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AN INVESTIGATION OF KINASES RESPONSIBLE FOR SITE-SPECIFIC PHOSPHORYLATION OF p70S6K1 IN SKELETAL MUSCLE FOLLOWING CHRONIC FUNCTIONAL OVERLOADING

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ABSTRACT

The protein kinase p70S6K1 is a known regulator of cell growth as deletion of its gene results in smaller cell size and consequently reduced organ and body sizes. Its activity is modulated through multisite phosphorylation, some of which is mediated by the mechanistic target of rapamycin complex 1 (mTORC1) pathway. The present project was designed to investigate the roles of mTORC1 and that of other signaling pathways in the phosphorylation of p70S6K1 in an animal model of induced hypertrophic growth of skeletal muscle. Within 24 h of male Sprague Dawley rats undergoing unilateral tenotomy to induce chronic functional overloading of the plantaris muscle, phosphorylation of the Thr389 and the Thr421/Ser424 sites on p70S6K1 was significantly elevated compared to a control plantaris from the contralateral leg.

To further investigate regulation of phosphorylation of these sites on p70S6K1, a cell culture model system was employed for easier manipulation of signaling pathways. In HEK293E cells deprived of serum for 2.5 h to impair cell growth, treatment with insulin-like-growth factor 1 (IGF-1) for 30 min resulted in a robust stimulation of the phosphorylation of the Thr389 and the Thr421/Ser424 sites on p70S6K1. In this model system, rapamycin, an inhibitor of mTORC1, and TORIN2, an inhibitor of both mTORC1 and mTORC2, each prevented IGF-1-induced stimulated phosphorylation of the Thr389 site on p70S6K1 but not that of the Thr421/Ser424 sites. The JNK inhibitor SP600125 attenuated the IGF-1-induced stimulated phosphorylation of the Thr421/Ser424 sites on p70S6K1 but not that of the Thr389 site, and in combination with TORIN2 it blocked the effect of IGF1 on

both sites. In contrast, inhibitors of the MAP kinase signaling pathway including UO126 and SB203580 had no effect on the IGF-1-induced stimulated phosphorylation of the Thr 389 and the Thr421/Ser424 sites whereas anisomycin, an activator of the JNK pathway specifically stimulated phosphorylation of the Thr421/Ser424 sites but not the Thr389 site.

To assess a possible role for JNK in mediating phosphorylation of p70S6K1 on the Thr421/Ser424 sites in vivo, rats were treated with SP600125 6 h prior to tissue harvest following tenotomy. SP600125 attenuated the tenotomy-induced elevated phosphorylation of the Thr421/Ser424 sites on p70S6K1 but had no effect on that of Thr389. Phosphorylation of JNK was elevated following tenotomy and this was prevented by treatment with SP600125, providing additional evidence of a role for the JNK signaling pathway in mediating regulation of p70S6K1 in this experimental model of hypertrophic growth. Taken together, the results from these two experimental model systems demonstrate that both the mTORC1 and JNK signaling pathways contribute to the stimulation of cell growth by mediating site-specific phosphorylation of p70S6K1.

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And most importantly, I thank you Lord because……,

Chapter 1

Introduction

mTORC1 Activation and its Role in Skeletal Muscle Hypertrophy

Chronic exercise and over-loading of mammalian skeletal muscle are growth-promoting processes that often lead to increases in muscle fiber size and hypertrophy [1-3]. Stimulation of various pro-growth signaling pathways coupled with increased rates of protein synthesis is essential to these physiological changes [1-4]. Our understanding of the mechanisms and factors that are responsible for inducing skeletal muscle hypertrophy are key to developing therapeutics to treat muscle wasting conditions such as: diabetes, sarcopenia, cachexia, and disuse atrophy [5, 6].

One of the major signaling axes involved in exercise induced adaptive changes to skeletal muscle tissue is the mechanistic target of rapamycin complex 1 (mTORC1), a serine/threonine kinase that regulates protein synthesis and cellular growth [2]. Activation of mTORC1 enables the kinase to phosphorylate its most well-established and characterized downstream effectors: eukaryotic translation initiation factor 4E-binding protein (4E-BP1) and 70 kDa ribosomal protein S6 kinase (p70S6K1), two proteins that play vital roles in the regulation of mRNA translation, cellular proliferation/growth and muscle hypertrophy [7-11].

Phosphorylation of 4E-BP1 controls cellular proliferation [7] whereas phosphorylation of p70S6K1 regulates cell and organismal growth [8, 12]. In cells in culture, it has been shown that 4E-BP1 mediates mTORC1-induced cellular

proliferation, but not the growth of cells [7]. These phenomenon are supported by the observation that mice null for 4E-BP1 and 4E-BP2 are normal in size and develop naturally in comparison to wild-type animals [13]. In contrast, mice lacking p70S6K1 are smaller at birth and exhibit impaired growth, such that adults are significantly smaller compared to their wild-type littermates [14]. Montagne et al. [10] also reported that p70S6K1 is not only responsible for increasing cell size, it was also shown that Drosophila deficient of the S6Kinase gene (dS6K) presented with delays in development and a reduction in body size due to reduced cell size when compared to their wild type counterparts. Additionally, p70S6K1 regulates mRNA translation through phosphorylation of several downstream targets including eukaryotic initiation factor 4B (eIF4B), programed cell death protein 4 (PDCD4) and ribosomal protein S6 (rpS6) [9, 15]. Phosphorylation of these downstream substrates is required for maintaining optimal rates of protein synthesis in serum-deprived cells [9].

Characterization of mTORC1 and mTORC2

Many environmental cues are known to increase the activation and signaling of mTORC1 to its downstream substrates such as: nutrients, insulin-like growth factor 1 (IGF-1), resistance exercise, and chronic functional overloading of skeletal muscle [11]. Flux through the mTORC1 pathway has traditionally been linked to muscle hypertrophy, cellular growth/proliferation, and increased rates of protein synthesis in rodents and humans [2, 3, 5]. It has also been reported that stimulation

of this pathway leads to enhanced muscle fiber size and plasticity that is sustained for extended periods of time following resistance training and chronic overloading [2, 16].

However, these physiological changes and perturbations can be abrogated following administration of rapamycin, a macrocylic antibiotic and antagonist of mTORC1 isolated from bacteria found in the soil on Easter Island [2, 5, 17]. Rapamycin has also been shown to prevent liver re-growth following partial hepatectomy [18] and cardiac hypertrophy that occurs in response to constriction of the ascending aorta [19]. These findings support the vital role of mTORC1 in promoting growth and enhancing overall rates of global protein synthesis [5].

mTOR Complex 1 is composed of the following members: regulatory protein of mTOR (RAPTOR), which serves to increase the kinase activity of mTOR, mLST8/GβL, which binds the kinase domain of mTOR, DEP domain containing interacting protein (DEPTOR), an inhibitor of the complex, and proline-rich AKT substrate 40 (PRAS40) an additional inhibitor of the complex, although its mechanism of action is incompletely understood [11].

Additional studies in yeast and other model organisms led to the discovery of mammalian target of rapamycin complex 2 (mTORC2), which is composed of the following constituents: rapamycin-insensitive companion of mTOR (RICTOR), mLST8/GβL, mitogen-activated protein kinase associated protein 1 (MAPKAP1/mSin1), and pseudo-response regulator 5 (PRR5) [11]. The mTORC2 complex is not as sensitive to acute doses of rapamycin as mTORC1 and is activated

in response to growth factors such as insulin and insulin like growth factor 1 (IGF-1) [11].

Importance of Thr-421/Ser-424 Sites in the C-terminal Domain of p70S6K1

It is well established that mTORC1 is the protein kinase responsible for phosphorylating p70S6K1 on Thr389, which enhances translation, however, determination and establishment of the mechanisms that regulate this process, remain very controversial to date [12]. Previous studies have also provided data to suggest that Thr389 on p70S6K1 is rapamycin sensitive and responsive to fluctuations in nutrient levels within the cellular environment [12]. Schalm et al. [20] observed that the proline-directed sites Thr421/Ser424 located on the C-terminal domain of p70S6K1 are rapamycin insensitive, mitogen dependent, and potentially mTORC1 independent. It was further postulated that phosphorylation of these sites is not required for full activation of p70S6K1. Additionally, Schalm et al. [20] established that insulin-stimulated phosphorylation of Thr421/Ser424 is not blunted by rapamycin in HEK293 cells. It was also suggested by Schalm et al. [20] that stimulation of HEK293 cells with insulin, a mitogen, also promotes phosphorylation of a mutated form of p70S6K1 lacking a TOR signaling (TOS) motif. The TOS motif is a highly conserved sequence located on p70S6K1 that is necessary for mTORC1 activity. [20] Therefore, it was concluded that phosphorylation of the Thr421/Ser424 sites on p70S6K1 is a highly regulated process, which could potentially involve additional kinases other than mTORC1.

Regulation of p70S6K1 Through Site Specific Phosphorylation

Magnuson et al. [12] reported that full activation of p70S6K1 is a stepwise process, involving phosphorylation by various kinases on multiple sites located on the C-terminal domain of the protein by various kinases. Phosphorylation of the C-terminal residues located on p70S6K1 are thought to alter its confirmation which can be described in two models [12]. The conventional model proposes that the protein is in its inactive confirmation when the C-terminal domain is folded over the N-terminal domain which results in auto-inhibition of the kinase [12]. The introduction of mitogens or growth factors within the cellular environment allows the kinase to adapt to a more relaxed confirmation. This exposes the phosphorylation sites located on the C-terminal domain, and allows mTORC1 to phosphorylate Thr389 on the hydrophobic motif, which in-turn leads to the phosphorylation of Thr229 by phosphoinositide-dependent kinase 1 (PDK1) and full activation of p70S6K1 [12]. These ordered phosphorylation events establish that phosphorylation of the C-terminal residues is the rate controlling step in activation of the kinase.

Alternatively, Magnuson et al. [12] postulates that the protein exists in a more relaxed confirmation, and upon stimulation, the turn motif on the protein is phosphorylated at Ser371 by an unknown kinase. This further exposes the C-terminal domain of p70S6K1 and allows PDK1 and mTORC1 to phosphorylate Thr229 and Thr389 respectfully, thus fully activating the kinase. The kinase (s) that phosphorylates the C-terminal residues located on p70S6K1 are unknown to date.

However, in the literature, it is understood and accepted that mTORC1, at least in part, may phosphorylate some of the residues located on the C-terminal domain of p70S6K1.

MAPK's Role in Promoting Phosphorylation of the Thr421/Ser424 Sites

Thr421 and Ser424 are followed by a proline in the primary sequence of p70S6K1; this suggests the possible involvement of a mitogen-activated protein (MAP) kinase in the phosphorylation in one or both sites. The MAPK family of proteins are serine/proline directed kinases that are present in skeletal muscle in the following forms: extracellular signaling regulated kinase (ERK) 1 and ERK 2, p38 MAPK, ERK5, big MAPK and most noteworthy, c-Jun N-terminal kinase (JNK) [21]. Remarkably, chronic exercise can activate MAPK's and induce inflammatory mediated responses that contribute to physiological processes such as: muscle hypertrophy, lipid metabolism, insulin sensitivity and glucose homeostasis [21-23]. Thus in the present study, it was hypothesized that activation of both mTORC1 and one or more MAPK's is responsible for the phosphorylation of p70S6K1 on the Thr421/Ser424 sites in response to chronic functional overloading of the plantaris muscle in male Sprague Dawley rats.

Significance of the Present Study

The goal of the present study was to assess responses of p70S6K1 phosphorylation in a model of muscle hypertrophy (tenotomy) and to identify additional kinases involved in the observed changes. Tenotomy is a model of chronic functional overloading in which the tendons to the gastrocnemius and soleus muscles are severed at the Achilles, thus leading to hypertrophy of the plantaris muscle [24]. This model was chosen due to its relative noninvasiveness and simplicity in inducing chronic overloading of the plantaris muscle in male Sprague Dawley rats, when compared to other models. For example, we chose not to utilize synergist ablation in this study due to its undesirable disturbances to skeletal muscle tissue such as: inflammation, chronic blood loss and prolonged pain burden to experimental animals. We proposed that these negative physiological changes could adversely impact kinase signaling and rates of protein synthesis, which are crucial markers for this study.

We found that phosphorylation of Thr389 and the Thr421/Ser424 sites were enhanced within 24 h following tenotomy.

To further assess these changes we treated HEK293E cells with IGF-1 to identify the kinases involved in mediating phosphorylation of these sites and the order in which their phosphorylation occurs. It was noted that inhibition of mTORC1 blocked IGF-1 induced phosphorylation of both Thr389 and Thr421/Ser424 on p70S6K1. However, unlike that of Thr389, mTORC1 inhibition did not completely reduce phosphorylation of Thr421/Ser424 below the level

observed in serum-deprivation conditions. Thus, it was hypothesized that mTORC1 is at least in part responsible for the IGF-1 induced phosphorylation of the Thr421/Ser424 sites.

Of significance, we found that inhibition of JNK repressed phosphorylation of Thr421/Ser424 and also blunted maximal phosphorylation of these sites under conditions of IGF-1 treatment. This observation provided evidence to suggest that JNK could potentially be one of the kinases that phosphorylates p70S6K1 on the Thr421/Ser424 sites in conjunction with mTORC1. HEK293E cells treated with a JNK activator (anisomycin) provided additional evidence in support of this idea. Upon activation with anisomycin, phosphorylation of Thr421/Ser424 was enhanced to a greater extent compared to vehicle treated HEK293E cells in serum-deprived and IGF-1 treatment conditions, moreover, this effect was attenuated following administration of SP600125. Of particular interest was the finding that phosphorylation of p70S6K1 on Thr421/Ser424 was blunted following treatment with SP600125, a JNK inhibitor, in the plantaris of animals that received tenotomy when compared to the sham operated limbs.

Chapter 2

Objectives

The aim of this study was to investigate the mechanisms by which p70S6K1 is activated and what kinase (s) might be responsible for phosphorylating this growth promoting protein in an animal model that mimics resistance exercise and induces muscle hypertrophy.

It was hypothesized that both mTORC1 and one or more (MAPK) may phosphorylate p70S6K1 on Thr421/Ser424 in response to chronic functional overloading of the plantaris muscle in male Sprague Dawley rats.

Chapter 3

Methodology

Animal Care and Use

The Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine approved the animal facilities and experimental procedures used in this study. Adult male Sprague-Dawley rats (~ 200 – 260 g) were housed in temperature and humidity controlled holding facilities. Prior to the tenotomy procedure, animals were acclimated to a reversed 12:12 h light: dark cycle (lights off at 0700) for one week. Rats were provided standard rodent chow (Harlan-Teklad 8604, Indianapolis, IN) and water *ad libitum.*

Experimental Design of Animal Studies

One week prior to the experimental procedure, rats were provided standard rodent chow (Harlan Teklad 8604, Indianapolis, IN) for a period of 3 h at the start of the dark cycle; water was provided ad libitum. On the day of the surgery (following the 3 h feeding period) rats were anesthetized with isoflurane (3%) supplemented with oxygen via nasal inhalation. Unilateral tenotomy was performed on each rat as previously described [24], by severing the tendons of the soleus and gastrocnemius muscles at the Achilles to induce overloading of the plantaris muscle. As an internal control, a sham surgical procedure was performed on the contralateral leg of the animals by making a 0.5-inch incision in the skin to expose tendons of the

gastrocnemius, soleus, and plantaris muscles at the Achilles. Stainless steel surgical staples (Mik Ran Precision Inc.) and Vet-bond Tissue Adhesive (3M Animal Care Products, St. Paul MN) were then applied to each wound following the tenotomy or sham operation. Animals were administered a subcutaneous injection of an analgesic (Buprenex, 0.03mg/kg body mass) 12 h post surgery. Twenty-four hours after the tenotomy intervention, animals were again anesthetized, and the plantaris muscles from each leg of the animals were excised for subsequent analysis (see below).

Administration of Inhibitors

Animals underwent tenotomy procedure as described above. Six h prior to harvesting of muscle tissue, one-half of the rats received SP600125 (50mg/kg of body mass) dissolved in N-Methyl-2-Pyrrolidone (Sigma Aldrich, St. Louis, MO) and polyethylene glycol. Vehicle (saline) was administered to control animals.

Cell Culture mTOR/MAPK/SAPK-JNK Inhibition and Activation

HEK 293E cells were seeded in 12 well Cell-Bind plates (Corning Cell Bind Surfaces, Corning NY) or 100 mm culture dishes in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin for a 48 h period. On the day of the procedure, cells were incubated in serum free medium for 1.5 h, after which the following were added to the medium for 30 min: TORIN2 (10 nM) (TOCRIS Bioscience, Bristol,

United Kingdom), U0126 (10 μM) (Promega Corporation, Madison, WI), SP600125 (50 μM) (Sigma-Aldrich, St. Louis, MO) SB203580 (10 μM), anisomycin (5mg/ml) (Sigma-Aldrich, St. Louis, MO), PD98059 (20 μM) (Cell Signaling Technology Danvers, MA), or rapamycin (100 nM). Cells were then treated with IGF-1 (10 ng/ml) as indicated for 30 min and harvested in 1 x Laemmli sample buffer. The lysates were subjected to Western blot analysis as described below.

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Western Blot Analysis

The plantaris was excised, cleared of all connective tissue, and homogenized in 7 volumes of homogenization buffer as previously described [5]. Homogenization buffer consisted of HEPES (50mM), Triton-X (0.1%), EGTA (4 mM, pH 8), EDTA (10 mM, pH 8), sodium pyrophosphate (15 mM), β-glycerophosphate (100 mM) and sodium fluoride (25 mM). The homogenate was centrifuged at 2000 x *g* for 3 min at 4°C and the supernatant from the homogenate was combined with an equal volume of 2x Laemmli sample buffer and diluted with 1x Laemmli sample buffer to equal protein concentrations prior to Western blot analysis.

Proteins were separated using either 4-15% or 4-20% Bio-Rad Criterion gels with approximately 20 μg of protein loaded per lane and transferred to polyvinylidene difluoride membranes (PVDF), (Pall Corporation). PVDF membranes were blocked for 1 h at room temperature with 5% nonfat milk in Tris-Buffered Saline and Tween 20) (TBST) and placed on an oscillating platform. The membranes were then incubated with primary antibodies recognizing specific

phosphorylation sites, including p70S6K1 Thr421/Ser424, p70S6K1 Thr 389, c-Jun Ser63, Akt Ser473, p38 Thr180/Tyr182, ERK Thr-202/Tyr-204, and C-Jun N-terminal kinase Thr183/Tyr185 all of which were from Cell Signaling Technology (Danvers MA). Antibodies for GAPDH and alpha Tublin were from Santa Cruz Biotechnology. Blots were developed using a Fluor-Chem MultiFluor System (ProteinSimple, San Jose, CA) with the following reagents: ECL and ECL2, Pierce Biotechnology, Rockford IL.

Statistical analysis

Data are presented as means ± SEM. One-way analysis of variance and Student's *t* test were used to compare differences among groups. $p < 0.05$ was considered statistically significant.

Chapter 4

Results

Tenotomy Enhances Phosphorylation of p70S6K1

Phosphorylation of p70S6K1 on Thr389 was enhanced to 327% of the contralateral limb in the plantaris muscle of animals that underwent tenotomy (Fig 1A & B). Similarly, phosphorylation of p70S6K1 on Thr421/Ser424 was enhanced to 316% of the contralateral limb in the plantaris muscle of animals that underwent tenotomy (Fig 1A & C). Since p70S6K1 on Ser421/Thr424 is both rapamycin insensitive and occurs independent of the TOS-motif in p70S6K1 [20], we sought to identify the pathway/kinases responsible for phosphorylation of these residues in response to chronic functional overloading.

Inhibition of mTOR Enhances p70S6K1 Phosphorylation on Thr421/Ser424

To further investigate phosphorylation of p70S6K1 on Thr421/Ser424, HEK 293E cells were treated with rapamycin, which inhibits mTORC1, or TORIN2, which inhibits both mTORC1 and mTORC2. Following 2.0 h serum deprivation, phosphorylation of p70S6K1 on Thr389, an mTORC1 dependent site, and phosphorylation of Akt Ser473, a site that is directly phosphorylated by mTORC2, were undetectable (Fig 2A). However, when cells were administered IGF1, phosphorylation of both sites was enhanced (Fig 2A). While phosphorylation of

p70S6K1 on Thr421/Ser424 was still observed in cells following serum deprivation, IGF1 administration intensified phosphorylation of this site to 242% of the serum deprived condition (Fig 2B). Additionally, when cells were treated with rapamycin for 30 min prior to IGF1 administration, phosphorylation of Akt (which can activate mTORC1 through its downstream effectors) on Ser473 was still significantly increased by IGF1 administration, however phosphorylation of p70S6K1 on Thr389 was not observed (Fig 2A). To a large extent, rapamycin also prevented the IGF1 induced elevation in Thr421/Ser424 phosphorylation, as IGF1 only stimulated phosphorylation of these sites by 52% in the presence of rapamycin (Fig 2A). TORIN2 prevented IGF1-stimulated phosphorylation of both p70S6K1 Thr389 and Akt Ser473 phosphorylation following administration of IGF1 (Fig 2A). Of important note, treatment of serum-deprived cells with TORIN2 surprisingly enhanced phosphorylation of p70S6K1 on Thr421/Ser424 by 192% compared to vehicle, however IGF1-stimulation in the presence of TORIN2 did not further enhance phosphorylation of these sites (Fig 2A). These results suggest that phosphorylation of p70S6K1 on Thr421/Ser424 can occur independent of mTOR, however the kinase appears to facilitate the IGF1-mediated enhancement in phosphorylation of these sites.

Inhibition of c-Jun N-terminal Kinase Decreases Phosphorylation of p70S6K1

Thr421 and Ser424 are both followed by a proline in the primary sequence of p70S6K1, suggesting the possible involvement of a mitogen-activated protein (MAP)

kinase in the phosphorylation of one or both sites. To delineate whether or not a proline-directed kinase might be responsible for the phosphorylation of p70S6K1 at Thr421/Ser424, cells were serum deprived for 1.5 h and IGF1 treated in the presence of the following MAPK inhibitors: U0126 (MEK inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 inhibitor). As expected, p70S6K1 phosphorylation at Thr389 and Thr421/Ser424 was dampened in cells incubated in serum free medium and enhanced with the addition of IGF1 (Fig 3A). As compared to vehicle, UO126, SP600125, and SB203580 repressed the phosphorylation of extracellular signaling kinase (ERK) 1/2 phosphorylation at Thr202/Tyr204 (Fig 3B), c-Jun phosphorylation at Ser63 (Fig 3C), and p38 phosphorylation at Tyr180/Tyr182 (Fig 3D) respectively in serum deplete medium and with the addition of IGF1. Whereas IGF1 enhanced phosphorylation of ERK at Thr202/Tyr204 (Fig 3B) and p38 at Tyr180/Tyr182 (Fig 3D), the hormone did not enhance c-Jun phosphorylation at Ser63 (Fig 3C). IGF1-mediated enhancement of p70S6K1 phosphorylation at Thr389 was observed in the presence of all three MAPK inhibitors, however SP600125 and SB203580 repressed the degree of stimulation as compared to vehicle (Fig 3E). Importantly, SP600125 repressed p70S6K1 phosphorylation of Thr421/Ser424 by 42% during serum deprivation (Fig 3F). Further, neither U0126 or SB203580 altered p70S6K1 phosphorylation on Thr421/Ser424 in response to IGF1, whereas cells treated with SP600125 exhibited a 38% attenuation as compared to vehicle (Fig 3F). This observation suggests JNK, and potentially p38, contribute to the signaling pathways responsible for phosphorylation of p70S6K1 on Thr421/Ser424.

Inhibition of mTOR with TORIN2 Increases Phosphorylation of c-Jun on Ser63

To expand upon the observation that inhibition mTORC1 and JNK repressed phosphorylation Thr421/Ser424 on p70S6K1, cells were treated with TORIN2, SP600125, or with a combination of SP600125 and TORIN2 under the conditions previously described above. As previously observed, TORIN2 prevented the IGF1-mediated enhancement in phosphorylation of p70S6K1 on Thr389, however SP600125 diminished the effect (Fig 3G). Remarkably, TORIN2 administration produced a significant increase in the phosphorylation of c-Jun on Ser63, suggesting inhibition of mTOR serves to activate JNK (Fig 3C). Treatment with TORIN2 also enhanced phosphorylation of the Thr421/Ser424 sites upon serum deprivation, an effect that was blocked by the combined treatment with SP600125 (Fig 3H). Furthermore, whereas IGF1 enhanced the phosphorylation of Thr421/Ser424 in the presence SP600125, the combined treatment with TORIN2 prevented this effect (Fig 3H), as only a weak signal for Thr421/Ser424 phosphorylation was detected by Western blotting. We initially thought this weak antigenicity to be due to fidelity of the phospho-antibody (ie, a weak interaction with p70S6K1 in the absence of Thr421/Ser424 phosphorylation), however no signal was detected following treatment with lambda phosphatase (results not shown). Importantly, SP600125 did not completely ablate c-Jun Ser63 phosphorylation, thus the possibility exists that the means of JNK inhibition in the present study was not sufficient to completely inhibit JNK [25]. Alternatively, another MAPK, such as p38 could also be

responsible for the observed phosphorylation of c-Jun on Ser63 in the presence of SP600125, given that p38 and JNK share similar downstream substrates [22].

Activation of JNK Enhances mTORC1 Signaling

To further investigate the role of JNK in promoting phosphorylation of p70S6K1, cells were treated with anisomycin (a potent activator of JNK), TORIN2, SP600125, or a combination thereof following 2.5 h of serum deprivation and addition of IGF1 for 30 min. IGF1 was sufficient to enhance phosphorylation of p70S6K1 on Thr389 in all experimental conditions except when cells were treated with TORIN2, confirming its ability to inhibit mTORC1 without regard for the activation status of JNK (Fig 4A). In the absence of anisomycin, phosphorylation of c-Jun on Ser63 was not observed, however when cells were treated with anisomycin, phosphorylation of this site was enhanced (Fig 4A). TORIN2 further enhanced c-Jun Ser63 phosphorylation in the presence of anisomycin, suggesting that inhibition of mTOR promotes JNK kinase activity (Fig 4A compare lanes 5-8 with 13-16). Most strikingly, anisomycin enhanced Thr421/Ser424 phosphorylation of p70S6K1 by 571% under the serum-deprived condition, and another 71% in response to IGF1 treatment (Fig 4B). Further, SP600125 reduced phosphorylation of p70S6K1 on Thr421/Ser424 induced by anisomycin administration. Notably, combined treatment with both TORIN2 and SP600125 was additive in suppressing the anisomycin-induced increase in p70S6K1

Thr421/Ser424 phosphorylation (Fig 4B), further suggesting that both mTOR and JNK regulate these sites.

Chronic Functional Overload Enhances JNK Phosphorylation

To confirm the role of JNK in the phosphorylation of p70S6K1 on Thr421/Ser424 in vivo, rats were administered SP610025 at a time point that was both 24 h after chronic functional overloading (tenotomy) and 6 h prior to tissue harvest. As previously observed, phosphorylation of p70S6K1 on Thr421/Ser424 in the plantaris muscle was enhanced by tenotomy to 212% of that observed in a contralateral sham operated limb (Fig 5A-B). Notably, tenotomy also induced phosphorylation of JNK on Thr183/Tyr185 (Fig 5A & C). In rats treated with SP600125, p70S6K1 phosphorylation on Thr421/Ser424 was impaired by JNK inhibition with SP600125: 51% attenuation of p70S6K1 phosphorylation on Thr421/Ser424 upon sham operation as compared to a 30% attenuation following tenotomy (Fig 5A). Remarkably, the p70S6K1 Thr421/Ser424 phosphorylation mirrored similar attenuation of JNK phosphorylation on Thr183/Tyr185: 45% reduction upon sham operation and 31% reduction upon tenotomy (Fig 5C). Taken together, these data suggest that JNK contributes to the phosphorylation of p70S6K1 on Thr421 in response to chronic functional overloading.

Figure 1.

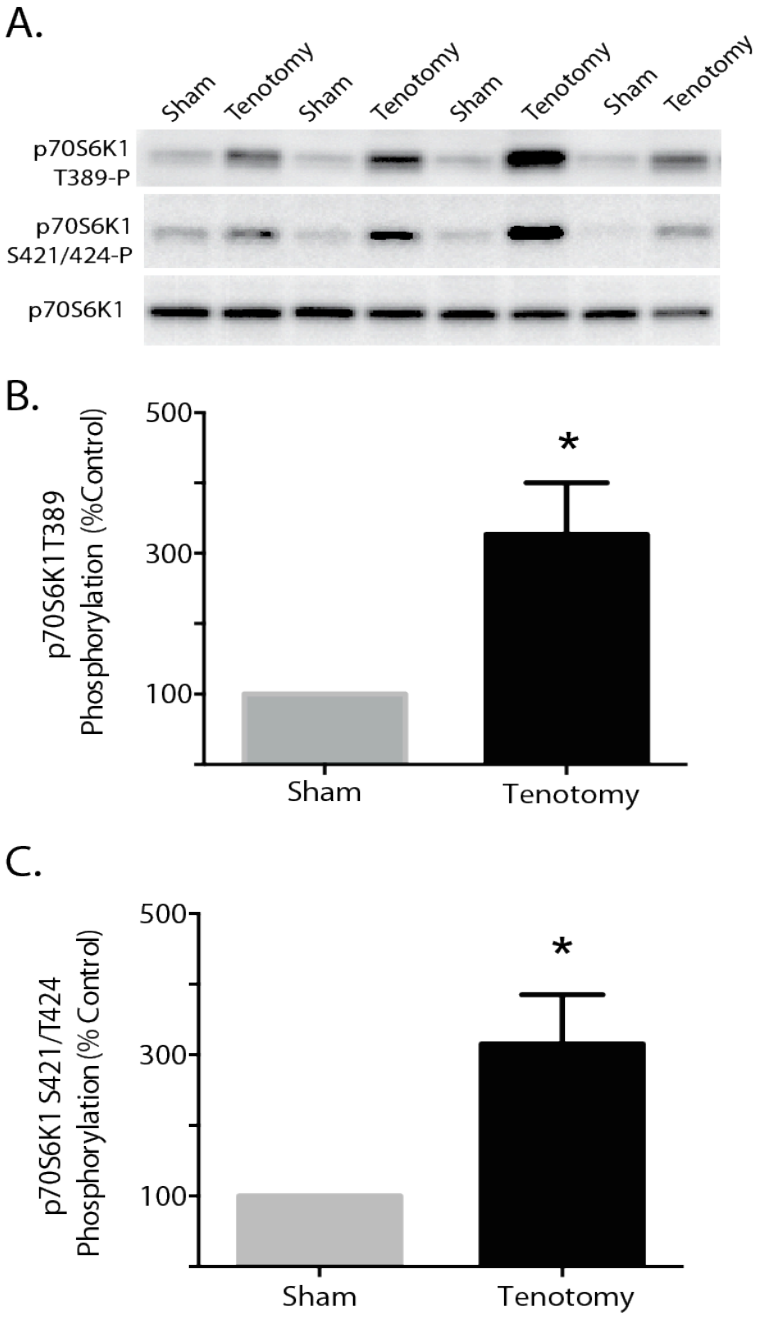

Figure 1: Tenotomy Enhances Phosphorylation of p70S6K1. The plantaris from a tentomized and the contralateral limb were harvested 24-h following the surgical procedure. Muscle samples were then prepared for analysis of p70S6K1 phosphorylation. (A) Western blot analysis of supernatant fractions of muscle homogenates using the phosphospecific anti Thr389 and Thr421/Ser424 p70S6K1 antibodies, as well as a total p70S6K1 antibody. Quantitation of the results for p70S6K1 phosphorylation on Thr389 (B) and Thr421/Ser424 (C) expressed relative to total p70S6K1. Results represent means + SEM (n = 7). Statistical significance is denoted by the presence of *p<0.05 vs. sham.

Figure 2.

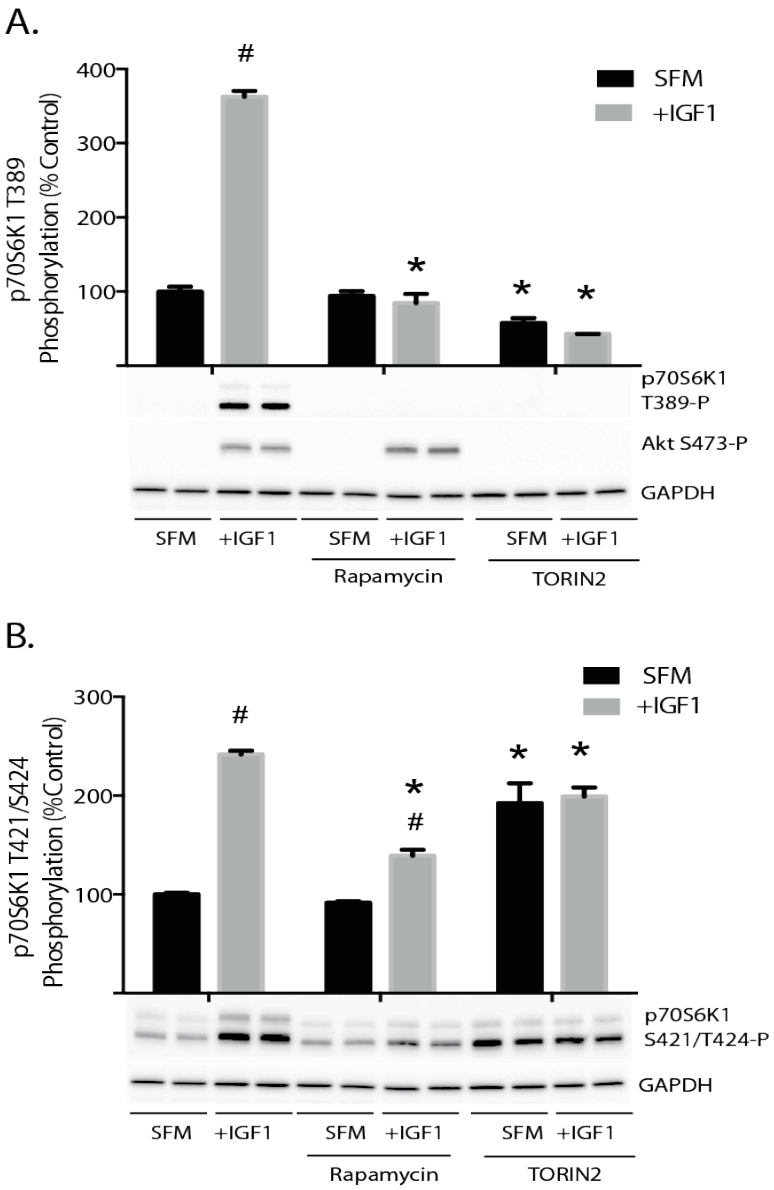

Figure 2: Phosphorylation of p70S6K1 on Thr424/Ser424 is Maintained in HEK293E Cells In the Presence of Rapamycin and TORIN2. HEK293E cells were incubated in serum-free medium (SFM) for 1.5 h to repress phosphorylation of p70S6K1. During the last 30 min of serum deprivation, cells were treated with rapamycin (100 nM) or TORIN2 (10 nM) as indicated. Activation of mTORC1 signaling was achieved by treating cells with IGF1 as indicated. Phosphorylation of p70S6K1 on Thr389 and Ser421/Thr424 and Akt on Ser473 was assessed by Western blot analysis with phosphospecific antibodies 15 min after the administration of IGF1. Quantitation of the results for p70S6K1 phosphorylation on (A) Thr389 and (B) Thr421/Ser424 expressed relative to GAPDH. Representative blots are shown. Values are means + SEM (n = 4). Statistical significance is denoted by the presence of #p<0.05 vs. SFM; *p<0.05 vs. vehicle.

Figure 3.

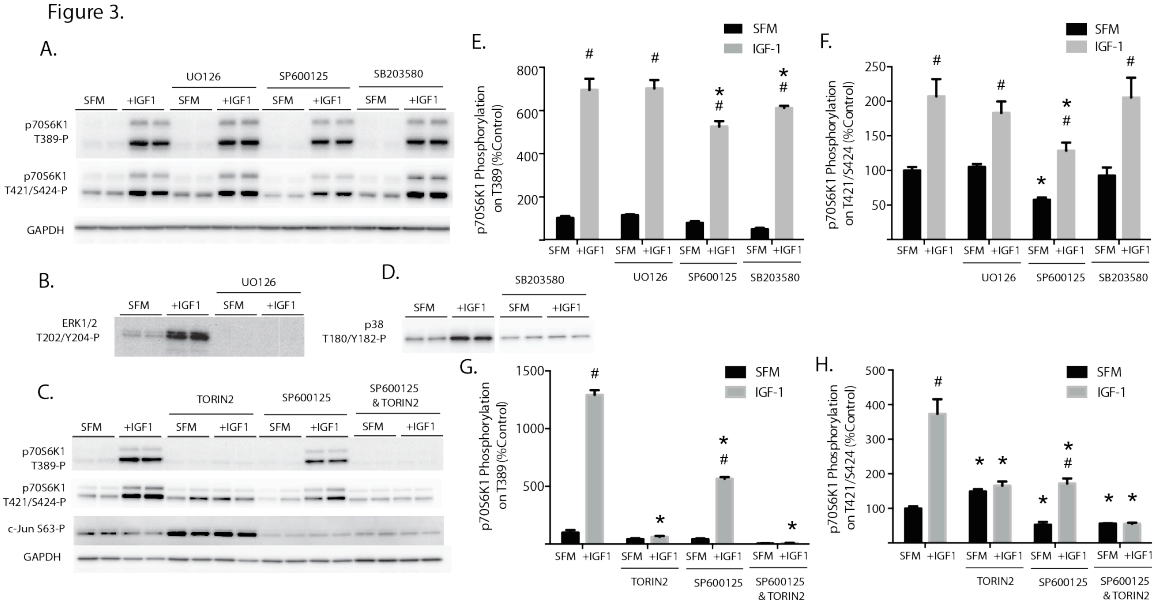

Figure 3: Treatment of HEK293E Cells with JNK Inhibitor Reduces Phosphorylation of p70S6K1. HEK293E cells were incubated in serum-free medium (SFM) for 1.5 h to repress phosphorylation of p70S6K1. During the last 30 min of serum deprivation, cells were treated with U0126 (10 μM), SP600125 (50 μM), SB203580 (10 μM), TORIN2 (10 nm) or a combination of SP600125 and TORIN2 as indicated. Activation of mTORC1 and mTORC2 signaling was achieved by treating cells with IGF1 as indicated. Phosphorylation of p70S6K1 on Thr389 and Ser421/Thr424 (A & C), ERK1/2 on Thr202/Tyr204 (B), c-Jun on Ser63 (F), and p38 on Thr180/Tyr182 (D) was assessed by Western blot analysis 30 min after the administration of IGF1 with phosphospecific antibodies. Results of p70S6K1 phosphorylation on Thr389 (E & G) and Ser421/Thr424 (F & H) were quantitated relative to GAPDH. Representative blots are shown. Values are means + SEM (n = 4). Statistical significance is denoted by the presence of #p<0.05 vs. SFM; *p<0.05 vs. vehicle.

Figure 4.

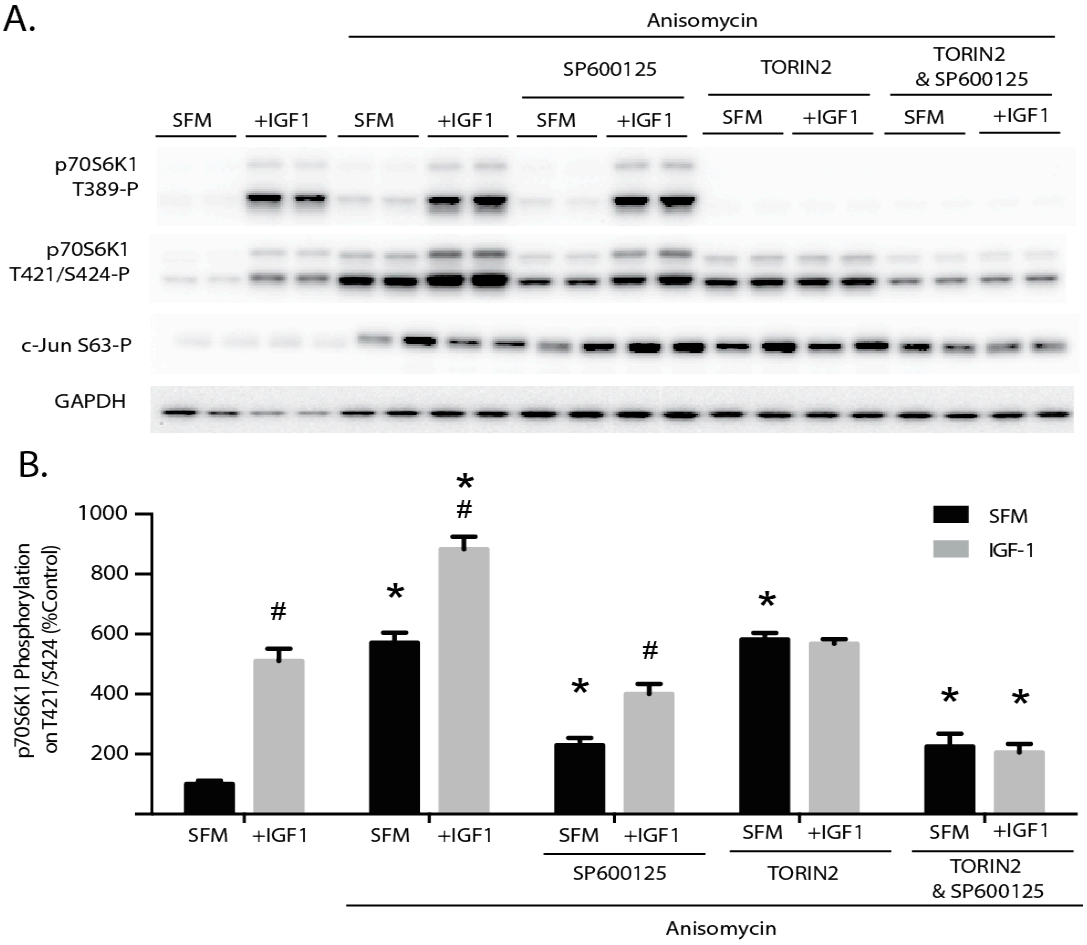

Figure 4: Activation of JNK Enhances Phosphorylation of p70S6K on Thr421/Ser424. HEK293E cells were incubated in serum-free medium (SFM) for 1.5 h to repress phosphorylation of p70S6K1. During the last 30 min of serum deprivation, cells were treated with anisomycin (5mg/mL), SP600125 (50 μM), TORIN2 (10 nm) or a combination as indicated. Activation of mTORC1 signaling was achieved by treating cells with IGF1 as indicated. (A) Phosphorylation of p70S6K1 on Thr389 and Ser421/Thr424, c-Jun on Ser63, and GAPDH expression was assessed by Western blot analysis 15 min after the administration of IGF1. (B) Quantitation of p70S6K1 phosphorylation on Ser421/Thr424 relative to GAPDH. Representative blots are shown. Values are means + SEM (n = 4). Statistical significance is denoted by the presence of #p<0.05 vs. SFM; *p<0.05 vs. vehicle.

Figure 5.

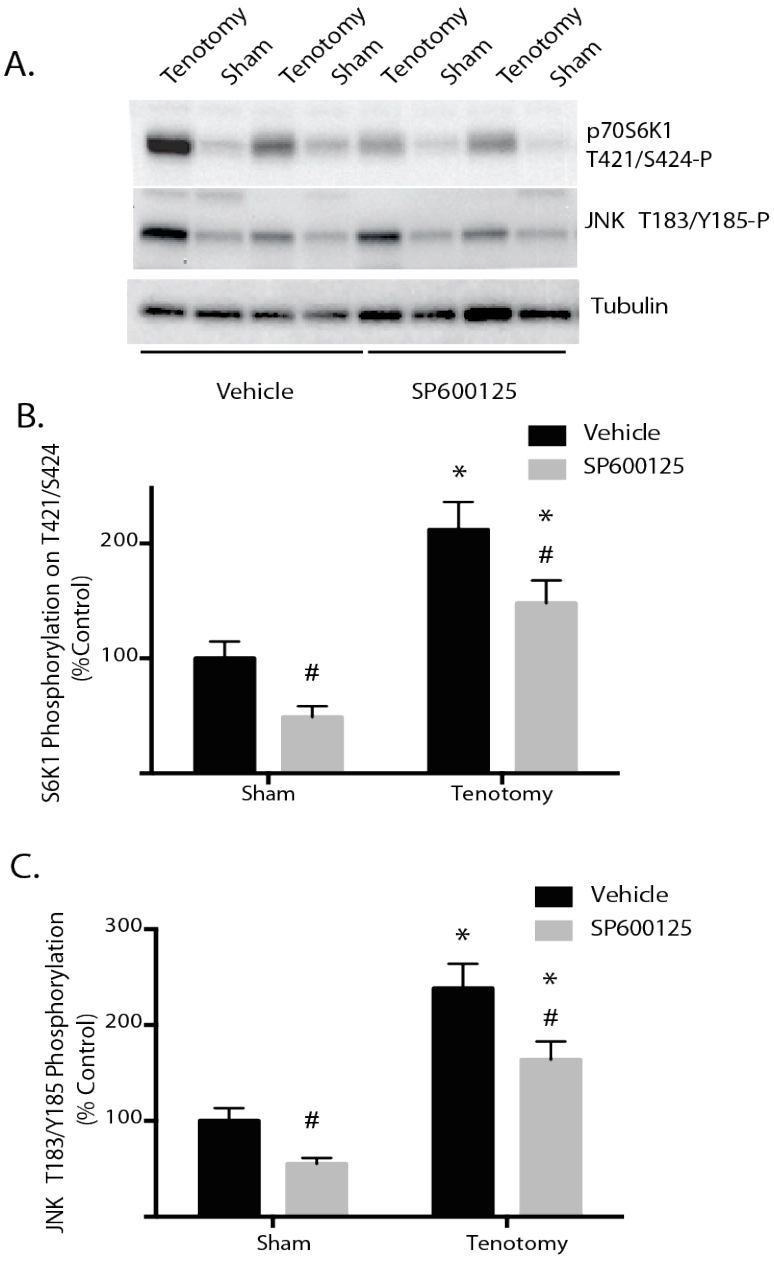

Figure 5: Chronic Functional Overload Enhances JNK Phosphorylation. The plantaris from a tenotomized and the plantaris from the contralateral limb were harvested 24-h following the surgical procedure. As indicated, rats received SP600125 (50mg/kg of body mass) 6 h prior to harvest of muscle tissue. Muscle samples were then prepared for analysis of p70S6K1 phosphorylation. (A) Western blot analysis of supernatant fractions of muscle homogenates using the phosphospecific anti Thr389 and Thr421/Ser424 p70S6K1 antibodies, as well as anti-tubulin. Quantitation of the results for p70S6K1 phosphorylation on Thr421/Ser424 (B) and JNK phosphorylation at Thr183/Tyr185 (C). Results represent means + SEM (n = 9). Statistical significance is denoted by the presence of #p<0.05 vs. vehicle; *p<0.05 vs. sham.

Figure 6.

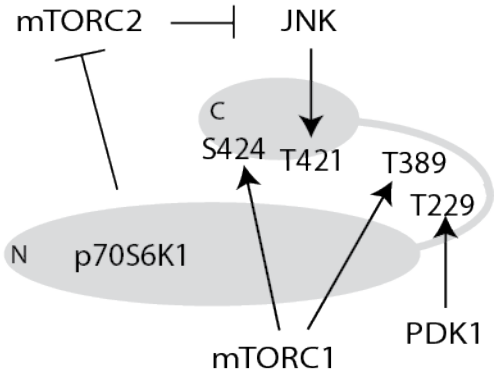

Figure 6: Proposed Model for the Regulation of p70S6K1 on Thr421/Ser421 Sites. Activation of p70S6K1 involves phosphorylation of multiple proline-directed sites including Thr421 and Ser424 located in a pseudo-substrate domain of the C-terminal lobe (C), which in the inactive confirmation serves an auto-inhibitory function by folding over the N-terminal lobe (N). JNK and mTORC1 participate in phosphorylation of this pseudo-substrate domain at Thr421 and Ser424 to induce a conformational change in p70S6K1 that facilitates phosphorylation at Thr389 by mTORC1, phosphorylation of Thr229 by phosphoinositide-dependent kinase 1 (PDK1) and ultimately full activation.

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Chapter 5

Discussion

Phosphorylation of p70S6K1 on the Thr389 and Thr421/Ser424 sites was enhanced following chronic functional overloading of the plantaris muscle in male Sprague Dawley rats (Fig. 1A & 5A). The data presented herein suggest the discovery of a novel mechanism by which p70S6K1 is phosphorylated on the Thr421/Ser424 sites by a kinase other than mTORC1. Given that the C-terminal domain of p70S6K1 is composed of proline directed phosphorylation sites [12], it was anticipated that a MAP kinase might be responsible for phosphorylating Thr421/Ser424 on the protein. This is especially significant and physiologically relevant, given that MAPK's have been shown to be activated in response to exercise in skeletal muscle and that p70S6K1 is a pro-growth kinase that is phosphorylated in a sequential, stepwise and highly regulatory fashion [12, 21, 23].

Reactive oxygen species have been known to increase in contracting skeletal muscle, which may lead to pathophysiological disease states such as: cachexia and diabetes [26]. The activation of MAPK's in skeletal muscle following resistance exercise stimulates inflammatory responses which could potentially ameliorate these processes and increase physiological activities such as: glucose homeostasis, insulin sensitivity, lipid metabolism and skeletal muscle hypertrophy [21]. These observations provide evidence that MAPK's can potentially serve as attractive pharmacological therapeutic interventions for pharmaceutical developers [21, 22].

In the present study we provide evidence to suggest that c-Jun N-terminal kinase, a MAPK, phosphorylation is enhanced following tenotomy (Fig. 1A & 5A). We also provide data to support the conclusion that JNK inhibition leads to a reduction in the phosphorylation of p70S6K1 on Thr389 and on Thr421/Ser424 (Fig. 3A & C) suggesting its role in phosphorylating this kinase.

Taken into account each model proposed by Magnuson et al. [12] for the activation of p70S6K1, phosphorylation of the proline directed sites located in its C-terminal domain seem to play a vital role in allowing for the protein to be fully active. p70S6K1 is involved in numerous processes within the cell that promotes the following: cellular proliferation, growth, hypertrophy of skeletal muscle and increased machinery required for translation. Additionally, p70S6K1 phosphorylation has been reported to be enhanced following bouts of resistance exercise training and still observed during periods of detraining [16]. This suggests that following attenuation of p70S6K1 phosphorylation the kinase can be reactivated very quickly with additional stimuli, thus providing evidence to the sensitivity of the protein, its rapid response to growth promoting stimuli within the cellular environment and its role in inducing skeletal muscle hypertrophy [8].

An additional finding of these data is the regulatory role, which mTORC1 and mTORC2 seem to play in the phosphorylation of p70S6K1 on Thr421/Ser424 and the identity of the additional kinase that may phosphorylate this site. We believe that inhibition of mTORC1 or mTORC2 relieves the suppression of c-Jun N-terminal kinase and allows for it to phosphorylate one or both of the Thr421/Ser424 sites located on p70S6K1 (Fig. 1,2,3,6). Treatment of HEK293E cells with rapamycin (a

mTORC1 inhibitor) does not completely abolish phosphorylation of these sites; this effect is also noted with administration of TORIN2, an inhibitor of both mTORC1 and mTORC2 (Fig. 2). Of principle importance is the effect of inhibiting mTORC1 and mTORC2 with TORIN2 and the phosphorylation of p70S6K1 on Thr421/Ser424 that persists (Fig. 3 & 4), which seems to be mitogen independent, phosphorylation is noted in serum deprivation conditions and in the presence of IGF-1 (Fig. 3.). This finding agrees with that of Schalm et al. [20]. However, Schalm et al. did not hypothesize that a MAPK could be responsible for phosphorylating one or both of these sites and that the kinase activity could potentially be regulated by mTORC1 or mTORC2, a finding that is novel to the present study.

Additionally, chronic functional overloading induces stress in skeletal muscle, and to maintain pro-growth conditions, a MAPK is activated, presumably JNK, which phosphorylates p70S6K1 on Thr421/Ser424 located on the proteins C-terminal domain, which is vital to its function. Hence it could be concluded that phosphorylation of this site is highly regulatory and maintained in conditions of stress and pro-growth in muscle such as: remodeling, hyperplasia, and hypertrophy following resistance training and chronic functional overloading.

It is with some degree of reservation that we can conclusively confirm that c-Jun NH2 terminal kinase is in part responsible for phosphorylating p70S6K1 on Thr421/Ser424 given that the data seem to suggest that p38 could play a role as well. When we treated HEK293E cells with anisomycin, a potent activator of the MAPK signaling axis, phosphorylation of C-Jun on Ser63 was noted in the presence of the JNK inhibitor SP600125. This seemed to suggest a possible role of the kinase

in phosphorylating the Thr421/Ser424 sites. Additionally, p38 and JNK share similar down stream effectors therefore some degree of cross talk may well exist between the kinases under these conditions [22].

In conclusion, the present study has provided data to suggest that a kinase other than mTORC1 may phosphorylate p70S6K1 on Thr421/Ser424, and this effect is mitogen independent and rapamycin insensitive. C-Jun NH2 terminal kinase could potentially be the kinase that is responsible for phosphorylating this site in conjunction with mTORC1. p70S6K1 when activated by mTORC1 is a pro-growth kinase that is essential to muscle hyperplasia and hypertrophy. Additionally, MAPK's are activated in skeletal muscle following resistance exercise and they have been shown to mediate inflammatory signaling that contributes to many key physiological processes that prevent and manage symptoms of chronic illnesses [21, 22].

Future studies will be conducted to further investigate JNK's role in phosphorylating p70S6K1 such as: site directed mutagenesis of Thr421/Ser424, and utilizing conditional knock out cell lines of RAPTOR/RICTOR components of the mTORC1 and mTORC2 complex. These studies will provide further evidence of the kinases responsible for phosphorylating this site and the role of mTOR in regulating the activity of the additional kinase involved in p70S6K1 activation.

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