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THÈSE

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Les facteurs de transcription de la famille p53 dans l'atrophie musculaire

Implications dans la Sclérose Latérale Amyotrophique et la cachéxie

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ABBREVIATIONS

AICAR	5-aminoimidazole-4-carboxamide-1-β-D-rib ofuranoside
ActRIIB	Activin receptor type-2B
ADP	Adenosine diphosphate
AEC	Ankyloblepharon-ectodermal defects-cleft lip/palate
AIF	Apoptosis-inducing factor
ALS	Amyotrophic lateral sclerosis
AMPK	AMP-activated protein kinase
ANG	Angiogenin
APAF1	Apoptotic protease activating factor 1
APE	AP endonuclease
ATM	Ataxia-telangiectasia mutated kinase
ATP	Adenosine triphosphate
Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma 2
bHLH	Basic helix-loop-helix
Bid	Bcl2 interacting protein
BiP	Binding immunoglobulin protein
BMP	Bone morphogenetic protein
C/EBP	CCAAT/enhancer binding protein
CDK	Cyclin-dependent kinase
c-IAP1/2	Cellular inhibitor of apoptosis protein-1/2
c-Myc	Cellular homolog of the myelocytomatosis viral oncogene
COP1	Constitutively photomorphogenic 1
CR	Cajal-Retzius
DBD	DNA-binding domain
Dlx5/6	Distal-less homeobox 5/6
DMD	Duchenne muscular dystrophy
DR5	Death receptor 5
EAAT2	Excitatory amino-acid transporter 2
EEC	Ectrodactyly Ectodermal dysplasia-Clefting syndrome
eIF3F	Eukaryotic translation initiation factor 3 subunit F
EMG	Electromyography
ER	Endoplasmic reticulum
ESCs	Embryonic stem cells
EVA1	Epithelial V-Like Antigen 1
FADH2	Flavin adenine dinucleotide reduced
FALS	Familial amyotrophic lateral sclerosis
FASN	Fatty acid synthase

FCCP	Trifluorocarbonylcyanide Phenylhydrazone
FGFR2b	Fibroblast growth factor receptor 2b
FGFs	Fibroblast Growth Factor Superfamily
FKHR	Forkhead transcription factor Foxo1
FoxO	Forkhead box class O
FUS	Fused in Sarcoma
G6P	Glucose 6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GADD45	Growth Arrest and DNA Damage-inducible 45
GAMT	Guanidinoacetate N-methyltransferase
GLS2	Glutaminase
GLUT	Glucose transporter
HGF	Hepatocyte growth factor
HOXC4	Homeobox C4
Hsp-70	70 kilodalton heat shock protein
IFNγ	Interferon gamma
IGF-1	Insulin-like growth factor 1
IKK	IκB kinase
IL-6	Interleukin 6
iNOS	Inducible nictric oxide synthase
Islet1	Insulin gene enhancer protein 1
KRT14	Keratin 14
LGMD	Limb girdle muscular dystrophy
LKB1	Liver kinase B1
LLN	N-acetyl-leucyl-leucyl-norleucinal
MAFbx	Muscle Atrophy F-box, Atrogin-1
MCK	Muscle-specific form of creatine kinase
MDM2	Mouse double minute 2 homolog
Mlc2v	Myosin light chain 2v
MPZL2	Myelin protein zero-like 2
MRF	Myogenic transcription factors
MRI	Magnetic resonance imaging
mTOR	Mammalian target of rapamycin
MuRF1	Muscle Ring Finger protein 1
myHC	Myosin heavy chain
NADH	Nicotinamide adenine dinucleotide dehydrogenase
NASA	National Aeronautics and Space Administration
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMJ	Neuro-muscular junction
OD	Oligomerization domain
PARP	Poly [ADP-ribose] polymerase

PAX	Paired-box transcription factors
Peg3	Paternally-Expressed Gene 3 Protein, PW1
PFK1	Phosphofructokinase 1
PI3k	Phosphatidyl-inositol 3-kinase
PIF	Proteolysis-inducing factors
Plk2	Serine/threonine-protein kinase
PML	Promyelocytic leukemia
PPP	Pentose phosphate pathway
pRb	Retinoblastoma protein
PUMA	p53 upregulated modulator of apoptosis
RMS	Rhabdomyosarcoma
ROS	Reactive oxygen species
SCD1	Stearoyl-CoA desaturase-1
SCG	Superior cervical ganglion
SCO2	Cytochrome oxidase deficient homolog 2
SHFM	Split-hand/foot-malformation
SMA	Spinal muscular atrophy
SMA	Sterile alpha motif
SOD1	Cytosolic Cu/Zn superoxide dismutase
Sox-17	Sry-related HMG box
SR	Sarcoplasmic reticulum
SUFU	Suppressor Of Fused Homolog
TARDBP	TAR DNA-binding protein 43 gene
Tbx5	T-box transcription factor 5
TDP-43	TAR DNA-binding protein 43
TGFβ	Transforming growth factor beta
TIGAR	TP53-inducible glycolysis and apoptosis regulator
TNFα	Tumor necrosis factor alpha
TRAF1/2	TNF receptor-associated factor 1/2
TSC2	Tuberous sclerosis 2
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VCP	Valosin containing protein
XPC/XPE	Xeroderma pigmentosum, complementation group C/E

I - BASIC MUSCLE PHYSIOLOGY

Muscles... They are really important, aren't they? After all, they allow us to move, breathe and communicate. But how do they work? What are the mechanisms involved when they degenerate? And why should the p53 family be involved? In this thesis, I will give insights into current knowledge and integrate my own research. But let's begin with a brief introduction on muscle function.

I - 1. Muscle function & anatomy

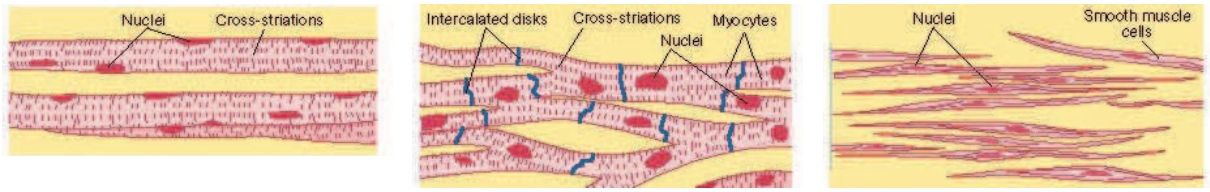
Muscles are one of the four major types of tissue in the body, alongside connective, neuronal and epithelial tissue. Motion and force are two physiological phenomena that are generated by contractile proteins in individual cells. As it will be introduced below, there are three different types of muscles that serve different purposes and thus having different macro- and microstructures (Figure1):

Skeletal muscles make up to 40% of body weight *(Zhang et al., 2007)* and enable movement as well as stabilisation of the body's posture. Additionally, they stabilize joints and can produce heat when needed. They can vary enormously in size - between just few millimetres (*stapedius,...*) to dozens of centimetres (*gluteus maximus*, *latissimus dorsi*,...) and strength. A large proportion of muscles are controlled voluntarily. Skeletal muscles are striated, meaning they have a parallel microstructure or organisation of fibers (Figure2). They attach to the bones by tendons *(van Mameren and Drukker, 1979)*. Skeletal muscle fibres are constituted by parallel syncytii of myocytes, containing up to 500 nuclei, depending on muscle size and required strength. Their contraction can vary from slow to very fast, depending on their type (See *Ch.I – 1.3. Different types of muscle fibres)*.

Cardiac muscle is a variant of striated muscle and is a major constituent of the heart, forming the myocardium. This tissue is constituted by mono-nucleated myocardiocytes that branch together to form a lattice. Their coordinated contraction enables the blood to be pumped through the aorta and pulmonary arteries. As it has to work permanently,

the cardiac muscle is controlled involuntary and is very resistant to fatigue. To achieve this endurance, the major type of myosin fibres are from type II (See *Ch.I – 1.3. Different types of muscle fibres).* Contrary to popular belief, cardiac muscle is able to regenerate, as it is estimated that young adults renew about 1% of cardiomyocytes per year *(Bergmann et al., 2009)*.

A



Skeletal muscle | Cardiac muscle | Smooth muscle

B

Property	Skeletal muscle	Cardiac Muscle	Smooth Muscle
Fiber dimensions	100 mm * <30 cm	10-20 mm * 50-100 mm	5-10 mm * 30-200 mm
Nuclei	Multiple, near sarcolemma	Generally single, centrally located	Single, centrally located
Filament organization	In sarcomere along myofibrils	In sarcomere along myofibrils	Scattered throughout sarcoplasm
Sarcoplasmic reticulum (SR)	Terminal cisternae in triads at zone of overlap	SR tubules contact T tubules at Z lines	Dispersed throughout sarcoplasm, no T tubules
Control mechanism	Neural, at single neuromuscular junction	Automaticity (pacemaker cells) neural or hormonal control	Automaticity (pacesetter cells)
Ca^{2+} source	Release from SR	Extracellular fluid and release from SR	Extracellular fluid
Contraction	Rapid onset; may be tetanized; rapid fatigue	Slower onset; cannot be tetanised; resistant to fatigue	Slow onset; may be tetanized; resistant to fatigue
Energy source	Aerobic metabolism at moderate levels of activity; glycolysis (anaerobic) during peak activity	Aerobic metabolism, usually lipid or carbohydrate substrates	Primarily aerobic metabolism

Figure 1 | **Comparison of muscle types**
A. Three types of muscle exist in the human body. Skeletal muscle is made of long fused muscle cells, cardiac muscle forms branched arrays of individual cells and smooth muscle is made of individual cells disposed in different layers to enable contraction in 2 dimensions.
B. Further differences in structure and function of the 3 different muscle types.

Smooth muscle is, like cardiac muscle, composed of mono-nuclear contractive cells with primarily type II myosin chains. Unlike skeletal muscle, the contraction of smooth muscles is slow and involuntary, because of their assigned tasks. Those muscles are

responsible for the contraction of blood and lymphatic vessels, the digestive tract, the bladder, the respiratory tract, the reproductive tract and the iris. Being wrapped around tubular structures like the intestine or the blood vessels, smooth muscles allow for peristaltic reduction of diameter, pushing forward to bolus or the blood.
As it is more relevant to my studies, I will focus on skeletal muscle, unless stated otherwise.

I - 1.1. Structure & contractility

Skeletal muscles basically work by creating tension between bones. They are attached to the bones by tendons, a solid fibrous tissue, mostly made out of collagen.
The smallest structural unit in a muscle is a "block" of myofilaments, a concatenation of thick myosin proteins, thin actin filaments and huge titin and nebulin proteins providing elasticity and protecting myofilament structure. The latter 3 proteins are attached at the Z-disc, while myosin links two actin/titin/nebulin together (Figure 2A). All filaments are grouped in an ordered and parallel manner, forming sarcomeres *(Hopkins, 2006; Luther, 2009)*. An array of many myofilaments forms a myofibril of which there are several per muscle fiber or cell (a syncythium with up to 500 nuclei). Myofilaments bathe in the sarcoplasm, containing mitochondria, the nuclei, and a muscle-specific variant of the endoplasmic reticulum, called sarcoplasmic reticulum. Dozens of myofibers (or myocytes), with muscle stem cells lodged in between them, form a superstructure called fascicle, limited by the perimysium (Figure 2B). Finally, fascicles are grouped and form the muscle "body" *(Purslow, 2010)*.

Motor neurons and contraction

Muscle contraction is regulated by neuronal influx. Smooth and cardiac muscles are stimulated by the autonomous nervous system via the para-sympathicus nerve, while skeletal muscles are stimulated by the central nervous system via dedicated motor neurons. Their nerve cells are located in the brain, the brain stem and the spinal cord and convey the action potentials for voluntary movements. The motor neurons in the brain are called upper motor neurons and pass the "message" on to the lower motor neurons in the brain stem and the spinal cord.

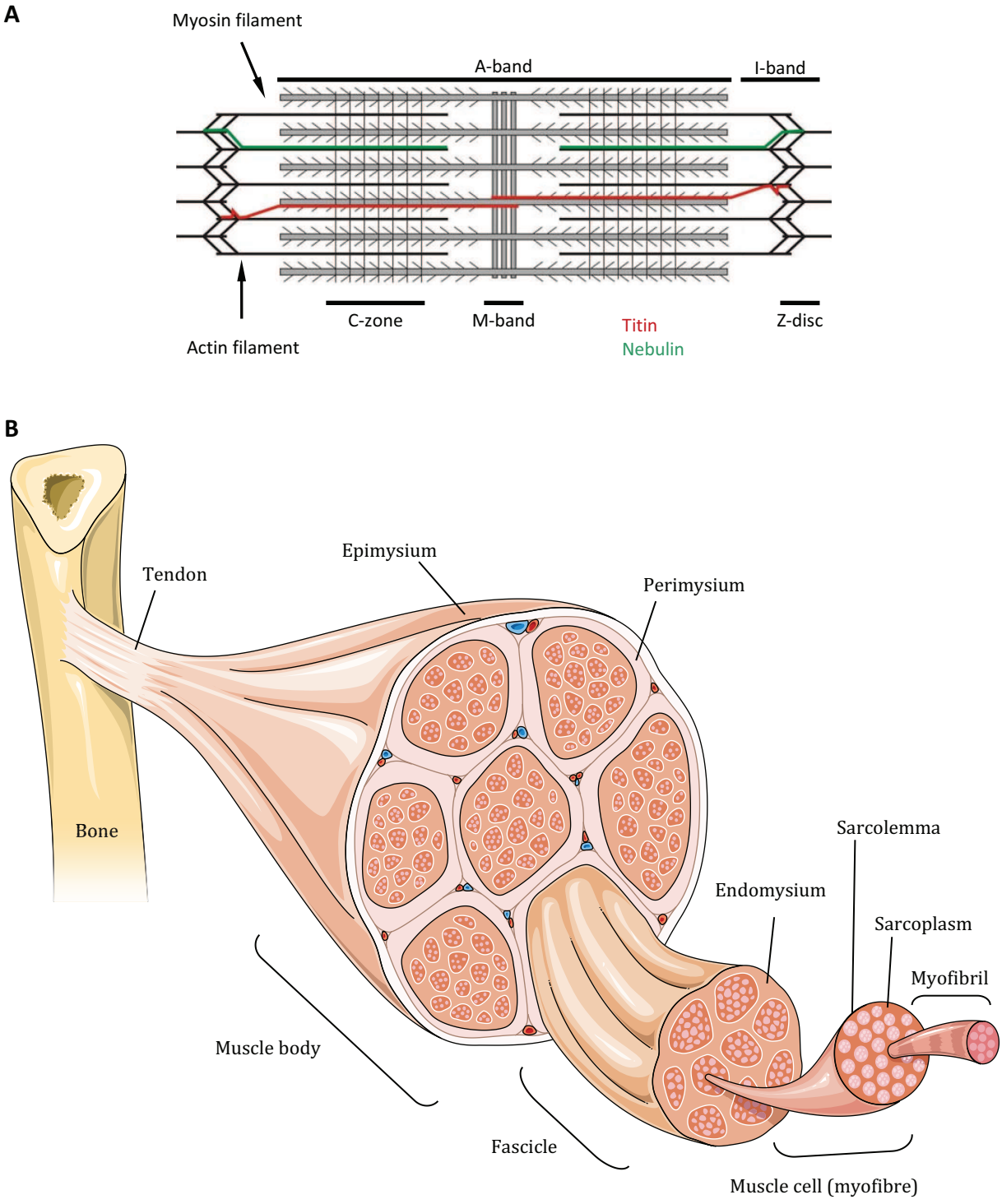
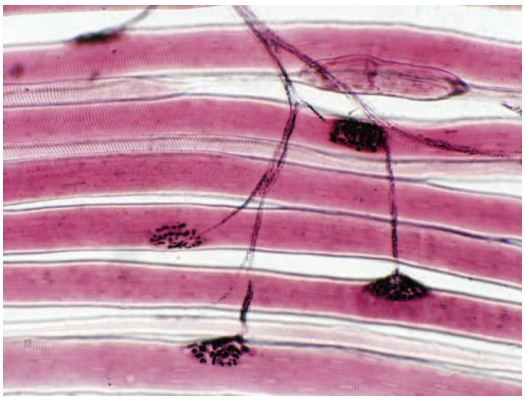


Figure 2 | **Structure of skeletal muscle**
(A) Structure of a muscle sarcomere. Only the main components are shown. Below the schematic diagram, an electron micrograph of a longitudinal muscle section, measuring approximately 2µm between Z-discs. Modified from *Luther, 2009* **(B)** Myofibres contain many myofibrils, their membrane is called sarcolemma. Muscle fibers assemble into fascicles surrounded by connective tissue. The whole muscle is composed by multiples fascicles. Modified from *Servier Medical Art*.

Muscles fibers attach to motor neurons individually or in groups, creating a structure called neuro-muscular junction (NMJ). Upon innervation (the action potential reaches the synapse), motor neurons release acetylcholine, which binds to postsynaptic receptors on the sarcolemma, the cell membrane of myofibres. This opens ion channels, leading to depolarization of the membrane (influx of sodium, efflux of potassium), triggering a muscular action potential that propagates by opening voltage-dependent sodium channels (Figure 3A). In turn, this leads to calcium release from the sarcoplasmic reticulum, enabling actin and myosin to interact and slide between each other, consuming ATP to shorten the myofibril *(Takamori, 2012; Hopkins, 2006)*.

A



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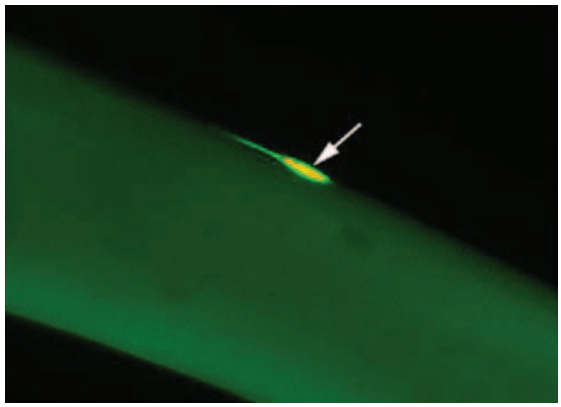

Figure 3 | **Myofibres from upclose**
(A) There is at least one neuromuscular junction per muscle fiber. From *Caceci's Group* **(B)** Staining of a Pax7-positive satellite cell (See *Ch. I – 1.2.*).. From *Relaix and Zammit, 2012*.

It is interesting to note that different types of myosin exist and that they help determining different types of fibres (See *Ch.I – 1.4*).

I - 1.2. Development of skeletal muscles and their maintenance

Embryonic development

Skeletal muscles are derived from the somitic mesoderm. In short, during embryogenesis, the mesoderm is divided into somites, which are aligned along the anterior-posterior axis of the nascent embryo. They further divide into sclero-, derma-, synde- and the myotome. Cells from the myotome express paired-box transcription factors (Pax3 and Pax7) and later migrate to different parts of the embryo where they express myogenic regulatory factors, proliferate and differentiate by fusion with each other to form muscle fibres *(Le Grand and Rudnicki, 2007; Mok and Sweetman, 2010)*.

In adult muscles

The actual muscular stem cells in developed muscles are called satellite cells. They are responsible for the formidable ability of growth and regeneration in response to various stresses (for examples, see Ch. *II - 1.*). Although the environment of adult muscles is profoundly different from the one during embryogenesis, regeneration and growth of muscle tissues resembles in many ways the steps observed during development.

Satellite cells are scattered around muscles in niches (between the sarcolemma and the basal lamina, in proximity to capillaries – Figure 3B) and remain quiescent in G_0 phase until activated. During this phase, satellite cells express the transcription factors Pax3 and Pax7 necessary for the regulation of myogenic commitment, but at the same time repress the expression of genes involved in differentiation. Pax7 in particular is widely used to identify satellite cells via immunohistochemisty *(Relaix and Zammit, 2012)*.

When needed (i.e. after muscle damage, exercise or disease), activation of the Notch pathway, followed by Wnt signalling, leads to expansion of the muscle progenitor pool of cells *(Brack et al., 2008)*. HGF, FGFs, TGFβ, myostatin and IGF-1 have been shown to contribute to this process *(Le Grand and Rudnicki, 2007)*. More specifically, a subset of about 10% of satellite cells keeps its "stemness" *(Bentzinger et al., 2012)*, while the majority of cells further proliferate (multiple rounds of mitosis) under control of basic helix-loop-helix (bHLH) myogenic regulatory factors (Myf5, MyoD). Through their basic domain, those transcription factors bind to DNA, while the HLH motif promotes dimerization with proteins that bind to promoters of muscle specific genes at so-called

"E-boxes" *(Rudnicki and Jaenisch, 1995)*. Some of these factors are so important and powerful that they are able to convince non-muscle cells like fibroblasts to exhibit myoblastic traits *(Braun et al., 1989)*. Interestingly, different knock-out mice have shown that Myf5 and MyoD can compensate each other's absence. However, absence of both factors failed to develop skeletal muscles, mice pups were thus born immobile and died soon after birth *(Rudnicki et al., 1993)*. By expressing Myf5 and MyoD, cells commit to the muscular lineage. Satellite cells are activated and start proliferating. They are now called myoblasts. Once the cells are in sufficient numbers, the expression of the "early" bHLH proteins leads to expression of more "terminal" bHLH myogenic transcription factors, called myogenin and Mrf6/MRF4. They trigger differentiation and the expression of myofibre specific genes. Cells exit the cell cycle and, by fusing with existing muscle fibres, can "repair" damaged fibres. Alternatively, the activated cells can fuse with each other, just like during development, to form new muscle fibres *(Bentzinger et al., 2012)*.

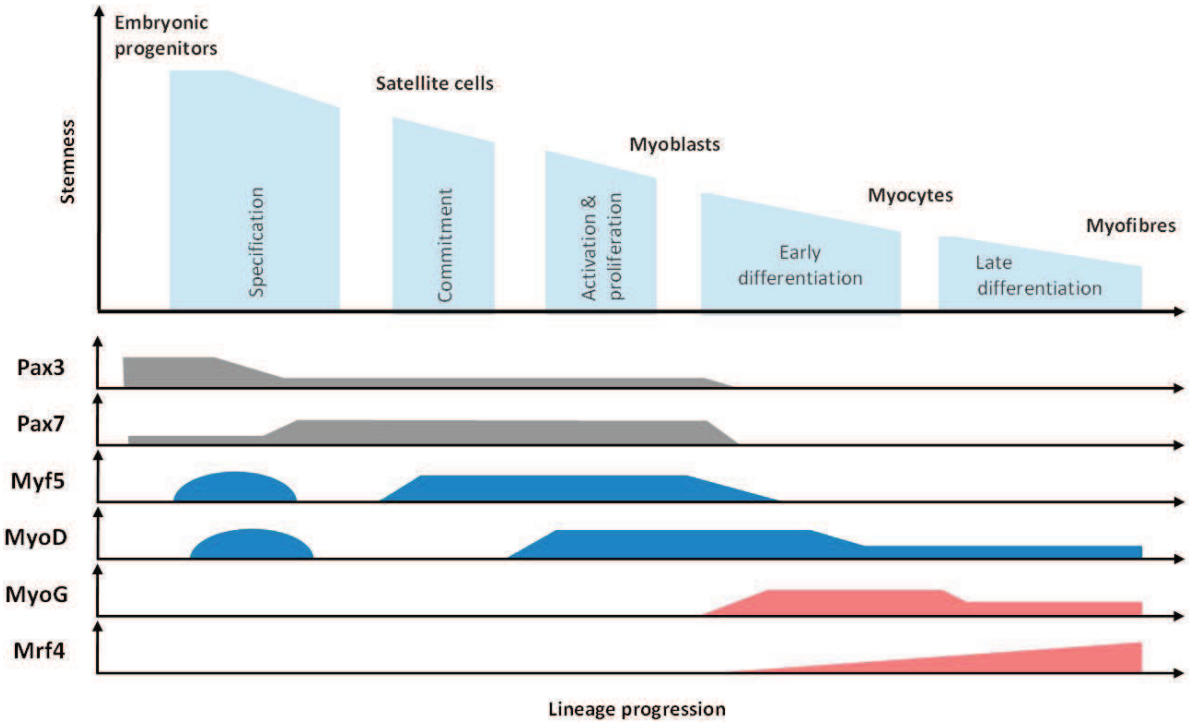

Figure 4 | **Muscle regulatory factors during myogenesis**
Timecourse of the expression of transcription factors that regulate proliferation and differentiation of muscle progenitor cells. Adapted from *Bentzinger et al., 2012*.

It seems likely that adult and growing muscles express hypertrophic and pro-survival proteins, such as the PI3 kinase, Akt (please see section I – 2.2.) and the cell cycle inhibitor p21 in order to grow or maintain their mass, as shown *in vitro* in myoblasts *(Ostrovsky and Bengal, 2003; Fujio et al., 1999)*.

I - 1.3. Energy production and storage

In order to get the energy needed by the muscles so desperately to contract, muscles primarily use the oxidation of carbohydrates and fat. This provides Adenosine Triphosphate (ATP) which is necessary for movement. Additionally, anaerobic metabolism can also be used, especially in fast twitch fibres. Like the liver, muscles also store glycogen.

Immediate access to ATP and glycolysis

ATP is a form of energy storage and transport used throughout the organism. By acting as a coenzyme, it is used in virtually all cellular processes that need some form of "energy", such as biosynthesis of DNA, RNA, fatty acids, proteins or the generation of movement. In muscles, ATP is also needed for the movement of the myosin heads. Unfortunately, the muscle stores are not sufficient for prolonged use of ATP. For this reason, muscles produce an energetic precursor, phospho-creatine, which can donate a phosphate group to ADP in order to produce ATP, thus constituting a second source of energy that can quickly be accessed. However, even in this situation, phospho-creatine quickly depletes and lasts less than 10 seconds under heavy load. This is where the carbohydrate glycogen, a form of branched and dense form of glucose, comes into play. When needed, it is catabolized into glucose monomers (glycogenolysis) that is then used for glycolysis, releasing ATP during the synthesis of pyruvate. A caveat of this method is the production of metabolites like lactic acid in anaerobic conditions (after glycolysis), thus tiring and aching the muscle *(Bonora et al., 2012)*.

Oxidative phosphorylation

Glycolysis does not require oxygen, it is immediately "available" but is not sufficient for long-term exercise or heavy workload. Instead, oxidative phosphorylation is much more

effective (about 20 times more ATP per glucose molecule), but only when oxygen is available. Briefly, this process uses protein complexes located in the mitochondrial inner membrane to transfer electrons from donors to acceptors, releasing energy to produce ATP.

The base of the Krebs, or citric acid cycle, remains glycolysis, which generates pyruvate. Coenzyme A and oxidation then produce Acetyl-CoA, a limiting metabolite for the Krebs cycle. Note that Acetyl-CoA is also produced by fatty acid oxidation and in extreme cases amino acid catabolism. The Krebs cycle generates NADH and $FADH_2$, which are oxidized by the mitochondrial respiration complexes I and II, respectively. The complexes III and IV then process the resulting products. During this process, large amounts of protons are released, building up in the intermembrane space of mitochondria and creating a pH gradient. The ATP synthase uses this gradient to phosphorylate ADP into ATP *(Bonora et al., 2012)*.

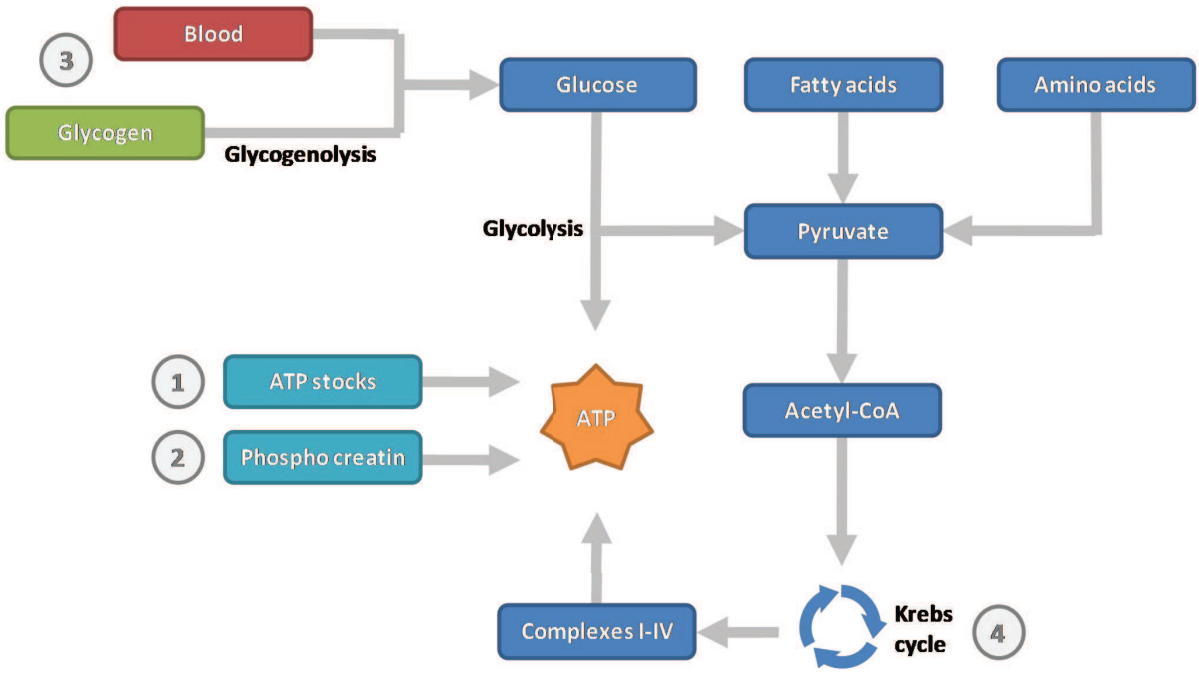

Figure 5 | ***Sources for ATP in the muscle***
When ATP stocks are depleted (1), phospho-creatin (2) provides a phosphate to generate ATP. For longer use, Glycogenolysis (3) provides glucose for glycolysis, also generating ATP. Under heavy load or sustained exercise, oxidative phosphorylation using metabolites from the Krebs cycle (4) provides the most ATP.

Mitochondrial defects that can arise in diseases like *Amyotrophic Lateral Sclerosis* (see chapter I - 3) inhibit this metabolic pathway, leading to energetic deficits in muscles. A pharmacological compound, Trifluorocarbonylcyanide Phenylhydrazone (FCCP), permeabilizes the inner mitochondrial membrane, thus destroying the proton gradient and uncoupling the electron transport from oxidative phosphorylation *(Kurvilla et al.,2003)*.

I - 1.4. The different types of muscle fibres

The mentioned succession of different sources for ATP (Figure 5) is somewhat idealized. In skeletal muscles, one can distinguish between two main types of muscle fibres: Type I and II. This classification is based on the subtype of myosin and importantly, the main source of energy. Simply put, type I fibres use "slow" myosin and an aerobic, oxidative metabolism (Figure 5[4]). In order to do so, they are vastly vascularised and contain higher numbers of mitochondria. Thus, they can stay active for long times and are resistant to fatigue. They are required for postural muscles and activities that base on endurance, such as running.

On the other hand, type II fibres use "fast" myosin and rely on anaerobic processing of phospho-creatine and glycogen (Figure 5[1, 2, 3]) to provide a quick but short-lasting response. They are less vascularised, contain less mitochondria, but contract faster. As such, those fibres are mainly used for activities like sprinting. It is important to note that type II fibres are further subdivided into class A (with an intermediate behaviour) and class B which are the fastest but less enduring fibres *(Scott et al., 2001)*.

Muscles are rarely composed just by one muscle fibre type. Instead, depending on use, the two main types are present in different proportions. The *gastrocnemius* muscle (in the posterior compartment of the lower leg in humans or hind limb in mice) for example, is involved in actions such as standing, as well as running and jumping. In mice and rats, it has been shown that only 5-10% of the muscle fibres are of type I. However, the *soleus* muscle, which is located just beneath the *gastrocnemius*, contains 40-55% of type I fibres *(Augusto et al., 2004, Cornachione et al., 2011)*. In humans, this number can rise up to 100% *(Gollnick et al., 1974, Edgerton et al., 1975)*, a beautiful example of how different morphologies and requirements lead to differences in muscle type. In humans,

this muscle exerts constant pulling, while standing to maintain body posture, hence the need for many fatigue resistant fibres.

II - INTRODUCTION TO MUSCULAR ATROPHY

In the last chapter, we have seen how muscles work and develop. During development and through exercise and use, muscles fibres increase in number as well as in diameter and therefore strength. However, in certain patho-physiological settings, muscles can waste away. A simple decrease of pure muscle mass is defined as "muscular atrophy". As developed muscle strength is related to muscle size, depending on the severity or progress, muscular atrophy inevitably leads to weakness and fatigue. However, there are different macrostructural and molecular phenotypes that define muscular atrophy, depending on the context. Concomitantly, underlying mechanisms can also be different. Taken together, all these results in a disbalance between muscle protein synthesis rate (for maintenance or renewal) and degradation (for maintenance also or in pathologic situations) which can be considered as the physical cause for atrophy *(Bonaldo and Sandri 2013)*.

II - 1. Disease independent muscular atrophy

I would like to introduce and define a few important pathological and representative settings that lead *in fine* to similar symptoms, but vary greatly in time, place, speed, aggressiveness and ability to recover (physiologically or pharmacologically). Namely, there are muscular atrophy directly due to disuse, ageing, starving and neuronal injuries, and muscular atrophy resulting from diseases such as cancer, muscular dystrophies or neuromuscular diseases.

II - 1.1. Muscle disuse

This cause of atrophy is usually the least dangerous or severe in comparison to other types of atrophy and has been widely studied. Almost everybody is aware that sporting a broken limb or being condemned to prolonged bed rest due to disease leads to reduced muscle size and weakness. After several days to weeks in these conditions, the unused muscles atrophy even in individuals with perfect health *(Ogawa et al., 2006; Brocca et al., 2012)*. This physiological effect is an adaptation to the decrease in muscle

activity. By decreasing muscle mass, the body does not spend excess energy in maintaining an under-used tissue. It basically reflects the disbalance of protein synthesis and degradation. In line with the first observation, that healthy people can suffer atrophy from disuse, there is also the notion of restoration. We have all noticed that humans can easily recover in a few weeks from such an episode. This phenomenon is also observed in rodents (Figure 6). Indeed, it has been shown that immobilized muscles lose mass by means of a reduction of individual muscle fibre diameter. The amount of fibres remains constant, which is more easily "repairable" later on *(Narici and Maffulli, 2010; Romanick et al., 2013)*. Interestingly, a switch in fibre type is also characteristic of a long-term disuse-setting. It has been previously shown that muscle fibres of type I (slow and oxidative) tend to switch to type II (fast and glycolytic) fibres *(Zhang et al., 2007)*. At the same time, type I fibres start atrophy earlier than type II fibres in disused muscles *(Ohira et al., 2002; Brocca et al., 2012)*.

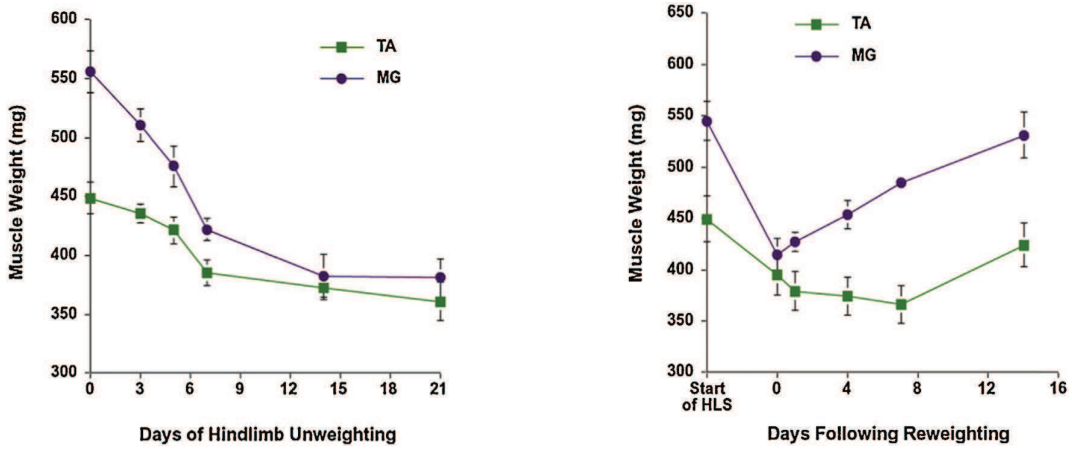

Figure 6 | **Effects of hind limb unloading/reloading on rat muscle mass**
Evolution of tibialis anterior (TA) and medial gastrocnemius (MG) muscle weight after hind limb unloading (left panel) and following reweighting (right panel), depicting the reversibility of disuse atrophy. From *Bodine, 2013*

Generally speaking, in conditions of "simple" disuse (i.e. without neuronal damage) in rodents, muscle atrophy starts quickly, but slows down after approximately 1 week, asymptotically approaching a plateau, until external stimuli lead to muscle regeneration: reloading of the muscle increases anabolic signals and fibres start to gain mass again *(Bodine, 2013)* (Figure 6).

In a pioneering and fascinating experiment, rats were sent into space for 2 weeks during a NASA space shuttle mission. Due to the absence of gravity, the animals didn't even have to support their own weight, let alone exercise. They experienced the aforementioned symptoms and structural changes (reduced muscle mass and fibre diameter) of muscular atrophy *(Ohira et al., 1992; Ohira et al., 2002)* (Figure 7A).

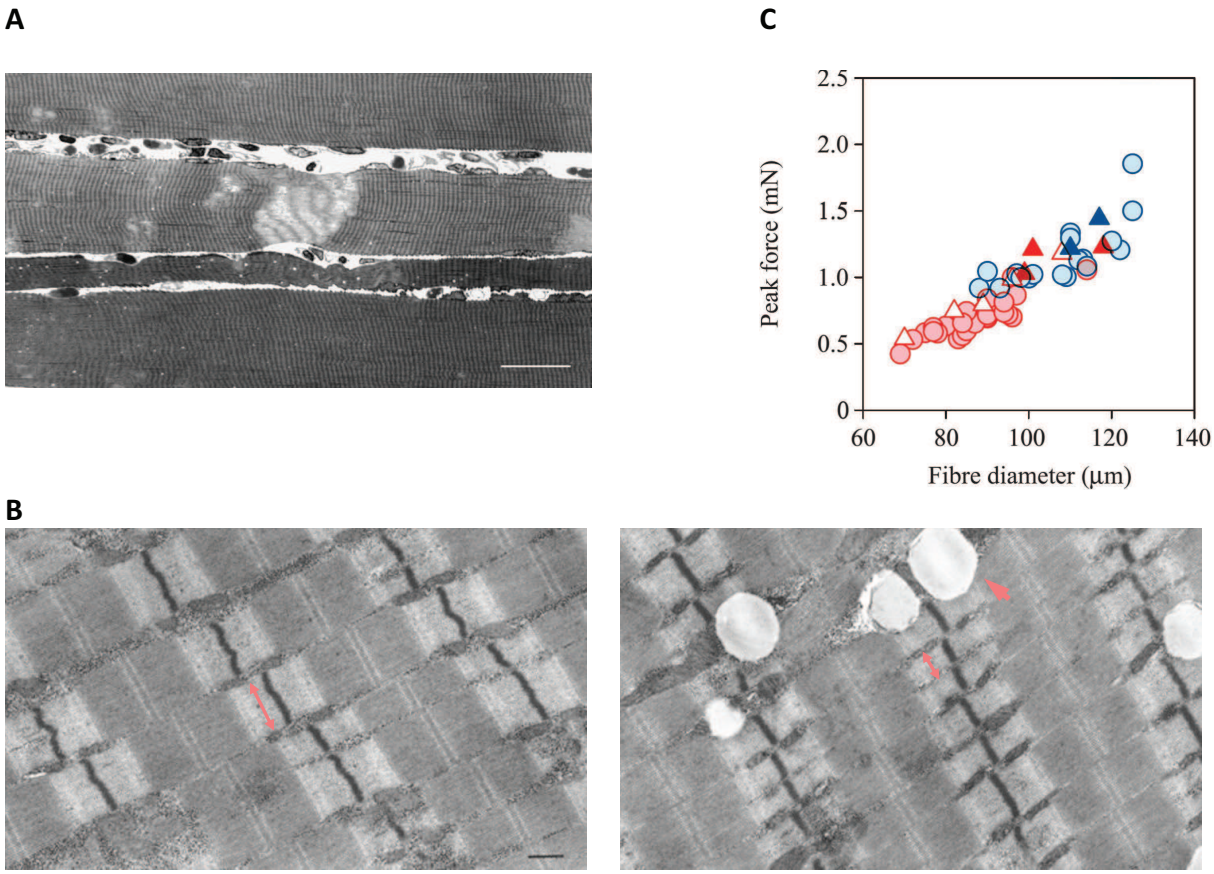

Figure 7 | **Effects of microgravity on muscles**
(A) Longitudinal section of a rat muscle fibre, after 2 weeks in space. The central muscle fibre exhibits myofilament disruption. (Scale bar: 42µm). From *Fitts et al., 2001*. **(B)** Microstructural effects of muscle unloading during space travel. Myofibril size (also reflected by the Z-bands) is reduced post-flight (right) vs. pre-flight (left). Furthermore, lipids (arrow) have infiltrated the muscle. (Scale bar: 1µm) From *Widrick et al. 1999*. **(C)** Representation of muscle fibre diameter and peak force before (blue) and after (red) space travel during 16 days. Modified from *Widrick et al. 1999*. This graph also nicely shows the reciprocity between muscle fibre size and developed strength.

Since the first experiments, more russian and american space flights were used to assess structural changes in muscles from rats, but also humans, actual cosmo- or astronauts. Most analyses were performed by two groups - Edgerton's and Fitts' lab. For

example, as reviewed by one group *(Fitts et al., 2001),* after one week in space, some human muscles lost as much as 37% in mass or strength (Figure 7 B, C). In a much more "down to earth" approach, animals were forced to disuse certain muscles by using approaches such as hind limb suspension or joint immobilisation. As you will see in the next chapter, muscular atrophy induced by disuse is based on several molecular alterations. For example, disuse has been associated with increased activity of the Ubiquitin proteasome pathway. *(Bodine et al., 2001a)*.

II - 1.2. The atrogenes - a muscle's worst nightmare

Before continuing the presentation of the different types of atrophy, I would like to pause and ask the following questions: How do muscles actually waste away? What are the molecular pathways? Who is the trigger-happy traitor inside the muscle?

As a first clue, an increase in activity of the ubiquitin-proteasome pathway has been shown in rats and mice during atrophy in diabetes, due to fasting and almost all conditions of atrophy *(Mitch and Goldberg, 1996; Lecker et al., 1999; Bodine et al., 2001a; Jagoe et al., 2002)*. This pathway takes part in regulating the protein content of a cell: the so-called E3 ubiquitin-ligases catalyse the addition of small ubiquitin proteins to lysine residues of specific proteins. Subsequently, this label is recognised by the 26S proteasome, which breaks down the labelled proteins *(Lecker et al., 2006)*. During atrophy, this degradation seems to be central: interestingly, the use of proteasome inhibitors such as LLN and MG-132 protects muscles from atrophy *(Tawa et al., 1997; Kadlcikova et al., 2004; Caron et al., 2011; Jamart et al., 2011)*.

In 2001, Glass' group identified two muscle-specific effectors of muscular atrophy termed MuRF1 (Muscle Ring Finger protein 1) and MAFbx (Muscle Atrophy F-box) *(Bodine et al., 2001a)*. Another group also identified MAFbx as an atrophy effector earlier the same year, but gave it another name – Atrogin-1 *(Gomes et al., 2001)*. By comparing the sequences, both presented similarities with known proteins (the presence of a RING-domain in MuRF1 and an F-box domain in MAFbx/Atrogin-1), prompting both groups to investigate further whether the identified proteins were E3 ubiquitin ligases. Additionally, several protein-protein interaction domains were also present (Figure 8). Glass' group was able to show *in vitro* that recombinant MuRF1

increased ubiquitination. However, both groups were initially unable to prove that Atrogin-1/MAFbx was an E3 ubiquitin ligase. It actually is part of a 3-protein "SCF" complex containing Skp1, Cullin1 and Atrogin-1/MAFbx. Both proteins were thus considered *bona fide* ubiquitin ligases *(Bodine et al., 2001a; Gomes et al., 2001)*. In order to credit both groups for their discoveries, our group decided to use the terms MuRF1 and Atrogin-1. Both proteins are commonly called "atrogenes". However, some groups use this term to include other proteins linked to atrophy, such as FoxO (see below).

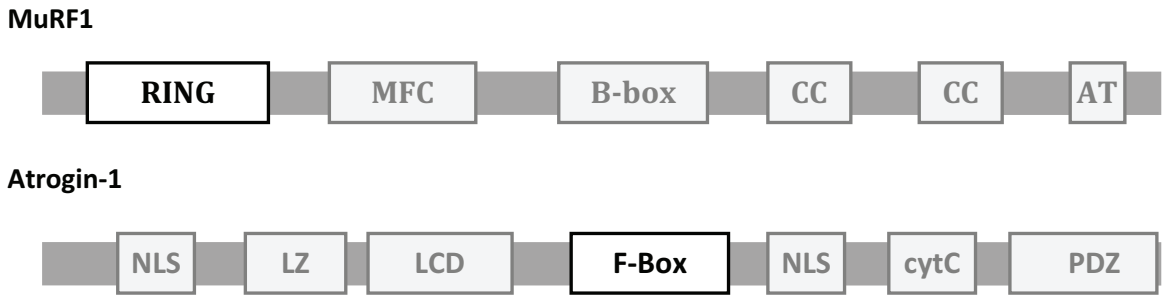

Figure 8 | **Schematic diagram of atrogene protein domains**
MuRF1 contains the RING domain, a conserved MuRF-family domain (MFC), a B-box, coiled-coiled (CC) domains and an acidic tail (AT). Total length is 353 amino acids
Atrogin-1 contains two potential nuclear localisation signals (NLS), a leucine zipper (LZ) a leucine-charged residue-rich domain (LCD), the F-box domain, a Cytochrome C family heme-binding site (cytC) and a PDZ domain. Total length is 355 amino acids. Modified from *Foletta et al., 2011*

In simple terms, MuRF1 and Atrogin-1 contribute to muscle atrophy by perturbing the balance of protein synthesis and breakdown by labelling specific proteins for degradation.

Role of the atrogenes

Not all physiological functions of MuRF1 and Atrogin-1 are known but many studies showed that both proteins are expressed specifically in muscles under a variety of conditions (i.e. disuse & microgravity, denervation, fasting, cancer, diabetes, kidney failure, glucocorticoid treatment... *Bodine et al., 2001; Lecker et al., 2004; Glass, 2005, Bonaldo and Sandri 2013)*.

In the article that first links both ubiquitin ligases with muscle wasting, Glass' group induced atrophy in mouse muscles by several techniques, mimicking different pathological settings. The authors forced muscle disuse by immobilizing a limb or by

axotomy of the sciatic nerve, effectively inhibiting muscle innervation and rapidly inducing atrophy. Finally, the group also induced atrophy by treating mice with glucocorticoids, which also induce muscular atrophy. In those contexts, the atrogenes rapidly accumulate (3 days) in muscles at the mRNA- and protein-level *(Bodine et al. 2001a; Lecker et al., 2004)*. The importance of those genes in atrophy has been underlined by knock-out mice. Individually deleting those genes protects from muscular atrophy. For example, after denervation of the tibialis anterior muscle, MuRF1 $^{-/-}$ or Atrogin-1 $^{-/-}$ muscles exhibit between 40 and 50% less atrophy than $^{+/+}$ muscles *(Bodine et al., 2001a)*.

Since more than a decade after their discovery, both enzymes remain the sole known widely recognised and studied effectors specifically induced during atrophy in order to reduce muscle size. In the meantime however, knowledge has grown about their targets and their regulation, but it has become increasingly difficult to present a unique and general function of these enzymes. In different settings of atrophy, they behave slightly differently. Furthermore, functional aspects have been studied in some patho-physiological settings, but not in others. Some findings are from mice, others from rats but were not confirmed in humans. Finally, many groups chose to study the implication of only one of the atrogenes, leaving uncertainty about the other's role in a given setting. As we all know, despite all similarities, important differences exist between rodents and humans. Not only are mice much smaller than us, they also scratch themselves with the hind limbs! On a molecular level, the difference is beautifully highlighted by differences in MuRF1 and Atrogin-1 expression during fasting. When deprived of food for a few days, mice show an increased expression of these enzymes, while humans do not *(Sandri et al., 2004; Larsen et al., 2006)*. This suggests that the fundamental regulation of the atrogenes is different in the human body. Unsurprisingly though, most experiments have since been performed with rodents. As it will be mentioned, the downstream targets and pathways that are affected are not the same for both proteins (Figure 9).

Molecular targets of MuRF1

It seems as though MuRF1 is responsible for the actual physical breakdown of the muscle *(Foletta et al., 2011)*. It has been shown that it binds to and dooms following proteins of the sarcomere: the myosin heavy chain *(Fielitz et al., 2007; Clarke et al.,*

2007), the myosin light chains 1 and 2, the myosin-binding protein C *(Cohen et al., 2009)*, as well as Troponin I *(Kedar et al., 2004)* by ubiquitination and subsequent proteasome-dependent degradation. Furthermore, it has also been shown that MuRF1 potentially mediates titin activity by binding next to its kinase domain *(Centner et al., 2001)*. However, this seems not to have been investigated further. In the heart, MuRF1 has been found to (indirectly) reduce myosin-binding protein C expression at the transcriptional level *(Mearini et al., 2010)*. Those proteins are essential for muscle contraction but also for structure and volume. When broken down, muscles lose in mass and strength. Muscle Creatine-Kinase is also another ubiquitination target of MuRF1 *(Koyama et al., 2008)*. This enzyme is crucial for rapid ATP production (See *Ch.I -1.3.*).

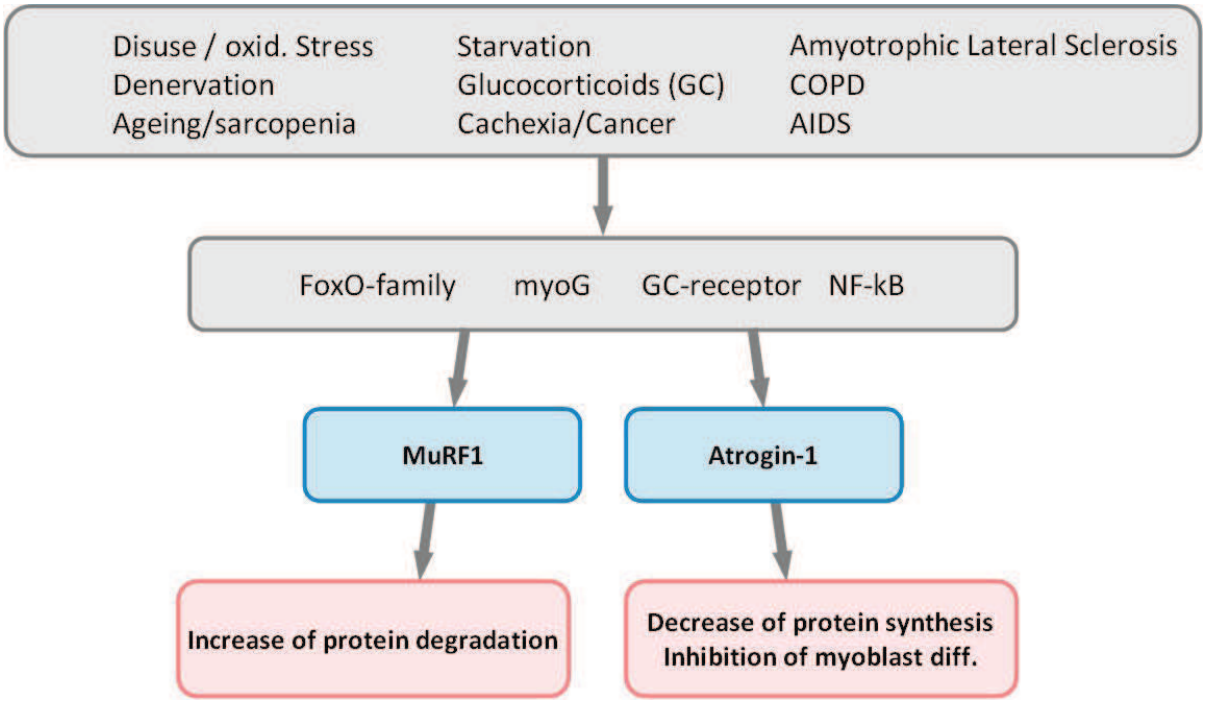

Figure 9 | **Induction and consequences of atrogene induction**
Multiple phenomenons or diseases can lead to activation of MuRF1 and Atrogin-1, leading to a disbalance in protein synthesis and inhibition of muscle repair.

Surprisingly, MuRF1 also seems to be involved in bone resorption. Inhibition of MuRF1 reduces osteoclast infiltration and reduces bone-loss during unloading-induced atrophy *(Kondo et al., 2011)*.

Molecular targets of Atrogin-1

Interestingly, Atrogin-1 seems to interact less with structural proteins and more with transcription and translation factors. It has been shown by a yeast two-hybrid assay that Atrogin-1 interacts with MyoD and eIF3f *(Lagirand-Cantaloube et al., 2008; Foletta et al., 2011)*. The importance of this interaction was confirmed by several findings: Both proteins are ubiquitinated and degraded during muscular atrophy. Protein synthesis is reduced when the elongation factor eIF3-f is lost *(Csibi et al., 2009)*, thus preventing replacement of degraded structural proteins. Additionally, when Atrogin-1 is overexpressed, the muscle regulatory factor MyoD is ubiquitinated *(Tintignac et al., 2005)*, preventing myoblast proliferation and differentiation. This means that effort of the muscle to repair itself is annihilated. However, inhibition of Atrogin-1 reversed the degradation of MyoD *(Lagirand-Cantaloube et al., 2009)*. Similar findings were described for MyoG, an important myogenic factor (Figure 4) which is another ubiquitination target of atrogin-1. Surprisingly, a layer of complexity is added as it has also been shown that myoG directly activates Atrogin-1 expression after denervation *(Moresi et al., 2010)*.

II - 1.3. Insights into MuRF1 and Atrogin-1 regulation

Analysis by several groups of the promoter region upstream of MuRF1 revealed the presence of response elements for the glucocorticoid receptor, as well as for FoxO, NF-κB and C/EBP transcription factors, which were already thought to upregulate ubiquitin and proteasome constituents *(Waddell et al., 2008)*. While glucocorticoids alone are sufficient to induce muscular atrophy, the identified potential response elements do not vary greatly when comparing their frequency in proximity to both atrogenes with other genes. However, in contrast to MuRF1 and Atrogin-1, other E3 ubiquitin ligases were not significantly induced during muscular atrophy, highlighting the central and specific role for those enzymes in this process *(Lecker et al., 2004)*.

FoxO transcription factors

Since then, the proteins of the FoxO family of transcription factors have emerged as potential transcriptional regulators in muscle atrophy. It has been shown that they are able to induce MuRF1 and Atrogin-1 expression *(Stitt et al., 2004; Sandri et al., 2004; Skurk et al., 2005)* and that transgenic mice overexpressing FoxO1 exhibited reduced

muscle mass caused by severe atrophy *(Southgate et al., 2007)*. However, it was unclear whether the FoxO transcription factors act alone when activating atrogene transcription. Bodine's group further investigated the role of the glucocorticoid receptor. Waddell et al. showed in cultured C2C12 myoblasts that synthetic glucocorticoid (dexamethasone) treatment or glucocorticoid receptor overexpression induces MuRF1 transcription. More than that, FoxO1 overexpression leads to the same effect. Both proteins physically bind to the promoter and their effect is synergistic and thus enhanced, suggesting that MuRF1 transcription is at least partially regulated by the activated glucocorticoid receptor and the FoxO family of transcription factors *(Waddell et al., 2008)*.

Similar findings for Atrogin-1 were reported by another group: The transcription factor FoxO3a directly binds the Atrogin-1 promoter and induces transcription *(Sandri et al., 2004)*. However, all findings regarding FoxO could, again, not be confirmed in humans in several settings *(Foletta et al., 2011)*.

IGF-1 / Akt signalling

A central pathway that regulates protein synthesis and breakdown is the PI3K/Akt/mTOR pathway. The PI3 kinase prohpsorylates Akt, which then promotes protein synthesis by activating the mTOR pathway. In turn, the ribosomal elongation factor eIF4E and the ribosomal protein S6 are activated, increasing the protein biosynthesis rate. The Insulin-like Growth Factor 1 (IGF1) is a circulation growth factor that is also produced by the muscle itself. Expression of IGF-1 and ensuing activation of Akt has a hypertrophic effect on muscles *(Lai et al., 2004)* and reduces protein degradation *(Rommel et al., 2001; Musaro et al., 2001).* In addition, its expression promotes regeneration or protects against atrophy following ageing (*Musaro et al., 2001)*, disuse *(Stitt et al., 2004)*, denervation *(Bodine et al., 2001b)*, or atrophy resulting from chronic heart dysfunction *(Schulze et al., 2005)*. As a matter of fact, Akt is at the crossroads of protein synthesis (via the aforementioned mTOR pathway) and protein degradation (via the FoxO family). Akt phosphorylates FoxO transcription factors, thus preventing their shuttling to the nucleus, where they should otherwise activate atrogene transcription.

In various settings of muscular atrophy, IGF-1 mRNA has been shown to be reduced, and MuRF1 and Atrogin-1 were induced *(Sacheck et al., 2004)*. During Amyotrophic

Lateral Sclerosis for example, atrophying muscles have a reduced activity of the PI3K/Akt pathway, with an associated FoxO3 induction *(Dobrowolny et al., 2011)*.

Akt activity can be inhibited through SMADs by myostatin. Through this action, myostatin is thought to contribute to satellite cell inhibition, thus preventing muscle regeneration *(Siriett et al., 2006)*. However, myostatin is reduced (and Akt thus not inhibited) upon exercise in rodents *(Kim et al., 2005)*.

Thus, activation (or rather, no inhibition) of the Akt pathway is enjoyable for a muscle (Figure 10).

The AMP-activated protein kinase

During the last 5 years, evidence has grown that the AMPK can act on atrogene activity via FoxO3 activation. On the contrary to Akt, which inhibits FoxO activity, AMPK phosphorylates other FoxO sites that stimulate its transcriptional activity *(Greer et al., 2007a,b)*. For example, in conditions of energy stress induced by mitochondrial fragmentation, FoxO3 is activated via the AMPK *(Romanello et al., 2010a,b)*. Furthermore, when stimulating AMPK with AICAR, the FoxO family is induced, leading to activation of Atrogin-1 *(Nakashima and Yakabe, 2007)* (Figure 10).

NF-κB signalling

The NF-κB transcription factor is an important regulator in response to cellular stress. This protein complex acts on inflammation by activating transcription of cytokines such as TNFα, IFN-γ or Interleukins 2, 6 and 8. Furthermore, NF-κB acts on cell fate (depending on its type) by promoting expression of anti-apoptotic genes encoding genes such as TRAF1/2 or c-IAP1/2. When inactive, NF-kB is bound to and inhibited by IκB proteins. After induction by TNFα (yes, the NF-κB target is also the activator), this inhibition is levered and NF-κB can activate gene transcription in the nucleus *(Baldwin, 2001; Tak and Firestein, 2001)* (Figure 10). The loss of inhibition or activation of the NF-κB pathway leads to increased muscular atrophy by increasing protein degradation via the ubiquitin-proteasome pathway with a concomitant increase of MuRF1 transcription. However, Atrogin-1 expression was not affected *(Cai et al., 2004)*. On the contrary, inactivation of NF-κB reduced protein degradation and promotes protein synthesis *(Hunter and Kandarian, 2004; Mourkioti et al., 2006; Judge et al., 2007)*.

The transcription factor NF-κB itself can be activated by TNFα via the PI3K/Akt pathway *(Ozes et al., 1999; Faurschou and Gniadecki, 2008)*. While this finding links several pathways, it also highlights the immense complexity of pathways regulating muscular atrophy. Indeed, this finding is not compatible with aforementioned findings (TNFα activates NF-κB and induces atrophy, PI3K reduces atrophy). Please read more on that on *Ch.II – 2.2*.

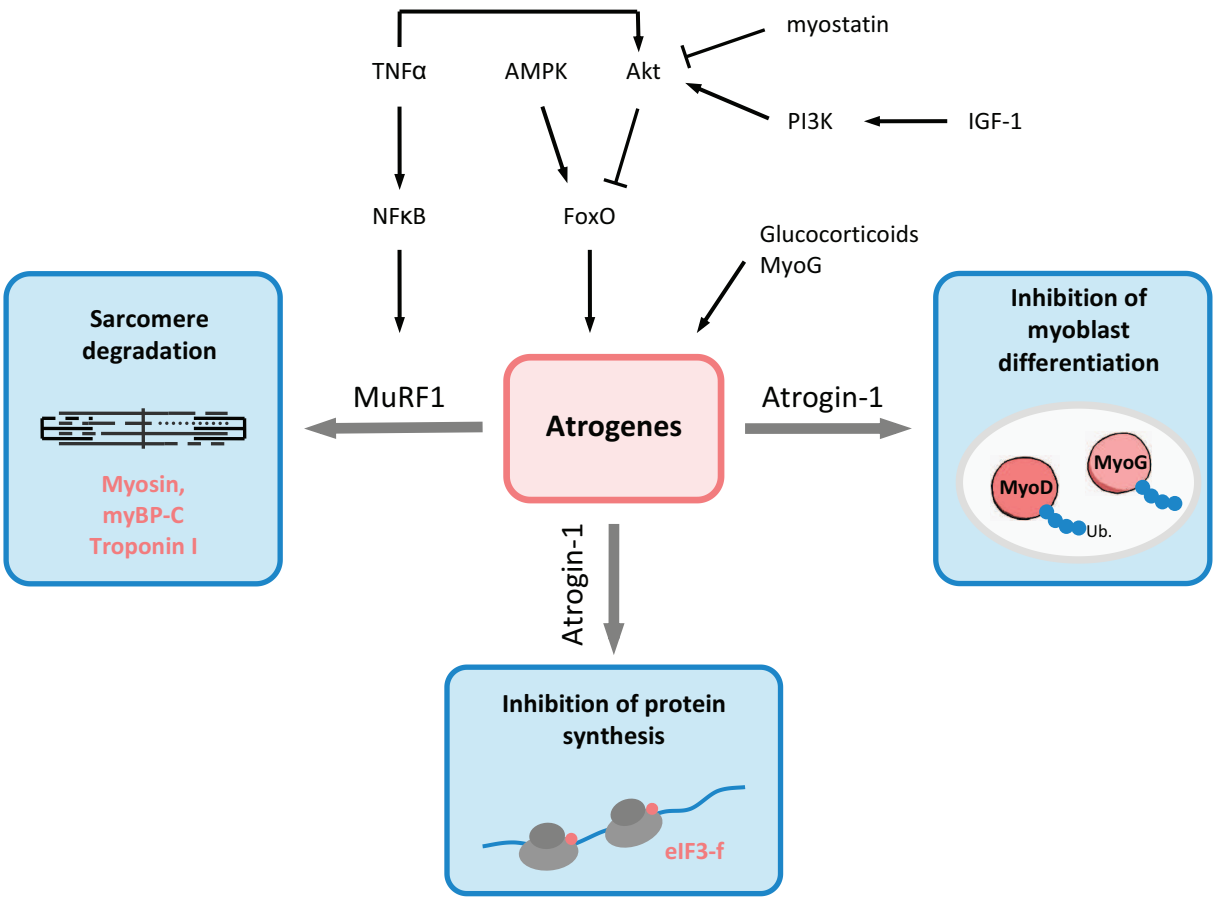

Figure 10 | **Signalling pathways leading to atrophy via MuRF1 And Atrogin-1**
Multiple pathways converge to activate the atrogenes. Their activation leads to different molecular outcomes, but all contribute to muscle wasting.

Are there plausible therapies against muscular atrophy?

If we take together all the evidence, it seems as though MuRF1 and Atrogin-1 are ideal candidates for symptomatic treatments for most types of atrophy. Despite all efforts, also including the upstream signalling, no specific inhibitors are clinically available. Knock out models have shown that inhibiting individual atrogenes does not completely protect against muscular atrophy *(Bodine et al., 2001a)*. So should we target the whole proteasome system? Several groups have shown that inhibiting the ubiquitin-proteasome pathway with inhibitors such as LLN or MG-132, greatly or at least partly, protects muscles from atrophy in rodents *(Tawa et al., 1997; Kadlcikova et al., 2004; Caron et al., 2011; Jamart et al., 2011)*. However, it is plausible that inhibiting the whole proteasome system, even if restricted to the muscles, could have severe deleterious effects on the long term. Indeed, the anticancer drug Bortezomib, also a proteasome inhibitor, induced cardiotoxicity *(Orciuolo et al., 2007)*.

Another way to potentially counteract muscular atrophy, is to act on upstream pathways, regulating protein degradations. IGF-1 has a proven role against muscular atrophy since it prevents protein degradation and promoting hypertrophy in healthy muscle *(Lai et al., 2004)*, it is also central to other cellular processes. However, uncontrolled activation of IGF-1 in other cell types could lead to accumulation of misfolded proteins and cell stress or even tumorigenesis.

Finally, there is the option to act on myostatin, a signalling molecule from the TGF-family that has only recently been investigated more thoroughly. This molecule induces hypertrophy when inactivated in dogs *(Mosher et al., 2007)* and humans *(Schuelke et al., 2004)* or protects against sarcopenia in old mice *(Siriett et al., 2007)*. Myostatin-targeted antibodies also protected from muscular disuse atrophy in mice *(Murphy et al., 2011)*.

Fortunately, as always, not all research was in vain: Our knowledge about the regulation of muscular atrophy and the balance between protein synthesis and breakdown has significantly improved over the last decade. However, many important points need to be addressed; one of the most important aspects is the difference between humans and rodents. It's obvious that healing without side effects a mouse suffering of severe muscular atrophy (that we induced!) would be fantastic… but pretty pointless when the pathways are not the same in humans.

Now that you know more about the most important molecular mechanisms underlying muscular atrophy, I feel it is a good time to continue to the more clinical aspects of muscular atrophy, where I will highlight or recall, when necessary, the implication of the two main atrogenes, MuRF1 and Atrogin-1.

II - 1.4. Denervation

Earlier, we have seen one reason why muscles could atrophy due to inactivity *(Ch.II – 1.1. Muscle disuse).* Another, more drastic reason for muscle inactivity is the inhibition of neuronal stimulation. This can occur in humans who, after a severe accident, will suffer from sectioned nerves in legs or the spinal cord. Denervation, or more specifically spinal cord injury, can also occur after ischemia, during cancer or in the context of diseases such as Amyotrophic Lateral Sclerosis. In rodents, denervation can be induced experimentally for example by sectioning or by crushing/squeezing the sciatic nerve that innervates the hind limbs *(Zhang et al., 2008)*. Naturally, as muscles fail to get stimulated, they become immobile; patients, as well as mouse models, become paralyzed and muscles atrophy. As such, denervation could be associated with disuse. Similarly, MuRF1 and Atrogin-1 are induced in muscles from mice and rats *(Bodine et al., 2001a; Sacheck et al., 2007)*. Inversely, inhibition of both atrogenes protects against denervation-induced atrophy. In human muscles, they are also induced in patients with spinal cord injury *(Urso et al., 2007)*. However, while this is true for the mRNA, protein increase has not been detected yet.

II - 1.5. Sarcopenia

As life-span increased over the last centuries, health-span really did not. We tend to live longer, but in which conditions? Diseases like cancer, heart/vascular conditions or Alzheimer's disease are just waiting for us at higher age. Additionally, the "syndrome" of sarcopenia is among the most serious and inevitable consequences of ageing. Hallmarks of sarcopenia are the progressive loss of muscle mass and quality. More specifically, muscular atrophy and fibrosis lead to reduced strength and fatigue. Interestingly, individual type II muscle fibres are more affected and have a reduced diameter.

Additionally, they slowly convert to type I fibres. Infiltration of fat and fibrous tissue or other non-contractile tissue (fibrosis) renders muscles stiffer. Furthermore, the neuromuscular junction gets dismantled. All those events together obviously lead to reduced mobility and frailty we see in the elderly *(Faulkner et al., 2007; Ryall et al., 2008)*.

The exact mechanisms are unknown, but current hypotheses propose that the satellite cells that are usually activated after injury or exercise (to proliferate and fuse into new or existing muscle fibres) fail to do so the more humans get older. Thus, the worn-out myofibres are not replaced or repaired, leading to decreasing muscle mass and strength. Those events seem to be caused by alterations in neuromuscular transmission, muscular metabolism and oxidative stress. In addition, extrinsic anabolic signals like testosterone or growth hormones are diminished or have less effect. Conversely, inflammation can be promoted by catabolic signals like cytokines.

The role of the atrogenes in this context is still a matter of discussion. While in atrophying muscles from elderly people no changes in MuRF1 or Atrogin-1 transcription has been observed *(Foletta et al., 2011)*, an increase of both genes was observed in rat *tibialis anterior* muscles only *(Clavel et al., 2006)*.

Interestringly, exercise has a beneficial effect on sarcopenia. Physically active elderly people suffer much less from sarcopenia symptoms. More so, their whole musculo-skeletal system (muscles, bones, tendons, ligaments, neurons...) remain healthy much longer *(Abate et al., 2007; Muscaritoli et al., 2010)*.

Basically then, it seems as though sarcopenia is less of a process in which muscular tissue is actively destroyed but instead, one in which normal homeostasis is reduced and the muscle is not renewed.

II - 1.6. Starvation

In normal conditions, muscles are under constant use. Incidentally, the proteins necessary for muscle structure and contractility need to be constantly replaced, similarly to the maintenance of other organs such as the intestine or the liver. It is just normal physiology – muscles and other tissues need energy and proteins in particular to remain healthy. However, when the body encounters a lack of nutrients, it gets tough.

It is no big surprise then that muscles atrophy occurs during food deprivation. Muscles account for up to 75% of a body's cellular mass and, as abundant source of amino acids, provide this substrate for tissue homeostasis, repair and gluconeogenesis. Thus, muscles rapidly and altruistically provide amino acids through the bloodstream to organs in need by breaking down their own proteins *(Koyama et al., 2008)*. After all, what is the point of being able to run when the brain is unable to command how to do it? However, there is hope for selfless muscles: it has been shown that atrophy due to nutrient deprivation is reversible; when nutrient intake returns to normal, muscles gain their initial mass again *(Jagoe et al., 2002)*.

In muscles of fasting mice, both MuRF1 and Atrogin-1 are rapidly induced at the mRNA level *(Gomes et al., 2001; Sandri et al., 2004; Koyama et al., 2008)*. Concomitantly, levels of IGF-1 are reduced, while glucocorticoids are elevated, leading to atrogene activation *(Mitch et al., 1999)*. However, as stated before *(Ch.II 1.2)*, this finding could not be confirmed for humans, suggesting different basic regulatory mechanisms or translating a higher resistance to fasting in humans *(Larsen et al., 2006)*. Another pathway that seems specific to muscular atrophy related to starvation, is the autophagy pathway. Simply put, cells cleanly consume themselves by fusing lysosomes with membranes encapsulating either cytoplasm or even organelles such as mitochondria. Proteases, nucleases, glycoside hydrolases and lipases then break down proteins, nucleic acids, glucides and fatty acids, respectively, which can then be used elsewhere *(Lira et al., 2013)*.

II - 2. Thoughts on muscle atrophy or: "can't we just call it apoptosis?"

You want a simple answer? *No, atrophy does not equal apoptosis*. Muscle atrophy is still a process which is poorly understood. In "normal", mononuclear cells from other tissues, the situation is mostly straightforward. When cells containing only one nucleus die, it is relatively easily to determine whether the process was for example apoptotic or not. For a brief explanation of apoptosis, please refer to *Ch. IV – 2.2. apoptosis.*

Using comparatively easy techniques such as western blotting and (quantitative) PCR, we can evidence an increase of pro-apoptotic genes (p53, Bax, Noxa, Puma…) or effectors of cell catabolism (Caspases 3, 6, 7, …). Additionally, agarose electrophoresis,

comet assays or the TUNEL technique detect DNA fragmentation, the sign that the entire cell is doomed. The point is that the muscles fibers, or myocytes, are not conventional cells at all. As explained before, they contain up to 500 so-called myonuclei, so in theory, 499 nuclei could die and a portion of the "cell" could still be considered alive! As you will see, the implication of apoptosis or "apoptosis-like" events in muscular atrophy is not straightforward. Interestingly, many reports covering apoptosis during muscular atrophy are based on rodent denervation studies or are linked to neuromuscular disorders: after denervation of the forelimbs of rats, genes associated with apoptosis are induced in muscles fibres (Fas, Caspase 8, c-myc, p53…).In addition, DNA laddering was also evidenced in total muscle extracts, and nucleus structure was altered *(Jin et al., 2001)*. In another study, apoptosis in hind limb gastrocnemius muscles was examined after denervation. Again, DNA fragmentation was observed and proteins associated with apoptosis were increased (AIF, cytochrome c, Caspase 3&9, PARP…) *(Siu and Alway, 2005)*, while normal skeletal muscles do not express those proteins at all *(Burgess et al, 1999)*. DNA fragmentation (laddering on agarose gel and ultrastructural changes of nuclei) and/or evidence of apoptosis (Caspase upregulation/activation, Bcl-2 family upregulation) were also evidenced during human diseases. As examples, one could name Duchenne Muscular Dystrophy and its corresponding animal models *(Sandri et al., 1998, 2001. Tews, 2002),* Spinal Muscular Atrophy *(Tews et al., 1997, 2005)* or patients with neurogenic muscular atrophy *(Tews et al., 2005)*.

Now here is the tricky part: Some studies could NOT evidence apoptosis in human dystrophies or myopathies *(Olivé et al., 1997; Inukai et al., 1997; Migheli et al., 1997).* This may be due to differences in the chosen disease stages and the methodology used *(Leeuwenburgh et al., 2005; Tews, 2005)*.

One purpose of apoptosis during atrophy, so it has been proposed, is to maintain a constant ratio of cytoplasm volume to nuclei. Apoptosis occurs locally in muscle fibres; only a subset of nuclei dies, while the majority of other nuclei remain intact *(Liu and Ahearn, 2001)*. Initially, when these fibres atrophy, the net cytoplasm volume decreases, while the number of nuclei remains the same *(Schwartz, 2008)*. By inducing "localized" apoptosis, individual nuclei can be removed, alongside the transcriptional and translational programs they control. In my opinion, this would imply that apoptosis is not a cause, but rather the consequence of atrophy (mediated by increase of protein degradation, atrogenes etc.). However, through a cunning experiment, a Norwegian

group claims that nuclei are not lost during atrophy. They induced atrophy in mice by muscle unloading, blocking neuronal innervation by axotomy of the sciatic nerve or treatment with tetrodotoxin. While observing severe muscular atrophy, Bruusgaard and Gundersen they did not observe loss of myonuclei using time-lapse microscopy. However, when examining histological sections, fragmented DNA was observed (TUNEL staining) in nuclei of satellite cells, sustaining the finding that atrophic muscles are not repaired efficiently *(Bruusgaard and Gundersen, 2008)*. Were all other reports based on false positives? We will never know. The fact is that evidence of apoptosis is there, but greatly depends on the type of atrophy and its progression. It seems established however, that apoptosis is not the main reason for muscle wasting, except for toxic insults, such as treatment with staurosporin *(McArdle et al., 1999)*.

Interestingly, the confusion and difficulty about the implication of apoptosis in muscular atrophy has lead to the existence of two completely separate subsets of research articles. Almost all original articles that are dealing with MuRF1 and Atrogin-1 or atrophy in general don't even mention the possibility of apoptosis. On the other hand, groups examining apoptosis in different disease settings seem to be afraid of mentioning the importance of the ubiquitin proteasome pathway. Efforts have to be made in bringing both "worlds" together.

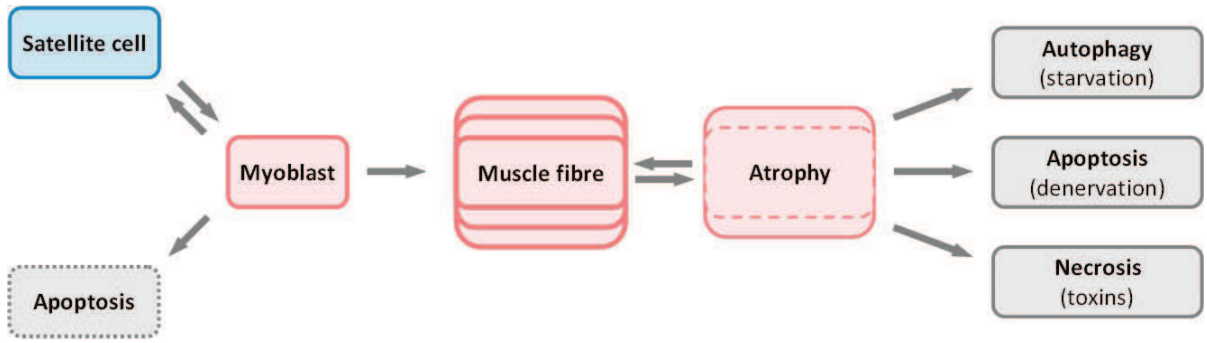

Figure 11 | **Death and development of muscles**
Myoblasts can either undergo apoptosis, revert to satellite cells of terminally differentiate into muscle fibres. Upon stimuli, muscle fibres can atrophy, a process which can be reversed for example after starvation or disuse. Depending on intensity, duration and atrophic conditions, muscle fibres will undergo autophagy, apoptosis or necrosis.

III - MUSCULAR ATROPHY AS A CONSEQUENCE OF DISEASES

We have seen how seemingly "simple" and "normal" biological events (i.e. immobility, ageing, starvation) can lead to muscular atrophy. Unfortunately, there are also some devastating diseases and syndromes out there: cancer, pulmonary disease, chronic heart dysfunction, sepsis, kidney failure, severe burn injury… Those diseases have more in common than being disabling, sometimes painful and potentially deadly: As a side-effect, patients can suffer from muscular atrophy, which further weakens their bodies and complicates treatment. There are also conditions that can be inherited, featuring muscular defects or atrophy as the main hallmark of the disease. In the following chapter, in order to give a faint insight into this vast domain, I would like to introduce muscular dystrophy as a representative of inherited myopathies, as well as cancer cachexia and Amyotrophic Lateral Sclerosis as two very different diseases we are currently working on. Because of their importance in my research project, the last two will be treated in separate chapters.

III - 1. Muscular dystrophy

Muscular dystrophies are a group of inherited diseases that share similar clinical features and are characterized by progressive muscle weakness of various muscles. The different types of dystrophy vary greatly in their severity, age of onset and progression rate.

III - 1.1. General Introduction

Muscular dystrophies have been historically classified depending on the main symptoms and the age of onset. One could discern Duchenne dystrophy, congenital dystrophy, limb girdle dystrophy or Emery-Dreifuss dystrophy to cite a few. Some, like limb girdle muscular dystrophy (LGMD), were then further subdivided into groups based on the inheritance and the responsible gene (LGMD1A, LGMD2B…), underlining the complexity of this group of diseases. Interestingly, almost all feature defects of proteins in the sarcolemma, the membrane of muscle fibres (See *Ch.I – 1.1*).

III - 1.2. Duchenne muscular dystrophy

I will focus on the most frequent and severe form of these inherited disorders: Duchenne muscular dystrophy (DMD) with one in 5000 male birth affected *(Leung and Wagner, 2013)*. DMD was first described in England by E. Meryon in 1851, but became associated with Duchenne in France several years later. The disease only affects boys in early childhood (diagnosed at around 4 years of age) and is hereditary (X-chromosome linked). After delay in learning to walk, patients experience difficulties in running and tend to fall frequently. Weakening of the leg muscles, associated with loss of mass, gradually increases until walking becomes almost impossible. Muscle fibres are eventually replaced by fat and fibrous tissue, limiting movement further; the young patients are wheelchair-bound by the age of 12. Wasting progresses to the upper limbs and in mid-adolescence, only fingers remain partly functional. Further difficulties arise when muscle weakness causes the spine into scoliosis (abnormally bent shape), which leads to difficulties in breathing. The weakening of chest-muscles further increases the problem. However, ventilation has dramatically increased survival (about 25-30 years of age). Finally, the heart also gets affected (cardiomyophathy) and needs pharmacological treatment *(Emery, 2002).*

This devastating phenotype results from mutations in the gene encoding dystrophin, a protein necessary to link the actin-myofilaments to the basal lamina/endomysium through the distroglycan complex in the sarcolemma *(Matsumura et al., 1993)*. The mutation inhibits dystrophin expression, thus leading to a failure in maintaining muscle structure and rendering the membrane vulnerable to mechanical and oxidative stress. This will lead to the activation of stretch-activated cation channel with abundant influx of electrolytes. In simple terms, this leads to inflammation and myofibre necrosis, inducing fibrotic tissue remodelling *(Klingler et al., 2012)*. Satellite cells proliferate and desperately try to help in restoring the muscle. But as they also harbour the dystrophin mutation, they fail to do so effectively – and their pool becomes exhausted *(Shi and Garry, 2006; Péault et al., 2007)*.

Muscle biopsies and immunohistochemistry against sarcolemmal-associated proteins, especially dystrophin, confirm genetic diagnosis. Histochemistry shows replacement of muscle cells by fat or fibrous tissue (Figure 12).

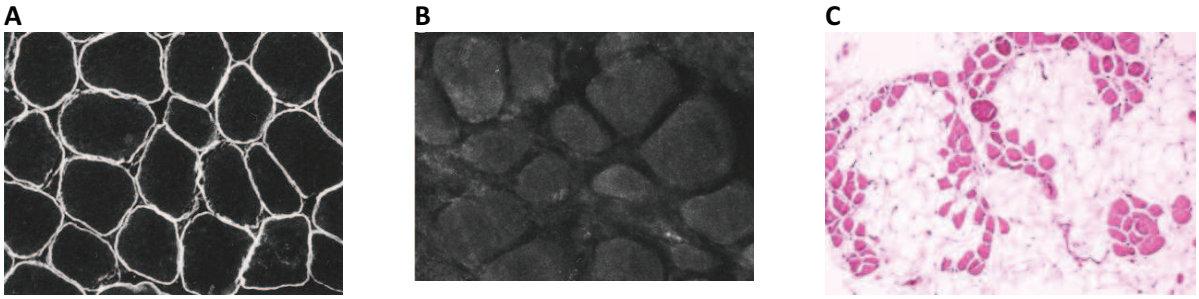

Figure 12 | **Loss of muscle integrity during Duchenne muscular dystrophy**
Detection of dystrophin on frozen microsections of control (A) or DMD (B) muscle. From *Muntoni et al., 1995*. (C) Invasion of a DMD patient *gastrocnemius* muscle (red) by adipocytes (white). From *Public Health Image Library – EP Ewing, 1972*

The implication of MuRF1 and Atrogin-1 is still unclear. First, only very few studies that examine this issue were published. While trying to decipher molecular differences in various stages of DMD, biopsies from different stages of the pathology were analyzed and no difference in Atrogin-1 expression was found between the earliest stages (foetus) and symptomatic patients *(Chen et al., 2005)*. While searching for more information, I found an abstract from a Chinese master's thesis claiming in 2011 that in biopsies from young DMD patients, MuRF1 and Atrogin-1 were induced (Zhang Ning's Lab, Department of Neurology, Xiangya Hospital, Central South University, Changsha, China). However, this finding doesn't seem to have led to publication. Further work is necessary to define the implication of the atrogenes in muscular dystrophy.

Therapeutic approaches for muscular dystrophy

In Duchenne muscular dystrophy, different mutations in the dystrophin gene lead to a lack in quantity or size of dystrophin protein due for example to nonsense mutations or splicing errors. For the moment, there is no cure, but current therapeutic approaches tend to go in three different and potentially complementary directions.

The first approach addresses the mutations of dystrophin in order to restore a correct expression *(Aoki et al., 2013; Ruegg, 2013; Leung and Wagner, 2013)*. The second

approach focuses on modulating signalling pathways that have been deregulated due to dystrophin mutation *(Ruegg, 2013)*. The third approach proposes restoring the muscle stem cell pool with satellite cells or myoblasts to repair damaged muscle with functional dystrophin *(Briggs and Morgan, 2013)*.

On the contrary to atrophy induced by disuse or starvation for example, that has a more "systemic" background, the atrophy arising in muscular dystrophy comes down to a mutation in a single protein: dystrophin.

III - 2. Cancer Cachexia

Cachexia is a severe metabolic complication arising in different diseases, prominently in cancer. It is characterized by a significant loss of body weight as a result of muscular atrophy and, but not necessarily, loss of adipose tissue. Although it may resemble disuse and starvation-induced atrophy, after all, cancer patients eat and move less, it is important to note that the skeletal muscle atrophy cannot be reversed by nutritional support *(Rolland et al., 2011)*. Thus the underlying signalling pathways seem to be very different.

III - 2.1. Clinical features

Yes, reduced food intake contributes to the cachexia symptoms, especially in patients with gastrointestinal cancers. However, abnormal metabolism, modified by tumor-secreted factors is also believed to be involved in cancer cachexia. For a given amount of lost weight, cancer patients lose more weight in muscles than healthy individuals do when on a diet or when starving *(Tisdale, 1997)*. The effects of cachexia on patients are profound: not only do they lose quality of life or autonomy due to loss of strength and increased fatigue, but cachexia also impairs their resistance to treatments. Cachexia affects about 25-30% of total cancer patients. This number rises up to more than 50% when considering patients in terminal stages *(Glass and Roubenoff, 2010)*.

About a fifth of cancer patients actually die because of the direct consequences of cachexia, prominently as a result of heart failure and respiratory insufficiency. Up to

30% of total body weight, corresponding to approximately 75% of muscle mass can be lost *(Tisdale, 2009; Burckart et al., 2010)*.

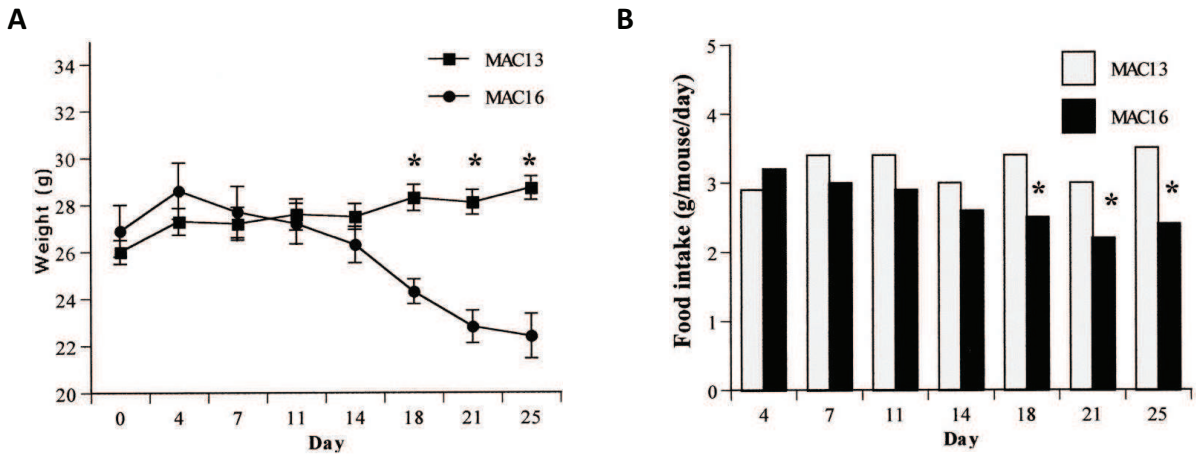

Figure 13 | **Tumor type versus weight loss and food intake in mice**
(A) Mice implanted with adenocarcinoma tumor MAC16 experience severe weight loss, while MAC13-implanted mice do not. (B) Weight loss of MAC16-implanted mice surpasses the probable effect of food intake reduction. From *Monitto et al., 2001*.

Initially, it was thought that metabolic changes and the actual symptoms were a consequence of increased energy needs of growing tumors. However, it has been shown that the tumor type (and thus sometimes also its location), and not its size is determining. For example, about 90% of patients with gastric or pancreatic carcinoma will suffer from severe cachexia. On the other hand, only 30% of patients with non-Hodgkin's lymphoma will be affected *(DeWys, 1985)*.

This data is backed up by experiments in mice (Figure 13): Even though bearing similar adenocarcinoma, mice with a MAC16 tumor lost up to 25% of their body weight, while mice with a MAC13 tumor did not *(Smith and Tisdale, 1993; Monitto et al., 2001)*. Finally, tumor mass frequently corresponds to 1% or less of total body mass, while cachexia is still induced. Strikingly, the genotype of patients will also highly affect susceptibility – patients with a very similar tumor burden will not necessarily suffer from the same intensity of cachexia. This has been related to polymorphisms in genes associated with cachexia *(Tisdale, 1997; Fearon et al., 2012)*. Efforts have been made to define precise criteria for diagnosis, as well as to classify patients in order to provide appropriate care (Table 2).

Recommended clinical criteria for diagnosis of cachexia

- **Weight loss of at least 5% in 6 to 12 months or less in the presence of underlying illness and absence of simple starvation**

- **BMI less than 20 with any degree of weight loss greater than 2%**

- **Three of the five criteria:**
  Decreased muscle strength
  Fatigue
  Anorexia (poor appetite or intake of less than 20 kcal/kg of body weight)
  Low fat-free mass index *(Appendicular skeletal muscle index less than 7.25kg/m² in males or 5.4kg/m² in females)*
  Abnormal biochemistry: *Increased inflammatory markers (C-reactive protein, IL-6)*
  Anemia (< 12g/dl)
  Low serum albumin (< 3.2 g/dl)

- **Suggested classification of cachexia depending on weight loss *(within the previous 12 months or less)***
  Mild cachexia / Precachexia if weight loss is less than 5%
  Moderate cachexia / Cachexia if weight loss is less than 10%, reduced food intake, inflammation
  Severe cachexia / Refractory cachexia if weight loss is less than 15%, resistant cancer

Table 2 | **Diagnosis and degree of cachexia**
Compilation based on panels of experts. *Evans et al., 2008, Fearon et al., 2011*

III - 2.2. Molecular mechanisms

Of course, not everything is known yet about the mechanisms leading to cancer cachexia. A very interesting experiment has proposed that the blood serum obtained from animals with induced cachexia contains circulating factors leading to cachexia. The authors transplanted mice with cells from adenocarcinoma tumors, inducing (*MAC16*) or not (*MAC13*) the symptoms of cancer cachexia. Blood serum was then used to incubate isolated gastrocnemius muscles. As assessed by tyrosine release, the muscles started atrophying when they were incubated serum from mice with MAC16 tumors *(Smith and Tisdale, 1993)*. Similar results were obtained with plasma from cancer patients *(Belizario et al., 1991)*. On the contrary to disuse atrophy for example, this suggested that the muscles, which are not connected to the tumor, are sensitive to external stimuli or "proteolysis-inducing factors". So let's start looking there…

The inflammatory pathway - TNFα

Once upon a time (in 1968), the Tumor Necrosis Factor alpha (TNFα) was identified by several groups as a cytotoxic antigen *(Kolb and Granger, 1968; Ruddle and Waksman, 1968)*, which has led to the discovery that it can act as a tumor suppressor, by inducing cell death of transformed/neoplastic cells, mediated by activated macrophages *(Carswell et al., 1975)*. Today, it is well known that TNFα is also able to induce apoptosis, inflammation and… cachexia. Interestingly, the 2011 Nobel Prize laureate Bruce Beutler contributed to the discovery of a "factor" inducing atrophy, while studying cachexia in rabbits *(Beutler et al., 1985a)* and termed it "cachectin". After cloning and sequencing this factor, the group realized that cachectin was the same protein than TNFα *(Beutler et al., 1985b)*.

When it was shown that tumor cells artificially overexpressing TNFα induced cachexia in mice *(Oliff et al., 1987)* researchers quickly hypothesised that inflammation, caused by this protein, plays a central role in muscle wasting. And indeed, *in vitro* as well as in animal models, TNFα leads to cachexia or cell death and induces MuRF1 and/or Atrogin-1. For example, TNFα treatment of cultured mouse C2C12 myoblasts induces Atrogin-1 *(Li YP et al., 2005)* and inhibits their differentiation by downregulating MyoD and MyoG (See *Ch.I – 1.2.*) *(Guttridge et al., 2000).* Rats *(Frost et al., 2007)* and mice treated with TNFα also display Atrogin-1 *(Li YP et al., 2003, 2005; Coletti et al., 2005)* and MuRF1 *(Adams et al., 2007)* induction. The addition of TNFα to cultured rat L6 myofibres induces expression of both atrogenes, with a concomitant downregulation of the PI3K/Akt pathway. The fibres further underwent apoptosis and necrosis *(Sishi and Engelbrecht, 2011)*. One would be tempted to assume that this atrogene-activation is mediated by the FoxO1/3 factors, which are induced when Akt is inhibited. However, recent findings (when count in cachexia-research-years) contradict this hypothesis. Moylan's results using C2C12 myoblasts rather suggest that TNFα bypasses FoxO1/3 signalling in order to activate Atrogin-1. Instead of reducing Akt phosphorylation (like during starvation for example – *Lecker et al., 2004*), Akt was actually activated, leading to inhibition of the FoxO family, while Atrogin-1 was still induced *(Moylan et al., 2008)*. Several articles support these findings: Treating cultured C2C12 myofibres with IGF-1, that should inhibit FoxO activation, does not protect against TNFα-induced atrophy *(Dehoux et al., 2007)*. Patients with atrophy as a consequence of pulmonary disease exhibit, induction of atrogene mRNA in muscles, while Akt is also induced *(Doucet et al.,*

2007). Finally, patients with amyotrophic lateral sclerosis (ALS) have normal FoxO1/3 levels, but suffer from skeletal muscle atrophy with increased Atrogin levels *(Leger et al., 2006)*.

In contrast to other types of atrophy, muscular atrophy resulting from inflammatory signalling does not seem to involve FoxO1/3. However, FoxO4 responds to TNFα activation: In its presence, FoxoO translocates to the nucleus and when repressed, Atrogin-1 transcription is inhibited *(Moylan et al., 2008)*.

Unfortunately, there is again a difference between rodents and humans. While experimentally induced tumors secrete TNFα in mice, its role for human muscles is unclear. Antibodies raised against TNFα have shown no benefit in patients with cachexia *(Jatoi et al., 2010)* and highly increased levels of TNFα in the body could not be found either *(Fearon et al., 2012)*, suggesting that TNFα is contributing to but not causing atrophy/cachexia alone.

Interleukin-6

On the contrary to TNFα, circulating pro-inflammatory cytokine interleukin-6 (IL-6) was detected in cancer patients witch cachexia. Several tumors secrete this factor and IL-6 levels correlate with the severity of atrophy/cachexia and reduced survival *(Moses et al., 2009)*. However, IL-6 alone only induces cachexia in doses much higher than physiological levels *(Baltgalvis et al., 2008)*, suggesting that is has to act in conjuction with other factors (like TNFα) in the host to induce muscular atrophy. Explaining the molecular effect of IL-6 is complicated. While blocking the IL-6 receptor, muscle protein breakdown was suppressed, however, synthesis did not return to normal levels *(White et al., 2011)*. Interestingly, using antibodies against IL-6 in patients resulted in better overall wellbeing – anorexia, fatigue and anemia were reduced. However, an effect on body or muscle mass could not be observed *(Bayliss et al., 2011)*.

III - 2.3. Treatment options

It has long been considered that cachexia is an inevitable side effect of cancer and not much effort has been made to prevent this, except acting on the syndrome of anorexia. This “symptomatic” treatment still is of utmost importance, especially in the early

stages *(Del Fabbro et al. 2011)*. After all, the muscle is not the only organ requiring proteins, carbohydrates and water. Providing necessary nutrients or pharmacologically stimulating appetite (for example *Megestrol* and *medroxyprogesterone acetate* or corticosteroids) are, and will still be important approaches to cachexia *(Simons et al., 1996; MacDonald et al., 2003)*. Unfortunately, those molecules seem to increase adipose tissue and promote retention of water with no effect on muscle tissue. But on longer-term use, corticosteroids could also contribute to muscle wasting while enhanced nutrition may stimulate tumor growth.

Exercise stimulates anabolic signalling, potentially counteracting muscle wasting *(Faulkner et al., 2007)* while improving general health of patients (cardiovascular system). However, exercise is naturally limited in cancer patients and doesn't necessarily mean the patient should go running and weigh-lifting, but rather undertake little walks to stop permanent bedrest and disuse of positional muscles.

Targeting inflammatory cytokines is another therapeutic option that directly addresses the underlying pathways of cachexia. However, the results are contrasted, at best. On one hand, while thalidomide is used to inhibit TNFα, reduce muscle loss and enhance strength, it has also been shown to produce severe adverse effects *(Dodson et al., 2011)*. On the other hand, antibodies against TNFα or IL-6 have shown no effect on cachectic muscles in other studies *(Jatoi et al., 2010; Bayliss et al., 2011).*

A recent study targeted the downstream pathway of TNFα (via iNOS – not discussed in this thesis) with a molecule that usually inhibits protein translation. At low, nontoxic doses, pateamine A enhanced cultured C2C12 myoblast differentiation and lead to inhibition of iNOS translation via its association with stress granules. This prevents the production of nitric oxyide and the expression of MyoD in cultured C2C12 and tumor-bearing mice *(Di Marco et al., 2012)*. However, treatment of mice with pateamine A concomitantly reduced tumor size. While this finding is perfect, it could also at least contribute to improvements in muscle after pateamine A treatment, blurring the results.

The concept of low doses of otherwise unadapted drugs may lead to new high throughput screenings using C2C12 cells and the reconsideration of many dismissed drugs.

Finally, there is the option to act on the TGF-family (See *Ch. II – 1.3. IGF-1 / Akt signalling*). Approaches targeting the ActRIIB receptor of the TGF-family (responding to

myostatin) or its ligand myostatin in tumor-bearing mice, performed rather well and reduced muscular atrophy and weakness *(Klimek et al., 2010; Zhou et al., 2010; Murphy et al., 2011)*.

III - 2.4. Animal models (we) used to study cachexia

There are several ways to induce cancer-related cachexia in rodents. The first method consists of implanting a tumor into the animal, which leads to cachexia. Although murine adenocarcinoma were used to induced more than 10 years ago *(Monitto et al., 2001)*, it has become common practice to use the C26 colorectal carcinoma *(Tanaka et al., 1990; Bonetto et al., 2009; Walter et al., 2010; Penna et al., 2010)*. Transplanting parts of an existing tumor (allograft) or injecting resuspended cells under the skin of mice induces growth of a C26 tumor, leading to the classic symptoms of cachexia: a significant loss of body weight, loss of muscle mass and strength and loss of adipose tissue *(Penna et al., 2010)*.
Using murine cells allows us to use "normal" mice, instead of Nude- or SCID-mice. However, there have been differences in tumor acceptance depending on the genetic background of the mice used. For example, C57BL/6 or Swiss mice do not keep the implanted tumor, while Balb/c mice do – and are now uniquely used.

The second method to induce cachexia is through cytotoxic drugs. Doxorubicin (also called Adriamycin or Hydroxy-Daunorubicin) is a synthetic anthracycline antibiotic. The closely related molecule Daunorubicin (without the added hydroxyl-group) is naturally produced by *Streptomyces peucetius*. Doxorubicin is used against a wide range of cancers, including leukaemia and neuroblastoma. Given its structure (Figure 14), Doxorubicin is able to intercalate DNA, inhibit topoisomerase II and thus, induces double strand breaks and basically stops replication *(Fornari et al., 1994; Zhang et al., 2012)*. It also leads to detachement of histones from DNA, notably H2AX, impairing transcriptional regulation and DNA repair *(Pang et al., 2013)*. Doxorubicin effects lead, like many anticancer drugs, to adverse effects in humans: its use is limited by dose-dependent cardiotoxicity, which is thought to be induced by excessive ROS generation and apoptosis *(Fu et al., 2010; Gilliam et al., 2011; Zhang et al., 2012)*. In mice,

Doxorubicin induces atrophy of skeletal muscle, including the diaphragm muscle *(Gilliam et al., 2009, 2011)*, making it a suitable model for cancer-related cachexia.

Both research models have their advantages: While grafting C26 tumors in mice closely mimicks cachexia in patients, treatment with doxorubicin is more controllable (dose) and can also be used *in vitro* in conjunction with cultured C2C12 myoblasts to decipher molecular mechanisms.

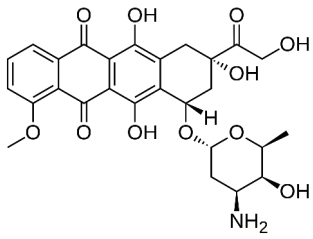

Figure 14 | **Structure of Doxorubicin**

Formula: $C_{27}H_{29}NO_{11}$
Molecular mass: 543,25 g/mol
CAS number: 23214-92-8

III - 3. Amyotrophic Lateral Sclerosis

ALS is the most widespread type of motor neuron disease. It is fatal but still poorly understood. In simple terms, adult-onset loss of motor neurons leads to muscular atrophy causing weakness, fatigue and general paralysis. Patients, mostly in their fifties usually pass away 2-3 years after diagnosis because of respiratory failure *(Gonzalez de Aguilar et al., 2007)*.

III - 3.1. Symptoms

As ALS sets on, upper motor neurons from the motor cortex, and lower motor neurons emerging from the brainstem and the the spinal cord are destroyed. Concomitantly, the neuromuscular junction is destroyed. This leads to paralysis and atrophy of skeletal muscles (Figure 15). However, the onset of ALS is subtle - symptoms may be misinterpreted or may even remain unnoticed, meaning that patients remain undiagnosed even after the disease is well set-on.

Many individuals observe first effects in the limbs - such cases are called "limb onset ALS". As hands and arms are heavily used everyday, spasticity and fasciculations render tasks such as writing or picking up smaller objects become increasingly difficult. Other individuals suffer from difficulties when walking and stumble more often. On the contrary, patients with "bulbar onset" first experience difficulties when speaking, chewing or swallowing. Even though the onset, the sequence of the symptoms and the speed of progression may vary between patients, the disease will spread to the whole body. Muscle weakness and atrophy become more obvious - as speaking and swallowing become increasingly impaired, patients will inevitably face progressive paralysis become wheelchair- and bed-bound. In later stages, as paralysis reaches the respiratory muscles and the diaphragm, breathing becomes difficult, leading to the requirement of respiratory support and ultimately death by respiratory failure *(Pasinelli and Brown, 2006; Gonzalez de Aguilar et al., 2007; Kiernan et al., 2011)*.

The presence of upper und lower motor neuron damage that are not linked to other causes, are clinical criteria of ALS. As there is no precise test for ALS, and the disease remains partly unknown, diagnosis relies on the symptoms and general tests (EMG, MRI,

biopsies, blood tests...) to rule out other diseases such as a viral infection, peripheral neuropathy, a myopathy or cervical spondylosis, to name a few. As such, diagnosis is based on the Airlie House *(Traynor et al., 2000)* and the El Escorial criteria from 1998, revisited in 2000 *(Brooks et al., 2000)* (Table 3).

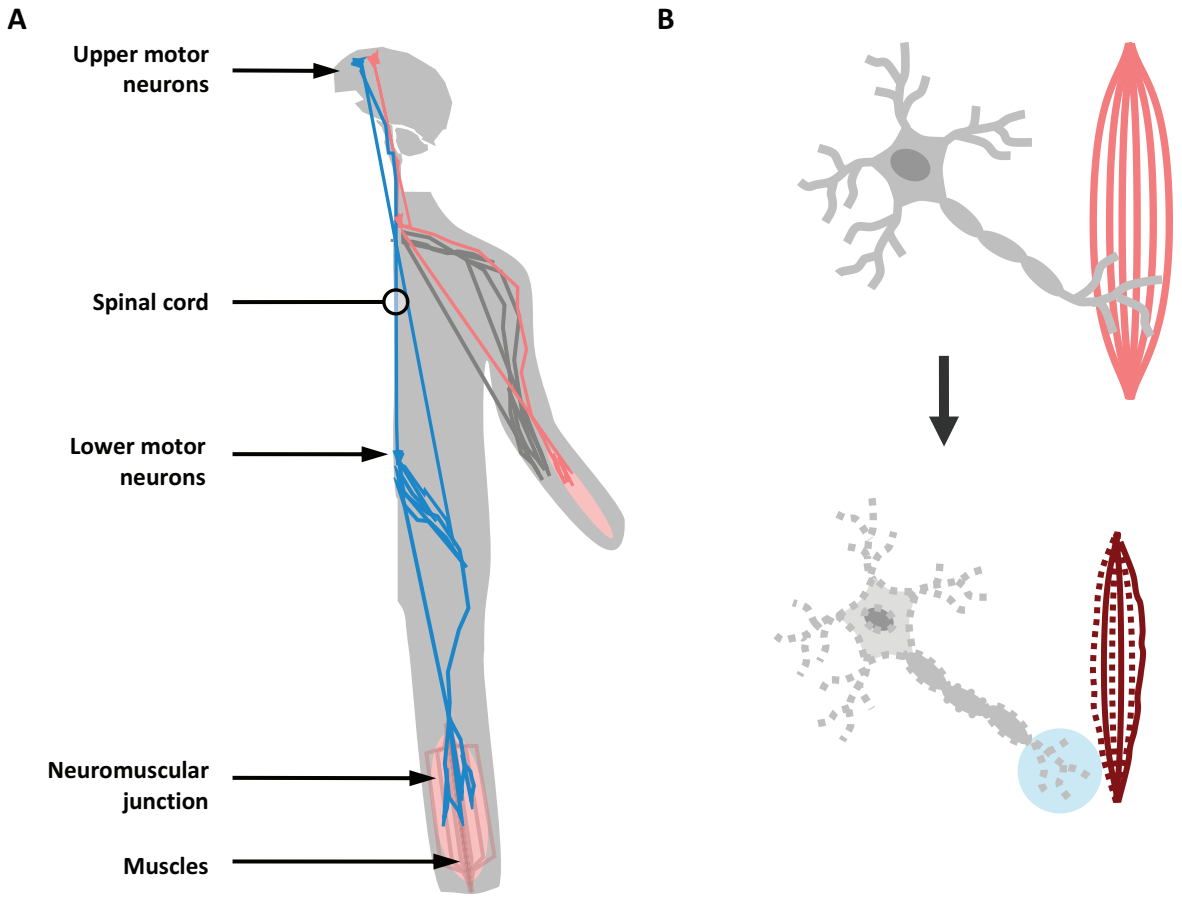

Figure 15 | **Main events during Amyotrophic Lateral Sclerosis**
(A) Motor axis within the body and (B) simplified view. During ALS, motor neurons die, the neuromuscular junction is dismanteled and muscles atrophy.

III - 3.2. Etiology

First scientifically described by Jean-Martin Charcot in 1869, ALS is among the most frequent neuromuscular diseases with a worldwide incidence of 1-2 per 100.000. There are, however, geographical differences, and statistics from many parts of the world are lacking. Prevalence is between 5-7 per 100.000, so yearly, there are about 5000 new cases in the US and 500 in France. The disease is diagnosed mainly for people between

40 and 60 years of age. Unfortunately, no cure exists and the only approved treatment for ALS, riluzole, merely prolongs survival by a few months *(McCombe and Henderson, 2010; Kiernan et al., 2011)*.

Diagnosis of ALS requires:

- The presence of: (a) evidence of lower motor neuron degeneration by clinical, electrophysiological, or neuropathological examination; (b) evidence of upper motor neuron degeneration by clinical examination; and (c) progression of the motor syndrome within a region or to other regions, as determined by history or examination; and
- The absence of: (a) electrophysiological and pathological evidence of other disease processes that might explain the signs of lower or upper motor neuron degeneration; and (b) neuroimaging evidence of other disease processes that might explain the observed clinical and electrophysiological signs.

El Escorial criteria

- Definite ALS: upper and lower motor neuron signs in three regions.
- Probable ALS: upper and lower motor neuron signs in at least two regions with upper motor neuron signs rostral to lower motor neuron signs.
- Possible ALS: upper and lower motor neuron signs in one region, upper motor neuron signs alone in two or more regions, or lower motor neuron signs above upper motor neuron signs.
- Suspected ALS: lower motor neuron signs only in two or more regions.

Airlie House (modified) criteria

- Clinically definite ALS: clinical evidence alone of upper and lower motor neuron signs in three regions.
- Clinically probable ALS: clinical evidence alone of upper and lower motor neuron signs in at least two regions with some upper motor neuron signs above (rostral) the lower motor neuron signs.
- Clinically probable/laboratory-supported ALS: clinical signs of upper and lower motor neuron dysfunction are in only one region, or upper motor neuron signs alone in one region with lower motor neuron signs defined by electromyography criteria in at least two limbs, together with proper application of neuroimaging and clinical laboratory protocols to exclude other causes.
- Possible ALS: clinical signs of upper and lower motor neuron dysfunction in only one region, or upper motor neuron signs alone in two or more regions; or lower motor neuron signs rostral to upper motor neuron signs and the diagnosis of clinically probable-laboratory-supported ALS cannot be proven.
- Suspected ALS: this category has been deleted from the revised El Escorial criteria.

Table 3 | **Diagnostic criteria for Amyotrophic Lateral Sclerosis**
Derived from modified Airlie House *(Traynor et al., 2000)* and the revisited El Escorial criteria *(Brooks et al., 2000)*

Epidemiological data about ALS is difficult to gather because the "real" onset date is unknown. Animal studies have shown that there is an important delay between the first

molecular changes and the appearance of measurable criteria such as reduced nerve conductance and the onset of clinical symptoms - which is the case when patients start experiencing problems and seek for medical attention. Finally, unreported cases and wrong diagnostics blur the comprehension of ALS.

Interestingly, about 90% of all reported cases are sporadic, with no hereditary history and known cause. However, about 5-10% of all patients inherited ALS (familial ALS or FALS) in an autosomal dominant fashion *(Byrne et al., 2011)*. Surprisingly, the average age of onset is different in both cases. While the age of onset peaks at around 60 years of age for sporadic ALS, onset for familial ALS peaks at around 50 years of age *(McCombe and Henderson, 2010; Kiernan et al., 2011)*.

III - 3.3. Genetics of ALS

It was long known that about 10% of all ALS cases are inherited. While an approximate location of a frequently mutated gene was determined on chromosome 21 in 1991, it was reported in 1993 that the most frequently mutated gene in familial ALS (until recently) was encoding the cytosolic Cu/Zn superoxide dismutase (SOD1). This protein accounts for 20% of inherited cases and participates in superoxide $O_2^{\cdot -}$ detoxification *(Rosen et al., 1993)*. Mutations in SOD1 also occur in sporadic ALS and have generally been studied to great depth. Interestingly, by studying affected families, other genes involved in completely different cellular functions like for example TARDBP, FUS or ANG (genes involved in RNA processing and metabolism), have been identified *(Strong et al., 2010)*. A total of 16 genes has currently been identified as sufficient to induce ALS (Table 2). The current challenge is to understand how genes involved in very different processes lead to common cellular events (neuronal death, muscle atrophy) and ultimately the same characteristic symptoms of ALS.

C9ORF72

I would like to quickly introduce a gene that has recently (by the end of 2011) become the most interesting new gene to work on in ALS. For the time being, not much is known about this gene, with the exception that it is located on the inherited, mutated, locus of chromosome 9p21. This region contains an open reading frame coding for a protein and

has thus been termed C9ORF72. The mutations in this gene result in the expansion of hexanucleotide repeats (GGGGCC) of different lengths. Sequence analysis has shown that the C9ORF72 sequence is highly conserved among species, yet its function remains unknown. In a back-to-back publication in Neuron, two groups have independently published similar results. One group analyzed the occurrence of this mutated gene in finnish families affected by ALS and found that about 46% of all inherited cases, as well as 21% of all sporadic cases, are due to a mutation in C9ORF72 *(Renton et al., 2011)*. The second group examined an independent set of patients in Canada and determined that in this population, about 24% of all inherited cases, and 4% of sporadic cases are due to C9ORF72 mutation *(DeJesus-Hernandez et al., 2011)*.

Gene	Locus	Protein	Found in cell. inclusions	ALS subtype	Other
Autosomal dominant FALS genes also implicated in SALS					
SOD1	21q22.1	Cu,Zn superoxide dismutase (SOD)	+	ALS1	Can be recessive in FALS
FUS	16p11.2	Fused in sarcoma (FUS)	+	ALS6	Can be recessive in FALS
ANG	14q11.1	Angiogenin (ANG)		ALS9	Autosomal Dominant or Haploinsufficient
TARDBP	1p36.22	TAR DNA Binding Prot.-43 (TDP-43)	+	ALS10	Can be recessive in FALS
OPTN	10p13	Optineurin	+	ALS12	Newly characterized
C9ORF72	9p21	C9ORF72	?	ALS-FTD	*Most frequent*
Autosomal dominant FALS genes					
ALS3	18q21	ALS3		ALS3	
SETX	9q34.13	Senataxin		ALS4	Can cause juvenile onset
ALS7	20p13	ALS7		ALS7	
VAPB	20q13.33	vAMP-associated protein B	+	ALS8	Can cause juvenile onset
FIG4	6q21	Phosphoinositide 5-phosphatase		ALS11	
VCP	9p13.3	valosin-containing protein		ALS14	
Autosomal recessive FALS genes					
ALS2	2q33.1	Alsin		ALS2	Can cause juvenile onset
SPG11	15q15.1	Spatacsin		ALS5	Can cause juvenile onset
X-linked dominant FALS gene					
UBQLN2	Xp11.2	Ubiquilin-2	+	ALS15	Can cause juvenile onset
Other genes					
ATXN2	12q24.1	Ataxin-2		ALS13	Increases ALS susceptibility

Table 4 | **Common genes involved in ALS**
Overview of genes causing ALS discovered over the years. From *Pratt et al., 2012*

Since the first reports of C9ORF72 mutations, the results have been confirmed in other countries, for example in Italy or Spain. New results suggest that translated dipeptide products from the repeated region (and not the protein itself) are toxic in neurons by forming aggregates *(Buchman et al., 2013)*. Given the high prevalence of C9ORF72 mutations, it will be very important to elucidate the function of this gene and to generate a new ALS mouse model based on it.

Cu/Zn superoxide dismutase

As mentioned above, SOD1 is particularly frequent in inherited ALS (about 20%). The enzyme catalyzes the dismutation of a toxic byproduct of oxidative phosphorylation, the superoxide radical anion ($O_2^{\cdot-}$) into oxygen and H_2O_2. Catalases or peroxidases then detoxify the latter molecule. Over the years, more than 150 different mutations of SOD1 have been identified in patients suffering from ALS *(Turner and Talbot, 2008)*. For example, the most frequent SOD1 mutation in northern America is the alanine 4 substitution to valine (A4V). Closer investigation has revealed that in general, such alterations lead to a gain-of-function phenotype. Indeed, mice deficient of SOD1 do not develop ALS *(Reaume et al., 1996)* but the expression of mutated SOD1 proteins leads to the ALS symptoms, while still retaining SOD1 activity (for most mutations) *(Gurney et al., 1994).* However, mice lacking SOD1 were much more sensitive to loss of nerves after induced injury. Thus, the enzyme appears to be essential for survival *(Reaume et al., 1996).*

Research has shown in mouse models of ALS harbouring an SOD1 mutation, that the enzyme forms toxic filamentous aggregates (Lewy body-like) in neurons and supporting glial cells, even before the onset on symptoms. The mutations cluster in regions, affecting structural integrity of the protein, thus reducing stability and promoting aggregation *(DiDonato et al., 2003)*. Those aggregates contain misfolded SOD1 and other proteins such as the 14-3-3 family and are thought to be the result of interaction between misfolded SOD1 and the heatshock protein Hsp70-8 *(Zetterström et al., 2011, Forsberg et al., 2011)*. The sequestration of anti-apoptotic proteins (like 14-3-3σ) is thought to contribute to neuronal death *(Okamoto et al., 2011)*.

Additionally, in patients with sporadic or hereditary ALS, SOD1 accumulates in "small, granular non-ubiquitin reactive inclusions" in spinal motor neurons. However, such inclusions were also found in the cytosol or the nucleus of patients with no SOD1

mutation *(Forsberg et al., 2010, 2011)*. Therefore, it is difficult to state that SOD1 aggregates are only the result of the mutations.

III - 3.4. Pathophysiology of SOD1 mutations

Although many mutated genes can cause ALS, the effects of mutated SOD1 remain the most studied field. Because my work was based on cellular and murine SOD1 models, I will now focus on the intracellular effects of SOD1 aggregates. Their effects are drastic and lead to many different problems (reviewed by *Ilieva et al., 2009; Turner et al., 2013*), that result in the death of motor neurons.

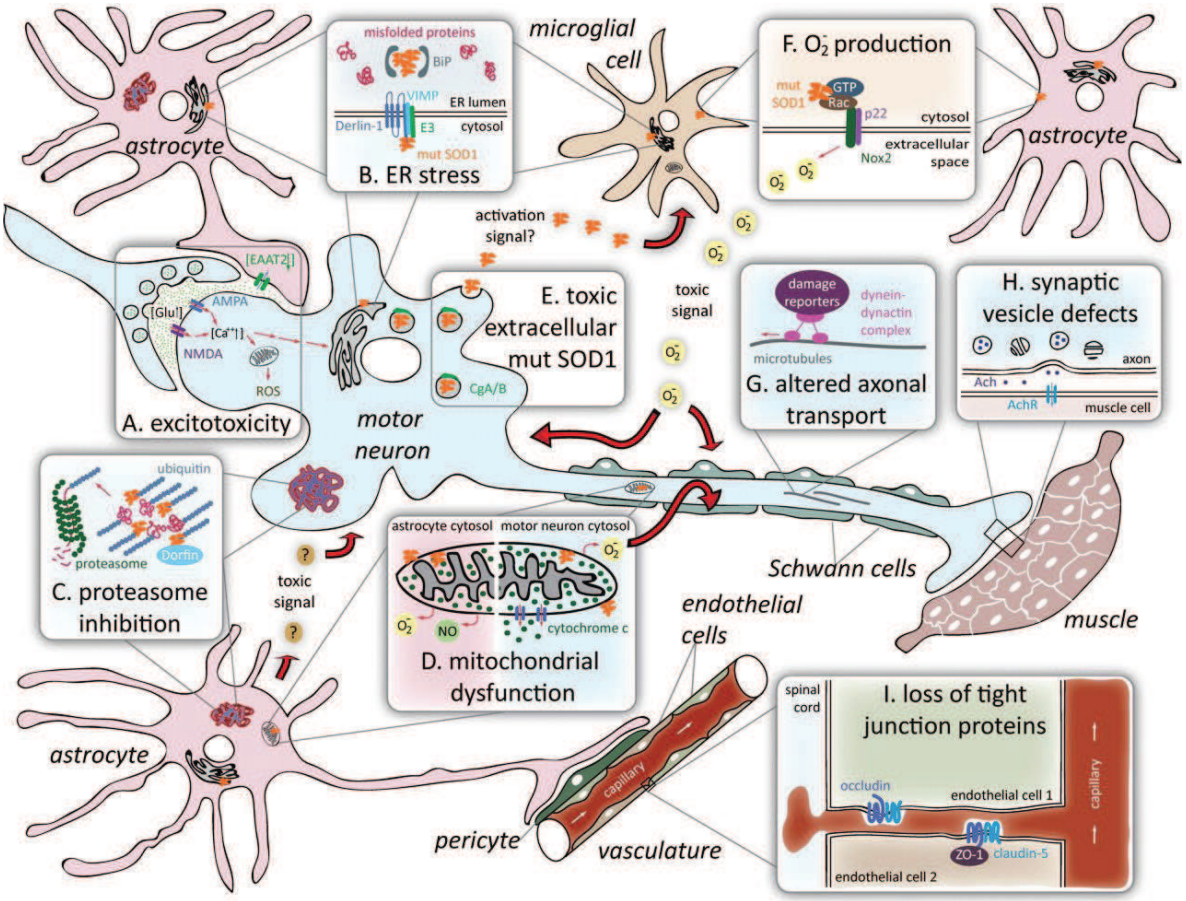

Figure 16 | **Mechanisms of mutated SOD1 toxicity in the nervous system**
Eight very different mechanisms can contribute to neuronal death when mutated SOD1 is present. From *Ilieva et al., 2009*

Aggregation of SOD1 proteins remains the number one hypothesis regarding the outcome of most known mutations. Several mechanisms of SOD1 toxicity have been proposed (Figure 16):

- An early proposed mechanism is glutamate excitotoxicity of the motor neurons. Glutamate is the excitatory neurotransmitter released by upper motor neurons. Losing the connectivity between upper and lower motorneurons reduces the expression of the EAAT2 glutamate transporters in supporting astrocytes. This leads to a failure in glutamate uptake from synapses to stop excitation. As a consequence, post-synaptic neurons get overactivated and their Calcium stocks overwhelmed, which leads to cell death *(Bruijn et al., 1997; Yang et al., 2009)*.
- Mutated SOD1 proteins aggregate in association with the endoplasmic reticulum (ER). Furthermore, ER stress is induced by abnormal interactions of mutant SOD1 with ER proteins such as BiP *(Kikuchi et al., 2006)* or derlin-1 *(Nishitoh et al., 2008)*. Additionally, this inhibits degradation of other proteins, usually transported out of the ER to be processed by the uqiquitin-proteasome system.

  To mimick ER stress *in vitro* (and actually also *in vivo*), it is possible to pharmacologically induce it with the compound *Tunicamycin*. This drug inhibits N-glycosylation, which leads to accumulation of misfolded proteins in the ER and thus induces the signalling cascade of ER stress.
- Mutations in SOD1 proteins can also lead to proteasome inhibition as a result of "overload" with ubiquitinated misfolded protein aggregates, which may damage astrocytes and motor neurons *(Bruijn et al., 1998)*. It is also possible to pharmacologically inhibit the proteasome with the compound *MG132*.
- Mitochondrial dysfunction mediated by mutant SOD1 deposition on the mitochondrial membrane in motor neurons provokes release of cytochrome c that can trigger apoptosis *(Mattiazzi et al., 2002; Vande Velde et al., 2008)*. In astrocytes, cytochrome C release leads to nitroxidative stress *(Cassina et al., 2008)*. The latter phenomenon, oxidative stress in general, can be pharmacologically induced with *Vitamin K* or *Menadione*.
- Microglial cells (from the immune system) can induce neuronal death when activated by extracellular mutant SOD1 *(Zhao et al., 2010)*. SOD1 is secreted from

motor neurons and astrocytes after binding with components of neurosecretory vesicles - chromogranins *(Urushitani et al., 2006)*.

- Mutant SOD1 can bind with Rac1 to activate the NADPH oxidase subunit Nox2. This leads to extracellular superoxide accumulation from microglia or astrocytes that can damage neighboring motor neurons *(Harraz et al., 2008)*.
- Altered SOD1 proteins can interfere with the organization of the cytoskeleton and alter axonal transport. Furthermore, cell stress and death related proteins are increasingly retrogradely transported *Perlson et al., 2009)*.
- Affinity of mutated SOD for membranes presumably leads to defects in synaptic vesicles (stalling and loss from distal synapse) in vulnerable motor neurons *(Pun et al., 2006)*.
- Damage of vasculatory cells by destabilization of tight junctions results in leakage of toxic products in surrounding tissue. Indeed, in the spinal cord, microhemorrhagies have been observed early in the disease *(Zhong et al., 2008)*.

It is important to note that the studies regarding the toxic effects of SOD1 have been extensively performed in neurons and their glial support-cells. Information of SOD1 effects in muscles however is scarce.

III - 3.5. Mouse models

Over the years, many mouse models of ALS have been introduced and used. The most studied ones, unsurprisingly, are mice harbouring a SOD1 mutation, as mis-sense mutations in the SOD1 gene were the first identified cause for ALS "back in the days" *(Rosen et al., 1993)*. Spirits were high as researchers finally had a mouse model to elucidate basic mechanisms. The basis of most of our knowledge of ALS arises from the first SOD1 models. However, more and more SOD1 mutations appeared in families and different ALS-SOD1 mice were created. At the same time, more genes that could lead to ALS were identified.

The most widely used mouse model for ALS, is the transgenic SOD1(G93A) mouse in a C57BL/6 background. It expresses approximately 20-24 copies of the human coding sequence, under control of the human promoter *(Gurney et al., 1994)*. This mouse model

was quickly adopted and until now over twenty other models have been created. The one we used in our experiments overexpresses in a FVB background a mutated SOD1 where a Glycine residue is substituted with Arginine in position 86, hence the name SOD1(G86R). In contrast to the SOD1(G93A) model, the SOD1 gene used in the SOD1(G86R) model is of murine origin and has been mutated at the equivalent position of a human (fALS) mutation. Furthermore, the promoter also originates from a mouse. The expression level is similar to the wild-type SOD1 *(Ripps et al., 1995),* thus closer mimicking expression levels found in ALS patients. An advantage of this model is that the mutated SOD1 is less stable that its G93A counterpart. Combined with lower expression levels, this leads to less SOD1 aggregates, while still leading to ALS symptoms with a similar time course *(Morrison et al., 1998a, b)*. Motor neuron loss does not start prior to the onset of symptoms.

At 75 days of age, SOD1(G85R) mice have no marked motor phenotype. At about 90 days of age, denervation is ongoing, mice start to exhibit altered hind limb extension reflexes and electromyogram analysis as well as Acetylcholine Receptor α confirm that the neuromuscular junctions get altered. The following two weeks are marked by progressive weakening, leading to full paralysis at about 105 days of age Up to 50% of motor neurons are lost at this point *(Morrison et al., 1998)*.

As pointed out earlier, as of today, 16 different mutations have been described to cause ALS in humans and several have a corresponding mouse model (SOD1, FUS, TDP-43, VCP). However, it seems as though only SOD1 mouse models reproduce the fuller spectrum of ALS symptoms. C9ORF72 models are currently under development.

IV - THE P53 FAMILY

IV - 1. Generalities

The p53 protein family comprises three members, conveniently named p53, p63 and p73. They play a central role in cellular processes such as apoptosis, cell cycle arrest, response to DNA damage, cellular differentiation and metabolism. The family owes of course its name to p53. It was discovered almost 35 years ago, when cancer researchers focused on viruses causing cancer, by 5 independent groups. It was at first considered an oncogene for its ability to interact with the oncogenic large T antigen from the SV40 virus *(Lane and Crawford, 1979; Linzer and Levine; 1979; Kress et al., 1979; Melero et al., 1979; Smith et al., 1979)*. As it had an apparent mass of 53kD, it was termed p53 in 1983 at the first *International p53 Workshop*. This is most fortunate because the real molecular mass of this protein is 43.7kD. No one would have liked the "p44" family... Many years of research later finally established p53 as a transcription factor that protects the genome *(Farmer et al., 1992; Lane, 1992)*. As such, its most prominent function is the induction of apoptosis. By inducing the death of overly mutated cells, it protects the body against tumors. Knockout mice lacking the p53 gene are viable but are more susceptible to spontaneous tumors *(Donehower et al., 1992)*. They may also have reduced fertility and altered testis *(Rotter et al., 1993)* or develop exencephaly, as the neural tube did not close during embryogenesis *(Sah et al., 1995)*.

IV - 1.1. Building the family

About 15 years ago, two similar proteins to p53 were discovered: the transcription factors p63 *(Yang et al., 1998)* and p73 *(Kaghad et al., 1997)*. They exhibit a high sequence similarity to p53 but their function seemed to be more on the development-side. When knocking down p63 in mice, the phenotype is severe: mice are born alive but die within a few hours of birth. Furthermore, they completely lack limbs, a stratified epidermis and epithelial tissues such as lachrymal glands, teeth or hair follicles and present abnormalities in heart development *(Mills et al., 1999; Yang et al., 1999; Rouleau et al., 2011)*. This points to a central role of p63 in epithelial development.

Mice lacking the p73 gene are also born alive; however, they develop slowly and have a high mortality rate during the first 2 months. They have a striking phenotype with hydrocephalus, dysgenesis of the hippocampus and defects in the vomeronasal organ, essential for detecting pheromones and thus crucial for social behaviour. For example, $p73^{-/-}$ mice do not mate. This phenotype suggests an important role of p73 in neuronal development. Furthermore, $p73^{-/-}$ mice suffer from chronic infections and inflammation *(Yang et al., 2000)*.

IV - 1.2. Structure

The proteins from the p53 family are homologous transcription factors. They feature a similar structure and each member has several isoforms (Figure 17).

a. The DNA-binding domain

The highest similarity between all members is found in the central DNA-binding domain (DBD). p63 and p73 share about 65% identity with p53, and about 85% of identity between themselves. The DNA-binding domain is crucial for sequence specific binding to response elements of the p53 family proteins in order to activate transcription of specific genes *(Vousden and Lu, 2002).* The high similarity in the DBD suggests that the members are able to bind similar DNA sequences. Indeed, while each member regulates its specific target genes, all 3 members are also able to activate the same subset of targets, such as genes implicated in DNA damage response and apoptosis (p21, GADD45α, Puma, Noxa, Bax…) *(Harms et al., 2004; Bernassola et al., 2005; Lin et al., 2009)*. Interestingly, they can also bind to each others promoter *(Stiewe, 2007)*. The isoform ΔNp73 for example, is able to bind the p53 response element *(Stiewe et al., 2002)*.

b. The oligomerization domain

Members of the p53 family actually bind to DNA as tetramers through an oligomerization domain (OD) in the carboxy-terminal region. Mutations in the OD of p53 inhibit tetramer formation and impede p53 function, indicating that tetramerization is a necessity *(Davison et al., 2001)*. The importance of the OD is also

highlighted by the fact that OD mutations have been linked to cancer formation *(Lomax et al., 1998)*. While wild-type p53 proteins cannot form heterotetramers with the other members, mutant forms of p53 can oligomerize with p63 or p73, inhibiting their function *(Davison et al., 2001; Gaiddon et al., 2001; Joerger et al., 2009)*. Interestingly, p63 and p73 are able to form functional heterotetramers by associating through their OD *(Davison et al., 1999; Coutandin et al., 2009)*.

c. The transactivation domain

Recruitment of the basic transcriptional machinery is achieved through the amino-terminal transactivation domain, which is surprisingly different between the members (about 25% identity between p53 and the others, and 40% between p63 and p73) *(Stiewe et al., 2007; Su et al., 2013)*. Each of the three genes has a second intronic promoter, leading to the generation of 2 main isoforms for each gene. The first one generates p53, TAp63 and TAp73, which all contain the transactivation domain and are thus capable of activating transcription. So called ΔN isoforms are generated through the second promoter and do not contain the transactivation domain. While they still bind to DNA, they can not interact with the basic transcription machinery and counteract, by competition, the activity of "TA" isoforms *(Yang et al., 2002)*. For example, it quickly became clear that ΔNp63 can inhibit DNA binding of TAp63 and p53 *(Yang et al., 1998)*. Similarly, ΔNp73 is able to inhibit TAp73 and p53 binding to their respective promoter *(Grob et al., 2001; Benard et al., 2003)*. However, more recent reports suggest that ΔNp63 or ΔNp73 still retain transcriptional activity, likely due to a small N-terminal domain that differs from the classic "TA" domain *(Lin et al., 2009)*.

For a very long time no other isoforms of p53 were known to arise from its gene. However, recent research has found that the p53 gene can also produce amino-terminal truncated isoforms through an intronic promoter (Δ133p53, Δ160p53). Furthermore, the p53 and p73 genes can generate further "TA" variants through alternative splicing (Δ40p53, ex2p73, ex2/3p73) *(Murray-Zmijewski et al., 2006; Marcel et al., 2011; Wei et al. 2012)*.

d. The carboxy-terminal domain

As if 3 family members and 2 main transcripts per gene leading to proteins with antagonistic functions were not enough, nature has come up with more isoforms.

Alternative splicing of p53, p63 and p73 transcripts at the 3' end gives rise to different transcriptional variants, translated into proteins of different size and composition in the carboxy-terminal region. For p53, 6 different variants are known (p53α, β, γ, δ, ε, ζ, ΔE6) resulting mainly from splicing in the exon 9 *(Bourdon et al., 2005; Hofstetter et al., 2010)*. For p63, there are 5 known splicing variants (p63α-ε) *(Mangiulli et al., 2009)*, whereas for p73, 9 different isoforms have been characterized (p73α to η1) *(de Laurenzi et al., 1999; Ueda et al., 1999; Wei et al., 2012)*.

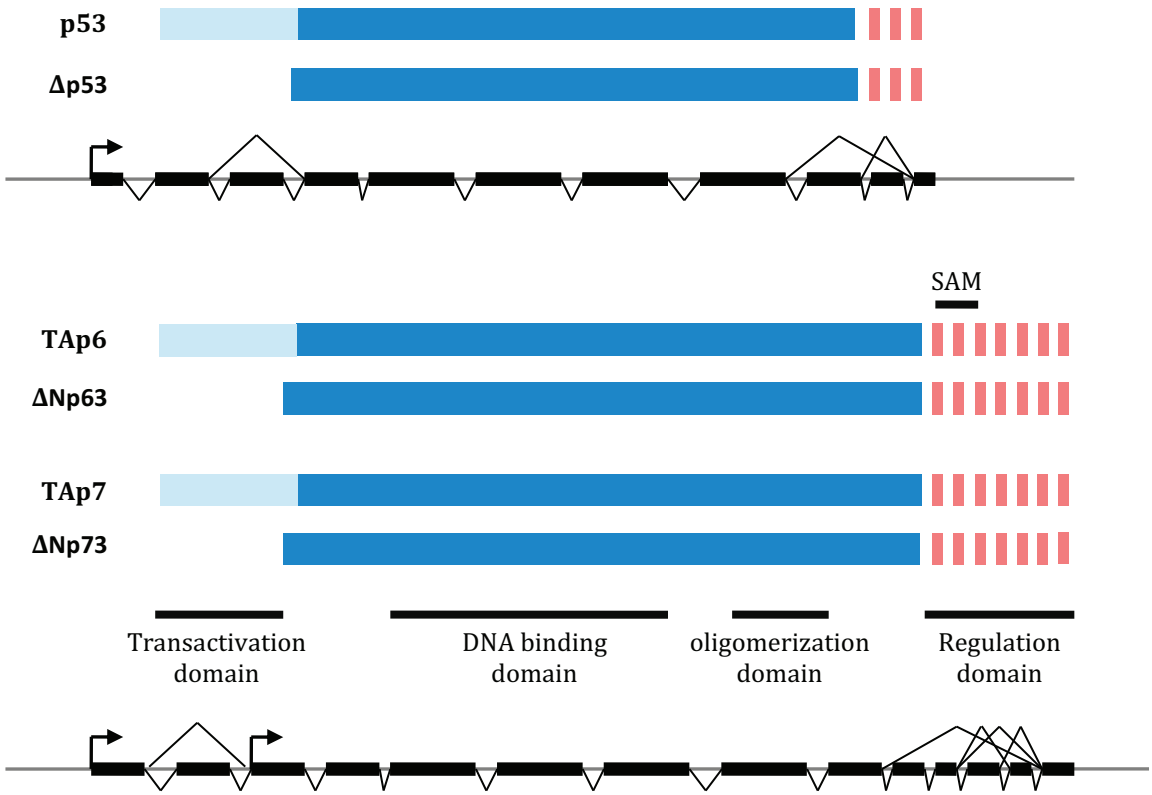

Figure 17 | **Schematic diagram of the main members of the p53 family**
All members of the p53 family contain a DNA binding domain and an oligomerization domain. In simple terms, presence or absence of the transactivation domain dictates their ability to activate or inhibit transcription. Finally, variants in the c-terminal domain confer the ability to fine-tune the response. Gene structure with alternative promoters and alternative splicing sites is simplified.

When combining the carboy-terminal with the amino-terminal variants, the p53, p63 and p73 genes could theoretically produce 28, 10 and 36 different transcripts and proteins, respectively. Among the longer isoforms of p63 and p73 (for example p63α),

several contain a protein-protein interaction domain called sterile alpha motif (SAM) that is not found in p53 *(Chi et al., 1999)*. While the different splicing variants have been characterized in sequence, complete information about their function is still missing. The vast majority of p53, p63, and p73 studies concentrate on the first known isoforms, α, β and γ and little is known about the other isoforms that have, in part, just recently emerged.

Even though the proteins of the p53 family share high identity, their central role in cells can be quite different. Differences in sequence and structure lead to different protein-protein interactions, differences in post-translational modifications and finally, of course, differences in the transcriptional activation of target genes. In the following section, I would like to introduce each member more in detail to give insight into the ever growing functions of the p53 family.

IV - 2. p53 – he was there first

p53 is a protein now known to play a role in cell cycle arrest, senescence, DNA damage response, apoptosis and metabolism. This is a myriad of very different functions, with the extreme of cell death one on side, and cell rescue on the other. How is this regulated?

IV - 2.1. Regulation of p53

In normal conditions, p53 is a protein with a short half-life and is thus usually undetectable in the absence of stress. There is no reason to keep a protein that may kill the cell. On the other hand, it's very useful for a cell to have somebody for "the dirty jobs" at hand quickly. So nature found a compromise: the p53 protein is mostly regulated at the post-translational level. Little is still known about the physiological transcriptional activation of p53 itself. A few transcription factors have been identified as positive upregulators of its transcription in response to cell stress. For example, the transcription factors HoxA5 and Pitx1 are able to activate p53 transcription, leading to apoptosis *(Liu and Lobie, 2007)*.

a. Taming p53 activity

p53 transcripts are translated normally but their life after that is in the hands of MDM2. This protein is p53's main regulator as it features E3 ubiquitin ligase activity targeted at p53. Ubiquitination of p53 after translation ensures a low level of p53 under unstress conditions of the cell, as p53 is targeted for degradation by the ubiquitin-proteasome way *(Toledo and Wahl, 2006)*. At the same time, MDM2 is also a transcriptional target of p53, providing a negative feedback loop *(Honda et al., 1997; Kubbutat et al., 1997)*. Additionally, MDM2 is capable of auto-ubiquitination allowing the p53-MDM2 complex to rapidly disengage in order to permit p53 activation *(Honda et al., 1997)*.

Pirh2 and COP1 are other, more recently discovered E3 ubiquitin ligases that regulate p53 activity in a similar feedback regulatory loop *(Leng et al., 2003; Dornan et al., 2004, Brooks and Gu, 2006)*.

b. Activating p53 – post-translational modifications

Not being ubiquitinated by MDM2 is not sufficient for transcriptional activation. Because of the importance of the processes that p53 governs, p53 undergoes additional steps to ensure that it works as it is supposed to. Extensive phosphorylation regulates p53 activity (for example by disrupting interaction with MDM2) and gene selectivity. Most phosphorylation sites are present in the amino-terminal transactivation domain *(Bode and Dong, 2004)*. For example, upon induction of DNA damage by γ-irradiation, the kinase ATM is induced and activates p53 by phosphorylating Serine 15. ATM also phosphorylates the Chk2 kinase, which in turn phosphorylates p53 on Serine 20 *(Shiloh, 2001; Dornan 2003)*.

Similarly to phosphorylation, p53 can also be acetylated to inhibit ubiquitination. It has been demonstrated that acetylation by Tip60 at Lysine 120 (in the DBD) and p300/CBP at Lysine 164 is essential for p53 transcriptional activity. Additionally, p53 can also be acetylated in the c-terminus domain, for example at Lysine 373 or 382 by p300/CBP *(Tang et al., 2008; Beckerman and Prives, 2010)* which activates p21 *(Zhao et al., 2006)*.

Finally, to ensure transactivation by p53, the promoter of the target genes must be accessible. Acetylation of histones promotes a relaxed state of chromatin and allows access to DNA binding sites. By interacting with p300/CBP, p53 promotes acetylation of histones surrounding its response elements *(Brooks and Gu, 2003)*.

Other post-translational modifications for p53 have been described: Neddylation and Sumoylation are processes similar to ubiquitination, that in the case of p53 influence its stability and transcriptional activity *(Brooks and Gu, 2003)*. For example, sumoylation of p53's lysine 386 (in the carboxy-terminal domain) increases its transcriptional activity *(Müller et al., 2000)*, while Neddlyation of lysines 370, 372 and 373 (also in the carboxy-terminal domain) mediated by MDM2, inhibit its transcriptional activity *(Xirodimas et al., 2004)*.

IV - 2.2. Stopping the cell cycle

p53 reacts to multiple signals such as DNA damage or oncogene expression in order to stop the cell cycle and to induce DNA repair and senescence or, on the contrary, cell death by apoptosis.
The choice depends on the phosphorylation and acetylation pattern of p53, modulating the binding affinity of p53 to certain promoters. Thus, promoter constitution and abundance of p53 proteins also influence the transcriptional activity *(Murray-Zmijewski et al., 2008)*. All those regulations are vast and I will thus present only the main findings, helping to understand the basic functions of p53.

Progression in the cell cycle is controlled by cyclins and cyclin-dependent-kinases (CDKs) *(Nurse, 2000)*. Upon UV radiation for example, p53 can stop the cell cycle in G1, when transcription errors have been detected, by transcriptionally activating p21, which will block the cell cycle through binding and inhibition of the CDK1, 2, 4 and 6 *(Harper et al., 1993; el-Deiry et al., 1993; Gartel and Radhakrishnan, 2005)*. The p53 protein can also stop the cell cycle in G2, when replication errors have been detected, by inducing transcription of GADD45α and 14-3-3σ, inhibiting cyclin B/Cdc2 activity *(Hermeking et al., 1997; Zhan et al., 1999; Jin et al., 2000)*.

DNA repair can be the next step after stopping the cell cycle. GADD45α, activated by p53, participates in this process by interacting with histones, thus altering chromatin configuration and facilitating access for proteins of the DNA-repair pathways *(Carrier et al., 1999)*. Furthermore, p53 activates the transcription of p48/XPE and XPC, which recognize DNA adducts and promote nucleotide-excision repair *(Hwang et al., 1999;*

Shimizu et al. 2003; Fitch et al., 2003). Interestingly, p53 is also required for base excision repair, where it interacts independently of transcription with the AP endonuclease (APE) and with DNA polymerase β (polβ), two enzymes that are crucial for this DNA-repair pathway *(Zhou et al., 2001)*. p53 stimulates the interaction of the APE with polβ and stabilizes the interaction of polβ with DNA.
Senescence can also be promoted by p53 through activation of p15, p16 and p21. In contrast to p21, p15 and p16 permanently bind to and inhibit CDK4 and CDK6, thus permanently inhibiting the cell cycle progression and preventing mutated cells to transmit their genomic content *(Di Leonardo et al., 1994; Hall et al., 1995; Serrano et al., 1997)*.

By stopping the cell cycle, p53 is thought to provide time to the cellular machinery to "sort out problems" and by inducing genes implicated in DNA-repair or inducing senescence. It directly contributes to the maintenance of genome integrity, before cell division and the spreading of potential harmful mutations through generations of cells. However, sometimes, even p53 has to give up... When accumulated DNA damage becomes too important, p53 induces apoptosis. This is demonstrated for example in experiments where DNA repair was induced when cells were treated with low doses of cisplatin (creating DNA adducts) or γ-irradiation (modifying bases or breaking DNA). On the contrary, at high doses, apoptosis was induced *(Offer et al., 2002)*. When taking together those effects, p53 can be seen as the protector of the somatic cell line.

IV - 2.3. Induction of apoptosis

One of the first and most thoroughly studied functions of p53 is the induction of apoptosis. In addition to the aforementioned functions, this capacity completes its features of tumor suppression. The importance of p53 in this progress is highlighted by the fact that over 50% of human cancers exhibit mutations or deletions of p53 *(Murray-Zmijewski et al., 2006)*. Additionally, apoptosis is lost in tumors of p53-/- mice *(Parant and Lozano, 2003; Symonds et al., 1994)*.
The apoptotic programme is rather complex and can be subdivided into two main pathways, extrinsic and intrinsic (Figure 18).

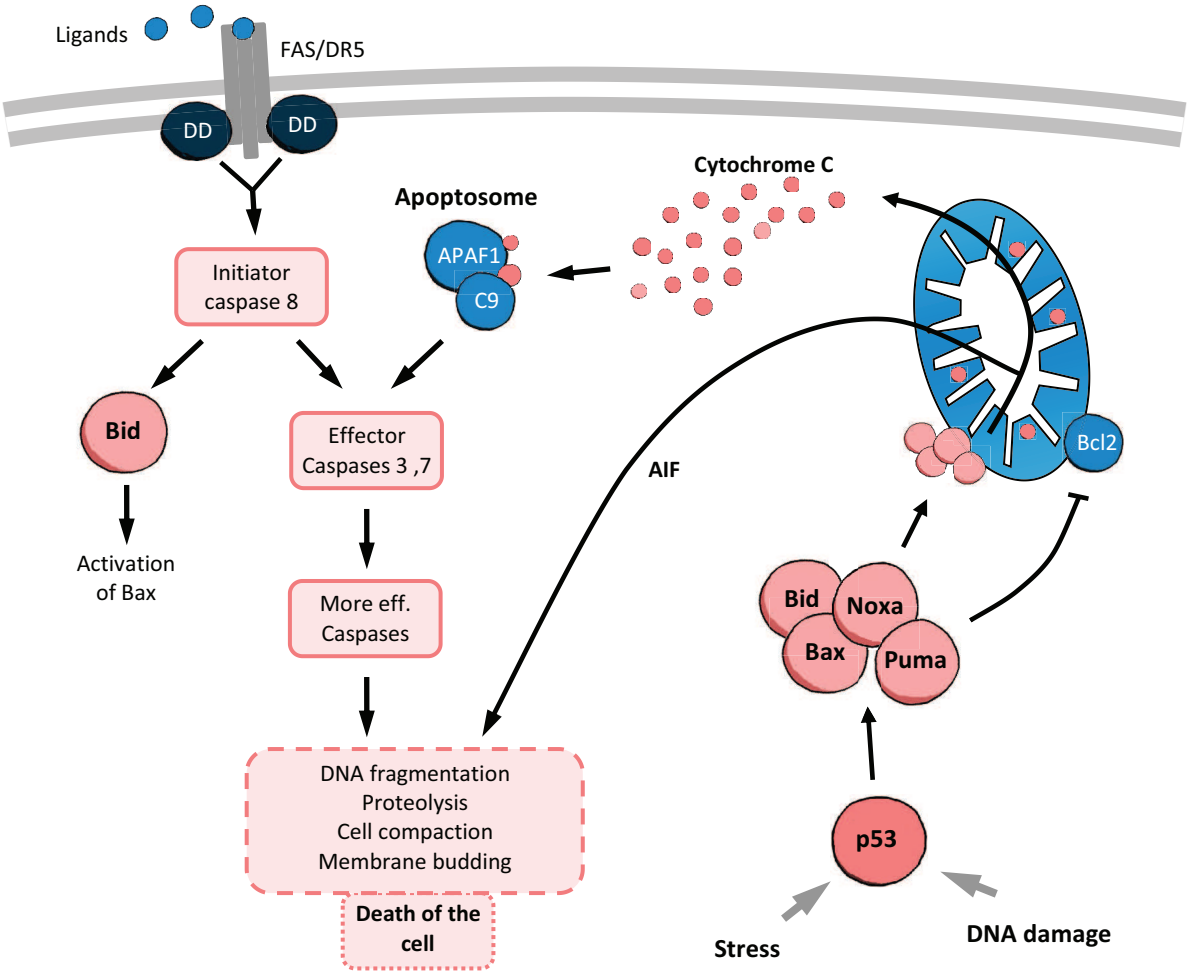

Figure 18 | **The extrinsic and intrinsic apoptosis pathway.**
Briefly, upon ligand-binding to death-domain receptors, the initiator caspase 8 is activated, leading in turn to activation of effector caspases, responsible for actual cell disruption.
Upon cellular stress such as DNA damage, p53 transcriptionally activates the pro-apoptotic Bcl-2 family members that will mediate cytochrome C and AIF release, leading to activation of caspases and cell disruption.

The extrinsic apoptosis pathway relies on cell-surface receptors with "death domains" such as DR5 and Fas to induce apoptosis by external stimuli (inflammation or immunological stimulation). Upon ligand binding (Fas ligand or TNFα), the death domain of the receptors binds to adapter proteins and activate the initiator (pro)caspase 8 by cleavage. The active caspase 8 subsequently activates effector caspases, such as Caspase 3 *(Thornberry and Lazebnik, 1998)*, triggering other caspases to achieve cellular degradation. Caspase 8 also communicates with the intrinsic

pathway, by cleaving and activating Bid, a pro-apoptotic Bcl-2 family member that participates in cytochrome c release from mitochondria by activating Bax *(Sax et al., 2002; Hail et al., 2006; Tait and Green, 2010)*. Interestingly, p53 participates in this pathway by transcriptionally activating the receptors Fas and DR5, as well as Caspase 8 *(Fukazawa et al., 1999; Wu et al., 1997; Liedtke et al., 2003)*.

The intrinsic apoptotic pathway relies on the mitochondria to release apoptotic factors. In order to induce apoptosis, p53 activates the transcription of several pro-apoptotic members of the Bcl-2 family: Bax, Bid, Puma and Noxa *(Fridman and Lowe, 2003)*. Bid activates Bax, while Noxa and Puma bind other, anti-apoptotic members (Bcl-2, Bcl-x) of the Bcl-2 family, increasing the ratio of pro- to anti-apoptotic proteins. This releases AIF which translocates to the nucleus to induce DNA fragmentation and condensation. More importantly, cytochrome C is also released from mitochondria inner membrane space, associates with APAF1 and the Caspase 9 to form the apoptosome. This complex then activates caspase 3 and other effector caspases (1, 3, 6, ...) *(Fridman and Lowe, 2003; Hail et al., 2006)*. This leads in turn to condensation and fragmentation of DNA, proteolysis, cell compaction and membrane budding (Figure 18). Cells stay impermeable while the cell membrane disrupts to encapsulate cellular components that will be cleared by macrophages *(Elmore, 2007).*

IV - 2.4. Regulation of Metabolism

In recent years, it has become apparent that p53 not only regulates cell life and death decision, but also metabolism. This actually nicely completes p53's tumor suppressor activity as tumors frequently have an altered metabolism that has to be tamed. Below are a few, non exhaustive examples of metabolic regulation by p53.

The mTOR protein is a central regulator of many cellular aspects including cell proliferation and growth, transcription and translation. It is part of two protein complexes called mTORC1 and 2. As a sensor of nutrient and oxygen availability, mTORC1 is active in "healthy" cells in presence of mitogens and promotes cell growth *(Howell and Manning, 2011)*. On the contrary, in inadequate growth conditions,

mTORC1 is inhibited by the TSC1/2 complex. p53 participates in this inhibition by activating Sestrin 1 and 2 expression upon genotoxic and oxidative stress *(Budanov and Karin, 2008)*. Both proteins are ROS scavengers aimed at protecting the cell, but they can also inhibit mTOR by promoting the activation of TSC2 through AMPK. Interestingly, upon genotoxic stress, p53 also directly induces the expression of mTOR negative regulators such as AMPKβ, TSC2 or Plk2 *(Feng et al., 2007; Matthew et al. 2009)*. In conditions of stress, the inhibition of mTOR helps the cells (and indeed p53) to reduce activity, thus minimizing the stress's negative consequences.

The regulation of glycolysis is central in cells. This process creates energy by breaking down glucose into ATP and pyruvate , which is later used for oxidative phosphorylation after processing in the Krebs cycle for further and efficient ATP production. Cancerous cells in majority favour to use glycolysis exclusively (Warburg effect) in order to proliferate faster. Metabolites of glycolysis can serve as precursors for the synthesis of nucleotides, amino acids and lipids *(Ward and Thompson, 2012)*. In line with its tumor-suppressor activity, p53 is able to repress glycolysis and promote oxidative phosphorylation, thus reducing tumor growth. The TP53-induced glycolysis and apoptosis regulator (TIGAR) is, as its name implies, activated by p53. This protein intervenes directly in glycolsis by inhibiting the rate-limiting enzyme PFK1. TIGAR dephosphorylates the intermediate sugar fructose-2,6-bisphosphate, an allosteric activator of PFK1. By doing so, TIGAR severely impairs glycolysis *(Bensaad et al., 2006; Li and Jogl, 2009)* and promotes the alternative pentose-phosphate pathway (PPP). However, p53 is also able to act upstream of glycolysis by decreasing intracellular glucose. p53 directly inhibits the expression of the Glucose transporters GLUT1 and GLUT4 and indirectly inhibits GLUT3 expression by modulating the NF-kB/IKK balance *(Schwartzenberg-Bar-Yoseph et al., 2004; Kawauchi et al., 2009)*.

In addition, p53 is also able to promote the Krebs cycle and oxidative phosphorylation. By activating the transcription of the glutaminase GLS2, p53 enhances production of glutamate and thus its metabolite α-ketoglutarate. This metabolite can directly enter the Krebs cycle and contribute to NADH production in mitochondria *(Suzuki et al., 2010, Hu et al., 2010)*. In order to contribute to oxidative phosphorylation, p53 transcriptionally activates SCO2, a key enzyme in the mitochondrial complex IV.

Finally, p53 is also responsible for some seemingly contradictory findings. By promoting hexokinase II expression *(Mathupala et al., 1997)*, a rate-limiting enzyme that transforms glucose into glucose-6-phosphate (G6P), p53 could promote glycolysis – which was ought to be inhibited. However, G6P simultaneously constitutes the entry point for the PPP, so a concomitant expression of TIGAR would still promote this pathway. Another peculiar finding is the inhibition of G6PDH, the first enzyme in the PPP pathway, by physical binding with p53 *(Jiang et al., 2011)*. Again, this would promote glycolysis and support tumor growth. But again, there could be a logical explanation: The Vousden lab proposes that these results reflect variations of the p53 response in consequence to different situations that may not occur at the same time and place. In cancer cells, p53 may downregulate the PPP in order to reduce NADPH production also limiting cell proliferations *(Berkers et al., 2013)*.

Finally, it has also been demonstrated that p53 is able to regulate the metabolism of creatine by inducing GAMT. This regulation is of importance when glucose is absent and fatty acid oxidation has to take place in order to produce necessary ATP *(Ide et al., 2009)*. This aspect has been greatly reviewed by *Zhu and Prives (2009)* and *Zhang et al. (2010)*.

Considering all findings and knowing that p53 is mutated or absent in about 50% of all cancers, the implications of p53 in metabolism could help to explain the Warburg effect in cancer cells or rather its absence in normal cells *(Zhang et al., 2010)*.

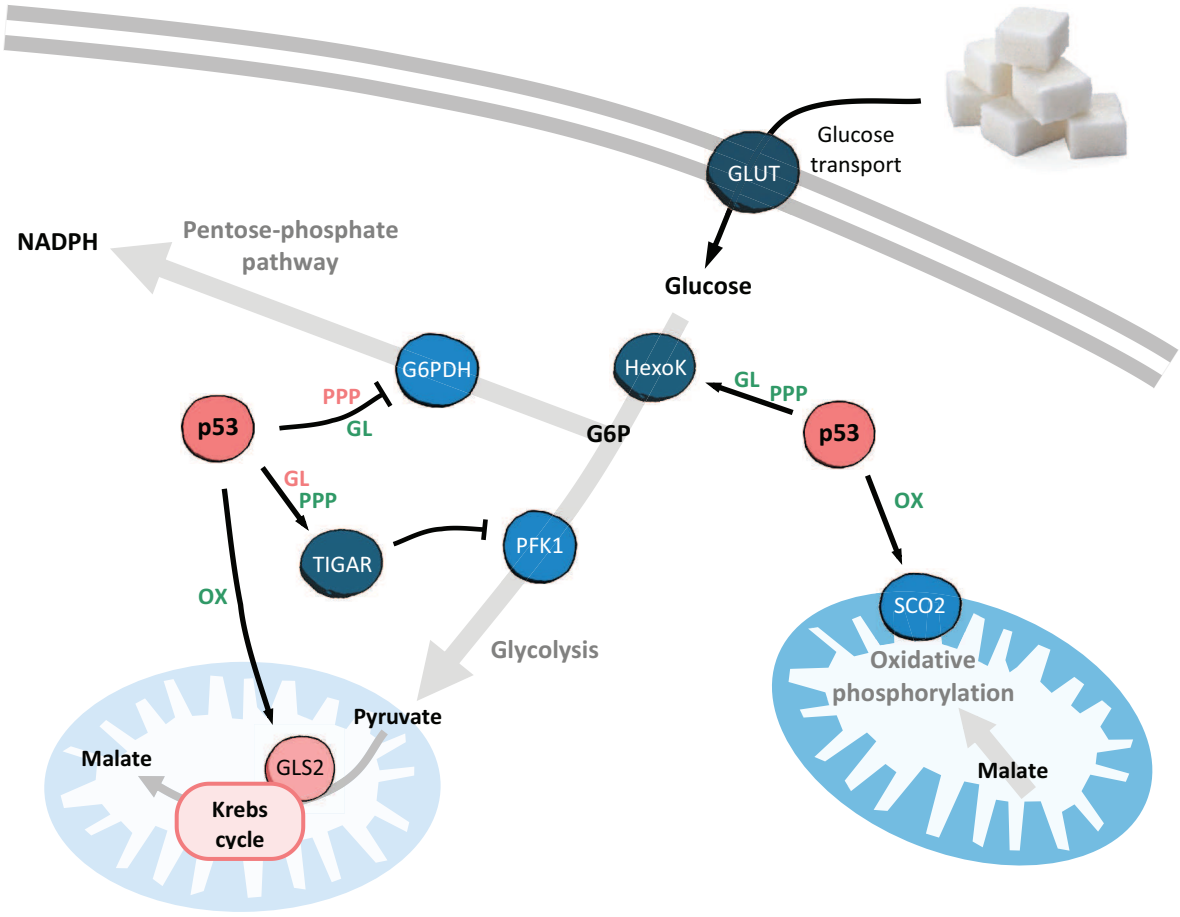

Figure 19 | **Insights into energetic metabolism regulation by p53**
By transcriptionally activating or physically blocking key proteins of different energetic pathways, p53 can influence metabolic balance. Stimulation (green) or inhibition (red) of metabolic pathways by p53 is highlighted (*GL: glycolysis, PPP: pentose-phosphate pathway, OX: oxidative phosphorylation).*

IV - 3. p63 – skinning me softly

The family member p63 is thought to be the actual ancestor of the p53 family as it has changed least during evolution, when comparing different species *(Belyi et al., 2010; Dötsch et al., 2010)*. Through its similarities, and despite its differences with p53, the transcription factor p63 activates a similar subset of target genes such as p21, Bax or MDM2 *(Harms et al., 2004)*. In total however, whole-genome microarray studies suggest that p63 can activate the transcription of over 1000 genes, one fifth of them being other transcription factors. Genes that have been identified are involved in cell proliferation, death, adhesion and signalling *(Yang et al., 2006)*. Of course the physiological relevance of those findings will have to be thoroughly verified. In this chapter though, I would like to present the more specific and exclusive functions of p63.

IV - 3.1. Skin development

Soon after the discovery of p63 *(Yang et al., 1998)*, mice in which the whole p63 gene was invalidated were created *(Mills et al., 1999; Yang et al., 1999).* These new models to study the function of p63 led to the first descriptions of its main function: the striking phenotype of single-layered epithelia and missing appendages like teeth and glands (See *Ch. IV. 1.1.*) suggested that p63 is crucial for the regulation of epidermal development *(Yang et al., 1998, 1999; Mills et al., 1999)*. In humans, mutations in the p63 gene also lead to developmental deficits which are, fortunately, less severe than the knockout of p63 in mice. For example, the Ectrodactyly Ectodermal dysplasia-Clefting syndrome (EEC) features 3 major symptoms: a) deficiency or absence of central digits from hands and feet (ectrodactyly), b) defects of ectodermal structures like hairs, teeth, nails and certain glands (ectodermal dysplasia) and c) clefting of the lip and palate *(Celli et al., 1999)*. Other, similar diseases have also been related to p63, such as the ADULT-, Limb-Mammary-, AEC-, Rapp-Hodgkin and split-hand/foot-malformation (SHFM) syndromes *(Van Bokhoven et al., 2001; Murray-Zmijewski et al., 2006)*. For example, in absence of functional p63 in mice, the expression of two essential genes for limb development (Dlx5 and Dlx6) is strongly reduced. Interestingly, Dlx5/Dlx6 double-

knockout mice develop symptoms closely related to SHFM and EEC, highlighting the importance of p63 in the pathogenesis of those diseases *(Lo Iacono et al., 2008)*.
Deeper analysis of p63 mouse models by the 2 groups that developed the first p63 knockout mice led to conflicting results. I will only present the main findings here.

On one hand, Frank McKeon's group claimed that p63 is required for maintenance and renewal of epidermal progenitor cells *(Yang et al., 1999; Pellegrini et al., 2001; Senoo et al., 2007)*. Indeed, proliferative epithelial cells express high levels of ΔNp63, which is necessary to maintain their proliferative potential *(Senoo et al., 2007)*.
On the other hand, Dennis Roop's group claimed that p63 is necessary for the commitment of embryonic ectoderm to epiderm and epithelial stratification *(Mills et al., 1999)*. Absence of p63 led to a failure in differentiation of epidermal cells *(Koster et al., 2004)* and forced expression of TAp63 restored differentiation *(Koster et al., 2007)*.
A common finding of both groups is the prevalence of ΔNp63 during epidermal differentiation *(Senoo et al., 2007)*. Indeed, during this process, ΔNp63 accounts for 99% of total p63 isoforms and locates in the basal layer of the epidermis *(Laurikkala et al., 2006)* where it expresses characteristic basal layer genes such as Keratin 14. TAp63 is found in the upper layer where it drives differentiation by expressing target genes such as Keratin 1 *(Candi et al., 2006)*.

Skin basically develops from the embryonic ectoderm. During embryonic development, cells derived from this pool become either neural or epidermal progenitor cells, depending on the balance between morphogenic factors they encounter: when stimulated by BMP-4, ectodermal cells undergo epidermal differentiation, while in absence of this factor (and presence of other factors such as noggin), they undergo neural differentiation *(Hogan et al., 1996; Gambaro et al., 2006)*. By using mouse embryonic stem cells *in vitro*, Daniel Aberdam's group showed that ΔNp63 is induced by BMP-4, while there is no effect on TAp63 expression *(Medawar et al., 2008)*. *In vivo*, in mouse embryonic ectodermal and epidermal cells, ΔNp63 messengers are present, while TAp63 is absent *(Mikkola, 2007)*. In response to activation by BMP-4, ΔNp63 allows for commitment of ectodermal cells to keratinocyte differentiation *(Medawar et al., 2008)* and induces epidermal-specific genes such as Keratin 14 *(Candi et al., 2006)*, BMP-7, FGFR2b and Notch-pathway actors Jag1 and Notch1 *(Laurikkala et al., 2006)*.

Taken together, these findings indicate that p63 is crucial in the development of the skin, by promoting proliferation of precursor cells via ΔNp63 and differentiation/ stratification via TAp63 (Figure 20[2]).

IV - 3.2. Protection of the female germ-line

The human body would not be such a marvellous product of evolution, if DNA mutations could easily accumulate and be passed on to the offspring. We humans accumulate more than enough mutations in single cells throughout our life and it would be an act of cruelty to pass accumulated mutations directly on to our children. Fortunately, this is not the case.

In contrary to males, females produce all their gametocytes (oocytes) during embryogenesis and "store" them until needed from puberty on. Oocytes are generated from oogonia by meiosis. However, instead of completing the process immediately like spermatogonia do, nascent oocytes pause during meiosis 1 in the prophase one (meiotic arrest). In this phase, oocytes are tetraploid and vulnerable to DNA damage. During the menstrual cycle, one day before ovulation, individual primary oocytes (or primordial follicles) finally complete both parts of meiosis. Interestingly, TAp63 is constitutively expressed during the phase of meiotic arrest, where it mediates oocyte death by apoptosis following genotoxic insult *(Suh et al., 2006; Livera et al., 2008; Gonfloni et al., 2009)*. When irradiating mouse ovaries, TAp63 was phosphorylated and apoptosis of primary oocytes was induced. Interestingly, mature oocytes did not express TAp63 and are resistant to γ-irradiation-induced apoptosis. In addition, inhibition of TAp63 expression protected the oocytes from irradiation-induced death *(Suh et al., 2006; Livera et al., 2008)*. Similarly, in mouse oocytes treated with the anticancer drug and DNA-intercalant cisplatin, TAp63 was overexpressed and oocytes died by apoptosis *(Gonfloni et al., 2009)*.

Intriguingly, TAp63 expression levels are already high in oocytes in absence of genotoxic stress, suggesting tight post-translational modifications. The activation of TAp63 occurred after induction of DNA double strand break repair. Additionally, TAp63 was found to be phosphorylated on tyrosines 149, 171 and 289 by c-Abl *(Gonfloni et al., 2009)*, which changes its conformation and induces tetramerization *(Deutsch et al.,*

2011). Mutants of those tyrosine residues failed to activate pro-apoptotic p63 target genes *in vitro* in different cell lines. Strikingly, inhibition of c-Abl protects oocytes from apoptosis, highlighting c-Abl as the main regulator of TAp63 *(Gonfloni et al., 2009)*.

Given this very specific role of protecting the oocytes of unwanted DNA-damage, by apoptosis p63 can be considered the "protector of the female germline" (Figure 20[4]).

IV - 3.3. Regulation of lipid and glucose metabolism

Recent studies suggest a role for p63 in energy metabolism. Several findings show that p63 participates in the regulation of lipid metabolism. First, in cancer cells, p63 modifies the expression the fatty acid synthase (FASN), an essential enzyme that synthesizes long-chain fatty acids. More specifically, ΔNp63 transcriptionally induces FASN *(D'Erchia et al., 2006)*. Additionally, inhibition of TAp63 or ΔNp63 represses FASN expression, suggesting a central role for p63 in this process *(Sabbisetti et al., 2009)*.
Secondly, mice specifically deficient for TAp63 have led to the discovery that on the long term, those mice become obese, confirming the suggested role of p63 in energy metabolism. However, these mice produce increased levels of leptin, (reflecting increased body fat), and adiponectin, FASN and SCD1 (reflecting increased fatty acid metabolism and oxidation) in the serum and in liver tissue. Indeed, besides increased fatpads, in the livers of obese mice, lipid deposits were dramatically increased *(Su et al., 2012)*. In this situation, TAp63 inhibited FASN expression, while Sabbisetti reported increase of expression and activity of FASN *(Sabbisetti et al., 2009)*. However, the implication of FASN goes further than metabolism as it promotes cell survival: inhibition of p63 increases cell death, while overexpression of FASN protected cells from apoptosis. This effect is thought to be due to Akt activation, mediated by FASN through p63. Indeed, p63 inhibition reduced Akt activation *(Sabbisetti et al., 2009).* How exactly this leads to increased survival still needs to be determined. Unfortunately, this is not discussed by Flores' group *(Su et al., 2012)*.

TAp63$^{-/-}$ mice also exhibit deficiencies in glucose metabolism. For example, blood glucose clearance is impaired as shown by glucose administration or insulin injection.

In each case, the removal of circulating glucose in blood is less efficient. In line with this finding, it has been shown that the insulin level is reduced in TAp63$^{-/-}$ mice. Additionally, glucose uptake in TAp63-deficient mouse embryonic fibroblasts is reduced, suggesting that TAp63$^{-/-}$ mice are insulin resistant and glucose intolerant *(Su et al., 2012)*.

The Flores group explains this effect by the p63-mediated activation of LKB1, Sirtuin1 and AMPK. Those 3 proteins are part of a pathway where LKB1 phosphorylates and thus actives AMPK, which stimulates fatty acid oxidation and acts as a sensor of energy balance in the cell. By doing so, it promotes energy conservation and glucose uptake *(Zheng et al., 2012)*. Sirtuin1 is an acetyltransferase that mediated cell survival through inactivation of p53 or FoxO1 *(Morris et al., 2011)*. In cultured keratinocytes and fibroblasts, TAp63 directly activates the transcription of those 3 metabolic regulators *(Su et al., 2012)*.

All in all, those new results significantly expand the realm of p63 activity to the domain of metabolism, but further efforts have to be made to better understand the implication of p63 in this newly identified subject (Figure 20[1]).

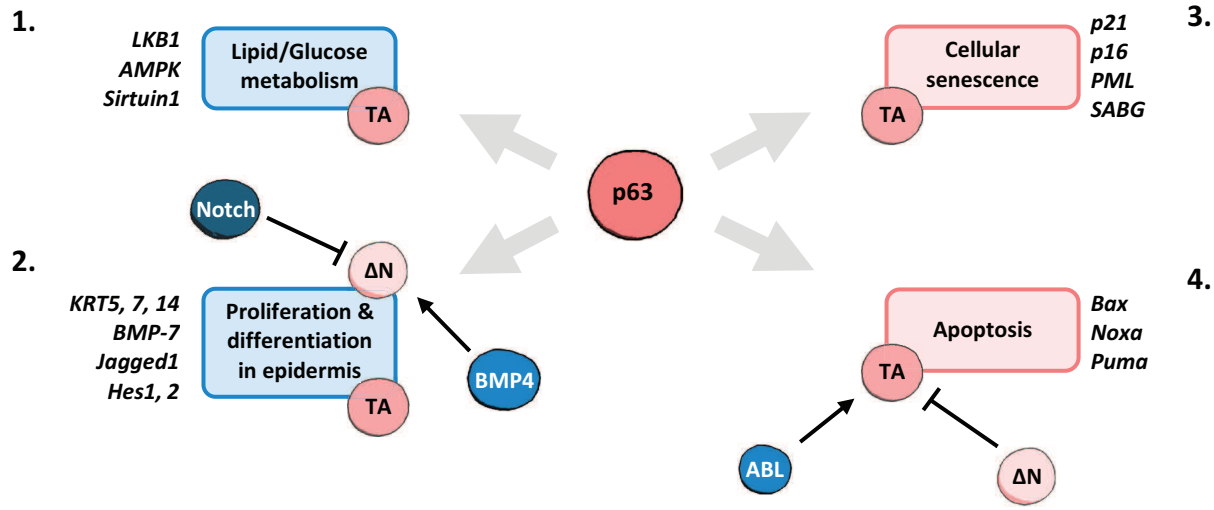

Figure 20 | **Main proposed mechanisms for p63**
p63 is able to control diverse pathways (energetic metabolism, developmental of the epidermis, senescence and apoptosis) by inducing different target genes.

IV - 3.4. Tumor suppression

Mice invalidated for all p63 isoforms die quickly after birth, making it difficult to assess whether p63, similarly to p53, is a tumor suppressor *(Mills et al., 1999; Yang et al., 1999)*. However, given its similarity with p53, and the existing of both antagonistic isoforms, it has long been suggested that TAp63 also acts as a tumor suppressor protein *(Mills, 2006)*. At least, it could contribute to p53-dependent tumor suppressor activity; right *(Flores et al., 2002)*? Indeed, in addition to the ability to activate some of the p53 target genes, such as p21, GADD45α, Puma, Noxa or Bax *(Harms et al., 2004; Bernassola et al., 2005; Lin et al., 2009)*, p63 can also be activated by the same pathways as p53. For example, the ASPP1 and 2 proteins and PML can bind to p53, p63 and p73 and promote their transcriptional activity *(Bergamaschi et al., 2004; Bernassola et al., 2005)*. Furthermore, in cells subjected to γ- or UV-radiation, TAp63 is induced concomitantly to p53 induction, while ΔNp63 is repressed *(Mills, 2006)*. On a functional level, TAp63 is able to induce apoptosis *(Gressner et al., 2005; Suh et al., 2006)*. This is, in a way, how it should work. In cancers such as squamous cell carcinoma, things can be a little different. While mutations in p63 are very rare, TAp63 can be upregulated. So far so good, this seems like a good response to tumor burden. However, ΔNp63 has also been shown to be upregulated both at the transcriptional and the translational level, exceeding TAp63 protein levels. The outcome of this disbalance, a tumor, suggests that in some situations, the balance between TA and ΔN isoforms is more determining that the absolute levels of p63 isoforms *(Sniezek et al., 2004; DeYoung et al., 2006)*. This result makes sense, knowing that ΔN isoforms can inhibit TA isoform-mediated transcription by competitive binding to DNA *(Westfall et al., 2003)*.

Speaking of isoforms… Recent results with isoform-specific p63-knockout mice have finally proven that p63 completes p53 in this respect. Mice harbouring hetero- or homozygous deletions of TAp63 were prone to spontaneous carcinomas and metastatic sarcoma. As tumors were also present (and actually more aggressive) in $TAp63^{+/-}$ mice, it was suggested that TAp63 is a haplo-insufficient tumor suppressor *(Su et al., 2010)*.
In another $p63^{+/-}$ mouse, produced in Alea Mills' group, spontaneous tumor formation was not enhanced, even when mice were treated with a powerful tumor-inducing chemical. However, mice showed signs of premature aging, like epithelia hyperplasia

that did not progress to tumors. Additionally, deficiency in p63 expression led to widespread cellular senescence and corresponding markers like p16, PML or SA-beta-gal *(Keyes et al. 2005, 2006)*. This difference has been attributed to the different genetic backgrounds of $p63^{+/-}$ mice *(Melino, 2011)*.
Interestingly, both groups crossed their mice with a $p53^{+/-}$ mouse. Elsa Flores' group observed that in the resulting offspring, tumor burden was higher than in $p53^{+/-}$ mice alone, as the propensity of metastatic tumors increased *(Iwakuma et al., 2005; Su et al., 2010)*. On the contrary, Mills' group observed fewer tumors than in $p53^{+/-}$ mice, suggesting that the induction of cellular senescence protects mice from tumors *(Keyes et al. 2006; Guo et al., 2009)*.

The function of p63 in a tumor context is complicated and remains controversial. It seems to depend on tissue type and protein balance with ΔNp63. Depending on the context, induction of senescence may protect the body against cancer. In other contexts, induction of cell death through the usual p53 family target genes may be the main mechanism of defence (Figure 20[3,4]).

IV - 4. p73 – essential for neurons

The transcription factor p73 is the third member of the p53 family which has been discovered after p53 *(Kaghad et al., 1997)*. A mouse model lacking p73 was generated *(Yang et al., 2000)* and highlighted one of the two main features of p73 known to date: the regulation of neuronal development. Later, it was shown that p73 is also involved in tumor suppression. In this chapter, I would like to briefly introduce the main findings regarding both aspects while giving insight in general transcriptional processes governed by p73.

IV - 4.1. Regulation of neuronal survival

The first generated $p73^{-/-}$ mouse had a striking phenotype. In addition to a high mortality rate, mice suffered from a hydrocephalus du to accumulation of cerebrospinal fluid (Figure 21A) with loss of cortical neurons, and dysgenesis of the hippocampus, a brain structure that is crucial for memory and spatial navigation (Figure 21B). In addition, the $p73^{-/-}$ mice suffered from chronic infections and inflammation, as well as behavioural and reproductive abnormalities, likely due to deficiencies in pheromone detection. In contrast to $p53^{-/-}$ mice, the knockout of p73 does not increase spontaneous tumor susceptibility *(Yang et al., 2000)*. In the developing brain, p73 is expressed in different regions. It is found in Cajal-Retzius (CR) cells (Reelin producing neurons in the marginal zone of the brain), in the ependyme (a type of glial cells lining the brain's ventricular system) and in the choroid plexus (producing cerebrospinal fluid). However, in $p73^{-/-}$ mice, the amount of CR cells is reduced *(Meyer et al., 2002)*.

As is the case with all p53 family members, deleting the p73 gene disrupts TAp73 and ΔNp73 expression, rendering difficult to assess each isoform's role in this syndrome. Recent advances in genetic engineering led to the development of a ΔNp73-specific knockout mouse, developed independently by two groups. A Belgian group was the first to describe that ΔNp73 has an important role for neuronal survival and development of different brain structures *in vivo*. In $\Delta Np73^{-/-}$ mice, a similar lot of brain structures as in $p73^{-/-}$ mice failed to develop correctly, namely the CR cells, the vomeronasal organ, the choroid plexus and others *(Tissir et al., 2009; Wilhelm et al., 2010)*. This suggests that

ΔNp73 is central to the development of the brain and nervous system. However, the observed effects were not as dramatic as with the total p73 knockout model. For example, the shorter lifespan, the hydrocephalus and the loss of cortical neurons are not reproduced in ΔNp73$^{-/-}$ mice.

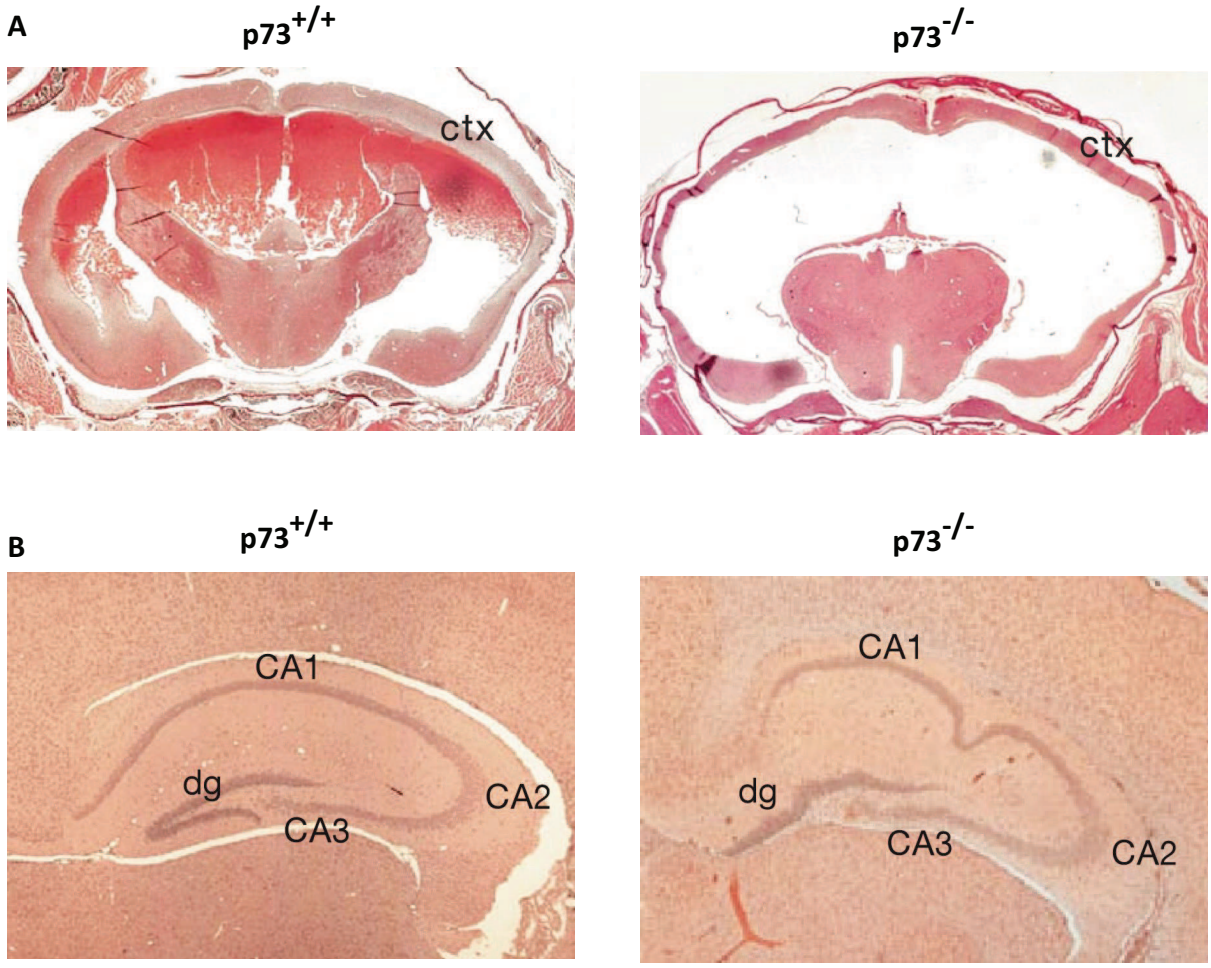

Figure 21 | **Developmental defects in brains of p73$^{-/-}$ mice**
Histological sections displaying **(A)** Hydrocephalus: Expansion of ventricles, intracranial haemorrhaging and compression of the cortex (ctx) and **(B)** Hippocampal disgenesis: lack of infrapyramidal blade and hypertrophied pyramidal blade in the dentate gyrus (dg) and unusual arrangements of the CA1-3 pyramidal cells. From *Yang et al., 2000*.

Interestingly, mice in which only TAp73 has been ablated develop a light neural phenotype with hippocampal dysgenesis *(Tomasini et al., 2008)*, implying that TAp73 is also necessary for normal development of the hippocampus.

Finally, neither of the individual knockout mice presented inflammation or chronic infection *(Tissir et al., 2009; Tomasini et al., 2008; Wilhelm et al., 2010)* as observed in

$p73^{-/-}$ mice, suggesting that the two p73 isoforms could compensate for each other loss in these processes.

Initially however, it has been proposed that TAp73 and ΔNp73 had antagonistic functions. For example, in dying mouse cortical neurons subjected to genotoxic stress, TAp73 was highly expressed along p53 family target genes Bax, Noxa and DR5. Concomitantly, ΔNp73 was reduced. However, overexpression of ΔNp73 protected cells against apoptotic death *(Benosman et al., 2011)*. Similarly, in response to ischemia, TAp73 levels were first induced in the cerebral cortex of mice, but were then repressed as ΔNp73 was induced. This effectively inversed the balance of TAp73 to ΔNp73 and protected the cortical neurons from death *(Bui et al., 2009)*. Finally, in cultured neurons of the superior cervical ganglion (SCG), ΔNp73 was detectable, in contrast to TAp73. Removal of the nerve growth factor induced p53-dependent apoptosis with a concomitant decrease of ΔNp73 expression. Overexpression of ΔNp73 efficiently protected SCG cells, *(Pozniak et al., 2000)*.

Taken together, these results underline the role of ΔNp73 in neuronal protection and survival. Thus, in a neuronal context, TAp73 and ΔNp73 may also have overlapping and redundant functions in addition to their opposing effect observed *in vitro*.

IV - 4.2. p73 in tumorigenesis

The first p73 impaired mouse did not display signs of increased susceptibility to spontaneous tumors *(Mills et al., 1999; Yang et al., 1999)*. However, soon after p73 discovery, it was described that p73 could induce apoptosis *(Jost et al., 1997; Yang et al. 1998)*. We know now that ΔNp73 in particular, is able to induce cell death *(Benosman et al., 2011)*. In addition, TAp73 is overexpressed, but rarely mutated, in many tumor types and complexes between TAp73 and ΔNp73 were found in cancerous cell lines, rendering TAp73 inactive *(Moll and Slade, 2004; DeYoung and Ellisen, 2007)*. p73's overall similarities with the other p53 family members made it thus very likely that p73 has a role in tumorigenesis. Indeed, new mouse models from Tak Mak's group where only TAp73 was ablated, displayed a predisposition to spontaneous tumors, as well as a higher susceptibility to chemically-induced carcinoma *(Tomasini et al., 2008)*.

In general, this phenotype is similar to the $p73^{+/-}$ knockout mice from McKeons' group *(Flores et al., 2005)*. However, in contrast to mice with homo- or heterozygous deletions of p73, TAp73-deficient mice are infertile. This seems to be due to genomic instability (aneuploidy) in oocytes, suggesting that TAp73 participates, like p63 in oocyte quality control *(Levine et al., 2011)*. Genomic instability caused by TAp73 depletion can also be the cause of susceptibility to spontaneous tumors and suggests that TAp73 is involved in basic molecular processes regulating cell division. Mak's group went on to show that TAp73 interacts with proteins of the mitotic spindle assembly checkpoint and acts on their cellular localization *(Tomasini et al., 2009; Vernole et al., 2009)*. Interestingly, genomic instability induced by loss of TAp73 seems to be tissue specific as it does not affect all types of cells. For example, in $TAp73^{-/-}$ mice spontaneous tumors developed in lungs and were aneuploid. In contrast, thymic cells had a normal karyotype and did not form spontaneous tumors *(Tomasini et al., 2008)*.

Mice lacking ΔNp73 expression did not develop spontaneous tumors. But interestingly, embryonic fibroblasts derived from those mice expressed a light transcriptional upregulation of the p53 family target genes p21, Puma and MDM2, suggesting that in normal cells, ΔNp73 represses transcription of these genes. Upon genotoxic stress (Cisplatin, Doxorubicin, Etoposide and γ-irradiation) emybryonic fibroblast and thymocyte viability was markedly reduced *(Wilhelm et al., 2010)*. These results are in accordance with prior results proposing that ΔNp73 competes with TAp73 and p53 by occupying DNA binding sites in cultured cells and primary tumors *(Zaika et al., 2002)*.

IV - 4.3. Cell fate decisions

Over the last 15 years, more and more has been found out about the members of the p53 family. Results, especially on a more molecular level, from one member have been inferred to the other ones to confirm hypotheses or propose new ones. In the case of p73, a body of interesting research articles confirms its role in the control of cell cycle and death, which is similar to p53 in this respect.

TAp73 is able to induce cell cycle arrest by transcriptionally activating target genes such as the cell cycle inhibitors p21 and p57 *(Bálint et al., 2002)*, or repressing cell cycle activators such as Cyclin B1/B2 and various Cdcs *(Allocati et al., 2012)*. The activity of

p73 is strongly regulated by post-translational modifications and protein-protein interactions. For example, MDM2 increases p73 activity by neddylating and addressing it to the nucleus *(Xirodimas et al., 2004)*. On the contrary, ubiquitination of p73 by Itch inhibits its activity and targets it to proteasomal degradation *(Rossi et al., 2005)*.

The activity of p73 is also of importance in mitosis. As shown earlier, TAp73 cooperates with proteins involved in the control of the mitotic spindle assembly *(Tomasini et al., 2009)*.

Finally, TAp73 is able to induce apoptosis in neurons and cancer cells, while ΔNp73 inhibts DNA binding of TA-isoforms, effectively protecting cells from apoptosis *(Benosman et al., 2011; DeYoung and Ellisen, 2007)*.

IV - 5. Concluding remarks on the p53 family

With implications in epidermal and neuronal development through phenomena like cell death or differentiation, regulatory functions in energetic metabolism, as well as undeniable importance in the protection against tumors, the p53 family has proven to be among the most important families of proteins. The amount of available information today is impressive. This is mirrored by the number of publications in Pubmed relating to “p53”. While until 1990 there were only 600 publications, there are now almost 70000. While the amount on available information steadily grows, the complexity of this family becomes more and more apparent. I mean, who would have knew in the early days of the p53 family that p63 could upregulate enzymes involved in the oxidation of fatty acids? Did Frank McKeon envision in 1997 that one day somebody would work on p73-regulated sperm cell adhesion?

Since new findings still emerge with the help of complete knockouts of the different p53 family members, I wonder what we can expect from the newly developed isoform-specific knock out mice.

V - ROLE OF THE P53 FAMILY IN MUSCLES

All generated mice, including isoform-specific deletions of 53 family members had different overt phenotypes. Muscular defects were not one of them. Indeed, all "surviving" mice had normal locomotion. However, in certain situations, muscles may fail to develop properly, develop tumors, or atrophy. In the following chapter, I would like to present the main findings concerning the role of p53 family proteins in muscular cells.

V - 1. Regulation of heart development by p63

Recently, a surprising new role of p63 in cardiogenesis was discovered in Daniel Aberdam's group. Rouleau et al. noticed that in $p63^{-/-}$ mice embryos (from Roop's group), cardiac development is severely impaired. Although hearths looked normal on a macroscopic level, detailed ultrastructural analysis from histological sections revealed a dilated cardiomyopathy. This developmental defect was chiefly characterized by thin ventricular walls and reduced trabeculation (Figure 23).

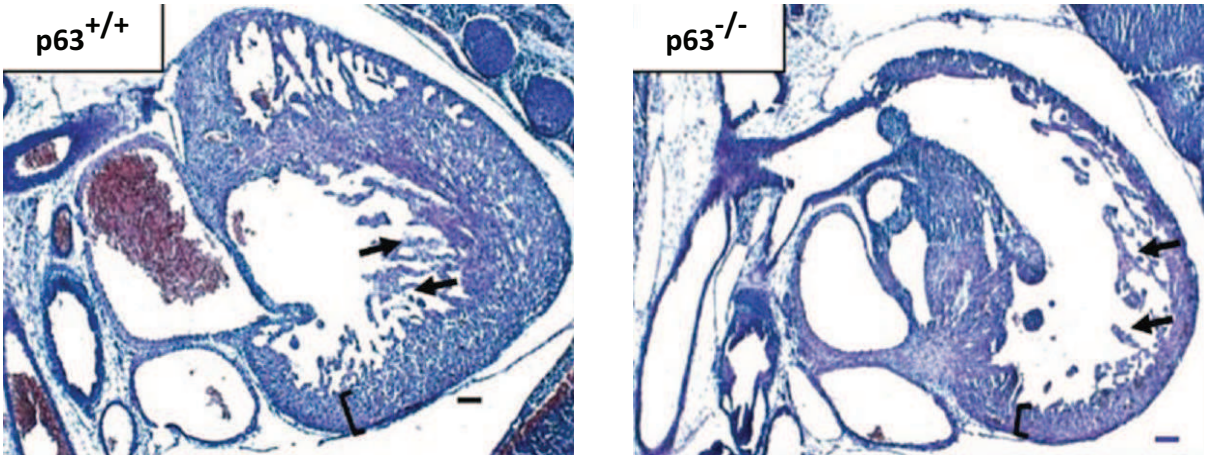

Figure 23 | **Developmental defects in p63 deficient heart**
The diameter of the ventricular wall is reduced (bracket) and trabeculation is altered (arrows). From *Rouleau et al., 2011*.

Additionally, in $p63^{-/-}$ embryonic hearts, myofibril content was reduced and disorganized. Interestingly, ablation of p63 also affected mitochondria biogenesis, leading to randomly distributed and swollen organites. These profound effects unsurprisingly altered cardiac function, resulting in impaired excitation-contraction coupling. Taken together, these results suggest impairment in progenitor cell proliferation or migration *(Rouleau et al., 2011)*.

To understand the underlying mechanisms, further experiments were carried out using $p63^{-/-}$ embryonic stem cells (ESCs). In accordance with the *in vivo* results, ablation of p63 resulted in downregulation of the early cardiac progenitor markers such as Tbx5, Nkx2.5 and islet1, as well as mature cardiomyocyte markers such as TroponinT2, α-Actinin and Mlc2v. Concomitantly, the ESCs were unable to differentiate into beating cardiomyocytes *(Rouleau et al., 2011)*.

In developing embryos, TAp63 is expressed in the endoderm in close vicinity to mesocardiac progenitors. The results obtained in Aberdam's group suggest that TAp63 expression promotes cardiac specification mainly through a cell-non-autonomous manner, by inducing the transcription factors Sox-17 and GATA-4, 6 *(Paris et al., 2012)*.

V - 2. Myoblast differentiation and implications in rhabdomyosarcoma

Muscle differentiation requires a cascade of muscle specific gene expression and its main events have been described earlier (See *Ch. I – 1.2*). The last step of muscle differentiation is the irreversible cell-cycle arrest of proliferating myoblasts that express MRFs such as myoD and myoG. The cell cycle arrest is mediated by the retinoblastoma protein pRb and the Cdk inhibitors p21 and p57 which remain expressed to prevent cell-cycle reentry *(Guo et al., 1995; Zacksenhaus et al., 1996; Novitch et al., 1996; Peschiaroli et al., 2002)*. After this arrest, new muscle differentiation-specific genes are induced, such as myosin heavy chain (myHC) or muscle creatine kinase. Finally, the myoblasts are able to fuse into myofibres. It has been shown that pRB-deficient mice almost completely lack myofibres and die immediately after birth *(Huh et al., 2004)* suggesting that pRb is essential for muscle development.

To gain insight in the regulation of cell-cycle withdrawal and muscle differentiation, Thorsten Stiewe's group examined the potential involvement of the p53 family in this process using cultured C2C12 myoblasts. While individual knockdown of p21 or pRb had no effect on differentiation, the double knockdown hindered the expression of myHC, reflecting a failure in differentiation. Interestingly, expression of ΔNp73 at near physiological levels in cultured, differentiating myoblasts effectively inhibited differentiation into myofibres. Instead, the myoblasts slowed their proliferation rate down but remained in a single-cell state. Furthermore, downstream targets of MyoG such as myHC were not expressed (as in pRb-deficient cells) and reduced pRb phosphorylation. This was caused by reduced levels of p21 and p57 that thus failed to inhibit Cdks, leading to hyperphosphorylation and inactivation of pRb. In addition, pRb expression was also reduced, suggesting that at least one member of the p53 family activates pRb during differentiation. Indeed, consistent with results from *Bálint et al., 2002,* upon overexpression of p53, TAp63 or TAp73, p21 and pRb were transcriptionally induced, while p57 was only induced by TAp73 and TAp63, but not p53 *(Cam et al., 2006)*.

In cultured C2C12 myoblasts, p53 is even necessary in order for myoblasts to differentiate. In absence of p53, pRb is less expressed but remaining proteins are active and sufficient to stop the cell cycle, while cells fail to differentiate *(Porrello et al., 2000)*. Stiewe's group essentially confirmed those results, but showed that some isoforms, namely TAp63γ and TAp73β, were also able to induce pRb transcription, suggesting a redundant function of p53 family members in this aspect during muscle development or regeneration. Increased activity of pRb (hypophosphorylation) was controlled by TAp63 and TAp73-dependent upregulation of p57 (Figure 24) *(Cam et al., 2006)*.

These results have a direct implication in the development of rhabdomyosarcoma (RMS), the most frequent paediatric soft tissue sarcoma. RMS is characterized by a failure of muscle precursor differentiation, contributing to aberrant growth of cells. Patients with alveolar RMS express gene fusions, for example Pax3-FKHR, that result from chromosome translocation *(Sorensen et al., 2002)*.

In Pax3-FKHR expressing nude mice, ΔNp73 is necessary to induce malignant transformation, suggesting that, in RMS, ΔNp73 is oncogenic. Interestingly, both TAp73 and ΔNp73 are expressed in RMS patient biopsies, suggesting that the ratio of both

proteins is important, not the absolute quantity. Furthermore, an RMS cell line in which p73 was inhibited, failed to form tumors when xenografted into mice and induced p57. Similarly, established mouse tumors stopped their cell cycle and stopped growing when p73 was inhibited by sh-RNA (Figure 24) *(Cam et al., 2006)*.

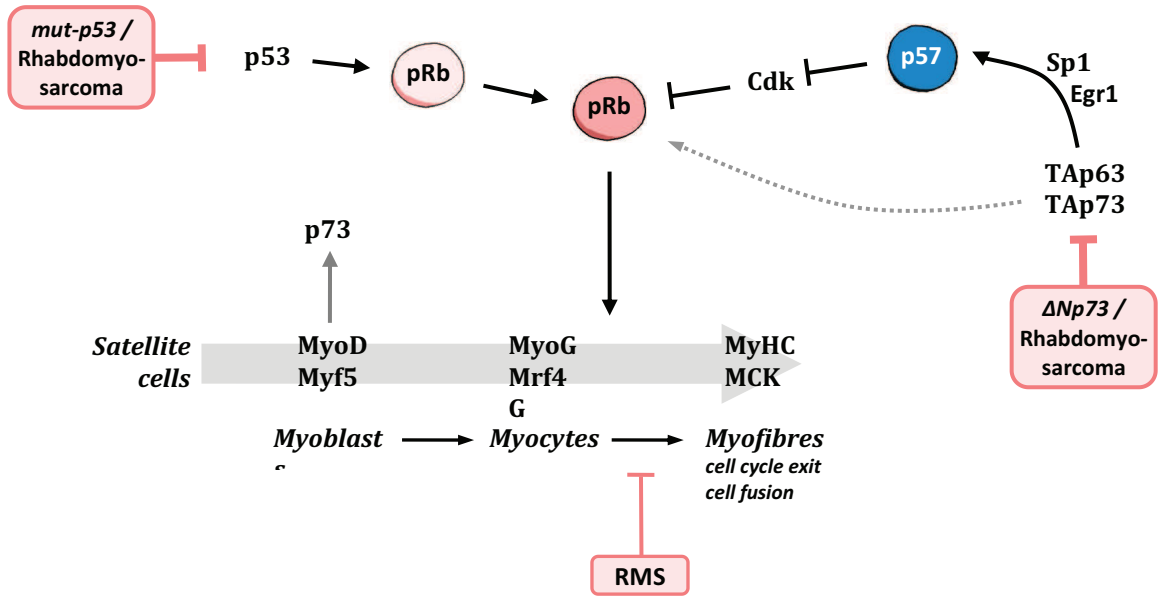

Figure 24 | **Complementary functions of the p53 family during myogenic differentiation**
Activation of pRb by the p53 family in proliferating myocytes induces cell cycle arrest and terminal differentiation. Alterations in the p53 family contribute to Rhabdomyosarcoma (RMS) by inhibiting cell cycle exit. Based on results from *Porrello et al., 2000; Cam et al., 2006* and *Figliola et al., 2008*

Interestingly, the same year, another group published similar data linking p73 with myoblast differentiation. In their hands, p73 was identified as an intermediary between MyoD and p57 expression. pRb, p21 and p57 are all transcriptional targets of MyoD. During myoblast differentiation, TAp73α, β and δ isoforms were transiently induced. Overexpression of those proteins induced p57 expression, while the overexpression of a dominant negative p73 variant inhibited p57 expression *(Vaccarello et al., 2006)*.

Since, the group realized that p57 transcription is mediated by a protein complex containing p73, Sp1 and Egr1. As the minimal promoter of p57 that responds to MyoD stimulation contains Sp1 and Egr1 binding sites, the authors propose that p73 promotes the binding of both transcription factors to their respective responsive element. *(Figliola et al., 2008)*. Interestingly, even in 2008, the authors never used the term

TAp73 and used only *p73* instead. Given the results and the materials and methods section, it is likely they used TAp73 expression vectors.
Those results complete a previous finding where p53 is necessary for MCK transcription mediated by MyoD *(Tamir and Bengal, 1998)*. In this study, a dominant negative mutant of p53 was used to repress the expression of MCK. Back in 1998, the other p53 family members just emerged and it is possible that the effects seen by expressing a dominant negative p53 (also) affected p63 and p73 activity. In addition, later findings stipulated that p53 function is not required for myogenic potential, as primary $p53^{-/-}$ myoblasts were able to differentiate into myofibres *(Schwarzkopf et al., 2006)*.

In contrast to Vaccarello's, Figliola's and Cam's results, a previous study reported that ectopic expression of TAp73α (but not TAp73β) inhibited myogenic differentiation. Concomitantly, the transcriptional activity of MyoD was also reduced, highlighted by a repression of its target genes, such as p21, myHC and MyoG, *(Li CY et al., 2005)*.
As Vaccarello et al. observed only transient expression of TAp73 at the beginning of differentiation, one could argue that its inhibiting role is limited to the beginning of differentiation only. Of note, the work of Stiewe's group focused on the very last step in myogenic differentiation when MyoD expression is already reduced *(Bentzinger et al., 2012)*. In many experiments, Cam et al. used the expression of myHC as the marker for terminal differentiation *(Cam et al., 2006)*.

Taken together, those results indicate that the members of the p53 family have to cooperate in a complex network to control myogenic differentiation. When their expression is altered, failure of cell cycle arrest may lead to tumor formation. The results presented by Stiewe's group *(Cam et al., 2006)* may help explaining why the different p53 family knockout mice have no developmental problems in muscles.
Unfortunately, neither of the groups seemed to have pursued their work on muscle differentiation and the p53 family since. Thus a straightforward mechanism of the involvement of the p53 family in muscle differentiation can not be proposed.

V - 3. p53 in muscular atrophy and cachexia

Various studies highlight the role of p53 in muscle cachexia and cardiotoxicity mediated by doxorubicin.

In vitro, as well as in regenerating muscles *in vivo*, TNFα inhibits myogenesis and differentiation, downregulates the myogenic factors MyoD and MyoG *(Guttridge et al., 2000)*, and upregulates Atrogin-1 expression, leading to muscular atrophy *(Coletti et al, 2002, 2005)*.

When treated with TNFα, primary or C2C12 $p53^{-/-}$ myoblasts were able to differentiate, while $p53^{+/+}$ myoblasts did not, suggesting that p53 activity is necessary for TNFα-induced inhibition of differentiation. One target gene of p53, Peg3/PW1, has been proven essential for muscle development *(Nicolas et al., 2005)* but also in TNFα-mediated inhibition of myogenesis *(Coletti et al., 2002)*. In cultured C2C12 myoblasts treated with Doxorubicin, Peg3 expression was necessary to inhibit myoblast differentiation. Surprisingly, in C2C12 cells, PW1 transcriptionally activated p53, suggesting a positive feedback loop between p53 and PW1, resulting in constitutive p53 expression in myogenic cells.

Upon C26 tumor-induced cachexia in p53-deficient mice (See *Ch.III – 2.4*), weight loss, muscular atrophy and Atrogin-1 expression was markedly reduced, proving that p53 activity is essential for muscular atrophy or cachexia in tumor-bearing mice. Finally, inhibiting Peg3 activity protected muscles in C26 tumor-bearing mice *(Schwarzkopf et al., 2006)*.

Similarly, it has been reported that p53 protein expression is upregulated in bird-muscles after unloading of the wings and induces apoptosis *(Siu and Alway, 2005)*.

These results highlight an important role of p53 in the regulation of muscular atrophy. The activation of a p53 response in muscle cachexia results in an opposite outcome than the mechanisms observed in muscle development. The p53 family activity seems necessary for correct differentiation and the prevention of aberrant cell proliferation, therefore exhibiting a beneficial function in muscles.

Thus, the role of the p53 family seems to be highly context-dependent in muscles. During differentiation, the p53 family is induced by myogenic signals and contributes to cell cycle arrest *(Cam et al., 2006)*. On the contrary, during muscular atrophy, p53 is

induced by inflammatory signals (TNFα) and inhibits muscle regeneration by blocking muscle differentiation *(Schwarzkopf et al., 2006)* and inducing cell death through Peg3, Bax and Caspase activation *(Coletti et al., 2002)*.

VI - AIMS OF THE THESIS

Muscular atrophy is a complex phenomenon that can be induced in many different situations such as disuse, ageing, starvation, cancer, neuromuscular diseases and more. As seen in the introduction, muscular atrophy is a severe condition, in particular in the context of Amyotrophic Lateral Sclerosis (ALS) and Cancer. While studied since decades, both diseases are still not entirely understood due to their variability and complexity of underlying causes.

The p53 family is most known for its role in tumor suppression and development of the skin and the nervous system. The mechanisms in use to achieve these functions rely on the control of cell fate by inducing target genes involved in DNA-repair, cell cycle arrest, differentiation or cell death. Although p53, p63 or p73 knockout mice have no overt muscular phenotype, several research articles highlighted the importance of the p53 family during muscle cell differentiation and atrophy.

In ALS, a lot of effort has been put into understanding how motor neurons degenerate. The ensuing muscular atrophy was or still is considered a mere consequence of motor neuron death.

We wanted to understand if the molecular mechanisms leading to muscular atrophy and cell death in the context of ALS involve proteins of the p53 family. In order to do this, I sought to answer the following questions:

- Is a p53 family pathway activated in atrophying muscles during ALS?
- What are the processes governed by this activation and which proteins of the p53 family induce them?
- What causes this activation?

In addition, we wanted to verify our findings in another, very different context of muscular atrophy. Many cancer patients suffer, depending on the tumor type, from cachexia, a condition where skeletal muscles progressively atrophy and weaken the patients. Actually, Cachexia is, in many cases, the cause of death for cancer patients. Again, the underlying causes are widespread and still poorly understood. Two murine models for cachexia are commonly used in research. The first mimicks cancer cachexia observed in patients, by implanting tumor cells subcutaneously in mice. The second one

addresses the secondary effect of an anticancer drug often seen in clinic, which also induces cachexia.
To contribute to the knowledge in muscular atrophy related to cancer, I just started to seek answers to the following questions:

- What is the developmental timecourse of atrophy and weakness in our two models for cancer-associated cachexia?
- Is the p53 family activated during cachexia?
- Are the findings similar to ALS?

A third aspect acts as a bridge between the two first parts of the project. As no cure is known yet for ALS and cancer cachexia, we wanted to assess the potential cytoprotective effect of flavonoid- and tocopherol-derivated molecules on cultured muscle cells and atrophic mouse muscles. These compounds were obtained through a new collaboration with the Biotech *AxoGlia* from Luxemburg.

- Do AxoGlia molecules have a beneficial effect on myoblasts in vitro?
- Are the compounds able to protect from muscular atrophy in ALS and cancer cachexia?
- Is there an effect on the p53 family?

In the following chapter, my results will be presented in the form of two manuscripts. Some more recent results will be presented in the chapter “discussion and perspectives”.

VII - RESULTS

During my time in Christian Gaiddon's Team in Strasbourg, I contributed to 3 publications. The first one has already been published and deals with the effects of TAp73 and ΔNp73 in neurons following genotoxic stress and APP-pathway induction *(Benosman et al., 2011)*.
The second two are submitted manuscripts that are in direct relation to my research project (first author) and will be therefore presented here.

VII - 1. p63 contributes to muscular atrophy in ALS and induces MuRF1

Amyotrophic Lateral Sclerosis (ALS) is a severe and lethal neuromuscular disease that affects adults in their fifties. To this date, no curative treatment has been developed and the mechanisms underlying this disease are diverse, complex and far from being fully understood. ALS is characterized by the loss of motor neurons and muscular atrophy leading to paralysis and death by respiratory failure within few years. Most research projects focus on the causes of motor neuronal death while muscle atrophy has been much less addressed.

Approach

We wanted to study the implication of the p53 family proteins during muscular atrophy in ALS as they are potent inducers of cell death, cell cycle arrest and differentiation, mechanisms that could contribute to muscle wasting and failure of regeneration.
To achieve this goal, we used several models for, or related to ALS. Among the 16 identified genes that can cause the disease, Cu/Zn Dismutase 1 (SOD1) is particularly frequently mutated in humans and has thus been studied extensively. We had access to an ALS mouse model that expresses a mutated form of this enzyme, SOD1(G85R), and replicated the symptoms observed in ALS patients. A well characterized timecourse of symptoms and molecular events enabled us to choose reference points before and at onsetting muscular atrophy.

Atrophy roughly starts at the time of muscle fibre denervation, which could be assimilated to denervation and disuse atrophy. To study this aspect, we transiently denervated the hindlimb of mice (nerve crush).
Finally, we used cultured C2C12 myoblasts to study the molecular relationships of p53 family and target gene activation.

Main findings

- p53 target genes are induced in atrophying muscles from ALS patients and the SOD1(G86R) murine ALS model
- The expression of p63 and some p53 family targets correlates with the severity of muscular atrophy in patients
- TAp63 is overexpressed and accumulates in muscle fibre nuclei during atrophy in muscles from the SOD1(G86R) mouse
- ALS-associated cellular stresses and mutated SOD1 induce TAp63 and cell death
- TAp63 induces MuRF1 expression by binding to its promoter

p63 contributes to murf-1 regulation and muscular atrophy in ALS

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Running title

Function of p53 proteins in muscular atrophy

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Abstract

Previous work has shown that p53 is induced during neuron cell death in a mouse model of amyotrophic lateral sclerosis (ALS). To further understand the function of the p53 family (p53, p63, p73) in the degenerative processes of the motor axis during ALS, we investigated their regulation in skeletal muscle. We found that the expression levels of p53 target genes correlated with the severity of muscle atrophy, both in ALS patients and in SOD1(G86R) mice that develop ALS symptoms. The up-regulation of p53 family target genes also correlated with an induction of TAp63 while ΔNp63 was repressed. Using *in vivo* and *in vitro* models, we demonstrated that the regulation of p63 expression involved both denervation and stresses intrinsic to muscles cells induced by the SOD1(G86R). At a functional level, our results showed that TAp63 exerted a pro-apoptotic role on myoblastic cells and reduced ΔNp63 expression, while ΔNp63 protected myoblastic cells against oxidative stresses. Finally, we showed that TAp63 induced the promoter and the expression of the E3 ubiquitin ligase MuRF1, an effector of muscle atrophy. Altogether, these results indicate that p63 proteins participate in the muscular atrophic processes in ALS by regulating multiple genes, including the muscle atrophy/remodeling gene *murf-1*.

Introduction

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 (*p21*, *bax*, *gadd45α*, *mdm2*...)[1, 2]. All three members encode for TA and ΔN isoforms that vary in their N-terminus due to alternate promoter usage, skipping the N-terminus transactivation domain (ΔN isoforms) [3, 4]. The differences in the amino-terminal region account for opposite functions, whereby the TA isoforms activate transcription, and the ΔN isoforms inhibit transcriptional activation [5]. Through their cellular activities, p53 proteins are involved in a broad variety of physiological functions that include tumor suppression and organ development [1]. Over 50% of human tumors exhibit mutations or inactivation of *p53*, further highlighting its 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), alteration of the energy metabolism)[6-8].

In addition to their role in cancer and development, several studies have highlighted the involvement of the p53 family 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 syndrome [9-16]. We have previously reported an induction of p53 in degenerating spinal cord motorneurons in an ALS mouse model expressing mutated Cu/Zn superoxide dismutase 1 (SOD1(G86R)) [17].

As for the degeneration of the nervous system, muscle atrophy is associated with aging, cancer, AIDS and neurodegenerative diseases such as ALS [18]. Several observations indicate that the p53 family members play an important role in muscle physiopathology. p53 is activated during myogenic differentiation, participates with MyoD to myogenesis, and mediates doxorubicin-induced muscle atrophy via its target gene *pw1* [19, 20]. Nonetheless, p53 expression is not essential for muscle development [21] or regeneration , 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 [23] [24] [25] [26] and ΔNp73 appears to protect muscle cells against stresses [27]. Finally, a recent study showed that p63 is important for the regulation of muscle cell metabolism via the regulation of Sirtuin 1 and AMPK [28].

Based on these studies, we sought to explore the role of p53 protein during muscle atrophy associated with ALS. ALS is a neurodegenerative disease with a loss motor neurons and progressive muscle atrophy leading to spasticity, paralysis and ultimately to death [29]. Around 20% of all inherited ALS cases can be linked to mutations in the gene encoding SOD1. Cellular events that have been shown to be triggered by these different mutations include aggregation of SOD1 proteins in the cytoplasm [30], increase in oxidative stress [31] and subsequent DNA damage [32], endoplasmic reticulum (ER) stress [33] or alterations in the mitochondrial function [34]. To date, the exact molecular mechanisms driving muscle catabolism in the symptomatic phase of ALS remain poorly understood. Because of the absence of pre-symptomatic markers, it is only during the symptomatic phase that the physicians can diagnose the pathology, highlighting the necessity of understanding the muscle catabolism processes for therapeutic purposes. Given that we previously showed that p53 is activated in the motor neurons of the mouse model of ALS SOD1(G86R) mouse, we sought to investigate whether the p53 response was also developed in the atrophic skeletal muscles during the pathology.

Results

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

We analyzed two transcriptome databases generated from muscular biopsies of patients with ALS [35] and from the ALS mouse model SOD1(G86R)[36]. This showed that a subset of genes with significant expression changes in ALS patients were related to apoptosis and DNA damage response (S1, S3). Notably, *p21* and *gadd45α,* two canonical p53 target genes were up-regulated (Fig. 1A) and their expression correlated with the degree of the pathology of the biopsied muscle. The induction of p53 target genes was verified with an independent group of ALS patients. RT-qPCR analyses confirmed a stronger up-regulation of *p21* and *gadd45α*, but also an up-regulation of other p53 target genes such as *noxa* in ALS patients (Fig. 1B, S1).

Microarray analysis made from muscles of the ALS mouse model SOD1(G86R) confirmed the up-regulation of *p21* and *gadd45α*, but also revealed the induction of additional targets and regulators of the p53 family (S1). The expression of many of these genes (*e.g. p21*, *gadd45α*, *rb1*, *myf6* and *txn1*) was already modified at the onset of motor symptoms, namely altered hind limb extension reflexes. To validate this result,

we examined the expression of several target genes in hind limb gastrocnemius muscle of SOD1(G86R) mice by RT-qPCR (Fig. 1C, S3). Groups taken at 60 days and 75 days of age correspond to the asymptomatic stage. 90 day-old groups correspond to an early or pre-symptomatic stage associated with established gene deregulation (e.g. nicotinic receptor, Fig. 1C). Finally, the symptomatic stage group (beginning after 105 days) is characterized by the onset of paralysis. Up-regulation of the p53 target genes *gadd45α, p21*, *bax*, *peg3* was observed at 90 days, further increasing at 105 days in SOD1(G86R) mice (Fig. 1C, S3). The induction of *gadd45α* partially correlated with the induction of *acetylcholine receptor α* (*achr α*, a molecular marker indicating the severity of muscular denervation. In addition to the genes found regulated in the microarray analysis, we demonstrated that other p53 family target genes like *noxa*, *perp* and *siva,* involved in apoptosis, were induced (S2).

p53 proteins have recently been linked to energy metabolism and endoplasmic reticulum (ER) stress pathway activation [6, 28, 37]. Analysis of the expression of p53 family target genes implicated in several metabolic pathways (*tigar*, *sestrins*, *sco2, Sirtuin1, AMPK*) [38] [28] or ER stress (*chop*, *bip*, *xbp1*...) [39], did not reveal a coherent regulation in respect to disease progression (S2). For example, the expressions of *sestrin* 2 and *tigar* [38] were regulated in opposite directions during the progression of the disease (S2). Therefore, our data strongly suggest that the correlation between ALS progression and p53 function is mostly due to cell growth arrest and cell death regulation.

We also confirmed by RT-qPCR an up-regulation of several upstream regulators of the p53 family, including *mdm2*, *myf6*, *mlf1*, and *thioredoxin* (S2) [1]. Taken together, our results suggest that a p53-like pathway is activated in ALS muscles both in patients and the murine ALS-model.

p53 family members are regulated in mouse skeletal muscles during ALS

In order to identify which of p53, p63 and/or p73, was responsible for transcriptional activation of the above-mentioned target genes, we investigated their levels in the muscles of SOD1(G86R) mice. Our analysis revealed an increased expression of TAp63 mRNA in SOD1(G86R) (Fig. 2A). Strikingly, the levels of TAp63 isoforms were strongly induced towards the end of the disease, while the ΔNp63 isoforms were down-regulated during the same time period (Fig. 2A). A similar

tendency was observed for p53, TAp73 and ΔNp73, albeit at a lower magnitude. This is in accordance with our data from ALS patient muscle biopsies, whereby the expression of p63 in ALS patients correlated with the degree of muscle pathology (Fig. 2B). Therefore, our findings point to p63 as the potential relay responsible for the induction of p53-like target genes in atrophic muscle.

p63 protein accumulates in muscle fibers during ALS

Immunoblotting with a TAp63 isoforms specific antibody revealed an important accumulation of proteins in muscles of SOD1 (G86R) mice that correlated with the progression of the disease (Fig. 3A). When probing with a ΔNp63 specific antibody, we did not observe any specific band (data not shown). The use of a p63 antibody directed against all p63 isoforms confirmed an up-regulation of p63 in muscles of SOD1(G86R) mice (Fig. 3B). Immunohistochemistry with a p63 antibody directed against all p63 isoforms revealed a marked increase of immunoreactivity in the nuclei of muscle fibers of SOD1(G86R) (Fig. 3C, S5). In contrast, there was no significant increase in p73 staining (S3). However, in both cases we noted a higher number of p63 or p73 positive nuclei. This effect appeared to be due to the atrophy of the muscle fibers, increasing the density of nuclei. Similar experiments to detect expression of p53 did not yield an analyzable signal. Our data suggests that modifications of p63 messenger and protein expression levels are important regulatory events in the skeletal muscles during ALS.

Muscle denervation induces a p53 family response

Activation of the p53 family in ALS muscles could be triggered mainly by two events: the denervation of the muscle fibers following the destabilization of the neuromuscular junction, or the expression of mutated SOD1 in the muscle cells. To verify the first hypothesis, we induced denervation in 80 days old wild type and SOD1(G86R) animals by sciatic nerve crushes, and 7 days later gastrocnemius muscles were analyzed. Our results showed that denervation up-regulated TAp63 mRNA levels 5-6-fold in wild-type mice (Fig. 4A). Concomitantly, ΔNp63 levels were down-regulated 0.4-fold (Fig. 4A). In SOD1(G86R) mice, nerve crush further accentuated TAp63 and ΔNp63 mRNA level changes. In addition, the TAp63 target genes p21 and GADD45α were found to be strongly induced after nerve crush (Fig. 4B). These results show that

an alteration of the neuro-muscular junction seems to be sufficient to activate the canonical p53 family response.

Mutated SOD1 is sufficient to induce the p53-like 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(G86R) on muscle cells via a transient overexpression of SOD1(G86R) in the mouse myoblast cell line C2C12. Total protein analysis revealed an augmentation of TAp63 in SOD1(G86R) transfected cells (Fig. 5A). Several target genes of the p53 family (*bax*, *p21*, *gadd45α*) were also induced (Fig. 5C and data not shown). As we were not able to detect protein expression of ΔNp63 in C2C12 cells, we used ΔNp63 reporter plasmids to analyze the promoter of ΔNp63. Over-expression of SOD1(G86R) reduced the transcriptional activity of the *ΔNp63* promoter (Fig. 5B) [40]. We also tested for a possible cross-regulation of ΔNp63 expression by the increased expression of TAp63 observed in ALS. We observed that TAp63 repressed the *ΔNp63* promoter activity (Fig. 5B), while it expectedly induces the *mdm2* promoter (data not shown). These results demonstrated that SOD1(G86R) was sufficient to trigger a p53-like response similar to our *in vivo* observations in atrophic muscle tissues.

We then further investigated how mutated SOD1 could trigger a p53-like response in muscle cells. We sought to pharmacologically reproduce the cellular stresses that have been associated with the presence of a mutated SOD1, namely: oxidative stress (induced by menadione) [31], DNA damage (induced by etoposide) [32], mitochondrial deregulation (induced by FCCP) [34] and ER Stress (induced by tunicamycin) [30, 33]. Treated cells revealed an increase of TAp63 in all stresses tested (Fig. 6A). Thus, the activation of the p53 family during ALS seems multifactorial, since muscular denervation or stresses directly caused by the expression of SOD1(G86R) in muscular cells are both capable to trigger a p53-like response.

ΔNp63 protects against cellular events observed in ALS

To examine whether the up-regulation of TAp63 and the down-regulation of ΔNp63 in atrophic muscles have any physiological significance, we transiently overexpressed them in C2C12 cells along with GFP, and concomitantly treated them with FCCP and menadione. Cell death was then measured by assessing the nuclear shape of GFP-

positive (i.e transfected) cells [15]. Expression of a TAp63 isoform or treatment with FCCP increased cell death, which was substantiated by an increase in the number of condensed and fragmented nuclei (S3). In treated cells, TAp63 overexpression further increased cell death caused by FCCP and menadione. In contrast, ΔNp63 overexpression reduced FCCP and menadione induced cell death, suggesting that under these conditions, ΔNp63 might counteract the activity of TAp63 in C2C12 cells (Fig. 6B).

TAp63 regulates MuRF1 expression, a specific muscle atrophy effector

To further understand how p63 could have an impact on muscle atrophy during ALS, we analyzed the expression of two documented effectors of muscular atrophy, namely Atrogin-1 and MuRF1. These proteins are E3 ubiquitin ligases that target muscular proteins for degradation during muscular atrophy or remodeling [41]. We found that these two genes are strongly up-regulated in muscles of SOD1(G86R) mice (Fig. 7A). Since their expression peaked when TAp63 expression was at its highest, we tested whether TAp63 could regulate *murf1* and *Atrogin-1* expression. Indeed, TAp63 overexpression in C2C12 cells strongly induced MuRF1 mRNA levels (Fig. 7B). *Atrogin-1* expression level was much less affected (data not shown). Importantly, p53 or p73 had little or no effect on MuRF1 expression (Fig. 7C). We found that p53 family members induced *murf1* promoter reporters that contained at least the fragment -500bp to -1000bp (Fig. 8A and B) [42]. Importantly, that fragment contains potential p63 binding sites with high probability scores, such as RE1/2 (-660/-690bp) and RE4 (-2530bp) (Fig. 8C). Chromatin immunoprecipitation experiments (ChIP) covering these binding sites showed that TAp63 proteins bound preferentially onto RE1/2 (Fig. 8D). Taken together, these results indicate that p63 is able to control cell fate in C2C12 muscular cells, and this may involve the regulation of *murf1 via* its direct binding on *murf1* promoter.

Discussion

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

Regulation of p63 expression during muscular atrophy

Our results demonstrate that there is a complex p53-like response developed by the atrophic muscle during ALS progression. In our study, multiple p53 target genes (*bax*, *noxa*, *p21*, *perp*...) were strongly induced during the atrophic phase of the disease, both in the ALS mouse model and in patients (Fig. 1, S1, S2). Although the number of patients was relatively limited (17 patients) due to the difficulties to obtain biopsies, we showed with two completely independent groups of patients and two different methods that the expression level of certain p53 target genes such as *GADD45α* correlated very well with the degree of atrophy (Fig. 1). Expression analysis of p53 family members indicated that in ALS p63 seems more likely to play a prominent role than p53 and p73 as the TAp63 isoforms are strongly up-regulated and localized in the nuclei of the fibers during the pathology (Fig. 2, 3, S3, S5). It was previously reported that p63 participates in muscles cells differentiation and metabolism, and contributes to cardiac muscle development [23, 25, 26, 28, 43]. However, by combining biopsies from ALS patients and an animal model for ALS, the present study provides the first solid evidence that p63 participates also in the muscular atrophy.

Although our results indicate that p63 participates in muscle atrophy, we cannot exclude the possible contribution of p53 and p73 due to the fact that their mRNA expression is regulated similarly to p63, although to a much weaker extent (Fig. 2). Several studies support this possibility by showing that p53 and p73 play a role in muscle cell differentiation, cachexia and survival [23] [44] [45] [19, 46] [47]. However, p53 genetic inactivation does not affect ALS progression, muscle development or muscle regenerative capacity [21] [22, 48, 49]. Furthermore and importantly, in our studies p53 and p73 proteins levels are not altered in SOD1(G86R) ALS mouse model.

The causes for p63 regulations during ALS muscle atrophy seemed complex. One is an intercellular origin represented by loss of interaction between the muscle and the nerve cells (Fig. 3). The other is an intrinsic origin due to the expression of the SOD1(G86R) in the muscle cells causing intracellular stresses (Fig. 5).

Function of the p63 response in muscular atrophy during ALS

Based on the literature, the p53 family could mediate different cellular outcomes in muscles and therefore on the 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 [30-34]. Our study showed that the majority of the p53/p63/p73 target genes up-regulated during ALS in the atrophic muscles were

connected to cell death (*gadd45α, peg3, perp, noxa, bax, siva, eda2r, wig1/pag608*...) (Fig. 1 and S2). Genes connected to other functions, such as ER stress (CHOP, Bip, XBP1, Scotin) or the energy metabolism (TIGAR, Sestrin 1, Sestrin 2, SCO2) seem to be less consistently regulated, as some are up-regulated (sestrin 1, sestrin 2, XBP1), while others are down-regulated (SCO2, TIGAR, CHOP, Bip,.. see S2). Therefore, it seems more likely that p63 function in ALS is connected to muscular atrophy via a control of muscle cell survival. This hypothesis is further supported by two of our results. First, over-expression of TAp63 induces cell death in C2C12 myoblasts (Fig. 6), which correlates with the ability of TAp63 to induce the muscle atrophy effector gene *murf1* via a direct binding on the *murf1* promoter (Fig. 7, 8). Second, over-expression of ΔNp63 protects myoblastic cells against stresses (Fig. 6). 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 physiopathological conditions.

ALS patients are currently diagnosed at a stage where denervation and muscular alterations already occurred, and because of the lack of curative treatment, lead to death within 2 to 5 years. The results presented here suggest that p63, via the regulation of p63 target genes such as *eda2r*, *peg3* but also, as we show, via *murf1*, contributes to muscle catabolism in these patients. It remains to be established whether this signalling pathway is critical for muscular atrophy during ALS and whether it is common to other muscular atrophies (cachexia, diabetes, etc.).

Methods

SOD1-G86R mice. SOD1(G86R) mice were genotyped as described in [50]. For surgery, 80-day-old FVB mice were anesthetized both sciatic nerves were exposed at mid thigh level and crushing was performed (or not – CT) with a forceps during 20 s ~5mm proximal to the trifurcation. Control animal used in the experiments were wild type littermates. Animal experimentations have been performed following the European guidelines and protocols validated by the local ethical committee (CREMEAS).

Cell culture. C2C12 cells were obtained from ATCC and grown in DMEM (Dulbecco's modified Eagle's medium; Life Technology) with 10% foetal bovine serum (Life technology, Germany) at 37 °C in a humidified atmosphere (5% CO_2).

Quantitative PCR. TRIzol (Invitrogen) was used to extract RNA. One µg of RNA was used for reverse transcription (iScript cDNA kit, BioRad) and qPCR was carried out (iQ SYBR Green, BioRad) **(S5)**. Expression levels were normalized using either 18S, TBP or RPB1.

Western blotting. Cells or tissue were lysed with LB (125mM Tris-HCl pH 6.7, NaCl 150mM, NP40 0.5%, 10% Glycerol). Proteins were denatured and deposed directly (75 g of proteins) on SDS-PAGE gel, or they were precipitated (2mg of proteins) with a p63 antibody and G sepharose beads before separation. Western blot were performed using antibodies raised against p53 (rabbit anti-p53, FL-393, Santa Cruz, CA), p63 (mouse anti-p63, 4A4, Santa Cruz, CA; p63, ABCAM, France) or TAp63 (Biolegend, CA). Secondary antibodies (anti-rabbit, anti-mouse: Sigma, MA) 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, CA).

Transfection and luciferase assay. Cells were transfected by a polyetylenimmine (PEI)-based or JetPrime (Polyplus, Strasbourg, France) as previously described [51]. For luciferase assays, cells were seeded in 24-well plates, and transfected with the expression vectors (200 ng) and the reporter constructs (250 ng). Luciferase activity was measured in each well 24 hours later and results were normalized with a CMV

driven reporter gene [16]. The -1584 ΔNp63 luc and -46 ΔNp63 luc constructs were previously described [40]. The *murf-1* luc constructs were previously described [42].

Transfection and apoptosis assay. C2C12 cells were grown on coverslips coated with poly-ornithine in 24-wells plates. Cells were co-transfected with the expression vectors (200 ng/well) and a GFP-expression vector (50 ng/well) as previously described [52]. Cells were cultured for 18 hours 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 33342 staining agent (1 µg/ml, Sigma, Germany). GFP positive cells were then observed with a microscope (Zeiss, Germany) to assess the nucleus morphology.

Chromatin Immunoprecipitation (ChIP) Assay. ChIP assays were performed by 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, 1µg of Antibody was used. P63 was immunoprecipitated with a mouse antibody raised against total p63 (4A4, SCBT). Mouse-anti-RAB11A was used as negative control (SCBT).

Acknowledgment

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Figures

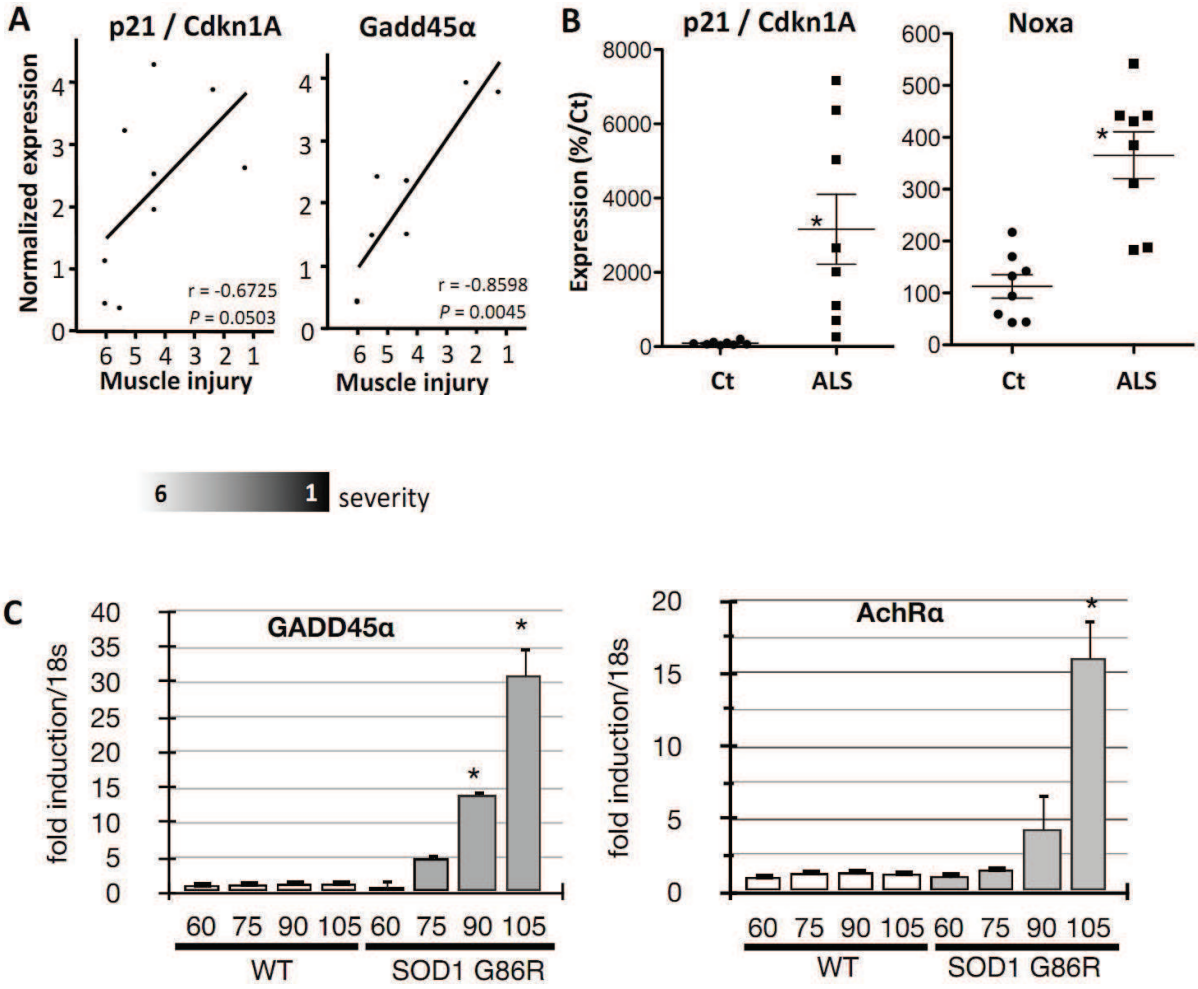


Figure 1 | Correlation of p53 family target gene expression in muscles from patients and an ALS mouse model with disease intensity.

(A) Expression data were generated using a gene profiling database deposited at ebi.ac.uk/arrayexpress (accession no. E-MEXP-3260). 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. **(B)** RNA from biopsies of control and ALS patients (n=8) were analyzed by RT-qPCR. **(C)** Expression levels of the p53 family target gene GADD45*α* were assayed by RT-qPCR in wild-type (WT) and SOD1(G86R) mouse gastrocnemius muscle samples and compared to AchR*α*. Bars are means of fold induction versus the condition WT 60 days-old and of three experiments containing animals with matching age (60, 75, 90, 105 days-old, n=5) 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

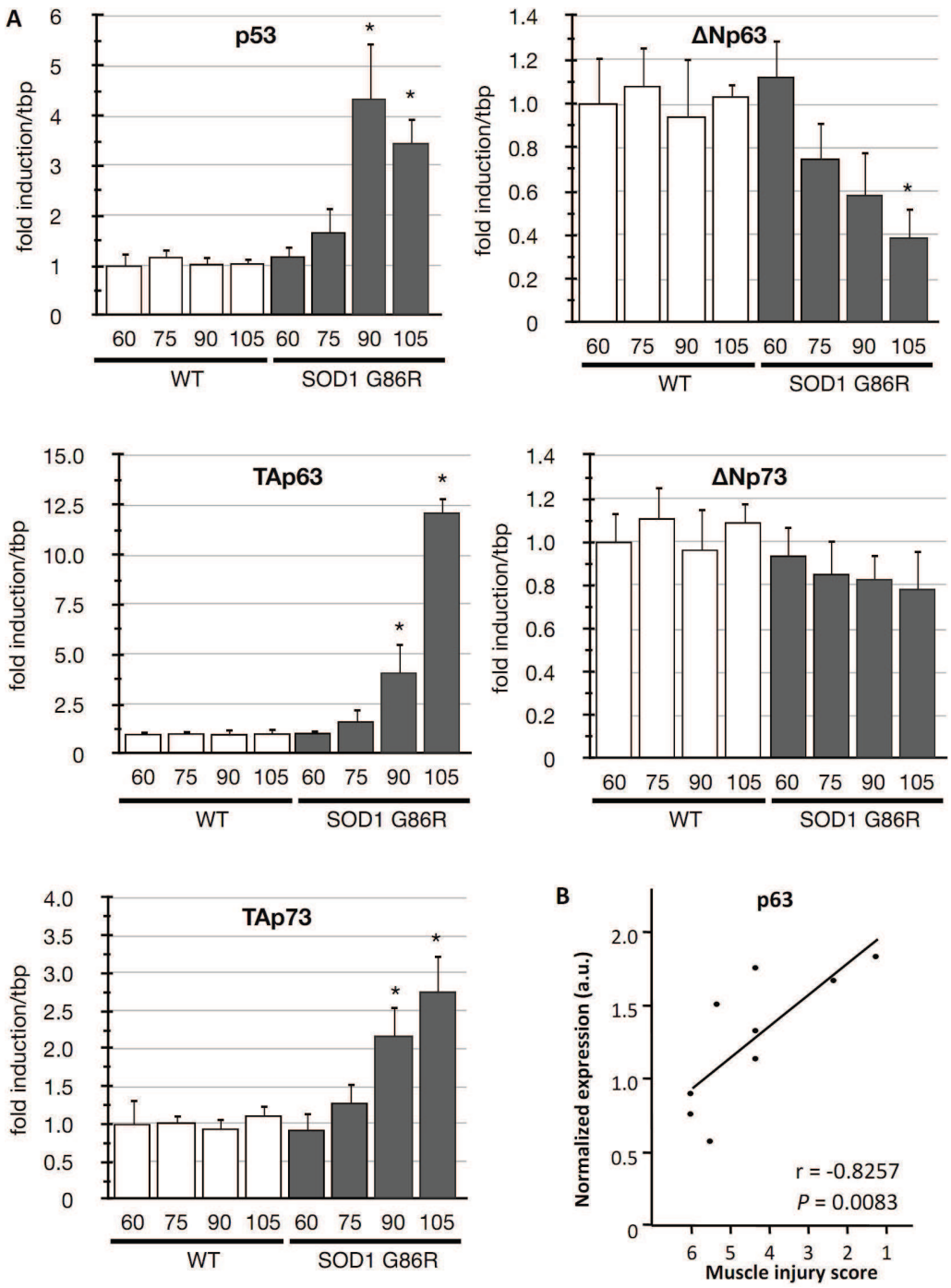


Figure 2 | Expression of p53 family members in ALS muscles.

(A) mRNA levels of p53 family members 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=5, 6) and experimental condition (wild-type, or SOD1(G86R)). **(B)** mRNA levels (total p63) from nine ALS patient deltoid muscles were assayed by DNA microarray, correlated with the intensity of muscle injury. Correlation coefficients (r) and P-values were determined by Spearman correlation test.

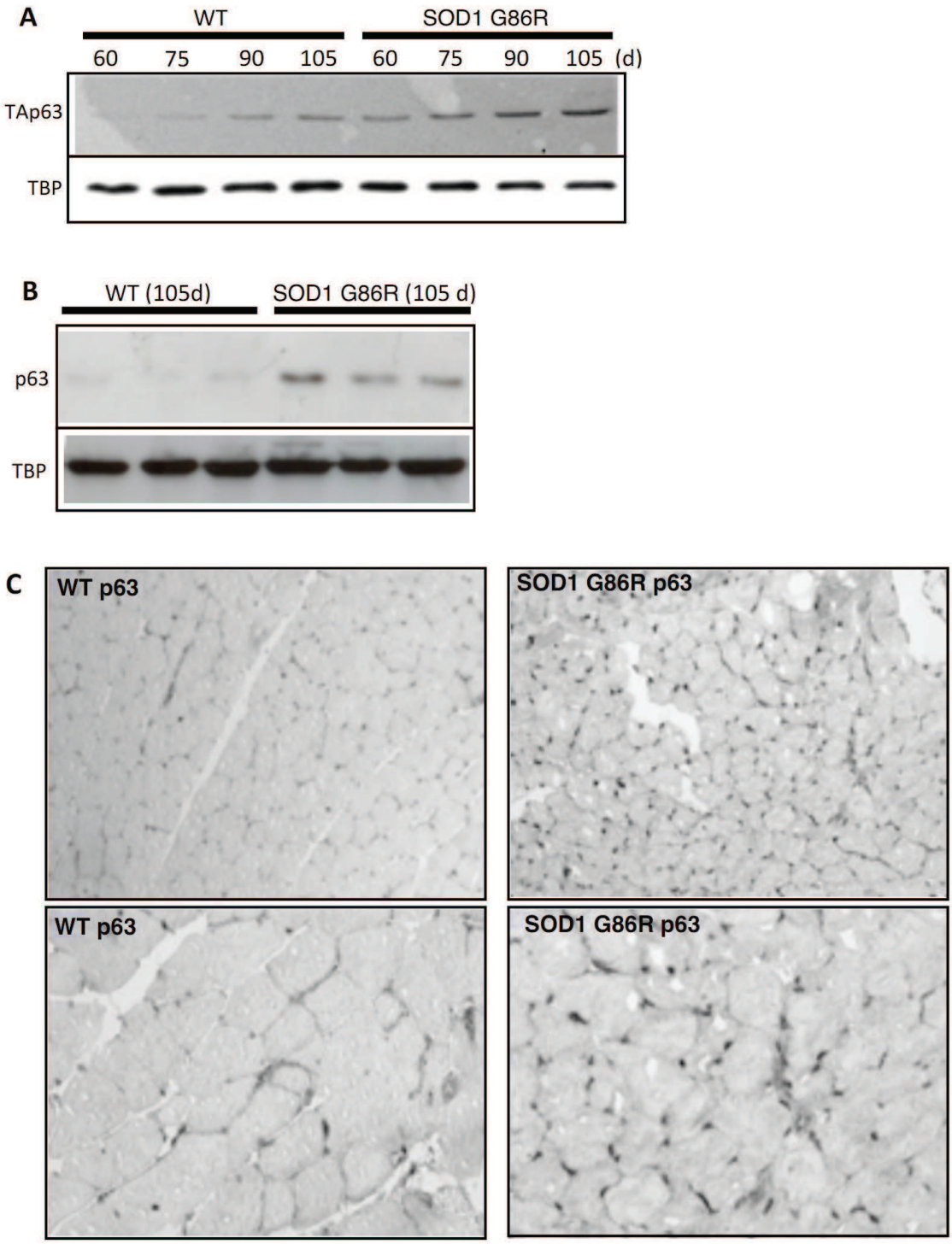


Figure 3 | p63 protein expression in SOD1(G86R) muscle.

(A, B) 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 (A) or total p63 (C). (A) Shows pools of proteins from 3 animals. (B) Shows p63 expression for 3 WT or SOD1(G86R) individuals at 105d. TBP was used as loading control. **(C)** Gastrocnemius muscles from wild-type or symptomatic SOD1(G86R) (105 days) mice were cryodissected and probed for total p63 protein.

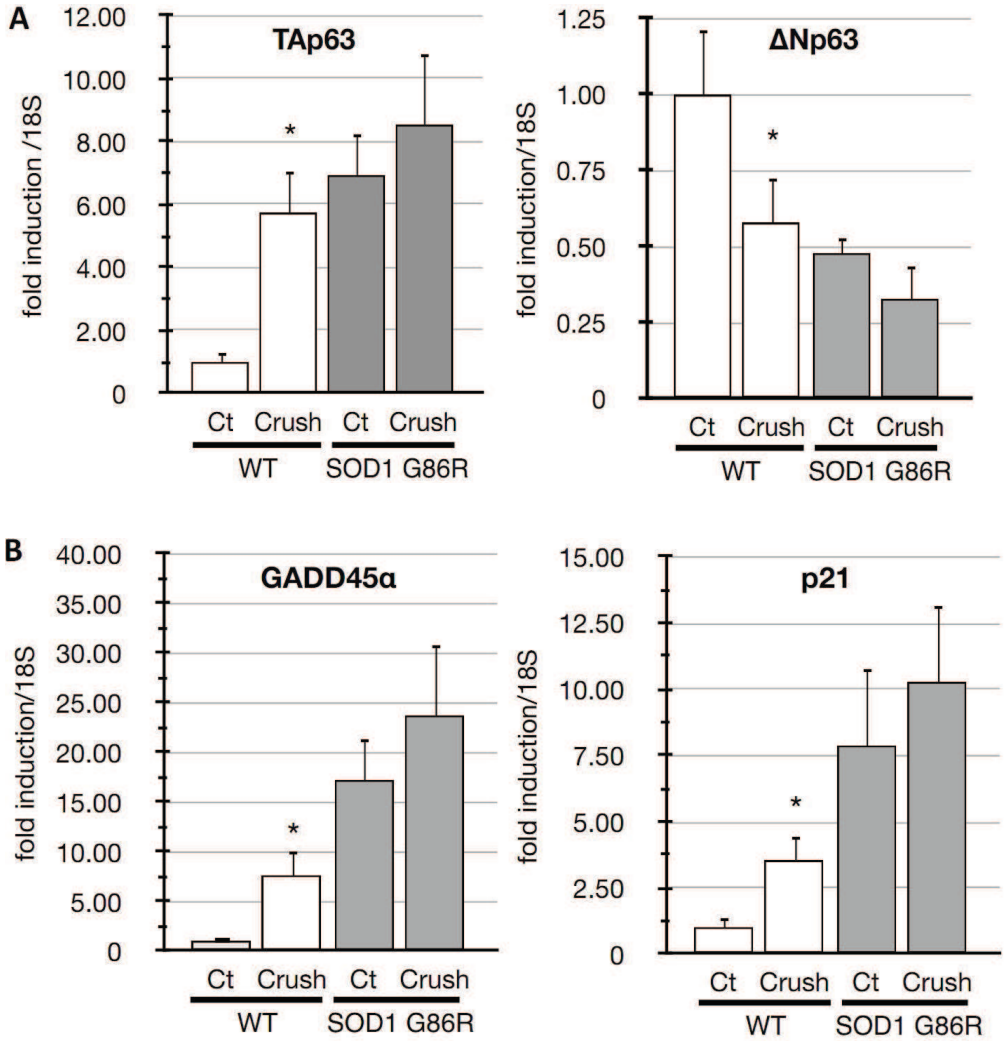


Figure 4 | Expression of p63 and p53 family target genes following sciatic nerve crush.

WT and SOD1(G86R) mice at 80 days of age were anesthetized and the sciatic nerve crushed. Sham-operated contra limbs served as control (Ct). After 7 days, gene expression of (A) p63 and (B) p53 family target genes was assayed by RT-qPCR (n=4). Values were normalized to the value of sham-operated WT muscles/animals.

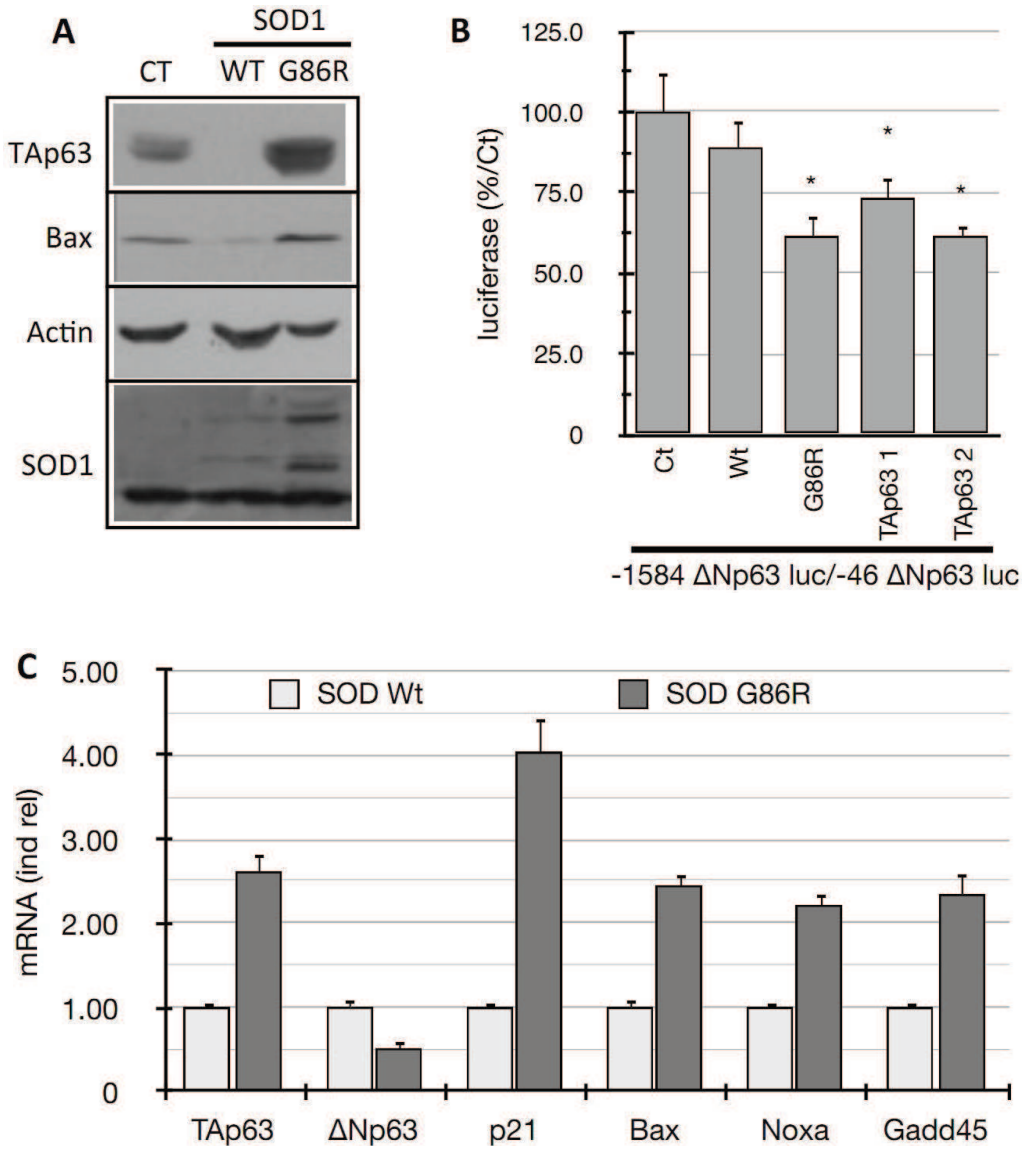


Figure 5 | p63 expression following ALS-associated insults in muscular cells. Proteins were extracted from C2C12 myoblasts **(A)** expressing WT or SOD1(G86R) after 5-day puromycin selection. **(B)** C2C12 myoblasts were transfected with expression vectors for SOD1 variants (WT or G86R) or TAp63γ (2 concentrations, 1, 2) and luciferase reporter genes containing deletions of the Δ*Np63* promoter (-1584-+32 or -46/+32). 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. **(C)** 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). *: $p < 0.01$.

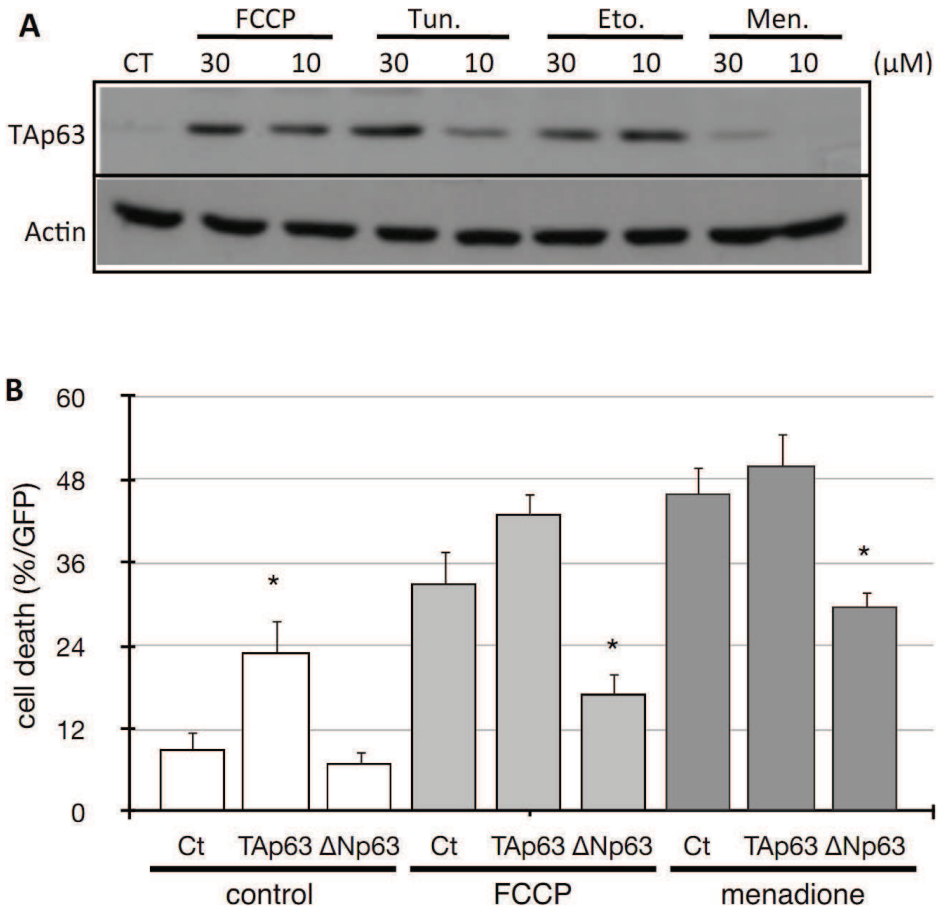


Figure 6 | Function of p63 in myoblastic cells.

(A) Proteins were extracted from C2C12 myoblasts treated with compounds (*FCCP*, *Tunicamycin*, *Etoposide*, *menadione*). Western blot analysis revealed TAp63 expression. **(B)** C2C12 were transfected with a GFP expression vector and either TAp63γ or ΔNp63γ expression vectors. After 24 hours, cells were left untreated (Ct) or treated with FCCP (1μM) or menadione (1μM) for 24 hours. Cells were stained with Hoechst and examined with a fluorescence microscope. Percentage of dead cells among the GFP-positive cells counted. Bars correspond to means with SD (n=3). *: $p < 0.01$.

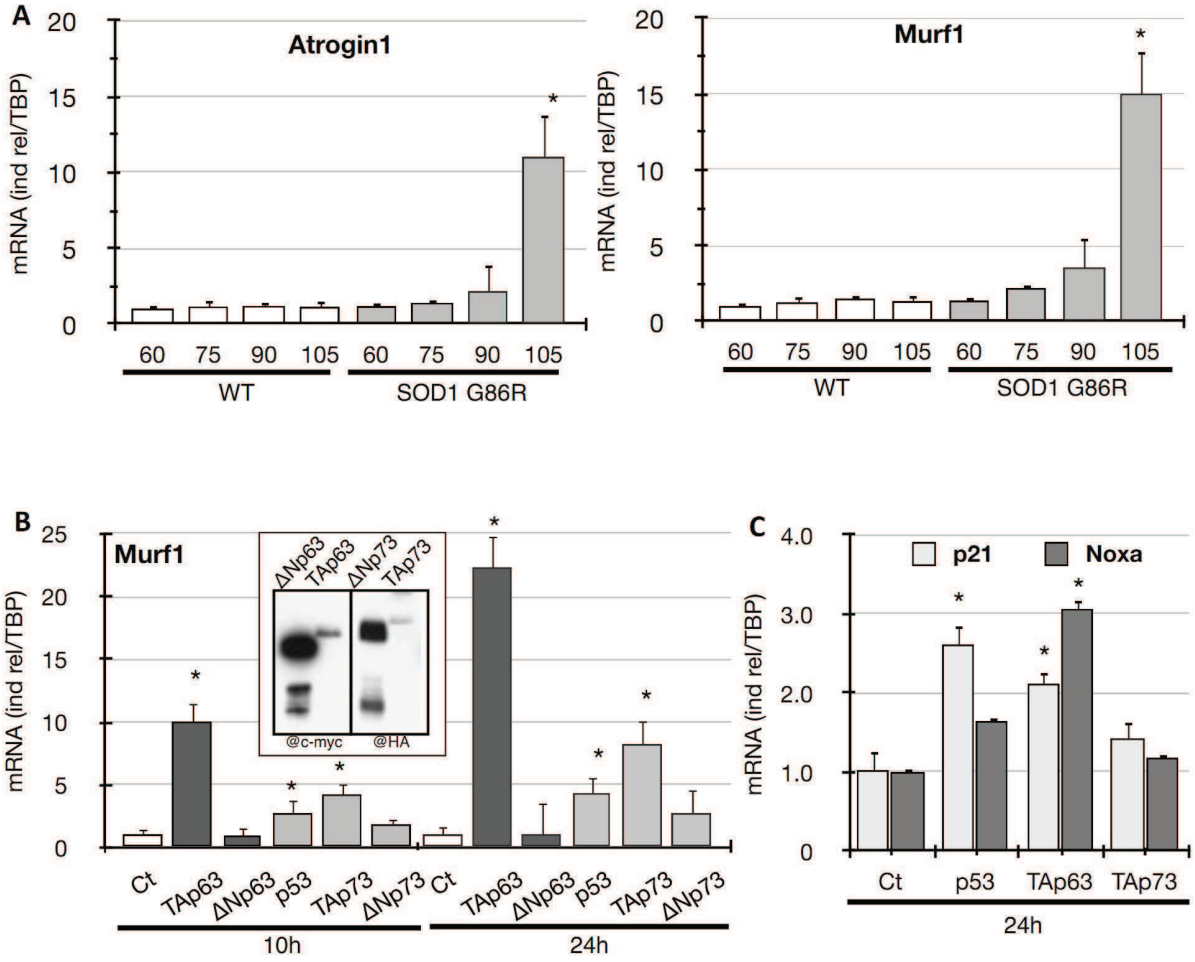


Figure 7 | Regulation of Murf1 and Atrogin in ALS and by p63.

(A) mRNA levels of *Atrogin* and *murf1* were assayed in SOD1(G86R) gastrocnemius muscle by RT-qPCR. Graphs represent mean and SD (n=3). **(B, C)** mRNA levels of *murf1*, *p21*, *noxa* were assayed in C2C12 cells expressing p53 variants (TAp63γ, ΔNp63γ, p53, TAp73β, ΔNp73β) by RT-qPCR. Bars are means of fold induction versus the control condition (Ct) with SD (n=3). *: $p < 0.01$. Inset panel shows p63 and p73 expression levels after transfection.

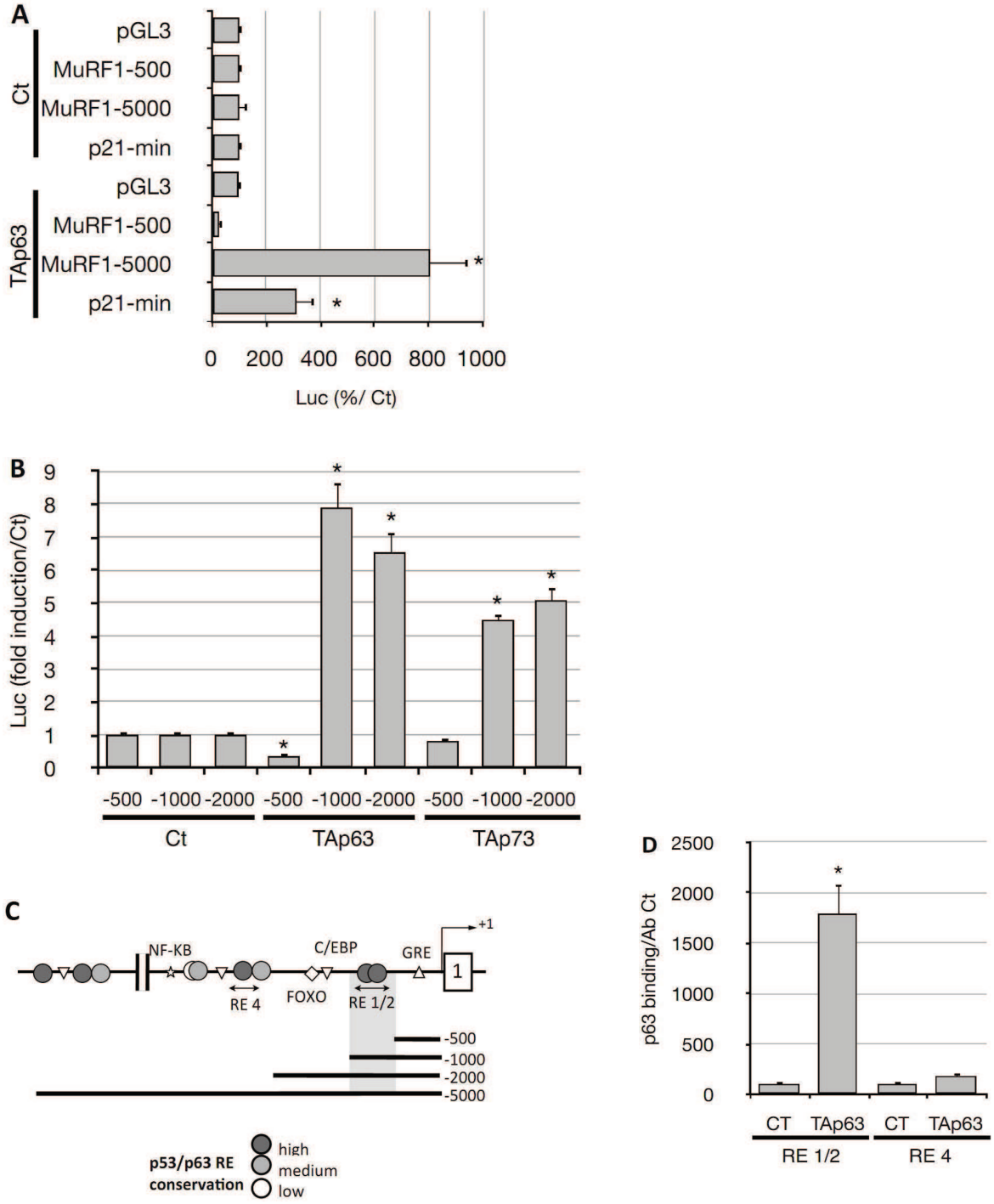


Figure 8 | Regulation of *murf1* promoter by p63.

(A, B) *Murf1* reporter genes were co-transfected with pCDNA3 or TAp63 in C2C12 cells and luciferase activity was assessed 16 hours later. pGL3 was used as negative control. Bars correspond to means with SD (n=3). *: $p < 0.01$. **(C)** *murf1* promoter with potential p63 binding sites and regulation of *murf1* reporter genes by p63. Progressive *murf1* promoter deletion reporter genes (-500; -1000; -2000; -5000 Murf1-luc) and putative p63 binding sites are indicated. **(D)** Chromatin immunoprecipitation assay on *murf1* promoter. Bars correspond to means with SD (n=3). *: $p < 0.01$.

Supplementary 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
Gadd45∝	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

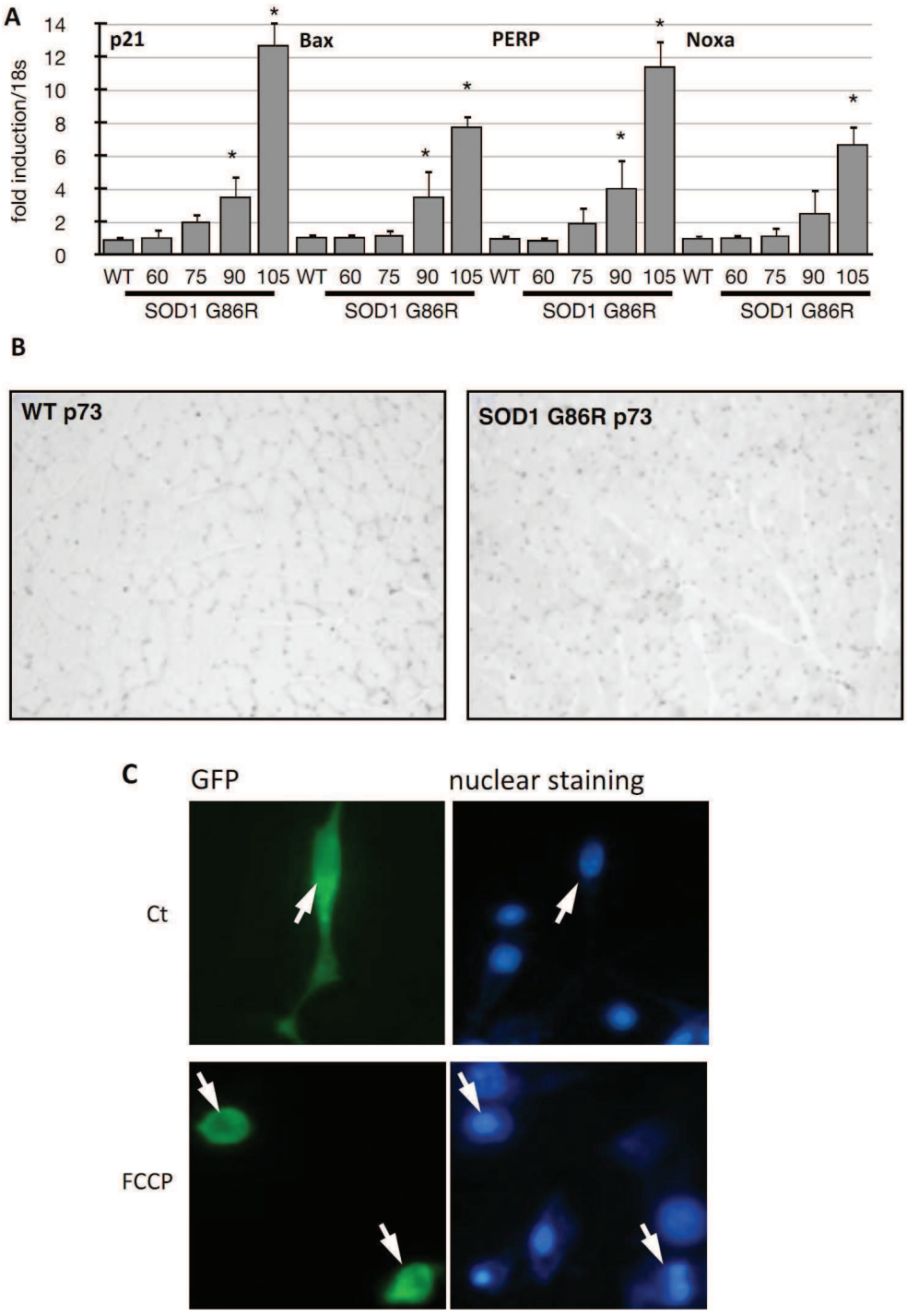
Table 1 | **Regulation of p53 family related genes in skeletal muscle of SOD1(G86R) and denervated mice.**

Data were generated using a gene expression database deposited at ebi.ac.uk/arrayexpress (accession no. 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.

Gene name	Function	90 d.	105 d.
p53-family target genes			
p21/Cdkn1a	Cell cycle arrest	4	13
Gadd45α	Cell cycle arrest	5.6	21
Peg3	Apoptosis inducing	3	7
Perp	Cell cycle arrest	4	12
Noxa	Apoptosis effector	5	12
Bax	Apoptosis effector	3	8
SIVA	Apoptosis inducing	3	5
Zmat3/Wig-1	Growth regulation	1.6	1.1
Eda2R	NF.Kb/JNK pathway	3.4	9.4
TIGAR	Glucose metabolism	0.75	0.2
Sestrin1	ROS homeostasis	-	16.3
Sestrin2	ROS homeostasis	1.27	1.46
SCO2	Glucose metabolism	1.18	0.91
CHOP/DDIT3	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			
ACh Receptor alpha	Neuromuscular junction	4.2	12.4

Table 2 | **Individual assessment of p53 family related gene regulation in skeletal muscle of SOD1(G86R) mice.**

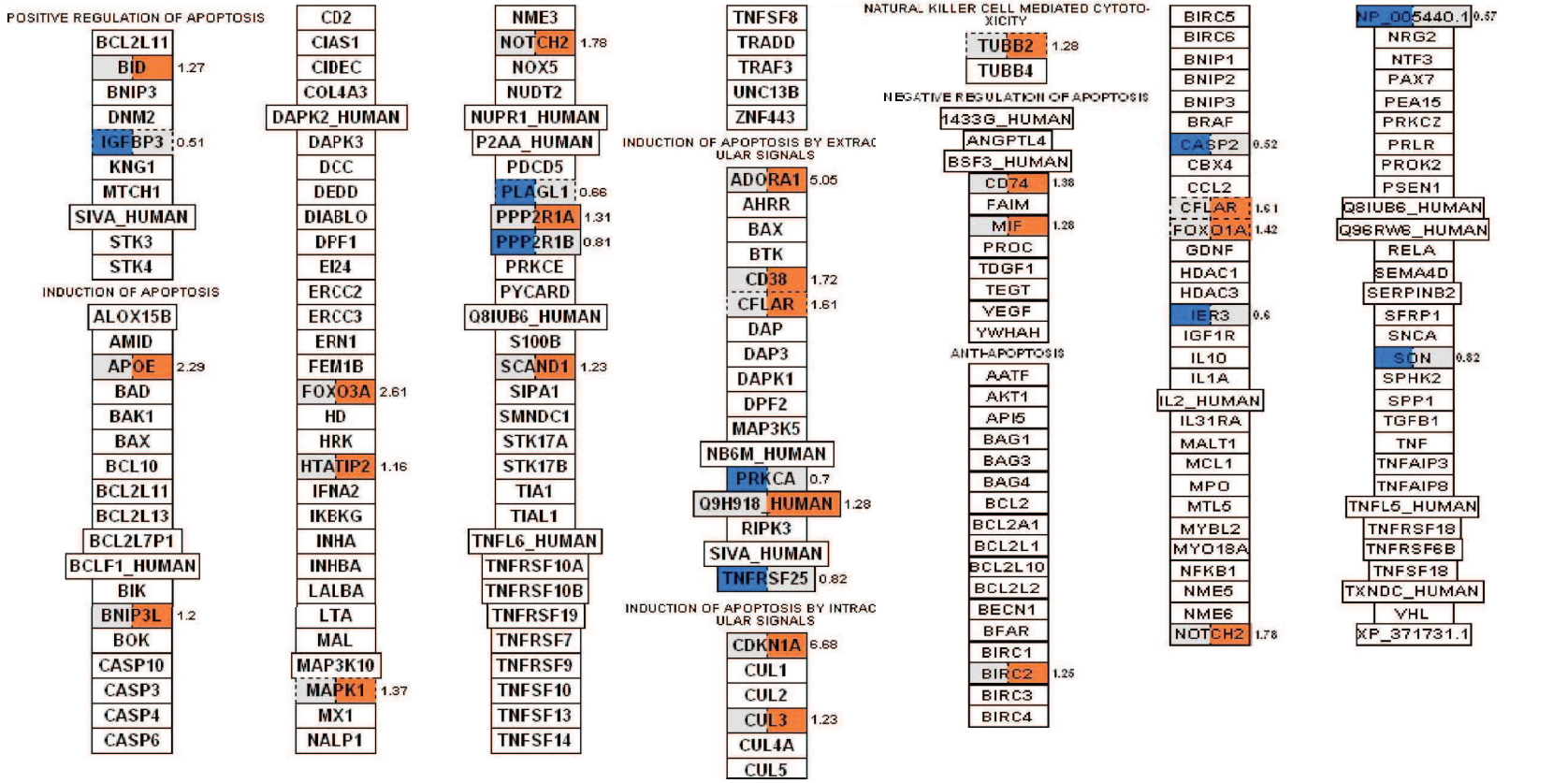
Summary of the regulation of p53 family target genes, regulators and members as assayed by quantitative PCR such as described in Figure 1 and 2. Results from additional genes that were tested as described in Figure 1 and 2 are presented (SIVA, Tigar, CHOP/DITT3...). Values from 90 day-old (Preparalysis) and around 105 day old symptomatic mice are presented.



S3 | **A. Correlation of p53 family target gene expression in muscles from patients and an ALS mouse model with disease intensity.**

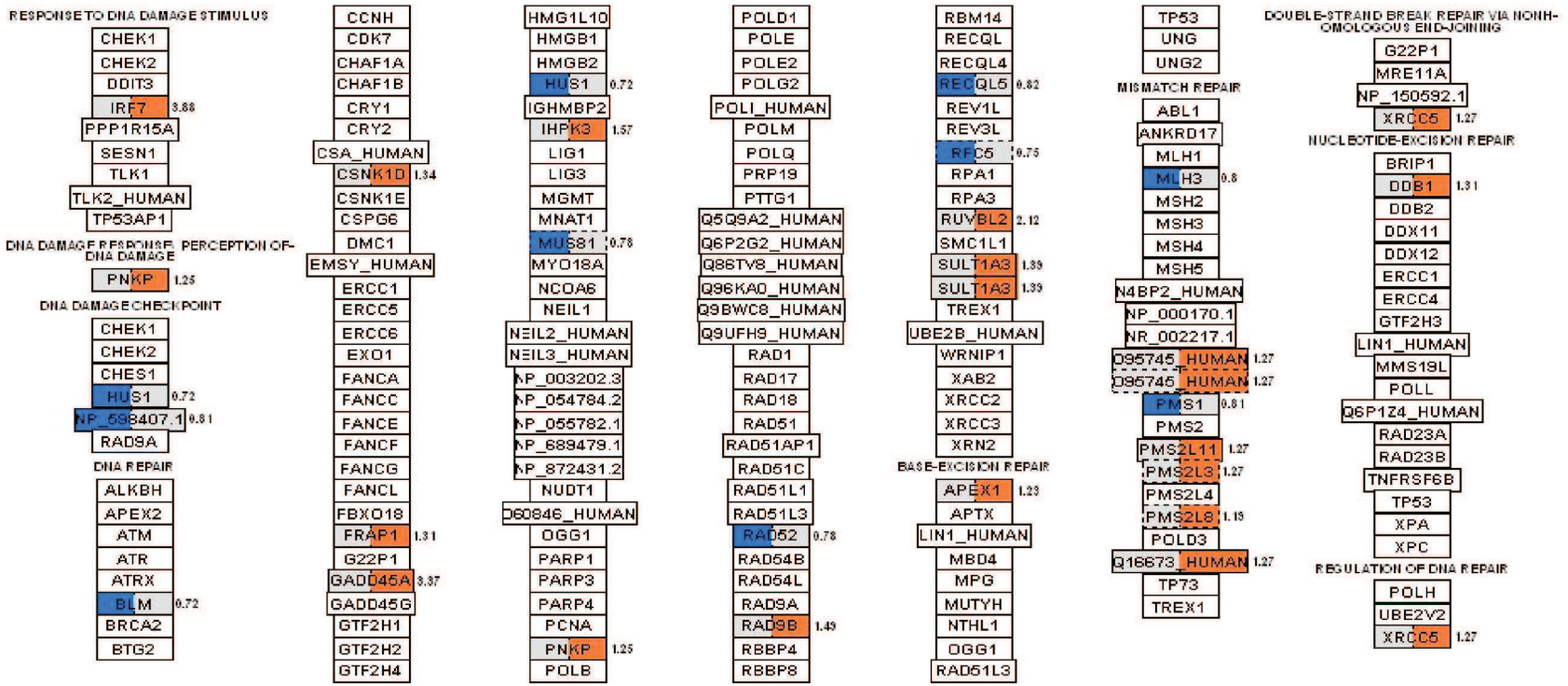
A. Expression levels of p53 family target genes (p21, Bax, Perp, Peg3) were tested. WT refers to 105 day-old WT animals, as inter-age variations were insignificant. Bars are means + SD of fold induction versus the condition WT 60 days old. *: p< 0.01 compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test **B**. Gastrocnemius muscles from wild-type or symptomatic SOD1(G86R) (105 days) mice

were cryodissected and probed for total p73 protein. **C**. C2C12 were transfected with a GFP expression vector and either TAp63γ or ΔNp63γ expression vectors. After 24 hours, cells were left untreated (Ct) or treated with FCCP (1μM) or menadione (1μM) for 24 hours. Cells were stained with Hoechst and examined with a fluorescence microscope. Shown is an example of this staining. Above, GFP-positive control cells (untreated). Below, dead GFP-positive cell treated with FCCP.



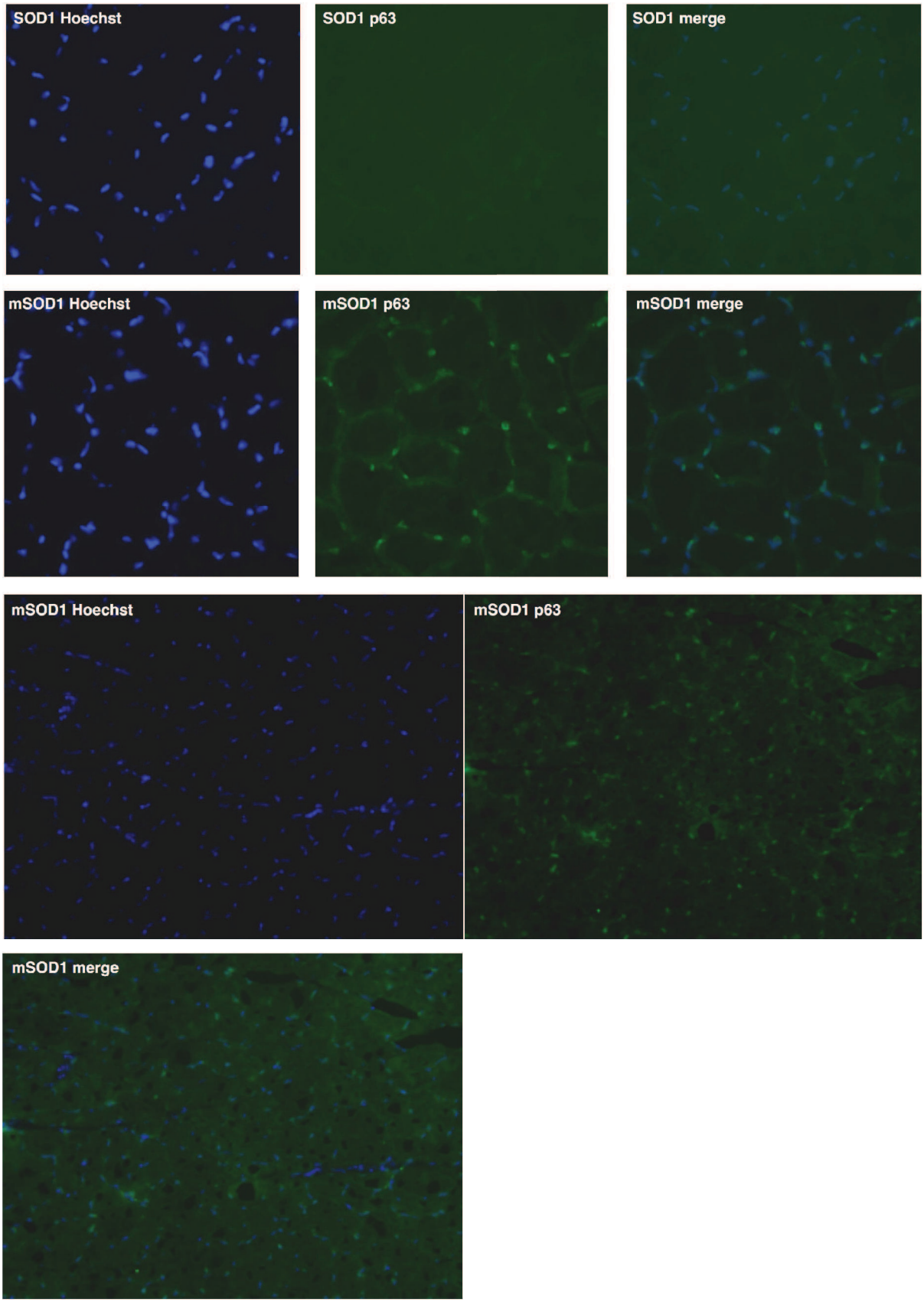
S4 | **Genes involved in regulation of apoptosis** (A)

...that showed differential expression in deltoid muscle biopsies from 9 patients with probable or definite ALS according to the revised El Escorial criteria, as compared to 10 control subjects undergoing a shoulder orthopaedic surgery. Raw data were generated by DNA microarray analysis and deposited at www.ebi.ac.uk/arrayexpress (accession no. E-MEXP-3260) [Pradat et al., 2011]. Gene expression changes were considered significant at $P<0.05$, as determined by two-sample T-test. Significant gene expression changes were visualised by means of GenMAPP version 2.1 software. Numbers next to boxes indicate the ratio of expression in ALS patients versus control subjects.



S4 | **Genes involved in response to DNA damage** (B)

...that showed differential expression in deltoid muscle biopsies from 9 patients with probable or definite ALS according to the revised El Escorial criteria, as compared to 10 control subjects undergoing a shoulder orthopaedic surgery. Raw data were generated by DNA microarray analysis and deposited at www.ebi.ac.uk/arrayexpress (accession no. E-MEXP-3260) [Pradat et al., 2011]. Gene expression changes were considered significant at $P<0.05$, as determined by two-sample T-test. Significant gene expression changes were visualised by means of GenMAPP version 2.1 software. Numbers next to boxes indicate the ratio of expression in ALS patients versus control subjects.



S5 | **p63 expression assessed in SOD1(G86R) mice by immuno-histochemistry**

Transcriptomics. Gene expression in muscles of SOD1(G86R) mice and ALS patients was analyzed at the transcriptome level using two databases previously generated and deposited at www.ebi.ac.uk/arrayexpress, as described [50, 51]. To identify differentially expressed genes, statistical assessment was achieved by applying two-sample T-test, as implemented in BRB-ArrayTools version 3.8 software (developed by Dr. Richard Simon and BRB-ArrayTools Development Team, and freely available from the National Cancer Institute at linus.nci.nih.gov). Significant gene expression changes were visualised in functional categories based on Gene Ontology classification, using GenMAPP version 2.1 software (developed by the J. David Gladstone Institutes at the University of California San Francisco, and freely available at www.GenMAPP.org). Correlation analysis was performed by means of non-parametric Spearman test, as implemented in Prism version 4.0b software (GraphPad Software Inc., San Diego, CA).

Immunohistochemistry. Mouse gastrocnemius muscles were sampled, submersed in freezing medium (Tissue-Tek O.C.T compound, Sakura) and immediately frozen in a nitrogen-cooled isopentane bath. Muscles were sliced in transversal axis at 14µm in a cryostat (Leica CM3050S) 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% para-formaldehyde for 10 min [72]. After a 5 minute wash with PBS, the samples were permeabilized with 3% Triton X-100 in PBS for 10 min, washed with TBS, incubated in 100mM 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) at 1:100 with 0.1% Triton X-100 in PBS (Triton buffer) overnight at room temperature. They were then washed thrice with Triton buffer for 10 min and incubated with cyanine 3-coupled goat anti-mouse antibody (Jackson Immunoresearch) at 1:1000, as well as with 1µg/ml Hoechst 33342 (Sigma), in Triton buffer at room temperature for 1 h. After washing thrice with Triton buffer, the slides are covered with mounting medium (Aqua-Poly/Mount, Polysciences) on glass slips and observed by confocal microscopy (Zeiss, Germany). Antibody specificity were verified with slides probed with only the secondary antibody

S6 | **Supplementary material and methods**

VII - 2. Biological activity of novel flavonoid and tocopherol derivatives on myoblast survival and protective effect towards cancer cachexia or amyotrophic lateral sclerosis

While parts of the many mechanisms leading to muscular atrophy are well understood, there still is confusion how they are activated and how they interact. Differences, or interplay between fundamental atrophy-inducing pathways complicate the finding of drugs against specific targets. Indeed, different settings of atrophy involve different mechanisms, for example fibre degradation versus the inhibition of myofibre renewal.
AxoGlia Therapeutics is a young biotech based in Luxembourg and consists of a team of chemists and biologists that synthesize and test flavonoid and tocopherol analogues in the context of neurodegeneration. AxoGlia evidenced that some of their molecules induce differentiation of neurons, protecting them from stress. In addition, treatment with these compounds repressed the Notch pathway in neuronal cells.
We were interested in the protective properties of the molecules and wondered if they could be of benefit in muscular cells.

Approach

In order to evidence the protective potential of the compounds, we challenged cultured C2C12 myoblasts with different drugs in combination with AxoGlia compounds and assessed cell survival. Cultured myoblasts were also used to start investigating the molecular mechanisms induced by AxoGlia compounds. Finally, we used the SOD1(G86R) mouse model of ALS and the doxorubicin-induced mouse model of cancer cachexia to assess the effects of AxoGlia compounds on muscular atrophy *in vivo*.

Main findings

- Two compounds, AGT48 and AGT251, induce hyperproliferation of myoblasts

- AGT48 and AGT251 protect cultured myoblasts and motor neurons from genotoxic stress
- The Notch pathway was perturbed in myoblasts treated with AGT251 and in SOD1(G86R) mice
- AGT251-treated SOD1(G85R) mice had an increased lifespan
- Treatment of mice with AGT251 reduced muscular atrophy after treatment with Doxorubicin

Biological activity of novel flavonoid and tocopherol derivatives on myoblast survival and protective effect towards cancer cachexia or amyotrophic lateral sclerosis

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Running title

Tocopherol and flavanoid derivatives biological activity on muscle atrophy via the Notch pathway

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Abstract

Muscular atrophy is a physio-pathological process associated with severe human pathologies such as Amyotrophic Lateral Sclerosis (ALS) or cancer. One of the molecular mechanisms proposed to play a major role is the production of reactive oxygen species (ROS). Based on their known anti-oxidant properties we explored the potential of flavonoid- and tocopherol-omega alkanol chain derivatives in protecting muscle against atrophy. All compounds showed a cytotoxic activity at high concentrations on myoblast cells. However, we identified two tocopherol-omega alkanol chain derivatives AGT48 and AGT251 protecting myoblastic cells against ROS productive agents, cisplatin and menadione. We also showed that these compounds regulated the Notch pathway that is important in controlling muscle cell fate. In particular, mRNA levels for *notch 3* and *4* were strongly repressed. *In vivo* experiments demonstrated that these compounds delayed death by respiratory muscle atrophy in a murine model of ALS. Interestingly, in this model the mRNA levels for *notch 3* and *4* were also repressed, while the mRNA level for the Notch target gene *hes 1* was transiently induced. Finally, we showed that the compound AGT251 partially opposed muscle atrophy and weight loss in a mouse model of cancer related cachexia. Altogether, we identified two compounds of the tocopherol family that protect against muscle atrophy in various models possibly through the regulation of the notch pathway.

1. Introduction

Muscle atrophy is associated with aging and severe human pathologies, such as degenerative diseases, cancers, AIDS, and nerve injury (von Haehling et al., 2010). For example, Amyotrophic Lateral Sclerosis (ALS) is characterized by the death of motoneurons and the atrophy of skeletal muscles, which leads to paralysis and death due to a lack of an efficient cure (Ludolph et al., 2012). The pathology is usually diagnosed between the ages of 40 and 70, and the incidence is of 1.5 for 100 000 people. During cancer, muscle atrophy (also called muscle wasting or cachexia in this context) may develop as a side effect of chemotherapy (cisplatin, doxorubicin) (Hydock et al., 2011), or in late stage of the pathology (Fearon et al., 2012). This muscle-wasting syndrome is strongly debilitating for the patients and drastically reduces the quality of life. Even more dramatically, it may force the clinician to stop the treatment and therefore cachexia is estimated to account for up to 30% of the cancer lethality. In these pathological settings, the lack of an efficient cure correlates with a poor knowledge of the molecular mechanisms involved.

One of the molecular mechanisms that has been proposed to play a key role in muscle atrophy is the abnormal production of ROS (Barbieri and Sestili, 2012). ROS are continuously produced by the cell metabolism and play a role in cell signaling and homeostasis. However, upon various physio-pathological conditions ROS levels are abnormally increased due to excess of production or lack of detoxification by enzymes such as GSH, SOD or catalase (Ray et al., 2012). In particular, elevated ROS production has been linked to the presence of mutations in the ROS detoxification enzyme SOD1 associated with hereditary ALS (Beckman et al., 2001). Similarly, cisplatin or doxorubicin, two widely used anticancer drugs have been shown to induce the production of ROS, which mediate their cytotoxicity (Chirino and Pedraza-Chaverri, 2009). ROS production has also been linked to the muscular atrophy induced by the presence of a tumor in mice, likely mediated by cytokines such as TNFalpha (Adams et al., 2008). At the cellular level, production of ROS induces lipid peroxidation, protein oxidation and DNA damage. These cellular alterations lead to activation of specific signaling pathways such as the unfolded protein response (UPR, for protein oxidation and aggregation) and/or transcription factors such as p53/p73 (for DNA damage), which in turn lead to apoptosis or autophagy and tissue degeneration (Barbieri and

Sestili, 2012; Benosman et al., 2007; Benosman et al., 2011; Gonzalez de Aguilar et al., 2000).

In order to counteract the atrophy process, several small molecules with antioxidant properties have been developed and tested for their cytoprotective properties. Two examples of these antioxidants are the tocopherol derivatives and the flavonoids. Interestingly, 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, fat-soluble vitamins, including alpha-tocopherol, are considered as important disease modulators in multiple sclerosis (MS) and show beneficial outcomes on white matter damage in experimental models (Blanchard et al., 2013). Flavanoids derivatives have also shown the ability of reducing ROS effects and have neuroprotective activity or cardioprotectivity (Hauss et al., 2007; Ribeiro et al., 2012; Thuaud et al., 2011). These compounds have been recently shown to interact with proinhibitin that mediates some of their effects (Polier et al., 2012).

In view of these cytoprotective effects, we decided to investigate the ability of novel derivatives presenting an omega-alkanol chain to protect or improve the health of animals that are affected by muscle atrophy.

2. Materials and methods

2.1 Chemicals and Synthesis

AGT048, AGT251 and AGT262 tocopherol derivatives synthesis were performed as described previously (Muller et al., 2006). AGT072 hydroquinone synthesis was also described previously (WO2010128038). AGT031, AGT171, AGT184 and AGT216 flavonoid derivatives were synthesized following the chemical scheme described in patent WO2009130253.

2.2 Cell cultures

Murine C2C12 myoblasts and murine motoneuron NSC34 cells were manipulated and cultured in DMEM with 10% FCS (Eurobio) and 1% Penicillin + Streptomycin (Sigma) at 37°C with 5% CO2 atmosphere as previously described (Sohm et al., 1999).

2.3 Cell survival

2000 cells were seeded per well in 96-well microplates (Falcon Multiwell), 48h prior to any treatment. Drugs were applied for 48h in fresh medium. MTT assay was performed as previously described by replacing the medium with fresh medium supplemented with 5mg/L MTT (Sigma) for 1h (Gaiddon et al., 1999). Cells were lysed in isopropanol with 0.04N HCl. Measurements were performed at 550nm.

2.4 Quantitative PCR.

Cultured cells were lysed with 1ml of TRIzol per $10cm^2$. Mice were sacrificed by cervical dislocation, hindlimb gastrocnemius muscles were sampled, immediately frozen in liquid nitrogen and stored at -80°C for later use. 1ml TRIzol (Invitrogen) per 150mg of muscle was used to extract RNA according to manufacturer's instructions. RNA samples were ethanol-precipitated twice and 1µg was used for reverse transcription (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems). qPCR was performed using 2ng/µl cDNA (RNA equivalent) according to manufacturer's instructions (SYBR Green PCR Master Mix, Applied Biosystems) and with 400µM of each primer (Supplemental data, table 1). Expression levels were normalized using average of 18S.

2.5 SOD1(G86R) mice

FVB mice were transduced as described with mutant SOD1(G86R) under constitutive promoter control. Transgene expression was monitored by PCR on SOD1 (F: GACATCATTGTTCATCC R: ATTGATGGAATGCTCTCCTGA), FspI digestion and agarose electrophoresis (Ripps et al., 1995). We distinguished several experimental groups: young asymptomatic mice (60 days), asymptomatic mice (75 days), presymptomatic mice (90 days, in certain cases further divided into normal and denervated) and symptomatic mice (105-110 days) when full paralysis in on.

2.6 Mice treatment

SOD1(G86R) mice were injected intraperitoneally 3x/week beginning at an age of 65 days. AGT251 (20mg/Kg) was administered in 0.9% NaCl with 6% propylene glycol, 4% DMSO and 2% Cremophor EL. Vehicle-treated littermates received the same formulation without AGT251. For doxorubicin treatment, 8 weeks-old mice were injected once intraperitoneally with doxorubicin (Bioaustralis) diluted at 20mg/kg in

0,9% NaCl. AGT251 treatment was performed as described above. *In vivo* experiments were repeated at least twice with a number of animals (between 5 and 12) recommended to optimize statistical analyses accordingly to the regional and national animal ethical committee. All animal manipulation were performed under appropriate supervision and following protocols validated by the regional and national animal ethical committee.

3. RESULTS

3.1 Tocopherol and flavonoid derivatives regulate cell cycle and cell survival in myoblastic and neuroblastoma cells

The effect on cell survival of seven AGT flavonoid or tocopherol derivatives was assayed on myoblast C2C12 and motoneuron NSC-34 cell lines. Dose-response relationships were established by means of an MTT assay and using a large window of concentrations (1nM-0.1mM) (Fig. 1). Most compounds were reducing cell number with an IC50 in the range between 50 and 125 μM, although 3 compounds were already active at lower concentrations (Fig. 1A, Table 1). The ability of these compounds to reduce cell numbers was also confirmed by the up-regulation of the expression of p21 and noxa, two genes respectively involved in cell growth arrest and apoptosis (Fig. 1B). Interestingly, two compounds did significantly increase total cell survival by 22% (AGT048) or 43% (AGT251) at concentrations preceding toxicity. AGT031 did not show a pro-survival effect and was more toxic than most compounds. Thus, it was used as a negative control in subsequent experiments.

3.2 AGT048 & AGT251 protect against oxidative and genotoxic stresses

As AGT048 and AGT251 compounds were inducing an increase in cell number, we assessed whether these compounds could protect cells against stresses that are associated with degenerative syndromes, including oxidative stresses. Therefore, C2C12 and NSC34 cells were treated with various chemical cytotoxic compounds that induce stresses associated with degenerative syndromes. We used: i) menadione that induces a strong production of ROS, ii) FCCP that provokes a mitochondrial dysfunction, iii) tunicamycin that causes protein aggregation, and iv) cisplatin that induces DNA

damages and mild ROS production. We chose concentration of the cytotoxic drugs corresponding at their IC75 (dose necessary to induce 75% of their maximal effect). We observed that AGT048 and AGT251 were able to partially rescue cell loss induced by cisplatin and menadione but not the others (Fig. 2 and data not shown). The protective effect was mostly observed with mild concentrations of the cytotoxic drugs. As expected, AGT031 did not protect cells.

Taken together, these experiments demonstrated that some AGT compounds, especially AGT251, have a positive effect on cell growth and protect myoblasts and motoneurons against genotoxic stress *in vitro*.

3.3 AGT251 represses the notch pathway

Previous work has shown that compounds sharing a similar architecture to our compounds were able to repress *notch 4* messenger RNA expression in neurospheres (Coowar et al., 2004). In addition, it has been suggested that the notch pathway is playing a role in muscle regeneration (Arthur and Cooley, 2012). To assess whether a similar mechanism was used by AGT251 in the C2C12 myoblastic cells we followed notch-receptor *(notch 1-4)* and -ligand (*jagged* and *delta*) expression levels by RT-qPCR (Guruharsha et al., 2012). We observed that in AGT251-treated C2C12 cells Notch 3 mRNA level was down-regulated, especially at concentrations of AGT251 that have a strong cytotoxic effect (30 and 60µM) (Fig. 3). In addition to *notch 3* down-regulation, we showed a decrease of *notch 1*, *2* and *4* receptor-, as well as of the notch-ligand *jag1* mRNA levels at cytotoxic concentrations of AGT251. On the contrary, and quite unexpectedly, the mRNA expression levels of the notch-ligands *delta 1* and *jagged 1* were significantly increased at high doses of AGT251. Another interesting observation was that the only gene expression patterns that clearly differentiated protective dose and cytotoxic doses of AGT251 were whose of *jag2* and *notch 4*. Indeed, the protective doses of AGT251 (7.5 and 15µM) increased the expression of *jagged 2* and *notch 4*, while the higher and cytotoxic doses of AGT251 (30 and 60µM) strongly decreased it. This differential effect of AGT251 on gene expression was also observed on two genes of cell cycle arrest and cell death, respectively *p21* and *noxa* (Fig. 3). Expression of both genes was unchanged or tended to diminish with cytoprotective doses of AGT251 and was strongly induced by cytotoxic doses.

Cisplatin treatment by itself affected relatively weakly the expression of the components of the notch pathway (Fig. 3). Variations in mRNA levels were not above 2 fold (*notch 4*) depending on the dose and the gene analyzed. In presence of cisplatin, the overall regulation of the notch pathway by AGT251 was maintained.

3.4 AGT251 increases survival in mouse model of ALS

Given its protective effect on muscle and neuronal cells *in vitro*, we wondered if treatment with AGT251 could be beneficial for mice showing a progressive degeneration of the motor-axis. Mice with a substitution mutation in the Cu/Zn Superoxide Dismutase 1 gene (G86R) are used as a model of the human pathology Amyotrophic Lateral Sclerosis (ALS) (Gonzalez de Aguilar et al., 2000). Those mice (subsequently referred as SOD1(G86R) mice) die at around 105 days following muscular atrophy and motoneuronal death. SOD1(G86R) mice of 60 or 75 days of age are in the asymptomatic stage and physical symptoms are starting around 90 days with some mice showing already a clear denervation (indicated by: 90N) of the gastrocnemius muscle.

As AGT affects the notch pathway, we first assessed whether in this model, the expression of components of this pathway were altered during the ALS pathology. We measured the expression levels for Notch receptors and for their target genes *hes* (Fig. 5A, B). Expression of the notch related genes was compared with that of a denervation marker (acetyl choline receptor alpha) and two effectors of muscle atrophy (MuRF1 and Atrogin-1) (Fig. 5C, D) (Foletta et al., 2011). We detected only the expression of *notch 3* and *4* and *hes 1*. Importantly, we observed a strong down-regulation of *notch 3* and *4* expression levels in SOD1(G86R) mice showing clear ALS symptoms characterized by denervation and muscular atrophy, which correspond to denervated animals (90N and 105 days-old) as indicated by the high expression of the *acetylcholine alpha-receptor*, *murf1* and *Atrogin*-1 genes. In contrast, the expression of the Notch target gene *hes1* was biphasic. We observed an induction of *hes1* at the age of 90 days that subsequently at 105 days decreased to a level comparable to that found in control mice (Fig. 5B). These observations established that in ALS atrophic muscle a notch-dependent response was taking place that might participate in the development of the pathology or in a compensatory process.

We then used these mice to assess whether AGT251 could prolong their life span. We treated those mice thrice a week with AGT251 starting at an asymptomatic age of 65 days. Treatments were performed until death. Results showed a statistically significant increase in survival of AGT251 treated SOD1(G86R) mice by 8% compared to vehicle treated mice (Fig. 5E). Vehicle injected animal had a median survival of 105 days while it was 112 days for AGT251-injected animals. This finding suggests that the beneficial effect of AGT251 on muscle and neuronal cells also occurs in mice, delaying death and the progression of muscular atrophy in our murine ALS model.

3.5 AGT251 protects muscle from doxorubicin-induced cachexia

ALS is a complex pathology and it is still hotly debated which cell population between motoneurons, astrocytes and muscles, participate to or are important for disease. As the beneficial effects of AGT251 on mouse survival may impact any of these cell types, we decided to further investigate the protective effect on a more specific animal model of muscle atrophy. For this, we chose a mouse model in which muscle atrophy is induced by the anticancer drug doxorubicin.

Cachexia is a severe syndrome with muscle atrophy observed in patients treated with doxorubicin or harboring tumors (Fearon et al., 2012). The muscular atrophy induced by doxorubicin treatment can be reproduced in mice (Romanick et al., 2013). To further assess the protective effects of AGT251 against muscle atrophy we used two groups of mice that were treated with doxorubicin (10mg/Kg) to induce cachexia. One group was simultaneously treated with AGT251 (20mg/Kg). After 15 days, total mice weight and skeletal muscle weight were measured and RNA was extracted from gastrocnemius muscles to quantify expression of muscle atrophy markers (MuRF1 and Atrogin-1).

We observed a loss of total animal and muscle weight upon doxorubicin treatment (Fig. 6A and data not shown). This effect correlated with an up-regulation of the two effectors and markers for muscle atrophy, MuRF1 and Atrogin-1. Very interestingly, we observed that the co-treatment with the tocopherol derivative AGT251 partially reversed the muscle atrophy as assessed by muscle weight. This effect is further confirmed by the measure of MuRF1 and Atrogin-1 expression that showed a repression upon AGT251 treatment. Based on our results AGT251 appeared to protect muscles against atrophy in cachexia conditions and improves animal survival in a neurodegenerative disease mouse model.

4. Discussion

Muscular atrophy is associated with several lethal human diseases such as neuro-muscular syndromes or cancers. In the case of ALS, respiratory muscle atrophy leads to death by suffocation. In cancer, it has been estimated that about 30% of the lethality of cancer is due to cachexia that includes muscle atrophy. 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 anti-oxidant and anti-inflammatory properties that have previously shown protective activity on the nervous system and the heart.

4.1 Cellular effects of novel flavonoid and tocopherol derivatives

The synthesized flavonoid and tocopherol derivatives hybrid with an omega-alkanol chain showed all *in vitro* biological activity on the C2C12 myoblast cell line. Above a specific concentration, each compound was able to drastically reduce cell growth as observed by MTT tests and on the induction of cell growth arrest and pro-apoptotic genes p21 and Noxa (Table 1, Fig. 1, 4). Similar results were obtained with the motoneuronal cell line NSC34 (Fig. 2 and data not shown). However, a moderate concentration of two tocopherol derivatives, AGT048 and AGT251 sharing a similar pharmacophore, favored the cell growth of C2C12 cells. In addition, at these sub-toxic concentrations AGT048 and 251 counteracted the toxic effect of cisplatin on both C2C12 cells and NSC34 cells (Fig. 2). The tested flavanoid and tocopherol derivatives share all the same omega-alkanol chain. However, only two tocopherol derivatives present the protective effect on C2C12 cells, which suggests that the protective effect is not mediated by the omega-alkanol chain and more likely by an intact tocopherol core.

4.2 Tocopherol derivatives regulate the notch signaling pathway in myoblasts

It was previously shown that tocopherol derivatives repressed the mRNA level for Notch 4 in neuronal cells (Coowar et al., 2004). In addition, a recent study showed that the Notch pathway is involved in muscle repair during aging or physical damage (Arthur and Cooley, 2012). Our study brings additional information on these two aspects. First, we showed that high concentration of tocopherol derivatives strongly repress not only *notch 4* but also *notch* 1, 2 and 3, as well as the notch ligands *jagged 2*

and *delta 1*. In contrast, Jagged 1 is up-regulated, indicating a switch between the two Notch ligands, jagged 1 been replaced by Jagged 2. Therefore, these novel data indicate that tocopherol derivatives induce a strong alteration of the notch pathway. However, these strong changes in Notch signaling are not observed at moderate concentration of tocopherol derivatives. Indeed, we observed only a mild up-regulation of *jagged* 2 and *notch 4* both in absence and in presence of stress (cisplatin). It remains to establish whether these alterations of the Notch pathway are playing a fundamental role in the tocopherol derivatives activity on muscle cells as observed in neurons (Coowar et al., 2004).

Our second interesting observation is that the Notch signaling pathway is altered in the mouse model of ALS harboring a severe muscular atrophy (Fig. 5). We observed a significant down-regulation of *notch 3* and *4* at the end stage of the pathology when muscular atrophy is the strongest. Interestingly, it follows a transient up-regulation of the expression of the Notch1 target gene *hes1*. The functional significance of these regulations is still unclear as the notch signaling pathway is complex and its role in muscle cell proliferation and differentiation is still debated. In particular, the exact role of the various receptor and ligands is not well established and the various receptors are often generically referred as the "Notch receptor". *In vitro* and *in vivo* data indicate that activation of the Notch pathway inhibits muscle cell differentiation (Buas and Kadesch, 2010) and is induced in satellite cells of the muscle when mechanically injured (Conboy and Rando, 2002). In addition, it has been shown that inhibition of the Notch pathway with soluble Jagged 1 reduces muscle regeneration while its activation with a Notch1 specific antibody facilitate the repair process (Conboy et al., 2003). Therefore the transient up-regulation of Hes1 in SOD1(G86R) mice might be explained by an attempt of the muscle to start a regenerative process. However, how exactly the Notch receptor(s) controls through Hes 1 the differentiation and the regenerative processes in muscle remains elusive. The existence of redundant and cross-regulated mechanisms at the receptor, the ligand and the target gene levels might explain the difficulty to understand the role of this/these pathways in muscle physiology. This is rather well illustrated in our conditions as we see a switch between *jagged 1* and *jagged 2* expression upon tocopherol treatment that correlate with its toxic affect on myoblast cells. Altogether our observations highlight the fact that the Notch pathway is significantly deregulated in stress conditions that affect the muscle physiopathology,

including neuromuscular diseases, and support the idea that molecules targeting the notch pathways might be interesting for muscle protection.

4.3 Tocopherol derivatives ameliorate mouse survival in ALS model and inhibit muscle cachexia

The trophic effect of tocopherol derivatives on myoblast and motoneuronal cell survival observed *in vitro* was validated using *in vivo* models. Indeed, sustained injection of AGT251 prolonged mouse survival of mice developing ALS with severe muscle atrophy (Fig. 5). This effect might be linked to an improved resistance of muscle fibers and/or motoneurons that are both primary tissues affected by the pathology. The protective effect of the tocopherol derivative AGT251 on muscles was further confirmed using a mouse model of cancer cachexia. In this model, AGT251 reduced the cachexia induced by doxorubicin both on the overall weight of the mice and more specifically on the muscular weight, and effectors and markers of muscular atrophy (MuRF1 and Atrogin-1).

Altogether these *in vivo* data indicate that specific tocopherol derivatives can be used as protective molecules for treating syndromes that involve skeletal muscle atrophy, such as observed in ALS or cancer-related cachexia. Therefore, tocopherol derivatives appear as interesting molecules that can protect against stresses in several types of tissues, such as heart (Prasad, 2011) and neurons (Sen et al., 2004), but also skeletal muscles.

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Figures

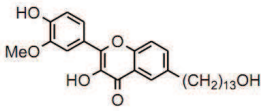
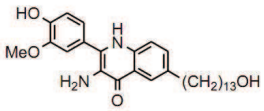
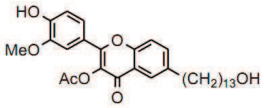
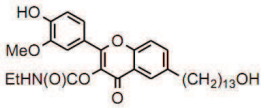
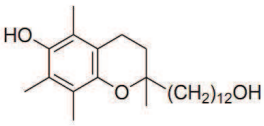
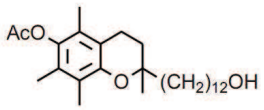
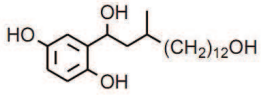
	compound	formula	IC50 for cytotoxicity (μM)	maximum cell number (%/Ct)	conc. for max. survival (μM)
Flavanoid derivatives	AGT031		10 - 25	100	0.5
	AGT171		75 - 125	100	0.5
	AGT184		25 - 50	100	0.5
	AGT216		10 - 25	100	0.5
Tocopherol derivatives	AGT048		50 - 75	122	0.5
	AGT251		50 - 75	143	12.5
	AGT072		50 - 75	100	0.5

Table 1 | **Biological activity of tocopherol and flavonoids 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.

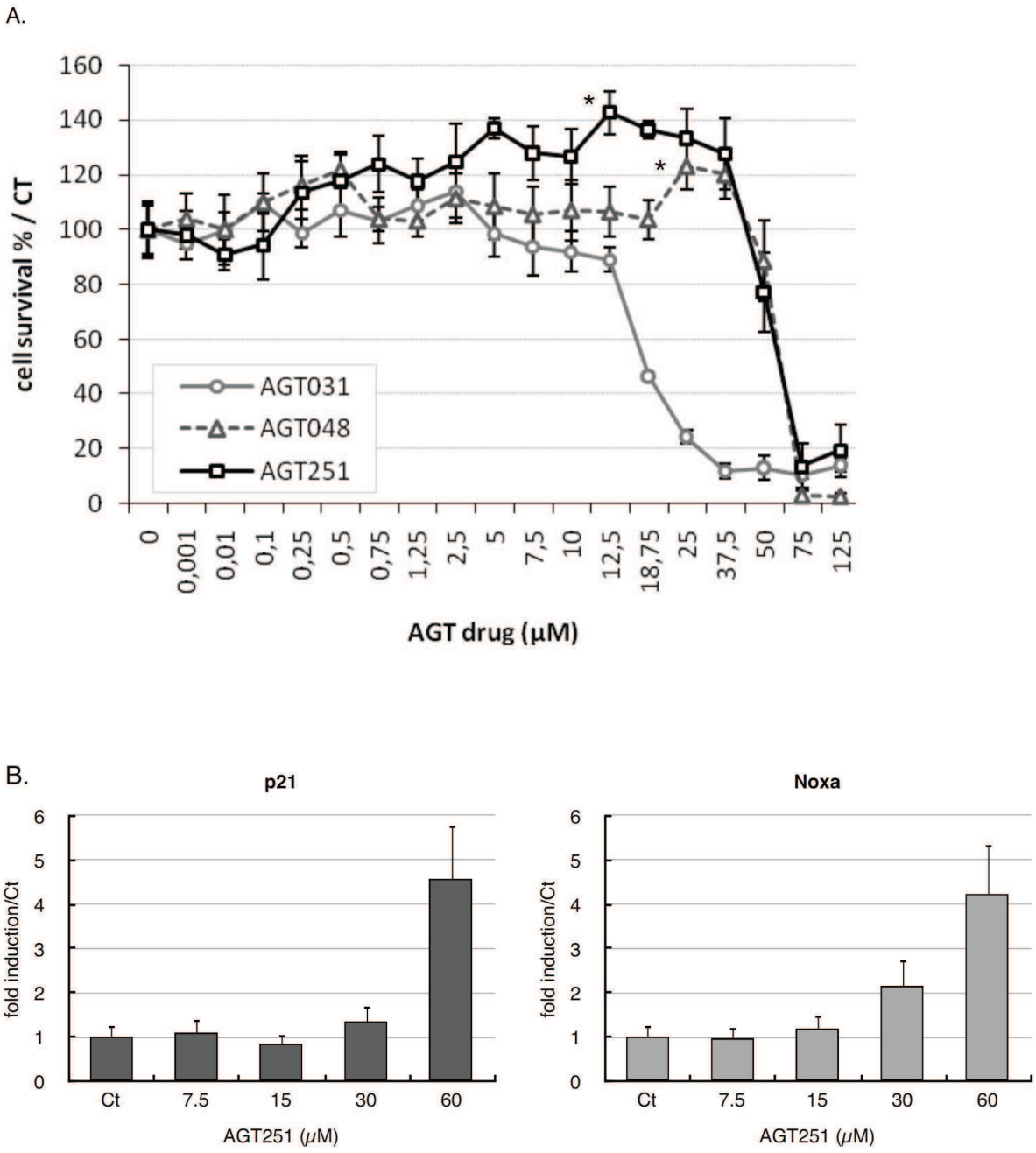


Figure 1 | **Tocopherol and flavonoids derivatives control myoblast cell survival.**
(A) C2C12 myoblast cells were cultured for 48 hours in presence of the indicated compounds (AGT031, AGT048, AGT251 in µM). Cell survival was evaluated using MTT test. Curves indicate mean ± SD (n=8). *: $p < 0.01$ compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test. **(B)** The Tocopherol derivative AGT251 regulates p53 targets genes involved in cell cycle arrest and apoptosis in myoblast cells. mRNA levels of p53 target genes p21 and noxa were assayed in C2C12 myoblast cells treated with AGT 251 (in µM) using RT-qPCR. Graphs are means (n=3) of fold induction versus Ct in absence of cisplatin.

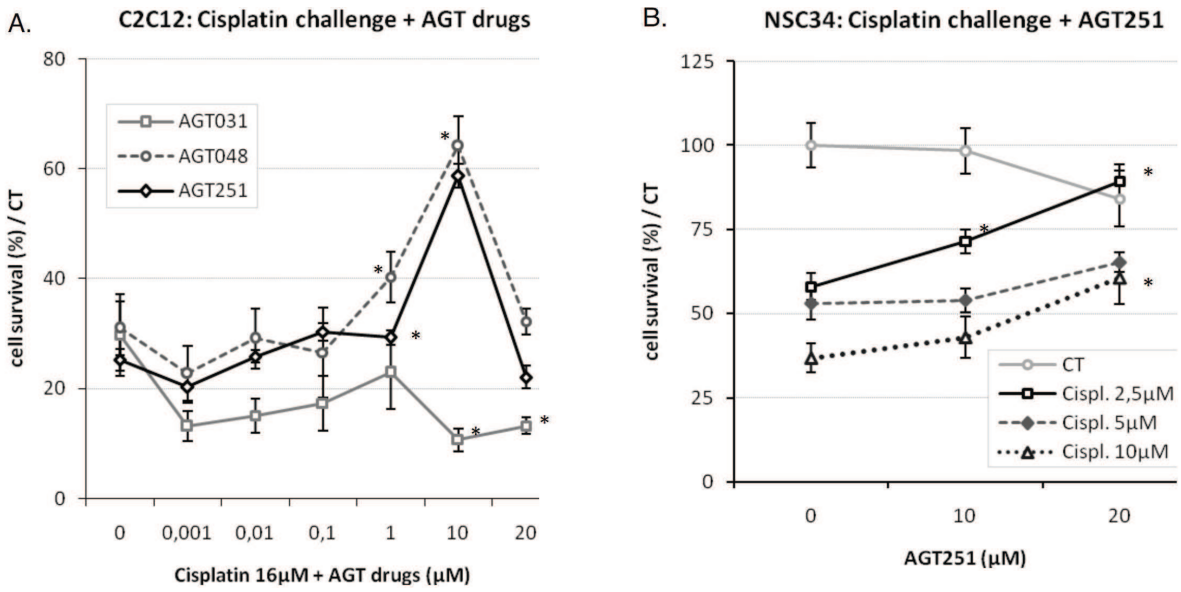
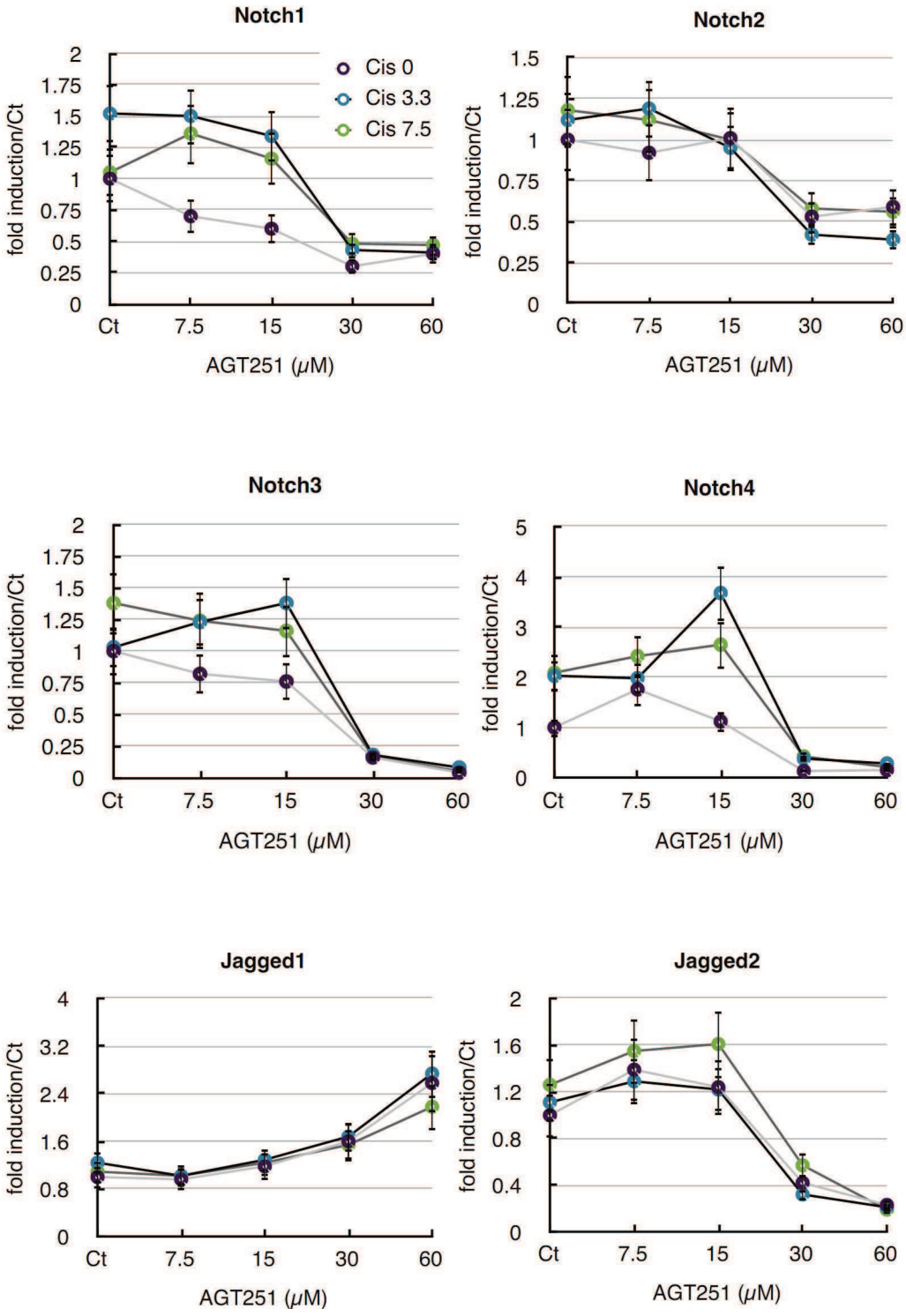


Figure 2 | **Tocopherol and flavonoids derivatives protect myoblast cells population against DNA damaging stress.**

(A) C2C12 myoblast cells and **(B)** NSC34 motoneuronal cells were cultured for 48 hours in presence of the indicated compounds (AGT031, AGT048, AGT251 in µM) in absence or in presence of cisplatin (at the concentration inducing 75% of the cytotoxicity of the cisplatin, IC75, or with concentration surrounding the IC50 in µM). Cell survival was evaluated using MTT test. Curves indicate mean ± SD (n=8) relative to control in absence of cisplatin. *: $p < 0.01$ compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test.



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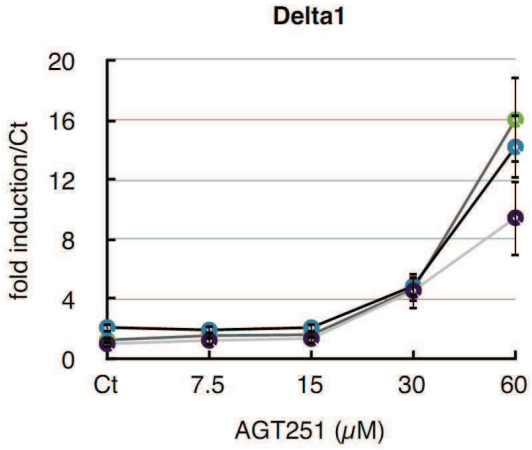


Figure 3 | **The tocopherol derivative AGT251 regulates the expression of components of the Noch pathway.**
mRNA levels of components of the notch signaling pathway were assayed in C2C12 myoblast cells treated with AGT251 (in µM) in absence or in presence of cisplatin (in µM) using RT-qPCR. Graphs are means of fold induction versus Ct in absence of cisplatin with SD (n=3).

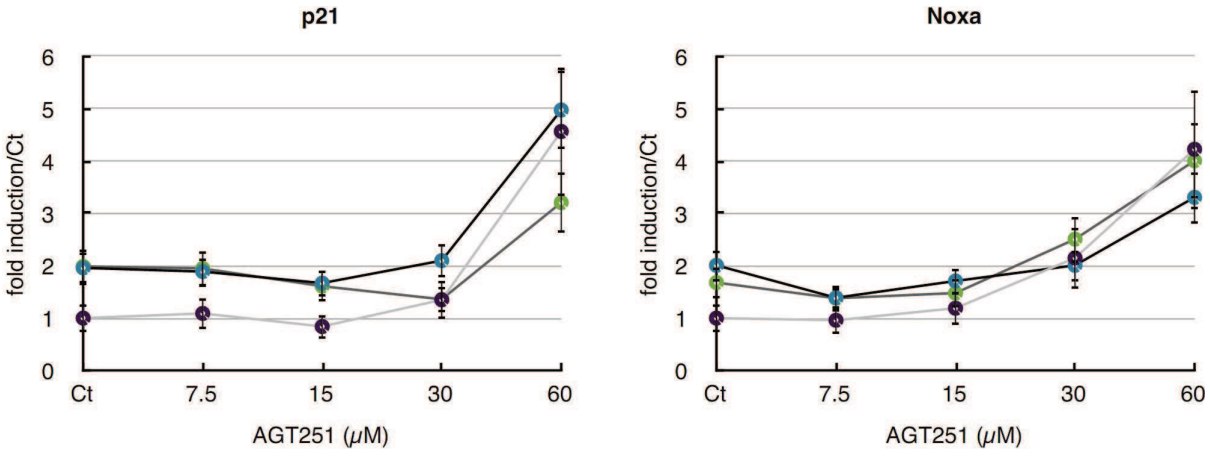


Figure 4 | **The tocopherol derivative AGT251 regulates p53 targets genes involved in cell cycle arrest and apoptosis in myoblast cells.**
mRNA levels of p53 target genes *p21* and *noxa* were assayed in C2C12 myoblast cells treated with AGT251 (in µM) in absence or in presence of cisplatin (in µM) using by RT-qPCR. Graphs are means of fold induction versus Ct in absence of cisplatin with SD (n=3).

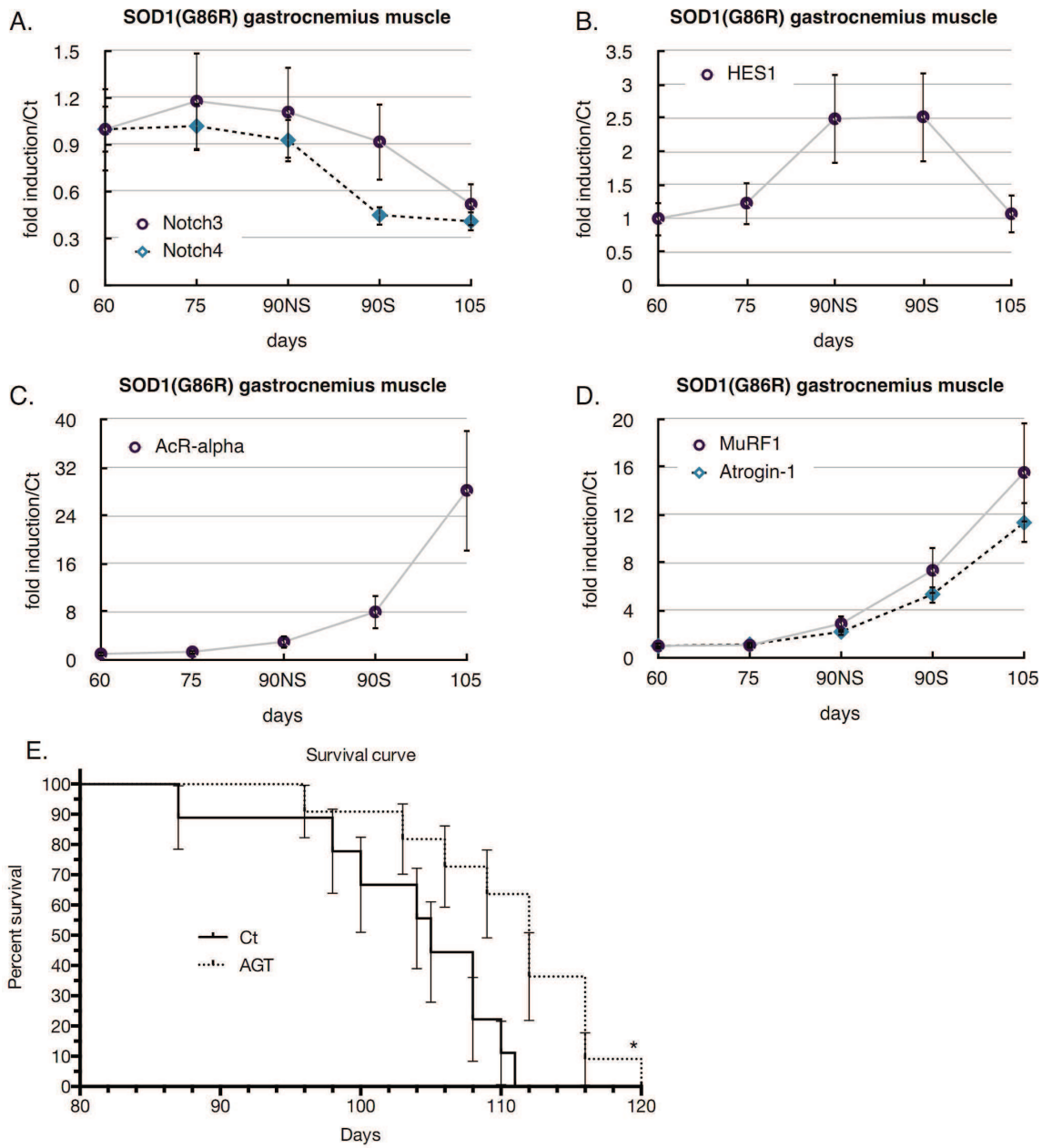


Figure 5 | **AGT251 favors mice survival in a model of ALS.**

The mRNA levels of components of the notch signaling pathway (**(A)** Notch 3 and 4, **(B)** Hes1 and markers of ALS disease progression (**(C)** Acetylcholine receptor alpha, AcR-alpha, **(D)** MuRF1 and Atrogin-1), were assayed in muscles of SOD1(G86R) mice using RT-qPCR. mRNA were extracted at different ages. Curves are means of fold induction versus the condition wild-type littermate 60 days-old and of three experiments containing animals with matching age (60, 75, 90, 105 days-old, n=5). **(E)** SOD1(G86R) 65 days old mice were injected 3 times a week with AGT251 until death. Survival curves relative to day of death of the untreated animal (n=12). * Indicates statistically significant differences established by Log-rank (Mantel-Cox) test ($p<0.001$) and Gehan-Breslow-Wilcoxon test ($p=0.0183$).

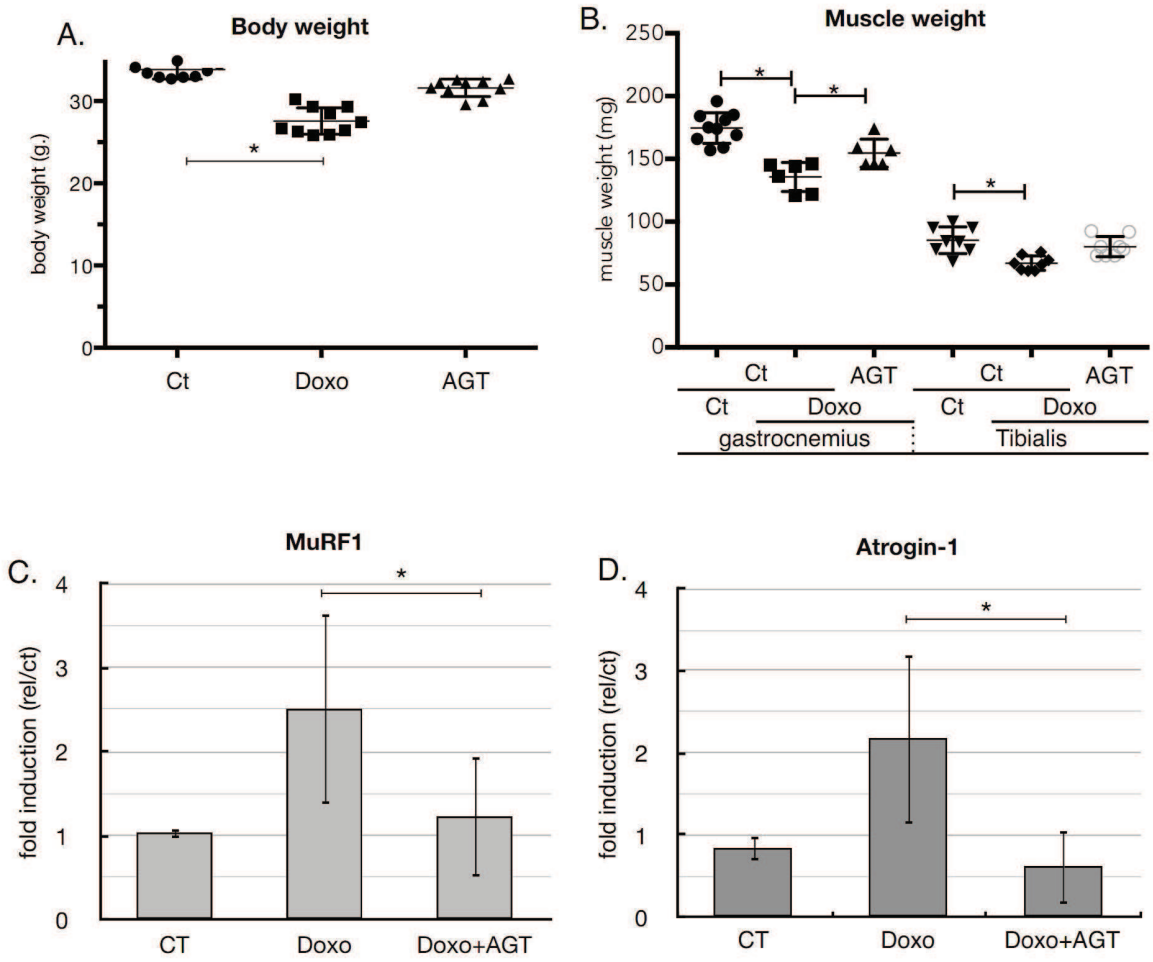


Figure 6 | **AGT251 reduces muscle cachexia.**

8-weeks-old mice were injected with doxorubicin (10mg/Kg) and AGT251 (20mg/Kg). **(A)** Body and **(B)** muscle weight was quantified after 15 days. Graphs represent means ± SD in mg of muscles (n=9). *: p< 0.01 compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test. **(C, D)** mRNA levels of the muscular atrophy markers MuRF1 and Atrogin-1 were assayed in muscles of mice treated with doxorubicin and AGT251 using RT-qPCR. Graphs represent means ± SD relative to untreated animals (Ct). *: p< 0.01 compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test.

VIII -DISCUSSION AND PERSPECTIVES

The work presented in this thesis manuscript comprises two main aspects. First, the implication of the p53 family of transcription factors in muscular degeneration during a severe neuromuscular disorder, Amyotrophic Lateral Sclerosis. And second, the identification of an interesting compound that increases survival in ALS and reduced muscular atrophy in the context of cancer associated muscular atrophy, or cachexia.
I would like to discuss 2 points: The relevance of our findings for understanding ALS and the possible implications of the p53 family in cancer cachexia.

VIII - 1. Implication of the p53 family in muscles during ALS

ALS is characterized by the loss of motor neurons and concomitant muscular atrophy. Thus, it was long believed that the effects of the mutated genes on ALS development (for example the toxicity of SOD1 mutants) was restricted to the motor neurons and was cell autonomous. However, in recent years, this belief was more and more contradicted and it is now known that the inherent cause of ALS does not lie only in motor neurons, but also involves neighbouring cells *(Ilieva et al., 2009)*. Indeed, restricting mutant SOD1 expression to motor neurons in mice does not lead to ALS symptoms *(Pramatarova et al., 2001)*. More recently, it has been shown that inhibiting mutant SOD1 expression in glial cells extended survival of mice *(Yamanaka et al., 2008)*. As more results show similar effects, it became apparent that the expression of mutant SOD1 in motor neurons alone is insufficient to induce ALS.

Interestingly, several findings propose a “dying-back” phenomenon *(Dadon-Nachum et al., 2011)* where neuromuscular junctions (NMJ) are destabilized and degradated before the actual death of the cell body. Indeed in patients, the NMJ is already altered at the onset of symptoms. So what about muscles? After all, they are directly connected to the motor neurons, at the source of NMJ degradation. Whether the muscle is involved in the pathogenesis of ALS is still a matter of debate. In 2006, Miller et al. demonstrated that muscle specific suppression of SOD1 by viral delivery of siRNA did not alter progression of ALS in mice. However, muscle mass and myofibre diameter and quantity were

increased in absence of mutated SOD1 *(Miller et al., 2006)*. In line with those results, other groups demonstrated that muscle-restricted expression of mutated SOD1 was enough to induce muscular atrophy, reduce muscular strength and lead to NMJ destabilization and apoptotic death in motor neurons *(Dobrowolny et al., 2008; Wong and Martin, 2010)*. Finally, in SOD1(G93A) mice, a loss of muscle mass is already measurable before other typical ALS symptoms appear *(Marcuzzo et al., 2011)*.

Taken together, those interesting findings show that the expression of a mutated SOD1 does not only directly affect and kill motor neurons, but also affects other cell types, including muscles, that can suffer and atrophy directly from mutated SOD1 toxic effects. However, the mechanisms leading to this effect are still incompletely understood.
A clue comes from the observation that the expression of Nogo-A, a neurite outgrowth inhibitor, is increased in muscles from ALS patients and an ALS mouse model. Interestingly, the inhibition of Nogo-A in SOD1(G86R) leads to reduced muscle denervation and increased survival of mice. Conversely, overexpression of Nogo-A in wild-type muscles leads to NMJ destabilization *(Jokic et al., 2006)*. This finding is in line with the dying-back hypothesis and suggests that muscles actively contribute to the development of ALS by promoting NMJ destabilization, leading to motor neuronal death.

Through our results, we think to have added interesting information to this ongoing discussion. Our results have shown that target genes that promote cell death such as Bax, Puma or Noxa are induced in muscles during ALS in patients and the SOD1(G86R) mouse model. These genes are likely induced by the members of the p53 family, which are also overexpressed in atrophying muscles, most prominently TAp63.
We have shown that in cultured C2C12 myoblasts, the overexpression of mutated SOD1(G86R) is sufficient to induce the expression of p53 family target genes and TAp63. Similarly, the induction of different cell stresses associated with the expression of mutated SOD1 in neurons and muscles also leads to TAp63 expression. In addition, the overexpression of TAp63 in C2C12 cells increases cell death.
These results strongly suggest that during ALS, the expression of mutated SOD1 leads to transcriptional activation of TAp63 through yet unknown mechanisms. This activation is likely to contribute to demise of skeletal muscles. However, these effects appear simultaneously to denervation and the loss of motor neurons in SOD1(G86R) mice. In

addition, TAp63 is similarly induced after transient denervation of healthy muscles. Given this timecourse of events, we cannot state that the activation of TAp63, mediated by a mutated SOD1, is strictly muscle-autonomous and takes place independently to the dying-back of motor neurons.
However, as stated before, the expression and the cellular effects of a mutated SOD1 in muscle cells is sufficient to induce the induction of TAp63 and its target genes. According to this finding, we may hypothesize that the modified expression of p63 in muscles directly contributes to muscular atrophy. At the same time, this process is amplified by the progressive denervation of the muscles which also leads to p63 deregulation.

While this is all interesting, you may ask: "Great, but what makes you say that p63 actually really contributes to muscular atrophy?"
When we first started this project and obtained the first results, we were asking similar questions. Is the observed induction of TAp63 and its corresponding target genes a cause or a consequence of muscular atrophy? What is the cellular consequence of this induction?
In simple terms, muscular atrophy can be caused by two separate events that can occur simultaneously. On one hand, there is the actual dismantling of muscle fibres through degradation of sarcomeric proteins, concomitant, or not, with localized apoptosis. For example, in muscular atrophy due to (experimental) denervation, the ubiquitin ligases MuRF1 and Atrogin-1 are induced and mediate the breakdown of muscle fibres *(Bodine et al., 2001a)*. On the other hand, muscular atrophy can be caused or enhanced by the failure to repair atrophying fibres. In non-pathological situations, satellite cells and myoblasts are activated, proliferate and differentiate to repair or replace damaged muscle fibres *(Bonaldo and Sandri, 2013)*. Different groups suggest that the expression of p53 family members is important for the differentiation of myoblasts. Conversely, inhibition of p53 family proteins can lead to the inhibition of differentiation *(Porrello et al., 2000; Cam et al., 2006; Vaccarello et al., 2006)*.

The induction of TAp63 in atrophic muscles during ALS could thus have two origins. First, TAp63 could be activated in myoblasts in order to promote differentiation and thus repair of the muscles. In this situation, the overexpression of TAp63 would have a

protective function for the muscle. In addition, we also observed the downregulation of ΔNp63, which could in turn inhibit differentiation of myoblasts.

Second, TAp63 activation in muscle fibres would probably lead to induction of known ubiquitous target genes involved in cell death or in activation of other, muscle-specific genes that could contribute to muscle fibre demise. Thus, TAp63 would be noxious for muscles.

When carefully analyzing the DNA microarray data and correlating them with our individual assessment of target genes by qPCR we had several findings. All main transactivating isoforms of the p53 family were transcriptionally induced (p53, TAp63, TAp73), with TAp63 being the most upregulated (about 12-fold) and ΔNp63 the most downregulated (about 3-fold). This prompted us to focus on p63 as its deregulation was most apparent. However, we did not dismiss p53 and p73 entirely, knowing that not only the absolute quantity of transcription factors, but also the balance between TA- and ΔN-isoforms is important. This effect is very apparent during tumorigenesis when p63 isoform levels are perturbated and enhance spontaneous tumor formation *(Sniezek et al., 2005; DeYoung et al., 2006)*. Further, many different target genes of the p53 family were upregulated in atrophying muscles, and most were involved in cell death processes (Bax, Noxa, Perp, Peg3…) or the arrest of cell cycle (p21, GADD45). To my knowledge, there are no reports that describe a cellular situation where transactivating p53 family members are upregulated alongside several inducers of apoptosis without a significant, noxious outcome for the cell…

So far, those results alone suggest that the deregulation of p63 expression is harmful for muscle cells and likely contributes to the observed atrophy in ALS.

In line with this finding, the transcriptional induction of TAp63 in muscles from SOD1(G85R) mice led to the accumulation of p63 proteins in myonuclei. Upon close inspection of transversal and longitudinal (not shown) sections of atrophic muscles from DOS(G86R) mice, we think that the stained, individual nuclei were located below the sarcolemma, suggesting that p63 was not expressed in satellite cells but in the muscle fibres. Unfortunately, several attempts of Pax7 staining were inconclusive (staining/specks did not co-localize with nuclei), prohibiting us from confirming this claim.

Finally, and most importantly, we have shown that TAp63 activates the transcription of MuRF1. When overexpressing TAp63 in cultured C2C12 myoblasts, MurF1 expression is rapidly induced (after 6-10 hours), while Atrogin-1 expression is barely affected (not shown). While especially p53 and TAp73 also activate the transcription of MurF1, the effect is more subtle in comparison to TAp63. Using a luciferase assay and Chromatin immunoprecipitation, we could show that TAp63 activates MuRF1 transcription by directly binding the promoter upstream of the transcription start site.
Together, these results show for the first time that p63 participates in degenerative processes of the muscle by specifically activating the transcription of the MuRF1 ubiquitin ligase. This finding confirms our initial suspicions, that TAp63 deregulation in ALS-muscles induces atrophy.
As mutated SOD1 expression is sufficient to induce TAp63, which in turn activates MuRF1, we can join other research groups in proposing that during ALS, muscles actively contribute to their own demise in addition to being subjected to denervation.

There is still a lot that could be done in order to understand the underlying mechanisms. In particular, we have not explored what exactly causes transcriptional activation of the p53 family members, especially TAp63. Furthermore, we do not know if TAp63 is implicated alone in the process of activating MuRF1. Genetic ablation of TAp63 would be one way to achieve this, for example using TAp63$^{-/-}$ mice. However, SOD1(G86R) mice have a FVB background while TAp63$^{-/-}$ mice are C57BL/6, which renders the process more tedious, especially when considering the reproductive problems that both mice can encounter. It would also imply completely re-evaluating the resulting phenotype. While crossing a TAp63 knockout mouse with an ALS mouse model would be very elegant, it would probably be necessary to also cross a p73 knockout mouse with an ALS-mouse. The p53 family members have been shown to compensate for each others absence in some situations, so it is plausible that in the absence of TAp63, TAp73 could “take over” and drive MuRF1 expression. As said before, we never really excluded TAp73 from our hypotheses. Indeed, while being about 3 times less efficient than TAp63 in transactivating MuRF1, TAp73 is still a potent inductor of MuRF1.
A technically faster, cheaper and simpler solution could be to microinject sh-RNA vectors targeting different p53 family isoforms directly into specific muscles of an ALS mouse, for example the large gastrocnemius muscle. In theory this should inhibit the

selected combinations of isoforms, however, in this context, the vectors only express sh-RNA during 1-2 weeks. This may not be enough to see an effect, as the development of ALS is a long term process, beginning on a molecular level before the first symptoms arise.

It would be interesting though to be granted access to muscle tissue or RNA extracts of mice specifically expressing mutant SOD1 in muscles *(Miller et al., 2006)*. This could help confirm our *in vitro* findings where mutated SOD1 activates TAp63 and TAp63 induces MuRF1 expression.

Finding the reasons for the activation of p63 is paramount in order to understand how and why TAp63 contributes to muscular atrophy. Several pathways may potentially regulate p63 activity in this context. For example, it has been shown that the AMPK is able to bind to p63 and p73, inducing the repression of their transcriptional activity *(Lee et al., 2009)*. As the interaction is mediated by the C-terminus, this could also inhibit ΔNp63/ΔNp73 activity. The regulatory mechanisms of this interaction are still unknown. Several articles have also now demonstrated an activation of p53 by the AMPK upon metabolic stress *(Nieminen et al., 2013)*. While this has not been demonstrated for p63 and p73 yet, a similar regulation of their activity is plausible, given the p53 family sequence homology and the latest results concerning the function of p63 in energetic metabolism *(Su et al., 2010)*.

Regulation of p53 family members on a post translational level is important for the actual regulation of their activity. However, we can't provide an explanation for the strong transcriptional induction of TAp63 in muscles during ALS.

NF-κB has been described to activate the transcription of TAp63 by direct binding to its promoter *(Wu et al., 2010)*. During ALS, the activation of the NF-κB has been observed in the spinal cord, in astrocytes, and contributes to motor neuron death *(Migheli et al., 1997)*. The status of NF-kB in muscles is unclear and thus, it would be important to verify whether the NF-kB pathway is activated in atrophying muscles. However, this is plausible givent that NF-kB activation induced muscular atrophy *(Cai et al., 2004)* (Also see *Ch.II – 1.3*). Thus, we can hypothesize that NF-kB activation may activate TAp63 transcription. Using C2C12 cells, overexpression or silencing experiments should be able to give first insights in this possible regulation.

Another pathway that can lead to p63 deregulation is the Notch pathway. This pathway is important for tissue morphogenesis and maintenance during development and adults. In simple terms, it controls differentiation in various tissue types. For example during muscle regeneration in adults, the Notch pathway regulates satellite cell activation, leading to increased proliferation and myogenic progression. After these differentiation steps myoblasts fuse with existing or new myofibres to encrease or maintain muscle mass *(Luo et al., 2005)*.

During skin development, Notch1 activation promotes keratinocyte differentiation by inhibiting their proliferation. During this differentiation, TAp63 expression is repressed. A crosstalk between p63 and Notch has been demonstrated and is fairly complicated and not fully understood. In several cell types, such as the keratinocytes, TAp63 is downregulated by Notch, whereas in fibroblasts, Notch activates p63 expression. Interestingly, during development, p63 is able to activate Notch transcription in keratinocytes, evidencing a complicated feedback loop *(Dotto, 2009)*.

Interestingly, in atrophying muscles from the SOD1(G86R) ALS model, we evidenced a repression of Notch receptors. This may have two consequences: On one hand, it inhibits the proliferation of satellite cells and prevents muscle regeneration. On the other hand, Notch signalling in myofibres could permit TAp63 transcription. The link between Notch and p63 in muscles and in particular whether the repression of the Notch pathway regulates TAp63 activation remains to be seen.

ALS is a hugely complicated, multifactorial disease. In order to study basic mechanisms of muscular atrophy it is probably wiser to rely on simpler mouse models, such as denervation and immobilisation or treatment by Doxorubicin and use muscular cells in vitro to decipher subtle mechanisms. Although very complicated until recently (due to the lack of potent transfection agents), it is now easy to overexpress or inhibit candidate genes in order to unravel the mechanisms of muscular atrophy.

VIII - 2. The p53 family in cancer cachexia

During muscular atrophy in ALS, we observed a strong upregulation of TAp63 transcription and a weaker, yet significant upregulation of TAp73. This in accompanied by the induction of several p53 family target genes such as p21, Bax or Noxa. On one hand, this could be the result of the net sum of activating and inhibiting factors, as ALS is a complicated syndrome involving different cell types and temporal events (denervation). On the other hand, it could be a general mechanism that is induced during muscular atrophy.

Indeed, verifying if a similar activation of p53 family members and target genes is induced in muscles in other degenerative conditions would help defining the importance of this event. We chose to address this question by using *in vivo* models of muscular atrophy that are least similar to ALS. Indeed, disuse atrophy for example could be associated, with denervation-induced atrophy, to the motor neuron degradation found in ALS. Furthermore, in global mechanistic terms, it resembles denervation and on a clinical scale, it is not that "important".

Cancer cachexia affects about a quarter of cancer patient. In terminal stages of cancer, about half of the patients are affected. The severity of this condition is highlighted by the fact that about a fifth of cancer patients dies of direct consequences of cancer-associated muscular atrophy, or cachexia *(Glass and Roubenoff, 2010)*. In addition, cachexia is though to be mainly driven by inflammatory mediators, which is not the main characteristic of ALS.

Recently, we started using the two aforementioned mouse models of cachexia (See *Ch. III – 2.4*), the induction of C26-tumors and Doxorubicin. First, we had to characterize those models in our hands.

Weight loss

After 1-2 weeks of C26 tumor development or Doxorubicin treatment, mice appeared much leaner. The skeleton was more visible and palpable than in control mice.

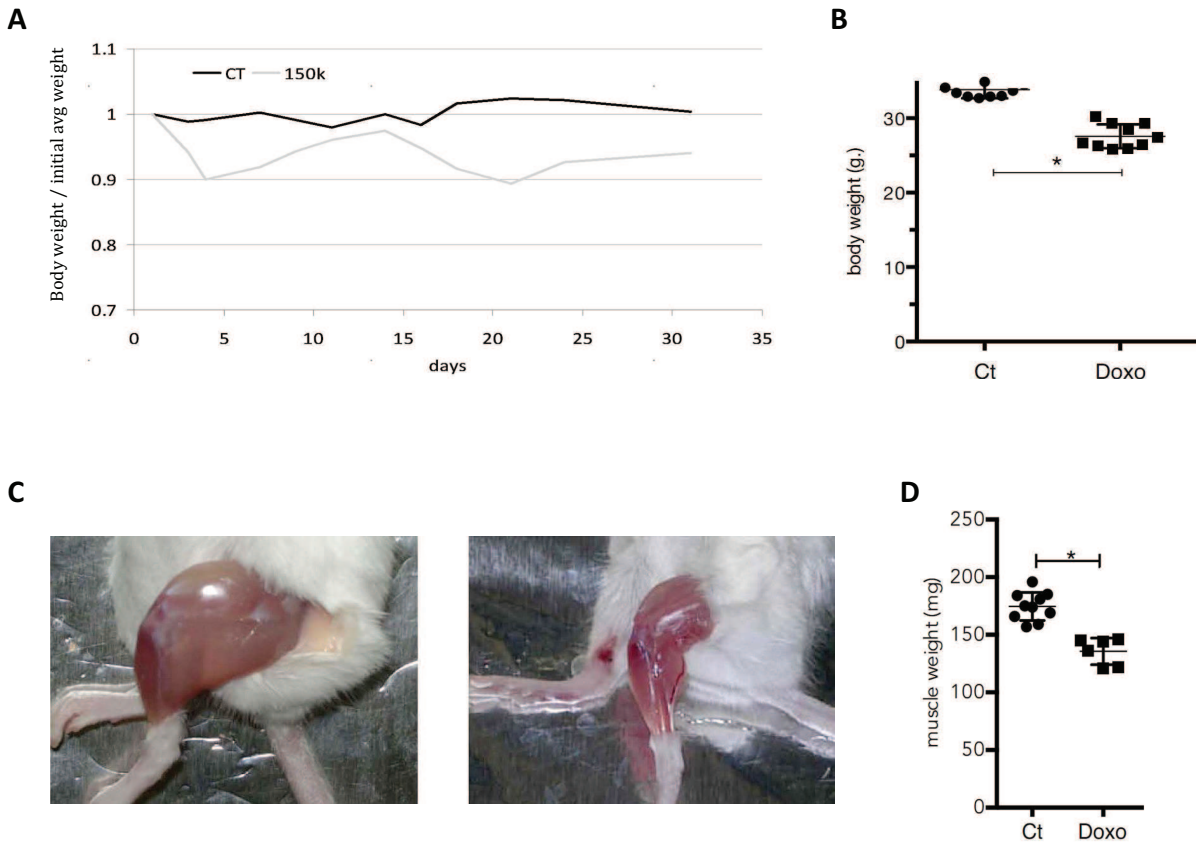
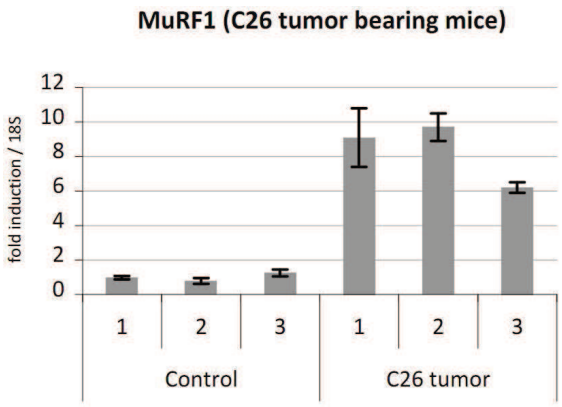
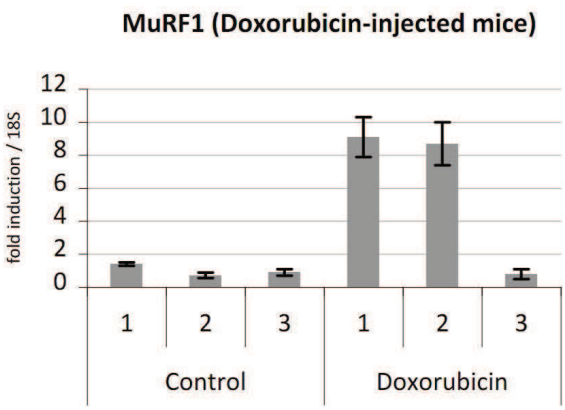


Figure 25 | **Muscular phenotype of mice with cachexia**
(A) Body mass evolution of mice bearing C26 tumors. 150000 C26 cells were injected at day 0. Tumors were palpable after approximately 7 days. **(B)** Body weight of mice with cachexia after Doxorubicin injection. **(C)** Appearance of the hindlimb in a normal (left panel) and a C26 tumor bearing mouse (right panel). From *Aulino et al., 2010.* **(D)** Gastrocnemius muscle weight of mice with severe muscular atrophy (about 20%) after Doxorubicin injection.

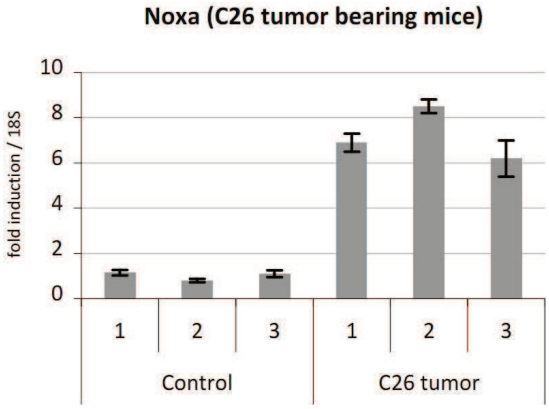
Interestingly, while mice appeared visibly "thinner" to the naked eye, the weighing did not necessary reflect this. Indeed, in mice which received a C26 tumor cell injection, weight loss at the end of the experiment appears to by only 5% (Figure 25A). Concomitant with the muscular atrophy, the injected C26 cells form a growing tumor mass which in the end probably accounts for up to 5 grams (about 15-20% of a mouse's total weight). The "carcass weight" is thus lower and more accurately reflects our visual impression. Dissected muscles showed severe loss of mass in C26-mice (Figure 25C) or mice treated with Doxorubicin (Figure 25D). Aulino et al. have extensively characterized the phenotype of C26-bearing mice and out results are in full accordance with theirs *(Aulino et al., 2010)*.

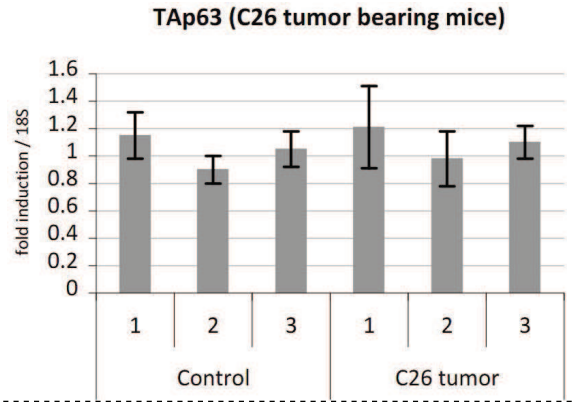
A


MuRF1 (C26 tumor bearing mice)
fold induction / 18S
12
10
8
6
4
2
0
1
2
3
1
2
3
Control
C26 tumor


MuRF1 (Doxorubicin-injected mice)
fold induction / 18S
12
10
8
6
4
2
0
1
2
3
1
2
3
Control
Doxorubicin

B


Noxa (C26 tumor bearing mice)
fold induction / 18S
10
8
6
4
2
0
1
2
3
1
2
3
Control
C26 tumor


TAp63 (C26 tumor bearing mice)
fold induction / 18S
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
1
2
3
Control
C26 tumor

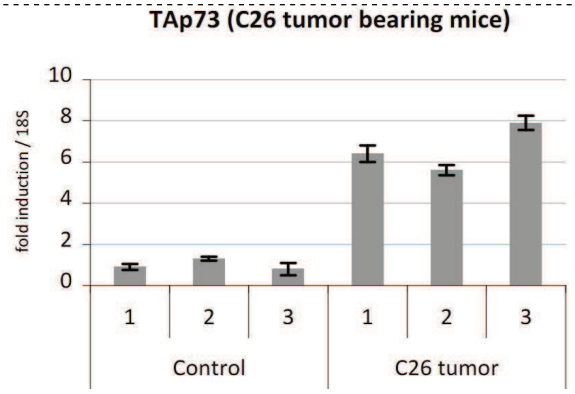


Figure 26 | **Expression of MuRF1 and the p53 family pathway in muscles during cachexia**
(A) Expression levels of MuRF1 in the 2 cancer cachexia models. **(B)** Expression levels of TAp63, TAp73 and Noxa in C26 tumor bearing mice. n=6, 2 mice pooled per group.

We also verified the presence of muscular atrophy on a molecular level by assessing MuRF1 and Atrogin-1 expression in both situations, C26 tumors and doxorubicin injection. The expression of MuRF1 (Figure 26A) and Atrogin-1 is indeed highly induced and confirms the induction of muscular atrophy on the molecular level. Naturally, we wanted to verify whether the expression of various p53 family target genes was also modified in this situation. Representatively, the expression of Noxa (Figure 26B) and p21 was highly induced. However, and to our great deception, the expression of TAp63 in muscles was not modified in both situations of cachexia. On the contrary, in muscles from C26 bearing mice, the expression of TAp73 was highly induced. This is a very interesting finding, suggesting that during muscular atrophy, modifications of p53 family expression are highly dependent on the context or origin of the muscular atrophy. It will be very interesting to understand the underlying mechanisms. What causes the modification of p63 and p73 expression? Do both family members have the same function during muscular atrophy? As we have demonstrated in C2C12 mypblasts, TAp73 is also able to activate MuRF1 transcription.

In ALS, the initial stimulation of TAp63 expression is mediated by the presence of a mutated SOD1 and by the denervation process. During cachexia, increased levels of inflammatory cytokines and in particular TNFα have been observed and suggested to drive the muscular atrophy *(Glass and Roubenoff, 2010)*. Interestingly, TNFα is able to induce the creation of ceramide, a type of sphingolipid, with various biological activities.

Of particular interest are its abilities to inhibit myogenic differentiation, protein synthesis in myotubes and the enhancement proteolysis through (NF)κB and autophagy *(De Larichaudy et al., 2012)*.

A recent study proposes that muscular atrophy in cachexia involves the metabolism of sphingolipids. Interestingly, muscular atrophy induced by C26 tumors or TNFα treatment was partially reversed by using inhibitors of ceramide synthesis, such as myriocin. Concomitantly, the expression of Atrogin-1 was repressed, while Akt wasactivated. However, no effect on MuRF1 expression could be observed *(De Larichaudy et al., 2012)*.

This finding suggests that the induction of Atrogin-1 observed in cachexia is at least partly mediated by ceramides, while MuRF1 is stimulated through another pathway. Indeed, through NF-κB, TNFα can induce the expression of MuRF1 (See *Ch II – 1.3*).

These finding could help us better understand the regulation of p63 and p73 in the context of cancer cachexia. Using a similar *in vivo* approach, it would be highly interesting to verify the modification of p53 family member expression depending on TNFα-induced ceramide production. In addition, or as a first and simple approach, we could treat C2C12 myoblasts or differentiated myofibres with TNFα and inhibitors of ceramide production.

In general, we can emit several hypotheses that could be easily verified:

First, we should assess if treatment of mice or muscle cells with TNFα alone causes activation of TAp73 and/or other p53 family members. Second, we can verify whether inhibition of ceramide synthesis inhibits TAp73 and if in its absence, TAp63 could take over and exert TAp73's function, i.e. activating p53 family target genes and MuRF1. In various settings, p53 family members have been shown to be redundant in function. For example, during developmental control of myogenesis, the p53 family members are thought to act redundantly *(Hüttinger-Kirchhof et al., 2006)*.

Third, ceramides inhibit Akt function, which has been shown to activate MuRF1 and Atrogin-1 transcription with the FoxO transcription factors. Could loss of Akt function induce the expression of TAp63 or TAp73?

Conclusion

The results I obtained during my thesis indicate that members of the p53 family are not only activated during ALS, but also in a totally differend kind of atrophy, cancer cachexia. In ALS, it seems that TAp63 is the dominant isoform, while TAp73 is also induced and could induce MuRF1 expression to contribute tu muscle wasting. On the contrary, in cancer-associated muscular atrophy, TAp63 expression is not altered, while TAp73 is highly induced. These findings are very interesting because they indicate an "atrophy-specific" expression pattern of the p53 family members that depends on the initial factors leading to atrophy. In ALS, one could propose the Notch pathway, and in cancer-associated muscular atrophy, the TNFα signalling.
Using a tocopherol-derivative produced by AxoGlia, we could increase the lifespan of mice affected by ALS. In our doxorubicin/cachexia model, we could markedly reduce muscular atrophy. While we do not know the exact mechanisms of this effect, use of tocopherol-derivated compound may help elucidating general mechanisms during muscular atrophy.

A new student recently joined our lab and will try to aswer our open questions. I am pleased that this research project will continue.

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X - SCIENTIFIC PRODUCTION

Research articles

Benosman S, Meng X, von Grabowiecki Y, Palamiuc L, Hritcu L, Gross I, Mellitzer G, Taya Y, Loeffler JP, Gaiddon C.
Complex regulation of p73 isoforms after alteration of amyloid precursor polypeptide (APP) function and DNA damage in neurons.
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von Grabowiecki Y, Benosman S, Blanchard O, Palamiuc L, Mériaux S, Devignot V, Mellitzer G, Gonzalez de Aguilar JL, Gaiddon C.
p63 contributes to murf-1 regulation and muscular atrophy in ALS.
Submitted.

von Grabowiecki Y, Licona C, Coowar D, Mellitzer G, Gaiddon C.
Biological activity of novel flavonoid and tocopherol derivatives on myoblast survival and protective effect towards cancer cachexia or amyotrophic lateral sclerosis.
Submitted.

Oral communication

von Grabowiecki Y, Benosman S, Gonzalez de Aguilar JL, and Gaiddon C.
Role of the p53 family of transcription factors in amyotrophic lateral sclerosis
5th p63/p73 workshop, 2011, Lyon

Poster presentations

- 10ème colloque de la Société des Neurosciences, 2011, Marseille
- Premières Journées scientifiques de la Fédération de Médecine Translationnelle de Strasbourg
- 20th EuCC Annual Meeting, 2013, Strasbourg
- 6th p63/p73 workshop, 2013, Kazusa/Japan
- 4ème Forum scientifique du Canceropôle Grand-Est, Strasbourg

MISCELLANEOUS

- Teaching for college/university students during second year of PhD
- Acquired diplomas in animal experimentation and rodent surgery
- Supervised 3 interns

Yannick VON GRABOWIECKI

Les facteurs de transcription de la famille p53 dans l'atrophie musculaire - Implications dans la Sclérose Latérale Amyotrophique et la cachéxie

Résumé

L'atrophie musculaire est un symptôme dangereux retrouvé dans plusieurs maladies. Dans la sclérose latérale amyotrophique (SLA), une maladie neuromusculaire rare, ainsi que dans le cancer (phénomène de cachexie), l'atrophie musculaire cause le décès des patients. Les facteurs de transcription de la famille p53 sont impliqués dans de nombreux processus cellulaires, faisant face à des situations de « stress » pour les cellules. Notamment, ils peuvent induire la mort cellulaire ou promouvoir la différentiation.
Nous avons constaté, à partir de modèles cellulaire et animaux de SLA et cachéxie cancéreuse, que des membres de la famille p53 sont activés dans les muscles atrophiques. Cette activation entraine l'expression de gènes cibles impliqués dans la mort cellulaire. De manière intéressante, TAp73, mais surtout TAp63, sont capables d'activer la transcription d'un effecteur de l'atrophie musculaire appelé MuRF1, démontrant que la famille p53 peut participer activement à l'atrophie en induisant la dégradation des fibres musculaires.
De plus, nous avons utilisé nos modèles animaux pour identifier une nouvelle approche contre l'atrophie musculaire. Ainsi, nous avons identifié un dérivé de tocophérol avec des propriétés thérapeutiques intéressantes.

Mots clés : atrophie musculaire, sclérose latérale amyotrophique, cachexie, p63, régulation transcriptionelle, MuRF1

Résumé en anglais

The p53 family of transcription factors in muscular atrophy
Involvements in Amyotrophic Lateral Sclerosis and cachexia

Muscular atrophy is a dangerous condition found in several diseases. In amyotrophic lateral sclerosis (ALS), a rare neuromuscular disease, as well as in cancer (phenomenon of cachexia), muscular atrophy can be fatal to patients.
The transcription factors from the p53 family are involved in several cellular processes, facing cellular "stress" situations. Most notably, they can induce dell death or promote differentiation.
We found, using cellular and mouse models of ALS and cachexia, that members of the p53 family are induced during muscular atrophy. This induction leads to the expression of canon nic target genes involved in cell death. Interestingly, TAp73, but especially TAp63, are able to activate the transcription of an effector or muscular atrophy called MuRF1. This proves that the p53 family cand participate in muscular atrophy by promoting the breakdown of muscle fibres.
In addition, we used our mouse models to identifiy a new approach agains muscular atrophy. Indeed, we identified a derivative of tocopherol with interesting therapeutical proprieties.

Keywords: muscular atrophy, amyotrophic lateral sclerosis, cachexia, p63, transcriptional regulation, MuRF1

Les facteurs de transcription de la famille p53 dans l'atrophie musculaire

Implications dans la Sclérose Latérale Amyotrophique et la Cachéxie

résumé de thèse en francais

Yannick von Grabowiecki

I - INTRODUCTION

Les muscles... Ils sont vraiment importants, non? Après tout, ils nous permettent de bouger, de respirer et de communiquer. Mais comment fonctionnent-ils? Quels sont les mécanismes impliqués quand ils se dégradent? Et pourquoi est-ce que la famille p53 pourrait-elle être impliquée? Dans cette thèse, je donne un aperçu des conaissances actuelles et y intègre mes propres résultats de recherche.
L'introduction (anglaise) est tout à fait détaillée et aborde les points suivants:

- **La physiologie de base des muscles**

  Structure et contractions, développement de muscles squelettiques et leur homéostasie, production et stockage d'énergie, les différents types de fibres musculaires.

- **Introduction à l'atrophie musculaire**

  L'inactivité, la dénervatin, la sarcopénie, le jeune, les "atrogènes" MuRF1 et Atrogin-1 et leur régulation, le lien avec l'apoptose.

- **L'atrophie musculaire comme conséquence de maladies**

  La dystrophie musculaire, la cachexie cancéreuse, la Sclérose latérale amyotrophique

- **La famille p53**

  Généralités, structure, p53 (régulation, arrêt de cycle cellulaire, apopotose, régulation du métabolisme), p63 (développement de peau, protection de lignée germinale, régulation du métabolisme, suppression de tumeur), p73 (régulation de survie neuronale, tumorigenèse, vie et mort cellulaire)

- **Le rôle de la famille p53 dans les muscles**

  Régulation du développement du coeur par p63, différentiation de myoblastes et implications dans le rhabdomyosarcome, p53 dans l#atrophie musculaire et la cachexie.

II - OBJECTIFS DE LA THÈSE

L'atrophie musculaire est un phénomène complexe qui peut etre induit par une multitude de situations différentes comme l'inactivité, le vieillissement, le jeunem le cancer, des maladies neuromusculaires etc.

Comme nous l#avons vu dans l'introduction, l'atrophie musculaire est un syndrôme sévère, en particulier dans le contexte de la Sclérose latérale amyotrophique (SLA) et le cancer. Alors qu'elles sont étudiées depuis des décennies, ces 2 maladies ne sont toujours pas entièrement maitrisées du fait de leur variabilité et momplexité de causes sous-jacentes.

Les facteurs de transcription de la famille p53 sont surtout connues pour leur rôle dans la suppression de tumeurs, le développement de la peau et du système nerveux. Les mécanismes mis en jeu pour obtenir ces fonctions se basent sur le contrôle du destin cellulaire via l'induction de gènes cibles impliqués dans la réparation de l'ADN, l'arrêt du cycle cellulaire, la différentiation ou la mort cellulaire. Alors que les souris invalidées pour p53, p63 ou p73 n'ont pas de phénotype lié aux muscles, certains articles de recherche soulignent l'importance de la famille p53 durant la différentiation et l'atrophie musculaire.

Dans le cas de la SLA, un grand effort a été fourni pour comprendre comment les neurones moteurs dégenèrent. L'atrophie musculaire qui en decoule était ou est toujours considére come une simple consequence de la mort neuronale.

Nous voulions comprendre les mécanismes moléculaires qui lient à l'atrophie musculaire et la mort cellulaire dans le contexte de la SLA, en lien avec la famille p53. Afin d'obtenir des éléments de réponse, j'ai voulu répondre aux questions suivantes:

- Est-ce que une voie de signalisation dépendante des membres de la famille p53 est activée dans les muscles durant la SLA?
- Quels sont les processus cellulaires régulés par cette potentielle activation et quelles sont les protéines de la famille p53 les induisent?
- Qu'est ce qui cause cette activation?

Pour approfondir nos recherches, nous avons voulu vérifier nos résultats dans un

contexte d'atrophie musculaire différent. Beaucoup de patients de cancer souffrent, en fonction du type de tumeur, de cachexie - un syndrome d'atrophie progressive des muscles squelettiques qui affaiblit beaucoup les patients. La cachexie est par ailleurs souvent la cause de décès des patients. Mais de nouveau, les causes sous-jacentes sont variées et peu compries.

Deux modèles murins de cachexie sont couramment utilisés en recherche. Le premier mime la cachexie cancéreuse observée chez les patients par implantation sous-cutanée de cellules tumorales dans les souris. La seconde adresse les effets secondaires de drogues anticancéreuses souvent observées en clinique, qui peuvent également induire la cachexie.

Dans le but de contribuer aux avancées de recherche dans l'atrophie musculaire liée au cancer, j'ai commencé par chercher des réponses aux questions suivantes:

- Quel est le décours temporel de faiblesse et atrophie musculaire dans nos deux modèles de cachexie cancéreuse?
- Est-ce que la famille p53 est activée durant la cachexie?
- Est-ce que les résultats obtenus sont comparables à ceux dans la SLA?

Finalement, un troisième aspect fait la liaison entre les 2 premières parties de mon projet de thése. Etant donné qu'il n'existe à ce jour pas de traitement pour la SLA et la cachexie cancéreuse, nosu avons voulu vérifier le potentiel cytoprotecteur de molécules dérivées de flavonoides et de tocopherols dans ces cellules musculaires en culture et des muscles murins en cours d'atrophie. Ces composés ont été obtenus grace a une nouvelle collaboration mise en place avec la compagnie biotech *AxoGlia* du Luxembourg.

- Est-ce que les molécules AxoGlia ont un effet bénéfique sur des myoblastes in vitro?
- Est-ce que les composés sont capables de protéger de l'atrophie musculaire dans la SLA et la cachexie cancéreuse?
- Retrouve-t'on un effet sur la famille p53?

Dans le chapitre suivant, je présenterai mes résultats sous forme de 2 manuscrits.

III - RÉSULTATS

Durant la période de préparation de thèse au sein de l'équipde de Christian Gaiddon à Strasbourg, j'ai contribué à 3 publications. La première est déjá publiée et concerne les effets de TAp73 et ΔNp73 dans les neurones suite a des stress genotoxiques et l'induction de la voie de signalisation APP (Benosman et al., 2011).
Pour les deux autres, il s'agit de manuscrits soumis en relation directe avec mon projet de recherche (premier auteur) et seront en conséquence, présentés ici.

III - 1. p63 contribue à l'atrophie musculaire dans la SLA et induit MuRF1

La Sclérose latérale amyotrophique (SLA) est une maladie neuromusculaire sévère et létale qui affecte les adultes agés d'environ 50 ans. A ce jour, aucun traitement curatif n'a été developpé et les mécanimes sous.jacents de cette maladie sont divers, complexes et loin d'être entièrement compris. La SLA est caractérisée par la perte de neurones moteurs et l'atrophie musculaire qui conduit à la paralysie et la mort par insuffisance respiratoire en l'espace de quelques années. La plupart des projets de recherche se concentre sur les causes de la mort des neurones moteurs alors que l'atrophie musculaire n'est que peu étudiée dans ce contexte.

Approche

Nous avons voulu étudier les implications des protéines de la famille p53 durant l'atrophie musculaire de la SLA, car elles sont de puissants activateurs de mort cellulaire ou d'arrêt de cycle cellulaire, des mécanismes qui pourrait contribuer à la dégradation musculaire et le manque de régénération.
Afin de pouvoir éclaircir cette problématique, nous avons utilisé plusieurs modèles de, ou reliés à la SLA. Parmis les 16 gènes actuellement identifiés comme causant ou contribuant à la maladie, la superoxide dismutase Cu/Zn (SOD1) est particulièrement souvent mutée chez l'homme et a donc été abondamment étudié. Nous avions accès à un modèle murin de la SLA qui exprime une forme mutée de cette enzyme - SOD1(G86R) - et qui reproduit les symptomes trouvés chez les patients humains. Un decours temporel bien caractérisé des symptomes ainsi que des évènements moléculaires nous a permi de choisir des points de référence avant et lors du début de l'atrophie musculaire.
L'atrophie débute approximativement lors de la dénervation des fibres musculaires. Ceci peut être assimilée à de la dénervation et une atrophie d'inactivité. Afin d'étudier cet aspect, nous avons transitoirement dénervé le train arrière des souris (écrasement de nerf sciatique).

Finalement, nous avons utilisé des myoblastes C2C12 en culture pour étudier les relations entre l'activation de membres de la famille p53 et l'expression de gènes cible.

Principaux résultats

- Des gènes de la famille p53 (p53, TAp63, TAp73) sont induits dans des muscles atrophiques de patients SLA comme chez les souris du modèle murin SOD1(G86R) (Figure 1)
- L'expression de p63 ainsi que de certaines cibles de la famille p53 corrèlent avec la sévérité de l'atrophie musculaire chez les patients (Figure 2).
- TAp63 est surexprimé et s'accumule dans les noyaux des fibres musculaires atrophiées dans les muscles de souris SOD1(G86R) (Figure 3).
- Des stress cellulaires associés à l'expression de SOD1 mutée induisent TAp63 et la mort cellulaire.
- TAp63 induit l'expression de MuRF1 en se fixant à son promoteur (Figure 4).

p63 contributes to murf-1 regulation and muscular atrophy in ALS

von Grabowiecki Y.[1.2], Benosman S.[3], Blanchard O.[1.2], Palamiuc L.[2.4], Mériaux S.[2.4], Devignot V.[1.2], Mellitzer G.[1.2], Gonzalez de Aguilar JL.[2.4], and Gaiddon C.[1.2]

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Running title
Function of p53 proteins in muscular atrophy

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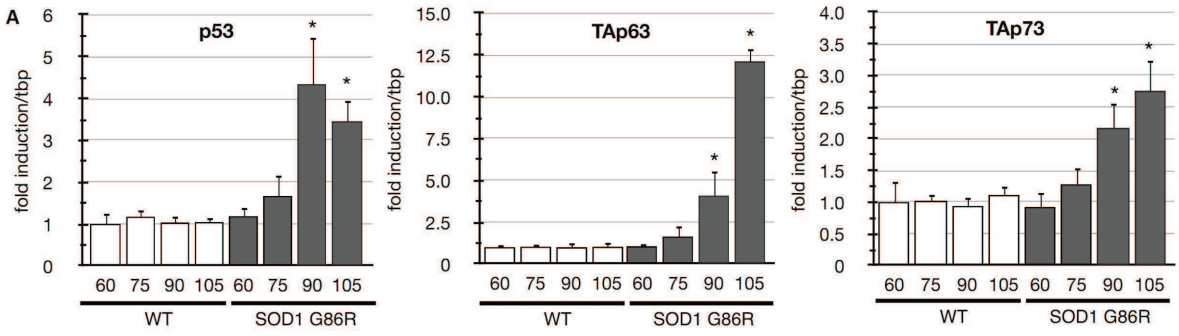

Figure 1 | Le niveau d'expression des ARNm de membres de la famille p53 dans les muscles de souris SOD1(G86R) par RT-qPCR. Les graphes représentent des moyennes d'induction par rapport au souris contrôle sauvages de 60 jours (n=5, 6).

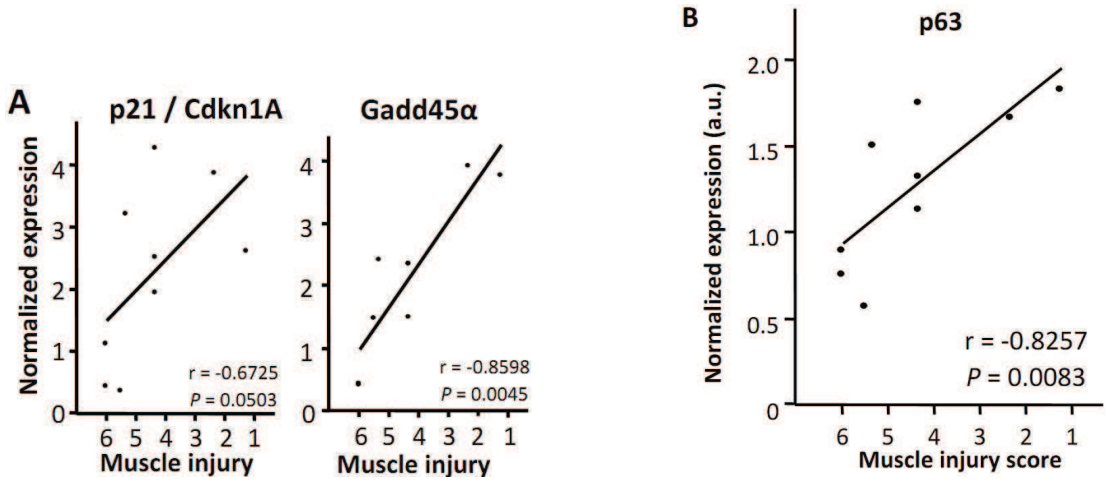

Figure 2 | Les données d'expression (A) de gènes cible de la famille p53 ou (B) de p63 ont été générées à partir d'expériences de puce à ADN (ebi.ac.uk/arrayexpress - accession E-MEXP-3260), obtenues par biopsies de muscles deltoides. Les scores de dommages musculaires sont déterminés par mesure de force musculaire et le degré d'atrophie musculaire (6: force normale et très faible atrophie, 1: paralysie totale et atrophie sévère). Chaque point représente un patient individuel. Les coefficients de corrélation et les p-valeurs ont été déterminées par test de Spearman.

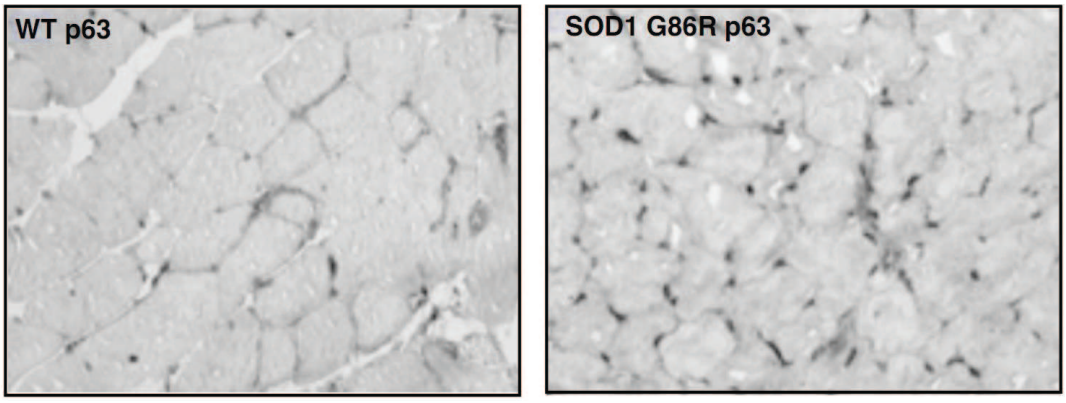

Figure 3 | Les muscles gastrocnémiens de souris sauvage ou SOD1(G86R) symptomatiques (105 jours) ont été cryodisséqués et immunohybridés pour détecter toutes les isoformes de p63.

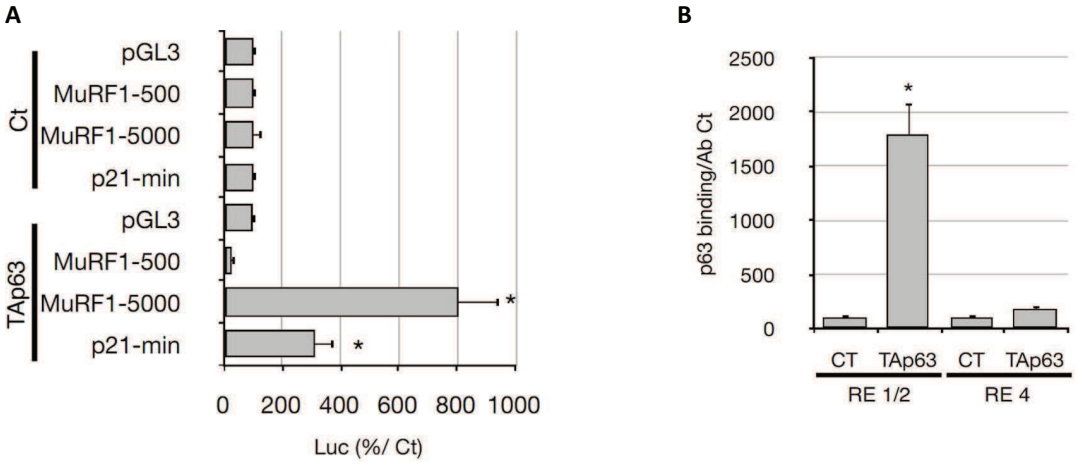

Figure 4 | (A) Des gènes rapporteur MuRF1 ont été co-transfectés avec pCDNA3 ou TAp63 dans des myoblastes C2C12 et l'activité luciférase a été mesurée après 16 heures. pGL3 a été utilisé comme contrôle négatif. (B) Immunoprécipitation de chromatine sur le promoteur de MuRF1. Les graphes représentent des valeurs moyennes avec écart-type (n=3).
* $p < 0.001$.

III - 2. Activité biologique de nouveaux dérivés de flavonoide et tocopherol sur la survie de myoblastes, et effet protecteur sur la cachexie cancéreuse ou la SLA

Alors que beaucoup de mécanismes qui conduisent à l'atrophie musculaire sont bien connus, il reste incertains comment ces derniers sont activés et comment ils interagissent. Les différences ou interactions entre les voies de signalisation d'atrophie compliquent la recherche de nolécules agissant sur des cibles spécifiques. En effet, différents types d'atrophie impliquent différents mécanismes, par exemple la dégradation active de fibres contre un défaut de renouvellement de myofibres.

AxoGlia est une jeune entreprise biotech basée au Luxembourg et consisten en une équipe de chimistest et de biologistes qui synthétise puis teste de dérivés de flavonoide et de tocopherol dans le contexte de la neurodégénéréscence. AxoGlia a mis en évidence que certaines de leurs molécules induit la différentiation de neurones ce qui les protège de certains stress cellulaires. De plus, un traitement avec ces composés réprime la voie de signalisation Notch dans ces cellules neuronales.
Nous étions intéressés par l'activité protectrice de ces molécules et nous somes demandé si elles pourrait être bénéfiques dans les cellules musculaires.

Approche

Afin de déterminer le potentiel protecteur de ces composes, nous avons traité des myoblastes C2C12 en culture avec différentes drogues, en combination avec les composés AxoGlia, pour évaluer la survie cellulaire. Les myoblastes ont également été utilisés pour comprendre les mécanismes induits par les composés AxoGlia. Finalement, nous avons utilisé le modèle murin de SLA SOD1(G86R) et le modèle de cachéxie cancéreuse par injection de doxorubicine pour évaluer les effets des composés AxoGlia sur l'atrophie musculaire *in vivo*.

Principaux résultats

- Deux composés, AGT48 et AGT251, induisent la prolifération de myoblastes (Figure 5).
- AGT48 et AGT251 protègent les myoblastes et des neurones moteurs en culture de stress cellulaires génotoxiques.
- La voie de signalisation Notch est perturbée dans les myoblastes traitées à l'AGT251 et dans des souris SOD1(G86R) (Figure 6).
- Des souris SOD1(G86R) traitées à l'AGT251 ont une survie prolongée (Figure 7).
- Le traitement de souris avec de l'AGT251 diminue l'atrophie musculaire après traitement à la Doxorubicine (Figure 8).

Biological activity of novel flavonoid and tocopherol derivatives on myoblast survival and protective effect towards cancer cachexia or amyotrophic lateral sclerosis

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Running title
Tocopherol and flavanoid derivatives biological activity on muscle atrophy via the Notch pathway

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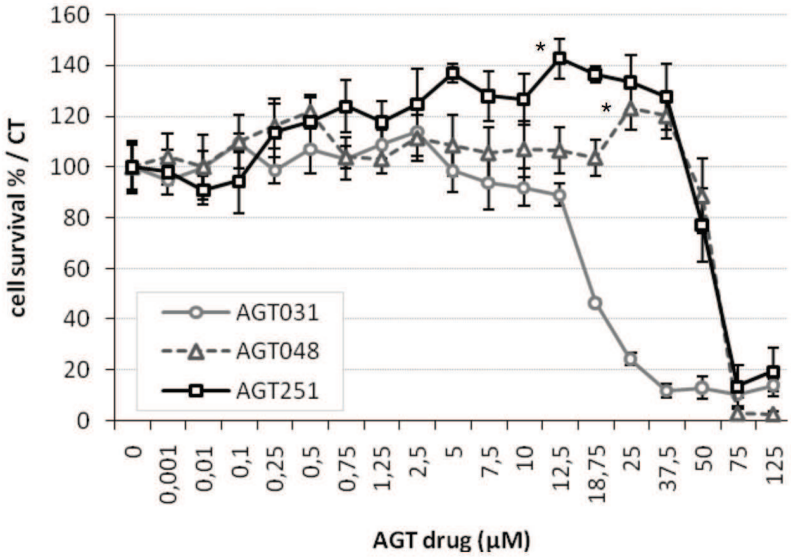

Figure 5 | Des myoblastes C2C12 ont été cultivés durant 48h en présence des composés et doses indiquées (AGT031, AGT048, AGT251, en µM). La survie cellulaire a éte évaluée par un test MTT. Les courbes indiquent une moyenne ± ecart-type (n=8).
* p< 0.01 en comparaison au contrôle, calculé par ANOVA et post-test de Tukey.

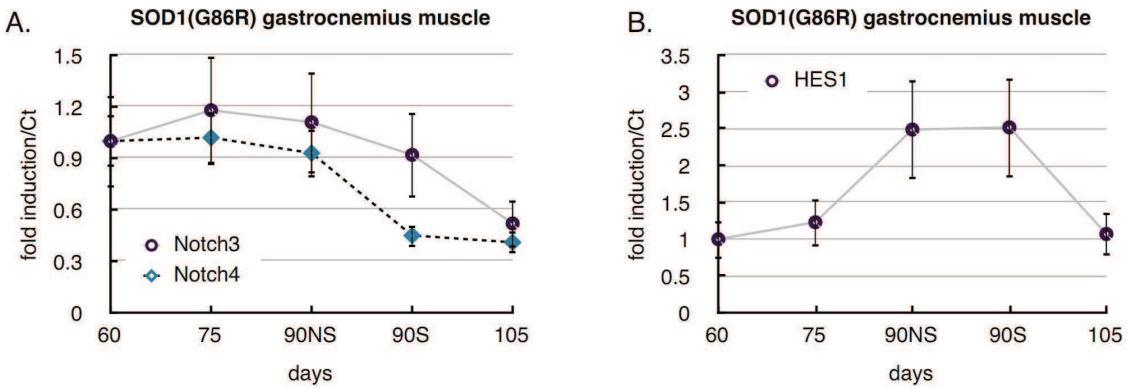

Figure 6 | Les niveau d'expression des ARNm de (A) Notch 3&4 et (B) Hes1 on été mesurés dans les muscles de souris SOD1(G86R) par RT-qPCR. L'ARNm a été extrait à différents ages. Les courbes indiquent une moyenne par rapport aux souris sauvages de 60 jours (n=5).

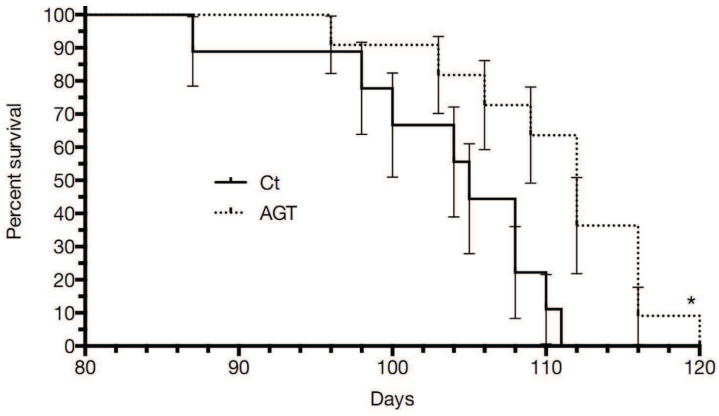

Figure 7 | Des souris SOD1(G86R) agées de 65 jours ont été injectées 3 fois par semaine avec de l'AGT251 jusqu'au décès. Les courbes de survie sont relatives au décès des souris traitées véhicule (n=12).
* différence statistique singificative établie par test Mantel-Cox ($p<0.001$) et Gehan-Breslow-Wilcoxon test ($p=0.0183$).

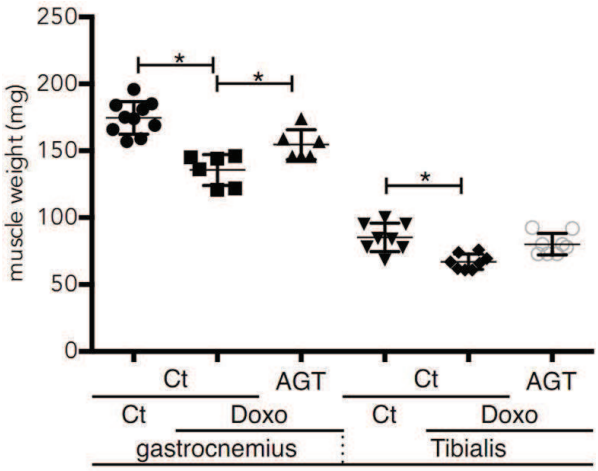

Figure 8 | Des souris sauvages agées de 8 semaines on été injectées une fois avec de la doxorubicine (10mg/Kg) et 3 fois par semaine avec de l'AGT251 (20mg/Kg). Le poids des muscles a été quantifié après 15 jours. Le graphe représente la moyenne en mg ± écart-type des muscles (n=9).
* $p< 0.01$ par rapport au contrôle, calculé par ANOVA et post-test de Tukey.

IV - CONCLUSION

Les résultats obtenus durant ma thèse indiquent que les membres de la famille p53 ne sont pas seulement activés durant la SLA, mais le sont egalement dans un type totalement différent d'atrophie musculaire, la cachéxie cancéreuse. Dans la SLA, il semblerait que TAp63 est l'isoforme la plus importante, alors que TAp73 est également surexprimé et pourrait induire l'expression de MuRF1 pour contribuer à la dégénérescence du muscle. Au contraire, dans l#atrophoe musculaire associée au cancer, l#expression de TAp63 n'est pas altérée, alors que TAp73 est hautement exprimé. Ces résultats sont très intéressants car ils indiquent que l'expression des membres de la famille p53 est spécifique au type d'atrophie en question et dépend des facteurs initiaux qui conduisent à cette dégradation musculaire. Dans la SLA, on pourrait proposer la voie de signalisation Notch, et dans l'atrophie liée au cancer, la signalisation dépendante du TNF-alpha.

En utilisant un dérivé de tocophérol produit par AxoGlia, nous avons pu augmenter la survie de souris affectés par la SLA. Dans notre modèle de doxorubicine/cachexie cancéreuse, nous avons significativemnet pu réduire l'atrophie musculaire. Alors que nous ne connaissons pas encore les mécanismes exacts de cet effet, l'utilisation dérivés de tocopherol pourraient aider à élucider les mécanismes gnéneraux de l'atrophie musculaire.