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RESEARCH ARTICLE

Raptor is critical for increasing the mitochondrial proteome and skeletal muscle force during hypertrophy

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Abstract

Loss of skeletal muscle mass and force is of critical importance in numerous pathologies, like age-related sarcopenia or cancer. It has been shown that the Akt-mTORC1 pathway is critical for stimulating adult muscle mass and function, however, it is unknown if mTORC1 is the only mediator downstream of Akt and which intracellular processes are required for functional muscle growth. Here, we show that loss of Raptor reduces muscle hypertrophy after Akt activation and completely prevents increases in muscle force. Interestingly, the residual hypertrophy after Raptor deletion can be completely prevented by administration of the mTORC1 inhibitor rapamycin. Using a quantitative proteomics approach we find that loss of Raptor affects the increases in mitochondrial proteins, while rapamycin mainly affects ribosomal proteins. Taken together, these results suggest that mTORC1 is the key mediator of Akt-dependent muscle growth and its regulation of the mitochondrial proteome is critical for increasing muscle force.

KEYWORDS
hypertrophy, mitochondria, mTOR, rapamycin, Raptor, skeletal muscle

Abbreviations: 4E-BP1, Eukaryotic translation initiation factor 4E-binding protein 1; AAV, Adeno Associated Virus; CAD, carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, dihydroorotase; CSA, cross sectional area; FKBP12, FK506-binding protein; GSK-3β, glycogen synthase kinase 3β; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; mTORC2, mammalian target of rapamycin complex 2; RPS6, ribosomal protein S6.

1 | INTRODUCTION

The size of adult skeletal muscle is a balance between protein synthesis and protein degradation. During certain conditions, like increased mechanical loading or postnatal growth, skeletal muscles undergo an increase in the size of their fibers, leading to muscle hypertrophy. While various signals can induce muscle hypertrophy, most models of muscle growth are accompanied by the activation of the intracellular serine/threonine kinase mammalian target of rapamycin (mTOR).[1] The kinase mTOR can be found in two different intracellular complexes, i.e. mTORC1 characterized by the presence of the scaffold protein Raptor, and mTORC2, containing Rictor.[2] The importance of mTORC1 for muscle hypertrophy was determined by the use of the highly specific allosteric mTORC1 inhibitor rapamycin. Indeed, most experimental models of muscle hypertrophy show a reduced or even completely blunted muscle growth when rapamycin is administered during the growth period.[3,4] Interestingly, while rapamycin reduces hypertrophy after the activation of the kinase Akt, an upstream activator of mTORC1, it doesn't completely prevent muscle growth.[5] These findings suggest the presence of alternative, mTORC1-independent, pathways that regulate skeletal muscle hypertrophy. Indeed, other downstream effectors of Akt, like the glycogen synthase kinase 3β (GSK-3β), have been shown to regulate size of myotubes.[6] In addition, while mTORC1 signaling is activated in most models of muscle hypertrophy, there are examples where treatment with rapamycin does not affect muscle growth.[7] On the other hand, while highly specific, rapamycin is known to not inhibit all mTORC1 targets with the same efficiency.[8] It is therefore possible that mTORC1 is absolutely necessary for muscle growth and that rapamycin only partially inhibits its downstream effectors. Something similar is observed in a high resistance exercise rat model, as the increase in protein synthesis after the exercise bout is not prevented by rapamycin, yet completely inhibited after administration of mTOR ATPase inhibitor.[9]

In order to understand the role of mTORC1 during muscle hypertrophy, we induced muscle hypertrophy in a genetic loss-of-function model of mTORC1 by ablating Raptor inducibly only in skeletal muscle. We find that Raptor is not critical for muscle hypertrophy after Akt activation, however, it is required for increasing mitochondrial function and muscle force. Unexpectedly, during hypertrophy, the proteome affected by rapamycin is different from the one regulated by Raptor, as the former is mainly required for the increase in ribosomal proteins, while the latter regulates increases in mitochondrial proteins. Interestingly, using rapamycin treatment in combination with Raptor deletion, we completely prevent the hypertrophy by Akt activation, suggesting that mTORC1 is the key mediator of muscle hypertrophy.

2 | MATERIALS AND METHODS

2.1 | Mice generation and treatments

Inducible, muscle-specific Raptor knock-out mice were generated as previously described.[10] Briefly, mice expressing LoxP sites flanking exon 6 of Raptor gene ($Raptor^{fl/fl}$) were crossed with a line carrying Cre recombinase fused to a mutated estrogen receptor (ER) domain under the control of human skeletal actin promoter (HSA). Cre-$Raptor^{fl/fl}$ mice were treated by oral administration with tamoxifen-containing chow (Tam400/Cre-ER, Harlan) for 3 weeks. Cre-negative mice were used as control. Both male and female mice were used. To generate Akt-Raptor knock-out mice, Cre-negative $Raptor^{fl/fl}$ mice were crossed with mice expressing Akt-ER only after the deletion of an upstream DNA sequence by the Cre recombinase. Gastrocnemius muscles were injected with a single injection containing 2×10^{10} vg of AAV9 expressing the Cre recombinase, brought to 30 μl with physiological solution. Recombinant AAV9-Cre vector used in this study was prepared by the AAV Vector Unit at the International Centre for Genetic Engineering and Biotechnology Trieste (http://www.icgeb.org/avu-core-facility.html), as described previously.[11]

Tamoxifen treatment by tamoxifen-containing chow oral administration started one month after the viral injection and lasted for 3 weeks. Each genotype was compared to wildtype animals of the same background injected with AAV-Cre and treated with tamoxifen. Rapamycin treatment was performed by intraperitoneal injections at 2 mg/kg body weight every day for 10 days or 3 weeks. In vivo protein synthesis was measured by intraperitoneal injection of 0.040 μmol/g puromycin.[12] Thirty minutes after the injection, mice were sacrificed by cervical dislocation and muscles were collected and frozen in liquid nitrogen for Western blot analysis. Mice of the same age (3 months-old) were used for each individual experiment. Experimental protocols were reviewed and approved by the local Animal Care Committee, University of Padova.

2.2 | In vivo electroporation

Control and Cre-positive $Raptor^{fl/fl}$ mice were treated for 3 weeks with tamoxifen-containing chow. One week after tamoxifen diet finished, mice were anesthetized and TA and GC muscles were transfected with a plasmid

coding for hemagglutinin (HA)-tagged myr-Akt. Muscles were injected with plasmid DNA (30 mg in 50 ml saline). Injection was followed by electroporation with stainless steel electrodes connected to an ECM830 BTX porator (Genetronics) with the following settings: six pulses of 20 ms each and 200-ms interval, voltage adjusted to the thickness of the leg (220 V cm). Muscles were analyzed 10 days after electroporation. Only areas which did not show signs of muscle damage were examined (no central nuclei or infiltration) to avoid examining fibers which have a re-expression of Raptor through addition of satellite cells.

2.3 | Antibodies

We used the following antibodies for Western blotting: P-AKT (S473; ref. 4060), P-S6 (S240/244; ref. 5364), S6 (ref. 2217), AKT (ref. 9272), GSK-3β (ref. 9315), P-GSK-3β (S9; ref. 9322), P-4E-BP1 (Thr37/46; ref. 2855), 4E-BP1 (ref. 9644), RAPTOR (ref. 2280) and P-CAD (S1859; ref. 12662) from Cell Signaling, puromycin (clone 12D10; ref. MABE343) from Millipore, GAPDH (ref. 8245) from Abcam, actin (ref. 56459) from Santa Cruz. For immunofluorescence, P-AKT (S473; ref. 4060) was from Cell Signaling and 488-conjugate Wheat Germ Agglutinin (WGA; ref. W11261) was from Invitrogen. For puromycin western blotting we used a Jackson ImmunoResearch polyclonal mouse secondary antibody (ref. 2338514).

2.4 | Immunohistochemistry

10 μm frozen cryosections were examined by fluorescence microscope. WGA staining was used to measure cross-sectional area (CSA) of transfected and surrounding non-transfected myofibers. For puromycin staining, muscles cryosections were treated with Methanol and 0.3% hydrogen peroxide for 30 min, blocked with mouse-on-mouse (MOM; Vector Laboratories) and incubated with anti-puromycin antibody (MABE343 from Millipore). After the washes, HRP-conjugated anti-mouse secondary antibodies (Bio-Rad) and a 3′-3′-diaminobenzidine (DAB; Sigma-Aldrich) solution were used.

2.5 | RNA-DNA quantification

Gastrocnemius muscles were homogenized in one volume of a phenol:chloroform mixture to separate nucleic acids. RNA and DNA content were then measured through Qubit 2.0 fluorometer (Invitrogen), using specific fluorescent dyes, selective for RNA (Qubit RNA Assay Kits, Invitrogen) or double-stranded DNA (Qubit dsDNA HS Assay Kits, Invitrogen), respectively.

2.6 | In vivo force measurements

Gastrocnemius muscle tension was measured in living mice as previously described.[13] Briefly, animals were anesthetized and a 305B muscle lever system (Aurora Scientific Inc.) was used for measuring in vivo muscle contractile performance. Force was normalized to the muscle mass as an estimate of specific force. Animals were then sacrificed by cervical dislocation and muscles were collected, weighted and frozen.

2.7 | Skinned fibers force measurements

Gastrocnemius muscles were collected and skinned as described previously,[14,15] with some adjustment in the buffer compositions. Freshly dissected muscle bundles were pinned on a polystyrene support and gently mixed at 4°C in skinning buffer 1 (potassium propionate 60 mM, TES [*N*- tris[hydroxymethyl]methyl-2-aminoethanesulphonic acid] 25 mM, Magnesium acetate 2 mM, EGTA 9 mM, sodium azide 1 mM, 0.05% Triton X-100, DTT 1 mM, ATP 4 mM, 1 Complete protease inhibitor tablet in 250 ml, 25% glycerol). After 4÷6 h, the solution was replaced by skinning buffer 2 (potassium propionate 60 mM, TES buffer 25 mM, Magnesium acetate 2 mM, EGTA 1 mM, sodium azide 1 mM, DTT 1 mM, ATP 4 mM, 1 Complete protease inhibitor tablet in 250 ml, 50% glycerol). Single fibers were dissected from bundles, transferred to an Aurora Scientific Permeabilized Fibers setup and attached to a tensiometer on one end and to a feedback motor at the other end. For Akt and Akt-Raptor ko muscles, infected fibers were significantly bigger than non-infected fibers allowing for their identification. Fibers were washed from glycerol and equilibrated in Relax buffer, pCa 8.97 (Imidazole 25 mM, EGTA 10 mM, potassium propionate 11.8 mM, magnesium acetate 6.89 mM, ATP 5.56 mM, N-[Imino(phosphonoamino) methyl]-N-methylglycine 20 mM, glutathione 10 mM). Sarcomere length was measured in Preactivating solution, pCa 8.97 (Imidazole 25 mM, EGTA 0.1 mM, HDTA 9.9 mM (TCI Europe), potassium propionate 11.7 mM, magnesium acetate 6.48 mM, ATP 5.56 mM, N-[Imino(phosphonoamino) methyl]-N-methylglycine 20 mM, glutathione 10mM) using a video optical system and adjusted to 2.4 to 2.6 μm at the beginning of the experiment. A full contraction in Activating buffer, pCa 4.69 (Imidazole

25 mM, Ca^{2+}-EGTA 10 mM, potassium propionate 11.7 mM, magnesium acetate 6.39 mM, ATP 5.65 mM, N-[Imino(phosphonoamino) methyl]-N-methylglycine 20 mM, glutathione 10 mM) was performed. The whole experiment was performed in a temperature-controlled bath set at 16°C.

2.8 | Mass spectrometry sample preparation

Frozen muscle samples were ground with mortar and pestle and resuspended in ice-cold lysis buffer (6M Guanidinium hydrochloride, 100 mM Tris pH 8.5) with protease and phosphatase inhibitor cocktail. After homogenization on a rotating wheel at 4°C for 30 min and sonication the proteins were precipitated with ice-cold acetone at −20°C over-night. Subsequently, the pellet was dissolved in digestion buffer (8M urea, 50 mM ammonium bicarbonate) and 50 μg of protein were reduced with 5 mM DDT for 60 min, followed by carbamidomethylation with 40 mM iodoacetamide for 45 min in the dark at room temperature. A predigest with the protease LysC (Wako Pure Chemicals Industries) was performed for 3 h. The samples were diluted to 2M Urea with ammonium bicarbonate buffer followed by a full digest with trypsin (Promega) for 16 h at room temperature. The resulting peptides were desalted and stored until measurement using the C18 based Stage tips.

2.9 | LC-MS/MS measurement and data analysis

For measurement, samples were eluted from Stage tips with elution buffer (60% acetonitrile, 1% ammonia), vacuum dried and resuspended in resuspension buffer (2% acetonitrile, 5% formic acid). Samples were measured on a QExactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled to an Easy nLC 1000 ultra-high-performance liquid chromatography (UHPLC) (Thermo Fisher Scientific). Peptides were separated by in-house made 50 cm columns packed with 1.7 μm C18 beads in a binary buffer system, consisting of Buffer A (0.1% formic acid) and Buffer B (80% acetonitrile in 0.1% formic acid). All samples were analyzed with a 240 min gradient.

Spectral matching of the resulting raw data was done in MaxQuant 1.5.3.8[16] using a Uniprot mouse proteome database (16.06.2017). Parameters were set to default with trypsin selected as protease, match-between runs and label free quantification (LFQ) were enabled.[16] Statistical tests, GO-term annotation and enrichment analysis as well as data visualization was done with Perseus (1.5.5.3)[17] and InstantClue.[18]

The MaxQuant output protein groups file was filtered for reverse hits, potential contaminants and hits only identified by site. LFQ values were log2 transformed and each sample assigned into condition specific groups. A two-samples Welch's test was performed to identify significantly regulated proteins between groups. We defined as significant each protein with a minimal 1.5-fold regulation based on Welch's test difference and a p-value below .05. Each protein was annotated with gene ontology (GO) terms and Pfam names. A one dimensional annotation enrichment analysis[19] was performed on the Welch's test difference to identify significantly enriched GO terms or Pfam names.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD028796.[20]

3 | RESULTS

3.1 | Raptor k.o. mice show reduced Akt-dependent fiber hypertrophy, which is prevented by rapamycin

The role of mTORC1 in muscle hypertrophy is generally determined by evaluating its sensitivity to the mTORC1 inhibitor rapamycin. However, it has been shown that rapamycin does not inhibit all mTORC1 downstream targets to the same level[8] and long-term rapamycin treatment also affects mTORC2.[21] In order to understand the role of mTORC1 during muscle hypertrophy, we used a genetic loss-of-function model for mTORC1 and determined its role during muscle hypertrophy.

We previously showed that activation of Akt leads to a rapid functional muscle hypertrophy, which is partially prevented by treating mice with the mTORC1 inhibitor rapamycin.[5] To determine the contribution of mTORC1 during muscle hypertrophy, we used a tamoxifen-inducible transgenic mouse in which we can delete the scaffold protein Raptor, and thus mTORC1 signaling, only from adult skeletal muscle.[10] We treated three-month old Raptor k.o. mice for three weeks with tamoxifen to delete the Raptor gene (Figure S1A,B), and one month after the beginning of the treatment, muscles were electroporated with a plasmid coding for the myristoylated form of Akt, i.e. myr-Akt. We stained muscles for the phosphorylated form of S6, which is induced upon Akt overexpression, to distinguish between transfected fibers and non-transfected ones from the same muscle. As can be seen in Figure 1A, in Raptor k.o. muscles, transfected myofibers are significantly

bigger than surrounding non-transfected fibers ten days after electroporation (Figure 1A). Performing a DAB staining for puromycin on serial sections, we found that the increased fiber size is accompanied by an increase in protein synthesis rates. Quantification of fiber cross sectional area (CSA) shows that hypertrophy in Raptor k.o. mice is partially impaired, as the fold increase in the CSA of transfected fibers compared to non-transfected ones is significantly reduced compared to that observed in wild-type muscles (1.75- and 2.35-fold increase, respectively) (Figure 1B,C). Next, we determined if hypertrophy in Raptor k.o. muscles is still sensitive to rapamycin. Mice

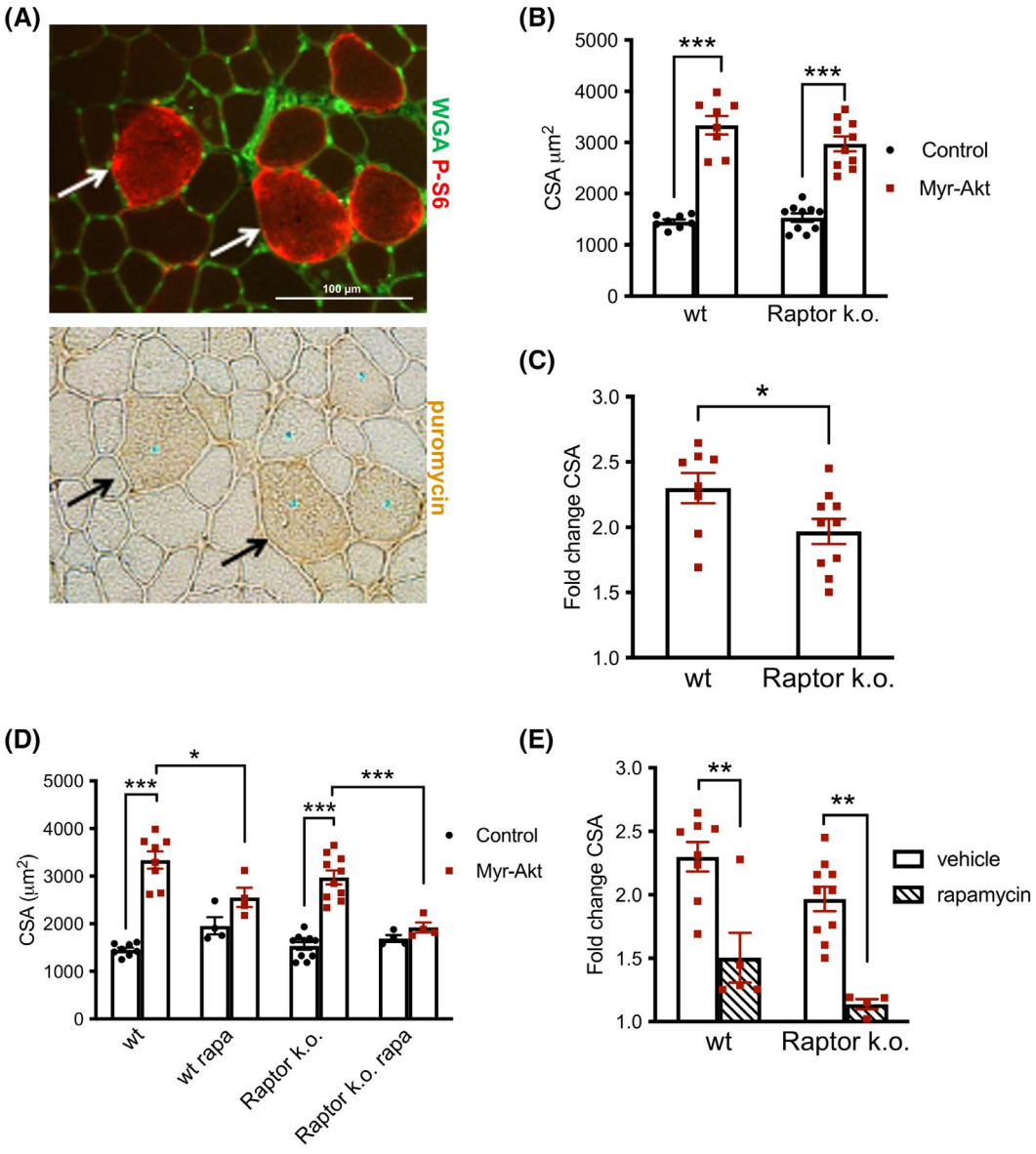

FIGURE 1 Raptor is not required for rapamycin-sensitive Akt-induced hypertrophy. (A) Electroporation of a plasmid coding for a constitutively active form of Akt (myr-Akt) induces significant fiber hypertrophy in Raptor k.o. muscles. Transfected fibers are stained with an antibody for P-S6 (S240/244), which is strongly induced by myrAkt. In the lower image a DAB staining for puromycin shows an increase in protein synthesis in hypertrophic fibers (black and white arrows; $n = 4$ muscles). (B) No significant changes in Akt-induced fiber hypertrophy between tamoxifen and oil treated littermates (control; $n = 4$ muscles (1100 fibers), k.o.; $n = 3$ muscles (400 fibers)). (C) The fold increase in fiber size in Akt transfected vs. non transfected fibers is similar in control and Raptor k.o. animals. (D) Myr-Akt transfected fibers show a significant reduction in fiber hypertrophy when treating mice with rapamycin (2 mg/kg) in both wildtype and Raptor k.o. mice ($n = 4$–10 muscles per group). (E) Fold increase of CSA of myr-Akt transfected fibers compared to surrounding non-transfected fibers shows a significant reduction (but does not prevent hypertrophy) in the rapamycin treated group. Data are shown as mean ± SEM. Statistical analysis was performed using two-tailed Student's *t*-test for 1C and two-way ANOVA with Sydàk's multiple comparison test for 1B, 1D and 1E. Statistical significance: $*p < .05$, $**p < .01$, $***p < .001$

were treated for ten days with rapamycin starting immediately after electroporation of myr-Akt. As was observed previously,[5] wildtype mice show a 50%–60% reduction in fiber hypertrophy compared to vehicle treated animals (Figure 1D,E). Very surprisingly, rapamycin treatment almost completely blocked hypertrophy in Raptor k.o. fibers, suggesting that Raptor deletion and rapamycin either have different intracellular targets or inhibit different downstream mediators of mTORC1.

3.2 | Raptor is critical for maintaining muscle histology and force during hypertrophy

We have shown previously that rapamycin treatment leads to a smaller, yet functional, Akt-dependent hypertrophy.[5] To understand if loss of Raptor affects muscle histology and function, we generated a new transgenic mouse line, which allowed us to activate Akt in Raptor k.o. muscles in a very high percentage of muscle fibers. To obtain the Akt-Raptor k.o. mouse, we crossed a line containing two LoxP sites flanking exon 6 of Raptor gene, with a line expressing a tamoxifen-inducible Akt (Akt-ER), only after the deletion of an upstream sequence by the Cre recombinase. Intramuscular injection of an AAV-Cre in the gastrocnemius muscle results in Raptor deletion and Akt-ER expression in infected fibers. While Akt-ER is being produced, without administration of tamoxifen it is not stable and therefore rapidly degraded. One month after AAV injection, when Raptor protein levels were strongly reduced by western blotting (Figure S2A), mice were subjected to tamoxifen treatment for 3 weeks to activate Akt (Figure 2A). We decided to activate Akt for this time period, as we have previously shown that this leads not only to an increase in muscle mass, but also in muscle force.[22] As can be seen in Figure 2B, Raptor protein is strongly reduced in Raptor k.o. and Akt-Raptor k.o. muscles after the injection. Residual Raptor protein, as observed in western blotting, is a consequence of normal Raptor levels in non-muscle tissue and in the non-infected muscle fibers. Immunohistochemistry staining for phosphorylated S6 revealed an efficiency of injection of about 80%–95%, giving further validation for the experimental model used (Figures 2C and S2B). As expected, no Akt-phosphorylation was observed in blood vessels or nerve endings, showing the tissue-specificity of the AAV-infection (Figure S2C). In order to understand if Akt can induce fiber growth when Raptor is deleted, we measured the ratio between muscle weight and body weight, and fiber cross sectional area. In line with the results found after electroporation, activation of Akt in the absence of Raptor showed a significant muscle hypertrophy (Figures 2D and S3A). Quantification of cross-sectional area showed a 2.4-fold increase in fiber size in Akt overexpressing myofibers, which is significantly reduced to 1.74 in fibers expressing Akt in the absence of Raptor, despite similar levels of protein synthesis rates (Figures 2E and S3B). Very different from hypertrophy after Akt activation in wildtype mice, muscle histology was significantly impaired in Akt-Raptor k.o. muscles, as evidenced by centrally localized nuclei, infiltrations of inflammatory cells, and necrotic fibers (Figure 2F). Considering the presence of central nuclei, we wondered if fusion of satellite cells could restore Raptor expression level, despite the continuous presence of the Cre protein in infected fibers. Therefore, we quantified fiber CSA in fibers with and without central nuclei. As can be seen in Figure S3C, we did not observe any difference in fiber size, suggesting re-introduction of Raptor through satellite cell fusion is not an important issue in this model.

Next, we aimed to understand if Raptor is required for an increase in muscle force after Akt activation. We measured in vivo strength of the gastrocnemius muscle and observed an increase in absolute tetanic force in Akt-overexpressing muscles (Figure 2G,H). Interestingly, when activating Akt in the absence of Raptor absolute muscle force is not increased, despite a 70% increase in muscle mass. Normalizing absolute force for muscle weight, we observed a significant decrease in muscle tension in Akt mice, which is even further decreased in Akt-Raptor k.o. animals (Figure S4A). Interestingly, normalized force is not affected compared to wildtype mice, when treating Akt mice with rapamycin (Figure S4B). As we showed previously,[22] when muscles show this very rapid increase in muscle mass, also muscle architecture is affected, leading to an underestimation of force production using in-vivo force measurements, due to changes in pennation angle. To better understand how the contractile apparatus is affected, while avoiding problems linked to muscle architecture, we performed force measurements on isolated skinned muscle fibers. As can be seen in Figure 2I, tension produced by skinned fibers from Akt muscle shows a significant increase as compared to control fibers. Fibers taken from Akt-Raptor k.o. muscles, on the other hand, do not show this increase in normalized tension, underlining an important role for Raptor in stimulating the function of the contractile apparatus during Akt-dependent hypertrophy.

3.3 | Rapamycin treatment in Akt-Raptor k.o. muscles prevents residual mTORC1 signaling and completely blocks muscle growth

Next, we performed a western blotting analysis of some canonical downstream targets of Akt-mTORC1 signaling to get some insight into the differences observed in muscle growth

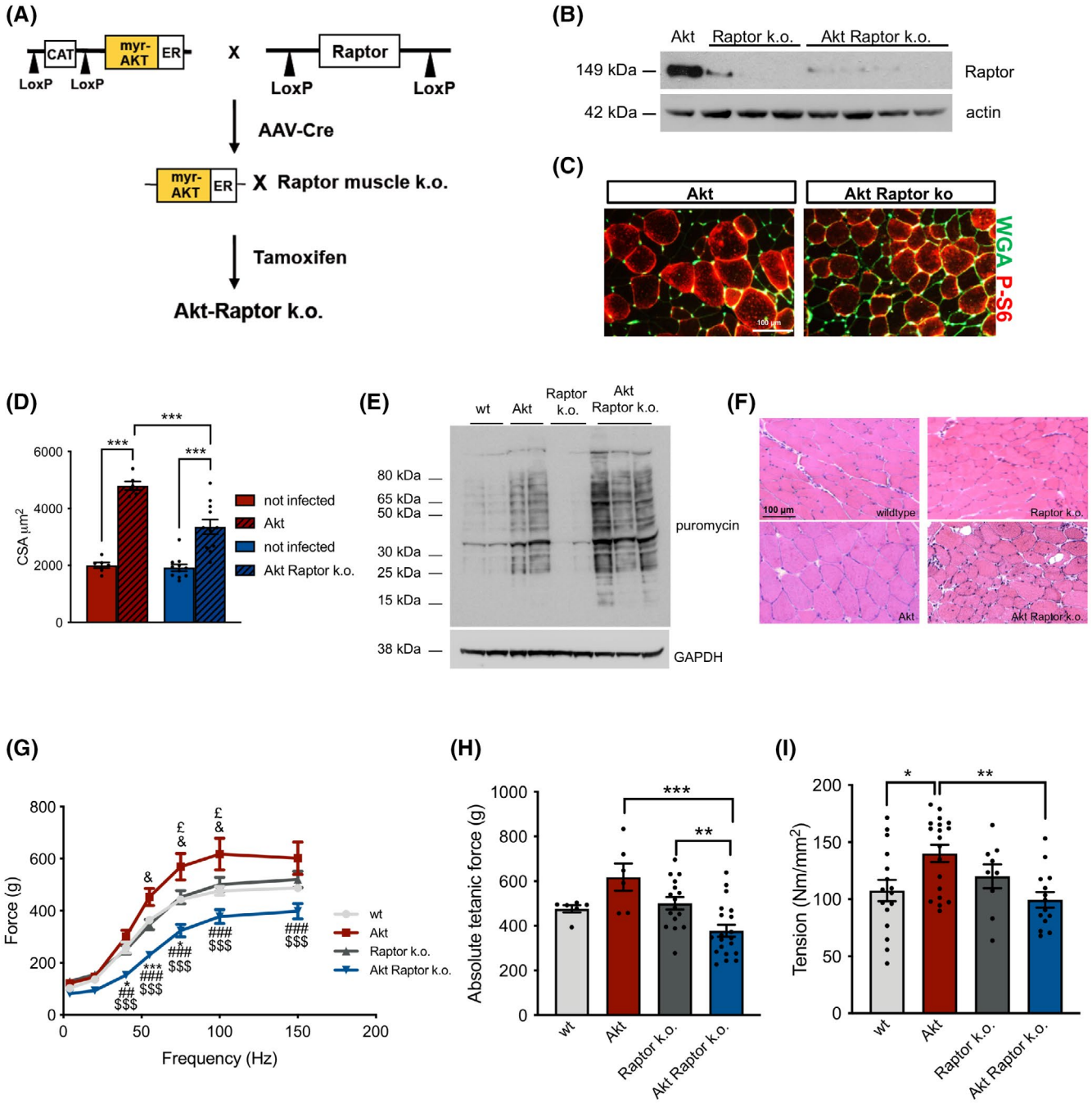

FIGURE 2 Generation of inducible muscle-specific Akt-Raptor knockout mice (Akt-Raptor k.o.). (A) Crossing the Akt-inducible line with a Raptor floxed line we obtained the Akt/Raptor floxed line. One single injection of an adeno-associated virus (AAV) leads to an infection of the Cre protein at very high efficiency in both the gastrocnemius and tibialis anterior muscle of adult mice. After one month, when Raptor is efficiently deleted, we injected tamoxifen to activate Akt. (B) Western blot of muscles taken out seven weeks after AAV-Cre infection show good knockdown of raptor protein. (C) IHC for P-S6 on serial sections show significant hypertrophy in infected (red) fibers. (D) Activation of Akt for three weeks leads to a significant increase in muscle weight independently of Raptor. Fiber cross-sectional area in infected and non-infected fibers in Akt and Akt-Raptor k.o. mice. (E) puromycin incorporation in wildtype and raptor k.o. muscles after Akt activation. (F) H&E staining showing altered histology in Akt-Raptor ko muscles with the presence of necrotic and regenerating fibers. (G) Absolute force-frequency curve shows a significant increase in absolute force in Akt muscles. Activation of Akt in the absence of Raptor does not increase absolute muscle force (n = 6–20 muscles per group). (H) Maximal tetanic force (force at 100 Hz) in the different groups. (I) Tension produced by skinned fibers taken from the different groups. Activation of Akt leads to an increase in normalized tension as observed previously.[22] Fibers taken from Akt-Raptor mice are significantly weaker than wildtype fibers (n = 14–20 fiber per muscle, 3 muscles per group). Data are shown as mean ± SEM. Statistical analysis was performed using one-way and two-way ANOVA with Tukey's multiple comparison test. Statistical significance: *$p < .05$, **$p < .01$, ***$p < .001$. With regard to Figure 2G, Akt-Raptor k.o. was compared with *, wildtype; #, Raptor k.o.; $, Akt; wildtype was compared with &, Akt

and function. As expected, phosphorylation of Akt and of its downstream target GSK3β shows a trend to increase in both Akt and Akt-Raptor k.o. mice compared to controls. However, very unexpectedly, we found that after Akt activation, phosphorylation of the ribosomal protein S6, a marker sensitive to mTORC1 activity, is unaffected by Raptor deletion (Figure 3A). We recently showed that little residual mTOR signaling is able to maintain muscle homeostasis for one month after Raptor deletion,[10] possibly explaining this phosphorylation of RPS6. To understand if rapamycin could inhibit the mTORC1 signaling and muscle growth in Akt-Raptor k.o. mice we treated mice for three weeks with tamoxifen while simultaneously administering rapamycin. As can be seen in Figure 3B, rapamycin is able to completely

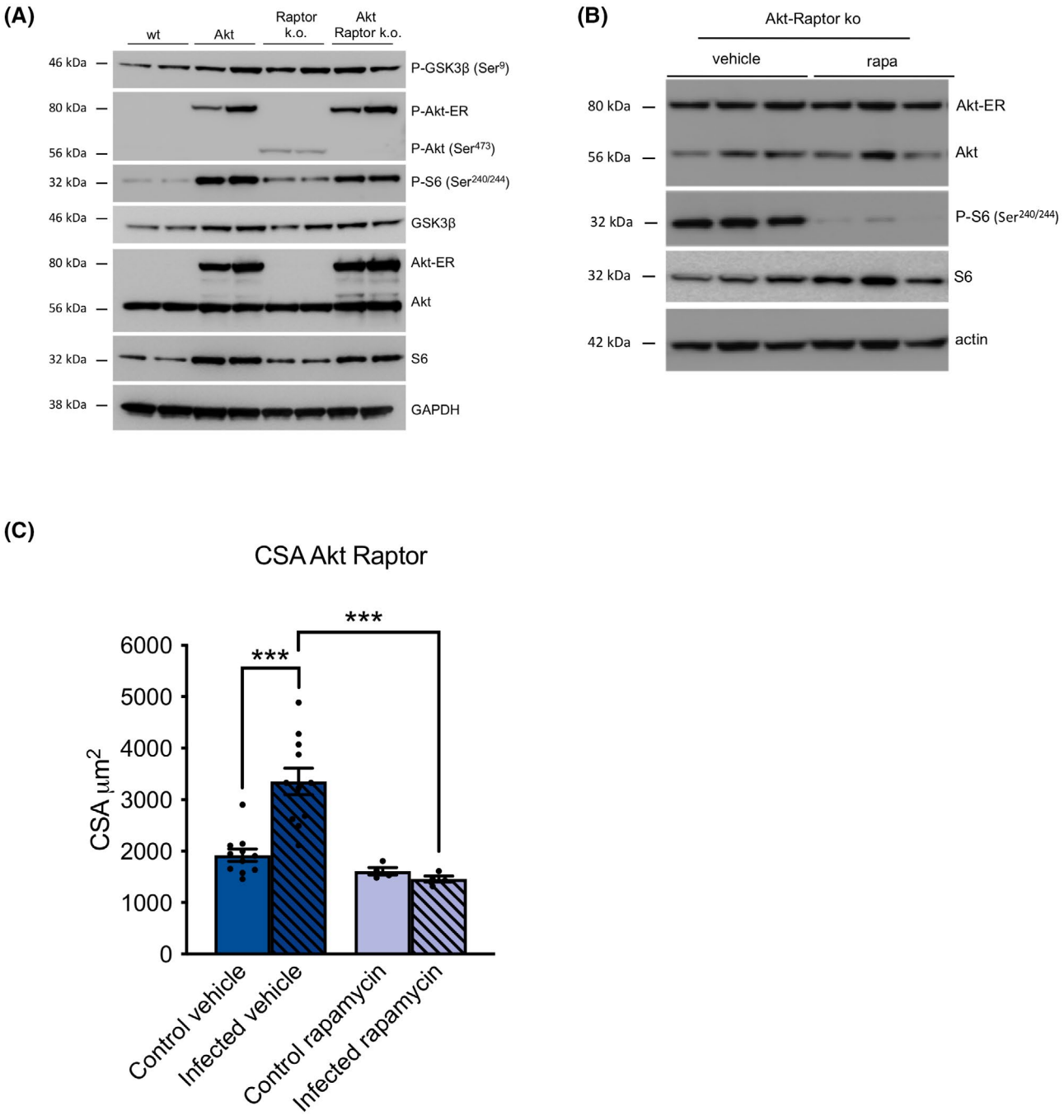

FIGURE 3 Hypertrophy in Akt-Raptor k.o. mice is completely prevented by rapamycin. (A) Akt activation in Raptor k.o. muscles is accompanied by a significant increase in phosphorylation of ribosomal protein S6 (Ser240/244). (B) Treatment of rapamycin can prevent the increase in RPS6 phosphorylation after Akt activation also in Raptor k.o. muscles. (C) Rapamycin treatment completely prevents hypertrophy in infected fibers of Akt-Raptor k.o. mice. Data are shown as mean ± SEM. Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparison test. Statistical significance: ***$p < .001$

prevent the increase in S6 phosphorylation found in Akt-Raptor k.o. muscles, suggesting that this phosphorylation is caused by residual mTORC1 signaling and not through the activation of mTORC1-independent kinases. Interestingly, when we analyze fiber CSA in these Akt-Raptor k.o. mice after rapamycin treatment, we see that fiber hypertrophy is completely prevented (Figure 3C). This lack of fiber growth is supported by analysis of puromycin incorporation, showing that rapamycin can completely block the increase seen in vehicle-treated Akt-Raptor k.o. animals (Figure S5A).

Considering that Akt-Raptor k.o. animals are significantly weaker compared to wildtype or Raptor k.o. mice, we wondered if rapamycin treatment could prevent muscle dysfunction in these mice, possibly by normalizing protein synthesis rates and protein quality. However, as can be seen in Figure 4A, rapamycin does not prevent the reduction in force production observed in Akt-Raptor k.o. mice, despite completely preventing muscle growth. In addition, muscle tension of Raptor k.o. animals is significantly decreased upon rapamycin administration to a similar extent as in Akt-Raptor k.o. muscles. The weakness observed in these animals is also observed at the level of the contractile apparatus, as shown by the force reduction in isolated skinned fibers (Figure 4B). To see if the loss of force is linked to muscle injury, we stained the gastrocnemius for mouse Immunoglobulins (IgG), which are indicative of membrane damage and fiber permeability. IgG-positive fibers are increased in Akt-Raptor k.o. muscles compared to Raptor k.o. mice. Interestingly, also Akt-Raptor k.o. muscles treated with rapamycin, which do not undergo muscle growth, show a similar amount of IgG-positive fibers (Figure 4C,D). Furthermore, we also observe the presence of central nuclei and necrotic fibers (Figure 4E). Taken together these results suggest that a complete block of mTORC1 signaling, by combining a genetic loss-of-function and a pharmacological inhibition, is sufficient to completely prevent Akt-dependent muscle growth and lead to a muscle pathology.

3.4 | Raptor is required for increases in mitochondrial proteins, while rapamycin inhibits the synthesis of ribosomal proteins

To better understand why force is reduced in Akt-Raptor k.o. mice we performed a quantitative proteomics analysis. We compared the quantitative proteome of Akt activation for three weeks in wildtype and Raptor k.o. mice. As can be seen in Figure 5A, we identified 2036 differentially regulated proteins comparing Akt and Akt-Raptor k.o. muscles, of which 245 were significant. Interestingly, a lot of the top ranked differentially expressed proteins are mitochondrial proteins. For example, amongst the top ranked differentially expressed proteins, we found COQ7 (5-demethoxyubiquinone hydroxylase), a protein critical for Coenzyme Q production, to be highly down-regulated. Interestingly, the regulation of this protein has already previously been linked to mTOR signaling.[23] When we perform a 1D-enrichment analysis on differentially expressed proteins we clearly see an enrichment of proteins related to mitochondrial function in the Raptor-dependent proteome (Figure 5B). Next, we performed western blotting analysis for various mitochondrial proteins to understand if there were important differences in total mitochondrial content between Akt and Akt-Raptor k.o. conditions (Figure 5C). While we didn't see major differences in the total number of mitochondria by western blotting, we did observe a very clear difference in mitochondrial histology with the appearance of centrally localized clusters, suggestive of mitochondrial dysfunction (Figure 5D). Indeed, a similar non-homogeneous distribution of mitochondria was observed after 7 months of Raptor deletion and was accompanied by mitochondrial dysfunction.[10] Since signaling of mTORC1 through 4E-BP1 is thought to be important for mitochondrial activity and biogenesis,[24] we checked the phosphorylation levels of 4E-BP1 in the different conditions. As can be seen in Figure S5B, after activation of Akt, we see a reduction in phosphorylation levels of 4E-BP1 in Akt-Raptor ko muscles, in line with the observed reduction of mitochondrial proteins, as seen in the 1D-enrichment analysis.

Considering the differential regulation of mitochondrial proteins and the striking effect of rapamycin treatment in blocking Akt-dependent hypertrophy completely in Raptor k.o. muscles, it was tempting to assume that rapamycin blocks a different group of proteins which are affected less, or not at all, by Raptor deletion. To address this, we performed the same analysis of the proteome, only this time comparing Akt activation in vehicle and rapamycin treated wildtype mice (Figure 6A). Of the 2036 differentially regulated proteins, 109 reached statistical significance. Somewhat surprisingly, there was very little overlap between the proteome affected by loss of Raptor or inhibition with rapamycin. Indeed, using a 1D-enrichment analysis to determine the main protein groups down-regulated by rapamycin treatment, we identified ribosomal proteins or proteins of translation machinery (Figures 6B and S6). That this regulation is quite different from that observed in Akt-Raptor ko mice is clear from the box plot in Figures 6C and S7A, which nicely show how mitochondrial and ribosomal proteins are regulated differently in both situations, when compared to Akt activation. This major difference in the affected proteome helps to explain why rapamycin treatment can still have major effects on Akt-dependent muscle growth in Raptor k.o. mice. The fact that Akt can still induce ribosome biogenesis also in the absence of Raptor is also in line with the

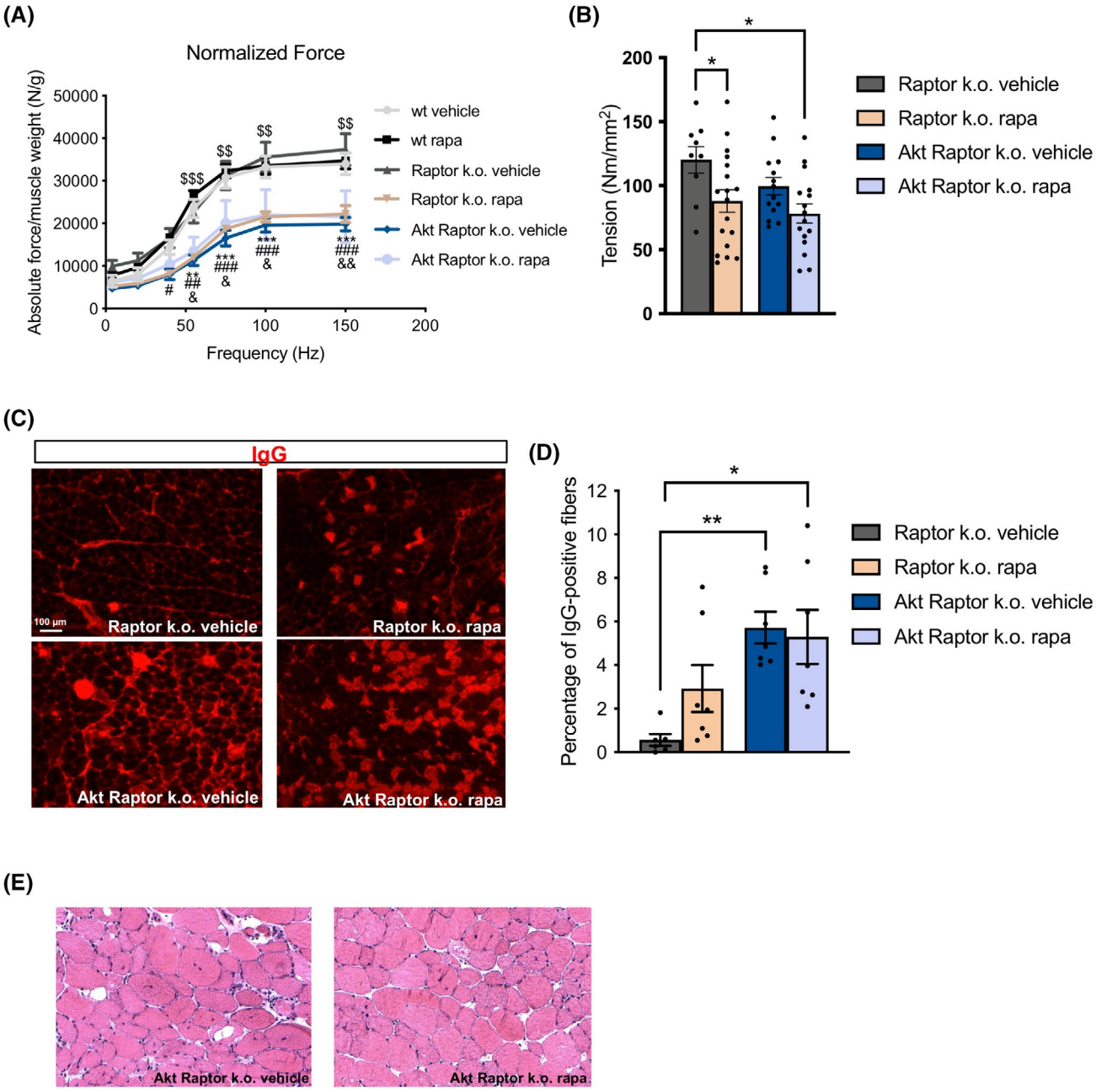

FIGURE 4 Rapamycin treatment in Akt-Raptor k.o. mice leads to muscle dysfunction and fiber necrosis. (A) Normalized force of the gastrocnemius in vivo shows a strong reduction in force production in Raptor mice after rapamycin treatment, which does not affect wildtype mice. Rapamycin treatment in Akt-Raptor k.o. mice impairs muscle force, despite a complete block in muscle growth ($n = 6–8$ per group). Statistical analysis was performed using two-way ANOVA with Tukey's multiple comparison test. (B) Tension production of skinned fibers show that part of the reduction observed in muscle force in vivo can be explained by an impaired performance of the contractile apparatus ($n = 10–15$ fiber per muscle, 3 muscles per group). Statistical analysis was performed using two-way ANOVA with Newman-Keuls's multiple comparison test. (C) Hypertrophic Akt-Raptor k.o. and non-hypertrophic Akt-Raptor k.o. mice after rapamycin treatment show a high number of IgG-positive fibers. (D) Quantification of IgG-positive fibers in the different groups ($n = 7$ per group). Statistical analysis was performed using two-way ANOVA with Sydàk's multiple comparison test for Figure 4D. (E) H&E staining showing central nuclei and necrotic fibers in both Akt-Raptor k.o. mice treated with vehicle or rapamycin. Note the presence of hypertrophic fibers in the vehicle group which are no longer present in the rapamycin treated mice. Data are shown as mean ± SEM. Statistical significance: $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$. With regard to Figure 4A, Akt-Raptor k.o. vehicle was compared with *, wildtype vehicle; #, Raptor k.o. vehicle; Akt-Raptor k.o. rapamycin was compared with $, wildtype rapamycin; Raptor k.o. rapamycin was compared with &, Raptor k.o. vehicle

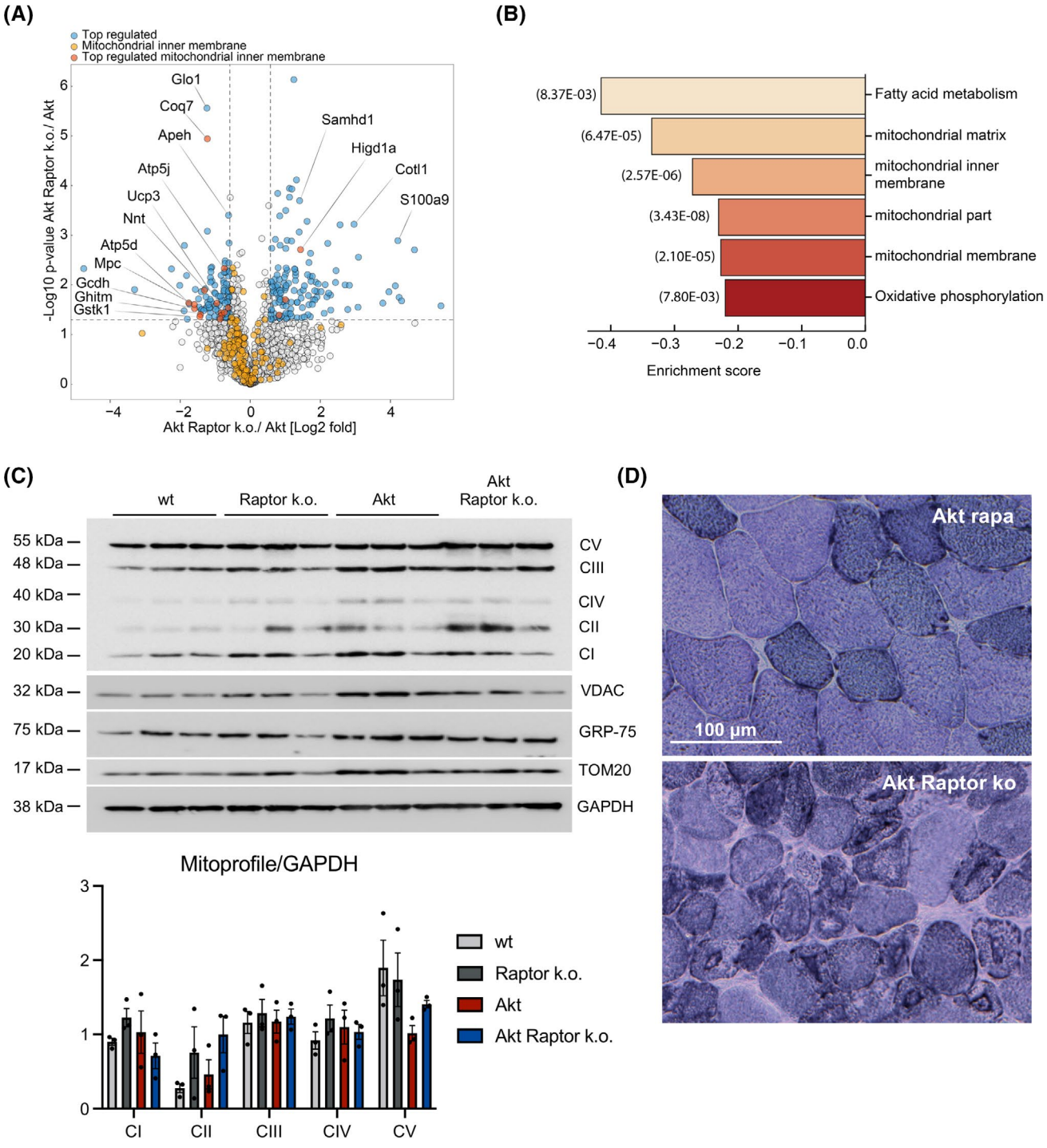

FIGURE 5 Raptor deletion impairs synthesis of mitochondrial proteins and histology. (A) Volcano plot showing the changes in the muscle proteome comparing three weeks of Akt activation in wildtype or Raptor k.o. mice ($n = 3$–6 muscles per group). (B) 1D enrichment analysis showing that the most highly downregulated groups of proteins belong to mitochondria and mitochondrial metabolism. On the left of the bar is the significance of the different groups. (C) Western blot analysis for respiratory complex proteins, VDAC and GRP-75 does not show major differences between total mitochondria in Akt and Akt-Raptor k.o. muscles. (D) SDH staining shows major differences in mitochondrial histology comparing Akt-Raptor to Akt rapa muscles

persistent phosphorylation of the multifunctional enzyme CAD, a target of the mTORC1-S6K1 axis[5] (Figure 6D). Also, the ratio of RNA/DNA is increased to similar extent in Akt and Akt-Raptor k.o. muscles, as compared to controls, suggesting that lack of Raptor does not impair ribosome biogenesis (Figure 6E).

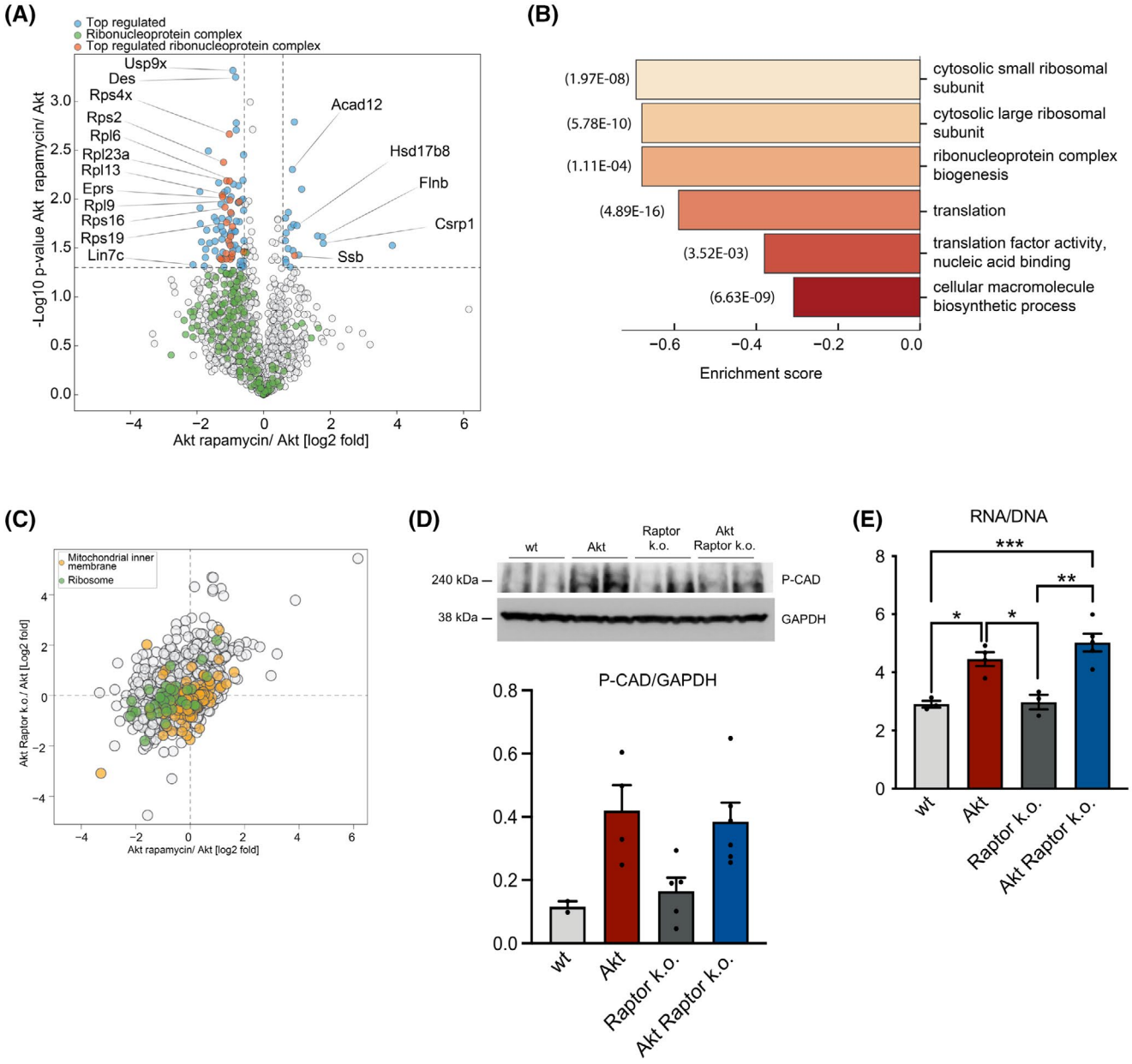

FIGURE 6 Rapamycin treatment reduces ribosome biogenesis. (A) Volcano plot showing the changes comparing three weeks of Akt activation in wildtype mice to Akt activation while treating mice with rapamycin for three weeks ($n = 3$ per group). (B) 1D enrichment analysis show that the most downregulated protein groups belong to ribosomal proteins and the translational apparatus. (C) Scatter plot showing the differential regulation of mitochondrial and ribosomal proteins in Akt-Raptor ko and Akt rapa muscles. (D) No difference in CAD phosphorylation comparing Akt to Akt-Raptor k.o. muscles. (E) Increases in RNA/DNA after activation of Akt, a good indication of ribosomal biogenesis, is not impaired in Akt-Raptor k.o. muscles. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparison test. Statistical significance: $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$

4 | DISCUSSION

It is well established that mTORC1 signaling is activated in most models of skeletal muscle hypertrophy.[1] Furthermore, treatment with the mTORC1 inhibitor rapamycin is generally sufficient to blunt, or even prevent muscle hypertrophy. Despite this critical role for mTORC1, it is not known which proteins are regulated by mTORC1 during muscle growth. In addition, the fact that rapamycin generally only blunts muscle growth, suggests the presence of mTORC1-independent mediators of muscle growth.

Here we address the functional role of mTORC1 during muscle hypertrophy, comparing the effect of pharmacological inhibition with rapamycin to the genetic inhibition by deletion of Raptor. Interestingly, we find that loss of Raptor only reduces muscle growth, but, unlike rapamycin treatment, completely impairs increases in muscle force production. Unexpectedly, treating Akt-Raptor k.o. mice

with rapamycin completely impairs muscle growth, suggesting an additive inhibitory effect on mTORC1 signaling. Indeed, performing a quantitative proteomics analysis we find that Raptor is critical for increasing the mitochondrial proteome, while rapamycin acts mainly on ribosomal proteins. Taken together, our data suggests that Akt-dependent hypertrophy in vivo is completely mediated by mTORC1 and Raptor is required for the synthesis of mitochondrial proteins and increasing muscle force (Figure 7).

4.1 | Loss of Raptor reduces Akt-dependent muscle growth, but remains rapamycin-sensitive

In this study we examined how loss of Raptor, only from skeletal muscle fibers, can influence Akt-dependent muscle hypertrophy. It was recently shown that loss of Raptor is sufficient to prevent muscle hypertrophy after mechanical overloading.[25] A major difference with our study is the fact that hypertrophy in this model of myotenectomy is less pronounced, showing a rapamycin-sensitive hypertrophy of 10%–22%, depending on the fiber type examined. It is likely that in our model the bigger increase in muscle mass allows to identify both a Raptor-dependent and -independent part of muscle growth. A major discrepancy between the two models are the observed rates of protein synthesis. It was reported that overload hypertrophy in Raptor k.o. mice leads to an increase in protein synthesis rates, without an increase in fiber size, different from what we observe after Akt activation. While these results are somewhat difficult to interpret, it is tempting to speculate that loss of Raptor leads to a shift in the proteome which, depending on the nature of the upstream stimulus, can affect muscle growth or not. Considering the results from our proteomics analysis, this would suggest that an increase in mitochondrial proteins is a pre-requisite for an increase in muscle size after mechanical loading, possibly by increasing its ATP-producing capacity.[24]

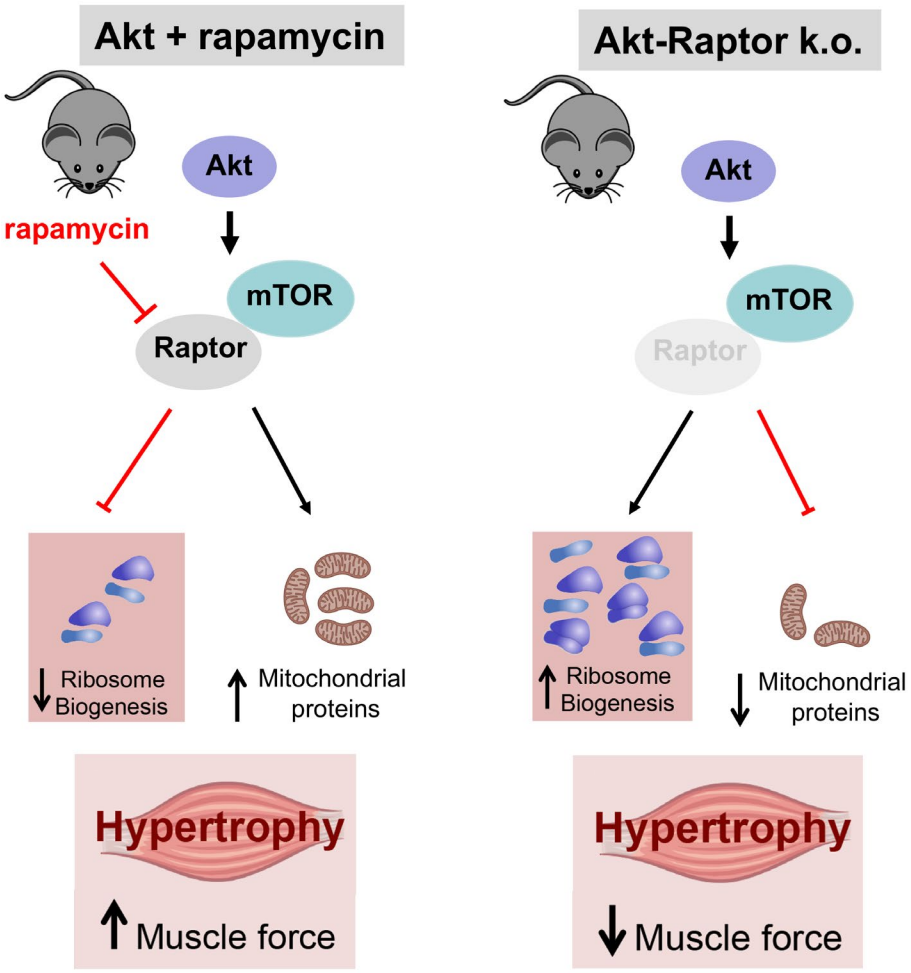

FIGURE 7 Scheme showing the difference of genetic or pharmacological inhibition of mTORC1 during skeletal muscle hypertrophy. Rapamycin treatment reduces mainly ribosome biogenesis, without affecting mitochondrial biogenesis, leading to a reduced and functional muscle hypertrophy. Loss of Raptor, on the other hand, does not impair ribosome biogenesis, but affects the mitochondrial proteome and histology leading to a reduced dysfunctional muscle growth

Rapamycin binds to FKBP12 and as a dimer allosterically inhibits mTORC1. It has been shown that rapamycin, while showing high affinity for mTOR, does not inhibit all downstream targets of mTOR signaling in the same manner.[8] It was suggested that the higher the affinity of mTOR for the specific phosphorylation motif, the less sensitive it becomes to the inhibiting action of rapamycin. Indeed, while S6K1 is strongly inhibited by rapamycin, this is much less pronounced for 4E-BP1 during long-term rapamycin treatments.[26] In line with this, we hypothesize that the residual hypertrophy in Akt mice after rapamycin treatment can be explained by rapamycin-insensitive mTORC1 signaling. Indeed, we find that loss of Raptor and rapamycin treatment are additive and each block a different part of the Akt-dependent proteome. While we cannot exclude a role for rapamycin on mTORC2, the fact that we do not find an increase in fiber size 10 days (Figure 1) or three weeks (Figure 2) after Akt activation in Raptor k.o. muscles, suggests that we completely prevent muscle growth from the beginning of Akt activation. This speaks against a role of mTORC2 inhibition in blocking muscle growth, as loss of mTORC2 activity only starts multiple days after the beginning of rapamycin treatment.[21] We also do not observe any changes in the level of Rictor comparing Akt and Akt-Raptor ko muscles (Figure S7B), suggesting a relatively stable mTORC2. The ability of mTOR to phosphorylate S6K1 independently from Raptor was already shown previously in vitro.[27] Interestingly, similar to what we observe in our Akt-Raptor mice, this phosphorylation remains rapamycin sensitive, suggesting that in certain contexts rapamycin can inhibit mTOR activity when Raptor is down-regulated. Furthermore, an additive effect of rapamycin in Raptor knockout cells was also reported for the translation of a specific subset of mRNAs.[28] A very interesting result in our study is the fact that treating Akt-Raptor mice with rapamycin is able to completely prevent muscle hypertrophy. This finding suggests that mTORC1 is absolutely required to increase muscle mass after Akt activation. The phosphorylation and inactivation of glycogen synthase kinase 3β (GSK-3β) is therefore likely not playing an important role in mediating Akt-dependent muscle growth, contrary to what was hypothesized previously.[6] This finding is also consistent with the fact that muscle-specific inactivation of GSK-3β does not alter muscle mass.[29]

4.2 | Raptor is required for synthesis of mitochondrial proteins and increasing muscle function

An important finding in this manuscript is the major role for Raptor in maintaining muscle function during muscle hypertrophy. Not only was this observation clear on a whole muscle level, but also increased force production in isolated skinned fibers was impaired. This impairment of the contractile apparatus, also when in the presence of an excessive amount of calcium, suggests a reduction of force production in most fibers, not just a reduction of total muscle force due to necrosis of a subpopulation of fibers. Similar reductions in maximal force production of skinned fibers has been observed in certain mouse models of muscular dystrophy and can possibly be related to increased oxidative stress and mitochondrial dysfunction.[30] Restoration of defective autophagy and mitochondrial function, as observed in mice lacking Col6a1, is sufficient to restore muscle function on both the whole muscle and the single fiber level.[31] As we observe a significant reduction in mitochondrial proteins after loss of Raptor, together with a major alteration in mitochondrial histology, it is tempting to assume that mitochondrial dysfunction plays an important role in the loss of force production.

An important role for Raptor in stimulating the synthesis of mitochondrial proteins has been suggested through its regulation of 4E-BP1.[24,32] Indeed, in most cell systems, long-term rapamycin treatment (days) does not prevent the phosphorylation of 4E-BP1, contrary to the more pronounced inhibitory effect of Raptor deletion.[26] Despite these reports, we were not able to observe any differences in 4E-BP1 phosphorylation levels when comparing Akt activation in Raptor ko mice or after administrating rapamycin (Figure S5B). This makes it likely there are other effectors of mTORC1 downstream of 4E-BP1, which are affected by loss of Raptor without undergoing changes after rapamycin treatment. A critical role for 4E-BP1 in mediating muscle function after a hypertrophic stimulus is also found in cardiac muscle. Indeed, loss of Raptor or mTOR leads to an impaired overload hypertrophy with cardiac dysfunction.[33,34] Interestingly, loss of mTOR signaling in 4E-BP1 ko mice preserves mitochondrial function and cardiac function during hypertrophy. In line with our results observed in skeletal muscle, treatment with rapamycin impairs overload cardiac hypertrophy, but does not impair contractility.[35]

Taken together, we show for the first time the critical role of Raptor for increasing skeletal muscle force during hypertrophy. Using a proteomics approach we find that Raptor is critical in mediating increases in mitochondrial proteins, while rapamycin treatment impinges on ribosomal proteins and the translational apparatus. These results suggest that increased mitochondrial biogenesis is critical for inducing or sustaining a functional muscle growth. Combining this pharmacological and genetic loss-of-function, we show that mTORC1 is the key downstream mediator of muscle anabolism after Akt activation.

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DISCLOSURES

Martina Baraldo, Leonardo Nogara, Achille Homère Tchampda Dondjang, Marco Scalabrin, Georgia Ana Dumitras, Alessia Geremia, Clara Türk, Frederik Telkamp, Lorena Zentilin, Mauro Giacca, Marcus Krüger and Bert Blaauw declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Bert Blaauw conceived the project and wrote the manuscript. Martina Baraldo, Leonardo Nogara, Georgia Ana Dumitras, Alessia Geremia, Achille Homère Tchampda Dondjang, Marco Scalabrin, Clara Türk, Frederik Telkamp, Lorena Zentilin, performed experiments and analyzed data. Lorena Zentilin, Mauro Giacca, Marcus Krüger contributed intellectually and provided technical advice. All authors discussed the results and commented on the manuscript.

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SUPPORTING INFORMATION

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