Deciphering the mechanisms that govern skeletal muscle plasticity is essential to understand its pathophysiological processes, including age-related sarcopenia. The voltage-gated calcium channel CaV1.1 has a central role in excitation-contraction coupling (ECC), raising the possibility that it may also initiate the adaptive response to changes during muscle activity. Here, we revealed the existence of a gene transcription switch of the CaV1.1 β subunit (CaVβ1) that is dependent on the innervation state of the muscle in mice. In a mouse model of sciatic denervation, we showed increased expression of an embryonic isoform of the subunit that we called CaVβ1E. CaVβ1E boosts downstream growth differentiation factor 5 (GDF5) signaling to counteract muscle loss after denervation in mice. We further reported that aged mouse muscle expressed lower quantity of CaVβ1E compared with young muscle, displaying an altered GDF5-dependent response to denervation. Conversely, CaVβ1E overexpression improved mass wasting in aging muscle in mice by increasing GDF5 expression. We also identified the human CaVβ1E analogous and show a correlation between CaVβ1E expression in human muscles and age-related muscle mass decline. These results suggest that strategies targeting CaVβ1E or GDF5 might be effective in reducing muscle mass loss in aging.

**INTRODUCTION**

A decrease in electrical activity, such as during neuromuscular disease, disuse, or aging, can cause massive muscle atrophy (1). Disuse atrophy after muscle denervation or immobilization is characterized by the activation of a compensatory response to counteract mass loss. In particular, the induction of the growth differentiation factor 5 (GDF5)/SMAD4 (an acronym from the fusion of Caenorhabditis elegans Smad genes and the Drosophila Mad, Mothers against decapentaplegic) pathway is essential not only for avoiding excessive muscle mass loss but also for promoting reinnervation after nerve crush (2, 3).

Aging muscles are characterized by progressive loss of mass and strength (4), suggesting an impairment of compensatory mechanisms. Currently, the best approaches to maintain aged muscle function and size are caloric restriction (limits autophagy and DNA damage) and exercise (restores muscle activity) (5–9). Yet, the evidence that muscle stimulation improves mass maintenance suggests that proteins sensing sarcolemmal depolarization might be suitable candidates for triggering a compensatory response. Among the five subunits of the L-type Ca2+ channel CaV1.1, the CaVα1s subunit displays voltage sensor activity (10, 11). The intracellular subunit of the complex, CaVβ1 (encoded by the Cacnb1 gene), plays a role in excitation-contraction coupling (ECC) by targeting CaVα1s to the membrane and regulating its activity (12). Currently, CaVβ1A is considered as the skeletal muscle–specific isoform (13). Furthermore, CaVβ1 can be anchored to membrane, free in the cytoplasm, or located to the nucleus of proliferating muscle cells where it acts as regulator of transcription factors (14). CaVβ1 is essential for the development of embryonic mouse muscles and for the prepatterning and development of neuromuscular junctions (NMJs), independently of ECC (14–16). Yet, no ECC-independent pathway involving CaVβ1 is known in adult muscle.

Here, we provided the link between electrical activity sensing protein and muscle adaptation that compensates for atrophy. First, we demonstrated how the innervation state of skeletal muscle regulates the CaVβ1 isoform transition epigenetically. We showed that CaVβ1D is the constitutively expressed isoform in adult mouse muscle, whereas CaVβ1E is the embryonic variant up-regulated upon impairment of electrical activity. Second, we highlighted CaVβ1E as a major player in limiting muscle mass loss in mice through its ability to trigger GDF5 signaling in denervated muscle. In addition, we revealed that muscle aging is associated with altered CaVβ1E/GDF5 axis and that overexpression of CaVβ1E drives the pathway necessary to counterbalance age-related muscle atrophy in rodents. Last, we provided evidence of a human hCaVβ1E isoform expressed in adult muscle and tightly linked to muscle decline in aging.

**RESULTS**

Embryonic CaVβ1E expression in adult mouse muscle

Resection of mouse sciatic nerve is a suitable model for measuring the response of adult skeletal muscle to alterations in activity (1, 17). To establish whether the expression of Cacnb1 could change in this model, we quantified its mRNA by reverse transcription quantitative polymerase chain reaction (RT-qPCR) using primers in exons 2 and 3. We observed a time-dependent increase in the amplification of this region in denervated tibialis anterior muscles (TAs) compared with innervated control muscles [day zero (D0)] (Fig. 1A). Western blot of CaVβ1 revealed the appearance of an extra 70-kDa band whose intensity increased with time after denervation, whereas the intensity of the band around 55 kDa was unchanged (Fig. 1, B and C).
Fig. 1. Expression of an embryonic CaVβ1 isoform by alternative first exon splicing after muscle denervation. (A) RT-qPCR for Cacnb1 from mouse tibialis anterior muscles (TAs) innervated [day zero (D0)] or after the indicated number of days of denervation. Primers were designed for exons 2 and 3 of the Cacnb1 coding region. (B and C) Western blot (B) and quantification (C) of CaVβ1 bands in innervated or denervated TAs after the indicated number of days of denervation. Actin was the loading control. (D) CaVβ1 protein and Cacnb1 gene and transcript variants (adapted from (40)). Cacnb1-A (NM_031173), Cacnb1-B (NM_145121), Cacnb1-C (NM_001159319), Cacnb1-D (NM_001159320), Cacnb1-E (NM_001282977), and Cacnb1-F (NM_001282978). Molecular weights (MW) are in brackets. (E) Cacnb1 gene showing two putative open reading frames (ATG1 and ATG2). Thin colored vertical lines indicate the start of transcription at ATG1 in innervated muscle and at ATG1 and ATG2 in denervated muscle. (F and G) RT-PCR (F) and quantification (G) of different Cacnb1 regions in innervated (Inn) or denervated (Den) TAs validating the RNAseq data. Primers were designed for different exons (Ex) of the predicted coding region of Cacnb1. Ribosomal phosphoprotein (PO) was the loading control. (H and I) RT-PCR (H) and quantification (I) of Cacnb1-E–specific region in exon 14 and the Cacnb1-D–specific region in exon 13 in Inn or Den TAs. PO was the loading control. (J) RT-PCR of the expression of Cacnb1 (Ex 5 to 8/9) in adult Inn and Den TAs and in the spinal cord (SC). PO was the loading control. (K) RT-PCR for the Cacnb1-E–specific region in exon 14 and the Cacnb1-D–specific region in exon 13 in embryonic and neonatal muscles (day 12.5 (E12.5) and 16 (E16) since fertilization; postnatal 0 (P0)), in adult TAs Inn or Den for 15 days. 3T3 cells were the negative control. PO was the loading control. (L) Western blot of CaVβ1 in TAs Inn or Den for 15 days and in embryonic muscle (E16; diluted 1:10) using AbCaVβ1 (central peptide). Actin was the loading control. (A, C, and I) Means ± SEM (n = 6 mice per group; (C) and (I), n = 3 independent experiments); *P < 0.05, **P < 0.001 (ordinary one-way ANOVA, Dunnett’s test). (G) Means ± SEM (n = 3 independent experiments); ***P < 0.001 (ordinary two-way, Sidak’s test).
The Cacnb1 gene (GSMG0007319) has 14 exons that can be spliced to give six transcript variants (Fig. 1D). To identify potential Cacnb1 splicing events occurring in denervated muscle, we performed a genome-wide transcriptomic analysis at the exon level on RNA extracted from innervated and denervated mouse TA s. We found 1022 differentially regulated alternative splicing events (from 706 distinct genes) (data file S1). Among these, Cacnb1 displayed a first exon splicing event, showing that the transcript started in a putative noncoding sequence at the 5′ end of exon 3 in innervated muscles. In denervated muscles, another transcript of Cacnb1 starting at exon 1 was found to be up-regulated (Fig. 1E and table S1), implying the transcription of two different splicing isoforms. RT-PCR confirmed that in innervated TAs, the Cacnb1 open reading frame is at the 5′ end of exon 3 (ATG1). By contrast, in denervated muscles, two Cacnb1 transcripts were expressed: one starting at exon 1 (ATG2) and another at ATG1–exon 3 (Fig. 1, F and G).

By searching Cacnb1 variants in the National Center for Biotechnology Information database, we found that Cacnb1-D is the only one starting immediately upstream the exon 3. On the basis of these results, our data suggest that the specific CaVβ1 isoform expressed in the adult mouse skeletal muscle is CaVβ1D and not CaVβ1A, as expected (14). Moreover, the size of the extra CaVβ1 band appearing in denervated muscle suggested that it could be CaVβ1B or CaVβ1E (66 and 70 kDa, respectively). However, CaVβ1 antibody targeting a central peptide of the protein [AbCaVβ1: sc-25689 (H-50)] raised against amino acids 211 to 260 L-type CaVβ1 human origin, NM_199247-NP_954855 recognized only CaVβ1E in mouse. To validate which isoform was up-regulated after denervation, we designed specific primers matching the two sequences at the level of the 3′ end of exon 13 for Cacnb1-D and exon 14 for Cacnb1-E. RT-PCR data confirmed that only Cacnb1-E increased in denervated muscle (Fig. 1, H and I), confirming protein expression data.

Next, we verified whether CaVβ1E was the only isoform induced in denervated muscle. Amplification of the region between exons 5 and 6/8 of Cacnb1 let the discrimination of the several variants depending on the amplicon size. RT-PCR showed that innervated and denervated muscle expressed only Cacnb1-D transcripts in mouse. To evaluate whether CaVβ1E was expressed in the adult mouse skeletal muscle, we performed an RT-PCR using primers matching the two exons at the level of the 3′ end of exon 13 for Cacnb1-D and exon 14 for Cacnb1-E. RT-PCR data confirmed that only Cacnb1-E increased in denervated muscle (Fig. 1, H and I), confirming protein expression data.

Several embryonic proteins are induced after muscle denervation (3, 21–25). We wondered whether CaVβ1E could be an embryonic isoform. RT-PCR (primers matching exon 14) (Fig. 1K) and RT-qPCR (primers in exons 2 and 3 for Cacnb1-D and exon 13 for Cacnb1-E) revealed that Cacnb1-D is the specific variant in embryonic and neonatal muscles, whereas Cacnb1-D is not expressed in the embryo (fig. S1, A and B). The Cacnb1-E transcript in embryonic muscle started at exon 1 (fig. S1, C and D) as Cacnb1-E expressed in denervated adult muscle. Western blotting showed the same bands in denervated adult and embryonic muscle protein extracts using AbCaVβ1 (Fig. 1L) or an antibody specific to CaVβ1E (fig. S1E). Last, we analyzed whether CaVβ1D and CaVβ1E could have different localization in muscle fiber. Immunofluorescence analysis of innervated and denervated muscle sections and isolated fibers with AbCaVβ1 showed that CaVβ1 staining and triadic localization most likely reflected expression of CaVβ1D (fig. S2, A to C). In contrast, CaVβ1E-specific staining was distributed at the Z-lines (fig. S2, D to F). Furthermore, CaVβ1E-specific staining localized mostly at the nuclei, consistently with the presence of a nuclear localization signal predicted by nLS mapper, and its intensity increased in denervated muscle compared with CaVβ1 staining (fig. S2, G to I).

In conclusion, our results show that an alternative first exon splicing is the source of the differential expression of mouse adult and embryonic Cacnb1 variants. CaVβ1D, not CaVβ1A, is expressed in innervated adult skeletal muscle, whereas embryonic muscle expresses CaVβ1E. CaVβ1E is low in innervated adult mouse muscle, but denervation specifically increases its expression. Moreover, CaVβ1D and CaVβ1E display different intracellular locations in adult muscle fibers.

CaVβ1E and GDF5 signaling activation after denervation

To evaluate whether CaVβ1E has a role in disuse atrophy, we injected into mouse TA an adeno-associated virus (AAV) vector carrying a short hairpin RNA targeting a sequence in Cacnb1 exon 2 (AAV-ShCaVβ1E) (26) and thereby abolishing specifically Cacnb1-E. Two months after injection, CaVβ1E expression induced after denervation was decreased by ~90% (Fig. 2, A to C, and fig. S3, A and B), without decreasing CaVβ1D protein expression (Fig. 2, B and C). Blocking the induction of CaVβ1E exacerbated muscle atrophy after denervation (Fig. 2, D to F), with decreased muscle fiber size (Fig. 2F) and increased fibrosis (fig. S3, C to F). This suggested a role for CaVβ1E in preserving disused muscle mass. The GDF5 pathway has been shown to be essential for limiting muscle loss under atrophic conditions (2). We thus evaluated Gdf5 expression in the absence of CaVβ1E and found a significant (P < 0.0001) reduction in the denervation-induced Gdf5 increase (Fig. 2G). In contrast, Gdf8 or Bmp7 transcription, which could be both implicated in increased atrophy (27, 28), was not modified upon CaVβ1E ablation (Fig. 2, H and I). Furthermore, all components of the GDF5 pathway, SMAD1/5 phosphorylation, SMAD4 nuclear translocation, and Id-2 transcription (29, 30), were inhibited with CaVβ1E down-regulation, suggesting the positive control of GDF5 signaling by CaVβ1E (Fig. 2, J to O). However, Id-1 expression was not modified after denervation in scrambled (Scra)- or AAV-ShCaVβ1E–treated muscle (Fig. 2N), suggesting that its activation might be very weak or transient (2).

CaVβ1 has been shown to inhibit myogenin signaling during myoblast proliferation (14); yet, there are no data about this regulation in adult muscle. In innervated or denervated TAs, we found no change in transcription of Myogenin after CaVβ1E knockdown (fig. S3G) and no alterations in Fbxo32, MuRF1 expression (fig. S3, H and I), indicating that the myogenin pathway is unlikely to participate in the increased atrophy after CaVβ1E down-regulation. However, Chrna1 transcription after denervation was significantly (P < 0.0001) reduced in the absence of CaVβ1E, suggesting its involvement in modulating genes associated with end-plate formation, independently of myogenin. A previous study showed that in proliferating muscle cells, canonical and noncanonical DNA E-box sequences (CANNNTG and CANNNTG) of several promoter regions could be targeted by CaVβ1 (14). Analysis of the 100 most differentially regulated genes in innervated and denervated TAs revealed the presence of one or more E-box sequences in the promoter of all these genes (table S2). However, among them, only Gdf5 and myogenin had a demonstrable role in muscle mass homeostasis (2, 31).

To further analyze the role of CaVβ1E on Gdf5 expression, we used the myogenic cell line C2C12 as tool in vitro. C2C12 cells expressed increasing Cacnb1-E during differentiation (fig. S4, A
Fig. 2. CaVβ1E down-regulation and increased muscle atrophy by reduced GDF5 signaling after denervation. (A) RT-qPCR for Cacnb1Δ-E (Ex2 and Ex3) in adult TAs Inn or Den for 15 days treated with AAV-Sh scrambled (Scra) or AAV-ShCaVβ1E (ShCaVβ1E). (B and C) Western blot (B) using AbCaVβ1 (central peptide) and quantification (C) of CaVβ1 expression in adult TAs Inn or Den treated with Scra or ShCaVβ1E. (D) Means ± SEM (n = 6 mice per group); *P < 0.05, **P < 0.01, and ***P < 0.001 [ordinary one-way ANOVA, Sidak’s test]. (E) Means ± SEM (n = 6 mice per group); *P < 0.05 [independent samples t test (two tailed)]. (F and P) Means ± SEM ((F) n = 3 cryosections quantified per condition; (O) n = 3 independent experiments); *P < 0.05, **P < 0.01, and ***P < 0.001 [ordinary two-way ANOVA, (F) Tukey’s test, (O) Sidak’s test].
and B) and only CaVβ1E protein (band at 70 kDa) as embryonic muscle. (fig. S4C). Moreover, the transcription of Gdf5, as well as Myogenin, also increased in differentiating C2C12 cells (fig. S4, D and E). Inhibiting Caemb1-E expression in differentiating C2C12 cells by transfecting a plasmid ShCaVβ1E (pCDNA3-ShCaVβ1E) (fig. S4F) prevented the expression of Gdf5 without affecting Myogenin transcription (fig. S4, G and H), mimicking its effect in vivo. We then measured in C2C12 the consequence of CaVβ1E down-regulation on the promoter activity of Gdf5. Thus, the sequence from ~312 to the Gdf5 transcription start site, which contains one CANNTG and two CANNNTG E-boxes, was cloned upstream of firefly luciferase in herpes simplex virus thymidine kinase (HSVTK)–Luc3–modified plasmid and transfected into C2C12 cells. Firefly/Renilla signal increased during differentiation, reflecting Gdf5 promoter activation, and this effect was abolished by the ShCaVβ1E (fig. 2P). These data indicated that CaVβ1E targets the Gdf5 promoter, consistently with the effect observed in vivo and in vitro.

CaVβ1E seems to precede CaVβ1D appearance in the embryo and in C2C12. We thus considered whether Caemb1-E and Caemb1-D mRNA expression could affect each other and have a role in muscle mass homeostasis via Gdf5. Although AAV-ShCaVβ1E injection prevented Caemb1-D rise after denervation, it also induced a slight decrease in Caemb1-D mRNA expression (fig. S5, A and B), yet with no impact on Caemb1D protein expression. However, only the rescue of CaVβ1E, and not CaVβ1D, restored Gdf5 transcription and atrophy after denervation in AAV-ShCaVβ1E–treated muscles (fig. S5, C and D).

To confirm that the increased atrophy upon CaVβ1E down-regulation was dependent on inadequate GDF5 activation, we overexpressed this factor in TAs together with ShCaVβ1E, via AAV gene transfer (fig. S5, E and F). The up-regulation of Id-1 and Id-2 proved the activation of a GDF5-dependent pathway (fig. S5, G and H) in double-treated muscles. As expected, GDF5 overexpression rescued the atrophy-induced CaVβ1E ablation and improved muscle histology by decreasing fibrosis (fig. S5, I to R).

**Aging muscles: A key role for CaVβ1E**

During aging, skeletal muscle shows denervation-like signs and progressive muscle wasting (4, 32). This suggests that impairment in compensatory response might occur. However, little is known about CaVβ1 expression and function (26), and nothing is known about Gdf5 amount in aging muscle.

Because our data indicate the involvement of CaVβ1E via Gdf5 in muscle maintenance, we investigated its role in age-related muscle wasting. In C57Bl/6 mice, we observed significant (P < 0.0001) TA muscle mass loss at 78 weeks relative to adult mice at 12 weeks (Fig. 3A). In addition, Caemb1-E basal expression was significantly lower in TAs of 78-week-old compared with 12-week-old mice (P < 0.0001) (Fig. 3B), whereas Caemb1-D transcription did not change (Fig. 3C). To evaluate whether the cross-talk between Caemb1-E and Gdf5 would also be affected, we quantified these transcripts after denervation in TAs from 12-, 52-, and 78-week-old mice. Although CaVβ1E expression increased in denervated young muscle, the up-regulation of transcript and protein in response to denervation was impaired since 52 weeks of age (Fig. 3, D, G, and H). Consequently, the Gdf5 increase was reduced (Fig. 3F), affecting SMAD1/5 phosphorylation (Fig. 3, J and K). Id-1 remained unchanged (Fig. 3L), whereas Id-2 transcription followed the altered Gdf5 induction (Fig. 3M). CaVβ1D expression did not change (Fig. 3, E, G, and I).

We explored some possible causes responsible for decreasing CaVβ1E during aging. Measuring the expression of NMJ components Chrna1, Chrne, Chrg, and Musk in 78-week-old mice muscles, we could not detect changes indicating end-plate alterations at this age (Fig. 4, A to D). Similarly, we checked whether age-related modifications in fiber-type composition occurred, maybe explaining CaVβ1E down-regulation. Expression of myosin heavy chain (MyHC) iso-types IIA and IX decreased significantly during aging (P = 0.006 and 0.013, respectively), as reported (9, 33, 34), whereas MyHC-I and MyHC-IIB did not change between 12 and 78 weeks of age (Fig. 4, E to H). To elucidate whether CaVβ1E expression could be associated to fiber-type modifications, we analyzed its amount in MyHC-I– and MyHC-II–positive fibers by immunofluorescence. In young muscle, CaVβ1E was expressed at higher intensity in MyHC-IIA– and MyHC-IIIX–positive fibers, although in old muscle, it was decreased in all fiber types (Fig. 4, I to K).

If CaVβ1E increased upon lack of nerve activity, for example, denervation, modifications in muscle activity could potentially affect basal Caemb1-E transcription. Hence, we measured Caemb1-E mRNA after acute exercise of young and old mice (35, 36). Exercise training restored Caemb1-E transcription in old muscles but not in young (Fig. 4L), with Caemb1-D expression unaffected (Fig. 4M), suggesting that increased muscle activity is able to rapidly normalize Caemb1-E transcript only when it is decreased. However, MyHC-IIA and MyHC-IIIX expressions were not restored, implying that contractile activity could regulate Caemb1-E expression independently of type IIA-IX fiber abundance (Fig. 4, N and O).

To evaluate whether CaVβ1E or GDF5 overexpression might mitigate age-related muscle mass loss, we injected AAV vectors carrying CaVβ1E or GDF5 into the TA of 78- to 80-week-old mice. A strong overexpression of CaVβ1E and GDF5 was observed 3 months after injection (Fig. 5, A, B, and D; and fig. S6A), without modifying Caemb1-D expression (Fig. 5C and fig. S6C), yet increasing expression of each other reciprocally (Fig. 5D and fig. S6B). The up-regulation of Caemb1-E in old muscle activated GDF5 signaling, as measured by SMAD1/5 phosphorylation and SMAD4 nuclear translocation. (Fig. 5, E to G). The increase in Gdf5 either by CaVβ1E or by its own overexpression induced Id-2 transcription, with no changes in Id-1, confirming a weaker or transient response of this factor to GDF5 (Fig. 5, H and I; and fig. S6, D and E). In addition, the rise of GDF5 signaling after Caemb1-E or Gdf5 overexpression was associated with preservation of aged muscle mass (Fig. 5J) and a gain of specific force (Fig. 5K) compared with control old muscles.

Subsequently, we investigated whether CaVβ1E overexpression could affect muscle mass of young mice (12 weeks). Ectopic expression of CaVβ1E (fig. S7, A, D, and E) had no effect on Caemb1-D, yet it induced a slight increase in Gdf5 expression (fig. S7, B to E). However, GDF5 signaling, measured by Id-1 and Id-2 transcription, was not or poorly activated (fig. S7, F and G). Consequently, muscle mass and the response to denervation were not different between Scra- and CaVβ1E-treated TAs of young mice (fig. S7, H to L). We also overexpressed GDF5 in young TAs (fig. S8, A, D, and E), which induced Caemb1-D expression in innervated TAs compared with scrambled (fig. S8, B, D, and E) without affecting Caemb1-D expression (fig. S8C). Nevertheless, GDF5 overexpression and its activated signaling, measured as Id-1 and Id-2 transcription (fig. S8, F and G), increased mostly innervated muscle mass (fig. S8, H to L). Overall, these data show that the loss of CaVβ1E/GDF5 cross-talk observed during aging may be critical.
**Fig. 3. Muscle mass loss during aging and alteration of the CaVβ1E/GDF5 axis.** (A) Muscle/body weight ratio of innervated adult TAs from 12-, 52-, and 78-week-old mice. (B and C) RT-qPCR for (B) Cacnb1-E (Ex2 and Ex3) and (C) Cacnb1-D (Ex13) in innervated TAs from 12-, 52-, and 78-week-old mice. (D to F) RT-qPCR for (D) Cacnb1 (Ex2 and Ex3), (E) Cacnb1-D (Ex13), and (F) Gdf5 in TAs Inn or Den for 15 days from 12-, 52-, and 78-week-old mice. (G to I) Western blot (G) using CaVβ1 (central peptide) and quantification (H and I) of CaVβ1E and CaVβ1D in Inn or Den TAs from 12-, 52-, and 78-week-old mice. Actin was the loading control. (J and K) Western blot (J) and quantification (K) of phosphorylated SMAD1/5 and SMAD5 in Inn or Den TAs from 12-, 52-, and 78-week-old mice. (L and M) RT-qPCR for (L) Id1 and (M) Id2 in Inn or Den TAs from 12-, 52-, and 78-week-old mice. (A to C) Means ± SEM (12, n = 8 mice per group; 52, n = 9 mice per group; and 78, n = 10 mice per group); *P < 0.05 (ordinary one-way ANOVA, Dunnett’s test). (D to F, L, and M) Means ± SEM (n = 6/7); *P < 0.05, **P < 0.01, and ***P < 0.001 (ordinary one-way ANOVA, Sidak’s test). (H, I, and K) Means ± SEM (n = 3; Western blots used for quantification are showed in raw data); *P < 0.05, **P < 0.01, and ***P < 0.001 (ordinary one-way ANOVA, Sidak’s test).
for muscle wasting, and its rescue counteracts the process of age-related muscle decline.

**Human muscle: A new CaVβ1E isoform implicated in skeletal muscle aging**

Given the apparent importance of CaVβ1E in mouse skeletal muscle, we wondered whether an analogous mechanism might be conserved in humans and, thus, if another unidentified CACNB1 isoform was expressed in adult human skeletal muscle. Three human CACNB1 (hCACNB1) variants have been identified corresponding to the mouse isoforms A (specific of skeletal muscle), B, and C (Fig. 6A) (18–20, 37). Human mRNA extracted from one quadriceps and two fascia lata (FL) muscle biopsies of healthy adult participants together with human mRNA extracted from the cervical SC (table S2), as positive control for hCACNB1-B (18–20), were probed for exons 13 and 5 to 9. Amplification of the sequence in exon 13 showed that all
muscles expressed hCACNB1-A. As in mouse muscle, amplification of the region between exons 5 and 9 demonstrated that only a 380 bp corresponding to the putative hCACNB1-A appeared and that the exon 7B–containing isoform, hCACNB1-B (245 bp), was solely expressed in SC. Furthermore, no transcription of hCACNB1-C, which also has the short 7B exon, was found in muscle (Fig. 6B). We further checked whether human muscle could express an hCACNB1 transcript analogous to mouse Cacnb1-E. Amplification of the region in exon 14 revealed that the human muscles expressed the previously unidentified variant that we have called hCACNB1-E (Fig. 6C). This isomorph corresponded to the predicted XM_006722072.2 variant, having a codon start (ATG2) upstream the exon 3 (Fig. 6, A and C). Western blot and immunofluorescence experiments confirmed its expression in two different human FLs (Fig. 6, D and E), with localization similar to that of mouse CaVβ1E (fig. S2, E and F).

Because we found that the altered CaVβ1E/GDF5 axis was associated to muscle wasting during aging in mice, we compared characteristics indicating muscle mass (lean mass percentage) and function (power)
Fig. 6. Expression of CaVβ1E in human muscle: Conserved compensatory mechanism? (A) Representation of human CaVβ1 protein and hCACNB1 gene and transcript variants [adapted from (40)]. hCACNB1-A (NM_199247), hCACNB1-B (NM_000723), hCACNB1-C (NM_199248), predicted hCACNB1-E (XM_006722072.2). MW are in brackets. (B) RT-PCR for different isoforms of hCACNB1 in human quadriceps (Q) and fascia lata (FL1 and FL2) muscle biopsies from three healthy adults. RNA from one human SC biopsy was the positive control for hCACNB1-B. Primers were designed for different exons (Ex) of the coding region of hCACNB1. Human ribosomal phosphoprotein (hPO) is the loading control. (C) RT-PCR for hCACNB1-E in human Q, FL1, and FL2 muscle biopsies from three healthy adults. RNA from SC biopsy was the positive control for the expression of exon 14 of hCACNB1-B. Primers were designed for different exons of the coding region of hCACNB1. hPO was the loading control. (D) Western blot using CaVβ1 (central peptide) in human FL1 and FL2 muscle biopsies from the same adult healthy participants as in (B) and (C). Actin was the loading control. (E) Hematoxylin/eosin (H/E) and immunofluorescence (IF) images of human FL1 and FL2 muscle used in (B), (C), and (D) stained with hCaVβ1E (yellow), Cav3 (magenta), and DAPI (cyan). Scale bars, 50 μm (H/E) and 10 μm (IF). (F and G) Distribution of (F) lean mass percentage and (G) power in human quadriceps biopsies from healthy young and aged volunteers (Table 1). (H and I) Distribution of (H) hCACNB1-E or (I) hCACNB1-A expression in human quadriceps biopsies from healthy young and aged volunteers (Table 1). (J) Linear regression between hCACNB1-E expression and lean mass percentage in human quadriceps biopsies from healthy young and aged volunteers (Table 1). (K) Distribution of hGDF5 (pink triangles, left y axis) and hCACNB1-E (blue circles, right y axis) expression in human quadriceps biopsies from healthy aged volunteers (Table 1) having increasing lean mass percentage. Dotted black line indicates the average of lean mass percentage of the young group. (F to I) Means ± SEM (young, n = 8; aged, n = 17); **P < 0.01, ***P < 0.001 [independent samples t test (two-tailed)].
in a cohort of healthy young (20 to 42 years) and aged (70 to 81 years) volunteers included in a previous study (Table 1) (39). The aged group displayed significantly lower lean mass and power than the young group ($P = 0.003$ and $P < 0.0001$, respectively) (Fig. 6, F and G). We then measured the hCACNB1-E transcript and found a significant reduction ($P = 0.0045$) of its expression in the aged group (Fig. 6H), whereas hCACNB1-A transcription did not differ between groups (Fig. 6I). A low hCACNB1-E expression was also associated with a low lean mass percentage (Fig. 6J). We detected a weak amount of hGDF5 in muscle biopsies; however, in aged muscle samples, we could associate low expression of both hCACNB1-E and hGDF5 with low lean mass percentage. In addition, participants with higher lean mass percentage displayed higher expression of both hCACNB1-E and hGDF5 (Fig. 6K). Overall these results suggest that the CaVβ1E/GDF5 axis in compensatory response and aging (fig. S9) might be a conserved mechanism between mice and humans.

DISCUSSION

In adult skeletal muscle, the mechanism connecting electrical activity sensing and changes in gene expression is unclear. Here, we showed that denervation boosts the expression of embryonic CaVβ1E isoform, which mediates transcriptional reprogramming in adult muscle. Many years ago, CaVβ isoforms were identified in humans (19, 20, 40), in rats (18), and, less clearly, in mice (13). We anticipated that the 55-kDa band corresponded to CaVβ1A, a previously identified muscle-specific isoform with this molecular weight (18, 19). However, we speculated that the 70-kDa band could be a previously unappreciated CaVβ1 isoform. Our results revealed that CaVβ1D, not CaVβ1A, is the main constitutive isoform specific to normal adult mouse muscle and that CaVβ1D and CaVβ1E have distinct roles. In silico analysis using the cNLS mapper confirmed the presence of a nuclear localization signal at the N terminus of CaVβ1E but not in CaVβ1D. CaVβA is not expressed in adult mouse muscle and C2C12; however, its N terminus is putatively identical with those of CaVβ1E, suggesting that the mechanism by which it translocates to the nucleus is the same described in past publications (14, 41). This finding also implicates CaVβ1D in regulating calcium channel assembly in the cytoplasm, whereas CaVβE could have a role in both modulating nuclear transcription factors and cooperating with cytoskeletal or T tubules at the Z-lines, as demonstrated for others proteins (42, 43).

Table 1. Characteristics of human muscles. Lean mass (%) was assessed by dual-energy x-ray absorptiometry. Power is expressed by watt on kilograms (W/kg). Y, young participants, from 20 to 42 years old; A, aged participants, from 70 to 81 years old; M, male; F, female; ND, not determined.

| Participant | Gender | Age (years) | Height (m) | Weight (kg) | Lean mass (%) | Power (W/kg) |
|-------------|--------|-------------|------------|-------------|---------------|--------------|
| Y1          | M      | 20.9        | 1.82       | 84.4        | 82.3          | 54.7         |
| Y2          | F      | 24.5        | 1.63       | 56.2        | 78.4          | 51.1         |
| Y3          | F      | 26.1        | 1.53       | 53.0        | 70.4          | 38.4         |
| Y4          | M      | 26.4        | 1.76       | 56.7        | 89.2          | 48.6         |
| Y5          | M      | 27.1        | 1.83       | 73.8        | 75.9          | 42.4         |
| Y6          | M      | 37.0        | ND         | ND          | ND            | ND           |
| Y7          | M      | 38.0        | ND         | ND          | ND            | ND           |
| Y8          | M      | 42.0        | ND         | ND          | ND            | ND           |
| A1          | F      | 70.8        | 1.68       | 70.2        | 58.9          | 22.3         |
| A2          | M      | 70.9        | 1.58       | 65.1        | 70.4          | 34.5         |
| A3          | M      | 71.4        | 1.76       | 90.7        | 64.5          | 29.7         |
| A4          | M      | 71.4        | 1.68       | 84.4        | 66.3          | 27           |
| A5          | F      | 71.5        | 1.61       | 53.3        | 67.6          | 21.2         |
| A6          | M      | 71.7        | 1.67       | 69.4        | 79.2          | 35.3         |
| A7          | M      | 72.5        | 1.67       | 74.3        | 70.4          | 34.7         |
| A8          | M      | 72.9        | 1.74       | 91.6        | 64.2          | 28.1         |
| A9          | M      | 73.8        | 1.67       | 76.0        | 71.7          | 36           |
| A10         | F      | 74.2        | 1.54       | 58.3        | 64.0          | 26.3         |
| A11         | M      | 75.0        | 1.69       | 80.0        | 68.3          | 29.4         |
| A12         | M      | 76.4        | 1.58       | 67.7        | 74.7          | 25.6         |
| A13         | M      | 76.7        | 1.76       | 85.5        | 67.4          | 25.8         |
| A14         | F      | 77.8        | 1.58       | 55.5        | 70.8          | 22.2         |
| A15         | M      | 78.0        | 1.75       | 80.3        | 68.5          | 22.6         |
| A16         | M      | 79.6        | 1.67       | 67.9        | 80.9          | 26.3         |
| A17         | F      | 80.6        | 1.59       | 56.3        | 71.2          | 29           |
We also discovered that Cavβ1E is the main Cavβ1 isoform in proliferating and differentiating C2C12 cells, whereas Cavβ1D and Cavβ1A isoforms are undetectable. Previous studies reporting expression, transcriptional activity, and nuclear localization of Cavβ1A in muscle fibers (41), muscle precursors, and C2C12 cells (14) were likely evaluating the as-yet-undiscovered Cavβ1E. An issue of our study could be deciphering mechanisms underlying the transition between Cavβ1E and Cavβ1D during myogenesis and identifying molecular factors regulating the implicated splicing events.

The major finding of this work is the central role of Cavβ1E in maintaining muscle mass. GDF5 is one of the main regulators of muscle mass homeostasis in response to disuse atrophy (2); however, few studies report progress on modulators of this factor (3, 44, 45). Our data validate previously described roles of GDF5 (2, 46) and add a positive upstream player controlling its signaling. Cavβ1E expression is needed for the activation of the Gdf5 promoter, probably by acting on specific E-box sequences, as described for other target genes (14).

In addition, we demonstrated that a compromised Cavβ1E/GDF5 axis during aging is associated to muscle wasting. In our study, Cavβ1E overexpression led to increased GDF5 expression and activation of its pathway, improving age-related muscle decline. By contrast, Cavβ1E overexpression did not affect muscle mass in young mice. This suggests that endogenous Cavβ1E induction after nerve withdrawal is sufficient to trigger the maximal compensatory response. In addition, when muscles express a physiological amount of GDF5, feedback mechanisms could be activated to prevent hypertrophy. GDF5 exerted hypertrophic effects mostly on innervated muscle, suggesting that nerve is important for its trophic signal. In this hypothesis, GDF5 could stimulate pre- and/or postsynaptic NMJ partners, improving neurotransmission. Thus, increased GDF5 signaling, induced by overexpression of Cavβ1E or GDF5 itself, might counteract age-related muscle decline also by preventing the associated denervation occurring later. Cavβ1 has been shown as important for correct synaptic patterning in the embryo (15). Although a role for GDF5 in this process has not been established yet, its relevance to mediate reinnervation in adult muscle is proven (3). We also demonstrated that an hCACNB1-E variant is expressed in human muscle, indicating that mechanisms governing skeletal muscle homeostasis might be conserved across mammalian species. Although the hCACNB1-E transcript is slightly different compared with mouse Cacnb1-E, we observed a similar localization and hypothesize the same function. hCACNB1-E expression was higher in young compared with aged human muscle with reduced lean mass and power, strongly suggesting a defect in compensatory mechanism contributing to age-related muscle mass loss. Despite weak transcription of hGDF5 in undamaged young human muscle, we demonstrated an association between its expression and muscle mass.

Other studies identified hypertrophic and potentially druggable factors reduced in sarcopenic muscle such as insulin-like growth factor 1 (7), growth hormone (47), leptin (48), and, recently, apelin (9). These molecules are dependent on muscle activity and aging. Here, we identified Cavβ1E as “sensor” of electrical activity defect, which could be a central player, upstream of all these factors.

Age-related muscle decline is a life-threatening condition (49) affecting a large population, which results in progressive loss of autonomy, increased mortality associated to frailty, and risk of falls in elderly individuals (50). GDF5 or GDF5-like molecules can be considered as potential therapeutic compounds not only to prevent muscle mass wasting during senescence but also, in a wider application, to ameliorate consequences of neuromuscular defects. Last, deciphering the fine regulation of Cavβ1E expression and GDF5 signaling would open a promising therapeutic field that will contribute to increasing the quality of life.

The study has some limitations. The events altering the correct basal expression of Cavβ1E in aging muscle remain unresolved. Previous studies have reported a link between elevated Cavβ1A and age-related muscle weakness (26, 51, 52), but Cacnb1 transcripts were not fully characterized nor antibodies used were well defined. Cavβ1E expression is higher in young type II A-IIIX fibers compared with IIb. The fiber type II decrease occurring during aging may thus have a role in Cavβ1E reduction. Still, other mechanisms, dependent on muscle activity per se, modifying DNA methylation or mitochondrial metabolism, could participate in altering the Cavβ1E/GDF5 axis.

MATERIALS AND METHODS
Study design
The primary objective of this study was to decipher the link between alteration of electrical activity and skeletal muscle mass maintenance. We focused on the role of Cavβ1, a subunit of the voltage sensor Cavβ1.1. RNA sequencing (RNAseq) data of innervated and denervated TAs led to the identification of an embryonic variant, Cavβ1E, up-regulated in mouse muscle by denervation. Validation of RNAseq data and characterization of Cavβ1 isoforms in mouse and human muscles have been performed by RT-PCR, qPCR, Western blot, and immunostaining experiments. To investigate the role of Cavβ1E in muscle mass homeostasis in mouse, we knocked down its local expression in vivo by AAV-shRNA. We quantified atrophy by measuring muscle weight and fiber size distribution. To determine fiber size, images were processed with a machine learning algorithm (Weka) under Fiji for accurate segmentation. Muscle histology was performed by hematoxylin and eosin and Sirius red staining. To evaluate the effect of Cavβ1E down-regulation on the transcriptional activity of the Gdf5 promoter, we performed a luciferase reporter-based assay in C2C12. To establish the effect of Cavβ1E or GDF5 in vivo on muscle mass, we overexpressed them in mouse by local AAV-gene transfer. To characterize the expression of Cavβ1 isoforms in humans, we used muscle biopsies of a cohort of healthy young and aged volunteers (39). Body composition and muscle function of volunteers included in the study (39) had been measured previously and provided for correlation analysis with hCACNB1-E. We estimated the sample size on the basis of known variability of assays. All mouse experiments were performed twice. Outliers not following normal distribution of samples were removed on the basis of Grubb’s test. All experiments using animals and cells were done in a nonblinded manner, yet investigators were blinded to allocation in using human samples for RT-qPCR experiments.

Statistical analysis
For comparison between two groups, two-tailed paired and unpaired Student’s t tests were performed to calculate P values and to determine statistically significant differences (significance was for P < 0.05, as detailed in the figure legends). For comparison among more than two groups, ordinary one- or two-way analysis of variance (ANOVA) tests followed by the appropriate multiple comparison tests (as detailed in the figure legends) were performed. All
experiments have been done twice with the same results. All statistical analyses were performed with the GraphPad Prism 7 software.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. Expression of embryonic Cavβ1 isoform.
Fig. S2. Cavβ1 isoform localization on adult skeletal muscle.
Fig. S3. Histological characterization and myogenin signaling in Cavβ1E knockout muscles.
Fig. S4. Expression of Catch21E and GDF5 in C2C12.
Fig. S5. Effects of Cavβ1E, Cavβ1ID, and GDF5 overexpression on AAV-ShCavβ1E–treated muscles.
Fig. S6. GDF5 overexpression in aged mouse muscle.
Fig. S7. Cavβ1E overexpression in young mouse muscle.
Fig. S8. GDF5 overexpression in young muscle mouse.
Fig. S9. Schematic representation of CaV1E overexpression in young mice muscle.
Table S1. Expression data.
Table S2. List of human samples.
Table S3. List of human samples.
Table S4. Primer list.
Data file S1. List of 1022 differentially regulated alternative splicing events.
Data file S2. Uncut Western blotting raw data.
Data file S3. Individual participant data.

View/request a protocol for this paper from Bio-protocol.

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