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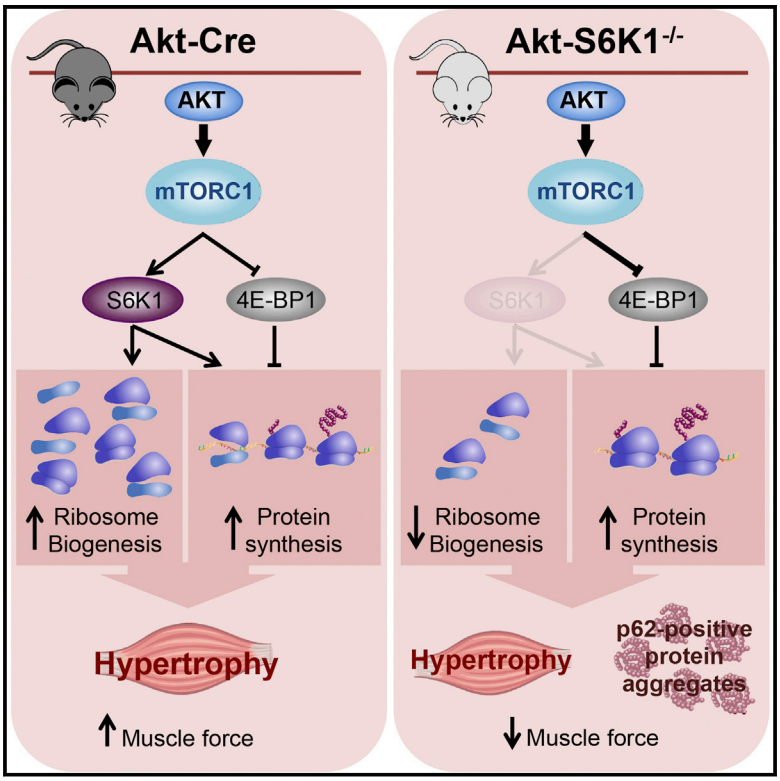
Cell Reports

S6K1 Is Required for Increasing Skeletal Muscle Force during Hypertrophy

Graphical Abstract



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In Brief

Marabita et al. describe the role of the kinase S6K1 during muscle hypertrophy. They show that, surprisingly, S6K1 is not required for muscle growth but is important for stimulating ribosome biogenesis, preventing the formation of protein aggregates, and increasing muscle force.

Highlights

- S6K1 is required for increasing adult muscle force, not muscle size
- 4E-BP1 and S6K1 can independently mediate rapamycin-sensitive muscle growth
- Hypertrophy in the absence of S6K1 leads to the formation of protein aggregates
- S6K1 is required for increases in ribosome biogenesis

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S6K1 Is Required for Increasing Skeletal Muscle Force during Hypertrophy

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SUMMARY

Loss of skeletal muscle mass and force aggravates age-related sarcopenia and numerous pathologies, such as cancer and diabetes. The AKT-mTORC1 pathway plays a major role in stimulating adult muscle growth; however, the functional role of its downstream mediators in vivo is unknown. Here, we show that simultaneous inhibition of mTOR signaling to both S6K1 and 4E-BP1 is sufficient to reduce AKT-induced muscle growth and render it insensitive to the mTORC1-inhibitor rapamycin. Surprisingly, lack of mTOR signaling to 4E-BP1 only, or deletion of S6K1 alone, is not sufficient to reduce muscle hypertrophy or alter its sensitivity to rapamycin. However, we report that, while not required for muscle growth, S6K1 is essential for maintaining muscle structure and force production. Hypertrophy in the absence of S6K1 is characterized by compromised ribosome biogenesis and the formation of p62-positive protein aggregates. These findings identify S6K1 as a crucial player for maintaining muscle function during hypertrophy.

INTRODUCTION

Better understanding of the signaling pathways that control muscle cell size and function is of great importance, as there are currently no therapeutic approaches that can prevent or reduce muscle wasting and frailty, as seen in aging and muscular dystrophy. While various studies have identified important regulators of adult skeletal muscle mass, surprisingly little is known about how these pathways can modulate muscle force. The two major signaling pathways regulating adult skeletal muscle mass are the AKT-mTORC1 (Mammalian Target of Rapamycin Complex 1) (Blaauw et al., 2013; Schiaffino et al., 2013) and the myostatin/BMP-Smad pathway (Sartori et al., 2013). Overexpression of a constitutively active form of the serine/threonine kinase AKT, or stimulation of Bone Morphogenic Protein (BMP) signaling, leads to a rapid increase in protein synthesis and significant muscle growth. Despite being regulated through the activation of different membrane receptors, hypertrophy in both cases is significantly reduced by treatment with rapamycin, a specific inhibitor of mTORC1 (Bodine et al., 2001; Winbanks et al., 2013). This key role of mTORC1 signaling in the regulation of adult muscle mass is further supported by the fact that rapamycin blocks muscle growth after synergist ablation, a model of overload-induced muscle hypertrophy (Bodine et al., 2001). Even though from these studies it is clear that most models of adult skeletal muscle hypertrophy are rapamycin sensitive, the key downstream mediators of mTORC1 signaling and their roles in improving muscle force during hypertrophy in vivo are not known.

One of the main rapamycin-sensitive downstream effectors of mTORC1 in the stimulation of protein synthesis in various cell types is the serine/threonine kinase p70S6K1 (S6K1). Overexpression of a constitutively active form of S6K1 in myotubes induces hypertrophy (Rommel et al., 2001), while mice lacking S6K1 are 20% smaller than their wild-type littermates (Pende et al., 2000). Furthermore, overexpression of a constitutively active form of AKT in myotubes lacking S6K1 and the closely related p70S6K2 (S6K2) does not lead to myotube hypertrophy at low doses of AKT activation and is rapamycin insensitive (Ohanna et al., 2005). In addition to these results obtained in cell culture, studies performed in human and murine muscle identified the activation of S6K1, and its downstream target ribosomal protein S6, as an in vivo marker of resistance exercise intensity, closely following the increase in muscle mass (Baar and Esser, 1999; Burd et al., 2010).

Despite these results, which suggest an important role for S6K1 in the regulation of muscle mass, S6K1/2 double-knockout mice have a normal global translational regulation, which remains sensitive to rapamycin treatment (Garelick et al., 2013; Pende et al., 2004). Furthermore, overexpression of a dominant-negative form of 5′ AMP-activated protein kinase (AMPK), which is strongly activated in S6K1/2 double-knockout mice, completely rescued fiber size in knockout (KO) animals in vivo, suggesting that S6K1 is not required for stimulating protein synthesis and maintaining fiber size (Aguilar et al., 2007).

Another potential mTORC1 mediator involved in the regulation of protein synthesis is the eIF4E-binding protein 1 (4E-BP1). Phosphorylation of 4E-BP1 by mTORC1 leads to its dissociation from eukaryotic initiation factor 4E (eIF4E), permitting it to bind to eukaryotic initiation factor 4G1 (eIF4G1) and forming an active initiation complex (eIF4F). Interestingly, knockdown of eIF4E or overexpression of a non-phosphorylatable mutant version of 4E-BP1, i.e., with a mutation in the four mTOR phosphorylation sites (4E-BP1-4A), is sufficient to prevent increases in cell growth, showing the importance of the eIF4E-eIF4G1 interaction in stimulating protein synthesis. On the role of 4E-BP1 in the regulation of skeletal muscle mass very little is known. Overexpression of either wild-type 4E-BP1 or the 4E-BP1-4A mutant did not modify myotube size (Ohanna et al., 2005), and mice lacking both 4E-BP1 and 2 have a normal muscle weight and a regular translational regulation (Steiner et al., 2014).

Here, we determine the relative contribution of S6K1 and 4E-BP1 in the regulation of a functional muscle hypertrophy. Our main findings show that S6K1 is sufficient, but not required, for rapamycin-sensitive muscle hypertrophy. Hypertrophy due to overexpression of AKT in muscles lacking S6K1 can occur due to compensatory signaling of mTORC1 to 4E-BP1. Importantly, while S6K1 is not required for muscle growth, it is necessary for AKT-induced increases in ribosome biogenesis and muscle force. The rapid increase in protein synthesis after AKT activation in mice lacking S6K1 leads to the appearance of myopathic features such as necrotic fibers, central nuclei, and p62-positive protein aggregates. Reducing protein synthesis rates by rapamycin prevents the formation of protein aggregates and induces a smaller, yet functional hypertrophy.

Taken together, our results show that S6K1 is required for the regulation of protein quality and muscle force, but not for increasing skeletal muscle mass.

RESULTS

AKT-Induced Muscle Growth Can Be Mediated Both by S6K1/2 and by mTOR Signaling to 4E-BP1

In order to identify the downstream mediators of AKT-mTORC1 during muscle hypertrophy, we first focused our attention on the two best-characterized mTORC1 targets, S6K1/2 and 4E-BP1. To determine the role of S6K1/2 in AKT-induced muscle hypertrophy, we electroporated a plasmid coding for a constitutively active form of AKT (myr-AKT) in the tibialis anterior (TA) muscle of wild-type, S6K1 knockout, and S6K1/2 double-knockout mice. As can be seen in Figure 1A, the cross-sectional area of the transfected fibers (in red) 10 days after electroporation are significantly bigger than the surrounding non-transfected fibers. We quantified the increase in fiber size in the three different groups, and as can be seen in Figure 1B, both transfected and non-transfected fibers are significantly bigger in wild-type as compared to S6K1 and S6K1/2 knockout mice. In contrast, no significant differences were observed in the size of transfected or non-transfected fibers between S6K1 and S6K1/2 knockout mice, confirming that S6K1 is the major kinase involved in the regulation of cell size. When comparing the relative increase in fiber size between myr-AKT-transfected and non-transfected fibers in the various groups (Figure 1C), no significant differences were observed, suggesting that S6K1 is not required for AKT-induced fiber hypertrophy. In mice lacking S6K1, S6K2 can compensate for the absence of S6K1 by phosphorylating ribosomal protein S6, as shown in the western blot in Figure 1D. As can be seen, in mice lacking both S6K1 and S6K2 the phosphorylation of ribosomal protein S6 is undetectable. The fact that the transfected fibers in mice lacking S6K1 and those lacking S6K1/S6K2 show the same level of hypertrophy suggests that phosphorylation of S6 on Ser240/244 is not required for AKT-induced fiber growth. Furthermore, using a small interfering RNA (siRNA) approach for S6K1/2 we find that hypertrophy also occurs when S6K is reduced in adult muscles, therefore excluding a possible role of compensation mechanisms in the global S6K1/2 KO animals (Figures 1E, 1F, S1A, and S1B).

Can this apparently minor role of S6K1 in the regulation of fiber size be explained by increased signaling through mTOR-dependent phosphorylation of the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1)? In order to elucidate this, we co-transfected wild-type and S6K1 KO animals with myr-AKT and a constitutively active mutant version of 4E-BP1, in which four mTORC1-dependent phosphorylation sites are changed to alanines (4E-BP1-4A). As can be seen in Figure 1G, co-expression of 4E-BP1-4A does not reduce myr-AKT-induced hypertrophy in wild-type animals, as transfected fibers show a 116% ± 13% increase in fiber size (n = 5 muscles per group). However, when co-transfecting myr-AKT with 4E-BP1-4A in S6K1 KO animals, muscle hypertrophy is reduced by 61% ± 8% (Figure 1G, n = 5), showing that after AKT activation, mTOR signaling to 4E-BP1 can indeed compensate for the lack of S6K1 in muscle growth. Importantly, this differential effect of 4E-BP1-4A on fiber growth is not due to changes in its protein stability in wild-type and the S6K1 KO mouse (Figure S1C).

S6K1 and 4E-BP1 Are Key Mediators of Rapamycin-Sensitive Hypertrophy

In order to better understand whether rapamycin reduces hypertrophy in a S6K-dependent manner, we electroporated wild-type and knockout mice with myr-AKT, while simultaneously injecting mice intraperiteonally with 2 mg/kg body weight of rapamycin. Muscles taken from mice treated with rapamycin showed no phosphorylation of S6, demonstrating a proper delivery of the drug to the muscles (Figure 2A). As can be seen in Figure 2B, transfected fibers in the vehicle-treated muscles showed a significant hypertrophy, which is partially prevented by rapamycin treatment. Quantification of fiber size revealed that a significant hypertrophy persists in rapamycin-treated wild-type mice yet is strongly reduced by 61% ± 7% when compared to vehicle-treated animals (Figures 2C and 2D, n = 6). This inhibitory effect of rapamycin on fiber growth is not dose dependent (Figure S2). To understand whether this strong reduction in fiber size of AKT-transfected fibers after rapamycin treatment in wild-type animals is due to a block on S6K and S6 phosphorylation, we repeated the same experiment in S6K1 and S6K1/2 knockout mice. As can be seen in Figures 2B–2D (n = 5), rapamycin treatment reduced hypertrophic growth in S6K1 and S6K1/2 knockout mice, respectively, by 73% ± 9% and 71% ± 7%. This reduction in fiber hypertrophy is not different from that seen in wild-type

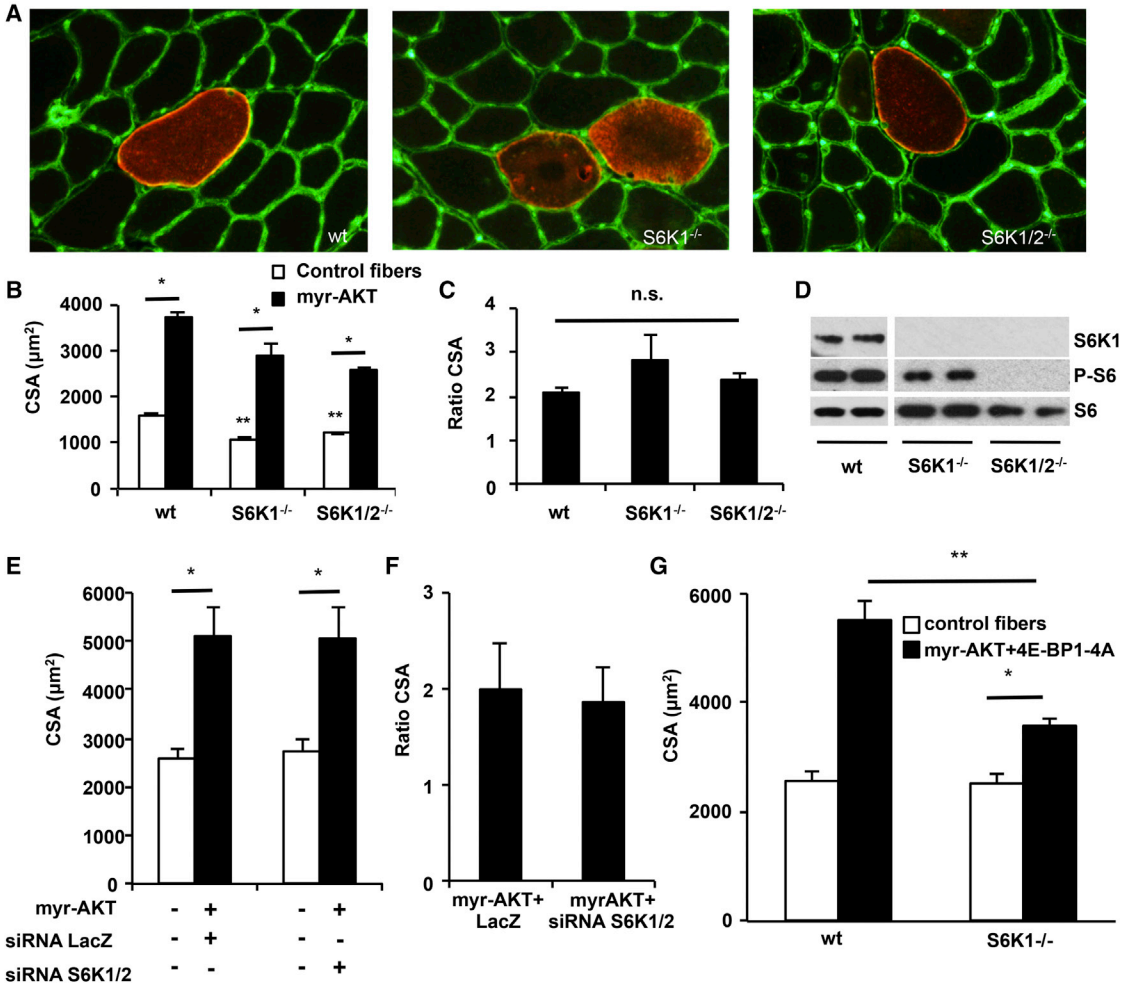

Figure 1. S6K and 4E-BP1 Phosphorylation Can Independently Mediate AKT-Induced Adult Skeletal Muscle Growth

(A) Sections taken from muscles electroporated with a plasmid coding for myr-AKT and taken out 10 days later. A significant fiber hypertrophy is found in transfected fibers (stained for anti-HA in red, anti-laminin in green) compared to non-transfected control fibers in wild-type (left), S6K1 knockout mice (middle), and S6K1/2 double-knockout mice (right).

(B) Cross-sectional area of myr-AKT-transfected and control non-transfected fibers (wild-type [WT]; n = 6 muscles, S6K1 KO; n = 7 muscles, S6K1/2 KO; n = 6 muscles; at least 200 fibers were counted per group, and more than 30 per muscle, *$p < 0.001$). Control fibers in S6K1 and S6K1/2 KO mice were significantly smaller compared to wild-type (**$p < 0.01$).

(C) Fold increases of fiber size in AKT-positive fibers compared to non-transfected control fibers in the same muscle.

(D) Western blot showing the phosphorylation of S6, which persists in the S6K1 KO mice, but is completely absent in the S6K1/2 KO.

(E) Average fiber size of fibers co-transfected with myr-AKT and a siRNA for S6K1/2 or for LacZ, compared to surrounding non-transfected fibers from the same muscle (n = 4 muscles for each group, *$p < 0.001$).

(F) Co-transfection of the siRNA for S6K1/2 and the plasmid coding for myr-AKT showed a 2-fold increase in transfected fibers, similar to that found in fibers co-transfected myr-AKT and a siRNA against LacZ.

(G) Cross-sectional area of fibers co-transfected with myr-AKT and a plasmid coding for a mutant version of 4E-BP1, in which four mTORC1-dependent phosphorylation sites are modified in alanines (4E-BP1-4A). Fiber hypertrophy is significantly reduced in S6K1 KO, but not in wild-type animals (*$p < 0.01$ **$p < 0.05$; n = 5 muscles per group).

All data represent mean ± SEM.

animals, showing that rapamycin can inhibit fiber hypertrophy in a S6K1/2-independent manner.

Considering the differential regulation of mTOR-dependent 4E-BP1 signaling in wild-type and S6K1 KO animals, we wondered whether rapamycin reduces muscle hypertrophy by acting on different mTORC1 mediators in wild-type and S6K1 KO animals. In order to answer this, we co-transfected wild-type and S6K1 KO animals with myr-AKT and 4E-BP1-4A, while treating animals with vehicle or rapamycin for 10 days. As can be seen in Figure 2E, rapamycin reduces fiber hypertrophy by 64% ± 10% in wild-type animals, similar to the results obtained when electroporating only myr-AKT (Figure 2D, n = 5), suggesting that rapamycin blocks fiber growth independently of 4E-BP1 phosphorylation. As shown in Figure 1G, co-transfection of myr-AKT with 4E-BP1-4A in S6K1

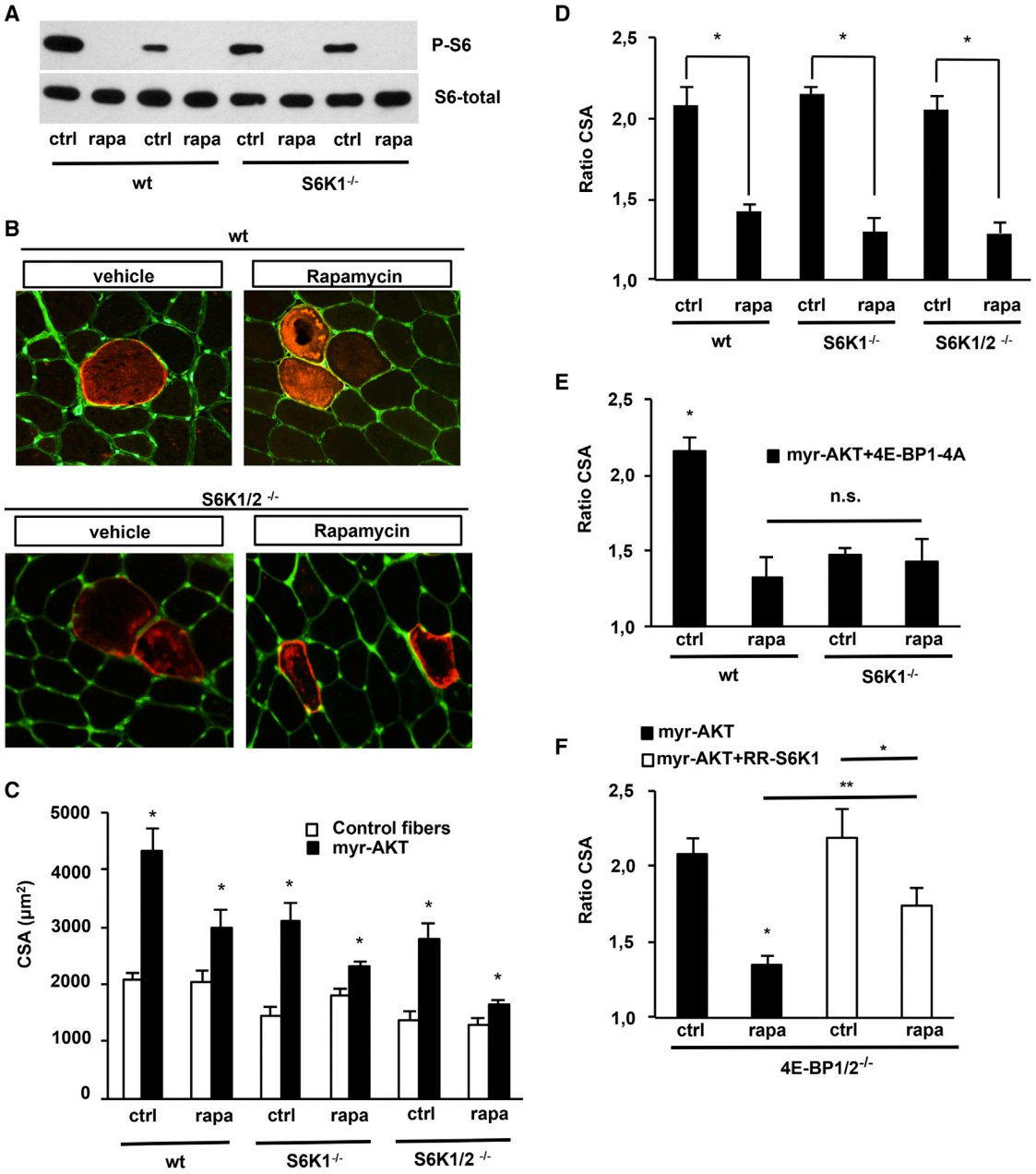

Figure 2. Rapamycin Reduces AKT-Induced Hypertrophy by Inhibiting S6K1 or 4E-BP1 Signaling

(A) Mice were electroporated with myr-AKT while being treated with rapamycin (2 mg/kg per day). As can be seen in the western blot in muscles taken out after 10 days of rapamycin treatment show an effective inhibition of the phosphorylation of S6 in wild-type and S6K1 KO animals.

(B) Representative images showing a significant reduction of hypertrophy in myr-AKT-transfected fibers compared to non-transfected fibers when comparing vehicle (left) or rapamycin-treated mice. Also in S6K1 KO (data not shown) and S6K1/2 KO (lower images), rapamycin significantly reduced AKT-induced hypertrophy.

(C) Cross-sectional area of fibers transfected with myr-AKT compared to non-transfected surrounding fibers. Rapamycin reduces fiber hypertrophy in WT, S6K1 KO, and S6K1/2 KO animals. A significant increase in fiber size of the transfected fibers persists (*$p < 0.005$, n = at least 6 for each group).

(D) Fold increase in CSA of AKT-positive fibers compared to untransfected control fibers in the same muscle show the same reduction in fiber hypertrophy in WT, S6K1, and S6K1/2 KO mice (*$p < 0.005$).

(E) Ratio of fiber size between fibers co-transfected with myr-AKT and 4E-BP1-4A compared to surrounding non-transfected fibers. Rapamycin treatment significantly reduces fiber size in co-transfected wild-type fibers, but does not in co-transfected S6K1 KO fibers (n = at least 5 muscles per group, with minimal 30 transfected fibers per muscle; *$p < 0.001$).

(F) Fold change in CSA of AKT-positive fibers in 4E-BP1/2 KO mice. Co-transfection of a RR-S6K1 and myr-AKT in 4E-BP1 KO mice renders hypertrophy partially resistant to rapamycin (n = at least 5 muscles per group, with minimal 20 transfected fibers per muscle; **$p < 0.01$).

All data represent mean ± SEM.

KO animals reduces fiber hypertrophy by 61% ± 8% (n = 5). Interestingly, performing the same co-transfection in S6K1 KO combined with a rapamycin treatment did not further reduce fiber hypertrophy (Figure 2E).

Next, we wanted to understand whether S6K1 and 4E-BP1 are the only rapamycin-sensitive targets involved in AKT-induced muscle hypertrophy. To render S6K1 and 4E-BP1 rapamycin resistant, we used, respectively, a rapamycin-resistant mutant plasmid of S6K1 (S6K1-RR) and 4E-BP1/2 double-knockout mice. Double-knockout mice were used to avoid the potential compensation by 4E-BP2 upon 4E-BP1 ablation. As can be seen in Figure 2F, overexpression of myr-AKT in 4E-BP1/2 KO mice leads to a significant 109% ± 9% increase in fiber size, which is reduced by 69% ± 3% after rapamycin treatment. In order to understand whether this reduction is due to S6K1, S6K1-RR was transfected together with myr-AKT in 4E-BP1/2 KO animals. As shown in Figure 2F, co-transfection of S6K1-RR does not further increase the hypertrophy found after overexpression of myr-AKT in 4E-BP1/2 KO mice, but it does render hypertrophy partially resistant to rapamycin, as it is reduced by only 38% ± 4% (n = 5 in all groups).

AKT Activation in S6K1 KO Mice Alters Muscle Structure and Function

Various examples of muscle hypertrophy exist in which the increase in muscle mass is not accompanied by an increase in muscle function (Amthor et al., 2007). Considering the fact that activation of S6K1 is linked with a functional muscle hypertrophy in humans (Burd et al., 2010), we wondered whether it might play a role in improving muscle function. To better assess the functional role of S6K1 during muscle hypertrophy induced by AKT activation, we generated a transgenic mouse in which AKT can be activated in an S6K1 KO background. To obtain the Akt-S6K1 KO mice, we crossed three different transgenic lines, i.e., a line expressing mlc1f-cre, a second expressing a myr-AKT-ER with a stop sequence floxed in front, and a third line lacking S6K1 (Figure 3A). Injection of tamoxifen in Akt-S6K1 KO mice leads to a rapid stabilization and phosphorylation of myr-AKT-ER and to the activation of downstream targets, such as the phosphorylation of ribosomal protein S6 (Figure 3B). Furthermore, measurement of puromycin incorporation 24 hr after one injection of tamoxifen showed a clear increase in protein synthesis in muscles taken from Akt-cre and Akt-S6K1 KO mice, as compared to controls (Figure 3C). Continued treatment of Akt-cre and Akt-S6K1 KO mice for 3 weeks with tamoxifen induces a significant hypertrophy in both fast and slow muscles (Figure 3D). Quantification of the wet weight of the gastrocnemius muscle showed, respectively, a 55% ± 6% and 70% ± 4% increase in Akt-cre and Akt-S6K1 KO mice after 3 weeks of tamoxifen treatment (Figures S3A and S3B, n = 8). In order to determine muscle histology in these hypertrophic muscles, we performed a succinate dehydrogenase and H&E staining (Figures 3E and S3C). Although overall muscle structure appeared normal in gastrocnemius muscles from tamoxifen-treated Akt-S6K1 KO mice, occasional areas with muscle damage and regenerating fibers were present (Figure 3F). Indeed, staining for embryonic myosin heavy chain, which is only expressed in early stages of regenerating fibers, showed various positive fibers in certain areas of Akt-S6K1 KO muscles (Figure 3G). Furthermore, there were fibers lacking staining for eosin, indicating fiber necrosis (Figure S3D), something very rarely observed in tamoxifen-treated Akt-cre mice, and absent in control S6K1 KO mice. In order to quantify the myopathic features in the hypertrophic muscles, the total number of central nuclei per muscle section was determined (Figure 3H, n = 8). While there was an increase in the number of centrally nucleated fibers in the gastrocnemius of Akt-cre mice (0.4% ± 0.1%), this number increased significantly in Akt-S6K1 KO muscles (1.8% ± 0.6%), confirming the presence of muscle fiber necrosis and subsequent regeneration. Furthermore, when we examined muscle ultrastructure by electron microscopy, we found four out of 23 fibers with large empty spaces in between individual myofibrils of KO mice (Figure S3E), confirming the presence of occasional fibers in a pathological state.

Considering these myopathic features in hypertrophic Akt-S6K1 KO muscles, we asked whether muscle function was compromised. We previously showed that the activation of AKT for 3 weeks is sufficient to increase muscle force (Blaauw et al., 2009). As can be seen in Figure 3I, absolute maximal force of the gastrocnemius muscle measured in vivo increased in both wild-type and KO mice after AKT activation. However, when normalizing muscle force for muscle weight, a significant decrease in muscle tension was found at all stimulation frequencies in hypertrophic Akt-S6K1 KO mice and not in wild-type animals (Figures 3J and 3K).

S6K1 Is Required for AKT-Induced Increases in Ribosome Biogenesis

In order to better understand the reason for the decrease in normalized muscle force in Akt-S6K1 KO muscles, despite only a relatively small percentage of necrotic fibers, we analyzed signaling changes in Akt-cre and Akt-S6K1 KO muscles. As can be seen in Figure 4A, many of the known downstream targets of Akt involved in protein synthesis, such as GSK-3β, mTOR, and RPS6, show a similar phosphorylation or expression level in both Akt-cre and Akt-S6K1 KO muscles after AKT activation. However, in line with findings from our electroporation experiments, phosphorylation of 4E-BP1 on Ser65 is significantly higher in Akt-S6K1 KO than in Akt-cre muscles (Figures 4A and 4B), suggesting an increase in mTOR signaling to 4E-BP1.

Recently, a role for S6K1 has been proposed in the stimulation of ribosome biogenesis after refeeding (Chauvin et al., 2014). An important step in ribosome biogenesis is the S6K1-dependent stimulation of pyrimidine synthesis by phosphorylating and activating the multifunctional enzyme CAD (carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, dihydroorotase) on serine 1859 (Ben-Sahra et al., 2013). In Akt-cre mice treated for 3 weeks with tamoxifen, there is a significant increase in CAD phosphorylation (Figure 4C, n = 6), corresponding to a 44% ± 11% increase in the ratio of RNA/DNA (Figure 4D). Normalizing total DNA and RNA for muscle weight showed that this is due to an increase in total RNA content, not due to changes in DNA content (Figures S4A and S4B). Considering the fact that 80%–85% of total RNA is ribosomal RNA, this increase in total RNA can be considered a marker of increased ribosome biogenesis. Interestingly, in Akt-S6K1 KO

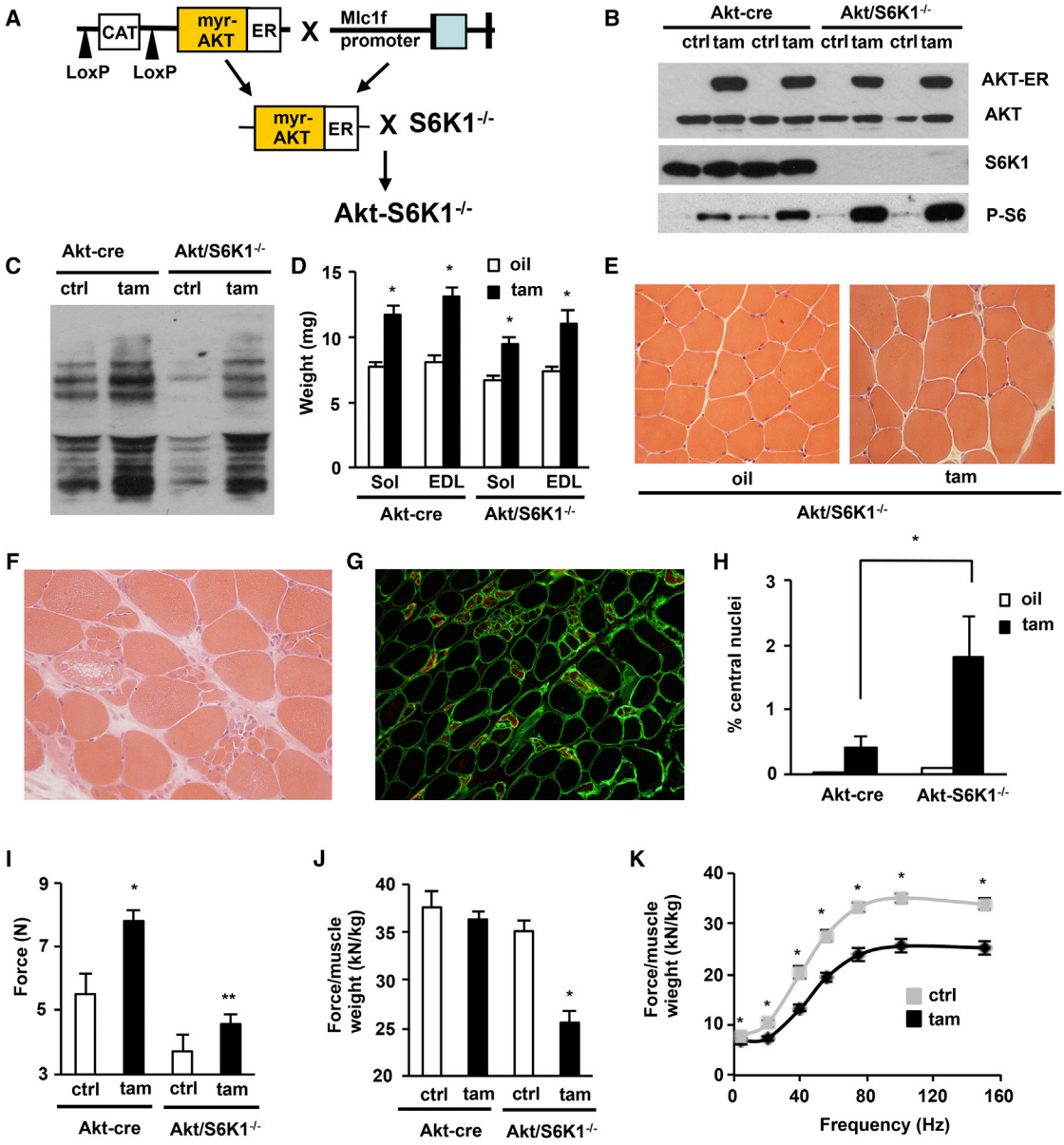

Figure 3. Lack of S6K1 during Muscle Hypertrophy Alters Muscle Structure and Function

(A) Schematic representation of the generation of a muscle-specific inducible AKT transgenic line and its crossing with S6K1 KO mice, giving rise to Akt-S6K1 KO mice.

(B) Immunoblot analysis of protein extracts from gastrocnemius muscle of Akt-cre and Akt-S6K1 KO mice treated with tamoxifen for 3 weeks. Tamoxifen treatment stabilizes AKT-ER, whereas Akt-ER is undetectable in oil-treated controls. Ribosomal protein S6 is phosphorylated after AKT activation independently of the presence of S6K1.

(C) Activation of AKT in wild-type and S6K1 KO animals leads to a significant increase in puromycin incorporation after 24 hr. Images are representative for four individual experiments.

(D) AKT activation for 3 weeks leads to muscle hypertrophy both in fast EDL muscles, as well as in the slow soleus muscle. Each bar represents the wet weight of muscles from tamoxifen-treated mice versus their respective controls (n = 8 for each muscle, *p < 0.005).

(E) H&E staining of muscles taken from Akt-S6K1 KO gastrocnemius muscles after oil or tamoxifen treatment.

(F) Areas showing muscle degeneration (eosin-negative fibers) and regeneration (central nuclei) are found in Akt-S6K1 KO muscles.

(G) Area of muscle showing numerous fibers expressing the embryonic myosin heavy chain isoform (red). In green the muscle lamina is stained.

(H) Number of central nuclei per section in the gastrocnemius of Akt-cre and Akt-S6K1 KO mice after 3 weeks of tamoxifen treatment (n = 8 per group, *p < 0.05).

(I) Force measurements performed in vivo show that AKT activation for 3 weeks leads to a significant increase in absolute tetanic force of the gastrocnemius muscle in both Akt-cre and Akt-S6K1 KO mice (stimulation frequency 100 Hz, n = 12 per group, *p < 0.001, **p < 0.05).

(J) When normalizing force for muscle weight Akt-S6K1 KO mice show a significant reduction in tetanic muscle tension after 3 weeks of tamoxifen treatment (*p < 0.001).

(K) Force-frequency curve of the normalized force in Akt-S6K1 KO mice after 3 weeks of tamoxifen treatment (n = 22 muscles per group; *p < 0.001).

All data represent mean ± SEM.

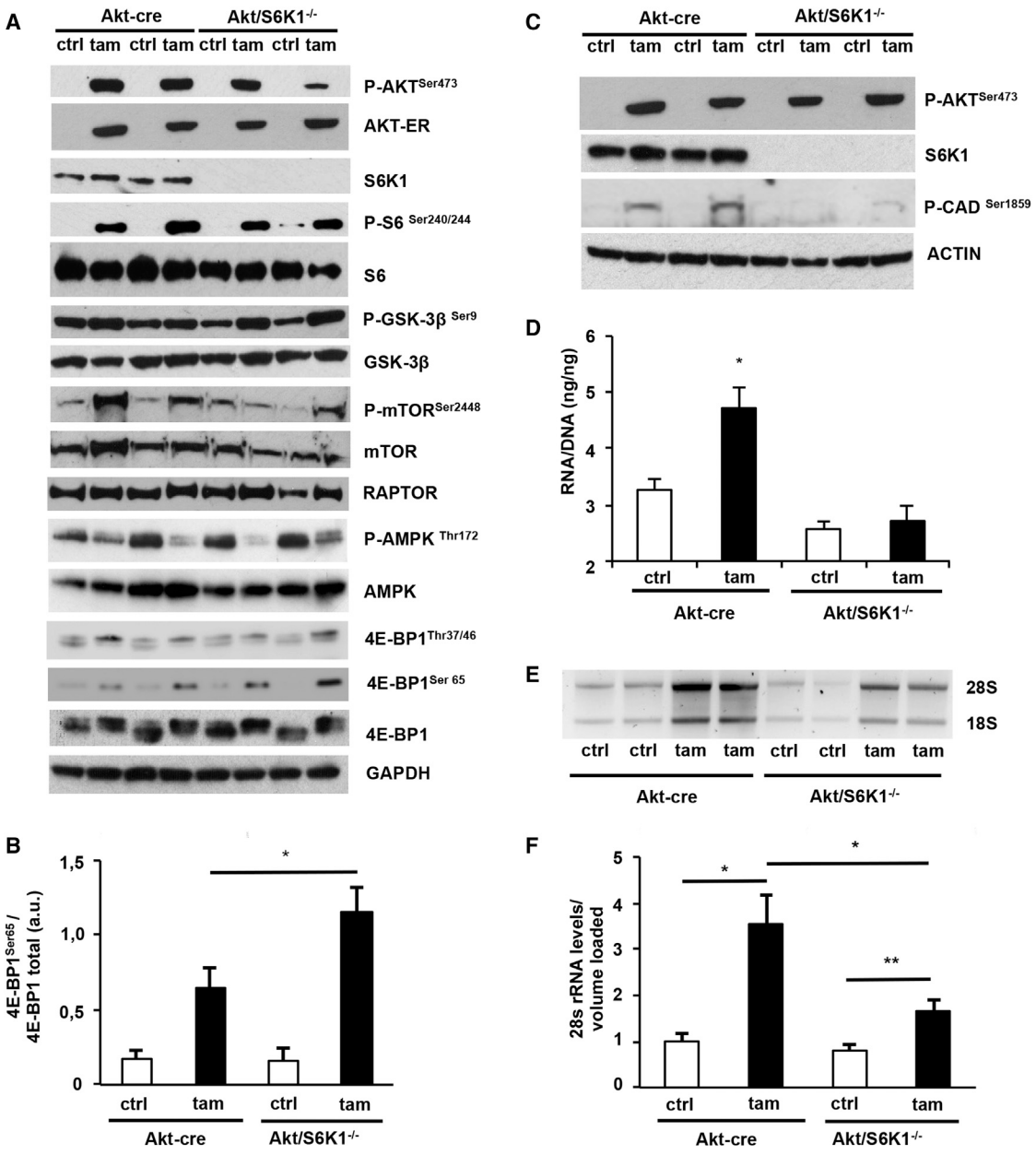

Figure 4. S6K1 Is Required for AKT-Induced Increases in RNA Synthesis

(A) Western blots of the AKT-mTORC1 pathway performed on gastrocnemius muscle taken after 3 weeks of tamoxifen treatment (representative images of multiple blots, n = 4 per group).

(B) Quantification of the phosphorylation of 4E-BP1 on serine 65 in Akt-cre and Akt-S6K1 shows a significant increase in mTORC1 signaling to 4E-BP1 in S6K1 KO mice (n = 4 per group, *p < 0.01).

(C) Phosphorylation of CAD on serine 1859 after 3 weeks of Akt activation.

(D) Quantification of the ratio of total RNA and total DNA in gastrocnemius muscles of Akt-cre and Akt-S6K1 after 3 weeks of tamoxifen treatment (n = 6 per group, *p < 0.01).

(E) Amount of 18S and 28S ribosomal RNA in Akt-cre and Akt-S6K1 KO mice show a blunted increase in RNA synthesis in Akt-S6K1 KO muscles.

(F) Quantification of 28S rRNA per loaded volume (n = 4 muscles per group, *p < 0.001; **p < 0.01).

All data represent mean ± SEM.

mice, activation of AKT is not able to increase phosphorylation of CAD on Ser1859 (Figure 4C) and also no significant increase in the ratio RNA/DNA is observed (Figure 4D). In order to understand whether the increase in RNA in Akt-cre animals corresponds to ribosomal RNA, we analyzed the changes in 18 s and 28 s rRNA. As can be seen in Figures 4E and 4F, AKT

activation in wild-type animals leads to a very strong increase in both 18 s and 28 s, which is strongly compromised in Akt-S6K1 KO mice (Figures 4E, 4F, and S4C).

S6K1 Is Required for Maintaining Protein Quality during High Rates of Muscle Growth

It has been suggested that the number of ribosomes and the speed of translational elongation can impinge on translation fidelity, leading to an increase of dysfunctional proteins (Conn and Qian, 2013; Orsolic et al., 2016). In other cell types, it has been shown that protein aggregates that form during protein synthesis, due to translational errors and folding problems, are positive for the scaffold protein p62 (Pankiv et al., 2007). Interestingly, when we examine the amount of p62 in Akt-S6K1 KO muscles, we find a more pronounced increase than that seen in Akt-cre muscles (Figures 5A and 5B). Using an immunohistochemistry analyses to evaluate the distribution of this increased amount of p62, we find that p62 is distributed homogenously in Akt-cre muscles without showing aggregates. However, in S6K1 KO muscles we observe a very strong accumulation of p62-positive aggregates in some fibers, but not all (Figure 5C). Quantification of the number of fibers presenting these aggregates reveals that they are present in 27% ± 0.6% of S6K1 KO fibers after 3 weeks of AKT activation (Figure 5D). Furthermore, fiber-type analyses showed that they are mainly found in a subset of type IIB fibers (Figure S5). These results are in line with the previous finding in which some but not all fibers showed a compromised ultrastructure (Figure S3B). In order to understand whether these aggregates are due to the high rate of protein synthesis and can be linked to the reduction of muscle tension, we treated mice for 3 weeks with tamoxifen, while reducing protein synthesis rate by co-treating mice with rapamycin. As can be seen in Figure 6A, rapamycin reduces, but does not prevent the increase in protein synthesis in Akt-S6K1 KO mice after 3 weeks of AKT activation, leading to a significant increase in the muscle weight/body weight ratio (Figure 6B). As can be seen in the western blot performed on these muscles, accumulation of p62 in S6K1 KO muscles is strongly reduced after rapamycin treatment (Figure 6C). Furthermore, immunohistochemistry analyses showed no fibers containing p62-positive aggregates in the rapamycin-treated S6K1 KO muscles (data not shown). Next, we determined muscle force in the gastrocnemius muscles after 3 weeks of co-treatment with tamoxifen and rapamycin. As can be seen in Figure 6D, normalized tetanic muscle force in the rapamycin-treated Akt-S6K1 KO group is completely preserved. Taken together, these results suggest that S6K1 is required for stimulating ribosome biogenesis and preserving translation fidelity, thereby maintaining proper muscle function during muscle hypertrophy (Figure 6E).

DISCUSSION

The AKT-mTORC1 pathway plays a major role in muscle hypertrophy in response to IGF-1 signaling, as shown by the inhibitory effect on muscle growth by rapamycin. Furthermore, this pathway also mediates the hypertrophic effects seen after loss of myostatin, or by overexpression of inhibitors of transforming growth factor β (TGF-β) signaling, such as follistatin (Winbanks et al., 2012). Despite this critical role of AKT-mTORC1 signaling in regulating adult muscle mass, the downstream mediators of this muscle hypertrophy and their functional role in adult skeletal muscle are not known.

Here, we show that one of the main downstream effector of mTORC1 signaling in other cell types, the kinase S6K1, is not required for AKT-induced increases in muscle mass. Furthermore, S6K-independent muscle growth remains sensitive to rapamycin, due to compensatory signaling of mTORC1 to 4E-BP1. While S6K1 is not required for muscle growth, we find that it is required for increasing muscle force. Muscle hypertrophy in the absence of S6K1 is accompanied by a defective ribosome biogenesis and formation of protein aggregates. Reducing rates of protein synthesis by rapamycin prevents the formation of protein aggregates and leads to smaller, yet completely functional hypertrophic muscles.

S6K1 and 4E-BP1 Can Independently Mediate Rapamycin-Sensitive Muscle Hypertrophy

Numerous studies have shown an important role for S6K1 in the regulation of organ size (Fenton and Gout, 2011). While its role on cell size during development has been well documented, results obtained in adult tissues are less straight forward. As mentioned in the introduction, the loss of S6K1/2 leads to an increase in AMPK activation, which reduces mTORC1 signaling (Aguilar et al., 2007). The reduction in fiber size found in S6K1/2 KO muscles can be completely rescued by the overexpression of a dominant-negative form of AMPK, restoring S6K-independent mTORC1 signaling. Furthermore, overexpression of follistatin in adult skeletal muscle, either by plasmid electroporation or by AAV-mediated delivery, leads to a 2- to 3-fold increase in fiber size, which requires the IGF1 receptor and is strongly reduced by co-transfecting a dominant-negative form of AKT (Kalista et al., 2012). Interestingly, this very significant increase in fiber size is completely unaffected by the loss of S6K1/2. Here, we show using various approaches that S6K1/2 are not required for AKT-induced skeletal muscle hypertrophy, due to compensatory mTORC1 signaling to 4E-BP1.

A possible explanation for these results is that S6K1 and 4E-BP1 compete for the binding to the core mTORC1 component RAPTOR, which subsequently increases the mTOR-dependent, rapamycin-sensitive phosphorylation of either S6K1 or 4E-BP1 (Hara et al., 2002). Lack of either S6K1 or 4E-BP1 signaling would therefore lead to an increase in signaling through the other remaining pathway, which then remains rapamycin sensitive, as it depends on mTOR-RAPTOR binding. Results obtained from the two knockout mice support this model in which S6K1 and 4E-BP1 compete for RAPTOR binding. In mice lacking 4E-BP1/2, an upregulation of the phosphorylation of S6K1 and S6 has been shown, possibly contributing to the insulin resistance seen in these mice (Le Bacquer et al., 2007). On the other hand, in S6K1 KO animals there is a slight increase in 4E-BP1 phosphorylation (Ben-Hur et al., 2013), which is possibly more pronounced in conditions of elevated mTORC1 signaling.

A similar competition between S6K1 and 4E-BP1 signaling might be active during cardiomyocyte hypertrophy. Increases

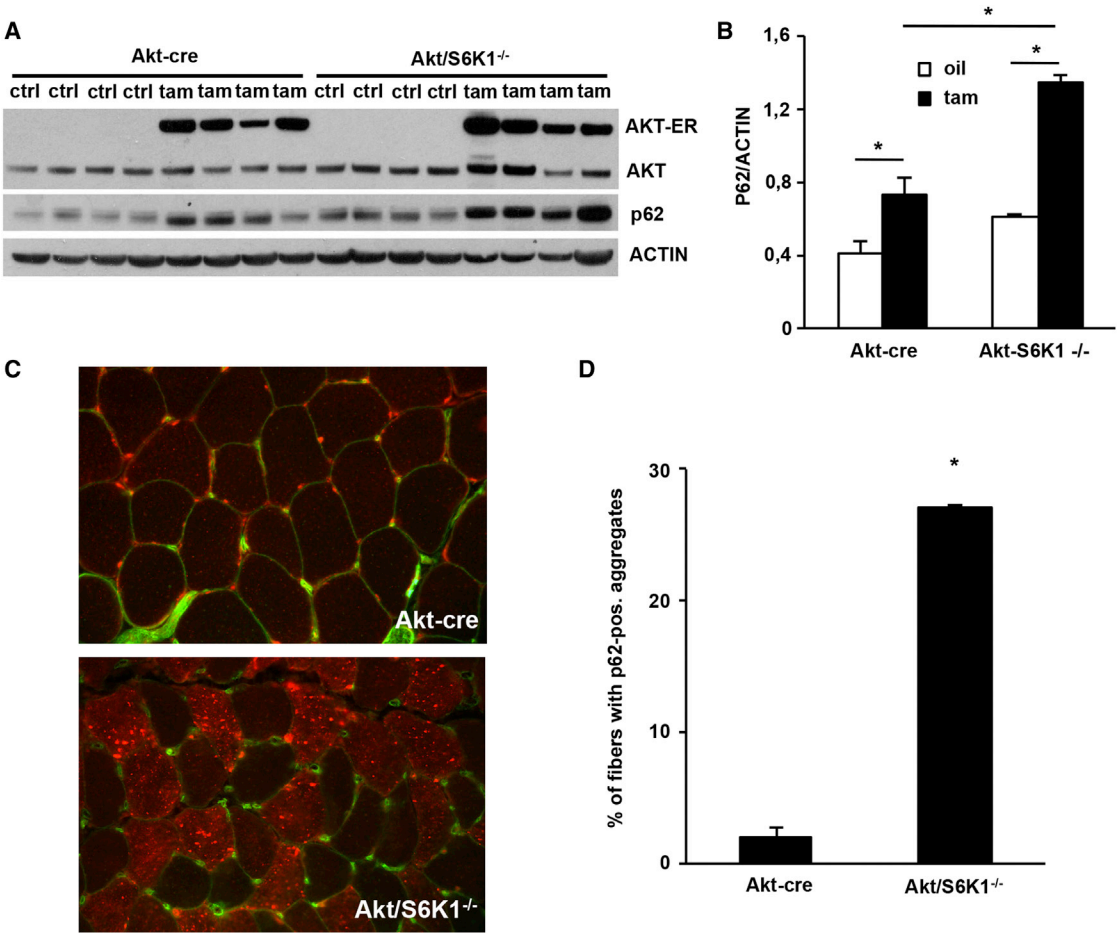

Figure 5. Strong Accumulation of p62 in Akt-S6K1 KO Muscles

(A) Three weeks of AKT activation leads to a significant increase in p62-protein content in Akt-S6K1 KO muscles as compared to Akt-cre muscles.

(B) Quantification of p62 in western blotting in Akt-cre and Akt-S6K1 KO muscles after 3 weeks of AKT activation (n = 5 per group, *p < 0.001).

(C) Immunohistochemistry images for p62 show the presence of fibers containing numerous p62-positive aggregates in Akt-S6K1 KO muscles. Staining for p62 in Akt-cre mice only showed a diffuse staining in almost all fibers.

(D) Quantification of the number of fibers showing these p62 aggregates shows a major increase in positive fibers in Akt-S6K1 KO gastrocnemius muscles (*p < 0.001, n = 6 muscles per group).

All data represent ± SEM.

in protein synthesis by phenylephrine in cardiac myocytes do not require the formation of the eIF4F-complex, as the overexpression of 4E-BP1 does not have any effect (Huang et al., 2009). Similarly, overload hypertrophy of cardiomyocytes by aortic constriction or prolonged swimming is unaffected by the lack of S6K1/2 (McMullen et al., 2004). Rapamycin treatment, on the other hand, does significantly reduce cardiac hypertrophy after overloading or activation of Akt, suggesting that inhibition of both S6K1-and 4E-BP1 signaling is sufficient to prevent cardiac hypertrophy (Shioi et al., 2003).

We show here that inhibition of mTOR signaling to 4E-BP1 and S6K1 is sufficient to reduce muscle growth and render it insensitive to rapamycin. However, overexpression of a rapamycin-resistant mutant of S6K1 in 4E-BP1/2 double-KO mice, which renders both mTORC1 targets resistant to the inhibitory effect of rapamycin, only partially prevents the inhibitory effect of rapamycin on muscle growth. The fact that rapamycin can only partially reduce hypertrophy in this situation underlines the importance of S6K1 and 4E-BP1 on rapamycin-sensitive muscle growth. However, that rapamycin can still reduce muscle hypertrophy significantly when compared to vehicle-treated mice, despite not being able to act on S6K1 or 4E-BP1, suggests that there are other permissive mTOR signaling events that are required for muscle growth. It has been shown that mTOR is the rapamycin-sensitive mediator of overload-induced hypertrophy (Goodman et al., 2011), suggesting that, besides S6K1 and 4E-BP1, other mTOR-dependent signaling pathways play a role in muscle growth. Future studies are required to address two major open issues raised by these results: (1) which are the additional signaling pathways involved in rapamycin-sensitive muscle growth and (2) which are the AKT-dependent, mTOR-independent mediators of muscle growth?

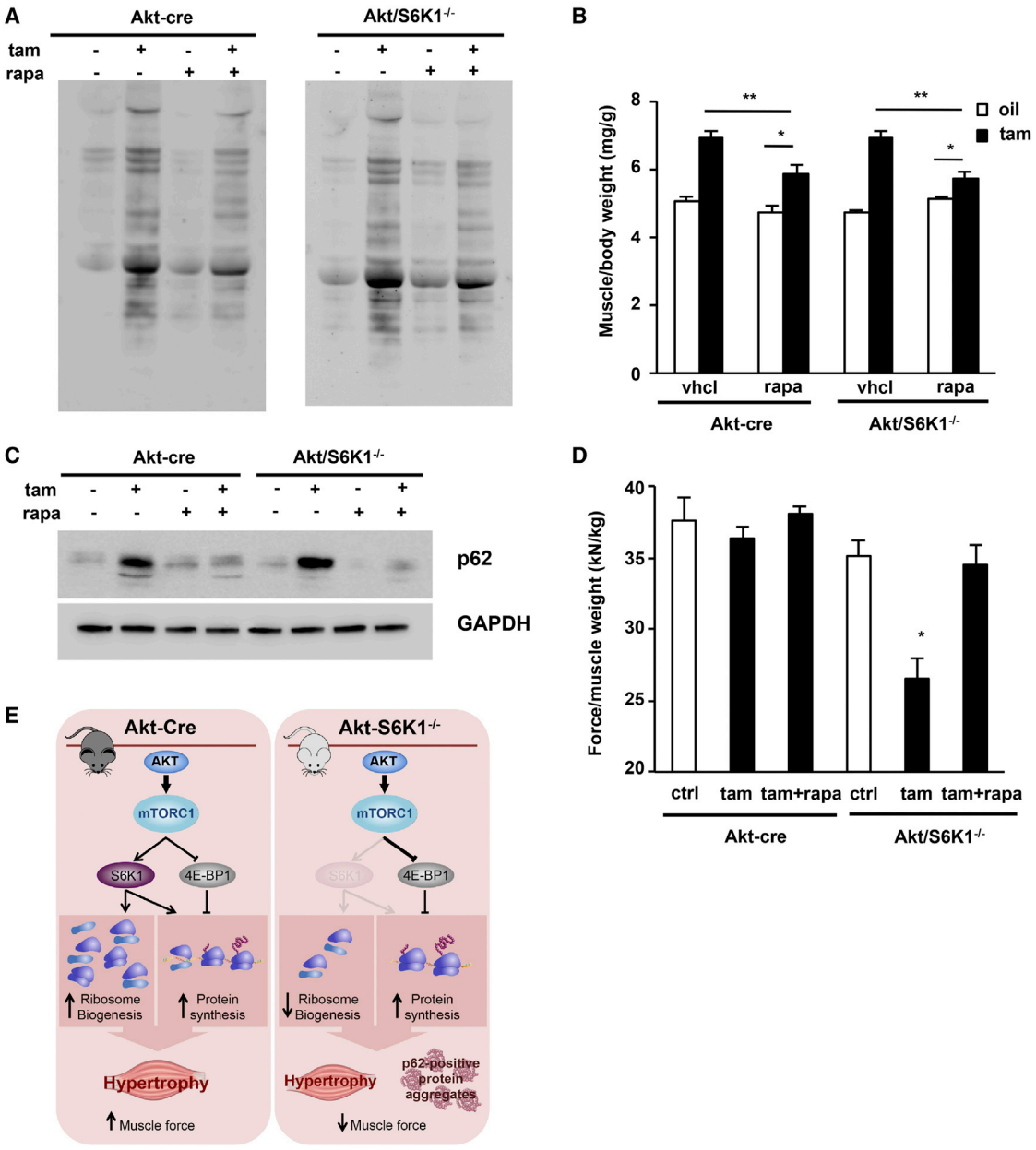

Figure 6. Rapamycin Treatment Prevents Muscle Myopathy in Akt-S6K1 Mice

(A) Representative blot of puromycin incorporation after treatment of Akt-cre and Akt-S6K1 KO mice for 3 weeks with tamoxifen while administering rapamycin (2 mg/kg body weight). As can be seen in the blot, rapamycin reduces protein synthesis in both Akt-cre and Akt-S6K1 KO gastrocnemius muscles.

(B) Three weeks of AKT activation while simultaneously treating mice daily with rapamycin reduces the ratio between the weight of the gastrocnemius muscle compared to whole-body weight (n = 12, *p < 0.005 comparing control and tam in rapamycin-treated animals; n = 12, **p < 0.01 comparing tam+vehicle and tam+rapa).

(C) p62 content after rapamycin treatment is strongly reduced after rapamycin treatment.

(D) The loss in normalized muscle force observed in gastrocnemius muscles from Akt-S6K1 KO is completely prevented by rapamycin treatment (*p < 0.001).

(E) Scheme depicting the functional role of S6K1 in increasing muscle force during hypertrophy.

All data represent mean ± SEM.

S6K1 Is Required for Ribosome Biogenesis and Increasing Skeletal Muscle Function

The phosphorylation and activation of S6K1 occurs in almost all forms of muscle growth and is even considered as a marker for the anabolic response of muscle to resistance exercise (Terzis et al., 2008). Since the activation of S6K1 is not required for the increase of adult muscle mass, we investigated whether S6K1 might be required for the induction of a functional muscle

hypertrophy. We have previously shown that activation of AKT for 3 weeks is sufficient to induce an increase in muscle mass and force (Blaauw et al., 2009). Despite the fact that hypertrophic growth seen in Akt-S6K1 KO mice was similar to that seen in Akt-cre mice, normalized muscle force in KO muscles was significantly repressed. A possible explanation for this reduction in muscle tension is the presence of numerous fibers with p62-positive aggregates in Akt-S6K1 KO muscles, as it is well known that muscles with an increase in protein aggregates show a decrease in force production (Masiero et al., 2009). This raises the question of how the lack of S6K1 can lead to the formation of p62-positive aggregates. When newly synthetized proteins are truncated or fail to fold correctly they tend to form aggresome-like induced structures (ALIS), which require p62 for their formation (Pankiv et al., 2007). Interestingly, the formation of these ALIS is more pronounced in conditions in which autophagy is inhibited (Wenger et al., 2012), as is the case for both Akt-cre and Akt-S6K1 KO muscles. Considering that protein synthesis rates are similar in both transgenic lines, this suggests that S6K1 influences the quality of newly formed proteins.

How can S6K1 influence protein quality and thereby muscle force? It has been shown that S6K1 regulates the biosynthesis of pyrimidines through the phosphorylation of the enzyme CAD, increasing the amount of DNA or RNA, independently of 4E-BP1 signaling (Ben-Sahra et al., 2013). Indeed, here we show that in hypertrophic Akt-S6K1 KO muscles the ratio of RNA/DNA does not increase, possibly due to a block of CAD-Ser1859 phosphorylation, suggesting a defective ribosome biogenesis. This lack of ribosome biogenesis in S6K1 KO animals could potentially influence the quality of mRNA translation. Translation is an error-prone process, with amino acid misincorporations occurring every 10^3 or 10^4 codons translated (Drummond and Wilke, 2009). Various factors influence translation fidelity, such as the total number of ribosomes (Belin et al., 2009) or the speed of translational elongation. S6K1 is thought to play an important role in both processes, namely, by stimulating ribosome biogenesis (Ben-Sahra et al., 2013; Chauvin et al., 2014) and by reducing the speed of translational elongation (Conn and Qian, 2013). It is tempting to assume that skeletal muscle, which requires the synthesis of multiple large sarcomeric proteins for muscle growth, is particularly sensitive to changes in translation speed and fidelity. Indeed, in a model of synergist ablation, where muscle undergoes an explosive increase in size, there is a very significant increase in ribosome biogenesis (Goodman et al., 2011; Kirby et al., 2015), possibly to sustain elevated rates of protein synthesis while maintaining protein quality. Future studies evaluating the content of the protein aggregates, and how these induce muscle dysfunction, will help to better understand in which way the presence of aggregates can impinge on muscle force.

Overall, these results suggest that in conditions of increased protein synthesis, activation of S6K1 is necessary to increase ribosome biogenesis and therefore maintain protein quality and muscle force.

Conclusions

The data reported here show that S6K1 and mTOR-dependent phosphorylation of 4E-BP1 can independently mediate AKT-induced muscle hypertrophy. Importantly, we show that they are two key targets downstream of mTOR for the inhibitory effect of rapamycin, a drug that inhibits most forms of muscle hypertrophy. The importance of S6K1 in maintaining muscle force during hypertrophy underlines the importance of better understanding not only the signaling pathways regulating muscle mass, but also the signaling pathways involved in the regulation of muscle force production. In certain muscular dystrophies, or during aging, a pivotal part of the pathology is due to the reduction in specific force, not just reduced muscle mass.

EXPERIMENTAL PROCEDURES

Transgenic Mice/Treatments

Generation of S6K1/2-deficient mice (C56BL/6-129/Ola) and S6K1 KO mice (C57BL/6) has been previously described (Pende et al., 2004). The generation of Akt-Cre transgenic mice was described previously (Blaauw et al., 2008, 2009). We crossed this Akt-Cre line with S6K1 KO mice in order to obtain the Akt-S6K1 KO mice. To activate AKT, animals were treated with 1 mg of tamoxifen dissolved in sunflower oil once every other day. Each genotype was compared with Cre-negative animals of the same genetic background injected with tamoxifen. Mice lacking 4E-BP1 and 4E-BP2 were described previously (Le Bacquer et al., 2007). Rapamycin treatment was performed by intraperitoneal injections at 2 mg/kg body weight every day. Puromycin was injected i.p. with 0.04 μmol/g puromycin exactly 30 min before removal of muscles. Muscle homogenates were processed for western blot analysis using an antibody specific for puromycin. Experimental protocols were reviewed and approved by the local Animal Care Committee, University of Padova.

Plasmid Constructs

We used hemagglutinin (HA)-tagged plasmid constructs to overexpress myr-AKT (Pallafacchina et al., 2002) and 4E-BP1-4A, mutated on the first four mTORC1-dependent phosphorylation sites (Yanagiya et al., 2012). The sequences used for knocking down S6K1/2 were described previously (Aguilar et al., 2007). The selected oligos were cloned into the pSUPER vector. We co-transfected a plasmid coding for Snap-GFP together with the pSUPER. As a control, we used pSUPER constructs targeting LacZ. The plasmid expressing a rapamycin-resistant mutant of S6K1 has been described previously (Ohanna et al., 2005).

In Vivo Electroporation

Adult mice were used in all experiments. TA muscles were transfected as described previously (Tothova et al., 2006). Muscles were analyzed 7 or 10 days after electroporation.

Antibodies for Western Blot and Immunohistochemistry

We used the following antibodies for immunohistochemistry; laminin from Sigma, Phospho-S6 from Cell Signaling Technology, HA from Santa Cruz Biotechnology, and embryonic MyHC from Developmental Studies Hybridoma Bank. For western blotting, P-AKT (S473), P-AKT (T308), P-S6 (S240/244), S6, S6K1, AKT, GSK-3β, P-GSK-3β (S9), 4E-BP1, RAPTOR, P-mTOR (S2448), mTOR, P-CAD (S1859), P-AMPK (T172), and AMPK were from Cell Signal, P62 was from Sigma, GAPDH was from Abcam, actin was from Santa Cruz, and puromycin from Millipore.

Histology

Cryosections of the TA and gastrocnemius muscles after electroporation, in wild-type animals, and S6K1 and S6K1/2 KO animals were examined by fluorescence microscope. Cross-sectional area was measured for TA as described previously (Pallafacchina et al., 2002) and compared with the area of surrounding non-transfected myofibers. Cross-sectional area of transfected fibers was measured in at least five muscles per group. Cryosections of TA and gastrocnemius muscles were stained for H&E and for succinate dehydrogenase in Akt-Cre and Akt-S6K1 KO animals.

In Vivo Muscle Mechanics

Force measurements performed in vivo on the gastrocnemius muscle were described elsewhere (Blaauw et al., 2009). Briefly, in vivo gastrocnemius muscle contractile performance was measured using a 305B muscle lever system (Aurora Scientific) in mice anaesthetized with a mixture of Xylotine and Zoletil.

RNA-DNA and Ribosomal RNA Quantification

The TA muscle was homogenized in 1 μl of a basic phenol/chloroform buffer to separate nucleic acids. RNA and DNA content were determined using specific fluorescent dyes selective for RNA (Qubit RNA Assay Kits, Invitrogen) or double-stranded DNA (Qubit dsDNA HS Assay Kits, Invitrogen), respectively. Measurements were realized using Qubit 2.0 fluorometer (Invitrogen). In order to highlight changes in rRNA content occurring during the 3-week treatment period, we used mice with exactly the same body weight before starting tamoxifen treatment. Next, we homogenized whole muscles in the same volume and loaded 10 μl of the RNA solution on a 1% agarose gel and viewed it under UV light. Densitometric measurements of the 28S and 18S ribosomal RNA (rRNA) were performed with ImageJ software.

Statistics

All data are expressed as means ± SEMs. Differences between groups were assessed using Student's t test or one-way ANOVA. Significance was defined as a p value of less than 0.05 (95% confidence).

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.020.

AUTHOR CONTRIBUTIONS

B.B. conceived the project and wrote the manuscript. B.B., M.M., M.B., F.S., J.J.M.C., R.S., H.N., and I.N. performed experiments and analyzed data. M.P. and M.K. contributed intellectually and provided technical advice. S.P. participated in manuscript revision. All authors discussed the results and commented on the manuscript.

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