

# Protein Turnover in Skeletal Muscle: Looking at Molecular Regulation towards an Active Lifestyle

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## ABSTRACT

Skeletal muscle is a highly plastic tissue, able to change its mass and functional properties in response to several stimuli. Skeletal muscle mass is influenced by the balance between protein synthesis and breakdown, which is regulated by several signaling pathways. The relative contribution of Akt/mTOR signaling, ubiquitin-proteasome pathway, autophagy among other signaling pathways to protein turnover and, therefore, to skeletal muscle mass, differs depending on the wasting or loading condition and muscle type. By modulating mitochondria biogenesis, PGC-1 $\alpha$  has a major role in the cell's bioenergetic status and, thus, on protein turnover. In fact, rates of protein turnover regulate differently the levels of distinct protein classes in response to atrophic or hypertrophic stimuli. Mitochondrial protein turnover rates may be enhanced in wasting conditions, whereas the increased turnover of myofibrillar proteins triggers muscle mass gain. The present review aims to update the knowledge on the molecular pathways implicated in the regulation of protein turnover in skeletal muscle, focusing on how distinct muscle proteins may be modulated by lifestyle interventions with emphasis on exercise training. The comprehensive analysis of the anabolic effects of exercise programs will pave the way to the tailored management of muscle wasting conditions.

## Introduction

Skeletal muscle (SkM) is the most abundant tissue of the body and the largest protein reservoir in vertebrates (50–75% of whole-body proteins). This is a key tissue in maintaining numerous metabolic functions with impact on the energy homeostasis of the organism [1, 2]. When compared with other tissues such as the liver and gut, SkM protein turnover is relatively slow [3], possibly reflecting the postmitotic nature of muscle fibers. The constant turnover or remodeling of SkM proteins allows the replacement of damaged or obsolete proteins by new ones. Therefore, proteome plasticity sup-

ports SkM adaptation to external stimuli, which may be noticed by changes in its mass and function [4]. Variations in muscle mass result from alterations in the protein amount and content and/or by changes in cell turnover. At the cellular level, the imbalance between myonuclear accretion and loss contributes to SkM hypertrophy and atrophy, respectively. Myonuclear accretion is guided by the stem cells resident on the periphery in close association with the sarcolemma, called satellite cells. Satellite cells are activated and fuse with muscle fibers for regeneration after muscle injury or

during conditions such as load-induced hypertrophy (reviewed by [5, 6]).

At the molecular level, SkM mass is roughly regulated by protein turnover that is driven by the balance between protein synthesis and breakdown, which affects proteins differently depending on their properties. SkM contains three classes of proteins according to their solubility and function. Sarcoplasmic proteins include most of the metabolic enzymes and contributes in 30–35% (by mass) of cases to the total muscle protein pool. This is the protein class with higher turnover. Myofibrillar proteins are the most abundant class of proteins (55–60% by mass) and include around 20 distinct proteins. Stroma proteins are extracellular and comprise collagen and some membrane proteins, and this is the most insoluble class of proteins (followed by myofibrillar proteins) [7]. The rates of SkM protein turnover are different among species, being higher in rodents compared to humans [8]. Moreover, the rates of protein turnover regulate differently the levels of specific proteins [9]. Half-lives of 10 days were reported for actomyosin [10], 54 days for myosin, 3–10 days for creatine kinase [11], 30 days for cytochrome c [12], and 45 days for collagen [13] in rats. Given their different properties, distinct regulatory mechanisms of protein synthesis vs. breakdown interact in protein turnover, with potential impact in muscle mass and functionality. For instance, the synthesis rate of myofibrillar proteins has been associated with strength capacity, whereas the metabolic protein synthesis rate, particularly of mitochondrial proteins, has been linked to maximal oxidative capacity [14].

SkM mass loss or gain reflects the relative contribution of protein synthesis and proteolysis rates [15]. SkM mass loss is usually associated to the decline of protein synthesis; however, some studies reported the increase of protein synthesis when there is a decrease in muscle mass [15, 16]. Enhanced synthesis of myofibrillar and mitochondrial proteins was associated with the age-related mass decrease of the plantaris and tibialis anterior from F344BN F1 rats (from 24 to 28 months of age) [16]. This anabolic response may represent an attempt of muscle fibers to maintain their mass. Gastrocnemius hypertrophy following resistance-loaded voluntary wheel running was linked with reductions in proteolysis rather than increases in protein synthesis levels [17]. Moreover, the relative contribution of protein synthesis and proteolysis to the net protein balance and, consequently, to SkM mass differs depending on the wasting or loading condition and muscle type [15]. Oxidative fibers possess a larger capacity for protein synthesis but also for proteolysis compared to less oxidative fibers. This suggests that oxidative fibers have a relatively higher rate of protein turnover, which may explain their small size compared to glycolytic fibers [18].

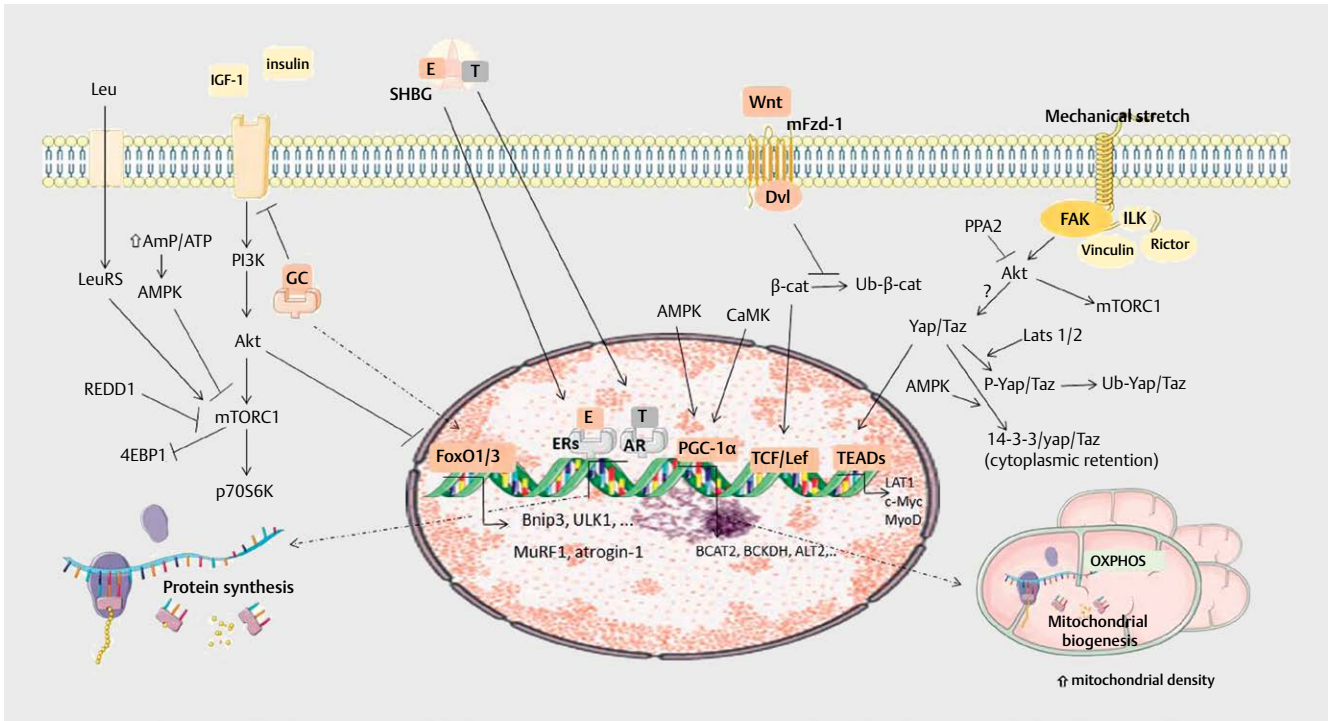
The advances made in recent decades on the comprehension of the molecular mechanisms regulating SkM mass, a major contributor to what is known as muscle plasticity [19], increased the awareness of the intricate network of pathways involved in such control. Contrarily to the ubiquitin-proteasome pathway (UPP) and the Akt/mTOR signaling that have been extensively assessed in loading and wasting conditions, the contribution of other pathways such as the ones mediated by Wnt, Notch and Hippo to SkM mass regulation remains less clear [20–22]. These pathways may also impact cell turnover, specifically satellite cells proliferation and activity. Interventions regulating these molecular mechanisms towards the preservation or increase of SkM mass have been pursued

to prevent and/or treat wasting conditions. Exercise (particularly resistance training) is an intervention that modulates protein turnover towards increased SkM mass [4, 19]. Their therapeutic value has been investigated in the set of aging and wasting conditions such as cachexia associated with chronic diseases [23]. Herein, we review and update the molecular players and signaling pathways implicated in the regulation of protein turnover during periods of increased or decreased mechanical loading.

## The triggering players of protein turnover in skeletal muscle

SkM mass adapts to changes in the pattern of neural stimulation, loading conditions, availability of substrates, and hormonal signals. The role of these signals on muscle plasticity can be perceived in several pathophysiological conditions. SkM responds to the lack of neural stimulation and contractile activity by atrophying due to an imbalance in protein turnover, which is paralleled by a decline of the resistance to fatigue [24]. SkM reacts to mechanical stimulation by changing protein turnover that encompasses integrin-mediated pathways [25]. These transmembrane glycoproteins make the link between extracellular matrix (ECM) and fiber's cytoskeleton. ECM composition is also affected in such conditions. For example, accelerated type IV collagen turnover was reported in SkM from spinal cord-injured subjects after electric stimulation [26]. Among hormones, anabolic testosterone is known to regulate the expression of thousands of genes through its binding to androgen receptors (membrane-bound or cytoplasmic). These genes impact SkM structure, fiber type, metabolism, and mass. Estrogen receptors also act as transcription factors after binding to estrogens, thus regulating SkM function and regrowth of atrophied SkM [27]. Insulin and insulin-like growth factor-1 (IGF-1) stimulate glucose and amino acid uptake and promote a net positive protein balance by upregulating protein synthesis. The anterior pituitary secreted growth hormone acts on membrane receptors activating signaling pathways that culminate in increased SkM mass [9, 24]. Glucocorticoids have a significant impact on SkM mass by blunting insulin-induced protein synthesis, amino acid transport into the fiber, and increasing proteolysis through gene expression regulation [28] (► **Fig. 1**). Pro-inflammatory cytokines act on membrane receptors triggering the activation of catabolic pathways, explaining the SkM wasting seen in chronic diseases such as heart failure or cancer [23, 29] (► **Fig. 2**). Understanding the interplay between the signaling pathways modulated by all these players on protein turnover paves the way to therapeutically mitigate SkM mass loss in aging, disuse, and chronic diseases.

Changes in contractile activity are capable of modulating the synthesis of new muscle proteins with effect on SkM phenotype [30]. The anabolic role of resistance training makes this exercise mode one of the most prescribed therapeutic approaches for SkM mass gain. The synthesis of myofibrillar protein and satellite cell activation are the main drivers of muscle hypertrophy [30]. The effect of endurance training on SkM mass is controversial and depends on exercise intensity. In fact, the health benefits of endurance exercise are mainly related to improved oxygen transport to SkM, increased capillarity, enhanced mitochondrial density, decreased insulin resistance, and myofibrillar remodeling character-



► **Fig. 1** Signaling pathways involved in the regulation of skeletal muscle protein turnover. The anabolic hormones insulin and IGF-1 activate PI3K/Akt/mTOR signaling, increasing protein synthesis. mTORC1 is inhibited by AMPK that senses low ATP levels, and by REDD1, and it is stimulated by amino acids such as Leu. Steroid hormones regulate gene expression with testosterone impacting several gene encodings for anabolic proteins. Akt phosphorylates and inhibits FoxO1 and 3, which regulate the expression of E3 ligases and autophagic proteins. AMPK or CaMK phosphorylate and activate PGC-1 $\alpha$  that regulates the expression of gene encoding for proteins involved in amino acid metabolism and mitochondrial biogenesis. Dvl is activated by the interaction between Wnt and the mFzd-1 receptor and then targets  $\beta$ -catenin, avoiding its translocation to the nucleus, and it activates TCF/Lef. Yap/Taz from Hippo signaling when in the nucleus activates the transcription factor TEADs, regulating the expression of c-Myc, MyoD, and LAT1. If phosphorylated, Yap/Taz is retained in the cytoplasm and may be targeted by UPP. Mechanical stress stimulates integrin signaling, which is mediated by Akt, but not by mTORC1. Parts of the figure were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License [CC BY 3.0]: (<https://creativecommons.org/licenses/by/3.0/>).; **Abbreviations:** 4EBP1, eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1;  $\beta$ -cat,  $\beta$ -catenin; ALT2, alanine transaminase 2; AMPK, 5' AMP-activated protein kinase; AR, androgen receptor; BCAT2, branched chain amino acid transaminase 2; BCKDH, branched-chain  $\alpha$ -ketoacid dehydrogenase; Bnip3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; CaMK, Ca<sup>2+</sup>/calmodulin-dependent protein kinase; Dvl, dishevelled; E, estrogen; ER, estrogen receptor; FAK, focal adhesion kinase; FoxO, Forkhead box protein O; GC, glucocorticoid; ILK, integrin-linked kinase; LAT1, Large Neutral Amino Acid Transporter 1; Lats 1/2, large tumor suppressor 1/2; Leu, leucine; LeuRS, leucyl-tRNA synthetase; mFzd, membrane Frizzled; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; OXPHOS, oxidative phosphorylation; P, phosphate; p70S6K, or S6K1, ribosomal protein S6 kinase beta-1; PI3K, phosphoinositide 3-kinase; PPA2, protein phosphatase A2; REDD1, regulated in development and DNA damage responses 1; SHBG, sex hormone-binding globulin; T, testosterone; TCF/Lef, T cell factor/lymphoid enhancer factor family; TEADs, transcriptional enhanced associate domain; ULK1, Unc-51 like autophagy activating kinase 1; Yap/Taz, Yes-associated protein/transcriptional co-activator with PDZ-binding motif; Ub, ubiquitin; UPP, ubiquitin proteasome pathway.

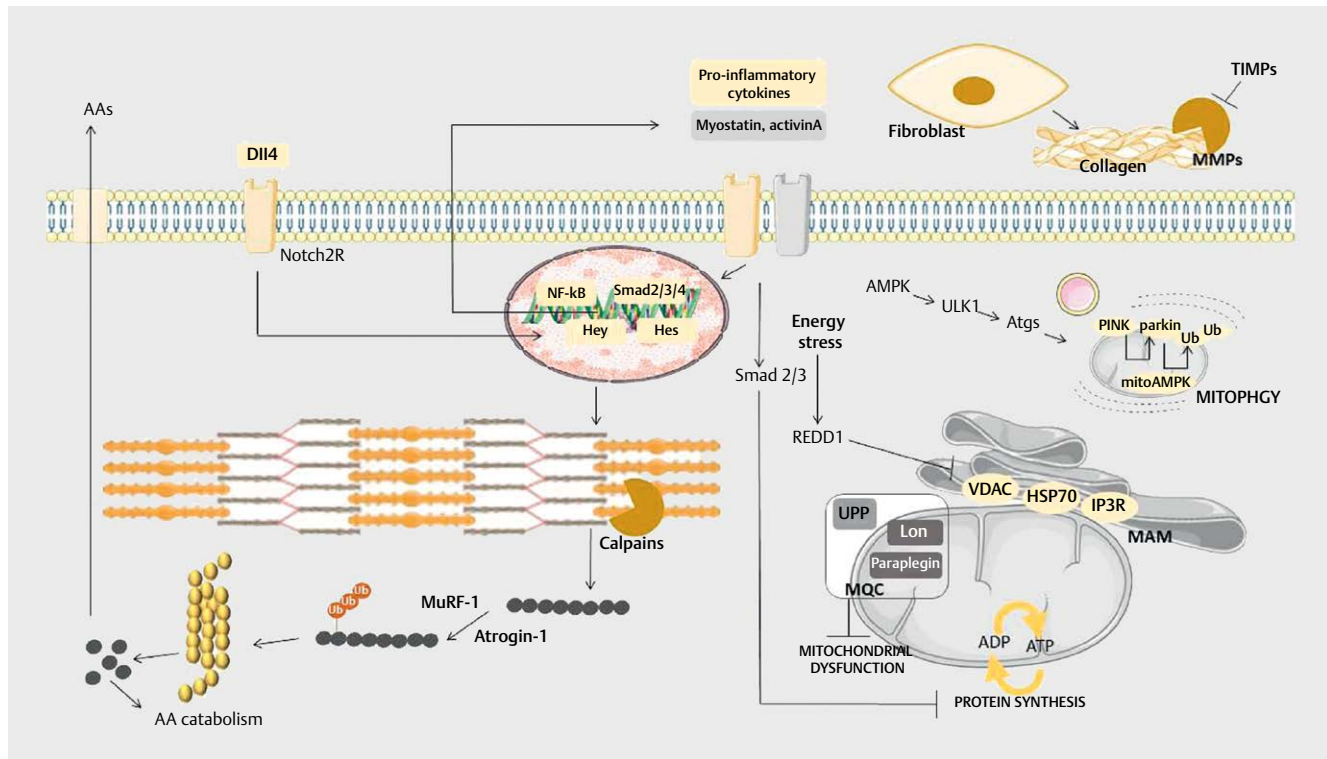
ized by fast-to-slow switch [3, 31]. The molecular mechanisms involved in protein turnover and how they are handled by exercise are reviewed in the following sections.

## The signaling pathways involved in the regulation of protein turnover in skeletal muscle

### Regulation of protein synthesis

Protein synthesis rates are determined by de novo production of ribosomes (increasing translational capacity) and by ribosome composition (determining translation selectivity). In fact, ribosome biogenesis is an early event in several conditions with an impact on

SkM growth and maintenance [20, 32]. The association between the accumulation of rRNA levels and muscle hypertrophy was reported in an incremental overload model [33] and after 4 weeks or 8 weeks of resistance training [34, 35]. Ribosomes are highly conserved supramolecular complexes involved in the translation of mRNA into protein. These structures consist of two subunits in eukaryotic cells: the small 40 S subunit (contains 33 ribosomal proteins and 18 S rRNA) and the large 60 S subunit (comprises 47 ribosomal proteins and 3 rRNAs) [32]. Mitochondria also harbor a small proportion of ribosomes (mitoribosomes) involved in the translation of mtDNA-encoded oxidative phosphorylation (OXPHOS) polypeptides. In response to defects in the assembly of mitoribosomes that leads to mitochondrial dysfunction, the upregulation of the transcription of cell-survival mechanisms takes place as a compen-



► **Fig. 2** Proteolytic systems involved in the regulation of muscle protein turnover. Calpains ( $\mu$ -,  $m$ - and  $-3$ ) target myofibrillar proteins, releasing peptides that are ubiquitinated and degraded by 26S proteasome. The E3 ligases MuRF-1 and atrogin-1 participate in the ubiquitination of myofibrillar-derived peptides and other protein classes such as the metabolic ones. The resultant amino acids may be secreted and metabolically support other organs like the liver. Catabolic and pro-inflammatory cytokines activate several pathways in the muscle fiber, including NF- $\kappa$ B and Smad ones, promoting a catabolic environment. Notch signaling is activated by DII4, activating the transcriptional repressors Hey and Hes. Collagen levels in the extracellular matrix are determined by the balance between its production and secretion by fibroblasts and by the activity of MMPs, which is modulated by TIMPs. Mitochondrial protein turnover is regulated by proteases located inside this organelle and at the outer membrane that make up part of the MQC system. The interaction of mitochondria with endoplasmic reticulum is regulated by several proteins at the MAM, whose role in  $Ca^{2+}$  homeostasis and mitochondrial function is modulated by REDD1. This protein acts as an energy sensor. The accumulation of dysfunctional mitochondria is avoided by mitophagy, which requires an intricate network of molecular players. Parts of the figure were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License [CC BY 3.0]: (<https://creativecommons.org/licenses/by/3.0/>);

**Abbreviations:** AA, amino acids; Atgs, autophagy-related proteins; DII4, Delta-like 4; HSP, heat shock protein; IP3R, inositol 1,4,5-trisphosphate receptor; MAM, mitochondria-associated membranes; MMPs, metalloproteinases; MQC, mitochondrial quality control; MuRF1, muscle RING-finger protein-1; NF- $\kappa$ B, nuclear factor kappa-light-chain-enhancer of activated B cells; Notch2R, receptor of neurogenic locus Notch homolog protein; PINK, PTEN-induced kinase; REDD1, regulated in development and DNA damage responses 1; TIMPs, tissue inhibitors of metalloproteinases; Ub, ubiquitin; ULK1, Unc-51 like autophagy activating kinase; UPP, ubiquitin-proteasome pathway; VDAC, voltage-dependent anion channel.

satory measure [36]. Still, the direct association between mitochondria and SkM mass is poorly understood despite the recognized role of mitochondria on the regulation of protein turnover, as analyzed below.

### mTOR is a key regulator of protein synthesis

Translation initiation is the most studied process of protein synthesis with a focus on the regulatory role of mTOR, which is also a key regulator of ribosome biogenesis [37]. From the two multi-protein complexes of mTOR, mTORC1 has been viewed as the main regulator of protein synthesis, linking energetic and hormonal signals to protein synthesis [38]. Once activated by increased loading or amino acid consumption, mTORC1 phosphorylates the downstream kinase ribosomal protein of 70 kDa S6 kinase 1 (p70S6K1) and the 4E-binding protein-1 (4EBP1) that promotes the initiation

of protein synthesis through ribosomal binding to mRNA [30]. mTOR is targeted by Akt [or protein kinase B (PKB)], which is activated by the phosphatidylinositol 3-kinase (PI3K), a downstream player of insulin signaling [39] (► **Fig. 1**). Akt also phosphorylates and inhibits glycogen synthase kinase (GSK)  $\beta$ , thus preventing the inhibition of translation initiation via eukaryotic initiation factor 2B $\epsilon$  (eIF2B $\epsilon$ ) [40]. The role of mTORC1 in regulating SkM mass has been extensively analyzed, being widely accepted that mTORC1 activation results in increased protein synthesis, whereas suppression of mTORC1 signaling and decreased protein synthesis contributes to SkM loss (reviewed by [40]). Indeed, Guridi et al. [41] showed that the inhibition of mTORC1 (in raptor muscle knock-out (RAMKO) mice) causes a significant reduction of muscle mass, paralleled by a decreased expression of proteins involved in glucose and fatty acid oxidation. In fact, the SkM phenotype of RAMKO was



characterized by a reduced oxidative capacity and decreased mitochondrial density. The same authors also demonstrated that the chronic activation of mTORC1 (in tuberous sclerosis protein 1 (TSC1) muscle knock-out (TSCmKO) mice) promotes severe metabolic changes characterized by glucose intolerance (due to decreased translocation of glucose transporters (GLUT) 4 to the sarcolemma) though without an impact on SkM structure. In either case, mice were unable to gain lean mass with age, suggesting that a proper balance in mTORC1 signaling is essential for SkM homeostasis [41].

Intriguingly, the activation of mTORC1/p70S6K signaling was reported in rat soleus muscle at the initial stage of hindlimb unloading (24 hours after mechanical unloading) and was associated with the upregulation of E3-ligases from the UPP [42]. The suppression of contractile activity at the onset of hindlimb unloading may cause a fast accumulation of high-energy phosphates (ATP, ADP, and creatine phosphate), which was reported to be elevated in rat soleus muscle after 24 hours [42] and 10 days of unloading [43]. Increased levels of high-energy phosphates inhibit AMP-activated protein kinase (AMPK) by decreasing its phosphorylation at Thr172 [44]. AMPK is a heterotrimeric holoenzyme comprising three subunits: a catalytic  $\alpha$  ( $\alpha 1$  or  $\alpha 2$ ), a scaffolding  $\beta$  ( $\beta 1$  or  $\beta 2$ ), and a regulatory  $\gamma$  ( $\gamma 1$ ,  $\gamma 2$ , or  $\gamma 3$ ) subunit [43]. AMP (or ADP) can bind to the  $\gamma$  subunit, changing its conformation and exposing the Thr172 site of the catalytic  $\alpha$  subunit. The phosphorylation of this amino acid residue activates AMPK [45]. Thus, AMPK phosphorylation at Thr172 affects protein synthesis in SkM by interfering with mTOR signaling through phosphorylation of TSC2 (mTORC1 inhibitor) and of Raptor [46–48]. Regulated in development and DNA damage 1 (REDD1) is also a negative regulator of mTORC1 and was proposed to inhibit protein synthesis in SkM during exercise [49]. In addition, AMPK impairs translation by detaching polysomes into free ribosomal subunits [37]. In cancer cachexia, the increase of AMPK activity and REDD1 content was associated with decreased mTOR activity in SkM [50, 51]. Treadmill exercise attenuated this trend by increasing the expression of IGF-1 and, therefore, mTORC1 signaling [50]. Indeed, exercise training has been recommended to preserve or increase the SkM mass in subjects at risk of muscle wasting. Resistance training is a well-established exercise mode that stimulates myofibrillar protein synthesis; however, endurance training has been more prescribed for preventing muscle wasting [23]. According to Jee et al. [52], only high intensity endurance training preserved mice SkM mass (compared to moderate intensity). Still, in a rat model of cancer that develops cachexia, treadmill exercise was shown to prevent and counteract muscle loss, an effect that was associated with exercise-induced lower tumor malignancy and the decrease of circulating pro-inflammatory cytokines [53]. Eccentric resistance exercise has a higher anabolic effect in type II fibers (the type of muscle fibers more susceptible to atrophy in cachexia) compared to concentric and to eccentric combined to concentric exercise (reviewed by [23]). Moreover, the type of protein that is synthesized depends on the type of exercise. In general, endurance training leads to an increase in the expression of mitochondrial genes, and ultimately to enhanced mitochondrial density, leading to a shift toward an oxidative phenotype and fatigue resistance. Resistance exercise mostly induces the transcription of gene encoding for myofibrillar proteins [30]. The ability of a muscle fiber

to alter the type and amount of protein is dependent on the protein's half-life; the ones with a shorter half-life and higher synthesis rates are capable of attaining a new steady state faster in response to a stimulus [54]. These attributes of protein synthesis contribute to the SkM phenotypes seen after regular exercise.

### Myostatin signaling inhibits protein synthesis in skeletal muscle

Myostatin, also known as growth and differentiation factor 8 (GDF-8), belongs to the transforming growth factor (TGF)- $\beta$  family and is a key regulator of SkM mass. This ligand of type IIb activin receptors (particularly ActRIIB) is expressed in SkM, and its upregulation has been reported in several catabolic conditions such as cancer cachexia [55]. Following ligand binding, ActRIIB recruits and activates the activin type I receptors (ALK4 and ALK5), which phosphorylate and activate Smad2 and Smad3. In the nucleus, Smad proteins regulate the expression of target genes [56]. Moreover, myostatin was shown to block Akt/TORC1 signaling, through Smad2 inhibition of Akt activity, thus reducing protein synthesis [57, 58]. In cultured human myotubes, the reduction of phosphorylated Akt resulted in the accumulation of active Forkhead box-O (FoxO) transcription factors that regulate the expression of E3 ligases from the UPP [59], as detailed in the section entitled "The ubiquitin-proteasome pathway oversees skeletal muscle proteolysis". Overexpression of myostatin was also reported to elicit the downregulation of muscle structural proteins (e. g., MHCIIb, troponin I, and desmin) and myogenic transcription factors (MyoD and myogenin), resulting in a significant loss of SkM mass [60]. Thus, blocking myostatin increases protein synthesis and SkM size, which involves not only the activation of Akt/mTOR signaling but of other pathways such as Hippo signaling [55]. Exercise training can be seen as a therapeutic strategy to downregulate myostatin expression in SkM, as recently reported in ovariectomized rats submitted to weight-bearing exercise for 8 weeks [61].

### Wnt and Hippo signaling interplay in the regulation of protein turnover

Other important regulators of SkM mass in response to exercise include Wnt/ $\beta$ -catenin signaling. This pathway also mediates the gain of SkM mass induced by electric stimulation following spinal cord injury [62] and is critical to SkM development, formation of the neuromuscular junction, and activation of stem cells. During overload-induced SkM hypertrophy, Wnt activates its receptor, mFrizzled (mFzd)-1, and the disheveled (Dvl)-dependent inhibition of  $\beta$ -catenin complex degradation. Consequently,  $\beta$ -catenin accumulates in the nucleus and acts as a transcription factor by interacting with T-cell factor/lymphoid enhancer factor (TCF/Lef) [21, 63] (► **Fig. 1**). Wnt/ $\beta$ -catenin interferes with other pathways such as Hippo signaling, which can inhibit Wnt pathway by blocking Dvl or avoiding  $\beta$ -catenin translocation to the nucleus [63]. Yes-associated protein (Yap) and its ortholog transcriptional co-activator with PDZ binding motif (Taz) are the main players of the Hippo signaling. Yap/Taz may bind to TEA domain (TEAD) transcription factors and regulate the expression of specific genes such as L-type amino acid transporter 1 (LAT1). However, when Yap/Taz are phosphorylated by the large tumor suppressor kinases (Lats) 1 and 2, they are maintained in the cytoplasm and targeted by the UPP. Yap can also

be phosphorylated by AMPK and sequestered in the cytoplasm [63, 64]. Yap was reported to accumulate in the nucleus and to interact with TEAD after disruption of the neuromuscular junction, regulating gene expression that maintains SkM mass [65]. Yap may be activated by integrin-mediated stress fiber formation in response to a mechanical stimulus [25]. In fact, increased mechanical loading (using the synergist ablation mice model) was shown to promote Yap overexpression in the plantaris muscle, which was associated with increased content of phosphorylated Akt (Thr308) and total Akt but not of mTORC1. Mechanical loading-induced overexpression of Yap was also associated with the increase of c-Myc expression, a potent activator of ribosomal biogenesis, and of MyoD, a myogenic regulator factor, and to decreased levels of Smad2/3 and MuRF1. Thus, Yap seems to induce SkM hypertrophy by increasing c-Myc and MyoD expression (► Fig. 1) and by suppressing protein breakdown rates through the inhibition of Smad-mediated E3 ligase expression [66]. However, in the SkM fiber-specific knock-in (MCK-tTA-hYAP1 S127A) mouse model, the overexpression of constitutively active Yap (hYAP1 S127A) resulted in SkM atrophy rather than hypertrophy. This atrophic phenotype was reverted after removing hYAP1 S127A expression, with mice recovering body and muscle weight [67]. These apparent contradictory findings on the impact of Hippo signaling in SkM mass regulation may be explained by the experimental models used. Still, this topic deserves further investigation.

### Notch signaling regulates skeletal muscle mass

Muscle fibers express Notch2 receptors that may be activated by the ligand Dll4, which is upregulated and released by the microvascular endothelium in atrophic conditions. The endothelial Dll4–SkM Notch2 axis was recently shown to be a central mechanism that regulates catabolic signals from mechanical and metabolic stimulation [22] (► Fig. 2). In addition, the Notch target gene *Hey1* was reported to be upregulated in the rat soleus muscle after 4 months of spinal cord transection (by approximately 1.5-fold), and further increased by 7 days of electric stimulation, suggesting a role for Notch signaling in the hypertrophy of atrophied muscle in response to exercise [62]. Data from the microarrays used to profile the transcriptome of the biceps brachii from young women and men after an acute unilateral resistance session followed by 12 weeks of progressive training highlighted the activation of atrophic factors from the Notch pathway (e. g., the transcriptional repressors of *Hey* and *Hes* family members) in women, concomitantly with the hypertrophic effect of resistance exercise seen in both sexes [68]. Thus, Notch signaling seems to be differently modulated by sex, being upregulated in women following resistance exercise.

Overall, there are multiple signaling pathways controlling protein synthesis during loading and wasting conditions. The stimulation of protein synthesis and SkM mass gain by exercise is dose- and threshold-dependent. Different loading patterns result in similar anabolic responses in untrained muscle; however, in trained subjects, muscle protein synthesis depends on the mode of exercise [69]. Resistance exercise training has been consistently reported to increase protein synthesis; however, no changes in protein synthesis were detected in rats' gastrocnemius after 6 weeks of progressive resistance-loaded voluntary wheel running, despite mass gain [17]. Both resistance and endurance exercise blunt muscle

protein synthesis during contraction [3, 69], perhaps to channel energy supplies for contraction. Contracting muscle itself and/or humoral factors seem to interplay to activate/suppress the signaling pathways involved in the regulation of protein synthesis in SkM, with AMPK and mTOR taking a key role in such regulation. The contribution of these pathways to the rate of protein turnover depends on their interaction with the pathways that control proteolysis and should be assessed in an integrated perspective.

### Regulation of muscle protein breakdown

Mammalian SkM contains four major proteolytic systems: lysosomes, UPP, calpains, and caspases [70]. The role of lysosomal proteases (e. g., cathepsins) in myofibrillar protein turnover is expected to be small, since low pH is required for their optimal activity, and engulfment of myofibrils would be necessary. Nevertheless, small autophagosomes were described in the perinuclear region and between myofibrils, and several mechanisms of autophagy are involved in bringing bulk cytoplasmic, long-lived proteins inside lysosomes [71]. Oxidative muscles exhibit higher levels of cathepsins, which are up-regulated (particularly cathepsin L) in wasting conditions such as cachexia [71, 72]. The caspase system is usually associated with apoptosis. Still, caspase-3 may initiate SkM proteolysis by cleaving myofibrillar proteins into smaller fragments, providing substrate for UPP [72, 73]. The contribution of calpains and the UPP to SkM protein turnover, particularly of myofibrillar proteins, is expected to be higher [24, 74]. In fact, the activation of calpains and UPP has been reported under conditions promoting muscle atrophy. However, increased contractile activity (e. g., in the early moments of exercise training) also stimulates these proteolytic systems, possibly to remove the damaged proteins resulting from the mechanical and metabolic challenges imposed on muscle fibers [74]. The relevance of UPP and calpains in SkM adaptation to pathophysiological conditions has been discussed in numerous reviews [70, 75]. Still, some issues on the role of calpains and UPP in SkM protein turnover are highlighted herein.

### Proteolysis mediated by calpains

Calpains (calcium-activated cysteine proteases) were shown in 1976 to initiate myofibrillar protein turnover by selectively releasing filaments from the outer layer of myofibril [76], making them available for degradation by other proteolytic systems, mainly UPP. Indeed, myofibrillar proteins to be degraded without affecting the contractile activity of muscle must be disassembled from the myofibril and then proteolytically decomposed [7]. Yet a small number of myofilaments can be easily released by myofilaments, primarily the ones lacking in or with lower amounts of  $\alpha$ -actinin, an actin crosslinking protein [10]. Calpains are concentrated in the Z disk of myofibrils [77], and they were suggested to penetrate the myofibrillar matrix and cleave sites inside the myofibril [7]. At least 15 different calpains were described in mammals; however, only six are expressed in SkM. Of these,  $\mu$ - and m-calpains (activated by  $\mu$ M and mM concentrations of  $\text{Ca}^{2+}$ , respectively) have been the isoforms most studied in SkM remodeling. Both  $\mu$ - and m-calpains (also known as calpain-1 and calpain-2, respectively) have nearly identical substrate specificities. Among their substrates are myofibrillar and signaling proteins such as protein kinase C (PKC), calcineurin, and calcium/calmodulin-dependent protein kinase IV

(CaMK) [70, 75]. GLUT4 was also shown to be a substrate of m-calpain, which supports the participation of calpains in the regulation of the SkM metabolism [78]. There is another calpain isoform, calpain-3 (or p94), with recognized physiological relevance in SkM protein turnover. This isoform works at sub- $\mu\text{M}$   $\text{Ca}^{2+}$  concentrations and it is not inhibited by calpastatin, the endogenous inhibitor of calpains. Calpain-3 mainly localizes in the sarcomere bound to titin (at two regions, N2A line and M line), the largest whole-body protein (3 million Dalton), and the third most abundant in SkM (following actin and myosin). Calpain-3 interaction with titin protects calpain-3 from auto-degradation and maintains the enzyme in an inactive state, despite being near its substrates [79]. Calpain-3 also binds at the Z-band to  $\alpha$ -actinin through the N-terminus [80]. Titin damage may be an important signal for the activation of this calpain isoform [79]. Calpain-mediated cleavage of sarcomere proteins such as titin was reported in muscle unloading and is associated with the decrease of force-generating capacity [81]. The activation of calpains in disuse-induced atrophy was shown to depend on time and muscle type. For example, increased calpain activation was observed in the soleus within 12 hours of hindlimb suspension, whereas in gastrocnemius calpain activity was not detected until the muscles had been unloaded for more than 3 days [82]. Calpain activation also occurs following a stimuli like eccentric exercise (but not isometric contractions). The increase of intracellular  $\text{Ca}^{2+}$  concentration activates calpain-3 that cleaves titin and other myofibrillar proteins [83–85]. Consequently, the transmission of force will tend to decrease. The activation of calpains following exercise is also time-dependent. For example,  $\mu$ -calpain was reported to be activated 30 minutes after eccentric exercise in humans [86], whereas calpain-3 activation was detected 24 hours after the activity [85]. Immediately after a single bout of aerobic exercise training, calpain activation was detected in mice plantaris muscle; however, 48 hours after the end of the exercise session, activity was no longer observed [74].

### The ubiquitin-proteasome pathway oversees skeletal muscle proteolysis

The peptides resulting from myofibrillar protein cleavage must be removed to avoid the toxicity of their accumulation. Thus, the cleavage products are targeted by ubiquitin for subsequent elimination by 26S proteasome (► **Fig. 2**). The UPP is an ATP-dependent process, linking proteolysis to energy availability. In SkM, this proteolytic system includes the E3-ligases MuRF1 (or Trim63) and Muscle Atrophy F-box (MAFbx), also known as atrogenin-1 [38]. These atrogenes have been seen as the gold-standard markers of SkM proteolysis, and their expression varies with the atrophic stimuli. For example, the peak of atrogenin-1 and MuRF1 mRNA expression in the gastrocnemius was reported 3 days after denervation, before the manifestation of atrophy, and returned to basal levels by 14 days (atrogenin-1) and 28 days (MuRF1) [87]. The expression of these E3-ligases in the soleus and plantaris was also observed to peak 3 days after hindlimb immobilization. Still, the expression of atrogenin-1 and MuRF1 was higher in plantaris than in soleus muscle; however, the percentage of muscle mass decrease was similar in both muscles, suggesting that E3-ligases have a higher contribution to the atrophy of fast-twitch than slow-twitch muscles [88].

The gene encoding for MuRF1 and atrogenin-1 seems to be up-regulated together under most atrophy conditions. In fact, the proximal promoter region of each gene contained several consensus Class O FoxO binding sites (FBE), capable of binding FoxO1, FoxO3a, and FoxO4 transcription factors, all of which are expressed in SkM and under Akt regulation [89]. Akt is determinant in the control of both anabolic and catabolic signals. Its activity may be inhibited by TRB3 (the mammalian *Drosophila* tribbles homolog 3), a pseudokinase that directly binds to Akt. The overexpression of TRB3 was associated to decreased mTOR/S6K1 phosphorylation and, consequently, protein synthesis rate, and to increased FoxOs activation and atrogenes expression, reducing SkM mass [90]. However, contradictory findings on the effect of TRB3 overexpression in SkM were reported [91], possibly justified by the mice model used and diet. Phosphorylation of FoxO transcription factors promotes their export from the nucleus to the cytosol, inhibiting their activity. However, the MuRF1 and atrogenin-1 promoters do not seem to be equally activated by these transcription factors. FoxO1 was reported to increase MuRF1 expression, whereas FoxO3a seems to increase atrogenin-1 [89]. Still, FoxO3 was suggested to be the most critical regulator of atrophy since its deletion in SkM was not compensated by other factors. FoxO3 activity is regulated by several molecules such as REDD1, which prevents its dephosphorylation in SkM from cachectic mice [51]. FoxO transcription factors were shown to regulate the expression of other E3-ligases besides MuRF1 and atrogenin-1, as for example the muscle ubiquitin ligase of the SCF complex in atrophy-1 (MUSA1), also known as Fbxo30 [92, 93]. This ligase was reported to be critical for denervation and fasting-induced SkM atrophy. FoxO-dependent atrogenes include autophagy-related genes such as LC3 and Bnip3 [93], whose role in protein turnover is discussed below.

The protein substrates of E3-ligases are mostly unknown. In fact, the identification of the substrates targeted for ubiquitination by an individual E3 ligase has been challenging. Moreover, ubiquitination of a protein not only can mark it for degradation by the proteasome but may also regulate its activity, change its cellular localization, and interfere in protein-protein interactions [89]. Still, MuRF1, and its paralog MuRF2, were reported to be located in the sarcomere, the sarcomere proteins being an expected substrate of these ligases [79]. In fact, MuRF1 (more abundant than its paralog) interacts with the giant muscle proteins titin and nebulin, cooperating with calpain-3 in sarcomere proteins turnover [2]. MuRF1 was also shown to ubiquitinate other myofibrillar proteins such as troponin I and myotilin [94], and metabolic proteins such as M-creatine kinase and aldolase A. By degrading and, consequently, down-regulating metabolic proteins, MuRF1 regulates energetic pathways [2]. Atrogenin-1 seems to regulate the activity of transcription factors such as c-Myc [95] and eukaryotic initiation factor 3f (eIF3f) [96] by directing them for proteasome breakdown. MuRF1 also controls protein synthesis through the ubiquitination of transcription factors, such as the glucocorticoid modulatory element binding protein-1 (GMEB1) [97]. Concomitantly, amino acids are provided from the degradation of these proteins and can be used as energetic fuel. Curiously, 3-hydroxyisobutyrate dehydrogenase (HIBADH), an enzyme involved in the valine catabolic pathway, is also a target of MuRF1, suggesting that valine, and eventually other branch-chain amino acids (BCAA), are not oxidized in SkM and may

be released to the bloodstream and used by other tissues [2, 48]. Therefore, the E3 ligases MuRF1 and atrogin-1 are key regulators of muscle protein turnover by controlling both protein synthesis and proteolysis. Even so, MuRF1 and atrogin-1 are under the control of mTORC1 signaling, which seems to involve the nuclear-cytoplasmic traffic of histone deacetylases (HDACs), particularly HDCA5. In unloaded soleus muscle, increased p70S6K activity resulted in the upregulation of E3 ligases via phosphorylation and nuclear export of HDAC5 [42]. Overall, the signaling pathways involved in the regulation of protein synthesis and breakdown interplay in the SkM remodeling induced by wasting and loading conditions, reflecting and contributing to the energetic status of this organ.

### The role of mitochondria in the control of protein turnover

The balance between protein synthesis and breakdown is tightly controlled by mitochondria, the powerhouses of SkM fibers. Mitochondria harbor the oxidative phosphorylation (OXPHOS) system, the major ATP supplier of SkM fibers. ATP is needed for several cellular processes, with protein synthesis being the major ATP-consuming process of myofibers that face high energy demands during contraction [31]. Thus, perturbations in mitochondrial homeostasis and, consequently, on ATP generation can have deleterious impact on the maintenance of SkM mass and function, as reported in several SkM wasting conditions [98, 99]. On the other hand, regulators of protein synthesis, such as mTORC1 and AMPK, control mitochondrial biogenesis and function [100–102]. mTORC1 selectively promotes the translation of nucleus-encoded mitochondria-related genes through the inhibition of the eukaryotic translation initiation factor 4E (eIF4E)-binding proteins (4EBPs) [101]. In SkM, rapamycin (mTOR inhibitor) decreased the expression of peroxisome proliferator-activated receptor- $\gamma$  coactivator (PGC)-1 $\alpha$ , estrogen-related receptor (ERR)  $\alpha$  and nuclear respiratory factors (NRFs), and, consequently, reduced mitochondrial gene expression and organelle functionality. The transcription factor yin-yang 1 (YY1) seems to be required for rapamycin-dependent repression of the gene encoding for these mitochondrial transcriptional regulators [103]. By controlling mTORC1, REDD1 affects mitochondrial functionality in SkM. REDD1 was detected in the mitochondrial fraction of mice SkM after endurance exercise and appears to interact with mitochondria-associated endoplasmic reticulum (ER) membranes (MAM, a functional interaction between mitochondria and ER) proteins, including mitochondrial heat shock protein (mtHSP) 70 (► **Fig. 2**). Thus, MAM contact sites are disrupted, decreasing ATP availability for ER-dependent protein synthesis [31, 49]. In this way, energy is saved to ensure cell survival during metabolic challenges [49].

A great amount of ATP is consumed by sarcoendoplasmic reticulum calcium ATPase (SERCA) pumps and actomyosin contraction. Transient changes in cytosolic Ca<sup>2+</sup> generated by physiological stimuli prompt large increases in the Ca<sup>2+</sup> concentration of the mitochondrial matrix, stimulating the Ca<sup>2+</sup>-sensitive dehydrogenases of the tricarboxylic acid (TCA) cycle (e. g., NAD-isocitrate dehydrogenase and oxoglutarate dehydrogenase). Ca<sup>2+</sup> entry into mitochondria is mediated by the mitochondrial calcium uniporter (MCU). Mitochondrial Ca<sup>2+</sup> accumulation via MCU was positively

associated with the size of myofibers. Moreover, MCU expression was correlated with PGC1- $\alpha$ 4, an isoform of the transcriptional regulator of mitochondria biogenesis PGC-1 $\alpha$  [104, 105]. This isoform is highly expressed in trained SkM but does not regulate most of the known targets of PGC-1 $\alpha$  (e. g., OXPHOS genes). PGC1- $\alpha$ 4 specifically stimulates the anabolic pathway IGF1/Akt and represses the catabolic route triggered by myostatin [104, 106]. PGC1- $\alpha$ 4 is particularly responsive to resistance exercise and drives muscle hypertrophy, whereas PGC-1 $\alpha$  induces many of the adaptations promoted by endurance training including mitochondrial biogenesis, fiber-type switching, upregulation of fatty acid oxidation and angiogenesis [106, 107]. PGC-1 $\alpha$  may be phosphorylated (at Thr177 and Ser538) by AMPK when ATP levels are depleted and the AMP/ATP ratio increases, which in turn induces PGC-1 $\alpha$  promoter and the transcription of many AMPK target genes (e. g., GLUT4, mitochondrial genes). Thus, anabolic pathways are inhibited, and catabolic ones are stimulated to restore the ATP content [108].

The translation of mtDNA-encoded genes into the corresponding proteins is also essential for mitochondrial homeostasis and is impacted by several pathophysiological conditions. Mitochondrial translation is divided into four phases: initiation, elongation, termination, and mitoribosome recycling. The mitochondrial translation factors involved are two mitochondrial initiation factors (mtlIF2 and mtlIF3), three mitochondrial elongation factors (mtEFTu, mtEFTs, and mtEFG1), one mitochondrial release factor (mtRF1L), and two mitochondrial recycling factors (mtRRF1 and mtRRF2) [73, 109]. Changes in the levels of these mitochondrial translation factors and, consequently, on mtDNA-encoded proteins were reported in wasting and loading conditions. Indeed, 3 days after denervation, a decrease was observed in the expression of mitochondrial initiation factor mtlIF2, elongation factor mtEFTu, recycling factor mtRRF1, and translational activator TACO1 in soleus muscle. After 7 days, other mitochondrial translation factors were downregulated, such as mtlIF3, mtEFTs, mtEFG1, mtRF1L, mtRRF1, and mtRRF2. This decrease in the expression of mitochondrial translational factors resulted in the diminished content of mtDNA-encoded proteins and reduced mitochondrial biogenesis [110], supporting the involvement of mitochondrial translation in the regulation of mitochondrial biogenesis and functionality. In opposition, endurance exercise has been consistently reported to increase mitochondrial biogenesis (meaning an increase in muscle mitochondrial number and volume, and changes in organelle molecular composition) by enhancing the translation of both nDNA and mtDNA-encoded mitochondrial proteins [30, 111]. Still, the impact of mitochondrial translation on SkM remodeling depends on muscle type, being higher in type I fibers that present higher mitochondrial density [109]. Overall, mitochondrial adaptations to regular exercise not only enhance exercise performance but also impact a broader range of health issues [111].

### Quality control systems balance biogenesis mechanisms to maintain mitochondrial health

Being a postmitotic tissue, SkM mass not only depends on mitochondrial biogenesis but also on mitochondria quality control (MQC) systems to keep mitochondrial proteostasis and function [99]. MQC systems require the activity of molecular chaperones and mitochondrial proteases (e. g., Lon and AAA proteases). Be-



sides, UPP degrades misfolded proteins located in the outer membrane of mitochondria. Mitochondrial proteases and UPP work together to remove oxidized and misfolded mitochondrial proteins (► **Fig. 2**), avoiding the toxicity of their accumulation [112]. In fact, the accumulation of oxidized proteins was associated with decreased mitochondrial content of Lon and m-AAA paraplegin in the gastrocnemius muscle from diabetic [113] and cancer cachectic rats [114]. Moreover, mitochondria are highly dynamic, being able to fuse, mix, and replenish their content. Mitochondrial fusion may be seen as an attempt to avoid autophagy and maximize ATP generation. However, when mitochondrial membrane potential is lost, fission occurs. This process provokes organelle removal through mitophagy, which is an important mechanism of MQC [112].

Mitophagy may be triggered by AMPK that phosphorylates and activates Unc-51-like autophagy activating kinase (ULK1), the most upstream known mitophagy protein. This kinase recruits other autophagy-related proteins (Atg) that form complexes [115, 116] and may promote the fusion of lysosome with mitochondria-containing autophagosome [44]. The activation of AMPK and ULK1 was reported in SkM 6 hours after acute treadmill exercise [117]. More recently, Drake et al. [45] demonstrated that tissue-specific isoforms of AMPK are localized on the outer mitochondrial membrane (known as mitoAMPK) and are required for mitophagy. This kinase becomes phosphorylated at Thr172 in SkM in response to treadmill exercise. When mitoAMPK activity is inhibited, exercise-induced mitophagy is attenuated. Data suggest that mitoAMPK acts as an energetic sensor to fine-tune mitochondrial functionality, thus maintaining the energetic homeostasis in SkM. Mitophagy may also be initiated by phosphatase and tensin homolog-induced putative kinase protein (PINK)1. When mitochondrial membrane potential is lost, PINK1 accumulates in the outer mitochondrial membrane and recruits its substrate, the E3 ligase parkin, which in turn is phosphorylated and activated by PINK1. Parkin mediates the ubiquitination of proteins from the mitochondrial surface [98, 118] (► **Fig. 2**). Ablation of PINK1 and parkin was reported to reduce mitophagy in SkM and to increase mitochondria susceptibility to oxidative stress [99]. In denervated gastrocnemius, PINK1/Parkin mediated mitophagy was observed and resulted in significant reductions in mitochondrial number [119]. An opposite effect was reported following exercise training and was characterized by increased mitophagy though balanced by increased mitochondria biogenesis [99]. Autophagy receptors are recruited and interact with autophagosome membrane protein light chain 3-I (LC3-I) from the autophagosome, which is converted in the phosphatidylethanolamine conjugated form LC3-II [98, 118]. Increased LC3-II and parkin levels were reported immediately following acute exhaustive exercise in mice SkM, suggesting enhanced mitophagy and mitochondrial proteins breakdown. These processes were compromised in mice lacking PGC-1 $\alpha$ , supporting the master role of PGC-1 $\alpha$  in the regulation of exercise-induced mitophagy [117]. Endogenous mitochondrial membrane-bound receptor proteins, such as BCL2/adenovirus E1B 19kD interacting protein 3 (Bnip3), may drive mitophagy by binding to LC3. Increased expression of this protein was observed in plantaris muscle after 4 weeks of voluntary physical activity and occurred in parallel with the overexpression of PGC-1 $\alpha$ , suggesting that mitophagy occurs in tandem with mitochondrial biogenesis to improve SkM functionality [120].

The fusion of lysosomes with autophagosome generates an autolysosome, allowing lysosomal enzymes to complete the mitophagy process. These autolysosomes are characterized by the presence of lysosomal-associated membrane protein 1 or 2 (LAMP1 or LAMP2) [118]. The accomplishment of all steps of mitophagy is crucial to avoid the accumulation of dysfunctional mitochondria. In fact, in age-related loss of SkM there is an accumulation of dysfunctional mitochondria due a decline in mitophagy process [115, 121].

Taken together, there are multiple molecular mechanisms involved in the continuous reshaping of the mitochondrial network towards the maintenance of healthy mitochondria. Collectively, these mechanisms represent a MQC system that recognizes and counteracts mitochondrial dysfunction, essential for protein turnover and SkM mass maintenance. Thus, the dysregulation of this system negatively impacts SkM mass and function, which is at the core of several pathophysiological conditions.

### Regulation of extracellular matrix protein turnover

Intramuscular ECM plays a key role in intercellular communication, force transmission, and structure maintenance. ECM mediates the transmission of contractile force, regulating the efficiency of muscular contraction and protecting muscle fibers from excessive stress and assisting in the healing following microtrauma [122]. ECM homeostasis is regulated by protein turnover, which in turn is reflected by the rates of protein synthesis (mostly of collagen) and breakdown, particularly those mediated by metalloproteinases (MMPs) [123]. Collagen represents the most abundant ECM protein within the muscle and SkM contains collagen types I, III, IV, V, VI, XII, XIII, XIV, XV, XVIII, and XXII (from the 28 members of the collagen superfamily) [122]. Of these, collagens I and III account for 75 % of total SkM collagen and are present in the form of fibrils [122, 124], with slow-twitch muscles containing more collagen than fast-twitch ones. In SkM, collagen is mostly produced by fibroblasts and is characterized by several posttranslational modifications of the polypeptide chains, which are enrolled in the triple-helical procollagen structures secreted by exocytosis to the ECM. After N-terminal cleavage, the collagens self-assemble into fibrils [125]. Collagen transcription is rather slow (around 3 days), whereas secretion takes less than 1 hour [122].

Collagens, independently of their type, are substrates of MMPs, whose activity is regulated by tissue inhibitors of metalloproteinases (TIMPs) (► **Fig. 2**). MMP-2 and MMP-9 (also known as gelatinases A and B, respectively) target type IV collagen, the major collagenous component of the basement membrane [122]. TIMP-1 forms a complex with MMP-9 and TIMP-2 with MMP-2. MMP-2 is constitutively expressed in fibroblasts and its activity is enhanced in inflammatory conditions. MMP-9 is secreted by endothelial and inflammatory cells. As regulators of ECM integrity, MMPs, particularly gelatinases, participate in the SkM remodeling in response to stimuli such as exercise training [126]. Despite similar substrate specificity, the expression pattern and transcriptional regulation of these MMPs are very different. For instance, increased expression of MMP-9 was reported after a single bout of exercise, whereas the content of MMP-2, MMP-14, and TIMP-1 was raised after 10 days of exercise training [127]. MMPs activity counteracts the increased collagen content reported in trained subjects, which ap-

pears to be due to the upregulation of posttranscriptional and translation events; however, the amount of collagen and other ECM proteins seems to be dependent on the exercise mode, particularly in aged individuals [124]. Four days of eccentric training promoted a greater upregulation of collagens I and III expression, as well as of TGF- $\beta$  and lysyl oxidase (that form cross-links in ECM proteins), in female rat gastrocnemius compared with 4 days of concentric or isometric training [128]. In human SkM biopsies, collagen synthesis was reported to increase 4.5 hours after maximal contractions (shortening or lengthening contractions), suggesting an increase of protein synthesis activity of SkM fibroblasts promoted by both types of contractions [129]. After forced lengthening contractions, the activity of prolyl 4-hydroxylase (which catalyzes the formation of 4-hydroxyproline in collagens) and type IV collagen concentration was enhanced in the rat tibialis anterior muscle and were paralleled by the activation of MMP-2 and MMP-9 and their inhibitors during recovery [130]. In aged SkM, increased collagen concentration with changed composition (shift towards higher type I to type III collagen) was reported. Alongside, hydroxyllysylpyridinoline cross-linking and advanced glycation products (AGE) were also raised. Decreased MMP activity may explain uncontrolled ECM expansion, causing enhanced muscle stiffness and reduced whole muscle function [122, 131]. During immobilization, SkM becomes fibrotic with increased collagen content and disorganization, which contribute to the reduced capacity of immobilized SkM to elongate in response to stretching without tissue damage [132].

Altogether, ECM remodeling is characterized by increased collagen synthesis, which is balanced by MMP activity following exercise but not in aging or other pathophysiological conditions, resulting in muscle fibrosis. In fact, fibrosis is a hallmark of maladaptive remodeling of SkM, which is a sign of muscle weakness and impaired regeneration after injury. Strategies for tackling SkM fibrosis entails the targeting of TGF- $\beta$ 1, a potent pro-fibrotic cytokine. This M2 macrophage secreted cytokine not only stimulates fibroblasts to produce and secrete collagen and other ECM proteins but also promotes the secretion of TIMPs, inhibiting MMP activity. This cytokine also fosters the transdifferentiation of several resident cell types into myofibroblasts, boosting a pro-fibrotic phenotype [133].

### Amino acids pool and protein turnover

The balance between protein synthesis and breakdown from and to the intracellular pool of free amino acids is determinant to protein turnover in SkM. Still, different SkM types present different protein amounts and turnover as well as amino acid composition, making it difficult to disclose the impact of changes in amino acid pools under different stimuli. Indeed, distinct SkM types are differently affected by wasting conditions and starvation that result in the release of amino acids. For instance, soleus (slow-twitch muscle) was reported to have higher concentrations of His and Lys and the most non-essential amino acids compared with EDL (fast-twitch muscle) in the postprandial state [134]. Greater activity of proteasome was also reported in the soleus and was associated with the release of 3-methylhistidine (a marker of proteolysis of myofibrillar proteins) [135, 136].

Changes in the protein balance occur daily, depending on food intake (particularly of essential amino acids) and physical activity, which mainly affect protein synthesis [88]. One day of starvation

blunts protein synthesis and proteolysis is enhanced, increasing the release of amino acids from SkM to feed liver gluconeogenesis. Still, the circulating levels of these amino acids decrease except for BCAA (meaning Val, Leu, and Ile). Why BCAA circulating levels increase during starvation is not clear, though it was suggested that their synthesis from branched-chain keto acids rises in the liver and their uptake is reduced due to decreased insulin production [134]. SkM is the main site of BCAA metabolism, with the highest activity of BCAA aminotransferase (BCAT2), the enzyme that catabolizes the first step of BCAA degradation. BCAA are essential amino acids used for protein synthesis and energy production, and perform signaling roles (e. g., via mTOR) [137]. BCAA, specifically the proteogenic amino acid Leu, activate Rag GTPase and, consequently, the translocation of mTORC1 from the cytoplasm to the surface of lysosomes, where it interacts with the heterodimer formed by GTP-loaded RagB and GDP-loaded RagD. Thus, the downstream players from mTOR pathway are activated [138]. However, Leu levels are not sensed directly by Rag GTPase or mTOR but by Leucyl-tRNA synthetase (LeuRS) (► **Fig. 1**) and sestrin2, which were identified as Leu sensors [139, 140]. Four weeks of resistance exercise training was reported to promote gastrocnemius hypertrophy, which was associated with the enhanced phosphorylation of mTOR, p70S6K and 4E-BP1, and the upregulation of LeuRS but not of sestrin2 [141]. Some authors reported that sestrin1, instead of sestrin2, is highly expressed in the SkM and mediates Leu activation of mTORC1 [142]. Indeed, the downregulation of sestrin1 expression was described in the mice soleus within 24 hours of hindlimb immobilization and was associated with diminished mTOR signaling [143]. Increased levels of sestrin1 were detected in human SkM biopsies following 12 weeks of resistance exercise [144]. Acute treadmill exercise increased sestrin1 accumulation and induced a discrete enhancement of sestrin2, whereas 4 weeks of aerobic exercise decreased the basal levels of both sestrins in mice quadriceps muscles. Moreover, the expression of sestrin1 and 2 was associated with AMPK activation in trained muscles [145]. Arg can also activate the mTOR pathway by binding to the cytosolic arginine sensor for mTORC1 subunit 1 (CASTOR1) and disrupts its interaction with GTPase-activating protein toward rags 2 (GATOR2), thereby activating mTORC1 [146].

The exercise-induced increase of BCAA metabolism in SkM is also mediated by PGC-1 $\alpha$ , which seems to enhance the expression of BCAT2 and branched-chain  $\alpha$ -keto acid dehydrogenase (BCKDH) (the enzyme that catalyzes the second step of BCAA metabolism) [146, 147]. PGC-1 $\alpha$  also activates alanine aminotransferase (ALT2) in SkM during fasting [148]. Likewise, PGC-1 $\alpha$  overexpression was associated with increased levels of GABA and Glu in the gastrocnemius muscle [149]. Thus, PGC-1 $\alpha$  seems to play a role in the regulation of amino acid metabolism in SkM.

Amino acids increase the synthesis of SkM proteins, either myofibrillar, sarcoplasmic and mitochondrial ones, probably in a dose-dependent manner. In fact, feeding acts synergistically with exercise to promote protein synthesis in SkM [69], and the gain of muscle mass after exercise is dependent on amino acid availability (particularly Leu) during the postexercise period. The maximum protein synthesis may be achieved with the intake of approximately 20 g of high-quality protein (such as whey proteins) [69]. Nevertheless, the ingestion of 40 g of whey protein after resistance exer-

cise resulted in greater stimulation of protein synthesis in SkM compared to 20 g of whey protein, though without affecting the p70S6K1 activity. This apparent disconnect between mTOR signaling and protein synthesis seems to be explained by a temporal discrepancy between both processes [150]. Still, BCAA supplementation did not improve muscle soreness and function during recovery of untrained young adult (average age of 24 years old) from resistance exercise [151]. Overall, more than BCAA supplements, the ingestion of high-quality protein boosts protein synthesis following exercise training, improving muscle health.

## Conclusions and future perspectives

Although many studies have highlighted the molecular mechanisms involved in the regulation of protein turnover and, consequently, in SkM mass and function, few advances have been made in their modulation, with a view toward the therapeutic management of wasting conditions. Different methodologies have been applied to assess the protein synthesis and breakdown levels in wasting and loading conditions, which may justify discrepancies among studies regarding the contribution of protein turnover to SkM mass variations. Isotope labeling is one of the methodologies used to assess protein turnover in SkM (e. g., [16, 150]), but the majority of studies measured the levels of markers of protein synthesis and proteolysis pathways and correlated with mass gain or loss and/or changes of fiber cross-sectional area (e. g., [42, 87]). Most of the studies on protein turnover have been conducted with animal models. They offer the advantage of controlling biasing factors such as age, diet, sex, comorbidities, and even genetic diversity (inbred vs outbred strains), and to obtain muscle samples representative of whole muscle and in high amounts compared to human muscle biopsies. However, one should be aware that there are differences in protein metabolism between species, as critically discussed by Phillips et al. [152]. In SkM from adult rats, total protein turnover is 3- to 4-fold higher and protein synthetic rates are approximately 2.5-fold greater than in adult humans. Rodents' growth take place during a great part of their life span, which means that the insulin sensitivity of SkM protein synthesis is not lost as in human adulthood, when growth stops. Still, data from these studies consistently support exercise as an excellent strategy against SkM loss by driven protein synthesis and blunting proteolysis.

The modulation of lifestyle through the implementation of exercise programs is challenging in aged subjects and patients with chronic diseases, the population group at higher risk of SkM wasting. Exercise program adherence is usually low among older and frail people, and patients with functional limitations. Moreover, the definition of the "optimal" exercise dose implies the adjustment of exercise intensity or volume, which may be time-consuming and unrealistic in the clinical setting. In fact, the effects of exercise programs on SkM mass and function depends on the subject's health status, as recently discussed by Bland et al. [153]. Thus, exercise prescriptions should be tailored, which requires the evaluation of this therapeutic approach on SkM protein turnover. Future work on the molecular comprehension of its impact on the regulation of SkM mass and function in distinct clinical scenarios is urgently required to envision the implementation of personalized interventions.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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