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Physiological role of Kvβ2 (AKR6) in murine skeletal muscle growth and regulation

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Abstract

Aim—Potassium channel accessory subunits (Kvβ) play a key role in cardiac electrical activity through ion channel modulation. In the present study we hypothesize that Kvβ2 regulates skeletal muscle growth and fiber phenotype via protein-protein interactions.

Methods—Kvβ2 knockout mouse model was used for morphometric, immunohistochemical and biochemical analysis to evaluate the role of Kvβ2 in skeletal muscle physiology.

Results—Deletion of Kvβ2 gene in mice (Kvβ2 knockout, KO) leads to significant decrease in body weight along with skeletal muscle size. Key hind limb muscles such as biceps, soleus, and gastrocnemius were significantly smaller in size in KO mice compared to that of wild type. Morphometric measurements and histological analysis clearly point that the fiber size is decreased in each of the muscle type in KO compared with wild type mice. In addition, Kvβ2 deletion contributes to fiber type switching from fast to slow fiber as indicated by more abundant MHCI expressing fibers in gastrocnemius and soleus muscles, which may underscore the smaller muscle size alongside increase in U3 ubiquitin ligase;NEDD4 expression. Using targeted siRNA knockdown approach, we identified that Kvβ2 knockdown do not affect the myoblasts proliferation. However, Pax7 expression was significantly decreased in 4 week old gastrocnemius muscle suggesting that cellular reserve for growth may be deficient in KO mice. This is further supported by decreased migratory capacity of C2C12 cells upon siRNA targeted Kvβ2 knockdown.

Conclusion—Overall, this is the first report identifying that genetic deletion of Kvβ2 leads to decreased skeletal muscle size along with isotype switching.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

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Keywords

Kvβ2; skeletal muscle; growth; MHC; regulation; fiber; NEDD4

1. INTRODUCTION

Skeletal muscle constitutes about 40–50% of the total body weight and is considered one of most energetically dynamic and plastically adaptive tissue in the body[1]. Skeletal muscle mass is determined by both the addition of new myonuclei supplied through activation of resident satellite cells and the net protein turnover [2]. A major physiological attribute for skeletal muscle comes from the metabolic adaptability in its phenotype to meet the work demands. Several factors including genetics, environment, physical activity, nutrition, metabolism and health status can modulate these growth processes and influence the size of skeletal muscle [3]. Because of the central role of skeletal muscle in both locomotion as well as in metabolism, chronic disease conditions such as diabetes, heart failure, cancer and obesity can lead to significant remodeling in skeletal muscle [4].

We previously showed that Kv auxiliary subunits couple cellular metabolism to cardiac structural and electrical complement, and modulates action potentials in response to changes in cellular redox balance [5]. We also showed that in murine hearts, Kvβ1.1 subunit binds to fast myosin (Myh6) protein, and genetic deletion of this subunit led to enhanced Myh6 expresison and cardiac hypertrophy in female hearts, suggesting a potential role of these subunits in modulating the metabolic phenotype of the heart as well as myocyte hypertrophy. Further, experimental studies in both heterologous expression systems and animals showed that Kvβ –subunits are dynamic and interact with many proteins including calcium handling (Ca^{2+} sensitive) calmodulin [6], myosin filaments [5] and ion channel interacting; (potassium channel association protein; KChAP [7]. High throughput proteomic analysis further show that Kvβ subunits can also bind to other proteins including E3 ubiquitin ligases such as NEDD4, suggesting a potential of these subunits in modulation of skeletal muscle size [8].

Both the postnatal and adult rodent skeletal muscles expresses variable, but abundant, levels of different isoforms of Kvβ1 and Kvβ2 genes [9]. Further, Kvβ subunits also bind to members of Kv1.x family of potassium channels [10], that are abundantly expressed in skeletal muscle [11]. Thus, it is plausible that Kvβ subunits may regulate skeletal myogenesis and postnatal muscle phenotype, and that these subunits may also modulate the effects of metabolism on skeletal muscle physiology. Therefore, we hypothesized that Kvβ2 regulates skeletal muscle growth and fiber phenotype via protein-protein interactions with NEDD4, PAX7 and calcium handling proteins.

In the past, we showed that Kvβ subunits binds to Kv channels such as the Kv1 and Kv4 [12,13]. However, with the discovery of multiple binding partners for Kvβ subunits, it is highly likely that Kvβ2 plays a key role in skeletal myogenesis by interacting with regulatory binding partners that are crucial for growth, and differentiation. Therefore, in the present study, we utilized Kvβ2 knockout and identified its physiological relevance in murine hind limb muscles. Using a multi-pronged approach including protein–protein interaction and targeted siRNA knockdown, we probed the physiological roles of Kvβ2 in skeletal

myogenesis and its interactions with key underlying molecular mediators. Therefore, the present investigation identifies fundamental roles of Kvβ2 subunit in the skeletal myogenesis.

2. RESULTS

2.1 Kvβ2 deletion and body weights

Deletion of Kvβ2 gene in KO mice was confirmed using protein lysates isolated from gastrocnemius muscle. As shown in Fig. 1A, Wt mice showed abundant Kvβ2 protein in skeletal muscle whereas the KO mice showed complete absence of Kvβ2 protein, suggesting homozygous deletion of Kvβ2 in the KO mice. Body weights of KO mice recorded at 6, 12 or 16 weeks of age, were significantly ($p < 0.05$) less than that of age matched Wt mice (Fig. 1B). Depending on the age, the differences in body weight between the two groups ranged from 11–22%. Moreover, body weight normalized to tibial length in 16 week old mice was ~ 13% lesser than that of age matched Wt mice (Fig. 1C).

2.2 Skeletal muscle morphometry

Hind limb skeletal muscles; biceps, gastrocnemius and soleus, were isolated from 16 week old Wt and KO mice. Both the wet muscle weights, and weight normalized to tibia length were assessed. When compared to Wt mice, the ratio of major hind limb skeletal muscle weights/tibial length were markedly decreased in KO mice as compared to that of Wt group (Fig. 1D). All three muscles were analyzed for histology, and fiber cross-sectional area (AU) was measured (Fig. 2A–F). The distribution of fibers with larger cross-sectional area was altered in KO mouse muscles compared to Wt (Fig. 2G, H and I) to further test this we quantitatively assessed the cross sectional area of the myofibers (Fig. 2J, K and L), which was significantly decreased in KO muscles vs. Wt. These results are consistent with the idea that Kvβ2 deletion regulates muscle size, suggesting an essential role of this subunit in skeletal myogenesis and/or maintenance.

2.3 Kvβ2 modulates myogenesis and injury in C2C12 cells

Expression of Kvβ2 gene was assessed in proliferating myoblasts. 5-bromo-2′-deoxyuridine: BrdU assay showed no detectable differences in proliferation (Fig. 3A–G) at 72 hours post-transfection, when Kvβ2 was knocked down to 80% lower than the control (Fig. 3H). The mRNA expression levels of Pax7, P21 and P27 were not significantly altered compared with control, however, the expression levels of MyoD were significantly decreased in Kvβ2 knockdown compared with control (Fig. 3H). Expression levels of Kvβ2 were also assessed in differentiated C2C12 cells. Differentiation of C2C12 in low serum media for 3 days led to a significantly decreased expression of Pax7 mRNA ($p < 0.05$) (Fig. 3I), while the expression of myogenin increased ($p < 0.05$) (Fig. 3J). Kvβ2 mRNA expression decreased when assessed after 3 day differentiation ($p < 0.05$) (Fig. 3K). To test the role of Kvβ2 in C2C12 skeletal muscle myoblasts migration, we utilized targeted siRNA deletion of Kvβ2 using the *in vitro* scratch injury model. As shown in Fig. 3, we identified that knockdown of Kvβ2 leads to decreased area of C2C12 migration in the scratched area in Kvβ2 knockdown cell groups as compared to control cells, identifying the role of Kvβ2 in cell migration and injury repair (Fig. 3L–N).

2.4 Kvβ2 deletion altered myosin isoform expression in skeletal muscle

Quantitative real-time PCR assay was performed to assess the mRNA expression of myosin heavy chain isoforms in biceps femoris, gastrocnemius, and soleus muscles. Both the biceps femoris (Fig. 4A) and gastrocnemius (Fig. 4B) of KO mice had significantly ($p < 0.05$) higher expression of MHCIIa and MHCIIx mRNA relative to that recorded in corresponding Wt group. Further in the hind limb muscles of KO mice, the mRNA levels of MHCI and MHCIIb did not show statistically significant differences, when compared with Wt group (Fig. 4A & B). The slow-twitch soleus of KO mice (Fig. 4C) had a significant ~6.5 fold higher MHCI mRNA levels when compared with that of Wt, whereas the MHCIIa and MHCIIx were not different. Western blot analysis further indicated that mRNA expression changes in myosin isoforms translate into altered protein levels. Immunoblot analysis showed significantly higher expression of MHCIIa protein in both biceps and gastrocnemius of KO mice as compared to that of matched Wt group (Fig. 4D–G).

2.5 Deletion of Kvβ2 enhanced the proportion of slow-twitch fibers

Both transcriptome and protein expression changes suggested that Kvβ2 deletion significantly alters myofiber distribution towards slower phenotype (MHCIIb → MHCIIx → MHCIIa → MHCI). Hence, an immunohistochemistry analysis for type I fibers was conducted in the soleus (Fig. 5A–F) and gastrocnemius muscles (Fig. 5G–L). When compared to Wt group, KO group had significantly higher proportion of MHCI fibers in both soleus and gastrocnemius muscle (Fig. 5P and Q), which indicates that normal fiber type distribution is altered in the KO skeletal muscle, and that deletion of Kvβ2 supports a predominant oxidative muscle phenotype.

2.6 Enhanced Calcineurin and decreased Pax7 expression in Kvβ2 knockout skeletal muscle

Increased frequency of type I fibers and higher expression profiles of slow muscle specific genes suggested that Kvβ2 effects on skeletal muscle fiber type may be Calcineurin mediated. Immunoblot analysis of (Fig. 6A, B) protein lysates showed significant increases in Calcineurin A expression in KO mice as compared to Wt group. Western blot analysis of Pax7 in gastrocnemius skeletal muscle showed significant downregulation of Pax7 in Kvβ2 KO compared with wild type group, suggesting that Pax7 plays a role in decreased muscle size in KO mouse hind limb muscles (Fig 6. C, D). As noted in the figure, we utilized the Pax7 specific band identified between 50–60kDa for analysis and comparison between wildtype and Kvβ2 KO groups.

2.7 Kvβ2 binds to E3 ubiquitin ligase, Nedd4

High throughput studies in the past have indicated that Kvβ2 binds to Nedd4, which is one of the major E3 ubiquitin ligases. Given the significant reduction in the body weights of KO mice, we assessed Kvβ2-Nedd4 interaction in the skeletal muscle. Kvβ2-Nedd4 interaction was verified with a co-immunoprecipitation utilizing IgG magnetic beads (SureBeads-BioRad) incubated with Kvβ2 antibody. As shown in Fig. 7A, gastrocnemius expresses abundant Nedd4 protein (lane1; IN), and Kvβ2 successfully pulled down Nedd4 protein (lane 2; +). Absence of band in lane 3 (−) shows that IgG did not interact with Nedd4.

Overall the co-immunoprecipitation assay and the pull-down assay clearly identify that Kvβ2 interacts with Nedd4 in gastrocnemius muscle. A secondary pull-down assay was performed using gastrocnemius tissue lysates with $Kv\beta2^{(DDK\text{-}tag)}$ as bait. Protein-protein interaction between Nedd4 and Kvβ2 was determined, as shown in Fig. 7B, gastrocnemius expresses abundant Nedd4 protein (lane1; IN), and Kvβ2-DDK successfully pulled down Nedd4 protein (lane 2; +). Negative control (lane 3;-) excludes non-specific Nedd4 binding to DDK-agarose beads, suggesting that Kvβ2 interacts with Nedd4 in gastrocnemius muscle. Presence of Kvβ2-DDK in lysates of gastrocnemius (lane 1; IN) and Kvβ2 pulled down complexes (lane 2; +) was also confirmed (Fig. 7C).

2.8 Kvβ2 deletion enhances Nedd4 expression in skeletal muscle

Deletion of Nedd4 in mice was shown to confer resistance against limb unloading induced skeletal muscle atrophy. We sought to examine if the expression and or localization of Nedd4 is altered in KO mice. Immuno-histochemistry of biceps and gastrocnemius cross-sections (Fig. 8A–O) was performed using a specific antibody against Nedd4. Image analysis revealed that Nedd4 is expressed in considerable quantities in skeletal muscle, Nedd4 localizes with sarcoplasmic membrane. Interestingly both in soleus and gastrocnemius, Nedd4 expression significantly increased in KO group as compared with Wt group (Fig. 8P and Q), suggesting that Kvβ2 interaction with Nedd4 is essential to cell volume/size regulation in skeletal muscle, thus providing a potential biochemical link for Kvβ2 deletion and small muscle size in mice.

3.0 DISCUSSION

In the present study, we identify and report the physiological significance of Kvβ2 to skeletal muscle growth and phenotype. The major findings of this study are that genetic deletion of Kvβ2 in mice leads to: (1) significant decrease in body weight and skeletal muscle mass, (2) increased frequency of type I fibers and MHCI and MHCIIa expression in KO mouse hind limb muscles suggesting the emergence of a predominant oxidative fiber type (3) increased Nedd4 and decreased Pax7 expression suggesting decreased muscle growth contributed by satellite cells in the young mice and (4) enhanced Calcineurin expression, offering potential links for the increased slow fiber type prevalence.

Since Kvβ2 deletion led to both reduced muscle weights and decreased body weight, one reason could be reduced skeletal mass. However, tibial lengths are comparable in both Wt and KO mice, and the major tibial normalized hind limb skeletal muscle weights are significantly decreased in KO mice. Besides, the smaller muscle size in KO mice was also reflected at the myofiber level where fiber cross-sectional area decreased and the proportion of smaller fibers in hind limb muscles increased. This change may be attributed to predominance of smaller sized, slow-oxidative muscle fiber, as compared to relatively larger fast-glycolytic muscle fibers.

Hence, the smaller body weights in KO mice are very likely because of the deficient skeletal myogenesis, thus supporting Kvβ2 direct contribution to skeletal muscle phenotype determination. Adult skeletal muscle mass is determined by both the addition of new myonuclei from activated satellite cells as well as protein accretion in the muscle tissue [14].

Therefore, it is also possible that overall protein synthesis is reduced in the KO mice. Altered expression of myogenic regulatory factors [15] may underlie the skeletal muscle changes in KO mice. Our data both in 4 week old mice and C2C12 cells suggested that Kvβ2 deletion may result in smaller myotube formation, and the fact that both Pax7 and Kvβ2 expression declined simultaneously after 3 days of differentiation in C2C12 cells suggest that Kvβ2 deletion may serve as a permissive factor for differentiation. This is further supported by the data in four week KO gastrocnemius muscles where the Pax7 expression levels were reduced to around 50% of that of Wt group. Therefore, Kvβ2 offers a key role in skeletal muscle differentiation, and that deletion may compromise skeletal myogenesis.

Postnatal skeletal myofibers are categorized mainly into type I, IIa, IIx or IIb, which differ both by the predominant type of contractile myosin isoform/s expressed, as well as the primary mode of in situ metabolism for ATP supply [16]. The remarkable diversity in the work capacity, endurance or fatigability of different vertebrate skeletal muscles arises in part from the differential distribution of fiber types in the individual muscles. It is the dynamic shift in these muscle fiber types in response to diverse functional and contractile demands such as that seen with aging and/or exercise[17], physical inactivity [18] and metabolic conditions [19], that underscores skeletal muscle plasticity. The biceps and gastrocnemius muscles in rodents predominantly consist of type II fibers expressing MHCIIa, MHCIIx or MHCIIb [20]. Soleus muscle however have a much higher percentage of MHCI type and MHCIIa, and therefore have lower contractile velocity and higher oxidative metabolism. Our mRNA expression analysis indicate that myosin isoform expression in KO mice lean towards predominantly slow myosin isoforms expression in all muscles tested, and increased MHCIIa protein expression in gastrocnemius and biceps further supports the emergence of a slow phenotype. Transcriptome data from KO soleus muscles further suggest that myosin isoform switching effect of Kvβ2 deletion as more MHCI expression was noted while MHCIIb isoform expression decreased in KO mice as compared to Wt mice. Immunohistochemistry data confirmed that the expression changes in mRNA and proteins translated into increased proportion of type 1 fiber in hind limb muscles, supporting the idea that Kvβ2 deletion promotes slow-oxidative muscle phenotype. Taken together, these data clearly show that Kvβ2 differentially regulates the muscle fiber type in the three major muscles: gastrocnemius, biceps and soleus. Further support that Kvβ2 deletion promotes the emergence of increased slow fiber phenotype can be conferred from smaller cross sectional area of myofibers as well as increased frequency of smaller fibers in hind limb muscle in KO mice, as oxidative fibers are generally considered smaller than glycolytic type 2 fibers[21]. Hence, we find that Kvβ2 deletion leads to a decrease in muscle size and a shift to predominantly slow fiber types in hind limb muscles, indicating that Kvβ2 inhibition may be a key regulator of fiber type maintenance in skeletal muscle, and that deletion may promote oxidative phenotype.

Calcineurin is considered a key regulator of postnatal skeletal muscle phenotype [22]. Because Kvβ2 deletion has resulted in significantly increased slow myosin isoform expression, we assessed the expression of Calcineurin in hind limb muscles and identified increased Calcineurin expression both in gastrocnemius and biceps of KO mice, suggesting that the enhanced slow fiber phenotype may be in part mediated through activation of Calcineurin

signal transduction. Past studies support this inference because activation of Calcineurin signaling was shown to increase transcriptional activity of slow fiber-specific gene promoters both in C2C12 cells [23] as well as in mouse skeletal muscle [24]. Further, transgenic overexpression of Calcineurin was also shown to enhance slow myofiber numbers [24], while the pharmacological inhibition of Calcineurin by cyclosporine A increased fast (type 2) fibers, in mice [23]. Moreover, Calcineurin has been indicated in fiber-type switching in skeletal muscle in response to conditional stimuli such as mechanical overloading or endurance exercise [25]. Hence, it is plausible that increased expression of Calcineurin, which is a calcium-dependent calmodulin binding serine/protein phosphatase, may in part explain the role of Kvβ2 in myofiber specification. The present study identifies the influence of Kvβ2 in calcineurin expression in muscle, however the causal role remains unclear.

The ubiquitin ligase NEDD4 (Neural precursor cell expressed developmentally down-regulated protein 4) was previously shown to positively regulate skeletal muscle weight after denervation by preventing and delaying atrophy [26]. In the present study we identify that in Kvβ2 KO skeletal muscle there is decreased muscle size compared with wild type. Therefore we asked the question if Nedd4 plays a role in the decreased muscle size in Kvβ2 KO skeletal muscle. Deletion of Kvβ2 leads to increased expression of Nedd4 which is likely a compensatory mechanism in response to genetic deletion of Kvβ2 in the mouse skeletal muscle. The post-translational regulation of Kvβ2-Nedd4 based interactions was evaluated by studying the protein-protein based interactions. Based on our hypothesis that Kvβ2 plays a physiological role in modulation of skeletal muscle growth, differentiation and proliferation, we evaluated the potential role of Nedd4 based regulation for Kvβ2 and performed the pull down assay to identify novel interaction between Kvβ2 and Nedd4 in the skeletal muscle. We investigated the binding of Kvβ2 with Nedd4 and identified that Kvβ2 displays tight binding to Nedd4 in skeletal muscle suggesting that in wild type the Kvβ2 helps regulate the Nedd4 mediated muscle size. Based on our findings we clearly identify increased Nedd4 expression and strong interactions between Kvβ2-Nedd4, these interactions are conceptually novel and point to the idea that Kvβ2-Nedd4 based interactions may be relevant for neuromuscular activity for influencing the size and growth of muscles. Although, we identified the link for Kvβ2 in skeletal muscle growth, differentiation and fiber type changes mediated due to its interactions with Nedd4 and affecting Calcineurin and Pax7 expression, the present study however does not evaluate the mechanisms for these associations. It is well established that Pax7 plays a key role in skeletal myogenesis [27]. We identified in this study that both Kvβ2 and Pax7 are coregulated in myoblasts and that deletion of Kvβ2 leads to decrease in Pax7 expression in gastrocnemius muscle, suggesting that Kvβ2 may have a stabilizing or potentiating roles on Pax7 expression and satellite cell determination. Moreover, past evidence shows that Pax7 binds to Nedd4, which results in Pax7 downregulation[28]. Overall in the present study, we identified an increase in Nedd4 expression along with its binding to Kvβ2 suggesting that Pax7-Nedd4-Kvβ2 are co-regulated in skeletal muscle leading to decreased muscle size with altered fiber type in the absence of Kvβ2.

Conclusion

In conclusion, this is the first report highlighting the physiological importance of Kvβ2 in skeletal muscle. Using a combination of immunohistochemistry, protein-protein interaction and molecular biology techniques we identified the key partners of Kvβ2 and the links for smaller muscle size. The present study provides a link between isoform switching from fast to slow fiber type in which the interactions are likely mediated by Kvβ2 and Nedd4. Deletion of Kvβ2 further resulted in decreased Pax7 expression in young skeletal muscle. In addition, Kvβ2 binds with Nedd4, and Kvβ2 deletion leads to enhanced Nedd4 expression in adult muscle fibers. In vitro studies in C2C12 suggested that Pax7 co-regulates with Kvβ2, and that knockdown of Kvβ2 in C2C12 cells decreases recovery from injury, suggesting compromised migration of myoblasts.

4.0 MATERIALS AND METHODS

4.1 Animals

Age matched, littermate wild type (Wt) or Kvβ2 knockout (KO), male mice from at least two or more litters were randomly chosen for the present study. Hemizygous ($Kv\beta2^{+/-}$) KO mice were obtained from Dr. Geoffrey Murphy at the University of Michigan and then KO mice were generated as described previously [29]. Use of animals in research and experimental protocol were approved by the Institutional Animal Care and Use Committee at the University of South Florida (Tampa, FL, USA), in accordance with the US National Institutes of Health guidelines. Mice were maintained under 12h:12h; day: night cycle, food and water were supplied *ad libitum*. Kvβ2 deletion in KO mice was confirmed by probing for the expression of Kvβ2 protein in gastrocnemius protein lysates using an anti-Kvβ2 antibody (NeuroMab, Davis, CA).

4.2 Body weights and Histo-morphological analysis

Body weights of 6, 12 and 16 week old Wt and KO mice were determined, and tibial length was assessed in 16 weeks old mice at necropsy. Biceps femoris (Biceps), gastrocnemius (Gas) and soleus (Sol) muscles were collected from each mouse, and after removing any adjoining fat and fibrous tissue, muscle weights were recorded. Subsequently, muscle tissue were frozen in isopentane bath immersed in liquid N_2, and stored at −80°C until preparing cryosections (15 μm) using a cryostat (Microm HM505 E, Walldorf, Germany). Gross histological examination of muscle cross-sections was conducted by performing Hematoxylin and Eosin (H & E) staining. Images were acquired using Olympus IX73 light microscope. Muscle fiber cross-sectional area (CSA) of nearly 100 random myofibers was determined from at least 3 serial sections of each sample using Image J software. Additionally, fiber size distribution as a factor of CSA (arbitrary units) was compared between the Wt and KO muscles.

4.3 Quantitative Real-Time-PCR (qRT-PCR)

Gene expression analysis was performed following our previously published procedures[30]. Total RNA was isolated from Wt and KO skeletal muscle tissue by using the Exiqon miRCURY RNA Isolation kit (Exiqon, Woburn, MA), following the manufacturers protocol.

RNA concentration was determined by utilizing the NanoDrop ND-1000 UV-Vis Spectrophotometer (ThermoScientific Inc, USA). Employing iScript cDNA Synthesis Kit (BioRad), 1μg of total RNA was converted into complimentary DNA (cDNA). Using gene specific primers[31], qRT-PCR analysis was performed using iTaq universal SYBR green supermix and a CFX96 system (Bio-Rad, Hercules, CA). Hypoxanthine Phosphoribosyl transferase (HPRT) was used an endogenous reference because it was similar between groups in vitro and muscle tissue. CT (cycle threshold) values between Wt/control and KO/siRNA demonstrated <1% difference. HPRT expression between C2C12 (control) and siRNA Kvβ2 were 19.99 vs. 19.81, respectively. For all the candidate genes (Table 1), the quantities of the mRNA expression relative to HPRT mRNA levels were obtained. Data was normalized to that of Wt and expressed as mean (fold) ± SEM.

4.4 Western Blot analysis

Protein expression was assessed by Western blotting as previously published[30]. Skeletal muscle; Biceps femoris, Gastrocnemius, Soleus were isolated and homogenized in 1:10 volumes of T-per protein extraction reagent (ThermoScientific, USA) supplemented with 10 mM DDT, protease inhibitor (1:100, Sigma-Aldrich, St. Louis, MO), and phosphatase inhibitor cocktail II (1:100, Sigma-Aldrich). Protein was extracted on ice for 10 min by frequent mixing of the sample. Tissue lysate was then centrifuged at 10,000x g for 10 minutes at 4°C, and the supernatant collected. Protein quantification was performed using the Pierce 660 assay (Thermo Fisher Scientific, Waltham, MA). Approximately, 25–50 μg of protein was loaded into each well and resolved on an appropriate SDS-PAGE gel (Bio-Rad Laboratories). Proteins were transferred overnight onto a PVDF membrane at a constant voltage of 30 V. Successful transfer of proteins was confirmed by Poncoeu-S stain. Blots were washed in 1x Tris buffered saline containing 0.1% Tween-20 (TTBS), blocked for 1 hour at room temperature in 1xTTBS containing 5% non-fat milk, and probed with anti-Kvβ2 (Antibodies Incorporated), anti-Myh1 (SantaCruz), anti-Myh7 (Sigma-Aldrich), anti-calicneurin A (Abcam), anti-NEDD4 (BD Biosciences), antibodies following our previously reported protocols [30]. GAPDH was used as loading control for quantification. Relative protein expression was quantified as Ratio of wild type and Knockout for target protein.

4.5 Cell culture and transfections

C2C12 cells were purchased from American Tissue and Cell culture; ATCC (Manassas, VA, USA). Cell culture was carried following our pervious published protocols[30]. Cells were cultured in DMEM medium containing 10% fetal bovine sera and 1% penicillin and Streptomycin. For gene targeting experiments, cells were grown to 60% confluence in 6-well plates. SiRNA-targeted knockdown of Kvβ2 was accomplished by applying 20 nM Kvβ2 siRNA (Origene, Rockville, MD, USA) diluted in silentFect reagent (BioRad). Control group of cells were transfected with scramble siRNA (SantaCruz, USA). Differentiation of C2C12 cells was initiated when the cells were 90% confluent by adding the DMEM media supplemented with 2% horse serum, 1% penicillin and Streptomycin. Cell proliferation assay was conducted using 5-bromo-2′-deoxyuridine (BrdU), following manufacturer's instructions (Thermofisher Scientific, USA). After 72 hours of transfection with either scrambled siRNA control (Santa Cruz) or Kvβ2 siRNA (Origene), BrdU solution (10μM) was added to cells and incubated for 4 hours. Following fixation, permeabilization and

washing of the cells, BrdU inclusion into the new DNA was detected by immunofluorescence. Coverslips were mounted with ProLong® Gold Antifade Mountant containing DAPI (Molecular Probe, USA), and images were acquired by employing Fluorescent microscopy. Scratch assay was conducted in 35 mm cell culture plates with control or Kvβ2 siRNA transfected C2C12 cells at 80% confluence. Scratch injury was performed by using a sterile pipette tip at the center of the cell culture plate and images were acquired at 0 and 12 hour time points. The area not covered by cell growth was quantified by Image J analysis and cell migration index as % (percentage) was assessed using the ratio of area un-occupied by cells (12 hour time point)/ initial open area (0 hour).

4.6 Pull-down assay

The interaction of Kvβ2 and Nedd4 within the skeletal muscle was conducted utilizing a pull-down assay with gastrocnemius tissue lysate. The protocol for the use of DDK-coated Agarose beads for pull-down assay has been described in detail in previous research [12]. Briefly COS-7 cells were transiently transfected with a DDK-tagged Kvβ2 plasmid (Origene, Rockville, MD, USA) of which cellular protein extracts were utilized. COS-7 DDK-tagged Kvβ2 protein extracts were incubated with anti-DDK agarose beads (Origene) for 3 hrs. at 4°C, followed by addition of gastrocnemius protein extracts and incubated overnight at 4°C. Bound proteins were eluted and immunoblot analysis was performed using Nedd4 antibody (1:2000) and Kvβ2 antibody (1:100). To further validate the interaction of Kvβ2 and Nedd4 an immunoprecipitate (IP) pull-down was performed utilizing the Kvβ2 antibody. Briefly SureBeads G (BioRad) were allocated and washed with PBS (x3) and incubated with Kvβ2 antibody (40μg) for 4 hrs. at 4°C. Excess antibody was removed and coated beads were washed with PBS (x3) followed by addition of gastrocnemius protein extracts incubated overnight at 4°C. Bound proteins were eluted and immunoblot analysis was performed using Nedd4 antibody (1:2000).

4.7 Immunohistochemistry, confocal microscopy and quantification

The frozen muscles in isopentane from soleus and gastrocnemius were sectioned into 15 μm thickness and fixed into 4% formaldehyde. The sections were blocked with SuperBlock™ T20 (TBS) blocking buffer (Thermo scientific, USA) at room temperature for 30 min and incubated with Nedd4 antibody (1:50, BD Biosciences, USA) and monoclonal anti-myosin (skeletal, slow) (1:100, Sigma, USA). Alexa fluor 568 (Invitrogen, USA) fluorescent dye conjugated to goat anti-mouse IgG was used as secondary antibody for visualization, and slides were mounted with ProLong® Gold Antifade Mountant containing DAPI (Molecular Probe, USA). Nuclei were labeled by DAPI. Images were visualized and captured with a confocal multiphoton laser scanning microscope (Olympus FV1000 MPE, Olympus America Inc., USA). Negative control group was utilized to identify the background staining and reactivity. Images were quantified and fibrous cross sectional areas were calculated using Image J software.

4.8 Statistical analysis

Data were reported as mean ± SEM. Average mean differences between the groups were compared by unpaired Student's t-test. A value of $p \leq 0.05$ was considered statistically significant.

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References

1. Janssen I, Heymsfield SB, Wang ZM, Ross R. Skeletal muscle mass and distribution in 468 men and women aged 18–88 yr. J Appl Physiol (1985). 2000; 89(1):81–88. [PubMed: 10904038]
2. Brotto M, Abreu EL. Sarcopenia: pharmacology of today and tomorrow. J Pharmacol Exp Ther. 2012; 343(3):540–546. [PubMed: 22929991]
3. Frontera WR, Ochala J. Skeletal Muscle: A Brief Review of Structure and Function. Calcified Tissue International. 2015; 96(3):183–195. [PubMed: 25294644]
4. Sakuma K, Aoi W, Yamaguchi A. Molecular mechanism of sarcopenia and cachexia: recent research advances. Pflügers Archiv - European Journal of Physiology. 2017:1–19.
5. Tur J, Chapalamadugu KC, Katnik C, Cuevas J, Bhatnagar A, Tipparaju SM. Kvbeta1.1 senses pyridine nucleotide changes in the mouse heart and modulates cardiac electrical activity. American journal of physiology Heart and circulatory physiology. 2016 ajpheart.00281.02016.
6. Swain SM, Sahoo N, Dennhardt S, Schönherr R, Heinemann SH. Ca(2+)/calmodulin regulates Kvβ1.1-mediated inactivation of voltage-gated K(+) channels. Scientific Reports. 2015; 5:15509. [PubMed: 26487174]
7. Kuryshev YA, Wible BA, Gudz TI, Ramirez AN, Brown AM. KChAP/Kvbeta1.2 interactions and their effects on cardiac Kv channel expression. American journal of physiology Cell physiology. 2001; 281(1):C290–299. [PubMed: 11401852]
8. Persaud A, Alberts P, Amsen EM, et al. Comparison of substrate specificity of the ubiquitin ligases Nedd4 and Nedd4-2 using proteome arrays. Molecular Systems Biology. 2009; 5:333–333. [PubMed: 19953087]
9. Grande M, Suàrez E, Vicente R, et al. Voltage-dependent K+ channel β subunits in muscle: Differential regulation during postnatal development and myogenesis. Journal of Cellular Physiology. 2003; 195(2):187–193. [PubMed: 12652645]
10. Pongs O, Schwarz JR. Ancillary Subunits Associated With Voltage-Dependent K^+ Channels. Physiological Reviews. 2010; 90(2):755–796. [PubMed: 20393197]
11. Villalonga N, Martínez-Mármol R, Roura-Ferrer M, et al. Cell cycle-dependent expression of Kv1.5 is involved in myoblast proliferation. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 2008; 1783(5):728–736. [PubMed: 18230363]
12. Tur J, Chapalamadugu KC, Padawer T, et al. Deletion of Kvbeta1.1 subunit leads to electrical and haemodynamic changes causing cardiac hypertrophy in female murine hearts. Experimental physiology. 2016; 101(4):494–508. [PubMed: 27038296]
13. Tipparaju SM, Li X-P, Kilfoil PJ, et al. Interactions between the C-terminus of Kv1.5 and Kvβ regulate pyridine nucleotide-dependent changes in channel gating. Pflugers Archiv. 2012; 463(6): 799–818. [PubMed: 22426702]
14. Schiaffino S, Dyar KA, Ciciliot S, Blaauw B, Sandri M. Mechanisms regulating skeletal muscle growth and atrophy. The FEBS journal. 2013; 280(17):4294–4314. [PubMed: 23517348]
15. Olguin HC, Yang Z, Tapscott SJ, Olwin BB. Reciprocal inhibition between Pax7 and muscle regulatory factors modulates myogenic cell fate determination. The Journal of Cell Biology. 2007; 177(5):769–779. [PubMed: 17548510]
16. Schiaffino S, Reggiani C. Fiber types in mammalian skeletal muscles. Physiol Rev. 2011; 91(4): 1447–1531. [PubMed: 22013216]
17. Mosole S, Carraro U, Kern H, et al. Long-Term High-Level Exercise Promotes Muscle Reinnervation With Age. Journal of Neuropathology & Experimental Neurology. 2014; 73(4):284–294. [PubMed: 24607961]
18. Borina E, Pellegrino MA, D'Antona G, Bottinelli R. Myosin and actin content of human skeletal muscle fibers following 35 days bed rest. Scandinavian Journal of Medicine & Science in Sports. 2010; 20(1):65–73. [PubMed: 19883388]

19. Hyatt JP, Nguyen L, Hall AE, et al. Muscle-Specific Myosin Heavy Chain Shifts in Response to a Long-Term High Fat/High Sugar Diet and Resveratrol Treatment in Nonhuman Primates. Front Physiol. 2016; 7:77. [PubMed: 26973542]
20. Quiat D, Voelker KA, Pei J, et al. Concerted regulation of myofiber-specific gene expression and muscle performance by the transcriptional repressor Sox6. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108(25):10196–10201. [PubMed: 21633012]
21. van Wessel T, de Haan A, van der Laarse WJ, Jaspers RT. The muscle fiber type–fiber size paradox: hypertrophy or oxidative metabolism? European Journal of Applied Physiology. 2010; 110(4):665–694. [PubMed: 20602111]
22. Hudson MB, Price SR. Calcineurin: a poorly understood regulator of muscle mass. The international journal of biochemistry & cell biology. 2013; 45(10):2173–2178. [PubMed: 23838168]
23. Chin ER, Olson EN, Richardson JA, et al. A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. Genes & Development. 1998; 12(16):2499–2509. [PubMed: 9716403]
24. Naya FJ, Mercer B, Shelton J, Richardson JA, Williams RS, Olson EN. Stimulation of Slow Skeletal Muscle Fiber Gene Expression by Calcineurin in Vivo. Journal of Biological Chemistry. 2000; 275(7):4545–4548. [PubMed: 10671477]
25. Parsons SA, Millay DP, Wilkins BJ, et al. Genetic Loss of Calcineurin Blocks Mechanical Overload-induced Skeletal Muscle Fiber Type Switching but Not Hypertrophy. Journal of Biological Chemistry. 2004; 279(25):26192–26200. [PubMed: 15082723]
26. Nagpal P, Plant PJ, Correa J, et al. The Ubiquitin Ligase Nedd4-1 Participates in Denervation-Induced Skeletal Muscle Atrophy in Mice. PLOS ONE. 2012; 7(10):e46427. [PubMed: 23110050]
27. Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA. Pax7 is required for the specification of myogenic satellite cells. Cell. 2000; 102(6):777–786. [PubMed: 11030621]
28. Bustos F, de la Vega E, Cabezas F, et al. NEDD4 Regulates PAX7 Levels Promoting Activation of the Differentiation Program in Skeletal Muscle Precursors. Stem cells (Dayton, Ohio). 2015; 33(10):3138–3151.
29. McCormack K, Connor JX, Zhou L, et al. Genetic analysis of the mammalian K+ channel beta subunit Kvbeta 2 (Kcnab2). The Journal of biological chemistry. 2002; 277(15):13219–13228. [PubMed: 11825900]
30. Chapalamadugu KC, Panguluri SK, Bennett ES, Kolliputi N, Tipparaju SM. High level of oxygen treatment causes cardiotoxicity with arrhythmias and redox modulation. Toxicology and applied pharmacology. 2015; 282(1):100–107. [PubMed: 25447406]
31. Burch N, Arnold AS, Item F, et al. Electric pulse stimulation of cultured murine muscle cells reproduces gene expression changes of trained mouse muscle. PLOS ONE. 2010; 5(6):e10970. [PubMed: 20532042]
32. Wen Y, Bi P, Liu W, Asakura A, Keller C, Kuang S. Constitutive Notch activation upregulates Pax7 and promotes the self-renewal of skeletal muscle satellite cells. Mol Cell Biol. 2012; 32(12):2300–2311. [PubMed: 22493066]
33. Gündogdu MS, Liu H, Metzdorf D, et al. The haematopoietic GTPase RhoH modulates IL3 signalling through regulation of STAT activity and IL3 receptor expression. Molecular Cancer. 2010; 9(1):225. [PubMed: 20738848]

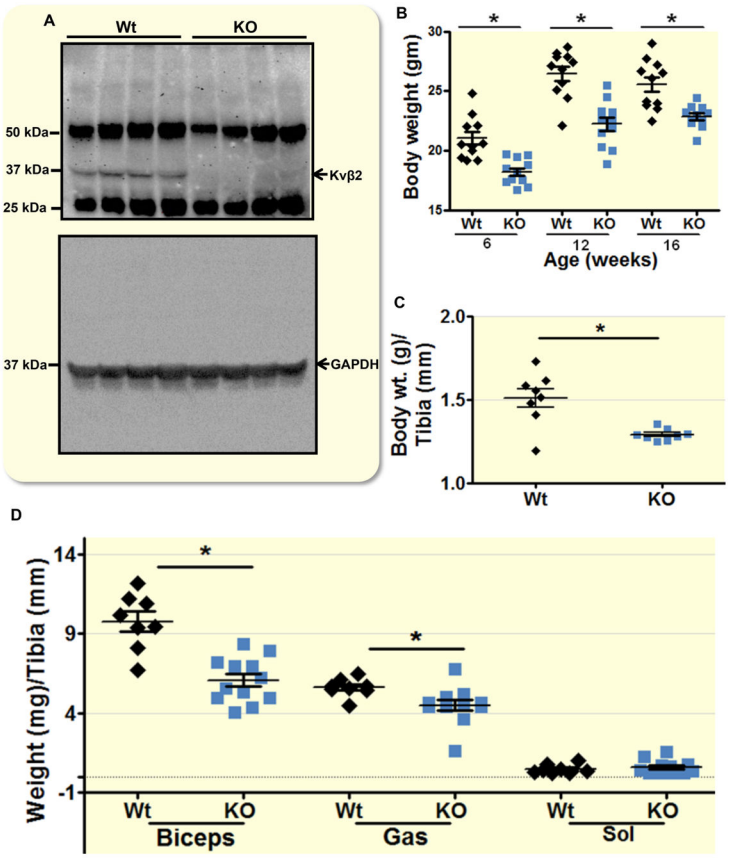

Figure 1. Genotypic and phenotypic analysis of Kvβ2 knockout (KO) mice

A) Western blot analysis of KO skeletal muscle showing complete absence of Kvβ2 expression, n=4 drawn from different mouse litter, B) comparison of wild type or knockout mouse body weights from 6, 12 or 16 week old mice (n=11) each. C) Age matched, body weights of 16 week old mice normalized to tibia length Wt and KO (*n=8*) mice and D) weights of biceps femoris, gastrocnemius (Gas) and soleus (Sol) muscles normalized to tibia length in Wt (*n=8*) and KO (*n=12*) mice. Dot plot represent mean ± SEM. Significant mean differences between groups were identified by a Student's t-test, and a $p \leq 0.05$ was considered statistically significant.

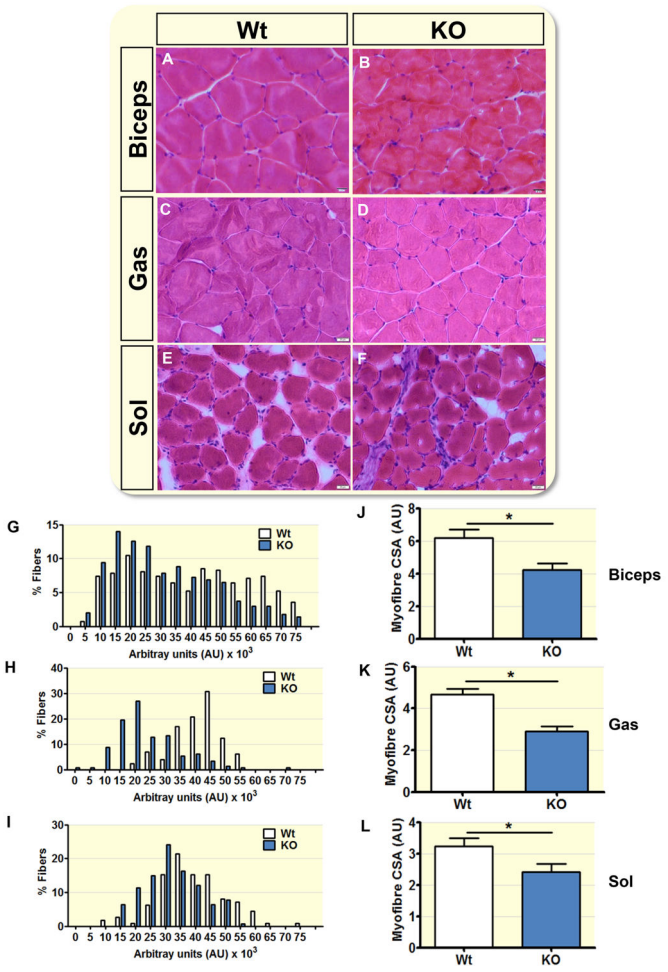

Figure 2. Skeletal muscle phenotype in Wt and Kvβ2 knockout (KO) mice

Representative images of Hematoxylin and Eosin (H & E) stained cross-sections of A–B) biceps femoris, C–D) gastrocnemius (Gas) and E–F) soleus (Sol) muscles. Myofiber % (fiber size distribution as a % of total counted fibers, arbitrary units, AU) and cross-sectional area (arbitrary units, AU) were measured, G, J) biceps femoris, H, K) gastrocnemius and I, L) soleus, respectively, using image J software. Scale Bar = 20μm. Bars represent mean ± SEM, *n=6*. Significant mean (AU) differences in cross-sectional area (CSA) between Wt and KO mouse were identified by a t-test, $p \leq 0.05$.

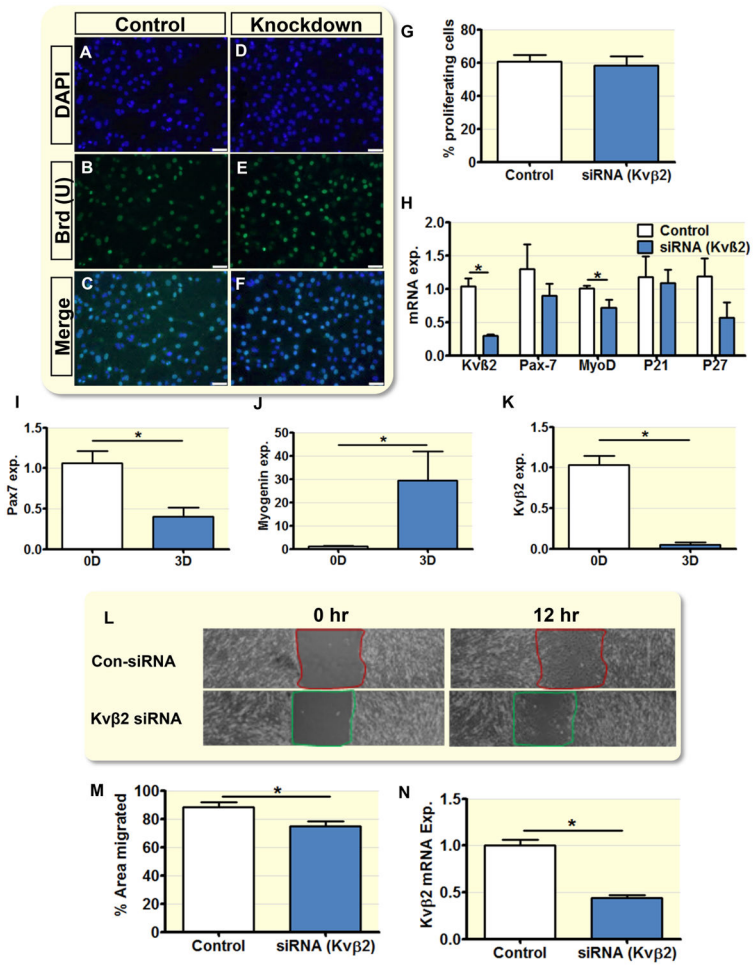

Figure 3. Kvβ2 in C2C12 myogenesis

Kvβ2 was knocked-down in C2C12 cells and Brd(U)-DAPI staining was conducted, A–C) control siRNA, D–F) Kvβ2 siRNA and G) cell proliferation was assessed, x 20 magnification, Scale Bar = 50μm. H) Knockdown of Kvβ2 by siRNA treatment was confirmed by Kvβ2 mRNA expression by qRT-PCR. Transcript expression levels of Pax7, MyoD and P21 and P27 were also confirmed. C2C12 cells were differentiated for 3 days and temporal mRNA expression pattern of genes is presented for I) Pax7, J) Myogenin, K) Kvβ2. L) Representative images for C2C12 cells with area injured at 0 and 12 hour point using control siRNA or Kvβ2 knockdown. M) and N) show the quantification of area migrated in 12 hours and Kvβ2 expression in C2C12 cells, respectively. Mean differences between the two groups were identified by Students' t-test, *n=6*, $p \leq 0.05$.

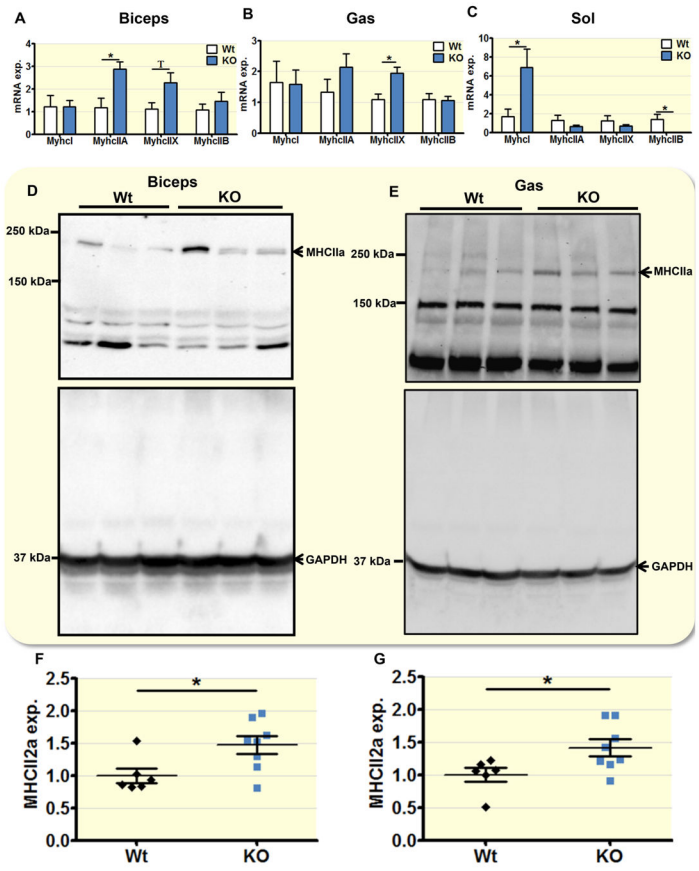

Figure 4. mRNA expression of myosin isoform in skeletal muscles

In age-matched, 12 week old, wild type (Wt) and Kvβ2 knockout (KO) mouse, tissue expression of MHCI, MHCIIa, MHCIIx and MHCII2b mRNA levels were measured. A) Biceps, B) Gastrocnemius (Gas) and C) soleus (Sol), *n=6*; D) and E) Protein levels of MHCIIa were measured in biceps and gastrocnemius (Gas) using immunoblotting, respectively. F) and G) show the quantification (Mean ± SEM) of MHCIIa expression in biceps and Gas after normalization to GAPDH expression, *n=6.* Significant mean difference was identified by Students t-test, $p \leq 0.05$.

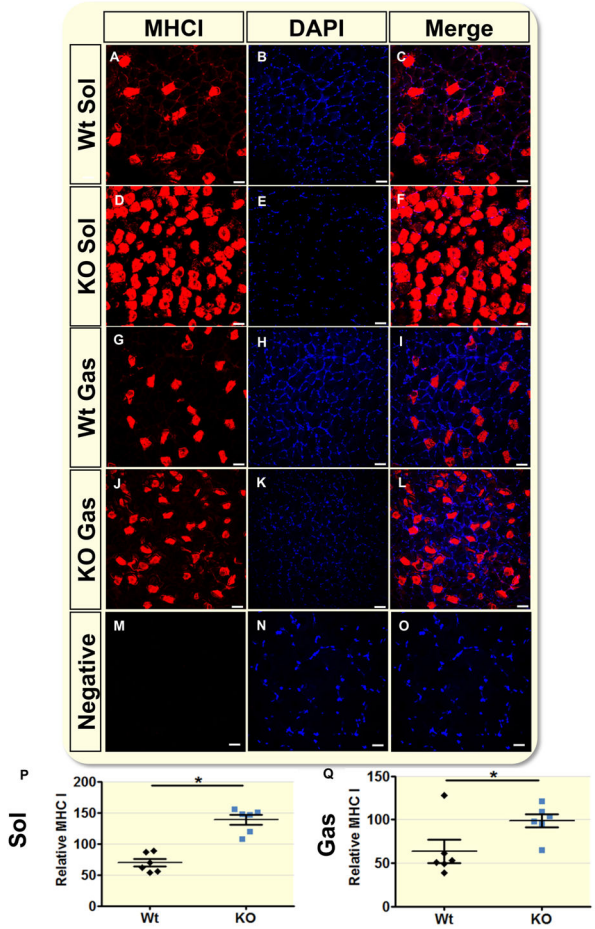

Figure 5. Immunohistochemistry of MHC I and Kvβ2 in hind limb muscle

MHC I protein localized in sarcolemmal region of soleus and gastrocnemius region. Tissue expression and cellular distribution of MHC I was assessed in soleus (A–F) and gastrocnemius (G–L) muscle cross sections (15 μm) of wild type (Wt) or Kvβ2 Knockout (KO) mice. Negative controls (M–O) using secondary antibody for gastrocnemius muscle from wild type skeletal muscle. Data are expressed as means±SEM; $n = 6$/group in soleus (P) and gastrocnemius (Q). * $p < 0.05$ vs. Kvβ2 knockout (KO) mice. Scale bar = 50μm, magnification x 20.

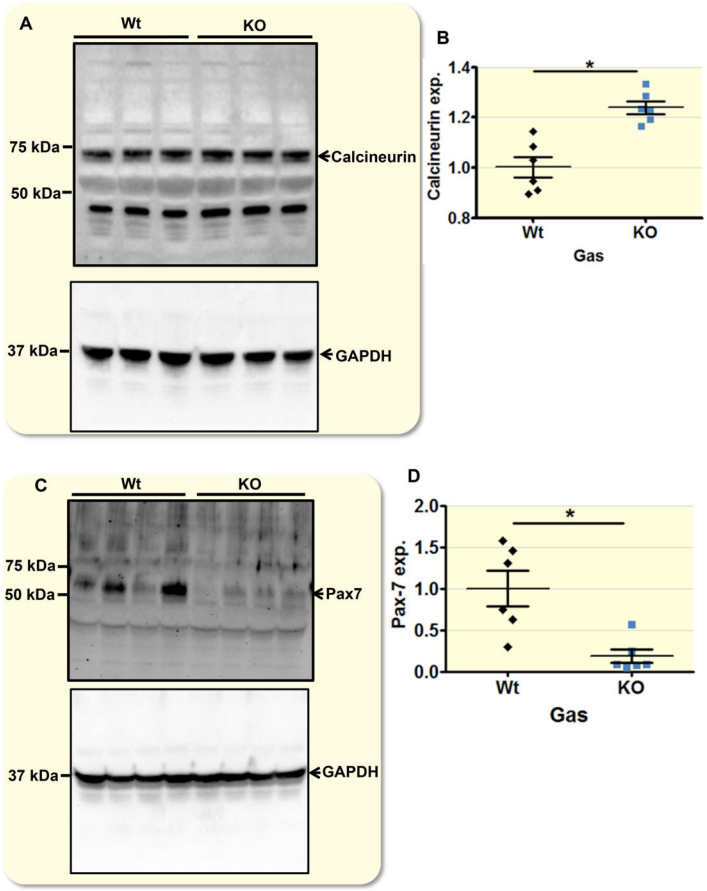

Figure 6. Calcineurin and Pax7 expression in skeletal muscles

Tissue expression of Calcineurin A was measured in the skeletal muscles of 4 week old age-matched wild type (Wt) and Kvβ2 knockout (KO) mouse: A–B) Gastrocnemius; Gas (*n=6*) Data are expressed as means±SEM and C–D) Pax7 expression was measured in Gastrocnemius; Gas (*n=6*). Data are expressed as means±SEM. Significant mean differences between the groups were identified by Students's t-test, $p \leq 0.05$.

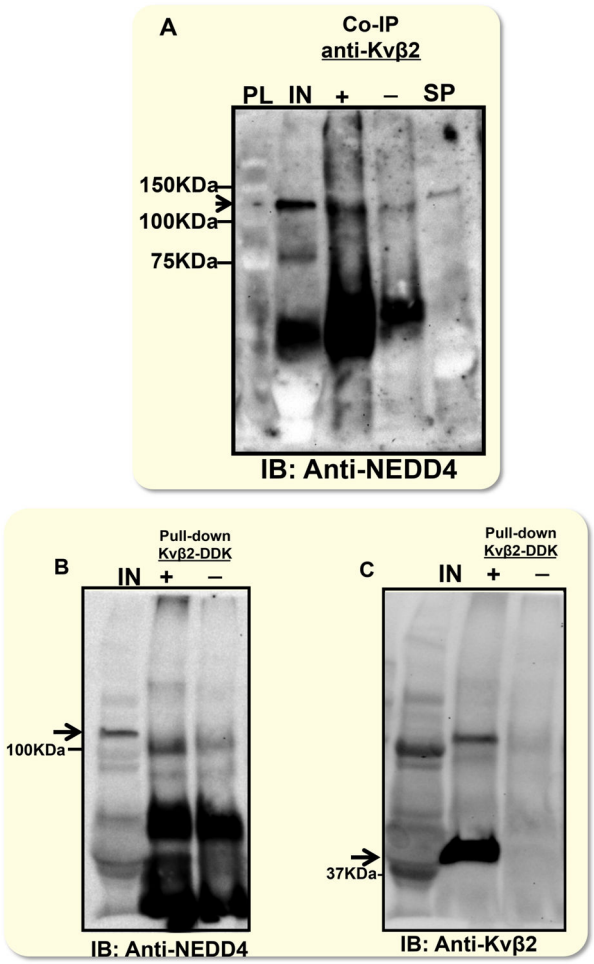

Figure 7. Kvβ2 Pull down

A) Western blot image of Co-IP. Wt *gastrocnemius* muscle with IgG magnetic beads coated with Kvβ2 antibody. PL is protein ladder followed by lane 1 (IN-input) is *gastrocnemius* muscle; lane 2 (+) is *gastrocnemius* muscle co-incubated with IgG beads coated with Kvβ2 antibody (40μg) overnight; Lane 3 (−) is *gastrocnemius* muscle co-incubated with IgG beads for 16 hours. SP is supernatant from (−) lane. Nedd4 primary antibody (1:1000 dilution) was incubated overnight with blot and a ~115 kDa band was noted in lane 1 and lane 2, however was absent in lane 3. A total of 3 separate Co-IP experiments were performed using gastrocnemius muscles from different mice. B) Western blot image of WT *gastrocnemius* muscle and Cos-7 cells transfected with Kvβ2-DDK plasmid. Lane 1 (IN-input) is *gastrocnemius* muscle; lane 2 (+) is *gastrocnemius* muscle co-incubated with Kvβ2-DDK homogenate from Cos-7 cells transfected with Kvβ2-DDK with DDK-coated agarose beads overnight; Lane 3 (−) is *gastrocnemius* muscle co-incubated with solely DDK-coated agarose beads overnight. Nedd4 primary antibody (1:1000 dilution) was incubated overnight with blot and a ~115 kDa band was noted in lane 1 and lane 2, however was absent in lane 3. C) Western blot image of WT *gastrocnemius* muscle and Cos-7 cells transfected with Kvβ2-DDK plasmid. Lane 1 (IN-input) is *gastrocnemius* muscle; lane 2 (+) is *gastrocnemius* muscle co-incubated with Kvβ2-DDK homogenate from Cos-7 cells transfected with Kvβ2-

DDK with DDK-coated agarose beads overnight; Lane 3 (−) is *gastrocnemius* muscle co-incubated with DDK-coated agarose beads overnight. Kvβ2 primary antibody (1:100 dilution) was incubated for 16 hours with blot and a 40 kDa band was noted in lane 1 and lane 2, however was absent in lane 3. For identifying protein-protein interactions, 3 separate experiments each were performed for co-immunoprecipitation or 3 pull down assays with a total of 12 mice for reproducibility.

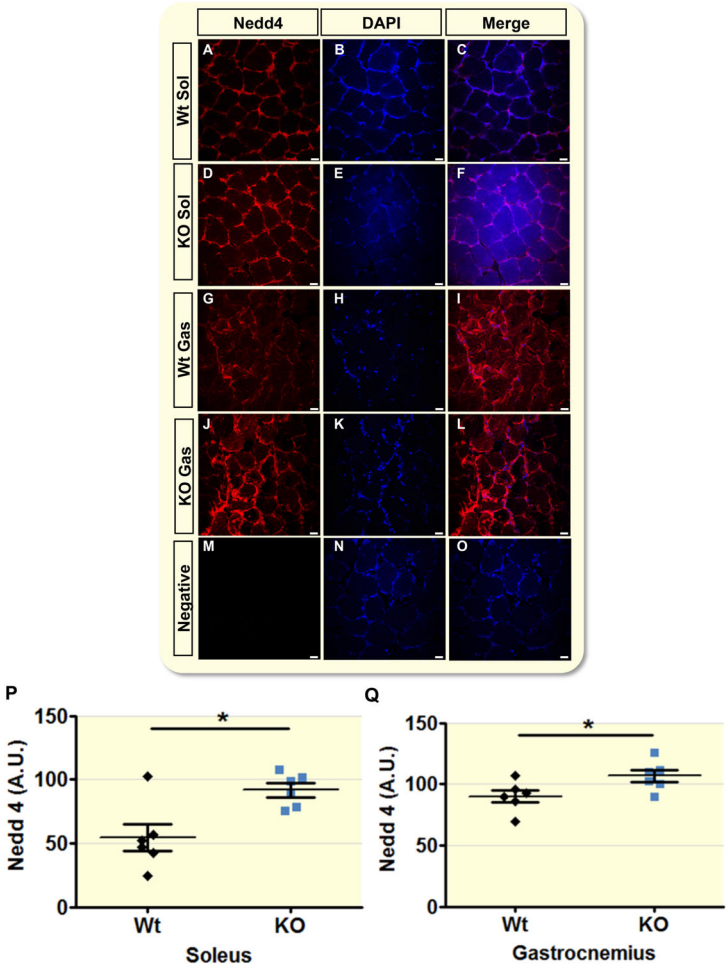

Figure 8. Immunohistochemistry of Nedd4 in soleus and gastrocnemius muscle

Nedd4 protein localized in sarcolemma region of soleus and gastrocnemius region. Tissue expression and cellular distribution of Nedd4 was assessed in soleus (A–F) and gastrocnemius (G–L) muscle cross sections (15 μm) of Wt or Kvβ2 knockout (KO) mice. Negative controls (M–O) using secondary antibody for gastrocnemius muscle from wild type skeletal muscle. Data are expressed as means±SEM; *n*= 6/group in soleus (P) and gastrocnemius (Q). * p <0.05 vs. Kvβ2 knockout (KO) mice. Scale bar = 50μm and magnification x 40.

Table 1

Primer sequences used for qRT-PCR

Gene	Primers	Sequence (5′-3′)
Pax7 [32]	Forward	CTGCTGAAGGACGGTCAC TG
	Reverse	GGA TGC CAT CGA TGC TGT GT
MyoD [32]	Forward	GGCTACGACACCGCCTACTA
	Reverse	CGA CTC TGG TGG TGC ATC TG
Myogenin [32]	Forward	TGCCCAGTGAATGCAACTCC
	Reverse	TTGGGCATGGTTTCGTCTGG
P21^{Cip1} [33]	Forward	GCAGACCAGCCTGAC
	Reverse	GCAGGCAGCGTATATACAGGA
P27^{kip1} [33]	Forward	AGG GCC AAC AGA ACA GAA GA
	Reverse	CTCCTGGCAGGCAACTAATC
MHCI [31]	Forward	CCTTGGCACCAATGTCCCGGCTC
	Reverse	GAAGCGCAATGCAGAGTCGGTG
MHCIIa [31]	Forward	ATGAGCTCCGACGCCGAG
	Reverse	TCTGTTAGCATGAACTGGTAGGCG
MHCIIx [31]	Forward	AAGGAGCAGGACACCAGCGCCCA
	Reverse	ATCTCTTTGGTCACTTTCCTGCT
MHCIIb [31]	Forward	GTGATTTCTCCTGTCACCTCTC
	Reverse	GGAGGACCGCAAGAACGTGCTGA