammalian target of rapamycin (mTOR), and thus, many have concluded that mTOR plays a key role in CML-induced growth. However, direct evidence that mTOR confers the CML-induced activation of growth promoting events such as hypertrophy, hyperplasia and ribosome biogenesis is lacking. This study addressed that gap in knowledge by using a specialized line of transgenic mice. Surprisingly, the results indicate that only a few of the growth promoting events induced by CML are fully dependent on mTOR signalling (e.g. hypertrophy). These results advance our understanding of the molecular mechanisms that regulate skeletal muscle mass and should help future studies aimed at identifying targets for therapies that can prevent the loss of muscle mass during conditions such as bedrest, immobilization, and ageing.

Abstract Chronic mechanical loading (CML) of skeletal muscle induces compensatory growth and the drug rapamycin has been reported to block this effect. Since rapamycin is considered to be a highly specific inhibitor of the mammalian target of rapamycin (mTOR), many have concluded that mTOR plays a key role in CML-induced growth regulatory events. However, rapamycin can exert mTOR-independent actions and systemic administration of rapamycin will inhibit mTOR signalling in all cells throughout the body. Thus, it is not clear if the growth inhibitory effects of rapamycin are actually due to the inhibition of mTOR signalling, and more specifically, the inhibition of mTOR signalling in skeletal muscle cells. To address this issue, transgenic mice with muscle specific expression of various rapamycin-resistant mutants of mTOR were employed. These mice enabled us to demonstrate that mTOR, within skeletal muscle cells, is the rapamycin-sensitive element that confers CML-induced hypertrophy, and mTOR kinase activity is necessary for this event. Surprisingly, CML also induced hyperplasia, but this occurred through a rapamycin-insensitive mechanism. Furthermore, CML was found to induce an increase in FoxO1 expression and PKB phosphorylation through a mechanism that was at least partially regulated by an mTOR kinase-dependent mechanism. Finally, CML stimulated ribosomal RNA accumulation and rapamycin partially inhibited this effect; however, the effect of rapamycin was exerted through a mechanism that was independent of mTOR in skeletal muscle cells. Overall, these results demonstrate that CML activates several growth regulatory events, but only a few (e.g. hypertrophy) are fully dependent on mTOR signalling within the skeletal muscle cells.

(Received 10 August 2011; accepted after revision 26 September 2011; first published online 26 September 2011)

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Abbreviations AMPK, AMP-activated protein kinase; CSA, cross-sectional area; FoxO, forkhead box containing proteins, O-subclass; IGF-1, insulin like growth factor 1; IRS-1, insulin receptor substrate 1; MHC-emb, embryonic myosin heavy chain; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; mTORC2, mammalian target of rapamycin complex 2; p70^{S6k}, ribosomal S6 kinase 1; PI3K, phosphatidylinositol-3-kinase; PKB, protein kinase B; PLT, plantaris muscle; RR-mTOR, rapamycin-resistant mTOR; RRKD-mTOR, rapamycin-resistant kinase dead mTOR; SA, synergist ablation; UBF, upstream binding factor.

Introduction

Skeletal muscles play a critical role in whole body metabolism (Pedersen & Febbraio, 2008; Srikanthan & Karlamangla, 2011), and as such, the maintenance of skeletal muscle mass is important for disease prevention and quality of life (Hurley *et al.* 2011; LeBrasseur *et al.* 2011). Over the last century it has become apparent that skeletal muscle mass can be regulated by a variety of different stimuli, and one of the most well recognized of these stimuli is mechanical loading. Evidence that mechanical loads regulate skeletal muscle mass is derived from numerous studies which have shown that a chronic elevation in mechanical loading results in an increase in muscle mass, while chronic mechanical unloading results in a decrease in muscle mass (Goldberg *et al.* 1975). Although it is well recognized that mechanical loads play a critical role in the regulation of skeletal muscle mass, the molecular mechanisms that control this process remain poorly defined (Sandri, 2008). However, mechanical load-induced alterations in muscle mass are ultimately driven by an alteration in the balance between the rates of protein synthesis and protein degradation. Thus, identifying the molecules that regulate mechanical load-induced changes in protein synthesis and protein degradation should provide fundamental insight into how mechanical loads regulate skeletal muscle mass.

One molecule that has been widely implicated in the regulation of protein synthesis is a kinase called the mammalian target of rapamycin (mTOR). Indeed, recent studies have shown that muscle hypertrophy resulting from increased mechanical loading is associated with an increase in mTOR signalling, whereas atrophy resulting from a decrease in mechanical loading is associated with decreased mTOR signalling (Baar & Esser, 1999; Hornberger *et al.* 2001). Furthermore, studies with rapamycin, an inhibitor of mTOR, have suggested that signalling through mTOR is necessary for the induction of protein synthesis and ultimately the growth that is induced by increased mechanical loading (Bodine *et al.* 2001; Hornberger *et al.* 2004; Drummond *et al.* 2009).

mTOR exists in two functionally distinct multi-protein signalling complexes called mTORC1 and mTORC2 and, in general, signalling by mTORC1, but not mTORC2, is inhibited by rapamycin (Zoncu *et al.* 2011). Thus, it has been widely assumed that the growth inhibitory effects of rapamycin are exerted through an mTORC1-dependent

mechanism (Bodine, 2006; Nader, 2007). However, rapamycin can potentially exert mTOR-independent actions. For example, rapamycin can bind and sequester the FKBP12 protein and this could interfere with the important role that the FKBP12 plays in the function of the ryanodine receptor and signalling by members of the transforming growth factor- β superfamily (Avila *et al.* 2003; Wang & Donahoe, 2004; Osman *et al.* 2009). Furthermore, systemic administration of rapamycin would be expected to inhibit mTOR signalling in all cells throughout the body. Therefore, it is not clear if the growth inhibitory effects of rapamycin are actually due to inhibition of mTOR signalling, and more specifically, due to the inhibition of mTOR signalling within skeletal muscle cells *per se*.

Not only has it been widely assumed that mTOR is the rapamycin-sensitive element that confers mechanical load-induced growth, but it has also been widely assumed that rapamycin exerts its growth inhibitory effects by blocking the ability of mTOR to phosphorylate downstream substrates (e.g. ribosomal S6 kinase (p70^{S6k})). In other words, it is thought that mTOR regulates mechanical load-induced growth via a kinase-dependent mechanism. However, even moderately high concentrations of rapamycin do not inhibit the intrinsic kinase activity of mTOR (Oshiro *et al.* 2004). Thus, a rapamycin-sensitive event does not necessarily imply an mTOR kinase-dependent event. For example, rapamycin has been shown to inhibit myogenesis, and the expression of either rapamycin-resistant mTOR, or rapamycin-resistant kinase-dead mTOR, can rescue myogenesis from the inhibitory effects of rapamycin (Erbay & Chen, 2001). Hence, it also remains to be determined if mTOR kinase activity is necessary for mechanical load-induced growth.

In this study we have attempted to fill many of the aforementioned gaps in knowledge by employing transgenic mice with muscle specific expression of various rapamycin-resistant mutants of mTOR. These mice were subjected to chronic mechanical loading using the synergist ablation model and then the effects of rapamycin on various growth related events such as hypertrophy, hyperplasia, ribosome biogenesis, and associated molecular signalling events, were examined. Surprisingly, the results demonstrate that only a few of the events examined (e.g. hypertrophy) were fully dependent on mTOR signalling within the skeletal muscle cells.

Methods

Materials

Rabbit anti-laminin antibodies were purchased from Sigma-Aldrich (St Louis, MO, USA). Anti-embryonic myosin heavy chain antibodies were purchased from the Developmental Studies Hybridoma Bank at the University of Iowa (Ames, IA, USA). Anti-phospho p70^{S6k}(389), anti-total UBF (F-9), TRITC-conjugated anti-rabbit and FITC-conjugated anti-mouse antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Anti-total Rb antibodies were purchased from BD Biosciences (San Jose, CA, USA). Peroxidase-conjugated anti-rabbit and peroxidase-conjugated anti-mouse antibodies were purchased from Vector Laboratories (Burlingame, CA, USA). All other antibodies were purchased from Cell Signaling (Danvers, MA, USA).

Animals

FVB/N mice with human skeletal actin promoter driven expression of a FLAG tagged rapamycin-resistant (Ser2035Thr) mutant of mTOR (RR-mTOR), or a FLAG tagged rapamycin-resistant kinase dead (Ser2035Thr/Asp2357Glu) mutant of mTOR (RRKD-mTOR) have been previously described (Ge *et al.* 2009). These transgenic mice were bred with wild-type (WT) FVB/N mice and the offspring employed were either null (wild-type) or hemizygotes. Genotypes were confirmed with tail snips by PCR and 8- to 10-week-old males were used for all experiments. Before all surgical procedures, mice were anaesthetized with an intraperitoneal (i.p.) injection of ketamine (100 mg kg⁻¹) and xylazine (10 mg kg⁻¹). After tissue extraction, the mice were killed by cervical dislocation. All methods were approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison and conform to the principles of UK regulations, as described in Drummond (2009).

Synergist ablation of the plantaris muscle

Bilateral synergist ablation (SA) surgeries were performed by removing the soleus and distal half of the gastrocnemius muscle as previously described (Goodman *et al.* 2011a). Mice in the control group were subjected to a sham surgery where an incision was made on lower leg and then closed. Following the surgeries, incisions were closed with Vetbond surgical glue (Henry Schein, Melville, NY, USA). Mice were allowed to recover for 7 or 14 days and then the plantaris (PLT) muscles were collected and subjected to the various measurements described below.

Rapamycin injections

Rapamycin was purchased from LC laboratories (Woburn, MA, USA) and was dissolved in DMSO to generate a 5 µg µl⁻¹ stock solution. The appropriate volume of the stock solution needed to inject mice was dissolved in 200 µl of PBS. For the vehicle control condition, mice were injected with an equivalent amount of DMSO dissolved in 200 µl of phosphate-buffered saline (PBS). Immediately following the SA procedure, vehicle or rapamycin solutions were administered via intraperitoneal injections and these injections were repeated every 24 h for up to 14 days.

Sample preparation for immunoprecipitations and Western blot analysis

Upon collection, PLT muscles were immediately frozen in liquid nitrogen. Frozen muscles were homogenized with a Polytron for 20 s in ice cold buffer A (40 mM Tris (pH 7.5), 1 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 25 mM β-glycerophosphate, 25 mM NaF, 1 mM Na₃VO₄, 10 µg ml⁻¹ leupeptin and 1 mM PMSF) and the whole homogenate was used for further analysis. Sample protein concentration was determined with a DC protein assay kit (Bio-Rad, Hercules, CA, USA), and unless otherwise noted (e.g. immunoprecipitation), equivalent amounts of protein from each sample were dissolved in Laemmli buffer, heated to 100°C for 5 min and then subjected to Western blot analysis as described below.

Immunoprecipitation

Immunoprecipitation of FLAG tagged mTOR was performed with slight modifications of our previously described method (Hornberger *et al.* 2007). Briefly, whole muscle homogenates were centrifuged at 8200 g for 5 min and 300 µg of protein from the supernatant was diluted to a volume of 0.5 ml with fresh ice cold buffer A. This sample was then incubated with 20 µl of EZview red ANTI-FLAG M2 agarose affinity gel beads (Sigma-Aldrich) with gentle rocking at 4°C for 2 h. Following the incubation, the beads were pelleted by centrifugation at 500 g for 30 s and washed with fresh ice cold buffer A. After four washes, the pellets were dissolved in 2× Laemmli buffer and heated to 100°C for 5 min. The beads were again pelleted by centrifugation at 500 g for 30 s and the supernatant was subjected to Western blot analysis as described below.

Western blot analysis

Western blot analyses were performed as previously described (Goodman *et al.* 2011a). Briefly, samples were subjected to electrophoretic separation on SDS-PAGE

acrylamide gels. Following electrophoretic separation, proteins were transferred to a PVDF membrane, blocked with 5% powdered milk in Tris-buffered saline, 0.1% Tween 20 (TBST) for 1 h followed by an overnight incubation at 4°C with primary antibody dissolved in TBST containing 1% bovine serum albumin (BSA). After an overnight incubation, the membranes were washed for 30 min in TBST and then probed with a peroxidase-conjugated secondary antibody for 1 h at room temperature. Following 30 min of washing in TBST, the blots were developed on film using regular enhanced chemiluminescence (ECL) reagent (Pierce, Rockford, IL, USA) or ECL plus reagent (Amersham, Piscataway, NJ, USA). Once the appropriate image was captured, the membranes were stained with Coomassie Blue to verify equal loading in all lanes. Densitometric measurements were carried out using ImageJ (NIH; <http://rsb.info.nih.gov/nih-image/>). It should be noted that the vast majority of the Western blot analyses in this study were run with the samples grouped according to the genotype (i.e. WT samples were not run with RR-mTOR or RRKD-mTOR samples on the same blot). In these cases, every blot for a given genotype contained at least three vehicle sham samples and all of the remaining samples on the blot were expressed relative to the mean of those samples. Furthermore, in many cases, multiple separate Western blots were needed to obtain all of the data for a given molecule. In an effort to reduce variability between these separate Western blots, extensive care was taken to ensure that the vehicle sham samples from all of the separate blots laid within the same region of the grey scale.

Immunohistochemical analysis

PLT muscles were excised and immediately submerged in optimal cutting temperature compound (Tissue-Tek; Sakura, Torrance, CA, USA) at resting length and frozen in liquid nitrogen chilled isopentane. Mid-belly cross-sections (10 μm thick) were taken perpendicular to the long axis of the muscle with a cryostat and immediately fixed in -20°C acetone for 10 min. Sections were warmed to room temperature for 5 min and then incubated in PBS for 15 min followed by a 20 min incubation in a buffer B (PBS containing 0.5% BSA and 0.5% Triton X-100). Sections were then incubated with the indicated primary antibodies dissolved in buffer B for 1 h at room temperature. Sections were washed with PBS and then incubated with the appropriate fluorophore conjugated secondary antibodies dissolved in buffer B for 1 h at room temperature. Finally, the sections were washed with PBS and fluorescence images were captured with a Nikon DS-QiMc camera on a Nikon 80i epi-fluorescence microscope with TRITC (laminin) and FITC (MHC-emb) cubes. The monochrome images were merged with Nikon

NIS Elements D image analysis software and the average cross-sectional area (CSA) of 150 randomly selected fibres per sample were measured by tracing the periphery of individual fibres along the laminin stain. The total number of fibres per muscle cross-section, and the total number of fibres that were positive for MHC-emb per muscle cross-section were manually counted. All analyses were performed by investigators that were unaware of the sample identification.

Total and ribosomal RNA

The PLT muscles were weighed and then total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The resulting RNA pellet was resuspended in 4 μl of nuclease free, DEPC treated water (Genemate, Kaysville, UT, USA), per mg of muscle weight. The concentration of the RNA in this solution ($\mu\text{g } \mu\text{l}^{-1}$) was then determined with spectrophotometric analysis at 260 nm. The amount of total RNA per muscle was then calculated by multiplying the RNA concentration of the solution by the total volume of the solution. The value for total RNA was then divided by the muscle weight to obtain (μg of total RNA per mg muscle). In addition, 10 μl of the RNA solution was run on a 1% agarose gel and viewed under UV light. Densitometric measurements of the 28S and 18S ribosomal RNA (rRNA) were performed with ImageJ software as described above.

Statistical analysis

All values are expressed as means (+SEM in graphs). Statistical significance was determined by using ANOVA, followed by Student–Newman–Keuls *post hoc* analysis. Differences between groups were considered significant if $P \leq 0.05$. All statistical analyses were performed on SigmaStat software (Systat Software Inc., San Jose, CA, USA).

Results

Dose dependent effect of rapamycin on mechanical load-induced hypertrophy

In order to determine the minimum concentration of rapamycin required to inhibit mechanical load-induced hypertrophy, wild-type (WT) mice were subjected to sham or SA surgeries and administered daily injections of rapamycin at concentrations of 0.3, 0.6, 1.0 or 3.0 mg (kg body mass) $^{-1}$, or the solvent vehicle, for 14 days. After 14 days, PLT muscles were excised, muscle sections were immunohistochemically stained for laminin and fibre CSA was measured (Fig. 1). The results

demonstrated that rapamycin inhibited the mechanical load-induced hypertrophy in a dose-dependent manner (Fig. 1E). Furthermore, a rapamycin dose of 0.6 mg kg^{-1} was determined to be the minimum needed to fully inhibit mechanical load-induced hypertrophy, and thus, this dosage of rapamycin was used for all subsequent experiments.

Characterization of transgenic mice with skeletal muscle specific expression of rapamycin-resistant mTOR or rapamycin-resistant kinase dead mTOR

To define the role of mTOR in various growth regulatory processes, transgenic mice with muscle specific expression of a FLAG-tagged rapamycin-resistant mTOR (RR-mTOR), or FLAG-tagged rapamycin-resistant kinase dead mTOR (RRKD-mTOR) were employed. Both of these transgenes contain the Ser2035Thr mutation which prevents the interaction of mTOR with the FKBP12-rapamycin complex, and in turn, induces rapamycin resistance (Brown *et al.* 1995; Lorenz & Heitman, 1995). As previously reported, expression of these transgenes could not be detected in the liver or kidneys of the mice (data not shown) (Ge *et al.* 2009; Goodman *et al.* 2010). Total mTOR expression in PLT muscles from the RR-mTOR and RRKD-mTOR mice was significantly higher (>4-fold) than that observed in muscles from WT mice (Fig. 2A and B; note these values result from a combination of both endogenously expressed WT mTOR and the transgenically expressed RR-mTOR or RRKD-mTOR). FLAG immunoprecipitation of PLT muscle samples was also used to confirm the appropriate expression of the RR-mTOR and RRKD-mTOR constructs. As expected,

the immunoprecipitates demonstrated the presence of the FLAG-tagged mTOR in muscles from both the RR-mTOR and RRKD-mTOR mice. Furthermore, phosphorylation of mTOR on the Ser2481 residue (an mTOR auto-phosphorylation site; Peterson *et al.* 2000) could be detected in the immunoprecipitates from RR-mTOR, but not RRKD-mTOR, mice (Fig. 2A).

To confirm that the PLT muscles from the RR-mTOR and RRKD-mTOR mice displayed the appropriate phenotypes, WT, RR-mTOR and RRKD-mTOR mice were subjected to sham or SA surgeries and administered daily injections of rapamycin or the solvent vehicle for 7 days. The PLT muscles were then assessed for changes in phosphorylation status of the mTOR substrate p70^{S6k}. As shown in Fig. 2C, SA induced a large (22-fold) increase in the phosphorylation of p70^{S6k} on the threonine 389 residue (p70(389)) in vehicle treated WT mice, and rapamycin completely eliminated this effect. This observation verifies that the rapamycin treatment effectively eliminated a well recognized mTOR-dependent signalling event in WT mice. More importantly, it was determined that the SA-induced increase in p70(389) phosphorylation was largely rescued from the inhibitory actions of rapamycin in RR-mTOR mice, but no rescue effect was observed in RRKD-mTOR mice (Fig. 2C). Combined, these results demonstrate that the muscles from the RR-mTOR and RRKD-mTOR transgenic mice conveyed the expected phenotypes. However, it is worth noting that in SA muscles from RR-mTOR mice, the amount of p70(389) phosphorylation was still partially reduced by rapamycin, which is likely to be due to the effects of rapamycin on the endogenous WT mTOR that exists in the muscles of these mice. Nevertheless, it can be concluded that mTOR, within the skeletal muscle cells, is the primary rapamycin-sensitive element that confers the mechanical

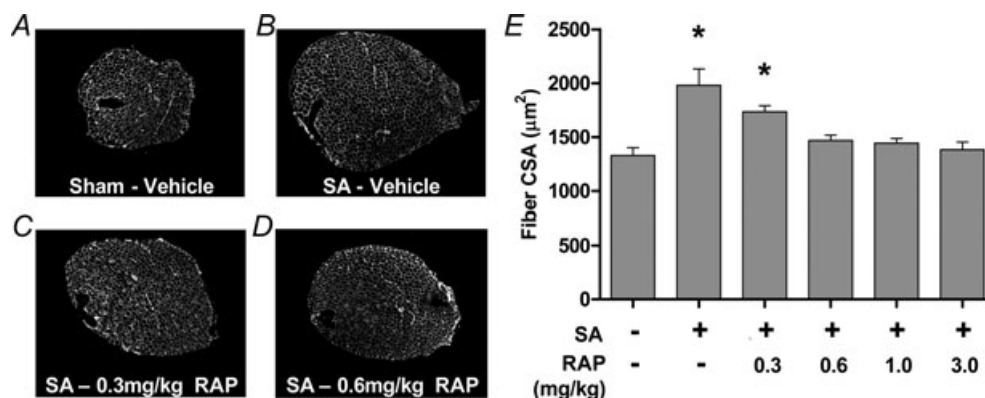


Figure 1. Dose-dependent effect of rapamycin on mechanical load-induced hypertrophy

Plantaris muscles from wild-type mice were subjected to sham or synergist ablation (SA) surgeries. Following surgery, the mice were administered a daily regime of vehicle (RAP⁻) or 0.3–3.0 mg kg^{-1} rapamycin (RAP⁺) injections. A–D, after 14 days, plantaris muscles were collected and mid-belly cross-sections were subjected to immunohistochemistry for laminin. E, the average fibre cross-sectional area (CSA) for each muscle was determined from 150 randomly selected fibres. Values represent the mean \pm SEM, $n = 3$ –6 muscles per group. *Significantly different from the vehicle sham condition, $P \leq 0.05$.

load-induced increase in p70(389) phosphorylation and mTOR kinase activity is necessary for this event.

The p70^{S6k} kinase contains numerous phosphorylation sites and it has been suggested the phosphorylation of the threonine 421/serine 424 residues p70(421/424), located in the auto-inhibitory domain of p70^{S6k}, invokes a conformational change that allows other sites to be phosphorylated and fully activate the enzyme (Dennis *et al.* 1998). We have previously shown that *ex vivo* mechanical stretch induces an increase in p70(421/424) phosphorylation and this effect is partially sensitive to rapamycin (Hornberger *et al.* 2004). However, to date, very little is known about the regulation of the p70(421/424) phosphorylation in skeletal muscle *in vivo*. Thus, we also examined the role of mTOR in regulating SA-induced changes in p70(421/424) phosphorylation. As shown in Supplemental Fig. 1, SA induced a large increase in p70(421/424) phosphorylation and this effect was partially inhibited by rapamycin treatment. Similar to p70(389), in the presence of rapamycin, the SA-induced

increase in p70(421/424) was rescued in RR-mTOR, but not in RRKD-mTOR mice. These data suggest that the mechanical load-induced increase in p70(421/424) phosphorylation is at least partially regulated by mTOR, within the skeletal muscle cells, and mTOR kinase activity is necessary for this effect. The mechanism behind mTOR's regulation of p70(421/424) phosphorylation, and the functional consequence of this event in skeletal muscle remain to be explored.

The role of mTOR in mechanical load-induced hypertrophy

To define the role of mTOR in mechanical load-induced hypertrophy, we subjected WT, RR-mTOR and RRKD-mTOR mice to sham or SA surgeries and administered the mice daily injections of rapamycin or the solvent vehicle for 14 days. After 14 days, PLT muscles were removed and muscle fibre CSA was analysed

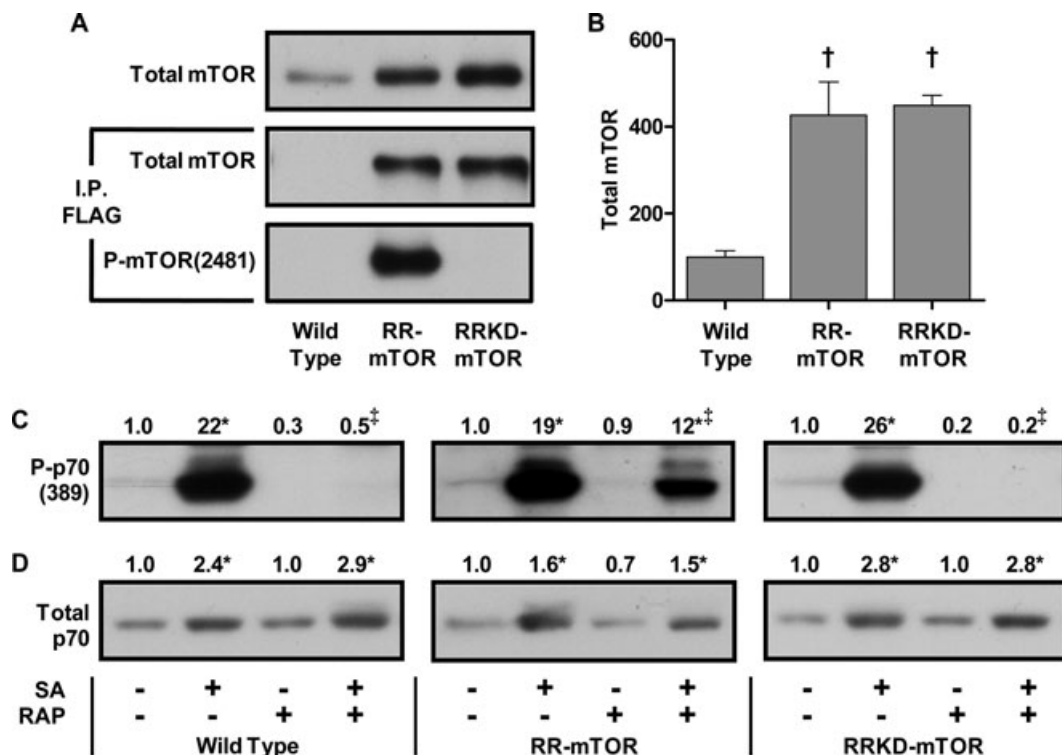


Figure 2. Confirmation of the phenotypes in RR-mTOR and RRKD-mTOR transgenic mice

A, lysates from muscles of wild-type, RR-mTOR and RRKD-mTOR mice were subjected to Western blot analysis or immunoprecipitation (i.p.) for the FLAG-tag followed by Western blot analysis with the indicated antibodies. B, quantification of total mTOR with the data expressed as a percentage of the values obtained in wild-type samples. C and D, mice were subjected to sham or synergist ablation (SA) surgeries. Following surgery, the mice were administered a daily regime of either vehicle (RAP⁻) or 0.6 mg kg⁻¹ rapamycin (RAP⁺) injections. At 7 days post-surgery, the plantaris muscles were collected and subjected to Western blot analysis. Representative images and quantification of the Western blot analysis for phosphorylation of the p70^{S6k} on the threonine 389 residue (P-p70(389)) (C) and total p70^{S6k} (D). The values at the top of each blot represent the amount of each protein when expressed relative to the values obtained in the vehicle treated sham muscles of the respective genotype. Values are expressed as the mean (+SEM in graphs), *n* = 3–11 per group. †Significantly different from wild-type, *Significant effect of SA within a given drug treatment, ‡significant effect of RAP within the SA groups, *P* ≤ 0.05.

(fibre size distributions for all genotypes with and without rapamycin are shown in Supplemental Fig. 2 and muscle weight to bodyweight ratios are shown in Supplemental Fig. 3). It was determined that in vehicle treated WT animals, SA induced a hypertrophic response as revealed by an increase in fibre CSA. Treating WT animals with rapamycin did not significantly alter the CSA of muscle fibres from sham animals, but it abolished the SA-induced hypertrophic response (Fig. 3A–D and M). These results are consistent with previous studies which have demonstrated that rapamycin can prevent mechanical load-induced hypertrophy (Bodine *et al.* 2001).

We next subjected RR-mTOR transgenic mice to sham or SA surgeries to determine if mTOR, in skeletal muscle cells, is the rapamycin-sensitive element that confers the SA-induced hypertrophic response. The results indicated that unlike in WT mice, a SA-induced hypertrophic response could be detected in both the vehicle and rapamycin treated animals (Fig. 3E–H and M). It was also determined that, similar to the results observed with p70(389) phosphorylation, rapamycin caused a partial inhibition of the hypertrophic response (33% increase in

vehicle vs. 26% increase in rapamycin treated animals). As previously suggested, the partial sensitivity to rapamycin is likely to be due to the effects of rapamycin on the endogenous WT mTOR that exists in the muscles of the RR-mTOR mice. Nonetheless, the results with the RR-mTOR mice indicate that mTOR, within skeletal muscle cells, is the primary rapamycin-sensitive element that confers the mechanical load-induced hypertrophic response.

To determine if the kinase activity of mTOR was necessary for mechanical load-induced hypertrophy, we subjected RRKD-mTOR transgenic mice to sham or SA surgeries. As shown in Fig. 3I–L and M, SA induced a significant increase in the CSA of muscle fibres from vehicle treated RRKD mice; however, the magnitude of the hypertrophic response was relatively small when compared to what was observed in muscles from vehicle treated WT and RR-mTOR mice. This observation suggests that the expression of RRKD-mTOR conferred a partially dominant negative effect on SA-induced hypertrophy. Unexpectedly, rapamycin treatment induced a small, but significant, increase in the fibre CSA of sham RRKD-mTOR mice (Fig. 3M). Moreover, in rapamycin

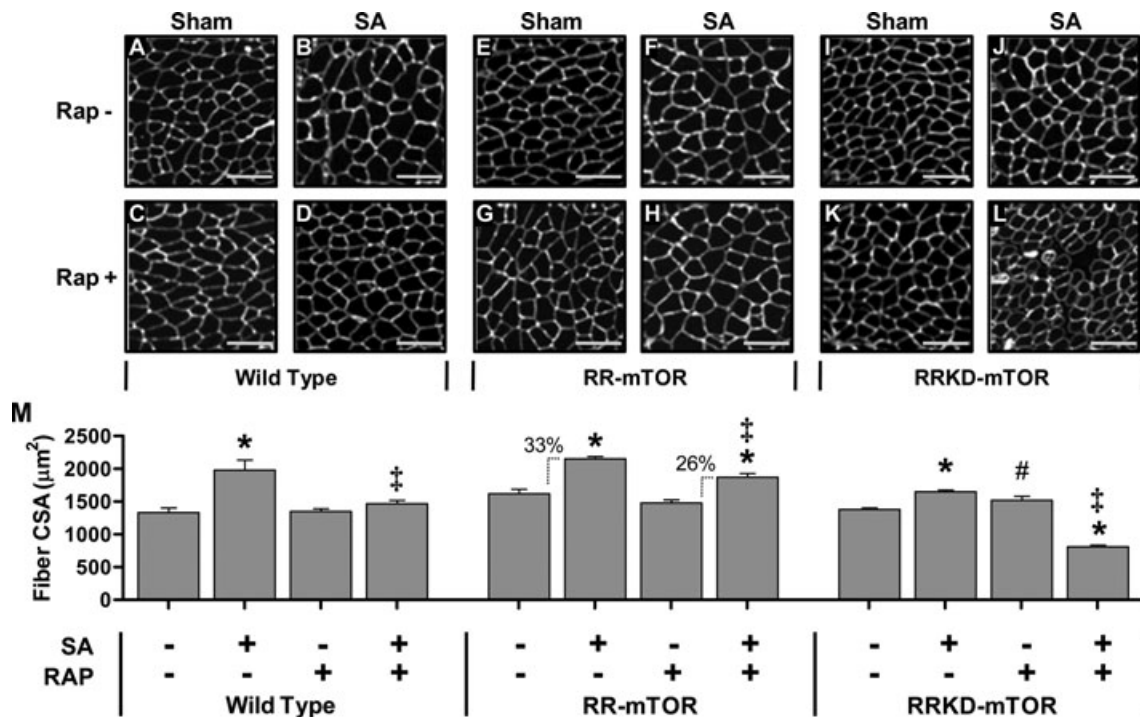


Figure 3. The role of mTOR in mechanical load-induced hypertrophy
 Plantaris muscles from wild-type, RR-mTOR, and RRKD-mTOR mice were subjected to sham or synergist ablation (SA) surgeries. Following the surgery, the mice were administered a daily regime of either vehicle (RAP⁻) or 0.6 mg kg⁻¹ rapamycin (RAP⁺) injections. A–L, at 14 days post surgery, the plantaris muscles were collected and cross-sections from the mid-belly were subjected to immunohistochemistry for laminin. M, the average fibre cross-sectional area (CSA) for each muscle was determined from 150 randomly selected fibres. All values are expressed as the mean + SEM, n = 4–7 muscles/group. *Significant effect of SA within a given drug treatment, #significant effect of RAP within the sham groups, ‡significant effect of RAP within the SA groups, P ≤ 0.05. Scale bars in the images represent a length of 100 μm.

treated RRKD-mTOR mice, SA actually resulted in a substantial reduction in the fibre CSA. It was also noted that these muscles frequently revealed considerable spacing between individual fibres and we suspect that this is a result of oedema. Nevertheless, when taken together, our results indicate that the expression of RRKD-mTOR in conjunction with rapamycin treatment causes mild hypertrophy when muscles are subjected to sham conditions, but atrophy when the muscles are subjected to SA.

The role of mTOR in mechanical load-induced hyperplasia

While determining the concentration of rapamycin required for inhibiting the SA-induced hypertrophic

response, it was noticed that rapamycin eliminated the mechanical load-induced hypertrophy of individual fibres, but it only partially inhibited the increase in the CSA of the whole muscle (Fig. 1A–D). One possibility for this observation is that SA induced not only an increase in fibre size, but also an increase in the number of fibres. Thus, we explored whether the fibre number was altered by SA and, if so, whether this effect was regulated by an mTOR-dependent mechanism. To this end, we first counted the total number of fibres per muscle cross-section in vehicle treated WT sham and SA muscles. The results indicated that SA induced a 59% increase in the number of fibres per cross-section (Fig. 4A, B and M). We then stained the sections for embryonic myosin heavy chain (MHC-emb) to explore whether the increase

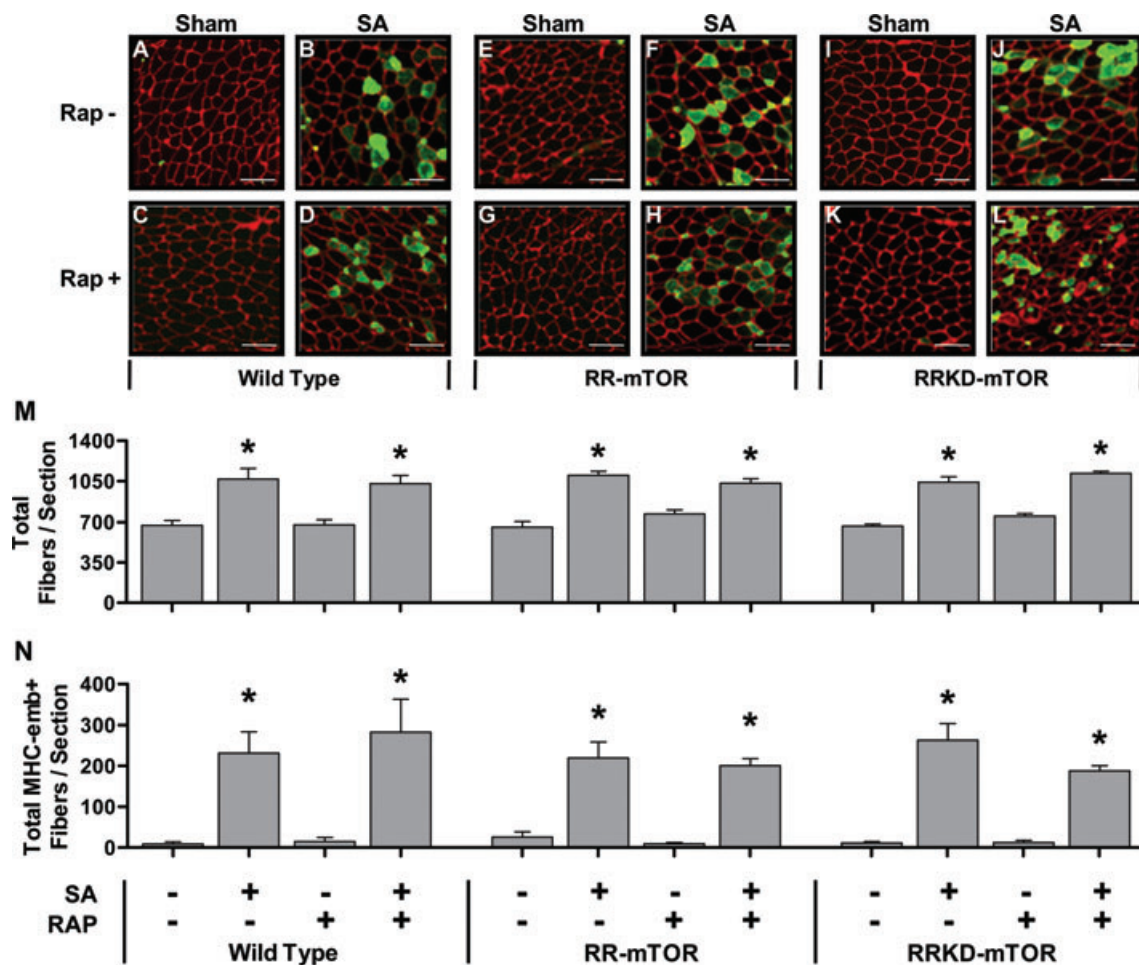


Figure 4. The role of mTOR in mechanical load-induced hyperplasia

Plantaris muscles from wild-type, RR-mTOR, and RRKD-mTOR mice were subjected to sham or synergist ablation (SA) surgeries. Following the surgery, the mice were administered a daily regime of either vehicle (RAP⁻) or 0.6 mg kg⁻¹ rapamycin (RAP⁺) injections. A–L, at 14 days post-surgery, the plantaris muscles were collected and cross-sections from the mid-belly were subjected to immunohistochemistry for laminin (red) and embryonic myosin heavy chain (MHC-emb⁺, green). M and N, the total number of fibres per muscle cross-section (M) and the total number of MHC-emb⁺ fibres per muscle cross-section (N) were manually counted. All values are expressed as the mean + SEM, *n* = 4–6 muscles per group. *Significant effect of SA within a given drug treatment, *P* ≤ 0.05. Scale bars in the images represent a length of 100 μm.

in fibre number may have resulted from the formation of new muscle fibres. Our results revealed that SA induced a large increase in the number of MHC-emb positive fibres (Fig. 4A, B and N). Interestingly, the number of MHC-emb positive fibres in SA muscles was similar to the increase in fibre number. This result is consistent with the notion that SA induced an increase in fibre number via new fibre formation (i.e. hyperplasia, see Discussion).

To determine whether the presumed SA-induced hyperplasia occurred through an mTOR-dependent mechanism, we measured the total number of fibres and the number of MHC-emb positive fibres in WT animals treated with rapamycin. As shown in Fig. 4, rapamycin had no effect on the SA-induced increase in fibre number or the number of MHC-emb positive fibres. Rapamycin also had no effect on these parameters in either the RR-mTOR or RRKD-mTOR mice (Fig. 4E–N). Thus, when combined, these results indicate that SA induces hyperplasia through a rapamycin-insensitive mechanism.

The role of mTOR in mechanical load-induced signalling events

To investigate some of the molecular signalling events associated with the phenotypes induced by mechanical loading, we performed Western blot analysis on signalling molecules that have previously been implicated in the regulation of skeletal muscle growth. In these experiments WT, RR-mTOR and RRKD-mTOR mice were subjected to SA for 7 days and treated with either rapamycin or the vehicle control conditions.

Protein kinase B. Protein kinase B (PKB) is a known effector of insulin/IGF-1 signalling and has the potential to induce muscle hypertrophy through a combination of increasing protein synthesis via the stimulation of mTOR signalling and inhibition of the glycogen synthase kinase 3β (Rommel *et al.* 2001), and by inhibiting protein degradation via the repression of forkhead box containing proteins O-subclass (FoxO) transcriptional activity (Stitt *et al.* 2004). As such, we were interested in determining the effects of mechanical loading and rapamycin on PKB in the WT, RR-mTOR and RRKD-mTOR mice. As shown in Fig. 5A, SA induced an increase in total PKB protein in all genotypes regardless of the presence or absence of rapamycin treatment, which indicates that SA induces an increase in PKB protein expression via a rapamycin-insensitive mechanism.

Next, we examined the phosphorylation of PKB on the threonine 308 residue (P-PKB(308)), which can serve as marker of signalling by phosphatidylinositol-3-kinase (PI3K) (Alessi *et al.* 1997). In vehicle treated WT mice, SA induced an increase (6.8-fold) in P-PKB(308), and this effect was significantly amplified (to 11-fold) when the

mice were treated with rapamycin (Fig. 5B). In vehicle treated RR-mTOR mice, SA also induced an increase in P-PKB(308), but the amplification of this effect by rapamycin did not occur in the RR-mTOR mice (Fig. 5B). In vehicle treated RRKD-mTOR mice, SA again induced a significant increase in P-PKB(308), and similar to WT mice, the magnitude of this effect was amplified by rapamycin treatment. Taken together, these data suggest that rapamycin enhances P-PKB(308) in SA muscles via an mTOR-dependent mechanism that requires mTOR kinase activity. Interestingly, we also found that in RRKD-mTOR sham mice, rapamycin alone induced a large elevation in P-PKB(308), which may, in part, help to explain the mild hypertrophic response that was observed in this condition.

We also examined the phosphorylation of PKB on the serine 473 residue (P-PKB(473)). It was determined that, similar to P-PKB(308), P-PKB(473) was elevated by SA in all three vehicle treated genotypes (Fig. 5C). However, unlike P-PKB(308), the amount of P-PKB(473) in SA samples was not significantly increased by rapamycin treatment in the WT or RRKD-mTOR mice, but it was in RR-mTOR mice (Fig. 5C). Furthermore, unlike P-PKB(308), P-PKB(473) was not altered in rapamycin treated RRKD-mTOR sham mice. Thus, the phosphorylation of PKB on the threonine 308 and serine 473 residues can be regulated by distinct mechanisms and our results indicate that these mechanisms vary in their dependence on signalling by mTOR.

FoxO1 and FoxO3 transcription factors. A direct downstream target of PKB is the FoxO transcription factors. PKB can phosphorylate and inhibit FoxO nuclear translocation, and thus FoxO transcriptional activity (Stitt *et al.* 2004). Furthermore, increases in FoxO expression have been reported in various models of atrophy, and a decrease in FoxO expression can induce hypertrophy (Goodman *et al.* 2011b). We therefore examined the effect of mechanical loading and rapamycin on the phosphorylation state and total protein levels of FoxO1 and FoxO3 in WT, RR-mTOR and RRKD-mTOR mice.

First, we investigated total FoxO1 protein expression levels in sham and SA mice treated with the vehicle control condition. As shown in Fig. 5D, SA induced an increase in total FoxO1 protein in all genotypes. In WT and RRKD-mTOR mice, the amount of FoxO1 in the SA muscles was significantly reduced by rapamycin treatment, but this effect was not observed in RR-mTOR mice. These results indicate that FoxO1 protein expression in SA muscles is regulated, in part, by an mTOR-dependent mechanism that requires mTOR kinase activity.

We next examined the phosphorylation state of FoxO1 on the threonine 24 residue (P-FoxO1(24)) in sham and SA mice treated with or without rapamycin. As shown in Fig. 5E, the amount of P-FoxO1(24) was increased

(~3-fold) in SA muscles from all three genotypes and rapamycin did not significantly alter this response. Thus, it appears that the amount of P-FoxO1(24) in SA muscles is regulated through a rapamycin-insensitive mechanism.

Total FoxO3 protein expression was also increased (~4-fold) with SA in all three genotypes of vehicle treated mice (Fig. 5F). However, unlike the results

observed with FoxO1, rapamycin did not significantly alter this effect. We also examined the phosphorylation of FoxO3 on two different phosphorylation sites (serine 252 (P-FoxO3(252)) and serine 317/320 (P-FoxO3(317/320))) and found both to be increased with SA to a similar extent in all three genotypes. Furthermore, rapamycin had no effect on amount of P-FoxO3(252) or P-FoxO3(317/320)

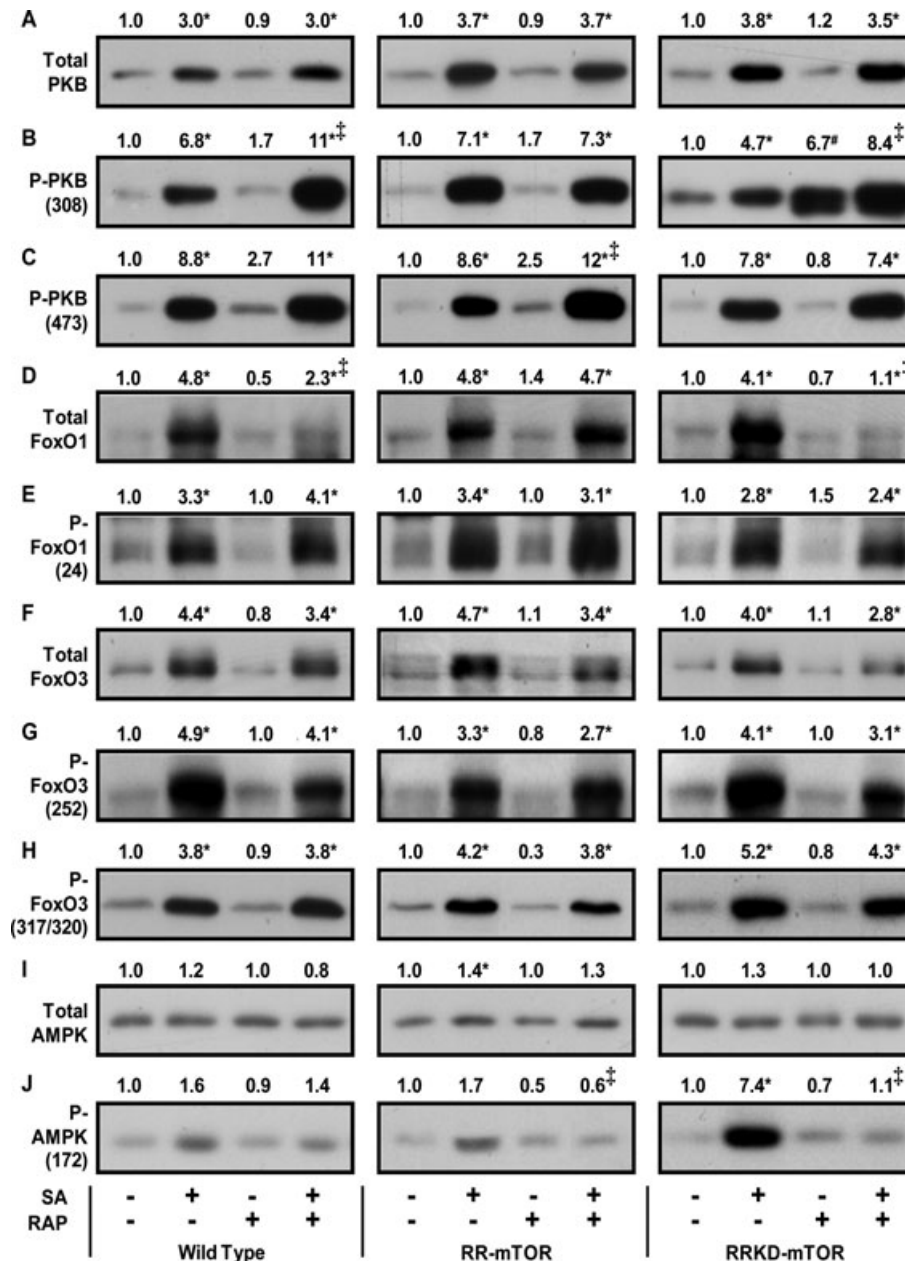


Figure 5. The role of mTOR in mechanical load-induced signalling events

Plantaris muscles from wild-type, RR-mTOR and RRKD-mTOR mice were subjected to sham or synergist ablation (SA) surgeries. Following surgery, the mice were administered a daily regime of either vehicle (RAP⁻) or 0.6 mg kg⁻¹ rapamycin (RAP⁺) injections. At 7 days post-surgery, the plantaris muscles were collected and subjected to Western blot analysis with the indicated antibodies. The values at the top of each blot represent the mean amount of each protein when expressed relative to the values obtained in the vehicle treated sham muscles of the respective genotype, $n = 3-6$ per group. *Significant effect of SA within a given drug treatment, #significant effect of RAP within the sham groups, ‡significant effect of RAP within the SA groups, $P \leq 0.05$.

in SA muscles from any of the genotypes (Fig. 5G and H). Combined, these results indicate that SA induces an increase in total FoxO3, P-FoxO3(252) and P-FoxO3(317/320) via a rapamycin-insensitive mechanism.

AMP-activated protein kinase. AMP-activated protein kinase (AMPK) is a cellular energy sensor that is activated by phosphorylation on its threonine 172 residue (P-AMPK(172)). Active AMPK can, in turn, inhibit energy consuming anabolic pathways (e.g. protein synthesis) and activate energy producing catabolic pathways (e.g. protein degradation) (Steinberg & Kemp, 2009). In skeletal muscle, AMPK has been shown to inhibit mTOR signalling and activate autophagy- and proteasome-dependent proteolysis (Goodman *et al.* 2011b). As such, we were interested in examining changes in AMPK that might be induced by mechanical loading and the potential effects that rapamycin might have on these changes. Our results indicated that total AMPK protein was largely unaffected by SA or treatment with rapamycin. The only exception to this occurred in vehicle treated RR-mTOR mice in which it was determined that SA induced a small (1.4-fold), but significant, increase in total AMPK (Fig. 5I). Similarly, SA and rapamycin had very little effect on P-AMPK(172) in WT and RR-mTOR mice. To our surprise, however, SA induced a large (7.4-fold) increase in P-AMPK(172) in vehicle treated RRKD-mTOR mice (Fig. 5J), and this effect was eliminated when the RRKD-mTOR mice were treated with rapamycin. The SA-induced activation of AMPK in vehicle treated RRKD-mTOR mice may help to explain the smaller hypertrophic response that occurred in these mice, however, it is unlikely that activation of AMPK played a significant role in the atrophic response to SA that was observed when RRKD-mTOR mice were treated with rapamycin (Fig. 3).

The role of mTOR in mechanical load-induced events associated with ribosome biogenesis

To determine the role of mTOR in mechanical load-induced ribosome biogenesis, we first measured total RNA in muscles of WT, RR-mTOR and RRKD-mTOR mice that were subjected to sham or SA surgeries for 7 days and treated with either rapamycin or the vehicle control conditions. Our results demonstrated that in WT vehicle treated mice, SA induced a 2.3-fold increase in total RNA (Fig. 6A). It has been reported that > 85% of total RNA is composed of ribosomal RNA (rRNA) which suggests that the changes in total RNA were mostly likely to be due to changes in rRNA (Young, 1970). Indeed, it was determined that in vehicle treated WT mice, SA induced a 2.8-fold increase in the 28S + 18S rRNA subunits (Fig. 6B). Moreover, in WT mice, the

amount of RNA and the 28S + 18S rRNA subunits found in the SA muscles was partially reduced by rapamycin treatment. This observation suggested that the SA-induced increases in rRNA may occur, in part, through an mTOR-dependent mechanism. To further examine this possibility we measured the effect of SA and rapamycin in RR-mTOR mice. The results were highly comparable to those observed in WT mice and demonstrated that rapamycin still significantly reduced the amount of total RNA and 28S + 18S rRNA in the SA muscles. Similar observations were also made in the RRKD-mTOR mice (Fig. 6). Taken together, our results show that SA induces an increase in rRNA through a combination of both rapamycin-insensitive and rapamycin-sensitive mechanisms. Since the effects of rapamycin were not altered in RR-mTOR mice, our findings also suggest that rapamycin reduces the rRNA in SA muscles via the inhibition of mTOR in non-skeletal muscle cells and/or through an mTOR-independent mechanism.

We next examined total UBF protein levels in an attempt to define the mechanisms through which SA induces an increase in rRNA. We were interested in this measurement because previous studies indicate that ribosome biogenesis is highly regulated by the ability of UBF to induce rDNA transcription (Hannan *et al.* 2003). Furthermore, it has been shown that growth promoting stimuli such as serum stimulation can induce an increase in the abundance of UBF, and consequently rDNA transcription, and this occurs through a rapamycin-sensitive mechanism (Hannan *et al.* 2003). Thus, a reduction in UBF expression might be a component of the mechanism through which rapamycin reduced the rRNA levels in SA muscles. Our results demonstrated that total UBF protein levels increased 5.5-fold with SA in WT vehicle treated mice, but rapamycin treatment did not significantly alter this effect (Fig. 6C). Similar responses of UBF to SA and rapamycin were also observed in RR-mTOR and RRKD-mTOR mice. These results indicate that SA induces an increase in total UBF and this response is driven through a rapamycin-insensitive mechanism. Therefore, contrary to our hypothesis, an increase in total UBF may actually be a component of the rapamycin-insensitive mechanism via which SA induces an increase in rRNA.

Previous studies have shown that the ability of UBF to induce rDNA transcription can be regulated through its interaction with the retinoblastoma protein (Rb) (Cavanaugh *et al.* 1995). Furthermore, it has been reported that in L6 myotubes, the Rb-UBF interaction can be regulated by an mTOR-dependent phosphorylation of Rb (Nader *et al.* 2005). The phosphorylation state of Rb can be measured by changes in the gel mobility of the protein and, as shown in Fig. 6D, SA induced a 2-fold increase in the ratio of hyperphosphorylated Rb (ppRb) to total Rb (ppRb + pRb) in WT vehicle treated mice, and this effect was not altered by rapamycin treatment. Similar

overall observations were also made in the RR-mTOR and RRKD-mTOR mice (Fig. 6D). These results indicate that SA induces an increase in Rb phosphorylation via a rapamycin-insensitive pathway, and hence, this may be another component of the rapamycin-insensitive mechanism through which SA induces an increase in rRNA.

Discussion

Mechanical load-induced hypertrophy

Over a decade ago, it was reported that rapamycin can prevent mechanical load-induced hypertrophy (Bodine *et al.* 2001). Since rapamycin is considered to be a highly specific inhibitor of mTOR, it has become widely concluded that rapamycin prevents mechanical load-induced hypertrophy by inhibiting mTOR signalling. Recently, however, it has become clear that rapamycin only inhibits a subset of mTOR signalling (e.g. signalling by the mTORC1 complex) and rapamycin can exert

non-specific/mTOR-independent effects (Avila *et al.* 2003; Wang & Donahoe, 2004; Osman *et al.* 2009; Zoncu *et al.* 2011). Furthermore, systemic administration of rapamycin would be expected to inhibit mTOR signalling in all cells throughout the body. Hence, it is not clear if the growth inhibitory effects of rapamycin are actually due to inhibition of mTOR signalling, and more specifically, due to the inhibition of mTOR signalling within skeletal muscle cells. In other words, there is strong evidence to support the conclusion that signalling through a rapamycin-sensitive mechanism is necessary for mechanical load-induced hypertrophy, but there is essentially no direct evidence that mTOR is the rapamycin-sensitive element that confers this response. Since a vast number of studies have relied on the assumption that the inhibitory effects of rapamycin are exerted through mTOR, we felt that it was imperative that this assumption be tested. To accomplish this goal, we employed transgenic mice with skeletal muscle specific expression of a various rapamycin-resistant mutants of mTOR. Using these mice we were able to demonstrate

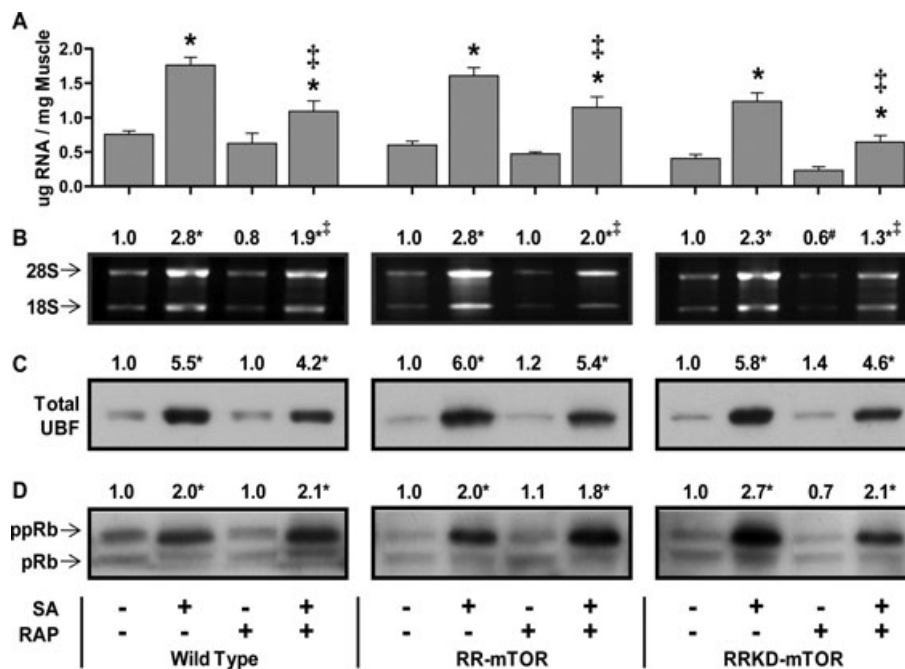


Figure 6. The role of mTOR in mechanical load-induced events associated with ribosome biogenesis Plantaris muscles from wild-type, RR-mTOR, and RRKD-mTOR mice were subjected to sham or synergist ablation (SA) surgeries. Following surgery, the mice were administered a daily regime of either vehicle (RAP⁻) or 0.6 mg kg⁻¹ rapamycin (RAP⁺) injections. At 7 days post-surgery, the plantaris muscles were collected and subjected to RNA isolation or Western blot analysis. *A*, total RNA concentration was determined by spectrophotometric analysis. *B* and *C*, representative images and quantification of the 28S + 18S rRNA bands (*B*) and Western blot analysis for expression of UBF (*C*). The values at the top of the blots represent the amount of rRNA or UBF when expressed relative to the values obtained in the vehicle treated sham muscles of the respective genotype. *D*, representative image and quantification of the Western blot analysis for Rb protein. The values at the top of the blot represent the ratio of hyperphosphorylated Rb (ppRb) to total Rb (ppRb + pRb) and were expressed relative to the values obtained in the vehicle treated sham muscles of the respective genotype. All values are expressed as the mean (+SEM in graphs), $n = 4-6$ per group. *Significant effect of SA within a given drug treatment, #significant effect of RAP within the sham groups, ‡significant effect of RAP within the SA groups, $P \leq 0.05$.

that mTOR, specifically within the skeletal muscle cells, is the rapamycin-sensitive element that confers mechanical load-induced hypertrophy. We also obtained evidence that mTOR kinase activity is necessary for this event (Fig. 3).

Work from our lab has previously shown that the activation of mTOR signalling, within skeletal muscle cells, is sufficient to induce hypertrophy (Goodman *et al.* 2010). We have now also provided evidence that mechanical loading is sufficient to induce mTOR signalling within the skeletal muscle cells (Fig. 2C). Thus, it appears that the mechanical load-induced activation of mTOR signalling, in skeletal muscle cells, is both necessary and sufficient for the induction of a hypertrophic response. Therefore, identifying how mechanical loads activate mTOR signalling in skeletal muscle cells, and defining the downstream targets through which mTOR induces hypertrophy, should provide fundamentally important insight into how mechanical loads ultimately regulate skeletal muscle mass.

Mechanical load-induced hyperplasia

As stated above, rapamycin blocked the mechanical load-induced hypertrophy of individual fibres, but as shown in Fig. 1, it only partially blocked the increase in the CSA of the whole muscle. Based on our results, it appears that the residual increase in muscle CSA is primarily due to an increase in number of fibres per cross-section. Although the exact mechanism that caused the increase in fibre number is not known, there are several potential explanations that could account for it. For example, previous studies have shown that hypertrophy induced changes in the fibre pennation angle can lead to an overestimation of the number of fibres in the muscle cross-section (Maxwell *et al.* 1974; Gollnick *et al.* 1981). However, this only appears to be true when the cross-sections are taken perpendicular to the long axis of the fibres. On the other hand, it has been shown that when cross-sections are taken perpendicular to the long axis of the muscle, the hypertrophy-induced increase in fibre pennation angle actually leads to an underestimation of the total fibre number (Maxwell *et al.* 1974). Since the cross-sections in this study were taken perpendicular to the long axis of the muscle, it does not appear that hypertrophy induced changes in fibre pennation angle could explain the large increase in fibre number that was observed. An alternative possibility is that SA induced splitting/branching of the existing fibres (Vaughan & Goldspink, 1979; Tamaki *et al.* 1996). However, previous studies have shown that the existence of these types of fibres in muscles subjected to SA is very low ($\leq 2.5\%$) (Tamaki *et al.* 1996). Hence, it is unlikely that split/branched fibres account for the $\sim 60\%$ increase in fibre number that was observed. In our opinion,

the most likely explanation for the increase in fibre number is hyperplasia. In support of this possibility, we detected the presence of a significant pool of fibres expressing the developmental MHC-emb isoform, and the number of MHC-emb positive fibres was similar to the increase in fibre number that was induced by SA. We also determined that, in muscles from WT mice, the size of the MHC-emb+ fibres was substantially reduced by rapamycin, and this effect was largely eliminated in muscles from RR-mTOR, but not RRKD-mTOR, mice (Supplemental Fig. 4). Importantly, rapamycin does not reduce the size of fibres in sham muscles (Fig. 3). Therefore, it can be argued that SA induces the formation of MHC-emb+ fibres and these fibres undergo a rapid mTOR-dependent hypertrophic response that requires mTOR kinase activity. Interestingly, rapamycin had no effect on the SA-induced increase in fibre number, or the number of MHC-emb positive fibres, indicating that the increase in fibre number is driven through a rapamycin-insensitive mechanism. This is important because previous studies have shown that myogenesis following myotoxin-induced injury occurs through a rapamycin-sensitive mechanism (Ge *et al.* 2009). Thus, the mechanical load-induced increase in fibre number may be driven through a mechanism that is distinct from that employed during the degeneration/regeneration that occurs in response to myotoxin-induced injury. Further studies will be needed to explore the potentially exciting differences that exist in these two models of new fibre formation.

Mechanical load-induced signalling events

In this study, we have made several novel observations with regards to the role of mTOR in mechanical load-induced signalling events. For example, we show for the first time that SA induces an increase in FoxO1 protein expression and this is regulated, in part, by an mTOR-dependent mechanism that requires mTOR kinase activity. SA also induced an increase in FoxO3 protein expression, but this occurred through a rapamycin-insensitive mechanism. Thus, SA induces an increase in the expression of both FoxO1 and FoxO3 and these effects are, at least partially, regulated by distinct mechanisms. Interestingly, an increase in proteins involved in protein degradation, such as the FoxOs, suggest a mechanical load-induced increase in protein turnover. Indeed, it has recently been shown that SA induces not only an increase in protein synthesis, but also an increase in protein degradation (Miyazaki *et al.* 2011). Thus, our FoxO1 data suggest that mTOR may play a role in mediating the mechanical load-induced increase protein degradation.

Another novel finding was the response of PKB(308) phosphorylation to SA and rapamycin. As shown in

Fig. 5, we determined that SA induced an increase in PKB(308) phosphorylation, and this effect was significantly amplified when the mice were treated with rapamycin. Importantly, the amplification by rapamycin was observed in muscles from WT and RRKD-mTOR mice, but not RR-mTOR mice. Thus, the amplification of PKB(308) phosphorylation appears to be regulated by an mTOR-dependent mechanism that requires mTOR kinase activity. One such mechanism could involve the previously described negative feedback loop through which activated p70^{S6k} phosphorylates the insulin receptor substrate-1 (IRS-1) and, in-turn, causes inhibition of signalling through PI3K/PKB (Tzatsos & Kandrór, 2006; Um *et al.* 2006). Under this mechanism, SA would promote an mTOR kinase-dependent increase in p70^{S6k} activity (e.g. Fig. 2) which subsequently activates the IRS-1 negative feedback loop, and consequently, represses the SA-induced increase in PKB(308) phosphorylation. When WT and RRKD-mTOR, but not RR-mTOR, mice are treated with rapamycin, the SA-induced increase in p70^{S6k} activity is eliminated, and hence, the IRS-1 negative feedback loop is not activated. Consequently, no repression occurs and amplification of signalling through PI3K/PKB is observed. If this mechanism is correct, it would indicate that the mechanical load-induced activation of mTOR represses signalling through the IRS-1/PI3K/PKB pathway. This is potentially significant because such a mechanism could explain how high intensity resistance exercise (which can activate mTOR) can lead to impaired insulin sensitivity (Kirwan *et al.* 1992; Howlett *et al.* 2007).

Interestingly, we observed that rapamycin treatment in sham RRKD-mTOR mice also led to increased PKB(308) phosphorylation, but this effect was not observed in WT or RR-mTOR sham mice. This 'hyperphosphorylation' of PKB(308) has also been observed in skeletal muscle specific Raptor (Bentzinger *et al.* 2008) and mTOR (Risson *et al.* 2009) knockout mice, and suggests that the combination of the RRKD-mTOR construct and rapamycin produce a phenotype that is similar to these knockout models. However, unlike the Raptor and mTOR knockout models, PKB(473) phosphorylation remained unaltered in rapamycin treated sham RRKD-mTOR mice (Bentzinger *et al.* 2008; Risson *et al.* 2009). Thus, the rapamycin treated sham RRKD-mTOR mice appear to be somewhat different than the Raptor and mTOR knockout models. The reasons for both the similarities and differences in these models remain to be determined.

Another interesting finding in the RRKD-mTOR mice was that SA induced a large (>7-fold) increase in AMPK(172) phosphorylation, an effect that was not observed in WT or RR-mTOR mice (Fig. 5). This suggests that the expression of RRKD-mTOR induced changes that made the muscle less capable of dealing with the metabolic demands of SA. Such an effect would not be surprising because mTOR has recently been shown to play a role in

the regulation of both glycolytic and oxidative metabolism (Cunningham *et al.* 2007; Düvel *et al.* 2010). Interestingly, rapamycin treatment eliminated the SA-induced increase in AMPK(172) phosphorylation, which suggests that SA muscles from rapamycin treated RRKD-mTOR mice had an improved energetic status. In this context, it is also worth reiterating that SA muscles from rapamycin treated RRKD-mTOR mice had a significantly greater amount of PKB(308) phosphorylation when compared to SA muscles from vehicle treated RRKD-mTOR mice. Activated PKB has previously been shown to increase ATP production through increased glucose metabolism and oxidative phosphorylation (Gottlob *et al.* 2001; Hahn-Windgassen *et al.* 2005). Thus, the rapamycin-induced increase in PKB(308) phosphorylation may have increased PKB activity sufficiently enough to improve energy supply and, in-turn, prevent the increase in AMPK(172) phosphorylation. However, further investigation will be needed to test this mechanism and other potential interactions between mTOR, PKB, AMPK and regulators of metabolism.

In summary, we have identified some novel and unexpected signalling events that occur in response to mechanical loading and rapamycin treatment, particularly in the RRKD-mTOR mice. Interestingly, unlike in WT and RR-mTOR mice, we observed an atrophic response in muscles from RRKD-mTOR mice that were subjected to SA in conjunction with rapamycin treatment. The mechanism(s) behind this phenotype are not clear, as the atrophic response occurred despite elevated growth promoting PKB signalling and the absence of the potentially catabolic AMPK activation seen in the vehicle treated SA RRKD-mTOR mice. Further studies will be needed to fully characterize the signalling events underlying this atrophic response, the results of which may provide novel insights into the role of mTOR in the regulation of skeletal muscle mass.

Mechanical load-induced ribosome biogenesis

Numerous studies have implicated mTOR in the regulation of ribosome biogenesis (Mayer & Grummt, 2006). It has also been shown that increasing the mechanical loads on skeletal muscle can induce ribosome biogenesis (Goodman *et al.* 2011a). However, to date, the potential role of mTOR in regulating mechanical load-induced ribosome biogenesis has not been reported. In this study we demonstrate that SA induces a significant accumulation of rRNA, and this occurs through both rapamycin-sensitive and rapamycin-insensitive mechanisms. Interestingly, the rapamycin-sensitive component of this mechanism appears to be exerted through mTOR in non-skeletal muscle cells and/or through an mTOR-independent mechanism (Fig. 6). The

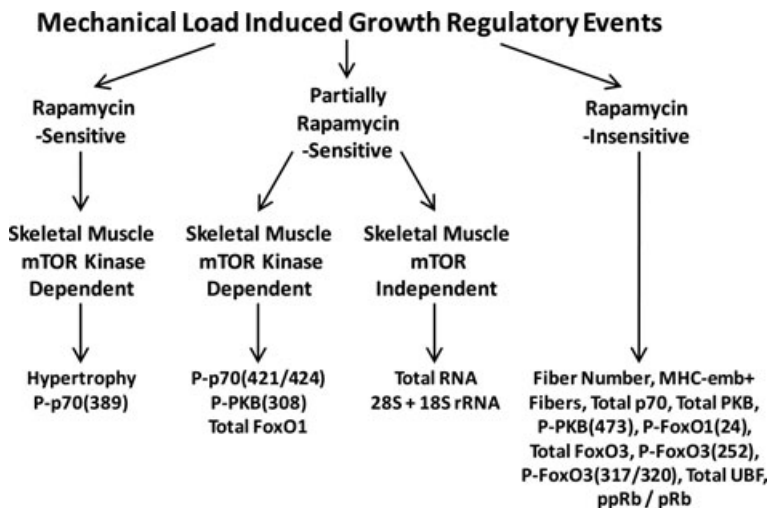


Figure 7. Summary of the effect of rapamycin on various mechanical load-induced growth regulatory events

Growth regulatory events activated by mechanical loading in wild-type animals were categorized as rapamycin sensitive, partially rapamycin sensitive or rapamycin insensitive. The events that showed sensitivity to rapamycin were further separated by their dependence of signalling by mTOR, within the skeletal muscle cells, as determined by the outcomes observed with RR-mTOR and RRKD-mTOR mice. Note, all mTOR-dependent events that were identified in this study required mTOR kinase activity and are therefore referred to as mTOR kinase-dependent events.

exact reason for the rapamycin-sensitive effect is not entirely clear. However, previous studies have shown that SA induces the influx of inflammatory cells into the muscle, and it is likely that these cells contribute to the SA-induced increase in rRNA (DiPasquale *et al.* 2007). Furthermore, rapamycin is a drug with potent immunosuppressive properties (Saunders *et al.* 2001). Hence, we suspect that the reduction of rRNA in SA muscles from rapamycin treated mice results from a suppression of the SA-induced inflammatory response, rather than from inhibition of the ribosome biogenesis that occurs within the muscle fibres. Specifically, we propose that rapamycin attenuates the SA-induced influx of rRNA containing inflammatory cells and this leads to a reduction in the total rRNA per muscle, but not necessarily a reduction in total rRNA within the muscle fibres themselves.

In this study we also explored the potential roles that Rb and UBF might play in the mechanical load-induced increase in rRNA. Our results show that SA induces an increase in Rb phosphorylation and total UBF expression, and it is likely that both of these events contribute to the induction of ribosome biogenesis by SA. Interestingly, the increase in Rb phosphorylation and total UBF was not significantly affected by rapamycin treatment. Thus, both of these events are likely to be part of the rapamycin-insensitive mechanism that contributes to SA-induced ribosome biogenesis. When combined, we interpret the results of this study to indicate that mTOR, within the skeletal muscle cells, does not play a major role in the mechanical load-induced ribosome biogenesis. Although unexpected, this conclusion is consistent with results from a recent study which demonstrated that rRNA synthesis and total rRNA levels were reduced in denervated skeletal muscles despite elevated mTOR signalling (Machida *et al.* 2011). Therefore, it appears that future studies aimed at understanding how mechanical loads regulate ribosome biogenesis should be focused on investigating

pathways that signal through a rapamycin-insensitive mechanism.

Conclusions

As summarized in Fig. 7, the results of this study demonstrate that chronic mechanical loading activates several growth regulatory events, but surprisingly, only a few (e.g. hypertrophy) of these are fully dependent on mTOR signalling within the skeletal muscle cells. In the future, it will be important to understand how mechanical loads activate mTOR signalling in skeletal muscle cells, and to define the downstream targets through which mTOR induces hypertrophy. Identifying the rapamycin-insensitive mechanisms that regulate growth regulatory events such as hyperplasia and ribosome biogenesis will also be an important topic for future studies. A combined understanding of these mechanisms will provide fundamental insight into how mechanical loads regulate skeletal muscle mass and may ultimately lead to the development of therapies that can prevent atrophy during conditions associated with decreased mechanical loading such as spaceflight, bedrest and ageing.

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Author contributions

All of the work performed on this study was conducted in Troy A. Hornberger's laboratory. C.A.G. – conception/design of experiments, collection/analysis of data, writing. J.W.F. – conception/design of experiments, collection/analysis of data, writing. D.M.M. – collection/analysis of data. H.C.L. – collection/analysis of data. J.-S.Y. – collection/analysis of data. B.L.J. – collection/analysis of data. T.A.H. – conception/design of experiments, collection/analysis of data, writing.

Acknowledgements

Special thanks are extended to Dr Jie Chen (Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign) for providing the RR-mTOR and RRKD-mTOR transgenic mice. This work was supported by National Institutes of Health grants AR053280 and AR057347 to T.A.H.