Autophagy in Muscle Stem Cells

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Abstract

Muscle stem cells, also known as satellite cells, are responsible for the regenerative capacity of adult muscle tissue in response to stress and injury. Upon regenerative stimuli, satellite cells are activated and undergo myogenic commitment. Myogenic progenitors, which are termed myoblasts, undergo rapid proliferation, propagation, and differentiation into myocytes, which then fuse with each other to form new myotubes or to a pre-existing myotube. This process of myogenic differentiation is metabolically demanding and involves cellular remodeling of organelles and cellular architecture. Autophagy, a catabolic mechanism involving the sequestration of cellular contents into double membrane autophagosome vesicles, is strongly implicated at various stages during myogenesis; from the satellite stem cell to the mature muscle tissue. Moreover, aberrant autophagy (both the overstimulation and inhibition of autophagy) in both satellite cells and mature muscle cells can be detrimental for muscle health and physiology. This chapter outlines the importance of autophagy in maintaining skeletal muscle tissue homeostasis and satellite cell regenerative capacity.

Keywords

Autophagy, Muscle stem cell, Myoblast, Myogenesis, Satellite cell, Skeletal muscle

Abbreviations

AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AMPK	AMP-activated protein kinase
BAG3	BAG cochaperone 3
CASA	chaperone-assisted selective autophagy
DM1	myotonic dystrophy type 1
ECM	extracellular matrix
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
HSPA8/HSC70	heat shock 70 kDa protein 8
LD	lipid droplet
LSM	lipid storage myopathy
MFN1/2	mitofusin 1/2
miRNA	microRNA
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
NAF-1	nutrient-deprivation autophagy factor-1
NMJ	neuromuscular junction
p62/SQSTM1	autophagy receptor p62/sequestome 1
PGC-1α	proliferator-activated receptor-γ coactivator-1α
ROS	reactive oxygen species
SIRT1	sirtuin-1
TALEN	transcription activator-like effector nuclease
T tubules	transverse tubules

1 Introduction

Skeletal muscle is a highly organized contractile tissue making up roughly 40% of human whole body lean mass (Neel et al. 2013). The human musculoskeletal system is responsible for allowing movement, the maintenance of posture, body position and body temperature (Frontera and Ochala 2015). While both skeletal and cardiac muscle are forms of striated muscle, cardiac muscle functions as a self-stimulating and non-fatiguing group of muscle cells (Mukund and Subramaniam 2020). In contrast, the motor activity of skeletal muscle is voluntary and the tissue itself exhibits fatigue and has high energy requirements (Mukund and Subramaniam 2020).

1.1 Skeletal muscle architecture

Skeletal muscles are supported by the cytoskeleton network composed of bundles of fascicles, which consists of bundles of muscle fibers, termed myofibers (Mukund and Subramaniam 2020). Single myofibers are multinucleated and of variable lengths and shapes containing several myofibrils arranged in parallel and units of sarcomere, the basic contractile unit of muscles, arranged in series (Roy and Edgerton 2009). Single myofibers are encased by the sarcolemma, which acts as the muscle plasma membrane and is directly involved in synaptic transmission, action potential propagation, and excitation-contraction coupling in response to stimulation (Frontera and Ochala 2015). The sarcolemma is connected to extracellular matrix (ECM) that surrounds the skeletal muscle fibers and is encapsulated by the basal lamina, an outer membrane layer that defines the anatomical length and boundary of a myofiber (Frontera and Ochala 2015). The sarcoplasmic reticulum, which is the main calcium store in skeletal muscles, forms a network with transverse (T) tubules that surrounds the myofibrils (Rossi et al. 2008). The connection between T tubules and the sarcoplasmic reticulum is critical for the release of calcium leading to

muscle contraction in response to the action potential generated by motor neurons (Rossi et al. 2008). The ECM surrounding the muscle fibers is composed of various types of collagens, laminins, fibronectin, and proteoglycans providing mechanical support to the myofibers during contraction (Rossi et al. 2008).

1.2 Satellite cells: muscle-resident stem cells

Adult skeletal muscles are stable under normal conditions and have remarkable capacity for regeneration after injury due to the presence of satellite cells, which are muscle resident somatic stem cells accounting for 3-6% of all myonuclei (Yin et al. 2013). Satellite cells were first discovered in 1961 by Alexander Mauro upon examination of the peripheral region of myofibers dissected from the tibialis anterior muscles of the frog by electron microscopy (Mauro 1961). Notably, while fused myonuclei are scattered across the myofibers, satellite cells reside along host myofibers directly above the sarcolemma and under the basal lamina (Mukund and Subramaniam 2020; Mauro 1961). Satellite cells can be identified by their unique anatomical location with electron microscopy, which also reveals their morphological characteristics: a large nuclear-to-cytoplasmic ratio, few organelles, small nucleus, and condensed interphase chromatin (Mauro 1961; Schultz et al. 1978). This morphology supports the notion that most satellite cells in healthy, unstressed muscles are mitotically quiescent and transcriptionally inactive (Yin et al. 2013). Satellite cells can also be identified by immunofluorescence using antibodies specific to satellite cell markers such as the paired box transcription factor, PAX7 (Schultz et al. 1978; Seale et al. 2000). *In situ* hybridization analyses in skeletal muscle tissues demonstrated that *Pax7* mRNA is expressed exclusively in satellite cells (Seale et al. 2000). Moreover, *Pax7* is expressed in

proliferating myoblasts derived from the satellite cell lineage and its expression is downregulated during myogenic differentiation (Seale et al. 2000).

Cell surface protein markers such as ITGA7, ITGB1, caveolin1, CD34, M-cadherin, CXCR4, N-CAM, syndecan 3/4 and VCAM-1 can be used for identifying satellite (Dumont et al. 2015). Fluorescence-activated cell sorting (FACS) is a widely used method to isolate satellite cells from freshly harvested muscle (Maesner et al. 2016). Using *Pax7-zsGreen* transgenic reporter mice, FACS with the cell surface markers ITGA7, ITGB1, CXCR4 and CD34 were shown to allow for successful prospective isolation of *Pax7*-expressing satellite cells (Maesner et al. 2016). Moreover, a combination of CD34 and ITGA7 enables the isolation of a more restricted, namely quiescent, subset of satellite cells (Maesner et al. 2016). As well, VCAM-1 is expressed in satellite cells from young and old mice during quiescence and upon injury, and may be used for isolating activated satellite cells (Liu et al. 2015).

1.3 The role of satellite cells

Satellite cells are responsible for postnatal growth of skeletal muscles (Gattazzo et al. 2020). During this period, satellite cells differentiate and contribute to muscle growth at varying rates; they exist in heterogenous pools comprised of 80% fast-dividing and 20% slow-dividing populations (Schultz 1996). The slow-dividing population spends more time in G_0-phase between divisions and serves as a source for resident stem cells (Schultz 1996). Flow cytometry analyses assessing various myogenic markers through stages of postnatal development demonstrated that postnatal growth is accompanied by waves of satellite cell commitment and differentiation (Gattazzo et al. 2020). Specifically, by assessing markers of a cycling signature $PAX7^+/Ki67^+$, non-cycling $PAX7^+/Ki67^-$, and the quiescence marker CD34, it was found that the levels of cycling

satellite cells is highest in the early postnatal stage and becomes less prevalent as the muscles develop until adulthood when cycling satellite cells are absent and satellite cells express CD34 (Gattazzo et al. 2020).

While satellite cell contribution to muscle is most apparent during early muscle development, satellite cells continue to contribute to muscle throughout life (Keefe et al. 2015). Genetic lineage tracing experiments have demonstrated that satellite cells in adult muscles contribute to muscle homeostasis in sedentary conditions (Keefe et al. 2015). *Pax7*-expressing satellite cells were labeled upon tamoxifen induction in $Pax7^{CreERT2}$:$Rosa^{mTmG}$ mice, allowing visualization of membrane-bound GFP following Cre-mediated recombination (Keefe et al. 2015). All myofibers examined over a 12- or 20-month period were GFP-positive indicating that satellite cells have contributed to these myofibers despite the lack of muscle injury or stress (Keefe et al. 2015). These results demonstrate that satellite cells actively participate in maintaining normal steady-state muscle homeostasis.

1.4 Myogenesis: Quiescence, Activation, Proliferation, Differentiation and Self-Renewal

In resting muscle, satellite cells are kept in a G_0-quiescent and mononucleated state within their niche (Yin et al. 2013). Maintenance of the quiescent state is highly regulated and dependent on the expression of specific quiescence genes and post-transcriptional regulation of differentiation genes (Fukada et al. 2007). Indeed, microRNA (miRNA) pathways play a critical role in maintaining the quiescent state (Cheung et al. 2012). Microarray analysis of quiescent satellite cells revealed 22 highly expressed quiescence-specific miRNAs (Cheung et al. 2012). Specifically, miRNA-489 retains satellite cell quiescence by supressing activation as the overexpression of miRNA-489 inhibited muscle regeneration (Cheung et al. 2012). Additionally, microarray analysis

of quiescent satellite cells revealed 507 genes, including cell cycle down-regulators and myogenic inhibitory factors, that were highly expressed in the quiescent state (Fukada et al. 2007). Analysis of histone modifications via chromatin immunoprecipitation followed by sequencing have demonstrated that quiescent satellite cells are primed for rapid activation rather than staying strictly dormant (Liu et al. 2013). An overwhelming majority of genes in quiescent satellite cells are marked by tri-methylated histone H3 lysine K4 (H3K4), a marker of active transcription, while only a few are marked with tri-methylated histone H3 lysine K27 (H3K27), a marker of gene repression, at the transcription start site (Liu et al. 2013; Wysocka et al. 2006; Bogliotti and Ross 2012).

Further to maintaining muscle homeostasis, satellite cells are responsible for the regenerative capacity of adult muscle tissue in response to stress and injury (Yin et al. 2013). Using *Pax7*^{CreERT2} mice to genetically label satellite cells and characterize their response to muscle injury, Murphy and colleagues found that all regenerated muscle derives from *Pax7*-expressing satellite cells (Murphy et al. 2011). Importantly, genetic ablation of satellite cells via Cre-mediated expression of diphtheria toxin A results in a complete and irreversible loss of muscle regeneration (Murphy et al. 2011). Similar conclusions were drawn using an alternative mouse model with a different *Pax7*^{CreERT2} allele (Lepper et al. 2011). In a separate and complementary approach to deplete satellite cells that were genetically engineered to express the human diptheria toxin receptor following local intramuscular injection of diptheria toxin, it was confirmed that elimination of satellite cells resulted in loss of muscle tissue and a failure to regenerate damaged muscle (Sambasivan et al. 2011). Moreover, expression of *Pax7* in satellite cells is required for satellite cell function and regenerative myogenesis (von Maltzahn et al. 2013). These studies altogether corroborate that satellite cells and *Pax7* expression are essential for muscle regeneration.

Following stress or injury to the muscle, satellite cells become activated and are recruited to the cell cycle (Relaix and Zammit 2012). Activated satellite cells undergo commitment to become myogenic progenitors, known as myoblasts, that are capable of undergoing rapid proliferation, propagation and differentiation (Petrany and Millay 2019). Myoblasts may fuse to pre-existing muscle fibers or fuse with each other to form new myotubes (Petrany and Millay 2019). The activation and commitment of satellite cells to myogenesis are dependent and regulated by a hierarchy of transcription factors including PAX7 and the myogenic regulatory factors (MRFs), which include MYF5, MYOD, MRF4 and myogenin (Seale et al. 2000; Hernández-Hernández et al. 2017). Notably, *Myf5* is transcribed in quiescent satellite cells but *Myf5* transcripts are sequestered within mRNP granules along with their antagonist miRNA-31 (Crist et al. 2012). Upon activation, mRNP granules dissociate and release *Myf5* mRNA, thus allowing its translation and the rapid accumulation of MYF5 protein to promote myogenesis (Crist et al. 2012). *Myod* mRNA in quiescent satellite cells is regulated by the mRNA decay factor tristetraprolin which binds to the 3' UTR of the transcript, promoting its decay (Hausburg et al. 2015). Upon activation, *Myod* transcripts are stabilized by the inactivation of tristetraprolin by the p38α/β MAP kinase pathway (Hausburg et al. 2015). Thus, unlike satellite stem cells, myoblast progenitors are characterized by their abundant expression of MYF5 and MYOD transcription factors (Yin et al. 2013). MYF5 drives proliferation while MYOD drives early differentiation (Rudnicki et al. 2008). During myoblast differentiation, MYF5 and MYOD are downregulated followed by enhanced expression of myogenin and MEF2 while MRF4 expression marks terminally differentiated and fused myotubes (Yin et al. 2013).

Recent studies indicate that the satellite cell population within adult muscle exists as a heterogeneous population where some satellite cells are in a more committed state compared to

others in a more stem cell-like state (Tierney and Sacco 2016). Upon examination of isolated satellite cells from *Myf5-nLacZ* mice it was found that 13% of quiescent satellite cells are LacZ$^-$, and do not express *Myf5* in comparison to the majority of LacZ$^+$/*Myf5*$^+$ satellite cells (Kuang et al. 2007). Upon activation, these *Myf5*$^-$ satellite cells undergo either symmetric expansion, wherein two identical *Myf5*$^-$ daughter cells are generated, or asymmetric division, yielding one *Myf5*$^-$ satellite cells and one committed *Myf5*$^+$ satellite cell (Kuang et al. 2007). Satellite cell heterogeneity can also be characterized by differential PAX7 expression where satellite cells with high levels of PAX7 are less primed for differentiation and require more time for activation in comparison to satellite cells that express low levels of PAX7 (Rocheteau et al. 2012). Similarly, satellite cells vary in their expression of CD34 as quiescent satellite cells with low levels of CD34 exist in a more committed state compared to those with high levels of CD34 that exist in a more stem cell-like state (García-Prat et al. 2020).

1.5 Satellite cells contribute to muscle health

Muscle satellite cells are critical for both muscle homeostasis in the resting state as well as muscle regenerative capacity upon injury (Yin et al. 2013). Disruptions in satellite cell functions are associated with impairments in the ability of muscle to launch an effective regenerative response as observed in muscle wasting during aging and disease (Brooks and Faulkner 1994). Age-related muscle deterioration, known as sarcopenia, is characterized by a decrease in muscle mass and strength and contributes significantly to a decrease in quality of life and morbidity in the elderly (Karakelides and Nair 2005). During aging, muscle stem cells are numerically and functionally compromised while their niche also becomes less supportive (Blau et al. 2015). Immunofluorescence staining against PAX7 in freshly isolated myofibers harvested from young

and old mice indicated an age-associated decrease in satellite cell number (Shefer et al. 2006). Moreover, aged satellite cells break quiescence under homeostatic conditions, further depleting the muscle resident satellite cell pool (Chakkalakal et al. 2012). In addition to aging, satellite cell function is altered in muscle degenerative diseases such as Duchenne muscular dystrophy (DMD), which is a progressive and fatal neuromuscular disease resulting from the loss of dystrophin (Yiu and Kornberg 2015; Filippelli and Chang 2021). In contrast to aging, satellite cell numbers are elevated in dystrophic muscles, however defective regulation of stem cell commitment and other cellular abnormalities contribute to the reduced regenerative capacity of dystrophic satellite cells (Kottlors and Kirschner 2010; Chang et al. 2018).

Maintenance of the satellite stem cell population is essential to sustain muscle homeostasis and tissue plasticity in response to movement, exercise, injury, stress, aging and disease. One well known cellular pathway that contributes to the health and fitness of tissues and cells is autophagy. Autophagy is a catabolic program that is responsible for the degradation and recycling of cellular components in a lysosome-dependent manner (Mizushima 2007). Macroautophagy, one of the main types of autophagy, involves the sequestration of cytoplasmic constituents into double membraned structures known as autophagosomes (Mizushima 2007). Autophagosomes subsequently fuse with lysosomes, where their contents are broken down by lysosomal enzymes and the resulting macromolecules are released back to the cytoplasm for utilization (Mizushima 2007). In this chapter, we highlight the contribution of autophagy in muscle health and satellite stem cell function.

2 Autophagy in skeletal muscle

The daily voluntary movements of skeletal muscle place a high demand for energy production on the mitochondria of muscle cells (Sandri 2010). Muscle contractions cause mechanical and metabolic alterations of proteins and organelles within muscle cells (Sandri 2010). Additionally, this process results in an accumulation of reactive oxygen species (ROS), such as peroxidases, superoxides, and hydroxyl radicals (Neel et al. 2013; Powers et al. 2011). The presence of such ROS not only inhibits the phosphatidylinositol-3-kinase/Protein kinase B and the mammalian target of rapamycin (mTOR) signaling pathway, thereby suppressing protein synthesis, but may also result in the damage of cellular components (Neel et al. 2013; Powers et al. 2011). Thus, the skeletal muscle machinery is equipped with a waste removal mechanism to eliminate misfolded proteins and dysfunctional organelles (Sandri 2010). Autophagy contributes to this quality control assurance in skeletal muscle through the sequestration of aberrant entities within autophagosomes that are subsequently delivered to the lysosome for degradation (Sandri 2010).

2.1 Basal autophagy is required to maintain muscle mass

Autophagy occurs at basal levels in all eukaryotic cells (Levine and Kroemer 2008). With respect to skeletal muscle, autophagy is required to clear dysfunctional organelles and other forms of cellular waste as their accumulation leads to the activation of catabolic pathways, resulting in muscle atrophy and consequential muscle weakness (Masiero et al. 2009). The role of autophagy in skeletal muscle has been examined through the deletion of autophagy genes in mouse models. As shown in mice harbouring a muscle-specific deletion of *Atg7*, an essential autophagy gene, the loss of autophagy causes an accumulation of abnormally large mitochondria and a dilated sarcoplasmic reticulum (Masiero et al. 2009). Ultimately, this leads to an unfolding protein response which suppresses protein synthesis alongside a simultaneous production of ROS from

the dysfunctional mitochondria and, finally, cell death via apoptosis (Masiero et al. 2009). Phenotypically, disrupting autophagy in muscle manifests as a loss of muscle mass and muscle strength (Masiero et al. 2009). Similarly, in a model of attenuated autophagy via reduced expression of *Atg16l1*, a gene that is important for autophagosome biogenesis, muscle fibers of hypomorphic *Atg16l1* mice were smaller than their wild-type counterparts (Paolini et al. 2018). Moreover, the recovery and regeneration from a muscle injury is significantly slower in *Atg16l1* mice (Paolini et al. 2018).

On the other hand, the loss of a negative autophagy regulator, which results in enhanced constitutive autophagy also has detrimental effects on skeletal muscle health. Nutrient-deprivation autophagy factor-1 (NAF-1, also known as CISD2) is an endoplasmic reticulum (ER) BCL-2 interacting protein that promotes the ability of BCL-2 to antagonize Beclin 1-dependent autophagy (Chang et al. 2012; Chang et al. 2010). Of note, homozygous mutations of *NAF-1* cause Wolfram syndrome type 2, an autosomal recessive neurodegenerative disease (Amr et al. 2007). The skeletal muscles of *Naf-1* null mice exhibit signs of degeneration, a dramatic reduction in force-generating capacity, dysregulated calcium flux, and elevated levels of autophagy (Chang et al. 2012; Chen et al. 2009). Furthermore, the mitochondria of *Naf-1* deficient muscle tissue and myoblasts are enlarged, suggesting an adaptative response to augmented autophagy (Gomes et al. 2011; Chang et al. 2012). The brain and muscle tissues of *Naf-1* knockout mice exhibit mitochondrial breakdown and dysfunction as well as autophagic cell death (Chen et al. 2009). The mitochondrial dysfunction in *Naf-1* null mice exacerbates with age and is accompanied by increased autophagy, which is characteristic of premature aging, thus implicating NAF-1 as a longevity factor (Chen et al. 2009).

Altogether these studies demonstrate that both the inhibition and augmentation of basal autophagy in skeletal muscle contributes to myofiber damage and may underlie certain muscle disorders (Masiero et al. 2009). Thus, maintaining a critical level of autophagy is essential for muscle homeostasis and health.

2.2 Role of selective autophagy in muscle homeostasis

In addition to general macroautophagy, selective autophagy has also been shown to play critical roles in maintaining muscle homeostasis. Chaperone-assisted selective autophagy (CASA) is a form of macroautophagy that mediates the specific degradation of ubiquitinated protein aggregates (Kaushik and Cuervo 2012). CASA involves encapsulation of protein aggregates within autophagosomes via chaperones and cochaperones that interact with the autophagy receptor p62/sequestosome 1 (SQSTM1) (Kaushik and Cuervo 2012). BAG cochaperone 3 (BAG3) and heat shock 70 kDa protein 8 (HSPA8/HSC70) chaperones induce CASA through direct binding of protein aggregates (Kaushik and Cuervo 2012). Mutations in BAG3 (Starvin in *Drosophila*), which colocalizes with the Z-disk marker α-actinin in adult fly muscle fibers, are associated with childhood muscle dystrophy (Arndt et al. 2010). BAG3, together with the dual-function co-chaperone/ubiquitin ligase CHIP, p62, HSC70 and the small heat shock protein HSPB8, promote the degradation of damaged Z-disk protein components including filamin to preserve muscle integrity (Arndt et al. 2010). Impairment of CASA-mediated proteostasis leads to Z-disk disintegration and progressive muscle weakness (Arndt et al. 2010). These findings demonstrate that CASA, which contributes to cellular proteostasis is required for Z-disk maintenance in muscle (Arndt et al. 2010).

Lipophagy is the selective autophagic degradation of lipid droplets (LDs), a eukaryotic, intracellular lipid organelle responsible for the storage of triacyclglycerols, cholesterol esters, and retinyl esters (Kounakis et al. 2019). The lipids stored within LDs are utilized for the synthesis of macromolecules, lipid membrane components such as phospholipids, and, most relevantly, for energy production (Kounakis et al. 2019). Upon energetic demand, lipids can be accessed via lipolysis which is the degradation of lipids within LDs by cytosolic lipases (Kounakis et al. 2019). Lipophagy is another method for the cell to access LD content using one or more cargo adaptors, such as p62 (Kounakis et al. 2019). Lipid accumulation is characteristic of a variety of pathologies related to metabolic disorders (Kounakis et al. 2019). Notably, lipid storage myopathy (LSM) is a group of clinically heterogenous diseases that are characterized by an accumulation of LDs in skeletal muscle, often adjacent to the mitochondria (Angelini et al. 2016). Lipophagy is critical in reducing this accumulation in LSM patients (Angelini et al. 2016). Interestingly, a major consequence of the treatment for insulin resistance, such as bariatric surgery, is the disappearance of LDs from skeletal muscle (Leichman et al. 2008; Zhou et al. 2000; Unger and Orci 2001). While the mechanism behind the reduction in LDs is unknown, it has been proposed that p62-mediated lipophagy is responsible for the breakdown of LDs (Lam et al. 2016).

Given the high energetic demands of skeletal muscle tissue, maintenance of mitochondrial homeostasis is critical for ATP biogenesis and muscle function (Triolo and Hood 2021). Mitophagy, the selective autophagic degradation of mitochondria, helps to maintain the mitochondrial pool in muscle cells while releasing a relatively low amount of ROS (Triolo and Hood 2021). Upon signs of mitochondrial dysfunction, such as high ROS production, loss of membrane potential, or respiratory impairment, mitochondria are targeted for degradation within the lysosome (Triolo and Hood 2021). This degradative process is balanced with mitochondrial

biogenesis, allowing for efficient organelle turnover that maintains the metabolic needs of muscle tissue (Triolo and Hood 2021; Hood et al. 2019). Thus, forms of selective autophagy, including CASA, lipophagy and mitophagy, contribute to muscle maintenance and health.

2.3 Autophagy in muscle regeneration and exercise

Exercise training throughout life has numerous benefits for the body. During exercise, cellular remodeling of muscle tissue is activated to meet the exercise-induced elevation in energetic demands (Vainshtein and Hood 2016). This remodeling process involves the synthesis of new organelles and proteins to replace cellular components that may have become oxidized and damaged during exercise (Vainshtein and Hood 2016). Specifically, this includes the activation of both of the major cellular proteolytic programs: the ubiquitin proteasome and autophagy pathways (Vainshtein and Hood 2016). Autophagy is triggered by metabolic stresses including nutrient insufficiency, oxidative stress, and calcium imbalance (Vainshtein and Hood 2016; Rahman et al. 2014; Mofarrahi et al. 2013). These stresses are caused inadvertently as by-products of exercise, which may explain why exercise triggers autophagy (Barry et al. 2011; Vainshtein and Hood 2016). A singular instance of endurance training can change protein turnover markers in a training level-dependent manner (Sanchez et al. 2014). On a cellular level, an elevated production of ROS, higher NAD+ levels, and a general increase in the AMP-to-ATP ratio activate AMP-activated protein kinase (AMPK) in response to exercise (Vainshtein and Hood 2016; Canto et al. 2009; Hardie 2011). AMPK induces autophagy in part by activating ULK1, a key player within the autophagy induction complex that is responsible for autophagosome formation (Vainshtein and Hood 2016; Kim et al. 2011).

Interestingly, recent work has shown that the induction of autophagy in response to exercise and other forms of stress may proceed in two distinct phases (Vainshtein and Hood 2016; Pietrocola et al. 2013). The first phase involves a rapid increase in autophagic flux within minutes or hours of exposure to the stressor and is mediated by post-translational protein modifications of stress-responsive factors already present within the cell (Pietrocola et al. 2013). Contrastingly, the second phase is delayed and relies on transcription factors, such as p53, NF-kB, and STAT3 that activate transcriptional programs to synthesize stress-responsive factors that ensure long-term adaptation to stress (Pietrocola et al. 2013).

Importantly, not only does physical exercise induce autophagy, but autophagy is essential to support muscle plasticity in response to exercise as it ensures exercise-induced metabolic responses and skeletal muscle adaptation to exercise training, allowing for an improvement in physical performance (Sanchez et al. 2014; Lira et al. 2013). BCL-2, which regulates Beclin 1-dependent autophagy, also controls autophagy induced by exercise (Pattingre et al. 2005; He et al. 2012). Mutant *Bcl2* mice that are deficient in autophagy activation exhibit impaired endurance and glucose metabolism during exercise (He et al. 2012). These results suggest that the beneficial metabolic effects of exercise may be due in part to exercise-induced autophagy. Of note, nutritional availability is important as it has direct effects on autophagy, and thus exercise-induced autophagy is more evident when exercise is performed in a fasted state (Sanchez et al. 2014).

During acute exercise, the transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) orchestrates mitochondrial biogenesis to aid skeletal muscle energetically (Vainshtein et al. 2015). PGC-1α, which regulates muscle oxidative capacity, also promotes exercise-induced autophagy (Halling et al. 2016). Additionally, exercise increases the phosphorylation of the mitochondrial fission protein DRP1, indicating an increase in

mitochondrial fission, which triggers mitophagy (Tanaka et al. 2010; Jamart et al. 2012). Thus, mitochondrial biogenesis and muscle homeostasis are balanced by mitochondria and protein turnover during exercise. Disruptions in this equilibrium, which is evident in muscle disuse and aging, has negative impacts on cellular mitochondrial health (Triolo and Hood 2021; Hood et al. 2019; Marzetti et al. 2013; Ziaaldini et al. 2017; Kim et al. 2017).

Autophagy also plays an important role in the regeneration of skeletal muscle tissue that follows strenuous exercise or muscle injury, during which damage to proteins and organelles occur (Call et al. 2017). Autophagic flux increases during muscle regeneration (Call et al. 2017). This indicates that myotubes acquire a higher metabolic capacity as they differentiate and suggests that autophagy is required to mediate remodeling of the mitochondrial network during regeneration (Call et al. 2017; Drake et al. 2016; Yan et al. 2012). After injury, two phases of mitochondrial remodeling occur: the first phase consists of mitochondrial degradation, where mitochondria appear fragmented and there is a clear absence of mitochondria around contractile units of the muscle, while the second phase involves reorganization of the mitochondrial network, where the network becomes reinstated despite the total mitochondrial content still being lower than the pre-injury state (Call et al. 2017). As shown in *Ulk1* knockout mice, *Ulk1* is not only required for mitophagy but also for mitochondrial remodeling during the maturation phase of regeneration as *Ulk1*-deficient mice exhibited delayed mitochondrial remodeling after induced injury (Call et al. 2017; Kundu et al. 2008). Despite the importance of autophagy in muscle regeneration, it is also possible that autophagy alone does not adequately clear the abundance of damaged proteins and organelles which result from muscle damage (Call and Nichenko 2020). This creates an autophagy "bottleneck" wherein autophagosomes accumulate in injured skeletal muscle cells (Call and Nichenko 2020). This effect is not exclusive to skeletal muscle, as it has also been reported in

cardiomyocytes (Ma et al. 2012). The accumulation of autophagosomes within the tissue have been proposed to have a negative effect on satellite cells and their regenerative potential and could contribute to muscle pathologies (Call and Nichenko 2020).

2.4 Autophagy in muscle health

Autophagy is under the tight regulation of many signalling pathways in skeletal muscle, where it is essential for both energy production and consumption, as well as for the clearance of waste products and the turnover of macromolecules (Xia et al. 2021). Autophagy is critical for maintaining skeletal muscle integrity under physiological and stress conditions, as a basal level of autophagy is necessary for muscle homeostasis (Xia et al. 2021). However, both deficient and excessive autophagy disturbs this homeostasis which may contribute to cell damage, muscle weakness, and muscle atrophy (Xia et al. 2021). Moreover, mutations in genes that mediate autophagy underlie muscle pathologies (Xia et al. 2021). Thus, autophagy is necessary for muscle health.

The loss of skeletal muscle throughout the course of aging, known as sarcopenia, is well-documented and inevitable (Park et al. 2019; Carnio et al. 2014). Initially, sarcopenia was thought to result from a general decline in the synthesis of proteins alongside an enhancement of protein degradation (Carnio et al. 2014). Sarcopenia manifests phenotypically as muscle fiber atrophy and degeneration, muscle weakness, dysfunctional mitochondria, and increased oxidative stress (Carnio et al. 2014; Cesari et al. 2014). For this reason, the elderly population experiences a lowered quality of life as they are pre-disposed to an increased risk of morbidity, disability, and mortality (Carnio et al. 2014; Visser and Schaap 2011). Interestingly, this degeneration is not attributable to the loss of motor neurons in the brain or spinal cord (Carnio et al. 2014; Chai et al.

2011; Morrison and Hof 1997). Rather, during aging, the interaction between neuromuscular junctions (NMJs) and myofibers is altered, ultimately leading to a loss of muscle innervation (Carnio et al. 2014; Chai et al. 2011; Valdez et al. 2010). Studies in aged mice show that aged NMJs exhibit axonal swelling, sprouting, synaptic detachment, withdrawal of axons from postsynaptic sites, and fragmentation of the postsynaptic specialization (Valdez et al. 2010). Furthermore, autophagy also declines during aging (Carnio et al. 2014). As observed in *Atg7*$^{-/-}$ autophagy-deficient mice, aged mice show higher levels of atrophy, centrally-nucleated fibers, and inflammation when autophagy is inhibited (Carnio et al. 2014). Remarkably, the NMJs of *Atg7* knockout mice are more fragmented and unstable than age-matched control mice (Carnio et al. 2014). *Atg7* knockout mice also exhibit elevated level of mitochondrial dysfunction and, consequentially, oxidative stress (Carnio et al. 2014). Therefore, autophagy, which declines with age, is required for proper muscle and nerve function maintenance of the integrity of NMJs (Carnio et al. 2014).

Mitofusin 2 (MFN2), alongside mitofusin 1 (MFN1), are proteins located at the outer mitochondrial membrane that are important mediators of mitochondrial dynamics, i.e., fusion and fission, mitochondrial network architecture, and mitochondrial metabolism (Sebastian et al. 2016; Bach et al. 2003). MFN2 also regulates autophagy, mitophagy, the unfolded protein response, oxidative metabolism, and general cell proliferation (Sebastian et al. 2016; Munoz et al. 2013; Hailey et al. 2010; Ngoh et al. 2012; Chen and Dorn 2013). During aging, MFN2 protein expression decreases (Sebastian et al. 2016). Further, *Mfn2* deficiency in young mice impairs autophagy and reduces mitochondrial quality, leading to an aggravated state of pre-mature sarcopenia and metabolic deficiency (Sebastian et al. 2016). Similarly, muscle-specific loss of AMPK, which normally activates both autophagy and mitophagy, resulted in exacerbated age-

related myopathy and mitochondrial dysfunction (Bujak et al. 2015). These studies suggest that inducing autophagy via the activation of AMPK may act to prevent mitochondrial disease, hypoglycemia, and myopathy during aging (Bujak et al. 2015).

Overall, there is a clear contribution between impaired autophagy and the decline of skeletal muscle mass and function during aging. For this reason, it is not surprising that re-establishing autophagy in muscles is a potential treatment for sarcopenia both by pharmacological and exercise-induced modulation (Joseph et al. 2019; Zeng et al. 2020; Park et al. 2019). A partial inhibition of mTOR complex 1 (mTORC1), using rapamycin-related drugs known as rapalogs, counteracts sarcopenia in rats, as evidenced by an increase in muscle mass and fiber cross-sectional area (Joseph et al. 2019). Moreover, treatment with rapalogs led to enhanced levels of autophagy and the downregulation of senescence markers $p16^{INK4a}$ and $p21^{CIP1}$ (Joseph et al. 2019). Further, exercise-induced autophagy, such as through treadmill and resistance exercise, enhanced mitochondrial function, suppressed muscle mass loss, and enhanced AMPK phosphorylation to better modulate autophagic flux (Zeng et al. 2020).

Another example that illustrates the importance of a regulated autophagy program in muscle health is Pompe's disease. Pompe's disease is a highly heterogeneous and devastating disorder caused by deficiencies in the *GAA* gene which encodes for alpha-glucosidase, a lysosomal enzyme which breaks down glycogen to glucose (Raben et al. 2007). Pompe's disease manifests in both infants and adults, the former being the most severe form with symptoms including cardiomegaly, hypotonia, and death by cardiorespiratory failure within the first year of life (Raben et al. 2007; Kishnani et al. 2006). Pompe's disease also affects the autophagic pathway (Raben et al. 2007). Muscle fibers isolated from the knockout mouse model of Pompe's disease exhibit large amounts of autophagic accumulation, especially in type II fibers (Raben et al. 2007). Enzyme

replacement therapy is now available to Pompe's disease patients (Raben et al. 2007). It utilizes a recombinant human GAA and has allowed infantile patients to survive significantly longer than untreated patients and has improved cardiac function (Raben et al. 2007). However, only a small percentage of clinical trial patients saw improvements in mortality and skeletal muscle function (Raben et al. 2007). Moreover, Pompe's disease type II fibers are more therapeutically resistant than their wild-type counterparts (Raben et al. 2007; Raben et al. 2005). Interestingly, and conversely to sarcopenia, the suppression of autophagy in skeletal muscle eases the large induction of autophagy that is characteristic of Pompe muscle fibers, as shown in muscle-specific autophagy-deficient Pompe mice (Raben et al. 2008).

To conclude, both the excess of autophagy, as is the case in Pompe's disease, and impaired autophagy, which causes a gradual decrease in skeletal muscle mass during aging, results in muscle pathology. Thus, maintaining a balanced and homeostatic control of autophagy is crucial for skeletal muscle health.

3 Autophagy in muscle stem cells

Muscle satellite cells are key players in maintaining muscle tissue homeostasis and facilitating regeneration (Relaix and Zammit 2012). These tissue resident stem cells are kept in a quiescent state until stimulated by stress or damage to activate, enter the cell cycle, and either expand, differentiate, or self-renew (Cheung and Rando 2013; Montarras et al. 2013; Yin et al. 2013; Comai and Tajbakhsh 2014). Satellite cells maintain their quiescence through cytoprotective and cellular quality control mechanisms that inhibit irreversible withdrawal from the cell cycle; these mechanisms are lost in advanced age, during which satellite cells switch to a senescence-like state (Sousa-Victor et al. 2014). As a result, the number and function of satellite cells decline with age

and the regenerative capacity of skeletal muscle is profoundly compromised (Price et al. 2014; Brack et al. 2005; Hwang and Brack 2018).

3.1 Homeostatic maintenance of muscle stem cells

Satellite cell quiescence is preserved via active regulation of organelle and protein homeostasis, implicating basal autophagy as a cellular quality control mechanism in satellite stem cells (Garcia-Prat et al. 2016). By enabling the recycling of macromolecules to provide energy-rich macromolecules as well as eliminate damaged proteins, organelles, and toxic compounds, autophagy allows cells and tissues to continually adapt to stress (Jiang and Mizushima 2014; Mariño et al. 2011). Thus, the contribution of autophagy in satellite cells parallels the importance of autophagy in muscle tissue, which is essential for maintenance of muscle mass and integrity (Sandri 2010). As autophagic activity declines with age or due to genetic impairment, toxic cellular waste accumulates and satellite cell functions are disturbed, accompanied by perturbations in mitochondrial function and ATP production, and entry into senescence (Garcia-Prat et al. 2016; Tang and Rando 2014). As with muscle tissue, a critical balance in the levels of basal autophagy is essential for satellite stem cell integrity, as excessive autophagy can also lead to stem cell defects (Fernandes et al. 2020).

Transcriptomic analysis of quiescent satellite cells compared to activated satellite cells uncovered that autophagy is the most prevalent pathway during quiescence and autophagic genes are downregulated in association with age (Garcia-Prat et al. 2016). Aged satellite cells display common traits of deficient autophagy, including formation of p62 aggregates, accumulation of autophagic vesicles, ubiquitin-positive inclusions, and reduced accumulation of LC3-II upon treatment with bafilomycin A1 (an autophagy-flux inhibitor which prevents lysosome

degradation), which altogether indicates reduced capacity for autophagosome formation (Garcia-Prat et al. 2016). The block in autophagic flux increases progressively with age in mice from young (3 months) to old (20-24 months) to geriatric (over 28 months) (Garcia-Prat et al. 2016). Geriatric satellite cells exhibited increased co-localization of p62 and ubiquitin aggregates in non-degraded autophagosomes, thus indicating a block in autophagosome clearance (Garcia-Prat et al. 2016).

Genetic impairment of autophagy by specific deletion of *Atg7* in quiescent satellite cells of young mice severely reduced satellite cell numbers, while the remaining satellite cells exhibited signs of premature aging such as induction of senescence genes $p16^{INK4a}$, $p21^{CIP1}$, and $p15^{INK4b}$, as well as evidence of DNA damage (Garcia-Prat et al. 2016). Young *Atg7*-deficient satellite cells and aged satellite cells shared similar phenotypes, including accumulation of mitochondria, lysosomes, and p62 and ubiquitin-positive aggregates, and a lower proportion of healthy mitochondria (Garcia-Prat et al. 2016). These results indicate that basal autophagy is required for maintaining satellite cell integrity and fitness and to preserve the pool of quiescent satellite cells (Garcia-Prat et al. 2016). Following muscle injury, satellite cells from these mice displayed reduced activation and proliferation capacity, evidence of cell-intrinsic regenerative failure, as well as accelerated entry into senescence (Garcia-Prat et al. 2016). Thus, the decline in autophagy in satellite cells may underlie the physical loss and functional exhaustion of muscle satellite cells associated with aging. These findings also implicate defective autophagy as a cause of senescence, rather than a consequence rising from senescence.

In addition, enhanced levels of ROS and mitochondrial dysfunction are a common phenotype observed in both geriatric satellite cells as well as *Atg7*-deficient satellite cells (Garcia-Prat et al. 2016). Notably, ROS inhibition in geriatric satellite cells with Trolox, a vitamin E analogue, prevented the induction of senescence markers and induced autophagic flux, resulting

in a reduction of ubiquitin and p62 aggregates as well as mitochondria-ROS colocalization (Garcia-Prat et al. 2016). Moreover, treatment with Trolox restored satellite cell expansion, and rescued satellite cell defects in proliferation and regenerative capacity (Garcia-Prat et al. 2016).

The reinstatement of basal autophagy using either genetic or pharmacological approaches in geriatric mice reversed senescence in satellite cells and rescued regenerative function (Garcia-Prat et al. 2016). Reactivation of autophagy with the mTOR inhibitor rapamycin or ectopic expression of *Atg7* in geriatric satellite cells prior to transplantation into pre-injured muscles of young recipient mice restored their capacity for expansion and engraftment and prevented senescence (Garcia-Prat et al. 2016). Of note, similar defects in protein and organelle clearance were observed in aged human satellite cells, alongside increased ROS levels and markers of senescence (Garcia-Prat et al. 2016). Restoration of autophagy and organelle homeostasis in aged human satellite cells with rapamycin was able to rescue cells from entering senescence, prevent abnormal mitochondrial content and ROS levels, as well as protein aggregation (Garcia-Prat et al. 2016). Thus, inducing autophagy may serve as a therapeutic avenue to improve aged satellite cell function to prevent age-related regenerative decline in muscle.

3.2 Autophagy in satellite cells is under circadian regulation

The day-night oscillation of genes that maintain tissue homeostasis have been observed in numerous tissues, suggesting that adult stem cells are subject to circadian control (Janich et al. 2014). Circadian rhythms segregate cellular functions throughout the 24-hour day to minimize potential exposure to harmful situations and maximize cellular performance and energetic efficiency (Janich et al. 2014). Gene ontology analysis of whole transcriptome gene expression data from satellite cells revealed that adult quiescent satellite cells expressed many transcripts

required for homeostasis in an oscillatory manner, such as myotube differentiation and cell proliferation (Solanas et al. 2017). This "rhythmic transcriptome" encompasses genes within the transforming growth factor-beta/bone morphogenetic protein and fibroblast growth factor signaling pathways, which regulate maintenance of satellite cell quiescence and readiness for activation (Solanas et al. 2017). Additionally, transcripts involved in DNA double-strand break repair, including *Rad23a*, *Ercc4*, and *Xpa*, were rhythmically expressed, consistent with previous findings indicating that quiescent satellite cells compared to differentiated muscle cells, are more predisposed to repairing this type of damage (Ferdousi et al. 2014; Solanas et al. 2017). Intriguingly during aging, the oscillatory transcriptome of satellite cells is dramatically reprogrammed (Solanas et al. 2017). Aged satellite cells exhibited a distinct program of oscillatory genes, including those involved in mitochondrial DNA repair, cytokine production, and inflammation (Solanas et al. 2017).

Intriguingly, Solanas et al. found that the expression of key autophagy-related genes, including *Becn1*, *Flcn*, *Atg13*, and *Svip*, were under rhythmic control in adult satellite cells (Solanas et al. 2017). The expression of these genes peaked late at night or early in the morning, resulting in higher levels of autophagic activity during the day (Solanas et al. 2017). In comparison, autophagy genes in aged satellite cells were not under circadian control and consequently autophagy levels were significantly reduced throughout the day (Solanas et al. 2017). These findings suggest that aged satellite cells lose their capacity to rhythmically recycle damaged cellular components that are produced in the cell. Thus, the age-associated loss of rhythmic regulation of autophagy leads to an overall decline in autophagy, ultimately impairing the cell's intracellular quality control mechanism to sustain organelle and protein homeostasis, maintain quiescence and preserve stemness (Garcia-Prat et al. 2016; Solanas et al. 2017).

3.3 Quiescent satellite cells exist in two distinct metabolic states

Molecules and pathways responsible for regulating cellular energy status and metabolism, such as the nutrient sensing mTOR pathway, have been shown to influence different aspects of stem cell function, including pluripotency, differentiation, proliferation, and self-renewal (Murakami et al. 2004; Chen et al. 2008; Sampath et al. 2008; Folmes et al. 2012). The downstream targets of these metabolic pathways include those relevant to the autophagic process and can act to induce autophagy during conditions of increased energetic demand and stress.

Satellite stem cells within the quiescent state can exist in two functional phases; G_0 and G_{Alert} (Rodgers et al. 2014). Cells in G_{Alert} are considered to be in an intermediate "alert" phase while still in quiescence (Rodgers et al. 2014). Metabolically, these cells have higher mitochondrial activity, larger cellular volumes, enhanced differentiation kinetics, and a higher propensity to cycle than those in G_0 (Rodgers et al. 2014). The mTOR pathway, which inhibits autophagy, has been shown to be required for the transition of satellite cells from G_0 to the G_{Alert} (Rodgers et al. 2014). Thus, autophagy in this context may contribute to a "deeper" quiescent satellite cell, while the transition to G_{alert} requires active mTOR and concomitant inhibition of autophagy.

3.4 Contribution of autophagy in satellite cell activation

As stem cells activate and exit quiescence, different bioenergetic requirements exist during the differentiation process (Folmes et al. 2012). Quiescent stem cells initiate the activation process from a position characterized by low mitochondrial content and activity, metabolism, and translation rates. Thus during activation, stem cells need to fulfill a high demand for energy and

nutrients in order to support cellular growth (Lunt and Vander Heiden 2011). An increase in ATP production is associated with progression through G_1 of the cell cycle as it is necessary to fuel DNA replication and cellular growth processes (Lunt and Vander Heiden 2011; Folmes et al. 2012). During muscle regeneration, Fiacco and colleagues reported that autophagy is induced upon satellite cell activation following injury and autophagy levels return to baseline by the end of the regeneration process (Fiacco et al. 2016). Pharmacological induction of autophagy led to enhanced satellite cell activation and proliferation, while inhibiting autophagy resulted in impaired satellite cell regenerative capacity (Fiacco et al. 2016).

Another study by Tang and Rando found that autophagy in satellite cells was induced early during the activation process from quiescence (Tang and Rando 2014). Autophagy levels remained elevated during satellite cell proliferation and were subsequently reduced during self-renewal (Tang and Rando 2014). In contrast to the study conducted by Garcia-Prat and colleagues, Tang and Rando did not detect basal autophagic flux in quiescent satellite cells (Tang and Rando 2014; Garcia-Prat et al. 2016). Autophagy was detected in more than half of activated satellite cells 1.5 days following muscle injury, which increased to over 80% of activated satellite cells by 2.5 days post-injury (Tang and Rando 2014). These results illustrate the increase in autophagic flux required during satellite cell activation upon muscle injury *in vivo* (Tang and Rando 2014). Upon examination of single myofiber-associated satellite cells *ex vivo*, autophagy was found to be induced early in activated satellite cells prior to the initiation of DNA synthesis (Tang and Rando 2014).

The inhibition of autophagy through chemical inhibition with either chloroquine or 3-methyladenine, or siRNA-mediated knockdown of essential autophagy genes *Atg5* and *Atg7*, resulted in delayed satellite cell activation (Tang and Rando 2014). Moreover, ATP content was

greatly reduced following autophagy inhibition, suggesting that autophagy contributes bioenergetic resources to facilitate satellite cell activation (Tang and Rando 2014). The delay in satellite cell activation was partially rescued upon supplementation with exogenous sodium pyruvate (Tang and Rando 2014). Sirtuin-1 (SIRT1), an NAD-dependent deacetylase that responds to changes in cellular metabolism, was required for autophagy induction during satellite cell activation (Tang and Rando 2014). In satellite cells, SIRT1 interacts with and deacetylates ATG7 (Tang and Rando 2014). Chemical inhibition of SIRT1 reduced autophagic flux and satellite cell-specific deletion of *Sirt1* resulted in delayed satellite cell activation (Tang and Rando 2014). Autophagic flux is therefore upregulated during satellite cell activation to meet the necessary metabolic requirements for satellite cells to proceed through the myogenic program.

3.5 Autophagy prevents apoptosis in satellite cells

Activated cells whose energetic needs are not met by autophagy become susceptible to apoptosis, a form of programmed cell death (Jejurikar et al. 2006). During cell fate decisions, young satellite cells induce autophagy over apoptosis, whereas aged satellite cells are more likely to induce apoptosis (Jejurikar et al. 2006). Indeed, autophagy and apoptosis have opposing correlations related with satellite cell aging. Unlike autophagy that decreases with age, apoptosis is increased across the lifespan (Schultz and Lipton 1982). When compared with young and middle-aged satellite cells, old and geriatric satellite cells displayed enhanced levels of cleaved poly(ADP-ribose) polymerase (PARP), a marker of apoptosis, and were progressively positive for TUNEL and annexin V labelling (White et al. 2018). Moreover, young and aged satellite cells exhibited differential susceptibility to apoptosis in the absence of autophagy (White et al. 2018). Upon inhibition of autophagy mediated by *Atg5* knockdown, apoptosis was not induced in young satellite

cells but was increased two-fold in geriatric satellite cells resulting in enhanced cell death (White et al. 2018). Apoptosis induced by the suppression of autophagy occurred via the canonical apoptotic pathway, as a pan-caspase inhibitor was able to prevent cell death and *Bcl2* overexpression reduced onset of apoptosis (White et al. 2018). Moreover, in young satellite cells, *Atg5* depletion delayed cell proliferation, while geriatric satellite cells failed to proliferate (White et al. 2018).

AMPK regulates autophagy and apoptosis in part through its ability to phosphorylate the cyclin inhibitor $p27^{Kip1}$ and has been shown to play an important role in satellite cell-mediated muscle regeneration (Liang et al. 2007; Fu et al. 2015; Theret et al. 2017). $p27^{Kip1}$ can prevent apoptosis by inhibiting the activation of Cdk2 and the activity of the pro-apoptotic factor BAX (Gil-Gómez et al. 1998; Hiromura et al. 1999). AMPK-dependent phosphorylation of $p27^{Kip1}$ at Thr198 promotes its stability and cytoplasmic translocation, leading to increased autophagy and decreased apoptosis (Liang et al. 2007; White et al. 2018). Both AMPK and $p27^{Kip1}$ phosphorylation were reduced in old mice and to a greater extent in geriatric mice (White et al. 2018). Restoration of AMPK activity using the AMP analog, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) in geriatric satellite cells prevented cell death (White et al. 2018). Compared to control young satellite cells, which exhibited high transplantation efficiency, satellite cells in which AMPK activity was genetically suppressed prior to transplantation showed a significant decline in engraftment (White et al. 2018). In contrast, geriatric satellite cells, which displayed inherently poor transplantation efficiency compared to young cells, exhibited improved engraftment upon constitutive activation of AMPK with AICAR (White et al. 2018). AICAR treatment of geriatric satellite cells led to reduced levels of senescence and expression of the senescence genes $p16^{INK4a}$ and $p21^{CIP1}$ (White et al. 2018). Thus, the AMPK/$p27^{Kip1}$ signaling axis

controls the autophagy/apoptosis balance in satellite stem cells and activating this pathway may improve aged satellite cell function and muscle regeneration.

3.6 Satellite cell function and regenerative capacity

Calorie restriction is well known to have a positive effect on lifespan and prevents age-related deterioration (Hursting et al. 2003). Calorie restriction is also a potent inducer of autophagy and is a non-genetic and non-chemical method used to stimulate autophagy in animal models (Bagherniya et al. 2018). Intriguingly, short term calorie restriction in both young and old mice enhanced satellite cell numbers and improved muscle stem cell function (Cerletti et al. 2012). Satellite cells from calorie restricted mice exhibited enhanced mitochondrial content, suggesting a switch to fatty acid oxidation and oxidative phosphorylation for energy production (Cerletti et al. 2012). Moreover, satellite cells from calorie restricted mice exhibited increased expression of SIRT1 and FOXO3, both of which mediate autophagy (Cerletti et al. 2012). Mice maintained on a calorie restricted diet displayed enhanced muscle repair in response to injury and improved satellite cell transplantation and engraftment efficiency (Cerletti et al. 2012).

Metformin is a drug that mimics calorie restriction and is used for the treatment of type 2 diabetes (Wang et al. 2017). Metformin has been shown to activate autophagy through its ability to activate AMPK and inhibit mTOR signaling (Shi et al. 2012). Treatment of immortalized C2C12 mouse myoblasts with metformin prevented terminal differentiation and permanent exit from the cell cycle (Pavlidou et al. 2017). In satellite cells, treatment with metformin resulted in a delay in satellite cell activation in association with delayed downregulation of PAX7 and differentiation (Pavlidou et al. 2019). Thus, metformin retains satellite cells in a more stem-like and pre-differentiation state. Upon muscle injury, metformin treatment delayed activation from quiescence

(Pavlidou et al. 2019). This effect was attributed to a reduction in phosphorylation of ribosomal S6 kinase, a downstream target and readout of mTOR signaling, indicating an inhibition in mTOR activity and protein synthesis (Pavlidou et al. 2019). Altogether, these studies indicate that factors mediating metabolism and autophagy play an important role in stem cell function and regenerative capacity.

3.7 The role of mTOR signaling in myogenesis

Upon the presence of an external stimuli, quiescent satellite cells undergo chronological stages of myogenesis; they re-enter the cell cycle and give rise to proliferative myoblasts, which subsequently differentiate into myocytes, and fuse to form myofibers (Bentzinger et al. 2012). Adult myogenesis, which relies on PAX7-positive satellite cells, recapitulates many mechanisms present during embryonic muscle development (Rion et al. 2019; Dumont et al. 2015). Both embryonic and adult myogenesis are highly dependent on mTORC1, an established regulator of cellular growth (Rion et al. 2019). Inactivation of mTORC1 via genetic deletion of *Raptor* (a component of mTORC1) in mouse embryonic muscle progenitors impaired muscle development and resulted in perinatal lethality (Rion et al. 2019). *Raptor*-deficient embryos exhibited a 50% reduction in *Myf5* expression, indicating that the inactivation of mTORC1 directly influences the early stages of myogenesis (Rion et al. 2019). In adult myogenesis, mTORC1 is activated in satellite cells following muscle injury and remains high during the proliferative phase of myogenesis (Rion et al. 2019). In contrast, myocytes and myotubes do not exhibit phosphorylation of ribosomal S6 kinase, indicating low mTORC1 activity during late myogenesis (Rion et al. 2019). Of note, mTORC2 appears to be dispensable for myogenesis (Rion et al. 2019). Thus, early

stages of embryonic and adult myogenesis are highly dependent on mTORC1 and inhibiting mTORC1 is detrimental to myogenesis (Rion et al. 2019).

Myotonic dystrophy type 1 (DM1) is a neuromuscular disease caused by mutated transcripts of the myotonic dystrophy protein kinase harboring expanded CTG repeats that results in the formation of nuclear RNA foci and disturb RNA-binding proteins (Gagnon et al. 2018; Lee and Cooper 2009; Song et al. 2020). DM1 manifests phenotypically as muscle atrophy and decreased skeletal muscle regeneration (Gagnon et al. 2018; Lee and Cooper 2009; Song et al. 2020). Moreover, DM1 satellite cells exhibit reduced proliferative capacity and enhanced levels of autophagy, which are thought to contribute to the muscle regeneration defect (Song et al. 2020). Intriguingly, satellite cells differentiated from induced pluripotent stem cells obtained from DM1 patients that were subsequently edited by transcription activator-like effector nucleases (TALENs) to target the CTG repeats showed reduced levels of autophagy, increased levels of phosphorylated mTOR, and enhanced cell proliferation rates (Song et al. 2020). Accordingly, this rescue in cell proliferation in DM1 TALEN-edited satellite cells was abrogated upon treatment with the mTOR inhibitor rapamycin (Song et al. 2020). Thus, the proliferation defect in DM1 satellite cells was rescued via the activation of mTOR and inhibition of autophagy (Song et al. 2020; Bargiela et al. 2015).

3.8 Coordination of myogenic differentiation and p53-dependent autophagy

In addition to the temporal coordination of myogenic proteins during differentiation, autophagy during myogenesis is also regulated in a differentiation stage-dependent manner (Bentzinger et al. 2012). While autophagy levels are reduced during myoblast proliferation, autophagy is required

during the later stages of myogenic differentiation to protect myoblasts from apoptosis during differentiation (McMillan and Quadrilatero 2014). The onset of autophagy during differentiation is mediated by an initial increase in apoptosis, which induces the activation of autophagy to subsequently prevent excessive apoptosis (Jiang et al. 2021).

Myocytes, which are fully differentiated myoblast-derived cells that have not yet undergone fusion, exhibit decreased autophagic flux (Fortini et al. 2016). In contrast, autophagy is required for myocyte fusion. This is evidenced by an increase in the transcript levels of autophagy-related genes and positive immunofluorescent staining for autophagosome and lysosome proteins during fusion (Fortini et al. 2016). Moreover, when autophagy is genetically inactivated by siRNA silencing of Beclin 1 *in vitro*, the fusion index, a quantifiable readout for myocyte fusion and thus differentiation efficiency, is reduced by 1.7-fold (Fortini et al. 2016). Accordingly, myotubes, but not myocytes, exhibit accumulated levels of autophagic LC3-II in the presence of lysosomal inhibitors (Fortini et al. 2016). Moreover, chemical inhibition of autophagy during differentiation resulted in reduced expression of myosin heavy chain and myogenin, which are markers of terminal differentiation, and delayed formation of myotubes (McMillan and Quadrilatero 2014).

p53 is a multifaceted protein which can shuffle between both the nucleus and cytoplasm to achieve different outcomes (Phelan et al. 1998; Mrakovcic and Frohlich 2018). In addition to its role as a tumor suppressor gene, it also acts as an activator or inhibitor of autophagy, dependent on its subcellular localization (Mrakovcic and Frohlich 2018; Maiuri et al. 2010; Tasdemir et al. 2008). When p53 is localized to the nucleus, it activates autophagy induced by exogenous stress, leading to either a pro-death or pro-survival outcome (Maiuri et al. 2010; Tasdemir et al. 2008). In contrast, cytoplasmic p53 inhibits autophagy induced by ER stress or nutrient deprivation (Maiuri

et al. 2010; Tasdemir et al. 2008). Myoblasts derived from *p53* null mice exhibited a reduction in basal autophagy and impaired ability to terminally differentiate into myotubes (Fortini et al. 2016). Moreover, mitochondrial biogenesis, which occurs during myogenic differentiation, is impaired in the absence of p53 (Fortini et al. 2016). Thus, p53-mediated autophagy is required for metabolic remodeling and myoblast fusion during differentiation (Fortini et al. 2016). Interestingly, p53 also imposes a quality control mechanism during differentiation and is activated upon genotoxic stress (Yang et al. 2015). p53 binds directly to the myogenin promoter to repress myogenin expression and delay differentiation (Yang et al. 2015). This ultimately protects terminally differentiated muscle cells from post-mitotic nuclear abnormalities (Yang et al. 2015). Thus, p53-mediated autophagy and differentiation contribute to proper myotube fusion.

3.9 Mitophagy is required for mitochondrial biogenesis and myogenesis

During myogenesis, as myoblasts differentiate into myotubes, the mitochondrial network is altered to adapt to different metabolic needs. Mitochondria are remodeled during differentiation, including alterations in their abundance, morphology and functional properties (Wagatsuma and Sakuma 2013). Additionally, there is a switch from glycolysis, which serves as the main energy source for myoblasts, to oxidative phosphorylation at the terminal differentiation stage (Wagatsuma and Sakuma 2013). Thus, remodeling of the mitochondrial network, which includes their degradation and biogenesis, is under tight regulation to ensure the proper advancement of differentiation (Wagatsuma and Sakuma 2013). During the early stages of myogenic differentiation, an increase in dynamin 1-like-mediated mitochondrial fragmentation, and removal of mitochondria via p62/SQSTM1-mediated mitophagy were observed (Sin et al. 2016). Mitochondria biogenesis is subsequently upregulated via a PGC-1α-dependent pathway, resulting in a myotube freshly

populated with new mitochondria that are better primed for oxidative phosphorylation (Sin et al. 2016). Additionally, CRISPR-Cas9-mediated deletion of *Bnip3*, a member of the BCL-2 protein family that is upregulated during mitophagy and which modulates mitochondrial membrane permeability, impairs myoblast differentiation (Baechler et al. 2019).

The E3 ubiquitin ligase Parkin is recruited to mitochondria upon the loss of mitochondrial membrane potential to initiate of mitophagy in several cell types (Panicker et al. 2017). Interestingly, inducing Parkin-mediated mitophagy by uncoupling mitochondria during *in vitro* myogenesis resulted in excessive mitophagy and myotube atrophy (Peker et al. 2018). Loss of Parkin function via siRNA-mediated knockdown resulted in impaired mitochondrial turnover as well as myotube atrophy (Peker et al. 2018). Similarly, *Parkin* knockout mice exhibit enhanced fibrosis and decreased myofiber cross-sectional area following cardiotoxin injury, suggesting an impairment in regeneration (Esteca et al. 2020). In the absence of Parkin, satellite cells exhibit reduced differentiation capacity and increased proliferation (Esteca et al. 2020). Altogether, these studies indicate that mitophagy is an important contributor to the myogenic program to support the differentiation process and meet the metabolic needs of the tissue.

4 Conclusion

Autophagy is clearly important for muscle health and contributes to the maintenance of the integrity and plasticity of the muscle tissue as well as the regenerative capacity and fitness of muscle stem cells. Constitutive basal levels of autophagy are important for the homeostatic maintenance of cells and tissues, while induced autophagy mediates cellular response to stress and enhanced metabolic requirements. Impaired autophagy has detrimental effects on muscle health

and is the underlying cause of age-related muscle decline and various myopathies including muscular dystrophies. Importantly, muscle stem cells, which contribute to both muscle homeostasis and muscle regenerative capacity throughout life, are also dependent on a dynamic autophagy program (Fig. 1). An interplay of metabolic sensing pathways that include regulators such as mTOR and AMPK, thus control autophagy to ensure that the level of autophagy is finely tuned to the metabolic needs of the satellite cell as it transitions through the myogenic differentiation process (Fig. 2). Ultimately, the ability of satellite cells to contribute to muscle repair and sustain future rounds of regeneration is an important determinant of muscle health.

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Compliance with Ethical Standards

Disclosure of Interests: All authors declare they have no conflict of interest.

Figures

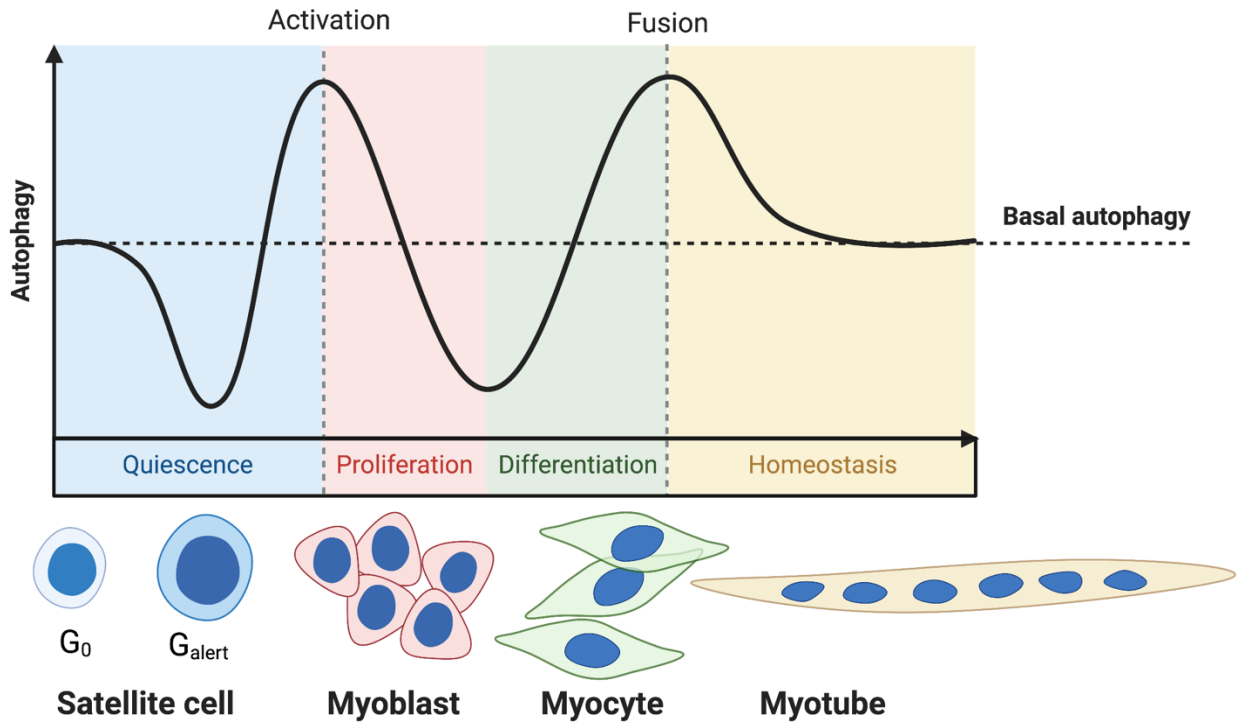

Fig. 1 Dynamic contribution of autophagy during myogenic differentiation. Basal autophagy is required to maintain satellite stem cell homeostasis, preserve stemness and prevent the accumulation of factors that can lead to senescence. Quiescent satellite cells in G_{alert} are metabolically distinct from G_0 satellite cells, and are dependent on mTORC1, which inhibits autophagy. Following a regeneration stimulus, autophagy is upregulated in satellite cells to provide sufficient energy to facilitate the transition from quiescence to activation. Myoblasts exhibit low autophagic flux, as cells prioritize cell proliferation and growth to expand the progenitor population. Myocyte fusion requires autophagy for remodeling of the mitochondria network. Finally, mature muscle cells maintain a basal level of autophagy to ensure homeostatic maintenance of muscle mass and health.

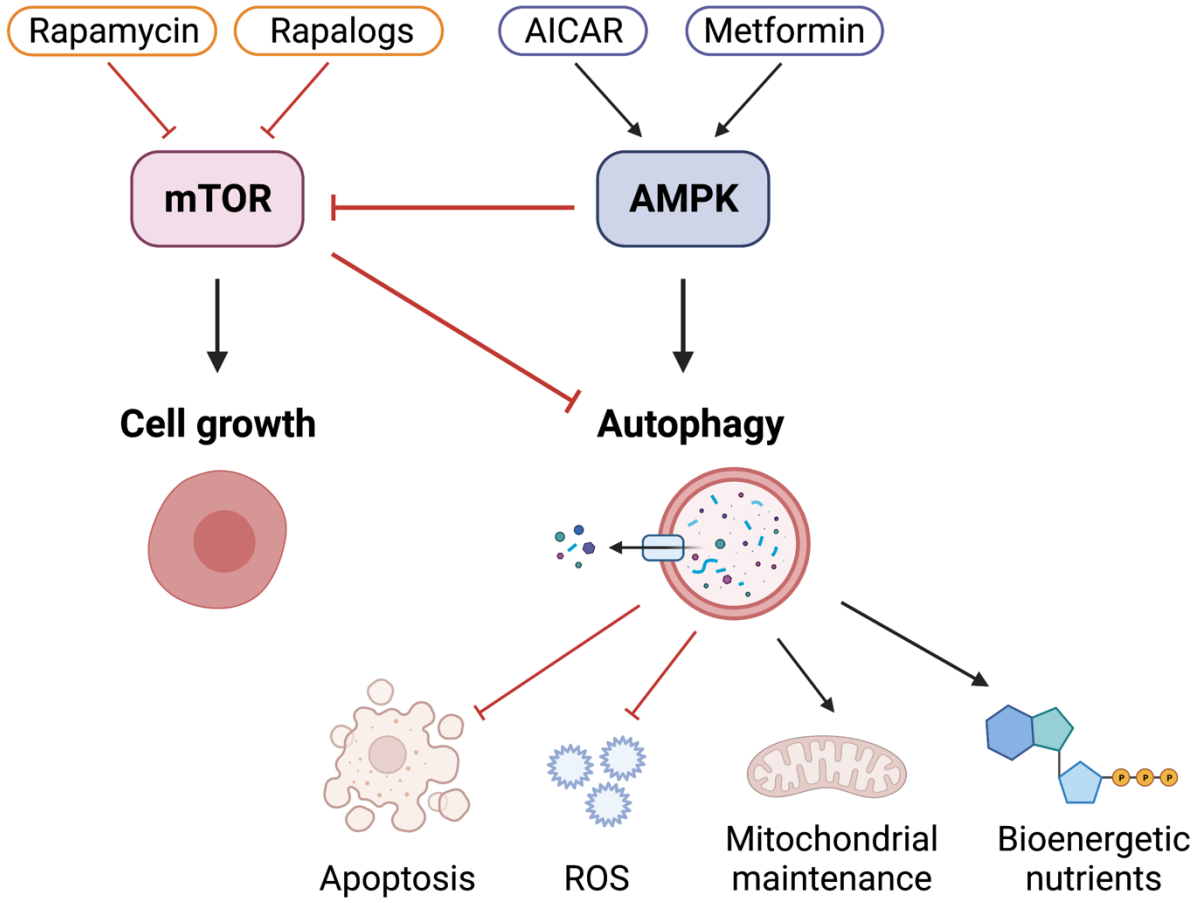

Fig. 2 Nutrient sensing pathways regulate autophagy. Autophagy is regulated by mTOR and AMPK protein kinase complexes. mTOR inhibits autophagy and promotes cell growth, while AMPK inhibits mTOR and induces autophagy. Compounds that modulate autophagy include rapamycin and rapologs that inhibitor mTOR, as well as AICAR and metformin that activate AMPK. In muscle cells, autophagy inhibits apoptosis and prevents the accumulation of reactive oxygen species (ROS). Autophagy also maintains the mitochondrial network and provides metabolic bioenergetic nutrients.

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