Role of autophagy and mitophagy in exercise-induced skeletal muscle remodeling in human

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Table of content

List of abbreviations

Ac	acetylation
Akt/PKB	protein kinase B
AMPK	AMP-activated kinase
ATF2	activating transcription factor-2
Atg	autophagy related protein
Atg	autophagy related gene
ATP	adenosine triphosphate
V-ATPase	V-adenosine triphosphatase

Bcl-2	B cell lymphoma-2
Bcl-X_L	B cell lymphoma-extra large
BNIP3	Bcl2/adenovirus E1B 19 kDa interacting protein 3
BNIP3L/Nix	Bcl2/adenovirus E1B 19 kDa interacting protein 3 Like
Ca^{2+}	calcium ion
CaMK2	calcium/calmodulin-dependent protein kinase 2
CaMK1α	calcium/calmodulin-dependent protein kinase 1 α
CaMKKα	calcium/calmodulin-dependent protein kinase kinase α
Cdk1-cyclin B	cyclin-dependent-kinase B
CK2	casein kinase 2
CLPP	Ser protease ATP-dependent Clp protease proteolytic subunit
CLPX	ATP-dependent Clp protease ATP-binding subunit ClpX-like
COx	cytochrome c oxidase
CREB	cAMP activated-response element-binding protein
CS	citrate synthase

DNA	deoxyribonucleic acid
Drp1	dynamin-related protease 1
ERK1/2	extracellular signal-regulated kinases 1/2
ERRα	estrogen-related receptor α
ETC	electron transport chain
Fis1	fission protein 1
FIP200	focal adhesion kinase family interacting protein of 200 kDa
FoxO3	Forkhead box O3
FUNDC1	Fun14 domain-containing protein 1
Gabarap	GABA type A receptor-associated protein
GabarapL1	GABA type A receptor-associated protein-like 1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HMGB1	high mobility group box 1
IMM	inner mitochondrial membrane
IR	insulin receptor
IRS1	insulin receptor substrate 1
LC3	microtubule-associated protein 1 light chain 3

LAMP1/2	lysosome-associated membrane proteins 1/2
LONP	Lon protease homolog
MDV	Mitochondria-derived vesicles
MEF2	myocyte enhancer factor-2
MIEF1/MID51	mitochondrial elongation factor 1/mitochondrial dynamic protein 51
Mff	mitochondrial fission factor
Mfn1/2	mitofusins 1/2
mtDNA	mitochondrial deoxyribonucleic acid
MTERF	mitochondrial transcription termination factor 1
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
nDNA	nuclear deoxyribonucleic acid
NRF1	nuclear respiratory factor 1
NRF2	nuclear respiratory factor 2
OMM	outer mitochondrial membrane
OPA1	optic atrophy protein 1
P	phosphorylation

PARKIN	E3 ubiquitin protein ligase parkin
PDH	pyruvate dehydrogenase
PDK1	phosphoinositide-dependent kinase 1
PE	phosphatidylethanolamine
PGC1α	peroxisome proliferator-activated receptor gamma alpha co-activator-1 alpha
PIP2	phosphatidylinositol 3-4,5-bisphosphate
PIP 3	phosphatidylinositol 3-4,5-trisphosphate
PI3K	p150, phosphatidylinositol-3-phosphate kinase
PINK1	PTEN-induced putative kinase 1
PolG	DNA polymerase gamma
POLRMT	RNA polymerase mitochondrial
PPAR	peroxisome proliferator activated receptor
p38 MAPK	p38 mitogen-activated protein kinase
p53	tumor protein 53
p62/SQSTM1	p62/sequestosome 1
qRT-PCR	quantitative real-time polymerase chain reaction
Rab7	ras-related protein 7

Rag	recombination-activating gene protein
Rev-erb-α/NR1D1	nuclear receptor Rev-erbα-/NR1D1
Rheb	Ras homolog enriched in brain
RIP140	receptor interacting protein of 140 kDa
ROS	reactive oxygen species
SDH	succinate dehydrogenase
Ser	serine
SIRT1	sirtuin 1
SIRT2	sirtuin 2
SRC	proto-oncogene tyrosine-protein kinase Src (SRC)
TKB1	TANK-binding kinase 1
Tfam	mitochondrial transcription factor A
TFB1M	mitochondrial transcription factor B1
TF2BM	mitochondrial transcription actor B2
TFEB	transcription factor EB
Thr	threonine
TIM	translocase of inner membrane
TOM	translocase of outer membrane
TSC1/2	tuberous sclerosis complex 1/2
Tyr	tyrosine

Ub	ubiquitination
ULK1/2	UNC-51-like kinases 1/2
UPS	ubiquitin-proteasome system
UVRAG	UV-resistance associated gene protein
VDAC	voltage-dependent anion channel
YY1	YingYang 1

Chapter 1: Introduction

Skeletal muscle is involved in fundamental functions of the body such as breathing, posture and locomotion. Muscle mass is fine-tuned by the balance between anabolism and catabolism of the proteins from which it is built. For instance, a decrease in protein synthesis or an increase in protein degradation both result in a loss of muscle mass. Important muscle atrophy is associated with muscle weakness, leading to a loss of autonomy when performing daily activities (Janssen et al. 2002). Representing nearly 40% of total body mass, skeletal muscle consumes substantial amounts of glucose and fatty acids and, in case of energy deficit, provides a significant reservoir of amino acids. Therefore, loss of muscle mass also accounts for metabolic disturbances such as glucose intolerance and worsens chronic diseases like obesity and type 2 diabetes (Booth et al. 2000).

Growing evidences support that physical exercise is certainly the most effective and the cheapest approach to maintain muscle mass. To a larger extent, it preserves a healthy body metabolism together with a good quality of life (Booth et al. 2000). Exercise is a physiological stimulus that challenges muscle plasticity. Skeletal muscle is able to optimally adapt its phenotype in response to increased contractile and metabolic activities induced by exercise. The type and the intensity of physical exercise could imply mechanical and metabolic alterations (contractile protein damages, reactive oxygen species overproduction harming mitochondrial integrity), the accumulation of which could disrupt muscle function, hence the necessity for skeletal muscle to have an efficient apparatus dedicated to

the prompt disposal of defective cellular elements. Skeletal muscle is endowed with two major proteolytic systems: whereas the ubiquitin-proteasome is able to degrade single proteins or small protein aggregates, the autophagy-lysosome machinery is recruited to eliminate larger cellular components like protein aggregates and entire organelles. So, mitochondria are removed by a specific autophagy process called mitophagy. The present thesis focuses on the activation of autophagy and mitophagy induced by acute endurance exercise and endurance training in human skeletal muscle.

A tight regulation of protein catabolism is essential to avoid excessive disposal of muscle proteins, which could as well disturb whole body homeostasis. But, inhibition of autophagy seems also deleterious for health. In mice, it leads to muscle weakness and atrophy, accumulation of reactive oxygen species (ROS), protein aggregates and defective organelles, as well as premature senescence (Raben et al. 2008, Masiero et al. 2009, Lo Verso et al. 2014, Garcia-Prat et al. 2016). Moreover, activation of autophagy during exercise seems to be a key player for the adaptations to endurance training and the beneficial effects of regular exercise on muscle function and metabolism. For example, exercise training fails to protect autophagy-deficient murine muscle from the deleterious effects of a high-fat diet (He et al. 2012). Moreover, exercise-trained mice with an impaired autophagy activation ($Atg6^{+/-}$) show attenuated increases of mitochondrial content and angiogenesis in skeletal muscle, as well as impaired improvement of endurance capacity (Lira et al. 2013). All this suggests that

autophagy could participate in benefits of exercise on muscle and whole body health. However, most of the studies dedicated to the activation of autophagy during exercise were carried out on animal models, and apart from the works of our labo (Jamart et al. 2012, Jamart et al. 2012), little is known in humans. Therefore, the research reported in this thesis focuses on the activation of autophagy and mitophagy in human skeletal muscle during a single endurance exercise. Since these catabolic pathways can also be regulated by the nutritional state, we have more specifically studied the combination of exercise and nutrition on the activation of autophagy and mitophagy. The results of these studies are reported in the following publications: Schwalm et al. 2015, Schwalm et al. 2017. In a third study that is not yet completed, we checked whether, as in mice, autophagy and mitophagy are more activated in the skeletal muscle of regularly exercised human subjects compared to sedentary ones. We also wanted to know if this activation is dependent on age. But, before presenting our results, it is necessary to put this research in the context of current knowledge on this topic. The next pages will be devoted to this in order to facilitate the understanding of our results, but without making an exhaustive review.

1. Maintenance of cellular homeostasis by autophagy-lysosomal system in mammals

1.1. Autophagy machinery

1.1.1. Phagophore formation and autophagy initiation

Membranes necessary for phagophore formation probably originate from plasma membrane (Ravikumar et al. 2010) or membrane structures of organelles, among which endoplasmic reticulum (ER), Golgi, endosomes and mitochondria (Tooze and Yoshimori 2010). Even though origins of autophagic membranes are still under debate, two protein complexes have been identified as essential for phagophore formation and the onset of autophagy.

The p150-PI3KIII-Beclin-1-Atg14 complex

Autophagy induction requires the association of p150, phosphatidylinositol-3-phosphate kinase class III (PI3KIII), Beclin-1 and Atg14 proteins. The stabilization of this complex is challenged by a variety of proteins, among which anti-apoptotic proteins B-cell lymphoma-2 (Bcl-2) and B-cell lymphoma extra-large (Bcl-X_L) that compete with PI3K to bind Beclin-1, therefore affecting autophagosome membrane synthesis by disrupting the complex. Conversely, a wide variety of binding partners of Beclin-1, such as UV-resistance associated gene protein (UVRAG), high mobility group box 1 (HMGB1) and Atg14L/Barkor, have been evidenced to promote the initiation of autophagy (Kang et al. 2011).

The FIP200-ULK1/2-Atg13-Atg101 complex

A second complex is recruited to the phagophore during first steps of autophagy, which gathers the focal adhesion kinase family interacting protein of 200 kDa (FIP200), UNC-51-like kinases 1/2 (ULK1/2), Atg13 and Atg101. Upon autophagy induction, Atg13 and ULK1/2 are dephosphorylated, entailing an increased activity of UKL1/2. The subsequent phosphorylation of FIP200, Atg13 and ULK1/2 itself ends in phagophore membrane formation (Hosokawa et al. 2009, Jung et al. 2009).

1.1.2. Autophagosome membrane elongation

Atg12-Atg5-Atg16L complex

Elongation of the pre-autophagosomal membrane is controlled by ubiquitin-like proteins conjugation system: Atg12 is first activated by Atg7 (E1 ubiquitin activating enzyme-like) before being transferred to Atg10 (E2 ubiquitin conjugating enzyme-like) and associating with Atg5 (Mizushima et al. 1998). Additional anchor of Atg5 with Atg16L results in the final assembly of the Atg12-Atg5-Atg16L complex (Mizushima et al. 2003). The complex associates with the phagophore until its elongation is fully completed, then dissociates from the autophagosome.

LC3 conjugation system

A second ubiquitin-like conjugation system acts in conjunction with the Atg12-Atg5-Atg16L complex during the elongation process. The microtubule-associated protein 1 light chain 3 (LC3) is cleaved by Atg4, a

protease, to form the LC3 I cytosolic isoform. Successive conjugations of LC3 I with Atg7 (E1 ubiquitin activating enzyme-like), Atg3 (E2 ubiquitin conjugating enzyme-like) and phosphatidylethanolamine (PE) generates the LC3 II isoform, which tethers to both external and internal membrane of the autophagosome (Kabeya et al. 2000, Tanida et al. 2004).

Cross-talk between Atg12-Atg5-Atg16L and LC3 conjugation systems has been reported. On the one hand, PE conjugation to LC3 is facilitated by Atg10 (Nemoto et al. 2003) and Atg12-Atg5-ATG16L complex (Hanada et al. 2007). On the other hand, Atg3 promotes Atg5-Atg12 conjugation process (Tanida et al. 2002). Yet, LC3 II role differs from Atg12-Atg5-Atg16L in that it remains anchored from early (autophagosome genesis) to final stages of autophagy (degradation of the autolysosome content) (Tanida et al. 2004). LC3 is endowed with a domain allowing the anchor of autophagy receptors, as p62/sequestosome 1 (p62/SQSTM1). p62/SQSTM1 targets ubiquitinated substrates and also contains a LC3-interacting motif, which facilitates its recruitment to autophagosomes (Pankiv et al. 2007). The closure of the newly synthesized autophagosomal double-membrane features autophagosome genesis completion.

1.1.3. Maturation, fusion and degradation

Unlike phagophores, autophagosomes are mobile organelles moving along microtubules in a bidirectional manner, with the help of dynein and kinesin motor proteins (Reed et al. 2006, Kimura et al. 2008). This trafficking system enables autophagosomes to fuse with endosomes, then lysosomes (Kochl et al. 2006, Kimura et al. 2008, Liang et al. 2008).

Endosome incorporation is suggested to mediate autophagosome maturation, even though the underlining mechanisms of this process are still unclear (Aad et al. 2010).

Proper fusion of mature autophagosomes with lysosomes depends on the GTPase ras-related protein 7 (Rab7) and lysosome-associated membrane proteins 1 and 2 (LAMP1/2) (Huynh et al. 2007). V-adenosine triphosphatase (V-ATPase)-dependent acidification of the lysosome lumen activates hydrolases catalyzing the degradation of the autolysosome content (Yamamoto et al. 1998, Settembre et al. 2013).

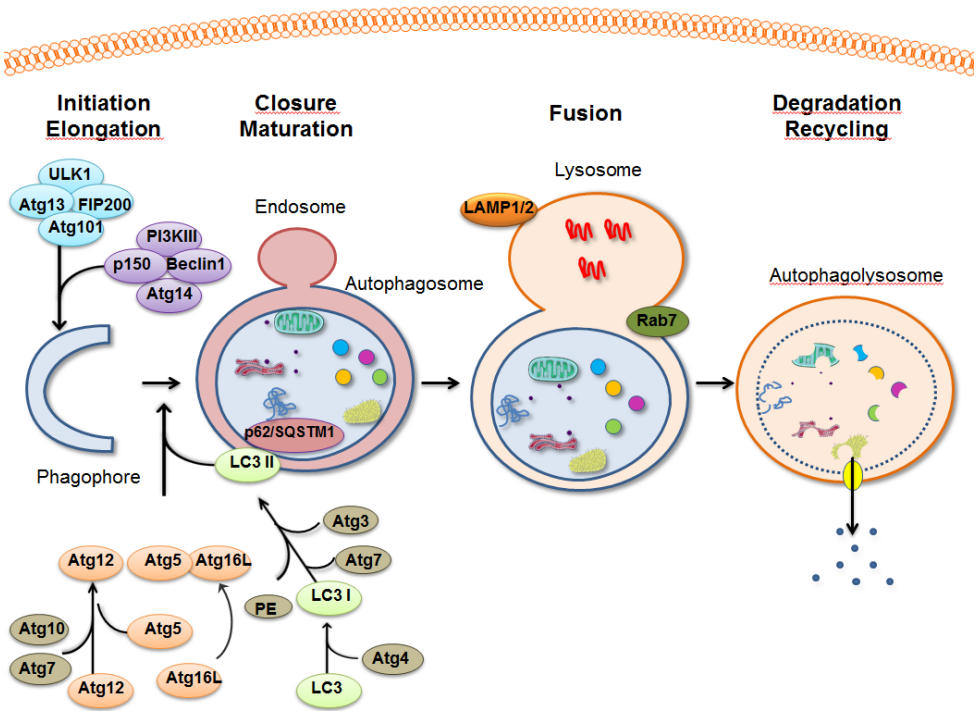

Figure 1. The autophagy machinery. Atg3/4/5/7/10/12/13/14/16L/101: autophagy related protein; FIP200: focal adhesion kinase family interacting protein of 200 kDa; LC3 I/II: microtubule-associated protein 1 light chain 3 I/II; LAMP1/2: lysosome-associated membrane proteins 1/2; PE: phosphatidylethanolamine; PI3K III: p150, phosphatidylinositol-3-phosphate kinase class III; p62/SQSTM1: p62/sequestosome 1; Rab7: ras-related protein 7; ULK1/2: UNC-51-like kinases 1/2. See details in the text.

1.2. Autophagy signaling pathways involved in physiological states

Autophagy relies on coordinated transcriptional and post-translational regulations. Here, we will focus on signaling events triggered by exercise and nutritional state that are reported in skeletal muscle of rodents and humans (Figure 2).

1.2.1. Negative regulation of autophagy: mTOR signaling pathway

The mammalian target of rapamycin (mTOR) pathway is implied in a broad range of cellular functions (cell growth and proliferation, metabolism, cytoskeleton organization, initiation of translation…), including autophagy. mTOR pathway acts as a repressor of autophagy in conditions of nutrient sufficiency. Binding of insulin or growth factors to insulin receptor (IR) promotes its tyrosine-kinase activity and recruitment of insulin receptor substrate 1 (IRS1), which activates phosphoinositide-3-kinase (PI3K). Active PI3K converts phosphatidylinositol 3-4,5-bisphosphate (PIP2) to PIP3 phosphatidylinositol 3-4,5-trisphosphate, attracting phosphoinositide-dependent kinase 1 (PDK1) and Akt/PKB to the cell membrane (Cantley 2002). Akt is both phosphorylated on Thr308 and Ser473 (in mice and humans) by PDK1 and mTOR-rictor complex, respectively (Alessi et al. 1996). Activated Akt then phosphorylates tuberous sclerosis complex 1/2, (TSC1/2), leading to the inhibition of the latter. This allows the successive activation of Ras homolog enriched in brain (Rheb) and mTOR complex 1 (mTORC1), the latter being phosphorylated on Ser2448 (mice and humans). In the presence of amino

acids, such as L-leucine, L-glutamine and arginine, mTORC1 can be alternatively activated by recombination-activating gene (Rag) GTPases, through tethering with the regulatory associated protein of mTOR (raptor) subunit of mTORC1 (Nicklin et al. 2009, Rebsamen et al. 2015, Chantranupong et al. 2016). This interaction is thought to favor the localization of mTOR in the vicinity of its activator Rheb (Sancak et al. 2008). Active mTORC1 meets the FIP200-ULK1/2-Atg13-Atg101 complex and phosphorylates ULK1 on Ser757, resulting in the blocking of autophagy (Hosokawa et al. 2009, Kim et al. 2011).

1.2.2. Positive regulation of autophagy: AMPK signaling pathway

AMP-activated kinase (AMPK) pathway governs autophagy under nutrient deficit. Drop of cellular [AMP + ADP / ATP] ratio activates the AMPKα catalytic subunit *via* its phosphorylation on Thr172. Active AMPK then phosphorylates TSC2$^{Ser1387/Thr32}$ (mice and humans) and raptor (Inoki et al. 2003, Gwinn et al. 2008, Zoncu et al. 2011). The interaction of mTORC1 with the FIP200-ULK1/2-Atg13-Atg101 complex is disrupted and the mTORC1-mediated phosphorylation of ULK1/2^{Ser757} (mice and humans), which is inhibitory for autophagy, is repressed. This leaves AMPK to bind and phosphorylate ULK1/2 at multiples sites as Ser317, Ser467, Ser555, Ser574, Ser637 and Ser777 to initiate autophagy (mice and humans) (Egan et al. 2011, Kim et al. 2011, Shang et al. 2011). Next, activated ULK1 phosphorylates and activates Beclin1 on Ser14 (mice) or Ser15 (humans) to initiate autophagy (Russell et al. 2013). On the contrary, AMPK^{Thr172}-induced autophagy can be indirectly blunted by Akt, when the latter

phosphorylates AMPKα1 on Ser485 (rat) and AMPKα1/α2$^{Ser487/Ser491}$ (human) (Dagon et al. 2012, Hawley et al. 2014).

1.2.3. Transcriptional regulation of autophagy

Autophagic transcription program is governed by distinct transcription factors, among which members of Forkhead box O (FoxOs). Nuclear import of FoxOs initiates the transcription of genes coding for autophagy, such as *LC3*, *Gabarap*, *Bnip3, and Bnip3L* (Mammucari et al. 2007). The transcription of autophagy-related genes is prevented by Akt-mediated phosphorylation of FoxO1$^{Thr24/Ser256}$ and FoxO3$^{Thr32/Ser253}$, retaining FoxOs in the cytosol (mice and humans) (Mammucari et al. 2007). Conversely, AMPK-mediated phosphorylation of FoxO3a on Ser588 induces its nuclear translocation (mice) (Sanchez et al. 2012). Furthermore, constitutive activation of FoxO1 in mouse skeletal muscle was recently evidenced to trigger the transcription of *cathepsin L*, suggesting a role for FoxO1 in the final stages of autophagy, when proteins are delivered to the lysosomes for degradation (Yamazaki et al. 2010).

FoxOs activity is also controlled by sirtuins: under nutrient starvation, FoxO1 is acetylated and dissociates from sirtuin-2 (SIRT2) in the cytoplasm. This allows FoxO1 to tether Atg7 to induce autophagy (Zhao et al. 2010). In addition, the transcriptional activity of FoxO3 is promoted by sirtuin-1 (SIRT1)-dependent deacetylation, in conditions of caloric restriction, activating BNIP3-mediated autophagy (Kume et al. 2010). Transcription factor EB (TFEB) was first described as a key regulator of lysosomal biogenesis through mTOR pathway. In the presence of nutrients, mTORC1

associates and phosphorylates TFEB at Ser142 and Ser211 (in mice) in a Rag-GTPAse-dependent manner. TFEB is then retained in the cytosol and lysosome biogenesis is interrupted (Settembre et al. 2011, Settembre et al. 2012). Extracellular-regulated kinases 2 (ERK2)-mediated phosphorylation of TFEB at Ser142 (in mice) also results in cytosolic location of TFEB in nutrient-rich medium (Settembre et al. 2011). Interestingly, TFEB was also found to upregulate the transcription of autophagy genes (Palmieri et al. 2011). In addition, TFEB overexpression increases the number of autophagosomes, promotes fusion between autophagosomes and lysosomes and degradation of autophagy substrates (Settembre et al. 2011). Altogether, these observations suggest a common transcriptional regulation of autophagy and lysosome biogenesis mediated by TFEB.

Nuclear receptor Rev-erb-α/NR1D1 inhibits the expression of a wide panel of autophagy-related genes as *Becn1*, *Ulk1* and *Atg5* in mouse oxidative muscle, including the ones dedicated to the removal of dysfunctional mitochondria like *Bnip3*, *Park2/Parkin* (Woldt et al. 2013).

1.2.4. Others pathways potentially regulating autophagy during physiological conditions

Autophagy is known to be driven by p38 MAPK and NFκβ signaling during pathological states such as cachexia or sepsis, all featured by chronic oxidative stress and inflammation (McClung et al. 2010, Mofarrahi et al. 2012). Alternatively, both pathways are also induced during physiological conditions, like acute exercise. The latter also implicates oxidative and inflammatory stresses, depending on the intensity and the

mode of exercise, but in a transient way (Kramer and Goodyear 2007). Based on the previous finding that pathological oxidative stress activates autophagy through p38 MAPK axis, two studies speculated that exercise-induced autophagy would be partly regulated by that pathway (McClung et al. 2010, Jamart et al. 2013). Despite both autophagy and p38 MAPK were activated by the protocol, those processes were not dependent on oxidative stress. To date, evidences clearly linking physiological stimuli to p38 MAPK and NFκβ -induced autophagy are lacking.

1.2.5. Crosstalk between autophagy and apoptosis

Several reports indicate that markers of autophagy-dependent degradation removal could be also involved in apoptosis-mediated degradation, suggesting a potential cross-talk between autophagy and apoptosis. The tumor protein 53 (p53) would serve as a hub between cell survival (*i.e.* autophagy) and cell death (*i.e.* apoptosis). On the one hand, the nuclear translocation of p53 would stimulate autophagy, through the transcription of genes coding for pro-autophagy effectors, among which *AMPK*, *TSC2* and *Bcl2/adenovirus E1B 19 kDa interacting protein 3* (*BNIP3*) (Maiuri et al. 2010). *ULK1/2* would be another transcriptional target of p53 as well (Gao et al. 2011). On the other hand, cytosolic localization of p53 would rather repress autophagy, thus facilitating apoptosis (Maiuri et al. 2010). Nuclear abundance of p53 was demonstrated to occur 3h after acute exercise in human skeletal muscle, but in that study, autophagy gene expression was not analyzed to confirm or not the role of p53 in autophagy (Tachtsis et al. 2016). On the opposite, nuclear presence of p53 is reduced

in mouse muscle after exercise, highlighting the complexity of p53 regulation (Saleem and Hood 2013). In humans, exercise-induced nuclear translocation of p53 is hypothesized to be facilitated by its phosphorylation on Ser15 during the recovery period (Bartlett et al. 2012). Possibly, reduced carbohydrate availability would also increase the phosphorylation of $p53^{Ser15}$, both at rest and after acute exercise (Bartlett et al. 2013). However, in the two studies of Bartlett and collaborators, phosphorylation state of p53 was only quantified in total muscle extracts and changes in autophagy-related gene expression were not assessed. Besides, a very recent report underlined the role of AMPK/$p27^{Kip1}$ signaling in the regulation of the autophagy/apoptosis balance during aging in murine muscle satellite cells. Whereas senescent satellite cells exhibited increased apoptosis, lower autophagy and decreased phosphorylation of both AMPK and $p27^{Kip1}$, restored activation of AMPK/$p27^{Kip1}$ axis prevented senescence and cell death. In particular, cell survival relied on nuclear translocation of $p27^{Kip1}$ (White et al. 2018).

A possible connection between selective autophagic-degradation of organelles like mitochondria and apoptosis has also been reported, and is briefly described in below sections 2.4.1 and 2.4.2 of the present chapter.

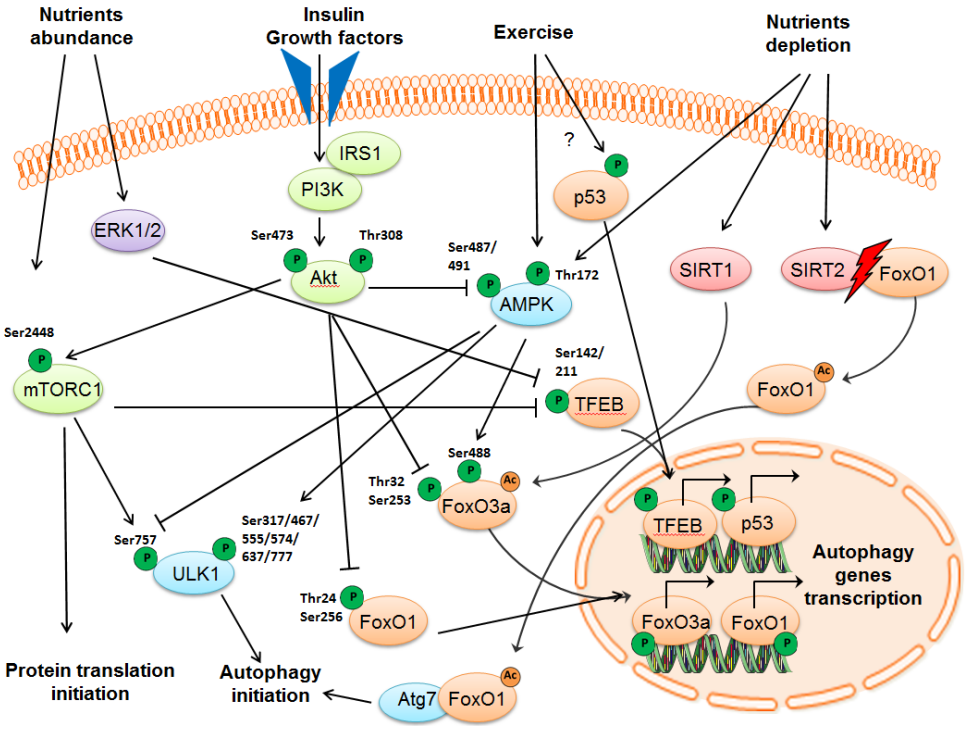

Figure 2. Overview of autophagy signaling pathways mediated by nutrition and exercise, two stimuli on which the present work will be focused in skeletal muscle of mice and humans. See details in the text. Ac: acetylation; Akt/PKB: protein kinase B; AMPK: AMP-activated kinase; Atg7: autophagy related protein 7; ERK1/2: extracellular signal-regulated kinases 1/2; FoxO1: Forkhead box O1; FoxO3: Forkhead box O3; IRS1: insulin receptor substrate 1; mTOR: mTOR complex 1; P: phosphorylation; PI3K: p150, phosphatidylinositol-3-phosphate kinase; p53: tumor protein 53; Ser: serine; SIRT1: sirtuin 1; SIRT2: sirtuin 2; TFEB: transcription factor EB; Thr: threonine; Ub; ubiquitination; ULK1/UNC-51-like kinases 1/2.

1.3. Role of autophagy in skeletal muscle homeostasis and myopathies

A fine-balanced regulation of autophagy is crucial for muscle homeostasis, since deficient or excessive activation of autophagy can lead to detrimental consequences as muscle atrophy and metabolic disturbances (Figure 3).

The specific role of autophagy in skeletal muscle has been first highlighted by loss-of-function experiments. Muscle-specific Atg5 and Atg7 knockout mice, characterized by altered autophagy, exhibit muscle weakness and atrophy, and accumulation of ROS, abnormal organelles and protein aggregates (Raben et al. 2008, Masiero et al. 2009, Lo Verso et al. 2014). The critical impact of autophagy failure is even more obvious in conditions when autophagy is required for prolonged energy need and removal of damaged contractile proteins and ROS-induced dysfunctional organelles, such as during eccentric endurance exercise: exacerbated myofibers degeneration is observed in exercised mice deficient in collagen VI, an alternative model of muscle dystrophy due to compromised autophagy (Grumati et al. 2011). Furthermore, eccentric exercise-induced ROS production was significantly higher in Atg7 knockout mice than in wild type animals (Lo Verso et al. 2014).

Defective autophagy has pathological consequences in diverse forms of muscle diseases. In particular, abnormal accumulation of autophagic vacuoles is the hallmark of inherited autophagic vacuolar myopathies, a subset of myopathies featured by genetic mutations affecting lysosomal

function. For instance, Pompe disease originates from a defect in the gene coding for lysosomal enzyme acid α-glucosidase, which compromises the hydrolysis of glycogen to glucose, and leads to an overload of glycogen in defective lysosomes (Katzin and Amato 2008). Danone disease is caused by mutations of the *Lamp2* gene, which alters the maturation of autophagosomes and the fusion of autophagosomes with lysosomes (Endo et al. 2015). X-linked myopathy with excessive autophagy is due to mutations of the *VMA21* gene, which consequently prevents the assembly of V-ATPases required for acidification of the lysosome and diminishes the activity of lysosomal hydrolases, leading to accumulation of autophagolysosomes (Dowling et al. 2015). Autophagic vacuolar myopathies, as well as cardiomyopathies, can develop much more commonly secondary to treatment with inhibiting-autophagy drugs (chloroquine, hydroxychloroquine, colchicine) used to investigate autophagic flux, or else used in clinical therapy for malaria, rheumatological diseases and gout (Klionsky et al. 2016).

Nonetheless, autophagy induction is suggested to participate to the beneficial effects of exercise on skeletal muscle function: autophagy-deficient mice have lessened exercise endurance and an impaired glucose tolerance. Furthermore, while long-term exercise protects control mice against deleterious metabolic effects of a high-fat diet, autophagy-deficient animals are unable to reverse high-fat diet metabolic abnormalities after training (He et al. 2012). Training also improves basal autophagy levels in mixed-fibers type muscle of mice. Importantly, that increased basal

autophagy is mandatory for exercise-induced muscle adaptations and improvement of physical performance (Lira et al. 2013).

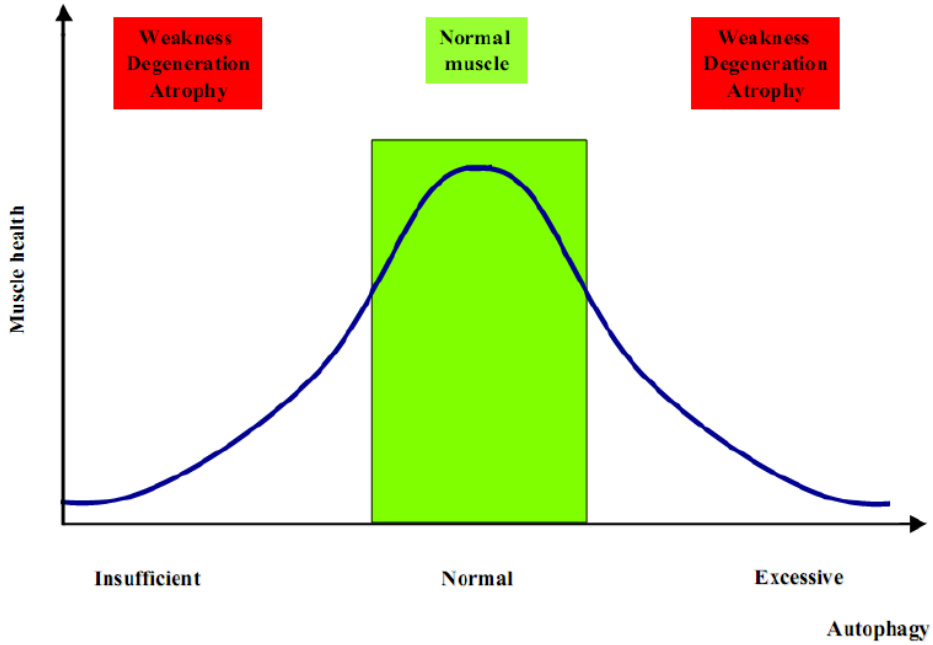

Figure 3. Insufficient or exaggerated activation of autophagy are both harmful for muscle health (Grumati and Bonaldo 2012).

1.4. Interpretation of autophagy markers in humans studies

In this work, we investigated changes in markers previously identified in autophagy signaling pathways in response to exercise, nutrition and aging in human skeletal muscle biopsies. The particularity of this experimental model is that only static measurements of autophagy markers can be obtained and multiple biopsies samples within subjects are ethically limited. Autophagy activity is commonly investigated in living cells or small organisms using pharmacological inhibitors, which targets different stages of autophagy process. In humans, measuring direct autophagy activity is not possible. Even though pharmacological substances to repress autophagy are clinically approved to treat specific pathologies, the resort to these molecules in a physiological context is ethically questionable because they are associated to detrimental side effects (see chapter 5, section 7: limitations) (Klionsky et al. 2016). Therefore, in human studies, the variety of analyses tools is more restricted than in other models. However, the use of quantitative and qualitative analyses of markers involved in different stages of autophagy facilitates the interpretation of experimental observations.

The protein expression of LC3 II is commonly referred as a marker of mature autophagosomes abundance (Klionsky et al. 2016). In particular, LC3b isoform is widely expressed in human skeletal muscle (He et al. 2003). The lipidation of LC3, through conversion of LC3 I to LC3 II, governs the generation of mature autophagosomes, and allows their fusion with

lysosomes. The comparison of LC3 I protein levels with LC3 II protein expression by Western Blot, using LC3 II/LC3 I ratio, is helpful to assess the amounts of mature autophagosomes susceptible to fuse with lysosomes. Both LC3 I and II should be normalized to one or several housekeeping proteins, which are easily detectable in the tissue and whose expression is not affected by experimental conditions. The resort to Coomassie blue as a loading control, which stains for total cellular proteins, is an alternative method for normalizing protein expression (Klionsky et al. 2016). LC3 II remains anchored to the double membrane of autophagosomes from their formation until their degradation (Tanida et al. 2004). Thus, decreased LC3 II may reflect decreased autophagosomes turnover through repressed maturation of autophagosomes or inefficient clearance of the cargo of autophagolysosomes. On the opposite, increased LC3 II can be explained by the formation of new mature autophagosomes, or else, by the accumulation of autophagosomes resulting from the blocking of their fusion with lysosomes. It can also be due to inefficient degradation of autophagolysososmes. Therefore, amounts of LC3 II protein must be compared with variations of autophagy markers, which intervene exclusively in early steps of autophagy, and markers involved in final steps of autophagy only (Klionsky et al. 2016).

p62/SQSTM1 recognizes ubiquitinated protein aggregates and binds to LC3 II before being degraded with the autophagolysosome cargo (Johansen and Lamark 2011). Therefore, reduced p62/SQSTM1 protein content is thought to evidence autophagic degradation, when observed in parallel to

increased LC3 II/ LC3 I ratio, at least for dynamic measurements (Klionsky et al. 2016).

Assessment of the phosphorylation state of autophagy proteins involved in autophagy signaling gives clues on upregulation or downregulation of autophagy according to experimental conditions. For instance, ULK1, involved in first steps of autophagy only, is inhibitory for autophagy when phosphorylated on Ser757. Conversely, phosphorylation of ULK1 on Ser317 has an activating effect on autophagy. Changes in phosphorylation of ULK1 can be supplemented with expression of phospho-proteins acting upstream of ULK1. For example, analyzing phospho-Akt in parallel to phospho-AMPK is relevant because they belong to antagonistic pathways of autophagy, which converge on ULK1. Phosphorylation of $Akt^{Ser473/Thr308}$ is supposed to repress autophagy *via* increased $ULK1^{Ser757}$ level, whereas phosphorylation of $AMPK^{Thr172}$ is likely to stimulate autophagy through enhanced $ULK1^{Ser317}$ content. In the case of mitophagy, tissue fractionation for Western-Blot analyses offers the opportunity to clarify the cellular location of specific proteins, which migrate from the cytosol to the mitochondria surface when mitophagy is increased, like dynamin-related protease 1^{Ser616} ($Drp1^{Ser616}$) or $PARKIN^{Ser65}$. Furthermore, simultaneous quantification of mitochondrial and cytosolic amounts of a given mitophagy protein allows to refine data quantification, in comparison to analyses performed on total muscle extracts.

Other technical assays, used in parallel to Western Blot, improve the interpretation of changes in autophagy protein expression. Among them,

immunofluorescent or histochemical staining of muscle cryosections, to analyze the colocalization of well-chosen autophagy/mitophagy proteins, provides visual indications of the state of progress of autophagy/mitophagy (Klionsky et al. 2016). In the case of mitophagy, 3D-colocalization of proteins specific to the membranes of autophagosomes (as LC3), mitochondria (like TOM20) and lysosomes (as LAMP2b) allows to estimate whether mitochondria are engulfed in mature autophagosomes (early mitophagy event) or predisposed to be eliminated by autophagolysosomes (late mitophagy event).

Because autophagy is subject to transcriptional regulation, analysis of autophagy-related gene expression, through quantitative real-time polymerase chain reaction (qRT-PCR), gives additional arguments to state whether autophagy is induced or not. In addition, this is valuable for validating data from Western Blot analyses (Klionsky et al. 2016).

2. Maintenance of mitochondria homeostasis in mammals

2.1. Mitochondrial biogenesis

Mitochondrial biogenesis (mitogenesis), *i.e.* increase in mitochondrial mass and mitochondria copy number, is achieved through synthesis of new mtDNA and proteins involved in mitochondria structure and function. Indeed, in human, only 13 mitochondrial proteins are encoded by the mitochondrial genome, while the nuclear genome codes for 99% of the mitochondrial proteins. Mitochondrial-encoded genes encompass transfer ribonucleic acid (tRNA), ribosomal ribonucleic acid (rRNA) and most of

electron-transport chain (ETC) complexes, except complex II. Nuclear-encoded mitochondrial proteins are synthetized as loosely folded precursors by ribosomes in the cytosol. Thereby, they need to be imported and processed within mitochondria by import and proteases machineries. The translocase of the outer mitochondrial membrane (TOM) complex is the major import gateway of mitochondrial proteins. According to their function and structural properties, mitochondrial proteins are inserted into the inner mitochondrial membrane (IMM) by translocases of the inner mitochondrial membrane (TIM), or are cleaved on the outer mitochondrial membrane (OMM) by sorting and assembly machinery (SAM) complex, or reach the inner membrane space by intermembrane space receptors (Mia proteins), or else translocate to the mitochondrial matrix thanks to TIM complexes and translocases-associate membrane (PAM) proteins (Wiedemann and Pfanner 2017). Heat shock proteins contribute to complete mitogenesis inasmuch as they insure functional conformation of mitochondrial proteins (Voos and Rottgers 2002).

2.2. Mitochondrial quality control systems

Being the place of oxidative phosphorylation in the cell, mitochondria spontaneously generate ROS, which are necessary for mitochondria signaling, but which could jeopardize the integrity of these organelles and whole cell if they are overproduced. Indeed, the vicinity of mitochondrial DNA (mtDNA), membranes, proteins and lipids with the ROS-producing electron transport chain make them particularly vulnerable to excess of ROS. Alternatively, ROS-altered mitochondria release accrued amounts of

ROS, worsening organelles malfunction (Balaban et al. 2005). Thus, mitochondria comprise different quality control mechanisms specific to the nature and the extent of the mitochondria insult, which work in conjunction to prevent the accumulation of harmful mitochondrial components while leaving the entire organelle intact (Quiros et al. 2015).

2.2.1. Micro-proteases: AAA complexes

Mitochondria house proteolytic systems that intervene in the degradation of non-assembled, misfolded, oxidized proteins, the AAA proteases complexes. They are either included in the intermembrane space (intermembrane AAA (iAAA) proteases; Ser protease HTRA2/Omi (HTRA2/Omi)), in the inner mitochondrial membrane, or in the mitochondrial matrix (matrix AAA (mAAA) proteases; Ser protease ATP-dependent Clp protease proteolytic subunit (CLPP); ATP-dependent Clp protease ATP-binding subunit ClpX-like (CLPX); Lon protease homolog (LONP)). These micro-proteases are suggested to be the first mean of defense against defective mitochondrial proteins (Quiros et al. 2015).

2.2.2. Ubiquitin-proteasome system

The clearance of dysfunctional OMM proteins is operated by the ubiquitin-proteasome system (UPS): E3 ligases are recruited to the surface of mitochondria, for ubiquitination of the target proteins, which are finally degraded through the UPS (Karbowski and Youle 2011). In addition, the UPS tags severely oxidized IMM proteins, which are retransferred to the OMM for ubiquitination and subsequent degradation. The vicinity of the

IMM proteins with the respiratory chain makes them likely to be oxidized by free radicals (Azzu and Brand 2010). Beyond removal of unwanted mitochondrial membrane proteins, the UPS is thought to prevent fusion of faulty mitochondria with healthy mitochondrial pool and facilitates their degradation by mitophagy. For example, E3 ubiquitin protein ligase parkin (PARKIN) has been shown to switch to mitochondria harboring a low membrane potential in order to ubiquitinate mitochondrial fusion proteins Mitofusins 1/2 (Mfn1/2). The latter are then extracted from the OMM by the chaperone p97 to be degraded by the UPS, while depolarized organelles are specifically degraded by mitophagy (Tanaka et al. 2010). Clustered ubiquitinated mitochondrial proteins associate to the p62/SQSTM1 adaptor, which binds to LC3 autophagic receptor to finalize the removal of mitochondrial proteins (Pankiv et al. 2007, Geisler et al. 2010). Conversely, PARKIN-mediated ubiquitination and the subsequent degradation of the fission protein Drp1 inhibit fission (Wang et al. 2011).

2.2.3. Mitochondria-derived vesicles

In addition, oxidized mitochondrial proteins can be delivered to late endosomes or lysosomes for local degradation by vesicles directly budding from mitochondria microtubules. Basal production of mitochondria-derived vesicles (MDV) is enhanced by oxidative stress, and the specificity of the cargo of MDV would rely on the origin of ROS. Sources of ROS external to mitochondria give birth to single-membrane MDV containing OMM proteins only, such as TOM20 or VDAC. Conversely, mitochondrial sources of ROS generate double-membrane MDV, which trap IMM and

matrix proteins such as pyruvate dehydrogenase (PDH) and complex III subunit core 2 (Soubannier et al. 2012, McLelland et al. 2014). Here, the abundance of MDV enriched in OMM proteins is suggested to be mediated by the recruitment of PTEN-induced putative kinase 1 (PINK1) and PARKIN to the OMM, while the amounts of MDV enriched in IMM and mitochondrial matrix markers would be dependent on the abnormal accumulation of DRP1 at the surface of mitochondria (McLelland et al. 2014, Wang et al. 2016).

2.2.4. Mitochondrial dynamics

Mitochondria constitute a dynamic reticulum, the size of which, morphology and motility are ceaselessly reorganizing to preserve cell function in response to metabolic and environmental stress.

Fusion brings mitochondria together in a connected network to merge mitochondrial DNA, membrane constituents, proteins or metabolites (Nakada et al. 2001, Arimura et al. 2004). This form of cross-complementation would alleviate cellular stress by preventing partially defective mitochondria from removal, as in the case of minor DNA mutations (Yoneda et al. 1994, Nakada et al. 2001). In addition, fusion would help mitochondria to maintain oxidative phosphorylation despite nutrient deficit or starvation and to protect them from autophagic removal (Rossignol et al. 2004, Gomes et al. 2011, Rambold et al. 2011). Proper mitochondria fusion of OMM and IMM is achieved by the GTPases Mfn1/2 and the optic atrophy protein 1 (OPA1), respectively (Ding and Yin 2012). Beyond governing fusion, Mfn2 and OPA1 are reported to play additional

key roles in mitochondria remodeling: phosphorylation of Mfn2 by PINK1 entails recruitment of PARKIN on the OMM by tethering Mfn2 and promotes autophagic degradation (Chen and Dorn 2013). Furthermore, OPA1 determines mitochondrial cristae architecture and assembly of respiratory chain complexes into super-complexes (*i.e.* physical interaction between respiratory chain complexes) (Cogliati et al. 2013). The latter drive the electron flux in the ETC and are crucial for using available substrates optimally (Lapuente-Brun et al. 2013).

Fission divides mitochondrial network into fragments of the isolated organelle to get rid of severely damaged mitochondria because they need to consume more energy to maintain their membrane potential. Fission fragments one mitochondrion in two daughter organelles with transient uneven membrane potential: the depolarized one is targeted by selective autophagy, *i.e.* mitophagy, whereas the hyperpolarized mitochondrion recovers its membrane potential before it rejoins the healthy mitochondrial network by fusion (Twig et al. 2008). Even though they appear to be opposite mechanism processes at first sight, fusion and fission finally turn out to act in a complementary fashion (Figure 4).

In mammals, fission relies on the phosphorylation of cytosolic DRP1 at Ser616 (in humans) by Cyclin-dependent-kinase B (Cdk1-cyclin B) and calcium-dependent/calmodulin-dependent protein kinase 2 (CaMK2), which tethers OMM proteins Fis1, mitochondrial fission factor (Mff) or MIEF1/Mid51 (Yoon et al. 2003, Taguchi et al. 2007, Zhao et al. 2011, Xu et al. 2016). However, DRP1 has been reported to have a preferential affinity

for Mff, while Fis1 is more prone to bind mitochondrial elongation factor 1/mitochondrial dynamic protein 51 (MIEF1/MID51) (Otera et al. 2010, Zhao et al. 2011). In addition, phosphorylation of DRP1 at Ser637 by the calcium/calmodulin-dependent protein kinase 1 α (CaMK1 α), following calcium influx, also promotes fission through the interaction between DRP1 and Fis1 (Han et al. 2008). Conversely, phosphorylation at Ser637 (in humans) by the cAMP-dependent-protein kinase A (PKA) docks DRP1 in the cytosol, preventing mitochondrial degradation (Cribbs and Strack 2007). Besides, rise of cytosolic calcium levels (Ca^{2+}) leads to calcineurin-induced dephosphorylation of Drp1 at the same residue allows the mitochondrial translocation of Drp1 (Cereghetti et al. 2008).

2.2.5. Autophagic degradation of mitochondria: mitophagy

Removal of entire mitochondria is not limited to a bulk degradation process within the whole cell, but can be specifically mediated at organelle level through mitophagy, while leaving other cellular components intact. Mitophagy aims to suppress impaired mitochondria and to adjust the number of organelles within the cell by eliminating superfluous ones, which are energy-consuming. Mitophagy distinguishes from macro-autophagy insofar as it is sensitive to mitochondrial morphology and membrane potential, and requires distinct effectors. Fission of the mitochondrial network and loss of mitochondrial membrane potential are both pre-requisite events that flag mitochondria and attract autophagic machinery (Twig et al. 2008). Targeted mitochondria are engulfed into

autophagosomes and finally degraded through specific autophagy (Figure 4).

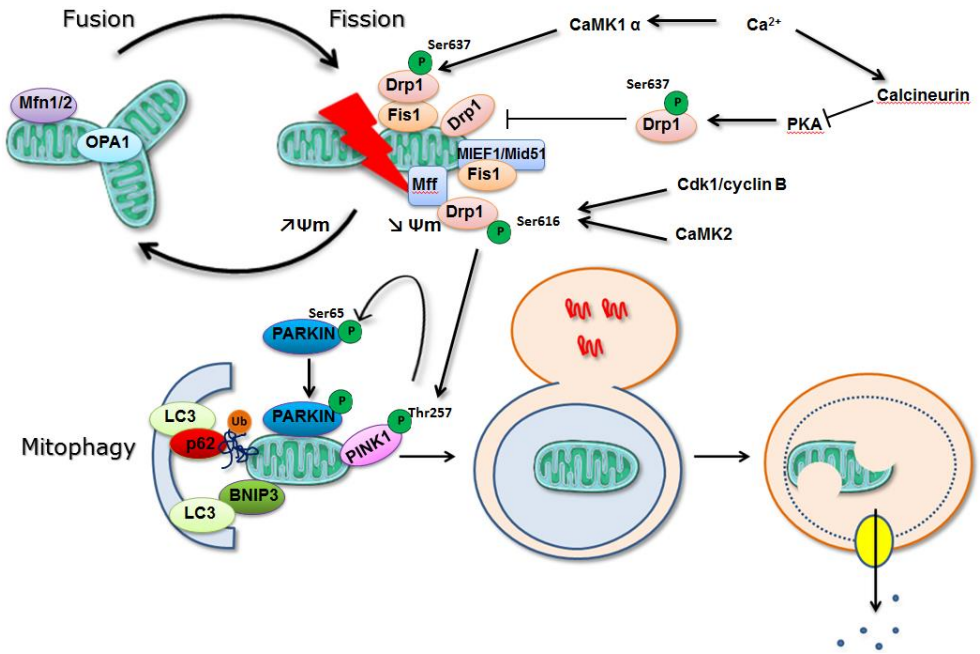

Figure 4. Mitochondrial dynamics and mitophagy. BNIP3: Bcl2/adenovirus E1B 19 kDa interacting protein 3; Ca^{2+}: calcium ion; CaMK1α: calcium/calmodulin-dependent protein kinase 1 α; CaMK2: calcium/calmodulin-dependent protein kinase 2; Cdk1-cyclin B: cyclin-dependent-kinase B; Drp1: dynamin-related protease 1; Fis1: fission protein 1; LC3: microtubule-associated protein 1 light chain 3; MIEF1/MID51: mitochondrial elongation factor 1/mitochondrial dynamic protein 51; Mff: mitochondrial fission factor; Mfn1/2: mitofusins 1/2; OPA1: optic atrophy protein 1; P: phosphorylation; PARKIN: E3 ubiquitin protein ligase parkin; PINK1: PTEN-induced putative kinase 1; p62/SQSTM1: p62/sequestosome 1; Ser: serine; Ψm: mitochondrial membrane potential. See details in the text.

2.3. Molecular pathways regulating mitochondrial biogenesis and functions

2.3.1. Nuclear-encoded genes transcription

Mitogenesis is partly driven by the expression of nuclear-encoded genes. The family of peroxisome proliferator activated receptors (PPARs) coactivators potentiates the activity of a vast array of mediators as nuclear hormones receptors, nuclear respiratory factors, transcription factors and sirtuins, all involved in mitogenesis and mitochondrial function.

Peroxisome proliferator-activated receptor gamma alpha co-activator-1 alpha (PGC1α) binds to nuclear respiratory factor 1/2 (NRF1/2) to initiate the transcription of several nuclear-encoded respiratory genes (Islam et al. 2017). The PGC1α-NRF1/2 pathway also governs the expression of nuclear-encoded genes for mitochondrial transcription factors A (TFam), B1 (TFB1M) and B2 (TFB2M), all being essential for the transcription and replication of mtDNA (Falkenberg et al. 2002, Wright et al. 2007, Hood et al. 2016).

PGC1 coactivators directly interacts with PPARs, which are important for mitochondrial metabolism since they upregulate the expression of genes implicated in fatty acids oxidation, in particular in skeletal muscle (Vega et al. 2000, Fan and Evans 2015). PPAR members are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. PPARs form heterodimers with retinoid X receptors (RXRs) and bind as a complex to the peroxisome proliferator response elements,

located in the regulatory region of their target genes. The activity of PPARs is governed by ligand binding, as well as phosphorylation, SUMOylation and ubiquitination events (Phua et al. 2018). PPARs are important mediators of glucose and lipid homeostasis, and also regulate proliferation, differentiation, and postnatal development (Phua et al. 2018). PPAR members are differently distributed amongst tissues of the body. PPARγ is mostly found in adipose tissue, where it regulates adipogenesis and adipocyte metabolism (Lehrke and Lazar 2005). PPARα is expressed in active metabolic tissues, like liver, kidney, heart, and skeletal muscle, and PPARβ/δ is ubiquitously expressed because of its implication in diverse systemic and basic cellular functions (energy regulation in metabolically active tissues, inflammation, wound healing, keratinocyte and intestinal cell differentiation) (Phua et al. 2018). PPARβ/δ is particularly abundant in skeletal muscle. It is important for muscle regeneration since ablation of PPARβ/δ impedes proliferation and differentiation capability of murine satellite cells (Angione et al. 2011). PPARβ/δ expression is elevated by endurance training in human muscle, ensuing increased transcription of oxidative and lipoprotein metabolism genes and PGC1α protein amounts (Greene et al. 2012). In muscle of mice, endogenous activation of PPARβ/δ upregulates oxidative genes, mitochondrial biogenesis and oxidative myofibers content, and spares glucose utilization, leading to better endurance performance (Wang et al. 2004, Fan et al. 2017). PPARs are pharmaceutical targets for treatment of type 2 diabetes and dyslipidemia: synthetic ligands of PPARs are clinically used to improve insulin sensitivity, lipid uptake and oxidation (Phua et al. 2018). Furthermore, PPARβ/δ

agonist has been shown to restore impaired exercise endurance in mice model of ischemic cardiomyopathy (Zizola et al. 2015). A role for PPARs in autophagy has been recently suggested in the liver of mice, since activation of PPARγ by irbesartan administration has been reported to induce autophagy in the context of hyperlipidemia and hepatic steatosis (Zhong et al. 2017). To the best of our knowledges, a possible implication of PPARs in skeletal muscle autophagy has not been determined yet.

PGC1α and PGC1β orchestrate the activity of the estrogen-related receptors (ERRs), another set of nuclear hormones receptors. ERRs are highly expressed in tissues with elevated metabolic demand, like skeletal muscle (Eichner and Giguere 2011). They are implicated in the transcription of genes implicated in oxidative phosphorylation, fatty acid oxidation, Krebs cycle and factors regulating mitochondrial fusion/fission (Dominy and Puigserver 2013). They also participate to PGC1α-mediated activation of antioxidant enzymes by linking to *SIRT3* promoter (Kong et al. 2010). The PGC1 coactivators/ERRs axis is positively mediated by SIRT1-dependent deacetylation events. Alternatively, ERRs activity would be attenuated by the receptor interacting protein of 140 kDa (RIP140) (Wilson et al. 2010).

PGC1α was also found to function in conjunction with YingYang1 (YY1) transcription factor in the transcriptional control of mitochondrial oxidative phosphorylation (Cunningham et al. 2007).

PGC1α activity is mediated by cAMP activated-response element-binding protein (CREB) transcription factor, the latter taking part in

gluconeogenesis, β-oxidation and the expression of respiratory chain subunits (Gopalakrishnan and Scarpulla 1994, Herzig et al. 2001).

Beyond playing a key role in lysosome biogenesis and autophagy, nuclear translocation of TFEB was recently reported to enhance the expression of a plethora of mitogenesis-related genes, including *PGC1α, TFam* and *NRF1/2*. In addition, TFEB promotes mitochondrial enzyme expression and activities of citrate synthase (CS), (succinate dehydrogenase (SDH), cytochrome c oxidase (COx) and respiratory chain complexes (Mansueto et al. 2017).

Sirtuins are NAD^+-dependent deacetylases located either in the cytoplasm, nucleus or mitochondrion. Nuclear SIRT1 is first known to deacetylate many transcription factors to upregulate genes playing a role in mitochondrial respiration (Dominy and Puigserver 2013). In mouse skeletal muscle and in fasting conditions, SIRT1 deacetylates PGC1α and target genes related to fatty acid oxidation (Gerhart-Hines et al. 2007). SIRT1 is thought to contribute in mitogenesis and oxidative metabolism, according to experiments where mice were fed with resveratrol, an inducer of SIRT1 (Lagouge et al. 2006). SIRT3 also seems to govern skeletal muscle mitochondrial oxidative metabolism, given that its overexpression enhances muscle oxidative capacity and lipid utilization in mice, but the molecular mechanisms remain unclear (Lin et al. 2014). Furthermore, the upregulation of mitochondrial SIRT3 by PGC1α/ERR axis activates anti-oxidant enzymes (Kong et al. 2010).

Similarly to the PGC1α-NRF1/2 pathway, the transcriptional expression of TFam depends on the transcription factor p53 (Saleem et al. 2014). p53 also operates the transcription of genes coding for mitogenesis and oxidative phosphorylation-associated proteins such as NRF1 and cytochrome *c* oxidases (Matoba et al. 2006, Saleem and Hood 2013). As the *PGC1α* gene promoter contains a binding site for p53, and because loss of p53 reduces PGC1α expression, mitogenesis may alternatively rely on interaction between p53 and PGC1α (Irrcher et al. 2008, Saleem et al. 2009).

Rev-erbα/NR1D1 was demonstrated to play an important role in mitochondrial respiration and function in oxidative muscle of mice: Rev-erbα/NR1D1 augments mitochondrial DNA content, as well as the expression of genes encoding subunits of the ETC. This process was dependent of AMPK/SIRT1/ PGC1α signaling (Woldt et al. 2013).

Beside mitochondrial biogenesis, PGC1 coactivators control mitochondrial dynamics: indeed, PGC1α and PGC1β upregulate *Mfn1/2* genes in an ERRα-dependent fashion in muscle of mice and humans (Russell et al. 2013). Conversely, muscle specific knock-down of PGC1α and PGC1β decrease the expression of *Mfn1/2* and Drp1 in mice (Zechner et al. 2010).

PARIS (ZNF746) was identified as a transcriptional repressor of PGC1α and its target gene NRF1. Because PARIS activity is under PARKIN control, PARIS was suggested to link mitophagy to mitogenesis: Parkin activation

degrades PARIS in the cytosol through the ubiquitin-proteasome system. This avoids nuclear import of PARIS and subsequent inhibition of PGC1α. In this way, loss of mitochondria number due to mitophagy could be restored with synthesis of new organelles (Shin et al. 2011).

2.3.2. Mitochondria-encoded genes transcription

Mitogenesis also results from the up-regulation of genes encoded on mtDNA. mtDNA comprises a light and a heavy strand, and the majority of mitochondrial genes are found on the heavy strand. The promoter of both strands and the origin of replication are located on the D-loop, a non-coding region of the mtDNA. Bidirectional mtDNA transcription begins with the assembly of a complex, which comprises TFam, TFB2M and RNA polymerase mitochondrial (POLRMT), and occurs in the D-loop region. Mitochondrial transcription termination factor (MTERF) proteins also contribute to mtDNA transcription as they bind to promoters. TFAM, TFB2M, MTERF, and POLRMT, are regulated by the interaction of PGC1α and PGC1β with NRFs, ERRs, and YY1 (Dominy and Puigserver 2013). The transcription of mtDNA is dependent on the translocation of TFam in the mitochondrial matrix, which is facilitated by TFam acetylation (Dinardo et al. 2003, Safdar et al. 2011). On the opposite PKA–mediated phosphorylation of Tfam in the mitochondria impedes TFam binding to mtDNA and reduces transcriptional activation (Lu et al. 2013). Besides nucleus, PGC1α can localize within mitochondria to interact directly with TFam and induce the transcription of mtDNA (Safdar et al. 2011). Similarily, p53 would be able to enter mitochondria to form complexes with TFam,

since knock-down of p53 diminishes the abundance of mtDNA transcripts during exercise (Saleem and Hood 2013).

2.3.3. Mitogenesis-associated kinases and deacetylases

Mitogenesis transcription program is controlled by upstream phosphorylation and deacetylation events. Among kinases involved in mitogenesis, AMPK is known to stimulate mitochondrial content and function. For instance, AMPK enhances amounts and activity of mitochondrial enzymes like cytochrome *c*, succinate dehydrogenase (SDH) and citrate synthase (CS) (Winder et al. 2000). Several downstream targets of AMPK would be brought into play: indeed, AMPK directly phosphorylates p53 at Ser15 and PGC1α at Thr177 and Ser538 (Jager et al. 2007, Saleem et al. 2009). AMPK is probably responsible for an indirect upregulation of PGC1α expression as well, forasmuch as AMPK-mediated phosphorylation of GATA4 favors binding of the latter to *PGC1α* gene promoter, entailing a higher PGC1α expression (Irrcher et al. 2008). Recent phosphoproteomic analyses discovered a novel potential substrate for AMPK in the frame of mitogenesis. Indeed, AMPK-mediated phosphorylation of the A kinase anchor protein 1 (AKAP1) at Ser103 coincides with improved mitochondrial respiration (Hoffman et al. 2015).

p38 MAPK is another kinase brought into play in mitogenesis, since it directly phosphorylates PGC1α at Ser262, 265, 298 (Puigserver et al. 2001). p38 MAPK also activates PGC1α transcription through phosphorylation of the transcription factors myocyte enhancer factor-2 (MEF2) and activating transcription factor-2 (ATF2) (Yan et al. 2007). Activation of p38 MAPK axis

is dependent of upstream activation of CaMKII in response to rise in cytosolic Ca^{2+} levels (Wright et al. 2007). In addition, p38 MAPK controls p53 content by direct phosphorylation at Ser15 (Saleem et al. 2009).

Conversely, activation of mitogenesis transcription program is counteracted by protein kinase A (PKA) and extracellular signal-regulated kinases 1 & 2 (ERK1/2) when they phosphorylate TFam at Ser55 and Ser177, respectively (Lu et al. 2013, Wang et al. 2014). In parallel to the aforementioned kinases, mitogenesis is proposed to be under the control of the deacetylase sirtuin 1 (SIRT1). SIRT1-dependent deacetylation of p53 induces p53 migration to the nucleus (Philp et al. 2011) and p53 was reported to play a role in mitochondrial biogenesis and metabolism (2.4.1 section), despite no causal link has been established between these two phenomena yet. On the other hand, even though SIRT1 is reported to interact with and deacetylate PGC1α, numerous discrepancies reported in the literature gave no convincing evidences of implication of SIRT1 in mitogenesis in skeletal muscle. Actually, nuclear activity of SIRT1 would be preponderant over its protein content to stimulate PGC1α-related mitogenesis, in particular in the frame of exercise training (Tang 2016).

Although molecular mechanisms of mitogenesis have been extensively studied in cellular and animals models, the paradigm of a synchronized regulation of PGC1α expression and the one of mitogenesis-related genes (nuclear and mitochondrial-encoded) is still questioned in human skeletal muscle (Islam et al. 2017).

2.4. Molecular pathways regulating mitophagy

The selectivity of mitophagy relies on mitochondria dynamics, as described above, and on specific regulatory proteins.

2.4.1. PINK1-PARKIN-induced mitophagy

Under basal physiological conditions, PINK1 is imported to the IMM and degraded inside the mitochondria. When mitochondrial membrane potential collapses, PINK1 accumulates on the OMM, and autophosphorylates on Thr257, shifting the E3-ubiquitin ligase Parkin from the cytosol to the OMM and recruiting ubiquitins. Activation of PARKIN, which contains an ubiquitin-like domain, is stimulated by $PINK1^{Thr257}$-phosphorylation of ubiquitins at Ser65 residue (Kondapalli et al. 2012, Shiba-Fukushima et al. 2012, Koyano et al. 2014). PARKIN promotes ubiquitination and proteasome-dependent degradation of a vast repertoire of OMM proteins involved in mitochondrial shaping, motility, protein import and folding (Chan et al. 2011). This process facilitates the recognition of mitochondria by the autophagic machinery. *In vitro* experiments reported that the p62/SQSTM1 adaptor is needed for PARKIN-induced aggregation of depolarized mitochondria, but is dispensable for mitochondria removal (Narendra et al. 2010, Lazarou et al. 2015). The phosphorylation of p62/SQSTM1 at Ser403 by casein kinase 2 (CK2) and/or TANK-binding kinase 1 (TKB1) facilitates its binding to the ubiquitin chains on damaged mitochondria (Matsumoto et al. 2011, Pilli et al. 2012). In parallel, ULK1-mediated phosphorylation of p62/SQSTM1 at Ser407 is necessary for autophagosome formation (Lim et al. 2015). Beyond its

function of mitophagy receptor, p62/SQSTM1 acts as a signaling hub for amino acids sensing and for oxidative stress defense mechanisms (Katsuragi et al. 2015).

PINK1/PARKIN pathway is proposed to have both cytoprotective (mitophagy) and cytotoxic (apoptosis) roles, determining mitochondria outcome according to the degree of mitochondria insult. On the one hand, limited or transient mitochondrial depolarization would result in PARKIN-mediated mitophagy, promoting mitochondrial pool health and cell survival. On the other hand, high or sustained mitochondrial depolarization would engage PARKIN-dependent apoptosis to eliminate cells harboring an important number of defective mitochondria (Carroll et al. 2014). Despite the threshold of mitochondrial damage required to selectively trigger mitophagy or apoptosis is blurred, the cytosolic presence of p53 would have an inhibitory effect on PARKIN-induced mitophagy (Hoshino et al. 2013).

2.4.2. BNIP3-BNIP3L/Nix-induced mitophagy

BNIP3 and BNIP3L/Nix are receptors which belong to the OMM. BNIP3 participates in the processing of LC3 to its active form LC3b II, and therefore fosters autophagosome formation (Mammucari et al. 2007). BNIP3 preferentially tethers to LC3 (Rikka et al. 2011). Conversely, in yeast, BNIP3L/Nix was reported to have a higher affinity for the GABA type A receptor-associated protein (Gabarap), a member of LC3 proteins, (Novak et al. 2010). Roles of BNIP3 and BNIP3L/Nix in mitophagy may be somewhat different since LC3 intervenes during autophagosome

elongation, whereas Gabarap insures autophagosome closure and maturation (Weidberg et al. 2010). However, that observation was not confirmed in mammals yet.

BNIP3 represses the inhibitory interaction of Bcl2 on Beclin1 on autophagy, allowing Beclin 1 to activate PI3K complex and subsequent autophagic degradation. Alternatively, the association of BNIP3 and BNIP3L with Bcl2 can induce cell death (Zhang and Ney 2009). Phosphorylation state of BNIP3 is also proposed to determine the induction of pro-survival mitophagy or mitochondrial apoptosis *in vitro*: indeed, phosphorylation of BNIP3 at Ser17 and 24 (unknown kinases) enhances its binding to LC3b in a Bcl-X_L-dependent manner, and fosters mitophagy. This entails a lower cytochrome c release capacity of mitochondria, which counteracts apoptosis (Zhu et al. 2013). In addition, the interaction of BNIP3 with OPA1 is suggested to influence mitochondrial dynamics and apoptosis in cellular models: association of BNIP3 with OPA1 inhibits mitochondrial fusion and causes fission, activating apoptosis (Landes et al. 2010).

Lastly, it is currently unclear whether PINK1-PARKIN and BNIP3 are distinct or complementary mitophagy pathways. On the one hand, BNIP3-mediated mitophagy is known to be induced by hypoxia, including in rodent skeletal muscle (Band et al. 2009). However, to our knowledge, the participation of PARKIN in mitophagy in hypoxic environment is not clarified in skeletal muscle. On the other hand, one reported that PARKIN is recruited during BNIP3-dependent mitophagy in cardiac myocytes and both gene transcription and protein expressions of PARKIN and BNIP3 were

showed to be downregulated by the common mediator Rev-erba/NR1D1 in murine skeletal muscle (Lee et al. 2011, Woldt et al. 2013).

2.4.3. FUNDC1-induced mitophagy

Apart from BNIP3, emerging attention is paid to Fun14 domain-containing protein 1 (FUNDC1) mitochondrial receptor in the frame of mitophagy. Interaction of FUNDC1 with LC3 recruits the latter to mitochondria and lead to mitophagy (Liu et al. 2012) . Conversely, mitophagy is inhibited following phosphorylation of FUNDC1 at Tyr18 and Ser13 by proto-oncogene tyrosine-protein kinase Src (SRC) and casein kinase 2 (CK2), respectively (Chen et al. 2014). The potential role of FUNDC1 in mitophagy has been recently reinforced since dephosphorylated FUNDC1 was found to associate to Drp1, a fission protein required to initiate mitophagy (Chen et al. 2016).

2.5. Role of mitochondrial dynamics and mitophagy in skeletal muscle homeostasis and myopathies

Mitochondrial dynamics appears to be essential for the maintenance of proper skeletal muscle metabolism. Loss of fusion-related gene *Mfn2* has numerous deleterious consequences in skeletal muscle of mice, including muscle atrophy, severe mtDNA depletion, fragmented mitochondria, lower expression of respiratory chain subunits, increased ROS concentrations and disturbed insulin signaling. This model is more susceptible to develop insulin resistance following a high-fat diet, in comparison to wild-type animals (Chen et al. 2010, Sebastian et al. 2012). Furthermore,

mitochondrial dysfunctions and morphological alterations typical to *Mfn2*-KO mice are exacerbated during aging (Sebastian et al. 2016). In humans, *Mfn2* mRNA expression is proportional to insulin sensitivity and inversely correlated to body mass index. In particular, *Mfn2* mRNA is reduced in skeletal muscle of lean and obese type 2 diabetes patients (Bach et al. 2005). Dominant-negative mutation of fission-related gene in C2C12 muscle cells and primary myoblasts dramatically hinders myogenic differentiation (Kim et al. 2013). On the opposite, muscle-specific overexpression of *Drp1* in mice induces mitochondrial structural alterations, reduced mtDNA abundance and disturbs muscle protein synthesis. This is accompanied with loss of muscle mass and lower exercise performance (Touvier et al. 2015).

Loss-of-function of *Mfn2* and *OPA1* is involved in Charcot-Marie Tooth type 2A and dominant optic atrophy, respectively. These myopathies are both featured by small mitochondria, disorganized mitochondrial cristae and accumulation of lipid droplets in skeletal muscle (Grumati and Bonaldo 2012). Moreover, oxidative phosphorylation is weakened in dominant optic atrophy, resulting in a decline of ATP production (Amati-Bonneau et al. 2009).

Impairment of the mitophagy machinery is also well established to be implicated in congenital muscular dystrophies. They are linked to mutations of genes coding for extracellular matrix proteins and their receptors, which alters cellular basement membranes, and therefore,

negatively impedes cell survival and differentiation. For example, Bethleem myopathy and Ulrich congenital muscular dystrophies are characterized by genetic mutations of collagen IV (Collins and Bonnemann 2010). In muscle of mice deficient in collagen IV, failure of autophagy coincides with abnormal mitochondria morphology and reduced BNIP3 amounts. As BNIP3 helps to the recruitment of autophagosomes to mitochondria by tethering LC3, this suggests that selective elimination of defective mitochondria is inefficient in the absence of BNIP3. Increased number of deficient mitochondria is observed when these mice are confronted to mechanical and metabolic stresses, such as eccentric exercise (Grumati et al. 2011).

The role of mitophagy effectors, as PINK1 and PARKIN, has been extensively studied in various cell types in the frame of neurodegenerative disorders, and genetic mutations have been demonstrated to be determinant in the development of Parkinson's and Alzheimer's diseases. However, much less is known about their implication in skeletal muscle homeostasis. Direct evidences of the likely contribution of mitophagy to the maintenance of functional and healthy mitochondria in skeletal muscle were very recently observed in PARKIN-KO mice: these animals showed decreased specific force, reduced mitochondrial respiration and augmented ROS production. Moreover, they had lower fusion but higher fission protein content (Chen et al. 2018, Gouspillou et al. 2018). Despite a similar basal mitophagy compared to wild-type mice, PARKIN-KO mice exhibited a lower activation of mitophagy in response to acute exercise,

which basically requires an efficient mitochondrial respiration and is likely to augment more ROS generation. (Chen et al. 2018). This suggests that impaired mitophagy alters basal muscle contractile function, compromises mitochondria integrity and disturbs muscle adaptations to exercise.

3. Physiological triggers of autophagy/mitophagy in skeletal muscle

3.1. Regulation of autophagy/mitophagy by nutrient depletion

To date, few studies examined the effect of nutrient availability on autophagy and mitophagy in skeletal muscle, particularly in human. Nonetheless, BNIP3 seems to be mandatory for autophagy induction when nutrients are lacking, since knock-down of BNIP3 almost abolishes autophagosome formation in response to fasting (Mammucari et al. 2007). Our laboratory recently confirmed in healthy mice that fasting not only results in increased BNIP3 protein levels, but also enhances PARKIN expression (Jamart et al. 2013).

A 24h-fasting causes accrued expression of many autophagy and mitophagy-related genes in mice, among which *LC3*, *GabarapL1*, *Bnip3*, *Bnip3L*, *Vps34* (PI3K class III), *Atg4b* and *Cathepsin L.* In this model, autophagy transcription is signaled *via* Akt/mTORC2/FoxOs axis, which implies dephosphorylation of Akt^{Ser473} and $FoxO3a^{Thr32}$. Furthermore, this mechanism does not rely on mTORC1 but partly requires the presence of

mTORC2. Indeed, knockdown of the mTORC1-downstream target S6 kinase (S6K) blocked autophagy, while knockdown of rictor, member of mTORC2 complex, did not prevent FoxO-dependent autophagy. In particular, the expression of *LC3*, *Bnip3* and *Bnip3L* is governed by FoxO3a activation (Mammucari et al. 2007). In addition to FoxO3a, FoxO1 was demonstrated to elevate the expression of *cathepsin L* in fasted murine skeletal muscle (Yamazaki et al. 2010)

The transcription of fission-related genes is sensitive to nutrient paucity since *Drp1* mRNA is upregulated in response to fasting in mice (Jamart et al. 2013). Although nutrient depletion raises TFEB content in many cell lines, no studies investigated the regulation of TFEB in fasted skeletal muscle yet. However, it is conceivable that TFEB is sensitive to conditions of high energy demand in this tissue, since exhaustive exercise upregulates TFEB in skeletal muscle of mice (Mansueto et al. 2017).

Taken together, those evidences go along with an upregulation of autophagy and mitophagy in response to nutrient deficit in skeletal muscle. However, further investigations are needed to determine the molecular mechanisms beyond autophagy and mitophagy in fasted skeletal muscle. It also remains unclear whether fasting-induced autophagy is non-selective or whether this process may be specifically directed towards mitochondria.

3.2. Regulation of autophagy/mitophagy by endurance exercise

Exercise is identified as a strong inducer of autophagy in skeletal muscle. In mice exhibiting a normal basal autophagy, mutations in phosphorylation sites of autophagy proteins, as well as allelic or partial loss of autophagy-related genes, prevent autophagy to increase in response to exercise (He et al. 2012). As a consequence, increase in exercise-induced insulin sensitivity is blunted, glucose uptake is impaired and exercise endurance is lower.

Autophagy genes and proteins are upregulated by AMPK and FoxO3 pathways during ultra-endurance running in human, a mode of exercise that requires high energy demand and is known to entail mechanical and metabolic alterations of cellular constituents (Jamart et al. 2012, Jamart et al. 2012). Interestingly, autophagy is also increased in mice in response to shorter sessions of endurance exercise coupled to fasting, this mechanism relying on mTOR pathway downregulation (Jamart et al. 2013). However, at the time where the present thesis work began, autophagy regulation during more conventional forms of exercise was still not investigated in human. Moreover, the influence of the nutritional state during exercise was not directly evidenced in human.

Acute endurance exercise enhances both mRNA and protein markers for fission in whole muscle extracts of mice. The effect of exercise was persistent within the hours following the trial (Ding et al. 2010, Jamart et al. 2013). In human, a previous study of our lab observed higher fission protein $Drp1^{Ser616}$ after one session of ultra-endurance running (Jamart et

al. 2012). However, no data for fission or mitophagy were available within the scope of shorter acute exercise protocols.

High intensity interval training (HIIT) exerts a gradual elevation of Fis1 protein in human while Drp1 total content increases the very first days and then plateaus during the rest of the trial (Perry et al. 2010). Yet, evidences of mitophagy activation in such conditions are lacking since phosphorylation of Drp1 was not measured and because fission proteins were measured in total muscle extracts. Moreover, it remains to be determined whether downstream mitophagy proteins are influenced by HIIT in human.

Both acute and long-term exercise favor the nuclear localization of TFEB in skeletal muscle of mice, and enhance the expression of genes and proteins associated with mitogenesis and mitochondrial function. Importantly, this mechanism was independent of PGC1α (Mansueto et al. 2017).

3.3. Regulation of autophagy/mitophagy during aging

Aged skeletal muscle is associated with a gradual loss of mass and function. The latter manifests as loss of strength, reduced mobility and metabolism disorders, which directly impinge daily life activities (Nair 2005). Impaired muscle function is even a strong predictor of mortality (Newman et al. 2006).

Detrimental mtDNA mutations and exacerbated oxidative stress within mitochondria have long been considered as the more plausible theory of cellular aging, even though the correlation between these two processes reached no consensus yet (Pinto and Moraes 2015).

Skeletal muscles of old rodents and human display accumulation of damaged mitochondrial proteins, which could partly originate from defective mitochondria quality control system (Choksi et al. 2008, Wohlgemuth et al. 2010, Beltran Valls et al. 2015). More precisely, accrued ROS formation, mtDNA alterations and lowered oxidative capacity are reported in human mitochondrial fraction, suggesting that both mitochondria quality control and function are jeopardized during aging (Conley et al. 2000, Tonkonogi et al. 2003, Bua et al. 2006, Ghosh et al. 2011). For now, the poor specificity of the markers measured does not allow to assert whether and how autophagy and mitophagy are affected during aging in skeletal muscle, especially in human. In addition, failure of autophagy in aged murine cells is suspected to precipitate satellite cells into senescence, through increased oxidative stress and mitochondrial dysfunction, therefore preventing muscle ability to regeneration (Garcia-Prat et al. 2016). Senescence of muscle satellite cell is thought to foster cell apoptosis-mediated cell death over autophagy-mediated cell survival (White et al. 2018). Nonetheless, these observations remain to be confirmed in human skeletal muscle.

4. Aims of the thesis

Autophagy is a major actor in the quality control system of the cell. Its fine regulation is mandatory to insure cell homeostasis; in the case of skeletal muscle, an optimal level of autophagy contributes to maintain muscle mass and a healthy metabolism. Therefore, exploring strategies the goal of which is a physiological up-regulation of autophagy is important for health sciences. While pharmacological components have long been used to trigger autophagy in the frame of pathological states, less attention has been paid to physiological stimuli, such as exercise or nutrition, especially in human.

Autophagy is known to be induced by extreme forms of endurance exercise in human skeletal muscle, probably due to high mechanic and metabolic disturbances of muscle homeostasis. Though, whether more conventional forms of exercise, which do not necessarily imply muscle damage, could promote autophagy was uncharted when we started the present thesis work. Therefore, chapter 2 aims to evaluate whether endurance exercise activates short-term autophagy in human skeletal muscle, in the frame of a low and high intensity cycling protocol. Because exercise-induced autophagy was previously described to be driven by insulin-Akt pathway in mouse, we also explore the regulatory pathways involved in autophagy in human skeletal muscle during acute exercise.

In the line of chapter 2, the purpose of chapter 3 is to determine whether autophagy is selectively directed towards mitochondria in human

during the same protocol of acute endurance exercise. As fission markers were previously found to be enhanced with endurance exercise combined with fasting in mouse skeletal muscle, we investigate whether mitophagy is transitorily activated through fission during exercise in human, depending on the nutritional state.

Physical activity decreases progressively with ageing. Concomitantly, mitochondrial structure and activity are altered, even if a correlation between these two processes is not clearly established. Mitochondria provide the main source of energy for skeletal muscle. Therefore, developing strategies to preserve mitochondrial integrity is probably decisive for lifelong muscle health. The first goal of chapter 4 is to elucidate whether mitochondrial function and mitophagy are altered with physical inactivity during aging in human skeletal muscle. Exercise training maintains muscle mass, mitogenesis and mitochondrial activity. However, whether and how mitophagy could be influenced at long-term by an active lifestyle remains uncharted in human skeletal muscle. We secondly investigated whether regular endurance exercise could counteract a potential decline in mitochondrial function and mitophagy during aging.

Chapter 2: Activation of autophagy in human skeletal muscle is dependent on exercise intensity and AMPK activation

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Abstract

In humans, nutrient deprivation and extreme endurance exercise both activate autophagy. We hypothesized that cumulating fasting and cycling exercise would potentiate activation of autophagy in skeletal muscle. Well-trained athletes were divided into control (n = 8), low intensity (LI, n = 8) or high intensity (HI, n = 7) exercise groups and submitted to fed and fasted sessions. Muscle biopsy samples were obtained from the *vastus lateralis* before, at the end and 1 h after a 2 h LI or HI bout of exercise. Phosphorylation of ULK1^{Ser317} was higher after exercise ($P < 0.001$). In both the fed and the fasted state, LC3bII protein level and LC3bII/I were decreased after LI and HI ($P < 0.05$) while p62/SQSTM1 was decreased only 1h after HI ($P < 0.05$), indicating an increased autophagic flux after HI. The autophagic transcriptional program was also activated as evidenced by the increased level of LC3b, p62/SQSTM1, GabarapL1 and cathepsin L mRNAs observed after HI, but not after LI. The increased autophagic flux after HI could be due to increased AMP-activated protein kinase a (AMPKα) activity, as both AMPKα^{Thr172} and ACC^{Ser79} had a higher phosphorylation state after HI ($P < 0.001$). In summary, the most effective strategy to activate autophagy in human skeletal muscle seems to rely on exercise intensity more than dietary supply.

Key Words: *cathepsin — cycling — fasting — LC3b — p62/SQSTM1*

Introduction

Regular physical exercise is widely recognized as a primary prevention tool to fight against many chronic diseases (Booth et al. 2000). Acute physical exercise induces a powerful but brief stress that challenges the whole body homeostasis. An appropriate recovery period leads to adaptations aiming at reducing homeostasis disturbances when the same stimulation is applied again, as it is the case during a training program. Over the past decades, intensive work has been dedicated to unravel the molecular mechanisms behind exercise training-induced health benefits, specifically in skeletal muscle as a result of its major role during exercise (Rockl et al. 2008, Garber et al. 2011).

Among the cellular processes contributing to the maintenance of homeostasis, macro-autophagy – hereafter called autophagy – has emerged as a crucial mechanism for maintaining muscle structure and preserving muscle mass (Masiero et al. 2009). Autophagy is a bulk degradation process through which intracellular components such as soluble proteins, protein aggregates and organelles are wrapped inside double membrane vesicles called autophagosomes, which fuse with endosomes and lysosomes, the latter containing hydrolases necessary for autophagosome degradation. The products of this proteolytic process are then transferred back to the cytoplasm, where they can be reused.

The autophagic flux is featured by autophagic initiation and resolution events, namely LC3b (microtubule associated protein-1 light chain 3 b) I

lipidation associated to p62/SQSTM1 (p62/sequestosome 1) degradation (Barth et al. 2010). LC3b II, the lipidated form of LC3b I, directly reflects the presence of autophagosomes (Barth et al. 2010): LC3b II is recruited both at the inner and outer membrane during vesicle elongation, where it remains bound until autophagosomes fuse with lysosomes (Ravikumar et al. 2010). Thus, the LC3b II/LC3b I ratio, is recognized as a reliable marker of autophagosomes synthesis (Klionsky 2007). p62/SQSTM1 binds both to aggregated proteins and to LC3b II, and is degraded with the autophagosome content following fusion with lysosome (Pankiv et al. 2007). Because LC3b II is involved both in early and later stages of autophagy, from autophagosomes formation to lysosomal degradation, the assessment of additional autophagic markers acting upstream or downstream LC3b II is helpful to better interpret changes in autophagic flux (Barth et al. 2010).

Autophagy is constitutively active in skeletal muscle cell and plays a predominant role in cellular remodelling by controlling quality and clearance of intracellular altered proteins and organelles (Mizushima and Komatsu 2011, Weidberg et al. 2011). Besides its role in basal homeostasis, autophagy is also part of a cytoprotective process in the myofiber response to intracellular and extracellular stresses by supplying alternative sources of energy and preventing accumulation of damaged and/or aberrantly folded proteins and organelles (Ravikumar et al. 2010). Recently, strenuous endurance running has been evidenced as a stress leading to autophagy activation and higher autophagic flux in mice skeletal muscle (Grumati and

Bonaldo 2012, He et al. 2012), which is possibly dependent on the eccentric component of the contractions (Grumati et al. 2011). Contrary to studies reporting a chronic increase of autophagy in various muscle atrophy models (Schiaffino and Hanzlikova 1972, Wang et al. 2005, Sandri 2008, O'Leary et al. 2012), studies dealing with exercise rather consider the potential beneficial effects of a transitory increase of autophagy for muscle metabolism. Indeed, those studies suggest that activation of autophagy in skeletal muscle by running probably aims at eliminating the damaged and/or aberrantly folded proteins generated to prevent accumulation of toxic waste that would challenge cellular homeostasis (Grumati et al. 2011, Jamart et al. 2012, Jamart et al. 2012) and that autophagy is required for improving insulin sensitivity, mitochondrial biogenesis and angiogenesis (He et al. 2012, Lira et al. 2013).

Most of the work on endurance exercise-induced autophagy has been performed in mice. Animal studies are generally carried out on small, immature mammals with a high basal metabolic rate and a weak capacity to maintain homeostasis. These studies are difficult to transfer to human adults, whose body weight is supposed to remain stable over years, with consequently slower protein turnover. In human, two studies from our laboratory showed that an ultra-marathon activates autophagy both by transcriptional and post-translational regulatory mechanisms in human skeletal muscle (Jamart et al. 2012, Jamart et al. 2012). The aforementioned studies evidenced autophagy activation in response to strenuous running, the nature of which involves eccentric contractions and

consequently muscle damage. We are unaware of any experiment showing the activation of autophagy in human muscle in response to endurance exercises, like cycling, which implicate only concentric contractions. Indeed, beyond muscle damage, autophagy can be activated while the energetic status of the cell is impaired or in response to changes in hormonal environment (Kahn et al. 2005, Schiaffino and Mammucari 2011). This suggests that eccentric contractions and subsequent muscle damage might not be required to activate autophagy during exercise.

In the present study, we thoroughly evaluated how endurance exercise regulates short-term autophagy in human skeletal muscle, by the use of a low and a high intensity cycling protocol. Because we previously showed in mice that cumulating both fasting and exercise running led to a higher activation of autophagy (Jamart et al. 2013), we tested the hypothesis that autophagy activation could be potentiated by the association of fasting with exercise in human. Finally, we aimed to elucidate the signaling mechanisms implicated in the regulation of autophagy after such a concentric exercise protocol in human skeletal muscle.

Materials and methods

Subject characteristics and preliminary testing

Twenty-three healthy young men whose characteristics are described in Table 1 were recruited for the study. The protocol was approved by the local Ethical Committee of the Université catholique de Louvain and

conducted in accordance with the Declaration of Helsinki. Subjects were all informed about the experimental procedure before their written consent was obtained. They were selected on voluntary basis and were all experienced cyclists or triathletes. The minimal level of maximal oxygen consumption ($VO_{2\ peak}$) for inclusion in the protocol was 50 $ml \cdot min^{-1} \cdot kg^{-1}$. Before the study began, subjects reported to the laboratory for a medical examination that aimed to exclude subjects with any underlying pathology. Afterwards, height, weight and percentage of body fat were measured using the Jackson and Pollock method (Jackson and Pollock 1978). A maximal incremental exercise test was performed on a cycle ergometer (Cyclus III; RBM electronics, Leipzig, Germany) for determining $VO_{2\ peak}$ and maximal power output (W_{max}). The starting load was 70 W, incremented by 40 W every 3 min until exhaustion. Heart rate (Polar Team System 2; Polar Electro, Kempele, Finland) and respiratory exchanges (Metalyzer 2; Cortex, Leipzig, Germany) were continuously monitored.

Participants were randomly divided into 3 groups: a low intensity exercise group (LI, *n* = 8), a high intensity exercise group (HI, *n* = 7) and a non-exercising control group (*n* = 8). One week after the maximal test, subjects took part in a familiarization session to ensure they could handle the workload requested of them during the protocol in the fasted state. A 2-weeks period separated the familiarization session and the first experimental session. Subjects had to refrain from strenuous exercise 3 days before each experimental session.

Table 1. Subjects characteristics.

Group	Age (y)	Height (cm)	Weight (kg)	Body fat (%)	$V_{O_2\ peak}$ (L/min)	$V_{O_2\ peak}$ (ml/min/kg)	W_{max} (W)	W_{max} (W/kg)
Control ($n = 8$)	23 ± 1	182 ± 2	72.7 ± 2.2	10.2 ± 1.6	4.44 ± 0.17	61.2 ± 2.5	328 ± 15	4.5 ± 0.2
LI ($n = 8$)	22 ± 1	182 ± 2	77.2 ± 3.8	10.7 ± 1.2	4.78 ± 0.16	62.5 ± 2.7	347 ± 12	4.5 ± 0.2
HI ($n = 7$)	24 ± 1	179 ± 3	68.5 ± 4.1	8.0 ± 1.3	4.44 ± 0.33	64.5 ± 1.7	323 ± 20	4.7 ± 0.1

Dietary control

The day preceding each experiment, a standardized carbohydrate-rich diet was given to the subjects (63% carbohydrate, 13% protein and 24% fat). During the fed sessions, a standardized breakfast was given immediately after the first biopsy was performed. During the exercise period, fasted subjects received 150 ml of water every 15 min, whereas during the fed session they consumed 150 ml of a 6% carbohydrate energy drink every 15 min (Sports Drink Orange, 3 Action; Tessenderlo, Belgium). Participants were asked not to consume medication or dietary supplement during the 3 days preceding each experiment.

Exercise protocol

Each subject undertook 2 experimental sessions, fasted and fed, interspersed with a 2-week period (Fig. 1). For each session, they arrived at 6:00 AM to the laboratory after an 8-h overnight fast. A flexible catheter was placed into the antecubital vein, from which a first venous blood sample of 5 ml was collected (time 0). Then, a first biopsy sample, with the needle pointing proximally, was taken from the mid portion of the *vastus lateralis* muscle under local anaesthesia (1 ml of Xylocaine 2%; AstraZeneca, Belgium) (baseline). The leg on which the first biopsy was performed was chosen at random. Samples were immediately frozen in liquid nitrogen then stored at -80°C before further analysis. Ninety minutes later, a second venous blood sample was collected (time 90). A second

muscle biopsy, with the needle pointing distally, was performed, with the sample taken through the same incision as the first preexercise sample.

Still under local anaesthesia, a second incision was made 3 cm distally from the first incision site, to be able to perform the third biopsy within 30 s of the end of the exercise. The exercise bout then began (time 105). The LI group cycled for 2 h at 55% of $VO_{2\ peak}$, whereas the HI group cycled for 2 h at 70% of $VO_{2\ peak}$. A venous blood sample was taken immediately before the start of, every 30 min during, and during the last 5 min of the exercise period (times 135, 165, 195, 225). With the needle pointing proximally, a third muscle biopsy was performed, with the sample taken from the second incision site made prior to the beginning of the exercise. One hour after the end of the exercise bout, a last venous blood sample was taken and the last biopsy - with the needle pointing distally in the incision made before exercise- performed. Pointing the needle in the opposite direction was intended to reduce potential activation of inflammatory signalling pathways, as recommended by Van Thienen *et al*. (Van Thienen et al. 2014). All subjects underwent the same protocol 2 weeks after the first session, receiving the crossed-over nutritional treatment (fasted *vs* fed).

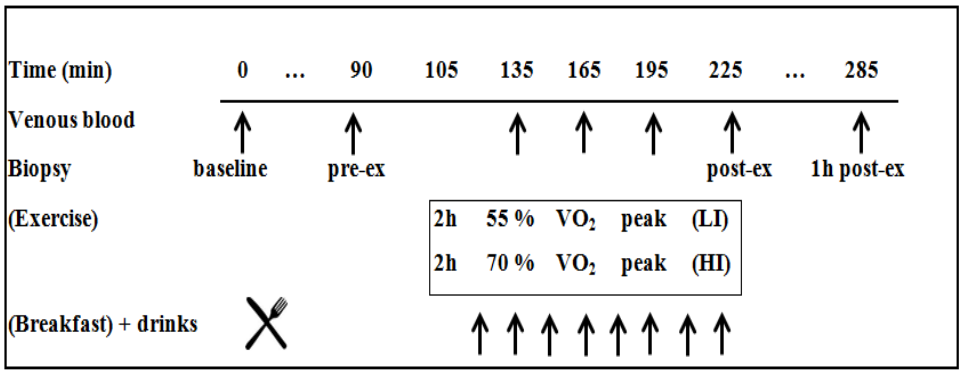


Figure 1. Schematic view of experimental protocol. One muscle biopsy was performed and one venous blood sample was taken at time 0 (baseline). After 90 min, a second biopsy was performed and one venous blood sample was taken. Subjects then either rested for 2 h or cycled for 2 h at 55% (LI) or at 70% of $VO_{2\ peak}$ (HI). Venous blood samples were taken every 30 min during the exercise bout. A third biopsy was performed and one venous blood sample was taken at the end of the exercise period. One hour later, a last biopsy was performed and one venous blood sample was taken. Fed subjects received a breakfast immediately after baseline measurements as well as 150 ml of a 6% carbohydrate drink every 30 min during the exercise bout, while fasted subjects received the same quantity of water.

Protein extraction

A total of ~30 mg of muscular biopsy sample was homogenized with an Ultra-Turrax homogenizer (Ika, Staufen, Germany) for 2 x 10 s at 13,500 rpm in an ice cold buffer containing 20 mM Tris, pH 7.0, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM DTT (1,4-dithiothreitol) and a protease inhibitor

cocktail containing 1 mM EDTA (Roche Applied Science, Vilvoorde, Belgium). Homogenates were centrifuged for 10 min at 10,000 *g*, at 4°C. Supernatants were collected and immediately stored at -80°C before further analysis. Protein contents were determined using the DC kit for protein dosage (Bio-Rad Laboratories, Hercules, CA, USA).

SDS-PAGE and immunoblotting

A total of ~50 µg muscle proteins were mixed with Laemmli sample buffer and warmed for 5 min at 95°C. Proteins were separated by SDS-PAGE during 2 h at 40 mA, then transferred on polyvinylidene fluoride (PVDF) membranes for 2.5 h at 80 V. After blocking for 1 h in a Tris-buffered saline plus 0.1% Tween-20 (TBST) containing 5% nonfat dry milk, membranes were incubated at 4°C overnight with one of the following antibodies: phospho-4E-BP1$^{Thr37/46}$ (4E-BP1, factor 4E binding protein 1) (#2855), 4E-BP1 total (#9644), phospho-ACC^{Ser79} (ACC, acetylCoA carboxylase) (#11818), ACC total (#3676), phospho-Akt^{Ser473} (#9271), phospho-Akt^{Thr308} (#9275), Akt total (#2920), phospho-AMPKα^{Thr172} (AMPKα,AMP-activated protein kinase α) (#2535), AMPKα total (#2603), phospho-FoxO1/3a$^{Thr24/32}$ (FoxO1/3, forkhead box containing protein O subclass 1/3) (#9464), FoxO3a total (#24975), phospho-ULK1^{Ser317} (#12753), phospho-ULK1^{Ser757} (ULK1, unc-51-like kinase 1) (#6888), ULK1 total (#8054) (Cell Signaling Technology, Danvers, MA, USA), LC3b (L7543) or p62/SQSTM1 (P0067) (Sigma-Aldrich, St. Louis, MO, USA). Membranes

were washed three times for 10 min in TBST, then incubated for 1 h at room temperature with a secondary antibody conjugated to horseradish peroxidase. Three additional washes were done before chemiluminescence detection with the ECL-Plus Western blott kit (Amersham Biosciences, GE Healthcare, Waukesha, WI, USA). The bands were captured with GeneSnap and quantified with the GeneTool software (G-Box; Syngene, Cambridge, United Kingdom). When the electrophoretic migration of a protein leaded to two bands, both of them were considered in the quantification process. Phosphorylated proteins were reported to their respective total form; LC3b results were reported as the LC3b II/I ratio; and FoxO3a total, LC3b II/I , p62/SQSTM1 and ULK1 total expression were reported to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam, Cambridge, United Kingdom), which remained unchanged in all experimental conditions. An internal standard was used to limit variations in protein expression when protein samples were separated on different SDS-PAGE gels. If not mentioned, the total forms of the phosphoproteins were unchanged by exercise or diet.

RNA extraction and quantitative Real-Time PCR

A total of ~30 mg of muscle biopsy was homogenized in 1ml Trizol reagent (Invitrogen, Merelbeke, Belgium), using the TissueLyser II from Qiagen (Venlo, Netherlands). RNA isolation was achieved according to the manufacturer's instructions. RNA quality and quantity were assessed by Nanodrop spectrophotometry. Reverse transcription was performed from 1 µg RNA using the iScript cDNA Synthesis Kit from Bio-Rad, following

manufacturer's instructions. The following primers were used : *Cathepsin L* forward sequence 5'-GTGAAGAATCAGGGTCAGTGTG-3'and reverse sequence 5'-GCCCAGAGCAGTCTACCAGAT-3'; *GabarapL1* forward sequence 5'-GTGCCCTCTGACCTTACTGTTG-3' and reverse sequence 5'-CATTTCCCATAGACACTCTCATC-3'; *Gapdh* forward sequence 5'-CATGGTCGTCATGGGTGTGAACCA-3' and reverse sequence 5'-AGTGATGGCATGGACTGTGGTCAT-3'; *LC3b* forward sequence 5'-AATCCCGGTGA- TAATAGAACGA-3'and reverse sequence 5'-GGAGACGCTGACCATGCTGT-3' and *p62/Sqstm1* forward sequence 5'-CCTCTGGGCATTGAAGTTG-3' and reverse sequence 5'-TATCCGACTCCATCTGTTCCTC-3'. Experiments were conducted using the following conditions: 3 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C. All samples were run in triplicate with an internal standard on each plate to correct for interplate variability. Each reaction was processed in a 10 µl volume containing 4.8 µl IQ SybrGreen SuperMix (Bio-Rad), 0.1 µl of each primer (100 nM final) and 5 µl cDNA at the appropriate dilution. Melting curves were systematically performed for quality control. Relative mRNA expression level were normalized to GAPDH, the expression of which was unaffected by diet or exercise.

Blood samples analyses

Samples were collected in heparin tubes and kept on ice before centrifugation for 6 min at 3,000 *g* at 4°C. Supernatants were collected and stored at -20°C before further analysis. Plasma insulin concentration was

measured using ELISA kits (Mercodia, Uppsala, Sweden), following the manufacturer's instructions.

Statistical analysis

All values are expressed as means ± SEM. A mixed ANOVA model was used with the subjects as a random variable and groups (control, LI and HI) and conditions (fasted, fed) as fixed independent variables. When appropriate, contrast analyses were performed to compare means. Statistical significance was set at $P < 0.05$.

Results

Plasma insulin levels

Baseline plasma insulin concentrations were approximately 3-5 mU/l and were not different among the 3 groups and the nutritional conditions (Table 2). In the fasted conditions, insulin levels remained stable throughout the experiments, independent of resting or exercise intensity. Fed subjects displayed an increased plasma insulin concentration after breakfast (90 min) in each group ($P < 0.001$). Compared to control conditions, in which insulin levels remained elevated until 165 min, insulin levels in LI and HI rapidly returned to levels near to basal ($P < 0.05$). No effect of exercise intensity was observed at any time point.

Group	State	Time (min)							
		0	90	135	165	195	225	285
Control	Fasted	4.2 ± 0.4	3.7 ± 0.2	4.5 ± 0.3	6.1 ± 0.4	5.6 ± 0.3	4.4 ± 0.3	3.4 ± 0.2
	Fed	3.8 ± 0.2^{g}	36.8 ± 2.2^{f}	35.9 ± 1.1^{f}	39.1 ± 2.8^{f}	22.2 ± 0.8^{a,g}	24.1 ± 2.0^{f,g}	5.0 ± 0.2^{d,g}
LI	Fasted	4.6 ± 0.3	4.5 ± 0.3	4.1 ± 0.1	3.6 ± 0.1^{a}	2.3 ± 0.1^{b}	1.9 ± 0.2^{a}	2.8 ± 0.2
	Fed	4.3 ± 0.3^{g}	23.8 ± 1.4^{f}	11.4 ± 1.2^{f,g,c}	11.8 ± 1.5^{f,g,c}	6.7 ± 0.5^{e,g,a}	6.0 ± 0.6^{b,g,e}	4.3 ± 0.3^{g}
HI	Fasted	5.2 ± 0.7	4.8 ± 0.3	2.4 ± 0.3^{a}	1.7 ± 0.3^{b}	1.1 ± 0.3^{b}	0.5 ± 0.2^{a}	2.4 ± 0.4
	Fed	4.6 ± 0.6^{g}	34.0 ± 3.9^{f}	6.8 ± 0.8^{d,g,c}	9.0 ± 2.1^{e,g,c}	4.5 ± 1.0^{d,g,b}	3.5 ± 0.7^{g,c}	10.7 ± 3.1^{f,g,b}

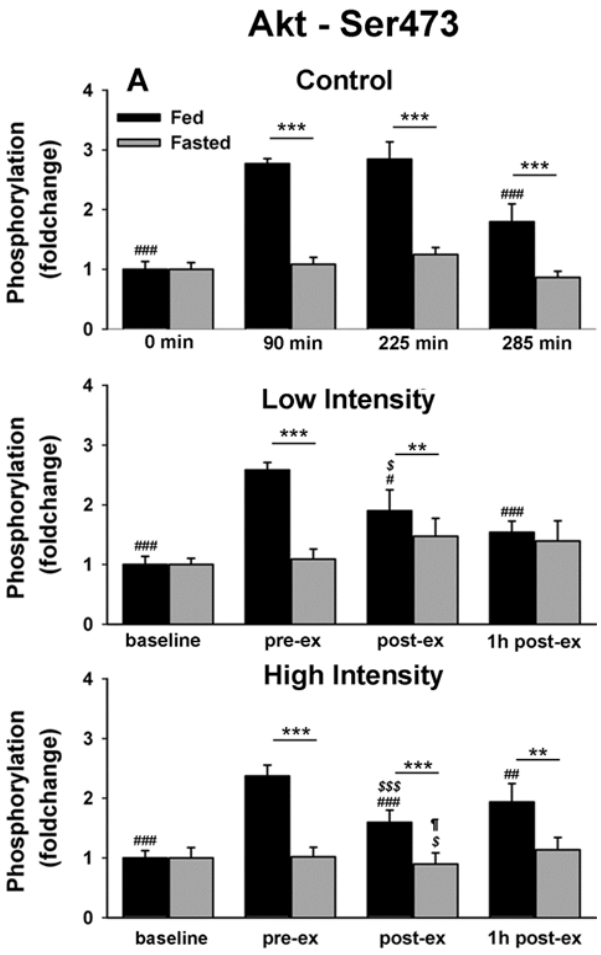
Table 2. Plasma insulin concentrations. Values are presented as mean ± SEM of the control, LI and HI groups, measured during fasted or fed sessions at 0, 90, 135, 165, 195, 225 and 285 min. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* control group (exercise effect); $^{d}P < 0.05$, $^{e}P < 0.01$, $^{f}P < 0.001$ *vs* fed (nutrition effect); $^{g}P < 0.001$ *vs* pre-exercise (time effect).

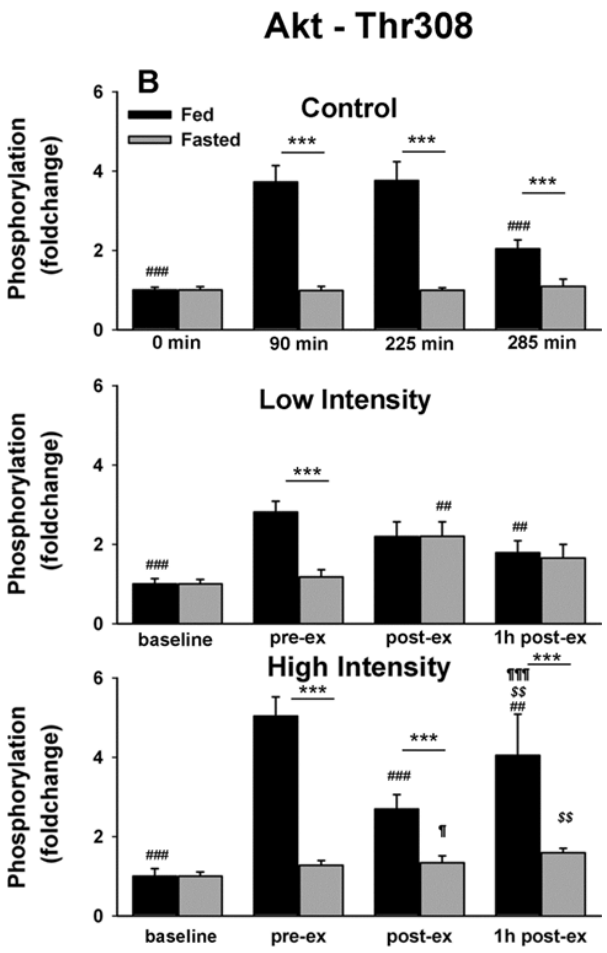
mTORC1-dependent control of autophagy is not regulated by cycling exercise

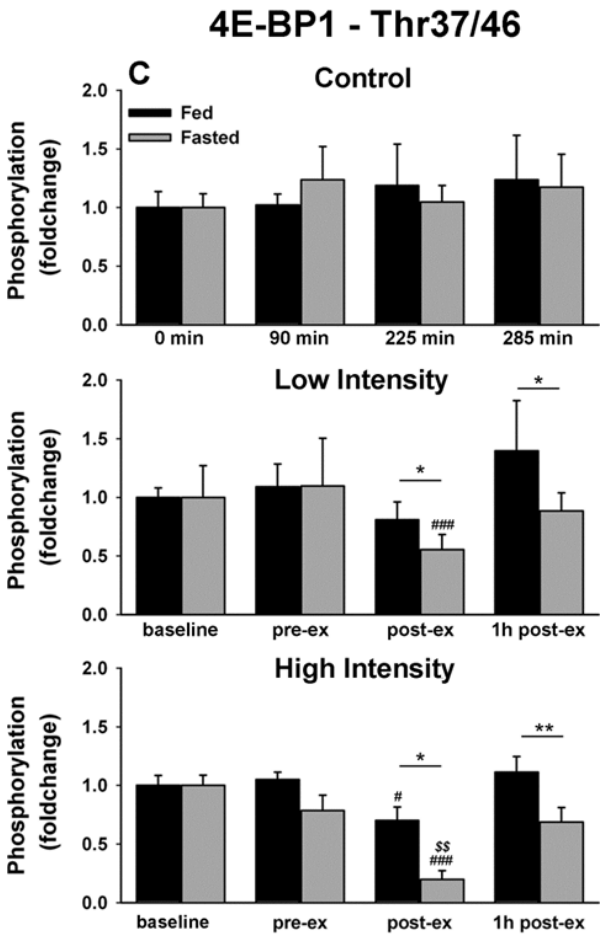
An increase in plasma insulin levels activates Akt, which acts both as an activator of protein synthesis *via* its downstream target mTORC1 (mammalian target of rapamycin complex 1), and as a repressor of protein degradation through the ubiquitin-proteasome and autophagy-lysosomal pathways (Schiaffino and Mammucari 2011). After the peak in insulin concentration after breakfast, the phosphorylation state of Akt^{Ser473} (Fig. 2*A*, Supplemental Fig. S2*A*) and Akt^{Thr308} (Fig. 2*B*, Supplemental Fig. S2*B*) was more than doubled after food intake and remained higher in the fed compared to the fasting group until the end of the trial in control ($P < 0.001$). Compared to control, feeding-induced increase in Akt^{Ser473} phosphorylation was reduced immediately after exercise both in LI ($P < 0.05$) and HI ($P < 0.001$). In the fasted state, exercise did not modify Akt^{Ser473} or Akt^{Thr308} phosphorylation in HI, but an increased Akt^{Thr308} phosphorylation was observed after exercise in LI ($P < 0.01$).

We then evaluated mTORC1 activity by measuring the phosphorylation state of two of its downstream targets, the translation repressor 4E-BP1 at $Thr^{37/46}$ (Ma and Blenis 2009) and the autophagic inductor ULK1 at Ser757 (Kim et al. 2011). Phospho-4E-$BP1^{Thr37/46}$ was decreased when exercise was performed in the fasted state ($P < 0.001$), and even more in HI (-80%) than in LI (-44%) ($P < 0.01$, Fig. 2*C*, Supplemental Fig. S2*C*). In fed subjects, a dephosphorylation of 4E-$BP1^{Thr37/46}$ was observed after exercise as well, but only in HI, thus providing an argument for decreased mTORC1 activity.

Feeding had no effect on 4E-BP1$^{Thr37/46}$ phosphorylation. Similarly to Akt, an increase in ULK1^{Ser757} was measured after feeding ($P < 0.001$), but contrary to Akt, ULK1^{Ser757} returned to baseline within 2 h independently of exercise, as the control group showed the same pattern of phosphorylation as the exercised groups (Fig. 2*D*, Supplemental Fig. S2*D*).




Akt - Thr308
B
Control
Fed
Fasted
Phosphorylation (foldchange)
0 min
90 min
225 min
285 min
Low Intensity
baseline
pre-ex
post-ex
1h post-ex
High Intensity


4E-BP1 - Thr37/46
C
Control
Fed
Fasted
Phosphorylation (foldchange)
2.0
1.5
1.0
0.5
0.0
0 min
90 min
225 min
285 min
Low Intensity
*
*
###
baseline
pre-ex
post-ex
1h post-ex
High Intensity
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baseline
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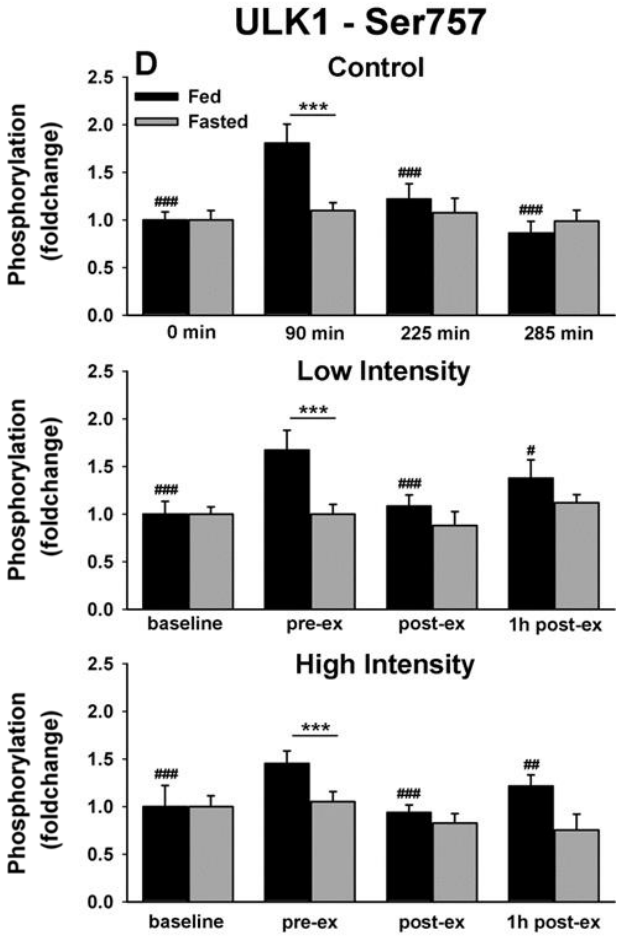


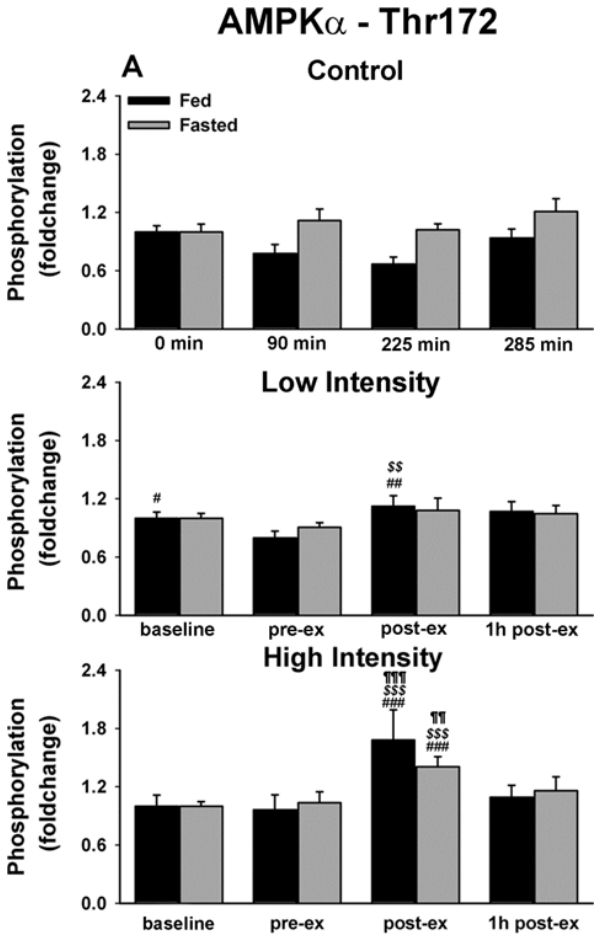
Figure 2. Phosphorylation changes in the insulin-dependent autophagy pathway in response to concentric endurance exercise as a function of intensity, nutritional state and time. Phosphorylation of (A) AktSer473; (B) AktThr308; (C) 4E-BP1Thr37/46 and (D) ULK1Ser757 at baseline, before, immediately after and 1 h after cycling exercise at LI (55% $VO_{2\ peak}$, LI) or HI (70% $VO_{2\ peak}$, HI) or in control conditions. Values are presented as mean ± SEM. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ *vs* control group (exercise effect); $^{¶}P < 0.05$, $^{¶¶¶}P < 0.001$ *vs* LI (intensity effect); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P <$

0.001 *vs* fed (nutrition effect); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* preexercise (time effect).

AMPK-dependent autophagy pathway is regulated by cycling exercise

In addition to its regulation by insulin, autophagy is also controlled by AMPK (Kim et al. 2011), a master regulator of cellular energy homeostasis activated by nutrient deprivation and strenuous exercise, among others (Kahn et al. 2005). The phosphorylation state of AMPKα^{Thr172} was increased immediately after exercise, especially when performed at HI ($P < 0.01$ in LI and $P < 0.001$ in HI), but was independent of nutritional state (Fig. 3*A*, Supplemental Fig. S2*E*). To ascertain the activation of AMPK after exercise, we measured the phosphorylation state of ACC at Ser^{79}, a downstream target site for AMPK (Kahn et al. 2005). Similar to AMPKα^{Thr172}, phosphorylation of ACC^{Ser79} was increased after exercise, but contrary to AMPK, the effect seems to be more persistent. Indeed, ACC^{Ser79} was still doubled 1 h after exercise compared to preexercise levels in HI ($P < 0.001$, Fig. 3*B*, Supplemental Fig. S2*F*). The phosphorylation state of ACC was independent of the nutritional state. ULK1 has multiple AMPK-dependent sites of phosphorylation, among which the Ser^{317}, thereby entailing autophagy initiation (Kim et al. 2011). Exercise increased phosphorylation of ULK1^{Ser317} in both fed and fasted subjects ($P < 0.05$, Fig. 3*C*, Supplemental Fig. S2*G*). Similar to ACC^{Ser79}, exercise-induced ULK1^{Ser317} was higher and more persistent in HI than in LI, as 1 h post-exercise ULK1^{Ser317} was still more phosphorylated than pre-exercise ($P < 0.01$). The combination of HI with fasting potentiated the phosphorylation of

ULK1^{Ser317} because a 2-fold increase *vs* a 50% increase in the fed state was observed immediately after exercise ($P < 0.01$). Interestingly, compared with preexercise levels in HI, ULK1^{Total} expression was higher immediately after HI exercise in both fasted and fed subjects ($P < 0.01$) and lower at 1 h after exercise, but only in fasted subjects ($P < 0.05$, Fig. S1*A*, Supplemental Fig. S2*G*). No variations in AMPK^{Thr172}, ACC^{Ser79} or ULK1^{Ser317} were measured in control rested conditions. These findings collectively indicate that cycling endurance exercise up-regulates the AMPK-dependent autophagy pathway in human skeletal muscle, especially when performed at HI, without any additional effect of fasting.


AMPKα - Thr172
A
Control
Fed
Fasted
Phosphorylation (foldchange)
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1.8
1.2
0.6
0.0
0 min
90 min
225 min
285 min
Low Intensity
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baseline
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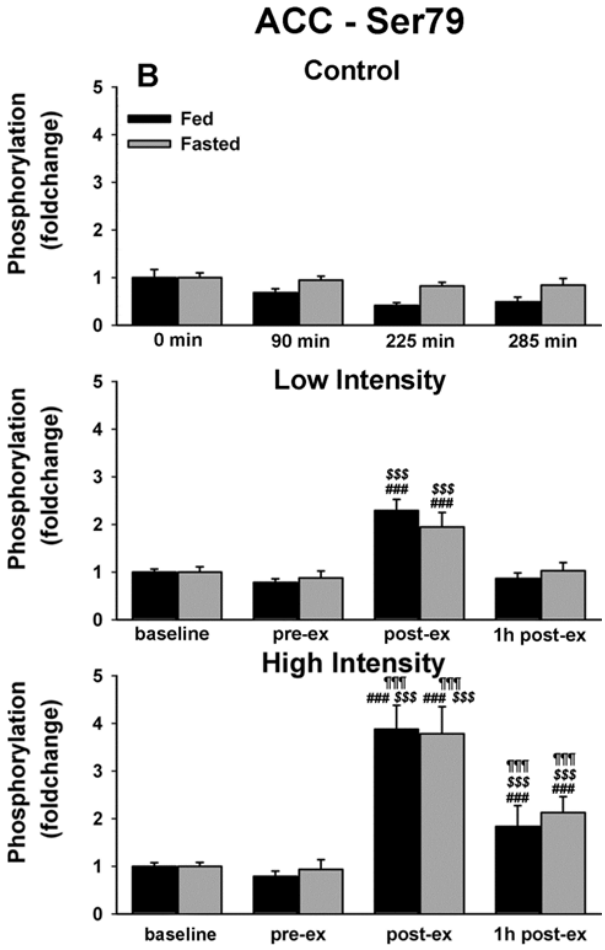
ACC - Ser79
B
Control
Fed
Fasted
Phosphorylation (foldchange)
0 min
90 min
225 min
285 min
Low Intensity
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baseline
pre-ex
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1h post-ex
High Intensity
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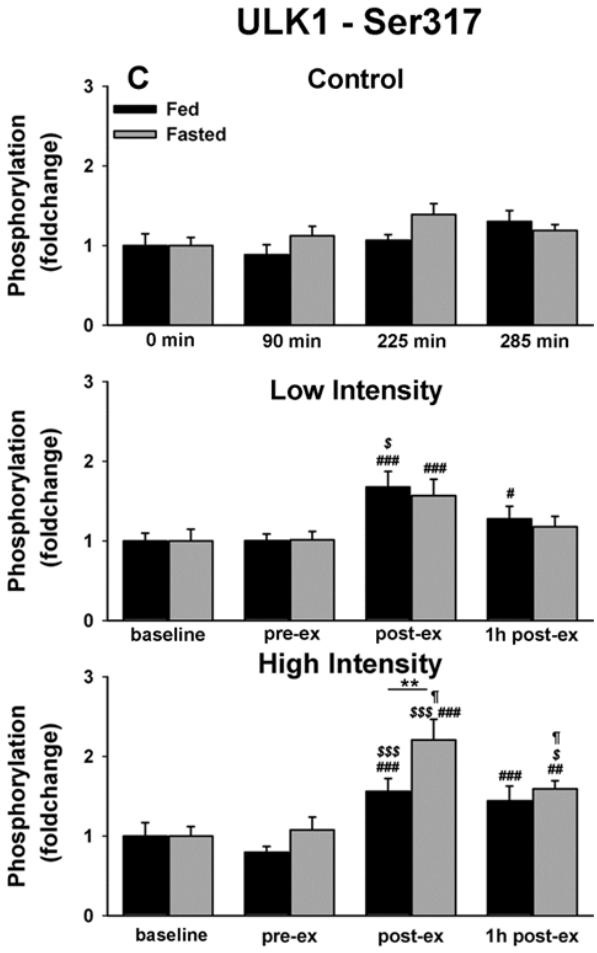


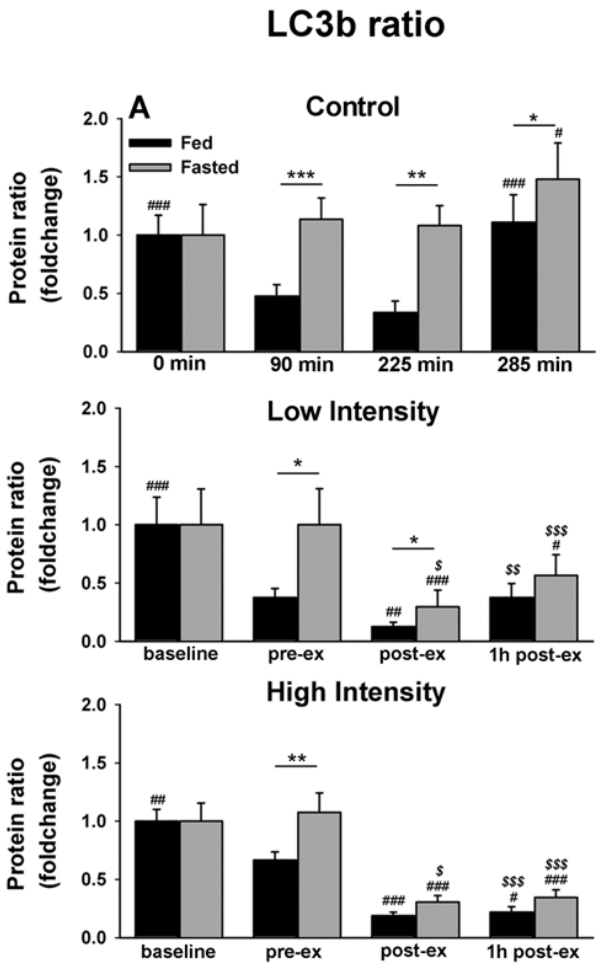
Figure 3. Phosphorylation changes in the AMPK-dependent autophagy pathway in response to concentric endurance exercise as a function of intensity, nutritional state, and time. Phosphorylation of (A) AMPK^{Thr172}, (B) ACC^{Ser79}, and (C) ULK1^{Ser317} at baseline, before, immediately after, and 1 h after cycling exercise at LI (55% $VO_{2\ peak}$) or HI (70%$VO_{2\ peak}$) or in control conditions. Values are presented as mean ± SEM. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ vs. control group (exercise effect); $^{¶}P < 0.05$, $^{¶¶}P < 0.01$, $^{¶¶¶}P < 0.001$ *vs*. LI (intensity effect); $^{**}P < 0.01$ *vs.* fed (nutrition effect); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* preexercise (time effect).

Autophagic flux is activated by cycling exercise in an intensity-dependent way

In light of our previous findings, we investigated whether the autophagic flux was activated, which is featured by an enhanced LC3b II protein level and LC3b II/I ratio associated to a decreased p62/SQSTM1 protein content (Barth et al. 2010). To have better interpretation of changes in autophagic flux, changes in LC3b II, LC3b II/I ratio and p62/SQSTM1 were referred to phosphorylation state of ULK1^{Ser317}, which acts upstream of LC3b II. In fasted control conditions, the LC3b II/I ratio, recognized as a reliable marker of autophagosomes synthesis (Klionsky 2007), was gradually increased throughout the trial ($P < 0.05$) while in fed conditions, this ratio decreased after breakfast ($P < 0.001$) and returned to baseline level 3 h later ($P < 0.001$, Fig. 4*A*, Supplemental Fig. S2*H*).

Compared with control, the LC3b II/I ratio in LI and HI was lower immediately after exercise in fasted conditions (approximately -70%, $P < 0.05$) and 1 h after exercise in both fasted and fed conditions (approximately -70% and -75%, respectively, $P < 0.01$). This exercise-induced decrease in LC3b II/I ratio was independent of the intensity and was due to a decreased LC3b II expression rather than a change in LC3b I (data not shown). p62/SQSTM1 binds both to aggregated proteins and to LC3b, and is degraded with the autophagolysosome content (Pankiv et al. 2007). In control subjects, p62/SQSTM1 expression was about 10% less in fasted than in fed conditions at time 225 min, corresponding to the end of the nutrition protocol for the fed subjects ($P < 0.05$) and approximately

60% less in fasted *vs* fed 1 h after ($P < 0.01$, Fig. 4*B*, Supplemental Fig. S2*I*). In LI, p62/SQSTM1 was not modified by any condition while in HI, its expression 1 h after the end of the exercise was lower than before exercise, as well as lower than in LI conditions at the same time, both in fed and fasted conditions ($P < 0.05$). Taking into account AMPK-dependent autophagy activation, these findings support the notion that Hi is the most potent way to increase autophagic flux during cycling exercise in human skeletal muscle, independent of the nutritional condition.



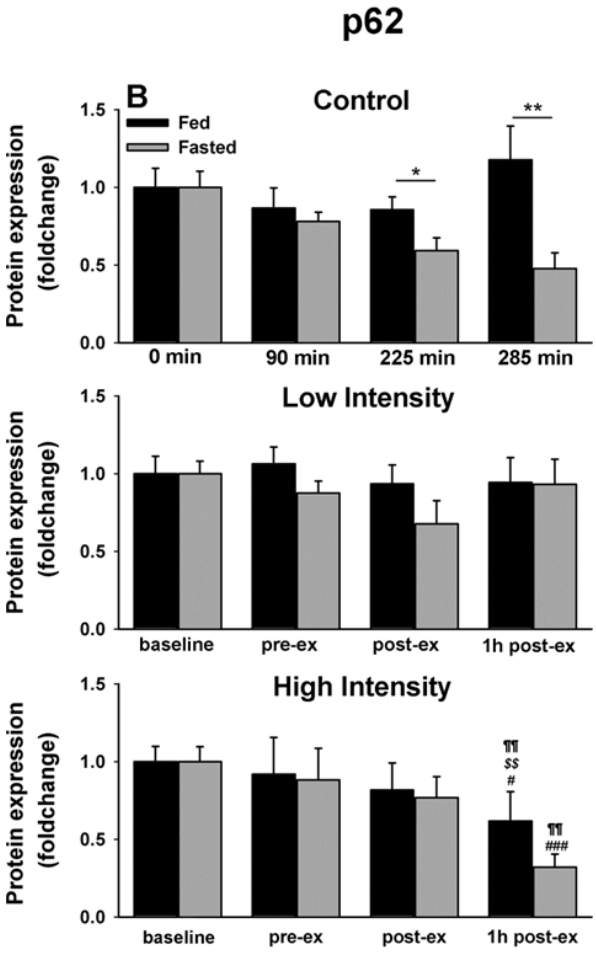
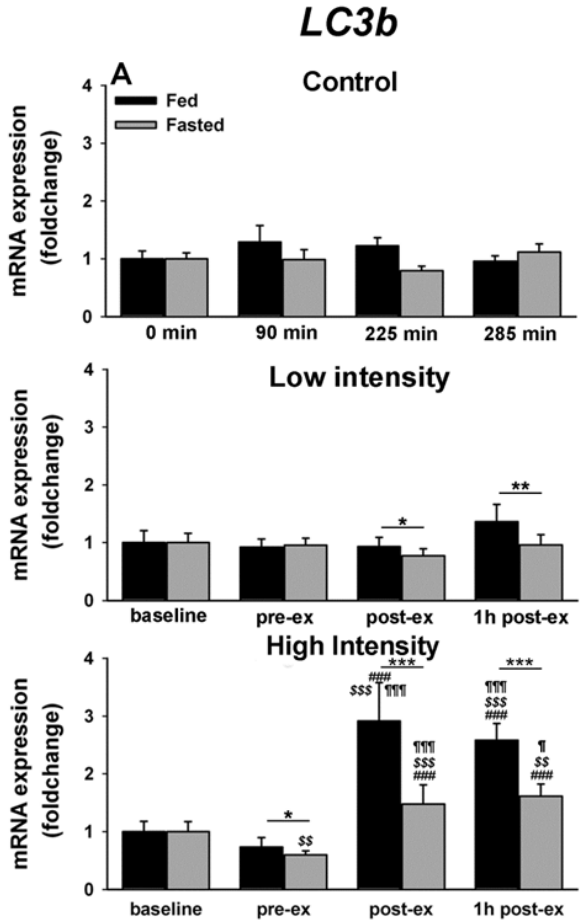


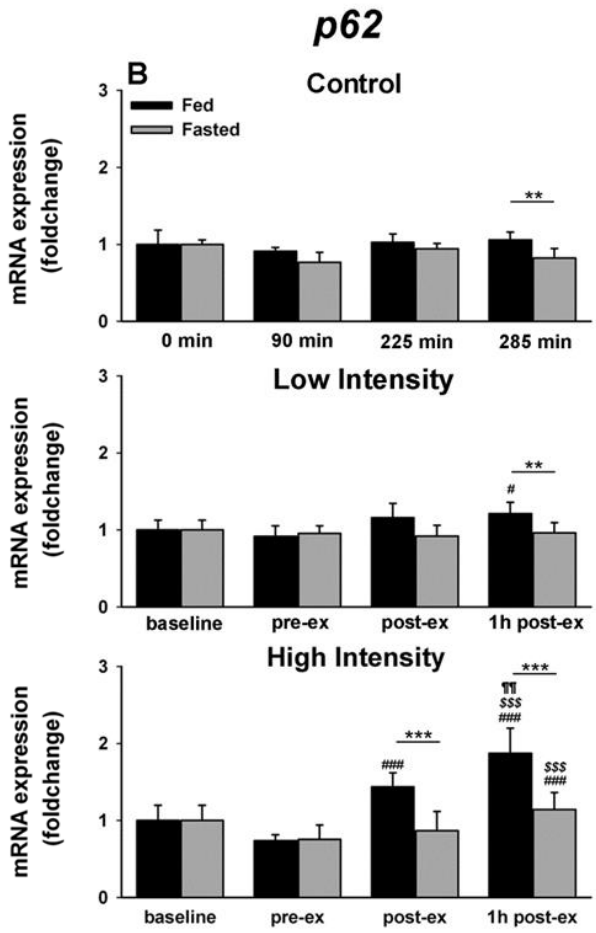
Figure 4. Changes in the expression of autophagic flux-related proteins in response to concentric endurance exercise as a function of intensity, nutritional state and time. Phosphorylation of (A) LC3b II/I ratio and (B) protein expression of p62/SQSTM1 at baseline, before, immediately after and 1 h after cycling exercise at LI (55% $VO_{2\ peak}$) or HI (70% $VO_{2\ peak}$) or in control conditions. Values are presented as mean ± SEM. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ *vs* control group (exercise effect); $^{¶¶}P < 0.01$, *vs* low intensity (intensity effect); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* fed (nutrition effect); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* preexercise (time effect).

Autophagy-related genes expression is activated by cycling exercise in an intensity-dependent way

To gain further insight on the regulation of autophagy at the transcriptional level with concentric endurance exercise, we measured the phosphorylation state of the transcription factor FoxO1/3a, which, when dephosphorylated translocates from the cytosol to the nucleus and promotes the transcription of autophagic genes (Greer and Brunet 2005). Overall, FoxO1/3a$^{Thr24/32}$ decreased over time in fasted conditions, being different from preexercise levels in control ($P < 0.05$) and HI ($P < 0.01$) groups and from fed conditions immediately after exercise ($P < 0.05$) and 1 h after exercise ($P < 0.01$) in the LI group (Supplemental Fig. S1*B* and Fig. S1*D*). No changes were observed among fed subjects. It is of note that a decrease of FoxO3 total was measured 1 h after HI exercise in both fed and fasted subjects ($P < 0.01$, Supplemental Fig. S1*C* and Fig. S1*D*). The changes observed at the level of FoxO1/3a$^{Thr24/32}$ were only partially followed by changes in mRNA expressions of the following autophagy-related genes, *LC3b* (Fig. 5*A*), *p62/Sqstm1* (Fig. 5*B*), *GabarapL1* (Fig. 5*C*) and *cathepsin L* (Fig. 5*D*), as the latter were increased over time only in HI exercise subjects ($P < 0.001$). In addition, contrary to what expected from FoxO1/3a$^{Thr24/32}$ results, higher *LC3b* ($P < 0.001$) and *p62/Sqstm1* ($P < 0.001$) mRNA levels were observed in fed than in fasted subjects after exercise. However, fasting potentiated *GabarapL1* mRNA expression after exercise ($P < 0.01$ after exercise and $P < 0.001$ 1 h after exercise), indicating a different impact of diet on the transcription of those autophagy-related genes. Taken together, these results indicate a transcriptional activation of

autophagy in human skeletal muscle with concentric endurance exercise performed at HI, yet only partially regulated by FoxO1/3a$^{Thr24/32}$ and without additional effect of fasting.




p62
B
Control
Fed
Fasted
mRNA expression (foldchange)
0 min
90 min
225 min
285 min
**
Low Intensity
baseline
pre-ex
post-ex
1h post-ex
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High Intensity
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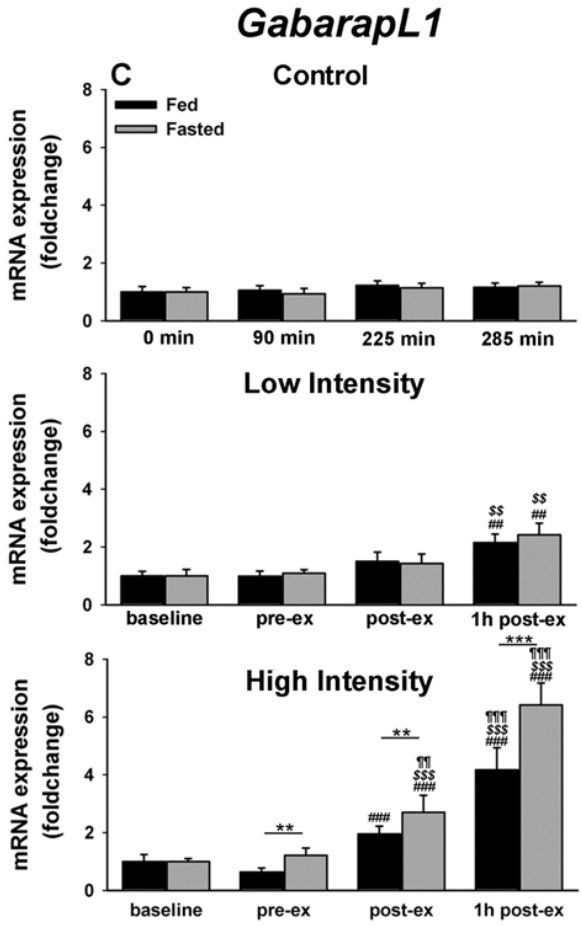

GabarapL1
C
Control
Fed
Fasted
mRNA expression (foldchange)
0 min
90 min
225 min
285 min
Low Intensity
baseline
pre-ex
post-ex
1h post-ex
High Intensity

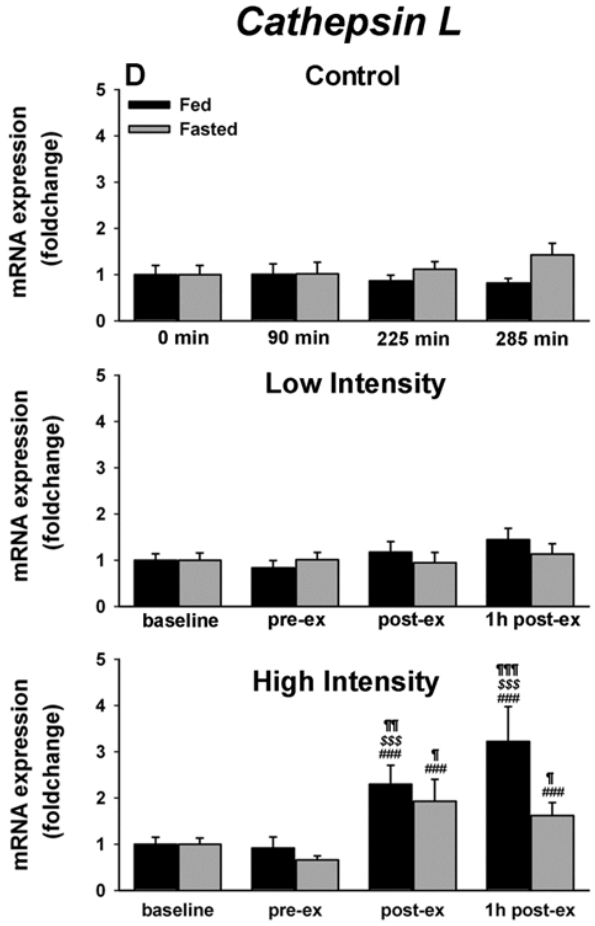


Figure 5. Changes in autophagy-related gene expression in response to concentric endurance exercise as a function of intensity, nutritional state and time. mRNA levels of (A) *Lc3b*; (B) *p62/sqstm1*; (C) *GabarapL1* and (D) *Cathepsin L* at baseline, before, immediately after and 1 h after cycling exercise at LI (55% $VO_{2\ peak}$, LI) or HI (70% $VO_{2\ peak}$, HI) or in control conditions. Values are presented as mean ± SEM. $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ *vs* control group (exercise effect); $^{¶}P < 0.05$, $^{¶¶}P < 0.01$, $^{¶¶¶}P < 0.001$ *vs* LI (intensity effect); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* fed (nutrition effect); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* preexercise (time effect).

Discussion

Food deprivation and exercise are commonly identified as major stimuli of autophagy, although this has only been demonstrated in murine models and in human skeletal muscle after ultraendurance running, which is characterized by large muscle damage (Mizushima et al. 2004, Grumati et al. 2011, Jamart et al. 2012, Jamart et al. 2012, Kim et al. 2012, Jamart et al. 2013). Hence, the possible activation of autophagy in human skeletal muscle during other kind of exercise is still uncharted. In this study, we used cycling to show for the first time that autophagy is also activated by concentric muscle contractions, probably *via* the activation the AMPK pathway and its downstream effector ULK1. This process seems to rely more on the intensity of exercise than the nutritional state.

The strength of the present study is the inclusion of a control resting group, whether fasted or fed. Many studies dealing with the regulation of autophagy, most of which were performed in mice, measured the changes in autophagic markers before and after exercise as well as during the recovery period. Without a resting group, it is not possible to isolate the effects due to exercise itself, the effects due to the nutritional state and those combined. As mentioned above, nutritional state and exercise can each regulate autophagy independently, but both have never been tested together in a single study in humans. The present methodology allows conclusions to be drawn for the first time on the activation of autophagy in human skeletal muscle by exercise and even on the intensity of exercise, the nutritional state and the combination of both. Thanks to the rested

conditions, the effect of the nutritional status on the regulation of autophagy could be clearly interpreted and isolated from the effect of exercise.

Based on the literature (Zhao et al. 2007), the lowest level of autophagy was expected in the fed conditions, in which high phosphorylation states were measured at the level of Akt^{Ser473}, Akt^{Thr308} and $ULK1^{Ser757}$, paralleling the elevated insulinemia after feeding. In this condition, the unchanged level of $ULK1^{Ser317}$ and p62/SQSTM1, together with a lower LC3b II/I ratio, were arguments in favor of a repressed autophagic flux due to food intake. Opposite results were hypothesized in fasted conditions, as reduced Akt phosphorylation and higher autophagosome number has been previously documented in skeletal muscle of mice in response to fasting (Mizushima et al. 2004, Mammucari et al. 2007). However, in the present study, insulin concentration and Akt phosphorylation in rested conditions remained stable over time. At the end of the experimental trial at rest and in a fasted state, an increase in the LC3b II/I ratio and a tendency of the p62/SQSTM1 level to decrease can be interpreted as an accelerated autophagic flux over time even if the phosphorylation state of $ULK1^{Ser317}$ remained stable. Interestingly, the subjects were fasted for 8 h already, which could have been postulated as sufficient to activate autophagy. On the basis of our data, it seems that autophagy in skeletal muscle can still be further activated during the 5 h after an overnight fast and that this occurs independently of the Akt and AMPK pathways, as neither was modified in rested fasted conditions. Of note, neither AMPK nor ACC phosphorylation

was changed in rested conditions, indicating that it is unlikely that autophagy relies on AMPK-dependent signal transduction in nonexercised conditions. Taken together, our results suggest that feeding can repress autophagy in rested human skeletal muscle, likely through the insulin/Akt pathway, while autophagy can be further activated after 8 h of fasting by a mechanism that remains to be determined but that is independent of the Akt and AMPK pathways.

A single session of LI concentric endurance exercise led to a decrease of LC3b II and LC3b II/I ratio in all subjects, indicating a reduction of autophagosomes synthesis in skeletal muscle cells, but did not alter p62/SQSTM1 level, suggesting that autophagosomes degradation did not occur. However, we cannot exclude the notion that activation of autophagy was delayed during LI exercise and therefore was not observable in the studied time frame. Two previous studies concluded to an increased autophagic flux after acute LI exercise in mice, despite the opposite fluctuations of LC3b II level and no significant changes in p62/SQSTM1 content (Kim et al. 2012, Jamart et al. 2013). When the exercise was performed at HI, the decreased LC3b II level and LC3b II/I ratio suggests *a priori* a lower autophagic flux. However, it must be kept in mind that LC3b II depicts the presence of autophagosomes in the cell and LC3b II/I ratio is an indicator of autophagosome synthesis, which taken alone, do not allow to assert that the autophagic flux is activated or inhibited. In order to get a better picture of the changes in autophagic flux, which cannot be measured *per se* in humans, LC3b measurements need to be combined

with other markers of autophagy like ULK1, which initiates this process and p62/SQSTM1, which reflects autophagosome degradation. Clearly, 1 h after intensive exercise, the ULK1^{Ser317} phosphorylation state was increased and p62/SQSTM1 was decreased, thus providing an argument for increased autophagic flux. Together, these data support the idea that eccentric contractions are not required to activate autophagy in skeletal muscle during exercise.

Our data in humans are somewhat different to previous work in mice as the LC3b II/I ratio was found to be enhanced after acute exhausting exercise (Grumati et al. 2011). Interestingly, these autophagic markers appeared to be poorly affected by the nutritional state during exercise, contrary to our recent findings carried out in mice, which showed a higher autophagic flux in the fasted state compared to the fed state (Jamart et al. 2013). One could postulate that exercise added to food deprivation probably results in a much intense cellular stress in mice than in humans, likely as a result of their higher metabolism rate and insulin sensitivity. Overnight fasting is thus likely not long enough to enhance exercise-induced autophagy in human skeletal muscle. These findings support the notion that HI is the most potent stimulus to increase the autophagic flux during cycling exercise in human skeletal muscle compared to the nutritional state.

We then tried to understand further how autophagy was regulated by exercise and the nutritional state by analyzing the posttranslational autophagic initiation, mainly controlled by ULK1 (Kim et al. 2011). ULK1 is

activated by AMPK, which phosphorylates the latter at several sites, including Ser^{317}, while ULK1 is inhibited by mTORC1, which phosphorylates it at Ser^{757}, thereby preventing ULK1 activation by AMPK. Initiation of autophagy can therefore be regulated by AMPK-dependent and mTORC1-dependent pathways. Because mTORC1 is highly regulated by insulin, the latter pathway is also called the insulin-dependent pathway. Although we found that phosphorylation of AMPKα^{Thr172} was dispensable for LI exercise-induced autophagy in skeletal muscle of mice, which instead relied on the insulin/Akt/mTORC1 signaling (Jamart et al. 2013), the present study is to our knowledge the first to show that in humans, HI cycling exercise induces autophagy concomitantly with an increase in AMPKα^{Thr172} and ULK1^{Ser317} phosphorylation, indicating a likely involvement of the AMPK-dependent pathway. In accordance with previous studies conducted in skeletal muscle, we confirmed that AMPKα^{Thr172} phosphorylation is increased with exercise intensity (Egan et al. 2010, Tadaishi et al. 2011, He et al. 2012), but was not affected by fasting (Gonzalez et al. 2004), as supported by substantial ACC^{Ser79} and ULK1^{Ser317} phosphorylation state after HI exercise, whatever the diet. Importantly, this effect was long-lasting, as phosphorylation of ACC and ULK1 were still higher 1 h after exercise completion. Of note, the insulin/mTORC1 pathway did not contribute to the activation of autophagy during endurance exercise, as ULK1^{Ser757} was not regulated differently in the 2 exercised groups than in the control group. Without a control group, we could have concluded that exercise reduced ULK1^{Ser757} in both LI and HI fed groups. But a closer look at the control rested group in the fed state reveals that ULK1^{Ser757} showed exactly

the same pattern of regulation, indicating that exercise was not involved in the decrease in ULK1^{Ser757} phosphorylation between the preexercise and postexercise time points. The increase between baseline and preexercise values and the subsequent return to baseline 1 h after seem to be attributable to the normal kinetics of regulation of ULK1^{Ser757} after feeding. This result highlights the real added value of having a nonexercised group. Our results show that concentric endurance exercise induced-autophagy relies mostly on AMPK activation, which is dictated by the intensity of exercise rather than by nutrient depletion in human skeletal muscle.

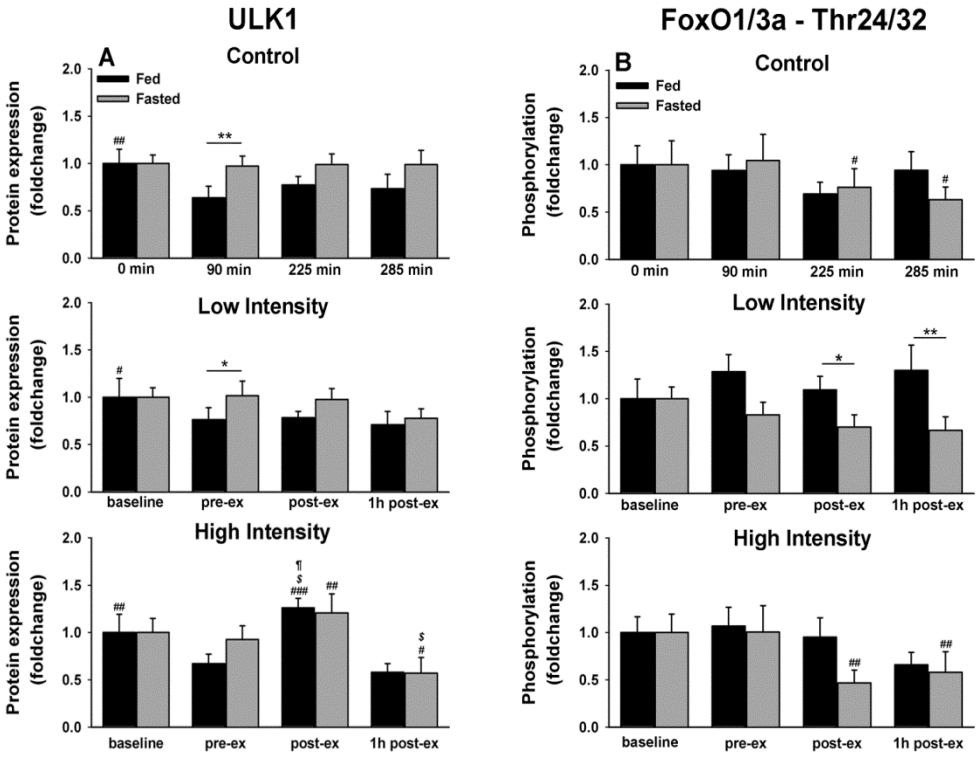
Autophagy can be regulated at the posttranslational level, as mentioned above, but transcriptional mechanisms participate in the regulation of autophagy as well (Sandri 2010). Both Akt and AMPK play a key role in the transcriptional modulation of autophagy as antagonist regulators of a common downstream substrate, FoxO3a (Mammucari et al. 2007, Sanchez et al. 2012). Expression of autophagic genes was previously stated to be enhanced further to fasting or diverse exercise conditions in both mice and human skeletal muscle (Mammucari et al. 2007, Jamart et al. 2012, Moresi et al. 2012, Jamart et al. 2013). Having found that HI cycling exercise promoted autophagic gene expression, particularly in the fed condition, we addressed the question of which one of the AMPK or Akt pathway was brought into play to induce autophagy at the transcriptional level. FoxO3a activation was reported to directly stimulate autophagy *via LC3b* and *GabarapL1* transcription in rested fasted mice (Mammucari et al. 2007, Moresi et al. 2012). Unexpectedly, the lower phosphorylation of

FoxO1/3a$^{Thr24/32}$, and thereby the higher activity of FoxO1/3a (Greer and Brunet 2005) with fasting failed to increase the expression of *LC3b* and *GabarapL1* in control conditions. Combination of fasting with HI exercise also reduced FoxO1/3a$^{Thr24/32}$ levels, but compared to control conditions, the activation of FoxO1/3a was probably high enough to induce, or at least to contribute to, transcription of *LC3b* and *GabarapL1*. Although Akt is a known kinase for FoxO1/3a at Thr$^{24/32}$ (Brunet et al. 1999), another undefined kinase (or phosphatase) should be responsible for the decrease in FoxO1/3a phosphorylation, as Akt phosphorylation at Ser^{473} remained stable during the 3 trials in the fasted state.

Those observations in the fasted state suggest that fasting *per se* is insufficient to trigger FoxO1/3a-dependent autophagy, yet additional cell stresses, such as HI exercise, are necessary to allow transcriptional autophagy to occur in human skeletal muscle. Interestingly, our results show that other transcription factors should activate the transcriptional autophagic program in fed conditions, as FoxO1/3a$^{Thr24/32}$ was not modified in these conditions. Possible candidates are NFκβ (Mofarrahi et al. 2012) or unknown transcription factors downstream of p38 mitogen-activated protein kinase (McClung et al. 2010), or we could have missed phosphorylation of FoxO1/3a by AMPK. Indeed, FoxO3a is controlled by AMPK *via* different phosphorylation sites than Akt (Brunet et al. 1999, Greer et al. 2007). Measuring the AMPK-dependent activity of FoxO3a would have given us helpful clues to figure out and clarify the underlying mechanisms of transcriptional autophagy activation in our experimental

protocol. A potential sensitivity of FoxO3a to HI exercise would be an attractive hypothesis to link, at least in part, AMPK activation to a larger autophagic genes expression with HI exercise. However, no FoxO3a antibodies specific to the sites phosphorylated by AMPK are currently available. In addition, it must be kept in mind that cytosolic levels of FoxO3a are also under proteasome control (Bertaggia et al. 2012). Therefore, the decrease in the total form of FoxO3a, which ensued 1 h after HI exercise completion, may be interpreted as a higher proteasomal degradation of the latter after its acetylation, which we did not assess. Our findings, although strongly evidencing a short-term transcriptional activation of autophagy with HI concentric endurance exercise, emphasize the necessity of further investigations to decipher the molecular mechanisms responsible for autophagy transcriptional regulation in human skeletal muscle during exercise.

In summary, the present study is to our knowledge the first to provide evidence that exercise-induced autophagy relies on the activation of the AMPK pathway in human skeletal muscle. In addition, our findings indicate that the most effective strategy to activate autophagy depends on exercise intensity rather than dietary supply, thus providing interesting clues to improve the understanding of remodeling in both healthy and pathological human skeletal muscles.


ULK1
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Fed
Fasted
Protein expression (foldchange)
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225 min
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FoxO1/3a - Thr24/32
B
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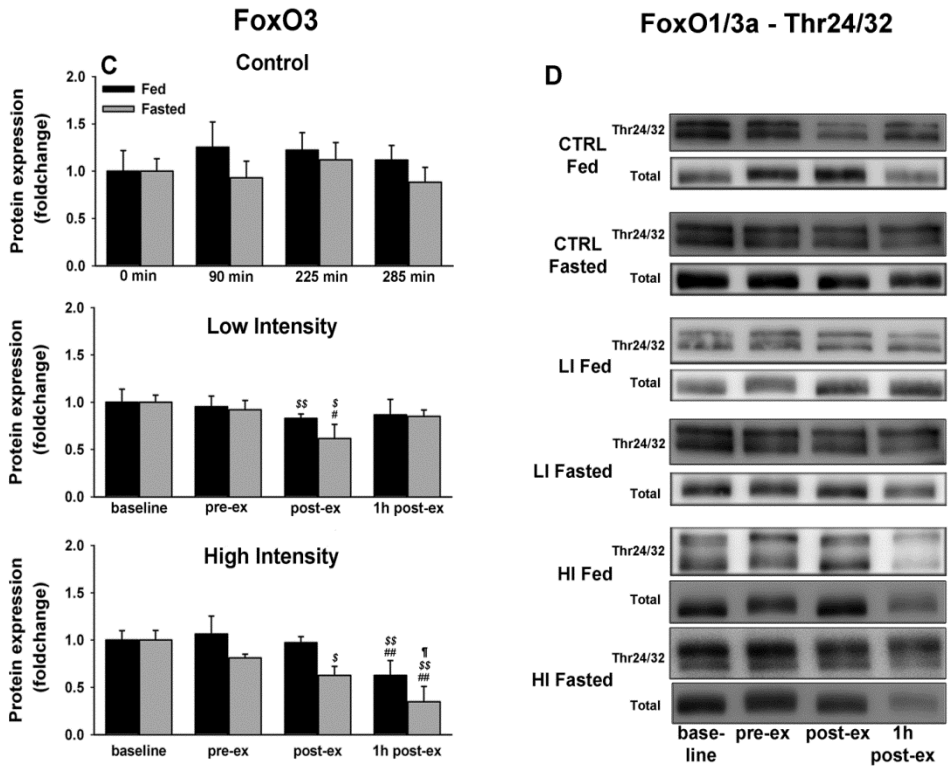
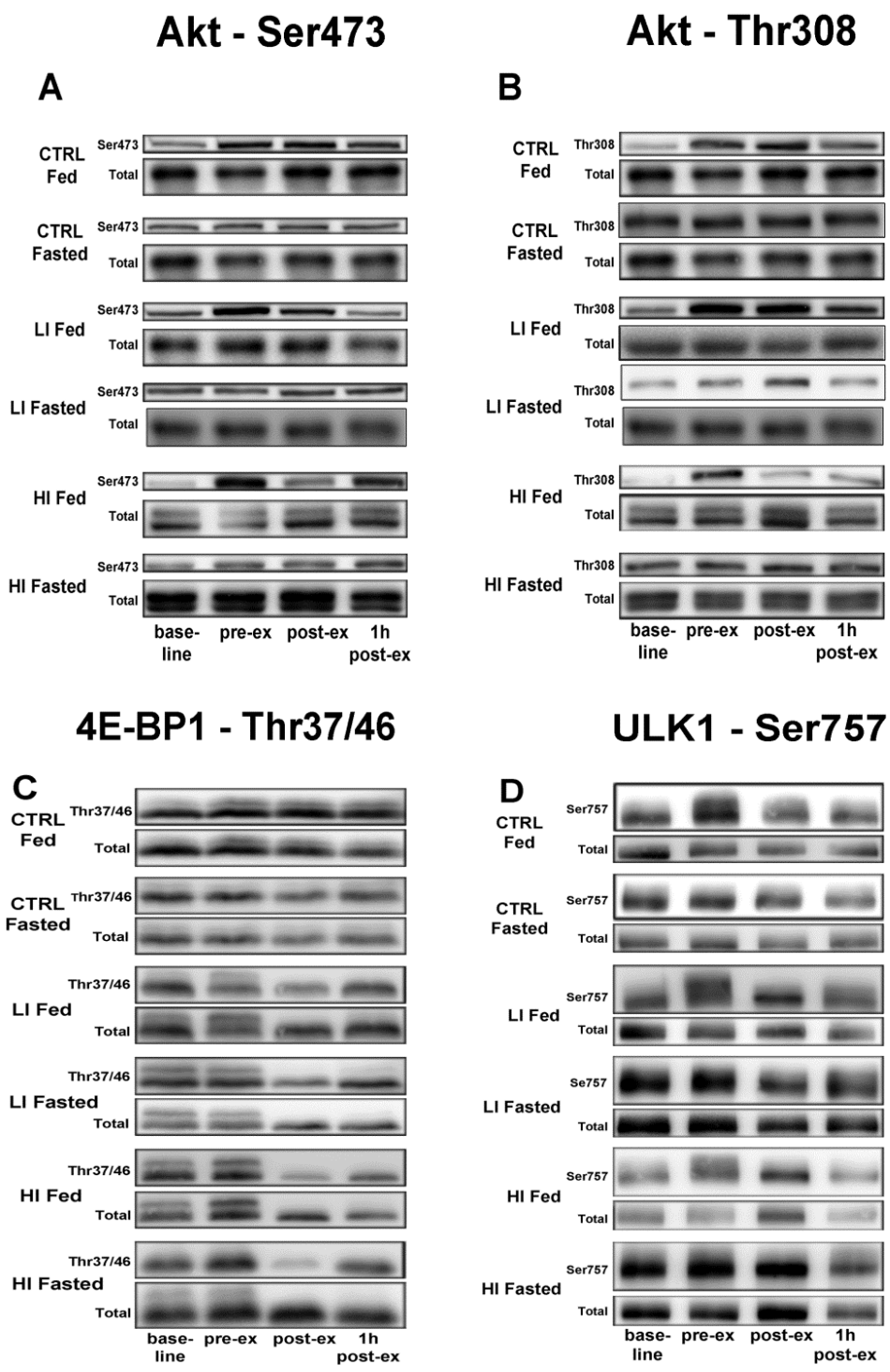


Supplementary figure 1. Changes in A) ULK1^{Total}, B) FoxO1/3a$^{Thr24/32}$ and C) FoxO3a^{Total} in response to concentric endurance exercise as a function of intensity, nutritional state and time. Data were collected at baseline, before, immediately after and 1 h after cycling exercise at low (55 % $VO_{2\ peak}$, LI) or high intensity (70 % $VO_{2\ peak}$, HI) or in control conditions (CTRL). D) Representative western blots for FoxO1/3a$^{Thr24/32}$ and FoxO3a^{Total}. Values are presented as the means ± SEM. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ *vs* control group (exercise effect); $^{¶}P < 0.05$ *vs* low intensity (intensity effect); $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* fed (nutrition effect); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* pre-exercise (time effect).


Akt - Ser473
A
CTRL Fed
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LI Fed
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HI Fed
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Ser473
Total
base-line
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post-ex
1h post-ex
Akt - Thr308
B
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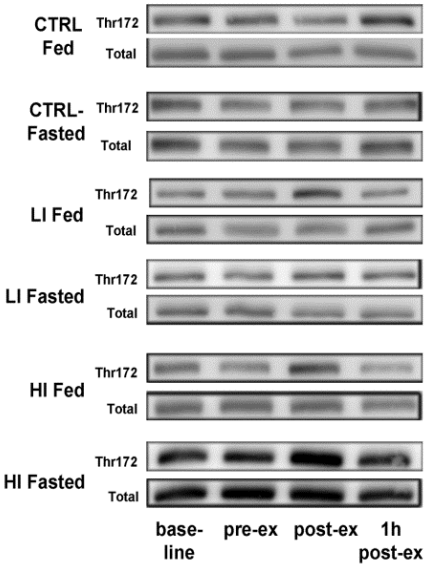
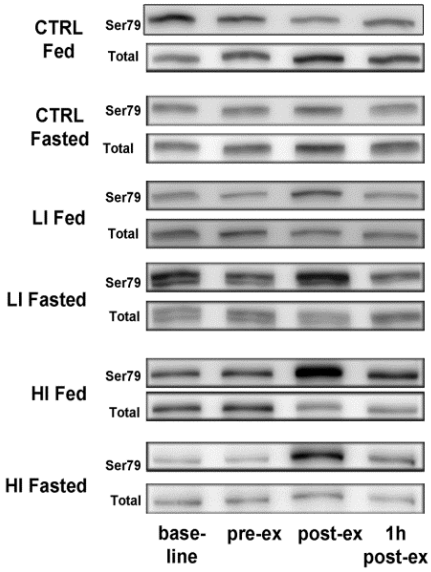
4E-BP1 - Thr37/46
C
Thr37/46
ULK1 - Ser757
D
Ser757
Se757

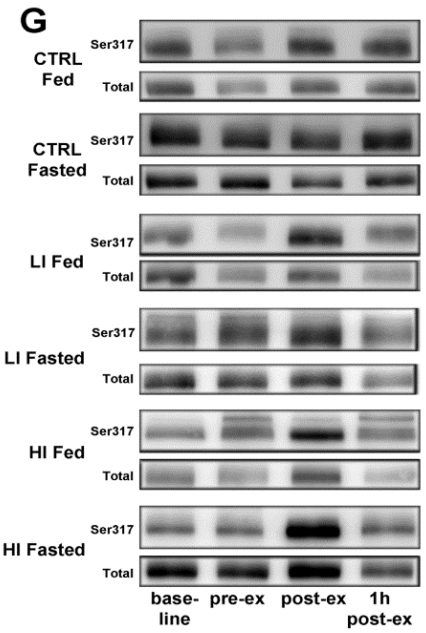
AMPKα - Thr172



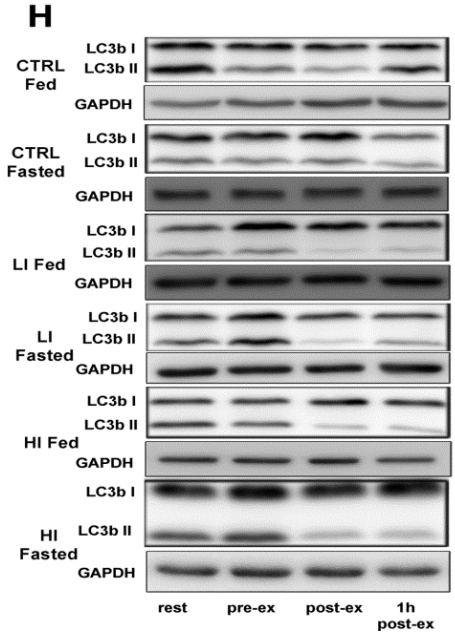
ACC - Ser79

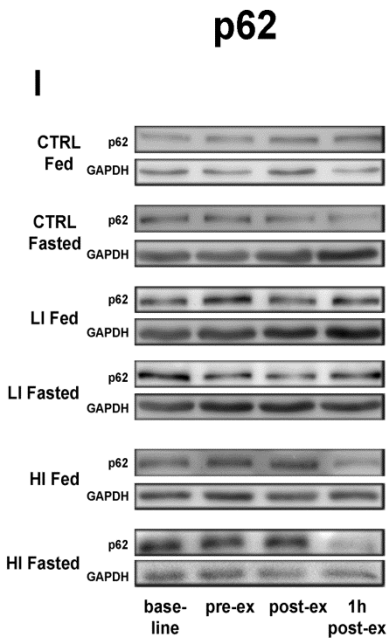


ULK1 - Ser317



LC3b ratio





Supplementary figure2. Representative western blots for A) Akt^{Ser473} and Akt^{Total}; B) Akt^{Thr308} and Akt^{Total}; C) 4E-$BP1^{Thr37/46}$ and 4E-$BP1^{Total}$; D) $ULK1^{Ser757}$ and $ULK1^{Total}$; E) $AMPK^{Thr172}$ and $AMPK^{Total}$; F) ACC^{Ser79} and ACC^{Total}; G) $ULK1^{Ser317}$ and $ULK1^{Total}$; H) LC3b I and LC3b II; I) p62/SQSTM1 and GAPDH.

Chapter 3: Lack of activation of mitophagy during endurance exercise in human

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Abstract

This study aimed to determine whether fission and mitophagy are activated by acute endurance exercise in human skeletal muscle and to investigate if this activation is dependent upon the nutritional state. Trained athletes (*n* = 7) cycled for 2 h at 70% $_{VO2peak}$ in a fed or fasted state. Vastus lateralis muscle biopsies were obtained at baseline, before, immediately after, and 1 h after exercise. Protein and mRNA markers for mitophagy, mitochondrial biogenesis, fission and fusion were analyzed using quantitative real-time polymerase chain reaction and Western blot. Fission, assessed by phospho-DRP1^{Ser616} in the mitochondrial fraction, increased postexercise and 1 h postexercise only in the fed state. LC3b II and p62/SQSTM1 in the mitochondrial fraction were unchanged, whereas the LC3b II/I ratio was decreased only postexercise in the fasted state (*P* = 0.019), indicating a reduced mitophagy. Genes implicated in fission and mitophagy, such as *Drp1*, *Bnip3*, and *Bnip3L*, and proteins involved in fission (Fis1) or mitophagy (BNIP3) were all more expressed after exercise in the fed state (*P* < 0.05). As expected, the mRNA levels of *PGC1α*, *Tfam*, and *Hsp60*, all markers of mitogenesis, were increased after endurance exercise, but to a larger extent in the fed than that in the fasted state. The present study provides the very first evidence that mitophagy is not activated during and early after high-intensity endurance exercise in human, whatever the nutritional state, despite a selective activation of fission in the fed state. However, when nutrient availability is optimal, muscle cells seems capable of preparing mitochondria for lysosomal

degradation. Thus, we may not exclude an activation of mitophagy at a later stage after exercise.

Key Words: *BNIP3 — Drp1 — cycling — fasting — mitochondria — PGC1α*

Introduction

Mitochondria are organelles providing a large part of energy to cells via oxidative phosphorylation. They form a dynamic network, the abundance, size, distribution, and morphology of which are continuously rearranged through biogenesis, motility, fusion, and fission events. Fusion and fission are complementary quality control strategies, which help the cell to cope with metabolic and environmental disturbances, making possible the maintenance of optimal energetic capacity of the cell, together with the maintenance of a healthy mitochondria population (Westermann 2012, Youle and van der Bliek 2012). Fusion gathers individual mitochondria within an elongated interconnected mitochondrial network (Karbowski and Youle 2011), whereas fission selectively fragments mitochondrial network into isolated mitochondria (Karbowski and Youle 2011). The outer mitochondrial membrane protein mitofusin 2 (Mfn2) and the inner membrane protein optic atrophy 1 (OPA1) are both GTPases that play a key role in the fusion process (Ding and Yin 2012).

Fission is usually consecutive to fusion, giving birth to mitochondria harboring uneven membrane potential for a transitory period. Upon

mitochondria depolarization, the cytosolic GTPase dynamin-related protein 1 (DRP1) is phosphorylated at serine 616 and translocates to the outer mitochondria membrane to initiate fission (Otera and Mihara 2011). Mitochondrial fission 1 protein (Fis1) is another fission protein embedded in the outer membrane of mitochondria, which is believed to interact with DRP1 (Yoon et al. 2003).

Severely depolarized mitochondria are removed through mitophagy, namely, a selective form of autophagy (Twig et al. 2008). Autophagy traps targeted cell constituents in a double membrane vesicle called autophagosome, which fuses with lysosomes containing hydrolases, allowing the entire degradation of the autophagosome. At a molecular level, activation of autophagy is featured by increased conversion of microtubule-associated protein-1 light chain 3 (LC3b) I protein to its active form LC3b II, parallel to a lower p62/SQSTM1 protein level (Barth et al. 2010). Both proteins are cleaved on autophagosome membrane. Although LC3b II intervenes from autophagosome formation to their degradation (29), p62/SQSTM1 is a receptor for proteins aggregates, which is eliminated with autophagosome content after it fuses with lysosome (Pankiv et al. 2007). Both LC3b II and p62/SQSTM1 facilitate recruitment of damaged mitochondria to autophagosome, either by LC3b II interaction with Bcl-2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) or by p62/SQSTM1-dependent clustering of ubiquitinated mitochondria (Ding et al. 2010, Narendra et al. 2010, Novak et al. 2010, Rikka et al. 2011). Depolarized mitochondria might be specifically tagged by the E3-ubiquitin

ligase Parkin, which ubiquitinates mitochondrial outer membrane proteins after recruitment by PTEN-induced kinase-1 to mitochondria (Narendra et al. 2010). In addition, the complex BNIP3/BNIP3-Like (Nix) can interact with and bind to LC3 (Novak et al. 2010, Rikka et al. 2011). Both Parkin and BNIP3 facilitate the engulfment of mitochondria into autophagic vesicles before fusion with lysosomes and subsequent proteolytic degradation.

Mitochondrial remodeling (*i.e.*, fusion and fission) seems to be closely related to mitochondrial turnover (*i.e.*, the balance between mitochondria biogenesis and removal). The fine tuning of these two processes has been proposed to participate at the basal integrity of the mitochondrial network and in the maintenance of muscle mass (Romanello et al. 2010). Yet, little is known about the role of mitochondrial machinery in the regulation of mitochondrial homeostasis under stressful conditions, such as exercise. Most of the studies exploring the potential benefits of exercise on murine or human muscle metabolism mainly focused on mitochondria biogenesis by measuring, among others, the expression of the peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α) and mitochondrial transcription factor A (Tfam), two key regulators of this process (Lira et al. 2010, Ljubicic et al. 2010). Nonetheless, mitochondria plasticity and clearance (mitophagy) are emerging as essential mechanisms in exercise-induced metabolic adaptations in skeletal muscle (Yan et al. 2012, Lira et al. 2013).

Nutrient deprivation may disturb cell homeostasis, leading to catabolism of cellular constituents, including mitochondria, to provide alternative

sources of energy and sustain cell function. Although starvation has been reported to induce mitochondria fusion elongation in vitro, sparing these organelles from autophagic removal (Gomes et al. 2011, Rambold et al. 2011), increased mitophagy markers were found in skeletal muscle of fasted mice (Jamart et al. 2013). In human, however, the regulation of mitophagy under these conditions has not been documented yet. Therefore, it remains to determine to what extent nutrients deficit can affect mitochondrial remodeling to face energetic challenges.

On the basis of the aforementioned background, we hypothesized that, in human skeletal muscle, mitophagy is transitorily activated during endurance exercise in a fission-dependent manner and that this transient mitophagy is preferentially activated when exercise is performed in a fasted state.

Materials and methods

Subject characteristics and preliminary testing

The samples analyzed in the present work were collected during a previous study (Schwalm et al. 2015). Here, we used the remaining muscle samples of the seven healthy young volunteers cycling at high-intensity (HI group: age = 24 ± 1 yr, body mass index = 21.3 ± 2.3 $kg \cdot m^{-2}$, VO_{2peak} = 64.5 ± 1.7 $mL \cdot min^{\cdot -1 \cdot} kg^{-1}$, Wmax = 4.7 ± 0.1 $W \cdot kg^{-1}$). The protocol was approved by the local ethical committee (Université catholique de Louvain) and conducted in accordance with the Declaration of Helsinki. The subjects

were fully informed about the experimental procedure before their written consent was obtained. They were selected on a voluntary basis and were all experienced cyclists or triathletes. The minimal level of maximal oxygen consumption (VO_{2peak}) for inclusion in the protocol was 50 $mL \cdot min^{-1} \cdot kg^{-1}$. Before the beginning of the study, subjects reported to the laboratory for a medical examination aiming to exclude any underlying pathology. Afterward, height, weight, and percentage of body fat were measured according to the Jackson and Pollock method (Jackson and Pollock 1978). A maximal incremental exercise test was performed on a cycle ergometer (Cyclus II; RBM Electronics, Leipzig, Germany) for determining VO_{2peak} and maximal power output (Wmax). The starting load was 70 W, incremented by 40 W every 3 min, until exhaustion. Heart rate (Polar Team System 2; Polar Electro, Kempele, Finland) and respiratory exchanges (Metalyzer 2; Cortex, Leipzig, Germany) were continuously monitored.

One week after the maximal test, subjects took part in a familiarization session to ensure they could handle the workload asked during the protocol in the fasted state. A 2-wk period separated the familiarization session and the first experimental session. Subjects were randomly chosen to perform exercise sessions either in a fed or in a fasted state. Subjects had to refrain from strenuous exercise 3 d before each experimental session.

Dietary control

The day before each experiment, a standardized carbohydrate-rich diet was given to the subjects (63% carbohydrate, 13% protein, and 24% fat). During the fed session, a standardized breakfast was given immediately after the first biopsy. During the exercise period in the fasted state, 150 mL of water was ingested every 15 min, whereas in the fed state, 150 mL of a 6% carbohydrate energetic drink was consumed every 15 min (Sports Drink Orange, 3 Action, Tessenderlo, Belgium). Participants were asked not to consume medication or dietary complement during the 3 d preceding each experiment.

Exercise protocol

Each subject undertook two experimental sessions, a fasted and a fed one, randomly assigned and interspersed with a 2-wk period (Fig. 1). For each session, they arrived at 6:00 AM to the laboratory after an 8-h overnight fast. A first biopsy sample, with the needle pointing proximally, was taken from the mid portion of the vastus lateralis muscle under local anesthesia (1 mL of Xylocaine 2%; AstraZeneca, Belgium) (baseline). The leg of the first biopsy was chosen at random. Samples were immediately frozen in liquid nitrogen then stored at -80°C before further analysis. Thereafter, the fed group received a standardized breakfast, whereas the fasted group only received water (see previous section). Ninety minutes after the first biopsy, a second muscle biopsy, with the needle pointing distally, was taken through the same incision as the first one (preexercise).

Still under local anesthesia, a second incision was made 3 cm distally from the first incision site, to be able to take the third biopsy within 30 s at the end of the exercise. Fifteen minutes after, subjects started to cycle for 2 h at 70% of VO_{2peak}. With the needle pointing proximally, a third muscle biopsy was taken from the second incision site made before the beginning of the exercise (postexercise). One hour after the end of the exercise bout, a last biopsy—with the needle pointing distally in the incision made before exercise—was taken (1 h postexercise). Pointing the needle in the opposite direction was intended to reduce potential activation of inflammatory signaling pathways as recommended by van Thienen et al. (Van Thienen et al. 2014). All subjects underwent the same protocol 2 wk after the first session, receiving the crossover nutritional treatment (fasted vs fed).

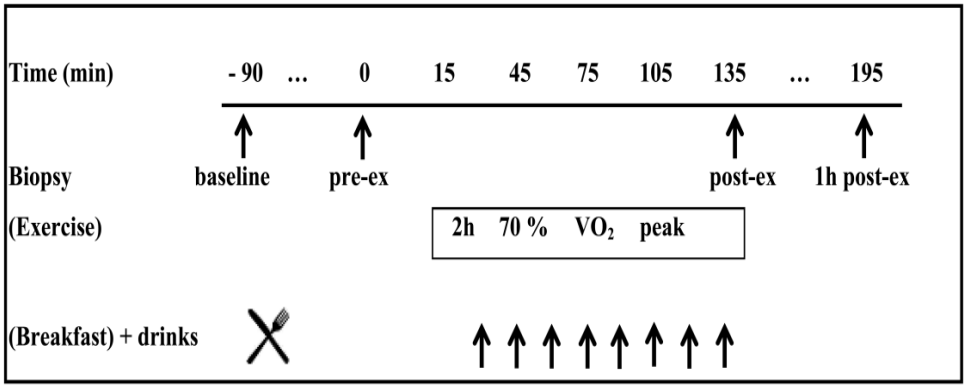


Figure 1. Schematic view of experimental protocol. A first muscle biopsy was taken at baseline (-90 min) and a second biopsy 90 min after (preexercise, 0 min). Fifteen minutes after the second biopsy, subjects cycled for 2 h at 70% of VO_{2peak} (15 min). A third biopsy was taken at the end of the exercise period (postexercise, 135 min) and a last biopsy 1 h postexercise (195 min). Fed subjects received a breakfast immediately after baseline measurements as well as 150 mL of a 6% carbohydrate drink every 30 min during the exercise bout while fasted subjects received the same amount of water.

Cytosolic and mitochondrial protein fractionation

Muscular biopsy sample (~30 mg) was dedicated to cytosolic and mitochondrial protein fractionation following an adapted protocol of Quadrilatero and Rush (Quadrilatero and Rush 2006). Muscle samples were homogenized with a TissueLyser II (Quiagen, Hilden, Germany) for 2 × 3 min at 15 Hz in ice-cold buffer containing 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 MgCl2, 1 mM ethyleneglycotetraacetic acid, 1 mM ethylenediaminetetraacetic acid, pH adjusted to 7.4, supplemented with

200 mM sodium orthovanadate, 1 mM 1,4 dithiothreitol, and a protease inhibitor cocktail containing 1mMethylenediaminetetraacetic acid (Roche Applied Science, Vilvoorde, Belgium). Homogenates were centrifuged for 10 min at 10,000g at 4°C. Supernatants (S1) were collected and centrifuged for 20 min at 16,000g at 4-C. Resulting supernatants (S2) were removed from pellets (P1) to be centrifuged for 20 min at 16,000g at 4°C and stored at -80°C (cytosolic protein fraction). P1 were washed in ice-cold buffer and centrifuged for 2 20 min at 16,000g at 4°C, each time carefully removing supernatants. Resulting pellets (P2) were resuspended in ice-cold buffer supplemented with 0.1% Triton X-100 (mitochondrial protein fraction), sonicated with Ultrasonic Cleaner (VWR, Radnor, PA) and immediately stored at -80°C before further analysis. Cytosolic and mitochondrial protein contents were determined using the DC kit for protein dosage (Bio-Rad Laboratories, Hercules, CA). The sole presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and lysosome-associated membrane protein 2b (LAMP2b) protein in the cytosolic fraction and cytochrome c oxidase (COx IV) and mitochondrial 20 kDa outer membrane protein (TOM20) protein in the mitochondrial fraction were assessed to verify the purity of our extracts. GAPDH and LAMP2b were detected in the cytosolic fraction only, whereas COx IV and TOM20 were measured exclusively in the mitochondrial fraction, excluding any possible contamination between the two different fractions (Fig. 2).

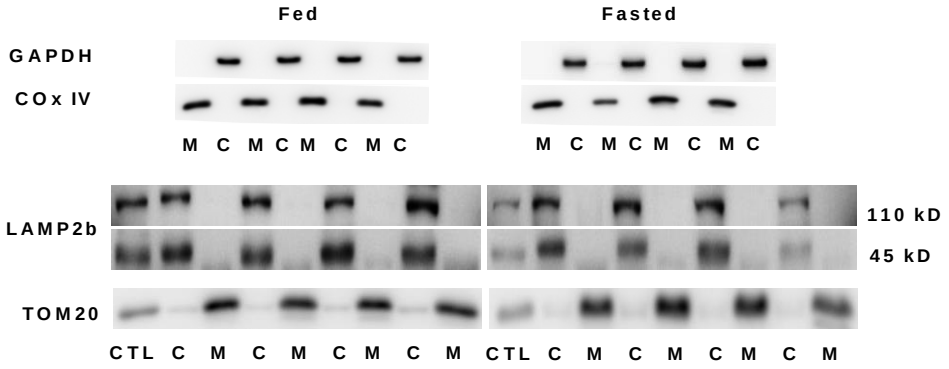


Figure 2. Assessment of the purity of cytosolic and mitochondrial fractions. GAPDH, COx IV, LAMP2b, and TOM20 were all measured in the cytosolic and mitochondrial extracts by western blotting to ascertain the absence of contamination between the different cellular fractions. GAPDH/ LAMP2b and COx IV/TOM20 proteins were chosen as representative markers of the purity of cytosolic and mitochondrial fractions, respectively. For LAMP2b, two bands were detected at approximately 110 (glycosylated form) and 45 kDa (unglycosylated form) and are therefore displayed here (Eskelinen 2006). C, cytosolic fraction; M, mitochondrial fraction; CTL, whole cell lysate.

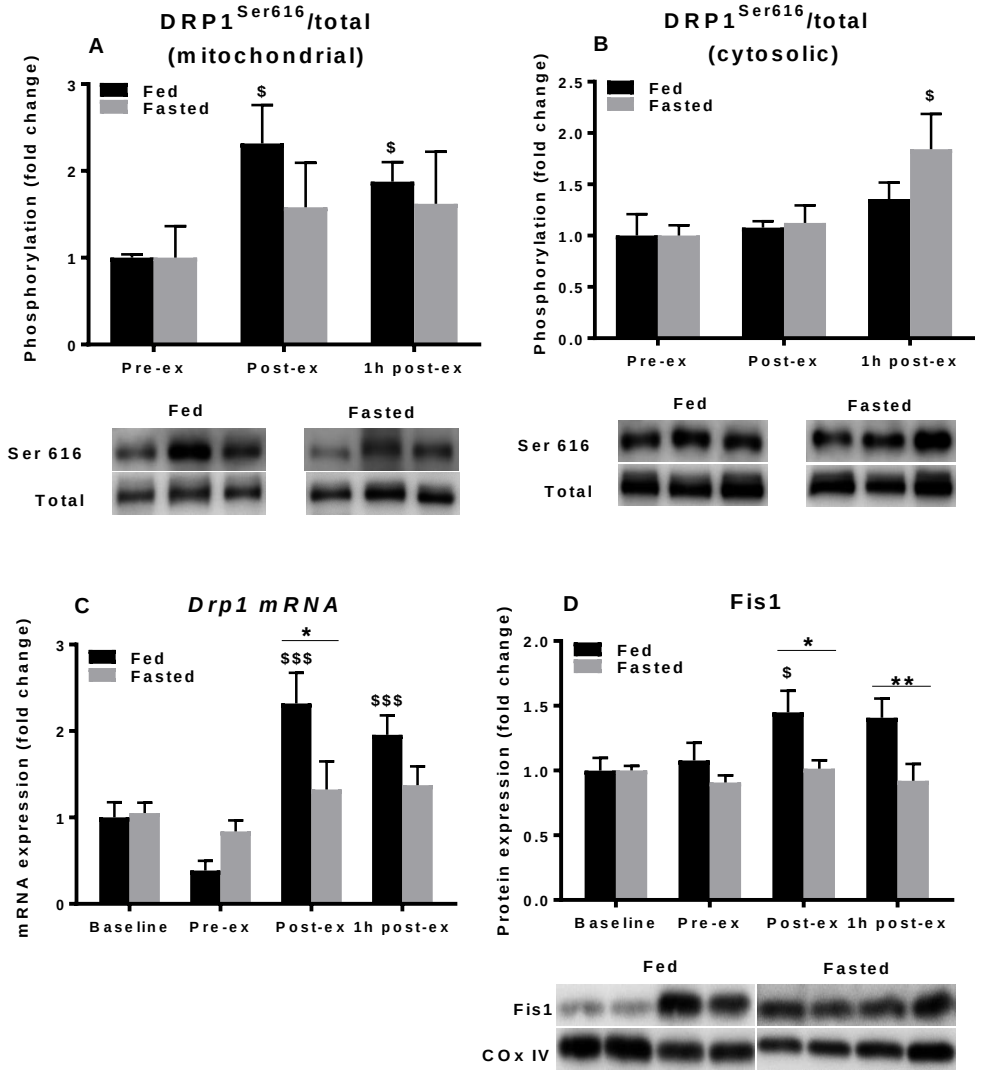
Statistical analysis

All values are expressed as the mean ± SEM. Statistical analyses were performed using GraphPadPrism 7.0. Data were analyzed by two-way repeated-measures ANOVA to test the main effect of exercise and nutrition as well as the interaction of these two factors. When a significant main effect was found, Fisher *post hoc* tests were performed to test potential differences between fed and fasted subjects at each time point (asterisks) as well as differences between preexercise and each other time point in

the same nutritional state (dollar signs). Statistical significance was set at $P < 0.05$.

Acute endurance exercise and the nutritional state regulate markers for fission

Compared with preexercise, mitochondrial DRP1^{Ser616} increased postexercise ($P = 0.013$) and 1 h postexercise in the fed state ($P = 0.052$, Fig. 3*A*), whereas cytosolic DRP1^{Ser616} increased 1 h postexercise in the fasted state ($P = 0.019$, Fig. 3*B*). Drp1 mRNA increased immediately postexercise ($P < 0.001$) and 1 h postexercise ($P = 0.006$) in the fed conditions only (Fig. 3*C*). Immediately postexercise, *Drp1* mRNA levels were twofold higher in fed compared with fasted state ($P = 0.018$). Similarly to *Drp1* mRNA, mitochondrial Fis1 protein expression increased postexercise in the fed conditions only ($P = 0.038$, Fig. 3*D*), with a ~50% higher level in fed compared with fasted state immediately postexercise ($P = 0.018$) and 1 h postexercise ($P = 0.009$). *Fis1* mRNA was neither impinged by exercise nor the nutritional state (Fig. 3*E*).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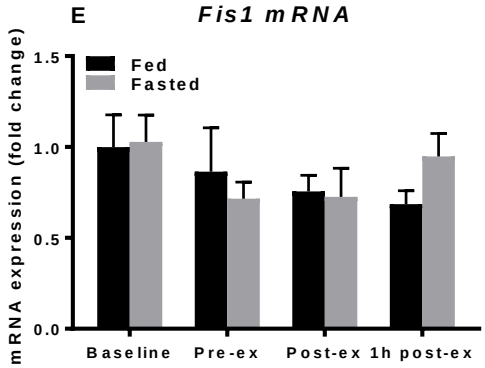
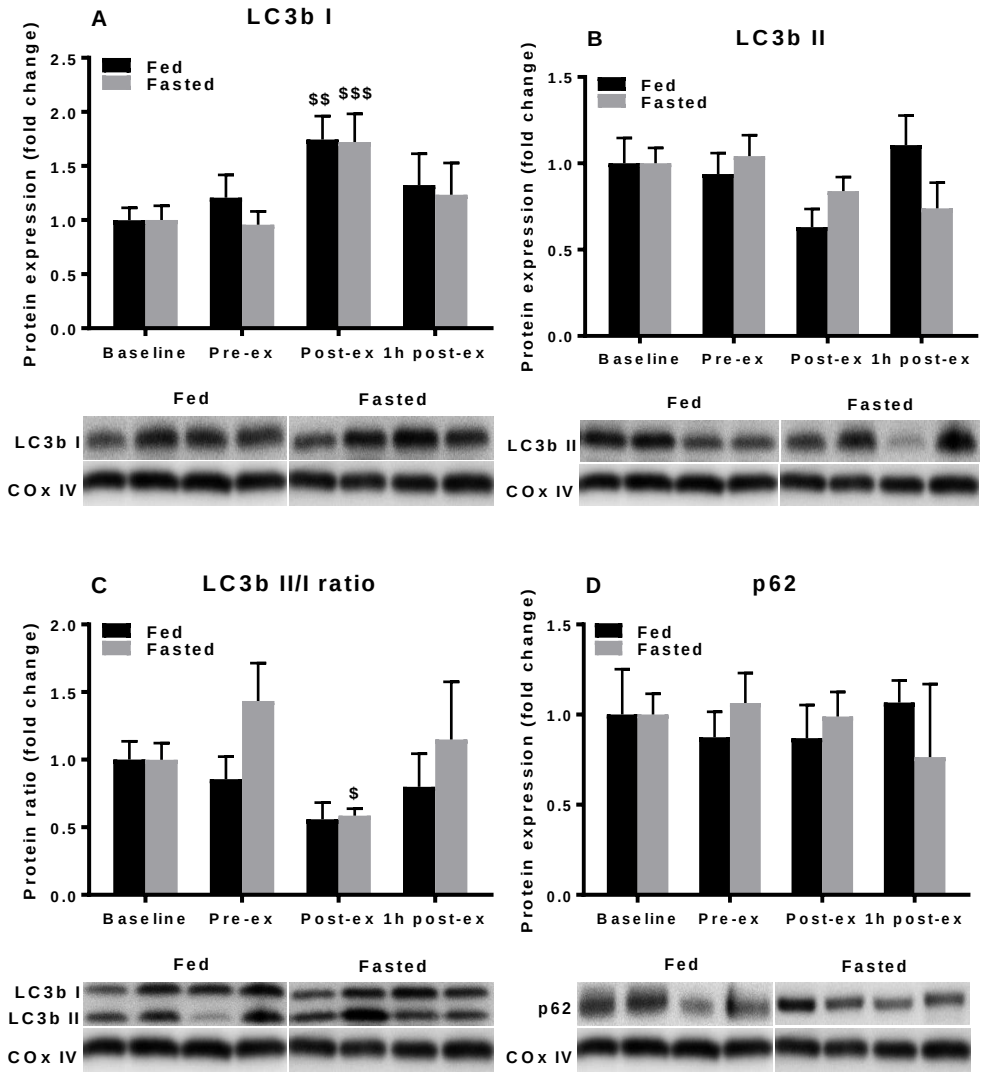


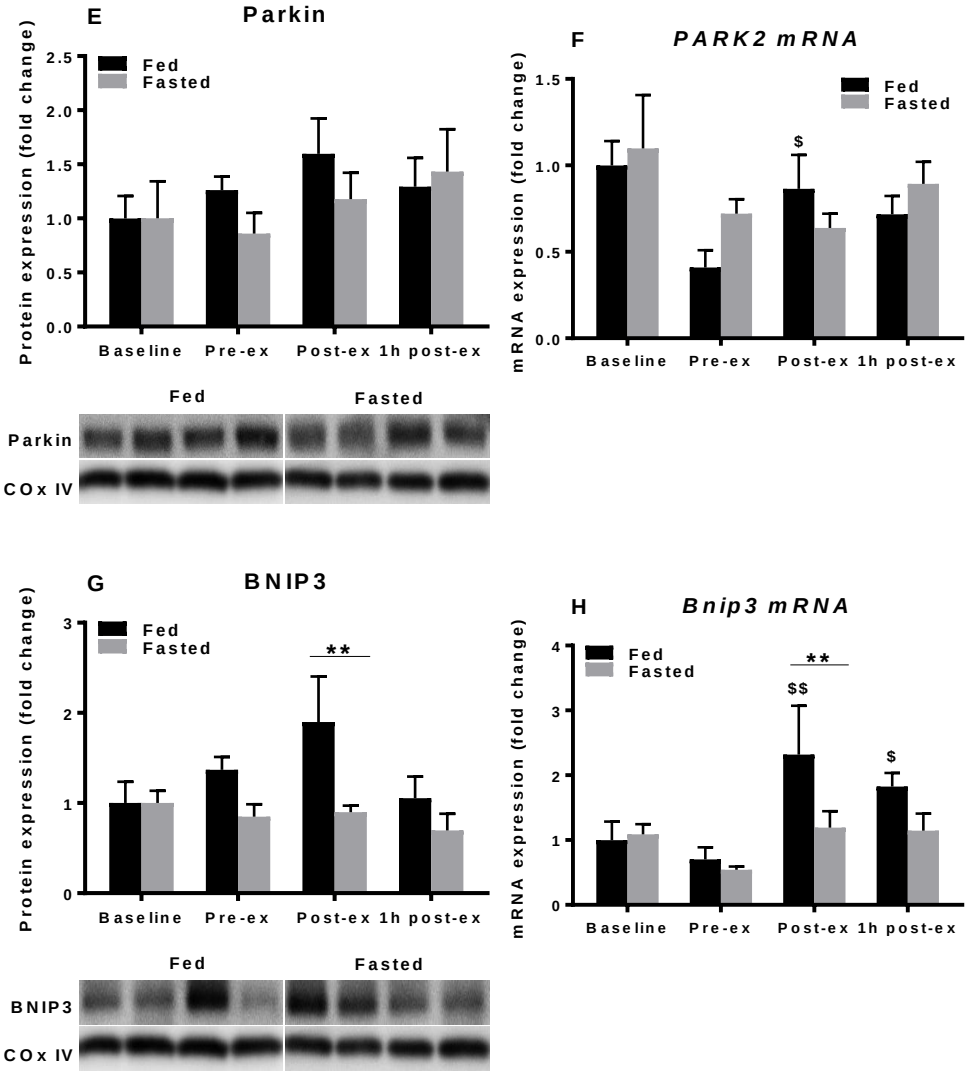
Figure 3. Regulation of fission markers by exercise and the nutritional state in human skeletal muscle. Phosphorylation of mitochondrial (A) and cytosolic DRP1^{Ser616} (B), mRNA levels of *Drp1* (C), protein expression of Fis1 (D), and mRNA levels of *Fis1* (E) in response to high-intensity concentric endurance exercise at baseline, preexercise, immediately postexercise, and 1 h after exercise in the fasted and fed states. Values are expressed as mean ± SEM. A and B, n = 4; C–E, n = 7. $^{\$}P < 0.05$, $^{\$\$\$}P < 0.001$ vs preexercise (time effect); $^{*}P < 0.05$, $^{**}P < 0.01$ vs fasted (nutrition effect).

Canonical autophagic markers are not activated in the mitochondrial fraction after endurance exercise

Independently of the nutritional state, the mitochondrial protein content of LC3b I was approximately 1.7-fold more elevated immediately postexercise than preexercise ($P < 0.01$, Fig. 4*A*). In accordance with increased LC3b I and stable LC3b II levels postexercise (Fig. 4*B*), the LC3b II/I ratio was decreased immediately after exercise ($P < 0.001$, ANOVA; Fig. 4*C*). More specifically, the ratio decreased by a half postexercise compared with preexercise in the fasted conditions ($P = 0.019$). In addition, at

preexercise, the LC3b II/I ratio tended to be different between the fed conditions, after having received a breakfast, and the fasted conditions (*P* = 0.095). The mitochondrial level of p62/SQSTM1, however, was unvarying throughout the trial (Fig. 4*D*).

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Parkin
Protein expression (fold change)
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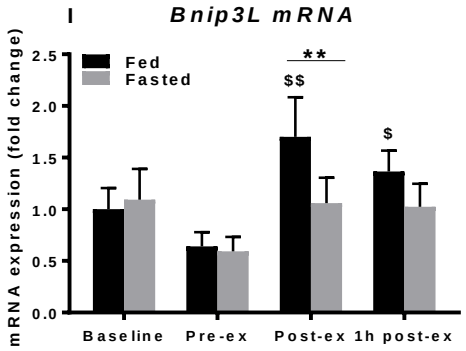


Figure 4. Regulation of mitophagy markers by exercise and the nutritional state in human skeletal muscle. Protein expression of LC3b I (A), LC3b II (B), LC3b II/I ratio (C), p62/SQSTM1 (D), Parkin (E), mRNA levels of *Park2* (F), protein expression of BNIP3 (G), and mRNA levels of *Bnip3* (H) and *Bnip3L* (I) in response to high-intensity concentric endurance exercise at baseline, preexercise, immediately postexercise, and 1 h after exercise in the fasted and fed states. Values are expressed as mean ± SEM. A–I, $n = 7$. $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ vs preexercise (time effect); $^{*}P < 0.05$, $^{**}P < 0.01$ vs fasted (nutrition effect).

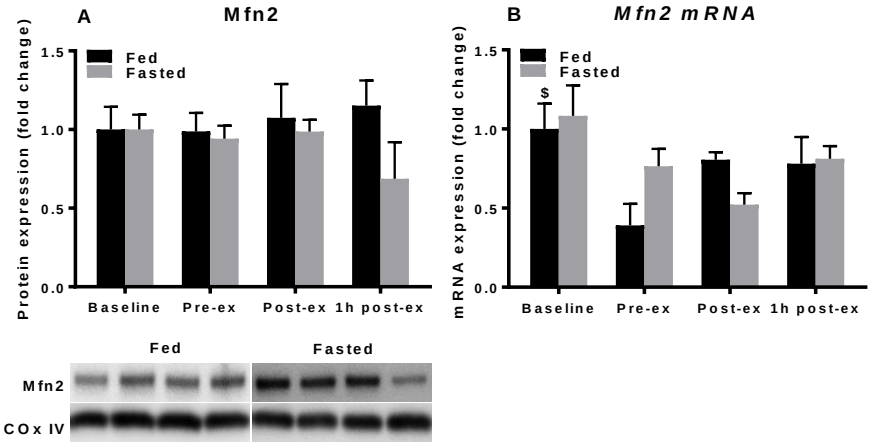
BNIP3 expression is increased whereas Parkin is not affected in response to acute endurance exercise in the fed state

No changes in mitochondrial Parkin amount were detected as a result of exercise or the nutritional state (Fig. 4*E*). Nonetheless, in the fed conditions, Park2 mRNA levels were approximately 50% higher at baseline

and postexercise compared with preexercise (P < 0.05, Fig. 4*F*). The presence of BNIP3 in the mitochondrial fraction tended to increase postexercise in the fed conditions only (P = 0.080), resulting in a twofold higher expression in fed compared with fasted state (P = 0.003, Fig. 4*G*). Accrued mitochondrial content of BNIP3 was associated with elevated *Bnip3* and *Bnip3L* mRNA levels in the fed state postexercise (P < 0.01) and 1 h postexercise (P < 0.05), reaching a twofold difference with the fasted conditions postexercise (P < 0.05, Fig. 4*H* and *I*).

Markers for mitochondrial fusion are not modified by endurance exercise or the nutritional state

Preexercise Mfn2 mRNA levels were lower compared with baseline in the fed state only (P = 0.019, Fig. 5*B*), whereas no variations in Mfn2 (Fig. 5*A*) and OPA1 (Fig. 5*C*) protein expression were observed in any condition, suggesting that fusion was not altered either by exercise or by the nutritional state.


A
Mfn2
Protein expression (fold change)
1.5
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Post-ex
1h post-ex
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B
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mRNA expression (fold change)
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Pre-ex
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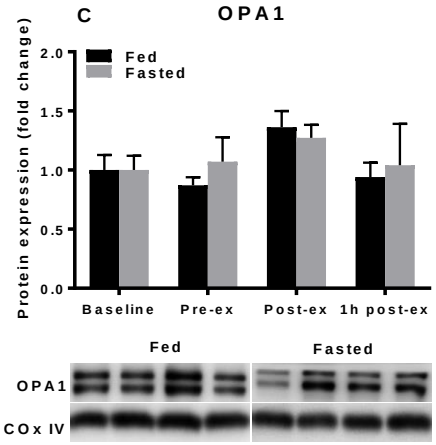
C
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Protein expression (fold change)
2.0
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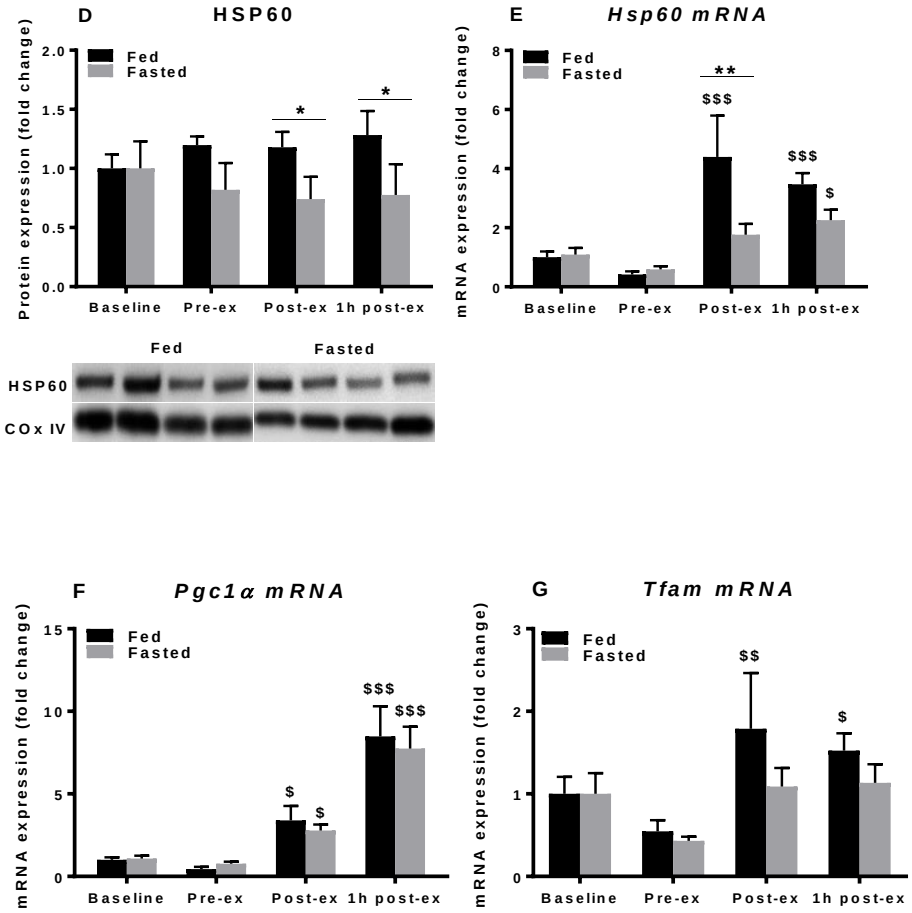

Figure 5. Regulation of fusion and mitogenesis markers by exercise and the nutritional state in human skeletal muscle. Protein expression of Mfn2 (A), mRNA levels of *Mfn2* (B), protein expression of OPA1 (C), HSP60 (D), mRNA levels of *Hsp60* (E), *PGC1α* (F), and *Tfam* (G) in response to high-intensity concentric endurance exercise at baseline, preexercise, immediately postexercise, and 1 h after exercise in the fasted and fed states. Values are expressed as mean ± SEM. A, B, D–G, *n* = 7; C, n = 4. $^{\$}P$ < 0.05, $^{\$\$}P$ < 0.01, $^{\$\$\$}P$ < 0.001 vs preexercise (time effect); $^{*}P$ < 0.05, $^{**}P$ < 0.01 vs fasted (nutrition effect).

The nutritional state influences endurance-induced mitochondrial biogenesis

An indirect marker of mitochondrial biogenesis is the HSP60, which belongs to the matrix chaperones of the mitochondrial protein folding system (Voos and Rottgers 2002). HSP60 protein expression was higher in the fed state than that in the fasted state immediately (P = 0.041) and 1 h (P = 0.020) after exercise (Fig. 5*D*). Similarly, *Hsp60* mRNA levels was higher in the fed state than that in the fasted state immediately after exercise (P = 0.003, Fig 5*E*). In addition, compared with preexercise levels, *Hsp60* mRNA was higher immediately postexercise (P < 0.001) and 1 h postexercise (P = 0.001) in the fed state and 1 h postexercise in the fasted state (P = 0.044). *PGC1α* mRNA levels increased after exercise (P < 0.05), independently of the nutritional state (Fig 5*F*). Following a similar pattern of regulation as *HSP60* mRNA, the mRNA levels of Tfam increased after exercise only in the fed group (P < 0.05, Fig. 5*G*). Together our results show that markers for mitogenesis were upregulated after exercise, more in the fed state compared with the fasted state.

Discussion

This study is the first to analyze the regulation of the fission, mitophagy, and fusion processes by endurance exercise in the mitochondrial fraction of human muscle biopsies. Contrary to our hypothesis, which was based on the activation of autophagy in human skeletal muscle during high-intensity endurance exercise (Schwalm et al. 2015), the results indicate that

mitophagy is not activated during the same exercise protocol, and even inhibited while exercise is performed in a fasted state. This provides arguments in favor of a differential regulation of autophagy and mitophagy in skeletal muscle during exercise.

Regulation of fission

During an acute bout of high intensity endurance exercise, increased fission markers were found in murine skeletal muscle, concurrently with decreased fusion markers (Ding et al. 2010). Forasmuch as these changes were all the most evident when the trial was prolonged, a redistribution of the mitochondrial network was thought to occur within muscle cell in response to higher local energy needs. Here, we quantified DRP1^{Ser616} phosphorylation in the mitochondrial fraction to assess fission as activation of DRP1 is a mandatory process for mitochondrial fission to take place. Indeed, DRP1 controls the final step by splitting the membrane and leading to the birth of daughter mitochondria (Twig et al. 2008). In our experiment, mitochondrial DRP1^{Ser616} was increased postexercise and 1 h postexercise in the fed state only, which suggests that fission was activated during exercise itself in a nutrition-dependent way. Interestingly, 1 h postexercise, cytosolic DRP1^{Ser616} was upregulated only in the fasted state, which might underline a delayed effect of exercise on fission protein kinetics in that nutritional state. DRP1^{Ser616} is initially phosphorylated in the cytoplasm before migrating to the mitochondria (Taguchi et al. 2007). It can therefore be hypothesized that the regulation of fission in the fasted state is somewhat delayed compared with the fed state. Unfortunately, no biopsy

was taken beyond 1 h postexercise, and this hypothesis cannot be tested. Supporting the increase in mitochondrial DRP1^{Ser616} during exercise in the fed state, we also observed an elevated mitochondrial content of Fis1 protein during the recovery period only in the fed state. Fis1 is known to instigate a molecular network favoring a fission process directed preferentially toward dysfunctional mitochondria rather than fragmented ones (Gomes and Scorrano 2008). Our results suggest therefore a role of endurance exercise in the mitochondrial quality control. Nevertheless, it is surprising to observe that Fis1 increases only when exercise is performed in a fed state. This could reflect a protective mechanism of the cell to avoid disposal of damaged mitochondria when the nutrient availability is reduced (fasting) and the energy expenditure increased (exercise).

In summary, as phosphorylation of mitochondrial DRP1^{Ser616} and appearance of Fis1 in the mitochondrial compartment were concomitant in the fed state, our results support an increased mitochondrial fission during high intensity exercise selectively in that nutritional state.

Regulation of mitophagy

Because we previously reported an activation of autophagy in the same condition (Schwalm et al. 2015), we hypothesized that mitophagy would be activated during endurance exercise and more in the fasted state compared with the fed state. The presence of autophagosomes in the mitochondrial compartment is depicted by LC3b II, whereas the LC3b II/I ratio is an indicator of autophagosome turnover, which must be

interpreted together with p62/SQSTM1 changes. LC3b protein is involved in the turnover of autophagosomes from their formation to their lysosomal degradation, whereas p62/SQSTM1 is only associated with later stages of autophagy, i.e., with autophagosomes removal. Thus, the analysis of upstream and downstream markers of autophagy process helps to better understand whether autophagy is directed specifically to mitochondria. In our experiment, LC3b II did not change, whereas the LC3b II/I ratio was decreased immediately postexercise in the fasted state, p62/SQSTM1 remaining unchanged. This means that mitophagy was globally unchanged during our experiment except during exercise in the fasted statewhere it was decreased (decreased LC3b II/I and unchanged p62/SQSTM1). These conclusions do not corroborate our initial hypothesis but are compatible with the lack of fission activation as the latter is known to be permissive for mitophagy (Twig et al. 2008). Nevertheless, similarly to fission, we may not rule out any activation of mitophagy in the hours after the exercise.

The selectivity of mitophagy relies in particular on Parkin and BNIP3 effectors (Romanello et al. 2010). Like Fis1, a higher content of BNIP3 in the mitochondrial compartment after exercise in the fed state constitutes an argument in favor of the formation of a selective molecular network dictated by the nutritional state. In other words, even if mitophagy does not seem to be activated during and 1 h after a high-intensity endurance exercise in human, muscle cells seemed to prepare mitochondria for lysosomal degradation in conditions where nutrients availability was not impaired. This regulation in the fed state is reinforced by augmented *Drp1*,

Bnip3, and *Bnip3L* mRNA levels after exercise, showing that the transcriptional mitophagic program was activated as well. Parkin is another key mediator of mitophagy. It has been advanced to be phosphorylated by PTEN-induced kinase 1 at serine 65 to onset mitophagy upon mitochondria membrane depolarization (Kondapalli et al. 2012, Shiba-Fukushima et al. 2012). Hence, we cannot exclude that the phosphorylation state of Parkin might have been modified without displaying changes in total protein content in the outer membrane of the mitochondria. Unfortunately, currently, no commercially available phosphoantibody is validated in human. Whether DRP1 and Parkin can regulate mitophagy independently is still under debate. Parkin is supposed to be dispensable for mitophagy in the presence of DRP1 (Kageyama et al. 2014), whereas both DRP1-dependent fission and Parkin recruitment have been shown to be involved in BNIP3-induced mitophagy in cardiac myocytes (Lee et al. 2011).

Forkhead box O 3a (FoxO3a) regulates the transcription of *Bnip3* and *Bnip3L* genes in skeletal muscle (Mammucari et al. 2007). As we previously documented that our protocol activated FoxO3a in the fasted state (Schwalm et al. 2015), we expected to observe increased mRNA levels of *Bnip3* and *Bnip3L* in similar conditions. Contrarily, an increase in *Bnip3* and *Bnip3L* mRNA with exercise was found after food intake, but no variations were spotted with fasting. These results contrast with those of Jamart et al. (Jamart et al. 2012) in human, as the transcription of those genes was augmented after ultraendurance exercise, either ensuing from nutrients stocks in deficit or owing to muscle damages after eccentric muscle

contractions. Here, any implication of high-energetic stress or muscle damage in the regulation of *Bnip3* and *Bnip3L* after exercise may be ruled out as the upregulation was specific to the fed conditions and cycling implies mainly concentric muscle contractions. It remains to determine the mechanisms responsible for the selective increased expression of *Bnip3* and *Bnip3L* in the fed state. BNIP3 is needed for autophagosomes generation, helping the processing of LC3b I to LC3b II (Mammucari et al. 2007). Regardless of enhanced mitochondrial BNIP3 content after exercise in the fed state, mitochondrial LC3b II was not increased, and mitochondrial p62/SQSTM1 was not attenuated, suggesting that the basal autophagic flux might have been likely sufficient to remove any undesirable mitochondria, if there is any. Besides, increased LC3b I amounts in the mitochondrial fraction is somewhat surprising because this precursor form of LC3b is supposed to be located in the cytosol (Kabeya et al. 2000). This cannot originate from any contamination of the mitochondrial fraction by the cytosolic fraction because we confirmed the high purity of both subcellular extracts. Interestingly, we previously found that autophagy was activated in whole cell extracts following the same protocol (Schwalm et al. 2015). It seems therefore that the mitochondrial fraction does not reflect what happens at the whole cell level. These observations are compatible with Frank et al. (Frank et al. 2012), who evidenced that specific degradation of mitochondria did not rely on increased autophagic flux, but was triggered by fission activation. In that study, mitophagy was triggered by a mild and transient oxidative stress, resembling the one encountered during endurance exercise. Moreover, exercise-induced production of

reactive oxygen species may account for the augmented presence of Fis1 protein we found in the mitochondrial fraction, Fis1 targeting preferentially mitochondria that become dysfunctional (Gomes and Scorrano 2008).

The nutritional state influences the exercise-induced mitochondrial biogenesis

Either no change or a marked decrease of fusion at the mRNA level during endurance exercise has been reported in murine skeletal muscle (Ding et al. 2010, Jamart et al. 2013). The various exercise durations and intensities chosen probably account for these controversial data. Conversely, transcription of fusion-related genes was importantly and durably stimulated during the hours after the trial (Ding et al. 2010). Here, in human, cycling endurance exercise did not enhance Mfn2 or OPA1 expression at short term, regardless of the nutritional state. Mfn2 expression was not modified 24 h after a similar modality of exercise either (Cartoni et al. 2005). The only variations previously detected in mitofusins were found at the transcriptional level 24 h after exercise (Cartoni and Lincoln 2005). This delayed activation of the fusion program after exercise was assumed to be controlled by mitochondrial biogenesis, via upregulation of *PGC1α* (Cartoni et al. 2005). Although we confirmed an increase in *PGC1α*, *Tfam*, and *Hsp60* mRNA early during the recovery period after exercise, we were not in position to corroborate the reliance of fusion on those markers of mitochondria biogenesis as we did not take samples a few hours or the day after exercise.

AMPK has been described to regulate PGC1α via SIRT1-dependent deacetylation of PGC1α during fasting and exercise (Canto et al. 2010). The increase in *PGC1α* mRNA observed during exercise, independently of the nutritional state, fits with AMPK activation previously confirmed in our exercise protocol (Schwalm et al. 2015). Although AMPK activation was not influenced by the nutritional state, the markers for mitogenesis were globally more increased when the exercise was performed in the fed state. We have no definitive explanation for this observation, but here again the nutritional state enhanced mitochondrial adaptation induced by exercise, which, together with the regulation observed at the fission level, seems to indicate a higher remodeling rate in the fed state. Although no ultimate conclusion may be drawn, this observation challenges the relevance of training in the fasted state for increasing muscle mitochondrial content, which is commonly used by athletes. It also brings an argument in favor of a decoupling between the processes regulating mitophagy and mitochondrial biogenesis. Indeed, in the light of our results, mitophagy does not seem mandatory to increase key markers of mitochondrial biogenesis. Importantly, no changes in the mRNA levels of *cytochrome c oxidase subunit I*, *cytochrome b*, and *ATPase subunit 6* were detected in any condition, suggesting that the transcriptional regulation of mitochondrial biogenesis was not modified during the early recovery in our conditions (data not shown). These results were expected, as the first hour after exercise seems a too short period to observe any change in mitochondrial content.

In conclusion, the present study provides the very first evidence that mitophagy is not activated during and early after high-intensity endurance exercise in human, whatever the nutritional state and despite a selective activation of fission in the fed state. However, when nutrient availability is optimal, muscle cells seem capable of preparing mitochondria for lysosomal degradation. Thus, we may not exclude an activation of mitophagy at a later stage after exercise.

Chapter 5: Discussion, limitations, perspectives and conclusion

1. Role of autophagy, mitophagy and mitogenesis during acute endurance exercise in human skeletal muscle

1.1. Regulation of autophagy by acute endurance exercise and nutrition

Chronic increase in autophagy reported in various muscle atrophy models is considered as a deleterious mechanism challenging muscle mass (Grumati and Bonaldo 2012). On the contrary, transitory activation of autophagy in response to exercise is suggested to be beneficial to improve muscle metabolism, notably in the context of insulin resistance (Yang et al. 2010). Indeed, autophagy is activated in response to strenuous or ultra-endurance running exercise in skeletal muscle, probably to prevent the accumulation of damaged proteins induced by repeated eccentric contractions. A second hypothesis would be that autophagy activation would provide alternative substrates in conditions of high and prolonged energy demand (Jamart et al. 2012, Jamart et al. 2012, Jamart et al. 2013). Yet, studies performed in mouse reported that autophagy is also activated by insulin, growth factors or prolonged fasting *per se*, suggesting that mechanic stress is not necessary to activate autophagy during exercise (Kahn et al. 2005, Schiaffino and Mammucari 2011). Interestingly, combination of endurance exercise with fasting accentuated autophagy activation in mice (Jamart et al. 2013). In the study presented in chapter 2, we assumed that autophagy would be activated by concentric endurance

exercise, and even more when exercise was performed under nutrient deprivation. To test this hypothesis, male athletes performed a single bout of cycling endurance exercise at low (LI) or high intensity (HI), either in a fed or a fasted state, while a control group stayed at rest all along the trail in both nutritional conditions. Fasting duration was selected on the basis of the study of Emberson and collaborators, who showed that the lowest concentrations of insulin, glucose and triglycerides is expected after 6 to 8h-overnight fasting (Emberson et al. 2002). The plasma glucose and insulin concentrations observed in the fasting state remained within the range of physiological values expected in healthy subjects, since the fasted control group had an insulinemia below 20 mU/L and a glycemia below 100 mg/dl (data not shown)(American Diabetes 2018). Importantly, those values remained unchanged in fasted subjects all along the trial.

The *vastus lateralis* muscle was preferentially chosen for biopsies due to its mixed fiber type composition, accessibility, trainability and low pain sensitivity (Staron et al. 2000). The transfer to humans of conclusions based on experiments using animals as an exercise model should be done with caution. Running is often the exercise model used in mice. However, it must be kept in mind that running biomechanics of mice differs from humans, and consequently, the type and the way muscles are recruited differs between the two models. In the frame of our study, fiber type and recruitment during cycling exercise in humans cannot be pertinently compared to running exercise in mice.

Exercise studies conducted in mouse select the type of muscles analyzed according to their fiber composition, which differs in metabolic, contractile and structural properties. For instance, *soleus* muscle is predominantly composed of type I oxidative, fatigue-resistant fibers, and is therefore more prone to be recruited for endurance exercise than other muscles (Bloemberg and Quadrilatero 2012). Voluntary exercise training fosters basal autophagy flux and expression of autophagy proteins in *plantaris* muscle of mixed fiber types in mice, but only increases autophagy protein level in oxidative *soleus* muscle (Lira et al. 2013). Because the subjects recruited for our study were endurance-trained athletes, their *vastus lateralis* muscle probably contained a higher percentage of oxidative, fatigue-resistant fibers, as type I and type IIa fibers (Egan and Zierath 2013). To check adequately whether autophagy activation is dependent on muscle fiber type, one should ensure that all muscle fibers are recruited during exercise. This is only possible when high levels of strength and speed are developed by well-trained subjects during a resistance exercise, but not during endurance exercise. Moreover, exercise intensities chosen for our trial are known to favor the recruitment of type I and type IIa fibers (Egan and Zierath 2013).

We observed that autophagy was activated by cycling endurance exercise, but was not influenced by fasting, as confirmed by the control group. Activation of $Ulk1^{Ser317}$, upstream effector of LC3b II, together with reduced p62/SQSTM1 level, downstream effector of LC3b II, helped us to reliably interpret decreased LC3b II/I ratio as a higher degradation of

autophagosomes. Despite LC3b II/I ratio is commonly assessed to feature autophagosome abundance, its reliability may be questioned because it is involved from autophagosome formation to their degradation. In our experiment, either increased or decreased LC3b II/I ratio would mean that autophagy is activated, when compared to higher Ulk1^{Ser317} expression. However, the state of progress of autophagy would slightly differ depending on the one or the other observation. Indeed, increased Ulk1^{Ser317} and LC3b II/I ratio would reflect activation of early steps of autophagy, followed by higher autophagosome presence. On the other hand, increased Ulk1^{Ser317} amounts and decreased LC3b II/I ratio (as noticed in our study) would be interpreted as higher degradation of autophagosomes subsequent to initiation of autophagy. Here, it may be considered that our measures were obtained at later stages of the autophagy process.

To investigate by which signaling mechanisms endurance exercise mediated autophagy activation, effectors of autophagy signaling pathways sensitive to nutrient availability and exercise, were analyzed. Our results revealed that autophagy activation occurred in parallel to the activation of AMPK signaling, proportionally to the intensity of exercise. Our data corroborated previous reports mentioning the relationship between AMPK activation and exercise intensity in human skeletal muscle (Wojtaszewski et al. 2000, Stephens et al. 2002, Chen et al. 2003, Wojtaszewski et al. 2003, Egan et al. 2010). However, in human skeletal muscle, it has never been

demonstrated that autophagy activation relied on exercise intensity before.

Capable to sense cellular energy deficit, through increased [AMP/ATP] and glycogen depletion, AMPK was expected to be more activated with nutrient deficit, and so do autophagy. Indeed, autophagy was reported to be driven by circadian rhythm in murine skeletal muscle, where increased basal autophagy inversely correlated with feeding periods of rodents (Ma et al. 2011). Neither AMPK, nor autophagy, were activated in subjects at rest and in a fasted state within the duration of our experimental protocol (12h-fasting). Actually, AMPK and autophagy were even not affected in 72-fasted human biopsies, meaning that nutrient deprivation may not be the main trigger of AMPK-dependent autophagy in human skeletal muscle (Vendelbo et al. 2014). In HI-exercised group, AMPK was as well activated in the fasted as in the fed state. Yet, given that subjects were all experienced triathletes or cyclists, they probably benefit from higher muscle energy stores, which optimally deliver fuel sources, even during prolonged exercise in the absence of exogenous food intake (Costill et al. 1977, Essen et al. 1977, Greiwe et al. 1999). In this view, trained subjects are certainly less sensitive to nutrient depletion than sedentary ones to maintain exercise. Stable insulinemia and glycemia were observed in fasted condition all along the trial, suggesting that glycogen may not be the sole source of energy during exercise in the fasted state. Indeed, subjects cycled at duration and exercise intensities where the contribution of carbohydrates and fatty acids is nearly equivalent (Egan and Zierath 2013).

Furthermore, elevated ACC^{Ser79} expression is an additional argument in favor of the use of fatty acids during the trial. Indeed, phosphorylation and subsequent inhibition of $ACC1^{Ser79}$ by AMPK, blocks fatty acid synthesis and leads to their oxidation (Lage et al. 2008).

Alternatively to AMPK, PPARβ/δ is positively regulated during nutrient scarcity, including in human skeletal muscle: PPARβ/δ targets *pyruvate dehydrogenase kinase* (*PDK*) gene to shift muscle substrate utilization from glucose to fatty acids (Phua et al. 2018). During endurance exercise, PPARδ agonists are also reported to be augmented, likely via AMPK. For now, exercise-mediated increase in AMPK has only been observed to stimulate PPARδ expression at the transcriptional level, but not at the protein level (Watt et al. 2004, Mahoney et al. 2005). PPARδ agonists are thought to increase PGC1α protein expression, the latter inducing the transcription of PPARs-governed genes implicated in fatty acid oxidation (Phua et al. 2018). Furthermore, PGC1α is suspected to be a key mediator of exercise-induced autophagy, as revealed by PGC1α-KO mice, which shows blunted autophagy activity in response to exercise, as well as lessened transcription of autophagy-related genes (Vainshtein et al. 2015, Vainshtein et al. 2015, Halling et al. 2016). Even so, direct implication of PPARδ/PGC1α signaling in exercise-induced autophagy remains to clarify, particularly in human skeletal muscle.

Autophagy has previously been reported to be directed towards lipids (lipophagy) in many cell types (Singh and Cuervo 2012). In skeletal muscle, excessive lipophagy was even observed in mice exposed to high sugar diet

or in the context of lipid storage diseases in human (Angelini et al. 2016, De Stefanis et al. 2017). Lipophagy was proposed to compensate for excessive accumulation of fatty acids and lipotoxic molecules involved in insulin resistance. Moreover, a very recent report observed that a ketogenic amino acid rich replacement ameliorated high-fat diet induced impairment of autophagy in mice muscle, suggesting a role for ketone bodies in autophagy regulation (Li et al. 2018). For now, in physiological models such as exercise, we are unaware of studies dealing with lipophagy, or showing that fatty acids or ketone bodies could regulate global autophagy in human skeletal muscle. At the moment we designed our experimental protocol, insulin was known to regulate autophagy in skeletal muscle, but not fatty acids or ketone bodies. That is why we assessed insulin concentration instead of measuring fatty acids or ketone bodies levels.

Besides cellular ATP rate, another potential stimulus for AMPK activation during our exercise protocol is the rise of intracellular Ca^{2+}due to the contractile activity of skeletal muscle. Indeed, Ca^{2+} activates Ca^{2+}/calmodulin-dependent protein kinase kinase α (CaMKKα), which, in turn, phosphorylates $AMPK^{Thr172}$ (Richter and Ruderman 2009). The cytosolic release of Ca^{2+} from the endoplasmic reticulum was demonstrated to promote autophagy (Decuypere et al. 2011). However, whether Ca^{2+} released from sarcoplasmic reticulum (*i.e.* muscle Ca^{2+} tank) may have a direct role in autophagy activation in skeletal muscle is unclear.

Our exercise protocol also initiated the transcriptional autophagic program, since HI exercise groups exhibited an increased expression of

autophagy-related genes, especially when nutrient availability was optimal. Surprisingly, that transcriptional regulation of autophagy partially coincided with the activation of FoxO transcription factor, which was only activated in the fasted state after HI exercise. Two of the phosphorylation sites of FoxO analyzed (FoxO1^{Thr24} and FoxO3a^{Thr32}) are downstream targets of Akt (Brunet et al. 1999). Any implication of the Akt axis in autophagic transcription may be rejected because Akt^{Ser473} was not phosphorylated in the fasted state after HI exercise. Those findings stand out from the ones identified in exercised mice, where AMPK-governed activation of FoxO3 was noticed in optimal nutrient availability (Pagano et al. 2014).

p38 MAPK and NFkβ may be other potential mediators of autophagy at the transcriptional level, even though their role is probably predominant in pathologic states such as cachexia and sepsis (McClung et al. 2010, Mofarrahi et al. 2012).

TFEB has recently gained further attention as a transcriptional regulator of autophagy, independent of canonical autophagy pathways. TFEB was found to drive the expression of autophagic and cells lysosomal genes in response to starvation (however *in vitro*), as well as in skeletal muscle of exercised mice, through exercise-induced calcium influx (Settembre et al. 2011, Medina et al. 2015). To our knowledge, no study focused on the role of TFEB during fasting or exercise in human skeletal muscle yet. Interestingly, we found two genes which are under the control of TFEB to be more expressed during and shortly after exercise: *GabarapL1* and *p62* (Palmieri, 2011). Thus, because TFEB seems to be activated when cellular

energy needs are challenged (for instance, during endurance exercise) or in response to increased calcium levels (for instance, with muscle contraction), it is conceivable that TFEB would be brought into play to transcribe autophagy-related genes analyzed in our experiment.

The study described in chapter 2 demonstrated for the first time that autophagy markers are increased in parallel to changes in AMPK phosphorylation state and exercise intensity in human skeletal muscle. This suggests that autophagy could be regulated by AMPK during cycling exercise. Furthermore, it brings into question the stimuli behind AMPK and autophagy activation commonly admitted in mammals, as autophagy and AMPK activation were observed to be poorly sensitive to nutrient deficit in human skeletal muscle. However, the mechanisms underlying the transcriptional activation of autophagy during exercise remain to be elucidated in human. The more recent findings suggest that AMPK or TFEB signaling deserve to be further explored. Lastly, we confirmed that conventional form of exercise, which does not induce excessive mechanical or metabolic stress, is able to activate autophagy. This suggests that adapted exercise can even benefit to patients with altered muscle structure or function. In this view, adapted physical activity may be considered as a potential tool to restore impaired autophagy in myopathies or metabolic disorders like insulin resistance or obesity.

1.2. Regulation of mitophagy by acute endurance exercise and nutrition

Based on our first findings that autophagy was activated during and early after conventional endurance exercise in human skeletal muscle, we assumed that mitophagy would be transitorily activated as well in the same exercise protocol. In particular, we postulated that mitophagy would be preferentially activated when exercise was performed in the fasted state. We exploited remaining biopsies samples obtained in HI- exercised subjects during fed and fasted sessions, where mitochondrial proteins were isolated. Mitophagy was not activated during and early after HI endurance exercise. Fission, the prerequisite step of mitophagy, as well as the transcriptional program of mitophagy were both stimulated during and in the hour following endurance exercise in conditions of optimal nutrient availability. In contrast, mitophagy was not affected by fasting, even when that nutritional state was associated to HI exercise. Canonical markers of autophagy measured in the mitochondrial fraction were not upregulated by exercise, feeding or fasting, attesting that mitochondria were not the main cellular component targeted by autophagic degradation. This independent regulation of autophagy and mitophagy within the same experimental conditions was previously observed in *vitro*: fission-dependent mitophagy was induced in the absence of global autophagy (Frank et al. 2012). Here, we showed that the opposite phenomenon may also occur: an increased autophagy free of mitophagy activation at short-term.

Even though mitophagy is often cited as a protective mechanism set up when mitochondria integrity is seriously jeopardized, it has been evidenced that a moderate and transient oxidative stress, like the one which can be met during endurance exercise, is sufficient to stimulate mitophagy in human HeLa cells (Frank et al. 2012). To the best of our knowledges, this has not been reported in human myotubes. Explaining the regulation of mitophagy in a whole organism *in vivo* is more complex than in isolated cultured cells. Our protocol and exercise and nutrition failed to activate more mitophagy at short-term compared to baseline because it might not entail a high enough metabolic stress (ROS overproduction, disruption of mitochondrial potential, critical depletion of energy substrates) and severe mitochondrial damages (ROS-induced structural alterations of mitochondrial membranes and mtDNA). In other words, basal mitophagy of healthy athletes was probably efficient enough to maintain mitochondria integrity during HI endurance exercise or fasting.

The regulatory mechanisms driving the transcriptional activation of mitophagy during exercise remain to be elucidated. We already excluded any implication of FoxO3 in the chapter 3. Despite FoxO3 is known to be closely connected to BNIP3 expression during fasting-induced autophagy in skeletal muscle of mice (Mammucari et al. 2007), this seemed not to be the case in our experiment, even though we based on indirect observations of FoxO3 variations. Indeed, FoxO3 was activated with exercise in the fasted state while *Bnip3* and *Bnip3L* expressions were heightened in response to exercise in the fed state only.

Even if we mentioned the potential role of TFEB in autophagy regulation during exercise in the section above, to the best of our knowledge, direct connection of TFEB with mitophagy signaling have not been ascertained yet (Palmieri et al. 2011, Romanello and Sandri 2015).

PINK/PARKIN signaling is an alternative axis to induce mitophagy. The role of PINK1/PARKIN has been first identified in pathological models: for instance, mutations of PINK1/PARKIN genes are responsible for neurodegenerative diseases, and a very recent study also highlighted their involvement in skeletal muscle atrophy (Peker et al. 2018). Nonetheless, whether PINK1/PARKIN axis is essential for the regulation of mitophagy in physiological conditions is currently unclear, particularly in human skeletal muscle. Neither fission, nor mitophagy seemed to be influenced by PARKIN in the context of acute endurance exercise, since PARKIN abundance in the mitochondrial fraction did not vary in our exercise protocol. Diet did not impacted on PARKIN amounts either. It is conceivable that we could have missed phosphorylation changes of PARKIN at Ser65 because the specific anti-body was not commercially available at the time we performed the study. Yet, for now, such phosphorylation has been observed in mammalian cells only, and following genetic or pharmacological induction of mitophagy (Rub et al. 2017). On the other hand, increased presence of $PARKIN^{Ser65}$ in the mitochondrial compartment of our human muscle samples is unlikely, otherwise we should had noticed decreased protein levels of Mfn2, the latter being degraded when PARKIN is recruited to the mitochondrial surface (Narendra et al. 2010).

The present work evidenced a differential regulation autophagy and mitophagy at short-term within the same exercise protocol, where mitochondria are not exclusive targets of the autophagic degradation system. Is there a physiological relevance to remove the main sites of energy supply in skeletal muscle when energy demand is accrued and energy stores depleted? On the other hand, the activation of fission and mitophagic transcriptional program would indicate that mitophagy would come under long-term regulatory mechanisms, contrary to autophagy. In this view, alternative mitophagy control systems as micro-proteases, ubiquitin-proteasome system or mitochondria-derived vesicles might act as the first line of defense to maintain mitochondria integrity during transitory physiological stresses.

1.3. Regulation of mitogenesis by acute endurance exercise and nutrition

Numerous reports stated that endurance exercise stimulated mitogenesis in skeletal muscle (Egan and Zierath 2013). Here, we had clues that mitogenesis preferentially happened when the energy status of muscle cell is optimal during exercise. Indeed, acute HI endurance exercise stimulated the transcription of direct and indirect markers of mitogenesis. Because the mitochondrial translocation of PGC1α and TFam proteins fosters the transcription of mtDNA, the possibility to assess their presence in the mitochondrial compartment would have been helpful to confirm that mitogenesis was definitely induced by acute endurance exercise in the fed state (Safdar et al. 2011). Some studies bases on the ratio between

mitochondrial DNA/nuclear DNA as a complementary measure to determine mitochondrial mass (and therefore mitogenesis); still, the use of this tool needs to be nuanced with proteomic data reporting that the ratio does not reflects mitogenesis protein levels in various tissues, even skeletal muscle has not been assessed yet (Mootha et al. 2003).

We hypothesized in the section 3 of our manuscript that the expression of *PGC1α* could be upregulated by the activation of AMPK in our protocol. Indeed, AMPK-mediated phosphorylation of GATA4 was showed to upregulate *PGC1α* expression in murine skeletal muscle cells (Irrcher et al. 2008). In addition to AMPK, very recent investigations pointed to a role of TFEB in the regulation of mitogenesis genes (Mansueto et al. 2017). If we keep in mind that TFEB seems to intervene when energy needs are accrued, as during HI endurance exercise, TFEB would therefore appear as an attractive candidate driving the transcription of mitogenesis genes during acute endurance exercise (Settembre et al. 2011, Medina et al. 2015).

Finally, we showed that acute endurance exercise globally increased the expression of protein and genes involved in mitochondrial removal and synthesis in the fed state. For all that, we have no evidence that mitochondrial turnover (*i.e.* increased mitophagy associated to increased mitogenesis) is fostered by acute endurance exercise at the post-translational level. However, the transcriptional program of mitochondrial turnover would be activated during nutrient-rich conditions, as suggested

by the higher expression of mitophagy and mitogenesis-related genes with acute endurance exercise.

2. Role of mitophagy, mitogenesis and mitochondrial function in regular endurance exercise in human skeletal muscle

2.1. Mitophagy and regular endurance exercise

Mitochondrial turnover and/or function are suspected to deteriorate during aging. In human skeletal muscle, which of those mechanisms causes mitochondrial aging has not been fully solved. Physical inactivity in elderly would be an additional factor involved in the impairment of mitochondrial health (Johannsen et al. 2008). Conversely, endurance exercise, beyond improving mitochondrial content and metabolism, is emerging as a potential strategy to sustain mitochondrial integrity (Cartoni et al. 2005, Egan and Zierath 2013, Konopka et al. 2014, Schwalm et al. 2017). We hypothesized that, in human skeletal muscle, mitophagy would be impaired with physical inactivity during aging and conversely, the maintenance of regular physical activity would help to maintain mitochondria quality control. Biopsies were obtained from young and old subjects who have been sedentary or physically active for at least 10 years.

In the present work, we demonstrated that both acute (chapter 3) and repeated (chapter 4) endurance exercise enhances protein markers for fission in the mitochondrial fraction of human skeletal muscle. However, fission did not seem affected by a sedentary lifestyle during aging. Acute endurance exercise did not upregulate mitophagy markers in young

athletes (chapter 3) within the first hour after exercise completion. The up-regulation of *Bnip3* and *BnipL3* mRNA led us to consider the regulation of mitophagy at a longer-term, *i.e.* in response to regular exercise and during aging. As reported in chapter 4, the expression of markers of mitophagy BNIP3, LC3b and p62/SQSTM1, quantified in the mitochondrial fraction, were only slightly affected by regular physical activity and age. We detected an increased presence of PARKIN in the mitochondrial fraction of active subjects, independently of their age. These data are in line with a very recent study, where phospho-PARKIN^{Ser65}, which is recruited to mitochondria to induce mitophagy, was found to be augmented following endurance training, despite it was measured in total extracts of muscle biopsies (Kondapalli et al. 2012, Tarpey et al. 2017). Therefore, we may not rule out that mitophagy is activated in the hours following exercise, as already observed in mice 6h after acute exercise (Laker et al. 2017), or else in response to repeated endurance exercise bouts, (*i.e.* endurance training) through a PARKIN-dependent regulation.

2.2. Mitogenesis and regular endurance exercise

Endurance exercise is known to stimulate mitogenesis markers in skeletal muscle (Egan and Zierath 2013). Whereas increased *PGC1α* and *Tfam* expression was found in our acute exercise protocol, no difference in *PGC1α*, *Tfam* and *NRF1/2* was noticed at rest between sedentary and trained subjects. This is in line with a previous study, which did not observed any differences in *PGC1α* expression between untrained and trained subjects at rest (Pilegaard et al. 2003). *PGC1α* mRNA expression

was demonstrated to be sensitive to the intensity of exercise, being rapidly and highly increased after the first bout. However, the response was blunted and delayed from 7 weeks of training in spite of adapted power output over the training period (Perry et al. 2010). Thus, the fact that the physically active subjects recruited for our experiment exercised for years may explain why we did not detect any significant changes in *PGC1α* and its downstream targets in comparison to sedentary subjects.

A recent study performed in murine skeletal muscle showed that high-intensity endurance training upregulates *NRF1/2* in a TFEB-dependent fashion, but does not rely on *PGC1α* expression (Mansueto et al. 2017). Besides, exercise-induced TFEB activation did not affect autophagy-related genes, suggesting a dual but independent role of TFEB on mitogenesis and autophagy. In our human protocol, the quantification of TFEB presence in skeletal muscle nuclei by Western-Blot or immunofluorescence analyses would have been interesting to confirm what was observed in mice.

Active subjects displayed higher mtDNA/nDNA than sedentary ones. Because mitochondria have their own DNA, amounts of mtDNA are considered to be proportional to the number of mitochondria; therefore, this ratio is commonly referred as a mitogenesis indicator (Medeiros 2008). Moreover, mtDNA replication and repair is stimulated by increased activity of the nuclear encoded DNA polymerase gamma (PolG) (Krasich and Copeland 2017). Thus, increased mtDNA copy number in active subjects may be associated with improved mitochondria content, enhanced mtDNA replication and preserved mitochondria integrity with long-term endurance

exercise. On the contrary, mitochondrial copy number was not different between young and aged subjects; disputing that mitochondria content, mtDNA replication and mitochondria integrity would be altered during aging in healthy adults. Because mtDNA/nDNA does not reflect mitochondrial function, changes in mitochondria copy number have to be nuanced with assessments of mitochondrial activity, using respirometry or enzymology assays. We assessed COx activity by histochemical analysis, an indirect indicator of mitochondrial activity. In spite of invariant mitochondrial copy number, old sedentary subjects exhibited a lower COx activity than old trained subjects, suggesting that mitochondrial function is lessened with physical inactivity during aging. Conversely, the maintenance of regular physical activity during aging would improve both mitochondrial content and function.

2.3. Mitochondrial functions and regular endurance exercise

Oxidative phosphorylation was documented to be impaired in human during aging (Conley et al. 2000, Tonkonogi et al. 2003). Here, we did not find any effect of age on protein levels of mitochondrial respiratory chain complexes (OXPHOS) or citrate synthase, probably because our subjects were only 67 years old. Nevertheless, regular endurance exercise enhanced the expression of OXPHOS in both young and old subjects, though the effect of regular exercise on enzyme expression and activities was especially visible during aging, since CS expression and COx activity were higher in old active adults than their sedentary counterparts. Although

regular endurance exercise is well identified to improve mitochondrial oxidative phosphorylation, a potential role of TFEB in mitochondrial function is currently emerging in the context of exercise. Indeed, TFEB-transfected muscles of mice exhibited higher expression and activities of OXPHOS. Yet, TFEB deletion only reduced expression and activity of complex II of OXPHOS. In addition, TFEB was necessary for exercise-induced metabolism adaptations (Mansueto et al. 2017). It remains to be determined whether, in human skeletal muscle, TFEB would contribute to improved mitochondrial function in response to exercise, beyond participating in mitochondria integrity.

In the same way as TFEB, PPARβ/δ might be responsible for mediating the expression of mitochondrial enzymes and OXPHOS. Overexpression of PPARβ/δ in mouse skeletal muscle was recently showed to upregulate OXPHOS protein level, before increasing PGC1α protein content (Koh et al. 2017). Those changes were not accompanied by higher *PGC1α* mRNA (Wang et al. 2004). Moreover, PPARβ/δ does not target *TFam* or *NRF1/2* genes (Scarpulla 2008). In accordance with that aforementioned study, we found augmented protein amounts of OXPHOS and CS, without any changes in *PGC1α*, *TFam* and *NRF1/2* mRNA expression. Thus, quantifying PPARβ/δ and PGC1α protein expression would have provided us interesting clues to clarify the hypothetic role of PPARβ/δ in mitochondrial function.

3. Limitations and perspectives

The present work is part of pioneering investigations on autophagy and mitophagy conducted in human skeletal muscle. Even so, as most of human studies interpreting data from biopsy samples only, one major limitation of this model lies in the fact that it inevitably provides indirect evidences on the way exercise influences autophagy and mitophagy. Indeed, we assessed dynamic processes (fission, fusion, mitophagy, autophagy) based on consecutive static measurements obtained at a given time point. The autophagic flux, *i.e.* increased formation of mature autophagosomes combined to augmented degradation of autophagolysosomes, is commonly measured in real-time in living cells or small organisms, using pharmacological agents to stimulate or block autophagy (Klionsky et al. 2016). In human skeletal muscle, the rate of appearance and removal of typical autophagy structures is not possible to assess in real-time. Even if some anti-autophagy drugs are clinically approved in humans (chloroquine, hydroxychloroquine and colchicine) to treat specific diseases (malaria, rheumatological diseases, and gout), they are not devoid of deleterious side-effects, as myopathies. Therefore, their experimental use is debatable (Klionsky et al. 2016). For now, the variety of technical tools used to measure the expression of autophagy effectors from muscle biopsies is quite limited (see section 1.4 of chapter 1), and depict static events only. We are aware that they provide indirect evidences of autophagy progress and cannot reflect directly autophagy activity. Nonetheless, the resort to diverse experimental procedures, among the ones feasible for human studies, permitted us to refine analyses

of the data obtained for a same autophagy marker (in particular for LC3 II, involved in different autophagy stages) and diminishes the probability of misinterpreting the results. Furthermore, the harvest of several biopsy samples over time (in studies described in chapter 2 and 3), even though number-limited for ethical reasons, gave us an indirect overview of the state of advancement of autophagy in response to physiological triggers (as exercise or nutrition). Our future investigations should consider electron microscopy to analyze biopsy samples at different time points. Even though this technique requires a high technical expertise for proper processing of the samples, and a high experience for the correct characterization of autophagy structures, electron microscopy will permit to visualize the morphology of autophagic structures at a high resolution (nanometer scale), allow their correct identification, show the degree of selectivity of autophagy and complement data from quantitative assays (Klionsky et al. 2016). Besides, the use of stable isotope labelling to assess the degradation of autophagy/mitophagy proteins (synthesis/degradation ratio) will be a complementary tool providing comparative data between different physiological conditions (Holm and Kjaer 2010).

A second limitation of our work relates to the design of our exercise protocols. In the study presented in the chapter 2 (autophagy, acute exercise and nutrition), subjects were selected on the basis of their VO_{2peak} but were not matched for workload. However, we confirmed that power output and maximal power output were not different between groups (presented in chapter 2, table 1: subjects characteristics). Secondly, in the

study of chapter 3 (mitophagy, acute exercise and nutrition), it was not possible to include a control group. Indeed, because we used remaining muscle biopsies of the 1st experiment described in chapter 2, the number of muscle samples of the control group was not significant to be compared with the HI-exercised group. Data obtained from HI-exercised subjects cannot differentiate accurately the effects of nutrition, exercise and time, contrary to the autophagy study. Thirdly, despite a similar modality of exercise (cycling) was chosen between the different protocols, exercise intensity was not monitored for the long-term exercise study (chapter 4). We observed that autophagy activation was potentiated when acute exercise was performed at high intensity of endurance, whereas mitophagy seemed not activated. However, we are not aware whether mitochondria remodeling (*i.e.* fission and fusion) and turnover (*i.e.* mitophagy and mitogenesis) are stimulated by repeated sessions of high intensity endurance exercise.

Regarding the two studies assessing changes of mitophagy markers following exercise (chapter 3 and 4), mitochondria integrity following tissue fractionation was estimated on the basis of fraction purity, by Western-Blot analyses. The exclusive presence of specific mitochondrial proteins in the mitochondrial fraction, as well as their absence of the cytosolic compartment, was an argument in favor a preserved mitochondrial structure during mitochondrial isolation procedure. However, we cannot be in position to ascertain that mitochondrial function is intact. Thus, using respirometry assays from small fresh muscle samples, which examine the

correct function of the different mitochondrial respiration states, will help to guarantee that mitochondrial function is unharmed by our protocol of mitochondrial isolation (Boutagy et al. 2015, Boutagy et al. 2015).

The study described in chapter 4, where a single biopsy was obtained, provides a descriptive overview of mitochondrial fate during aging according to physical activity status, which deserves to be further investigated. Considering mitochondrial fate from the angle of a longitudinal study, where multiple muscle samples will be collected over years in a large cohort of sedentary and trained subjects, will help us to better understand the mechanisms of mitochondria ageing in skeletal muscle according to lifelong physical activity. Including groups trained with different modalities of exercise will be useful to design accurate protocols aiming at preserving mitochondria integrity and muscle health.

Lastly, the mechanisms explored during this thesis were described in healthy humans only. It will be an interesting perspective to compare healthy with pathological populations within the same protocol. Could exercise itself be helpful to restore impaired quality control systems in patients? Could patients respond enough to exercise to improve deficient autophagy/mitophagy or supplemental tools would be needed?

4. Conclusion

In the context of chronic diseases and aging, the maintenance of muscle mass and function appears crucial to proper quality of life and exercise constitutes an attractive strategy for physiological muscle remodeling in human. Mechanisms by which exercise induces autophagy and mitophagy were explored in human skeletal muscle. In the present work, we show that acute endurance exercise promotes autophagy probably through the activation of AMPK signaling pathway. In addition, exercised-induce autophagy relied more on exercise intensity than nutrient availability. However, that same protocol of acute endurance exercise did not activate mitophagy during and in the first hour following exercise, despite a selective activation of fission in the fed state. Nonetheless, when nutrient status was optimal, increased transcription of mitophagy-related genes suggested that muscle cells are preparing mitochondria for lysosomal degradation at latter stages after exercise completion. Furthermore, we evidenced that physical inactivity during aging does not necessarily impairs mitochondrial integrity and function in healthy adults. However, endurance training fosters mitochondria quality control and function through improved mitophagy and expression of oxidative enzymes and respiratory chain complexes.

References

Aad, G., B. Abbott, J. Abdallah, A. A. Abdelalim, A. Abdesselam, O. Abdinov, . . . A. Collaboration (2010). "Search for new particles in two-jet final states in 7 TeV proton-proton collisions with the ATLAS detector at the LHC." Phys Rev Lett 105(16): 161801.

Alessi, D. R., M. Andjelkovic, B. Caudwell, P. Cron, N. Morrice, P. Cohen and B. A. Hemmings (1996). "Mechanism of activation of protein kinase B by insulin and IGF-1." EMBO J 15(23): 6541-6551.

Amati-Bonneau, P., D. Milea, D. Bonneau, A. Chevrollier, M. Ferre, V. Guillet, . . . P. Reynier (2009). "OPA1-associated disorders: phenotypes and pathophysiology." Int J Biochem Cell Biol 41(10): 1855-1865.

American Diabetes, A. (2018). "2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2018." Diabetes Care 41(Suppl 1): S13-S27.

Angelini, C., A. C. Nascimbeni, G. Cenacchi and E. Tasca (2016). "Lipolysis and lipophagy in lipid storage myopathies." Biochim Biophys Acta 1862(7): 1367-1373.

Angione, A. R., C. Jiang, D. Pan, Y. X. Wang and S. Kuang (2011). "PPARdelta regulates satellite cell proliferation and skeletal muscle regeneration." Skelet Muscle 1(1): 33.

Arimura, S., J. Yamamoto, G. P. Aida, M. Nakazono and N. Tsutsumi (2004). "Frequent fusion and fission of plant mitochondria with unequal nucleoid distribution." Proc Natl Acad Sci U S A 101(20): 7805-7808.

Arribat, Y., N. T. Broskey, C. Greggio, M. Boutant, S. Conde Alonso, S. S. Kulkarni, . . . F. Amati (2018). "Distinct Patterns of Skeletal Muscle Mitochondria Fusion, Fission and Mitophagy upon Duration of Exercise Training." Acta Physiol (Oxf): e13179.

Ashrafi, G. and T. L. Schwarz (2013). "The pathways of mitophagy for quality control and clearance of mitochondria." Cell Death Differ 20(1): 31-42.

Azzu, V. and M. D. Brand (2010). "Degradation of an intramitochondrial protein by the cytosolic proteasome." J Cell Sci 123(Pt 4): 578-585.

Bach, D., D. Naon, S. Pich, F. X. Soriano, N. Vega, J. Rieusset, . . . A. Zorzano (2005). "Expression of Mfn2, the Charcot-Marie-Tooth neuropathy type 2A gene, in human skeletal muscle: effects of type 2 diabetes, obesity, weight loss, and the regulatory role of tumor necrosis factor alpha and interleukin-6." Diabetes 54(9): 2685-2693.

Balaban, R. S., S. Nemoto and T. Finkel (2005). "Mitochondria, oxidants, and aging." Cell 120(4): 483-495.

Band, M., A. Joel, A. Hernandez and A. Avivi (2009). "Hypoxia-induced BNIP3 expression and mitophagy: in vivo comparison of the rat and the hypoxia-tolerant mole rat, Spalax ehrenbergi." FASEB J 23(7): 2327-2335.

Barth, S., D. Glick and K. F. Macleod (2010). "Autophagy: assays and artifacts." J Pathol 221(2): 117-124.
Bartlett, J. D., C. Hwa Joo, T. S. Jeong, J. Louhelainen, A. J. Cochran, M. J. Gibala, . . . J. P. Morton (2012). "Matched work high-intensity interval and continuous running induce similar increases in PGC-1alpha mRNA, AMPK, p38, and p53 phosphorylation in human skeletal muscle." J Appl Physiol (1985) 112(7): 1135-1143.
Bartlett, J. D., J. Louhelainen, Z. Iqbal, A. J. Cochran, M. J. Gibala, W. Gregson, . . . J. P. Morton (2013). "Reduced carbohydrate availability enhances exercise-induced p53 signaling in human skeletal muscle: implications for mitochondrial biogenesis." Am J Physiol Regul Integr Comp Physiol 304(6): R450-458.
Beltran Valls, M. R., D. J. Wilkinson, M. V. Narici, K. Smith, B. E. Phillips, D. Caporossi and P. J. Atherton (2015). "Protein carbonylation and heat shock proteins in human skeletal muscle: relationships to age and sarcopenia." J Gerontol A Biol Sci Med Sci 70(2): 174-181.
Bento, C. F., M. Renna, G. Ghislat, C. Puri, A. Ashkenazi, M. Vicinanza, . . . D. C. Rubinsztein (2016). "Mammalian Autophagy: How Does It Work?" Annu Rev Biochem 85: 685-713.
Bertaggia, E., L. Coletto and M. Sandri (2012). "Posttranslational modifications control FoxO3 activity during denervation." Am J Physiol Cell Physiol 302(3): C587-596.
Birgisdottir, A. B., T. Lamark and T. Johansen (2013). "The LIR motif - crucial for selective autophagy." J Cell Sci 126(Pt 15): 3237-3247.
Bloemberg, D. and J. Quadrilatero (2012). "Rapid determination of myosin heavy chain expression in rat, mouse, and human skeletal muscle using multicolor immunofluorescence analysis." PLoS One 7(4): e35273.
Booth, F. W., S. E. Gordon, C. J. Carlson and M. T. Hamilton (2000). "Waging war on modern chronic diseases: primary prevention through exercise biology." J Appl Physiol (1985) 88(2): 774-787.
Boutagy, N. E., E. Pyne, G. W. Rogers, M. Ali, M. W. Hulver and M. I. Frisard (2015). "Isolation of Mitochondria from Minimal Quantities of Mouse Skeletal Muscle for High Throughput Microplate Respiratory Measurements." J Vis Exp(105).
Boutagy, N. E., G. W. Rogers, E. S. Pyne, M. M. Ali, M. W. Hulver and M. I. Frisard (2015). "Using Isolated Mitochondria from Minimal Quantities of Mouse Skeletal Muscle for High throughput Microplate Respiratory Measurements." J Vis Exp(105): e53216.
Brunet, A., A. Bonni, M. J. Zigmond, M. Z. Lin, P. Juo, L. S. Hu, . . . M. E. Greenberg (1999). "Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor." Cell 96(6): 857-868.

Bua, E., J. Johnson, A. Herbst, B. Delong, D. McKenzie, S. Salamat and J. M. Aiken (2006). "Mitochondrial DNA-deletion mutations accumulate intracellularly to detrimental levels in aged human skeletal muscle fibers." Am J Hum Genet 79(3): 469-480.
Cantley, L. C. (2002). "The phosphoinositide 3-kinase pathway." Science 296(5573): 1655-1657.
Canto, C., L. Q. Jiang, A. S. Deshmukh, C. Mataki, A. Coste, M. Lagouge, . . . J. Auwerx (2010). "Interdependence of AMPK and SIRT1 for metabolic adaptation to fasting and exercise in skeletal muscle." Cell Metab 11(3): 213-219.
Carroll, R. G., E. Hollville and S. J. Martin (2014). "Parkin sensitizes toward apoptosis induced by mitochondrial depolarization through promoting degradation of Mcl-1." Cell Rep 9(4): 1538-1553.
Cartoni, A. and N. B. Lincoln (2005). "The sensitivity and specificity of the Middlesex Elderly Assessment of Mental State (MEAMS) for detecting cognitive impairment after stroke." Neuropsychol Rehabil 15(1): 55-67.
Cartoni, R., B. Leger, M. B. Hock, M. Praz, A. Crettenand, S. Pich, . . . A. P. Russell (2005). "Mitofusins 1/2 and ERRalpha expression are increased in human skeletal muscle after physical exercise." J Physiol 567(Pt 1): 349-358.
Cereghetti, G. M., A. Stangherlin, O. Martins de Brito, C. R. Chang, C. Blackstone, P. Bernardi and L. Scorrano (2008). "Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria." Proc Natl Acad Sci U S A 105(41): 15803-15808.
Chan, N. C., A. M. Salazar, A. H. Pham, M. J. Sweredoski, N. J. Kolawa, R. L. Graham, . . . D. C. Chan (2011). "Broad activation of the ubiquitin-proteasome system by Parkin is critical for mitophagy." Hum Mol Genet 20(9): 1726-1737.
Chantranupong, L., S. M. Scaria, R. A. Saxton, M. P. Gygi, K. Shen, G. A. Wyant, . . . D. M. Sabatini (2016). "The CASTOR Proteins Are Arginine Sensors for the mTORC1 Pathway." Cell 165(1): 153-164.
Chen, C. C. W., A. T. Erlich and D. A. Hood (2018). "Role of Parkin and endurance training on mitochondrial turnover in skeletal muscle." Skelet Muscle 8(1): 10.
Chen, G., Z. Han, D. Feng, Y. Chen, L. Chen, H. Wu, . . . Q. Chen (2014). "A regulatory signaling loop comprising the PGAM5 phosphatase and CK2 controls receptor-mediated mitophagy." Mol Cell 54(3): 362-377.
Chen, H., M. Vermulst, Y. E. Wang, A. Chomyn, T. A. Prolla, J. M. McCaffery and D. C. Chan (2010). "Mitochondrial fusion is required for mtDNA stability in skeletal muscle and tolerance of mtDNA mutations." Cell 141(2): 280-289.
Chen, M., Z. Chen, Y. Wang, Z. Tan, C. Zhu, Y. Li, . . . Q. Chen (2016). "Mitophagy receptor FUNDC1 regulates mitochondrial dynamics and mitophagy." Autophagy 12(4): 689-702.
Chen, Y. and G. W. Dorn, 2nd (2013). "PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria." Science 340(6131): 471-475.

Chen, Z. P., T. J. Stephens, S. Murthy, B. J. Canny, M. Hargreaves, L. A. Witters, . . . G. K. McConell (2003). "Effect of exercise intensity on skeletal muscle AMPK signaling in humans." Diabetes 52(9): 2205-2212.
Choksi, K. B., J. E. Nuss, J. H. Deford and J. Papaconstantinou (2008). "Age-related alterations in oxidatively damaged proteins of mouse skeletal muscle mitochondrial electron transport chain complexes." Free Radic Biol Med 45(6): 826-838.
Cogliati, S., C. Frezza, M. E. Soriano, T. Varanita, R. Quintana-Cabrera, M. Corrado, . . . L. Scorrano (2013). "Mitochondrial cristae shape determines respiratory chain supercomplexes assembly and respiratory efficiency." Cell 155(1): 160-171.
Collins, J. and C. G. Bonnemann (2010). "Congenital muscular dystrophies: toward molecular therapeutic interventions." Curr Neurol Neurosci Rep 10(2): 83-91.
Conley, K. E., S. A. Jubrias and P. C. Esselman (2000). "Oxidative capacity and ageing in human muscle." J Physiol 526 Pt 1: 203-210.
Costill, D. L., E. Coyle, G. Dalsky, W. Evans, W. Fink and D. Hoopes (1977). "Effects of elevated plasma FFA and insulin on muscle glycogen usage during exercise." J Appl Physiol Respir Environ Exerc Physiol 43(4): 695-699.
Cribbs, J. T. and S. Strack (2007). "Reversible phosphorylation of Drp1 by cyclic AMP-dependent protein kinase and calcineurin regulates mitochondrial fission and cell death." EMBO Rep 8(10): 939-944.
Cunningham, J. T., J. T. Rodgers, D. H. Arlow, F. Vazquez, V. K. Mootha and P. Puigserver (2007). "mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex." Nature 450(7170): 736-740.
Dagon, Y., E. Hur, B. Zheng, K. Wellenstein, L. C. Cantley and B. B. Kahn (2012). "p70S6 kinase phosphorylates AMPK on serine 491 to mediate leptin's effect on food intake." Cell Metab 16(1): 104-112.
De Stefanis, D., R. Mastrocola, D. Nigro, P. Costelli and M. Aragno (2017). "Effects of chronic sugar consumption on lipid accumulation and autophagy in the skeletal muscle." Eur J Nutr 56(1): 363-373.
Decuypere, J. P., G. Bultynck and J. B. Parys (2011). "A dual role for Ca(2+) in autophagy regulation." Cell Calcium 50(3): 242-250.
Dinardo, M. M., C. Musicco, F. Fracasso, F. Milella, M. N. Gadaleta, G. Gadaleta and P. Cantatore (2003). "Acetylation and level of mitochondrial transcription factor A in several organs of young and old rats." Biochem Biophys Res Commun 301(1): 187-191.
Ding, H., N. Jiang, H. Liu, X. Liu, D. Liu, F. Zhao, . . . Y. Zhang (2010). "Response of mitochondrial fusion and fission protein gene expression to exercise in rat skeletal muscle." Biochim Biophys Acta 1800(3): 250-256.
Ding, W. X., H. M. Ni, M. Li, Y. Liao, X. Chen, D. B. Stolz, . . . X. M. Yin (2010). "Nix is critical to two distinct phases of mitophagy, reactive oxygen species-mediated

autophagy induction and Parkin-ubiquitin-p62-mediated mitochondrial priming." J Biol Chem 285(36): 27879-27890.
Ding, W. X. and X. M. Yin (2012). "Mitophagy: mechanisms, pathophysiological roles, and analysis." Biol Chem 393(7): 547-564.
Dominy, J. E. and P. Puigserver (2013). "Mitochondrial biogenesis through activation of nuclear signaling proteins." Cold Spring Harb Perspect Biol 5(7).
Dowling, J. J., S. A. Moore, H. Kalimo and B. A. Minassian (2015). "X-linked myopathy with excessive autophagy: a failure of self-eating." Acta Neuropathol 129(3): 383-390.
Egan, B., B. P. Carson, P. M. Garcia-Roves, A. V. Chibalin, F. M. Sarsfield, N. Barron, . . . D. J. O'Gorman (2010). "Exercise intensity-dependent regulation of peroxisome proliferator-activated receptor coactivator-1 mRNA abundance is associated with differential activation of upstream signalling kinases in human skeletal muscle." J Physiol 588(Pt 10): 1779-1790.
Egan, B. and J. R. Zierath (2013). "Exercise metabolism and the molecular regulation of skeletal muscle adaptation." Cell Metab 17(2): 162-184.
Egan, D. F., D. B. Shackelford, M. M. Mihaylova, S. Gelino, R. A. Kohnz, W. Mair, . . . R. J. Shaw (2011). "Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy." Science 331(6016): 456-461.
Eichner, L. J. and V. Giguere (2011). "Estrogen related receptors (ERRs): a new dawn in transcriptional control of mitochondrial gene networks." Mitochondrion 11(4): 544-552.
Emberson, J. R., P. H. Whincup, M. Walker, M. Thomas and K. G. Alberti (2002). "Biochemical measures in a population-based study: effect of fasting duration and time of day." Ann Clin Biochem 39(Pt 5): 493-501.
Endo, Y., A. Furuta and I. Nishino (2015). "Danon disease: a phenotypic expression of LAMP-2 deficiency." Acta Neuropathol 129(3): 391-398.
Eskelinen, E. L. (2006). "Roles of LAMP-1 and LAMP-2 in lysosome biogenesis and autophagy." Mol Aspects Med 27(5-6): 495-502.
Essen, B., L. Hagenfeldt and L. Kaijser (1977). "Utilization of blood-borne and intramuscular substrates during continuous and intermittent exercise in man." J Physiol 265(2): 489-506.
Falkenberg, M., M. Gaspari, A. Rantanen, A. Trifunovic, N. G. Larsson and C. M. Gustafsson (2002). "Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA." Nat Genet 31(3): 289-294.
Fan, W. and R. Evans (2015). "PPARs and ERRs: molecular mediators of mitochondrial metabolism." Curr Opin Cell Biol 33: 49-54.
Fan, W., W. Waizenegger, C. S. Lin, V. Sorrentino, M. X. He, C. E. Wall, . . . R. M. Evans (2017). "PPARdelta Promotes Running Endurance by Preserving Glucose." Cell Metab 25(5): 1186-1193 e1184.

Frank, M., S. Duvezin-Caubet, S. Koob, A. Occhipinti, R. Jagasia, A. Petcherski, . . . A. S. Reichert (2012). "Mitophagy is triggered by mild oxidative stress in a mitochondrial fission dependent manner." Biochim Biophys Acta 1823(12): 2297-2310.
Gao, W., Z. Shen, L. Shang and X. Wang (2011). "Upregulation of human autophagy-initiation kinase ULK1 by tumor suppressor p53 contributes to DNA-damage-induced cell death." Cell Death Differ 18(10): 1598-1607.
Garber, C. E., B. Blissmer, M. R. Deschenes, B. A. Franklin, M. J. Lamonte, I. M. Lee, . . . M. American College of Sports (2011). "American College of Sports Medicine position stand. Quantity and quality of exercise for developing and maintaining cardiorespiratory, musculoskeletal, and neuromotor fitness in apparently healthy adults: guidance for prescribing exercise." Med Sci Sports Exerc 43(7): 1334-1359.
Garcia-Prat, L., M. Martinez-Vicente, E. Perdiguero, L. Ortet, J. Rodriguez-Ubreva, E. Rebollo, . . . P. Munoz-Canoves (2016). "Autophagy maintains stemness by preventing senescence." Nature 529(7584): 37-42.
Geisler, S., K. M. Holmstrom, D. Skujat, F. C. Fiesel, O. C. Rothfuss, P. J. Kahle and W. Springer (2010). "PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1." Nat Cell Biol 12(2): 119-131.
Gerhart-Hines, Z., J. T. Rodgers, O. Bare, C. Lerin, S. H. Kim, R. Mostoslavsky, . . . P. Puigserver (2007). "Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha." EMBO J 26(7): 1913-1923.
Ghosh, S., R. Lertwattanarak, N. Lefort, M. Molina-Carrion, J. Joya-Galeana, B. P. Bowen, . . . N. Musi (2011). "Reduction in reactive oxygen species production by mitochondria from elderly subjects with normal and impaired glucose tolerance." Diabetes 60(8): 2051-2060.
Gomes, L. C., G. Di Benedetto and L. Scorrano (2011). "During autophagy mitochondria elongate, are spared from degradation and sustain cell viability." Nat Cell Biol 13(5): 589-598.
Gomes, L. C. and L. Scorrano (2008). "High levels of Fis1, a pro-fission mitochondrial protein, trigger autophagy." Biochim Biophys Acta 1777(7-8): 860-866.
Gonzalez, A. A., R. Kumar, J. D. Mulligan, A. J. Davis, R. Weindruch and K. W. Saupe (2004). "Metabolic adaptations to fasting and chronic caloric restriction in heart, muscle, and liver do not include changes in AMPK activity." Am J Physiol Endocrinol Metab 287(5): E1032-1037.
Gopalakrishnan, L. and R. C. Scarpulla (1994). "Differential regulation of respiratory chain subunits by a CREB-dependent signal transduction pathway. Role of cyclic AMP in cytochrome c and COXIV gene expression." J Biol Chem 269(1): 105-113.

Gouspillou, G., R. Godin, J. Piquereau, M. Picard, M. Mofarrahi, J. Mathew, . . . S. N. A. Hussain (2018). "Protective role of Parkin in skeletal muscle contractile and mitochondrial function." J Physiol 596(13): 2565-2579.
Greene, N. P., J. D. Fluckey, B. S. Lambert, E. S. Greene, S. E. Riechman and S. F. Crouse (2012). "Regulators of blood lipids and lipoproteins? PPARdelta and AMPK, induced by exercise, are correlated with lipids and lipoproteins in overweight/obese men and women." Am J Physiol Endocrinol Metab 303(10): E1212-1221.
Greer, E. L. and A. Brunet (2005). "FOXO transcription factors at the interface between longevity and tumor suppression." Oncogene 24(50): 7410-7425.
Greer, E. L., P. R. Oskoui, M. R. Banko, J. M. Maniar, M. P. Gygi, S. P. Gygi and A. Brunet (2007). "The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor." J Biol Chem 282(41): 30107-30119.
Greiwe, J. S., R. C. Hickner, P. A. Hansen, S. B. Racette, M. M. Chen and J. O. Holloszy (1999). "Effects of endurance exercise training on muscle glycogen accumulation in humans." J Appl Physiol (1985) 87(1): 222-226.
Grumati, P. and P. Bonaldo (2012). "Autophagy in skeletal muscle homeostasis and in muscular dystrophies." Cells 1(3): 325-345.
Grumati, P., L. Coletto, A. Schiavinato, S. Castagnaro, E. Bertaggia, M. Sandri and P. Bonaldo (2011). "Physical exercise stimulates autophagy in normal skeletal muscles but is detrimental for collagen VI-deficient muscles." Autophagy 7(12): 1415-1423.
Gwinn, D. M., D. B. Shackelford, D. F. Egan, M. M. Mihaylova, A. Mery, D. S. Vasquez, . . . R. J. Shaw (2008). "AMPK phosphorylation of raptor mediates a metabolic checkpoint." Mol Cell 30(2): 214-226.
Halling, J. F., S. Ringholm, M. M. Nielsen, P. Overby and H. Pilegaard (2016). "PGC-1alpha promotes exercise-induced autophagy in mouse skeletal muscle." Physiol Rep 4(3).
Han, X. J., Y. F. Lu, S. A. Li, T. Kaitsuka, Y. Sato, K. Tomizawa, . . . M. Matsushita (2008). "CaM kinase I alpha-induced phosphorylation of Drp1 regulates mitochondrial morphology." J Cell Biol 182(3): 573-585.
Hanada, T., N. N. Noda, Y. Satomi, Y. Ichimura, Y. Fujioka, T. Takao, . . . Y. Ohsumi (2007). "The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy." J Biol Chem 282(52): 37298-37302.
Harbauer, A. B., R. P. Zahedi, A. Sickmann, N. Pfanner and C. Meisinger (2014). "The protein import machinery of mitochondria-a regulatory hub in metabolism, stress, and disease." Cell Metab 19(3): 357-372.
Hawley, J. A., M. Hargreaves, M. J. Joyner and J. R. Zierath (2014). "Integrative biology of exercise." Cell 159(4): 738-749.

Hawley, S. A., F. A. Ross, G. J. Gowans, P. Tibarewal, N. R. Leslie and D. G. Hardie (2014). "Phosphorylation by Akt within the ST loop of AMPK-alpha1 down-regulates its activation in tumour cells." Biochem J 459(2): 275-287.
He, C., M. C. Bassik, V. Moresi, K. Sun, Y. Wei, Z. Zou, . . . B. Levine (2012). "Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis." Nature 481(7382): 511-515.
He, H., Y. Dang, F. Dai, Z. Guo, J. Wu, X. She, . . . L. Yu (2003). "Post-translational modifications of three members of the human MAP1LC3 family and detection of a novel type of modification for MAP1LC3B." J Biol Chem 278(31): 29278-29287.
Herzig, S., F. Long, U. S. Jhala, S. Hedrick, R. Quinn, A. Bauer, . . . M. Montminy (2001). "CREB regulates hepatic gluconeogenesis through the coactivator PGC-1." Nature 413(6852): 179-183.
Hoffman, N. J., B. L. Parker, R. Chaudhuri, K. H. Fisher-Wellman, M. Kleinert, S. J. Humphrey, . . . D. E. James (2015). "Global Phosphoproteomic Analysis of Human Skeletal Muscle Reveals a Network of Exercise-Regulated Kinases and AMPK Substrates." Cell Metab 22(5): 922-935.
Holm, L. and M. Kjaer (2010). "Measuring protein breakdown rate in individual proteins in vivo." Curr Opin Clin Nutr Metab Care 13(5): 526-531.
Hood, D. A., L. D. Tryon, H. N. Carter, Y. Kim and C. C. Chen (2016). "Unravelling the mechanisms regulating muscle mitochondrial biogenesis." Biochem J 473(15): 2295-2314.
Hoshino, A., Y. Mita, Y. Okawa, M. Ariyoshi, E. Iwai-Kanai, T. Ueyama, . . . S. Matoba (2013). "Cytosolic p53 inhibits Parkin-mediated mitophagy and promotes mitochondrial dysfunction in the mouse heart." Nat Commun 4: 2308.
Hosokawa, N., T. Hara, T. Kaizuka, C. Kishi, A. Takamura, Y. Miura, . . . N. Mizushima (2009). "Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy." Mol Biol Cell 20(7): 1981-1991.
Huynh, K. K., E. L. Eskelinen, C. C. Scott, A. Malevanets, P. Saftig and S. Grinstein (2007). "LAMP proteins are required for fusion of lysosomes with phagosomes." EMBO J 26(2): 313-324.
Inoki, K., T. Zhu and K. L. Guan (2003). "TSC2 mediates cellular energy response to control cell growth and survival." Cell 115(5): 577-590.
Irrcher, I., V. Ljubicic, A. F. Kirwan and D. A. Hood (2008). "AMP-activated protein kinase-regulated activation of the PGC-1alpha promoter in skeletal muscle cells." PLoS One 3(10): e3614.
Islam, H., B. A. Edgett and B. J. Gurd (2017). "Coordination of mitochondrial biogenesis by PGC-1alpha in human skeletal muscle: a re-evaluation." Metabolism.
Jackson, A. S. and M. L. Pollock (1978). "Generalized equations for predicting body density of men." Br J Nutr 40(3): 497-504.

Jager, S., C. Handschin, J. St-Pierre and B. M. Spiegelman (2007). "AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha." Proc Natl Acad Sci U S A 104(29): 12017-12022.
Jamart, C., N. Benoit, J. M. Raymackers, H. J. Kim, C. K. Kim and M. Francaux (2012). "Autophagy-related and autophagy-regulatory genes are induced in human muscle after ultraendurance exercise." Eur J Appl Physiol 112(8): 3173-3177.
Jamart, C., M. Francaux, G. Y. Millet, L. Deldicque, D. Frere and L. Feasson (2012). "Modulation of autophagy and ubiquitin-proteasome pathways during ultra-endurance running." J Appl Physiol (1985) 112(9): 1529-1537.
Jamart, C., D. Naslain, H. Gilson and M. Francaux (2013). "Higher activation of autophagy in skeletal muscle of mice during endurance exercise in the fasted state." Am J Physiol Endocrinol Metab 305(8): E964-974.
Janssen, I., S. B. Heymsfield and R. Ross (2002). "Low relative skeletal muscle mass (sarcopenia) in older persons is associated with functional impairment and physical disability." J Am Geriatr Soc 50(5): 889-896.
Johannsen, D. L., J. P. DeLany, M. I. Frisard, M. A. Welsch, C. K. Rowley, X. Fang, . . . S. Louisiana Healthy Aging (2008). "Physical activity in aging: comparison among young, aged, and nonagenarian individuals." J Appl Physiol (1985) 105(2): 495-501.
Johansen, T. and T. Lamark (2011). "Selective autophagy mediated by autophagic adapter proteins." Autophagy 7(3): 279-296.
Jung, C. H., C. B. Jun, S. H. Ro, Y. M. Kim, N. M. Otto, J. Cao, . . . D. H. Kim (2009). "ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery." Mol Biol Cell 20(7): 1992-2003.
Kabeya, Y., N. Mizushima, T. Ueno, A. Yamamoto, T. Kirisako, T. Noda, . . . T. Yoshimori (2000). "LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing." EMBO J 19(21): 5720-5728.
Kageyama, Y., M. Hoshijima, K. Seo, D. Bedja, P. Sysa-Shah, S. A. Andrabi, . . . H. Sesaki (2014). "Parkin-independent mitophagy requires Drp1 and maintains the integrity of mammalian heart and brain." EMBO J 33(23): 2798-2813.
Kahn, B. B., T. Alquier, D. Carling and D. G. Hardie (2005). "AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism." Cell Metab 1(1): 15-25.
Kang, R., H. J. Zeh, M. T. Lotze and D. Tang (2011). "The Beclin 1 network regulates autophagy and apoptosis." Cell Death Differ 18(4): 571-580.
Karbowski, M. and R. J. Youle (2011). "Regulating mitochondrial outer membrane proteins by ubiquitination and proteasomal degradation." Curr Opin Cell Biol 23(4): 476-482.
Karunadharma, P. P., N. Basisty, Y. A. Chiao, D. F. Dai, R. Drake, N. Levy, . . . P. S. Rabinovitch (2015). "Respiratory chain protein turnover rates in mice are highly

heterogeneous but strikingly conserved across tissues, ages, and treatments." FASEB J 29(8): 3582-3592.
Katsuragi, Y., Y. Ichimura and M. Komatsu (2015). "p62/SQSTM1 functions as a signaling hub and an autophagy adaptor." FEBS J 282(24): 4672-4678.
Katzin, L. W. and A. A. Amato (2008). "Pompe disease: a review of the current diagnosis and treatment recommendations in the era of enzyme replacement therapy." J Clin Neuromuscul Dis 9(4): 421-431.
Kim, B., J. S. Kim, Y. Yoon, M. C. Santiago, M. D. Brown and J. Y. Park (2013). "Inhibition of Drp1-dependent mitochondrial division impairs myogenic differentiation." Am J Physiol Regul Integr Comp Physiol 305(8): R927-938.
Kim, J., M. Kundu, B. Viollet and K. L. Guan (2011). "AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1." Nat Cell Biol 13(2): 132-141.
Kim, T. N. and K. M. Choi (2013). "Sarcopenia: definition, epidemiology, and pathophysiology." J Bone Metab 20(1): 1-10.
Kim, Y. A., Y. S. Kim and W. Song (2012). "Autophagic response to a single bout of moderate exercise in murine skeletal muscle." J Physiol Biochem 68(2): 229-235.
Kimura, S., T. Noda and T. Yoshimori (2008). "Dynein-dependent movement of autophagosomes mediates efficient encounters with lysosomes." Cell Struct Funct 33(1): 109-122.
Klionsky, D. J. (2007). "Autophagy: from phenomenology to molecular understanding in less than a decade." Nat Rev Mol Cell Biol 8(11): 931-937.
Klionsky, D. J., K. Abdelmohsen, A. Abe, M. J. Abedin, H. Abeliovich, A. Acevedo Arozena, . . . S. M. Zughaier (2016). "Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition)." Autophagy 12(1): 1-222.
Kochl, R., X. W. Hu, E. Y. Chan and S. A. Tooze (2006). "Microtubules facilitate autophagosome formation and fusion of autophagosomes with endosomes." Traffic 7(2): 129-145.
Koh, J. H., C. R. Hancock, S. Terada, K. Higashida, J. O. Holloszy and D. H. Han (2017). "PPARbeta Is Essential for Maintaining Normal Levels of PGC-1alpha and Mitochondria and for the Increase in Muscle Mitochondria Induced by Exercise." Cell Metab 25(5): 1176-1185 e1175.
Kondapalli, C., A. Kazlauskaite, N. Zhang, H. I. Woodroof, D. G. Campbell, R. Gourlay, . . . M. M. Muqit (2012). "PINK1 is activated by mitochondrial membrane potential depolarization and stimulates Parkin E3 ligase activity by phosphorylating Serine 65." Open Biol 2(5): 120080.
Kong, X., R. Wang, Y. Xue, X. Liu, H. Zhang, Y. Chen, . . . Y. Chang (2010). "Sirtuin 3, a new target of PGC-1alpha, plays an important role in the suppression of ROS and mitochondrial biogenesis." PLoS One 5(7): e11707.
Konopka, A. R., M. K. Suer, C. A. Wolff and M. P. Harber (2014). "Markers of human skeletal muscle mitochondrial biogenesis and quality control: effects of age and aerobic exercise training." J Gerontol A Biol Sci Med Sci 69(4): 371-378.

Koyano, F., K. Okatsu, H. Kosako, Y. Tamura, E. Go, M. Kimura, . . . N. Matsuda (2014). "Ubiquitin is phosphorylated by PINK1 to activate parkin." Nature 510(7503): 162-166.
Kramer, H. F. and L. J. Goodyear (2007). "Exercise, MAPK, and NF-kappaB signaling in skeletal muscle." J Appl Physiol (1985) 103(1): 388-395.
Krasich, R. and W. C. Copeland (2017). "DNA polymerases in the mitochondria: A critical review of the evidence." Front Biosci (Landmark Ed) 22: 692-709.
Kume, S., T. Uzu, K. Horiike, M. Chin-Kanasaki, K. Isshiki, S. Araki, . . . D. Koya (2010). "Calorie restriction enhances cell adaptation to hypoxia through Sirt1-dependent mitochondrial autophagy in mouse aged kidney." J Clin Invest 120(4): 1043-1055.
Lage, R., C. Dieguez, A. Vidal-Puig and M. Lopez (2008). "AMPK: a metabolic gauge regulating whole-body energy homeostasis." Trends Mol Med 14(12): 539-549.
Lagouge, M., C. Argmann, Z. Gerhart-Hines, H. Meziane, C. Lerin, F. Daussin, . . . J. Auwerx (2006). "Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha." Cell 127(6): 1109-1122.
Laker, R. C., J. C. Drake, R. J. Wilson, V. A. Lira, B. M. Lewellen, K. A. Ryall, . . . Z. Yan (2017). "Ampk phosphorylation of Ulk1 is required for targeting of mitochondria to lysosomes in exercise-induced mitophagy." Nat Commun 8(1): 548.
Landes, T., L. J. Emorine, D. Courilleau, M. Rojo, P. Belenguer and L. Arnaune-Pelloquin (2010). "The BH3-only Bnip3 binds to the dynamin Opa1 to promote mitochondrial fragmentation and apoptosis by distinct mechanisms." EMBO Rep 11(6): 459-465.
Lapuente-Brun, E., R. Moreno-Loshuertos, R. Acin-Perez, A. Latorre-Pellicer, C. Colas, E. Balsa, . . . J. A. Enriquez (2013). "Supercomplex assembly determines electron flux in the mitochondrial electron transport chain." Science 340(6140): 1567-1570.
Lazarou, M., D. A. Sliter, L. A. Kane, S. A. Sarraf, C. Wang, J. L. Burman, . . . R. J. Youle (2015). "The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy." Nature 524(7565): 309-314.
Lee, Y., H. Y. Lee, R. A. Hanna and A. B. Gustafsson (2011). "Mitochondrial autophagy by Bnip3 involves Drp1-mediated mitochondrial fission and recruitment of Parkin in cardiac myocytes." Am J Physiol Heart Circ Physiol 301(5): H1924-1931.
Lehrke, M. and M. A. Lazar (2005). "The many faces of PPARgamma." Cell 123(6): 993-999.
Li, J., M. Kanasaki, L. Xu, M. Kitada, K. Nagao, Y. Adachi, . . . D. Koya (2018). "A ketogenic amino acid rich diet benefits mitochondrial homeostasis by altering the

AKT/4EBP1 and autophagy signaling pathways in the gastrocnemius and soleus." Biochim Biophys Acta 1862(7): 1547-1555.
Liang, C., J. S. Lee, K. S. Inn, M. U. Gack, Q. Li, E. A. Roberts, . . . J. U. Jung (2008). "Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking." Nat Cell Biol 10(7): 776-787.
Lim, J., M. L. Lachenmayer, S. Wu, W. Liu, M. Kundu, R. Wang, . . . Z. Yue (2015). "Proteotoxic stress induces phosphorylation of p62/SQSTM1 by ULK1 to regulate selective autophagic clearance of protein aggregates." PLoS Genet 11(2): e1004987.
Lin, L., K. Chen, W. Abdel Khalek, J. L. Ward, 3rd, H. Yang, B. Chabi, . . . Q. Tong (2014). "Regulation of skeletal muscle oxidative capacity and muscle mass by SIRT3." PLoS One 9(1): e85636.
Lira, V. A., D. L. Brown, A. K. Lira, A. N. Kavazis, Q. A. Soltow, E. H. Zeanah and D. S. Criswell (2010). "Nitric oxide and AMPK cooperatively regulate PGC-1 in skeletal muscle cells." J Physiol 588(Pt 18): 3551-3566.
Lira, V. A., M. Okutsu, M. Zhang, N. P. Greene, R. C. Laker, D. S. Breen, . . . Z. Yan (2013). "Autophagy is required for exercise training-induced skeletal muscle adaptation and improvement of physical performance." FASEB J 27(10): 4184-4193.
Liu, L., D. Feng, G. Chen, M. Chen, Q. Zheng, P. Song, . . . Q. Chen (2012). "Mitochondrial outer-membrane protein FUNDC1 mediates hypoxia-induced mitophagy in mammalian cells." Nat Cell Biol 14(2): 177-185.
Ljubicic, V., A. M. Joseph, A. Saleem, G. Uguccioni, M. Collu-Marchese, R. Y. Lai, . . . D. A. Hood (2010). "Transcriptional and post-transcriptional regulation of mitochondrial biogenesis in skeletal muscle: effects of exercise and aging." Biochim Biophys Acta 1800(3): 223-234.
Lo Verso, F., S. Carnio, A. Vainshtein and M. Sandri (2014). "Autophagy is not required to sustain exercise and PRKAA1/AMPK activity but is important to prevent mitochondrial damage during physical activity." Autophagy 10(11): 1883-1894.
Lu, B., J. Lee, X. Nie, M. Li, Y. I. Morozov, S. Venkatesh, . . . C. K. Suzuki (2013). "Phosphorylation of human TFAM in mitochondria impairs DNA binding and promotes degradation by the AAA+ Lon protease." Mol Cell 49(1): 121-132.
Ma, D., S. Panda and J. D. Lin (2011). "Temporal orchestration of circadian autophagy rhythm by C/EBPbeta." EMBO J 30(22): 4642-4651.
Ma, X. M. and J. Blenis (2009). "Molecular mechanisms of mTOR-mediated translational control." Nat Rev Mol Cell Biol 10(5): 307-318.
Mahoney, D. J., G. Parise, S. Melov, A. Safdar and M. A. Tarnopolsky (2005). "Analysis of global mRNA expression in human skeletal muscle during recovery from endurance exercise." FASEB J 19(11): 1498-1500.

Maiuri, M. C., L. Galluzzi, E. Morselli, O. Kepp, S. A. Malik and G. Kroemer (2010). "Autophagy regulation by p53." Curr Opin Cell Biol 22(2): 181-185.
Mammucari, C., G. Milan, V. Romanello, E. Masiero, R. Rudolf, P. Del Piccolo, . . . M. Sandri (2007). "FoxO3 controls autophagy in skeletal muscle in vivo." Cell Metab 6(6): 458-471.
Mansueto, G., A. Armani, C. Viscomi, L. D'Orsi, R. De Cegli, E. V. Polishchuk, . . . A. Ballabio (2017). "Transcription Factor EB Controls Metabolic Flexibility during Exercise." Cell Metab 25(1): 182-196.
Masiero, E., L. Agatea, C. Mammucari, B. Blaauw, E. Loro, M. Komatsu, . . . M. Sandri (2009). "Autophagy is required to maintain muscle mass." Cell Metab 10(6): 507-515.
Matoba, S., J. G. Kang, W. D. Patino, A. Wragg, M. Boehm, O. Gavrilova, . . . P. M. Hwang (2006). "p53 regulates mitochondrial respiration." Science 312(5780): 1650-1653.
Matsumoto, G., K. Wada, M. Okuno, M. Kurosawa and N. Nukina (2011). "Serine 403 phosphorylation of p62/SQSTM1 regulates selective autophagic clearance of ubiquitinated proteins." Mol Cell 44(2): 279-289.
McClung, J. M., A. R. Judge, S. K. Powers and Z. Yan (2010). "p38 MAPK links oxidative stress to autophagy-related gene expression in cachectic muscle wasting." Am J Physiol Cell Physiol 298(3): C542-549.
McLelland, G. L., V. Soubannier, C. X. Chen, H. M. McBride and E. A. Fon (2014). "Parkin and PINK1 function in a vesicular trafficking pathway regulating mitochondrial quality control." EMBO J 33(4): 282-295.
Medeiros, D. M. (2008). "Assessing mitochondria biogenesis." Methods 46(4): 288-294.
Medina, D. L., S. Di Paola, I. Peluso, A. Armani, D. De Stefani, R. Venditti, . . . A. Ballabio (2015). "Lysosomal calcium signalling regulates autophagy through calcineurin and TFEB." Nat Cell Biol 17(3): 288-299.
Menshikova, E. V., V. B. Ritov, L. Fairfull, R. E. Ferrell, D. E. Kelley and B. H. Goodpaster (2006). "Effects of exercise on mitochondrial content and function in aging human skeletal muscle." J Gerontol A Biol Sci Med Sci 61(6): 534-540.
Miller, B. F., M. M. Robinson, M. D. Bruss, M. Hellerstein and K. L. Hamilton (2012). "A comprehensive assessment of mitochondrial protein synthesis and cellular proliferation with age and caloric restriction." Aging Cell 11(1): 150-161.
Mizushima, N. and M. Komatsu (2011). "Autophagy: renovation of cells and tissues." Cell 147(4): 728-741.
Mizushima, N., A. Kuma, Y. Kobayashi, A. Yamamoto, M. Matsubae, T. Takao, . . . T. Yoshimori (2003). "Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate." J Cell Sci 116(Pt 9): 1679-1688.

Mizushima, N., H. Sugita, T. Yoshimori and Y. Ohsumi (1998). "A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy." J Biol Chem 273(51): 33889-33892.
Mizushima, N., A. Yamamoto, M. Matsui, T. Yoshimori and Y. Ohsumi (2004). "In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker." Mol Biol Cell 15(3): 1101-1111.
Mofarrahi, M., I. Sigala, Y. Guo, R. Godin, E. C. Davis, B. Petrof, . . . S. N. Hussain (2012). "Autophagy and skeletal muscles in sepsis." PLoS One 7(10): e47265.
Mootha, V. K., J. Bunkenborg, J. V. Olsen, M. Hjerrild, J. R. Wisniewski, E. Stahl, . . . M. Mann (2003). "Integrated analysis of protein composition, tissue diversity, and gene regulation in mouse mitochondria." Cell 115(5): 629-640.
Moresi, V., M. Carrer, C. E. Grueter, O. F. Rifki, J. M. Shelton, J. A. Richardson, . . . E. N. Olson (2012). "Histone deacetylases 1 and 2 regulate autophagy flux and skeletal muscle homeostasis in mice." Proc Natl Acad Sci U S A 109(5): 1649-1654.
Mourier, A., E. Motori, T. Brandt, M. Lagouge, I. Atanassov, A. Galinier, . . . N. G. Larsson (2015). "Mitofusin 2 is required to maintain mitochondrial coenzyme Q levels." J Cell Biol 208(4): 429-442.
Nair, K. S. (2005). "Aging muscle." Am J Clin Nutr 81(5): 953-963.
Nakada, K., K. Inoue, T. Ono, K. Isobe, A. Ogura, Y. I. Goto, . . . J. I. Hayashi (2001). "Inter-mitochondrial complementation: Mitochondria-specific system preventing mice from expression of disease phenotypes by mutant mtDNA." Nat Med 7(8): 934-940.
Narendra, D., L. A. Kane, D. N. Hauser, I. M. Fearnley and R. J. Youle (2010). "p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both." Autophagy 6(8): 1090-1106.
Narendra, D. P., S. M. Jin, A. Tanaka, D. F. Suen, C. A. Gautier, J. Shen, . . . R. J. Youle (2010). "PINK1 is selectively stabilized on impaired mitochondria to activate Parkin." PLoS Biol 8(1): e1000298.
Nemoto, T., I. Tanida, E. Tanida-Miyake, N. Minematsu-Ikeguchi, M. Yokota, M. Ohsumi, . . . E. Kominami (2003). "The mouse APG10 homologue, an E2-like enzyme for Apg12p conjugation, facilitates MAP-LC3 modification." J Biol Chem 278(41): 39517-39526.
Newman, A. B., V. Kupelian, M. Visser, E. M. Simonsick, B. H. Goodpaster, S. B. Kritchevsky, . . . T. B. Harris (2006). "Strength, but not muscle mass, is associated with mortality in the health, aging and body composition study cohort." J Gerontol A Biol Sci Med Sci 61(1): 72-77.
Nicklin, P., P. Bergman, B. Zhang, E. Triantafellow, H. Wang, B. Nyfeler, . . . L. O. Murphy (2009). "Bidirectional transport of amino acids regulates mTOR and autophagy." Cell 136(3): 521-534.

Novak, I., V. Kirkin, D. G. McEwan, J. Zhang, P. Wild, A. Rozenknop, . . . I. Dikic (2010). "Nix is a selective autophagy receptor for mitochondrial clearance." EMBO Rep 11(1): 45-51.
O'Leary, M. F., A. Vainshtein, H. N. Carter, Y. Zhang and D. A. Hood (2012). "Denervation-induced mitochondrial dysfunction and autophagy in skeletal muscle of apoptosis-deficient animals." Am J Physiol Cell Physiol 303(4): C447-454.
Otera, H. and K. Mihara (2011). "Molecular mechanisms and physiologic functions of mitochondrial dynamics." J Biochem 149(3): 241-251.
Otera, H., C. Wang, M. M. Cleland, K. Setoguchi, S. Yokota, R. J. Youle and K. Mihara (2010). "Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells." J Cell Biol 191(6): 1141-1158.
Pagano, A. F., G. Py, H. Bernardi, R. B. Candau and A. M. Sanchez (2014). "Autophagy and protein turnover signaling in slow-twitch muscle during exercise." Med Sci Sports Exerc 46(7): 1314-1325.
Palmieri, M., S. Impey, H. Kang, A. di Ronza, C. Pelz, M. Sardiello and A. Ballabio (2011). "Characterization of the CLEAR network reveals an integrated control of cellular clearance pathways." Hum Mol Genet 20(19): 3852-3866.
Pankiv, S., T. H. Clausen, T. Lamark, A. Brech, J. A. Bruun, H. Outzen, . . . T. Johansen (2007). "p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy." J Biol Chem 282(33): 24131-24145.
Peker, N., V. Donipadi, M. Sharma, C. McFarlane and R. Kambadur (2018). "Loss of Parkin Impairs Mitochondrial Function and Leads to Muscle Atrophy." Am J Physiol Cell Physiol.
Perry, C. G., J. Lally, G. P. Holloway, G. J. Heigenhauser, A. Bonen and L. L. Spriet (2010). "Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle." J Physiol 588(Pt 23): 4795-4810.
Philp, A., A. Chen, D. Lan, G. A. Meyer, A. N. Murphy, A. E. Knapp, . . . S. Schenk (2011). "Sirtuin 1 (SIRT1) deacetylase activity is not required for mitochondrial biogenesis or peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1alpha) deacetylation following endurance exercise." J Biol Chem 286(35): 30561-30570.
Phua, W. W. T., M. X. Y. Wong, Z. Liao and N. S. Tan (2018). "An aPPARent Functional Consequence in Skeletal Muscle Physiology via Peroxisome Proliferator-Activated Receptors." Int J Mol Sci 19(5).
Pilegaard, H., B. Saltin and P. D. Neufer (2003). "Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle." J Physiol 546(Pt 3): 851-858.

Pilli, M., J. Arko-Mensah, M. Ponpuak, E. Roberts, S. Master, M. A. Mandell, . . . V. Deretic (2012). "TBK-1 promotes autophagy-mediated antimicrobial defense by controlling autophagosome maturation." Immunity 37(2): 223-234.
Pinto, M. and C. T. Moraes (2015). "Mechanisms linking mtDNA damage and aging." Free Radic Biol Med 85: 250-258.
Puigserver, P., J. Rhee, J. Lin, Z. Wu, J. C. Yoon, C. Y. Zhang, . . . B. M. Spiegelman (2001). "Cytokine stimulation of energy expenditure through p38 MAP kinase activation of PPARgamma coactivator-1." Mol Cell 8(5): 971-982.
Quadrilatero, J. and J. W. Rush (2006). "Increased DNA fragmentation and altered apoptotic protein levels in skeletal muscle of spontaneously hypertensive rats." J Appl Physiol (1985) 101(4): 1149-1161.
Quiros, P. M., T. Langer and C. Lopez-Otin (2015). "New roles for mitochondrial proteases in health, ageing and disease." Nat Rev Mol Cell Biol 16(6): 345-359.
Raben, N., V. Hill, L. Shea, S. Takikita, R. Baum, N. Mizushima, . . . P. Plotz (2008). "Suppression of autophagy in skeletal muscle uncovers the accumulation of ubiquitinated proteins and their potential role in muscle damage in Pompe disease." Hum Mol Genet 17(24): 3897-3908.
Rambold, A. S., B. Kostelecky, N. Elia and J. Lippincott-Schwartz (2011). "Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation." Proc Natl Acad Sci U S A 108(25): 10190-10195.
Ravikumar, B., K. Moreau, L. Jahreiss, C. Puri and D. C. Rubinsztein (2010). "Plasma membrane contributes to the formation of pre-autophagosomal structures." Nat Cell Biol 12(8): 747-757.
Ravikumar, B., S. Sarkar, J. E. Davies, M. Futter, M. Garcia-Arencibia, Z. W. Green-Thompson, . . . D. C. Rubinsztein (2010). "Regulation of mammalian autophagy in physiology and pathophysiology." Physiol Rev 90(4): 1383-1435.
Rebsamen, M., L. Pochini, T. Stasyk, M. E. de Araujo, M. Galluccio, R. K. Kandasamy, . . . G. Superti-Furga (2015). "SLC38A9 is a component of the lysosomal amino acid sensing machinery that controls mTORC1." Nature 519(7544): 477-481.
Reed, N. A., D. Cai, T. L. Blasius, G. T. Jih, E. Meyhofer, J. Gaertig and K. J. Verhey (2006). "Microtubule acetylation promotes kinesin-1 binding and transport." Curr Biol 16(21): 2166-2172.
Richter, E. A. and N. B. Ruderman (2009). "AMPK and the biochemistry of exercise: implications for human health and disease." Biochem J 418(2): 261-275.
Rikka, S., M. N. Quinsay, R. L. Thomas, D. A. Kubli, X. Zhang, A. N. Murphy and A. B. Gustafsson (2011). "Bnip3 impairs mitochondrial bioenergetics and stimulates mitochondrial turnover." Cell Death Differ 18(4): 721-731.
Rockl, K. S., C. A. Witczak and L. J. Goodyear (2008). "Signaling mechanisms in skeletal muscle: acute responses and chronic adaptations to exercise." IUBMB Life 60(3): 145-153.

Romanello, V., E. Guadagnin, L. Gomes, I. Roder, C. Sandri, Y. Petersen, . . . M. Sandri (2010). "Mitochondrial fission and remodelling contributes to muscle atrophy." EMBO J 29(10): 1774-1785.
Romanello, V. and M. Sandri (2015). "Mitochondrial Quality Control and Muscle Mass Maintenance." Front Physiol 6: 422.
Rooyackers, O. E., D. B. Adey, P. A. Ades and K. S. Nair (1996). "Effect of age on in vivo rates of mitochondrial protein synthesis in human skeletal muscle." Proc Natl Acad Sci U S A 93(26): 15364-15369.
Rossignol, R., R. Gilkerson, R. Aggeler, K. Yamagata, S. J. Remington and R. A. Capaldi (2004). "Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells." Cancer Res 64(3): 985-993.
Rub, C., A. Wilkening and W. Voos (2017). "Mitochondrial quality control by the Pink1/Parkin system." Cell Tissue Res 367(1): 111-123.
Russell, A. P., S. Wada, L. Vergani, M. B. Hock, S. Lamon, B. Leger, . . . T. Akimoto (2013). "Disruption of skeletal muscle mitochondrial network genes and miRNAs in amyotrophic lateral sclerosis." Neurobiol Dis 49: 107-117.
Russell, R. C., Y. Tian, H. Yuan, H. W. Park, Y. Y. Chang, J. Kim, . . . K. L. Guan (2013). "ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase." Nat Cell Biol 15(7): 741-750.
Safdar, A., J. M. Bourgeois, D. I. Ogborn, J. P. Little, B. P. Hettinga, M. Akhtar, . . . M. A. Tarnopolsky (2011). "Endurance exercise rescues progeroid aging and induces systemic mitochondrial rejuvenation in mtDNA mutator mice." Proc Natl Acad Sci U S A 108(10): 4135-4140.
Safdar, A., J. P. Little, A. J. Stokl, B. P. Hettinga, M. Akhtar and M. A. Tarnopolsky (2011). "Exercise increases mitochondrial PGC-1alpha content and promotes nuclear-mitochondrial cross-talk to coordinate mitochondrial biogenesis." J Biol Chem 286(12): 10605-10617.
Saleem, A., P. J. Adhihetty and D. A. Hood (2009). "Role of p53 in mitochondrial biogenesis and apoptosis in skeletal muscle." Physiol Genomics 37(1): 58-66.
Saleem, A., H. N. Carter and D. A. Hood (2014). "p53 is necessary for the adaptive changes in cellular milieu subsequent to an acute bout of endurance exercise." Am J Physiol Cell Physiol 306(3): C241-249.
Saleem, A. and D. A. Hood (2013). "Acute exercise induces tumour suppressor protein p53 translocation to the mitochondria and promotes a p53-Tfam-mitochondrial DNA complex in skeletal muscle." J Physiol 591(14): 3625-3636.
Sancak, Y., T. R. Peterson, Y. D. Shaul, R. A. Lindquist, C. C. Thoreen, L. Bar-Peled and D. M. Sabatini (2008). "The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1." Science 320(5882): 1496-1501.
Sanchez, A. M., A. Csibi, A. Raibon, K. Cornille, S. Gay, H. Bernardi and R. Candau (2012). "AMPK promotes skeletal muscle autophagy through activation of forkhead FoxO3a and interaction with Ulk1." J Cell Biochem 113(2): 695-710.

Sandri, M. (2008). "Signaling in muscle atrophy and hypertrophy." Physiology (Bethesda) 23: 160-170.
Sandri, M. (2010). "Autophagy in skeletal muscle." FEBS Lett 584(7): 1411-1416.
Scarpulla, R. C. (2008). "Transcriptional paradigms in mammalian mitochondrial biogenesis and function." Physiol Rev 88(2): 611-638.
Schiaffino, S. and V. Hanzlikova (1972). "Studies on the effect of denervation in developing muscle. II. The lysosomal system." J Ultrastruct Res 39(1): 1-14.
Schiaffino, S. and C. Mammucari (2011). "Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models." Skelet Muscle 1(1): 4.
Schwalm, C., L. Deldicque and M. Francaux (2017). "Lack of Activation of Mitophagy during Endurance Exercise in Human." Med Sci Sports Exerc 49(8): 1552-1561.
Schwalm, C., C. Jamart, N. Benoit, D. Naslain, C. Premont, J. Prevet, . . . M. Francaux (2015). "Activation of autophagy in human skeletal muscle is dependent on exercise intensity and AMPK activation." FASEB J.
Sebastian, D., M. I. Hernandez-Alvarez, J. Segales, E. Sorianello, J. P. Munoz, D. Sala, . . . A. Zorzano (2012). "Mitofusin 2 (Mfn2) links mitochondrial and endoplasmic reticulum function with insulin signaling and is essential for normal glucose homeostasis." Proc Natl Acad Sci U S A 109(14): 5523-5528.
Sebastian, D., E. Sorianello, J. Segales, A. Irazoki, V. Ruiz-Bonilla, D. Sala, . . . A. Zorzano (2016). "Mfn2 deficiency links age-related sarcopenia and impaired autophagy to activation of an adaptive mitophagy pathway." EMBO J 35(15): 1677-1693.
Settembre, C., C. Di Malta, V. A. Polito, M. Garcia Arencibia, F. Vetrini, S. Erdin, . . . A. Ballabio (2011). "TFEB links autophagy to lysosomal biogenesis." Science 332(6036): 1429-1433.
Settembre, C., A. Fraldi, D. L. Medina and A. Ballabio (2013). "Signals from the lysosome: a control centre for cellular clearance and energy metabolism." Nat Rev Mol Cell Biol 14(5): 283-296.
Settembre, C., R. Zoncu, D. L. Medina, F. Vetrini, S. Erdin, S. Erdin, . . . A. Ballabio (2012). "A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB." EMBO J 31(5): 1095-1108.
Shang, L., S. Chen, F. Du, S. Li, L. Zhao and X. Wang (2011). "Nutrient starvation elicits an acute autophagic response mediated by Ulk1 dephosphorylation and its subsequent dissociation from AMPK." Proc Natl Acad Sci U S A 108(12): 4788-4793.
Shiba-Fukushima, K., Y. Imai, S. Yoshida, Y. Ishihama, T. Kanao, S. Sato and N. Hattori (2012). "PINK1-mediated phosphorylation of the Parkin ubiquitin-like domain primes mitochondrial translocation of Parkin and regulates mitophagy." Sci Rep 2: 1002.

Shin, J. H., H. S. Ko, H. Kang, Y. Lee, Y. I. Lee, O. Pletinkova, . . . T. M. Dawson (2011). "PARIS (ZNF746) repression of PGC-1alpha contributes to neurodegeneration in Parkinson's disease." Cell 144(5): 689-702.
Singh, R. and A. M. Cuervo (2012). "Lipophagy: connecting autophagy and lipid metabolism." Int J Cell Biol 2012: 282041.
Soubannier, V., G. L. McLelland, R. Zunino, E. Braschi, P. Rippstein, E. A. Fon and H. M. McBride (2012). "A vesicular transport pathway shuttles cargo from mitochondria to lysosomes." Curr Biol 22(2): 135-141.
Staron, R. S., F. C. Hagerman, R. S. Hikida, T. F. Murray, D. P. Hostler, M. T. Crill, . . . K. Toma (2000). "Fiber type composition of the vastus lateralis muscle of young men and women." J Histochem Cytochem 48(5): 623-629.
Stephens, T. J., Z. P. Chen, B. J. Canny, B. J. Michell, B. E. Kemp and G. K. McConell (2002). "Progressive increase in human skeletal muscle AMPKalpha2 activity and ACC phosphorylation during exercise." Am J Physiol Endocrinol Metab 282(3): E688-694.
Strait, J. B. and E. G. Lakatta (2012). "Aging-associated cardiovascular changes and their relationship to heart failure." Heart Fail Clin 8(1): 143-164.
Tachtsis, B., W. J. Smiles, S. C. Lane, J. A. Hawley and D. M. Camera (2016). "Acute Endurance Exercise Induces Nuclear p53 Abundance in Human Skeletal Muscle." Front Physiol 7: 144.
Tadaishi, M., S. Miura, Y. Kai, E. Kawasaki, K. Koshinaka, K. Kawanaka, . . . O. Ezaki (2011). "Effect of exercise intensity and AICAR on isoform-specific expressions of murine skeletal muscle PGC-1alpha mRNA: a role of beta(2)-adrenergic receptor activation." Am J Physiol Endocrinol Metab 300(2): E341-349.
Taguchi, N., N. Ishihara, A. Jofuku, T. Oka and K. Mihara (2007). "Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission." J Biol Chem 282(15): 11521-11529.
Tanaka, A., M. M. Cleland, S. Xu, D. P. Narendra, D. F. Suen, M. Karbowski and R. J. Youle (2010). "Proteasome and p97 mediate mitophagy and degradation of mitofusins induced by Parkin." J Cell Biol 191(7): 1367-1380.
Tang, B. L. (2016). "Sirt1 and the Mitochondria." Mol Cells 39(2): 87-95.
Tanida, I., Y. S. Sou, J. Ezaki, N. Minematsu-Ikeguchi, T. Ueno and E. Kominami (2004). "HsAtg4B/HsApg4B/autophagin-1 cleaves the carboxyl termini of three human Atg8 homologues and delipidates microtubule-associated protein light chain 3- and GABAA receptor-associated protein-phospholipid conjugates." J Biol Chem 279(35): 36268-36276.
Tanida, I., E. Tanida-Miyake, M. Komatsu, T. Ueno and E. Kominami (2002). "Human Apg3p/Aut1p homologue is an authentic E2 enzyme for multiple substrates, GATE-16, GABARAP, and MAP-LC3, and facilitates the conjugation of hApg12p to hApg5p." J Biol Chem 277(16): 13739-13744.

Tanida, I., T. Ueno and E. Kominami (2004). "LC3 conjugation system in mammalian autophagy." Int J Biochem Cell Biol 36(12): 2503-2518.
Tarpey, M. D., K. P. Davy, R. P. McMillan, S. M. Bowser, T. M. Halliday, N. E. Boutagy, . . . M. W. Hulver (2017). "Skeletal muscle autophagy and mitophagy in endurance-trained runners before and after a high-fat meal." Mol Metab 6(12): 1597-1609.
Tezze, C., V. Romanello, M. A. Desbats, G. P. Fadini, M. Albiero, G. Favaro, . . . M. Sandri (2017). "Age-Associated Loss of OPA1 in Muscle Impacts Muscle Mass, Metabolic Homeostasis, Systemic Inflammation, and Epithelial Senescence." Cell Metab 25(6): 1374-1389 e1376.
Tonkonogi, M., M. Fernstrom, B. Walsh, L. L. Ji, O. Rooyackers, F. Hammarqvist, . . . K. Sahlin (2003). "Reduced oxidative power but unchanged antioxidative capacity in skeletal muscle from aged humans." Pflugers Arch 446(2): 261-269.
Tooze, S. A. and T. Yoshimori (2010). "The origin of the autophagosomal membrane." Nat Cell Biol 12(9): 831-835.
Touvier, T., C. De Palma, E. Rigamonti, A. Scagliola, E. Incerti, L. Mazelin, . . . S. Brunelli (2015). "Muscle-specific Drp1 overexpression impairs skeletal muscle growth via translational attenuation." Cell Death Dis 6: e1663.
Twig, G., A. Elorza, A. J. Molina, H. Mohamed, J. D. Wikstrom, G. Walzer, . . . O. S. Shirihai (2008). "Fission and selective fusion govern mitochondrial segregation and elimination by autophagy." EMBO J 27(2): 433-446.
Vainshtein, A., E. M. Desjardins, A. Armani, M. Sandri and D. A. Hood (2015). "PGC-1alpha modulates denervation-induced mitophagy in skeletal muscle." Skelet Muscle 5: 9.
Vainshtein, A., L. D. Tryon, M. Pauly and D. A. Hood (2015). "Role of PGC-1alpha during acute exercise-induced autophagy and mitophagy in skeletal muscle." Am J Physiol Cell Physiol 308(9): C710-719.
Van Thienen, R., G. D'Hulst, L. Deldicque and P. Hespel (2014). "Biochemical artifacts in experiments involving repeated biopsies in the same muscle." Physiol Rep 2(5): e00286.
Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe and F. Speleman (2002). "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes." Genome Biol 3(7): RESEARCH0034.
Vega, R. B., J. M. Huss and D. P. Kelly (2000). "The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes." Mol Cell Biol 20(5): 1868-1876.
Vendelbo, M. H., A. B. Moller, B. Christensen, B. Nellemann, B. F. Clasen, K. S. Nair, . . . N. Moller (2014). "Fasting increases human skeletal muscle net

phenylalanine release and this is associated with decreased mTOR signaling." PLoS One 9(7): e102031.
Voos, W. and K. Rottgers (2002). "Molecular chaperones as essential mediators of mitochondrial biogenesis." Biochim Biophys Acta 1592(1): 51-62.
Wang, H., P. Song, L. Du, W. Tian, W. Yue, M. Liu, . . . Q. Chen (2011). "Parkin ubiquitinates Drp1 for proteasome-dependent degradation: implication of dysregulated mitochondrial dynamics in Parkinson disease." J Biol Chem 286(13): 11649-11658.
Wang, K. Z., J. Zhu, R. K. Dagda, G. Uechi, S. J. Cherra, 3rd, A. M. Gusdon, . . . C. T. Chu (2014). "ERK-mediated phosphorylation of TFAM downregulates mitochondrial transcription: implications for Parkinson's disease." Mitochondrion 17: 132-140.
Wang, W., X. Wang, H. Fujioka, C. Hoppel, A. L. Whone, M. A. Caldwell, . . . X. Zhu (2016). "Parkinson's disease-associated mutant VPS35 causes mitochondrial dysfunction by recycling DLP1 complexes." Nat Med 22(1): 54-63.
Wang, X., C. Blagden, J. Fan, S. J. Nowak, I. Taniuchi, D. R. Littman and S. J. Burden (2005). "Runx1 prevents wasting, myofibrillar disorganization, and autophagy of skeletal muscle." Genes Dev 19(14): 1715-1722.
Wang, Y. X., C. L. Zhang, R. T. Yu, H. K. Cho, M. C. Nelson, C. R. Bayuga-Ocampo, . . . R. M. Evans (2004). "Regulation of muscle fiber type and running endurance by PPARdelta." PLoS Biol 2(10): e294.
Watt, M. J., R. J. Southgate, A. G. Holmes and M. A. Febbraio (2004). "Suppression of plasma free fatty acids upregulates peroxisome proliferator-activated receptor (PPAR) alpha and delta and PPAR coactivator 1alpha in human skeletal muscle, but not lipid regulatory genes." J Mol Endocrinol 33(2): 533-544.
Weidberg, H., E. Shvets and Z. Elazar (2011). "Biogenesis and cargo selectivity of autophagosomes." Annu Rev Biochem 80: 125-156.
Weidberg, H., E. Shvets, T. Shpilka, F. Shimron, V. Shinder and Z. Elazar (2010). "LC3 and GATE-16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis." EMBO J 29(11): 1792-1802.
Westermann, B. (2012). "Bioenergetic role of mitochondrial fusion and fission." Biochim Biophys Acta 1817(10): 1833-1838.
White, J. P., A. N. Billin, M. E. Campbell, A. J. Russell, K. M. Huffman and W. E. Kraus (2018). "The AMPK/p27(Kip1) Axis Regulates Autophagy/Apoptosis Decisions in Aged Skeletal Muscle Stem Cells." Stem Cell Reports 11(2): 425-439.
Wiedemann, N. and N. Pfanner (2017). "Mitochondrial Machineries for Protein Import and Assembly." Annu Rev Biochem 86: 685-714.
Wilson, B. J., A. M. Tremblay, G. Deblois, G. Sylvain-Drolet and V. Giguere (2010). "An acetylation switch modulates the transcriptional activity of estrogen-related receptor alpha." Mol Endocrinol 24(7): 1349-1358.

Winder, W. W., B. F. Holmes, D. S. Rubink, E. B. Jensen, M. Chen and J. O. Holloszy (2000). "Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle." J Appl Physiol (1985) 88(6): 2219-2226.
Wohlgemuth, S. E., A. Y. Seo, E. Marzetti, H. A. Lees and C. Leeuwenburgh (2010). "Skeletal muscle autophagy and apoptosis during aging: effects of calorie restriction and life-long exercise." Exp Gerontol 45(2): 138-148.
Wojtaszewski, J. F., C. MacDonald, J. N. Nielsen, Y. Hellsten, D. G. Hardie, B. E. Kemp, . . . E. A. Richter (2003). "Regulation of 5'AMP-activated protein kinase activity and substrate utilization in exercising human skeletal muscle." Am J Physiol Endocrinol Metab 284(4): E813-822.
Wojtaszewski, J. F., P. Nielsen, B. F. Hansen, E. A. Richter and B. Kiens (2000). "Isoform-specific and exercise intensity-dependent activation of 5'-AMP-activated protein kinase in human skeletal muscle." J Physiol 528 Pt 1: 221-226.
Woldt, E., Y. Sebti, L. A. Solt, C. Duhem, S. Lancel, J. Eeckhoute, . . . H. Duez (2013). "Rev-erb-alpha modulates skeletal muscle oxidative capacity by regulating mitochondrial biogenesis and autophagy." Nat Med 19(8): 1039-1046.
Wright, D. C., P. C. Geiger, D. H. Han, T. E. Jones and J. O. Holloszy (2007). "Calcium induces increases in peroxisome proliferator-activated receptor gamma coactivator-1alpha and mitochondrial biogenesis by a pathway leading to p38 mitogen-activated protein kinase activation." J Biol Chem 282(26): 18793-18799.
Wright, D. C., D. H. Han, P. M. Garcia-Roves, P. C. Geiger, T. E. Jones and J. O. Holloszy (2007). "Exercise-induced mitochondrial biogenesis begins before the increase in muscle PGC-1alpha expression." J Biol Chem 282(1): 194-199.
Xu, S., P. Wang, H. Zhang, G. Gong, N. Gutierrez Cortes, W. Zhu, . . . W. Wang (2016). "CaMKII induces permeability transition through Drp1 phosphorylation during chronic beta-AR stimulation." Nat Commun 7: 13189.
Yamamoto, A., Y. Tagawa, T. Yoshimori, Y. Moriyama, R. Masaki and Y. Tashiro (1998). "Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells." Cell Struct Funct 23(1): 33-42.
Yamazaki, Y., Y. Kamei, S. Sugita, F. Akaike, S. Kanai, S. Miura, . . . Y. Ogawa (2010). "The cathepsin L gene is a direct target of FOXO1 in skeletal muscle." Biochem J 427(1): 171-178.
Yan, Z., P. Li and T. Akimoto (2007). "Transcriptional control of the Pgc-1alpha gene in skeletal muscle in vivo." Exerc Sport Sci Rev 35(3): 97-101.
Yan, Z., V. A. Lira and N. P. Greene (2012). "Exercise training-induced regulation of mitochondrial quality." Exerc Sport Sci Rev 40(3): 159-164.
Yang, L., P. Li, S. Fu, E. S. Calay and G. S. Hotamisligil (2010). "Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance." Cell Metab 11(6): 467-478.

Yoneda, M., T. Miyatake and G. Attardi (1994). "Complementation of mutant and wild-type human mitochondrial DNAs coexisting since the mutation event and lack of complementation of DNAs introduced separately into a cell within distinct organelles." Mol Cell Biol 14(4): 2699-2712.
Yoon, Y., E. W. Krueger, B. J. Oswald and M. A. McNiven (2003). "The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1." Mol Cell Biol 23(15): 5409-5420.
Youle, R. J. and A. M. van der Bliek (2012). "Mitochondrial fission, fusion, and stress." Science 337(6098): 1062-1065.
Zechner, C., L. Lai, J. F. Zechner, T. Geng, Z. Yan, J. W. Rumsey, . . . D. P. Kelly (2010). "Total skeletal muscle PGC-1 deficiency uncouples mitochondrial derangements from fiber type determination and insulin sensitivity." Cell Metab 12(6): 633-642.
Zhang, J. and P. A. Ney (2009). "Role of BNIP3 and NIX in cell death, autophagy, and mitophagy." Cell Death Differ 16(7): 939-946.
Zhao, J., J. J. Brault, A. Schild, P. Cao, M. Sandri, S. Schiaffino, . . . A. L. Goldberg (2007). "FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells." Cell Metab 6(6): 472-483.
Zhao, J., T. Liu, S. Jin, X. Wang, M. Qu, P. Uhlen, . . . M. Nister (2011). "Human MIEF1 recruits Drp1 to mitochondrial outer membranes and promotes mitochondrial fusion rather than fission." EMBO J 30(14): 2762-2778.
Zhao, Y., J. Yang, W. Liao, X. Liu, H. Zhang, S. Wang, . . . W. G. Zhu (2010). "Cytosolic FoxO1 is essential for the induction of autophagy and tumour suppressor activity." Nat Cell Biol 12(7): 665-675.
Zhong, J., W. Gong, L. Lu, J. Chen, Z. Lu, H. Li, . . . L. Wei (2017). "Irbesartan ameliorates hyperlipidemia and liver steatosis in type 2 diabetic db/db mice via stimulating PPAR-gamma, AMPK/Akt/mTOR signaling and autophagy." Int Immunopharmacol 42: 176-184.
Zhu, Y., S. Massen, M. Terenzio, V. Lang, S. Chen-Lindner, R. Eils, . . . N. R. Brady (2013). "Modulation of serines 17 and 24 in the LC3-interacting region of Bnip3 determines pro-survival mitophagy versus apoptosis." J Biol Chem 288(2): 1099-1113.
Zizola, C., P. J. Kennel, H. Akashi, R. Ji, E. Castillero, I. George, . . . P. C. Schulze (2015). "Activation of PPARdelta signaling improves skeletal muscle oxidative metabolism and endurance function in an animal model of ischemic left ventricular dysfunction." Am J Physiol Heart Circ Physiol 308(9): H1078-1085.
Zoncu, R., L. Bar-Peled, A. Efeyan, S. Wang, Y. Sancak and D. M. Sabatini (2011). "mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase." Science 334(6056): 678-683.