Misregulation of autophagy and protein degradation systems in myopathies and muscular dystrophies

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Summary

A number of recent studies have highlighted the importance of autophagy and the ubiquitin-proteasome in the pathogenesis of muscle wasting in different types of inherited muscle disorders. Autophagy is crucial for the removal of dysfunctional organelles and protein aggregates, whereas the ubiquitin-proteasome is important for the quality control of proteins. Post-mitotic tissues, such as skeletal muscle, are particularly susceptible to aged or dysfunctional organelles and aggregation-prone proteins. Therefore, these degradation systems need to be carefully regulated in muscles. Indeed, excessive or defective activity of the autophagy lysosome or ubiquitin-proteasome leads to detrimental effects on muscle homeostasis. A growing number of studies link abnormalities in the regulation of these two pathways to myofiber degeneration and muscle weakness. Understanding the pathogenic role of these degradative systems in each inherited muscle disorder might provide novel therapeutic targets to counteract muscle wasting. In this Commentary, we will discuss the current view on the role of autophagy lysosome and ubiquitin-proteasome in the pathogenesis of myopathies and muscular dystrophies, and how alteration of these degradative systems contribute to muscle wasting in inherited muscle disorders. We will also discuss how modulating autophagy and proteasome might represent a promising strategy for counteracting muscle loss in different diseases.

Key words: Autophagy, Duchenne, Dystrophy, Muscle wasting, Myopathy, Skeletal muscle

Introduction

The ubiquitin-proteasome and autophagy lysosome are the major proteolytic systems of the cell and they have a crucial role in the removal of proteins, aggregates and organelles (Lecker et al., 2006). In the ubiquitin-proteasome system, proteins are targeted for degradation by the 26S proteasome through a covalent attachment of a chain of ubiquitin molecules (Box 1). In muscles, there are two specific ubiquitin ligases, atrogin-1 (also known as F-box only protein 32, FBXO32) and muscle RING finger-1 (MuRF1, also known as E3 ubiquitin-protein ligase TRIM63), which are considered the main markers of muscle atrophy because they are strongly upregulated in different types of muscular atrophy, and mice that are lacking either one of these genes are resistant to atrophy (Bodine et al., 2001a; Gomes et al., 2001). The expression of *Fbxo32* and *Trim36* is under the control of Forkhead box O (FoxO) transcription factors, which are negatively regulated by the AKT kinase (Bodine et al., 2001b; Sandri et al., 2004). Moreover, *Trim36* expression is also regulated by nuclear factor κB (NF-κB) signaling (Cai et al., 2004).

Lysosomes are composed of membrane-bounded vesicles of low pH that contain different acidic hydrolases that can degrade a variety of molecules (Bechet et al., 2005). The lysosome is the end point of several cellular pathways, including endocytosis, pinocytosis, phagocytosis and autophagy. The docking of ubiquitylated proteins to the proteasome is crucial for substrate recognition and sequestration, and delivery of cargos to the lysosome is required for a proper degradation through autophagy machinery. Three different mechanisms have been described in mammals for the delivery of autophagic cargo to lysosomes: microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy (Mizushima et al., 2008) (Box 2). Sequestered organelles and proteins are then docked onto the lysosomes for their degradation. The fusion of the outer membrane of the autophagosome with the lysosomal membrane also determines the degradation of the inner membrane and of the proteins that are associated with it.

A number of recent findings have underlined the crucial role of macroautophagy (hereafter referred to simply as autophagy) in the control of muscle mass (reviewed in Sandri, 2011). Indeed, the autophagic process plays a crucial role in the turnover of cell components, both under constitutive conditions and in response to various stimuli, such as cellular stress, nutrient deprivation, amino acid starvation and cytokines. Thus, it is no surprise that the abnormal regulation of autophagy is detrimental for myofiber health and is involved in a number of inherited muscle diseases (Fig. 1). Defects in the autophagy flux were first described in a group of lysosomal storage diseases that primarily affect muscle, generally referred to as autophagic vacuolar myopathies (AVM) (Malicdan and Nishino, 2012), which are characterized by the engulfment of autophagosome inside myofibers. These diseases are caused by mutations of genes coding for proteins that are

Box 1. The ubiquitylation process

The ubiquitylation process is a multistep pathway that involves first the activation of the small ubiquitin protein by an ATP-dependent activating enzyme (E1), which then transfers the highly reactive ubiquitin to one of the ubiquitin-conjugating enzymes (E2). Subsequently, an ubiquitin protein ligase (E3) binds the protein substrate and the ubiquitin–E2 thio-ester and catalyzes the formation of an ubiquitin chain on the target protein. This reaction is the rate-limiting step in the ubiquitylation process and therefore affects the kinetics of protein degradation. In fact, once the protein is ubiquitylated, it is docked onto the proteasome for degradation, unless the polyubiquitin chain is removed by the de-ubiquitylating enzymes (Lecker et al., 2006). Different E2–E3 enzyme pairs function in the degradation of different proteins, and the specificity of the different E3 enzymes for specific groups of proteins provides an exquisite selectivity to this degradation process.

involved in lysosome function or in the fusion between autophagosomes and lysosomes, but that do not directly contribute to the autophagic machinery. Only recently it was demonstrated that defective autophagy also contributes to the pathogenesis of different forms of muscular dystrophies. Dystrophic muscles can display accumulation of altered organelles inside myofibers, when autophagy is impaired, or excessive degradation of myofiber components, when there is an excessive autophagic flux. Interestingly, modulation of autophagy, in order to re-establish a proper flux, is able to rescue several muscle alterations in these pathologies (reviewed in Bonaldo and Sandri, 2013). However, as for AVMs, no muscular dystrophies have been primarily associated with mutations of genes that code for proteins that are directly involved in the autophagy process. So far, the Vici syndrome is the only disease that is characterized by a muscular phenotype and has been linked to a mutation in an autophagy gene, namely the gene coding for the key autophagic regulatory protein EPG5 (Cullup et al., 2013).

The role of autophagy in AVMs

The central role of lysosomes in myofiber homeostasis is highlighted by AVMs, a group of muscle disorders that are characterized by defective function of lysosomes (Box 3) and show accumulation of autophagic vesicles in skeletal myofibers because of defects in their clearance. In fact, the pathogenic mechanisms of all of these muscle disorders have been attributed to the functional impairment of lysosomes, which ultimately affects the cargo delivery by autophagy. In AVMs, autophagic vesicles are abundant and are thought to contribute to the pathogenesis of the disease; however, the precise relevance of autophagic vesicles in each of these disorders and the mechanisms by which they are formed remain to be clarified (Malicdan and Nishino, 2012). Two primary AVMs that share some common features are Pompe disease and Danon disease.

Pompe disease, also known as glycogen storage disease (GSD) type II, is an autosomal recessive disorder caused by the absence or deficiency of acid α-glucosidase (GAA), a lysosomal enzyme that is responsible for the hydrolysis of glycogen to glucose (Raben et al., 2002). Without GAA, glycogen that is delivered to the lysosome accumulates and results in different clinical phenotypes. In the severe early-onset form of GSDII, reduced GAA activity, or lack thereof, causes hypotonia, feeding difficulties and cardiomyopathy, leading to death from cardiac failure within the first year of life (Raben et al., 2002). Partial GAA defects induce late-onset forms of GSDII that are mainly characterized by progressive skeletal muscle weakness, while cardiac muscle is spared. The animal model of Pompe disease,

Box 2. Different types of autophagy

Microautophagy

Microautophagy is the direct engulfment of small portion of cytosol into lysosomes. Although it is still unknown whether microautophagy is present in skeletal muscle, electron microscopy observations indicate that microautophagy can participate in the uptake of glycogen into lysosomes (Raben et al., 2008; Takikita et al., 2010).

Macroautophagy

Macroautophagy is characterized by membranes that are committed to growth, which then become double-membraned vesicles that surround a portion of the cytoplasm, organelles, glycogen and protein aggregates. In macroautophagy, small ubiquitin-like molecules (LC3, GABARAP, GATE16, ATG12) are transferred from the conjugation system to membranes, which marks them for growth and commitment to become a double-membrane vesicle, called an autophagosome (Levine and Kroemer, 2008; Mizushima et al., 2008). This reaction requires the recruitment and assembly of different components of the autophagy machinery on phospholipids, but only the ubiquitin-like components are covalently bound to the phospholipid phosphatidylethanolamine (PtdEth) (Tanida et al., 2004a; Tanida et al., 2004b). This covalent bond occurs both on the outer and inner membranes of the autophagosome. The Beclin 1 complex plays a key role in the initial steps of autophagy. In fact, autophagosome formation requires a protein complex that recruits the class III phosphoinositol-3-kinase VPS34 to produce phosphatidylinositol 3-phosphate (Mizushima and Komatsu, 2011). This activity is coordinated by the interaction of Beclin 1 with several other proteins, including VPS34, VPS15, UVRAG, AMBRA1, Bif-1 (also known as ZBTB24), ATG14 (also known as Barkor) and Rubicon, to form different protein complexes that mediate distinct functions (He and Levine, 2010).

Chaperone-mediated autophagy

In contrast to macro- and micro-autophagy, delivery of cargo by CMA does not require the formation of intermediate vesicle compartments, membrane fusion or membrane deformity of any type. The substrates for CMA are translocated directly from the cytosol into the lysosomal lumen across the membrane in a process that is mediated by a translocation protein complex that requires substrate unfolding (Bandyopadhyay et al., 2008). Because of the particular characteristics of this delivery, only soluble proteins, but not complete organelles, can be degraded through CMA. For a protein to be amenable for lysosomal degradation by CMA, the presence of a pentapeptide KFERQ motif in its amino acid sequence is absolutely necessary (Dice et al., 1990).

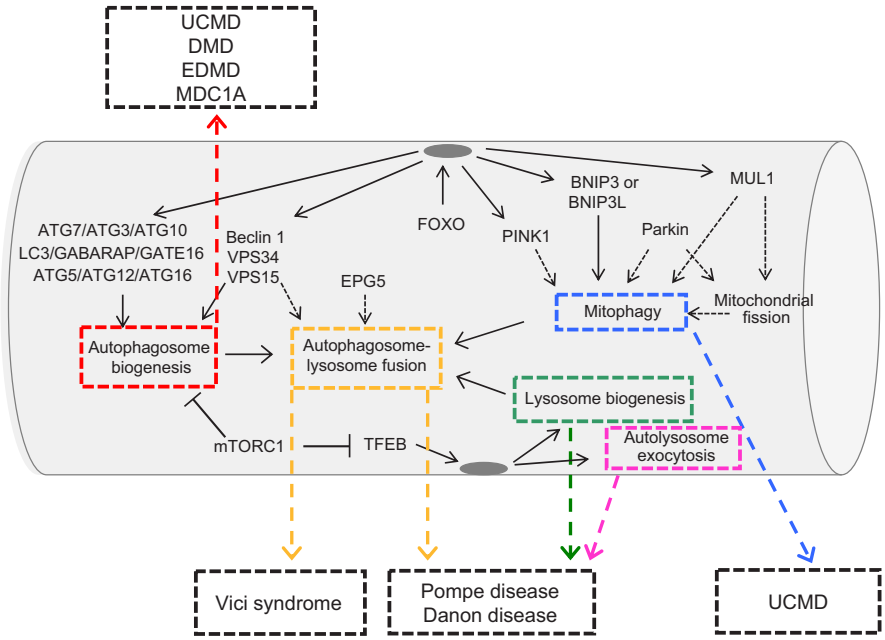

Fig. 1. Schematic illustration of the correlation between defects in autophagy and proteasome systems and inherited muscle diseases. The dashed boxes inside the myofiber highlight the key stages of the autophagy process that have been shown to be impaired in different muscle disorders; these include autophagosome biogenesis (red), fusion between autophagosomes and lysosomes (yellow), selective autophagic removal of mitochondria (blue), lysosome biogenesis (green) and autolysosome exocytosis (pink). Gray ovals represent the myofiber nuclei.

Gaa-knockout mice, mainly recapitulates the infantile or early childhood forms of this disease, owing to the complete lack of the enzyme (Raben et al., 2002).

The presence of large glycogen-filled lysosomes is a hallmark of GSDII, but other pathological features have also been reported for skeletal muscles, such as failure of autophagosomal turnover

Box 3. Muscle diseases and autophagy deregulation

Autophagic vacuolar myopathies

AVMs are a group of lysosomal storage diseases primarily affecting cardiac and/or skeletal muscle. Pompe disease (or GSD II) is owing to a defect in lysosomal GAA enzyme (Raben et al., 2002), Danon disease (or GSD IIb) is caused by the lack of LAMP2 (Nishino et al., 2000), and X-linked myopathy with excessive autophagy (XMEA) is triggered by mutations in an essential assembly chaperone of the V-ATPase, the main mammalian lysosomal proton pump complex (Ramachandran et al., 2013). Other forms of AVMs, caused by different conditions, have also been described. For instance, chloroquine-mediated inhibition of lysosome function induces a myopathy characterized by autophagosome accumulation followed by contracture and tissue necrosis (Schmalbruch, 1980).

LAMP2 and Danon disease

Alternative splicing of *LAMP2* transcripts gives rise to three different protein variants, LAMP2A, LAMP2B and LAMP2C. LAMP2A is the lysosomal receptor for CMA and, therefore, this type of autophagy is affected in Danon patients. LAMP2 is required for the correct maturation of early autophagic vesicles by their fusion with endosomes and lysosomes. LAMP2-deficient cells exhibit accumulation of early autophagic vesicles, intracellular mistargeting of lysosomal enzymes and of LAMP1, abnormal retention of mannose 6-phosphate receptors in autophagosomes and reduced degradation of long-lived proteins, as well as a lower extent of degradation of autophagic substrates after starvation (Tanaka et al., 2000). Deficiency in LAMP1 and LAMP2 leads to a delayed recruitment of Rab7, a key factor involved in the late endocytic pathway, to autophagosomes, thus altering the progression of the autophagic process (Huynh et al., 2007).

Collagen VI and its related diseases

Collagen VI has a key role in muscle homeostasis, as demonstrated by the fact that mutations of any of the three collagen VI genes, *COL6A1*, *COL6A2* and *COL6A3*, cause several diseases with different degrees of clinical symptoms and progression, such as UCMD, Bethlem myopathy and congenital myosclerosis (Bönnemann, 2011). Studies in collagen-VI-null (*Col6a1*$^{-/-}$) mice and in patients that are affected by collagen VI deficiency have allowed the identification of a number of patho-physiological defects, which include ultrastructural alterations of organelles, mitochondrial dysfunction and spontaneous apoptosis of muscle fibers (Bonaldo et al., 1998; Irwin et al., 2003; Angelin et al., 2007).

The beneficial effects of reactivation of autophagy in *Col6a1*-null mice

In vivo transfection of the tibialis anterior muscle with a plasmid expressing Beclin 1 was sufficient to activate autophagy and to counteract apoptosis in *Col6a1*-null mice. Similar effects were elicited in *Col6a1*-null mice by prolonged fasting, as well as by treatment with rapamycin, an mTOR inhibitor that it is widely used to induce autophagy (Klionsky et al., 2012), or with cyclosporine A, a drug acting on mitochondria membrane potential (Irwin et al., 2003). Of note, these treatments are detrimental for wild-type muscles because they lead to excessive activation of autophagy that results in a noticeable degree of myofiber damage and muscle wasting. Our group can achieve a more physiological and long-term stimulation of autophagy by feeding *Col6a1*-null mice with a specifically designed low-protein diet, where the protein amount was decreased to a quarter of its normal content; this led to sustained increase of Beclin 1 and formation of autophagosomes, allowing for the clearance of dysfunctional organelles and restoration of myofiber survival, with normalization of muscle morphology and recovery of muscle strength (Grumati et al., 2010; Grumati et al., 2011).

and extensive buildup of autophagic cargo (Raben et al., 2009; Raben et al., 2010). Different hypotheses have been formulated over the years to explain the pathogenesis of this disease, including energy crisis due to alteration of glucose homeostasis, mechanical damage and rupture of the glycogen-filled lysosome, excessive autophagy that disrupts sarcomere structure and, finally, an inhibition of the autophagic flux that impacts on endosome trafficking and on maturation or delivery of GAA (Malicdan and Nishino, 2012; Malicdan et al., 2008; Nascimbeni et al., 2012a; Nascimbeni et al., 2012b; Raben et al., 2008). The precise contribution of each of these mechanisms in the onset of GSDII remains unclear. Recent studies have mainly focused on the development of new strategies to enable the re-establishment of appropriate autophagic flux in affected muscles. Although these results, which were obtained using *in vitro* tools and animal models, have been encouraging, further studies are needed to fully elucidate the pathogenesis of Pompe disease. For instance, the genetic suppression of autophagy in skeletal muscles significantly reduces the glycogen burden in *Gaa*-knockout mice, but does not improve clinical phenotypes, such as kyphosis, muscle wasting and decreased life span (Raben et al., 2010).

Research in Pompe disease is also focusing on other molecular targets. Peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) is known to promote the expression of genes that are involved in mitochondrial biogenesis and oxidative metabolism. In skeletal muscles, PGC1α converts fast glycolic fibers to slow oxidative fibers and, in addition, is able to slow protein degradation and protect from atrophy-induced ageing (Takikita et al., 2010). PGC1α overexpression in *Gaa*-deficient muscles prevents the formation of autophagic buildup through an upregulation of the autophagy flux, but fails to improve the clearance of glycogen; instead, it increases the lysosomal glycogen load (Takikita et al., 2010). Recently, it has been proposed that transcription factor EB (TFEB) could be an effective therapeutic target for Pompe disease (Spampanato et al., 2013). TFEB is a master regulator of lysosomal biogenesis and autophagy, and it also elicits the production of new lysosomes and increases the number of autophagosomes in a variety of cell types (Sardiello et al., 2009; Settembre et al., 2011). Transient overexpression of TFEB in myotubes and myofibers results in fusion of the lysosome with the plasma membrane and its exocytosis, as well as a significant reduction in glycogen (Spampanato et al., 2013). Moreover, *in vivo* overexpression of TFEB in Pompe mouse models ameliorates the pathologic phenotype by decreasing lysosomal glycogen stores and attenuating morphological and ultrastructural alterations, for example, leading to a significant decrease in the size and number of lysosomes containing glycogen stores. These findings indicate that modulation of TFEB activity might be a promising target for the development of a better therapy for Pompe disease (Spampanato et al., 2013).

Our previous work showed that muscle-specific inhibition of autophagy leads to muscle weakness and myofiber degeneration (Masiero et al., 2009; Masiero and Sandri, 2010). To address the role of autophagy in GSDII, we subsequently monitored autophagy in muscle biopsies and myotubes of early- and late-onset GSDII patients at different points during disease progression (Nascimbeni et al., 2012a). We also analyzed muscles from patients treated with enzyme replacement therapy (ERT). Our data suggest that autophagy is a protective mechanism that is required for myofiber survival in late-onset forms of GSDII. Notably, our findings indicate that a normal autophagy flux is important for the correct maturation of GAA, as well as for the uptake of recombinant human GAA during ERT (Nascimbeni et al., 2012a; Nascimbeni et al., 2012b).

Danon disease is also known as GSD type IIb because one of its key features is the accumulation of glycogen. However, in contrast to Pompe disease, this disorder is not directly linked to defects of enzymes that are involved in glycogen breakdown, but is caused by mutations of the *LAMP2* gene that encodes the lysosome-associated membrane protein (LAMP)-2 (Malicdan and Nishino, 2012) (Box 3). Danon is inherited through the X chromosome and is characterized by muscle weakness, cardiomyopathy and mental retardation (Malicdan and Nishino, 2012). Skeletal muscles of Danon patients have large vacuolar structures that are positive for sarcolemmal proteins, such as dystrophin and associated proteins, extracellular matrix molecules and acetylcholinesterase, and that are surrounded by lysosomes (Sugie et al., 2005). These structures are also known as 'autophagic vacuoles with sarcolemmal features' (AVSF). Morphological studies have shown that AVSFs appear to be accumulations of lysosomes, which, when observed by electron microscopy, consist of clusters of autophagic vacuoles that are indicative of autolysosomes (Sugie et al., 2005). Considering all these results, the pathogenic mechanism of Danon disease could thus be considered as a builtup autophagic machinery that is caused by the failure of lysosome biogenesis, maturation and function (Fig. 1).

A recent study demonstrated that mice lacking the vacuolar protein sorting 15 (VPS15, also known in mammals as PIK3R4), a crucial component of the autophagy machinery, develop a severe myopathy with hallmarks of AVMs and lysosomal storage diseases, as well as accumulation of autophagosomes and glycogen (Nemazanyy et al., 2013). Importantly, overexpression of the VPS34–VPS15 complex (VPS34 is also known as PIK3C3), which is required for autophagy, in myoblasts of patients that are affected by Danon disease partially decreases the protein levels of LC3, thereby stimulating autophagy flux and reducing the accumulation of glycogen (Nemazanyy et al., 2013).

An important but largely unexplored aspect of lysosomal disorders is the impact of lysosomes on signaling pathways. For instance, the Rag GTPase complex, which senses lysosomal amino acids, promotes the translocation to the lysosomal surface of the mammalian target of rapamycin complex 1 (mTORC1), a protein kinase complex that acts as a nutrient sensor in the cells (Zoncu et al., 2011). Accumulation of amino acids inside the lysosomal lumen generates an activating signal that is transmitted to the Rag GTPases through the vacuolar H^+-ATPase (v-ATPase), which then recruits mTORC1, thus initiating protein synthesis (Zoncu et al., 2011). Activation of mTORC1 also induces phosphorylation and relocalization of TFEB onto the lysosomal membrane, therefore inhibiting its transcriptional activity (Settembre et al., 2012). These findings indicate that the lysosome senses its content and activity, and regulates its own biogenesis by a lysosome-to-nucleus signaling mechanism through the mTOR–TFEB axis. However, to what extent this crucial pathway is altered in Pompe and Danon disease is still an unexplored field.

Vici syndrome, an inherited disease affecting muscle and caused by mutation of an autophagy gene

Vici syndrome is a recessive inherited multisystem disorder that is characterized by cardiomyopathy, callosa agenesis, cataracts

and combined immunodeficiency (Cullup et al., 2013). Notably, skeletal muscles of Vici patients display consistent myopathic features, including atrophy of type 1 fibers, centrally nucleated fibers and abnormal glycogen accumulation (Cullup et al., 2013). Electron microscopy analyses of myofibers revealed the presence of exocytic vacuoles, as well as numerous vacuole-like areas and dense bodies that resemble lysosomes and abnormal mitochondria (Cullup et al., 2013). The underlying genetic defect has been identified as mutations of the *EPG5* gene, which encodes a protein that is predominantly expressed in cardiac and skeletal muscle, the central nervous system, thymus, immune cells, lung and kidney (Cullup et al., 2013). *EPG5* is the human homolog of an autophagy gene that was first identified in *Caenorhabditis elegans* and encodes the key autophagy regulator ectopic P-granules autophagy protein 5, which has an essential role in starvation-induced autophagy (Tian et al., 2010). Therefore, Vici syndrome is the first multisystemic disorder that has been shown to be directly associated with a defect in an autophagy-related gene (Fig. 1). In agreement with the role of EPG5, myofibers and fibroblasts of Vici patients display an accumulation of the autophagy adaptors p62 (also known as SQSTM1) and NBR1, and of lipidated LC3, confirming that the autophagy pathway is blocked (Cullup et al., 2013). Furthermore, the presence of puncta that are positive for LC3 and p62, together with a reduced colocalization of LC3 with the lysosomal receptor for CMA, LAMP1, suggests that the fusion of autophagosomes with lysosomes is blocked in these patients (Cullup et al., 2013). These data have been confirmed by the phenotype of *Epg5*-knockout mice; deletion of *Epg5* leads to selective damage of cortical layer 5 pyramidal neurons and spinal cord motor neurons, which result in muscle degeneration, myofiber atrophy and reduced survival (Zhao et al., 2013a). Interestingly, morphological studies of gastrocnemius muscle showed centrally nucleated and vacuolated fibers, whereas ultrastructural analyses revealed misalignment of Z-lines and the accumulation of abnormal enlarged mitochondria. Consistently with observations in Vici patients, the autophagy flux is blocked in the gastrocnemius muscle of *Egp5*-null mice, although several phenotypic features of Vici syndrome are absent in these mice (Zhao et al., 2013b).

Muscular dystrophies: roles of the autophagy lysosome versus the ubiquitin-proteasome

Role of autophagy

As discussed above, the involvement of the autophagy lysosome system in inherited diseases that affect skeletal muscles has been clearly established in disorders that are characterized by mutations in lysosomal genes, such as Pompe disease and Danon disease. Until recently, surprisingly little was known with regard to the role of autophagy in muscular dystrophies. Nonetheless, a number of studies have indicated that abnormal regulation of autophagy is also occurring in different forms of muscular dystrophies, including collagen-VI-related diseases, such as Bethlem myopathy and Ullrich congenital muscular dystrophy (UCMD), Duchenne muscular dystrophy (DMD), merosin-deficient congenital muscular dystrophy (MDC1A) and Emery–Dreyfuss muscular dystrophy (EDMD) (Fig. 1).

The first demonstration of a role of defective autophagy in muscular dystrophies was provided by our studies in mice and in patients with mutations of genes that code for collagen VI (Grumati et al., 2010), an extracellular matrix protein that forms a distinct microfilamentous network in the periphery of muscle fibers (Bönnemann, 2011) (Box 3). We found that a failure of the autophagy machinery is responsible for the inefficient removal and the persistence of altered organelles in muscles of *Col6a1*-null mice (Grumati et al., 2010). The ensuing accumulation of dysfunctional mitochondria elicits apoptosis, which in turn leads to muscle wasting and weakness. Lack of collagen VI in *Col6a1*-null mice has a major impact on factors and signaling pathways that are involved in the regulation of autophagy; for instance, it results in persistent activation of the AKT kinase even after fasting (Grumati et al., 2010). Although the nature of the receptor(s) that transduce collagen VI signals from the extracellular matrix to the autophagic machinery remains unknown, we found that the Beclin 1 complex and the AKT–mTOR pathway are strongly affected by lack of collagen VI, pointing to a role of these factors (Grumati et al., 2011). The abnormal AKT activation in muscles of *Col6a1*-null mice causes a block of the initial steps of autophagy through the constitutive activation of mTOR and cytosol sequestration of FoxO transcription factors, which are involved in the transcriptional regulation of several autophagy genes, such as *Bnip3* (Mammucari et al., 2008; Sandri, 2010). BNIP3 is known to contribute to the selective removal of aged or dysfunctional mitochondria in mitophagy, a specialized subtype of autophagy, which also involves other factors such as Parkin, PINK1, MUL1 and BNIP3L (Youle and Narendra, 2011; Lokireddy et al., 2012).

The protein levels of Beclin 1 and BNIP3 are not only reduced in muscles of *Col6a1*-null mice, but also in muscle biopsies of patients that are affected by collagen-VI-related diseases (Grumati et al., 2010). Notably, the forced activation of autophagy in *Col6a1*-null mice by genetic, pharmacological or nutritional means is effective in eliminating altered organelles

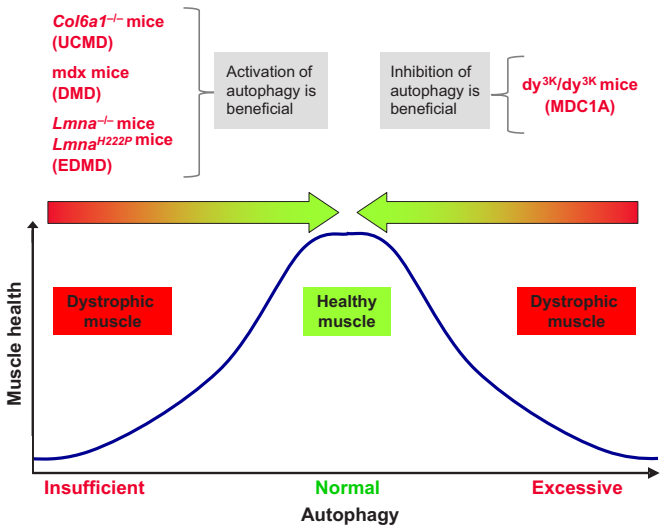

Fig. 2. Hypothetical model of the pathogenic role of deregulated autophagy in muscular dystrophies. A basal autophagy flux is fundamental for the clearance of damaged organelles and contributes to the homeostasis of healthy muscle fibers. An insufficient autophagy flux leads to an inefficient removal of altered organelles in mouse models for DMD (mdx), EDMD ($Lmna^{-/-}$ and $Lmna^{H222P}$), and collagen VI-related diseases ($Col6a1^{-/-}$). Conversely, lack of laminin α2 in dy^{3K}/dy^{3K} mice, a mouse model for MDC1A, leads to an excessive autophagy buildup that, in this case, contributes to muscle pathology.

and restoring myofiber homeostasis (Fig. 2), with a concomitant rescue of the myopathic phenotype and recovery of muscle strength (Grumati et al., 2010) (Box 3). These findings might open novel therapeutic venues to counteract muscle wasting and weakness in collagen-VI-related diseases, and a pilot clinical trial using low-protein diets is currently being conducted in Bethlem myopathy and UCMD patients (see also http://clinicaltrials.gov, study identifier, NCT01438788).

The studies in *Col6a1*-null mice and in patients that are affected by collagen-VI-related diseases paved the way for also investigating autophagy in other muscular dystrophies (Tolkovsky, 2010). For example, a recent study has revealed that autophagy also has a role in the pathogenesis of DMD (De Palma et al., 2012), the most common type of muscular dystrophy in children, which is caused by mutations of the gene encoding dystrophin, a protein involved in the linking the cytoskeleton to the plasma membrane in muscle fibers (Rahimov and Kunkel, 2013). Indeed, autophagy was found to be impaired in muscles of both dystrophin-deficient *mdx* mice, a mouse model of DMD, and DMD patients, with accumulation of damaged organelles. As is the case in collagen VI deficiency, the defective activation of autophagy is accompanied by a persistent activation of the AKT–mTOR axis, and treatment of mdx mice with a low-protein diet is also able to reactivate autophagy in muscle fibers, with concomitant normalization of AKT and mTOR signaling and significant recovery of muscle structure and function (De Palma et al., 2012) (Fig. 2). The beneficial effects of activating autophagy in mdx mice have been independently confirmed by another recent study, which showed that treatment of mdx mice with an agonist drug against the energy sensor AMP-activated protein kinase (AMPK), which also activates autophagy, results in an improvement of the structural and functional properties of the diaphragm (Pauly et al., 2012). Further studies have also demonstrated an impairment of the autophagic flux in mice with mutations in the *Lmna* gene, which codes for lamin A and C, two essential components of nuclear lamina. In humans, *LMNA* mutations cause a varied spectrum of dystrophic and progeroid syndromes, including EDMD and dilated cardiomyopathy (Puckelwartz and McNally, 2011). Studies of *Lmna*-null mice and of a knock-in mouse model that carries a lamin A/C point mutation that recapitulates EDMD have revealed that impaired autophagy due to elevated mTOR signaling has a major role in the onset of cardiac and skeletal muscle defects (Choi et al., 2012; Ramos et al., 2013). Furthermore, pharmacological inhibition of mTOR by rapamycin or its analogs could restore autophagic flux and improve the cardiac and skeletal muscle function of both *Lmna* mouse models (Choi and Worman, 2013; Ramos et al., 2013) (Fig. 2). Taken together, these findings that have been obtained in different types and models of muscular dystrophies strongly point to deregulated autophagy as a pathogenic factor in these diseases.

It is quite extraordinary that similar molecular defects, that is, excessive activation of the AKT–mTOR pathway and defective autophagosome formation, are shared by muscles that have defects in a wide array of different proteins whose localizations and functions range from extracellular matrix (collagen VI), over cytoskeleton (dystrophin) to nuclear lamina (lamins). However, the effect of deregulated autophagy in muscular dystrophies is not always owing to excessive AKT–mTOR signaling or defective autophagic flux. In fact, studies in the dy^{3K}/dy^{3K} mutant mouse model for laminin α2, a protein that participates in the formation of the extracellular basal lamina that surrounds myofibers, indicated that autophagy is increased in these mice (Carmignac et al., 2011a) (Fig. 2). In humans, mutations in the gene encoding laminin α2 cause MDC1A, a severe form of congenital muscular dystrophy (Gawlik and Durbeej, 2011). The increased autophagic flux of dy^{3K}/dy^{3K} muscles is due to the inactivation of AKT, which in turn leads to enhanced expression of several autophagy genes that are under control of FoxO proteins. Increased activation of autophagy was also detected in primary myotubes and in muscle biopsies from two MDC1A patients (Carmignac et al., 2011a). Furthermore, pharmacological inhibition of autophagy in dy^{3K}/dy^{3K} mice significantly improves their dystrophic phenotype (Carmignac et al., 2011a).

Taken together, these findings highlight that an appropriate and tight control of the autophagic flux is needed to maintain skeletal muscle homeostasis. Therefore, both failure to activate autophagy and excessive buildup of the autophagic machinery are detrimental for the health of myofibers and contribute to the onset of muscle pathology in different types of muscular dystrophies (Fig. 2).

Role of the proteasome

As discussed above, besides the autophagy machinery, the ubiquitin-proteasome system also has an important role in myofibrillar protein degradation and maintenance of muscle mass (Sandri, 2010). A proper function of the ubiquitin-proteasome system is required for correct muscle homeostasis, and proteasome dysfunction has been associated with muscle pathologies. For example, the studies described above on mouse models for MDC1A and DMD also provided evidence that the proteasome system is strongly upregulated in these mice (Carmignac et al., 2011b; Bonuccelli et al., 2003).

Because proteolysis can be increased in the muscles of both mdx mice and DMD patients, protease inhibitors have been considered as potential therapeutic strategies (Bonuccelli et al., 2003; Assereto et al., 2006). Indeed, several proteins that are involved in linking the cytoskeleton to the plasma membrane, such β-dystroglycan, α-dystroglycan, α-sarcoglycan and dystrophin, are either absent or found at only very low levels in myofibers from mdx mice and DMD patients, and restoring their physiological levels therefore might alleviate the phenotypes. Indeed, use of proteasome inhibitors, such as MG-132 and Velcade, has been found to result in the restoration of the normal protein levels and subcellular localization of β-dystroglycan, α-sarcoglycan and dystrophin (Bonuccelli et al., 2007; Gazzerro et al., 2010). Moreover, MG-132 and Velcade treatment of mdx mice leads to a decrease of myofiber membrane damage and the amelioration of the histopathological signs of the disease. However, when freshly isolated skeletal muscle biopsies from DMD patients were treated in this manner, not all of the explants examined showed signs of phenotype rescue (Bonuccelli et al., 2003; Bonuccelli et al., 2007; Assereto et al., 2006; Gazzerro et al., 2010). Although the inhibition of the proteasome appears to be an attractive strategy to combat DMD, other observations in the mdx animal model demonstrate that doing so might be detrimental. For instance, it has been shown that the reported histological effects of inhibiting the proteasome do not translate into functional effects and that the continued inhibition of the proteasome is instead deleterious and outweighs any therapeutic benefits (Selsby et al., 2012). The concept of inhibiting the general proteolysis is certainly interesting, but

the risks that can derive from the accumulation of proteins that are mutated in these diseases and of proteolysis cleavage products needs to be considered. For example, mutations in the dystrophin gene can generate a truncated form of the protein that is usually degraded, and it is questionable whether the accumulation of such truncated dystrophin forms, which have been found after MG-132 treatment, might have a therapeutic effect (Selsby et al., 2012).

The MDC1A mouse model (dy^{3K}/dy^{3K}) also displays an upregulation of proteasome activity in dystrophic limb muscles, as well as an overall increase in ubiquitylated proteins (Carmignac et al., 2011b). However, in this case, it is accompanied by reduced levels of AKT phosphorylation, which not only controls autophagy but also proteasome-mediated protein degradation. As AKT phosphorylation negatively modulates FoxO transcription factors, this results in a significant increase in the mRNA levels of FoxO target genes, such as the muscle-specific E3 ubiquitin ligases atrogin-1 and MuRF1, in dy^{3K}/dy^{3K} mice. Inhibition of the proteasome with MG-132 in these mice appears to improve their muscle shape and physiology; it has been shown to restore AKT phosphorylation and to decrease the amount of apoptotic nuclei, leading to a longer lifespan and a better locomotion (Carmignac et al., 2011b).

A deregulation of the ubiquitin-proteasome system has also been reported for other muscular dystrophies, including limb girdle muscular dystrophy (LGMD) type 2H, type 2A and type 2B (Frosk et al., 2002). LGMDs are a group of muscular dystrophies that affect the muscles of the shoulders and pelvic girdles. A total of 15 genetically defined LGMDs have been identified, and most of the affected genes encode cytoskeletal or structural muscle proteins. For instance, mutation of the *TRIM32* gene, which encodes an E3 ubiquitin ligase, has been linked to LGMD2H and to sarcotubular myopathy (Frosk et al., 2002). TRIM32 is a member of the tripartite motif (TRIM) family of proteins that have ubiquitin ligase activity in their RING finger domain. TRIM32 is localized in the Z-line of the sarcomere and is able to interact with the head and neck region of myosin IIA and can ubiquitylate actin *in vitro* (Kudryashova et al., 2005). Several muscle-specific substrates and interacting partners of TRIM32 have been identified (Frosk et al., 2002). TRIM32 differs from most of the other TRIM family members because its C-terminus contains a specific NHL domain that mediates protein–protein interactions. All TRIM32 mutations that result in LGMD2H and in sarcotubular myopathy occur in this domain. Mutated TRIM32 behaves like the wild-type protein with respect to its ability to associate with dysbindin, a protein involved in the trafficking of proteins to lysosomes and lysosome-related organelles, but its ubiquitin ligase activity is abrogated (Locke et al., 2009). Interestingly, dysbindin and TRIM32 are both located in the Z-line, and Z-line streaming is a pathological feature of LGMD2H and of *Trim32*-knockout animals (Kudryashova et al., 2009). Recently, it has been demonstrated that TRIM32, in contrast to atrogin-1 and MuRF1, is not involved in muscle atrophy itself, but instead participates in muscle regeneration after atrophy. In fact, muscles of *Trim32*-knockout mice display substantially fewer satellite cells, reduced muscle growth and premature senescence (Kudryashova et al., 2012). These findings point to a new mechanism of muscular dystrophy that is due to a reduced proliferation and subsequent differentiation of satellite cells into myoblasts, which in turn results in an impaired capacity for muscle growth.

The ubiquitin-proteasome system might also have a crucial role in LGMD2A; here, the mRNA and protein levels of MuRF1 are found to be higher than those of atrogin-1 (Fanin et al., 2013), a finding that might correlate with the role of MuRF1 in muscle proteolysis (Attaix and Baracos, 2010). Mutations of dysferlin, a transmembrane protein implicated in sarcolemma repair, cause different forms of inherited muscle diseases, including Miyoshi myopathy, LGMD2B and distal anterior compartment myopathy (Azakir et al., 2012). For instance, the R555W dysferlin mutant is functional, but is rapidly degraded by the proteasome in skeletal muscle, and treatment with the proteasome inhibitor Velcade restores the resealing of the plasma membrane in patient-derived myoblasts (Azakir et al., 2012). These findings indicate that treatment with proteasome inhibitors might represent a therapeutic strategy for LGMD2B patients that are affected by missense mutations that nevertheless are capable of producing functional dysferlin.

Conclusions

Our understanding of the mechanisms that control the autophagy-lysosome and ubiquitin proteasome systems has greatly advanced during the past few years. Major milestones in this progress have been the identification of the transduction pathways that control them, such as the AKT pathway and its main downstream effectors mTOR and FoxO3, and the understanding of the involvement of autophagy in important cellular functions, including glycogen homeostasis, organelle turnover and protein quality control. The crucial function of protein homeostasis (proteostasis) in cell survival and maintenance of cellular homeostasis has prompted investigations of the role of these degradation systems in the muscle wasting that occurs in inherited muscle diseases. It is now clear that there is no common mechanism that applies to all muscular dystrophies or muscle-wasting conditions, and an important objective for future studies will be to define the exact contribution of these systems in the specific disease of interest. To that end, future studies of the pathogenic mechanisms that contribute to muscle loss should consider the concept that an excessive activation, as well as a strong inhibition of protein degradation, can contribute to muscle degeneration by different means. The detailed dissection of these pathways and the identification of the genes that control proteostasis might pave the way for the development of interventions that are able to boost muscle growth and/or prevent muscle wasting. Current research efforts already actively pursue this aim and have great promise in becoming important for the therapeutic treatment of neuromuscular diseases.

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