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Influence of ageing and essential amino acids on quantitative patterns of troponin T alternative splicing in human skeletal muscle

Joel Coble[#1], **Rudolf J. Schilder**[#2], **Arthur Berg**[3], **Micah J. Drummond**[4], **Blake B. Rasmussen**[4], and **Scot R. Kimball**[1,6]

[1]Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, Hershey, PA 17033

[2]Department of Biology, The Pennsylvania State University, University Park, PA 16802

[3]Department of Public Health Sciences, The Pennsylvania State University College of Medicine, Hershey, PA 17033

[4]Department of Nutrition and Metabolism, University of Texas Medical Branch, Galveston, TX 77555

[#] These authors contributed equally to this work.

Abstract

Ageing is associated with a loss of skeletal muscle performance, a condition referred to as sarcopenia. In part, the age-related reduction in performance is due to a selective loss in muscle fiber mass, but mass-independent effects have also been demonstrated. An important mass-independent determinant of muscle performance is the pattern of expression of isoforms of proteins that participate in muscle contraction, e.g. the troponins. In the present study we tested the hypothesis that ageing impairs alternative splicing of the pre-mRNA encoding fast troponin T (*Tnnt3*) in human vastus lateralis muscle. Furthermore, we hypothesized that resistance exercise alone or in combination with consumption of essential amino acids will attenuate age-associated effects on *Tnnt3* alternative splicing. Our results indicate that ageing negatively affects the pattern of *Tnnt3* pre-mRNA alternative splicing in a manner that correlates quantitatively with age-associated reductions in muscle performance. Interestingly, whereas vastus lateralis *Tnnt3* alternative splicing was unaffected by a bout of resistance exercise 24 hour prior to muscle biopsy, ingestion of a mixture of essential amino acids after resistance exercise resulted in a significant shift in the pattern of *Tnnt3* spliceform expression in both age groups to one predicted to promote greater muscle performance. We conclude that essential amino acid supplementation after

[6]Correspondence: Dept. of Cellular and Molecular Physiology, H166, The Pennsylvania State University College of Medicine, 500 University Dr., Hershey, PA 17033. Tel.: 717-531-8970; Fax: 717-531-7667; skimball@psu.edu.

Competing Interests

The authors have no competing or financial interests.

Author Contributions

SRK conceived the experiment and JC, MD, SRK, BBR, and RJS contributed to the design. Human studies were performed by and cDNA was prepared by MD in the laboratory of BBR. DNA fragment analysis was performed by JC in the laboratories of SRK and RJS. Data was analyzed by RJS and statistical analyses were reviewed and approved by AB. JC created the original draft of the article and RJS and SRK contributed critical revisions. All authors approved the final version of the manuscript.

resistance exercise may provide a means to reduce impairments in skeletal muscle quality during ageing in humans.

Keywords

Age; resistance exercise; amino acids; muscle; mTOR; troponin T

Introduction

Reduced muscle strength and performance are important contributors to the development of physical frailty that is commonly observed with advanced age (Mitchell et al. 2012). In part, impaired performance is a consequence of a loss of muscle mass, a condition referred to as sarcopenia (Rosenberg 1989). Recent studies have shown that sarcopenia is associated with the development in the elderly of resistance to anabolic stimuli that potently promote muscle mass accretion in younger individuals. For example, the ability of either amino acids or resistance exercise to stimulate muscle protein synthesis is attenuated in muscle of older compared to younger individuals (Guillet et al. 2004; Cuthbertson et al. 2005; Katsanos et al. 2005; Kumar et al. 2009; Durham et al. 2010; Fry et al. 2011). Similar observations have been made in rodents (Mosoni et al. 1995; Dardevet et al. 2000; Dardevet et al. 2002). In both humans and rodents, the decreased response of muscle protein synthesis to anabolic stimuli is associated with reduced signaling through the mechanistic target of rapamycin in complex 1 (mTORC1), an important regulator of cell growth and metabolism (Funai et al. 2006; Drummond et al. 2008b).

Although a loss of muscle mass plays an important role in the development of physical frailty in the elderly, it appears it is not solely responsible for the reduction in muscle performance (e.g. Newman et al. 2006; Delmonico et al. 2009). In addition to age-related neural impairments affecting locomotion (e.g. Guillet et al. 1999; Manini et al. 2013), both the velocity and force of muscle contraction appear to decrease to a greater extent than would be predicted based solely on the loss of mass (Brooks et al. 1994; Yu et al. 2007; Mitchell et al. 2012; Miller et al. 2013; Moore et al. 2014), i.e. the force per cross sectional area is reduced. At a molecular level, muscle contraction is mediated by the calcium-induced interaction of actin and myosin, a process that is modulated by the troponin complex. As part of the complex, troponin T transduces to tropomyosin the signal generated by the binding of calcium to troponin C, thereby allowing myosin to interact with actin to produce force. Three homologous troponin T genes have been identified in mammals: slow (*Tnnt1*), cardiac (*Tnnt2*), and fast (*Tnnt3*) (Stefancsik et al. 2003). The pre-mRNAs for all three genes are alternatively spliced, leading to the production of mRNAs encoding multiple protein variants that exhibit differential functional characteristics. Consequently, changes in the pattern of troponin T pre-mRNA alternative splicing results in variations in muscle force, power output, and calcium sensitivity in both insects (Fitzhugh et al. 1997; Marden et al. 2001; Schilder et al. 2007) and mammals (Pan et al. 1992; Briggs et al. 1996; Chaudhuri et al. 2005; Sancisi et al. 2014). Although little is known about mechanisms involved in regulating alternative splicing of the troponin T pre-mRNA in general, the pattern of rodent *Tnnt3* splice variant expression is rapidly altered in young rats in response to changes in

effective body mass, e.g. in animals wearing a weighted vest for up to 5 d (Schilder et al. 2011), resulting in expression of splice variants associated with greater force output and calcium sensitivity. This finding suggests that changes in muscle loading promote changes in *Tnnt3* pre-mRNA alternative splicing. Interestingly, signaling through mTORC1 has been implicated in the control of alternative splicing (Ma et al. 2008; Ma et al. 2009), although whether or not it is directly involved in modulating *Tnnt3* pre-mRNA alternative splicing is unknown. However, it is important to note that mTORC1 regulates alternative splicing of another important sarcomere gene, cardiac titin (Linke et al. 2010), and experimental manipulation of the activity of a protein kinase that functions upstream of mTORC1, Akt (a.k.a. protein kinase B), was recently found to alter the pattern of *Tnnt3* splice variant expression in response to cyclic loading in C2C12 myotubes in culture (Schilder et al. 2012).

The present study tested the overall hypothesis that alternative splicing of human *Tnnt3* (*hTnnt3*) pre-mRNA is altered with age resulting in production of splice variants associated with reduced muscle force production and calcium sensitivity in muscle from older compared to younger individuals. Furthermore, it is also predicted that resistance exercise and/or consumption of essential amino acids in combination with resistance exercise alter alternative splicing of *hTnnt3* pre-mRNA, resulting in a shift in the pattern of *hTnnt3* pre-mRNA splice variant expression in the elderly to one more closely aligned with that observed in younger subjects. The results support the overall hypothesis and demonstrate that expression of *hTnnt3* splice variants positively associated with increased force production and calcium sensitivity is reduced with age. They also show that ingestion of a bolus of a mixture of essential amino acids, but not a bout of resistance exercise alone, leads to an increase in the proportion of a *hTnnt3* spliceform associated with increased force production and calcium sensitivity. Together, these data provide a possible avenue through which the mass-independent loss of force production that occurs with age can occur.

Materials and methods

Samples

The cDNA used in the present study was generated from samples obtained in previous studies (Drummond et al. 2008a; Fry et al. 2011) summarized in Table 1. In those studies, young (27 ± 2 yrs) and older (68 ± 2 yrs) male subjects were studied following an overnight fast. In the morning, a muscle biopsy was taken from the vastus lateralis muscle and two hours later each subject in both studies performed 8 sets of 10 repetitions of bilateral leg extensions (Cybex-VR2, Medway, MA) at 70% of their one-repetition maximum (1RM), as determined in two previous sessions occurring at least one week prior to the studies. In study 2 (Drummond et al. 2008a), the subjects were administered a 20g essential amino acid (EAA) bolus 1 h post exercise. In study 1 (Fry et al. 2011), biopsy samples were collected from each subject 3, 6, and 24 h post exercise while in study 2 biopsies were taken at 3 and 6 h post exercise only (see Table 1). All subjects gave informed, written consent before participating in the study, which was approved by the Institutional Review Board of the University of Texas Medical Branch (which is in compliance with the Declaration of Helsinki). Young and older subjects were not participating in an organized aerobic or

resistance exercise training program and were considered to be recreationally active. All subjects were considered healthy and were not diabetic, as evaluated with a clinical history, physical examination, and laboratory blood and urine tests during the screening process.

Characterization and quantification of hTnnt3 splice form abundance

Quantitation of *hTnnt3* splice form abundance in cDNA samples involved PCR-based DNA fragment analysis using a fluorescein-labeled forward primer F1 (5′-TCACCATGTCTGACGAGGAA-3′) and two unlabeled reverse primers αR1 and βR2 (5′-CTGAGCGTGGTGATGTCATA-3′ and 5′-CCAGCCTTCTTGCTGAACTT-3′, respectively). The fluorescein-labeled forward primer F1 hybridized with the constitutively expressed *hTnnt3* exon 2, while the reverse primers spanned mutually exclusive exon 16 and constitutive exon 18 (αR1) or mutually exclusive exon 17 and constitutive exon 18 (βR2) respectively. This strategy allowed for amplification of all, and size resolution of most, *hTnnt3* splice forms (see Results).

PCR was performed using the following cycling protocol: 5 min at 95°C, followed by 4 cycles of 30s at 94°C, 30s at 65°C (−1.0°C/cycle), followed by 1 min 15s at 72°C. This was followed by 29 cycles of 30s at 94°C, 30s at 60°C, 1 min 15s at 72°C, with a final 15 min at 72°C to end. The fluorescein-labeled PCR samples were diluted 1:20 and 1 μl of each diluted sample was quantitatively assessed by capillary electrophoresis using an ABI DNA analyzer (Applied Biosystems, Foster City, CA). Amplicon fragment size (i.e. using an internal size standard) and quantity (i.e. fluorescence peak height) were determined using the software Peakscanner (Applied Biosystems). Amplicons had to be present in each muscle sample and in sufficient abundance (i.e. >1% relative abundance) to be included in the study. The relative abundance of each *hTnnt3* splice form in the cDNA samples was determined by dividing individual *hTnnt3* splice form peak heights by the combined peak height for all splice forms.

hTnnt3 splice form nucleotide sequences were confirmed by cloning and sequencing using an unlabeled PCR reaction as described above and using a pooled cDNA sample (i.e. young and older subject cDNA samples combined). The amplicons were resolved on a 1.5% agarose gel, extracted from the gel using a Promega Wizard SV Gel and PCR Clean-Up System (Promega, Fitchberg, WI), and then cloned into One Shot® *E. Coli* cells using a TOPO TA Cloning kit (Invitrogen, Carlsbad, USA). Sequencing (ABI Hitachi 3730XL DNA Analyzer, Applied Biosystems) was then performed for 100 colonies to verify the identity of predicted amplicons.

Statistical Analyses

Statistical analyses were performed using R (http://www.R-project.org/) and JMP statistical software (JMP v9, SAS Institute Inc., Cary, NC). All *hTnnt3* splice form relative abundance data were first arcsine-transformed to meet normality assumptions of statistical tests applied.

Preliminary analyses (by means of one-way ANOVA within each age group) of mean *hTnnt3* splice form relative abundance at the different post-exercise timepoints (see also Table 1) revealed no significant differences in *hTnnt3* splice form relative abundance and we

therefore collapsed these data to create a new mean relative abundance datapoint for each *hTnnt3* splice form across the respective post-exercise timepoints. We used these collapsed means in subsequent analyses of effects of exercise and EAA treatment.

Effects of Age on *hTnnt3* alternative splicing (two-way ANOVA) as well as correlations of *hTnnt3* splice form relative abundance and muscle performance (Table 2, Fig 2) were analyzed using only the combined "Rest" data (see Table 1) from study 1 and 2.

Effects of exercise and its interaction with Age were examined independently for study 1 and 2 independently using the "Rest" and collapsed mean *hTnnt3* splice form abundances across the respective timepoints (see Table 1) using repeated measures ANOVA (package *aov* in R).

Effects of EAA treatment were examined (two-way ANOVA) by comparing the collapsed mean *hTnnt3* spliceform abundances between the "Exercise only" and "Exercise +EAA" studies (see Table 1), but excluding the "Rest" data.

Results

Characterization of human Tnnt3 spliceform expression

Previous studies determined the nature and expected expression pattern of human *Tnnt3* exon structure (Stefancsik et al. 2003) and significantly added to the known number of expressed *Tnnt3* spliceforms in rat and mouse muscle (Schilder et al. 2011; Sancisi, Germinario et al. 2014). The number and relative expression of *Tnnt3* spliceforms present in human skeletal muscle has to our knowledge not been determined. We therefore set out to characterize *hTnnt3* spliceform expression in vastus lateralis muscle by means of fluorescently labeled, primer-assisted PCR followed by DNA Fragment analysis (see Methods). This procedure consistently revealed twelve fluorescent peaks (Fig 1) corresponding to what we have determined to be a minimum of nine *hTnnt3* spliceforms. The discrepancy between the number of identified peaks and spliceforms is a consequence of two sequenced cDNA clones, corresponding to *hTnnt3 α5a* or *hTnnt3 α5b*, generating spliceforms of equal size that cannot be resolved using our PCR strategy. Moreover, we were unable to sequence cDNA clones with sizes corresponding to peaks labeled with *α1-α4* (Fig 1) after screening approximately 100 clones; therefore these four spliceforms remain classified as "predicted" (and denoted in grey in Fig 1). However, given that these four peaks are consistently detected (i.e. in all samples examined here) with relative abundance >1%, they were included in our subsequent analyses of *hTnnt3* spliceform expression relative abundance.

Effects of age on vastus lateralis muscle hTnnt3 expression

Given the published differences in vastus lateralis muscle performance between the older and younger subjects examined here (Drummond et al. 2008a; Fry et al. 2011) and established general correlations of troponin T spliceform expression patterns and muscle performance elsewhere (e.g. Pan and Potter 1992; Briggs and Schachat 1996; Gomes et al. 2004; Brotto 2005) we tested the hypothesis that age affected vastus lateralis *hTnnt3* alternative splicing. Based on our previous findings on effects of body weight variation on

Tnnt3 alternative splicing in rat gastrocnemius muscle (Schilder et al. 2011), we accounted for possible effects of body weight using a two-way ANOVA. Table 2 shows that the first 3 principal components of *hTnnt3* spliceform abundance were significantly affected by age, but not by body weight. No significant interaction term (i.e. age × body weight) was detected during our analyses and this interaction term was dropped from the ANOVA model. Previous findings on these subjects revealed no significant differences in body weight, BMI, or lean mass (Fry et al. 2011) suggesting that there may not have been enough body weight variation to detect an effect on vastus lateralis muscle *hTnnt3* alternative splicing. For these reasons, the factor body weight was not taken into account in any subsequent analyses listed below. Individually, the relative abundance of seven out of thirteen *hTnnt3* spliceforms was significantly affected by age.

Correlation between muscle performance and vastus lateralis hTnnt3 alternative splicing

We next examined whether the observed changes in *hTnnt3* correlated with the previously observed (Drummond et al. 2008a; Fry, Drummond et al. 2011) difference in muscle performance in old and young subjects. *Tnnt3* spliceforms containing the 3′ α exon were previously found to convey higher calcium sensitivity and force output to muscle compared to those containing the 3′ β exon (Gallon et al. 2006). Here we found in the "Rest" dataset (Table 1) that overall vastus lateralis *hTnnt3 α* spliceform abundance was significantly reduced in older compared to younger individuals and thus, because of the relative nature of the method used to express spliceform abundance, we necessarily found a corresponding increase in overall *hTnnt3 β* spliceform abundance (Fig 2, top). Interestingly, the variability in *hTnnt3* alpha and beta spliceform relative abundance was much higher in the old than the young groups. One possible reason for this finding could be greater variation in body weight in individuals in the old compared to the young group, given that we previously showed that body weight is an important determinant of *hTnnt3* spliceform expression (Schilder et al. 2011). However, when body weight-associated variation was taken into account in a two-way ANOVA of *hTnnt3 alpha* and *beta* summed relative abundances (with Age and body weight as factors), higher variation in the old groups was still evident, although three data points in particular appeared to drive this greater level of variation. Exploration of the role of the three samples by means of exclusion from the dataset revealed that even without these datapoints, there was still a significant difference ($p=0.047$) between old and young summed *hTnnt3* alpha/beta abundance. Therefore, although we cannot at this point account for the apparent age-related difference in variation, our main result is robust to the presence/absence of 3 low-abundance samples. In addition, we found that for *hTnnt3 α7*, the most abundant *hTnnt3* spliceform, and *hTnnt3 β1* there were statistically significant ($p =0.02$) positive and almost significant ($p = 0.058$) negative correlations respectively with human skeletal muscle performance (Fig 2, bottom). These results agree with earlier findings (Gallon et al. 2006) in that increased relative abundance of *Tnnt3* α spliceforms in muscle is associated with higher performance and given that these subjects did not significantly differ in lean mass (Drummond et al. 2008a; Fry et al. 2011) suggest that reduction of muscle performance associated with ageing may be initiated by impaired regulation of sarcomere composition prior to significant muscle atrophy typically associated with ageing.

Effects of resistance exercise on hTnnt3 alternative splicing

Repeated measures ANOVA in R using the *aov* package revealed no effects of exercise (i.e. comparing "Rest" means and post-exercise collapsed means) in study 1 nor study 2, taking into account any effects of Age (Tables 3 and 4).

Effects of EAA treatment of hTnnt3 alternative splicing

Assessing the effects of EAA and age on *hTnnt3* alternative splicing required comparison of results across studies 1 and 2. To insure that any changes observed were not a consequence of differences in baseline levels between studies, we determined whether young and old groups from the two studies differed in "Rest" expression levels for any of the *hTnnt3* spliceforms (mean comparisons using Student's t tests). As presented earlier (i.e. the effects of Age; Table 2) "Rest" expression of some *hTnnt3* spliceforms differed between young and old groups but importantly we found no significant differences (Supplementary Table S1) in "Rest" levels of hTnnt3 expression between study 1 and 2 for the two age groups. Thus, our subsequent analyses of effects of EAA treatment were not confounded by any study-associated baseline differences in expression in young or old groups. We next examined effects of EAA (and Age) on *hTnnt3* alternative splicing by comparing the collapsed mean *hTnnt3* spliceform abundances between study 1 and 2, while excluding the "Rest" data. A two-way ANOVA on effects of Age, EAA treatment and interaction term showed significant effects of EAA treatment on *hTnnt3* α7 and α4 that were independent of any effects of Age (Table 5). Of particular interest was the effect of EAA on *hTnnt3 α7* relative abundance, which according to Figure 2 data is indicative of enhanced muscle performance (i.e. an increase in *hTnnt3 α7* due to EAA treatment; Fig 3A). The lack of a significant interaction term (i.e. Age × EAA treatment; Table 5) indicates that this effect of EAA treatment was equally strong in both age groups.

Even though no significant overall effects of EAA on *hTnnt3* β spliceform level were apparent from the two-way ANOVA analysis (Table 5), post-hoc contrasts analysis demonstrated that there were age-specific significant effects of EAA treatment on *hTnnt3* β splice form abundance (Fig 3B). EAA treatment resulted in decreased relative abundance of both *hTnnt3 β1* and *β3* spliceforms in vastus lateralis muscle of study 2 old subjects compared to those in study 1, but had no effect in young subjects. Overall, these results indicate that EAA treatment has both general (i.e. in young and old muscle) and age-specific effects on *hTnnt3* splicing patterns that may (over time) benefit muscle function.

DISCUSSION

In addition to muscle mass loss (e.g. Thompson 2009; Piccirillo et al. 2014), ageing muscle typically shows a shift in fiber type distribution towards a more slow oxidative, type I fiber-rich phenotype due to selective loss of fast type II fibers (Lexell 1995; Thompson 2009; Venturelli et al. 2013). This can cause a shift in the general force-frequency relationship in muscle (Allman et al. 2004; Horner et al. 2011) as well as metabolic characteristics of the tissue (e.g. Thompson 2009), the latter of which is under significant debate recently (Ortega 2013; Venturelli and Richardson 2013). How variation in molecular composition (i.e. quality) of skeletal muscle fibers may contribute to these functional changes is less well

known but has been examined to some extent at the level of myosin-actin cross-bridge kinetics (Thompson 2009; Miller, Bedrin et al. 2013). The effect of age on expression patterns and interactions of other sarcomere gene products, and how environmental factors such as diet may modulate such age-related effects, remains poorly understood.

Although a variety of studies have reported that relative force production is impaired in muscle of older compared to younger individuals, it is interesting that in some studies using isolated skinned muscle fibers no age-related difference in relative force production (i.e. force normalized to fiber diameter or cross-sectional area) was observed (Trappe et al. 2003; Reid et al. 2012). In part, the discrepancy between results using intact muscle and isolated fiber results may be a consequence of alterations in neural function (e.g. impaired neuromuscular activation) that develop with age (e.g. Reid et al. 2014). Such changes would have little or no effect on measurements made using isolated muscle fibers, but could dramatically alter the response of intact muscle to neural stimulation. It has also been proposed that surviving muscle fibers in the elderly may undergo compensatory changes that serve to maintain function in older individuals (Reid et al. 2014). In this regard it is interesting that the average age of the older subjects in the present study was 10-20 years younger than that of the individuals in the previous studies (Trappe et al. 2003; Reid et al. 2012). Moreover, in contrast to the earlier studies, little or no sarcopenia was evident in the subjects in the present study. It will be interesting in future studies to assess whether relative force production is also unchanged in older individuals prior to manifestation of sarcopenia as has been reported to individuals in which loss of muscle mass is evident. In such studies, it will be important to assess the sensitivity of contraction to calcium stimulation over a range of concentrations, rather than simply using a concentration that induces maximal contraction, based on the known function of the troponins in calcium signaling.

Our studies examining effects of body weight, obesity, and mechanical loading on quantitative regulation of *Tnnt3* (i.e. fast troponin T) pre-mRNA alternative splicing in rodent muscle (Schilder et al. 2011; Schilder et al. 2012) recently stimulated another group to examine in a very similar fashion how resistance exercise affects alternative splicing of slow troponin T (*Tnnt1*) in vastus lateralis muscle of older humans (Zhang et al. 2014). In that study, long-term (i.e. 5 month) resistance exercise exposure was found to significantly change *Tnnt1* alternative splicing to produce a pattern that correlated with muscle fiber performance. However, no comparisons between muscles from older and younger subjects were made. Nonetheless, taken together these studies on skeletal muscle *Tnnt3* and *Tnnt1* highlight the importance of appropriate regulation of quantitative alternative splicing responses in muscle to changes in experienced load. In addition, they suggest important roles for mechano-sensitive signaling through protein kinase cascades such as Akt/ERK/mTORC1 (Schilder et al. 2012) in controlling these types of homeostatic adjustments by skeletal muscle. These pathways are of particular interest because they are known to promote protein synthesis in muscle in response to both resistance exercise and amino acid consumption. Indeed, inhibition of mTORC1 by rapamycin blocks both exercise (Drummond et al. 2009) and amino acid-induced (Dickinson et al. 2011) stimulation of muscle protein synthesis. However, in the elderly, both mTORC1 signaling and protein synthesis are refractory to the stimulatory effect of exercise compared to younger

individuals (Fry et al. 2011). In contrast, the stimulation of muscle mTORC1 and protein synthesis by amino acids is maintained in the elderly, although the response is delayed in older compared to younger individuals (Drummond et al. 2008a). These signaling pathways have only recently been linked to the control of quantitative alternative splicing (Lynch 2007), and consequently their role in modulating alternative splicing of pre-mRNA is currently unexplored. However, the finding in the present study that ingestion of a mixture of amino acids after resistance exercise, but not a bout of resistance exercise, leads to a shift in the pattern of alternative splicing of the *Tnnt3* pre-mRNA is intriguingly similar to the response of mTORC1 and protein synthesis to the two inputs. Finally, these findings reinforce the notion that patterns of alternative splicing of thin filament proteins such as TNNT3 and TNNT1 are important molecular markers for skeletal muscle structure-function relationships in healthy and diseased phenotypes, as was recently demonstrated for facioscapulohumeral muscular dystrophy (Gabellini et al. 2006; Sancisi et al. 2014).

In the present study, we compared *hTnnt3* alternative splicing in vastus lateralis muscles from young (i.e. 27±2 yrs old) and older (i.e. 68±2 yrs old) individuals and demonstrate that quantitative patterns of alternative splicing of *hTnnt3*, an important regulator of muscle contractile dynamics and force output (e.g. Pan and Potter 1992; Briggs and Schachat 1996; Ogut et al. 1999; Brotto 2005), were significantly altered in muscle from older compared to younger individuals. Ageing was associated with a *hTnnt3* spliceform expression profile that is predictive of lower muscle performance (Fig 2). Moreover, we demonstrate that administration of essential amino acids following resistance exercise rapidly changed the vastus lateralis *hTnnt3* spliceform expression pattern to one that is predicted to promote higher muscle performance in both young and old subjects (Table 2), while a single bout of resistance exercise prior to muscle biopsy had no effect on *hTnnt3* spliceform pattern during a 24 h recovery period. In a future study it will be interesting to assess the effect of multiple, intermittent doses of EAA on *hTnnt3* alternative splicing. In addition, in a future study it would be interesting to assess alternative splicing in a group of individuals with greater variation in body weight, since in the present study, young and older subjects did not differ significantly in body mass or lean mass (Drummond et al. 2008a; Fry et al. 2011) and our findings therefore suggest that impaired regulation of quantitative alternative splicing of *hTnnt3* is an important factor contributing to age-related muscle function decline occurring independently of (and prior to) overall muscle mass loss. In other words, in addition to selective loss of fast (type II) fibers, the intrinsic contractile characteristics of these fibers are also affected during ageing.

The decrease in cellular plasticity associated with human ageing has recently been linked to significant transcriptome-wide changes in splice factor expression and alternative splicing events in several human cell types (Harries et al. 2011; Holly et al. 2013), suggesting that ageing has systemic effects on the regulation of alternative splicing. Transcriptomics studies in ageing skeletal muscle have demonstrated significant changes in overall gene (Lombardi et al. 2009; Liu et al. 2013) and micro-RNA expression (Drummond et al. 2008b; Drummond et al. 2011), but to our knowledge skeletal muscle alternative splicing has not yet been examined in this fashion. Given that alternative splicing of more than 90% of human genes is regulated by a relatively small set of splice factors our observations at the

level of *hTnnt3* alternative splicing likely represent the tip of the iceberg in terms of age-related changes to skeletal muscle alternative splicing that occur at different levels of muscle organization. Indeed, a well known feature of ageing skeletal muscle is the altered alternative splicing of Insulin-like Growth Factor (IGF-I) pre-mRNA that results in lower levels of the Mechano Growth Factor (MGF) that controls skeletal muscle growth, maintenance, and repair (Goldspink 2005; Goldspink 2012). These effects are thought to be mediated by ageing-induced impaired functionality of the extracellular matrix-linked mechanotransduction system in muscle (Goldspink 2012), as has also been found in ageing cardiac muscle (Burgess et al. 2001). Nutritional intervention may be one avenue to counteract such impairments as evidenced by a recent comprehensive meta-analysis of the beneficial effects of protein supplementation on human skeletal muscle mass and strength gains in both young and old individuals (Cermak et al. 2012). Similarly, in cardiac muscle, alternative splicing-mediated switching between expression of N2BA and N2B isoforms of the giant protein titin, the ratio of which determines cardiomyocyte stiffness and predicts heart disease, is controlled by the nutrient-sensitive protein kinases Akt and mTORC1 (Linke and Kruger 2010). The findings of our current study further underscore the importance of dietary inputs to muscle health and suggest that essential amino acid supplementation effects on alternative pre-mRNA splicing may provide an avenue to enhance skeletal muscle quality during ageing, when normal mechanosensitive inputs controlling this phenotype may become impaired.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EAA	essential amino acids
hTnnt3	human fast skeletal muscle troponin T
mTORC1	mechanistic target of rapamycin complex 1
Tnnt	troponin T

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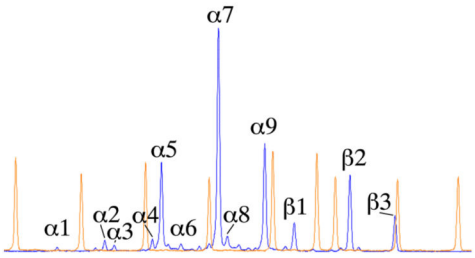
Key Points

- Loss of muscle mass and function are important contributors to physical frailty in the elderly
- Prior studies have shown that muscle function is in part controlled by the pattern of spliceform expression of proteins such as troponin T
- In this study we show that the pattern of alternative splicing of the pre-mRNA encoding fast troponin T (*Tnnt3*) is altered in older compared to younger individuals, and that the change in the pattern of *Tnnt3* alternative splicing is quantitatively correlated with age-associated reductions in muscle performance
- Ingestion of a mixture of essential amino acids following resistance exercise resulted in a shift in the pattern of Tnnt3 alternative splicing to one predicted to promote greater muscle performance
- The results suggest that essential amino acid supplementation effects on alternative pre-mRNA splicing may provide an avenue to enhance skeletal muscle quality during ageing



Splice form	exon composition											Seq.	Rel. abundance
	1-3	4	5	6	7	8	f	9-15	16	17	18		
hTnnt3 α1			X						X			N	0.015±0.001
hTnnt3 α2			X			X			X			N	0.032±0.001
hTnnt3 α3		X	X						X			N	0.022±0.001
hTnnt3 α4		X	X		X				X			N	0.019±0.001
hTnnt3 α5a		X	X			X			X			Y	0.278±0.015
hTnnt3 α5b			X	X	X				X			Y	
hTnnt3 α6		X	X	X					X			Y	0.011±0.001
hTnnt3 α7		X	X	X	X				X			Y	0.305±0.015
hTnnt3 α8		X	X	X		X			X			Y	0.045±0.005
hTnnt3 α9		X	X	X	X	X			X			Y	0.173±0.007
hTnnt3 β1		X	X			X				X		Y	0.034±0.008
hTnnt3 β2		X	X	X	X					X		Y	0.043±0.008
hTnnt3 β3		X	X	X	X	X				X		Y	0.024±0.004
*hTnnt3 αf**		X	X	X	X	X	X		X			Y	n/a

Figure 1.

Characterization and quantification of *hTnnt3* alternative splicing in vastus lateralis muscle. Top: Fluorescently labeled DNA fragment peaks showing *hTnnt3* splice form diversity and abundance (i.e. peak height) of each splice form in vastus lateralis muscle of an older subject. Internal size standards are represented by orange trace. Bottom: *hTnnt3* pre-mRNA comprises 18 exons, including a 5′ alternatively spliced cassette containing exons 4–9 (white boxes), and the mutually exclusive exons 16 and 17 (‘X’ denotes inclusion of specific exons). Twelve different hTnnt3 peaks were detected by RT-PCR while cDNA sequencing identified 10 unique spliceforms. Splice forms containing exon 16 or exon 17 were given an ‘α’ or ‘β’ designation, respectively. **hTnnt3 αf* is an embryonic spliceform containing an additional 5′ exon “f” that was sequenced but is only very rarely found in muscle samples.

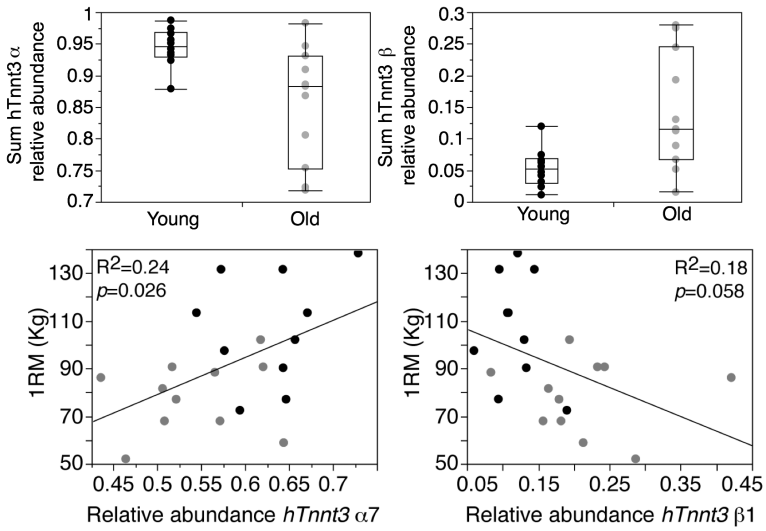


Figure 2.

Top: Sum of relative abundance for all *hTnnt3* α (left panel) and β (right panel) spliceforms in vastus lateralis muscle of old and young subjects (p = 0.0084). Bottom: least squares regression of muscle performance (1RM) and the relative abundance of splice forms *hTnnt3 α7* (left panel) and *hTnnt3 β1* (right panel). Fitting parameters as follows: *hTnnt3 α7*: R^2 = 0.24, p = 0.026; *hTnnt3 β1*: R^2=0.18, p = 0.058. N = 21. Grey symbols, older subjects; black symbols, young subjects.

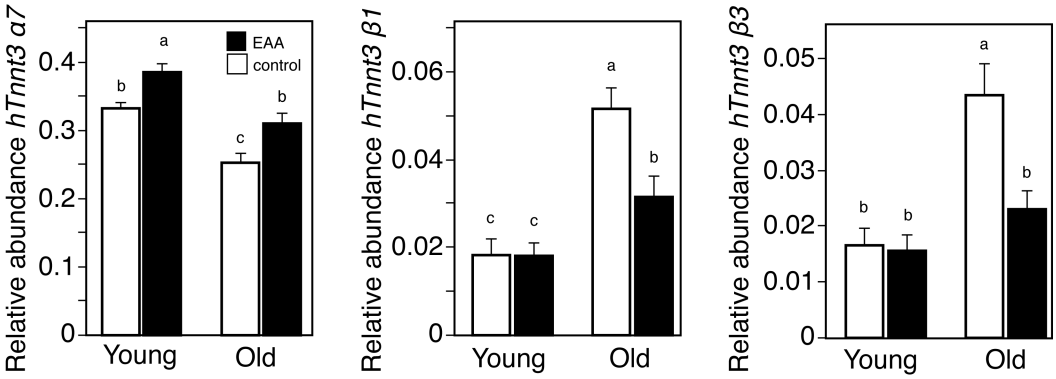


Figure 3.

Effects of EAA supplementation on *hTnnt3* alternative splicing. A. Significant increases were observed in both young and old subjects for *hTnnt3 α7* relative abundance (letters indicate significantly different means at α=0.05) whereas EAA affected *hTnnt3 β* spliceform abundance in old subjects only (B); N = 21. Time-averaged mean (excluding the "Rest" data) comparisons, rather than absolute spliceform abundance changes among the studies, are presented here to illustrate how EAA treatment in older individuals appears to revert their muscle phenotype to resemble that of a younger individual.

Table 1

Study design and sample distribution

Time point	Exercise Only (study 1)	Exercise + EAA (study 2)
Rest	N =12 (6 young + 6 old)	N=10 (5 young + 5 old)
3 hr post exercise	N =12 (6 young + 6 old)	N=10 (5 young + 5 old)
6 hr post exercise	N =12 (6 young + 6 old)	N=10 (5 young + 5 old)
24 hr post exercise	N =12 (6 young + 6 old)	No samples collected

Table 2

Two-way ANOVA on the effects of Age and body weight on *hTnnt3* splice form relative abundance. Statistically significant (after Benjamini-Hochberg false discovery rate correction) *p* values are highlighted in bold typeface. False discovery rate correction was not performed on principal components (PC1-4).

hTnnt3 splice form	% variation	Age		Body weight	
		***F* statistic**	***P* value**	***F* statistic**	***P* value**
α1		6.4879	**0.0202**	0.0083	0.9286
α2		0.2643	0.6134	0.3280	0.5739
α3		6.7550	**0.0181**	0.0374	0.8488
α4		2.0746	0.1669	2.0705	0.1673
α5		0.1680	0.6868	0.1027	0.7523
α6		4.7410	0.0430	0.9146	0.3516
α7		10.2526	**0.0049**	2.4593	0.1342
α8		0.1838	0.6732	1.4722	0.2407
α9		1.8620	0.1892	1.4798	0.2395
β1		10.1991	**0.0050**	0.5540	0.4663
β2		4.4963	0.0481	0.9754	0.3364
β3		7.8092	**0.0120**	2.9346	0.1039
PC1	39.5	4.9711	**0.0387**	0.1772	0.6787
PC2	23.5	4.9899	**0.0384**	3.6046	0.0738
PC3	17.0	4.8112	**0.0416**	0.9599	0.3402
PC4	9.3	2.2805	0.1484	0.2296	0.6376

Table 3

Study 1 - Effect of exercise (no EAA treatment) using a repeated measures ANOVA taking into account the factor Age (R package *aov*: aov.out=aov(hTnnt3 splice form abundance ~ age*exercise+Error (subject/test))

hTnnt3 splice form	Age		Exercise		Exercise*Age	
	***F* statistic**	***P* value**	***F* statistic**	***P* value**	***F* statistic**	***P* value**
α1	3.499	0.0942	1.298	0.284	0.766	0.404
α2	0.01	0.923	0.107	0.751	0.014	0.909
α3	6.642	0.0298	1.861	0.206	1.174	0.307
α4	6.24	0.034	2.607	0.141	0.005	0.947
α5	0.117	0.74	0.001	0.974	0.396	0.545
α6	2.981	0.118	0.023	0.882	2.455	0.152
α7	6.327	0.033	0.167	0.693	0.252	0.628
α8	0.052	0.825	0.415	0.535	1.806	0.212
α9	1.541	0.246	0.165	0.694	0102	0.756
β1	12.73	0.00604	0.252	0.628	0.307	0.593
β2	3.964	0.0777	0.188	0.675	0.027	0.873
β3	6.345	0.0328	0.015	0.905	0.055	0.820

Table 4

Study 2 - Effect of exercise (all received EAA treatment) using a repeated measures ANOVA taking into account the factor Age (R package *aov*: aov.out=aov(hTnnt3 splice form abundance ~ age*exercise+Error (subject/test))

hTnnt3 splice form	Age		Exercise		Exercise*Age	
	***F* statistic**	***P* value**	***F* statistic**	***P* value**	***F* statistic**	***P* value**
α1	1.354	0.278	1.823	0.214	1.319	0.284
α2	2.061	0.189	0.075	0.791	2.311	0.167
α3	1.22	0.301	0.963	0.355	0.386	0.552
α4	0.647	0.444	0.464	0.515	0.270	0.617
α5	1.029	0.34	1.036	0.338	0.602	0.460
α6	1.229	0.3	0.238	0.639	1.524	0.252
α7	9.079	0.0167	3.791	0.0874	0.657	0.441
α8	2.005	0.194	0.375	0.557	0.008	0.931
α9	0.247	0.632	0.286	0.608	0.000	1.000
β1	3.891	0.084	1.401	0.2706	4.402	0.0692
β2	0.473	0.511	1.24	0.298	3.014	0.121
β3	2.301	0.168	1.393	0.272	1.750	0.22

Table 5

Two-way ANOVA on the effects of Age and EAA (and interaction term) on *hTnnt3* splice form relative abundance comparing data obtained from study 1 and 2, post-exercise (see text). Statistically significant (after Benjamini-Hochberg false discovery rate correction) *p* values are highlighted in bold typeface. False discovery rate correction was not performed on principal components (PC1-4).

hTnnt3 splice form	Age		EAA		Age*EAA	
	***F* statistic**	***P* value**	***F* statistic**	***P* value**	***F* statistic**	***P* value**
α1	2.4253	0.1378	1.7105	0.2083	0.3003	0.5908
α2	1.7730	0.2006	0.5312	0.4760	1.3029	0.2695
α3	6.0321	0.0251	1.2538	0.2784	0.9736	0.3376
α4	5.3080	0.0341	14.5057	**0.0014**	0.4427	0.5147
α5	1.8653	0.1898	0.0000	0.9970	0.2077	0.6543
α6	0.7308	0.4045	2.7261	0.1171	0.9190	0.3512
α7	16.6591	**0.0008**	10.8661	**0.0043**	0.1301	0.7228
α8	1.6938	0.2105	3.6962	0.0715	0.9883	0.3341
α9	0.6447	0.4331	0.5622	0.4636	0.0295	0.8656
β1	11.6368	**0.0033**	3.3515	0.0847	2.3247	0.1457
β2	1.5897	0.2244	0.6225	0.4410	1.7386	0.2048
β3	6.2125	0.0233	3.0363	0.0995	1.5912	0.2242
PC1	2.7610	0.1149	0.1103	0.7438	1.9706	0.1784
PC2	9.5282	**0.0067**	13.7314	**0.0018**	0.0029	0.9578
PC3	4.0787	0.0595	1.8529	0.1912	0.0302	0.8642
PC4	1.1624	0.2960	0.1430	0.7100	0.2313	0.6367

Results were obtained using the collapsed means across the timepoints, but excluding the “Rest” data.