UNIVERSIDADE DE COIMBRA

# SKELETAL MUSCLE ATROPHY: THE ROLE OF miRNAs

# ATROFIA DO MÚSCULO ESQUELÉTICO: A FUNÇÃO DOS miRNAs

**Universidade de Coimbra**

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# ATROFIA DO MÚSCULO ESQUELÉTICO: A FUNÇÃO DOS miRNAs

Dissertation presented to the Faculty of Medicine of the University of Coimbra for the fulfillment of the requirements for a Doctoral degree in Health Sciences, branch of Biomedical Sciences.

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# Acronyms and Abbreviations

19S – Regulatory subunit of the proteasome

20S – Core particle of the proteasome

3'UTR – 3' Untrasnlated Region

40S – Ribosomal subunit

4E-BP1 – Eukaryotic Translation Initiation Factor 4E-Binding Protein 1

5'UTR – 5' Untranslated Region

60S – Ribosomal subunit

aaUTP – 5-(3-amminoallyl)-UTP

ActRIIB – Activin receptor IIB

Ago – Argonaute

AKT – V-AKT Murine Thymoma Viral Oncogene Homolog

ALk4 – Activin Receptor-like Kinase 4

ALk5 – Activin Receptor-like Kinase 5

ALS – Amyotrophic Lateral Sclerosis

AMPK – AMP-Activated Protein Kinase

AP-1 – Activator Protein 1

Atg – Autophagy-related gene

ATP –Adenosine triphosphate

$BaCl_2$ – Barium Chloride

Bcl-2 – B-cell lymphoma 2

C2C12 – mouse myoblast cell line

Ccr4 – Chemokine, CC Motif, Receptor4

CDC25A – Cell division cycle 25a

CDC34 – Cell division cycle 34 (ubiquitin conjugating enzyme)

CDK6 – Cyclin dependent kinase 6

CMA – Chaperone-mediated autophagy

CMV – Cytomegalovirus

Col6a1 – Collagen VI

CreER – Cre recombinase fused with the estrogen receptor

CSA – Cross Section Area

Cx43 – connexin-43

$CXMD_j$ – canine X-linked muscular dystrophy in Japan

Cy3 – Cyanine Dye 3

Cy5 – Cyanine Dye 5

DAPC – dystrophin-associated protein complex

DAVID – Database for Annotation, Visualization and Integrated Discovery

DCP2 – Deccaping enzyme 2

DGCR8 – DiGeorge Syndrome critical region gene 8

DMD – Duchene Muscular Dystrophy

D-MEM – Dulbecco's modified Eagle's medium

DNA – Dexoxyribonucleic acid

DTA – Diphtheria toxin A

DUB – Deubiquitinating enzymes

EDL – Extensor digitalis longus

eIF3f – Eukaryotic translation initiation factor 3, subunit F

eIF4E – Eukaryotic translation initiation factor 4E

eIF4G – Eukaryotic translation initiation factor 4-gamma

eIF6 – Eukaryotic translation initiation factor 6

Ezh2 – Enhancer of Zeste, drosophila, homolog 2

FBS – Fetal bovine serum

FGF – Fibroblast growth factor

FGFBP1 – Fibroblast growth factor-binding protein

FMRP – Fragile X Mental Retardation Protein

FO – Functional Overload

FoxO – Forkead Box O

Fstl1 – Follistatin-like 1

Gabarap – Gabba receptor-associated proteins

GATE16 – Golgi associated ATPaseEnhancer 16 KDa)

GFP – Green Fluorescence protein

GSK3β - Glycogen synthase kinase 3β

GW182 – Trinucleotide repeat-containing gene 6a

HDAC4 – Histone deacetylase 4

Hox-A11 – Homeobox A11

HU – Hindlimb unloading

HWA448 – Torbafylline

IGF-1 – Insulin growth factor-1

IGF-II – Insulin-Like growth factor II

IKKβ – Inhibitor of Kappa Light chain gene enhancer in B cells, Kinase

IL-6 – Interleukin 6

IREs – Internal Ribosome Entry Site

IRS – Insulin receptor sunstrate

Jumpy – myotubularin-related protein

JunB – V-Jun Avian sarcoma virus 17 oncogene homolog

KDa – KiloDalton

KEGG – Kyoto Encyclopedia of Genes and Genomes

LAMP-2A – Limbic system-associated membrane protein

LLN – N-acetylleucyl-leucyl-norleucidal

MAFbx – Muscle atrophy F-box

MAP1LC3 – Microtubule-associated protein 1. Light chain3

MEF2 – Mads Box transcription enhancer factor 2

MG132 – CBZ-leucyl-leucyl-leucidal

MHC – Myosin heacy chain

Mib1 – drosophila homolog of Mindbomb 1

MRF4 – Muscle regulatory factor 4

mRNA – messenger ribonucleic Acid

mTOR – mammalian target of rapamycin

Murf-1 – muscle-specific ring finger protein

Myf5 – myogenic factor 5

MyH – Myosin Heavy Chain

MyoD – Myogenic differentiation antigen 1

NF-kB – Nuclear factor kappa-B

NMJs – neuromuscular junctions

nPTB – polypirimidine tract.binding protein

N-Ras – Neuroblastoma Ras Viral oncogene

NUMB – Drosophila homolog of Numb

ORF – Open Reading Frame

P27 – Cyclin dependent Kinase inhibitor 1B

PA200 – Proteasome activator, 200 Kda

PA28 – Proteasome activator 28-alpha

Pax3 – Paired box gene 3

Pax7 – Paired box gene 7

PAZ – Piwi, Argonaut and Zwille domain

PcG – Polycomb Group proteins

PDCD10 – Programmed Cell Death 10

PDH – Plant homeo domain

PDK1 – Phosphoinositide-dependent kinase

PDK2 – Pyruvate dehydrogenase kinase, isoenzyme 2

PDK4 – Pyruvate dehydrogenase kinase, isoenzyme 4

PE – Phosphatidylethanolamine

PGC1α – Peroxisome proliferator-activated receptor-gamma, coactivator 1, alpha

PI3K – Phosphatidylinositol-3-kinase

PI3P – Phosphatidyl inositol triphosphate

PIP3 – Phosphoinositide-3, 4, 5-triphosphate

PKB – Protein Kinase B

PKM2 – Muscle piruvate kinase 2

Pola1 – DNA polymerase alpha1

PolE4 – DNA polymerase epsilon 4

PolK – DNA polymerase kappa

PS-341 – Velcade

PTEN – Phosphatase and tensin homolog

PTX – Pentoxyfylline

Purβ – Purine-rich element-binding protein B

PYGM – Muscle glycogen phosphorylase

Ran-GTP – Ras-related nuclear protein

RISC – RNA-induced silencing complex

RNAi – RNA interference

Roc1 – Regulator of cullins 1

RT-PCR – Real Time Polymerase Chain Reaction

Runx1 – Runt-Related transcription factor1

S6K1 – Ribosomal protein S6 kinase

SAM – Significance Analysis of micro-arrays

SCF – Complex composed of Skp1, Cullin and F-box

SH-EP1 – Neuroblastoma cell line

SHIP2 – SH2-containing inositol phosphatase 2

shRNA – Short hairpin RNA

Skp1 – S-phase kinase-associated protein

SMAD – Sma- and Mad-related proteins

SOD1 – Superoxide diemutase

Sox6 – Sry-box6

Sp3 – Transcription factor sp3

SR – Sarcoplasmic reticulum

SRF – Serum response factor

STZ –Streptozotocin

TA – Tibialis Anterior

TGF-β – Transforming growth family - β

THRAP1 – Thyroid Hormone Receptor Associated Protein 1

TNF-α – Tumor necrosis factor - α

TOR – Target of rapamycin

TR – Thyroid Hormone Receptor

TRBP – Tar RNA Binding Protein

TWEAK – TNF-Like weak promoter of apoptosis

U2OS – Human osteosarcoma cell line

U6 – Small nuclear RNA U6

UCP2 – Uncoupling protein 2

Utrn – Utrophin

UVRAG – Ultraviolet irradiation resistance associated gene

VMP1 – Vacuole Membrane Protein 1

Vps34 – phosphatidylinositol 3-kinase class 3

Xrn1 – 5’-3’-exoribonuclease

YY1 – Yin Yang 1

# Thesis Organization

This thesis is organized as a scientific paper. It contains seven chapters preceded by an abstract in English and Portuguese.

The **First Chapter** consists of an introduction to the molecular mechanisms implicated in the skeletal muscle atrophy. How is the skeletal muscle organized? What is muscle hypertrophy and atrophy? Which are the degradative mechanisms implicated during muscle atrophy? Which are the molecular regulators of this catabolic process? All these questions are approached in this chapter. Furthermore, there is also a detailed introduction on miRNAs. Their origins, their biogenesis, their mechanism of action and their role in muscle cells and adult muscle.

In the **Second Chapter** the major aims of the thesis are presented.

A detailed description of the methods used in this thesis is presented in the **Third Chapter**.

In the **Fourth and Fifth Chapter**, the results obtained are presented. The fourth chapter consist on the identification and functional characterization of the miRNAs altered during the atrophic conditions. The fifth chapter describes the identification and functional validation of the target genes of the altered miRNAs.

The **Sixth Chapter** comprises a general discussion and main conclusions of this thesis.

In the **Seventh chapter**, the future perspectives of this work are presented.

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# Abstract

Skeletal muscle atrophy is a condition associated to loss of muscle mass in many diseases. Atrophy is a characteristic response to starvation, aging and disuse conditions (such as immobilization, denervation or unloading) but it also occurs as a complication in several chronic diseases such as cancer, diabetes, sepsis, AIDS, renal and heart failure and others. Independently of the cause, the main feature of muscle wasting is the enhancement of protein degradation that overcomes protein synthesis.

Skeletal muscle atrophy is a transcriptionally regulated process (Lecker et al., 2004; Sandri et al., 2004a; Stitt et al., 2004). FoxOs are critical transcription factors involved in the regulation of critical rate-limiting enzymes belonging to the two most important degradative pathways: the ubiquititn/proteasome (Gomes et al., 2001; Sandri et al., 2004a) and the autophagy/lysosome (Mammucari et al., 2007; Zhao et al., 2007). Also NF-kB is involved in transcriptional regulation during muscle wasting (Cai et al., 2004; Hunter and Kandarian, 2004). Furthermore, a restricted group of genes, called atrophy-related genes or atrogenes, are commonly up- or down-regulated to all the atrophic conditions (Lecker et al., 2004). These findings suggest the presence of a shared molecular mechanism that controls muscle atrophy. Among the atrogenes there are genes involved in several fundamental biological processes that may require an additional regulation to fine-tune their action during muscle wasting. This action might be accomplished by a new class of regulatory molecules, the miRNAs. miRNAs are predicted to regulate several genes from the same pathway. Their role in adult skeletal muscle is largely unknown.

miRNAs are small non-coding RNAs with approximately 22 nucleotides that regulate post-transcriptionally gene expression. They are highly conserved among species and they are predicted to regulate the expression of approximately 60% of protein coding genes. Conventionally, miRNAs are known to regulate gene expression by binding to the 3'-Untranslated Regions (3'UTRs) of the mRNAs and, therefore, blocking translation or inducing mRNA degradation. Each miRNA has the potential to target hundreds of different mRNAs. On the other hand each mRNA can be targeted by different miRNAs creating in this way complex regulatory networks. One of the hallmarks of miRNAs is their specificity. In fact, several miRNAs are involved in developmental and physiological processes that require tissue- and stage-specific expression. The tight regulation of miRNAs expression is crucial and alterations are correlated with pathological conditions.

The essential role of these regulators in skeletal muscle was clearly demonstrated in several animal models in which the miRNA pathway was blocked leading to a compromised myogenic

development (Kwon et al., 2005; O'Rourke et al., 2007; Sokol and Ambros, 2005). Furthermore, several miRNA show a muscle-specific expression and are called myomiRs. This group is composed by miRNA-1, miRNA-133, miRNA-206, miRNA-208a, miRNA-208b and miRNA-499. Muscle specific miRNAs are involved in several processes of the muscle physiology including myogenesis, fiber type establishment and muscle regeneration. Besides myomiRs, other miRNAs were shown to be involved in the regulation of these processes. The involvement of several miRNAs in the regulation of several aspect of muscle biology creates a complex regulatory network increasing the complexity of muscle biology. Moreover miRNA deregulation is associated with muscle disease (De, V et al., 2010; Eisenberg et al., 2007; McCarthy and Esser, 2007a; Williams et al., 2009; Yuasa et al., 2008a). However, regardless of the growing evidences on miRNAs function few studies have addressed their biological role *in vivo*. In this thesis, we studied the role that miRNAs play in skeletal muscle atrophy. We have used an *in vivo* approach supported by bioinformatic analyses to identify some of the mechanisms controlled by miRNAs.

In the first part of the thesis we have established the miRNA expression signature of several atrophic conditions by microarray analysis. According to our results, each atrophic condition has a specific miRNA expression profile. Only middle-to-late atrophic conditions showed a significant alteration of the miRNAs expression levels. Although no common miRNA was found between the different conditions, two highly up-regulated miRNAs were found in denervation, miRNA-206 and miRNA-21. Thus, we decided to address their biological role *in vivo*. Our studies showed, for the first time, that *in vivo* over-expression of these two miRNAs leads to an atrophic phenotype, while inhibition of these miRNAs induced hypertrophy.

In the second part of this thesis, we performed mRNA expression profile by using the same samples used for miRNA profile. A bioinformatic approach based on gene expression data allowed us to identify genes that were both predicted targets of the miRNAs and down-regulated in the mRNA expression arrays. This approach allowed the identification of a set of target genes that were directly down regulated by the miRNAs. In particular, we decided to study YY1, eIF4E3 and PDCD10 because they were predicted targets of both miRNAs. Indeed, luciferase experiments together with over-expression experiments confirmed that YY1 is a target gene of miRNA-21 and eIF4E3 and PDCD10 are targets of both miRNA-206 and miRNA-21. Although the role of these genes during skeletal muscle atrophy is still not clear, they are clearly down-regulated during denervation and the function of this down-regulation is currently under study.

Our results indicate that the atrophic process, apart from the transcriptional regulation, is also under a miRNA fine tuning. Furthermore, our data point to miRNA-206 and miRNA-21 as important modulators of the atrophic process suggesting that they are potential therapeutic targets.

# Resumo

A atrofia do músculo esquelético é uma condição associada à perda de massa muscular que ocorre em várias doenças. Esta atrofia é uma resposta característica ao jejum, envelhecimento e a condições de desuso ( como imobilização, desenervação  e falta de carga no músculo) mas também ocorre como complicação associada a diversas doenças crónicas como cancro, diabetes, sepsia, SIDA e falha renal e cardíaca entre outras. Independentemente da causa, a maior característica da atrofia muscular é um aumento da degradação proteica em relação à síntese proteica.

A atrofia do músculo esquelético é um processo regulado a nível da transcrição  transcrição (Lecker et al., 2004; Sandri et al., 2004a; Stitt et al., 2004). Os factores de transcrição da família FoxO são fundamentais para a regulação de enzimas limitantes que pertencem aos dois mecanismos catabólicos mais importantes do músculo: o sistema ubiquitina/proteassoma proteassoma (Gomes et al., 2001; Sandri et al., 2004a) e o sistema autofagia/lisossoma (Mammucari et al., 2007; Zhao et al., 2007). O factor de transcrição NF-kB também está envolvido na regulação que ocorre durante o processo de atrofia muscular (Cai et al., 2004; Hunter and Kandarian, 2004). Para além disso, um grupo restrito de genes, chamados “atrogenes” ou genes relacionados com a atrofia, encontram-se frequentemente sobre- ou sub-expressos em todas as condições de atrofia. Estas evidências sugerem que existem mecanismos moleculares partilhados que controlam a atrofia muscular. Entre os “atrogenes” encontram-se genes envolvidos em diversos processos biológicos fundamentais que podem requerer um nível adicional de ajustamento durante a perda de massa muscular. Este ajustamento pode ser conseguido através de uma nova classe de moléculas reguladoras chamadas miRNAs. Os miRNAs são hipoteticamente capazes de regular vários genes da mesma via de sinalização. O seu papel no músculo adulto é amplamente desconhecido.

Os miRNAs são pequenos RNAs não codificantes com aproximadamente 22 nucleotídeos que regulam a expressão de genes. São altamente conservados entre espécies e é previsto que sejam capazes de regular a expressão de aproximadamente 60% dos genes codificantes. Convencionalmente, os miRNAs regulam  a expressão génica ligando-se à região 3’-UTR (região não traduzida) dos RNAs mensageiros (mRNA), e posteriormente bloqueando a sua tradução ou induzindo a sua degradação. Cada miRNA tem o potencial de regular centenas mRNAs. Por seu lado, cada mRNA pode ser regulado por centenas de miRNAs diferentes, criando-se deste modo redes reguladoras complexas. Uma das principais características dos miRNAs é a sua especificidade. De facto, diversos miRNAs estão envolvidos em processos fisiológicos  e ligados ao desenvolvimento, e requerem, por isso, uma expressão que é específica para determinado tecido e

fase de desenvolvimento. Uma apertada regulação da expressão dos miRNAs é essencial e alterações na sua expressão estão associadas a situações patológicas.

O papel essencial que estas moléculas reguladoras têm no músculo esquelético foi claramente demonstrado em diversos modelos animais nos quais a via dos miRNAS foi bloqueada, comprometendo o desenvolvimento miogénico. (Kwon et al., 2005; O'Rourke et al., 2007; Sokol and Ambros, 2005). Para além destas evidências, existe um grupo de miRNAs específicos do músculo – são chamados myomiRs. Este grupo é composto pelos miRNA-1, miRNA-133, miRNA-206, miRNA-208a, miRNA-208b e miRNA-499. Estes myomiRs estão envolvidos em diversos processos da fisiologia muscular, incluindo miogénese, estabelecimento do tipo de fibra e regeneração muscular. Na regulação destes processos estão envolvidos outros miRNAs para alem dos myomiRs. O envolvimento de diversos miRNAs na regulação de vários aspectos da biologia do músculo cria uma rede regulatória, aumentando deste modo a complexidade da biologia do músculo. Para além disso a desregulação da expressão de miRNAs está associada a doenças musculares (De, V et al., 2010; Eisenberg et al., 2007; McCarthy and Esser, 2007a; Williams et al., 2009; Yuasa et al., 2008a). Independentemente do aumento do numero de evidências relacionadas com a função dos miRNAS, poucos estudos abordaram o seu papel biológico *in vivo*. Nesta tese, nós estudamos o papel dos miRNAs no processo de atrofia do músculo esquelético. Foi utilizada uma aproximação *in vivo*, que se baseou em análises bioinformáticas, para identificar alguns dos mecanismos controlados pelos miRNAs.

Na primeira parte desta tese, nós estabelecemos o perfil de expressão de miRNAs em diversas condições de atrofia, usando para isso análise de micro-arrays. De acordo com os nosso resultados, cada condição atrófica possui um perfil de expressão de miRNAs específico. Apenas condições atróficas a médio-longo termo apresentam uma significativa alteração nos níveis de expressão dos miRNAs. Apesar de não ter sido encontrado nenhum miRNA comum a todas as condições de atrofia, identificamos dois miRNAs, miRNA-206 e miRNA-21, fortemente aumentados após desenervação. Decidimos por isso estudar o seu papel biológico *in vivo*. Os nossos estudos demonstraram, pela primeira vez, que a sobre-expressão destes miRNAs induzem um fenótipo atrófico enquanto a inibição *in vivo* induz um fenótipo hipertrófico.

Na segunda parte desta tese, estabelecemos o perfil de expressão de mRNAs das mesmas amostras que tinham sido usadas para estabelecer o perfil de expressão de miRNAs. Uma aproximação bioinformática baseada nos dados de expressão génica permitiu-nos identificar os genes que eram simultaneamente alvos previstos dos miRNAs e que se encontravam sub-expressos nos arrays de expressão de mRNA. Esta metodologia permitiu-nos identificar um grupo de genes alvo cuja expressão é regulada negativamente pelos miRNAS. Em particular, decidimos estudar

YY1, eIF4E3 e PDCD10 porque estes três genes são previsivelmente regulados pelos dois miRNAs. De facto, experiências de luciferase juntamente com experiências de sobre-expressão confirmaram que YY1 é um alvo do miRNA-21 e que eIF4E e PDCD10 são alvos do miRNA-206 e miRNA-21. Apesar do papel destes genes durante o processo de atrofia muscular não ser conhecido, sabemos que estão sub-expressos durante a desenervação. A relevância da diminuição na expressão de YY1, eIF4E3 e PDCD10 durante a desenervação está presentemente a ser investigada.

Os nosso resultados indicam que o processo atrófico, para além da regulação a nível da transcrição, é também modulado por miRNAs. Para além disso os nossos dados apontam para o miRNA-206 e miRNA-21 como importantes moduladores do processo atrófico, sugerindo que estes miRNAs podem ser potenciais alvos terapêuticos.

# Chapter 1 - Introduction

## 1.1- Skeletal Muscle: the main player

To walk or to run, to sit or to lie down, to listen or to look and even to breath, are all one contraction away from achieving their goals. This is the major function of muscles – to contract, to generate force, to produce movement. However, this does not mean that locomotion is the only muscle function. Skeletal muscles are highly specialized in producing energy and subsequently generating heat. They do so not only as a consequence of exercise, but also independently, and this constitutes a great adaptive advantage of several species throughout the animal kingdom. Most importantly, skeletal muscle plays an essential role in the maintenance of body's energy homeostasis. In periods of fasting, through catabolic processes, muscles are capable of releasing amino acids (alanine and glutamine) into the blood that are then used by the liver to maintain the glucose levels (gluconeogenesis). The main focus of this thesis are the molecular mechanisms implicated in the catabolic processes that ultimately lead to skeletal muscle atrophy.

### 1.1.1- Skeletal Muscle Architecture

Skeletal muscle is a highly organized tissue. According to the task they perform, they present different size, shape and properties. However their basic structure is the same. Skeletal muscles are surrounded by the epimysium, a coat of connective tissue that separates different muscles. Each muscle is divided into bundles or fascicles of fibers which are also coated by connective tissue – the perimysium. Finally the endomysium envelops each fiber (Fig 1).

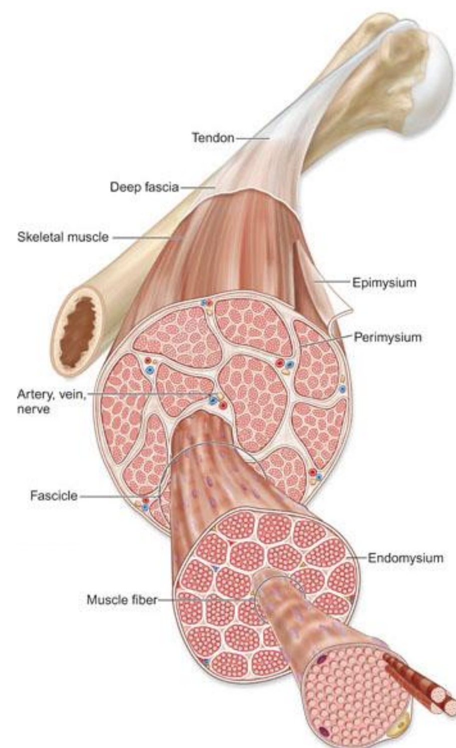



**Fig 1: Schematic representation of the skeletal muscle architecture.** Skeletal muscle is composed of fascicles of muscle fibers. Each fiber is composed of myofibrils.

In the intramuscular region a highly vascularized system of arterioles and venules allows the supply of oxygen and substrates to produce energy. This system penetrates deep into the perimysium to form a dense capilar network that maintains a constant nutrient environment in the vicinity of the fiber. Additionally, in the intramuscular region we can also find the nerve branches that innervate the muscles. These nerves, composed of motoneurons, ramify into the perimysium. Each motoneuron establishes contact with a

single fiber through the neuromuscular junction.

Skeletal muscle fibers are multinucleated cylindrical cells, in which the nuclei are located in the periphery underneath the plasma membrane – also called sarcolemma. Representing only 1% of the nuclei present in the muscle and almost undistinguishable are the satellite cells – the stem cells of the muscle. These are normally quiescent cells in adult muscle, but are able to proliferate in response to injury and give rise to regenerated muscle and to more satellite cells (Morgan and Partridge, 2003).

Apart from the nuclei another organelle of great importance present in the cytoplasm are the mitochondria. Skeletal muscle contains two populations of mitochondria, the subsarcolemmal and the intermyofibrillar mitochondria. Apart from their localization they also possess different biochemical properties but their basic function is to supply the energetic demands of the fiber. Apparently they seem to be differentially affected by disease and exercise training (Koves et al., 2005).

With the aid of electron microscopy it was shown that muscle fibers possess a tubular system. It is composed of T-Tubules that are required for the conduction of impulses to the interior of the fiber. Another tubular structure is the sarcoplasmic reticulum (SR) that is responsible for the Ca2+ handling during contraction/relaxation.

The striated appearance of the muscle is due to the organization of the myofibril, the units responsible for the contraction. These myofibrils are disposed in parallel arrangement and run the entire length of the fiber. Myofibrils consist of bundles of myofilaments. These filaments are mainly composed of actin and myosin organized in thin and thick filaments, respectively. The myofilaments are arranged in functional units called sarcomeres. As we can see from Fig 2 each sarcomere is delimited by the Z-disk. This structure is composed mainly of α-actinin, desmin, vimentin and, together, with nebulin provides positional support for the actin filaments. The I-band (light band), which is mainly composed of actin filaments, surrounds the Z-disk. These actin filaments expand to a dark anisotropic band called A-band. This A-band is also composed of myosin thick

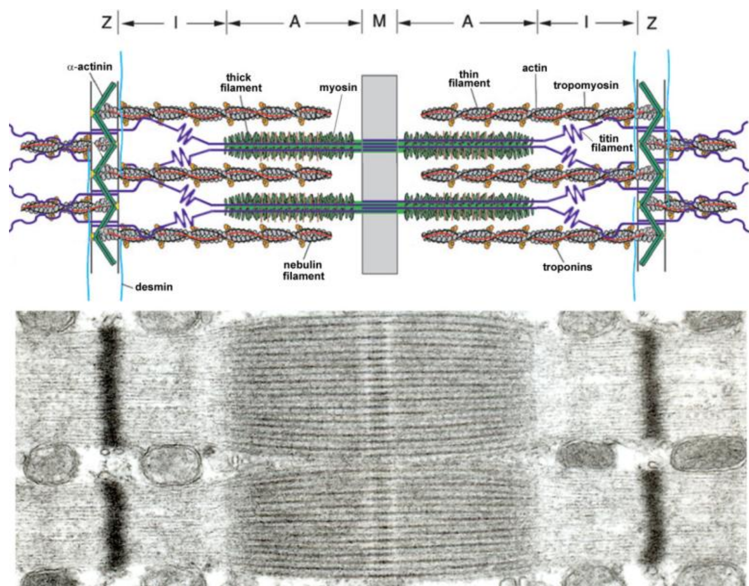



**Fig 2: Sarcomere Structure.** Schematic representation and electron microscopy image of the sarcomere.

filaments and is interrupted in the middle by a darker band, the M-band, only composed of myosin filaments. Spanning from the Z-disk to the M-band there is a filament of titin (connectin) which serves not only as structural support but also for the assembly of the contractile machinery.

### 1.1.2- Skeletal muscle fiber type

Although the basic structure of the muscle fiber is the same, the proteic composition varies, giving different properties to the muscles. Skeletal muscle fibers are classified having in consideration their metabolic, biochemical and structural properties. In this way skeletal muscle fibers can be classified as type I (oxidative/slow) or type II fibers (glycolytic/fast). They are usually identified according to the myosin heavy chain (MyH) isoform they preferentially express, since myosins are the main determinant of the contractile properties. Type I fibers express mainly MyH-7 and have a slow velocity of shortening. They are rich in mitochondria and mainly use oxidative metabolism for energy production. This provides a stable and long-lasting supply of ATP, thus rendering these fibers fatigue-resistant. Type II fibers on the other hand can be subdivided into three subtypes: IIa, IIx, and IIb according to the MyH isoform they express (MyH-2, MyH-1 and MyH-14 respectively). They have a lower mitochondrial content and their metabolic properties are graded, being the IIb the fastest, most glycolitic and thus most susceptible to fatigue and the IIa the slowest and most oxidative. Since in most cases both fiber types coexist in one muscle, the type of contraction (slow or fast) results from the percentage of different fiber types present in the muscle. Muscles containing mainly type I fibers are slow muscles highly vascularized, containing high levels of myoglobin, in opposition to muscles containing mainly type II fibers.

Although being a post-mitotic tissue, adult skeletal muscle has a great capability to adapt to a wide variety of stimuli. The amount and pattern of muscle activity together with stimuli received during pathological conditions may lead to alterations in fiber size and fiber type and consequently alterations in its properties

## 1.2- Skeletal Muscle Hypertrophy and Atrophy

Skeletal muscle is the most abundant tissue in the human body accounting for ≈50% of the total body mass. It is not only the major site of metabolic activity but it is also the largest protein reservoir, serving as a source of amino acids to be utilized for energy production during periods of

food deprivation (Nader et al., 2005). Accordingly, amino acids generated from muscle protein breakdown are utilized by the liver to produce glucose. Muscle is a dynamic tissue that continuously adapts its size not only to the nutritional status but also to a variety of external stimuli, including mechanical load, neural input, hormones/growth factors and stress. In normal conditions the balance between protein synthesis and protein degradation dictates the maintenance of skeletal muscle mass. Whenever this balance is shifted we can have one of the two situations: muscle hypertrophy or muscle atrophy.

### 1.2.1- Skeletal Muscle Hypertrophy

Skeletal muscle hypertrophy is characterized by an increase in the muscle mass and in the cross section area of the fibers. Two types of muscular hypertrophy can occur: sarcoplastic hypertrophy or myofibrilar hypertrophy. While the first one is associated to an increase of the sarcoplasmic fluid without any increase in muscle force, the second implies an increase in the contractile components of the fiber with increase in muscle strength. Generally, muscle hypertrophy is associated to strength training, however it can also be the consequence of genetic conditions, as it will be discussed later. In any case, there is an increase in protein synthesis.

Simultaneously to the increase in fiber size there is also incorporation of new myonuclei in the fibers to maintain the proportion between cytoplasm and nuclei. This myonuclei derive from the activation of satellite cells – the stem cells of the muscle. (Zammit, 2008). Satellite cells play a major role during embryonic and pos-embryonic period, contributing for muscle growth. In adult muscle they become quiescent and therefore have a minor role in muscle growth. However there are some studies that state the importance of satellite cells during muscle hypertrophy. In fact, muscle hypertrophy, induced by loading or IGF-1, was partially or totally prevented by muscle irradiation, thus eliminating all satellite cells (Adams, 2006) (Barton-Davis et al., 1999). In the same way, depletion of satellite cells by knocking out Pax7, a transcription factor required for satellite cell differentiation, severely blunted postnatal muscle growth (Oustanina et al., 2004). However a recent study completely contradicts these arguments and present evidences that adult muscle growth is completely independent of satellite cells. The authors develop a genetic mouse model to conditionally ablate satellite cells in adult muscles. Basically they have generated a mouse model in which the diphtheria toxin A (DTA) gene is under the control of the Pax7-CreER. After tamoxifen injection the Pax7 expressing cells start to produce DTA leading to an ablation of almost 90% of the satellite cells. When submitted to synergistic ablation, a model of hypertrophy, Pax7 depleted

muscle underwent hypertrophy. The authors further showed that although satellite cells are not required for muscle hypertrophy, they are requested for the formation of new fibers and for muscle regeneration (McCarthy et al., 2011). Therefore, it is now believed that muscle hypertrophy results mainly from an increase in protein synthesis rather than an activation of satellite cells.

At the molecular level, Insulin Growth Factor-1 (IGF-1) was shown to be one of the most important growth factor implicated in muscle hypertrophy. Although it is mainly produced by the liver, there are some muscle specific isoforms (Hameed et al., 2004), such IGF1Ea that was shown to promote proliferation and differentiation of satellite cells (Adi et al., 2002; Allen and Boxhorn, 1989; Doumit et al., 1996). During overload-induced muscle hypertrophy the expression of this growth factor was increased suggesting a role in muscle growth (DeVol et al., 1990). This data was confirmed by muscle-specific transgenic mice that showed muscle hypertrophy, increased regeneration and increased muscle strength (Coleman et al., 1995; Musaro et al., 2001).

The binding of IGF-1 or insulin to its receptor leads to a cascade of events that culminate in the activation of AKT. This serine/threonine kinase is a critical component of the anabolic pathways and is activated by various stimuli, including growth factors, mechanical stimuli, and insulin. There are 3 AKT isoforms, being AKT1 and AKT2 the ones with highest expression in skeletal muscle (Yang et al., 2004). Several lines of evidence support the idea that AKT is essential to muscle growth. In fact, mice that lack AKT1 are smaller than their littermates (Yang et al., 2004). Accordingly, over-expression of a heart-specific active form of AKT1 results in a hypertrophic phenotype (Matsui et al., 2002; Shioi et al., 2002). The same is observed in skeletal muscle with a constitutively or inducible active form of Akt (Blaauw et al., 2009; Lai et al., 2004). On the other hand the over-expression of 2 upstream phosphatases, PTEN and SHIP2, that prevent the docking of AKT to the plasma membrane, inhibits muscle growth (Bodine et al., 2001c; Goberdhan et al., 1999; Huang et al., 1999) confirming the essential role of AKT in muscle hypertrophy.

Several molecules, downstream of Akt, were shown to be involved in cell size regulation. Both IGF-1 and nutritional stimuli leads to the phosphorilation (through AKT) and activation of mTOR (mammalian target of rapamycin), a kinase that is selectively inhibited by rapamycin. It was shown that in hypertrophic conditions, both AKT and mTOR are phosphorilated, and this hypertrophy could be blocked by rapamycin (Bodine et al., 2001c; Pallafacchina et al., 2002; Rommel et al., 2001). In fact rapamycin was able to block the effects of AKT in muscle growth in the inducible AKT transgenic mice (Izumiya et al., 2008). The activation of mTOR leads to an increase in protein synthesis by two different mechanisms: the activation of p70S6 kinase, a known positive regulator of protein translation, and the inhibition of 4E-BP1, a negative regulator of the

protein initiation factor eIF-4E (Hara et al., 1997; Proud, 2004). Ohanna et al (Ohanna et al., 2005) showed that p70S6 kinase is required for muscle fibers to achieve normal size.

Finally, activation of AKT phosphorylates and inhibits the activity of GSK3β (Glycogen synthase kinase 3 beta) (Cross et al., 1995) leading to hypertrophy. It was demonstrated that a dominant negative, kinase dead form of GSK3β induces dramatic hypertrophy in skeletal myotubes (Hardt and Sadoshima, 2002; Rommel et al., 2001). Since GSK3β blocks protein translation initiated by the eIF2B protein (Hardt and Sadoshima, 2002), it's possible that GSK3β stimulates protein synthesis independently of the mTOR pathway.

But AKT is not always required in order to have an hypertrophic phenotype. In fact, Rafaello et al (Raffaello et al., 2010) showed that JunB, a transcription factor markedly down-regulated in muscle of several atrophic conditions, is sufficient to promote muscle growth independently of mTOR and AKT. Furthermore, JunB over-expression reduced denervation-induced muscle atrophy by inhibiting MAFbx/Atrogin-1 and Murf-1. This inhibition was the result of a physical interaction between JunB and FoxO3 that prevented the binding of FoxO3 to the promoter region of MAFbx/Atrogin-1 and Murf-1. Finally, the authors have shown that JunB over-expression leads to a reduction in the myostatin levels, therefore releasing muscle growth from the inhibitory action of myostatin.

As discussed briefly, another important player in skeletal muscle hypertrophy is myostatin, a negative regulator of muscle growth that is secreted mainly by the muscle. Myostatin is a member of the transforming growth family-β (TGF-β), and at the molecular level, binds to the activin receptor IIB (ActRIIB), a type II TGF-β receptor, which, in turn, activates activin receptor-like kinase 4 (ALK4) or ALK5, both type I serine/threonine kinase receptors. The downstream targets of this pathway are still to be elucidated. Mutations in the myostatin gene were shown to be responsible for the hypertrophic phenotype in cattle (Charlier et al., 1995) and in humans (Schuelke et al., 2004). Furthermore, a similar phenotype was observed in knock-out mice (McPherron et al., 1997). Surprisingly this increase of mass, observed in myostatin null mice, was not accompanied by an increase of force (Amthor et al., 2007). Similarly, the treatment of adult mice with anti-myostatin antibody led to an increase of muscle mass of approximately 12% (Whittemore et al., 2003a). This hypertrophic phenotype was the result not only of hypertrophy but also hyperplasia, increase in the number of the muscle fibers, suggesting an activation of muscle satellite cells. In fact myostatin influence the expression of myogenic transcription factors such as Pax7, MyoD and myogenin, inhibiting satellite cell activation and differentiation (McFarlane et al., 2006; McFarlane et al., 2008). Given the capacity to activate satellite cells and to improved muscle regeneration blockage of myostatin soon became an attractive therapeutic target. In fact several studies used different

strategies to inhibit myostatin in order to revert the phenotype of muscle associated pathologies. The identification of a Myostatin pro-peptide that has a inhibitory role on the biological activity of myostatin was the starting point for this therapeutic approach (Bogdanovich et al., 2005; Thies et al., 2001). Soon after, another approach was used. The administration of intra-peritoneal injections of blocking antibodies for three months in Mdx-/- mouse resulted in an increase in body weight, muscle mass, muscle size and absolute muscle strength (Bogdanovich et al., 2002; Whittemore et al., 2003b). This approach was also used in amyotrophic lateral sclerosis (SLA) (Holzbaur et al., 2006). Later on, the use of DNA vaccines (Tang et al., 2007) and retrovirus-based RNAi system were also used efficiently in order to down-regulate the myostatin expression levels (Yang et al., 2008).

Regardless of the cause, physiological or genetic, skeletal muscle hypertrophy is always associated with an increase in protein synthesis. But, what happens when protein synthesis is overtaken by protein degradation?

### 1.2.2- Skeletal Muscle Atrophy

Skeletal muscle atrophy is a condition associated to loss of muscle mass without any genetic cause, in contrast to dystrophy. It is characterized by an overall decrease in protein content, fiber diameter, force production, and fatigue resistance. It is a characteristic response to starvation, aging and disuse conditions (such as immobilization, denervation or unloading) but it also occurs as a complication in several chronic diseases such as cancer, diabetes, sepsis, AIDS, renal and heart failure and others. In all this situations protein synthesis is overtaken by protein degradation.

Sarcopenia, the loss of muscle mass in the elderly, is predicted to occur in 5-10% of over-60-year old population. Apart from the loss of lean mass, it is characterized by the loss of the neuromuscular junctions, and a reduction of type II fibers that ultimately leads to an increase of frailty. On the other hand, cachexia is a wasting syndrome occurring as a complication associated to chronic diseases or it’s treatments. It is estimated to occur in 55% of all cancer patients. It is a multifactorial syndrome accompanied by loss of fat and muscle mass. In cachexia the chronic lesions produce cytokines, such as TNF-α (tumor necrosis factor-alpha), TWEAK (TNF-like weak promoter of apoptosis), IL-6, and others, that will affect systemically the whole body. Cachexia may also result from the direct effect of the treatments used in these conditions. Most importantly, cachexia is often associated with a poor prognostic.

Severe muscle wasting leads to death. This was clearly seen during the II World War by Jewish physicians and also before the advent of antiretroviral therapy for AIDS patients. But also mild atrophy has consequences as it was shown to suppress the immune system and to increase cancer mortality by decreasing the response and tolerance to therapy (Glass and Roubenoff, 2010). Different types of atrophy have distinct causes, and the molecular mechanisms implicated are also distinct and only recently started to be clarified.

Two independent pioneering studies (Bodine et al., 2001a; Gomes et al., 2001) toke advantage of gene expression profiling to identify important players in muscle wasting. These authors compared, by gene expression analysis, muscles from normal animals with muscles from different atrophic models: denervation, cachexia, diabetes and renal failure. They have identified a group of genes that were up- or down-regulated in all atrophic models studied, suggesting a common transcriptional program implicated in muscle atrophy. The authors have called this genes atrogenes or atrophy-related genes and among these, we could find proteins implicated in protein degradation, ATP production, extracellular matrix and proteins involved in transcription and translation control.

A close analysis of the atrogenes, revealed the presence of two groups of genes belonging to the two major degradative pathways of the muscle: the ubiquitin/proteasome and the autophagy/lysosome pathway.

#### *1.2.2.1- The Ubiquitin/Proteasome system*

The ubiquitin-proteasome is one of the major mechanisms involved in protein breakdown during muscle atrophy. It is a highly selective process that requires a tight regulation. Although the main function is to control protein turnover and to eliminate abnormal proteins, it also plays an important role in signal transduction, class I antigen presentation, cell cycle regulation, and transcription control (Glickman and Ciechanover, 2002). Not surprisingly, deregulation of this pathway has been implicated in cancer and in several inherited diseases.

### 1.2.2.1.1- The Proteasome

The proteasome is a large multicatalytic protease that mediates protein degradation. It is composed of two multimeric complexes: the 20S core particle and the 19S regulatory particle (Fig 3). The 20S core particle is a barrel-shaped structure arranged as a stack of four rings, two α and two β, each of them composed of 7 subunits. The general structure is thus αββα, being that the catalytic sites are located in the β rings. In fact, among the 7 residues that compose the β-rings three have catalytic properties, β1, β2, and β5, which mediate caspase-like, tryptic-like or chimotryptic-like activity, respectively. On each extremity of the 20S proteasome is attached a regulatory 19S particle. It is composed of at least 19 units, being that 9 form the lid while 10 constitute the base. At the base, and in close contact with the α-ring of the 20S proteasome we can find 6 ATPases, responsible for generating energy for the degradative process. While some of these ATPAses are responsible for the opening of an orifice in the α-ring of the 20S proteasome to allow substrate entry, others mediate the recognition of the substrate-linked poly-ubiquitin chain. The other non-ATPase subunits of the base are also reported to bind to the poly-ubiquitin chains and even to E3-ligases. The function of the lid subunits is less clear. It was shown that it is required for the unfolding of ubiquitin-tagged proteins (Glickman et al., 1998) and also for the de-ubiquitination of the proteins, an essential process for recycling ubiquitin (reviewed in (Glickman and Ciechanover, 2002; Navon and Ciechanover, 2009; Pickart and Cohen, 2004).

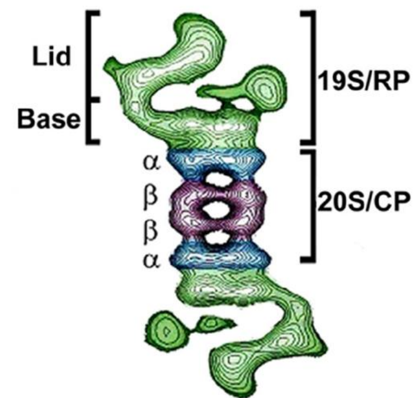



**Fig 3: Proteasome Structure.** Composed of two multimeric particles: the 20S core particle and the 19S regulatory article

Two other alternative proteasome regulatory complexes were identified, the11S (also called PA28) and the PA200. These two complexes are highly conserved and they can be found from worms to humans. While the 11S is a homo- or heteromeric complex composed of 7 subunits, the PA200 is a single chain protein of 200 KDa. Structural studies revealed that a central channel was formed in the 20S proteasome upon binding to the 11S regulatory complex, suggesting a diffusion model in which the proteins pass from the open regulatory unit into the 20S core proteasome. In contrast to what happens with the 19S regulatory unit, it was shown that the association of the 11S particle to the 20S core proteasome is ATP-independent and is responsible for the degradation of peptides and non ubiquitinated proteins. Although the biochemical properties of these complexes are now clear, little is known about their biological functions. It is however known that 11S regulatory complex is mainly expressed in the immune system and it is up-regulated after IFN-γ

treatment (Stratford et al., 2006). On the other hand, PA200 was shown to be located within the nucleus and it was proposed to be involved in DNA repair. (Reviewed in Rechsteiner and Hill, 2005). In skeletal muscle there is little information on these alternative regulatory units of the proteasome. However it is known that chronic contractile activity in skeletal muscle induces an impressive increase in the protein levels of the PA28 and PA200 complex (Ordway et al., 2000) and that several types of skeletal muscle wasting induces an increase expression of the PA200 complex (Lecker et al., 2004), suggesting that in fact they do play a role in skeletal muscle and that further studies are required to clarify their function.

#### 1.2.2.1.2- Ubiquitin and the ubiquitin conjugating cascade

Ubiquitin is a highly conserved 76-amino-acid polypeptide that is ubiquitously expressed in all eukaryote cells. No clear function has been attributed to free ubiquitin. It is found linked to substrate proteins through an isopeptide bond that is established between the carboxyl group of the final amino acid of ubiquitin and the ε-amino group found in one or more lysine residues of the cellular protein. Ubiquitin itself contains seven lysines, and each of these can be further conjugated by the carboxyl terminus of another ubiquitin to form a polyubiquitin chain. The way ubiquitin became linked to the proteins will determine their fate. Proteins can remain monoubiquitinated or additional ubiquitin moieties can be attached forming poly-ubiquitin chains. While monoubiquitination of proteins are thought to have a role in their localization and in their degradation via lysosome, the role of polyubiquitination depends on the lysine involved in the chain formation. Lysine 48 is the most studied and important in muscle wasting since it is responsible for directing the targeted proteins for degradation through the multimeric proteasome.

Protein ubiquitination is a multistep process in which ubiquitin has to be initially activated, in an ATP-dependent way (Fig 4). In this process an E1-activating enzyme forms a thiol-ester bond with the carboxy-terminal glycine of the ubiquitin. Activated ubiquitin is then transferred to the E2-conjugating enzyme. This E2-ubiquitin complex binds to E3 ubiquitin ligases which are specifically coupled with the substrate protein. These E3 ubiquitin ligases are responsible for transferring the ubiquitin to the target protein (Reviewed in (Weissman, 2001). In eukaryotic cells there is only one E1-activating enzyme that provides activated ubiquitin to a family of several E2-conjugating enzymes. Typically each of these E2 interacts with several E3-ligases. But it is in the large number of E3 ligases that reside the specificity of this reaction. In fact there are several types of ubiquitin ligases: those that bind directly to the substrate and those that require the formation of a protein

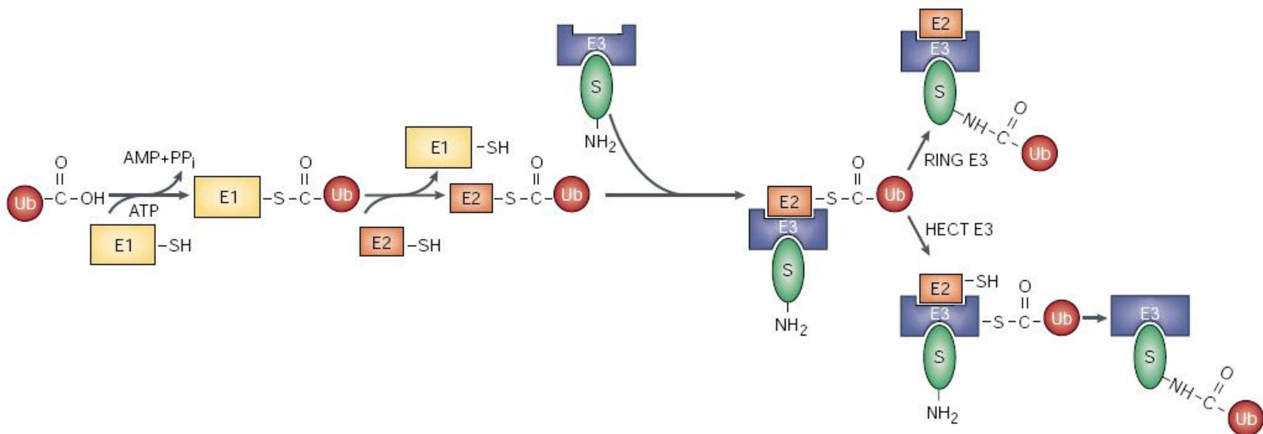



**Fig 4: Ubiquitination Machinery.** Free ubiquitin (Ub) is activated by a E1-activating enzyme and transferred to an E2-conjugating enzyme. These E2 associate with an E3-ligase that, directly or indirectly, will transfer the ubiquitin to the target substrate (Weissman, 2001).

complex, those who function as scaffold to bring E2 and the substrate together and those that receive the activated ubiquitin from the E2 and then transfer it to the substrate. Although they are a heterogeneous group of proteins they can be classified in two major groups: HECT-domain and Ring Finger-domain. The HECT-domain contains a COOH-terminal domain to which the activated ubiquitin is transferred from the E2, and a variable NH2-domain that specifically recognize and binds the substrate. The Ring Finger-domain family of E3 can be further divided in two distinct groups: those monomers or homomeric, in which the same protein contains the Ring Finger and the substrate recognition site, and those multisubunit proteins in which the Ring Finger is only responsible for the binding to the E2 and for the recruitment of the other proteins to the complex. (Reviewed in Glickman and Ciechanover, 2002).

Another group of proteins important in the regulation of this system are the deubiquitinating (DUB) enzymes. There are almost 70 different DUB enzymes in the human genome with several functions being documented (Reviewed in Wing, 2003). First they are essential as processors of the ubiquitin gene products. In the genome, ubiquitin is encoded as fusion proteins, with either additional copies of ubiquitin arranged in tandem or as a fusion with either the L40 or S27a ribosomal subunits. DUBs are therefore responsible for processing the ubiquitin gene products into mature ubiquitin. Secondly, DUBs have an important role as editing enzymes in situations in which protein ubiquitination has to be reverted. This can happen when ubiquitinated proteins are to be rescued from proteasomal degradation or in cases in which the signaling function of ubiquitin has been accomplished. Finally, DUBs play a crucial role in recycling the ubiquitin from the ubiquitinated proteins that are degraded in the proteasome. This process of ubiquitin recycling is helped by other proteins that, although not being DUBs, possess deubiquitinating activity under

certain situations. This is the case of RPN11 from the lid of the 19S proteasome that only when integrated in the proteasome has this capacity.

During the last years, the ubiquitin/proteasome pathway has been implicated in several human pathologies from neurodegenerative disorders to cancer. Therefore a better understanding of each of these different components of the pathway, in each particular system, will help the finding of active molecules that can be used as therapeutic approach.

#### 1.2.2.1.3- The Ubiquitin-Proteasome System in skeletal muscle

In skeletal muscle, the accumulation of ubiquitinated proteins in an ATP-dependent way was initially shown by (Wing et al., 1995), during denervation and starvation and it was latter confirmed in other muscle wasting-associated conditions: cancer cachexia (Baracos et al., 1995), and sepsis (Tiao et al., 1996; Tiao et al., 1997). Additional studies have demonstrated increased rates of ubiquitination in extracts prepared from atrophying muscles (Combaret et al., 2004; Kee et al., 2003; Solomon et al., 1998).

Gene expression analysis of atrophic human samples revealed an increased transcription of ubiquitin and proteasome related genes. In fact, sepsis, was sufficient to induce an increase in the mRNA levels of Ubiquitin and HC3, a component of the 20S proteasome, in the human rectus abdominis muscle (Tiao et al., 1997) Also head trauma patients, 8 days after the incident, show a clear activation of the ubiquitin/proteasome system, as shown by the increased levels of ubiquitin, HC2, a component of the 20S proteasome and of an E2 conjugating enzyme (Mansoor et al., 1996).

But, it was clearly from studies in murine models of atrophy that the major advances in understanding these conditions were achieved. A comparative study analysing different types of skeletal muscle atrophy identified several proteasome and ubiquitin related genes that were part of a common program of transcriptional adaptation to muscle atrophy (Lecker et al., 2004). Not surprisingly, the two most induced genes, in all the atrophic conditions, were two muscle specific E3-ligases, MAFbx/atrogin-1and Murf-1. These two genes had already been shown to be up-regulated during three atrophic conditions – denervation, immobilization and unweighting (Bodine et al., 2001a). The authors of this study went further, generating the respective knock-out mouse models of these two genes in order to understand their biological role. Under basal conditions both of these animals were normal when compared to WT. However, when submitted to atrophic stimuli, such as denervation, MAFbx and Murf-1 knock out animals were spared from atrophy, by 50 and 30 % respectively. But, what is it known about these two genes?

MAFbx, also known as Atrogin-1, is a E3-ligase that contains a F-box domain. This means that MAFbx/Atrogin-1 belongs to the family of E3-ligases that function as a SCF complex (for Skp1, Cullin, F-box) (Gomes et al., 2001; Jackson and Eldridge, 2002). In these complexes the F-box protein interacts with the substrates, while Cul1-Roc1 components associate with the E2 Ub-conjugating enzymes. Skp1 is an adaptor protein that brings together the F-box protein and the Cul1-Roc1-E2 complex. Murf-1, on the other hand, contains a canonical N-terminal RING domain characteristic of RING-containing E3 ligases (Borden and Freemont, 1996) which are required for ubiquitin-ligase activity (Kamura et al., 1999).

The role these two proteins play in muscle atrophy is unquestionable. However, and despite all the effort, little is known about their true targets. The first approach took advantage of the yeast-2-hybrid assay in order to identify the binding partners of MAFbx/Atrogin-1(Li et al., 2004). One of the proteins that came out from this study was α-Actinin-2. Co-immunoprecipitation experiments confirmed that these two proteins in fact interact. However, the protein levels of α-Actinin-2 did not change upon MAFbx/Atrogin-1 over-expression suggesting that it is not a target of ubiquitination. Since α-Actinin-2, like MAFbx/Atrogin-1, co-localize in the Z-disk of cardiomyocytes, the authors looked for proteins that also localize in the Z-disk and could interact with α-Actinin-2. They found out that Calcineurin-A, which play an important role in coordinating myocyte gene expression program and that determines cell size, could also interact with MAFbx/Atrogin-1 and that in fact was ubiquitinated and degraded in a MAFbx/Atrogin-1 dependent way. Three other proteins were shown to be direct targets of mAFbx/Atrogin-1 dependent ubiquitination. One of these proteins was MyoD, a key myogenic transcription factor involved in muscle differentiation and muscle repair. In fact, over-expression of MAFbx/Atrogin-1 results in polyubiquitination of MyoD and in inhibition of MyoD-induced myotube differentiation (Lagirand-Cantaloube et al., 2009). Also myogenin, another myogenic transcription factor, was shown to be polyubiquitinated by MAFbx/Atrogin-1 and degraded during dexamethasone-induced myotube atrophy (Jogo et al., 2009).The last of the known ubiquitination target of MAFbx/Atrogin-1 is eIF3-f (elongation initiation factor3 subunit 5). eIF3-f has an important role in translation initiation (Hinnebusch, 2006). The over-expression of this initiation factor was shown to induce hypertrophy, both in myotubes and *in vivo*, and to prevent starvation-induced atrophy in myotubes. Furthermore, repression of eIF3-f in myotubes induces atrophy. Using yeast two-hybrib assay MAFbx/Atrogin-1 was shown to directly interact with eIF3-f leading to its ubiquitination and degradation (Lagirand-Cantaloube et al., 2008). Considering these evidences, the first steps to understand how MAFbx/Atrogin-1 actually induces atrophy were done.

Also Murf-1 has some known targets, and the majority of them are structural proteins. One of the first to be identified was titin, a giant protein responsible for the elasticity of the muscle.

Although being a structural protein, it also contains several domains that anticipate an important role in signal transduction. According to Centner et al (Centner et al., 2001), Murf-1 interacts with the repeats A168/A169 adjacent to the titin kinase domain and therefore might modulate the kinase activity of titin. Also myosin heavy chain (MyH) isoforms are targeted by Murf-1 and -3. In fact, the generation of a double knock-out mouse model of Murf-1 and Murf-3 lead to a myopathy with MyH accumulation (Fielitz et al., 2007). These accumulating MyH were smaller than normal MyH suggesting a process of partial degradation. The authors further show, *in vitro* and *in vivo*, that Murf-1 and Murf-3 directly interact and ubiquitinate different MyH isoforms. Additionally, MuRF1 degrades myosin-binding protein C and myosin light chain-1 (MLC1) and MLC2 during denervation and fasting conditions (Cohen et al., 2009). In order to obtain these *in vivo* results, the authors have generated a knock-in mouse model in which the Ring-finger of Murf-1 had been deleted. Curiously, these animals were spared from denervation-induced atrophy suggesting that deletion of this portion of the protein is similar to the loss of the entire proteins. Altogether these results suggest that Murf-1 interacts and ubiquitinate preferentially structural proteins. Contesting this idea, Hirner S et al, (Hirner et al., 2008), show that MuRF1 is predicted to interact with several proteins associated with glucose production and glycogen metabolism. Yeast two-hybrid assay has demonstrated the interaction of MuRF-1 with PDH, PDK2, PDK4, PKM2 (all participating in glycolysis) and with phosphorylase β (PYGM) and glycogenin (both regulating glycogen metabolism). In the light of these data, it is plausible that these two proteins, MAFbx/Atrogin-1 and Murf-1, up-regulated during several atrophic conditions, can regulate distinct aspects of the atrophic process.

Considering the relevance of the ubiquitin/proteasome system in skeletal muscle atrophy, it is appealing to think that its inhibition can protect from loss of muscle mass. In this line of thought, several proteasome inhibitors were tested in order to see whether they were effective attenuating muscle atrophy. Early studies in which atrophic muscle were collected and incubated *ex vivo* with N -acetylleucyl-leucyl-norleucinal (LLN), or with CBZ-leucyl-leucyl-leucinal (MG132) showed a 50% reduction in the overall rate of proteolysis (Tawa, Jr. et al., 1997). This study anticipates the potential role of proteasome inhibitors in attenuate the consequences of muscle wasting. Combaret et al (Combaret et al., 1999) used Pentoxifylline (PTX), which is a xanthine derivative widely used in humans, in order to prevent muscle wasting induced in Yoshida sarcoma-bearing rats. Apparently, daily administration of PTX blocked the ubiquitin pathway by suppressing the enhanced expression of ubiquitin, the 14-kDa ubiquitin conjugating enzyme E2, and the C2 20S proteasome subunit in muscle from cancer rats. Also Torbafylline (HWA 448) was shown to spare rat skeletal muscles from wasting induced by cancer or sepsis (Combaret et al., 2002). Another

proteasome inhibitor, Velcade, also known as PS-341 and bortezomib, when administrated *in vivo*, lead to a 50% reduction in atrophy associated with denervation (Beehler et al., 2006). Also the administration of MG132 (1 mg/kg/48 h) *in vivo*, during hindlimb unloading (HU), partially prevents muscle atrophy.

Although effective, the inhibition of the proteasome only partially prevents muscle atrophy, suggesting that another mechanism might be involved in loss of muscle mass during catabolic conditions.

### *1.2.2.2- The Autophagy/Lysosome Pathway*

Another mechanism implicated in skeletal muscle atrophy is the Autophagy/Lysossome pathway. It is mainly implicated in the lysosomal degradation of long-lived proteins and damaged or supernumerary organelles. Degradation by this mechanism occurs normally at basal levels for the routine turnover of cytoplasmatic components, and it plays an important role during development, differentiation and tissue remodeling. On the other hand, alterations in the autophagic flux are also implicated in several diseases, from neurodegenerative to cancer, passing by neuromuscular.

Autophagy is a ubiquitous and well conserved process from yeast to mammals. Due to its characteristics can be divided into three different types (Fig 5): macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). Macroautophagy implicates the formation of a C-shaped double-membrane vacuole (phagophore) that wraps cytoplasm and organelles to be

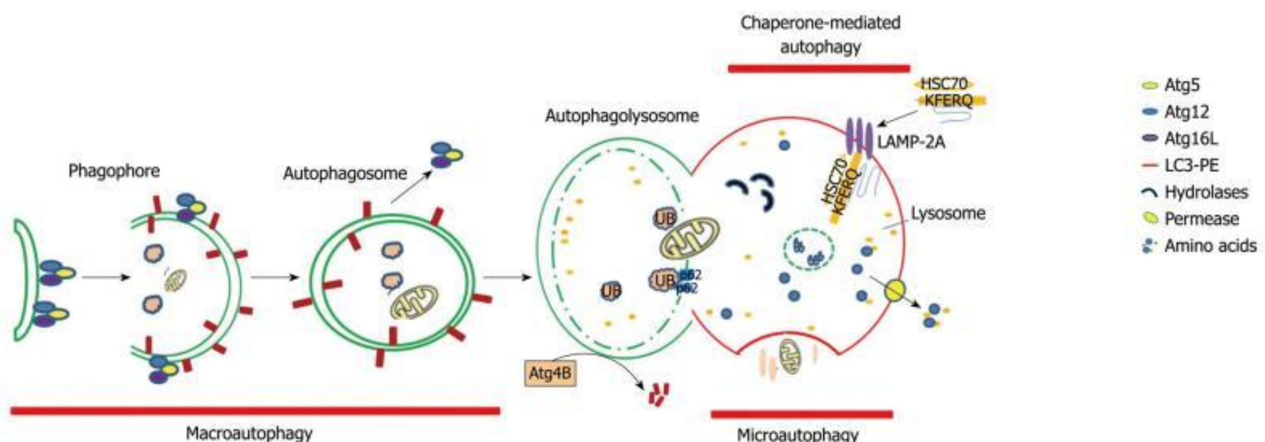



**Fig 5: Types of autophagy: Macroautophagy, Microautophagy and Chaperone-Mediated Autophagy**. During macroautophagy occurs the formation of a double-membrane vesicle called autophagosome that engulfs the cytoplasmatic components to be degraded. The autophagosome then fuses with the lysosome where the degradation occurs, forming in this way the autophagolysosome. During microautophagy, on the other hand, the components to be degraded are engulfed directly by an invagination of the lysosome membrane. Chaperone-mediated autophagy is a selective process in which the proteins to be degraded a peptide motif that is recognized by the chaperones. These proteins are then transported by LAMP-2A to the interior of the lysosome (Ding, 2010).

degraded. This vacuole then fuses with the lysosomal membrane and the autophagic body enters the lysosome were the components are degraded and recycled (reviewed in (Yang et al., 2005). On the other hand, during microautophagy the cytoplasmatic material is incorporated in the lysosome through a direct invagination of the lysosomal membrane forming an internal vacuolar vesicle where the degradation occurs. CMA is different from these two types of autophagy because it does not imply the formation of vesicular traffic and also because is a much more selective autophagic process. In fact, only cytosolic proteins with particular peptide motifs are recognized by the complex system of molecular chaperones and co-chaperones. These proteins are then unfolded and translocate across the lysosomal membrane helped by a group of lysosomal proteins. The most important of which is LAMP-2A that is required for binding the targeted proteins to the lysosomal membrane and also for its translocation into the lumen of the lysosome. In this introduction we will focus mainly in macroautophagy that will be called autophagy for the sake of simplicity.

Apart from the CMA, autophagy was initially thought to be mainly a non selective process in which bulk cytoplasm was randomly sequestered into the cytosolic autophagosomes. However, there are evidences that autophagy can selectively eliminate damaged organelles such as mitochondrias, endoplasmatic reticulum and peroxissomes (Eskelinen, 2008). Furthermore, recent studies have shown that LC3, one of the key molecules in the formation of the autophagosome, may act as a membrane receptor and interact with p62 or NBR1 (multi-functional adaptor proteins that bind to poly-ubiquitinated proteins) to promote the selective elimination of poly-ubiquitinated proteins/aggregates (Glick et al., 2010).

Autophagy is a process normally induced during nutrient-poor condition and it is responsible for the degradation of superfluous material, in order to provide amino-acids for adaptive protein synthesis. The molecular steps of this process are well studied in yeast and are conserved in mammals, and will be discussed below.

#### 1.2.2.2.1- Autophagic machinery

One of the open questions related to autophagy is the origin of the membranes that forms the autophagosome. What is known is that a double-layer membrane starts surrounding the cytoplasmatic cargo until it completely engulfs it, forming in this way the autophagosome. These autophagosomes then fuse with the lysosome allowing the degradation of its content by the lysosomal proteases. Nutrients are then recycled back to the cytosol through lysosomal permeases.

The autophagosome starts to form at the phagophore assembly site/ pre-autophagosome structure (PAS) and it is orchestrated by a tightly regulated machinery comprising two highly conserved ubiquitin-like conjugation systems: the Atg12-Atg5 and the Atg8-phosphatidylethanolamine (PE) system (Fig 6). Both systems are inter-connected since if one is defective the other cannot target proteins to the PAS.

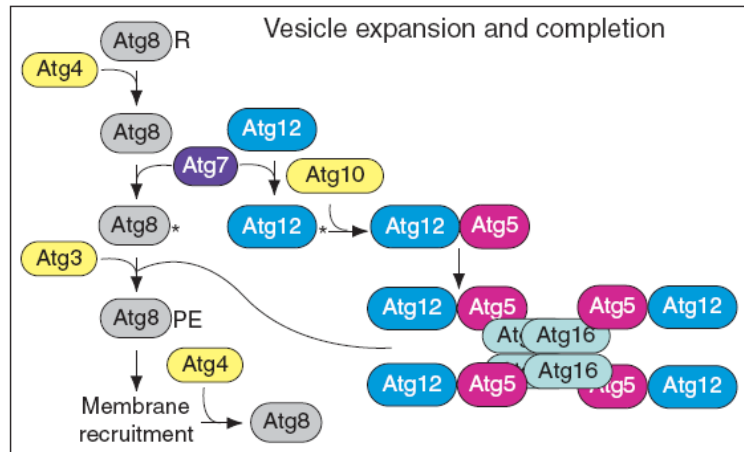



**Fig 6: Two ubiquitin-like conjugation systems are involved in autophagosome formation**. Atg8 and Atg12 are ubiquitin-like proteins that are activated by the E1-like enzyme Atg7. Atg8 and Atg12 are then transferred to the E2-like enzymes Atg3 and Atg10, respectively. While Atg8 becomes conjugated to phosphatidylethanolamine (PE), Atg12 binds to Atg5. Atg8-PE is integrated in the forming autophagosome. The complex Atg12-Atg5 binds Atg16 and this allows the multimerization of the complex (Klionsky, 2005).

In the Atg12-Atg5 conjugation system, Atg12 behaves like ubiquitin. It is initially activated, in an ATP-dependent way, by Atg7 that functions as an E1-activating enzyme. It does so by creating a thioester bond between the C-terminal glycine of Atg12 and a cysteine residue in Atg7. Atg12 is then transferred to another cysteine residue in Atg10, forming a new thioester bond and releasing Atg7. Atg10 functions as an E2-conjugation enzyme and potentiates the covalent linkage of Atg12 to Atg5. Conjugated Atg12-Atg5 complexes binds to Atg16 and this multimeric complex is required for the elongation of the expanding pre-autophagosomal membrane. The formation of this complex is thought to induce the curvature of the autophagosomal membrane through asymmetric recruitment of Atg8.

Atg8 is also an ubiquitin-like molecule, and it is present in early isolation membranes, autophagosome and autophagic bodies. It is initially proteolytically cleaved by Atg4, exposing a glycine residue that will allow it's binding to Atg7. This activated Atg8 is then transferred to Atg3, an E2-like enzyme that will mediate the conjugation of Atg8 with phosphatidylethanolamine (PE), an abundant membrane phospholipid. This Atg8 lipidation can be reverted by Atg4, which cleaves lipid-protein linkage, and this allows cycles of conjugation/deconjugation important for the normal progression of autophagy. There are at least 4 mammalian Atg8 homologs, MAP1LC3 (LC3), GATE16 (Golgi Associated ATPase Enhancer 16KDa), Gabarap (Gabba Receptor-Associated Proteins) and Atg8L. Among them the most abundant in autophagosome membrane is LC3. It can be detected in two different forms: LC3-I and LC3-II. While the first correspond to the free cytosolic form, the second correspond to the membrane bound, lipidated form. The proportion

between LC3-I and LC3-II reflects the abundance of autophagosome and is one of the best methods to assay autophagy levels and flux.

The autophagosome formation is regulated by several mechanisms. The complex Atg1/Atg13/Atg17 is required for the initial formation of the phagophore, possibly, by recruiting Atg9, a transmembrane protein that is thought to be involved in the recruitment of lipids to the growing membrane. The phosphorylation status of Atg13, which is regulated by TOR (Target of rapamycin), influences the binding to Atg1 and the formation of the above mentioned complex. In this way, growth factor and the nutrient status of the cell, through TOR, are capable of controlling autophagy. Another protein implicated in autophagosome elongation is the class-III PI3 kinase Vps34. This protein uses phosphatidylinositol (PI) as substrate to generate phosphatidyl inositol triphosphate (PI3P) that is essential to the process of phagophore elongation. Furthermore, the interaction of Vps34 with Atg6 (Beclin-1 in mammals) increases significantly its catalytic activity to generate PI3P (Funderburk et al., 2010; Furuya et al., 2005). In mammals, other regulatory proteins that complex with Beclin-1/Vps34 have been identified, such as the stimulatory Ambra-1, Atg14L and UVRAG (ultraviolet irradiation resistance associated gene) (an homologue of Vps38, that is part of yeast Vps34 complex II) or the inhibitory Rubicon (RUN domain and cystein-rich domain containing, BECN1-interacting) and Bcl-2 (Itakura et al., 2008; Liang et al., 2006; Maiuri et al., 2007; Matsunaga et al., 2009; Pattingre et al., 2005; Sun et al., 2008a; Zhong et al., 2009). Ultimately, mature autophagosome fuses with the lysosomal compartment forming the autophagolysosome. This maturation step is essential for the autophagic flux and any blockage will result in the accumulation of autophagosomes. There are several proteins that seem to be involved in the regulation of this essential process. Rubicon and UVRAG alternatively interact with the complex Beclin-1/Vps34 and while the first down-regulate the endocytic trafficking and the maturation of the autophagosome the latter has the opposite effect.

#### 1.2.2.2.2- Autophagy Regulation

The autophagic response occurs as a consequence of diverse external and/or internal stimuli. Therefore the signaling pathways implicated are also diverse. One of the most important conditions associated with increase autophagy is nutrient depletion. In mammals, the main signaling pathway implicated in this condition is the mTOR pathway.

mTOR, the mammalian Target of Rapamycin, is a serine/threonine kinase capable of sensing growth factors, nutrient signals and energetic status of the cell. Growth factor regulates

mTOR through insulin/insulin-like growth factor 1 (IGF1) - PtdIns3K - PKB (protein kinase B)/ AKT pathway. In the presence of extracellular growth factors, this signalling pathway activates mTOR and consequently inhibits autophagy (Inoki et al., 2002; Manning et al., 2002). Whenever growth factors are removed from the medium, even in the presence of nutrients the cells activate autophagy (Lum et al., 2005) in order to maintain energy homeostasy. On the other hand, amino acid starvation, or nutrient deprivation, is known to inhibit mTOR and in this way induce autophagy. Rapamycin, an inhibitor of mTOR, is able to induce autophagy even in the presence of nutrients suggesting that mTOR has a negative regulatory role in autophagy (Noda and Ohsumi, 1998). Finally, mTOR senses the energetic status of the cell through AMPK. When the ATP/AMP ratio decreases, for instances under glucose deprivation or if mitochondria are dysfunctional, AMPK is activated, inhibiting mTOR and thus inducing autophagy (Corradetti et al., 2004; Shaw et al., 2004), that will increase the ATP production via recycling of nutrients.

mTOR can inhibit autophagy by two different mechanisms: by controlling transcription and translation acting on 4E-BP1 and p70S6 kinase, and by directly or indirectly interact with Atg proteins, such as Atg13, in the autophagosome formation, as it was already described before.

Although much is already known about the regulation of autophagy, the cross-talk between different pathways requires further studies.

### 1.2.2.2.2- Autophagy in skeletal muscle

Early biochemical studies, by zonal centrifugation, in skeletal muscle of starved rats revealed the existence of two populations of lysosome-like particles: one, less representative, originated from macrophages and connective tissue and another, much more representative, originated from muscle fibers. The authors further stated that although evidences had been provided for the existence of a lysosomal system they could not explain “why morphological studies have repeatedly failed to observe lysosomes in normal muscles cells” (Canonico and Bird, 1970). Two years later the first evidences for the presence of autophagosomes in skeletal muscle were reported. Electron microscopy

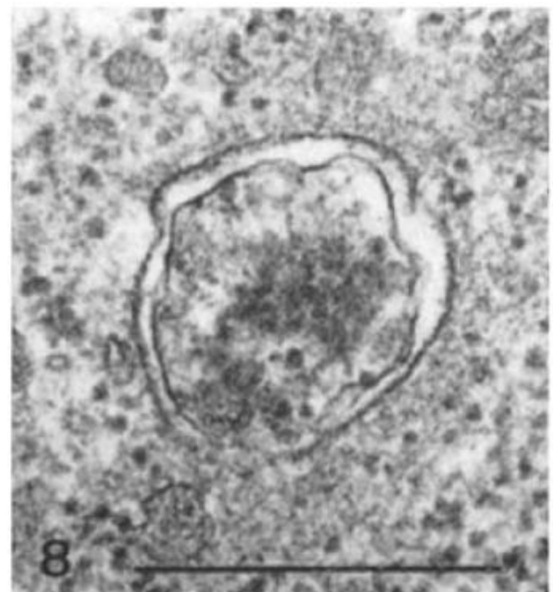



**Fig 7: Autophagic vacuoles engulfing glycogen.** Diaphragm of rats, 3h after birth. Scale mark 0,5 µm (Schiaffino and Hanzlikova, 1972a).

studies, in neonatal skeletal muscle, revealed the presence of double layer vacuoles filled with glycogen (Fig 7) suggesting a functional role of the lysosomal system in mobilizing glycogen (Schiaffino and Hanzlikova, 1972a). However, the role autophagy plays in skeletal muscle was difficult to prove and extremely controversial (Furuno et al., 1990; Schiaffino and Hanzlikova, 1972b) mainly due to technical limitations that relied in enzymatic assays and in the use of unspecific pharmacological inhibitors.

In the last years, several tools have been developed that allow a better study of the autophagic flux *in vivo* and in particular in skeletal muscle. Apart from the technical advances in the imaging techniques, the generation of a transgenic animals in which the LC3 gene (Atg8 in yeast), involved in the autophagosome formation, was fused with the GFP gene, allowed the *in vivo* visualization of the autophagosomes (Mizushima et al., 2004). Analyzing the effect of starvation-induced autophagy *in vivo*, it was observed that fast skeletal muscle has a higher induction of autophagosome formation than slow skeletal muscle. Furthermore, GFP positive dots (autophagosomes) in skeletal muscle are much smaller than those of other tissues such as liver, pancreas or even heart, providing a possible explanation for the previous difficulty in observing autophagy related structures in skeletal muscle. Using a similar approach, (Mammucari et al., 2007) showed that autophagosome formation is implicated in muscle atrophy induced by FoxO3, one of the most important transcription factor implicated in muscle atrophy. In fact co-electroporation of the tibialis anterior muscle with LC3-GFP and with constitutively active FoxO3 (c.a.Foxo3) increased the number of GFP positive foci, representing autophagosomes. Furthermore the authors provided evidences that FoxO3 is directly regulating the expression levels of LC3 by binding to its promoter region.

The role of autophagy in muscle wasting was further confirmed by a genetic model of Amyotrophic Lateral Sclerosis (ALS). In this transgenic model the expression of $SOD1^{G93A}$, the mutated form of the protein, is known to induce an increase in ROS levels that results in muscle atrophy with consequent muscle weakness. In this model, muscle atrophy was partially reduced when the autophagic flux was blocked by the over-expression of shRNAs against LC3, thus preventing autophagosome formation (Dobrowolny et al., 2008). Furthermore, the contribution of autophagy in the loss of muscle mass that occurs during ageing, also called sarcopenia, was recently demonstrated (Wenz et al., 2009; Wohlgemuth et al., 2010). These reports showed an increase in the expression levels of several components of the autophagic machinery during ageing, suggesting that in the elderly there is a higher requirement of this system to degrade and recycle damage components of the fibers. The authors went further on showing that PGC1a, a key regulator of

mitochondrial biogenesis, was sufficient to ameliorate loss of muscle mass and to decrease the age-induced autophagy.

These studies clearly demonstrated that autophagy is a process implicated in muscle wasting. Additionally, autophagosomes are characteristic of a class of muscle disorders called Autophagic Vacuolar Myopathies. Altogether, these evidences might suggest that autophagy is detrimental for muscle physiology. However it is also possible that autophagy is a required mechanism to increase cell survival and to eliminate damaged organelles. The fact that several adquired or genetic muscle disorders present abnormal mitochondrias and aggregated proteins might suggest that autophagic flux is impaired instead of exacerbated. Therefore, in order to address the basal role of autophagy in skeletal muscle, (Masiero et al., 2009; Masiero and Sandri, 2010), generated muscle specific knock-out mice for Atg7, rendering them unable to form the autophagosome. The authors observed that instead of preventing muscle loss, these mice had increased muscle atrophy and presented several features of myopathy, such as accumulation of protein aggregates, abnormal mitochondrias and concentric membranous structures. A similar phenotype was observed in muscle-specific Atg5 knock-out mice, another genetic model of blockage of the autophagy (Raben et al., 2008). Altogether these results suggest that autophagy is required at basal levels for normal muscle physiology and that its blockage is detrimental and leads to muscle degeneration.

In agreement with these results, (Grumati et al., 2010) showed that in skeletal muscles of collagen VI–knockout (Col6a1-/-) mice, an animal model of Bethlem myopathy and Ullrich congenital muscular dystrophy, autophagy flux is impaired. Furthermore, forced activation of autophagy by genetic, dietary and pharmacological approaches restored myofiber survival and ameliorated the dystrophic phenotype of Col6a1-/-mice. Therefore, autophagy seems to be an essential mechanism that is activated under stress conditions, such muscle atrophy or genetic disorders. Defective activation or blockage of autophagy is detrimental since it leads to the accumulation of aggregated proteins and damaged organelles.

Another important point to consider is the regulation of autophagy in skeletal muscle since it is particular when compared with other tissues such as liver or pancreas. While most tissues show a transient activation of autophagy, in skeletal muscle there is a persistent generation of autophagosomes (Mizushima et al., 2004) that requires transcriptional regulation. This suggests that in skeletal muscle there might be different pathways that govern short-term and long-term induced autophagy. In spite of the little amount of information regarding this topic, some inhibitors and activators of autophagy were already identified. The first of which was Runx1, a DNA-binding

protein, that it is highly up-regulated in denervation, a condition of induced autophagy although to a less extent than fasting. Wang X et al (Wang et al., 2005), generated a knock-out model of Runx1 and have demonstrated that it is essential to prevent disused myofibers from undergoing autophagy, myofibrillar disorganization, and severe muscle wasting. Although the mechanism of action is not clear a recent study in hepatic cells showed that Runx1 interacts with FoxO3 promoting the FoxO3 recruitment to the Bim promoter (Wildey and Howe, 2009). Another regulator of autophagy in skeletal muscle is Jumpy, a PI3P phosphatase associated with sporadic cases of centronuclear myopathy. It was shown that in C2C12 myoblasts, reduction of Jumpy by siRNA lead to an induction of autophagosome formation and increased rate of proteolysis (Vergne et al., 2009). The authors went further demonstrating that Jumpy co-localizes transiently with the autophagosome and proposed a model in which the balance between the PI3P production (regulated by VPS34) and the PI3P hydrolysis (regulated by Jumpy) determines the induction and baseline levels of autophagy. But the most potent autophagy inhibitor in skeletal muscle is the kinase AKT. Acute activation of Akt in adult mice or in muscle cell cultures completely inhibits autophagosome formation and lysosomal-dependent protein degradation during fasting (Mammucari et al., 2007; Mammucari et al., 2008; Zhao et al., 2007) However the role of mTOR, a downstream target of AKT, in opposition to other tissues, is much less important. In fact, biochemical studies have determined that rapamycin-mediated mTOR inhibition only barely (10%) increases protein breakdown in differentiated myotubes (in opposition to the 50% protein breakdown induced by AKT inhibition) (Zhao et al., 2007), and it is not sufficient to induce autophagosome formation *in vivo* (Mammucari et al., 2007; Sartori et al., 2009; Zhao et al., 2007). Moreover, deletion of S6K1, a downstream target of mTOR, and of S6K2 does not affect the autophagic flux in cultured myotubes (Mieulet et al., 2007). Therefore the starvation induced autophagy resulting from inactivation of the IGF1-Akt pathway does not seem to be dependent of mTOR in contrary to what happens in other tissues in which mTOR is the main inhibitor of autophagy.

As it was already described, skeletal muscle atrophy can result from several conditions, pathological or not. All these conditions lead to a decrease in protein synthesis and an increase in protein degradation. Protein degradation results from the activation of the two main degradative systems in the muscle: the ubiquitin/proteasome system and the autophagy/lysosome system. But which are the molecular pathways that activate these mechanisms?

### *1.2.2.3- The main orchestrators of skeletal muscle atrophy*

The molecular pathways involved in muscle atrophy only recently started to be unraveled. One of the most important pathway is the IGF-1/Akt/FoxO (Fig 8). Briefly, this pathway is activated by Insulin or Insulin-like Growth Factor (IGF-1) that binds to their receptors. This leads to the autophosphorilation of these receptors and generates the docking sites for Insulin Receptor Substrate (IRS) that becomes phosphorilated by the receptor. Phosphorilated IRS recruits and activates phosphatidylinositol-3-kinase (PI3K) which phosphorylates membrane phospholipids, generating phosphoinositide-3,4,5-triphosphate (PIP3). In turn, PIP3 acts as a docking site for two kinases, phosphoinositide-dependent kinase 1 (PDK1) and Akt that leads to their activation. Activated Akt inhibits protein degradation by phosphorylating the transcription factors of the FoxO family, and thus maintaining them in the cytoplasm. Simultaneously, activated Akt stimulates protein synthesis via the mammalian target of rapamycin (mTOR) and glycogen synthase kinase 3β (GSK3β). Whenever the IGF-1/Akt pathway becomes shutdown, such as in atrophic conditions, FoxO transcription factors are no longer phosphorylated by Akt and enter the nuclei where they can transcribe the atrogenes and activate an atrophic program (Sandri et al., 2004b). There are several evidences stating the relevance of this pathway in skeletal muscle atrophy. To start with, inhibition of PI3K or over-expression of a dominant-negative Akt reduces the size of C2C12 myotubes (Rommel et al., 2001). Furthermore, the activation of Akt during denervation partially prevents atrophy (Bodine et al., 2001a; Pallafacchina et al., 2002). And this is consistent with the fact that

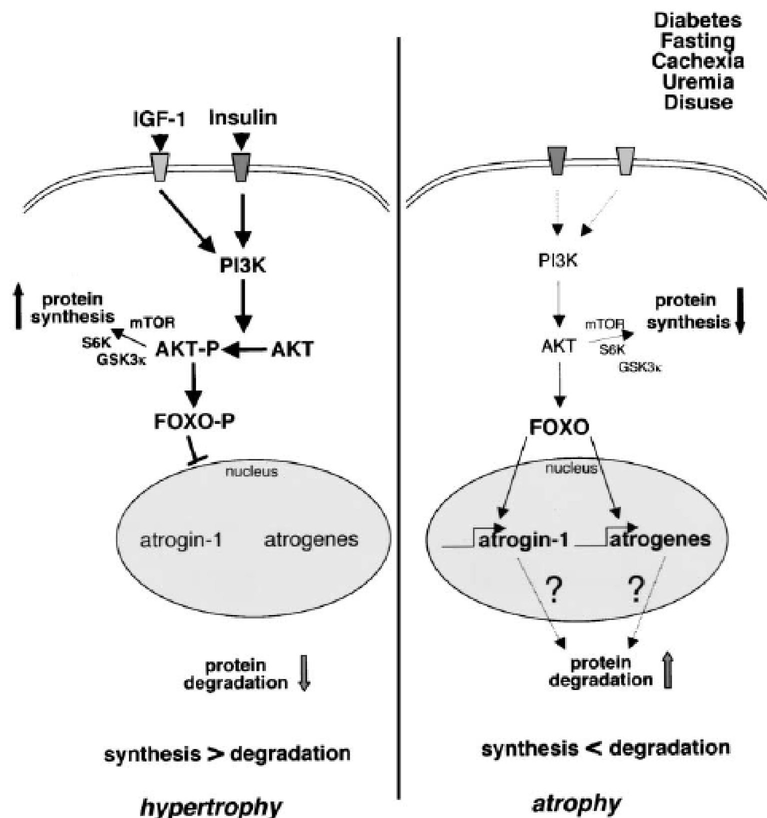



**Fig 8: IGF-1/Akt Pathway and FoxO in Muscle Atrophy and Hypertrophy** (Sandri et al., 2004a).

muscles from mice lacking Akt1 and Akt2 are smaller than their WT littermates (Peng et al, 2003). Additionally *in vitro* administration of insulin or IGF-1 was sufficient to blunt the atrophic response to dexamethasone, a glucocorticoid known to induce muscle atrophy (Sacheck et al 2004). Moreover, fasting and glucocorticoids induce the expression of FoxO factors both in the liver and in the muscle (Furuyaa et al, 2003; Imae et al 2003). Not surprisingly, FoxO1 was found significantly increased in several conditions of muscle atrophy (Lecker et al., 2004). Altogether these data indicate that during muscle atrophy this pathway is regulated both at the transcriptional and pos-translational level in order to orchestrate a complex atrophic program.

As it was shown, FoxO family of transcriptional factors are key elements in the atrophic process. They belong to the Forkhead family of proteins that are present in all eukaryotes, and they are characterized by the presence of a conserved DNA binding domain called Forkhead Box (Calnan and Brunet, 2008). In invertebrates, FoxO subfamily is composed of four members: FoxO1 (FKHR), FoxO3 (FKHRL1), FoxO4 (AFX) and FoxO6. While the first 3 are ubiquitously expressed, FoxO6 is expressed mainly in the brain (Jacobs et al., 2003). While playing different roles in different tissues, in the skeletal muscle FoxO1 and FoxO3 are mainly involved in the atrophic process (Brunet et al., 1999; Sacheck et al., 2004; Sandri et al., 2004a; Stitt et al., 2004). In fact, muscles from transgenic mice over-expressing FoxO1 are smaller than WT controls (Kamei et al., 2004) and knockdown of FoxO expression by RNAi is able to block the upregulation of atrogin1/MAFbx expression during atrophy (Liu et al., 2007; Sandri et al., 2004a), indicating that FoxO is sufficient to promote muscle loss.

Another important pathway during skeletal muscle atrophy is the NF-kB pathway. This transcription factor play major roles as mediators of inflammation and immunity, and in the muscle appear to mediate the effect of several cytokines, such as TNF-α, during muscle wasting and cachexia. In the inactive state NF-κB is sequestered in the cytoplasm by a family of inhibitory proteins called IκB. In response to TNFa, the IκB kinase (IKK) complex phosphorylates IκB resulting in its ubiquitination and proteasomal degradation. This leads to nuclear translocation of NF-κB and activation of NF-κB-mediated gene transcription. Muscle-specific over-expression of IKKß in transgenic mice leads to severe muscle wasting, that is mediated, at least in part, by the ubiquitin-ligase MuRF1 but not by atrogin-1/MAFbx (Cai et al., 2004). On the contrary, muscle-specific inhibition of NF-κB by transgenic expression of a constitutively active IκB mutant partially prevents denervation induced atrophy. Likewise, lack of p105/p50 NF-kB gene, in knock-out mice, abolished muscle atrophy induced by hindlimb unloading (Hunter and Kandarian, 2004). The exact mechanism by which NF-kB becomes activated is still unknown. However, it is known that NF-κB

is transiently activated after an acute bout of physical exercise, although it is not known whether this has any effect on activity-dependent gene regulation (Ho et al., 2005; Ji et al., 2004).

As it was already seen, myostatin is a member of the TGFß family, expressed and secreted predominantly by the skeletal muscle that functions as a negative regulator of muscle growth. It was also showed that lack of myostatin induces muscle growth. So it was hypothesized that it could also play a role in muscle atrophy. However the results are conflicting. The systemic over-expression of myostatin, through injection of Chinese hamster ovary cells engineered to express myostatin, was found to induce profound muscle and fat loss, analogous to that seen in human cachexia syndromes (Zimmers et al., 2002). However the generation of muscle specific myostatin transgenic animals showed that this atrophy was mild and only present in males (Reisz-Porszasz et al., 2003). The same mild atrophy was also observed when myostatin was electroporated into adult T.A. muscles (Durieux et al., 2007). In order to clarify this issue, Sartori et al (Sartori et al., 2009) used a similar approach. The TGF-β pathway was activated by electroporating muscles with c.a.ALK5 or c.a.ALK4, the two receptors of this pathway, leading to a reduction of 20% in the cross section area of the muscles. This atrophy was dependent on the activation of the SMADs 2 and 3, two transcription factors downstream of the TGF-β pathway. This indicates that activation of the myostatin pathway has a role in muscle atrophy and that its inhibition might be beneficial to prevent muscle loss.

Until now, it was shown that skeletal muscle atrophy is a process that requires the activation of two degradative processes: the ubiquitin/proteasome and the autophagy/lysosome system. The activation of these two systems is tightly regulated at the transcriptional level, but also at the post-translational level. But a new class of master regulators have emerged in the recent years that can regulate entire pathways. They operate at the post-transcriptional level and their role in skeletal muscle atrophy is barely unknown. They are called miRNAs.

## 1.3- miRNAs: from their origins to their functions in muscle atrophy

miRNAs are small non-coding RNAs with approximately 22 nucleotides that can regulate post-transcriptionally gene expression. They are highly conserved among species and they are present in animals, plants, and virus. Currently there are more than 1000 human miRNAs and almost 700 murine miRNAs. They are predicted to regulate the expression of approximately 60% of protein coding genes. Conventionally, miRNAs are known to regulate gene expression by binding to the 3'-Untranslated Regions (3'UTRs) of the mRNA, and in this way block translation or induce mRNA degradation. Each miRNA has the potential to target hundreds of different mRNAs, and on the other hand each mRNA can be targeted by different miRNAs creating in this way complex regulatory networks. One of the hallmarks of miRNAs is their specificity. In fact, several miRNAs are involved in developmental and physiological processes that require tissue- and stage-specific expression. The tight regulation of miRNAs expression is crucial and alterations are correlated with pathological conditions.

In 1969, Roy J. Britten and Eric H. Davidson, wrote a theory about the organization of the genome of higher organisms. They based their theory in some observations: "There exists a significant class of genomic sequences which are transcribed in the nuclei of higher cell types but appear to be absent from cytoplasmic RNA's", "This genome differs strikingly from the bacterial genome due to the presence of large fractions of repetitive nucleotide sequences which are scattered throughout the genome. Furthermore, these repetitive sequences are transcribed in differentiated cells according to cell type-specific patterns." Their theory can be summarized by the statement "Batteries of producer genes are regulated by activator RNA molecules synthesized on integrator genes." These were, perhaps, the foundations for the appearance of the miRNAs (Britten and Davidson, 1969).

But it was only in 1993 that Lee et al (Lee et al., 1993) have discovered in *C. elegans* the first miRNA, *lin*-4. It was seen that *lin*-4 disrupt the timing of post-embryonic development in *C. elegans* by repressing the expression of *lin-14*, which encodes a nuclear protein (Lee et al., 1993). The negative regulation of *lin-14* by *lin-4* requires partial complementarity between *lin-4* and the 3'UTR of *lin-14* mRNA (Ha et al., 1996; Olsen and Ambros, 1999).

Seven years later a second miRNA, *let-7*, was discovered, again in nematodes (Reinhart et al., 2000). *let-7* functions in a manner similar to *lin-4*, repressing the expression of the *lin-41* and *hbl-1* mRNAs by binding to their 3'UTRs (Lin et al., 2003; Reinhart et al., 2000; Vella et al., 2004).

The dawn of miRNAs was followed by large scale screenings that established miRNAs as a new and large class of ribo-regulators (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001).

### 1.3.1- Genomic distribution and transcription regulation

Human and murine miRNAs are distributed along the entire respective genomes except in the Y chromosome. miRNA Transcriptional Units (TUs) can be scattered individually along the chromosomes or they can be arranged in clusters. Two or more miRNA located in the same chromosome at close distance of each other (usually it is considered between 3Kb and 10 Kb) are defined as clusters. It is predicted that approximately 50% of the known miRNAs are clustered. These miRNAs clusters are often transcribed into polycistronic primary transcripts, and the miRNAs belonging to one cluster are often related to each other. However there are also clusters in which the members belong to different miRNAs families and this might be important for the regulation of a specific target gene or several target genes of the same pathway. (reviewed in (Davis and Hata, 2009; Kim and Nam, 2006; Olena and Patton, 2010; Zhang et al., 2009).

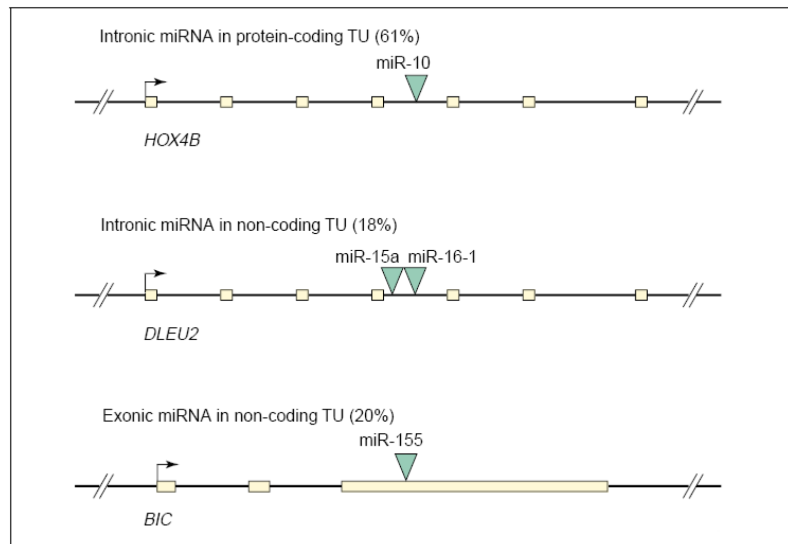



**Fig 9:Genomics organization and structure of miRNAs genes.** (Kim and Nam, 2006)

Initially miRNAs were thought to be mainly intergenic. However a closer analysis showed that almost 70 % of the known miRNAs were located in defined TUs. The vast majority of these miRNAs located in TUs are located in introns of protein coding genes, although they can also be located in introns of non-coding RNAs. Some miRNAs may also be located in exons of non-coding genes while only a few have a mixed localization depending on the splice variant transcribed in the tissue (Fig 9).

One question that we can pose regards the transcriptional regulation of these miRNAs. Initially, several reports indicated that miRNAs and the host genes had similar profiles suggesting that the regulatory mechanism were the same (Baskerville and Bartel, 2005; Combaret et al., 2004;

Lagos-Quintana et al., 2001; Lau et al., 2001; Rodriguez et al., 2004). However there are other recent studies in which the expression profiles of miRNA and the host gene are independent indicating that these miRNAs are under the regulation of their own promoter (Fujita et al., 2008). In fact it is now believed that 25-33% of intronic miRNAs are transcribed form independent promoters (Corcoran et al., 2009; Ozsolak et al., 2008).

The analysis of miRNAs regulatory regions revealed that they are, in everything, similar to the ones of protein coding genes. The relative frequency of CpG islands, TATA box, TFIIB recognition, initiator elements, and histone modifications clearly suggests that these promoters, like the ones from mRNA, are regulated by transcription factors, enhancers and silencing elements. This is essential for the time-, developmental- and tissue-specific expression pattern of miRNAs. (reviewed in (Davis and Hata, 2009; Krol et al., 2010). Repression of miRNAs to ensure their tissue-specificity has also been reported. In fact miRNA-124, a miRNA abundant in the brain, is negatively regulated by the REST factor, which is highly expressed in precursor neuronal cells and in non neuronal cells. During development the REST factor is silenced allowing the expression of miRNA-124 in the post-mitotic neuronal cells. Simultaneously, it is now thought that miRNAs are particularly involved in the regulation of transcription factors (Shalgi et al., 2007). This, creates double feedback loops in which the miRNA regulates the expression of a transcription factor which modulates the expression of the miRNA itself, contributing for the fine tuning of the miRNA expression levels.

### 1.3.2- Overview of miRNAs Biogenesis

miRNAs are transcribed into long transcripts mainly by RNA polymerase II, although there are also evidences implicating RNA polymerase III in the transcription of some miRNAs (Borchert et al., 2006). Most of them are polyadenylated in its 3'end and capped at its 5'extremity, like mRNAs. In the canonical pathway (Fig 10) these primary-miRNAs (pri-miRNAs) folds into a stem loop structure that will be further processed by two RNAse III endonuclease, Drosha and Dicer. In the nucleus, the hairpin structure is cleaved from the flanking regions originating the precursor-miRNA (pre-miRNA) that is ≈70 nucleotide long. This first processing step is catalyzed by Drosha that is helped by a cofactor, DGCR8 (DiGeorge syndrome critical region gene 8) (Pasha in Drosophila). This complex is called the Microprocessor. DGCR8 contains two dsRNA-binding domains that directly interact with the stem-loop and with the flanking region, serving as a molecular anchor to Drosha that carries out the cleavage reaction. The cleavage produces highly

exact extremities and is highly regulated. The production of pre-miRNAs not always requires the participation of the microprocessor complex. In fact, a rare alternative pathway has been identified initially in fly and nematodes, but also present in mammals (Berezikov et al., 2007; Okamura et al., 2007; Ruby et al., 2007). This pathway uses the splicing machinery to liberate introns that mimic the features of pre-miRNAs. These structures are called mirtrons. After being spliced they enter the normal miRNA processing pathway.

Pre-miRNAs are then exported to the cytoplasm by Exportin-5 in a Ran-GTP dependent way, where they will be further processed. In the cytoplasm the terminal loop of the pre-miRNA is cleaved originating a mature dsRNA of approximately 22 nucleotides of length. This step is carried out by Dicer. The PAZ domain of Dicer binds to the 3'overhangs of the pre-miRNAs and this binding determines the cleavage site since that Dicer's

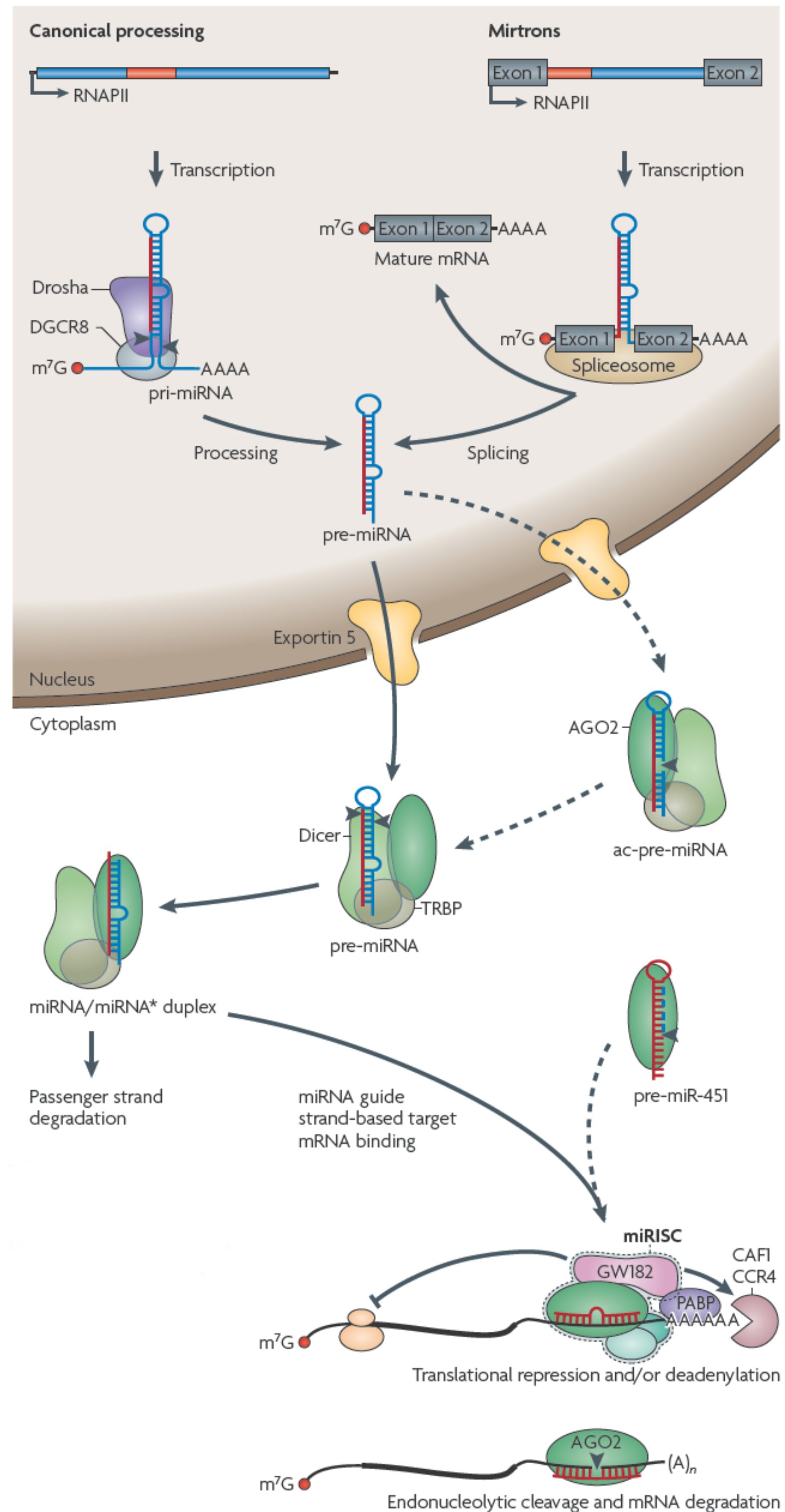



**Fig 10: miRNA Biogenesis.** (Krol et al., 2010)

catalytic sites are located precisely two helical turns away from the PAZ domain (bound to the pre-miRNA). In this step, Dicer is assisted by the Tar RNA Binding Protein – TRBP (know as Loquacious in Drosophila), another dsRNA binding protein. At the end of this last processing step Argonaute 2 is recruited to the complex Dicer/TRBP leading to the unwinding of the duplex. At this stage one of the strands, the mature miRNA, is preferentially incorporated into the complex that

will repress target gene expression – the RNA-Induced Silencing Complex (RISC). The choice of the mature strand is based on the thermodynamic stability of the two ends of the duplex. The complementary strand (miRNA*) in most cases is degraded. However there are growing evidences that both strands can be incorporated into the RISC complex in a functional way (Guo and Lu, 2010).

The key proteins of the RISC complex are the Argonautes (AGO). These proteins contain three highly conserved domains, PAZ, MID and PIWI domains, that interact with the miRNAs. In mammals there are four AGO that function in miRNA repression (Ago1 to Ago4). Different AGO proteins seems to have different specificity to the miRNA or siRNA pathway. Another crucial factor for miRNA-repression is the GW182 protein. This protein interact directly with AGO proteins and are thought to be the effectors of AGO. There are other proteins interacting with RISC to modulate miRNA function. This is the case of Fragile X Mental Retardation Protein (FMRP), which binds RNA molecules and might modulate translation. Also RNA Helicase RCK/p54, which is a p-body component, is thought to be essential to induce repression. Finally TRIM32 was recently seen to bind the RISC components enhancing in this way its activity. However further studies are required for a better understanding of the proteins that modulate this complex process of miRNA-mediated repression (Carthew and Sontheimer, 2009; Fabian et al., 2010; Krol et al., 2010).

### 1.3.3- Prediction of miRNAs Targets

One of the most challenging fields in the study of miRNAs is the identification of the target genes they regulate. The study of the molecular mechanism implicated in target recognition, together with computational approaches were soon translated into basic principles used in the development of bioinformatic tools that could predict miRNAs targets.

The majority of animal miRNAs displays only modest base-pairing to their targets in contrast to what happens in plants, where the base pairing is perfect. Historically, miRNAs are known to regulate the 3’UTR of the target genes. This was experimentally demonstrated with the first miRNAs identified and it was also assumed as an *in silico* convenience that was further confirmed experimentally. But the use of these predictive algorithms has left underestimated the possibility that miRNAs might regulate other regions, such as the 5’UTR or even the coding region of the mRNAs. In fact, experiments involving artificial and natural mRNAs have shown that their 5’UTR can be targeted by miRNAs (Lytle et al., 2007). Recent reports have also started to address the possibility that miRNAs can target the Open Reading Frame (ORF) of certain genes.

Accordingly, three independent studies have identify three miRNAs that functionally target the coding region of DNMT3B (Duursma et al., 2008), p16 $^{INK4A}$ (Lal et al., 2008) and several zinc finger family members (Huang et al., 2010). Moreover, several computational analysis either in Drosophila either in Vertebrates genomes revealed that miRNA complementarities in conserved coding regions are more common than predicted (Forman et al., 2008; Stark et al., 2007; Tay et al., 2008). Even more surprising is the fact that miRNAs seems to have the capacity to activate the translation of certain mRNAs by binding to their 5'UTR as it was demonstrated by Orom et al and Henke et al (Henke et al., 2008; Orom et al., 2008). These recent facts suggest that animal miRNAs might function in a very similar way to Plants miRNAs with no significant mechanistic differences in target recognition.

But which are the principles currently assumed for target recognition? The interaction between miRNA and mRNA are through base pairing - most of the times imperfect base pairing. The most important region of the miRNA is the so called "seed" region. According to the seed "rule", the interaction between miRNA and mRNA requires a contiguous and perfect (or nearly perfect) Watson-Crick base pairing of the 5' nucleotides 2-8 of the miRNA. Although this seems to be a rather general and consensual rule, there are some exceptions in which mismatches or even bulged nucleotides have been identified in the seed region. Another observation is that the presence of an A in the $1^{st}$ nucleotide and a A or U in the position 9 seams to improve miRNA activity although it's not required. Another characteristic of the miRNA/mRNA interaction is the presence of mismatches and bulged nucleotides in the position 10-12. As for the 3' extremity of the miRNA, the complementarity is quite relaxed although it stabilizes the interaction mainly when the seed matching is not perfect.

Another point to take into consideration when discussing the miRNA/mRNA interaction is the presence of multiple sites in the same 3'UTR. In fact this seems to lead to a more efficient mRNA repression. Some algorithms also take into account the conservation between related species of the miRNA-binding site. Finally one must consider that mRNAs have a secondary structure that might block miRNAs binding.(reviewed in (Brodersen and Voinnet, 2009; Du and Zamore, 2005; Fabian et al., 2010).

Giving different weight to each of these parameters, several algorithms were developed to predict miRNAs targets. The most known and robust ones are: TargetScan, PicTar, Miranda and PITA.

TargetScan was the first to be developed (Lewis et al., 2003). It takes into consideration the conservation between species and for this aligns the sequences of the 3'UTR of zebrafish, mouse, rat and human. This procedure reduces the probability of finding false positives. This algorithm

requires a perfect complementarity between the seed region of the miRNA and the binding site. Finally, it takes advantage of the RNAFold from the Vienna package (Hofacker, 2003) in order to introduce a thermodynamic parameter and to further narrow down the list of predicted targets. This algorithm was redefined in 2005 (Lewis et al., 2005) in order to further increase the fidelity of the predictions. In this direction, the authors have decreased the complementarity of the seed region to 6 nucleotides. They now require the presence of an A in the first position (5') of the miRNA binding site. The relative position of the miRNA binding site in the interior of the 3'UTR is also taken into account. Altogether, this modifications lead to the development of a very stringent algorithm in order to avoid false positives.

Also PicTar (Krek et al., 2005) uses the 3'UTR regions from multiple species as initial data. However, it is not as stringent as TargetScan since it does not take into consideration the relative position of the binding site inside the 3'UTR. Then it looks for the binding site between miRNAs and 3'UTR, but also here is less stringent since it also considers imperfect bindings. Finally it takes into account the binding free energy for each possible interaction that comes out from the previous analysis.

MiRanda (Enright et al., 2003) is a program that does not take into consideration the conservation between species of the target genes. It was develop taking into account the importance of the binding between the seed and target mRNA, but it does not require a perfect binding. Also this algorithm takes into account the thermodynamic stability of the miRNA-mRNA duplex, according to the Vienna package. An updated version (John et al., 2006) increases the stringency of this algorithms mainly by requesting a almost complete complementarity (allowing only a G:U instable binding). Simultaneously, this updated version takes into account the number of binding sites for the same miRNAs in the 3'UTR of the gene.

PITA (Probability of Interaction by Target Accessibility) takes into consideration the accessibility of the target site in order to predict the interactions miRNA-RNA (Kertesz et al., 2007). To accomplish this, it considers only mRNAs with perfect complementarity in the 6-8 nucleotides of the seed region of the miRNA, allowing only one mismatch. The final classification of the miRNA/mRNA interaction indicates the energetic cost to release the mRNA from the binding with the miRNA. To calculate this, it takes from the free energy of the mRNA, the energy implicated in the formation of the duplex miRNA-mRNA. This algorithm does not take into consideration the conservation between species.

Although the principles used for target recognition are quite well established and accepted, and considering that different algorithms have different sensibilities, validation of the predicted targets is always required.

## 1.3.4- Mechanisms of mRNA posttranscriptional repression by miRNAs

Mature miRNAs are loaded into the RISC complex and together this complex is able to bind the target mRNA and regulate the expression of the target gene. However the mechanisms that miRNAs use to regulate gene expression are still very controversial and poorly understood. It is widely accepted that the degree of complementarity between miRNA and mRNA dictate the regulatory mechanism implicated in gene silencing. Accordingly, perfect complementarity induces Ago-catalyzed cleavage of the mRNA, while bulges and mismatches are believed to block translation of the mRNA into protein. But, is this a universal rule that functions for all miRNAs/mRNAs? The advances made in the last decade in understanding the molecular mechanisms involved in miRNA-induced silencing are not conclusive. On the contrary, they have generated a series of hypothesis on how miRNAs influence mRNA translation/stability.

### *1.3.4.1- Post-transcriptional Repression by miRNAs*

The initial observations concerning miRNAs functioning revealed that protein level of the target gene was decreased while mRNA levels remained unchanged, suggesting the involvement of a translational repression mechanism. Translation of mRNA is a highly complex process that involve multiple protein factors and that can be divided in three stages: initiation, elongation and termination. During translation, proteins interacting with the poly-A tail (such as PABPC) bind to proteins interacting with the 5'cap of the mRNA (such as eIF4E and the eIF4G complex) giving rise to a circular mRNA that is protected from degradation and it's translated. So how do miRNAs repress translation? Does it occur at the initiation stage or at the elongation stage? Several *in vitro* and *in vivo* studies were preformed giving rise to several hypotheses that still require further confirmation.

One of the first theories that came out suggested that the nascent polypeptide chain might be degraded co-translationally, and this hypothesis was supported by the fact that miRNAs and target mRNAs were associated with polysomes without any *de novo* protein being detectable (Nottrott et al., 2006). An alternative model proposed to explain these facts was the ribosome drop-off model in which the ribosomes engaged in the translation of miRNA-associated mRNAs were prone to dissociate prematurely (Petersen et al., 2006). This model was further supported by the fact that miRNA-associated mRNAs tend to dissociate faster from the polysomes than unrepressed mRNAs

after treatment with a translational inhibitor. All these two theories suggested that miRNA block translation at a post-initiation step.

However, several studies have suggested that miRISC complex represses mRNA translation at the initiation step. For example, studies using mRNAs translated in a cap independent manner (such as using an Internal Ribosome Entry Site - IRES) failed to be repressed by miRNAs (Humphreys et al., 2005; Pillai et al., 2005). Always supporting a cap-dependent mechanism, it was seen that, in cell extracts, an artificial mRNA containing a $m^7$Appp-cap structure fail to be repressed by miRNAs while the normal $m^7$Gppp-capped mRNAs were silenced (Mathonnet et al., 2007; Wakiyama et al., 2007). Soon several competing models came out trying to explain how miRNAs could induce translational repression at the initiation stage. In the first model, miRISC is proposed to compete with eIF4E for the binding of the mRNA 5' cap. It was speculated that Ago2, GW182 or another downstream factor could compete directly with eIF4E for cap binding. Based on the fact that several repressed mRNAs are deadenylated, a second model was proposed in which miRISC, through GW182 protein, stimulates the deadenylation of the mRNA tail. However there are also reports indicating that nonpolyadenylated mRNAs can be targeted by miRNAs. Finally, miRISC might be implicated in the recruitment of eIF6, a factor that prevents the premature association of the 60S ribosomal subunit with the 40S pre-initiation complex, to the mRNA, preventing in this way the assembly of translationally competent ribosomes at the start codon. Currently, and taking into account the available data, it is more accepted that translational repression occurs at the initiation step. (reviewed in Carthew and Sontheimer, 2009; Fabian et al., 2010; Huntzinger and Izaurralde, 2011).

#### *1.3.4.2- Degradation of the target mRNAs*

Although the initial studies reported a block in translation, soon the evidences for target mRNA cleavage started to appear. Microarray experiments after over-expression of specific miRNA showed a massive down-regulation of the predicted targets (Farh et al., 2005; Guo et al., 2010; Lim et al., 2005; Selbach et al., 2008). Furthermore, depletion of miRNAs from the cells (Baek et al., 2008; Krutzfeldt et al., 2005; Selbach et al., 2008) or even depletion of essential components of the miRNA pathway lead to an increase abundance of the predicted mRNA targets (Eulalio et al., 2007; Eulalio et al., 2009; Giraldez et al., 2006; Rehwinkel et al., 2005), indicating that miRNAs also regulate mRNA stability and degradation.

It is widely accepted that fully complementary targets leads to endonucleolytic cleavage. But what happens to partially complementary targets? It is believed that in these cases mRNAs degradation is not due to AGO2-mediated cleavage but rather due to deadenylation, decapping and exonucleolytic digestion of the mRNAs. In this mRNA decay pathway, mRNAs are primarily deadenylated by the Caf1-Ccr4-Not1 deadenylase complex in a process that requires the protein GW182. Subsequently mRNAs are decapped by the DCP2 enzyme, and finally degraded by the 5'-to-3' exonuclease XRN1. Depletion of AGO2, GW182 or depletion of the deadenylation complex lead to the stabilization of the miRNAs targets indicating that miRNA-bound to AGO2 in a complex with GW182 might recruit deadenylases to induce mRNA degradation. One question that remains open is whether mRNA degradation is a consequence of translational blockage or whether mRNA deadenylation renders mRNA silenced for translation.

Despite the evidences supporting the several modes of action of miRNA mediated mRNA repression, it remains controversial which is the prevailing mechanism. There are several recent studies indicating that target degradation is the main mode of regulation. Using transfection or depletion of a specific miRNAs allowed Selbach et al. and Baek et al. to study the effects at the level of protein and mRNA in simultaneous. The authors have concluded that the main mechanism was miRNA induced mRNA degradation and only rarely block of translation (Baek et al., 2008; Selbach et al., 2008). Another study showed that, in HEK-293T cells transfected with miRNA-124, mRNA degradation accounted for 75% of the changes observed at the protein level. These authors have co-immunoprecipitated mRNAs bound to AGOs and identified 600 transcripts that were differentially bound after the expression of miRNA-124, but analyzing the translational rates they couldn't find evidences for translational blockage (Hendrickson et al., 2009). But the same doubt remains – is translational repression the cause of these degradation? Regardless, it is now well accepted that mRNA degradation is an important and well documented mechanism trough which miRNAs exert their functions. (reviewed in Carthew and Sontheimer, 2009; Fabian et al., 2010; Huntzinger and Izaurralde, 2011; Pillai et al., 2007).

#### *1.3.4.3- P-Bodies and the compartmentalization of miRNA-mediated repression*

Repressed mRNAs can be found in discrete cytoplasmatic foci known as P-bodies, GW-bodies or stress granules (Fig 11). These foci are enriched in factors involved in mRNA decay mechanism and translational repression. They contain deadenylases, decapping enzymes and the

exonuclease Xrn1 involved in mRNA decay. They lack ribosomes and translation initiation factors and this is consistent with the idea that p-bodies contain repressed mRNAs. As expected, p-bodies also contain components of the miRNAs pathway such as AGO2 and GW182 proteins. Several evidences showed that a functional miRNA pathway is required for p-bodies formation. However, disruption of these cytoplasmatic structures, by silencing certain components, does not interfere with the functioning of the miRNA pathway.

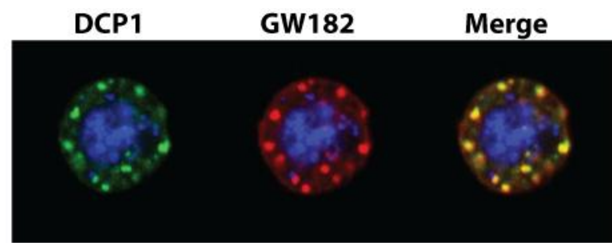



**Fig 11: P-bodies in Drosophila cells.** P-bodies are cytoplasmatic foci where proteins involved in mRNA degradation and translational repression co-localize. Co-localization of the decapping protein DCP1 with GW182

One interesting observation is that mRNAs located in the p-body are not necessarily committed to degradation. In fact under certain conditions mRNAs can exit the p-body and re-enter the translational process, indicating that p-bodies can function as a temporary storage for repressed mRNAs (reviewed in (Fabian et al., 2010; Pillai et al., 2007). Although little is known about this mechanism, it is appealing to think that cells can have a ready-to-use stock of mRNAs that can be immediately translated upon request and that miRNAs can contribute to this repression state.

### 1.3.5- Functions of miRNAs

During the last two decades a large amount of information has been gathered concerning miRNAs biology. Taking into consideration the large amount of genes that can be regulated by miRNAs it is reasonable to think that they play a major role in several biological processes. In fact, they modulate crucial aspects of cell metabolism and identity. They can induce cell death, but they can also increase cell viability. They are responsible for important regulatory events on development and organogenesis. They can interfere with temporal and spatial determination. They are involved in fine-tuning delicate processes such as neuronal synaptic plasticity and heart functioning. They take part on the response to viral infections. And obviously…as everything that is important in basic physiological mechanisms, they are also important in disease. Consistently it has been reported that miRNAs are deregulated in oncogenesis, in heart failure, in hepatitis, in diabetes, in autoimmunity, in neurodegeneration and in mental disorders. And if they are deregulated in disease they are a potential therapeutical target or at least can be used as a prognostic factor. And this has been intensively studied in the last few years. Furthermore, loss and gain of function experiments have started to unravel the striking functions of miRNAs in numerous facets of muscle

biology. But what is known about the role of miRNAs in the model that we study, the skeletal muscle?

### *1.3.5.1- miRNAs in skeletal muscle*

Skeletal muscle, together with the cardiac muscle, is enriched of a subset of miRNAs called myomiRs (Fig 12). The first group of myomiRs identified was miRNA-1, miRNA-133 and miRNA-206 (Sempere et al., 2004). These miRNAs are encoded in bicistronic miRNAs genes located in different chromosomes. miRNA-1-1 and miRNA-133a-2 are clustered on mouse chromosome 2, miRNA-1-2 and miRNA-133a-1 are clustered on mouse chromosome 18 and

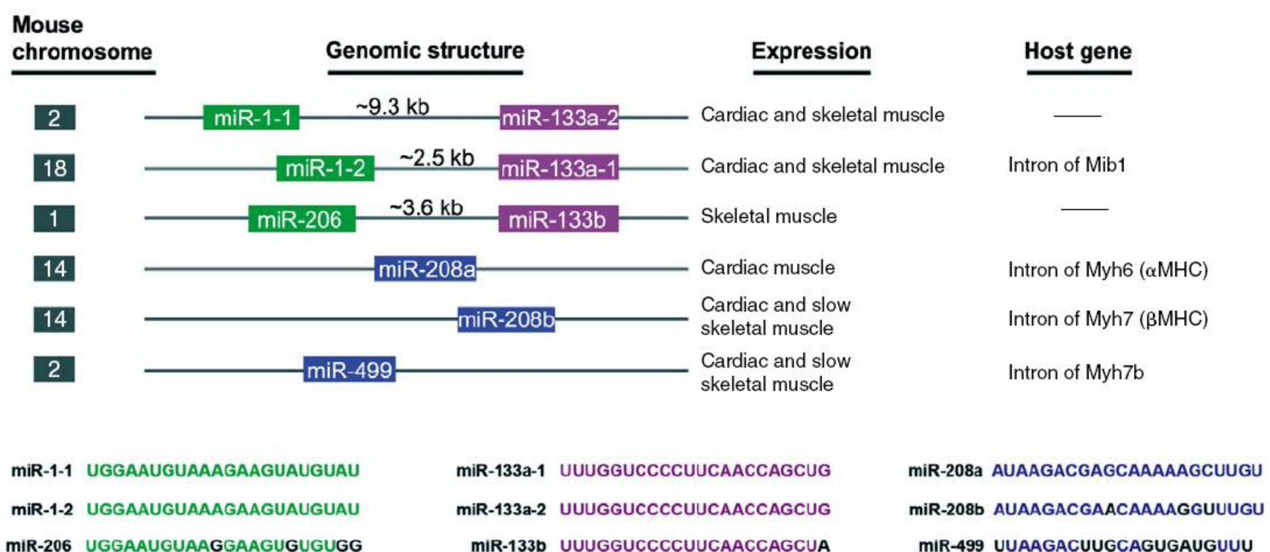



**Fig 12: Genomic organization of the muscle-specific miRNAs and their sequence homologies**. Genomic localization of the muscle-specific miRNAs, their host genes and tissues where they are mainly expressed. Comparison of the miRNAs sequences (from 5' to 3'). Matching color indicates sequence homology while black nucleotides represent differences.(Chen et al., 2009a)

miRNA-206 and miRNA-133b are located in mouse chromosome 1. While the cluster containing miRNA-1-2/miRNA-133a-1 is located in the intron of a protein coding gene – Mib1, a E3 ubiquitin ligase ubiquitously expressed, the other two clusters are derived from non-coding regions of the genome. miRNA-1-1 and miRNA-1-2 share the same nucleotide sequence, while miRNA-206 only differs from them in 4 nucleotides. miRNA-133a-1, miRNA-133a-2 and miRNA-133b have almost the same sequence. This suggests that these three miRNAs might have a common ancestral origin, and that they might regulate similar target genes. As for their expression profiles, the cluster containing miRNA-206 is expressed almost exclusively in the skeletal muscle, while the other two cluster are expressed both in skeletal and cardiac muscle.

Another family of myomiRs is composed by miRNA-208a, miRNA-208b and miRNA-499. The first two miRNAs are located on chromosome 14 and are both encoded by an intron of their host genes, Myh6 and Myh7 respectively. Several evidences indicate that their expression and the expression of their myosin host genes share common regulatory elements. Likewise, miRNA-499, another myomiRs, is also located in the intron of a myosin gene – Myh7b, and is also co-expressed with its host gene. Considering that the first two miRNAs are very similar among them and that miRNA-499 contains the exactly same seed region it is expected that these three miRNAs regulate the expression of common target genes. It is interesting to see that myosin genes not only encode for the major contractile proteins of the muscle but also encode miRNAs that have major functions in regulating muscle gene expression.

#### *1.3.5.2- miRNAs and the differentiation of muscle cells*

The differentiation of muscle cells is a highly regulated process in which transcription factors such as MyoD, Myogenin, Myf5, MRF4, MEF2 and SRF are key players. The sequential activation of these factors blocks the proliferative potential of myoblasts inducing their differentiation into multinucleated myotubes. This is a complex process that is tightly regulated at the transcriptional level. Recently it was shown that an additional post-transcriptional level of regulation was also implied, being miRNAs the new players in this fine tuning.

Using C2C12 muscle cells transfected with the first myomiRs identified, miRNA-1, miRNA-133 and miRNA-206, allowed the study of these miRNAs *in vitro* (Chen et al., 2006a; Kim et al., 2006). While miRNA-1 and miRNA-206 induced myogenesis, miRNA-133 promoted myoblast proliferation. The fact that miRNA-1 and miRNA-133 have opposing effect on C2C12 differentiation was quite unexpected since they are originated from the same polycistronic transcript but it is easily understood when we take in consideration some of the validated targets. In fact miRNA-1 was shown to target Histone Deacetylase 4 (HDAC4), a transcription repressor of muscle differentiation that blocks MEF2 activity. Additionally, miR-1 also represses the translation of Hand2, a transcription factor that promotes ventricular cardiomyocytes expansion during embryogenesis (Zhao et al., 2005). On the other hand, miRNA-133 was shown to repress SRF, an essential regulator of muscle differentiation (Chen et al., 2006b). Furthermore, miRNA-133 was also shown to repress the translation of the polypyrimidine tract-binding protein (nPTB), an essential factor involved in alternative splicing that influences the muscle differentiation program (Boutz et al., 2007). Also uncoupling protein 2 (UCP2), an important regulator of energy

expenditure and thermogenesis in various organisms, was shown to be repressed at the translation level by miRNA-133 during C2C12 differentiation (Chen et al., 2009b). Altogether, these results may explain the opposite effects of these two miRNAs.

The role of miRNA-206 in muscle differentiation was also studied in detailed. Initial reports (Anderson et al., 2006; Kim et al., 2006; Rao et al., 2006) identified p180subunit (Pola1) of DNA polymerase alpha and the Gap junction protein connexin43 (Cx43) as being functional targets of miRNA-206. The first is essential for DNA synthesis and therefore its down-regulation by miRNA-206 is believed to contribute to exit from cell cycle proliferation during myotube differentiation. Gap junctions, on the other hand, are hypothesized to be required in embryonic skeletal muscle to allow passage of signaling molecules and metabolites and for the coordinated maturation of contractile capabilities. Cx43 is known to be down-regulated at the late stages of myoblast differentiation, and this down-regulation is now believed to occur as a consequence of the miRNA-206 modulation (Anderson et al., 2006). Also stating the important role of miRNA-206 in C2C12 differentiation, Rosenberg et al (Rosenberg et al., 2006) showed that this miRNA targets follistatin-like 1 (Fstl1) and Utrophin (Utrn). Although the role of Fstl1 remains elusive, it is known that repression of Utrn is an essential step during differentiation, since it's replaced by Dystrophin in the Dystrophin Associated Glycoprotein Complex.

Considering the role miRNAs play during myogenic differentiation, it is not surprising that myogenic transcription factors are involved in their regulation. In fact it's known that the induction of miRNA-206 is affected in part for the binding of MyoD to its promoter region (Rosenberg et al., 2006). Chip-on-Chip analyses also showed that MyoD and Myogenin can bind to the up-stream regions of miRNA-1, miRNA-133 and miRNA-206 inducing their expression (Rao et al., 2006). Also SRF, together with MyoD and Mef2, is able to bind to the promoter region of miRNA-1 as it was seen in the heart (Zhao et al., 2005). Therefore, miRNA expression levels are regulated by myogenic transcription factors, and, to fine tune this process, some miRNAs can modulate the levels of transcription factors.

Apart from the myomiRs, other miRNAs are also implicated in muscle differentiation. This is the case of miRNA-181, a broadly expressed miRNA. miRNA-181 expression levels dramatically increases in the TA muscle after cardiotoxin-induced injury and in C2C12 cells during differentiation (Naguibneva et al., 2006). C2C12 cells depleted of miRNA-181 had compromised differentiation mainly due to a decrease expression of MyoD and myogenin. Bioinformatics analysis of the possible targets of miRNA-181, together with *in vitro* experiments revealed that homeobox protein Hox-A11, a MyoD repressor, is in fact a target of miRNA-181. Taken together,

the results of this study showed that miRNA-181 is required for the differentiation process mainly due to its capacity to regulate a Hox-A11, a repressor of the differentiation process.

Another miRNA involved in the differentiation process is miRNA-214. This miRNA was shown to be expressed during early segmentation stages in zebrafish somites (Flynt et al., 2007), and to be involved in cardiac hypertrophy (Watanabe et al., 2008). A detailed study in C2C12 cells showed that this miRNA promotes cell differentiation, mainly by facilitating the cells from exiting cell cycle. This miRNA does so by targeting N-Ras, a proto-oncogen that is known to target muscle differentiation (Liu et al., 2010). An independent study showed that the transcription of this miRNA is repressed by Polycomb Group proteins (PcG). During muscle cell differentiation this repression is relieved mainly by the binding of MyoD and myogenin inducing an up-regulation of miRNA-214. This miRNA, through a negative feed-back loop, represses the levels of Ezh2, a catalytic component of the PcG complex, ensuring the maintenance of a differentiation program (Juan et al., 2009).

Different members of the TGF-β pathway were shown to inhibit myogenesis mainly by suppressing the expression of MRFs. This is the case of myostatin and also SMAD3. The SMAD inhibitors of the pathway, such as SMAD7, on the other hand are enhancers of myogenesis since they interact with MyoD and block the signals from myostatin. Recently miRNA-24 has been shown to modulate TGF-β inhibition of myogenesis. Sun *et* al (Sun et al., 2008b) showed that SMAD3 is able to bind to the promoter region of miRNA-24 and to repress its expression. Furthermore it was shown that over-expression of miRNA-24 was sufficient to induce myogenesis and that it was able to rescue the TGF-β inhibition of differentiation. The authors proposed a mechanism in which miRNA-24, under the regulation of TGF-β, controls the expression levels of early and late genes of the differentiation process.

An additional miRNA involved in muscle myogenesis is the miRNA-125b (Ge et al., 2011). The levels of this miRNA decline considerably during C2C12 differentiation and during muscle regeneration induced by $BaCl_2$. Introduction of this miRNA in C2C12 cells dampened myoblast differentiation, as shown by the decrease in the differentiation index (number of MHC positive cells) and in the fusion index (percentage of nuclei in myocytes with at least two nuclei). The authors went further showing that miRNA-125b is able to regulate the expression of IGF-II, a critical inducer of skeletal muscle myogenesis, and that the expression of miRNA-125b is under the control of mTOR, although in a kinase independent-way.

Skeletal muscles are quite responsive to mechanical stress. It is known that mechanical forces are transduced by surface receptors into intracellular signals. In fact, an important factor that is known to delay C2C12 differentiation is cyclic stretch (Kook et al., 2008). *In vitro* studies

revealed that cyclic stretch decreases the levels of myogenin and stimulates proliferation of C2C12 cells and recently it was shown to influence the levels of miRNAs in differentiating cells. One of this miRNAs is miRNA-146a (Kuang et al., 2009). Its expression levels were increased after 48-h of cyclic stretch (5% elongation/10cycles/min) in differentiating C2C12 cells. Furthermore the authors showed that cyclic stretch induced the proliferative state of C2C12 cells. To elucidate the functional role of the up-regulation of this miRNA the authors showed that NUMB is a target of miRNA-146a. NUMB is a key negative regulator of the Notch pathway, an important pathway for muscle development. It is known that Notch pathway allows the expansion of progenitor cells by repressing the differentiation program. It is then possible that cyclic stretch, trough the regulation of specific miRNAs, can alter the proliferative/differentiation potential of C2C12 cells.

Also miRNA-221 and miRNA-222 seem to be implicated in the differentiation process. Cardinali et al (Cardinali et al., 2009) showed that these two miRNAs are down-regulated during differentiation and that ectopic expression of these two miRNAs lead to a decreased exit from cell cycle and decreased myogenin expression. Among the predicted targets for these miRNAs one could find the cell cycle inhibitor p27. Since it had been shown that p27 interferes with myoblast fusion and expression of myosin, and considering that its expression levels were reduced during differentiation the authors confirmed that it was a true target by luciferase assay. This implicates miRNAs in the regulation of cell cycle during the differentiation process.

Apart from the known regulatory mechanisms implicating transcription factors, a new layer of complexity has been added to the process of muscle cell differentiation. The discovery of miRNAs that fine tune protein dosages anticipates an important role of these tiny molecules during muscle development and regeneration.

#### *1.3.5.3- Role of miRNAs in adult skeletal muscle*

One of the first evidences that miRNAs might play a crucial role in adult skeletal muscle came from a study in sheep (Clop et al., 2006). The aim of the study consisted in identifying the gene responsible for the hypertrophic phenotype of the Texel sheep. The authors found a point mutation in the 3'UTR of the myostatin gene that creates a new binding site for the miRNA-1 and miRNA-206. In these breed of animals, myostatin, a negative regulator of muscle growth, is down-regulated by these two muscle-specific miRNAs inducing an exacerbated muscle growth.

Another important evidence highlighting the essential role that miRNAs play in muscle came from loss-of-function experiments. In this case, O'Rourke et al (O'Rourke et al., 2007)

generated a muscle specific conditional knock-out of Dicer, the RNAse III enzyme required for miRNA maturation. In these mice, the expression of Cre recombinase was under the control of MyoD regulatory elements, and therefore started to be expressed from embryonic day 9.5. All Dicer skeletal muscle mutants died just after birth. They showed severe defects in skeletal muscle embryonic development that was mainly translated in muscle hypoplasia with hypertrophy of the few remaining fibers. This hypoplasia was attributed to an increase apoptosis rather than a defect in myofibers formation. These results are similar to the ones obtained by (Kwon et al., 2005; Sokol and Ambros, 2005) in which the knock-down of the muscle specific miRNA-1 in Drosophila caused arrest in embryogenesis with disorganized muscle development and aberrant expression of muscle-specific genes. Altogether these results anticipate a fundamental role of miRNAs in different aspects of skeletal muscle biology.

#### 1.3.5.3.1- Role of miRNAs in fiber type switch

As discussed previously, skeletal muscle is composed of fibers with different structural and metabolic properties. The expression of different MHC isoforms, together with the activity of mitochondrial enzymes dictates the classification of muscle fiber in Type I, IIa, IIx and IIb. Several lines of evidences suggest that these properties can be changed by miRNAs.

In early studies, (McCarthy and Esser, 2007a) analyzing the expression levels of muscle-specific miRNAs in both plantaris, a fast muscle, and soleus, a slow muscle, realized that miRNA-206 was 7 fold increased in the soleus muscle compared to plantaris. Taken in consideration this fact, the authors proposed that miRNA-206 might be fiber type-I specific, and this was further supported by the fact that functional overload, a condition known to induce a fast-to slow switch, induced an increase in the expression levels of miRNA-206. Although no mechanistic explanation was proposed, the authors hypothesized that miRNA-206 might be repressing the fast phenotype of muscle fibers.

More recently, van Rooij et al (van et al., 2007; van et al., 2009), showed that three miRNAs, miRNA-208a, miRNA-208b and miRNA-499, encoded in the introns of muscle myosin genes are involved in a network that is able to regulate MHC expression levels itself and hypothetically influence fiber type. As discussed previously, these myomiRs, encoded by Myh6 (α-MHC), Myh7 (β-MHC), and Myh7b respectively, seem to be co-regulated with their host genes. Using knock-out and transgenic mice for miRNA-208a, the authors showed that the expression of the slow β-MHC was influenced by this miRNA. Furthermore they showed that this regulation

involved the thyroid hormone receptor (TR), since miRNA-208a targets thyroid hormone receptor associated protein 1 (THRAP1), a modulator of the TR involved in the recruitment of RNA Polimerase II and general initiation factors. Furthermore, double knock-out animals for both miRNA-208b and miRNA-499 showed a substantial reduction in type I myofibers in soleus muscle. Conversely, forced expression of miRNA-499 in the soleus muscle induced a complete conversion of all fast myofibers into type I fibers. Altogether these results suggest that myosin genes, through the expression of miRNAs, can influence a myriad of other functions in muscle fibers. In fact miRNA-208 and miRNA-499 were shown to directly down-regulate the expression of Sox6, Purβ, Sp3 and HP1β, four known repressors of the slow phenotype of muscle fibers (Bell et al., 2010; McCarthy et al., 2009; van et al., 2009).

But miRNAs can also regulate essential factors for the metabolic properties of the fibers. This is the case of PGC1-α, an important transcriptional co-activator involved in adaptive thermogenesis, fatty acid oxidation, gluconeogenesis, and mitochondrial biogenesis. It was shown by Aoi et al (Aoi et al., 2010) that miRNA-696 can block the translation of PGC1-α. The expression levels of this miRNA, which is markedly affected by both exercise and immobilization in opposing ways, is inversely correlated with the protein levels of PGC1-α. Furthermore, the over-expression of this miRNA in myoblasts leads to a decrease in mitochondria biogenesis and fatty acid oxidation confirming the role this miRNA play in the fiber type switch observed during exercise/immobilization.

#### 1.3.5.3.2- Role of miRNAs in muscle regeneration

Skeletal muscle regeneration is a complex process in which muscle satellite cells became activated, proliferate, migrate to the injured area and differentiate into new myofibers or fuse to injured fibers. Although the precise molecular mechanisms are not well defined, it is now known that miRNAs play a role in these processes. In fact Nakasa et al (Nakasa et al., 2010) showed that local injection of a mixture of double strand miRNAs (miRNA-1, miRNA-133 and miRNA206) into a lacerated muscle accelerate muscle regeneration and prevents fibrosis. Although no targets were identified, this effect was associated to an increased expression of myogenic factors such as MyoD, myogenin and Pax7. To clearly demonstrate the role of miRNA-206 on muscle regeneration, Yuasa et al (Yuasa et al., 2008a) preformed in situ hybridization on cardiotoxin-injected muscles. They observed a marked increase in the staining for miRNA-206 in newly formed

myofibers with centralized nuclei but not on intact pre-degenerated fibers. These results suggest that miRNA-206, together with miRNA-1 and miRNA-133, do have a role in the regenerative process.

Confirming the involvement of miRNAs in regeneration and differentiation of satellite cells Crist et al (Crist et al., 2009) showed that miRNA-27b is able to directly down-regulate Pax3, an essential transcription factor that has to be down-regulated in order to allow satellite cell to differentiate. *In vivo* over-expression of a miR-27b in Pax3-positive cells in the embryo lead to down-regulation of Pax3, resulting in interference with progenitor cell migration and in premature differentiation. Furthermore, injection of inhibitors of miRNA-27b in the injury site resulted in altered levels of Pax3 and in defective regeneration, reflecting the key role miRNA-27b plays in this process.

As it was seen before, miRNA-181 is up-regulated during differentiation of C2C12 cells. Likewise, its expression levels are increased during the regenerative process induced by cardiotoxin injection. Consistently, Hox-A11, one of the know target of miRNA-181, is down-regulated during this process. Another miRNAs that seems to affect muscle regeneration is miRNA-125b (Ge et al., 2011). *In vivo* experiments revealed that the expression of this miRNA is decreased during $BaCl_2$-induced muscle regeneration. Co-injection of this miRNA negatively affects this process while co-injection of antagomir against miRNA-125b promoted muscle regeneration. This data, together with the fact that miRNA-125b targets IGF-II, and with the fact that miRNA-125b inhibits myoblast differentiation, sustain a model in which miRNA-125b has to be repressed in order to allow muscle regeneration to occur.

Although the exact role of miRNAs in the regenerative process is still poorly unknown, the fact they are deregulated anticipates a potential therapeutic application for conditions associated with defective regeneration such as dystrophies and sarcopenia.

#### 1.3.5.3.3- Role of miRNAs in sarcopenia

Sarcopenia is defined as the slow but progressive loss of muscle mass with advancing age and is characterized by a deterioration of muscle quantity and quality leading to a gradual slowing of movement and a decline in strength. The role of miRNAs during this process only now starts to be unraveled. The first approach used by Drummond et al (Drummond et al., 2008) consisted in analyzing by Taqman RT-PCR the expression levels of three muscle specific myomiRs, miRNA-1, miRNA-133 and miRNA-206, in human samples. According to their data the expression of the pri-miRNA-1 and pri-miRNA-133 increased significantly although no change was observed in the

mature miRNAs. To further investigate the role of miRNAs during sarcopenia, the same authors, profiled miRNA expression patterns in aging human skeletal muscle using a miRNA array. Among the 18 differentially expressed miRNAs the Let-7 family members Let-7b and Let-7e were the most up-regulated. Among the possible targets of these miRNAs the authors found three cell cycle regulators, CDK6, CDC25A and CDC34, to be down-regulated at the mRNA level. Although a direct validation was not performed the authors propose that Let-7 expression may be an indicator of impaired cell cycle function possibly contributing to reduced muscle cell renewal and regeneration in older human muscles (Drummond et al., 2010).

#### 1.3.5.3.4- Role of miRNAs in muscle hypertrophy and atrophy

As discussed before, the role that miRNAs play in muscle hypertrophy was clearly demonstrated by the point mutation in the 3'UTR of the myostatin gene found in the hypertrophic Texel sheep. But other studies that followed confirmed the role of miRNAs in the control of muscle mass.

McCarthy and Esser (McCarthy and Esser, 2007a) studied functional overload (FO) of the plantaris muscle, a condition that leads to an increase of 45% in muscle mass after 7 days. They showed an increased transcription of miRNA genes as indicated by the elevated levels of pri-miRNA-1-2, pri-miRNA-133a2 and pri-miRNA-206. While the expression levels of the first two mature miRNAs were reduced by 50%, the mature miRNA-206 did not change after FO. Although the reasons for this discrepancy between pri-miRNA and mature miRNA were not found, these data clearly demonstrate that transcription of miRNAs is influenced by hypertrophic stimuli.

But miRNAs can also negatively regulate hypertrophic stimuli. As it was already seen, one of the most important growth factor implicated in muscle hypertrophy is IGF-1. What Elia et al (Elia et al., 2009) showed is that there is a reciprocal regulation of miRNA-1 and IGF-1 either in cardiac or skeletal muscle. In fact, miRNA-1 is able to block the translation of IGF-1. On the other hand, down-regulation of IGF-1 leads to a decrease phosphorylation of AKT and Foxo3. Dephosphorylated Foxo3 enters the nucleus where it binds to two responsive elements in the regulatory region of miRNA-1 inducing its transcription. These evidences imply Foxo3, one of the most important transcription factor involved in skeletal muscle atrophy, in the regulation of miRNA transcription. But also Foxo3 itself can be targeted by miRNAs. This is the case of miRNA-155 (Yamamoto et al., 2011) and miRNA-96 (Lin et al., 2010). While the first was down-regulated in a regulatory T-cell line, the second is up-regulated in human breast cancer to induce cell proliferation.

Both of them were shown to bind and modulate the 3'UTR of Foxo3, but their role in skeletal muscle atrophy is unknown.

Skeletal muscle atrophy is a characteristic response to microgravity exposure caused by spaceflight. After 11 days of spaceflight the expression levels of miRNA-206 were significantly reduced whereas the expression levels of miRNA-1 and miRNA-133 showed a trend towards reduction (Allen et al., 2009). This decrease in the expression of miRNA-206 was accompanied by an increase expression of Atrogin-1 and myostatin, two genes involved in muscle atrophy. Whether miRNA-206 plays a direct or indirect role in the repression of these atrogenes is still unknown. Somehow contradictory results were obtained by Williams et al (Williams et al., 2009) studying the expression levels of miRNA-206 in denervation, another disuse condition. In this case miRNA-206 was robustly increased in fast-twitch fibers, extensor digitorum longus (EDL), tibialis anterior (TA), and gastrocnemius/plantaris, after 10 days of denervation. The authors confirmed the results from McCarthy et al (McCarthy and Esser, 2007a) in which the expression of this miRNA was considerably over-expressed in slow muscle and denervation only modestly increased miRNA-206 expression levels in this muscle.

Increased protein degradation and reduced protein synthesis can also be the result of pro-inflammatory cytokines. One of these cytokines that is known to play an important role during muscle wasting is TWEAK – TNF-like weak inducer of apoptosis (Dogra et al., 2007). Incubation of C2C12 myoblasts with this cytokine lead to a decrease in the expression levels of miRNA-1, miRNA-133, miRNA-23 and miRNA-206 and to an increase in the expression levels of miRNA-146a and miRNA-455 (Panguluri et al., 2010). On the other hand, *in vivo* experiments showed that only miRNA-1 and miRNA -133 were significantly repressed in TWEAK transgenic mice.

Although very preliminary, these studies confirm the role that miRNAs play in the control of muscle mass. A more broad approach to identify the most important miRNAs involved in these processes together with the identification and validation of their targets will help in identifying possible therapeutic targets.

#### *1.3.5.4- miRNAs in muscle pathology*

In the previous section we have addressed the role of miRNAs in several aspects of muscle physiology. However in the recent years some studies started to unravel their role in muscle pathology.

One of the first approaches, performed by Eisenberg et al (Eisenberg et al., 2007), consisted in analyzing by miRNA microarray the expression profiles of human samples from 10 different primary muscle disorders. Among these disorders one could find muscular dystrophies, inflammatory myopathies and congenital myopathies. They have identified 185 miRNAs that were deregulated in these 10 disorders among which 55 were commonly deregulated in more than 5 disorders. Furthermore, they have found five miRNAs, miRNA-146b, miRNA155, miRNA-214, miRNA-221 and miRNA-222, common to all disorders suggesting the involvement of common regulatory mechanisms. The authors have further found a sub-group of 18 miRNAs that correctly predict and distinguish the various diseases from the normal muscle tissue.

Among the muscular disorders the most studied one in terms of miRNAs is, with no doubts, the Duchene Muscular Dystrophy (DMD). This is an X-linked recessive disease caused by mutations in the dystrophin gene. Loss of dystrophin protein in DMD leads to membrane destabilization and subsequent activation of pathophysiological processes, resulting in inflammation, necrosis, and fibrosis. Studies in human samples as well as in DMD animal models, such as the mdx mouse model and the $CXMD_j$ dog model, revealed the presence of miRNAs with altered expression. In fact, McCarthy et al (McCarthy et al., 2007) showed that the diaphragm of mdx mice presented increased expression of miRNA-206, while the plantaris showed decreased expression of this myomiR. Also miRNA-133 was decreased in the soleus muscle of mdx mice. Although it is not clear the reason why different muscles behave differently, it is interesting to find the dramatic increase in miRNA-206 in the diaphragm since it is the muscle that better recapitulate the human disease in the mdx mice. Also Yuasa et al (Yuasa et al., 2008b) reported that miRNA-206 is increased in T.A. muscle from mdx mice, while there was a slight decrease in the T.A. muscles of $CXMD_j$ dog. The proposed reason for this discrepancy comes from the fact that miRNA-206 is mainly expressed in newly formed fibers and in $CXMD_j$ dog muscles there is much more inflammatory infiltrate fibroblasts and degenerating fibers reducing in this way the quantity of miRNA-206 producing fibers.

A wider approach was used by Greco et al (Greco et al., 2009). In order to identify miRNAs involved in the pathological pathways of DMD they compared the miRNA expression profile of adductor muscles derived either from mdx or wt mice. They have observed a striking increase in the expression levels of miRNA-31, miRNA-34c, miRNA-206, miRNA-222, miRNA-223, miRNA-335, miRNA-449 and miRNA-494 while miRNA-1, miRNA-29c and miRNA-135a were significantly decreased in mdx muscles. To confirm the role of these miRNAs in the pathology, their expression levels were analyzed in muscle samples from DMD patients. The vast majority of

the analyzed miRNAs were deregulated in a similar manner in mdx mice and in DMD patients suggesting a conserved pathological mechanism.

Another miRNA that was recently implicated in DMD is miRNA-222. Using a bioinformatics approach De Arcangelis et al (De, V et al., 2010) identified three miRNAs, miRNA-24, miRNA-222 and miRNA-339 predicted to target β1-Syntrophin, a key component of the dystrophin-associated protein complex (DAPC) that is significantly reduced in mdx muscles at the protein level, suggesting a post-transcriptional regulation. Studies with the 3'UTR of β1-Syntrophin revealed that it was in fact modulated in mdx muscles. Furthermore, northern blot analysis revealed that miRNA-222 was up regulated up to 3 fold in gastrocnemius muscle of mdx mice. Interestingly the over-expression of this miRNA in C2C12 cells lead to a down-regulation of muscle specific miRNAs such as miRNA-1, miRNA-133 and miRNA-206, suggesting that miRNA-222 might have a role in the regulation of others miRNAs.

The role of miRNAs was also addressed in amyotrophic lateral sclerosis (ALS). This is a progressive neurodegenerative disease that results in loss of motor neurons, denervation of target muscles fibers, muscle atrophy and paralysis. To gain further insights in the molecular mechanisms implicated in the disease, Williams et al (Williams et al., 2009) compared the miRNA expression profiles of muscles from Wt mice and from G93A-SOD1 transgenic mice that express a low copy number of a mutant form of superoxide dismutase (SOD1) in which glycine-93 is replaced with alanine (G93A-SOD1), as seen in a subset of human ALS patients. The most up-regulated miRNA was miRNA-206, the muscle specific miRNA. To understand the role of this miRNA the authors have generated miRNA-206 specific knock-out mice. Despite the important role of this miRNA in muscle physiology homozygous for the deletion of miR-206 showed no obvious abnormalities. However when these mice were crossed with the G93A-SOD1 transgenic mice the resulting mutants showed an acceleration in the progression of the disease, suggesting that miRNA-206 is required to counteracts, although ultimately unsuccessfully, the pathogenesis of ALS. The authors focused on the neuromuscular junctions (NMJs), and they saw that miRNA-206 was required for the formation of new NMJs after injury. Furthermore they showed that miRNAs-206 inhibits the translation of HDAC4, a factor implicated in the control of neuromuscular gene expression. They further showed that the opposing effects of miRNA-206 and HDAC4 on NMJs innervation was indirectly mediated by FGFBP1, a secreted factor that interacts with FGF7/10/22 family members which are known regulators of synapse formation.

As we have seen, miRNAs are involved in different aspects of muscle biology. The identification of miRNAs deregulated in different conditions, together with a better understanding of their targets will help us understanding the molecular mechanisms implicated in the normal and diseased muscle, and ultimately will allow the development of new and specific therapeutic tools.

# Chapter 2 – Aims

The major aim of this thesis was to address the biological role of miRNAs during the process of skeletal muscle atrophy.

Skeletal muscle atrophy is a condition associated to food deprivation, aging and disuse. It is also a complication of several chronic diseases, such as cancer diabetes, sepsis, AIDS, renal and heart failure among others. In all these situations protein synthesis is overtaken by protein degradation. This increased catabolism is achieved by the activation of two degradative pathways: the ubiquitin/proteasome and the autophagy/lysosome that are tightly regulated at the transcriptional level by specific molecular pathways.

Recently a new regulatory mechanism, capable of regulating gene expression post-transcriptionally, has been discovered. miRNAs, small RNA molecules with approximately 22 nucleotides in length, bind to the 3'UTR of the target genes and either repress protein translation or induce mRNA degradation. They are predicted to regulate almost 60% of protein coding genes. Several miRNAs can target the same 3'UTR and the same miRNA can target several mRNAs, thus creating a complex regulatory network. Their expression is tightly regulated by transcription factors and this contributes for their tissue and developmental specificity. Deregulation of their expression patterns is often associated with pathological conditions and it is now used as a prognostic tool. Given their tissue specificity, their therapeutical potential is now being studied.

In skeletal muscle, the role of miRNAs only recently started to be approached. The existence of a group of myomiRs anticipates an active role of these regulatory molecules. Furthermore, the elimination of the miRNA pathway, in the muscle, leads to a lethal phenotype. It is now known, mainly by *in vitro* studies, that muscle specific miRNAs are involved in the process of muscle differentiation. *In vivo* studies revealed that miRNAs are also involved in fiber type switch, in muscle regeneration and in the normal functioning of the neuromuscular junction. Finally, the expression of miRNAs is altered in different muscle disorders. But nothing is known about the role of miRNAs during skeletal muscle atrophy, the major subject studied in our laboratory.

Lecker et al (Lecker et al., 2004), showed that during different atrophic conditions a group of genes, called atrogenes, were commonly transcriptionally regulated, suggesting the existence of common regulatory mechanisms. Therefore, we have hypothesized that miRNAs could also be transcriptionally regulated during the atrophic process and that a common miRNA could govern atrophy. To address this hypothesis, we intended to characterize the miRNA signature in atrophying muscles. This would allow the identification of condition-specific miRNAs and, possibly, miRNAs common to all the atrophic conditions (AtromiRNA). We were also interested in studying the biological role of the most deregulated miRNAs. With loss and gain of function, we could understand if the miRNAs under study do have an active role in the atrophic program. This would

be one of the first *in vivo* approaches to the function of miRNAs in the skeletal muscle. Simultaneously, we were interested in understanding through which targets these miRNAs were modulating atrophy. To accomplish that, we were interested in developing innovative approaches that could facilitate the identification of the possible targets. We were also interested in study the biological role of the targets, in the particular context of muscle wasting.

Given that muscle atrophy is a transcriptionally regulated process, and that miRNAs are transcriptionally regulated but simultaneously regulate transcription factors, the understanding of this new layer of regulation might open new doors to the understanding of the molecular mechanism governing atrophy. Additionally, and taking in consideration the advances made in the last decade in the miRNAs biology, it is not unrealistic to think on miRNAs as new potential therapeutic tools or targets.

# Chapter 3 - Methods

## 3.1- Animal models and surgical procedures

All experimental procedures were approved by the Ethics Committee of the University of Padova and authorized by the Italian Ministry of Health. Mice were housed in individual cages in an environmentally controlled room (23 °C, 12-h light-dark cycle) and provided food and water *ad libitum.* Adult male CD1 mice that were 2 or 3 month old were used.

Three different models of skeletal muscle wasting were used: starvation, denervation and diabetes. For starvation experiments chow was removed in the morning and mice stayed with free access to water but with no food for a period of 24h or 48h and then they were sacrificed. In denervation experiments the sciatic nerve of one leg was cutted. The other leg was used as control. Mice were sacrificed after 3, 7 or 14 days. Diabetes-induced muscle wasting was accomplished by one single acute intraperitoneal (IP) injection of streptozotocin (Sigma S-0130). Briefly, STZ (180mg/Kg) was dissolved in freshly prepared sodium citrate buffer (pH 4.4) (Sigma S-4641), and administered as a single dose IP immediately after preparation. Concurrent controls received an equal volume of vehicle. Mice were sacrificed 7 days after IP injection. Total RNA was extracted from atrophic EDL muscles to access the expression levels of Atrogin-1, an atrophy-related ubiquitin ligase that is up-regulated during different models of muscle loss.

Adult male CD1 mice (28-34g) were used in all over-expression, silencing and luciferase experiments. Tibialis Anterior (TA) muscles were electroporated as described previously (Fig 13) (Dona et al., 2003; Sandri et al., 2004a). Briefly, TA muscles were isolated through a small hind limb incision and plasmid DNA was injected along the muscle length. Plasmid DNA was diluted in a total volume of 40ul of 0.9% NaCl (final concentration) solution. Electric pulses were then applied by two stainless steel spatula electrodes placed on each side of the isolated muscles belly (21 V/cm for five pulses at 200-ms intervals) with the Electro Square Porator (ECM 830, BTX). Electroporated muscles were collected 7, 10 or 14 days later. No gross or microscopic evidence for necrosis or inflammation as a result of the electroporation procedure was noticed.

**Fig 13: *In vivo* electroporation allows the generation of transgenic muscles**. A saline physiological solution containing the desired combination of DNA is injected in the exposed T.A. muscles. Five electrical pulses of 21V are applied to the muscles to allow the entry of the DNA into the muscle fibers

The following constructs were electroporated or co-electroporated in T.A. muscles of at least three different animals:

- pcDNA 6.2-GW/EmGFP-miR-Negative Control (Negative Control) – 20 μg
- pcDNA 6.2-GW/EmGFP-miR-miRNA-206 (miRNA-206) – 20 μg
- pcDNA 6.2-GW/EmGFP-miR-miRNA-21 (miRNA-21) – 20 μg
- miRNA-206 (10 μg) + miRNA-21 (10 μg)
- pmiRZIP lentivector (ZIP NULL) – 10 μg
- pmiRZIP lentivector anti-miRNA-206 (ZIP-206) – 10 μg
- pmiRZIP lentivector anti-miRNA-21(ZIP-21) – 10 μg
- Zip-206 (10 μg) + Zip-21 (10 μg)
- Zip-21 (10 μg) + Zip-206 (10 μg)
- pMIR206-Luc (10 μg ) + Renilla Null (5 μg)
- pMIR21-Luc (10 μg ) + Renilla Null (5 μg)
- pMIR206-Luc (10 μg ) + Renilla Null (5 μg) + miRNA-206 (20 μg)
- pMIR21-Luc (10 μg ) + Renilla Null (5 μg) + miRNA-21 (20 μg)
- pMIR206-Luc (10 μg ) + Renilla Null (5 μg) + ZIP-206 (25 μg)
- pMIR21-Luc (10 μg ) + Renilla Null (5 μg) + ZIP-21 (25 μg)
- pMIR-YY1-3’UTR-Luc (10 μg ) + Renilla Null (5 μg)
- pMIR-eIF4E3-3’UTR-Luc (10 μg ) + Renilla Null (5 μg)
- pMIR-PDCD10-3’UTR-Luc (10 μg ) + Renilla Null (5 μg)
- pMIR-PolK-3’UTR-Luc (10 μg ) + Renilla Null (5 μg)
- pMIR-YY1-3’UTR-Luc (10 μg ) + Renilla Null (5 μg) + Negative control (20 μg)
- pMIR-YY1-3’UTR-Luc (10 μg ) + Renilla Null (5 μg) + miRNA-206 (20 μg)
- pMIR-YY1-3’UTR-Luc (10 μg ) + Renilla Null (5 μg) + miRNA-21 (20 μg)
- pMIR-YY1-3’UTR-Luc (10 μg ) + Renilla Null (5 μg) + miRNA-206 (10 μg) + miRNA-21(10 μg)
- pMIR-eIF4E3-3’UTR-Luc (10 μg ) + Renilla Null (5 μg) + Negative control (20 μg)
- pMIR-eIF4E3-3’UTR-Luc (10 μg ) + Renilla Null (5 μg) + miRNA-206 (20 μg)
- pMIR-eIF4E3-3’UTR-Luc (10 μg ) + Renilla Null (5 μg) + miRNA-21 (20 μg)
- pMIR-eIF4E3-3’UTR-Luc (10 μg ) + Renilla Null (5 μg) + miRNA-206 (10 μg) + miRNA-21 (10 μg)
- pMIR-PDCD10-3’UTR-Luc (10 μg ) + Renilla Null (5 μg) + Negative control (20 μg)
- pMIR-PDCD10-3’UTR-Luc (10 μg ) + Renilla Null (5 μg) + miRNA-206 (20 μg)

- pMIR-PDCD10-3'UTR-Luc (10 μg ) + Renilla Null (5 μg) + miRNA-21 (20 μg)
- pMIR-PDCD10-3'UTR-Luc (10 μg ) + Renilla Null (5 μg) + miRNA-206 (10 μg) + miRNA-21 (10 μg)
- pMIR-PolK-3'UTR-Luc (10 μg ) + Renilla Null (5 μg) + Negative control (20 μg)
- pMIR-PolK-3'UTR-Luc (10 μg ) + Renilla Null (5 μg) + miRNA-206 (20 μg)
- pMIR206-Luc (10 μg ) + Renilla Null (5 μg) + pcDNA3 (20 μg)
- pMIR206-Luc (10 μg ) + Renilla Null (5 μg) + FoxO3 TM (20 μg)
- pMIR206-Luc (10 μg ) + Renilla Null (5 μg) + Ikkβ (20 μg)
- pMIR21-Luc (10 μg ) + Renilla Null (5 μg) + pcDNA3 (20 μg)
- pMIR21-Luc (10 μg ) + Renilla Null (5 μg) + FoxO3 TM (20 μg)
- pMIR21-Luc (10 μg ) + Renilla Null (5 μg) + Ikkβ (20 μg)
- pcDNA3.1/V5-His-Empty (20 μg)+ YFP-LC3 (10 μg)
- pcDNA3.1/V5-His-YY1 (20 μg) + YFP-LC3 (10 μg)
- pcDNA3.1/V5-His-eIF4E3 (20 μg) + YFP-LC3 (10 μg)
- pGL2-Murf-1 promoter (5,5Kb)-Luc (10 μg) + Renilla Null (5 μg) + Negative Control (20 μg)
- pGL2-Murf-1 promoter (5,5Kb)-Luc (10 μg) + Renilla Null (5 μg) + miRNA-206 (20 μg)
- pGL2-Murf-1 promoter (5,5Kb)-Luc (10 μg) + Renilla Null (5 μg) + miRNA-206 (20 μg) + miRNA-21 (20 μg)
- pGL3-Atrogin-1 promoter (3,5Kb)-Luc (10 μg) + Renilla Null (5 μg) + Negative Control (20 μg)
- pGL3-Atrogin-1 promoter (3,5Kb)-Luc (10 μg) + Renilla Null (5 μg) + miRNA-206 (20 μg)
- pGL3-Atrogin-1 promoter (3,5Kb)-Luc (10 μg) + Renilla Null (5 μg) + miRNA-206 (20 μg) + miRNA-21 (20 μg)

## 3.2- Cell Culture

For *in vitro* experiments we used MEFs (Mouse embryonic fibroblasts) and C2C12 (Mouse myoblast) cells. Cells were maintained in culture with D-MEM (Dulbecco's modified Eagle's medium) (Invitrogen # 41966-029) containing 1% penicillin-streptomycin (Gibco # 15070) and 10% FBS (Fetal bovine serum) (Gibco # 10106-169). C2C12 cells were maintained in proliferation and were not differentiated into myotubes. Transfection was performed using the Lipofectamine 2000 reagent (Invitrogen # 11668-027) according to the manufacturer's protocol. One day prior to

the transfection, the cells were plated onto 6-well plates at a density of 100000 cells per well. The next day, cells were transfected with a total of 4 μg of plasmidic DNA per well if 6-well plate were being used or with 1.6 μg of plasmidic DNA per well if 12-well plate were being used. The cells were cultured for 4 h in Optimem (Invitrogen # 31985-047) without serum or antibiotics. Afterwards, the medium was changed, and the cells were incubated in Optimem supplemented with FBS and penicillin-streptomycin. Cells were used 24 or 48 h according to the experiments.

## 3.3- RNA and miRNA purification

For the micro array experiments mRNA and miRNAs were isolated form frozen gastrocnemius muscles from animals placed under different atrophic conditions and controls. The isolation of miRNAs/mRNAs was achieved by an initial purification with Trizol (Invitrogen #15596-026) followed by a subsequent purification and fractionation with the Purelink miRNA-Isolation Kit (Invitrogen #K1570-01). Briefly, frozen muscles were powdered in a mortal and were then homogenized in 1,5ml of trizol with the polytron. The aqueous phase (0,6 ml) was isolated according to the Trizol instructions and transferred to a new eppendorf. Ethanol 100% was added to arrive to a final concentration of 35% (0,32 ml). This solution was added to the first spin column from the Purelink miRNA Isolation Kit**.** After a centrifugation at 12000g for 1min at RT the flow through was kept, since it contains the miRNA fraction (RNA smaller than 200 nucleotides), as well as the first spin column that contains the mRNA fraction (RNA higher than 200 nucleotides). Ethanol 100% was added to the flow through in order to arrive to a final concentration of ethanol of 70% (1,07 ml). This solution was added to a second spin column and centrifuged at 12000g for 1 min at RT. The second column contains the miRNA fraction**.** Both spin columns were washed twice with washing buffer. miRNAs were eluted from the second column with 55 μl of DNAse/RNAse free water while mRNA were eluted from the first column with 200 ul of DNAse/RNAse free water. In this way miRNA and mRNAs were extracted from the same sample.

For the Real-Time PCR (RT-PCR) experiments total RNA was extracted from T.A., Gastrocnemius, EDL and Soleus muscle using the Trizol reagent according to the manufacturer's instructions. The same protocol was used for RNA purification from cells.

The quantification of the miRNA/mRNA was done in a NanoVue Plus (G&E Healthcare) and RNA integrity was assessed with the NanoRNA 6000 LabChip kit with a Agilent 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer's instructions.

## 3.4- Micro Array of miRNAs and mRNAs

For the microarray of miRNAs the *mirVana™miRNA Probe set* platform (1564 V1, October 2005)(Ambion) was used. This platform contains approximately 400 probes for human, murine and rat microRNAome present in the miRNA Registry (miRBase - Release 9). The probes were spotted on SCHOTT Nexterion® Slide E slides by a robotic station Microgrid II Biorobotics present at the CRIBI - Padova University. Each miRNA probe was represented in quadruplicate in the array. The array also contains at least two different spike probes in order to normalize the labeling procedure. Micro arrays of miRNAs were done by competitive hybridization in which the miRNA population of the atrophic samples was compared to the miRNA population of a pool of control samples mixed in equal amounts. Dye swap was performed in order to avoid dye bias. At least three biological replicates were performed for each atrophic condition. To label the miRNAs we used the mirVana™ miRNA Labeling Kit (Ambion #AM1562) and amine-reactive dyes (GE Healthcare) following the manufacturer's instructions. Briefly, 2 μg of purified miRNAs of each sample, together with 800 pg of spikes per sample, were poly-adenylated, purified, conjugated with Cy3 or Cy5 and purified for a second time. After this procedure, labeled miRNAs (and spikes) were incubated in the spotted slides for 21h at 42°C. Array acquisition was performed using a *ScanArray™ Lite*, (Perkin Elmer). In order to avoid false positives, a very stringent analysis was preformed. To achieve this, only the fluorescent values 40% above the background were utilized. Furthermore, only the values present in at least 65% of the microarray experiments were considered. Doing so, from the 384 probes present in the array only 159 were considered for further analysis. Some experiments were completely removed from the analysis since the results obtained with the inversion of the fluorophore were not consistent. In order to obtain a more up-dated information regarding the probes present in the array, miRNA probes were re-annotated using the miRbase database (http://microrna.sanger.ac.uk). At the end of this process from the 159 probes that passed the qualitative analysis only 118 matched murine miRNA. These miRNAs were analyzed using the software MacOSX MeV v4.2 (MultiExperiment Viewer) that allows the visualization, clusterization, classification and statistical analysis of the data .

The mRNA microarray experiments were conducted at the CRIBI by Matteo Silvestrin and Cristiano de Pittà. For the microarray, the Operon Mouse Oligo Set, version 1.1 was used. This platform contains approximately 13500 probes for the mouse genome. The probes were designed based on almost 14000 UNIGENE clusters from *M musculus*, mainly at the 3' terminal region. The probes were spotted on MICROMAX Glass Slides SuperChip I (PerkinElmer Life Science, Inc) using a robotic station Microgrid II Biorobotics present at the CRIBI - Padova University. Total

RNA was labeled using the MessageAmp aRNA Kit (Ambion). Briefly, 1 μg of total RNA was amplified using a T7 RNA polymerase, followed by a retro-transcription using an ArrayScript and a oligo($dT_{15}$) primer. This double-strand cDNA was then used to synthesize the antisense mRNA using the MEGAscript IV, a T7 RNA Polimerase that incorporates the 5-(3-ammnioallyl)-UTP (aaUTP). This aaUTP was used in the labeling reaction since it contains a reactive ammine group that binds to the NHS ester group of the Cy3 or Cy5 fluorophore (GE Healthcare). After labeled, these aRNAs were hybridized with the pre-spotted slides for 48h. Array acquisition was performed using a *ScanArray*™ *Lite*, (Perkin Elmer). Data was analyzed using the M.I.D.A.W Microarray Data Analysis Web Tool (Romualdi et al, 2005). This software allows background correction, global and local mean and variance normalization. The expression data was subsequently analyzed using the software MacOSX MeV v4.2 (MultiExperiment Viewer) that allows the visualization, clusterization, classification and statistical analysis of the data. In order to identify altered genetic pathways DAVID (Database for Annotation, Visualization and Integrated Discovery) database was used.

### 3.5- Integration of the data obtained with the mRNA expression profile with those of the miRNA expression profile.

In order to reduce the number of predicted targets, Matteo Silvestrin and Cristiano de Pittà from the CRIBI, developed a software that integrates the data from the mRNA micro-array and the data from the miRNA micro-array. Briefly, using the freely available predictive database (Miranda, TargetScan, Pictar and PITA), a list of all the predicted targets of the altered miRNAs was downloaded. The obtained list was crossed with the list of mRNAs whose expression was altered in the mRNA microarray. In order to cross this information the GeneMIR++ algorithm was used. This algorithm runs under the MatLab 7.4 (MathWorks) software and basically performs the statistical analysis of the biological conditions in which the expression levels of a certain mRNA is under the control of one or more miRNAs (Huang and Chow, 2007). This program consider only the miRNAs and the mRNAs that are expressed above the average expression level and gives back the highest probabilistic punctuation when the expression levels of the miRNAs and of the mRNAs are strongly inversely correlated and the lowest probabilistic punctuation when both miRNA and mRNA have the same expression pattern. In this way the huge list of predicted targets is reduced in a biological way since it includes the data from the mRNA expression analysis.

## 3.6- Validation of the microarray results

miRNA expression profiles were validated using the TaqMan® MicroRNA Assays (Applied Biosystems). According to the manufacturer's instructions, each 15 µl RT reaction contained 5 ng of total RNA, 3.0 µl of 5× stem-loop RT primer, 1× RT buffer, 0.25 mM of dNTPs, 50 U MultiScribe™ reverse transcriptase and 3.8 U RNase inhibitor. Reaction mixes were incubated in a MJ Mini Personal Thermo Cycler (BioRad) for 30 min at 16°C, 30 min at 42°C, followed by 5 min at 85°C, and then held at 4°C. RT products were diluted three times with 45 µl of $H_2O$ prior to setting up PCR reaction. For each sample RT-PCR was carried out in triplicates. Each 10 µl reaction mixture included 1 µl of diluted RT product, 5 µl of 2 × TaqMan® Universal PCR Master Mix and 0.5 µl of 20× TaqMan® MicroRNA Assay. The reaction was incubated in a 7900 HT Real-Time PCR System (Applied Biosystems) in 96- well plates at 95°C for 10 min, followed by 40 cycles of the following steps: 95°C for 15 sec and 60°C for 1 min. To evaluate differences in miRNA expression, we used a relative quantification method. The expression of the miRNA target was standardized by a non-regulated small non-coding RNA that works as reference. U6 small nuclear RNA (U6 snRNA) (Applied biosystems - Assay ID 001973) was used as endogenous control because the level of this small RNA remains constant in the different atrophic conditions. The miRNA studied were miRNA-21 (Applied biosystems - Assay ID 000397), miRNA-206 (Applied biosystems - Assay ID 000510) and miRNA-133b (Applied biosystems - Assay ID 002247). The relative expression ratio was calculated using the $2^{-\Delta\ \Delta Ct}$ (RQ, relative quantification) method.

Gene expression analysis was performed by RT-PCR. Complementary DNA was generated using Superscript III Reverse transcriptase (Invitrogen #18080044). Brifley, 400ng of total RNA was incubated at 65°C for 5 minutes together with 1µl Random primer hexamers (50 ng/µl random) and 1µl dNTPs (10 mM each). This mix was then incubated for 2 minutes on Ice to allow the primers to align to the RNA. After this incubation the following reagents were added: 4µl of First strand buffer 5x, 1µl of DTT 100mM, 1µl of RNase Out (Invitrogen #11777019) and 0,5µl of Superscript III Reverse Transcriptase. This mixture was then incubated in a MJ Mini Personal Thermo Cycler (BioRad) for 5 min at 25°C, 60 min at 50°C, followed by 15 min at 70°C, and then held at 4°C. At the end of the RT reaction, the volume of each sample was adjusted to 50µl with RNase free water. 1 µl of diluted cDNAs were amplified in 10 µl PCR reactions in an ABI Prism 7900HT (Applied Biosystem) thermocycler in 96-well plates. The Power SYBR Green PCR Master Mix (Applied Biosystem #4367659) was used for the Real-Time PCR reaction. Briefly, 5µl of

SYBR Green was mixed with 0,2μl of gene specific primers (50 μM each) and with 4μl of water. The PCR mix was then incubated in a 7900 HT Real-Time PCR System (Applied Biosystem) in 96-well plates at 95°C for 10 min, followed by 40 cycles of the following steps: 95°C for 15 sec and 60°C for 1 min.

| **Primer** | **Sequence** |
|---|---|
| Atrogin-1-Fw | 5'-GCAAACACTGCCACATTCTCTC-3' |
| Atrogin-1-Rv | 5'-CTTGAGGGGAAAGTGAGACG-3' |
| GAPDH-Fw | 5'-CACCATCTTCCAGGAGCGAG-3' |
| GAPDH-Rv | 5'-CCTTCTCCATGGTGGTGAAGAC-3' |
| Pan-Actin-Fw | 5'-CTGGCTCCTAGCACCATGAAGAT-3' |
| Pan-Actin-Rv | 5'- GGTGGACAGTGAGGCCAGGAT-3' |
| YY1-Fw | 5'-TGAGAAAGCATCTGCACACC-3' |
| YY1-Rv | 5'-CGCAAATTGAAGTCCAGTGA-3' |
| eIF4E3-Fw | 5'-AACATCCCTCCTGTGACCAG -3' |
| eIF4E3-Rv | 5'-TCCAATGGTCGCTAACAACA-3' |
| PDCD10-Fw | 5'-GGGCACTTGAACACCAAAAG-3' |
| PDCD10-Rv | 5'-CAGGCCACAGTTTTGAAGGT-3' |
| PolK-Fw | 5'-AGATCCCAGACACACCCTTG-3' |
| PolK-Rv | 5'-GTCACGTAGGGGAGGCATTA-3' |

Table 1: List of primers used in Real Time experiments

To evaluate differences in miRNA expression, a relative quantification method was chosen where the expression of the mRNA is standardized by a non-regulated gene used as reference. Two genes were used as a reference: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pan-Actin. The relative expression ratio was calculated using the $2^{-\Delta\ \Delta Ct}$ (RQ, relative quantification) method. The oligonucleotide primers used are described in Table 1.

## 3.7- Cross-sectional area measurements

Cross-sectional area of electroporated fibers was measured as described previously (Sandri et al., 2004a) and compared with the surrounding non electroporated myofibers (control). The pictures of the electroporated muscles were taken at 20x magnification. Fiber cross-sectional area

was measured using IMAGE software (Scion, Frederick, MD). All data are expressed as the mean ± SEM. Comparison were made by using the student's t test, with $p<0.05$ being considered statistically significant.

### 3.8- Luciferase assays

Luciferase measurements in muscles electroporated with reporter constructs were performed using the Dual-Luciferase Reporter Assay System (Promega # E1910) adapting the manufacturer's instructions. Briefly, muscles were powdered in liquid nitrogen before the addition of the lysis buffer. This suspension was submited to repeated cycles of freazing/thawn after which they are centrifuged (Serrano et al., 2001). The supernatant was analyzed according to the manufacturer's instructions. To control for transfection efficiency, firefly luciferase activity was divided by renilla luciferase activity. Results are expressed as means ± SD of at least three different animals.

In cells, luciferase assay was performed according to the manufacturer's instructions.

### 3.9- Cloning and Plasmids

Transfection and electroporation experiments were preformed with the following constructs. pmiRZIP lentivector (ZIP NULL), pmiRZIP lentivector anti-miRNA-206 (ZIP-206) and pmiRZIP lentivector anti-miRNA-21(ZIP-21) were acquired from System Bioscience. pMIR206-Luc and pMIR21-Luc were acquired from Signosis BioSignal. Renilla Null and Renilla TK were acquired from Promega (pRL-null # E2271, pRL-TK # ). FoxO3 TM was kindly provided by M.E. Greenberg**,** and Ikkβ was kindly provided by P. M. Cànoves.

The mature sequences of miRNA-206 and miRNA-21 were cloned into the BLOCK-iT PolII miR RNAi Expression Vector Kit with EmGFP (Invitrogen #K4936-00). The oligos used are shown in Table 2. As negative control the pcDNA6.2-GW/EmGFP-miR-neg control was used. This vector forms an hairpin structure that is processed into mature miRNA that is predicted not to target any known vertebrate gene.

| **Name** | **Sequence** |
|---|---|
| miRNA-206 – Top | 5’-TGCTGTGGAATGTAAGGAAGTGTGTGGGTTTTGGCCACTGACTGACCCACACACCC TACATTCA-3 |
| miRNA-206 – Bottom | 5’-CCTGTGAATGTAGGGTGTGTGGGTCAGTCAGTGGCCAAAACCCACACACTTCCTTACATTCCAC-3 |
| miRNA-21 – Top | 5’-TGCTGTAGCTTATCAGACTGATGTTGAGTTTTGGCCACTGACTGACTCAACATCTCTGATAAGCTA-3’ |
| miRNA-21 – Bottom | 5’-CCTGTAGCTTATCAGAGATGTTGAGTCAGTCAGTGGCCAAAACTCAACATCAGTCTGATAAGCTAC-3’ |
| YY1-3’UTR-Fw | 5’-GCCTCTTCAGGAGTGTGATTG-3’ |
| YY1-3’UTR-Rv | 5’-CAATTTCTGGGAGGCTCAAG-3’ |
| eIF4E3-3’UTR-Fw | 5’-TCTGCCATCGTATCACTTGC-3’ |
| eIF4E3-3’UTR-Rv | 5’-GCCTCTTACGCTCTGACCAC -3’ |
| PDCD10-3’UTR-Fw | 5’-ACTAGTCCAGGATGTTGAATGGGATT -3’ |
| PDCD10-3’UTR-Rv | 5’-GCGGCCGCAAGTAAAGAAATGTTTAACA-3’ |
| PolK-3’UTR-Fw | 5’-ACTAGTCCTTTAAGGAAGACAAGTGCAA-3’ |
| PolK-3’UTR-Rv | 5’-GCGGCCGCCAACAAAAATAAACTTCAGATGGAA-3’ |
| YY1-CR-Fw | 5’-GCCATGGCCTCGGGCGACACCCTCTAC-3’ |
| YY1-CR-Rv | 5’-CTGGTTGTTTTTGGCTTTAGCGTG-3’ |
| eIF4E3-CR-Fw | 5’-AACATGGCGCTGCCCCCGGCTGCC-3’ |
| eIF4E3-CR-Rv | 5’-GTGTTTTCCACGTCCACCTTCAAA-3’ |

Table 2: List of primers used for the cloning experiments

To clone the 3’UTR of the different analyzed genes the following strategy was taken. RNA from muscle was retro-transcribed into cDNA and the region of interest was amplified by PCR. The primers were design to contain SpeI or NotI restriction sites as shown in Table2. The PCR product was purified and cloned into the pCR2.1-TOPO Vector (Invitrogen #45-0641). Both pCR2.1-TOPO vector and the pMIR206-LUC were opened with the restriction enzymes DpeI+NotI. The insert was cloned into the pMIR-LUC. All Constructs were sequenced to confirm the insert and the absence of mutation.

| Primer | Sequence |
|---|---|
| **eIF4E3_mut-206_forw** | 5'-AAAGTCAGGGGCCTCCACTTGAAGCGCTAAACAGGAAGCCAAATTA-3' |
| **eIF4E3_mut-206_rev** | 5'-TAATTTGGCTTCCTGTTTAGCGCTTCAAGTGGAGGCCCCTGACTTT-3' |
| **eIF4E3_del-206_forw** | 5'-GGGCCTCCACTTGCGCTAAACAGGAAGC-3' |
| **eIF4E3_del-206_rev** | 5'-GCTTCCTGTTTAGCGCAAGTGGAGGCCC-3' |
| **eIF4E3_mut-21_forw** | 5’-GCCTAGCAAAACCCTTTTTCTGCTGCATTGTTGTGACACTTCCCTGCA-3' |
| **eIF4E3_mut-21_rev** | 5'-TGCAGGGAAGTGTCACAACAATGCAGCAGAAAAAGGGTTTTGCTAGGC-3' |
| **eIF4E3_del-21_forw** | 5'-TAGCAAAACCCTTTTTCTGAATTGTTGTGACACTTCCCTG-3' |
| **eIF4E3_del-21_rev** | 5'-CAGGGAAGTGTCACAACAATTCAGAAAAAGGGTTTTGCTA-3' |
| **Yy1_mut-1_forw** | 5'-GTGCATATTGTACACTTTTTGGGGATCTTATTAGTAATGCTGTGTGATTTTCTGGA-3' |
| **Yy1_mut-1_rev** | 5’-TCCAGAAAATCACACAGCATTACTAATAAGATCCCCAAAAAGTGTACAATATGCAC-3' |
| **Yy1_mut-2_forw** | 5'-GCTGTGTGATTTTCTGGAGGTTGATCGCTGTGCTTGCGGTAGATTTTCTTT-3' |
| **Yy1_mut-2_rev** | 5'-AAAGAAAATCTACCGCAAGCACAGCGATCAACCTCCAGAAAATCACACAGC-3' |
| **Yy1_del-1_forw** | 5'-CATATTGTACACTTTTTGGGGATATTAGTAATGCTGTGTGATTTTCT-3' |
| **Yy1_del-1_rev** | 5'-AGAAAATCACACAGCATTACTAATATCCCCAAAAAGTGTACAATATG-3' |
| **Yy1_del-2_forw** | 5'-GTGATTTTCTGGAGGTTGACTTTGCTTGCGGTAGATTT-3' |
| **Yy1_del-2_rev** | 5'-AAATCTACCGCAAGCAAAGTCAACCTCCAGAAAATCAC-3' |

Table 3: List of primers used for the mutagenesis experiments

In order to confirm the specific binding of the respective miRNAs on the 3’UTR of eIF4E3, the binding sites for miRNA-206 and for miRNA-21 were mutated. The approach used consisted in mutate or delete 3 nucleotides of the seed region of each miRNA-binding site. Therefore the following mutants were created: eIF4E3 Mut206, eIF4E3 Del206, eIF4E3 Mut21 and eIF4E3 Del21. For the mutagenesis of the eIF4E3-3’UTR-Luc vector the QuickChangeII site-directed Mutagenesis Kit (Stratagene #200524) was used. Briefly, 20ng of eIF4E3-3’UTR-Luc vector was used per reaction. A 50 µl reaction contained 5 µl of 10xReaction buffer, 250 ng of each primer, 1 µl of dNTP mix, 2.5 µl of DMSO (5% final concentration) and 1 µl of PFU ultra DNA polymerase. This mixture was then incubated in a MJ Mini Personal Thermo Cycler (BioRad) for 30 sec at 95°C to denature the vector followed by 20 cycles of 30 sec at 95°C, 1 min at 55°C, and 15 min at 68 °C and then held at 4°C. After this amplification step with the mutated primers, this reaction was digested for 2h with 1 µl of DpnI enzyme in order to degrade the original template. After this step, 4 µl of this digested reaction mix was transformed into XL-Gold Ultracompetent bacterias (Stratagene #200314) according to the instruction manual. All the mutated constructs were sequenced to confirm the presence of the desired mutations and the absence of other unspecific

mutations. The primers used for the mutagenesis are shown in Table 3. The same approach is currently being taken for the YY1-3’UTR-Luc vector.

The coding regions of YY1 and of eIF4E3 were cloned into the pcDNA3.1/V5-His TOPO (Invitrogen #K4800-01). Briefly, the coding regions of these genes were PCR amplified from skeletal muscle cDNA using the primers shown in Table 2. PCR products were purified and cloned into the pcDNA3.1/V5-His TOPO Vector according to the manufacturer’s instructions. All Constructs were sequenced to confirm the insert and the absence of mutation.

## 3.10- Protein extraction and Western blotting

Total proteins were extracted from MEFs or C2C12 by solubilization in lysis buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 1 mM EDTA, 0.5% Triton X-100, Phosphatase inhibitor cocktail 2 (Sigma #P5726) and Phosphatase inhibitor cocktail 3 (Sigma #P0044) and Complete EDTA-free protease inhibitor mixture (Roche # 11836145001). Protein concentration was quantified using the Bradford method (Bio-Rad Protein Assay - BioRad #500-0006).

Total proteins were extracted from whole muscle or from 20 slices of 20µm of thickness by using the following lysis buffer: 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 2% SDS, 1% Trirton X-100, 1mM DTT, Phosphatase inhibitor cocktail 2 and Phosphatase inhibitor cocktail 3 and Complete EDTA-free protease inhibitor mixture. Protein concentration was quantified using the BCA method ( BCA Protein Assay Kit – Thermo Fisher Scientific #23227).

50 µg of the whole-cell lysates was loaded and separated on 4–12% precast Bis–Tris NuPAGE gels (Invitrogen #NP0323). Proteins were transferred to Hybond-ECL Nitrocululose membrane (GE Healthcare #RPN303D) and stained with Ponceau S solution (Sigma-Aldrich) to verify the efficiency of the transfer. The blots were incubated in blocking buffer (TBS, 0.1% Tween 20, and 5% nonfat milk) for 1 h at room temperature. Membranes were washed in washing buffer (TBS and 0.1% Tween 20) three times for 5 min each, probed with the primary antibody in TBS, 0.1% Tween 20, and 2% nonfat milk overnight at 4°C, and then probed with the secondary antibody for 1 h in TBS, 0.1% Tween 20, and 2% nonfat milk. The antibody reaction was analyzed using the ECL method (SuperSignal West Pico Chemiluminescent substrate - Thermo Fisher Scientific #34080).

The following antibodies from Santa Cruz Biotechnology were used: anti-YY1 (C-20) (# sc-281) and anti-eIF4E3 (V-22) (sc-133542).

# Chapter 4 – miRNA expression signature in different atrophic condition

## 4.1- Overview of the miRNA expression profiles in different atrophic conditions.

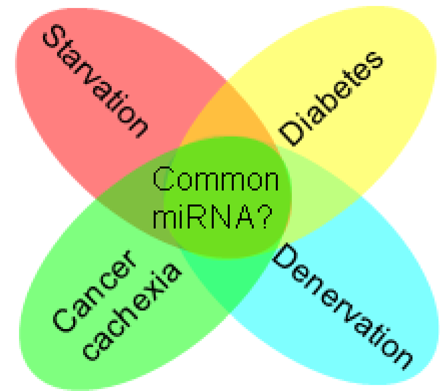



**Fig 14: Do multiple types of skeletal muscle atrophy share common miRNAs?**

Gene expression analysis of muscles resulting from different atrophic conditions allowed the identification of a group of atrophy-related mRNAs that are transcriptionally regulated. To understand if these catabolic conditions also regulate miRNAs expression and if atrophy-related miRNAs exist, we carried out comparative miRNA expression profiles. Briefly, a pool of miRNAs obtained from control gastrocnemius muscles was hybridized, in a competitive way, with miRNAs obtained from gastrocnemius muscles of diabetic-, starved- and denervated-mice. This approach identified miRNAs specific of each catabolic condition and miRNAs that are shared in more than one condition.

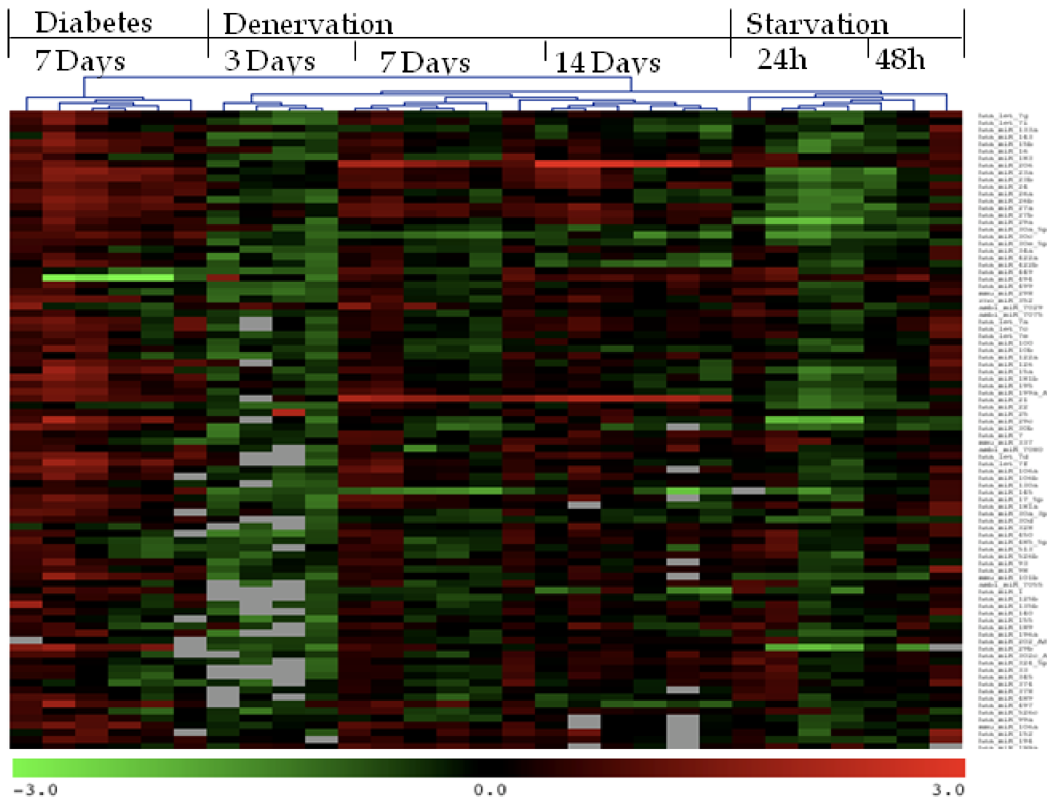



**Fig 15: miRNA expression profile in different atrophic condition**. Hierarchical clustering of atrophic samples compared to the respective controls. Ninety miRNAs were differently expressed between controls and atrophic gastrocnemius (n≥3). A color code represents the relative intensity of the expression signal when compared to the control samples, with red indicating higher expression levels and green indicating lower expression levels.

A platform that contains approximately 400 known miRNA (miRBase - Release 9) was used for the miRNA expression microarray. Importantly, starvation and denervation were analyzed at different time points to understand the kinetic of the miRNA response in these atrophic conditions.

The miRNAs expression profiles obtained for each atrophic condition were hierarchically clustered using the Pearson correlation coefficient. Using this approach a significant association of the different atrophic conditions was obtained. According to the hierarchical clustering dendrogram (Fig 15.) the two different starvation time points grouped together. These two conditions were extremely complicated to analyze since the different biological replicates were very variable among them. Still, according to the obtained results there is no significant variation between the two conditions. Also denervation experiments grouped together, mainly at 7 and 14 days. Short term denervation, 3 days, gave a peculiar expression profile since the majority of the analyzed miRNAs were unaltered or were down-regulated when compared to the controls. A completely independent profile was obtained from the diabetic muscles (7 days after treatment with STZ) in which the vast majority of the studied miRNAs appeared up-regulated. Altogether these results show that each atrophic condition has a particular miRNA expression profile. Furthermore, the results seem to indicate that the miRNA regulation is delayed compared to mRNA control. In fact mRNA changed their expression profile at earlier time points, immediately after the catabolic condition appears, while miRNA changes require a certain time. For instance, in denervated muscle the peak of mRNA changes happens at 3 day of denervation while the peak of miRNA alteration occurs at 14 day of denervation.

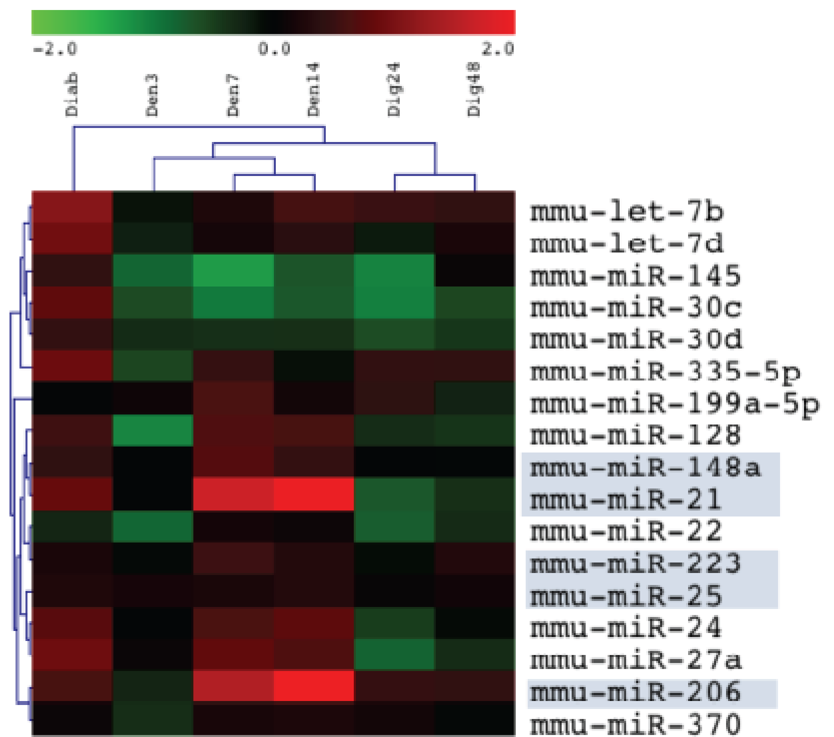



**Fig 16: List of miRNAs that are significantly deregulated in atrophic conditions ($p<0.05$).** The expression value results from the average of the Ln (atrophic sample/control) and represented in a colorimetric scale.

A more detailed statistical analysis was performed to try to identify possible miRNAs that change their expression in all the atrophic conditions. This analysis was not conclusive as we can see from Fig 16 that represents the miRNAs commonly altered in all atrophic conditions. Although the expression of these miRNAs was deregulated, their expression pattern is not the same in all the studied conditions.

Nevertheless, the analyses of each atrophic condition allowed the identification of the miRNAs that are persistently up- or down-regulated at different times and allow to determine whether these miRNAs are shared by another atrophic condition. Using this approach a group of 52 miRNAs were found to be deregulated in at least 3 of the 6 conditions studied (Fig 17). According to this analyze most of the deregulated miRNAs are up-regulated in long lasting atrophic conditions. Indeed, there are 21 miRNAs up-regulated after 7 days of denervation, 32 miRNAs up-regulated after 14 days of denervation and 61 miRNAs up-regulated after 7 days of STZ-induced diabetes. Among the up-regulated miRNAs two of them are particularly over-expressed during denervation. These are miRNA-21 and miRNA-206. Indeed, these two miRNAs are also up-regulated during STZ-induced diabetes and miRNA-206 is also induced after 48h of starvation. Also the Let-7 family is induced in 3 atrophic conditions, 14 days of denervation, diabetes and 48h of starvation. This is also the case of miRNA-181a that is induced in 7 days of denervation, diabetes and 48h of starvation.

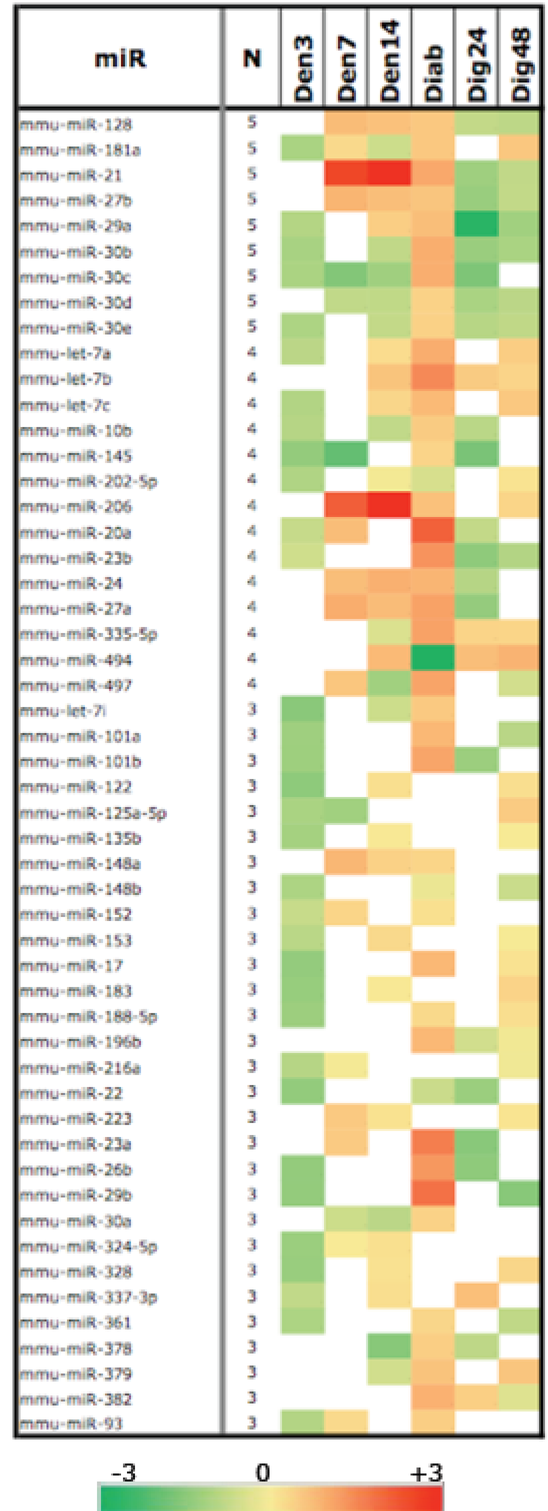



**Fig 17: Schematic representation of the miRNAs differently expressed in at least 3 of the 6 atrophic conditions studied** ($p<0.05$)

About the down-regulated miRNAs, the family of miRNA-30 is interesting since the expression of several members is reduced during denervation (7 and 14 days) and during starvation. Also miRNA-145 is strongly inhibited during these conditions. Conversely, miRNA-494 is peculiar since it is the only miRNAs down-regulated during STZ-induced diabetes.

## 4.2- Validation of the microarray results.

To obtain a more accurate quantification and to validate the results from the miRNA expression profile we decided to use TaqMan quantitative real time RT-PCR.

The previous analyses have underlined that the two most and commonly up-regulated miRNAs were miRNA-21 and miRNA-206. In the microarray, miRNA-206 expression levels were increased in denervation, mainly after 7 and 14 days from the cut of the sciatic nerve, diabetes and 48h of starvation. On the other hand, miRNA-21 was only up regulated in denervation, 7 and 14 days, and diabetes while it is down-regulated in starvation.

When we monitored the expression of miRNA-206 in denervated muscles by quantitative RT-PCR we confirmed a progressive up-regulation of its expression that reaches its maximum of 20 fold induction at day 14 after denervation (Fig 18). 48 hours of starvation led to a two-fold up-regulation of miRNA-206 expression levels while STZ-induced diabetes didn't significantly altered miRNA-206 expression levels.

MiRNA-21 showed a different pattern of expression being up-regulated already at 3 days of denervation and then remaining constantly at this level during the other time points (Fig 19).

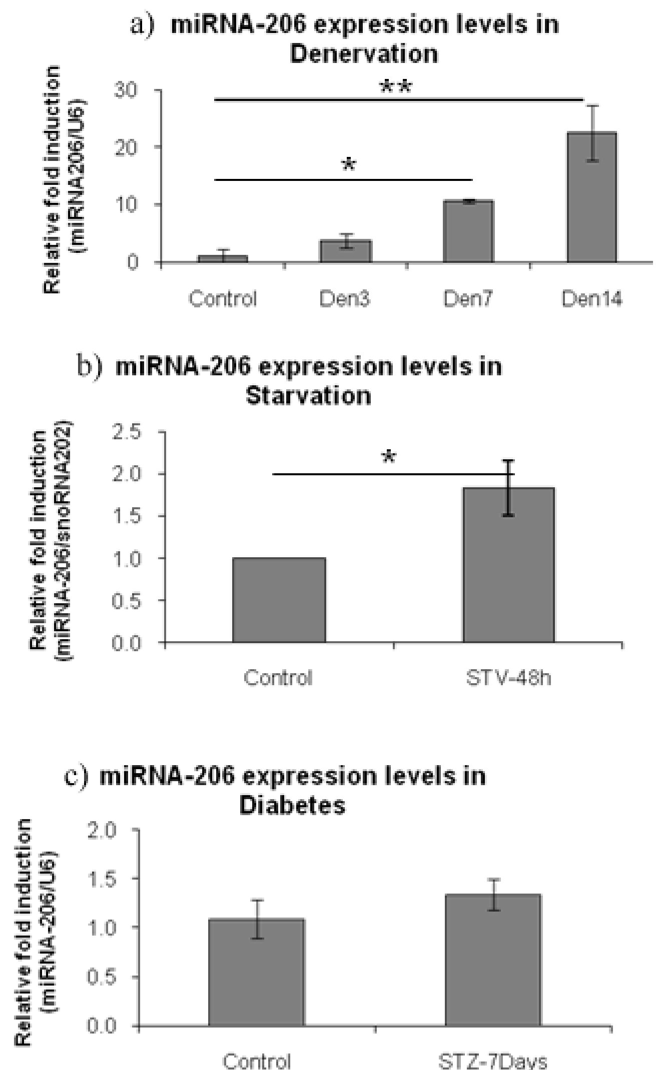



**Fig 18: miRNA-206 expression levels during different atrophic conditions quantified by TaqMan RT-PCR**. (a) Quantification of miRNA-206 expression level during different time points of Denervation (n=3 for each time point). U6 was used to normalize the expression levels. (b) Quantification of miRNA-206 expression level after 48h of Starvation (n=3). SnoRNA202 was used to normalize the expression levels (c) Quantification of miRNA-206 expression level during Streptozotocin–induced diabetes (n=5). U6 was used to normalize the expression levels. Values represent mean ± STD. * $p<0.05$, ** $p<0.01$ by T-Test.

Starvation on the other hand leads to a 50% decrease in the expression level of this miRNA while diabetes didn't significantly change its expression level. Altogether, these results confirmed the data obtained in the microarray except for the diabetes condition.

Considering these results we decided to focus on denervation since it is the atrophic condition that induces a more robust up-regulation of these two miRNAs. In order to better validate the functional meaning of this up-regulation we decided to use a luciferase assay. This approach reveals the activity of endogenous miRNAs. Briefly, we have used a vector that, in the 3'UTR of the Luciferase gene contains a binding site for the studied miRNA. In this way, an up-regulation of the miRNA expression level leads to a reduction in the luciferase protein. These miRNA-Luc sensors were electroporated into TA muscles of adult mice and then animals were denervated. As shown in Fig 20, Luciferase activity of miRNA21 and miRNA 206 sensors is significantly reduced after 7 days of denervation. These findings support the results of the expression profiling and indicate an increase activity of both miRNAs during denervation.

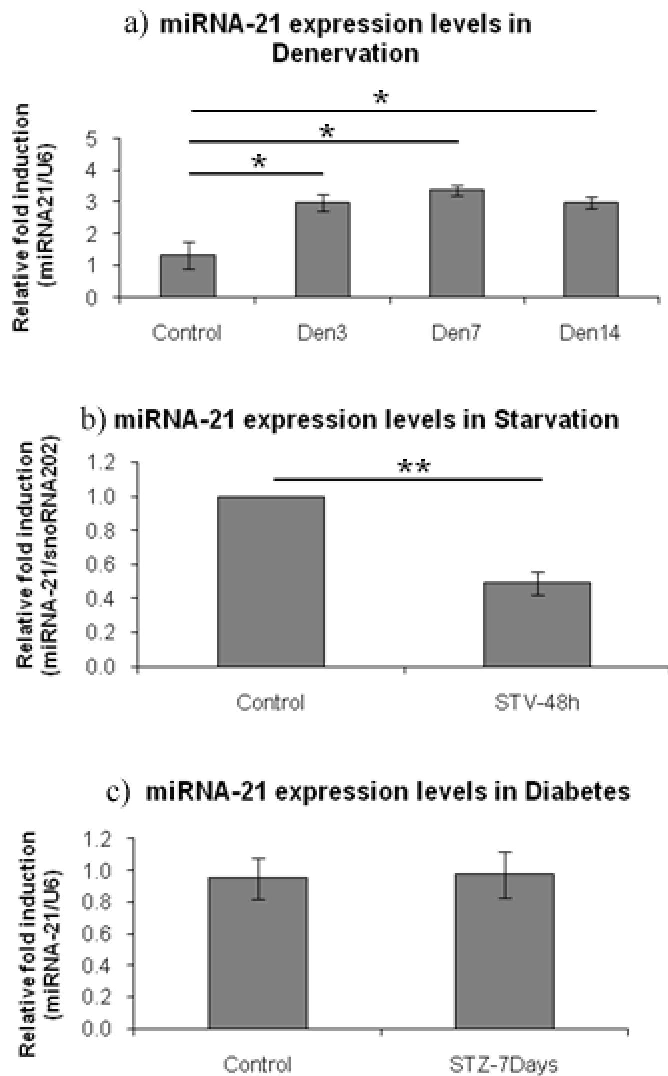
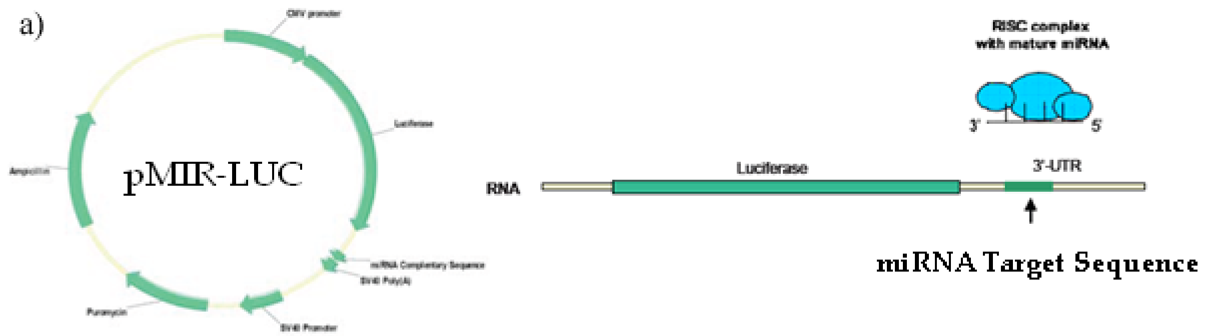



**Fig 19: miRNA-21 expression levels during different atrophic conditions quantified by TaqMan RT-PCR.** (a) Quantification of miRNA-21 expression level during different time points of Denervation (n=3 for each time point). U6 was used to normalize the expression levels. (b) Quantification of miRNA-21 expression level after 48h of Starvation (n=3). SnoRNA202 was used to normalize the expression levels. (c) Quantification of miRNA-21 expression level during Streptozotocin–induced diabetes (n=5). U6 was used to normalize the expression levels. Values represent mean ± STD. * $p<0.05$, ** $p<0.01$ by T-Test.



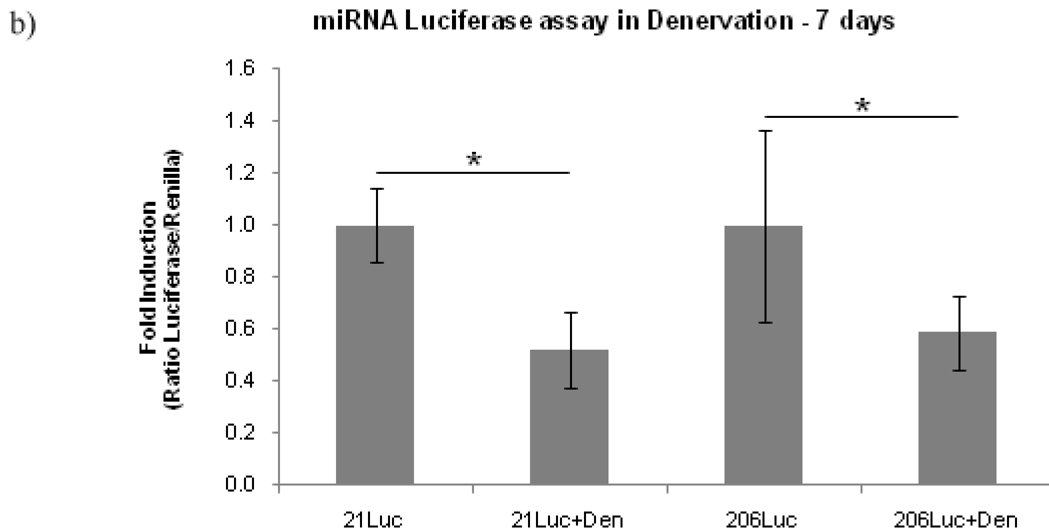




**Fig 20: miRNA-206 and miRNA-21 are up-regulated during Denervation**. (a) Schematic representation of the pMIR-LUC vector used to quantify the miRNA expression levels by luciferase assay. These vectors contain a CMV promoter, a firefly Luciferase Gene and a unique miRNA binding site at the 3'UTR region of the Luciferase gene . Whenever these miRNA is expressed, it binds to the perfectly complementary sequence resulting in repression of the luciferase gene expression. More than detecting the mature miRNAs levels this method measures miRNAs activity. (b) Quantification by Luciferase Assay of the miRNA-206 and miRNA-21 activity after 7 days of denervation. T.A. muscles were electroporated with 10ug of Luciferase sensor (206Luc or 21Luc) and with 5ug of Renilla Null (to normalize electroporation). One of the legs was denervated by severing the sciatic nerve. Luciferase levels were measured 7 days after denervation. ($n \geq 4$) Values represent mean ± STE. * $p<0.05$ by T-Test.

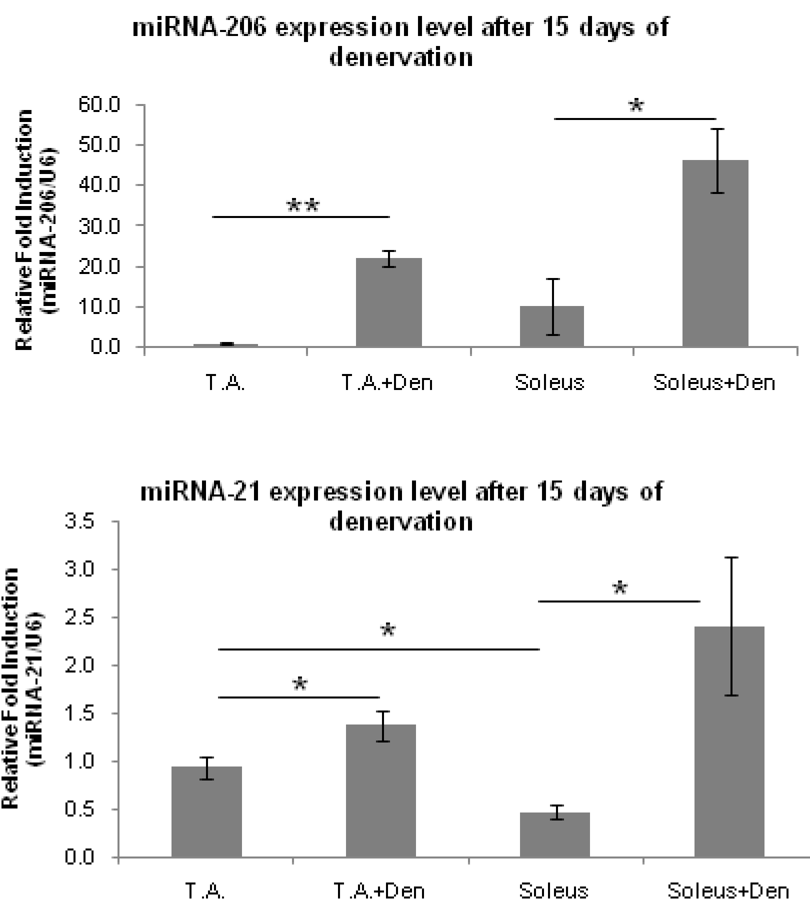



**Fig 21: Denervation leads to an up-regulation of miRNA-206 and miRNA-21 both in fast and slow muscles. (a)** Quantification of miRNA-206 expression level by TaqMan RT-PCR after 15 days of denervation in T.A and in Soleus muscle. (n=4). U6 was used to normalize the expression levels. **(b)** Quantification of miRNA-21 expression level by TaqMan RT-PCR after 15 days of denervation in T.A and in Soleus muscle. (n=4). U6 was used to normalize the expression levels. Values represent mean ± STE. * $p<0.05$, ** $p<0.01$ by T-Test.

These results confirm a functional induction of miRNA-21 and mRNA-206 in atrophying muscles. However, different muscles have different properties and therefore differently respond to catabolic signals. In order to understand whether the miRNA response after 15 days of denervation was similar between fast and slow muscles we evaluated the expression level of miRNAs in TA, a glycolitic muscle, and Soleus, an oxidative muscle. Importantly, the expression of miRNA-206 significantly increased in both TA and Soleus denervated muscles Fig 21. Interestingly, the basal levels of miRNA-206 in the Soleus was higher than in the TA. To what concerns miRNA-21, 15 days of denervation also induced its expression in both types of muscles. The basal expression of miRNA-21 was slightly lower in Soleus than in TA but the increase was more important in slow than fast muscle. Altogether these results suggest an involvement of miRNA-206 and miRNA-21 in muscle atrophy during denervation.

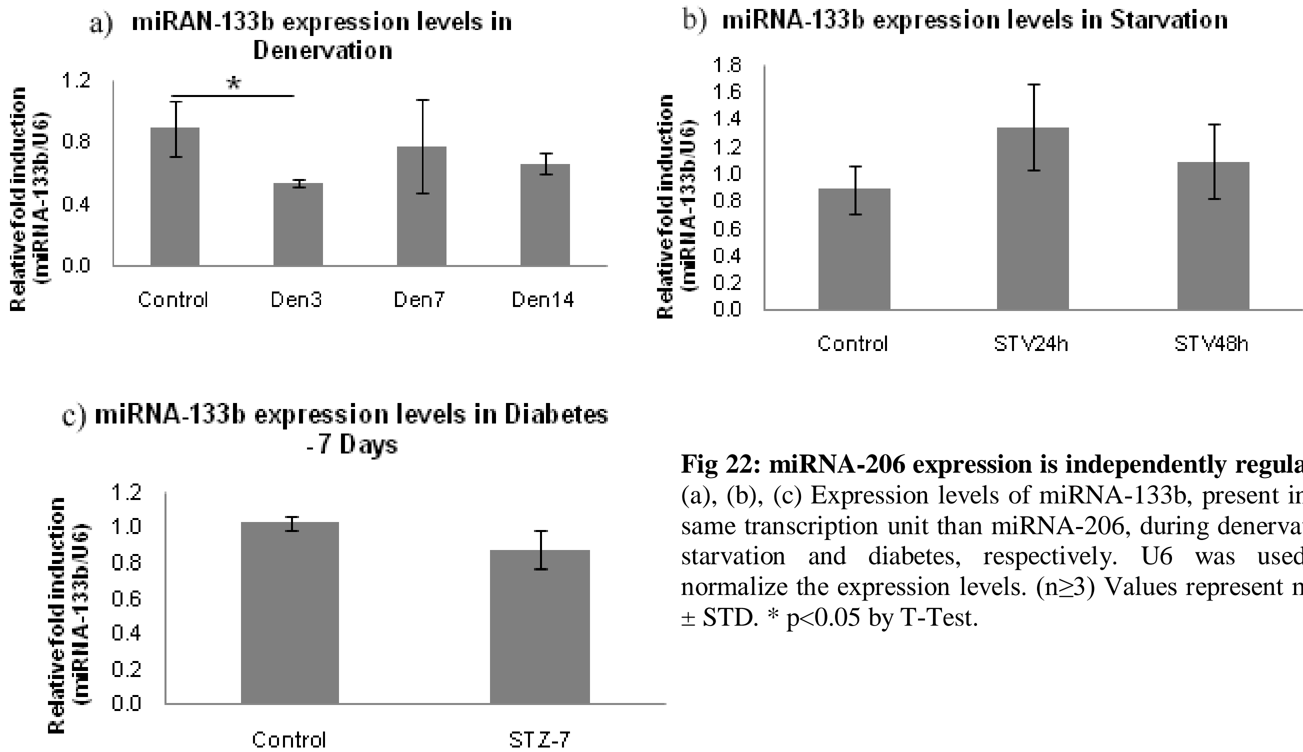



**Fig 22: miRNA-206 expression is independently regulated**. (a), (b), (c) Expression levels of miRNA-133b, present in the same transcription unit than miRNA-206, during denervation, starvation and diabetes, respectively. U6 was used to normalize the expression levels. (n≥3) Values represent mean ± STD. * p<0.05 by T-Test.

## 4.3- miRNA-21 and miRNA-206 expression is independent of its host genes

A close analysis at the genomic localization of these miRNAs, revealed that miRNA-206 is a intergenic polycistronic miRNA encoded together with miRNA-133b, while miRNA-21 is located on the $10^{th}$ intron of the protein coding gene Vacuole Membrane Protein 1 (VMP1/TMEM49).

Considering that miRNA-133b is embedded in the same genomic region of miRNA-206 and that shows the same muscle-specific pattern of expression of miRNA206, we monitored whether its expression changes in response atrophy. Fig 22 shows that miRNA-133b was significantly down–regulated after 3 days of denervation, returning later on to basal level. During starvation and diabetes miRNA133b did not significantly change the level of expression. These results suggest that miRNA-206 is regulated independently of its polycistronic companion, miRNA-133b.

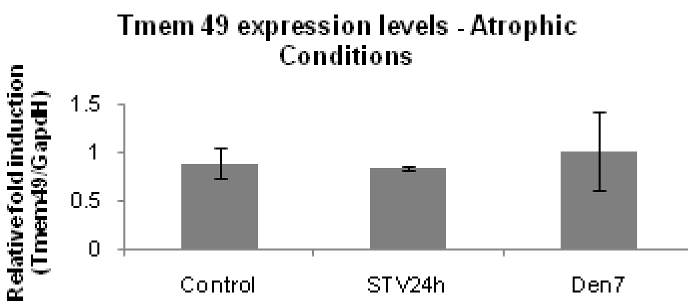



**Fig 23: miRNA-21 expression is independently regulated.** Expression levels of Tmem49, the host gene of miRNA-21, during starvation (24h) or denervation (7 days). GAPDH was used to normalize the expression levels of TMEM49. (n≥3) Values represent mean ± STD. * p<0.05 by T-Test.

Interestingly VMP1, is a protein implicated in the autophagic process since it is localized in the autophagossome and interacts with Beclin-1 (Vaccaro et al., 2008). As discussed already, autophagy is

one of the processes implicated in the protein degradation during skeletal muscle atrophy and consequently we decided to analyze the expression levels of this protein in denervation and starvation. Since the mRNA levels of VMP1 remain constant in all the atrophic conditions studied, we concluded that miRNA-21 is transcribed independently from its host gene confirming previous published data. (Fujita et al., 2008).

## 4.4- miRNA-21 and miRNA-206 expression levels are affected by FoxO3 and NF-κB

Skeletal muscle atrophy is a tightly regulated process at the transcriptional level, being FoxO3 and NF-κB two of the most important players. On the other hand miRNAs transcriptional regulation is a process poorly understood. Thus we decided to investigate whether FoxO3 and NF-κB transcriptional factors are also implicated in the up-regulation of these two miRNAs. Initially, the consensus binding sites for FoxO3, G/ATAAAT/CA., and for NF-κB, GGGA/GNNT/CT/CCC, were searched in the 10 Kb region upstream pre-miRNA-21 and pre-miRNA-206. As documented in Fig 24 the putative promoter region of miRNA-206 contains 4 binding sites for FoxO and 1 for

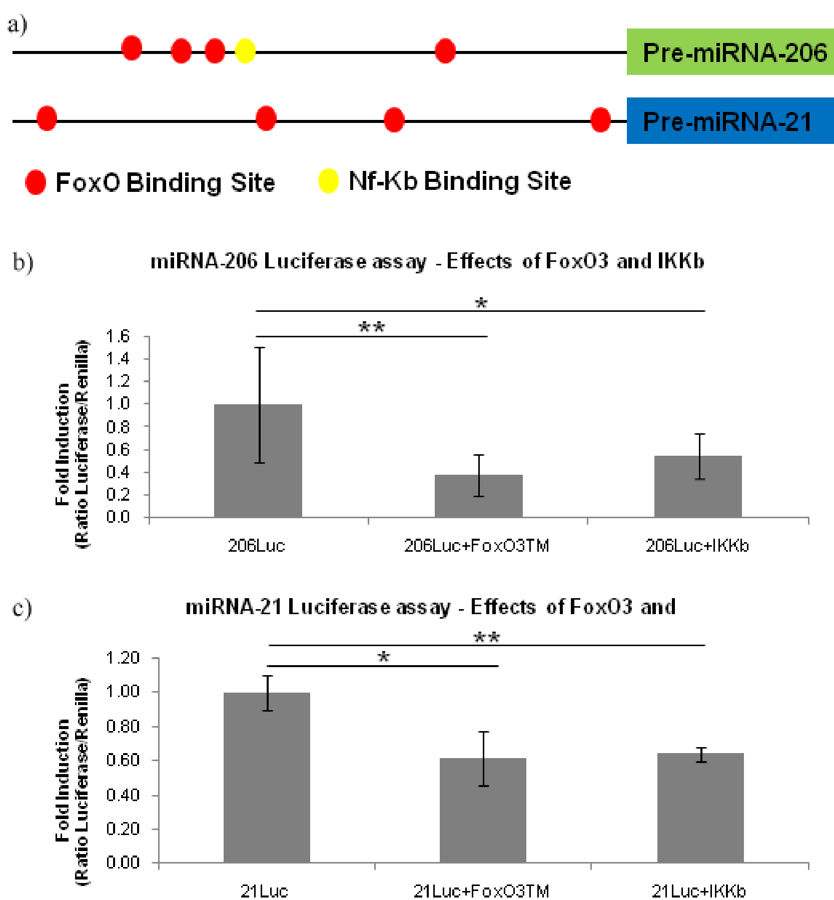



**Fig 24: miRNA-21 and miRNA-206 expression levels are affected by FoxO3 and NF-Kb**. (a) Schematic representation of the regulatory region of the miRNA-206 and miRNA-21. The binding site for FoxO and Nf-Kb were searched in the 10Kb up-stream each of these miRNAs. (b) FoxO3TM and IKKb increase the activity of miRNA-206. 10µg of the luciferase sensor for miRNA-206 (206Luc) were electroporated in T.A. muscle in the presence of 20µg of pcDNA3 (empty vector), FoxO3TM or IKKb expressing vector. A Renilla vector (5µg) was cotransfected to normalize for transfection efficiency. Fifteen days later, Luciferase/Renilla levels were measured. (n=8) Values represent mean ± STD. * $p<0.05$, ** $p<0.01$ by T-Test. (c) FoxO3TM and IKKb increase the activity of miRNA-21. 10µg of the luciferase sensor for miRNA-21 (21Luc) were electroporated in T.A. muscle in the presence of 20µg of pcDNA3 (empty vector), FoxO3TM or IKKb expressing vector. A Renilla vector (5µg) was cotransfected to normalize for electroporation efficiency. Fifteen days later, Luciferase/Renilla levels were measured. (n=4) Values represent mean ± STD. * $p<0.05$, ** $p<0.01$ by T-Test.

NF-κB. On the other hand miRNA-21 contains 4 binding sites for FoxO and no predicted binding site for NF-κB.

To understand whether these transcription factors can affect miRNA expression TAs were electroporated either with c.a.FoxO3 or with IKKβ, an activator of NF-κB (Cai et al., 2004), together with the Luciferase sensor for miRNA-206 or for miRNA-21. The presence of FoxO3 or NF-κB significantly decreased the levels of luciferase sensor for both miRNA-206 and miRNA-21, consistent with an up-regulation of both miRNAs (Fig 24). However, these experiments can't distinguish between a direct regulation of miRNAs by these transcription factors and an indirect effect consequent to muscle atrophy. To further investigate these two possibilities we have taken advantage of muscle specific FoxO1,3,4 KO animals that have been generated in the Sandri Lab. These mice show low levels of FoxO1, FoxO3 and FoxO4 proteins. We denervated these animals and the expression levels of these miRNAs were measured. Denervation led to a similar increase of

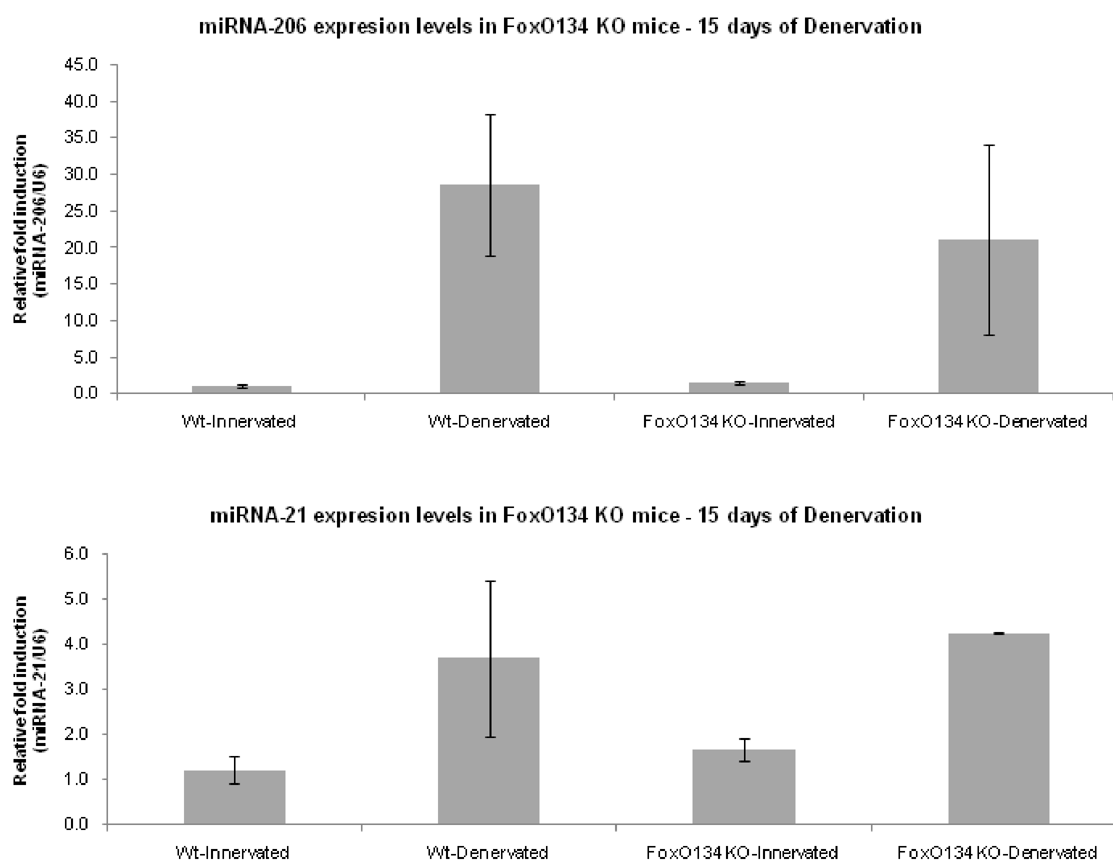



**Fig 25: FoxO is not responsible for the denervation-induced up-regulation of miRNA-21 and miRNA-206** (a) FoxO is not required for the up-regulation of miRNA-206 expression levels. TaqMan RT-PCR for miRNA-206 was performed on RNA extracted from T.A. muscles of WT and FoxO 1, 3 and 4 triple knock-out mice. Denervated muscles, 15 days after sciatic nerve severing, were compared to the contra-lateral muscles. (n=2) Values represent mean ± STD. * $p<0.05$, ** $p<0.01$ by T-Test. (b) FoxO is not required for the up-regulation of miRNA-21 expression levels. TaqMan RT-PCR for miRNA-21 was performed on RNA extracted from T.A. muscles of WT and FoxO 1, 3 and 4 triple knock-out mice. Denervated muscles, 15 days after sciatic nerve severing, were compared to the contra-lateral muscles. (n=2) Values represent mean ± STD. * $p<0.05$, ** $p<0.01$ by T-Test.

both miRNAs in Wt and KO animals (Fig 25), indicating that FoxOs are not necessary for the transcriptional regulation of these 2 miRNAs. The same analysis was impossible to do for NF-κB since we don't have available the respective knockouts mice.

## 4.5- miRNA-206 and miRNA-21 cooperate synergistically to induce skeletal muscle atrophy

In order to address the biological role of miRNA-206 and miRNA-21 in adult skeletal muscle we have cloned the mature sequence of these miRNAs into a co-cistronic vector that also express the GFP under the control of the same CMV promoter (Fig 26). When used *in vivo*, GFP fluorescence allows the recognition of the electroporated fibers over-expressing the mature miRNA. The over-expression of these vectors in C2C12 myoblasts lead to a significant up-regulation of the mature miRNAs, as detected by TaqMan RT-PCR (Fig 27) In order to deliver these vectors *in vivo*, we used the electroporation technique. This approach is designed to deliver DNA vectors specifically into adult skeletal muscle fiber, without transfecting other cell types present in the muscle, including the satellite cells, the muscle stem cell. To confirm that mature

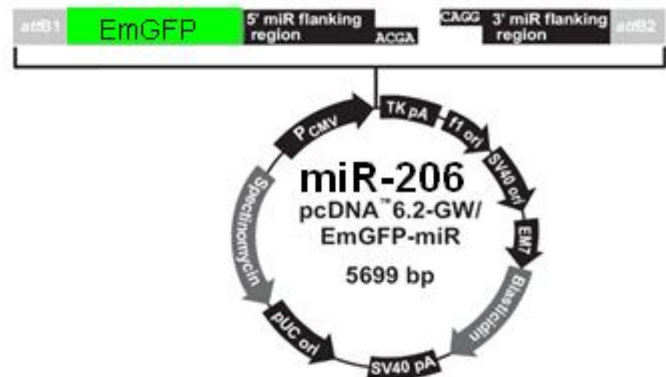



**Fig 26: miRNA overexpressing vector.** The pcDNA6.2-GW/EmGFP-miR vector makes possible the over-expression of the pre-miRNAs. Under a CMV promoter, this co-cistronic vector allows the expression of the GFP together with the desired pre-miRNA. When electroporated *in vivo*, the presence of the GFP permits the identification of the electroporated fibers.

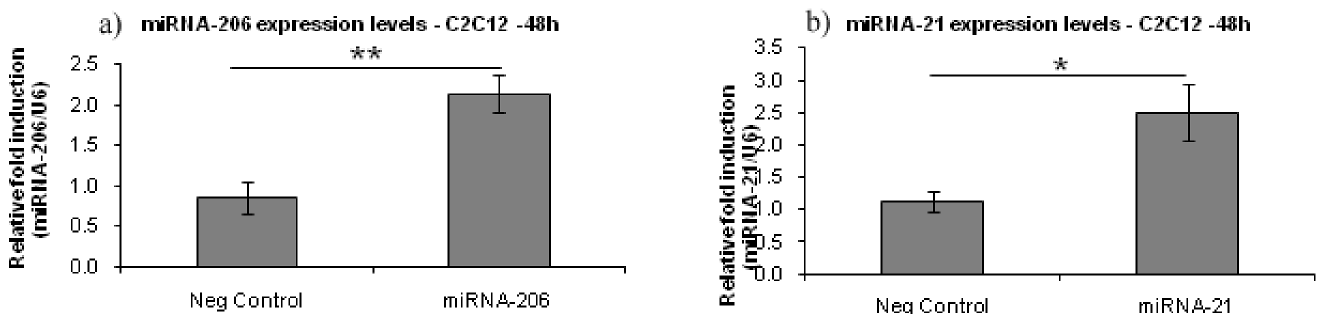



**Fig 27: Over-expressing vectors efficiently induce an *in vitro* up-regulation of the miRNAs levels** (a), (b) Transfection of the miRNAs- over-expressing vectors into C2C12 myoblasts leads to an effective increase in the mature miRNAs levels. TaqMan RT-PCR for miRNA-206 (b) or for miRNA-21 (c) was performed on RNA extracted 48h after the transfection with the respective over-expressing vectors (4µg). U6 was used to normalize the expression levels of the miRNAs. (n=3) Values represent mean ± STD. * $p<0.05$, ** $p<0.01$ by T-Test.

miRNAs are expressed only in adult fibers we stained the transfected muscles with a α-dystrophin antibody. Dystrophin is expressed only in adult myofiber and is localized immediately below the plasma membrane. As expected, GFP expression has been found only within dystrophin positive fibers (Fig 28 a,b) . Importantly, no mononucleated cells dystrophin negative were positive for GFP, indicating that only adult skeletal muscle fibers were electroporated. We then monitored the level of expression of mature miRNA in transfected muscles. Indeed, we found an up-regulation of 2-3 fold in these muscles. (Fig 28 c,d).

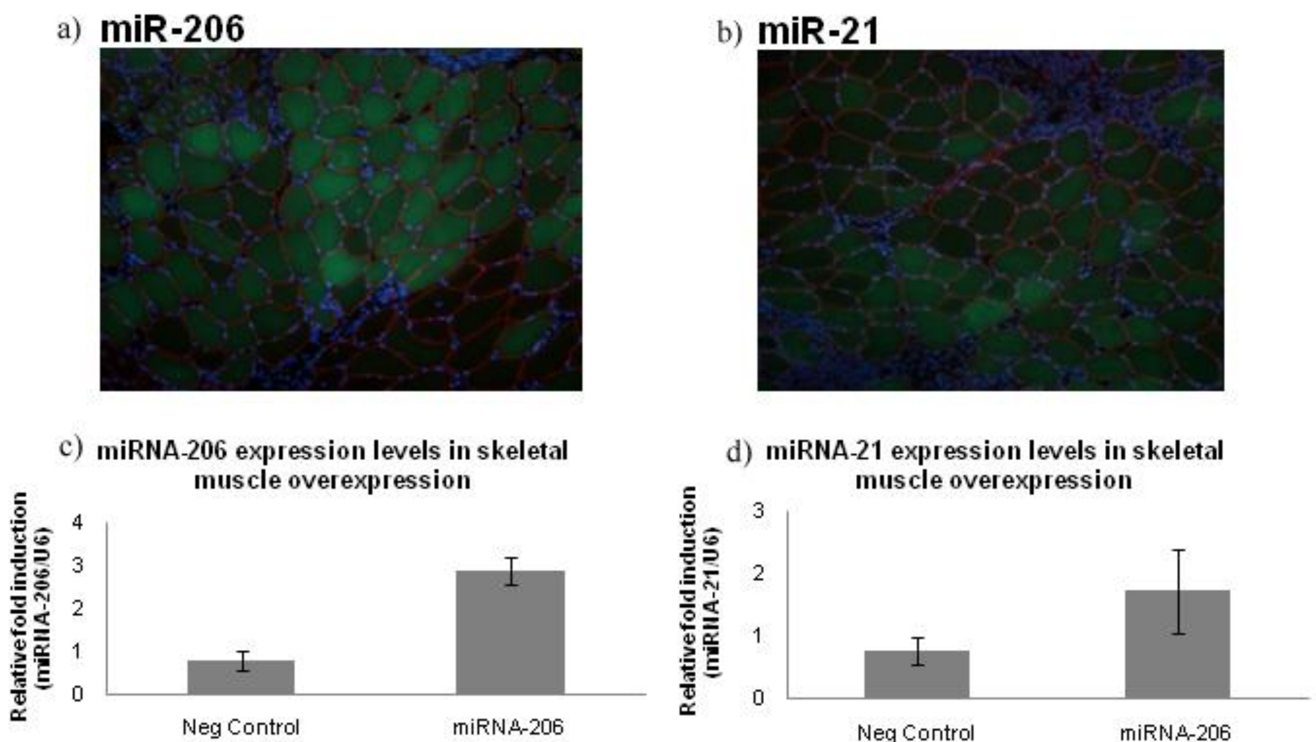



**Fig 28: Over-expressing vectors efficiently induce an *in vivo* up-regulation of the miRNAs levels** (a), (b) Adult muscle fibers are efficiently electroporated with the miRNAs over-expressing vectors (20µg). GFP staining denotes the presence of the EmGFP-miRNA over-expressing vector for miRNA-206 (a) or miRNA-21 (b). In red, anti-dystrophin staining delineates the fiber boundaries. In blue, DAPI staining evidences the fiber nuclei. (c), (d) Electroporation of the miRNAs over-expressing vectors into adult T.A. muscles leads to an effective, but variable, increase in the mature miRNAs levels. TaqMan RT-PCR for miRNA-206 (c) or for miRNA-21 (d) was performed on RNA extracted from T.A. muscles, 10 days after electroporation with the respective over-expressing vectors (20µg). U6 was used to normalize the expression levels of the miRNAs. (n=2) Values represent mean ± STE.

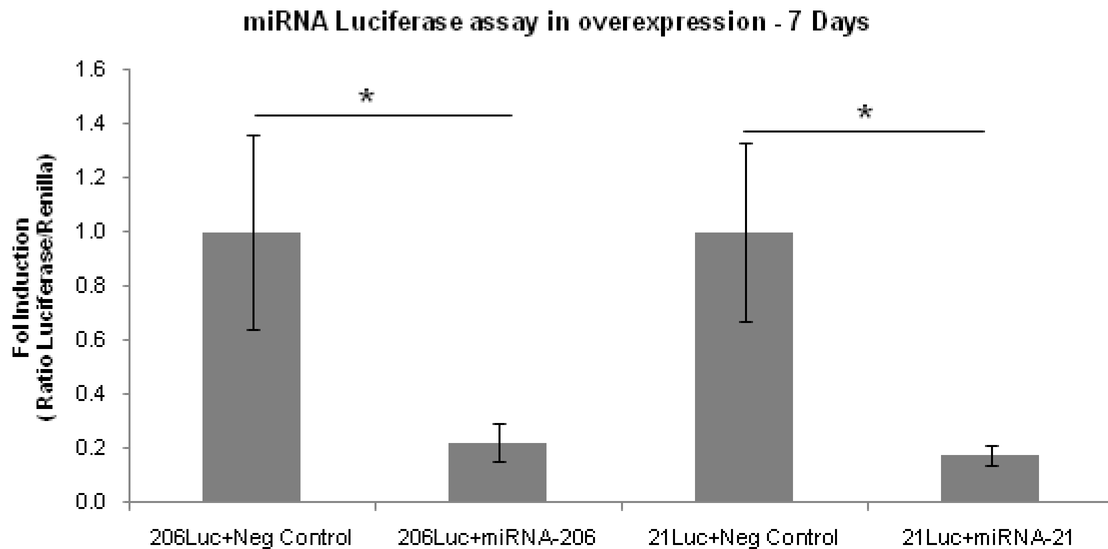



**Figure 29: *In vivo* electroporation of miRNA-206 and miRNA-21 efficiently increase the activity of these two miRNAs**. Electroporation of the Luciferase sensor (10µg) for each miRNAs in the presence of the respective miRNAs (20µg) decrease the Luciferase levels, 7 days after electroporation. Renilla (5µg) was cotransfected to normalize for electroporation efficiency. (n=5) Values represent mean ± STE. * $p<0.05$, by T-Test.

To further prove that the over-expressed miRNA were also functional and able to suppress translation of target transcript in vivo we used the luciferase miRNA sensor (Fig 29). Muscles were electroporated with the specific sensor of each miRNA in the presence or absence of the miRNA vector. Over-expression of miRNA-206 or miRNA-21 dramatically reduced by 80% the luciferase of their specific sensors confirming that these vectors are able to produce functional miRNAs *in vivo*.

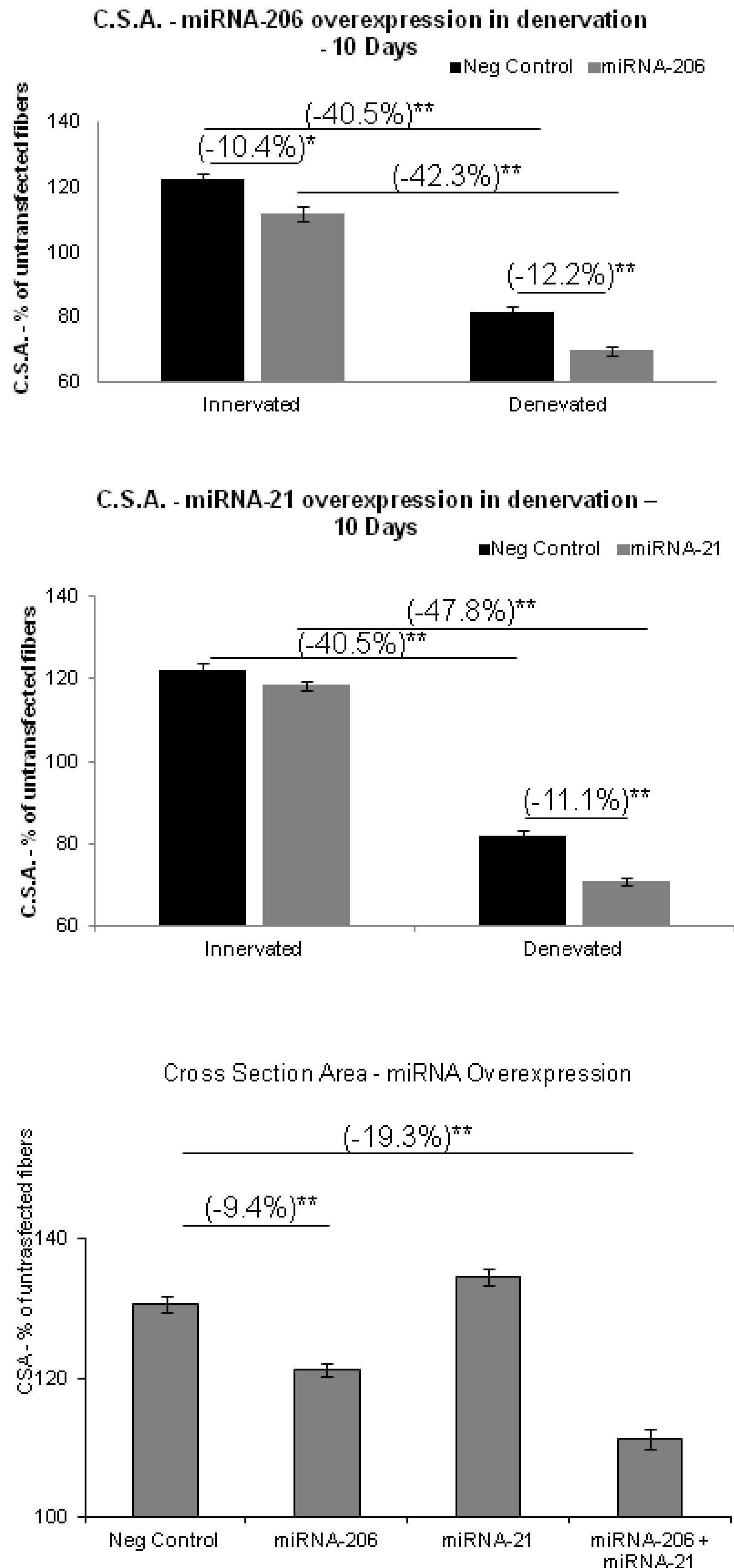



**Fig 30: miRNA over-expression does not protect from denervation induced atrophy but rather it induces an atrophic phenotype.** (a) Over-expression of miRNA-206 during denervation induces a reduction in the C.S.A. of the fibers. Adult T.A. muscles were electroporated with the Neg. Control (20µg) or with the miRNA-206 (20µg) over-expressing vector. Simultaneously, the right leg was denervated by severing the sciatic nerve. Cross-sectional area of transfected fibers, identified by the presence of GFP, was measured 10 days after electroporation (n≥3 muscles) Values represent mean ± STE. ** $p<0.01$, by T-Test. (b) Over-expression of miRNA-21 in denervated muscles induces a reduction in the C.S.A. of the fibers. Adult T.A. muscles were electroporated with the Neg. Control (20µg) or with the miRNA-21 (20µg) over-expressing vector. Simultaneously, the right leg was denervated by severing the sciatic nerve. Cross-sectional area of transfected fibers, identified by the presence of GFP, was measured 10 days after electroporation (n≥3 muscles) Values represent mean ± STE. ** $p<0.01$, by T-Test. (c) Over-expression of miRNA-206, at basal conditions, induces a reduction in the C.S.A. of the fibers and the presence of miRNA-21 potentiates this effect. Adult T.A. muscles were electroporated with the Neg. Control (20µg), miRNA-206 (20µg), miRNA-21 (20µg) or both miRNAs (20µg each). Cross-sectional area of transfected fibers, identified by the presence of GFP, was measured 10 days after electroporation (n≥3 muscles) Values represent mean ± STE. * $p<0.05$, ** $p<0.01$, by T-Test.

Next we used these vectors to over-express these miRNA in normal muscle, in order to mimic the denervated muscles, and in denervated muscle to anticipate their expression. In fact, we have shown that the miRNA response to an atrophic stimulus is delayed when compared to the transcriptional response. In fact, atrogin-1 and Murf-1 mRNA reach the peak of their expression at 3 days of denervation and start to return to basal expression

immediately. Conversely, the expression of miRNA-206 is elevated at 7 days of denervation and reaches its peak at 14 days while miRNA 21 reach a plateau at 3 days of denervation that is maintained afterward. Therefore, we have hypothesized that these miRNAs might slow down the atrophic program by down regulating critical atrophy-related transcripts. In fact muscle loss occurs mainly during the first 7 days of denervation and then it is progressively reduced especially after 14 days of denervation. To test this hypothesis we have electroporated miRNA expressing vectors in denervated and respective controlateral muscles and then we have monitored myofiber size. Interestingly, the expression of miRNA206 was sufficient to induce a 10% decrease of CSA when compared to controls (Fig 30 a, c). Moreover miRNA 206 over-expression did not protect from atrophy (Fig 30a). Denervated muscle fibers over-expressing miRNA-206 are 10% smaller than denervated controls. These findings suggest that miRNA-206 does not interfere with the atrophy program but instead is part of this process. When miRNA-21 was expressed in control innervated muscles, no major differences were observed when compared with negative controls. However, after denervation, miRNA21 expression enhanced muscle atrophy by 11% (Fig 30b). These results suggest that these 2 miRNAs don't have a role in slowing down the atrophic process, but rather might contribute to potentiate the atrophic program.

In order to understand whether they can have a synergistic action we expressed the two miRNAs separately or in combination in normal muscles (Fig 30c). When we measured the myofiber size we could confirm that miRNA-206 induced a significant reduction in the CSA while miRNA-21 didn't show any effect on CSA. However, the co-expression of both miRNAs showed a synergistic effect, leading to a 20 % reduction in the CSA.

### 4.6- miRNA-206 activates the promoter of Atrogin-1

Given that these two miRNAs contributes to myofiber atrophy we monitored whether they were able to regulate the expression of Atrogin-1 and Murf-1, the two E3-ligases involved in protein breakdown. Thus, we transfected adult TA with the luciferase sensors that contain the promoter region of Atrogin-1 or MuRF1 in presence or absence of the miRNAs. As shown in Fig 31, only miRNA-206 was able to activate the Atrogin-1 promoter. Surprisingly, the co-expression of both miRNAs did not alter the activity of the promoter. On the other hand, the promoter of Murf-1 was repressed by the presence of both miRNAs. Therefore, it is possible that miRNA-206 causes atrophy at least partially via activation of atrogin-1.

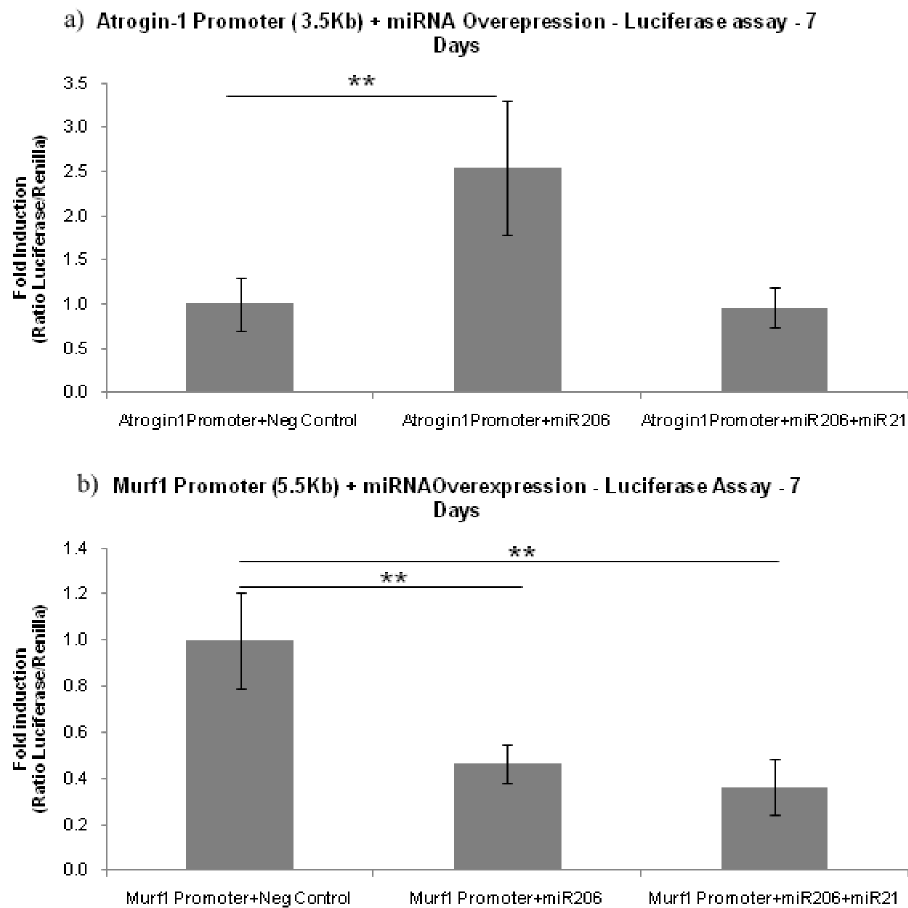



**Fig 31: Effect of the over-expression of miRNAs on the promoter activity of atrogin1 and murf1**. Electroporation of the promoter fused with the luciferase sensor (10µg) for atrogin1 and murf1 in the presence of miRNA206 (20µg) or miRNA-206+miRNA-21(20µg each). Renilla (5µg) was co-electroporated to normalize for electroporation efficiency. 7 days after electroporation muscles were analyzed (n=4) Values represent mean ± STD. ** $p<0.01$, by T-Test.

## 4.7- Inhibition of miRNA-206 and miRNA-21 partially protects from denervation-induced atrophy.

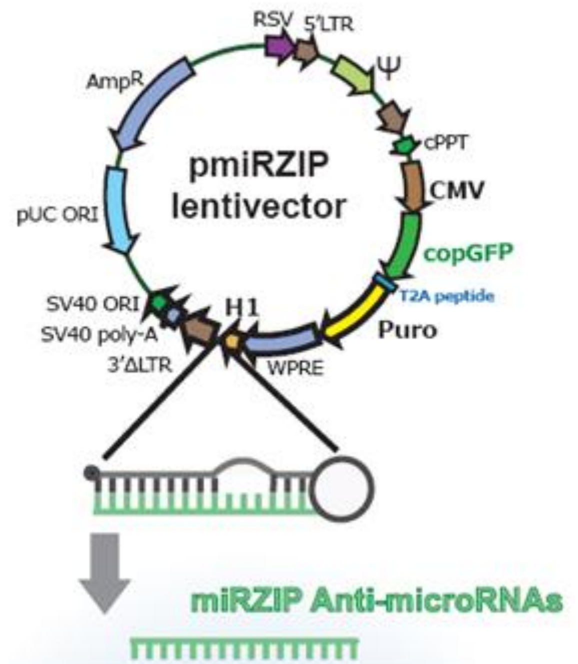



**Fig 32: Vector to over-express the antisense of specific miRNAs**. The pmiRZIP lentiviral vector express a hairpin interfering RNA that is design to generate a full-length antisense miRNA that specifically target and antagonize a specific endogenous miRNA. These hairpins are transcribed from a constitutive H1 promoter. In order to identify the transfected/electroporated fibers these vector also express a copGFP, under a CMV promoter.

Since the over-expression of these miRNAs induces atrophy, we wondered whether their inhibition was protective against denervation-induced atrophy.

The inhibition of miRNAs can be accomplished by the use of RNA molecules with different steric modifications. These molecules can be systemically delivered by intra-venous or sub-cutaneous injection, however the side effects of this systemic inhibtion are not well known. To avoid this problem we used a DNA vector, called miRZIP (Fig 32), that continuously produces the antisense RNA molecule for the miRNA of interest. Therefore, the produced anti-sense RNA molecules bind to the miRNA and act as sponge reducing the amount of free miRNA that can bind to the targets. This vector also expresses the GFP under a CMV promoter, therefore allowing the visualization of the electroporated fibers. To validate the function of these vectors we performed a luciferase assay in C2C12 muscle cell line. Briefly, a luciferase sensor for miRNA-21 or for miRNA-206 was transfected together with an empty miRZIP (Zip Null) vector

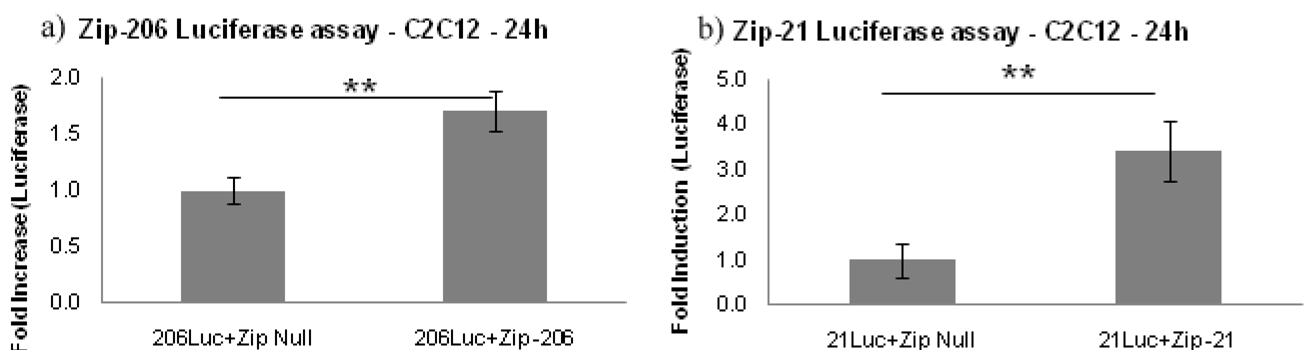



**Fig 33: Vectors to inhibit specific miRNAs efficiently reduce the miRNAs levels *in vitro***. (a), (b) Transfection of the miRZIP over-expressing vectors into C2C12 myoblasts leads to an effective decrease in the activity of the mature miRNAs. Luciferase assay for miRNA-206 (b) or for miRNA-21 (c) was performed on C2C12 myoblasts. Cells were transfected with the luciferase sensor for the respective miRNAs (0.5µg) in the presence of the empty vector or in the presence of the specific miRZIP vector (3.25µg). Renilla (0.25µg) was co-transfected to normalize for transfection efficiency. 24h after transfection Luciferase levels were measured. (n=3) Values represent mean ± STD. ** $p<0.01$ by T-Test.

or with miRZIP-206 or with miRZIP-21 respectively. Each specific miRZIP successfully increased the luciferase activity (Fig 33). These data are consistent with a reduction of the free miRNAs and therefore confirm that these vectors can efficiently block the target miRNA.

In order to test whether these vectors could down-regulate endogenous miRNAs *in vivo*, we transfected innervated and denervated muscles with the sensor for miRNA-206 together with Zip Null or with miRZIP-206. As expected, denervated muscles expressing the Zip Null showed a decrease in the luciferase activity that is consistent with an increase in the miRNA-206 expression level. However the muscles expressing the miRZIP-206 displayed an increase in the Luciferase activity both in innervated and denervated muscles confirming that we efficiently blocked free miRNA-206 in normal and atrophic muscles (Fig 34). It was not possible to perform the same analysis for miRNA-21 since miRZIP-21, *per se*, affects the expression of the Renilla, the vector that was used to normalize the efficiency of transfection.

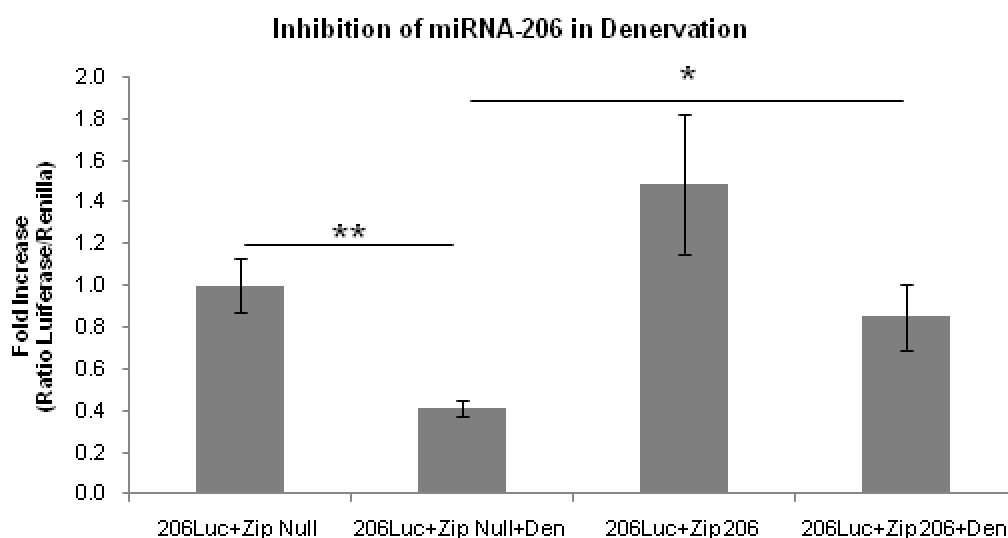



**Fig 34: Vectors to inhibit specific miRNAs efficiently reduce the miRNAs activity *in vivo*.** Expression of pmiRZIP lentiviral vector *in vivo,* during denervation, efficiently reduce the endogenous levels of miRNAs. Adult T.A. muscles were electroporated with the Luciferase sensor for miRNA-206 (10µg) together with the empty miRZIP (10µg) or with miRZIP-206 (10µg). A Renilla vector (5µg) was co-electroporated to normalize for electroporation efficiency. Simultaneously, mice were submitted to unilateral transection of the sciatic nerve. 7 days later the Luciferase/Renilla levels were measured. (n=6) Values represent mean ± STE. *$p<0.05$ and ** $p<0.01$ by T-Test.

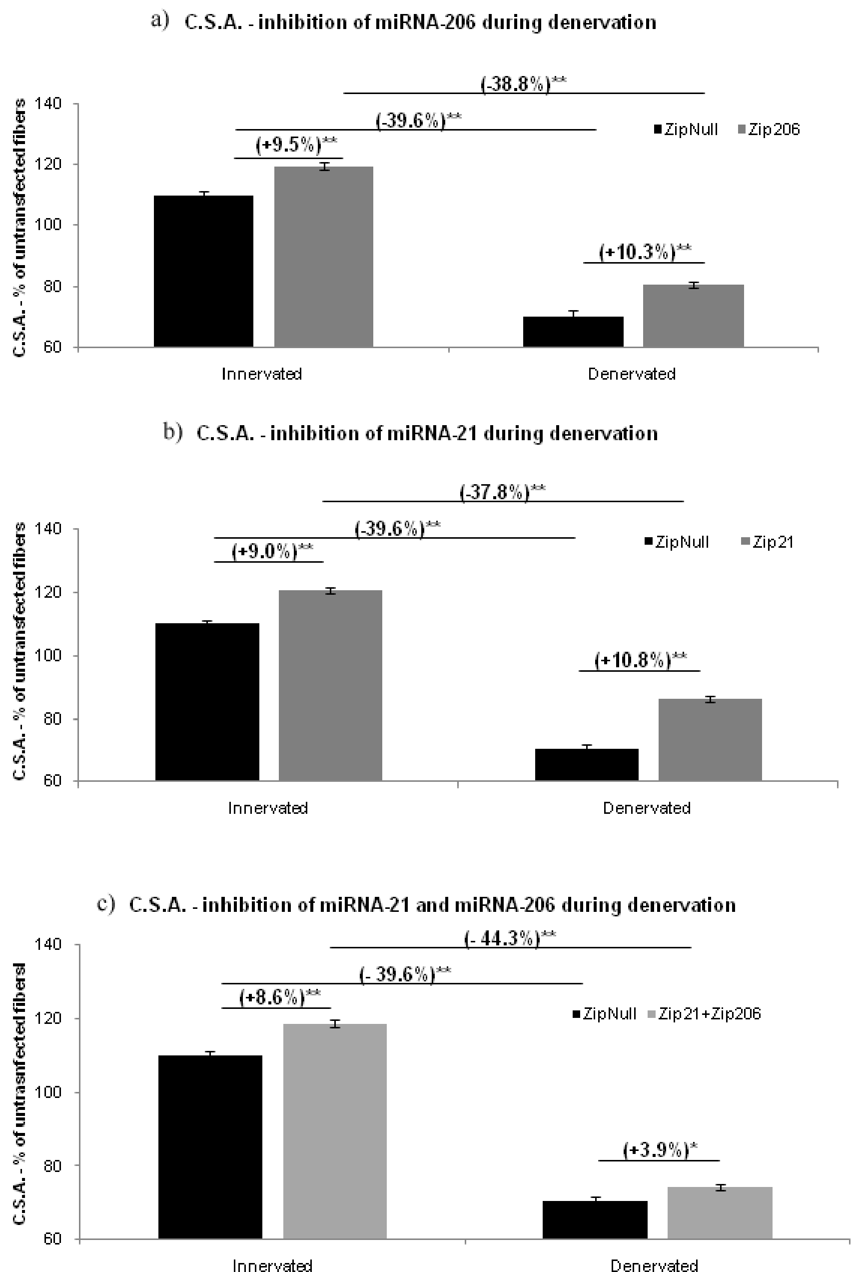



**Fig 35: Inhibition of miRNA-206 and miRNA-21 partially protects from denervation induced atrophy by promoting a 10% hypertrophy.** (a) Inhibition of miRNA-206 during denervation increases the C.S.A. of the fibers. Adult T.A. muscles were electroporated with the empty vector (10µg) or with the miRZIP-206 (10µg). Simultaneously, the right leg was denervated by severing the sciatic nerve. Cross-sectional area of transfected fibers, identified by the presence of GFP, was measured 7 days after electroporation. (n≥3 muscles) Values represent mean ± STE. ** p<0.01, by T-Test. (b) Inhibition of miRNA-21 during denervation increases the C.S.A. of the fibers. Same conditions as in a). (n≥3 muscles) Values represent mean ± STE. ** p<0.01, by T-Test. (c) Inhibition of both miRNA-206 and miRNA-21 during denervation increases the C.S.A. of the fibers. Adult T.A. muscles were electroporated with the empty vector (10µg) or with the miRZIP-206 (10µg) together with miRZIP-21 (10µg). Same conditions as in a). (n≥3 muscles) Values represent mean ± STE. *p<0.05 and ** p<0.01, by T-Test.

Having demonstrated that these miRZIP vectors are able to reduce significantly the levels of endogenous miRNAs, we then monitored whether the inhibition of miRNA-206 and miRNA-21 could reduce atrophy in denervated muscles and promote muscle growth in normal muscles. Thus, we transfected the different miRZIP vectors in innervated and denervated TA. The CSA was measure after 7 days of denervation. The inhibition of miRNA-206 and miRNA-21 per se was sufficient to induce a 10% hypertrophy in the innervated and denervated muscles. However, the simultaneous inhibition of these miRNAs was not additive (Fig 35).

The fact that miRNA over-expression induces atrophy and their inhibition induces hypertrophy is consistent and suggests that these miRNAs are modulating the expression of a negative regulator of the atrophic process.

# Chapter5 – Identification of target genes regulated by miRNA-206 and miRNA-21

We have shown that two miRNAs, miRNA-206 and miRNA-21, were particularly over-expressed during denervation-induced muscle atrophy. The over-expression of these two miRNAs in adult muscle, per se, was sufficient to induce muscle atrophy and their inhibition, always *in vivo*, induced an increase in the fiber size. In order to understand the molecular mechanisms that are underneath this regulation, we need to understand which target genes these miRNAs are regulating. In the second part of the results of this thesis we describe the approach that we used to identify the most probable target genes. We have also validated the most interesting ones as true targets of miRNA-206 and miRNA-21. The next step will be to address the biological role of these targets during the process of skeletal muscle atrophy.

## 5.1- Skeletal muscle atrophy is a transcriptionally regulated process

The prediction of the miRNAs targets is one of the most challenging aspects of the miRNA biology since each miRNA can have hundreds or thousands of predicted targets. As discussed before, one of the mechanisms by which miRNAs post-transcriptionally modulate gene expression is by inducing target mRNA degradation. Being supported by several evidences, this mechanism facilitates the identification of the miRNAs targets. If this mechanism prevails, one has only to consider the mRNAs that show an opposite regulation of the miRNAs. Despite the fact that miRNAs can also regulate the translation of the target genes, we decided to focus on the identification of the miRNA-induced mRNA degradation. This approach allowed a significant reduction of the huge number of predicted targets.

The first step of this process was to perform the mRNA micro-array on the same samples that have been used to characterize the miRNA expression profile. From the 13439 probes present in the array, only 8519 had a fluorescence signal above the background in all the 18 samples analyzed. In order to update the annotation of the different probes, the Gene ID conversion tool from DAVID (http://david.abcc.ncifcrf.govconversion.jsp) was used. At the end of the re-annotation only 6833 probes were associated to a single GeneSymbol, and these probes were used for further analyzes.

An hierarchical cluster analyze was performed using the Pearson correlation index that is the most robust method to group genes and samples that present similar expression motifs independently of the expression levels. As shown in the dendrogram each atrophic condition had a particular expression profile that allowed to group them in different clusters. In fact, and as it happened for the miRNA expression profile, samples collected 7 and 14 days after denervation grouped together when compared to the ones collected 3 days after denervation. Also 24 and 48h of starvation gave similar expression profiles that clustered together. Again, diabetes induced by streptozotocin is the most divergent condition that seems to be unrelated to any of the previous condition.

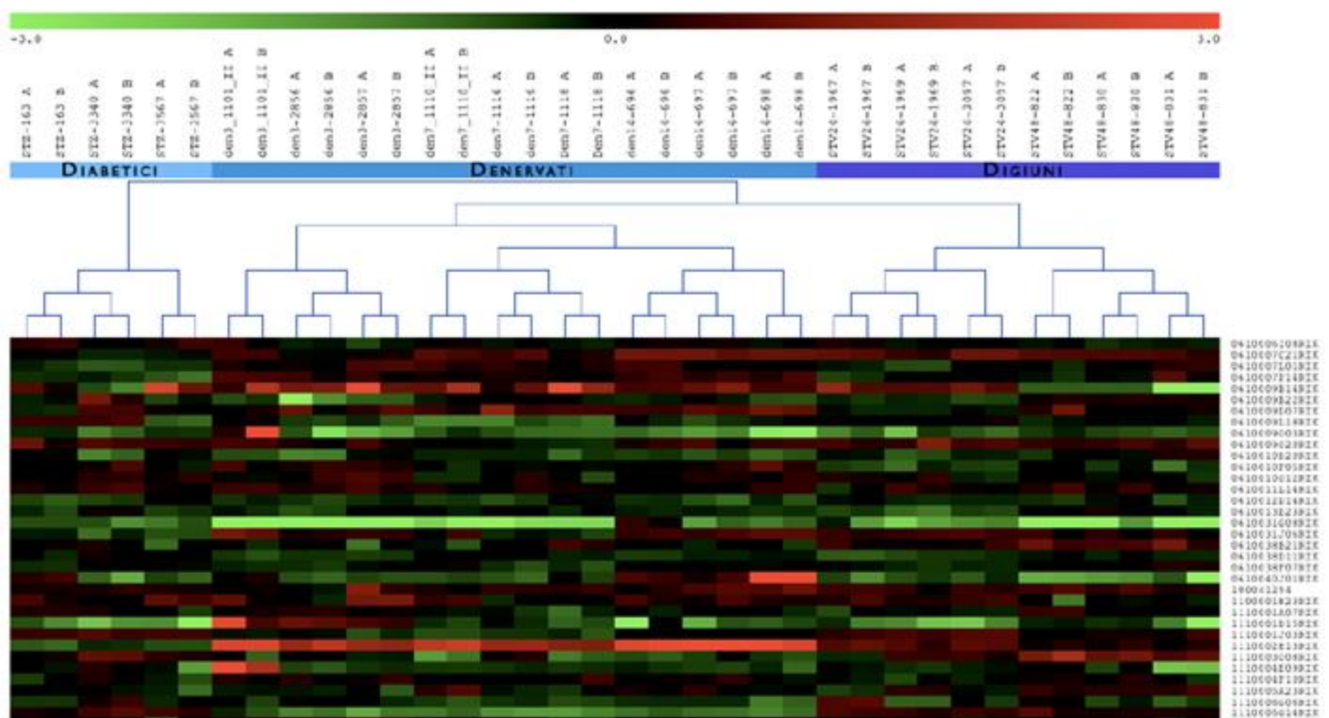



**Fig 36: Hierarchical cluster analysis obtained from the gene expression profile of several models of skeletal muscle atrophy**. A color code represents the relative intensity of the expression signal when compared to the control samples, with red indicating higher expression levels and green indicating lower expression levels.

Performing a SAM one class analysis of all the atrophic samples, a list with 1462 differently expressed genes was obtained (false discovery rate of 0.0%). In order to understand which were the biological processes mostly implicated in the atrophic process these genes were grouped in functional categories taking advantage of the freely available software DAVID, mainly using the KEGG pathway (Kanehisa et al, 2002) database. Among the biological processes that are more significantly enriched one should highlight the Proteasome and the Ribosome. It is also interesting to see that several genes of the Insulin signaling pathway are downregulated. This is the case of Insr (insulin receptor) and Igf-1r (Insulin growth factor 1 receptor). These results are in agreement with the findings obtained previously (Lecker et al., 2004).

**Fig 37: Significantly enriched pathways among the differently expressed genes (FDR=0%) in all atrophic conditions**. The percentage of enrichment represents the number of genes present in a pathway over the total number of differently over- (579) or under-expressed (883) genes. The enrichment P-value for each pathway is also reported.

| KEGG pathway | % | PValue |
|---|---|---|
| Proteasome | 3.11% | 2.96E-16 |
| Ribosome | 4.67% | 1.08E-15 |
| Naphthalene and anthracene degradation | 0.69% | 3.24E-02 |
| Type II diabetes mellitus | 0.79% | 4.49E-02 |
| Insulin signaling pathway | 1.70% | 1.96E-02 |
| Dorso-ventral axis formation | 0.57% | 5.76E-02 |
| Cytokine-cytokine receptor interaction | 2.49% | 3.39E-02 |
| Metabolism of xenobiotics by cytochrome P450 | 1.02% | 4.13E-02 |
| Propanoate metabolism | 0.57% | 8.87E-02 |
| Valine, leucine and isoleucine degradation | 0.68% | 8.87E-02 |
| Glycolysis / Gluconeogenesis | 0.79% | 7.37E-02 |

| PATHWAY | GeneSymbol | EntrezID | Descrizione | DEN3 | DEN7 | DEN14 | DIA8 | DIG24 | DIG48 |
|---|---|---|---|---|---|---|---|---|---|
| Insulin signaling pathway | **Eif4e** | **13684** | **EUKARYOTIC TRANSLATION INITIATION FACTOR 4E** | | | | | | |
| | Foxo1 | 56458 | FORKHEAD BOX O1 | | | | | | |
| | **Insr** | **16337** | **INSULIN RECEPTOR** | | | | | | |
| | Mapk10 | 26414 | MITOGEN ACTIVATED PROTEIN KINASE 10 | | | | | | |
| | Phka1 | 18679 | PHOSPHORYLASE KINASE ALPHA 1 | | | | | | |
| | Slc2a4 | 20528 | SOLUTE CARRIER FAMILY 2 (FACILITATED GLUCOSE TRANSPORTER), MEMBER 4 | | | | | | |
| | Socs1 | 12703 | SUPPRESSOR OF CYTOKINE SIGNALING 1 | | | | | | |
| | Socs2 | 216233 | SUPPRESSOR OF CYTOKINE SIGNALING 2 | | | | | | |
| | Srebf1 | 20787 | STEROL REGULATORY ELEMENT BINDING FACTOR 1 | | | | | | |
| Metabolism of xenobiotics by cytochrome P450 | Gstm3 | 14864 | GLUTATHIONE S-TRANSFERASE, MU 3 | | | | | | |
| | Cyp2c39 | 13098 | CYTOCHROME P450, FAMILY 2, SUBFAMILY C, POLYPEPTIDE 39 | | | | | | |
| | Cyp2c55 | 72082 | CYTOCHROME P450, FAMILY 2, SUBFAMILY C, POLYPEPTIDE 55 | | | | | | |
| | Cyp2c38 | 13097 | CYTOCHROME P450, FAMILY 2, SUBFAMILY C, POLYPEPTIDE 38 | | | | | | |
| | **Gstk1** | **76263** | **GLUTATHIONE S-TRANSFERASE KAPPA 1** | | | | | | |
| | Mgst1 | 56615 | MICROSOMAL GLUTATHIONE S-TRANSFERASE 1 | | | | | | |
| | **Dhdh** | **71755** | **DIHYDRODIOL DEHYDROGENASE (DIMERIC)** | | | | | | |
| | Cyp2f2 | 13107 | CYTOCHROME P450, FAMILY 2, SUBFAMILY F, POLYPEPTIDE 2 | | | | | | |
| Cytokine-cytokine receptor interaction | Acvr2b | 11481 | ACTIVIN RECEPTOR IIB | | | | | | |
| | Ccl11 | 20292 | SMALL CHEMOKINE (C-C MOTIF) LIGAND 11 | | | | | | |
| | Ccr6 | 12458 | CHEMOKINE (C-C MOTIF) RECEPTOR 6 | | | | | | |
| | Cntfr | 12804 | CILIARY NEUROTROPHIC FACTOR RECEPTOR | | | | | | |
| | Cxcl1 | 14825 | CHEMOKINE (C-X-C MOTIF) LIGAND 1 | | | | | | |
| | Edar | 13608 | ECTODYSPLASIN-A RECEPTOR | | | | | | |
| | Flt1 | 14254 | FMS-LIKE TYROSINE KINASE 1 | | | | | | |
| | Flt4 | 14257 | FMS-LIKE TYROSINE KINASE 4 | | | | | | |
| | Il15 | 16168 | INTERLEUKIN 15 | | | | | | |
| | Il17a | 16171 | INTERLEUKIN 17 | | | | | | |
| | Kdr | 16542 | KINASE INSERT DOMAIN PROTEIN RECEPTOR | | | | | | |
| | Kitl | 17311 | KIT LIGAND | | | | | | |
| | Pdgfd | 71785 | PLATELET-DERIVED GROWTH FACTOR, D POLYPEPTIDE | | | | | | |
| | Vegfa | 22339 | VASCULAR ENDOTHELIAL GROWTH FACTOR A | | | | | | |
| Glycolysis / Gluconeogenesis | Bpgm | 12183 | 2,3-BISPHOSPHOGLYCERATE MUTASE | | | | | | |
| | Hk2 | 15277 | HEXOKINASE 2 | | | | | | |
| | Ldhb | 16832 | LACTATE DEHYDROGENASE B | | | | | | |
| | **Pdha2** | **18598** | **PYRUVATE DEHYDROGENASE E1 ALPHA 2** | | | | | | |
| | **Pdha1** | **18597** | **PYRUVATE DEHYDROGENASE E1 ALPHA 1** | | | | | | |
| | **Dlat** | **235339** | **DIHYDROLIPOAMIDE S-ACETYLTRANSFERASE** | | | | | | |
| | Tpi1 | 21991 | TRIOSEPHOSPHATE ISOMERASE 1 | | | | | | |
| Propanoate metabolism | Ehhadh | 74147 | ENOYL-COENZYME A, HYDRATASE/3-HYDROXYACYL COENZYME A DEHYDROGENASE | | | | | | |
| | **Sucla2** | **20916** | **SUCCINATE-COENZYME A LIGASE, ADP-FORMING, BETA SUBUNIT** | | | | | | |
| | Ldhb | 16832 | LACTATE DEHYDROGENASE B | | | | | | |
| | Aldh6a1 | 104776 | ALDEHYDE DEHYDROGENASE FAMILY 6, SUBFAMILY A1 | | | | | | |
| | Mcee | 73724 | METHYLMALONYL COA EPIMERASE | | | | | | |
| Proteasome | Psma2 | 19166 | PROTEASOME (PROSOME, MACROPAIN) SUBUNIT, ALPHA TYPE 2 | | | | | | |
| | Psma5 | 26442 | PROTEASOME (PROSOME, MACROPAIN) SUBUNIT, ALPHA TYPE 5 | | | | | | |
| | Psma7 | 26444 | PROTEASOME (PROSOME, MACROPAIN) SUBUNIT, ALPHA TYPE 7 | | | | | | |
| | Psmb2 | 26445 | PROTEASOME (PROSOME, MACROPAIN) SUBUNIT, BETA TYPE 2 | | | | | | |
| | Psmb3 | 26446 | PROTEASOME (PROSOME, MACROPAIN) SUBUNIT, BETA TYPE 3 | | | | | | |
| | Psmb4 | 19172 | PROTEASOME (PROSOME, MACROPAIN) SUBUNIT, BETA TYPE 4 | | | | | | |
| | Psmb7 | 19177 | PROTEASOME (PROSOME, MACROPAIN) SUBUNIT, BETA TYPE 7 | | | | | | |
| | Psmc1 | 19179 | PROTEASE (PROSOME, MACROPAIN) 26S SUBUNIT, ATPASE 1 | | | | | | |
| | Psmc3 | 19182 | PROTEASOME (PROSOME, MACROPAIN) 26S SUBUNIT, ATPASE 3 | | | | | | |
| | Psmc4 | 23996 | PROTEASOME (PROSOME, MACROPAIN) 26S SUBUNIT, ATPASE, 4 | | | | | | |
| | Psmd2 | 21762 | PROTEASOME (PROSOME, MACROPAIN) 26S SUBUNIT, NON-ATPASE, 2 | | | | | | |
| | Psmd4 | 19185 | PROTEASOME (PROSOME, MACROPAIN) 26S SUBUNIT, NON-ATPASE, 4 | | | | | | |
| | Psmg2 | 107047 | PROTEASOME (PROSOME, MACROPAIN) ASSEMBLY CHAPERON 2 | | | | | | |
| Rybosome | Rpl10a | 19896 | RIBOSOMAL PROTEIN L10A | | | | | | |
| | Rpl13 | 270106 | RIBOSOMAL PROTEIN L13 | | | | | | |
| | Rpl15 | 66480 | RIBOSOMAL PROTEIN L15 | | | | | | |
| | Rpl19 | 19921 | RIBOSOMAL PROTEIN L19 | | | | | | |
| | Rpl26 | 19941 | RIBOSOMAL PROTEIN L26 | | | | | | |
| | Rpl28 | 19943 | RIBOSOMAL PROTEIN L28 | | | | | | |
| | Rpl38 | 67671 | RIBOSOMAL PROTEIN L38 | | | | | | |
| | Rpl7a | 27176 | RIBOSOMAL PROTEIN L7A | | | | | | |
| | Rps10 | 67097 | RIBOSOMAL PROTEIN S10 | | | | | | |
| | Rps14 | 20044 | RIBOSOMAL PROTEIN S14 | | | | | | |
| | Rps17 | 20068 | RIBOSOMAL PROTEIN S17 | | | | | | |
| | Rps18 | 20084 | RIBOSOMAL PROTEIN S18 | | | | | | |
| | Rps7 | 20115 | RIBOSOMAL PROTEIN S7 | | | | | | |
| | Rps9 | 76846 | RIBOSOMAL PROTEIN S9 | | | | | | |

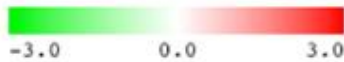



**Fig 38: DAVID analysis on the differently expressed genes revealed the presence of certain enriched pathways or cellular compartments.** For each gene, green represents under-expression when compared to the normal control and red represents over-expressed when compared to the normal control.

## 5.2- Skeletal muscle atrophy is a transcriptionally regulated process fine tuned by miRNAs.

Having established that each atrophic condition has a unique mRNA and miRNA signature, the obvious question was which mRNAs were regulated by which miRNAs. To address this question, Matteo Silvestrin and Cristiano di Pittà, from the CRIBI, developed an algorithm, that runs under GenMiR++ (Huang et al 2007), and that integrates these two information. This algorithm highlights the interaction miRNA- target mRNA that present opposite expression levels. In this way, from the list of possible targets predicted by several algorithms, it was possible to identify the most probable interactions.

This algorithm to run under GenMiR++ requires three matrixes: the information related to the mRNA expression level (X), the information related to the miRNA expression levels (Z), and a binary matrix with the information of the predicted interaction mRNA-miRNA (C). There are several algorithms that can predict the interaction miRNA-mRNA-target. The most used ones are, miRanda, TargetScanS, Pictar and PITA among others. The prediction program miRanda (Enright

| TARGET | miR | GenMiR | miRanda | PITA |
|---|---|---|---|---|
| 0610007C21RIK | mmu-miR-378 | 0.5461 | HIGH | |
| 0610007C21RIK | mmu-miR-125a-5p | 0.491 | MEDIUM | |
| 0610007C21RIK | mmu-miR-199a-3p | 0.491 | MEDIUM | |
| 0610007L01RIK | mmu-miR-214 | 0.523 | | MEDIUM |
| 0610009D07RIK | mmu-miR-338-3p | 0.4888 | MEDIUM | |
| 0610009L18RIK | mmu-miR-25 | 0.4893 | MEDIUM | |
| 0610009L18RIK | mmu-miR-142-3p | 0.4883 | MEDIUM | |
| 0610009L18RIK | mmu-miR-337-3p | 0.4859 | MEDIUM | |
| 0610009O03RIK | mmu-miR-122 | 0.5118 | HIGH | |
| 0610009O03RIK | mmu-miR-140 | 0.5003 | HIGH | |
| 0610009O03RIK | mmu-miR-370 | 0.4953 | MEDIUM | |
| 0610010D20RIK | mmu-miR-379 | 0.5004 | HIGH | |
| 0610031J06RIK | mmu-miR-17 | 0.4985 | HIGH | |
| 0610031J06RIK | mmu-miR-194 | 0.49 | MEDIUM | |
| 0610031J06RIK | mmu-miR-296-5p | 0.4877 | MEDIUM | |
| 0610031J06RIK | mmu-miR-125a-5p | 0.4864 | MEDIUM | |
| 0610040J01RIK | mmu-miR-335-5p | 0.5048 | HIGH | |
| 0610040J01RIK | mmu-miR-30e | 0.4951 | MEDIUM | |
| 0610040J01RIK | mmu-miR-30d | 0.4903 | MEDIUM | |
| 0610040J01RIK | mmu-miR-16 | 0.4867 | MEDIUM | |
| 0610040J01RIK | mmu-miR-496 | 0.4863 | MEDIUM | |
| 1110001D15RIK | mmu-miR-27b | 0.629 | HIGHEST | |
| 1110001D15RIK | mmu-miR-27a | 0.5944 | HIGHEST | |
| 1110001D15RIK | mmu-miR-223 | 0.4981 | HIGH | |

**Fig 39: Exel screen with a list of the interactions miRNA-mRNA target obtained with GenMIR++.** This approach integrates the predicted interactions, that result from miRanda and PITA (two predictive algorithms), with the data from the miRNA and mRNA expression profiles. This list contains 9876 interactions miRNA-mRNA target with a probability score above the 75° percentile.

*et al.*, 2003), considers that the most important region of the interaction miRNA-mRNA is the seed region, however it also evaluate the 3' interaction of the miRNA. Furthermore this program also considers the thermodynamic stability of the interaction miRNA-mRNA using the Vienna work-package. The last version of this program (John *et al.*, 2006) also takes into consideration the presence of multiple sites of binding in the same 3'UTR for the same miRNA. TargetScanS considers the complementarities within the seed region, but it takes also in consideration the conservation between species. Pictar (Krek *et al.*, 2005) analyzes the conservation among species of the miRNA-binding site as well. After searching for orthologus 3'UTR, this program looks for binding within the seed region of the miRNAs allowing some mismatch. Finally this program results in a score that considers the free energy of the miRNA/mRNA binding. One of the last developed programs is PITA (Probability of Interaction by Target Accessibility) (Kertesz et al 2007). This algorithm considers a perfect complementarity between the seed-region and the 3'UTR of the mRNA, allowing a single mismatch. It also considers the accessibility of the target to the miRNA. This program does not take into consideration the conservation between species.

PITA is the most updated predictive algorithm that takes in consideration not only the binding miRNA-mRNA but also the accessibility of the RISC complex to the target mRNA. On the other hand miRanda, not only considers the binding of the 3' of the miRNA, but it is also the most sensible (Sethupathy et al, 2006) algorithm considering the predicted targets that were validated. For these reasons these two algorithms were chosen to create two different binary matrixes to use in the GenMiR++. We then decided to join the lists of miRNA/mRNA-targets resulted by miRanda (32789 predictive interactions) and by PITA (8091 predictive interactions). This integrated list was loaded into GenMir++ together with the expression levels of the miRNAs and of the mRNAs. Then, GenMir++ generates a probabilistic score based on the anti-correlation between miRNA and mRNA expression levels. Taken in consideration the probabilistic score given by GenMir++ only 9000 interaction were considered (>75° percentile). The most significant interactions are displayed in Excel format and may be consulted in different ways: select a miRNA to see which are their targets, or select a gene to see which miRNAs can regulate it (Fig 39).

Since we are studying miRNA-206 and miRNA-21 we have focused on the interaction of these two miRNAs and their targets. The analysis with GenMir++ generated 322 possible targets for miRNA-206 and 121 possible targets of miRNA-21. All these target genes were down-regulated in mRNA array.

## 5.3- PolK, YY1, eIF4E3 and PDCD10 are possible targets of miRNA-206 and miRNA-21.

| | miRNA-21 | | miRNA-206 | |
|---|---|---|---|---|
| Gene | # of binding sites | dG duplex | # of binding sites | dG duplex |
| PolK | 0 | | 2 | -10.6<br>-12.1 |
| YY1 | 2 | -6<br>-8.2 | 2 | -14.9<br>-8.2 |
| eIF4E3 | 1 | -10.9 | 1 | -14.7 |
| PDCD10 | 1 | -10.1 | 2 | -12.7<br>-10.4 |

**Fig 40: Group of genes that were selected from the GenMIR++ list for further analysis.** Most of the selected genes are, predictably, targets of both miRNAs. The energy of the duplex miRNA-mRNA is also shown

A closer look on the list of predicted targets that came out from the GenMir++ analysis revealed the presence of some interesting genes. At the top of the list of the possible targets of miRNA-206 there was DNA polymerase Kappa (PolK). In the same list there was other two DNA polymerases, alpha 1 and epsilon 4 (PolA1 and PolE4 respectively). PolK is a low-fidelity enzyme that is involved in the extension step of lesion bypass (Lone et al., 2007). PolA1 is one of the most important DNA polymerase since is the only enzyme capable of initiating DNA replication at chromosomal origin sites and at sites of initiation of discontinuous synthesis of Okazaki fragments on the lagging side of the replication fork (Srivastava et al., 2000). Finally, PolE4 is a histone-fold protein that interacts with other histone-fold proteins to bind DNA in a sequence-independent manner. These histone-fold protein dimers combine within larger enzymatic complexes for DNA transcription, replication, and packaging. Although their role in the adult skeletal muscle, a post-mitotic tissue, is not clear, it is known that PolA1 is regulated by miRNA-206 in C2C12 myoblast (Kim et al., 2006). Considering that PolK is the target gene with the highest score and that contains 2 binding sites for miRNA-206 we decided to further study whether this polymerase was in fact modulated during denervation specifically by miRNA-206.

Another gene that came out as a target of miRNA-206 was yin-yang 1 (YY1). This is a zinc-finger transcription factor that has been shown to regulate different functions in skeletal muscle. In fact YY1 competes for the binding site of Serum Response Factor (SRF) (Gualberto et al., 1992), a known skeletal muscle transcription activator. Similarly, it binds and represses the promoter of the Dystrophin gene (Galvagni et al., 1998). Furthermore, NF-kB, one of the most important transcription factor during skeletal muscle atrophy, is known to activate YY1 transcription in order to inhibit fusion in C2C12 cells (Wang et al., 2007). YY1 is also known to interact directly with PGC1a in order to orchestrate mitochondrial biogenesis in a mTOR-dependent way (Cunningham et al., 2007). Considering these evidences and the fact that, according to PITA algorithm, YY1 has 2

binding sites for miRNA-206 and 2 binding sites for miRNA-21 we decided to further study the role of this transcription factor during skeletal muscle atrophy.

Also eIF4E3 can be targeted by both miRNA-206 and miRNA-21. This initiation factor is a rate limiting component of translation. EIF4E, together with eIF4G and eIF4A forms the eIF4F complex that is required for the recruitment of the 60S ribosomal subunit into the 43S pre-initiation complex to form a functional 80S initiation complex (Jackson et al., 2010). While eIF4E1 and eIF4E2 are ubiquitously expressed, eIF4E3 is expressed mainly in the heart and skeletal muscle. The role of this initiation factor in the muscle was never addressed however it is known that the over-expression of eIF4E3 in U2OS cells increased cell size and rescues the rapamacyn-induced decrease in cell size (Fingar et al., 2002).

Finally, the last gene we decided to study was Program Cell Death 10 (PDCD10). It is a possible target of both miRNA-206 and miRNA-21, containing a binding site for each miRNA. The tissues that present the highest expression levels of this gene are the heart, skeletal muscle and placenta. Considering that PDCD10 is involved in the cerebral cavernous malformations strongly suggests that it is a new player in vascular morphogenesis and/or remodeling (Bergametti et al., 2005). Again, the role of PDCD10 in the skeletal muscle atrophy is unknown. However this gene fulfills the criterion of having at least a binding site for both of the miRNAs under study.

The initial task was to validate the gene expression profile of microarrays by using quantitative RT-PCR. The analyses confirmed that YY1 was significantly down-regulated at 3 and 7 days but not at 14 days of denervation (Fig 41a). Importantly, eIF4E3, PDCD10 and PolK transcripts were down-regulated during early and late stages of denervation (Fig 41b, c, d). These results confirmed the data obtained with the micro-array.

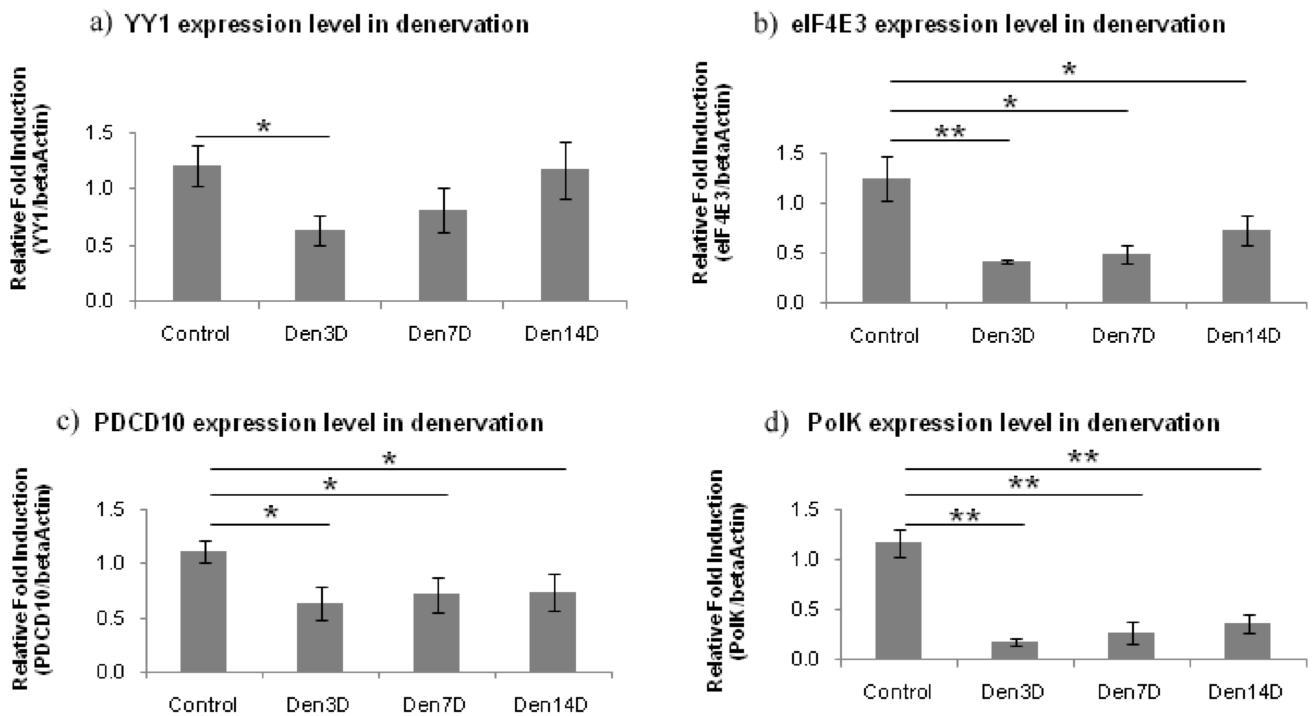



**Fig 41: All predicted targets are down regulated during denervation**. Expression levels of (a) YY1, (b) eIF4E3, (c) PDCD10 and (d) PolK during different time points of denervation. After 3, 7 or 14 days of denervation, induced by unilateral transection of the sciatic nerve, gastrocnemius muscle was collected, total RNA was extracted and gene expression analyzed by Real Time Quantitative PCR. β-Actin was used to normalize the expression levels of each gene. (n=3) Values represent mean ± STD. * $p<0.05$, ** $p<0.01$ by T-Test.

## 5.4- The 3'UTR of PolK, YY1, eIF4E3 and PDCD10 are modulated during denervation.

To confirm that these genes are true targets of the predicted miRNAs, their 3'UTR was cloned into the pMIR vector, at the 3'UTR of the Luciferase gene. These luciferase sensors quantify the degree by which the 3'UTR of a gene is modulated by miRNAs. Whenever there is a decrease in the luciferase level, it indicates that there is an increase in the miRNAs that regulate the 3'UTR of the sensor. In order to analyze whether these genes are under miRNA regulation in denervated muscle, we transfected these sensors into control and denervated T.A. muscles. After 15 days of denervation, the luciferase activity was measured. Indeed two weeks of denervation induced a 10 fold reduction of luciferase activity (Fig 42). These findings are consistent with a miRNA modulation of these 3'UTRs.

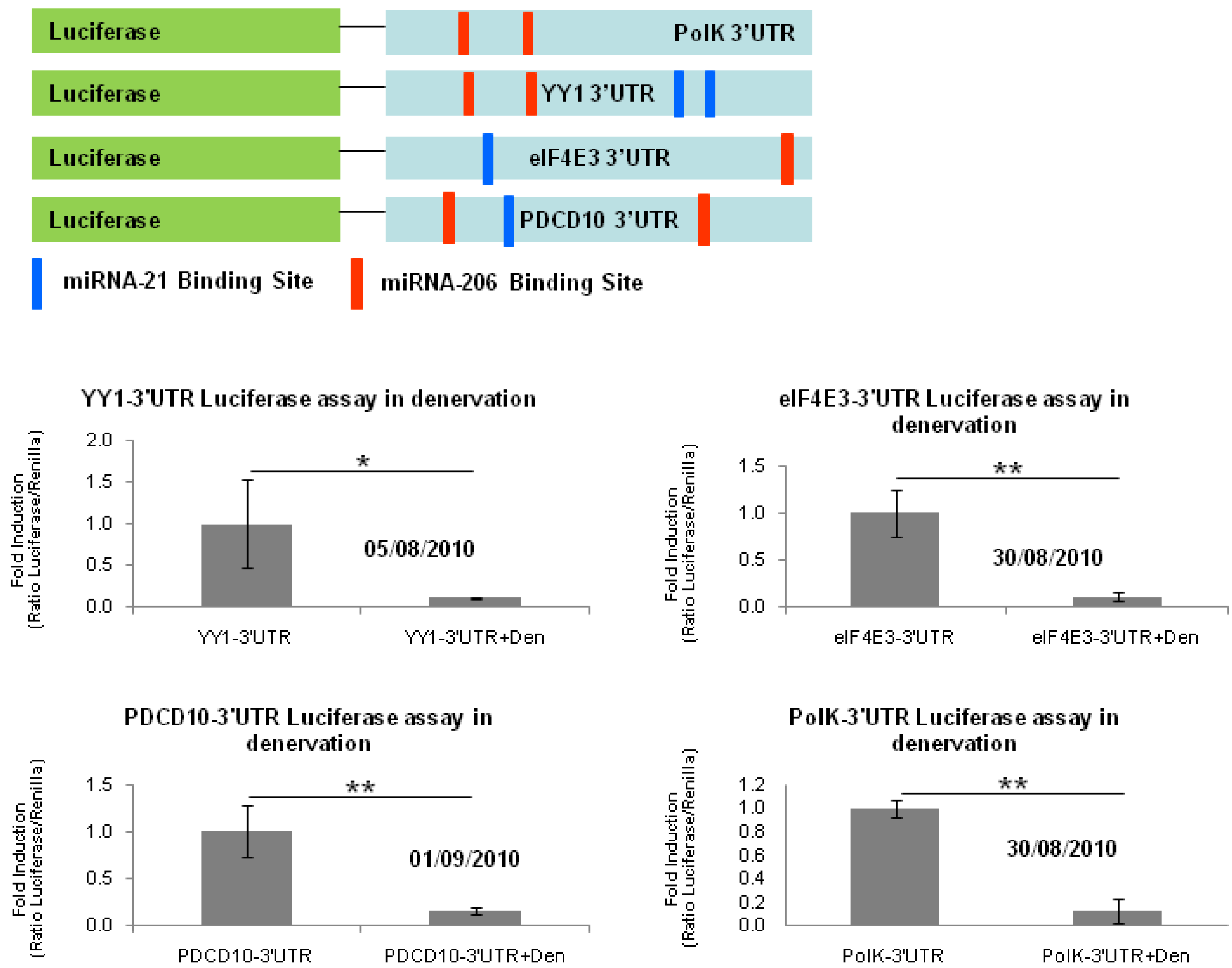



**Fig 42: Denervation down-modulates the 3'UTR of the selected genes.** (a) Schematic representation of the Luciferase sensors generated to access the effect of the miRNAs on the 3'UTR of the selected genes. The 3'UTR of each target gene was PCR amplified and cloned into the pMIR-LUC vector in the 3'UTR region of the Luciferase gene. The binding site for each miRNA are depicted - in blue the binding sites for miRNA-21 and in red the binding sites for miRNA-206. Denervation down-modulates the 3'UTR of (b) YY1, (c) eIF4E3, (d) PDCD10 and (e) PolK. The luciferase sensors for the 3'UTR of each gene (10µg) were electroporated into adult T.A. muscles. A Renilla vector (5µg) was co-electroporated to normalize for electroporation efficiency. Electroporated animals were simultaneously submitted to unilateral transection of the sciatic nerve. 7 days later the Luciferase/Renilla levels were measured. ($n \geq 3$) Values represent mean ± STD. *$p<0.05$ and ** $p<0.01$ by T-Test.

## 5.5- The 3'UTR of PolK, YY1, eIF4E3 and PDCD10 are modulated by miRNA-206 and miRNA-21.

These results strongly suggest that miRNAs can regulate the expression of these genes. To further prove that miRNA-206 and -21 are involved in such control we transfected the 3'UTR sensors together with the miRNA over-expressing vectors. After 7 days, muscles were collected and luciferase levels measured. Interestingly expression of miRNA 206 did not affect the YY1-3'UTR. However, the expression of miRNA-21 was able to reduce to half the luciferase levels of YY1-3'UTR. No additive effects were observed when miRNA-206 and miRNA-21 were co-expressed (Fig 43a). The same analysis was performed on the 3'UTR of eIF4E3 and PDCD10. The 3'UTR of these genes were down-regulated by both miRNA-206 and miRNA-21 (Fig 43 b, c). Furthermore, the co-expression of miRNA-206 and miRNA-21 led to a further suppression of the 3'UTR of eIF4E3 indicating an additive effect. To what concerns the 3'UTR of PolK, there was no effect on luciferase activity when miRNA-206 (Fig 43 d) was over-expressed.

In conclusion, the data obtained with the 3'UTR luciferase sensors suggest that YY1 is modulated by miRNA-21, while eIF4E3 and PDCD10

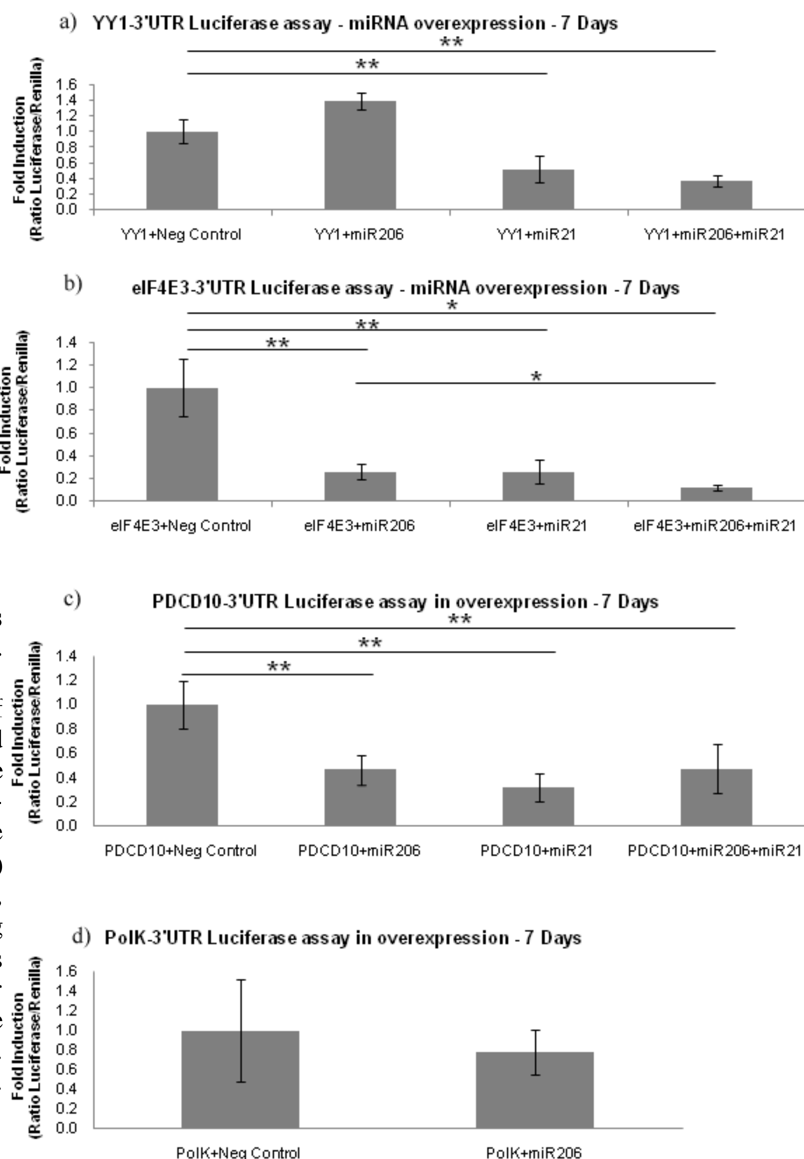



**Fig 43: Over-expression of miRNAs modulates the 3'UTR of the selected genes**. (a) YY1-3'UTR is modulated by miRNA 21 but not by miRNA-206. The 3'UTRs of eIF4E3 (b) and PDCD10 (c) are modulated by both miRNA-206 and miRNA-21. (d) The 3'UTR of PolK is not modulated by miRNA-206 as it was predicted. The luciferase sensors for the 3'UTR of each gene (10µg) were electroporated into adult T.A. muscles, together in the miRNAs over-expressing vectors (20µg). A Renilla vector (5µg) was co-electroporated to normalize for electroporation efficiency. 7 days later the Luciferase/Renilla levels were measured. (n≥3) Values represent mean ± STD. *$p<0.05$ and ** $p<0.01$ by T-Test.

are modulated by both miRNA-206 and miRNA-21. PolK is not modulated by miRNA-206.

To further prove the specificity of these miRNAs, we have mutated the 3'UTR binding sites of eIF4E3. Two different approaches were applied: three nucleotides of the seed region of the predicted miRNA binding site were either mutated (Mut206 and Mut21) or deleted (Del206 and Del21). Theoretically, if these are the only binding sites of these miRNAs, their mutation or deletion should rescue the luciferase activity when co-express with miRNA206 or 21. Thus, we co-transfected C2C12 muscle cells with the mutated 3'UTR and the respective miRNA-expressing vector. As expected, the WT eIF4E3 3'UTR is strongly reduced by miRNA-206 and miRNA-21 (Fig 44a, b). Importantly, the mutated/deleted eIF4E3 3'UTR were significantly less inhibited by miRNA 206 or miRNA21. These results confirm that eIF4E is controlled by these microRNAs. However, since the rescue was not complete, it may indicate that another non-predicted binding site for the miRNA of interest may exist in this region or that other miRNAs can regulate its expression. The same approach is currently under study for the 3'UTR of YY1.

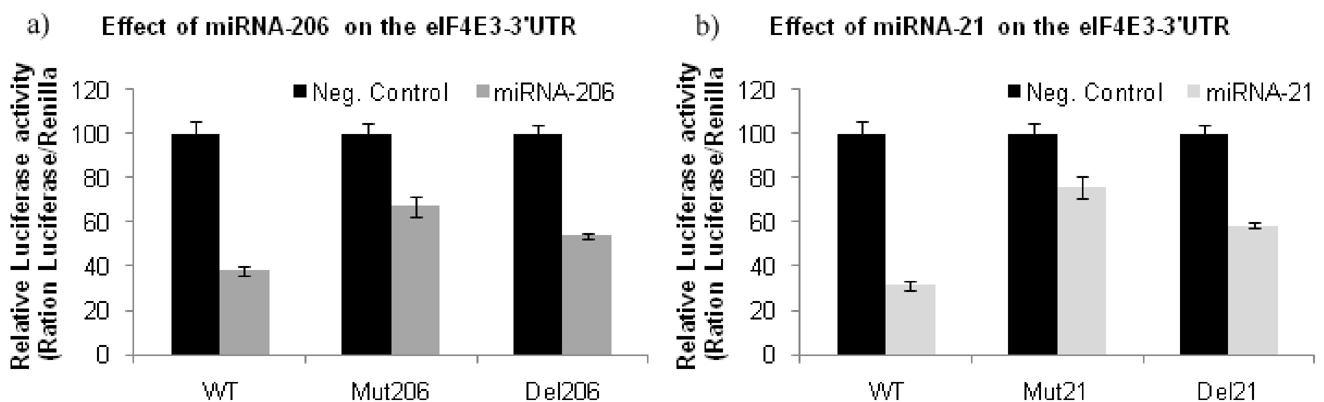



**Fig 44: Mutations/Deletions on the seed region of the 3'UTR of eIF4E3 partially prevent the miRNA-specific regulation**. Mutations/Deletions in the binding site for miRNA-206 (a) or miRNA-21 (b) partially prevent the regulation of these miRNAs on the 3'UTR of eIF4E3. (a) The luciferase sensors (WT, mutated or deleted) for the 3'UTR of eIF4E3 (0,64µg) were co-transfected with negative control (0,64µg) or miRNA-206 (0,64µg). A Renilla vector (0,32µg) was co-transfected to normalize for transfection efficiency. C2C12 myoblasts were collected 48h after transfection and Luciferase/Renilla levels were measured. ($n \geq 3$) Values represent mean ± STD

## 5.6- Over-expression of miRNA-206 and miRNA-21 down-regulate eIF4E3, PDCD10 and YY1 in C2C12 myoblasts.

To confirm that these genes are true targets of miRNA-206 and miRNA-21 one should observe a direct correlation between the over-expression of the miRNAs and a down-regulation of their targets. To achieve this, C2C12 myoblasts were transfected with the negative control, miRNA-206 or miRNA-21. After 48h total RNA was extracted and the expression of these genes was analyzed by real time RT-PCR. Interestingly, miRNA-206 significantly down-regulated the transcript of PDCD10 while miRNA-21 reduced mRNAs of eIF4E3 and PDCD10 (Fig 45). There is a trend of down-regulation of the eIFE3 transcript by miRNA206 that did not reach the statistical significance that could be achieved by additional experiments. Our previous data suggest that miRNA-21, but not miRNA-206, controls the 3'UTR of YY1. However, we couldn't see this regulation at the mRNA level. Thus, we hypothesized that, in cells, the regulation could be at the protein level. We then performed Western blot to detect YY1 protein in miRNA21 transfected cells. MiRNA-21 was sufficient to reduce the protein level of YY1 (Fig 46).

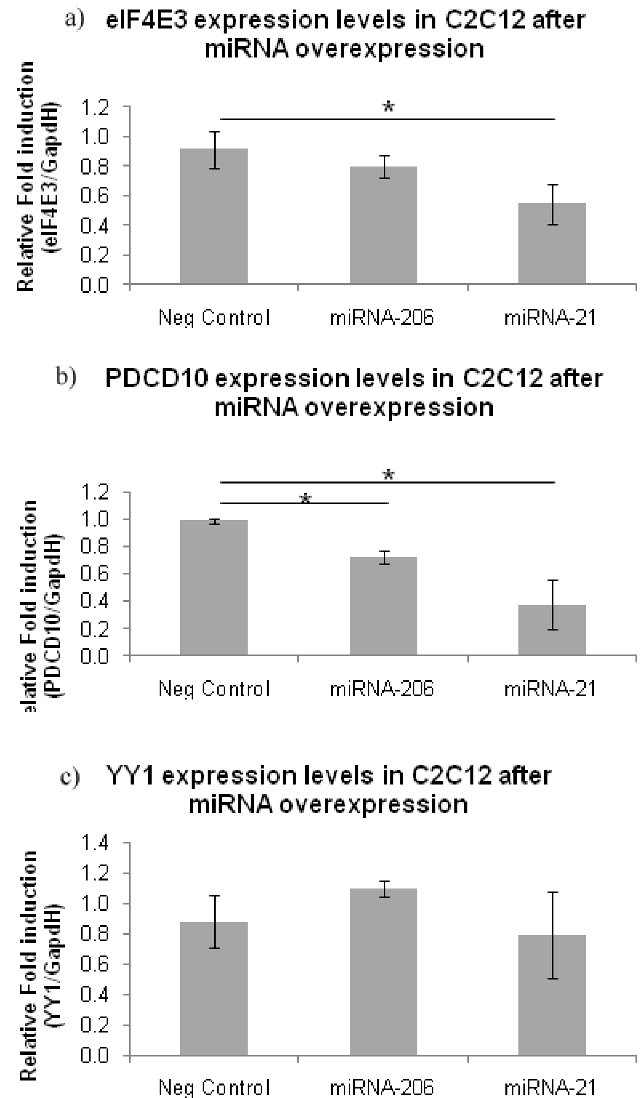



**Fig 45: miRNA over-expression alters the expression levels of the target genes.** Expression levels of (a) eIF4E3, (b) PDCD10 and (c) YY1. C2C12 myoblasts were transfected with 4 μg of miRNA expressing vector. 48h after transfection cells were collected and total RNA was extracted using Trizol. Gene expression analyzed by Real Time Quantitative RT-PCR using GapdH to normalize the expression levels of each gene. (n=3) Values represent mean ± STD. * $p<0.05$, ** $p<0.01$ by T-Test.

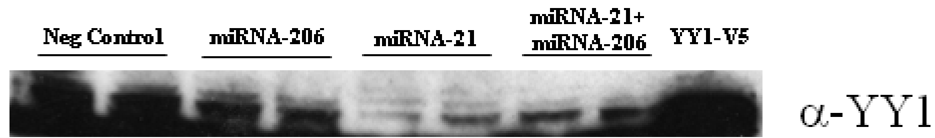



**Fig 46: miRNA-21 over-expression down-regulate YY1 at the protein level.** Western blot analysis on the YY1 protein levels after transfection of C2C12 myoblasts with miRNAs over-expressing vectors (4 µg). Cells were harvested 48h after transfection.

Altogether these results demonstrate for the first time that miRNA-206 regulates eIF4E3 and PDCD10, while miRNA-21 controls YY1, eIF4E3 and PDCD10.

# Chapter 6 – General Discussion and Conclusions

Skeletal muscle atrophy is a transcriptionally regulated process (Lecker et al., 2004; Sandri et al., 2004a; Stitt et al., 2004). FoxOs are key transcription factor that control the expression of rate-limiting enzyme of the two most important degradative pathways: the ubiquititn/proteasome (Gomes et al., 2001; Sandri et al., 2004a) and the autophagy/lysosome (Mammucari et al., 2007; Zhao et al., 2007) pathway. Also NF-kB is involved in the atrophy program (Cai et al., 2004; Hunter and Kandarian, 2004). Furthermore, there is a restricted group of genes, called atrogenes, which are commonly up- or down-regulated in all the atrophic conditions studied (Lecker et al., 2004). These findings suggest that basic mechanisms of atrophy are shared among diseases. Atrogenes belong to several fundamental biological processes, ranging from ATP production to nitrogen metabolism and from transcription/chromatin remodeling to translation control. The varieties of processes that are involved in atrophying muscles suggest highly regulated and coordinated mechanisms to adapt the myofiber to the catabolic condition. Therefore the level of control might not be only at transcriptional stage and might involve additional regulation to fine-tune the atrophy program. Recently, a new class of regulatory molecules has been discovered. They are called miRNAs and are predicted to regulate several genes from the same pathway. Their role in the skeletal muscle physiology has just been started to be unraveled. The aim of this thesis was to determine their role during muscle atrophy.

Our data strongly suggest that miRNAs are changing their expression and are contributing to the molecular mechanisms implicated in muscle atrophy. We have found several novel and un-expected findings that contribute to the understanding of muscle loss. Firstly, despite the presence of a set of gene, the atrophy-related genes, that are commonly regulated at transcriptional level during atrophy, the miRNA expression profile is peculiar for each atrophic condition. Therefore, we could not identify any atrophy-related miRNA or AtromiRNA. A second important point was the finding that the changes in miRNA expression are delayed when compared to the transcriptional regulation. This result suggests that miRNA are involved in fine-tuning the atrophy program that was previously activated at transcriptional level.

Although we were not able to identify a common miRNA to all the atrophic conditions studied, we have identified and characterized the two most induced miRNAs of denervated muscles. These two miRNAs, miRNA-206 and miRNA-21, are up-regulated both in fast and slow muscles. The role of these miRNA in regulation of muscle mass have been studied *in vivo* by developing vectors and techniques that allow the over-expression or the inhibition of these miRNA in adult TA muscles. These functional studies revealed that, together, these two miRNAs can account for almost half of the reduction in the CSA of the fibers that occurs during denervation-induced atrophy. Gain-of-function studies have found that miRNA-206, per se, was able of activate Atrogin-1, a key E3-

ligase involved in protein degradation. Furthermore, loss of function analyses found that the inhibition of both miRNA-206 and miRNA-21 promotes muscle growth and partially prevents the denervation-induced atrophy. Finally, we have identified some of the targets of these two miRNAs and we have started to unravel their biological role in the control of muscle mass and metabolism.

## 6.1- Different atrophic conditions activate specific miRNA programs.

To gain insight on the involvement of miRNAs in muscle wasting, miRNA expression profiles of atrophic muscles were performed. From the approximately 400 probes present in the array, ninety were significantly differentially expressed between controls and atrophic samples. However the bioinformatic analyses to reveal miRNAs that were commonly up or down-regulated in all the studied atrophic conditions was unsuccessful. This was unexpected since several studies in the muscle field indicate that common miRNAs can regulate common mechanisms. In fact, a study in cardiac muscle found miRNAs that are commonly induced in different conditions of cardiac hypertrophy. These shared miRNAs were also altered in human samples of heart failure (van et al., 2006). Also analyses on different muscular dystrophies found a group of miRNAs that are commonly altered (Eisenberg et al., 2007). Altogether, these results underline the existence of common regulatory mechanisms involved in miRNA expression. Although surprising, the lack of a common miRNA in our study can be justified by the fact that the atrophic conditions studied are quite different from each other. Accordingly, the same genomic study that identified a restrict group of genes common to all the atrophic conditions, also showed that the vast majority of differentially expressed genes were specific of each condition (Lecker et al., 2004). This is in agreement with the fact that the bioinformatic analysis hierarchically clustered the different samples according to their atrophic condition. In fact, samples from different time points of denervation clustered together, like the samples from different time points of starvation. The miRNA expression profile from diabetic samples indicated that this is a completely independent condition.

The realization of the miRNA expression profile at different time points revealed that at early stages of atrophy there is a general suppression of miRNAs expression. This was particularly evident at 3 days of denervation. The samples of 24h and 48h starved muscles were extremely difficult to analyze due to variability among samples. However, even in this condition the majority of the miRNAs were down-regulated at 24 h of fasting. These findings suggest that miRNA up-regulation is delayed and occurs in a second time when compared to the transcription of atrophy-related genes. In fact, 3 days of denervation and 24 h of starvation are the time of the strongest

induction of Atrogin-1 and LC3 gene expression while miRNAs induction requires at least 7 days of atrophy. It is therefore possible that the changes of miRNAs expression might be under the regulation of some atrophy-related genes. Another possibility is that these miRNAs can be involved in slowing down the atrophic process or in the metabolic and functional adaptation to the new atrophic condition.

Considering the absence of common atrophy-related miRNAs or atromiRNA, we decided to focus our attention on the two most induced miRNAs, miRNA-206 and miRNA-21, in denervated muscles.

Quantitative RT-PCR analyses and functional assays confirmed that both miRNA-206 and miRNA-21 were significantly up-regulated during denervation. This response of both miRNAs was independent of the type of muscle analyzed since denervation induced their expression levels both in slow and fast muscles. It was interesting to see that the basal levels of miRNA-206 were 10 fold higher in slow muscles when compared to fast muscles, as it was previously reported (McCarthy and Esser, 2007b). Perhaps, this justifies the fact that the fold increase of this miRNA upon denervation was higher in fast muscles (10 fold increase) when compared to slow muscles (4 fold increase). Since miRNA-206 expression is higher in slow muscle, it was proposed that its regulation is under the control of a transcription factor specific of slow program (McCarthy and Esser, 2007b). Other studies indentified MyoD as the critical regulator of miRN-206 expression (Rao et al., 2006; Rosenberg et al., 2006), Interestingly, MyoD is more expressed in fast muscles than in slow muscles (Voytik et al., 1993), suggesting that at least another transcription factor is responsible for the different expression of miRNA-206 between slow and fast muscles. Myogenin is another transcription factor that binds to the regulatory region of miRNA-206 (Rao et al., 2006) and it is highly expressed in adult slow muscles, being therefore a possible regulator of miRNA-206 in the slow muscles.

Another interesting observation is that miRNA-206 expression levels progressively increased during denervation, while miRNA-21 remains constant. It is interesting that both transcription factors that we have mentioned before, MyoD and Myogenin, are strongly induced during denervation (mainly after 2days of denervation) (Voytik et al., 1993) suggesting that they might be involved on the regulation of these miRNAs. However, this does not exclude that another atrophy-related transcription factor might be regulating miRNA-206 expression.

As for miRNA-21, one of the most important transcriptional regulator is AP-1 (Fujita et al., 2008). Data from our laboratory indicates that during skeletal muscle atrophy, DNA binding activity of nuclear extracts to the AP-1 binding sites decreases (Sandri et al., 2004a), suggesting that another transcription factor may be involved in the up-regulation of this miRNA.

In this thesis we showed that over-expression of c.a.FoxO3 was sufficient to up-regulate miRNA-206 and miRNA-21 but it was not required. In fact we have generated a knock-out animal in which FoxO1, FoxO3 and FoxO4 (FoxO1,3,4 -/-) were simultaneously deleted specifically in skeletal muscle. The findings clearly show that upon denervation miRNA-206 and miRNA-21 are still up-regulated in FoxO1,3,4 null muscles, indicating that their transcription is not FoxO dependent. Furthermore, it indicates that the miRNA induction during FoxO3 over-expression may be dependent of downstream targets of FoxO3 or products of the atrophy process. These *in vivo* results are in contrast with the ones obtained by Wang et al (Wang and Li, 2010). In this study, performed in A549 human lung cancer cells and in human neuroblastoma cell line SH-EP1, FoxO3 binds one of the two identified binding sites in the promoter region of miRNA-21. In fact over-expression of c.a.FoxO3a represses miRNA-21 expression while RNAi against FoxO3a increases the levels of miRNA-21. It is possible that the cellular context and the binding partners of FoxO3 can account for these discrepancies. Although, in skeletal muscle, FoxO family members are described as transcription factors that induce gene expression, there are reports that they act as repressor as well (for example (Karadedou et al., 2011)).

Similarly, over-expression of Ikkβ, an activator of the NF-kB pathway, in adult skeletal muscle, was sufficient to induce the expression of miRNA-206 and miRNA-21. However, it was not possible to discriminate between a direct transcriptional regulation or a indirect activation of an atrophic program, since we don't have available the specific knock-out model for this pathway.

As discussed in the introduction, miRNAs can be transcribed from intergenic locus but also from introns of coding genes. The transcripts can generate a single miRNA or more than two miRNAs when miRNA are poly-cystronic. While miRNA-206 is originated from an intergenic bi-cystronic locus, miRNA-21 is transcribed from the $10^{th}$ intron of the TMEM49 gene. Our results indicated that their processing was independent of their host genes, suggesting that they have independent promoters, as it was already demonstrated for miRNA-21 (Fujita et al., 2008). The fact that miRNA-206 is transcribed independently from its bi-cystronic companion is surprising, but the increased expression of miRNA-133b was not revealed neither by the array neither by quantitative RT-PCR. This is consistent with Chip-on-Chip experiments in which MyoD and Myogenin were found to be bound to the promoter region of miRNA-206 but not to the promoter region of miRNA-133b (Rao et al., 2006), and it may explain why miRNA-133 induces myoblast proliferation (Chen et al., 2006a) while miRNA-206 induces myoblast differentiation (Kim et al., 2006; Rosenberg et al., 2006).

We now know that different atrophic conditions activate a peculiar miRNAs signature. We also know that slow and fast muscles respond in similar ways, at least to what concerns miRNA-

206 and miRNA-21. Finally, the activation of key transcription regulators of the atrophic program, although indirectly, are sufficient to alter the miRNA expression levels.

## 6.2- miRNA-206 induces atrophy in adult skeletal muscle.

The fact that miRNA-206, a skeletal muscle-specific miRNA, was up-regulated during denervation was quite interesting. Despite several evidences addressing the role of this miRNA during C2C12 differentiation, little is known about the true function of this miRNA in adult muscle. It has been shown that miRNA-206 is highly expressed in slow muscles (McCarthy and Esser, 2007b), suggesting that it is required for the structural or metabolic characteristic of these muscles. It was also implicated in muscle regeneration (McCarthy et al., 2007; Nakasa et al., 2010; Yuasa et al., 2008c) although the molecular mechanisms were not addressed. Despite the predicted importance of this miRNA, no obvious abnormalities in weight, behavior or in the architecture and fiber-type distribution of skeletal muscles were found in miRNA 206 knockout mice (Williams et al., 2009). Therefore further *in vivo* studies are required to better understand the biological role of this miRNA.

On the contrary, the role of miRNA-21 in the skeletal muscle is still unknown. There are extensive studies on its role in cancer, being considered an onco-miR and its over-expression was shown in most cancer types (reviewed in (Kumarswamy et al., 2011). Moreover, it also regulates immunological and developmental mechanisms. Importantly, recent studies showed a role of this miRNA in cardiovascular biology. miRNA 21 was up-regulated in a variety of models of cardiac hypertrophy, a common pathological response to cardiac problems (reviewed in (Cheng and Zhang, 2010)). However, specific miRNA-21 expression in cardiomyocyte didn't have any direct effect on the regulation of cell size. The major correlation was revealed with cardiac fibrosis which was efficiently reversed by miR-21 knockdown *in vivo* (Thum et al., 2008). Also in this case, more in vivo studies are required to understand the role of this miRNA, mainly in muscle wasting.

To address their biological role in adult muscles, plasmids expressing these miRNAs were electroporated in adult muscles, to generate transgenic muscles. This approach avoided the adaptation phenomenon's typical of constitutive transgenic or knock-outs animals. The up-regulation of these two miRNAs, mainly at late stages of the muscle wasting, can have two explanations: it can indicate that they are required for the slowing down of the atrophic program or indicate that they are required for the adaptations to the new atrophic condition, and therefore are also implicated in the atrophic program.

To address the first hypothesis we expressed these miRNAs in denervated muscles. According to our data both of these miRNAs do not block the denervation-induced loss of muscle mass. Conversely, they further decrease the C.S.A. of the denervated fibers suggesting that these miRNAs exacerbate the atrophic program, giving strength to the second hypothesis. To confirm this view, we over-expressed these miRNA in control muscles. While miRNA-206 was sufficient to induce atrophy, miRNA-21 didn't alter the C.S.A. of the fibers. Importantly, electroporation of both miRNAs induce a more severe atrophy suggesting that miRNA-21, although unable to induce atrophy, exacerbates the atrophic effect of miRNA-206. This is the first report presenting functional evidences on the involvement of miRNAs in skeletal muscle atrophy.

Interestingly, the atrophy observed in the presence of miRNA-206, is followed by an increased activation of the atrogin-1 promoter activity. However, the co-expression of these miRNAs, although inducing a more severe atrophy, do not activate atrogin-1 promoter. Moreover, Murf-1 promoter is repressed by miRNA-206 alone or in the presence of miRNA-21, suggesting that this E3-ligase might not be involved in the atrophic process induced by the miRNAs. These two E3-ubiquitin ligases were shown to be highly induced during denervation (Bodine et al., 2001b), and their transcription was shown to be FoxO dependent (Sandri et al., 2004a). The exact mechanism by which miRNA-206 induced an increase of atrogin-1 promoter activity is not known, but it is possible that it regulates some transcriptional inhibitor. Although not addressed, it is also possible that these miRNAs can also act on the autophagic regulation of muscle mass.

The inhibition of miRNA-206 and miRNA-21 during denervation leads to hypertrophy and partially prevented the denervation-induced atrophy. This is in agreement with the fact that over-expression of these miRNAs leads to a decrease CSA of the fibers and may indicate that they are regulating key inhibitors of the atrophic process. Since miRNA-1 is able to repress IGF-1 (Elia et al., 2009), a well known anabolic signal (Coleman et al., 1995; Musaro et al., 2001), and considering that the sequence of miRNA-206 is highly similar to the sequence of miRNA-1, it is possible that inhibiting miRNA-206, IGF-1 becomes de-repressed and can induce muscle hypertrophy.

In order to better understand the function of miRNA-206 and miRNA-21, it is important to find the other target genes.

## 6.3- Atrophy related miRNAs regulate gene expression mainly by inducing degradation of target mRNAs.

As discussed in the introduction, miRNAs can regulate gene expression by two different post-transcriptional mechanisms: blocking the translation or inducing the mRNA degradation. Initially, the data supported the translational control as the prevailing mechanism of miRNA regulation. However, recent evidence underlines the mRNA degradation as an important mechanism of control as well. In order to simplify the definition of the targets, we have developed tools to define the transcripts that are reduced by miRNAs. To identify the possible targets a genomic approach was used. We started by establish the mRNA expression profile of the same samples that were previously analyzed by miRNA micro-array. This approach identified 1462 genes that were differentially expressed between atrophic and control conditions. We found a significant enrichment of the proteasome and of the ribosome pathway among the up-regulated genes. Furthermore, the insulin pathway was down regulated in the atrophic samples. These, confirmed the results previously obtained by others (Bodine et al., 2001a; Lecker et al., 2004).

Crossing the list of the up- or down-regulates mRNAs in each atrophic condition, with the list of the predicted targets of the altered miRNAs and looking for the inversely correlation between miRNA and mRNA we significantly reduced the list of predicted targets. Since we are focusing on miRNA-206 and miRNA-21, we found 322 and 121 possible targets, respectively. Considering the fact that target mRNA can be regulated simultaneously by more than one miRNA, and that both of these miRNAs are up-regulated during denervation, it was decided to focus mainly on targets that were commonly regulated by miRNA-206 and miRNA-21. Therefore, the number of potential target was reduced to 4: PolK, YY1, eIF4E3 and PDCD10.

In C2C12 myoblasts it was shown that DNA polymerase alpha1 (PolA1) is down- regulated by miRNA-206 (Kim et al., 2006). The author proposed that down-regulation was required for the exit from the cell cycle, and for the activation of a differentiation program. Although the role of DNA polymerases in adult skeletal muscle is not known, it is interesting to note that the list of the down-regulated predicted targets include three DNA polymerases (PolK, PolA1 and PolE4), being Polk the gene with the highest predictive scores. The reasons for this suppression is unknown, but our data has indicated that during denervation the 3'UTR of this genes is down-regulated,

suggesting the involvement of miRNAs in this regulation. However the two binding site for miRNA-206 were not sufficient to modulate the 3'UTR of this gene suggesting that another miRNA is responsible for the modulation of PolK.

Another predicted target down-regulated during denervation is YY1. This is a transcription factor which has been already described to modulate different function in the skeletal muscle. In fact, it has been shown to block several myogenic processes (Cunningham et al., 2007; Galvagni et al., 1998; Gualberto et al., 1992). Importantly, in adult muscles YY1 is involved in mitochondrial biogenesis (Cunningham et al., 2007) and in dystrophin expression (Galvagni et al., 1998). Considering that during atrophy there are metabolic and structural adaptations, this gene becomes of interest for further study. According to our data, the 3'UTR of this gene is modulated during denervation. Furthermore, miRNA-21 was shown to directly modulate the 3'UTR of YY1, while miRNA-206 doesn't have any effect. This data suggests that miRNA-21, by repressing YY1, might activate metabolic changes in adult muscle. Interestingly, YY1 might be also involved in the regulation of miRNA-206 and miRNA-21. In fact, it is known that YY1 binds to AP1, and repress the AP1-dependent transcription of miRNA-206 (Song and Wang, 2009). This is possibly a mechanism that may contribute for the up-regulation of miRNA-206 during denervation. On the other hand, YY1 repress the binding of SRF to some of muscle specific promoters (Gualberto et al., 1992). Considering that the regulatory region of miRNA-21 has a functional SRF binding site (Zhang et al., 2011) it is possible that the induction of miRNA-21 leads to a reduction of YY1, liberating SRF from its repressive effect and inducing the expression of miRNA-21.

During denervation, protein synthesis is altered, shifting the metabolic and structural properties of the fibers. Although requiring further confirmation, data from our laboratory, indicate that the rate of proteins synthesis might be decreased. In fact, Eukaryotic translation initiation factor 4E-binding protein 1 (eIF4E-BP1 or simply 4EBP1), a repressor of protein synthesis, is an atrophy-related gene (Lecker et al., 2004) that is up-regulated in atrophying muscles. Furthermore at the protein level it is highly up-regulated and hyper-phosphorylated during denervation suggesting a block of the pathways that activates proteins synthesis. 4EBP1 binds and repress eIF4E, the most critical initiation factor for the beginning of translation. Interestingly, in the list of the down-regulated predicted target genes, an isoform of eIF4E, eIF4E3, is one of the most repressed genes possibly by miRNA-206 and miRNA-21. Although there are no studies on the role of this gene in skeletal muscle, we have found that its 3'UTR is suppressed by miRNA-206 and miRNA-21. It is therefore possible that these two miRNAs act together and synergistically to slow down the rate of protein synthesis during denervation.

Also PDCD10 fulfill the standard to be a target of both miRNA-206 and miRNA-21. Although the role in skeletal muscle is not known, it is involved in the cerebral cavernous malformation for its role in vascular morphogenesis (Bergametti et al., 2005). This gene is highly expressed in skeletal muscle and during denervation is strongly down-regulated. According to our data, this down-regulation results from a down-modulation of its 3'UTR that occurs under the control of both miRNA-206 and miRNA-21. The function of this gene and the reason for this down-regulation during denervation requires further studies.

To confirm that these genes were real targets two approaches were taken. The first approach analyzed the expression levels of these genes in C2C12 myoblasts after transfection with miRNAs. This would indicate whether there was a direct regulation of target genes by the miRNAs. While miRNA-206 expression significantly decreased the mRNA level of PDCD10, miRNA-21 decreased the transcripts of eIF4E3 and PDCD10. Surprisingly, mRNA level of YY1 was not affected but a significant reduction of the protein was found. The regulation of eIF4E3 at the protein level still requires further studies. These results confirm that YY1 is a target of miRNA-21 and that PDCD10 is a target of both miRNA-206 and miRNA-21. To further confirm the miRNA-dependent regulation of eIF4E3, we mutagenize the miRNAs sites at 3'UTR. In fact, the mutagenesis of the miRNA-206 and miRNA-21 binding sites partially prevented the action of these miRNAs on the 3'UTR. The same approach is now being taken for the 3'UTR of the YY1 gene, in order to further confirm the previous results.

Altogether, these results indicate that the bioinformatic approach used to identify target genes of miRNAs was valid. Since the role of the majority of these target is not known in adult skeletal muscle or during the atrophic process, further studies are required to elucidate their function.

One of the most interesting results from this thesis concerns the inhibition of both miRNAs *in vivo*. In fact, this inhibition leads to an hypertrophic phenotype and partially prevents the denervation-induced loss of CSA. In a period in which miRNAs are gaining strength as therapeutic agents these results are quite promising. Several studies demonstrated that miRNAs can be inhibited *in vivo*. Recently, it was shown that systemic delivery, by subcutaneous injection, of antisense oligonucleotides was able to block specific miRNAs in the heart. For example, the inhibition of miRNA-208a, was sufficient to prevent cardiac remodeling and to improve cardiac function and serviva (Montgomery et al., 2011). This is an example of the therapeutic potential of the miRNAs. Using a sponge vector to block miRNA 206, we were able to partially prevent muscle atrophy induced by denervation, suggesting that it may be a useful treatment for spare muscle mass during

catabolic conditions or aging. Considering the tissue specificity of miRNA-206 no major side effects are expected. However a better understanding of the targets and of the molecular mechanisms that miRNAs are regulating is necessary.

In conclusion, the results presented in this thesis show that miRNA expression is deregulated during atrophic conditions. Focusing on muscle denervation we show that two miRNAs, miRNA-206 and miRNA-21 are strongly up-regulated. Although the exact mechanisms implicated were not fully elucidated we showed that FoxO3 and NF-kB, the two most important transcriptional regulators of muscle wasting, are sufficient to up-regulate these two miRNAs. The role of these miRNAs was also addressed *in vivo*. We showed, for the first time, that these miRNAs regulate muscle mass. Most importantly we have shown that inhibition of these two miRNAs induced muscle growth and prevent atrophy-induced muscle loss. Some targets of these miRNAs were also identified. We found that miRNA-206 regulates the expression of eIF4E3, an important regulator of muscle growth, while miRNA-21 could regulate the expression of eIF4E3 and YY1, an important regulator of mitochondria biogenesis. Altogether, these results indicate that increasing the knowledge on miRNA biology, specifically in the skeletal muscle, may help the understanding the molecular mechanisms that govern the atrophic conditions and may contribute for the developing of miRNA-based therapies.

# Chapter 7 – Future Perspectives

The work present in this thesis clearly demonstrated that each atrophic condition has a specific miRNA expression signature. As usual, from micro-arrays a huge number of information was retrieved. For practical reasons, and also because they were appealing, only two miRNAs were studied. Other studies are required to elucidate the role that the other deregulated miRNAs play during each specific atrophic condition. This will bring a better understanding on the complex regulatory role of miRNAs.

As it was shown in the results of this thesis, the role of the target genes is still unclear. Several approaches are currently being taken in order to address this point. We need to address whether the over-expression of this two genes induces a phenotype in the skeletal muscle. It was also shown that miRNA-21 requires an atrophic stimulus in order to develop an atrophic phenotype. Since, YY1 and eIF4E3 are targets of miRNA-21, and since they are down-regulated during denervation-induced atrophy, it is possible that their inhibition is a required event for the activation or commitment of an atrophic program. Therefore we are currently analyzing denervated muscles in which YY1 and eIF4E3 were over-expressed. The main question is whether these two proteins are capable of preventing muscle atrophy.

As it was already discussed, miRNAs can target hundreds of mRNAs and therefore block their translation or induce the degradation of the mRNAs. In this thesis we have shown that degradation of the target mRNA is an important mechanism. For practical reasons only a limited number of targets were studied. However, a broader knowledge of the miRNA target is required. In order to identify the mRNAs that are targeted by the miRNA machinery, we are currently optimizing an immunoprecipitation technique that allows the immunoprecipitation of the mRNAs bound to the Ago2 protein, a key protein in the recognition miRNA/target mRNA. Briefly, Ago2 Protein is immunoprecipitated under specific conditions allowing the maintenance of the biding mRNA/protein. In this way, all the mRNAs bound to Ago2 will then be purified and analyzed either by RT-PCR either by micro-array. By performing this experiment in innervated Vs denervated muscle, we will obtain a list of mRNAs regulated by the miRNA pathway during denervation. Crossing this list with the list of the possible targets of each deregulated miRNA, will allow the identification of hundreds of targets, and it will allow to understand which metabolic pathways are being post-transcriptionally regulated by miRNAs.

It was demonstrated that over-expression of the two miRNAs under study, miRNA-206 and miRNA-21, induces hypertrophy and partially prevent denervation-induced atrophy. Therefore, it will be important to address the effect of systemic delivery of these miRNAs on the atrophic program. A condition that deserves considerably study is the atrophy induced by ageing. This approach may contribute for the development of specific therapies in the following years.

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