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# Role of oxidised protein repair for skeletal muscle homeostasis

Sofia Lourenço dos Santos

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Thèse de doctorat de Biologie Intégrative

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L'école doctorale ED515 - Complexité du Vivant

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L'unité de recherche UMR 8256 UPMC-CNRS, ERL INSERM U1164

Adaptation Biologique et Vieillesse

Equipe : Vieillesse Cellulaire Intégrée et Inflammation

## *Importance de la réparation des protéines oxydées pour l'homéostasie du muscle squelettique*

Présentée par

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Pour obtenir le grade de Docteur de l'Université Pierre et Marie Curie

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*“The greatest enemy of knowledge is not ignorance, it is the illusion of knowledge.”*

Stephen William Hawking



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## ABSTRACT

Muscle regeneration and the stem cells, also known as satellite cells, that are engaged in this process are a major biomedical issue, notably for treatment of muscular dystrophies. In homeostasis conditions, satellite cells are in a quiescent state, however, in response to a muscle injury they become activated and start proliferating to undergo myogenic differentiation or renew the stem cell pool. Although signalling pathways of muscle regeneration have been intensively studied, other aspects of this regulation, in particular the redox state remains unclear. The cellular redox state is characterised by a balance between the production of reactive oxygen species (ROS) and their elimination by the antioxidant systems. Methionine sulfoxide reductases (Msr) enzymes are responsible for the reduction of methionine oxidation, being important regulators of protein function. Importantly, this system is also involved in oxidative stress protection by preventing protein irreversible oxidation damages, which have been described in some muscular dystrophies as well as in aged muscle.

The objective of this work is to determine, for the first time, the role of the Msr enzymes in satellite cells function during regeneration as well as their role in skeletal muscle fibres protection during oxidative stress conditions, such as muscle ageing or some muscular dystrophies. Using human primary myoblasts as a model of myogenesis *in vitro*, we found that, by neutralising excessive ROS levels and intracellular protein damage accumulation, MsrA enzymes counteract myoblasts senescence. We observed also that replicative senescent myoblasts or myoblasts derived from a late onset muscular dystrophy present reduced levels of MsrA enzymes, suggesting that modulation of Msr enzymes could prevent cellular ageing as well as represent a valuable approach in complement to the cellular therapies already in use for the treatment of muscular dystrophies. Secondly, using muscle biopsies derived from adult or geriatric mice, we found that Msr activity decreases with age and would partly contribute to the accumulation of protein oxidative damages observed in geriatric muscle. This suggests that Msr, through protein quality control, could participate to muscle fibre function and homeostasis. Finally, Msr enzymes were found up-regulated in dystrophic muscle comparing to healthy ones, which could be a response to the increased dystrophic-associated oxidative stress, although not sufficient to counteract the pathological symptoms.

To conclude, our results suggest that Msr enzymes are important for skeletal muscle tissue homeostasis, being involved in muscle stem cell redox regulation during myogenesis as well as in muscle fibres protection against oxidative stress.



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## ABBREVIATIONS

3MA	3-methyladenine
4-HNE	4-hydroxynonenal
ACE	angiotensin I converting enzyme
ACTN3	alpha-actinin-3
AGE	advanced glycation end-products
ALE	advanced lipid peroxidation end products
AP-1	activator protein-1
Atg	autophagic gene
bHLH	basic-helix-loop-helix
BMD	Becker muscular dystrophy
CaM	calmodulin
CaMKII	calmodulin-dependent protein kinase II
CAT	catalase
CEL	carboxyethyllysine
CMD	congenital muscular dystrophy
CML	carboxymethyllysine
CNTF	ciliary neurotrophic factor
CRISPR	clustered regularly interspaced short palindromic repeats
CuZnSOD	mitochondria intermembrane copper-zinc SOD
DAP	dystrophin-associated protein
DCFDA	2,7-dichlorofluorescein diacetate
DD	distal muscular dystrophy
DIGE	difference gel electrophoresis
DM1	myotonic dystrophy type 1
DMD	Duchenne muscular dystrophy
DNP	2,4-dinitrophenylhydrazone
DNPH	2,4-dinitrophenylhydrazine
DTT	dithiothreitol
ECSOD	extracellular copper-zinc SOD
EDMD	Emery-Dreifuss muscular dystrophy
EdU	5-Ethynyl-2'-deoxyuridine
ERTS	endoplasmic reticulum targeting signal
FGF	fibroblast growth factors
FOXO	forkhead box O
FSHD	facioscapulohumeral muscular dystrophy
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFD11	growth differentiation factor 11
GFP	green fluorescent protein
GPx	glutathione peroxidase

GR	GSH reductase
Grx	glutaredoxin
GS	glutathione synthetase
GSH	glutathione
GSNO	S-nitrosoglutathione
GSSG	glutathione disulfide
HDAC	histone deacetylases
HeyL	hairy/enhancer-of-split related with YRPW motif-like protein
HGF	hepatocyte growth factor
HNE	4-hydroxynonenal
IGF	Insulin growth factor
INI	intra-nuclear inclusions
I $\kappa$ B $\alpha$	inhibitor of kappa B-alpha
Jak2	Janus kinase 2
keap1	kelch-like ECH associated protein 1
LGMD	limb girdle muscular dystrophy
LMNA	lamin A/C
MACS	magnetic-activated cell sorting
MAFbx	muscle atrophy F-box
MAPK	mitogen-activated protein kinase
MD	muscular dystrophies
MDA	malondialdehyde
MetRO	methionine-R-sulfoxide
MetSO	methionine-S-sulfoxide
MetO	methionine sulfoxide
MetO <sub>2</sub>	methionine sulfone
MHC, MLC	myosin heavy chain, myosin light chains
MMD	myotonic muscular dystrophy
MnSOD	manganese SOD
MRF	myogenic regulatory factors
MRF4, Myf6	myogenic factor 6
Msr	methionine sulfoxide reductase
MSTN	myostatin
mtDNA	mitochondrial DNA
MTS	mitochondrial targeting signal
MuRF	muscle Really Interesting New Gene Finger
Myf5	myogenic factor 5
MyoD	myogenic differentiation 1
NAC	N-acetyl-L-cysteine
NADH	nicotinamide adenine dinucleotide reduced
NADPH	nicotinamide adenine dinucleotide phosphate
NF- $\kappa$ B	nuclear factor-kappaB
NFAT	nuclear factor of activated T-cells
NOS	nitric oxide synthase

NOX	NADPH oxidase
Nrf2	nuclear factor-erythroid 2 related factor 2
OPMD	oculopharyngeal muscular dystrophy
ORF	open reading frame
OXPHOS	oxidative phosphorylation
<i>PABPN1</i>	poly(A) binding protein nuclear 1
Pax	paired box protein
PGC-1 $\alpha$	peroxisome proliferator activated receptor $\gamma$ coactivator-1 $\alpha$
PGPH	peptidylglutamyl-peptide hydrolase
PI3K	phosphatidylinositol-3-kinase
PKB	protein kinase B
Prx	peroxiredoxins
<i>Rbpj</i>	recombining binding protein suppressor of hairless
RNS	reactive nitrogen species
ROS	reactive oxygen species
RYR1	ryanodine receptor 1
SA- $\beta$ gal	senescence-associated beta-galactosidase
SECIS	selenocysteine insertion sequence
SEM	standard error of the mean
SEPN1	selenoprotein N
shRNA	short hairpin RNA
siRNA	small interfering RNA
SNO	S-nitrosothiol
SOD	superoxide dismutase
<i>Spry1</i>	sprouty1
SSG	S-glutathionylates
Stat3	signal transducer and activator of transcription 3
T-tubules	transverse tubules
TGF-b	transforming growth factor beta
Tie2	TEK tyrosine kinase
TNF-a	tumour necrosis factor-a
TR	thioredoxin reductase
Trx	thioredoxin
Ub	ubiquitin
UPS	ubiquitin-proteasome system
VDAC	voltage-dependent anion channel
VDR	vitamin D receptor
W.T.	wild type
X-Gal	5-Bromo-4-chloro-3-indolyl $\beta$ -D-galactopyranoside
XO	xanthine oxidase





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# **INTRODUCTION**



# **I. The Skeletal Muscle**

Within muscle tissues, three different types of muscles, namely cardiac, smooth and skeletal muscles can be distinguished. The contraction of both cardiac and smooth muscle occurs involuntarily, thus being controlled by the autonomic nervous system. Their main functions are the insurance of blood circulation in the case of cardiac muscle while smooth muscles act to maintain the elasticity and movement of internal organs.

Skeletal muscles represent on average 40% of the human body weight. Their contraction can be voluntary when finely controlled by the central nervous system or involuntary when their innervation comes from the spinal cord. Skeletal muscles, anchored to bone by tendons, generate the force and movement needed to locomotion, postural behaviour and breathing. If the main function of skeletal muscles is the production of force and movement needed for contraction, it also participates to the thermoregulation, to metabolic homeostasis and represents a major source of proteins for the whole organism.

## **Skeletal Muscle Anatomy**

The skeletal muscle tissue is composed of several cell types and is well organised in different layers. The epimysium, a layer of connective tissue, covers the entire muscle and protects it from friction against other muscles and bones (Figure 1). This layer is continuous with tendons, where it becomes thicker, and composed of collagen to allow the formation of the muscular tendinous junction. Arteries, required for muscle oxygenation and nutrients intake, and other structures like nerves and the lymph are also enveloped by this structure. Continuous with the epimysium is the perimysium, a sheath of connective tissue that encloses fibre's bundles or fascicles of up to 150 muscle fibres (Figure 1). The last connective tissue is a thin elastic layer called endomysium or basal lamina that separates skeletal muscle fibres or myofibres (Figure 1). This structure consists mainly of reticular fibres composed of collagen that protect muscle fibres and drive muscle contraction within a given direction.

Myofibres are giant cells that can reach 30 cm long with a diameter from 10 to 100  $\mu\text{m}$  (Figure 1). They are post-mitotic multinucleated cells that originate from the fusion of a large number of mono-nucleated progenitor cells. Although adult skeletal muscle tissue is mainly composed of post-mitotic muscle fibres, it also contains fibroblasts, endothelial cells and myogenic adult stem cells called satellite cells (described later in chapter II) (Figure 1).



Vertebrate's skeletal muscles have a striated appearance due to myofibrils, the cross-striated muscle contractile proteins (Figure 1 and 2). Muscle contractile apparatus is the main constituent of the myofibre cytoplasm called sarcoplasm. The others organelles represent only 20% of the total sarcoplasm volume and are mostly: mitochondria – important to give the necessary ATP for the muscular contraction; nuclei – between 40 and 500 depending on the fibre size; sarcoplasmic reticulum – sandwiched between sarcolemma membranous invaginations called transverse tubules (T-tubules) and participating in muscular contraction; and cytoskeleton elements – which join myofibrils to the extracellular matrix.

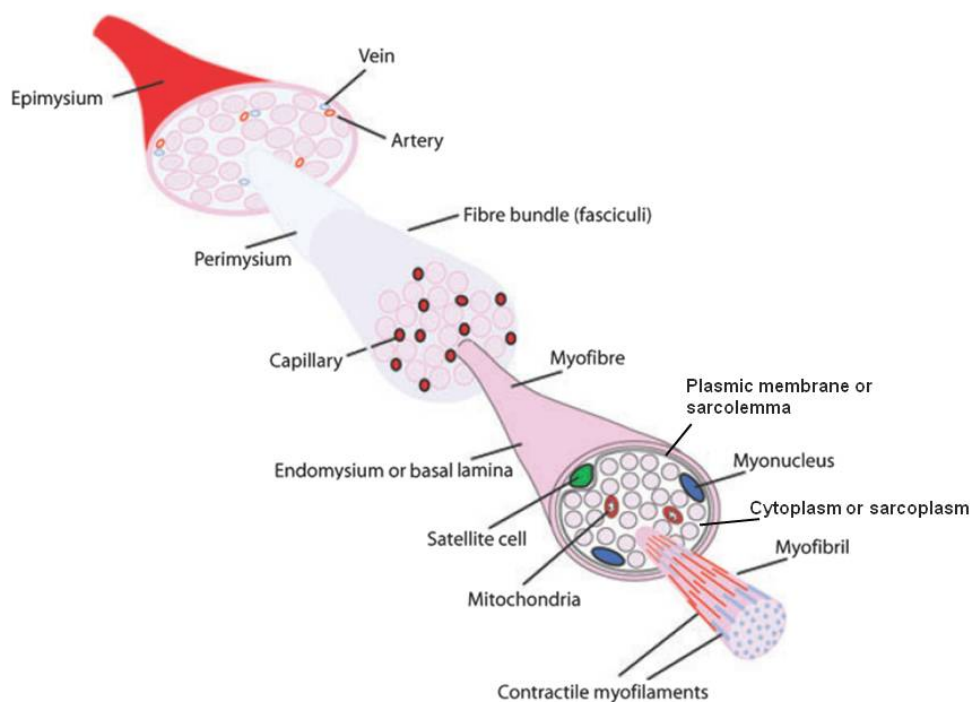


Figure 1 - **Skeletal muscle anatomy**. Muscle tissue is surrounded by the epimysium, which groups myofibres bundles (fasciculi) and blood vessels. These myofibre bundles are delimited by a connective tissue layer, the perimysium and assemble various myofibres infiltrated with a large capillary network. Individual myofibres, enveloped by an endomysium or basal lamina, are composed of multiple myonuclei, mitochondria and myofibrils that contain the contractile proteins. Between the sarcolemma and the basal lamina of the myofibres lie the skeletal muscle adult stem cells, the satellite cells. Adapted from (Otto, Collins-Hooper et al., 2009).

Myofibrils can measure from 1 to 2  $\mu\text{m}$  in diameter and are composed of structural and contractile elements. Between the structural elements are titin - an elastic protein that stabilizes the sarcomere and is implicated on the driving force and nebulin - a stiff protein used as matrix by the contractile elements. Two types of contractile filaments exist within

myofibrils: the thin myofilaments, measuring 7 nm in diameter and composed of actin, and the thick myofilaments, essentially composed of myosin and having 15 nm in diameter (Figure 2a and b). Repeats of thin and thick filaments between successive Z-bands (Z-discs) along the myofibril are called sarcomeres (Figure 2b and c). Electron microscopy and low-angle X-ray fibre diffraction helped to define the structure of these muscle repeating units (Huxley, 1957, Huxley, 1969). In vertebrate muscles sarcomeres are approximately 2  $\mu\text{m}$  long in a resting muscle but vary in length as the muscle stretches. Within sarcomeres, myosin filaments are crosslinked into dark arrays at the M-line of the A-bands, while the overlapping actin filaments are crosslinked in the Z-disk within the light I-band (Figure 2b and c) (Gautel, 2011).

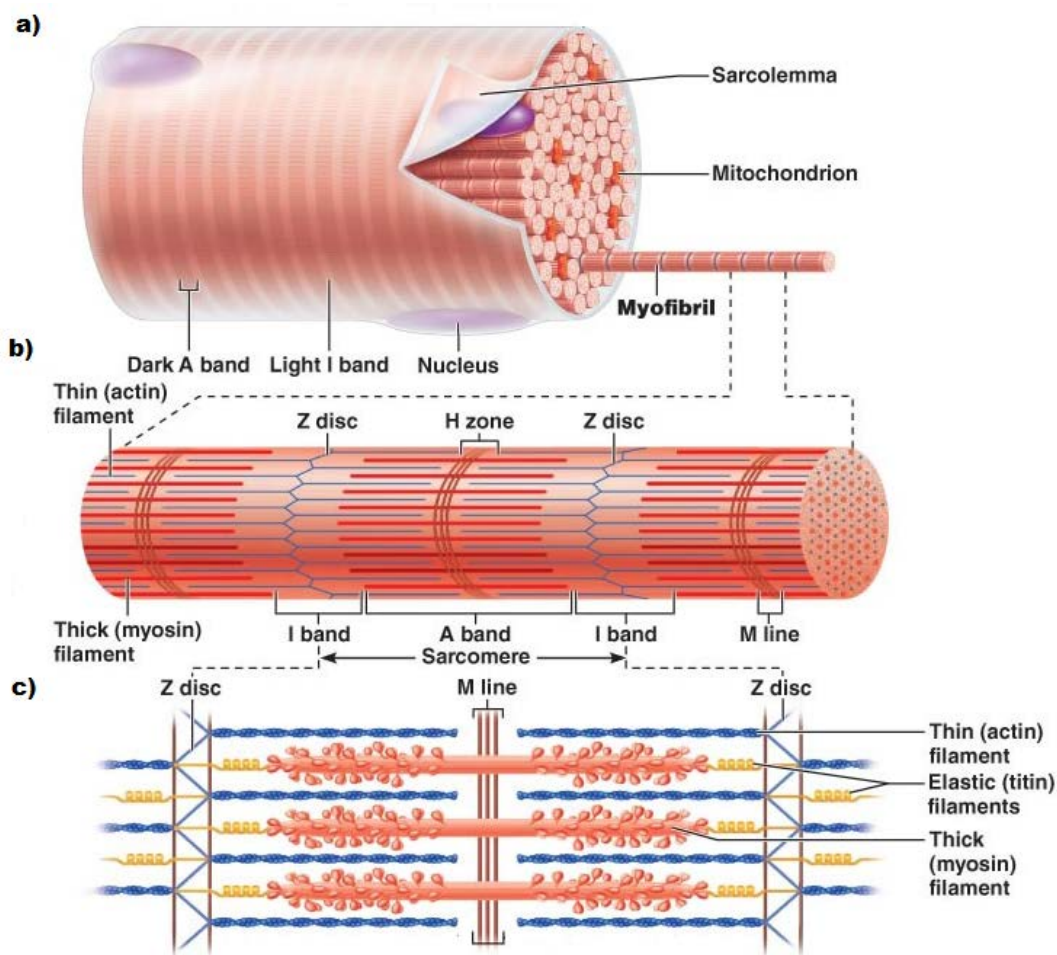


Figure 2 – **Levels of functional organisation of skeletal muscle.** a) **Muscle Fibres:** muscle fibres are cylindrical multinucleated cells bounded by a plasmatic membrane (sarcolemma) and composed by myofibrils; b) **Myofibrils:** muscle myofibrils are composed of thin and thick myofilaments containing mainly actin and myosin contractile proteins, respectively; c) **Sarcomere:** the myofibril repeating unit is compromised between two Z-bands. Adapted from © 2013 Pearson and Education, Inc.

## Skeletal Muscle Contraction

Muscles contract by sliding the thin actin and thick myosin filaments along each other (Squire, Al-khayat et al., 2005). Besides myosin and actin, muscle filaments have also regulatory proteins needed for contraction, such as troponin and tropomyosin which regulate muscle contraction in response to fluctuations in intracellular calcium concentration (Scott, Stevens et al., 2001). Tropomyosin is a two-stranded alpha-helical coiled coil homo or heterodimer protein that lies along the alpha-helical groove of actin filaments, covering its binding sites (Figure 3). Skeletal muscle troponin relies at each tropomyosin extremity and consists of 3 subunits: troponin I (TnI), which inhibits the myosin  $Mg^{2+}$ -ATPase activity; troponin C (TnC), which binds calcium ions ( $Ca^{2+}$ ) and abolishes TnI-mediated inhibition of actin-myosin cross-bridge formation; and troponin T (TnT), which is the tropomyosin-binding subunit (Figure 3) (Leavis & Gergely, 1984, Wei & Jin, 2011).

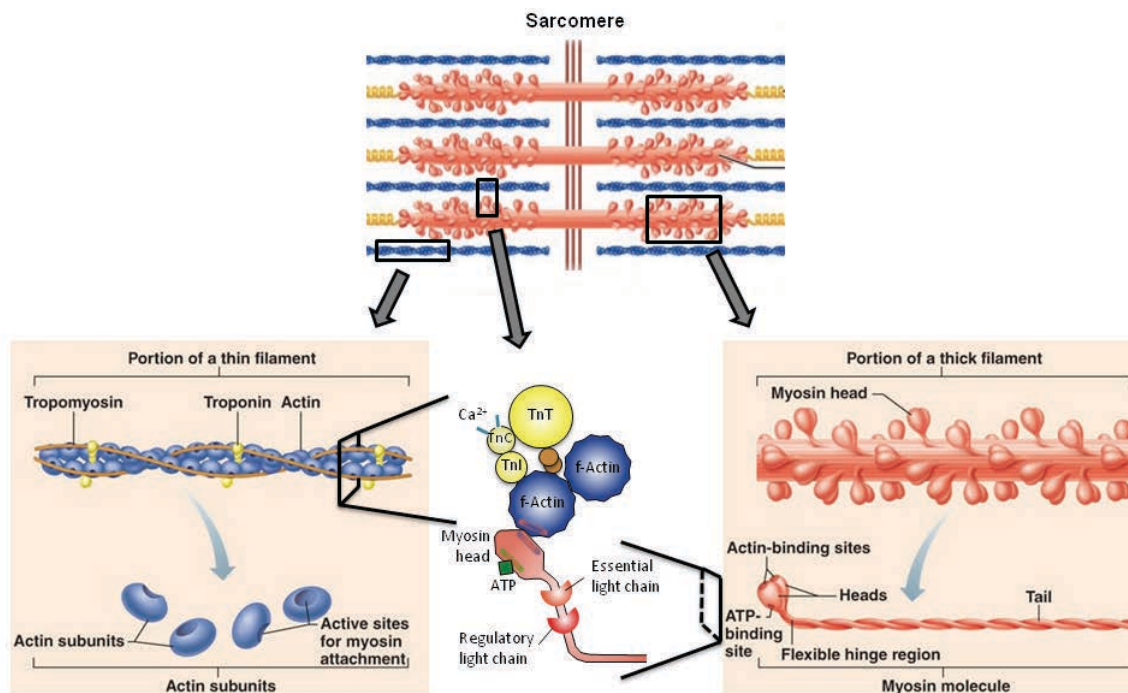


Figure 3 - **Structure and composition of thin actin and thick myosin filaments.** Thin filaments consist of two strands of actin subunits forming a helix in close relation with two regulatory proteins, tropomyosin and troponin. Binding of calcium ions ( $Ca^{2+}$ ) on troponin C (TnC) subunit results in myosin binding site exposing, allowing actin and myosin coupling. Thick filaments are composed of many myosin molecules whose heads bend in all opposite directions. Myosin light chains situated on the neck of myosin heavy chains regulate myosin ATPase activity. Adapted from © 2013 Pearson and Education, Inc and from (Boron & Boulpaep, 2012).

Within thick filaments, myosin is a hexamer composed of 4 myosin light chains (MLC) and 2 enrolled myosin heavy chains (MHC). Between the 4 light chains, two of them

stabilize the myosin head region and the other two regulate the MHC ATPase activity via their phosphorylation by  $\text{Ca}^{2+}$ -dependent and independent kinases (Figure 3). The two MHC are divided in 3 regions: the tail, the neck or hinge and the head. The head of the myosin molecule has a site that binds to actin to form crossbridges and an ATPase site that hydrolyses ATP (Goody, Hofmann et al., 1977). When ATP binds to myosin head, it causes myosin releasing from actin (step 1 on Figure 4). Hydrolysis of ATP into adenosine diphosphate (ADP) and inorganic phosphate (Pi) by the myosin ATPase releases the energy necessary to give myosin a high-energy conformation (step 2 on Figure 4) necessary for subsequent actin binding (step 3 on Figure 4). Pi release will make myosin head turn into the middle of the sarcomere taking actin thin filaments with it through its attachment into the adjacent actin subunit (cross-bridge formation; step 4 on Figure 4). Finally, ADP is also released, myosin returns back to its low energy state and the actin-myosin bridge acquires a rigid state (step 5 on Figure 4). A new cross-bridge cycle can then re-start by binding of a new ATP on myosin head. Indeed, muscle contraction depends on high-energy fluxes. The renewal of ATP used by the myosin ATPase for muscle contraction cycles arises mainly from three sources: creatine phosphate, glycogen / glycolysis and ATP generated directly from cellular respiration in muscle fibres mitochondria (McLeish & Kenyon, 2005).

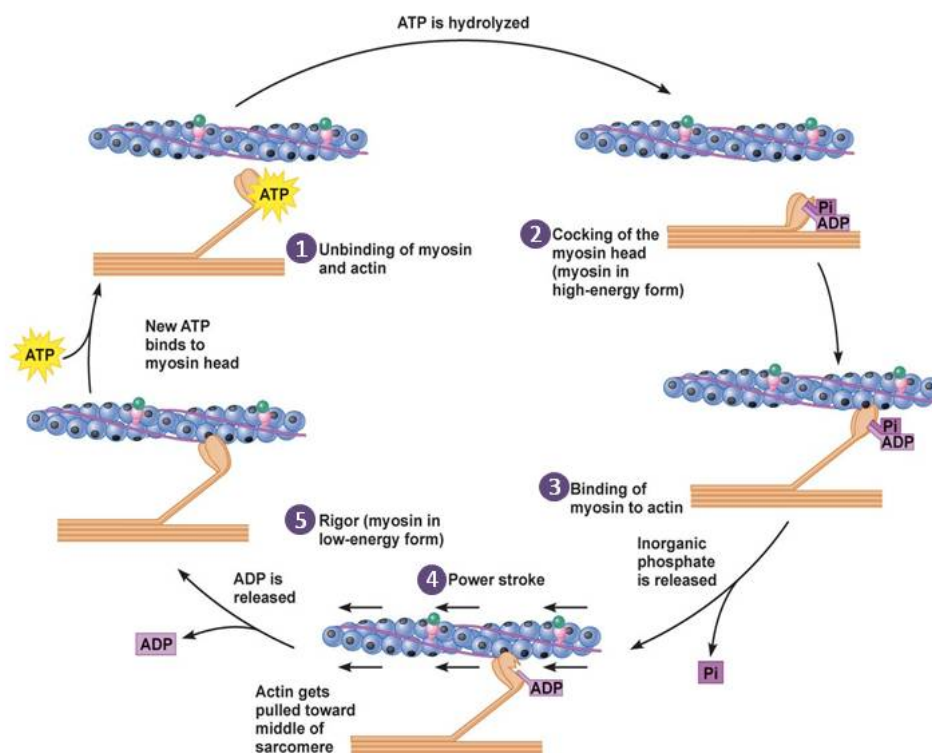


Figure 4 - **The cross-bridge cycle in skeletal muscle contraction.** During muscle contraction, myosin contractile protein converts the energy of ATP hydrolysis in mechanical energy. From © 2011 Pearson and Education, Inc.

## Skeletal Muscle Fibre Types

The heterogeneity of mammalian skeletal muscle fibres is the base of the flexibility that allows the same muscle to be used for various tasks such as continuous low-intensity activity (for example, posture), repeated small contractions (e.g. locomotion), or fast and strong contractions (such as jumping). The diversity of muscle fibres resides on different functional muscle cells compartments such as contractile machinery, cytoskeleton scaffold, energy supply systems, membrane excitation and excitation-contraction coupling (Table 1).

The first fibre type classification grouped two types of muscle fibres distinguished on the basis of their colour, red or white, and their contractile properties, fast and slow. Slow red muscles (type I) were the ones composed of fibres rich in myoglobin and mitochondria, with an oxidative metabolism and involved in continuous, tonic activity. On the other hand, fibres with small amounts of myoglobin and mitochondria, relying more on glycolytic metabolism and involved in phasic activity, were named fast white muscles (type II) (Needham, 1926). Years after, this classification was replaced by another based on muscle fibres mitochondrial content, MHC isoforms and metabolic enzymatic activities, giving rise to three major fibre types: slow (type I), fast highly oxidative and relatively fatigue resistant (type IIA) and fast weakly oxidative or fast-glycolytic with low fatigue resistance (type IIB). A third phase of muscle fibre type classification appeared with the discovery in rodents and later in humans of a new MHC fast type, called IIX that had properties between the MHC IIA and IIB (Schiaffino, Gorza et al., 1989, Smerdu, Karsch-Mizrachi et al., 1994). In addition, based on histochemical analysis of muscle sections and single fibres biochemical and physiological studies (Bottinelli, Betto et al., 1994, DeNardi, Ausoni et al., 1993, Gorza, 1990, Pette & Staron, 1990), seven types of skeletal muscle fibres were defined with pure or hybrid MHC composition: I, I/IIA, IIA, IIA/2X, IIX, IIX/IIB and IIB from slowest to fastest (Pette, Peuker et al., 1999, Scott et al., 2001, Staron, 1997). However, due some inconsistencies such as the discovery of fibres coexpressing type I and IIX but not IIA MHCs (Caiozzo, Baker et al., 2003, Schiaffino et al., 1989) or the fact that, depending on the method used, different laboratories would classified the same fibre differently, the actual reference to distinguish mammalian muscles fibre heterogeneity and plasticity is based only on four major fibre types (one slow and three fast fibre types) (Table 1) (Schiaffino & Reggiani, 2011).

Regardless of the classification system used to describe muscle fibres, there is overwhelming evidence that muscle fibres not only change in size in response to demands,

but also convert one type to another depending on different stimulus (e.g., training and rehabilitation, ageing). This plasticity in contractile and metabolic properties in response to stimuli allows for adaptation to different functional demands. Fibre conversions between type IIB and type IIA are the most common, but type I to type II conversions are possible (Ricoy, Encinas et al., 1998). For example, in humans, a decrease in muscle use after a previously high activity leads to slow to fast conversion, with shifts from MHC IIA to MHC IIX and MHC I to MHC IIA (Bogdanis, 2012). With this, there is also a concomitant decrease in the enzymes associated with aerobic-oxidative metabolism. Furthermore, during ageing, there are also adaptations of the MHC isoforms content with an overall shift occurring in fast to slow direction: MHC IIX to MHC IIA or MHC IIA to MHC I, mainly due to denervation of fast fibres and their reinnervation by slow motor units (Schiaffino & Reggiani, 2011).

Table 1 - Muscle fibre types characteristics.

	<i>Type I</i>	<i>Type IIA</i>	<i>Type IIX</i>	<i>Type IIB</i>
<b><i>Denomination</i></b>	Slow	Fast resistant, Fast oxidative	Fast intermediate	Fast fatigable, Fast glycolytic
<b><i>Colour</i></b>	red	red to pink	white	white
<b><i>Capillary density</i></b>	high	intermediate	low	low
<b><i>Mitochondria density</i></b>	very high	high	intermediate	low
<b><i>Metabolic preference</i></b>	oxidative	oxidative & glycolytic	glycolytic & oxidative	glycolytic
<b><i>Myosin heavy chain protein</i></b>	MHC-I/ $\beta$	MHC-IIA	MHC-IIX	MHC-IIB
<b><i>Myosin heavy chain gene</i></b>	MYH7	MYH2	MYH1	MYH4
<b><i>Myosin ATPase activity</i></b>	low	high	high	high
<b><i>Major ATP source</i></b>	triglycerides	glycogen & creatine phosphate	glycogen & creatine phosphate	glycogen & creatine phosphate
<b><i>Peak force</i></b>	low	intermediate	large	extremely large
<b><i>Resistance to fatigue</i></b>	high	relatively high	intermediate	low
<b><i>Contraction velocity</i></b>	slow	rapid	rapid	very rapid

## Skeletal Muscle Ageing

Skeletal muscle ageing is an inevitable biological process usually associated with a decrease in mass, strength and velocity of contraction. In humans, the age-associated loss of muscle strength and power starts at the age of 30 and 40 years, respectively (Deschenes, 2004). Skeletal muscle mass, particularly in muscles essential for locomotion, decreases of 25% until 60 years old (Deschenes, 2004, Janssen, Heymsfield et al., 2000). Between 60 and 80 years old, humans lose about 40% of their muscle mass and more than 50% after 90 years (Deschenes, 2004, Doherty, 2003, Janssen et al., 2000). The loss of muscle mass associated with ageing was named sarcopenia (from the greek: *sarx* which means “flesh” and *penia* which means “loss”) by Dr. Irwin Rosenberg (Rosenberg, 1997). Since this first classification, different definitions of sarcopenia have been promulgated (Cruz-Jentoft & Morley, 2012, Drescher, Konishi et al., 2016, Fielding, Vellas et al., 2011, Morley, Abbatecola et al., 2011, Rosenberg, 2011). They all clearly refer to an age-associated quantitative and qualitative loss of muscle function, meaning a loss of muscle mass and the appearance of muscle weakness and/or impaired performance (slow walking speed). Sarcopenia may also be associated with the loss of muscle power (force x velocity), particularly in older people with mobility limitations.

At tissue level, sarcopenia is accompanied by morphological changes of skeletal muscle including a remodelling of muscle fibres spatial arrangement (Thom, Morse et al., 2007) and an increase of 6 % to 15 % in the non-contractile muscle mass fraction (Taaffe, Henwood et al., 2009). Regarding muscle fibres, the loss of muscle mass involves a decrease in both muscle fibre size (atrophy) and number (hypoplasia). Although skeletal muscle ageing is commonly associated with a reduction in the number of both type I and type II skeletal muscle fibres, type II fibres are more atrophy vulnerable during ageing than type I muscle fibres (Schiaffino & Reggiani, 2011). However, in terms of muscle contraction, MHC I positive fibres showed low velocity of contraction with age, while those expressing MHC IIA did not presented any changes (Korhonen, Cristea et al., 2006). Thus, the increased type II fibre atrophy and the reduced velocity of contraction in type I fibres play a main role in the decline of muscle force during ageing. In parallel to muscle fibre atrophy and hypoplasia, sarcopenic muscle is characterised by an accumulation of intramuscular fat (myosteatorsis) and tissue fibrosis (collagen infiltration called myofibrosis) (Budui, Rossi et al., 2015, Taaffe et al., 2009, Zoico, Corzato et al., 2013). Both processes result in a decrease of muscle strength and contractility and to an increased rigidity of the tissue (Goodpaster,

Carlson et al., 2001). In addition, losses of  $\alpha$ -motoneurons by apoptosis together with the reduction of the conduction velocity lead to the progressive denervation of muscle fibres (Doherty, 2003, Edstrom, Altun et al., 2007, Kwan, 2013). Taken together, these events not only result in a decrease of coordination and strength but also trigger muscle fibres apoptosis by reducing the production of trophic factors.

At cellular and molecular levels, a number of modifications have been reported in sarcopenic muscle fibres that can explain the morphological and functional changes cited above. Thus, altered structure of the sarcoplasmic reticulum resulting in segregation of the calcium stores ultimately leads to impairment of the excitation-contraction coupling (Weisleder, Brotto et al., 2006). Similarly, several studies have reported in aged muscle a decrease in the level of structural and metabolic muscle proteins, such as myosin heavy chains (mainly MHC IIA and MHC IIX isoforms) (Balagopal, Schimke et al., 2001, Karakelides & Nair, 2005, Rooyackers, Adey et al., 1996).

It is important to note that in addition to the events affecting the muscle fibre, sarcopenic muscle is also characterised by a main reduction of its ability to regenerate compared to young muscle. This phenotype, which is due to reduction of both the number and the regenerative capacities of satellite cells will be discussed further in detail in chapter II (Blau, Cosgrove et al., 2015, Sousa-Victor, Garcia-Prat et al., 2015).

Sarcopenia is now recognised as a major clinical problem for older people and research in the area is expanding exponentially. However, despite this growing interest, its etiology is still unknown. In order to counteract modifications occurring in both muscle structure and function during ageing, it is therefore essential to understand the mechanisms involved in the maintenance of skeletal muscle.

Among the different hypotheses that have been suggested, a reduction in the number of satellite cells as well as a decline of their function has been proposed (Cosgrove, Gilbert et al., 2014, Sousa-Victor, Gutarra et al., 2014). However, a recent study has clearly refuted this hypothesis, demonstrating that mice whose skeletal muscles are depleted of satellite cells exhibit similar features of sarcopenia than control mice (Fry, Lee et al., 2015). Therefore, satellite cells are dispensable for the maintenance of normal adult and ageing muscles.

One of the most evident explanations for the loss of muscle mass observed during ageing is an imbalance between protein synthesis and degradation (Figure 5). Indeed, major age-dependent alterations in muscle proteolysis have been observed in aged muscle. Most of them are due to a lack of responsiveness of the ubiquitin-proteasome-dependent proteolytic



pathway to anabolic and catabolic stimuli (Karakelides & Nair, 2005, Nair, 1995, Toth, Matthews et al., 2005) and to alterations in the regulation of autophagy (Combaret, Dardevet et al., 2009).

Researchers have also been interested in a potential role for reactive oxygen species (ROS) in the process of ageing for over 60 years. ROS can cause oxidative damage to surrounding structures, and at a cellular level, the accumulation of oxidative damages occurring during life may contribute to the ageing phenotype, especially in those tissues in which ROS generation is more pronounced (Jackson, 2009). Skeletal muscle is one of the most metabolically active tissues in the organism. Given its elevated energetic demand, this tissue is characterised by a high mitochondrial mass and a constant mitochondrial biogenesis. Mitochondria are the main producers of cellular energy through oxidative phosphorylation. By consequence, they also constitute the major source of ROS essentially produced by the electron transport chain. Transcriptomic and proteomic analysis of young and ageing human, rat and mouse muscles have revealed progressive alterations related to oxidative metabolism, including increased mitochondrial ROS production, impaired mitochondrial function, and accumulation of oxidative damaged proteins (Barreiro, Coronell et al., 2006, Choksi, Nuss et al., 2008, Marzani, Felzani et al., 2005, Siu, Pistilli et al., 2008, Snow, Fugere et al., 2007, Thompson, Durand et al., 2006). Notably, the accumulation of damaged proteins leads to their aggregation and to the formation of proteolysis-resistant species. Such products have been shown to inhibit the proteasome activity, resulting into accumulation of misfolded, non-functional proteins (Friguet & Szweda, 1997, Grune, Jung et al., 2004). The consequences of excessive ROS and of accumulation of damaged and non-functional proteins in muscle tissue are multiples. Several studies have revealed their negative impacts on action-potential conduction, excitation–contraction coupling, the function of contractile proteins and the efficiency of mitochondrial respiration in muscle fibre (Andersson, Betzenhauser et al., 2011, Malinska, Kudin et al., 2012, Merry & McConell, 2012, Reid, 2001, Schoneich, Viner et al., 1999, Thompson et al., 2006). While these studies do not show that oxidative damage plays a role in the skeletal muscle ageing process directly, they clearly argued that it is important in contributing to several aspects of muscle ageing, including muscle atrophy and loss of muscle strength and function (Howard, Ferrucci et al., 2007, Min, Smuder et al., 2011, Powers, Smuder et al., 2011, Powers, Smuder et al., 2012). As previous mentioned, mitochondria are considered as the main source of ROS produced during aerobic metabolism. In view of their close proximity to ROS, these organelles, and notably their DNA are also their primary targets. Accumulation of

mitochondrial DNA and protein oxidative damages has been shown to lead to decreased mitochondria number, size and function in aged muscle (Barazzoni, Short et al., 2000, Wanagat, Cao et al., 2001). Resulting mitochondrial dysfunction leads to an impaired metabolic rate and energy production, together with increased ROS production, which in turn affect cell viability through necrosis and/or apoptosis of muscle fibres (Bua, McKiernan et al., 2002, Dirks, Hofer et al., 2006, Herbst, Pak et al., 2007, Wanagat et al., 2001). Finally, muscle fibres death accounts for the loss of muscle mass occurring in sarcopenia. To conclude, while these results clearly support the notion that muscle ageing correlates with deregulation of the myofibre redox state, whether such deregulation is a cause or a consequence of the muscle ageing process is still a subject of debate.

In addition to the intrinsic muscle events described above, a large panel of extrinsic factors have been proposed to contribute to the loss of muscle mass and strength during ageing (Figure 5). Among them, hormonal deregulations play a central role: while in men, the loss of muscle mass is significantly associated with a reduction of testosterone serum levels, in women it is mainly associated with oestrogen serum levels (Baumgartner, Waters et al., 1999). Similarly, changes in hormones such as insulin, growth hormone, prolactin, thyroid hormones, catecholamines and corticosteroids have been correlated to the loss of muscle mass and strength (Rolland, Czerwinski et al., 2008), and growth hormone and/or estrogens replacement therapies were shown efficient to lower fat mass and to increase lean body mass (Dionne, Kinaman et al., 2000, Rolland, Perry et al., 2007). Several non-muscular age-associated diseases have been proposed to contribute to the process of sarcopenia. Thus, high levels of pro-inflammatory cytokines as well as reduction of the vascular endothelial function (Degens, 1998) were associated with an increased risk of muscle mass and strength loss and to fatigue resistance failure (Giovannini, Onder et al., 2011, Harris, Ferrucci et al., 1999, Schaap, Pluijm et al., 2006). Physical activity is also an important predictor of muscle mass maintenance in both sexes (Baumgartner et al., 1999), with less physically active older adults being more likely to gain sarcopenic features compared with physically active older individuals (Freiberger, Sieber et al., 2011, Lee, Auyeung et al., 2007, Rolland et al., 2008). Similarly, healthy young and old volunteers lose almost 1 kg of muscle leg mass after 14 and 10 days of bed rest respectively and this was accompanied by a decrease in muscle strength, protein synthesis and aerobic capacity (Kortebein, Ferrando et al., 2007, Kortebein, Symons et al., 2008, Paddon-Jones, Sheffield-Moore et al., 2006). Another cellular consequence of physical activity is the age-associated loss of mitochondrial content and function that can be partially reversed by exercise training (Broskey, Greggio et al., 2014). Apart from physical

activity, other life style related factors are known to affect muscle metabolic homeostasis in the elderly. This is the case of nutrition, smoking and alcohol consumption (Figure 5). Indeed, while almost 40 % of individuals of more than 70 years old do not meet the recommended dietary allowance in proteins (Houston, Nicklas et al., 2008), protein supplementation trials in the elderly led to improvements in physical performance (Daly, O'Connell et al., 2014, Tieland, van de Rest et al., 2012), in lean body weight (body weight minus body fat weight), in muscle strength as well as in muscle protein synthesis (Dillon, Sheffield-Moore et al., 2009, Kim, Suzuki et al., 2012). Lastly, smoking and alcohol consumption also increase the risk of sarcopenia by impairing muscle protein synthesis (Petersen, Magkos et al., 2007) as well as proteases activity (Preedy, Adachi et al., 2001, Reilly, Mantle et al., 1997).

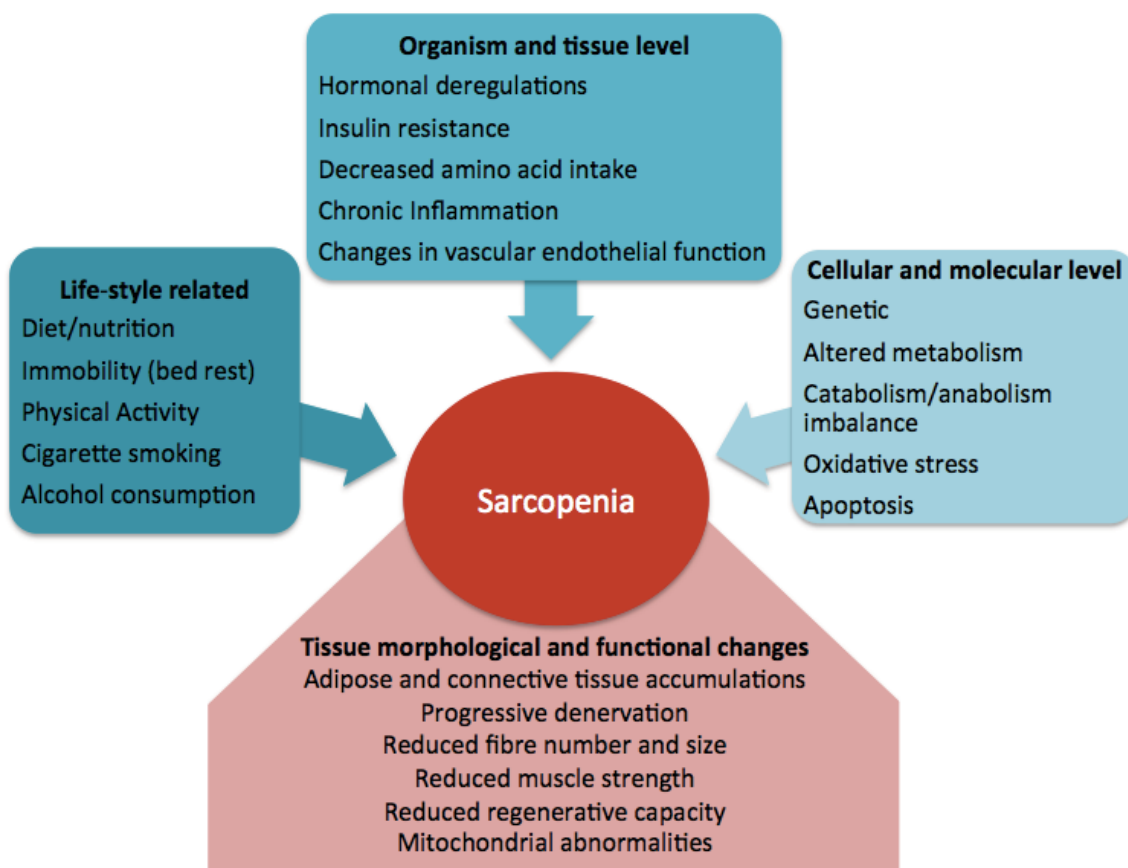


Figure 5 - **Multifactorial bases of sarcopenia and its phenotype.** Different causes of muscle ageing are grouped at different levels (life-style related, organism and tissue level as well as cellular and molecular level). Sarcopenic phenotype presents different skeletal muscle tissue morphological and functional.

Finally, although a strong genetic determination has been reported for muscle mass and muscle strength features (Huygens, Thomis et al., 2004, Kostek, Hubal et al., 2011, Thomis, Beunen et al., 1998, Tiainen, Sipila et al., 2004, Zhai, Stankovich et al., 2004), only a few studies have explored the role of potential candidate genes in determination of muscle strength. To date, five genes (*ACE*, *ACTN3*, *MSTN*, *CNTF* and *VDR*) have been associated with sarcopenia (Garatachea & Lucia, 2013, Tan, Liu et al., 2012). A great interest has been shown towards the *MSTN* gene, which encodes a specific myokine, called myostatin since its gene expression is up-regulated in old individuals compared to young subjects (Leger, Derave et al., 2008), and increase on its protein level in serum was correlated with reduced muscle mass in the elderly (Yarasheski, Bhasin et al., 2002).

It is clear now that sarcopenia is a result of different intrinsic and extrinsic changes occurring during ageing and the fact that muscle ageing is a result of multifactorial causes is hampering the research for efficient treatments to improving muscle function.

## Muscular Dystrophies

Muscular dystrophies (MD) are a group of highly heterogeneous inherited myopathies belonging to the family of neuromuscular diseases. They include over 30 different diseases caused by different genetic mutations but are all characterised by progressive skeletal muscle weakness, atrophy and muscle replacement by fat and/or connective tissue (Emery, 2002). Furthermore, most of these diseases are characterised by repeated cycles of muscle degeneration/regeneration, leading to chronic mobilization of the stem cells pool and in some cases to its final exhaustion.

MD can be transmitted in a dominant or recessive manner, with variable frequency depending on the disease, or they can be sporadic, caused by *de novo* mutations. Apart from their mode of inheritance and the prevalence of the disease, a large variability is observed between the different types of MD for the age of onset, the muscles affected, the severity, the rate of progression and the subsequent complications. Thus, while muscle weakness mainly affects limb, axial and facial muscles, in specific types of MD, other muscles (e.g. respiratory or swallowing skeletal muscle) as well as other organs (cardiac and smooth muscles, eye) can also be affected. MD can be very debilitating, eventually leading to loss of ambulation, difficulty of eating, and, for the most severe cases, to respiratory complications, cardiac defects and eventually to death (Shin, Tajrishi et al., 2013).

According to the distribution of predominant muscle weakness, eight major forms of MD can be defined, six of them being illustrated in Figure 6: Duchenne and Becker muscular dystrophy (DMD and BMD), Emery-Dreifuss muscular dystrophy (EDMD), limb girdle muscular dystrophy (LGMD), facioscapulohumeral muscular dystrophy (FSHD), Distal muscular dystrophy (DD), oculopharyngeal muscular dystrophy (OPMD), congenital muscular dystrophy (CMD) in which muscle weakness is more generalised and the myotonic muscular dystrophy (MMD or DM), which can affect other tissues than skeletal muscles. Heterogeneous groups such as LGMD or CMD can be further sub-classified according to their inheritance mode and the genetic defect responsible for the individual forms (Mercuri & Muntoni, 2013).

MD have also been designated according to their molecular defects (mutated gene or defective/missing protein), such as laminopathies, titinopathies, dystrophinopathies or dysferlinopathies. An increasing number of genes associated to different forms of MD have been identified. However, the main default of this classification comes from the clustering of pathologies caused by different mutations in the same gene but leading to heterogeneous

clinical features. For example, mutation in the titin gene cause both tibial muscular dystrophy (which belongs to DD) and the most severe type of LGMD, the LGMD 2J (Hackman, Vihola et al., 2002, Nigro, 2003). On the other hand, mutations in different genes can result in the same phenotype. For instance, EDMD may be due to an autosomal dominant mutation in the LMNA gene, a gene coding nuclear envelope proteins lamin A and C, or in the gene encoding the nuclear envelope protein emerin, EMD gene (Bione, Maestrini et al., 1994, Bonne, Di Barletta et al., 1999).

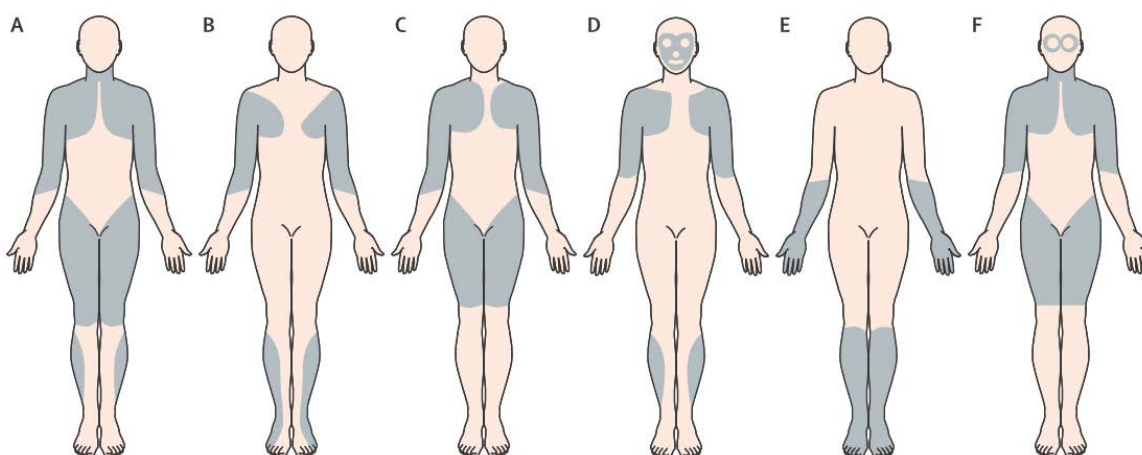


Figure 6 - **Patterns of distribution of muscle weakness in muscular dystrophies.** A) Duchenne and Becker muscular dystrophy; B) Emery-Dreifuss muscular dystrophy; C) Limb girdle muscular dystrophy; D) Facioscapulohumeral muscular dystrophy; E) Distal muscular dystrophy; F) Oculopharyngeal muscular dystrophy. Muscles mainly affected by each MD are highlighted in gray. From (Mercuri & Muntoni, 2013).

### ***Duchenne Muscular Dystrophy***

DMD is the most common childhood MD (frequency of 1:3500 male births) and affects all types of muscles, skeletal, cardiac and in some severe case smooth muscles. It is caused by recessive mutations in the *dystrophin* gene, located on the X chromosome, locus Xp21. *Dystrophin* gene is the largest known human gene, measuring 2.4 Mb and representing around 1% of the X chromosome. Mutations in this gene lead to the absence of functional dystrophin (Koenig, Monaco et al., 1988). The severity of the disease is not correlated with the size of the mutation but rather with the maintenance of the open reading frame (ORF) (Monaco, Bertelson et al., 1988). Genetic deletions in DMD patients are shown to shift the translational ORF resulting in a truncated abnormal dystrophin protein (Monaco,

Bertelson et al., 1987). A milder phenotype is seen in deletions that maintain the translational ORF giving rise to a smaller, semi-functional dystrophin protein, which causes the Becker muscular dystrophy (BMD) (Simard, Gingras et al., 1992).

In skeletal muscle, dystrophin protein is located under the sarcolemma. It is part of a protein complex called dystrophin-associated protein (DAP)<sup>1</sup> complex that connects the actin filaments of muscle fibres to the surrounding extracellular matrix through the sarcolemma (Ehmsen, Poon et al., 2002). DAP complex is composed of several proteins and glycoproteins localised in the cytoplasm side (dystrophin,  $\alpha$ -dystrobrevin and syntrophins), in the sarcolemma (dystroglycans, sarcoglycans, sarcospan and caveolin-3) and in the intermediate filaments (syncoilin, laminin-2 and synemin). The DAP complex have two main roles: i) a structural one, which consists on maintaining the integrity of the sarcolemma during repeated cycles of contraction (Claflin & Brooks, 2008, Petrof, Shrager et al., 1993) and ii) a regulatory role, by modulating signalling pathways via its interactions with others proteins such as calmodulin or the plasma membrane  $\text{Ca}^{2+}$ -ATPase (Constantin, 2014, Rando, 2001). The importance of the DAP complex in maintaining muscle integrity is supported by the various muscle diseases caused by absence of functional DAP (Mercuri & Muntoni, 2013). In the absence of dystrophin protein, the DAP complex is unstable thereby weakening the sarcolemma and muscle cytoskeleton and leading to muscle cell damages after muscle contraction cycles (Petrof et al., 1993). In addition, several aberrant intracellular signalling pathways due to the absence of dystrophin ultimately yield pronounced myofibre necrosis (degeneration). Muscle degeneration triggers the regeneration process orchestrated by adult muscle stem cells (further described in Chapter II). At the onset of the pathology, regeneration capacities can compensate muscle degeneration, leading to mild symptoms. However, when degeneration overcomes the regeneration process, dystrophic muscle fibres are gradually replaced by connective tissue and fat (Kharraz, Guerra et al., 2014, Kim, Merrow et al., 2013), resulting into progressive muscle weakness and fatigability (Flanigan, 2014, Wein, Alfano et al., 2015). As a result, most DMD patients become wheelchair-dependent early in their life, and the gradual development of cardiac hypertrophy typically results in premature death occurring in the 2<sup>nd</sup> or 3<sup>rd</sup> decades of life.

No effective treatment currently exists for DMD. Conventional treatments (such as surgery or physiotherapy) and pharmacological strategies (administration of corticosteroids, creatine, carnitine, gentamicin, myostatin, etc) that delay symptoms by tackling the

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<sup>1</sup> also known as dystrophin-glycoprotein complex (DGC)

secondary effects of DMD are being used in patients (Voisin & de la Porte, 2004). However, most of these strategies are only partially effective because they treat just one aspect of the pathogenesis of the disease and they may be toxic for the patients at long term. For these reasons, a great interest has been given to cellular therapies and genetic approaches for DMD treatment. Gene therapy consists in the introduction of a therapeutic nucleic acid (DNA, RNA or oligonucleotides) into targeted cells for gene replacement, gene repair or control of gene expression, via the use of viral, non-viral or cell-based vectors (Fairclough, Wood et al., 2013). Although this strategy has been very effective in trials with animals models using adeno-associated virus vectors (Pichavant, Chapdelaine et al., 2010), a recent clinical trial with DMD patients did not show an efficient dystrophin expression restoration and led to induction of an uncontrolled immune response (Mendell, Campbell et al., 2010). On the other hand, cell therapy is based on the delivery of autologous or heterologous precursor that will contribute to the regeneration of muscle fibres allowing tissue repair (Negroni, Bigot et al., 2016). One double-blind phase I clinical trial based on stem cell therapy was proved to efficiently increase the ratio of capillary per muscle fibres with a switch from slow to fast myosin-positive myofibres and no systemic side effects (Torrente, Belicchi et al., 2007). Several other trials are still in progress in France (for details see [clinicaltrials.gov](http://clinicaltrials.gov)). A first limitation of this therapy is the delivery method: as muscle progenitors are not able to cross the vessel wall, they cannot be injected intravenously. Similarly, when delivered by intra-peritoneal injection, cells are not able to colonize the skeletal muscle in order to regenerate the injured fibre (Partridge, 1991). Therefore, the only way to deliver cells to the site of injury is the use of intramuscular injections. However, while this approach was shown efficient in some dystrophies affecting a reduced number of muscles, it is not feasible in the case of DMD, in which a large number of muscles including diaphragm are affected.

Recently, the exon skipping approach has gained attention since it could target up to 83% of DMD patients characterised by deletions (Aartsma-Rus, Fokkema et al., 2009). This mutation-specific approach relies on synthesised antisense oligonucleotides, which alter splicing either by sterically blocking splice enhancer sequences or by altering secondary mRNA structure folding. Use of this technique improved the phenotypes of two mice models of DMD, the dystrophin deficient *mdx* mice and the *utrophin/dystrophin* double-knockout mice (Alter, Lou et al., 2006, Goyenville, Griffith et al., 2015). Several completed or ongoing clinical trials targeting exon 51 has demonstrated an efficient restoration of dystrophin expression at the sarcolemma, as well as a good tolerability of the drug (Goemans, Tulinius et al., 2010, Shrewsbury, Cirak et al., 2010). However, a poor cellular uptake, a rapid



clearance from the circulation, a variable skipping efficiency between muscles and a poor skipping efficiency in the heart are some of the challenges that have to be resolved for the use of this therapy (Goyenvalle & Davies, 2011). Among the recent efforts that have been recently done, a new exon skipping technology based on the CRISPR/Cas-9-mediated non homologous end joining called myo-editing have been proved efficient to restore dystrophin expression in the heart within a range expected to provide therapeutic benefit (Long, Amoasii et al., 2016).

### ***Oculopharyngeal Muscular Dystrophy***

OPMD is an autosomal dominant rare disease (frequency of 1:100 000 births) caused by a genetic mutation in the poly(A) binding protein nuclear 1 (*PABPN1*)<sup>2</sup> gene, located on the 14q11.1 chromosomal region (Brais, Bouchard et al., 1998). PABPN1 is an ubiquitous nuclear protein that binds poly(A) tails of mRNA with high affinity and is involved in mRNA polyadenylation. Wild-type PABPN1 protein has a 10 alanine stretch in its N-terminal domain, due to a (GCG)<sub>6</sub> repeat<sup>3</sup> on the *PABPN1* gene. The mutated PABPN1 protein (mPABPN1) has 11 to 17 alanine residues due to an extension of (GCG)<sub>7-13</sub> repeats on its gene. Although the size of the mutation has not yet been correlated with the severity of the phenotype, expansions of (GCG)<sub>8-13</sub> cause autosomal dominant OPMD, whereas homozygote (GCG)<sub>7</sub> alleles lead to autosomal recessive OPMD. In contrast to the majority of muscular dystrophies, OPMD symptoms usually appear in the 5<sup>th</sup> decade of age, with appearance of progressive swallowing difficulties (dysphagia), eyelid drooping (ptosis) and proximal limb weakness.

From a molecular point of view, OPMD skeletal muscles are characterised by an altered ubiquitin-proteasome pathway, mitochondrial dysfunction, modified gene expression, the presence of protein aggregates as well as intra-nuclear inclusions (INI) (Anvar, t Hoen et al., 2011, Chartier, Klein et al., 2015). These INI, which are composed of tubular filaments up to 250 nm in length contain the mutated PABPN1 protein and have been a hallmark of this disease for a long time. INI have been shown to contain also ubiquitin-proteasome subunits, heat shock proteins 40 and 70, Ski-interacting protein and abundant poly(A)-mRNA (Abu-Baker, Messaed et al., 2003, Bao, Cook et al., 2002, Calado, Tome et al., 2000,

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<sup>2</sup> also known as polyadenylate-binding protein 2 (PABP-2)

<sup>3</sup> encoding the first 6 alanine residues of the 10-length homopolymeric stretch

Kim, Noguchi et al., 2001, Wang, Mosser et al., 2005). In addition to INI, all biopsies from clinically affected OPMD muscles show dystrophic changes such as variation in the diameter of muscle fibres, atrophic angulated muscle fibres, fragmented red fibres and rimmed vacuoles (Gidaro, Negroni et al., 2013, Tomé & Fardeau, 1994). Furthermore, the *cricopharyngeus* muscle, one of the most affected muscles in OPMD presents a high level of both fibrosis and atrophy, together with an increased number of satellite cells, suggesting chronic regeneration/degeneration cycles (Gidaro et al., 2013). Various INI dependent and independent mechanisms have been proposed to explain the OPMD phenotype (Rankin, Wytttenbach et al., 2000). However, the exact mechanism responsible for polyalanine toxicity in OPMD still remains unknown.

Although not totally efficient, some medical interventions are possible for OPMD. Surgery is recommended when ptosis interferes with vision or cause cervical pain (Codere, 1993, Rodrigue & Molgat, 1997), and when dysphagia is accompanied by weight loss, near-fatal choking or recurrent pneumonia (Perie, Eymard et al., 1997, St Guily, Moine et al., 1995). Cricopharyngeal myotomy can be easily performed and efficiently improves swallowing as well as the quality of life of OPMD patients (Duranceau, 1997, Duranceau, Forand et al., 1980, Duranceau, Beauchamp et al., 1983, St Guily et al., 1995, St Guily, Perie et al., 1994). Nevertheless, in most of the patients a progressive dysphagia recurs within years (Coiffier, Perie et al., 2006). Based on the demonstration that muscle stem cells from non-affected muscles of OPMD patients maintained normal proliferative capacity and differentiate normally compared to muscle stem cells isolated from affected muscles (Perie, Mamchaoui et al., 2006), a cell-based autologous transplantation appeared as a valid therapeutic strategy. A phase I/IIa clinical study has recently been described (Perie, Trollet et al., 2014) in which myoblasts from clinically unaffected muscles of OPMD patients were isolated and grafted into their respective affected pharyngeal muscles. This autologous cell-based therapy was proven to be safe and efficient since it reduced the dysphagia by both reinforcing motor ability and restoring contractility of *cricopharyngeus* muscle (Perie et al., 2014). Similarly to other muscular dystrophies, pharmacological approaches have also been tested in OPMD patients. In this case, anti-PABPN1 antibodies or other pharmacological products that interfere with protein aggregation were proven to be efficient in reducing INI formation, cell death and other OPMD symptoms in different OPMD animal models such as *Drosophila* (Barbezier, Chartier et al., 2011, Chartier, Raz et al., 2009), *C. elegans* (Catoire, Pasco et al., 2008) or mouse (Davies, Rose et al., 2010, Davies, Sarkar et al., 2006, Davies, Wang et al., 2005).

### *Oxidative Stress in Muscular Dystrophies*

While MD are all characterised by progressive loss of muscle mass and function, they are associated with mutations in different genes encoding a wide variety of proteins: extracellular matrix proteins, transmembrane and membrane associated proteins, cytoskeletal proteins, cytoplasmic enzymes and nuclear matrix proteins (Cohn & Campbell, 2000, Mercuri & Muntoni, 2013). In addition to the genetic mutations leading to absence of protein expression or function, the contribution of other pathogenic mechanisms to the phenotype observed in MD, and notably to its progression, have been explored. Among them, the oxidative damage hypothesis has gained a particular interest (Canton, Menazza et al., 2014, Rando, 2002, Terrill, Radley-Crabb et al., 2013). Regulation of a balance between the production of ROS, mainly by mitochondria, and their elimination by the antioxidant systems is crucial for cellular function. Although at low levels, ROS act as signalling molecules regulating protein function associated with growth, proliferation and differentiation, when ROS levels overcome the capacity of cellular antioxidant systems, they can damage cellular components such as nucleic acids, lipids and in particular proteins, inflicting alterations to cell structure and function (see Chapter III for further details).

During the last decade, evidences have been accumulated suggesting that increased levels of ROS present within the skeletal muscle tissue may contribute to the pathology of muscular dystrophies (Terrill et al., 2013). Accordingly, oxidative stress has been clearly implicated in FSHD, laminopathies, SEPN1-related and RYR1-related myopathies, with at least two markers including increased levels of ROS, lipid peroxidation, protein carbonylation or higher expression of antioxidant enzymes being reported for each dystrophy (Arbogast, Beuvin et al., 2009, Dowling, Arbogast et al., 2012, Sieprath, Darwiche et al., 2012, Turki, Hayot et al., 2012). Similarly, oxidative damages due to glutathione (GSH) depletion and decreased GSH peroxidase and GSH-S-transferase antioxidant activities were also found in sarcoglycanopathy and in dysferlinopathy (Renjini, Gayathri et al., 2012).

Mitochondrial morphological changes and inclusions were described in OPMD patients, without molecular genetic abnormalities in mitochondrial DNA (Gambelli, Malandrini et al., 2004, Schroder, Krabbe et al., 1995, Wong, Dick et al., 1996). Analysis of a mice model of this pathology has revealed a decreased activity of mitochondrial respiratory chain complex I (Trollet, Anvar et al., 2010), suggesting an uncontrolled ROS production

that could lead to oxidative stress. Although this hypothesis has not yet been investigated, a study by Anvar *et al.* has showed a loss of activity of the ubiquitin-proteasome system (UPS) in both OPMD patients and mouse models, a defect that could possibly lead to defective protein homeostasis (Anvar et al., 2011).

The role of oxidative stress has been extensively investigated in DMD. By-products of lipid peroxidation as well as specific antioxidant activities were found significantly higher in muscles from DMD patients and mouse models (Haycock, Mac Neil et al., 1998, Hunter & Mohamed, 1986, Kar & Pearson, 1979, Menazza, Blaauw et al., 2010). Likewise, higher levels of antioxidants, vitamin E and coenzyme Q were found in muscle extracts of DMD patients (Renjini et al., 2012, Touboul, Brunelle et al., 2005). In agreement with these results obtained in humans, using the *mdx* mice, Dudley and colleagues showed that the sarcolemmal damage was modulated by synergistic interactions between mechanical and oxidative/nitrosative stresses (Dudley, Danialou et al., 2006). Collectively, these findings provide strong support that oxidative stress may contribute to the phenotype of several muscle dystrophies. However, the exact mechanism and the identity of key molecules that may be affected by the excessive ROS levels still remained unidentified.

In agreement to the oxidative damages reported in muscle fibres of both DMD patients and in the *mdx* mice, several studies have shown that antioxidant treatments can delay muscle wasting in *mdx* mice (Carlson, Samadi et al., 2005, Hnia, Hugon et al., 2007, Messina, Altavilla et al., 2006, Messina, Bitto et al., 2006, Whitehead, Pham et al., 2008). However, clinical trials using vitamin E, selenium or antioxidant enzymes treatments in DMD patients yielded no clinical improvement (Gebre-Medhin, Gustavson et al., 1985, Stern, Ringel et al., 1982, Tidball & Wehling-Henricks, 2007). To explain the failure of these antioxidant treatments in reducing pathology of muscular dystrophy, several hypothesis have been raised i) the observed oxidative damage is a consequence of the pathology but is not involved in its progression ii) systemic treatments with antioxidants may be too broad and non-specifically targeted or regulated to provide clinical improvements iii) the fact that antioxidants can have multiple functions, independently of their antioxidant properties, and when administrated systemically may induce detrimental effects, such as tissue injury; iv) the *mdx* mice may not be an optimal model for the study of DMD pathology in humans.



## II. Satellite Cells, the skeletal muscle stem cells

In mammal, skeletal muscle represents a paradigm of tissue regeneration. Indeed, while this postmitotic tissue is stable with low turnover under homeostatic conditions, it is therefore characterised by a remarkable regenerative capacity in situations where acute or chronic injuries occur. Muscle regenerative capacity is known since the second half of the 19<sup>th</sup> century. However, it took almost a century until the discovery of the cells responsible for this capacity. These cells were observed for the first time by A. Mauro in 1961 by electron microscopy of adult frogs muscle sections and were named satellite cells due to their peripheral positioning on muscle fibres (Mauro, 1961) (Figure 7). Some years later, they were observed in human muscle, occupying an identical position on the surface of muscle fibres between the basal lamina and the sarcolemma (Cravioto & Merker, 1963, Laguens, 1963, Shafiq, Gorycki et al., 1967). Satellite cells have a large nuclear-to-cytoplasmic ratio, a reduced organelle content and a small nucleus displaying increased amounts of heterochromatin compared with fibre nuclei (Charge & Rudnicki, 2004). As many tissue-specific stem cells, they remain mostly quiescent through life under homeostatic conditions. When stimulated by environmental signals (damage or stress) or in pathological conditions (muscular dystrophies), these cells activate, migrate to the regeneration site (Schultz, Jaryszak et al., 1985) and enter the cell cycle to proliferate and either undergo differentiation to form new fibers or self-renew to reconstitute the satellite cell pool (Alderton & Steinhardt, 2000). Each step of this regenerative process is regulated through the coordinated expression of specific transcription factors including Pax3/7 homeodomain transcription factors, and the myogenic regulatory factors (MRFs) MyoD, Myf5, Myogenin, and MRF4 (Alderton & Steinhardt, 2000).

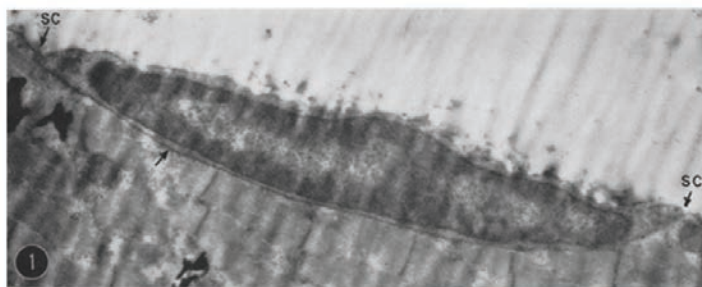


Figure 7 - **The first longitudinal view of a satellite cell.** Electronic microscopy of a frog *tibialis anticus* with 10 000 x of magnification. Sc arrows point the satellite cell extremities. The third arrow indicates the sarcolemma of the muscle fibre. From (Mauro, 1961).

The Pax family of paired-domain transcription factor plays key roles during tissue specification and organ development. In the context of skeletal muscle, Pax3 and Pax7 are important upstream regulators, which expression and activity are required for normal myogenesis.

The MyoD family of myogenic regulatory factors (MRF) is composed of four genes *MyoD* (Davis, Weintraub et al., 1987), *Myf5* (myogenic factor 5, (Braun, Buschhausen-Denker et al., 1989), *Myogenin* (Wright, Sassoon et al., 1989) and *MRF4* (also known as *Myf6*, (Rhodes & Konieczny, 1989), which expression is restricted to skeletal muscle cells. More than 25 years ago it was shown that members of this family of basic-helix-loop-helix (bHLH) transcription factors, when overexpressed both *in vitro* (Weintraub, Davis et al., 1991) and *in vivo* (Santerre, Bales et al., 1993) in non muscle cells, will activate the myogenic program, with suppression of other cell fates and formation of differentiated muscle. Since then the possibility of converting one cell type to another by transdifferentiation has become a major issue in the stem cell field. However, the phenomenon of myogenic conversion remains remarkable in that a single transcription factor can exert this overriding effect.

The four MRF family members control the formation of skeletal muscle via their binding to DNA consensus sequences (CANNTG) named E-box, which are present in specific skeletal muscle genes (Sabourin & Rudnicki, 2000). Mutations of *MyoD*, *Myf5*, and *MRF4* in the mouse and their expression in non-muscle cells have shown that these MRF function as myogenic determination factors. In other words, these experiments showed that in the absence of these three MRF there is no myogenesis during development and that these three MRF, contrary to the fourth MRF member *Myogenin*, are able to drive the myogenic cellular fate. This is due to the greater efficiency of myogenic conversion by the three MRF determination factors compared to *Myogenin*, which results of the presence of a C-terminal domain that recruits chromatin remodelling complexes (Fong & Tapscott, 2013). Thus, *Myogenin* acts only as a differentiation factor, as can *MRF4* and *MyoD*, controlling the differentiation of myoblasts into skeletal muscle fibres (Moncaut, Rigby et al., 2013).

### ***Emergence of satellite cell population***

Using murine embryos *Pax3*<sup>GFP/+</sup> and *Pax7*<sup>LacZ/+</sup>, two groups showed that Pax3/Pax7-positive cells provide the reserve cell population for muscle growth during embryonic and foetal development (Gros, Manceau et al., 2005, Relaix, Rocancourt et al., 2005). Co-expression of Pax3 and Pax7 is required both for maintenance of this stem cell population (Relaix et al., 2005) and for activation of *Myf5* and *MyoD*, that control entry into the skeletal muscle programme (Davis et al., 1987, Weintraub et al., 1991). As a consequence, in double *Pax3/Pax7* mutants, these cells fail to activate the myogenic program and assume other cell-fates or die, leading to a major muscle deficit. While Pax3 is highly expressed during embryonic myogenesis, its transcription is down-regulated in foetal muscle when Pax7 becomes the dominant factor in all myogenic progenitor cells. At E18.5 in mice, just before birth, the Pax3/Pax7 positive cells that have not entered into differentiation adopt a satellite position under the basal lamina of the skeletal muscle fibres. While at birth, these cells, so-called satellite cells account for 30 % of the total skeletal muscle tissue nuclei, this proportion decreases during the first month of life when contribute to postnatal growth (Charge & Rudnicki, 2004). At the end of postnatal growth, satellite cells cease proliferation and enter quiescence (Cheung & Rando, 2013). At this stage, they constitute no more than 7 % of the total skeletal muscle nuclei, and this proportion remains unchanged during the whole life, with the exception of ageing (Blau et al., 2015).

Postnatal and adult satellite cells are all marked by Pax7 expression, with continuing transcription of Pax3 in trunk muscles such as the *diaphragm*, *abdominals*, *pectoralis* and in some limb muscles such as *biceps* (Montarras, L'Honore et al., 2013, Montarras, Morgan et al., 2005, Relaix, Montarras et al., 2006). Prior to birth, Pax7 is not essential for myogenesis, presumably because Pax3 can compensate. After birth, on the other hand, Pax7 mutants lose their satellite cells by apoptosis, and Pax3 cannot compensate even in trunk muscles such as the diaphragm, perhaps because the protein is present at too low level or because of divergent Pax3 and Pax7 functions by this stage. As a consequence, *Pax7*<sup>-/-</sup> mice have a severe growth defect, leading to severe muscle atrophy (Oustanina, Hause et al., 2004, Seale, Sabourin et al., 2000); 50% decrease in body weight 7 days after birth and around 70% after two weeks).



## Satellite Cells, the mandatory actors of skeletal muscle regeneration

Adult skeletal muscle is a very stable tissue with low turnover under homeostatic conditions. However, despite its stability, this post-mitotic tissue is therefore characterised by a remarkable regenerative capacity in situations where acute or chronic injuries occur.

The notion that satellite cells function as muscle stem cells was established by clonal analysis, tissue culture experiments and *in vivo* regeneration studies. Culture of satellite cells isolated from muscle biopsies showed their proliferative and differentiation properties leading to formation of myotubes *in vitro* (Konigsberg, 1963). In parallel, tissue culture of isolated skeletal muscle fibres allowed to monitor satellite cells activation by phase contrast microscopy and provided conclusive proof that these cells could directly give rise to muscle precursor cells (myoblasts), which are able to fuse into multinucleated myotubes *in vitro* (Bischoff, 1975, Konigsberg, Lipton et al., 1975). Experiments based on use of radioactively labelled thymidine demonstrated that satellite cells could survive muscle injury or transplantation and become regenerating myoblasts when most of muscle fibres undergo necrosis (Moss & Leblond, 1970, Snow, 1977). In 2005, two main studies have revealed the potential of satellite cells as muscle stem cells *in vivo*. Using graft experiments of a single freshly isolated muscle fibre with its associated satellite cells in the *tibialis anterior* muscle of *mdx*-nude mice, Collins *et al.* showed that as low as 7 satellite cells could give rise to more than 100 new muscle fibres as well as replenish the satellite cell population (Collins, Olsen et al., 2005). While this study did not exclude the role of muscle fibres in regulating satellite cell function, a parallel study using purified satellite cells isolated by flow cytometry showed their contribution to muscle regeneration *in vivo* (Montarras et al., 2005). This experiment clearly demonstrated that satellite cells are able to differentiate into new muscle fibres as well as to replenish their reservoir, thus behaving as stem cells. These experiments have revealed the muscle stem cell properties of satellite cells, however, their mandatory role during regeneration was recently demonstrated by three elegant studies: mice in which Pax7-positive cells have been ablated were used for skeletal muscle regeneration experiments, demonstrating the complete absence of regeneration in absence of satellite cells (Lepper, Partridge et al., 2011, Murphy, Lawson et al., 2011, Sambasivan, Yao et al., 2011).

Muscle regeneration can be studied using several mice models of muscle injury. (Hardy, Besnard et al., 2016). In addition to models of muscular dystrophies such as the *mdx* mice, muscle injury can be experimentally induced by intramuscular injection of myotoxins

(bupivacaine, cardiotoxin and notexin), or of the chemical agent barium chloride. Alternatively, mechanical injuries by crush, freeze or by denervation-devascularization of skeletal muscle can also be used to trigger muscle regeneration process.

Although it cannot be considered as muscle regeneration process, the culture of satellite cells *ex vivo* and *in vitro* has also helped to characterise some important aspects of their function. In the case of *ex vivo* approaches, satellite cells can be purified from human or mice muscles by flow cytometry or cell separation columns thanks to the use of various combinations of antibodies directed against membrane satellite cells molecular markers (Liu, Cheung et al., 2015). In addition, the use of reporter mice, such as *Pax3<sup>GFP/+</sup>* (Montarras et al., 2005) and *Pax7-nGFP* mice (Sambasivan et al., 2011) also permits the direct satellite cells purification by flow cytometry. Regarding *in vitro* cell culture models, myoblasts cell lines such as human (Bigot, Jacquemin et al., 2008, Renault, Thornell et al., 2002), mouse C2C12 (Richler & Yaffe, 1970) and rat L6 (Yaffe, 1968) are routinely cultured.

The use of these models had contributed to the notion that muscle regeneration is characterised by two phases: a degenerative phase and a regenerative phase (Figure 8). The degeneration phase begins with necrosis of muscle fibres, triggered by disruption of sarcolemma. This phase is characterised by an increased of muscle proteins levels such as creatine kinase in serum of muscle-injured patients (Lott & Stang, 1980). Degeneration is followed by muscle repair, called regeneration phase. Quiescent satellite cells activate, and after several rounds of proliferation exit cell cycle, differentiate and fuse each other or to existing fibres to form new fibres (Yin, Price et al., 2013).

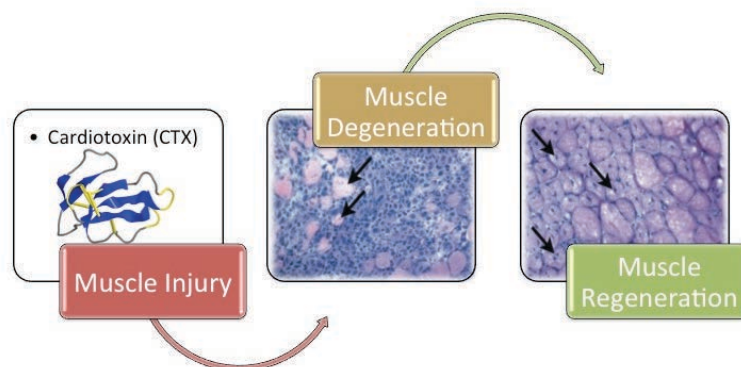


Figure 8 - **Skeletal muscle repair process.** Muscle injured induced by cardiotoxin injection results in rapid necrosis of fibres (arrows) (muscle degenerative phase), this triggers the activation of satellite cells that will proliferate, differentiate and fuse to either necrotic fibres repairing them, either between each other to form new fibres (muscle regeneration phase). Newly formed fibres have centrally located nuclei (arrows) and are often basophilic due to high protein synthesis and express embryonic forms of MHC. Adapted from (Charge & Rudnicki, 2004).

On muscle cross-sections, this can be followed by the presence of newly formed fibres with centrally located nuclei (Figure 8). Once fusion of myogenic cells is completed, newly formed fibres increase in size, and myonuclei migrate to the periphery of myofibres. In this chapter, I will revise the most important steps of the muscle regeneration process, including satellite cells proliferation, differentiation and fusion, as well as the signalling pathways and the secreted factors involved in its regulation.

### ***Satellite cells in quiescence***

As most of long-lived tissue-resident stem cells, adult satellite cells are quiescent (Cheung & Rando, 2013). This quiescence is required for their long-term maintenance, as its disruption leads to their spontaneous activation in absence of injury and to the progressive depletion of their reservoir (Montarras et al., 2013). The quiescent state, which is maintained during healthy resting periods, is marked and maintained by cell-intrinsic properties such as low metabolic activity, and by the cellular environment of the quiescent cell, including the extracellular matrix, which constitutes its niche, blood vessels and muscle fibre innervations (Montarras et al., 2013). Quiescence is a balance between high expression of cell cycle inhibitors and factors that will activate cell cycle. Cell adhesion molecules and some signalling pathways reinforce its quiescent state, whereas other signals lead to activation. Satellite cells are surrounded by the basal lamina, a collagen IV network containing laminins that anchor the satellite cell through interaction with  $\alpha7/\beta1$ -integrins on its surface. It also contains extracellular matrix proteins such as fibronectin, decorin and proteoglycans that bind inactive growth factor precursors and thus buffer the effects of signalling, maintaining satellite cell quiescence.

Satellite cells tend to be located in close proximity to capillaries and are characterised by a low metabolism, marked by reduced mitochondrial and organelle content (Montarras et al., 2013, Tang & Rando, 2014) and by fatty acids oxidation metabolism (Ryall et al. 2015), in contrast to the situation in activated cells where the greater ATP energetic output of aerobic glycolysis is required to support high-level macromolecular synthesis of proliferating cells. This metabolic status is a hallmark of quiescence stem cells and is important in limiting the production of reactive oxygen species that may represent a positive signal for satellite cell activation.

Notch signalling has been implicated in promotion of satellite cell quiescence, preventing spontaneous activation. Quiescent satellite cells are characterised by high Notch

signalling marked by elevated levels of Notch targets, such as HeyL (Figure 9). According to this result, conditional deletion of *Rbpj*<sup>4</sup> in adult satellite cells resulted in disruption of their quiescence and their progressive exhaustion, most of which underwent spontaneous differentiation (Bjornson, Cheung et al., 2012, Mourikis, Sambasivan et al., 2012). Similarly, Sprouty1, an intracellular inhibitor of the tyrosine kinase-signalling pathway, is highly expressed in quiescent satellite cells and down-regulated in proliferating satellite cells after injury. Analysis of *Spry1* mice mutants has revealed the critical role of this gene in maintenance of satellite cells quiescence (Chakkalakal, Jones et al., 2012).

*Pax7* is a general marker of adult quiescent satellite cells and its role at this stage has been controversial. As *Pax7* mutant mice die within the first 2 to 3 weeks of life, inducible conditional *Pax7* mutants have been generated. A first report in which regeneration experiments were performed just after *Pax7* deletion indicated that the satellite cell population was still present and that muscle regeneration could take place in the absence of *Pax7* (Lepper, Conway et al., 2009). Since then this view has been modified. In two other studies in which regeneration experiments were performed one month after *Pax7* ablation, muscle regeneration was shown to be severely impaired (Gunther, Kim et al., 2013, von Maltzahn, Jones et al., 2013). Gunther *et al.* showed that this defective regeneration was due to depletion of the satellite cell pool: absence of *Pax7* leads to disruption of satellite quiescence and to their spontaneous activation and differentiation in the absence of injury (Gunther et al., 2013).

### ***Satellite cells activation and proliferation***

Muscle regeneration starts with the activation of satellite cells all along the damaged fibre (Schultz *et al.*, 1985). After several rounds of proliferation, the resulting myogenic precursor cells differentiate and fuse to form new fibres or to repair damaged ones, while the others will re-enter into quiescence to renew the stem cell pool (Figure 9).

The importance of the MRFs expression for skeletal muscle regeneration process was revealed by their mutations in mice models in which muscle regeneration was induced. The *MyoD*<sup>-/-</sup> mouse model presented a reduced and inefficient regenerative capacity characterised by an increase in the precursor cell proliferation at the expense of their differentiation. Such defects lead to a decrease in the number of regenerated myotubes and as a consequence of

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<sup>4</sup> the main effector of canonical Notch signalling

branched fibres *in vivo* (Cornelison, Olwin et al., 2000, Megeney, Kablar et al., 1996). In agreement, *in vitro* experiments have revealed a defective differentiation potential of *MyoD*<sup>-/-</sup> cells eventually leading to a reduced number of mono-nucleated differentiated myocytes (Megeney et al., 1996, Sabourin, Girgis-Gabardo et al., 1999). In addition, expression of M-cadherin, which is required for myoblasts differentiation and fusion, is decreased in *MyoD*<sup>-/-</sup> cells (Megeney et al., 1996, Sabourin et al., 1999). Overall, these data demonstrate a critical role for MyoD in the control of satellite cell differentiation during muscle regeneration.

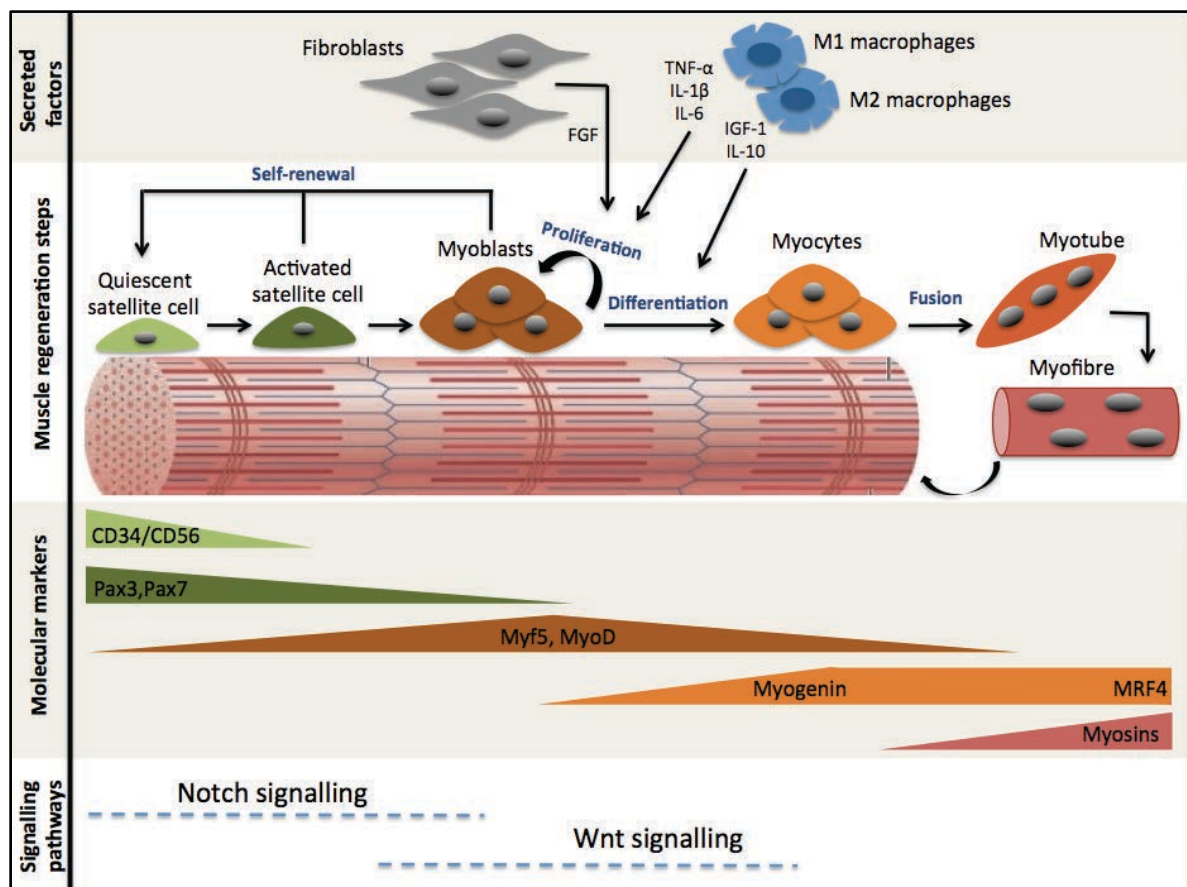


Figure 9 - **Skeletal muscle regeneration process.** In adult muscle, satellite cells, which reside between the basal lamina and the sarcolemma, are in a quiescent state and marked by the expression of the membrane protein CD34 in mouse or CD56 in humans and the transcription factors Pax3/7. Following muscle injury, satellite cells activate through Notch signalling pathways and start expressing Myf5 and MyoD transcription factors. They enter cell cycle to proliferate as myoblasts, and either self-renew to reconstitute their reservoir, or undergo differentiation. Different growth factors and cytokines secreted by fibroblasts and macrophages are required during the process of myoblasts proliferation and differentiation. Down-regulation of *Pax* genes, up-regulation of Myogenin protein and activation of the Wnt pathway are required for myoblasts differentiation. Early-differentiating myocytes will then fuse to form myotubes, which then mature into myofibres. Later stages of differentiation are marked by the assembly of sarcomeres and the expression of structural proteins such as myosins. Adapted from (Wang & Rudnicki, 2012, Zammit, Partridge et al., 2006). FGF, fibroblasts growth factors; IGF-1, insulin growth factor 1; TNF- $\alpha$ , tumour necrosis factor  $\alpha$ ; IL-1 $\beta$ , -6 and -10, interleukin 1 $\beta$ , 6 and 10.

Identification of a role for Myf5 in muscle regeneration had taken more time since no apparent phenotype in the Myf5 deficient mice adult muscles had been reported. The first defects related to the absence of this gene were evidenced by *in vitro* experiments showing reduced proliferative capacities and premature differentiation of cultured mice Myf5 null myoblasts compared to wild-type myoblasts (Montarras, Lindon et al., 2000). In agreement with these results, delayed muscle regeneration marked by fat accumulation, fibrosis and significant hypotrophy of newly regenerated muscle fibre was observed in Myf5 null mice (Gayraud-Morel, Chretien et al., 2007, Ustanina, Carvajal et al., 2007). This role of Myf5 in regulation of satellite cell proliferation was confirmed in Myf5<sup>-/-</sup>:mdx mice revealing aggravated dystrophic changes (Gayraud-Morel et al., 2007, Ustanina et al., 2007).

Overall, these results demonstrate that Myf5 and MyoD play distinct roles during adult muscle regeneration, with Myf5 promoting myoblasts proliferation, whereas MyoD promotes myoblasts differentiation.

### ***Early steps of satellite cells differentiation***

Myoblasts differentiation requires a high level of expression of MyoD together with Myogenin and MRF4 whose expression appears in differentiated myotubes (Smith, Janney et al., 1994) (Figure 9). MyoD expression persists in myotubes and collaborates with Myogenin for the myoblasts commitment to the differentiation program and to regulate the expression of muscle-specific genes necessary for terminal differentiation (Fuchtbauer & Westphal, 1992, Grounds, Garrett et al., 1992, Yablonka-Reuveni & Rivera, 1994). The serious defects in embryonic muscle development of mutant mice for Myogenin or MRF4 have hampered their study in adult muscle regeneration (Hasty, Bradley et al., 1993, Knapp, Davie et al., 2006). To investigate the role of Myogenin in postnatal muscle growth, Klein's laboratory created an inducible Myogenin conditional knockout mice (Knapp et al., 2006). Analysis of this mutant revealed that while Myogenin is absolutely required for skeletal muscle development and survival until birth, it is dispensable for postnatal life. However, Myogenin deletion after birth led to reduced body size implying a role for Myogenin in regulating body homeostasis. Despite a lack of skeletal muscle defects in Myogenin-deleted mice during postnatal life and the efficient differentiation of cultured Myogenin-deleted adult muscle stem cells, the loss of Myogenin profoundly altered the pattern of gene expression in cultured muscle stem cells (Liu, Zha et al., 2012) and adult skeletal muscle (Meadows, Cho et al., 2008). Remarkably, these changes in gene expression were distinct

from those found in Myogenin-null embryonic skeletal muscle, indicating that Myogenin has separate functions during postnatal life.

### ***Terminal differentiation during muscle regeneration***

At the end of the regeneration process, myocytes fuse to each other or with newly-formed myofibres (Figure 9). This fusion is ensured by semi-stable intercellular junction structures that mediate cell-cell adhesion and regulate intracellular cytoskeleton architecture as well as membrane rupture. Regulation of calcium flux together with various calcium dependent proteins, such as sarcolemmal calcium potassium channel Kir 2.1 or M-cadherin, have been shown to be important to myoblast fusion during regeneration (Bernheim & Bader, 2002, Zeschmick, Kozian et al., 1995). This morphological differentiation operates in parallel with biochemical differentiation marked by the activation of terminally differentiated muscle specific genes, encoding for MHC and other sarcomeric proteins.

While the role of MRF4 is still unclear, the observation that this transcription factor is expressed in the nuclei of young newly myotubes suggests a role in fibre maturation and in regulation of terminally differentiated genes (Zhang, Behringer et al., 1995, Zhou & Bornemann, 2001). Mice lacking MRF4 are viable and showed increased levels of Myogenin expression in their muscles (Zhang et al., 1995). One can think that Myogenin and MRF4 could have compensatory roles, which would also explain the small defects of the Myogenin conditional mutant. However, compensatory roles of these two MRFs remain to be studied.

### ***Satellite cells self-renewal***

Satellite cells, as other adult stem cells, have a self-renewal capacity, which allow them to prevent the depletion of their pool (Gross & Morgan, 1999, Heslop, Beauchamp et al., 2001). Several research groups have proposed mechanisms to explain this capacity. Some authors propose a model of asymmetric division where the mother cell gives rise to two daughters that have different fates from one another: one will differentiate and give rise to new fibres and the other will return to quiescent state to renew the stem cell pool. Supporting this model, Numb, a plasma membrane-associated cytoplasmic protein that represses Notch signalling, was shown to be asymmetrically segregated within dividing satellite cells (Conboy & Rando, 2002). Since activation of Notch-1 appears to promote the proliferation of satellite cells, whereas its inhibition in cultured satellite cells leads to their

differentiation (Conboy & Rando, 2002), the non-inheritance of Numb during asymmetric division may lead to satellite cell self-renewal through the Notch signalling. Indeed, knockout mice for two target genes of this pathway, Hey1 and HeyL, showed a remarkable reduction in satellite cell number probably due to an incapacity in keeping them at a quiescent state (Fukada, 2011). On the other hand, other studies have suggested a model of symmetric division in which a mother cell gives rise to two cells with identical fates, either differentiation and fusion, either returning to mitotically inactive state (Olguin & Olwin, 2004, Shinin, Gayraud-Morel et al., 2006, Zammit, Golding et al., 2004). While more studies are needed to understand the mechanism of satellite cells self-renewal, some authors suggest a mix of the two models with nearly 90% of satellite cells divisions being symmetric while 10 % of them assuming an asymmetric model (Kuang, Kuroda et al., 2007).

### ***Signalling pathways involved in regulation of the regeneration process***

In parallel to gene regulatory networks for transcription factors, several mechanisms involving cell-cell and cell-matrix interactions as well as extracellular secreted factors have been identified as regulators of satellite cell behaviour and function during muscle regeneration process.

The Hepatocyte Growth Factor (HGF) is a key activator of satellite cells playing a role in the early phase of skeletal muscle regeneration. Injection of this factor *in vivo* to an injured muscle is sufficient to activate the quiescent satellite cells (Tatsumi, Anderson et al., 1998), while its administration in late phases of regeneration has no impact on regeneration or even results in its inhibition (Miller, Thaloor et al., 2000). HGF-dependent activation of quiescent satellite cells is done by binding to its tyrosine receptor c-Met present on the surface of satellite cells (Cornelison & Wold, 1997, Gal-Levi, Leshem et al., 1998). Conversely, Yamada and colleagues have shown that high doses of HGF can induce satellite cells back to a quiescent state along with a decrease in MyoD and Myogenin expression (Yamada, Tatsumi et al., 2010). Such result denotes the importance of the concentration levels of these growth factors on controlling myogenic regulation of muscle progenitors.

During the early phase of muscle regeneration, proliferative precursor cells are characterised by moderate activity of Notch signalling. In order to progress into differentiation, proliferative precursor cells need to repress this activity. Activation of the Wnt/ $\beta$ -catenin signalling, which antagonized Notch pathway, is critical for myoblasts



adhesion and differentiation by directly interacting with MyoD (Kim, Neiswender et al., 2008), during adult muscle regeneration (Yin et al., 2013).

Fibroblast Growth Factors (FGF) are detected in skeletal muscle *in vivo* and in differentiated myoblasts *in vitro* (Allen, Dodson et al., 1984, Grass, Arnold et al., 1996, Hannon, Kudla et al., 1996). While exogenous administration of FGF-1, FGF-4 and FGF-6 enhances primary myoblasts proliferation *in vitro*, their inhibition by interference RNA induces myogenic differentiation (Kastner, Elias et al., 2000, Sheehan & Allen, 1999). In agreement, constitutive expression of a full-length FGF receptor-1 increased myoblasts proliferation and delayed their differentiation (Scata, Bernard et al., 1999). In the absence of FGF-6, mice exhibit severe defects of muscle regeneration, including fibrosis, collagen infiltration and reduced expression of both MyoD and Myogenin (Floss, Arnold et al., 1997). On the other hand, high levels of FGF-2 in aged stem cell niche led to loss of stem cell quiescence ultimately leading to impaired regeneration (Chakkalakal et al., 2012).

Insulin Growth Factors (IGFs) have also been implicated in regulation of skeletal muscle regeneration capacity through their anti-necrosis role (Shavlakadze, White et al., 2004), their positive role on muscle reinnervation (Vergani, Di Giulio et al., 1998) and on satellite cell migration, proliferation and differentiation (Allen & Boxhorn, 1989, Philippou, Halapas et al., 2007). Intramuscular injection of IGF-1 in injured aged rats increase satellite cells proliferation and muscle mass (Chakravarthy, Booth et al., 2001). IGF-2, on its turn, was not shown to enhance satellite cells proliferation but rather to favour myogenic differentiation (Florini, Ewton et al., 1996, Vandeburgh, Karlisch et al., 1991). Although IGFs implication in skeletal muscle regeneration is well proved, the molecular mechanisms underlying their role are still unclear. Some clues are the calcineurin/nuclear factor of activated T-cells (NFAT), involved in muscle development and growth, the PI3K/Akt/mTOR pathway, involved in cell survival and protein synthesis, and the p38 $\alpha$ ,  $\beta$  and  $\gamma$  MAPK signalling, which plays critical roles in satellite cells proliferation, differentiation and self-renewal (Bernet, Doles et al., 2014).

### ***Role of Inflammation during the muscle regeneration process***

If satellite cells are the major actors of muscle regeneration, they are not the solely ones. The early phase of muscle injury is accompanied by the activation of inflammatory cells that reside within the muscle tissue and that provide the chemotactic signals required to recruit circulating inflammatory cells (Tidball, 2011). Neutrophils are the first inflammatory

cells to invade the injured muscle, with a significant increase in their number being observed a few hours after induction of muscle damage (Fielding, Manfredi et al., 1993, Orimo, Hiyamuta et al., 1991). Two days after muscle injury, macrophages, which are important for the phagocytosis of muscle debris, become the predominant immune cell type within the injury site. These immune cells induce a pro-inflammatory phase during which pro-inflammatory cytokines such as IL-1, IL-6 and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) stimulate activation and proliferation of satellite cells (Bencze, Negroni et al., 2012) (Figure 9). During the second phase of regeneration, M1 macrophages acquire an anti-inflammatory phenotype through trans-differentiation into M2 macrophages. Such cells have been proved to inhibit satellite cell proliferation and to stimulate their differentiation and fusion thanks to the secretion of TGF $\beta$ , IGF-1 and the cytokine IL-10 (Chazaud, 2014).

## **Satellite Cells during ageing**

Aged skeletal muscle is characterised by a progressive loss of muscle mass and a decline of muscle functional properties, including a decline in regenerative potential (Sousa-Victor et al., 2014). This decline has been shown to derive from both decreased satellite cell number and age-associated satellite cell loss of function and it is generally accepted that these two events are due to both extrinsic and intrinsic changes.

Although it is now well established that sarcopenia progress independently of satellite cell contribution (Fry et al., 2015), several studies have demonstrated the major contribution of the niche and the systemic environment to the process of satellite cells ageing. The prevalent view that the age-dependent decline of muscle regeneration can be reversed by exposure to an external younger environment came from heterochronic grafting and parabiosis experiments with rodents. Indeed, when muscle explants of aged rat were grafted into young rats, increased muscle mass and contractile strength was observed (Carlson, Dedkov et al., 2001). Conversely, young grafts introduced in aged rats did not improved muscle function, with angiogenesis and innervation of the grafted muscle being markedly impaired. Parabiotic experiments were performed using young and aged mice. This experiments clearly showed that old mice placed in heterochronic parabiosis with young mice exhibited improved regenerative capacities after injury (Conboy, Conboy et al., 2005). These studies reveal that muscle environment and notably circulating growth factors present in young mice can reverse the loss of function of aged satellite cell. Among the circulating

factors that have been reported to decline with age, GDF11, a member of the TGF- $\beta$  superfamily appears as a good candidate. Supplementation of geriatric mice by systemic delivery of recombinant GDF11 led to a more robust regenerative capacity compared to aged non-treated mice (Sinha, Jang et al., 2014). Similarly, the age-related decrease of Delta-1 expression by muscle fibres together with the increased levels of Wnt3a in blood circulation have been shown to both decrease the Notch pathway and activate  $\beta$ -catenin signalling (Brack, Conboy et al., 2007) within satellite cells, thus impairing their activation and self-renewal.

In addition to extrinsic factors, recent findings have shown that geriatric satellite cells exhibit decreased regenerative properties when grafted into a young muscle, suggesting that exposure to a young environment is not sufficient to restore young regenerative capacities (Cosgrove et al., 2014, Sousa-Victor et al., 2014). Several studies have shown that with age, satellite cells suffer intrinsic alterations in cellular signalling pathways that affect their function. The abnormal activation of both Jak2-Stat3 (Price, von Maltzahn et al., 2014, Tierney, Aydogdu et al., 2014) and p38/MAPK signalling pathways (Bernet et al., 2014, Cosgrove et al., 2014) observed in geriatric quiescent satellite cell have been shown responsible for their acquisition of a pre-senescent phenotype, and as a consequence, for their decreased self-renewal properties and their defective proliferation and differentiation. Pharmacological inhibition of Jak2 and Stat3 activity or the use of siRNA strategies to knockdown their expression markedly enhanced satellite cells ability to repopulate their niche after transplantation into a regenerating *Tibialis Anterior* muscle (Price et al., 2014). In the case of p38/MAPK, it was shown that its activation could repress *Pax7* expression through recruitment of histone deacetylase (HDAC) to its promoter (Palacios, Mozzetta et al., 2010) and lead to de-repression of the cell cycle inhibitor p16 and p21 expression in geriatric satellite cells (Cosgrove et al., 2014, Sousa-Victor et al., 2014). Another hypothetical cause for defective differentiation capacities of geriatric cells may be the acquisition of oxidative damage due to both increased ROS production in a sarcopenic muscle and decreased antioxidant capacities (Bigot et al., 2008, Lorenzon, Bandi et al., 2004, Pietrangelo, Puglielli et al., 2009). However, further studies are needed to investigate the contribution of this potential cause to the impaired satellite cell function with ageing.

### III. The Reactive Oxygen Species

#### ROS Nature and Generation

Oxygen is one of the major components of the air. It is produced by plants during photosynthesis and is necessary for aerobic respiration. Because molecular oxygen ( $O_2$ ) has a high oxidation/reduction (redox) potential, it is an ideal electron acceptor. In fact, reactive oxygen species (ROS) are reactive oxygen intermediates created by electron acceptance. Oxygen molecule can accept up to four electrons (Figure 10). The addition of one single electron to molecular oxygen creates a single unpaired electron on it, forming the so-called superoxide anion ( $O_2^{\bullet -}$ ). The transfer of two electrons to oxygen yields the formation of peroxides ( $O_2^{2-}$ ). A simple and stable peroxide formed within cells is the hydrogen peroxide ( $H_2O_2$ ), which is produced by dismutation of  $O_2^{\bullet -}$  by superoxide dismutase enzymes. The three electron reduction state of oxygen is the hydroxyl radical ( $HO^{\bullet}$ ).  $HO^{\bullet}$  is extremely reactive with a short half-life probably reacting with the first molecule it encounters. It can be produced by Fenton reaction<sup>5</sup>, Haber-Weiss reaction<sup>6</sup> or by decomposition of peroxynitrite ( $ONOO^-$ )<sup>7</sup>. Finally, the addition of four supplementary electrons to oxygen will form water.

Peroxynitrite and nitric oxide ( $NO^{\bullet}$ ) belong to another family of reactive species produced within cells named reactive nitrogen species (RNS). ROS and RNS can be classified into radical and non-radical species. Radical species, such as  $O_2^{\bullet -}$ ,  $HO^{\bullet}$  or  $NO^{\bullet}$ , cannot cross lipophilic membranes but since they are very unstable, they are the most reactive ones. Non-radical species, like  $H_2O_2$ , are more stable with a longer half-life and are able to diffuse through biological membranes. These properties allow  $H_2O_2$  to activate a wide number of signalling pathways, making it the most relevant ROS signalling molecule in cells. In contrast, by Fenton reaction,  $H_2O_2$  gives rise to  $HO^{\bullet}$  which exerts most of the deleterious effects associated with oxidative stress, since it is able to react immediately with surrounding biomolecules, oxidising them.

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<sup>5</sup> Fenton reaction is the catalytic decomposition of  $H_2O_2$  by free iron bivalent ions ( $Fe^{2+}$ ) leading to the generation of  $HO^{\bullet}$ ,  $HO^-$  and  $Fe^{3+}$  ( $H_2O_2 + Fe^{2+} \rightarrow HO^{\bullet} + HO^- + Fe^{3+}$ ).

<sup>6</sup> Haber-Weiss reaction ( $H_2O_2 + O_2^{\bullet -} \rightarrow HO^{\bullet} + HO^- + O_2$ ) results from the decomposition of two reactions: iron reduction by  $O_2^{\bullet -}$  ( $Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$ ) and Fenton reaction.

<sup>7</sup> Peroxynitrite is a relatively reactive molecule resulting from the reaction of  $O_2^{\bullet -}$  with nitric oxide ( $NO^{\bullet}$ ).

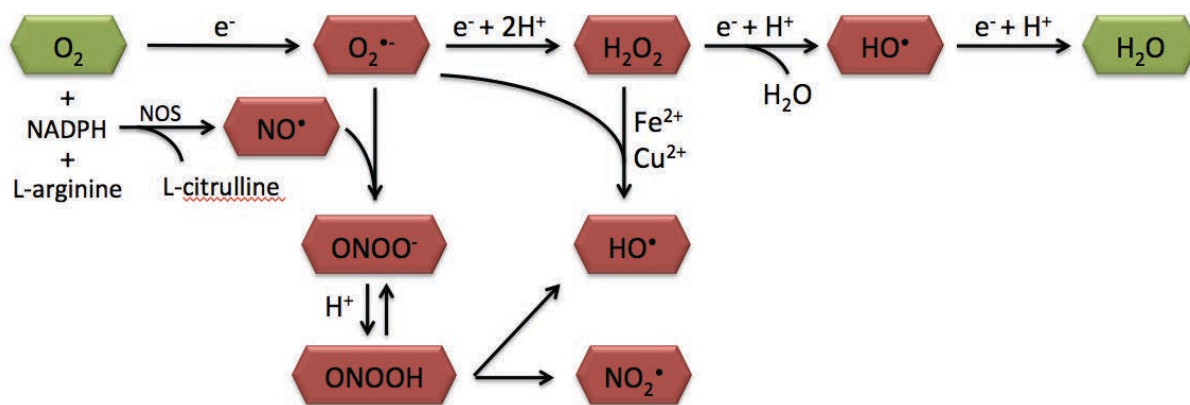


Figure 10 - **Production of reactive oxygen species.** The complete reduction of molecular oxygen ( $O_2$ ) needs the addition of 4 electrons, forming water. If less than 4 electrons are accepted by molecular oxygen, unpaired electrons rise and the reactivity of this molecule increases. In the presence of oxygen, NADPH and co-factors, nitric oxide synthase (NOS) catalyses the oxidation of the amino acid L-arginine to form L-citrulline and nitric oxide ( $NO^{\bullet}$ ).  $NO^{\bullet}$  conjugated with superoxide anion ( $O_2^{\bullet-}$ ), give rise to peroxynitrite ( $ONOO^-$ ), whose decomposition will produce nitrogen dioxide ( $NO_2^{\bullet}$ ) and hydroxyl radical ( $HO^{\bullet}$ ). In acid conditions,  $HO^{\bullet}$  can also result from the reaction of hydrogen peroxide ( $H_2O_2$ ) with metals (Fenton and Haber-Weiss reactions).

### **ROS Production Sites**

Under aerobic condition, oxidative phosphorylation (also known as OXPHOS) within mitochondria is the major endogenous and continuous physiological process for energy production. During energy transduction, a small number of electrons escape from mitochondrial electron transport chain complex I (NADH:ubiquinone oxidoreductase) and III (ubiquinol:cytochrome c oxidoreductase) and can then prematurely interact with oxygen forming the oxygen free radical superoxide ( $O_2^{\bullet-}$  in its anionic form), which is released into the mitochondrial intermembrane space (Chen, Vazquez et al., 2003, Kushnareva, Murphy et al., 2002). Mitochondrial superoxide radicals can also be produced in the outer mitochondrial membrane (Figure 11). Indeed, ROS concentration within the mitochondrial matrix and cytosol can be increased by the  $H_2O_2$  production that occurs during deamination of primary aromatic amines by flavoprotein monoamine oxidase on the outer mitochondrial membrane (Cadenas & Davies, 2000).

It is considered that 2 to 5 % of the total oxygen consumed by mitochondria will lead to the generation of  $O_2^{\bullet-}$  and that 0.15 % to 2 % of the electron flow during OXPHOS will generate  $H_2O_2$  (Finaud, Lac et al., 2006, St-Pierre, Buckingham et al., 2002). The production of ROS by mitochondria is, therefore, intrinsically connected to the cellular metabolic state. In skeletal muscle mitochondria, under resting conditions, only 0.15 % of electron flow

gives rise to  $H_2O_2$  (St-Pierre et al., 2002). However, during exercise, increased oxygen consumption needed for muscle contractions are responsible for a 50 to 100-fold increase in ROS generation (Kanter, 1998).

Although the vast majority of cellular ROS is generated in mitochondria (Balaban, Nemoto et al., 2005), this organelle is not the only source of ROS production within muscle cells. Other relevant sources of ROS production within muscle cells are NADPH (nicotinamide adenine dinucleotide phosphate) oxidases (NOX) located within sarcoplasmic reticulum, transverse tubules and sarcolemma (Figure 11) (Chan, Jiang et al., 2009). NOX generate  $O_2^{\bullet-}$  by transferring electrons from NADPH to molecular oxygen. An isoform of these enzymes, NOX4 is present in the endoplasmic reticulum, thus leading to the production of mainly  $H_2O_2$  in this organelle. Degradation of xenobiotics or unsaturated fatty acids by the Cytochrome P450 located in the membrane of the endoplasmic reticulum is another source of  $O_2^{\bullet-}$  and  $H_2O_2$  production (Seliskar & Rozman, 2007). Furthermore, the activity of various cytosolic enzymes such as xanthine oxidases (XO) or nitric oxide synthases (NOS) is also responsible for the formation of ROS. In skeletal muscle fibres under physiologic conditions, hypoxanthine and xanthine are oxidised to uric acid by xanthine dehydrogenase. However, during exercise, ischemic or hypoxic conditions, xanthine dehydrogenase is converted to xanthine oxidase, which further converts xanthine to uric acid, using molecular oxygen as electron acceptor producing  $O_2^{\bullet-}$  (Engerson, McKelvey et al., 1987, Gomez-Cabrera, Borrás et al., 2005, Gomez-Cabrera, Pallardo et al., 2003). The different isoforms of NOS, NOS1, NOS2 and NOS3, are able to synthesized  $NO^{\bullet}$  by the oxidation of the terminal guanidinyll nitrogen of the amino acid L-arginine in the presence of oxygen, NADPH and co-factors. Lipid metabolism that takes place within peroxisomes can also contributes to the production of  $H_2O_2$  within muscle cells. In fact, even if the primary function of peroxisomes is to consume cytosolic  $H_2O_2$ , they can produce significant quantities of ROS during degradation of fatty acids by the action of flavine oxidase enzymes. Finally, extracellular ROS production can also be at the origin of the increased ROS levels within skeletal muscle cells. Importantly, once activated, immune phagocytic cells increase their oxygen consumption by 10-fold, producing increased levels of  $O_2^{\bullet-}$  at the surface of their cellular membranes that can eventually diffuse into their neighbourhood cells. This very important defence mechanism from infectious agents releases also inflammatory cytokines that can bind to muscle membrane receptors and activate specific ROS-generating enzymes, such as NOX and XO (Ji, 2007). In addition to cytokines, the intracellular levels of ROS in skeletal muscle cells can be increased when cells are exposed to growth factors,

hormones, toxins (such as lipopolysaccharides), but also X-rays or pollutants (Ji, 2007). Furthermore, apart from intense contractile activity and external factors, skeletal muscle cells produce transient fluxes of ROS in response to an array of diverse stimuli, such as heat stress, acute hypoxia or acute osmotic stress.

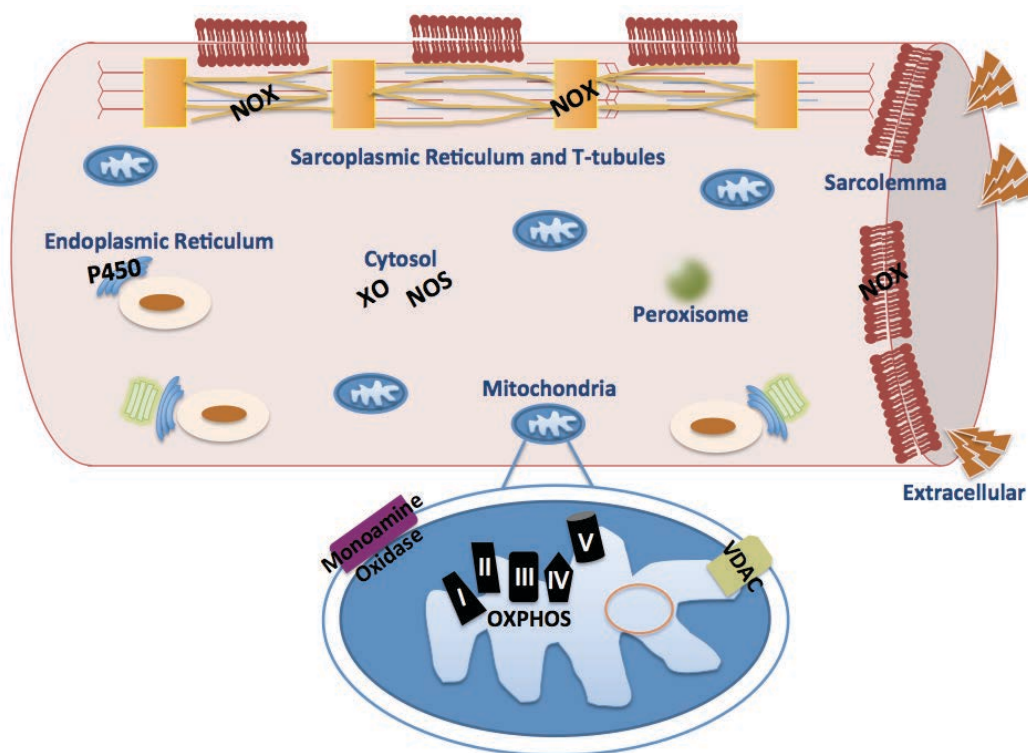


Figure 11 - **Sites and complexes of ROS production within skeletal muscle cells.** Mitochondria is an organelle with different sources of ROS production: complexes I and III of the electron transport chain (OXPHOS), monoamine oxidases and voltage-dependent anion channel (VDAC). Cytochrome P450 is the source of ROS production within endoplasmic reticulum. NADPH oxidases (NOX) are proteins responsible for transporting electrons across sarcolemma, sarcoplasmic reticulum and transverse tubules (T-tubules) membranes, thereby producing superoxide anion. In the cytosol, superoxide anion is produced by xanthine oxidases (XO) and nitric oxide is produced by nitric oxide synthases (NOS). Lipid metabolism is also source of ROS production within peroxisomes. Finally, extracellular cells also provide ROS for skeletal muscle cells.

## ROS Elimination by the Antioxidant Systems

In order to counteract the deleterious effects of ROS on biological macromolecules, cells have developed several enzymatic and non-enzymatic antioxidant systems. These mechanisms are dependent on the oxidant species, their production site and their biological targets.

### *Non-Enzymatic defence systems*

Although the enzymatic antioxidants are considered as the most efficient and specific regulators of the cell redox state, cells display also several other low-molecular weight non-enzymatic antioxidant systems, known as scavengers, that react directly with oxidising agents inhibiting them. Among these molecules there are cysteine, cysteamine, cysteine containing peptides, methionine, albumin, taurin, bilirubin, vitamins C and E, glucose, carotenoids flavones, hydroquinones, alpha-lipoic acid and uric acid.

**Carotenoids** (such as  $\beta$ -carotene) are lipid soluble antioxidants located primarily in cell membranes. They are capable of scavenging numerous ROS species including superoxide and peroxy radicals and due to their cellular location, they are efficient against lipid peroxidation (Stahl & Sies, 2003).

**Vitamin C (ascorbate**, at physiological pH) is an essential water-soluble antioxidant not synthesised by humans. Thus, it is acquired by human cells through dietary sources. Apart from being a cofactor for several metabolic enzymatic reactions, it acts also as a reducing agent providing electrons to various enzymatic or non-enzymatic reactions. Indeed, it has important antioxidant functions, directly scavenging ROS and RNS (Carr & Frei, 1999, Padayatty, Katz et al., 2003). In addition, vitamin C plays an important role in the recycling of vitamin E. The one- and two-electrons oxidised forms of vitamin C, semidehydroascorbic acid and dehydroascorbic acid, respectively, can be reduced in the body by GSH or by other NADPH-dependent enzymatic mechanisms (Meister, 1994). Although its antioxidant properties have been well characterised, it is not considered a major antioxidant because it has also pro-oxidant properties (Carr & Frei, 1999). This can also be an explanation why vitamin C supplementation does not seem to affect skeletal muscle redox state (Mason, Baptista et al., 2014).

**Vitamin E ( $\alpha$ -tocopherol**, its most reactive form) is a lipophilic compound that easily incorporates cellular membranes being able to stop the lipid peroxidation chain reaction by scavenging the lipid peroxy radicals. In this reaction, vitamin E becomes a radical that may function as a pro-oxidant when at high concentrations, although with less reactivity than lipid peroxy radicals (van Acker, Koymans et al., 1993). Oxidised  $\alpha$ -tocopheroxyl radicals can be then recycled back to their reduced active form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol (Packer, Weber et al.,



2001). In skeletal muscle, vitamin E protects cells from lipid peroxidation and neutrophil infiltration during surgical ischemia-reperfusion (Novelli, Adembri et al., 1997). In addition, vitamin E supplementation prevented oxidative stress-induced muscle in rats *soleus* muscle (Appell, Duarte et al., 1997) as well as oxidative stress-induced cell death of chicken skeletal muscle cells (Nunes, Gozzo et al., 2005). In agreement, vitamin E deficiency in mice and rats renders them more susceptible to peroxidative damage when stressed with iron or ascorbic acid and more prone to damage during contractile activity exercise (Jackson, Jones et al., 1983).

Among the cysteine containing antioxidants, there are the glutathione, the N-acetyl-L-cysteine (NAC) and the metallothioneins. **Glutathione (GSH)** is an ubiquitous tripeptide ( $\gamma$ -glutamyl-cysteinyl-glycine). It is synthesised by two enzymatic ATP-dependent reactions, the second of which is catalysed by the glutathione synthetase (GS). It is the most abundant thiol peptide found in cells, reaching concentrations of millimolars (Meister & Anderson, 1983). GSH content varies across organs depending on their function. For example, tissues with high exposure to oxidants such as the eye lens or the liver contain high levels of GSH. Similarly, the concentration of GSH found in skeletal muscle fibres varies across fibre types with slow type fibres containing higher GSH content (i.e. 2 to 3 mM) compared with fast fibres (i.e. 0.5 mM) (Leeuwenburgh, Hollander et al., 1997a). Upon reaction with its targeted molecules such as antioxidant enzymes, ROS or electrophiles, GSH becomes oxidised to glutathione disulfide (GSSG), which can then be reduced to GSH by the GSH reductase (GR) enzyme. Thus, the GSH/GSSG ratio reflects the cellular redox state. Studies indicate that skeletal muscle fibres adapt to regular bouts of high-intensity endurance exercise by increasing the levels of GSH due to an increased activity of key enzymes involved in GSH synthesis (Leeuwenburgh, Fiebig et al., 1994, Leeuwenburgh et al., 1997a, Marin, Kretzschmar et al., 1993, Sen, Marin et al., 1992). Furthermore, GSH supplementation improved lipid metabolism and acidification during exercise leading to less muscle fatigue (Aoi, Ogaya et al., 2015), while GSH depletion increased the levels of the peroxisome proliferator activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ )<sup>8</sup>, affecting mitochondrial function (Strobel, Matsumoto et al., 2014).

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<sup>8</sup> A key regulator of mitochondrial biogenesis and function

**N-acetyl-L-cysteine (NAC)** is a potent unspecific antioxidant molecule that is commonly used to increase the redox-reduced status of cells or model organisms. It is also being used in the treatment of some diseases in which an increased oxidative stress is involved, with more or less efficiency.

**Metallothioneins** are low molecular weight cysteine-rich polypeptides that bind a number of trace metals such as zinc, cadmium, mercury, platinum and silver. These unique proteins are found in all eukaryotes and in some prokaryotes and participate in diverse intracellular functions including maintenance of essential metal ion homeostasis as well as detoxification of heavy metals (Nordberg & Nordberg, 2000). Because of its cysteinyl thiolate groups, metallothioneins have been shown to scavenge different ROS and RNS (RuttKay-Nedecky, NejdI et al., 2013).

### *Enzymatic defence systems*

**Superoxide dismutases (SOD)** are widely distributed antioxidant enzymes found in nearly all living cells exposed to oxygen. Three types of SOD are expressed in human cells: two copper-zinc SOD, one mainly present in the cytoplasm but also present in mitochondria intermembrane space (CuZnSOD or SOD1) and another secreted into the extracellular space (ECSOD or SOD3) and a manganese SOD (MnSOD or SOD2) present in mitochondria. All SOD are capable of dismutating two molecules of  $O_2^{\cdot -}$  into molecular oxygen and  $H_2O_2$  (chemical reaction:  $2 O_2^{\cdot -} + 2 H^+ \rightarrow H_2O_2 + O_2$ ) (Figure 12). However, since MnSOD is present in the mitochondrial matrix, where oxygen is mostly consumed and ROS production most evident, it has been considered as one of the most important antioxidant enzyme in mammals. In fact, whereas CuZnSOD and ECSOD were shown non-essential for survival of mice lacking these enzymes (Sentman, Granstrom et al., 2006), knockout mice for MnSOD showed severe metabolic acidosis, degeneration of neurons and cardiomyocytes, and died between 1 to 24 days after birth (Huang, Carlson et al., 2001, Lebovitz, Zhang et al., 1996). In skeletal muscle, although SOD activity is higher in oxidative muscles compared with muscles with low mitochondrial content, 65 to 85 % of the total SOD activity is in the cytosol, with only the 15 to 35 % of remaining activity in the mitochondria (Powers, Criswell et al., 1994). Thus, although both MnSOD and CuZnSOD mutant mice exhibited oxidative damages (Elchuri, Oberley et al., 2005, Van Remmen, Ikeno et al., 2003), only

mice lacking CuZnSOD showed a loss of muscle mass similar to what is observed during sarcopenia (Muller, Song et al., 2006).

**Catalase (CAT)** is a homotetramer enzyme that contains four porphyrin heme groups. It catalyses the decomposition of  $\text{H}_2\text{O}_2$  into water and molecular oxygen (chemical reaction:  $2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2$ ) (Figure 12). CAT is an ubiquitous protein mainly present in peroxisomes, organelles that produce high quantities of  $\text{H}_2\text{O}_2$  by various oxidases. Although mice lacking catalase are phenotypically normal, their tissues were more sensitive to oxidant injuries (Ho, Xiong et al., 2004). Overexpression of CAT protected cells against oxidative damages (Erzurum, Lemarchand et al., 1993, Lindau-Shepard & Shaffer, 1993). When overexpression of CAT is targeted to mitochondria, it extends murine lifespan by reducing oxidative damage to DNA and proteins as well as protection against aconitase inactivation (Schriner, Linford et al., 2005). In skeletal muscle, CAT protein levels are highest in highly oxidative muscle fibres (Laughlin, Simpson et al., 1990, Leeuwenburgh et al., 1994, Powers et al., 1994). However, whether CAT expression increases or not in response to chronic exercise is still controversial, with studies showing an increase, decrease or no change in muscle CAT activity following training (Laughlin et al., 1990, Leeuwenburgh et al., 1994, Powers et al., 1994, Vincent, Powers et al., 2000).

**Glutathione Peroxidases (GPx)** are enzymes with peroxidase activity that use glutathione (GSH) as electron donor to reduce peroxides such as  $\text{H}_2\text{O}_2$  to water or organic hydroperoxides to their corresponding alcohols (e.g. chemical reaction:  $\text{ROOH} + 2 \text{GSH} \rightarrow 2 \text{ROH} + \text{GSSG}$ ) (Figure 12). To date, eight different isoforms of this enzyme (GPx1-8) have been identified in humans, encoded by different genes and varying in their cellular location and substrate specificity (Brigelius-Flohe & Maiorino, 2013). GPx1 is the most abundant one, being found in the cytosol and mitochondria of nearly all mammalian tissues and having  $\text{H}_2\text{O}_2$  as preferred substrate. Like GPx1, GPx2 (mainly present in the gastrointestinal system) and GPx3 (present in the plasma and kidney) are water soluble enzymes, whereas GPx4 protects membranes from oxidative damages. In fact, GPx4 exhibits a high preference for lipid hydroperoxides. Mammalian GPx1-4 are selenoproteins with a selenocysteine (Sec) instead of a cysteine (Cys) in their catalytic centre; while GPx6 is a selenoprotein only in humans. GPx7 and GPx8 are CysGPxs with low GPx activity. SecGPx overexpression in NIH3T3 fibroblasts and human MCF-7 breast carcinoma cells demonstrated the importance of these enzymes in protection against damages caused by

paraquat (Kelner, Bagnell et al., 1995). The various roles of the different GPx isoforms were also analysed by *in vivo* studies using mice animal models. K.O. mice for GPx1, GPx2 or GPx3 have a normal embryonic development and growth, while mice deficient in GPx4 die during early embryonic development (Esworthy, Aranda et al., 2001, Olson, Whitin et al., 2010, Yant, Ran et al., 2003). Although mice deficient in Gpx1 survive, they present high mitochondrial levels of H<sub>2</sub>O<sub>2</sub>, develop cataracts at an early age and exhibit defects in muscle satellite cell proliferation characterised by a reduced body mass (Esposito, Kokoszka et al., 2000, Reddy, Giblin et al., 2001). Furthermore, numerous studies showed that both in physiological or in endurance exercise training conditions, there is an increase in cytosolic and mitochondrial GPx activities, mainly in highly oxidative skeletal muscles containing primarily type I and IIa fibers (Criswell, Powers et al., 1993, Leeuwenburgh et al., 1994, Pereira, Costa Rosa et al., 1994, Powers et al., 1994).

**Peroxiredoxins (Prx)** are an ubiquitous family of cysteine-dependent peroxidase enzymes that like other peroxidases, regulate the peroxide levels within cells, by reducing hydroperoxides to water. These enzymes share the same basic catalytic mechanism, in which a cysteine of their active site, the peroxidatic cysteine, is oxidised to sulfenic acid by the peroxide substrate (e.g. chemical reaction: H<sub>2</sub>O<sub>2</sub> + Prx (reduced) → 2 H<sub>2</sub>O + Prx (oxidised)). The 6 Prx members are classified through their mechanism of sulfenic acid recycling. For a minority of Prx, termed “1-Cys” Prx, the sulfenic acid formed by oxidation is reduced directly by an intracellular reductant such as glutathione or ascorbate. On the other hand, the majority of Prx, called “2-Cys” Prx, have a second cysteine, which forms a disulfide bond with peroxidatic cysteine. This disulfide bond is then reduced by thioredoxin (Trx) enzymes or a thioredoxin-like proteins (Figure 12). Inactivation of these enzymes by over-oxidation of the active thiol to sulfinic acid can be reversed by sulfiredoxin (Biteau, Labarre et al., 2003). Prx are widely distributed within the cell, being located in the cytosol (Prx1, 2, 5 and 6), nucleus (Prx1) mitochondria (Prx3 and 5), peroxisomes (Prx4 and 5), endoplasmic reticulum and Golgi apparatus (Prx4) (Rhee, Chae et al., 2005, Wood, Schroder et al., 2003). Prx4 is also present in the extracellular space. These antioxidant enzymes regulate ROS levels and protect cells from oxidative stress damages as well as cell death (De Simoni, Goemaere et al., 2008, Kropotov, Serikov et al., 2006, Tavender & Bulleid, 2010). The study of mice lacking Prx1, 2 and 6 revealed their antioxidant roles, such as protection against oxidative stresses and protein oxidative damages, as well as their importance for cellular viability and lifespan (Lee, Kim et al., 2003, Neumann, Krause et al., 2003, Wang, Phelan et

al., 2003). In particular, Prx1<sup>-/-</sup> mice showed reduced lifespan associated with an increase in oxidative damage to both DNA and protein (Egler, Fernandes et al., 2005, Neumann et al., 2003). Prx3 K.O. mice displayed an aberrant mitochondrial redox state, resulting in abnormal adipocyte differentiation and impaired glucose tolerance and insulin resistance (Huh, Kim et al., 2012). In addition, it has been shown that Prx3 plays a crucial role in skeletal muscle fibres function by controlling mitochondrial homeostasis (Lee, Shin et al., 2014b).

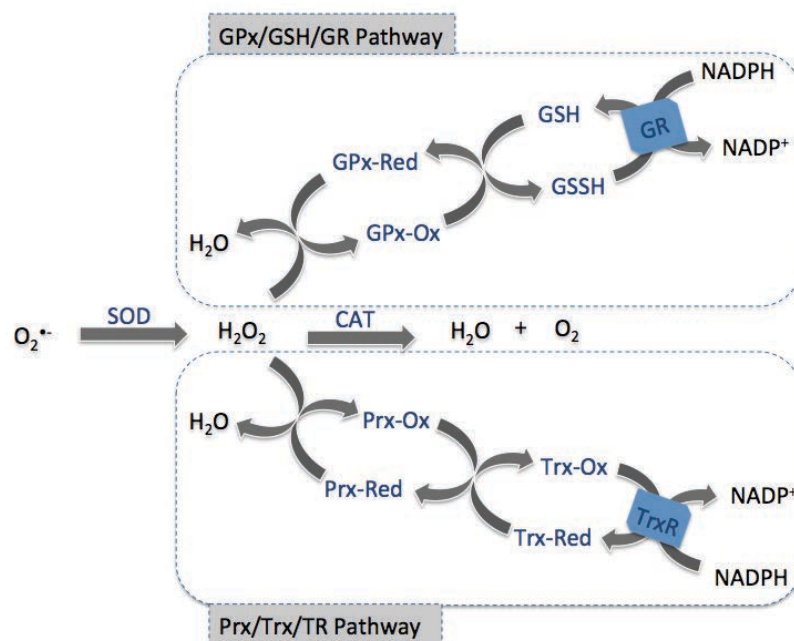


Figure 12 - **Cellular antioxidant defence systems.** Superoxide anion ( $O_2^{\cdot-}$ ) is dismutated in hydrogen peroxide ( $H_2O_2$ ) by the superoxide dismutase enzymes (SOD). Hydrogen peroxide ( $H_2O_2$ ) can be then reduced back to water by the catalase (CAT) enzyme producing molecular oxygen and water. Two other enzymatic pathways are able to reduce hydrogen peroxide ( $H_2O_2$ ) using NADPH as ultimate reducing power: the glutathione peroxidase (GPx) / glutathione (GSH) / glutathione reductase (GR) pathway and the peroxidase (Prx) / thioredoxin (Trx) / thioredoxin reductase (TR) pathway.

## Oxidative Damage to Macromolecules

Low amounts of ROS or RNS are required for cell signalling pathways and defence mechanisms against pathogens. However, when their concentrations exceed the cellular ability to remove them and/or to repair their oxidative damage, accumulation of oxidative stress-induced cell damage occurs. This is characterised by the oxidation of biomolecules

such as nucleotides, lipids and proteins, which will be reviewed in this chapter, with particular focus on protein oxidative damage.

### ***DNA oxidative damage***

Amongst free radical species, the highly reactive HO<sup>•</sup> can generate a large number of modifications in DNA by a variety of mechanisms. These damages include: i) single or double strand breaks; ii) sugar and/or base modifications; iii) DNA-DNA or DNA-protein cross-links such as thymine-tyrosine cross-link (Oleinick, Chiu et al., 1987). Among the four DNA bases, guanine is said to be the most reactive. In fact, its oxidised form, 8-oxoguanine, a well-known biomarker of DNA damage, adopts a conformation that allows it to pair with adenine, leading to transversion mutations (Cooke, Evans et al., 2003). Oxidation of thymine gives rise to thymine glycol, which is another known DNA oxidative damage indicator that blocks DNA replication and transcription (Cooke et al., 2003). Furthermore, persistence of DNA damage can lead to three major consequences: evolution of species, carcinogenesis and ageing (Cooke et al., 2003).

The fact that DNA is easily susceptible to alterations seems to be contradictory to the stability of genetic information from one generation to another. This paradigm results from the existence of highly efficient DNA repair systems. Thus, a rise in DNA oxidative damage could be due to increase damage and/or decreased repair capacity. In fact, mitochondrial DNA (mtDNA) is particularly susceptible to oxidative damage due to its location near the O<sub>2</sub><sup>•-</sup> production site but also due to its lack of protective histones, proofreading and complete repair mechanisms. Therefore, the basal oxidative level in mtDNA is 10 to 20-fold higher than in nuclear DNA (Richter, Park et al., 1988). Since mtDNA encodes essential proteins involved in the OXPHOS process, alterations in the mtDNA will lead to a respiratory chain dysfunction, further stimulating mitochondrial ROS generation and oxidative mtDNA damage, in a sequence of events that will end with the induction of apoptosis.

### ***Lipid oxidative damage***

The most common form of lipid oxidation is the free radical-mediated peroxidation of polyunsaturated fatty acids. It is produced in three steps that can be summarised as follows: a hydrogen atom is transferred from fatty acids, these then react with molecular oxygen to give rise to a lipid peroxy radical, which extracts a hydrogen from another lipid molecule generating a lipid hydroperoxide as well as a new radical (that continues the chain

reaction) (Porter, Caldwell et al., 1995). Cleavage of this cyclic peroxide formation process can result in the release of secondary toxic products, such as the reactive carbonyl compounds malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). These products are precursors of advanced lipid peroxidation end products (ALE), which are shown to accumulate during ageing and chronic diseases (Negre-Salvayre, Coatrieux et al., 2008). Since they are capable of reacting with DNA bases or being conjugated with proteins, the concentration of “free” HNE and MDA is very low *in vivo* (Esterbauer, Schaur et al., 1991). Their cross-link with proteins progressively alters protein structure and function (Friguet, Szweda et al., 1994). This will have consequences on signal transduction pathways related with the inflammatory status, cell proliferation and viability. For instance, at low concentrations, 4-HNE can act as a growth factor promoting cell proliferation, while at high concentrations it inhibits cell proliferation mediated by growth factor receptors. Thus, the reduction of lipid hydroperoxides is necessary not only to prevent further oxidation and decomposition but also for cell signalling. For these reasons, cells dispose of diverse antioxidant systems, such as selenocysteine-containing enzymes like GPx, to eliminate this kind of damages (Ayala, Munoz et al., 2014). Besides enzymatic systems, antioxidants such as vitamin E can also inhibit lipid peroxidation.

### ***Protein oxidative damage***

As the main components of biological systems, proteins are major targets of oxidation, scavenging up to 75% of ROS molecules (Davies, Fu et al., 1999). Protein oxidative damage, underestimated for a long time, is now assuming an important place in biological processes and is considered as a hallmark of ageing and some diseases such as neurodegenerative disorders. In fact, protein oxidation can lead to loss of protein function, which has important consequences to cells. On the other hand, reversible oxidative damages to proteins may have dual protective roles: controlling protein activity and protection against irreversible oxidative damages (discussed on Chapter IV).

### ***Reversible oxidative damage to proteins***

Contrary to other amino acids, oxidation of the two sulfur containing amino acids, cysteine and methionine, is reversible and repaired enzymatically. This property confers to these two amino acids an important redox power and allows them to be implicated in different signalling redox pathways.

Cysteines possess a high reactive thiol group (SH), which makes this amino acid an important structural and functional component of many proteins and enzymes. The first cysteine oxidation product is sulfenic acid (SOH), which is an intermediate in disulfide bond formation (Figure 13) (Rehder & Borges, 2010). At the physiological level, intra- or inter-molecular disulfide bridge formation is the most likely result of cysteine oxidation. This modification can be easily reduced back to thiols by a variety of enzymes systems such as thioredoxin or glutaredoxin (discussed on Chapter IV). Sulfenic acid can be converted to sulfinic acid (SO<sub>2</sub>H) by a second oxidation in cysteine residue. To date, the reduction of sulfinic acid by sulfiredoxins has been only described in oxidised cysteines located in “2-Cys” Prx active sites (Biteau et al., 2003). The thiol group of cysteines can also react covalently with RNS, such as NO<sup>•</sup> forming S-nitrosothiols (SNO) or with oxidised glutathione (GSSG) forming S-glutathionylates (SSG), which can be enzymatically reversed by thioredoxin or glutaredoxin enzymes (Figure 13). Finally, a third oxidation reaction on cysteine is possible by strong oxidants that will generate sulfonic acid (SO<sub>3</sub>H), which can not be reduced enzymatically (Figure 13).

Reversible oxidation in cysteine amino acid has been shown to play important regulatory roles on the function of several proteins. In particular, loss of sulfhydryl groups are involved in protein misfolding, catalytic inactivation of enzymes and loss of binding of metals (Klomsiri, Karplus et al., 2011). In addition, reversible oxidation of catalytic cysteine is involved in several redox enzymatic systems such as peroxiredoxins, glutaredoxins, thioredoxins, glutathione-S-transferases and methionine sulfoxide reductases (Brandes, Schmitt et al., 2009, Lowther, Brot et al., 2000a, Murphy, 2012, Rhee, Yang et al., 2005). Apart from regulating antioxidant enzymes activity, these oxidation-reduction cycles involving cysteines play important roles in several other cellular pathways, involving transcription factors such as AP-1 (activator protein-1), NF-κB (nuclear factor-κB), keap1 (Kelch-like ECH associated protein 1) and metabolic proteins including p53, creatine kinase, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as well as some protein tyrosine phosphatases (Brandes et al., 2009, Chung, Wang et al., 2013).

Methionine is an essential amino acid not synthesised by humans; hence it must be supplied through diet. Methionine alone or within proteins is a very reactive amino acid that can react with different kinds of ROS such as O<sub>2</sub><sup>•-</sup>, H<sub>2</sub>O<sub>2</sub>, OH<sup>•</sup>, ONOO<sup>-</sup>, chloramine or hypochloric acid (Schoneich, 2005). Due to its high reactivity towards almost all kinds of ROS, both free and surface exposed methionine on proteins, are excellent ROS scavengers (Levine, Mosoni et al., 1996). Methionine oxidation, by the addition of an extra oxygen



atom, leads to the generation of two diastereoisomeric forms of methionine sulfoxide (MetO), named methionine-S-sulfoxide (MetSO) and methionine-R-sulfoxide (MetRO) (Figure 13). These two oxidised forms of methionine can be reduced back to native methionines by methionine sulfoxide reductase (Msr) system (further described in Chapter V). Further oxidation of MetO by ROS leads to the generation of irreversible methionine sulfone (MetO<sub>2</sub>).

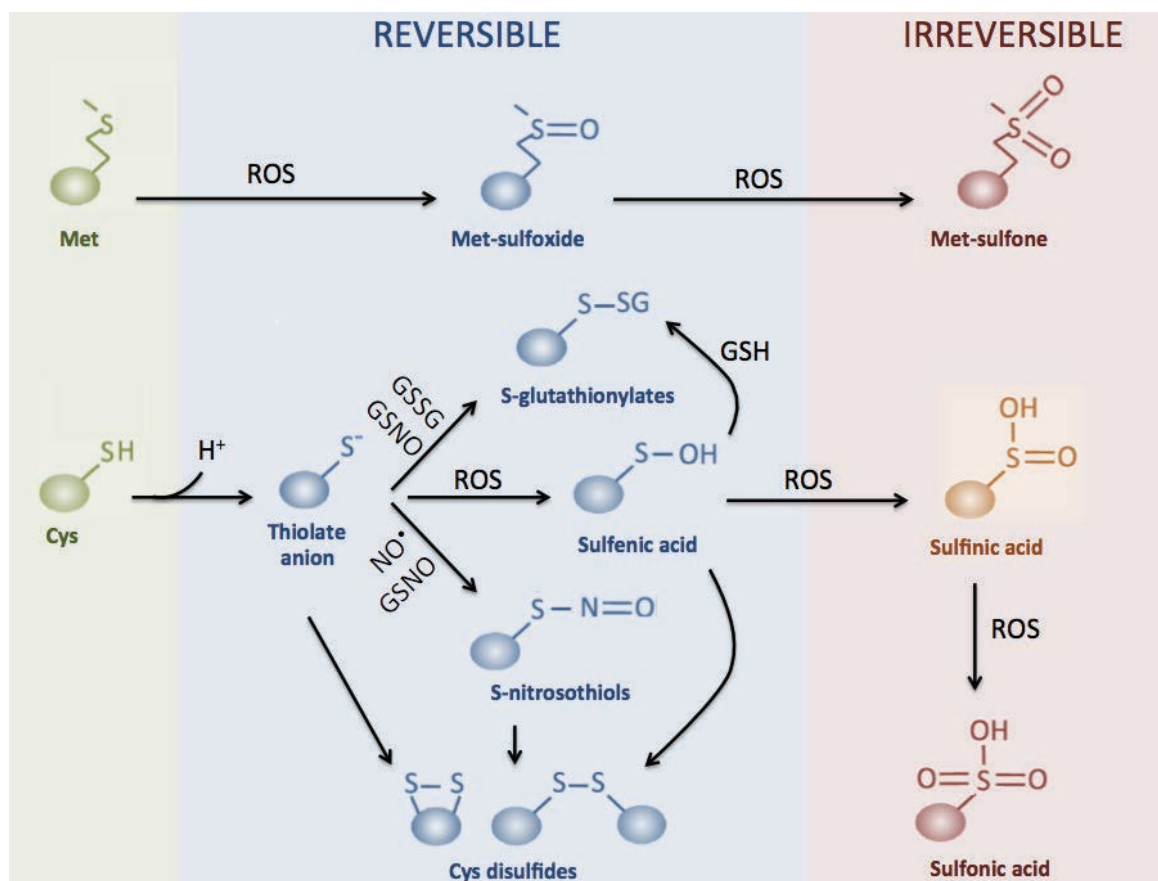


Figure 13 - **Oxidative modifications on sulfur containing amino acids.** When reacting with ROS, methionine (Met) sulfur gives rise to methionine sulfoxide (Met-SO), which can be reduced back to Met by the methionine sulfoxide reductases enzymes (Msr). However, further oxidation on Met-sulfoxide, forming Met-sulfone is irreversible in cells. Cysteine (Cys) thiol groups (SH) are very reactive groups that can give rise to thiolate anions (S<sup>-</sup>) within proteins. These anions can further react with ROS or RNS producing sulfenic acid (SOH) or S-nitrothiols (SNO). The last ones can also be formed enzymatically by S-nitrosoglutathiones (GSNO) in a reaction called S-nitrosylation. All thiolate anions, sulfenic acid and S-nitrothiols can be interconverted into disulfide bonds. Disulfides can be reduced back to cysteine by thioredoxin (Trx) and glutaredoxin (Grx) enzymes in a NADPH dependent manner. Grx enzymes are also responsible the reduction of the S-glutathionylates (SSG) formed by reaction of thiolate anions with oxidised glutathione (GSSG) or sulfenic acid with glutathione (GSH). Further oxidation of sulfenic acid by ROS results in the formation of sulfinic acid (SO<sub>2</sub>H), which was for long consider an irreversible modification. However, when occurring within peroxiredoxins, sulfinic acid can be reversed back to thiol by sulfiredoxins (Srx) in an ATP-dependent manner. Finally, a third oxygen introduction in cysteines by ROS leads to the formation of sulfonic acid (SO<sub>3</sub>H), which has been shown to be irreversible. Adapted from (Ma, 2013, Rojo, McBean et al., 2014).

The preferential targeting of methionine amino acids by ROS protects other amino acid residues from oxidation, particularly those located at the catalytic sites of the proteins. Such antioxidant property confers damage protection to vital proteins and thus confers to Met oxidation/reduction cycles an important role on protein homeostasis. This is made possible since oxidation of surface exposed methionine does not always affect protein function. Indeed, oxidation of methionine residues in the plasminogen activator inhibitor (Strandberg, Lawrence et al., 1991), in interferon  $\alpha$ -2b (Gitlin, Tsarbopoulos et al., 1996) and in glutamine synthetase enzyme (Levine et al., 1996) do not alter their biological activities. However, in some instances, methionine oxidation does cause impairment of protein structure and/or function, with reduction of MetO being associated with a recovery of protein function. In these cases, Msr enzymes by reducing MetO play important roles in regulation of protein function (discussed in detail in Chapter V).

### ***Irreversible oxidative damage to protein***

If cysteine and methionine residues are, by far, the most sensitive to oxidation by almost all kinds of ROS, other amino acids can also be prone to oxidation within proteins. However, contrary to the above-cited amino acids, there is no enzymatic systems capable of reversing their oxidation. Protein carbonyls and tyrosine nitration are the main types of irreversible oxidations within proteins. These modifications are often associated with oxidative damage and have been used as biomarkers for assessment of oxidative stress in ageing and diseases (Stadtman, 2001).

Tyrosine oxidation induced by oxidants such as hypochlorite or peroxynitrite leads to the formation of tyrosyl radicals, which may form intra- or inter-molecular cross links or react with nitric oxide forming a 3-nitrotyrosin group on proteins' tyrosine side chains in a reaction called protein nitration (van der Vliet, Eiserich et al., 1996). Although the reversibility of protein nitration has been questioned, there is no clearly defined mechanism, other than protein degradation, to remove this modification (Abello, Kerstjens et al., 2009). Current development of methods for detection and quantification of this protein nitrotyrosine modification has allowed the investigation of its deleterious effects as well as its role in redox signalling (Dekker, Abello et al., 2012, Weber, Kneschke et al., 2012). In fact, its detection under normal physiological conditions such as healthy pregnancy indicates that formation of protein nitrotyrosine may have also a physiological function (Webster, Roberts et al., 2008). However, tyrosyl radicals have also been found in various diseases, in aged

skeletal and cardiac mouse muscle (Leeuwenburgh, Wagner et al., 1997b) as well as in proteins derived from aged non-cataractous human lens (Wells-Knecht, Huggins et al., 1993). Protein nitration is not only restricted to tyrosine residue but also limited to specific proteins, thus acting as a highly selective process (Abello et al., 2009).

Conversely, protein carbonylation is a more abundant and more random protein non-repairable modification. For this reason, protein carbonyl content is the most commonly used marker of protein oxidation (Baraibar, Ladouce et al., 2013). Oxidative modifications of proteins can be classified into those that modify the side chains and those that cleave the peptide bond, leading to the formation of protein derivatives or peptide fragments both containing highly reactive carbonyl groups: aldehydes and ketones (Stadtman & Levine, 2003). Carbonyl groups in proteins can occur through different pathways: 1) ROS can directly react with proteins; 2) metal cations such as iron and copper can react with proteins catalysing their reaction with ROS; or 3) ROS can react with other macromolecules such as lipids and sugars generating reactive groups that will further attack proteins (Figure 14). Direct oxidation of proteins by ROS will result in oxidative cleavage of peptide bonds by either  $\alpha$ -amidation pathway or by oxidation of the glutamyl side chains leading to the generation of highly reactive carbonyl derivatives, such as the  $\alpha$ -ketoacid peptide (Figure 14, step 3) (Stadtman & Berlett, 1998). Among all amino acids, the most susceptible amino acids to metal catalysed oxidative attack are lysine, arginine, proline and threonine (Figure 14, step 2). The most representative products of this type of carbonylation are  $\alpha$ -amino adipic semialdehyde from lysyl residue, glutamic semialdehyde from arginyl and prolyl residue, 2-pyrrolidone from prolyl residue, and 2-amino-3-ketobutyric acid from threonyl residue (Requena, Levine et al., 2003). In sugars-derived protein oxidation, secondary reactions on amino groups of lysine or arginine residues with reducing sugars or their oxidation products, derived from glycation or glycoxidation reactions, will generate reactive carbonyls in proteins (Figure 14, step 4). In more detail, sugar aldehydes or ketones can spontaneously, non-enzymatically and reversibly, react with the amino groups of protein side chains giving rise to Schiff base adducts. These initial reactions representing the early-stage glycation processes and their products are called early-stage glycation adducts or Amadori products. Further irreversible modification of these adducts through rearrangement, dehydration, oxidation, fragmentation and cross linking with proteins yields stable end-stage adducts called Advanced Glycation End-products (AGE) (Cho, Roman et al., 2007). Although glucose and fructose are present in significant concentrations *in vivo*, they react slowly with proteins to form AGE. In contrast, aldehydes such as glyoxal and methylglyoxal that are

present in very low concentrations *in vivo*, react more rapidly with proteins in a glycation process. Only few glycation products are known, including carboxymethyllysine (CML), carboxyethyllysine (CEL), pentosidine, glucosepane, 3-deoxyglucosone-derived lysine dimers, glyoxal-derived lysine dimers and methylglyoxal-derived lysine dimers (Rabbani & Thornalley, 2008). CML and pentosidine contents are the most common used biomarkers for intracellular glycoxidative stress. These AGE have been initially related to diabetes but their formation have recently been involved in human skin ageing, rat liver ageing and neurodegenerative diseases (Bakala, Ladouce et al., 2013, Radjei, Gareil et al., 2016). Peroxidation of polyunsaturated fatty acids results in the formation of  $\alpha,\beta$ -unsaturated reactive aldehydes that can react with lysine, cysteine or histidine residues within proteins leading to protein carbonyl formation (Figure 14, step 1). More precisely, free radicals generated in the cell under oxidative stress conditions can directly attack cellular membranes, resulting in the decomposition of membrane polyunsaturated acids into a variety of oxidised products, including 4-HNE, acrolein and MDA (Ayala et al., 2014). 4-HNE is an highly reactive  $\alpha,\beta$ -unsaturated aldehyde that can react with protein lysine, cysteine, and histidine residues of proteins to form Michael adducts (Sayre, Lin et al., 2006). MDA, on its turn, form Schiff base adducts when reacting with lysine residues, affecting protein function (Slatter, Paul et al., 1999). The reaction of these lipid peroxidation products with proteins, will lead to the formation of the Advanced Lipid peroxidation End products (ALE), which are also known to accumulate during ageing and in certain age-related diseases.

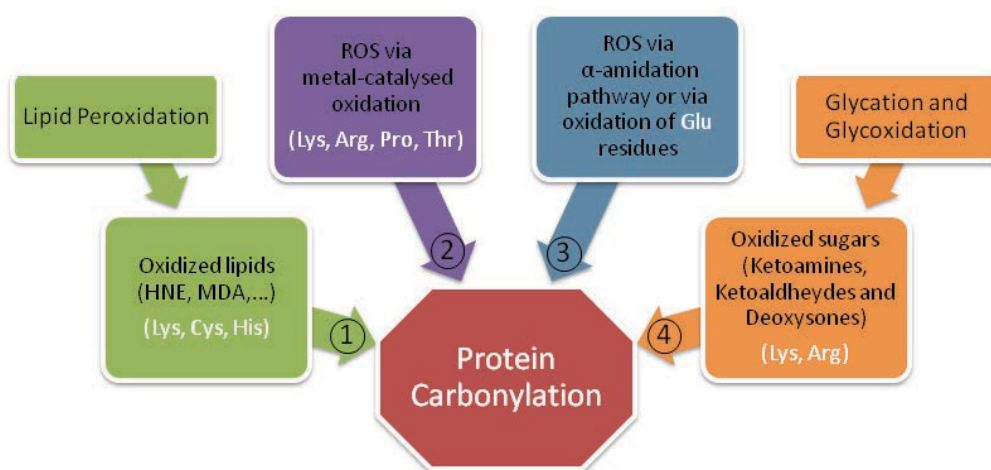


Figure 14 - **Sources of protein carbonyls.** Carbonylation on proteins can be triggered by oxidised lipids (1); directly by ROS via metal catalysed oxidation (2); via the  $\alpha$ -amidation pathway (3); or by oxidised sugars (4).

Due to its greater magnitude comparing to other types of protein oxidative damage, protein carbonylation is usually considered as an indicator of the level of susceptibility or resistance to oxidative stress. This protein modification causes unfolding of the target protein domains and subsequently exposition of the interior hidden hydrophobic amino acids to the surface of the polypeptide chains (Chao, Ma et al., 1997, Friguet et al., 1994, Pacifici, Kono et al., 1993). This rearrangement increases the protein hydrophobicity and enhances its recognition and degradation by different protease systems (discussed in Chapter IV), such as the proteasome (Grune et al., 2004). Indeed, besides protein degradation, no other convincing mechanism has been found to reverse or eliminate this modification. When protein degradation is defective, excessive oxidation may result in stable aggregate formation due to covalent cross-links or hydrophobic interactions between these unfolded proteins. These structures were shown to accumulate within the cells since they are resistant to degradation (Grune et al., 2004) and they can even inhibit proteasome function (Friguet & Szweda, 1997, Sitte, Huber et al., 2000). The accumulation of these protein oxidation-derived aggregates can be deleterious to cellular function and was already observed during ageing and diverse pathological situations such as neurodegenerative disorders, diabetes and atherosclerosis (Baraibar & Friguet, 2013, Baraibar et al., 2013, Berlett & Stadtman, 1997, Friguet, 2006, Squier, 2001). In the case of skeletal muscle tissue, protein carbonylation has been associated with skeletal muscle dysfunction and sarcopenia for long time (discussed in Chapter I), however, it is only recently that the protein targets of this oxidative damage are being revealed (Lourenço Dos Santos, Baraibar et al., 2015).

## **ROS in Skeletal Muscle Myogenesis**

It is now clear that ROS play dual opposite roles in skeletal muscle tissue: a deleterious one leading to many muscle diseases and sarcopenia and another more physiological role, controlling different important signalling pathways relevant for muscle function. The regulation of this balance between beneficial and detrimental ROS effects will depend mostly on their concentrations but also on their nature, origin and targets as well as on the antioxidant status of the cell. Thus, it is important to keep in mind all these aspects when studying ROS in biological functions. Among the diverse ROS-dependent signalling pathways relevant to skeletal muscle cellular homeostasis and function, I will particularly

focus on those described as important for muscle regeneration in *in vivo* models of regeneration as well as *in vitro* experiments using muscle progenitor cells.

As for other adult stem cells, ROS are believed to play important roles in myoblasts function. Studying muscle progenitors cells *in vitro*, different authors showed that their differentiation is accompanied by an increase in the number of mitochondria and ROS levels (Malinska et al., 2012, Piao, Seo et al., 2005, Won, Lim et al., 2012). Increased ROS levels can result from higher activity of mitochondrial respiratory chain complexes but also from the activity of cytosolic and membrane enzymes, such as NOS and NOX, respectively. Independently of the source of ROS, studies suggest that ROS are important for the myoblasts differentiation step (Figure 15). Indeed, the use of mitochondrial specific antioxidants, the inhibition of complex I of the mitochondrial respiratory chain or the suppression of NOX2 activity blocked myotube formation (Lee, Tak et al., 2011, Piao et al., 2005). In agreement, overexpression of antioxidant enzymes such as Heme oxygenase 1 (Kozakowska, Ciesla et al., 2012) or Prx2 (Won et al., 2012) promotes myoblasts proliferation and delays their differentiation as revealed by increased levels of Pax7 and decreased Myogenin expression, respectively. In contrast, increased intracellular ROS levels through *Gpx1* inactivation (Lee, Shin et al., 2006) or in the Nrf2 K.O. mice (Al-Sawaf, Fragoulis et al., 2014) led to decreased myoblasts proliferation capacities and premature differentiation resulting in the formation of hypotrophic myotubes or to immature muscle regeneration with less MyoD expression.

Among the different signalling pathways that are modulated by ROS and that could mediate a positive role on differentiation, NF- $\kappa$ B and p38 $\alpha$  mitogen-activated protein kinase (MAPK) have been the most studied in myogenesis (Figure 15). Contrary to other p38 isoforms ( $\beta$ ,  $\delta$  and  $\gamma$ ), p38 $\alpha$  plays an important role in adult skeletal muscle regeneration (Brien, Pugazhendhi et al., 2013, Ruiz-Bonilla, Perdiguero et al., 2008). Indeed, deletion of p38 $\alpha$  in primary myoblasts delayed cell cycle exit and differentiation via continuous proliferation (Perdiguero, Ruiz-Bonilla et al., 2007). A recent transcriptomal study using primary and C2C12 myoblasts in the presence or absence of p38 $\alpha$  also revealed its role as an activator of myogenic differentiation (Segales, Islam et al., 2016). Myoblasts fusion was also shown to require NF- $\kappa$ B-dependent expression of NOS enzymes, with the NO $\cdot$  produced by these enzymes acting as a messenger molecule for myoblast fusion in a Ca $^{2+}$ -dependent manner (Choi, Baek et al., 1992, Lee, Baek et al., 1994, Lee, Kim et al., 1997). Indeed, Ca $^{2+}$  influx was important for the expression of Myf5 in C2C12 myoblasts (Furutani, Murakami

et al., 2009). It should be noticed that although NF- $\kappa$ B has mostly been associated with a negative regulation of skeletal muscle differentiation (further described below), there are also evidences that it promoted myogenic differentiation downstream the p38/MAPK pathway (Baeza-Raja & Munoz-Canoves, 2004, Lee et al., 2011). Indeed, if ROS production by NOX2 enzymes leads to muscle differentiation via activation of the NF- $\kappa$ B pathway, the inhibition of phosphatidylinositol-3-kinase (PI3K) and p38/MAPK signalling suppressed the NOX2/NF- $\kappa$ B effects on muscle differentiation (Piao et al., 2005). In conclusion, although there is still some open questions concerning the precise mechanisms through which ROS may trigger muscle signalling pathways important for myogenesis, it has become accepted that ROS, in particular H<sub>2</sub>O<sub>2</sub> and NO<sup>•</sup>, are important signalling molecules that mediate muscle differentiation through the activation of p38 $\alpha$ /MAPK and NF- $\kappa$ B pathways, which in some cases may involve regulation of Ca<sup>2+</sup> influx.

Contrariwise, numerous evidences indicate that excessive ROS levels due to elevated ROS production and/or their deficient elimination by the antioxidant enzymes will create an oxidative state that will impair myogenic differentiation (Figure 15). Indeed, ROS generated by the inflammatory cytokine TNF- $\alpha$  induced cachexia and inhibited mice skeletal muscle regeneration after injury (Coletti, Moresi et al., 2005). In agreement, *in vitro* treatment of murine C2C12 myoblasts with TNF- $\alpha$  depleted the levels of GSH thus increasing ROS levels, which impaired myogenic differentiation via redox-dependent and independent pathways (Langen, Schols et al., 2002). An example of a redox sensitive pathway is the redox-dependent blockage of the formation of functional catenin-cadherin interacting complexes, which are known to be involved in the early steps of myogenic differentiation (Goichberg, Shtutman et al., 2001). Different teams showed that the oxidative stress state and the inhibition of myoblasts differentiation induced by TNF- $\alpha$  treatment or GSH deletion were achieved via NF- $\kappa$ B signalling (Ardite, Barbera et al., 2004, Guttridge, Mayo et al., 2000, Langen, Schols et al., 2001, Langen, Van Der Velden et al., 2004). Indeed, NF- $\kappa$ B was shown to be constitutively active in proliferative myoblasts and to inhibit cell cycle exit thus blocking differentiation by promoting a mitogenic activity via cyclin D1 and Pax7 up-regulation as well as by decreasing MyoD mRNA and protein levels (Guttridge, Albanese et al., 1999, Guttridge et al., 2000, He, Berardi et al., 2013, Langen et al., 2004). Moreover, treatment of primary myoblasts with an NF- $\kappa$ B inhibitor (curcumin) stimulates myoblast fusion thereby enhancing myogenesis and repair (Thaloor, Miller et al., 1999). Thus, if under homeostatic conditions NF- $\kappa$ B signalling promotes myogenic differentiation, in conditions of oxidative stress it will impair myoblast fusion and differentiation. One explanation to its

opposite functions may come from the existence of an alternate and a canonical NF- $\kappa$ B pathway which are differentially regulated depending on the biological context (Bakkar & Guttridge, 2010). In addition, the dual effects of ROS on NF- $\kappa$ B activity could come from the oxidation of different proteins of this signalling pathway: the oxidation of I $\kappa$ B leads to its subsequent degradation, and by consequence, to NF- $\kappa$ B stimulation, while the direct oxidation of NF- $\kappa$ B proteins leads to a decrease of its activity by reduction of its DNA binding affinity.

In order to further clarify the consequences of an oxidative stress state in skeletal muscle myogenesis, different laboratories used exogenous oxidants, such as high doses of H<sub>2</sub>O<sub>2</sub>, menadione or paraquat to treat myoblasts models *in vitro*. If the vast majority of these studies led to the generally accepted view that an oxidant treatment is deleterious for myoblasts survival, proliferation and differentiation, in some cases, it would only affect one of these myogenic stages. For instance, treatment with relatively high concentrations of H<sub>2</sub>O<sub>2</sub> resulted in a dramatic decrease in cellular viability and lifespan of proliferative human-derived myoblasts, but did not affect their myogenicity neither their ability to differentiate or fuse to form multinucleated myotubes *in vitro* (Renault et al., 2002). Apart from the increased apoptosis, which is dependent on the activation of caspase proteins (Primeau, Adhietty et al., 2002), increased cell atrophy and sensitivity to mechanical stresses as well as decreased cell stiffness and adhesion capacities were found in human and/or mouse-derived myoblasts in oxidative stress conditions (Dargelos, Brule et al., 2010, El Haddad, Jean et al., 2012, McClung, Judge et al., 2009, Mougeolle, Poussard et al., 2015, Renault et al., 2002, Sun, Wong et al., 2014). Other studies observed that viable oxidative-injured myoblasts presented impaired differentiation *in vitro* characterised by late protein synthesis recovery and reduced MyoD, Myogenin and MRF4 expression (Furutani et al., 2009, Hansen, Klass et al., 2007). These oxidative stress-derived myotubes presented reduced diameter and less abundance of muscle important proteins such as myosin, actinin and troponin I (McClung et al., 2009). Thus, an oxidative stress state would affect mainly the survival, proliferative and adhesion capacities of myoblasts *in vitro*, although it could also impair differentiation of the surviving cells. Protein kinases such as PI3K and Protein kinase B (PKB or Akt) were shown to be involved in L6 rat myotubes resistance to a menadione-mediated oxidative stress (Lim, Woo et al., 2008) as well as for C2C12 myoblast protection against H<sub>2</sub>O<sub>2</sub>-induced apoptosis (Matheny & Adamo, 2010).

If an oxidative state has been proved harmful for muscle function, antioxidant molecules were shown to play protective roles on muscle myogenesis. Indeed, natural



antioxidants such as Oligopin<sup>9</sup> (Dargelos et al., 2010), *Schisandrae Fructus* ethanol extract (Kang, Han et al., 2014), *Sargassum horneri* methanol extract (Kang, Choi et al., 2015) or creatine (Sestili, Barbieri et al., 2009) prevented cultured myoblasts and myotubes from cell death after oxidative treatment via modulation of heat shock protein B1 phosphorylation (Poussard, Pires-Alves et al., 2013) or via Nfr2-dependent up-regulation of the antioxidant enzyme heme oxygenase-1 (Kang et al., 2015, Kang et al., 2014). Another antioxidant enzyme that was shown to mediate the effects of antioxidant molecules is the GPx3, which mediated the antioxidant effects of retinoid acid treatment on human primary myoblasts cultures (El Haddad et al., 2012). Moreover, a proteomic study on creatine-treated C2C12 myoblasts showed increased levels of Prx3 and Prx4 (Young, Larsen et al., 2010), suggesting an indirect antioxidant capacity of creatine, mediated by these thioredoxin-dependent peroxide reductases. Indeed, the presence of antioxidant enzymes was proved necessary to counteract oxidative state conditions. While the myotubes sensitivity to exogenous oxidative stresses was attributed to a reduced expression and activity of antioxidant enzymes at these later stages of *in vitro* differentiation (Franco, Odom et al., 1999), the high levels of SOD and GSH found in muscle-derived stem cells, conferred them greater regenerative capacities when implanted *in vivo* comparing to myoblasts, which presented less antioxidant enzymes (Urish, Vella et al., 2009). Moreover, the study of myoblasts derived from K.O. mice for the mitochondrial Prx (Prx3) revealed a deregulation of myotubes mitochondrial network and membrane potential with higher ROS levels and less ATP production compared to the W.T. myotubes (Lee et al., 2014b).

Taken together, these studies suggest that tiny regulated ROS levels are necessary for cell cycle exit and myoblasts differentiation. This is achieved by an increase in the activities of different ROS producing enzymes such NOX, NOS and enzymes of the mitochondrial respiratory chain at early states of differentiation. In order to avoid excessive ROS production, some antioxidant enzymes follow the ROS increase during differentiation. This is the case for Prx2, whose expression increases from early differentiation steps in a PI3K-dependent activation of NF- $\kappa$ B manner (Won et al., 2012). Indeed, ROS signalling in muscle progenitors is mediated by p38 $\alpha$ /MAPK and NF- $\kappa$ B pathways to allow MyoD expression, myoblasts fusion and differentiation. On the other hand, elevated ROS levels creating an oxidative state on myoblasts will lead to mitochondrial degeneration, oxidative damages and less adhesion capacities, ultimately leading to cell death. Such an oxidative stress state is

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<sup>9</sup> a natural pine bark extract containing members of the flavenoids family

created by exogenous ROS and its effects on myoblasts function are also mediated by the NF- $\kappa$ B pathway. Depending on the cases, elevated ROS content could also lead to impaired differentiation of the surviving cells. In contrast, antioxidant treatments, by increasing the expression of specific antioxidant enzymes, will allow myoblasts survival and enhanced adaptive myogenic capacity in regard to an oxidative stress state.

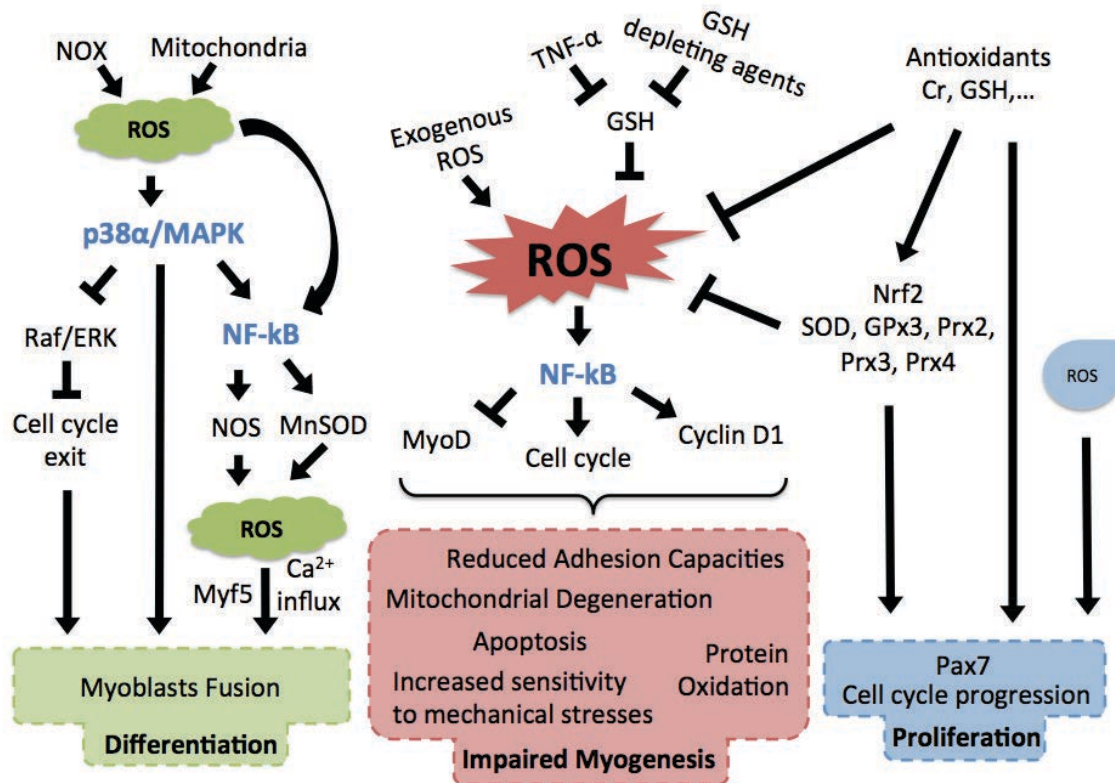


Figure 15 – **Critical role of ROS in myogenesis.** While low levels of ROS are observed in proliferative myoblasts, moderate ROS levels are required for cell cycle exit, fusion and differentiation. However, excessive ROS, not balanced by antioxidant systems are deleterious for myogenesis. Thus, for correct myogenesis, myoblasts redox state should be tightly regulated.



## IV. Protein Homeostasis

Protein homeostasis (also known as proteostasis) is a major process that needs to be tightly regulated in order to preserve cellular and organismal physiological function. Since skeletal muscle is the major protein reservoir in the body, the study of protein synthesis, maintenance and breakdown within muscle cells gains even more importance. In this chapter, I will review proteolytic systems as well as protein repair systems that deal with oxidative modifications to proteins, as part of protein homeostasis.

### Protein Degradation Systems

When protein oxidation results in irreversible modifications, these proteins have to be eliminated to avoid their accumulation and subsequent cell dysfunction. This elimination is done by proteolytic systems that will degrade these proteins, generating amino acids that can be further used for *de novo* protein synthesis. Among these protein degradation systems are Lon and Clp proteases present in the mitochondria, the ubiquitin-proteasome system present in the nucleus and in the cytoplasm, and various other proteases present in the lysosomes. More recently, evidences suggest that some cysteine proteases such as caspases and the  $\text{Ca}^{2+}$ -dependent calpains are also able to degrade sarcomeric proteins with oxidative modifications (Chen, Huang et al., 2014, Smuder, Kavazis et al., 2010) but the mechanisms for this kind of oxidised protein degradation are not yet elucidated.

#### *Mitochondrial proteases*

Mitochondrial proteases are critically important for mitochondrial protein maintenance and have been implicated in ageing and some diseases such as cancer (Hamon, Bulteau et al., 2015, Quiros, Langer et al., 2015). These proteases are responsible for the elimination of damaged, oxidised or unfolded proteins being distributed within the different mitochondrial compartments (Figure 16). In the mitochondrial matrix, protein quality control is mainly achieved by two ATP-dependent serine proteases: Lon and Clp. Lon protease (LonP) is a soluble protein encoded by a single nuclear gene and responsible not only for the degradation of misfolded or damaged proteins but also of normal mitochondrial proteins promoting correct regulation of mitochondrial metabolic processes (Wang, Maurizi et al.,

1994). Activation of LonP requires the formation of a single homo hexameric ring with 3 functional domains in each subunit: a N-terminal domain, involved in substrate recognition; an ATPase domain that binds and hydrolyses ATP; and a protease domain, which hosts the proteolytic active site (Hamon et al., 2015). Its proteolysis mechanism is similar to that of other ATP-dependent proteases: it starts with substrate recognition and binding, unfolding of the substrate, translocation onto the proteolytic chamber and final protein cleavage (Licht & Lee, 2008). Regarding Clp protease, its active form includes a proteolytic core (named ClpP in humans) made of two heptameric rings and sandwiched between two ATPase-active chaperone rings (called ClpX in humans), forming the so-called ClpXP complex (Kress, Maglica et al., 2009). The chaperone rings consist of hexameric rings assembled around the central pore. Mechanistically, misfolded, damaged proteins or specific metabolic enzymes are recognised and unfolded by the chaperone subunits before being transferred through their central pore to the protease compartment where they are hydrolysed into peptides of 5 to 10 amino acids (Yu & Houry, 2007).

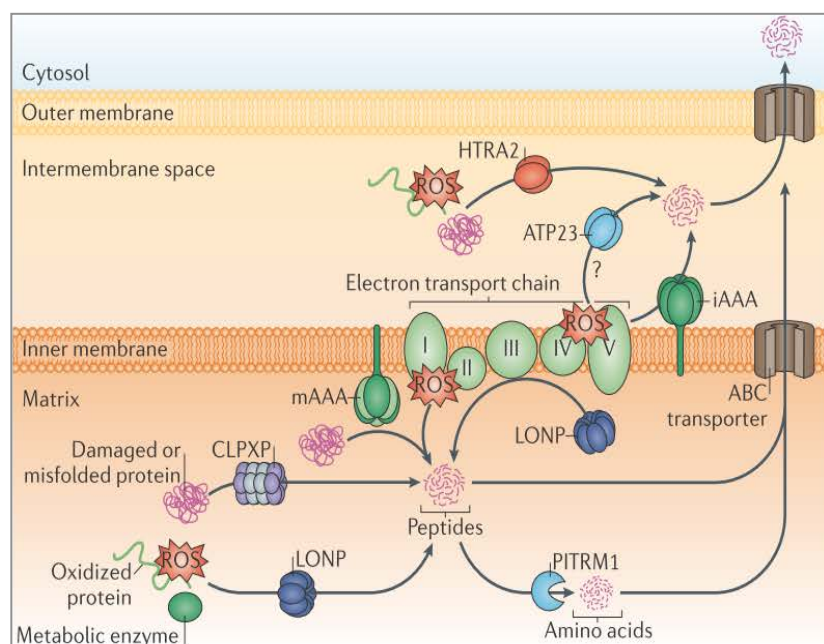


Figure 16 - **Mitochondrial proteases.** Mitochondria have some different proteases responsible for protein quality control, which are distributed within different mitochondrial compartments. For the degradation of misfolded, damaged and oxidised proteins, as well as for the turnover of some metabolic enzymes, mitochondria possesses the two ATP-dependent proteases, Clp (ClpXP) and Lon (LonP) in their matrix and the matrix ATPases associated with diverse cellular activities (mAAA) proteases in their inner membrane. In the intermembrane space, the serine protease HTRA2 degrades misfolded, damaged and oxidised proteins, while intermembrane AAA (iAAA) proteases participate in the maintenance of the respiratory chain complexes along with the mitochondrial inner membrane protease ATP23. In the matrix, the maintenance of the respiratory chain is made by the LonP and the mAAA proteases. Finally, the peptides generated by proteolysis can be exported to the cytosol or further degraded into amino acids by the oligopeptidase presequence protease (PITRM1). (Quiros et al., 2015)

### *The ubiquitin-proteasome system*

The ubiquitin-proteasome system (UPS) is a major proteolysis system, being responsible for the tiny regulated elimination of intracellular proteins such as abnormal, denatured, damaged (i.e. oxidised) as well as short-lived proteins (such as regulatory proteins) (Ciechanover, 2005, Goldberg, 2007). It is an enzymatic multicomplex composed of two main components: the ubiquitin system and the proteasome system. The ubiquitin system is responsible for the protein recognition and for its labelling with ubiquitin (Ub), thanks to an enzymatic cascade involving three different enzymes named E1 (Ub-activating enzyme), E2 (Ub-conjugating enzyme) and E3 (Ub-ligase) (Figure 17). The specificity of this ubiquitylation reaction is mainly due to the E3 enzymes, which are responsible for substrate recruitment. Thus, it is not surprising that numerous E3-ligases exist within cells.

In a second step, ubiquitin tagged proteins are then recognized by the proteasome 26S, which proceeds to protein hydrolysis into inactive peptides (Figure 17). The 26S proteasome is composed of two constituents: the 20S proteasome, its proteolytic core, and the 19S proteasome, the regulatory component responsible for the binding of the poly-ubiquitinated substrates. The 19S proteasome confers a poly-ubiquitin recognition and ATPase activity dependence to the 26S proteasome. In fact, 20S proteasome, in contrast to 26S proteasome, does not require tagging by Ub or ATP activity (Shringarpure, Grune et al., 2003). Instead, it is capable of recognising modified secondary and tertiary protein structures as well as oxidised proteins (Shringarpure et al., 2003). In eukaryotic cells, the 20S proteasome consists of 7 different  $\alpha$ - and 7 different  $\beta$ - subunits that form four heptameric rings, which are disposed in a cylindrical manner (Figure 17). The  $\alpha$ -subunits constitute the outer rings that contain the binding site for regulatory proteins and the  $\beta$ -subunits are in the middle of the rings and contain the active proteolytic sites. Among the 7  $\beta$ -subunits, only 3 exhibit catalytic activities named  $\beta$ 1 responsible for the peptidylglutamyl-peptide hydrolase (PGPH) or caspase-like (C-L) activity,  $\beta$ 2 caring the trypsin-like (T-L) activity and  $\beta$ 5 for the chymotrypsin-like (CT-L) activity (Goldberg, 2007). The CT-L activity seems to be the initial and rate limiting step in protein degradation by 20S proteasome. It cleaves proteins after amino acids with large and hydrophobic side chains, generating smaller fragments that will further be cleaved after basic residues by  $\beta$ 2-subunit and/or after acidic amino acids by the PGPH activity of the  $\beta$ 1-subunit (Orlowski & Wilk, 2000).

At least 19 different subunits give rise to the 19S regulatory particle, also known as

RP/PA700. In the presence of high salt concentrations, it can be dissociated into two subcomplexes, namely the lid (involved in substrate uptake and de-ubiquitylation) and the base (involved in the recognition, unfolding and further translocation of the substrate) (Figure 17). Apart from the one or two 19S complexes attached at either one or both ends of 20S core, other proteasome regulators can also bind 20S proteasome, such as the 11S complex (also known as PA28, REG or PA26) (Stadtmueller & Hill, 2011).

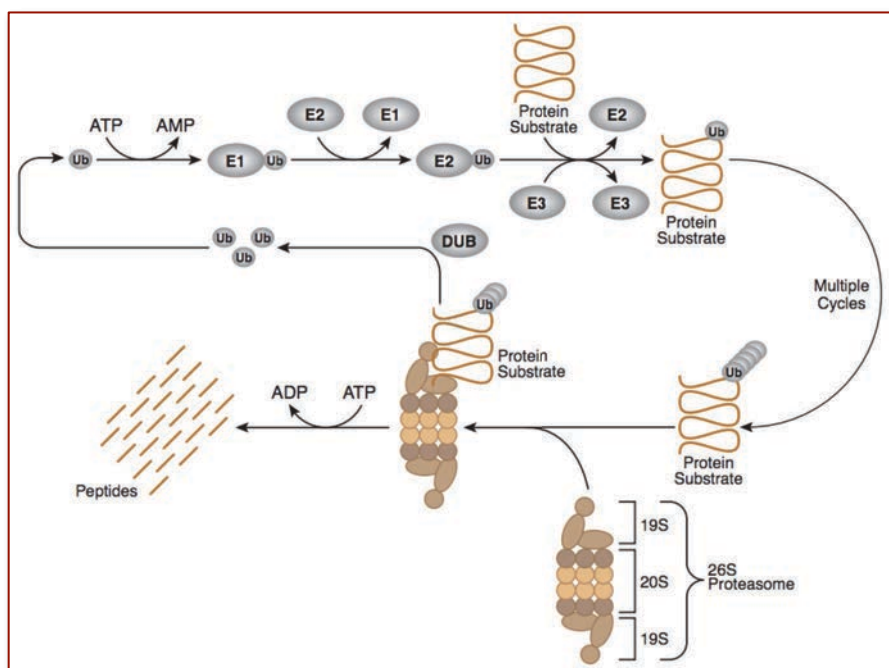


Figure 17 - **The ubiquitin-proteasome system (UPS)**. UPS enzymes mediate a series of reactions responsible for the ubiquitylation and degradation of intracellular proteins. In the activation reaction, ubiquitin (Ub) is transferred to an E1 enzyme in an ATP-dependent manner. The activated Ub is subsequently transferred to an E2 enzyme via a conjugating reaction. The E2 enzyme then carries the Ub to the E3 enzyme, which is important not only because it covalently ligates Ub to lysine residues on the substrate protein, but also because it mediates substrate specificity. This process of Ub ligation may be repeated with a lysine of the Ub protein itself serving as the substrate, leading to the formation of a poly-Ub chain on the target protein. Poly-Ub proteins are then recognized by the 26S proteasome, which mediates their regulated degradation into peptides. 26S protein degradation is achieved by the proteolytic core of the 20S proteasome and regulated by the 19S proteasome in an ATP-dependent manner. Finally, de-ubiquitylation enzymes (DUB) enzymes can reverse substrate protein ubiquitylation, thus increasing the regulation of this type of protein elimination. (From *Cell Signaling Technology - Ubiquitin/Proteasome Pathway*)

### ***Lysosomal system***

Lysosomes are organelles filled with various acidic hydrolases such as peptidases, lipases and nucleases responsible for the degradation of various biological macromolecules, thereby recycling their biochemical components for energy production and other anabolic

reactions (Fitzwalter & Thorburn, 2015). The optimal activity of lysosomal hydrolases requires an internal acidic pH, which is maintained by proton pumps present on its single or double membranes and that actively transport  $H^+$  ions into the lumen. While the UPS is a highly regulated protein degradation system responsible for the degradation of soluble short-lived cytosolic proteins; large protein aggregates, soluble long-lived proteins, as well as entire organelles are targeted to lysosomes by autophagy. Three types of autophagy exist based on the mechanism of cargo delivery to the lysosomes: microautophagy, chaperone-mediated autophagy and macroautophagy (Figure 18). In the case of microautophagy, the cytoplasmic components to be degraded are engulfed by the lysosome directly through invaginations or protrusions of its membrane (Li, Li et al., 2012). Chaperone-mediated autophagy is a more selective pathway, being responsible for the degradation of a particular group of soluble cytosolic proteins. Proteins containing a specific sequence signature are first recognized by HSC-70 (heat shock cognate 70) and other co-chaperones leading to its deliverance to the lysosome where they are further degraded (Orenstein & Cuervo, 2010). Between all forms of autophagy, macroautophagy remains the best-characterised one and for this reason, it is often simply referred to as autophagy. In this type of autophagy, a double-membrane vesicle sequesters a small portion of the cytoplasm to be degraded (e.g. soluble materials and/or organelles) giving rise to the so-called autophagosome (Figure 18). The autophagosome then fuses directly or indirectly with a lysosome, forming an autolysosome, where its content is degraded by lysosomal enzymes (Klionsky & Emr, 2000, Mizushima, Levine et al., 2008). It is important to note that lysosomal formation is also functionally connected with heterophagic pathways (Figure 18). Indeed, extracellular material and plasma membrane proteins can be delivered to the lysosomes for degradation via the endocytic pathway. In this case, the extracellular material is first integrated within endocytic vesicles derived from the plasma membrane, which then fuse with early endosomes that gradually mature into late endosomes. These late endosomes will subsequently fuse with transport vesicles, originated from the trans-Golgi network, to constitute the lysosome (Luzio, Pryor et al., 2007).

Although the UPS and autophagy have long been studied as two separate proteolytic pathways with distinct functions, recent findings suggest that, at least within certain conditions, these two systems are interconnected and might play compensatory functions in some cases. In fact, not only proteins with short half-life have been shown to be degraded by autophagy, but also long-lived proteins can be eliminated by the UPS (Fuertes, Martin De Llano et al., 2003, Li, 2006). In addition, recent findings show that Ub recognition can also



be present in the autophagic pathway of protein degradation (Khaminets, Behl et al., 2015). However, although proteasome inhibition may induce autophagy, autophagy inhibition may not induce proteasomal protein degradation but leads to impaired degradation of specific UPS targets (Korolchuk, Mansilla et al., 2009). These could be due to the formation of heavily oxidised and cross-linked proteins, which are poor substrates for the proteasome (Ravikumar, Duden et al., 2002).

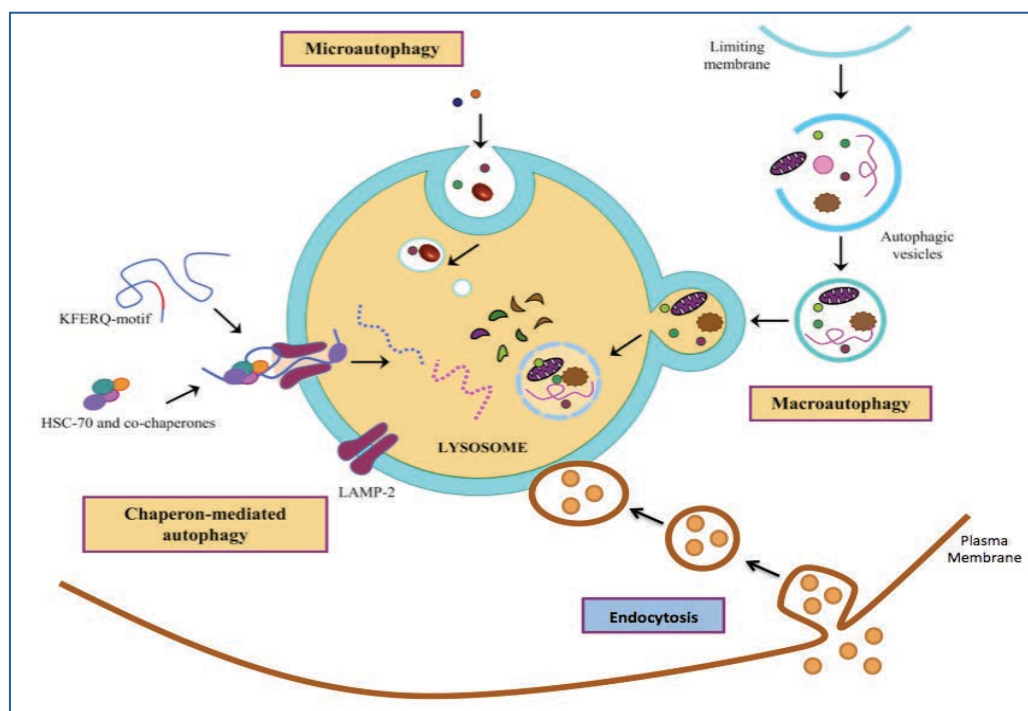


Figure 18 - **The lysosomal system.** Proteases of lysosomes are able to degrade proteins and organelles from cytosol (autophagy) but also from the extracellular environment (heterophagy). Three types of autophagy can be distinguished. In macroautophagy, a double membrane envelops a small portion of the cytoplasm containing the material to be degraded, forming the autophagosome. This structure then fuses with lysosome, forming the autolysosome where the cytoplasmic content is degraded. In microautophagy, the cytoplasmic material is swallowed by the lysosome directly through invaginations or protrusions of its membrane. In chaperon-mediated autophagy, soluble cytosolic proteins are recognized by their KFERQ-like motif and delivered to the lysosome by HSC-70 and co-chaperones. In the case of extracellular components, there are delivered to the lysosome for degradation through the endocytic pathway, where a vesicle is formed from the cytoplasmic membrane. Adapted from (Chondrogianni, Petropoulos et al., 2014).

## Proteolytic Systems in Skeletal Muscle

Apart from their roles in degradation of oxidative and non-functional misfolded proteins, lysosomal proteases and UPS, together with calpains and caspases constitute the main muscle proteolytic systems, which are involved in metabolic protein turnover and

breakdown of specific proteins. Activation of skeletal muscle proteolytic systems is transcriptionally regulated in specific conditions mainly related to muscle atrophy, such as fasting, ageing as well as some diseases, including cancer, diabetes, sepsis and renal failure (Kandarian & Jackman, 2006, Lecker, Goldberg et al., 2006). Indeed, in atrophying skeletal muscle, the rapid loss of muscle mass occurs primarily through an activation of protein breakdown (Bonaldo & Sandri, 2013, Paco, Ferrer et al., 2012, Sandri, 2013, Shenkman, Belova et al., 2015, Talbert, Smuder et al., 2013). For this reason, the subset of transcripts belonging to proteolytic systems that have been identified in these conditions have been designated atrophy-related genes or "atrogens" (Sacheck, Hyatt et al., 2007).

In skeletal muscle, the UPS plays a major role in the control of muscle mass and is required to remove sarcomeric proteins upon changes in muscle activity. While its highly activation is responsible for the loss of muscle mass in muscle wasting conditions (Nury, Doucet et al., 2007), the deletion of its components was also shown to contribute to myofiber degeneration and weakness in muscle disorders characterised by the accumulation of abnormal inclusions (Kitajima, Tashiro et al., 2014). Thus, appropriate proteasomal activity is important for muscle growth and for maintaining myofiber integrity. During sarcopenia for instance, a loss of myofibrillar proteins has been associated with a decrease in proteasome expression levels (Husom, Peters et al., 2004). As described above, the specificity and the rate-limiting step of proteasomal degradation are achieved by the E3 ubiquitin ligases. Two muscle-specific E3 ligases, MuRF1 (for Muscle Really Interesting New Gene Finger 1, also known as TRIM63) and MAFbx (for Muscle Atrophy F-box, also known as atrogin-1 or FBXO32), are thought to be key regulators of proteasomal proteolysis in skeletal muscle, especially in atrophic conditions (Bodine & Baehr, 2014, Foletta, White et al., 2011). Indeed, overexpression of MAFbx in skeletal myotubes leads to atrophy, while mice deficient in either MAFbx or MuRF1 were found to be resistant to atrophy induced by denervation or activity fasting (Bodine, Latres et al., 2001, Cong, Sun et al., 2011). MuRF1 ubiquitinates several muscle structural proteins, including troponin I (Kedar, McDonough et al., 2004), actin (Polge, Heng et al., 2011), myosin heavy chains (Clarke, Drujan et al., 2007, Fielitz, Kim et al., 2007), myosin light chains and myosin binding protein C (Cohen, Brault et al., 2009). Although it can also interact with sarcomeric proteins such as myosin heavy and light chains, desmin and vimentin (Lokireddy, Wijesoma et al., 2012), well-known molecular substrates of MAFbx are MyoD, calcineurin and eukaryotic translation initiation factor 3 subunit f (eIF3-f, a critical component in protein translation) (Csibi, Leibovitch et al., 2009, Tintignac, Lagirand et al., 2005).

Regarding autophagy, in skeletal muscle, it is a highly regulated pathway for the degradation of non-myofibril cytosolic proteins or other types of macromolecules and organelles (Mizushima, 2007, Sandri, 2010). Its regulation is done by the autophagic gene (Atg) family: some of these genes being involved in autophagy initiation (Atg1), induction (Atg3, 7, 10, 13) or in autophagosome formation (Atg2, 5, 6, 8, 9, 12) (Mizushima, 2007). Emerging evidences suggest that a baseline level of autophagy in skeletal muscle tissue is required for the maintenance of normal muscle function and mass and that an increase in autophagy above the baseline will contribute to skeletal muscle atrophy (Bechet, Tassa et al., 2005, Bonaldo & Sandri, 2013, Masiero, Agatea et al., 2009, Sandri, 2013, Schiaffino, Dyar et al., 2013). Indeed, specific inactivation of Atg7 in mice muscle tissue led to a dramatic skeletal muscle atrophy and weakness due to decreased autophagosome formation (Masiero et al., 2009). But also excessive activation of autophagy by numerous signals such as exercise, starvation, caloric restriction, hypoxia, oxidative stress and DNA or mitochondrial damage (Kim, Kim et al., 2013, Kroemer, Marino et al., 2010, Liu, Wise et al., 2008, Mazure & Pouyssegur, 2010, Wohlgemuth, Seo et al., 2010) aggravates muscle wasting by removing cytoplasm portions, organelles and proteins (Dobrowolny, Aucello et al., 2008, Mammucari, Milan et al., 2007, Zhao, Brault et al., 2007). Studying the mechanisms that control the autophagic/lysosomal pathway in skeletal muscle, Mammucari and his colleagues identified the FOXO3 transcription factor, already known for playing critical roles in activating UPS and muscle atrophy (Sandri, Sandri et al., 2004), as necessary and sufficient for the induction of autophagy in skeletal muscle *in vivo* (Mammucari et al., 2007). More precisely, they have found that FOXO3 activation controls the transcription of autophagy-related genes, including LC3 (a mammalian Atg8 ortholog) (Mammucari et al., 2007). Attenuation of autophagy by inhibition of LC3 preserved muscle mass in a mouse model of oxidative stress (mice with muscle-specific expression of a mutant SOD1) that presented muscle atrophy due to autophagy activation (Dobrowolny et al., 2008). This study suggests an important link between oxidative stress, autophagy and muscle atrophy, which have been reviewed recently (Lee, Giordano et al., 2012, Pellegrino, Desaphy et al., 2011, Powers et al., 2012).

## Protein Repair Systems

Enzymatically repair of protein oxidative damage is only possible on certain oxidation products of the sulfur-containing amino acids, cysteine and methionine. In the case of cysteine, the major systems involved in reversing the oxidation of disulfide bridges and sulfenic acid include the reduced forms of small proteins such as thioredoxin and glutaredoxin. Methionine sulfoxide, on its turn, is reduced back to methionine by the methionine sulfoxide reductases enzymes that are then recycled by the thioredoxin/thioredoxin reductase system.

### *Thioredoxin system*

Thioredoxins (Trx) are small ubiquitous proteins with two catalytic redox active cysteines (Cys-XX-Cys), which catalyse the reversible reduction of protein disulfide bonds. Subsequently, oxidised thioredoxins are reduced back enzymatically by the NADPH-dependent thioredoxin reductase (TR) enzymes, which together with NADPH and Trx constitute the thioredoxin system (Lu & Holmgren, 2014) (Figure 19). Two Trx enzymes have been identified to date, the Trx1, which is present in the cytosol and can be translocated into the nucleus in oxidative stress conditions and the Trx2, which is present in the mitochondria. The antioxidant activity of these enzymes consists of providing electrons to thiol-dependent peroxidases, allowing the recycling of these Prx enzymes for the continuous removal of ROS and RNS. Furthermore, Trx enzymes will also be involved in the protection against protein oxidative damages by reducing methionine sulfoxide reductases, enzymes capable of repairing oxidised methionines. In mammals, Trx also regulate the activity of many redox-sensitive transcription factors, such as NF- $\kappa$ B, Nrf2 and p53 (Lu & Holmgren, 2014).

### *Glutaredoxin system*

Glutaredoxins (Grx) are found in almost all living organisms and collaborate with thioredoxins for the reduction of protein disulfides and S-glutathionylated proteins. Four Grx isoenzymes (Grx1, Grx2, Grx3 and Grx5) exist in mammals. In terms of structure, they belong to the Trx superfamily having a dithiol or monothiol active motive, Cys-XX-Cys or

Cys-XX-Ser, respectively (Lillig, Berndt et al., 2008). The dithiol isoenzyme Grx1 is localised mainly in the cytoplasm, in the nucleus, in intermembrane space of mitochondria and in the extracellular space. On the other hand, the dithiol Grx2 and the monothiol Grx5 are mostly mitochondrial enzymes, even if Grx2 have been also found in the cytoplasm and nucleus of tumor cells. Finally, the monothiol isoenzyme Grx3 is present in the nucleus as well as in the cytoplasm (Kalinina, Chernov et al., 2014). In contrast to Trx, Grx are reduced back non-enzymatically by glutathione, which is recovered by the glutathione reductase enzyme in the presence of NADPH (Figure 19). An exception was observed with the Grx2 isoenzyme, which was shown to be reduced back by Trx2 in mitochondria (Johansson, Lillig et al., 2004). The reduction of disulfides and the participation on protein deglutathionylation states the importance of Grx enzymes in defence against oxidative stress as well as in redox regulation of signal transduction (Song, Rhee et al., 2002, Starke, Chock et al., 2003). This allows them to be involved in cellular functions such as differentiation of macrophage-like cell lines (Takashima, Hirota et al., 1999) and in apoptosis prevention of cardiac myoblasts (Murata, Ihara et al., 2003).

### ***Methionine sulfoxide reductase system***

Methionine sulfoxide reductases (Msr) constitute a group of four ubiquitous enzymes that catalyse the reduction of free and protein-derived methionine sulfoxides (MetO) to methionine. Two diastereoisomers of methionine sulfoxides can be formed after protein methionine oxidation, MetRO and MetSO. MsrA, which is present in the cytoplasm, in the nucleus and in the mitochondria reduces specifically the MetSO. In contrary, the three MsrB are responsible for the reduction of the diastereoisomer R of MetO. In terms of their intracellular localisation, MsrB1 is present in the cytoplasm as well as in nucleus, MsrB2 is only present in the mitochondria and MsrB3 is present in the mitochondria as well as in the endoplasmic reticulum of eukaryotic cells (Lee, Dikiy et al., 2009). Msr enzymes possess one cysteine (a selenocysteine in the case of MsrB1) in their catalytic site responsible for MetO reduction and whose recycling involves the formation of a disulfide bond with a second Msr cysteine. The disulfide bond can be further reduced restoring Msr activity by the Trx system (Figure 19). Msr have been intensely studied for their antioxidant roles as well as their protection against oxidative stress and apoptosis (Cabreiro, Picot et al., 2008, Salmon, Perez et al., 2009, Ugarte, Petropoulos et al., 2010). Furthermore, they have been suggested

as involved in longevity modulation of some model organisms such as *D. melanogaster* (Chung, Kim et al., 2010) and *C. elegans* (Minniti, Cataldo et al., 2009). More recently, these enzymes have been considered as regulators of protein function (Lee, Peterfi et al., 2013) and as being involved in redox regulation of cellular signalling (Bigelow & Squier, 2011). These proteins as well as their functions will be further discussed in Chapter V.

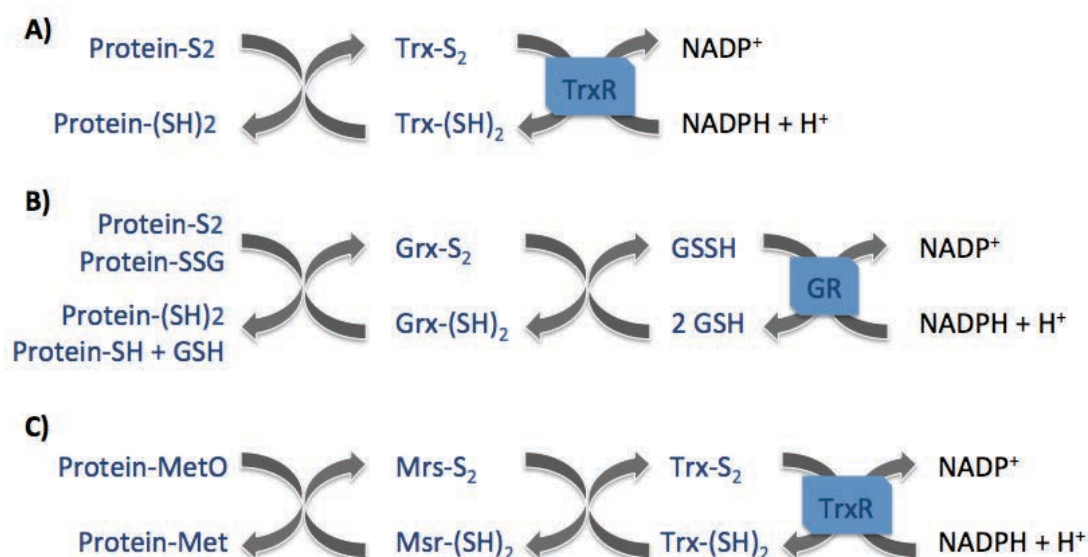


Figure 19 - **The 3 major protein repair systems.** A) Thioredoxin system participates to the reduction of protein disulfide (Protein-S<sub>2</sub>); B) glutaredoxin system reduces protein disulfide (Protein-S<sub>2</sub>) as well as protein glutathione mix disulfide (Protein-SSG); and C) methionine sulfoxide reductase system reduces methionine sulfoxides (MetO).

## Protein Homeostasis in Skeletal Muscle Myogenesis

The proteolytic systems previously cited are essential for the maintenance of muscle progenitor cells homeostasis during remodelling of skeletal muscle tissue. Apart from their importance for myoblasts viability (McMillan & Quadrilatero, 2014, Xing, Shen et al., 2014), the mitochondrial proteases, the UPS and macroautophagy have been reported to be important for muscle stem cells activation, proliferation or differentiation processes. Indeed, while autophagy is induced during mice satellite cells activation, its inhibition in satellite cells resulted in their delayed activation *ex vivo* (Tang & Rando, 2014). The importance of protein degradation systems for satellite cells activation seems to be related to the control of Pax family proteins homeostasis. Indeed, during adult satellite cells activation, Pax3 protein was shown to be degraded by the proteasome catalytic subunits (Boutet, Disatnik et al.,

2007), after being poly-ubiquitinated by Taf1 (for TATA-binding protein-associated factor), a protein that has both E1 Ub-activating and E2 Ub-conjugating activities and that is down-regulated during myogenic differentiation (Boutet, Biressi et al., 2010). Furthermore, the degradation of the Pax3 paralogous protein Pax7, seems also to be achieved by proteasome activity with UPS-mediated degradation of Pax7 protein in mouse immortalized myoblasts being required for expression and accumulation of Myogenin protein and further myoblasts commitment to terminal differentiation (Olguin, Yang et al., 2007).

Moreover while myoblasts proliferation was recently shown to be dependent on the expression of the mitochondrial ClpXP protease (Deepa, Bhaskaran et al., 2016), myoblasts differentiation and fusion *in vitro* was accompanied by an increase in the expression of Ub-conjugates, autophagy-related proteins as well as proteasome activities (Ciechanover, Breitschopf et al., 1999, Cui, Hwang et al., 2014, Ebisui, Tsujinaka et al., 1995, Gardrat, Montel et al., 1997, Kim, Rhee et al., 1998, McMillan & Quadrilatero, 2014), suggesting their important roles in these steps of myogenesis. Indeed, treatment of C2C12 myoblasts with autophagy inhibitors such as 3MA (3-methyladenine) or using of short hairpin RNA (shRNA) against Atg7, also resulted in impaired myoblast fusion and differentiation with an increase in DNA fragmentation and a lower expression of myosin heavy chain proteins (McMillan & Quadrilatero, 2014). Similarly, the use of proteasome specific inhibitors or interference RNA against proteasome subunits led to an accumulation of Ub-conjugates preventing myoblasts fusion *in vitro* (Ciechanover et al., 1999, Gardrat et al., 1997, Ueda, Wang et al., 1998), while its removal allowed myoblasts to further proceed into differentiation (Kim et al., 1998). Inhibition of myoblasts fusion by proteasome specific inhibitor (bortezomib) was shown to be NF- $\kappa$ B-dependent and resulted in less myotube formation and reduced expression of muscle specific proteins, such as Myogenin and myosin heavy chains (Kim et al., 1998, Xing et al., 2014). Importantly, not only the proteasome catalytic activity but also other components of the UPS were proven important for myogenesis. Among the various E3 ligases identified in striated muscles, the supracited MuRF proteins have been intensively studied in myoblasts. As differentiation proceeds in culture, MuRF2 is the first MuRF isogene to be expressed (Perera, Mankoo et al., 2012) and it has its maximal expression in myotubes compared to proliferative myoblasts (Pizon, Rybina et al., 2013). Depletion in MuRF2A by specific siRNA directed against its isoforms (MuRF2A/p50 and MuRF2B/p60) reduced C2C12 myoblast fusion and myotubes formation, although this effect started to be recovered just after two days (Perera et al., 2012). Contrary to MuRF2, the expression of another muscle specific E3 Ub-ligase, MAFbx appears in late

stages of differentiation where it is required for MyoD poly-ubiquitination (Tintignac et al., 2005). In fact, early overexpression of MAFbx in C2C12 myoblasts suppresses their MyoD-dependent differentiation and impaired myotubes formation *in vitro* (Tintignac et al., 2005).

Globally, all these studies have demonstrated the role of the proteolytic systems in skeletal muscle myogenesis, either through the elimination of muscle specific proteins or by eliminating protein irreversible damages at precise differentiated states. Given the increasing evidence demonstrating the importance of controlled redox state for myoblast differentiation, the hypothesis of a redox mediated regulation of proteolysis activity in myogenesis, gains even more credits. Recent studies performed on C2C12 cells showed that inhibition of the immunoproteasome increased protein oxidation and proapoptotic proteins that impaired myoblasts differentiation (Cui et al., 2014). In addition, ClpP deficient cells showed increased ROS generation and mitochondrial alterations that lead to impaired myoblast differentiation and cell proliferation (Deepa et al., 2016).

In the same line of view, one could think of an even more rapid redox regulation of muscle protein function by the oxidised protein repair systems. However, concerning the systems involved in the reversing oxidised cysteines and methionines, which have proven to be important for protein homeostasis and consequently cellular function, their role on skeletal muscle myogenesis is very poorly studied. The only studies involving Grx protein repair systems in muscle tissue were done in embryonic cardiac H9c2 cells and revealed the importance of this system in the protection of cells from apoptotic cell death through the regulation of the Akt redox state (Murata et al., 2003, Urata, Ihara et al., 2006). Regarding the Trx system, it was reported that Trx1 and Trx2 expression did not appear to be modulated during C2C12 myoblasts differentiation while Trx reductases 1 and 2 expression decreases during differentiation (Dimauro, Pearson et al., 2012). Although the authors did not investigated their role in myogenesis, another studied with C2C12 cells had shown that TrxR2 is important for myoblasts survival under oxidative conditions (Rohrbach, Gruenler et al., 2006). Thus, studies are needed to investigate the importance of protein repair systems, especially the Msr system, the only one capable of reducing methionine oxidation, for skeletal muscle progenitors during differentiation.





## V. Methionine Sulfoxide Reductases

In this current chapter, I will describe in detail, the methionine sulfoxide reductase (Msr) system, composed by the stereospecific enzymes MsrA and MsrB and involved in the reduction of the diastereoisomeric forms of methionine sulfoxide (MetO). I will address the importance of the Msr system in the protection against oxidative stress, in ageing, as well as their role in the regulation of protein biological activities, all of which are most likely important for skeletal muscle homeostasis.

### Methionine sulfoxide reductases discovery

Due to the presence of a sulfur atom, methionine residues are very sensible to oxidation leading to a modification or loss of protein function. First evidences of the importance of keeping methionine in its reduced state for biological function appeared 70 years ago. Studying *Lactobacillus arabinosus*, Waelsch and colleagues found that methionine oxidation of glutamine synthetase inhibited the conversion of glutamic acid into glutamine, an essential step for bacterial growth (Waelsch, Owades et al., 1946). In addition, sporulation of *Bacillus subtilis* was also described to be affected by methionine oxidation (Krask, 1953). Few years after the identification of the first deleterious effects of MetO, a Msr activity, capable of reducing back MetO to Met was described, primarily in yeast (Black, Harte et al., 1960), later in bacteria (Ejiri, Weissbach et al., 1979) and in higher organisms such as plants (Doney & Thompson, 1966) and animals (Aymarda, Seyera et al., 1979). Msr activity was evidenced in *E. coli* by their ability to grow in a culture medium with L-MetO as the only source of methionine, thus capable of catalysing the reduction of MetO (Ejiri et al., 1979). In 1981, Brot and colleagues partially purified one of the enzymes responsible for the reduction of MetO within proteins and showed it as essential for restoring the activity of the ribosomal protein L12 in *E. coli* (Brot, Weissbach et al., 1981). The enzyme, later called MsrA, uses reduced thioredoxin (Trx) *in vivo* or dithiothreitol (DTT) *in vitro* as electron donor (Brot, Werth et al., 1982). MsrA is an ubiquitous protein, differentially expressed in mammalian tissues and capable of reducing a variety of substrates such as free MetO and peptides or proteins containing MetO (Moskovitz, Jenkins et al., 1996a). MsrA was found to be a stereospecific enzyme only capable of reducing the MetSO diastereoisomer of MetO, with an increased specificity for protein-bound MetO compared to

free MetO (Sharov, Ferrington et al., 1999, Sharov & Schoneich, 2000).

Twenty years after the purification of MsrA, Grimaud *et al.* discovered that full reduction of oxidised calmodulin can be done by the combined action of MsrA and another enzyme called MsrB (Grimaud, Ezraty et al., 2001). This new Msr is in fact responsible for the reduction of the MetRO diastereoisomer within proteins, which is not reduced by MsrA (Kryukov, Kumar et al., 2002, Olry, Boschi-Muller et al., 2002). MsrB, later called MsrB1, SelX or SelR in mammals, was discovered in 1999 by Lescure and colleagues as a novel selenoprotein, but at this time, its function was unknown (Lescure, Gautheret et al., 1999).

MsrB exclusively acts on peptidyl-MetO (Grimaud et al., 2001). Intrigued by this, different authors have identified a novel Msr, called fRMsr or MsrC, in *E. coli* which activity is specific for free MetO, being unable to reduce MetO present within proteins (Etienne, Spector et al., 2003, Lin, Johnson et al., 2007, Olry et al., 2002). Very recently, Gennaris and co-workers made another interesting and novel discovery: they found a new Msr system, named MsrPQ, present in the envelope of *E. coli* bacteria, that, contrary to the other known Msr, can reduce both MetO diastereoisomers using electrons directly from the respiratory chain, thus independently from Trx enzymes (Gennaris, Ezraty et al., 2015). A similar system may exist in eukaryotic subcellular oxidising compartments, such as the endoplasmic reticulum or lysosomes but, to date, fRMsr and MsrPQ systems were only found in prokaryotes or unicellular eukaryotes.

## **Methionine sulfoxide reductases phylogenetic, tissue and cellular distribution**

The phylogenetic distribution of *Msr* genes was revealed by genomic analyses made in different organisms. These studies show the presence of *MsrA* and *MsrB* genes in all eukaryotes without exception. Bacteria can possess only *MsrA* genes, the two *Msr* genes or a bifunctional *MsrA/B* fusion gene (Zhang & Weissbach, 2008). This universal presence of *Msr* genes supports their essential roles for cell function, either in protecting them against oxidative damages as well as in regulating protein function. The greatest exception to the universal *Msr* representation between life domains is its absence in 12 archaea representative genomes (Zhang & Weissbach, 2008). Several hypothesis for this, although none of them was already been clearly proven, are proposed: i) the development of a functionally

equivalent system; ii) the low O<sub>2</sub> solubility at high temperatures which would avoid ROS production within hyperthermophiles; iii) the observation of non-enzymatic MetO reduction at higher temperatures (Fukushima, Shinka et al., 2007); iv) the existence of protein structures that protect Met residues; v) the presence of *Msr*-containing plasmids in these archaea; vi) the discovery of fRMsr in some archaea including some of those lacking *Msr* genes (Kim, Kwak et al., 2014b) or finally vii) the existence of an efficient first line of defence against ROS production (CAT, SOD and Prx enzymes), which would create a low-ROS environment that diminishes the frequency of protein oxidation.

The fact that no *MsrB*-containing organism exists without *MsrA* gene suggests that these two genes evolved independently and that MsrA protein alone is sufficient to perform this specific protein repair process, while MsrB may have evolved to play other redox functions, increasing defences against greater oxidative damage seen in more complex life forms such as animals and plants. In addition, the organisation of the two *Msr* genes also differs, this can explain the massive *MsrB* gene duplication seen in the plant *Arabidopsis thaliana*: nine *MsrB* genes in contrast to five *MsrA* genes (Rouhier, Vieira Dos Santos et al., 2006). In contrast to plants and algae (Rouhier et al., 2006), animals and bacteria contain fewer *Msr* genes; mammals have one *MsrA* and three *MsrB* genes while *E. coli*, *S. cerevisiae*, *C. elegans* and *D. melanogaster* have only one *MsrA* and one *MsrB* gene (Kim & Gladyshev, 2004b, Kryukov et al., 2002). Such gene diversity underlines the biological importance of this system and gene redundancy may be explained by the necessity for organisms to respond to the modification of environmental conditions such as physiological, oxidative stress, thermal stress conditions...

In eukaryotes, Msr enzymes are ubiquitously expressed (Brot et al., 1981, Moskovitz et al., 1996a), with the only exception of leukemic cells that do not express MsrA (Kuschel, Hansel et al., 1999). Analyses of mouse, rat and human tissues revealed a maximal expression level of MsrA in kidney and liver tissues, followed by heart, lung, brain, skeletal muscle, retina, testis, bone marrow and blood (Kuschel et al., 1999, Moskovitz et al., 1996a). Highest Msr activities were found in rat kidney (Moskovitz, Weissbach et al., 1996b) and human neutrophils (Brot, Fliss et al., 1984), which in the case of neutrophils was later shown to be due mainly to MsrB type (Achilli, Ciana et al., 2008). In human skin, MsrA was shown to participate to tissue homeostasis and to be a sensitive target for UV (Ogawa, Sander et al., 2006, Picot, Moreau et al., 2007). MsrA and all three MsrB proteins are expressed in melanocytes (Schallreuter, Rubsam et al., 2006) and keratinocytes (Ogawa et al., 2006). A lower MsrA expression was found in dermal fibroblasts (Ogawa et al., 2006) while a greater

MsrA expression was found in sebaceous glands (Taungjaruwina, Bhawan et al., 2009). MsrB1 and MsrB3 were both expressed within vascular endothelial cells (Taungjaruwina et al., 2009).

Msr enzymes are differentially distributed in the mammal subcellular compartments (Figure 20), which indicate that each Msr may have an organelle-specific role. MsrA is present in mitochondrial matrix due to its N-terminal mitochondrial signal sequence (Hansel, Kuschel et al., 2002) (Figure 21) but was also found in rat liver cytosolic fractions (Vougier, Mary et al., 2003) and in the nucleus of mouse cells (Kim & Gladyshev, 2005b) (Figure 20). If its N-terminal peptide sequence was sufficient for mitochondrial targeting, other structural and functional elements present in the MsrA sequence will determine its intra-cellular distribution. Full folded MsrA is retained in the cytosol, while partially misfolded MsrA appears to be targeted to the mitochondria (Kim & Gladyshev, 2005b). An alternative first exon splicing generating an additional MsrA form lacking a mitochondrial signal (Figure 21), which resides in cytosol and nucleus, was also evidenced but no protein resulting from this alternatively spliced MsrA was detected in mouse tissues (Kim & Gladyshev, 2006). For the MsrB family, the situation is much more complex than for MsrA, due to the existence of four proteins resulting from the transcription and consequent translation of three different genes. The selenoprotein protein MsrB1 is present both in the nucleus and the cytosol, while MsrB2, also known as CBS-1, is present only in the mitochondria due to the presence of a N-terminal signal peptide (Kim & Gladyshev, 2004b) (Figure 20 and 21). Interestingly, MsrB3A and MsrB3B result from alternative splicing of the first exon of the *MsrB3* gene with MsrB3A displaying an endoplasmic reticulum signal peptide while MsrB3B showed a mitochondrial signal peptide at the N-terminus, in addition to an endoplasmic reticulum retention signal peptide at their C-terminus (Figure 20 and 21). In mouse, however, there is no evidence for *MsrB3* alternative splicing and the only MsrB3 protein is present in the endoplasmic reticulum, even though it has both the endoplasmic reticulum and the mitochondrial signal peptides at its N-terminus (Kim & Gladyshev, 2004a).

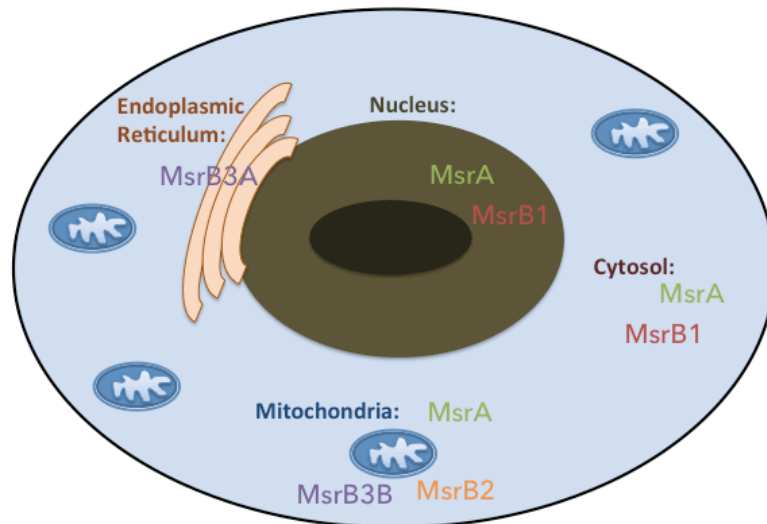


Figure 20 - **Subcellular distribution of methionine sulfoxide reductase enzymes in humans.** MsrA and MsrB1 enzymes are present in the cytoplasm and in the nucleus of human cells. Within mitochondria, there are MsrA together with MsrB2 and MsrB3B enzymes. The endoplasmic reticulum possesses also a MsrB type enzyme called MsrB3A.

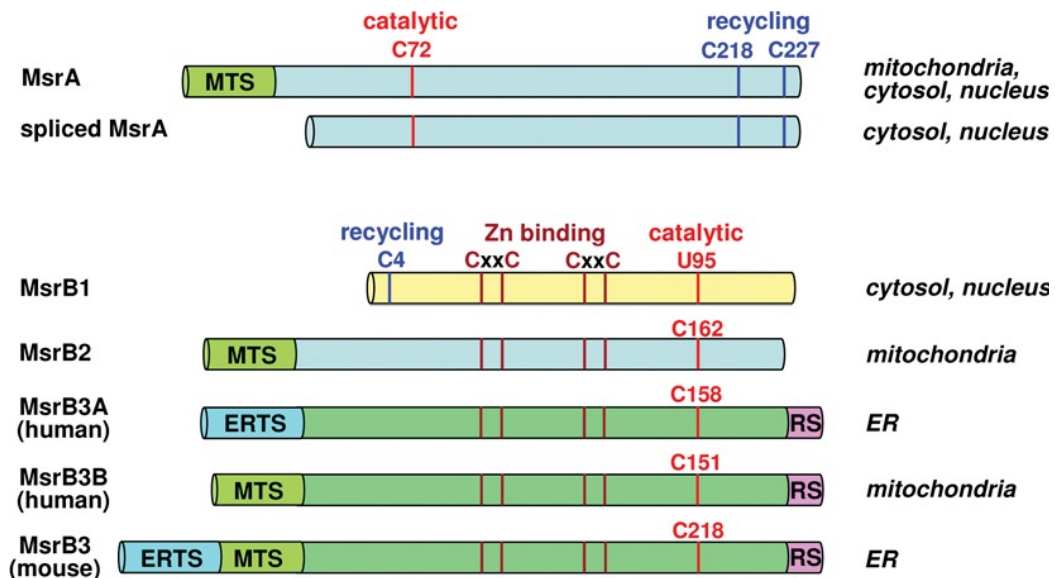


Figure 21 - **Genetic organisation of mammalian methionine sulfoxide reductases (Msr).** Two genes were identified for MsrA. One of them contains a mitochondrial targeting signal (MTS) in its N-terminal domain while the second gene corresponds to a spliced form of MsrA without MTS, generated by alternative first exon splicing. For MsrB, three different genes were identified. MsrB1 is the only presenting a selenocysteine (U) in the catalytic site and does not have any MTS, endoplasmic reticulum targeting signal (ERTS) or retention signal (RS). Instead, MsrB2 with a cysteine (C) in the catalytic site possess a MTS in the N-terminal domain of the protein. MsrB3 A and B are a result of alternative splicing and contain both a RS at the C-terminal domain while at the N-terminal domain MsrB3A possess only a MTS and MsrB3B a MTS and an ERTS. The Zn-binding domains that occur in two CxxC motifs of MsrBs are also shown. From (Kim & Gladyshev, 2007).

## Methionine sulfoxide reductases sequence, structure and catalytic activity

Sequence alignment between the primary structures of MsrA protein from different organisms showed that there is a high homology among them, with *E. coli* and *Bos taurus* MsrA having 67 and 88 % sequence identity to human MsrA (Lowther et al., 2000a). In addition, this alignment has put in evidence a conserved active-site sequence GCFWG in all organisms studied. The strictly conserved cysteine within this motif (Cys-51 in the case of *E. coli* MsrA, Cys-72 in the case of bovine MsrA and Cys-74 in the case of human MsrA) is essential for the MsrA reducing activity. Moreover, three other cysteine were shown to be conserved within 70 % of all MsrA proteins: Cys-86, Cys-198 and Cys-206 in the case of *E. coli*; Cys-107, Cys-218 and Cys-227 for bovine MsrA; and Cys-109, Cys-220 and Cys-230 in the case of human MsrA (Lowther et al., 2000a). Toward the C-terminus, the two last cysteines bracket a glycine-rich region on MsrA sequence and may serve as additional recycling cysteine for the catalytic mechanism.

MsrA three-dimensional (3D)-structures obtained by X-ray crystallography from *E. coli* (Tete-Favier, Cobessi et al., 2000), *Bos Taurus* (Lowther, Brot et al., 2000b), *Mycobacterium tuberculosis* (Taylor, Benglis et al., 2003) and *Populus trichocarpa* (Rouhier, Kauffmann et al., 2007) were also essential to determine the mechanistic aspects of MsrA catalysis. MsrA folding belongs to an  $\alpha/\beta$  class of proteins. The presence of the catalytic cysteine in the N-terminal  $\alpha$ -helix of the protein allows it to face MetO residues present in other proteins, while the two recycling cysteines are buried in the C-terminal region of the protein. Analysis of the 3D structures of the MsrA with MetO showed that the oxygen of the methionine sulfoxide is strongly stabilized by a hydrophilic subsite composed of a network of hydrogen bonding interactions including Tyr-82, Glu-94 and Tyr-134 in MsrA (numbers based on the *E. coli* MsrA sequence) (Ranaivoson, Antoine et al., 2008). Using NMR technology, a high degree of flexibility of the C-terminal region of oxidised MsrA was evidence, which favour the intramolecular disulfide bond between the two recycling cysteines (Coudeville, Antoine et al., 2007).

The catalytic mechanism for MsrA has been described around 15 years ago by Boschi-Muller and colleagues in the case of *E. coli* MsrA and by Lowther *et al.* for bovine MsrA (Boschi-Muller, Azza et al., 2000, Lowther et al., 2000a). Based on sulfenic acid chemistry, these two groups propose a reaction mechanism for MsrA catalysis consisting in several steps (Figure 22). First, the catalytic Cys-51/72 will act as a nucleophilic agent

attacking the sulfoxide moiety of the substrate leading to formation of a sulfenic acid on the catalytic cysteine with the concomitant release of 1 mol of methionine per mol of Msr. Subsequently, the recycling Cys-198/218 attack on the sulfenic intermediate will create an intramolecular disulfide bond between the catalytic and the recycling cysteine. In the case of another recycling cysteine, such as Cys-206 from *E. coli* and Cys-227 from *Bos taurus*, there is subsequent nucleophilic attack of Cys-206/227 on Cys-198/218 creating a new intramolecular disulfide bond between these two recycling cysteines. The last step involves reduction of this disulfide bond by Trx *in vivo* or other reducing agents, such as DTT, *in vitro*. Kinetic studies showed that the rate of formation of the sulfenic acid is high while the recycling process which reduces back the oxidised catalytic cysteine is overall rate-limiting (Olry, Boschi-Muller et al., 2004). Murine and human MsrA possess the same mechanism of action but in the case of other bacteria, such as *Neisseria meningitidis* (Antoine, Boschi-Muller et al., 2003) and *Mycobacterium tuberculosis* (Taylor et al., 2003), MsrA proteins possess only one recycling cysteine equivalent to Cys-198 from *E. coli*. Recently, MsrA has been shown to also have an oxidase activity towards methionines, producing Met-SO within proteins, including itself, or in free methionines (Lim, You et al., 2011), even in the presence of thioredoxin (Kriznik, Boschi-Muller et al., 2014).

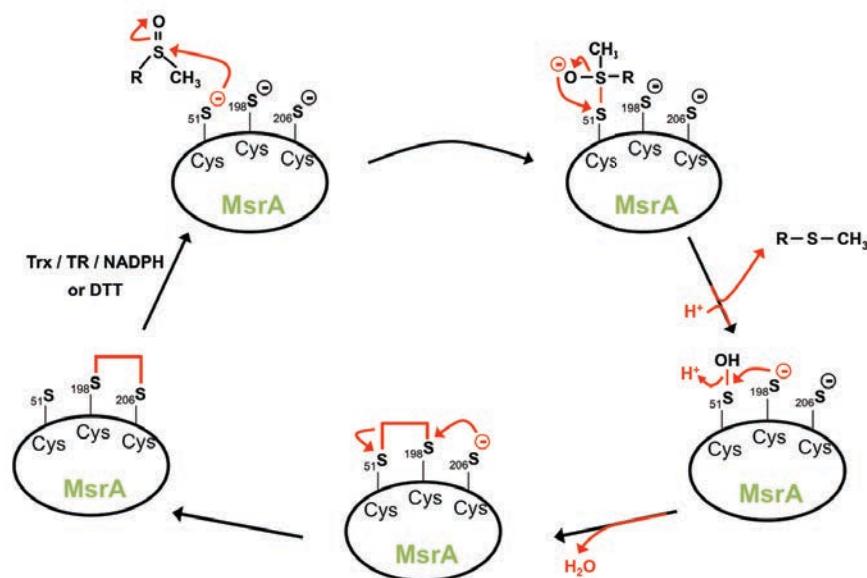


Figure 22 - **MsrA catalytic mechanism.** Bacterial MsrA is used in this representation. The nucleophilic attack of the MsrA catalytic Cys-51 on the sulfur atom of the methionine sulfoxide substrate leads to the formation of an unstable intermediate (enzyme bound to the substrate). Ionic rearrangement leads to the formation of a sulfenate ion with the concomitant release of the methionine molecule and protonation of the sulfenate ion to produce a sulfenic acid intermediate on MsrA. The nucleophilic attack of the recycling Cys-198 on the sulfur atom of the sulfenic acid intermediate leads to the formation of an intramolecular disulfide bond. MsrA full native state recovery is achieved after another nucleophilic attack of Cys-198 on Cys-206 generating a second intramolecular disulfide bond, which can be reduced either by the Trx / Trx reductase / NADPH regenerating system or by DTT. Adapted from (Boschi-Muller et al., 2000, Lowther et al., 2000a).



The first protein showing a methionine-R-sulfoxide reductase activity was identified in *E. coli* and was named MsrB. It has none similarity with MsrA, presents 43 % sequence homology with PilB from *Neisseria gonorrhoeae* and contains a conserved signature sequence CGWP(S/A)F (Grimaud et al., 2001). Indeed, the PilB protein has a MsrA and a C-terminal MsrB-like domains that function with opposite substrate stereospecificity and a N-terminal thioredoxin-like domain that allows the regeneration of the both Msr active sites (Lowther, Weissbach et al., 2002, Olry et al., 2002, Wu, Neiers et al., 2005). The 3D-structures of MsrB obtained by X-ray crystallography of PilB did not show any similarity with the one from MsrA (Lowther et al., 2002). However, the active sites for both enzymes show an axial symmetry as if they were reflecting each other in a mirror, which can be explained by the stereospecificity of the two enzymes. This symmetry suggests a similar catalytic mechanism for both enzymes and indeed, the two catalytic cysteines of PilB, Cys-495 and Cys-440, function in a similar way to the *E. coli* MsrA Cys-51 and Cys-206: a nucleophilic attack by Cys-495 to MetO leads to the production of a trigonal intermediary compound that after ionic rearrangement and subsequent methionine release, will form a sulfenic acid on PilB Cys-440 (Lowther et al., 2002). As for MsrA, a series of proton exchanging events occurs leading to the formation of a disulfide intramolecular bond, which is consequently reduced by Trx (Lowther et al., 2002).

MsrB1 is a mammalian MsrB enzyme that shows less homology with MsrBs from invertebrates, with only 29 % of similarity between the sequences of human MsrB1 and the MsrB domain of *Neisseria gonorrhoeae* (Lescure et al., 1999). The presence of the selenium atom in its active site is critical for the catalytic function of this enzyme. In fact, the wild type selenoprotein MsrB1 is 800-fold more active than the corresponding cysteine-MsrB1 mutant form (Kim & Gladyshev, 2004b). Similarly, a cysteine to selenocystein mutation in mammalian MsrB2 and MsrB3 resulted in 100-fold increase in the catalytic activity of the enzymes (Kim & Gladyshev, 2005a). The incorporation of a selenocysteine into the primary sequence of MsrB1 protein is due to a SECIS (SelenoCysteine Insertion Sequence) element downstream to the UGA selenocysteine codon (Lescure et al., 1999). The presence of a recycling cysteine, present in the N-terminal region of the MsrB1 protein is important for resolving the selenic acid intermediate formed during catalysis (Figure 23) (Kim & Gladyshev, 2005a).

MsrB2 was first identified in humans as a 21 kDa protein composed of 202 amino acids and carrying the conserved MsrB sequence GTGWP (Jung, Hansel et al., 2002). It presents 59 % homology with *E. coli* MsrB and 42 % with the C-terminal domain of

*Neisseria gonorrhoeae* PilB. MsrB2 is four times less efficient than MsrA for the reduction of a specific synthetic substrate. Contrarily to the other 60 % of MsrB containing a recycling cysteine residue in the middle of their sequences, the mammalian MsrB2 proteins do not have this cysteine residue and the sulfenic intermediate could be directly reduced by Trx (Kim & Gladyshev, 2005a).

Another gene, that seems to be only present in the genome of mammals codes, by alternative splicing, two proteins of the MsrB family, MsrB3A and MsrB3B (Kim & Gladyshev, 2004b). As for the other MsrB proteins, they contain a catalytic cysteine and the characteristic CXXC motifs responsible for zinc binding in the protein (Figure 21). In terms of their catalytic activity, these enzymes act similarly to the MsrB2 enzymes (Figure 23). Despite of the presence of cysteine residues in their active sites, catalytic efficiencies of MsrB2 and MsrB3 are only slightly lower than the selenocystein-MsrB1 (Kim & Gladyshev, 2004b, Kim & Gladyshev, 2005a).

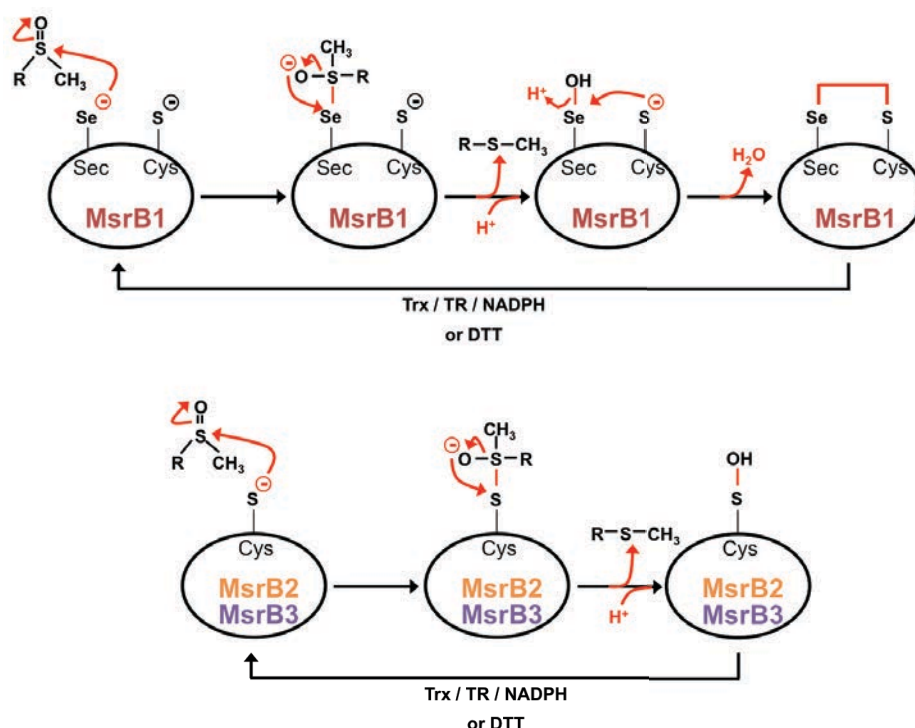


Figure 23 - **MsrB catalytic mechanism.** In the case of MsrB1 (upper part of the figure), methionine sulfoxide reduction starts with the nucleophilic attack of selenocystein (Sec) on the sulfur atom of the substrate leading to the formation of an unstable intermediate. Ionic rearrangement leads to the formation of a selenic acid intermediate with the concomitant release of a methionine molecule. The nucleophilic attack of the recycling cysteine (Cys) on this selenic acid intermediate leads to the formation of an intramolecular selenenylsulfide bond, which is subsequently reduced by the thioredoxin (Trx) / thioredoxin reductase (TR) / NADPH regenerating system or by dithiothreitol (DTT). In contrast, the sulfenic intermediate of MsrB2 and MsrB3 (lower part of the figure), formed on Cys after methionine release, can be directly reduced to its fully active state by one of these mechanisms. Adapted from (Kim & Gladyshev, 2005a, Lowther et al., 2002).

### ***Reducing power for the Msr system***

First studies on the biological reducing agents for MsrA revealed that reduced Trx, high levels of DTT or reduced lipoic acid as being able to reduce oxidised MsrA *in vitro*. If *E. coli* MsrA and MsrB and bovine MsrA efficiently use either Trx or DTT as reducing agents, human MsrB2 and MsrB3 showed less than 10 % of their activity with Trx as reducing agent when compared to DTT (Sagher, Brunell et al., 2006b). This suggests that in animal cells, Trx may not be the only reducing power for these two enzymes. Thionein, the reduced metal-free form of metallothionein, could also function as a reducing system for human MsrB3 and MsrB2 although with weaker activity (Sagher et al., 2006b). Furthermore, selenium compounds such as selenocystamine were found to act as reducing agents for human MsrB2 and MsrB3 (Sagher, Brunell et al., 2006a). More recently, it was found that a few Msr such as *A. thaliana* MsrB1, *Clostridium* Sec-containing MsrA or the red alga *G. gracilis* MsrA can be regenerated by a glutaredoxin (Grx)/ glutathione (GSH) system (Couturier, Vignols et al., 2012, Kim, Lee et al., 2011, Tarrago, Laugier et al., 2009, Vieira Dos Santos, Laugier et al., 2007). In the case of *A. thaliana* MsrB1, the sulfenic acid is reduced by glutathione forming a glutathionylated intermediate that is attacked by glutaredoxins (Tarrago et al., 2009). However, the rate of the recycling process is at least 10 to 100-fold lower compared to Trx acting in Msr with a recycling cysteine, suggesting that the Grx system would not be used in Msr in which a disulfide bond is formed. In agreement methionine auxotrophic *E. coli* is unable to grow in the presence of MetO when the *TrxI* gene is inactivated (Jacob, Kriznik et al., 2011).

### **Methionine sulfoxide reductases in protection against oxidative stress**

Several studies realised in different bacteria and eukaryotes revealed that Msr enzymes and methionine amino acids work together in cellular protection against oxidative stress. Surface-exposed methionine residues in proteins are more easily oxidised by ROS due to the presence of sulphur atoms. However, they are believed to be more resistant to oxidative inactivation (Levine et al., 1996), thus keeping protein structure and catalytic function. Through this mechanism, methionines are proposed to act as a threshold barrier for other amino acid oxidation, which would lead to loss of the protein activity (Hsu, Narhi et

al., 1996, Levine et al., 1996). Another mechanism through which methionine can act as antioxidant amino acid and observed in *E. coli* (Jones, Alexander et al., 2011), *S. cerevisiae* (Wiltrout, Goodenbour et al., 2012) but also in mammalian cells (Netzer, Goodenbour et al., 2009), is its misacylation, meaning the incorporation of methionines by non-methionyl-tRNAs during translation. To date, this translation infidelity was shown to be specific to methionines and its frequency of occurrence increases upon innate immune or chemically induced oxidative stress (Netzer et al., 2009), thus suggesting that it plays a role in protecting against this kind of stresses. Recently, nine Met-mistranslated forms of the  $\text{Ca}^{2+}$ /calmodulin-dependent kinase II (CaMKII) were observed under  $\text{Ca}^{2+}$  stress. Some of these Met-misacylations are located in the catalytic domain and result in increased catalytic activity and in alterations of proteins subcellular localisation (Wang & Pan, 2015). Finally, the antioxidant protection conferred by methionine residues is also due to their cyclic reduction by Msr enzymes, which by render methionines prone to new oxidation reactions, will lower ROS levels (Luo & Levine, 2009).

In agreement, the absence of MsrA expression leads to reduced *E. coli* as well as *M. tuberculosis* viability when treated with ROS and RNS (Moskovitz, Rahman et al., 1995, St John, Brot et al., 2001). This increased sensitivity to oxidants was reversed by transformation of the mutant strain with a plasmid containing the wild-type *MsrA* gene from the respective bacteria species (St John et al., 2001). This MsrA protection against an oxidative stress state induced by different oxidants has been also verified in other bacteria such as *Ochrobactrum anthropi* (Tamburro, Robuffo et al., 2004) and *Staphylococcus aureus* (Singh, Moskovitz et al., 2001). In agreement, MsrA was shown relevant for survival of *Mycobacterium smegmatis* within macrophages, which produce high levels of ROS and RNS to fight against microorganisms (Douglas, Daniel et al., 2004). Overall the studies indicate that bacteria are highly dependent of MsrA to counteract the damaging effects of oxidative stress and consequently for their survival in diverse natural niches.

MsrA functions also as an antioxidant in protection of the eukaryotic unicellular *S. cerevisiae*. MsrA null yeast mutants that were not capable of reducing protein-bound MetO when submitted to  $\text{H}_2\text{O}_2$  treatment, presented a significant protein carbonyl accumulation (Oien & Moskovitz, 2007) and growth alteration (Moskovitz, Berlett et al., 1997). In agreement, overexpression of MsrA in yeast that reduced the levels of free and protein-bound MetO led to increased resistance to toxic concentrations of  $\text{H}_2\text{O}_2$  (Moskovitz, Flescher et al., 1998). Not only MsrA, but also MsrB were proved to protect *S. cerevisiae* against oxidative damages mediated by the toxic metal chromium (Sumner,

Shanmuganathan et al., 2005).

Msr protection against oxidative stress is also important for higher organisms such as plants and animals. During the dark periods when *A. thaliana* produces more ROS, it has been shown that the Msr system was important to prevent protein oxidative damage, thus minimizing protein turnover in these conditions of limited energy supply (Bechtold, Murphy et al., 2004). MsrB3 was identified in a proteomic study as a cold-responsive protein in *Arabidopsis* (Bae, Cho et al., 2003) as essential to reduce oxidised methionine and to lower ROS level that accumulates in the endoplasmic reticulum during plant cold acclimation (Kwon, Kwon et al., 2007).

In the case of invertebrate animals, the most studied model organisms were the *D. melanogaster* and the *C. elegans*. Overexpression of MsrA in the nervous system of *D. melanogaster* leads to transgenic animals that are more resistant to paraquat-induced oxidative stress (Ruan, Tang et al., 2002). In addition, the induction of MsrA by ecdysone, protects *Drosophila* against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress (Roesijadi, Rezvankhah et al., 2007). Studying worms, Minniti *et al.* found that *MsrA* gene is expressed in most tissues, particularly in the intestine and the nervous system and that suppression of this gene resulted in animals more sensitive to paraquat treatment, presenting chemotaxis and locomotor failure, partly due to muscle defects (Minniti et al., 2009). In mammals, paraquat injection decreased the survival of MsrA<sup>-/-</sup> mice comparing to MsrA<sup>+/+</sup> or MsrA<sup>+/-</sup> mice (Salmon et al., 2009). MsrA null mutant mice present also higher level of protein carbonyls in the liver and kidney (Moskovitz, Bar-Noy et al., 2001) as well as in the heart tissue, correlated with mitochondria morphological changes in this tissue (Nan, Li et al., 2010). Our laboratory showed that the modulation of Msr activity in rat hearts observed along the course of ischemia/reperfusion may involve structural modification of the enzyme rather than a modification of MsrA protein level (Picot, Perichon et al., 2006). Indeed, Dr. Levine's laboratory has later shown that the MsrA cytosolic form needs to be myristoylated in order to confer heart protection against ischemia/reperfusion damages, suggesting that it must interact with a hydrophobic domain (Zhao, Sun et al., 2011). This protective role of MsrA against ischemia/reperfusion injuries was also evidenced in mouse kidney, with increased oxidative stress markers, inflammation and fibrosis observed in kidneys of MsrA<sup>-/-</sup> mice after injury comparing to wild type (Kim, Choi et al., 2013, Kim, Noh et al., 2015). In agreement to what have been found in MsrA<sup>-/-</sup> mice, increased GSH, protein and lipid oxidation were also found in the liver and kidney of the MsrB1 K.O mice (Fomenko, Novoselov et al., 2009). MsrB1 is a selenium protein, thus depending on selenium concentrations to be

produced. Interestingly, Dr. Moskowitz have found that MsrA K.O mice submitted to a selenium deficient diet presented decreased MsrB activity but also less Gpx and Trx activities in their brains (Moskovitz, 2007).

Msr protection against oxidative damages was also investigated in different mammalian cell types *in vitro*. This is the case for instance for human (Sreekumar, Kannan et al., 2005), monkey (Lee, Gordiyenko et al., 2006) and rat (Dun, Vargas et al., 2013) retinal pigment epithelial cells and for human lens cells (Kantorow, Hawse et al., 2004). As an example, overexpression of MsrA confers to human lens cells an increased resistance to H<sub>2</sub>O<sub>2</sub>-induced stress while *MsrA* gene silencing led to an increased sensitivity towards oxidative treatment and to a loss of viability even in the absence of exogenously added stress (Kantorow et al., 2004). The protecting role of MsrB against oxidative stress was also stressed out in human lens cells. Silencing of all or individual genes led to increased oxidative stress-induced cell death indicating that MsrB are also implicated in human lens epithelial cell viability (Marchetti, Pizarro et al., 2005). MsrB1 was the most studied MsrB in human lens epithelial cells. Silencing of this *Msr* gene resulted in increased ROS levels, lipid oxidation, ER stress, decreased mitochondrial potential and release of cytochrome c, ultimately leading to caspase-dependent apoptosis (Dai, Liu et al., 2016, Jia, Li et al., 2012, Li, Jia et al., 2013). Furthermore, peroxynitrite treatment to MsrB1-deficient human lens epithelial cells will aggravate the oxidative damages and F-actin disruption, that normally occurs after this nitric stress (Jia, Zhou et al., 2014), suggesting that MsrB1 protects lens cells from F-actin nitration.

We and others have shown that human T lymphocyte cells presented an increase resistance to H<sub>2</sub>O<sub>2</sub> or zinc treatments when transfected with MsrA and/or MsrB2 genes by reducing the levels of intracellular ROS species and protein oxidative damages that would lead to cell death (Cabreiro et al., 2008, Cabreiro, Picot et al., 2009, Moskowitz et al., 1998). The role for MsrA in the prevention against the accumulation of protein and cellular oxidative damage provoked by H<sub>2</sub>O<sub>2</sub>-induced oxidative stress was also studied in fibroblast cells (Picot, Petropoulos et al., 2005) and was associated in these cells to MsrA-dependent differentially expression proteins implicated in protection against oxidative stress, apoptosis and premature ageing (Cabreiro, Picot et al., 2007). If overexpression of MsrA in the endoplasmic reticulum of mammalian cells increases their resistance to oxidative and ER stresses (Kim, Kim et al., 2014c), the resistance to a ER stress is mainly conferred by MsrB3 (Kwak, Lim et al., 2012, Lim, Han et al., 2012). Finally, in human skin cells, the behaviour of the MsrA enzyme seems to be dependent on the type of UV exposure and the dose

applied, suggesting a hormetic response to environmental stress. In fact, low doses of UVA stimulate MsrA expression, while UVB or high doses of UVA contribute to decrease MsrA expression and increase protein carbonyl (Ogawa et al., 2006, Picot et al., 2007), that can be prevented by pre-treating the cells with MsrA (Pelle, Maes et al., 2012). In melanocytes, the absence of MsrA expression also increased sensibility to oxidative stresses and cell death even in the absence of exogenous stresses (Zhou, Li et al., 2009). Since it was found that Msr expression and activities are significantly decreased in the epidermis of patients with vitiligo, a depigmentation disorder (Schallreuter, Rubsam et al., 2008), one could think that their loss of pigmentation could be due an increased melanocytes cells death caused by their reduced Msr expression.

In conclusion, methionine amino acid residues together with the Msr system constitute a potent antioxidant ROS scavenging system, preserving macromolecules in their reduced state and thus protecting different cell types and organisms from different kinds of oxidative and ER stresses, ultimately contributing to protein homeostasis and cell survival in endogenous or exogenous stress conditions.

## **Methionine sulfoxide reductases in disease, ageing and longevity**

Given that Msr system protects proteins, the major cellular effectors, from irreversible oxidation products resulted from a severe oxidative stress and that protein carbonyls levels are usually assumed as a marker of oxidative stress in pathophysiological conditions and during ageing, it is expected that Msr would be implicated in diseases and in ageing process.

Indeed, it has been shown that oxidised proteins accumulate in tissues from patients exhibiting age-related diseases such as Alzheimer's, Parkinson's, Huntington's diseases and cataracts (Gil-Mohapel, Brocardo et al., 2014, Swomley & Butterfield, 2015). Reduced MsrA activity was found in the brains of Alzheimer's disease patients (Gabbita, Aksenov et al., 1999). In agreement, an important accumulation of MetO was observed in the brains of aged rats suffering from neurodegenerative diseases, accompanied by an important decrease in MsrA expression (Moskovitz et al., 1996a). Furthermore, the MsrA K.O. mice demonstrated behavioural abnormality (tip-toe walking) consistent with cerebellar dysfunction (Moskovitz et al., 2001), increased light scattering - a common cataract symptom (Brennan, Lee et al., 2009) and enhanced neurodegeneration with characteristic

features of neurodegenerative diseases (Pal, Oien et al., 2007). Methionine oxidation in amyloid  $\beta$ -peptide ( $A\beta$  peptide), more specifically Met-35, is thought to be critical for aggregation and neurotoxicity (Hou, Kang et al., 2002) and it was shown that the absence of MsrA modifies  $A\beta$  solubility properties and causes mitochondrial dysfunction in a mouse model of Alzheimer's disease (Moskovitz, Du et al., 2016, Moskovitz, Maiti et al., 2011). In the case of Parkinson's disease, oxidation of the methionine residues in  $\alpha$ -synuclein is thought to be the main reason of protein fibrillation causing the pathology (Glaser, Yamin et al., 2005). *In vivo*, MsrA overexpression inhibits development of the locomotor and circadian rhythm defects caused by ectopic expression of human alpha-synuclein in the *Drosophila* nervous system (Wassef, Haenold et al., 2007).

The importance of Msr in age-related diseases and the accumulation of modified proteins produced by the action of ROS as major causes of ageing, suggests that Msr would have also an important role in ageing phenotype. Indeed, the accumulation of oxidatively modified proteins during ageing has been largely attributed to declined efficacy of the systems involved in protein homeostasis such as protein degradation and protein repair (Chondrogianni et al., 2014). In fact, it has been shown in the laboratory that MsrA is down-regulated in aged rats (Petropoulos, Mary et al., 2001) and during replicative senescence of fibroblasts (Picot, Perichon et al., 2004). Increased MetO levels were found in membrane proteins of senescent erythrocytes (Brovelli, Seppi et al., 1990) and in senescent *E. coli* (Weissbach, Resnick et al., 2005).

Several studies have been done to elucidate the implication of the Msr system in regulating lifespan but they are still controversial. The first two groups having tried to test this hypothesis used MsrA K.O. mice and MsrA overexpressing *Drosophila* as models, respectively (Moskovitz et al., 2001, Ruan et al., 2002). Knockout of the MsrA gene in mice reduces its lifespan by 40% (Moskovitz et al., 2001) while, in contrast, its overexpression in *Drosophila* accounts for a 70% extension in healthy lifespan (Ruan et al., 2002). In both studies, MsrA-dependent lifespan modulation was related to its role in protection against oxidative stress but later, an other study showed that while the lack of MsrA in mice increases sensitivity to oxidative stress, it does not diminish lifespan (Salmon et al., 2009). However, MsrA overexpression was shown to increase lifespan of *S. cerevisiae* (Koc, Gasch et al., 2004) while its inhibition in yeast or in *C. elegans* is accompanied by a shorten lifespan (Minniti et al., 2009).

Overall these studies suggest that the importance of Msr system in ageing and neurodegenerative diseases is dependent of their role as an antioxidant enzyme protecting



cells and organisms from the deleterious effects of an increased oxidative stress.

## **Methionine sulfoxide reductases as regulators of protein and cellular functions**

There is increasing evidence that the cyclic interconversion between Met and MetO within proteins is implicated in the regulation of cell signalling functions. In fact, it has been demonstrated that the oxidation of certain methionine residues in proteins induces mainly the loss of their biological activity, while their reduction by Msr is capable of reversing it.

The types of proteins in which methionine oxidation has been involved in their function are very diverse: proteases or protease inhibitors, metabolic enzymes, cytoskeleton proteins, cytokines, heat shock proteins, hormones, heme proteins, proteins associated with neurodegenerative disorders, proteins involved in immunodefences as well as different bacterial proteins and snake toxins (see (Oien & Moskovitz, 2008) for review). Between the more than 50 proteins reported to have altered activity due to formation of methionine sulfoxide, only part of them was already described as being substrates of Msr enzymes either *in vitro* or *in vivo* experiments. Here I will focus on some examples of these Msr substrates.

The *E. coli* ribosomal protein L12 was the first characterised substrate of MsrA (Brot et al., 1981). The oxidation of three methionines by H<sub>2</sub>O<sub>2</sub> decreases its ability to bind to ribosomes and to interact with other ribosomal proteins such as L10, impairing protein synthesis (Caldwell, Luk et al., 1978). The activity of L12 can be regained by MsrA reduction of the oxidised methionines (Brot et al., 1981). Another *E. coli* protein involved in protein machinery is the Ffh component of the ubiquitous signal recognition particle. This protein contains a methionine-rich domain whose oxidation compromises Ffh interaction with a small RNA. Oxidised Ffh is a substrate for MsrA and MsrB enzymes and reduction of Ffh MetO residues allows the recovery of its RNA-binding abilities (Ezraty, Grimaud et al., 2004).

Regarding serine protease inhibitors, methionine oxidation was associated with a loss of function in  $\alpha$ 1-antitrypsin (Abrams, Weinbaum et al., 1981) and  $\alpha$ 2-antitrypsin (Stief, Aab et al., 1988). In particular,  $\alpha$ 1-antitrypsin protein is important for lung protection by preserving anti-neutrophil elastase activity, which is associated with the risk of developing emphysema. Oxidation of two methionines of  $\alpha$ 1-antitrypsin causes a loss of the anti-neutrophil elastase activity, which can be restored in part with the addition of MsrA *in vitro*

(Glaser, Karic et al., 1987). Methionine oxidation of Human Immunodeficiency Virus 2 (HIV-2) protease, which cleavages proteins involved in the HIV-2 reproductive cycle, also inhibits its proteolytic activity while the addition of only MsrA partially restores it (Davis, Newcomb et al., 2000). These findings could lead to new therapeutic strategies in order to fight against diseases such as pulmonary emphysema and AIDS.

In addition, an important protein participating in the immunological and oxidative stress response, the inhibitor of kappa B-alpha ( $I\kappa B\alpha$ ), named for its inhibition activity of the transcription factor nuclear factor- $\kappa B$  (NF- $\kappa B$ ), can also be oxidised a methionine residue thereby increasing its resistance to proteasomal degradation (Kanayama, Inoue et al., 2002). When  $I\kappa B\alpha$  is oxidised by taurine chloride or chloramines, it cannot dissociate from the NF- $\kappa B$ , thus preventing it from nucleus translocation and subsequent activation of its target genes (Midwinter, Cheah et al., 2006). Inhibition of NF- $\kappa B$  activation is prevented by MsrA (Mohri, Reinach et al., 2002).

Furthermore, methionine oxidation and its Msr-dependent reduction is important for the regulation of cellular excitability, in particularly through the regulation of the voltage-gated and the calcium ( $Ca^{2+}$ )-activated potassium channels (Chen, Avdonin et al., 2000, Ciorba, Heinemann et al., 1997) (Figure 24A).

Another protein involved in cellular excitability, and whose activity is regulated by Msr enzymes, is the  $Ca^{2+}$ -binding protein calmodulin (CaM) (Bigelow & Squier, 2005). This protein detects the calcium signal in cells and coordinates energy metabolism, which in turn produce ROS in the mitochondria. CaM loses its conformational stability upon Met oxidation (Lim, Kim et al., 2013) thus failing to activate the plasma membrane  $Ca^{2+}$ -ATPase (Figure 24B) (Yao, Yin et al., 1996). In contrast, its fully reduction by MsrA and MsrB leads to its binding to the inhibitory domain of the plasma membrane  $Ca^{2+}$ -ATPase, inducing helix formation within the CaM-binding sequence and releasing enzyme inhibition (Grimaud et al., 2001, Sun, Gao et al., 1999). Upon oxidative stress, increasing levels of cytosolic  $Ca^{2+}$  are probably the consequence of the oxidation of specific methionines in CaM that will be no longer able to activate the  $Ca^{2+}$ -ATPase in the plasma membrane. CaM oxidation accumulation will result in down-regulation of metabolism thus, controlling the generation of ROS.

One of the CaM proteins targets, the  $Ca^{2+}$ /calmodulin-dependent protein kinase II (CaMKII), is also regulated by methionine oxidation. Indeed, apart from  $Ca^{2+}$ /CaM regulation or its autophosphorylation at Thr-287, oxidation of one of its methionine residues leads also to its  $Ca^{2+}$ /CaM-independent activity (Figure 24C) (Erickson, Joiner et al., 2008).

This is consistent with the notion that Met oxidation does not invariably induce enzyme inactivation. The same authors have shown that MsrA enzymes are essential for reversing CaMKII oxidation in myocardium *in vivo* (Erickson et al., 2008). More recently, it was shown that oxidation of CaMKII mediates the cardiotoxic effects of aldosterone, while MsrA overexpression reversed its effects (He, Joiner et al., 2011).

If Met oxidations are involved in the regulation of biological functions, we could think that they cannot depend only on random oxidation by multiple forms of ROS. There should be also specialised oxidases that catalyse the oxidation of specific protein targets. Indeed, Hung and colleagues have found that a flavoprotein monooxidase, called Mical is able to bind to F-actin and to selectively oxidised 2 of its 16 methionine residues into the R stereoisomer of MetO, resulting on actin disassembly *in vitro* and *in vivo* (Hung, Yazdani et al., 2010). This Mical-dependent redox regulation of actin that can be reversed by MsrB1/SelR, was involved in bristles formation in *Drosophila* (Hung, Spaeth et al., 2013), in membrane trafficking in bone marrow-derived macrophages (Lee et al., 2013) (Figure 24D) and in lens epithelial cells (Jia et al., 2014).

The increasing work done in the reversibility of methionine oxidation *in vitro* as well as *in vivo* led to the accepted view that this sophisticated redox based mechanism must be assimilated to other covalent modifications on proteins such as phosphorylation, acetylation or glutathionylation, implicated in the regulation of many important protein and cellular functions.

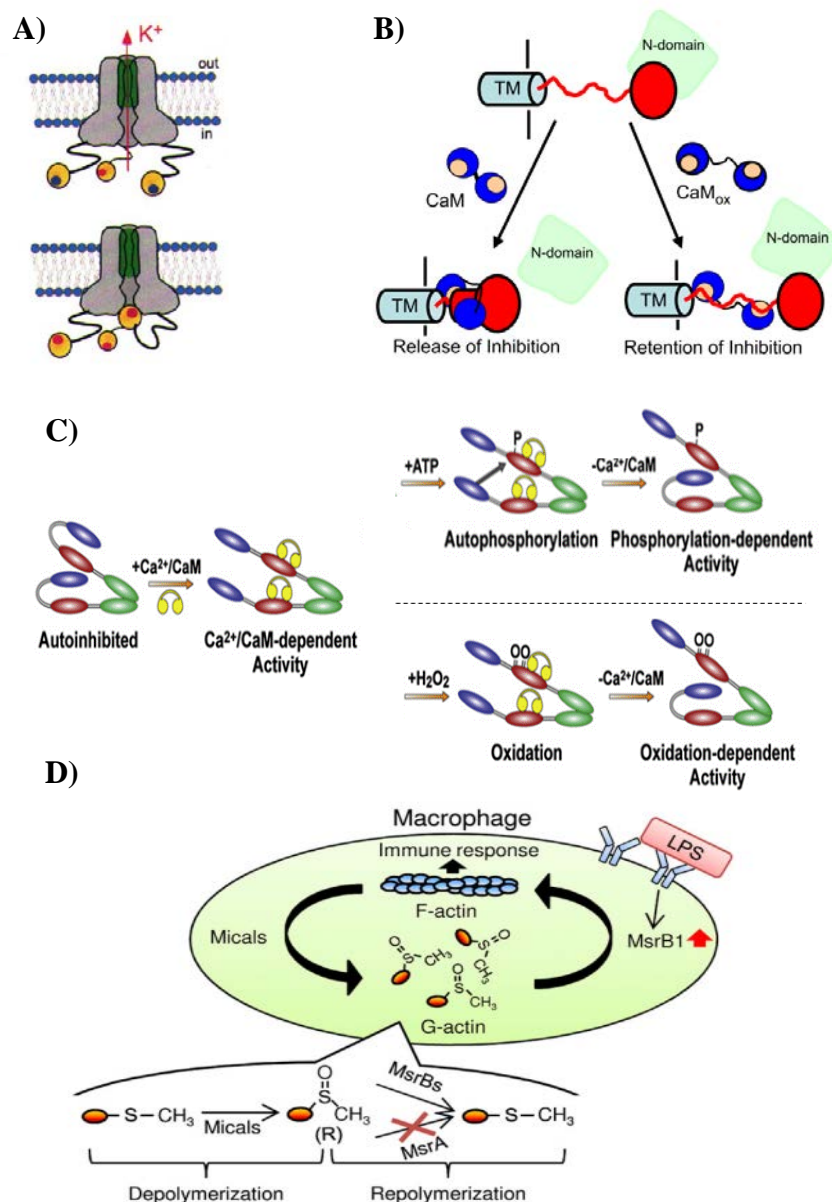


Figure 24 - **Representations of methionine and Msr regulation of protein function.** **A)** voltage-gated potassium channels. Oxidation of Met in the N-terminal of K<sup>+</sup> channels leads to their inactivation, while Msr reduction restores their function. **B)** CaM binding functions as a conformational switch to release enzyme inhibition (green) through association with the Ca-ATPase (red), whose transmembrane helix (TM) is presented in grey. In contrast, CaM methionine oxidation results in an altered binding interaction which retains the association between the inhibitory enzyme and the Ca-ATPase. Msr reduction of CaM will restore catalytically important domain interactions with Ca-ATPase associated with an optimal transport activity. **C)** Mechanism of CaMKII activation by autophosphorylation or by oxidation: after initial activation by binding of Ca<sup>2+</sup>/CaM to its regulatory domain (red), CaMKII can be phosphorylated at T287 yielding persistent activity even after the removal of Ca<sup>2+</sup>/CaM or oxidised at M281/282 blocking reassociation of its catalytic domain (blue), also yielding persistent CaMKII activity. **D)** Model of redox regulation of actin function via reversible stereoselective site-specific methionine oxidation and reduction and its importance for the macrophage immune response. From (Bigelow & Squier, 2005, Erickson et al., 2008, Hoshi & Heinemann, 2001, Lee et al., 2013).



## **OBJECTIVES**



In this thesis, we addressed for the first time the role of the methionine sulfoxide reductase (Msr) proteins, in skeletal muscle tissue, either their importance for satellite cell function as well as their role on skeletal muscle fibres.

The study of satellite cells function in the context of muscle regeneration represents a major biomedical issue, mainly for treatment of diseases characterised by a progressive muscle weakness and degeneration, such as muscular dystrophies. Although muscular dystrophies are inherited disorders caused by different genetic mutations and differ in the age of onset, in the mode of inheritance and in the severity of its progression, they share some certain common pathologic features such as the presence of an oxidative stress state and senescence of muscle progenitor cells associated with less differentiation capacity (Negroni et al., 2016). The study of important antioxidant defence mechanisms in this context could give some insights to new therapeutic targets for these muscle disorders. Moreover, muscle ageing has also been associated with high levels of oxidative damages in fibres (Jackson, 2016; Powers et al., 2012). In this context, we studied the Msr system which by repairing oxidative damage in methionines has been involved in the prevention protein irreversible oxidation damages during ageing and neurodegenerative disorders (Kim et al., 2014a; Ugarte et al., 2010), and which role has never been investigated in muscle cells.

This work is organised in two parts, the first one regards the study of Msr enzymes in muscle satellite cells. In a second part, we investigated whether Msr enzymes, by their antioxidant properties and/or their protein regulation functions, are involved in muscle fibre homeostasis.

## **I. Importance of the Msr system for skeletal muscle satellite cells**

To investigate the role of Msr system in myogenesis, we chose to a cell culture approach using human-derived myoblasts kindly given by Dr. Gillian Butler-Browne from the “Institut de Myology” (Paris). Since there is no information regarding the different Msr genes in myoblasts, we analysed Msr expression and activity profiles in proliferative myoblasts and differentiated myotubes. Furthermore, in order to establish a relationship between Msr activity, cellular redox state and protein homeostasis, expression of other antioxidant enzymes, proteasome activity, protein irreversible damages and ROS levels were assayed during myoblast differentiation. Finally, the importance of Msr enzymes in



myogenesis was further analysed by the study of MsrA-deficient myoblast's proliferation and differentiation capacities.

## **II. Importance of Msr enzymes for skeletal muscle fibres protection against oxidative stress under normal or pathological conditions**

To study the importance of Msr system for skeletal muscle fibres homeostasis, we used muscle biopsies obtained from mouse models as well as from human donors. First of all, we examined the Msr expression and activity in different types of muscle fibres (slow oxidative, fast glycolytic and mix type). Knowing in which fibre type these enzymes are more required, we investigated their function during skeletal muscle ageing. To clarify the role of Msr enzymes in muscle oxidative stress conditions, their expression in sarcopenic biopsies was compared with that from muscular dystrophies in which an important oxidative stress has been described.

## **MATERIALS AND METHODS**



## Human muscle biopsies

Human muscle biopsies were obtained from the MYOBANK BTR (Bank of Tissues for Research, a partner in the EU network EuroBioBank) or from neurologists during surgical procedures and after informed consent in accordance with European recommendations and French legislation.

Biopsies from OPMD patients were obtained after cricopharyngeal myotomy, a medical procedure that eliminates the obstacle generated by the dysfunctional upper esophageal sphincter musculature (Coiffier et al., 2006). All patients were aged between 54 and 83 years old and healthy donors between 39 and 91 years old at the time of muscle biopsy. OPMD patients exhibited a typical clinical phenotype and *PABPN1* mutations.

DMD patients were aged between 12 and 16 years old and healthy donors between 10 and 37 years old at the time of muscle biopsy. DMD patients showed a typical clinical phenotype and *Dystrophin* mutations.

cDNAs derived from total RNA of muscle biopsies from dystrophic (OPMD, DMD) or healthy subjects were kindly given by Dr. Capucine Trollet and Dr Anne Bigot from the Myology Institute (Paris). Two groups of muscle biopsies from 12 OPMD patients, 4 DMD patients and 15 healthy individuals were used for qPCR analysis as follows: i) 6 *cricopharyngeus* and 6 *sternocleidomastoid* muscle biopsies from OPMD patients and healthy individuals; ii) paravertebral muscles from 4 DMD patients and *quadriceps* from 3 healthy individuals.

## Mouse muscle biopsies

OPMD transgenic mice (Davies et al., 2005) and controls were generated by crossing the heterozygous carrier strain A17.1 obtained from Rubinsztein's group (Davies et al., 2005) with the wild type (W.T.) FvB background mice. The mice were genotyped after tail biopsies by PCR 3 to 4 weeks after birth. Total RNA was extracted from *Extensor Digitorum Longus* muscle of W.T. and A17.1 OPMD transgenic mice using Trizol (Life Technologies) according to the manufacturer's instructions. cDNAs derived from total RNA were kindly given by Dr. Capucine Trollet from the Myology Institute (Paris).

The *mdx/mdx* mice (Bulfield, Siller et al., 1984) were obtained from the Jackson laboratories. *Tibialis anterior* muscles dissected from 6 month old *mdx/Y* or wild-type mice were frozen in liquid nitrogen and conserved at -80°C before being used for mRNA

extraction.

*Tibialis Anterior*, *Soleus*, *Quadriceps* and *Gastrocnemius* muscles were dissected from adult (3 months) and geriatric (25 months) C57Bl6 mice. Muscles were frozen in liquid nitrogen and conserved at -80°C before being used for RNA and protein extraction.

All animal procedures were approved and conducted in accordance with the animal ethics committee following the regulations of the Ministry of Agriculture and the European Community guidelines.

### **OPMD-derived human myoblasts primary cultures**

Primary Human OPMD myoblasts were obtained as described previously (Bigot, Klein et al., 2009, Mamchaoui, Trollet et al., 2011) and cultivated in growth medium composed of mixed 199 Medium/DMEM (1/4 v/v) (Gibco/Invitrogen Life Technologies) supplemented with 20% foetal calf serum (Invitrogen Life Technologies), 2.5 ng/ml HGF (Invitrogen Life Technologies), 0.1 µM Dexamethasone (Sigma-Aldrich, St. Louis, MO) and 50 µg/ml Gentamycin (Invitrogen Life Technologies). The myogenic purity of the populations was monitored by immunocytochemistry using desmin as a marker. Enrichment of myogenic cell was eventually performed using immunomagnetic cell sorting system MACS (Miltenyi Biotec, Paris, France) according to the manufacturer's instructions. Briefly, cells were labelled with anti-CD56 microbeads, and separated in a MACS column placed in a magnetic field. The two populations CD56<sup>+</sup> and CD56<sup>-</sup> were kept, and myoblast purification was evaluated by immunochemistry using desmin as a marker.

### **Mouse satellite cells isolation**

Satellite cells were isolated from a pool of diaphragm, *pectoralis* and abdominal muscles of adult (6 weeks-old) *Pax3<sup>GFP/+</sup>* mice (Relaix et al., 2005), by flow cytometry (Astrios, Beckman Coulter) on the basis of size and granularity and GFP fluorescence as previously described (Montarras et al., 2005). The percentage of GFP-positive cells in each sample was 100%, as expected.

Further material and methods are described the article presented in “Results - Part I. Importance of the Msr system for skeletal muscle satellite cells”. All primers and antibodies used in this thesis are listed in **Table 2**, **Table 3** and **Table 4**, respectively.

**Table 2 -List of human primers used in this work.**

<b>Gene</b>	<b>Forward primer</b>	<b>Reverse primer</b>
<b>CAT</b>	5' CTGACTACGGGAGCCAC 3'	5' TGATGAGCGGGTTACACG 3'
<b>Emerin</b>	5' AAGAGGAGTGCAAGGATAGGG 3'	5' GGAGGAAGTAGGATAATAGGACAGG 3'
<b>GPx2</b>	5' ACAGTCTCAAGTATGTCCGT 3'	5' TGTCCCTCAGGTAGGCGA 3'
<b>GPx3</b>	5' CGGGGACAAGAGAAGTCG 3'	5' CCCAGAATGACCAGACCG 3'
<b>GPx4</b>	5' CTCACCAAGTTCCTCATCG 3'	5' GGGCAGGTCCTTCTCTATC 3'
<b>MHC-IIa</b>	5' GAAAGTCTGAAAGGGAACGCA 3'	5' CGCCACAAAGACAGATGTTTTG 3'
<b>MsrA</b>	5' GGGCAACTCGGCCCTCGAACA 3'	5' CCAGAAACATCCCATCCAAA 3'
<b>MsrB1</b>	5' GTGTGCCAAGTGTGGCTATGA 3'	5' CTTGCCACAGGACACCTTCAA 3'
<b>MsrB2</b>	5' CGGAGCAGTTCTACGTCACAA 3'	5' CAGAGCCAGACGTACCATGA 3'
<b>MsrB3</b>	5' CTCAGGAGAAAGGGACCGAAA 3'	5' GCCAACCTGAACCGGAGTCAA 3'
<b>MyoD</b>	5' AACTGCTCCGACGGCATG 3'	5' ACAGGCAGTCTAGGCTCGACA 3'
<b>Myogenin</b>	5' GAGTTCAGCGCCAACCCAG 3'	5' TGCCCGCTTGGGAAGACAAT 3'
<b>Prx2</b>	5' GACTACAGAGGGAAGTACGTGG 3'	5' TCAGTTGTGTTTGGAGAAGTATTCC 3'
<b>Prx3</b>	5' TGCCTGGATAAATACACC 3'	5' AGTCTCGGGAAATCTGCT 3'
<b>Prx5</b>	5' ATTCGCTGGTGTCCATCTTT 3'	5' GTGCCATCTGGTTCACATTCA 3'
<b>Prx6</b>	5' ATTCTCAGGGTAGTCATCTC 3'	5' TTTGGCTTCTTCTCAGG 3'
<b>SOD1</b>	5' TGCATCATGGCCGCA 3'	5' TTTCTTCATTTCCACCTTTGCC 3'
<b>SOD2</b>	5' GGAGATGTTACAGCCCAGATAG 3'	5' CAAAGGAACCAAAGTCAAG 3'
<b>Trx1</b>	5' TGGTGAAGCAGATCGAGAGCAAGA 3'	5' ACCACGTGGCTGAGAAGTCAACTA 3'
<b>Trx2</b>	5' TGTGGGCATCAAGGATGAGGATCA 3'	5' TGGCATGGAAGGGCTGAGTTCTAT 3'

**Table 3 - List of mouse primers used in this work.**

<b>Gene</b>	<b>Forward primer</b>	<b>Reverse primer</b>
<b>HPRT</b>	5' TCCTCATGGACTGATTATGGA 3'	5' GTAATCCAGCAGGTGAGCAAAA 3'
<b>MHC-I</b>	5' GAGGAGAGGGCGGACATC 3'	5' GGAGCTGGGTAGCACAAGAG 3'
<b>MHC-IIa</b>	5' ACTTTGGCACTACGGGAAAC 3'	5' CAGCAGCATTTCGATCAGCTC 3'
<b>MHC-IIx</b>	5' CAGCAGCATTTCGATCAGCTC 3'	5' CCTGCTCCTAATCTCAGCATCC 3'
<b>MsrA</b>	5' ACGCAATCCCACCTACAAAG 3'	5' CGGATGTGGGATAGACTGCT 3'
<b>MsrB1</b>	5' AGCGTTCACAACCCGACGACTT 3'	5' AGCGTTCAGTAAACCATCC 3'
<b>MsrB2</b>	5' CTGACCCCGGAACAGTTCTA 3'	5' GCCGTAAGCCTCAGAAAATG 3'
<b>MsrB3</b>	5' GCCATCGAGTTCACAGATGA 3'	5' CGAGGTCCATCGTCAAAAAT 3'
<b>Myogenin</b>	5' CAACCAGGAGGAGCGCGATCTCCG 3'	5' AGGCGCTGTGGGAGTTGCATTCACT 3'

**Table 4 – List of primary antibodies used in this work.**

<b>Protein</b>	<b>Purchased from</b>	<b>Reference</b>	<b>Host</b>	<b>Dilution</b>
<b>20S <math>\beta</math>1</b>	Enzo Life Sciences	BML-PW8140	mouse	1/1000
<b>20S <math>\beta</math>2</b>	Enzo Life Sciences	BML-PW9300	mouse	1/1000
<b>20S <math>\beta</math>5</b>	Enzo Life Sciences	BML-PW8895	rabbit	1/1000
<b>Caspase-3</b>	Cell Signaling	9662	rabbit	1/1000
<b>Cleaved Caspase-3</b>	Cell Signaling	9661	rabbit	1/1000
<b>CML</b>	R&D Systems	MAB 3247	mouse	1/1000
<b>DNP</b>	Sigma Aldrich	D9656	rabbit	1/5000
<b>Emerin</b>	Santa Cruz Biotechnologies	SC-25284	mouse	1/1000
<b>Emerin</b>	Santa Cruz Biotechnologies	SC-15378	rabbit	1/1000
<b>MsrA</b>	Abcam	ab-16803	rabbit	1/1000
<b>MsrB2</b>	Abcam	ab-101513	rabbit	1/1000
<b>MyoD1</b>	Dako	M3512	mouse	1/300
<b>Myogenin</b>	Santa Cruz Biotechnologies	SC-576	rabbit	1/300
<b>p16</b>	Santa Cruz Biotechnologies	SC-468	rabbit	1/1000
<b>Troponin C</b>	Santa Cruz Biotechnologies	SC-20642	rabbit	1/1000
<b>Ubiquitin</b>	Santa Cruz Biotechnologies	SC-8017	mouse	1/500

## **RESULTS**





## **Part I – Importance of the Msr system for skeletal muscle satellite cells**

### ***Background***

Muscle regeneration study represents a major biomedical issue, mainly for treatment of diseases characterised by a progressive muscle weakness and degeneration, such as muscular dystrophies. Despite its stability under homeostatic conditions, skeletal muscle tissue has a remarkable ability to regenerate after injury thanks to the presence of adult stem cells, called satellite cells. Under homeostatic conditions, these cells, located under the basal lamina that surrounds multinucleated muscle fibres, are characterised by a quiescent state. However, when stimulated by damage or by stress, satellite cells become activated and enter the cell cycle to proliferate and undergo differentiation to form new fibres or self-renew to reconstitute the satellite cell pool. Each step of this process is regulated through the coordinated expression of specific transcription factors including Pax3/7 and the myogenic regulatory factors (MRFs): MyoD, Myf5, Myogenin, and MRF4. Signalling pathways involving these transcription factors in the regulation of satellite cell function have been extensively studied (Moncaut et al., 2013). However, important aspects of myogenesis regulation, in particular the redox state remains less studied. The cellular redox state is characterised by a balance between the production of reactive oxygen species (ROS), mainly by mitochondria, and their scavenging by the antioxidant systems. The regulation of this balance is crucial for cellular function: although at low levels, ROS act as signalling molecules for the regulation of multiple cellular processes including growth, proliferation, differentiation and apoptosis, when ROS levels overcome the capacity of cellular antioxidant systems, they damage cellular components, in particular proteins, altering their structure and function.

To date, it is known that myoblasts differentiation is accompanied by an increased in mitochondria number and activity, leading to high ROS levels production (Malinska et al., 2012). NADPH oxydase (NOX) and nitric oxide synthase (NOS) enzymes are also responsible for ROS production needed for myoblasts fusion and differentiation in a Ca<sup>2+</sup>-dependent manner (Hidalgo, Sanchez et al., 2006, Piao et al., 2005). On the other hand, excessive ROS production through TNF- $\alpha$ -dependent signalling or their deficient

elimination by antioxidants will lead to impaired myogenic differentiation *in vitro* (Ardite et al., 2004, Langen et al., 2002) as well as muscle regeneration *in vivo* (Coletti et al., 2005, Langen et al., 2004). In agreement, natural antioxidants administration to myoblasts pre-treated with oxidative molecules will prevent myoblast cell death and differentiation arrest though the increase in antioxidant transcription factors and enzymatic systems (El Haddad et al., 2012, Kang et al., 2015, Kang et al., 2014, Sestili et al., 2009, Young et al., 2010). Between antioxidant enzymes such as Pxr2 and Prx3 have been shown to protect myoblasts from oxidative damages and to regulate myotubes ATP production by modulation of ROS levels (Lee et al., 2014b, Won et al., 2012). However, the mechanisms underlying this ROS-mediated modulation of myogenesis remain unclear. ROS are known to interact with macromolecules and in particular to proteins thus regulating their function. Indeed, protein oxidation was detected in myoblast treated with H<sub>2</sub>O<sub>2</sub> (Baraibar, Hyzewicz et al., 2011, Dargelos et al., 2010, Franco et al., 1999). While irreversible oxidative damages to proteins lead to protein degradation, the oxidation of sulfur-containing amino acids can be repaired by specific enzymatic systems. Myoblast fusion and differentiation was associated with increased expression of proteasome and autophagy-related proteins as well as higher proteasome activities (Cui et al., 2014, McMillan & Quadriatero, 2014). Indeed, proteasome subunits inhibition or knock down of genes coding proteins involved in the UPS or in the autophagic system will lead to reduced myoblasts fusion and less myotubes formation *in vitro* (Cui et al., 2014, McMillan & Quadriatero, 2014) and *in vivo* (Perera et al., 2012). The involvement of these two degradation pathways in myogenesis suggests that proteostasis is needed for the correct progress of skeletal muscle myogenesis. Protein repair systems constitute also an important part of protein homeostasis. Between them, methionine sulfoxide reductase enzymes, capable of reducing methionine oxidation on proteins, are involved in regulation of protein function (Kim et al., 2014a). In mammals, the Msr system is composed of a single MsrA and three MsrBs (MsrB1, B2 and B3) proteins with different intracellular locations (Kim, 2013). MsrA is thought to reduce the S form of MetO while MsrB proteins reduce the R form (Lee & Gladyshev, 2011). Importantly, Msr has also been involved in oxidative stress protection by preventing protein irreversible oxidation damages in different eukaryotic models, such as *S. cerevisiae* (Oien & Moskovitz, 2007, Sumner et al., 2005), *D. melanogaster* (Ruan et al., 2002), *C. elegans* (Minniti et al., 2009) or mice (Salmon et al., 2009) as well as in different mammalian cell types, including human lens cells (Li et al., 2013), T lymphocyte cells (Cabreiro et al., 2009), fibroblasts (Cabreiro et al., 2007) and skin cells (Pelle et al., 2012, Picot et al., 2007, Zhou et al., 2009).

We hypothesize that Msr enzymes have a key role in muscle regeneration by regulating protein function and / or by taking an essential place in protecting skeletal muscle satellite cells against oxidative stress conditions. For this study we used a cell culture approach. Human myoblasts derived from a 5 days old infant obtained through our collaboration with Dr. Gillian Butler-Brown from the “Institut de Myologie” (Renault et al., 2002), were grown and let to differentiate *in vitro*. The redox state of myoblasts at the different culture stages in relation with Msr expression and activity profiles were characterised. Then, the role of MsrA protein in myoblasts differentiation was investigated by the use of lentiviral-based myoblast transduction with shRNA sequences specific against MsrA mRNA. This work constitutes the first study on the Msr role in myogenesis and is presented here in a paper prepared for submission format. Supplementary results that further complete this study are presented afterwards.



***Down-regulation of methionine sulfoxide reductase A decreases human myoblasts  
proliferative capacities by inducing a senescent state***

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## **Abstract**

Methionine sulfoxide reductase (Msr) enzymes are important for the regulation of redox homeostasis in various cells, tissues and organs. While redox state is known to regulate myogenesis, the role of the Msr enzymes during myogenic differentiation still remains unknown. Using cultured human primary myoblasts, we show that Msr activity significantly decreases during myoblasts differentiation. Silencing of MsrA expression by the use of short hairpin RNA (shRNA) in myoblasts inhibited its expression up to 90%, without affecting other Msr gene expression, and resulted in a decrease of nearly 40% of the total Msr activity. Suppression of MsrA expression increased the levels of reactive oxygen species and protein irreversible damages in myoblasts, thereby reducing their proliferative capacities, and compromising their differentiation. In addition, in the absence of MsrA, myoblasts showed increased senescent-associated  $\beta$ -galactosidase activity, suggesting that the impaired proliferative and differentiation capacities are likely to occur because of the ROS-mediated senescence of myoblasts. These results demonstrate for the first time the role of MsrA as a critical regulator of skeletal muscle myogenesis.





## Introduction

Redox homeostasis is crucial to prevent oxidative stress in cells by maintaining reactive oxygen species (ROS) at physiological levels and to keep them as signalling molecules. ROS regulate specific cellular functions through the oxidation of specific protein residues such as cysteine (Cys) and methionine (Met). Such redox regulation is only possible due to the presence of proteins capable of reversing this kind of post-translational protein modifications, thus regulating protein function. Methionine sulfoxide reductase (Msr) proteins are the only known cellular enzymes able to reduce methionine oxidation (MetO) (Chondrogianni et al., 2014, Kim et al., 2014a). In mammals, this family of enzymes is composed of four members, one MsrA and three MsrB, which stereoselectively and respectively reduce the MetSO and the MetRO diastereoisomers produced by ROS (Ugarte et al., 2010). By doing so, both MsrA and MsrB enzymes have been implicated in the control of protein functions, including the inhibitor of kappa B-alpha ( $\text{I}\kappa\text{B}\alpha$ ) (Mohri et al., 2002), potassium channels (Chen et al., 2000, Santarelli, Wassef et al., 2006), the  $\text{Ca}^{2+}$ -binding protein calmodulin (CaM) (Lim et al., 2013), the calmodulin-dependent protein kinase II (CaMKII) (He et al., 2011) and actin (Hung et al., 2013, Jia et al., 2014, Lee et al., 2013).

Msr are also critical enzymes for the protection against an oxidative stress state, resulting from an aberrant ROS production and leading to irreversible oxidative damages to biomolecules if not scavenged by the cellular antioxidant systems. In this context, higher Msr enzymes expression increased survival and cellular resistance to an induced oxidative stress in different tissues and cell types such as, neurons (Yermolaieva, Xu et al., 2004), cardiac myocytes (Prentice, Moench et al., 2008), lens (Kantorow et al., 2004), T lymphocytes (Cabreiro et al., 2009), fibroblasts (Picot et al., 2005) and mouse embryonic stem cells (<http://www.ncbi.nlm.nih.gov/pubmed/20506347>). In contrast, Msr silencing in human melanocytes (Zhou et al., 2009) or human lens (Jia et al., 2012, Li et al., 2013) resulted in increased oxidative damages that could ultimately lead to cell death, and MsrA knockout mice presented increased oxidative stress markers and inflammation in kidneys after ischemic/reperfusion injuries (Kim et al., 2015). The importance of Msr enzymes is also supported by studies suggesting its role in protecting against neurodegenerative diseases (Wassef et al., 2007), in neuroinflammation (Fan, Wu et al., 2015), in atherosclerosis (Xu, Du et al., 2015) and in longevity of some model organisms including *C. elegans* (Minniti et al., 2009) and *D. melanogaster* (Ruan et al., 2002).

The study of muscle regeneration represents a major biomedical issue, mainly for treatment of diseases characterised by a progressive muscle weakness and degeneration, such as muscular dystrophies. Indeed, after damage or stress, muscle progenitor cells enter the cell cycle to proliferate and either undergo differentiation to form new fibres or self-renew to reconstitute the muscle stem cells pool (Buckingham & Montarras, 2008). Each step of this process is tightly regulated through the coordinated expression of specific transcription factors including the myogenic regulatory factors (MRFs) MyoD, Myf5, Myogenin, and MRF4 (Moncaut et al., 2013). Although signalling pathways involved in myoblasts function have been extensively studied (Buckingham & Rigby, 2014, Montarras et al., 2013), important aspects of its regulation, in particular the redox-dependent signalling still remains unclear.

If the antioxidant and regulation roles of MsrA enzymes have been intensively studied in different mammalian cells, here we give the first evidence for this role in skeletal muscle progenitors cells. We found that Msr activity is increased in proliferative myoblasts and significantly decreases during myoblasts differentiation, in parallel of increased oxidative damages to proteins. Furthermore, knockdown of MsrA expression in myoblasts altered their redox homeostasis and decreased their proliferative capacities through senescence induction.

## Material and Methods

### Cell culture

Human satellite cells were isolated from a biopsy of a 5-day-old infant *quadriceps* muscle as previously described (Edom et al., 1994) upon autopsy in accordance with the French legislation on ethical rules. The derived myoblasts were cultured at 37°C in a humid atmosphere containing 5% CO<sub>2</sub>. Growth medium consisted of Dulbecco's Modified Eagle Medium (DMEM with 4.5 mg/mL glucose, Gibco ThermoFisher Scientist) / Medium 199 (Gibco ThermoFisher Scientist) (4:1) supplemented with 20% (v/v) foetal bovine serum (Gibco ThermoFisher Scientist) and 50µg/mL of gentamicin (Gibco ThermoFisher Scientist). For differentiation studies, myoblasts were seeded at a density of  $9 \times 10^3$  cells/cm<sup>2</sup>. Cell differentiation was induced by placing confluent myoblasts in DMEM supplemented with 2% (v/v) foetal bovine serum and 50µg/mL of gentamicin. Differentiation was evaluated 2, 4 and 6 days after serum reduction by the expression of differentiation markers such as Myogenin, myosin heavy chain (MHC) and Troponin C (TnC) assessed by immunoblot experiments, qPCR or immunocytofluorescence assays.

OPMD-derived myoblasts cultures were obtained as described previously (Bigot et al., 2009, Mamchaoui et al., 2011) and cultured as described above with growth medium consisting of 1:4 vol 199 Medium / DMEM (4.5 mg/mL glucose, Gibco ThermoFisher Scientist) supplemented with 20% foetal calf serum (Invitrogen Life Technologies), 2.5 ng/ml HGF (Invitrogen Life Technologies), 0.1 µM Dexamethasone (Sigma-Aldrich, St. Louis, MO) and 50 µg/ml Gentamycin (Invitrogen Life Technologies). The myogenic purity of the populations was monitored by immunocytochemistry using desmin as a marker. Enrichment of myogenic cell was eventually performed using immunomagnetic cell sorting system MACS (Miltenyi Biotec, Paris, France) according to the manufacturer's instructions. Briefly, cells were labelled with anti-CD56 (a specific marker of myoblasts) microbeads, and then separated in a MACS column placed in a magnetic field.

### Generation of a stable MsrA deficient cell line

For generation of the stable cell line, human myoblasts were infected with short hairpin RNA (shRNA) lentiviral transduction particles containing a puromycin resistance gene for selection (MISSION<sup>TM</sup> TRC shRNA, Sigma-Aldrich). Five different sequences of shRNA for *MsrA* gene (Sigma-Aldrich product type SHCLNV-NM\_012331,

TRCN0000046454 (seq.1), TRCN0000046455 (seq.2), TRCN0000286336 (seq.3), TRCN0000293703 (seq.4), TRCN0000298176 (seq.5)) and one for control (Sigma-Aldrich product type SHC002V – shRNA non-target control) were used. Myoblasts were infected following manufacturer's instructions and obtained by puromycin selection. Briefly, 70 % confluent myoblasts plated in 6-wells plates were infected with  $2.5 \times 10^4$  TU/mL of shRNA lentiviral particles in a growth medium supplemented with 8  $\mu\text{g/mL}$  of hexadimethrine bromide. After 3 days, the transfection medium was replaced by fresh growth medium supplemented with 2  $\mu\text{g/mL}$  of puromycin for selection. Puromycin concentration was gradually decreased to a final concentration of 0.5  $\mu\text{g/mL}$ . Puromycin was removed before for further experiments.

### **RNA extraction, reverse transcription and real time qPCR**

Total RNA from cultured cells was extracted using a NucleoSpin® RNA kit (Macherey Nagel) following the manual's instructions. Reverse transcription was performed on 1  $\mu\text{g}$  of total RNA using a SuperScript® III First-Strand Synthesis System and random hexamers (Invitrogen Life Technologies) following the manufacturer's instructions and using random hexamers. Real time qPCR analysis was performed with a LightCycler® 480 Real-Time PCR System (Roche Diagnostics), using LightCycler® 480 SYBR Green I Master (Roche Diagnostics). Specific primers sequences used to amplify cDNA are listed in Table 1. The cycling program was set up as followed: 6 min at 95 °C and 50 cycles at 95°C for 15 sec, 60°C for 15 sec and 75°C for 15 sec. The amplified *Emerin* fragment was used as an internal control for RT-qPCR.

### **Western blot analysis**

Cells were lysed in a lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 % Triton-X100, 1 mM EDTA, 1 mM DTT) supplemented with protease inhibitor cocktail (Sigma-Aldrich), kept on ice for 30 min and centrifuged at 21,000 g for 25 min. The supernatant was recovered and protein concentration was determined by using Bio-Rad protein assay dye reagent (Bio-Rad). Forty  $\mu\text{g}$  of protein lysate were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4-15 % polyacrylamide gels (Criterion™ TGX™ precast gels, BioRad) and subsequently transferred onto a nitrocellulose membrane (GE Healthcare). Total protein profile on nitrocellulose membranes was stained with Ponceau red.

After blocking with Odyssey blocking buffer (Li-Cor Biosciences) or alternatively in PBS supplemented with 1 % BSA, membranes were incubated either 1 h at room temperature (R.T) or overnight at 4°C with the following primary antibodies: MsrA and MsrB2 (1:1000, Abcam), Troponin C, Ubiquitin and Emerin (1:1000, Santa Cruz Biotechnologies),  $\beta$ 1,  $\beta$ 2 and  $\beta$ 5 proteasome subunits (1:1000, Enzo Life Sciences), CML (1:1000, R&D Systems). For Oxyblots, 15  $\mu$ g of proteins were derivatized with 10 mM 2,4-dinitrophenylhydrazine (DNPH) diluted in HCl 2M for 15 min at R.T and further neutralized with a solution containing 2 M Trizma-Base and 30 % glycerol prior to SDS-PAGE and transfer. 2-4-dinitrophenylhydrazone (DNP) proteins adducts were revealed using anti-DNP antibodies (1:5000, Sigma-Aldrich).

Membranes were incubated 1 h at R.T with secondary antibodies conjugated with infrared dye (Li-Cor Biosciences) or with Horseradish Peroxidase. Membranes were scanned with an Odyssey infrared (Li-Cor Biosciences) or with LAS 3000 (Fujifilm Life Sciences) imaging systems. Quantification of Western blots was performed by densitometry using Image J software. Depending on the experiments, total protein profile or Emerin were used as loading control.

### **Immunocytofluorescence assays**

Cells cultured in 35mm dishes were washed in PBS, fixed with 4% formaldehyde in PBS for 10 min at RT and permeabilized with 0.5 % Triton X-100 in PBS for 15 min at RT. After 30 min blockage with 0.2 % gelatin in PBS, cells were incubated with primary antibodies, overnight at 4°C. Nuclei staining was done using 500 ng/mL DAPI (Invitrogen Life Technologies) during the secondary antibody incubation. Slides were mounted with the Fluorescence mounting medium (Dako).

Antibodies used were as follows: MyoD (1:100, Dako), Myogenin (1:100, Santa Cruz Biotechnology), MHC (1:100, MF20, Developmental Studies of Hybridome Bank), ki67 (1:200, Abcam) antibodies. For EdU staining, the Click-iT EdU Cell Proliferation Assay kit (Invitrogen) was used following the manufacturer's instructions; EdU was incubated at 10  $\mu$ M for 16 h in cell culture medium.

Secondary antibodies were coupled to a fluorochrome, either Alexa 488 or 546 (Invitrogen), and used at a 1:300 dilution. Images were acquired using a fluorescent Leica DMi8 microscope equipped with MetaMorph software (Leica Microsystems). Images were optimized globally for contrast and brightness and assembled using Image J software.

### **Senescent Associated $\beta$ -galactosidase activity**

For senescent associated  $\beta$ -galactosidase (SA- $\beta$ gal) staining,  $1 \times 10^5$  myoblasts cultured in 6 well-plates were rinsed twice with PBS, fixed with 0.2% glutaraldehyde / 2% formaldehyde for 5 min at R.T before being stained with 1 mg/mL of 5-Bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside (X-Gal, Sigma-Aldrich) in 2 mM  $MgCl_2$ , 150 mM NaCl, 5 mM  $K_3Fe(CN)_6$ , 5 mM  $K_4Fe(CN)_6$  and 40 mM  $Na_2HPO_4$  pH 6 for 16 h at 37°C. After staining, cells were rinsed in PBS, fixed for 10 min in 4 % formaldehyde, incubated 30 min 500 ng/mL of DAPI and mounted after washing in PBS. Images were acquired as described above. More than 400 cells were counted and the percentage of senescent cells was calculated by dividing the number of SA- $\beta$ gal positive cells by the total cells number.

### **Measurement of reactive oxygen species**

Intracellular ROS levels were measured using the 2,7-dichlorofluorescein diacetate (DCFDA, Sigma-Aldrich) fluorescent probe. Cells were loaded with 10  $\mu$ M DCFDA for 40 min and fluorescence was recorded with a Fluostar Optima plate reader (BMG Labtech) with wavelengths of 485 nm for excitation and 520 nm for emission. ROS levels were normalized by cellular viability, measured just after plate reading using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) following the manual's instructions.

### **Total methionine sulfoxide reductase activity**

Total Msr activity was assessed using N-acetyl-[3H]Met-R,S(O) (Perkin Elmer) as substrate. The enzymatic assay was performed as previously described (Brot et al., 1982). Briefly, 50  $\mu$ g of protein extracts was mixed with 25 mM Tris-HCl pH 7.4, 10 mM  $MgCl_2$ , 15 mM DTT and substrate (77.97 Ci/mmol). After 2h of incubation at 37°C, the reaction was stopped by adding 1 N HCl and the reduced substrate was dissociated from the oxidised substrate by extraction in 1.2 mL of ethyl acetate. 4 mL of scintillation liquid was added to the radioactivity ethyl acetate phase and CPM (counts per minute) values were calculated in a Tri-Carb Liquid Scintillation Counter 2100 TR (Packard Instrument Company, Inc.).

### **20S Proteasome subunits catalytic activities**

Peptidase activities of the proteasome were evaluated in protein lysates (without the anti-proteases cocktail) using appropriate fluorogenic peptide substrates. Caspase like activity (C-L) was monitored by the hydrolysis of Z-LLE-AMC peptide (Enzo Life Sciences), trypsin-like activity (T-L) by the hydrolysis of the Bz-VGA-AMC peptide (Enzo

Life Sciences) and chymotrypsin-like activity (CT-L) by the hydrolysis of the Suc-LLVY-AMC peptide (Enzo Life Sciences). The assay buffer was composed of 20 mM Tris-HCl pH 7.5, 10% (v/v) glycerol and 20  $\mu$ M of the appropriated peptide substrate dissolved in DMSO. Reactions were performed in the presence (100  $\mu$ M) and absence of the specific proteasome inhibitor MG132 (Enzo Life Sciences), to test the specificity of the measured activity. Enzymatic kinetics was carried out in 96 wells plates for 30 min at 37°C using 10  $\mu$ g of cytosolic protein fractions in a temperature-controlled microplate reader (Fluostar Galaxy, BMG Labtech) with wavelengths of 340 nm for excitation and 460 nm for emission. Proteasome activities were determined as the difference between total activity and the remaining activity of the crude extract in the presence of MG132.

### **Statistical analysis**

Results were expressed as the means  $\pm$  standard error of the mean (SEM) of at least three independent experiments. The difference between two mean values was analysed by two-tailed Student's t-test and considered statistically significant when  $p < 0.05$ .



## Results

### **Msr activity is higher in proliferative myoblasts than in differentiated myotubes**

To investigate the profile of Msr activity and expression during myogenic differentiation, human myoblasts were cultured and analysed in proliferation and 2, 4 and 6 days after induction of differentiation (Fig. 1A-E). While proliferative myoblasts, marked by EdU incorporation and MyoD expression (Fig. 1B) exhibited high Msr activity (Fig. 1C), early and late differentiation, evidenced by Myogenin and myosin heavy chain (MHC) expression (Fig. 1B) are accompanied by a significant decrease of this activity (Fig. 1C).

To identify which member of the Msr family may be responsible for this variation of activity, we investigated their gene expression profile during the time course of differentiation. We detected expression of the four members of Msr family (*MsrA*, *MsrB1*, *B2* and *B3*) in human myogenic cells, both in proliferation, marked by *MyoD* expression, and during differentiation, revealed by *Myogenin* expression (Fig. 1D). As we did not detect significant changes of expression for any of the four transcripts between proliferative and differentiated states (Fig. 1D), we then quantified their protein expression by western-blot analysis (Fig. 1E). Similarly to what we observed for transcripts, neither MsrA nor MsrB2 protein levels were altered during differentiation. These results therefore demonstrate that the level of Msr proteins does not account for the decrease of Msr activity during differentiation, and suggest that post-translational modifications may occur.

### **Proliferative myoblasts reveal less protein oxidative damages than myotubes**

As critical actors of redox regulation, members of the Msr family are involved in both modulation of reversible methionine oxidations (Achilli, Ciana et al., 2015, Chondrogianni et al., 2014, Kim et al., 2014a) and cell protection against ROS and oxidative damages to proteins (Cabreiro et al., 2009, Picot et al., 2005, Prentice et al., 2008). To correlate Msr activity with the oxidative status of myoblasts, ROS generation and protein oxidative damages were evaluated in proliferative and differentiated cells. Intracellular ROS were found to increase after 2 days of myoblasts differentiation and remained significantly higher during the late differentiation stages (Fig. 2A). This increase of ROS was associated with an accumulation of protein carbonyls (Fig. 2B) and advanced glycated end products such as carboxymethyllysine (CML) modified proteins (Fig. 2C) at the late stages of differentiation. To get more insight on protein homeostasis during myoblasts differentiation, we took a look on a major protein degradation system, the ubiquitin-proteasome system. We

found that protein ubiquitin labelling was more pronounced in differentiated myotubes compared to proliferative myoblasts (Fig. 2D). Proteins whose oxidation is irreversible, such as carbonyls are degraded by the 20S proteasome. Here we measured the 3 activities and analysed the expression of the catalytic subunits responsible for each of these activities (Fig. S1). While the expression of  $\beta 1$ ,  $\beta 2$  and  $\beta 5$  were not found to vary significantly during myoblasts differentiation (Fig. S1A), the proteasome trypsin-like activity was increased along differentiation (Fig. S1B). This up regulation of the 20S proteasome proteolytic activities is in agreement to what have been found by others during differentiation of C2C12 mouse myoblasts (Cui et al., 2014). These observations suggest that the accumulation of oxidised proteins in differentiated myotubes was not due to a defect of protein degradation systems but more likely by a change in redox homeostasis.

### **Down-regulation of MsrA decreases human myoblasts proliferative capacities *in vitro***

To further study the role of MsrA on myoblast protein redox homeostasis, we inhibited *MsrA* expression in human myoblasts using shRNA sequences within lentiviral particles. Five different sequences were tested for their ability to decrease *MsrA* mRNA expression and we found that, except for the sequence 4, all the other 4 sequences led to a significantly decrease of *MsrA* mRNA in transduced myoblasts compared to those transduced with the shRNA control (shCtrl) sequence (% *MsrA* inhibition comparing to control sequence determined by qPCR: 97,9 % for seq.1; 80,2 % for seq.2; 66,9 % for seq.3 and 93,6 % for seq.5) (Fig. S2). Seq.1 shMsrA displayed the higher inhibition efficacy but expression of the other *Msr* members was significantly up regulated in these myoblasts due to possible compensatory mechanisms (Fig. S2). Transduction with seq. 5 of shMsrA resulted to almost 94 % less expression of *MsrA* transcripts and no side effects in the expression of *MsrB1*, *MsrB2* or *MsrB3* (Fig. 3A). For this reason, we chose to continue our study with this shMsrA sequence. We analysed Msr protein expression and activity in shMsrA transduced myoblast comparing to myoblasts transduced with the shCtrl sequence. MsrA protein level was also 92 % and 88 % decreased compared to proliferative and differentiated control myoblasts, respectively, while the MsrB2 protein levels did not change from those of the shCtrl myoblasts as determined by western blot experiments (Fig. 3B). Interestingly, the knockdown of the MsrA protein results in 40 % decrease in Msr activity in proliferative cells and 50 % in differentiated myotubes (Fig. 3C) indicating that the reduction of the S diastereoisomer of the MetO is completely abolished in these cells and that the remaining 50 % of activity is due to the MsrB enzymes.

To evaluate the role of MsrA on myoblasts proliferation, shMsrA and shCtrl cells were assessed for 5-ethynyl 2' deoxyuridine (EdU) incorporation and for labelling with anti-Ki67 antibodies. While 47 % of MyoD control cells were revealed by Ki67 antibody and 51 % were able to incorporate EdU, only 38 % of MyoD shMsrA cells were positive for Ki67 and 30 % for EdU labelling, suggesting that the absence of MsrA inhibits cell proliferation and DNA synthesis (Fig 4A). The myogenicity of the shMsrA cells is maintained, as shown by the MyoD labelling positive myoblasts (Fig. 4A). To understand why mutated myoblasts had reduced proliferative capacities, we evaluated senescence by assessing the senescence-associated- $\beta$ -galactosidase (SA- $\beta$ -galactosidase) activity, a well-known senescence biomarker. As shown in Fig. 4B, inactivation of MsrA drove myoblasts into a senescent state as 31 % of the cells expressed this senescent biomarker while only 14 % of the control cells were positive. In contrast, no increase of cell death was evidenced when testing caspase 3 activation (data not shown). It is known that excessive ROS production can promote cellular senescence (Childs, Durik et al., 2015, Munoz-Espin & Serrano, 2014). As shown in Fig. 4E, inhibition of MsrA in myoblasts led to higher ROS levels and protein carbonyls adducts, suggesting that MsrA activity is important for maintaining low ROS levels in proliferative myoblasts in order to prevent senescence and to protect myoblast proteins against irreversible oxidative damages.

### **Myoblast differentiation is impaired in the absence of MsrA**

Since increased ROS levels we also found in shMsrA cells at 2 and 4 days after differentiation comparing to shCtrl cells (Fig. 5A), we investigated if this would impact myoblast differentiation. We observed an alteration in the differentiation program in cells lacking MsrA as evidenced by a reduced number of Myogenin and MHC expressing cells compared to control cells (Fig. 5B). The alteration in the expression of early and late differentiation markers was quantified by qPCR (Fig. 5C) revealing 83 % decreasing expression for *Myogenin* and 53 % for *MHC* at 2 and 4 days after differentiation, respectively. Western blot experiments using anti-troponin C (TnC) antibodies also evidenced a 35 % decrease in TnC expression of shMsrA cells comparing to shCtrl at 4 days of differentiation (Fig. 5D).

### **OPMD-myoblasts with reduced proliferative capacities exhibit decreased *Msr* expression**

In the late onset dystrophy, Oculopharyngeal Muscular Dystrophy (OPMD), the *cricopharyngeus* muscle is one of the most pathologically affected muscles, characterised by high levels of atrophy and fibrosis (Gidaro et al., 2013). To investigate whether MsrA could play a role in the premature proliferative arrest previously observed in OPMD-derived myoblasts (Perie et al., 2006), we analysed *MsrA* gene expression in myoblasts derived from OPMD patients or from healthy donors of the same age range. We found that myoblasts derived from the *cricopharyngeus* muscle of OPMD patients presented less *MsrA* expression comparing to cricopharyngeal myoblasts derived from healthy individuals (Fig.6), indicating that MsrA deficiency may contribute to their defective proliferation.

## Discussion

Redox regulation of stem cells function is gaining an increasing interest in the domain of regenerative medicine (Bigarella, Liang et al., 2014). Regarding skeletal muscle regeneration, the myoblasts redox state has been reported to participate in myogenesis but the mechanisms involved are still largely unexplored (Kozakowska, Pietraszek-Gremplewicz et al., 2015). Moreover, although recent studies have reported the reversible redox modifications of cysteine and methionine within proteins as a key mechanism that affects essentially all redox signalling pathways, in a rapid, compartmentalised, reversible and highly specific manner (Gellert, Hanschmann et al., 2015, Kim et al., 2014a), the role of the enzymes responsible for this redox switches of proteins have not yet been studied in the context of myogenesis.

Here we studied for the first time the role of Msr enzymes, which reverse the oxidation of methionine into proteins, in skeletal muscle myogenesis, especially its implications on proliferation and differentiation capacities of human myoblasts.

We found that Msr activity was maximal in proliferative human myoblasts and it progressively decreased during myoblasts differentiation *in vitro*. However, the decrease in activity was not explained by a modulation of the expression of MsrA or MsrB members, suggesting that possible post-translational modifications on Msr enzymes, alteration of its catalytic site or defective recycling process may occur during myoblasts differentiation. It is well known that cycles of methionine oxidation and its reduction by Msr enzymes function a ROS scavenger mechanism to avoid ROS-dependent oxidation on other amino acids (Cabreiro et al., 2008, Luo & Levine, 2009), which would have greater consequences for protein homeostasis since it leads their degradation. During myoblast differentiation *in vitro*, we observed an increase in ROS levels. If in the presence of high Msr activity, the redox switch on methionine residues in proteins will regulate protein function, when Msr activity is low, as we observed in differentiated myotubes, oxidised methionines cannot be reversed and are retained into proteins, thus impacting their function. Furthermore, increased ROS levels together with low Msr activity could also result in irreversible oxidative damages to proteins triggering them to proteasomal degradation or resulting in their accumulation. Indeed, it was found that the sustained methionine oxidation of calmodulin triggers it to degradation by the 20S proteasome (Balog, Lockamy et al., 2009). We found different markers of irreversible protein oxidation including protein carbonylation and advanced

glycation end products in myotubes, indicating that failure of Msr activity affects the redox-related protein homeostasis. Accordingly, the 20S proteasome, in charge of the elimination of oxidised proteins, was found more activated in myotubes comparing to myoblasts. Likewise, 20S- and immune-proteasome subunits were shown upregulated during *in vitro* differentiation of mouse C2C12 myoblasts leading to an increase of their activities, just after the increase in protein carbonyls groups (Cui et al., 2014). Thus, Msr enzymes, by regulating protein oxidation, seems to be important to control protein homeostasis during myoblasts differentiation, particularly to their proliferative state, where its activity is higher.

Msr enzymes are major protein repair enzymes involved in protein quality control, regulation of protein function and in cellular protection against oxidative stresses (Chondrogianni et al., 2014, Kim, 2013). Between them, MsrA was shown to be a major regulator of antioxidant defence in mammals (Luo & Levine, 2009). Methionine oxidation in proteins associated with MsrA deficiency has also been implicated in several diseases (Oien & Moskovitz, 2008), suggesting that high levels MsrA activity could directly improve cell therapies. As an example, prolonged oxidation of CaMKII the methionines leading to autonomous activation of the kinase has been suggested to participate to pathological signalling in heart failure, arrhythmias and vascular injury (Anderson, 2015, Erickson et al., 2008). Here we found that MsrA knockdown reduced human myoblasts proliferative capacities *in vitro*. In agreement, MsrA down-regulation has been shown to inhibit fibroblast cell proliferation (Choi & Kim, 2011). Moreover, we found that MsrA deficiency in human myoblasts led to increased ROS levels and protein carbonyl content, indicating an increase myoblasts oxidative stress, that resulted in cellular senescence. Consistent with this, Garcia-Prat et al. have found that due to autophagy inactivation, increased ROS levels in satellite cells lead to their senescence after activation (Garcia-Prat et al., 2016). In addition, several years ago, we showed that protein oxidation is increased during fibroblasts senescence associated with decreased MsrA expression and activity (Ahmed, Rogowska-Wrzesinska et al., 2010, Picot et al., 2004).

The low Msr activity due to MsrA knockdown resulted also in increased ROS levels in myotubes and impaired differentiation, evidenced by decreased *Myogenin* gene and protein expression. This resulted in a smaller number of differentiated cells with decreased *MHC* expression in later stages of differentiation. One possible explanation for this impaired differentiation is the oxidative stress-associated senescence created by the absence of MsrA protein in myoblasts, which impeded the induction of their differentiation. Indeed, it has been shown that senescence is detrimental to muscle regeneration not only by reducing the

number of progenitors, but also by inducing modifications in their differentiation capacities accompanied by a down-regulation of muscle specific proteins (Bigot et al., 2008).

Finally, given the importance of MsrA to myogenesis, one can think that the reduced expression of MsrA that we found in OPMD-derived myoblasts could participate to their observed defective proliferation (Perie et al., 2006).

In summary, this work provides evidence that Msr activity is higher in proliferative myoblasts and is reduced during muscle differentiation. Also, MsrA knockdown in myoblasts increased intracellular ROS levels and protein irreversible oxidations thereby resulting in a senescence state that impaired their differentiation. Taken together, these results suggest that MsrA is an important antioxidant enzyme for the control of ROS content as well as protein homeostasis in myoblasts. This makes of it a potential target for the treatment of muscle disorders characterised by impaired regenerative capacities.

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**Fig. 1 – Changes of intracellular Msr activity during differentiation of human myoblasts.**

**A-E)** Human myoblasts were cultured and analysed in proliferation and 2, 4 and 6 days after induction of differentiation. **B)** Cells were incubated with EdU and processed for DAPI staining, detection of EdU incorporation and MyoD, Myogenin and myosin heavy chain (MHC) expression by immunofluorescence. Representative images are shown. **C)** Msr activity during myoblasts differentiation was measured using radioactive labelling of substrate MetSO. **D)** At each stage of culture, cells were used for RNA purification. RNA samples were analysed by qPCR for myogenic differentiation markers and *Msr* members. Values are shown relative to the level of *Emerin* transcripts. **E)** Proteins were extracted at each stage of culture. Western-blot analysis indicated the level of MsrA, MsrB2 and TnC proteins. Graphs represent the mean  $\pm$  SEM values from at least 3 independent experiments. \*  $P < 0.05$  and \*\*  $P < 0.01$  in a paired student T test.

**Fig. 2 – Human myogenic differentiation is accompanied by increase of protein oxidative modifications.**

**A)** Intracellular reactive oxygen species (ROS) were detected using the DCFDA fluorescent sensor during human myoblasts differentiation. **B-D)** Proteins were extracted at each stage of culture. Western-blot analysis indicated the level of **B)** Protein carbonyl groups, **C)** carboxyl methyl lysine (CML) and **D)** Protein ubiquitin groups. Graphs represent the mean  $\pm$  SEM values from at least 3 independent experiments. \*  $P < 0.05$  and \*\*  $P < 0.01$  in a paired student T test.

**Fig. 3 – MsrA knock-down leads to significant decrease of Msr activity.**

**A-C)** shCtrl and shMsrA cells were analysed in proliferation and 6 days after induction of differentiation. **A)** RNA samples were analysed by qPCR for *MsrA*, *MsrB1*, *MsrB2* and *MsrB3* transcripts. Values are shown relative to the level of *Emerin* transcripts. **B-C)** Proteins were extracted, and **B)** Western-blot analysis indicated the level of MsrA and MsrB2 proteins in both shCtrl or shMsrA cells. **C)** Msr activity was quantified and presented as a percentage (%) of the total Msr activity of proliferative shCtrl cells. Graphs represent the mean  $\pm$  SEM values from 3 independent experiments. \*  $P < 0.05$  and \*\*  $P < 0.01$  in a unpaired student T test between shCtrl and shMsrA.

**Fig. 4 – Loss of MsrA leads to defective myoblast proliferation and to senescence.**

**A-D)** shCtrl and shMsrA cells were analysed in proliferation. **A)** shCtrl and shMsrA cells were incubated with EdU and processed for DAPI staining, detection of EdU incorporation and MyoD and Ki67 expression by immunofluorescence. Representative images are shown. Cells positive for each marker were counted and quantified as the percentage (%) of total cells. **B)** shCtrl and shMsrA cells in proliferation were assayed for senescence-associated- $\beta$ -galactosidase (SA- $\beta$ -gal) staining and processed for DAPI staining. Positive cells were counted and quantified as the percentage (%) of total cells. **C)** Intracellular reactive oxygen species (ROS) were detected using the DCFDA fluorescent probe on shCtrl and shMsrA proliferative cells. **D)** Protein carbonyl groups levels on shCtrl and shMsrA myoblasts were detected by oxyblot. All graphs represent the mean  $\pm$  SEM values from 3 independent experiments. \*  $P < 0.05$  and \*\*  $P < 0.01$  in a unpaired student T test.

**Fig. 5 – Effects of MsrA knock-down for myoblasts differentiation.**

**A-D)** shCtrl and shMsrA cells were analysed at 2 and 4 days after induction of differentiation. **A)** Intracellular reactive oxygen species (ROS) were detected using the DCFDA fluorescent sensor on shCtrl and shMsrA myotubes. **B)** Cells were processed for DAPI staining and detection of Myogenin and myosin heavy chain (MHC) expression by immunofluorescence. Representative images are shown. **C)** RNA samples were analysed by qPCR for *Myogenin* and *MHC-IIa* transcripts. Values are shown relative to the level of *Emerin* transcripts. **D)** Proteins were extracted and western-blot analysis indicated the level of Troponin C (TnC) expression. Graphs represent the mean  $\pm$  SEM values from at least 3 independent experiments. \*  $P < 0.05$  and \*\*  $P < 0.01$  in a unpaired student T test.

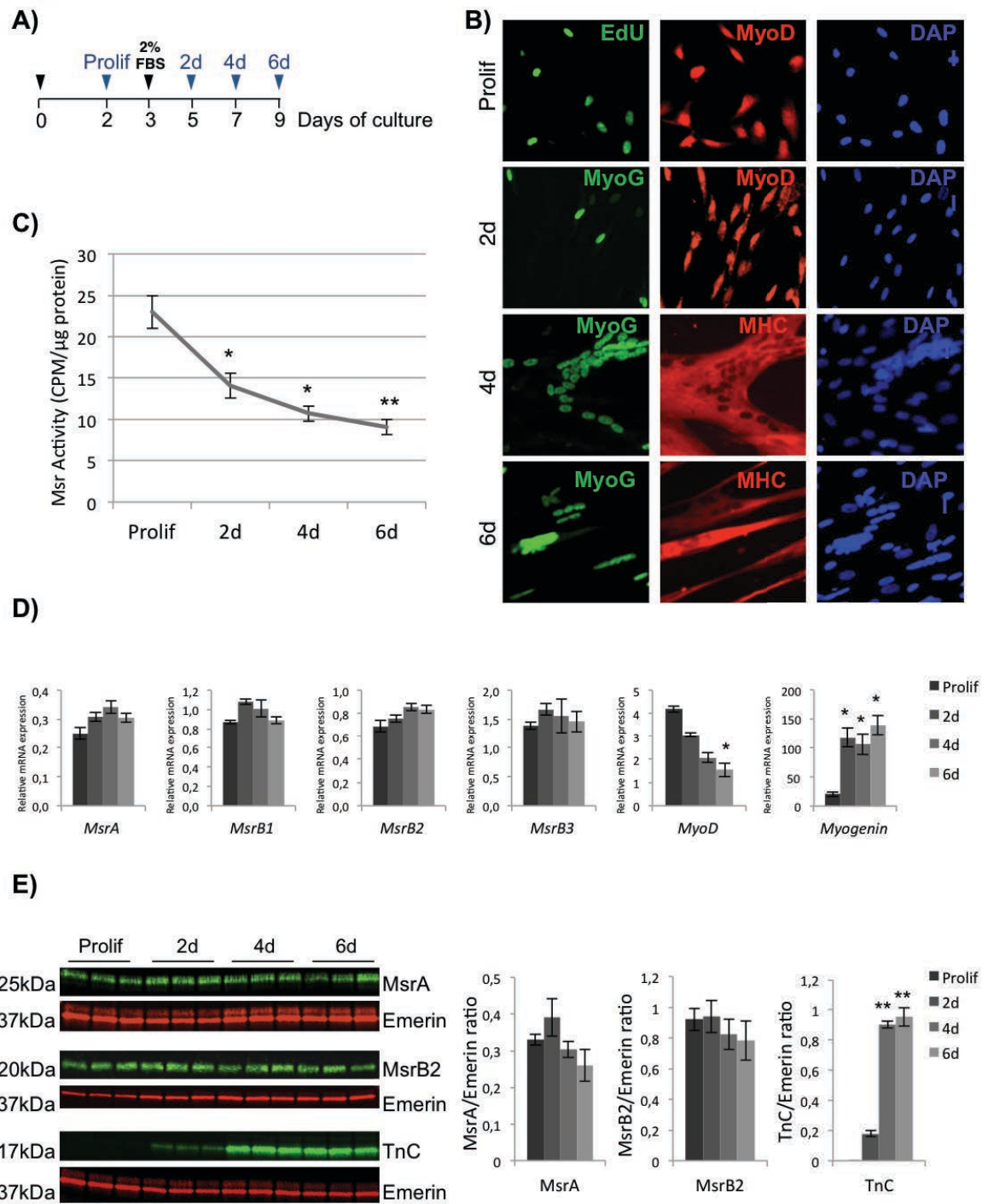
**Fig. 6 –MsrA expression is reduced in OPMD-derived myoblasts.**

Human myoblasts derived from the *cricopharyngeus* muscle of patients with oculopharyngeal muscular dystrophy (OPMD) and healthy donors of the same age range (Control) were culture and collected at proliferative state. RNA samples were analysed by qPCR for *MsrA* transcripts. Values are shown relative to the level of *Emerin* transcripts. Graph represents the mean  $\pm$  SEM values from 6 independent experiments. \*  $P < 0.05$  in a unpaired student T test.

Table 1 - List of primers used in the study.

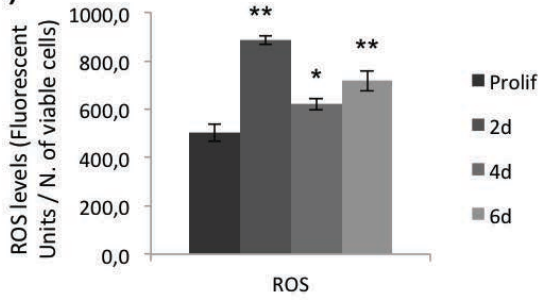
<b>Gene</b>	<b>Forward primer</b>	<b>Reverse primer</b>
<b>Emerin</b>	5' AAGAGGAGTGCAAGGATAGGG 3'	5' GGAGGAAGTAGGATAATAGGACAGG 3'
<b>MHC-IIa</b>	5' GAAAGTCTGAAAGGGAACGCA 3'	5' CGCCACAAAGACAGATGTTTTG 3'
<b>MsrA</b>	5' GGGCAACTCGGCCTCGAACA 3'	5' CCAGAAACATCCCATTCCAAA 3'
<b>MsrB1</b>	5' GTGTGCCAAGTGTGGCTATGA 3'	5' CTTGCCACAGGACACCTCAA 3'
<b>MsrB2</b>	5' CGGAGCAGTTCTACGTCACAA 3'	5' CAGAGCCAGACGTACCATGA 3'
<b>MsrB3</b>	5' CTCAGGAGAAAGGGACCGAAA 3'	5' GCCAACCTGAACCGGAGTCAA 3'
<b>MyoD</b>	5' AACTGCTCCGACGGCATG 3'	5' ACAGGCAGTCTAGGCTCGACA 3'
<b>Myogenin</b>	5' GAGTTCAGCGCCAACCCAG 3'	5' TGCCCGGCTTGGAAGACAAT 3'

Fig.1

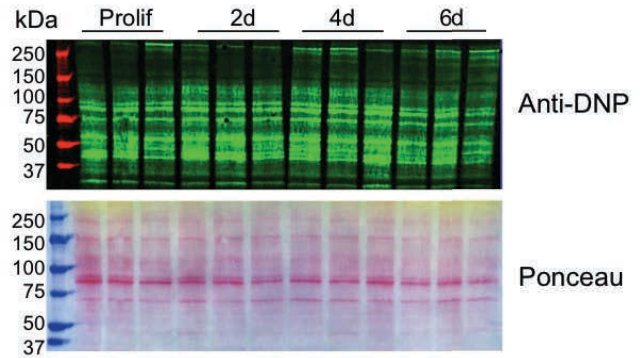
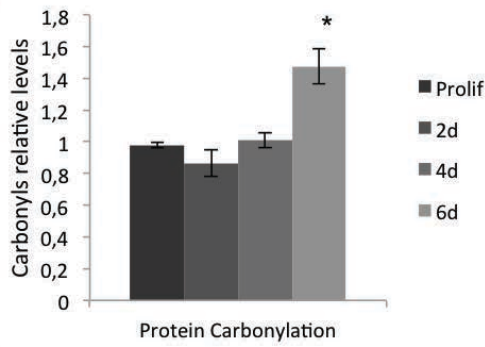


**Fig.2**

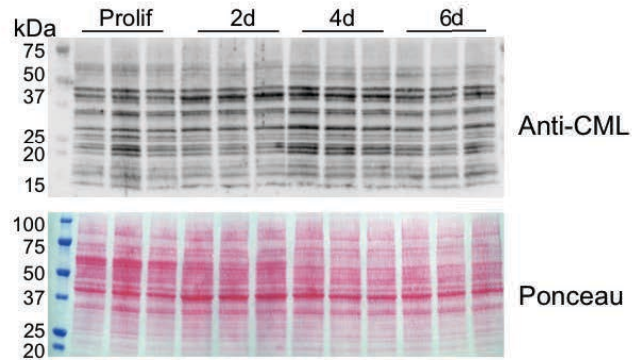
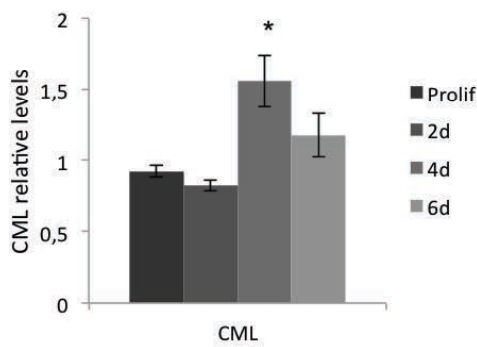
**A)**



**B)**



**C)**



**D)**

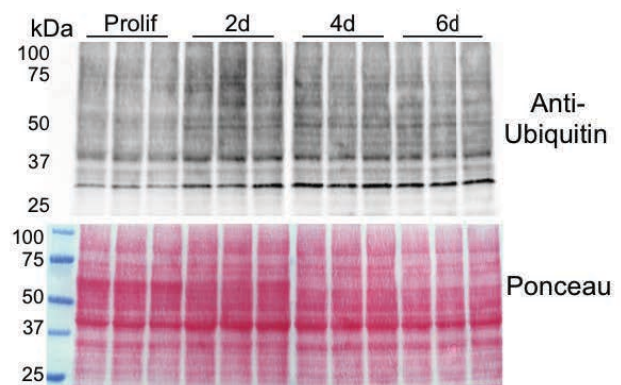
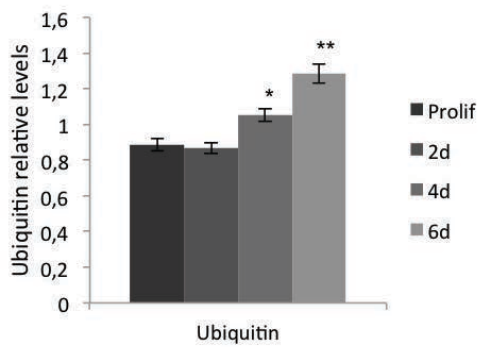
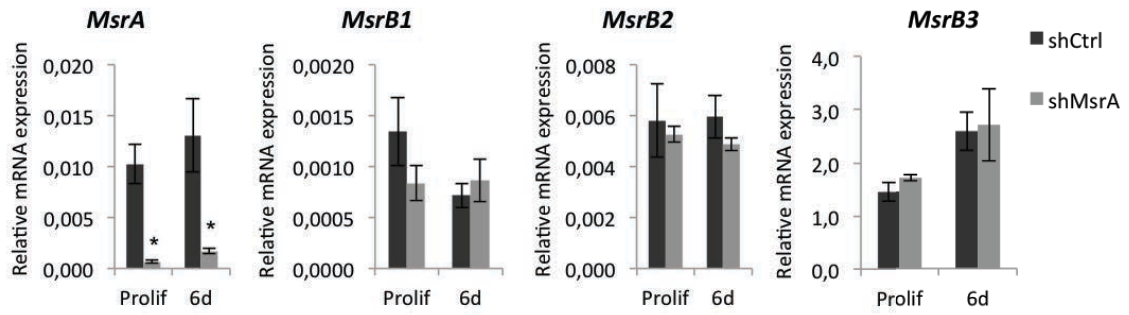


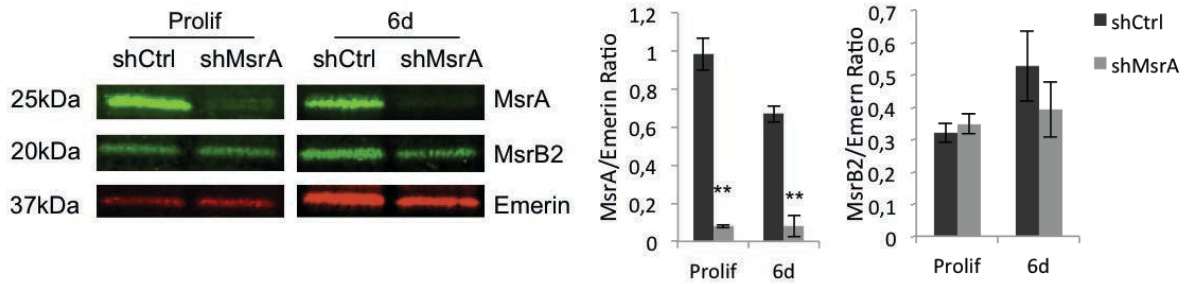


Fig.3

A)



B)



C)

Msr Activity	Prolif	6d
shCtrl	100 %	68,1 ± 7,4 %
shMsrA	60,9 ± 12,3 %	34,3 ± 6,5 %

Fig.4

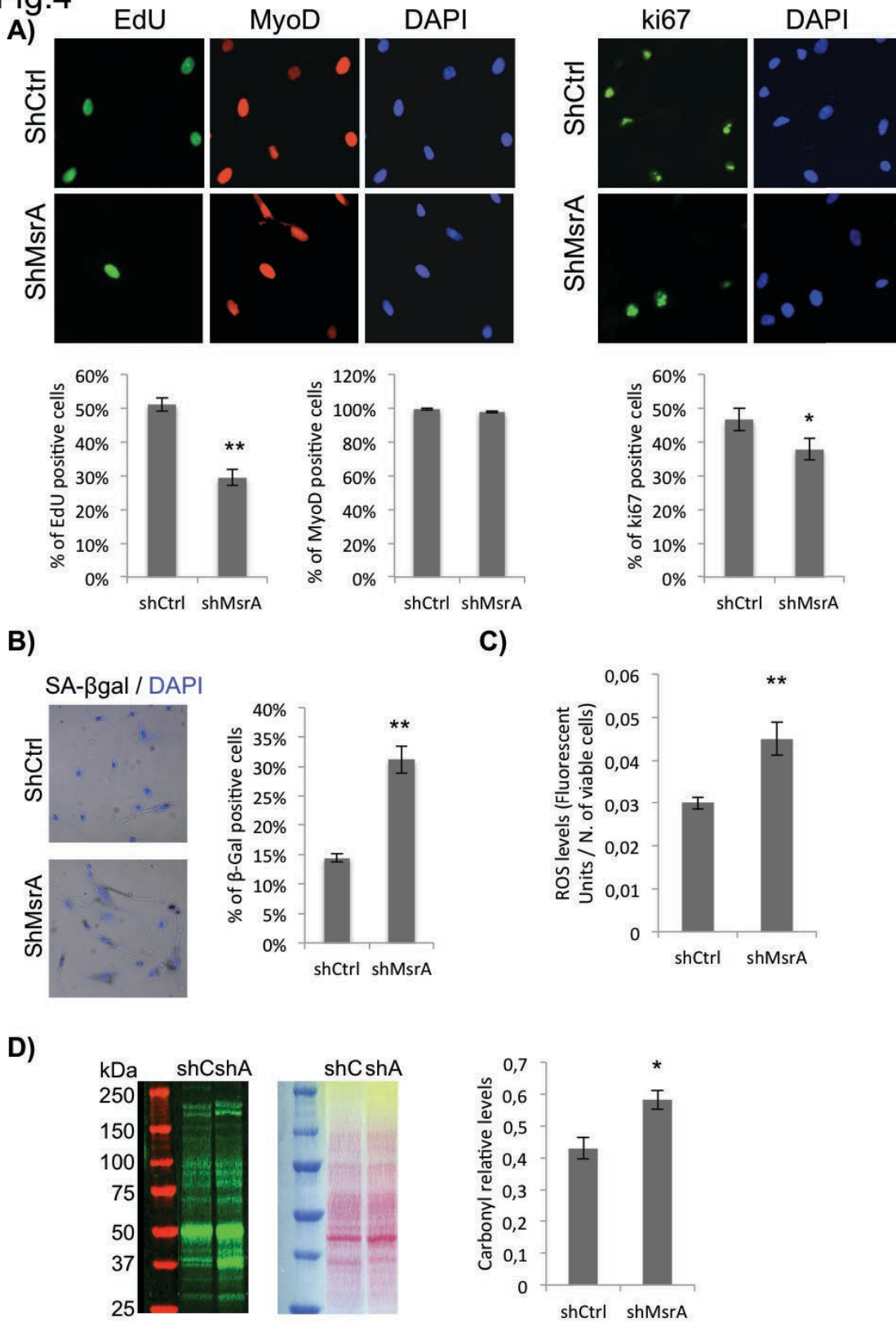
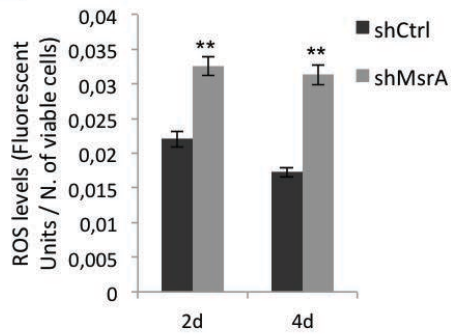
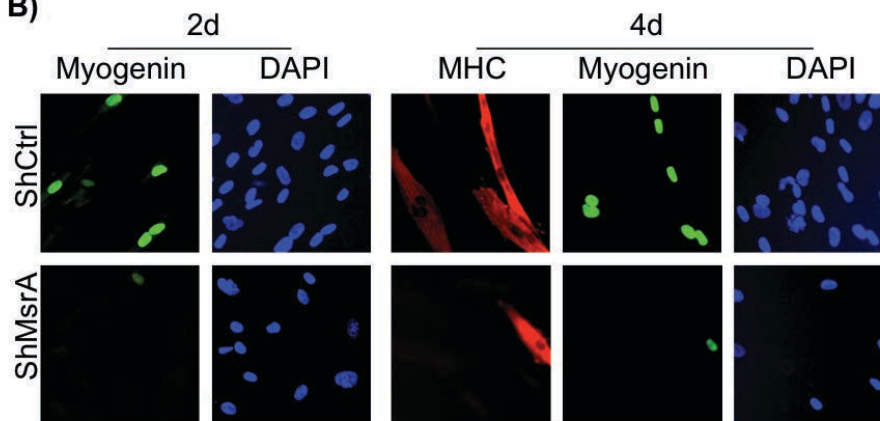


Fig.5

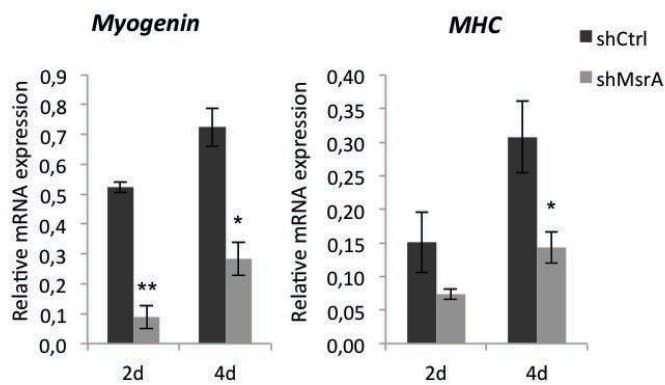
A)



B)



C)



D)

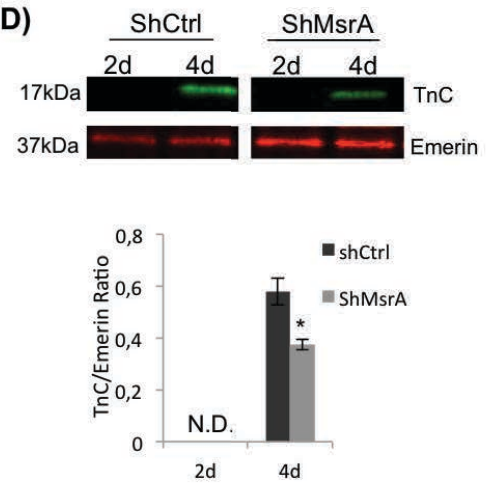
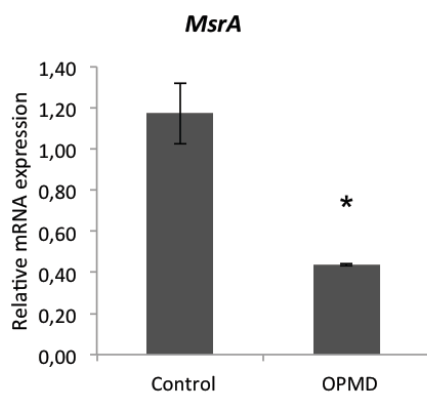


Fig.6



## Supplementary data

### **Fig. S1 – Variation of proteasome expression and activity during human myogenic differentiation.**

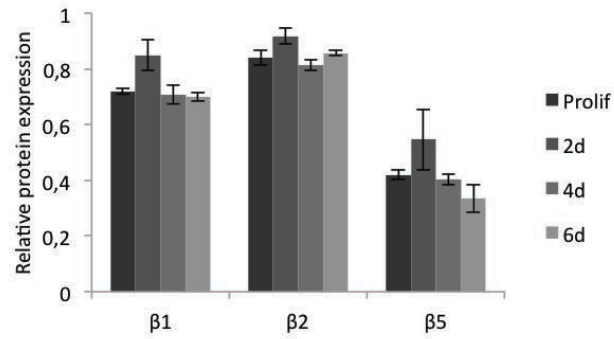
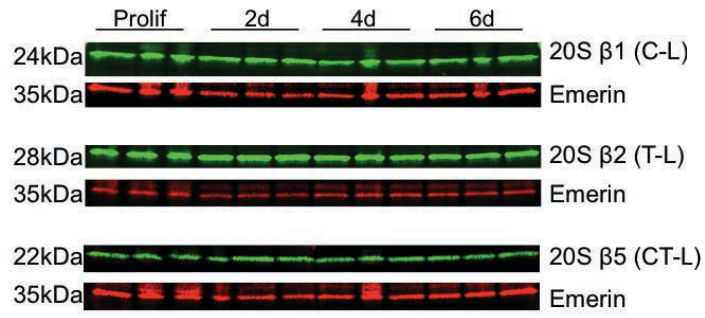
**A-B)** Proteins were extracted in proliferation and 2, 4 and 6 days after induction of differentiation. **A)** Western-blot analysis indicated the level of 20S proteasome catalytic subunits expression,  $\beta$ 1 corresponding to caspases-like activity (C-L),  $\beta$ 2 corresponding to trypsin-like activity (T-L) and  $\beta$ 5 corresponding to chymotrypsin-like activity (CT-L). **B)** Proteasome activities were assayed using selective fluorescent substrates for each catalytic subunit activity. Graphs represent the mean  $\pm$  SEM values from at least 3 independent experiments. \*  $P < 0.05$  and \*\*  $P < 0.01$  in a paired student T test (Prolif vs. 2d, 4d or 6d).

### **Fig. S2 – Msr transcripts expression between the different shMsrA myoblasts.**

At proliferative state, cells derived from myoblasts transfection with shCtrl sequence and from five different shMsrA sequences were used for RNA purification. RNA samples were analysed by qPCR for *MsrA*, *MsrB1*, *MsrB2* and *MsrB3* transcripts and normalized by the level of *Emerin* transcripts. Values are shown relative to the shCtrl cells transcripts expression.

Fig. S1

A)



B)

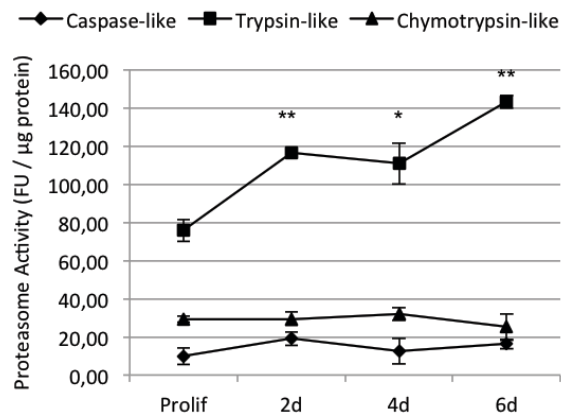
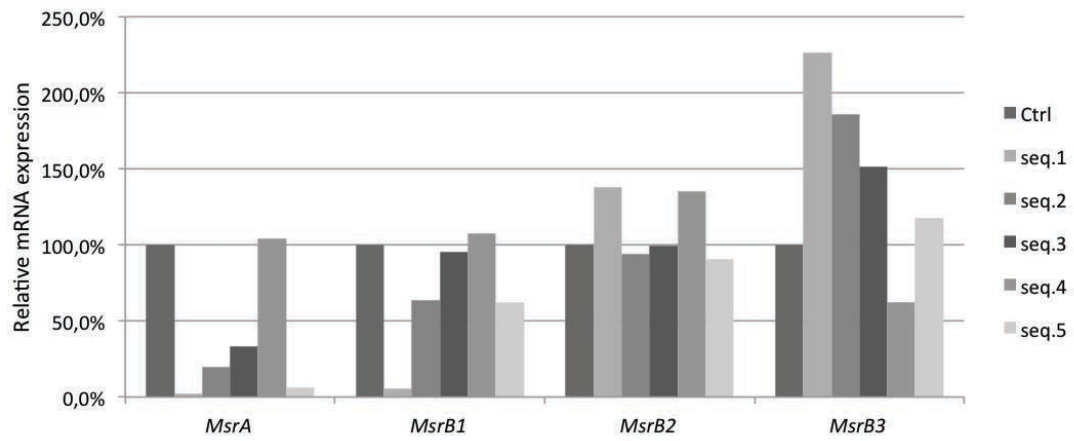


Fig. S2



## ***Complementary results***

### **Most antioxidant enzymes transcripts are increased during myoblast differentiation**

To further characterise the redox state during human myoblast differentiation, we analysed the gene expression of different antioxidant enzymes, known to directly react with ROS: catalase (CAT), superoxide dismutase (SOD), peroxiredoxin (Prx) and glutathione peroxidase (GPx), in proliferative myoblasts and in differentiated myotubes. We observed an increase in the antioxidant enzymes transcripts Prx2 and Prx3, as previously described in mouse myoblasts (Lee et al., 2014b, Won et al., 2012) and, except from the CAT, SOD1 and GPx3 enzymes, whose gene expression is greater in proliferative myoblasts, in SOD2, Prx5 Prx6, Gpx2, and Gpx4 at 4 and 6 days after induction of differentiation (Figure 25A). Some of these enzymes are regulated by the antioxidant transcription factor nuclear factor Nrf2 (erythroid 2-like factor 2) (see (Zhang, Davies et al., 2015) for review). As shown in Figure 25B, the increase expression of these enzymes in differentiated myotubes could result from a greater expression of this *Nrf2* transcription factor in later differentiation stages. Interestingly, the transcripts of *Trx* enzymes, which are responsible for the recycling of Msr and Prx enzymes and redox regulation of cellular signalling and stress, do not significantly vary during myoblasts differentiation (Figure 25C), as previous observed (Dimauro et al., 2012).



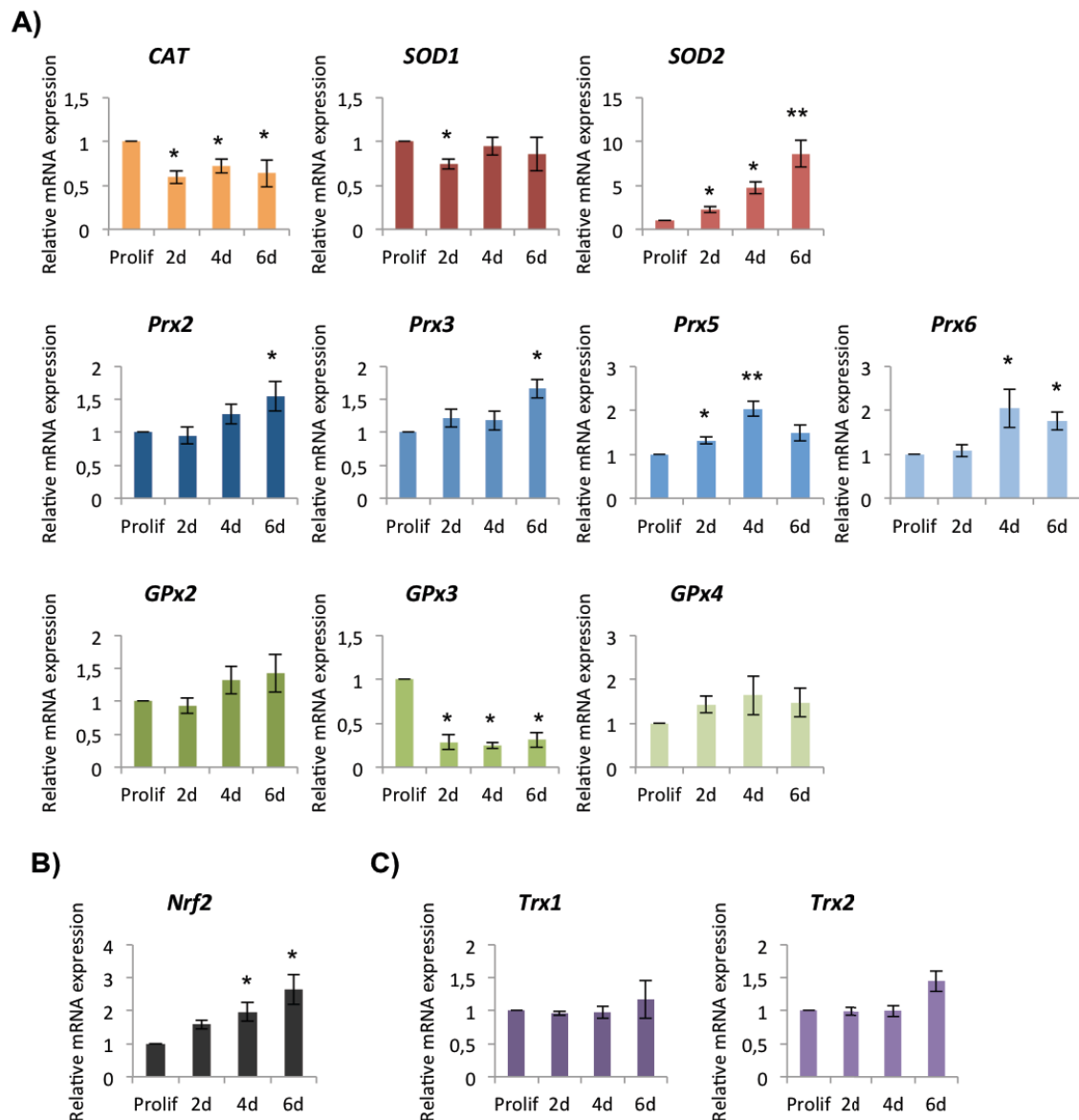


Figure 25 – **Antioxidant enzymes expression during human myoblasts differentiation *in vitro*.** Total mRNA was purified at each stage of culture and analysed by qPCR for the expression of (A) antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD1 and 2), peroxiredoxins (Prx2, 3, 5 and 6) and glutathione peroxidases (Gpx2, 3 and 4); for (B) the antioxidant transcription factor Nrf2 and for the (C) thioredoxin (Trx1 and 2). Transcripts expression was normalized to the level of *Emerin* mRNA. Graphs represent the mean  $\pm$  SEM values from at least 3 independent experiments. \*  $P < 0.05$  and \*\*  $P < 0.01$  in a paired student T test.

## p16<sup>INK4a</sup>-depend senescence in MsrA-deficient myoblasts

MsrA deficiency leads to proliferation arrest due mainly by induction of senescence as shown by the senescent morphology of the cell (Figure 26A) and the increased number of cells presenting a SA- $\beta$ -galactosidase activity (see Fig. 4B of the article) rather than apoptosis revealed by the absence of Caspase-3 cleavage (Figure 26B). To further characterise the mechanism inducing the altered proliferative capacities of the shMsrA compared to shCtrl cells, we analysed the expression of p16<sup>INK4a</sup>, a cyclin-dependent kinase inhibitor, in MsrA-deficient myoblasts. As shown in Figure 26C, the higher p16<sup>INK4a</sup> expression may explain the senescence state induced by the absence of MsrA.

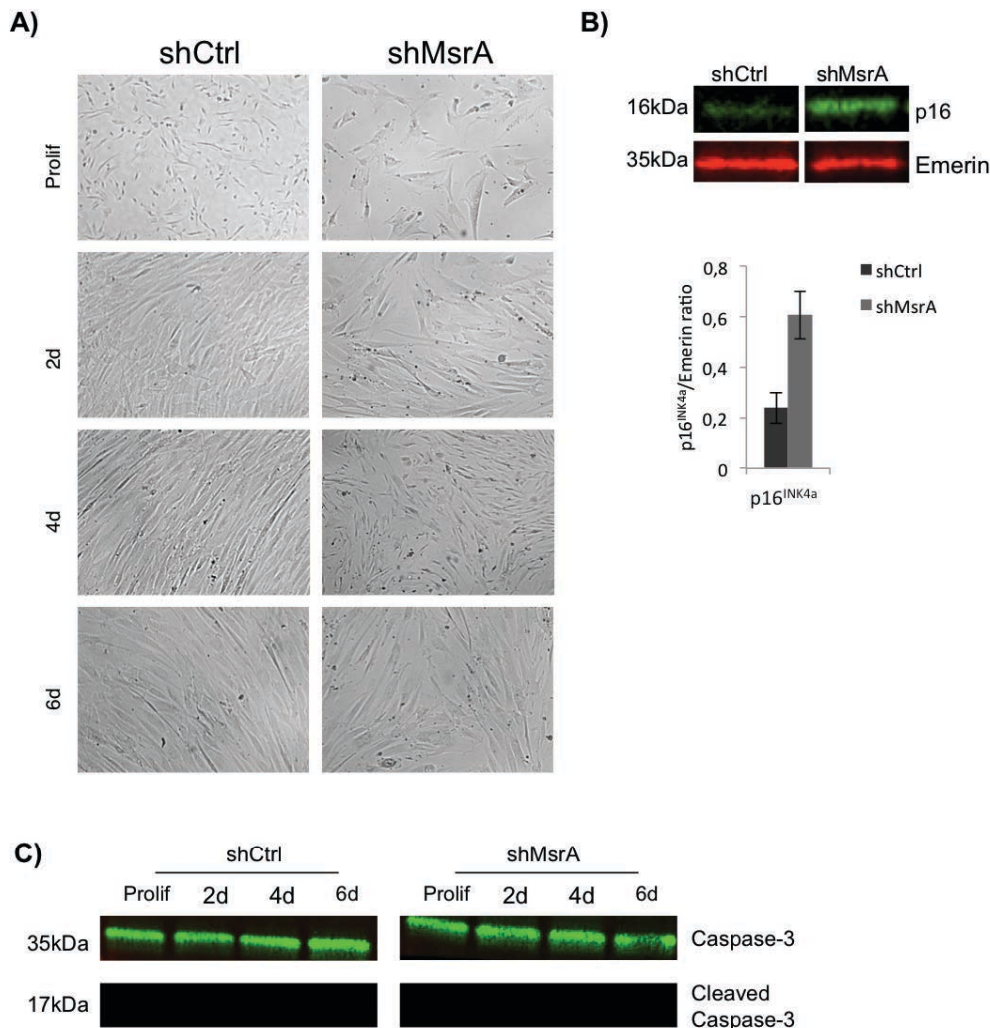


Figure 26 – **Senescence is induced in ShMsrA myoblasts.** **A)** Representative phase contrast images of human myoblast transfected with shCtrl or shMsrA lentiviral particles at different culture stages. **B-C)** Proteins from shCtrl and shMsrA cells were extracted at each stage of culture. Western-blot analysis indicate the level of Caspase-3, cleaved Caspase-3 (**B**) and p16<sup>INK4a</sup> proteins (**C**). Graphs represent the mean  $\pm$  SEM values from at least 3 independent experiments. Prolif - proliferation; d - days after induction of differentiation *in vitro*.

## Senescent myoblasts have less *MsrA* expression

As we have shown that *MsrA* inhibition led to the senescent of myoblasts, we wonder if replicative senescent myoblasts have decreased *Msr* expression. Myoblasts at 32 cumulative population doublings (CPD)<sup>10</sup> presented on average 11,1% of senescent cells, while 85,5% of myoblasts at 52 CPD were senescent as evidenced by SA- $\beta$ -galactosidase activity measurements (Figure 27A). As shown in Figure 27B, senescent myoblasts present two times less *MsrA* transcript expression that the proliferative ones while the expression of the other *Msr* genes do not significantly vary from young to senescent myoblasts (Figure 27B). This suggests that the myogenic defects found in the replicative senescent myoblast could be partly due to a reduced *MsrA* expression.

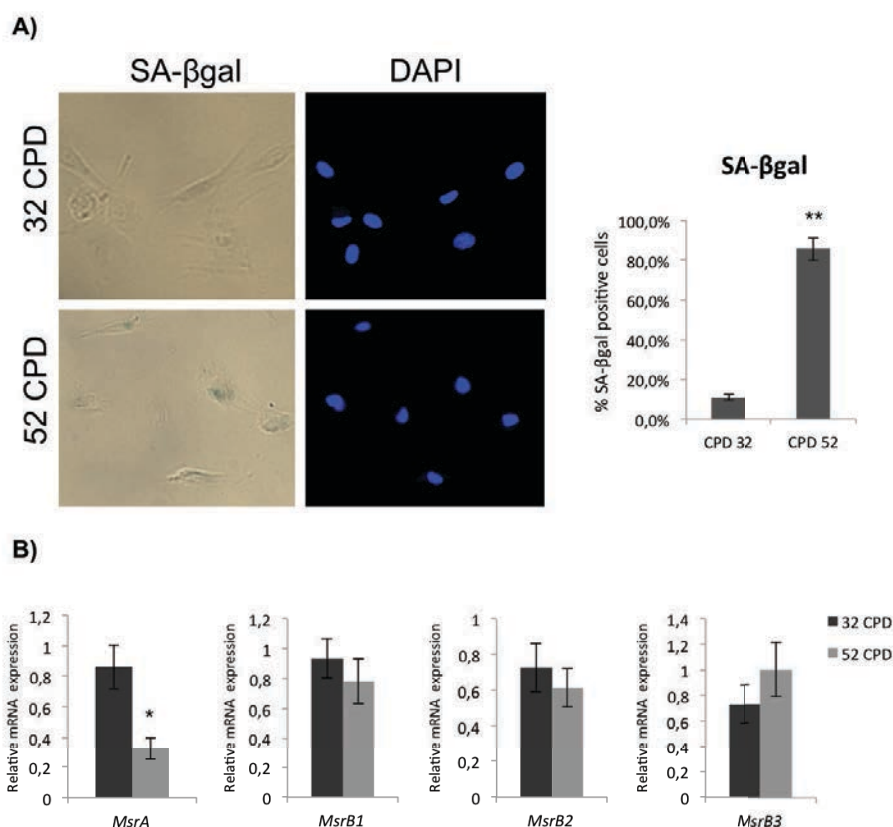


Figure 27 – **Senescent myoblasts had decreased *MsrA* expression. Myoblasts with 32 and with 52 cumulative population doublings (CPD) were analysed.** **A)** Cells in proliferation were assayed for senescence-associated- $\beta$ -galactosidase (SA- $\beta$ gal) staining and nucleus were evidenced by DAPI staining. Positive cells were counted and quantified as the percentage (%) of total cells. **B)** Total RNA from proliferative myoblasts was purified and analysed by qPCR for the expression of *MsrA*, *B1*, *B2* and *B3*. Transcripts are shown relative to the level of *Emerin* transcripts. Graphs represent the mean  $\pm$  SEM values from 6 independent experiments. \*  $P < 0.05$  and \*\* $P < 0.01$  in an unpaired student T test.

<sup>10</sup> CPD refers to the total number of times the cells in the population have doubled since their primary isolation *in vitro*

### **Fibroblast cells derived from pathologic fibrotic muscle have greater *Msr* expression than myoblasts**

The main muscle affected by the late onset muscular dystrophy, Oculopharyngeal Muscular Dystrophy (OPMD), are the *cricopharyngeus* and the elevator eyelid muscles whose progressive atrophy leads to dysphagia and ptosis (Luigetti, Lo Monaco et al., 2015). In particular, the *cricopharyngeus* muscle is characterised by a high level of fibrosis in healthy individuals that is exacerbated in muscle derived from OPMD patients (Gidaro et al., 2013). Thus, cultures derived from biopsies progressively lose their myogenicity, being enriched in fibroblasts. Isolation of the myogenic culture fraction that is CD56 positive (CD56<sup>+</sup>) from the non-myoblastic fraction (CD56<sup>-</sup>) can be done by microbeads conjugated with CD56 antibodies (see material and methods). While less *MsrA* expression was found in CD56<sup>+</sup> myoblasts derived from *cricopharyngeus* muscle from OPMD patients comparing to CD56<sup>+</sup> control cultures (Figure 28), in the case of CD56<sup>-</sup> cells, mainly composed of fibroblasts, we found more *MsrA* transcripts in fibroblasts derived from OPMD patients comparing to those from healthy donors (Figure 28). Furthermore, increased *MsrB2* and *MsrB3* expression was also observed in OPMD-derived fibroblasts comparing to OPMD-derived myoblasts (Figure 28). Whether the differential expression of *Msr* in myoblasts and fibroblasts is responsible for their differential proliferative capacities observed *in vitro* and resistance towards oxidative stress needs to be further investigated.

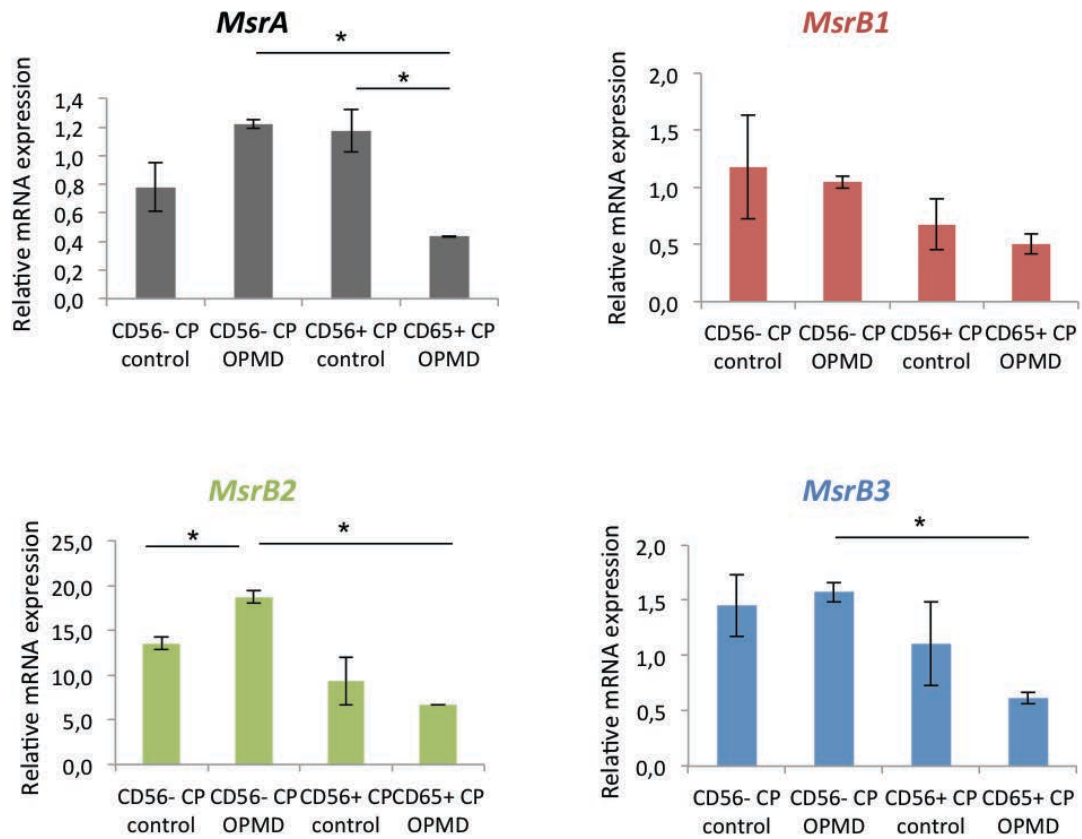


Figure 28 – **Msr expression in human OPMD-derived myoblasts.** Total RNA was purified from proliferative primary cellular fractions (CD56<sup>+</sup> representing the myoblastic fraction; CD56<sup>-</sup> representing the non myoblastic fraction). Transcripts are shown relative to the level of *Emerin* transcripts. Graphs represent the mean  $\pm$  SEM values from 6 independent experiments. \* P<0.05 in an unpaired student T test. CP – *cricopharyngeus* muscle.

## Msr family is expressed in quiescent and activated satellite cells

In homeostasis conditions, satellite cells of adult are in a quiescent state. Since cultured human myoblasts did not allow the study of Msr enzymes gene expression in quiescence, we used the *Pax3*<sup>GFP/+</sup> mice line to purify the satellite cells marked by *Pax3* (*GFP*) by flow cytometry ((Montarras et al., 2005) and materials and methods) from adult and compared them to satellite cells derived from 8 days post-birth mice in which almost 80% of them are activated to allow muscle growth (Pallafacchina, Francois et al., 2010). The *Msr* transcripts analysed by qPCR in adult quiescent cells, characterised by a low *Myogenin* expression and in activated satellites cells, marked by a high *Myogenin* levels (Figure 29) showed a higher expression for *MsrB1* and *MsrB3* genes in quiescent cells than in activated cells and no difference in *MsrA* and *MsrB2* gene expression.

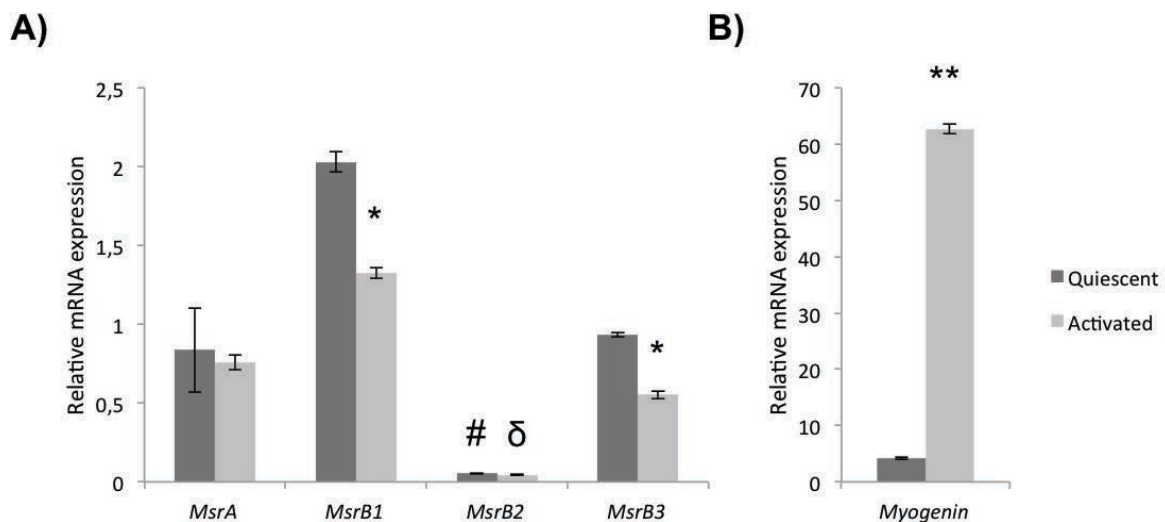


Figure 29 – **Msr expression profile in quiescent and activated satellite cells.** **A)** Expression of Msr transcripts in satellite cells isolated by flow cytometry from *Pax3*<sup>GFP/+</sup> muscles derived from adult mice (quiescent) or 8 days post birth mice (activated) was analysed by qPCR. **B)** Quiescent and activated stages were followed by *Myogenin* expression. Transcripts are shown relative to the level of *HPRT* transcripts. Graphs represent the mean  $\pm$  SEM values from 3 independent experiments. \*  $P < 0.05$  and \*\*  $P < 0.01$  in an unpaired student T test (Quiescent vs. Activated); #  $P < 0.05$  in a paired student T test (Quiescent cells: *MsrB2* vs. *MsrB1* and *MsrB3*);  $\delta$   $P < 0.05$  in a paired student T test (Activated cells: *MsrB2* vs. *MsrA*, *MsrB1* and *MsrB3*)

## ***Discussion***

The study of skeletal muscle regeneration represents a major biomedical issue, notably for the treatment of muscle degenerative disorders. In this work, we used human primary myoblasts as a culture model to investigate the role of Msr enzymes in myogenesis. By reducing methionine oxidation within proteins, these enzymes are involved in different redox signalling pathways (Gellert et al., 2015, Kim et al., 2014a). Although increasing evidence indicates that skeletal muscle myogenesis is controlled by ROS-dependent signalling, the role of protein homeostasis systems, in particular those responsible for the reduction of reversible oxidative modifications to proteins, still remains largely unexplored. In addition to their involvement in redox pathways, Msr enzymes are also recognised as important antioxidant enzymes (Luo & Levine, 2009). We thus hypothesise that they could play important roles during muscle regeneration.

We showed that ROS levels increase at early stages of myoblasts differentiation and that this increase is followed by an up-regulation of some antioxidant enzymes that directly interact with ROS, such as SOD and Prx. The increase in both ROS and antioxidant enzymes has also been reported other studies (Lee et al., 2014b, Malinska et al., 2012, Piao et al., 2005, Won et al., 2012). Between them, Prx2 and Prx3 enzymes have been postulated as critical regulators of myoblasts differentiation since their inhibition led to an accumulation of aberrant ROS levels and mitochondrial dysfunctions (Lee et al., 2014b, Won et al., 2012). In contrast, we found that CAT and some GPx enzymes expression significantly decrease during myoblasts differentiation, as observed several years ago (Franco et al., 1999). As opposed to the ROS eliminating enzymes, we found that Trx expression seems to be unchanged during myoblasts differentiation, as reported also by Dimauro *et al.* in a mouse model of muscle differentiation (Dimauro et al., 2012). Interestingly, although we did not observe a modulation of Msr expression during myoblasts differentiation, a significant decrease in Msr activity was found, suggesting that regulation of Msr activity is required for correct myogenesis. Both Msr and Trx enzymes depend on Trx reductase (TR) enzymes to be recycled and thus functional (Bigelow & Squier, 2011). Given that TR expression was found decreased in myotubes compared to myoblasts (Dimauro et al., 2012), one can think that, by regulating the expression of TR enzymes, myoblasts may control Msr and Trx enzymes activity. The specific regulation of Msr and Trx during myoblasts differentiation indicates that their functions in protein-reducing, and signal-transducing (Bigelow & Squier, 2011) are also important for correct myogenesis.

To further explore the importance of the Msr enzymes in myoblasts function, we used shRNA sequences against MsrA transcripts, the only Msr member able to reduce the MetSO diastereoisomer of MetO and which is widely distributed within cells: in the cytoplasm, nuclei and mitochondria (Kim, 2013). Deletion of this Msr member significantly reduced total Msr activity and induced a senescence state in myoblasts evidenced by increased SA- $\beta$ -galactosidase activity and up-regulation of p16<sup>INK4a</sup>. This senescent state can explain the decreased proliferative capacities found in MsrA-deficient myoblasts, since we did not observe caspase-dependent apoptosis. Accordingly, in geriatric mice, it has been shown that the decline in satellite cells number and self-renewal capacities with age was due to a switch to an irreversible senescent state, caused by de-repression of p16<sup>INK4a</sup> (Sousa-Victor et al., 2014). In the same year, Cosgrove *et al.* have shown that the high numbers of p16<sup>INK4a</sup>-related senescent satellite cells with age was due to an increased activation of the ROS-dependent p38 $\alpha$ /MAPK signalling cascade and that once this cascade is inhibited, satellite cells regenerative capacities *in vivo* were largely improved (Cosgrove et al., 2014). We found that MsrA inhibition leads to increased ROS content and to p16<sup>INK4a</sup>-dependent myoblasts senescence, whether this requires the activation of p38 $\alpha$ /MAPK signalling pathway remains to be confirmed. However, it is well known that Msr enzymes, by reducing MetO, function as ROS scavengers (Luo & Levine, 2009), supporting the idea that in its absence, different ROS signalling pathways could be turned on. Another possible ROS-dependent signalling pathway that has been implicated in myoblasts function is the ROS/NF- $\kappa$ B pathway which functions mainly downstream the p38 $\alpha$ /MAPK effectors (Baeza-Raja & Munoz-Canoves, 2004, Lee et al., 2011, Piao et al., 2005). Since MsrA has been recently shown to reduce inflammation in microglia through inhibition of the ROS/MAPK/NF- $\kappa$ B pathway (Fan et al., 2015), one could think that inhibiting MsrA may induce this pathway in myoblasts, thus impairing myogenesis.

Myoblasts from OPMD patients present low proliferative capacities in culture (Perie et al., 2006) and are rapidly overcome by intense proliferative fibroblasts. Interestingly higher *MsrA*, *MsrB2* and *MsrB3* expression was found in cultured fibroblasts comparing to myoblasts derived from OPMD-damaged muscle. Since we showed that MsrA play a critical role in regulating proliferative capacities of myoblasts, reduced expression of Msr enzymes observed in OPMD patients could explain their previously observed premature proliferative arrest (Perie et al., 2006). In contrast, the higher *MsrA*, *MsrB1* and *B3* expression in OPMD-derived fibroblast could be favouring their proliferation, explaining the higher fibrosis



occurring in *cricopharyngeal* muscle of dystrophic patients. Consistent with this, MsrA and MsrB3 were described as regulators of fibroblast proliferation via p53-p21 pathways (Choi & Kim, 2011, Lee, Kwak et al., 2014a). Whether OPMD-derived myoblasts proliferative arrest is due to the induction of a senescent state remains to be explored. However, the fact that this muscle dystrophy is characterised by chronic degeneration and regeneration cycles requiring the permanent activation of satellite cells (Gidaro et al., 2013), it is possible that the increase of satellite cell divisions ultimately leads to their senescence. Exhaustion of the satellite cells regenerative and self-renewal capacities by replicative senescence and changes in the environment are within the causes for the decrease in the satellite cell pool in other muscular dystrophies such as DMD (Decary, Hamida et al., 2000) and DM1 (myotonic dystrophy type 1) (Bigot et al., 2009). In myoblasts submitted to replicative senescence, we observed a reduction in MsrA expression compared to young culture myoblasts. Since replicative senescence could play a role in the regenerative defects observed in muscular diseases (Bigot et al., 2008), this suggests that targeting Msr expression could be an important tool for the treatment of these degenerative muscle disorders.

We propose that MsrA activity is important for maintaining low ROS levels in proliferative myoblasts and for protecting myoblast proteins against irreversible oxidative damages in order to prevent senescence and decreased proliferative capacities. Inhibition of another important protein homeostasis system, the autophagy-lysosomal system, in satellite cells resulted in mitochondrial dysfunctions and increased intracellular ROS content leading to senescence after satellite cells activation (Garcia-Prat et al., 2016). Moreover, these authors found that a basal level of autophagy seems to be required to maintain the murine satellite cells in a quiescent state (Garcia-Prat et al., 2016). Here we observed that quiescent satellite cells derived from adult mice have increased *MsrB1* and *B3* expression comparing to activated cells while *MsrA* expression remains higher in quiescent and activated satellite cells, suggesting that different Msr enzymes could participate to the cellular quality-control systems that actively repress senescence program, thereby preserving cellular proteotoxicity in quiescent and activated satellite cells.

In conclusion, we have shown that, by neutralizing excessive ROS levels and intracellular protein damage accumulation, Msr enzymes counteracts myoblasts senescence thereby preventing altered myogenesis, which is occurring in aged muscle or in muscle degenerative conditions such as in muscular dystrophies. This reinforces the notion that by modulating Msr enzymes, we could prevent cellular ageing and suggests that Msr could represent a valuable pharmacological target for the treatment of muscular dystrophies.

## **Part II - Importance of Msr enzymes for skeletal muscle fibres protection against oxidative stress under normal or pathological conditions**

### ***Background***

Skeletal muscle is an abundant tissue in mammals required for the production of force and movement needed for contraction. Nevertheless, skeletal muscle tissue is also the major site of fatty acid oxidation, participating on carbohydrate metabolism and maintenance of the body heat homeostasis. With age, however, a reduction in the number and size of muscle fibres is observed together with a loss of muscle strength and velocity of contraction. This age-dependent loss of muscle mass and force, commonly named sarcopenia, plays a major role in loss of independence and quality of life in the elderly. In addition, given that skeletal muscle plays central role as reserve for energy and amino acids, in the regulation of body temperature and in metabolic homeostasis, age-related loss of muscle mass and functions will also trigger severe metabolic side effects in the old populations.

Tissue ageing has been for long associated with an increased oxidative stress state and the skeletal muscle tissue is not an exception. Oxidative stress markers found in aged skeletal muscle fibres confirmed the causal role of oxidative stress, among the multifactorial bases of the sarcopenia, in the appearance of the muscle ageing (Powers, 2014). In fact, the redox state is of even greater relevance for skeletal muscle comparing to other tissues due to its high oxygen consumption rate. This can lead to an overproduction of ROS that, if not scavenged by antioxidant systems, will damage different kind of cellular biomolecules. Between them, skeletal muscle proteins, including those forming myofilaments, are very sensitive to redox modifications. The importance of protein irreversible damage accumulation and dysfunction of degrading systems, such as the proteasome, in the development of sarcopenia have been intensively studied (Andersson et al., 2011, Choksi et al., 2008, Lourenço Dos Santos et al., 2015, Snow et al., 2007, Thompson et al., 2006). However, although cysteine and methionine, the only protein residues in which oxidation is repaired by cellular enzymatic systems, are by far the most sensitive to oxidation by ROS, little is known about the role of reversible protein oxidation and their repairing systems for skeletal muscle homeostasis. In this regard, the study of the Msr systems deserves a special

attention since it is the only cellular enzymatic system capable of reversing methionine oxidation. Furthermore, Msr enzymes have been shown to play an important role in oxidative stress protection by preventing protein oxidative damages during ageing and neurodegenerative disorders (Kim et al., 2014c, Minniti et al., 2009, Moskovitz et al., 2011, Salmon et al., 2009). Although decreased MsrA expression and activity were found in different rat tissues (kidney, liver and brain) during ageing (Petropoulos et al., 2001), this important antioxidant system has never been studied in skeletal muscle ageing or pathologies.

Regarding skeletal muscle disorders, the study of Msr enzymes is also of particular interest since an increased oxidative state has already been suggested to participate to the pathology of some of them. This is the case for some muscular dystrophies such as Duchenne muscular dystrophy (DMD). Indeed, increased lipid oxidative damages, high levels of specific antioxidant enzymes and decreased GSH levels were found in muscle fibres from DMD patients or mouse models (review in (Canton et al., 2014, Terrill et al., 2013)), supporting a role of oxidative stress state in the development of this muscular dystrophy. The study of Msr enzymes in the context of the oculopharyngeal muscular dystrophy (OPMD) is also worthy of a special attention since, apart from increased mitochondrial and proteasome dysfunctions (Anvar et al., 2011, Trollet et al., 2010), this muscular dystrophy has a late onset of appearance meaning that premature ageing could play also a role in the phenotype of this muscular dystrophy.

As we hypothesised that Msr enzymes are involved in the protection of skeletal muscle tissue against oxidative stress conditions, such as during ageing, the aim of the second part of my thesis work was to investigate the expression and activity of Msr enzymes in skeletal muscle tissue. To clarify the role of Msr enzymes in pathological muscles, their expression will be also analysed in human muscle biopsies from DMD and OPMD patients and in skeletal muscle biopsies derived from dystrophic mouse models.

## ***Msr enzymes are involved in protection of skeletal muscle fibres from oxidative stress conditions***

### **Msr family is mainly expressed and activated in fast twitch muscle fibres**

Four main types of muscle fibres can be distinguished in skeletal muscle tissue in regard to their metabolic preference, varying from slow contracting fibres (type I) having the highest mitochondrial density and using oxidative phosphorylation for energy production to fast fatigable fibres (type IIb) with low mitochondrial content and producing ATP mainly through glycolysis. We wonder if Msr enzymes, which are differently distributed within cells, are differently expressed or active between the different types of muscle fibres. To investigate this, we measure Msr expression and activity in adult mouse hindlimb biopsies of different muscle types including *tibialis anterior*, a fast glycolytic muscle (fibres expressing mainly MHC-IIa and IIx isoforms), *soleus*, a slow oxidative type muscle (fibres expressing mainly MHC-I isoforms), *gastrocnemius* and *quadriceps*, two mixed muscle fibres (fibres expressing mainly MHC-I and IIa isoforms) (Figure 30A). Our results show that Msr activity is present in all three types of skeletal muscle, with fast glycolytic fibres having a maximal Msr activity comparing to slow and mixed muscle types (Figure 30B). The radioactive substrate we used in this study contains the two diastereoisomeric forms of the MetO and can be reduced by both MsrA and MsrB members. To identify which Msr members are responsible for this high activity in fast glycolytic muscles, we analysed their transcripts expression profile in the different mouse muscles. All *Msr* genes are expressed in the different muscles studied (Figure 30C), with *MsrB1* being the most expressed gene, especially in *tibialis anterior* muscle, followed by *MsrB3*. However, we did not find significant variations in the expression of these genes between the different types of muscle fibres, suggesting that the difference found in Msr activity should be due to post-transcriptional changes. Interestingly, *MsrB2*, whose expression is confined to mitochondria, is the least represented gene in all muscles analysed.

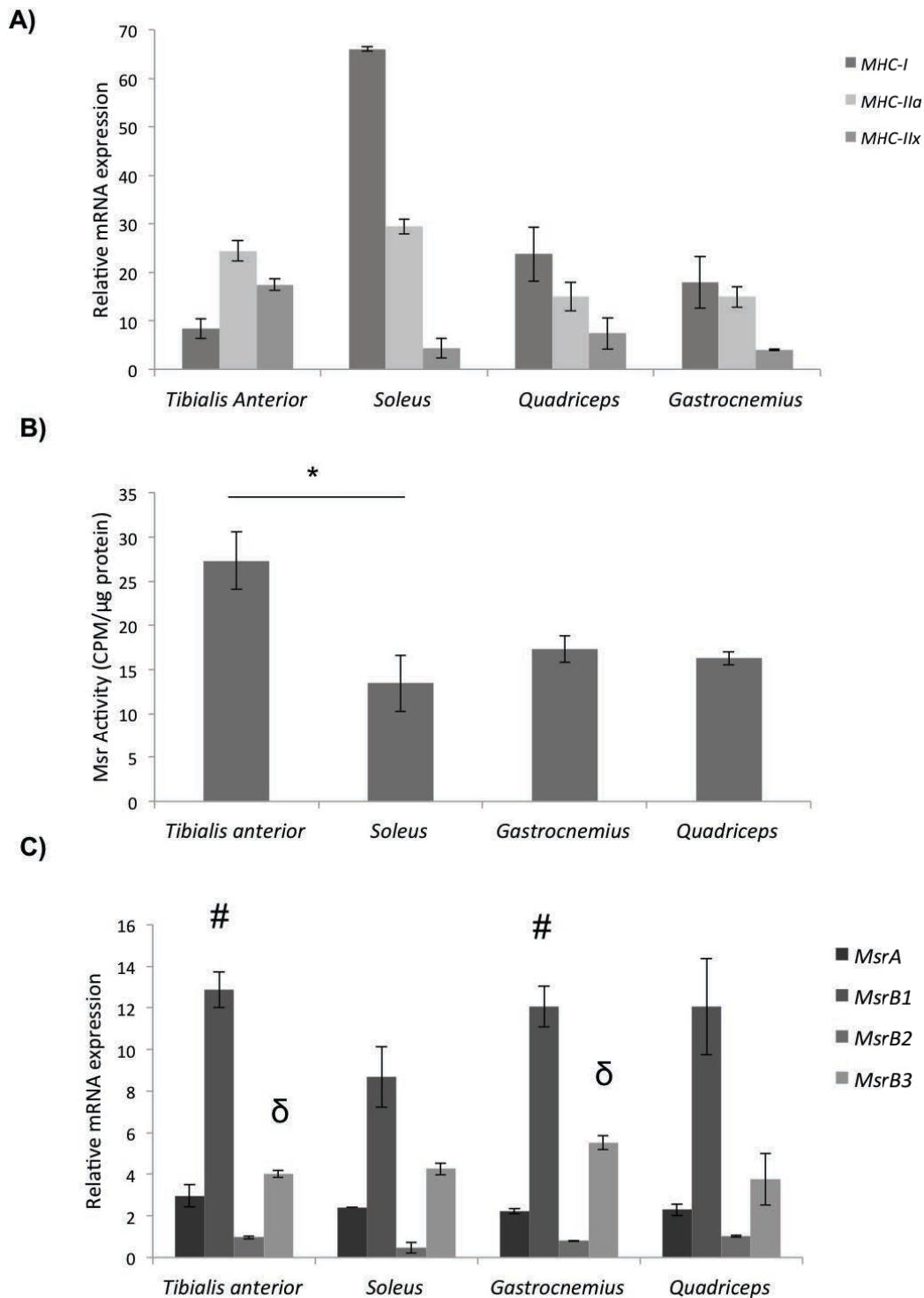


Figure 30 – **Msr activity and expression in different mouse skeletal muscle types.** **A)** For each muscle biopsy, myosin heavy chain (MHC) isoforms were analysed by qPCR after RNA purification. **B)** Msr activity was measured in protein extracts from different kinds of skeletal muscle using radioactive labelling of N-acetyl-[3H]Met-R,S(O) substrate. **C)** *Msr* transcripts expression was analysed by qPCR after RNA purification. Transcripts are shown relative to the level of *HPRT* transcripts. Graphs represent the mean  $\pm$  SEM values from at least 3 independent experiments. \*  $P < 0.05$  in an unpaired student T test; #  $P < 0.05$  in a paired student T test (MsrB1 vs. MsrA and MsrB2);  $\delta$   $P < 0.05$  in a paired student T test (MsrB2 vs. MsrB3).

### **Msr expression and activity are decreased in sarcopenic muscle**

To determine if an impaired expression and / or activity of the Msr system could be related to the oxidative stress state observed in sarcopenia, we investigated the Msr activity and expression in *tibialis anterior* biopsies obtained from young adults of 3 months of age and geriatric mice of 25 months of age. At this older age, fibre atrophy is already detected as well as irreversible damages to proteins (Figure 31A). Our results indicate a dramatic decrease in Msr activity during muscle ageing (Figure 31B). In order to determine which Msr is responsible for this decrease, we analysed the Msr expression profile by qPCR. As shown in Figure 31C, there is a significant decline in *MsrA* and *B1* gene expression with age while *MsrB2* and *B3* transcripts do not seem affected by sarcopenia. These results suggest that the reduction of *MsrA* and *B1* gene expression in geriatric muscle would be responsible for the significant decline observed in Msr activity during muscle ageing.

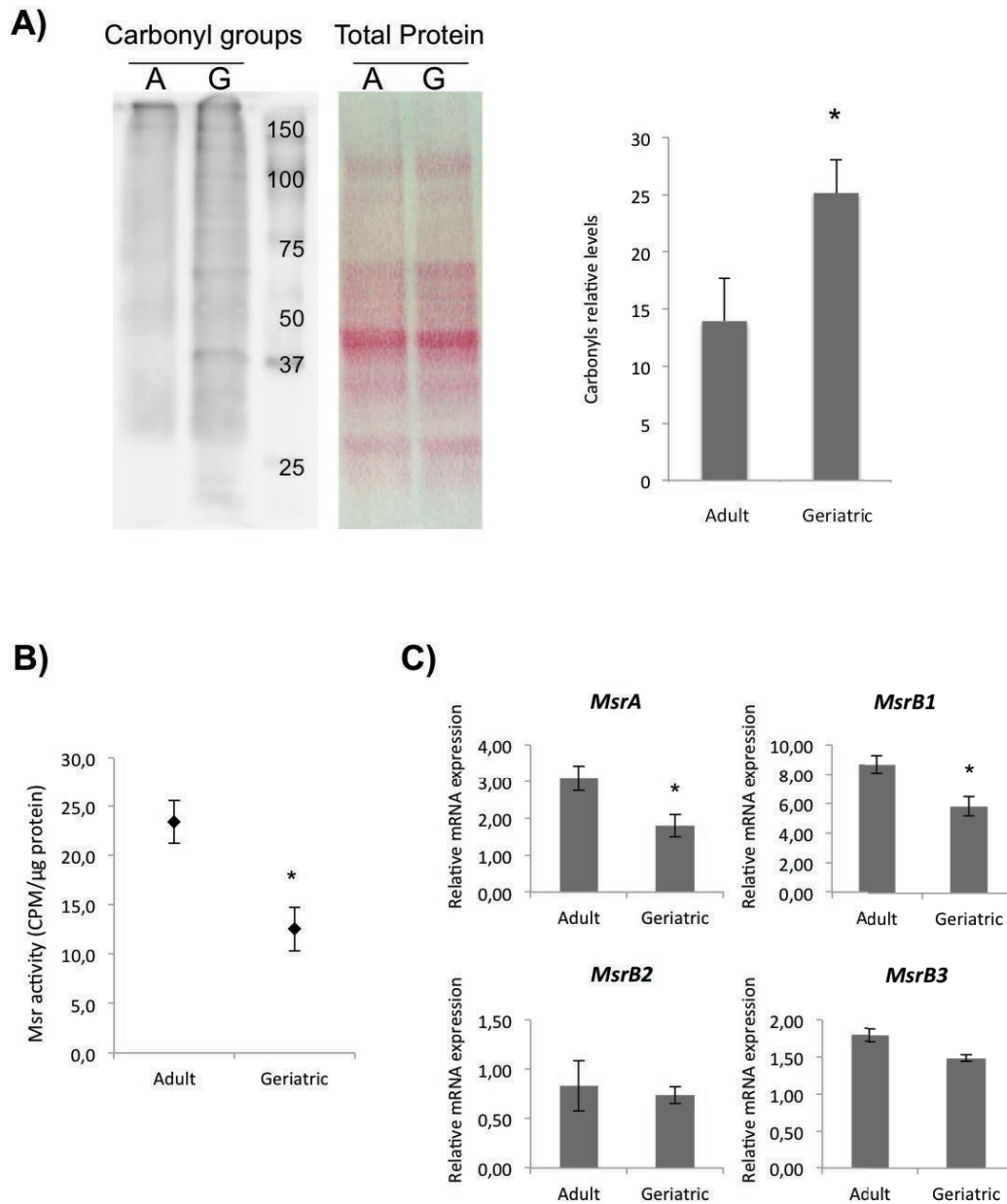


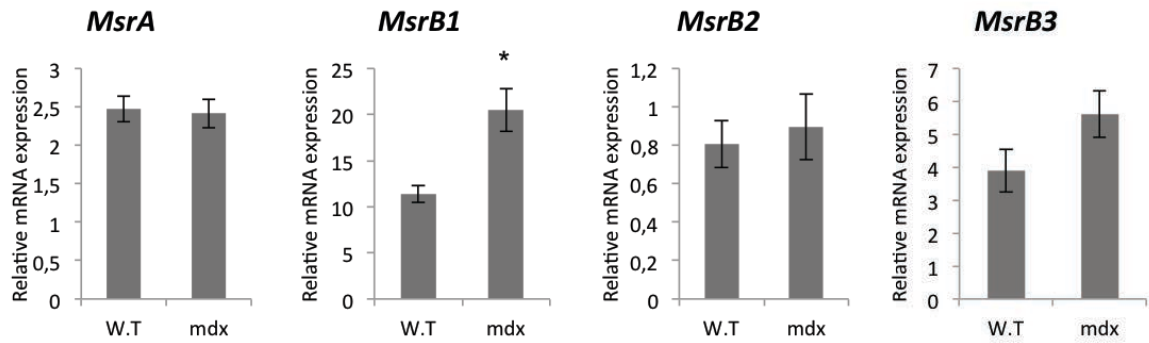
Figure 31 – **Msr activity and expression in adult (3 months) and geriatric (25 months) mouse skeletal muscle.** **A-B)** Proteins were extracted from adult and geriatric mouse *tibialis anterior* biopsies. **A)** Western-blot analysis indicated the level of protein carbonyl groups. **B)** Msr activity was measured in protein extracts from muscle biopsies using radioactive labelling of N-acetyl-[<sup>3</sup>H]Met-R,S(O) substrate. **C)** *Msr* transcripts expression was analysed by qPCR after RNA purification. Transcripts are shown relative to the level of *HPRT* mRNA. Graphs represent the mean  $\pm$  SEM values from at least 3 independent experiments. \*  $P < 0.05$  in an unpaired student T test.

### ***MsrA* and *MsrB1* expression are induced in muscle pathological conditions**

Recent reports have shown the presence of an important oxidative stress in some muscle dystrophies evidenced by an increase in protein irreversible and reversible oxidation (Canton et al., 2014, Terrill et al., 2013). In addition, different studies have shown the role of Msr proteins in resistance against oxidative stress during degenerative disorders (Chondrogianni et al., 2014). We hypothesise that the Msr system could be involved in fibre protection in muscular dystrophies where an oxidative stress is observed. We chose to study the DMD due to its elevated frequency of occurrence, its high degree of severity but in particular due to the important oxidative stress state found in muscle fibres of DMD patients. To determine if the Msr family is implicated in the pathology of this muscular dystrophy, we measured *Msr* transcripts expression in muscle biopsies of DMD patients compared to control donors of the age range as well as in muscle of *mdx*, a DMD mouse model, compared to control wild type (W.T.) mice. As shown in Figure 32A, we found a significant higher *MsrB1* gene expression in *tibialis anterior* muscle derived from the *mdx* mouse comparing to W.T. mice. In agreement, we observed the same pattern in *MsrB1* as well as *MsrA* gene expression in muscle biopsies derived from DMD patients comparing to muscle biopsies from healthy donors (Figure 32B). However, due to the great variability between DMD patients muscle samples (evidenced by the great error bars) and to the small number of biopsies studied (4 DMD patients), our data did not reach statistical significance. No difference in the *MsrB2* and *B3* expression was found between muscle biopsies from normal or DMD conditions in both human and mouse models (Figure 32).



**A)**



**B)**

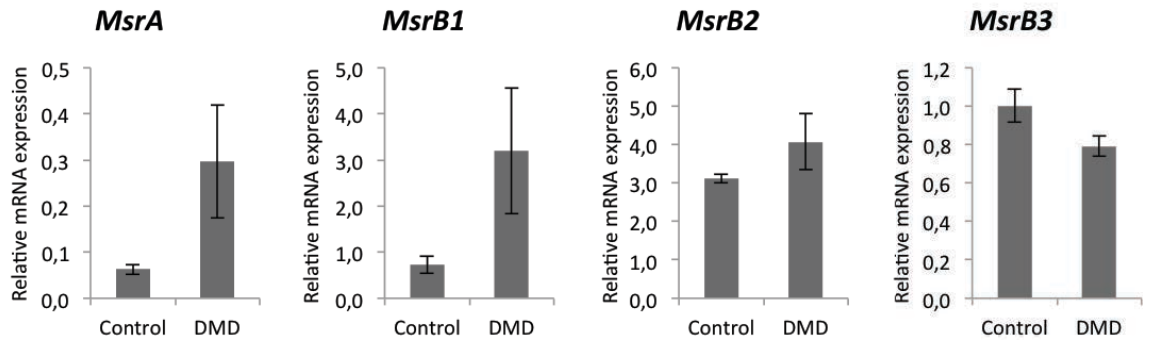
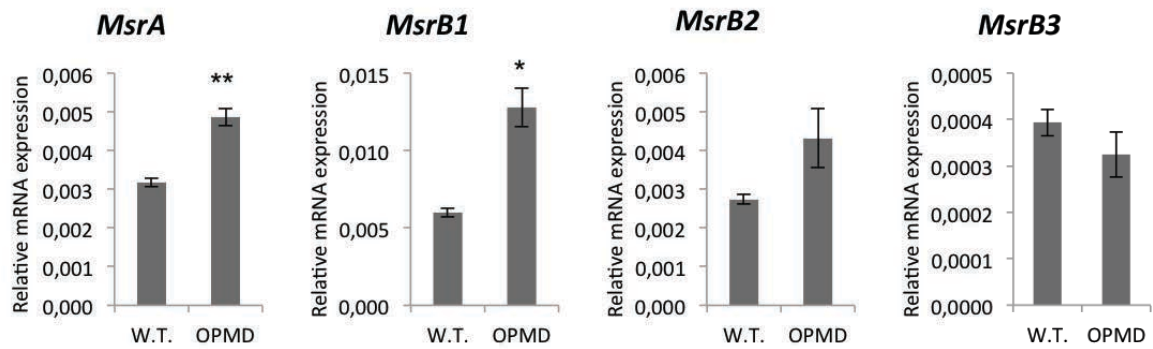


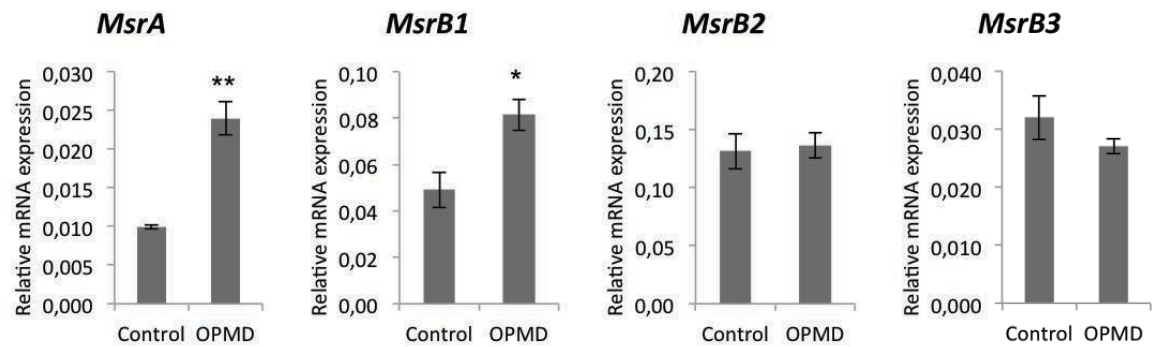
Figure 32 – *Msr* transcripts expression in Duchenne muscular dystrophy. *Msr* transcripts expression was analysed by qPCR after RNA purification from (A) mouse *tibialis anterior* muscle biopsies or (B) human *paravertebral* and *quadriceps* muscle biopsies. Transcripts are shown relative to the level of *HPRT* or *Emerin* mRNA. Graphs represent the mean  $\pm$  SEM values from at least 3 independent experiments. \* P<0.05 and \*\* P<0.01 in a unpaired student T test. **W.T** – wild type mice; **mdx** – DMD mouse model; **DMD** – Duchenne Muscular Dystrophy

In order to further understand role of *Msr* enzymes in muscular dystrophies, investigated their expression in the late-onset OPMD, in which some evidences of deregulated protein homeostasis were showed to be a predominant aspect of the pathology (Anvar et al., 2011). In human, the disease is characterised by dysphagia especially due to atrophy and fibrosis of the *cricopharyngeal* muscle. Regarding the OPMD mouse model, named A17.1 in relation with the number of alanine stretches evidenced in the mutated PABPN1 protein, the OPMD-related atrophy and intra-nuclear intrusions accumulation were restricted to fast glycolytic fibres (Trollet et al., 2010). For both human and mouse models, we used biopsies from the most affected muscles by the disease. Thus biopsies from human *cricopharyngeus* muscles and from mouse *extensor digitorum longus* (a fast twitch fibre) were used to analyse *Msr* expression. Higher *MsrA* and *B1* genes expression was found in muscles derived from the A17.1 mice comparing to the W.T. mice (Figure 33A). Consistent with these results, we found also a greater *MsrA* and *B1* gene expression in the *cricopharyngeus* muscle of OPMD patients compared to healthy individuals (Figure 33B). In both human and mouse biopsies, *MsrB2* and *MsrB3* expression was not affected by the OPMD phenotype (Figure 33A and B). Analysis of another pharyngeal muscle that does not present OPMD features in human patients, the *sternocleidomastoid* muscle (SCM), revealed no significant increase in any *Msr* transcripts in OPMD samples comparing to healthy individuals (Figure 33C). Thus suggesting that the increase in *MsrA* and *MsrB1* gene expression observed is specific to muscles affected by dystrophic mutations. However, one cannot exclude the fact that the higher in *MsrA* and *B1* expression could be a result of the increased fibrosis present in OPMD-affected muscles in contrast to the SCM (Gidaro et al., 2013).

A)



B)



C)

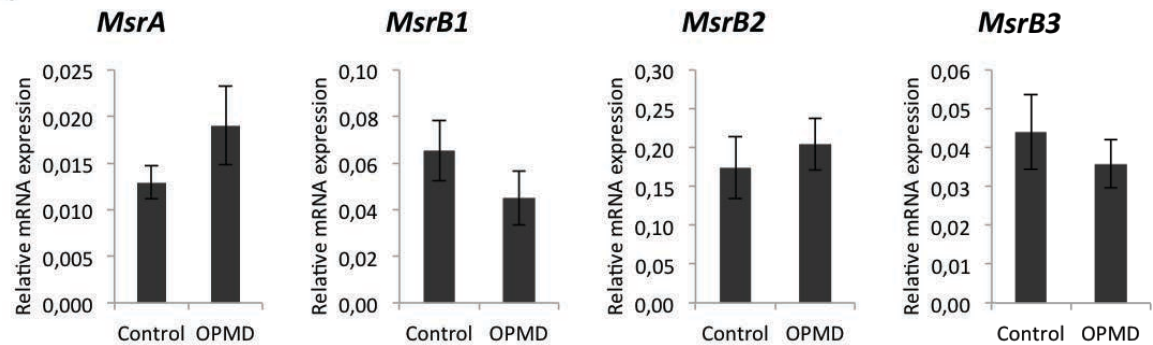


Figure 33 – *Msr* transcripts expression in oculopharyngeal muscular dystrophy. *Msr* transcripts expression was analysed by qPCR after RNA purification from (A) *extensor digitorum longus* muscle biopsies from 6 weeks wild type (W.T.) and A17.1 (OPMD) mice (B) *cricopharyngeus* muscle biopsies or (C) *sternocleidomastoid* muscle biopsies of human healthy individuals and OPMD patients. Transcripts are shown relative to the level of *HPRT* or *Emerin* mRNA. Graphs represent the mean  $\pm$  SEM values from at least 3 independent experiments. \*  $P < 0.05$  and \*\*  $P < 0.01$  in a unpaired student T test. **W.T** – wild type mice; **OPMD** – Oculopharyngeal Muscular Dystrophy.

## *Discussion*

Skeletal muscle fibres represent one of the most abundant cell types in mammals. Their highly specialised contractile and metabolic functions depend on large numbers of contractile proteins, structural proteins, but also on a large spectrum of enzymes whose optimum activity is required for proper muscle function. Thus, maintaining protein homeostasis is critical to prevent muscle fibres dysfunction and the subsequent pathological conditions. Proteostasis requires protein synthesis, degradation but also when possible, repair of damaged proteins. Indeed, the enzymatic repair of altered proteins appears as a faster and less energy consuming process compared to the degradation of damaged proteins since it does not require the subsequent protein synthesis in order to re-establish the lost function. In fact, immediately after repair, protein function is restored. The necessity of a proper proteostasis in skeletal muscle is especially acute due to its unique requirements. Indeed, these force-generating structures are constantly challenged by mechanical, heat and oxidative stress, which increase protein damage and require efficient protein turnover to maintain optimal performance. In this context, Msr proteins, which repair oxidised methionines within proteins, are of great interest to maintain muscle protein function.

Here we investigated, the Msr activity and expression in different skeletal muscle fibre types. Adult skeletal muscles contain slow and fast-twitch fibres according to their speed of contraction. Slow-twitch fibres, also known as oxidative type I fibres, express the slow type I isoform of myosin heavy chain (MHC I), whereas fast-twitch fibres may express three different types of MHC (MHC IIa, IIx or IIb). Differential expression of MHC isoforms affects muscle specific force and is the major determinant of its maximal velocity of contraction, which will also depend on the type of metabolism of each fibre type. Thus, type I (slow-twitch, oxidative) fibres are enriched in mitochondria and rely on oxidative phosphorylation for ATP production. In contrast, type IIb fibres (fast-twitch, glycolytic) fibres are poor in mitochondria and have very effective glycolytic ATP synthesis. Msr enzymes are differently distributed within cells: MsrA is widely distributed in nucleus, cytoplasm and mitochondria; MsrB1 being present only in the nucleus and cytoplasm; MsrB2 expression is restricted to mitochondria and MsrB3 is expressed in the nucleus and in the endoplasmic reticulum. The mitochondrial enzymes MsrA and MsrB2 were shown to protect mitochondria function (Cabreiro et al., 2008, Nan et al., 2010). Moreover, overexpressing of MsrA enhanced ATP synthesis by increasing specifically the activity of the respiratory chain in retinal pigment epithelial cells (Dun et al., 2013). Such contribution

of MsrA to energy metabolism raises the question as whether the Msr distribution is dependent on the different muscle fibres metabolism requirements. We observed that, in physiological conditions, total Msr activity is higher in *tibialis anterior* muscle mainly composed of fast-glycolytic fibres comparing to slow-twitch fibres of the *soleus* muscle (Figure 30). However, this difference does not seem to be due to higher Msr expression in type II fibres comparing to type I or mix fibre types (Figure 30), indicating that in basal conditions, Msr activity in muscle fibres is not dependent on *Msr* gene expression. In addition, we did not found differences in the pattern of expression of the different *Msr* genes in regard to the muscle fibre type (Figure 30), suggesting that it is Msr activity and not the *Msr* expression that will be tiny regulating depending on muscle energetic source, metabolism or velocity of contraction. Interestingly, we found that in all skeletal muscle fibres studied but mainly in MHC Iib containing fibres, the *MsrB1* gene coding to the Msr selenoprotein is the most expressed one (Figure 30), which could be related to the importance of maintenance of cytoplasmic protein functions in glycolytic fibres. Actually, it has been recently found that MsrB1 controlled assembly and disassembly of actin by reversing oxidation of its methionines that were oxidised by monooxygenases of the Mical family (Hung et al., 2013, Kaya, Lee et al., 2015, Lee et al., 2013).

Furthermore, it is well known that skeletal muscle atrophy occurring in ageing, inactivity and prolonged fasting states is predominantly restricted to type II glycolytic muscle fibres, while type I fibre size is largely unaffected (Frontera, Suh et al., 2000, Lexell, 1995, Nikolic, Malnar-Dragojevic et al., 2001). In addition, the oxidative stress observed in aged skeletal muscle has been proposed as a possible causative factor for muscle atrophy (Pellegrino et al., 2011, Powers, 2014). Since increasing evidence recognizes Msr enzymes as cellular protectors from an oxidative stress state associated with age (Kim et al., 2014c, Minniti et al., 2009, Moskovitz et al., 2011, Salmon et al., 2009), we further investigated their role in skeletal muscle ageing by studying their activity and expression in type II muscle fibres from aged mice. We found accumulation of oxidised proteins with age (Figure 31), in agreement to previous studies (Barreiro et al., 2006, Choksi et al., 2008, Lourenço Dos Santos et al., 2015, Marzani et al., 2005, Siu et al., 2008, Snow et al., 2007). In parallel, a significant decrease of Msr activities was observed and was attributed, at least in part, to a lower expression of *MsrA* and *MsrB1* (Figure 31). Decreased Msr expression and activities have been found in other mouse tissues with age (Petropoulos et al., 2001). Since methionine in proteins acts as ROS scavenger and avoiding oxidation of other aminoacids (Luo & Levine, 2009), the decreased Msr activity can explained, at least in part, the increase in

carbonyl content as well as the impaired protein homeostasis occurring in sarcopenic muscle tissue. However, a smaller activity of the 20S proteasome was also described with age in mouse type II muscle fibres (Ferrington, Husom et al., 2005), thus, we can not exclude that age-dependent defective proteasome activities in geriatric fast twitch muscle fibres take part in the accumulation of protein damages.

Redox homeostasis is also a critical factor in pathologies associated with excessive oxidative stress. In the case of skeletal muscle tissue, oxidative stress has been clearly evidenced in some muscular dystrophies, such as the DMD (Terrill et al., 2013). In this muscular dystrophy, the chronic degeneration of muscle fibres leads to a continuous inflammation state resulting in increased ROS levels and other markers of oxidative stress such as thiol oxidation, protein carbonylation and lipid peroxidation (Armstrong, Zerbes et al., 2011, Terrill, Radley-Crabb et al., 2012, Terrill et al., 2013). The causal role of an important oxidative stress in DMD is also supported by pre-clinical studies in *mdx* mice that reported increased dystrophin expression, decreased necrosis and improved muscle pathology following administration of different antioxidants including green tea, resveratrol, catalase or NAC, (Evans, Call et al., 2010, Hori, Kuno et al., 2011, Selsby, 2011, Terrill et al., 2012). We found higher *Msr* expression, mainly *MsrB1*, in muscle biopsies derived from *mdx* mice compared to W.T. mice as well as in muscle biopsies derived from DMD patients when compared to healthy donors of the same age, although in the case of humans, these differences were not statistically significant due to the small and heterogeneous group of analysed biopsies (Figure 32). In agreement, elevated levels of antioxidant enzymes such as catalase, glutathione peroxidase, superoxide dismutase 1 and 2, have been reported in muscles collected from *mdx* mice (Disatnik, Dhawan et al., 1998). Moreover, in regard to the other protein homeostasis systems, increased proteasome activity (Chen, Graber et al., 2014) and impaired autophagy (Spitali, Grumati et al., 2013) were also described in DMD mice models. Thus, there is numerous evidences that antioxidant systems are activated in dystrophic muscles but they seem to be insufficient to scavenge the increased ROS levels and to prevent accumulation of oxidative damages to biomolecules. In contrast, during ageing, impairment of the antioxidant systems hence participates to the accumulation of protein oxidation within cells.

The study of late onset muscular dystrophies constitute another interesting model since their phenotype appears with age and thus could be caused by age-related factors, such as a dysfunction of protein homeostasis systems. Indeed, an integrative transcriptome analysis using different affected muscles from mice models and patients of a late onset

muscular dystrophy, the oculopharyngeal muscular dystrophy (OPMD), emphasized the UPS as the major deregulated system (Anvar et al., 2011). Here, we observed an increased *Msr* expression in biopsies collected from the most affected muscles of an OPMD mice model or of OPMD patients comparing to muscle biopsies from W.T or healthy donors, respectively (Figure 33). Thus, contrary to the proteasome dysfunction, which is suggested to be a possible cause of this late onset muscular dystrophy, the increased *Msr* expression seems to be a consequence of a possible oxidative stress state, although there is yet a lack of evidence of oxidative damages in muscle fibres affected by OPMD dystrophy. We did not find increased *Msr* expression in muscle biopsies from non-affected muscle of OPMD patients comparing to muscle biopsies from healthy donors (Figure 33). This further indicates that the *Msr* increase in the most clinically affected muscles is related to the dystrophic phenotype rather than to the genetic mutation that is present in all muscles. One possible explanation for this *Msr* increase is also the higher level of fibrosis present in the *cricopharyngeus* muscle comparing to other pharyngeal muscles.

Overall these results suggest that in skeletal muscle, *Msr* activity decreases with age and would partly contribute to the accumulation of protein oxidative damages. This will lead to loss of protein function affecting muscle fibre activity and ultimately contributing to the sarcopenic phenotype. Contrary, in the case of some muscular dystrophies, for which the oxidative stress is well established, *Msr* and antioxidant systems are highly activated in response to the increased ROS, although this adaptation does not seem to be sufficient to counteract the pathological symptoms.

## **CONCLUSION AND PERSPECTIVES**





Our work represents the first study of Msr enzymes in skeletal muscle tissue, either in muscle fibres or in the adult stem cells. In addition, we had the opportunity to access muscle biopsies of muscles from human pathological conditions, DMD and OPMD, and from mouse models of these pathologies. Our results suggest that Msr enzymes are involved in muscle stem cell regulation during myogenesis as well as in muscle fibres protection against oxidative stress.

Considering our results obtained from skeletal muscle fibres, we can think that the decrease in Msr expression found in sarcopenic muscle possibly participates to some sarcopenic features such as the decrease in protein quality control, which leads to increased protein degradation and therefore up-regulates cellular catabolic systems that ultimately lead to fibre atrophy. To investigate this hypothesis, it would be interesting to study a MsrB1 null mouse model, since this Msr member was found to be the most expressed in muscle fibres. The consequences of MsrB1 deletion on fibre homeostasis during ageing would be assessed using markers of oxidative stress such as irreversible protein oxidation, measurement of the degree of muscle atrophy and fibrosis as well as analysis of fibre size and type (I or II) on muscle sections of mutant and control mice at different ages. In this context, it would be of a great interest to measure Met oxidation that could represent a particularly attractive biomarker for the assessment of oxidative stress. Unfortunately, its use as a marker of oxidative damage to proteins suffers from the lack of tools, such as specific antibodies, to detect and quantify MetO *in vitro* and *in vivo*. Otherwise, a two-dimensional study with proteins extracts derived from muscle biopsies from aged and young muscle searching for differential protein expression could give some clues on new signalling cascades, whose modulation could influence muscle antioxidant capacity and thus improve muscle function during ageing. Moreover, the results obtained from analysis of muscle biopsies derived from dystrophic muscles suggest the presence of an increased oxidative stress state in muscular dystrophies. Although this has been undoubtedly evidenced in the case of DMD, regarding the late onset OPMD, clear indications for the presence of an increased oxidative stress are needed and could help to complete the causes of this dystrophic phenotype. Finally, in order to get new insights into the consequences of an oxidative stress, a comparative 2D-DIGE<sup>11</sup> proteomic study optimized for the detection of oxidised proteins (OxyDIGE) could be done using biopsies from healthy and dystrophic muscle. After oxidised protein targets identification by mass spectrometry, the implication of these modifications in the

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<sup>11</sup> Two dimensional-differential gel electrophoresis

development of the disease would be further analysed in order to try to discover new mechanisms linked to the pathology of this diseases.

Regarding skeletal muscle stem cells, we found that high Msr activity levels are required to ensure myoblasts proliferation by repressing senescence associated signalling. Whether this is due to the ROS-dependent cascades that control myoblast differentiation, such as the p38 $\alpha$ /MAPK or NF- $\kappa$ B, is current under investigation in our laboratory. If possible, the comparison of protein-bound MetO content within shMsrA and shCtrl myoblasts and the subsequent identification of these proteins would also help to define the Msr-regulated signalling pathways important for myogenesis. In order to further demonstrate that senescence prevention is due to the Msr antioxidant properties, control and shMsrA myoblasts could be cultured in the presence or absence of an exogenous oxidant treatment and their proliferative capacities and senescence induction evaluated. Moreover, it would be interesting to overexpress MsrA enzymes in myoblasts and investigate if this could extend the number of myoblasts divisions in culture before becoming senescent. In view to make MsrA a possible therapeutic target for the treatment of muscular dystrophies, overexpression of MsrA in myoblasts derived from OPMD patients could be compared to their respective controls, as well as with myoblasts from healthy patients, in regard to their proliferative capacities in culture and subsequently their regenerative capacity *in vivo* by myoblasts transplantation in a mouse model of muscle regeneration. Moreover, the study of the MsrA<sup>-/-</sup> mice would also allow to further explore the role of MsrA in satellite cells during muscle regeneration *in vivo*. For this, proliferation and differentiation of mutant and control satellite cells could be analysed after muscle injury. Histological analysis of mutant and control muscles would also be performed at different times after muscle injury to investigate differences in the size of muscle fibres, thus giving insights about the regenerative phenotype. Assuming reduced proliferative capacities of MsrA<sup>-/-</sup> satellite cells, we expect to detect smaller myofibres at the end of muscle regeneration in tissue sections derived from mutant mice compared to controls. Finally, the crossing of MsrA<sup>-/-</sup> mice with the Pax3<sup>GFP/+</sup> would allow the purification of mutant satellite cells at different regeneration times and the measurement of their redox state and oxidised proteins levels.

In conclusion, with this work, we give the first insights into the importance of Msr enzymes for skeletal muscle tissue. We showed that their modulation could present a valuable approach to improve myogenesis. However, further studies are needed to assess their role in muscle regeneration in view to improve the current cellular therapies for muscular dystrophies or other degenerative muscle disorders.

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## **ANNEXES**





## Research Paper

## Oxidative proteome alterations during skeletal muscle ageing

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## ABSTRACT

Sarcopenia corresponds to the degenerative loss of skeletal muscle mass, quality, and strength associated with ageing and leads to a progressive impairment of mobility and quality of life. However, the cellular and molecular mechanisms involved in this process are not completely understood. A hallmark of cellular and tissular ageing is the accumulation of oxidatively modified (carbonylated) proteins, leading to a decreased quality of the cellular proteome that could directly impact on normal cellular functions. Although increased oxidative stress has been reported during skeletal muscle ageing, the oxidized protein targets, also referred as to the 'oxi-proteome' or 'carbonylome', have not been characterized yet. To better understand the mechanisms by which these damaged proteins build up and potentially affect muscle function, proteins targeted by these modifications have been identified in human *rectus abdominis* muscle obtained from young and old healthy donors using a bi-dimensional gel electrophoresis-based proteomic approach coupled with immunodetection of carbonylated proteins. Among evidenced protein spots, 17 were found as increased carbonylated in biopsies from old donors comparing to young counterparts. These proteins are involved in key cellular functions such as cellular morphology and transport, muscle contraction and energy metabolism. Importantly, impairment of these pathways has been described in skeletal muscle during ageing. Functional decline of these proteins due to irreversible oxidation may therefore impact directly on the above-mentioned pathways, hence contributing to the generation of the sarcopenic phenotype.

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## Introduction

Skeletal muscles require dynamic changes in energy supply and oxygen flux for contraction, making it prone to reactive oxygen species (ROS)-mediated damage as a result of an increase in electron flux and leakage from the mitochondrial respiratory

chain. Although physiological concentrations of ROS can function as signaling molecules that regulate proliferation, growth, differentiation, and apoptosis [1], when ROS levels overcome the capacity of cellular antioxidant systems, they become toxic, introducing oxidative modifications on cellular macromolecules such as nucleic acids, lipids and, in particular, proteins, inflicting alterations to normal cellular functions. In skeletal muscle, oxidative stress state has negative consequences on action-potential conduction, excitation–contraction coupling, satellite cell differentiation, muscle contraction and mitochondrial respiration [2].

Skeletal muscle ageing is associated with the gradual degenerative loss of skeletal muscle mass, quality, and strength, a condition known as sarcopenia. Oxidative stress contribute at least in part to muscle atrophy [3–6], and previous studies have addressed the extent of protein oxidative damage in the development of sarcopenia in mammalian models [7–10]. Although it is believed that oxidative stress contributes to skeletal muscle dysfunction

**Abbreviations:** 1D, one-dimensional; 2D, bi-dimensional; ATP, adenosine triphosphate; CK, creatine kinase; DNP, 2–4-dinitrophenylhydrazone; DNPH, 2,4-dinitrophenylhydrazine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPD1, Glycerol-3-phosphate dehydrogenase [NAD<sup>+</sup>], cytoplasmic; HSP70, Heat shock 70 kDa protein; IEF, Isoelectric focusing; MM-CK, muscle-type creatine kinase; MS, mass spectrometry; MyBPC, myosin-binding protein C; PCr-CK, phosphocreatine-creatine kinase; RA, *rectus abdominis*; RMI, relative modification index; ROS, reactive oxygen species; ZASP, LIM domain-binding protein 3

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through macromolecular damage, the molecular mechanisms remain elusive. Protein post-translational modifications induced by ROS are important features of oxidative stress [11]. Among them, protein carbonyl content is by far the most commonly used marker of protein oxidation [12,13]. In fact, protein carbonylation occurs when proteins directly react with ROS, leading to the formation of carbonyl groups (aldehydes and ketones) for instance on such amino acids side chains as arginine, lysine, threonine and proline. Introduction of carbonyl groups on proteins can also occur through the reaction of aldehydic products of lipid peroxidation and of dicarbonyl compounds upon glycation and glycooxidation. Since it often leads to a loss of protein function and an increased thermosensitivity or hydrophobicity of the targeted protein [14,15], protein carbonylation has been considered as an indicator of protein damage.

In human skeletal muscle, preliminary studies on *vastus lateralis* and external intercostal muscles have shown increased accumulation of protein carbonyls during ageing [16–19]. However, in most cases, the protein targets of these oxidative damages and their functional consequences have not been identified. Indeed, this is an essential step to get a complete view of protein oxidative modifications and to understand the mechanisms by which these oxidized proteins potentially contribute to muscle weakness and dysfunction during ageing.

Therefore, proteomic studies, including the analysis of protein abundance as well as protein carbonylation are expected to provide valuable information to unravel the key molecular pathways implicated. In fact, proteomics and in particular bi-dimensional (2D) gels represent appropriate tools for the detection and identification of specific carbonylated proteins in a complex mixture [2,13,20]. The identification of such oxidatively modified proteins (i.e. the oxi-proteome components), can give some insights into the mechanisms by which these damaged proteins accumulate and potentially affect cellular and/or tissular function during ageing or in disease conditions [21]. In this paper, the occurrence and characterization of carbonylated proteins was studied in human *rectus abdominis* muscle obtained from young and old healthy donors. Although no significant differences in global protein carbonylation was observed at the proteome level, we have used 2D gel electrophoresis based proteomic approaches to improve the resolution of individual proteins for the quantitative analysis of their carbonylation status and further identification of these major skeletal muscle proteins that are targeted by oxidative damage during human *rectus abdominis* skeletal muscle ageing.

## Material and methods

### Human biopsies

Human *rectus abdominis* muscle biopsies were obtained during surgery. Each biopsy used has the written consent of the volunteer donor. A total of 22 human muscle biopsies were used: 11 from healthy men individuals between 0 to 12 years old (named young samples) and 11 from healthy men individuals between 52 and 76 years old (named old samples) (Table 1). All muscle biopsies had an initial wet weight between 15 and 24 mg (Table 1). The study was approved by the Ethical Committee at the Uppsala University Hospital.

### Protein extraction for proteomics analyses

Proteins extracts from skeletal muscle biopsies were obtained by physical disruption of the sample biopsies using a ULTRA-TURRAX® T25 (IKA®) at 4 °C in a lysis buffer containing 10 mM Tris-HCl (pH 7.4), 8 M urea, 2 M thiourea, 4% CHAPS and 20 mM

**Table 1**  
Characteristics of the samples biopsies.

Sample no	Donor age (years)	Wet weight (mg)	Protein recovery ratio (w/w in %) <sup>a</sup>
1	0	24.00	7.0
2	1	20.80	4.3
3	5	20.00	7.6
4	9	17.00	9.3
5	6.5	15.00	7.1
6	10.75	21.90	7.6
7	12	22.00	9.1
8	7.1	22.00	9.7
9	76	20.30	9.5
10	70	18.00	8.3
11	74	19.10	7.8
12	66	19.60	8.0
13	74	19.20	8.4
14	65	18.66	5.2
15	65	18.78	6.6
16	71	24.00	10.8
17	12	20.00	15.6
18	3	20.50	3.6
19	9	20.38	11.0
20	56	20.47	7.4
21	61	20.53	17.1
22	52	20.13	27.2

<sup>a</sup> Protein recovery ratio corresponds to the protein amount in mass / biopsy mass.

DTT. After incubation on ice for 20 min, soluble proteins were recovered after clarification by centrifugation for 40 min at 21,000 g. Proteins were further precipitated using the 2D clean-up kit (GE Healthcare) and the resulting pellet was re-suspended into the same lysis buffer. Protein concentrations were determined by the Bradford Method [22] using the Bio-Rad Protein Kit Assay (Bio-Rad).

### Protein carbonyl immunodetection after derivatization with DNPH

Carbonylated proteins were derivatized with 2,4-dinitrophenylhydrazine (DNPH) to form 2–4-dinitrophenylhydrazone (DNP) proteins adducts [23]. For total carbonyl quantification, equal quantities of proteins were loaded and separated by SDS-PAGE 12% (v/v). Chemicals for SDS-PAGE were purchased from Bio-Rad. All other chemicals were of analytical grade and obtained from Sigma-Aldrich. For the detection of carbonylated proteins, gels were electrotransferred onto Hybond-C nitrocellulose membranes (GE Healthcare) and incubated with anti-DNP antibodies (1:5000, Sigma-Aldrich). Carbonylated proteins were revealed by a fluorescent anti-rabbit IgG 800CW (1:15,000) polyclonal antibody (LI-COR). Densitometry analyses were performed using NIH ImageJ software and the data are expressed as % volume in pixels.

For 2D gel electrophoresis, derivatization of proteins carbonyls was achieved on IPG strips after isoelectric focusing (IEF), with a 10 mM DNPH, 2 M HCl solution at room temperature (RT) as described previously [24]. 500 µg of protein were diluted on a re-hydration buffer (7 M urea, 2 M thiourea, 1% Amberlite, 4% CHAPS, 1.2% Destreak (GE Healthcare), 0.5% Pharmalyte pH 3–10 (GE Healthcare)) and loaded into 13 cm IPG strips pH 3–10 NL (GE Healthcare). Gel rehydration of the IPG strips was done overnight at RT in an Immobiline DryStrip rewelling tray. IEF was performed using an Ettan™ IPGphor™ 3 Isoelectric Focusing System (GE Healthcare) at 20 °C using the following electrical profile: step, 150 V for 11 h; grad, 1000 V for 3 h; grad, 8000 V for 2 h; step, 8000 V for 1 h. Neutralization step after derivatization with DNPH was performed with 2 M Trizma-Base containing 30% of glycerol (GE Healthcare). Before SDS-PAGE, all IPG strips were equilibrated with a 6 M urea, 10% SDS and 30% Glycerol (GE Healthcare), 0.5 M Tris-HCl (pH 8.8) solution for 2 steps of 15 min: the first one with

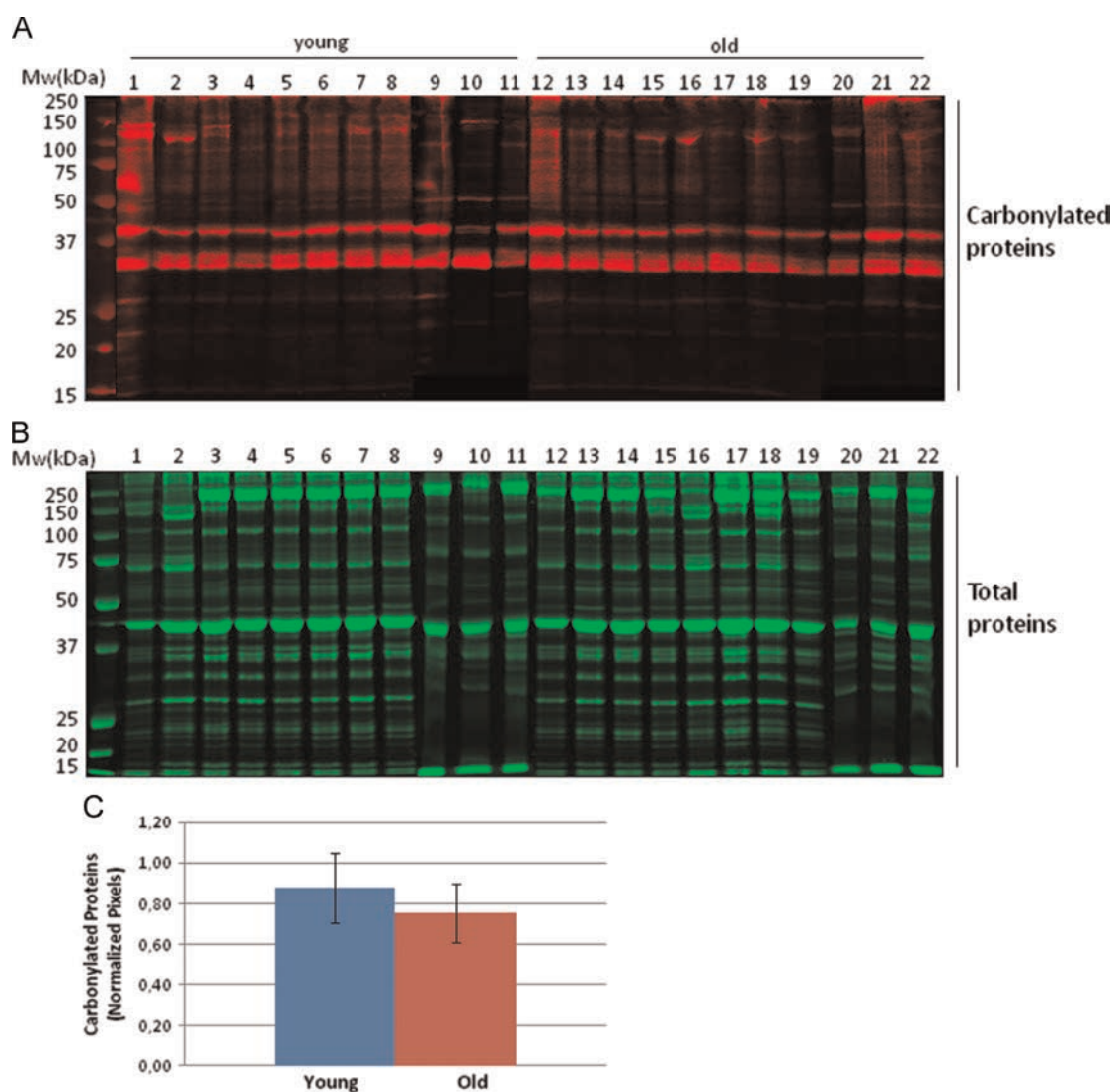
equilibration solution containing 1% DTT and the second with equilibration solution containing 3% iodoacetamide. SDS-PAGE was carried out using the Protean II system (Bio-Rad). For each sample, two 12% (v/v) polyacrylamide gels were performed in parallel: one for total protein stains with Coomassie Brilliant Blue G-250 (Bio-Rad) for further mass spectrometry analysis; and the other with derivatized protein residues for electrotransfer onto nitrocellulose membranes, where total amount of proteins were stained by a Fast Green solution (Sigma-Aldrich) prior to antibodies incubation for loading control. Membranes were then blocked with Odyssey blocking buffer (LI-COR) overnight at 4 °C. Primary anti-DNP antibodies (1:5000, Sigma-Aldrich) were incubated for 1 h at RT. Revelation was done by a fluorescent anti-rabbit IgG 800CW (1:15,000) polyclonal antibody (LI-COR). Washing steps were done with a PBS/0.1% Tween solution. Carbonylated proteins were revealed by the Odyssey Infrared Imaging System (LI-COR).

#### Proteomics data acquisition and analysis

Spot detection and quantification were carried out using the image Master 2D Platinum 7 software (GE Healthcare). For comparison, we used the data expressed as spot % volume (%vol) in pixels, which corresponds to a normalized value of the spot volume by considering the total volume of all the spots present in the membrane [25]. To take into account the total amount of loaded protein in each IPG strip, we defined a %vol per carbonylated spot (N%vol) by normalizing the %vol of each anti-DNP incubated membrane with its corresponding %vol on the Fast Green-stained membrane. The relative modification index ratio (RMI ratio) was obtained by dividing the N%vol of the old group of samples by the N%vol of the young one.

#### In-gel digestion and mass spectrometry

Spots of interest were manually excised from Coomassie Blue stained 12% (v/v) polyacrylamide gels and were automatically in-



**Fig. 1.** Analysis of total carbonylated proteins of human *rectus abdominis* muscle biopsies. (A) Carbonylated protein profiles from young and old human biopsies after 1D gel electrophoresis. Protein carbonyl were detected by Western-blotting against protein-DNP derivatives and monitored by fluorescent secondary antibody hybridization. (B) Total protein profiles stained with colloidal Coomassie brilliant blue G after 1D gel electrophoresis. (C) Densitometric analysis of carbonylated protein western-blot. Semi-quantitative assessment of modified proteins was done using total protein staining for normalization. Relative values are expressed as mean  $\pm$  S.D. ( $n=11$ ) and no significant difference was found between the young and old groups.

gel digested by trypsin [26] using a robot Freedom EVO 100 digester/spotter robot (Tecan). Resulted peptides were then desalted for mass spectrometry (MS/MS) analysis. Results obtained were subjected to a search on the SwissProt database using the MASCOT software (Matrix Science Ltd., London, UK). The software compares the MASCOT peptide sequences derived from spectra with those in libraries for protein identification. Search parameters were as follows: database, SwissProt; taxonomy, all entries or mammalian; enzyme, trypsin; allow up to one missed cleavage; fixed modifications, none; variable modifications, methionine oxidation; peptide mass tolerance, 70 ppm; and fragment mass tolerance, 500 ppm.

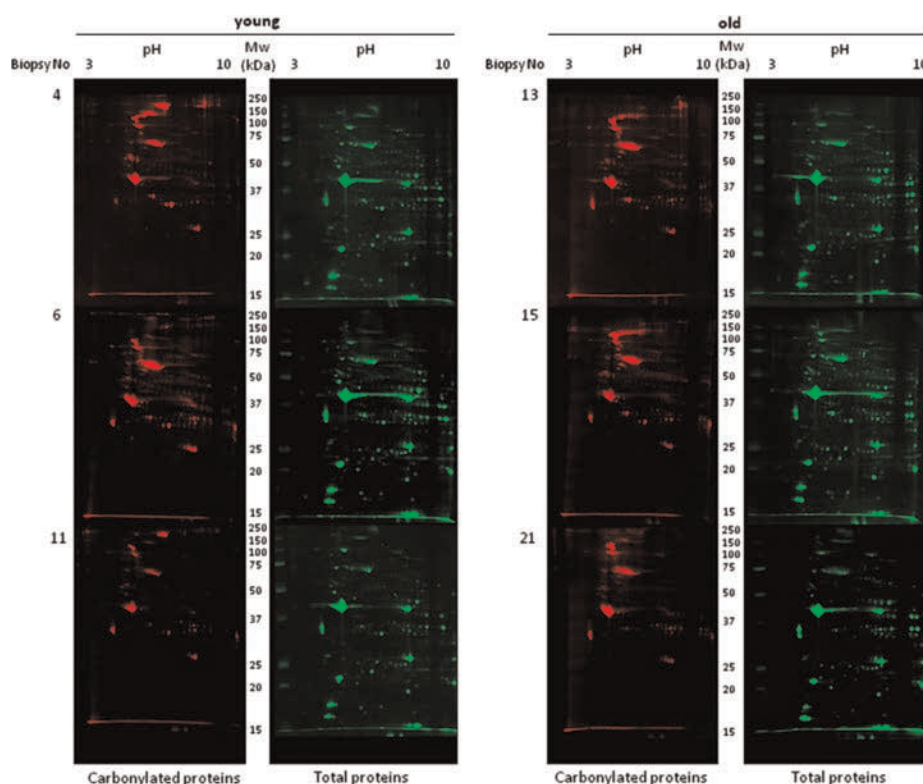
## Results and discussion

Initial screening looking at changes at the global proteome level of carbonylated proteins was performed after derivatization of protein carbonyls with DNP followed by immunodetection of DNP protein adducts after SDS-PAGE (Fig. 1A). Densitometry analysis was performed and normalized by total protein content for each sample (Fig. 1B and C). No significant differences at the global level in protein carbonyl content was detected between groups, in agreement with what was previously reported by Marzani et al. who did not find a statistical difference on protein carbonyl content during ageing in both *rectus abdominis* and *vastus lateralis* human muscles [18]. More recently, Fanò et al., using skeletal muscle biopsy samples obtained from *vastus lateralis*, found that protein carbonyls, showed a significant increase during ageing. However, this difference was not significant after splitting by gender [27]. Importantly, immunodetection of carbonylated proteins after one-dimensional (1D) electrophoresis separation has

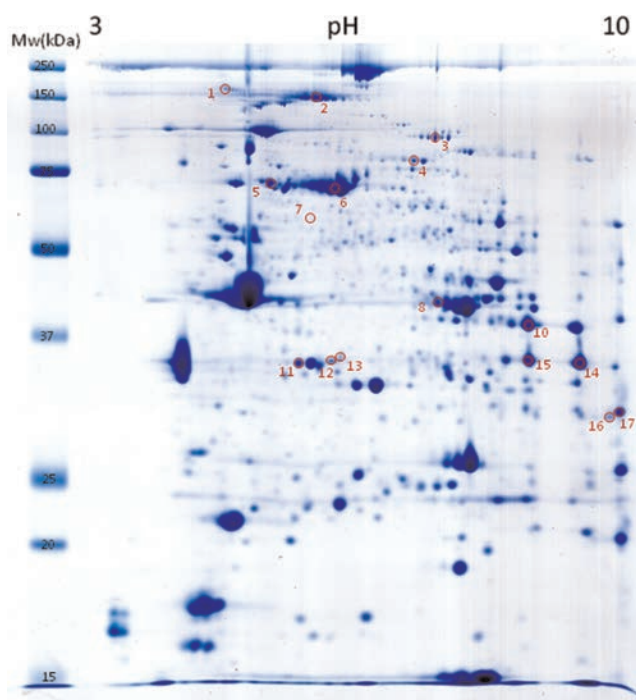
serious limitations for the resolution of single protein bands and can provide only restricted information.

To further analyze the occurrence of protein carbonylation at the single protein level, 2D gel electrophoresis separation of protein extracts was performed prior to immunodetection of carbonylated proteins. 2D gels are very appropriate to investigate protein isoforms since many post-translational modifications (such as carbonylation of arginine or lysine residues) often leads to changes in the isoelectric point of proteins, and thus shift the position of the protein in 2D gels. After electrotransfer onto nitrocellulose membranes total protein profiles were obtained by fast-green staining (Fig. 2). All the analyzed samples displayed a similar protein migration pattern, suggesting no drastic shifts in the protein profiles at the expression level (Fig. 2, right panels) between the two experimental groups. Immunodetection of carbonylated proteins was performed after their derivatization by DNP as described in Material and methods. Interestingly, the pattern of the modified proteins was not superimposable with the pattern obtained for the total protein staining, indicating that certain proteins represent preferential targets for these deleterious oxidative modifications. Several proteins of high molecular weight were found preferentially carbonylated (Fig. 2, left panel) while certain proteins with an isoelectric point higher than 5, also appeared to be preferentially carbonylated.

A relative modification index (RMI) per spot was calculated in order to evidence differentially carbonylated proteins between the two experimental groups taking into account their protein expression levels. Seventeen protein spots exhibited an RMI ratio consistently higher than 1.3 (increasingly carbonylated) in all biopsies from the aged group as compared with the young one. On the other hand, fourteen protein spots showed decreased carbonylation in the old group (RMI < 0.7). Protein spots evidenced as



**Fig. 2.** Oxi-proteome analysis of young and old human skeletal muscle samples. Protein extracts from young ( $n=3$ ) and old ( $n=3$ ) human skeletal muscle biopsies were separated by 2D gel electrophoresis. After the second dimension, gels were electrotransferred onto nitrocellulose membranes for subsequent immune detection of DNP-derivatized carbonylated proteins (left panels). Densitometry analysis was done by image Master 2D software (GE Healthcare) using total protein staining (right panels) as loading control.



**Fig. 3.** Coomassie blue staining of one representative 2D gel. The numbers and positions of the 17 selected spots identified by MS/MS correspond to those found as consistently increased on the old group (RMI > 1.3).

increasingly carbonylated were excised from Coomassie Blue stained 2D gels (Fig. 3) and analyzed by MS/MS for protein identification. Protein spots that were identified in the aged group are listed in Table 2. Among the identified proteins, 8 are muscle specific, 4 are ubiquitous in different tissues and organ systems, and 2 proteins belong from plasma (Table 2).

The identified skeletal muscle proteins were then analyzed and grouped by metabolic pathways and cellular functions. Major biological functions include muscle contraction, energy transduction and energy metabolism (Fig. 4). Among proteins involved in muscle contraction, we have found that myosin 7, troponin T, myosin-binding protein C (MyBPC), and LIM domain-binding protein 3 (ZASP) are increasingly carbonylated in aged muscle. Interestingly, the decreased speed of contraction observed in old age at both the muscle and motor protein levels is a hallmark of skeletal muscle ageing [28–31]. Myosin is a highly conserved protein that converts chemical energy into mechanical force and a key protein for muscle contraction. Previous studies have shown increased glycation of myosin in both fiber types of aged rats [32]. In addition, the carbonylated residues have been identified [33]. Interactions of myosin with cytoskeletal proteins such as titin, myomesin/M protein and MyBPC play an important role in thick filaments physiology. MyBPC contributes to the assembly and stabilization of thick filaments and modulates the formation of actomyosin cross-bridges, via direct interactions with both thick myosin and thin actin filaments [34,35]. The importance of MyBPC to muscle contraction is further emphasized by the discovery that mutations in genes encoding MyBPC cause myopathies in both skeletal [36,37] and cardiac muscles [38–40]. Increased oxidation of MyBPC, together with the previously reported age-dependent decrease of myosin isoforms and regulatory proteins like myosin binding proteins C and H [41–44], may contribute to the destabilization of muscle fibers. In addition, perturbations in the thin sarcomere filaments of muscle fibers have also been associated with differently expression of actin and its regulatory proteins such as troponin and tropomyosin [42,43,45,46]. Here we found

increased carbonylation levels of troponin T in the elderly. Troponin inhibits the actomyosin  $Mg^{2+}$ -ATPase and the  $Ca^{2+}$  release from the sarcoplasmic reticulum inducing a change in the troponin-tropomyosin conformation that exposes myosin binding sites on the actin filament and activates the myosin ATPase, thereby allowing muscle contraction [47,48]. Since muscle contraction depends on myofibrillar thin filament interactions [49], the oxidative damage to these proteins may well compromise this interaction and hence muscle contraction.

The motor functions of striated muscle crucially depend on the highly ordered arrays of thick myosin and thin actin filaments in sarcomeres. Although its detailed role has not been elucidated yet, the LIM domain-binding protein 3 (ZASP), found as increasingly carbonylated in aged *rectus abdominis* muscle, functions as an adapter to couple protein kinase C-mediated signaling to the cytoskeleton and to maintain Z-disc stability as well as cytoskeletal ultrastructure during contraction. Accumulation of this protein in its oxidized form may lead to protein aggregates formation and myofibril disintegration that may result in muscle weakness.

Muscle contraction depends also in high-energy fluxes where creatine kinase plays a central role. During muscle contraction, myosin hydrolyzes ATP into ADP upon filament sliding. The renewal of ATP is achieved mainly by the phosphocreatine-creatine kinase (PCr-CK) system. Among the creatine kinase (CK) isoenzymes, the muscle-type CK (MM-CK) specifically binds to the myofibril M-line and is associated with the action-activated myosin ATPase as an intramyofibrillar ATP regenerator [50–52]. Since this MM-CK was found irreversibly oxidized in old *rectus abdominis* muscle, this could alter muscle metabolism and compromise muscle contraction in the elderly. Besides the PCr-CK system, the glycolytic network and its closer interaction between mitochondria and organelles also provide energy within muscle cells [53]. In this study, 3 glycolytic enzymes appear as highly carbonylated in old slow oxidative skeletal *rectus abdominis* muscle: the fructose-bisphosphate aldolase A, the glycerol-3-phosphate dehydrogenase (GPD1) and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 2). Interestingly, several of these enzymes involved in anaerobic metabolism have been found decreased with age in murine and human skeletal muscle [54,55].

Although slow skeletal muscles uses mainly oxidative mitochondrial processes to generate the levels of ATP needed to maintain contractile activity for long time without showing fatigue [53], they also rely in glycolysis for their energy production [56]. For this reason, muscle metabolism and contraction in aged skeletal muscle would be affected by irreversible carbonylation on glycolytic proteins, as previously reported in certain diseases when these glycolytic enzymes are not functional [57–62]. Defects in the muscle form of glycogen phosphorylase, another protein found increased carbonylated in old *rectus abdominis* muscle, are involved in type 5 glycogen storage disease, also known as McArdle disease, a myopathy also characterized by exercise intolerance, such as easy fatigability, muscle cramps and contractures as well as muscle weakness [63,64]. Intramuscular glycogen acts as a readily available source of glucose-6-phosphate for glycolysis within skeletal muscle and glycogen phosphorylase catalyses the rate-limiting step in glycogenolysis [65]. Therefore, a deficiency in glycogen phosphorylase may result in the inability to mobilize muscle glycogen during anaerobic metabolism [66].

Although it is well recognized that ageing causes changes in the proteome, the nature and targets of these changes, their consequences on skeletal muscle function and how they may contribute to sarcopenia have not yet been completely elucidated. Our results suggest that oxidative stress during skeletal muscle ageing targets the contractile machinery, but also structural and regulatory proteins. In addition, the main mechanism of energy production, the phosphocreatine kinase system was affected by



**Table 2**

Localization of carbonylated proteins identified from old skeletal muscle biopsies.

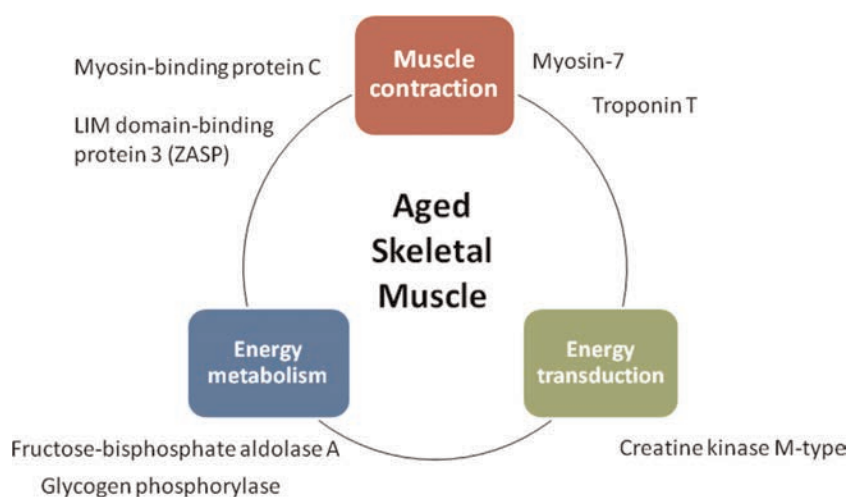
Protein name and localization	Swiss-Prot accession no	Protein spot no <sup>a</sup>	Mascot score <sup>b</sup>	Sequence coverage (%)	No. of sequenced peptides	Theoretical protein mass (kDa)	Theoretical PI	RMI ratio <sup>c</sup>
<b>Ubiquitous proteins:</b>								
Collagen alpha-1(VI) chain	CO6A1_HUMAN	1	205	7	5	108	5.3	4.34
Heat shock cognate 71 kDa protein	HSP7C_HUMAN	5	256	15	5	71	5.4	1.63
Glycerol-3-phosphate dehydrogenase [NAD <sup>+</sup> ], cytoplasmic (GPD1)	GPDA_HUMAN	13	214	22	4	38	5.8	1.52
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	G3P_HUMAN	14	243	12	3	36	8.6	1.67
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	G3P_HUMAN	15	424	25	5	36	8.6	1.68
Voltage-dependent anion-selective channel protein 1	VDAC1_HUMAN	16	685	39	8	31	8.6	1.88
<b>Muscle specific proteins:</b>								
Myosin-binding protein C, slow-type (MyBPC)	MYPC1_HUMAN	2	119	3	3	128	5.8	1.54
Glycogen phosphorylase, muscle form	PYGM_HUMAN	3	882	22	13	97	6.6	1.67
Myosin-7	MYH7_HUMAN	7	63	–	1	223	5.6	3.12
Creatine kinase M-type	KCRM_HUMAN	8	206	9	3	43	6.8	1.44
Creatine kinase M-type	KCRM_HUMAN	9	416	20	6	43	6.8	1.54
Fructose-bisphosphate aldolase A	ALDOA_HUMAN	10	611	31	7	39	8.3	1.65
Troponin T, slow skeletal muscle	TNNT1_HUMAN	11	512	22	6	33	5.9	1.87
Troponin T, slow skeletal muscle	TNNT1_HUMAN	12	244	22	5	33	5.9	1.42
LIM domain-binding protein 3 (ZASP)	LDB3_HUMAN	17	206	8	4	77	8.5	1.70
<b>Plasma proteins:</b>								
Serotransferrin	TRFE_HUMAN	4	399	17	10	77	6.8	1.31
Serum albumin	ALBU_HUMAN	6	986	29	13	69	5.9	1.33

Spots of interest were identified by MALDI-TO-FTOF-MS as described under Material and methods. For each spot, different parameters clarifying protein identification by MS are indicated.

<sup>a</sup> Protein spot number refers to the numbered spots in Fig. 3.

<sup>b</sup> Mascot protein scores greater than 56 are significant ( $P < 0.05$ ).

<sup>c</sup> RMI ratio represents the Relative Modification Index ratio.



**Fig. 4.** Functional grouping of muscle proteins increasingly oxidized with age. Increasingly oxidized muscle-specific proteins identified in aged *rectus abdominis* biopsies were grouped in three functional categories: muscle contraction, energy metabolism and energy transduction.

carbonylation in the elderly while other energetic metabolic pathways such as glycolysis appear to be also affected. Finally, the heat shock 70 kDa protein (HSP70), a key player in protein quality control with different identified roles in skeletal muscle [67], such as protection against oxidative stress, was also found highly carbonylated in old skeletal muscle. Up-regulation of heat shock proteins is a well known feature of muscle ageing [46,68,69]. Further studies should address the functional status of the identified carbonylated proteins and related cellular pathways associated

with muscle dysfunction in order to reveal their role in the development of the ageing phenotype.

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