

Eukaryotic initiation factor 2B epsilon induces cap-dependent translation and skeletal muscle hypertrophy

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Non-technical summary Skeletal muscle comprises ~40% of total body mass, and the control of muscle mass has significant effects on overall health. Skeletal muscle mass is determined by the balance of protein synthesis and degradation within muscle cells. We sought to determine which cellular proteins that control protein synthesis within muscle cells are associated with muscle growth after resistance exercise, a potent growth stimulus. We identified two proteins that were associated with muscle growth in humans: p70S6K and eIF2Bε. Follow up studies determined that eIF2Bε alone is sufficient to induce muscle growth. This is the first study to determine that this protein can induce skeletal muscle growth. These results further our understanding of how skeletal muscle responds to resistance exercise.

Abstract The purpose of this study was to identify signalling components known to control mRNA translation initiation in skeletal muscle that are responsive to mechanical load and may be partly responsible for myofibre hypertrophy. To accomplish this, we first utilized a human cluster model in which skeletal muscle samples from subjects with widely divergent hypertrophic responses to resistance training were used for the identification of signalling proteins associated with the degree myofibre hypertrophy. We found that of 11 translational signalling molecules examined, the response of p(T421/S424)-p70S6K phosphorylation and total eukaryotic initiation factor 2Bε (eIF2Bε) protein abundance after a single bout of unaccustomed resistance exercise was associated with myofibre hypertrophy following 16 weeks of training. Follow up studies revealed that overexpression of eIF2Bε alone was sufficient to induce an 87% increase in cap-dependent translation in L6 myoblasts *in vitro* and 21% hypertrophy of myofibres in mouse skeletal muscle *in vivo* ($P < 0.05$). However, genetically altering p70S6K activity had no impact on eIF2Bε protein abundance in mouse skeletal muscle *in vivo* or multiple cell lines *in vitro* ($P > 0.05$), suggesting that the two phenomena were not directly related. These are the first data that mechanistically link eIF2Bε abundance to skeletal myofibre hypertrophy, and indicate that eIF2Bε abundance may at least partially underlie the widely divergent hypertrophic phenotypes in human skeletal muscle exposed to mechanical stimuli.

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Abbreviations 4E-BP1, eIF4E binding protein 1; eIF, eukaryotic initiation factor; FITC, fluorescein isothiocyanate; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; GSK3, glycogen synthase kinase 3; GST, glutathione *S*-transferase; HA, haemagglutinin; MFA, mean fibre area; MR, modest responder; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; NR, non-responder; p70S6K, p70 ribosomal protein S6 kinase; PI3K, phosphatidylinositol 3-kinase; rpS6, ribosomal protein S6; Rheb, Ras homologue enriched in the brain; TRITC, tetramethylrhodamine isothiocyanate; XR, extreme responder.

Introduction

Translation initiation pathways, including PI3K/Akt/mTOR and eIF2B, are key determinates of cell size regulation in a number of model systems including skeletal muscle (Bodine *et al.* 2001; Rommel *et al.* 2001; Hardt *et al.* 2004; Lai *et al.* 2004). Furthermore, mechanical load, a stimulus that induces skeletal muscle cell (i.e. myofibre) hypertrophy, increases the activity of translation initiation pathways in both animal (Baar & Esser, 1999; Parkington *et al.* 2003) and human (Blomstrand *et al.* 2006; Coffey *et al.* 2006; Dreyer *et al.* 2006; Mayhew *et al.* 2009) muscle leading to increased functional and metabolic reserves of the tissue. It is therefore important to identify which endogenous proteins controlling mRNA translation initiation are most influential in determining myofibre hypertrophy in response to mechanical load.

Translation initiation is thought to be the rate limiting step in protein synthesis (Augert *et al.* 1986). An extensively studied signalling pathway that has been shown to increase translation initiation rates of 5'-capped mRNA is that involving the serine/threonine kinase mammalian target of rapamycin (mTOR). As a component of the mTOR complex 1 (mTORC1), mTOR phosphorylates at least two downstream targets that control translation: eIF4E binding protein 1 (4E-BP1) and the 70 kDa isoform of ribosomal protein S6 kinase 1 (p70S6K). Together, mTORC1-dependent signalling aids in the creation of a mature, translation-competent ribonucleoprotein complex.

Another component of the translational machinery that exerts rate-limiting influence over translation rates in some scenarios is eIF2 (Kimball *et al.* 1998). The GTP-bound form of this initiation factor aids in initiator methionine tRNA (Met-tRNA_i) binding to the AUG start codon, thereby supplying the initial amino acid in a nascent polypeptide chain. Recycling of GDP for GTP on eIF2 is performed by eIF2B, which is composed of α - ϵ subunits (Webb & Proud, 1997). Importantly, the cellular abundance of the catalytic ϵ subunit was shown to be regulated by mechanical load in skeletal muscle *in vivo* at the level of mRNA translation (Kubica *et al.* 2005), while exogenous overexpression of eIF2B ϵ was sufficient to increase eIF2B guanine nucleotide exchange factor (GEF) activity (Tuckow *et al.* 2010) and overall protein synthesis (Balachandran & Barber, 2004; Hardt *et al.* 2004). Phosphorylation of eIF2B ϵ can occur at numerous sites and by multiple kinases, and is generally considered to be inhibitory. Most well-characterized is the GSK3 β -directed inhibition of eIF2B ϵ by S539 (corresponding to S535 in mouse) phosphorylation. To date, the specific contribution of increased eIF2B ϵ abundance or activity to the overall phenotypic outcome of myofibre hypertrophy remains unclear.

The specific signalling proteins altered by an acute bout of unaccustomed mechanical load have been interpreted as implicit drivers of myofibre hypertrophy after a period of repeated exposures to mechanical stimuli (i.e. training) in humans (Dreyer *et al.* 2008; Wilkinson *et al.* 2008; Burd *et al.* 2010). However, the multifaceted nature of the signalling response of mechanically loaded skeletal muscle makes it particularly difficult to segregate mechanisms that are instructive for, as opposed to simply permissive of or coincident with, myofibre hypertrophy *in vivo*. We have previously applied K-means cluster analysis to delineate humans that experience robust (extreme responders, XR), moderate (moderate responders, MR), or no hypertrophy (non-responders, NR) in response to a standardized lower extremity resistance training programme (Bamman *et al.* 2007; Kim *et al.* 2007; Petrella *et al.* 2008). Given the limitations of human research, this cluster-centred model may be the most robust analytical approach available to reveal the regulatory mechanisms mediating mechanical load-induced hypertrophy as opposed to those induced by mechanical load *per se* but not instructive for hypertrophy.

The purpose of this study was to identify translational signalling components in skeletal muscle that are responsive to mechanical load and may be partly responsible for myofibre hypertrophy *in vivo*. To accomplish this, we employed a multilayered approach beginning with a human cluster model, followed by targeted studies *in vitro* and in mice *in vivo*. We found that of 11 translational signalling molecules examined in humans, only the response of p(T421/S424)-p70S6K phosphorylation and eIF2B ϵ protein abundance 24 h after a single bout of unaccustomed resistance exercise was coincident with myofibre hypertrophy following 16 weeks of resistance training. Overexpression of eIF2B ϵ alone was sufficient to induce cap-dependent translation *in vitro* and hypertrophy in mouse skeletal muscle *in vivo*. However, genetically altering p70S6K activity had no influence over eIF2B ϵ protein abundance. These are the first data that mechanistically link eIF2B ϵ abundance to skeletal myofibre hypertrophy, and that this may at least partially underlie the widely divergent hypertrophic phenotypes in human muscle exposed to mechanical stimuli.

Methods

Ethical approval

The project was conducted in accordance with the standards set forth by the *Declaration of Helsinki* and was approved by the Institutional Review Boards of both the University of Alabama at Birmingham and the Birmingham Veterans' Affairs Medical Center. Written informed consent was obtained from each volunteer prior to participation. All animal experiments in this study

followed protocols approved by the Animal Care and Use Committee at the University of Wisconsin – Madison, and were in accordance with the guidelines of *The Journal of Physiology* (Drummond, 2009).

Plasmid construction

Plasmid DNA containing mouse eIF2B ϵ (IMAGE clone ID 4211776) was purchased from Invitrogen (Carlsbad, CA, USA). Both the full-length and C-terminal deletion mutant (deleted after amino acid 529) were subcloned, each with the addition of a C-terminal HA-tag, into the pRK7 vector using *Sall/EcoRI* sites. Green fluorescent protein (GFP) was cloned into the same vector using identical sites. Myc-tagged p70S6K mutants (T389E and T389A) were kindly provided by Dr. George Thomas (University of Cincinnati) and have been described previously (Pearson *et al.* 1995). The p Δ EMCV luciferase reporter (Carter & Sarnow, 2000) was a gift from Dr. Sunnie Thompson (University of Alabama at Birmingham). The plasmids encoding GFP, HA-tagged Ras homologue enriched in the brain (Rheb), and glutathione S-transferase (GST)-tagged p70S6K used in mouse overexpression studies have been previously described (Goodman *et al.* 2010).

Human subjects

Sixty-six adults recruited from the Birmingham, Alabama metropolitan area completed the 16 week resistance training study. All participants were screened by a health history questionnaire and older subjects also passed a physical exam and graded exercise stress test. Subjects were not obese (BMI <30) and free of any musculoskeletal or other disorders that might have affected their ability to complete testing and/or resistance training. Subjects had not experienced any leg resistance training in the 5 years prior to initiating the study protocol. None of the subjects were being treated with pharmacological interventions that are known to influence muscle mass or that may have interacted with the exercise stimulus (such as testosterone, GH, IGF-1, or immunosuppressive therapy).

Using statistical (K-means) cluster analysis, subjects were classified *post hoc* into three clusters based on changes in vastus lateralis mean myofibre cross sectional area (MFA, μm^2) across 16 weeks training as described previously (Bamman *et al.* 2007). The resulting three clusters – XR ($n=17$; change in MFA \pm SEM = $+2,475 \pm 140 \mu\text{m}^2$), MR ($n=32$; $+1,111 \pm 46 \mu\text{m}^2$), and NR ($n=17$; $-16 \pm 99 \mu\text{m}^2$) – were characterized in detail elsewhere (Bamman *et al.* 2007). It is noteworthy for this report that the clusters did not differ in training intensity, volume, or weekly adherence to the programme. A random subset of

subjects ($n=8-10$ XR, $13-15$ MR, and 8 NR) was analysed for translation initiation markers in the current study.

Resistance training

A more extensive description of the resistance training programme performed by these subjects is provided elsewhere (Bamman *et al.* 2007). Briefly, participants completed a 16 week progressive training regimen consisting of three sets of 8–12 repetitions to volitional fatigue with 90 s recovery between sets on the squat, machine leg press, and knee extension exercises performed 3 times per week. All participants were supervised by a Certified Strength and Conditioning Specialist (National Strength and Conditioning Association) or Certified Health Fitness Instructor (American College of Sports Medicine).

Human tissue collection and processing

Fasted morning muscle biopsy samples were collected using routine methods (Evans *et al.* 1982) at baseline, 24 h after the first full bout of resistance exercise at week 1, and 24 h after a bout of resistance exercise at week 16 of the training programme. We chose to sample at 24 h post-exercise since muscle protein synthesis has been found to be elevated at this time point (Phillips *et al.* 1997), and indeed we have found this to be the case in a subset of the subjects examined here (Mayhew *et al.* 2009). Although many transient signalling events are likely to return to baseline levels by 24 h, we postulated that the 24 h time point would allow us to capture key events that drive protein synthesis during recovery without interference from the myriad processes activated during and shortly after resistance exercise. Approximately 70 mg of tissue were mounted cross-sectionally in liquid nitrogen-cooled isopentane for subsequent histological analysis and the remainder was weighed, divided, and snap frozen (30–35 mg per tube) in liquid nitrogen. Snap frozen samples were separated into cytosolic and membrane fractions as described previously (Mayhew *et al.* 2009), with Western blotting performed on the cytosolic fraction only.

Animals

Eighteen female FVB mice 8–10 weeks of age were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and randomly assigned to one of three experimental groups: GFP ($n=7$), eIF2B ϵ ($n=5$), and eIF2B ϵ -S535A ($n=6$). Mice used for expression of GFP ($n=3$), GFP + GST-p70S6K ($n=3$), or HA-Rheb + GST-p70S6K ($n=4$) were described previously (Goodman *et al.* 2010).

Skeletal muscle transfection

Mice were anaesthetized with 100 mg kg⁻¹ ketamine plus 10 mg kg⁻¹ xylazine and the tibialis anterior (TA) muscle was transfected using electroporation as previously described (Goodman *et al.* 2010) with 2.5 µg µl⁻¹ for GFP, 4.0 µg µl⁻¹ for eIF2Bε-HA, or 4.0 µg µl⁻¹ eIF2Bε-S535A-HA dissolved in 12 µl sterile phosphate buffered saline (PBS). Similar methods were used for the injection of GFP, HA-Rheb, and GST-p70S6K (Goodman *et al.* 2010). Mice were allowed to recover for 2 or 7 days before muscle harvest, depending upon the experiment.

Measurements of transfected muscle fibre cross sectional area

Mice were killed by cervical dislocation, after which muscles were excised and fixed in ice cold PBS containing 4% paraformaldehyde with gentle rocking at 4°C for 30 min. The fixed muscles were submerged in optimal cutting temperature compound and frozen in liquid nitrogen chilled isopentane. Cross sections (10 µm thick) from the mid-belly of the muscle were obtained with a cryostat and fixed in -20°C acetone for 10 min. Sections were warmed to room temperature for 5 min and then rehydrated with cool steam vapours. Under gentle rocking, the rehydrated sections were incubated in PBS for 15 min followed by a 20 min incubation in solution A (PBS containing 0.5% bovine serum albumin (BSA) and 0.5% Triton X-100). Sections were then incubated with rabbit anti-laminin (Sigma-Aldrich, St Louis, MO, USA) and rat anti-HA (Roche, Madison, WI, USA) antibodies dissolved in solution A for 1 h at room temperature. Sections were washed with PBS and then incubated with anti-rabbit TRITC-conjugated and anti-rat FITC conjugated secondary antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) dissolved in solution A for 1 h at room temperature. Finally, the sections were washed with PBS and mounted with Vectashield mounting media (Vector Laboratories, Burlingame, CA, USA). Transfected fibres (HA or GFP positive) and laminin were identified in dual fluorescence images captured through FITC and TRITC cubes on a Nikon 80i epi-fluorescence microscope. The images were merged with Nikon NIS Elements D image analysis software and the cross-sectional area (CSA) of 30–90 randomly selected transfected and non-transfected fibres per sample were measured by tracing the laminin stain of individual fibres. All CSA analyses were performed by investigators blinded to the treatment. The CSA of the transfected and non-transfected fibres were plotted on a histogram and the average CSA of each was calculated for each sample.

Cell culture

C2C12, L6 (ATCC, Manassas, VA, USA), and HEK293T cells (a gift from Dr. Sunnie Thompson, University of Alabama at Birmingham) were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM Glutamax (Invitrogen), 50 U ml⁻¹ penicillin, and 50 µg ml⁻¹ streptomycin. All cells were grown in a 37°C humidified incubator at 5% CO₂.

Transient transfections

C2C12 and L6 cells were grown to 80–90% confluence on six-well plastic plates. Cells were trypsinized, re-plated onto collagen-I (6.7 µg cm⁻²) coated plates, and immediately transfected with 2 µg of either constitutively active (T389E) or dominant negative (T389A) myc-tagged p70S6K, or pRK5 empty vector control using Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions. HEK293T cells were transfected while adherent. The day following transfection C2C12 and HEK293T were exposed to serum-free medium for 24 h prior to harvest, while L6 cells were exposed to 0.5% FBS-containing medium (complete serum starvation results in L6 cell death in our hands). Cells were harvested in modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS), to which was added 1:100 each Sigma P2714 (4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), *N*-(trans-epoxysuccinyl)-*L*-leucine-4-guanidinobutylamide (E-64), bestatin, leupeptin, aprotinin, and Na-EDTA), P2850 (microcystin LR, cantharidin, and bromotetramisole), and P5726 (sodium vanadate, sodium molybdate, sodium tartrate, and imidazole) and homogenized with repeated pipette tip flushing. Lysates were centrifuged at 5000 *g* for 5 min and equal protein loads of the soluble fraction, as determined by the BCA (bicinchoninic acid) protein assay, were subjected to Western blotting as described below. Luciferase reporter experiments were carried out on 12-well plates of L6 myoblasts transfected with 20 ng of pΔEMCV luciferase reporter and 800 ng of either wild-type mouse eIF2Bε-HA, eIF2Bε-ΔC-HA, or GFP. Twenty-four hours after transfection cells were cultured in 0.5% FBS-containing medium for an additional 24 h before determination of *Renilla* luciferase activity. All experiments were carried out in quadruplicate.

Immunoblotting

Immunoblotting was performed on lysates using established methods in our laboratory (Bamman *et al.* 2004). Samples were run on 4–15% Tris-HCl SDS-PAGE (Bio-Rad) or 4–12% Bis-Tris SDS-PAGE (Invitrogen)

gel matrices with 35 μg (*in vivo* human and mouse lysates) or 20 μg (*in vitro* cell lysates) total protein loaded into each well and transferred to PVDF membranes. Primary antibodies used included p(S473)- (no. 4051) and total (no. 9272) Akt, Rheb (no. 4935), p(S2448)- (no. 2971) and total (no. 2972) mTOR, p(T421/S424)- (no. 9204), p(T389)- (no. 9206), and total (no. 9206) p70S6K, p(S21/S9)-GSK3 α/β (no. 9331), total GSK3 β (no. 9315), p(T37/46)- (no. 9459) and total (no. 9452) 4E-BP1, p(S209)- (no. 9741) and total (no. 9742) eIF4E, p(S1108)- (no. 2441) and total (no. 2498) eIF4G, total eIF2B ϵ (no. 3595), p(S240/244)- (no. 4838) and total (no. 2217) rpS6, GFP (no. 2555), α -tubulin (no. 2125), myc-tag (no. 2278), mouse HA-tag (no. 2367), and p(T197/202)-Mnk1 (no. 2111), which were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies specific for the p85 subunit of PI3K (no. 06–497) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA), total Mnk1 (no. sc-28780) was from Santa Cruz Biotechnology, and rat HA-tag (no. 3F10) was from Roche Diagnostics Corp (Indianapolis, IN, USA). Ideal primary antibody concentrations were determined in preliminary experiments and were 1:2000 (v/v) for PI3K(p85) and GFP, 1:2500 for p(S21/S9)-GSK3 α/β , GSK3 β , p(S240/244)-rpS6, rpS6, p70S6K, and Rheb, 1:3000 for myc-tag, and rat HA-tag, 1:5000 for mouse HA-tag and α -tubulin, and 1:1000 for all other antibodies. HRP-conjugated secondary antibody was used at 1:50,000 (w/v) followed by chemiluminescent detection with SuperSignal West Dura or Femto substrate (Thermo Scientific, Rockford, IL, USA) in a Bio-Rad ChemiDoc imaging system with band densitometry performed using Bio-Rad Quantity One (software package 4.5.1). Parameters for image development in the ChemiDoc were consistent across all membranes using predefined saturation criteria for the CCD camera.

RT-PCR

Transfected L6 myoblasts were harvested in 1 ml Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) to which was added 100 μl BCP (Molecular Research Center, Inc.), vortexed for 1 min, and centrifuged at 13,000 g for 10 min at 4°C. An equal volume of isopropanol was added to the supernatant, pelleted at 13,000 g for 15 min, and washed twice with 75% ethanol in nuclease free water. The remaining pellet was air dried and resuspended in nuclease free water. One or three micrograms of RNA was reverse transcribed using SuperScript III (Invitrogen) and oligo-dT primers per the manufacturer's instructions. PCR of the cDNA product was carried out as described (Bamman *et al.* 2007) using *Renilla* luciferase primers published elsewhere (Butcher *et al.* 2005) and the following β -actin primers: forward 5'-GAGAGGGAAATCGTGCGTGAC-3', reverse 5'-CATCTGCTGGAAGGTGGACA-3'.

Luciferase activity

Transfected L6 cells plated on 12-well plates were lysed in 250 μl Passive Lysis Buffer (Promega, Madison, WI, USA). *Renilla* luciferase activity was determined with the Dual Luciferase Reporter assay kit (Promega) using a Berthold Lumat LB9507 luminometer.

Data analysis

Human response cluster data were subjected to 3×3 (response cluster \times time) repeated measures ANOVA for each protein to test differences in total protein expression levels and phosphorylation state among clusters and across time. Tukey's HSD test was performed *post hoc* to localize main effects, while Fisher's LSD test was used to localize interaction effects. For the *in vivo* mouse experiments, myofibre CSA data for transfected *vs.* non-transfected fibres in each condition were analysed using Student's *t* test. All other data were analysed by one-way ANOVA and, when appropriate, Tukey's HSD test performed *post hoc*. For all statistics, significance was set at the $P = 0.05$ level.

Results

Translation initiation signalling proteins not differentially regulated by human response cluster

We have previously reported on three distinct phenotypic groups of humans that differ in their hypertrophic responsiveness to 16 weeks of resistance training (Bamman *et al.* 2007). Further, it is established that mechanical load-induced myofibre hypertrophy is regulated in large part at the level of mRNA translation initiation (Augert *et al.* 1986; Kimball *et al.* 2002). We thus chose to investigate which signalling components that control translation initiation were differentially regulated among response clusters and may account for the observed phenotypic disparities.

Repeated measures ANOVA revealed main time effects ($P < 0.05$) for p(S473)-Akt, which increased 51% at week 1 (all percentage increases are with respect to baseline unless otherwise noted), and total Akt, which increased 37% at week 16 (Fig. 1A). These changes were noted without any change in the total content of the p85 regulatory subunit of PI3K, an upstream regulator of Akt (data not shown). GSK3 is a known downstream target of Akt and negative regulator of eIF2B ϵ catalytic activity (Bolster *et al.* 2004). Phosphorylation (inhibition) of the α isoform of this protein increased 15% at week 16 ($P < 0.05$); however it did so equally in all response clusters (Fig. 1B). Despite the increase in p(S473)-Akt at week 1, neither an mTOR phosphorylation site downstream of Akt, S2448, nor total mTOR levels were altered

at any time point (Fig. 1C). Downstream of mTOR, the phosphorylation state of the eIF4E binding protein, 4E-BP1, trended toward significance ($P = 0.052$), with a 23% increase occurring from baseline to week 1, while the total expression level of 4E-BP1 was significantly decreased by 25% at week 16 ($P < 0.05$) (Fig. 1D). Thus, while the levels of p(S473)-Akt, total Akt, p(S21)-GSK3 α , and total 4E-BP1 were altered significantly by mechanical load, these alterations occurred to an equal degree in XR, MR, and NR and could therefore not explain the differences in hypertrophy noted among these groups.

The phosphorylation of eIF4E on S209 increased 22% at week 1 in all clusters ($P < 0.05$) and returned to baseline levels by week 16 (Fig. 1E). Total protein expression of eIF4E increased 18% at week 1 and remained 14% elevated at week 16 ($P < 0.05$). eIF4G, a protein that complexes with eIF4E and eIF4A to form eIF4F (Vary & Lynch, 2006), associates with both the cytosolic and membrane fractions

in our hands; it is important to note that we report only the cytosolic compartment here. Phosphorylation (S1108) of cytosolic eIF4G increased 52% at week 1 and 39% at week 16 ($P < 0.05$) without any change in the total cytosolic expression level ($P > 0.05$) (Fig. 1F). Therefore the regulation of initiation factors 4E and 4G, while being responsive to mechanical load, were equally responsive in all response clusters at the 24 h time point and could not predict differential myofibre hypertrophy among clusters.

Translation initiation signalling proteins differentially regulated by human response cluster

Mnk1 has been shown to phosphorylate eIF4E and regulate eIF4E activity during stressed conditions (Chen *et al.* 2010). A significant time \times cluster interaction revealed that p(T197/202)-Mnk1 phosphorylation

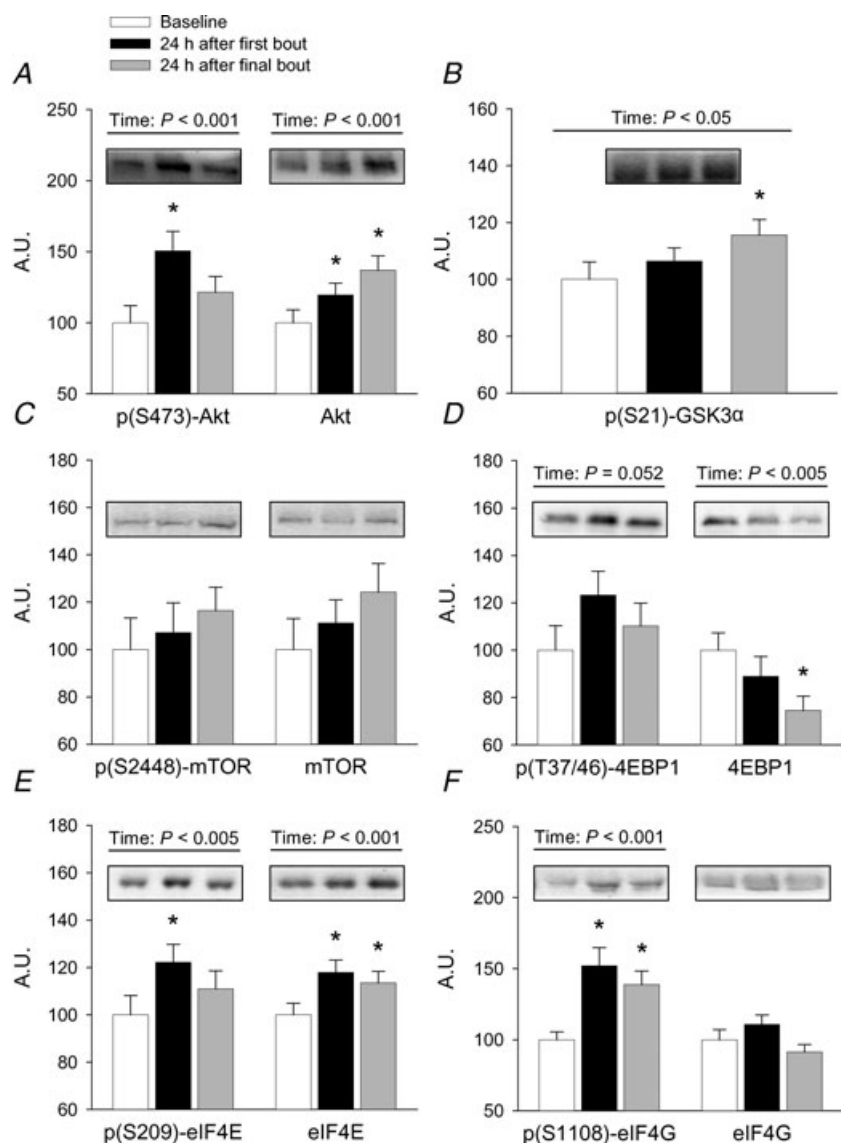


Figure 1. Resistance exercise-induced changes in translational signalling components not altered differentially by hypertrophy response cluster

Human subjects completed a 16 week resistance training study as described in the text, with skeletal muscle biopsies taken at baseline and 24 h after the first and last loading bouts. Western blotting was used to determine the phosphorylation and/or abundance of each protein. Since the data were not different among response clusters (non-significant response cluster \times time interaction term), the data are presented collapsed across all subjects. Values are mean \pm SEM arbitrary units based on optical density of immunoblotted protein bands, relative to baseline levels within each cluster, $n = 27-34$ / group. *Different from baseline, $P < 0.05$; †different from first bout response, $P < 0.05$.

increased 95% at week 16 with respect to week 1 in NR only (Fig. 2A). Total Mnk1 expression tended to increase 39% at 16 week in all clusters combined ($P = 0.051$, data not shown).

The regulation of GSK3 β seemed to occur by a different mechanism than the α isoform. Phosphorylation of GSK3 β on S9 showed a significant cluster \times time interaction; it increased slightly (17%), although significantly, at both week 1 and week 16 in MR ($P < 0.05$), and decreased 28% at week 16 with respect to week 1 in XR only ($P < 0.05$, Fig. 2B). The expression level of total GSK3 β was not altered at any time point ($P > 0.05$, data not shown). A clear trend between GSK3 β phosphorylation and hypertrophy was not evident. Therefore, while GSK3 β -dependent signalling may have been slightly altered by response cluster across time, the degree to which this modest change may have been physiologically relevant remains unclear at this time.

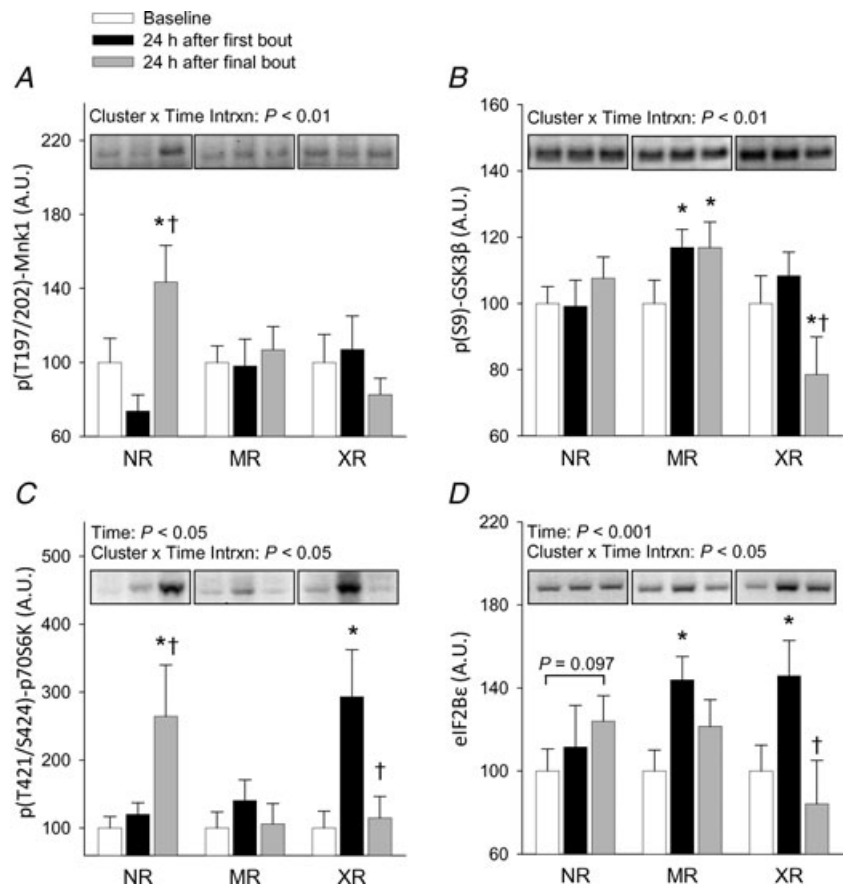
A downstream effector of mTOR, p70S6K, is regulated by phosphorylation at numerous sites including T421/S424 in the autoinhibitory domain, and the putative mTOR-specific phosphorylation site T389 in the linker domain (Han *et al.* 1995; Pearson *et al.* 1995). We were unable to reliably measure T389 phosphorylation using three commercially available phospho-specific antibodies, most likely due to the fasted state of the subjects. On the

other hand, T421/S424 phosphorylation was increased by 193% in XR at week 1 ($P < 0.05$) and returned to baseline by week 16, while in NR a 164% increase was not evident until week 16 ($P < 0.05$, Fig. 2C). The response of MR did not change at any time point. These observations occurred without a significant change in the expression level of total p70S6K protein in any group ($P > 0.05$, data not shown). Therefore, the response of T421/S424 phosphorylation appeared to effectively distinguish the three response clusters. This suggests that either activation of p70S6K signalling or a kinase responsible for T421/S424 phosphorylation early in a resistance training programme may partially underlie the hypertrophic phenotype seen in XR vs. MR and NR after 16 weeks training. p70S6K is a well characterized regulator of mRNA translation and cell size in various cell types, including skeletal muscle (Fingar *et al.* 2002; Ohanna *et al.* 2005). We thus chose not to further pursue the role of p70S6K in this response.

eIF2B ϵ is the catalytic component of the eIF2B holoenzyme, and its activity is required to exchange GDP for GTP on eIF2, thereby allowing Met-tRNA_i binding to the AUG start codon to initiate translation (Webb & Proud, 1997). Total eIF2B ϵ protein increased 45% and 44% at week 1 in XR and MR ($P < 0.05$), respectively, with XR fully and MR partially returning to baseline levels by week 16 (Fig. 2D). While eIF2B ϵ expression in NR

Figure 2. Resistance exercise-induced changes translational signalling components that were altered differentially by hypertrophy response cluster

Human subjects completed a 16 week resistance training study as described in the text, with skeletal muscle biopsies taken at baseline and 24 h after the first and last loading bouts. Western blotting was used to determine the phosphorylation and/or abundance of each protein. Each variable presented here demonstrated a significant response cluster \times time interaction term, in contrast to the data presented in Fig. 1, and were thus expressed by cluster. Values are mean \pm SEM arbitrary units based on optical density of immunoblotted protein bands, relative to baseline levels within each cluster, $n = 8$ –10 XR, 13–15 MR, 8 NR. *Different from baseline within cluster, $P < 0.05$; †different from first bout response within cluster, $P < 0.05$.



did not significantly change at any time point, a trend toward an increase from baseline to week 16 was noted ($P = 0.09$). Therefore, of the initiation factors studied here, eIF2B ϵ was regulated differentially among response clusters, while eIF4E and eIF4G were regulated similarly among all clusters.

Since protein, and particularly leucine, ingestion has been shown to stimulate mTORC1-dependent translation initiation irrespective of mechanical load (Anthony *et al.* 2000; Norton & Layman, 2006), we previously performed dietary analysis on the full human subjects cohort, and found that all response clusters had similar intakes of total carbohydrate, total fat, total protein and leucine (Thalacker-Mercer *et al.* 2009). Importantly, this finding is likely to rule out total protein or leucine intake as a causal mechanism for the differential hypertrophy and translation initiation signalling between response clusters.

eIF2B ϵ overexpression increases *in vitro* translation and *in vivo* myofibre hypertrophy

Since we observed a mechanical load-induced increase in eIF2B ϵ among only those subjects who experienced

subsequent myofibre hypertrophy (XR and MR), we sought to determine if increased eIF2B ϵ was sufficient to induce this response. We first transiently transfected L6 myoblasts with a *Renilla* luciferase reporter and either GFP, wild-type eIF2B ϵ , or an eIF2B ϵ mutant lacking the C-terminal catalytic domain (eIF2B ϵ - Δ C). The translation of the luciferase reporter is driven exclusively by a cap-dependent mechanism, and this technique has been shown previously to be reflective of overall protein synthesis rates (Balachandran & Barber, 2004). Overexpression of eIF2B ϵ resulted in an 87% increase in luciferase activity relative to GFP transfected controls ($P < 0.05$, Fig. 3), which is similar to what has been found previously in other cell types (Balachandran & Barber, 2004). This response was dependent upon the catalytic activity of the protein, as a mutant lacking the catalytic domain failed to alter luciferase activity from control levels ($P > 0.05$). Critically, the altered luciferase activity occurred at the post-transcriptional level, most likely due to increased translation rates, as the abundance of the reporter mRNA did not differ between conditions (Fig. 3). Thus, we interpret these results to indicate that increased eIF2B ϵ catalytic activity was sufficient to increase cap-dependent translation rates in L6 myoblasts *in vitro*.

We next wished to determine if the increased cap-dependent translation rates induced by eIF2B ϵ overexpression would result in myofibre hypertrophy in the intact animal. Overexpression of eIF2B ϵ *via* electroporation of plasmid DNA into mouse tibialis anterior muscle resulted in a 21% increase in cross-sectional area of transfected fibres 7 days after electroporation ($P < 0.05$), while expression of GFP had no effect relative to non-transfected fibres (Fig. 4). An eIF2B ϵ mutant (S535A) that has previously been reported to act in a constitutively active manner in cardiac myocytes (Hardt *et al.* 2004) had an effect equal to that of the wild-type protein ($P < 0.05$ vs. non-transfected fibres). The mutated residue is a phosphorylation target of GSK3, which could indicate that GSK3-dependent inhibition of eIF2B ϵ may play less of a role in skeletal muscle than in cardiac muscle (Hardt *et al.* 2004), or that overexpressed wild-type eIF2B ϵ may have been present in a quantity too great to be inhibited by endogenous levels of GSK3. In either case, the degree of overexpression of either wild-type or the S535A mutant was evidently sufficient to saturate the cellular need for eIF2B GEF activity, since we saw equal hypertrophy when either was overexpressed. These data demonstrate that increased abundance of eIF2B ϵ was sufficient to induce significant myofibre hypertrophy in mouse skeletal muscle in a short time (7 days), making it likely that the hypertrophy seen in XR and MR humans over 16 weeks was at least partially due to augmented protein synthesis secondary to increased abundance of eIF2B ϵ .

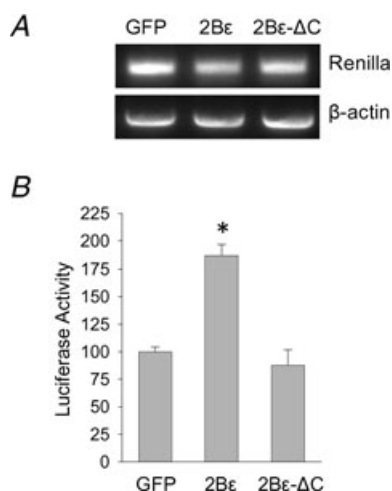


Figure 3. eIF2B ϵ activity is sufficient to increase cap-dependent translation *in vitro*

L6 myoblast cells were transfected with a CMV-driven *Renilla* luciferase reporter and either GFP, or wild-type eIF2B ϵ (2B ϵ), or a mutant lacking the C-terminal catalytic domain (2B ϵ - Δ C). Translation of the luciferase reporter is driven exclusively by a cap-dependent mechanism, and this technique has been shown previously to be reflective of overall protein synthesis rates (Balachandran & Barber, 2004). Twenty-four hours after transfection cells were cultured in 0.5% FBS for an additional 24 h and harvested for the assays indicated. *A*, RT-PCR showing similar mRNA expression of the luciferase reporter among conditions. *B*, *Renilla* luciferase activity was determined and expressed relative to GFP control. Values are means \pm SEM, $n = 4$. *Different from GFP control, $P < 0.05$.

p70S6K signalling does not induce increased eIF2B ϵ protein abundance *in vitro* or *in vivo*

It is known that p70S6K signalling is necessary for proper regulation of cell size (Fingar *et al.* 2002; Holz *et al.* 2005; Ohanna *et al.* 2005); however, the downstream effectors that mediate this process are not well defined. We observed increases in both p(T421/S424)-p70S6K phosphorylation and eIF2B ϵ abundance among XR (Fig. 2). Furthermore, Kubica *et al.* (2008) found that mTORC1 signalling was sufficient to induce increased translation of eIF2B ϵ , which was blocked by rapamycin administration. Together these data suggested that p70S6K may be the rapamycin-sensitive component downstream of mTORC1 that mediates this response. In order to test this hypothesis, we transfected multiple cell lines with constitutively active (T389E) or dominant negative (T389A) p70S6K in order to determine the effects of p70S6K signalling on eIF2B ϵ protein abundance. In C2C12 mouse myoblasts, alterations in p70S6K activity failed to exert an effect on eIF2B ϵ protein abundance ($P > 0.05$) despite the expected effects on rpS6 phosphorylation (a consequence of p70S6K signalling) relative to pRK5-transfected control cells (Fig. 5). Similar results were obtained in L6 myoblasts. Since C2C12 and L6 myoblasts have a propensity to differentiate under serum withdrawal, we also performed these experiments in the non-myogenic HEK293T cells. Although we obtained nearly 100% transfection efficiency and greater subsequent effects on rpS6 phosphorylation in HEK293T cells, there remained no effect on total eIF2B ϵ abundance ($P > 0.05$, Fig. 5). This lack of effect occurred despite the ability of each of these cell lines to increase eIF2B ϵ in response to other stimuli in our hands (data not shown), indicating that eIF2B ϵ levels are in fact malleable in these cell lines. Thus, in three different cell lines of myogenic and non-myogenic origins, p70S6K activity had no influence over the cellular steady state abundance, and therefore presumably the synthesis, of eIF2B ϵ .

Although we found no change in eIF2B ϵ abundance with altered p70S6K activity *in vitro*, the possibility existed that the behaviour of the cell lines chosen did not reflect the *in vivo* condition. Therefore, we chose to investigate whether increased p70S6K activity was sufficient to induce an increase in eIF2B ϵ abundance in mouse skeletal muscle *in vivo*. To do this, we overexpressed wild-type p70S6K with or without Rheb, an upstream activator of mTORC1 (Yang *et al.* 2006), via electroporation of plasmid DNA into mouse tibialis anterior muscle. Co-expression of Rheb with p70S6K caused significantly increased T389 phosphorylation of both endogenous and GST-tagged p70S6K (Fig. 6), an event that is synonymous with increased activity of the protein (Pearson *et al.* 1995; Weng *et al.* 1998). Increased p70S6K activity had no effect on eIF2B ϵ abundance relative to

GFP-transfected negative controls ($P > 0.05$, Fig. 6). Thus, increased p70S6K activity was not sufficient to induce increased eIF2B ϵ abundance under these conditions. Although the mechanical load-induced increase in eIF2B ϵ translation has been shown to be rapamycin sensitive (Kubica *et al.* 2005), we have shown here that p70S6K signalling alone is not sufficient to increase eIF2B ϵ abundance *in vitro* or *in vivo*. Combined, these data strongly suggest that p70S6K signalling is not involved in eIF2B ϵ translation.

Discussion

The main findings presented here are that mechanical load-induced increases in p(421/S424)-p70S6K phosphorylation and eIF2B ϵ abundance were associated with myofibre hypertrophy after 16 weeks training in humans (Fig. 2). We additionally demonstrated that ectopic expression of eIF2B ϵ , a factor required for all translation initiating at an AUG codon, was sufficient to induce skeletal muscle hypertrophy *in vivo* (Fig. 4). Together these data strongly indicate that eIF2B ϵ abundance, and by extension eIF2B activity, are key determinants of cellular hypertrophy *in vivo*. However, although we observed concurrent p70S6K phosphorylation and increased eIF2B ϵ abundance in the humans clustered as XR, p70S6K signalling was not sufficient to increase eIF2B ϵ abundance as confirmed both *in vitro* and *in vivo*.

Given the data presented here, we suggest that eIF2B ϵ exerted a greater influence over myofibre hypertrophy than eIF4E or eIF4G in response to mechanical load under these conditions, since neither the phosphorylation state nor the total protein abundance of eIF4E, 4E-BP1, or eIF4G responded differently by cluster at the 24 h post-exercise time points analysed (Fig. 1). While our human results are admittedly limited to this single post-exercise time point, this interpretation of our data is supported by previous work, where neither the phosphorylation state nor the association of eIF4E and eIF4G was altered in rat muscle in response to resistance exercise (Farrell *et al.* 1999; Farrell *et al.* 2000) while eIF2B activity was increased (Farrell *et al.* 1999). A similar dissociation of eIF4F regulation and protein synthesis was observed under a variety of conditions in cardiomyocytes (Huang *et al.* 2009), L6 myoblasts (Kimball *et al.* 1998), and mouse muscle *in vivo* (Anthony *et al.* 2002). The importance of eIF2B ϵ in translation and cell size regulation can be further emphasized by the dramatic impact of a dominant negative upstream regulator of eIF2B ϵ , GSK3 β , on C2C12 myotube diameter (Rommel *et al.* 2001), and a constitutively active eIF2B ϵ on rat neonatal cardiomyocyte surface area (Hardt *et al.* 2004). This is consistent with our data in which overexpression of eIF2B ϵ was sufficient

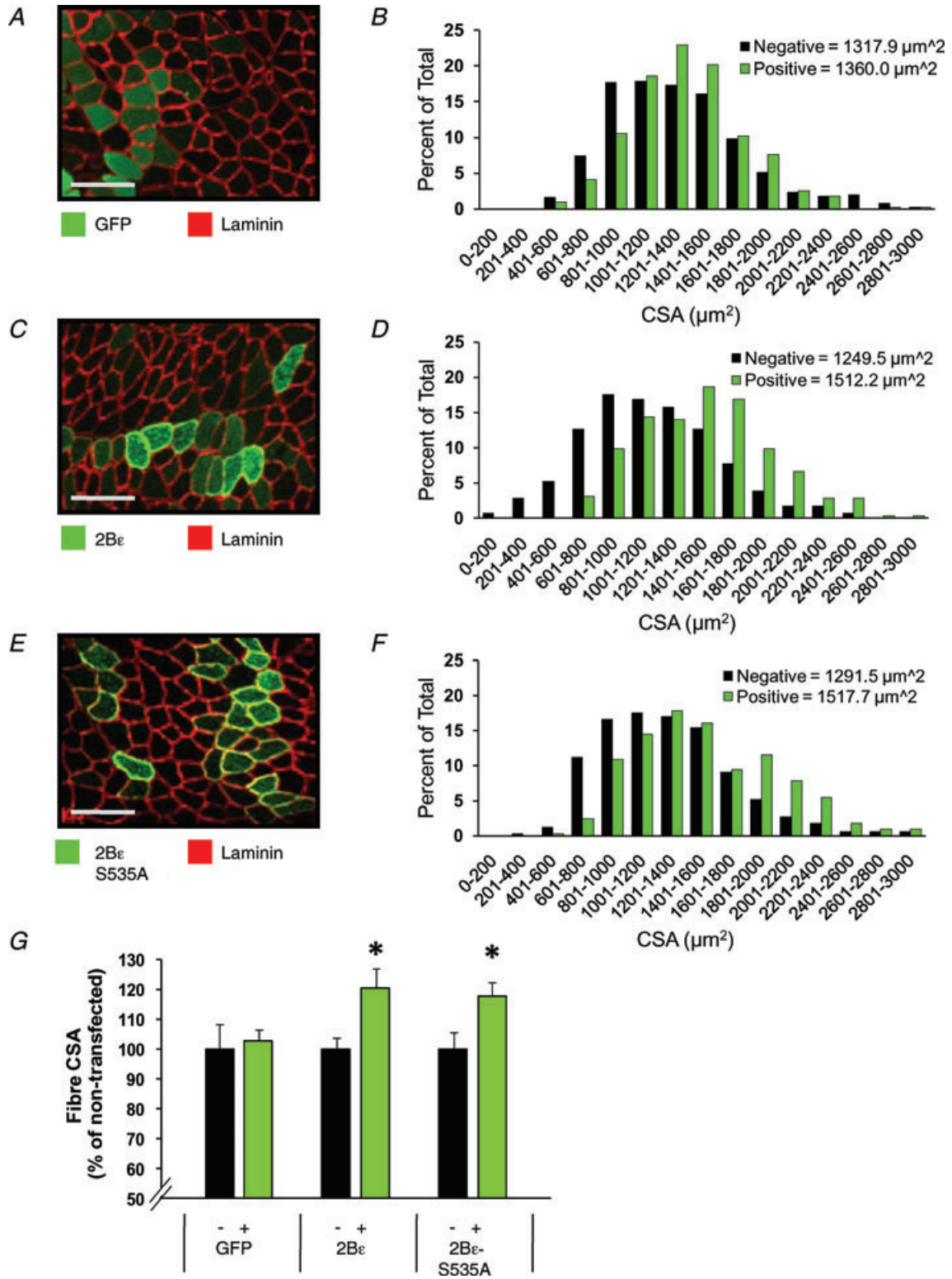


Figure 4. Overexpression of eIF2B ϵ or eIF2B ϵ -S535A induce skeletal muscle fibre hypertrophy
 Mouse TA muscles were transfected with GFP (A and B), eIF2B ϵ (2B ϵ) (C and D), or constitutively active eIF2B ϵ -S535A (E and F). At 7 days post-transfection, the muscles were collected and cross-sections from the mid-belly of the muscle were subjected to immunohistochemistry for GFP and laminin (A), 2B ϵ and laminin (C), or 2B ϵ -S535A and laminin (E). Scale bar = 100 μm . The CSA of transfected fibres (green bars) and non-transfected fibres (black bars) from GFP (B) 2B ϵ , (D), or 2B ϵ -S535A (F) transfected muscles was determined and plotted

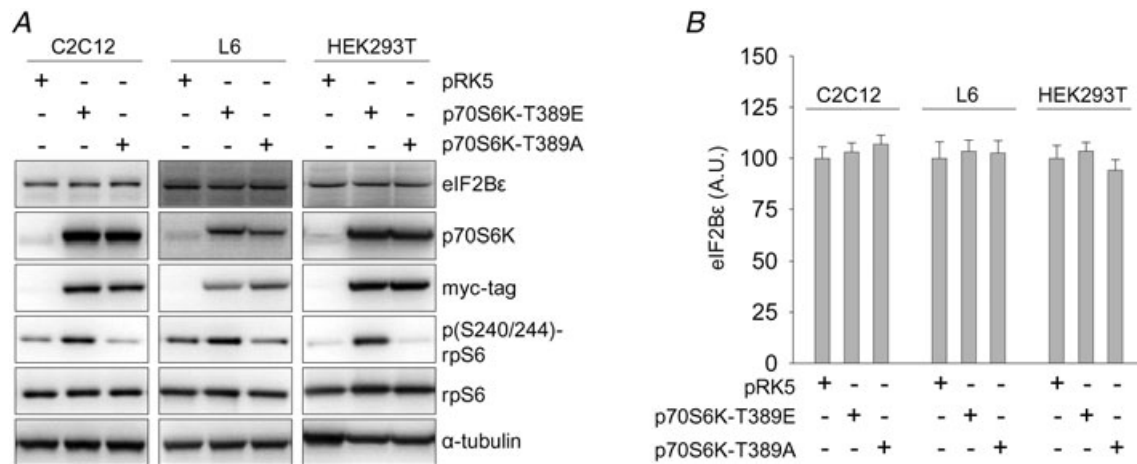


Figure 5. p70S6K signalling does not alter total eIF2Bε cellular abundance *in vitro*
 A, C2C12, L6 and HEK293T cells were transfected with myc-tagged constitutively active (T389E) or dominant negative (T389A) p70S6K, or empty vector control (pRK5). Twenty-four hours later cells were exposed to serum free (C2C12 and HEK293T) or 0.5% FBS medium (L6) for an additional 24 h, harvested and analysed by Western blotting with the indicated antibodies. B, values are means ± SEM for the eIF2Bε data shown in A, n = 4/group.

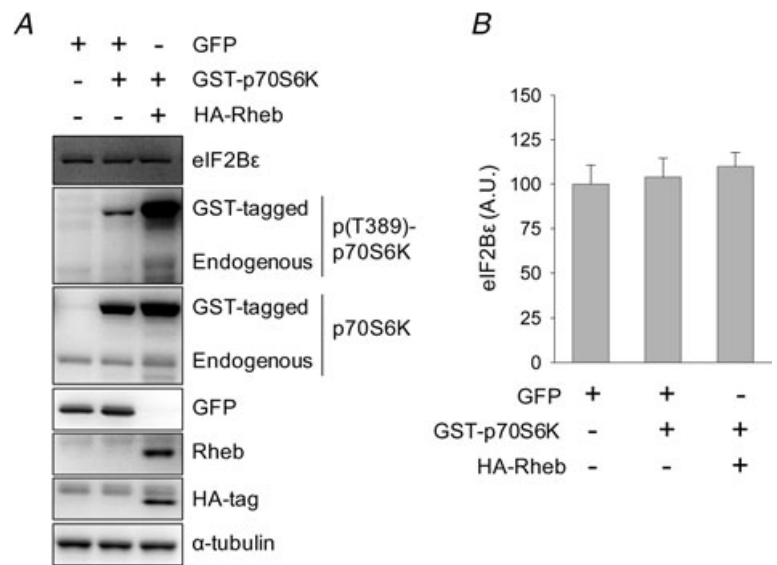


Figure 6. p70S6K signalling does not alter total eIF2Bε cellular abundance *in vivo*
 A, mouse TA muscles were transfected with GFP, GST-tagged p70S6K and/or HA-tagged Rheb. Mice recovered for 48 h at which time muscles were harvested and analysed by Western blotting with the indicated antibodies. B, values are means ± SEM for the eIF2Bε data shown in A, n = 3–4 muscles/group.

to significantly increase both cap-dependent translation *in vitro* (Fig. 3) and skeletal myofibre size in mice (Fig. 4). Therefore, it seems that eIF2 GTP recycling and Met-tRNA_i binding (as indexed by eIF2Bε abundance), but not 5'-capped mRNA binding (as indexed by eIF4G, eIF4E and 4E-BP1 phosphorylation or abundance), may be more critical at physiological levels to the *in vivo*

response to mechanical load, and may implicate eIF2B as a rate limiting factor. Indeed, the relative abundance of eIF2B compared to other initiation factors suggests that it may be rate limiting (Oldfield *et al.* 1994; Kimball *et al.* 1998; Singh *et al.* 2007). Based on the putative inhibition of eIF2Bε by GSK3β-mediated phosphorylation, and on prior acute resistance exercise findings showing decreased

on a histogram (n = 285–510 transfected and 285–510 non-transfected fibres/group). H, average CSA of the transfected (+) and non-transfected (–) fibres per muscle from samples transfected with GFP, 2Bε, or 2Bε-S535A. Values are means ± SEM, n = 5–7 muscles/group. *Different from the non-transfected fibres within a given condition, P < 0.05.

phosphorylation (Glover *et al.* 2008), we attempted to assess eIF2B ϵ S539 phosphorylation status in our human clusters. However, immunoblotting experiments with two separate primary phospho-antibodies failed to yield satisfactory results (see Supplemental Material).

Tuckow and colleagues recently found that eIF2B ϵ overexpressed in rat skeletal muscle resulted in no change in basal protein synthesis rates; however, it blunted the sepsis-induced decrease in eIF2B activity and overall protein synthesis (Tuckow *et al.* 2010). In a similar manner, the increased eIF2B ϵ abundance that resulted from cellular transformation also prevented the stress-induced suppression in both eIF2B GEF activity and protein synthesis (Balachandran & Barber, 2004). Others have found that mutations within the catalytic domain of eIF2B ϵ yielded no observable phenotype in yeast under normal conditions, but conferred cold sensitivity (Mohammad-Qureshi *et al.* 2007). Additionally, we found that differences in luciferase activity (i.e. cap-dependent translation) between myoblasts of differing eIF2B activity were only evident after serum reduction, a significant cell stress (Fig. 3 and D. L. Mayhew, unpublished observations). Taken together these data are consistent with a model in which eIF2B has the greatest impact on overall protein synthesis rates under stressed conditions. As basal protein synthesis rates generally appear unaltered by increased eIF2B ϵ abundance (Balachandran & Barber, 2004; Tuckow *et al.* 2010) (D. L. Mayhew, unpublished observations), we hypothesize eIF2B ϵ is not rate limiting for protein synthesis under basal conditions but becomes rate limiting in response to stress. A greater abundance of eIF2B ϵ may therefore blunt the suppression of protein synthesis during frequent transient stresses (e.g., temperature stresses, fasted state between feedings) in the free living animal *in vivo*. This hypothesis is consistent with the well characterized model of stress-induced eIF2B suppression by phosphorylated eIF2 α (Wek & Cavener, 2007), in which a greater abundance of eIF2B ϵ would require greater eIF2 α phosphorylation in order to be inhibited to a similar extent (i.e. greater stress resistance). Over time, greater eIF2B activity would result in greater muscle mass when compared to animals with less eIF2B activity in response to similar stresses, which may explain the hypertrophy we observed in myofibres overexpressing eIF2B ϵ for 7 days.

Nearly identical regulation of p70S6K to what we have observed here has been noted in a similar model of chickens that were pre-selected for divergent growth phenotypes (Duchene *et al.* 2008a). In that study, insulin stimulated an increase in T421/S424 phosphorylation only in skeletal muscle of fast growing chickens (similar to XR here), which increased p70S6K activity, while Akt and 4E-BP1 phosphorylation were increased to similar degrees in both fast and slow growing chickens (Duchene *et al.* 2008a). These data and our data together indicate that

the regulation of p(T421/S424)-p70S6K phosphorylation may be a phylogenetically conserved mechanism that results in increased p70S6K activity, translation initiation, and myofibre size in only a subset of animals that are prone to myofibre hypertrophy. Although this same group found that ERK2 signalling was indispensable for insulin induced p70S6K phosphorylation at T421/S424 (Duchene *et al.* 2008b), further work will be needed to determine the mechanism responsible for the variable sensitivity in mechanical load-induced p70S6K phosphorylation in humans.

In summary, we have observed that many proteins in translation initiation signalling pathways are responsive to mechanical load in humans. However, the regulation of the majority of the proteins examined here, including proteins previously shown to be important for hypertrophy in other models, were not sufficient to induce myofibre hypertrophy in NR. It is probable that many pathways are required for hypertrophy to occur *in vivo*, with some pathways providing a permissive environment while others provide directive influence over the multitude of cellular processes that result in mRNA translation and hypertrophy (Tang *et al.* 2001; Bolster *et al.* 2004). To our knowledge this is the first report to provide *in vitro* and *in vivo* evidence that eIF2B ϵ abundance is an important determinant driving protein synthesis and cellular hypertrophy in skeletal muscle, and that increased eIF2B ϵ abundance is not a functional consequence of p70S6K signalling. It is currently unknown whether p(T421/S424)-p70S6K phosphorylation and eIF2B ϵ abundance result from a common upstream event or independently regulated processes. Our results suggest that both p70S6K phosphorylation and eIF2B ϵ abundance may be used as potential signalling biomarkers for myofibre hypertrophy. These are the first data that mechanistically link eIF2B ϵ abundance to skeletal myofibre hypertrophy, and indicate that both eIF2B ϵ abundance and p70S6K phosphorylation may at least partially underlie the widely divergent hypertrophic phenotypes in human skeletal muscle exposed to mechanical stimuli.

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Author contributions

D.L.M., T.A.H. and H.C.L. contributed to study conception and design, and data collection, analysis and interpretation. M.M.B. contributed to study conception and design, and data analysis and interpretation. All human and *in vitro* work was conducted

in the lab of M.M.B., while all mouse work was conducted in the lab of T.A.H.. D.L.M., M.M.B. and T.A.H. contributed to the initial drafting the article, and all authors revised the article for intellectual content and approved the final version. The authors have no conflicts of interest to disclose.

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