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Review

“Get the Balance Right”: Pathological Significance of Autophagy Perturbation in Neuromuscular Disorders

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Abstract. Recent research has revealed that autophagy, a major catabolic process in cells, is dysregulated in several neuromuscular diseases and contributes to the muscle wasting caused by non-muscle disorders (e.g. cancer cachexia) or during aging (i.e. sarcopenia). From there, the idea arose to interfere with autophagy or manipulate its regulatory signalling to help restore muscle homeostasis and attenuate disease progression. The major difficulty for the development of therapeutic strategies is to restore a balanced autophagic flux, due to the dynamic nature of autophagy. Thus, it is essential to better understand the mechanisms and identify the signalling pathways at play in the control of autophagy in skeletal muscle. A comprehensive analysis of the autophagic flux and of the causes of its dysregulation is required to assess the pathogenic role of autophagy in diseased muscle. Furthermore, it is essential that experiments distinguish between primary dysregulation of autophagy (prior to disease onset) and impairments as a consequence of the pathology. Of note, in most muscle disorders, autophagy perturbation is not caused by genetic modification of an autophagy-related protein, but rather through indirect alteration of regulatory signalling or lysosomal function. In this review, we will present the mechanisms involved in autophagy, and those ensuring its tight regulation in skeletal muscle. We will then discuss as to how autophagy dysregulation contributes to the pathogenesis of neuromuscular disorders and possible ways to interfere with this process to limit disease progression.

Keywords: Autophagy, skeletal muscle, MAP1LC3, p62/SQSTM1, vacuole, myopathy, dystrophy, mTORC1, FoxO, mitophagy

ABBREVIATIONS

Atg Autophagy-related gene
AVM Autophagic vacuolar myopathy
AVSF Autophagic vacuole with
 sarcolemmal features

BM	Bethlem myopathy
CASA	Chaperone-assisted selective autophagy
CMA	Chaperone-mediated autophagy
(AD-)CNM	(Autosomal dominant) Centronuclear myopathies
COLVI-RM	Collagen VI-related myopathies
DM	Distal myopathies
DM1	Myotonic Dystrophy type I
DMD	Duchenne muscular dystrophy
EM	Electronic microscopy
ER	Endoplasmic reticulum
ERAD	ER associated protein degradation

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FoxO3	Forkhead box class O
GAA	Acid α -glucosidase
GSDII(b)	Glycogen storage disease type II(b)
Hsp	Heat shock protein
IBMPFD	Inclusion body myopathy associated with Paget's disease of the bone and frontotemporal dementia
LPD	Low protein diet
3-MA	3-methyladenine
MAP1LC3	Microtubule-associated protein light chain 3
MDC1A	Congenital muscular dystrophy Type 1
MiT/TFE	microphthalmia/transcription factor E
MFM	Myofibrillar myopathies
mTORC1	mammalian Target of Rapamycin Complex 1
OPMD	Oculopharyngeal Muscular Dystrophy
PE	Phosphatidylethanolamine
PI3P	Phosphatidylinositol-3-phosphate
RV	Rimmed vacuole
sIBM	sporadic Inclusion Body Myositis
SQSTM1	(p62) Sequestosome 1
UCMD	Ullrich congenital muscular dystrophy
UPR	Unfolding protein response
UPS	Ubiquitin-proteasome system
VCP	Valosin containing protein
Vps34	Vacuolar sorting protein 34
XMEA	X-linked myopathy with excessive autophagy
XLMTM	X-linked myotubular myopathies

INTRODUCTION

Muscle tissue accounts for approximately 40% of whole body mass. It displays an incredible plasticity, enabling it to adjust its size based on external stimuli. Numerous pathological conditions can threaten its maintenance and cause muscle dysfunction. Muscle mass relies heavily on the balance between catabolic and anabolic processes, and more specifically on the equilibrium between protein synthesis and degradation (i.e. proteostasis). While promoting protein synthesis increases muscle mass, blocking protein degradation is rather detrimental. Importantly, due to the central metabolic importance of muscle tissue, changes in muscle mass and defects in skeletal muscle proteostasis often affect

global body metabolism [1, 2]. Although these integrated perturbations may significantly impact the general health of patients suffering from neuromuscular disorders, this phenomenon has been only poorly explored. By contrast, the literature reporting pathogenic defects in protein degradation in muscle diseases has grown exponentially over the last decade. There are two major contributors to protein degradation in cells: The ubiquitin-proteasome system (UPS) and the autophagy-lysosome process. While involving different machineries and potentially targeting distinct proteins, the two systems are tightly connected and dysfunction of one impinges on the other [3]. Altered activity of the UPS contributes to muscle atrophy in neuromuscular disorders, such as MDC1A (*congenital muscular dystrophy type 1*) [4] and DMD (*Duchenne muscular dystrophy*) [5, 6]. Signs of impaired UPS include accumulation of misfolded and/or ubiquitinated proteins [7]. In parallel, autophagy coupled with the endocytic/lysosomal compartments permits recycling of proteins and organelles under basal conditions (referred to as constitutive autophagy) and upon different cellular stresses (adaptive, induced autophagy) [8]. Autophagy is essential for cell homeostasis as highlighted by the tissue alterations arising from its dysregulation. The role of autophagy impairment in the pathogenesis of neurodegenerative diseases [9, 10], cancers [11] or infectious diseases [12], as well as in age-associated homeostasis decline [13, 14] is now clearly documented. Most neuromuscular disorders have been linked to insufficient autophagy-mediated degradation, although some, such as MDC1A, have been shown to have enhanced autophagic flux. Based on these observations, autophagy has often been considered as a promising target for therapeutic strategies. In this review, the mechanisms at play in autophagy and the signalling involved in its regulation will be briefly presented; for further details, the reader is referred to reviews regarding specific autophagy-associated molecular mechanisms [15–18]. In the following sections, the pathological significance of autophagy perturbation in neuromuscular disorders will be discussed.

AUTOPHAGY AT A GLANCE

Autophagy is an evolutionary conserved homeostatic process, associated with unfavourable and conditions of stress (Fig. 1) [19]. Constitutive

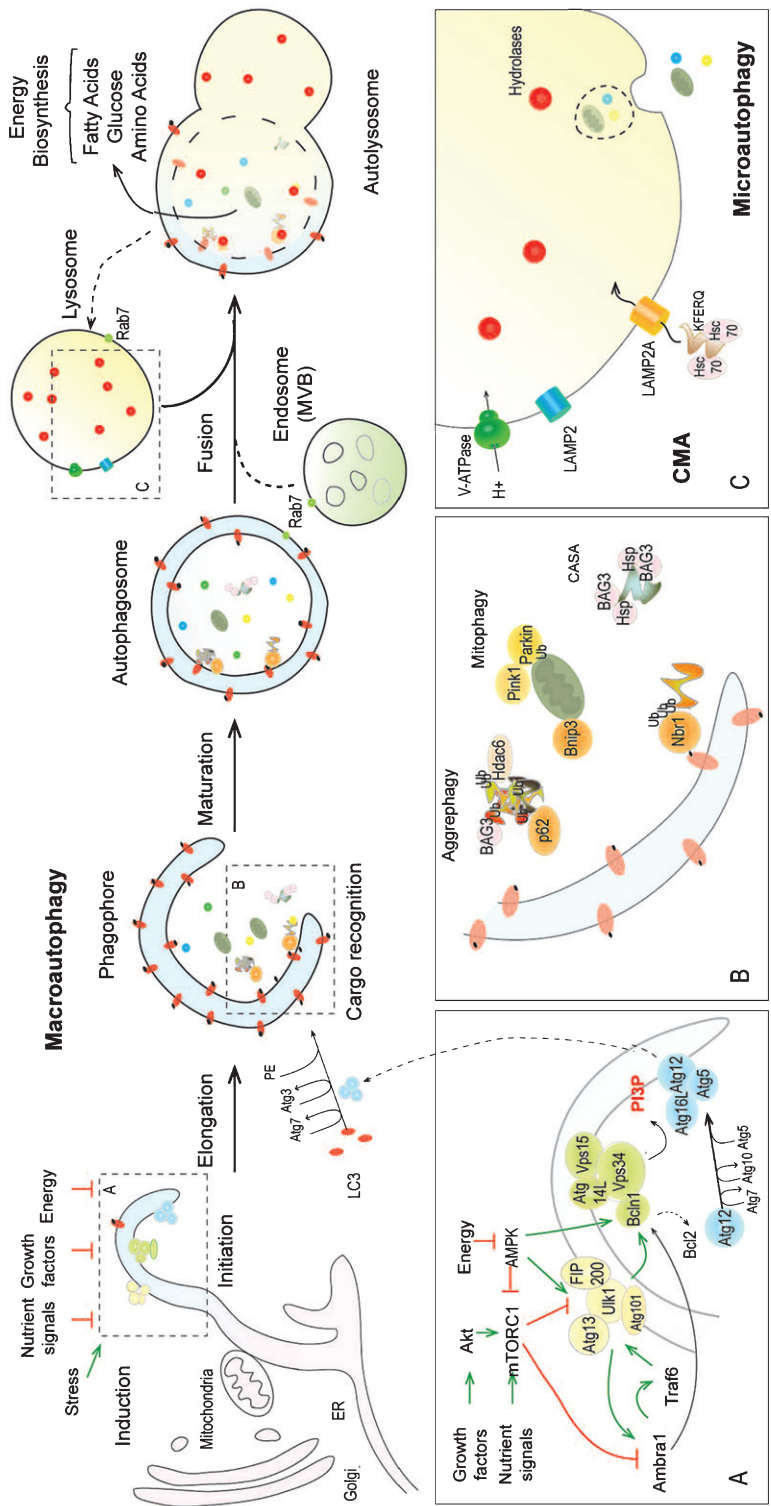


Fig. 1. Overview of mechanisms and proteins involved in autophagy in mammals. In (macro)autophagy, initiation leads to the formation of a phagophore, which engulfs large cytoplasmic parts and expands to give rise to autophagosomes. Autophagy induction depends on the balance of several regulatory pathways converging on the Utk1 complex (A). Autophagy ensures selective degradation of proteins and organelles, mediated by different autophagy cargo receptors (p62, Nbr1) and chaperone/co-chaperone proteins (Hsp, BAG3) (B). After fusion with the lysosomes and/or endosomes, degradation of the autolysosomal content by lysosomal enzymes permits recycling of metabolites. Of note, lysosomes are also involved in degradation associated with microautophagy and chaperone-mediated autophagy (CMA) (C). Red lines represent inhibition; green arrows show activation. Bcln1, Beclin1; MVB, multivesicular bodies; Ub, ubiquitin.

autophagy ensures basal turnover of proteins and organelles and is a key element of the quality control program in cells [20]. Induced autophagy is triggered by various stimuli, such as nutrient deprivation, energy depletion or growth factor withdrawal, as well as oxidative or endoplasmic reticulum (ER) stress. Autophagy is coupled to lysosomes, constituting the end point of the degradative process. There are three different types of autophagy: Macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA), which each differ by their mechanism of cargo delivery to the lysosomes [21, 22] (Fig. 1). Microautophagy involves direct invagination of a small cytosolic portion by lysosomes and may contribute to glycogen loading in these organelles. CMA relies on the selective translocation of soluble, unfolded proteins into lysosomes, after multimerization of the lysosomal membrane receptor LAMP2A [23, 24]. The KFERQ motif of the target proteins is recognized by chaperones (e.g. Hsc70) and co-chaperones [25]. Macroautophagy, also simply referred to as autophagy, proceeds through the formation of double membrane vesicles, called autophagosomes. They engulf large cytoplasmic portions and organelles, which are degraded after fusion with lysosomes [20, 21]. Autophagosome formation involves more than 30 Atg (*autophagy-related gene*) proteins (Fig. 1).

Autophagy induction

Nutrient sensing in cells is controlled by the large multiprotein complex mTORC1 (*mammalian Target of Rapamycin Complex 1*), which includes, as major constituents, mTOR and raptor. Amino acid-dependent activation of mTORC1 by the GTPase Rheb occurs at the lysosomal surface and is tightly controlled by Rag GTPases and the Ragulator complex [26–28]. Rheb is inhibited by the TSC1/TSC2 complex, which integrates energetic and growth factor signals through its phosphorylation by AMPK and Akt/PKB, respectively [29] (Fig. 2). In most cell types, autophagy induction is determined by mTORC1 inhibition, as seen upon treatment with rapamycin, a pharmacologic mTORC1 inhibitor [30]. mTORC1 inhibits autophagy by phosphorylating Ulk1 (Ser 757) and Atg13, in the Ulk1-Atg13-Fip200-Atg101 complex [31, 32]. mTORC1 activity disrupts the interaction of Ulk1 and AMPK [33, 34]. Upon energy deprivation, this interaction is restored, leading to Ulk1 phosphorylation at Ser317 and Ser777 and thereby to autophagy induction

[34–38] (Fig. 1). Ulk1 stabilization further depends on its ubiquitination by the E3 ubiquitin ligase Traf6. Inhibitory phosphorylation of Ambra1, a positive kinase for Traf6, by mTORC1 limits Ulk1 activation [39] (Fig. 1). ER stress, reactive oxygen species (ROS) and hypoxia induce autophagy by modulating mTORC1 and AMPK signalling, although their effect also depends on PERK/eIF2 α , IRE1/JNK, and HIF1/Bnip3 pathways [40].

Autophagy initiation

Activation of the Ulk1-Atg13-Fip200-Atg101 complex triggers assembly of the nucleation machinery at multiple sites, initiating *de novo* vesicle formation. Membranes to build the initial autophagy compartment (also called phagophore) are provided by ER, Golgi, mitochondria, and plasma membrane [41–46]. Nucleation requires production of phosphatidylinositol-3-phosphate (PI3P), which is reliant on the class III PI3 kinase, Vps34 (*Vacuolar Sorting Protein*), complexed with Vps15-Beclin1-Atg14L proteins [47, 48]. Activation of Vps34 complex is mediated by AMPK and Ulk1, through phosphorylation of Beclin1 and/or Ambra1, which binds to Bcl2 and prevents it from sequestering Beclin1 [49–53] (Fig. 1). PI3P formation permits the recruitment of Atg complexes at the phagophore [54].

Autophagosome maturation

Expansion and closure of autophagic vesicles involve transient attachment of the ubiquitin-like conjugation complex Atg12-Atg5 at the phagophore, following Atg12 activation by Atg7 (E1 activating enzyme) and its transfer to Atg10 (E2 conjugating enzyme) (Fig. 1). Interaction of Atg12-Atg5 complex with Atg16L facilitates the conjugation of LC3 (MAP1LC3 for *microtubule-associated protein 1 light chain 3*) to the lipid phosphatidylethanolamine (PE), after its activation by Atg7 and its transfer to Atg3 (E2 like enzyme) [54, 55]. LC3-PE (or LC3II) is inserted into autophagosome membranes and controls vesicle expansion and size [56]. Fusion between the lysosome and autophagosome, resulting in formation of the autolysosome, requires LAMP2 and Rab7 proteins and marks the final step of vesicle maturation. It correlates with autophagic vesicle acidification conferred by the lysosomal environment [57] (Fig. 1). Notably, both autophagosome maturation and lysosome biogenesis involve a tight interaction with the endocytic system, highly dynamic

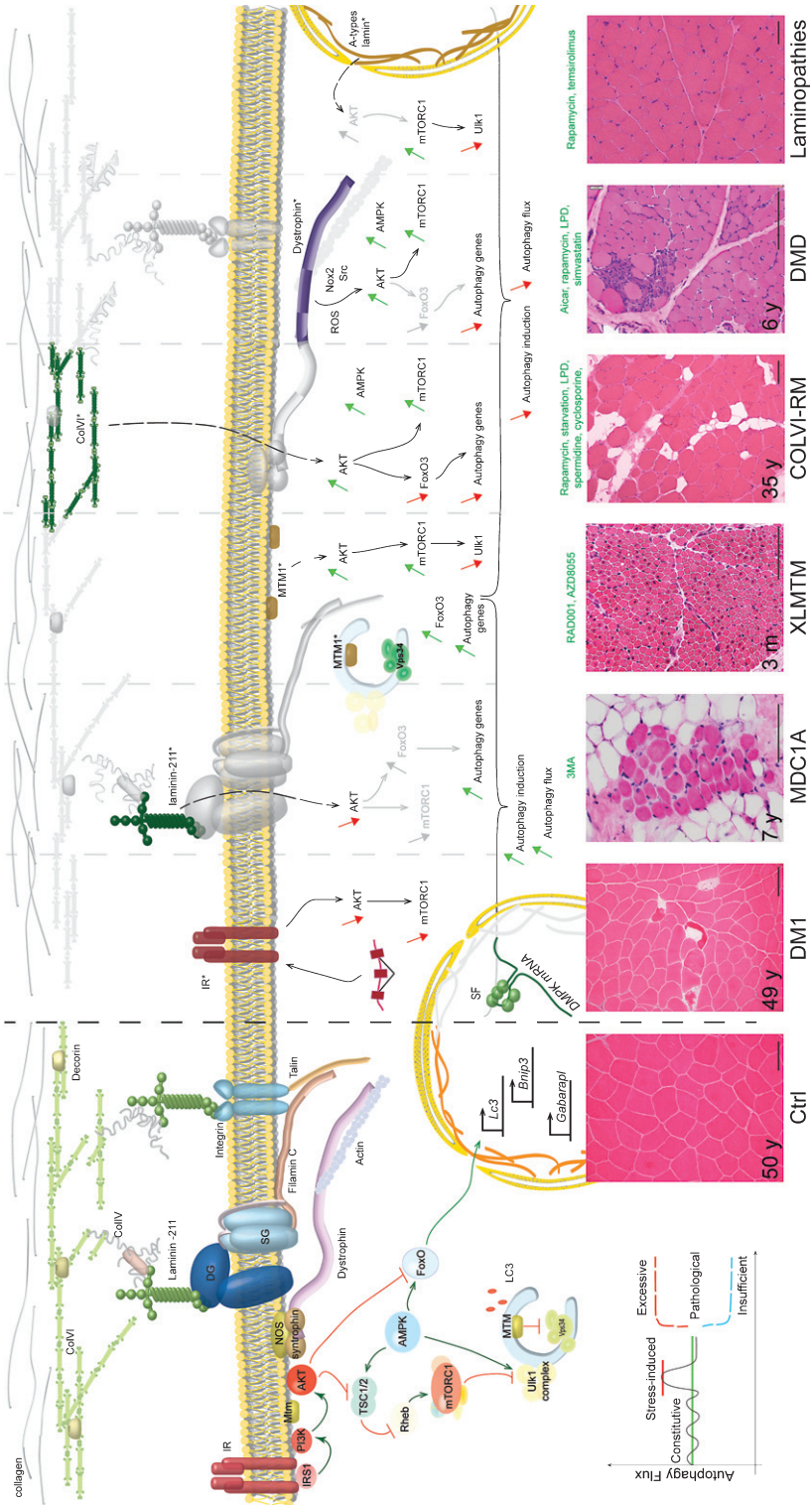


Fig. 2. Neuromuscular disorders related to dysregulation of autophagy induction. In control (Ctrl) muscle, autophagy induction is determined by the state of the Ulk1 complex, which is inhibited by Akt/PKB-mTORC1 signalling and activated by the AMPK pathway. The autophagic flux varies depending on nutritive and stress stimuli, which can promote prolonged autophagy induction via FoxO-dependent expression of autophagy genes. In pathological conditions, excessive or insufficient autophagic flux may contribute to muscle damage. In DM1 and MDC1A, Akt/PKB inhibition would promote autophagy induction, while Akt/PKB-mTORC1 activation seems to restrict autophagic flux in XLMTM, COLVI-RM, DMD and laminopathies. Of note, autophagy induction may be enhanced in XLMTM due to abnormal Vps34 activation caused by Mtm1 deficiency. Treatments, which proved some efficacy in animal models, are indicated in green. Red lines represent inhibition; green arrows show activation. In pathological conditions, red and green arrows indicate abnormal inhibition and activation of signalling pathways, respectively. Black and white arrowheads in the hematein & eosin staining of muscle cross-sections indicate fat and degeneration regions, respectively; arrows show vacuolated fibres. The mutated proteins are indicated by an asterisk. COL, collagen; DG, dystroglycan; IR, insulin receptor; IRS, insulin receptor substrate; m, month; PI3K, phosphoinositide-3 kinase; ROS, reactive oxygen species; SF, splicing factor; y, year. Scale bar, 100 μ m.

membrane remodelling and microtubule-dependent vesicle and cargo movements [58–60]. In particular, endosomes can fuse with autophagosomes to form amphisomes before fusing with lysosomes, which would facilitate aggregate removal [61]. Lastly, lysosome recycling from the autolysosome is essential to maintain autophagy and may depend on mTOR activity [62].

Cargo recognition and delivery

It was recently recognized that autophagy can proceed *via* selective degradation of cargoes. Clearance of specific substrates, including damaged organelles or ubiquitinated proteins, is mediated by receptors, such as p62/SQSTM1 or Nbr1, which recognize and bind to unfolded protein regions or poly/mono-ubiquitin chains of the cargoes and allow their translocation to the autophagy machinery by interacting with LC3/Gabap proteins [63–65] (Fig. 1). Notably, chaperones of the heat shock protein family (Hsp70 and HspB8) and the BAG3 co-chaperone coordinate selective degradation of ubiquitinated substrates upon autophagosome formation through a process called chaperone-assisted selective autophagy (CASA) [66, 67]. Moreover, p62, BAG3 and also HDAC6 participate in aggregate clearance by targeting misfolded proteins, which are clustered in inclusion bodies and large aggresomes, to autophagosomes (so called aggrephagy) [68–72] (Fig. 1).

Degradation steps

Lysosomal hydrolases, including cathepsin D and L, ensure degradation of the autolysosomal content and of the substrates of microautophagy and CMA. The resulting products (i.e. amino acids, free fatty acids and glucose) are released into the cytosol to be reused for energy production or in biosynthesis pathways (e.g. protein synthesis).

BALANCED AUTOPHAGY IS REQUIRED FOR MUSCLE HOMEOSTASIS

How to monitor autophagy in skeletal muscle

To best identify autophagy perturbation and determine its role in muscle disorders, one would need to combine *in vitro* analyses in human muscle cells, *in vivo* procedures in animal models of the pathology, as well as evaluation of autophagy markers in muscle

biopsies from patients. However, the latter is often difficult as sampling site and nutritive status of the patients at the time of biopsy are usually not defined. Further, heterogeneity in the results may arise from variability in the disease stages and quality of the muscle biopsy.

Quantification of LC3 and p62 protein levels constitutes the initial experiment when investigating autophagy. The amount of LC3II mirrors autophagosome accumulation in cells. Normalization of LC3II levels to the expression of housekeeping proteins is preferred over determination of LC3II/LC3I ratio, as the latter may be biased by LC3 transcriptional changes [16, 73]. Similarly, p62 accumulation is often considered as a relevant sign for autophagy impairment, but it may also be caused by transcriptional changes in the absence of autophagy blockade [74, 75]. Hence, transcript quantification of autophagy genes, including *Sqstm1/p62* and *Map1lc3 (Lc3)* needs to complement protein evaluation, but is not sufficient *per se* to assess the autophagic flux. Indeed, autophagy can be blocked at the induction or degradation steps, irrespective of any changes in gene expression [76]. Autophagosome accumulation can also be evaluated by quantifying the number of LC3-positive vesicles, an experiment that may require the use of GFP-LC3 transgenics or transfection [77].

A major drawback, often not considered in autophagy studies, is that LC3II levels or the autophagosome number are not sufficient to judge whether the autophagic flux is increased or blocked. Actually, accumulation of autophagic vesicles in muscle cells is more frequently associated with impaired autophagic flux than with increased autophagy induction. To characterize the flux, one needs to monitor the changes in autophagosome number or LC3II levels when lysosomal degradation is blocked. Such procedures should also allow discrimination between alterations in induction *vs.* degradation steps. While chloroquine and bafilomycin (both inhibitors of lysosomal acidification) have shown efficacy *in vitro*, colchicine, a microtubule depolarizing drug, is preferred for the targeting of muscle in mouse models [78, 79]. Complementary studies include electron microscopy (EM), which may clarify the nature of autophagic vacuoles. When possible, confocal microscopy monitoring - in cells or muscle - using the RFP/mCherry-GFP-LC3 reporter allows distinction between neutral, non-degradative vesicles (RFP⁺, GFP⁺) from acidic, functional autolysosomes (RFP⁺ only) [73]. Lastly, autophagy-mediated proteolysis

can be evaluated in cells and mice by measuring the degradation rate of labelled proteins in the presence of an autophagy and/or an UPS inhibitor [77, 80].

Of note, upon treatment of cell lines, mouse models or patients with candidate drugs that affect autophagy, evaluation of the effect of the treatment on autophagic flux is required in addition to the monitoring of phenotypic changes. In clinical trials, the nutritive status of patients should be clearly defined and ideally standardised at the time points of investigation. Furthermore, immunostaining and biochemical analyses of autophagy and lysosome markers should be combined to reduce the inherent limitations of sampling. Autophagy may be analysed in other cell types, such as melanocytes from patients, if those cells reproduce the autophagic perturbations observed in muscle [81, 82].

Conditions increasing autophagic flux in muscle

Exercise has been shown to induce autophagy in muscle and is thought to enable the tissue to dispose of altered contractile proteins and/or to adapt to increased energetic demand [83–87]. Nevertheless, the beneficial effect of autophagy induction in muscle on global glucose and lipid metabolism remains controversial [2, 83]. Similarly, fasting triggers autophagy in muscle (particularly in fast-twitch muscle) in response to nutrient or energy depletion [88, 89]. In these conditions, increased muscle breakdown not only ensures energetic maintenance in the tissue but also preserves global body metabolism by supplying amino acids to non-muscle tissues. Autophagy has also been implicated in denervation-induced muscle atrophy [78, 90–93] although mTORC1-dependent autophagy inhibition was suggested to limit muscle wasting upon nerve injury [94]. Additionally, in humans, the muscle wasting associated with sepsis [95, 96], cancer [97, 98], disuse [99] or cirrhosis [100] has been related to increased autophagic flux.

Impaired autophagic flux and atrophy

The development of mouse models deficient for autophagy-related proteins in muscle has revealed the deleterious effect of autophagy blockade on this tissue. In muscle depleted of Atg7 or Atg5, autophagy is blunted and mutant mice develop a progressive myopathy, characterised by muscle atrophy and weakness. Further, the loss of mass upon fasting and denervation is exacerbated in Atg7-deficient

mice compared to controls [101]. Autophagy impairment causes fibre vacuolization and intracellular accumulation of abnormal organelles, ubiquitinated proteins and p62-positive aggregates. In addition, increased oxidative and ER stresses were observed, which were likely responsible for muscle degeneration and dysfunction. Of note, Vps15 deficiency in mouse muscle does not alter autophagy induction but impinges on maturation steps and causes a severe myopathy. Pathological features in these mice include p62-positive aggregates and abnormal organelles, as seen in Atg7-deficient muscle, but also necrosis, inflammation, glycogen accumulation, autophagosome build-up and vacuoles lined with sarcolemmal proteins [102]. These alterations are reminiscent of those described in toxic vacuolar myopathies in humans, which can develop after long-term treatment with drugs interfering with autophagy, such as chloroquine (anti-malarial agent) or colchicine (used to treat gout) [103, 104]. Although recovery is obtained by ceasing treatment, adverse effects may come from the simultaneous administration of drugs promoting autophagy induction, such as rapamycin, which could dramatically aggravate muscle damage [105]. These pathological conditions are further evidence that autophagy imbalance is detrimental for skeletal muscle. Accordingly, a decline in autophagy capacity may contribute to aging-related muscle alterations, by reducing clearance of toxic organelles and products [106, 107]. Importantly, autophagy restriction in satellite cells also causes quiescence loss with age and thereby a decline in muscle regenerative capacity [108]. Together with the aforementioned data, these observations indicate that a fine-tuned balance of autophagy is required for muscle homeostasis.

Importance of mitophagy in muscle

Mitophagy refers to the selective degradation of mitochondria by autophagy and is essential to avoid accumulation of damaged mitochondria and excessive ROS generation during oxidative phosphorylation [109] (Fig. 1). It involves specific receptors, such as Bnip3, that are able to interact with depolarized mitochondria and the autophagic machinery [110, 111]. The E3 ubiquitin ligase Parkin and the kinase Pink1 also contribute to ubiquitin-dependent mitochondrial breakdown [112–115]. Mitophagy is induced in catabolic conditions and leads to remodelling of the mitochondria network, together with the fusion and fission machineries [116]. Importantly,

mitochondria fission is necessary and sufficient for muscle atrophy induction [117]. Conversely, accumulation of damaged mitochondria related to impaired mitophagy is detrimental for muscle tissue [118]. Hence, a tight regulation of mitophagy is also essential for muscle homeostasis, and to date, its pathogenic involvement in neuromuscular disorders probably remains underestimated [119].

AUTOPHAGY REGULATION IN SKELETAL MUSCLE

Transcriptional regulation of autophagy

In 2009, Mammucari et al. hypothesized that muscle ability to maintain stress-induced autophagy for a prolonged period requires autophagy gene up-regulation. By using transcriptomic analyses, they established that autophagy genes, such as *Map1lc3*, *Gabarapl1*, *Bnip3* and *Sqstm1*, are induced upon fasting and denervation, and that their regulation is driven by the FoxO3 (*Forkhead box class O*) transcription factor [120]. FoxO1 up-regulates *Cathepsin L* expression [121]. In mouse muscle, gene expression was increased after as little as 12 hrs of starvation, but remained unchanged from fed to basal conditions when constitutive autophagy was induced [76]. Interestingly, overexpression of *Bnip3* or of a constitutively active form of FoxO3 enhanced autophagosome formation, whereas expression of a dominant-negative form of FoxO3 or downregulation of either *FoxO3* or *Bnip3* by shRNA reduced autophagy induction upon fasting [120, 122]. FoxO3 phosphorylation by Akt/PKB, in response to growth factors, inhibits its nuclear import, while, upon energy deprivation, AMPK-mediated phosphorylation of FoxO3 activates its translocation [123–125]. In parallel, transcription factors of the MiT/TFE family, including TFEB, promote expression of both lysosomal (e.g. encoding V-ATPases, Cathepsin) and autophagy (e.g. *Map1lc3*, *Sqstm1*, *Atg9*) genes [126, 127]. mTORC1 regulates TFEB activity, and thus autophagy gene transcription, by phosphorylating TFEB and thereby modifying its nuclear translocation [128–130]. Lastly, epigenetic modulations likely contribute to autophagy gene regulation. For example, HDAC1/2 depletion in mouse muscle blunts autophagy gene expression and causes a myopathy in the surviving mutant mice. Whether this effect is independent of FoxO remains to be determined [131].

Role of mTORC1 in autophagy induction

In contrast to the well-established role of mTORC1 in autophagy regulation in most cell types, autophagy was suggested to be independent of mTORC1 in skeletal muscle [88, 132, 133]. In particular, rapamycin treatment and shRNA directed against *Mtor* failed to promote autophagy induction in muscle cells [120, 122, 133]. Further, acute Akt/PKB activation blocked LC3 lipidation and autophagy gene expression and caused severe muscle degeneration characterized by p62 aggregates and myofibre vacuolization [118, 120, 122]. Rapamycin did not restore autophagy induction in those conditions, suggesting that FoxOs were the determinant factors controlling autophagy. Nonetheless, TSCmKO mice, specifically depleted for TSC1 in muscle and characterized by constant activation of mTORC1 in the tissue, revealed new insights into the role of mTORC1 in autophagy control. Indeed, constitutive and fasting-induced autophagy is blocked in TSCmKO muscle and the mice show an age-dependent accumulation of autophagic vacuoles and debris [76]. Importantly, autophagy impairment occurs despite simultaneous FoxO-dependent up-regulation of autophagy genes and is mediated by mTORC1-dependent inhibition of Ulk1. In control muscle, mTORC1 inhibition correlates with induction of constitutive autophagy (from feeding to non-feeding time), while FoxO activation matches with starvation-induced autophagy. Such distinction between constitutive and stress-induced autophagy likely contributed to the initial oversight of mTORC1 involvement [134]. It is now clear that autophagy involves a dual regulation in muscle: While mTORC1 activation locks autophagy at the induction step, FoxOs may ensure prolonged, enhanced flux *via* transcriptional regulation of autophagy genes. Consistently, mTORC1 inactivation in raptor-depleted muscle increases autophagy induction, but concomitant FoxO inactivation seems to impinge on autophagosome processing [76]. It is worth noting that Fyn/STAT signalling was suggested to block the Vps34/Beclin1 complex and thereby autophagy, independently of mTORC1 [135]. However, mTORC1 is activated in Fyn-overexpressing transgenic muscle and likely contributes to autophagy-related atrophy of glycolytic muscle, as observed in TSCmKO muscle [135]. Autophagy regulation was also attributed to mTORC2 (a second mTOR complex characterized by its associated protein rictor) as *rictor*

knock-down induced autophagy in an Akt/PKB- and FoxO-dependent, but mTORC1-independent manner in muscle fibres [120]. However, perturbation in autophagic flux was detected in mice deficient for rictor in skeletal muscle, suggesting that mTORC2 does not contribute to autophagy control in muscle [76]. Lastly, it should be noted that differences exist between muscles regarding their sensitivity to autophagy and the corresponding regulatory signalling [76, 118, 136].

NEUROMUSCULAR DISORDERS RELATED TO ABNORMAL AUTOPHAGY INDUCTION

In the last decade, growing evidence has pointed to a converging role of autophagy perturbation in muscle diseases. Suspicion of autophagic defects was guided by characteristic histological features, including vacuoles, protein inclusions and damaged organelles in muscle fibres, although less specific alterations, such as fibre degeneration, can also arise from abnormal flux. In the following section we will discuss the pathologies related to Akt/PKB, mTORC1 and/or FoxO dysregulation with potential increased autophagic flux or impaired autophagy induction (Table 1).

Muscle pathologies with increased autophagic flux

Congenital Muscular Dystrophy type I – MDC1A is caused by mutations in the *LAMA2* gene, leading to deficiency in the laminin- $\alpha 2$ chain, which assembles with the $\beta 1$ and $\gamma 1$ chains to form laminin-211 [137] (Fig. 2). Utilising the severely affected, laminin- $\alpha 2$ -deficient, *dy^{3K}/dy^{3K}* mouse model, Carmignac et al. (2011) suggested that muscle atrophy and degeneration are related to increased autophagic flux, caused by Akt/PKB inhibition in diseased muscle (Fig. 2). Consistently, expression of the autophagy genes was increased in *dy^{3K}/dy^{3K}* muscle. The authors further showed that two successive injections of 3-methyladenine (3-MA), an inhibitor of Vps34, were sufficient to ameliorate muscle morphology, locomotion and lifespan of the mice [138]. However, the efficacy of 3-MA to inhibit autophagy induction was not assessed in the study. In fact, others reported that 3-MA may induce autophagy and inhibit Akt/PKB by interfering with class I PI3 kinase activity [139, 140]. Moreover, questions remain as to how laminin-211 deficiency leads to Akt/PKB dysregula-

tion, and as to how 3-MA administration normalizes Akt/PKB activation and autophagy gene expression.

Myotonic Dystrophy type I – DM1 is a multisystemic neuromuscular disorder, characterized - amongst other symptoms - by pronounced muscle atrophy. It is caused by a CTG repeat expansion in the *DMPK* gene; the expanded RNA leads to mis-splicing of numerous genes due to the nuclear sequestration of splicing factors [141, 142] (Fig. 2). Muscle wasting in DM1 was recently related to enhanced autophagy, based on the accumulation of LC3II and/or of autophagic vesicles in primary DM1 myoblasts [143–145], in muscle biopsies from patients [146–149] and in flight muscles of a DM1 *Drosophila* model [145]. However, it is still unclear whether these features arose from increased autophagy induction or from a defect in the degradation steps. Interestingly, altered expression of the insulin receptor, which correlates with glucose intolerance in DM1 patients [150–152], may cause Akt/PKB-mTORC1 dysregulation in diseased muscle. Akt/PKB down-regulation and/or mTORC1 inhibition were reported in muscle from *Dmpk*^{-/-} mice [152] and DM1 flies [145], as well as in DM1 human ES-derived neural stem cells [153]. In muscle biopsies and myoblasts from DM1 patients, results have not been consistent [144, 145, 154, 155]. Of note, approaches aiming at inhibiting autophagic flux tended to limit muscle atrophy in DM1 flies [145]. However, these results need to be reproduced in DM1 mouse models and in human muscle cells. Whether autophagy perturbation in DM1 only depends on Akt/PKB changes or involves other splicing-dependent and -independent events (such as p53 [143] or Tweak/Fn14 [156]) still needs to be addressed.

Muscle pathologies with impaired autophagy induction

Collagen VI-related myopathies – COLVI-RM are caused by mutations in genes encoding one of the three collagen VI α chains, and range from a severe, congenital form (*Ullrich congenital muscular dystrophy* - UCMD) to milder forms (*Bethlem myopathy* - BM) [157]. In 2010, Grumati et al. established that the autophagic flux is blocked at the induction step in muscle of *Col6a1*^{-/-} mice, which would contribute to pathological accumulation of abnormal organelles (e.g. dysfunctional mitochondria), increased fibre apoptosis and eventually muscle degeneration [118]. The autophagy defect relied, at least in part, on the

Table 1
Neuromuscular disorders related to autophagy defects – Signs, type of deregulation and possible therapeutic strategies

Disorder	Mutated gene	Animal model used	Histology signs	Autophagy features			References
				Genes/proteins	Flux in muscle	Regulatory signaling	
CMD	MDC1A	<i>dy^{3K}/dy^{3K}</i> mice	Degeneration	↑ Transcript, ↑ LC3II	Increased?	↓ Akt	137–140
	ColVI-RM	<i>Col6a1^{-/-}</i> mice	Abnormal organelles (mitochondria and SR), apoptosis, degeneration	↓ Transcript ↓ LC3II, (↑ p62)	Blocked at induction	↑ Akt, ↓ FoxO, ↑ mTORC1, ↑ AMPK LPD, rapamycin, cyclosporin1, spermidine	87, 118, 157–160
Dystrophies	Laminopathies	<i>LMNA</i>	Vacuoles, aggregates	↑ LC3, ↑ Ulk ^{P757}	Blocked at induction?	↑ mTORC1, ↑ Akt	196–199
	DMD, BMD	<i>DMD</i>	Abnormal organelles, (aggregates, vacuoles)	↓ Transcript ↓ LC3II, (↑ p62)	Blocked at induction	↑ Akt, ↑ mTORC1, ↑ AMPK, ↑ Traf6	161–186
	OPMD	<i>PABPN1</i>	Aggregates, (RV)	–	–	–	160,265,266
	LGMD1D/E	<i>DNAJB6</i>	Aggregates, vacuoles	–	–	–	267–269
	DM1	<i>Dmpk^{-/-}</i> mice, DMI flies	Vacuoles, apoptosis	↑ LC3II	Impaired CASA?	↓ Akt/mTORC1	141–156
CNM	XLMTM	<i>MTM1</i>	AV, glycogen, aggregates, abnormal organelles	↑ Transcript ↑ LC3, ↑ p62, ↑ Ulk ^{P757}	Increased then blocked at maturation/fusion?	↑ Akt, mTORC1 ↑ FoxO RAD001, AZD8055	187–195
	AD-CNM	<i>DNM2</i>	–	↑ LC3II (St)	Blocked at induction/maturation?	–	244–247

(Continued)

GSDIIb	LAMP2	<i>Lamp2</i> ^{-/-} mice	AVSF, glycogen, aggregates	↑LC3II	<i>Blocked at maturation/fusion? Altered lysosome biogenesis? CMA?</i>	-	-	102, 201–209
GSDII	GAA	<i>GAA</i> ^{-/-} mice	AV, glycogen, autophagic build-up, abnormal organelles	↑LC3, ↑p62	Blocked at fusion, altered lysosome biogenesis	-	ERT+autophagy inhibition	210–226
Vici synd.	<i>EPG5</i>	<i>Epg5</i> ^{-/-} mice	AV, glycogen, abnormal organelles, degeneration	↑LC3, ↑p62/Nbr1	<i>Blocked at fusion/degradation?</i>	↓Akt, ↑FoxO	-	227, 228
XMEA	<i>VMA21</i>	-	AVSF	(↑ Transcript) ↑LC3II, ↑p62	<i>Blocked at degradation? Increased induction?</i>	↓mTORC1, ↑TFEB	-	248–252
IBMPFD	<i>VCP</i>	<i>VCP</i> ^{R155H} mice	RV, aggregates	↑LC3II, ↑p62	Blocked at fusion, increased induction	↓mTORC1	Exercise, HFD	229–243
sIBM	-	-	RV, aggregates, abnormal organelles	↑LC3II, ↑p62/Nbr1	<i>Blocked at fusion/degradation?</i>	↓mTORC1	-	271–280
MFMD	<i>BAG3</i> , <i>CRYAB</i> <i>FLNC</i> , ...	<i>Bag3</i> ^{-/-} mice, <i>Bag3</i> ^{P209L} zebrafish, <i>CryAB</i> ^{R120G} mice	Aggregates, RV	↑LC3, ↑p62	<i>Blocked? Impaired CASA? Increased induction?</i>	-	Rapamycin (CryAB ^{R120G} - heart)	253–264
GNE myopathy	<i>GNE</i>	-	RV, aggregates, abnormal organelles	-	-	-	-	281–285

AV, autophagic vacuole; AVSF, AV with sarcolemmal features; CASA, chaperone-assisted selective autophagy; CMA, chaperone-mediated autophagy; CMD, congenital muscular dystrophy; CNM, centronuclear myopathy; ERT, enzyme replacement therapy; HFD, high fat diet; LPD, low protein diet; 3-MA, 3-methyladenine; RV, rimmed vacuoles. Italics indicate elements remaining to be confirmed in skeletal muscle; brackets indicate non predominant pathological features.

restricted, FoxO3-dependent expression of *Beclin1* and correlated with an abnormal Akt/PKB activation (Fig. 2). Although *Beclin1* expression was shown to be necessary and sufficient to restore autophagy in mutant muscle, the role of Akt/PKB dysregulation remains to be clarified. Indeed, while prolonged starvation normalized *Beclin1* expression and the autophagic flux, Akt/PKB activation was only slightly changed in mutant muscle. As AMPK signalling was also altered in *Col6a1*^{-/-} muscle, further investigations on the activation state and pathogenic involvement of FoxO, mTORC1 and Ulk1 would be of interest. Although similar reduction in Beclin1 protein levels has been reported in muscle biopsies from patients, analysis of the autophagic flux in human muscle cells would provide a better insight into the pathogenic role of autophagy. Interestingly, autophagy induction also seems to be impaired in COLVI-deficient zebrafish, but was not related to abnormal Akt activation [158].

Importantly, forcing autophagy induction with prolonged starvation, long-term low protein diet (LPD), or treatments with rapamycin, cyclosporin or spermidine ameliorated the phenotype of mutant muscle. Improvements included normalization of muscle ultrastructure, decreased apoptosis, increased muscle strength and/or reduced muscle degeneration [118, 159]. By contrast, physical exercise failed to promote autophagic flux in mutant mice and led to major aggravation of muscle pathology [87]. Notwithstanding, a clinical trial in which 8 BM and UCMD patients were treated with LPD over 12 months has been initiated in 2011 (Merlini et al., NCT01438788). A preliminary report indicates that autophagy seems to be re-induced based on the comparison of autophagy markers in two patients before and after treatment [160].

Duchenne muscular dystrophy – Mutations in the gene encoding dystrophin, a cytoskeletal scaffolding protein, cause Duchenne or Becker muscular dystrophies (DMD and BMD, for the severe and milder forms, respectively) (Fig. 2). DMD is characterized by severe muscle atrophy and weakness, caused by progressive muscle degeneration, which is insufficiently compensated for by regeneration. Hence, with time, muscle is progressively replaced by connective tissue and fat [161]. Similar features are observed in *mdx* and *mdx/utrophin*^{-/-} mouse models [162–164]. The presence of damaged organelles and protein aggregates in DMD and *mdx* muscle suggest impaired autophagic flux; vacuoles could also be detected in muscles from BMD patients [165]. A detailed analy-

sis of the autophagic flux was conducted by De Palma et al. [166] in *mdx* mice and later confirmed by others [167–170]. In these studies, autophagy induction was altered in adult *mdx* mice as indicated by increased p62 levels and impaired LC3II formation in basal and fasted conditions. These autophagy markers were similarly affected in DMD biopsies [166]. One report also suggested a potential defect in lysosomal biogenesis in *mdx* muscle, based on LAMP1 downregulation [169].

Interestingly, as for COLVI-RM, autophagy blockade was related to Akt/PKB activation in *mdx* muscle, in mouse primary muscle cells and in DMD muscle biopsies [166, 167, 169, 171, 172]. Akt/PKB dysregulation was detected prior to necrosis and peaked during the necrotic and hypertrophic phases [166]. Consecutively, mTORC1 hyperactivation and restricted FoxO3-dependent autophagy gene expression could impair autophagy induction [166–168] (Fig. 2). Whether phosphorylation of FoxO3 and Ulk1 was altered in *mdx* mice was not described. There is evidence that imbalance in Akt/PKB-mTORC1 signalling in dystrophin-deficient muscle relies on Nox2 (NADPH oxidase)-dependent oxidative stress and activation of the associated Src kinase. Consistently, Src or Nox2 inhibition normalized Akt/PKB-mTORC1 activation and restored autophagosome formation and lysosome biogenesis [169]. It is important to note that other authors reported that Akt/PKB-mTORC1 activation is unchanged in *mdx* mice [173, 174].

Increased Akt/PKB activation may constitute an adaptive compensation to muscle damage in young mice [175]; this would promote regeneration, cause muscle hypertrophy and reduce myofibre death. Accordingly, Akt/PKB-mTORC1 stimulation improved muscle histology and/or function in *mdx* mice [173, 176–181], which may, at least partly, rely on the compensatory expression of cytoskeleton molecules, such as utrophin [182, 183]. Conversely, Akt/PKB-mTORC1 hyperactivation in *mdx* muscle may in fact be detrimental, and strategies that aim to normalize the pathways, such as rapamycin treatment, long-term LPD or Nox2 genetic inhibition, are sufficient to restore autophagic flux and ameliorate the dystrophic phenotype in *mdx* muscle [166, 169, 174, 184]. Similarly, AMPK activation in *mdx* muscle may constitute an adaptive response to the energetic stress associated with the opening of the mitochondrial permeability transition pore; reinforcing its activation with the AMP analog, AICAR, improved diaphragm muscle structure and force, likely by

increasing mitochondria integrity and autophagic flux [168]. Recently, treatment with the lipophilic statin, simvastatin, also improved muscle pathology in adult and old *mdx* mice, at least partly by re-inducing autophagic flux, although its effect on Akt/PKB-mTORC1 and AMPK signalling was not tested [185]. In 2013, Hindi et al. reported a dual effect of Traf6, depending on disease stage, in dystrophin-deficient muscle. Increased Traf6 levels, detected early in *mdx* muscle, may contribute to the massive inflammatory response in the diseased muscle. Consistently, Traf6 depletion improved muscle function in young *mdx* mice, likely by limiting tissue inflammation and promoting its regeneration [170]. Conversely, with age, Traf6 inhibition worsened muscle degeneration: This deleterious effect was associated with autophagic flux reduction, despite Akt/PKB-mTORC1 inhibition in *mdx:Traf6*^{-/-} mice [170]. Traf6-dependent autophagy induction would thus have a predominant, protective effect during late stages of the disease by promoting the clearance of by-products and damaged organelles. Hence, therapeutic strategies targeting autophagy in DMD muscle may be promising, especially during advanced stages of the disease. In contrast, a proper balance would be required in earlier phases to equilibrate muscle homeostasis, regeneration, growth, and to limit tissue inflammation [186].

X-linked myotubular myopathies – Congenital XLMTM are caused by mutations in the *MTM1* gene, encoding myotubularin 1. They are characterized by the predominance of centronucleated myofibres and thus part of the centronuclear myopathies (CNM). Pathological features observed in muscle biopsies from patients and/or muscle from Mtm1-deficient mice and dogs include abnormal mitochondria, ubiquitinated proteins, p62-positive aggregates, glycogen accumulation and/or autophagic vesicles [187–190]. While LC3 levels were increased in *Mtm1*-null mouse muscle, LC3II lipidation was impaired upon starvation of the mutant mice [187], suggesting that autophagy induction is blocked. Akt/PKB-mTORC1 activation, which was detected in Mtm1-deficient muscle as well as upon acute AAV-induced Mtm1 depletion, may contribute to autophagy blockade in the diseased muscle, as indicated by increased phosphorylation of Ulk1 at Ser757 [187, 189, 191]. In contrast to COLVI-RM, simultaneous FoxO3 activation was associated with increased expression of autophagy genes (e.g. *Map1lc3* or *Sqstm1*), which may participate in their accumulation in mutant muscle [189]. Intriguingly, while treatment with an mTORC1 inhibitor restored autophagy and improved

the muscle phenotype in Mtm1-deficient mice, AAV-mediated delivery of Mtm1 normalized autophagy markers independently of the FoxO and mTORC1 pathways [187, 191]. Moreover, the pre-symptomatic accumulation of autophagosomes in Mtm1-deficient mouse muscle, in the absence of FoxO/mTORC1 alteration, suggests that Mtm1 depletion may primarily cause an abnormal induction of the autophagic flux [189]. This hypothesis is consistent with the role of Mtmr14 (Myotubularin-related protein 14 - Jumpy) to negatively regulate autophagy. The PI3P phosphatase activity of Mtm1 and Mtmr14 is supposed to counteract the function of Vps34 at the phagophore stage. *Mtmr14* knock-down in C2C12 cells resulted in a loss of autophagy control and increased proteolysis [192]. Since mutations in the *MTMR14* gene were associated with CNM, one can hypothesize that uncontrolled, excessive autophagic flux may be part of the pathomechanisms leading to myotubularin-related disease [193]. Notwithstanding, Mtmr14 deficiency in mice or zebrafish only led to a slight perturbation of autophagy although combined knock-down of *mtm1* and *mtmr14* in zebrafish caused a severe phenotype marked by increased autophagic flux [194, 195]. Hence, the role of myotubularins and their potential redundancy need to be further analysed to better understand the consequences of their deficiency in patients.

Laminopathies – Mutations in the *LMNA* gene, encoding A-type lamins, are responsible for several disorders (e.g. *Emery-Dreifuss Muscular Dystrophy* or *Limb-Girdle Muscular Dystrophy type 1B*) affecting, among other tissues, skeletal muscle and heart (Fig. 2). Growing evidence suggests that A-type lamins have distinct functions besides their structural role in the nuclear lamina [196]. Autophagic vacuoles described in muscle biopsies of patients and in skeletal muscle of lamin A/C-deficient mice first suggested that increased autophagy (i.e. nucleophagy) may constitute an adaptive mechanism to remove abnormal nuclei in diseased myofibres [197]. Alternatively, such vacuolar structures may arise from impaired autophagy. As for COLVI-RM, DMD and XLMTM, mTORC1 activation was reported in skeletal muscle and heart from lamin A/C-deficient mice [198, 199]. While this correlates with Akt/PKB hyperactivation in cardiac muscle of patients and mutant mice, the status of Akt/PKB and FoxO signalling has not yet been analysed in the skeletal muscle of *Lmna*-knock-in (KI) or knockout (KO) mice. Similarly, although results obtained in diseased heart indicate that autophagy induction is

impaired [199], few data have been collected in skeletal muscle. Levels of some autophagy markers (e.g. LC3 or Atg7) are increased, but only Ulk1 inhibition, as shown by its elevated mTORC1-dependent phosphorylation in muscle from mutant mice, suggests that autophagy induction may be hampered in skeletal muscle [198] (Fig. 2). Treatment with rapamycin or temsirolimus, which both inhibit mTORC1, increased lifespan of lamin A/C-deficient mice and improved heart function, likely by restoring autophagic flux [198, 199]. Despite the failure of dietary administration of rapamycin to reduce mTORC1 in mutant skeletal muscle and consequently to normalize autophagy, intriguingly, the treatment reduced desmin accumulation in the tissue and increased motor coordination (assessed by a rotarod test) of the mice. Hence, whether autophagy dysregulation contributes to pathogenic alterations of the tissue remains to be investigated in more detail.

PATHOGENIC ROLE OF AUTOPHAGY IN AUTOPHAGIC VACUOLAR MYOPATHIES

Muscle disorders discussed in the following sections belong to the group of AVM, characterized by the accumulation of autophagic vacuoles in muscle biopsies from patients. AVM include i) lysosomal storage diseases (LSD; e.g. Danon disease), in which vacuoles result from primary lysosomal dysfunction, and ii) myopathies with rimmed vacuoles (RV; e.g. IBMPFD), interpreted as autophagic vacuoles due to their reactivity with lysosomal markers. Although the mechanisms underlying their formation are not known, RV may only secondarily arise from lysosomal defects, which may lead to autophagosome proliferation and enlargement. An alternative, non-exclusive hypothesis is that RV derives from defective nuclear breakdown [200].

Muscle pathologies related to impaired maturation of autophagic vesicles

Glycogen storage disease type IIb – GSDIIb (or Danon disease) is an X-linked multisystemic disorder characterized by severe hypertrophic cardiomyopathy, normally alongside a mild myopathy with predominant involvement of proximal muscle and potential mental retardation [201]. Although dominant mutations in *LAMP2* were first shown to cause the disease in 2000, there is still little data on the pathomechanism underlying skeletal muscle alterations [202]. *LAMP2* is a transmembrane protein mainly

found in lysosomes and late endosomes, suggesting that its deficiency may affect lysosome-related processes, such as autophagy. Indeed, vacuoles containing glycogen and autophagic debris, surrounding acid phosphatase-positive basophilic granules recognized as lysosomes, accumulate in muscle biopsies from Danon patients [202]. Vacuoles display a single membrane with sarcolemmal and extracellular matrix proteins, forming at their inner surface a layer of basal lamina (so called AVSF for autophagic vacuole with sarcolemmal features); their occurrence increases with age but their formation remains poorly understood (Fig. 3). It is noteworthy that such vacuoles are also found in cardiac muscle from Danon patients, in muscle and non-muscle tissues from *LAMP2*-deficient mice, and in *Lamp1/2*-double KO embryonic cells [203–205]. *LAMP2* depletion in hepatocytes and cardiomyocytes delays autophagosome maturation, restricts long-lived protein degradation and increases the half-life of autophagic vesicles [203]. Accordingly, *LAMP2* and/or *LAMP1* seem to be required for the efficient recruitment of Rab7 and the fusion of (auto)phagosomes with lysosomes in macrophages, hepatocytes, and mouse embryonic fibroblasts (MEFs) [206, 207]. Nonetheless, the proteolysis rate in *Lamp1/2*-KO MEFs was unaffected, suggesting that the role and importance of *LAMP2* differ between cell types [204]. Further, *LAMP2* deficiency in hepatocytes altered the recycling of the mannose-6-phosphate receptor from endosomes to the trans-Golgi network, which would cause mis-targeting and secretion of lysosomal enzymes (e.g. Cathepsin D) [208]. It remains unsolved whether autophagic vacuoles accumulate in muscle fibres because of defective fusion with lysosome or restricted lysosome degradative capacity. Interestingly, overexpression of Vps15/Vps34 in Danon human myoblasts reduced glycogen and LC3 accumulation, suggesting that compensation by other functional proteins may constitute a therapeutic option [102]. Lastly, although the *LAMP2B* splice isoform is predominantly expressed in skeletal muscle, *LAMP2A* deficiency may impair the degradation of specific cytosolic substrates targeted for chaperone-mediated autophagy (CMA). No major alteration of CMA was detected in *LAMP1/2*-deficient MEFs [205] or neuronal cells [209].

Glycogen storage disease type II – As for Danon disease, GSDII (or Pompe disease) is characterized by glycogen accumulation in lysosomes of muscle fibres. The defect arises from genetic deficiency in

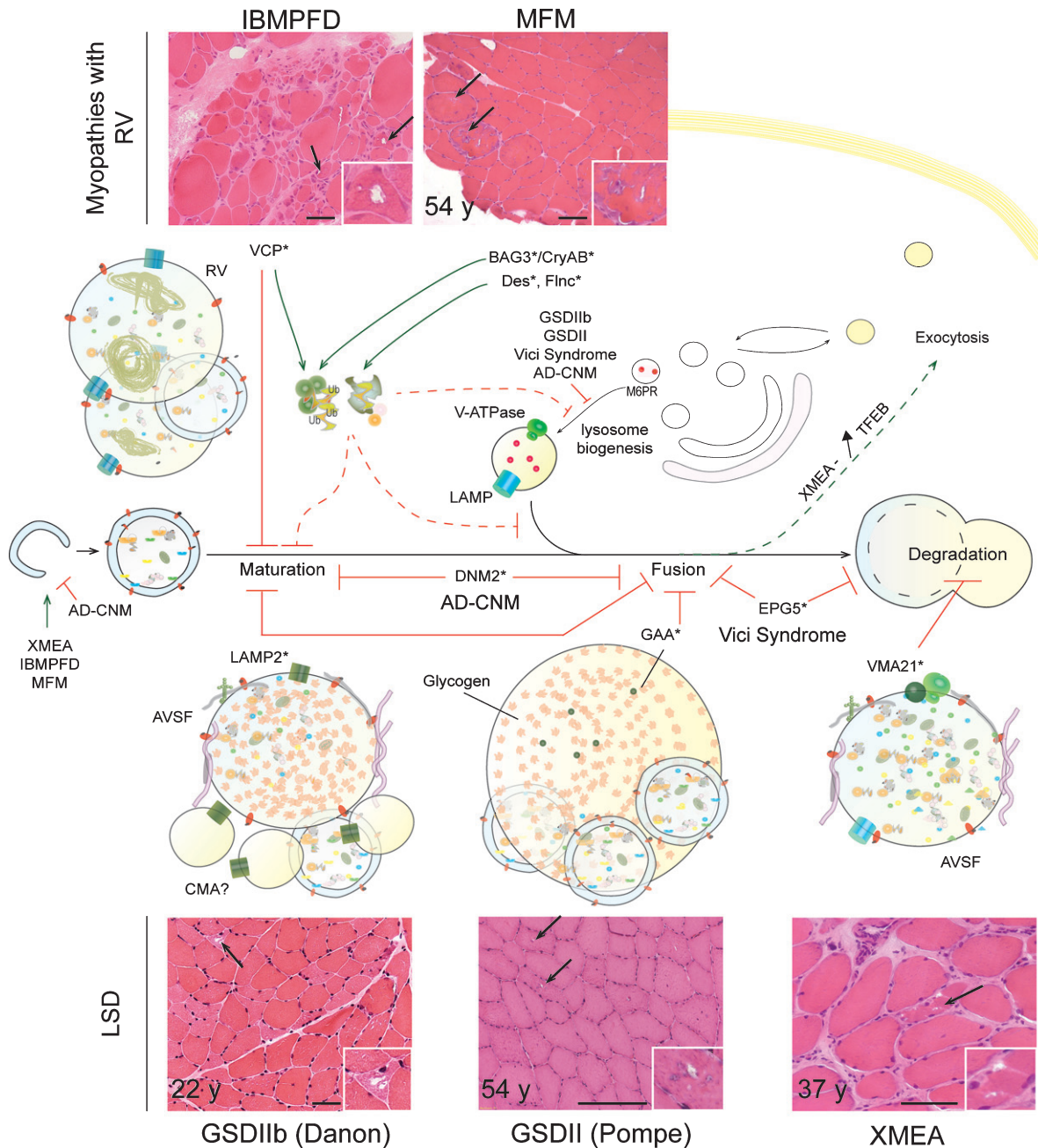


Fig. 3. Neuromuscular disorders related to defective autophagosome maturation or lysosomal dysfunction. In myopathies with rimmed vacuoles (RV) (e.g. IBMPFD and MFM), autophagic vesicles would enlarge due to blockade of the maturation and fusion steps, likely caused by the accumulation of protein aggregates. In IBMPFD, VCP deficiency may also directly alter autophagosome maturation. In lysosomal storage disorders (LSD), defective autophagosome maturation/fusion and/or altered degradation steps lead to the formation of autophagic vacuoles with sarcolemmal features (AVSF) or enlarged lysosomes; glycogen massively accumulates in these vesicles in GSDIIb and GSDII diseases. Centronuclear myopathies related to DN2 (AD-CNM) deficiency may also involve a defect in maturation/fusion steps. Of note, lysosome biogenesis and autophagy induction seem to be perturbed in some of these diseases. Red lines represent inhibition; green arrows show activation. Arrows in the H&E-stained muscle cross-sections indicate vacuoles. The pathogenic protein is indicated by an asterisk. CryAB, α -crystallin B chain; Des, desmin; Flnc, filamin C; M6PR, mannose-6 phosphate receptor; Ub, ubiquitin; y, year. Scale bar, 100 μ m.

acid α -glucosidase (GAA), the enzyme responsible for glycogen hydrolysis. While earlier hypotheses proposed that glycogen-filled lysosome enlargement and rupture led to muscle alterations, recent observations suggest that autophagy impairment may contribute to the loss of muscle homeostasis in GSDII [210, 211]. Autophagic vacuoles and debris, protein aggregates, as well as increased levels of LC3II, p62 and ubiquitinated proteins have been described in muscle biopsies from GAA-deficient patients [74] and GAA-KO mice [212–214] (Fig. 3). Confocal microscopy further confirmed the presence of large areas of autophagic build-up in the central region of muscle fibres of mutant mice, suggesting inefficient autophagosome disposal [215]. Elegant time-lapse analysis revealed defective autophagosome-lysosome fusion in GAA-KO fibres, which could be responsible for the observed defects [216] (Fig. 3). It remains to be answered whether altered fusion and concomitant restriction in lysosome biogenesis are caused by glycogen accumulation, lysosome rupture or insufficient acidification of endocytic/lysosomal vesicles [213, 217]. When glycogen accumulation was restricted by knocking-down or depleting glycogen synthase in GAA-deficient muscle, autophagic build-up was reduced and muscle function improved [218, 219]. In light of the increased levels of autophagy-related proteins, including LC3, Atg7 or Beclin1, in GAA-deficient muscle, some authors suggested that simultaneous enhanced autophagy induction, related to GSK3 β activation, may aggravate autophagic build-up [215, 220]. However, in the few experiments that tested the autophagic flux using lysosomal inhibitors, no major difference was detected between control and mutant cells [74, 221]. Importantly, numerous reports have established that resistance of skeletal muscle to enzyme replacement therapy (ERT) with recombinant human (rh) GAA is caused by autophagic build-up, which restricts trafficking and processing of rhGAA [222]. ERT was shown to be efficacious in heart tissue of severely affected patients but did not reduce skeletal muscle alterations, specifically because of its restricted effect on type II fibres [211]. Confocal microscopy revealed that rhGAA enzyme was trapped in autophagic vesicles in GAA-KO single fibres, rather than efficiently translocated into lysosomes as seen in control cells [223].

Attempts to modulate autophagy in order to limit muscle alterations have led to divergent results. TFEB or TFE3 overexpression in GSDII myotubes and GAA-KO muscle was sufficient to

restore autophagosome-lysosome fusion, promote exocytosis and clear mutant fibres from enlarged lysosomes, glycogen and vacuoles [216, 224, 225]. Functional autophagy constituted a prerequisite for TFEB-mediated lysosome exocytosis therapy [216]. Similarly, PGC1 α overexpression cleared autophagic build-up, likely by improving lysosome biogenesis, but did not impact on glycogen accumulation [226]. Moreover, rapamycin-treated primary myotubes from patients displayed reduced vacuolization [74], whereas addition of torin1/2, another inhibitor of mTOR, failed to reduce lysosome enlargement despite autophagy induction [216]. Conversely, genetic suppression of autophagy by depleting Atg7 prevented glycogen accumulation and autophagic build-up in GAA-KO mice, while Atg5 depletion in mutant mice [214] and Atg7 knock-down in primary mouse myotubes [213] had no effect on glycogen storage. Moreover, Atg7-GAA double KO mice displayed no major change in their overall phenotype [220] and Atg5 loss even worsened the muscle phenotype of GAA-KO mice. Combining ERT with autophagy inhibition led to significant improvements in glycogen removal and lysosomal function, which were related to decreased autophagic build-up with Atg7 depletion, but not Atg5 suppression [220]. However, there was no evidence for improvement of muscle function. Hence, although the pathogenic role of autophagy blockade is well established in GSDII, whether strategies should target or limit its induction in combination with other therapeutic options remains to be discussed and functionally analysed.

Vici syndrome – Vici syndrome is a rare disorder including neurologic, ocular, cardiac and muscular symptoms; mutations in the *EPG5* gene (ectopic P-granules autophagy protein 5) were reported in 2013 [227]. Epg5-deficient mice reproduced, among other alterations, the myopathy described in Vici patients, with fibre atrophy and degeneration [228]. Interestingly, accumulation of vacuoles, enlarged mitochondria and glycogen, as well as increased levels of LC3II, p62 and Nbr1 were detected in muscle from patients and/or *Epg5*-KO mice [227, 228]. In patient fibroblasts and *Epg5*^{-/-} MEFs, autophagic flux restriction was related to altered maturation of autophagic vesicles (at the fusion or degradation steps) and processing of endo/lysosomal compartments (Fig. 3). Whether these defects contribute to pathology in muscle and non-muscle tissues from Vici patients, and whether they can be rescued by modulating autophagy induction or expressing potentially compensating proteins, remains to be investigated.

Inclusion body myopathy associated with Paget's disease of the bone and frontotemporal dementia – IBMPFD is an autosomal dominant multi-systemic disorder caused by mutations in the *VCP* gene, encoding a type II member of the AAA+-ATPase family, p97/VCP (Valosin Containing Protein), located in the endoplasmic reticulum of all cells. Most IBMPFD patients suffer from muscle weakness and characteristic alterations, including rimmed vacuoles (RV) and tubulofilamentous depositions are observed in muscle cells. Accumulation of p62 and ubiquitinated proteins points to autophagy defects in IBMPFD [229, 230]. By functioning as an ubiquitin-selective segregase, VCP is involved in protein processing, recycling and degradation [231]. VCP deficiency impairs protein quality control by altering the unfolded protein response (UPR) and endoplasmic reticulum-associated protein degradation (ERAD) [232] (Fig. 3). VCP mutants may also limit the clearance of the resulting protein aggregates by altering the formation of aggresomes and their delivery to the autophagy machinery [233]. Moreover, several reports suggest that VCP plays a role in endosome and autophagic vesicle trafficking: Autophagosomes accumulate in muscle from IBMPFD patients and VCP-deficient mice, as well as in primary human myoblasts [229, 234–237]. Ju et al. further established *in vitro* that VCP deficiency impairs autophagosome maturation and fusion, hence leading to non-degradative vesicles [238] (Fig. 3). Nonetheless, a recent paper indicated that, although immature, autophagic vesicles are acidified and contain lysosomal enzymatic activity, suggesting a defect in the ultimate stages of their maturation [239].

Interestingly, autophagy and endo-lysosomal trafficking defects in IBMPFD seem to impair mTORC1 activation and thereby worsen muscle alterations by promoting autophagy induction. Hence, whilst rapamycin treatment aggravated muscle weakness and atrophy of VCP^{R155H}-transgenic mice, electroporation of an active form of Rheb or Akt/PKB in mutant muscle increased fibre size [240]. Alternative strategies aiming at normalizing autophagic flux in muscle, such as exercise or a lipid-enriched diet, ameliorated the phenotype of VCP^{R155H}-KI homozygous and heterozygous mice [241–243]. However, even if changes in LC3 and p62 levels were reported in the treated mice, further analyses are required to establish whether those strategies impact on the autophagic flux, and whether they do so by limiting autophagy induction or by releasing the degradation steps. This

may be essential to identify optimal therapeutic targets and strategies for IBMPFD.

Autosomal-Dominant Centronuclear Myopathy – Dominant mutations in the *DNM2* gene, encoding the GTPase dynamin 2, cause AD-CNM which are histologically recognized by the prominent internalization of myonuclei, the presence of sarcoplasmic radial strands and a predominance of type I fibres [244, 245]. AD-CNM is not included in AVM, and no hallmark of autophagy blockade is present in muscle biopsies from patients. However, as DNM2 is involved in intracellular membrane trafficking, endocytosis and cytoskeleton modulation, its deficiency may impact on autophagy and on the endo/lysosomal system. Data pointing in this direction were obtained in KI (p.R465W) mutant mice, a recognized model for the disease [246, 247]. Early lethality of homozygous mutant mice was related to defects in global body metabolism consistent with defective autophagic flux in metabolic organs during the neonatal period [246]. A slight increase in LC3II and p62 levels was detected in liver, but not in muscle, from homozygous neonates. Accumulation of p62 and non-degradative autophagosomes was also found in DNM2-deficient MEFs [246]. The presence of abnormal autophagosome maturation was supported by reduced acidification of autophagic vesicles, which may involve impaired fusion between autophagosome and endo/lysosomal compartments and/or defective lysosomal biogenesis (Fig. 3). Accumulation of LC3II and of autophagic vesicles in muscle from starved, 6 month-old heterozygous mice further pointed to altered autophagosome maturation in diseased muscle. However, the autophagic flux also seems compromised at the induction steps in mutant MEFs, with reduced LC3II accumulation observed upon bafilomycin treatment [246]. Hence, DNM2 deficiency may lead to combined defects in autophagosome formation and maturation. Since there was no drastic accumulation of autophagy vacuoles in diseased muscle, one may assume that reduced induction may predominate in DNM2-deficient muscle. This would be consistent with the clinical and histological similarity to XLMTM, related to impaired autophagy induction (see above).

Muscle pathologies related to impaired degradation steps

X-linked myopathy with excessive autophagy – XMEA specifically affects skeletal muscle and ranges

from severe neonatal to slowly progressive late-onset forms; it was only recently linked to mutations in the *VMA21* gene, which encodes a major chaperone of the lysosomal proton pump V-ATPase [248–250]. Muscle histology revealed fibre splitting with internalized capillaries, complement C5b-9 deposition and AVSF, as described for Danon diseased muscle [251] (Fig. 3). While there is currently no mouse model for the disease, analyses conducted in fibroblasts and lymphoblasts from XMEA patients have brought information regarding potential pathomechanisms. Ramachadran et al. established that *VMA21* deficiency restricts assembly and activity of the V-ATPase, thereby leading to increased lysosomal pH, altered lysosomal enzymatic activity and strongly reduced long-lived protein degradation [248]. Colocalization of autophagosome and lysosome markers suggests an effective fusion of these vesicles but a defect in autophagy degradation steps (Fig. 3). Hence, (macro)autophagy, but also microautophagy and CMA may be impaired in XMEA. Based on the increased expression of some autophagy genes (e.g. Beclin1, LC3), the authors further suggested that autophagy blockade may lead to mTORC1 inhibition by reducing the release of amino acids, and thereby to increased autophagy induction (Fig. 3). This may aggravate autolysosome proliferation and enlargement [248]. Hence, mTORC1 dysregulation as well as potential activation of TFEB, which may explain the observed autophagic vesicle extrusion at the plasma membrane still need to be documented in XMEA muscle cells. Lastly, development of a mouse model for the pathology is needed to compare autophagy impairment in skeletal muscle *vs* unaffected tissues (including cardiac muscle [252]) and to determine the consequences of modulating autophagy, mTORC1 and/or TFEB on the muscle phenotype.

Muscle diseases with autophagy perturbation related to abnormal protein aggregation

Myofibrillar myopathies – MFM are rare neuromuscular disorders characterized by muscle weakness, related to focal myofibril disintegration and caused by mutations in genes encoding structural proteins (e.g. desmin or filamin C) or chaperones (BAG3, α B-crystallin) found in the Z disk region [253]. Protein aggregates and RV constitute a hallmark of MFM and point to autophagy perturbation [254, 255] (Fig. 3). This is supported by the presence of autophagy-related proteins (e.g. p62, LC3) in

inclusions, revealed by proteomic analysis following laser-microdissection [256]. Whether accumulation of autophagy-related proteins reflects an adaptive induction of autophagy in response to protein aggregates or is rather a consequence of autophagy impairment, remains unclear. Of note, some mutations in structural proteins favour the aggregation of their mutated form, in the absence of an autophagy defect [257]. In cardiomyocytes, aggregate-prone mutated forms of desmin or α B-crystallin target autophagy induction in the early stages of the disease, but probably insufficiently to prevent their accumulation over a prolonged time period [258, 259]. Interestingly, BAG3 and α B-crystallin function as chaperones for structural proteins, and either prevent their aggregation or favour their degradation. In particular, BAG3 is involved in CASA, which is induced upon mechanical tension during muscle contraction and permits to degrade damaged contractile components, such as filamin C [67]. BAG3-deficient mice develop a lethal myopathy marked by myofibrillar degeneration and myofibre apoptosis [260]. Abnormal aggregation of mutated BAG3^{P209L} also reproduced myofibrillar disintegration in zebrafish, likely by trapping wild-type BAG3 [261]. In these conditions, aggregates may perturb the endo/lysosomal function as well as the maturation of the autophagosome, thereby leading to a vicious cycle where increased induction and impaired autophagic degradation accelerate aggregate and vacuole formation. If so, therapeutic interventions aiming at inducing autophagy should be applied in the early stages of the disease, before aggregates accumulate. Beneficial effects of such strategies have been obtained in desmin-related cardiomyopathy but remain to be tested in skeletal muscle [257, 262–264].

Similar mechanisms may underlie *Oculopharyngeal Muscular Dystrophy* (OPMD) with the expanded, mutated form of PABPN1 (Poly(A) Binding Protein Nuclear 1) being prone to aggregate in myonuclei. Whether dysregulated expression of autophagy-related proteins detected in OPMD reflects an induction and/or a blockade of autophagy remains to be established [160, 265, 266]. As for MFM related to BAG3 or α B-crystallin deficiency, muscle alterations caused by dominant mutations in *DNAJB6* (associated to *Limb-girdle muscular dystrophy 1D/E*), encoding another co-chaperone likely involved in CASA, may imply excessive protein aggregation, compensatory autophagy induction and/or perturbation of the autophagic flux, leading to Z-disk degradation, debris accumulation and fibre

vacuolization [267–269]. Mutations in the *SIL1* gene, which encodes a co-chaperone for BiP, a central quality controller for ER proteins, lead to a degenerative, vacuolar myopathy (as part of the *Marinesco-Sjögren syndrome*). Pathogenesis may also include induction of inefficient autophagy, in response to nuclear/ER stress and misfolded protein accumulation [270].

Muscle disease with abnormal autophagy features but no molecular mechanisms established

Sporadic Inclusion Body Myositis (sIBM) is an inflammatory, degenerative muscle disorder clinically marked by atrophy and weakness of distal muscle. Histologically, sIBM is characterized by lymphocytic infiltration, RV and intracellular amyloid deposition [271]. Although the pathomechanisms remain unclear, accumulation of autophagic vacuoles, ubiquitinated protein aggregates and abnormal mitochondria led to the hypothesis that autophagy impairment contributes to the disease [272, 273]. Consistently, reduced activity of lysosomal enzymes, as well as increased levels of p62 and LC3II were found in sIBM muscle biopsies and in human myofibres subjected to sIBM-mimicking culture conditions [274, 275]. Reduced GSK3-dependent phosphorylation of Nbr1 may promote the aggregation of ubiquitinated proteins, despite its increased expression in sIBM biopsies [276, 277]. Further, altered CMA leading to α -synuclein accumulation and reduced mitophagy due to lysosomal dysfunction may contribute to mitochondrial abnormalities detected in diseased muscle [271, 278]. Notwithstanding, elevated expression of some autophagy- and CMA-related components was reported in sIBM muscle biopsies, which may reflect increased induction of the process [278, 279]. Although therapeutic strategies potentiating lysosomal activity (e.g. sodium phenylbutyrate) are proposed to limit myofibre vacuolization and toxic product accumulation, further experiments overcoming the limits of the current *in vivo* and *in vitro* disease models are needed to assess the involvement of autophagy in sIBM onset and progression [280].

GNE myopathy (also reported as hIBM2 or *Distal Myopathies with Rimmed Vacuoles*) is an autosomal recessive disorder caused by mutations in the *GNE* gene and predominantly affecting distal muscle. The gene encodes an enzyme essential for sialic acid production; its deficiency may lead to glucoconjugate hypo-sialylation, which may impact on several

cell signalling pathways [281]. RV, cytoplasmic and nuclear inclusions, and abnormal mitochondria accumulate in muscle biopsies from patients. A knock-in mouse model expressing a mutated form (D176V) of the enzyme reproduced the muscle phenotype observed in *GNE* myopathy [282]. In this model, accumulation of A β peptide and of its protein precursor (A β PP) preceded RV formation, suggesting that autophagy defects are secondary events in the disease. Accordingly, perturbation in hypo-sialylated protein folding and trafficking may cause ER stress and target autophagy induction, thereby contributing to muscle degeneration [283, 284]. Sialic acid supplementation alleviated muscle alterations in mutant mice [285] and is currently being tested in clinical trials [281]. Other distal myopathies (DM), such as *Welander DM* (caused by deficiency in TIA1 mRNA binding protein), *Vocal Cord and Pharyngeal DM* (linked to mutations in the nuclear matrix gene *MATR3*) or *Tibial Muscular Dystrophy*, caused by mutations in *TTN* (encoding Titin), are marked by RV and may involve autophagy perturbation similar to *GNE* myopathy [286, 287].

For several additional myopathies, such as *dysferlinopathies* [288] or the rare *megaconial type congenital muscular dystrophy* [289, 290], autophagy perturbation has been suggested but additional investigations are needed to assess whether these perturbations are part of the pathomechanism leading to muscle alterations and how they can be linked to the genetic origin of the disease.

CONCLUSION

Abnormal autophagic flux has now been suggested to be involved in the pathogenesis of numerous muscle disorders, in which it may significantly contribute to muscle atrophy. However, many of the studies lack a comprehensive picture of the state of the flux in the diseased muscle and thus conclusions on the pathological significance of autophagy impairment often seem premature. In particular, efforts should be made to clarify whether the flux is enhanced in cases where autophagosomes or autophagy markers accumulate. The use of animal models, combined with *in vitro* analyses of human muscle cells, is required before a conclusion can be made on the pathogenic role of autophagy in a disease. To this end, a comprehensive analysis of the autophagic flux in muscle remains to be conducted, if possible, at different stages of the disease, in the cases of MDC1A, DM1,

XLMTM, laminopathies, GSDIIb, AD-CNM and GNE myopathy. Another drawback is the need to elucidate whether a given autophagy defect constitutes a primary pathogenic event or rather a consequence (or adaptive response) to primary pathogenic muscle alterations. Autophagy perturbations in AVM are primarily or secondarily related to lysosome dysfunction and have an established link to the genetic cause (with the exception of sIBM). By contrast, it is delicate, although essential, to decipher the molecular mechanisms leading to signalling dysregulation and abnormal autophagy induction in the case of diseases caused by deficiency in components of the extracellular matrix (e.g. COLVI; laminin) or of the cytoskeleton (e.g. A-type lamins). Once autophagy dysfunction is established, one needs to consider the efficacy of therapeutic approaches aiming at normalizing the flux, both on muscle phenotype and the restoration of the autophagy flux. Along these lines, further investigations to identify signalling pathways at play in autophagy control, or those which may be dysregulated in diseases, will help to develop specific strategies for individual disorders. A major challenge will be to avoid excessive correction of the flux, which may result in another extreme with too much (or alternatively insufficient) protein and organelle disposal. Therapeutic options should aim at balancing the flux, and therefore, the long-term side effects on muscle mass and force should always be considered. Although out of the scope of this review, we would like to emphasize that other pathological conditions associated with skeletal muscle atrophy involve autophagy perturbation. Examples include neurodegenerative disorders, such as spinobulbar muscular atrophy, in which autophagy induction may contribute to muscle wasting [291–293]. Excessive autophagy seems also to contribute to cachexia-induced muscle atrophy [98], while a decline in the autophagic capacity may contribute to age-related muscle alterations [14, 108]. Further understanding of the consequences of autophagy defects in neuromuscular disorders will help to better elucidate the mechanisms at play in these conditions and help to guide us toward therapeutic strategies to counteract the loss of muscle homeostasis.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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