Role of mTOR kinase activity in skeletal muscle integrity and physiology

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ABSTRACT

Mammalian target of rapamycin (mTOR) is a key protein kinase that integrates and coordinates diverse signaling information mediated by growth factors, nutrient availability and energy status. It forms at least two functionally distinct signaling complexes, named mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2). mTOR regulates a great deal of cellular activities such as translation, transcription, autophagy, etc. The knockout of mTOR in mice is embryonic lethal. Mice with skeletal muscle-specific deletion of mTOR suffer from a progressive myopathy that causes premature death, and exhibited muscle atrophy, impaired mitochondrial function and increased glycogen content.

My study aims to characterize the role of mTOR kinase activity in muscle integrity and function. To this aim, I have generated a mouse line named mTOR MKOKI which is defective in endogenous muscle mTOR (mTOR MKO), while expressing a FLAG-tagged muscle-specific kinase-inactive allele (Asp2357Glu) of mTOR (FLAGmTOR MKI), thereby mimicking the effects of a potent, bioavailable and selective mTOR catalytic inhibitor in muscle.

Here I describe the phenotype of MKOKI and compared it with MKO phenotype. I found that: 1) a mTOR kinase dead allele did not rescue any altered parameters associated to the loss of the mTOR protein, but in contrast exacerbates these alterations. 2) Muscle mTOR kinase activity was required for oxidative metabolism maintenance and dystrophin expression. 3) MKOKI muscles show a hyper-accumulation of glycogen that gave rise to myofibrillar disorganization, a feature not observed in MKO. 4) Glycogen hyper-accumulation is due to a stronger Akt hyperactivation that leads to myophosphorylase downregulation. Glycogen degradation inhibition gave rise to myofibrillar disorganization following glycogenosis. Sustained muscle Akt1 and Akt2 activation actually improved glucose uptake and storage as glycogen but also compromised its utilization further leading to severe Glycogen Storage

Disease. 5) Autophagy inhibition in mTOR MKOKI, in contrast to MKO further leaded to pronounced atrophy, dystrophy. 6) mTOR catalytic inhibitors might have severe mutiple effects in postmitotic tissues such as skeletal muscles, which are able to activate the feedback loop on Akt due to mTOR kinase inhibition.

Keywords: *mTOR kinase, Akt, autophagy, exercise, skeletal muscle, mice*

RESUME

Cible de la rapamycine chez les mammifères (mTOR) est une protéine kinase importante, qui intègre et coordonne les informations de multiples voies de signalisation, sert de médiateur des facteurs de croissance, de la quantité de nutriment disponible et du statut énergétique. Il a au moins deux complexes de signalisation, fonctionnellement différents, appelés mTOR complexe 1 (mTORC1) et mTOR complexe 2 (mTORC2). mTOR régule de nombreux processus moléculaires et cellulaires tels que la transcription, la traduction, l'autophagie, et ainsi de suite. Les souris avec l'inactivation du gène mTOR sont mortels dans la période embryonnaire. Les souris qui est knock-out conditionnels de mTOR dans les muscles squelettiques subissent une myopathie progressive, conduisant à une mort prématurée, et présentent une atrophie musculaire, une altération de la fonction mitochondriale et à augmenter le glycogène.

Mes études sont pour caractériser le rôle de l'activité de la kinase mTOR dans l'intégrité musculaire et les fonctions musculaires, pour démontrer des fonctions de mTOR dans des muscles qui demandent ou demandent pas l'activité de la kinase, et donc qui ne sont pas distribuées par mTOR dans le corps. Dans ce but-là, on génère une ligne de souris nommée mTOR MKOKI qui est défectueux dans des muscles endogènes mTOR (mTOR MKO), tout en exprimant une allèle kinase-inactive, muscle-spécifique et drapeau-étiquetable (Asp2357Glu) de mTOR (FLAGmTOR MKI), imitant ainsi les effets dans le muscle d'un inhibiteur catalytique mTOR, puissant, biodisponible et sélective.

Ici, je décris le phénotype des souris MKOKI et MKO. Je trouve que 1) un allèle mort de kinase mTOR ne sauve pas des paramètres modifiés associés à la perte de la protéine mTOR, mais au contraire exacerbe ces altérations. 2) l'activité de la kinase mTOR dans les muscles est nécessaire pour l'entretien du métabolisme oxydatif et l'expression de la dystrophine. 3) MKOKI muscles ont

hyper-accumulation de glycogène qui a provoqué la désintégration de myofibrilles et n'est pas observé sur le MKO. 4) l'hyper-accumulation de glycogène est à cause de plus de l'hyper-activation de l’Akt qui provoque une baisse de la phosphorylase musculaire. L’inhibition de la dégradation de glycogène augmente la désorganisation de la myofibrille suite à la glycogénèse. L’activation entretenue des muscles Akt1 et Akt2 effectivement améliore l'absorption et le stockage du glucose comme du glycogène mais également compromet son utilisation entraînant davantage à la maladie plus sévère du stockage du glycogen. 5) l’inhibition de l'autophagie des souris MKOKI davantage conduit à l'atrophie prononcée et la dystrophie par rapport à MKO. 6) mTOR inhibiteurs catalytiques ont peut-être de multiples effets graves dans les tissus postmitotiques comme les muscles squelettiques, qui sont capables d'activer la boucle de réaction sur l’Akt malgré l'inhibition de la kinase mTOR.

MOT-CLE: *kinase mTOR, Akt, autophagie, exercise, muscle squelettique, souris*

INDEX

1. INTRODUCTION

1.1. Overview of skeletal muscle

1.1.1. Structure and function of skeletal muscle

Skeletal muscle is a form of striated muscle tissue that is controlled by the somatic nervous system. It is responsible for voluntary movements, and also the automatic movements (e.g. standing, holding up head, and breath). Skeletal muscle, cardiac muscle and smooth muscle are the three major muscle types. Taken literally, most skeletal muscles are attached to bones via tendons, which are bundles of collagen fibers. Skeletal muscles function as the motors, brakes and also the shock absorbers. They can be used as heaters when shivering and also being an important store of protein.

Skeletal muscle is made up of large numbers of components known as myocytes, or "muscle cells", sometimes called "muscle fibers" or myofibers, which are long, cylindrical, multinucleated cells. They are formed from the fusion of developmental myoblasts (a type of embryonic progenitor cell) in myogenesis process.

They are arranged in bundles, separated by sheets of connective tissue containing collagen. Each muscle fibre is surrounded by a cell membrane, which allows the contents of the fibres to be quite different from that of the body fluids outside them. The myofibers are composed of myofibrils. The myofibrils are in turn composed of actin and myosin filaments repeated in units defined as

a sarcomere, which is the basic functional unit of the myofiber and forms the basic machinery necessary for muscle contraction. Just because of the sarcomere, the skeletal muscle has its striated appearance. The term muscle actually refers to numerous bundles of muscle fibers gathered by connective tissue.

The segment between two neighbouring Z-lines is defined as the sarcomere. With electron micrographs, we noticed the Z-line is a series of dark lines. Surrounding the Z-line, it is the light band region—I-band. After the I-band, it is the dark band—A-band. Then, there is a paler region called the H-band inside the A-band. The names of A- and I-band come from their properties of reflecting the polarized light under a polarization microscope. And within the H-band, it is the middle of the sarcomere, called M-line.

Actin filaments are the main component of the I-band and extend into the A-band. Myosin filaments extend throughout the A-band and overlap in the M-band. The titin (or known as connectin) protein binds to the thin filament system in the Z-line. It extends from the Z-line to the M-band, where it interacts with the thick filaments. In the Z-line and in the M- band of the sarcomere, multiple proteins critical for the stability of the sarcomeric structure are found. Actin filaments and titin protein are cross-linked in the Z-line by the Z-line protein α-actinin. The M-band myosin and the M- proteins build the thick filament bridge system to the M-band part of titin (the stretchy filaments) (Figure 1).

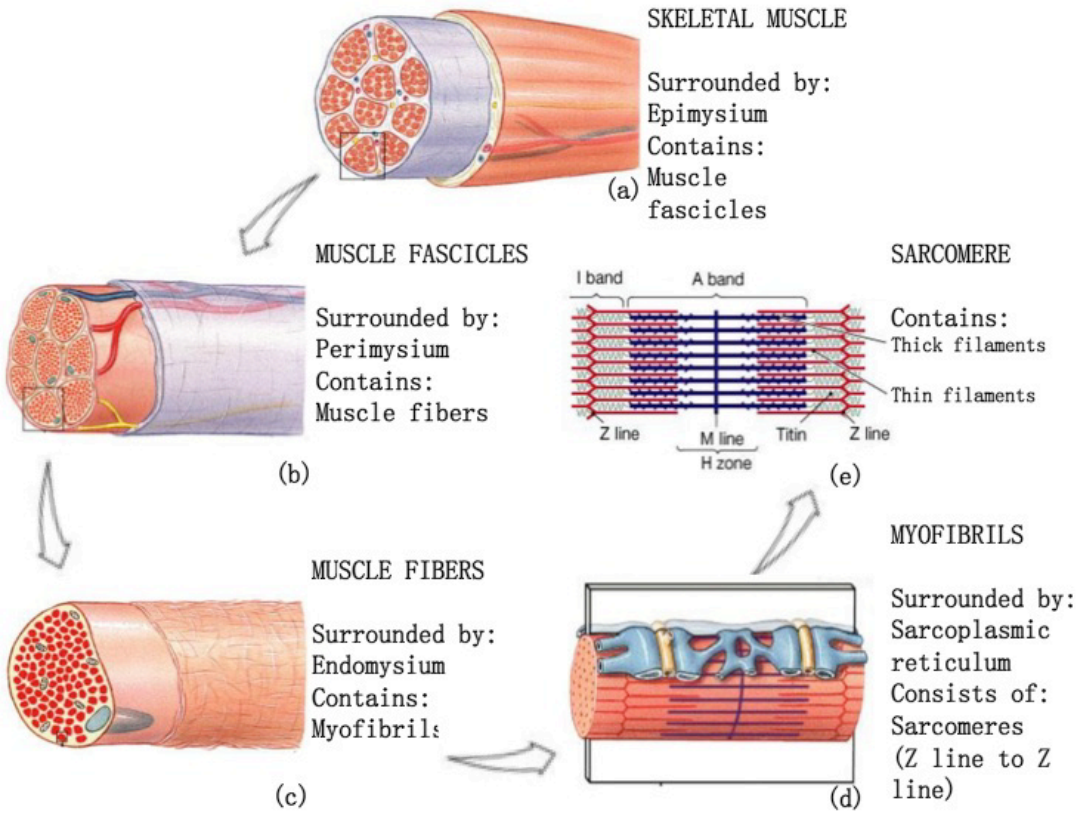


Figure 1. Schematic representation of skeletal muscle structure.

The muscle contraction occurs through an interaction of the actin with the myosin molecules (called crossbridges). Each of the crossbridges can produce about 5×10^{-12} Newtons force, meanwhile，it can pull the thin filament along past the thick filament by about 10×10^{-9} metres. Numerous of these tiny movements and tiny forces will shorten the myofibrils, and thus the whole muscle; therefore, some part of the skeleton is moved by the contraction of

attached muscles which are linked directly or via tendons to the bone.

As the body carries out a movement, events in the brain and the spinal cord produce action potentials in the axons of the motor neurons. Every axon branch sends action potentials to multiple muscle fibres. At the nerve terminals of each axon branch (neuromuscular junction) acetylcholine is released through the coming action potential, and this associates with receptors on the muscle fibre membrane, leading to generate an action potential. This action potential spreads over the whole surface of the fibre and is subsequently conducted into the interior via an extensive network of T-tubules.

Thereby, the permeability of the sarcoplasmic reticulum is changed by action potential, leading the sarcoplasmic reticulum of muscle to release ionic calcium, which then interacts with the regulatory protein troponin. Calcium-bound troponin changes its conformation, facilitating the movement of tropomyosin, thus exposing the myosin-binding sites on actin. This promotes myosin and actin ATP-dependent cross-bridge cycling and shortening of the muscles—the muscle contraction.

As soon as calcium is released from the sarcoplasmic reticulum, the calcium pumps in the membranes of the sarcoplasmic reticulum start to move the calcium back inside, therefore bringing to an end of a muscle twitch. More sustained periods of activity are the amount of movements we make; a series of action potentials are required to be sent to the muscle. The contractions generated in this way are stronger than a single twitch.

The muscle contraction is an ATP- dependent process. Mitochondria, which are located closed to Z- line, provided the energy. Muscle contraction needs energy to drive the crossbridges by means of their cyclic interactions with actin. The myosin molecule does work in moving the thin filament in each cycle. Energy is also utilized for the process of calcium pumping via the sarcoplasmic reticulum. When muscles are used to do external work, the energy consumption is highest. For example, the energy comsumption is high when the body weight has to be lifted in the movement of climbing stairs. However energy is also used when holding up a weight without doing work on it (e.g. isometric contraction). Less energy is need when muscles are used to lower weight, such as descending stairs.

1.1.2. Skeletal muscle fiber type diversity

In mammals, skeletal muscle constitutes about 40% of total body mass, meanwhile, it is responsible for about 30% the resting metabolic rate in human adult [1]. Skeletal muscle has a key role in regulating glycemic and metabolic homeostasis. It is the main (about 80%) site of glucose disposal under insulin-stimulated conditions [2]. Besides, skeletal muscle is the biggest glycogen storage organ. Moreover, exercise increases skeletal muscle glucose uptake by an insulin-independent pathway [3].

Skeletal muscle fibers are classified as slow- or fast-twitch according to the contractile property. This classification accords with histochemical staining for myosin ATPase as type I (slow-twitch) and type II (fast-twitch), which has the

highest ATPase activity. Type I, slow-twitch muscles are red in appearance. They are specialized for a more continuous activity and a fatigue-resistant phenotype. They mainly develop an oxidative metabolism, having high mitochondrial content and rich blood capillaries. Similarly, type IIa fibers are rich in mitochondria and capillaries, so that they also appear red. Although stain strongly for succinate dehydrogenase (SDH), type IIa fibers are also rich in glycolytic enzymes, thus they are classified as fast-twitch oxidative glycolytic fibers and form fast fatigue-resistant units. Type IIx fibers, also called IId, are characterized by a more glycolytic metabolism; in rat skeletal muscle, Type IIx fibers have strong staining for SDH. They has characteristic of a velocity of shortening intermediate between Type IIa and IIb. However, in humans their SDH staining is the weakest, because that they mostly rely on the glycolytic metabolism. Therefore, Type IIx fibers are the fastest and the most fatigable ones in human. In rodents, Type IIb fibers constitute the most glycolytic and fastest fibers (white, fast- twitch glycolytic fibers); in humans, MHC IIb is not expressed.

Immunohistochemical staining and protein electrophoretic separation are able to determine myosin heavy chain (MHC) isoform protein expression independently. This is another important criterion of fiber type classification. Each isoform has different contractile properties that parallel ATPase activity and twitch characteristics. Uniform fibers containing MHC1, 2A, and 2X presence, while mixture fibers containing 1-2A and 2A-2X isoforms have also been found. Muscle fiber type is genetically determined during development, but the

adaptive transformation of muscle fibers is controversial [4]. Under appropriate training stimulus, the plasticity of muscle allows changes in metabolic potential and morphology [5].

1.1.3. Formation of skeletal muscle

Skeletal muscle is formed in various of species from a pool of muscle precursor cells (myoblasts). Myoblasts proliferate, differentiate and then fuse together to form multinucleated muscle cells—myotubes. These myotubes become larger, innervated, and finally mature into myofibers.

In mammals (e.g. mouse), myofibers are formed through fusion of myoblast during the period of utero embryogenesis. There are big differences between skeletal muscles of the head, limbs, and body, with different sources of precursor cells and expression modes of regulatory genes [6]. The myofiber number is generally fixed by birth and subsequently is the postnatal growth and muscle maturation processes.

In mature mammals, there exist satellite cells on the surface of the mature myofiber (outside the sarcolemma and beneath the basement membrane), which are a small part of quiescent myoblasts. They are often referred to as myogenic stem cells. These satellite cells are activated when the myofiber homeostasis is disturbed, especially in the condition of myofibers necrosis, to generate new muscle to replace the damaged segment [7, 8].

Skeletal myogenesis is initiated in the embryo due to signalling molecules from surrounding tissues that specify the myogenic cell fate. Transgenic analysis of

DNA sequences that regulate Myf5 (myogenic determination factor 5) demonstrates the complexity of elements required to regulate the spatio-temporal expression of this gene. Myf5 and MyoD are essential for the acquisition of myogenic identity. The signalling pathways that influence the onset of myogenesis lead to activation of Myf5 and Myod either directly or indirectly [9].

The myogenic regulatory factors also function during the formation of adult muscle fibres. Myf5 (Myf5- nlacZ) is transcribed in most satellite cells [10], as these cells differentiate, MyoD accumulates. Without MyoD, muscle growth and regeneration are affected [11]. Pax7, the orthologue of Pax3, plays an important role in the formation of adult skeletal muscle [12]. This Pax gene is expressed in satellite cells. In addition, satellite cells are absent in Pax7 mutant mice.

Fetal myoblasts and embryonic satellite cells are maintained in an undifferentiated state through the TGF-β (Transforming growth factor) and BMP (Bone morphogenetic protein) family members. Moreover, FGF (fibroblast growth factor) and PDGF (platelet-derived growth factor) may regulate the proliferation and growth of satellite cells [13]. However, additional signals important for specifying embryonic muscle are still required to be identified, particularly during human development.

1.1.4. Regulation of skeletal muscle anabolism and mass

The mass and integrity of skeletal muscle is very important to the body health. Skeletal muscle plays major roles in whole-body disposal of glucose, amino acids and fatty acids, the accumulation of which can worsen the metabolic state [14-16]. Defects in skeletal metabolism and muscle growth will give rise to or aggravate many diseases, such as diabetes, cancer, and HIV, chronic kidney diseases, congestive heart failure, peripheral artery disease, rheumatoid arthritis [17-20]. In multiple diseases, muscle mass is a key predictor of disease prognosis, length of hospital stay, and treatment outcomes [16, 21].

There are many factors that regulate muscle mass and function. Physical activity and nutrition are the two key factors. The anabolic effects of resistance exercise on muscle protein synthesis and mass are well demonstrated [18]. Among macronutrients, proteins and branched-chain amino acids have remarkable effects in activating muscle protein anabolism [18, 22]. At the molecular level, the nutrient or energy sensor mTORC1 appears to mediate the anabolic effect of amino acids and resistance exercise [23, 24].

1.1.5. Central role of muscle protein in whole- body metabolism

Protein content maintenance of certain tissues and organs is fundamental for survival, such as the skin, brain, heart, and liver. These tissues and organs depend on a stable supply of amino acids through the blood to being as precursors for the protein synthesis to balance the protein breakdown rate in all

tissues in the postabsorptive condition. Moreover, muscle protein functions as the key reservoir to replace blood amino acid taken up by other tissues when there is no nutrient intake [25-27]. In the fasting condition, blood amino acids serve as precursors for both the protein synthesis and hepatic gluconeogenesis [28]. Therefore, the protein content of essential tissues and organs, as well as the concentration of necessary plasma glucose, can be maintained in a relatively homeostasis even that the nutritional intake is absent, provided muscle mass is sufficient to provide the necessary amino acids.

The breakdown of net muscle protein remarkably maintains plasma amino acid concentrations, providing sufficient muscle mass. For example, obese individuals who had increased muscle mass can maintain normal plasma amino acids concentrations after ≥60 days of fasting [29]. By comparison, depletion of muscle mass is detrimental for life. For example, there is firm correlation between the depletion of body muscle mass and the survival time of seriously ill patients with AIDS [30].

1.1.6. Role of muscle in chronic disease

Chronic diseases caused by unhealthy lifestyle account for more than two-thirds of deaths in the world. Studies focused on daily diet and physical activity and measure indexes to predict risk of disease, such as blood lipids, body mass index, and bone biomarkers. However, few studies have assessed muscle mass or physical or metabolic function to make clear the role of muscle. Nonetheless, changings in muscle has a crucial role in the most common conditions and

diseases. In the United Stats, heart disease and cancer are the major chronic diseases [31]. Both cancer and cardiac failure are often implicated with rapid and massive loss of muscle mass, strength, and metabolic function. Under these cachexia conditions, the muscle mass is a key determinant of survival [32, 33]. Sarcopenia is the degenerative loss of skeletal mass, quality, strength and function associated with aging. Sarcopenia is known as a widespread syndrome that has a destructive effect on the life quality and survival. Sarcopenia is a component of the frailty syndrome, and behaves impairment of the ability to perform activities of daily living.

1.1.7. Skeletal muscle and physical exercise

Physical activity is beneficial to human health. It can prevent many preventable death and premature death. About one in three adults and four in five adolescents around the world do not achieve the recommended quantity and quality of daily exercise. Regular exercise and physical activity are the basis in the prevention, management, and treatment of many diseases, including coronary heart disease, hypertension, obesity, type 2 diabetes mellitus (T2DM), and age-related muscle wasting (sarcopenia) [34, 35]. Actually, regular exercise combined with dietary intervention is more effective than pharmacological intervention in the prevention and treatment of T2DM [36] and sarcopenia [37].

1.1.7.1. Exercise and skeletal muscle mass

Skeletal muscle structure and function can adapt to environmental changes and to various stimuli, including stimuli modifying its contractile activity (inactivity,

denervation, endurance exercise, electrical stimulation), stimuli modifying applied load (resistance exercise, unloading, microgravity) and other environmental factors such as growth factors, nutrient availability, hypoxia, heat, and inflammation mediators. There are 3 potential approaches to maintain or improve muscle mass and function: exercise, and nutrition, hormonal therapy. Here the review focuses on the exercise.

Exercise can improve muscle function and increase muscle mass. Improved function may include both the muscle contractile properties and muscle metabolism. Exercise training improves the insulin sensitivity [38]. Progressive loss of strength [39] and muscle mass [40] happens throughout life, and the rate of loss is accelerated in the middle age and maintained until the old age [41]. Therefore, earlier intervention (e.g. in middle age or younger ages) is imperative to prevent and decrease the deleterious effects of sarcopenia in old age.

According to either increasing muscle endurance or increasing muscle strength, physical exercises modifying the muscle plasticity are generally classified into two categories. Muscle endurance, as the ability of muscle to sustain repeated relatively low intensity affairs for long periods of time, is also linked to the aerobic capacity.

On the other side, muscle strength, the ability of muscles to overcome a resistance, is built up by increasing muscle load rather than by repeating low intensity exercise. This is supported by muscle fiber hypertrophy. It mostly depends on the glycolytic metabolism, thus resistance training only slightly

improves the aerobic capacity and assists only marginally in the maintenance of cardiovascular health [42].

1.1.7.2. The Molecular Basis of Skeletal Muscle Adaptation to Exercise

Skeletal muscle displays remarkable plastic in functional adaptation and remodeling in response to contractile activity. Training-induced adaptations are demonstrated by changes in contractile protein and function [43], metabolic regulation [44], mitochondrial function [45], transcriptional responses [46], and intracellular signaling [47]. The molecular mechanisms that govern the adaptation to exercise training comprise a gradual alteration in protein content and related enzyme activities. These progressive changes show activation and/or repression of specific signaling pathways that regulate the process of transcription and translation, and exercise-responsive gene expression.

Transient postexercise changes in gene transcription include immediate early genes, myogenic regulators, carbohydrate (CHO) genes metabolism, lipid mobilization, mitochondrial metabolism and oxidative phosphorylation, transport and oxidation, and transcriptional regulators of gene expression and mitochondrial biogenesis [46, 48]. A single bout of exercise alters the DNA binding activity of many transcription factors, including HDACs [49], MEF2 [50], and NRFs [51]. Both protein stability and subcellular localization of transcriptional factor complexes within the nucleus and mitochondria are also influenced [52, 53].

Then, review focuses on cellular energy status, ATP Turnover, and AMP-

Activated Protein Kinase Signaling. AMPK modulates cellular metabolism by the phosphorylation of metabolic enzymes [54] and by transcriptional regulation [55]. AMPK activation is regulated through a cellular energy deficit, which is revealed by increases in the AMP/ATP and Cr/PCr (creatine-phosphocreatine) ratios. Beside intense exercise, cellular stresses that deplete ATP or increase the cellular AMP/ATP ratio (such as glucose deprivation or oxidative stress) also facilitate the activation of AMPK [56]. Given the rate of ATP turnover during muscle contraction, by responding to an altered cellular energy status, AMPK plays the role as a signal transducer for metabolic adaptations. Acute exercise increases the phosphorylation of AMPK and enzymatic activity in an intensity-dependent manner [57], showing intensity-dependent effects of physical exercise on ATP turnover and adenine nucleotide concentrations [58].

AMPK activation acts to conserve ATP by suppressing biosynthetic and anabolic pathways, while stimulating catabolic pathways simultaneously to restore cellular energy [56]. In skeletal muscle, acute AMPK activation inhibits glycogen [54] and protein synthesis [59], but improves glucose transport [60] and lipid metabolism [61]. Chronic AMPK activation changes metabolic related gene expression and induces mitochondrial biogenesis [62], partly through AMPK-induced regulation of the DNA binding activity of transcription factors including HDACs, MEF2, and NRF-1[62].

1.1.7.3. Regulation of Skeletal Muscle Gene Expression and Adaptation

An increasing network of transcription factors and co-regulator proteins has emerged. Through integrating signals from physiological stimuli and

coordinating metabolic adaptation it regulates the skeletal muscle phenotype. This network applies molecular control on contractile, metabolic, and mitochondrial adaptation, clarified by an ability to change the expression of key enzymes in CHO and lipid metabolism, and also the coordination of myogenesis and mitochondrial biogenesis in answer to exercise. The complicated and highly regulated process of mitochondrial biogenesis requires the co-ordination and co-expression of both the nuclear genomes and the mitochondrial genomes for the assembly and expansion of the reticulum, and a dynamic mitochondrial network.

By using transgenic animals and pharmacological manipulation, mechanistic studies have demonstrated the roles of key regulators of skeletal muscle phenotype. Various regulators are sufficient to activate mitochondrial biogenesis, fiber-type transformation, and reprogramming of skeletal muscle metabolism, but many of them are not indispensable for exercise-induced skeletal muscle adaptation. For instance, PGC-1α acts as a transcriptional coactivator through recruitment and co-regulation of numerous transcription factors that regulate skeletal muscle gene NRF-1, NRF-2, ERRα, and Tfam expression [63]. PGC-1α activity is highly controlled by many posttranslational modifications: phosphorylation and deacetylation [55, 64]. Through acute exercise, key upstream kinases and deacetylases regulating these modifications are activated [51, 57, 64], corresponding with changes in protein stability, functional activity, and subcellular localization [52, 53]. Abnormal expression of PGC-1α in muscle increases mtDNA expression and mitochondrial biogenesis [65], whereas

changing the PGC-1α activity induces molecular adaptations that enable the cell to meet the changing energy demands, augmenting cellular respiration rates and substrate utilization [66]. The phenotype of PGC-1α overexpression in rodent skeletal muscle is similar to aerobically trained muscle, characteristic of increased mitochondrial density, ATP synthesis, respiratory capacity, antioxidant defense in type II muscle fibers, and improved exercise performance [66]. Whereas, in muscle-specific PGC-1α KO mice, various of these adaptations are reversed, including that muscle fibers exhibit a more glycolytic phenotype, reduced exercise capacity, impaired mitochondrial respiratory function, and impaired recovery from exercise [67]. Nevertheless, after exercise training, myoglobin, metabolic gene expression, and mitochondrial biogenesis increase, which are similar to wild-type animals, whereas training-induced alterations in mitochondrial enzyme expression are only properly reduced. Remarkably, exercise-induced angiogenesis is reduced in PGC-1α KO mice [68]. Thus, despite strong effects on skeletal muscle phenotype, PGC-1α is not *essential* for the majority of adaptive responses to exercise training.

Numerous proteins play roles in the regulation of metabolic gene expression and mitochondrial biogenesis in skeletal muscle. Exercise-induced modulation of these pathways is critical to skeletal muscle adaptation and modulation of the mitochondrial phenotype [69].

In addition, Dysfunctional mitochondria have been involved in sarcopenia [70] and insulin resistance [71], however, long-term aerobic exercise training may

prohibit the age-related declines in health that proceed as a function of mtDNA mutations, as found in mtDNA mutator mice [72]. Therefore, mitochondrial biogenesis and adaptation with regular exercise have implications for a broader range of health issues, not only benefit exercise performance.

1.1.7.4. Regulation of Skeletal Muscle Protein Synthesis by Contraction

The control of protein translation and synthesis produces protein during the exercise-induced hypertrophy, while the activation and incorporation of satellite cells add the newly formed myofibrils to the contractile machinery. The muscle hypertrophy induced by resistance exercise is strongly implicated with the $p70^{S6K}$ phosphorylation [73]. mTOR mediates nutrient and metabolic stimuli to regulate cell growth and proliferation. Contraction-induced $p70^{S6K}$ activation is dependent on mTOR. Activation of this pathway by contraction drives translational processes, and mediates muscle hypertrophy by protein accretion. Activation of mTOR is critical to load-induced muscle growth. This is demonstrated through the attenuation of hypertrophic responses and protein synthesis by rapamycin-the mTOR inhibitor [74]. The mTOR pathway controls mechanisms of protein synthesis at multiple levels including translation capacity, translation efficiency etc. through increases of specific mRNAs translation. mTORC1, which contains raptor and confers rapamycin sensitivity, is need for signaling to $p70^{S6K}$ and 4E-BP1. And meanwhile, mTORC2, which contains rictor and is rapamycin insensitive, is needed for signaling to Akt-FoxO [75]. The function of mTOR activity on downstream regulators of protein synthesis is mainly achieved by contraction-induced regulation of mTORC1 [76].

Nutrient-dependent regulation of muscle growth is accomplished through insulin- and Akt-dependent activation of the mTOR pathway. These pathways exhibit cooperativity to promote muscle growth in athletes and in disease states. They can be enhanced through adequate nutritional intake such as post exercise carbohydrate (CHO) and amino acid ingestion or increased dietary protein [77].

On the other side, both insulin and the systemic IGF-1 isoforms are involved in mTOR activation in active muscle. Then the activated mTOR upregulates protein synthesis [78]. Both insulin and IGF-1 activate PI3K, which generates PIP3 (phosphatidylinositol-3, 4, 5-triphosphate). Subsequently, PIP3 recruits Akt/PKB to the plasma membrane, where at least two distinct kinases, PDK1 and the TORC2 complex phosphorylate it. Of the three isoforms in mammals, exercise is related with the Akt1 (PKBα) activation, which is upregulated by IGF1 and by still unclear contraction signals. Insulin can activate Akt2. Akt/PKB upregulates protein synthesis through activating the TORC1 pathway and through inhibiting GSK3β (glycogen synthase kinase 3β). The GSK3β downregulates protein synthesis via eIF2B (eukaryotic initiation factor 2B) inhibition [75].

1.2. Catching a glimpse of mTOR

1.2.1. TOR kinase

TOR is a conserved serine/threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family. The discovery of TOR

kinases originate from the study of rapamycin (sirolimus as the trade name), a natural compound first isolated from the *Streptomyces Hygroscopicus* bacteria. These bacteria were found in Easter Island (called Rapa Nui in the native language) in the South Pacific Ocean. Rapamycin was originally developed as an anti-fungal agent. To date, it is used as an FDA (Food and Drug Administration) -approved immunosuppressant and chemotherapeutic agent. The potent anti-proliferative properties and specificity of rapamycin have also proven to be very useful characteristics for studying cell growth regulation. As early as the beginning of 1990s, genetic screens in budding yeast identified TOR1 and TOR2 as mediators of the toxic effects of rapamycin. Soon after, biochemical approaches in mammals succeeded in purification of mTOR and its discovery as the target of rapamycin[79-81].

In the cell, the dimer that formed by rapamycin interacts with FKBP12 (FK506-binding protein 12 kDa) binds directly to TOR, inhibiting TOR activity [82]. From *Drosophila* to mammals, being a single gene product, TOR is commonly referred to as dTOR (*Drosophila* TOR) and mTOR (*mammalian* TOR). TOR kinase has putative orthologs throughout eukaryotes and possesses a marked conservation of its core cellular functions despite limited sequence similarities.

mTOR, with a predicted molecular weight of 289 kDa has a physiological important role of mTOR which is confirmed by the fact that the knockout of mTOR in mice is embryonic lethal [83-85]. mTOR contains 20 tandem HEAT

(a protein-protein interaction structure motif of two anti-parallel α-helices that found in *H*untingtin, *E*longation factor 3, PR65/*A* and *T*OR) repeats at the amino-terminal region, followed by a *F*AT (*F*RAP, *A*TM, and *T*RRAP) domain (Figure 2) [86]. The HEAT repeats functions to mediate protein-protein interactions. The C-terminal to the FAT domain is the FRB (*F*KBP12/rapamycin *b*inding) domain, which offers a docking site for the FKBP12/rapamycin complex. FATC (*FAT C*-terminus) domain is at the C-terminus of the protein. The kinase domain is between the FRB and FATC domain. The mechanism that FAT and FATC domains modulate mTOR kinase activity is not clear yet.

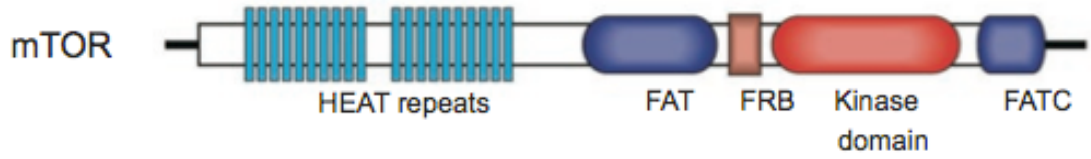


Figure 2. The domain structure of mTOR.

HEAT: a protein-protein interaction structure motif of two tandem anti-parallel α-helices structures found in *H*untingtin, *E*longation factor 3, PR65/*A* and *T*OR; FAT: a domain structure shared by *F*RAP, *A*TM and *T*RRAP, all of which are from PIKK family; FRB: *F*KBP12/*r*apamycin *b*inding domain; FATC: *FAT C*-terminus

1.2.2. mTOR complexes

mTOR interacts with multiple of proteins to form two distinct complexes, which are mTORC1 (mTOR complex 1) and mTORC2 (mTOR complex 2). The mTORC1 and mTORC2 complexes have different sensitivities to rapamycin as

well as upstream and downstream outputs (Figure 3, top panel) [87]. mTORC1 and mTORC2 share the catalytic mTOR subunit, mLST8 (mammalian lethal with sec13 protein 8, or G protein beta subunit-like, GβL) [88, 89], DEPTOR (DEP domain containing mTOR-interacting protein) [90], and also the Tti1/Tel2 complex [91]. By comparison, raptor (regulatory-associated protein of mTOR) [92, 93] and PRAS40 (proline-rich Akt substrate 40 kDa) [94-97] are specific to mTORC1, whereas rictor (rapamycin-insensitive companion of mTOR) [88, 98], mSin1 (mammalian stress-activated map kinase-interacting protein 1) [99, 100], and protor1/2 (protein observed with rictor 1 and 2) [96, 101, 102] are only components of mTORC2. The known molecular functions of the mTOR complex are described in Figure 3 (Figure 3, bottom panels) [87].

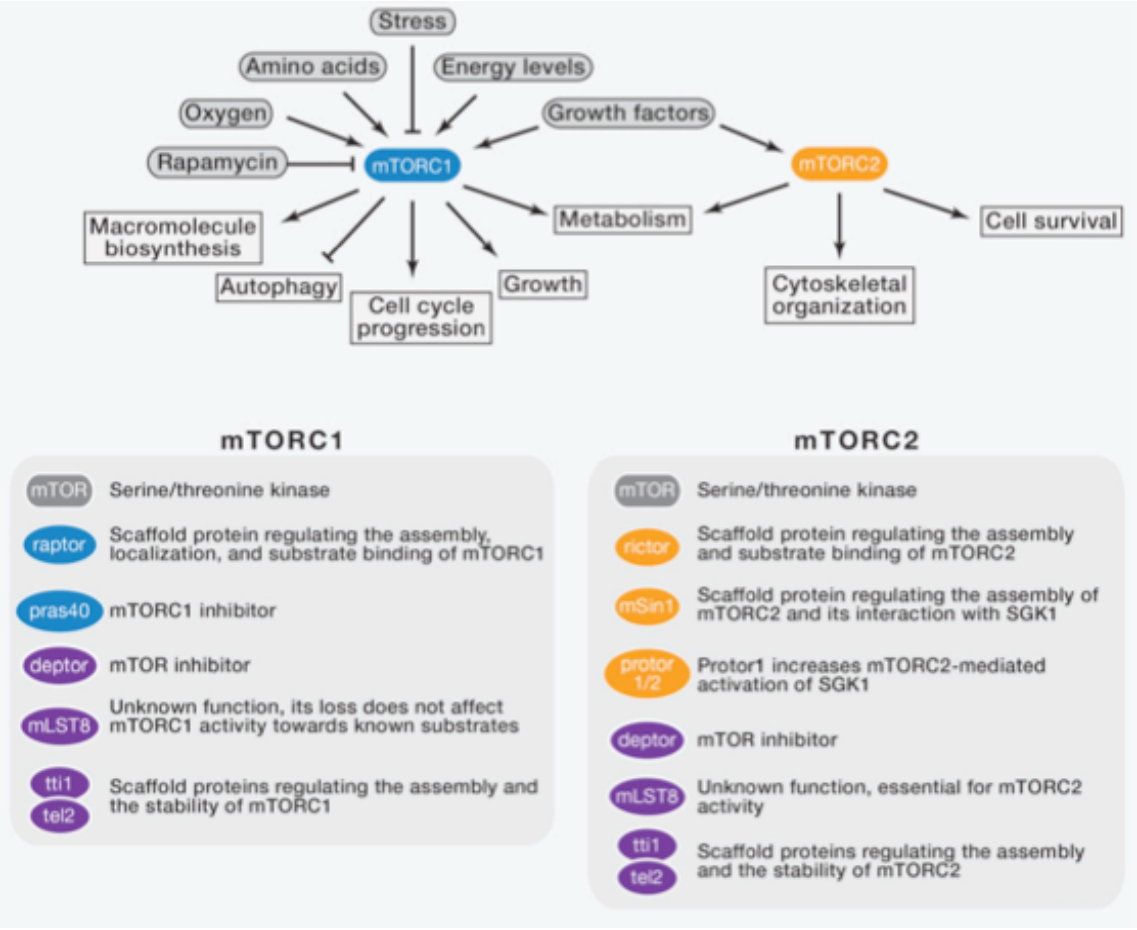


Figure 3. mTORC1 and mTORC2 Complexes. The mTOR kinase has two protein complexes termed mTORC1 and mTORC2 separately. mTORC1, acutely sensitive to rapamycin, responds to amino acids, growth factors, stress, oxygen, and energy. It facilitates cell growth through inducing and inhibiting anabolic and catabolic processes respectively, and drives cell-cycle progression. mTORC2 is insensitive to acute rapamycin treatment but its structure can be disrupted by chronic exposure to the drug. It responds to growth factors and regulates cell survival, metabolism and the cytoskeleton. The lower panel demonstrates the known functions of the protein components of the mTOR complexes and the bottom panel schematically depicts their interaction sites.

1.2.3. Functions of mTOR in metabolic tissues

mTOR signaling is found in all tissues, however, it is probably particularly significant in metabolic tissues. Metabolic organs including liver, muscle and adipose tissue are sensitive to nutrients, insulin/IGF-1, and energy, the three inputs that regulate mTOR. Liver, muscle and adipose tissue regulate the whole body glucose and lipid homeostasis.

1.2.3.1. mTOR in the liver

In the fasting condition, the liver produces glucose through glycogenolysis (glycogen breakdown) or through gluconeogenesis (glucose synthesis), to prevent hypoglycemia. In the feeding condition, the liver reduces blood glucose levels through various manners including glycolysis (blood glucose consumption), glycogenesis (conversion of glucose to glycogen) and lipogenesis (triglyceride). Mice with mTOR signaling defective in the liver are glucose intolerant, hyperglycemic, hyper- insulinemic and have decreased glycogen content [103-107], demonstrating that mTOR plays a major role in glucose

homeostasis in liver. The above defects are similar to those observed in type 2 diabetes patients, suggesting that defective hepatic mTOR signaling is related with the pathophysiology of type 2 diabetes.

Lipogenesis is activated through the transcription factor SREBP (sterol regulatory element-binding protein) [108-110]. As first demonstrated in mouse embryonic fibroblasts (MEFs) and retinal pigment epithelial cells, mTORC1 mediates SREBP-1 maturation in an S6K1-dependent manner to stimulate de novo synthesis of lipid [111, 112]. However, mTORC1 stimulates SREBP-1 expression in an S6K-independent manner in primary hepatocytes [113]. Above studies shows that mTORC1 activates SREBP expression and maturation, but through two separate effector pathways.

The S6K-independent pathway involves the phosphatidic acid phosphatase lipin-1, which is the mTORC1 substrate and functions as a negative regulator of SREBP-1 activity [114]. mTORC1 directly phosphorylates lipin-1 in response to nutrients and growth factors. This blocks the translocation of lipin-1 into the nucleus, hence allowing SREBP transcriptional activity. Studies demonstrate well that mTORC1 is essential to activate SREBP-1 and lipid synthesis in cultured cells, however, the mTORC1 function in lipogenesis in vivo is still less clear. Liver-specific mTORC1 deficient (raptor knockout) mice present decreased triglyceride content in liver and a reduction in plasma cholesterol levels only in the condition of a high fat diet [114]. Therefore, mTORC1

signaling is likely essential for the accumulation of hepatic triglyceride in vivo only in the pathological conditions.

Study shows that protection against the accumulation of hepatic lipid in LTsc1KO (liver-specific tsc1 knockout) mice is due to the Akt signaling attenuation, as restoration of Akt2 (the main hepatic isoform of Akt) restores the lipogenesis [107]. This demonstrates that mTORC1 and Akt are independently essential for lipogenesis. Decreased Akt signaling in LTsc1KO mice is due to the mTORC1-mediated negative feedback loop [107]. Akt is proposed to prevent expression of Insig2a encoding an SREBP inhibitor [107]. mTORC1 is essential in the activation of SREBP as shown above. Therefore, both Akt and mTORC1 are required for lipogenesis, and the molecular mechanism of selective hepatic insulin resistance still need to be further studied. However, there was data showed that mTORC1 is not necessary for the accumulation of hepatic lipid, because the treatment of rapamycin does not prevent high-fat diet or Pten deletion- induced hepatic steatosis [106].

mTORC2 is also insulin-stimulated and is important for lipid and glucose homeostasis in the liver. LiRiKO (Liver-specific rictor knockout) mice are hypolipidemic and hyperglycemic [103-105], showing total hepatic insulin resistance. This is due to the loss of phosphorylation on Akt Ser473 site [104]. LiRiKO mice, like the LTsc1KO mice, present reduced SREBP-1c activity. Akt signaling restoration suppressed the defects in SREBP-1c activity and de novo lipogenesis [104].

LiRiKO mice shows defects in SREBP-1c activity and hepatic lipogenesis, and mTORC2 but not mTORC1 is impaired. These mean that Akt regulates SREBP-1c at least partly independently of mTORC1. The regulation of Insig2a was not changed in the liver of LiRiKO mice, indicating that the phosphorylation of Akt Ser473 is not necessary for Insig2a inhibition. To sum up, mTORC1, mTORC2, and Akt are required for hepatic lipogenesis.

Hepatic mTORC2 regulates glucose homeostasis through the glycolysis activation and gluconeogenesis inhibition [104]. mTORC2 stimulates glycolysis by the activation of glucokinase and the transcription factor ChREBP. mTORC2 inhibits gluconeogenesis through inhibiting the FoxO1nuclear accumulation. The regulation of glucokinase and FoxO1 are through the Akt Ser473phosphorylation. These studies demonstrate that hepatic mTORC2 tightly regulates Akt to regulate glucose and lipid homeostasis and thus the whole body metabolism. A defect of mTORC2 signaling in the liver may lead to the development of diabetes.

1.2.3.2. mTOR in adipose: Regulation of adipogenesis and lipogenesis

mTOR signaling plays a essential role in adipogenesis [115]. Adipogenesis leads to the adipose tissue formation, the most important storage site of energy in mammals. In vitro, mTORC1 inhibition blocks adipogenesis and impairs the fat cells [116-118]. In contrast, mTORC1 overactivation promotes adipogenesis [119]. There are numerous downstream effectors implicated in the regulation of adipogenesis. S6K1, through regulating the expression of early adipogenic

transcription factors, regulates the commitment of embryonic stem cell to adipogenic progenitors [120]. The 4E-BPs regulate the terminal differentiation of adipocytes by the translational control of PPAR-g, the master regulator of adipogenesis [120, 121].

Mice with adipose-specific knockout of the mTORC1 are lean and have smaller and fewer adipocytes. The high-fat diet cannot induce obesity of these mice [118]. On the other side, mice with adipose-specific knockout of mTORC2 have normal fat mass but a defect in adipose tissue phosphorylation of Akt that leads to an increase in lipolysis and circulating free fatty acids (FFA) [122].

mTORC1 is greatly active in the tissues of obese and high-fat-fed rodents [123-125]. Elevated circulating insulin levels, nutrients (e.g. branch-chain amino acids and glucose), and proinflammatory cytokines, probably promote the activity of mTORC1 in obese animals. In addition to directly contributing to the expansion of adipose tissue by the activation of adipogenic/lipogenic factors in adipose tissue, mTORC1 promotes insulin resistance by the S6K1-mediated inhibition of insulin signaling [125]. The reduction of insulin action in adipose tissue probably exacerbates systemic insulin resistance through promoting the release of FFA via adipocytes, ectopic fat deposition, and lipotoxicity [126].

Studies show that the high rate of protein synthesis linked with mTORC1 activation probably induces insulin resistance through promoting ER (Endoplasmic reticulum) stress and the UPR (unfolded protein response) [127]. ER stress is prevalent in enlarged adipocytes. ER stress impairs insulin signaling

by the destabilization of IRS1 via JNK (c-Jun N-terminal kinase) [128]. Further studies are required to know what extend the activation of mTORC1 in the adipose tissue of obese individuals promotes the ER stress and insulin resistance.

Rictor$^{ad-/-}$ (adipose-specific rictor knockout) mice present an increase in body size because of an increase in lean mass and largely unaffected fat mass [122, 129]. This can be explained through the study that mTORC2 in WAT negatively regulates IGF-1 and the production of insulin via the liver and pancreas, respectively, thus controlling systemic growth and glucose and lipid metabolism [129]. In the adipose tissue, a negative feedback endocrine loop may attribute to the mTORC2-mediated regulation of IGF-1 and insulin, considering that these hormones active mTORC2 itself. Further studies are needed to identify the possible factor(s) that signal from adipose to the liver and pancreas as part of such a loop. And this loop might maintain the hormone homeostasis.

1.2.3.3. mTOR in muscles

mTOR or raptor knockout mice have been generated to study the function of mTORC1 signaling in skeletal and cardiac muscle. Skeletal muscle-specific knockout mice present progressive muscle dystrophy, decreased oxidative capacity and increased glycogen content [130, 131]. Skeletal muscle of S6K1 deficient mice develops atrophic and accumulates glycogen, showing that mTORC1 regulates muscle mass and physiology via at least S6K1 [132, 133]. Muscle of S6K1 deficient mice exhibit increased mitochondrial activity not

decreased, implying that mTORC1 may control mitochondrial oxidative capacity by a substrate other than S6K1 [133]. Mice with cardiac-specific mTOR or raptor knockout have dilated cardiomyopathy due to the loss of 4E-BP1 inhibition and subsequently reduced protein synthesis [134, 135].

Studies show that the increased glycogen accumulation in skeletal muscle-specific mTOR or raptor knockout mice is mediated through the Akt hyperactivation due to the loss of the negative feedback loop [130, 131]. Although Akt hyperactivation, muscle-specific raptor knockout mice present slightly glucose intolerant. This is beyond expectation because Akt activates glycolysis and glucose uptake. Reduction in PGC-1α leads to the decrease in mitochondrial oxidative capacity in the raptor knockout mice. The defect is suppressed through the restoration of PGC-1α expression [136].

Although with reduced Akt Ser473 phosphorylation, skeletal muscle-specific rictor knockout mice have no obvious phenotype [131, 137]. The slightly glucose intolerant of them may be due to loss of Akt-medited AS160 phosphorylation. AS160 (Akt substrate of 160 kDa) is a main Akt substrate essential for insulin-stimulated translocation of the glucose transporter GLUT4 (Glucose transporter type 4) to the plasma membrane [137].

1.3. Downstream effects of TOR signaling

1.3.1. TOR and functions of TOR kinase

1.3.1.1. Biological functions of TOR kinase mediated by TORC1

Amino acids or growth factors can induce mTORC1, activated mTORC1 promotes diverse cellular processes, including ribosome biogenesis, stimulation of mRNA translation, and inhibition of apoptosis [138]. This leads to increased size and number of cells. Those outcomes are caused by the action of mTORC1 on its substrates, especially 4E-BP1 and ribosomal S6 kinase (S6K1) (Figure 4.) [138, 139]. Likewise, inhibition of TORC1 activity leads to a decrease in cell size and a reduction in proliferation. Other substrates of mTORC1 are involved in regulating the gene transcriptions required for the ribosome production.

mTORC1 is also been implicated in regulating miRNA (micro RNA). This class of small RNAs regulates gene expression through binding to the 3′ end of mRNA, thus inhibiting translation and (or) destabilizing the target mRNA [140]. Among these small RNA, miR-1 is a muscle-specific miRNA involved in myogenesis regulation. In myotubes, mTORC1 regulates the transcription of this miRNA, as rapamycin treatment reduces its abundance [141].

The best-characterized functions of mTORC1 in skeletal muscle are those mediated by the actions of the complex on 4E-BP1 (eukaryotic initiation factor 4E-binding protein) and S6K1 [81]. Nutrients and growth factors are the upstream signaling of TORC1. They are transported through TORC1 to the translation machinery by eIF4E (eukaryotic initiation factor 4E) and 4E-BP1. 4E-BP1 and S6K act upon the regulatory complexes bound to the untranslated regions of mRNAs [81]. eIF4E is inhibited by 4E-BP1. This inhibition is relieved by mTORC1 through phosphorylating 4E-BP1, thus promoting

formation of eIF4F complex and mRNA translation [142]. Under nutrient limitation conditions, 4E-BP negatively regulates the assembly of initiation factors through binding and isolating eIF4E, thereby the function of eIF4E to recruit the translation initiation complex to the 5' mRNA cap structure is suppressed [139, 143].

TORC1 activation also promotes initiation of translation through phosphorylation of S6K [142]. S6K phosphorylates various substrates implicated in translation initiation, including the eIF4B regulatory subunit of RNA helicase (eIF4A), leading to the progression of the small ribosomal subunit towards the start codon (Figure 4) [139, 143].

S6K is also implicated in a negative-feedback loop that acts to decrease the activity of TORC1 (Figure 4); the phosphorylation and inactivation of IRS1 (insulin receptor substrate 1) by S6K results in a decrease in PI3K activity and thus reduces AKT activation and hence the TORC1 signaling [144, 145].

In $TSC1^{-/-}$ or $TSC2^{-/-}$ MEFs with hyperactivation of mTORC1 and S6K1, PI3K-Akt signaling cannot be affected by insulin [146-149]. However, this can be rescued through the prolonged treatment of rapamycin. Similarly, the Akt activity of drosophila $TSC1^{-/-}$ larvae is attenuated, which can be restored via the knockout of dS6K [150].

These studies suggest that active mTOR-S6K1 impinges on Akt signaling. IRS1 (insulin receptor substrate 1) connects insulin receptors with PI3K through direct binding [151, 152]. Interestingly, the expression of IRS1 was reduced in cells

with an elevated S6K1 activity [153]. Further studies indicated that active S6K1 phosphorylates IRS1 on many inhibitory sites and promotes its degradation [144, 145, 154]. Therefore, the activation of mTOR-S6K1 can attenuate Akt signaling [125]. This auto-regulatory pathway is recognized as S6K1-dependent negative feedback inhibition [155]. This may explain why TSC tumors are normally benign, because over-activation of Akt, such as in the condition of PTEN mutations, often gives rise to malignancy. With TSC mutations, mTOR and S6K1 activities are enhanced, leading to overgrowth of tissue; meanwhile, they restrain other pathways responsible for reduced apoptosis and increased proliferation through inhibiting Akt signaling. Besides the modulation on PI3K-Akt signaling, S6K1 also can directly phosphorylate mTOR on a C-terminal site [156]. It is not clear of the phosphorylation consequence. It may increase the intrinsic kinase activity of mTOR. This provides a mechanism for mTORC1 to regulate its function through S6K1.

Moreover, TORC1 has been demonstrated to regulate ribosome biogenesis and tRNA production. In a nutrient-sensitive manner, TORC1-dependent phosphorylation of the key RNA polymerase regulatory subunit TIFIA regulates the outcomes of RNA gene transcripts [157].

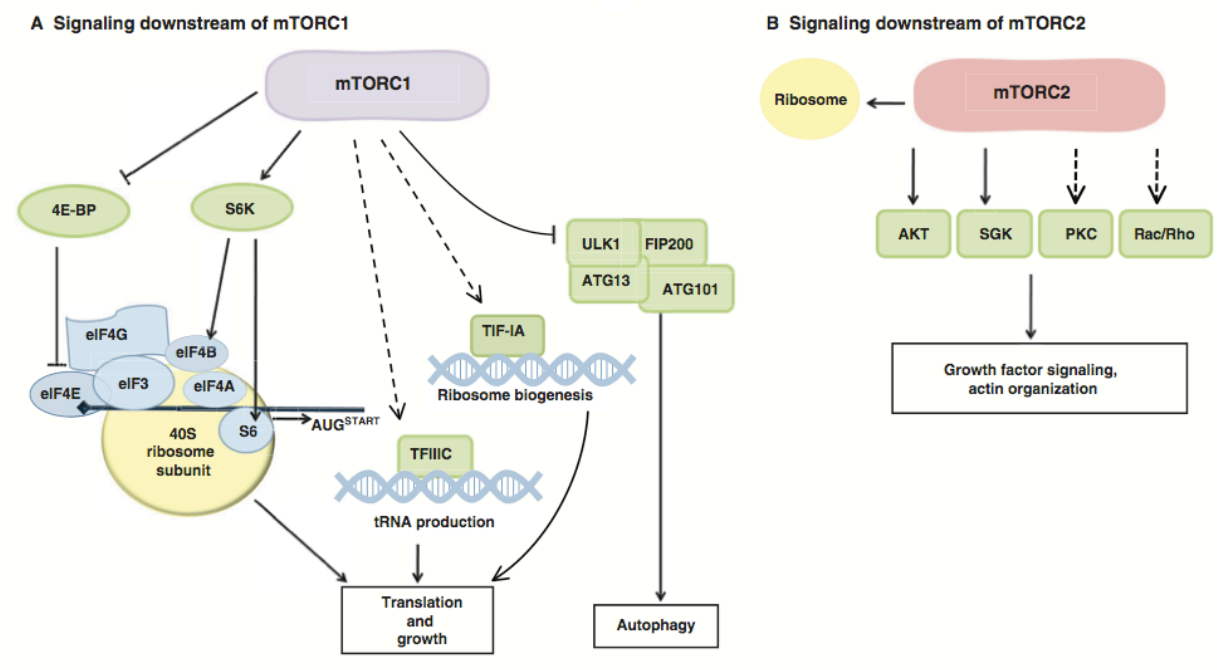


Figure 4. Downstream of mTORC signaling. (A) mTORC1 (Mammalian target of rapamycin complex 1) phosphorylation of eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP) breaks the interaction of 4E-BP with eIF4E, which releases eIF4A to facilitate the binding of ribosomes to the transcriptional initial site. mTORC1 also activates ribosomal S6K, which activates the eukaryotic translation initiation factor 4B (eIF4B) and the S6 ribosomal protein through direct phosphorylation. mTOR can also function with general transcription factor III C (TFIIIC) and relieve its inhibitor Maf1, and thus lead to increased tRNA production. TORC1 activation also promotes association between the transcription initiation factor 1A (TIF-1A) and polymerase I (PolI), then promoting rRNA synthesis. At last, mTORC1 signaling can phosphorylate Unc-51-like kinase 1 (ULK1) and autophagy related 13 homolog (ATG13), consequently inhibit autophagy. (B) mTORC2 activates AKT and serum/glucocorticoid regulated kinase (SGK) by phosphorylation, and is involved in signaling via protein kinase C (PKC) and Rac/Rho. Overall, these mTORC2-mediated activities promote growth factor signaling and cytoskeletal reorganization. mTORC2 can also interact with ribosomes in response to insulin signaling. Abbreviation: ATG101, autophagy-related protein 101; FIP200, FAK family interacting protein of 200 kDa.

1.3.1.2. Kinase-independent function of mTOR in myofiber formation and growth

mTOR kinase activity is required for almost all mTOR functions. However, studies found that mTOR regulation of the initiation of C2C12 myoblast differentiation was kinase activity independent, and this is mediated through IGF-II [158, 159], which is implicated in both satellite cell proliferation and differentiation [160]. Ge et al. generated and characterized transgenic mice expressing two mTOR mutants with the control of HSA (human skeletal actin) promoter: rapamycin-resistant (RR) and RR/kinase-inactive (RR/KI). They demonstrated that the kinase-inactive RR/KI-mTOR appears myofiber formation with the treatment of rapamycin as in RR-mTOR. Moreover, the expression of Igf2 mRNA during regeneration is inhibited by rapamycin treatment and partially rescued through the expression of both RR- and RR/KI-mTOR transgene [161]. These data are consistent with a kinase-independent function of mTOR in the process of myofiber initial formation during regeneration.

The myofiber growth requires the kinase-active form of mTOR. This is supported by RR- mTOR, but not RR/KI-mTOR, in the treatment of rapamycin. These findings fully accord with studies in vitro, that rapamycin-sensitive mTOR signaling is essential for myotube maturation and hypertrophy [162, 163]. *s6k1*$^{-/-}$ mice displayed that growth of regenerating myofibers is impaired but the regenerating fiber number is similar to that in WT mice. These studies demonstrate the requirement of S6K1 for muscle growth [132] and also validate in vivo for the S6K1-independent initiation of myoblast differentiation in vitro [158, 159]. These are consistent with the kinase-independent role of mTOR in regeneration as S6K1 is a main substrate of the mTOR kinase.

1.3.2. TORC1 mediates repression of autophagy

TORC1 is a potent and important inhibitor of autophagy. Autohagy is a very important lysosomal- dependent cellular degradation process that regulates and generates energy and nutrients to maintain essential cellular activities upon nutrient starvation condition. The TORC1 ability to regulate autophagy is highly conserved, as the process of autophagy itself. Accumulating research evidence suggests that ULK1 (Unc-51-like kinase 1), a serine/threonine-protein kinase involved in autophagy in response to starvation, the mammalian homolog of the yeast protein kinase autophagy-specific gene 1 (ATG1). ULK1 plays an important role in the regulation of autophagy initiation. ULK1 is directly phosphorylated by the energy-sensing kinase TORC1 [164-166]. It is known that TORC1-mediated phosphorylation of ULK1 inhibits its activation following the limitation of energy and leads to the whole decrease in autophagy [167]. AMPK can activate ULK1, but mTORC1 is capable of disrupting the interaction between ULK1 and AMPK by phosphorylation of ULK1 [167, 168].

1.4. mTOR complex 2 signaling network

1.4.1. mTORC2 and its function

Owing to the specific inhibition of TORC1 by rapamycin, much less is known about upstream and downstream signaling of mTORC2 compared to mTORC1. mTORC2 complex is insensitive to cellular energy or nutrients conditions. However, it can be regulated by insulin and growth factors through a not

well-understood mechanism requiring PI3K [169].

In addition, active mTORC2 assoicates with the ribosome, and insulin promotes mTORC2-ribosome binding in a PI3K- dependent mode, the recruitment of ribosome seems essential for mTORC2 signaling [170]. The TSC1/TSC2 complex is able to control mTORC2 in a large range of different cell types.

The TSC1/TSC2 complex that downregulates mTORC1 is required for proper mTORC2 activation [171, 172]. This tumor suppressor complex physically interacts with mTORC2 via rictor subunit, but its regulation of mTORC2 is independent of its GTPase-activating protein activity toward Rheb, which is different from mTORC1 [171, 173]. Furthermore, the impaired mTORC2 activity in TSC2- deficient cells cannot be restored by downregulating mTORC1. This suggests that, in addition to the mTORC1-mediated feedback regulation of IRS-1, mTORC2 can be more directly regulated by TSC1/TSC2 complex.

Besides, phospholipase D (PLD) can activate mTORC2 [174]. Studies in vitro have demonstrated that IKK (inhibitor of nuclear factor κ-B kinase) regulates the activity of mTORC2 through physically affecting mTORC2 assembly. Inactivated IKK interacts with Rictor and competes with mTOR, therefore reducing mTORC2 activity [175].

Activated mTORC2 phosphorylates Akt, SGK1 (serum- and glucocorticoid-induced protein kinase 1) and PKCα (protein kinase C α). mTORC2 phosphorylates Akt at Ser473 site, and this phosphorylation is necessary for the full activation of Akt [176]. Phosphorylation at Ser473 site is a post-translational modification and proceeds upon Akt recruitment to the

membrane through affinity of its PH domain to membrane lipids. This means that mTORC2 and Akt would colocalize at the membrane. In fact, mTORC2 has been localized to membrane compartments [177, 178].

mTORC2 might thus active mTORC1 and, by interacting with the 60S ribosome subunit directly, promote protein synthesis [179]. Meanwhile, mTORC1 can negatively regulate the activation of mTORC2. S6K1 can phosphorylate mTORC2 component mSin1, thus dissociate it from mTORC2 [180]. SGK1 can be directly activated by mTORC2 to regulate ion transport and cell growth [181]. In addition, mTORC2 activates the PKCα, thus regulates cell shape through affecting the actin cytoskeleton [115]. Remarkably, recent studies have proved that mTORC2 together with mTORC1 participates in the regulation of insulin signaling through regulating IRS-1 degradation in cells [182].

1.4.2. mTORC2 and autophagy regulation

While mTORC1 is clearly associated in lysosomal signaling and autophagy, there is much less experimental evidence showing that mTORC2 is involved. However, mTORC2 can indirectly repress autophagy through Akt1. As well as repressing autophagy through Akt1/mTORC1 activation, mTORC2 also represses autophagy through the Akt1/forkhead box O3 (FoxO) 3A signaling pathway [183]. In skeletal muscle, Akt1 activation inhibits the transcription of autophagy genes, such as MAP1LC3A, GABARAPL1 and Bnip3 (BCL2/adenovirus E1B 19kDa interacting protein 3), which are normally induced by fasting [184]. This effect was independent of rapamycin, indicating

no mTORC1 involvement. However, inhibition of mTORC2 through Rictor knockdown allowed FoxO3A nuclear translocation and formation of autophagic vesicles, suggesting that mTORC2 activation was essential for autophagy suppression [184].

On the basis that involvement of Akt1 and mTORC1 as negative regulators of autophagy, stimulation with insulin-like growth factor (IGF)-1 would be expected to repress autophagy. However, beyond our expectation, knockdown of the IGF-1 receptor and impaired IGF-1 signaling, actually inhibit autophagy process [185]. This effect was due to the influence of mTORC2 on the actin cytoskeleton and endocytic pathways. Strategies of Rictor knockdown to block mTORC2 signaling reduced the phosphorylation of PKC-α/β and strongly disrupted the actin cytoskeleton. As a result, this reduced endocytosis and impaired the formation of the early autophagosome precursors [185]. This suggests that a controlled appropriate level of mTORC2 signaling is necessary for formation and trafficking of autophagosomes. Considering that mTORC2 seems to have a dual role as a negative and positive regulator of autophagy, caution is required when applying the dual mTORC1/2 kinase inhibitors, as long-term treatment will likely disturb normal autophagic processes.

Muscle- specific mTOR knockout mice display reduced postnatal growth, due to reduced size of fast muscle fibers and severe myopathy [130]. Muscle-specific loss of rictor (RImKO) mice has minimal change on muscle physiology [131, 137], and raptor/rictor muscle-specific double knockout (DmKO) possess similar pathological phenotypes as RAmKO mice. These indicate that mTOR

functions require only mTORC1 in adult skeletal muscle [131]. Nevertheless, one study shows that some functions of mTOR (e.g. the activation of terminal oligopyrimidine mRNA translation) could be independent of both mTORC1 and mTORC2 [186].

Whether mice specifically depleted for rictor in skeletal muscle (RImKO) would show changes in autophagy? Rencent study shows that there is no difference of the GFP-LC3 puncta number in RImKO mice muscles versus the control mice in basal and starved conditions [187]. Moreover, in 3- and 6-month-old RImKO mice, the levels of LC3I, LC3II, and p62 were similar to control mice [187]. These data demonstrate that autophagy in skeletal muscle is not regulated via mTORC2.

1.5. Upstream regulation of TORC signaling

As described before, two TOR complexes have been identified in yeast [188-190]. However, because of the availability and wide use of rapamycin, the potent and specific mTORC1 inhibitor, most of our current knowledge of mTOR is concentrated on mTORC1.

mTORC1 is the better characterized of the two mTOR complexes—mTORC1 and mTORC2. An obvious characteristic of this branch of the pathway is the diversity and number of upstream signals it senses. The mTORC1 pathway integrates inputs from numerous intracellular and extracellular cues, including growth factors, amino acids, stress, energy status and oxygen, to control various major processes such as protein and lipid synthesis and autophagy (Figure 5).

For years, massive and interesting progress has been made in identifying mTOR-associated proteins, enabling to the discovery of mTORC2. Compared to mTORC1, mTORC2 has a distinctive physical structure and physiological functions compared to mTORC1 [191, 192]. These findings extend our knowledge in regarding mTOR biology. TORC2 can be stimulated by growth factors and PI3K [88]. PI3K inhibitors are able to inhibit TORC2-mediated target phosphorylation. The best-characterized target of TORC2 is Akt, also known as protein kinase B (PKB), is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes including cell proliferation, transcription, cell migration glucose metabolism, apoptosis etc. AKT is phosphorylated at Serine 473 under TORC2 activation condition [176]. Phosphorylation of Akt on Serine 473 site strengthens the activation phosphorylation at T308, which is required for AKT activity [193]. TORC2 also phosphorylates SGK (Serum and Glucocorticoid Induced Kinase) and PKC alpha (Protein kinase C alpha) [181, 194-196]. Further studies are necessary to clarify whether all these proteins represent direct targets of the TORC2 kinase, and to illuminate their physiological function in TORC2 signaling. The TORC2-mediated AKT activation set TORC2 on the upstream of TORC1 in the TOR signaling (Figure 5) [197]. One study emphasized a role for ribosomes in the activation of TORC2 [170]. The full activation of TORC2 by insulin required the interaction of TORC2 with NIP7 (nuclear import 7), which is a protein responsible for ribosome biogenesis and rRNA maturation). This raises many interesting questions regarding the regulation of TORC2 and its ribosomal

interactions, however, it also shows that additional levels of interaction between TORC1 and TORC2 might exist, because both complexes associated with the process of ribosome biogenesis. TORC2 is also able to regulate actin cytoskeletal organization via Rho GTPases, a function conserved with yeast TORC2 [88].

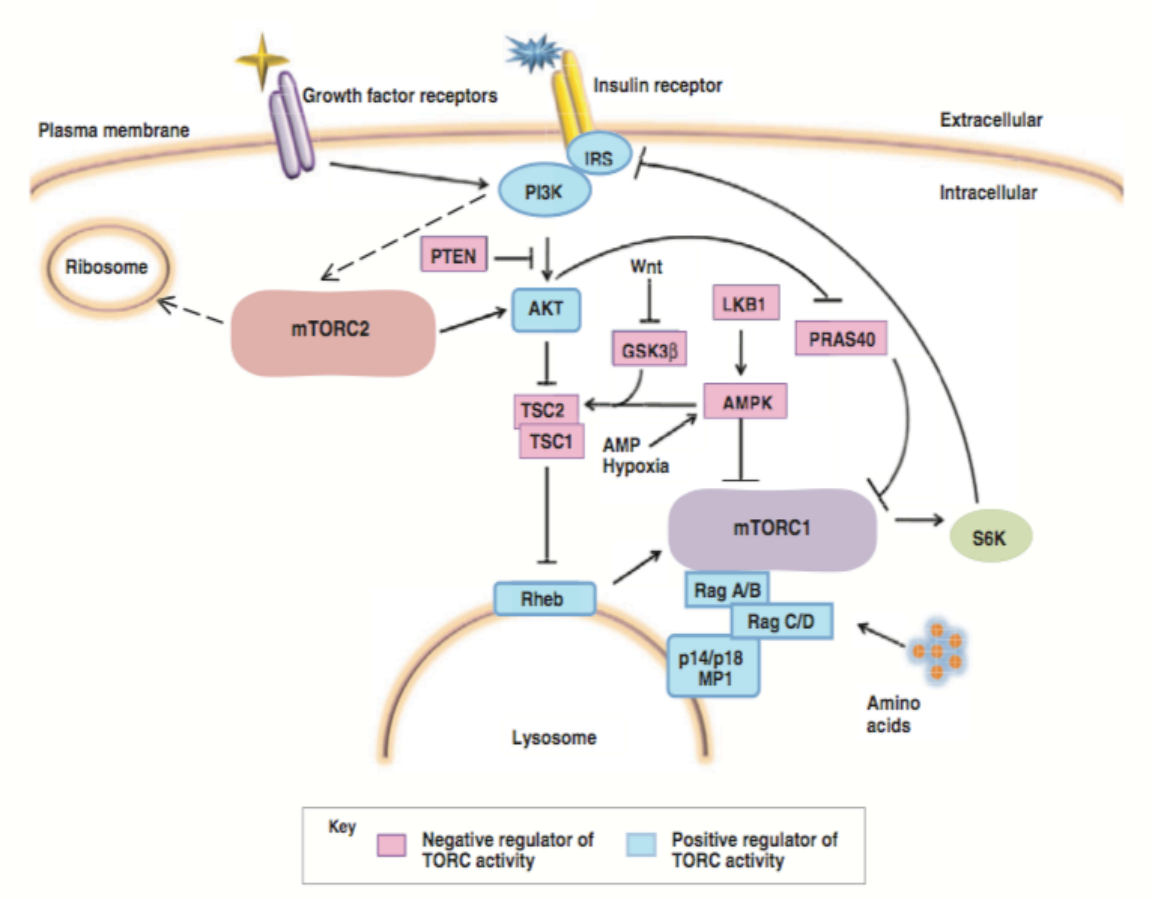


Figure 5. Upstream regulation of mTORC signaling. mTORC (Mammalian target of rapamycin complex) activity is influenced by numerous positive (shown in blue) and negative (shown in pink) regulators. mTORC1 is activated through growth factors and insulin signaling. This activation is mediated by PI3K (phosphatidylinositol 3-kinase) and AKT, which suppress the TSC (tuberous sclerosis complex) 1/2 complex, consequently relieving the TSC1/2-mediated repression of Rheb (Ras homolog enriched in brain) and activating TORC1. In contrast, low cellular energy levels (conveyed through AMP) and hypoxia activate AMP kinase. This kinase inhibits mTORC1 both by direct phosphorylation of TSC2 and by Raptor

(regulatory associated protein of mTOR) inhibition. The TSC1/2 complex acts to integrate intracellular and extracellular signals, and under unfavorable cellular conditions inhibits the activity of mTORC1. At the lysosomal membrane, mTORC1 can also be activated by amino acids, which act through the Rag proteins and the Ragulator complex (which consists of p14, p18 and MP1). mTORC2 can also be activated by growth factors through PI3K and some undetermined effectors (indicated by the broken arrow). In turn, mTORC2 can regulate AKT, thus placing it upstream of TORC1. Besides, mTORC2 can also be activated through its association with ribosomes. Abbreviations: IRS, insulin receptor substrate; GSK3β, glycogen synthase 3β; LKB1, liver kinase B1; PTEN, phosphatase and tensin homolog protein; PRAS40, proline-rich AKT substrate 40 KDa; S6K, ribosomal S6 kinase; MP1, MEK partner 1.

1.5.1. TSC complex

The heterodimer consisting of TSC1 (tuberous sclerosis 1; also known as hamartin) and TSC2 (also known as tuberin) is a key upstream regulator of mTORC1 and functions as a GTPase-activating protein (GAP) for the Rheb (Ras homolog enriched in brain) GTPase. The GTP-bound form of Rheb directly interacts with mTORC1 and thus strongly activates its kinase activity. TSC1/2, as a Rheb GAP, negatively regulates mTORC1 by converting Rheb into its inactive GDP-bound state [198, 199].

TSC1/2 suppresses mTOR to limit cell growth under stress conditions, and relieves its inhibition when conditions are favorable for growth. The TSC1/2 complex is inactive under growth conditions, hence facilitating Rheb-GTP to activate TORC1. Rheb overexpression enables the increasing TORC1 target

phosphorylation, which can be reversed by rapamycin treatment or by mTOR inactivation, suggesting that Rheb primarily functions through TORC1 [147].

In TSC syndrome patients, loss of mTOR inhibition causes a hyperactive mTOR, leading to cell overgrowth and tumor formation. In many other hamartoma syndromes, elevated mTOR activity has been detected. Thereby, these results implicate the possible common cause underlying diverse benign tumor syndromes, and highlight the role of mTOR as an anti-cancer drug target. Rapamycin naturally became the ideal drug to treat TSC syndrome due to its specific and potent inhibition of mTOR.

1.5.2. PI3K/Akt

mTORC1 can sense the growth factor signals, and subsequently regulate cell growth. Many growth factors (such as insulin) launch their intracellular signaling cascades by activating PI3K through cell surface receptors [200]. Akt is a major effector of PI3K, also called PKB (serine/threonine protein kinase B). Akt is one of the most crucial survival kinases, associated in regulating a wide range of cellular processes, including growth, metabolism, proliferation and apoptosis [201-203]. At the cell membrane, active PI3K gives rise to the generation of PIP3, the lipid second messengers or PtdIns(3,4,5)P3 (phosphatidylinositol-3,4,5-trisphosphate) and PIP2 (PtdIns(3,4)P2) [204, 205]. PIP3 recruits Akt through its PH (*p*leckstrin *h*omology) domain. The activation loop site T308 (Threonine 308) and the hydrophobic motif site S473 (Serine 473) are phosphorylated by PDK1 (3-phosphoinositide-dependent protein kinase-1)

and PDK2 respectively by Akt at the plasma membrane [206, 207]. Through the binding of its PH domain to PIP3, PDK1 is also recruited to the membrane [208]. Akt is completely activated by dual phosphorylation by PDK1 and PDK2. In Drosophila, TSC1/2 is determined to be the downstream of PI3K and Akt but upstream of S6K1 by Genetic epistasis analysis. And in fly, the fact that Akt directly phosphorylates TSC2 on multiple sites associates PI3K-Akt to TSC-mTORC1 [146, 209-211]. These phosphorylations inhibit the function of TSC1/2, and subsequently the mTOC1 activity is upregulated. Basing on this finding, the extracellular growth factor signals and intracellular TSC-mTORC1 regulation is linked, building the growth factor (insulin)- PI3K- Akt- TSC-mTORC1- S6K1/ 4EBP1 signaling pathway that explains how mTOR regulates cell growth under growth factor stimulation.

Akt is also capable of activating TORC1 by the direct phosphorylation of PRAS40, reducing the ability of PRAS40 to inhibit TORC1 [212, 213] [95]. From above, these findings highlight Akt as a potent upstream activator of TORC1 activity. These also provide a mechanistic understanding of the elevated TORC1 activity levels that are observed in most of cancers in which PI3K-Akt signaling is elevated.

1.5.3. Amino acids

Amino acids are essential for protein synthesis, and for being substrates for energy production. In both unicellular and multicellular organisms, there are

mechanisms to sense amino acids, transport them into the cell, and synthesize new ones.

For decades, the importance of amino acids in the growth and homeostasis of organisms was gradually revealed. In a dramatic series of studies, depriving rats of a single amino acid, leucine, gave rise to profound weight loss and muscle waste, followed by death [214]. Besides, amino acid withdrawal from cells and organisms triggered autophagy, a process of cellular self-eating where proteins and organelles are degraded into simple metabolites and recycled [215].

Withdrawal of amino acids from the culture media strongly suppressed mTORC1 signaling in mammalian cells and yeast alike; additionally, starvation or using its chemical inhibitor rapamycin potently suppressed mTORC1 and induced autophagy [215]. Basing on this, a feedback loop started to emerge, linking amino acids, mTORC1, and autophagy in a mechanism that drives growth under nutritional sufficiency and mediates growth arrest under starvation conditions, replenishing amino acid stores. Acting independently of insulin and TSC, amino acids appeared to be distinct from the insulin/PI3K pathway [216-218].

Lots of studies showing the discovery of the nutrient-dependent Rag GTPase pathway has proved the strongest relationship between amino acids and TORC1 stimulation. Rag A-D (Rag family members) is part of the Ras family of GTPases. They are specific in the dimerization ability through long C-terminal extensions. The dimeric Rag complex is composed of a Rag A/B monomer and a

Rag C/D monomer. In the existence of amino acids, it binds to TORC1 [219-221]. On the basis that during amino acid signaling the Rag-mediated activation of TORC1 still needs Rheb, Rag complexes is the upstream of Rheb. TORC1's activation mediated by amino acid also depends on the right subcellular localization of both the TORC1 components to the lysosomal membrane and the Rag activators. While Rag A/B is in the GTP-bound state and Rag C/D is in the GDP-bound state, the GTP-loading of Rag A/B is controlled by amino acids, and binding to TORC1 is found most strongly under nutritional sufficient conditions [219, 220]. However, there are many critical points of TORC1 regulation by amino acids that still to be uncovered.

1.6. mTOR signaling and muscle

1.6.1. mTOR and protein synthesis

mTORC1 regulates protein synthesis through inducing ribosome biogenesis and mRNA translation [139]. mTORC1 phosphorylates and activates the hydrophobic motif (Thr389) of S6K, which phosphorylate the S6 ribosomal protein (a component of the 40S ribosomal subunit) at its five Ser residues (Ser235, Ser236, Ser240, Ser244 and Ser247). mTORC1 also phosphorylates the translation inhibitor 4E-BP, facilitating the release of 4E-BP from eIF4E (eukaryotic translation initiation factor 4E). This enables eIF4G to bind eIF4E at its 5′-end of mRNAs to promote the cap-dependent translational initiation. Recently, Chauvin et al. showed that 78% of ribosome biogenesis mRNAs were

downregulated in both whole- body S6K1- and S6K2-double-knockout mice[222]. Moreover, $S6^{P-/-}$ (P indicates phosphorylation) knock-in mice in which the five S6K target serines in S6 are mutated to alanine are also defective for ribosome biogenesis mRNA expression. These studies demonstrate that the mTORC1–S6K–S6 signaling cascade indirectly promotes protein synthesis through the increasing ribosome biogenesis. S6K may regulate protein synthesis through phosphorylating eIF4B[223] and eEF2K (eukaryotic elongation factor 2 kinase) [224], and through physically interacting with eIF3 [143]. Further study is needed to know how these events affect global translation.

However, S6K-deficient muscle and liver cells do not have a defect in global protein synthesis, even the evidence showing that S6K promotes protein synthesis [222, 225]. The mTORC1 inhibitor—rapamycin only weakly inhibits global translation in cells [226, 227]. On the contrary, treatments of Torin1 or INK128, two ATP-competitive inhibitors of mTOR, inhibit overall mRNA translation [226, 227]. The stronger inhibition of translation is probably because the Torin1 and INK128 can inhibit mTORC1 and mTORC2 completely, whereas rapamycin only weakly inhibits mTORC1 not mTOCR2 [226, 228]. Torin1 only slightly affect global translation in 4E-BP-deficient cells [227]. These data show that mTORC1 regulates global translation mainly through suppressing 4E-BP. $S6k1^{-/-}$ $S6k2^{-/-}$ and $S6^{P-/-}$ mice activate a compensatory mechanism by inhibition of 4E-BP, which upregulates protein synthesis in the absence of the transcription of ribosome biogenesis genes, thus maintain global translation [222].

Besides the function on the global translation, mTORC1 preferentially promotes the translation of a range of mRNAs that contain a 5′ oligopyrimidine tract (named a 5′ TOP). 5′ TOP-containing mRNAs is responsible to encode components of the translation machinery including ribosomal proteins and elongation factors. Most mRNAs that are translated in an mTOR- dependent manner contain a 5′ TOP [226, 227]. Thoreen et al. showed that mTORC1 promotes the translation of 5′ TOP-containing *eEF2* mRNA through inhibiting 4E-BP [227]. These ribosome profiling studies also identified a 5′ TOP-like motif [227] and PRTE (pyrimidine- rich translational element) [226] within the 5′ untranslated region of mRNAs, which are controlled by mTORC1.

5′ TOP and/or PRTE motifs are enriched in proteins that are involved in cell invasion and metastasis, such as YB1/ NSEP1 (Y-box binding protein), vimentin, MTA1 (metastasis associated 1) and CD44 [226]. Importantly, acute inhibition of mTORC1 by INK128 decreased the synthesis of these invasion- and metastasis- related proteins before the inhibition of global protein synthesis. Translation of *YB1* mRNA, which contains a PRTE, is dependent on the mTORC1-mediated inhibition of 4E-BP. Thus, the mTORC1– 4E-BP axis regulates the translation of mRNAs with 5′ TOP and PRTE motifs. This model is consistent with data demonstrating that 4E-BP inhibits certain mRNAs translation selectively [229].

Comparing to TORC1, the function of mTORC2 in protein synthesis is less well-defined. mTORC2 can associate with translating ribosomes to

co-translationally phosphorylate and stabilize substrates. Study shows that mTORC2 co-translationally phosphorylates Akt at Thr450 site, which blocks the Akt ubiquitylation [230]. It has been demonstrated that mTORC2 promotes the production of IGF2 and thus the cell proliferation, by also co-translationally phosphorylating IMP1 (IGF2 mRNA-binding protein 1) at Ser181 site [231]. mTORC2 is activated as it associates with the ribosome [170]. The molecular mechanism of the ribosome– mTORC2 association is required to be elucidated, and additional substrates that are co-translationally phosphorylated by mTORC2 also need to be identified. All of these should provide more insight into the function of mTORC2 in protein synthesis.

1.6.2. Role of mTOR in hypertrophy and atrophy

1.6.2.1. Muscle Hypertrophy

The growth of skeletal muscle mass depends on protein turnover and cell turnover [232]. Cellular turnover is very important during muscle development in embryo. Besides, the incorporation of satellite cell into the growing fibers occurs during postnatal muscle growth [233] accompanied with increased protein synthesis. The activation of satellite cells plays an important role in maintaining the quantity of cytoplasm or number of nuclei within that cytoplasm. However, in adult fibers, the contribution of cellular turnover to homeostasis of fibers is minor. And there still exists controversy of its role in hypertrophy [234, 235]. In adult muscle, the physiological conditions of increasing protein synthesis and decreasing protein degradation mainly promote muscle growth.

Skeletal muscle hypertrophy can be induced in many experiment models. IGF1 is one of the best-characterized muscle growth-promoting factors. In one work-induced hypertrophy, transcription of the gene encoding IGF-1 (insulin-like growth factor 1) was increased [236]. Activation of IGF-1 in muscle cells is sufficient to induce hypertrophy [237].

Muscle-specific transgenic mice with increased IGF-1 expression have muscles that are twofold greater in mass than the wild-type mice [238, 239]. And the growth of muscle mass is accompanied with muscle strength increase. In addition, acute ectopic expression of IGF1 in adult muscles through electroporation is sufficient to promote muscle hypertrophy [240].

The activation of Akt is induced by IGF1 and insulin via the phosphatidylinositol- 3,4,5- triphosphates, which is produced by PI3K. At the membrane, Akt is phosphorylated on different residues by at least two distinct kinases, mTOR-Rictor complex and PDK1.

In mammals, there are three Akt genes, Akt1 (PKBα), Akt2 (PKBβ), and Akt3 (PKBγ), which have distinct functions. In skeletal muscle, both Akt1 and Akt2 are expressed at higher levels compared with Akt3, which is mainly expressed in the brain. Akt1-null mice showed growth retardation and muscle atrophy, whereas Akt2-null mice had a Type 2 diabetes- like syndrome, and Akt3-null mice presented impaired brain development [241].

mTOR pathway and GSK3βare the two major downstream of the Akt pathway, which are activated or blocked by Akt separately; both of them regulate protein synthesis. GSK3β is inhibited by Akt and thus blocks the eIF2B (eukaryotic

initiation factor 2B) that is implicated in protein synthesis. Studies show that hypertrophy is induced by the expression of a dominant negative kinase inactive form of GSK3β in skeletal myotubes [242].

The kinase mTOR (mammalian target of rapamycin) is a key regulator of cell growth that integrates signals from nutrients, growth factors, and energy status to regulate protein synthesis and other cell functions [243, 244]. As the name implies, mTOR can be selectively inhibited by rapamycin. Rapamycin binds to members of the FKBP (FK binding protein) family, and then the rapamycin/FKBP complex binds to mTOR and suppresses its activity. The role of mTOR in muscle growth was certified by in vivo studies that rapamycin blocked overload hypertrophy and regenerating muscle growth [74, 245]. Actually, rapamycin completely blunts the effects of Akt on muscle growth in tetracycline-inducible Akt transgenic mice [246]. Akt phosphorates and inhibits TSC2 (tuberous sclerosis 2), and thus actives mTOR indirectly. TSC2 is a GAP (GTPase activating protein) protein, which functions with TSC1 to inhibit the Rheb (a small G protein) that in turn activates mTORC1. Transgene mice that have overexpressing of TSC1 specifically in skeletal muscle present muscle growth defect [247].

There two multiprotein complexes of mTOR: mTORC1, which is required for signaling to S6K and 4EBP1 and mTORC2, which is required for signaling to Akt-FoxO. The function of mTOR on the translation machinery and protein synthesis is mediated through TORC1-dependent phosphorylation of the S6K1/2 and of 4E-BP1, which represses the cap-binding protein eIF4E. S6K1 is an

important substrate of Akt pathway. In S6K1- null mice, the muscle fibers are smaller, however there is no impairment in polysome formation, in protein synthesis, and in protein degradation [225]. In addition, TORC1 negatively regulates the IGF1 pathway through S6K1 [125, 133]. Therefore, mTORC1 and mTORC2 may play opposite roles on Akt activity: TORC1 negatively regulates IGF1 signaling, while TORC2 enhances Akt activity. Notably, long-term treatment of rapamycin in vitro can suppress not only mTORC1 but also the mTORC2 complex and thus potentially influence the Akt-FoxO signaling in some cell types. By this, rapamycin may induce transcriptional regulation through an indirect transcriptional effect by inhibiting Akt and thus derepressing FoxO under certain circumstances, instead of a direct effect, e.g., by blocking mTOR phosphorylation of downstream targets. Therefore, FoxO activity/localization may always need to be monitored in studies involving rapamycin treatment [248].

1.6.2.2. Muscle atrophy

Atrophy is a decrease in cell size mainly caused by loss of organelles, proteins, and cytoplasm. It is an active process regulated by specific signaling pathways and transcriptional programs.

Proteolysis, as observed in atrophy, has been demonstrated to occur partially due to the activation of ubiquitin- mediated proteasomal degradation [249]. Many models of muscle atrophy, including immobilization, denervation, high-dose dexamethasone treatment, and treatment with inflammatory cytokines, all lead to transcriptional upregulation of MuRF1 and MAFbx (also named atrogin-1)

genes, which encode for E3 ubiquitin ligases [250, 251]. Moreover, mice deficient in either MAFbx (Muscle atrophy Fbox protein) or MuRF1 (Muscle RING finger-containing protein 1) gene were showed to be resistant to atrophy as compared to wild-type control littermates [23].

Importantly, studies showed that myosin heavy chains were ubiquitinated and degraded by MuRF1 [252, 253]. Till now, these two genes are the best markers for muscle atrophy. And they could be considered as master genes for muscle wasting. Meanwhile, many other atrophy-related genes are of potential interest, including genes coding for regulators of protein synthesis, enzymes of metabolic pathways, lysosomal protease and transcription factors. However, their specific roles in muscle wasting need more exploration.

MuRF1 protein contains four domains. The most NH3-terminal domain is a RING-finger [254, 255], which is necessary for the ubiquitin ligase activity of MuRF1. This domain binds to an E2 protein, which in turn facilitates transfer of ubiquitin to the substrate [256]. The next B-box domain, downstream of the RING, can mediate self-association process – the B-box domain in MuRF1 self-associates into dimers with high affinity [257]. Next, there is a ‘‘coiled-coil domain’’ in MuRF1, which may be needed for the formation of heterodimers between MuRF1 and itself [258]. Additional myosin domain- containing proteins in the muscle thick filament, including myosin light chain and myosin binding protein C, were also degraded by MuRF1 [259]. Therefore, MuRF1 induces muscle atrophy, at least partially, by directly attacking the thick filament of the sarcomere and leading to the proteolysis of myosin proteins.

MAFbx contains an Fbox domain, a motif seen in a family of E3 ubiquitin ligases named SCFs (Skp1, Cullin, and Fbox). The Fbox containing proteins are not enzymes themselves. They bring substrates to the E2 through the Fbox binding to the Skp1-Cullin complex. Rbx1, a RING-containing protein, also belongs to this complex, which functions in activating the E2 [260]. Fbox containing proteins generally bind a substrate only after that substrate has first been modified post-translationally – e.g. by the phosphorylation of serine or tyrosine [261].

MyoD [262] and calcineurin [263] are the substrates of MAFbx. However, it has not been shown whether protein is ubiquitinated by MAFbx in skeletal muscle or under atrophy conditions. MAFbx has been proved as an E3 ligase for eIF3-f, a protein initiation factor [264]. This demonstrated that MAFbx activity leaded to muscle atrophy by the downregulation of protein synthesis.

Activation of Akt can inhibit the transcriptional upregulation of MAFbx and MuRF1 during atrophy [265]. Their upregulation was demonstrated to require the FoxO transcription factors [266, 267]. When phosphorylated by Akt, FoxO transcription factors are excluded from the nucleus, and upon dephosphorylation condition, they translocate to the nucleus [268].

The translocation and activity of FoxO transcription factors are needed for upregulation of MuRF1 and MAFbx. Study showed that the activation of FoxO3 was sufficient to induce atrophy [184, 269]. The transgenic expression of FoxO1 also leaded to an atrophic phenotype [270, 271].

By isolating a set of genes regulated by dexamethasone and inversely regulated

by IGF-1, a FOXO-independent, anti-atrophy checkpoint downstream of Akt activation was demonstrated. Inhibitors of PI3K could block the genes, which were controlled by IGF-1 in skeletal muscle. This is consistent with the previous work demonstrating an essential role for the PI3K/Akt/FoxO pathway. However, the inhibition of Akt downstream, at the level of mTOR, can block most of changes induced by IGF-1, including the inverse regulation of atrophy-stimulated genes [248].

It was further demonstrated that the mTOR signaling did not involve phosphorylation of the FoxO transcription factors, because treatment with rapamycin did not perturb FoxO translocation [248]. Therefore, there may be many checkpoints of atrophy signaling downstream of PI3K/Akt activation. But it is important to notice that the study of mTOR signaling was not in the context of a simultaneous atrophy and hypertrophy signals, only that inhibition of mTOR suppressed the transcriptional changes induced by IGF-1 (including genes inversely regulated by dexamethasone). Therefore, it still needs to check if mTOR is necessary for the dominant inhibition of dexamethasone-induced transcriptional changes by IGF-1.

1.6.3. mTOR and Ubiquitin/proteasome system

Two major pathways for regulating protein catabolism in eukaryotic cells are the ubiquitin–proteasome system (UPS) and the autophagy–lysosomal system.

The UPS functions as the main route for degrading thousands of short-lived proteins and numerous regulatory proteins. UPS-mediated catabolism is also

necessary to maintain amino acid pools in acute starvation and plays an important role in the degradation of defective proteins.

The majority of membrane-bound or organelle- associated proteins are degraded through the lysosomal pathway, via either endocytosis or autophagy mechanisms. Studies also show that the UPS and lysosomal degradation pathways function in a cooperation manner [272, 273]. For instance, oligo/mono-ubiquitination of receptor and other membrane proteins is also needed for efficient endocytosis [274, 275].

The majority of cellular proteins are degraded through the UPS, which includes both substrate-recruiting and substrate-degrading machinery. The former is composed of three enzymes: E1, E2 and E3. Ubiquitin (Ub) is a small 8 kDa protein. Firstly, E1 activates the polypeptide ubiquitin in an ATP dependent manner, facilitating its transfer onto the ubiquitin carrier enzyme E2. Activated ubiquitin is subsequently transferred via the ubiquitin protein ligase E3 to a substrate protein [276]. The substrate-recruiting machinery then promotes the formation of an isopeptide bond between the ubiquitin's C-terminal glycine residue and the ε-amino group of lysine residue, leading to the formation of a polyubiquitin chain. Repeated addition of ubiquitin moieties onto the first one forms a polyubiquitylated substrate protein that is recognized by the 26S proteasome— the proteolytic machinery of the UPS, and is destroyed in an ATP- dependent manner. DUBs (Deubiquitinating enzymes) can rescue proteins from degradation and recycle ubiquitin or ubiquitin oligomers from ubiquitin–protein conjugates.

The requirement for the addition of numerous ubiquitin molecules may be part of a proof-reading mechanism, as a large number of deubiquitylating enzymes (DUBs) are also found within cells [277]. DUBs remove ubiquitin molecules before a sufficiently large branch structure has been synthesised to activate proteasomal destruction (Figure 6).

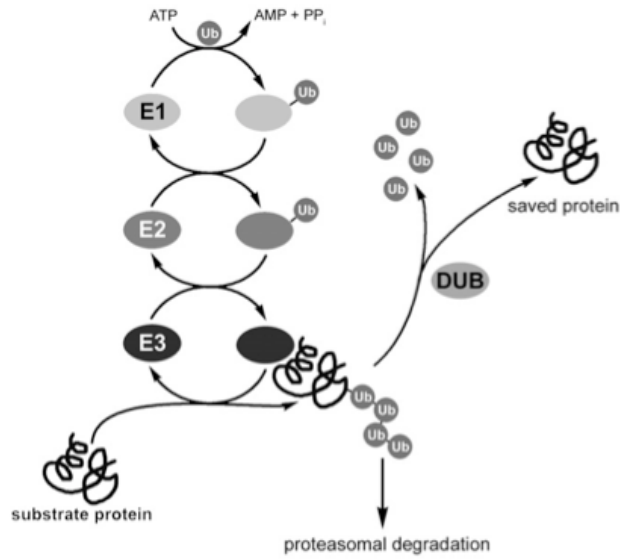


Figure. 6. The ubiquitylation cascade. Ub, ubiquitin; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; DUB, deubiquitinating enzyme.

The ubiquitin system plays important roles in pathophysiological processes in muscle. Muscle degeneration that follows denervation, long-term immobilization, and many catabolic states, such as in sepsis and cancer-induced cachexia diseases, results in activation of the UPS and induction of multiple of its enzymatic components. This leads to abundant degradation of proteins in muscle [249, 278, 279]. Additionally, the N-end rule pathway is very important in stress-induced proteolysis of muscle [280]. An important development in this area is the discovery of two, degeneration-induced ubiquitin ligases, Murf1 (Atrogin 1) and Murf2 [250, 251]. However, the substrates of these induced E3s

have not been identified yet, and the characteristics of signaling mechanisms implicated in regulating muscle hypertrophy and atrophy is still not well clarified. Cytokines (e.g. TNFα and IL-6) are not implicated, at least not directly. It appears that Akt/mTOR signaling pathway is up-regulated during muscle hypertrophy and down-regulated during atrophy. Consistent with these, in vivo studies showed that genetic activation of the Akt/mTOR pathway induced hypertrophy and prevented atrophy, meanwhile genetic blockade of this pathway inhibited hypertrophy [74].

1.7. Autophagy

Living organisms from yeast to humans are able to eat parts of themselves to survive. This comprises the degradation of cellular components, either because they are detrimental to the organism (e.g., damaged organelles and microbial invaders) or because the resulting breakdown products are required to support metabolism. This process was properly defined autophagy from the Greek language “auto” or oneself and “phagy” or to eat. It has drawn attention as an essential contributor to human health and disease. Autophagy or self-eating is a highly conserved cellular process that mediates degradation of intracellular components of all types including proteins, organelles, particulate structures and even pathogens in lysosomes [281]. There are numerous forms of autophagy, each of which implicates transporting intracellular cargo to lysosomes for degradation. The three most common forms are macroautophagy, microautophagy and chaperone-mediated autophagy (CMA).

Among them, macroautophagy uses the intermediate organelle ‘‘autophagosome’’. It is formed by an isolation membrane (also termed phagophore), which isolates a small portion of the cytoplasm, including soluble materials and organelles. Then the autophagosome fuses with the lysosome to be an autolysosome and degrade the materials within it. Autophagosomes may also fuse with endosomes before fusion with lysosomes.

In microautophagy process, the lysosome itself engulfs small components of the cytoplasm by the lysosomal membrane inward invagination (Figure 7) [282]. Membrane dynamics during microautophagy may be quite similar to that of endosomal sorting complex needed for transport (ESCRT)-dependent multivesicular body (MVB) formation, which occurs in the stage of late endosome. Actually, significant amounts of cytosolic proteins are involved into the endosomal lumen during MVB formation both in bulk and selectively [283].

Lastly, the chaperone-mediated autophagy does not implicate membrane reorganization; on the contrary, substrate proteins directly translocate across the lysosomal membrane during the chaperone-mediated autophagy (Figure 7) [282]. The chaperone protein heat shock cognate 70 (Hsc70) and cochaperones specifically recognize cytosolic proteins that include a KFERQ-like pentapeptide [284]. Lamp-2A, the transmembrane protein that is an isoform of Lamp-2, functions as a receptor on the lysosome, and unfolded proteins are transported into the lysosomal lumen by a multimeric translocation complex.

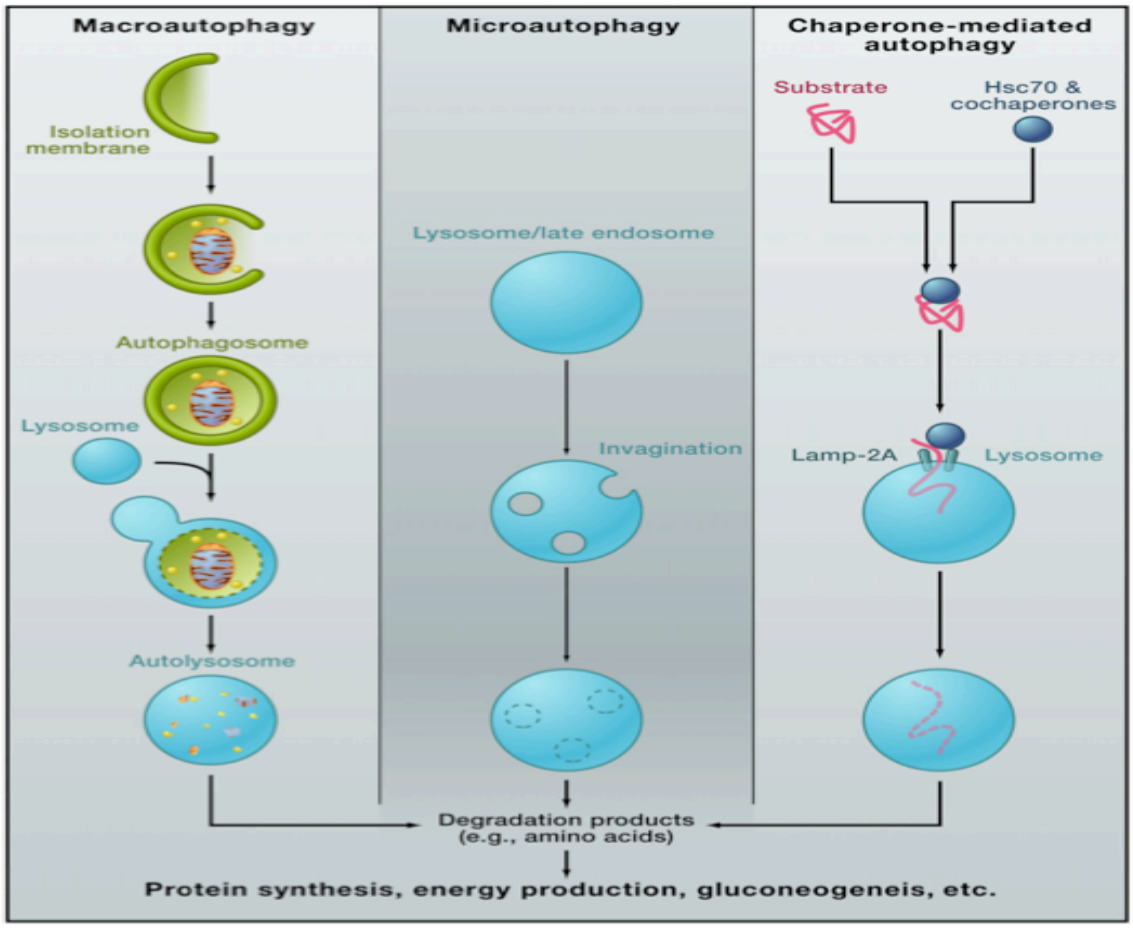


Figure 7. Macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy: A portion of cytoplasm, including organelles, isolated by membranes (also called phagophore) to form the autophagosomes. The autophagosome outer membrane fuses with the lysosome, and the interior material is degraded in the autolysosome. Microautophagy: Small fragments of the cytoplasm are directly engulfed by the lysosomal or late endosomal membrane inward invagination. Chaperone-mediated autophagy: substrate proteins with a KFERQ-like pentapeptide sequence are firstly recognized by cytosolic Hsc70 and cochaperones. After that, they are translocated into the lysosomal lumen after binding with Lamp-2A in lysosomes. After all three types of autophagy, the resultant degradation products are utilized for different purposes, such as protein synthesis, energy production, and gluconeogenesis.

According to the type of cytosolic component preferentially degraded, variations of these autophagic pathways have been described. For instant, selective degradation of mitochondria by macroautophagy is now termed mitophagy, or

lipophagy which degrade lipids, or ER-phagy which degrade regions of the endoplasmic reticulum (ER) [285, 286].

Macroautophagy (autophagy hereafter), the predominant form, produces autophagosomes vesicles that capture and deliver cytoplasmic materials and organelles to lysosomes [287]. The autophagy-related genes (the atg genes) are conserved from yeast to mammals and regulate the renewal of intracellular cytoplasm, proteins, and organelles.

As is known, autophagy is the only mechanism to degrade large structures including organelles and protein aggregates. Basal autophagy serves a housekeeping function in the absence of stress. It services as a routine “garbage disposal” to cells, discarding damaged components that could otherwise become toxic. Such kind cellular refreshing is particularly significant in quiescent and terminally differentiated cells, where damaged components are not attenuated by cell replication. Autophagy provides a nutrient source in starvation, promoting survival. Autophagy is induced by several of other stressors and can degrade proteins, oxidized lipids, damaged organelles, and also intracellular pathogens. Although it is not always possible to determine the metabolic and garbage disposal roles for autophagy, it is clearly known that autophagy prevents disease. Defects in autophagy are associated to many diseases, such as neurodegeneration, liver disease, aging, cancer, Crohn’s disease, and metabolic syndrome.

1.7.1. Process of macroautophagy

After autophagy induction, a double-membrane vesicle begins to come into being in the cytosol, leading to the sequestration of cytoplasmic components. The origin of the isolating membrane is not known, but for mammalian cells, it is generally thought coming from the endoplasmic reticulum, as it elongates it seals and isolates entire cytosolic regions (Figure 8) [288].

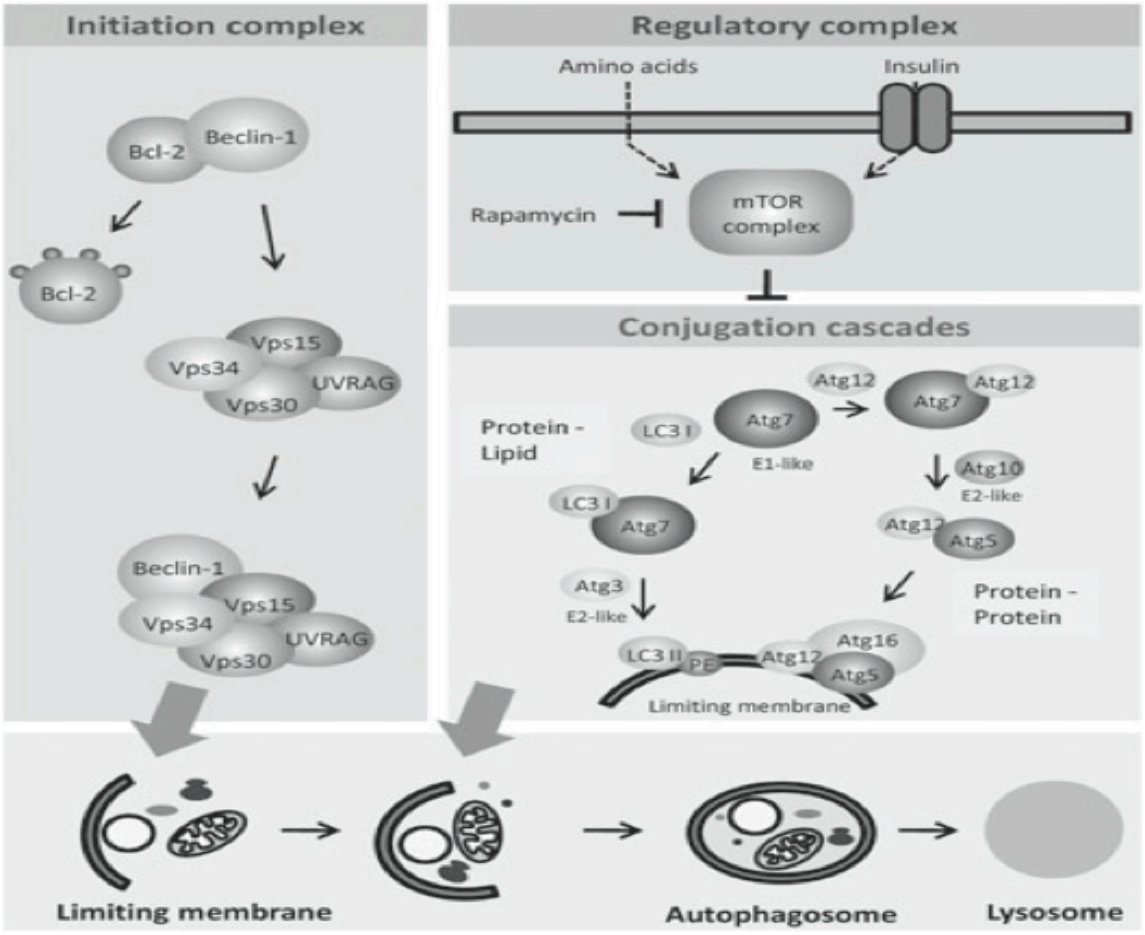


Figure 8. Autophagy pathways in mammalian cells. Three different types of autophagy exist at the same time in most types of mammalian cells. The predominant molecular components that take part in the execution and regulation of each of these autophagic pathways are described here. (A) Macroautophagy implicates the sequestration of cytosolic regions by a *de novo* formed membrane that engulfs into a double membrane vesicle or autophagosomes. Autophagosomes fuse with the lysosomes, which is required for cargo

degradation. Macroautophagy induction gives rise to the mobilization of the initiation complex - a protein kinase type III complex towards the autophagosome formation sites. Lipid phosphorylation of this kinase complex is fundamental for the recruitment in these regions of the two conjugation cascades components that mediate the formation and elongation of the autophagosome membrane. The mTOR protein kinase complex, the major negative regulator of macroautophagy, is also described.

The double membrane vesicle producing from this autophagosomes process is then moved towards the lysosomes and on membrane fusion, the enzymes within the lysosomes get access to the autophagosome cargo and degrade it [286]. In the early studies, macroautophagy was first identified and characterized in mammals, specifically in rodent animal liver, using morphological approaches that quantified changes in the size and number of autophagosomes and lysosomes [289].

Nevertheless, we appreciate the yeast screening studies that facilitate the recent advances in the molecular dissection of this process. These study methods have aided to identify more than 30 different genes generically known as autophagy-related genes (ATG), which encode for protein products implicated in the execution and regulation of macroautophagy [290]. Briefly considered, Atg proteins organize functional complexes that mediate macroautophagy in each step: induction/initiation, nucleation, membrane elongation, cargo recognition, sealing, and finally fusion with lysosomes.

Series of protein complexes composed of atg gene products coordinate the autophagosome formation. The Atg1 in yeast or ULK1 in mammals is an

necessary positive regulator of autophagosome formation [287]. Under nutrient-sufficient conditions, autophagy is blocked by mTORC1 (mammalian target of rapamycin complex 1) through its binding with the ULK1 complex. As is well known, mTORC1 is a key regulator of cell growth and metabolism. It contains five subunits that include Raptor, which binds ULK1, and mTOR, a serine-threonine kinase. mTOR inhibits autophagy initiation via phosphorylating ULK1 and another complex member (the mammalian homolog of yeast Atg13). In starvation condition, mTORC1 dissociates from the ULK1 complex, relieving it to trigger autophagosome nucleation and elongation process.

Autophagosome nucleation needs a complex containing Atg6 or its mammalian homolog, Beclin 1, that facilitates the recruitments of the class III phosphatidylinositol 3-kinase VPS34 to produce phosphatidylinositol 3-phosphate [291]. Expansion of autophagosome membranes implicates two ubiquitin-like molecules, Atg12 and Atg8 (named LC3 in mammals), and also two associated conjugation systems. To form pre-autophagosomal structures, the E1-like Atg7 and E2-like Atg10 covalently link Atg12 with Atg5, which bind Atg16L1 together. In the second ubiquitin-like reaction system, LC3 is cleaved by the protease Atg4. And phosphatidylethanolamine is conjugated to cleaved LC3 by Atg7 and Atg3 (a second E2-like enzyme). This lipidated LC3-II associates with autophagosome membranes that are newly forming. LC3-II stays on mature autophagosomes until after fusion with lysosomes and is intensively used to monitor autophagy.

The process initiating with the Beclin 1 complex leads to nascent autophagosome membranes. These membranes gather around cargo, sealing the cargo in a vesicle that fuses with a lysosome subsequently, producing an auto-lysosome. The contents are afterwards degraded by proteases, lipases, nucleases, and glycosidases. The breakdown products including amino acids, lipids, nucleosides, and carbohydrates are released by the lysosomal permeases into the cytosol, where they are available for synthetic and metabolic pathways.

1.7.2. Regulation of autophagy

Cells integrate information concerning nutrient availability, growth factor, stress, hormonal receptor activation, and internal energy through an elaborate network of signaling pathways (Figure 9) [292]. In mammals, insulin, the crucial hormone of the fed state, blocks autophagy.

A main intracellular hub for integrating autophagy-related signals is mTORC1 [293]. In the existence of abundant nutrients and growth factors involving insulin, mTORC1 accelerates cell growth and metabolic activity while inhibiting the ULK1 complex and autophagy. In deprivation or stress conditions, various signaling pathways inactivate mTORC1 kinase activity. This both inhibits cell growth to reduce energy demand and induces autophagy to promote stress adaptation and survival. The second mTOR complex, mTORC2, positively regulates mTORC1. The cellular energy–sensing pathway controlled by adenosine monophosphate–activated protein kinase (AMPK) is the upstream of

mTORC1 [294]. High concentrations of AMP signal, energy depletion activate AMPK, and suppress mTORC1, hence promoting autophagy (Figure 9) [292].

Regulation of autophagy also proceeds by the forkhead box or FOXO transcription factors, whose activation gives rise to transcription of atg genes [269]. Likewise, the transcription of mitophagy-specific genes and mitophagy are induced by hypoxia and activation of hypoxia-inducible factors, or HIFs (Figure 9) [292, 295]. There also exist less well-characterized mTOR-independent regulators of autophagy. One is ammonia, which is a by-product of amino acid catabolism that stimulates autophagy, likely in less perfused tissues and tumors [296]. Glucagon, an important hormone in the fasted state, also causes autophagy in the liver. Like glucagon activates adenylate cyclase and cyclic adenosine monophosphate (cAMP) production, adrenergic receptor activation also activates liver autophagy.

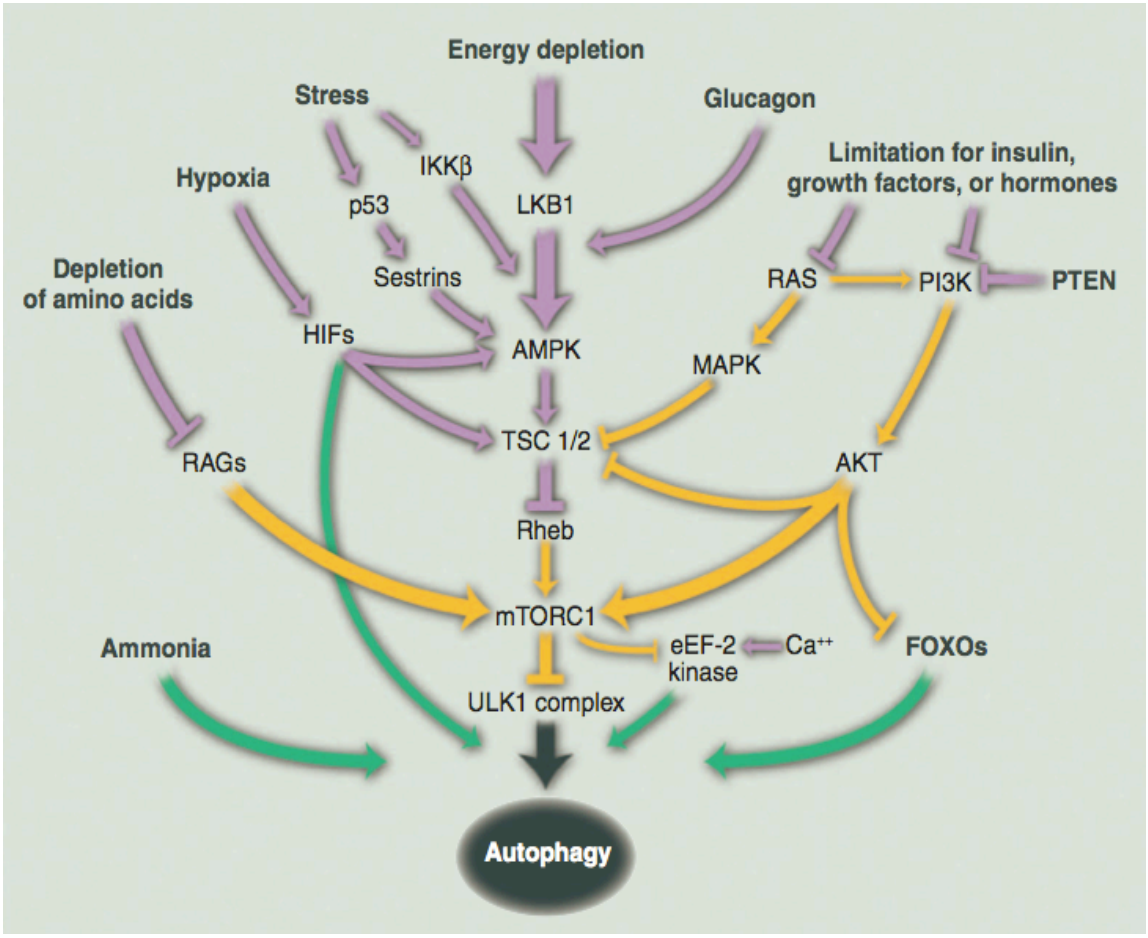


Figure 9. Signaling pathways that regulate autophagy. Nutrient, growth factor, hormone, and stress signals regulate autophagy. Purple lines describe events that positively regulate autophagy. Yellow lines describe those events that negatively regulate autophagy. Numerous pathways converge on the AMPK-mTORC1 axis. Green lines describe pathways that are mTOR-independent. IKKβ, inhibitor of nuclear factor kB kinase β; TSC1/2, tuberosclerosis complexes 1 and 2; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol-3 kinase; PTEN, phosphatase and tensin homolog; and EF, elongation factor.

1.7.3. Autophagy as a regulator of metabolism

Autophagy is crucial for regulating cellular metabolic capabilities. A highlighted example comes from yeast capable of living off of fatty acids or methanol, substrates that are degraded in peroxisomes. When more attractive forms of carbon become available, the peroxisomes are no longer needed and are

eliminated through autophagy process. The function of autophagy in clearing needless peroxisomes is conserved in mammals. Peroxisomes are induced in liver by numerous hydrophobic chemicals, known as peroxisome proliferators. Removal of peroxisome proliferators gives rise to restoration of normal peroxisome abundance by autophagy [297].

Autophagy also regulates the abundance of liver lipid droplets through their constitutive degradation. In mice, defective autophagy causes larger and much more lipid droplets, increased hepatic triglycerides and cholesterol concentrations, and increased gross liver size. Lipophagy is selectively decreased by a high-fat diet and by free fatty acids in vitro [298]. Therefore, in addition to promoting lipid droplet growth, free fatty acids may decrease lipid droplet breakdown. Free fatty acids are released by lipophagy, so there exists the feedback inhibition of free fatty acids on lipophagy. But this feedback mechanism may react oppositely in the condition of a chronic high-fat diet or obesity.

Comparing to the role of autophagy in clearing lipid droplets from the liver, autophagy is needed in the production of the large lipid droplets that possess characteristic of white adipose tissue [299, 300]. White adipose refers to the typical fat storage tissue that expands in obesity. By contrast, brown adipose is a mitochondria-rich tissue that catabolizes glucose and lipids to produce heat rather than ATP. Brown adipose tissue involves uncoupling protein 1, which enables protons to leak across the inner mitochondrial membrane (IMM),

short-circuiting oxidative phosphorylation. Inhibition of autophagy prohibits white adipocyte differentiation, and adipose-specific knockout of atg7 causes white adipocytes manifest features typical of brown adipose tissue in mice. In accordance with the rapid energy consumption of brown adipocytes, these mice are lean but they are not healthy—when served either a normal or high-fat diet, their risk of early death increases [299].

Autophagy also plays important roles in both insulin secretion and physiological sensitivity to the hormone. It is necessary for the health of pancreatic β cells and also for the expansion of β cells mass that takes place in response to a high-fat diet [301, 302]. Defective autophagy leads to insulin resistance in liver [303]. For reasons that are not yet fully revealed, hepatic autophagy is decreased and its restoration by retroviral expression of atg7 alleviates obese mice insulin resistance in.

1.7.4. Autophagy in skeletal muscle homeostasis

Skeletal muscles with bones are the main agents of the body locomotion. Precise and coordinated movements are realized by the highly organized structure of the cytosol of muscle fibers, the multinucleated and highly specialized cells of skeletal muscles implicated in contraction. Contractile proteins are assembled into repetitive structures. The sarcomere is the basal unit. These structures are well packed into the myofiber cytosol. Under the basal lamina, myonuclei are located at the edge of the myofibers, whereas many organelles such as sarcoplasmic reticulum and mitochondria are embedded among the myofibrils.

An appropriate organization and function of sarcoplasmic reticulum and mitochondria is essential for a correct release of calcium and a rapid supply of ATP, respectively. This exact and finely regulated arrangement of contractile proteins and organelles is a specific characteristic of muscle cells.

Skeletal muscle is a plastic tissue that rapidly responds and adapts to various different physiological conditions, such as physical exercise, loading, diet modification, and hormonal stimulation. During catabolism, numerous different changes occur in the cytosol of myofibers: proteins are mobilized, organelles are reorganized to rapidly supply energy needs, and also the location of myonuclei can be modified. In addition, strenuous physical activity, improper dietary regimens and aging will cause mechanical and metabolic damages of myofiber organelles, particularly mitochondria, and contractile proteins. For instance, physical exercise needs a higher level of energy produced by mitochondria. Meanwhile, the production of reactive oxygen species (ROS) and the mechanical damage of mitochondria are the side effects of excessive physical exercise [304, 305]. The protein turnover is slowed down during aging, thus aggregates of dysfunctional proteins is easier to accumulate inside the cytosol of different cells, including myofibers [306]. Considering these reasons, highly dynamic tissues such as skeletal muscle needs a prompt and efficient system for the elimination of protein aggregates, the removal of altered organelles, and the disposal of toxic products that may cause cell death, thus facilitating the proper contraction of sarcomeres. On the other side, protein degradation in skeletal muscles requires to be regulated precisely, because it would be extremely

detrimental for the homeostasis of the entire body if protein degradation is induced excessively [305].

The ubiquitin-proteasome and the autophagy-lysosome pathways are the two major proteolytic systems in muscle. The transcription of the two ubiquitin ligases—atrogin-1 and MuRF1 and the ubiquitination of the substrates are necessary for the proteasome system [307]. Hence, the ubiquitin-proteasome system can eliminate single proteins or small aggregates rapidly. On the contrary, the autophagic system can degrade large proteins aggregates and entire organelles. In the autophagy-lysosome system, autophagosomes, which are double-membrane vesicles, are able to engulf a part of the cytosol and fuse with lysosomes, where lytic enzymes will completely degrade these content. The lysosomal machinery realizes the recovery of amino acids, which supply the energy requirements of muscle cells [281, 308, 309]. By the difficulties to detect the autophagosomes *in vivo* within the myofiber structure, insight into the role of autophagy in skeletal muscle was delayed. With the development of new biochemical and imaging tools, it is able to follow autophagy flux, from the formation of autophagosomes to their fusion with lysosomes. All these greatly prompted the characterization of autophagy in muscle homeostasis and under pathological conditions [310, 311]. LC3 lipidation and p62 degradation is able to monitor the autophagy flux biochemically. LC3 is the mammalian homolog of the yeast Atg8 gene. It is lipidated when recruited for the double-membrane commitment and growth [312]. The polyubiquitin-binding protein p62 (SQSTM-1) is implicated in the proteasome system and that can either locate

free in the cytosol and nucleus or exist within autophagosomes and lysosomes [311, 313]. p62 is degraded by lytic enzymes after the autophagosome fuse with the lysosome [311, 312]. The first imaging tool to study the autophagosome structures was electron microscopy [314]. The high image resolution of this technique enables to observe the double membranes of the autophagosomes and their content. This tool allows the analysis of a small area of the tissue; its usefulness is limited [311, 312, 314]. The generation of the GFP-LC3 transgenic mouse provides a great improvement in the analysis of autophagy *in vivo*. This animal model allows easy detection of autophagosomes by monitoring the presence of bright GFP-positive puncta inside the myofibrils and under the plasma membrane of the myofibers. This tool has enabled to study the autophagy activation in skeletal muscles with diverse contents of slow and fast-twitching myofibers and react to stimuli such as fasting. For instance, in the fast-twiching muscle—*extensor digitorum longus*, few GFP-LC3 puncta were observed before starvation, whereas lots of small GFP-LC3 puncta came out between myofibrils and in the perinuclear regions after 24 h starvation. On the contrary, in the slow-twitching muscle—*soleus*, autophagic puncta were almost absent in standard condition and barely induced after 24 h starvation [314]. Even the function of autophagy in skeletal muscle is not yet completely understood, it is becoming clear that autophagy may have both beneficial and detrimental effects. This depends on the specific tissue condition and the level of autophagic process activation. Therefore, autophagy can contribute to muscle loss during

atrophy [184] and sarcopenia [315], however, on the other side, a correct autophagy flux is essential for myofiber survival [316, 317].

Because of the lack of tools and the intrinsic difficulty to study a tissue such as skeletal muscle, the physiological role of autophagy in muscles is still not well clarified. The autophagic flux was revealed to be increased during certain catabolic conditions, such as denervation [318], atrophy [75], and fasting [281, 310, 319], hence contributing to protein breakdown.

Food deprivation is one of the strongest stimuli for the induction of autophagy in muscle. Actually, skeletal muscle, after the liver, is the most responsive tissue to the activation of autophagy during food deprivation. Muscles are the biggest reserve of amino acids in the body, therefore has the vital role to maintain the amino acid pool by digesting muscular protein and organelles during fasting autophagy [319]. In mammalian cells, mTORC1, which is composed of mTOR and Raptor, is the nutrient sensor that regulates autophagy negatively. In the atrophy condition, protein breakdown is mediated by atrogenes, which are controlled by the forkhead box O (FoxO) transcription factors [266]. Activation of autophagy seems to exacerbate muscle loss during atrophy. *In vivo* and *in vitro* studies revealed that many genes coding for components of the autophagic machinery, such as LC3, Atg12, Vps34, GABARAP and Bnip3, are regulated by FoxO3 transcription factor [184, 269]. FoxO3 is capable of regulating the ubiquitin-proteasome system and the autophagy-lysosome machinery independently *in vivo* and *in vitro* [184, 269]. Denervation also can induce

autophagy in skeletal muscle, even at a slower rate than fasting. This is mediated by RUNX1, a transcription factor upregulated during autophagy. Scarcity of RUNX1 leads to excessive autophagic flux in denervated muscle and causes atrophy [320].

On the other hand, the characteristic of aged protein accumulation, dilated sarcoplasmic reticulum and dysfunctional mitochondria are typical feature of many muscle diseases, suggesting a detrimental scenario in the situation of an impairment of autophagy flux. For example, protein aggregates positive for ubiquitin and p62/SQSTM1 protein have been observed inside myofibers of patients affected by sporadic inclusion body myositis [321]. Besides, accumulation of dilated sarcoplasmic reticulum and dysfunctional mitochondria were founded in two inherited muscle diseases, Ullrich congenital muscular dystrophy and Bethlem myopathy [322].

Atg5 and Atg7 muscle-specific knockout mice generation has been the first step to better demonstrate the role of autophagy in muscle physiology. These knockout models revealed that autophagy suppression is not beneficial for the whole muscle homeostasis. In fact, both models has the characteristic of muscle weakness and atrophy [323, 324], with also a important reduction of the body weight, which is strictly correlative with the important loss of muscle tissue because of an atrophic condition [324-326]. These animal models have improved our knowledge of autophagy and skeletal muscle, but many aspects remain to be uncovered. Increased autophagy is crucial during catabolic

conditions such as denervation and fasting, but at the same time it is detrimental when completely blocked. These two opposite conditions determine an unhealthy consequence in terms of muscle homeostasis. So, an unbalanced autophagy flux is extremely detrimental for muscle, as too much induces atrophy whereas too little causes muscle weakness and degeneration.

One difference in these two opposite situation is the timing. Muscle wasting associated to the inhibition of autophagy becomes evident and symptomatic only after numerous altered proteins and dysfunctional organelles are accumulated within the myofiber, hence interfering with the normal physiological functions. This pathological condition becomes evident and phenotypically remarkable only after a few months or even for years. Conversely, the excessive increase of autophagy flux enable to induce a rapid loss of muscle mass, during days or weeks [305]. Although the proper balance of the autophagic flux appears to be the crucial point for muscle health, there are still various open questions about the mechanisms that regulate autophagy in skeletal muscles. Solving these questions is significant both for understanding the mechanisms underlying muscle homeostasis in physiological conditions and also for muscle disorders since a series of findings indicate that autophagy alterations are implicated in the pathogenesis of many myopathies and dystrophies.

There are several pathological conditions of activation or impairment of the autophagic flux. Meanwhile, modifications of the autophagy-lysosomal system also happen in different tissues of healthy subjects during many physiological

processes, such as aging. Numerous reports indicate that Atg proteins, as well as other proteins needed for the induction of autophagy, have decreased expression in aged tissues and that autophagy diminishes during aging [306].

There are only few evidences about the involvement of autophagy in muscular changes as a result of the aging. Studies in rat muscles showed an age-related decline in autophagic degradation and an accompanied age-related increase in oxidative damage and apoptosis conditions, which both correlate negatively with autophagy. In addition, a constant autophagic stimulus such as caloric restriction may ameliorate the physiological state of muscles [315, 327]. Muscle aging is characterized by the progressive protein aggregates accumulation that involves with impaired muscle function in drosophila. Multiple data indicate that the molecular mechanisms of protein quality control are in charge of the age-related muscle weakness during aging. Particularly, increased activity of the FOXO/4E-BP axis is sufficient to delay protein aggregates accumulation and to maintain muscle function, an effect that is mediated at least partially by promoting the activity of the autophagy-lysosome system [328].

1.7.5. Physical exercise and autophagy

Multiple reports proposed how a particular dietary regimen is proper to stimulate autophagy and to prevent some common diseases related with the aging and sedentary life [306, 327]. Besides, as well known, physical exercise has beneficial effects on health. It strengthens muscles, helps to control body weight, protects against diabetes, cancer, and age-related muscle loss (or

sarcopenia). However, the cellular and molecular mechanisms underlying the beneficial effects of physical exercise are still poorly understood. Some recent studies proved how physical exercise is useful for autophagy induction in muscle and how this mechanism is an interesting manner to clarify the positive effect of physical activity [329-332]. Even though the effects of training were limited to running exercise in mice in these studies, it is obvious that autophagy is strongly induced after an acute run on the treadmill and also after an endurance exercise. Physical activity represents a main stress condition for skeletal muscles, which require a huge amount of energy to supply contraction activity. Besides, during exercise contractile proteins and organelles, such as mitochondria, are loaded an intense work and this may cause their exhaustion and the production of toxic substances and catabolites like ROS in the case of mitochondria. The autophagic machinery represents a convenient and efficient way to supply the increased vigorous needs and to exclude the dysfunctional organelles. The autophagosomes can selectively engulf dysfunctional organelles. Amino acids can be recycled as a new source of energy after the fusion with lysosome and degradation by lytic enzymes [282].

In a recent work, Grumati et al. reported the first evidence of the important role of the autophagy flux during physical exercise. Mice were subjected to two different protocols of exercise, one is an endurance spontaneous training on running wheels and the other is a short but forced exercise on treadmill. For the first protocol, after spontaneous exercise on running wheels for three months, there were no significant evidences of LC3 lipidation but there was an obvious

decrease in Akt phosphorylation. With this long-term running wheel exercise, there is an indirect evidence of autophagy induction through analyzing the responses of collagen VI null (*Col6a1*$^{-/-}$) mice, which have a dystrophic phenotype implicated to a damage of the autophagy flux [317]. After this form of exercise, *Col6a1*$^{-/-}$ mice showed a strongly exacerbation of their muscle phenotype. Comparing to wild-type controls subjected to the same treatment, muscle of these mice still showed a high level of Akt activity [329]. It seems that such a long-term protocol of voluntary exercise causes an adaptation of muscle tissue, therefore induction of autophagy becomes hard to reveal.

There is an intense indication that autophagy is activated during endurance running in humans. In fact, two recent studies showed that muscle biopsies obtained from healthy subjects after ultra-endurance exercise display a significant increase of several autophagy-related genes in the mRNA levels [330, 332]. Interestingly, like the mice subjected to long-term running wheel exercise, human muscles after ultra-endurance exercise also show a decrease in Akt phosphorylation and a accompanied increase in AMPK activity [332]. The other protocol, acute exercise by one-hour forced running on treadmill for investigating the relationship between autophagy and physical activity in mice. Interestingly, this protocol gave rise to a remarkable activation of the autophagic flux activation in muscles of wild-type mice. Actually, after the treadmill exercise, hindlimb muscles revealed a marked lipidation of LC3 and multiple autophagy puncta within myofibers [329]. The impairment of autophagy is probably detrimental because it does not facilitate the supply of amino acids and

the mobilization of glucose, therefore determining a detrimental overload of work charged on mitochondria. Besides, physical exercise is capable for promoting mitochondria biogenesis and for improving mitochondrial function, including the old mitochondria replacement with new and energy-efficient ones [333].

He *et al.* confirmed observations above on autophagy induction by physical exercise, and showed that exercise-induced autophagy plays a significant and previously unrecognized role in metabolism. This work demonstrated that autophagy is triggered by exercise in tissues associated in glucose and energy metabolism, such as liver and pancreas. The activation of autophagy also contributes to the metabolic effects of long-term training. In a high-fat diet model of obesity and glucose tolerance, different from wild-type mice, exercise is unable to improve glucose tolerance in autophagy-impaired mice, which are fed with a high-fat diet [332]. These findings demonstrated a link between exercise and autophagy and prove that autophagy is a crucial contributor to the metabolic benefits of both acute and long-term training.

The above studies have revealed a significant link between autophagy and physical exercise, however，there are still multiple questions. It is still not clear whether the effects of physical exercise on autophagy in muscle are cell- and/or tissue-autonomous, and physical exercise may determine the release of cytokines that possibly play an important role. Further studies are required to

fully make it clear that the molecular mechanisms and functions of autophagy during exercise [334].

1.7.6. Autophagy in disease

There are many evidences that link macroautophagy with human disease. For instance, increased levels of autophagy are related to neurodegenerative diseases such as Parkinson's disease [335]. The mechanism is not well understood, by which neuronal degeneration occurs. It calls for more study to prompt the effective treatments development. Lowered autophagy levels are also indefinite in many other diseases. LAMP-2 (lysosomal associated membrane protein-2) is a transmembrane protein in the late endosome/lysosome. Autophagy associates directly with heart disease, revealed through the fact that a deficiency of LAMP-2 is associated with the cardiomyopathic Danon's disease [336]. In the maturation of autophagosomes, LAMP-2–deficient mice are defective [337]. Reduced autophagy levels are also found in certain cancers [338]. *Beclin 1*, the human homolog of the yeast autophagy gene *APG6*, is a tumor suppressor gene. By means of research on it, the crucial role for autophagy in controlling the unregulated cell growth implicated with tumor development has been uncovered [339]. *Beclin* interacts with the antiapoptotic protein Bcl-2, thus prohibits the Bax-dependent release of mitochondrial cytochrome c. Studies show that decreased Beclin 1 levels associated with the breast tumors development or progression. Autophagy has also been related to programmed cell death in multicellular organisms. Autophagy is specifically associated with type II

programmed cell death (nonapoptotic cell death program) [340, 341], however, there is an indication that early autophagy stages may function in apoptosis, the type I programmed cell death [342]. And a human apoptosis-specific protein was clarified to be homologous to the product of *APG5* gene [343]. Probably, inhibition of autophagy might be therapeutic in some neurodegenerative diseases; even its selective activation in specific cells could offer a method for the treatment of cancer.

Studies also find an intriguing link between apoptosis and autophagy in the coordinated regulation of PKB/Akt and p70S6 kinase. The p70S6 kinase activity is regulated by mTOR, meanwhile it is also regulated by the action of PDK1 (phosphoinositide-dependent protein kinase-1) [344, 345]. Multifunctional effector PDK1 controls various kinases [204, 346]. mTOR or probably PDK1 can phosphorylate p70S6 kinase, thus inhibits autophagy. The activity of class I phosphatidylinositol 3-kinase (PI3K) facilitates the membrane recruitment of Akt by its pleckstrin homology domain. Phosphorylation by PDK1 activates Akt and inhibits apoptosis [347]. So, class I PI3K may inhibit apoptosis and autophagy via PDK1. Therfore, certain signals that trigger apoptosis may induce autophagy in the meantime [348]. Activated autophagy may give rise to cell death even when the inhibitors of apoptosis present. These revealed that autophagy is a second mechanism for PCD (programmed cell death). Moreover, autophagy-related PCD might actually have evolved ahead of apoptosis [340].

1.7.7. LC3, p62 and autophagy

1.7.7.1 Atg8/LC3 lipidation system essential for autophagy

Over 30 ATG genes have been identified through yeast genetics and biochemical analysis [312, 349, 350]. The molecular function of each Atg protein is almost conserved from yeasts to mammals. The ubiquitin-like protein Atg8 plays an important role in autophagosome formation. There are three families of Atg8 homologues in mice and humans called LC3s (microtubule-associated protein 1 light chain 3), GABARAPs, and GABARAP-like proteins. The structure of Atg8 includes ubiquitin fold and N-terminal two α-helices [351].

In yeast, Atg8 is expressed with a C-terminal arginine residue, which is removed by the Atg4, a cysteine protease, leaving a glycine residue at the C-terminus [352]. There exists another ubiquitinylation-like conjugation system [353]. E1-like enzyme Atg7 activates the C-terminal glycine residue of Atg8. Then an E2-like enzyme Atg3 with an Atg12-5-16 complex catalyzes jointly the transfer of the activated Atg8 to the target lipid substrate, phosphatidylethanolamine (PE). This way Atg8 associated membrane tightly. Atg8 thus can be applied as a marker of the autophagosomal membrane and a crucial molecule during the formation of autophagosome. The conjugation of Atg8 to and its removal from phosphatidylethanolamine are necessary for autophagy.

LC3 (the mammalian homolog of the yeast Atg8) is processed after its synthesis by Atg4B, a cysteine protease, which exposes the C-terminal glycine residue (called LC3-I). Then, LC3-I is activated by Atg7 (E1) and transferred to Atg3

(E2) [354]. Thereafter, the C-terminal glycine of LC3-I conjugates to the amino group of PE in an Atg12–Atg5•Atg16-dependent manner [355]. LC3-II is localized in the inner and outer membranes of the isolation membrane/phagophore. It is essential for membrane biogenesis [351]. LC3-II localized on the outer membrane is re-cleaved efficiently by Atg4B following the autophagosome formation and recycled. Finally, lysosomal proteases degrade LC3-II and other cellular constituents in the inner membrane. As a result, LC3 is utilized as an important autophagosome marker [354].

1.7.7.2. p62 function in autophagy

p62 protein (also known as sequestosome 1; SQSTM1; ubiqutin-binding protein) has been found in many tissues and cells, including muscles, lymphoid cells, serving probably a common cellular signal transduction mechanism (e.g. ubiquitin- associated degradation and autophagy). Autophagy substrates are targeted for degradation by associating with p62. p62 is a multifunctional protein participating in various cellular functions. p62 has an important role and is originally discovered as a scaffold in cell signal transduction cascades and in the degradation of proteins and organelles [356]. p62 may also play a critical role in many diseases (e.g. cancer, obesity, neurodegenerative diseases, and other age-associated pathologies).

It is also examined in ubiquitinylated-protein aggregates [357]. p62 consists of a C-terminal UBA (Ubiquitin Binding-Associated) domain [358] and a short LIR (LC3-interacting region) sequence acting for LC3 interaction [359]. With a PB1 domain, p62 facilitates self-aggregation and associates with other adaptors such

as NBR1 (neighbour of BRCA1 gene 1) [360]. p62 knockout mice and Drosophila studies revealed that p62 was needed for the ubiquitinylated proteins aggregation and therefore was essential for their autophagic clearance [361, 362]. p62 levels usually conversely correlate with autophagic degradation. The loss of Atg genes or factors needed for the fusion of autophagosomes with lysosomes all lead to a marked increase of p62-positive aggregates [363]. Another adaptor in selective autophagy is NBR1. With its PB1 domain, NBR1 can interact with p62, and it also able to participate in the recruitment and autophagosomal degradation of ubiquitinylated proteins through its own UBA domain and LIR [364].

Because LC3-II in the inner autophagosome membrane is degraded with other cellular contents by lysosomal proteases, p62 captured by LC3 is selectively transported into the autophagosome. The impaired autophagy is accompanied by accumulation of p62.

p62 expresses ubiquitously in cell. It is conserved in metazoa but not in fungi and plants [365]. p62 functions as a scaffold protein for several signal transductions by interaction with multiple signaling proteins such as ERK, aPKC, RIP, caspase-8, and TRAF6 [366, 367]. Significantly, p62 has been uncovered as a component of inclusion bodies in many human diseases, including neurodegenerative diseases (e.g., Parkinson’s disease, Alzheimer’s disease, and amyotrophic lateral sclerosis) and liver diseases (e.g., hepatic steatosis, alcoholic hepatitis, and hepatocellular carcinoma). An the N-terminal PB1 (Phox and Bem1p) domain presents self-aggregation, and the C-terminal

UBA (ubiquitin-associated) domain can bind to ubiquitinated proteins, involving the p62 in disease-related inclusion formation [368].

Because p62 is removed from the cytoplasm mainly through autophagy, p62 is generally considered to conversely correlate with autophagic activity [363]. p62-positive inclusions accumulation during immunocytochemistry or increased p62 protein levels are frequently utilized as impairment signs of autophagy. Transgenic p62 reporter systems are sometimes used to monitor the degradation rate of autophagy, but this needs caution as overexpressed p62 tends to self-aggregate and may not indicate the activity of autophagy [369]. Additionally, long-term starvation may influence positively the amount of p62 in certain mammalian cell types through its transcriptional upregulation and facilitating de novo p62 protein synthesis by providing autophagy-derived amino acids [370].

1.7.7.3. p62-mTORC1-Autophagy connections on fat metabolism

p62 coordinates the processes required for metabolic homeostasis partly by its connections with autophagy. Notably, p62 binds proteins for disposal by autophagy; meanwhile, autophagy also constitutively degrades it. This has important functional influences in vivo. For example, in the liver, genetic inhibition of autophagy leaded to poorly characterized hepatotoxicity and the accumulation of p62. And this was rescued through the genetic inactivation of p62 [361].

p62 generally represses obesity and enhances energy consumption, whereas PKC ζ represses the proinflammatory actions in obesity conditon. Accord with

this, PKC ζ /IL-6 double-knockout mice showed normal insulin responses, glucose tolerance, and reduced hepatosteatosis, even with a high-fat diet [371]. Therefore, these signaling proteins may act positively in preventing metabolic syndrome and type II diabetes.

Multiple evidences demonstrated that p62 repressed adiposity in an ERK1-dependent cell-autonomous manner [372]. In p62-deficient mice and 3T3-L1 cells lacking p62, embryo fibroblasts presented high activated ERK levels accompanied with increased adipogenesis [372]. p62 interacted with ERK1 preferentially over ERK2, and the reduction of ERK1 completely reverses adipogenesis in cultured cells. Significantly, in vivo with p62/ERK1 double-knockout mice, which had normal adipogenesis and no obesity, insulin resistance, or hepatosteatosis, also behaved the same as in vitro [357].

1.7.7.4. p62 and the amino acid sensing of mTOR

Amino acid levels are associated to mTOR through a mechanism that is dependent, at least to some extent, on p62 [373]: mTORC1 links with the amino acid sensing Rag family of small GTPases [219, 220] on the surface of lysosomes [221]. p62 mediates this process by associating with Raptor and the Rag GTPases, thus forming a high molecular weight complex that transfer the signal from amino acids to the mTORC1 signaling pathway [373]. It is possible that the p62-mTORC1 complex responds locally to amino acids produced by the autophagy-lysosome system to inhibit excessive autophagy. Therefore, p62 levels may play a role as a positive feedback for cell growth and autophagy. Low p62 would reduce the activity of mTORC1; increase autophagy, and further

lower its own levels, hence sustaining autophagic degradation processes [374]. On the contrary, high p62 would increase the activity of mTORC1, reduce autophagy, and further increase p62 levels, thus sustaining cell growth [375]. It must be pointed out that the regulation through amino acid levels is currently the only input known to require p62 when mTORC1 is activated via various different inputs. Insulin, for example, is able to activate mTORC1 in the absence of p62 [373]. Therefore, p62 levels may sense the availability of free amino acid in the cell, activate anabolic pathways via mTORC1 when amino acids are sufficient, and slowing them down when there are insufficient nutrients for protein synthesis.

1.7.7.5. p62 and protein degradation of autophagy

Autophagy has been considered to be a non-selective, bulk process, however, studies have also presented that the autophagosome membrane can selectively recognize certain organelles and proteins [376, 377]. In mammals, p62 is the best-known autophagy-specific substrate [359]. It functions in many processes including obesity, bone metabolism, and inflammatory signal transduction [357, 378, 379]. p62 interacts with LC3, preferentially degraded by autophagy. Studies showed that p62 massively accumulated in autophagy-deficient cells and tissues [313, 361].

It is very important that p62 can be degraded by autophagy. p62 is a key molecule that forms inclusion bodies. In liver- and neuron-specific, autophagy-deficient mice, p62 is found in the ubiquitin-positive aggregates [361]. Interestingly, in these Atg knockout mice, when p62 is simultaneously

deleted, the ubiquitin-positive aggregates no longer accumulate. Therefore, p62 overexpression can lead to the formation of the protein aggregates [361].

1.7.7.6. p62 is recruited to the autophagosome formation site

The relationships among Atg and Atg-related proteins have been explored in both yeast [380] and mammals [381]. These studies demonstrated that the Atg1/ULK1 protein complex was the most upstream unit and was followed by three complexes including: Atg14/PI3-kinase complex, Atg12–Atg5–Atg16L1 complex and Atg8/LC3–PE. These proteins accumulate at a perivacuolar structure called PAS (preautophagosomal structure) in yeast. These proteins were recruited to the endoplasmic reticulum (ER) in mammals, which may be equal to the yeast PAS [381, 382].

As the interactions between p62 and ubiquitin are very weak, the initiation of the polyubiquitinylated aggregate formation is probably the self-oligomerization of p62 through its PB1 domain [383]. These aggregates containing p62 and ubiquitinylated proteins may act as a nucleating scaffold for the biogenesis of autophagosome, potentially through binding various Atg proteins [383-385]. Moreover, in Drosophila cells, phagophores may preferentially form at p62 aggregates near lysosomes. This is very similar to the location of PAS near the vacuole/lysosome in yeast [386, 387].

mTORC1 is active when bound to lysosomes and promotes cell growth and inhibits autophagy through phosphorylating Atg1 (ULK1/2) [221, 388]. These data suggest the interactions between p62 and upstream Atg proteins may direct assembly of early autophagic structures on the surface of protein aggregates.

Subsequently, Atg8/LC3 will be recruited to the forming phagophore, and the double membrane will enclose the p62-containing aggregate facilitated by interactions between p62, Atg8/LC3, and other Atg proteins [389, 390].

Generally p62 is recruited to preexisting isolation membrane in a LC3-dependent manner [376]. However, p62 and protein NBR1 were demonstrared to localize to the ER-associated autophagosome formation site, which was independent of LC3 localization to membranes [391]. Even as autophagosome formation was blocked by wortmannin treatment or genetic ablation of FIP200, p62 co-localized with upstream autophagy factors such as ULK1 protein. Although p62 is not essential for autophagy [361], p62 is possibly able to recruit Atg proteins and also to decide where autophagosome should be fabricated.

In bacteria, p62 has been suggested to be crucial for pexophagy [392] and elimination [393]. Therefore, p62 may function in recruiting Atg proteins to initiate the formation of autophagosome. The interaction between p62 and LC3 might be important for appropriate incorporation of p62 inside autophagosomes and/or the recruitment of additional p62 into autophagosomes.

p62 associates with the proteasome [394] and is also a proteasome substrate [395]. Intriguingly, either an accumulation [394] or a reduction [273] of p62 give rise to impaired degradation of proteasome substrates. This apparent conflict showed that while single p62 molecule can deliver specific substrates to the proteasome, they can also bind ubiquitin-tagged proteins and isolate them into p62 bodies, as the p62 concentration increased beyond normal physiological

levels. Moreover, this would also suggest that when proteasome activity is impaired, p62 could deliver proteasome substrates to the lysosome via the autophagy machinery. p62 facilitates selective autophagy of proteins, organelles, and bacteria [359, 396, 397].

Notably, p62 must oligomerize to localize the aggregates to autophagosome-forming structures and for efficient engulfment and delivery of autophagy substrates [391]. These studies demonstrated that p62 was more than only an adaptor between autophagy and its substrates, but is an active component participating in the autophagic process [361, 362].

1.7.7.7. p62 in Autophagy Regulation

The function of p62 in the autophagy regulation is controversial. It was suggested to promote the activation of mTORC1 through contributing to its translocation to the lysosome. The reduction of p62, similar to the inactivation of mTORC1, may activate autophagy [373]. However, p62 was found to relaease Beclin1 (an Atg6 homologue) through disrupting the association of Bcl-2 and Beclin1 in HEK293 and HeLa cells, and therefore p62 may positively regulate the bulk autophagy induction [398]. Moreover, p62 interacted with and controls the deacetylase activity of HDAC6, a modifier of F-actin network implicated in selective autophagy [399]. When p62 silencing suppressed cell proliferation and induced autophagy in carcinoma cells, abnormal autophagosomes appeared and the inhibition of p62 leaded to autophagic cell death [400]. Recent study found that in Drosophila fat body cells p62 was not required for proteasome inhibition-induced autophagy [401]. Collectively, the

role of p62 in autophagy induction is complicated and maybe context-dependent. In the nucleus, p62 is able to recruit proteasomes to nuclear polyubiquitinylated protein aggregates, meanwhile, it can even transfer ubiquitinylated-substrates from the nucleus into the cytosol, where exists autophagy with a more robust degradative capacity [402].

1.7.8. AMPK regulates autophagy via FoxOs

FoxOs play a role in the regulation of autophagic/ lysosomal protein degradation [184, 269]. The expression of multiple autophagy related genes is up regulated in numerous models of skeletal muscle atrophy [184, 403, 404]. FoxO3 binds to several key autophagy genes (e.g. LC3, Bnip3) [184, 269]. Overexpression of FoxO3 increases the formation of LC3 positive autophagosomes and stimulates the degradation of lysosomal protein [184, 269]. FoxO3 is both necessary and sufficient for autophagy induction in skeletal muscle [184]. Thus, several evidences demonstrate that FoxO3 plays an important role in the regulation of protein degradation through autophagy in skeletal muscle.

There are fewer studies on whether FoxO1 plays a role in the induction of autophagy in skeletal muscle. Muscles with overexpressing FoxO1 have elevated levels of the lysosomal protease cathepsin L [405]. Study has shown that the expression of cathepsin L is a direct target of FoxO1 in skeletal muscle, by fasting-induced expression of cathepsin L being inhibited in FoxO1 knockout mice and by expression of a dominant negative FoxO1 mutant [406]. Therefore, FoxO1 may function in the degradation of lysosomal protein in skeletal muscle.

Nevertheless, whether it functions in the autophagosome formation and the redundancy degree between FoxO1 and 3 are yet to be further studied. The role of FoxO4 in the regulation of skeletal muscle autophagy is also not clear.

AMPK-induced proteolysis is by means of the autophagy activation. AMPK is required for autophagy-induced proteolysis in non-muscle cells [407]. AMPK can directly phosphorylate and activate ULK1, leading to the formation of the ULK1/Atg13/FIP200 protein complex involved in the early autophagosome formation [167, 168]. This shows a direct mechanism for cellular energy stress to cause the catabolism of cellular proteins and provide substrates for production of energy and survival.

AMPK phosphorylates multiple Akt-independent sites on FoxO3 to stimulate its transcriptional activity [408, 409]. Study shows that treatment of muscle cultures with AICAR, an activator of AMPK, can increase the protein breakdown and atrogin-1 expression through the FoxOs [410]. AMPK activates FoxO3 in myofibers to induce the expression of atrogin-1 and MuRF1 in conditions of energy stress [411, 412]. AMPK activation also induces some autophagy-related genes expression, including those coding for LC3 and Bnip3.

From above, FoxO3 plays an important role in the autophagy regulation of skeletal muscle [184, 269], AMPK can induce activation of the autophagic/lysosomal pathway via FoxO3.

1.7.9. Akt regulates autophagy via GSK3/NBR1

GSK3 (glycogen synthase kinase 3) activity is inhibited through the PI3K-Akt pathway, a well-known regulator of nonselective autophagy. Akt activates mTOR through a cascade of phosphorylations, and mTOR blocks autophagy by the inhibitory phosphorylation of ULK1 and ATG13, proteins required for the induction of autophagy [388]. GSK3 is part of this regulatory cascade. In conditions of serum deprivation, GSK3 can activate autophagy by the phosphorylation and activation of the KAT5/ TIP60 (K [lysine] acetyltransferase 5), which in turn acetylates and activates ULK1 [413].

Nicot et al. showed that GSK3-mediated phosphorylation of NBR1 (neighbor of BRcA1 gene 1) was increased in serum-deprived cells and that phosphorylation of NBR1 inhibited selective autophagy of ubiquitinated proteins [414].

GSK3 might thus affect the balance between activation of nonselective and inhibition of selective autophagy during starvation periods. This would favor random bulk autophagy that will provide the essential nutrients for cell survival. Moreover, through GSK3, this PI3K-Akt pathway could also modulate selective autophagy.

The autophagy receptor NBR1 binds Ubiquitin and the autophagosome-conjugated MAP1LC3/LC3 (microtubule-associated protein 1 light chain 3) proteins, thus facilitating ubiquitinated protein degradation. Various neurodegenerative and neuromuscular diseases are linked with inappropriate aggregation of ubiquitinated proteins. GSK3 activity is associated

in multiple proteinopathies. Study shows NBR1 is a substrate of GSK3 [414]. GSK3 phosphorylates NBR1 at Thr586 site, thus prevents the aggregation of ubiquitinated proteins and their selective autophagic degradation [414].

2. EXPERIMENT PART

2.1. Introduction

The structure, mass, and composition of muscle are crucial for body's motility, metabolism, and viability. Skeletal muscle is composed of various myofibers with different metabolic and functional properties, contraction rates, and resistance to fatigue [415, 416] but presents prominent morphological and metabolic capability of adapting to several physiological (e.g. exercise) and pathological (e.g. myopathy, aging) conditions. These diverse adaptive processes, including hypertrophy, atrophy, regeneration, fiber type conversion, or mitochondrial biogenesis, showed powerful plasticity of muscle. In mammals, skeletal muscle constitutes about 40% of total body mass, meanwhile, it is responsible for about 30% the resting metabolic rate in human adult [1]. Skeletal muscle has a key role in regulating glycemic and metabolic homeostasis. It is the main (about 80%) site of glucose disposal under insulin stimulated conditions [2]. Besides, skeletal muscle is the biggest glycogen storage organ. Moreover, exercise increases skeletal muscle glucose uptake by an insulin-independent pathway [3]. The mass and integrity of skeletal muscle is very important to the body health. Skeletal muscle plays major roles in whole-body disposal of

glucose, amino acids and fatty acids, the accumulation of which can worsen the metabolic state [14-16]. Defects in skeletal metabolism and muscle growth will give rise to or aggravate many diseases, such as diabetes, cancer, and HIV, chronic kidney diseases, congestive heart failure, peripheral artery disease, rheumatoid arthritis [17-20].

Mammalian target of rapamycin (mTOR) is a protein kinase that integrates and coordinates diverse signaling information mediated by growth factors, nutrient availability and energy status. It has two functionally distinct signaling complexes, called mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2) [417].

The IGF1/Akt/mTOR signaling pathway is a major regulator of skeletal muscle mass through regulating atrophy and hypertrophy. Importantly, mTOR regulates skeletal muscle mass by controlling (i) protein synthesis via S6K and 4EBP1, (ii) protein degradation via the control of Akt anti-atrophic functions, (iii) autophagy, a process which when excessive or impaired is detrimental to muscle mass. In addition, aberrant mTOR signaling activity has been linked to multiple diseases including cancer and metabolic disorders, such as obesity and type 2 diabetes. As a result, a great lot of research has focused on the development of multiple classes of mTOR inhibitors.

Rapamycin has widely clinical uses in organ transplantation, cardiology, and oncology. However, rapamycin and rapalogs had limited clinical success due to their inability to maintain a complete inhibition of mTOR activity. This has been addressed by the development of ATP-competitive kinase domain inhibitors that

inhibit both mTORC1 and mTORC2 catalytic activities, whose approach exhibits a clear advantage for therapeutics. Unfortunately, toxicity is a possible caveat to consider if all mTOR catalytic functions are inhibited.

Accord with this, our laboratory generated muscle specific mTOR knockout mice and demonstrated that loss of muscle mTOR caused a myopathy characterized by reduced muscle mass and suppression of mitochondria biogenesis. mTOR MKO mice presented muscle atrophy, suppression of mitochondria biogenesis and increased glycogen content similar to the phenotype of RAmKO mice in which the mTORC1 component Raptor was specifically inactivated in skeletal muscle [131].

Collectively, the ongoing extensive development and clinical use of mTOR inhibitors, together with the major recent findings on the importance of mTOR signaling in the maintenance of muscle mass and integrity demonstrate the necessity to completely understand muscle mTOR functions at the physiological, cellular and molecular levels as my PhD research project in order to develop appropriated clinical approach and to avoid dangerous side effects.

At the molecular level, my laboratory has demonstrated that mTORC1 regulates mitochondrial activity through the PPAR-PGC1α pathway by applying the PPAR pan-agonist bezafibrate and by transgenic overexpression of PGC1α in mTOR MKO and RAmKO mice, respectively [136]. However, none of these experimental paradigms did prevent the progressive lethal myopathy. We also found that mTORC1 regulates glycogen levels through PKB/Akt. Indeed, both mTOR MKO and RAmKO myopathic mice present a remarkable increase in the

phosphorylation of protein kinase B/Akt (PKB/Akt) at threonine 308 and serine 473. Increased phosphorylation of Akt at threonine 308 arises from the loss of feedback inhibition from S6 kinase on IRS-1. And increased phosphorylation of Akt on serine 473 arises from activation of a kinase distinct of mTORC2. In spite of the similarities between mTOR MKO and RAmKO muscles, mTOR MKO mice present more severe dystrophic features including fiber regeneration/degeneration extending to both glycolytic and oxidative muscles. A possible explanation for this observation is the finding by my laboratory that mTOR MKO muscles, in contrast to RAmKO muscles, exhibit dystrophin downregulation. mTOR binds to dystrophin promoter, likely via the YY1 transcription factor, and regulates dystrophin transcription in a cell-autonomous, rapamycin resistant, raptor and kinase-independent manner. Collectively, our findings demonstrate that mTOR, mainly via mTORC1, is important for the maintenance of skeletal muscle oxidative properties, glucose utilization, growth, integrity and function. Through interaction with DNA-binding transcription, mTOR acts a transcriptional coactivator on dystrophin promoter. mTOR possesses kinase independent activities and may function independently of mTORC1 and mTORC2 complexes, through an unidentified complex or a complex-independent fashion. The loss of muscle mTOR complexes initiates a mTOR-independent activation of Akt. Lastly, the progressive lethal myopathy in mTORC1 mutant mice is independent of altered mitochondrial activity. According to all above, impaired mTOR-mediated processes could contribute to human myopathies and, clinical use with mTOR inhibitors might be detrimental

to muscle function.

My thesis project aims to address multiple crucial points regarding mTOR functions in skeletal muscles as well as the underlying mechanisms.

(i) What are the mTOR-regulated functions that lead to muscular dystrophy when inhibited? mTOR controls various activities which have not yet been well-studied in vivo including gene transcription, protein synthesis and degradation, autophagy. Alterations of these processes may contribute to the severity of the mTOR MKO and MKOKI (defective in endogenous muscle mTOR but expressing a kinase-inactive mutant of mTOR) muscle phenotype.

(ii) What is the contribution of mTOR kinase activity in muscle physiology and integrity and what are the consequences of inhibition of mTOR kinase activity? It is well established that mTOR displays myogenic functions that do not need its kinase activity such as in the regulation of IGF-II and dystrophin expression as well as in the initiation of differentiation and nascent myotube formation [130, 158, 159, 161]. But it remains unknown at which level muscle homeostasis needs mTOR kinase functions in vivo, including metabolism, structural integrity etc. Actually, many mTOR functions have been identified using rapamycin which does not inhibit mTOR's intrinsic catalytic activity but partially blocks mTORC1 function through destabilizing the mTOR-Raptor, thereby uncoupling mTOR from its substrates. Thus mTOR rapamycin sensitive functions do not suggest a requirement for mTOR kinase activity.

In this study, I demonstrated that these MKOKI mice developed a severe myopathy, displaying characteristics of muscular dystrophy and metabolic

myopathy, leading to premature death. mTOR MKOKI mice did not survive more than 12 weeks, ultimately died between 10 and 12 weeks of age while mTOR MKO mice died between 24 and 38 weeks of age. The mTOR kinase dead allele does rescue any altered parameters associated to the loss of the mTOR protein, but in contrast exacerbated these alterations. Muscle mTOR kinase activity is required for the maintenance of oxidative metabolism and dystrophin expression. Besides, the severe dystrophic and metabolic MKOKI phenotype as compared to MKO phenotype is likely due to additional: (i) The stronger PGC1a downregulation in MKOKI muscles possibly because the hyper-activation of Akt. (ii) The earlier and stronger Akt1 and Akt2 hyperactivation in MKOKI muscles leaded to dramatic increased glycogen stores. (iii) Glycogen degradation inhibition leaded to myofibrillar desorganization following glycogenosis. (iv) Autophagy inhibition further leaded to pronounced atrophy, dystrophy and metabolic crisis (e.g. glucongenesis hepatic).

2.2. Results and Discussion

2.2.1. Generation of mTOR MKOKI mice

In order to characterize the role of mTOR kinase activity in muscle integrity and function, we have generated a mouse line named mTOR MKOKI which is defective in endogenous muscle mTOR (mTOR MKO), while expressing a FLAG-tagged muscle-specific kinase-inactive allele (Asp2357Glu) of mTOR

(FLAGmTOR MKI), thereby mimicking the effects of a potent, bioavailable and selective mTOR catalytic inhibitor in muscle (Figure 1). The phenotype of the mTOR MKOKI mice will be analyzed and compared to the phenotype of mTOR MKO littermates. By using the mTOR MKOKI and mTOR MKO mouse model, I will clarify the role of muscle mTOR in the maintenance of muscle integrity at the level of the regulation of protein synthesis, the regulation of protein degradation, notably in the regulation of muscle autophagy.

For this purpose, we crossed muscle-specific mTOR knockout mice (HSA-Cre+; mTORflox) with HSA-FLAGmTOR MKI transgenic mice previously generated and characterized by the laboratory of Jie Chen [161]. Importantly, the HSAFLAGmTOR MKI transgenic line did not display any obvious phenotype. Indeed, despite the higher levels of transgene expression compared to the endogenous protein, the kinase inactive mTOR did not behave as a dominant negative mutant on mTOR substrates. In mTOR MKOKI mice, expression of both Cre recombinase and FLAGmTOR MKI allele is driven by the muscle specific promoter HSA (human skeletal actin). Therefore inactivation of the endogenous mTOR locus in differentiated myotubes of the developing embryos is conjunctively associated with the expression of the kinase inactive allele of mTOR. In parallel we have generated a transgenic line used as a control for this genetic setting, named mTOR MKOWT, which is defective in endogenous muscle mTOR (mTOR MKO), while expressing a FLAG- tagged muscle-specific wild type allele of mTOR (FLAGmTOR MWT). This line was obtained by crossing muscle-specific mTOR knockout mice (HSA-Cre+;

mTORflox) with HSA-FLAGmTOR MWT transgenic mice previously generated and characterized by the laboratory of Jie Chen [161].

Expected litters composition from these two genetic settings is respectively: 1) Control genetic setting 25% mTOR flox (Control), 25% mTOR flox; mTOR WT (Transgenic mTOR MWT), 25% HSA-Cre+; mTORflox (mTOR MKO), 25% HSA-Cre+; mTORflox; mTOR WT (mTOR MKOWT).

2) Mutant genetic setting 25% mTOR flox (Control), 25% mTOR flox; mTOR KI (Transgenic mTOR MKI), 25% HSA-Cre+; mTORflox (mTOR MKO), 25% HSA-Cre+; mTORflox; mTOR KI (mTOR MKOKI).

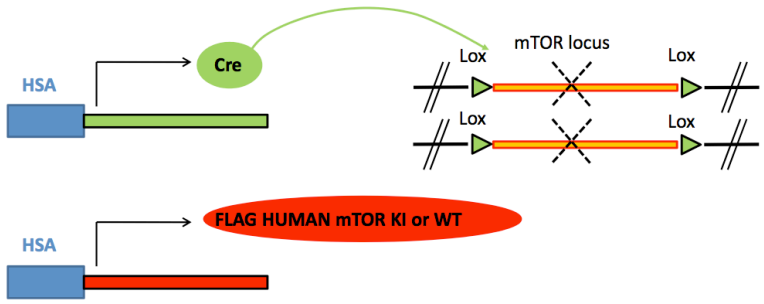


Figure 1. Schematic of strategy of MKOKI mice generation.
Mouse lines abbreviations:
1) mTOR MKO mice: Muscle specific mTOR Knock Out mice
2) mTOR MKI mice: transgenic mice expressing a Muscle-specific FLAG-tagged mTOR Kinase-Inactive allele
3) mTOR MWT mice: transgenic mice expressing a Muscle-specific FLAG-tagged mTOR Wild Type allele
4) mTOR MKOKI mice: Muscle specific mTOR Knock Out mice expressing a Muscle-specific FLAG-tagged mTOR Kinase-Inactive allele
5) mTOR MKOWT mice: Muscle specific mTOR Knock Out mice expressing a Muscle-specific FLAG-tagged mTOR Wild Type allele

2.2.2. Metabolism in mTOR MKOKI mice

2.2.2.1. Growth defects and premature death of MKOKI mice

mTOR MKOKI mice develop a more severe dystrophic phenotype than mTOR

MKO mice. Our genetic approach was validated since the human mTOR WT allele totally rescued the growth, muscle pathology and viability of mTOR MKO mice. While mTOR MKOKI mice were born at expected Mendelian ratios. While mTOR MKOKI mice were normal at birth, they rapidly developed severe growth retardation and an early onset muscular dystrophy 3 weeks after birth and die within 4-10 weeks. Pronounced sign of kyphosis in mTOR MKOKI mice was observable at 4 weeks of age, while mTOR MKO mice were still morphologically similar to Control mice at the same age, and only started to exhibit spinal deformation about 13 weeks of age (Figure 2A).

I found that the FLAG human mTOR WT allele completely rescued the mTOR MKO phenotype and viability thereby validating our genetic approach. From the very beginning, MKOKI mice started to behave differently from mice of other genotypes. From 3 weeks old, the difference became obviously large. The body weight of MKOKI mice only increased slightly, in comparison, mice of other genotypes grew fast and significantly (Figure 2B). Tibialis anterior (TA) and soleus muscles of 4-wk-old (data not shown) and 6-wo-old male MKOKI mice (Figure 2C) presented severe atrophy phenotypes.

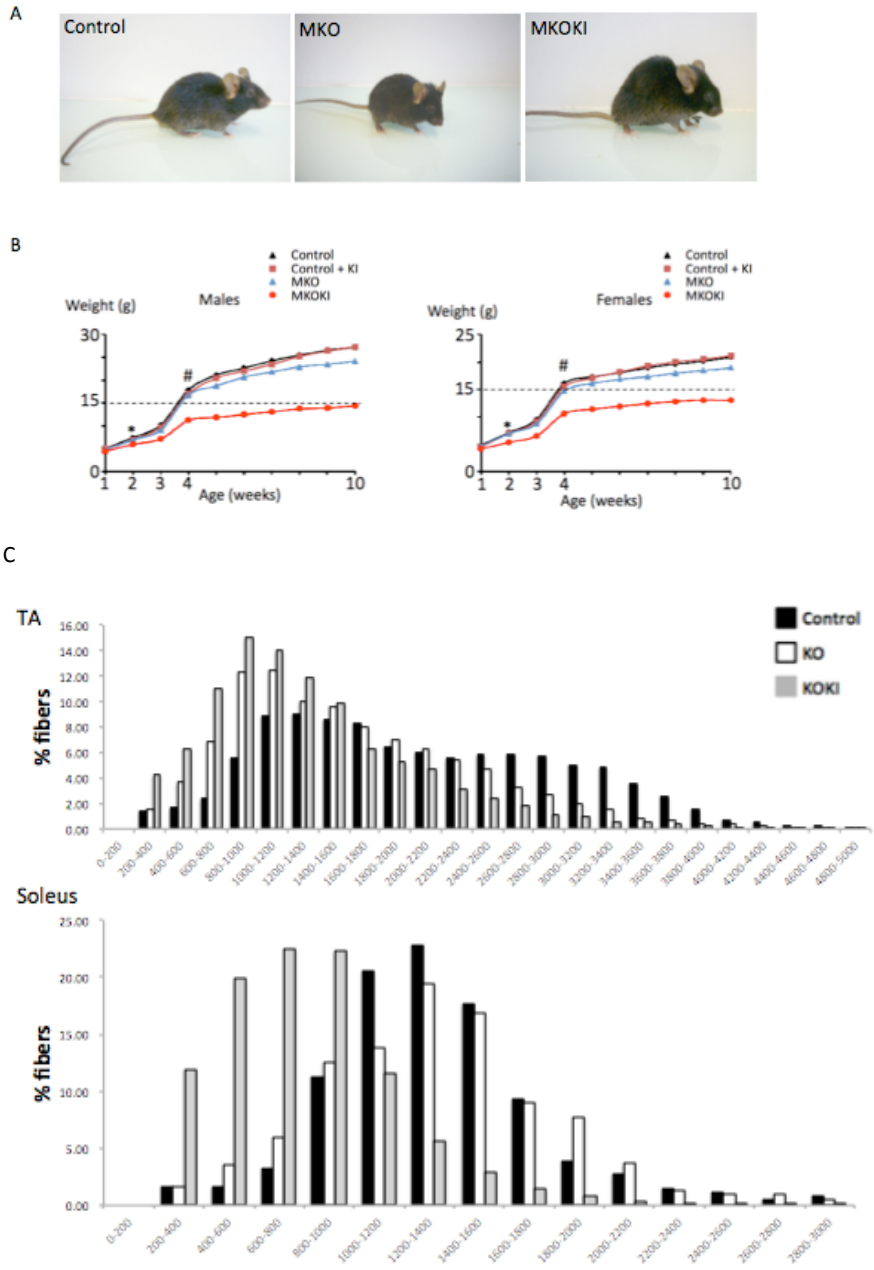


Figure 2. mTOR MKOKI defective in endogenous muscle mTOR but expressing a kinase-inactive mutant of mTOR. (A) Morphology of 4-wk-old male mice. (B) Growth curves of mTOR mutant mice (n = 10-15 mice; # P<0.05 Control versus MKO; * P<0;01 Control versus KOKI). (C). Fiber distribution analysis on TA and Soleus muscles from 6-wk-old male mice (n=6).

Table 1. Relative weight of skeletal muscles, organs and length of femur and tibia in 6-wk-old mice

	Control	MKO	MKOKI
Body weight	100%	95%	70%
TA	100%	92%	60%

GC	100%	92%	63%
Soleus	100%	90%	45%
Kidney	100%	99%	90%
Liver	100%	98%	76%
Heart	100%	101%	85%
White fat	100%	107%	60%
Brown fat	100%	98%	85%
Spleen	100%	99%	76%
Femur length	100%	99%	99%
Tibia length	100%	102%	99%

2.2.2.2. High regeneration levels in MKOKI muscles

With HE staining, we know MKOKI soleus was smaller than KO and control (Figure 3). Increased nuclei density was shown in MKOKI soleus. Comparing the Control mice, relative cross section area (CSA) of MKOKI and MKO soleus is 65% and 95% separately. Similarly, the relative CSA of MKOKI and MKO of TA (tibialis anterior) muscle is 55% and 83% separately compared to the Control mice. Nuclei density increased in KOKI soleus. However, despite the severe phenotype, there were very few CN (centrally located nuclei) in MKOKI TA (Figure 3A).

Ongoing muscle regeneration was further confirmed at the molecular level. Expression of perinatal muscle myosin heavy chain (MHC) MyH8, IGF-II, and myogenin were significantly higher in 6-wk-old mTOR mutant TA and soleus

muscles as compared to mTOR Control muscles (Figure 3B).

We previously showed that mTOR controls dystrophin transcription. Using an electroporation based- approach, we found that mTOR kinase activity was not required for dystrophin regulation. Surprisingly, dystrophin mRNA level was strongly reduced in mTOR MKOKI TA and soleus indicating that mTOR kinase activity is required for proper dystrophin expression (Figure 3B).

A

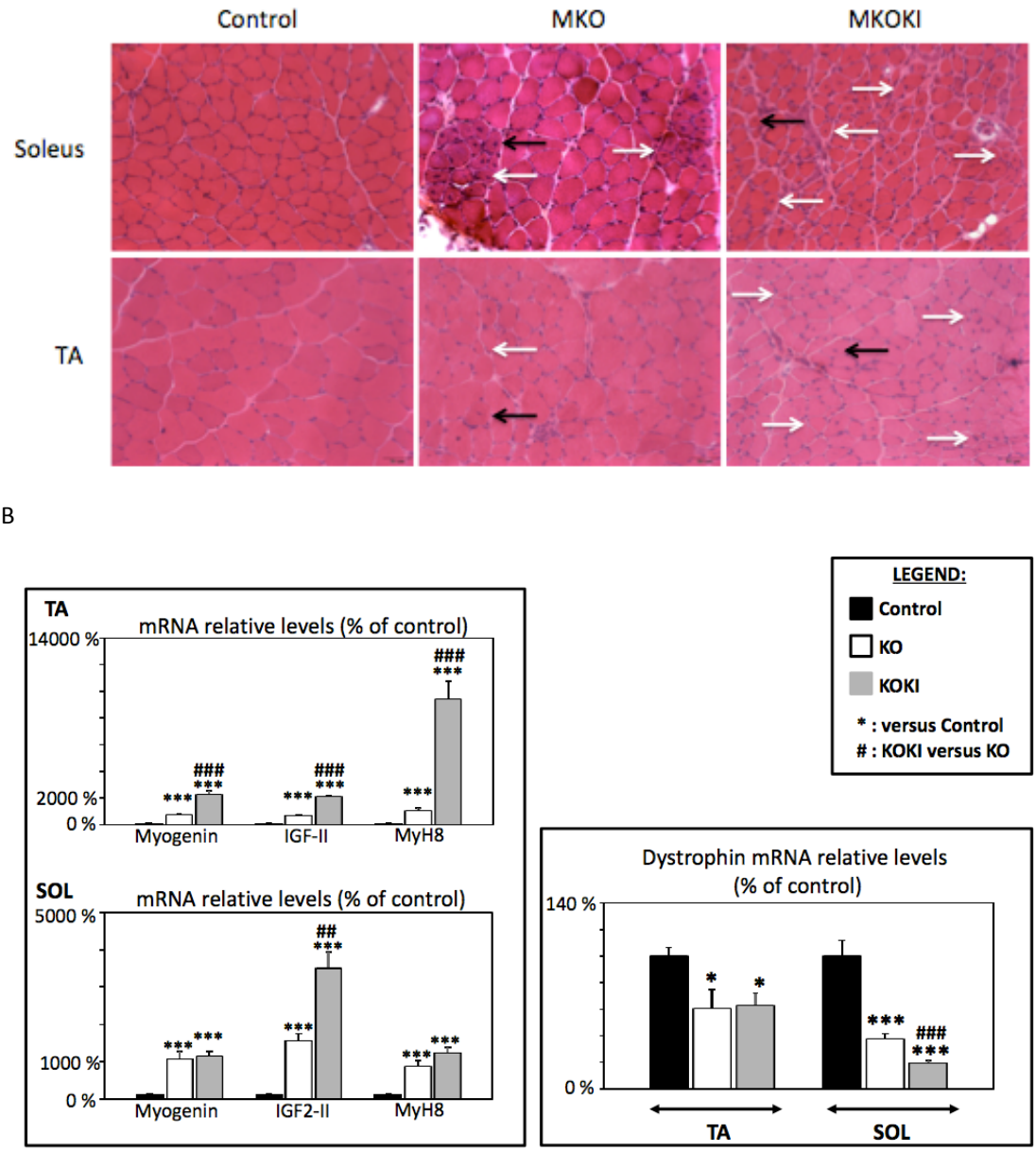


Figure 3. High regeneration levels in MKOKI muscles. (A) H&E-stained transverse sections of soleus and TA from 6-wk-old mice. Variation in fiber size with small atrophic fibers (white arrows), regenerated muscle fibers with centrally located nuclei (black arrows) are shown. Bar, 50 µm. (B) Relative mRNA levels of muscle regeneration related genes and dystrophin gene from 6-wk-old mice (n=6 sample sets). *, P < 0.05; **, P < 0.01; ***, P < 0.001; #, P < 0.05; ## < 0.01; ###, P < 0.001. Data indicate mean ± SEM.

2.2.2.3. Characterisation of glucose homeostasis in MKOKI mice

Periodic acid Schiff staining of soleus, PLA (plantaris) and GC (gastrocnemius) muscle sections from 6-wk-old mice showed glycogen accumulation in mTOR mutant muscles. Glycogen content was similarly increased in mTOR MKO and mTOR MKOKI muscles (Figure 4A), consistent with the loss of the negative

mTORC1/S6K1 negative feedback loop onto IRS. Additionally, the electronic microscopy study found that massive glycogen accumulation in large areas of freely dispersed as well as dense clustered cytoplasmic granules impairing myofilament and sarcomeric organization in MKOKI (Figure 4C).

On the basis of these, we proceeded glycogen quantification. Preliminary experiments indicated: 44- fold increase in KOKI muscles versus Control, while only 11-fold increase in MKO versus Control. The fold changes were consistent. Likely there were problems with glycogen extraction efficiency from muscles versus contaminating free glucose, no problem with glycogen extraction from liver. These observations indicate that PAS staining should not be used for glycogen quantification at least for large amount.

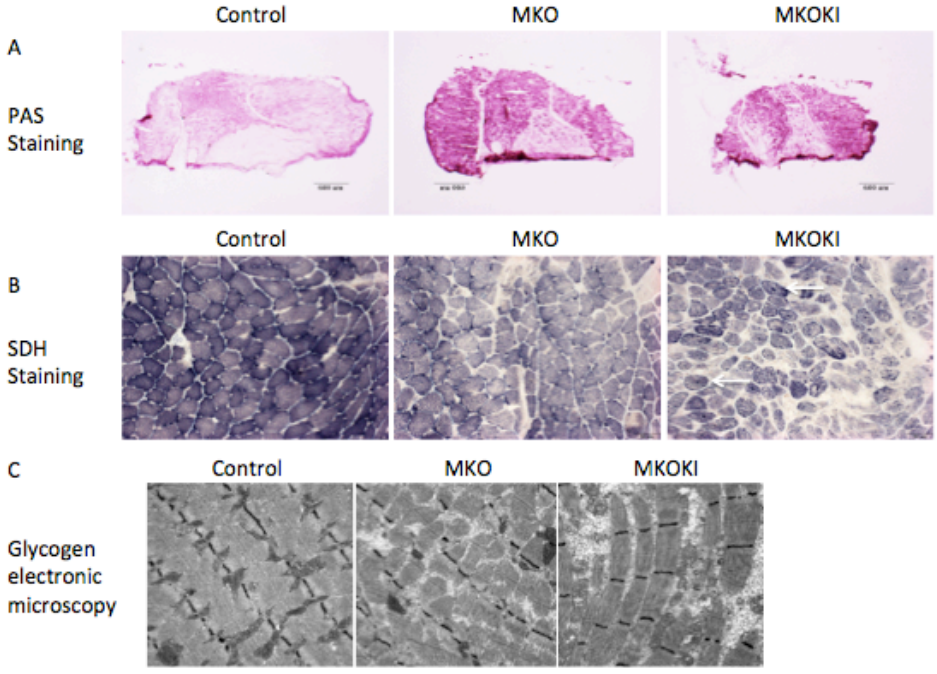


Figure 4. Mitochondrial activity, oxidative metabolism and glycogen content of muscles from mutant mice. (A) PAS staining for glycogen content on TA muscles from 6-wk-old mice. Bar, 600 µm. (B) SDH staining for mitochondrial activity on TA muscles from 6-wk-old mice. Bar, 50 µm. Mitochondrial accumulated in the center of the muscle fiber (white arrow). (C) Glycogen electronic microscopy on TA muscles from 6-wk-old mice. Bar 1 µm.

2.2.2.4. Mitochondrial activity and Oxidative metabolism

Mitochondria content and activity is similarly altered in mTOR MKO and mTOR MKOKI SOL but also PLA and GC to a lower extent. Mitochondrial accumulated in the center of the muscle fiber (Figure 4B).

To study the oxidative metabolism, I analyzed the mRNA level of PGC1α in soleus muscle, mRNA levels for PGC-1α and protein levels for the Complex IV were strongly downregulated in mTOR MKOKI, while it was significantly higher in MKO than in MKOKI (Figure 5A).

Only mTOR MKOKI muscles from 6-wo-old mice lacked the red color characteristic of oxidative muscles. Consistent with this, myoglobin protein levels was strongly downregulated in mTOR MKOKI muscles (Figure 5B).

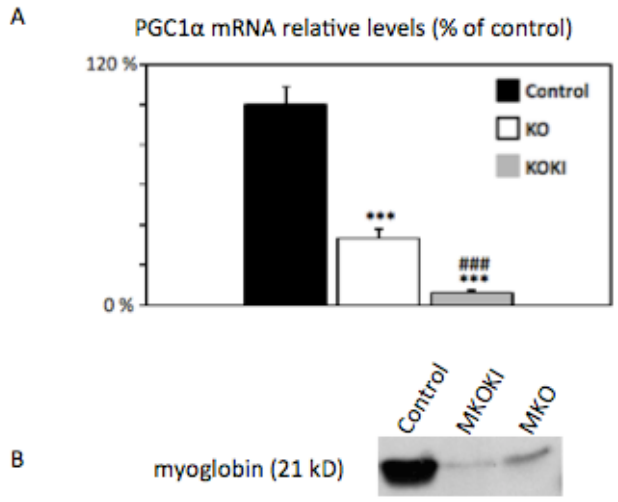


Figure 5. PGC-1α and myoglobin analysis for oxidative metabolism. (A) Relative mRNA levels of PGC-1α in mTOR mutant soleus muscles from 6-wk old female mice. (n=6 sample sets). ***, P < 0.001: mutant versus control; ###, P< 0.001: MKO versus MKOKI. Data indicate mean ± SEM. (B) Western blot analysis showing reduced protein levels for myoglobin in Control, MKO and MKOKI soleus muscles. Equal protein loading was controlled by measuring total protein content and Coomassie blue staining.

In conclusion, expression of the mTOR inactive kinase protein in skeletal muscles exacerbated the mTOR MKO phenotype. This means that mTOR inactive kinase protein has early lethality to the mice. These mice presented

severe growth failure: Pronounced muscle atrophy & Growth defects of several internal organs (Table 1). Dramatic increase in muscle glycogen content leaded to myofibrillar disorganization. Reduced PGC1a levels demonstrated the lower oxidative metabolism of MKOKI muscles. mTOR kinase activity is required for PGC1-a expression. Increase MKOKI muscle regeneration levels were confirmed by the relative mRNA levels of the Myogenin, IGF-II, and MyH8.

2.2.2.5. Characterization of growth defects of MKOKI mice

2.2.2.5.1. Growth failure not due to impaired muscle IGF1 production

IGF-1 is expressed ubiquitously and is essential for normal growth and development. The research on total IGF1 KO mice verify that liver contributed to 75% of the circulating IGF-1 level, 25% extra hepatic, but liver specific IGF1 KO has no defect in growth or development.

IGF1 transgene muscle mice display muscle hypertrophy and increased bone mass. There are factors that could contribute to growth failure of KOKI mice. GRP94 (94 kDa glucose-regulated protein) is member of the HSP90 family of ER stress response proteins. GRP94 muscle KO mice displayed that its muscle, liver, kidney and heart mass decreased about 25%, the length of femur reduced by 4%~7%. This was attributed to impaired muscle IGF1 production [5]. In addition, SRF (Serum response factor) KO mice displayed severe growth retardation, reductions of muscle mass and of other tissues, and myopathy, leading to early death, only 50% alive at 6-wk-old, this was attributed to impaired IL4 (responsible for myoblast recruitment) and IGF1 expression.

So firstly, I explored the post-natal growth failure to know whether there is

impaired muscle IGF1 production.

However, our MKOKI mice had normal femur and tibia length. There was no significant change of mutant mice for the IGF1 mRNA level (Figure 6A). This exclude impaired muscle IGF1 production in the growth defect of mTOR MKOKI mice.

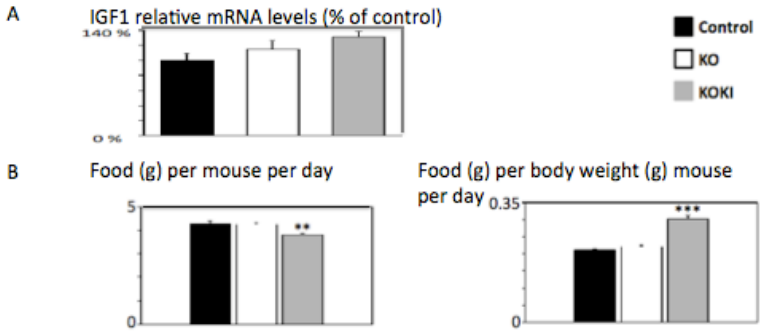


Figure 6. Post-natal growth failure of MKOKI mice. (A) Relative mRNA levels of IGF1 in mutant muscles from 6-wo-old mice. (B) Food intake per mouse per measured over 14 days between 4 and 6 weeks of age (n = 6 males per genotype; ** P < 0.01; *** P<0.001) or normalized by body weight (n = 6). (C) Fed blood glucose concentrations (n=10 males per genotype; **P<0.01 KOKI versus Control).

2.2.2.5.2. Growth failure not due to Starvation/caloric restriction

Food intake was measured each day for each mouse over 14 days between 4- and 6-wk-old (Figure 6B). In addition, food intake was normalized by body weight, data showed that MKOKI mice are slightly hypophagic, however food intake is increased when normalized to body weight, reduced nutrient intake unlikely contributes to MKOKI growth defect(Figure 6B).

2.2.2.5.3. Growth failure due to altered glucose homeostasis

Fed blood glucose concentrations were measured. Data demonstrated that MKOKI mice have lower fed glucose levels (Figure 7A). This is likely due to strong uptake by muscles.

Meanwhile, I analyzed whether MKOKI muscles are unable to provide precursors for hepatic gluconeogenesis or not? As we know, Alanine plays an important role in glucose–alanine cycle between tissues and liver. In muscles that can degrade amino acids for fuel, amino acids are collected in the form of glutamate through transamination. Then glutamate can transfer its amino acids through the alanine aminotransferase (ALT) to pyruvate, a product of muscle glycolysis, producing alanine and α-ketoglutarate. The alanine is then passed into the blood and transported to the liver, where a reverse of the alanine aminotransferase reaction occurs. Pyruvate regenerated forms glucose by gluconeogenesis, which returns to muscle by the circulation system. In the glucose–alanine cycle, pyruvate and glutamate are removed from the muscle and transferred to the liver. Glucose is regenerated from pyruvate and then sent back to muscle: the energetic burden of gluconeogenesis is thus imposed on the liver instead of the muscle. All available ATP in muscle is devoted to muscle contraction.

Alanine aminotransferase (ALT) is also known as glutamate pyruvate transaminase (GPT). The mRNA levels of GPT2 was analyzed by RT-QPCR, data showed it reduced by 5% in MKOKI muscles whereas reduced by 28% in MKO muscles (Figure 7B). Western blot studies displayed that protein levels of GPT2 in MKOKI muscles were strongly reduced whereas slightly reduced in MKO muscles compared to Control muscle (Figure 7C). The downregulation of GPT2 indicate the reduced capacity to form the gluconeogenic precusors alanine.

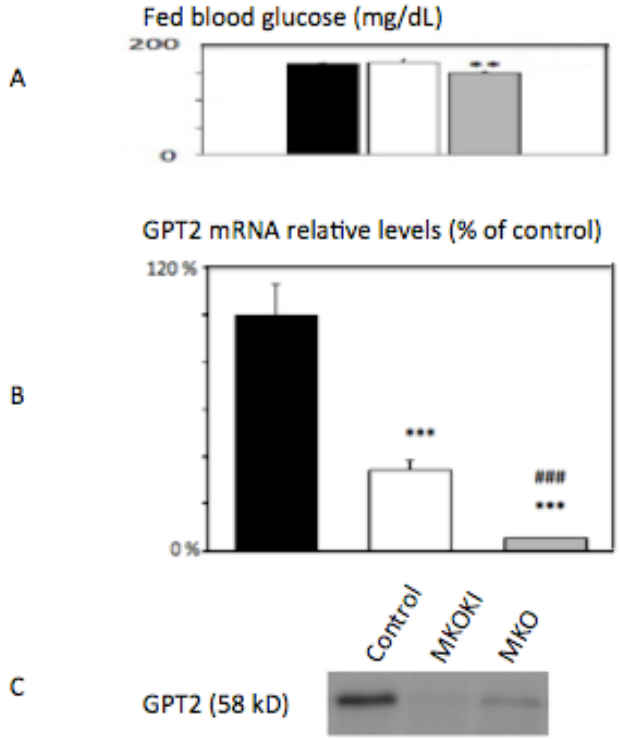


Figure 7. Growth failure due to altered glucose homeostasis.
(A) fed blood glucose concentrations (n=10 males mice per genotype; **P<0.01 KOKI versus Control) (B) Relative mRNA levels of GPT2 in mTOR mutant TA muscles from 4-wk old MALE mice. (n = 6 sample sets). ***, P < 0.001: mutant versus control; ###, P< 0.001: MKO versus MKOKI. Data indicate mean ± SEM. (C) Western blot analysis showing GPT2 protein level in TA muscles from 4-wk-old mice.

2.2.2.6. Molecular characterization of MKOKI muscle

2.2.2.6.1. myofibrillar disorganization due to glycogen accumulation is the implication of PKB/Akt signaling

Loss of S6K/IRS Feedback loop: IRS1, Akt-p308, pGSK3 were more upregulated in MKOKI than MKO mice (Figure 8). mTORC2 signaling showed upregulation: Akt-P473 was more phosphorylated in MKOKI than MKO mice (Figure 8). MKOKI mice displayed glycogen storage disease type V features (mTOR MKO mice similar to a much lower extent). Glycogen storage disease type V (GSDV) is a metabolic disorder, more specifically a glycogen storage disease, caused by a deficiency of myophosphorylase. GSDV is also named as McArdle disease or muscle phosphorylase (myophosphorylase)

deficiency.

Glycolysis is an evolutionarily conserved series of ten chemical reactions that produce pyruvate and ATP from glucose by utilizing eleven enzymes. Phosphofructokinase 2 (PFK2) or fructose bisphosphatase 2 (FBPase2) stimulates the synthesis and degradation of fructose 2,6-bisphosphate. GP (Glycogen phosphorylase) is an important enzyme in carbohydrate metabolism. The activity of GP is regulated by either noncovalent binding of metabolites or by covalent modification. GP catalyzes the phosphorylation of glycogen to Glc-1-P (Glucose-1-phosphate). There are three genes that encode the brain, liver and muscle forms of GP including PYGB, PYGL and PYGM.

Genetic sequencing of the PYGM gene codes for the muscle isoform of glycogen phosphorylase. Genetic sequencing can be done to determine the existence of gene mutations, and if GSDV is present. GSDV exhibits the characteristic of fatigue, cramps, and muscle pain during the first few minutes of exercise (exercise intolerance). There are significant variations of GSDV signs and symptoms in affected individuals. The features typically start in teens or twenties. In most people affected with GSDV, the muscle weakness becomes worse over time. About 50% of people with GSDV undergo breakdown of muscle tissue (rhabdomyolysis) and myoglobinuria. Knock-in mice for the R50X mutation in the PYGM gene appear McArdle disease [418]. The increase (27-fold) of muscle glycogen content appeared in p.50X/p.50X mice were actually much higher than that observed in patients with McArdle disease, which only increases more than 2-fold [419].

Our data showed that PYGM was strongly downregulated in MKOKI muscles versus MKO, demonstrating the remarkable defects in glycogen degradation in MKOKI mice (Figure 8).

Akt1 transgenic mice did not display such a glycogen accumulation phenotype (Figure 8). Is this due to Akt2 hyperactivation? By western blot analysis, we found the strong Akt2 hyperactivation in MKOKI likely explained the glycogen accumulation phenotype (Figure 8).

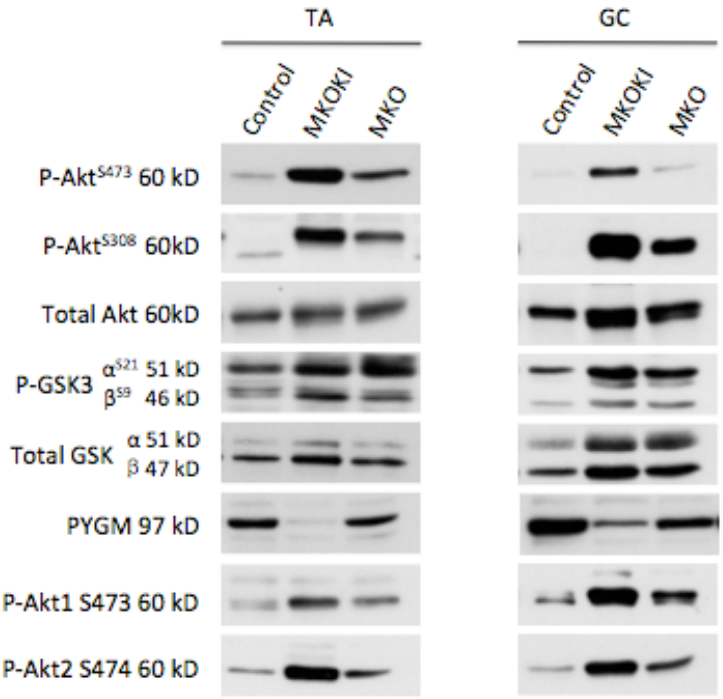


Figure 8. Signal transduction on TA and GC muscle from 6-wk-old mTOR mutant mice.

2.2.2.6.2. The identification of the kinase phosphorylating Akt on S473 in skeletal muscle

When mTOR is inhibited, the identification of the kinase phosphorylating Akt on S473 in skeletal muscle is an important but difficult task.

Kinases phosphorylating Akt on S473 include integrin-linked kinase (ILK), ataxia telangiectasia mutated (ATM), DNA-dependent kinase (DNA-PK),

MAPKAP kinase (MK) 2, protein kinase C (PKC) α, PKCβII, p21-activated kinase (PAK) 1 and PAK2, and TBK. Among these putative PDK2s, ILK has drawn much attention upon the mechanistic link between aberrant ILK upregulation and tumor progression in multiple types of human malignancies. Armelle A. Troussard et al. showed that conditional knock-out of Integrin-linked Kinase demonstrates that ILK play an essential role in PKB/Akt activation [420].

Interestingly, we found the that ILK protein expression profil was similar to Akt 473 phosphorylation (Figure 9A). However, experimental evidence verified that the kinase-like domain of ILK lacked catalytic activity [421].

We also found that the expression profil of TBK was similar to Akt 473 phosphorylation (Figure 9A).

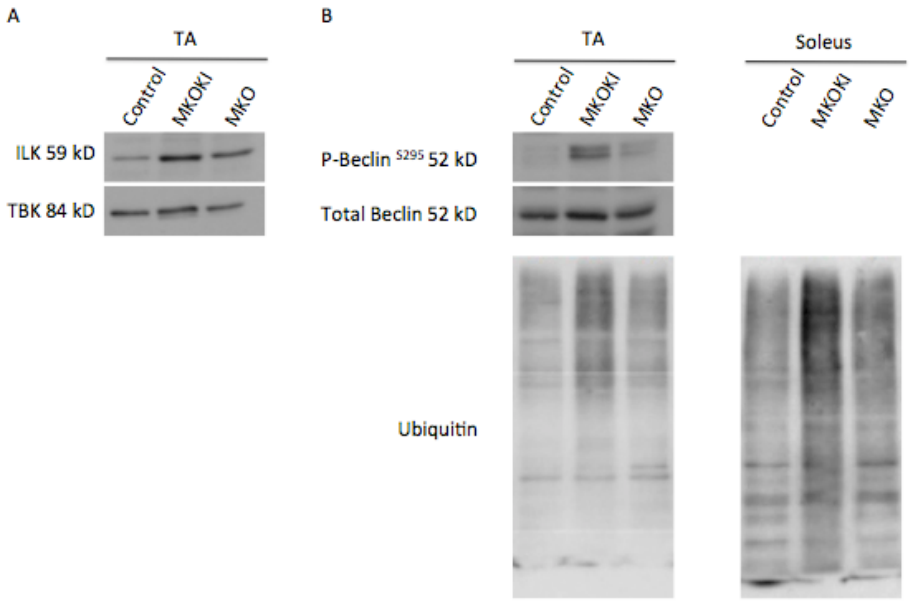


Figure 9. Beclin and the identification of the kinase phosphorylating Akt on Ser473. (A) Western analysis for ILK and TBK antibodies on TA muscles from 6-wo-old mice. (B) Western analysis for Beclin 1 (phospho S295), Total Beclin and Ubiquitin antibodies on TA and Soleus muscles from 6-wo-old mice.

2.2.2.6.3. Implications for Therapies Targeting the PI3K/Akt/mTOR Pathway

For the traditional therapies, as we know, 1) Rapamycin and rapalogs, partial mTORC1 inhibitors, can arrest cells in the G1 phase. However, monotherapy with rapalogs has shown limited antitumor efficacy due to cell resistance including: incomplete 4EBP1 dephosphorylation, feedback loop activation of the survival pathways: PI3K/Akt, MAPK/ERK, and Mnk/eIF4E. 2) Combined PI3K inhibitors/rapalogs, also as partial mTORC1 inhibitors, have functions of robust growth suppression, induction of cell cycle arrest and apoptosis of tumor cells. The effects of this combined PI3K/mTOR inhibitors have been attributed to the lack of activation of PI3K/Akt signaling. 3) mTOR catalytic inhibitors which inhibit mTORC1 and mTORC2, rapidly and efficiently lead to cell cycle arrest and apoptosis of actively proliferating cancer cells due to the lack of feedback loop activation of PI3K/Akt signaling.

My thesis study indicate that mTOR catalytic inhibitors might have severe mutiple effects in postmitotic tissues such as skeletal muscles, which are able to activate the feedback loop on Akt despite mTOR kinase inhibition.

Moreover, activation of the feedback loop on IRS/PI3K/AKT is thought to be a promising therapeutic avenue for the treatment of insulin resistance. My data demonstrated that sustained muscle Akt1 and Akt2 activation actually improved glucose uptake and storage as glycogen but also compromised its utilization further leading to severe Glycogen Storage Disease.

There are also limits of my study. Because the MKOKI mice were very weak, according to the rules of National and European legislation on animal experimentation, there is no possible for proceeding MKOKI challenge by

starvation to study autophagy and also the exercise effects on mTOR kinase function. And there is no effective chloroquine assays applied on starved mice. There is also no possible to electroporate MKOKI mice with LC3. Therefore, we need to develop new strategy to study the relationship of mTOR kinase functions with autophagy, and with exercise.

2.2.3. Autophagy in mTOR MKOKI mice

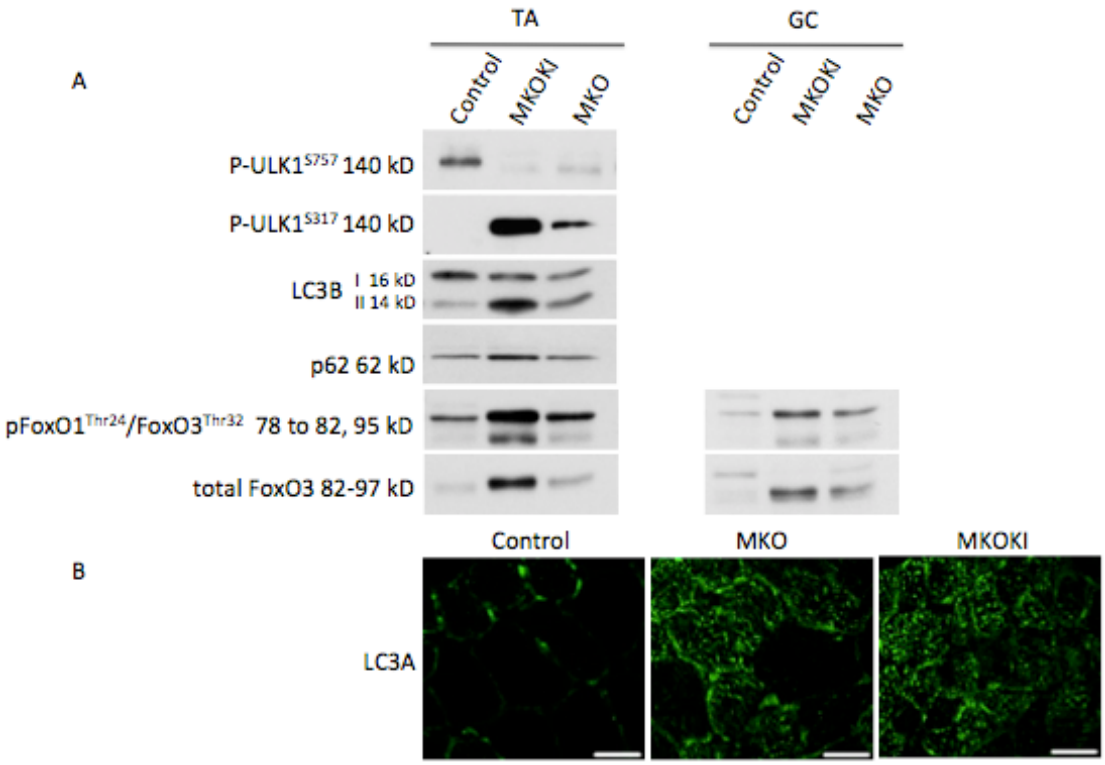
2.2.3.1. Implication of PKB/Akt signaling on autophagy

Because the MKOKI's characteristic of muscle atrophy and dystrophy, we further considered the implication of PKB/Akt signaling on autophagy.

Strong increased in LC3, p62 and Phospho-ULK1 (Ser317) protein levels in MKOKI muscles. These verified the Autophagy induction in MKO muscles and autophagy inhibition in MKOKI muscles (Figure 10A). The immunostaining of LC3 showed that there were more LC3A dots gathered in TA from MKOKI mice (Figure 10B). The immunostaining of p62 is ongoing. There is proof showed that the sustained induction of autophagy in RAmKO mice, in spite of the strong activation of Akt, indicates that LC3 lipidation is dictated by mTORC1 inactivation and does not require FoxO3 activation. So I wondered that does the strong Akt hyperactivation and FOXO inhibition in KOKI as compared to MKO more severely effect on autophagy related genes expression and thereby autophagic flux?

Data presented that despite stronger Akt activation and FoxO phosphorylation in MKOKI muscles, expression of autophagy–related gene was downregulated to

similar levels between MKOKI and MKO (Figure 10C). Therefore, differential Akt-mediated FoxO inhibition between MKOKI and MKO cannot explain opposite autophagic flux.



C

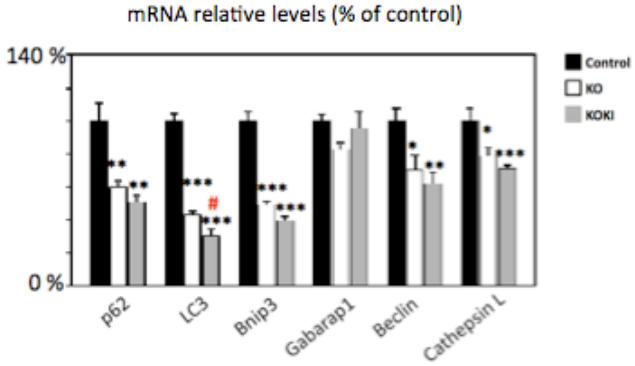


Figure 10. PKB/Akt signaling on autophagy. (A) Muscle extracts from 6-wo-old mice were immunoblotted with the indicated antibodies to examine mTOR signaling in TA and GC muscles. (B) Immunofluorescence staining for LC3A (Green) on Control, MKO and MKOKI TA muscle sections from 6-wk-old mice. Bars, 30 μm. (C) Relative mRNA levels of autophagy–related genes in TA muscles from 6-wk-old (n=6). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, $P < 0.05$. Data indicate mean ± SEM.

2.2.3.2 Beclin and autophagy flux

Study showed that Akt-mediated regulation of autophagy and tumorigenesis through Beclin1 phosphorylation; the regulation of Beclin1/vimentin interactions by active Akt may be a mechanism for inhibition of autophagy by intermediate filaments. So is there similar mechanism in skeletal muscle? Could this explain opposite autophagic flux between MKOKI and MKO? Data showed that Beclin 1 (phospho S295) protein levels increased remarkably in MKOKI mice as compared to MKO (Figure 9B). However, we still have no idea if Beclin 1 is sequestered in MKOKI or not. So for the next, immunofluorescence staining of beclin1 on muscle sections to assess potential beclin1 intermediate filaments interactions that might explain autophagy inhibition in MKOKI muscles. In addition, Amount of total ubiquitinated proteins was increased in 6 w/o KOKI mice soleus and TA muscles (Figure 9B).

2.2.4. Conclusion and perspective

2.2.4.1. Conclusion

In conclusion, my study demonstrate that:

1) a mTOR kinase dead allele did not rescue any altered parameters associated to the loss of the mTOR protein, but in contrast exacerbates these alterations.

2) Muscle mTOR kinase activity was required for oxidative metabolism maintenance and dystrophin expression.

3) MKOKI muscles show a hyper-accumulation of glycogen that gave rise to myofibrillar disorganization, a feature not observed in MKO.

4) Glycogen hyper-accumulation is due to a stronger Akt hyperactivation that leads to myophosphorylase downregulation. Glycogen degradation inhibition gave rise to myofibrillar disorganization following glycogenosis. Sustained muscle Akt1 and Akt2 activation actually improved glucose uptake and storage as glycogen but also compromised its utilization further leading to severe Glycogen Storage Disease.

5) Autophagy inhibition in mTOR MKOKI, in contrast to MKO further leaded to pronounced atrophy, dystrophy.

6) mTOR catalytic inhibitors might have severe mutiple effects in postmitotic tissues such as skeletal muscles, which are able to activate the feedback loop on Akt due to mTOR kinase inhibition.

2.2.4.2. Perspective

While mTOR KOKI mice were normal at birth, they rapidly develop severe growth retardation and an early onset muscular dystrophy 3 weeks after birth and die within 4-10 weeks. We found that the growth defects of mTOR MKOKI mice were associated with a strong reduction in muscle mass as well as internal organs mass, but we need more data of muscle mass and organs weight to be sure the results. mTOR MKOKI mice presented exacerbated phenotype features than mTOR MKO mice, including muscle atrophy, dystrophy, dystrophin downregulation altered mitochondrial functions as well as increased glycogen content. We also need to collect more data: dystrophin protein levels via western

blots and glycogen content via glycogen quantification. They are important supplemental proofs to show that mTOR kinase activity inhibition more detrimental for muscle integrity than loss of the protein, and mTOR kinase activity required for mitochondrial functions and dystrophin.

We found that inhibition of mTOR kinase activity leads to a strong reduction in muscle mass, which impairs whole body metabolism and organ growth. We concluded that reduced muscle mass compromised its role in fueling other organs with precursors for neoglucogenesis. For this conclusion, we need to examine whether the mRNA levels of G6Pase (Glucose 6-phosphatase) and PEPCK (Phosphoenolpyruvate carboxykinase) in liver decrease. Otherwise, active neoglucogenesis maybe due to lack of muscle mass and the liver glycogen stores.

We also found that glycogen phosphorylase expression was almost not detectable in MKOKI muscle. MKOKI mice displayed Mc Ardle/Type V glycogenolysis like phenotype. Therefore, we also plan to do further molecular characterization of MKOKI mice. For the next, the studies of glycogen phosphorylase mRNA level and protein level are required through the overexpression of gag-Akt1 and Akt2 and both in myoblast. With the overexpression of FoxO in myoblast, we will check the mRNA level and protein level of glycogen phosphorylase, to study whether the FoxO3 binding site is in glycogen phosphorylase. In addition, we will use FoxO 3 ChiP and probe to get the glycogen phosphorylase DNA information.

With these, we will infer whether Akt 1 or Akt2 or both regulates GP expression

through FoxO, thus affect the phenotype of MKOKI mice, or like our lab have showed there was a correlation Akt activity with GP expression; mTORC1 Regulates Glycogen Content Independently of PGC-1α Through PKB/Akt; the levels of glycogen phosphorylase were lower at the mRNA and protein level in PGC1α- TG (expressed increased levels of PGC-1α mainly in the fast-twitch extensor digitorum longus muscle) mice; moreover, the same down- regulation of glycogen phosphorylase also was found in RAmKO (raptor muscle-knockout) mice although the levels of PGC-1α are low in those mice; hyperactivation of PKB/Akt was sufficient to regulate the synthesis and degradation of glycogen independently of PGC-1α. In both case, this suggest mechanism Akt dependant GP expression. This shows the implication for therapies aiming to activate Akt, or leading to increased Akt (loss of negative feedback using mTOR inhibitors) as well as for TypeV but also type VI glycogenolysis.

For the autophagy studies, we found in MKOKI muscle p62 and LCII increased, total Ubiquitin protein also increased. These suggested that autophagy was blocked in MKOKI muscle in contrast to RAmKO. We still need to repeat the electronic microscopy and immunogold labelling study on new samples; and finish the immunostaining of p62 and LC3A.

We also found the possible mechanism may not involved transcriptional program but involved Akt mediated phosphorylation and sequestration of Beclin 1, as shown in cancer cells, never shown in muscle cells. We found Beclin 1 phosphorylation on Akt sites strongly increased. Therefore, for the next, we need to do the Beclin 1/14-3-3 immunostaning on intermediary filaments. On

the basis of these, we would make the potential conclusion that Akt mediated inhibition of autophagy in MKOKI muscle through Beclin1 phosphorylation and sequestration.

3. SUPPLEMENTAL MATERIALS AND METHODS

3.1. Animals

Muscle-specific mTOR knockout mice (HSA-Cre+; mTORflox) crossed with HSA-FLAGmTOR MKI transgenic mice previously generated and characterized by the laboratory of Jie Chen [161]. In mTOR MKOKI mice, expression of both Cre recombinase and FLAGmTOR MKI allele is driven by the muscle specific promoter HSA (human skeletal actin). Inactivation of the endogenous mTOR locus in differentiated myotubes of the developing embryos is conjunctively associated with the expression of the kinase inactive allele of mTOR. In parallel we have generated a transgenic line used as a control for this genetic setting, named mTOR MKOWT, which is defective in endogenous muscle mTOR (mTOR MKO), while expressing a FLAG- tagged muscle-specific wild type allele of mTOR (FLAGmTOR MWT). This line was obtained by crossing muscle-specific mTOR knockout mice (HSA-Cre+; mTORflox) with HSA-FLAGmTOR MWT transgenic mice previously generated and characterized by the laboratory of Jie Chen [161].

Expected litters composition from these two genetic settings is respectively: 1) Control genetic setting 25% mTOR flox (Control), 25% mTOR flox; mTOR WT

(Transgenic mTOR MWT), 25% HSA-Cre+; mTORflox (mTOR MKO), 25% HSA-Cre+; mTORflox; mTOR WT (mTOR MKOWT).

2) Mutant genetic setting 25% mTOR flox (Control), 25% mTOR flox; mTOR KI (Transgenic mTOR MKI), 25% HSA-Cre+; mTORflox (mTOR MKO), 25% HSA-Cre+; mTORflox; mTOR KI (mTOR MKOKI).

3.2. Muscle histology, morphometric measurements, and imaging

Skeletal muscles were collected, embedded in tragacanth gum, and frozen quickly in isopentane cooled in liquid nitrogen. 10-µm-thick sections were obtained from the middle portion of frozen muscles and processed for histological and immunohistochemical analysis according to standard protocols. The fiber CSA and the number of centrally nucleated fibers were determined for Lectin (from Triticum vulgaris TRITC Conjugate) & DAPI - stained sections using Metamorph software.

Fluorescence microscopy was performed using Zeiss Axiovert 100M microscope. Light microscopy were performed by AxioImager (ex-Axioplan Zeiss CCD) microscope and CP Achromat 5x/ 0.12, 10x/ 0.3 Ph1, or 20x/0.5 Plan NeoFluor objectives (Carl Zeiss, Inc.). Images were captured using a charge-coupled device monochrome camera (Coolsnap HQ; Photometrics) and MetaMorph software. For all imaging, exposure settings were identical between compared samples. All samples for microscopy were viewed at room temperature.

Fibers size and central nuclei are measured by ImageJ and attached macros

(Macros is programed by David Cluet and Brian Rudkin, LBMC, ENS Lyon, France).

Confocal microscopy was performed using a spectral confocal laser-scanning microscope (TCS SP5; Leica) on an upright microscope (DM6000 B; Leica). Confocal software (LAS AF; Leica) was used for acquisition with a Plan Apo 63× NA 1.4 oil objective (HCX; Leica). Fluorescence and light microscopy were performed using an upright microscope (Axioplan2; Carl Zeiss, Inc.) and 10× NA 0.3 (Ph1), 20× NA 0.5, or 40× NA 0.75 Plan NeoFluor objectives (Carl Zeiss, Inc.). Images were captured using a charge-coupled device monochrome camera (Coolsnap HQ; Photometrics) and MetaMorph software. For all imaging, exposure settings were identical between compared samples. All samples for microscopy were viewed at room temperature.

3.2.1. HE staining (Hematoxylin & Eosin)

i). Slides dry in the air at room temperature, 30min

ii). 1st BATHING: Harris Hematoxylin Solution (Hematoxyline de Harris, Sigma Aldrich), 10min. Rinse with H2O (tap water)

iii). 2nd BATHING: Eosin Solution0.5% (Eosin Y disodium salt, Sigma Aldrich), 5min. Wash in distilled H2O, 2times.

iv). Dehydrate:

(1) 1min, EtOH 70%

(2) 1min, EtOH 95%

(3) 1min, EtOH 100%(bain I)

(4) 1min, EtOH 100%(bain II)

(5) 1min, Xylene (bain I) under the hood.

(6) 1min, Xylene (bain II) under the hood.

v). Mount slides with Aquatex (Merk)

3.2.2. SDH (Succinic dehydrogenase) staining

Phosphate solution (5ml)

(1) KH2PO4 0.2M 1ml (2) Na2HPO4.7H2O 0.2M 4ml (3) Succinic acid 0.27 g

(4) Nitrotetrazolium Blue chloride 5mg

Procedure:

i). Slides dry in the air at room temperature, 30min

ii). Dako pen surround the sections

iii). Phosphate solution incubate 37°C, 2h

iv). Wash with distilled water, 3 times

v). Mount with Auatex

vi). Seal the edge with Nail polish

3.2.3. COX (Cytochrome Oxidase) staining

Prepare Solution

i). Prepare solution A (use NaOH30% to ajust pH to pH7.4)

(1) 0.1M NaH2PO4 (Sigma-Aldrich) (40ml)

(2) 0.1M Na2HPO4 (Sigma-Aldrich) (about 9.6 ml)

ii). Phosphate solution

(1) 10ml solution A (pH7.4)

(2) 1 tablet (10mg) DAB (3,3'-DIAMINOBENZIDINE Tetrahydrochloride) (Sigma-Aldrich)

(3) 10mg cytochrome C (Sigma-Aldrich)

iii). Filtrate the Phosphate solution (filter 0.22mm)

Procedure:

i). Slides dry in the air at room temperature

ii). Dako pen surround the sections

iii). Phosphate solution, incubate 37°C, 4h

iv). Wash with distilled water, 3 times

v). Mount with Aquatex

vi). Seal the edge with Nail polish

3.2.4. PAS (Periodic acid-schiff)

Procedure:

i). Fix with Carnoy2, 5min

Carnoy2 (100ml) content:

(1) Ethanol 100%, 60ml

(2) Chloroform (VWR) 30ml

(3) Acetic acid 100% (VWR) 10ml

ii). Wash with distilled H2O

iii). Periodic acid (SIGMA-ALDRICH), 5min

iv). Wash with distilled H2O

v). Schiff' reagent (Merck), 30min(drop reagent on slides)

vi). Wash with tap water, 10min

vii). Dehydrate slides

viii). Mount with Aquatex (Merck)

ix). Seal with nail polish

3.3. Quantitative real-time PCR

Total RNA was isolated from muscle tissues using TRI Reagent (TRI Reagent RNA Isolation Reagent trizol) (Sigma).

RNA was analyzed by quantitative real-time PCR using SYBR Green (Qiagen). The data were normalized to cyclophilin B mRNA levels.

Primers used for Quantitative PCR Analyses

Gene	Forward primer (5'->3')	Reverse primer (5'->3')
28s	GGG-TGG-TAA-ACT-CCA-TCT-AA	AGT-TCT-TTT-CAA-CTT-TCC-CT
36B4	GGC-CCT-GCA-CTC-TCG-CTT-TC	TGC-CAG-GAC-GCG-CTT-GT
47s	CTC-CTG-TCT-GTG-GTG-TCC-AA	TGA-TAC-GGG-CAG-ACA-CAG-AA
5s	GGC-CAT-ACC-ACC-CTG-AAC-GC	CAG-CAC-CCG-GTA-TTC-CCA-GG
Acetyl choline receptor gamma	GGC-AGT-ATT-GGA-GAA-GCT-AGA-GAA	CAC-CAG-CAA-CCA-CTC-CTC-GT
AChR	GTC-CAG-AAA-TGA-GGC-AGA-GC	CCA-CTC-CTC-ATT-CCC ACT-GT
Beclin1	TCT-CTC-GAA-GAT-TCA-TCC-CC	CTG-TGC-ATT-CCT-CAC-ACA-GC
Bnip3	AGC-TTT-GGC-GAG-AAA-AAC-AG	GGG-GGA-ATA-TTT-TCT-GGT-CG
CathepsinL	GTG-GAC-TGT-TCT-CAC-GCT-CA	TCC-GTC-CTT-CGC-TTC-ATA-GG
Cyclophillin B	GAT-GGC-ACA-GGA-GGA-	AAC-TTT-GCC-GAA-AAC-C

(mCycloB)	AAG-AG	AC-AT
Dystrophin	TGC-GCT-ATC-AGG-AGA-CAA-TG	TTC-TTG-GCC-ATC-TCC-TTC-AC
FOXO1	ATT-CGG-AAT-GAC-CTC-ATG-GA	GTG-TGG-GAA-GCT-TTG-GTT-GG
Gabarapl1	CAT-CGT-GGA-GAA-GGC-TCC-TA	ATA-CAG-CTG-GCC-CAT-GGT-AG
IGF1	TGG-ATG-CTC-TTC-AGT-TCG-TG	GCA-ACA-CTC-ATC-CAC-AAT-GC
IGF2	ACC-CGA-CCT-TCG-GCC-TTG-TG	AAG-CCG-CGG-TCC-GAA-CAG-AC
IL6	TGC-AAG-AGA-CTT-CCA-TCC-AGT-TGC-C	TGT-GAA-GTA-GGG-AAG-GCC-GTG-GT
LC3B	AGT-GGA-AGA-TGT-CCG-GCT-C	AGC-GCC-GTC-TGA-TTA-TCT-TG
Mac-1	TTC-GAC-CAA-CGG-GGG-CAA-CG	TAT-CCC-TTG-GCC-CGC-CGG-AA
MAFbx (atrogin-1)	CTC-TGT-ACC-ATG-CCG-TTC-CT	GGC-TGC-TGA-ACA-GAT-TCT-CC
mTOR	CAA-ACC-ACA-GGG-TGA-GGA-CT	AGG-GCA-GCA-ACA-GTG-AGA-GT
MurF1	ACC-TGC-TGG-TGG-AAA-ACA-TC	AGG-AGC-AAG-TAG-GCA-CCT-CA
Myogenin	CTA-CAG-GCC-TTG-CTC-AGC-TC	AGA-TTG-TGG-GCG-TCT-GTA-GG
Myosin heavy chain perinatal (mMyh8)	CAA-GGA-TGG-AGG-GAA-AGT-GA	GGT-TCA-TGG-GGA-AGA-CTT-GA
p62	CGG-CTT-CCA-GGC-GCA-CTA-CC	GTT-CCC-GCC-GGC-ACT-CCT-TC
PGC-1	TCA-CAC-CAA-ACC-CAC-A	TCT-GGG-GTC-AGA-GGA-

	GA-AA	AGA-GA
Sarcolipin	ACT-GAG-GTC-CTT-GGT-AGC-CT	CAT-GGC-CCC-TCA-GTA-TTG-GT
Serca1	CTG-ACC-GAA-AGT-CAG-TGC-AA	GTC-AGG-GAC-AGG-GTC-TGT-GT
Serca2a	TGG-TGA-CAA-AGT-TCC-TGC-TG	CAC-CAC-CAC-TCC-CAT-AGC-TT
SRPK3	CGG-ACA-TCA-AGC-CTG-AGA-ACA	ACA-GCT-TGC-CAA-TCA-AGA-CCT
TNFalpha	CAA-CGC-CCT-CCT-GGC-CAA-CG	TCG-GGG-CAG-CCT-TGT-CCC-TT

3.3.1. Total RNA extraction

The principal phases of this procedure are the following:

(1). Pipette 400µl Tri reagent (Sigma) in Fast Prep tubes

(2). Cut muscle with scissors into pieces

(3). Fast Prep, 4m/s, 40s

(4). Incubate 5min, 30°C

(5). Centrifuge 5min, 14000rpm

(6). Pipette the supernatant into a new 1.5 ml eppendorf tube

(7). Pipette 350µl Tri reagent, rinse the beads, centrifuge 5min, 14000rpm

(8). Pool the supernatant in the same tube

(9). Add 0.2ml chloroform, mix vigorously

(10). Incubate 15min, 30°C

(11). Centrifuge 15min, 12000g, 5°C. Carefully transfer the top (aqueous) layer into a new 1.5 ml eppendorf tube

(12). Add 1µl GlycoBlue (Ambion), 0.5ml isopropanol (2-propanol) to precipitate, shake gently, and incubate 10min, room temperature

(13). Centrifuge 10min, 12000g, 4°C. Discard the supernatant very carefully

(14). 0.5ml EtOH (Ethyl Alcohol) 70%, rinse and vortex to suspend the pellet

(15). Centrifuge 15min, 12000g, 4°C. Remove the supernatant

(16). Add 200µl 0.5M NaCl, vortex, then add 200ul Chloroform, vortex

(17). Centrifuge 10min, 12000g, 4°C

(18). Take supernatant, add 1µl GlycoBlue, 0.7volume isopropanol (140µl), and shake gently, incubation 10min, room temperature

(19). Centrifuge 10min, 12000g, 4°C

(20). Pellet +0.5ml EtOH 70%, vortex

(21). Centrifuge 15min, 12000g, 4°C, remove the supernatant

(22). Dry the pellet naturally

(23). Add 25µl DNase/RNase free H2O (1~5mg tissue) or 50ul DNase/RNase free H2O (10mg tissue), dissolve the pellet

(24). Incubate 10min, 50°C

(25). Store at -80°C

3.3.2. First Strand cDNA Synthesis

i). Add the reagents into a sterile, nuclease-free tube on ice:

	Template RNA (total RNA)
Con. (ng/µl)	C
Template RNA 100~500ng	=100~500 (ng) / C
Random Hexamer Primer 0.2 ug/µl	1µl

Nuclease-free H2O	=12-[(100~500)/C]-1
Total volume A	12µl

ii). PCR machine

(Preheat lid: on, 95degree, block type: 48well)

Step 1. 70°C, 5min

Step 2. 4°C, pause

Open PCR machine; add the following reagents into in the indicated order:

Mix		Each tube
	5x Reaction buffer (Fermentas)	4µl
	RNase Inhibitor (20U/ul) (Promega)	1µl
	dNTP Mix (10mM each)	2µl
	Revert Acid H Minus Reverse Transcriptase 200u/ul (Fermentas)	1µl
	Total volume B	8µl
	Total volume (A+B)	20 µl

Step 3. 25°C，15min

Step 4. 42°C，1h

Step 5. 70°C，2min

Step 6. 4°C，PAUSE

iii). Stock cDNA products at -20°C

3.3.3. Amplification of the PCR Product to prepare the standards

Primer Mix (forward and reverse primers)

Primer Stock solution 100µm each

Mix at 5µM each: 10µl forward primer + 10µl reverse primer + 180µl Nuclease-free water

PCR reaction (50µl) / gene:

5µl buffer (Qiagen) red 10X

2.5µl mix primer 5µm

0.5ul dNTP (10mM each dNTP)

0.3µl Taq polymerase (Qiagen)

4µl cDNA (dilution 1/40)

37.7 µl Nuclease free H2O

Program in the PCR machine:

Step 1: 95 °C lid, preheat on

Step 2: 95 °C, 30s

Step 3: 55 °C, 30s (depend on the Tm value of primers)

Step 4: 72 °C, 30s, go back to step 2, 35 cycles

Step 5: 72 °C, 10min

Step 6: 4 °C, pause

3.3.4. Purification of the PCR product after electrophoresis

Nucleic Acid and Protein Purification kit, NucleoSpin Extract II (250preps), MACHEREY-NAGEL

i). Solubilize gel slice

(1) For 100mg agarose gel, add 200ul Buffer NT (Binding Buffer)

(2) 5~10min, 50°C

(3) Vortex briefly every 2~3min, gel completely dissolved

ii). Bind DNA

(1) NucleoSpin Extract II Column into a Collection Tube (2ml)

(2) Load sample

(3) Centrifuge, 1min, 11000g

(4) Discard flow-through; place the column back into Collection tube

iii). Wash silica membrane

(1) Add 700ul Buffer NT3 (Wash buffer) (When open this kits for the first time, add 160ml ethanol in NT3)

(2) Centrifuge 1min, 11000 g

(3) Discard flow-through; place the column back into Collection tube

iv). Dry silica membrane

Centrifuge 2min, 11000 g, to remove Buffer NT3.

v). Elute DNA

(1) Put the column into a clean 1.5ml DNA Low Binding tube

(2) Add 50ul nuclease-free H2O, incubate 1min, RT to increase the yield of eluted DNA.

(3) Centrifuge 1min, 11000g

vi). -20°C stock.

3.3.5. Q-PCR reaction

i). Standards dilution and sample dilution

Use the cDNA extracted from electrophoresis gel above to prepare the Standard dilutions

Point 1: 1µl cDNA + 99µl nuclease-free H2O

Point 2: 10µl Point 1 + 90µl nuclease-free H2O

Point 3 to 10: repeat as for point 2

Vortex for each step, store in ice

ii). Sample cDNA diluted by 1/40, vortex and store in ice or at -20°C for long term storage

iii). Q-PCR reaction (Rotorgene) (Quantifast SYBR Green PCR kit 4000)

Reaction mix:

5µl Qiagen Mix (2x QuantiFast SYBR Green PCR Master Mix)

1µl primer mix (both forward and reverse primer are 5µm)

iv). Add 6µl mix solution in Rotorgene tubes, add 4µl of sample (diluted by 1/40) or diluted standards

v). Rotor-gene 6000 to run Q-PCR.

vi). Analysis

Level of expression of gene X:

Gene X level/Housekeeping gene level

3.4. Protein immunoblot

3.4.1. Protein extraction

On frozen tissue (-80°C) or fresh tissue

Do everything in ice to avoid protein degradation and preserve phosphorylation signal

Lysis buffer: Stock solution (100 ml)

Reagent	Final concentration	Content
Tris(hydroxymethyl) aminomethane 2-Amino-2-hydroxymethyl-1,3-propandiol (Euromedex)	20mM	2ml of 1M Tris-HCl solution pH8
Glycerol (Euromedex)	5%	5 ml
NaCl (Euromedex)	138mM	0.8g
KCl (Sigma)	2.7mM	0.02g
Nonidet P40 (Fluka)	1%	1ml 100% stock solution
Sterile Distilled water		Complete to 100ml

Add the reagent below just before the lysis:

For 10 ml of lysis buffer

Reagent	Final concentration	Content
EDTA (Euromedex)	5 mM	100µl 0,5M stock solution
DTT (Euromedex)	1 mM	10µl 1M solution
phosphostop (Roche)		1 tablet/10ml of lysis buffer

Protease inhibitors (Sigma)		200µl (1 tablet in 2ml of lysis buffer)

Finally the Lysis Buffer Plus is ready to use

Procedure:

(1). Pippet cold Lysis Buffer Plus in eppendorf tube

(2). Add muscle inside and cut into small pieces with scissors, keep in ice

(3). Put dry ice in FastPrep (MP*FastPrep-24) blue rotor to precool

(4). Shake tubes in FastPrep, speed 4m/s, 20s

(5). Tubes in ice, re-shake if necessary

(6). Wait 10-15 min in ice to continue the lysis

(7). Centrifuge 5 min at Vmax (14000rpm) at 4°C

(8). Pipet maximum supernatant and transfer in a new 1.5ml tube

(9). Centrifuge 15 min at Vmax

(10). Pipet supernatant and transfer in a new tube

(11). Store in ice or freeze in liquid nitrogen, then store at -80°C for long term storage

3.4.2. Protein Dosage

Use Biorad protein Dc assay

i). Prepare standard curve with BSA (bovin serum albumin) at 10mg/ml (prepare 20mg in 2ml Lysis Buffer Plus)

Point 1: 5 mg/ml: 15 µl stock + 15 µl Lysis Buffer Plus

Point 2: 2.5 mg/ml: 15 µl Point 1 + 15 µl Lysis Buffer Plus

Point 3: 1.25 mg/ml: 15 µl Point 2 + 15 µl Lysis Buffer Plus

Point 4: 0.625 mg/ml: 15 µl Point 3 + 15 µl Lysis Buffer Plus

Point 5: 0.313 mg/ml: 15 µl Point 4 + 15 µl Lysis Buffer Plus

ii). Prepare 25µl solution A' / well: 25µl of solution A + 0.5µl solution S

In 96-well plate:

Load 5µl of sample /standard or blank (Lysis Buffer Plus) on duplicate

Add 200µl of solution B

Wait about 15min (less than 1 hour) at room temperature.

iii). Spectrophotometer (Versamax Microplate reader)

Read wavelength 750 nm

3.4.3. Western blot

i). Gel

1.5mm glass plate (spacer), 1.5mm comb

(1) Prepare resolving gel

Protein size (kDa)	Gel percentage (%)
4-40	20
12-45	15
10-70	12.5
15-100	10
25-200	8

10ml resolving gel solution:

1) Distilled H2O

2) 30%Acrylamide Mix (commonly 29.2% acrylamide and 0.8% N,

N'-Methylene-bis-acrylamide (MBAM)

3) 1.5M Tris (pH8.8)

4) 10% SDS

Vortex them, add the following two reagents at the last minute to pour gel.

5) 10% APS

6) TEMED (N, N, N', N'-Tetramethylethylenediamine, Sigma)

(2) Prepare stacking gel (3ml/gel):

1) Distilled H2O

2) 30%Acrylamide Mix (commonly 29.2% acrylamide and 0.8% N, N'-Methylene-bis-acrylamide (MBAM)

3) 1.5M Tris (ph6.8)

4) 10% SDS

Vortex them, add the following two reagents at the last minute to pour gel

5) 10% APS

6) TEMED

ii). Load sample

(1) Prepare samples (protein sample: 5x blue=1:4)

5x Blue preparation

Reagent	Content	Final concentration
Tris 1M pH6.8	3ml	300 mM
SDS	1 g	10 %
DTT	0.78 g	500 mM
Glycerol 100%	3 ml	30 %

Bromophenol blue	1 mg	0.01 %
dH2O	About 3.5ml to get buffer final total volume10ml	

(2) Prepare the same amount of samples (15~50ug/gel well), 95degree, 5min

(3) Fill the tank with running buffer Laemmli1x, load 5ul~8ul prestained protein ladder (Euromedex), or 12ul HiMark Prestained High Molecular Weight Protein standard (Invitrogen) for high molecular weight protein

(4) Run electrophoresis in running buffer Laemmli1x at 150V constant for 1 hour to 2 hour by the protein size

iii). Transfer

(1) Transfer buffer: 1) 200ml 100%Ethanol 2) 100ml 10x transfer buffer (Tris base 60.4g, Glycine 288g, fill dH2O to 2L) Add H2O to 1L

(2) PVDF membrane immerses in 100%Ethanol, then immerses in transfer buffer

(3) From Cathode (Negative pole) to Anode (positive pole):

·Sponge

·2 filter papers

·Gel

·PVDF

·2 filter papers

·Sponge

(4) Apply a current of 200mA for 1 gel for 1 hour and 30 minutes to 2 hours by

the protein size at 4°C

iv). Blocking

5% w/v BSA, 1X TBS, 0.1% Tween 20 at 4°C with gentle shaking, overnight

v). Primary antibody

Use the right antibody dilution according to the antibody datasheet at 4°C with gentle shaking, overnight

vi). Washing

The 2nd day, wash membrane with TBS1x/0.1% Tween (1X TBS, 0.1% Tween 20) for 10min x 3 times, swing bed, fast speed

vii). Secondary antibody (dilute by 1/10000 in TBS1x/Tween 0.1%/5% BSA) at room temperature with gentle shaking, 1h

viii). Wash with TBS1x/Tween0.1%, 10min x 3times, at room temperature with shaking

ix). Detection

Method 1: ECL (reagent A: reagent B=1:1) (GE Healthcare Life Sciences), 1ml/membrane, incubation 1min

Method 2: ECL prime (reagent A: reagent B=1:1) (GE Healthcare Life Sciences), 1ml/membrane, incubation 5min

3.4.4. Antibody list

Antibody	**Supplier**	**Reference**
4E-BP1	Cell Signaling (OZYME)	#9452

AKT	Cell Signaling (OZYME)	#9272
Anti-Beclin 1	abcam	ab62472
Anti-Beclin 1 (phospho S234)	abcam	ab183335
Anti-Beclin 1 (phospho S295)	abcam	ab183313
Anti-BiP/GRP78	BD Transduction Lab	610978
Anti-Flag M2 (mTOR)	Sigma	F1804-1MG
Anti-phospho-p38 alpha (Thr180/Tyr182) clone 8.78.8	Millipore	#05-1059
Anti-α-Tubulin	Sigma	T6074
Desmin	Cell Signaling (OZYME)	#5332
Dystrophin	Novocastra	NCL-Dys1
eIf3f	ROCKLAND	600-401-934
eIF4B	Cell Signaling (OZYME)	#3592
eIF4E	Cell Signaling (OZYME)	#9742
eIF4G	Cell Signaling (OZYME)	#2498
FoxO3a	Cell Signaling (OZYME)	#2497
GAPDH	Cell Signaling (OZYME)	#2118
GPT2	ProteinTech	16757-1-AP
GSK-3α/β (0011-A)	Santa Cruz	sc7291
ILK	Millipore	#05-575
IRS1	Cell Signaling (OZYME)	#2382
LAMP-1	Developmental Studies Hybridoma Bank	1D4B
LAMP-2	Santa Cruz	sc-20004
LC3B	Cell Signaling (OZYME)	#2775
MDM2	abcam	ab38618
Mnk2	abcam	ab55145
Mouse Cathepsin D Biotinylated Antibody	R&D Systems Europe	BAF1029
mTOR	Cell Signaling (OZYME)	#2972

p62	Progen	GP62-C
Phospho-p44/42 MAPK (Erk1/2) (Thr202/ Tyr204)	Cell Signaling (OZYME)	#4370
Phospho-4E-BP1 (Thr70)	Cell Signaling (OZYME)	#9455
Phospho-Akt (Ser473)	Cell Signaling (OZYME)	#9271
Phospho-Akt (Thr308)	Cell Signaling (OZYME)	#4056
Phospho-Akt (Thr450)	Cell Signaling (OZYME)	#12178
phospho-Akt1 (ser473)	Cell Signaling (OZYME)	#9018
phospho-Akt2 (ser474)	Cell Signaling (OZYME)	#8599
Phospho-AMPKa (Thr172)	Cell Signaling (OZYME)	#2531
Phospho-eEF2 (Thr56)	Cell Signaling (OZYME)	#2331
Phospho-eIF2a (Ser51)	Cell Signaling (OZYME)	#9721
Phospho-eIF4B (Ser422)	Cell Signaling (OZYME)	#3591
Phospho-eIF4G (Ser1108)	Cell Signaling (OZYME)	#2441
Phospho-FoxO1 (Thr24)/FoxO3a (Thr32)	Cell Signaling (OZYME)	#9464
Phospho-GSK-3α/β (Ser21/9)	Cell Signaling (OZYME)	#9331
Phospho-Mnk1 (Thr197/202)	Cell Signaling (OZYME)	#2111
Phospho-mTOR (Ser2481)	Cell Signaling (OZYME)	#2974
Phospho-S6 (Ser235/236)	Cell Signaling (OZYME)	#2211
Phospho-S6 (Ser240/244)	Cell Signaling (OZYME)	#2215
Phospho-Ulk s317	Cell Signaling (OZYME)	#6887
Phospho-Ulk s757	Cell Signaling (OZYME)	#6888
PYGB/L/M	Santa Cruz	sc-66913
Raptor	Cell Signaling (OZYME)	#4978
Ribosomal Protein L11 (N-17)	Santa Cruz	sc-25931
Rictor	Cell Signaling (OZYME)	#2140
S6	Cell Signaling (OZYME)	#2212
TBK1/NAK	Cell Signaling (OZYME)	#3504
Ubiquitin-protein conjugates,	ENZO	UG9510

rabbit pAb		
Utrophin (n-terminus)	Novocastra	NCL-DRP2

4. LIST OF ABBREVIATIONS

Full Name	Abbreviation
(PtdIns(3,4)P2)	phosphatidylinositol-3,4-trisphosphate
4E-BP1	eukaryotic initiation factor 4E-binding protein
ATG	autophagy-related genes
BMP	Bone morphogenetic protein
Bnip3	BCL2/adenovirus E1B 19kDa interacting protein 3
CMA	chaperone-mediated autophagy
DEPTOR	DEP domain containing mTOR-interacting protein
dTOR	Drosophila TOR
EDL	extensor digitorum longus
eIF4E	eukaryotic initiation factor 4E
FAT	FRAP, ATM, and TRRAP
FKBP12	FK506-binding protein 12 kDa
G6Pase	Glucose 6-phosphatase
GAP	GTPase-activating protein
GβL	G protein beta subunit-like
HEAT	Huntingtin, Elongation factor 3, PR65/A and TOR
IKK	inhibitor of nuclear factor κ-B kinase
IMM	inner mitochondrial membrane
IRS1	insulin receptor substrate 1
LC3	microtubule-associated protein 1 light chain 3
LIR	LC3-interacting region
MHC	myosin heavy chain
miRNA	micro RNA
mLST8	mammalian lethal with sec13 protein 8
mSin1	mammalian stress-activated map kinase-interacting protein 1
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
MVB	multivesicular body
Myf5	myogenic determination factor 5
NBR	neighbour of BRCA1 gene 1
NI7	nuclear import 7
PAS	preautophagosomal structure
PB1	Phox and Bem1p
PDGF	platelet-derived growth factor
PDK1	3-phosphoinositide-dependent protein kinase-1

PEPCK	Phosphoenolpyruvate carboxykinase
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PLD	phospholipase D
Pol I	polymerase I
PRAS	proline-rich Akt substrate 40 kDa
PtdIns(3,4,5)P3	phosphatidylinositol-3,4,5-trisphosphate
Raptor	regulatory associated protein of mTOR
Rheb	Ras homolog enriched in brain
Rictor	rapamycin-insensitive companion of mTOR
ROS	reactive oxygen species
SGK	serum and glucocorticoid induced kinase
TFIIIC	transcription factor III C
TGF-β	Transforming growth factor β
TIF-1A	transcription initiation factor 1A
TSC1	tuberous sclerosis 1
UBA	Ubiquitin Binding-Associated
ULK1	Unc-51-like kinase 1

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