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Modulation of alternative splicing of trafficking genes by genome editing reveals functional consequences in muscle biology

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Abstract

Alternative splicing is a regulatory mechanism by which multiple mRNA isoforms are generated from single genes. Numerous genes that encode membrane trafficking proteins are alternatively spliced. However, there is limited information about the functional consequences that result from these splicing transitions. Here, we developed appropriate tools to study the functional impact of alternative splicing in development within the most *in vivo* context. Secondly, we provided evidence of the physiological implications of splicing regulation during muscle development. Our previous work in mouse heart development identified three trafficking genes that are regulated by alternative splicing between birth and adulthood: the clathrin heavy chain, the clathrin light chain- α , and the trafficking kinesin binding protein-1. Here, we demonstrated that alternative splicing regulation of these three genes is tissue- and developmental stage-specific. To identify the functional consequences of splicing regulation *in vivo*, we used genome editing to block the neonatal-to-adult splicing transitions. We characterized the phenotype of one of these mouse lines and demonstrated that when splicing regulation of the clathrin heavy chain gene is prevented mice

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COMPETING INTERESTS

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exhibit an increase in body and muscle weights which is due to an enlargement in myofiber size. The significance of this work has two components. First, we revealed novel roles of the clathrin heavy chain in muscle growth and showed that its regulation by alternative splicing contributes to muscle development. Second, the new mouse lines will provide a useful tool to study how splicing regulation of three trafficking genes affects tissue identity acquisition and maturation *in vivo*.

Keywords

Alternative splicing; Membrane trafficking; Development; CRISPR-Cas9; Muscle; Clathrin

1. INTRODUCTION

Numerous trafficking genes are regulated by alternative splicing (Blue et al., 2018; Brinegar et al., 2017; Dillman et al., 2013; Giudice et al., 2014; Hannigan et al., 2017; Irimia et al., 2014), a post- and co-transcriptional mechanism by which single genes produce multiple transcripts and therefore multiple protein isoforms. Approximately 95% of human genes undergo alternative splicing (Pan et al., 2008; Wang et al., 2008) and coordinated splicing networks regulate organ development as well as tissue identity acquisition and maintenance (Baralle and Giudice, 2017). We previously demonstrated that intracellular trafficking and membrane dynamic genes are the most significantly regulated by alternative splicing in mouse postnatal heart development (Giudice et al., 2014). Among these trafficking genes, we found three splicing events of interest: (a) an alternative cassette exon in the clathrin heavy chain (*Cltc*) gene, (b) two consecutive cassette exons in the clathrin light chain-a (*Clta*) gene, and (c) alternative polyadenylation (polyA) site selection in the trafficking kinesin binding protein-1 (*Trak1*) gene.

Membrane trafficking controls numerous functions within the cells including cell communication during organ development, internalization of ion channels, receptors and ligands to control homeostasis and signaling, the transport of newly synthesized proteins, and the dynamics of organelles such as mitochondria, endosomes, lysosomes, among others (Blue et al., 2018). Intracellular trafficking is especially important in heart and skeletal muscle because of the large size of the cells that comprise these tissues. Therefore, it is vital to understand how these genes are regulated throughout development.

Clathrin mediated endocytosis is one of the most important internalization pathways in the cell. Clathrin is composed of trimerized heavy chains (CLTC) with bound light chains (CLTA, CLTB) (triskelion) and assembles to form a membrane coat. Formation of the clathrin lattice on cellular membranes is initiated after nucleation by clathrin adaptors that trigger clathrin-coated vesicle budding (Brodsky, 2012; Kirchhausen, 1999; Pearse and Robinson, 1990). Once invagination has initiated, dynamin proteins associate with the neck of the forming pit and recruit additional proteins including actin, which allow for the scission of the coated vesicle (Merrifield et al., 2002) and endocytosis. Although CLTC is the main driver of clathrin mediated endocytosis, CLTC also has structural roles in skeletal muscle where it regulates the formation and maintenance of the costamere (Vassilopoulos et al., 2014). Still, nothing is known about the functions of CLTA and CLTB in heart and/or

skeletal muscle. On the other hand, TRAK1 regulates endosome-to-lysosome trafficking (Sagie et al., 2018; Stephenson, 2014; Webber et al., 2008) but is also an adaptor protein that links the kinesin heavy chain to mitochondria. Through the interaction with mitofusins, TRAK1 contributes to mitochondrial trafficking and fusion. When the formation of the TRAK1-kinesin adaptor is inhibited in axons of hippocampal pyramidal neurons, mitochondrial mobility is decreased (Brickley and Stephenson, 2011). Furthermore, TRAK1 depletion leads to mitochondrial fragmentation (Lee et al., 2017) and a recent report identified mutations in the *TRAK1* gene in individuals with neurodevelopmental delay, seizures, and fatal encephalopathy (Barel et al., 2017). Fibroblasts derived from these individuals exhibit damaged mitochondria distribution due to impaired mitochondrial motility and reduced respiration capacity (Barel et al., 2017). Still, the function of TRAK1 in cardiac and skeletal muscle tissue has not been described.

The alternative splicing field has made tremendous progress in describing the mechanisms regulating splicing decisions. However, the functional implications of splicing have been significantly under-investigated. We and others have used antisense oligonucleotides to redirect splicing in skeletal muscles of adult mice to identify muscle-specific splicing isoforms required for adult muscle function (Brinegar et al., 2017; Giudice et al., 2016; Vecellio Reane et al., 2016). To study the physiological implications of splicing during fetal-to-adult development a different strategy is required. Therefore, the first aim of this work was to create an appropriate *in vivo* approach to block the fetal-to-adult splicing transitions that occur in *Cltc*, *Clta*, and *Trak1* genes. We thus applied CRISPR/Cas9 genome editing to generate mouse lines that are unable to undergo normal splicing transitions of *Cltc*, *Clta*, or *Trak1* genes during postnatal development. The second aim of this work was to demonstrate the capacity of the approach to reveal novel physiological information relevant to the resulting isoform transitions. To do that, we chose one of the genes of interest (clathrin heavy chain) and showed that failure to gradually express the isoform containing the alternative exon during development resulted in an increase in body and muscle weights in adulthood.

2. MATERIALS AND METHODS

2.1. Materials.

Chemicals and cell culture reagents were obtained from GIBCO, Life Technologies, ThermoScientific, and Sigma. HeLa cells were purchased from ATCC.

2.2. Cell transfections.

HeLa cells were seeded to six well plates (5×10^5 cells/well) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, #100–106) and 2 mM glutamine until they reached 70% confluence. The next day, transfections were performed using RNAiMax Lipofectamine (Invitrogen, #13778030) and Stealth siRNAs (ThermoFisher Scientific), following manufacturer protocols. siRNA sequences for the Luciferase reporter control (#12935146) were GCACUCGAUUGACAAAUACGAU (sense) and AAAUCGUAUUUGUCAUACAGAGUGC (antisense). siRNA sequences for the *Clta* (si-

Clta) (#MSS236195) were GGAUCGAUUGCAGUCAGAGCCUGAA (sense) and UUCAGGCUCUGACUGCAAUCGAUCC (antisense). RNA and protein were extracted 48 h after transfection.

2.3. Animals.

All animals were in the FVB background and were handled following the NIH Guidelines for Use and Care of Laboratory Animals that were approved by the Institutional Animal Care and Use Committees (IACUC) at Baylor College of Medicine and The University of North Carolina at Chapel Hill.

2.4. Generation of mouse lines using CRISPR/Cas9.

For each splicing event, two independent mouse founder lines were generated using CRISPR/Cas9 technology in the Genetically Engineered Mouse (GEM) Core at Baylor College of Medicine. For each splicing event, two guide RNAs (sgRNAs) targeting two intronic sequences flanking the alternative exons were designed using available bioinformatics tools (<http://crispr.mit.edu>) by the Embryonic Stem Cell Core (Baylor College of Medicine) (Table 1). The sgRNAs and Cas9 mRNA were synthesized by the Embryonic Stem Cell Core and injected into the pronuclear stage of mouse embryos by the Genetically Engineered Mouse Core (Baylor College of Medicine). Injected embryos were cultured at 37 °C under 5% CO₂ and then transferred into the ampulla of the oviduct of a pseudo-pregnant female. To screen pups for the genome modifications, tail DNA was utilized as template for PCR reactions using primers flanking the target region (Table 2). PCR products were analyzed by gel electrophoresis revealing the presence or absence of the targeted exons. Some tail DNA PCR products were sequenced to identify the fusion generated by NHEJ. Screening of several F1 animals identified the individual F1 mice that were used to establish the lines with the deletion to then make the homozygotes.

2.5. Mouse genotyping.

Genomic DNA was extracted from tail biopsies by Direct PCR lysis reagent (Viagen, #102-T) following manufacturer protocols or by boiling them in 50 mM NaOH for 10 min followed by neutralization with 1 M Tris-HCl at a 1:10 dilution before pelleting at 13,000 r.p.m. for 6 min. The supernatant containing the DNA was utilized to perform PCR using GoTaq green master mix (Promega, #M7123) and specific primers (0.5 µM) (Sigma) (Table 2). PCR amplification conditions were as follows: (i) 95 °C for 5 min, (ii) 33 cycles of 95 °C for 30 s, 61 °C for 30 s, (iv) 72 °C for 40 s (iii) 72 °C for 5 min, and 4 °C pause. Amplification products were analyzed by 1.5% agarose gel electrophoresis in Tris-borate-EDTA (TBE) solution (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) for 75 min at 120 V. Gels were prepared with 0.5 µg/mL ethidium bromide and visualized using the ChemiDoc™ XRS+ (Biorad) imaging system.

2.6. Tissue harvest.

Animals were anaesthetized and after cervical dislocation (adults) or decapitation (neonates) tissues were removed, flash frozen in liquid nitrogen, and stored at -80 °C prior to usage.

2.7. RNA extraction and cDNA synthesis.

RNA was extracted from homogenized tissues or cells using TriZol and following manufacturer protocols. When tissues were used for RNA isolation, frozen tissues were placed in tubes with Lysing Matrix D 1.4mm ceramic beads (MP Biomedicals, #6913) with TriZol (Invitrogen, #15596018) and then homogenized at 6,500 r.p.m. in 20 s intervals using the Precellys 24 homogenizer (Bertin Instruments, #P000669-PR240-A) until complete homogenization. Then RNA was extracted following TriZol manufacturer protocols. RNA concentration was measured using a nanodrop lite spectrophotometer (ND-LITE, ThermoScientific). RNA was reverse transcribed into cDNA using the suggested protocol from the High-capacity cDNA reverse transcription kit (Applied Biosystems, #4368813). The cDNAs were reverse transcribed as follows: (i) 25 °C for 10 min, (ii) 37 °C for 120 min, (iii) 85 °C for 5 min, (iv) 4 °C pause.

2.8. Analysis of alternative splicing by RT-PCR.

The cDNA was used to perform alternative splicing PCRs using primers (0.5 µM) targeting the constitutive exons flanking the alternative regions (Table 3). PCRs were performed using the GoTaq green master mix. The amplification conditions for Clta and Cltc were as follows: (i) 95 °C for 1 min 15 sec, (ii) 28 cycles of 95 °C for 45 s, 57 °C for 45 s, 72 °C for 1 min, (iii) 72 °C for 10 min, (iv) 4 °C pause. The amplification conditions for Trak1 were as follows: (i) 95 °C for 1 min 15 sec, (ii) 28 cycles of 95 °C for 45 s, 59 °C for 45 s, 72 °C for 1 min, (iii) 72 °C for 10 min, (iv) 4 °C pause. PCR products were separated by 6% polyacrylamide gel or 1.5% agarose gel electrophoresis in TBE. After electrophoresis, gels were stained with 0.4 µg/mL ethidium bromide for 10 min. Gels were visualized using the ChemiDoc™ XRS+ imaging system. Quantification of gels was performed by densitometry using Image Lab™ 6.0.1 (Biorad) software for analysis. The Percent Spliced In (PSI) (Wang et al., 2008) was estimated using the following equation:

$$PSI = 100 \times \frac{\text{inclusion band}}{\text{inclusion band} + \text{skipping band}}$$

2.9. Protein extraction.

Frozen tissues were placed in tubes with Lysing Matrix D 1.4mm ceramic beads and RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM Tris, 5 mM EDTA, pH 8.0) supplemented with protease (Sigma Aldrich, #11697498001) and phosphatase (Sigma Aldrich, #4906845001) inhibitors. Tissues were homogenized at 6,500 r.p.m. in 20 s intervals using the Precellys 24 homogenizer until complete homogenization. Tissue lysates were incubated at 4 °C for 2 h with gentle agitation before sonication for 3 min (30 s bursts) at 75 V. After spinning at 14,000 r.p.m. for 10 min at 4 °C, supernatants were collected. When protein was extracted from HeLa cells, cells were lysed in RIPA buffer, sonicated for 3 min (30 s bursts) at 75 V, centrifuged at 14,000 r.p.m. for 10 min at 4 °C, and supernatants were collected. Protein concentration was measured using the Pierce BCA protein assay kit (ThermoScientific, #23225).

2.10. Western blot assays.

Protein samples (25–40 µg) prepared in loading buffer (62.5 mM Tris-HCl, 10% glycerol, 2% SDS, 0.02% bromophenol blue, 143 mM beta-mercaptoethanol, 5 mM dithiothreitol, pH 6.8) were boiled for 5 min and then loaded into 4–20% Mini-Protean TGX Stain-Free Gels (Biorad, #456–8093). Electrophoresis was performed in running buffer (25 mM Tris, 190 mM glycine, 0.1% w/v SDS, pH 8.3) for 30 min at 90 V, then 1.5 h at 130 V. After electrophoresis, gels were exposed to UV light for 2.5 min to activate TGX within the gel for total protein visualization. Proteins were transferred onto 0.2 µm PVDF Immobilon-P^{SQ} membranes (Merck Millipore, #ISEQ00010) and visualized using the ChemiDoc XRS+ imaging system. Membranes were then blocked with 5% non-fat dried milk in a buffer containing 19 mM Tris, 2.7 mM KCl, 137 mM NaCl, 0.1% Tween20, pH 7.4 (TTBS) for 1 h, washed, and incubated overnight at 4 °C with the primary antibodies diluted in 5% bovine serum albumin (BSA) in TTBS. Primary antibodies were as follows: (a) polyclonal rabbit anti-LCA H55 (Santa Cruz Biotechnology, #sc28276) diluted 1:500, (b) polyclonal rabbit anti-TRAK1 (ATLAS Antibodies, #HPA005853) diluted 1:500, (c) monoclonal mouse anti-CLTC (BD Biosciences, # 610499) diluted 1:1,000, (d) monoclonal rabbit anti-GAPDH (14C10) (Cell Signaling, #2118) diluted 1:1,000, and (e) polyclonal rabbit anti-sarcomeric alpha actinin (ACTN2) (abcam, #ab72592) diluted 1:500. The next day, the membranes were washed three times (10 min each) with TTBS and then incubated for 1–1.5 h at room temperature in the darkness with secondary antibodies. Secondary antibodies were as follows: (a) polyclonal goat anti-rabbit IgG (H+L) secondary antibody DyLight 800 4X PEG (Invitrogen, #SA5–35571) diluted 1:10,000 in 5% BSA in TTBS, (b) polyclonal goat anti-mouse IgG (H+L) secondary antibody DyLight 800 4X PEG (Invitrogen, #SA5–35521) diluted 1:10,000 in 5% BSA in TTBS, (c) goat anti-rabbit HRP linked antibody (Cell Signaling, #7074) diluted 1:1,000 in 5% non-fat dried milk in TTBS. Membranes were then washed three times (10 min each) with TTBS. Fluorescent signal was detected using the Odyssey CLx Blot Imager (Li-Cor). HRP signal was detected using the SuperSignal West Pico PLUS Chemiluminescent substrate kit (ThermoScientific, #34580) and a G:Box (Syngene) containing a GeneSys imaging software.

2.11. Flexor digitorum brevis (FDB) myofiber isolation, T-tubule staining, and imaging.

Excised FDB muscles were digested with dissociation medium (4 mg/mL Collagenase A (Sigma, #11088793001) dissolved in DMEM supplemented with 1% penicillin/streptomycin for 2 h at 37 °C under 5% CO₂. After the initial dissociation, myofibers were isolated from intact muscle via trituration with glass pipettes with decreasing tip diameters. Myofibers were placed in fresh DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, and incubated at 37 °C under 5% CO₂ overnight. The following day, myofibers were washed with Hank's Balanced Salt Solution (HBSS) and stained with 5 µg/mL FM4–64fx (Life Technologies, #F34653) for 20 min at room temperature. Myofibers were washed twice with HBSS, fixed in 4% paraformaldehyde, and counterstained with 2 µM DAPI in PBS. Samples were imaged using a Zeiss LSM 880 confocal microscope with a 10x Plan Apochromat objective (0.45 WD). An argon multiline laser at 561 nm (FM4–64fx) and a 404 nm diode laser (DAPI) at 38 mW were used at 2% power. The emission filters were as follows: (a) 570–695 nm (FM4–64fx) and (b) 410–514 nm (DAPI). Image analysis was performed using ImageJ software.

2.12. MRI body composition analysis.

Live animals were weighed and immediately analyzed for whole body fat mass and lean mass using an EchoMRI-3n1–100 with ACQ-SYS version 2014 EchoMRI proprietary software prior to tissue isolation.

2.13. Statistics.

Statistical analysis was performed using the GraphPad Prism 5.0 GraphPad Software. Data is presented as the mean $\pm$ S.E.M. The unpaired t-test was used for data presented with non-littermate pairs. For data from littermate pairs a Wilcoxon paired t-test was used. A Bonferroni posttest correction was applied to all *P* values when multiple comparisons were performed. All t-tests were two-tailed. *P* 0.05 was considered statistically significant.

3. RESULTS

3.1. Alternative splicing of *Cltc* and *Clta* is developmental stage- and tissue-specific

Using unbiased RNA-sequencing (RNA-seq) methods, we previously found that *Cltc*, *Clta*, and *Trak1* genes undergo alternative splicing transitions during postnatal mouse heart development (Giudice et al., 2014). To determine whether these splicing transitions were cardiac specific or also present in other tissues, we performed reverse transcription PCR (RT-PCR) assays on heart, skeletal muscle, brain, testis, uterus, kidney, and liver at postnatal day 1 (PN1) and/or adult stages and quantified the PSI of the alternative region by densitometry.

The *Cltc* gene contains 33 exons. Exon 31 contains 21 nucleotides (Fig. 1A), is included in adult heart (PSI=47), skeletal muscle (PSI=71) and brain (PSI=29) (Fig. 1B), and is included at a lower level in the testis (PSI=18) and uterus (PSI=5) (Supplementary Fig. 1A). In contrast, inclusion of exon 31 is not detected in liver (Fig. 1B) and adult kidney (Supplementary Fig. 1A).

The *Clta* gene contains seven exons of which exons 5 and 6 are alternatively spliced giving rise to four transcripts (Fig. 2A). Exons 5 and 6 encode protein regions located close to the C-terminus, between the region that binds to CLTC and the calmodulin binding domain (Brodsky, 2012). Exon 5 contains 54 nucleotides, encodes 18 amino acids, and is mostly brain-specific (Fig. 2B). Exon 6 contains 36 nucleotides, encodes 12 amino acids, and is included at a low level at birth in brain, heart, and skeletal muscle. Exon 6 inclusion increases in adulthood (Fig. 2B). Inclusion of *Clta* exons 5 and 6 was not detected in liver (Fig. 2B) or other tissues tested (Supplementary Fig. 1B).

3.2. *Trak1* polyA selection is tissue- and developmental stage- specific

The *Trak1* gene contains 18 exons and our previous RNA-seq studies (Brinegar et al., 2017; Giudice et al., 2014) suggested that adult cardiomyocytes and skeletal muscle primarily utilize a proximal polyA site while neonatal cardiomyocytes and embryonic skeletal muscle primarily utilize two distal polyA sites (Fig. 3A-B). To validate the RNA-seq data, we designed a forward primer (Trak1-polyA-f) that binds to the constitutive exon 13 and two reverse primers (Trak1-polyA-r1 and Trak1-polyA-r2) shown in Fig. 3C. Use of the

proximal or distal polyA sites will generate PCR products of 425 or 498 base pairs (bp), respectively. We performed RT-PCR analysis on RNA isolated from neonatal and adult tissues using these three primers and observed a change in the ratio from favoring the 498 bp to the 425 bp band in neonatal and adult striated muscles (Fig. 3D) confirming the RNA-seq data (Brinegar et al., 2017; Giudice et al., 2014). In contrast, other adult tissues such as the brain, kidney, liver, testis, and uterus mostly showed the 498 bp band (Fig. 3D). Further assays were performed to discriminate the usage of distal-1 versus distal-2 polyA sites with another set of primers (Supplementary Fig. 2A). These experiments revealed that in neonatal muscles the distal polyA sites are used in approximately equal proportions consistent with the RNA-seq data (Supplementary Fig. 2B). In contrast, adult muscles utilize mostly the proximal polyA site and thus the reverse primers of this set did not bind leading to the observance of light bands (Supplementary Fig. 2B, *compare lanes 1 and 3*).

Taken together, these results led us to conclude that the proximal polyA site is used predominantly in adult striated muscles and not in the other tissues that we have tested.

3.3. Modulation of endogenous alternative splicing *in vivo* using CRISPR/Cas9 technology

We used CRISPR/Cas9 to produce genomic modifications in mouse zygotes of *Cltc*, *Clta* and *Trak1* genes to prevent expression of isoforms that predominate in several adult tissues with the goal of identifying postnatal splicing transitions with robust physiological impact. To modulate endogenous *Cltc* splicing we designed a pair of guide RNAs (sgRNAs) targeting Cas9 nuclease to intronic sequences flanking the alternative exon 31. The genomic segment containing the exon is deleted and the cut sites joined by nonhomologous end joining (NHEJ) (Fig. 4A). Therefore, the allele containing the deletion is expected to express only the isoform lacking exon 31 at all stages of development. Similarly, to modulate endogenous *Clta* splicing we designed two guide RNAs targeting the intronic sequences that flank both alternative exons 5 and 6 (Fig. 4B). After editing, both exons were deleted at the genomic level and the allele is expected to express only the isoform lacking both exons.

For *Trak1*, the strategy was to delete the proximal polyA site that is primarily used in adult striated muscle. We designed two sgRNAs targeting sequences upstream and downstream of the proximal polyA signal (Fig. 4C). After editing, the proximal polyA signal was deleted and it is expected that only the distal polyA sites are used throughout development.

Since F0 animals can be chimeric for multiple deletions around the target site due to NHEJ, F1 animals from different F0 animals were used to generate two independent founder lines for each of the *Cltc*, *Clta*, and *Trak1* genes. The junctions for each of the six lines were determined by the sequence of the PCR product using primers that flank the deleted region and animals homozygous for the deleted allele were generated and confirmed by genotyping (Fig. 4D, E, F).

3.4. Animals homozygous for the *Clta* exon-deleted allele express only the isoform lacking exons 5 and 6

We performed RT-PCR using RNA from brain, skeletal muscle, and heart tissue from *Clta* homozygous (*Clta*-HO) and wild type (*Clta*-WT) animals. While *Clta*-WT mice showed the

inclusion of exons 5 and 6 in the brain, *Clta*-HO animals do not include these exons as expected (Fig. 5A). Similarly, the heart and skeletal muscle of *Clta*-WT mice partially include exon 6, while in *Clta*-HO animals exon 6 is absent from the mRNA (Fig. 6A). Importantly, RT-PCR analysis did not detect aberrant use of cryptic splice sites resulting from the genome deletion that removed exons 5 and 6. We conclude that exons 4 and 7 are spliced at the correct splice sites.

We next aimed to evaluate the protein expressed from the wild type and deleted alleles. To first validate a specific antibody, we depleted CLTA in HeLa cells using small interfering RNAs (si-RNAs) and observed the reduction of the detected band only when cells were treated with si-Clta and not with the luciferase control siRNA (si-Luc) (Supplementary Fig. 3). This data confirmed the specificity of the antibody we utilized in the following experiments. In adult brain of *Clta*-WT mice, we clearly detected almost exclusive expression of the Clta isoform containing exons 5 and 6 (Clta[+ex5+ex6], 248 amino acids). A faint band revealed the presence of a low amount of Clta isoform containing exon 5 and lacking exon 6 (Clta[+ex5-ex6], 236 amino acids), which is the most abundant variant in wild type neonatal brains (compare the first and third lanes in Fig. 5B). Adult brains from *Clta*-HO mice showed a single band migrating slightly faster than the neonatal band (Fig. 5B, *second and third lanes*), which should be the Clta isoform lacking both exons 5 and 6 (Clta[-ex5-ex6]) (218 amino acids). This data led us to conclude that adult *Clta*-HO mice exclusively express the CLTA isoform lacking exons 5 and 6 consistent with our RT-PCR data shown in Fig. 5A. We performed similar Western blots using skeletal muscle protein extracts from adult *Clta*-WT and *Clta*-HO and neonate wild type tissues, but we could not detect the band revealing the presence of Clta isoform including only exon 6 in the adult *Clta*-WT (Fig. 5C). Based on our RT-PCR results shown in Fig. 5A, the isoform containing exon 6 and lacking exon 5 is expressed at the mRNA level leading us to conclude that this protein isoform might be less stable or less efficiently translated.

3.5. Animals homozygous for the *Trak1* genome editing use only the distal polyA sites

We used RT-PCR on RNA isolated from adult heart and skeletal muscle tissue from animals homozygous for the *Trak1* deleted allele to confirm exclusive use of a distal polyA site in both tissues (Fig. 6A). When we evaluated TRAK1 protein expression in skeletal muscles by Western blot, we observed that *Trak1*-HO mice exhibit two bands at approximately 100 kDa similar to PN4.5 tissues (Fig. 6B, *first and third lanes*). In contrast, *Trak1*-WT adult mice exhibited a major isoform of 75 kDa (Fig. 6B, *second lane*). We conclude that neonatal wild type animals Trak1 express two TRAK1 isoforms of 100–120 kDa from use of the distal polyA sites, and adult animals transition to the usage of the proximal polyA site which results in the expression of a shorter TRAK1 isoform. Consistent with the loss of the proximal polyA site and the inability to express the shorter isoform, *Trak1*-HO animals exhibit similar splicing patterns than neonatal mice (Fig. 6A and Supplementary Fig. 2B) and express the long protein isoforms in adulthood.

3.6. Animals homozygous for the *Cltc* deleted allele have increased body weight due to increased muscle mass

We performed RT-PCR using RNA from brain, skeletal muscle, and heart of the *Cltc* homozygous (*Cltc*-HO) and wild type (*Cltc*-WT) animals. These are the tissues in which the alternative exon 31 is included in adults (see Fig. 1B). While *Cltc*-WT mice showed a partial inclusion of exon 31 in the brain, heart, and skeletal muscle, the exon is absent from the mRNA of *Cltc*-HO animals as expected (Fig. 7A). Western blot experiments confirmed the expression of CLTC protein in both *Cltc*-WT and *Cltc*-HO mice (Fig. 7B).

Cltc-HO mice are viable and exhibit slight but significant higher body weights than their *Cltc*-WT littermates in both males and females at different postnatal developmental stages (Fig. 8A). This finding was unique for the *Cltc*-HO (*versus Cltc*-WT) since we did not observe the same differences in *Clta*-HO (*versus Clta*-WT) (Supplementary Fig. 4A) or *Trak1*-HO (*versus Trak1*-WT) (Supplementary Fig. 4B). We next performed whole-body magnetic resonance imaging (MRI) to evaluate lean and fat mass in *Cltc*-HO *versus Cltc*-WT animals. Analysis revealed that while fat mass was not significantly different between these animals, lean mass was higher in *Cltc*-HO mice than in *Cltc*-WT littermates (Supplementary Fig. 5). Isolation of different skeletal muscles revealed that *Cltc*-HO mice have significantly heavier *tibialis anterior*, *gastrocnemius*, and *quadriceps* muscles than *Cltc*-WT mice, both in males and females (Fig. 8B). We next asked whether the difference in muscle weights was due to the presence of larger myofibers. We isolated FBD myofibers (Fig. 9A) and measured their area by microscopy, confirming that muscle fibers from *Cltc*-HO mice were larger than those isolated from *Cltc*-WT littermates ($25,117 \pm 1,853 \mu\text{m}^2$ *versus* $19,394 \pm 1,234 \mu\text{m}^2$) (Fig. 9B). Specifically, while the fiber length was not different between genotypes, fiber width was greater in *Cltc*-HO mice than in *Cltc*-WT littermates ($51 \pm 3 \mu\text{m}$ *versus* $41 \pm 2 \mu\text{m}$) (Fig. 9C). These observations led us to conclude that failure to express the *Cltc* isoform that includes exon 31 results in increased skeletal muscle mass due to an enlargement of the myofibers.

4. DISCUSSION

Alternative splicing regulates numerous membrane-trafficking proteins which are involved in clathrin-mediated endocytosis, secretory pathways, and membrane dynamics (Blue et al., 2018). Alternative splicing and membrane trafficking are fundamental cellular processes; however, we do not know how splicing-trafficking networks modulate tissue identity acquisition and maintenance, or intracellular architecture. Therefore, investigation of the interaction between alternative splicing and membrane trafficking in striated muscle physiology was the goal of the present work.

The progress in the splicing and trafficking fields pinpoints two questions that are still open: (a) how the pre-mRNA splicing patterns are translated into the expression of different protein isoforms in specific tissues, and (b) the physiological roles of numerous splicing events in trafficking proteins are still unknown within *in vivo* contexts. We first characterized the splicing patterns of *Clta*, *Cltc*, and *Trak1* pre-mRNAs and demonstrated that all three genes are regulated by alternative splicing in a tissue- and developmental stage-specific manner in mice (Figs. 1–3). To address the second question, we used CRISPR/Cas9-

mediated genome modification to generate mouse lines that permit a molecular dissection of the physiological implications of the splicing regulation of *Clta*, *Cltc*, and *Trak1* alternative exons within an *in vivo* developmental context (Figs. 4–7).

Alternatively spliced variants are typically expressed as a dynamic ratio that could change during development rather than exclusive expression of one isoform at a specific developmental stage. When we manipulated splicing decisions using CRISPR/Cas9, we eliminated one spliced variant, forcing the exclusive expression of one protein isoform. Therefore, a caveat with this approach is that CRISPR-mediated changes do not exactly recapitulate postnatal stage specific alternative splicing pattern. To the best of our knowledge, it is not possible to fine tune the modulation of splicing to recapitulate an exact ratio of expression that completely matches a particular developmental stage. However, the splicing transitions we targeted undergo clear fetal to adult protein isoform transitions and we blocked this transition, resulting in the expression of the fetal isoform in adult tissue. Therefore, we are using this approach to identify critical protein isoform transitions that are required for tissue function in the adult.

Despite its developmental transition, nothing is known about the physiological impact of *Cltc* splicing regulation. Notably, studies investigating CLTC functions in endocytosis have been centered on the short isoform that lacks exon 31. Several studies have proposed unconventional roles for clathrin in actin organization that are distinct from those involved in coated vesicle formation (Bonazzi et al., 2012, 2011; Saffarian et al., 2009; Schafer et al., 2002; Veiga et al., 2007). CLTC directly interacts with alpha-actinin and vinculin, which are focal adhesion proteins crucial for striated muscle function and mechanotransduction (Burridge et al., 1980; Fausser et al., 1993; Merisko, 1985; Schook et al., 1979). In skeletal muscle, CLTC controls the structure of the costamere (Vassilopoulos et al., 2014), which is the attachment site between the plasma membrane and the sarcomere. *Cltc* depletion in the *tibialis anterior* of adult mice led to a loss of contractile force due to the detachment of sarcomeres from the plasma membrane (Vassilopoulos et al., 2014). We demonstrated that *Cltc* splicing regulation contributes to the development of muscle mass since we observed that when *Cltc* splicing is blocked in the *Cltc*-HO mice, which express only the short isoform, muscle mass is increased (Fig. 8) due to an enlargement of the myofibers (Fig. 9). Our observations that *Cltc*-HO mice exhibit higher muscle weights than the *Cltc*-WT littermates led us to propose that *Cltc* splicing might contribute to the formation and maintenance of the contractile apparatus through interactions with different costamere proteins (Vassilopoulos et al., 2014). Another scenario is that *Cltc* splicing might control endocytosis of key growth factors that in turn regulate specific signaling pathways involved in muscle growth such as myostatin and mTOR cascade.

Overall, we present the results from these mouse lines to demonstrate the utility of using CRISPR/Cas9-mediated genome modification to identify functional consequences of alternative splicing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. *Cltc* splicing transitions are both developmental stage- and tissue-specific.

A. Exon 31 of *Cltc* gene contains 21 nucleotides and is alternatively spliced. Primers targeting the constitutive flanking exons were designed to evaluate the PSI of exon 31. **B.** Inclusion of exon 31 was evaluated by RT-PCR assays in mouse tissues at postnatal day 1 (PN1) and adult stages. See also Supplementary Fig. 1. The PSI values were measured by densitometry. ** $P < 0.01$ and *** $P < 0.001$ by t-test (two-tailed, unpaired), $n = 2-3$ (PN1) and $n = 3-4$ (adults). bp: base pairs, nt: nucleotides.

Fig. 2. *Clta* splicing transitions occur during postnatal development in a tissue specific manner.
A. The *Clta* gene has two consecutive alternative exons, one containing 54 nucleotides (exon 5) and one containing 36 nucleotides (exon 6). Primers targeting the constitutive flanking exons were designed to evaluate the PSI of each exon by RT-PCR. **B.** Inclusion of exons 5 and 6 was evaluated by RT-PCR assays in mouse tissues at postnatal day 1 (PN1) and adult stages. See also Supplementary Fig. 1. The PSI values were measured by densitometry.
 * $P < 0.05$ by t-test (two-tailed, unpaired), $n = 2-3$ (PN1) and $n = 3-4$ (adults).

Fig. 3. Alternative polyA selection in *Trak1* pre-mRNA is developmental stage- and tissue-specific.

A. USCS tracks from RNA-seq studies in fetal-neonatal and adult samples of mouse cardiomyocytes and skeletal muscle (Brinegar et al., 2017; Giudice et al., 2014) revealed three polyadenylation (polyA) sites used within the *Trak1* gene. **B.** The region between the exons 12 and 18 in panel A is shown. Adult striated muscles primarily use the proximal polyA site following exon 14, while fetal and neonatal striated muscles use also two distal polyA sites following exons 15 (distal-1 polyA) or 18 (distal-2 polyA). **C-D.** Specific primers (**C**) were designed to confirm polyA site usage by RT-PCRs in different neonatal (PN1) and adult mouse tissues (**D**). * $P < 0.05$ by t-test (two-tailed, unpaired), $n = 2$ (PN1) and $n = 4$ (adults). bp: base pairs, chr: chromosome, E18: embryonic day 18, PN1: postnatal day 1.

Fig. 4. Genetically engineered mouse lines lack splicing transitions in *Cltc*, *Clta*, and *Trak1*. **A-C.** Schematic for CRISPR/Cas9-mediated genomic deletions to prevent adult splicing patterns of *Cltc* (**A**), *Clta* (**B**), and *Trak1* (**C**). For genotyping, primers were designed to bind upstream and downstream of the guide RNA (shown in red) binding sites. **D-E.** Genotyping of wild type (WT), heterozygous (HET), and homozygous (HO) animals for *Cltc* (**D**), *Clta* (**E**), and *Trak1* (**F**) mouse lines. bp: base pairs.

Fig. 5. Homozygous *Clta* animals express mRNAs lacking exons 5 and 6.

A. PCR products of reverse transcribed RNA from heart, skeletal muscle (*gastrocnemius*), and brain isolated from adult (4 months old) *Clta*-HO versus *Clta*-WT animals. Splicing was quantified by measuring the PSI of each exon by gel densitometry. **B-C.** Protein extracts from brain (**B-C**) and skeletal muscle (**C**) tissues from adult *Clta*-HO versus *Clta*-WT animals were analyzed by Western blot assays. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by t-test (two-tailed, paired), $n = 3$ littermate pairs (*Clta*-HO, *Clta*-WT). ex: exon, HO: homozygous, WT: wild type.

Fig. 6. *Trak1*-HO animals exhibit exclusive usage of the distal polyA sites.

A. PCR products of reverse transcribed RNA from heart and skeletal muscle (*quadriceps*) isolated from adult *Trak1*-HO versus *Trak1*-WT animals. **B.** Skeletal muscle tissues isolated from neonatal FvB wild type animals, adult *Trak1*-HO mice, and adult *Trak1*-WT littermates were evaluated by Western blot assays. *** $P < 0.001$ by t-test (two-tailed, unpaired), $n = 4-6$ for *Trak1*-HO and $n = 3-8$ for *Trak1*-WT (no littermates). HO: homozygous, WT: wild type.

Fig. 7. *Cltc*-HO animals express mRNAs lacking exon 31.

A. RT-PCR was performed using RNA extracted from heart, skeletal muscle (*quadriceps*), and brain tissues from adult *Cltc*-HO or *Cltc*-WT animals. Splicing was quantified by measuring the PSI of the alternative exon by gel densitometry. **B.** Protein lysates from skeletal muscles (*gastrocnemius*) of *Cltc*-HO and *Cltc*-WT adult animals were analyzed by Western blot assays. *** $P < 0.001$ by t-test (two-tailed, unpaired), $n = 8$ per genotype (heart and *quadriceps*), $n = 6$ per genotype (brain) (no littermates). ACTN2: sarcomeric alpha actinin, HO: homozygous, WT: wild type.

Fig. 8. *Cltc*-HO animals exhibit increased body weight and larger skeletal muscle mass compared with *Cltc*-WT controls.

A. Body weight was measured at different postnatal developmental stages for *Cltc*-HO and *Cltc*-WT littermate animals in both males and females. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ by t-test (two-tailed, paired) followed by the Bonferroni correction for multiple comparisons for body weight time points, $n = 6-38$ littermate pairs per gender (*Cltc*-HO, *Cltc*-WT). **B.** *Cltc*-HO and *Cltc*-WT animals were sacrificed at adult stages and the weight of the *tibialis anterior*, *gastrocnemius*, and *quadriceps femoris* muscles were measured and

normalized to the tibia length. * $P<0.05$ and ** $P<0.01$ by t-test (two-tailed, unpaired) followed by a Bonferroni posttest correction for multiple comparisons (the different types of muscles), $n=6$ per gender for *Cltc*-HO, $n=6$ per gender for *Cltc*-*WT* (no littermates).

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Fig. 9. Myofibers from *Cltc*-HO animals are larger than those from *Cltc*-WT littermates.

A. FDB myofibers were isolated from *Cltc*-HO and *Cltc*-WT littermates and T-tubules were stained. Myofibers were imaged by confocal microscopy. Scale bars 50 μm . **B-C.** Myofiber area (**B**) and width (**C**) were measured using ImageJ software. * $P < 0.05$ and ** $P < 0.01$ by t-test (two-tailed, unpaired), $n = 8$ myofibers from two *Cltc*-HO mice and $n = 14$ myofibers from two *Cltc*-WT animals.

Table 1.

Sequence of the guide RNAs (sgRNAs) designed to generate the mouse models by CRISPR/Cas9 technologies. PAM (protospacer adjacent motif) sequences are underlined.

sgRNA name	5' to 3' sequence	quality score
Cltc 5' sgRNA	CTCGCTCCTCCCTTTT <u>TTAGTGG</u>	66
Cltc 3' sgRNA	CTAATCGGCTAATAGGCTT <u>TAGG</u>	80
Clta exon 5 – 5' sgRNA	CTGGAGTGCCCA <u>CCGCTGGAAGG</u>	81
Clta exon 6 – 3' sgRNA	CAATGGAGATGACCGGTCTGT <u>G</u>	90
Trak1 5' sgRNA	AAGCTACTTCTGGATAACCCAGG	81
Trak1 3' sgRNA	GGGTCACCTATGTTACGTCTT <u>G</u>	94

Table 2.

Sequence of the genotyping primers.

gene name	gene symbol	primer name	5' to 3' sequence
Clathrin heavy chain	Cltc	Cltc P1	TGCTGTCCATTTTCCTAGCC
Clathrin heavy chain	Cltc	Cltc P2	ACGTTCCAGATACCCTGACA
Clathrin light chain-a	Clta	Clta P1	TGAGCCTCAGTCACCTTGC
Clathrin light chain-a	Clta	Clta P2	GCAAGCAGGCAGGAAAAA
Clathrin light chain-a	Clta	Clta P3	ACCAGCTCAGCCAACAGTCT
Trafficking kinesin-binding protein-1	Trak1	Trak1 P1	GTCGTGGCTTGAGCAGTACA
Trafficking kinesin-binding protein-1	Trak1	Trak1 P2	GGGGACAGAGGAAACAGTGA
Trafficking kinesin-binding protein-1	Trak1	Trak1 P3	AGGGCACAAGGTCAGAGAAA

Table 3.

Sequence of the primers utilized to evaluate the alternative splicing patterns of Cltc, Clta, and Trak1 pre-mRNAs. f: forward, r: reverse, polyA: polyadenylation site.

gene name	gene symbol	primer name	5' to 3' sequence
Clathrin heavy chain	Cltc	Cltc-21-f	GAAACCGCATGGAGACATAA
Clathrin heavy chain	Cltc	Cltc-21-r	AAACAATGGGCTGTGTCTCTG
Clathrin light chain-a	Clta	Clta-54-36-f	GCGATAAAGGAGCTGGAAGA
Clathrin light chain-a	Clta	Clta-54-36-r	GTTTGCTGGACTGGGGTTA
Trafficking kinesin-binding protein-1	Trak1	Trak1-polyA-f	CAACGTGGTCCTCGATAACA
Trafficking kinesin-binding protein-1	Trak1	Trak1-polyA-r1	AGTCGCAGAGGAGGACAGAG
Trafficking kinesin-binding protein-1	Trak1	Trak1-polyA-r2	TCATCCAGGTCAACATCCAA
Trafficking kinesin-binding protein-1	Trak1	Trak1-distal-f	GCTTCTCTGGCATGTCCTTC
Trafficking kinesin-binding protein-1	Trak1	Trak1-distal-r1	CTGAGGCTGGCTGGAGTAAC
Trafficking kinesin-binding protein-1	Trak1	Trak1-distal-r2	GGAGCTGGGGCAGAGTTAAG
Glyceraldehyde-3-phosphate dehydrogenase (loading control)	Gapdh	Gapdh-f	CGTCCCGTAGACAAAATGGT
Glyceraldehyde-3-phosphate dehydrogenase (loading control)	Gapdh	Gapdh-r	TTGATGGCAACAATCTCCAC