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**An embryonic CaV $\beta$ 1 isoform promotes muscle mass maintenance via GDF5 signaling in adult mouse.**

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**One sentence summary:** CaV $\beta$ 1E boosts down-stream Growth Differentiation Factor 5 signaling to counteract muscle mass loss in denervated or aged mouse muscles.

## **Abstract**

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 transcription switch of the CaV1.1 beta subunit (CaV $\beta$ 1) gene 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 CaV $\beta$ 1E. CaV $\beta$ 1E boosts down-stream 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 CaV $\beta$ 1E compared to young muscle, displaying an altered GDF5-dependent response to denervation. Conversely, CaV $\beta$ 1E over-expression improved mass wasting in aging muscle in mice by increasing GDF5 expression. Crucially, we also identified the human CaV $\beta$ 1E analogous and show a correlation between CaV $\beta$ 1E expression in human muscles and age-related muscle mass decline. These results suggest that strategies targeting CaV $\beta$ 1E or GDF5 might be effective in reducing muscle mass loss during senescence.

## **Introduction**

A decrease in electrical activity, such as during neuromuscular disease, disuse, or aging can cause massive muscle atrophy (1). Disuse atrophy following muscle denervation or immobilization is characterized by the activation of a compensatory response to counteract mass loss. In particular, the induction of Growth Differentiation Factor 5 (GDF5)/SMAD4 (an acronym from the fusion of *Caenorhabditis elegans Sma* genes and the *Drosophila Mad*, Mothers against decapentaplegic) pathway is essential 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  $\text{Ca}^{2+}$  channel  $\text{CaV}1.1$ ,  $\text{CaV}\alpha 1s$  subunit displays voltage sensor activity (10, 11). The intracellular subunit of the complex,  $\text{CaV}\beta 1$  (encoded by *Cacnb1* gene), plays a role in (excitation–contraction coupling) ECC by targeting  $\text{CaV}\alpha 1s$  to the membrane and regulating its activity (12). Currently,  $\text{CaV}\beta 1A$  is considered as the skeletal muscle specific isoform (13). Furthermore,  $\text{CaV}\beta 1$  can be anchored to membrane, free in the cytoplasm, or located to nucleus of proliferating muscle cells where it acts as regulator of transcription factors (14).  $\text{CaV}\beta 1$  is essential for the development of embryonic mouse muscles and for the pre-patterning and development of neuromuscular junctions (NMJ), independently of ECC (14–16). Yet, no ECC-independent pathway involving  $\text{CaV}\beta 1$  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  $\text{CaV}\beta 1$  isoform transition epigenetically. We showed that  $\text{CaV}\beta 1D$  is the constitutively expressed isoform in adult mouse muscle, whereas  $\text{CaV}\beta 1E$  is the embryonic variant up-regulated upon impairment of electrical activity. Second, we highlighted  $\text{CaV}\beta 1E$  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  $\text{CaV}\beta 1E$ /GDF5 axis and that over-expression of  $\text{CaV}\beta 1E$  drives the pathway necessary to

counterbalance age-related muscle atrophy in rodents. Finally, we provided evidence of a human hCaV $\beta$ 1E isoform expressed in adult muscle and tightly linked to muscle decline in aging.

## **Results**

### **Embryonic CaV $\beta$ 1E 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 if the expression of *Cacnb1* could change in this model we quantified its mRNA by 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 to innervated control muscles (D0) (Fig 1A). Western blot of CaV $\beta$ 1 revealed the appearance of an extra 70 kDa band whose intensity increased with time after denervation while the intensity of the band around 55 kDa was unchanged (Fig 1B, C).

The *Cacnb1* gene (GSMG0007319) has 14 exons that can be spliced to give 6 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 TAs. We found 1022 differentially regulated alternative splicing events (from 706 distinct genes) (Datafile S1). Among these, *Cacnb1* displayed a first exon splicing event showing that the transcript started in a putative non-coding 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 upregulated (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 1F, G).

By searching *Cacnb1* variants in the National Center for Biotechnology Information (NCBI) database, we found that *Cacnb1-D* is the only one starting immediately upstream the exon 3. Based on these results, our data suggest that the specific CaV $\beta$ 1 isoform expressed in the adult mouse skeletal muscle is CaV $\beta$ 1D and not CaV $\beta$ 1A, as expected (14). Moreover, the size of the extra CaV $\beta$ 1 band appearing in denervated muscle suggested that it could be CaV $\beta$ 1B or CaV $\beta$ 1E (66 and 70 kDa, respectively). However, CaV $\beta$ 1 antibody targeting a central peptide of the protein (AbCaV $\beta$ 1: sc-25689 (H-50) raised against amino acids 211-260 L-type CaV $\beta$ 1A of human origin - NM\_199247-NP\_954855) recognized only CaV $\beta$ 1E in mouse. To validate which isoform was upregulated 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 1H, I) confirming protein expression data.

Next, we verified if CaV $\beta$ 1E was the only isoform induced in denervated muscle. Amplification of the region between exons 5 and 8/9 of *Cacnb-1* let the discrimination of the several variants depending on the amplicon size. RT-PCR showed that innervated and denervated muscle expressed only *Cacnb1* variants containing exon 7A, corresponding to *Cacnb1-D* and *Cacnb1-E* (381 bp), and not *Cacnb1-B* (246 bp), expressed in spinal cord (SC) (18–20) nor *Cacnb1-C* (246 bp) and *Cacnb1-F* (222 pb) (Fig 1J).

Several embryonic proteins are induced after muscle denervation (3, 21–25). We wondered whether CaV $\beta$ 1E could be an embryonic isoform. RT-PCR (primers matching exon 14) (Fig 1K) and RT-qPCR (primers in exons 2-3 for *Cacnb1-E* and exon 13 for *Cacnb1-D*) revealed that *Cacnb1-E* is the specific variant in embryonic and neonatal muscles, while *Cacnb1-D* is not expressed in embryo (Fig S1A, B). *Cacnb1-E* transcript in embryonic muscle started at exon 1 (Fig S1C, D) as *Cacnb1-E* expressed in denervated adult muscle. Western blotting showed the

same bands in denervated adult and embryonic muscle protein extracts using AbCaV $\beta$ 1 (Fig 1L) or an antibody specific to CaV $\beta$ 1E (Fig S1E). Finally, we analyzed if CaV $\beta$ 1D and CaV $\beta$ 1E could have different localization in muscle fiber. Immunofluorescence analysis of innervated and denervated muscle sections and isolated fibers with AbCaV $\beta$ 1 showed that CaV $\beta$ 1 staining and triadic localization most likely reflected expression of CaV $\beta$ 1D (Fig S2A-C). In contrast, CaV $\beta$ 1E-specific staining was distributed at the Z-lines (Fig S2 D-F). Furthermore, CaV $\beta$ 1E-specific staining localized mostly at the nuclei, consistently with the presence of a Nuclear Localization Signal predicted by cNLS mapper, and its intensity increased in denervated muscle compared to CaV $\beta$ 1 staining (Fig S2G-L).

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. CaV $\beta$ 1D, not CaV $\beta$ 1A, is expressed in innervated adult skeletal muscle, whereas embryonic muscle expresses CaV $\beta$ 1E. CaV $\beta$ 1E is low in innervated adult mouse muscle but denervation specifically increases its expression. Moreover, CaV $\beta$ 1D and CaV $\beta$ 1E display different intracellular locations in adult muscle fibers.

### **CaV $\beta$ 1E and GDF5 signaling activation after denervation**

To evaluate whether CaV $\beta$ 1E has a role in disuse atrophy, we injected into mouse TA an Associated-Adeno-virus (AAV) vector carrying a short-hairpin RNA targeting a sequence in *Cacnb1* exon 2 (26) (AAV-ShCaV $\beta$ 1E) and thereby abolishing specifically *Cacnb1*-E. Two months after injection, CaV $\beta$ 1E expression induced after denervation was decreased by ~90% (Fig 2A-C; Fig S3A, B), without decreasing CaV $\beta$ 1D protein expression (Fig 2B, C). Blocking the induction of CaV $\beta$ 1E exacerbated muscle atrophy after denervation (Fig 2D-F), with decreased muscle fiber size (Fig 2F) and increased fibrosis (Fig S3C-F). This suggested a role for CaV $\beta$ 1E 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 absence of CaV $\beta$ 1E 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 CaV $\beta$ 1E ablation (Fig 2H, I). Furthermore, all components of the GDF5 pathway, SMAD1/5 phosphorylation, SMAD4 nuclear translocation, and *Id-2* transcription (29, 30), were inhibited with CaV $\beta$ 1E down-regulation suggesting the positive control of GDF5 signaling by CaV $\beta$ 1E (Fig 2J-O). However, *Id-1* expression was not modified after denervation in Scra or AAV-ShCaV $\beta$ 1E treated muscle (Fig 2N), suggesting that its activation might be very weak or transient (31).

CaV $\beta$ 1 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 CaV $\beta$ 1E knock-down (Fig S3G) and no alterations in *Fbxo32*, *MuRF1* expression (Fig S3H-I), indicating that the myogenin pathway is unlikely to participate in the increased atrophy following CaV $\beta$ 1E down-regulation. However, *Chrna1* transcription after denervation was significantly ( $P < 0.0001$ ) reduced in absence of CaV $\beta$ 1E, suggesting its involvement in modulating genes associated with endplate formation, independently of myogenin. A previous study showed that in proliferating muscle cells canonical and non-canonical DNA E-box sequences (CANNTG and CANNNTG) of several promoter regions could be targeted by CaV $\beta$ 1 (14). Analysis of the 100 most differentially regulated genes in innervated and denervated TA 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 (31, 32).

To further analyze the role of CaV $\beta$ 1E on *Gdf5* expression, we used the myogenic cell line C2C12 as tool in vitro. C2C12 cells expressed increasing *Cacnb1*-E during differentiation (Fig



S4A, B) and only CaV $\beta$ 1E protein (band at 70kDa) as embryonic muscle. (Fig S4C). Moreover, the transcription of *Gdf5*, as well as *Myogenin*, also increased in differentiating C2C12 cells (Fig S4D, E). Inhibiting *Cacnb1*-E expression in differentiating C2C12 cells by transfecting a plasmid ShCaV $\beta$ 1E (pCDNA3-ShCaV $\beta$ 1E) (Fig S4F) prevented the expression of *Gdf5* without affecting *Myogenin* transcription (Fig S4G, H), mimicking its effect in vivo. We then measured in C2C12 the consequence of CaV $\beta$ 1E 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 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 ShCaV $\beta$ 1E (Fig 2P). These data indicated that CaV $\beta$ 1E targets *Gdf5* promoter, consistently with the effect observed in vivo and in vitro.

CaV $\beta$ 1E seems to precede CaV $\beta$ 1D appearance in embryo and in C2C12. We thus checked if *Cacnb1E* and *Cacnb1D* mRNA expression could affect each other and have a role in muscle mass homeostasis via GDF5. Although AAV-ShCaV $\beta$ 1E injection prevented *Cacnb1E* rise after denervation, it also induced a slight decrease of *Cacnb1D* mRNA expression (Fig S5A, B), yet with no impact on CaV $\beta$ 1D protein expression. However, only the rescue of CaV $\beta$ 1E, and not CaV $\beta$ 1D, restored *Gdf5* transcription and atrophy after denervation in AAV-ShCaV $\beta$ 1E treated muscles (Fig S5C, D).

To confirm that the increased atrophy upon CaV $\beta$ 1E down-regulation was dependent on inadequate GDF5 activation, we overexpressed this factor in TAs together with ShCaV $\beta$ 1E, via AAV-gene transfer (Fig S5E, F). The up-regulation of *Id1* and *Id2* proved the activation of GDF5-dependent pathway (fig S5G, H) in double treated muscles. As expected, GDF5 over-

expression rescued the atrophy induced CaV $\beta$ 1E ablation and improved muscle histology by decreasing fibrosis (Fig S5I-R).

### **Aging muscles: a key role for CaV $\beta$ 1E**

During aging skeletal muscle shows denervation-like signs and progressive muscle wasting (4, 33). This suggests that impairment in compensatory response might occur. However, little is known about CaV $\beta$ 1 expression and function (26) and nothing is known about *Gdf5* amount in aging muscle.

Because our data indicate the involvement of CaV $\beta$ 1E 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, *Cacnb1*-E basal expression was significantly lower in TAs of 78 compared to 12 week-old mice ( $P < 0.0001$ ) (Fig 3B), whereas *Cacnb1*-D transcription did not change (Fig 3C). To evaluate if the cross-talk between *Cacnb1*-E and *Gdf5* would also be affected, we quantified these transcripts after denervation in TAs from 12-, 52- and 78-week-old mice. Although CaV $\beta$ 1E 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 3D, G, H). Consequently, the *Gdf5* increase was reduced (Fig 3F) affecting SMAD1/5 phosphorylation (Fig 3J, K). *Id1* remained unchanged (Fig 3L) whereas *Id-2* transcription followed the altered *Gdf5* induction (Fig 3M). CaV $\beta$ 1D expression did not change (Fig 3E, G, I).

We explored some possible causes responsible for decreasing CaV $\beta$ 1E during aging. Measuring the expression of NMJ components *Chrna1*, *Chrne*, *Chrng* and *Musk* in 78 week-old mice muscles, we could not detect changes indicating endplate alterations at this age (Fig 4A-D). Similarly, we checked if age-related modifications in fiber type composition occurred, maybe explaining CaV $\beta$ 1E down-regulation. Expression of myosin heavy chain (MyHC) isoforms IIA

and IIX decreased significantly during aging ( $P= 0.00616652$  and  $P= 0.01394505$ , respectively), as reported (9, 34, 35), whereas *MyHC-I* and *-IIB* did not change between 12 and 78 weeks of age (Fig 4E-H). To elucidate if  $\text{CaV}\beta 1\text{E}$  expression could be associated to fiber type modifications, we analyzed its amount in *MyHC-I* and *-II* positive fibers by immunofluorescence. In young muscle,  $\text{CaV}\beta 1\text{E}$  was expressed at higher intensity in *MyHC-IIA* and *-IIX* positive fibers, although in old muscle it was decreased in all fiber types (Fig 4I-K).

If  $\text{CaV}\beta 1\text{E}$  increased upon lack of nerve activity, for example denervation, modifications in muscle activity could potentially affect basal *Cacnb1E* transcription. Hence, we measured *Cacnb1E* mRNA after acute exercise of young and old mice (36, 37). Exercise training restored *Cacnb1E* transcription in old muscles but not in young (Fig 4L), with *Cacnb1D* expression unaffected (Fig 4M), suggesting that increased muscle activity is able to rapidly normalize *Cacnb1E* transcript only when it is decreased. However, *MyHC-IIA* and *IIX* expressions were not restored, implying that contractile activity could regulate *Cacnb1E* expression independently of type IIA-X fiber abundance (Fig 4N, O).

To evaluate whether  $\text{CaV}\beta 1\text{E}$  or GDF5 over-expression might mitigate age-related muscle mass loss, AAV vectors carrying  $\text{CaV}\beta 1\text{E}$  or GDF5 were injected into the TA of 78–80-week-old mice. A strong over-expression of  $\text{CaV}\beta 1\text{E}$  and GDF5 was observed 3 months after injection (Fig. 5A, B, D and S6A) without modifying *Cacnb1-D* expression (Fig 5C and S6C), yet increasing expression of each other reciprocally (Fig 5D and S6B). The up-regulation of *Cacnb1-E* in old muscle activated GDF5 signaling, as measured by SMAD1/5 phosphorylation and SMAD4 nuclear translocation. (Fig 5E-G). The increase of *Gdf5* either by  $\text{CaV}\beta 1\text{E}$  or by its own over-expression induced *Id-2* transcription, with no changes in *Id-1*, confirming a weaker or transient response of this factor to GDF5 (Fig 5H, I and S6D, E). In addition, the rise of GDF5

signaling following *Cacnb1*-E or *Gdf5* over-expression was associated with preservation of aged muscle mass (Fig 5J) and a gain of specific force (Fig 5K) compared to control old muscles. Subsequently, we investigated if CaV $\beta$ 1E over-expression could affect muscle mass of young mice (12 weeks). Ectopic expression of CaV $\beta$ 1E (Fig S7A, D, E) had no effect on *Cacnb1*-D, yet it induced a slight increase of *Gdf5* expression (Fig S7B, C, D, E). However GDF5 signaling, measured by *Id-1* and *Id-2* transcription, was not or poorly activated (Fig S7F, G). Consequently, muscle mass and the response to denervation were not different between Scra- and CaV $\beta$ 1E-treated TAs of young mice (Fig S7H-L). We also overexpressed GDF5 in young TAs (Fig S8A, D, E) which induced *Cacnb1*-E transcription in innervated TAs compared to scrambled (Fig S8B, D, E) without affecting *Cacnb1*-D expression (Fig S8C). Nevertheless, GDF5 over-expression and its activated signaling, measured as *Id-1* and *Id-2* transcription (Fig S8F, G), increased mostly innervated muscle mass (Fig S8H-L).

Overall, these data show that the loss of CaV $\beta$ 1E/GDF5 cross-talk observed during aging may be critical for muscle wasting and its rescue counteracts the process of age-related muscle decline.

### **Human muscle: a new CaV $\beta$ E isoform implicated in skeletal muscle aging**

Given the apparent importance of CaV $\beta$ 1E in mouse skeletal muscle, we wondered whether an analogous mechanism might be conserved in humans and, thus, if another unidentified *CACNBI* isoform was expressed in adult human skeletal muscle. Three human *CACNBI* (h*CACNBI*) variants have been identified corresponding to the mouse isoforms A (specific of skeletal muscle), B, and C (Fig 6A) (18–20, 38). Human mRNA extracted from one quadriceps and two fascia lata (FL) muscle biopsies of healthy adult subjects together with human mRNA extracted from the cervical spinal cord (Table S2), as positive control for h*CACNBI*-B (18–20), were probed for exons 13 and 5-9. Amplification of the sequence in exon 13 showed that all muscles expressed h*CACNBI*-A. As in mouse muscle, amplification of the region between exons 5 and 9

demonstrated that only a 380 bp corresponding to the putative *hCACNBI-A* appeared and that the exon 7B-containing isoform, *hCACNBI-B* (245 bp), was solely expressed in spinal cord (SC). Furthermore, no transcription of *hCACNBI-C*, which also has the short 7B exon, was found in muscle (Fig 6B). We further checked if human muscle could express a *hCACNBI* 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 *hCACNBI-E* (Fig 6C). This isoform corresponded to the predicted XM\_006722072.2 variant, having a codon start (ATG2) upstream the exon 3 (Fig 6A, C). Western blot and immunofluorescence experiments confirmed its expression in two different human FLs (Fig 6D, E), with localization similar to that of mouse CaV $\beta$ 1E (Fig S2E, F).

Since we found that altered CaV $\beta$ 1E/GDF5 axis was associated to muscle wasting during aging in mice, we compared characteristics indicating muscle mass (lean mass percentage) and function (power) (39, 40) in a cohort of healthy young (20-42 years) and aged (70-81 years) volunteers, included in a previous study (40) (Table 1). Aged group displayed significantly lower lean mass and power than young group ( $P= 0.003255$  and  $P<0.0001$ , respectively) (Fig 6F, G). We then measured *hCACNBI-E* transcript and found a significant reduction ( $P= 0.0045$ ) of its expression in aged group (Fig 6H), whereas *hCACNBI-A* transcription did not differ between groups (Fig 6I). A low *hCACNBI-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 *hCACNBI-E* and *hGDF5* with low lean mass percentage. In addition, participants with higher lean mass percentage displayed higher expression of both *hCACNBI-E* and *hGDF5* (Fig 6K).

Overall these results suggest that CaV $\beta$ 1E/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 CaV $\beta$ 1E isoform which mediates transcriptional reprogramming in adult muscle. Many years ago CaV $\beta$ 1 isoforms were identified in humans (19, 20, 41), rat (18) and, less clearly, in mouse (13). We anticipated that 55 kDa band corresponded to CaV $\beta$ 1A, 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 CaV $\beta$ 1 isoform. Our results revealed that CaV $\beta$ 1D, not CaV $\beta$ 1A, is the main constitutive isoform specific to normal adult mouse muscle and that CaV $\beta$ 1D and CaV $\beta$ 1E have distinct roles. Indeed, *in silico* analysis using cNLS mapper confirmed the presence of a Nuclear Localization Signal at the N-terminus of CaV $\beta$ 1E but not in CaV $\beta$ 1D. CaV $\beta$ A is not expressed in adult mouse muscle and C2C12, however, its N-terminus is putatively identical with those of CaV $\beta$ E, suggesting that the mechanism by which it translocates to the nucleus is the same described in past publications (14, 42). This finding also implicates CaV $\beta$ D in regulating calcium channel assembly in the cytoplasm, whereas CaV $\beta$ E 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 (43, 44).

We also discovered that CaV $\beta$ 1E is the main CaV $\beta$ 1 isoform in proliferating and differentiating C2C12 cells, while CaV $\beta$ 1D and CaV $\beta$ 1A isoforms are undetectable. Previous studies reporting expression, transcriptional activity and nuclear localization of CaV $\beta$ 1A in muscle fibers (42), muscle precursors and C2C12 cells (14) were likely evaluating the as-yet undiscovered CaV $\beta$ 1E. An interesting issue of our study could be deciphering mechanisms underlying the transition

between CaV $\beta$ 1E and CaV $\beta$ 1D during myogenesis and identifying molecular factors regulating the implicated splicing events.

The major finding of this work is the central role of CaV $\beta$ 1E in maintaining muscle mass. GDF5 is one of the main regulators of muscle mass homeostasis in response to disuse atrophy (31), however few studies report progress on modulators of this factor (3, 45, 46). Our data validate previously described roles of GDF5 (31, 47) and add a positive upstream player controlling its signaling. Indeed, CaV $\beta$ 1E expression is needed for the activation of *Gdf5* promoter, probably by acting on specific E-box sequences as described for other target genes (14).

In addition, we demonstrated that a compromised CaV $\beta$ 1E/GDF5 axis during aging is associated to muscle wasting. In our study, CaV $\beta$ 1E over-expression led to increased GDF5 expression and activation of its pathway improving age-related muscle decline. By contrast, CaV $\beta$ 1E over-expression did not affect muscle mass in young mice. This suggests that endogenous CaV $\beta$ 1E induction following nerve withdrawal is sufficient to trigger the maximal compensatory response. In addition, when muscles express physiological amount of GDF5, feedback mechanisms could be activated to prevent hypertrophy. Interestingly, 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 post-synaptic NMJ partners improving neuro-transmission. Thus, increased GDF5 signaling, induced by over-expression of CaV $\beta$ 1E or GDF5 itself, might counteract age-related muscle decline also by preventing the associated denervation occurring later. CaV $\beta$ 1 has been shown as important for correct synaptic patterning in embryo (15). Even though a role for GDF5 in this process has not been established yet, its relevance to mediate re-innervation in adult muscle is proven (3). Importantly, we also demonstrated that a hCACNB1-E variant is expressed in human muscle, indicating that mechanisms governing skeletal muscle homeostasis might be conserved across mammalian species. Although hCACNB1-E transcript is

slightly different compared to mouse *Cacnb1*-E, we observed similar localization and hypothesize the same function. In fact, hCACNB1-E expression was higher in young compared to 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 as IGF-1 (7), GH (48), leptin (49) and, recently, apelin (9). These molecules are dependent on muscle activity and aging. Here, we identified CaV $\beta$ 1E 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 (50) affecting a large population, which results in progressive loss of autonomy, increased mortality associated to frailty and risk of falls in elderly subjects (51). GDF5 or GDF5-like molecules can be considered as potential therapeutic compounds to prevent muscle mass wasting during senescence but also, in a wider application, to ameliorate consequences of neuromuscular defects. Finally, deciphering the fine regulation of CaV $\beta$ 1E 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 CaV $\beta$ 1E in aging muscle remain unresolved. Previous studies have reported a link between elevated CaV $\beta$ 1A and age-related muscle weakness (26, 52, 53) but *Cacnb1* transcripts were not fully characterized nor antibodies used well defined. CaV $\beta$ 1E expression is higher in young type IIA-IIX fibers compared to IIB. The fiber type II decrease occurring during aging may thus have a role in CaV $\beta$ 1E reduction. Still, other mechanisms, dependent on muscle activity *per se*,



modifying DNA methylation or mitochondrial metabolism, could participate in altering the CaV $\beta$ 1E/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 CaV $\beta$ 1, a subunit of the voltage sensor CaV1.1. RNAseq data of innervated and denervated TAs led to identify an embryonic variant, CaV $\beta$ 1E, up-regulated in mouse muscle by denervation. Validation of RNAseq data and characterization of CaV $\beta$ 1 isoforms in mouse and human muscles have been performed by RT-PCR, qPCR, western blot and immunostaining experiments. To investigate the role of CaV $\beta$ 1E 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 Haematoxylin&Eosin and Sirius Red staining. To evaluate the effect of CaV $\beta$ 1E down-regulation on the transcriptional activity of *Gdf5* promoter, we performed a Luciferase reporter-based assay in C2C12. To establish the effect of CaV $\beta$ 1E or GDF5 *in vivo* on muscle mass, we over-expressed them in mouse by local AAV-gene transfer. In order to characterize the expression of CaV $\beta$ 1 isoforms in humans, we used muscle biopsies of a cohort of healthy young and aged volunteers (40). Body composition and muscle function of volunteers included in the study (40) 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 based

on Grubb's test. All experiments using animals and cells were done in a non-blinded 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 Anova tests followed by the appropriated 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 GraphPad Prism7 software.

### **Supplementary material list**

#### **Materials and Methods**

**Figure S1: expression of embryonic CaV $\beta$ 1 isoform**

**Figure S2: CaV $\beta$ 1 isoforms localization on adult skeletal muscle**

**Figure S3: Histological characterization and myogenin signaling in CaV $\beta$ 1E knock-down muscles**

**Figure S4: Expression of *Cacnb1-E*, *myogenin* and *Gdf5* in C2C12**

**Figure S5: Effects of CaV $\beta$ 1E, CaV $\beta$ 1D and GDF5 over-expression on AAV-ShCaV $\beta$ 1E treated muscles**

**Figure S6: GDF5 over-expression in aged mice muscle**

**Figure S7: CaV $\beta$ 1E over-expression in young mice muscle**

**Figure S8: GDF5 over-expression in young mice muscle**

**Fig S9: Schematic representation of CaV $\beta$ 1E/axis in young and old mouse muscle**

**Table S1: Expression data**

## Table S2: 100 top up-regulated genes in innervated and denervated samples

## Table S3: List of human samples

## Table S4: Primer list

## Datafile S1: List of 1022 differentially regulated alternative splicing events

## Datafile S2: Uncut Western Blotting raw data

## Datafile S3: Individual subject data

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### **Author Contributions**

F.P.R. and S.F. proposed the concept, designed experiments and wrote the manuscript; S.F. and C.G. conceived and conducted *in vivo* experiments, cloning and GeneString design; M.T. designed and conducted experiments to validate RNAseq data presented and to demonstrate hCACNBI-E expression and hCaV $\beta$ 1E localization in human muscles; C.B. performed most of qPCR and western blotting. C.G., M.T. and C.B. performed the remaining experiments and, together with S.F., analyzed the data; P.dG. performed RNAseq analysis; A.F. and M.L. conducted the force measurement; B.C. performed morphometric analysis using WEKA; S.B.Z. and L.J. provided AAV production; J.Y.H. gathered the human muscle biopsies of the European project MyoAge, maintained the blinded data, oversaw the evaluations, provided all corresponding data only after getting the results and analyzed data together with F.P.R., S.F., M.T. and C.G.; M.T. and C.G. performed separately the blinded to allocation qPCRs of MyoAge samples. No modifications, additions or exclusions were made to the validation data set from the point at which the model was locked down neither the validation data nor any subset of it had ever been used to assess or refine the model being tested.

### **Competing Interests**

S.F. and F.P.R. hold a patent (#18 184861.5) "Compositions for the treatment of sarcopenia or disuse atrophy". All other authors declare that they have no competing interests. Each of the authors

confirms that this manuscript has not been previously published and is not currently under consideration by any other journal. Additionally, all of the authors have approved the contents of this paper and have agreed to the *Science Translational Medicine* submission policies.

**Data and materials availability**

All data associated with this study are available in the main text or the supplementary materials.



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

**Table 1: Characteristics of human muscles:**

Young, from 20 - 42 years old (Y) and aged, from 70 - 81 years old (A) participants; gender male (M) and female (F). Lean mass (%) was assessed by dual-energy X-ray absorptiometry. Power is expressed by Watt on kilograms (W/kg); ND: not determined.

## Figure Legends

### **Fig 1: Expression of an embryonic CaV $\beta$ 1 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 *Cacnb1* coding region.

**B, C:** (B) Western blot and (C) quantification of CaV $\beta$ 1 bands in innervated or denervated TAs after the indicated number of days of denervation. Actin was the loading control.

**D:** CaV $\beta$ 1 protein and *Cacnb1* gene and transcript variants (adapted from (41)). *Cacnb1*-A (NM\_031173); *Cacnb1*-B (NM\_145121); *Cacnb1*-C (NM\_001159319); *Cacnb1*-D (NM\_001159320); *Cacnb1*-E (NM\_001282977); *Cacnb1*-F (NM\_001282978). Molecular weights (MW) in brackets.

**E:** *Cacnb1* gene showing two putative ORFs (ATG1-ATG2). Thin colored vertical lines indicate the start of transcription at ATG1 in innervated muscle and at ATG1 and ATG2 in denervated muscle.

**F, G:** (F) RT-PCR and (G) quantification 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, I:** (H) RT-PCR and (I) quantification 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 5-8/9) in adult TAs Inn and Den and in 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; post-natal 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 CaV $\beta$ 1 in TAs Inn or Den for 15 days and in embryonic muscle (E16; diluted 1:10) using AbCaV $\beta$ 1 (central peptide). Actin was the loading control.

**A, C, I:** Means  $\pm$  s.e.m (A: n=6 mice per group; C, I: n=3 independent experiments) \*P < 0.05; \*\*\*P < 0.001, (ordinary one-way Anova - Dunnett's test).

**G:** Means  $\pm$  s.e.m (n=3 independent experiments) \*\*\*P < 0.001, (ordinary two-way - Sidak's test).

**Fig 2: CaV $\beta$ 1E down-regulation and increased muscle atrophy by reduced GDF5 signaling after denervation.**

**A:** RT-qPCR for *Cacnb1*-E (Ex 2-3) in adult TAs innervated (Inn) or denervated for 15 days (Den) treated with AAV-Sh scrambled (Scra) or AAV-ShCaV $\beta$ 1E (ShCaV $\beta$ 1E).

**B, C:** (B) Western blot using AbCaV $\beta$ 1 (central peptide) and (C) quantification of CaV $\beta$ 1 expression in adult TAs Inn or Den treated with Scra or ShCaV $\beta$ 1E. Actin was the loading control.

**D, E:** (D) Inn or Den muscle/body-weight ratio and (E) percentage of atrophy after denervation of adult TAs treated with Scra or ShCaV $\beta$ 1E.

**F:** Frequency distribution of fiber size of TAs Inn or Den treated with Scra or ShCaV $\beta$ 1E.

**G-I:** RT-qPCR for (G) *Gdf5*, (H) *Gdf8* and (I) *Bmp7* in Inn or Den adult TAs treated with Scra or ShCaV $\beta$ 1E.

**J:** Western blot (top) and quantification (bottom) of phosphorylated SMAD1/5 and SMAD5 in adult TAs Inn or Den treated with Scra or ShCaV $\beta$ 1E.

**K, L, M:** (K, L) Immunofluorescence images and (M) quantification of TAs Inn or Den, treated with (K) Scra or (L) ShCaV $\beta$ 1E, stained with AchR (Magenta), SMAD4 (Yellow), Cav3 (Gray), DAPI (Cyan). Bar: 10  $\mu$ m.

**M, N:** RT-qPCR for (M) *Id-1* and (N) *Id-2* in adult TAs Inn or Den treated with Scra or ShCaV $\beta$ 1E.

**P:** Firefly/renilla ratio in 24- or 48h-differentiated C2C12 cells co-transfected with a renilla and HSVTK-Gdf5 promoter -Luc3' with either pCDNA3-Scrambled (Scra) or pCDNA3-ShCaV $\beta$ 1E (ShCaV $\beta$ 1E).

**A, C, D, G, H, I, J, M, N, O:** Means  $\pm$  s.e.m. (n = 4-7 mice per group; M: n=3 cryosections quantified), \*P < 0.05, \*\* P < 0.01, \*\*\*P < 0.001 (ordinary one-way Anova-Sidak's test).

**E:** Means  $\pm$  s.e.m. (n=6 mice per group), \*P < 0.05, (independent samples t-test (two tailed)).

**F, P:** Means  $\pm$  s.e.m. (F: n=3 cryosections quantified per condition; O: n=3 independent experiments), \*P < 0.05, \*\* P < 0.01, \*\*\*P < 0.001, (ordinary two-way Anova- F:Tukey's test; O: Sidak's test).

### **Fig 3: Muscle mass loss during aging and alteration of CaV $\beta$ 1E-GDF5 axis**

**A:** Muscle/body-weight ratio of innervated adult TAs from 12-, 52-, 78-week-old mice.

**B, C:** RT-qPCR for (B) *Cacnb1*-E (ex 2-3) and (C) *Cacnb1*-D (ex13) in innervated TAs from 12-, 52-, 78-week-old mice.

**D-F:** RT-qPCR for (D) *Cacnb1* (ex 2-3), (E) *Cacnb1*-D (ex13) and (F) *Gdf5* in TAs innervated (Inn) or denervated for 15 days (Den) from mice at 12-, 52-, 78-week-old mice.

**G-I:** (G) Western blot using CaV $\beta$ 1 (central peptide) and (H, I) quantification of CaV $\beta$ 1E and D in Inn or Den TAs from 12-, 52-, 78-week-old mice. Actin was the loading control.

**J, K:** (J) Western blot and (K) quantification of phosphorylated SMAD1/5 and SMAD5 in Inn or Den TAs from 12-, 52-, 78-week-old mice.

**L, M:** RT-qPCR for (L) *Id-1* and (M) *Id2* in Inn or Den TAs from 12-, 52-, 78-week-old mice.

**A, B, C:** Means  $\pm$  s.e.m. (12: n=8 mice per group, 52: n=9 mice per group, 78: n=10 mice per group) \*P < 0.05, (ordinary one-way Anova-Dunnet's test).

**D-F, L, M:** Means  $\pm$  s.e.m. (n=6/7), \*P < 0.05, \*\* P < 0.01, \*\*\*P < 0.001 (ordinary one-way Anova-Sidak's test).

**H, I, K:** Means  $\pm$  s.e.m. (n=3; western blots used for quantification are showed in raw data), \*P < 0.05, \*\* P < 0.01, \*\*\*P < 0.001 (ordinary one-way Anova-Sidak's test).

**Figure 4: NMJ-associated genes and MyHC expression and effect of exercise on CaV $\beta$ 1E expression in aged muscle**

**A-D:** RT-qPCR for (A) *Chrna1* (B) *Chrne* (C) *Chrng* and (D) *Musk* in innervated TAs from 12-, 52-, 78-week-old mice.

**E-H:** RT-qPCR for (E) *MyHC-I* (F) *MyHC-IIA* (G) *MyHC-IIX* and (H) *MyHC-IIB* in innervated TAs from 12- and 78-week-old mice.

**I, J:** Immunofluorescence images of TAs from (I) 12- and (J) 78-week-old mice stained with MyHC-IIA (Cyan), MyHC-IIB (Magenta) CaV $\beta$ 1E (Gray). MyHC-IIX are black (unstained).  
Bar: 25 $\mu$ m.

**K:** Fluorescence intensity of CaV $\beta$ 1E staining in MyHC-IIA -IIB -IIX positive fibers in TA muscles from 12- and 78-week-old mice.

**L, M:** RT-qPCR for (L) *Cacnb1-E* and (M) *Cacnb1-D* in innervated TAs from 12- and 78-week-old mice in resting or exercised conditions.

**N, O:** RT-qPCR for (N) *MyHCII-A* and (O) *MyHCII-X* in innervated TAs from 78-week-old mice in resting or exercised conditions.

**A-D, K, L, M:** Means  $\pm$  s.e.m. (A-D, L, M: n=6/8; K: MyHC-IIA n=24, MyHC-IIX n= 65, MyHC-IIB n=41) \*P < 0.05, \*\*\*P < 0.001, \*P < 0.05, \*\* P < 0.01, \*\*\*P < 0.001, (ordinary one-way Anova, A-D: Dunnett's test; K: Tukey's test; L, M: Sidak's test).

**E-H, N, O:** Means  $\pm$  s.e.m. (n=6) \*P < 0.05, \*\* P < 0.01, \*\*\*P < 0.001, (independent samples t-test (two tailed)).

**Fig 5: CaV $\beta$ 1E and GDF5 over-expression in aged muscle.**

**A, C, D:** RT-qPCR for (A) *Cacnb1*-E (ex 2-3), (C) *Cacnb1*-D (ex13), and (D) *Gdf5* in innervated TAs from 92-week-old mice treated with Scra or AAV-CaV $\beta$ 1E (CaV $\beta$ 1E). A: Minimum top axis= 15.

**B:** Immunofluorescence images of innervated TAs from 92-week-old mice treated with Scra or CaV $\beta$ 1E stained with: Cav3 (Magenta), CaV $\beta$ 1E (Yellow), DAPI (Cyan). Bar: 10  $\mu$ m.

**E, F:** Western blot (E) and quantification (F) of phosphorylated SMAD1/5 and SMAD5 in innervated TAs from 92-week-old mice treated with Scra or CaV $\beta$ 1E.

**G:** Immunofluorescence images of innervated TAs from 92-week-old mice treated with Scra or CaV $\beta$ 1E stained with SMAD4 (Yellow), Cav3 (Magenta), DAPI (Cyan). Bar: 10  $\mu$ m.

**H, I:** RT-qPCR for (H) *Id-1* and (I) *Id-2* in innervated TAs from 92-week-old mice treated with Scra or CaV $\beta$ 1E.

**J:** Muscle/body-weight ratio of innervated TAs from 12 and 92-week-old mice treated with Scra or from 92-week-old mice treated with CaV $\beta$ 1E.

**K:** Specific force generated by innervated TAs from 12 and 92-week-old mice treated with Scra or from 92-week-old mice treated with CaV $\beta$ 1E

**A, C, D, H, I:** Means  $\pm$  s.e.m. (Scra n=8; CaV $\beta$ 1E: n=8), \*P < 0.05, \*\* P < 0.01, \*\*\*P < 0.001 (independent samples t-test (two tailed)).

**F:** Means  $\pm$  s.e.m. (n=2-3), \*P < 0.05, (independent samples t-test (two tailed)).

**J, K:** Means  $\pm$  s.e.m. (J: Scra 12 weeks: n= 12; Scra 92 weeks: n= 11; CaV $\beta$ 1E 92 weeks n= 25; GDF5 92 weeks n= 11; K: Scra 12 weeks: n= 7; Scra 92 weeks: n= 15; CaV $\beta$ 1E 92 weeks n= 19; GDF5 92 weeks n= 6), \*P < 0.05, \*\* P < 0.01, \*\*\*P < 0.001 (ordinary one-way Anova-Sidak's test).

**Fig 6: Expression of CaV $\beta$ 1E in human muscle: conserved compensatory mechanism?**

**A:** Representation of human CaV $\beta$ 1 protein and hCACNBI gene and transcript variants (adapted from (41)). hCACNBI-A (NM\_199247); hCACNBI-B (NM\_000723); hCACNBI-C (NM\_199248); predicted hCACNBI-E (XM\_006722072.2). MW are in brackets.

**B:** RT-PCR for different isoforms of hCACNBI in human quadriceps (Q) and fascia lata (FL1, FL2) muscle biopsies from three healthy adults. RNA from one human spinal cord (SC) biopsy was the positive control for hCACNBI-B. Primers were designed for different exons (Ex) of the coding region of hCACNBI. Human Ribosomal phosphoprotein (hPO) is the loading control.

**C:** RT-PCR for hCACNBI-E in human Q, FL1, FL2 muscle biopsies from three healthy adults. RNA from SC biopsy was the positive control for the expression of exon 14 of hCACNBI-B. Primers were designed for different exons (Ex) of the coding region of hCACNBI. hPO was the loading control.

**D:** Western blot using CaV $\beta$ 1 (central peptide) in human FL1 and FL2 muscle biopsies from the same adult healthy subjects as in B and C. Actin was the loading control.

**E:** Hematoxylin/eosin (H/E) and immunofluorescence images of human FL1 and FL2 muscle used in B, C and D stained with hCaV $\beta$ 1E (Yellow), Cav3 (Magenta), DAPI (Cyan). Bar H/E: 50  $\mu$ m; bar IF: 10  $\mu$ m.

**F, G:** Distribution of (F) lean mass percentage and (G) Power in human quadriceps biopsies from healthy young and aged volunteers (Table 1).

**H, I:** Distribution of (H) *hCACNBI-E* or (I) *hCACNBI-A* expression in human quadriceps biopsies from healthy young and aged volunteers (Table 1).

**J:** Linear regression between *hCACNBI-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 *hCACNBI-E* (blue circles, right y axis) expression in human quadriceps biopsies from healthy aged volunteers (Table 1) having increasing lean mass percentage. Dot black line indicates the average of lean mass percentage of the young group.

**F-I:** Means  $\pm$  s.e.m. (Young: n=8; Aged: n=17) \*\*  $P < 0.01$ , \*\*\* $P < 0.001$ , (independent-samples t-test (two-tailed)).