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Proteolysis in illness-associated skeletal muscle atrophy: from pathways to networks

Simon S. Wing¹, Stewart H. Lecker², and R. Thomas Jagoe³

¹Departments of Medicine, McGill University and McGill University Health Centre Research Institute, Montreal, Quebec, Canada

²Department of Medicine, Renal Division, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts, USA

³Departments of Medicine, and Oncology, McGill University Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, Quebec, Canada

Abstract

Improvements in health in the past decades have resulted in increased numbers of the elderly in both developed and developing regions of the world. Advances in therapy have also increased the prevalence of patients with chronic and degenerative diseases. Muscle wasting, a feature of most chronic diseases, is prominent in the elderly and contributes to both morbidity and mortality. A major research goal has been to identify the proteolytic system(s) that is responsible for the degradation of proteins that occurs in muscle atrophy. Findings over the past 20 years have clearly confirmed an important role of the ubiquitin proteasome system in mediating muscle proteolysis, particularly that of myofibrillar proteins. However, recent observations have provided evidence that autophagy, calpains and caspases also contribute to the turnover of muscle proteins in catabolic states, and furthermore, that these diverse proteolytic systems interact with each other at various levels. Importantly, a number of intracellular signaling pathways such as the IGF1/AKT, myostatin/Smad, PGC1, cytokine/NFκB, and AMPK pathways are now known to interact and can regulate some of these proteolytic systems in a coordinated manner. A number of loss of function studies have identified promising therapeutic approaches to the prevention and treatment of wasting. However, additional biomarkers and other approaches to improve early identification of patients who would benefit from such treatment need to be developed. The current data suggests a network of interacting proteolytic and signaling pathways in muscle. Future studies are needed to improve understanding of the nature and control of these interactions and how they work to preserve muscle function under various states of growth and atrophy.

Address for correspondence: Dr. Simon S. Wing, Polypeptide Laboratory, Strathcona Anatomy and Dentistry Bldg, Room W315, 3640 University St., Montreal, Quebec, H3A 2B2 Canada. simon.wing@mcgill.ca; Dr. Stewart H. Lecker, Renal Division, Beth Israel Deaconess Medical Center, FR832, 185 Pilgrim Road, Boston MA 02215 USA. slecker@bidmc.harvard.edu; Dr. R. Thomas Jagoe, Segal Cancer Centre, Room E-541, Lady Davis Institute for Medical Research, Jewish General Hospital, 3755 Côte Ste Catherine, Montreal, Quebec, H3T 1E2 Canada. thomas.jagoe@mcgill.ca.

Referee William E. Mitch, Professor of Medicine and Chief of Nephrology, Baylor College of Medicine, Houston, TX, USA

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Understanding muscle wasting - exploring the roles of proteolytic systems

Over the last 20 years the view of skeletal muscle as an inert peripheral tissue that performs voluntary contractile activity has been revised and replaced with the concept that muscle mass and muscle metabolism are highly dynamic and are modulated by multiple influences. Indeed one of the defining characteristics of skeletal muscle tissue is its plasticity in the face of changing metabolic and contractile demands, which in turn reflects natural growth and aging, as well as the impact of environmental influences including acute and chronic disease.

Both developed and developing countries have seen a sustained rise in the number of older people in their populations and an increase in the prevalence of people suffering from chronic and degenerative diseases such as cancer and chronic heart, lung, renal and rheumatological conditions¹. Aging and these diverse diseases are associated with weight loss and muscle wasting², and, moreover, low muscle mass is associated with increased morbidity and poorer survival³⁻⁶. The prognostic importance of muscle wasting may reflect simply an association with more active or debilitating disease; however there is evidence that muscle atrophy contributes directly to morbidity and mortality in a number of ways. For example, several studies suggest that those with lowest muscle mass are at highest risk of chemotherapy drug toxicity⁷. Also muscle wasting has functional consequences and contributes to reduced mobility, social dependence and specific complications such as falls⁸. In the extreme, muscle atrophy of the respiratory muscles can compromise respiratory function and predispose to pneumonia and death⁹. Thus there is increasing interest in developing ways to identify and limit muscle atrophy, so as to improve function in general and reduce specific symptoms and complications.

As muscle atrophy is an important prognostic sign in many diseases, the study of underlying mechanisms may offer insights into ways to improve outcomes for affected patients. This critical adaptation of muscle tissue is achieved largely through changes affecting the dynamic balance between protein synthesis and proteolysis in multinucleate myofibers, which form the bulk of muscle tissue, rather than by cellular apoptosis and replacement. Not only does the balance of net synthesis and proteolysis in muscle define overall skeletal muscle mass, but also there is differential control of this balance at the level of individual muscles that can allow one muscle to atrophy while another hypertrophies. To achieve this, there is exquisite control of expression of individual muscle proteins at the myofiber level, which in turn establishes the phenotypic features of the muscle tissue including its contractile and metabolic properties. In contrast, different mechanisms come into play in the response to muscle damage, where the proliferation and maturation of latent muscle satellite cells is more important¹⁰.

Because combating muscle atrophy may be a route to deliver significant improvements in outcomes for large numbers of patients, approaches to identify and treat affected individuals must be considered. Recent advances in accurate measurement of total muscle mass using computed tomography (CT) image analysis in some diseases, including cancer¹¹, have become extremely important, but such approaches are still not widely applied and may not be the optimum way to identify the earliest changes in atrophying muscle tissue and thus to guide preventative therapies. As a result one priority is to develop more sophisticated biomarkers of impending pathological changes in muscle mass or phenotype. Candidate biomarkers include expression levels of molecules that reflect key aspects of the balance between protein synthesis and proteolysis within myofibers. To interpret such biomarkers and to rationally design new therapies to inhibit muscle wasting, there is a need for a better understanding of the different pathways underpinning the balance between protein synthesis and proteolysis for bulk protein and specific substrates, and an improved appreciation of the integration of the activities of these pathways.

This review will outline current knowledge on the proteolytic pathways in skeletal muscle, including autophagy, which has re-emerged as a highly regulated route for organelle degradation with specific importance in muscle. Changes in intramuscular protein synthesis and proteolysis pathways are initiated or modulated by a variety of extracellular signals, and the integration of these signaling pathways and their effects on different proteolytic pathways will be discussed. In particular the molecular control points which allow cross-talk between proteolytic and protein synthesis and the evidence of interactions between different proteolytic pathways will be highlighted.

Proteolytic systems in muscle wasting

Ubiquitin proteasome system

General features of the UPS—A large body of evidence indicating an important role for the ubiquitin proteasome system (UPS) in mediating muscle wasting has now accumulated. Ubiquitin, an abundant 8 kDa protein found in all eukaryotic cells, exerts its effects by serving in post-translational modification of other proteins (Figure 1) (reviewed in¹²). In this modification, the C-terminal end of ubiquitin is usually attached to the ϵ -amino group of the side chains of lysine residues within the target protein. Additional ubiquitin moieties can be added to produce a polyubiquitin chain in which each moiety is connected via an isopeptide bond between the C-terminal glycine of the distal ubiquitin and a lysine residue of the more proximal ubiquitin. Ubiquitin has seven lysine residues, and a proteomic analysis in yeast indicates that all seven lysines can be used to form ubiquitin–ubiquitin linkages, though to varying degrees¹³. Use of different lysine residues results in different conformations of the polyubiquitin chain, which are believed to confer distinct functions. Lys48-linked chains are well recognized to target substrates for degradation by the 26S proteasome¹⁴. However, recent evidence indicates that some other chain linkages can also target proteins to the proteasome^{15–17}.

Conjugation of ubiquitin to proteins involves a sequence of reactions¹⁸ catalyzed by three enzymes—ubiquitin activating enzyme (E1)¹⁹, ubiquitin conjugating enzyme (E2, UBC) (reviewed in²⁰) and ubiquitin ligase (E3)²¹. For some substrates, a fourth enzyme, E4,

lengthens short ubiquitin chains²². A single E1 gene appears to exist in somatic cells and supplies activated ubiquitin to a larger family of E2s^{23–24}. Approximately 30 genes encode E2s in mammalian cells (reviewed in²⁰). Each E2 appears to interact with distinct E3s and different E3s recognize distinct substrates. Where multiple E2s can interact with an E3, the different E2s can mediate formation of different types of ubiquitin chain linkages²⁵. Thus, there are multiple pathways of ubiquitin conjugation leading to precise ubiquitination of specific proteins. E3s can be organized into two major classes. One class (~90 human genes) contains a conserved C-terminal HECT domain (Homologous to E6-AP Carboxy-Terminus - named after E6AP, the first E3 described in this class)²⁶ and functions by first accepting ubiquitin from E2 onto a cysteine residue and then conjugating the ubiquitin to the substrate²⁷. The other E3 class (~800 human genes) contains a conserved RING finger motif^{28–29} and functions by binding both substrate and the E2²¹ and activating E2's conjugating activity^{30–31}. Ligases can exist as monomeric proteins or as multi-subunit complexes such as the family of cullin-RING ligases in which the substrate recognition and E2 binding functions are located on distinct subunits of the complex.

As in other post-translational modifications, ubiquitination is reversible and is mediated by ~100 deubiquitinating enzymes present in the mammalian genome (reviewed in³²). Besides reversing the effects of ubiquitination and releasing free ubiquitin for re-use, these enzymes also play an essential role in processing the products encoded by the four ubiquitin genes. Two of these genes encode polyubiquitin (multiple copies of ubiquitin in tandem) while the other two encode ubiquitin-ribosomal protein fusions. Deubiquitinating enzymes can be subclassified into 5 families based on the presence of conserved sequence motifs³². The largest subfamily of deubiquitinating enzymes are the ubiquitin specific proteases (USP).

The 26S proteasome consists of a 20S cylindrical core bound on one or both ends by 19S caps (reviewed in³³). The 20S core is comprised of four stacked rings of seven subunits each and contains in the interior of the rings, the active sites involved in peptide bond hydrolysis. The 19S caps serve to identify substrates for the proteasome and to translocate them to the proteolytic machinery in the 20S core complex. They recognize polyubiquitin chains either directly or via intermediary adaptor proteins which can bind ubiquitinated substrates as well as 19S subunits³⁴. The 19S cap contains deubiquitinating enzyme activities. Rpn11 is an integral subunit of the 19S cap and appears to be able to cleave ubiquitin chains from the substrate³⁵. Associated with the 19S cap are two other deubiquitinating enzymes USP14 and UCH37 (UCH, ubiquitin C-terminal hydrolase)^{36–37}. The 19S cap also contains ATPase activities which are involved in opening the gate to the 20S core as well as unfolding the substrate for insertion into the channel of the 20S complex.

The UPS is activated in atrophying skeletal muscle—One of the earliest findings demonstrating a role for the UPS in muscle wasting was the observation of increased steady state levels of ubiquitinated proteins in atrophying muscle^{38–42}. This suggested that ubiquitination is activated and increases flux through the pathway by increasing availability of ubiquitinated substrates for the proteasome. Supporting this model is increased expression of many components involved in ubiquitin conjugation in atrophying skeletal muscle (Table 1) (reviewed in⁴³). Genes encoding ubiquitin are induced as are genes encoding several

ubiquitin conjugating enzymes and ubiquitin ligases. The functional roles of these, particularly the ubiquitin ligases, will be discussed below.

Increased mRNA expression of many subunits of the 20S proteasome has been observed in atrophying skeletal muscle (Table 1)⁴⁴⁻⁴⁷. Increased expression of some 19S cap subunits have also been reported but is not as consistent a finding^{44,48-50}. Proteasome activity in muscle, as measured by *in vitro* assays, has also been found to be increased in some catabolic conditions⁵¹⁻⁵³. Overall the data are consistent with increased expression and activity of the proteasome in atrophying muscle.

To date only two deubiquitinating enzymes have been reported to be regulated in atrophying skeletal muscle. Both are up-regulated, indicating that they are not involved in mediating the overall increase in levels of ubiquitinated proteins seen in atrophy. *Usp14* mRNA has been demonstrated to be up-regulated in muscle in rodents in response to fasting, uremia, diabetes and cancer⁴⁴. This deubiquitinating enzyme is associated with the proteasome and so increased expression may be important for removing the ubiquitin chain prior to substrate degradation by the proteasome. However, mutational analysis of the yeast ortholog *UBP6* suggest that its deubiquitinating activity may actually serve to protect proteins from degradation,⁵⁴ so further studies are required to delineate more precisely the role of USP14 in muscle.

USP19 has also been shown to be up-regulated at the mRNA level in skeletal muscle in multiple catabolic conditions⁵⁵. Silencing of USP19 in L6 muscle cells results in increased expression of myofibrillar proteins⁵⁶. The finding that this is dependent on increased expression of myogenin implies that USP19 acts on signaling pathways that regulate myogenin and thereby a transcriptional program regulating myofibrillar protein genes. This suggests an additional novel mechanism in which the UPS can mediate muscle wasting - by suppressing the transcription and synthesis of myofibrillar proteins. Recently, mice lacking USP19 expression have been found to lose less muscle mass upon denervation,⁵⁷ indicating that USP19 is indeed a gene required for the full catabolic response.

Inactivation of ubiquitin ligases confers resistance to muscle wasting—

Although many UPS genes are induced in muscle in wasting conditions, only a limited number have been tested by genetic inactivation and found to be essential for mediating atrophy. Because most of these genes encode ubiquitin ligases, the subsequent discussion will focus on these enzymes. Deletion of the MuRF-1 (MuRF, muscle RING finger containing ligase) or MAFbx/Atrogin-1 (muscle atrophy F-box containing protein/Atrogin-1) ubiquitin ligases (58) results in ~35–50% sparing of muscle wasting following two weeks of denervation. These two ligases are muscle specific with expression in skeletal, cardiac and uterine (59) muscle. MuRF-1 is a monomeric ligase with a tripartite RING finger:B box:coiled-coiled motif. MuRF-2 and MuRF-3 are two other similar RING finger ligases with muscle and cardiac specific expression. MAFbx/Atrogin-1 is an F-box containing protein and, like other F-box subunits of ligases, is part of a cullin-RING ligase (CRL) complex (58, 60). The F-box containing subunit is the critical substrate recognition component of the complex. Multiple studies (see Table 1) have demonstrated marked induction (as much as 50-fold) of the mRNAs encoding MuRF-1 and MAFbx/Atrogin-1 in

many conditions of muscle atrophy, usually either simultaneously or just prior to activation of wasting. In denervation or spinal cord isolation forms of disuse atrophy, MAFbx/Atrogin-1 is highly induced within a few days of loss of neural stimulation, but returns to baseline by two weeks⁶¹. MuRF-1 ligase is elevated and remains so as long as 4 weeks.

In vivo and *in vitro* studies have provided insights into possible mechanisms by which these two ligases mediate muscle wasting. Yeast two-hybrid studies using MuRF-1 or MuRF-2 as baits identified eight myofibrillar proteins as interactors and possible substrates— titin, nebulin, nebulin related protein (NRAP), troponin-I, troponin-T, MyLC2 (MyLC, myosin light chain), myotilin and T-cap⁶². Isolation of MuRF-1 from muscle extracts revealed co-binding of a number of thick filament proteins including myosin binding protein C (MyBP-C), MyLC1, MyLC2, myosin heavy chain (MyHC) isoforms 1, 4, 8, and filamin⁶³. Incubation of these isolates with E1, E2, and ubiquitin demonstrated that these proteins can indeed be ubiquitinated. Furthermore MyBP-C, MyLC1 and MyLC2 are selectively lost upon denervation prior to the loss of MyHC and actin; however this does not occur in the mice expressing an inactive form of MuRF-1, which, like the *MuRF-1* knock-out mice, show sparing of loss of muscle mass upon denervation⁶³. Similarly there is less loss of MyHC in MuRF-1 deficient mice upon denervation or dexamethasone treatment, and MyHC can be ubiquitinated *in vitro*⁶⁴. Troponin-I has similarly been shown to be a substrate of MuRF-1 in cardiac muscle⁶⁵. In contrast though, levels of several thin filament proteins are not perturbed by loss of MuRF-1⁶³. These data support a model in which MuRF-1 plays a key role in ubiquitination and degradation of thick filament proteins.

Although MuRF-1 deficient mice have normal muscle structure or function under basal conditions, inactivation of an additional MuRF ligase leads to hypertrophy of both skeletal and cardiac muscle. Most *MuRF-1/MuRF-2* double knock-out mice do not survive beyond the perinatal period. The few that do survive show marked cardiac hypertrophy associated with decreased ventricular function and altered sarcomeric and mitochondrial ultrastructure⁶⁶. *MuRF-1/MuRF-3* double knock-out mice manifest skeletal and cardiac hypertrophy⁶⁷. In these mice, force generation is abnormal in both cardiac and skeletal muscle and is associated with accumulation of both b/slow myosin as well as MyHCIIa myosin. Thus, further loss of MuRF activity actually leads to myopathy with poorer muscle function despite increased mass.

The mechanisms by which the MAFbx/Atrogin-1 ligase might be involved in muscle wasting are less clear. MAFbx/Atrogin-1 was found to interact with myoblast determination protein (MyoD) in a yeast two-hybrid assay, and over-expression of MAFbx/Atrogin-1 in muscle cells results in degradation of MyoD and decreased muscle cell differentiation⁶⁸. MAFbx/Atrogin-1 recognizes a LXXXLL motif in MyoD. A similar motif exists in myogenin and indeed MAFbx/Atrogin-1 can also modulate myogenin levels in C2C12 cells⁶⁹. Finally, MAFbx/Atrogin-1 can target the eukaryotic initiation factor eIF3-f for degradation in C2C12 myotubes, which results in myotube thinning⁷⁰. eIF3-f is a regulatory subunit of eIF3 which plays an important role in initiation of translation. Over-expression of eIF3-f in C2C12 cells increases myotube size, and electroporation of eIF3-f into mouse muscle results in fiber hypertrophy. All of these results are consistent with increased expression of MAFbx/Atrogin-1 causing decreased transcription and translation of muscle

myofibrillar proteins. However, the precise relevance of these substrates *in vivo* remains unknown since they have not yet been carefully examined in *MAFbx/Atrogin-1* knock-out mice.

Other substrates have been identified through studies of the role of MAFbx/Atrogin-1 in the development of cardiac hypertrophy. In cardiomyocytes, calcineurin interacts with calmodulin to promote the activity of NFAT, a transcriptional activator of genes involved in mediating cardiac hypertrophy. MAFbx/Atrogin-1 can target calcineurin for ubiquitination and degradation⁷¹. Accordingly, mice over-expressing this protein in the heart show resistance to hypertrophy induced by IGF1 (insulin-like growth factor 1)/growth hormone. In addition, MAFbx/Atrogin-1 can polyubiquitinate FOXO1 and FOXO3a (FoxO, forkhead box protein), which are transcriptional activators of *MAFbx/Atrogin-1* and *MuRF-1*⁷². However, the ubiquitin chains formed are via Lys63 linkages and do not target the FoxO isoforms for degradation, but rather increase the transcriptional activity of FoxO; this results in increased expression of these two ligases, which, in turn, leads to a feed forward amplification that promotes atrophy or resistance to hypertrophy.

Recent experiments have shown that MAFbx/Atrogin-1 is also expressed in statin-induced muscle injury and suggests similarities between atrophy and myopathy mechanisms⁷³. *MAFbx/Atrogin-1* mRNA levels were significantly higher in muscle biopsies from a set of statin-treated patients with muscle fatigue symptoms than in control biopsy samples (in age-matched patients undergoing knee replacement)⁷³. Similarly, MAFbx/Atrogin-1 induction was found in both cultured muscle cells and in the muscles of whole zebrafish exposed to pharmacologic levels of statins but not in statin-treated muscle cells and fish lacking MAFbx/Atrogin-1⁷³. As well as inhibiting cholesterol synthesis, statins inhibit other biochemical reactions, including isoprenylation of small G-proteins (geranylgeranylation, farnesylation) and formation of coenzyme Q, which is required for oxidative phosphorylation. While it does not appear that statin inhibition of cholesterol or coenzyme Q biosynthesis is responsible for atrogin-1 induction, statin-induced MAFbx/Atrogin-1 expression and muscle damage can be prevented by geranylgeranol⁷⁴. Furthermore, inhibitors of the transfer of geranylgeranyl isoprene units to protein targets cause statin-like muscle damage and MAFbx/Atrogin-1 induction in cultured cells and in fish⁷⁴. These findings support the concept that dysfunction of small GTP-binding proteins lead to statin-induced muscle damage since these molecules require modification by geranylgeranyl moieties for their cellular localization and activity.

Another ligase that may be involved in disuse atrophy is Cbl-b (a ubiquitin ligase). This RING finger containing ligase is involved in the down-regulation of a number of plasma membrane receptor tyrosine kinases (reviewed in⁷⁵) and results in decreased signaling. Atrophy induced by unloading has been observed to induce expression of Cbl-b and association of this ligase with IRS-1 (IRS, insulin receptor substrate), a key downstream signaling mediator of insulin and IGF1⁷⁶. This results in degradation of IRS-1, decreased growth factor signaling and activation of MuRF-1 and MAFbx/Atrogin-1 expression. Transduction of muscle with plasmid expressing Cbl-b induces atrophy. Finally, *Cbl-b* knock-out mice show sparing of muscle wasting and myofiber atrophy upon unloading, a finding that confirms an essential role of Cbl-b in this form of wasting⁷⁶.

All three ligases involved in the N-end rule pathway of protein degradation (Ubr1, Ubr2, Ubr3) are up-regulated in atrophying skeletal muscle (44, 77). These ligases recognize specific amino acids at the N-termini of some of their substrates⁷⁸. Ubr2, which has been characterized in more detail⁷⁷, is induced in muscle of tumor-bearing animals as well as in muscle cells treated with TNF- α (tumor necrosis factor) or IL-6 (IL, interleukin), cytokines that are often increased in cancer. Over-expression of Ubr2 in muscle cells stimulates overall ubiquitin conjugation.

Although ubiquitination can clearly target proteins for degradation by the proteasome, it has been generally difficult to reconstitute degradation of a substrate *in vitro* simply with the enzymes involved in ubiquitination and purified proteasomes. This suggests the existence of factors that mediate delivery of ubiquitinated proteins to the proteasome. Indeed a number of such adaptor proteins have been identified. Members of the RAD23 family contain ubiquitin-like (Ubl) domains that can bind the Rpn10 subunit of the 19S cap of the proteasome as well as UBA (C-terminal ubiquitin-associated) domains that can bind polyubiquitinated proteins³⁴. The adaptor protein, ubiquitin binding protein ZNF216, has been shown to be induced in atrophying muscle⁷⁹. It contains a Zn finger A20 motif that binds polyubiquitin chains. The finding that mice lacking ZNF216 lose approximately 30% less muscle mass upon denervation confirms a role for this protein in muscle wasting.

Together, these studies provide compelling evidence for an essential role of the UPS in muscle wasting. A role in the targeting of thick filament proteins confirms as expected that the UPS is involved in degradation of myofibrillar proteins. Interestingly, recent evidence also suggests a role of the UPS in mediating the fall in protein synthesis seen in most catabolic conditions. The numerous components of the UPS that are involved suggest that the UPS mediates atrophy through multiple mechanisms; this may explain why inactivation of single genes in the UPS typically leads to only partial protection against wasting.

Autophagy

Lysosomal proteolysis—Lysosomes are membrane-bound organelles that contain a variety of hydrolases, including over a dozen proteases whose functions together are able to thoroughly digest proteins. The organellar sequestration of these hydrolases is critical in preventing autodigestion of the cell. Four major mechanisms exist to control the entry of substrates into lysosomes: endocytosis, by which plasma membrane proteins can be internalized into endosomes and trafficked to the lysosome for degradation⁸⁰; a chaperone mediated direct import pathway that involves transport of selected proteins across the lysosomal membrane⁸¹; microautophagy, in which small amounts of cytoplasm are sequestered via budding and internalization of the lysosomal membrane⁸²; and macroautophagy (quantitatively the most important mechanism), a process in which cytoplasmic contents including organelles such as mitochondria and endoplasmic reticulum are sequestered by double membrane vesicular structures termed autophagosomes⁸³. Maturation of these autophagosomes involves fusion with endosomes which then traffic to lysosomes.

Mechanisms of macroautophagy—In the late 1960s and 1970s, formation of autophagosomes was shown to be activated in the liver under catabolic conditions such as glucagon stimulation and insulin or amino acid deficiency^{84–85}. However, the molecular mechanisms by which this process occurred remained essentially unknown until the late 1990s, when Ohsumi and colleagues identified a large number of genes in yeast that were required for autophagy⁸⁶. Surprisingly, molecular analysis of the genes indicated that a number of them were involved in a system of protein conjugation remarkably similar to the ubiquitin system (Figure 2)⁸⁶. In yeast, two ubiquitin-like (Ubl) proteins, ATG12 and ATG8 (ATG, autophagy system protein), are activated by an activating enzyme ATG7 that, like E1, forms a high energy thiolester linkage between the activating enzyme and the carboxy terminus of the ubiquitin-like molecule. The Ubl proteins, ATG12 or ATG8, are then transferred to the cysteine residue of ATG10 or ATG3, respectively, which function analogously to ubiquitin conjugating enzymes (E2). ATG10 conjugates ATG12 to a lysine residue on its substrate ATG5. The ATG12/ATG5 then interacts with ATG16 to form oligomers that associate with membranes; this complex has E3-like activity, supporting the conjugation by ATG3 of ATG8 to membrane bound phosphatidylethanolamine. This conjugation pathway identified in yeast is highly conserved in mammalian cells. However, there are several orthologs of the Ubl protein ATG8 - LC3 (microtubule-associated proteins 1A/1B light chain 3A), GABARAP, GATE16, and conjugation of these onto phosphatidylethanolamine has been observed⁸⁷.

The mechanisms by which the proteins in these conjugation pathways and those encoded by other autophagy genes mediate autophagosome formation are becoming clearer (Figure 2). The autophagosome membranes appear to be derived mainly from the ER⁸⁸ and the outer mitochondrial membrane⁸⁹. Initial nucleation to form a phagophore can be induced by two signaling complexes—one that contains ULK1 kinase/ATG13/FIP200 and another that contains Beclin-1/ATG14/VPS34 class III phosphatidylinositol 3-kinase. This latter complex promotes the formation of PI3P which in turn can recruit the ATG5/ATG12/ATG16 complex to the phagophore membrane. Oligomerization of this complex promotes the formation of a coat and elongation of the autophagosome membrane. The conjugation of LC3 (ATG8) to phosphatidylethanolamine can then promote budding of the membrane and closure of the autophagosome⁹⁰. Such closure results in release of the LC3 from the membrane by the ATG4 deconjugating enzyme. The autophagosome then appears to merge initially with endocytic compartments and ultimately with lysosomes⁹¹.

Macroautophagy in skeletal muscle—Until recently, lysosomal proteolysis was generally viewed as having a minor role in the degradation of skeletal muscle proteins. Studies in isolated muscles from denervated⁹² or fasted rodents⁹³ indicated that although lysosomal proteolysis could be activated in these catabolic conditions, it appeared to make a minor contribution to the increase in proteolysis seen under these conditions. Most of the increase in muscle proteolysis could be ascribed to the UPS^{93–94} and so attention was focused on this pathway. However, more recent studies have clearly implicated a role for macroautophagy in the activation of protein breakdown. Firstly, many autophagy related genes—*LC3*, *Gabarap*, *Bnip3*, *Atg12*, *Atg4*^{95–96} as well as the lysosomal protease, cathepsin L^{44,97}, are induced in muscle in catabolic states. Secondly, suppression of the IGF1/AKT/

FoxO (AKT, serine/threonine kinase involved in insulin/IGF1 signaling) pathway, a major signaling pathway that activates protein breakdown (see below), also activates autophagic pathways. Indeed transduction of constitutively active FoxO3 into muscle myofibers is sufficient to activate autophagosome formation⁹⁵. Transgenic mice that express GFP-LC3 fusion proteins in which activation of macroautophagy is associated with increased GFP signal from autophagosomal membranes has revealed that there is marked increase in autophagosome formation in skeletal muscle upon fasting and that this is more prominent in the fast glycolytic muscles which manifest a larger degree of atrophy⁹⁸. Finally and most importantly, transduction of myofibers by electroporation with plasmids expressing shRNA against LC3 is able to partially block the atrophy induced by FoxO3 expression, indicating that at least part of the muscle wasting occurs via an autophagic mechanism⁹⁵).

However, chronic inactivation of autophagy does not render mice resistant to muscle wasting. In fact, skeletal muscle specific loss of the activating enzyme ATG7 leads to a form of myopathy in which there is myofibrillar disorganization, accumulation of cytoplasmic and sarcomeric aggregates, abnormal mitochondria and myofiber atrophy⁹⁹. Thus, autophagy is required for maintenance of normal muscle fiber function. From studies in other cellular systems (reviewed in¹⁰⁰), it is clear that autophagy plays a variety of homeostatic functions—clearance of protein aggregates, turnover of ribosomes, mitochondria and endoplasmic reticulum, and a role in programmed cell death. Inactivation of autophagy can lead to disorganized sarcomeric structure¹⁰¹, and abnormal accumulation of damaged organelles and protein aggregates, which in turn lead to cellular toxicity. Autophagy also plays a normal role in cellular energy economy. Low levels of amino acids result in decreased mTOR (mammalian target of rapamycin) activity which results in activation of macroautophagy and consequently hydrolysis of proteins and fatty acids to generate ATP in the cell. Therefore, it is not surprising that chronic blockade of this pathway will lead to myofiber damage.

The activation of macroautophagy in muscle wasting is now well established. However, the relative role of autophagy in relation to other proteolytic systems, in particular the UPS, remains to be established. Importantly, the substrates that are targeted by macroautophagy in muscle remain unclear. One established target is mitochondria. Recent studies¹⁰² indicate that fasting, denervation or expression of constitutively active FoxO3 result in altered organization of the mitochondrial network with possibly increased turnover. Furthermore, inhibiting loss of mitochondria from fission can offer partial protection from muscle wasting due to fasting. Finally, inducing a decrease in mitochondrial number and function can induce muscle wasting. Thus, activation of macroautophagy may lead to wasting not just by targeting proteins for degradation, but also by targeting organelles such as mitochondria.

Calpains

The calpain (CAPN) superfamily of Ca²⁺-dependent cytosolic cysteine proteases is encoded by 14 genes¹⁰³ and can be categorized into different subgroups. μ (μ)-calpain (CAPN1) and m-calpain (CAPN2) differ in their sensitivity to Ca²⁺. ‘Typical’ calpains (1, 2, 3, 8a, 9, 11, 12, and 13) have a canonical domain structure on the 80 kDa catalytic subunit and three of these (CAPN1, 2 and 9) can form heterodimers with a smaller 30 kDa regulatory subunit.

The ‘atypical’ calpains (CAPN5, 6, 7, 8b, 10, and 15) have altered domain structure including absent domain IV containing an EF-hand motif. Finally some calpains are ubiquitous (1, 2, 5, 7, 10, 13, and 15), whereas others are more tissue-restricted in expression [3a (striated muscle), 6 (placenta), 8 (stomach), 9 (digestive tract), 11 (testis), and 12 (hair follicle cells)]¹⁰⁴.

Calpains undergo autolytic activation though the unautolysed proteins still retain some proteolytic activity¹⁰⁵. Localization and autolytic activation seem to be important for controlling calpain activity. In some cases binding to membranes or other proteins may stabilize the calpains, limit autolysis and help direct activity to specific substrates, but other evidence suggests membrane binding and specifically exposure to membrane phospholipids reduces the Ca²⁺ activation threshold¹⁰³. In addition, activity of dimeric calpains (CAPN1, 2 and 9) is also specifically inhibited by calpastatin¹⁰⁴, a monomeric protein that has recently been shown to bind calpain in the presence of Ca²⁺ and assumes a structure which allows inhibition of the calpain active site while avoiding proteolytic cleavage itself^{106–107}.

Ubiquitous calpains are modulated by contractile activity—Approximately 7 different calpains are expressed in muscle, and post-mortem calpain mediated proteolysis in livestock muscle is a major determinant of meat tenderness, which is of importance to the food industry and the subject of intense research¹⁰⁸. However, CAPN expression and activity are recognized as an important component of adaptive changes in muscle related to contractile activity. Thus myofibrillar-bound CAPN1 activity is increased early in electrical low frequency stimulation-induced fast-to-slow myofiber type transition, whereas preferential increases in CAPN2 myofibrillar-bound activity are seen in response to direct muscle injury¹⁰⁹. Human gene expression studies showed no change in mRNAs encoding CAPN1, 2 and calpastatin as a result of disuse, but increases in all three genes were observed during remobilizing¹¹⁰. In addition other studies that have demonstrated increased calpain activity in human muscle after eccentric, *i.e.* muscle-damaging, exercise, particularly in the myofibrillar fraction, suggest that calpain-mediated proteolysis is involved in the myofibrillar disruption observed after this type of exercise¹¹¹.

The endogenous calpain inhibitor, calpastatin, which is active against CAPN1 and 2 but not CAPN3 in muscle, is also highly regulated in this tissue. For example, during myoblast fusion calpastatin levels are dramatically reduced, allowing calpain-dependent degradation of certain membrane-associated muscle proteins¹¹². Muscle-specific calpastatin over-expression also reduced muscle fiber atrophy by 30% and inhibited slow-to-fast myosin isoform transition in an unloading model¹¹³. Few studies have looked at the post-translational control of calpastatin activity, but other proteases, such as CASP1 (caspase-1), can degrade or inactivate calpastatin¹¹⁴.

Calpain 3 is muscle-specific and required for muscle homeostasis—CAPN3a (p94) has muscle-specific expression and mutations lead to limb-girdle muscular dystrophy type 2A (LGMD2A). In contrast to data on CAPN1 and 2, mRNA levels for CAPN3 are down-regulated acutely in animal models of muscle wasting such as cancer¹¹⁵, and denervation¹¹⁶, and in human disuse atrophy¹¹⁰. However CAPN3 down-regulation may not be a universal occurrence in atrophying muscle as CAPN3 was not identified as a

consistently regulated atogene in other studies of gene expression in muscle atrophy⁴⁴. In contrast, CAPN3 activation is increased in response to eccentric exercise¹¹⁷.

The mechanism by which CAPN3 mutation leads to dystrophy is still not entirely clear but the dystrophy phenotype is due to reduced CAPN3 activity rather than hyperactivation. CAPN3 binds to the giant titin molecule in the backbone of the myofibril; many studies have suggested it has a wide range of roles including remodeling of contractile proteins, control of apoptosis and modulation of gene expression¹¹⁸.

As described below, there is evidence that the transcription factor NF κ B (nuclear factor kappa-B) is important in mediating muscle atrophy in different conditions, particularly those associated with effects of cytokines such as TNF. NF κ B has pleotropic effects and also promotes transcription of inhibitor of apoptosis proteins that counteract the pro-apoptotic effects of cytokines¹¹⁹. CAPN3 substrates include I κ B α (NF κ B inhibitor alpha) and cardiac ankyrin repeat protein (CARP) which bind NF κ B. Lower levels of active CAPN3 can lead to increased myonuclear apoptosis indirectly by increasing NF κ B cytoplasmic sequestration through binding to I κ B α ¹²⁰ or CARP¹¹⁸ with consequent reduced anti-apoptotic effects of NF κ B.

Inhibiting calpain activity in muscle can reduce muscle wasting—Despite some evidence for a general up-regulation of calpain activity in muscle in disease models such as acute diabetes¹²¹, most of the earlier studies could demonstrate only a small fraction of overall proteolysis as being calpain-dependent. Many of these studies used isolated muscle preparations and compared the effects of inhibitors of different classes of proteases to demonstrate the role of specific proteolytic pathways. These studies were limited by their *in vitro* nature, the lack of specificity of some of the protease inhibitors used, and a failure to appreciate the inter-dependence or cross-talk between different proteolytic pathways. This may explain why, despite prior evidence for little effect of calpain-inhibition on bulk proteolysis, calpain inhibition does appear to have useful functional effects in a variety of clinically-relevant models of muscle wasting. These findings suggest that calpain-inhibition may still be a potentially useful therapeutic approach. Thus over-expression of calpastatin or use of exogenous calpain inhibitors has been shown to ameliorate or reduce: a) sepsis-induced muscle (diaphragm¹²² and heart¹²³) weakness, b) post-MI cardiac muscle remodeling¹²⁴, c) disuse atrophy¹²⁵, and d) hyperglycemia-related cardiac muscle dysfunction¹²⁶.

Calpain proteolysis links with other proteolytic pathways—The success of calpain inhibitors in slowing muscle wasting in many experimental conditions suggests a sequential process of coordinated protein cleavages by different classes of proteases, including calpain-dependent steps. There is now further experimental evidence for such interaction between calpains and other pathways related to proteolysis and protein synthesis in muscle. Thus in studies of proteolysis in rat diaphragms, calpain activation increased UPS proteolysis and inhibited AKT and mTOR phosphorylation, whereas proteasome inhibition prevented the effects of calpain-activation on overall proteolysis¹²⁷. Other studies of interaction between calpains and UPS include those in *CAPN3* knock-out mice which have smaller muscle fibers, slower rate of atrophy in response to unloading, but also markedly impaired recovery

from unloading compared to wild type. CAPN3-deficient animals, which lack the normal marked increase in CAPN3 on reloading, also failed to demonstrate the normal accumulation of high molecular weight ubiquitin-protein conjugates in muscle during reloading. This suggests that CAPN3 has an important role in remodeling during recovery from disuse and that the overall process involves UPS degradation of products of CAPN3 cleavage¹²⁸. Protein cleavage, including calpain-dependent steps upstream of UPS, may also have a role in enhancing proteasome degradation by providing protein fragments with NH₂-terminus amino acid residues that are particularly favored for ubiquitination by the Ubr family of ubiquitin ligases and targeted for proteasomal degradation¹²⁹.

A different type of cross-talk was demonstrated in a recent study in cancer cell lines: I κ B α is a calpain and UPS substrate but proteasome inhibition led to a counter-regulatory increase in calpain-dependent degradation of I κ B α and increased NF κ B signaling, rather than the expected stabilization of I κ B α ¹³⁰. Calpains can also interact with caspase proteases in a variety of different ways to activate^{131–132} or inhibit apoptosis, depending on conditions¹³³.

In summary calpains contribute to proteolytic cleavage of substrates prior to proteasomal degradation in muscle atrophy and have an additional important role in responses to muscle injury and remodeling after disuse. Calpain activity may be especially important to accelerate release of components of large multiprotein structures such as the myofibril. CAPN inhibition shows promise as a means to reduce muscle wasting in certain types of acute illness, but longer term inhibition of CAPN3 may lead to impaired remodeling and muscle dysfunction or dystrophy.

Caspases

In humans, the caspases (CASP) comprise a family of 12 cysteine-dependent proteases which cleave proteins preferentially after aspartic acid residues. Caspases have a variety of roles but as a group are best known for their involvement in programmed cell death pathways (apoptosis). Classical caspase-dependent apoptosis involves initiator caspases (CASP2, 8, 9, 10) which activate effector caspases (CASP 3, 6, 7) through proteolytic cleavage. The effector caspases then cleave a variety of substrates as part of an orchestrated process of cellular destruction¹³⁴. However some caspases (CASP1, 4, 5) are not thought to be directly involved in apoptosis, but rather to play roles in inflammatory cell and cytokine maturation¹³⁵.

In muscle atrophy early experimental results showing a dominant role for the ATP-dependent UPS in accelerated proteolysis suggested little or no role for caspase-dependent proteolysis (reviewed in¹³⁶). However there is evidence from experimental models linking caspase activity with specific types of muscle atrophy, *e.g.* in aging¹³⁷ and in cardiac muscle remodeling after myocardial infarct¹³⁸. Myonuclear apoptosis appears to be a feature of many models of muscle wasting, particularly those involving denervation. Other data in such diseases as chronic obstructive pulmonary disease¹³⁹ and congestive heart failure¹⁴⁰ have suggested that myonuclear apoptosis is also increased in association with muscle atrophy. Whether this limited form of apoptosis of nuclei without cell death reflects a response to muscle atrophy to reduce the number of nuclei and preserve the size of myonuclear domains

within smaller (atrophied) myofibrils, or indicates that apoptosis is a driving pathological process in atrophy in chronic disease is still uncertain.

Caspases may also affect muscle wasting through non-apoptotic pathways. In muscles of diabetic and uremic rats, CASP3 activation is increased and is associated with increased muscle levels of a 14 kDa actin cleavage fragment¹⁴¹. Inhibiting caspase activity in isolated muscles and cultured muscle cells reduces production of the 14 kDa fragment and inhibits the accelerated proteolysis due to diabetes. Moreover insulin and IGF1 inhibit the production of the 14 kDa fragment through a PI3K-dependent mechanism (PI3K, phosphoinositide-3-kinase), suggesting that these hormones have a direct effect on CASP3 in addition to their inhibition of the UPS via FoxO as described below¹⁴¹. The nature of the activation of UPS proteolysis by CASP3 in catabolic conditions has been studied further; in addition to potentially cleaving and releasing substrates for the UPS, there is evidence that in muscle tissue CASP3 cleavage of two ATPase-containing subunits on the 19S proteasome regulatory particle [PSMC1 (RPT2) and PSMC5 (RPT6)] amplifies proteasome activity¹⁴².

Genetic inactivation of CASP3 in mice results in partial protection from denervation-induced muscle atrophy. However, CASP3-deficient mice have similar levels of the 14 kDa actin fragment and activation of the UPS, suggesting that the initial actin degradation step may be performed by other caspases or indeed other proteases. However pro-apoptotic signaling and myonuclear apoptosis was reduced after denervation in muscle from *CASP3* knock-out animals. This suggests the primary role for CASP3 in denervation atrophy may be in promoting apoptosis rather than in actin cleavage¹⁴³. The functional impact of CASP3 activity was further demonstrated by CASP3 knock-down in rat heart muscle, which led to inhibition of sepsis-induced changes in contractile properties, an increase in selected contractile proteins, and a reduction in markers of DNA damage¹⁴⁴.

The precise roles and targets of caspase activation are unclear. Like calpains, caspases appear to work upstream of the UPS to facilitate the release of myofibrillar proteins, and in some studies there is evidence of parallel activation of both caspases and calpains contributing to muscle weakness and wasting¹²². In addition, caspase cleavage promotes atrophy by initiating myonuclear apoptosis. Caspases interact with other proteolytic systems including calpains as described above, and can also enhance proteasome activity¹⁴². Furthermore there is now evidence from studies in other cell types suggesting that caspase activity may have a role in actively directing a cell away from autophagy in favor of apoptosis through caspase cleavage of Beclin-1¹⁴⁵.

Major signaling pathways regulating muscle proteolysis

The IGF1/PI3K/AKT/FoxO pathway

Great strides have been made in the last decade in uncovering the intracellular signaling pathways that control muscle fiber size. A central pathway involves IGF1 and insulin, which are most important in promoting net protein accumulation and resulting growth/hypertrophy of adult muscle fibers. In skeletal muscle, the binding of IGF1 or insulin to the IGF1 receptor activates two major signaling pathways: the Ras-Raf-MEK-ERK pathway and the PI3K/AKT pathway. The Ras-Raf-MEK-ERK pathway affects fiber type composition but

has little effect on muscle fiber size¹⁴⁶, while activation of the PI3K/AKT pathway induces muscle hypertrophy by increasing translation (protein synthesis) through phosphorylation/activation of GSK (glycogen synthase kinase) and mTOR kinases^{58,147}. In addition to its activation through AKT phosphorylation, mTOR is also controlled by nutrient supply¹⁴⁸. During fasting or nutrient insufficiency, low levels of amino acids lead directly to inhibition of mTOR. This inhibition is augmented by the fall in insulin and IGF1 in these states, which decrease nutrient transport into muscle, further amplifying the reduction in mTOR activity. mTOR can thus integrate signals from the IGF1/PI3K/AKT pathway with information about the cell's nutritional status, both of which then influence mTOR activity. The latter is mediated by two multi-subunit mTOR complexes, the major one being mTORC1, in which mTOR complexes with raptor, and the less important one, mTORC2, in which mTOR complexes with rictor¹⁴⁹. The TORC1 complex is primarily the controller of protein synthesis through its phosphorylation of S6 kinase, while mTORC2 is postulated to regulate cytoskeletal organization. Interestingly, skeletal muscle lacking raptor (but not rictor) develops a dystrophic phenotype with smaller, damaged fibers¹⁵⁰. This phenotype seems to stem from a complex combination of dysregulated pathways downstream of mTORC1 that includes decreased translation resulting from reduced S6K activity, suppression of PGC-1 α (PGC-1, peroxisome proliferator-activated receptor gamma coactivator), and loss of feedback inhibition of IRS-1 that leads to hyperphosphorylation of AKT.

Several recent findings suggest that decreased activity of the IGF1/PI3K/AKT signaling pathway leads to muscle atrophy. Inhibition of PI3K and expression of dominant negative (inhibitory) forms of AKT reduce myotube diameter in culture¹⁵¹, and muscles from mice lacking AKT1 or AKT2 are smaller than their wild type littermates¹³. Conversely, stimulation of AKT can prevent denervation atrophy^{152–153}. As noted above, much of the IGF1-stimulated growth in muscle results from an increase in protein synthesis. Reduced AKT activity when IGF1 is low presumably causes decreases in translation that contribute to the loss of mass seen in muscle atrophy. However there is now evidence that strongly suggests the IGF1/PI3K/AKT pathway not only promotes protein synthesis but also induces reciprocal changes in protein degradation. This is demonstrated in part by evidence from experiments in tissue culture showing insulin and IGF1 inhibition of glucocorticoid-induced activation of proteolysis¹⁵⁴. These experiments strongly suggest that the IGF1/PI3K/AKT pathway, in addition to affecting translation mechanisms, suppresses protein degradation.

One downstream target of the IGF1/PI3K/AKT pathway that could mediate these IGF1 effects on protein degradation is the FoxO class of transcription factors, a subfamily of the large group of forkhead transcription factors. Mammalian cells contain three members of this family, FoxO1 (FKHR), FoxO3 (FHKRL1), and FoxO4 (AFX)¹⁵⁵. AKT blocks the function of all three transcription factors by phosphorylation of three conserved residues, leading to their sequestration in the cytoplasm away from target genes¹⁵⁶. Dephosphorylation of FoxO factors leads to nuclear entry and transcription of downstream genes that promote growth-suppression or apoptosis¹⁵⁷. FoxO gene expression is also tightly regulated. Fasting and glucocorticoids induce the expression of FoxO factors in muscle, while refeeding suppresses FoxO transcription¹⁵⁸. In seminal papers by both the Goldberg and the Glass groups^{159–160}, manipulation of FoxO1 and FoxO3 levels was shown to have dramatic effects on muscle fiber size: expression of constitutively active forms led to

dramatic atrophy while dominant negative (inhibitory) forms prevented denervation-induced atrophy. Dephosphorylation/activation of FoxO leads to induction of a conserved set of atrophy-specific genes (atrogenes) including the muscle-specific ubiquitin ligases, MAFbx/Atrogin-1 and MuRF-1 as well as the FoxO factors themselves, and promotes increased rates of protein degradation. Recent data has further demonstrated that activation of FoxO factors also promotes autophagy in skeletal muscle^{95–96}. The parallel activation of ubiquitin-mediated and autophagic protein degradation may promote the concerted dismantling of muscle during atrophic states in which different proteolytic pathways target different cellular constituents; the UPS for soluble and myofibrillar proteins and the autophagy system for organelles such as mitochondria. Thus, suppression of IGF1/PI3K/AKT signaling, leading to reduced levels of AKT and FoxO phosphorylation, FoxO nuclear translocation and increased FoxO-dependent transcription, appears to be the dominant atrophy signaling pathway in skeletal muscle.

The PGC-1 α transcriptional coactivator suppresses FoxO transcription

The PGC-1 family of transcriptional coactivators has been recently identified as promoters of the transcriptional program for mitochondrial biogenesis and oxidative metabolism that is critical to the maintenance of energy homeostasis in muscle^{161–162}. Transgenic expression of PGC-1 α in fast-twitch, glycolytic muscles transforms the type IIb muscle fibers into a more oxidative, mitochondria-rich phenotype^{163–164}. PGC-1 α therefore appears to be an important mediator in regulating mitochondrial metabolic properties in skeletal muscle and is a prime candidate as a target regulator. Interestingly, it has been found that PGC-1 α mRNA falls dramatically in various types of atrophying muscle¹⁶⁵. Furthermore, in transgenic mice over-expressing PGC-1 α , denervation and fasting cause a much smaller decrease in muscle fiber diameter and a smaller induction of MAFbx/Atrogin-1 than in control mice¹⁶⁵. Importantly, when the levels of PGC-1 α are maintained artificially by electroporation of PGC-1 α cDNA into adult muscle fibers, muscles are protected from the atrophy induced by denervation, fasting, or expression of FoxO3. This protective effect can explain how exercise, which induces expression of PGC-1 α , maintains muscle mass and retards atrophy, even in the face of circulating catabolic factors¹⁶⁶. Furthermore, recent findings using chromatin immunoprecipitation (ChIP) assays indicate that PGC-1 α directly or indirectly inhibits FoxO-dependent binding to and transcription of the atrogin-1 promoter¹⁶⁵. Mechanistically, an alternative explanation for the protection afforded by PGC-1 α is that it may lead to expression of an inhibitor of FoxO transcription. PGC-1 α therefore is a prime anti-atrophy target, the activity of which might be enhanced to protect against muscle atrophy through its ability to augment mitochondrial number and function.

AMPK also acts on the IGF1 signaling pathway and promotes atrophy signaling

Another intracellular signaling molecule that seems, at least in part, to feed into the IGF1/AKT/FoxO pathway is AMPK (AMP activated kinase). AMPK phosphorylation and activation are triggered by falling energy content (increased AMP/ATP ratio)¹⁶⁷, a state often present in catabolic conditions in which muscles atrophy. Recent studies have shown that an increase in muscle p-AMPK stimulates MAFbx/Atrogin-1 expression in mice and in cultured muscle cells^{102,168}. In mice treated with the AMPK agonist, AICAR, the agonist raises phospho-AMPK and increases MAFbx/Atrogin-1 expression, which is blocked by the

AMPK inhibitor, Compound C. AMPK activation promotes increased FoxO expression as well as its dephosphorylation and activation; this provides a mechanism by which AMPK leads to atrogenic expression^{102,169}. This pathway provides a potential means to link mitochondrial dysfunction and its consequent decrease in ATP production with atrophy development and to further amplify atrophy-promoting signaling pathways. Energy insufficiency can lead to FoxO activation directly via increased AMPK activity, and in addition, FoxO-mediated autophagy promotes mitochondrial loss and further energy imbalance through increased dependence on glycolytic as opposed to oxidative metabolism.

NF κ B and p38: other signaling pathways that act in parallel to the IGF1 signaling pathway

The secreted cytokine TNF¹⁷⁰ is well known as a cachectic factor that, along with the related factor TWEAK (TNF-related weak inducer of apoptosis)¹⁷¹ promotes muscle atrophy through multiple mechanisms¹⁷². Fundamentally, TNF binding to its receptor leads to activation of NF κ B and its downstream targets¹⁷³. Recent studies have demonstrated the role of NF κ B in cytokine-induced loss of myofibrillar proteins such as myosin¹⁷⁴. Since NF κ B is also activated by sepsis¹⁷⁵ and disuse¹⁷⁶, it likely plays a general role in development of muscle atrophy. Indeed, in culture models, blocking NF κ B lowers rates of protein degradation,¹⁷⁷ and conversely TNF treatment can attenuate insulin-stimulated protein synthesis¹⁷⁸. TNF-mediated NF κ B activation can also inhibit myocyte differentiation through the suppression of myogenic transcription factors like MyoD^{174,179–180}. This added effect of NF κ B on myogenesis may suppress the ability of muscle precursor, or satellite, cells to be recruited into atrophying muscle, amplifying the loss of muscle mass¹⁷⁹. Recent studies have demonstrated that the cytokine, TWEAK, has even more robust effects in causing muscle wasting than its related cousin, TNF¹⁷¹. In cultured cells or mice treated with TWEAK and in muscle-specific TWEAK transgenic animals, dramatic reductions in fiber size were seen. These effects may be due to the concomitant stimulation of multiple processes contributing to atrophy.

Important recent studies in mice highlight the importance of NF κ B activation in promoting muscle atrophy. Using transgenic mice that constitutively activate NF κ B (MIKK mice, for muscle specific expression of IKK, which phosphorylates I κ B α and leads to activation of NF κ B) or repress NF κ B (MISR mice, for muscle specific “super-repressor”, which expresses a dominant negative form of I κ B that prevents NF κ B activation), Shoelson and coworkers demonstrated corresponding atrophied and hypertrophied muscles respectively¹⁸¹. Interestingly, the expression of the ubiquitin-ligase MuRF-1, but not MAFbx/Atrogin-1, was up-regulated in the MIKK skeletal muscle, a finding that provided the first functional dissociation of the signals that induce MuRF-1 and MAFbx/Atrogin-1¹⁸¹. The fact that MAFbx/Atrogin-1 is up-regulated in essentially all types of atrophying muscle is strong support for activation of a second parallel pathway, distinct from NF κ B, (*e.g.* FoxO activation), during atrophy. Further experiments showed that NF κ B is also involved in mediating atrophy and remodeling due to unloading; *NF κ B1* knock-out animals demonstrated less atrophy following disuse (unloading) as well as a blockade of the fiber-type switching usually associated with atrophying muscle¹⁸².

In addition to activating NF κ B, TNF can also mediate signaling through p38. Interestingly, in cultured muscle cells, TNF acts to induce MAFbx/Atrogin-1 expression, and this up-regulation can be blocked by pharmacologic inhibitors of p38¹⁸³. Whether or not p38 has any effect on FoxO or MuRF-1 induction or on the NF κ B pathway is not known.

Myostatin

Myostatin is a member of the TGF α family that may also promote atrophy. Loss of function mutations in myostatin lead to skeletal muscle hypertrophy in multiple species including humans^{184–187}, whereas infusion of cells expressing myostatin into adult mice leads to muscle wasting¹⁸⁸. Forced over-expression of myostatin in skeletal muscle, however, does not show quite the same robust effects as the genetic mutants and infusion experiments above. In muscle-specific myostatin expressing transgenic mice, only males have reduced muscle and myofiber size¹⁸⁹ and in experiments in which myostatin was electroporated into skeletal muscle, only 10–20% reduction in fiber cross-sectional area was seen¹⁹⁰. Despite this, anti-myostatin peptibodies are capable of blocking muscle atrophy in mice with chronic kidney disease¹⁹¹. Myostatin binds to the high affinity activin type 2 receptor (ActRIIB) and oligomerizes with the co-receptors ALK4 or ALK5, which mediate its signaling through phosphorylation of Smad2 and 3 (Smad, mothers against decapentaplegic homolog)^{185,192}. Expression of dominant negative ActRIIB in transgenic mice results in skeletal muscle hypertrophy and suggests a role of the ActRIIB pathway in limiting muscle growth¹⁸⁵. Exciting new experiments demonstrate that antagonizing this receptor can dramatically prevent cancer-induced cachexia¹⁹³. Administration of an ActRIIB decoy receptor (sActRIIB) in multiple cancer cachexia models prevents further wasting, but also fully reverses skeletal muscle loss and atrophy of the heart, thereby dramatically prolonging survival of the tumor-bearing animals. The potent effects of the ActRIIB decoy may be augmented by inhibition of other circulating ligands like activin that bind to and stimulate ActRIIB. Thus, the ActRIIB pathway inhibition appears to have therapeutic potential for treating various types of muscle wasting, especially cancer cachexia.

Integrated control of signaling pathways that promote atrophy

As described above, in atrophying muscle, suppression of IGF1 signaling and activation of AMPK, NF κ B and Smad signaling through ActRIIB all contribute to the loss of muscle mass (Figure 3). It appears quite likely that these pathways act in concert with cross-talk between them. For example, AMPK activation has been shown to suppress, through dephosphorylation, the FoxO transcription factors¹⁰². The cytokine TWEAK both activates NF κ B and suppresses IGF1 signaling¹⁷¹, and signaling through ActRIIB may also contribute to reduced activity of mTOR¹⁹². Recent experiments also demonstrate that FoxO activation leads to IRS-2-mediated stimulation of the MEK/ERK signaling pathway and promotes expression of poly-ubiquitin¹⁹⁴. Yet another example of cross-talk between signaling pathways is a recent study that shows glucocorticoid-induced glucocorticoid receptor sequestration of PI3K promoting reduction of IGF1 signaling in acutely diabetic mice¹⁹⁵. Finally, the muscle wasting associated with inflammation involves IL-6 and serum amyloid A protein-induced impairment of IGF1 signaling¹⁹⁶.

The contribution of multiple signaling pathways to overall control of muscle mass is also consistent with the requirement of muscle to be able to respond to multiple extra-cellular signals. These include circulating factors (*e.g.* insulin, IGF1, thyroxine, corticosteroids and pro-inflammatory cytokines), paracrine molecules (*e.g.* mechano-growth factor (MGF) and myostatin), the type and concentration of metabolic substrates (*e.g.* branched-chain amino acids), environmental conditions (*e.g.* oxygen, acidosis), neuronal stimulation frequency or pattern and tension or workload. The integration of these multiple inputs and signaling pathways in muscle defines muscle mass, energy and protein metabolism, and fiber phenotype. In conditions leading to muscle atrophy, the balance among these different factors is disturbed, and changes typically occur in multiple components of this complex network with stimulation or inhibition of overlapping intracellular signaling pathways. In this way it is clear that net muscle wasting will show wide variation among individuals with the same disease, due to the combined effects of the many factors which impact on muscle mass and function.

Biomarkers and barriers to their use

The improved molecular understanding of the process of muscle wasting has the potential to produce better biomarkers for diagnosis and to gauge treatment. Clinically, the release of muscle enzymes like creatine kinase, aldolase and aspartate aminotransferase into the bloodstream is commonly used to diagnose muscle damage (myopathy) as opposed to atrophy or wasting, in which elevated concentrations of these substances are typically not found. In fact circulating factors that correlate with atrophy are lacking. Reduced concentrations of IGF1 might be expected in atrophy conditions, but since most IGF1 is produced locally, levels in the circulation correlate poorly with disease. Likewise, cytokines such as IL-1 and IL-16 and other factors such as TNF- α and myostatin have potential as biomarkers, but to date have not been well correlated with atrophying muscle. On the other hand, gene expression data has been used successfully, at least in research studies, to define the atrophy process. Muscle-specific ubiquitin protein ligases such as MAFbx/Atrogin-1 and MuRF-1 that are dramatically up-regulated as atrophy occurs have been used in a large number of animal and human¹⁹⁷ studies as sensitive markers of atrophy. The major barrier to their more generalized clinical use is the invasiveness of the muscle biopsy that is required to obtain the material necessary to measure gene expression. To date, attempts to identify trace levels of MAFbx/Atrogin-1 or MuRF-1 in the circulation of patients or animals with atrophying muscle have been unsuccessful (S.L., unpublished). One additional potential biomarker mentioned above is the caspase-derived 14 kDa actin fragment identified from atrophying muscles by Mitch and coworkers¹⁴¹. Though this fragment has recently been observed in atrophying muscles from hemodialysis and burn patients¹⁹⁸, its broad use suffers from the same pitfalls as the gene expression markers above, namely, that it also requires a muscle biopsy to be performed.

Current potential drug targets and strategies

It is clear from the above that we now have a more comprehensive understanding of muscle proteolytic pathways, their functions and their regulation. Loss of function studies have identified a number of genes within these pathways whose inactivation reduces muscle

wasting (Table 2). Because most of these are enzymes, the development of pharmacological inhibitors to them is an attractive and feasible approach towards the prevention and treatment of muscle atrophy. These targets presently include the ubiquitin ligases MuRF-1, MAFbx/Atrogin-1, Cbl-b, the deubiquitinating enzyme USP19, and CASP3. MuRF-1 and MAFbx/Atrogin-1 are particularly interesting because their expression is restricted to striated muscle tissues and they show very high induction of expression in a broad array of catabolic conditions that include local disuse as well as systemic diseases such as cancer¹⁹⁷. Thus, even modest inhibition of these muscle-specific E3 ligases may be sufficient to have a significant protective effect and their tissue-specific expression may limit the risk of systemic toxicity. In fact, strong and prolonged inhibition of these enzymes may result in myopathy, a suggestion that is supported by the observation of muscle damage and decreased function upon genetic inactivation of two of the three MuRF ligases⁶⁶⁻⁶⁷.

Other candidates as drug targets include genes with more general tissue expression. These include Cbl-b and USP19, which also regulate receptor tyrosine kinase trafficking²⁸ and cell cycle¹⁹⁹, respectively, but the general lack of a major phenotype in mouse knock-outs of those genes indicate that they may still be viable drug targets. Whole body inactivation of CASP3, an effector caspase in apoptosis, leads to a high proportion of embryonic death, a finding which makes this a less attractive candidate for inhibition. As discussed above, acute inhibition of autophagy can also inhibit muscle wasting, but chronic inhibition such as occurs in gene inactivation studies of an important autophagy gene leads to severe myopathy. Furthermore inhibition of CAPN1 and 2 through calpastatin over-expression also ameliorates muscle atrophy, so calpains may be useful therapeutic targets^{113,125}. Despite concerns regarding inhibition of broadly expressed drug targets with multiple cellular functions, there is evidence that inhibition of such pathways in adults or mature tissues may prove less toxic than expected. For example, given the universal importance of the UPS for protein turnover and regulation of multiple cellular pathways in all eukaryotic cells, it is surprising that the proteasome inhibitor Velcade (Bortezomib), the only drug inhibitor of the UPS that is approved for clinical use (in the treatment of multiple myeloma), is well tolerated. However, sadly, to date there is no evidence that Velcade has beneficial effects on human muscle wasting.

An inhibitor of any of these targets could possibly be effective if it possessed a pharmacokinetic profile that lowered its toxicity and/or was administered for only short periods of time when catabolic stimuli were maximal (*e.g.* episode of sepsis, short periods of fasting, peri-operatively). Such limited treatment could avoid toxicity arising from inhibition of other important functions of the targeted enzymes (*e.g.* muscle recovery, remodeling, or quality control of proteins and organelles).

The discussion in this review also suggests strongly that drug manipulation of signaling pathways such as the IGF1/AKT, cytokine-NFκB, and myostatin/activin pathways may be a feasible approach to the treatment of muscle wasting. Many of these pathways modulate both protein degradation and protein synthesis in a reciprocal manner to mediate changes in muscle size. As a result, targeting signaling pathways may be more effective than attempts at inhibiting proteolysis alone. However, once again signaling pathways which modulate overall protein synthesis and proteolysis are also broadly utilized in most cell types and, as

discussed above, targeting such fundamental processes to control muscle atrophy may be limited by systemic toxicity. In the case of the IGF1/AKT pathway, one would be concerned about the stimulatory effects of the administration of growth hormone, IGF1 or activators of downstream signaling molecules on growth in other tissues or in cancerous cells. Finally from a drug design perspective, identifying specific activators of enzymes is much more challenging than identifying specific inhibitors. However, the recent report of dramatic protection against cancer cachexia in mice by administration of a decoy receptor for activins demonstrates that targeting signaling pathways is indeed promising and that systemic toxicities may be avoided when administered for a limited period of time.

Conclusions - from pathways to networks and beyond

The last 20 years have witnessed major advances in our understanding of proteolysis in general as well as in skeletal muscle in particular. The application of gain of function and loss of function approaches to evaluating the roles of specific genes in proteolytic systems has clearly demonstrated that the UPS and the autophagic/lysosomal systems play important roles in mediating muscle protein catabolism. More limited studies suggest roles for calcium-dependent proteases and caspases, but further studies are required to better define the mechanisms by which these proteolytic systems may exert their effects on muscle protein balance. Nonetheless, the involvement of multiple proteolytic systems is concordant with the notion that as the myofiber atrophies, multiple cellular components (*e.g.* myofibrillar proteins, mitochondria, sarcoplasmic reticulum, nuclei) need to be degraded. Since all proteolytic systems have some degree of substrate specificity, a requirement for the coordinate involvement of multiple proteolytic systems is not surprising. Interestingly, as described in this review, the proteolytic systems interact with each other directly—*e.g.* the ubiquitination of some damaged proteins can lead to binding to the p62 adaptor protein and targeting to the autophagy pathway; calpains may cleave a subunit of the proteasome and thereby activate the enzyme.

The recent advances in our understanding of the signaling pathways that modulate skeletal muscle fiber growth and atrophy also support the concept of coordinate regulation of multiple proteolytic systems. As described in this review, the IGF1/AKT/FoxO pathway modulates in a coordinate fashion both the ubiquitin proteasome system and the autophagic pathway. Other pathways such as myostatin /activin or AMPK signaling can exert at least some of its stimulatory effects on protein degradation via inhibition of AKT and activation of FoxO. PGC-1 α can antagonize FoxO action at the promoters of its target genes, and recent evidence indicates that stress activated MAP kinases can stimulate export of FoxO from the nucleus. The ability of diverse signaling pathways to cross-talk at various levels between the initial signal and its target proteolytic system indicates clearly that proteolytic systems function within complex networks rather than as independent pathways.

The analysis of the functions of such signaling pathways as the AKT pathway also suggests a coordinate control of protein synthesis and protein degradation. Intriguingly, though, recent evidence suggests that coordinate control of protein synthesis and degradation also occurs through more intricate mechanisms in which induction of the MAFbx/Atrogin-1

ligase can lead to degradation of myoD, myogenin and eIF3-f that result in decreased rates of transcription and translation of important muscle cell proteins.

Despite the progress made in the last 20 years, a number of deficits in our understanding remains: (a) The structure of the network that is becoming apparent needs to be characterized more completely. In particular, more of the key nodes and the signalling molecules in the network need to be identified; (b) The functions of each proteolytic system need to be better characterized—*e.g.* what are all of the substrates (or organelles in the case of autophagy) of each proteolytic system? (c) How is the degradation of the various myofiber components regulated to maintain correct proportions of each of the components? (d) How important is myofiber loss in atrophy as well as new myofiber formation, or myoblast fusion to existing fibers, in hypertrophy or recovery from atrophy? (e) What are the contributions of the various proteolytic systems and protein synthesis to overall protein balance at various time points in the muscle wasting process? What are the implications of this with respect to the usefulness of therapies targeted to specific proteolytic systems? (f) Can longer term treatments to limit or prevent muscle atrophy be devised which enhance muscle function and preserve muscle tissue physiological plasticity? Although many questions remain, advances in recent years offer significant hope that effective treatment to prevent or reverse muscle wasting and sarcopenia can be found.

Abbreviations

ActRIIB	activin type 2 receptor
AKT	serine/threonine kinase involved in insulin/IGF1 signaling
AMPK	AMP activated kinase
Atg or ATG	autophagy system gene or protein
CASP	caspase
CAPN	calpain, Ca ²⁺ -dependent cytosolic cysteine protease
Cbl-b	a ubiquitin ligase, functions as a negative regulator of many signaling pathways downstream of receptor tyrosine kinases
CRL	cullin-RING ligase
CT	computed tomography
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme, UBC
E3	ubiquitin protein ligase
E4	ubiquitin chain elongating enzyme
eIF3	eukaryotic initiation factor for translation

FoxO	forkhead box protein, transcriptional activator
GSK	glycogen synthase kinase
IGF1	insulin-like growth factor 1
IκBα	NF κ B inhibitor alpha
IL	interleukin
IRS	insulin receptor substrate
LC 3	microtubule-associated proteins 1A/1B light chain 3A, protein involved in elongation of autophagosome
MAFbx/Atrogin-1	muscle atrophy F-box containing protein/Atrogin-1, ubiquitin protein ligase
mTOR	mammalian target of rapamycin
MuRF	muscle RING finger containing ligase
MyBP-C	myosin binding protein C
MyHC	myosin heavy chain
MyLC	myosin light chain
MyoD	myoblast determination protein, muscle differentiation factor
NFκB	nuclear factor kappa-B, pleiotropic transcription factor involved in many biological processes including inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis
PGC-1	peroxisome proliferator-activated receptor gamma coactivator, stimulator of transcription factors and nuclear receptors activities
PI3K	phosphoinositide-3-kinase
Smad	mothers against decapentaplegic homolog, intracellular signal transducer and transcriptional modulator activated by TGF-beta (transforming growth factor) and activin type 1 receptor kinases
TNF	tumor necrosis factor, inflammatory cytokine
TWEAK	TNF-related weak inducer of apoptosis, inflammatory cytokine
Ubl	ubiquitin-like

UCH	ubiquitin C-terminal hydrolase
UPS	ubiquitin proteasome system
USP	ubiquitin specific protease

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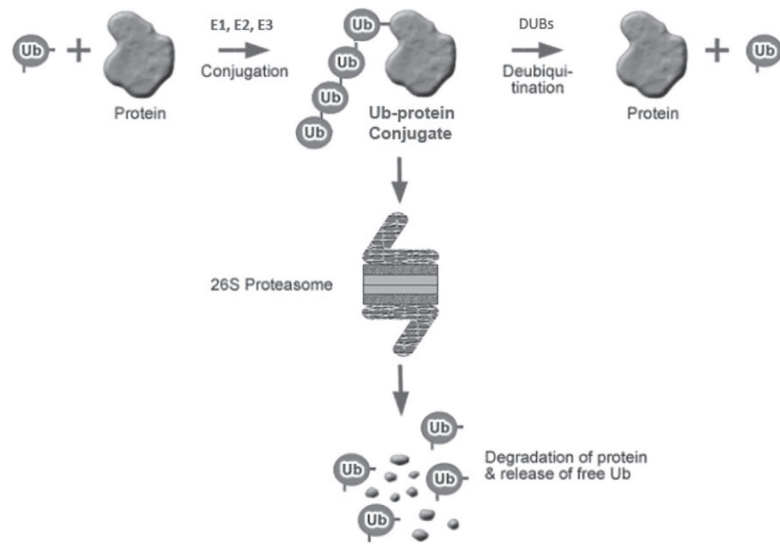
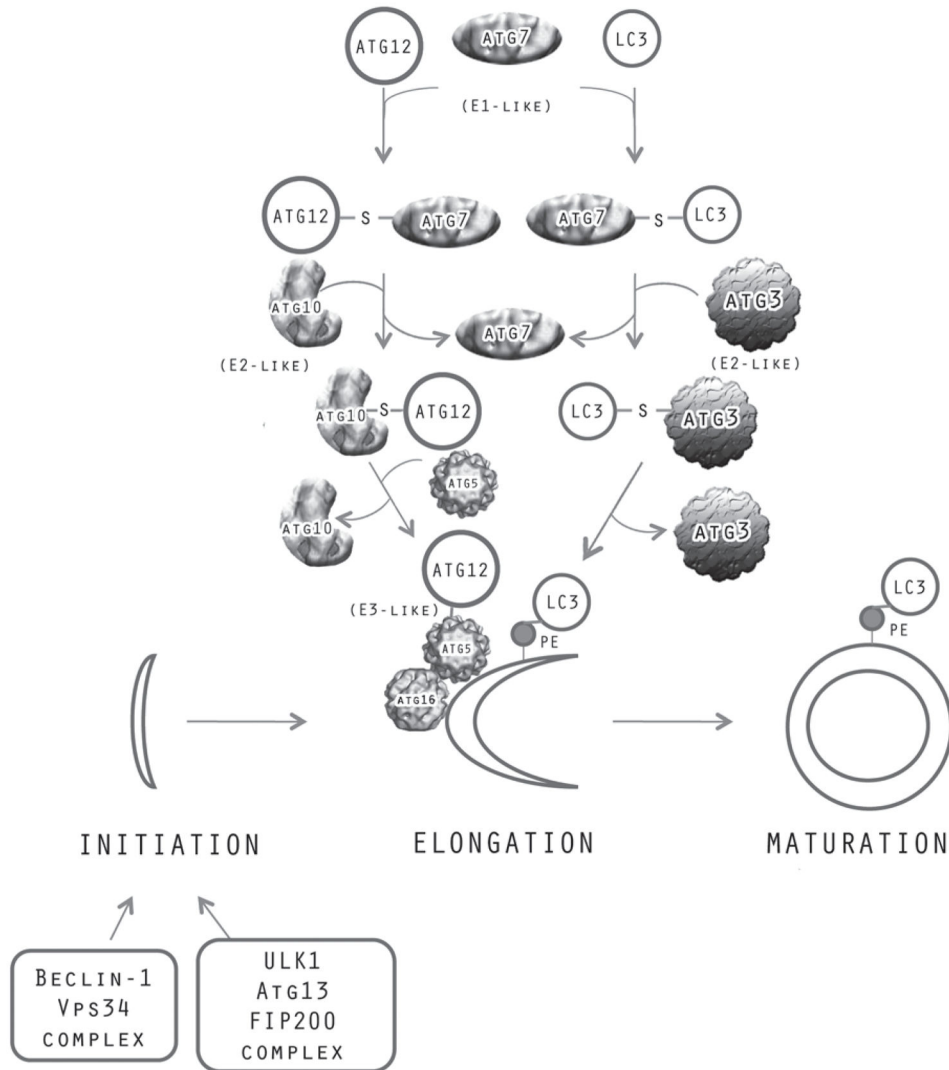


Figure 1.

The ubiquitin-proteasome system. Conjugation of ubiquitin (Ub) chains to proteins in which each ubiquitin is linked to the other via Lys 48 targets the protein substrate for recognition and degradation by the 26S proteasome. Conjugation is mediated by the sequential action of three enzymes - E1 (ubiquitin activating enzyme), which activates and transfers Ub to E2 (ubiquitin conjugating enzyme), which then works in concert with E3 (ubiquitin protein ligase) to mediate ubiquitination. E3s recognize substrates. The conjugation can also be reversed by deubiquitinating enzymes (DUBs).

**Figure 2.**

Formation of autophagosomes in autophagy. Initiation of the phagophore can be induced by two signaling complexes—one containing Beclin-1/Vps34 and the other Atg13/ULK1/FIP200. Elongation /maturation of the phagophore is mediated by two protein conjugation pathways—one in which Atg12 becomes conjugated to Atg5 through a pathway mediated by the Atg7 activating enzyme and the Atg10 conjugating enzyme. In the other pathway, LC3 or its paralogs, GABARAP and GATE16, become conjugated to phagophore bound phosphatidylethanolamine through a pathway mediated by the Atg7 activating enzyme, the Atg3 conjugating enzyme and the Atg12-Atg5/Atg16 ligase-like complex.

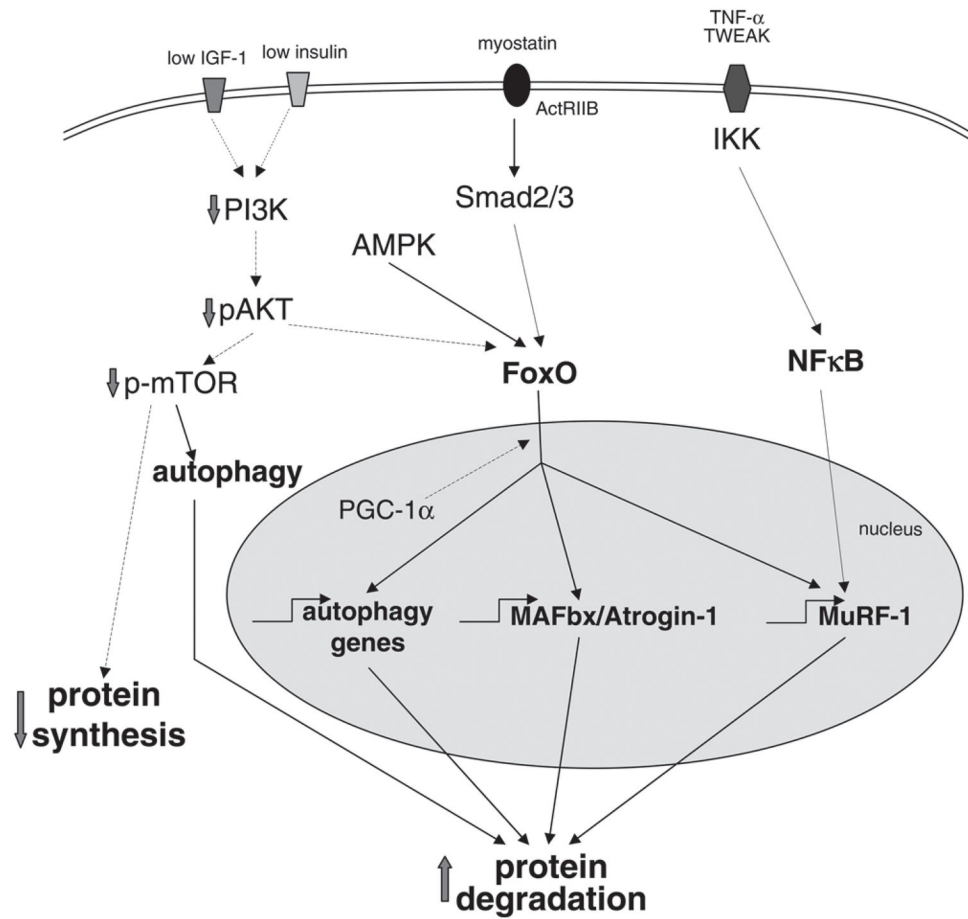


Figure 3. Signaling pathways involving MAFbx/Atrogin-1 and MuRF-1 that promote muscle wasting. Three major intracellular signaling pathways act to increase muscle protein degradation and suppress synthesis, leading to the loss of mass seen in atrophying muscle. Suppression of signaling through PI3K and AKT, caused by reduced insulin, insulin resistance or reduced IGF1, leads to FoxO translocation to the nucleus and transcription of the atrogenes MAFbx/Atrogin-1 and MuRF-1 as well as activation of autophagy. Myostatin binding to the ActRIIB leads to signaling through Smad intermediates that stimulates FoxO and other downstream targets. Cytokines such as TNF and TWEAK promote NFκB activation and lead to transcriptional activation of atrogenes such as MuRF-1. AMPK activation can augment atrophy signaling through phosphorylation of FoxO while PGC-1α can suppress these pathways also at the level of FoxO. (dashed lines-inhibition; solid lines - stimulation).

Table 1

List of genes in the UPS reported to be regulated in skeletal muscle wasting (examples of references in parentheses).

Ubiquitin genes	Ubiquitin conjugating enzymes	Ubiquitin ligases	Deubiquitinating enzymes	Proteasome subunits
UbB (93, 200–201)	E2 _{14k} /HR6B/UBC2 (46, 202)	MAFbx/Atrogin-1 (58, 60)	USP14 (44)	PsmA1 (39, 208–209)
UbC (93, 200–201)	E2 _{20k} (203) UBC4/UBC5 (44, 204)	MuRF-1 (58)	USP19 (55)	PsmA2 (200, 210–211)
UBA52 (44)		Cbl-b (76)		PsmA3 (39, 47, 200)
Ub S27A (44)		E4 (44)		PsmA4 (39, 46, 209)
		E3 α /UBR1 (205–206)		PsmA5 (44)
		E3 α -II/UBR2 UBR3 (77, 207)		PsmB1 (200, 212)
				PsmB2 (212–213)
				PsmB3 (44)
				PsmB4 (44)
				PsmC1 (51)
				PsmC2 (214–215)
				PsmC4 (51)
				PsmC5 (48) (51)
				PsmD1 (48, 51)
				PsmD3 (48)
				PsmD4 (51)
				PsmD8 (51) (44)
				PsmD11 (44)
				PsmE1 (48)
				PsmE3 (48)

Table 2

Genetic gain- and loss-of-function studies in muscle wasting.

Molecule	Function	Effect on muscle atrophy	References
MAFbx/Atrogin-1	Muscle-specific E3 ligase	Promote	(58)
MuRF-1	Muscle-specific E3 ligase	Promote	(58)
Cbl-b	E3 ligase	Promote	(76)
Calpastatin	Endogenous calpain inhibitor	Protect	(113, 125)
Caspase 3	Protease	Promote	(143)
AKT	Kinase	Protect	(147, 153)
TORC1	Kinase	Promote	(150)
FoxO	Transcription factor	Promote	(95–96, 159–160, 216)
PGC-1 α	Transcriptional co-activator	Protect	(165)
JunB	Transcription factor	Protect	(217)
NF κ B	Transcription factor	Promote	(181–182)
TWEAK	Cytokine	Promote	(171)
ActRIIB	Cytokine receptor	Promote	(193)
Myostatin	Cytokine	Promote	(184–189) (190)