Autophagy is required for cellular survival and for the clearance of damaged proteins and altered organelles. Excessive autophagy activation contributes to muscle loss in different catabolic conditions. However, the function of basal autophagy for homeostasis of skeletal muscle was unknown. To clarify this issue we have generated conditional and inducible knockout mice for the critical gene Atg7, to block autophagy specifically in skeletal muscle. Atg7 null muscles reveal an unexpected phenotype which is characterized by muscle atrophy, weakness and features of myofiber degeneration. Morphological, biochemical and molecular analyses of our autophagy knockout mice show the presence of protein aggregates, abnormal mitochondria, accumulation of membrane bodies, sarcoplasmic reticulum distension, vacuolization, oxidative stress and apoptosis. Moreover, autophagy inhibition does not protect skeletal muscles from atrophy during denervation and fasting, but instead promotes greater muscle loss. In conclusion, autophagy plays a critical role for myofiber maintenance and its activation is crucial to avoid accumulation of toxic proteins and dysfunctional organelles that, in the end, would lead to atrophy and weakness.

Skeletal muscles are the protein reservoir of our body. In fact, skeletal muscles are at least 40% of the body mass and the cytosol of the muscle cells is filled by contractile proteins, which determine the cellular size and morphology. Therefore, protein breakdown can greatly affect muscle mass and performance. The ubiquitin-proteasome and autophagy-lysosome systems control the half-life of 90% of cellular proteins. In atrophying muscle, FoxO transcription factors coordinate the activation of these proteolytic systems. Excessive activation of these pathways leads to important muscle wasting and to cachexia (a loss of body mass that cannot be reversed through nutritional intervention). Despite the well-known detrimental effects of lysosome inhibitors, such as chloroquine, on muscle function, the role of autophagy in skeletal muscle at basal state has never been explored. Moreover defining the role of autophagy inhibition in skeletal muscle homeostasis during catabolic conditions is critical for understanding the mechanisms of muscle loss and for developing new therapies against muscle wasting.

**Autophagy Inhibition Induces Muscle Loss and Myofiber Degeneration**

To clarify these issues we have generated conditional knockout mice for the Atg7 gene to block autophagy specifically in skeletal muscle. The expectation was to preserve muscle mass and eventually to gain more contractile proteins and improve muscle strength. However, the first analysis of autophagy-deficient mice shows a slight reduction of body growth, which starts to differ from the wild type after the first month from birth. The decrease in body weight reflects an important atrophy during denervation and fasting, but instead promotes greater muscle loss. In conclusion, autophagy plays a critical role for myofiber maintenance and its activation is crucial to avoid accumulation of toxic proteins and dysfunctional organelles that, in the end, would lead to atrophy and weakness.
and MuRF1 expression, two muscle-specific atrophy-related ubiquitin ligases, and accumulation of polyubiquitinated proteins in detergent soluble and insoluble protein fractions. Importantly, proteasome function, revealed in vivo by expressing a fluorescent proteasome substrate, is not impaired in Atg7 null muscles. Therefore, accumulation of ubiquitinated proteins is not caused by substantial proteasome inhibition. These data strongly suggest that ubiquitinated proteins are specifically targeted for lysosomal degradation via autophagy. We actually do not know which muscle proteins are ubiquitinated and delivered to lysosomes instead of to the proteasome and whether differences in the covalent binding of the ubiquitins can drive the fate of the protein toward one or the other pathway.

The important muscle atrophy is confirmed by another genetic model of autophagy knockout in which the deletion of the Atg7 gene is acutely induced by tamoxifen injection in adult animals. Three weeks of autophagy inhibition is sufficient to promote muscle atrophy and weakness. In fact physiological measurements of force in the two models of Atg7 knockout animals show that autophagy-deficient muscles are extremely weak when compared to control littermates. Moreover, when the strength is normalized for the muscle mass, an index called specific force, the Atg7 null muscles are still weaker than controls. Thus, Atg7 null muscles not only are smaller than controls, but also show an impairment in force transmission. Ultrastructural studies of autophagy-deficient muscle display accumulation of abnormal mitochondria, which are unusually big and can span from one to the next Z-line, distension of sarcoplasmic reticulum and appearance of concentric membranous structures that assemble between the myofibrils or just beneath the sarcolemma. These abnormalities alter the normal disposition of sarcomeric proteins, curving the contractile proteins, impairing the normal actin-myosin interaction, which explains the extreme weakness. These features are associated with the presence of centrally-nucleated fibers, whose numbers increase with age. Altogether, the features of atrophy, central nuclei, abnormal mitochondria, sarcoplasmic reticulum distension and the presence of membranous inclusions are reminiscent of different myopathies and distrophies whose pathogenetic mechanisms remain unknown.

**Figure 1.** During normal physiological conditions autophagy flux removes damaged mitochondria, altered proteins and unfolded proteins that are prone to aggregate, controls the quality of the folding process in the endoplasmic reticulum, and affects DNA stability. This clearing action of the autophagy system keeps myofiber in a healthy state. When autophagosomes are reduced or abolished, then abnormal mitochondria accumulate, damaged proteins aggregate, protein aggregates are not removed and an unfolded protein response is induced. Together, these abnormalities induce different signaling pathways that suppress protein synthesis and activate protein degradation leading to atrophy. Protein aggregates can contribute to toxicity and to degeneration. Dotted lines depict actions whose molecular mechanisms and role in adult skeletal muscle have yet to be completely defined.
Protein Aggregates, Unfolded Protein Response, Oxidative Stress and Apoptosis are Induced by Autophagy Inhibition in Adult Skeletal Muscle

To understand why Atg7 null muscles are smaller and weaker than controls, we better characterized these muscles at the biochemical level. Indeed, inhibition of autophagy results in accumulation of protein aggregates that are positive for p62 and ubiquitin. Protein aggregates are described in different muscle disorders including sporadic inclusion body myositis, but their role in the pathogenesis of mitochondrial damage and in myopathic phenotype is unclear. Interestingly, the toxic role of p62 aggregates is tissue specific. In fact, suppression of p62 abrogates protein aggregates and normalizes the pathological phenotype of liver-specific Atg7 knockout mice, but has no beneficial effect on neuron specific Atg7 knockout animals. Therefore, it will be crucial to understand the pathogenetic role of protein aggregates in muscle phenotype. The alteration in mitochondria morphology prompted us to analyze the presence of oxidative stress. Inhibition of autophagy causes oxidative stress that is revealed by the presence of higher amounts of carboxylated proteins and by the induction of many genes involved in ROS detoxification such as metallothioneins, thioredoxin reductase and sulfiredoxin. The presence of an oxidative stress can explain why autophagy impairment triggers FoxO activation, expression of the atrophy-related genes atrogin-1 and MuRF1, and muscle atrophy. Another interesting aspect that attracted our attention is the morphological evidence of abnormalities on sarcoplasmic reticulum. We then studied whether an endoplasmic reticulum (ER) stress is present in Atg7 null muscles. Indeed, the ER chaperone Bip/Grp78 is strongly upregulated in autophagy-deficient muscles. This finding strongly suggests a problem in the control of protein folding. Interestingly, the critical initiation factor eIF2α is strongly phosphorylated, and therefore inhibited, in autophagy-deficient muscles. The simultaneous presence of Bip upregulation together with phosphorylation of eIF2α confirms the induction of an unfolded protein response. This is the first evidence of a correlation between inhibition of initiation factors, and muscle weakness and atrophy. Protein aggregates, abnormal mitochondria, oxidative stress and an unfolded proteins response together induce a condition of stress that culminates with activation of apoptotic signals and, finally, leads to myofiber degeneration (Fig. 1).

Atg7 Null Muscles are not Protected from Muscle Loss during Catabolic Conditions

The morphological, biochemical, physiological and molecular findings corroborate a critical role of autophagy to maintain myofiber clear from toxic proteins and dangerous organelles. However it is unclear whether the same function is critical during catabolic conditions or whether autophagy inhibition can protect against muscle loss. Thus, we studied how autophagy-deficient muscles respond to fasting and denervation. Importantly, both catabolic states cause myofiber degeneration and important muscle atrophy. However, the two different atrophic conditions show several dissimilarities at biochemical and morphological levels that suggest a different role of the autophagy system during denervation and fasting. Interestingly, denervated muscles display a severe atrophic phenotype, accumulation of inclusions, enlargement of protein aggregates, the presence of vacuoles and membrane instability.

Together, these findings propose an important role of autophagy during denervation that was unknown or underestimated. The presence of few necrotic fibers prompted us to study whether a defect/alteration of sarcomeric proteins is present in Atg7 null muscles. Analyses of myosin heavy chain composition show no major difference between wild-type and knockout mice. Paradoxically, dystrophin protein is more abundant in autophagy-deficient muscles than controls. Thus, autophagy may regulate directly or indirectly the half-life of dystrophin and associated proteins. This aspect requires more detailed analysis to dissect the substrates of autophagy in skeletal muscle in order to open new perspectives for developing alternative therapeutic strategies for muscular dystrophies. In conclusion, autophagy failure/exhaustion can play a role in the pathogenesis of different myopathies, including aging sarcopenia, characterized by late-onset muscle disorders and by slow progression. The features that should indicate a problem in the autophagy system are: the presence of protein-aggregates, accumulation of abnormal mitochondria and distension of the sarcoplasmic reticulum. This concept may change the actual view that proteolysis is detrimental for muscle economy and may open the field to a new vision that would consider occasional activation of the proteolytic systems as helpful to prevent accumulation of toxic proteins and dangerous dysfunctional organelles.

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