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Role of p53 family in muscle wasting

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Abstract

Muscle atrophy in cachexia results from the imbalance between protein synthesis and degradation due to activation of the ubiquitin-proteasome pathway. Literature suggests that p53 family members play a role in controlling proliferation, differentiation and death of precursors and muscle fibers. Here we characterize the expression profile of the p53 family members in muscle atrophy in ALS (Amyotrophic Lateral Sclerosis) and in doxorubicin induced cachexia model. We revealed an increased expression of the p53 family members and atrogenes in a correlated manner on both models and a transcriptional activation of Trim63 by p53, p63 and p73. In addition, we tested whether compounds of tocopherol harboring antioxidant activity might reduce muscle atrophy. We showed that this compound counteracts the induction of the Notch pathway, important to muscle development and regeneration.

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Abbreviations

ALS	Amyotrophic lateral sclerosis
AMPK	AMP-activated protein kinase
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma 2
CDK	Cyclin-dependent kinase
DBD	DNA-binding domain
ER	Endoplasmic reticulum
FASN	Fatty acid synthase
FoxO	Forkhead box class O
GADD45	Growth Arrest and DNA Damage-inducible 45
IFNγ	Interferon gamma
IGF-1	Insulin-like growth factor 1
IL-1	Interleukin 1
IL-6	Interleukin 6
LKB1	Liver kinase B1
MAFbx	Muscle Atrophy F-box, Atrogin-1
MDM2	Mouse double minute 2 homolog
MRFs	Myogenic regulatory factors
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1

MuRF1	Muscle Ring Finger protein 1
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OD	Oligomerization domain
PAX	Paired-box transcription factors
Peg3	Paternally-Expressed Gene 3 Protein, PW1
PI3k	Phosphatidyl-inositol 3-kinase
pRb	Retinoblastoma protein
PUMA	p53 upregulated modulator of apoptosis
ROS	Reactive oxygen species
S6K1	p70 ribosomal protein S6 kinase 1
SOD1	Cytosolic Cu/Zn superoxide dismutase
TIGAR	TP53-inducible glycolysis and apoptosis regulator
TNF-α	Tumor necrosis factor alpha
Trim63	Tripartite Motif Containing 63

Introduction

I. Muscles – Physiology, anatomy and myogenesis.

Movement is indispensable for the organisms' life. The simple fact of breathing or moving an arm would be impossible to accomplish without muscle. Because of its essential role, muscles are one of the most abundant and energy-consuming tissues present in animals. Muscles fibers are formed by actin and myosin filaments that enable the contraction mechanism and, due to the different specialized tasks, they evolved in specific manners and were classified into two categories: the smooth and the striated muscles, of which later one is further divided in skeletal and cardiac muscles.

- **I - 1. Skeletal muscle**

Skeletal muscles are the ones involved with the voluntary movements. They are attached to the bones by tendons and are formed by multinucleated cells named muscle fibers or myofibers responsible for both fast and slow twitch. Each myofiber contains several myofibrils wrapped in a membrane called sarcolemma. Furthermore, they can be slow muscle fibers (type I) or fast muscle fibers (type II). The first ones are more efficient at continuously transforming oxygen in fuel by an aerobic metabolism and that is why they have more mitochondria and a rich blood supply. Slow muscle fibers are, for example, especially important for athletes who run marathons. On the other hand, fast muscle fibers, as the name also suggests, are required for rapid and powerful contractions. Even though they fatigue more quickly due to their anaerobic metabolism, type II fibers are better for bursts of speed and strength (Hill & Olson, 2012). The variable mixture of both fiber types composing the muscles determines its overall contractile property.

- **I – 2. Cardiac muscle**

Although part of the category of the striated muscle tissue, the cardiac muscle or myocardium consists in branched individual cardiomyocytes and is involuntarily controlled. This tissue is found specifically in the heart walls and plays an important role

in the contraction for the heart (heart beating). Because of its function to pump blood into the arteries, the heart must contract permanently and in a coordinated manner. Nature solved this problem by connecting cardiomyocytes by structures called intercalated discs in order to form a syncytium, while gap junctions assure electrical and molecular communication from cell to cell and form a network that allows a synchronized and efficient contraction.

• I – 3. Smooth muscle

Smooth muscle lacks striations and contains less myosin than the striated ones. Characterized by slow and involuntary movements, they are responsible for the digestive tract, urinary bladder, uterus, blood and lymphatic vessels contractions.

• I – 4. Muscle development (myogenesis) and regeneration

Like most of other tissues, muscle development takes place during embryogenesis and the big majority of this tissue derives from the mesodermal germ layer. Since my work is particularly focused in striated skeletal muscles, I will not go into details about cardiac and smooth muscles development.

I – 4.1. Myogenesis

According to its position relative to the midline, mesoderm is divided into lateral, intermediate and paraxial. The mesoderm alongside the neural tube, named paraxial mesoderm, will be divided in blocks called somites during embryonic development, which will subsequently originate practically to all skeletal muscles in the body (Bentzinger *et al.,* 2012). During the process of myogenic differentiation in skeletal muscle, the embryogenic progenitor cells, under influence of the transcriptions factors Pax7 and Pax3, can either stay in a quiescent undifferentiated stated as satellite cells or can be activated and differentiate to become myoblasts cells via the activity of the myogenic regulatory factors (Mrfs), Myf5 and MyoD. Later on, during the process of cell differentiation, MyoG (or myogenin) and Mrf4 are required for myocytes, differentiated forms of myoblast, to fuse and form the contractile units - myofibers - that will have their contraction controlled by a motor neuron (Figure 1) (Tajbakhsh, 2009).

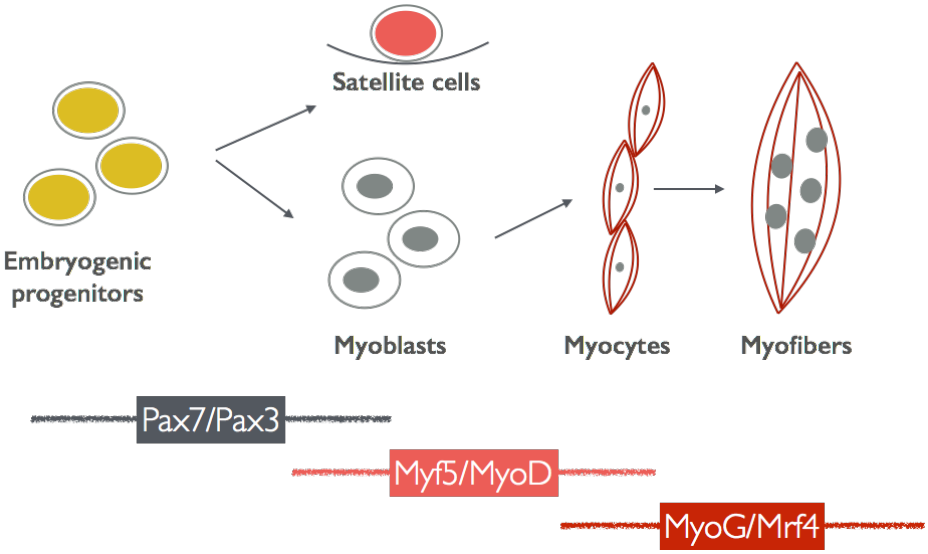

Figure 1 – Scheme of myogenic differentiation in skeletal muscle and the respective transcription factors involved in each step of myogenesis. Adapted from Zammit *et al.*, 2006 and Bentzinger *et al.*, 2012.

I – 4.2. Muscle regeneration

Of course muscles, like any other tissue, are susceptible to damage, pathological conditions and ageing; processes that can impair its function and lead to weakening on muscle strength, contraction, body stability and decrease in quality of life. Providentially, the muscle somatic stem cell or satellite cells, situated close to the sarcolemma, gives to muscle tissue the remarkable ability to regenerate in response to some of these harmful or physiological conditions. Interestingly, the regeneration mechanism shares some features with the one that drivers embryogenic myogenesis (Karalaki *et al.*, 2009). However, two distinct phases have to be carefully coordinated during the regeneration process.

The first one involves a brief muscle fiber necrosis that will deliberately induce inflammation, which results from disruption of the myofiber sarcolemma and myofiber integrity, leading to recruitment and infiltration of inflammatory cells. In the second phase of the regeneration process, tissue reconstitution takes place and the satellite cells are required to activate and induce myoblast proliferation. Afterward the cells proceed until their complete differentiation into myofibers like described above (Chargé & Rudnicki, 2004; Tajbakhsh, 2009).

II. Balance between hypertrophy and atrophy.

Muscle is a tissue remarkably plastic. As it was mentioned, they are perfectly able to regenerate, but in addtion, muscle fibers can adapt according to the metabolic and functional demands in the body, which means that the more they are necessary the more myofibers will develop, however the contrary is also true. Because of this dynamic environment, the rate of protein synthesis and protein degradation is always in a delicate balance and must be tightly regulated. Increase of muscle cells proliferation by satellite cells (hyperplasia) is the main process how muscle mass is increased during embryogenesis or maintained in adults (Fernandes *et al.,* 2012). In contrast, under stimuli like physical activity, growth factors or availability of nutrients, skeletal muscle mass increases by increasing the size of the pre-existing myofibers, process called hypertrophy. In fact, hypertrophy settles down when the overall rate of protein synthesis exceeds the rate of protein degradation in the muscle cells. Inversely, muscle loss occurs when the rate of protein degradation exceeds protein synthesis. Importantly, the molecular mechanisms that drive hypertrophy and atrophy are quite different and unique to each process (Sandri, 2008).

• II – 1. Molecular pathways in muscle hypertrophy

The main pathways in muscle atrophy are the IGF-Akt-mTOR pathway and the myostatin–Smad2/3 pathway, which collaborate to the regulatation of muscle hypertrophy in a positive or in a negative manner respectively (Schiaffino *et al*., 2013).

II – 1.1. The IGF-Akt mTOR pathway

In 2001, Musaro and colleagues had shown by using transgenic mice that skeletal muscle-specific overexpression of IGF-1 leads to muscle hypertrophy. Muscle growth was also observed when Akt was constitutively active in adult skeletal muscle (Lai *et al.,* 2004). In succinct words, IGF-1 (Insulin-like growth factor 1) activates Akt though phosphorylation by the PI3 kinase (PI3K). Thereafter, Akt stimulates protein synthesis by activating mTOR and its downstream effectors (Figure 2), like S6K1 and mTORC1 (Sandri, 2008). Indeed, *in vivo* studies showed that the selective mTOR inhibitor,

rapamycin, blocks hypertrophy (Pallafacchina *et al*., 2002), underlinying the importance of this pathway to muscle hypertrophy. The kinase mTOR responds to signals of growth factors, nutrients, and energy status, integrating different signaling pathways to control protein synthesis (Kim *et al*., 2002).

II – 1.2. The myostatin–Smad2/3 pathway

Conversely to the IGF-Akt-mTOR pathway, which regulates muscle hypertrophy, there is the myostatin–Smad2/3 pathway. Myostatin is produced by the skeletal muscle itself and negatively regulates muscle growth by Akt inhibition through SMADs proteins (Figure 2). Mutation in myostatin, for example, causes muscle hypertrophy in animal models as well as in humans (Lee, *at al*., 2001; Schuelke *et al*., 2004). Myostatin also plays a role during muscle regeneration since it is able to regulate Pax7, MyoD and myogenin expression, inhibiting satellite cells activation and consequently cell differentiation (Iezzi *et al*., 2004; McFarlane *et al*., 2007).

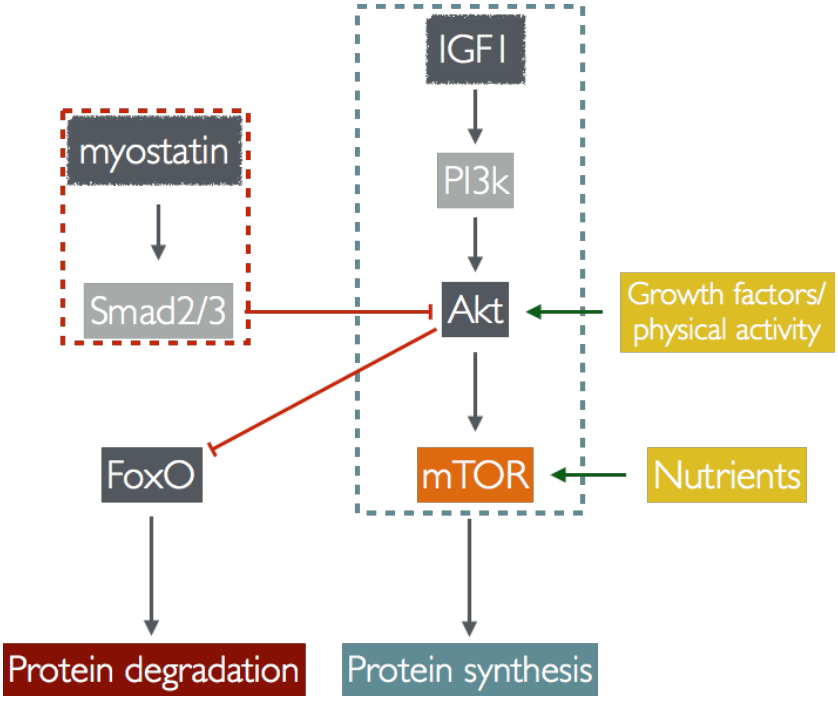

Figure 2 – Scheme of the major molecular pathways involved in the regulation of protein balance in skeletal muscle during hypertrophy. Adapted from Sandri 2008 and Schiaffino *et al*., 2013.

• II – 2. Molecular pathways in muscle atrophy

Although muscle atrophy is also a result of protein synthesis and degradation imbalance, it is not merely the inverse process of muscle hypertrophy. Not only because it can be stimulated by diverse conditions such as muscle disuse, starvation, denervation and aging, but also because muscles can play the "noble" role of serving as energy and amino acids body reservoir, which can be mobilized in stressful circumstances like cancer, uncontrolled diabetes, heart failure and sepsis (Foletta *et al.,* 2011; Wray *et al.,* 2013). During distinct catabolic conditions, proteins are mobilized from the muscles cells, which can be associated with remodeling of mitochondrial and sarcoplasmic networks and lost of myonuclei. These complex processes caused muscle atrophy and involve different molecular pathways that are also specific to the cause of the atrophy.

Due to these complex and contrasting situations leading to or influencing muscle atrophy, the molecular protein degradation mediators during atrophy process were hard to identify. However, in 2001, a group of scientists compared the gene expression profile of different muscle atrophy models in order to identify a subset of genes that are frequently up- or downregulated in atrophying muscle. The outcome of this experiment was the identification of two novel muscle specific ubiquitin ligases (E3): atrogin-1/MAFbx (Muscle atrophy F-box) and Trim63 (MuRF1-Muscle ring finger protein 1). These two genes were responsible for the protein degradation increase through the ubiquitin-proteasome system (Bodine *et al*., 2001; Gomes *et al.,* 2001) and found to be consistently upregulated in distinct models of muscle atrophy (diabetes, cancer cachexia, chronic renal failure, fasting, and denervation) (Lecker *et al.,* 2004).

II – 2.1. The ubiquitin-proteasome pathway

The ubiquitin-proteasome pathway is a proteolysis system responsible for the majority of intracellular protein breakdown in all cells of the body. In particular the 26S proteasome is the primary pathway of protein degradation in muscles during atrophy (Rock *et al.,* 1994). The proteasome basically recognizes ubiquitinated proteins and degrades them into small peptides through a multicatalytic protease complex. To ubiquitinate proteins, three enzymatic components are required to link chains of ubiquitin onto proteins destined for degradation (Figure 3) (Cao *et al.,* 2005; Sandri, 2013).

In an initial step, x (Figure 3) (Cao *et al*., 2005; Sandri, 2013).

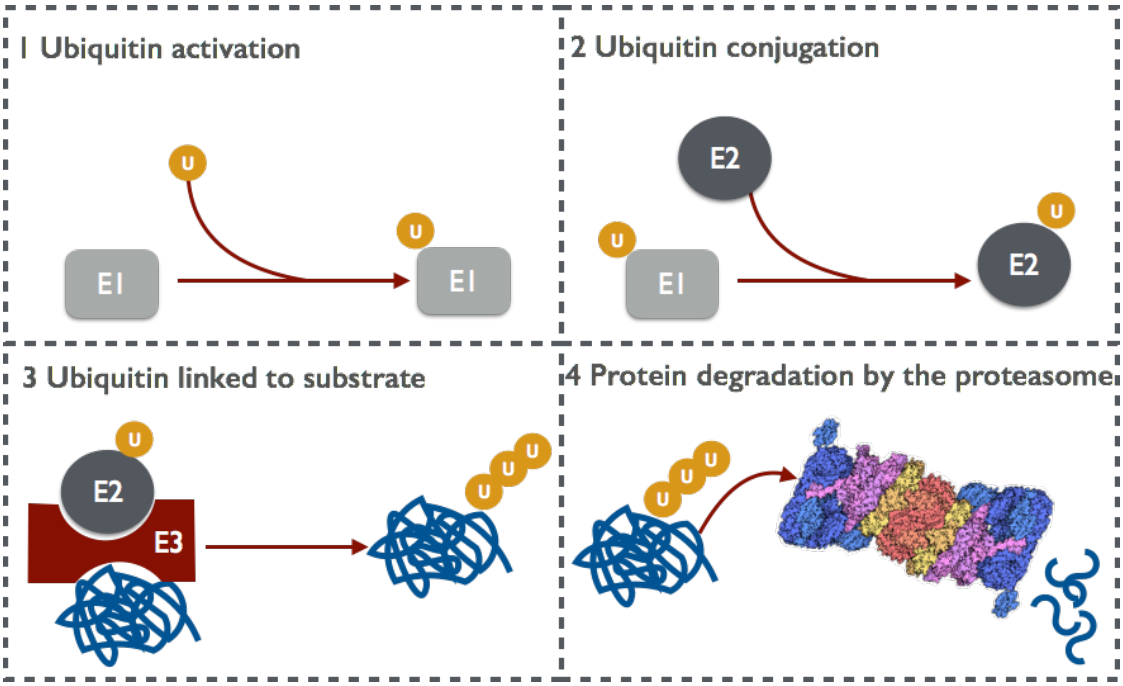

Figure 3 – Schematic diagram of the ubiquitylation system.

II – 2.2. Trim63 (Murf1) and Atrogin-1 E3 ubiquitin ligase

Among thousands of E3 ubiquitin ligase, Trim63 and Atrogin-1 stand out for being both muscle-specific and upregulated during muscle loss. The vast majority of E3s contains RING (really interesting new gene) finger domains are typically found in small proteins and can exist as monomers, dimers, or multisubunit complexes and, when assembled together, can carry the ubiquitin ligase activity (Deshaies, 1999).

Trim63 (Murf1) belongs to the TRIM superfamily of multidomain (RING domain, zinc-finger B-box domain, and leucine-rich coiled-coil domain) (Spencer *et al*., 2000; Centner *et al*., 2001; McElhinny *et al*., 2002); the E3 ubiquitin ligases and can be found as a monomer or dimer like its two other homologues, Murf2 and Murf3. They are all present in muscle cells but the Trim63 is the only family member shown to be related to muscle atrophy, while the others play a role in muscle development (Perera *et al*., 2012).

Atrogin-1 belongs to the cullin-RING E3 ligase (CRL) superfamily, which also contains a RING finger protein that permits it to associate with a cullin protein (CUL1) and recruit the ubiquitin-charged E2s. Unlike Trim63, Atrogin-1 is in a multisubunit complex, consisting of a SKP1 and a F-box protein that serve as the substrate adaptor element (Cardozo & Pagano, 2004).

Mice lacking Atrogin-1 and/or Trim63 showed to be resistant to denervation, fasting and dexamethasone-induced muscle atrophy (Bodine *et al.,* 2001; Baehr *et al.,* 2011; Cong *et al.,* 2011). Other experiments demonstrated their increased expression in a wide range of atrophy-inducing conditions and diseases like, immobilization, hindlimb suspension, denervation, ageing, sepsis, fasting, cancer, HIV, chronic obstructive pulmonary disease, diabetes and due to the use of glucocorticoids, (Lecker *et al.,* 1999, 2004; Bodine *et al.,* 2001; Gomes *et al.,* 2001; Jagoe *et al.,* 2002; Jones *et al.,* 2004). Taken together, these results highlight the relevance of the atrogenes as key regulators of muscle atrophy. However, the fact that Trim63 and Atrogin-1 are both transcriptionally upregulated under most atrophy-inducing conditions does not mean that they always behave the same way. Indeed, each one of them has specific targets and is differently regulated in distinct types of atrophy, playing distinct roles.

Trim63, for example, is associated to the degradation of myofibrillar protein through ubiquitination of muscle structural proteins, like troponin I (Kedar *et al.,* 2004), myosin heavy chains (Clarke *et al.,* 2007) and actin (Polge *et al.,* 2011), while Atrogin-1 is related to growth processes, since it promotes degradation of MyoD (Tintignac *et al.,* 2005). In response to exercise and muscle injury, MyoD is triggered and promotes satellite cells activation and proliferation, contributing to myoblast differenciation and muscle regeneration (Fuchtbauer *et al.,* 1992; Sabourin *et al.,* 1999). Moreover, Atrogin-1 also interacts with eIF3-f, an activator of protein synthesis (Csibi *et al.,* 2009). In simple terms, Atrogin-1 controls protein synthesis whereas Trim63 regulates protein degradation molecular pathways.

II – 2.3. IGF-PI3K-Akt-FoxO signaling

In addition to what was showed in the section II – 1.1., the IGF-1/PI3K/Akt pathway also regulates the transcriptional regulators of the atrogenes belonging to the

FoxO genes' family. The class O-type forkhead transcription factors (FoxO) is expressed in skeletal muscle and consist in four genes; FoxO1, FoxO3, FoxO4 and FoxO6 (Biggs *et al.,* 2001). These factors are usually increased during muscle waste and promote *in vitro* and *in vivo* muscle atrophy by directly binding to the Trim63 and Atrogin-1 promoter (Sandri *et al.,* 2004; Waddel *et al.,* 2008). Transgenic mice overexpressing FoxO1 in skeletal muscle showed reduction in muscle mass (Kamei *et al.,* 2004). In energy stress, FoxO3 is activated via AMPK in myofiber, leading to Trim63 and Atrogin-1 upregulation (Sanchez *et al.,* 2012), whereas FoxO4 induces Atrogin-1 expression in response to TNF-α treatment (Moylan *et al.,* 2008).

To counteract these mechanisms, illustrating the complexity of protein balance interconnection pathways, Akt negatively regulates FoxO transcription factors through IGF-1/PI3K/Akt pathway (see Figure 2) (Calnan & Brunet, 2008). In type 2 diabetic mice, where PI3K and Akt activities are reduced, increased on FoxO phosphorylation was observed paralleling to Atrogin-1 and Trim63 upregulation (Wang *et al.,* 2006). This suggests that the Akt pathway promotes muscle growth and simultaneously blocks protein degradation (Stitt *et al.,* 2004).

II – 2.4. Inflammatory cytokines and NF-κB signaling

Inflammatory cytokines, notably TNF-α, are potent triggers of muscle wasting. Actually inflammation is present in certain conditions that lead to muscle atrophy, such as in cancer cachexia (discussed later). Such relationship is so evident that when TNF-α was originally described, it was called "cachectin" (Beutler *et al.,* 1985). Importantly, one of its mechanisms of action is through the activation of the *NF-κB* family of transcription factors.

It has been shown in muscles that NF-κB responds to pro-inflammatory signals, like TNF-α (Li & Reid, 2000), and activates Trim63 promoter, inducing muscle atrophy via the ubiquitin-proteasome (Cai *et al.,* 2004). Contrarily from Trim63, TNF-α stimulates Atrogin-1 expression not directly but via the p38-MAP kinase pathway (Li *et al.,* 2005). Interestingly, NF-κB is induced in muscle atrophy associated situations like ageing and cancer cachexia (Bar-Shai *et al.,* 2008; Rhoads *et al.,* 2010). In addition, it

was shown to be required for cytokine-induced loss of skeletal muscle (Ladner *et al*., 2003) and mice lacking NF-κB subunits are resistant to muscle atrophy (Hunter & Kandarian 2004). Finally, *in vitro* studies suggest that TNF-α also inhibits myocyte differentiation through a NF-κB activation, further enhancing the muscle atrophy (Langen *et al*., 2001).

II – 2.5. Glucocorticoids-induced muscle atrophy

It is described that glucocorticoid treatment induces Atrogin-1 and Trim63 expression and muscle wasting *in vitro* and *in vivo*. Curiously, even though glucocorticoid levels are elevated in many pathological conditions associated with muscle loss (Clarke *et al*., 2007; Schakman *et al*., 2008) and the glucocorticoid receptor activates Trim63 promoter (Waddel *et al*., 2008), most of its effects are indirect, frequently affecting pathways previously described (section *II – 1.1.)*.

For example, glucocorticoids are described to decrease IGF1 and increase myostatin production (Ma *et al*., 2001; Schakman *et al*., 2008). More downstream in the same pathway, glucocorticoids induce REDD1 expression, an inhibitor of mTOR activity (remember Figure 2) (Wang *et al*., 2006). Taken together, the ability of the different signaling pathway and transcription factors to cooperate in the modulation of Trim63 and Atrogin-1 may explain the variable expression patterns observed depending on the source of the muscle atrophy (Figure 4).

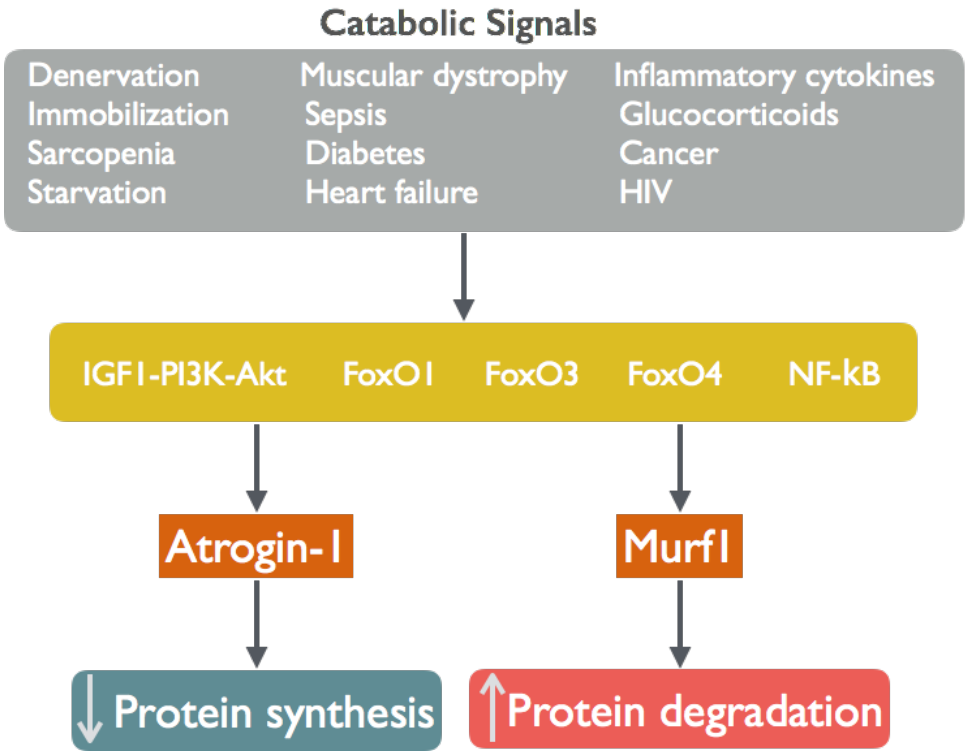

Figure 4 – Catabolic signaling pathways involved in Atrogin-1 and Trim63 regulation. Adapted from Foletta *et al*., 2011.

II – 2.6. The autophagy-mediated protein breakdown

In combination with the ubiquitin-proteasome pathway, the autophagic–lysosomal pathway form the two major pathways involved in protein breakdown (Sandri, 2013). Autophagy is a regulated process that allows degradation and recycling of damaged or non-functional cellular components. During this process there is a formation of autophagosome vesicles, which isolate parts of the cytoplasm and cell organelles supposed to be destroyed. Then, microtubule-associated protein 1 (LC3) is recruited to the autophagosomal membranes, fuses with lysosomes to form autolysosomes and the intra-autophagosomal components are then degraded by lysosomal acid proteases. The increase in LC3 synthesis makes it an important marker during autophagy (Glick *et al*., 2010).

It is described in the literature that autophagy is activated in skeletal muscle during fasting (Mizushima *et al*., 2004), disuse (Brocca *et al*., 2012), denervation (Zhao

et al., 2007), sepsis (Mofarrahi *et al.,* 2012) and cancer (Penna *et al.,* 2013). Moreover, genes related to the autophagic–lysosomal pathway, like LC3 and Gabarap, are upregulated during muscle atrophy, reinforcing its relation with muscle waste (Lecker *et al.,* 2004).

Interestingly, autophagy is regulated by mTOR through the Akt/mTOR pathway in response to nutrients and growth factors availability (see Figure 2) (Noda & Ohsumi, 1998). In addition, Mammucari and co-workers (2007) demonstrated that FoxO3 positively controls the activation of the autophagic/lysosomal pathway during muscle atrophy. This is in line with the fact that Akt negatively modulates FoxO (See section II – 2.3) (Brunet *et al.,* 1999), which improves protein degradation targeting the ubiquitin-proteasome pathway. Curiously, studies show that autophagy inhibition does not protect skeletal muscle from atrophy, it is indeed important to recycle organelles and prevent dysfunction accumulation (Masiero *et al.,* 2009; Masiero *&* Sandri, 2010). It is still unclear whether autophagy is part of the mechanisms that induce atrophy and would have a detrimental influence for the muscle or if it is a compensatory mechanism for cell survival.

III. Muscle atrophy in pathological conditions.

Loss of skeletal muscle mass is a physiological process that contributes to the protein balance in the cells. However, it can occur as part of certain diseases or simply due to the natural process of aging (sarcopenia). In these contexts, muscle loss leads to muscle weakness, impairing quality of life and increasing mortality.

As you noticed so far, the molecular pathways that orchestrate protein balance in muscles are extremely interconnected and some mechanisms are still poorly understood. Likewise, each of the conditions characterized by muscle waste (denervation, immobilization, sarcopenia, starvation, muscular dystrophy, ALS, sepsis, diabetes, heart failure, HIV and cancer) share common but also slight different features in the characteristics of loss of muscle mass (Romanick *et al.,* 2013), as I will outline with some examples below:

- **III - 1. Sarcopenia**

Sarcopenia is a progressive muscle mass loss that accompanies the physiological process of aging (Rosenberg, 1997). Due to the increasing life span, age-related diseases and disabilities have become a major health issue thus, decrease in muscle mass leading to fatigue and muscle weakness are a recurring problem affecting over 3% of the total world population (Iannuzzi-Sucich *et al.,* 2002).

The elevation in the rate of protein degradation starts approximately after the age of 50 and progresses at a percentage of 1–2% of muscle mass per year. Differently from disuse-induced muscle wasting where only fiber size is decreased, sarcopenia-induced atrophy is characterized by a loss in both fiber size and number (Lexell, 1993; Deschenes, 2004). Furthermore, decline in the density and regenerative capacity of satellite cells in elderly people impairs the regenerative capacity and consequently prolongs muscle recovery (Conboy & Rando, 2005).

The role of Trim63 and Atrogin-1 remain controversial in this specific situation. Experiments in aged rats show that expression of both atrogenes is suppressed in gastrocnemius (mixed fiber-type muscle) (Edstrom *et al.,* 2006); on the other hand, their levels are increased in tibialis, a fast-twitch muscle that contains predominantly fast muscle fibers (type II), suggesting a fiber-type-specific regulation of Trim63 and Atrogin-1 in sarcopenia (Clavel *et al.,* 2006). It is important to point out that in elderly people, release of growth factors and physical activity are usually diminished. Moreover, associated conditions like prolonged bed rest (muscle disuse) or diseases associated with the release of inflammatory cytokine, such as cancer, can enhance muscle atrophy. Currently, the most effective and used approaches to prevent sarcopenia are exercise and nutrition care. Regardless age, skeletal muscles respond to physical activity by increasing protein synthesis and muscle hypertrophy leading to increased muscle strength (Kosek *et al.,* 2006).

- **III – 2. Denervation**

Denervation is the loss of nerve connection and so, muscle ceases to be stimulated and are unable to contract. In other words, muscle paralysis is the immediate consequence of denervation. This condition can be caused either by physical damage like

in spinal cord injury or it can occur as consequence of disease as in amyotrophic lateral sclerosis (ALS) (Carvalho & Swash, 2006). In both case, decrease in muscle mass is associated with increased Trim63 and Atrogin-1 mRNA levels (Zeman *et al*., 2009; von Grabowiecki *et al*., 2016). In addition, it has been shown that knockout of both atrogenes protects skeletal muscle from denervation-induced atrophy (Bodine *et al*., 2001).

Experiments using denervated muscles models are convenient since denervation can be easily induced for example by sciatic nerve crush injury (Bauder & Ferguson, 2012). In one of the first denervation experiments done by Sarah Tower in cats (1935), the process was divided in three phases: muscle atrophy, acute degeneration and fibrotic dedifferentiation. It is important to point out that the rate of atrophy does not affect all muscle fibers homogeneously and also vary between different animal species (Gutmann & Zelená, 1962; Lewis, 1972). After denervation, loss of contractive tissue (muscle waste) is followed by loss of force, meaning a progressive twitch force reduction as atrophy settles down in the muscular tissue. Interestingly, it seems that the neuromotor activity is essential to maintain muscle physiological properties since fibrillation is not present in disused but innervated muscles. Apparently, electrical stimulation is able to preserve contractile properties (Pette, 2001)

III – 2.1. Amyotrophic lateral sclerosis – ALS

Regarding denervation associated to disorder, Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease marked by the degeneration of motor neurons, which leads to muscle denervation and consequently strong muscle atrophy and Atrogin-1 upregulation (Léger *et al*., 2006). The overall survival after diagnosis is about 3 years, since, in later stages, the progressive muscle waste leads to paralysis of respiratory muscles and death (Rowland & Shneider, 2001). The symptons of this incurable disorder usually appear between the ages of 50 and 65 and can happen without an obvious genetically inherited component in about 90% of the cases (the sporadic form of ALS) or be associated to genetic dominant inheritance factor in 10% of the cases (the familial form of ALS) (Greenway *et al*., 2006; Valdmanis & Rouleau, 2008).

It is a consensus in the literature that the main genes affected in ALS are C9orf72 and *SOD1*, the gene encoding for the antioxidant enzyme Cu/Zn superoxide dismutase 1

(Pasinelli *et al.,* 2004; Velde *et al.,* 2008; Ivanova *et al.,* 2014). Different mutations linked to these genes, like A4V or D90A in *SOD1*, contribute to the variability and aggressiveness of the disease (Andersen *et al.,* 1995; Luigetti *et al.,* 2009).

Mutated in about 20% of the cases of inherited ALS, the superoxide dismutase enzyme, as the name suggests, catalizes dismutation, which are basically redox reactions that transform the superoxide radical (O_2^-) into molecular oxygen (O_2) or H_2O_2, assigning to SOD a key antioxidant role. Indeed, oxidative stress is an important feature during the pathogenesis of ALS, since accumulation of ROS is toxic to neurons and muscle.

- **III – 3. Cachexia**

Cachexia is not simply characterized by muscle atrophy but it is a multifactorial complex syndrome accompanied by adipose tissue loss, fatigue, weakness, anemia, loss of appetite and alterations in carbohydrate, lipid and protein metabolism (Argilés *et al.,* 1997). Cachexia comes associated with a large number of chronic diseases such as AIDS (Grinspoon *et al.,* 2003), cancer (Argilés *et al.,* 2005), chronic renal failure (Cano, 2000), chronic obstructive pulmonary disease (COPD) (Reid, 2001), rheumatoid arthritis (Roubenoff *et al.,* 1994) and heart failure (Anker *et al.,* 2003) and is correlated with bad prognosis, reducing patient survival

The early signs of cachexia are sometimes difficult to recognize, that is why, in 2006, several international specialists gathered by the Society for Cachexia and Wasting Disorders created and characterized the term "pre-cachexia" in order to facilitate diagnosis. Pre-cachexia is described as a condition associated with chronic diseases, accompanied by inflammation and 5% or less of body weight loss within 6 months (Argilés *et al.,* 2011). Typically, cachexia can be recognized if the patient fits into three parameters: presence of systemic inflammation, less than 1500 kcal of food intake per day (normally it should be 2500 kcal in average men and 2000 kcal in women) and have lost more than 10% of total body weight (Tan & Fearon, 2008) (Figure 5). However, despite efforts, the diagnosis normally comes when the symptoms appear and the syndrome is already established.

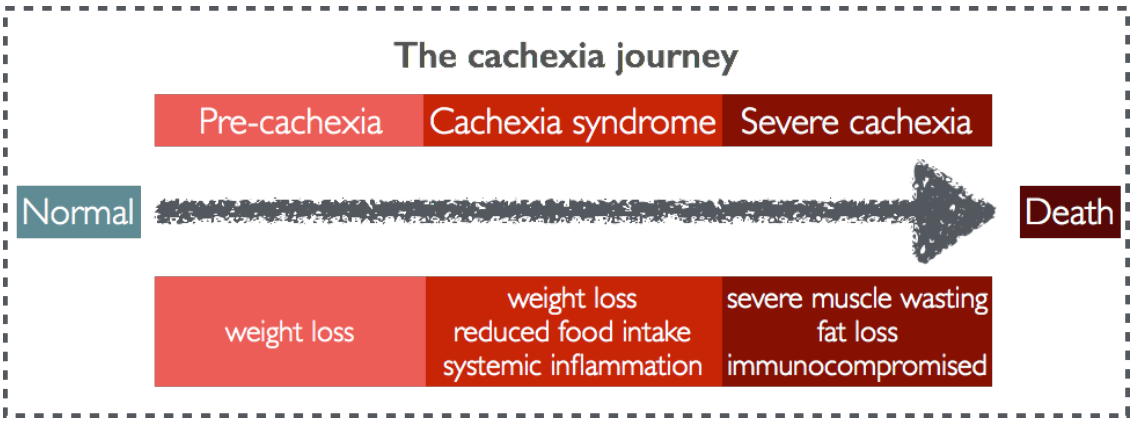

Figure 5 – The cachexia progression according to severity of muscle atrophy. Adapted from Tan & Fearon, 2008.

Recent attempts to "quantify" cachexia were validated by the cachexia score (CASCO) in order to classify patients according to the severity of the syndrome and perhaps improve treatment approaches. The categorization is made by using a numeric scale which classifies cachexia into mild (0–25), moderate (26–50), severe (51–75), and terminal (76–100), based on diverse features like body weight loss and lean body mass, inflammation, metabolic disturbances, immunosuppression, physical performance, anorexia, and quality of life. Each parameter accounts in a different way, proportionally to its relevance within the cachexia syndrome. For example, body weight loss accounts for up 40% while physical performance accounts for up 15% of CASCO (Argilés *et al.*, 2011).

Due to its heterogeneity, it is particularly difficult to underline the molecular mechanisms of cachexia. Nevertheless, it is well established that this syndrome is marked by systemic inflammation. Thus cytokines play a fundamental role in the pathogenesis of cachexia, especially TNF-α ("cachectin"), IL-6, IL-1 and IFNγ (Oliff *et al.*, 1987; Kotler, 2000; McNamara *et al.*, 1992; Carson *et al.*, 2010). In general, cytokines activate NF-κβ pathway, which subsequently induces protein degradation through the ubiquitin-mediated proteolytic system (as described above in Figure 4). TNF-α and interferon-γ act specifically inhibiting synthesis of myosin heavy chain, a molecule that forms the structure of skeletal muscles and generate force (Acharyya *et al.*, 2008).

The negative energy balance in cachexia is also a product of cytokines activity. TNF-α induces lipolysis and β-oxidation, a catabolic process that breaks fatty acid molecules through regulation of lipid metabolism (Ryden *et al.,* 2008). In the inflammatory context, IL-6 and IL-1 administration *in vivo* reduces body weight, decreases food intake and induces lipolysis and indeed, some of these effects were confirmed in humans (Opara *et al.,* 1995; Lyngso *et al.,* 2002; Wallenius *et al.,* 2002). In addition, induction of hypertriglyceridemia due to increase of VLDL synthesis and decrease in lipoprotein lipase activity are also linked with cytokines increase during cachexia (Grunfeld & Feingold, 1996).

Depending on the associated disease, other cachectic markers not related with inflammation can be altered and may play a less significant role. In COPD patients, for example, testosterone is reduced as well as its normal hyperthophic effect (Kimischke *et al.,* 1998; Bhasin *et al.,* 2003). In patients with AIDS, myostatin is elevated, contributing to protein degradation (Gonzalez-Cavidad *et al.,* 1998).

III – 3.1. Cancer cachexia

Cancer associated cachexia is a very well known and relevant syndrome though the molecular pathways are not completely understood. This association is particularly significant because it counts for more than 20% of death in cancer patients and affects more than half of them. The incidence of cancer cachexia can reach up to 80% depending on the cancer type and the stage of the disease. In particular patients with lung, pancreas, prostate and colon cancer are the most affect ones. Moreover, weight loss in cancer patients is directly proportional to morbidity and mortality. Cancer is the leading cause of death worldwide counting for more than 14 million cases according to the World Health Organization, in 2012. Therefore, the incidence of cancer cachexia is a problem that cannot be ignored. Indeed, it can lead to reduction of chemotherapy response, decrease in quality of life and is inversely correlated with patient survival (Dewys *et al.,* 1980; Teunissen *et al.,* 2007; von Haehling & Anker 2010).

Added to the habitual heterogeneity of the syndrome, in cancer-associated cachexia muscle homeostasis struggles with the tumor and the chemotherapy; which will necessarily contribute to the reduction in patient's physical activity. Besides the classical

symptoms, cachectic cancer patients also show insulin resistance and increased levels of glucagon and glucocorticoid, since tumor requires large amounts of nutrients to maintain cell growth. Importantly, insulin resistance is not favorable to muscles since insulin normally stimulates protein synthesis via the PI3K/Akt pathway (Tisdale, 2009).

Furthermore, the tumor is not a passive agent during cancer-associated cachexia, since it has the ability to secrete cytokines, potent muscle atrophy inductors. The host–tumor interaction creates a loop of inflammatory response, contributing to the overall loss of skeletal muscle. The presence of the tumor induces a systemic inflammatory response in the host; moreover, the cancer cells release pro-inflammatory cytokines that stimulates the host systemic inflammatory response. TNF-α, IL-6, IL-1, and INF-γ, all promote the activation of transcription factors associated with wasting in both adipose and skeletal muscle tissues (Espat *et al*., 1995; Argilés *et al*., 2003; Deans *et al*., 2006;). Cytokines can also act in the central nervous system (CNS) inducing appetite loss by stimulating release of leptin ("the satiety hormone") (Figure 6) (Maury & Brichard, 2010). Indeed, animals that received TNF-α administration show decrease in food intake and muscle loss (Perboni & Inui, 2006). Failure in food intake is considered a relevant component for the pathophysiology of cancer cachexia, since protein synthesis is seriously impaired due to the insufficient nutrient (Espat *et al*., 1995).

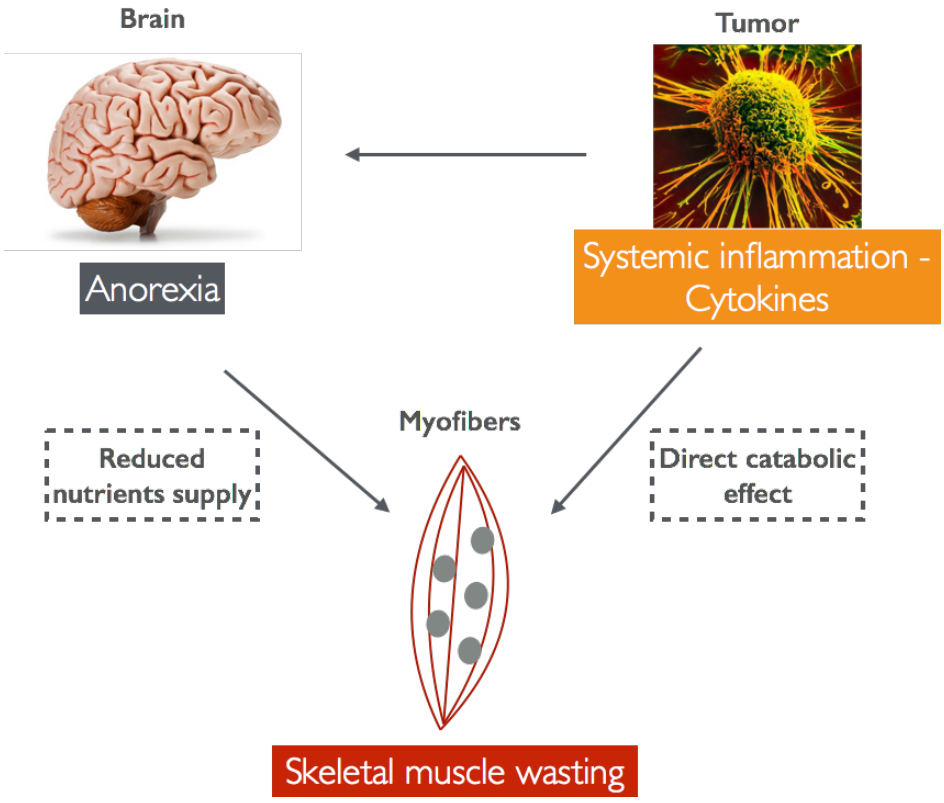

Figure 6 – Inflammatory loop mediated by the tumor. Cytokines can stimulate muscle wasting either directly or indirectly. Adapted from Skipworth *et al.*, 2007.

Another aggravating factor of cancer cachexia is the treatment itself. Since a long time it is notorious that chemotherapy treatments cause loss of appetite (McAnema & Daly, 1986). Besides that, cancer chemotherapy presents known side effects that might contribute to cachexia, like anorexia, nausea and diarrhea, but more than that, chemotherapeutical agents have a direct negative effect on protein metabolism. However, the catabolic mechanisms by which chemotherapy affects muscle protein wasting in cancer cachexia are not yet fully elucidated (Le Bricon *et al.*, 1995; Barracos, 2001).

From a molecular point of view, chemotherapeutic agents are known to induce NF-κB activity (Camp *et al.*, 2004), which plays a role in catabolic signaling pathways triggering Atrogin-1 and Trim63 (See section II – 2.4.). Cisplatin, for example, is able to reduce muscle size and mass in both *in vivo* and *in vitro* models through activation of the NF-κB signaling pathway (Damrauer *et al.*, 2008). In another study, administration of a

combination of drugs (cyclophosphamide, doxorubicin, 5-fluorouracil) induced a rapid inflammatory response with increase on glucocorticoid levels and significant loss of skeletal muscle mass in mice (Braun *et al*., 2014).

In this respect, doxorubicin most evident side effect is cardiotoxicity, where oxidative stress is the main cause (Takemura & Fujiwara, 2007). Doxorubicin acts mainly via interaction with topoisomerase II and subsequently inducing double stranded DNA breaks, defects in mitochondrial biogenesis and inducing oxidative stress through production of reactive oxygen (ROS) (Tewey *et al*., 1984). The mitochondria is the primary source of doxorubicin-induced oxidants, like superoxide anion, hydrogen peroxide and hydroxyl radical (Chaiswing *et al*., 2004; Gilliam *et al*., 2012). Administration of N-acetylcysteine (NAC), a precursor of the antioxidant glutathione, before doxorubicin exposure, protected rodents and dogs from doxorubicin-induced cardiotoxicity and myocardial lesions (Doroshow *et al*., 1981; Herman *et al*., 1985; Zhang *et al*., 2012). Furthermore, doxorubicin also stimulates systemic inflammatory, in particular by inducing the cytokine TNF-α (Mukherjee et al., 2003; Supriya *et al*., 2016). This cytokine is able to indirectly increase ROS levels in striated muscles; in fact, its inhibition prevents doxorubicin-induced cardiotoxicty (Mohamed *et al*., 2004). Such ROS stimulation can be linked to apoptotic signaling pathways as a contributing factor to muscle catabolism, since doxorubicin is a known stimulator of apoptosis in cardiac myocytes and differentiated myoblast cells. Indeed, oxidative stress is recognized as a therapeutic target in muscle atrophy (Takemura & Fujiwara, 2007; Arthur *et al*., 2008).

Doxorubicin induced muscle loss was also described in skeletal muscle of rats, primates and humans (McLoon *et al*., 1991; 1993; Cullu *et al*., 2003) and the ubiquitin-proteasome pathway seems to play a role during doxorubicin-induced muscle atrophic. Indeed, doxorubicin stimulates Atrogin-1 upregulation through the p38-MAP kinase pathway and Trim63 through a pathway partially dependent on FoxO in cardiac myocytes and skeletal muscles (Yamamoto *et al*., 2008; Sishi *et al*., 2013; Kavazis *et al*., 2014).

Overall, muscular atrophy in cancer cachexia is a product of several cumulative factors: abnormal metabolism (insulin resistance and lipolysis increase), reduction of physical activity and food intake, activation of proteolytic pathways (ubiquitin-proteasome pathway), increase on myostatin expression, elevated levels of pro-

inflammatory cytokines, testosterone decrease as well as tumor and chemotherapy influence. Nevertheless, variable aspects like age and co-existing morbidities also linked to cancer associated cachexia, contributing to the severity and enormous heterogeneity and complicating treatments (Figure 7) (Johns *et al.*, 2013).

Such multi-factorial syndrome requires a multidirectional treatment. The therapeutic approach should take into account both the cancer and the cachexia stages. It can include nutritional advising and supplements (Isenring & Teleni, 2013), exercise, appetite stimulants and selective androgen receptor modulators (Dalton *et al.*, 2013). Cytokine pathway inhibitors, catabolic pathways blockers or stimulating anabolic pathways in skeletal muscle are also an interesting option. In general, an effective treatment should combine nutritional care and exercise with pharmacological agents that block the breakdown of skeletal muscle, since only nutritional intervention is not completely efficient to reverse muscle wasting associated to cachexia.

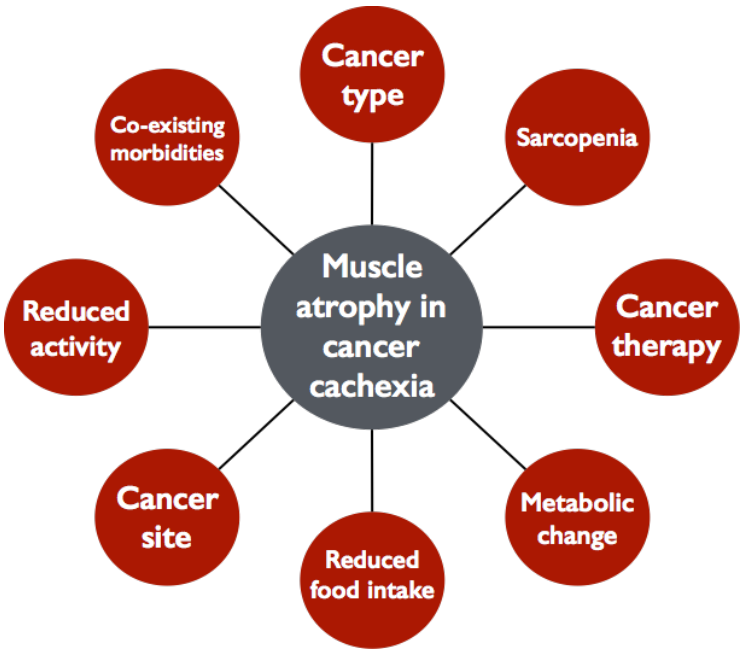

Figure 7 – Heterogeneity in cancer cachexia. Adapted from Johns *et al.*, 2013.

Currently there are no therapeutic approved drugs to treat cancer-associated cachexia but a variety of potential targets are in clinical trials studies. The "selective

androgen receptor modulators" (SARMs), for example, were developed from testosterone to have anabolic properties without testosterone adverse consequences and showed incouraging results in Phase III clinical trial by increasing lean body mass in cancer patients (Dalton *et al*., 2013). There are also the appetite regulator ghrelin (analogs) in trials, which increase food intake and also seem to have an anti-inflammatory effect in cachexia (Hanada *et al*., 2003; Granado *et al*., 2005) and the synthetic progesterone derivate appetite stimulant, megestrol acetate (Loprinzi *et al*., 1998; Garcia *et al*., 2013).

Unfortunatly, the most predictable approach, inhibition of synthesis or release of cytokines (eicosapentaenoic acid-EPA) and proteasome inhibitor (bortezomib), showed surprisingly disappointing results in human clinical trials, despite their encouraging effects in animal models (Jatoi *et al*., 2005; Dewey *et al*., 2007). Corticosteroids are frequently used and indeed show improvement in appetite. However, long-term exposure to corticosteroids leads to contradictory side effects, such as protein breakdown and insulin resistance (Loprinze *et al*., 1999). Drugs well established in the market, like selective COX-2 inhibitors and thalidomide, are also candidates to be used in the treatment of cancer cachexia due to their anti-inflammatory features (Gordon *et al*., 2005; Lai *et al*., 2008). Clearly cancer associated cachexia remains a field that needs to be explored, as despite numerous efforts it is still a non-curable syndrome. That is why it is necessary to further profoundly investigate the molecular pathways in order to gain a better and comprehensive understanding of the underlying mechanisms.

An extremely well described family, which in relationship to muscle wasting still almost unexplored, is the p53 family and its up- and downstream effectors. Besides a huge amount of studies showing its involvement in the most indispensable molecular pathways regulating development and cellular and tissular homeostasis, increasing evidence point out to the p53 family essential role as regulator or modulator of development and muscle atrophy.

IV - The p53 family.

P53 is the founding member of the p53 transcription factor family, including p63 and p73, discovered almost 20 years later. In collaboration, they modulate the most diverse and essential molecular pathways in the organisms, such as cell cycle, metabolism and response to stress and cell death. Indeed, experiments using deficient embryos for p63 and p73 beautifully showed that both isoforms were actually required for the p53-dependent apoptosis (Flores *et al.,* 2002). P63 and p73 also have nonredundant functions in normal developmental biology and are rarely mutated in human cancers (Irwin and Kaelin, 2001; Di Como *et al.,* 2002).

• IV – 1. Structure

Evolutionarily, p53 gene is highly conserved among different species. In terms of structure, p53 shares similarities with the other family members, p63 and p73. Their structure basically comprises a N-terminal transactivation domain (TA), followed by a central DNA-binding domain (DBD) and a C-terminal oligomerization domain (OD). In addition, p63 and p73 have a longer C-terminal sterile-a motif (SAM), which allows additional alternative splicing variants (Chi *et al.,* 1999) (Figure 8). In addition, the p63 and p73 exclusive SAM domain is important for protein interactions between the family members and with other proteins, since they mediate protein homodimerization. These interactions are particularity important in p53-mutated cancer cells because mutants of p53 can strongly interact with p63 and p73 and inhibit their activities in transformed cells (Gaiddon *et al.,* 2001).

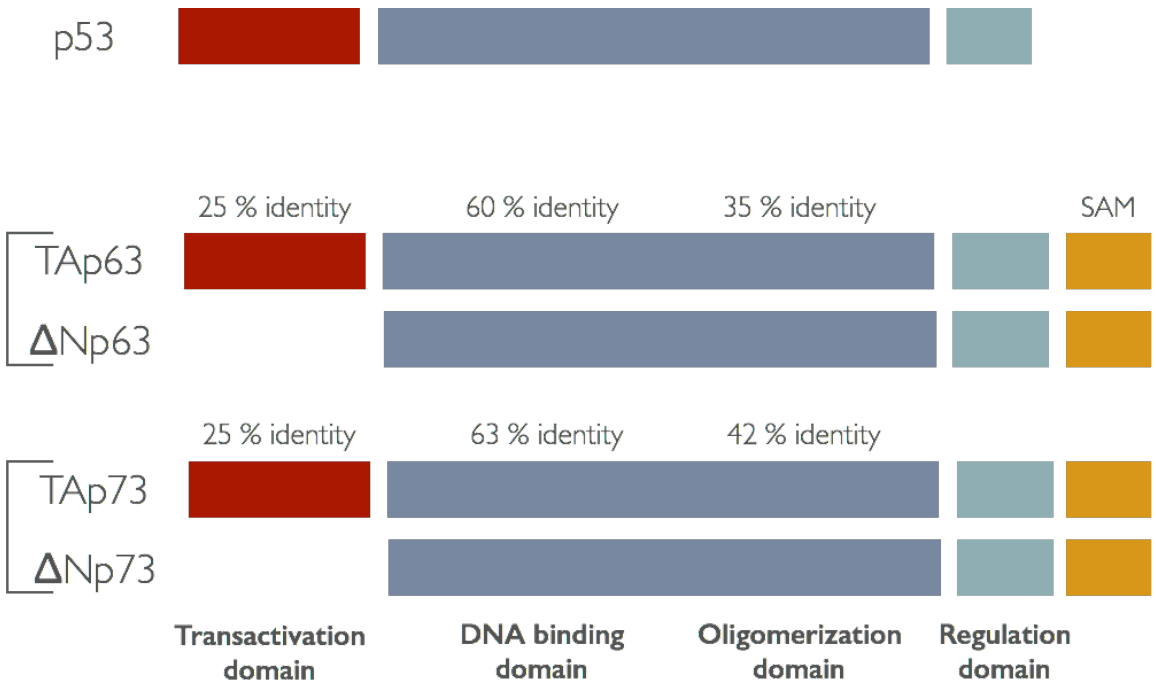

Figure 8 – Schematic structure of the members of the p53 family: p53, p63, p73 and their main isoforms, showing structural homology with p53.

The DBD recognizes and binds to a specific DNA sequence, which involves a molecular complementarity, followed by transactivation of the specific gene through the recruitment of the transcriptional machinery by the TA domain. The three family members share a significant similarity, in particular within the DBD, around 63% of homology between p53 and p73. This suggests redundant function whitin the p53 family since it enables all three members to bind to similar DNA sequences to transactivate the promoters, having thus potentially the same target genes. Before being able to bind to DNA, the oligomerization domain (OD) allows p53, p63 and p73 to form homotetramers and likewise, the similarities of OD between the different p53 family members raise the possibility of physical interactions between themselves (Ueda *et al.,* 2001), which will vary according to the different isoform interactions as it will be exposed on the below.

P63 and p73 contain two independent and distinct promoters that generate two different groups of isoform: one containing a complete TA domain whereas the other group lacks the TA domain and is described as being transcriptionally less active (ΔN isoforms). Moreover, TA and ΔN are generally attributed with antagonistic function, pro-

apoptotic versus pro-survival activity, respectively (Yang *et al.*, 1998; Pozniak *et al.*, 2000; Stiewe & Putzer, 2001). Importantly, it has been described that p63 and p73 ΔN isoforms can, under certain circumstances, be transcriptional active (Dohn *et al.*, 2001; Lin *et al.*, 2009). Nevertheless, their main function seemed to be targeting gene inhibition, which can occur either by competition between ΔN isoforms and TA proteins for the DNA binding sites, preventing active interaction of p53 or TAp63/p73 isoforms with the target gene; or the truncate isoform can physically interact with the other p53 family members in order to form transactivation incompetent hetero-oligomers (Figure 9) (Yang & McKeon, 2000; Stiewe et al., 2002).

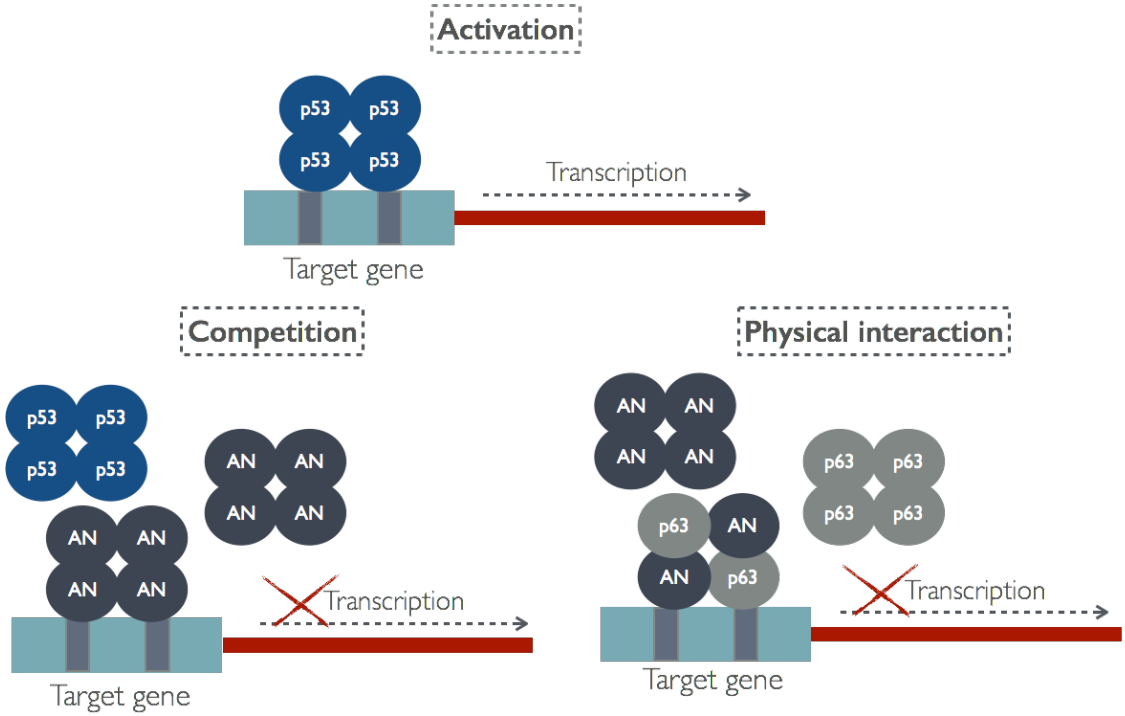

Figure 8 – Mechanism of inhibition of p53 and TA isoforms by ΔN p63/p73 proteins. Adapted from (Yang & McKeon, 2000).

P63 and p73 transcripts are also propitious to alternative splicing at the C-terminus region, which generates a combinatorial diversity of isoforms. In the case of p53, this domain is essentially subject of posttranslational modifications and regulation by phosphorylation and acetylation for example. For human and mouse p63 gene, there are at least three alternatively spliced C-terminal isoforms (α, β, γ), resulting in six

mRNA variants derived from the p63 gene and encoding for: TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β, and ΔNp63γ (Mangiulli *et al.,* 2009). P73 genes can be descript at least in seven alternatively spliced C-terminal isoforms (α, β, γ, δ, ε, ζ and η) (De Laurenzi *et al.,* 1999; Ueda *et al.,* 1999), which can homodimerize and heterodimerize with other splice variants. Unlike p63 and p73, p53 has a single promoter that drivers the synthesis of one transcript, encoding a single protein (Yang & McKeon, 2000).

The large number of splicing variants, with their potential to interact with each other make the p63 and p73 relationship immensely complex. They give rise to proteins that can functionally resemble but also counteract the actions of p53. TAp63γ and TAp63β, for example, are able to transactivate p53 target genes at levels comparable to wild-type p53 (Osaka *et al.,* 1998). Indeed, several studies consistently described p53-like activities such as apoptosis induction and activation of traditional p53 target genes such p21 and Bax by p63 and p73 (Shimada *et al.,* 1999). Likewise, ΔNp63 proteins are powerful antagonists of p53, TAp63 (Yang *et al.,* 1998), whereas the ΔNp73 couteracted p53 pro-apoptotic function (Pozniak *et al.,* 2000).

- **IV – 2. p53**

In humans, p53 is a nuclear protein that weights 53 KDa. P53 is known as the "guardian of the genome" due to its tumor suppressor role in mammals. Indeed, it is the most frequently mutated gene in cancer (more than 50% of all human cancer) with most of these mutations localized in the DNA biding domain (Cho *et al.,* 1994; Lane, 1992). Its potential tumor suppressor function was confirmed by the observation that p53 knockout mice spontaneously developed spontaneous tumors (Donehower *et al.,* 1992). Overall, p53 is one of the most suited genes and it controls differentiation, cell cycle, apoptosis, gene amplification, DNA recombination and cellular senescence and almost 100 genes have been reported to be activated by p53 (Shaw *et al.,* 1992; Brugarolas *et al.,* 1995; Wang *et al.,* 1998; Oren & Rotter, 1999; Vousden & Lu, 2002).

IV – 2.1. Regulation of p53 level and activity

P53 activity needs to be extremely regulated, and this is done through complex networks and posttranslational modifications that control the lengthening of its half-life (Vogelstein *et al.,* 2000). In absence of stress, p53 is normally maintained in an inactive form and in very low concentrations within the cells (Levine, 19997) through the action of several proteins like MDM2 (Haupt *et al.,* 1997), JNK (Fuchs *et al.,* 1998) and PARP-1 (Wesierska-Gadek *et al.,* 2003).

MDM2, an ER ubiquitin-ligase, is one of the main p53 negative regulators and together with p53, they establish an auto-regulatory feedback loop. MDM2 is able to block p53 transcriptional activity by favoring its exportation from the nucleus to the cytoplasm. In addition, due to MDM2 intrinsic E3 ubiquitin-ligase activity, it mediates both the ubiquitylation and proteasome-dependent degradation of p53 (Honda *et al.,* 1997). Curiously, MDM2 gene itself contains a p53-dependent promoter and is transcriptionally regulated by the p53 protein (Wu *et al.,* 1993).

Increase of p53 protein levels is not sufficient for its transcriptional activation. P53 conformational changes ensure its stability and activity. The main p53 post-translational modifications are ubiquitination, sumoylation, neddylation, cytoplasmic sequestration, ADP-ribosylation; and especially phosphorylation and acetylation (Oren, 1999). Those last ones usually enhance p53 transcriptional ability by increasing its stability (Xu, 2003). Most of these modifications occur in the N- and C-terminal regions of p53, also called regulatory domain. Under stress conditions, posttranslational modifications can avoid p53 degradation through ubiquitination, abolishing then its interaction with MDM2 (Brooks & Gu, 2003).

Independent pathways can initiate the p53 activation network. For example, DNA damage quickly activates p53 protein through phosphorylation mediated by ATM and Chk2 (Carr, 2000). Likewise, Ras and Myc activate p53 through $p14^{ARF}$ in response to abnormal growth signals (Sherr *et al.,* 2000). At the end, both pathways lead to increased concentration of p53 by either degradation inhibition and/or stabilization and thus, p53 can fulfill its role.

In the following I will describe some of the main functions of p53:

IV – 2.2. Cell cycle regulation

The cell cycle must be tightly regulated since it will promote life's continuity. It basically consists in mitosis phase (M phase), Gap1 between M phase and S phase (G1 phase), synthesis phase (S phase) and Gap2 between S phase and M phase (G2 phase). Cell-cycle progression is orchestrated by cyclins and cyclin-depedent-kinases (CDK) while checkpoints situated in the gaps phase allow ascertaining ideal conditions for cell duplication, such as enough amounts of nutrients or accuracy of DNA replication. In this context, p53 family of transcription factors prevents cell cycle progression via cell cycle arrest in response to a variety of stress signals. At that point, the cell has distinct alternatives: senescence, repairing the potential DNA damage, or, if it is not possible, the cell is driven to apoptosis (Nurse, 2000).

In response to cellular stresses or DNA damage, for example, p53 upregulates p21, which lead to cell cycle arrest through CDK inhibition (Xiong *et al.,* 1993). Overexpression of p21, for example, blocks the cyclin E/CDK2 complex and prevents expression of certain genes required to undergo S phase. P53-driven G2 arrest can be mediated through increased expression of Gadd45, which will then bind to the cyclin B/CDK1 complex and block cell cycle, allowing the cell to fix the error (Zhan *et al.,* 1999). In this circumstance, overexpression of Gadd45 induced by p53 facilitates topoisomerase relaxing of the chromatin and nucleotide excision repair in order to fix DNA possible mistakes (Carrier *et al.,* 1999).

IV – 2.3. Apoptosis induction

In case of cellular stress or an unrecoverable damage during cell cycle, cell fate is again guided by p53. Pro-apoptotic genes induced by p53 including Bax (Miyashita *et al.,* 1995), PUMA (Nakano & Vousden, 2001) and Noxa (Oda *et al.,* 2000), have distinct and fundamental functions during apoptosis. To understand how p53 controls the programmed cell death, it is important to have in mind that two distinct pathways carry out apoptosis: an extrinsic pathway, mediated by death receptors and an intrinsic pathway, mediated by the mitochondria.

The extrinsic signaling pathway involves transmembrane receptor of the tumor necrosis factor (TNF) receptor gene superfamily, including TNF-α/TNFR1 receptors and

FasL/FasR, which indeed can be transcriptionally activated by p53 (Fukazawa *et al*., 1999). The death receptors have an extracellular and a cytoplasmic domain responsible for transmitting the death signal detected on the cell surface to trigger intracellular signaling pathways (Ashkenazi & Dixit, 1998; Locksley *et al*., 2001). After ligand binding to the receptor on the cell surface, cytoplasmic proteins and procaspase-8 are recruited, forming the death-inducing signaling complex (DISC). Caspase 8 subsequently activates other members of the caspase family, like caspase 3, 6 and 7 (Cohen, 1997; Gupta *et al*., 2001; Liedtke *et al*., 2003).

The mitochondria triggers apoptosis by an internal mechanism controlled by the proteins of the Bcl-2 family. Among them, we can mention Bax, PUMA, all regulated by p53. Deficiency of certain growth factors, cytokines, radiation, toxins, hypoxia and free radicals can trigger loss of the normal mitochondrial transmembrane potential, increasing its permeability and causing release of pro-apoptotic factors into the cytosol, such as cytochrome c. This process is facilitated by Bcl-2, localized to the outer part membrane of mitochondria. Once in the cytosol, cytochrome c binds and activates the Apoptotic protease activating factor-1 (Apaf-1) and pro-caspase 9, forming then the apoptosome and activating effector caspases (Polyak *et al*., 1997; Cory and Adams, 2002).

P53 apoptosis-induction is directly correlated with its ability to suppress malignant transformation, since such capacity eliminates defective or unwanted cells in order to protect the organism from malignancies. Indeed, p53-null cells are more resistant to radiation and to chemotherapeutic agents inducing apoptosis (Lowe *et al*., 1993).

IV – 2.4. p53 and Metabolism

The metabolic responses mediated by p53 are also associated with its ability to promote cell survival. In response to metabolic stress (nutrient deprivation signals, glucose starvation, low energy levels, O_2 availability), LKB1 mediates p53 activation through AMP-activated protein kinase (AMPK). Concomitantly, the AKT–mTOR pathway ceases MDM2 activation, which will promote p53 accumulation, leading to a decrease in cell growth via p21 expression (Mayo & Donner, 2002; Zeng & Berger 2006). In addition, AMPK efficiently activates p53 by increasing its transcription and through direct phosphorylation and consequent p53 stabilization, contributing to the

induction of a reversible cell-cycle checkpoint in response to glucose limitation (Imamura *et al*., 2001; Jones *et al*., 2005). Interestingly, ratios of ATP and ADP can also modulate the ability of p53 to bind to DNA, ADP in a positive and ATP negative manner (Okorokov & Milner, 1999).

Variations in O_2 availability also provoke activation of p53 through different pathways. For example, during oxidative stress p53 is activated in response to the reactive oxygen species induced DNA damage (Augustyn *et al*., 2007). Reduced oxygen contents (hypoxia) condition induced p53 accumulation. P53 is stabilized by the hypoxia-inducible-factor 1 (HIF-1) while MD2M2, a p53 negative regulator, is decreased (Won *et al*., 1998; Alarcon *et al*., 1999). In addition to the oxidative stress responses, p53 can also modulate the intracellular ROS level in cells by triggering both pro- and antioxidant pathways. The pro-oxidant p53 activity contributes to cell death via apoptosis and is a product of the increased expression of pro-oxidant genes, like PIG3, and inhibition of antioxidant genes, such as TIGAR and sestrins (Johnson *et al*., 1996; Budanov *et al*., 2004; Bensaad *et al*., 2006).

Activation of p53 during metabolic stress allows the interconnection with the other molecular pathways driven by this gene, like inhibition of cell proliferation and apoptosis induction, resulting in a well-synchronized response integrating its functions (Figure 9). Furthermore, these p53 induced metabolic adaptations explain some outcomes of the p53 mutation within cancer cells, which may contribute to malignant progression and explain the differenct metabolism in cancer cells, like the increase in aerobic glycolysis characteristic to cancers, also known as the Warburg effect (Kim & Dang, 2006).

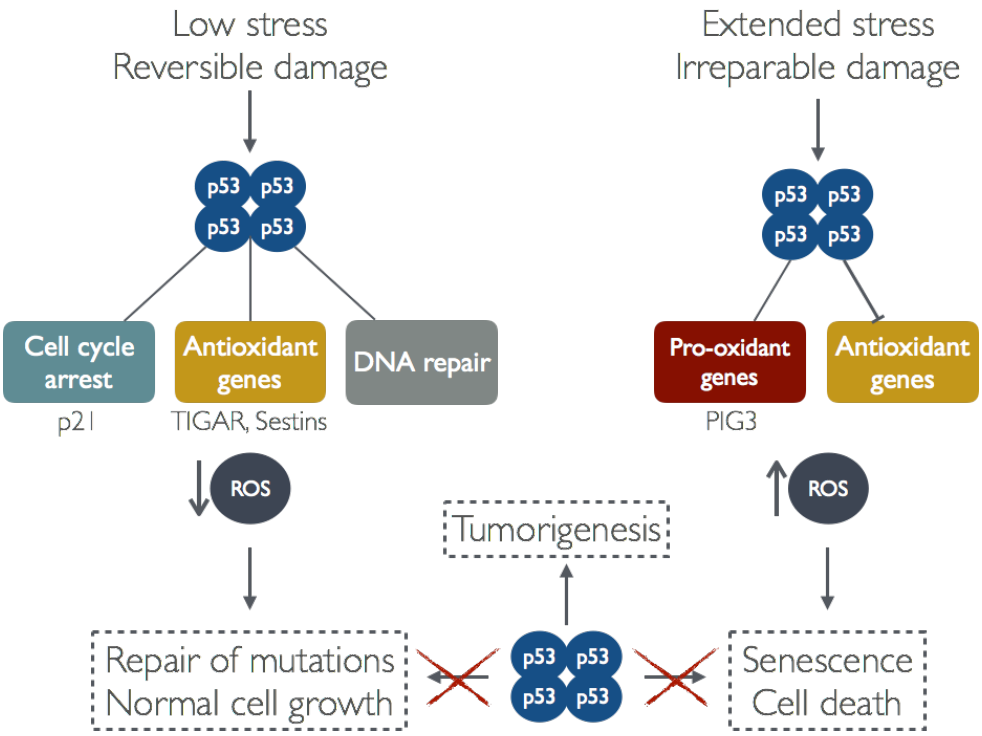

Figure 9 – Scheme of p53 molecular pathways during different levels of stress and how p53 mutations plays a role in tumorigenesis. Adapted from (Bensaad & Vousden, 2007).

- **IV – 3. p63**

As described in the section IV – 1., homology between p63 and p53 explain redundancy of function (apoptosis induction and tumor suppression) and their ability to transactivate the same responsive elements, like p21, REDD1, Bax and GADD45 (Osada *et al.,* 1998; Ellisen *et al.,* 2002; Gressner *et al.*, 2005). Indeed, some TA isoforms are known to induce apoptosis while the truncated ones (ΔN) have a predominant anti-apoptotic effect (Gressner *et al.,* 2005).

In terms of individual functions, ΔNp63 is essential for maintaining stem and progenitor cells while TAp63 plays a role in female germ cell preservation, senescence, aging, metabolism and preventing metastasis (Flores *et al.,* 2005; Suh *et al.,* 2006; Senoo *et al.,* 2007; Melino, 2011; Su *et al.,* 2012). Regarding particularities of the different p63 variants, TAp63γ is the most potent transactivator (Yang *et al.,* 1998) whereas ΔNp63 is the predominant p63 isoform expressed in epithelial cells, indispensable for its development and differentiation (Nylander *et al.,* 2000; Romano *et al.,* 2012).

Due to similar DNA binding specificity between p53 and p63 proteins, it is not so easy to determinate specific p63 target genes. For instance: PERP and IGFBP-3 were originally identified as p53 target genes but after a more recent approach (such as siRNA technologies) both are now considered to be p63 target genes (Barbieri *et al*., 2005; Ihrie *et al*., 2005). More recently, caspase-1, a proinflammatory caspase that modulates tumor suppression; was described to have biding sites for p63, joining the list of the p63 target genes (Celardo *et al*., 2013). Jagged1 (JAG1) is one of the p53 family target genes preferencially activated p63 (Sasaki *et al*., 2002). JAG1 plays a role during limb and craniofacial development through the Notch signaling pathway (Bao *et al*., 1997; Vargesson *et al*., 1998; Shimizu *et al*., 1999).

Besides functions shared with p53, p63 roles during development are critical. Mice completely lacking p63 exhibit striking defects in embryonic epidermal morphogenesis. They are born with craniofacial abnormalities, have severe limb truncations and defects in the development of stratified epithelia, dying shortly after birth (Mills *et al*., 1999; Yang *et al*., 1999). In human, it is possible to see the consequences of p63 mutation in ectodermal dysplastic syndrome (EEC), where the carries of this mutation exhibit ectrodactyly and cleft palates (Celli *et al*., 1999). A recent work highlighted TAp63's importance in regulating energy metabolism. Experiment using $TAp63^{-/-}$ mice demonstrated that the animals develop insulin resistance, obesity and glucose intolerance (Su *et al*., 2012).

In order to understand regulation of p63, we need to have in mind some important isoform specific functions. ΔNp63α, for example, maintain viability and proliferative capacity of basal epithelial cells, thus it is controlled by signals involved in survival and proliferation. Indeed, the epidermal growth factor (IGFR) and the PI3K, a mediator of survival and differentiation in keratinocytes; regulate expression of ΔNp63α (Cantley *et al*., 2002; Matheny *et al*., 2003). Moreover, this isoform is downregulated and phosphorylated under genotoxic stress in order to alleviate its negative p53 regulation (Westfall *et al*., 2005). Interestingly, stability increase via post-translational modifications appears to be inversely correlated with the transactivation ability of p63 (Ghioni *et al*., 2005).

IV – 3.1. Epithelial development

P63 is essential for epithelial development. During this process, TAp63 is firstly expressed in the single-layered surface ectoderm and is required for epithelial stratification initiation. In fact, TAp63 is particularly important for initiation of an epithelial stratification program. Then, during later stages of embryonic development, ΔNp63 is required for epidermal maturation and counteracts the effects of the TAp63 proteins (Koster & Roop, 2004).

IV – 3.2. p63 and metabolic regulation

p63 is an important regulator of lipid and glucose metabolism. Su and colleagues (Sue *et al*., 2012) showed that TAp63 null mice presented metabolic disorders and lipid deposits in several tissues. In particular, TAp63γ variant was upregulated and required to the cell response to metformin, a drug used to treat Type 2 diabetes. Basically, TAp63 regulates energy metabolism through transcription of the key metabolic regulators Sirt1, AMPKα2 and LKB1. Importantly, re-expression of Sirt1 and AMPKα2 on these TAp63 knockout mice was able to rescue normal metabolism.

P63 ensures cells energy supply in order to assist and prolong survival. Under starvation signals, Sirt1 is transcriptionally activated by TAp63, which will then promote ATP production via the tricarboxylic acid (TCA) cycle. AMPK controls lipid and glucose by balancing catabolic and anabolic activity besides, in collaboration with Sirt1, it modulates metabolic adaptation in skeletal muscles (Towler & Hardie, 2007; Cantó *et al*., 2010). TAp63 regulates both mRNA levels of AMPKα2 and activity. On the other hand, ΔNp63α has been shown to transcriptionally activate the enzyme that catalyzes fatty acid synthesis (FASN), enhancing p63 cell survival activity during development (Sabbisetti *et al*., 2009).

• IV – 4. p73

Like p63, p73 is rarely mutated in cancer (fewer than 0.5% of human cancers) and it is essential for the organism's development, in particular during neurogenesis (Ikawa *et al*., 1999). It is the only p53 family member with a fundamental role in CNS general

development and maintenance (Abraham *et al*., 2010). Mice deficient for all p73 isoforms exhibit neuronal and developmental abnormalities, malfunctions in the pheromone sensory pathway, chronic infections and inflammation. However, unlike the knockout mice for p53 and p63, $p73^{-/-}$ mice can survive until adulthood though with a high rate of mortality. Interestingly, they do not show increased susceptibility to spontaneous tumorigenesis (Yang *et al*., 2000).

Following the logic that the TA isoforms pro-apoptotic effect is counterbalanced by the truncate variants', in neuronal context, ΔNp73 can function as a prosurvival factor for differentiated neurons and it is indeed required for maintenance of CNS neurons (Pozniak *et al*., 2000). In addition, selective TAp73 or ΔNp73 knockdown confirmed that the truncate isoform (ΔN) is indispensable for neuronal survival, whereas TAp73 is required for neuronal stem cells maintenance and differentiation (Pozniak *et al*., 2000; 2002).

Given p73 high homology degree with p53 (even more than p63), it was not surpring that p73 is induced in response to DNA damaging and that it also plays a role in tumorigenesis (Gong *et al*., 1999). TAp73 is able to activate PUMA, p21 and Bax, modulating growth arrest and cell death (Jost *et al*., 1997; Ueda *et al*., 2001). On the other hand, increasing level of the anti-apoptotic variant, ΔNp73, is associated with cancer development. Indeed, tumour growth was observed in mice models overexpressing ΔNp73 (Stiewe *et al*., 2002).

Furthermore, the ΔNp73 isoform is an important negative regulator of p53 and TAp73 and it does so by competing for its target DNA-binding sites. In addition, ΔN and TA forms assemble less-active TAp73–ΔNp73 heterodimers and compromise the transcriptional activation of target genes. P53 can directly induce expression of ΔNp73 by its transcriptional activation, creating thereby a complex regulatory loop that, when disturbed (in the case of cancer), might result in persistent ΔNp73 expression, leading to inability to activate p53 and inhibition of apoptosis, contributing to tumor development (Kartasheva, *et al*., 2002; Stiewe *et al*., 2002; Vossio *et al*., 2002). So, ΔNp73 is notably associated with poor prognosis in neuroblastoma human cancers (Casciano *et al*., 2002). Interestingly, TAp73 is capable to promote apoptosis in a p53-independent manner due to its activation through the transcription factor E2F1 in response to DNA damage. This fact

may explain p53-mutated cell response to chemotherapy (Stiewe *et al.,* 2000; Irwin *et al.,* 2003).

In terms of regulation, despite the similarities with p53, MDM2 fails to ubiquitinate p73 (Zeng *et al.,* 1999). Instead, Itch is the ubiquitin-protein ligase that targets and degrades p73. Curiously, in the case of p73, ubiquitination is not a mandatory signal for its degradation and it can actually increase p73 stability, as it was demonstrated after NEDL2 p73 ubiquitination (Miyazaki *et al.,* 2003; Rossi *et al.,* 2005). In response to DNA damage, for example, p73 is activated through posttranscriptional modifications, like phosphorylation by c-Abl and acetylation by p300 (Costanzo *et al.,* 2002; Sanchez-Prieto *et al.,* 2002).

• IV – 5. P53 family and muscles

In addition to all functions of the p53 family members described above, studies show that they are also important for muscles cell development, differentiation and homeostasis. Concerning muscle development, p63 is particular significant during heart development. Indeed, TAp63 is expressed in endodermal cell during early phases of development and modulates the proliferation and survival of cardiac progenitors (Paris *et al.,* 2012; Gonfloni *et al.*, 2015). For instance, knockout mice for p63 develop less thick ventricular walls (Rouleau *et al.,* 2011).

During myogenic differentiation the p53 family cooperates to the activation of the retinoblastoma tumor suppressor protein (RB) (Cam *et al.,* 2006), which controls skeletal muscle proliferation, differentiation and cell death during embryogenesis (Zacksenhaus *et al.,* 1996). P53 induces transcription of the RB gene, whereas ΔNp73α inactivate it through phosphorylation and impairs cell differentiation. Porrello and colleagues (2000) showed that p53-deficient myoblasts fail to induce RB, and TAp63γ and TAp73β are capable to transactivate RB *in vitro*, suggesting a compensatory effect in absence of p53 (Cam *et al.,* 2006). Another study reported also an upregulation and involvement of p73 during muscle differentiation (Fontemaggi *et al.,* 2001).

Surprisingly, experiments with p53 null mice show that p53 is not essential for muscle development and regeneration (Donehower *et al.,* 1992; White *et al.,* 2002). However, p53 makes a link between differentiation and metabolism in muscles: P53

transcriptional regulates Sirt1, a gene particularly significant for normal development, metabolism and aging (Zhang & Kraus 2010). During proliferation and myogenesis, Sirt1 is increased, promoting satellite cells proliferation (Pardo & Boriek, 2011). In addition, in response to nutrient deprivation, Sirt1 has a p53-dependent negative effect on myogenesis (Nemoto *et al*., 2004).

Works support that TAp63 also regulates Sirt1. Interestingly, TAp63-/- mice showed lower levels of Sirt1 in muscle but not in skin, suggesting that TAp63 regulates Sirt1 expression in metabolic tissues, in particular, in muscles (Su *et al*., 2012). Likewise, the key metabolic factor TIGAR was decreased in p53- and TAp73-deficient muscle. During low or mild levels of stress, p53 activates TIGAR, which leads to inhibition of glycolysis and overall decrease of intracelular ROS, promoting cell survival (Bensaad *et al*., 2006; Lee *et al*., 2015). In addition, p53 contributes to metabolism modulation in muscles by decreasing expression of glucose transporters in myotubes (Schwartzenberg-Bar-Yoseph *et al*., 2004).

IV – 5.1. Muscle atrophy and the p53 family.

All p53 family members contribute to muscle developmental, but p53 family members modulate muscle waste in distinct contexts. Indeed, p53 was found to be overexpressed in immobilized muscles (Fox *et al*., 2014). In presence of pro-inflamatory cytokines, Tang and colleagues (2004) described p73 as being a pro-apoptotic factor in vascular smooth muscle cells. Interestingly, during TNF-α treatment, p73β was induced and required for the TNF-α-mediated apoptosis. This cytokine is also able to inhibit muscle differentiation, regeneration and myogenesis, through a p53-mediated apoptotic pathway (Coletti *et al*., 2002; Schwarzkopf *et al*., 2006). In response to genotoxic stress, p53 impairs the late stage of muscle differentiation via transcriptional repression of myogenin (MyoG) (Yang *et al*., 2015). This pathway could explain muscle atrophy in cancer cachexia, where p53 is usually mutated or chronic activated. Indeed, it was demonstrated that p53 is required for muscle waste in cachexia and that its hyperactivation promotes muscle atrophy *in vivo* (Schwarzkopf *et al*., 2006; 2008).

Additionally, it was reported that doxorubicin induces p53 activity in myocytes (Morimoto *et al*., 2008). As pointed out before, doxorubicin is proven to cause muscle

atrophy (Gilliam *et al*., 2011; Hydock *et al*., 2011; Kavazis *et al*., 2014) through different mechanisms: induction of Atrogin-1 via p38-MAPK pathway (Yamamoto *et al*., 2008), by ROS production (Gilliam *et al*., 2012), TNF-α elevation and activation of its receptor subtype 1 (TNFR1) (Gilliam *et al*., 2009), contribution of the endoplasmic reticulum (ER)-stress, upregulation of Trim63 expression (Sishi *et al*., 2013), ceramide accumulation (Delpy *et al*., 1999) and induction of FoxO transcription (Kavazis *et al*., 2014).

Hypothesis

Bioinformatics analyses performed in the laboratory on muscle of patients with ALS pointed out deregulations in the activity of 7 transcription factors, among them, p53, MyoD and myogenin. Based on this observation and the literature indicating a role of the p53 family in muscle physiology, the laboratory hypothesized that the p53 family may be important relay in muscle atrophy. Based on this hypothesis, the laboratory decided to investigate the function of the p53 family in muscular atrophy in two different models: 1) ALS and 2) cachexia induced by the cancer-related therapy (doxorubicin).

Objectives

The objectives of my thesis were focused on understanding the role of the p53 family in muscle atrophy by using cellular and animal models that mimic it. This led us also to use these models in order to identify therapeutic approaches. Hence, my project had 3 complementary objectives:

1. Characterize the expression profile of p53 family members in muscle atrophy during ALS and doxorubicin-induced cachexia.
2. Understand how the p53 family may impact on muscle atrophy, in particular by regulating pro-atrophic factors, such as TRIM63/Murf1
3. Identify novel therapeutic approach for muscle atrophy.

Results

During my Phd I contributed with three publications, two of them were already accepeted in eLife and in the Journal of Pharmacology and Experimal Therapeutics.

The first publication highlights Trim63 (Murf1) modulation by the p53 family during muscle atrophy, in particular p63, in muscle atrophy induced by ALS **(1)**. Bioinformatics analyses on 4 independent transcriptomic experiments performed with muscles of ALS patients or mouse models of ALS identified activation of a p53-like response. Further analyses performed on the patients' biopsies and in animal models showed upregulation of p53 and TAp63. Indeed, their expression correlated with the severity of the pathology.

The second publication is about to be submmited and comprises the role of the p53 family in muscle atrophy induced by doxorubicin **(2)**. We characterized the expression profile and the physical/functional interactions between p53 family members and the pro-atrophic ubiquitin ligases (Trim63 and Atrogin-1) in murine myoblast cells (C2C12) and mice treated with doxorubicin. Additionally, we also investigated other molecular pathways involved on doxorubicin induced muscle atrophy.

Finally, the third publication concerns the cytoprotective effects of antioxidant molecules in two different muscle atrophy context: ALS and cachexia **(3)**. The laboratory aimed to find innovative therapies to reduce muscle atrophy. To do so, we collaborated with a start-up (Axoglia) to test molecules with potential cytoprotective activity in muscles. These molecules are derived from flavagline or tocopherol and show an antioxidant activity. Following *in vitro* screening for the cytoprotective activity of the compounds, we used *in vivo* models to validate the ability of the compound to reduce muscle atrophy. We also characterized some of the molecular mechanisms underlying this effect. For instance, we were able to establish a deregulation of the Notch pathway in atrophic muscle.

1 - Transcriptional activator TAp63 is upregulated in muscular atrophy during ALS and induces the pro-atrophic ubiquitin ligase Trim63

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Abstrasct

Mechanisms of muscle atrophy are complex and their understanding might help finding therapeutic solutions for pathologies such as amyotrophic lateral sclerosis (ALS). We meta-analyzed transcriptomic experiments of muscles of ALS patients and mouse models, uncovering a p53 deregulation as common denominator. We then characterized the induction of several p53 family members (p53, p63, p73) and a correlation between the levels of p53 family target genes and the severity of muscle atrophy in ALS patients and mice. In particular, we observed increased p63 protein levels in the fibers of atrophic muscles via denervation-dependent and -independent mechanisms. At a functional level, we demonstrated that TAp63 and p53 transactivate the promoter and increased the expression of Trim63 (MuRF1), an effector of muscle atrophy. Altogether, these results suggest a novel function for p63 as a contributor to muscular atrophic processes via the regulation of multiple genes, including the muscle atrophy gene Trim63.

Introduction

Muscle atrophy is associated with aging, cancer, AIDS and neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) (von Haehling *et al.,* 2010). Although muscle atrophy is not necessarily the primary target of the pathology, it is often an important cause of lethality. For example, atrophy and dysfunction of respiratory muscles lead to death in ALS patients (Rothstein, 2009). As muscle atrophy is associated with complex pathologies, the exact mechanisms inducing muscle atrophy are varied and still debated.

Typically, ALS has been considered a neurodegenerative pathology specifically causing alteration in motor neurons, but more recent findings indicate that the etiology of the pathology is more complex. Indeed, a number of additional cell types, such as astrocytes (Yamanaka *et al.,* 2008), microglia (Boillée *et al.,* 2006) and muscle cells (Wong and Martin, 2010), have been described to be directly affected by the pathology and therefore to participate in the muscle atrophy.

Around 20% of all inherited ALS cases can be linked to mutations in the gene encoding SOD1. Cellular events (Pansarasa *et al.,* 2014) that have been shown to be triggered by these different mutations include aggregation of SOD1 proteins in the cytoplasm (Hart, 2006), increase in oxidative stress (Barber and Shaw, 2010) and subsequent DNA damage (Aguirre *et al.,* 2005), endoplasmic reticulum (ER) stress (Nishitoh *et al.,* 2008) or alterations of mitochondrial function (Manfredi and Xu, 2005). In addition, novel mutated genes (FUS, TARDBP) have been linked to ALS with differences in the pathophysiological outcomes (Chen *et al.,* 2013). These differences might be linked to the different impacts of the mutated proteins at the molecular level. Indeed, protein aggregates or other alterations induced by SOD1 mutants have been characterized in muscle cells, while other mutated proteins linked to ALS seem to not directly affect muscles (Pansarasa *et al.,* 2014).

To date, the exact molecular mechanisms driving muscle catabolism in the symptomatic phase of ALS remain poorly understood. It is also only during the symptomatic phase that ALS pathology can be diagnosed. The absence of pre-symptomatic markers highlights the need for understanding the muscle catabolic processes for therapeutic purposes. Several observations indicate that the p53 family members (p53, p63, p73) play an important role in muscle physiopathology and might therefore represent actors of the muscle atrophy (Schwarzkopf *et al.,* 2006; Mazzaro *et al.,* 1999; Cam *et al.,* 2006; Fontemaggi *et al.,* 2001; Martin *et al.,* 2011; Rouleau *et al.,* 2011; Belloni *et al.,* 2006; Su *et al.,* 2012).

The p53 family of transcription factors is a central regulator of cellular processes such as apoptosis, cell cycle arrest, metabolism or cellular differentiation through the regulation of several target genes (CDKN1A, BAX, GADD45A, MDM2 and others) (Arrowsmith, 1999; Menendez *et al.,* 2009). All three members encode TA and ΔN isoforms that vary in their N-terminus due to alternate promoter usage where TA has a canonical transactivation domain. ΔN isoforms lack such a domain and can serve as dominant negatives versus the TA isoforms in some cases, although they are also capable of transactivating certain genes (De Laurenzi *et al.,* 1998; Casciano *et al.,* 1999; Murray-Zmijewski *et al.,* 2006).

Through their cellular activities, p53 proteins are involved in a broad variety of

physiological functions that include tumor suppression and organ development (Arrowsmith, 1999). For example, p53 plays a role in the response against tumor-inducing events such as DNA damage, oncogene activation, and a variety of additional cellular stresses (hypoxia, reactive oxygen species (ROS), or alteration of energy metabolism) (Marcel *et al.,* 2011; Rufini *et al.,* 2013; Gonfloni *et al.,* 2014). In addition, several studies have highlighted the involvement of the p53 family members in neurodegenerative diseases. P53 as well as p63 and p73 have been shown to regulate neuronal apoptosis and their activation has been observed in various neurodegenerative diseases, such as Alzheimer, Parkinson and Angelman syndromes (Jiang *et al.,* 1998; de la Monte *et al.,* 1997; Seidl *et al.,* 1999; Bui *et al.,* 2009; Benosman *et al.*, 2007; Benosman *et al.,* 2011). We have previously reported an induction of p53 in degenerating spinal cord motor neurons in an ALS mouse model expressing mutated Cu/Zn superoxide dismutase 1 (SOD1[G86R]) (Gonzalez de Aguilar *et al.,* 2000).

In muscles, p53 is activated during myogenic differentiation, participates with MyoD to induce myogenesis, and mediates doxorubicin-induced muscle atrophy via its target gene pw1 (Schwarzkopf *et al.,* 2006; Mazzaro *et al.,* 1999). Nonetheless, p53 expression is not essential for muscle development (Donehower *et al.*, 1992) or regeneration (White *et al.,* 2002), which could be explained by compensatory mechanisms involving p63 and p73. Indeed, more recent studies have shown that p63 and p73 are also involved in myoblast differentiation (Cam *et al.,* 2006; Fontemaggi *et al.,* 2001; Martin *et al.,* 2011; Rouleau *et al.,* 2011) and ΔNp73 appears to protect muscle cells against stresses (Belloni *et al.,* 2006). Finally, a study showed that p63 is important for the regulation of muscle cell metabolism via the regulation of Sirtuins and AMPK (Su *et al.,* 2012).

In this study, we investigated the regulation and the role of the transcription factors of the p53 family in muscular atrophy during ALS based on a meta-analysis we performed with 4 microarray experiments obtained with biopsies of muscles from ALS patients or with muscles from ALS mouse models.

Materials and Methods

SOD1-G86R mice: SOD1 (G86R) mice were genotyped as described in (Ripps *et al.,* 1995). For surgery, 80-day-old FVB mice were anesthetized and both sciatic nerves were exposed at mid thigh level and crushing was performed (or not – CT) with a forceps during 20 s ~5 mm proximal to the trifurcation. Control animals used in the experiments were wild-type littermates. Randomization was performed based on body weight. Time course for animal pathology was performed based on a previous study on denervation and muscle atrophy (von Grabowiecki *et al.,* 2015). Animal experiments were performed

following the European guidelines and protocols validated by the local ethical committee.

Cell culture: C2C12 cells were obtained from ATCC (ATCC CRL-1772) and grown in DMEM (Dulbecco's modified Eagle's medium; Life Technology, Carlsbad, CA) with 10% fetal bovine serum (Life Technology) at 37˚C in a humidified atmosphere and 5% CO2. Mycoplasma contamination has been tested negatively using PlasmoTest (Invivogene, San Diego, CA). Differentiation of C2C12 cells was performed using 2% horse serum at 90% cell confluence.

Quantitative PCR: TRIzol (Invitrogen, Carlsbad, CA) was used to extract RNA. One μg of RNA was used for reverse transcription (iScript cDNA kit, Bio-Rad, France) and qPCR was carried out (iQ SYBR Green, Bio-Rad) (Supplementary file 1). Expression levels were normalized using either 18S, TBP or RPB1 as previously described (Vidimar *et al*., 2012).

Western blotting: Cells or tissue were lysed with LB (125 mM Tris-HCl pH 6.7, NaCl 150 mM, NP40 0.5%, 10% glycerol). Proteins were denatured and deposited directly (75 μg of proteins) onto a SDS-PAGE gel, or they were precipitated (2 μg of proteins) with a p63 antibody and G Sepharose beads before separation. Western blotting was performed using antibodies raised against p53 (rabbit anti-p53, FL-393, Santa Cruz Biotechnology, Dallas, TX), p63 (mouse anti-p63, 4A4, Santa Cruz Biotechnology; p63, Abcam, France) or TAp63 (Biolegend, CA). Secondary antibodies (anti-rabbit, anti-mouse: Sigma, France) were incubated at 1:1000. Loading was controlled with actin (rabbit anti-β-actin, Sigma, 1:4000) or TBP (anti-TBP 1:1000, Santa Cruz Biotechnology) (Antoine *et al*., 1996).

Transfection and luciferase assays: Cells were transfected by polyethylenimmine (PEI)-based or JetPrim (Polyplus, Strasbourg, France) as previously described (Gaiddon *et al*., 1999). For luciferase assays, cells were seeded in 24-well plates, and transfected with the indicated expression vectors (200 ng) and reporter constructs (250 ng) (Sohm *et al*., 1999). Luciferase activity was measured in each well 24 hr later and results were normalized with a CMV-driven reporter gene (Benosman *et al*., 2011). The -1584 ΔNp63 luc and -46 ΔNp63 luc constructs were previously described (Romano *et al*., 2006). The Trim63 luc constructs were previously described (Waddell *et al*., 2008). SiRNA tranfection was performed using 30 nM of siRNA and with RNAiMAX protocol as described by the provider (Life Technology). TAp63 siRNA sequences were covering the sequence: GAA CUU UGU GGA UGA ACC UCC GAA.

Chromatin immunoprecipitation (ChIP) assay: ChIP assays were performed using the standard protocol from the Magna ChIP G kit (Millipore). C2C12 lysates were sonicated 12 times at 10% power. For each 1 million cells, 1 μg of antibody was used. P63 was immunoprecipitated with a mouse antibody raised against total p63 (4A4, Santa Cruz Biotechnology). Mouse-anti-RAB11A was used as negative control (Santa Cruz

Biotechnology).

Microarrays analyses: ECL files from microarray experiments (E-MXP-3260; E-GEOD-41414; E-TABM-195; E-GEOD-16361) were obtained form the Array Express database (EMBL-EBI). Each experiment was first analyzed individually using AltAnalysis software (Emig *et al.,* 2010). Deregulated gene were identified based on two fold change expression and t-test p-value <0.05. Deregulated genes were then analyzed by GO-Elite with Prune Ontology term using Z-score (cutoff 1.96, p-value 0.05) and Fisher's Exact Test for ORA (2000 permutations) for over-representation in selected biological processes in several resources: Gene Ontology, MPhenoOntology, Disease Ontology, GOSlim, PathwayCommons, KEGG, Transcription Factor Targets, miRNA Targets, Domains, BioMarkers, RVista Transcription Sites, DrugBank, BioGrid.

Immunohistochemistry: Mouse gastrocnemius muscles were sampled, submersed in freezing medium (Tissue-Tek O.C.T compound, Sakura, Japan) and immediately frozen in a nitrogen-cooled isopentane bath. Muscles were sliced in transversal axis at 14 μm in a cryostat (Leica CM3050S, Leica, France) and placed on slides covered with 0.5% gelatine. The samples were then dried for 20 min on a hot plate and fixed in 4% paraformaldehyde for 10 min. After a 5 min wash with PBS, the samples were permeabilized with 3% Triton X-100 in PBS for 10 min, washed with TBS, incubated in 100 mM glycine in TBS for 20 min and finally washed again in PBS. The samples were incubated with mouse antibody raised against p63 (p63 clone 4A4, Santa Cruz Biotechnology) at 1:100 with 0.1% Triton X-100 in PBS (Triton buffer) overnight at room temperature. They were then washed three times with Triton buffer for 10 min and incubated with cyanine 3-coupled goat anti-mouse antibody (Jackson ImmunoRe- search, West Grove, PA) at 1:1000, as well as with 1 μg/ml Hoechst 33,342 (Sigma, France), in Triton buffer at room temperature for 1 hr. After washing three times with Triton buffer, the slides were covered with mounting medium (Aqua-Poly/Mount, Polysciences, Warrington, PA) on glass slips and observed by confocal microscopy (Zeiss, Germany). Antibody specificity was verified with slides probed with only the secondary antibody.

Results

P53-target genes and p53 regulators are induced in atrophic muscles during ALS

To identify the molecular mechanisms involved in muscle atrophy during ALS we performed a meta-analysis using four independent microarray experiments deposited at the Array Express database (EMBL-EBI). Two experiments contained gene expression data for the muscle of ALS patients and control individuals (E-MEXP-3260; E-GEOD-41414, [Pradat *et al.,* 2012; Bernardini *et al.,* 2013]). One experiment contained gene expression data for muscles of SOD1 (G86R) mice that represents an ALS model in

which the onset of the pathology is at 105 days of age (E-TABM-195 [Gonzalez de Aguilar *et al.,* 2008]). The last experiment contained gene expression data for muscles of SOD1 (G93A) mice in which onset of the pathology occurs at 14 weeks of age (E-GEOD-16361, [Capitanio *et al.,* 2012]). Beside the better pathophysiological relevance, data obtained from biopsies of ALS patients also provided a better representation of the diversity of the genetic anomalies observed in patients. In addition, patients were at various stage of the pathology, hence establishing a representative scale of muscle alterations. The panel of datasets we chose also included two different mouse models of ALS, allowing us to pinpoint common and specific deregulations. Importantly, the SOD1 mouse models are well characterized for their muscular phenotype alterations. In particular, it has already been established that SOD1 mutants present altered functions in muscles, in contrast to other mutated proteins linked to ALS (TARDBP, FUS etc) (Pansarasa *et al.,* 2014).

After standard normalization and statistical analyses, each experiment was independently subjected to gene ontology, signaling pathway, transcription factor, and miRNA analyses. Fold induction between control individuals and ALS individuals was set to two fold change and raw p value inferior to 0.05. We decided to focus on transcription factor deregulations. The bioinformatic analyses we performed pinpointed to only 7 transcription factors whose activity, indicated by coherent changes in expression of their target genes, was potentially deregulated in at least two out of four experiments (Figure 1A). The activity of one transcription factor, NFκβ, appeared deregulated only in experiments done with the mouse models. Deregulation of STAT1 activity was identified in three experiments. Interestingly, the activity of only three transcription factors, MyoD, Myogenin and p53, was identified to be commonly deregulated in all four experiments that included biopsies from patients and animal models. MyoD and Myogenin are muscle specific transcription factors involved in muscle cell differentiation (Zanou and Gailly, 2013). P53 was the transcription factor with the highest number of deregulated genes (51 genes). Notably the p53 target genes CDKN1A, GADD45A and PMAIP1, among others, were found induced in all four experiments.

As one of the experiments using biopsies of ALS patients included a scale (von Haehling *et al.,* 2010; Rothstein, 2009; Yamanaka *et al.,* 2008; Boillée *et al.,* 2006; Wong and Martin, 2010; Pansarasa *et al.,* 2014) of muscle alteration, we analyzed whether the expression of some of these genes might correlate with the severity of the pathology. We found that CDKNA1, GADD45A and PMAIP1 expression correlated with the degree of the pathology of the muscle from ALS patients (Figure 1B).

Besides the bioinformatic analysis on the deregulation of transcription factors, the signaling pathway analyses also indicated alterations in the p53 pathway characterized by deregulations in upstream regulators of p53, such as MDM2 and thioredoxin, and a p53

family member, P63 (Table 1, Figure 1B, Figure 1—figure supplement 1). In particular, the expression of P63 correlated with the severity of the pathology in muscles biopsies from ALS patients (Figure 1B).

In order to validate the bioinformatic analyses we performed RT-qPCR experiments with RNA from muscle biopsies of an independent group of ALS patients. We confirmed that CDKN1A, GADD45A and PMAIP1 were induced in the muscle biopsies of ALS patients (Figure 2A, B, C). Similarly, we analyzed the expression of these genes using muscle samples of independent groups of SOD1 (G86R) mice. Groups analyzed at 60 days and 75 days of age correspond to the asymptomatic stage, while 90 day-old groups correspond to an early or pre-symptomatic stage associated with established gene deregulations (von Grabowiecki *et al.,* 2015). Finally, the symptomatic stage group (beginning after 105 days) is characterized by the onset of paralysis and marked muscle atrophy (Figure 2—figure supplement 1). Upregulation of the p53 target genes Gadd45a, Cdkn1a, Bax, Pmaip1 and Perp was observed at 90 days and further increased at 105 days in SOD1 (G86R) mice (Figure 2D, E, F, Figure 2—figure supplement 2). In addition to these genes, we also analyzed by RT-qPCR the expression of additional p53 target genes and regulators of p53 by RT-qPCR (Table 1). In particular, the expression of p53 target genes involved in apoptosis (Pmaip1, Peg3 and Siva) was also induced.

P53 proteins have recently been linked to energy metabolism and endoplasmic reticulum (ER) stress pathway activation (Su *et al.,* 2012; Ramadan *et al.,* 2005; Zhu and Prives, 2009). Analysis of the expression of p53 family target genes implicated in several metabolic pathways (Tigar, sestrins, Sco2, Sirtuin1 or Prkaa1) (Su *et al.,* 2012; Vousden and Ryan, 2009) or ER stress (Chop, Bip, or Xbp1) (Stavridi and Halazonetis, 2004), did not reveal coherent regulation in respect to disease progression (Table 1). For example, the expressions of Sesn2 and Tigar (Vousden and Ryan, 2009) were regulated in opposite directions during the progression of the disease. Therefore, our data suggest that the correlation between ALS progression and p53 function might mostly be due to cell growth arrest and cell death regulation.

We also confirmed by RT-qPCR an upregulation of several upstream regulators of the p53 family, including Mdm2, Myf6, Mlf1, and Txn (Table 1) (Arrowsmith, 1999). Taken together, our results suggest that a p53-like pathway is activated in ALS muscles both in patients and the murine SOD1 ALS-models.

P53-family members are regulated in mouse skeletal muscles during ALS

As we observed in the muscle biopsies of ALS patients a correlation between p63 expression and the severity of the pathology, we investigated the expression levels of p53

family members in the muscles of SOD1 (G86R) mice. Our analysis revealed an increased expression of TA isoforms of Trp63 in SOD1 (G86R) (Figure 3). Strikingly, the mRNA levels of TA isoforms of Trp63 were strongly induced towards the end of the disease (105 day), while the mRNA levels for ΔN isoforms of Trp63 were downregulated during the same time period. A similar tendency was observed for p53, TA and ΔN isoforms of p73, albeit at a lower magnitude. The expression of TA isoforms of Trp63 correlated with acetylcholine receptor alpha (Chrna1) expression, a molecular marker indicating the severity of muscular denervation. In addition, we analyzed the expression of two documented effectors of muscular atrophy, namely Fbxo32 (Atrogin-1) and Trim63 (MuRF1). These proteins are E3 ubiquitin ligases that target muscular proteins for degradation during muscular atrophy or remodeling (Murton *et al.,* 2008). Importantly, the deregulation of Trp63 expression also correlated with the upregulation of these two markers. This is in accordance with our data from ALS patient muscle biopsies, whereby the expression of p63 also correlated with the degree of muscle pathology (Figure 1E).

P63 protein accumulates in muscle fibers during ALS

Based on the observed deregulation of p63 expression in ALS patients and the stronger upregulation of TAp63 in SOD1 (G86R) mice, we further analyzed p63 protein levels. Immunoblotting with a TAp63 isoforms specific antibody revealed a striking accumulation of p63 proteins in muscles of SOD1 (G86R) mice that correlated with the progression of the disease (Figure 4A). When probing with a ΔNp63 specific antibody, however, we did not observe any specific band. The use of a p63 antibody directed against all p63 isoforms confirmed an upregulation of p63 in muscles of SOD1 (G86R) mice (Figure 4—figure supplement 1). Immunohistochemistry with the same antibody also revealed markedly increased immunoreactivity in the nuclei of muscle fibers of SOD1 (G86R) (Figure 4B, Figure 4—figure supplement 3). In contrast, there was no significant increase in p73 staining (Figure 4—figure supplement 2). In this case, the apparent higher number of p73 positive nuclei appeared to be due to the atrophy of the muscle fibers, increasing the density of cells/nuclei. Similar experiments to detect expression of p53 did not yield a specific staining. However, we observed by western blot some slight increase in p53 protein levels in protein extract of muscle from SOD1 (G86R) mice (Figure 4—figure supplement 1). Taken together, our data indicated a complex regulation of p53 family members during muscular atrophy, highlighted by significant increase of TAp63 messenger and protein expression levels in the skeletal muscles during ALS.

Muscle denervation induces a p63 response

We then further investigated the possible cause of the deregulation of Trp63 expression. Several studies showed that the ALS etiology is complex and multifactorial, involving different cell types and molecular mechanisms. One established cause of muscular atrophy is motor neuron degeneration that leads to muscle denervation. However, it has also been shown that SOD1 mutants can also directly cause alteration in muscle cells such as SOD1 protein aggregates and mitochondrial abnormalities (Pansarasa *et al.,* 2014). To verify the first hypothesis, we induced denervation in 80 day-old wild-type and SOD1 (G86R) mice by sciatic nerve crush, and gastrocnemius muscles were analyzed 7 days later. Our results showed that denervation upregulated TAp63 mRNA levels five-to six fold in wild-type mice (Figure 5A). Concomitantly, ΔNp63 levels were downregulated 0.4-fold (Figure 5B). In SOD1 (G86R) mice, nerve crush further accentuated changes in mRNA levels for TA and ΔN isoforms of Trp63. In addition, the TAp63 target genes Cdkn1a and Gadd45a were found strongly induced after nerve crush (Figure 5C, D). These results show that nerve injury leading to alteration of the motor axis seems to be sufficient to activate a TAp63 response.

Mutated SOD1 is sufficient to induce the p63 response in myoblasts

Although it remains a challenge to reproduce in vitro the long-term development of ALS, we tried to assess the effect of the mutated SOD1 on muscle cells via an overexpression of SOD1 (G86R) in the mouse myoblast cell line C2C12. Several target genes of the p53-family (Bax, Cdkn1a, Gadd45a) were induced upon overexpression of SOD1 (G86R) (Figure 5E). Similarly TAp63 expression was increased at the mRNA level and the protein level (Figure 5E and Figure 5—figure supplement 1). In contrast, the mRNA levels as well as the promoter activity of ΔN isoforms of P63 were downregulated (Figure 5E, Figure 5—figure supplement 1) (Romano *et al.,* 2006). However, we were not able to confirm this result on ΔNp63 at the protein level. We also tested for a possible cross-regulation of ΔNp63 expression by the increased expression of TAp63 observed in ALS. We observed that TAp63 represses the promoter activity of the ΔN isoforms of P63, while it expectedly induces the Mdm2 promoter (Figure 5—figure supplement 1). These results demonstrated that expression of SOD1 (G86R) was sufficient to trigger a p53-like response similar to our *in vivo* observations in atrophic muscle tissues.

We then investigated whether TAp63 could be induced by different stresses related to the cellular damages caused by SOD1 mutants. We used pharmacological inductors for oxidative stress (menadione) (Barber and Shaw, 2010), DNA damage (etoposide) (Aguirre *et al.,* 2005), mitochondrial deregulation (FCCP) (Manfredi and Xu, 2005) and

ER Stress (tunicamycin) (Hart, 2006; Nishitoh *et al.,* 2008). Treated cells revealed an increase of TAp63 upon the four stresses (Figure 5F, Figure 5—figure supplement 2). Mitochondrial and ER stress triggers specific signaling pathways that involve a complex network of transcription factors such as ATF4, ATF6, XBP1 and CHOP (Senft and Ronai, 2015). Interestingly, overexpression of ATF4 and ATF6 induces the RNA level for TAp63 and TAp73 respectively, but not p53 (Figure 5—figure supplement 2). This result indicated that upregulation of TAp63 expression might be involved in the muscle cell response to diverse stresses including stresses related to SOD1 mutants.

TAp63 regulates Trim63 (MuRF1) expression, a specific muscle atrophy effector

As the expression profile of the TA isoforms of Trp63 correlated with the expression of the muscle atrophy effectors Fbxo32 and Trim63 (Figure 3), we hypothesized that TAp63 could regulate them directly. Bioinformatic analyses revealed the presence of several putative p63-binding sites in the promoter of Trim63 (Figure 6A). Therefore, we tested whether TAp63 could regulate Trim63 expression. Indeed, TAp63 overexpression in C2C12 cells strongly induced Trim63 mRNA levels (Figure 6B). Fbxo32 expression level was much less affected (data not shown). Note that under this condition p53 or p73 had less effect on Trim63 expression (Figure 6B). Under the same experimental conditions, other p63 target genes, Cdkn1a, Pmaip1, Casp1 and Prkaa1 were less induced (Figure 6C).

To further characterize the regulation of Trim63 by TAp63, we used luciferease reporter constructs containing progressive deletions of the Trim63 promoter. We found that p53 family members induced Trim63 promoter reporters that contained at least the fragment -500 bp to -1000 bp (Figure 7A and B) (Waddell *et al.,* 2008). Interestingly, that fragment contains potential p63 binding sites with high probability scores, such as RE1/2 (-660/-690 bp). We then assessed the capacity of p63 to bind the Trim63 promoter on binding sites that have high probability scores. Chromatin immunoprecipitation experiments (ChIP) covering RE1/2 and RE4 binding sites showed that TAp63 proteins bound preferentially onto RE1/2 (Figure 7C). Similarly, ChIP experiments indicated that p73 and p53 bound to RE1/2 (Figure 7—figure supplement 1). However, p73 seemed also to bind RE4.

To assess the physiological importance of TAp63 in Trim63 expression we used TAp63-specific silencing RNA (siRNA). Transfection in C2C12 cells of TAp63 siRNA diminished the expression of TAp63 at the protein and mRNA levels (Figure 7—figure supplement 1). TAp63 silencing or overexpression of ΔNp63 had a partial protective effect on C2C12 (Figure 7—figure supplement 2). Importantly, silencing of TAp63

reduced Trim63 mRNA levels in both basal state and following stress induced by FCCP (Figure 7D). SiRNA against p53 also diminished Trim63 RNA level, while siRNA against p73 had not significant effect (Figure 7—figure supplement 2). The combination of siRNA against TA isoforms of Trp63, TA isoforms of P73 and P53 diminished further Trim63 RNA level up to ~50%, but did not abolish it. Taken together, these results indicate a complex regulation of the Trim63 promoter, in which the direct binding of p63 and p53 correlates with the modification of gene expression in C2C12 muscular cells.

Discussion

In this study, we developed a comprehensive approach combining biopsies from ALS patients, transgenic animal model of ALS and myoblastic cell lines to analyse the expression and the possible function of P63, a member of the p53 family, in muscle atrophy.

Regulation of p63 expression during muscular atrophy in ALS patients and in ALS murine models

Our results demonstrate that there is a complex p53-like response developed by the atrophic muscle during ALS progression. This assertion is first based on the bioinformatic signalling pathway analyses of 4 independent microarray experiments performed on muscle biopsies of ALS patients as well as two different mouse models of ALS. These analyses pointed out a deregulation of p53 as one of the only three transcription factors deregulated in all four experiments, and common between mouse and human patient samples. Moreover, detailed expression profile analyses of several p53 target genes (Cdkn1a, Gadd45a, Pmaip1) or the p53 family member, P63, showed that their expression correlated significantly with the severity of the pathology in humans. The signalling pathway analyses were confirmed with groups of individuals independent of those used for the microarrays and by additional experimental methods. RT-qPCR confirmed the induction of multiple target genes of the p53 family. In addition, expression analysis of p53 family members indicated that in ALS the P63 gene seems more likely to play a regulatory role as the TAp63 isoforms are strongly upregulated and localized in the nuclei of the fibers during the ALS pathology (Figures 1, 3, 4, 5, figure supplement 2, 3, 4, 7). Our observation that the deregulation of p63 and p63 target genes occurs in muscle of ALS patients that have not been selected for a particular genetic alteration indicates that these deregulations are likely to be a common feature in ALS, independently of whether it is SOD1 that is mutated or another gene. Additional

experiments using other mouse models for TDP43 or FUS might confirm that. It was previously reported that p63 participates in muscle cell differentiation and metabolism, and contributes to cardiac muscle development (Cam *et al.,* 2006; Martin *et al*., 2011; Rouleau *et al.,* 2011; Su *et al.,* 2012; Osada *et al.,* 1998). Now, by combining biopsies from ALS patients and an animal model for ALS, the present study provides the first solid evidence that p63 might also participate in muscular atrophy.

Although our results indicate that TAp63 is strongly induced in muscle atrophy during ALS, we cannot exclude the possible contribution of p53 and p73 proteins due to the fact that their mRNA expression is upregulated, although to a much weaker extent than TAp63 (Figure 3—figure supple- ment 4). In addition, we detected p53 and p73 protein expression in muscles tissues. Several studies support this possibility by showing that p53 and p73 play a role in muscle cell differentiation, cachexia and survival (Schwarzkopf *et al.,* 2006; Cam *et al.,* 2006, Soddu *et al.,* 1996; Tamir and Bengal, 1998; Porrello *et al.,* 2000; Weintraub *et al.,* 1991). However, genetic inactivation of p53 does not affect ALS progression, muscle development or muscle regenerative capacity (Donehower *et al.,* 1992; White *et al.,* 2002; Kuntz *et al.,* 2000; Prudlo *et al.,* 2000). Nevertheless, our results suggest that the absence of p53 could be compensated by p63 or even p73. The slight increased in p53 protein levels observed in protein extracts of muscle from SOD1 (G86R) mice might be caused by the production of ROS that stabilized p53 through post-translational modifications as previously described in other stresses (Vurusaner *et al.,* 2012). In addition, the slight increase in p53 RNA level we observed might also contribute.

The causes of p63 regulation during ALS muscle atrophy seemed complex and reflect the debated etiology of the pathology (Yamanaka *et al*., 2008; Boillée *et al.,* 2006; Wong and Martin, 2010; Chen *et al.,* 2013). For instance, we showed that P63 deregulation could have an intercellular origin represented by the loss of interaction between the muscle and the nerve cells, as provoked in the nerve crush experiment (Figure 5). Hence, the degeneration of the motor neurons that is characteristic of ALS would be sufficient to explain the increased expression of TAp63 and its target genes in ALS. However, we also observed that P63 deregulation can have an intrinsic origin resulting from expression of SOD1 (G86R) in muscle cells causing intracellular stresses (Figure 5). Indeed, ALS-associated SOD1 mutations have been shown to induce SOD1 protein aggregates and mitochondrial dysfunction in muscle cells (Pansarasa *et al.,* 2014). Interestingly, we observed that both activation of a protein aggregate stress response pathway or mitochondrial dysfunction could induce a TAp63 response. This finding is supported by a previous report showing that tp63 is an effector of the ER stress pathway in zebrafish allowing the regulation of the pro-apoptotic gene bbc3 (puma) (Pyati *et al.,* 2011).

Protein aggregates, mitochondrial stress and oxidative stress triggered selective complex

stress pathways named ER stress (or UPR, unfolded response) or mitochondrial stressed pathways that utilize several common transcription factors as effectors, such as ATF4, ATF6, CHOP and XBP1 (Senft and Ronai, 2015; Broadley and Hartl, 2008; Lee, 2015; Michel *et al.,* 2015). Therefore, we investigated whether these effectors could drive the expression of p53 family protein. We showed that some of these transcription factors, notably ATF4 and ATF6, were able to induce the RNA levels of TA isoforms of Trp63 and P73 in C2C12 cells (Figure 5—figure supplement 2). However, bioinformatic analyses did not reveal potential canonical binding sites for these transcription factors neither in promoters of TA isoforms of P63 nor P73, suggesting that the regulation might occur through indirect mechanisms that remain to be identified.

Function of the p53 response in muscular atrophy during ALS

Based on the literature, the p53 family could mediate different cellular outcomes in muscles and therefore on muscle pathology. p53/p63/p73 proteins have been linked to cell death, differentiation, metabolism, ER stress induction and ROS defence, which have all been reported during ALS (Hart, 2006; Barber and Shaw, 2010; Aguirre *et al.,* 2005; Nishitoh *et al.,* 2008; Manfredi and Xu, 2005). Our study revealed that the majority of the p53/p63/p73 target genes upregulated during ALS in the atrophic muscles are connected to cell death (Gadd45a, Peg3, Perp, Pmaip1, Bax, Siva, Eda2r, Wig1/Pag608) (Figure 1 and Table 1). Genes connected to other functions, such as ER stress (chop, bip, xbp1, scotin) or energy metabolism (Tigar, Sesn1, Sesn2, Sco2) seem to be less consistently regulated, as some are upregulated (Sesn1, Sesn2, Xbp1), while others are downregulated (Sco2, Tigar, Chop, Bip, see Table 1). Therefore, it seems more likely that p53 family members, notably TAp63, function in ALS is connected to muscular atrophy via control of muscle cell survival and catabolism. This hypothesis is further supported by three of our results.

First, the p53 family members TAp63, p53 and TAp73 induce the muscle atrophy effector gene Trim63 (MuRF1), most likely via a direct binding to the Trim63 promoter (Figure 7, Figure 7—figure supplement 1). Second, overexpression of TAp63 induces cell death in C2C12 myoblasts (Figure 7— figure supplement 1). Third, overexpression of ΔNp63 protects myoblastic cells against stresses (Figure 7—figure supplement 2). Although these results were obtained in a myoblastic cell line, they are consistent with numerous other studies describing the ability of p63 to control cell death in various pathophysiological conditions. To establish the exact pathophysiological importance of TAp63 upregulation in ALS represent a difficult challenge. Indeed, we already observed that *in vitro* the silencing of TAp63 with siRNA does not entirely abolish the expression of Trim63 (Figure 7) and does not significantly reduce cell death induced by stresses

(Figure 7, Figure 7—figure supplement 2). This has several reasons. The first is that the expression of Trim63 involves several transcription factors, such as FOXO1 and the glucocorticoid receptors that certainly participate in the regulation of Trim63 during ALS. Indeed, the coordinated silencing of p53, TAp63 and TAp73 did not completely abolish Trim63 RNA levels (Figure 7—figure supplement 1), supporting the involvement of other types of transcription factors. The second reason is intrinsic to the p53 family. Indeed, we already know that p53, p63 and p73 have some redundant functions and target genes. We have also already established that p53 and p73 are expressed in muscles during the pathology (Figure 4—fig- ure supplements 1,2) and also bind to the Trim63 promoter (Figure 7—figure supplement 1). Therefore in absence of TAp63, p53 and/or TAp73 might replace it in some conditions. For example, we observed an upregulation of TAp73 in C2C12 cells when TAp63 is silenced. This compensatory mechanism might therefore also explain why TAp63 siRNA do not protect C2C12 cells from death, in contrast to the expression of the ΔNp63 isoform that could inhibit p53, TAp73 and TAp63 function altogether (Figure 7—figure supplement 2).

ALS patients are currently diagnosed at a stage where denervation and muscular alterations already are established and, because of the lack of curative treatment, lead to death within 2 to 5 years. The results presented here suggest that p53 family members, via the regulation of selected target genes such as Eda2r, Peg3 but also, as we show, via Trim63, might contribute to muscle catabolism in these patients. It remains to be established whether this signalling pathway is uniquely critical for muscular atrophy during ALS or whether it is common to other muscular atrophies occurring in pathologies such as cachexia, diabetes, and others.

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1– Figures and tables

Table 1 – Fold induction of p53-related genes in the ALS model SOD1 (G86R).

Gene name	Function	90 d.	105 d.
p53-family target genes			
Cdkn1a (p21)	Cell cycle arrest	4	13
Gadd45a	Cell cycle arrest	5,6	21
Peg3	Apoptosis inducing	3	7
Perp	Cell cycle arrest	4	12
Pmaip1	Apoptosis effector	5	12
Bax	Apoptosis effector	3	8
Siva	Apoptosis inducing	3	5
Zmat3	Growth regulation	1,6	1,1
Eda2R	NF.Kb/JNK pathway	3,4	9,4
Tigar	Glucose metabolism	0,75	0,2
Sens1	ROS homeostasis	-	16,3
Sens2	ROS homeostasis	1,27	1,46
Sco2	Glucose metabolism	1,18	0,91
Ddit3 (Chop)	ER stress	1,14	0,35
Bip (Grp78)	ER stress	1,25	1,08
Xbp1	ER stress	2	2,51
p53-family regulators			
Mlf1	Cell cycle arrest/differentiation	0,9	0,2
Myf6	Differentiation	4	7
Mdm2	p53 degradation	4	6
Txn1	Oxidative stress response	4	6
Id2	Inhibition of differentiation	2	3,1
p53-family members			
P53		4	3
TAp63		4	12
ΔNp63		0,5	0,3
TAp73		2	3
ΔNp73		0,9	0,8
Denervation/atrophy markers			
Chrna1 (ACh Receptor alpha)	Neuromuscular junction	4,2	12,4

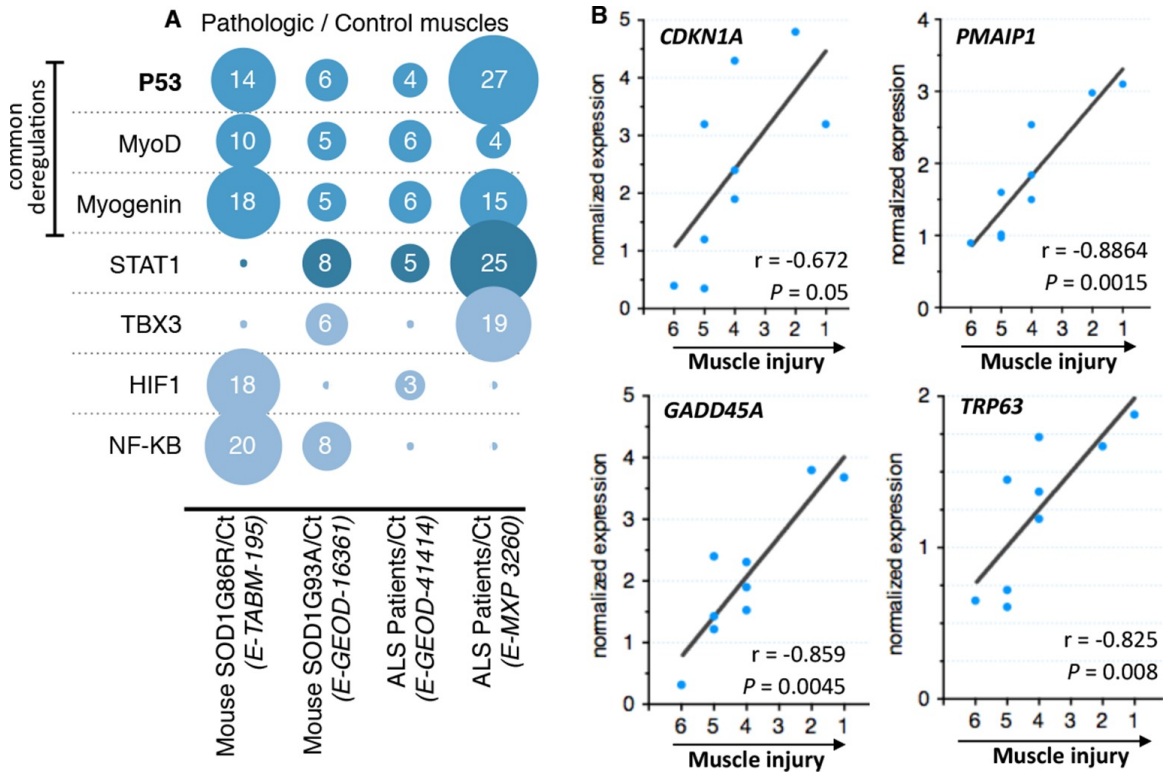

Fig. 1 – Microarray meta-analysis highlights links between the deregulation of p53 family related genes and ALS. (A) Representation of the number of deregulated target genes of the indicated transcription factors. Data were obtained using the indicated datasets from the Array Express database (EMBL-EBI) and quantification was carried out from AltAnalyze software analyses on transcription factor databanks (complete data in Supplementary file 2A, B, C, D. (B) mRNA levels from nine ALS patient deltoid muscles as by DNA microarray were correlated with the intensity of muscle injury. Expression data were generated using a murine gene profiling database deposited at ebi.ac.uk/arrayexpress (accession number E-MEXP-3260). In the corresponding study, muscle injury was estimated according to a composite score combining manual testing of strength of shoulder abductors and the degree of myofiber atrophy. This score ranges from 6 (normal strength and very low level of atrophy) to 1 (total paralysis and high level of atrophy). Each point represents an individual. Correlation coefficients (r) and p-values were determined by Spearman correlation test.

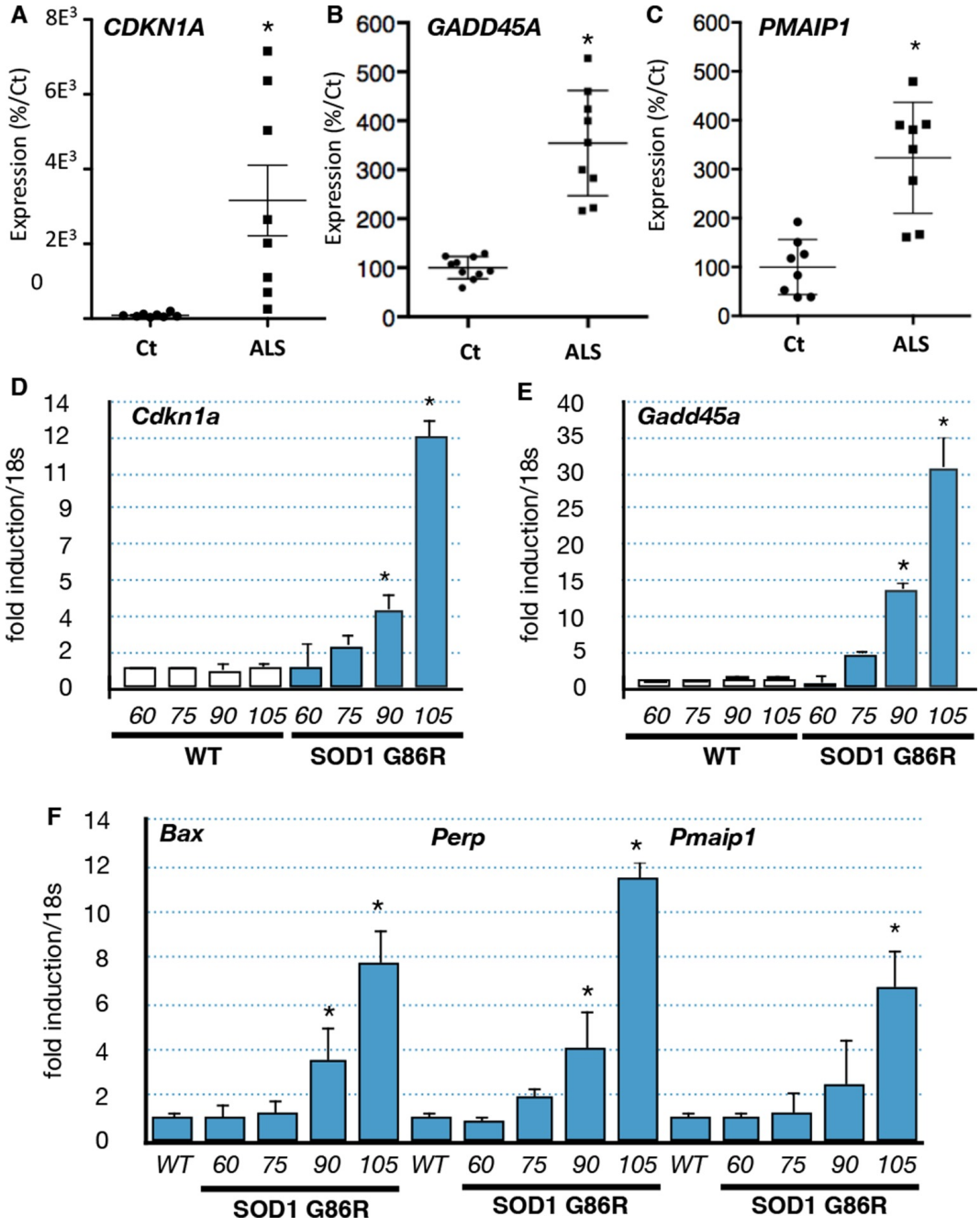

Fig. 2 – p53-family target gene expression in muscles from ALS patients and in an ALS mouse model correlates with disease intensity. (A–C) RNA from muscle biopsies of control and ALS patients (n = 8, Neuromuscular Unit [BioBank of Skeletal Muscle, Nerve Tissue, DNA and cell lines]) was extracted and analyzed by RT-qPCR. Absolute levels are normalized against the average of the control group. (D–F) p53 family target genes mRNA levels were assayed in SOD1 (G86R) mouse gastrocnemius muscle by RT-qPCR. Graphs are means of fold induction versus 60 days-old WT and of matching age (60, 75, 90, 105-days-old, n = 6) and experimental condition (wild-type, or SOD1 (G86R)). *p<0.01 compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test.

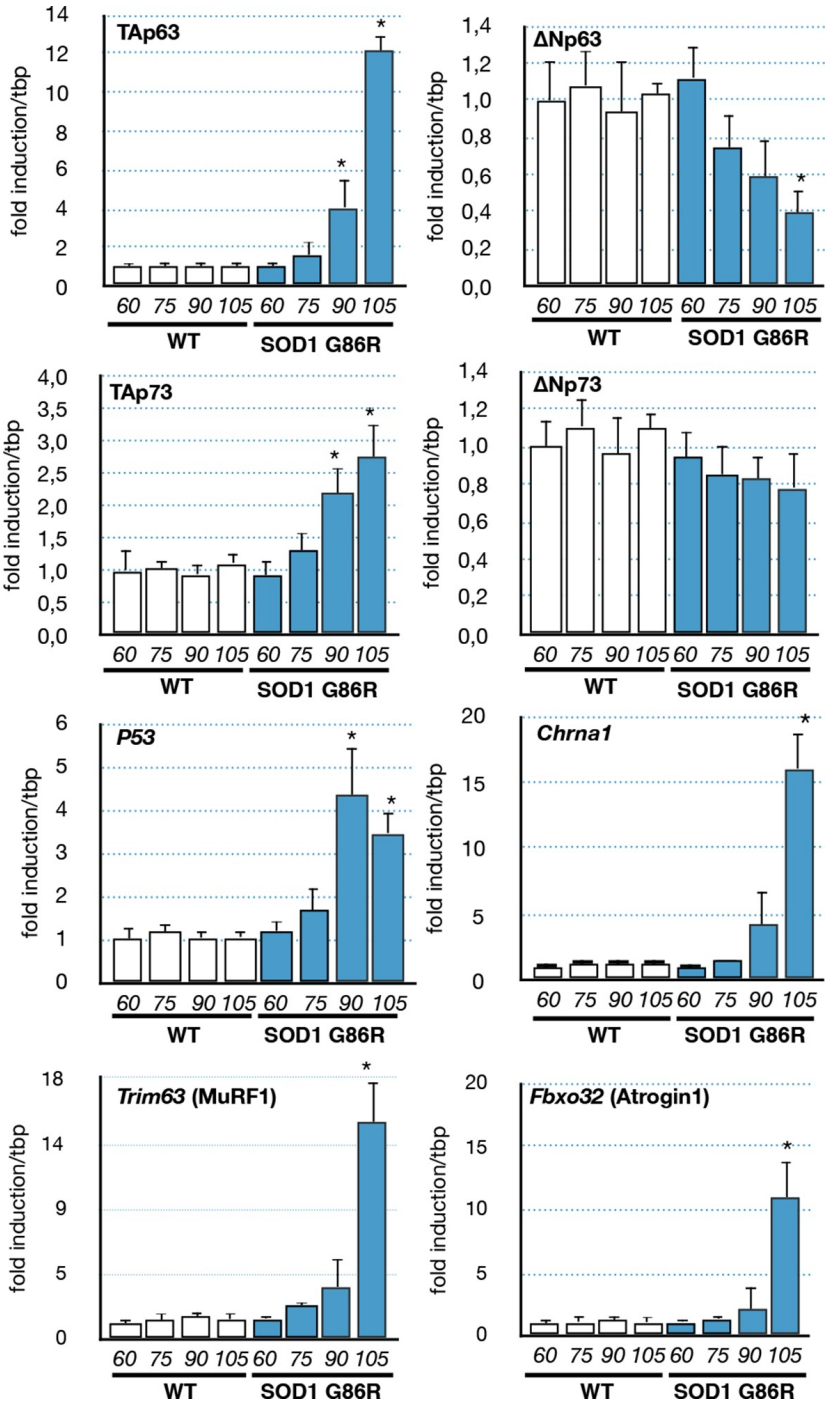

Fig. 3 – Expression of p53-family members in SOD1 (G86R) muscles. p53 family members, Chrnα1 (Acetylcholine receptor subunit alpha) or muscle atrophy effectors Trim63 (MuRF1) and Fbxo32 (Atrogin1) mRNA levels were assayed in SOD1(G86R) mouse gastrocnemius muscle by RT-qPCR. Bars are means of fold induction versus 'WT 60 days-old' and of matching age (60, 75, 90, 105 days-old, n = 6) and experimental condition (WT or SOD1 (G86R)). *$p<0.01$ compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test.

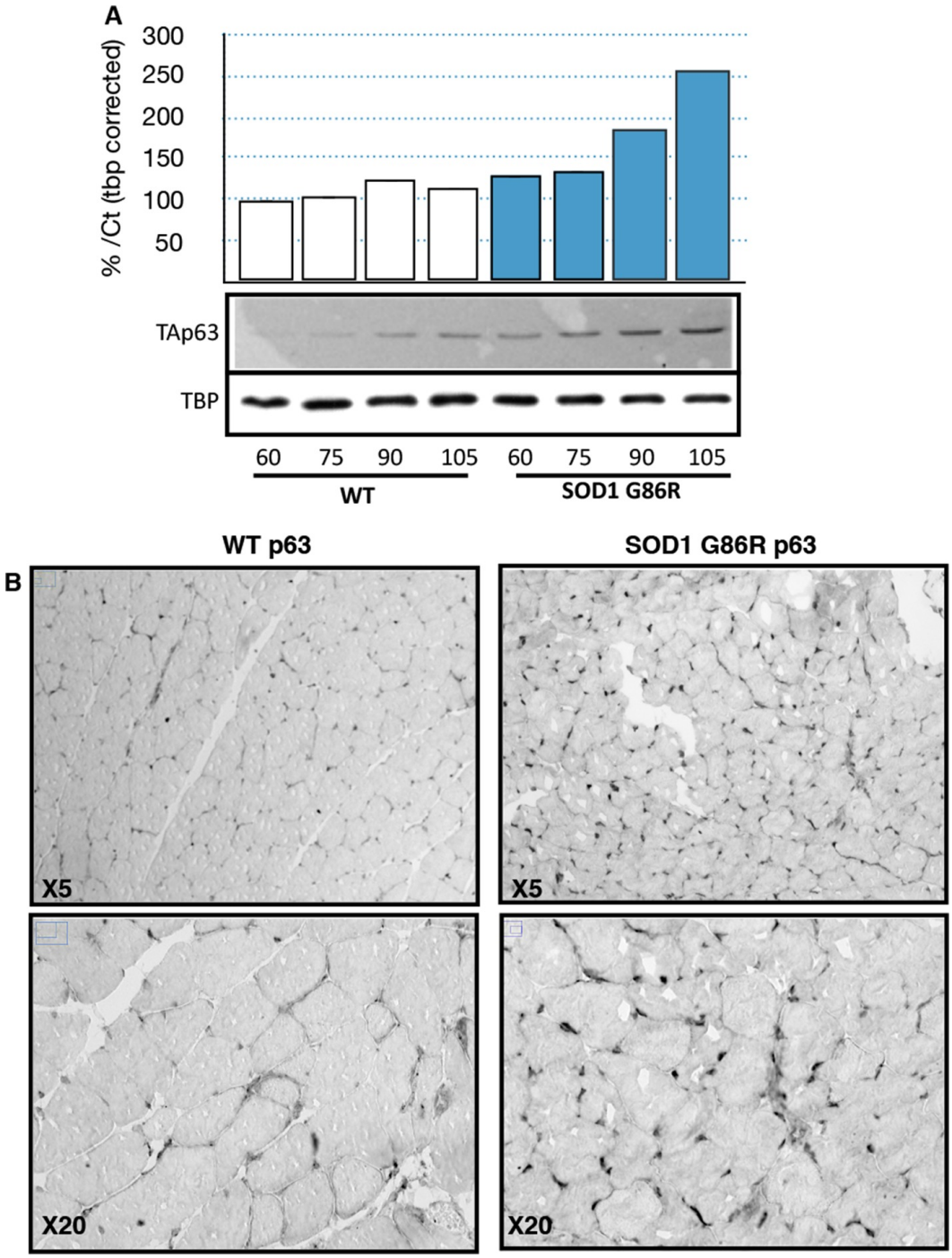

Fig. 4 – p63 protein expression in SOD1 (G86R) muscle. (A) Proteins from muscles were immuno-precipitated with a p63 antibody and then separated on a 10% SDS PAGE gel. Western blot experiment was performed using an antibody against TAp63. Each experimental point is a pool of proteins from 6 animals. Graph represents quantification of the blot using ImageJ image analyzer software indicated a %/WT 60 day-old animals. (B) Gastrocnemius muscles from wild-type or symptomatic SOD1 (G86R) (105 days) mice were cryodissected and probed for total p63 protein.

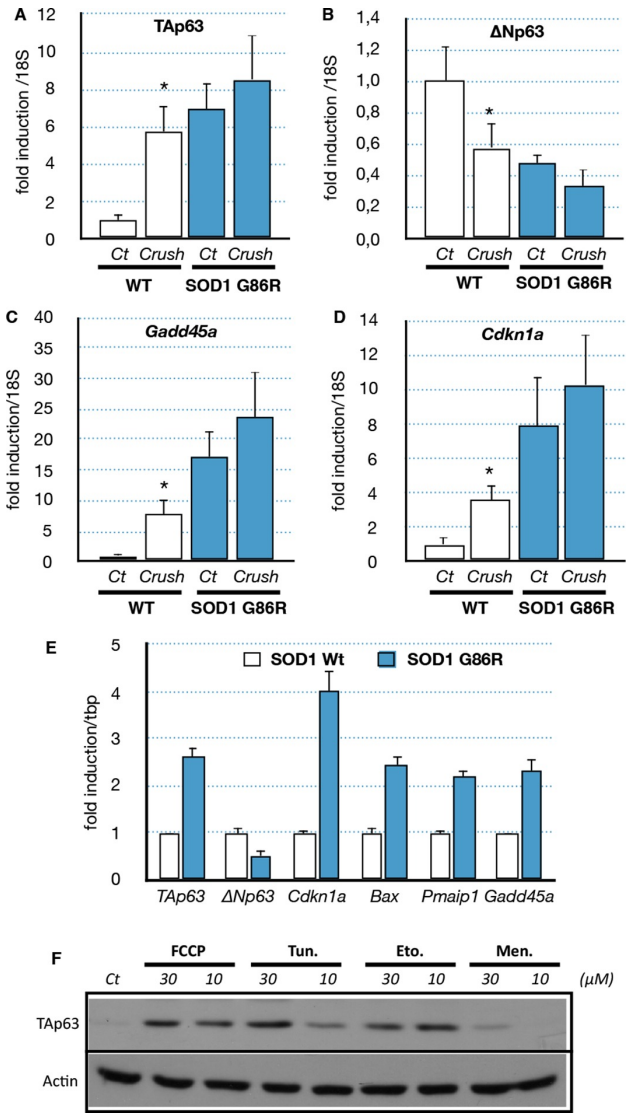

Fig. 5 – Expression of p63 and p53-family target genes following sciatic nerve crush, SOD1 expression of induction of stress (A–D) WT and SOD1 (G86R) mice (80 days of age) were anesthetized and the sciatic nerve crushed. Sham-operated contra limbs served as control (Ct). After 7 days, expression of TA isoforms of Trp63 (A, TAp63), ΔN isoforms of Trp63 (B, ΔNp63), Gadd45a (C) and Cdkn1a (D) was assayed by RT-qPCR (n = 6). Values were normalized to the value of sham-operated WT muscles/animals. Bars represent means (relative induction versus Ct) with standard deviation (n = 3). *p<0.01 as calculated by a one-way ANOVA test followed by a Tukey post-test. (E) C2C12 myoblasts were transfected with expression vectors for SOD1 variants (WT or G86R). mRNA from SOD1 transfected cells were analyzed by RT-qPCR for p63 and p63 target gene expression. Bars represent means (relative induction versus Ct) with standard deviation (n = 3). *p<0.01 as calculated by a one-way ANOVA test followed by a Tukey post-test. (F) Proteins were extracted from C2C12 myoblasts treated with compounds: FCCP, Tunicamycin (Tun), Etoposide (Eto), menadione (Men). Western blot analysis revealed TAp63 expression.

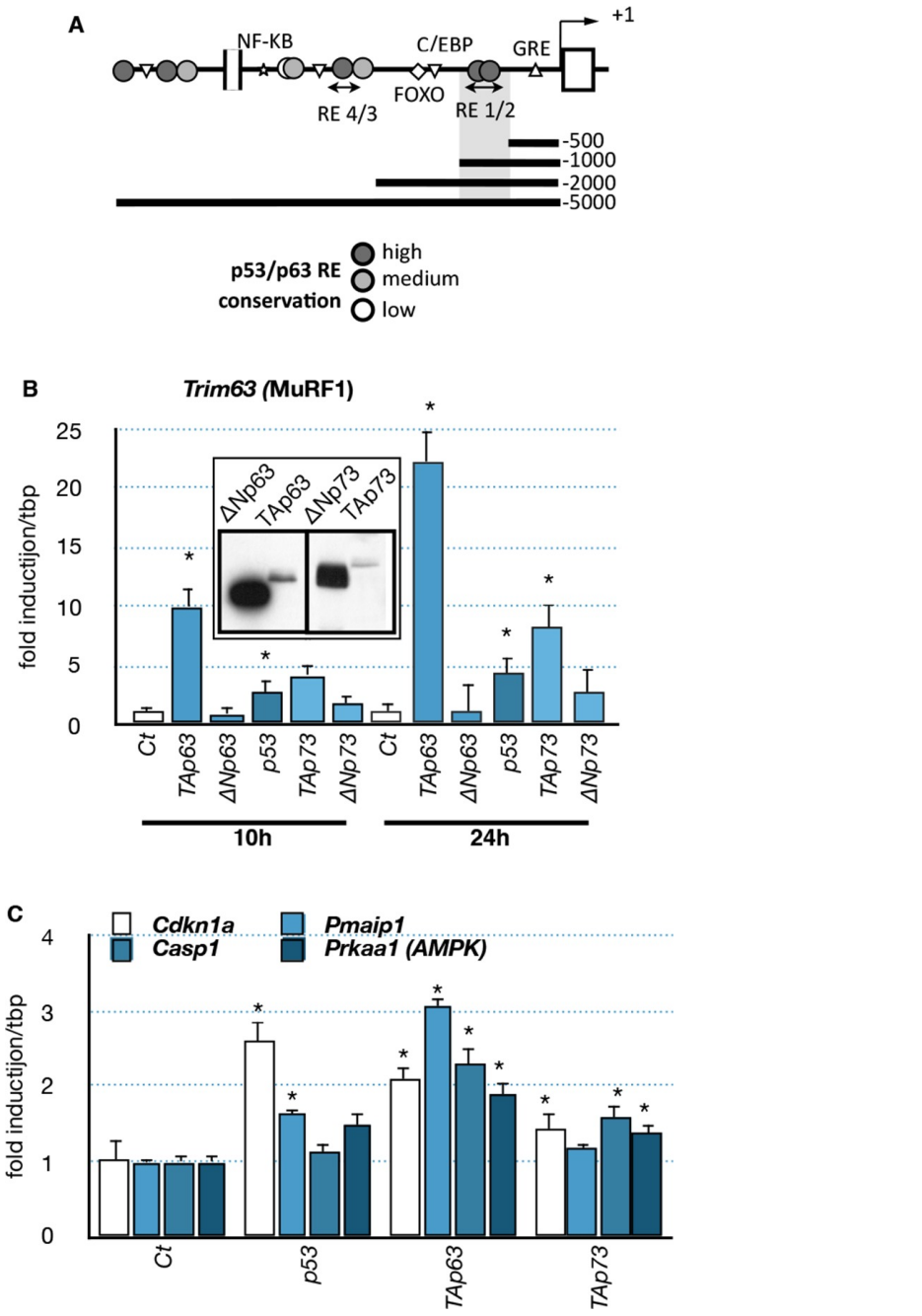

Fig. 6 – Effects of p53-family expression on Trim63 and p53-family target genes. (A) Schematic representation of the Trim63 promoter indicating the location of putative p53/p63 binding sites. (B, C) C2C12 myoblasts were transfected (inserted panel: western blot) with various p53-family members (TAp63γ, ΔNp63γ, p53, TAp73β, ΔNp73β). Total C2C12 RNA was subjected to RT-qPCR after 10 hr or 24 hr of transfection and Trim63 (B) or p63 target (C, Cdkn1a, Pmaip1, Casp1, Prkaa1) expressions are shown relative to control-transfected cells. Bars are means of fold induction versus the control (Ct) with SD (n=3). *p<0.01 as calculated by a one-way ANOVA test followed by a Tukey post-test.

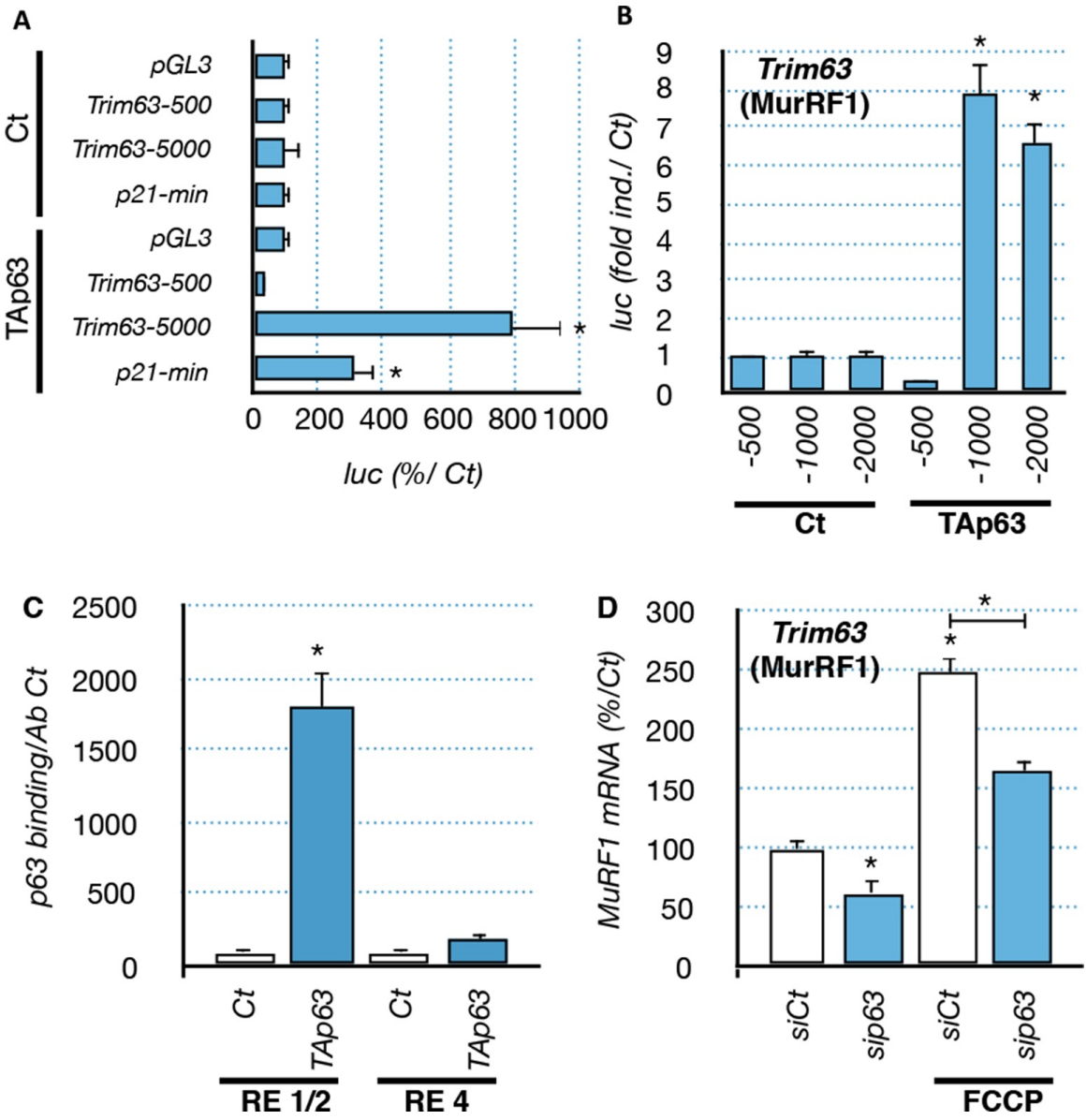

Fig. 7 – Regulation of Trim63 promoter by p63. (A, B) Trim63 promoter reporter constructs were co-transfected with pCDNA3 (Ct) or TAp63 into C2C12 cells and luciferase activity was assessed 16 hr later. pGL3 was used as a negative control. Bars correspond to means with SD (n = 3). *p<0.01 as calculated by a one-way ANOVA test followed by a Tukey post-test. (C) Chromatin immunoprecipitation assay was performed on the Trim63 promoter using RT-qPCR on RE1/2 and RE4 (see Figure 6A). Bars correspond to means with SD (n = 3). *p<0.01 as calculated by a one-way ANOVA test followed by a Tukey post-test. (D) Trim63 mRNA levels were assayed in C2C12 cells by RT-qPCR after TAp63 silencing by siRNA for 36 hr and after treatment with FCCP for 12 hr. Bars correspond to means with SD (n=3). *p<0.01 as calculated by a one-way ANOVA test followed by a Tukey post- test.

1– Sumplementary data

Gene	Function	Control	Healthy	Pre-paralysis	Paralysis	Denervated
p53-family target genes						
Cdkn1a	Cell cycle arrest	0.96	1.12	4.26	12.94	14.11
Gadd45a	Cell cycle arrest	0.96	1.25	12.91	45.00	35.61
Peg3	Apoptosis inducer	0.77	1.05	1.73	6.23	5.18
Rb1	Cell cycle arrest	0.96	0.98	2.28	6.73	5.26
Perp	Apoptosis effector	1.07	0.90	1.05	3.72	0.68
Sesn1	ROS homeostasis	0.97	0.93	0.90	3.86	0.90
p53-family regulators						
Mlf1	Cell cycle arrest/differentiation	1.10	1.19	0.64	0.07	0.13
Myf6	Differentiation	0.94	1.06	2.36	5.21	4.40
Mdm2	p53 degradation	0.95	1.05	1.39	2.34	2.24
Txn1	Oxidative stress response	0.89	1.06	2.13	3.93	5.01

Figure 1—figure supplement 1. Regulation of p53-family related genes in skeletal muscle of SOD1(G86R) and denervated mice. Data were generated using a gene expression database deposited at ebi.ac.uk/arrayexpress (accession number E-TABM-195). Gastrocnemius muscle samples from male SOD1(G86R) mice with no symptoms (Healthy, at 75 days of age), altered hind limb extension reflexes (Preparalysis, at 90 days of age), and at the onset of hind limb paralysis (symptomatic mice, at about 105 days of age) were analyzed by DNA microarray. Denervated muscles were obtained from wild-type mice after 7 days of sciatic nerve axotomy. Non-transgenic male littermates served as controls. 3–4 animals were pooled per group, and each condition was done in duplicate. Values are expressed as means of normalized expression levels.

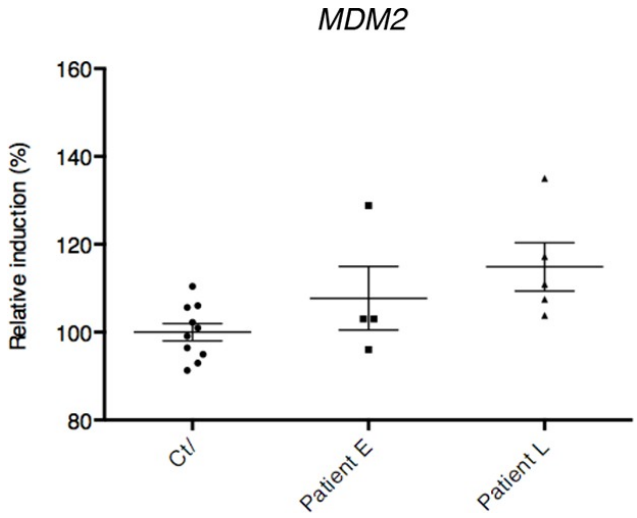

Figure 1—figure supplement 2. mRNA levels from control and ALS patient deltoid muscles as by DNA microarray were correlated with the intensity of muscle injury. Expression data were generated using a murine gene profiling database deposited at ebi.ac.uk/ arrayexpress (accession number E-MEXP-3260). In the corresponding study, muscle injury was estimated according to a composite score combining manual testing of strength of shoulder abductors and the degree of myofiber atrophy. This score ranges from 6 (normal strength and very low level of atrophy) to 1 (total paralysis and high level of atrophy). Each point represents an individual. In this experiment, patients with high level of atrophy (L, score 1–3) and low degree of atrophy (E, score 4–6) were grouped.

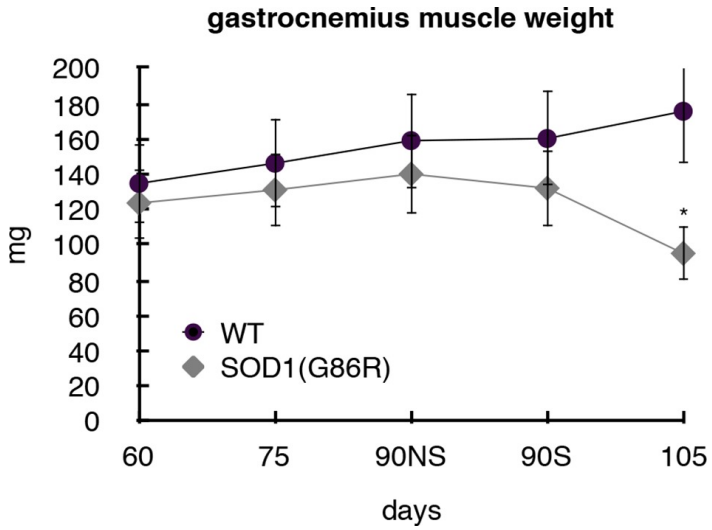

Figure 2—figure supplement 1. Gastrocnemius muscles from wild-type or symptomatic SOD1(G86R) (105 days) mice were dissected and weighted. Graph represents the weight (n = 5). *p<0.01 compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test. NS: non denervated, S: denerveted as assessed by acetylcholine alpha receptor (AchRα) expression.

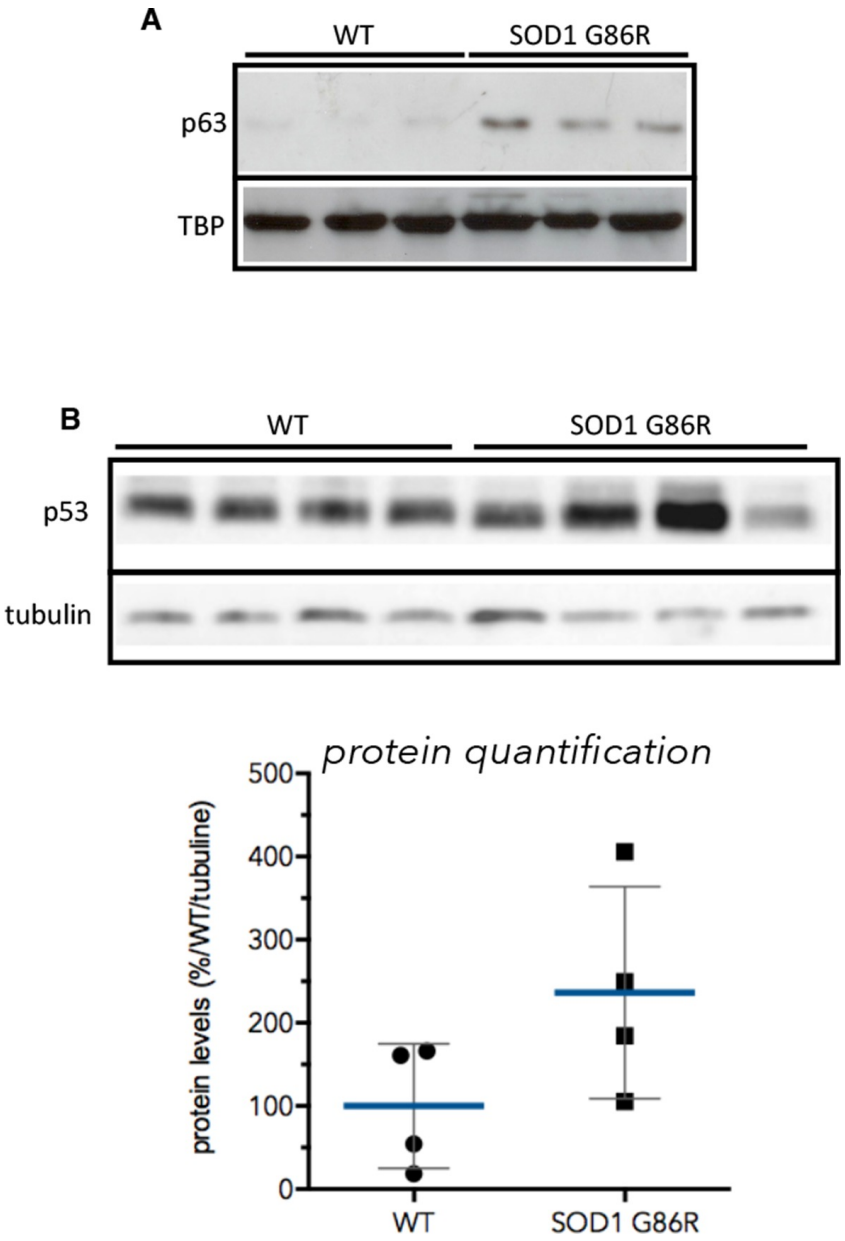

Figure 4—figure supplement 1. p53 and p63 protein expression in muscles of SOD1(G86R) mice. (A) Proteins from muscles were immuno-precipitated with a p63 antibody and then separated on a 10% SDS PAGEgel. Western blot experiment was performed using an antibody against p63 total. Shows pools of proteins from 3 animals at 105d. TBP was used as loading control. (B) Proteins (40 µg) from muscles were separated on 10% SDS PAGE gel. Western blot probing was performed with p53 antibody (IC12, 1/2000, Cell Signaling, Danvers, MA) and True Blot (Rockland Immunochemicals, Pottstown, PA) secondary antibody avoiding Ig heavy chain recognition. Tubilin was used as loading control. Graph below shows % of induction relative to the mean of p53 expression level in WT animals normalised with tubulin.

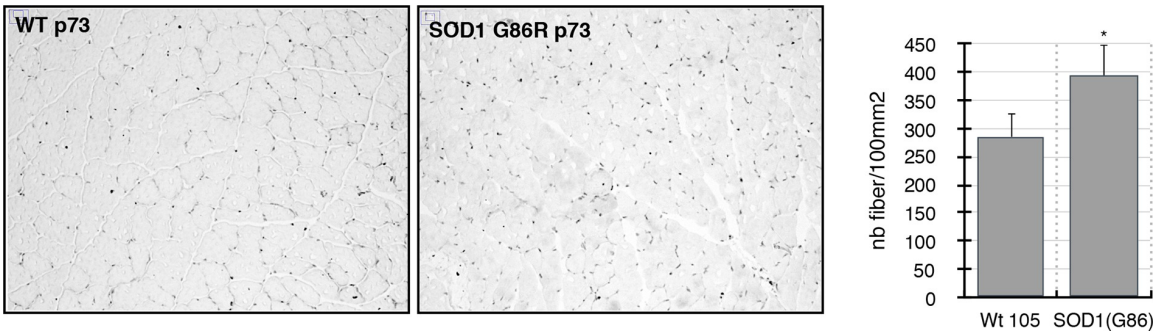

Figure 4—figure supplement 2. Gastrocnemius muscles from wild-type or symptomatic SOD1(G86R) (105 days) mice were cryodissected and probed for total p73 protein. Graph represents the number of fibers per surface unit as indicated (n = 5). *p<0.01 compared to control, as calculated by a one- way ANOVA test followed by a Tukey post-test.

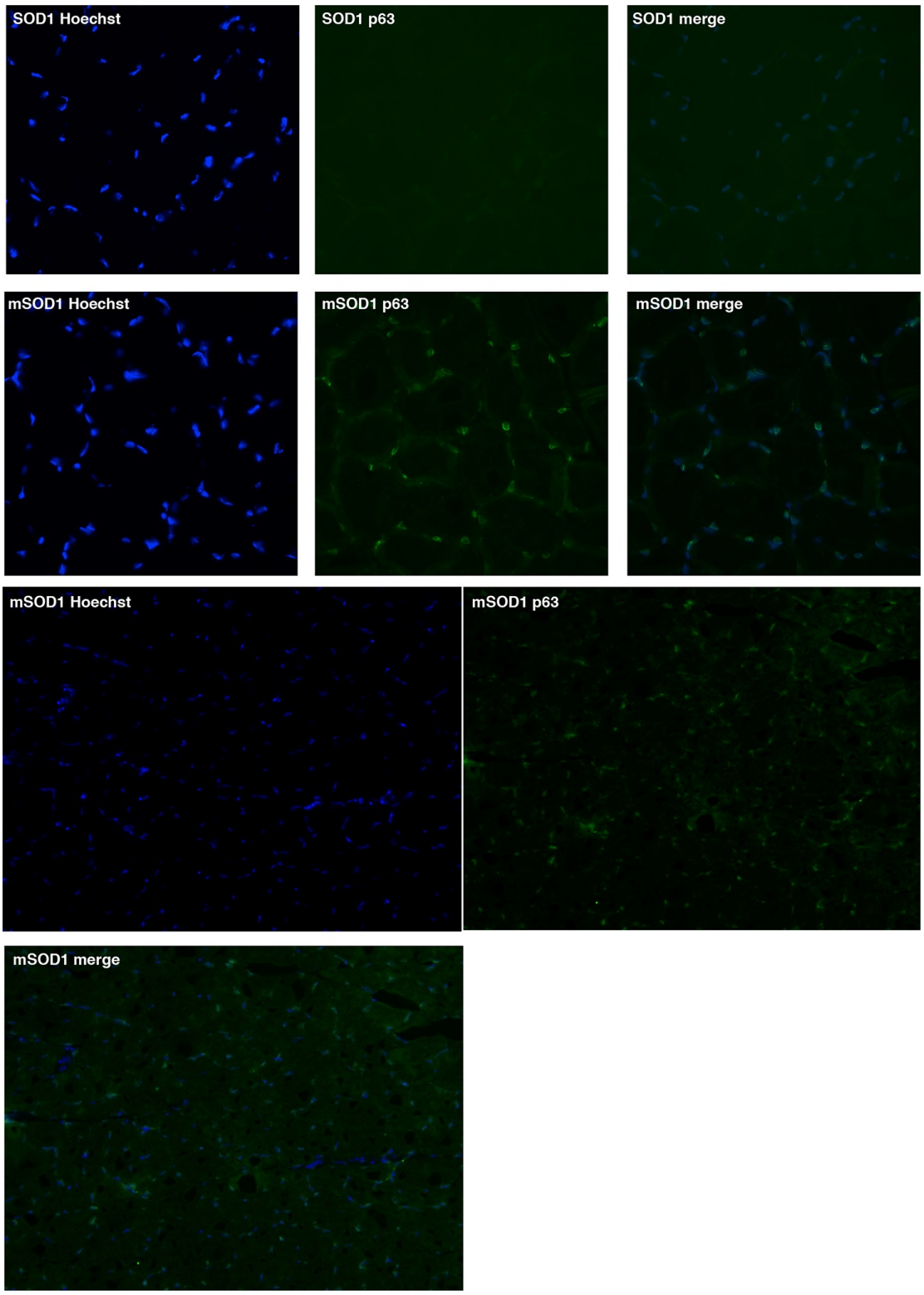

Figure 4—figure supplement 3. Gastrocnemius muscles from wild-type or symptomatic SOD1(G86R) (105 days) mice were cryodissected and probed for total p63 protein and nuclei (Hoechst).

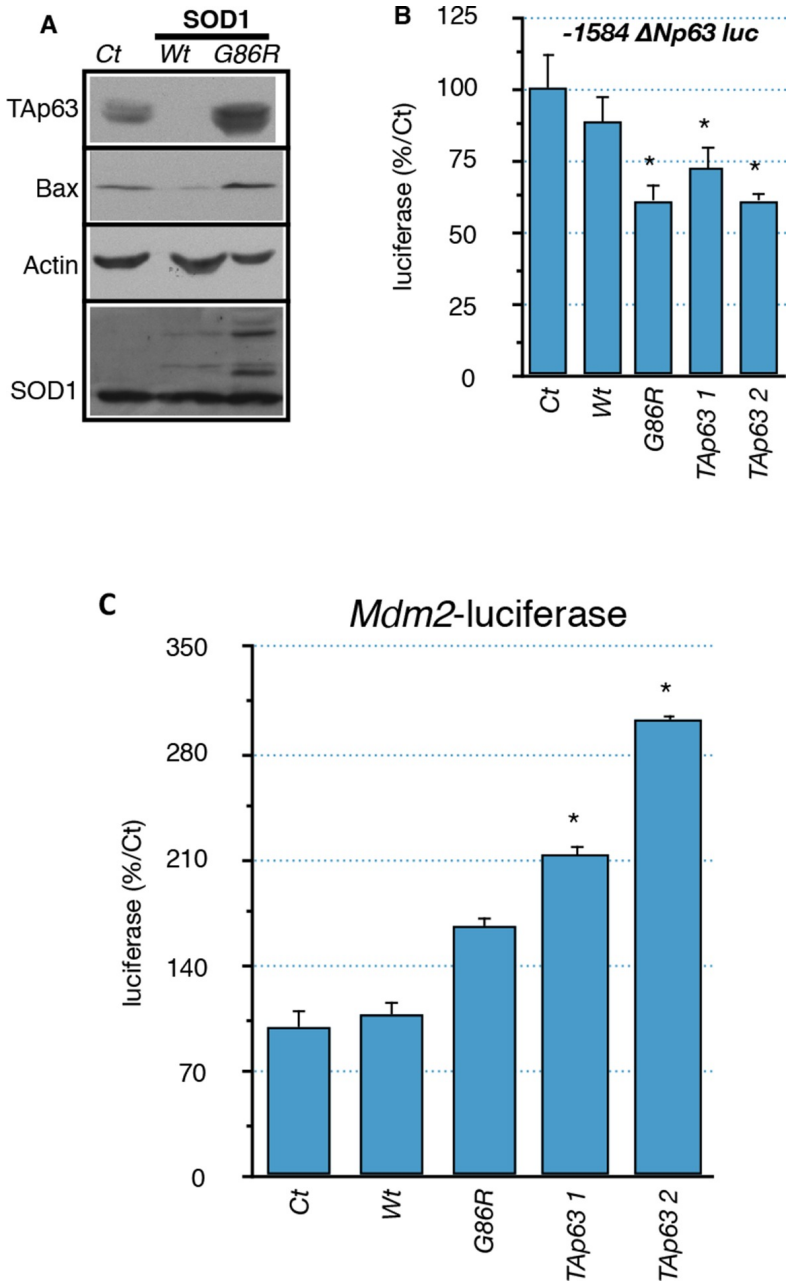

Figure 5—figure supplement 1. Regulation of p63 and Mdm2 expression by SOD1 (G86R). (A) Protein were extracted from C2C12 myoblasts expressing WT or SOD1(G86R) after 5-days puromycin selection. Western blot analysis revealed TAp63, Bax or SOD1 expression. Actin was used as loading control. (B, C) C2C12 myoblasts were transfected with expression vectors for SOD1 variants (WT or G86R) or TAp63γ (2 concentrations, 1, 2) and luciferase reporter genes containing deletions of the promoter of the ΔN isoforms of P63 (-1584-+32 or -46/+32) or Mdm2 promoter. Bars represent means (relative induction versus Ct) with standard deviation (n = 3). Results are standardized with the 'minimal' promoter reporter gene -46/+32-luc. Ct = cells transfected with an empty vector. Bars represent means (relative induction versus Ct) with standard deviation (n = 3). *p<0.01.

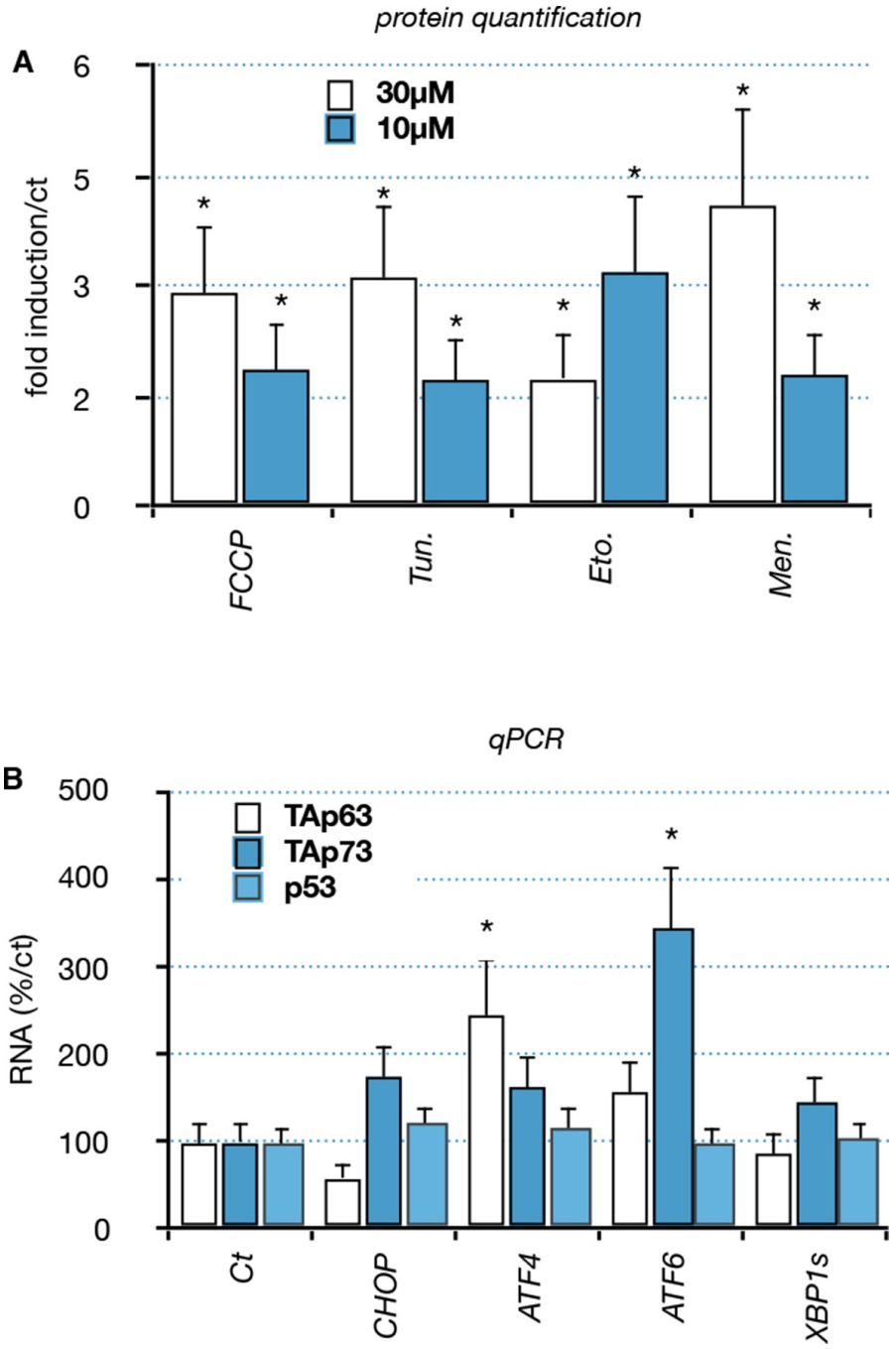

Figure 5—figure supplement 2. Functional interaction between members of the p53 family and ER or mitochondrial stress. (A) Quantification: Proteins were extracted from C2C12 myoblasts treated with compounds: FCCP, Tunicamycin (Tun), Etoposide (Eto), menadione (Men). Western blot analysis revealed TAp63 expression. Bars correspond to means with SD (n = 3). *p<0.01. (B) C2C12 myoblasts were transfected with expression vectors encoding transcription factors involved in the ER or mitochondrial stress pathway (CHOP, ATF6, ATF4, XBP1s). RNA levels for TA isoforms of Trp63, TA isoforms of P73 and P53 were followed by RT-qPCR. Bars represent means (relative induction versus Ct) with standard deviation (n = 3).*p<0.01.

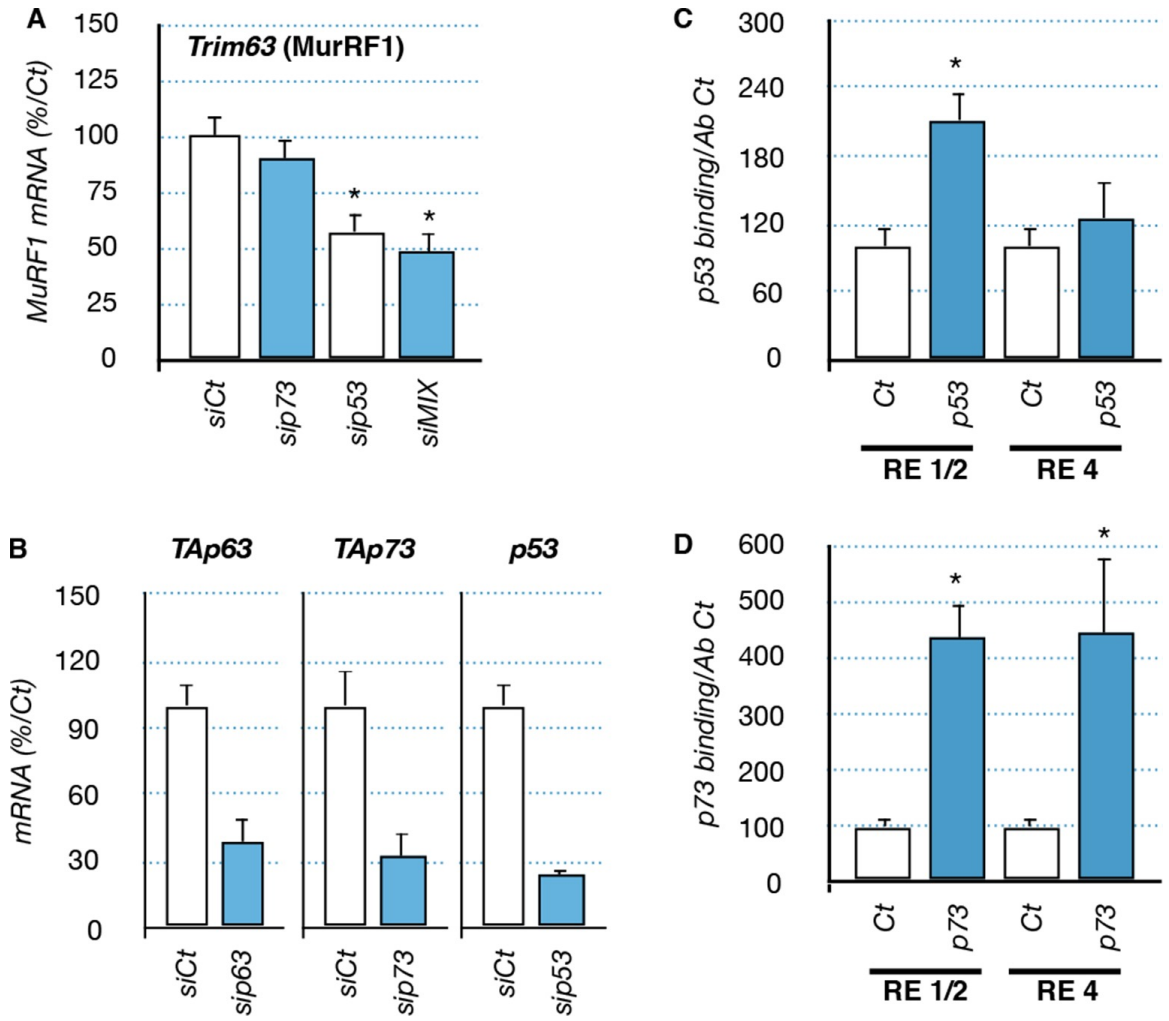

Figure 7—figure supplement 1. Regulation of Trim63 by p53 and p73 proteins. (A) mRNA levels of Trim63 in C2C12 cells following transfection with siRNA control and siRNA directed against p73, p53 and a mix of siRNA against P53, and the TA isoforms of Trp63 and P73 (siMIX). Bars represent means (relative induction versus Ct) with standard deviation (n = 3). *p<0.01. (B) mRNA level for TA isoforms of Trp63, TA isoforms of P73 and P53 in C2C12 cells following transfection with siRNA control and siRNA directed against p63, p73, and p53. Bars represent means (relative induction versus Ct) with standard deviation (n = 3). *p<0.01. (C, D) Chromatin immunoprecipitation (ChIP) assay was performed on the Trim63 promoter using RT-qPCR on RE1/2 and RE4. p53 immunoprecipitation (C) was performed using p53 antibody IC12 (Cell Signalling), p73 immunoprecipitation was performed using p73 antibody IMG-259a (Imgenex). Bars correspond to means with SD (n = 3). *p<0.01 as calculated by a one-way ANOVA test followed by a Tukey post-test.

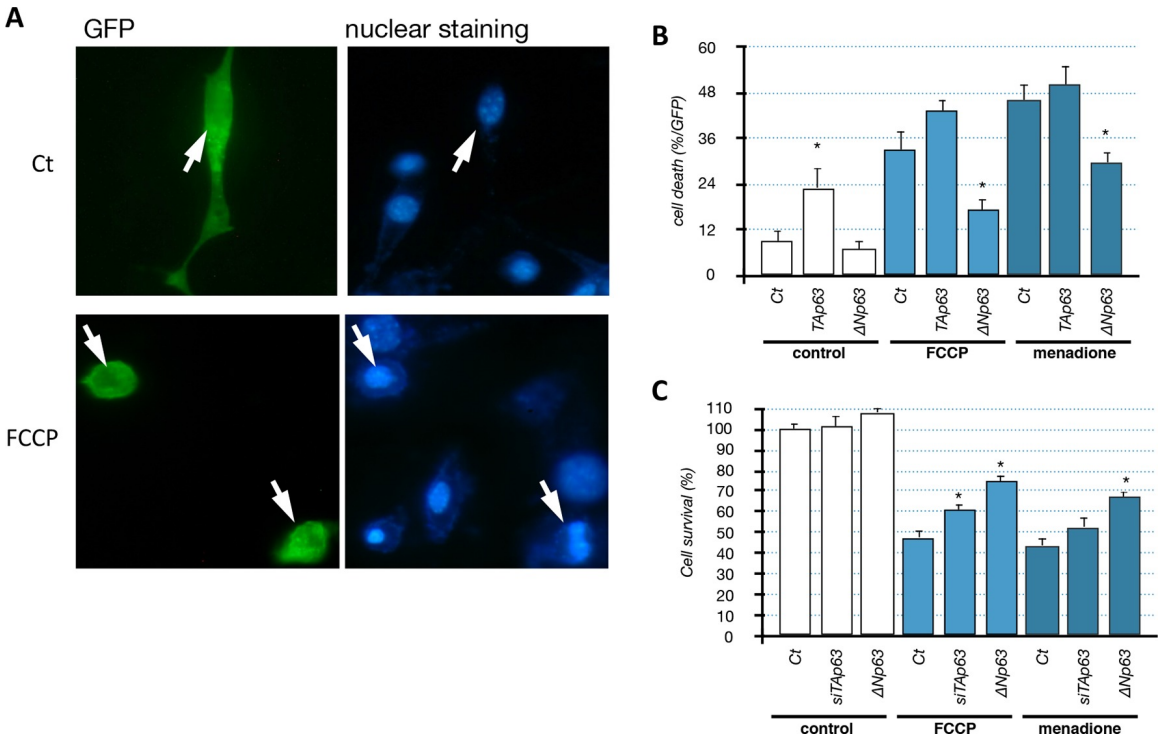

Figure 7—figure supplement 2. Impact of p63 on C2C12 cell survival. (A, B) C2C12 were transfected with a GFP expression vector and either TAp63γ or ΔNp63γ expression vectors. After 24 hr, cells were left untreated (Ct) or treated with FCCP (1 μM) or menadione (1 μM) for 24 hr. Cells were stained with Hoechst and examined with a fluorescence microscope (B). Above, GFP-positive control cells (untreated). Below, dead GFP-positive cell treated with FCCP. C2C12 cells were grown on coverslips coated with poly-ornithine in 24-wells plates. Cells were co-transfected with the indicated expression vectors (200 ng/well) and a GFP-expression vector (50 ng/well) as previously described (Broadley and Hartl, 2008). Cells were cultured for 18 hr with the indicated agents. Cells were subsequently washed with PBS and fixed with 4% paraformaldehyde for 15 min. After two washes, cells were incubated for 10 min with the Hoechst 33,342 staining agent (1 mg/ml, Sigma, Germany). GFP positive cells were then observed with an epi-fluorescent microscope (Zeiss, Germany) to assess the nucleus morphology. (C) C2C12 cells were transfected either with the ΔNp63γ expression vector or siRNA directed against the TA isoforms of Trp63. Cell survival was evaluated using MTT assay after 48 hr of treatment with the indicated drugs at 1 μM. *$p<0.01$ compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test.

2 - Role of the p53 family in the regulation of Trim63 in doxorubicin induced cachexia.

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Abstract

Muscle atrophy in cachexia results from the imbalance between protein synthesis and degradation due to activation of the ubiquitin-proteasome pathway. Literature suggests that p53 family members (p53, p63, p73) play a role in controlling proliferation, differentiation and death of precursors and muscle fibers. Here we characterize the expression profile of the p53 family members in muscle atrophy induced by the anticancer drug doxorubicin. We revealed that this compound induced expression of the p53 family members and atrogenes in a correlated manner, both *in vivo* and *in vitro*. In addition, we revealed a transcriptional activation of Trim63 by p53 and p73. Importantly, we also show that ROS and ceramide accumulation are important for Trim63 induction by doxorubicin. Overall, our data suggest that doxorubicin induces Trim63 through a pathway dependent on the p53 family, ROS and ceramide.

Introduction

Muscle atrophy can occur as part of many diseases and is often an indicator of bad prognostic, impairing quality of life and increasing mortality (Cano *et al.,* 2000; Anker *et al.,* 2003; Argilés *et al.,* 2005; Grinspoon *et al.,* 2003). In addition, several conditions like aging, starvation, disuse and immobilization (Jackman & Kandarian, 2004) can lead to muscle loss and, depending on the cause, different molecular pathways can be involved in the muscle atrophy process. However, the imbalance between protein synthesis and degradation mediated by the proteasome pathway and modulated by the muscle specific ubiquitin ligases Trim63 (Murf1) and Atrogin-1 (MAFbx) (Foletta *et al.,* 2011) are a common feature among the diverse causes of muscle loss. These muscle specific ubiquitin ligases - Trim63 and Atrogin-1 - are described to be consistently upregulated in a range of skeletal muscle atrophy models (Glass, 2005; Gumucio & Mendias, 2013;

Bodine & Baehr, 2014). The question that remains to be lightened is whether there is a common or specific molecular mechanism that triggers Trim63 or Atrogin-1 expression in certain muscle atrophy events.

Cancer cachexia is a multifactorial syndrome characterized by pronounced muscle atrophy, systemic inflammation and metabolic alterations (Tsoli & Robertson, 2013). Importantly, cachexia affects between 50 to 80% of cancer patients and is responsible for about 20% of their death (Dewys *et al.,* 1980). Increasing evidences point out the impact of the tumors and/or the therapies on muscle function and integrity in cancer patients. Studies suggest that the tumor itself plays an active role in muscle wasting due to its ability to secrete pro-inflammatory cytokines and the large requirement of nutrients to maintain cell growth, creating a tumor-host interaction that aggravates the condition of the patient (Tisdale, 2009). At the same time, cancer chemotherapy presents side effects that could contribute to cachexia, like anorexia, nausea and diarrhea. But more than that, it has been shown that chemotherapeutic agents have a direct negative effect on protein metabolism (Le Bricon *et al.,* 1995; Barracos, 2001), leading to cachexia. In this context, the anticancer drug doxorubicin is well recognized for having muscle catabolic effects (Hydock *et al.,* 2011). Some works suggest that its atrophic effect would be due to production of ROS (Gilliam *et al.,* 2012), TNF-α (Adams *et al.,* 2008) or ceramides (Larichaudy *et al.,* 2012). In addition, Morimoto and colleagues, 2008, suggested that doxorubicin is able to induce p53 accumulation in cardiac myocytes, which can contribute to muscle wasting (Schwarzkopf *et al.,* 2006).

Despite the classical roles of the p53 family of transcription factors (p53, p63 and p73) in tumorigenesis, cell cycle, apoptosis regulation, epithelial and neuronal development, numerous elements in the literature suggest that the p53 family modulates myogenesis and differentiation (Porello *et al.,* 2000; Fontemaggi *et al.,* 2001). For example, p63 is particularly important during heart development (Paris *et al.,* 2012), while p73 modulates myoblast differentiation (Cam *et al.,* 2006). In addition, p53 and p63 play a role during muscle regeneration and metabolism by controlling Sirt1, TIGAR and AMPK expression (Bensaad *et al.,* 2006; Pardo & Boriek, 2011; Su *et al.,* 2012). Within the p53 family, distinct promoters generate two classes of isoforms generally recognized for having antagonist function, containing (TA) or not (ΔN) the N-terminal transactivation domain (Murray-Zmijewski *et al.,* 2006). Importantly, within skeletal muscles, the balance between TA and ΔN-isoform expression influence on developmental and differentiation processes (Romano *et al.,* 2012). Interestingly, p53 has been shown to be induced during immobilization (Fox *et al.,* 2014). In addition, we underlined the p53 family relation with muscle atrophy showing that p63 is upregulated in amyotrophic lateral sclerosis (ALS) and able to directly modulate *Trim63* (Murf1) expression (von Grabowiecki *et al.,* 2016).

The lack of efficient therapies to treat muscle wasting associated to chronic diseases highlights the insufficient understanding of the complex mechanisms underlying muscular atrophy. Hence, in this work, we investigate whether the p53 family members can modulate muscle wasting in a cancer-associated cachexia model induced by doxorubicin and if it could be a common regulatory mechanism of muscle atrophy associated to diseases.

Materials and Methods

Cell culture: C2C12 cells were obtained from ATCC (ATCC CRL-1772) and grown in DMEM (Dulbecco's modified Eagle's medium; Life Technology, Carlsbad, CA) with 10% fetal bovine serum (Life Technology) at 37˚C in a humidified atmosphere and 5% CO2. Mycoplasma contamination has been tested negatively using PlasmoTest (Invivogene, San Diego, CA). Cells were treated with 0.4 or 0.6 μM of doxorubicin (Pfizer 50mg) and, 0.1mM of Myriocin (Myriocin from *Mycelia sterilia*, Sigma-Aldrich) for 24h or pretreatment with 2mM of N- acetyl-cysteine (NAC) for 1 hour.

Quantitative PCR: TRIzol (Invitrogen, Carlsbad, CA) was used to extract RNA. One μg of RNA was used for reverse transcription (iScript cDNA kit, Bio-Rad, France) and qPCR was carried out (iQ SYBR Green, Bio-Rad). Expression levels were normalized TBP as previously described (Vidimar *et al.,* 2012).

Western blotting: Cells or tissue were lysed with LB (125 mM Tris-HCl pH 6.7, NaCl 150 mM, NP40 0.5%, 10% glycerol). Proteins were denatured and deposited directly (35 μg of proteins) onto a SDS-PAGE gel. Western blotting was performed using antibodies raised against p53 (mouse monoclonal anti-p53, 1C12, Cell Signaling Technology, France), p63 (mouse anti-p63, 4A4, Santa Cruz Biotechnology; p63, Abcam, France), p73 (rabbit monoclonal anti-p73, EP436Y, Epitomics Abcam Company, France) and Trim63 (rabbit anti-Trim63, D01, Abnova; Taipei, Taiwan). Secondary antibodies (anti-rabbit, anti-mouse: Sigma, France) were incubated at 1:1000. Loading was controlled with actin (rabbit anti-β-actin, Sigma, 1:10000) (Antoine *et al.,* 1996).

Transfection assays: Cells were transfected by JetPrim (Polyplus, Strasbourg, France) as previously described (Gaiddon *et al.,* 1999). SiRNA transfection was performed using 10 nM of siRNA against p63-pan (Trp63 Stealth RNAi MSS212111, Invitrogen) and 50 nM of siRNA against p53 (Trp53 s75474, Ambion), using RNAiMAX or Dharmafect as described by the protocol provider (Life Technology and Dhamacon™ respectively). Trp53 sequence: GCU UCG AGA UGU UCC GGG Att

Trp63-pan sequence: CCG AGG UUG UGA AAC GAU GCC CUA A.

Chromatin immunoprecipitation (ChIP) assay: ChIP assays were performed using the standard protocol from the Magna ChIP G kit (Millipore). C2C12 lysates were sonicated 12 times at 10% power (Bioruptor™ UCD-200, Diagenode). For each 1 million cells, 1 µg of antibody was used. P53 and p73 were immunoprecipitated with a mouse antibody raised against total p53 (mouse monoclonal anti-p53, 1C12, Cell Signaling Technology, France) and p73 (mouse monoclonal anti-p73, E-3, Santa Cruz Biotechnology; Abcam, France). Mouse-anti-RAB11A was used as negative control (Santa Cruz Biotechnology).

Wild-type mice and doxorubicin treatment. In total forty wild-type (WT) male mice C57BL/6 background 5 months old were randomly assigned to four groups of 10 mice each. The animals received a single intraperitoneal injection of doxorubicin (Pfizer 50mg) diluted at 18mg/kg in 0.9% NaCl, during 1, 3 or 5 days. Vehicle-treated littermates received the same formulation without doxorubicin. In vivo experiments were repeated twice with a number of animals recommended to optimize statistical analyses according to the regional and national animal ethics committee. All animal manipulations were performed under appropriate supervision and observing protocols validated by the regional and national animal ethics committee.

TAp63$^{+/Cre}$ROSA26^{Tomato} reporter mouse line: Pr. Daniel Aberdam from the Institut National de la Santé et de la Recherche Médicale U898, University of Nice Sophia Antipolis, kindly donate us a TAp63$^{+/Cre}$*ROSA26*^{Tomato} mouse line, which express a Tomato-GFP upon Cre-mediated deletion of STOP cassete in the ROSA locus. So the Tomato reporter is under the control of the TAp63 promoter. Hence, tissues that express TAp63 isoforms will appear with a red fluorescence.

Statistical Analyses. Statistical analyses were performed using a one-way analysis of variance test followed by a Turkey post-test to allow a comparison between all the conditions. In the graphs, an asterisk indicates a statistically significant difference. Tests confirmed a statistically significant difference between control and treated mice. Statistical analyses were performed using Prism (GraphPad Software, San Diego, CA).

Results

P53 family members are induced by doxorubicin and their expression correlate with Trim63 (Murf1) and Atrogin-1 expression in vitro and in vivo.

Because the p53 family modulates essential pathways in cells and direct play roles during myogenesis, muscle cell differentiation and ALS associated muscle wasting, we aimed to investigate if it could also contribute to the regulatory molecular mechanism underlying muscle atrophy induced by the anti-cancer drug doxorubicin. Firstly, we characterized the expression profile of the p53 family members in our doxorubicin induced cachexia model. To do so, we treated murine myoblast cells (C2C12) with two concentration of

doxorubicin (0.4 and 0.6 μM) over different time and then analyzed mRNA and protein levels of the p53 family members (p53, TAp63, ΔNp63, TAp73), Trim63 and Atrongin-1 by RT-qPCR and western blot, respectively. This time course experiment revealed that doxorubicin strongly induced TAp63 mRNA level after 18 hours of treatment, which was maintained at 24 hours (Figure 1C). In contrast, in the protein level, doxorubicin only inducted p63 after 6h of treatment (Figure 1E). Interestingly, TAp73 mRNA level was already weakly induced after 6 hours of treatment, showing then a strong induction at 18 hours decreasing again at 24 hours after treatment (Figure 1D). Doxorubicin treatment strongly induced p73 protein accumulation after 24h of treatment (Figure 1E). Similarly, p53 protein expression was induced after 6 hours of treatment and progressively increased over time (Figure 1E). The induction of p53 protein expression and nuclear localization was confirmed by immunohistochemistry (Supplementary Figure 1). Interestingly, increased expression of p53 family members induced by doxorubicin correlated with Trim63 (Figure 1A and 1E) and Atrogin-1 upregulation (Figure 1B). These results show that doxorubicin induces the expression of p53 family members and atrogenes in myoblast cells in a similar time and dose dependent manner, suggesting that they might be related.

To see if the *in vitro* effect of doxorubicin on the expression of p53, p63, p73, Trim63 and Atrogin-1 in muscle cells are also observed *in vivo*, we treated C57BL/6 mice with 18 mg/kg of doxorubicin for 1, 3 and 5 days and analyzed their RNA and protein expression by RT-qPCR and western blot in the gastrocnemius muscle. The animals showed a reduction of gastrocnemius muscle weight after three days of doxorubicin injection, (Figure 2C), suggesting that the treatment induced muscle atrophy *in vivo*. The results show that doxorubicin treatment upregulated Trim63, Atrogin-1 and TAp63 (Figure 2A, B and E) mRNA expression in a correlative and significant manner after the third day of doxorubicin injection. Similar to the effects we observed *in vitro*, *in vivo* p53 protein (Figure 2D) accumulates with time after treatment. However and in contrast to C2C12 cells, TAp73 mRNA expression in muscle of mice was significantly downregulated within one day after doxorubicin treatment (Figure 2F). To address TAp63 expression in muscular tissue we used *TAp63*$^{+/Cre}$*ROSA26*$^{\text{Tomato}}$ mice, which show a red fluorescent (Tomato-GFP) when TAp63 is present. We confirmed then TAp63 expression in gastrocnemius muscle (Figure 2G), whereas control wild-type mice did not show any fluorecence (data not shown). Our results suggest that doxorubicin induces muscle atrophy in myoblast cells and in C57BL/6 mice through the activation of the ubiquitin-proteasome pathway possibly via the p53 family.

Functional interaction between p53 family member and the atrogenes - Trim63 (Murf1) and Atrogin-1

Based on the correlation between the expression of the different p53 family members and atrogenes, we aimed to characterize separately the modulation of Trim63 and Atrogin-1 by each member of the p53 family. To do so, we performed gain of function experiments in murine myoblastic cells (C2C12), treated or not treated with 0.6 μM of doxorubicin for 24 hours, and subsequently analyzed for the expression of Trim63 and Atrogin-1 mRNA by RT-qPCR. The results showed that overexpression of TAp63 and TAp73 strongly induce the expression of Trim63, which was further potentiated by the treatment with doxorubicin (Figure 3A). Interestingly, ΔNp63 and ΔNp73, normally described in the literature for having a dominant negative effect, also stimulated Trim63 expression, which in the case of ΔNp73 was further potentiated by the treatment with doxorubicin (Figure 3A). Importantly, overexpression of the different p53 family members did not affect Atrogin-1 expression (data not shown). These results confirmed our previous data suggesting that the p53 family regulates Trim63 expression. However, they also indicate that the p53 family members do not modulate Atrogin-1 upregulation by doxorubicin in C2C12 cells. For that reason, we decided to further focus our analysis on Trim63.

To investigate if the expression of Trim63 is not only induced but also depends on the expression of the different p53 family members we transfected C2C12 cells with p53 or p63 specific siRNA, treated cells or not with 0.6 μM of doxorubicin for 24 hours and analyzed again for the expression of Trim63 by RT-qPCR (Figure 3D-E). The efficacy of the p53 and p63 siRNAs was analyzed by RT-qPCR (Figure 3B and 3C) and Western blot (Suplementary Figure 2A and B) and show a decrease of p53 and p63 expression. In addition, some p63 target genes were deregulated upon p63 repression (AMPK, Sirt1 and REDD1) (Supplementary Figure 3A, 3B and 3D). As previously observed, doxorubicin consistently induced the expression of p53 and Trim63, respectively (Figure 1B, 1D and 1E). Transfection of siRNA against p53 or p63 strongly decreased Trim63 expression in the presence of doxorubicin (Figure 3D and 3E) but did not annihilate it. This suggests that doxorubicin induced expression of Trim63 in parts depends on p53 and p63.

Physical interaction between the p53 family members and Trim63 (Murf1).

Our results support the hypothesis that all three p53 family members may participate in the regulation of Trim63 expression during muscle atrophy induced by doxorubicin. Indeed, we have previously shown that TAp63 can bind and transactivate Trim63 promoter (von Grabowiecki *et al.,* 2016). In order to evaluate if doxorubicin might influence the p53 family biding to the Trim63 promoter we performed CHIP assays

covering two possible p53 family members binding sites in the *Trim63* promoter (Figure 4A) (von Grabowiecki *et al.,* 2016) in presence of doxorubicin. The CHIP assay using a p73 specific antibody showed that p73 was able to strongly bind to the two identified binding sites in the Trim63 promoter with a similar affinity (Figure 4B). Surprisingly, although our gain of function analyses showed that p53 could induce the expression of Trim63, the CHIP data suggest that p53 weakly bind to the Trim63 promoter (Figure 4B). Interestingly, doxorubicin did not increase the p53 family binding to the promoter.

Interaction between p53 family and other regulatory pathways involved in cancer-related muscle atrophy.

Our results show that the members of the p53 family modulate Trim63 expression (Figure 1, 2 and 3), but also suggest that other regulatory mechanisms might be involved in Trim63 induction by doxorubicin. It is described that doxorubicin atrophic effects in muscular cells might in part be due to the generation of reactive oxygen species (ROS) (Gilliam *et al.,* 2012) or ceramides (Delpy *et al.,* 1999) production. Therefore, we investigated if ROS contribute to doxorubicin induced Trim63 expression and how this relates to the expression of TAp63. To counteract the oxidative stress induced by doxorubicin we used N-acetylcysteine (NAC). C2C12 cells were first treated with NAC for one hour followed by 24 hours treatment with two different concentrations of doxorubicin (0.4 and 0.6 μM). Trim63 and TAp63 mRNA levels were analyzed by RT-qPCR (Figure 5). As expect, treatment with doxorubicin induced Trim63 and TAp63 expression (Figure 5A, B). Interestingly, whereas NAC reduced the doxorubicin-induced expression of Trim63 (Figure 5A), it had a positive effect on TAp63 expression (Figure 5B). These results suggest that oxidative stress induced by the treatment with doxorubicin does not alone account but contributes to the induction of Trim63 expression.

Another possible mechanism by which doxorubicin could induce Trim63 expression could be through ceramide accumulation, known to contribute to doxorubicin induced cardiomyopathy (Delpy *et al.,* 1999). To evaluate if ceramides participate in doxorubicin stimulation of Trim63 expression, we used a ceramide synthesis inhibitor (myriocin) (Larichaudy *et al.,* 2012) in addition to doxorubicin treatment. C2C12 cells were treated during 24 hours simultaneously with doxorubicin and myriocin and the expression of the different p53 family members and atrogenes were analyzed by RT-qPCR. Co-treatment with myriocin clearly reversed doxorubicin induced expression of Trim63 (Figure 6A). However, myriocin had no effect on the expression of Atrogin-1 (Figure 6B). Similarly, in combination with low dose of doxorubicin, myriocin showed no effect on the expression of TAp63 (Figure 6D) or the p53 family target gene p21 (Figure 6E) and a small upregulation on TAp73 (Figure 6D). Surprisingly, at a high doxorubicin

concentration myriocin diminished TAp63 but stimulated TAp73 expression (Figure 6C and 6D). These results suggest that ceramide might also contribute to the modulation of Trim63 expression, however in a p53 family independent manner.

Discussion

In the present work we investigated the functional relationship between p53 family members and atrogenes in doxorubicin-induced muscle atrophy.

Doxorubicin induces Trim63 (Murf1) expression via the p53 family.

Our results show a correlative induction of the p53 family and ubiquitin ligases Trim63 and Atrogin-1 by doxorubicin in a time and dose dependent manner in C2C12 myoblast cell line and in gastrocnemius muscle of mice. These results suggest that the cachexia side effect of the anti-cancer drug doxorubicin on skeletal muscle might be mediated via the induction of the p53 family members and their transcriptional activity on Trim63 expression. Indeed, several observations support this hypothesis. It has been shown in different rodent models that doxorubicin exercises an atrophic effect on skeletal muscle (McLoon et al., 1998; Hydock *et al.,* 2011; Gilliam et al., 2012). Two independent studies in cardiac myocytes showed that doxorubicin induces the expression of Atrogin-1 (Yamamoto *et al.,* 2008) and p53 (Morimoto et al., 2008). However, a study by Dimitrakis and co-workers (Dimitrakis et al. 2012) showed that in cultivated rat ventricular myocytes doxorubicin treatment repressed Trim63 expression. Considering this, the doxorubicin effect on Trim63 expression seamed to be cell type dependent as we see in C2C12 cells or in skeletal muscle of mice an induction of its expression by doxorubicin. This potential context dependent activation or repression of atrogenes is further supported by our findings that overexpression of the different p53 family members in C2C12 myoblast specifically induced the expression of Trim36 and not Atrogin-1. In addition, the data presented here show that not only all p53 family members can induce the expression of Trim63 but that this effect is potentiated by doxorubicin. In this respect, Latella and colleagues (Latella *et al.,* 2004) have shown that doxorubicin leads to p53 phosphorylation and stabilization, thereby possibly improving its transactivation activity. Overall, our data show a clear difference in the ability of the different p53 family members to induce Trim63 expression, with p53 showing the weakest and TAp63 the strongest effect. Curiously, both ΔNp63 and ΔNp73 isoforms induced Trim63 expression and, in the case of ΔNp73, doxorubicin treatment potentiated its effect to almost similar levels as TAp73 isoform. These data suggest that the ΔN variants, which lack the transactivation domain and thought to exercise a dominant negative function on p53 target genes activation, could actually promote Trim63

expression. Indeed, a similar observation was made by Liu and colleagues (2004) showing that ΔNp73 transactivates target genes such as p21, 14-3-3σ and GADD45. Importantly, we have previously demonstrated that p63 and Trim63 in atrophic skeletal muscles of ALS patients, or a mouse model of it (SOD1(G86R) mice), not only show a correlative expression but that TAp63 binds to and transactivates the Trim63 promoter (von Gabrowiecki 2016). Here we show that in addition to TAp63, p73 can also bind to the binding sites in the Trim63 promoter whereas p53 does it weakly. Although, these data show that the different p53 family members can directly transactivate Trim63 expression and might therefore relay doxorubicin-induced transcription of this atrogene, our data also suggests that they are important but not necessary for it. Indeed, inhibition of p53 or p63 only reduced Trim63 expression induced by doxorubicin treatment suggesting that they might more function as modulators. This is supported by the findings that inhibition or absence of p53 partially protects mice from the muscle atrophy provoked by cancer therapy (Komarov *et al.,* 1999) and tumor load (Schwarzkopf *et al.,* 2006), respectively.

Different regulatory pathways are involved in doxorubicin induced Trim63 expression.

Our data also suggest that other signaling pathways cooperated in doxorubicin-induced expression of Trim63. It has been shown that doxorubicin induces the production of reactive oxygen species and ceramides, playing an important role in the induction of muscular atrophy possibly via the activation of atrogenes (Gilliam 2012; Gouaze 2001; Li 2003; Kavazis 2014; Larichaudy 2012). Our results show that doxorubicin induced Trim63 expression in part depends on ROS production as co-treatment with N-acetylcysteine (NAC) diminished Trim63 by half. At the same time, co-treatment with NAC further stimulated doxorubicin induced TAp63 expression, suggesting different underlying mechanisms regulating their expression. However, our data do not exclude that ROS regulates TAp63 activity at the protein level or that doxorubicin induced ROS production is in part TAp63 dependent, like Ellisen and co-workers have observed (2002).

As mentioned above, another possible activation pathway of Trim63 by doxorubicin would be through ceramide synthesis (Reynolds *et al.,* 2004; Liu *et al.,* 2008). Importantly, co-treatment with the ceramide synthesis inhibitor "myriocin" completely abolished doxorubicin induced expression of Trim63, whereas it had a slight negative and positive effect on TAp63 and TAp73 RNA expression, respectively (Figure 7). Thus, similar to ROS, doxorubicin induced expression of the p53 family does not depend on ceramide production. Interestingly, co-treatment with "myriocin" had no effect on doxorubicin induced Atrogin-1 expression (Figure 7A), whereas it was shown to inhibit TNFα induced Atrogin-1 expression (Larichaudy 2012). That different mechanisms

regulating the expression of Trim63 and Atrogin-1 in C2C12 cells is further supported by our finding that the different p53-family members stimulate Trim63 expression but show no effect on Atrogin-1 RNA level.

Altogether, our data indicate that doxorubicin upregulates p53 family members, which induce Trim63 expression by direct transcriptional activation. In addition, our results suggest that doxorubicin is able to stimulate Trim63 through two additional pathways, ROS and ceramide. One hypothesis is that NF-κβ or FoxO might act downstream of ROS (Barbieri & Sestili, 2012) triggering Trim63 transcriptional activation. However, it remains to be established which factors precisely activate Trim63 and the p53 family members during doxorubicin treatment.

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2– Figures and Tables

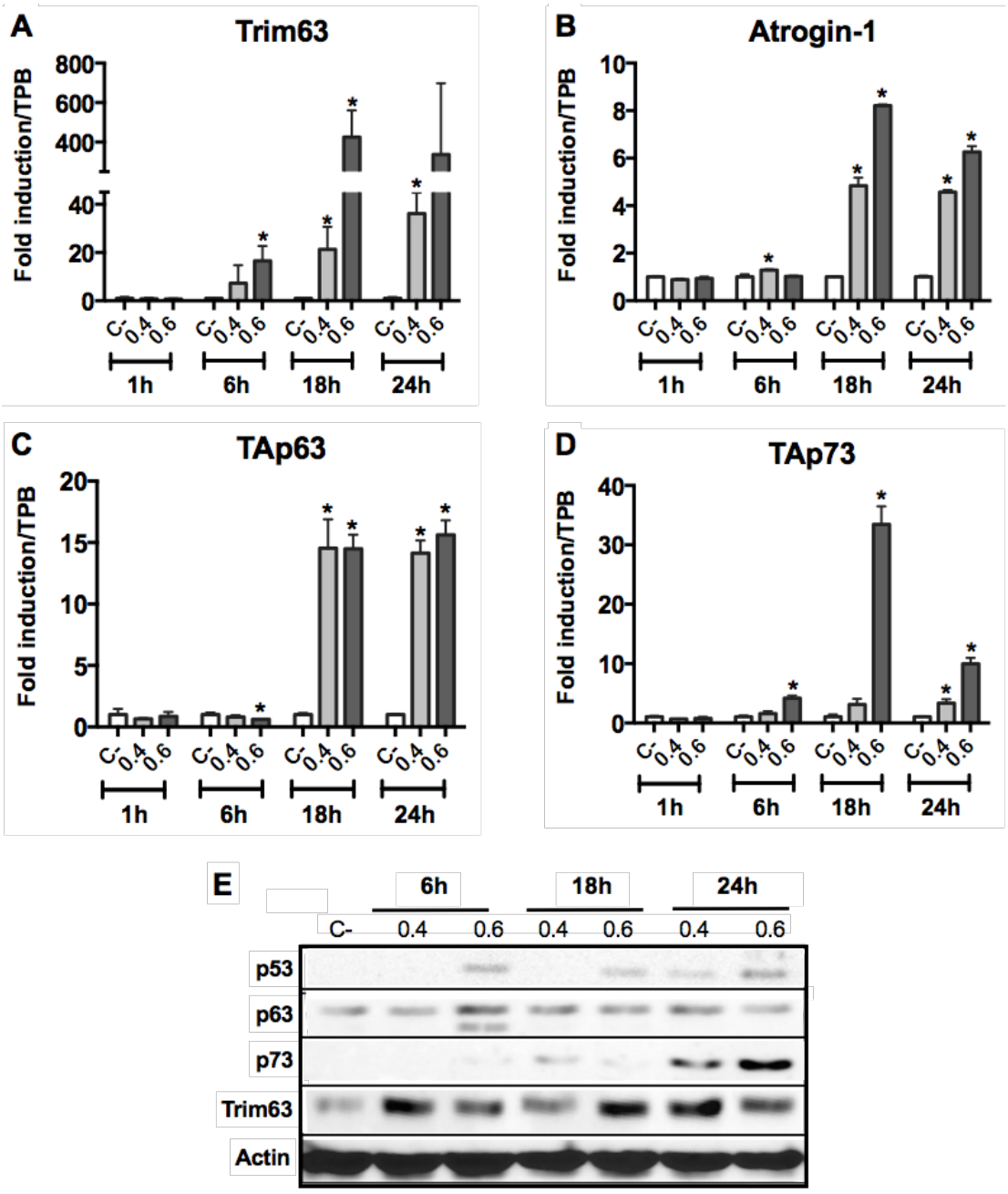


Figure 1: Effects of doxorubicin on atrogenes (**A, B**) and p53 family members (**C-E**). C2C12 cells were treated with two concentration of doxorubicin (0.4 and 0.6 µM) for 1, 6, 18 and 24h. Trim63 (**A**), Atrogin-1 (**B**), TAp63 (**C**) and TAp73 (**D**) mRNA levels were analyzed by RT-qPCR, while p53, p63 and p73 were detected by Western blot (**E**). C-

untreated control cells. Bars correspond to means with SD (n = 3). *p<0.05 as calculated by a one-way ANOVA test followed by a Turkey post-test.

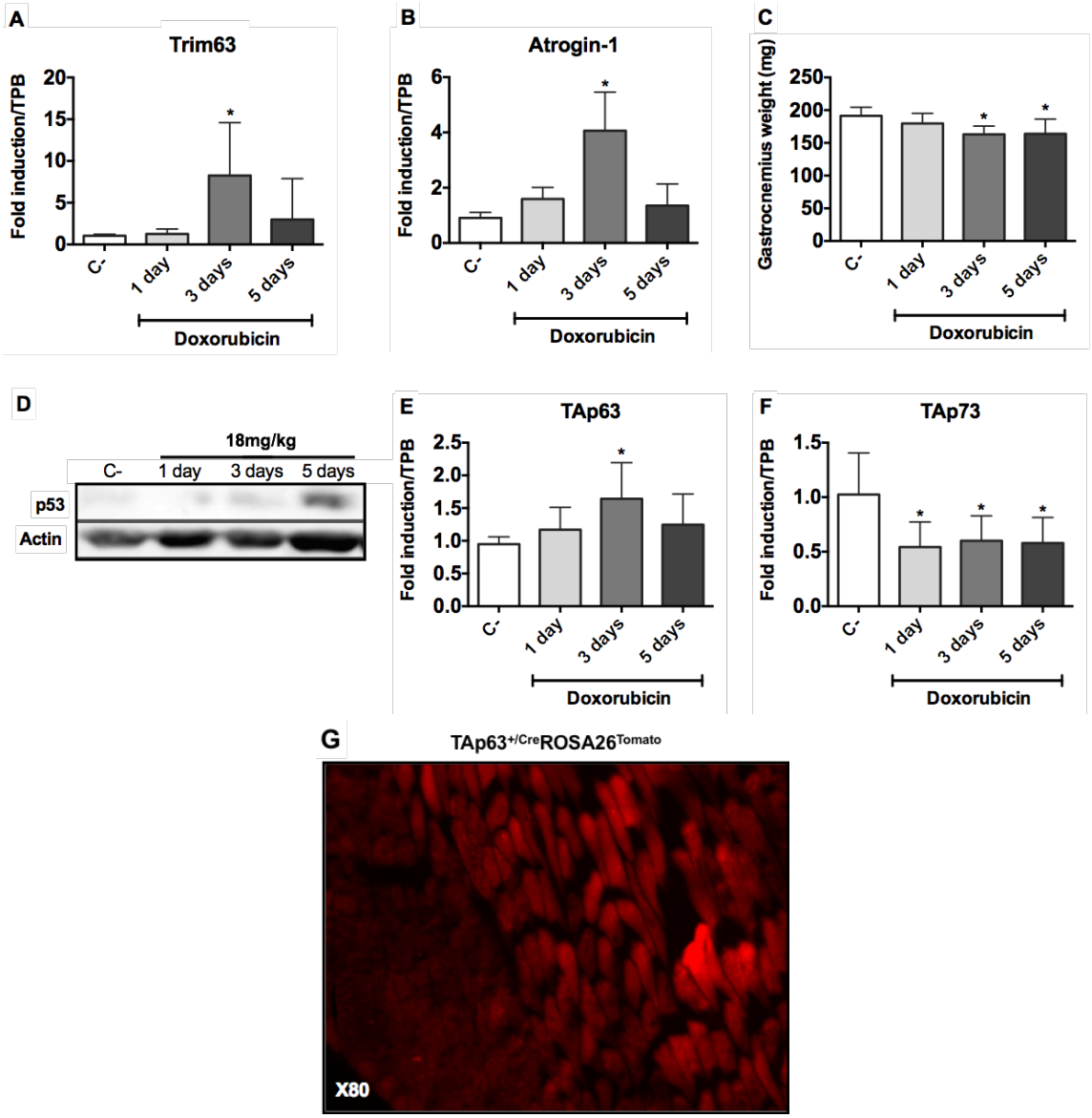


Figure 2: Atrophic effect of doxorubicin (**C**), expression of atrogenes (**A, B**) and p53 family members (**D-F**) in gastrocnemius muscle and p63 fluorescence in gastrocnemius muscles of *TAp63*$^{+/Cre}$*ROSA26*^{Tomato} mice (**G**). Wild type mice were treated with doxorubicin (18mg/kg) for 1, 3 and 5 days. mRNA and protein were extracted from gastrocnemius muscle and analyzed by RT-qPCR and Western blot respectively. C- untreated control mice. Bars correspond to means with SD (n = 10). *p<0.05 as calculated by a one-way ANOVA test followed by a Turkey post-test.

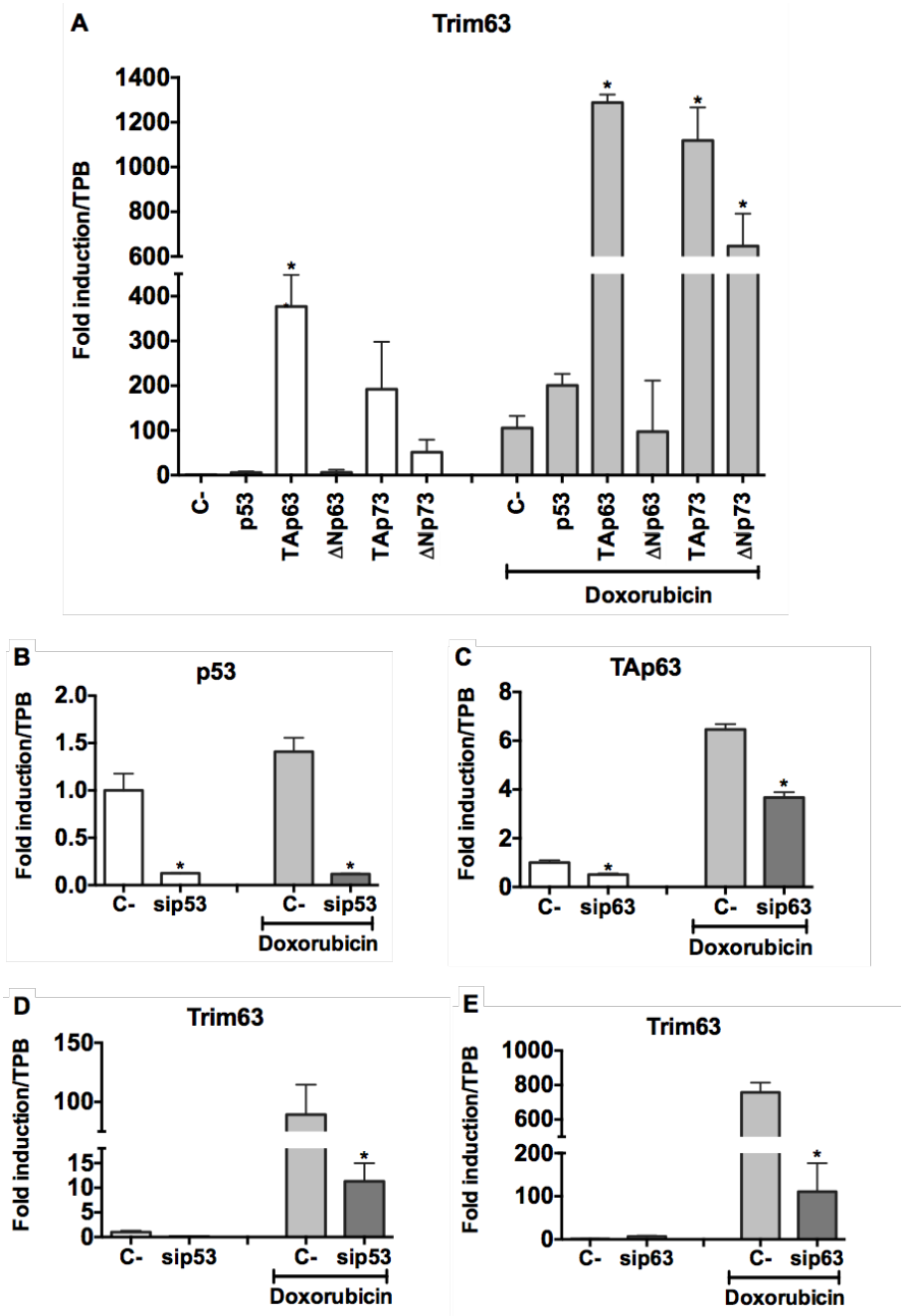


Figure 3: Effect of p53 family members gain of function on Trim63 expression (**A**) and p53 family dependent expression of Trim63 (**B-E**). C2C12 cells were transfected for 32h with plasmids encoding for different p53 family members (**A**) or either for 48h with control siRNA (C-) or siRNA (si) for p53 (**B, D**) or 72h for p63 (**C, E**). Total RNA was extracted and analyzed for the expression of Trim63 RT-qPCR. Expression levels are compared to empty vector or siRNA (C-) transfected cells. The grey bars represent Trim63 mRNA levels after 24 hours of doxorubicin (0.6μM) treatment. Bars correspond to means with SD (n = 3). *$p<0.05$ as calculated by a one-way ANOVA test followed by a Turkey post-test.

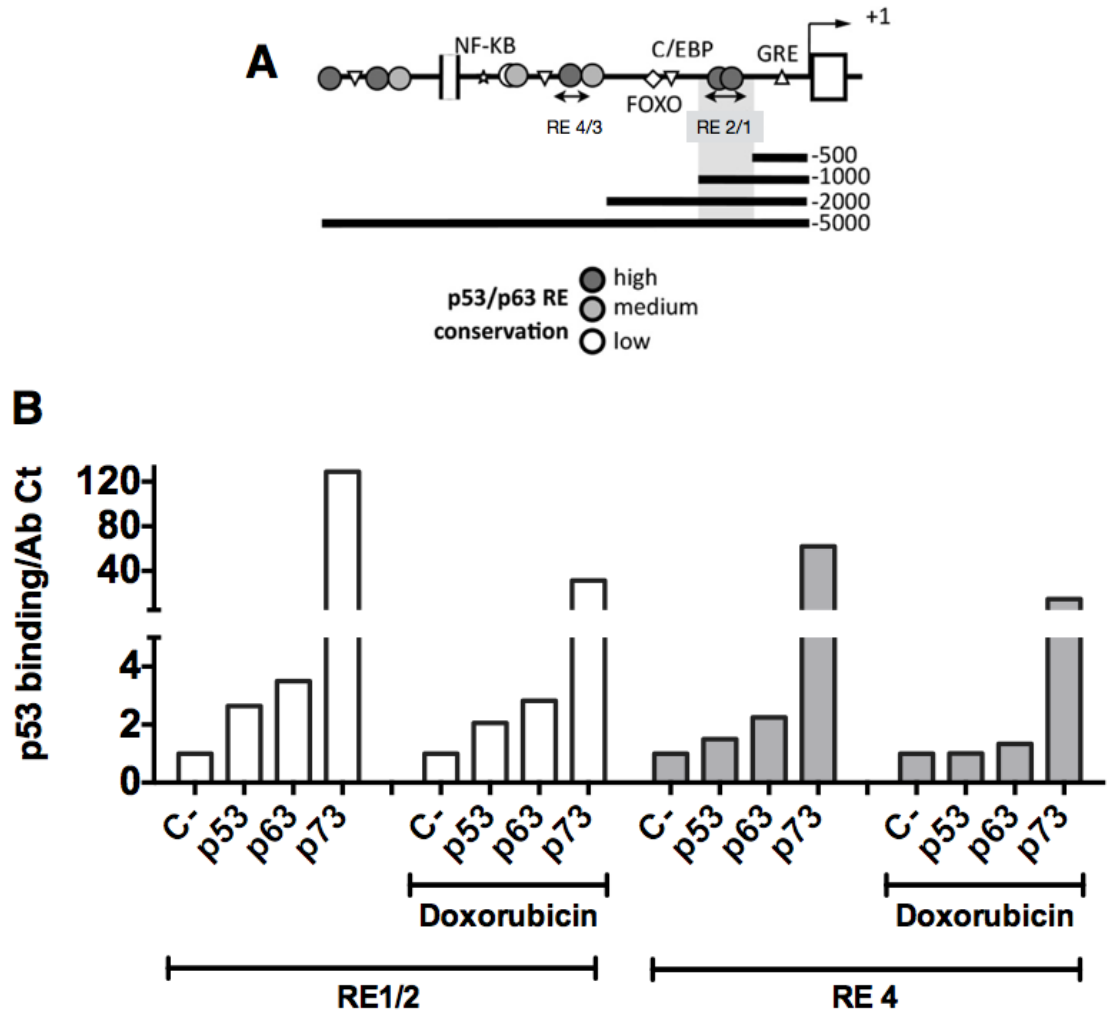


Figure 4: Regulation of Trim63 promoter by p53, P63 and p73. (**A**) Schematic representation of the Trim63 promoter indicating potential p53 family biding sites. (**B**) Chromatin immunoprecipitation assay was performed on the Trim63 promoter using p53, p63 and p73 specific antibodies followed by RNA analysis by RT-qPCR for RE1/2 and RE4. Mouse-anti-RAB11A was used as negative control.

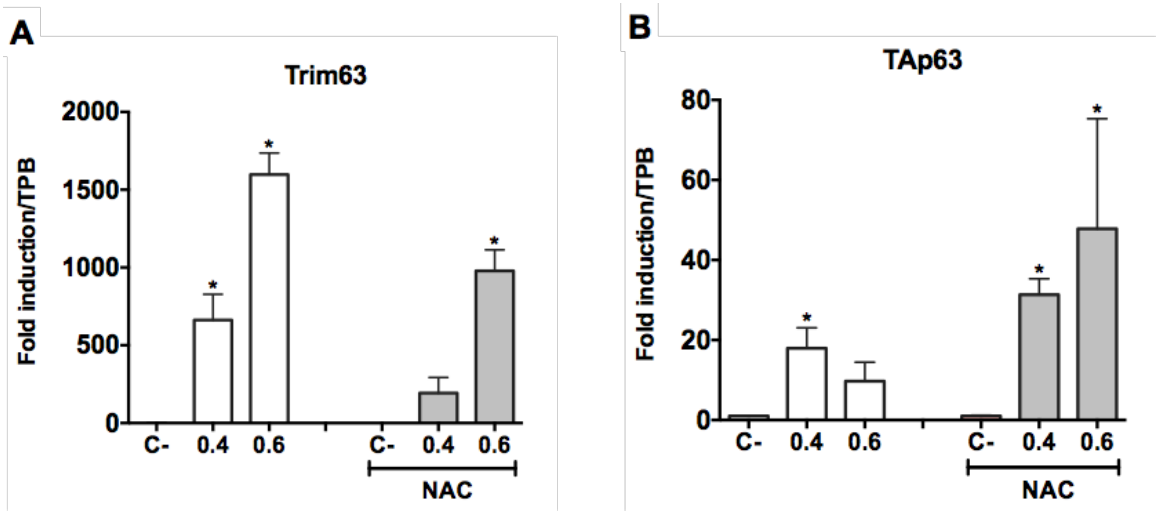


Figure 5: Impact of NAC (N-acetylcysteine) on doxorubicin induced Trim63 and TAp63 expression (**A, B**). C2C12 cells were pre-treated or not with NAC for one hour followed by doxorubicin (0.4 and 0.6µM) treatment for additional 24h or left untreated (C-). C2C12 total RNA was extracted and analyzed by RT-qPCR for the expression of Trim63 (**A**) and TAp63 (**B**). Bars correspond to means with SD (n = 3). *p<0.05 as calculated by a one-way ANOVA test followed by a Turkey post-test.

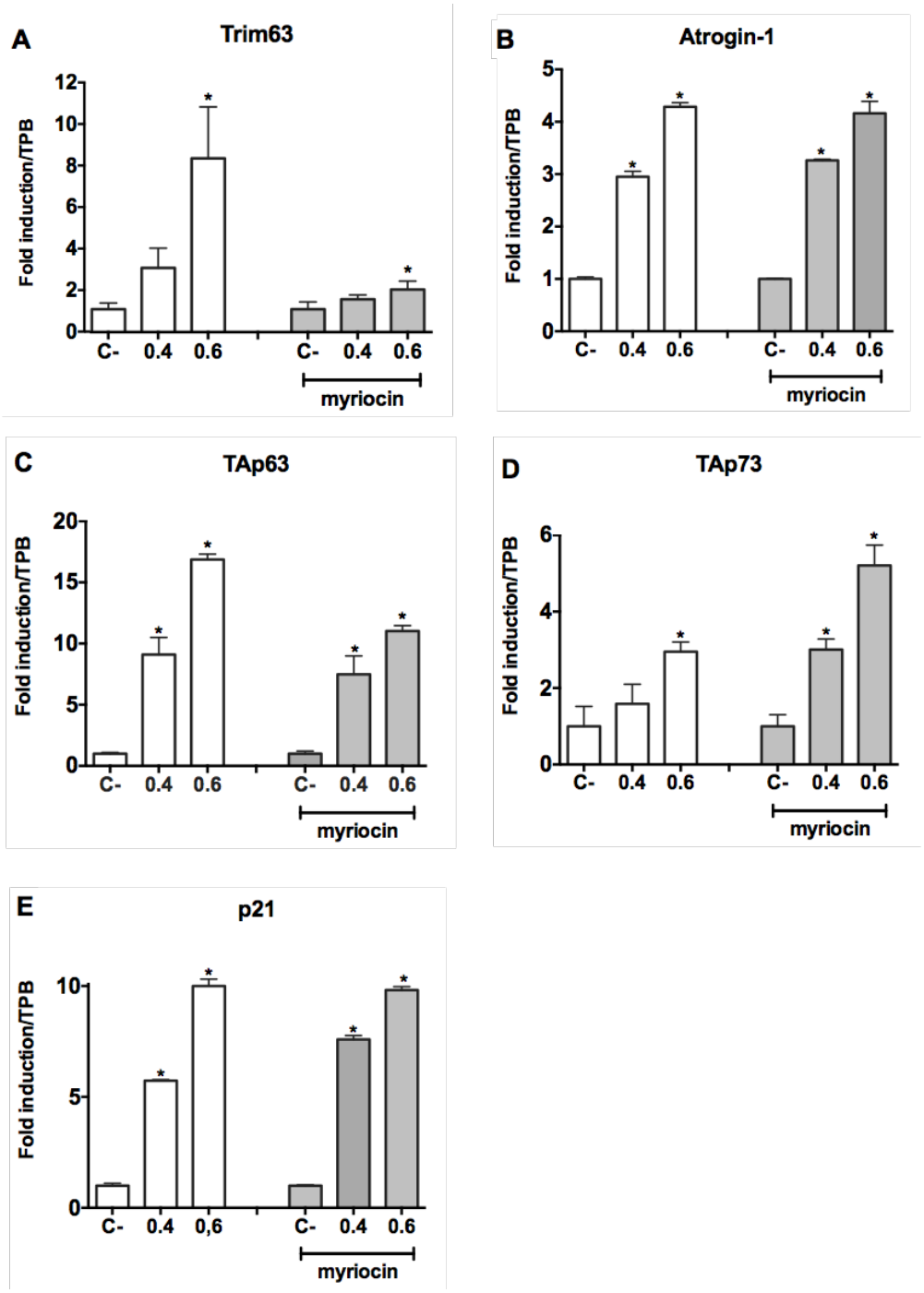
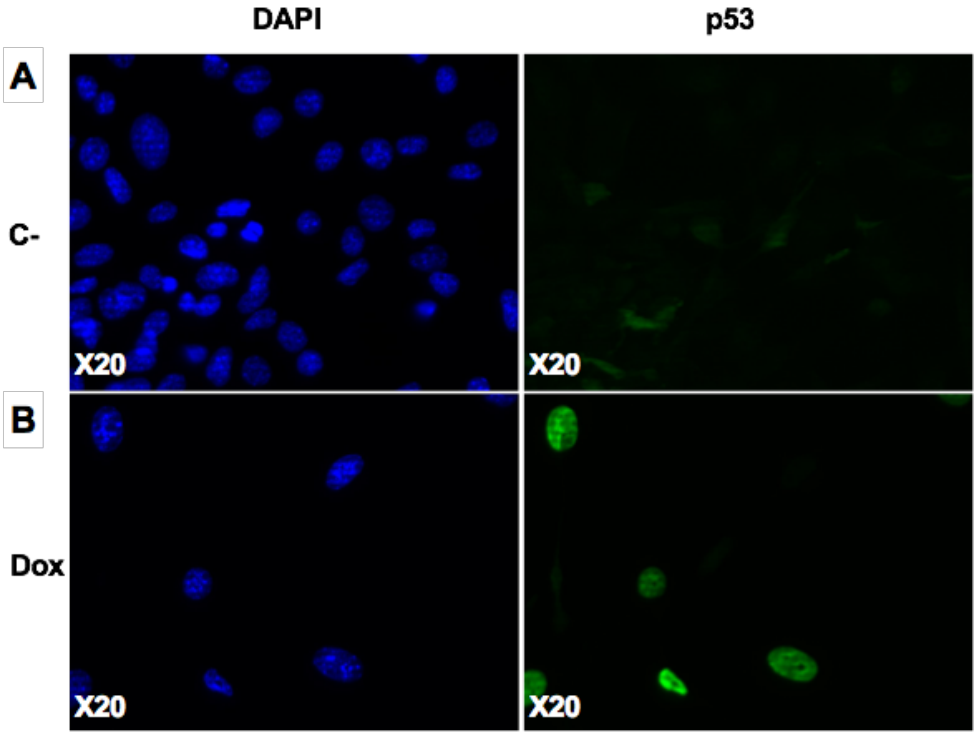


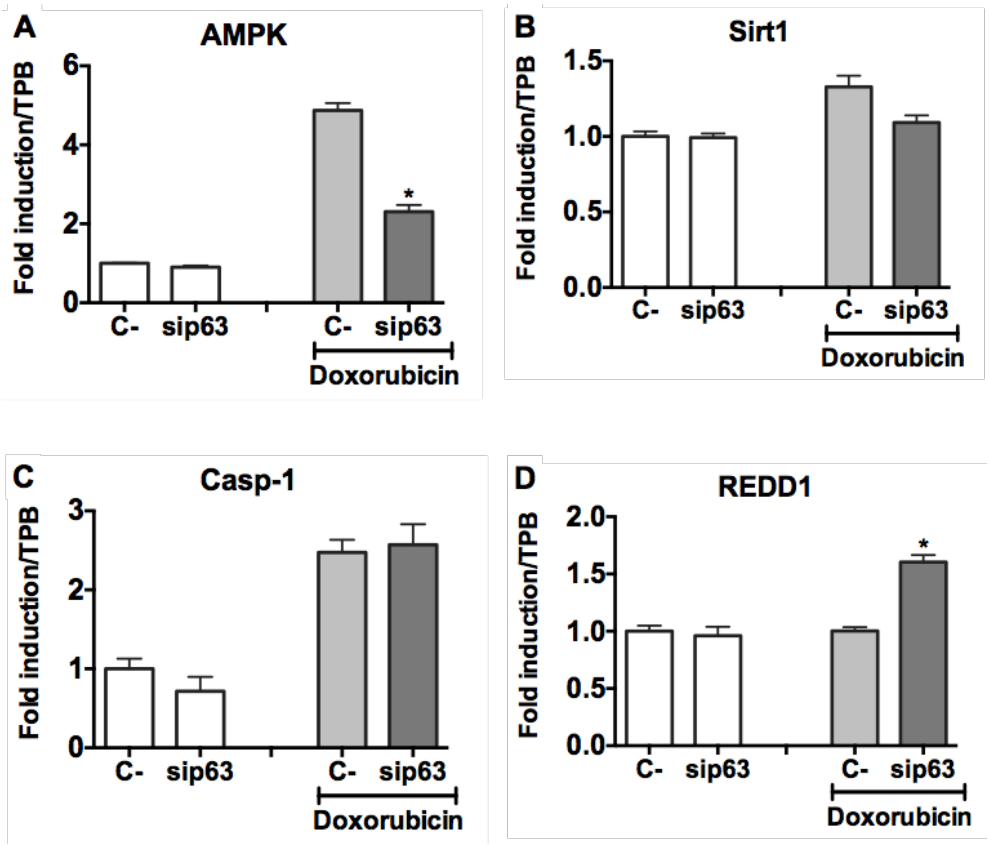
Figure 6: Impact of myoricin on doxorubicin-induced expression of atrogenes (**A, B**), p53 family members (**C-D**) and p53 target gene p21 (**E**). C2C12 cells were co-treated with doxorubicin (0.4µM or 0.6µM) and myriocin (100nmol/L) for 24 hours. Total RNA was extracted and analyzed by RT-qPCR for the expression of indicated genes (**A-F**). Bars correspond to means with SD (n = 3). *p<0.05 as calculated by a one-way ANOVA test followed by a Turkey post-test.

2– Suplementary data



Suplementary Figure 1: Effect of doxorubicin on p53. C2C12 cells were non-treated (**A**) or treated (**B**) with doxorubicin (0.6 μM) for 24h and p53 was detected by immunohistochemistry. Cells were fixed and stained with antibody against p53 and DNA was stained with DAPI.

Suplementary Figure 2: Silencing of p53 and p63 on C2C12 cells. P53 and p63 protein expression was detected by Western blot after 48h of transfection with either siRNA control (C-) or siRNA (si) for p53 (**A**) or p63 (**B**).



Suplementary Figure 3: Effect of doxorubicin on p63 target genes after p63 supression. C2C12 cells were transfected for 72h with control siRNA (C-) or siRNA for p63 (si). Total RNA was extracted and analyzed for the expression of AMPK (**A**), Sirt1 (**B**) and Caspase-1 (**C**) RT-qPCR. Expression levels are compared to empty vector or siRNA (C-) transfected cells. The grey bars represent p63 target genes mRNA levels after 24 hours of doxorubicin (0.6μM) treatment. Bars correspond to means with SD (n = 3). *$p<0.05$ as calculated by a one-way ANOVA test followed by a Turkey post-test.

3 - Regulation of a Notch3-Hes1 Pathway and Protective Effect by a Tocopherol-Omega Alkanol Chain Derivative in Muscle Atrophy

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Abstract

Muscular atrophy, a physiopathologic process associated with severe human diseases such as amyotrophic lateral sclerosis (ALS) or cancer, has been linked to reactive oxygen species (ROS) production. The Notch pathway plays a role in muscle development and in muscle regeneration upon physical injury. In this study, we explored the possibility that the Notch pathway participates in the ROS-related muscular atrophy occurring in cancer-associated cachexia and ALS. We also tested whether hybrid compounds of tocopherol, harboring antioxidant activity, and the omega-alkanol chain, presenting cytoprotective activity, might reduce muscle atrophy and impact the Notch pathway. We identified one tocopherol-omega alkanol chain derivative, AGT251, protecting myoblastic cells against known cytotoxic agents. We showed that this compound presenting antioxidant activity counteracts the induction of the Notch pathway by cytotoxic stress, leading to a decrease of Notch1 and Notch3 expression. At the functional level, these regulations correlated with a repression of the Notch target gene Hes1 and the atrophy/remodeling gene MuRF1. Importantly, we also observed an induction of Notch3 and Hes1 expression in two murine models of muscle atrophy: a doxorubicin-induced cachexia model and an ALS murine model expressing mutated superoxide dismutase 1. In both models, the induction of Notch3 and Hes1 were partially opposed by AGT251, which correlated with ameliorations in body and muscle weight, reduction of muscular atrophy markers, and improved survival. Altogether, we identified a compound of the tocopherol family that protects against muscle atrophy in various models, possibly through the regulation of the Notch pathway.

Introduction

Muscle atrophy is associated with severe human pathologies, such as degenerative diseases and cancer (von Haehling *et al.,* 2010). For example, amyotrophic lateral sclerosis (ALS) is characterized by the death of motor neurons and the atrophy of skeletal muscles, which leads to paralysis and death due to a lack of an efficient cure (Ludolph *et al.,* 2012). During cancer, muscle atrophy may develop as a side effect of the chemotherapy (cisplatin, doxorubicin) (Gilliam and St. Clair, 2011; Hydock *et al.,* 2011; Garcia *et al.,* 2013) or at the late stage of the pathology (Fearon *et al.,* 2012). This muscle-wasting syndrome (or cachexia) drastically reduces the patient's quality of life and may force the clinician to stop the treatment. The lack of an efficient cure against muscle atrophy correlates with a poor knowledge of the mechanisms involved.

Although the origin of muscle atrophy differs between pathologies, common features can be identified that play an important role in fiber and myoblast alterations, such as an abnormal production of reactive oxygen species (ROS) (Barbieri and Sestili, 2012; Gilliam and St Clair, 2011; Ray *et al.,* 2012). In particular, elevated ROS production has been linked to the presence of mutations in the ROS detoxification enzyme superoxide dismutase 1 (SOD1) associated with hereditary ALS (Beckman *et al.,* 2001). Similarly, cisplatin and doxorubicin have been shown to induce the production of ROS (Chirino and Pedraza-Chaverri, 2009; Gilliam and St. Clair, 2011; Hydock *et al.,* 2011; Garcia *et al.,* 2013; Gilliam *et al.,* 2013). ROS production has also been linked to the muscular atrophy induced by the presence of a tumor, likely mediated by cytokines, such as tumor necrosis factor-α (Adams *et al.,* 2008). At the cellular level, production of ROS induces lipid peroxidation, protein oxidation, DNA damage, and mitochondrial dysfunctions. These cellular alterations activate specific signaling pathways, such as the unfolded protein response, and/or transcription factors, such as p53/p73, which in turn lead to cell death and tissue degeneration (Gonzalez de Aguilar *et al.,* 2000; Benosman *et al.,* 2007, 2011; Barbieri and Sestili, 2012).

To counteract the atrophy process, small molecules have been tested for their cytoprotective properties. Two examples of these drugs are tocopherol and flavonoid derivatives. Flavonoid derivatives can reduce ROS effects and present neuroprotective or cardioprotective activity (Hauss *et al.,* 2007; Thuaud *et al.,* 2011; Ribeiro *et al.,* 2012). Several molecules that act on regeneration and neuroprotection are derived from tocopherol, a member of the vitamin E family, which is widely used in clinical practice because of its antioxidant and anti-inflammatory properties (Singh and Jialal, 2004). Moreover, α-tocopherol derivatives are considered disease modulators in multiple sclerosis and show beneficial outcomes after white matter damage in experimental models (Coowar *et al.,* 2004; Blanchard et al., 2013).

The Notch pathway is an intercellular developmental signaling cascade with fundamental roles in muscles (Buas and Kadesch, 2010; Bjornson *et al.,* 2012; Guruharsha *et al.,* 2012). This pathway comprises four receptors (Notch 1–4) and several ligands (δ-like, Jagged) that mediate their biologic function through induction of target genes, like Hes1 and HeyL (Buas and Kadesch, 2010; Bjornson *et al.,* 2012). Several studies have shown that Notch signaling favors myoblast proliferation, and when sufficient myoblasts are produced, Notch signaling needs to be switched off again to allow myoblast differentiation (Buas and Kadesch, 2010). This sequential regulation of the Notch pathway plays a central role in muscle development and muscle regeneration. For example, Notch3 silencing leads to muscle hypertrophy due to excess regenerative processes (Kitamoto and Hanaoka, 2010). Inversely, the expression of a general dominant negative Notch peptide leads to severe muscle defects that show similarities with muscular dystrophy (Lin *et al.,* 2013). These apparently contradicting results suggest that individual Notch components have a timely and cell-specific function in muscle development and repair that still remains to be defined more precisely.

In view of the cytoprotective effects of tocopherol and flavonoid derivatives, we decided to investigate the ability of novel derivatives presenting an omega-alkanol chain to improve the health of animals that are affected by muscle atrophy. We chose the omega-alkanol part, a fatty alcohol chain, as it was previously shown to provide cytoprotective properties on neurons (Borg *et al.,* 1987; Azzouz *et al.,* 1996; Hauss *et al.,* 2007). We hypothesized that the hybrids would combine the antioxidant properties of the tocopherol and the activity of the fatty alcohol chain, providing interesting cytoprotective activity on muscles. In addition, based on the role of the Notch pathway in muscle regeneration, we tested whether these hybrid compounds might regulate this pathway during the pathology associated with muscular atrophy.

Materials and Methods

Chemicals and Synthesis. AGT048, AGT251, and AGT262 tocopherol derivatives synthesis was performed as described previously (Muller *et al.,* 2006). AGT072 hydroquinone synthesis was also described previously (Coowar *et al.,* 2010). AGT031, AGT171, AGT184, and AGT216 flavonoid derivatives were synthesized following the chemical scheme described in the patent (Coowar *et al.,* 2009). All structures are shown in Table 1. Cell Cultures. Murine C2C12 myoblasts were obtained from the American Type Culture Collection (Manassas, VA). C2C12 cells were manipulated and cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Dominique Dutcher, Brumath, France) and 1% penicillin 1 streptomycin (Sigma-Aldrich, St. Louis, MO) at 37°C with 5% CO2 atmosphere as previously described (Sohm *et al.,* 1999). Cell

Survival. We seeded 2000 cells per well in 96-well micro-plates (Falcon Multiwell; Thomas Scientific, Swedesboro, NJ) 48 hours before any treatment. Cisplatin and menadione (Sigma-Aldrich) were applied for 48 hours in fresh medium. An MTT [3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide] assay was performed as previously described elsewhere by replacing the medium with fresh medium supplemented with 5 mg/l MTT (Sigma-Aldrich) for 1 hour (Gaiddon *et al.,* 1999). Cells were lysed in isopropanol with 0.04NHCl. Measurements were performed at 550 nm.

Quantitative Polymerase Chain Reaction. Cultured cells were lysed with 1 ml of TRIzol (Invitrogen/Life Technologies, Carlsbad, CA) per 10 cm^2. Mice were sacrificed by cervical dislocation; hindlimb gastrocnemius muscles were sampled, immediately frozen in liquid nitrogen, and stored at 280°C for later use. We used 1 ml of TRIzol (Invitrogen/Life Technologies) per 150 mg of muscle to extract RNA according to the manufacturer's instructions. RNA samples were ethanol-precipitated twice, and 1 mg was used for reverse transcription (High-Capacity cDNA Reverse Transcription Kit; Applied Biosys- tems, Foster City, CA). We performed quantitative polymerase chain reaction (qPCR) using 2 ng/μl cDNA (RNA equivalent) according to the manufacturer's instructions (SYBR Green PCR Master Mix; Applied Biosystems) and with 400 μM of each primer (Supplemental Table 2). Expression levels were normalized using an average of 18S.

SOD1 Mice*. SOD1* mice are FVB transgenic mice expressing the missense mutation G86R (human G85R equivalent) in the SOD1 gene (Ripps *et al.,* 1995). Transgene expression was monitored by PCR on SOD1 (forward GACATCATTGTTCATCC; reverse ATTGATG- GAATGCTCTCCTGA), FspI digestion, and agarose electrophoresis (Ripps *et al.,* 1995). We distinguished several experimental groups based on age and the presence of symptoms: asymptomatic 60-day-old mice, asymptomatic 75-day-old mice, early symptomatic mice (90 days; presenting signs of denervation as indicated by upregulation of the mRNA for acetylcholine receptor α-subunit), and symptomatic mice (105–110 days) when full paralysis in on.

Mice Treatment. SOD1* mice were injected intraperitoneally 3 times a week beginning at an age of 65 days (Rene *et al.,* 1996). AGT251 (20 mg/kg) was administered in 0.9% NaCl with 6% propylene glycol, 4% dimethylsulfoxide, and 2% Cremophor EL. Vehicle-treated littermates received the same formulation without AGT251. For doxorubicin treatment, 8-week-old mice were injected once intraperitone-ally with doxorubicin (BioAustralis, Smithfield, NSW, Australia) diluted at 18 mg/kg in 0.9% NaCl. AGT251 treatment was performed as described earlier. In vivo experiments were repeated at least twice with a number of animals (between 5 and 12) recommended to optimize statistical analyses according to the regional and national animal ethics committee. All animal manipulations were performed under appropriate supervision and observing protocols

validated by the regional and national animal ethics committee.

Statistical Analyses. Statistical analyses were performed using a one-way analysis of variance test followed by a Tukey post-test to allow a comparison between all the conditions. In the graphs, an asterisk indicates a statistically significant difference. For in vivo survival analysis of SOD mice, two statistical protocols were used: Mantel-Cox test and Gehan-Breslow-Wilcoxon. Both tests confirmed a statistically significant difference between control and AGT251-treated mice. Statistical analyses were performed using Prism (GraphPad Software, San Diego, CA).

Transfection and Luciferase Assays. Cells were transfected by a polyethylenimine-based or JetPrim (Polyplus, Strasbourg, France) as previously described elsewhere (Gaiddon *et al.,* 1996). For luciferase assays, cells were seeded in 24-well plates and transfected with the indicated expression vectors (200 ng) and reporter constructs (250 ng). Luciferase activity was measured in each well 24 hours later, and the results were normalized with a cytomegalovirus-driven reporter gene (Benosman *et al.,* 2011). The MuRF1-luc constructs were previously described by Waddell *et al*. (2008). The Hes1 reporter gene contains the 3-kb promoter of Hes1. Hes1 reporter gene and NICD and CBF expression vectors were a generous gift of Dr. Kadesch (Ross and Kadesch, 2001).

Results

Tocopherol and Flavonoid Derivatives Impact on Myoblastic Cell Survival.

The effect on cell survival of several flavonoid or tocopherol derivatives (AGT) was assayed on C2C12 cells, a commonly used in vitro model for myoblast cells. Dose-response survival relationships were established by means of an MTT assay and using a large window of concentrations (1 nM–125 μM) (Fig. 1A). Most compounds were reducing survival, with an IC50 between 50 and 125 μM, although three compounds were already active at lower concentrations (Fig. 1A; Table 1). The toxic effect of these compounds at high concentrations correlated with the upregulation of p21 and noxa expression (Fig. 1B). We chose p21 and noxa as they represent markers for cell growth arrest and apoptosis, respectively (Benosman *et al.,* 2011). Interestingly, two compounds significantly increased cell survival by 22% (AGT048, 25 μM) or 41% (AGT251, 15 μM) at concentrations preceding toxicity. AGT031 did not show any prosurvival effect and was more toxic than most compounds. Thus, it was used as a control in subsequent experiments.

AGT048 and AGT251 Protect against Oxidative and DNA Damage-Induced Stresses.

As AGT048 and AGT251 compounds were increasing cell survival, we assessed whether these compounds could also improve viability upon stresses associated with degenerative syndromes. Therefore, C2C12 were treated with 1) menadione that induces a strong production of anion superoxide (Rosen and Freeman, 1984) and 2) cisplatin that induces DNA damage and mild ROS production (Supplemental Fig. 1A) (Vidimar *et al.,* 2012). We observed that both AGT048 and even more AGT251 were able to increase cell survival in the presence of cisplatin and to a lesser extent of menadione (Fig. 2, A and B). Under the same conditions, AGT251 reduced the ROS production induced by cisplatin and menadione (Supplemental Fig. 1B). As expected, AGT031 did not increase cell survival.

To further assess the prosurvival activity of AGT251 at the molecular level, we analyzed the expression of p21 and noxa. We observed that in C2C12, cisplatin induced p21 and noxa expression (Fig. 2C). At the dose of AGT251 that favored survival (10 μM), the cisplatin-induced expression of p21 was unchanged, whereas the expression of the proapoptotic gene noxa was decreased. Taken together, these experiments demonstrated that AGT251 protects myoblast cells against DNA-damaging stress in vitro.

AGT251 Modulates the Notch Pathway.

To AGT251 Modulates the Notch Pathway. To further understand the biologic properties of the AGT251 tocopherol derivative, we then focused on the Notch pathway for two reasons. First, previous work has shown that compounds sharing a similar structure to our compounds were able to repress Notch4 RNA expression in neurospheres correlating with an increased neuronal survival (Coowar *et al.,* 2004; Blanchard *et al.,* 2013). Second, it has been shown that the Notch pathway plays a key role in muscle regeneration and myoblast survival (Buas and Kadesch, 2010; Bjornson *et al.*, 2012) and is regulated by the physiologic status of the mitochondria (Arthur and Cooley, 2012; Kasahara *et al.,* 2013).

Before evaluating the effect of AGT251 on the Notch pathway in stressed myoblastic cells, we first characterized the impact of cisplatin on the expression of Notch receptors (Notch 1–4) using reverse-transcription real-time quantitative polymerase chain reaction (Fig. 3A). A cytotoxic dose of cisplatin (7.5 μM) induced upregulation of Notch1, Notch3, and Notch4 mRNA levels. It is of note that the expression level of Notch4 in C2C12 cells was low (threshold cycle 6 30). To assess the functionality of these regulations, we also evaluated the expression of Notch target genes and observed an increase of Hes1 and HeyL mRNA upon cisplatin treatment.

We then tested how AGT251 might affect the cisplatin-induced activation of the Notch pathway. We found that prosurvival doses of AGT251 (Figs. 1A and 3B) caused a decrease of cisplatin-induced expression of Notch1 and Notch3 expression while Notch4 was further induced (Fig. 3C). Importantly, the repression of Notch1 and Notch3 correlated with a diminished expression of Notch target genes, such as Hes1. Taken together, these results indicated that the Notch pathway was induced by a cytotoxic dose of cisplatin, and that this induction was reduced by AGT251. In particular, we observed a correlation between the expression of Notch1, Notch3, and Hes1 upon treatment with AGT251.

AGT251 Protects Muscles from Doxorubicin-Induced Cachexia.

To assess whether our observations with C2C12 cells *in vitro* were physiologically relevant, we decided to use a murine model for cachexia. Cachexia is a severe syndrome including muscle atrophy observed in cancer patients with certain cancer localizations (such as pancreatic cancer) and in patients treated with doxorubicin (Fearon *et al.,* 2012). In patients, doxorubicin treatment is associated with muscle weakness and fatigue and with muscle atrophy (fiber types 1 and 2) (Bonifati *et al.,* 2000; Tozer *et al.,* 2008). The exact causes of muscle weakness and atrophy induced by doxorubicin remain poorly understood, and doxorubicin-treated rodents have been used to investigate these phenomena. In these models, muscle weakness and fiber alterations (proteolysis, autophagy, apoptosis) have been described, with variations depending on the mode of administration (intraperitoneal versus intravenous) and the type of muscle (soleus, extensor digitorum longus, heart, diaphragm) (Gilliam *et al.,* 2009, 2011, 2012, 2013; De Angelis *et al.,* 2010; Smuder *et al.,* 2011a,b; Dirks-Naylor *et al.,* 2013; Kavazis *et al.,* 2014; Yu *et al.,* 2014). These studies show evidence that muscle fibers as well as the progenitor pool seem to be affected and that the induction of ROS is a proposed cause of doxorubicin-induced myopathy.

To assess the effect of AGT251 on atrophy, two groups of mice were both treated once with doxorubicin (18 mg/kg) to induce cachexia, and one of them was also treated 3 times a week with AGT251 (20 mg/kg). The AGT251 dose was established as the maximal dose tolerated by the mice without noticeable adverse effects based on the body weight and behavior. After 15 days of treatment, the total mouse weight and skeletal muscle weight were measured, and RNA was extracted from muscles to quantify the expression of muscle atrophy markers (MuRF1 and Atrogin-1). We observed a loss of total animal and muscle weight with doxorubicin treatment (Fig. 4, A and B), confirming its atrophic effect on the muscle. This effect correlated with an upregulation of the two effectors and markers for muscle atrophy, MuRF1 and Atrogin-1 (Fig. 4C). It was very

interesting that we observed that cotreatment with AGT251 partially reversed the muscle atrophy, as assessed by muscle weight (Fig. 4A). This effect was further confirmed by the repression of MuRF1 and Atrogin-1 expression after AGT251 treatment (Fig. 4C). Based on our results, AGT251 appeared to protect muscles against atrophy under conditions inducing cachexia.

To investigate the impact of muscle atrophy and AGT251 on the Notch signaling pathway *in vivo*, we measured the expression of Notch receptors and Notch target genes. Doxorubicin treatment induced expression of Notch1 and Notch3 (Fig. 4D). Expression of the Notch target gene Hes1 was also increased by doxorubicin, in contrast with other Notch target genes, such as HeyL, which remained unaffected (Fig.4E). Importantly, administration of AGT251 led to a reduction of Notch1, Notch3, and Hes1 expression in doxorubicin-treated mice (Fig. 4, D and E). To further investigate the potential functional relationship between the Notch pathway and muscular atrophy, we used a reporter gene containing the MuRF1 promoter and a reporter gene containing the promoter of the Notch target gene Hes1. As expected, overexpression of the intracellular domain of Notch3 (NICD), alone or with its coactivator CBF, led to an increase in the Hes1 promoter activity (Fig. 4F). Interestingly, NICD also stimulated the activity of the MuRF1 promoter.

Taken together, these results confirmed that the activity of the Notch pathway is induced upon muscle injury, such as occurs with doxorubicin treatment, and that AGT251 is able to partly counteract this effect.

AGT251 Increases Survival in a Murine Model of ALS.

Given its protective effect on myoblasts in vitro and the protection against doxorubicin-induced muscular atrophy, we wondered whether treatment with AGT251 could be beneficial in mice showing progressive muscle degeneration leading to death. In addition, by using another model we wanted to assess whether AGT251 might have a broad protective effect independent of the etiology of the disease. Thus, we next studied mice with a substitution mutation in the Cu/Zn superoxide dismutase 1 gene (SOD1*), which is used as a model of the human pathology ALS (Ripps *et al.,* 1995; Gonzalez de Aguilar *et al.,* 2000). Although the G86R mutation in the SOD1 (SOD1*) does not affect SOD1 activity, the mice carrying the SOD1* transgene develop oxidative stress (Ripps *et al.,* 1995; Gonzalez de Aguilar *et al.,* 2000). On average, SOD1*mice die at around 105 days following muscular atrophy and motoneuronal death. SOD1* mice of 60 or 75 days of age are in the asymptomatic stage; their physical symptoms start at around 90 days, with some mice already showing a marked muscular denervation (group 90S) while others do not (group 90NS) (Ripps *et al.,* 1995; Gonzalez de Aguilar *et al.,* 2000). The denervation

of the animal can be followed by the expression of the acetylcholine α-receptor gene in the muscles (Fig. 5A). In addition, the level of muscular atrophy can be assessed using markers such as the MuRF1 and Atrogin-1 genes (Foletta *et al*., 2011) (Fig. 5B).

We first assessed whether in this model the expression of components of the Notch pathway were altered in the muscles during the ALS pathology (Fig. 5, C and D). We observed a marked increase in Notch3 expression at the early stage of the pathology (60 and 70 days), followed by a decrease upon disease progression (90S, animals with denervation at 90 days; 105 days) (Fig. 5, A and C). Interestingly, expression of Hes1 also increased at 90 days in all animals, regardless of their denervation status, while other Notch target genes, such as HeyL, remained unchanged (Fig. 4D). These observations established that in ALS atrophic muscle a Notch-dependent response takes place that might participate in the development of the pathology or in an attempt of a regenerative process.

We used then this mouse model to assess whether AGT251 could prolong the life span. We treated SOD1* mice 3 times a week with AGT251 (20 mg/kg) starting at an asymptomatic age of 65 days. The treatments were performed until death. The efficacy of the AGT251 treatment was indicated by a decreased expression of the muscle atrophy marker MuRF1 and Notch3-Hes1 components (Supplemental Fig. 3). But, more importantly, the results showed a mild but statistically significant increase in survival of AGT251-treated SOD1* mice compared with the vehicle-treated mice (Fig. 4E). Indeed, the vehicle-injected animals had a median survival of 105 days, but this number rise to 112 days (18%) for AGT251-injected animals. Altogether, this result indicated that AGT251 exerts a protective effect on diseases with muscle atrophy and that it also inhibits Notch3-Hes1 during this process.

Discussion

Muscular atrophy occurs in several lethal human diseases, including neuromuscular syndromes such as ALS or cancer. In cancer, it has been estimated that about 30% of the mortality is due to the cachexia that includes muscle atrophy. In the case of ALS, respiratory muscle atrophy is also the cause of death. This dramatic clinical status is a direct reflection of the absence of a curative treatment for muscle atrophy. In this study, we investigated the beneficial effect of flavonoid and tocopherol derivatives on skeletal muscles based on their antioxidant and anti-inflammatory properties, which have previously been shown to have protective activity in the nervous system and the heart. In addition, we investigated some of the molecular mechanisms potentially involved in muscle protection by focusing on the Notch pathway.

Cellular Effects of Novel Flavonoid and Tocopherol Derivatives.

The synthesized flavonoid and tocopherol derivatives and hybrids with an omega-alkanol chain showed *in vitro* biologic activity on the C2C12 myoblast cell line. Above a specific concentration, each compound reduced cell survival as observed by MTT tests and by the induction of cell growth arrest and the proapoptotic genes p21 and noxa (Fig. 1; Table 1). However, a moderate concentration of two tocopherol derivatives, AGT048 and AGT251, sharing a similar pharmacophore, stimulated survival. Importantly, at these subtoxic concentrations AGT048 and AGT251 partly counteracted the toxic effect of cisplatin or menadione on C2C12 cells (Fig. 2), which correlated with a reduced level of the proapoptotic gene noxa (Fig. 2C). In addition, AGT251 restored the expression level of two myoblastic cell markers, Pax7 and MyoD, which were both downregulated by cisplatin (Supplemental Fig. 4).

All the tested flavonoid and tocopherol derivatives share the same omega-alkanol chain. However, only two tocopherol derivatives present the protective effect on C2C12 cells, which suggests that this effect is not mediated by the omega-alkanol chain but more likely by an intact tocopherol core. This is further supported by the fact that the protective effect of AGT251 correlates with the reduction of ROS production in C2C12 cells (Supplemental Fig. 1B).

Tocopherol Derivatives Diminish the Stress-Induced Expression Levels of Notch1, Notch3, and Hes1 in Muscles.

A recent study showed that the Notch pathway is involved in muscle repair during aging or physical damage (Arthur and Cooley, 2012). In addition, it was previously shown that tocopherol derivatives repressed mRNA level for Notch4 in neuronal cells (Coowar *et al.,* 2004). Our study brings novel information on these two aspects.

Concerning the first aspect, we uncovered the first evidence of regulation of the Notch pathway during ALS or cachexia. Indeed, we observed both *in vitro* (C2C12 myoblastic cells) and *in vivo* (murine models of ALS and doxorubicin-induced cachexia) the induction of the Notch pathway after various stressors. In particular, we showed a correlation between the upregulation of Notch1 and Notch3 expression and their target gene Hes1. Based on the complex nature of the muscle—which includes fibers, muscle progenitors, vessels, and neuro-muscular junctions, for example—it is difficult to definitively conclude that the regulation of Notch observed *in vivo* is solely due to muscle cells. We can only hypothesize that part of this regulation does indeed involve muscle cells, based on our results with C2C12 and on the literature that has indicated that the Notch pathway is regulated in myoblastic cells and participates in the control of muscle

development and regeneration.

The consequences of this regulation in the muscles, and more specifically in the progenitors and the fibers, also remain to be established. As indicated by the literature, the Notch pathway seems to play a role in maintaining muscle stem cell quiescence (Bjornson *et al.,* 2012; Mourikis *et al.,* 2012). Therefore, the increase of Hes1 expression during the pathologies, or after *in vitro* treatment with anticancer drugs, might contribute to muscle atrophy by maintaining at least a subpopulation in a quiescent state. The exact and respective contribution of Notch1 and Notch3 in this process needs to be more clearly established.

In addition, our observation that NICD regulates the promoter of the atrophic/remodeling factor MuRF-1 might suggest that the Notch pathway could play a role in fibers. We also observed some differences in response between the models. For example, we found *in vitro* an induction of HeyL expression that we did not see *in vivo*. This is surprising because two recent publications indicated that HeyL might play a more important role than Hes1 in the maintenance of satellite cell number in the muscles (Buas and Kadesch, 2010; Fukada *et al.,* 2011). In contrast, our results might indicate that in injury/repair processes, such as those taking place in response to doxorubicin and in ALS-related syndromes, the opposite is the case, with Hes1 being more important than HeyL. However, the exact role of Notch1, Notch3 and Hes1 in the atrophic process and whether they are related to the regulation of the differentiation and proliferation equilibrium of satellite cells or fiber atrophy remains to be established. Interestingly, our study also suggested that Notch might be involved in muscle atrophy via induction of the atrophic/remodeling effector MuRF1, which provides a novel possible avenue in the overall function of the Notch pathway in muscle development and repair.

Concerning the second aspect, our data confirmed that tocopherol derivatives such as AGT251 affect the activity of the Notch pathway. Indeed, AGT251 treatment partly counteracted both *in vitro* and *in vivo* stress-induced Notch pathway activation. However, in C2C12 myoblastic cells and in muscles of ALS or doxorubicin-treated animals, the repression was mostly observed on Notch1 and Notch3 but not on Notch4 as previously seen in neurons (Coowar *et al.,* 2004). In addition, here we also provided the first evidence that regulation of the Notch receptor by tocopherol derivatives impacts the Notch target genes.

It is not yet precisely understood how AGT251 might act on the Notch pathway. As indicated earlier, one possibility is that due to its antioxidant properties, AGT251 protects the cells against the oxidative stress induced by cisplatin, doxorubicin, or mutant SOD1 (Supplemental Fig. 1B), hence reducing the induction of Notch. This suggests that AGT251 might help to restore the properties of the myoblastic cells. This is partly

supported by the fact that AGT251 reduces the inhibition of Pax7 and MyoD expression caused by cisplatin in C2C12 cells (Supplemental Fig. 4). Alternatively, the antioxidant properties of AGT251 might also protect the fibers from atrophy. In addition, one recent interesting observation has been that the Notch pathway is a relay toward myocyte differentiation induced by variations in mitochondria physiologic activity (Kasahara *et al.,* 2013). Therefore, based on the antioxidant activity of the tocopherol derivative (Supplemental Fig. 1B), one hypothesis might be that by controlling the mitochondrial redox status, AGT251 might regulate the Notch pathway. In addition, a more recent study indicates that the Notch pathway is partly controlled partly by Nrf2, which is a Redox sensitive factor (Paul *et al.,* 2014). Therefore, one hypothesis might be that regulation of the Notch pathway by the antioxidant AGT251 or during ROS-related muscle atrophy could involve Nrf2.

A Tocopherol Derivative Ameliorates Mouse Survival in ALS Model and Reduces Muscle Cachexia.

The *in vitro* trophic effect of tocopherol derivatives on myoblast cell line survival was validated using *in vivo* murine models. Indeed, sustained injection of AGT251 in a model of cachexia (doxorubicin-treated mice) ameliorated mice health, both on the overall weight of the mice and more specifically on the muscular weight, going along with the downregulation of effectors and markers of muscular atrophy (MuRF1 and Atrogin-1) and an upregulation of muscle fiber diameter (Supplemental Figs. 3 and 5). The protective effect of the tocopherol derivativeAGT251 on muscles was further confirmed as AGT251 prolonged slightly but significantly the survival of mice developing ALS with severe muscle atrophy.

Of note, the observed beneficial effect is close to what was previously observed for Riluzol in mice, the only treatment for ALS approved by the US Food and Drug Administration (Gurney *et al.,* 1996). This effect might be linked to an improved resistance of muscle fibers and/or motoneurons, which are both primary tissues affected by the pathology. More generally, the pro-atrophic effect of doxorubicin and mutated SOD1 as well as the protective effect of AGT251 might involve several components of the muscle that ultimately lead to an improvement or alteration of muscle and fiber size, respectively. For example, it is likely that the changes in the levels of ROS caused by doxorubicin, mutant SOD1, and AGT251 in the fibers and the progenitors might impact muscle atrophy.

Altogether, our *in vitro* and *in vivo* data indicate that specific tocopherol derivatives can improve syndromes that involve skeletal muscle atrophy, including in ALS or cancer-related cachexia. It also points out that the Notch pathway is regulated during muscle

atrophy, with specificities depending on the pathology. Our data suggest that Notch1, Notch3, and Hes1 seem to be potential players in these processes. However, the exact contribution of Notch1, Notch3, and Hes1 in the atrophic and regenerative processes remains to more precisely established. Consequently, designing molecules that target the Notch pathways might be an interesting avenue in the development of drugs aimed at the protection of muscle against various stresses.

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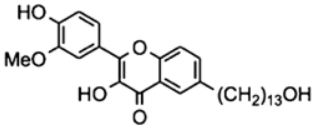
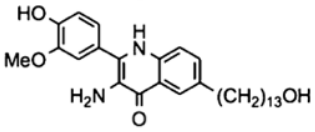
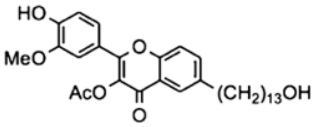
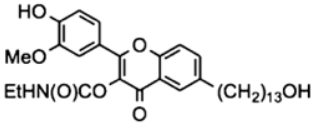
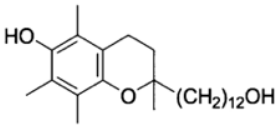
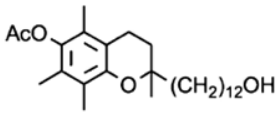
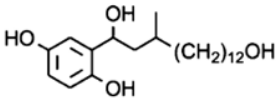
3– Figures and tables

Table 1 – Biologic activity of tocopherol and flavonoid derivatives on myoblast cells survival C2C12 myoblast cells were cultured for 48 hours in presence of the indicated compounds. Cell survival was evaluated using MTT test.

Derivative	Compound	Formula	IC_{50} for Cytotoxicity	Concentration for Maximum Survival	Maximum Survival
				μM	% of control
Flavanoid	AGT031		10–25	0.5	105
	AGT171		75–125	0.5	103
	AGT184		25–50	0.5	102
	AGT216		10–25	0.5	103
Tocopherol	AGT048		50–75	25	122
	AGT251		50–75	12.5	141
	AGT072		50–75	0.5	100

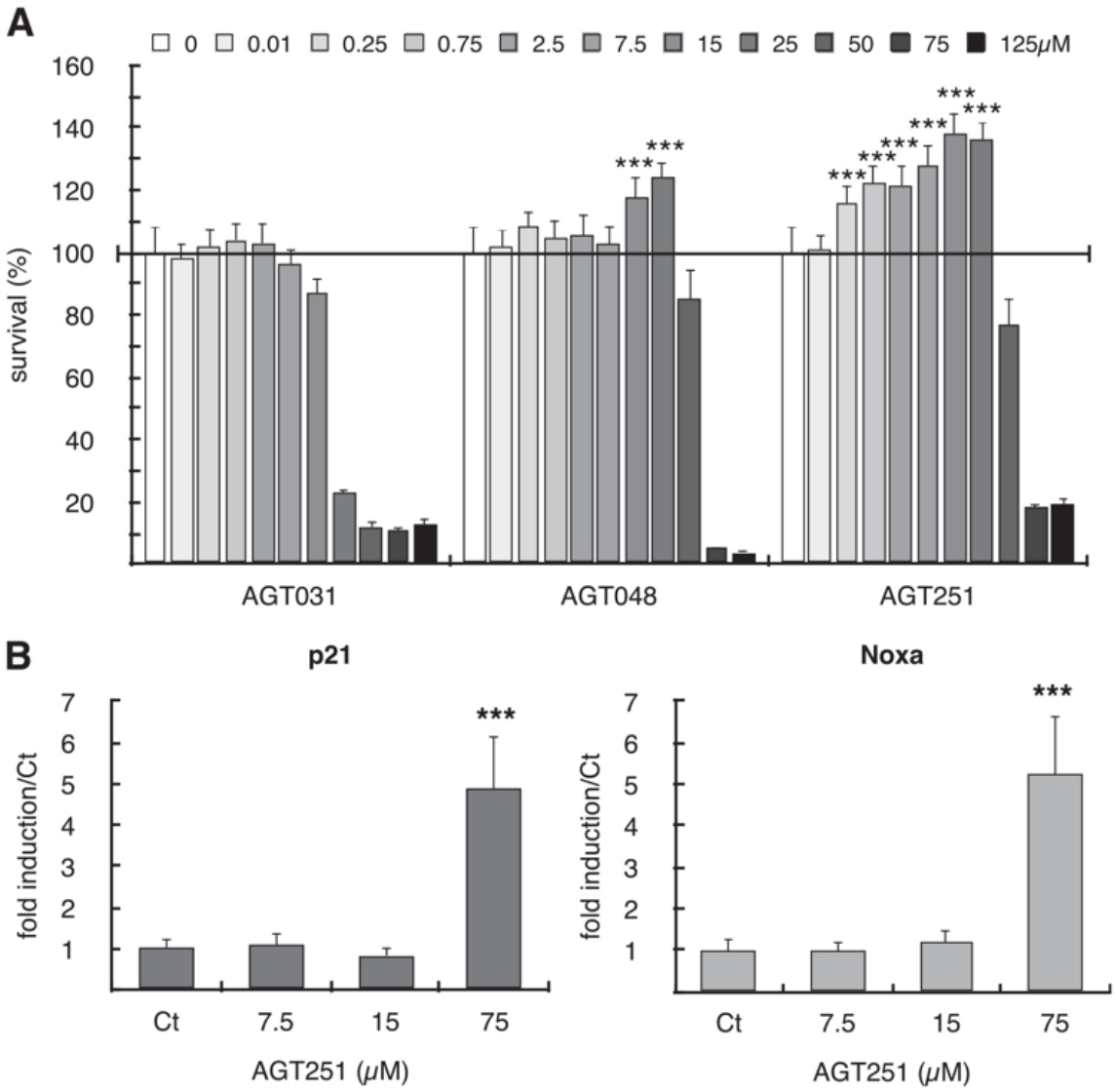


Fig. 1 – Tocopherol and flavonoids derivatives control myoblast cells survival. (A) C2C12 myoblast cells were cultured for 48 hours in presence of the indicated compounds (AGT031, AGT048, AGT251 in μM). Cell survival was evaluated using MTT test. Bars indicate mean 6 S.D. (n = 8). ***P, 0.001 compared with control, as calculated by a one-way analysis of variance test followed by a Tukey post-test. (B) mRNA levels of p53 target genes p21 and noxa were assayed in C2C12 myoblast cells treated with AGT251 (in μM) using reverse-transcription real-time quantitative polymerase chain reaction. Graphs are the mean (n = 3) of fold induction versus control in absence of cisplatin.

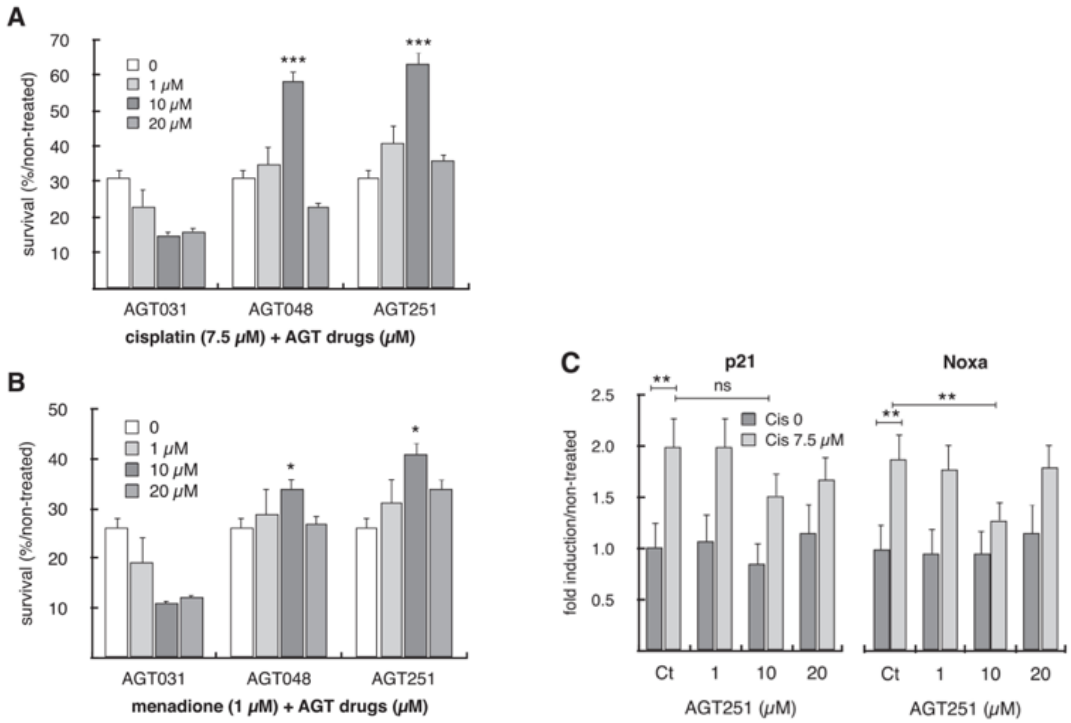


Fig. 2 – Tocopherol and flavonoids derivatives protect myoblast cell population against DNA damaging stress. C2C12 myoblast cells (A and B) were cultured for 48 hours in the presence of the indicated compounds (AGT031, AGT048, AGT251 in μM) in the absence or presence of (A) cisplatin (Cis; 7.5 μM) or (B) menadione (1 μM). Cell survival was evaluated using the MTT test. Bars indicate mean6 S.D. (n = 8) relative to control in the absence of cisplatin. *P<0.05, **P, 0.01, ***P<0.001 compared with control, as calculated by a one-way analysis of variance test followed by a Tukey post test. (C) mRNA levels of p21 and noxa were assayed in C2C12 myoblast cells treated with AGT251 (in μM) in the absence or presence of cisplatin (7.5 μM) using reverse-transcription real-time quantitative polymerase chain reaction. Graphs are the mean of fold induction versus control in the absence of cisplatin with S.D. (n =3).

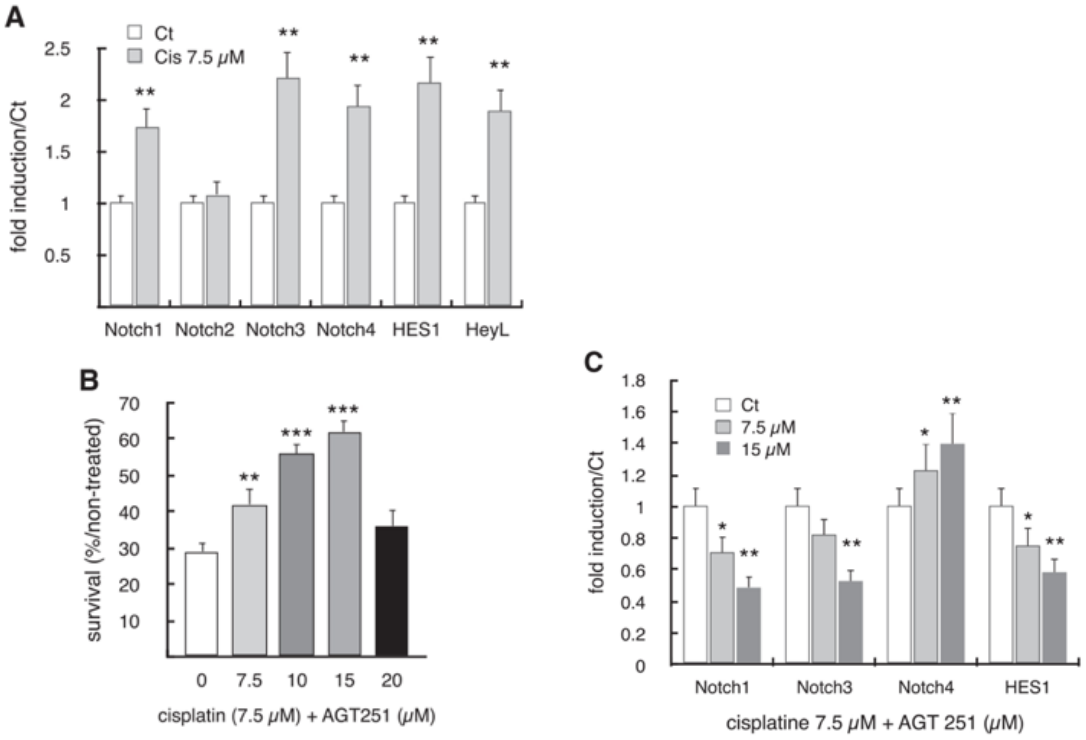


Fig. 3 – The tocopherol derivative AGT251 regulates the expression of components of the Notch pathway. (A) mRNA levels of components of the Notch signaling pathway were assayed in C2C12 myoblast cells treated with cisplatin (Cis; 7.5 μM) for 24 hours using reverse-transcription real-time quantitative polymerase chain reaction (RT-qPCR). Graphs are the mean of fold induction versus control with S.D. (n =3). **P<0.01 as calculated by aone-way analysis of variance (ANOVA) test followed by a Tukey post test. (B) C2C12 myoblast cells were cultured for 48 hours in the presence of cisplatin (7.5 μM) in the absence or presence of AGT251 (in μM). Cell survival was evaluated using the MTT test. Curves indicate mean 6S.D. (n =8) relative to control in the absence of cisplatin. **P<0.01, ***P<0.001 compared with control, as calculated by a one-way ANOVA test followed by a Tukey post test. (C) mRNA levels of components of the Notch signaling pathway were assayed in C2C12 myoblast cells treated for 24 hours with cisplatin (7.5 μM) in the absence or presence of AGT251 (in M) usingRT-qPCR. Graphs are the mean of fold induction versus control with S.D. (n =3). *P<0.05, **P<0.01 as calculated by a one-way ANOVA test followed by a Tukey post test.

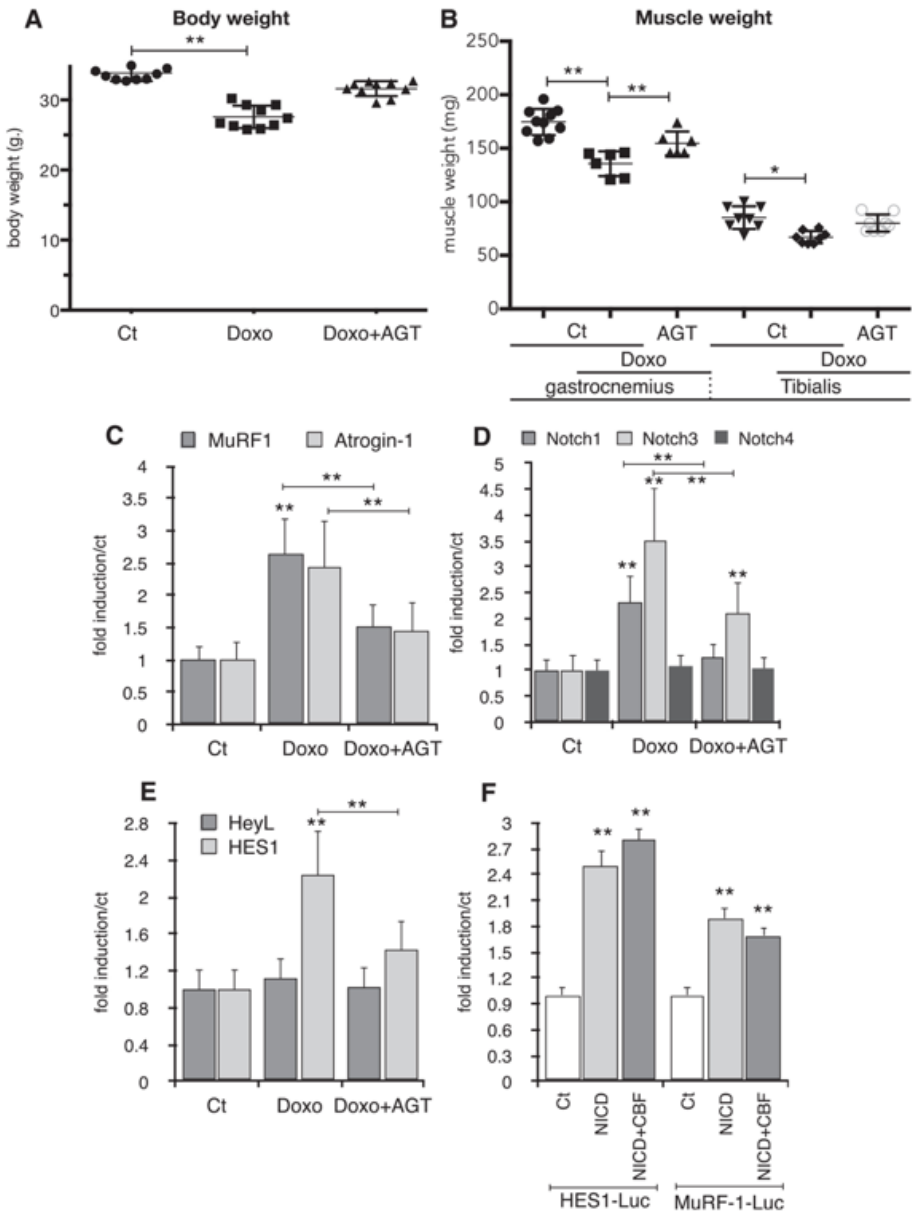


Fig. 4 – AGT251 reduces muscle cachexia. Eight-week-old littermate mice were injected with doxorubicin (Doxo; 18 mg/kg) and AGT251 (20 mg/kg). (A) Body and (B) muscle weight was quantified after 15 days. Graphs represent mean 6S.D. in mg of muscles (n = 9). *P<0.05, **P<0.01 compared with control, as calculated by a one-way analysis of variance (ANOVA) test followed by a Tukey post test. (C and D) mRNA levels of the muscular atrophy markers MuRF1, Atrogin-1, Notch1, Notch3, Notch4, Hes1, and HeyL were assayed in muscles of mice treated with doxorubicin and AGT251 using reverse-transcription real-time quantitative polymerase chain reaction. Graphs represent mean 6 S.D. relative to untreated animals (control). **P, 0.01 compared with control, as calculated by a one-way ANOVA test followed by a Tukey post-test.

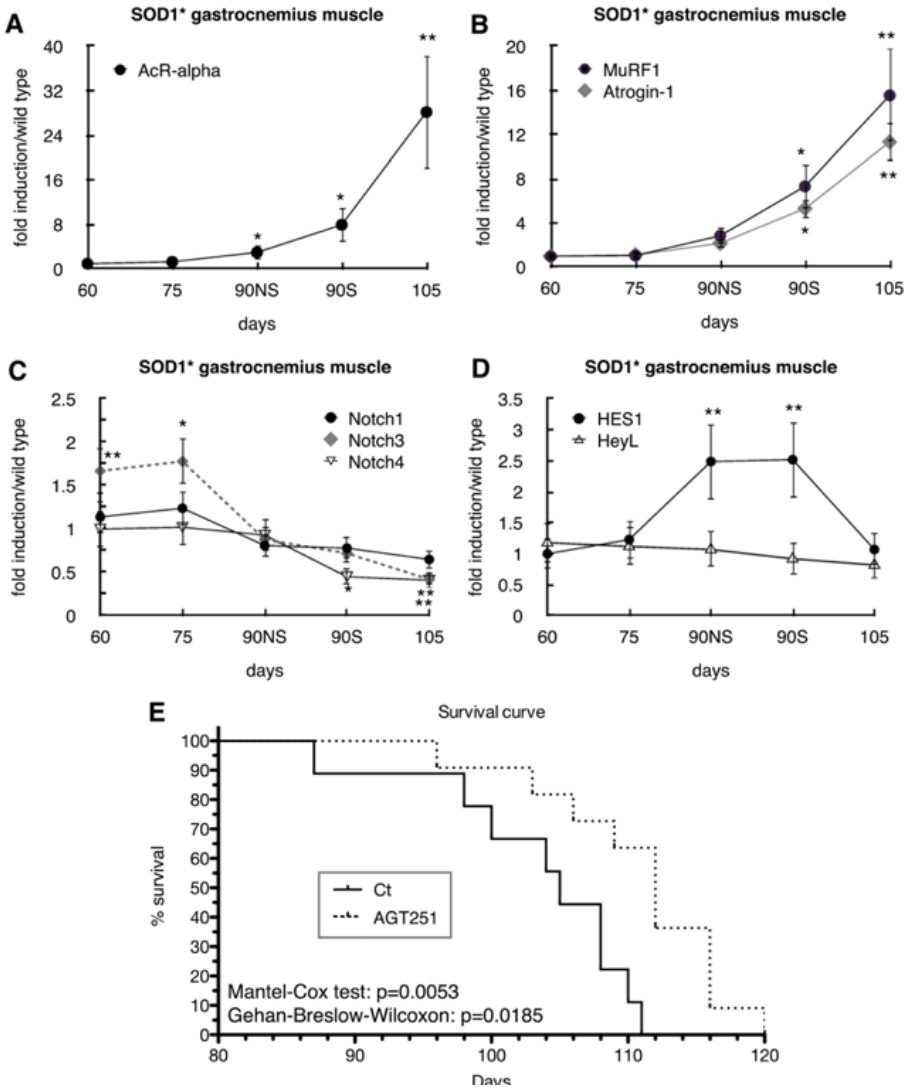


Fig. 5 – AGT251 favors mice survival in a model of ALS. The mRNA levels of markers of ALS disease progression—acetyl choline receptor α, AcR-α (A) MuRF1, and atrogin-1 (B)—and components of the Notch signaling pathway—Notch1, -3, and -4 (C), Hes1, HeyL (D)—were assayed in muscles of SOD1* mice using reverse-transcription real-time quantitative polymerase chain reaction. mRNA were extracted at different ages. Curves are themean of fold induction versus the condition of wild-type littermate 60-day-old mice and of three experiments containing animals of matching age (60, 75, 90NS, 90S, 105-day-old, n = 5). 90NS and 90S populations are differentiated based on the level of expression of the AcR-α gene. *P<0.05, **P<0.01 compared with control, as calculated by a one-way analysis of variance test followed by a Tukey post test. (E) SOD1* 650-day-old mice were injected 3 times aweek with AGT251 (20 mg/kg) until death. Survival curves are relative to the day of death of the untreated animals (n = 12). *Statistically significant differences established by log-rank (Mantel-Cox) test (P = 0.0053) and Gehan-Breslow-Wilcoxon test (P = 0.0183).

Discussion

My work consisted on investigating the molecular mechanisms behind muscle atrophy in ALS and doxorubicin induced cancer-associated cachexia. We identified a p53 family common pathway in these distinct muscle atrophy models and pointed out its specific mechanisms. Although p63, p73 and notably p53 functions in cell homeostasis, development and cancer are largely described in the literature, there is much more to investigate about p53 family involvement in muscle atrophy mechanisms.

To address p53 family function in different syndromes we used biopsies from ALS patients and mutated mice for SOD1 (an ALS linked mutation) (Hart, 2006), as well as murine myoblast cells (C2C12) and mice treated with doxorubicin in order to induce cachexia. We found that p53 family members (p53, TAp63 and TAp73) were induced within SOD1 mutated mice muscle and by the doxorubicin treatment. These alterations correlated with atrogenes upregulation in both models, indicating that p53 family may play a role on muscle atrophy induced by ALS and doxorubicin induced cachexia via atrogenes modulation. Confirming this observation, our results showed that p53 family members overexpression upregulated Trim63 but not Atrogin-1 in C2C12 cells. These results are in agreement with Schwarzkopf and colleagues' (2008) showing that constitutive activation of p53 *in vitro* induces muscle atrophy but does not increase Atrogin-1 expression, suggesting that the upregulation of Atrogin-1 in ALS and doxorubicin induced cachexia is not dependent on the p53 family signaling pathways.

Interestingly doxorubicin potentiated the p53 family members Trim63 induced expression, which suggests that doxorubicin might improve p53 family transactivity. Indeed, we showed that the p53 family members can bind to the Trim63 promoter. Importantly, we also showed that p53 and p63 are important but not indispensable for doxorubicin induced Trim63 upregulation. Similarly, p63 was required for Trim63 optimal activation after FCCP (a mitochondria depolarizing) treatment. These results suggest that the p53 family members (p53 and p63) may modulate Trim63 expression in distinct context of stresses.

In addition, we found upregulation of p53 family target genes linked to cell death in ALS and it is described that doxorubicin can induce apoptosis through a p53-dependent pathway (Wang *et al*., 2004). Moreover, studies have shown that apoptosis is activated in several conditions associated to muscle atrophy, (Allen *et al*., 1997a; Adams et al., 1999; Borisov & Carlson, 2000; Smith *et al*., 2000; Dirks & Leeuwenburgh, 2002), including in muscle biopsies from cancer patients (Busquets *et al*., 2007), contributing to muscle atrophy. It can be inferred then that the upregulation of Trim63 and the p53 family members in ALS and doxorubicin induced cachexia could be linked to induction of apoptosis. Indeed, doxorubicin induced DNA damage activates p53 and increases proapoptotic proteins in muscle cells (L'Ecuyer *et al*., 2006). Moreover, it was shown that FoxO1, a Trim63 transactivator, induces apoptosis and Trim63 upregulation in C2C12 cells, suggesting that the proteasome-mediated protein degradation could be associated with apoptosis increase (McLoughlin *et al*., 2009). However, this hypothesis needs to be confirmed.

According to our observations, both ALS and doxorubicin-induced cachexia have their particular molecular pathways that contribute to Trim63 modulation. In doxorubicin-induced cachexia for example, we found that ceramide accumulation normally induced by doxorubicin treatment contribute to Trim63 upregulation partially independent of the p53 family. Ceramide is described to induce muscle atrophy and indeed, blocking its synthesis in tumor-bearing mice has an anti-atrophic effect and decreasing on expression of atrogenes and Foxo3, a Trim63 activator (Larichaudy *et al*., 2012). Moreover, ceramide reduces protein synthesis by inhibiting Akt/mTOR signaling pathway (Hyde et al., 2004) and is able to activate the Trim63 transcription factor NF-κβ (Demarchi et al., 2005). Altogether, ceramide plays a role in both modulation of protein synthesis and protein degradation. However, in our doxorubicin-induced cachexia model, ceramide synthesis inhibition by myriocin diminished doxorubicin-induced Trim63 but did not impact on Atrogin-1 upregulation by doxorubicin. In addition, this regulation seems to be independently of the p53 family, suggesting that, upon doxorubicin treatment, ceramide might be relevant for the increase of protein degradation regulated by Trim63 and it could possibly trigger Trim63 activation via FoxO or NF-κβ induction.

In the ALS model, we showed that several stresses related to the ALS-linked mutation (SOD1) activated p63 and p73 (oxidative and ER stress, DNA damage and mitochondrial deregulation). Interestinly, some of these effects are also triggered by doxorubicin, like the oxidative stress. However, in our model of doxorubicin-induced cachexia, we showed that the pre-treatement with NAC upregulated TAp63 and only weakly diminished Trim63 upregulation, suggesting that oxidative stress might not be the main doxorubicin triggering factor of p53 family Trim63 modulation. Though, we still need to investigate the expression of other family members in the same condition. Although oxidative stress is a common feature found in ALS and doxorubicin induced cachexia, it plays distinc roles in both syndromes. In the case of ALS, ROS is related to the neurodegeneration process (Barber *et al.,* 2006), linked with mutations in the ROS detoxification enzyme superoxide dismutase 1 (SOD1) (Beckman *et al.,* 2001). The oxidative stress in ALS leads to DNA and mitochondria damage, activation of p53 and cell death (Shibata & Kobayashi, 2008). Whereas in cancer-associated cachexia, ROS is directly generated by doxorubicin and/or the tumor itself (Pelicano *et al.,* 2004; Gilliam *et al.,* 2012), and has a toxic effect in the muscles (Gilliam et al., 2012).

In order to protect muscles from waste and alleviate muscle weakness associated with these chronic syndromes, we investigated promising tocopherol derivatives and revealed that they improved survival by protecting against oxidative stress. Importantly, the Notch pathway mediates ROS-related muscular atrophy, a comum features in cancer-associated cachexia (Adams *et al.,* 2008; Gilliam *et al.,* 2009; 2012). We showed that the Notch pathway was regulated during ALS and doxorubicin induced cachexia and stimulated upon muscle injury stimuli. In corroboration with our work, Mu and coworkers (Mu *et al.,* 2016) demonstrated that the Notch pathway is overactivated in skeletal muscles from tumor-bearing mice, a well-recognized model of cancer-associated cachexia. Inversely, inhibition of this signaling pathway reduced muscle atrophy.

Interestingly, we also showed that the overexpression of the intracellular domain of Notch3 (NICD) regulated Trim63 (Murf1), suggesting the activation of the proteasome machinery by the Notch pathway during ALS and doxorubicin induced cachexia. The Notch pathway is described to contribute to myogenesis and muscle regeneration by increasing myoblast proliferation, activating satellite cells and contributing to cell

differenciation (Conboy *et al.,* 2003; Buas *et al.,* 2010). Basically, this pathway keeps muscle stem cells in an undifferentiated state by repressing myogenesis. This way, muscle tissue has a backup cell supply in case the muscle needs them for regeneration (Kopan *et al.,* 1994; Mourikis *et al.,* 2012). Doxorubicin inhibits myogenic differenciation by blocking MyoD (Kurabayashi *et al.,* 1993), suggesting a relation with the Notch pathway and a possible explanation to the doxorubicin induced Trim63 expression throught the p53 family, since the Notch pathway is dually regulated by p63 during cell differenciation (Nguyen *et al.,* 2006). In addition, p63 conection with JAG1 within the Notch pathway suggests its involvement in muscle atrophy regulation.

Added to our findings, the current results point to an encouraging direction towards to the development of new therapeutic approaches to treat muscle atrophy related to chronic disease throught the Notch pathway. However, it also highlights the necessity of better understanding the pathways underlying doxorubicin induced atrogenes expression.

Conclusion and perspectives

In this work we have discussed about the differences and similarities between ALS and doxorubicin-induced muscle atrophy. Taken together, our results demonstrate that the p53 family modulates Trim63 in different muscle atrophic conditions. Unfortunately, I could not address the impact of the specific downregulation ΔNp63 and ΔNp73 on doxorubicin induced Trim63 expression. Furthermore, we would like to validate our findings in muscle biopsies from cancer pantients but unfortunately they are difficult to obtain, because, contrary to ALS, the muscle biopsy collection is not part of the diagnosis procedure of the disease.

Our next step would be to confirm the role of the p53 family on Trim63 regulation within a tumor bearing mice model. Like doxorubicin administration, colon 26 adenocarcinoma implantation in mice is an efficient way to induce cancer-associated cachexia (Tanaka *et al.,* 1990). For that reason we have been working on a new mice colony where we crossed knockout mice for TAp63 with $APC_{\Delta 14}$ mutated mice. Loss of APC function is observed in about 80% of human colon tumors (Kwong & Dove, 2009), and so the APC mutation triggers molecular pathways that will give rise to spontaneous colorectal cancer (Fodde, 2002). This model will then allow us to mimic clinical condition *in vivo*.

Of course, there are still questions remaining to be answered. We would like to investigate which transcription factors are responsible for ROS and ceramide modulation during doxorubicin induced Trim63, and if they are completely independent of the p53 family Trim63 upregulation. In addition, we still do not know which doxorubicin feature (DNA damage, ROS, ER stress, Notch pathway) is the most relevant to Trim63 increase through the p53 family and whether doxorubicin increases p53 family members transcriptional activation of the Trim63 promoter.

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Résumé de Thèse en français

Rôle de la famille p53 dans l'atrophie musculaire

Paula Araujo de Abreu

Introduction

L'atrophie musculaire peut se produire dans le cadre de nombreuses maladies et est souvent un indicateur de mauvais pronostic, qui nuit à la qualité de vie et augmente la mortalité. En outre, plusieurs conditions comme le vieillissement, la famine, la désuétude et l'immobilisation peuvent entraîner une perte musculaire et, selon la cause, différentes voies moléculaires peuvent être impliquées dans le processus d'atrophie musculaire. Cependant, le déséquilibre entre la synthèse protéique et la dégradation médiée par la voie du protéasome et modulé par les ubiquitine ligases spécifiques du muscle Trim63 (Murf1) et Atrogin-1 (MAFbx) sont une caractéristique courante parmi les diverses causes de perte musculaire. Ces ubiquitine ligases spécifiques aux muscles - Trim63 et Atrogin-1 - sont décrites comme étant régulièrement régulées positivement dans une gamme de modèles d'atrophie des muscles squelettiques. Dans ce contexte, notre laboratoire s'est particulièrement intéressé à deux de ces modèles la SLA et les modèles de cachexie induite par le cancer. La question qui reste à éclaircir est de savoir s'il existe un mécanisme moléculaire commun ou spécifique qui déclenche l'expression de Trim63 ou d'Atrogin-1 dans certains événements d'atrophie musculaire.

La cachexie associée au cancer est un syndrome multifactoriel caractérisé par une atrophie musculaire prononcée, une inflammation systémique et des altérations métaboliques. Il est important de noter que la cachexie affecte entre 50 et 80% des patients cancéreux et est responsable d'environ 20% de leur décès. Des preuves croissantes mettent en évidence l'impact des tumeurs et / ou des thérapies sur la fonction musculaire et l'intégrité chez les patients atteints de cancer. Des études suggèrent que la tumeur elle-même joue un rôle actif dans la perte musculaire en raison de sa capacité à sécréter des cytokines pro-inflammatoires et la grande exigence de nutriments pour maintenir la croissance cellulaire, créant une interaction tumeur-hôte qui aggrave l'état du patient. Aussi, la chimiothérapie anticancéreuse présente des effets secondaires qui pourraient contribuer à la cachexie, comme l'anorexie, les nausées et la diarrhée. Mais plus encore, il a été montré que les agents chimiothérapeutiques ont un effet négatif direct sur le métabolisme des protéines, conduisant à la cachexie. Dans ce contexte, le

médicament anticancéreux doxorubicine est bien reconnu pour avoir des effets cataboliques musculaires.

Une autre pathologie extrêmement affectée par l'atrophie musculaire est la SLA, une pathologie neurodégénérative causée par une altération des motoneurones. Environ 20% de tous les cas de SLA héréditaire peuvent être liés à des mutations dans le gène codant SOD1. En outre, de nouveaux gènes mutés (FUS, TARDBP) ont été reliés à la SLA avec des différences dans les résultats physiopathologiques. Ces différences pourraient être liées aux différents impacts des protéines mutées au niveau moléculaire. En effet, les agrégats protéiques ou d'autres altérations induites par les mutants SOD1 ont été caractérisés dans les cellules musculaires, alors que d'autres protéines mutées liées à la SLA ne semblent pas affecter directement les muscles. À ce jour, les mécanismes moléculaires exacts conduisant au catabolisme musculaire dans la phase symptomatique de la SLA restent mal compris et l'absence de marqueurs pré-symptomatiques souligne la nécessité de comprendre les processus cataboliques musculaires à des fins thérapeutiques.

Plusieurs éléments de la littérature indiquent que les membres de la famille p53 (p53, p63, p73) jouent un rôle important dans la physiopathologie musculaire et pourraient donc représenter des acteurs de l'atrophie musculaire. Malgré les rôles classiques de la famille p53 des facteurs de transcription (p53, p63 et p73) dans la tumorigénèse, le cycle cellulaire, la régulation de l'apoptose, le développement épithélial et neuronal, la famille p53 module la myogenèse et la différenciation. Par exemple, p63 est particulièrement important pendant le développement cardiaque, alors que p73 module la différenciation des myoblastes. De plus, p53 et p63 jouent un rôle au cours de la régénération musculaire et du métabolisme en contrôlant l'expression de Sirt1, TIGAR et AMPK. Dans la famille p53, des promoteurs distincts génèrent deux classes d'isoformes généralement reconnues pour avoir une fonction antagoniste, contenant (TA) ou non (ΔN) le domaine de transactivation N-terminal (Murray-Zmijewski et al., 2006). Il est important de noter que, dans les muscles squelettiques, l'équilibre entre TA et l'expression de l'isoforme ΔN influence les processus de développement et de différenciation. Il est intéressant de noter que p53 s'est révélé être induit pendant l'immobilisation.

L'absence de thérapies efficaces pour traiter la perte musculaire associée à des maladies chroniques met en évidence la compréhension insuffisante des mécanismes complexes sous-jacents à l'atrophie musculaire. Dans ce travail, nous étudions si les membres de la famille p53 peuvent moduler la perte musculaire dans la SLA et dans le modèle de cachexie associée au cancer induite par la doxorubicine et si elle pourrait être un mécanisme régulateur commun de l'atrophie musculaire associée à des maladies.

Objectifs

Les objectifs de ma thèse étaient axés sur la compréhension du rôle de la famille p53 dans l'atrophie musculaire en utilisant des modèles cellulaires et animaux qui l'imitent. Cela nous a amenés à utiliser ces modèles pour identifier les approches thérapeutiques. Par conséquent, mon projet avait 3 objectifs complémentaires:

1. Caractériser le profil d'expression des membres de la famille p53 dans l'atrophie musculaire pendant la SLA et la cachexie induite par la doxorubicine.
2. Comprendre comment la famille p53 peut avoir un impact sur l'atrophie musculaire, en particulier en régulant les facteurs pro-atrophiques, tels que Trim63 / Murf1
3. Identifier une nouvelle approche thérapeutique pour l'atrophie musculaire.

Résultats

Pendant mon doctorat j'ai contribué à trois publications, dont deux d'entre elles sont déjà acceptées dans eLife et Journal of Pharmacology and Experimental Therapeutics.

La première publication met en évidence la modulation Trim63 (Murf1) par la famille p53 pendant l'atrophie musculaire, en particulier la p63, dans l'atrophie musculaire induite par la SLA. Des analyses bioinformatiques sur 4 expériences transcriptomiques indépendantes réalisées avec des muscles de patients atteints de SLA ou des modèles murins de SLA ont identifié l'activation d'une réponse de type p53.

D'autres analyses effectuées sur les biopsies des patients et sur des modèles animaux montrent une régulation positive de p53 et de TAp63. En effet, leur expression est en corrélation avec la sévérité de la pathologie.

La deuxième publication est sur le point d'être soumise et montre le rôle de la famille p53 dans l'atrophie musculaire induite par la doxorubicine. Nous avons caractérisé le profil d'expression et les interactions physiques / fonctionnelles entre les membres de la famille p53 et les ubiquitine ligases pro-atrophiques (Trim63 et Atrogin-1) dans les cellules myoblastiques murines (C2C12) et les souris traitées avec la doxorubicine. De plus, nous avons également étudié d'autres voies moléculaires impliquées sur l'atrophie musculaire induite par la doxorubicine.

Enfin, la troisième publication concerne les effets cytoprotecteurs des molécules antioxydantes dans deux contextes d'atrophie musculaire différents : la SLA et la cachexie. Le laboratoire visait à trouver des thérapies innovantes pour réduire l'atrophie musculaire. Pour ce faire, nous avons collaboré avec une start-up (Axoglia) pour tester des molécules potentiellement cytoprotectrices dans les muscles. Ces molécules sont dérivées de flavagline ou de tocophérol et montrent une activité antioxydante. Après le criblage *in vitro* de l'activité cytoprotectrice des composés, nous avons utilisé des modèles *in vivo* pour valider l'aptitude du composé à réduire l'atrophie musculaire. Nous avons également caractérisé certains des mécanismes moléculaires qui sous-tendent cet effet. Par exemple, nous avons pu établir une déréglementation de la voie Notch dans le muscle atrophique.

Discussion

Mon travail consistait à étudier les mécanismes moléculaires derrière l'atrophie musculaire dans la SLA et la cachexie associée au cancer induite par la doxorubicine. Nous avons identifié une voie commune de la famille p53 dans ces modèles distincts d'atrophie musculaire et avons souligné ses mécanismes spécifiques. Pour traiter la fonction de la famille p53 dans différents syndromes, nous avons utilisé des biopsies de patients souffrant de SLA et de souris mutées pour la SOD1 (mutation liée à la SLA), ainsi que des myoblastes murins (C2C12) et des souris traitées avec la doxorubicine pour

induire la cachexie. Nous avons trouvé que des membres de la famille p53 (p53, TAp63 et TAp73) ont été induits dans le muscle de la souris mutée SOD1 et par le traitement par la doxorubicine. Ces modifications sont en corrélation avec la régulation positive des atrogènes dans les deux modèles, ce qui indique que la famille p53 peut jouer un rôle sur l'atrophie musculaire induite par la SLA et la cachexie induite par la doxorubicine via la modulation des atrogènes.

Il est intéressant de noter que la doxorubicine a potentialisé l'expression induite par Trim63, ce qui suggère que la doxorubicine pourrait améliorer l'activité de p53 de se lie a Trim63. En effet, nous avons montré que les membres de la famille p53 peuvent se lier au promoteur Trim63. Fait important, nous avons également montré que p53 et p63 sont importants mais pas indispensables pour que la doxorubicine induise la surexpression de Trim63. De même, p63 était nécessaire pour l'activation optimale de Trim63 après un traitement par FCCP (une dépolarisation des mitochondries). Ces résultats suggèrent que les membres de la famille p53 (p53 et p63) peuvent moduler l'expression de Trim63 dans un contexte distinct du stress.

Selon nos observations, la SLA et la cachexie induite par la doxorubicine ont leurs voies moléculaires particulières qui contribuent à la modulation Trim63. Dans la cachexie induite par la doxorubicine par exemple, nous avons constaté que l'accumulation de céramides normalement induite par le traitement à la doxorubicine contribue à une régulation positive Trim63 partiellement indépendante de la famille p53. Dans le modèle SLA, nous avons montré que plusieurs stress liés à la mutation connue de la SLA (SOD1) activent les gènes p63 et p73 (stress oxydatif et ER, les dommages à l'ADN et la dérégulation mitochondriale). De manière intéressante, certains de ces effets sont également déclenchés par la doxorubicine, comme le stress oxydatif. Cependant, dans notre modèle de la cachexie induite par la doxorubicine, nous avons montré que le prétraitement avec le NAC avait une régulation positive TAp63 et seulement une diminution faible de la régulation positive Trim63, suggérant que le stress oxydatif pourrait ne pas être le principal facteur de déclenchement de la p53 Trim63.

Le stress oxydatif est une caractéristique courante de la SLA et de la cachexie induite par la doxorubicine et joue un rôle distinct dans les deux syndromes. Afin de protéger les muscles des déchets et d'atténuer la faiblesse musculaire associée à ces

syndromes chroniques, nous avons étudié les dérivés prometteurs du tocophérol et révélé qu'ils amélioraient la survie en protégeant contre le stress oxydatif par la voie Notch, qui favorise l'atrophie musculaire liée au RLO (radicaux libres de l'oxygène). Nous avons montré que la voie Notch était régulée au cours de la SLA et de la cachexie induite par la doxorubicine et stimulée par des stimuli des blessures musculaires. Inversement, l'inhibition de cette voie de signalisation a réduit l'atrophie musculaire. De manière intéressante, nous avons également montré que la surexpression du domaine intracellulaire de Notch3 (NICD) régulait Trim63 (Murf1), ce qui suggère l'activation de la machinerie protéasome par la voie Notch pendant la SLA et la cachexie induite par la doxorubicine.

Les résultats actuels indiquent une orientation encourageante vers le développement de nouvelles approches thérapeutiques pour traiter l'atrophie musculaire liée aux maladies chroniques dans la voie Notch. Cependant, ils soulignent également la nécessité de mieux comprendre les voies sous-jacentes à l'expression des atrogènes induite par la doxorubicine.

Conclusion et perspectives

Dans ce travail, nous avons discuté des différences et des similarités entre la SLA et l'atrophie musculaire induite par la doxorubicine. Pris ensemble, nos résultats démontrent que la famille p53 module Trim63 dans différentes conditions atrophiques musculaires. Ces résultats mettent en évidence une cible moléculaire qui peut être explorée dans le champ de l'atrophie musculaire afin de développer des solutions thérapeutiques pour traiter les déchets musculaires dans des conditions distinctes.

Cependant, il reste encore des questions à résoudre. Nous aimerions étudier quels facteurs de transcription sont responsables de RLO et de la modulation de céramide au cours de la doxorubicine induite Trim63, et si elles sont complètement indépendantes la surexpression de Trim63 par la famille p53. En outre, nous ne savons pas encore quel effet de la doxorubicine (lésions de l'ADN, RLO, stress ER, Notch) est le plus pertinent pour augmenter Trim63 via la famille p53 et si la doxorubicine augmente l'activation transcriptionnelle du promoteur Trim63 par les membres de la famille p53.

Paula ABREU

Rôle de la famille p53 dans l'atrophie musculaire

Résumé

L'atrophie musculaire de la cachexia provient du déséquilibre entre la synthèse et la dégradation de protéines. La littérature suggère que les membres de la famille p53 (p53, p63, p73) jouent un rôle dans le contrôle des processus de prolifération, différenciation et mort des précurseurs et des fibres musculaires. Ici nous avons caractérisé le profil d'expression de ces membres dans l'atrophie musculaire de la SLA (Sclérose Latérale Amyotrophique) et dans un modèle de cachexie induite par la doxorubicine. Nous avons montré une augmentation de l'expression des membres de la famille p53 et des atrogènes de manière corrélée sur ces deux modèles ainsi qu'une activation transcriptionnelle de Trim63 par p53, p63 et p73. Aussi, nous avons voulu savoir si les composés de tocophérol possédant une activité antioxydante pouvait réduire l'atrophie musculaire et avons montré que ce composé neutralise l'induction de la voie Notch, importante pour le développement musculaire et la régénération.

Résumé en anglais

Muscle atrophy in cachexia results from the imbalance between protein synthesis and degradation due to activation of the ubiquitin-proteasome pathway. Literature suggests that p53 family members play a role in controlling proliferation, differentiation and death of precursors and muscle fibers. Here we characterize the expression profile of the p53 family members in muscle atrophy in ALS (Amyotrophic Lateral Sclerosis) and in doxorubicin induced cachexia model. We revealed an increased expression of the p53 family members and atrogenes in a correlated manner on both models and a transcriptional activation of Trim63 by p53, p63 and p73. In addition, we tested whether compounds of tocopherol harboring antioxidant activity might reduce muscle atrophy. We showed that this compound counteracts the induction of the Notch pathway, important to muscle development and regeneration.