A Novel Actin mRNA Splice Variant Regulates ACTG1 Expression

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Abstract

Cytoplasmic actins are abundant, ubiquitous proteins in nucleated cells. However, actin expression is regulated in a tissue- and development-specific manner. We identified a novel cytoplasmic-γ-actin (Actg1) transcript that includes a previously unidentified exon (3a). Inclusion of this exon introduces an in-frame termination codon. We hypothesized this alternatively-spliced transcript down-regulates γ-actin production by targeting these transcripts for nonsense-mediated decay (NMD). To address this, we investigated conservation between mammals, tissue-specificity in mice, and developmental regulation using C2C12 cell culture. Exon 3a is 80% similar among mammals and varies in length from 41 nucleotides in humans to 45 in mice. Though the predicted amino acid sequences are not similar between all species, inclusion of exon 3a consistently results in the in the introduction of a premature termination codon within the alternative Actg1 transcript. Of twelve tissues examined, exon 3a is predominantly expressed in skeletal muscle, cardiac muscle, and diaphragm. Splicing to include exon 3a is concomitant with previously described down-regulation of Actg1 in differentiating C2C12 cells. Treatment of differentiated C2C12 cells with an inhibitor of NMD results in a 7-fold increase in exon 3a-containing transcripts. Therefore, splicing to generate exon 3a-containing transcripts may be one component of Actg1 regulation. We propose that this post-transcriptional regulation occurs via NMD, in a process previously described as “regulated unproductive splicing and translation” (RUST).

Citation: Drummond MC, Friderici KH (2013) A Novel Actin mRNA Splice Variant Regulates ACTG1 Expression. PLoS Genet 9(10): e1003743. doi:10.1371/journal.pgen.1003743

Editor: Carol A. Otey, University of North Carolina at Chapel Hill, United States of America

Received December 7, 2012; Accepted July 10, 2013; Published October 3, 2013

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Funding: This work received funding from the NIDCD (www.nidcd.nih.gov), grant R01DC4568. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

All mammals express six isoforms of actin: α-cardiac (Acta1, NM_009600), α-skeletal (Acta1, NM_009606), α-aortic (Acta2, NM_007392), γ-entric (Actg2, NM_009610), β-cytoplasmic (Actb, NM_007393), and γ-cytoplasmic (Actg1, NM_009609). Each actin is encoded on a separate chromosome but the coding sequence of the actins are 71% identical and there is 92% amino acid sequence identity between actin proteins. This degree of conservation is indicative of intolerance of these proteins to changes in amino acid composition, presumably because of the large number of proteins that interact directly with actin. Although the coding sequences are similar between actin isoforms, the genomic architecture of actin isoforms differs between the cytoplasmic (six exons), smooth muscle (nine exons), and cardiac and skeletal isoforms (seven exons). The genomic sequence of Actg1 was first described in 1986 by Erba and colleagues [1] and no splice variants of this gene have been reported.

In most dividing cells the two cytoplasmic actins are expressed at high levels. For example, mature skeletal and cardiac muscle derive from myoblasts, which express high levels of β- and γ-actin in their undifferentiated form. However, during differentiation, and in mature skeletal and cardiac muscle, the cytoplasmic actins are down-regulated to comprise only a small fraction of the total actin content, and α-skeletal and α-cardiac actins, respectively, become the predominant isoforms [2–4]. Nevertheless Actg1-null mice demonstrate that γ-actin is crucial for the normal function of mature skeletal muscle, as its complete absence results in a progressive myopathy in adult mice [5,6].

C2C12 mouse myoblast cell culture is widely used to study the expression and regulation of genes during skeletal muscle development. In this system, myoblasts proliferate until induced to differentiate either via serum-starvation or substitution of horse serum into the growth medium. Differentiation of myoblasts involves exit from the cell cycle, fusion with neighboring cells, elongation into myotubes, and movement of the nuclei to the periphery of the myotubes. Subsequent maturation is characterized by bundling of α-actin thin filaments to form myofibrils. Down-regulation of Actb in differentiated C2C12 cells was previously attributed to a 40 nucleotide long conserved element in the 3’ UTR of the Actb transcript [7]. In contrast, Actg1 down-regulation was proposed to involve inhibition of splicing of intron 3 from the primary Actg1 transcript, thus preventing the production of a mature RNA [2].

A potentially relevant mechanism for post-transcriptional down-regulation is Regulated Unproductive Splicing and Translation (RUST). RUST occurs by alternative splicing to include a regulatory exon which either contains, or creates via frameshift, a premature termination codon (PTC). Introduction of a PTC results in subsequent degradation of the mRNA by nonsense-mediated translation.
Author Summary

Actin is a well-studied protein that plays an essential role in nearly all cell types. Cytoplasmic actins are considered to be ubiquitously expressed in most tissues of the body with the exception of developing skeletal muscle, where muscle specific actins are up-regulated and γ-actin is repressed. Interest in the regulation of this transcript led to the hypothesis that intron retention is responsible for downregulation of cytoplasmic γ-actin in skeletal muscle during development. Since the publication of the sequence of γ-actin cDNA over two and a half decades ago, no additional splice variants or cDNAs of this gene have been described. In this paper, we identify an alternatively spliced transcript in muscle that allowed us to elucidate how the γ-actin is down-regulated during the important transition from myoblast to differentiated muscle cells. This is the first description of regulation of an actin transcript by regulated unproductive splicing and translation.

Results

Identification of a novel Actg1 transcript

While investigating γ-actin expression in a knock-in mouse model harboring a targeted mutation in exon 4 of Actg1, we identified a novel, alternatively spliced Actg1 transcript in wild-type animals. PCR amplification of skeletal muscle cDNA designed to amplify mouse Actg1 exon 3 to 4 (Figure 1A, Table 1), but to not allow amplification of Actb and Acta1, yielded an unexpected product. In addition to the predicted 102 bp product, an ampiclon of 147 bp was observed in skeletal muscle, heart and diaphragm (Figure 1B). Sequencing of the 147 bp product from skeletal muscle revealed an alternatively spliced transcript that includes a 45 bp exon located in intron 3, which we designate exon 3a (Figure 2A,B). It should be noted that in addition to the PCR products corresponding to the two alternatively spliced transcripts, larger amplicons that were not present in the no-RT controls were observed. Gel extraction and Sanger sequencing of these products revealed these were partially spliced products which included regions of intron 3 upstream and downstream of exon 3a (Figure 2C). In mouse, exon 3a is flanked by canonical splice acceptor and donor sites, and inclusion predicts introduction of an in-frame termination codon (Figure 2B,D). A BLAST search of the NCBI mouse EST database revealed transcripts that included exon 3a (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Exon 3a-containing transcripts are enriched in muscle

To determine if this transcript was present in other tissues of the adult mouse, cDNA was prepared from diaphragm, skeletal muscle, heart, intestine, spleen, kidney, testis, eye, lung, and brain from adult mice. Only skeletal and cardiac muscle were positive for the presence of alternatively spliced transcripts in addition to the normal Actg1 transcript, which was the major product amplified (Figure 1B).

To quantify expression of Actg1 isoforms, we designed primers compatible with qPCR to specifically amplify transcripts resulting from either an exon 3 - exon 4 (normal transcript) or an exon 3a - exon 4 splice (alternative transcript) (Figure 1A). Unlike competitive end-point PCR, the qPCR assay permits detection of low levels of the alternatively spliced Actg1 3a transcript. Using this method, we were able to obtain the relative abundance of normal Actg1 and alternative Actg1 transcript levels across tissues (Figure 1C,D). We found that brain exhibited the highest level of normal Actg1, whereas skeletal muscle and liver had the least. As expected from the results of the end-point PCR, skeletal muscle had the highest level of alternative Actg1.

The relative level of the alternative transcript was compared to that of the normal transcript using combined data from three qPCR experiments. The efficiencies of the PCR reactions for the normal and alternative transcript were within <1% of each other and the threshold was set at 0.2 for both reactions. This analysis revealed average ΔCt values of 20.8 for the normal and 22.7 for the alternative transcripts in skeletal muscle corresponding to a 3.5:1 ratio of normal to alternatively spliced Actg1 (Table 2).

Exon 3a is highly conserved among mammals

Evolutionary conservation of nucleotide sequence is typically indicative of functional significance. While no conservation of intron 3 is detected in fish or chicken cytoplasmic actin, the nucleotide sequence of Actg1 intron 3 is highly conserved among mammals. Specifically, the region containing the 45 bp alternatively spliced exon and flanking splice sites are 80% identical between humans and mice (Figure 2A,B). To determine if splicing of the Actg1 transcript is an evolutionarily conserved event in vivo, we prepared cDNA from human, dog, and cat skeletal muscle total RNA. Species- and isoform-specific primers were designed similar to the competitive end-point PCR assay for mouse described above and outlined in Figure 1A. Splicing to include exon 3a was observed in skeletal muscle cDNA from the species assayed (Figure 2C). All PCR products were sequenced to confirm the imputed exon 3a sequence (Figure 2B, human, cat, dog). Alignment of sequences obtained from the UCSC genome browser (http://genome.ucsc.edu) indicates that inclusion of exon 3a is predicted to introduce an in-frame PTC in non-primate mammals. In humans and rhesus, exon 3a is 41 nt in length and results in a frameshift of the ACTG1 coding sequence, thus creating a PTC in exon 4. While the amino acid sequence of the predicted polypeptide generated by inclusion of exon 3a is highly conserved in non-primate mammals, the frameshift generated by the 4 nt deletion in primates results in a complete loss of this conservation (Figure 2D).

Developmental regulation of Actg1 alternative splicing

To investigate the potential function of the alternative Actg1 transcript in a relevant tissue, we utilized the well-characterized C2C12 mouse myoblast cell line as a proxy for skeletal muscle
Figure 1. Splicing to include exon 3a into the Actg1 transcript is tissue specific. (A) Two PCR-based assays were employed to screen for the presence of Actg1 – competitive end-point PCR and splice-specific qPCR. Actg1-specific primers were designed to amplify either both the normal and alternative transcripts in a single reaction (competitive end-point PCR), or specifically the normal or alternative transcripts in separate reactions (splice-specific qPCR). (B) The competitive end-point PCR assay was used to amplify all Actg1 transcripts in various tissues from adult mouse. The larger PCR products at ~390 bp and ~290 bp are intermediate spliceforms of Actg1. The 147 bp product represents alternatively spliced, exon 3a-containing Actg1 transcripts, while the 102 bp product represents normally spliced Actg1 transcripts. (C) Expression data from qPCR to amplify normal Actg1 (exon 3 – exon 4) shows a high degree of variability of γ-actin expression between tissues. (D) Splicing to include exon 3a, as measured by qPCR is primarily limited to skeletal and cardiac muscle, with very low levels in the brain, eye, and intestine. All qPCR data is normalized to Rplp0 and Rrn18s expression and presented as a fold-difference to skeletal muscle.

doi:10.1371/journal.pgen.1003743.g001
transcripts increase during differentiation (Figure 3B).

Exon 3a-containing transcripts are exported to the cytoplasm, but no corresponding protein product is detected. Given the lack of conservation in the amino acid sequence generated by inclusion of exon 3a (Figure 2D), we hypothesized that a protein product is not produced from the alternative Actg1 transcript. To address this, we first sought to determine if the alternative transcript is exported to the cytoplasm and therefore available for translation. Total RNA was isolated from both cytoplasmic and nuclear fractions of mature myotube cultures. Using competitive end-point PCR, we found that the nuclear fraction contained all splice products including putative splicing intermediates, whereas only the normal and alternatively spliced transcript were present in the cytoplasmic fraction (Figure 4B).

Knowing that the alternative transcript is exported to the cytoplasm, we used western blotting to detect a protein product corresponding to either the use of the premature stop codon or a read-through of the stop codon. Because the levels of alternative transcript in the skeletal muscle samples exceed that of the normal transcript (Table 2), we probed for the presence of a protein product from alternatively spliced Actg1 transcripts. Based on the qPCR data, there should be a 3:1 ratio of normal ACTG1 to alternative ACTG1 (Table 2). We loaded 1:2 serial dilutions to determine our lower-limit of detection by western blot, beginning with 10 μg of total protein. No band corresponding either to usage of the termination codon (15 kDa) or a read-through of the termination codon in exon 3a (45 kDa) was detected (Figure 4C). We did observe a larger, 52 kDa protein in these samples which is consistent in size with previously described modifications of cytoplasmic and skeletal muscle actins, specifically mono-sumoylation [14] or mono-ubiquitination [15].

Inhibition of nonsense-mediated decay results in an increase of exon 3a-containing transcripts

We reasoned that exon 3a is alternatively spliced to post-transcriptionally down-regulate expression of Actg1. To address the hypothesis that exon 3a represses translation of Actg1 by targeting the transcript for NMD, we treated cells with cycloheximide to block translation. Cycloheximide targets the small ribosomal subunit and can be used to inhibit translation-dependent NMD of PTC-containing transcripts [16]. Cultures of proliferating myoblasts and mature myotubes were treated with either 40 μg/mL cycloheximide in ethanol or an ethanol-only control for three hours in otherwise standard growth conditions. A three-hour treatment with cycloheximide resulted in an approximately 7-fold increase of exon 3a-containing transcripts as measured by qPCR (Figure 5). These data strongly suggest that exon 3a targets the transcript for translation-dependent NMD. Furthermore, they indicate that splicing to include exon 3a is a frequent event in mature myotubes, given the rapid increase in the relative abundance of the alternatively spliced product.

Discussion

In this study, we identified a novel Actg1 splice variant enriched in cardiac and skeletal muscle. We propose that production of this alternative transcript is regulated and functional. Despite the fact

### Table 1. The sequence of primers used in this study.

| Primer | Sequence (5' → 3') | Target |
|--------|------------------|--------|
| MD003F | AGGCCAAACAGGAGAGATGACT | Hs ACTG1 |
| MD003aR | CGTCTCCAGATCCATGACA | Hs ACTG1 |
| MD004F | GAACCCCAAAGCTAACAGGAG | Mm Actg1 |
| MD004R | CAGATGATCAAGACAGACAC | Mm Actg1 |
| MD006F | AACAACGGTGAAGATGACT | Cf/Fc Actg1 |
| MD006R | ATCTCCAGATCCTGAGCACAATA | Cf/Fc Actg1 |
| MD013F | CAGAGGAAGATGAGCGAGATA | Mm Actg1 - normal splice only |
| MD013R | CATGACATGGCGACTGGTG | Mm Actg1 |
| MD014F | TCCCTGAAGCCTCCAGATAA | Mm Actg1 - alternative splice |
| MD014R | ACTGTCGCTTTTGGCG | Mm Ppia |
| 2musPpiaF | ACTGTCGCTTTTGGCG | Mm Ppia |
| 2musPpiaR | GCTGTCTTTGGAACCTTGTCG | Mm Ppia |
| MD015F | ACAACAAGCTGCTGAGGAC | Mm Rn1B1s |
| MD015R | CAGTGCTTGTGAGTGCTGA | Mm Rn1B1s |
| MD016F | GCGACCTGGAGAATGCACTA | Mm Rpdp0 |
| MD016R | GCTCCCAATGGAAGCATT | Mm Rpdp0 |

Primers were designed using Primer3 to amplify cDNA from either human (Hs), mouse (Mm), or dog (Cf) and cat (Fc). doi:10.1371/journal.pgen.1003743.t001

development. C2C12 cells are frequently used to study transcriptional and proteome changes during the differentiation of myoblasts into myotubes (shown in Figure 3A) [12,13]. Using these cells, we first asked if Actg1 alternative splicing is developmentally regulated. Total RNA was isolated from myoblasts prior to addition of differentiation medium and at 2 day intervals after addition of differentiation media. qPCR was used to determine expression levels of normal and alternatively spliced Actg1 transcripts were present in the cytoplasmic fraction (Figure 3B). These data also reveal that concurrent with the decrease in normal Actg1 expression, alternatively spliced Actg1 transcripts increase during differentiation (Figure 3B).
Table 2. Ct values for a skeletal muscle cDNA control demonstrate no plate-to-plate variability.

| Experiment          | Threshold | Avg Ct for Normal Splice | Avg Ct for Alternative Splice |
|---------------------|-----------|--------------------------|------------------------------|
| Tissue Panel        | 0.2       | 20.75                    | 22.62                        |
| C2C12 Timecourse    | 0.2       | 21.01                    | 22.70                        |
| C2C12 CHX Treatment | 0.2       | 20.95                    | 22.65                        |
| Combined Average    | –         | 20.90                    | 22.70                        |

For each qPCR plate, a skeletal muscle cDNA control sample was included to assess plate-to-plate variability for every primer pair. For the Tissue Panel experiment Ct values represent three skeletal muscle cDNA biological replicates in addition to the control skeletal muscle cDNA sample. Alternatively spliced transcripts constitute approximately 30% of steady-state Actg1 mRNA in adult skeletal muscle. Two technical replicates were averaged for every biological replicate in qPCR experiments.

doi:10.1371/journal.pgen.1003743.002
that cytoplasmic actins are well-studied and widely used as reference genes, this is the first report of alternative splicing for any actin transcript. Why might exon 3a containing transcripts have been overlooked? The most divergent sequence for distinguishing between actin transcripts reside in the 5'UTR which is sufficient for down-regulation of γ-actin during differentiation of myoblasts. PCR primers designed to specifically amplify one isoform are typically in the UTRs and inclusion of a small exon, such as the 41–45 nt 3'UTR, is likely to be overlooked in a large, >1 kb PCR product. Furthermore, short PCR products in which a 41–45 nt difference could be resolved are unlikely to involve exons 3–5 of the actin transcript because the high degree of nucleotide similarity between actin isoforms in these exons. Given that the alternative exon appears to be specific to γ-actin, a product corresponding to the inclusion of exon 3a in these exons. Given that the alternative exon appears to be specific to γ-actin, a product corresponding to the inclusion of exon 3a is the result of ‘noisy splicing’. Large-scale analyses indicate that the majority of alternatively spliced transcripts are likely generated in error because of their low abundance across multiple tissues and lack of correlation with expression differences in the genes examined [9,21,22]. However, several examples of RUST as a mechanism of post-translational regulation of the NMD pathway. Therefore, we hypothesize that γ-actin is down-regulated via alternative splicing to introduce a PTC, leading to the degradation of Actg1 transcripts via NMD [8,20].

We considered the possibility that the presence of the alternatively spliced Actg1 transcript is the result of ‘noisy splicing’. Large-scale analyses indicate that the majority of alternatively spliced transcripts are likely generated in error because of their low abundance across multiple tissues and lack of correlation with expression differences in the genes examined [9,21,22]. However, several examples of RUST as a mechanism of post-translational regulation of Actg1 transcripts is likely to be spurious [11,22]. A primary feature of ‘noisy splicing’ is lack of conservation of the alternative splice form in other species. However, we find that, similar to the normal coding exons of Actg1, exon 3a is highly conserved in mammals. Furthermore, in agreement with criteria for functional splice variants, we demonstrated that the alternative splice form is

Figure 3. Splicing to include exon 3a is a developmentally regulated event in skeletal muscle. (A) Microscopy of cell cultures before, during and after differentiation. C2C12 myoblasts were grown to 70% confluence and induced to differentiate in DMEM+10% horse serum. Partially differentiated cultures containing both myoblasts and myotubes were observed by 2 days post-differentiation. After 48 hours, medium was replaced with DMEM+2% horse serum and 10 μM Ara-C and cultured for an additional 4 days. (B) qPCR of RNA harvested in Trizol showed that concurrent with a decrease in normal γ-actin, splicing to generate alternative Actg1 increases during differentiation into myotubes. Expression of both the normal and alternative transcripts was normalized to Ppia and is presented as fold-difference compared to skeletal muscle. A two-tailed type 2 Student's T-test was used to compare expression differences between time points. For all time points compared, p<0.0001. doi:10.1371/journal.pgen.1003743.g003

Figure 4. Exon 3a containing alternatively spliced Actg1 is exported to the cytoplasm but does not produce a stable protein. Nuclear (A) and cytoplasmic (B) RNA fractions were harvested from C2C12 myotubes and evaluated for the presence of alternative Actg1 using competitive, end-point PCR. All Actg1 spliceforms, including partially spliced transcripts, were present in the nuclear fraction, however, only the normal and exon 3a transcripts were observed in the cytoplasmic fraction. (C) Western blot using an anti-γ-actin specific antibody was used to probe mouse skeletal muscle lysate for the presence of a protein product corresponding to the inclusion of exon 3a. Usage of the in-frame stop codon in exon 3a would generate a 15 kDa protein. Alternatively, read-through of the stop codon would increase the size of the ACTG1 protein by 2 kDa, resulting in a 44 kDa protein. Neither of these protein products is present. A γ-actin protein at 52 kDa is observed and likely represents a post-translational modification. Molecular weight marker is prestained Low Range (BioRad). doi:10.1371/journal.pgen.1003743.g004

A Novel γ-Actin Transcript Regulates Expression
have a similar genomic structure and similar to utilizing the addition, it was recently demonstrated that an expression construct has high degree of conservation in a portion of intron 3 [27]. In mechanism of regulation for the non-cytoplasmic actin isoforms. In skeletal and cardiac muscle, exon 3a splicing may be regulated by splicing enhancer or splicing repressor elements. The intronic region immediately adjacent to the 5' donor site of exon 3a is well conserved among mammals (Figure 6). During differentiation of myoblasts, multiple transcription and splicing factors are required to coordinate changes in gene expression crucial for differentiation. As such, the intronic conservation immediately 3' of exon 3a may contain recognition motifs for splicing enhancers or repressors. Indeed, an exon splicing enhancer prediction software, ESE Finder v3.0, identified several splicing enhancer recognition motifs (Figure 6). Of particular interest are the recognition motifs for SF2/ASF and SC35, which were shown previously to effect alternative splicing of β-tropomyosin in a tissue-specific manner [33].

In conclusion, this report documents the first identification and characterization of an alternatively spliced actin transcript. These data provide evidence for the dynamic regulation of Actg1 and further functional evidence for RUST.
Materials and Methods

Bioinformatics

Sequence ascertainment and analysis was performed using UCSC Genome Browser/BLAT (http://www.genome.ucsc.edu/), Ensembl! (http://www.ensembl.org), Sequencher 6.0 (Gene Codes Corp.), and Clustal X2 (http://www.clustal.org). Primers were designed using Primer3 software (http://frodo.wi.mit.edu/).

Potential exon splicing enhancers were identified using ESEFinder v3.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi)

Animals and tissue preparation

All animals were maintained according to Michigan State University IACUC and NIH guidelines. Tissue samples were harvested from three 1 year old C57Bl/6J mice, snap frozen on dry ice, and stored at −80°C. Prior to use for RNA or protein isolation, samples were chopped into 100–200 mg pieces. Cat and dog skeletal muscle samples were provided courtesy of Dr. John Fyfe (Michigan State University). Human skeletal muscle total RNA was purchased from Ambion (catalog AM7982; Austin, TX).

Cell culture

All cell culture media and supplements were purchased from Invitrogen (Carlsbad, CA), unless noted otherwise. C2C12 myoblast cells were purchased from ATCC (Manassas, VA; CRL-1772) and propagated in DMEM containing 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine. Differentiation of myoblasts into myotubes was achieved by culturing cells 70% confluent in DMEM supplemented with 10% horse serum in place of fetal bovine serum. Forty-eight hours post differentiation, DMEM with 2% horse serum and 10 μM Ara-C (Sigma, St. Louis, MO) was used to maintain differentiated myotubes and inhibit the proliferation of myoblasts. Cells were maintained at 37°C in 5% CO₂.

RNA isolation and cDNA synthesis

Total RNA isolation from tissue and cell culture samples was achieved using TRIzol (Invitrogen, Carlsbad, CA) purification followed by a DNaseI treatment using RNasea mini-columns (Qiagen, Hilden, Germany). Total RNA was quantified using a NanoDrop (Thermo, Wilmington, DE). cDNA was synthesized using SuperScriptIII reverse transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. Reaction volume was 20 μl; for qPCR, 300 ng of RNA was used per reaction, and for endpoint PCR, 1 μg of RNA was used. Following incubation at 55°C for 1 hour, samples were heat inactivated at 75°C for 20 minutes then stored at −20°C until used.

Nuclear and cytoplasmic RNA isolation

Myotube cultures were lysed rapidly with 1% Triton X-100 in PBS, cell debris and nuclei were gently scraped from the culture dishes and evaluated by brightfield microscopy for the presence of intact nuclei. Human skeletal muscle total RNA was purchased from Ambion (catalog AM7982; Austin, TX).

Competitive end-point PCR amplification

Acgt1 cDNA was amplified using Promega GoTaq polymerase per manufacturer’s instructions (Madison, WI) with 5 μM each of species- and isof orm-specific primers located in exons 3 and 4 of...
Actg1 (Table 1). For most reactions, 28 cycles were sufficient to amplify within the perceived linear range. Products were evaluated on 3% agarose gels containing 0.3 μg/mL ethidium bromide and visualized using BioRad GelDoc System (Hercules, CA). Pixel intensity of the PCR products was quantitated using GelDoc software (BioRad, Hercules, CA) for semi-quantitative analysis.

qPCR
Quantitative PCR (qPCR) was performed on an ABI7000, using Power SYBR Green (Invitrogen, Carlsbad, CA) as the reporter dye, and data were collected using StepOne Plus software (Applied BioSystems, Carlsbad, CA). Data were analyzed using qbasePLUS software (Biogazelle, Zwijjaarde, Belgium). Actg1 transcripts were normalized to Pia for experiments using C2C12 cells and to β2m and Rnl18s for experiments using mouse tissues. See Table 1 for primer sequences. Averaged data represent two technical replicates for each of three biological replicates. A two-tailed type 2 Student’s T-test was used to compare expression differences between samples.

Sequencing
Sequence data for exon 3a were obtained by Sanger sequencing on an ABI Prism 3700 DNA Analyzer at the Research Technology Support Facility (Michigan State University) using competitive, end-point PCR (Table 1).

Cycloheximide treatment
Cycloheximide (Sigma, St. Louis, MO) was dissolved in 100% ethanol at a stock concentration of 40 mg/mL and added to growth medium at a final concentration of 40 μg/mL. Cells were incubated in cycloheximide containing medium for 3 hours and immediately harvested in TRIzol for RNA isolation and cDNA synthesis as described above. Each experiment was repeated three times.

Protein isolation
Approximately 100 mg of skeletal muscle from a 1 year old mouse was lysed using a Polytron rotor homogenizer in lysis buffer containing 100 mM KCl, 10 mM PIPES, 5 mM EGTA, 1% Triton X-100 and Complete Protease Inhibitors (Roche, Basel, Switzerland), and incubated on ice for 1 hour to allow further lysis. Protein content in the total lysate was determined using a Bradford assay (BioRad, Hercules, CA).

Western blotting
Proteins were separated via SDS-PAGE on discontinuous 12% bis-acrylamide gels [10]. Proteins were transferred in 10 mM Tris pH 7.4, 100 mM glycine, 15% methanol (transfer buffer) at 4°C overnight at a constant current of 5 mAmp onto polyvinylidene difluoride (PVDF) membranes (BioRad, Hercules, CA). Membranes were incubated in PBS (pH 7.4) containing 5% non-fat milk and 0.025% Tween-20 (blocking buffer) for one hour at room temperature. A previously validated rabbit polyclonal anti-γ-actin specific antiserum raised against the first 15 amino acids of the polypeptide [5] was diluted 1:10,000 in blocking buffer. Membranes were incubated with primary antiserum for 2 hours at room temperature. Goat polyclonal anti-rabbit IgG-HRP conjugated secondary antibody (Sigma, St. Louis, MO) was used at 1:3,000 in blocking buffer for one hour at room temperature. Proteins were detected using an ECL Detection Kit (GE Healthcare, Waukesha, WI) with Amersham Hyperfilm MP autoradiography film (GE Healthcare, Waukesha, WI).

Acknowledgments
We thank Drs. Ronald Patterson, Richard Schwartz, Julie Schultz, Thomas Friedman, and Dennis Drayna for critical review of this manuscript and thoughtful discussions.

Author Contributions
Conceived and designed the experiments: MCD KHF. Performed the experiments: MCD. Analyzed the data: MCD KHF. Contributed reagents/materials/analysis tools: MCD KHF. Wrote the paper: MCD KHF.

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