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XXI CICLO

Identification and Characterization of FoxO-target genes by Chromatin Immunoprecipitation

Direttore della Scuola: Ch.mo Prof. Tulio Pozzan

Supervisore: Ricercatore Ordinario Dott. Marco Sandri

Dottorando: Milan Giulia

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RIASSUNTO:

Diverse patologie sistemiche, quali il diabete la sepsi, la cachessia neoplastica, e condizioni cataboliche, come la denervazione, l'immobilitazione, i trattamenti con glucocorticioidi o digiuno, portano conseguenza sono caratterizzate dalla perdita di massa muscolare, definita come atrofia muscolare. Questa perdita di massa muscolare è dovuto all'aumento dell'attività di sistemi proteolitici, come il sistema dell'ubiquitina-proteosoma e il sistema autofagico-lisosomiale. Studi di espressione genica hanno evidenziato l'esistenza di un gruppo comune di geni, sovra espressi o repressi in molte condizioni atrofiche. Questi geni sono stati chiamati geni dell'atrofia o *atrogenes* (Jagoe and Goldberg, 2001; LECKER et al., 2004). Tra questi spiccano due geni, MuRF-1 and atrogin-1, altamente espressi in molte condizioni atrofiche, che codificano per due ubiquitine ligasi muscolo specifiche. L'induzione induzione di atrogin-1 and MuRF-1 precede la perdita di massa muscolare e continua per tutto il processo. L'identificazione di queste due ubiquitine ligasi tra i geni dell'atrofia è importante in quanto esse costituiscono il la componente limitante del processo di ubiquitinizzazione, e, quindi, sono cruciali per la regolazione della degradazione proteica dipendente dal proteasome (Gomes et al., 2001). Atrogin-1 è regolato dai fattori trascrizionali FoxO che sono a loro volta inibiti dalla via di segnale di IGF1-PI3K-Akt. L'assenza di stimoli della crescita, come IGF1 o l'insulina, rende AKT non attivo e quindi non in grado di bloccare i fattori FoxO, che possono traslocare nel nucleo e interagire con i promotori dei geni bersaglio (Sandri et al., 2004). Nel tessuto muscolare dei mammiferi sono espressi tre fattori FoxO: FoxO1, FoxO3 and FoxO4, caratterizzati da un "forkhead box", che comprende il dominio di legame con il DNA. I fattori FoxO riconoscono e legano, come monomeri, una stessa sequenza consensus sul DNA. Precedenti studi hanno dimostrato che FoxO1 e FoxO3 sono coinvolti nell'atrofia muscolare (Kamei et al., 2004; Sandri et al., 2004; Stitt et al., 2004). Non sono ancora stati resi noti quali siano i geni controllati dai entrambi i fattori FoxO o in modo specifico da uno dei due, che determinano la perdita di massa muscolare. L'identificazione di geni bersaglio dei FoxO è oggetto di numerosi studi, dato il coinvolgimento di

questi fattori nel regolare svariate funzioni cellulari. Nell'ottica di comprendere in vivo l'interazione tra i FoxO e promotori dei geni bersaglio, ho applicato la tecnica della Chromatin immunoprecipitatetion (Das et al., 2004). Questa tecnica combina la specificità dell'immunoprecipitazione, per purificare proteine di interesse, con la potenza della reazione della PCR, per amplificare sequenze genomiche potenzialmente legate alla proteina studiata. Ho iniziato con l'analisi dell'interazione di FoxO1 e FoxO3 con una regione promotore di atrogin-1 contenente 14 siti potenziali per il legame di FoxO. Dopo una prima serie di esperimenti *in virto* per verificare l'efficienza della ChIP nel rilevare i legami dei fattori FoxO con il DNA, sono passata a studi *in vivo*, prima in condizioni di sovra espressione dei fattori FoxO e successivamente in un modello di atrofia. Mediante elettroporazione in muscoli tibialis anteriori di topi CD1, ho indotto l'espressione delle forme costitutivamente attive di FoxO1 e FoxO3 entrambi con all'epitopo HA. Dopo 7 giorni dall'operazione, i muscoli sono stati prelevati, lisati e trattati per essere utilizzati in esperimenti di ChIP. A seguito d'immunoprecipitazione, i frammenti di cromatina legati ai fattori FoxO sono stati amplificati mediante PCR utilizzando primer specifici per i 14 i siti potenziali di legame sul promotore di atrogin-1. Da questi esperimenti è emerso che FoxO3 si lega in molti siti sul promotore di atrogin-1, mentre per FoxO1 ho individuato una debole interazione su un unico sito. Per comprendere meglio quali siti sono fisiologicamente rilevanti nella regolazione di atrogin-1, ho analizzato l'interazione FoxO-promotore di atrogin-1 durante l'atrofia. Come modello di atrofia è stato scelto il digiuno, modello in cui c'è una notevole attivazione dei fattori FoxO e un incremento dell'espressione di atrogin-1 di circa 10 volte, rispetto alla condizione di controllo. Ho utilizzato per studi di ChIP il lisati muscolari di topi a 24 ore di digiuno e di topi di controllo. Questa serie di studi di ChIP hanno dimostrato che, durante il digiuno, FoxO3 lega in modo preferenziale tre regioni del promotore di atrogin-1, in cui aumentano i livelli di acetilazione della cromatina. L'analisi per FoxO1 rilevato solo una debole interazione di FoxO1 su una regione del promotore di atrogin-1. Per indagare la capacità di entrambi i fattori FoxO nel promuovere la trascrizione del gene, indipendentemente dal legame al promotore, ho utilizzato il sistema del gene reporter luciferasi fuso al promotore di atrogin-1. Dall'analisi al luminometro è emerso che FoxO3 promuove fortemente l'attività

promotoriale, mentre FoxO 1 non ha alcuna capacità di transattivare atrogin-1. Questi risultati hanno suggerito il coinvolgimento di altri geni regolati dai FoxO nel programma atrofico. Come conferma di questa ipotesi è stato osservato che la sovra-espressione di atrogin-1 per sè non è sufficiente a indurre la perdita di massa muscolare. Ho preso in esame altri due geni del gruppo degli *atrogenes*, LC3 e Bnip3, compresi tra il gruppo degli *atrogenes* maggiormente indotti. Entrambi appartengono al sistema dell'autofagia mediata dai lisosomi. Le rispettive proteine codificate, LC3 and Bnip3, costituiscono due componenti cruciali per la formazione degli autofagosomi, strutture vescicolari deputate alla degradazione di porzioni citoplasmatiche. L'analisi dei promotori di LC3 e Bnip3 ha evidenziato molti siti potenziali per l'interazione con i fattori FoxO. Gli esperimenti condotti di ChIP hanno dimostrato che in condizioni di digiuno FoxO3 lega regioni specifiche di questi promotori. Gli studi funzionali dell'attività promotoriale hanno confermato che il legame di FoxO3 con il promotore li LC3 è essenziale per attivare la trascrizione del gene. Inoltre, mediante l'espressione di FoxO3 costitutivamente attivo e del dominante negativo, è emerso che FoxO3 regola i livelli proteici di Bnip3. Tuttavia questi risultati non hanno chiarito ancora quali sono i geni regolati da FoxO1 e se ci sono altri *atrogenes* controllati da FoxO3. Nell'obiettivo di estendere gli studi d'interazione proteina-DNA a più regioni promotori del genoma, è stato scelto di applicare la tecnica della ChIP on Chip. Questa tecnica combina la ChIP con l'ibridazione su microarrays genomici. Gli esperimenti di ChIP on Chip sono stati condotti in colture cellulari, utilizzando miotubi infettati con adenovirus esprimenti la forma costitutivamente attiva di FoxO3 con l'epitopo HA. I risultati ottenuti hanno messo in evidenza che FoxO3 interagisce con i promotori di geni che apprtengono a diverse vie del segnale e sono implicati in importanti processi metabolici e nella regolazione trascrizionale. E' stato interessante notare che tra i promotori genici bersaglio di FoxO3 otto di questi sono promotori di *atrogenes*. Dall'analisi dei livelli di espressione di alcuni di questi geni bersaglio, è emerso il ruolo chiave di FoxO3 nella loro regolazione. Il mio lavoro svolto durante il dottorato pone le basi tecniche per l'identificazione di geni fondamentali al programma atrofico e costituisce un contributo per lo sviluppo di nuove strategie farmacologiche contro la perdita di massa muscolare.

SUMMARY:

Loss of muscle mass occurs in many debilitating diseases including sepsis, burn injury, cancer, diabetes, heart and renal failure, but also in other catabolic conditions like denervation, immobilization, glucocorticoid treatment, or fasting. The reduction in muscle cell size is caused by an increase of the activity of proteolytic systems, such as the ubiquitin-proteasome system and the lysosome autophagic system. Gene expression studies revealed a set of genes which are commonly up- and down-regulated in different atrophying conditions. These genes are called *atrophic related genes* or *atrogenes* (Jagoe and Goldberg, 2001; LECKER et al., 2004). Two of the most induced genes, whose activation precedes muscle loss, are muscle-specific and encode for ubiquitin ligase. These two enzymes, named MURF1 and Atrogin-1, are rate-limiting in the ubiquitination process and therefore, regulate the proteasomal-dependent protein breakdown (Gomes et al., 2001). Atrogin-1 is controlled by FoxO transcription factors, which are in turn inhibited by the IGF1-PI3K-Akt pathway. In the absence of growth factors, like IGF1 or insulin, AKT is not activated and does not block FoxOs which can translocate into the nuclei and interact with promoters of their target genes (Sandri et al., 2004). In mammalian muscle, there are three FoxO transcription factors: FoxO1, FoxO3 and FoxO4. All FoxOs share the same "forkhead box" which contains the DNA-binding domain. Previous studies showed FoxO1 and FoxO3 to be involved in muscle atrophy (Kamei et al., 2004). However, it is completely unknown how many genes are required for FoxO-mediated muscle loss and whether these genes are or are not commonly regulated by both FoxO1 and FoxO3. In this study we proprosed to understand which are the atrophy-related genes target of FoxO1 and FoxO3. We set up ChIP technology in order to dissect in vivo, the interaction between FoxOs and promoters of FoxO's target genes. This technique combines the specificity of immunoprecipitation in purifying specific proteins with the power of PCR to amplify specific sequences of DNA. First, we identified the potential FoxO1 and FoxO3 potential binding sites on the atrogin-1 promoter. Bioinformatic analysis of atrogin1 promoter showed that there are 14 potential FoxO binding sites.

Second, we used a cell culture system to establish different parameters for a successful ChIP . We then moved to in vivo studies of both overexpressed FoxOs and endogenous FoxO proteins. Tibialis Anterior (TA) of adult CD1 mice were transfected with constitutively active HA tagged: ca-FoxO3 or ca-.FoxO1. After seven days muscles were collected, lysated and analysed for ChIP. ChIP studies demonstrated that FoxO3 strongly binds many sites of atrogin-1 promoter, while FoxO1 weakly interacts with only one site. To better understand which sites are physiologically relevant for atrogin-1 regulation endogenous FoxO1 and FoxO3 were immunoprecipitated. Fasting was used as a model of atrophy, since this model strongly activates FoxOs increasing atrogin-1 levels to more than 10 fold. ChIP experiments were performed on TA muscles of fed and starved mice. Interestingly, we could demonstrate by ChIP that FoxO3 preferentially binds chromatin at three sites of atrogin-1 promoter. These sites showed also an increase of histone acetylation during fasting. FoxO1 displayed a weak interaction with atrogin-1 promoter. To understand if both FoxOs are able to regulate atrogin-1 expression, we used a functional assay in which the atrogin-1 promoter was cloned upstream the luciferase gene. This reporter was transfected together with FoxO1 or FoxO3 in the tibialis anterior muscle and seven days later the assay was performed. This approach confirmed that FoxO3, but not FoxO1, could transactivate the atrogin-1 promoter. Since both FoxOs can induce muscle loss, the probability that other genes are required for muscle atrophy was seriously considered. Indeed overexpression of atrogin-1 per sè is not sufficient to induce muscle wasting. To understand how FoxOs cause muscle loss we focused on the atrophy-related genes which belong to other proteolytic systems and especially to autophagy-lysosome system. In fact LC3 and Binp3 are among the upregulated atrogenes and are rate limiting proteins in autophagosome formation. Their promoters contain several potential FoxO binding sites and ChIP experiments showed that FoxO3 binds chromatin of LC3 and Bnip3 promoters in specific sites during starvation. Functional promoter assay confirmed that FoxO3 binding on LC3 promoter is essential to activate transcription. Moreover, gain and loss of function experiments showed that FoxO3 regulates Binp3 protein levels. Altogether these findings show that FoxO3, but not FoxO1, coordinates the two major proteolytic systems of the cell by regulating the promoter activity of

atrogin-1, LC3 and Binp3 genes. However these results have not clarified yet which atrogenes are regulated by FoxO1 and if there are other atrogenes under FoxO3 control. For this reason, we extend the ChIP assay to a genome wide analysis using a ChIP-on-Chip approach. This technique combines the Chromatin immunoprecipitation with hybridization on genomic microarray to allow a global analysis of protein–DNA interaction. The technique was set up using a cell culture system. Differentiated C2C12 myotubes were infected with adenovirus expressing HA tagged c.a.FoxO3 and 48 hours later a ChIP was performed using anti-HA antibody. The results of ChIP on Chip identified several binding sites on genome. Many of these regions are promoters for transcription factors, enzymes of different metabolic pathways and different kinases involved in regulation of different pathways. Importantly a FoxO3 binding site was detected in regulatory regions of other eight atrophy-related genes. When the function of this binding sites was validated it was found that 5 of these atrogenes were modulated by FoxO3. In conclusion, this PhD project establish the technical basis for the identification of the fundamental genes, under the control of a certain transcription factor, involved in the atrophy program. All these data will contribute to the development of new pharmacological strategy against muscle loss.

INTRODUCTION:

Skeletal muscle: structure and function

Skeletal muscle is constituted by cylindrical *multinucleated* cells, called muscle fibers, bundled together and wrapped by connective tissue. Each muscle is surrounded by a connective tissue sheath called the epimysium. Fascia, the connective tissue outside the epimysium, surrounds and separates the muscles. Portions of the epimysium project inward to divide the muscle into compartments. Each compartment contains a bundle of muscle fibers. Each bundle of muscle fiber is called a fasciculus and it is surrounded by a layer of connective tissue, called perimysium. Within the fasciculus, each individual muscle fiber is surrounded by connective tissue called the endomysium (**Fig1**).

Skeletal muscles have an important vascularisation to provide nutrients and oxygen and are innervated. Generally, an artery and at least one vein accompany each nerve that penetrates the epimysium of a skeletal muscle. Branches of the nerve and blood vessels follow the connective tissue components of the muscle reaching the single myofibers together with the capillaries network.

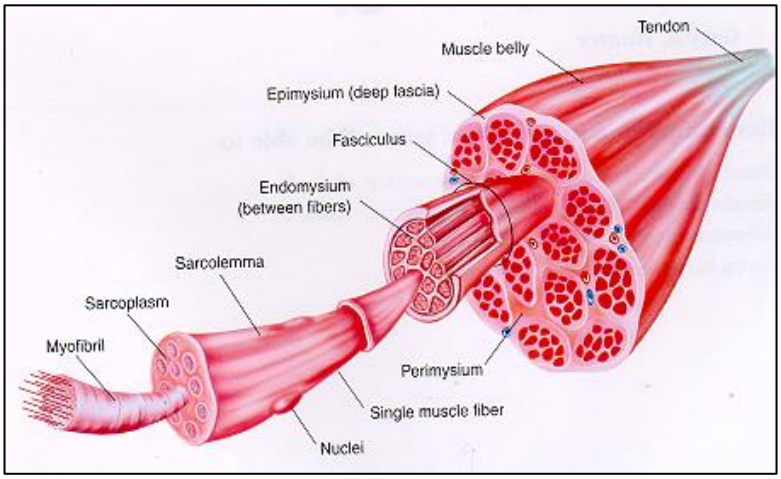


Figure 1 A Schematic representation of skeletal muscle structure.

The nuclei of muscle cells are located at the periphery of the muscle cell, just under plasma membrane, the sarcolemma. Conversely, the presence of central-located nuclei is pathological and it is consistent with a disease called centre nuclear myopathy. Mice have a peculiarity since regenerating muscles maintain the nuclei in a central position, therefore, only in mice the presence of central-nucleated myofibers reveal a precedent injury which was followed by regeneration.

Closed to muscle cell and surrounded by their own membrane, are preset the nuclei of satellite cell, the skeletal muscle stem cell.

Cytoplasm of muscle cells is filled by the contractile proteins organized in myofibrils (also called simply fibrils); they are all packed in a parallel arrangement. Each myofibril runs the entire length of the fibre In transversal section, myofibrils display a characteristic banding pattern of striated skeletal muscle. This aspect is owing to the lined up in series of sarcomeres , that are the structural units. The sarcomere comprises two sets of filament: thin ones, made mostly of actin, and thick filaments, made mostly of myosin. Several other important proteins, like troponin and tropomyosin, titin, nebulin, and desmin, help to hold these units together.

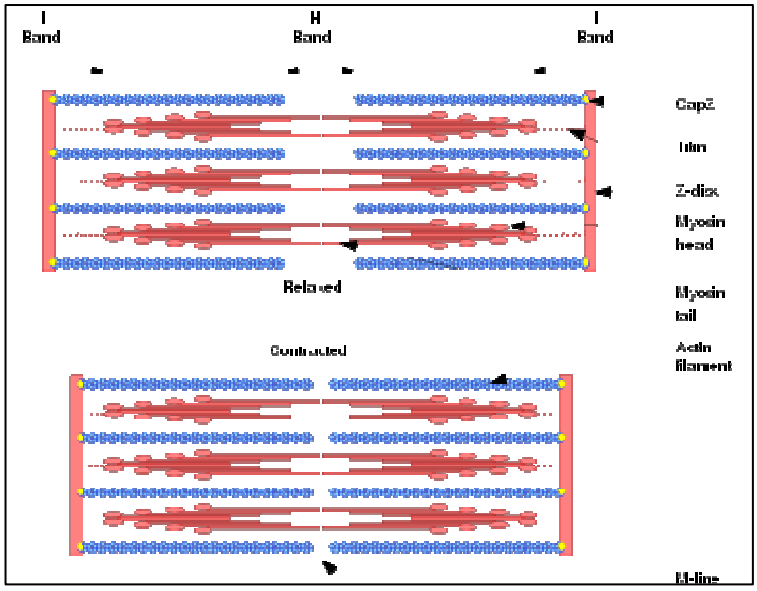


Figure 2 A scheme of muscle contraction.

The sarcomere is defined as the segment between two neighbour Z-lines. In electron micrographs the Z-line appears as a series of dark lines. Surrounding the Z-line, there is the region of the I-band (the light band). Following the I-band there is the A-band (the dark band). Within the A-band, there is a paler region called the H-band. The nomenclature of A- and I-band come form their properties to reflect the polarized light under a polarization microscope. Finally, inside the H-band is a thin M-line (*middle* of the sarcomere).

Actin filaments are the major component of the I-band and extend into the A-band.*Myosin* filaments extend throughout the A-band and are thought to overlap in the M-band. The giant protein, *titin* (connectin), extends from the Z-line of the sarcomere, where it binds to the thin filament system, to the M-band, where it is thought to interact with the thick filaments. Several proteins important for the stability of the sarcomeric structure are found in the Z-line as well as in the M-band of the sarcomere. Actin filaments and Titin molecules are cross-linked in the Z-disc via the Z-line protein alpha-Actinin. The M-band myosin as well as the M-proteins bridge the thick filament system to the M-band part of titin (the elastic filaments).

The interaction between actin and myosin filaments in the A-band of the sarcomere is responsible for the muscle contraction (*sliding filament model*).

Upon muscle contraction, the A-bands do not change their length, whereas the I-bands and the H-zone shorten.This is called the *sliding filament hypothesis* which is now widely accepted. There are projections from the thick filaments, called *cross-bridges* which contain the part (head) of myosin which binds to actin; and can hydrolyze ATP and convert chemical energy into mechanical energy. This process is quite efficient and 40-60% of energy is consumed for mechanical action while the remaining energy is lost as heat. These cross-bridges can be seen by electron microscopy. The cross bridges are mostly oriented transverse to the fibre axis in relaxed fibres and are angled at about 45 degrees in rigor. Contraction is a transient state, hard to capture because it is very fast, but it is assumed that these two states are representative of relaxed and contracted situations.

To allow the simultaneous contraction of all sarcomers, the sarcolemma penetrates into the cytoplasm of the muscle cell between myofibrils, forming membranous tubules called *t-tubules*. The t-tubules are electrically coupled with

the terminal cisternae which continue into the sarpolasmic reticulum. Thus the sarcoplasmic reticulum, which is the enlargement of smooth endoplasmic reticulum and which contains the majority of calcium ions required for contraction, extends from both sides of t-tubules into the myofibrils. Anatomically the structure formed by t-tubules surrounded by two smooth ER cisternal is called the triad and it allows the transmission of membrane depolarization from the plasmalemma to the endoplasmic reticulum.

The contraction starts when an action potential diffuses from the motor neuron to the sarcolemma and then it travels along t-tubules until it reaches the sarcoplasmic reticulum. Here the action potential changes the permeability of the sarcoplasmic reticulum, allowing the flow of calcium ions into the cytosol between the myofibrils. The release of calcium ions induces the myosin heads to interact with the actin, allowing the muscle contraction. The contraction process is ATP-dependent. The energy is provide by mitochondria which are located closed to Z-line.

Skeletal muscles contains several fiber types which differ for structural and functional properties. Mammalian muscle fibers are divided into two distinct classes: the **type I**, also called slow fibers, and **type II**, called fast fibers. This first classification considered only the mechanical properties. However the different fiber types show also peculiar myosin ATPase type, metabolism (oxidative or glycolitic), mitochondrial content revealed by succinate dehydrogenase (SDH) staining, resistance to fatigue(Pette and Heilmann, 1979; Pette et al., 1979; Schiaffino et al., 2007). Altogether these biochemical and molecular properties explain the contraction properties.

Since in most cases both fiber types coexist in one muscle, the type of contraction (slow or fast) results form the percentage of different fiber types present in the muscle. In this way, the skeletal muscle can be classified either in slow, if it contains more type I fibers, or in fast, if type II fibers are more abundant. The different fiber types contain also peculiar Myosin Heavy Chain (MHC) which are coded by different genes. The fiber type I expresses the slow isoform of MHC (MHCβ or MHC1), a great content of mitochondria, high levels of myoglobin, high capillary densities and high oxidative enzyme capacity. Muscles containing

many type I fibers display red colour for the great vascularisation and for the myoglobin content.

The type II, fast, myofibers are divided in three groups depending on which myosin is expressed. In fact distinct genes encode for MHC IIa, IIx (also called IId) and IIb. Type IIa myofibers are faster than type I, but they are still relatively fatigue-resistant. IIa fibers are relatively slower than IIx and IIb and have an oxidative metabolism due to the rich content of mitochondria (Schiaffino and Reggiani, 1996). Given all those characteristics, IIa fibers are also termed fast-oxidative fibers. They have been classically characterized as exhibiting a phenotype that has fast contraction, high oxidative capacity and a relative fatigue resistance. The IIx and IIb fiber types are called fast-glycolitic fibers and they show a prominent glycolitic metabolism containing few mitochondria of a small size, high myosin ATPase activity, expression of MHCIIB and MHCIIX proteins, the fastest rate of contraction and the highest level of fatigability.

The fiber type profile of different muscles is initially established, during development, independently of nerve influence. Nerve activity has a major role in maintenance and modulation of fiber type properties in adult muscle (Schiaffino, Sandri et al. 2007). Indeed type I fibers start to appear only after innervation and the absence of the nerve maintains a fast program and blocks the expression of slow isoform of myosin.

Plasticity of skeletal muscle: hypertrophy versus atrophy

Skeletal muscle comprise 40-50% of total body mass, and it is the major protein storage for the body. Being the largest protein reservoir, muscle serves as a source of amino acids to be utilized for energy production by various organs during catabolic periods (Lecker et al., 2006a). For instance, amino acids generated from muscle protein breakdown are utilized by the liver to produce glucose and to support acute phase protein synthesis (Lecker et al., 2006a). Skeletal muscle is a dynamic tissue continuously adapting its size in response to a variety of external stimuli, including mechanical load, neural, hormones/growth factors, stress and nutritional status (Waddell et al., 2008b). For instance, exercise

stimulates protein synthesis that leads to increase of fiber size and in general to increase muscle mass and force. This process is named hypertrophy.
Conversely muscle disuse causes a rapid loss of myofibrillar proteins that results in a decrease of fiber size and in general a decrease of muscle mass and force. This condition is called atrophy.

Hypertophy

The growth of skeletal muscle mass, like the mass of any other tissue, depends on protein turnover and cell turnover (Sartorelli and Fulco, 2004). Cellular turnover plays a major role during muscle development in embryo. Moreover satellite cell incorporation into the growing fibers takes place during post-natal muscle growth (Moss and Leblond, 1971) concomitantly with increased protein synthesis. The activation of satellite cells is important for maintaining a constant size of each nuclear domain (quantity of cytoplasm/number of nuclei within that cytoplasm). Unlike young muscle, the contribution of cellular turnover to homeostasis of adult fibers is minor and its role in hypertrophy has even been recently debated (McCarthy and Esser, 2007; Rehfeldt, 2007). In adult muscle the physiological conditions promoting muscle growth, therefore, do so mainly by increasing protein synthesis and decreasing protein degradation. The IGF1-AKT signaling is the major pathway which controls muscle growth. In addition to circulating IGF-1, mainly synthesized by the liver under GH control, local production by skeletal muscle of distinct IGF-1 splicing products has recently raised considerable interest. A specific IGF-1 splicing product is important for load- and stretch-induced adaptations in skeletal muscle (Goldspink, 1999) Muscle-specific over-expression in transgenic mice of an IGF1 isoform, locally expressed in skeletal muscle, results in muscle hypertrophy and, importantly, the growth of muscle mass matches with a physiological increase of muscle strength. Furthermore, the over-expression of a constitutively active form of Akt, a downstream target of IGF1, in adult skeletal muscle induced muscle hypertrophy. Similar results were obtained by the generation of conditional transgenic mice in which Akt is expressed in adult skeletal muscles only after tamoxifen or tetracycline treatment. Taken together with other observations, these results suggest that IGF1-Akt1 axis is a major mediator of skeletal muscle hypertrophy.

While it has been established that Akt plays a crucial role in muscle growth, the downstream targets involved in muscle hypertrophy remain to be defined.

Atrophy

Atrophy is a decrease in cell size mainly caused by loss of organelles, cytoplasm and proteins. A general loss of skeletal muscle mass occurs in many debilitating diseases including sepsis, burn injury, cancer, AIDS, diabetes, heart and renal failure.

In most types of muscle atrophy overall rates of protein synthesis are suppressed and rates of protein degradation are consistently elevated; this response accounts for the majority of rapid loss of muscular proteins. The identification of precise signaling cascade, that directs muscle wasting, is only at the beginning, although in recent years several pathways have emerged to be critical for the maintenance of muscle mass. A major contribution in understanding muscle atrophy comes from the pioneering studies on gene expression profiling performed independently by groups of AL Goldberg and DJ Glass. The idea to compare gene expression in different models of muscle atrophy leads to the identification of a subset of genes that are commonly up- or down-regulated in atrophying muscle. Since all the diseases used for the experiments of microarray (i.e. Diabetes, Cancer cachexia, chronic renal failure, fasting and denervation) have muscle atrophy in common, the commonly up- or down-genes are believed to regulate the loss of muscle components and are called *atrophy-related genes* or *atrogenes.* Together these findings indicate that muscle atrophy is an active process controlled by specific signaling pathways and transcriptional programs. These genes encode for proteins involved in different cellular processes like energy production, transcription factors, regulators or protein synthesis and enzymes of metabolic pathways. Among the upregulated atrophy-related genes there is a subset of transcripts related to protein degradation pathways. The major proteolytic systems responsible for protein breakdown in eukaryotic cells are the ubiquitin-proteasome system and the autophagy lysosome pathways. In diverse types of muscle wasting, the ubiquitin-proteasome pathway is activated, as shown by increased sensitivity to proteasome inhibitors; increased levels of ubiquitin

conjugates; enhanced rates of ubiquitin conjugation; and induction of genes for ubiquitin, several proteasomal subunits, and two critical ubiquitin ligases (E3s), atrogin-1/MAFbx and MuRF1 (Bodine et al., 2001; Lecker et al., 2004). An increased capacity for lysosomal autophagic proteolysis has been demonstrated in various types of atrophy (Bechet et al., 2005; Furuno et al., 1990). Electron microscopic studies have previously shown that autophagy is activated in denervation atrophy (Schiaffino and Hanzlikova, 1972b) and this system is stimulated in different conditions leading to muscle atrophy (Bechet et al., 2005).

The Ubiquitin-Proteasome System

Several evidences strongly support a major role of ubiquitin proteasome system (UPS) during muscle loss. Decrease in muscle mass is associates with: i) increase conjugation of ubiquitin to muscle proteins, ii) increase of proteasomal ATP-dependent activity, iii) increase protein breakdown which can be efficiently blocked by proteasome inhibitors and iv) upregulation of mRNA for ubiquitin, some ubiquitin-conjugating enzymes (E2), few ubiquitin-protein ligases (E3) and several proteasome subunits. The rate limiting enzyme of UPS is the E3 which catalyzes the transfer of ubiquitin from the E2 to the lysine in the substrate. This reaction is highly specific and the proteins, committed to ubiquitination and to proteasomal degradation, are recognized by the E3 (**Fig 3**). Thus the amount and the type of proteins degraded by the proteasome is largely determined by which E3 ligases are activated in the cell (Gomes et al., 2001)

Among the atrogenes, the two most induced genes are two novel ubiquitin ligases E3, atrogin1 (muscle atrophy F-box- MAFbx) and MuRF1(muscle ring finger1). These enzymes are up regulated in different models of muscle atrophy and are responsible for the increased protein degradation through the ubiquitin proteasome system (Sacheck et al., 2004).

Atrogin-1, also known as MAFbx, contains an F-box domain, a characteristic motif seen in a family of E3 ubiquitin ligases belonging to SCF complex (for Skp1, Cullin, F-box) (Gomes et al., 2001; Jackson and Eldridge, 2002). The F-box protein interacts with the substrates, while Cul1-Roc1 components associate with the E2 Ub-conjugating enzymes. Skp1 is an adaptor that brings F-box protein to

the Cul1-Roc1-E2 complex. Most substrates require the phosphorylation to interact with the F-box protein in an SCF complex (Jackson and Eldridge, 2002).

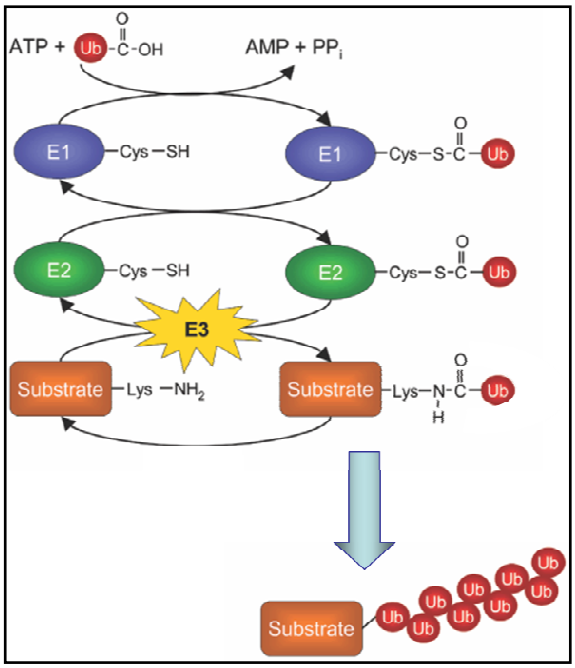


Figure 3: Ubiquitin –proteasome pathway Ubiquitin (Ub) is covalently attached to substrate proteins via a three-step mechanism involving the sequential actions of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase) enzymes. The attachment of multiple ubiquitin moieties by E3is the rate limiting step. *M*odified from *Lori A. Passmore1 and David Barford Biochem. J 2004.*

MuRF1 belongs to the RING finger E3 ligase subfamily, characterized by three RING-finger domains (Borden et al,1996) which are required for ubiquitin-ligase activity (Kamura et al., 1999). Those domains include a B-box, whose function is still unknown, and a coiled-coil domain, which may be required for the formation of heterodimers between MuRF1 and a related protein, MuRF2 The precise substrates of atrogin-1and MuRF1 have not been identified, but their expression is increased in many diseases (Price, 2003), even before the loss of muscle mass become evident, underling their important role. The strong induction of atrogin1 and MuRF1 at early stage of muscle wasting and their high expression suggests the role of both E3s in initiation and maintenance of accelerated proteolysis. Knockout animals lacking either MuRF1 or atrogin-1 show a reduced rate of

muscle atrophy after denervation (Bodine et al., 2001a), confirming that these liagases are necessary for the atrophy program. Up to now atrogin-1 and MuRF1 are actually the best markers for muscle atrophy and could be consider as master genes for muscle wasting.

Autophagy-Lysosome System

Autophagic Lysosome System involves dynamic rearrangements of membranes which engulf a portion of cytoplasm for its degradation in the lysosome. The portion of cytoplasm, including excess or abnormal organelles, is sequestered into double-membrane vesicles and delivered to the degradative organelle, the lysosome, for breakdown and for recycling the generated molecules (**Fig4**).

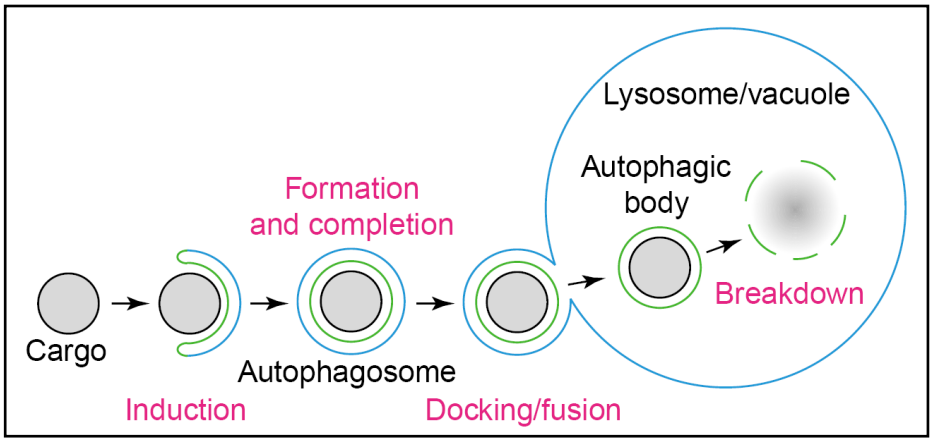


Figure 4: Principal steps of Autophagy Lysosome System pathway.

This process has an important role in various biological events, such as adaptation to environmental changes, cellular remodelling during development and differentiation and ageing. Autophagy is also involved in preventing certain types of disease, like Parkinson and Huntington, although it may contribute to some pathologies. Autophagy is also constitutively active in skeletal muscle, as shown by the accumulation of autophagosomes seen in human myopathies, caused by genetic deficiency of lysosomal proteins (Pompe's and Danon's diseases), or by pharmacological inhibition of lysosomal function, as in chloroquine myopathy

(Shintani and Klionsky, 2004). The generation of transgenic mice for LC3, the mammalian homolog of the essential autophagy gene Atg8, fused with GFP defined the amount of autophagy in different tissues and whether different organs respond to fasting by activating autophagosome formation (Kamura et al., 1999). Indeed skeletal muscle has been found to be one of the tissues with the highest induction of autophagy (Mizushima et al., 2004). In fast skeletal muscle of transgenic mice, food deprivation induced the rapid appearance of cytoplasmic fluorescent dots, corresponding to autophagosomes (Mizushima et al., 2004). In different cell systems, autophagy is activated by depletion of nutrients or of growth factors and, according to current views, in most cases this is mediated by the kinase mTOR (Lum et al., 2005). Autophagy is suppressed by mTOR, which is in turn controlled directly by the level of intracellular amino acids, by growth factors via Akt/PKB and by cell energy status via AMPK. Accordingly, rapamycin, a specific inhibitor of mTOR, activates autophagy. However, autophagy can also be induced by mTOR-independent mechanisms: leucine starvation has been reported to induce mTOR-independent autophagy in cultured myotubes (Mordier et al., 2000), and mTOR has also been found to be dispensable in other cell systems (Kochl et al., 2006; Sarkar et al., 2007; Yamamoto et al., 2006). Up regulation of autophagy and lysosomal genes has been documented at the transcript and protein level in different settings, but the mechanisms controlling this transcriptional regulation and their physiological relevance have not been characterized. The lysosomal proteinase cathepsin L is induced in muscle wasting (Sacheck et al., 2007), and microarray analyses suggest that this is also true for the autophagy-related genes LC3 and Gabarapl1 (Lecker et al., 2004). Several studies point to up regulation of autophagy genes in other cell systems and in different experimental conditions (Juhasz et al., 2007). However, the factors involved in the transcriptional regulation of autophagy genes have not yet been identified (Mammucari et al., 2007).

Signaling Pathways Controlling Muscle Atrophy Program

Given the important role of muscle loss in prognosis of many diseases, a subsequent crucial step was the identification of the signaling pathways which regulate the expression of the atrophy related genes (Sandri, 2008). Previous studies have already shown that **IGF1/insulin signalling** while promoting muscle growth is able to suppress protein breakdown (Bodine et al., 2001b). The insulin/IGF1 signaling cascade was the first example of a pathway which promotes protein synthesis and at the same time suppresses protein breakdown, indicating that the dynamic regulation of skeletal muscle mass is the result of a balance between hypertrophic and atrophic programs (**Fig 5**).

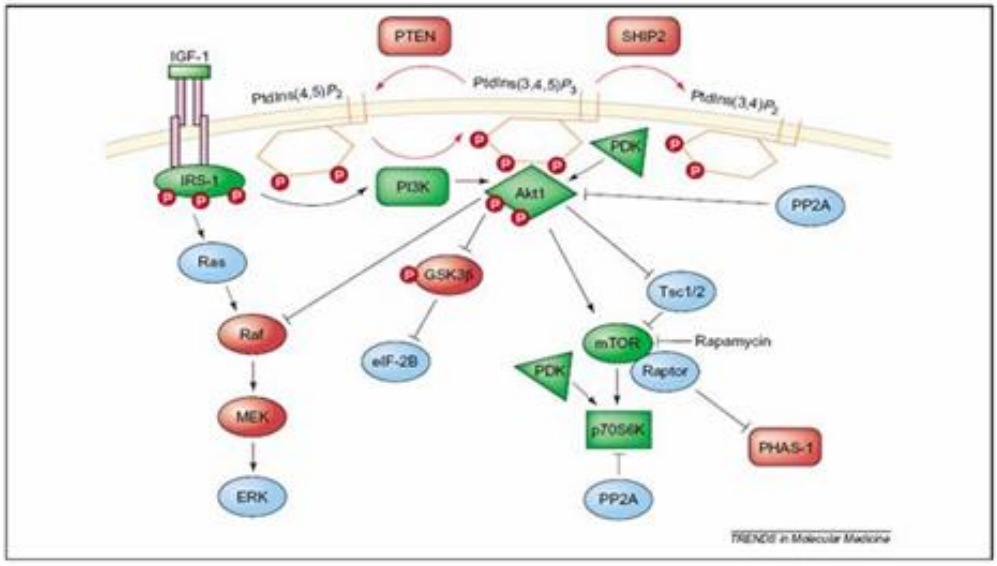


Figure 5. Insulin-like growth factor 1 (IGF-1)- mediated signalling pathways. Signalling molecules, that have been shown to have a negative effect on hypertrophy are red coloured, and the proteins, whose activation induces hypertrophy are green. Protein that have not been dissected their role are blue *(Glass,D J 2003).*

The binding of IGF1 or insulin to their membrane receptors activates two major signalling pathways: the Ras-Raf-MEK-ERK pathway and the PI3K/AKT pathway. The Ras-Raf-MEK-ERK is involved in controlling fiber type by promoting, in vivo, a nerve-dependent slow phenotype. Conversely the activation of PIK3/ AKT pathway induces muscle growth. Once AKT is activated, it initiates

a cascade of phosphorylation events targeting mammalian target of rapamycin (mTOR) and glycogen synthases kinase 3β (GSK3β). GSK3β is inhibited by Akt and, in turn, blocks the eukaryotic translation initiation factor 2B (eIF-2B), which is involved in protein synthesis. mTOR is part of two complexes: TORC1, which contains Raptor and is rapamycin sensitive. TORC1 signals to S6K1 and 4EBP1 and it controls proteins synthesis, while TORC2, which contains Rictor, phosphorylate, in a retrograde way, AKT, affecting also FoxO signalling. TORC2 complex is rapamycin insensitive and it does not affect protein synthesis (**Fig 6**).

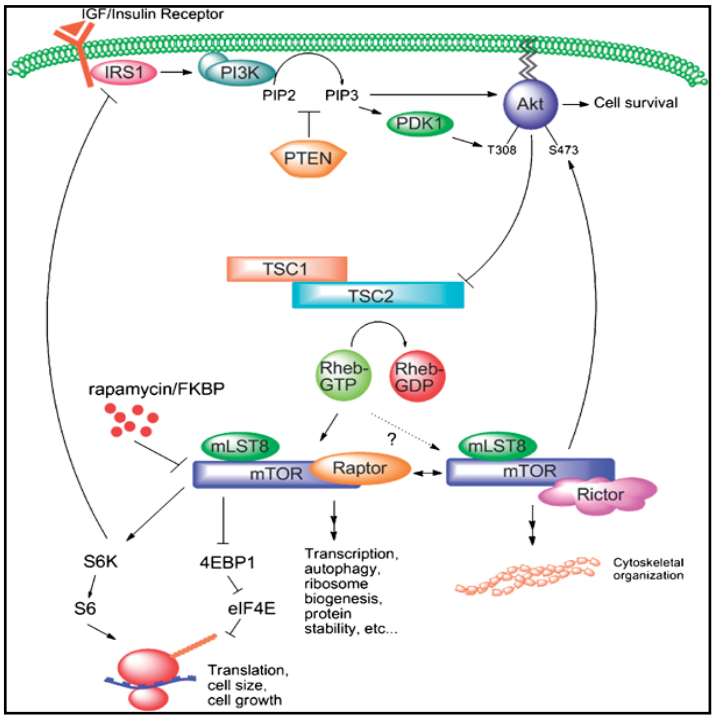


Figure 6: IGF-1 AKT m-TOR pathway

Several finding suggest that the decrease activity of IGF /PI3K /AKT signalling pathway can regulate the muscle atrophy program. In fact, the inhibition of PI3K activity and the expression of dominant negative of AKT reduces the mean size of myotubes and increases protein degradation and atrogin-1 expression. Conversely, both IGF1 transgenic mice and overexpression of constitutively active AKT in adult myofibers suppress protein degradation and muscle atrophy induced by denervation. Moreover the up regulation of MuRF1 and atrogin-1 is blocked by

IGF1 treatment or AKT activation. The up-regulation of atrogin-1/MAFbx and MuRF1 is normally blocked by Akt functioning through negative regulation of the family of transcription factors FoxO The FoxO family in skeletal muscle is comprised of three isoforms: FoxO1, FoxO3 and FoxO4

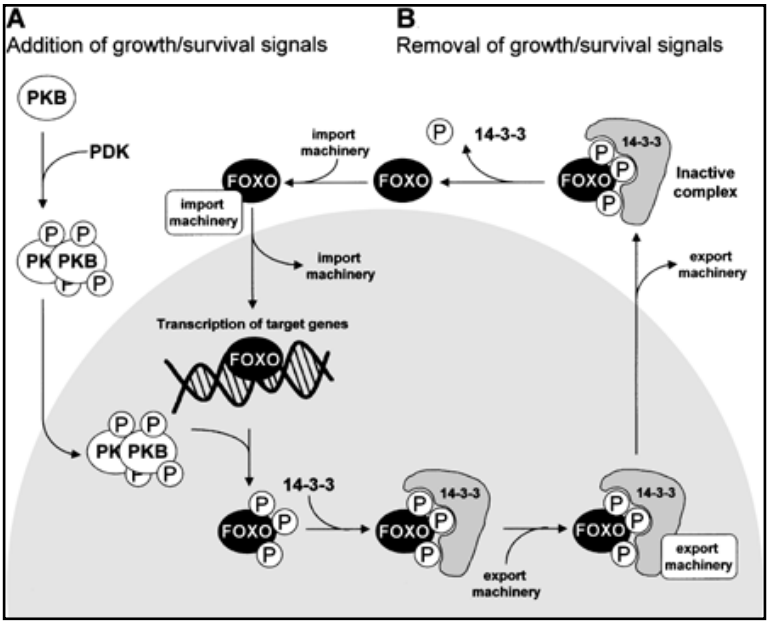


Figure 7: Regulation of FOXO transcriptional activity. (**A**) Growth/survival signals activate AKT/PKB, which then translocates into the nucleus. Phosphorylation of FOXO by AKT/PKB results in release from DNA and in binding to 14-3-3 proteins. This complex is then transported out of the nucleus, where it remains inactive in the cytoplasm. (**B**) Upon removal of growth/survival signals, FOXO is dephosphorylated, 14-3-3 is released and FOXO is transported back into the nucleus where it can bind target promoters and transcribe them *(Birkenkamp K.U. et al., 2003).*

Akt phosphorylates FoxOs, promoting the export of FoxOs from the nucleus to the cytoplasm. AKT blocks FoxO activity by phosphorylation of three conserved residues leading to 14-3-3 binding (**Fig 7**). The resulting complex prevents re-entry of FoxOs into the nucleus. As predicted, the reduction in the activity of the Akt pathway, observed in different models of muscle atrophy, results in decreased levels of phosphorylated FoxO in the cytoplasm and a marked increase of nuclear FoxO protein. The translocation and activity of FoxO members is required for the up-regulation of atrogin-1/ MAFbx and MuRF1, and FoxO3 was found to be

sufficient to promote atrogin-1/MAFbx expression and muscle atrophy when transfected in skeletal muscles *in vivo* (**Fig 8**).

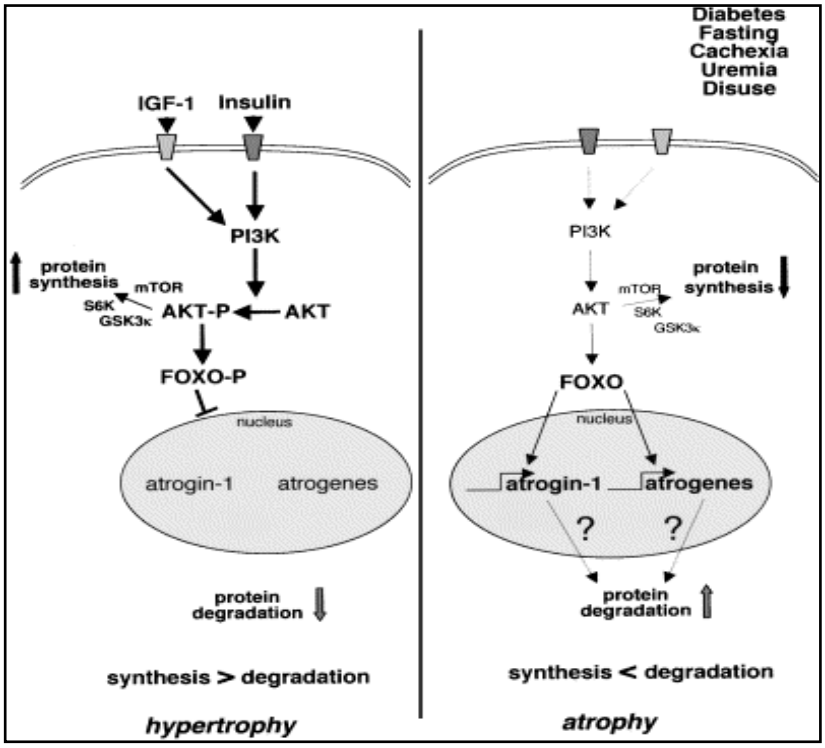


Figure 8: IGF-1/ AKT pathway and FoxO action during muscle atrophy (on the right) and hypertrophy (on the left) *(Sandri, Sandri et al. 2004)*

Recently the **NF-kB signaling cascade**, beside the IGF-1/PI3K/Akt/FoxO pathway, emerged to play an important role in the control of muscle mass. First hints on involvement of NF-kB in muscle wasting came from the up-regulation of this gene during disuse atrophy (Hunter et al., 2002) or sepsis (Penner et al., 2001). In the inactive state NF-κB is sequestered in the cytoplasm by a family of inhibitory proteins called IκB. In response to tumour necrosis factor-a (TNFα), 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.

Experiments in cultured myotubes demonstrated that the block of transcription factor NF-κB by over-expression of a mutant form of IκBα, that is insensitive to

degradation by the proteasome, inhibits protein loss induced by TNFα (Li and Reid, 2000). Interestingly, treatments with TNFα attenuates insulin stimulated protein synthesis (Williamson et al., 2005).

To further study the role of NF-kB in muscle wasting, Cai and co-workers created two transgenic mice in which the NF-κB pathway was activated or inhibited, through the muscle-specific expression of IKKα or a dominant inhibitory form of IκBα respectively (Cai et al., 2004). In these animals the activation of the NF-κB pathway is sufficient to induce significant muscle atrophy, marked by muscle weight loss, and the expression of the E3 ubiquitin ligase MuRF-1, but not atrogin-1, providing the first functional dissection of the roles of the two E3 ligases. On the other hand, while muscle-specific inhibition of NF-κB by transgenic expression of a constitutively active IκB mutant leads to no overt phenotype, denervation atrophy is substantially reduced. Similarly in another study, transgenic animals lacking NF-κB, undergo less atrophy induced by hind limb unloading than control littermates (Hunter and Kandarian, 2004). Finally conditional knockout mice for IKKβ are resistant to muscle atrophy confirming the role of this pathway in regulating muscle mass. However, despite the indirect evidence from genetic studies, up to now there is no evidence of a direct NFkB recruitment on MuRF1 promoter or on any other promoter of the atrophy-related genes.

FoxO Family members: role and functions

FoxO transcription factors belong to the large family of Forkhead proteins. Forkhead family is present in all eukaryotes. The member of Forkhead family are transcriptional regulators characterized by a conserved DNA binding domain termed the “forkhead box “(Calnan and Brunet 2008). The forkhead box is approximate 100-ammino acid, which contains the DNA binding domain (DBD) and it represents a variant of the helix-turn helix motif. Forkhead-DBD is constituted of three α helices and two characteristic large loops described as butterfly-like wings (Burgering, 2008). In humans, Forkhead proteins (Fox) have

been divided on the basis of sequence similarity, into 19 subgroups which are classified by alphabetic letters form A to S.

FoxO subfamily is conserved from *Caenorhabditis elegans* to mammalians. Invertebrates have only one gene whereas mammalians have four FoxO family members: FoxO1(FKHR) FoxO3 (FKHRL1), FoxO4 (AFX) and FoxO6. FoxO1,3 and 4 are ubiquitously expressed, while FoxO6 expression is restricted to brain.(Jacobs, van der Heide et al. 2003).

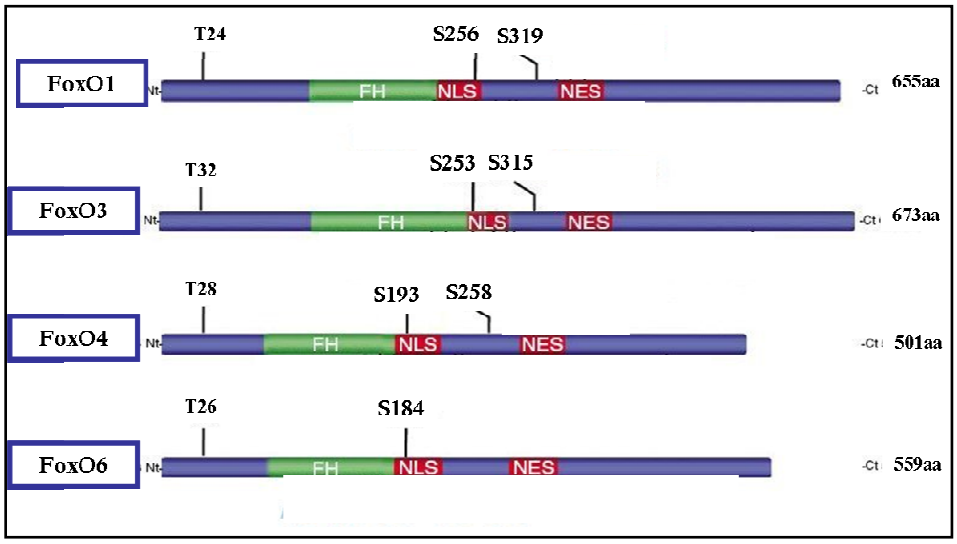


Figure 9: Structure of FoxO proteins. The Akt phosphorylation sites are shown (black). The green region depicts the Forkhead domain (FH), The nuclear localization signal (NLS) and the nuclear export sequence (NES) are shown in red *(EL Greer and et al 2005)*

Structure: FoxO proteins show four classical domains: a highly conserved DNA binding domain (DBD), a nuclear localization signal (NLS) located just downstream the DBD, a nuclear export sequence (NES) and a C-terminal trans-activation domain. (**Fig 9**)

FoxO 1 3 and 6 proteins have similar length of approximately 650 amino-acid residues, whereas FoxO4 sequence is shorter and contains about 500 amino-acid residues. Analysis of multiple sequence alignment shows that several regions of FoxO proteins are highly conserved. The regions displaying the highest sequence homology include the N-terminal region surrounding the AKT phosphorylation site (Thr32), the DBD, the region containing NLS and the COOH-terminal trans activation domain (Obsil and Obsilova 2008). Because of the shared DNA binding

domain, FoxOs are expected to bind similar or identical DNA sequences within the genome. The core consensus DNA sequence for FoxO binding has been determined by using gel shift experiments and it has the following sequence: 5'TTGTTTAC3'.

Regulation of FoxO factors: FoxO transcription factors are regulated by a wide range of external stimuli, such as insulin, IGF1, other growth factors, neurotrophins, nutrients, inflammatory cytokines, energy balance and oxidative stress . Altogether these stimuli control FoxO function by affecting protein stability, subcellular localization, DNA-binding and transcriptional activity. Most of these FoxO regulations are achieved by post-translation modification and include phosphorylation, acetylation, mono and poly-ubiquitination, N-acetylglucosamination and possibly other modifications that are not yet identified (Obsil and Obsilova, 2008). The subcellular localization of FoxO proteins is controlled by some post-transcriptional modifications, particularly phosphorylation and monoubiquitination.

Phosphorylation of FoxO at three conserved sites by the protein kinases AKT and SGK causes the sequestration of FoxO factors in cytoplasm. An exception is FoxO6, which is phosphorylated at only 2 of three phosphorylation sites and it is not regulated by nucleus cytoplasmic shuttling (Jacobs et al., 2003). In response to growth factors stimulation FoxOs can be phosphorylated at additional sites, and sequestered into cytoplasm. Anyway, phosphorylation has not only a negative effect on FoxOs activity. Through phosphorylation, stress stimuli trigger the relocalization of FoxO proteins into the nucleus. Stress activated kinases MST1 (Lethinen at al 2006) and JNK (Essers et al., 2004) phosphorylate FoxOs, disrupting the 14-3-3 /FoxO interaction and promoting the transcriptional adaptations to cell stress response. Oxidative stress stimuli caused the nuclear relocalization of FoxO via mono-ubiquitination at two lysines (van der Horst 2006). Furthermore, these lysines are also acetylated by CBP/p300 and can also be deacetylated by sir2/Sirt1. FoxO acetylation can either promote and accumulate FoxO in nuclear compartment or block the FoxO binding on target promoters (Brunet, Sweeney et al. 2004; Kitamura, Kitamura et al. 2005; Wang and Tissenbaum 2006). On the other hand, Sir 2/Sirt1 deacetylase contributes to life extension, promoting FoxO DNA binding (Tissenbaum and Guarente 2001;

Tsai, Sun et al. 2007). Thus the role of acetylation on FoxO activity is dependent on cell type and by the presence of other signalling events. In response to energy stress activated AMPK can phosphorylate FoxO3 at six sites triggering FoxO3 nuclear localization and transcriptional activity independently from Akt status (Greer et al., 2007). Genome wide microarray analysis pointed out that AMPK phosphorylation of FoxO3 induces changes in the expression of specific target genes, including energy metabolism and stress resistance genes (Greer et al., 2007).

Besides the post-transcriptional regulation, the level of FoxO expression is also tightly regulated. Fasting and glucocorticoid induce the expression of FoxO factors in mouse liver and in mouse muscle, while refeeding suppress FoxO gene induction (LECKER et al., 2004; Sacheck et al., 2007; Sandri et al., 2004) (**Fig 8**).

Functions: The roles of FOXO in whole organisms has been originally dissected by genetic studies in worms and, more recently, in flies in which there is only one FoxO gene (Greer and Brunet, 2005). In worms FoxO orthologue DAF 16 plays a crucial role downstream of the insulin-signalling pathway to regulate longevity. DAF-16 appears to induce a program of genes that coordinately extend life span by promoting resistance to oxidative stress, protection from protein unfolding-damage and resistance to pathogens. These findings suggest that one way in which DAF-16 activity leads to an increase in organism life-span is by increasing the cellular resistance to various stresses. Similarly, in drosophila, dFOXO plays an important role in conferring stress resistance (Junger et al., 2003). Interestingly, expression of a wild-type form of dFOXO is sufficient to increase longevity in flies (Giannakou et al., 2004) and to prevent the age associated decline of cardiac functions (Arden, 2008; Wessells et al., 2004). (Greer and Brunet, 2005)While the roles of mammalian FOXO transcription factors in cultured cells are well established, their targets in the whole organism are just beginning to be unravelled. Several studies have found that FoxO1,3 4 regulate important processes including cellular differentiation, tumour suppression, metabolism, skeletal muscle and heart size, cell cycle arrest, cell death and protection from stress (Arden, 2008). In proliferating cells, the main effect of the expression of active forms of FoxO family members is to promote cell cycle arrest at the G1/S boundary (Medema et al., 2000) by both up regulating cell cycle

inhibitors (p21 and p27) and repressing cell cycles activators (cyclinD1/D2). Moreover FoxO factors mediate cell cycle arrest at the G2/M by regulating cycling G2. Consistent with this FoxO factors' role, the expression of active forms of FoxO proteins up regulates several genes involved in the cellular response to stress, such as DNA damage inducible protein 45 (GADD45) Mn-dismutase, and catalase.

In differentiation, FoxO factors can either inhibit and promote differentiation, depending on cell type and on which member of FoxO family has been studied. In adipocytes and myoblasts, the expression of a constitutively active form of FoxO1 inhibits differentiation in, *in vitro* differentiation assays, while FoxO3 appears to potentiate erythroid differentiation.

In neurons and in lymphocytes the expression of constitutively nuclear forms of FoxO proteins trigger cell death by inducing two proapotitic Bcl2 family member, BIM and Bnip3 and the death cytokines Fas ligand and TRAIL.

FoxO transcription factors also play an important role in up regulating genes that control both glucose and lipid metabolism. FoxOs promote a switch in skeletal muscle energy metabolism from glucose oxidation to lipid oxidation .

The classical studies of single FoxO family member deletion have suggested that FoxO1 is critical during embryonic development, while FoxO3 and FoxO4 ablation can be compensated by the other FoxOs. In fact FoxO1-null mice die at embryonic day 10.5, from defects in angiogenesis (Furuyama et al., 2004; Hosaka et al., 2004). FoxO3-null mice are viable and looks normal (Castrillon et al., 2003). The main defect of FoxO3-null mice is an age-dependent female infertility (Castrillon et al., 2003), due to the premature activation of the ovarian follicles. FoxO3 mutant mice exhibit defects in glucose uptake (Castrillon et al., 2003), consistent with a role for FoxO family members in glucose metabolism. FoxO3-null mice also display over proliferation of helper T cells, in line with FoxO3's role in promoting cell cycle arrest (Lin et al., 2004). FoxO4-null mice are viable and do not appear to have any phenotype (Hosaka et al., 2004) FoxO6-null mice have not been generated yet. Taken together, these results suggest that FoxO1, FoxO3, FoxO4, and possibly FoxO6 family members may have both distinct and overlapping functions.

Recently tissue specific FoxO knockout mice have been generated. The deletion of all FoxO1, FoxO3 and FoxO4 in hematopoietic stem cell determined a defect in ROS detoxification (Tothova et al., 2007). Moreover total FoxOs knockout engendered a progressive cancer-prone condition characterized by lymphomas and hemangiomas, demonstrating that FoxOs are bona fide tumor suppressors (Paik et al., 2007)

Foxo in Skeletal Muscle

Previous studies have shown that FoxO1 and FoxO3 are involved in muscle atrophy (Brunet et al., 1999),(Stitt et al., 2004) (Sacheck et al., 2004; Sandri et al., 2004). FoxO levels increase during starvation and glucocorticoid treatment, conditions that promote muscle atrophy (Furuyama, Kitayama et al. 2003; Imae, Fu et al. 2003) Genes profiling studies have relieved an up regulation of FoxO1 after denervation or spinal cord isolation and in many systemic wasting states, suggesting a fundamental role of FoxO1 in all types of atrophy (Lecker et al., 2004).

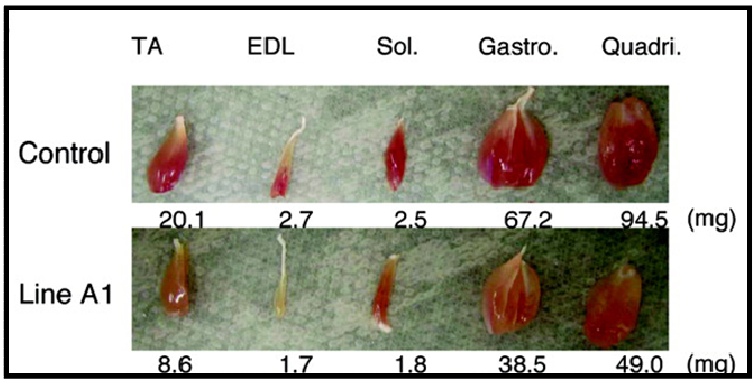


Figure 10: FoxO1 transgenic mice (lines A1) show muscle atrophy. Muscles were smaller in size and paler in color in FOXO1 transgenic mice than in control mice. (TA,tibialis anterior; Sol, soleus; Gastro, gastrocnemius; Quadri, quadriceps) (Kamei, Miura et al. 2004).

Since FoxO1 expression persists at high levels even 28 days after denervation, this transcription factor may have a role in the maintenance of muscle in atrophied states (Sacheck et al., 2007).Consistent with these observations, muscle specific transgenic mice which overexpressed FoxO1 weigh less than the wild-type control

mice and they show a reduced skeletal muscle mass (**Fig 10**). Their muscle are paler than wild type mice. Histological analyses have shown a marked decrease in size of both type I and type II fibers and a significant decrease in the number of type I fibers (Kamei et al., 2004). Both in C2C12 myoblasts and in FoxO1 transgenic mice, FoxO1 overexpression blocks protein synthesis through the up-regulation the translation inhibitor 4E-BP1 (Southgate et al., 2007). Moreover FoxO1 activation reduced mTOR signalling by blocking RAPTOR expression and by decreasing mTOR protein (Southgate et al., 2007). Finally in C2C12 myotubes glucocorticoid receptor and FoxO1 synergistically active MuRF1 gene (Waddell et al., 2008a)In fully differentiated skeletal and cardiac muscle cells, expression of a constitutively active form of FoxO3 causes atrophy (Sandri et al., 2004; Skurk et al., 2005; Stitt et al., 2004). Muscle fibers expressing a costively active form of FoxO3 appear smaller than the untransfected surrounding fibers (**Fig 11**).

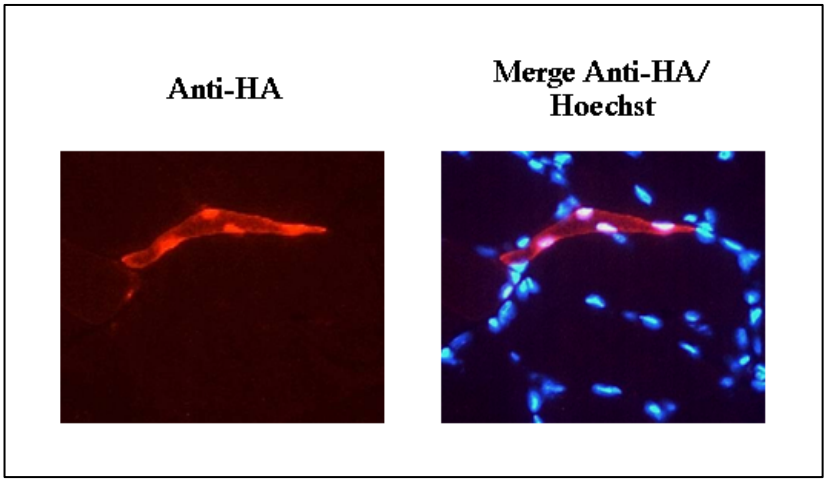


Figure 11: Foxo3 expression, revealed by immunohistochemistry staining for the HA tag (FoxO3 tag), caused important muscle atrophy.

Since there is no evidence of apoptosis, the reduction of fiber size inducted by FoxO3 activation is caused by activation of protein breakdown via atrogin-1 regulation (Sandri et al., 2004). Indeed different experiments of mutagenesis and deletion of atrogin-1 promoter have identified the critical promoter region where FoxO can interact and modulate atrogin-1 expression (Lecker et al., 2004).

AIM:

The main interest of the laboratory, where I performed my PhD program, is the characterization of the signalling involved in skeletal muscle atrophy and hypertrophy. It is well known that FoxO1 and FoxO3 regulate a muscle atrophy program, but it is still unclear the underlying molecular mechanisms and which are the FoxO target genes involved in muscle loss. The aims of my PhD project are:

1) to understand how many atrophy-related genes, *atrogenes*, are under FoxOs control.

2) to investigate which of these genes are commonly regulated by FoxO1 and 3.

3) to identify which atrogenes are specifically controlled by FoxO1 or FoxO3.

In this study we have characterized the FoxO target genes necessary for maintaining an atrophy program. In order to achieve this goal we set up the Chromatin Imunoprecipitation approach for in vivo studies. Our results are extremely important for the development of new therapeutic strategies in combating muscle loss.

MATERIALS AND METHODS:

Cell Culture

For *in vitro* experiments we used HEK (Human Embryonic Kidney) 293FT and C2C12 cells. HEK 293T were cultured in D-MEM (Dulbecco's modified Eagle's medium) (Invitrogen) 1% penicillin-streptomycin (Gibco), 10% FBS (Fetal bovine serum) (Gibco) in until the cells reached the 80% of confluence, than they were spited in another flask, using Tripsina-EDTA 1X (Invitrogen).
C2C12 mouse myoblasts were cultured in D-MEM (Dulbecco's modified Eagle's medium) (Invitrogen) 1% penicillin-streptomycin (Gibco), 10% FBS (Fetal bovine serum) (Gibco) until the cells reached the 70% of confluence. The medium was then replaced with DMEM 1% penicillin-streptomycin (Gibco) 2% horse serum (Gibco) and incubated for four days to induce myotubes formation.

Adenovirus infection

For infection, myotubes were incubated with adenovirus at a multiplicity of infection (MOI) of 250 in differentiation medium. The infection was typically greater than 90%. The efficiency of viral infection was directly revealed by observation at fluorescent microscope. In fact the adenoviral vector contains, into the viral backbone, the GFP under the control of a second CMV promoter. Thus each construct expresses both the cloned gene (c.a.FoxO3) and the GFP allowing us to follow the morphological changes in myotubes.
Infected cells are used for chromatin immunoprecipitation experiments or for RNA extraction. For those experiments we used adenovirus expressing constitutively active FoxO3 (ca-.FoxO3) (Brunet et al., 1999) and mock adenoviruses as control. The FoxO3 adenovirus used was created using the AdEasy strategy (Stratagene) (Luo et al., 2007). pECE c.a. FoxO3 was sub-cloned into the pAdTrack-CMV vector. Subsequent steps were performed according to the manufacturer's instructions.

Transfection of HEK 293 FT

To transfection of HEK 293 FT we used the LipofectamineTM2000 Reagent (Invitrogen) and we proceed as following described:

-500.000 cells were plated on 6-well plates(Falcon) in D-MEM (Dulbecco's modified Eagle's medium) (Invitrogen) 1% penicillin-streptomycin (Gibco), 10% FBS (Fetal bovine serum) (Gibco).

-24 hours late, the medium of the cells was discard and the cells were incubated with Opti-MEM® I Reduced Serum Medium (Invitrogen) and 10 % FBS. 4ug of the expression vector for well and 10 ul of LipofectamineTM2000 Reagent were added to a well.

The plasmids used for all those kind of experiments were the following:

- **pECE c.a.-FoxO3**: expression vector coding for the human full-length sequence of constitutively active form FoxO3. The three canonical Akt phosphorylation sites, T32, S253 and S315 are mutated in alanines (Brunet et al., 1999);
- **pECE dn-FoxO3:** expression vector coding for the human full-length sequence of FoxO3, lacking for the transactivation domain. (Brunet et al., 1999);

The transfected cells were grown for 24 hours in D-MEM and 10% FBS.

Mouse Tibialis Anterior Muscle Electroporation

Experiments were performed on 2 month old CD1 mice. Tibialis anterior muscles were electroporated as described previously by Sandri (Sandri et al., 2004) and Dona (Dona et al., 2003).The tibialis anterior muscle was isolated through a small hind limb incision. In the experiments performed for *chromatin immunoprecipitation* on overexpression of ca-FoxO1 and ca-FoxO3 in vivo, 20 ug of *Expression vecto***r** solubilizated in 40 ul of 0.9% NaCl water solution were injected along the muscle length.

In the *Reporter experiments*, the following mixture of DNA was injected along the muscle length:

Expression vector20 μg

Reporter plasmid10 μg

pRL-TK (Promega)5 μg

The volume was adjusted to 40 μl with 0.9% NaCl water solution. The plasmids used for all those kind of experiments were the following:

- **pECE ca.-FoxO3**: expression vector coding for the human full-length sequence of constitutively active form FoxO3. The three canonical Akt phosphorylation sites, T32, S253 and S315 are mutated in alanines (Brunet et al., 1999);
- **pCMV5 ca-FoxO1**: expression vector coding for the human full-length sequence of constitutively active form FoxO1.The three canonical Akt phosphorylation sites are mutated as follow: T24A, S253D and S316A (Nakae et al,2001).
- **pGL3-pAtrogin-1 3.5 kb**: reporter vector containing 3.5 kilo bases of atrogin-1 promoter sequence driving the firefly luciferase gene;
- **pRL-TK** (Promega): control vector driving the renilla luciferase gene;
- **pECE dn-FoxO3:** expression vector coding for the human full-length sequence of FoxO3, lacking for the transactivation domain. (Brunet et al., 1999 Shin et al 2001)

In the control electroporation experiments an equal amount of mock vector was injected.

Electric pulses were then applied by two stainless steel spatula electrodes placed on each side of the isolated muscle belly (20 Volts/cm, 5 pulses, 200 ms intervals) with the Electro Square Porator (ECM 830, BTX). No macrosccopic or microscopic evidence for necrosis or inflammation as a result of the transfection procedure has been noted. The experiments were approved by the veterinary office and the ethical institutional review boards.

Reporter Analysis On Electroporated Tibialis Anterior Muscles

Promoter activity can be studied by using the luciferase firefly gene. In our experiments we used *Dual-Luciferase®Reporter Assay Chemistry*-(Promega), that

includes “dual reporters”. The studied promoter region is cloned upstream and is driving the firefly luciferase expression. Renilla luciferase is under a minimal TK promoter control and it is used to normalize the firefly data for the efficiency of transfection, which can vary from animal to animal.

Both reports were co-transfected in tibialis anterior of 2 month old CD1 mice. After 7 days the muscle were collected, weighted and powdered in liquid nitrogen by a ceramic pestle and then were lysed in Passive Lysis Buffer 1x (Promega) accordingly to manufacturer instruction. An optimal lysis was achieved by 2 cycles of thawing at 4°C and freezing in liquid nitrogen. Firefly luciferase and renilla luciferase activities were measured sequentially in 5 µl of the muscle lisate.

Mutagenesis:

The LC3 promoter was mutated in the FoxO binding site using *QuikChange Multi Site-Directed* (*Stratagene*). The technique consists of a PCR amplification with a primer mutated at the nucleotide sites that we wanted to change. The PCR reaction amplifies only one DNA strand of the template.
The used primer was:

LC3	5'-AACAATGCAAAGCAAGCAACCCAAGGAAAGTAACCAGCC-3'
LC3 Mutated	5'-GGCTGGTTACTTTCCTTGGGTTGCTTGCTTTGCATTGTT-3'

Promoters Analyzes For Transcription Binding Sites

To investigate the FoxOs binding sites on several promoters we used *Genomatix* tool available on line (http://www.genomatix.de/products/

MatInspector). This bioinformatics tool is a software that utilizes a position weight matrices to find transcription factor binding sites along a genomic sequence. MatInspector gives a quality rating to the potential binding sites and thus allows quality-based filtering and selection of matches.We looked for FoxO binding sites on the following promoter sequences :

the 3.5 kilo bases promoter of atrogin-1

the 5.0 kilo bases promoter of Gadd 45α, LC3, Bnip3

Chromatin immunoprecipitation (ChIP) assay

The chromatin immunoprecipitation (ChIP) technique is the best tool to identify specific proteins associated with a region of the genome or, conversely, to find regions of the genome associated with specific proteins. Chromatin Immunoprecipitation experiments were performed using *ChIP assay kit* (UpState).

The principal phases of this procedure are the following:

1. Cross-link of the protein to the chromatin and lysis
2. Sonication of DNA
3. Immunoprecipitation (IP) of cross-linked Protein/DNA
4. Elution of Protein/DNA complexes
5. Reverse cross-link of Protein/DNA complexes to free DNA
6. DNA extraction with phenol/chloroform
7. PCR chromatin immunoprecipitated

Chromatin immunoprecipitation (ChIP) assay *in vitro*

We used one 10 cm dish plate of cell culture (HEL 293 FT and C2C12 myotubes) for each ChIP assay.

Cross-link of the protein to the chromatin and lysis

The proteins were crosslinked by adding 1% formaldehyde (Sigma-Aldrich) to cell culture medium for 10 minutes at room temperature. Formaldehyde is a reversible cross-linker which is used for the characterization of protein-protein and protein-DNA interactions. After bleaching formaldehyde action with 0.02M glycine (Sigma-Aldrich), the cells were washed twice with 4ml of cold Phosphate buffer saline (PBS)1x (GIBCO) containing 5ul Protease Inhibitor Cocktail (Roche).

The plates were collected by scraping and centrifuged for 5 min at 2000 rpm at 4° C. After removing the supernatant fraction, the cellular pellet was resuspended in 650 ul of SDS Lysis Buffer (Upstate Millipore).

Chromatin sonication

The cross-linked DNA was sonicated to shear the chromatin to an average size of 200-1000 bp in length. Efficient chromatin fragmentation was obtained by using 10 seconds pulse of a sonicator "Sonic Vibracell" on ice repeated for 6 times. After sonication the samples were centrifuged at 13000 rpm for 10 min at 4°C.

Immunoprecipitation (IP) of cross-linked Protein/DNA

The supernatant was collected and brought to final volume 2.2ml with Dilution Buffer (Upstate Millipore). 1ml of sheared chromatin was transferred in a 1.5ml tube for each immunoprecipitation experiment. An aliquot of fragmented chromatin (200ul) was stored at -80°C. This sample is called the "input" and is the control sample for PCR analyses. The remaining volume is used for the immunoprecipitation reaction. To remove proteins or DNA that could no-specifically bind to the immunoglobulins or can no-specifically bind Protein A-Agarose beads, the samples were pre-cleaned by 1 hour incubation at 4°C in rotation with 75ul of Protein A Agarose/Salmon Sperm DNA (Upstate Millipore). The beads were removed by centrifuging at 13000rpm for 5 minutes, at 4°C. Then 5ug of specific antibody or general IgG was added to supernatant and was incubated overnight at 4°C in rotation. 60ul of Protein A Agarose/Salmon Sperm DNA was added to the antibody/antigen/DNA complex and was incubated for 1 hour at 4° C in rotation. The agarose beads were collected by centrifugation at 1,000 rpm for 1 min at 4°C and the supernatant fraction was removed. Then the beads were washed for three times with increasing ionic-strength cold buffers (Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer, LiCl- Immune Complex Wash Buffer Upstate Millipore). Finally the beads were washed with 1ml of TE for two times.

Elution and Reverse cross-link of Protein/DNA complexes to free DNA

The samples were briefly centrifuged for 1 min at 13000 rpm and the supernatants were discarded. The beads were resuspended in 250 ul of fresh Elution Buffer (1%SDS, 0.1M $NaHCO_3$) and incubated for 15 min at room temperature in rotation. The samples were briefly centrifuged for 1 min at 13,000 rpm and the supernatants were collected. The same procedure was repeated another time to reach the final volume of 500 ul for each sample. The "input" control was thawed at room temperature and brought to 500ul with Elution Buffer. To reverse the formaldehyde cross-linking, 24ul of 5M NaCl was added to the samples and to the"input" control and was further incubated for 6 hours at 65°C. At this stage the samples could be used either for biochemical studies, including western blotting analysis to confirm the efficient immunoprecipitation, or molecular approaches to reveal which part of genome was pulled down.

For PCR analysis we added 10 ul 0.5 M EDTA, 20ul 1 M Tris-HCl, 20ug proteinase K (Gibco) for 1 hour at 45° C to remove proteins. The genomic DNA was purified by Phenol/Chloroform:/Isoamyl Alcohol 25:24:1 extraction (Sigma-Aldrich). Briefly the samples were mixed by vortexing and centrifuged at 13,000 rpm for 5 minutes at room temperature. The aqueous phases were collected in new tubes. DNA was precipitated by adding 50ul of 3M NaOAc, 20ug glycogen (Ambion), 1 ml of 100% EtOH (Sigma-Aldrich) and incubating overnight at -20°C. To collect the DNA, the samples were centrifuged at 12000 g for 30 min at 4° C and the pellet were resuspended in 50µl of H_2O (GIBCO).

PCR chromatin immunoprecipitated DNA

To avoid unspecific amplification the primers were designed to amplify regions around 200-300 pb. The quality of primers were checked with Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). 7 primer pairs were designed to specifically amplify the 14 FoxO binding sites on atrogin-1 promoter; three primer pairs for LC3, two for Gadd45α; four for Bnip3 were designed to specifically amplify the FoxO binding sites on different promoters.

PCR reaction was prepared as following:

Template DNA	2 µl
PCR buffer 10X (Invitrogen)	2.5µl
dNTPs 10mM	0.5 µl
MgCl2 1.5mM	0.75 µl
Primer forward 10µM1.25ul	
Primer reverse 10µM	1.25µl
Taq DNA Polymerase 5U/µl (Invitrogen)	0.25 µl
H2O pure (Gibco)	16.75ul
Total volume	25 µl

The following PCR reaction program was used:

step1:	94°C for 3 minutes
step2:	94°C for 30 seconds
step3:	58°C for 30 seconds
step4:	go to step 2 for 29 times
step5:	72°C for 45 seconds
step6:	94°C for 30 seconds
step7:	58°C for 30 seconds
step8:	72°C for 10 minutes

10ul of each PCR was analyzed by 2% gel electrophoresis. The size of the PCR products was determined by using 1 Kb DNA molecular marker (Invitrogen).

Chromatin immunoprecipitation (ChIP) assay *in vivo*

Since the muscle structure and in vivo experiments are more complex than myotubes and cell cultures, several changes have been performed to the protocol and specifically a further step of nuclear purification was added.

Extraction and lysis nuclei

We used 4 tibialis anterior muscles of 2 month old CD1 mice for each ChIP experiment. The muscles were frozen in liquid nitrogen and powdered with pestle and mortar in liquid nitrogen and resuspended in 4ml Homogenization Buffer

(18M Surcose, 10mM HEPES,1mM EDTA, 50mM KCl, 5% glycerol in distilled pure water). The muscles were homogenized with mini-Polytron for 5 seconds. The homogenation procedure was repeated 3 times. The efficiency of extraction was analyzed by spotting 20ul of homogenized sample together with 20ul of Trypan Blue 1X (Sigma-Aldrich) on a slide and by observing the released nuclei at light microscope. The volume of homogenized solution was brought to 18ml with cold Lysis Buffer (10mM HEPES pH 7.5, 1mM EDTA, 50mM KCl, 12.5mM NaCl, 5mM $MgCa_2$, 0.1mM EGTA in distilled and pure water).

Cross-link of the protein to the chromatin and lysis

The myonuclei were precipitated by centrifuging at 900rpm for 10 minutes at 4°C, were resuspended in 5ml of Lysis Buffer and treated with 1% formaldehyde for 10 minutes at room temperature. 0.02M glycine was added to the samples to quench the formaldehyde action. Then myonuclei are centrifuged at 900rpm for 10 minutes and resuspended in 600ul of Resuspention Buffer (10mM HEPES pH 7.5, 1mM EDTA,0.5%SDS, 1mM PMSF)

Chromatin sonication

An efficient chromatin fragmentation was obtained by using 10 seconds pulse of a sonicator “Sonic Vibracell” at 4°C repeated for 10 times. After sonication the samples were centrifuged at 13000 rpm for 10 min at 4°C.

The next steps were identical to the steps previously described for ChIP in vitro experiments.

Lysis and Protein extraction from transfected HEK 293 FT:

The transfected cells were mechanically stccate from the plate and were collected by centrifugation at 1250rpm for 5minutes. The pellet was resuspended in 200ul of the following lysis buffer:

5 mM Tris, pH 7.5

100 mM NaCl

5 mM $MgCl_2$

1 mM DTT

10% Glicerolo (Sigma)

0.5% Triton (Sigma)

1X Cocktail di inibitori di proteasi (Complete, Roche)

After a centrifugation at 10000 rpm for 5 min at 4°C we measured the protein concentration of the surnatant using BradFord assay kit (PIRCE) following the manufacture protocol.

Gel Electrophoresis And Western Blot

Electrophoretic run of proteins

The extracted protein from differentiated C2C12, from HEK 293 FT and from tibialis anterior (TA) were solubizated in Loading buffer made for esch semple as following:

1X NuPAGE® LDS Sample Buffer (Invitrogen)	4 µl
1X NuPAGE® Reducing Agent (Invitrogen)	1.6 µl

The volume of each sample was adjasted to 20 µl with 1% SDS. The samples were denaturated at 70 °C for 10 minutes. Samples were loaded on SDS 4-12 % precast polyacrylamide gels (NuPAGE Novex Bis tris-gels) (Invitrogen). The electrophoresis was run in 1X MOPS Running buffer (Invitrogen) for 1hour and 30 minutes at 150V constant.

Transfer of the protein to the nitrocellulose membrane

After electrophoretic run, proteins were transferred from gels to nitrocellulose membranes. The gel and the membrane were equilibrated in Transfer Buffer. The Transfer Buffer was prepared as following:

20X NuPAGE® Transfer buffer(Invitogen)	50 ml
10X NuPAGE® Antioxidant (Invitogen)	1ml
20% Methanol (Sigma-Aldrich)	200ml

The volume was brought to 1l with distilled water. The transfer was obtained by applying a current of 400mA for 1 hour and 30 minutes at 4°C. To evaluate the

efficiency of transfer, proteins were stained with Red Ponceau 1x (Sigma). The staining was easily reversed by washing with distilled water.

Incubation of the membrane with antibodies

Once the proteins were transferred on nitrocellulose membranes, the membranes were saturated with Blocking Buffer (5% no fat milk powder solubilizated in TBS 1X) for 1 hour at room temperature and were overnight incubated with various primary antibodies diluted in TBS 1X with 5% no fat milk powder at 4°C . Then membranes were washed 3 times with TBS-T, 1% no fat milk at room temperature and incubated with a 1:2000 dilution of a Goat Anti-Rabbit IgG (H + L)-HRP Conjugate (Bio-Rad) dissolved in TBS-T 5% no-fat milk, for 1 hour at room temperature. Membranes were washed 3 times in TBS-T, 1% no fat milk. Immunoreaction was revealed by ECL (Pierce) and followed by exposure to X-ray film (KODAK Sigma-Aldrich).

Antibodies

The following antibodies were used: anti-HA, anti-FoxO3 anti-FoxO1 (Santa Cruz), anti-Bnip3 (Calbiochem), anti-Acetyl-Histone3 (Upstate), anti-LC3 antibody (MBL International) and IgG (Upstate). Secondary antibody peroxidase-conjugated were from Bio-Rad..

ChIP on Chip

To identify the FoxO1- and FoxO3-target genes we extended the ChIP technology to genome analysis by using the ChIP on chip procedure. ChIP on Chip is a combination of standard ChIP and microarrays assay. This microarrays are DNA microarrays representing several promoter regions or hole genome. Following the ChIP immunoprecipitation, DNA sample was amplified using Ligation Mediated PCR method (LM-PCR). Subsequently, DNA fragments were labeled to hybridize onto Nimblegen’s promoter arrays. The arrays are covering the combined gene annotations from RefSeq (NM), UCSC known genes and the Mammalian Gene Collection (MGC). 4.4Kb (mouse). By comparing the hybridization signals generated by the immunoprecipitated sample versus the

"input", the binding sites of given protein were identified. ChIP on Chip experiments were supported by *Full ChIP-Chip Analysis Service* (NimbleGen) that provides to label the amplified samples, to hybridize, to scan the array and to perform data analysis.

The principal phases to amplify immunoprecipitated DNA and input are the following

- Blunting DNA Ends
- Ligation of linkers
- Ligation Mediated-PCR

Blunting DNA Ends

The immunoprecipitated sample and 200ng of its input and were treated with T4 DNA polymerase (New England Biolabs) in the following reaction mix:

10X T4 DNA polymerase buffer (New England Biolabs)	10 µl
BSA (10 mg/ml) (New England Biolabs)	0.5 µl
dNTP mix (10 mM each)	1 µl
T4 DNA polymerase (3U/µl) (New England Biolabs)	0.2 µl

The volume was adjusted to 50µl of final volume with H_2O. After a 20 minutes incubation at 12°C, 1/10 volume of 3M sodium acetate (pH 5.2) and 20µg of glycogen (Roche Applied Sciences) were added to the tube. The DNA sample is extracted by phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma-Aldrich) purification. After ethanol precipitation, the DNA was dissolved in 25 µl of distilled H_2O.

Ligation of linkers

The blunt-ended DNA was mixed in the ligation reaction:

Templete DNA	25 µl
10X ligase buffer (New England Biolabs)	10 µl
15mM annealed oligonucleotide *linkers*	6.7 µl
(oligo-1: GCGGTGACCCGGGAGATCTGAATTC,	

oligo-2: GAATTCAGATC,)	
T4 DNA ligase (New England Biolabs)	0.5 µl
of distilled H_2O	7.8 µl
total volume	50 µl

The ligation reaction was incubated overnight at 16°C. After ligation, the DNA was purified by ethanol precipitation and dissolved in 25 µl of distilled H_2O.

Ligation Mediated-PCR

The ligated DNA was added to Mix A:

10X ThermoPol reaction buffer (New England Biolabs)	4 µl
10X dNTP mix (2.5 mM each)	5 µl
oligo-1 (40 µM stock)	1.25 µl
distilled H_2O	4.75 µl
total volume	15 µl

into a 200 µl thin-walled PCR tube. The tube was first incubated at 55°C for 2 minutes in a thermal cycler, then 10 µl of MixB was added:

ThermoPol reaction buffer (New England Biolabs)	1 µl
Taq DNA polymerase (5U/µl)	0.5 µl
distille H_2O	8,5µl
total volume	10 µl

Subsequently, the following PCR protocol is performed:

step 1:	72°C for 3 minutes;
step 2:	95°C for 2 minutes;
step 3:	95°C for 30 seconds;
step 4:	60°C for 30 seconds;
step 5:	72°C for 1 minutes
step 6:	go to step 3, 24 times;
step 7:	72°C for 5 minutes;
step 8:	4°C indefinitely

After PCR, the DNA was purified by using the Qiaquick PCR purification kit (Qiagen) and eluted in 50 µl elution buffer. Prior to label samples, we check the quality of our DNA fragments. We used an Agilent Bioanalyzer and a Nanodrop spectrophotometer to quantity and to determine the size of our amplified DNA fragments.

Spectrophotometry of LM- DNA

1µl of each test and control LM-PCR sample were measured using NanoDropTM instrument.

All samples met the following requirements for acceptance:

concentration > 0.05 ug/ul

·A260/A280 > 1.7

·A260/A230 >1.5

Analysis of LM-DNA with Agilent 2100 Bioanalyzer:

The quality of LM-DNA samples was assayed with Agilent 2100 Bioanalyzer, using the NanoRNA 6000 LabChip kit (Agilent Technologies), following the manufacturer protocol.

NimbelGen Chip array:

The NimbleGen *Mouse Promoter Tow Array Sets 385K* were chosen for ChIP on Chip experiments. Those arrays cover the combined gene annotations from RefSeq, UCSC known genes and Mammalian Gene Collection. Each array contains 385.000 probes that represent 11.000 promoter regions of 5 kilo bases. There are 50-75mer probes per region, with roughly 100 basis pairs spacing, dependent on the sequence composition of the region.

Gene Expression Analysis

Quantitative Real-time PCR was performed with the SYBR Green chemistry (Applied Biosystems). SYBR green is a fluorescent dye that intercalates into double-stranded DNA and produces a fluorescent signal. The Real-Time PCR Instrument allows real time detection of PCR products as they accumulate during

PCR cycles and create an amplification plot, which is the plot of fluorescence signal versus cycle number. In the initial cycles of PCR, there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR products. A fixed fluorescence threshold can be set above the baseline. The parameter Ct (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. So the higher the initial amount of the sample, the sooner accumulated product is detected in the PCR process as a significant increase in fluorescence, and the lower the Ct value.

Quantification of the PCR products and determination of the level of expression

A relative quantification method were used to evaluate the differences in gene expression, as described by Pfaffl (Pfaffl, 2001). In this method, the expression of a gene is determined by the ratio between a test sample and a housekeeping gene.

The relative expression ratio of target gene is calculated based on PCR efficiency (E) and threshold cycle deviation(ΔCt) of unknown sample versus a control, and expressed in comparison to reference gene.

The mathematical model used of relative expression is represented in this equation:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta Ct}}{(E_{\text{reference}})^{\Delta Ct}}$$

The internal gene reference used in our real time PCR was b-actin, whose abundance did not change under the experimental conditions.

Primer pairs design

Gene-specific primer pairs were selected with Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi); sequences of distinct exons were chosen to avoid amplifying contaminant genomic DNA. Primer pairs were selected in a region close to the 3'-end of the transcript, and amplified fragments of 150-250 bp in length. To avoid the amplification of contaminant genomic DNA, the target sequences were chosen on distinct exons, separated by a long (more than 1000 bp) intron. The temperature of melting chosen was of about 58-60° C.

The sequences of the primer pairs are listed in the **Table Real Time PCR Primers**.

Extraction of total RNA:

Total RNA was prepared from differentiated myotubes using the *Promega SV Total Isolation kit.*

Synthesis of the first strand of cDNA

400ng of total RNA was reverse transcribed with SuperScript™ III (Invitrogen) in the following reaction mix

Random primer hexamers (50 ng/µl random)	1 µl
dNTPs 10 mM	1 µl
H_2O Rnase-free	8.5 µl

The samples were mixed by vortexing and briefly centrifuged and denaturated by incubation for 5 min at 70° C to prevent secondary structures of RNA.

Samples were incubated on ice for 2 minutes to allow the primers to align to the RNA; and the following components were added sequentially:

First strand buffer 5×(Invitrogen)	5 µl
DTT 100mM	2 µl
RNase Out (Invitrogen)	1 µl

SuperScript™ III (Invitrogen)	0.5 µl

The volume was adjusted to 20 ul with water.

The used reaction program was:

step1:	25°C for 5 minutes
step2:	42° C for 50 minutes
step3:	70°C for 15 minutes

At the end of the reaction, the volume of each samples was adjusted to 50ul with RNase free water.

Real-Time PCR reaction

1 µl of diluted cDNAs were amplified in 10 µl PCR reactions in a ABI Prism 7000 (Applied Biosystem) thermocycler, coupled with a ABI Prism 7000 Sequence Detection System (Applied Biosystems) in 96-wells plates (Micro Amp Optical, Applied Biosystems).

In each well it was added : 10ul Sample mix + 10ul reaction mix.

Sample mix was prepared as:

Template cDNA	1 µl
H_2O Rnase-free	9 µl
Total volume	10 µl

The SYBR® Green qPCR (Qiagen) was used for the Real-Time PCR reaction as following:

SYBR® Green qPCR (Qiagen)	10 µl
Mix Primer forward /reverse 50 mM	0.4 µl
Total volume	10 µl

The PCR cycle used for the Real-Time PCR was:

step 1:	95° C for 15 min

step2:	95° C for 25 sec
step3	60° C for 1 min
step4:	go to step2 for 34 times

Table PCR-Primers For ChIP experiments:

atrogin-1 promoter primerse		
Region	**Primer forward**	**Primer reverse**
1	5'-CAGGATCTTGGTGGCGACAGCTAC-3'	5'-CTCAGCATTCCCAGAGTCAGGAG-3'
2	5'-CAGGGAGGATCGCTCTGAGTTGAG-3'	5'-GAGCAGCGAGTGTAGCCTGAGA-3'
3	5'-TGGACACAGTGCTTGATGAACAGTC-3'	5'-GACAAACGTGAGCAAGGAAGACAC-3'
4	5'-GAATGCTGGGAGTTGTAGTCCTT-3'	5'-TGGACACAGTGCTTGATGAACAGTC-3'
5	5'-CCTGTGGTTTCTCCCTGGAAGGAG-3'	5'-GCAACACAGAACCCACAAGACACA-3'
6	5'-CTGGCAGGGAGGAGCCTAATGAATC-3'	5'-GAGACGGCTTTGCCACTCCC-3'
7	5'-TTCTCCAGGCCAGTAGGTGGTCTG-3'	5'-CAACTTGCTATCTCTGAGGCTAAC-3'

Table 1: atrogin-1 promoter primers

Gadd45α promoter primers		
Site	**Primer forward**	**Primer reverse**
1	5'-CCTCCTTCCAACCATAATACTC-3'	5'-GGTGCCCTCTGTTTTTGA-3'
2	5'-GGGCACCAAAAGACTACTACTG-3'	5'-GGTTCAAGTGTCCTCAGCTCT-3'

Table 2: Gadd45α promoter primers

LC3 promoter primersc		
Region	**Primer forward**	**Primer reverse**
1	5'-CATGCCTTGGGACACCAGAT3'	5'-ACCTTCTTCAAGTGCTGTTTGT-3'
2	5'-TATGTTTTCTAGGTTCACACTGC-3'	5'-AGGCAATGTTTGTGG AAG TGG-3'
3	5'-CTG GTC TAC AGA GTG AGT TC-3'	5'-GGC AGCGATGCAGCTAAT C-3'

Table 3: LC3 promoter primers

Bnp3 promoter primersc		
Region	**Primer forward**	**Primer reverse**
1	5'-CAGTCCGGGAGTGCTTATGT -3'	5'-GCCATTGATGCTGCAGTAGA-3
2	5'- GCCCTCGTATAACCTTAGCA-3'	5'-TGGGTCAGGTCACTAGAAGC-3'
3	5'-CCTGGCTTACATGGTGAGTT-3'	5'-ATTTGAAGGGGTTAGGGATG-3'
4	5'-CTCCACGACACCAGGATTAC-3'	5'-GCTGTAGGTCAGAGCCAAAA-3'

Table 4 Bnip3 promoter primers

Table Real Time PCR Primers:

atrogin-1	Forward	5'-GCAAACACTGCCACATTCTCTC-3'
	Reverse	5'-CTTGAGGGGAAAGTGAGACG-3'
p62	Forward	5'-CCCAGTGTCTTGGCATTCTT-3'
	Reverse	5'-AGGGAAAGCAGAGGAAGCTC-3'
Tgif	Forward	5'-TTTCCTCATCAGCAGCCTCT -3'
	Reverse	5'-CTTTGCCATCCTTTCTCAGC-3'
Atf4	Forward	5'-TCCTGAACAGCGAAGTGTTG-3'
	Reverse	5'-ACCCATGAGGTTTCAAGTGC-3'
Pmse4	Forward	5'-TGGCACAGATCAAGAGCAAC-3'
	Reverse	5'-AGAAAGGACCAGAACGCTGA-3'
GabarapL-1	Forward	5'-CATCGTGGAGAAGGCTCCTA-3'
	Reverse	5'-ATACAGCTGGCCCATGGTAG-3'
β-actin	Forward	5'-CTGGCTCCTAGCACCATGAAGAT-3'
	Reverse	5'-GGTGGACAGTGAGGCCAGGAT-3'

RESULTS:

Set Up of ChIP Technology And Validation In Vitro:

Analysis of atrogin-1 promoter in vitro

Previous studies have shown the role of FoxO transcription factors in regulating atrogin-1 expression, but the molecular mechanism underlying this regulation still remains unclear. Especially at which sites FoxO3 can bind and modulate atrogin-1 expression was unclear. We want to clarify this issue and develop a powerful technique to identify genes that are regulated by a certain transcription factor. We first analyzed the 3,5 kilo base long region of the atrogin-1 promoter with Genomatix bioinformatic tool to reveal putative FoxO binding sites. There are 14 potential FoxO binding sites in 5'UTR promoter region, suggesting a possible direct role of FoxO in controlling the activity of atrogin-1 (**Fig 12**).

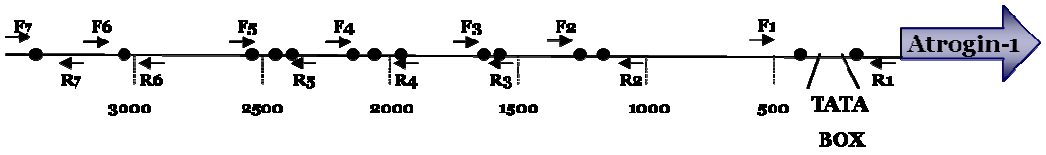


Figure12: Computational analysis of atrogin-1 promoter. The 3.5 kilo bases of atrogin-1 promoter were analyzed with the Genomatix bioinformatic tool (http://www.genomatix.de/) to identify putative FoxO binding sites. The black circles represent putative FoxO binding sites found by the program. Arrows represent the primers used for the ChIP analysis (**Table 1**). F: primer forward; R: primer reverse.

To study the possible direct interaction between various transcription factors and the atrogin1 promoter, we used the Chromatin Immunoprecipitation (ChIP) technique, which allows the detection of physical interaction between proteins and genomic DNA (Kuo, 1999 Das, 2004). The same approach is also widely used to reveal chromatin modifications. ChIP is a powerful tool to identify proteins associated with specific regions of the genome by using specific antibodies against either specific transcription factors or specific chromatin/histone

modifications. The initial step of the ChIP is the cross-linking between proteins and chromatin which is achieved by treating living cells with formaldehyde. After cross-linking, the cells are lysated and crude extracts are sonicated to break the chromatin into fragments of an average of 400-200 bp. The proteins, which are cross-linked to the DNA, are subsequently immunoprecipitated. Protein-DNA cross-links in the immunoprecipitated material are then reversed, and the co-purified DNA fragments are amplified by PCR (**Fig 13**).

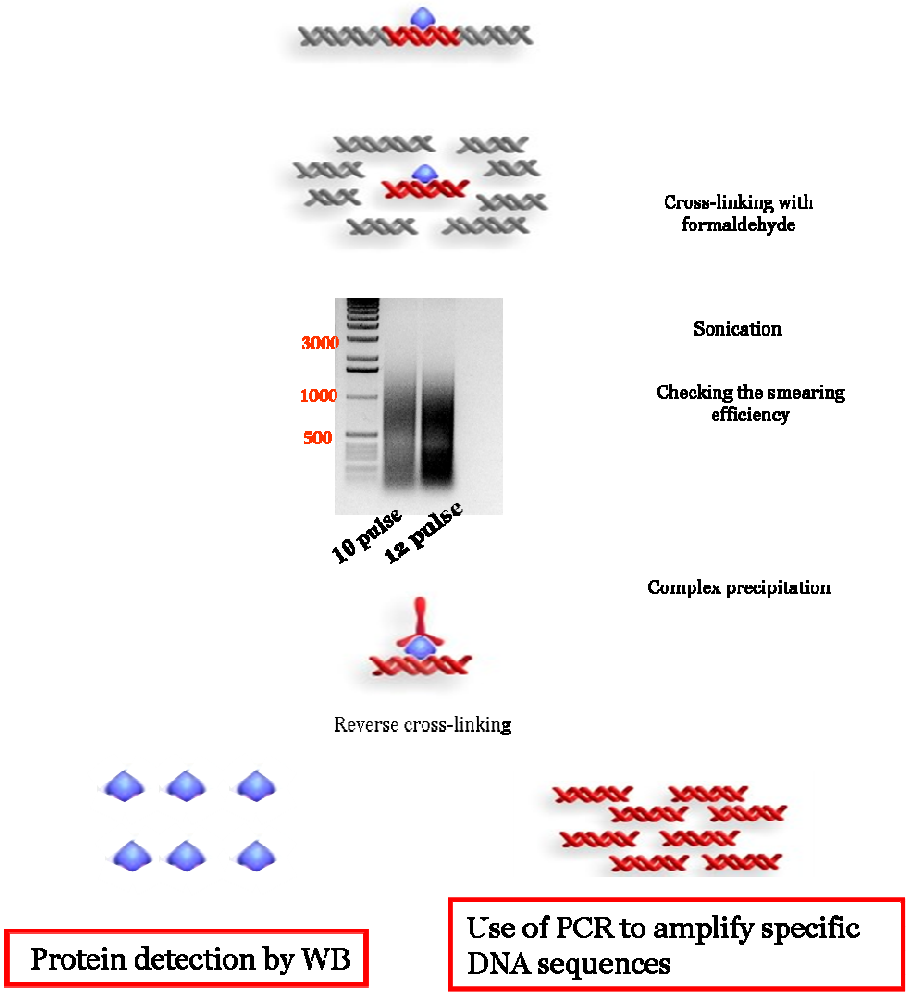


Figure 13 Chromatin immunoprecipitation (ChIP) principal steps. Schematic description of the major steps of the ChIP is depicted.

To identify which are FoxO1 and FoxO3 binding sites on atrogin-1 promoter, 7 primers pairs were designed to include all 14 potential FoxO sites.

In order to adjust the technique for our studies, we started with experiments *in vitro* using HEK 293T cells. A distinct advantage of HEK 293T is that they are easy to cell culture and they have a high efficiency of transfection. Using the Lipofectamine™ system, we contransfetced ca-FoxO1 or ca-FoxO3 with atrogin-1 promoter. Those two recombinant proteins have an HA-tag, and they are mutagenized at the Akt phosphorylation sites to get a nuclear localization. We co-transfected the atrogin-1 promoter because HEK 293T are an epithelial cell line which does not express atrogin-1 due to a hypercondensed and inaccessible chromatin at this locus.

24 hours after transfection cells were treated with formaldehyde in order to preserve DNA-FoxO interaction. The protein-DNA complexes were immunoprecipitated with 5ug of antibody against either the HA-tag or FoxO1 or FoxO3. As negative control, the same quantity of cellular lisate was immunoprecipitated with mouse IgG. The quality of immunoprecipitation was confirmed by 4-12% SDS page and by western blotting for anti-HA tag (**Fig 14**).

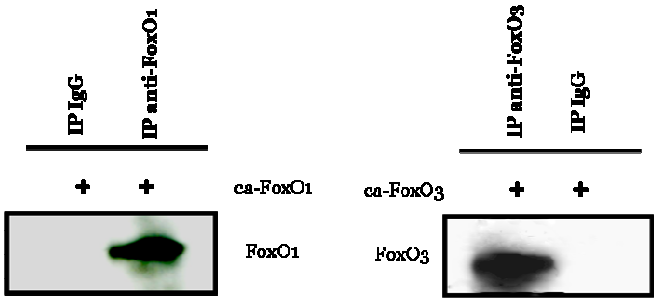


Figure 14: Western Blott to reveal the specificity of the immunoprecipitation. Crude lisates were immunoprecipitated with anti FoxO1 or FoxO3 and blotted for anti HA. In control experiment we used non specific IgG. Arrows point the specific FoxO protein, which are around 70 KD. IP: Immunoprecipitate. ID: Immunodetection..

Next, the immunoprecipitated DNA was amplified by PCR using the different primers pairs for FoxO binding sites on the atrogin-1 promoter. As a positive control for the PCR conditions and the primer design, we used the "input" which is the non-immunoprecipitated DNA. Input represents 10% of the total extracted chromatin. As a negative control, we used DNA, which was immunoprecipitated with non specific-IgG. The results from the PCR clearly show that overexpressed FoxO1 significantly binds regions 3 and 6, while weakly interacting with regions 4 and 5 (**Fig 15**).

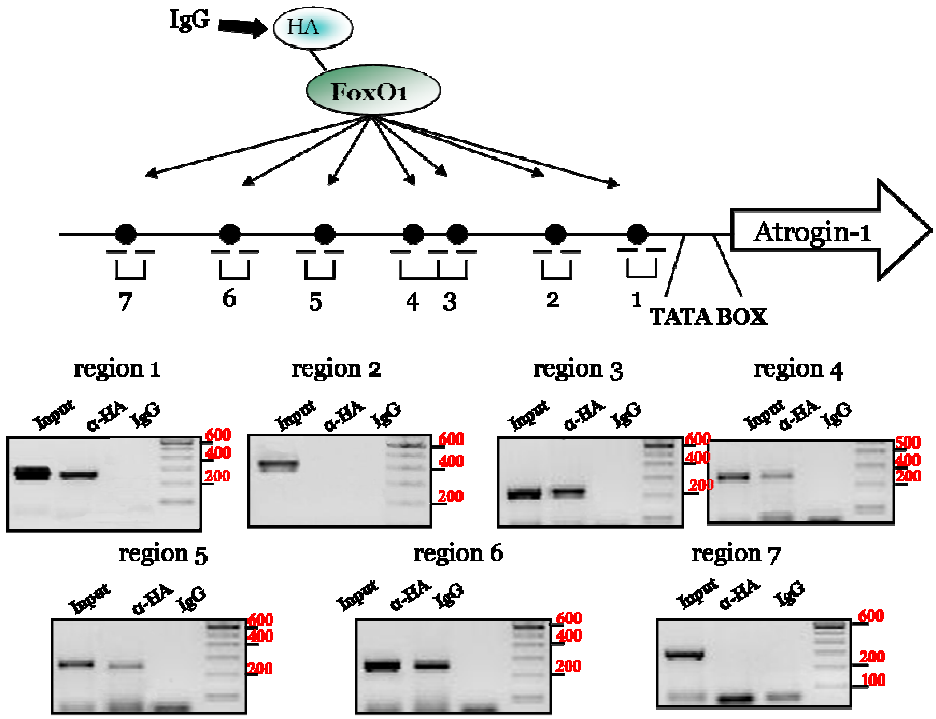


Figure 15 FoxO1 weakly binds atrogin-1 promoter. HEK293T were transfected with the ca-FoxO1expression vector together with 3.5 kb atrogin-1 promoter and after 2 days the chromatin was immunoprecipitated with anti-HA antibody or with mouse IgG. We used 7 primer pairs to amplify 7 regions of atrogin-1 promoter, which include all 14 FoxO binding sites. The PCR products were loaded in 2%agarose gel and run for 10 minutes at 100V. In each gel the PCR band on the left corresponds to amplification of input, the band in the middle is the immunoprecipitated DNA and the band on the right represents the negative control. The 1Kb DNA Ladder (Invitrogen) was used to determinate the correct size of the bands.

Conversely, FoxO3 is able to interact with all the studied regions of atrogin1 promoter, with the exception of the region 2 (**Fig 16**). The strength of FoxO binding can be determined by comparing the intensity of the immunoprecipitated DNA PCR band with its corresponding input. Bands that look similar indicate a strong interaction between the transcription factor and the chromatin while a lighter band in the immunoprecipitated DNA is consistent with a weak binding.

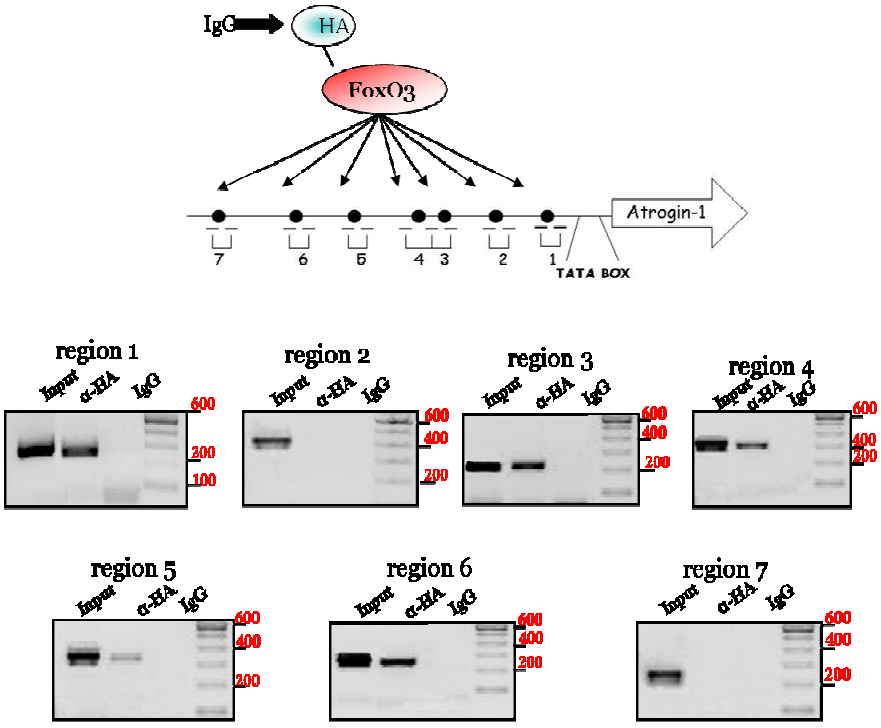


Figure 16: *In vitro* ChIP analysis showed the direct binding of FoxO3. HEK293T were transfected with the ca-FoxO3 expression vector and after 2 days the chromatin was immunoprecipitated with HA antibody or with normal mouse IgG. The seven gels represent the seven amplified regions of atrogin-1 promoter that include FoxO binding sites. In each gel the PCR products were loaded in the same order as described for FoxO1 in figure 15.

Analysis Of Atrigin-1 Promoter *In Vivo*

After having optimized the conditions for cell culture experiments, we moved to *in vivo*. Initially we used TA muscles transfected with FoxO

transcription factors. Four TA muscles were electroporated with either a plasmid encoding for ca-FoxO1, or a plasmid encoding for ca-FoxO3. Electroporation of plasmids into adult mouse skeletal muscles is a powerful tool to study the effects and function of the studied protein (Dona, et 2003 ; Sandri et al, 2004). This technique allows the over-expression of proteins in a normal muscle of a wild type animal giving results more quickly than transgenic animals. Furthermore, it avoids the compensatory effects that can occur in transgenic animals. Another important advantage of this technique is that the transfection happens only in a portion of muscle fibers (between 30% and 90%) (**Fig 17**). Therefore we can compare, in the same muscle, the size and morphology of the fibers expressing the transgene within the untransfected ones. Extensive control experiments have been conducted to prove that the process of electroporation does not affect the size of muscles and the expression of genes involved in atrophy-program (Murgia, et al 2000 ; Sandri,e al 2004).

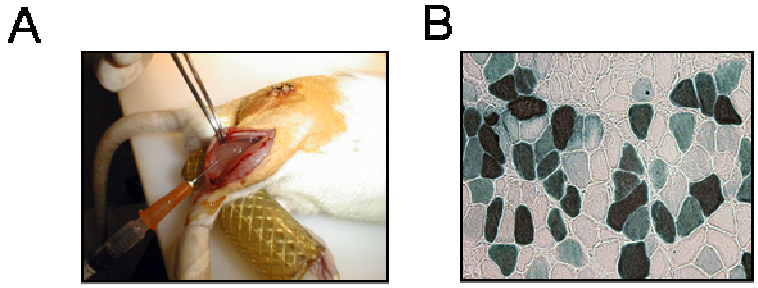


Figure 17: Transfection of adult Tibialis Anterior mouse muscles by electroporation. **A:** Mouse tibialis anterior (TA) muscles were isolated through a small hind limb incision and the plasmid DNA is injected along the muscle length. Electrical pulses were then applied through two stainless steel spatula electrodes placed on each side of the isolated muscle and induced the uptake of the DNA. **B** cross section of an electroporated TA. The blu fibers indicate the transfected ones which express β-Gal gene

Seven days after transfection, the muscles were collected and powdered with a pestle and mortar in liquid nitrogen. After homogenization and lysis the extracted

nuclei were fixed whit formaldehyde to preserve the protein-DNA interaction. Chromatin was sonicated and immunoprecipitated with 5ug of antibody either against specific FoxO factors, or against the HA-tag. As negative control, 5ug of mouse IgG were used. To check the quality of IP the protein fraction was loaded in a SDS 4-12 % precast polyacrylamide gel and western blotted with anti-HA antibody (**Fig 18**.**A**).

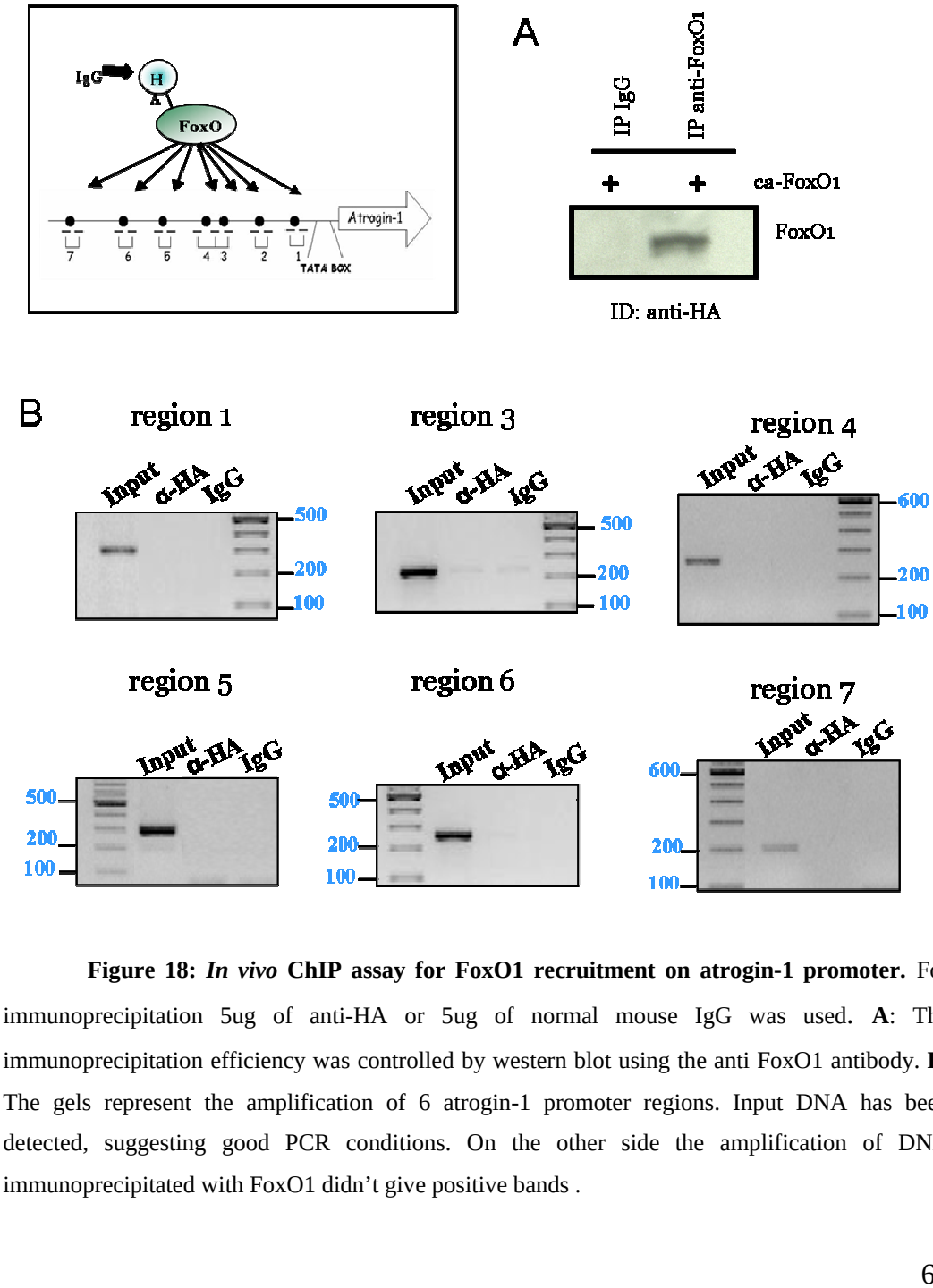


Figure 18: ***In vivo*** **ChIP assay for FoxO1 recruitment on atrogin-1 promoter.** For immunoprecipitation 5ug of anti-HA or 5ug of normal mouse IgG was used**. A**: The immunoprecipitation efficiency was controlled by western blot using the anti FoxO1 antibody. **B**: The gels represent the amplification of 6 atrogin-1 promoter regions. Input DNA has been detected, suggesting good PCR conditions. On the other side the amplification of DNA immunoprecipitated with FoxO1 didn't give positive bands .

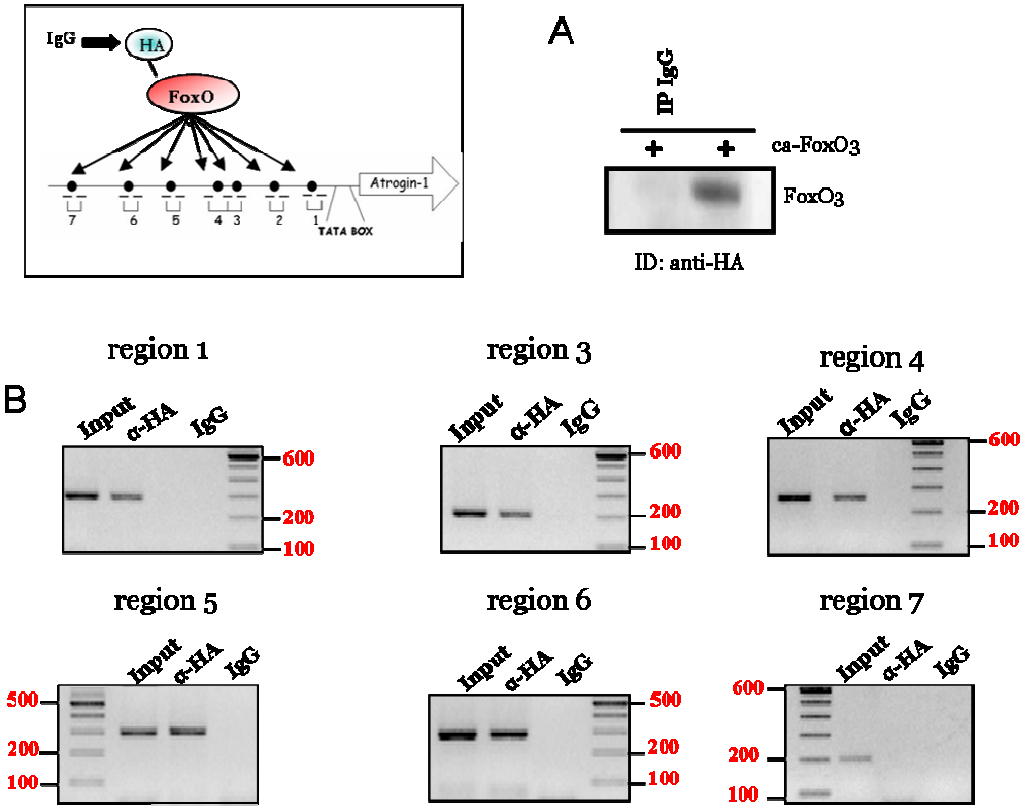


Figure 19: Analysis of FoxO3 recruitment on atrogin-1 promoter *in vivo*. Chromatin from tibialis anterior was, as for FoxO1 study, for immunoprecipitation we used 5ug of anti-HA or 5ug of normal mouse IgG **A:** The SDS 4-12 % precast polyacrylamide gel shows the FoxO protein immunoprecipitated. Anti FoxO3 antibody was used **B**: Immunoprecipitated DNA and input were amplified by PCR and the gel electrophoresis displays FoxO3 binding on every region except for the region 7.

Subsequently immunoprecipitated DNA and the input were amplified by PCR, with appropriate primers (**Fig 18 B** and **table 1**). Consistent with *in vitro* results, the primer pairs of region 2 didn't produce any PCR product, suggesting that the chromatin is either highly condensed or not an important binding site for FoxO transcription factors. In all our following experiments we didn't take into consideration this region, but we maintained the numeration for the other regions. As it can clearly be seen from the results obtained by PCR, transfected FoxO1 interacts weakly with region 3 of the atrogin-1 promoter, while the input was significantly amplified in each region.

Importantly we obtained completely different results for FoxO3. Except for region7, the PCR revealed an interaction in all the other regions studied (**Fig 19**).

Role Of Endogenous Foxo1 And Foxo3 During Fasting

The *in vitro* and *in vivo* results show a similar ability of FoxO3, when overxpressed, to bind different regions of atrogin-1 promoter. To investigate whether the endogenous FoxOs interact in the same chromatin regions revealed in overexpression experiments, we performed ChIP experiments using the TA muscles of mice kept in starvation for 24 hours. Fasting is a widely used model of acute muscle loss (LECKER et al., 2004; Mammucari et al., 2007; Sandri et al., 2004). During this catabolic condition the IGF1/PI3K/AKT pathway is suppressed and is not able to negatively regulate FoxOs. Thus, FoxO transcription factors can enter into the nucleus, interact with regulatory regions and activate target genes.

Positive control for FoxO1 and FoxO3 binding on the DNA: Gadd45α promoter

Since FoxO1 binding have not been detected in previous experiments, we wondered whether this failure was caused by an inefficient FoxO1 immunoprecipitation. Both FoxO1 and FoxO3 are reported to regulate GADD45α expression. Thus, we investigated the interaction of both transcription factors on the promoter of Gadd45α. (Kamei et al., 2004; Tran et al., 2002a). Using the Genomatix bioinformatic tool we identified two potential FoxO binding sites along the 3.5 kb upstream the TATA-box region (**Fig 20**).

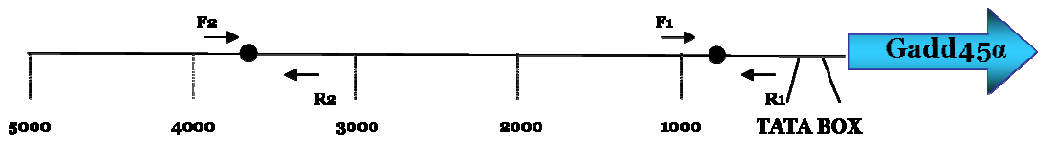


Figure 20: Bioinfromatics analysis of Gadd45α 5kb promoter for FoxO binding sites. To identify putative FoxO binding sites, the 5 kilo bases of Gadd45α promoter were analyzed with the Genomatix bioinformatic tool. The black circles represent putative FoxO binding sites. Arrows represent the primers used for the ChIP analysis (**Table 2**). F: primer forward; R: primer reverse.

We designed two specific primer pairs to include these sites and we performed the ChIP assay. For each experiment, 4 tibialis anterior muscles from both starved and fed mice were used. The immunoprecipitation of FoxO1 and FoxO3 was performed by using the specific antibodies previously tested in cell culture experiments.

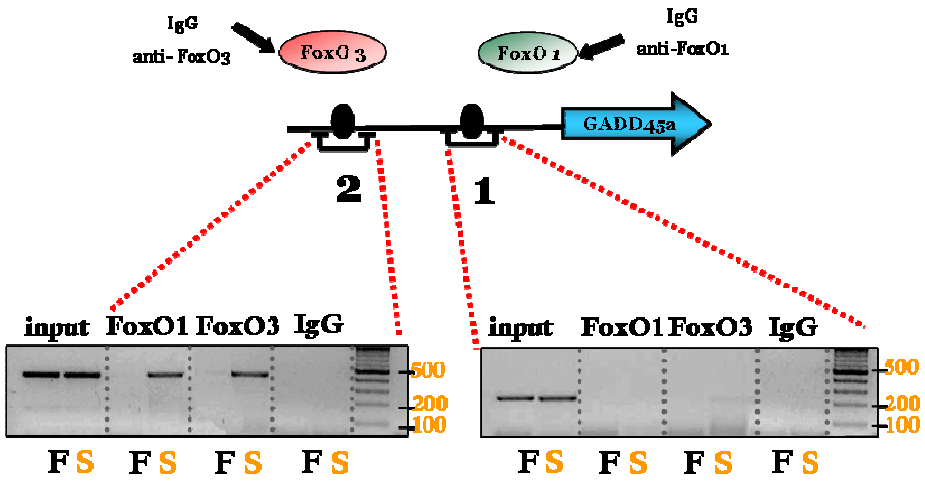


Figure 21:Chromatin Immunoprecipitation analysis (ChIP) of Gadd45α promoter shows the direct binding of the endogenous FoxOs. Chromatin was extracted from tibialis anterior of fed (**F**) and starved mice (S) and immunoprecipitated with antibodies against specific FoxO factors, or normal mouse IgG. We used 2 primer pairs to amplify the two putative (**table 2**) FoxO binding sites. Each gel electrophoresis is divided in four zones. Starting from the left the first region contains the PCR products from Input DNA. The second area show the PCR products from FoxO1-DNA interaction, the third region shows the PCR products from FoxO3-DNA interaction, and the in fourth one displays DNA immunoprecipitated with normal mouse IgG. The positive PCR products in the region 2 means that FoxO1 and FoxO3 are both able to bind the same region of Gadd45α.

The ChIP analyses show that both FoxOs bind chromatin of region 2, while the region 1 is not involved in any interaction. These findings validate our approach to detect both FoxO1- and FoxO3-DNA binding (**Fig 21**).

Study of endogenous FoxO1 and FoxO3 on the atrogin-1 promoter

To address whether both endogenous FoxO1 and FoxO3 directly interact with the atrogin-1 promoter, we performed ChIP *in vivo*. DNA immunoprecipitated with both FoxO1 and FoxO3 from fed and starved muscles were amplified by PCR using the seven primer pairs to include all 14 potential FoxO binding sites on atrogin-1 promoter (**Fig 22 and table 1**).

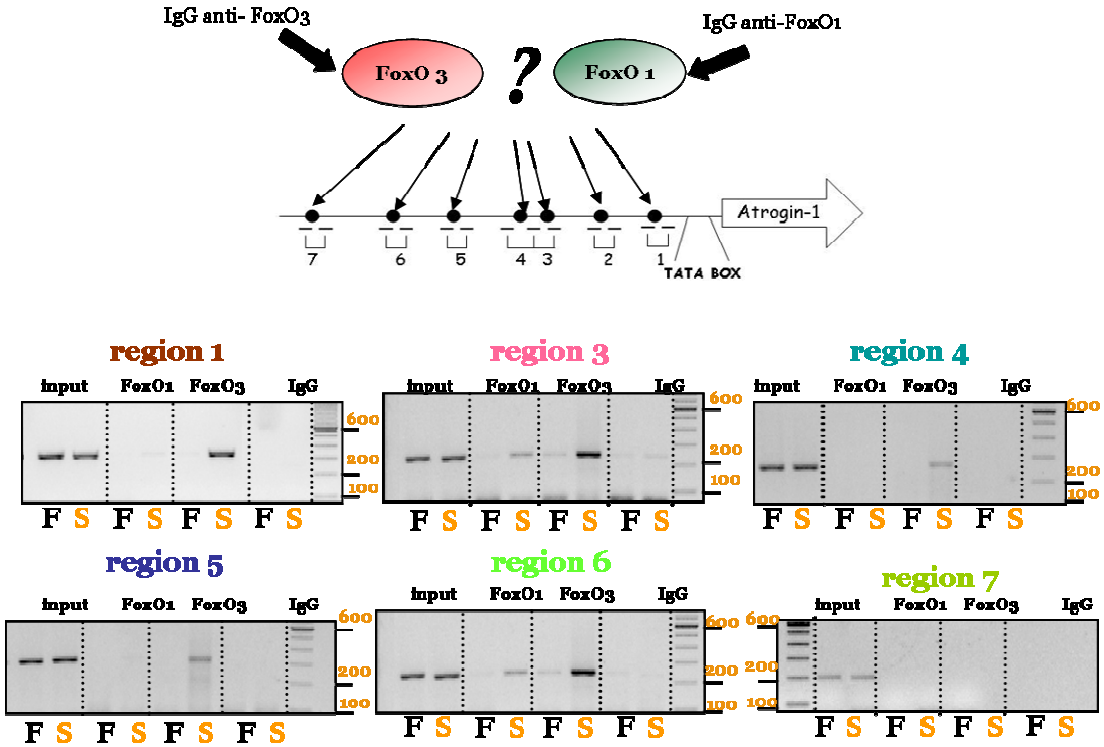


Figure 22: Recruitment of endogenous FoxO1 and FoxO3 on atrogin-1 promoter during muscle atrophy. To immunoprecipitate cross-linked chromatin we used 5ug of antibodies against specific FoxO factors, or normal mouse IgG. The DNA was amplified by PCR to include all 14 putative FoxO binding sites (**table 1**). The seven gel electrophoresis represent the amplified regions. FoxO3 is recruited on region 1 (-541/-238), 3 (-1747/-1537) and 6(-3228/-2985)., and more weakly on region 4 and 5. FoxO1 immunoprecipitation resulted in a weak interaction with chromatin of region 3 and 6.

The results show unexpected differences between endogenous and overexpressed FoxOs (**Fig 18** and **19**). Moreover FoxO1 and FoxO3 display a different pattern of binding. While FoxO1 interacts weakly with the sites of regions 3, and 6, FoxO3

strongly binds regions 1 ,3 and 6 and weakly to regions 5 and 4. The absence of any band in region 7 suggests that physiologically neither FoxO3 or FoxO1 interacts with this region of chromatin.

In order to investigate how FoxO binding on the atrogin-1 promoter can regulate the expression of this E3 ligase, we examined the levels of chromatin acetylation near the sites of FoxO3 binding. Acetylation is one of the most important chromatin structure modifications that occur in actively transcribing regulatory regions. (Williams et al., 2008) (**Fig 23**).

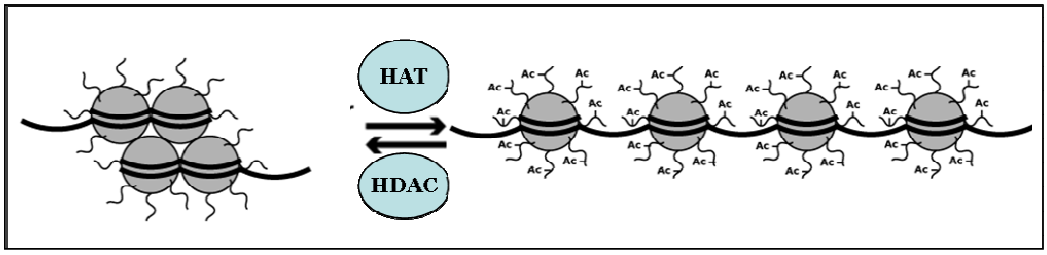


Fig 23 The relationship between histone acetylation and gene regulation. Eukaryotic DNA is packaged into chromatin by histone proteins, which assemble the DNA into an organized, higher-order structure. The organization of chromatin is considered to be regulated by a variety of post-translational modifications of histones, such as acetylation. Acetylation neutralizes the charge on histones, therefore, increasing chromatin accessibility to trans-acting factors and transcriptional machinery (Verdone et al., 2005).

Histone hyperacetylation is considered a marker of transcriptional activity. The positive charge of the acetyl groups disturbs the interaction between the histones and relax the chromatin in an open conformation. Consequentially, the chromatin becomes accessible to the transcriptional machinery. We performed ChIP experiments using a specific antibody against acetylated lysines of histone 3. The PCR data confirms that the three regions of FoxO3 binding are hyper-acetylated during muscle atrophy. The highest increase of acetylation occurs in the region3 (**Fig 24**).

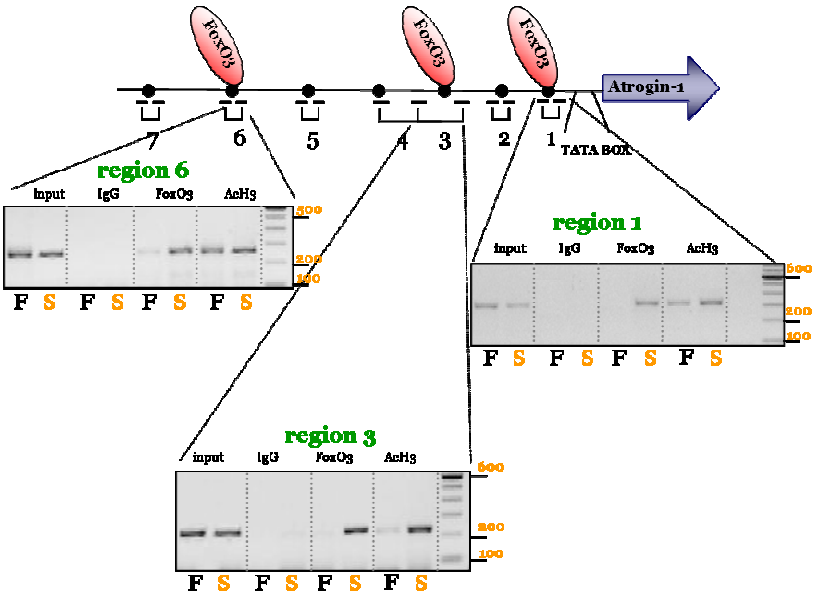


Figure 24: analysis of Histone acetylation of atrogin-1 promoter by ChIP assay. Chromatin from the tibialis anterior of fed (**F**) and starved mice (S). was immunoprecipitation with anti-acetyl histone 3, or with anti-FoxO3 or normal mouse IgG. The three gels correspond to the three regions of atrogin-1 which have been shown to greatly recruit FoxO3. The chromatin in all these regions show a certain level of acetylation which increases during fasting. The more dramatic increase occurs in region 3

This results suggest that FoxO3, during starvation, binds to chromatin of three specific sites of the atrogin-1 causing the histone hyperacetylation and the transactivation of this gene

To understand if the FoxOs binding on the atrogin-1 promoter has a functional role, we studied the effect of FoxO1 and FoxO3 overexpression on the atrogin-1 promoter activity. To address this issue, a dual reporter assay was used. By electroporation we overexpressed in TA muscles the atrogin1 reporter together with either ca FoxO1 or ca FoxO3. The atrogin1 reporter consists of 3.5 kilo bases upstream the TATA box of the atrogin-1 gene which was cloned upstream the firefly luciferase gene. We co-transfected this reporter gene with a renilla luciferase plasmid to normalize the data for the transfection efficiency. Seven days after the muscles were collected, and luciferase activity was measured (**Fig 25**)

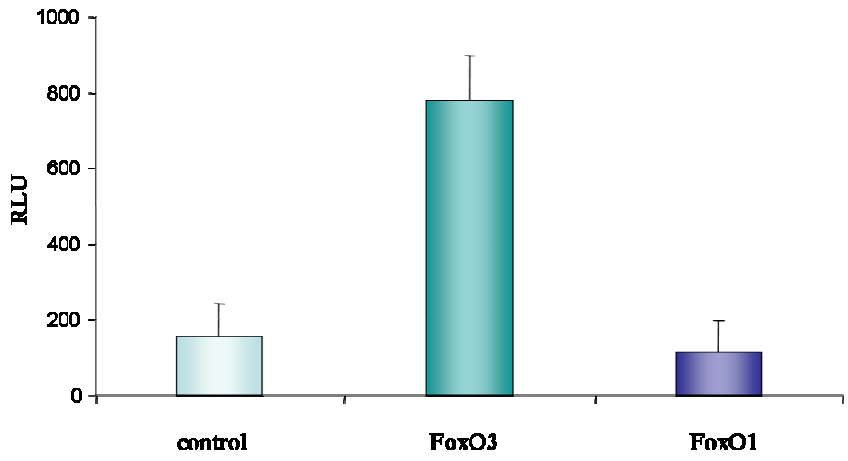


Figure 25: *In vivo* ca-FoxO3, but not FoxO1 activates the atrogin-1 promoter

Tibialis anterior were co-transfected with atrogin-1 reporter and the control renilla plasmid in presence or absence of ca-FoxO1 or ca-FoxO3. The activity of atrogin-1 promoter was tested measuring the firefly luciferase and normalizing for the renilla luciferase. Error bars represent SD.

The analysis of the data show that overexpression of FoxO1 does not activate the atrogin-1 promoter. On the other hand, overexpression of FoxO3 transactivate atrogin-1 promoter.

All these data confirm the different role of FoxO1 and 3 on atrogin-1 promoter and mirror the ChIP data. In fact during starvation FoxO1 weakly binds the atrogin-1 promoter and is not sufficient to strongly activate the transcription of the gene. Conversely, activated FoxO3 can bind specific sites of atrogin-1 promoter, modify the chromatin conformation and transactivate its expression.

Atrophy related genes under control of FoxO:

The results obtained using the atrogin-1 promoter don't explain how FoxO1 is able to induce muscle atrophy (Kamei et al., 2004). Moreover, when we overexpressed atrogin-1 protein in TA, we didn't induce a decrease in fiber size (**Fig 26**). Thus, atrogin-1 expression is required for muscle atrophy but it is not

sufficient to trigger muscle loss. This finding suggests that other genes have to be coordinately induced in order to loose muscle tissue.

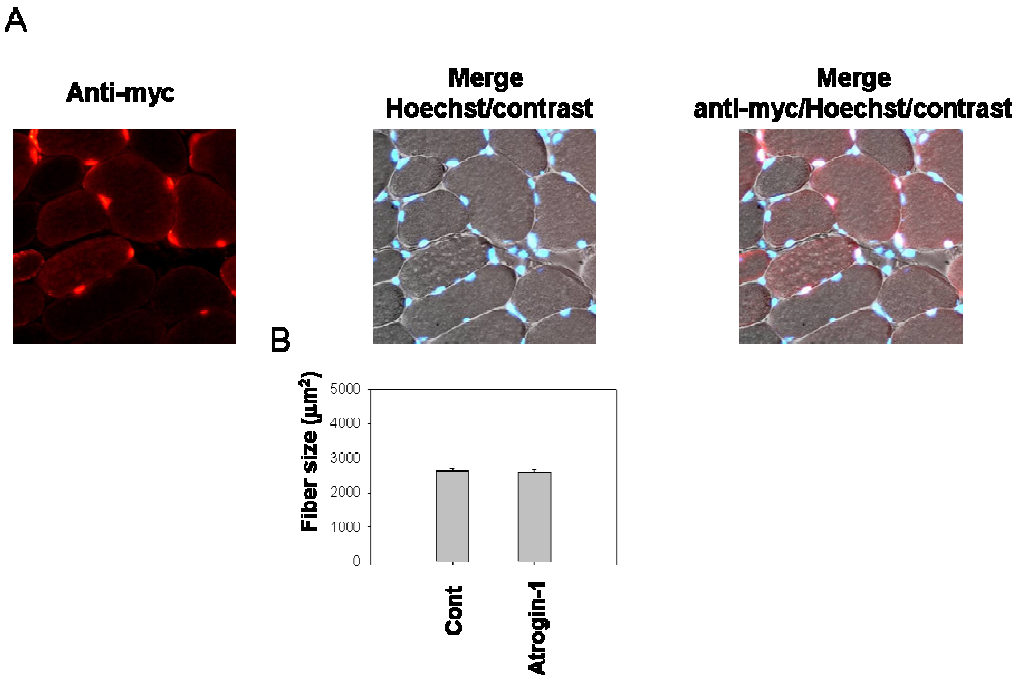


Figure 26: atrogin-1 overexpression in TA muscles does not affect myofiber size. A: The immuno-histochemestry for anti-myc (atrogin-1 tag) revealed the transfected fibers which did not differ in size compare to the surrounding untransfected fibers. **B**: The measure of cross-section area display that there is no difference of cross-sectional area between the fibers overexpressing atrogin-1 and untransfected ones

To investigate which are the other atrogenes that can be regulated by FoxOs and can contribute to muscle loss, we focused our attention to other atrogenes, which belong to the autophagy-lysosome system. Different models of muscle atrophy, including fasting and denervation, lead to an increased expression of different genes of the autophagic/lysosomal pathway. Interestingly, the most highly upregulated genes in atrophying muscles are two members of the Atg8 family, LC3, Gabarapl1, and two other genes involved in the regulation of autophagy, namely Bnip3 and Bnip3l. Atg8 proteins are required for vesicles formation and together with Bnip3 are among the upregulated atrogenes (LECKER, 2004; Mammucari, 2007). Evidence from our laboratory has showed that Akt actvation

suppressed the induction of autophagy-related genes during fasting. At this point we asked whether FoxO factors are directly involved in the transcriptional regulation of LC3 and Bnip3 genes.

The role of FoxO1 and FoxO3 on LC3 regulation

Initially we focused on LC3, the rate-limiting gene in autophagosome formation. In fact this protein has a fundamental role in the formation of autophagic vesicles and is lost when autophagosome fuses with lysosome. Therefore, LC3 protein level needs to be restored to maintain the autophagy flux during catabolic conditions. Bioinformatic analysis found three putative FoxO binding sites on the 5 kb LC3 promoter region (**Fig 27**).

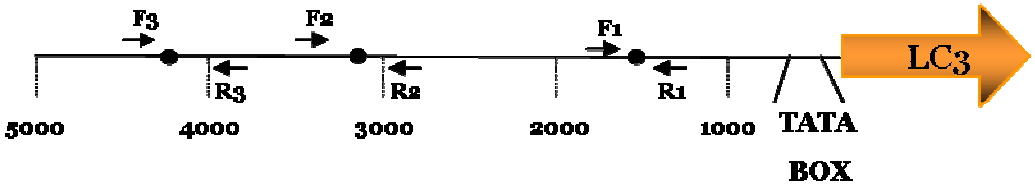


Figure 27: Analysis of 5 kb LC3 promoter by Genomatix bioinformatic tool. The dark circles represent putative FoxO binding sites. Three primer pairs were designed to include all the sites. Arrows represent the primers used for the ChIP analysis (**table 3**). F: primer forward; R: primer reverse.

To test the ability of FoxO1 and FoxO3 to bind these sites, ChIP experiments were performed on transfected or fasted TA muscles. PCR results showed that only the most proximal region is involved in the binding of FoxO transcription factors. In particular, overexpressed and endogenous FoxO3 stronlgy interacts with the site in region 1, while FoxO1 weakly binds this region of LC3 promoter (**Fig 28** and **Fig 29**).

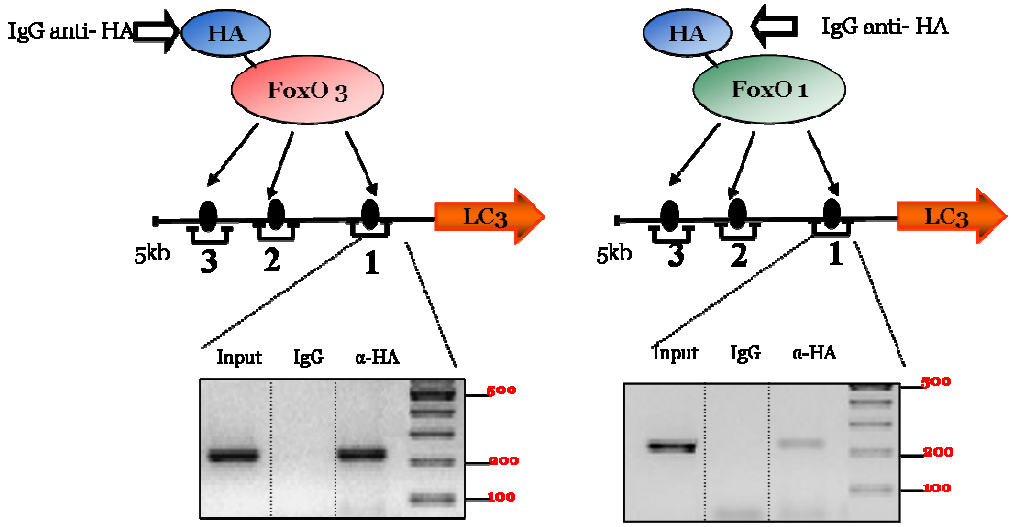


Figure 28: Analysis of FoxO1 and FoxO3 recruitment on LC3 promoter in transfected muscles. ca-FoxO1 and ca-FoxO3 were electroporated in tibialis anterior of adult CD1 mice. Chromatin from tibialis anterior was sonicated and immunoprecipitated with 5ug of anti-HA or 5ug of normal mouse IgG. The 2% agarose gels represent the most proximal region (-1608/-1379) of LC3, which has been found to recruit FoxOs. The left panel shows the PCR result of the ChIP of FoxO3 while the right panel reports the ChIP of FoxO1.

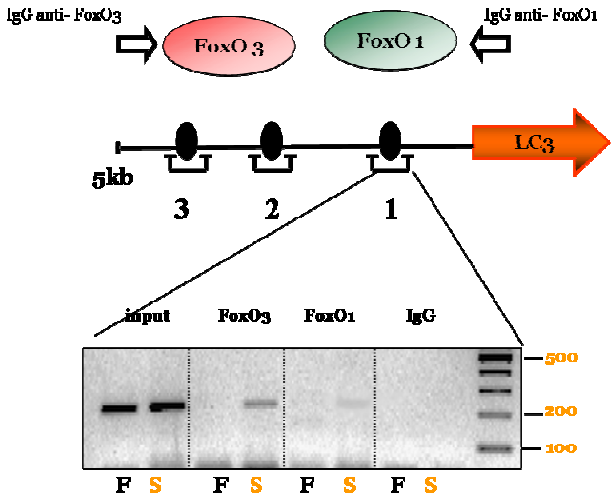


Figure 29: Analysis of endogenous FoxO1 and FoxO3 interaction with LC3 promoter in fed and starved muscles. For immunuprrecipitation we used 5ug of either anti-FoxO1 or anti-FoxO3 antibodies while in control experiments we used 5ug of normal mouse IgG. The 2% agarose gel shows the PCR analyses for region1 (-1608/-1379) of LC3 promoter. **F**=fed and S=starved.

To determine the functional role of FoxO3 binding, we used a Dual Luciferase Assay. First we cloned the studied region upstream the firefly gene. Then we disrupt the FoxO binding site by mutagenesis. We cloned both the wild type and the mutated region upstream a minimal SV40 promoter luciferase reporter vector. These constructs were transected in vivo into TA muscles together with c.a.FoxO3. The data from the luciferase assay showed that FoxO3 activates the wild type LC3 promoter, but not the promoter in which the FoxO site was mutated (**Fig 30**). In summary these data show that during starvation FoxO3 binds and activates the LC3 promoter.

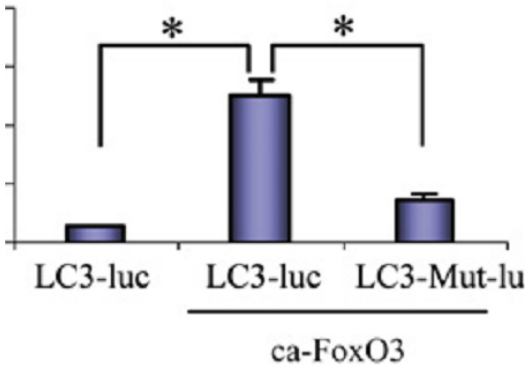


Figure 30 FoxO3 activates LC3 promoter-reporter, but not the promoter in which the FoxO binding site is mutated. A plasmid coding for the region of the LC3 promoter containing the proximal FoxO site or the mutated one was cloned upstram a minimal SV40 promoter of a luciferase reporter . The LC3 reporter was transfected into adult tibialis anterior muscle in presence or absence of ca-FoxO3. A renilla luciferase construct was co-transfected to normalize for transfection efficiency. Eight days later, firefly/renilla luciferase activity was determined. *$p < 0.001$. Error bars represent SD.

The role of FoxO1 and FoxO3 in Bnip3 regulation

We subsequently focused on Bnip3, which is among the most induced atrogenes in various types of muscle wasting and has been shown to control

autophagy in other cell systems (Hamacher-Brady et al, 2007; Tracy et al 2007). The overexpression of Bnip3 or Bnip3l is sufficient to induce autophagosome formation in cardiac and skeletal muscles (Mammucari et al.2007). Analysis of 5 kilo bases of Bnip3 promoter showed the presence of four FoxO binding sites (**Fig 31**)

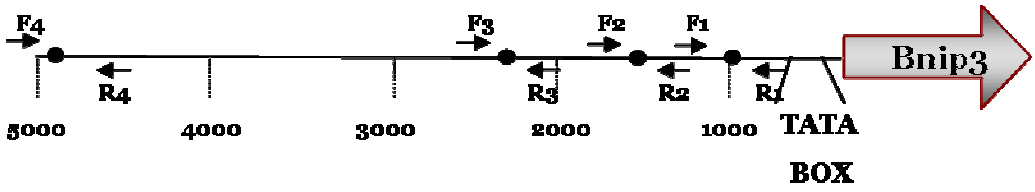


Figure 31: Bionformatics analysis of Bnip3 promoter. The 5 kilo bases of the Bnip3 promoter were analyzed using the Genomatix bioinformatic tool (http://www.genomatix.de/) in order to identify putative FoxO binding sites. The dark circles represent putative FoxO binding sites. Arrows represent the primers used for the ChIP analysis (**table 4**). F: primer forward; R: primer reverse.

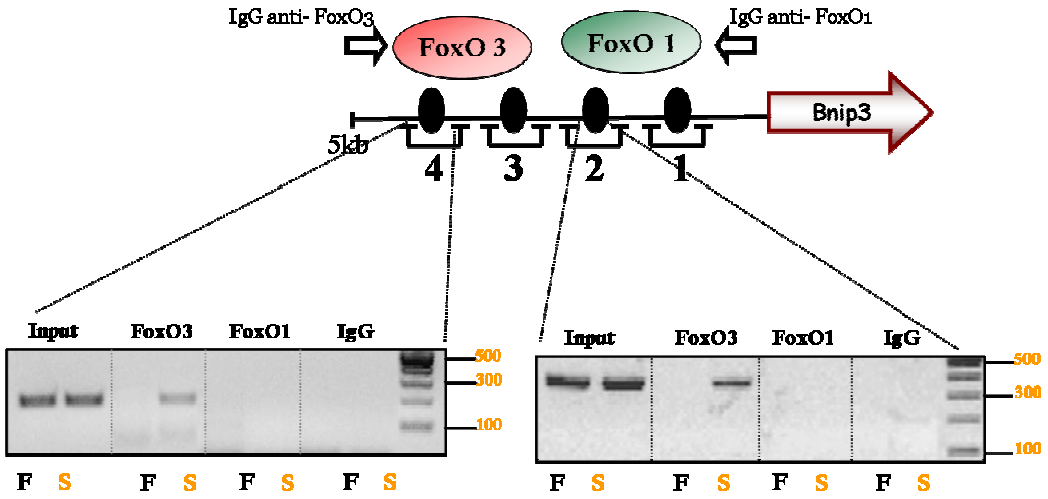


Figure 32: Analysis of FoxO1and FoxO3 binding on Bnip3 promoter in fed and starved muscles. Chromatin from fed (**F**) or starved (S) CD1 mice was immunoprecipitated with 5ug of anti-FoxO1 or anti-FoxO3 antibodies or with 5ug of normal mouse IgG. Here it has been reported the two sites involved in FoxO binding. The two sites found to recruit FoxOs are depicted The two panels show the PCR of region2 Fw2-Rv2 (-1236/-1029) and 4 (-4772/-4413).

To amplify these sites, we designed 4 specific primer pairs. Next we performed ChIP experiments using TA muscles of fed and starved mice. The results show that only FoxO3 interacts with chromatin of regions 2 and 4 (**Fig 32**).

To further investigate the role of FoxO3 in the regulation of Bnip3, we studied the effect of FoxO3 gain and loss of function on Bnip3 protein levels. We changed our strategy because we didn't succeed to clone the part of promoter which contains the two FoxO binding sites. FoxO3 overexpression increased Bnip3 protein levels in HEK 293 (**Fig 33**)

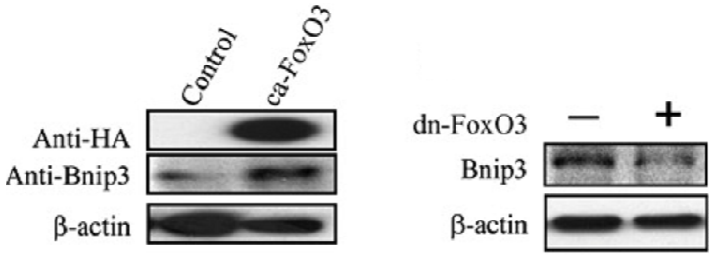


Figure 33: FoxO3 regulates the Bnip3 protein levels. The left panel show the western blot of transfected cells. FoxO3 gain of function caused the increase of Binip3 protein levels. The right panel show the reduction of Bnp3 protein when a dominant negative FoxO3 mutant was used.

Conversely, inhibition of FoxO3 activity, by expressing a dominant negative mutant, reduces Bnip3 protein levels in HEK293 cells.

Altogether the study on Bnip3 shows that, during starvation, endogenous FoxO3 binds the Bnip3 promoter at specific sites and induces an upregulation of Bnip3 protein.

Defining genes under FoxO control by ChIP on Chip approach:

In summary we demonstrated that FoxO3 binds the promoter of several atrophy related genes and it regulates their expression. Gain and loss of functions studies demonstrated (Mammucari, 2007) that the expression of atrogin1, LC3 and Bnip3 is essential to maintain the atrophy program, but not sufficient to induce it. We next wondered which are the target genes of FoxO3 responsible for mediating atrophy. To address this question we have chosen to extend ChIP technology to a genome-wide analysis (**Fig 34**). We decided to hybridize the immunoprecipitated chromatin with whole genomic promoters arrays.

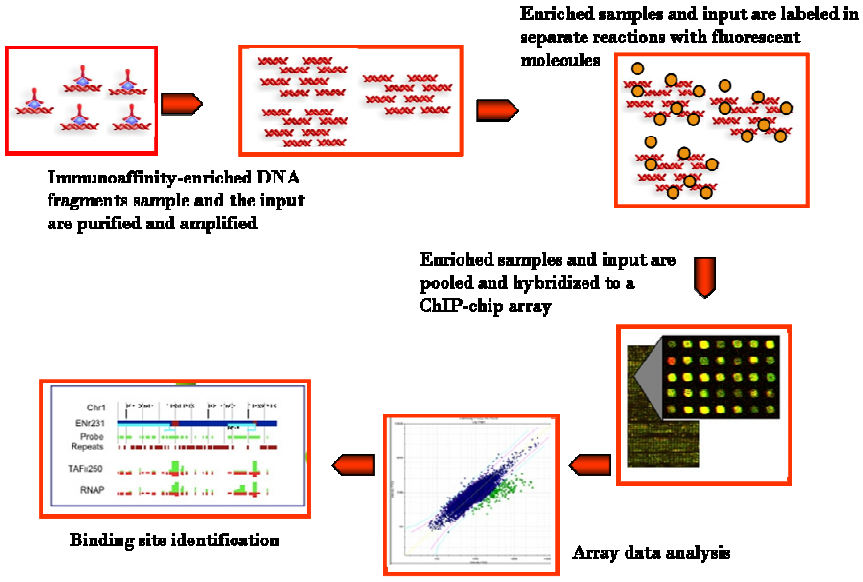
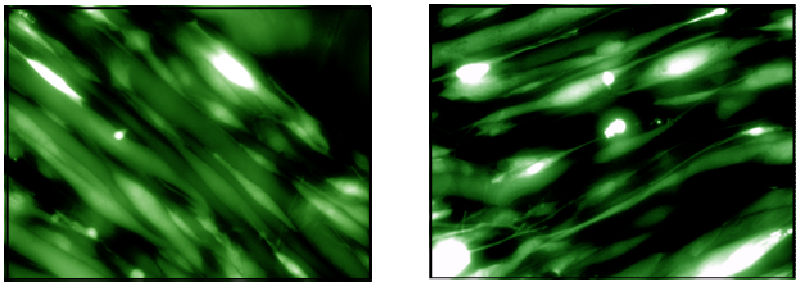


Figure 34: A schematic view of ChIP on Chip steps. ChIP-on-chip technique is a powerful approach to identify the chromatin which interacts with a specific protein. The immunoprecipitated DNA and its input are purified and amplified to get sufficient amount of DNA for the hybridization. The immunoprecipitated DNA in competition with the input DNA are hybridized with DNA microarrays, which contain non coding promoter regions of mouse genome. The statistical analysis of data show the promoters at which the studied protein was recruited.

Initial ChIP on chip tests were performed by using C2C12 myotubes infected with adenovirus expressing c.a.FoxO3. 48 hours after infection, myotubes, expressing FoxO3, started to shrink and to atrophy comparing to control myotubes which

were infected with mock construct. In fact the adenoviral plasmid that we have used for FoxO3 infection co-expresses a GFP protein under the control of an independent promoter. This vector allow to monitor the efficiency of infection and the morphology of the transfected cells (**Fig 35**).



GFP ca-FoxO3 GFP

Figure 35: FoxO3 overexpression caused a myotubes atrophy. Fluorescence microscopy of myotubes overexpressing pAdtrack-GFP(control) and pAdtrack caFoxo3 GFP. Myotube cultures were observed every 12 hours and photographed 48 hr after infection.

At this time cells were treated with formaldehyde, lysated, and immunoprecipitated with an antibody against HA. Several steps have been adjusted to get a sufficient amount of amplified chromatin for the hybridization step. In fact, a single ChIP sample does not provide enough DNA for the hybridization on promoters arrays. A general technique used to amplify the DNA obtained from the ChIP involves a ligation-mediated PCR (LM-PCR)**.** The quality and the quantity of the LM DNA was tested with RNA Nano Agilent Bioanalyzer and with NanoDrop spectrophotometer respectively. Chip arrays were performed by NimbelGen, which labelled the fragmented chromatin, hybridized the arrays and carried out a statistical analyses to determine the significant FoxO3 binding sites. The data revealed that FoxO3 binds chromatin of several genes implicated in various cell functions. Interestingly, eight atrophy-related genes showed a FoxO3 interaction on their regulatory regions (**Fig 36**).

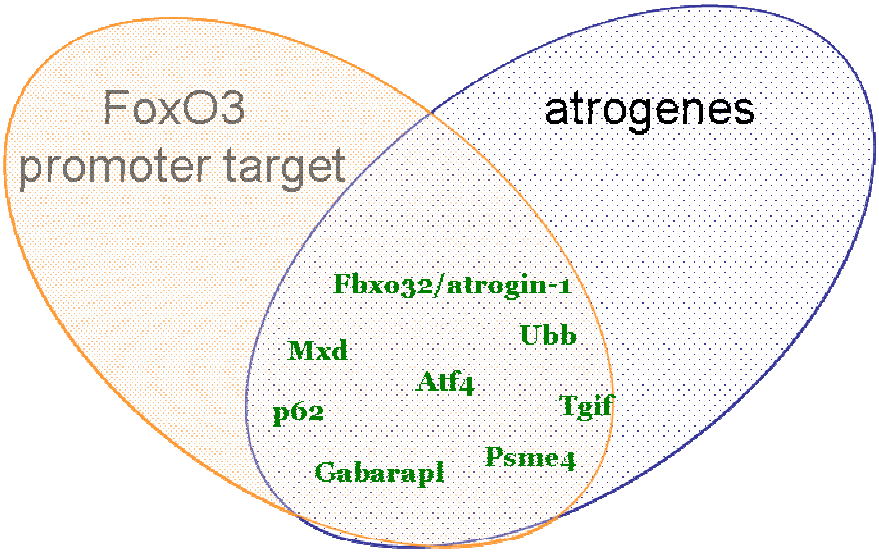


Figure 36: A schematic representation of the ChIP on chip result in C2C12 overexpressing ca-FoxO3. FoxO3 binds < 1000promoter genes that include eight atrogenes promoter. To understand the functional role of FoxO3 interaction we have started to analyse the mRNA levels of 6 of them in C2C12 overexpressing empty adenovirus vector or pAdca-FoxO3 by real time PCR.

Next we tested whether these interactions have a physiological relevance in controlling gene expression. We monitored the mRNA levels of these atrogenes in myotubes infected with FoxO3 or control virus.

Beside atrogin-1, FoxO3 interacts and induced expression of Tgif (transforming growth interacting factor), Pmse4 (a proteasome activator), p62 (a protein which clears ubiquitinated proteins via autophagy system) and Gabarapl, (one of three mammalian homologs of Atg8). Interestingly, FoxO3 represses the expression of ATF4 (activating transcription factor 4) (Lecker et al., 2006b; LECKER et al., 2004) (**Fig 37**). These results suggest that FoxO3, in C2C12 myotubes, is able to control the expression of 5% of the atrophy-related genes.

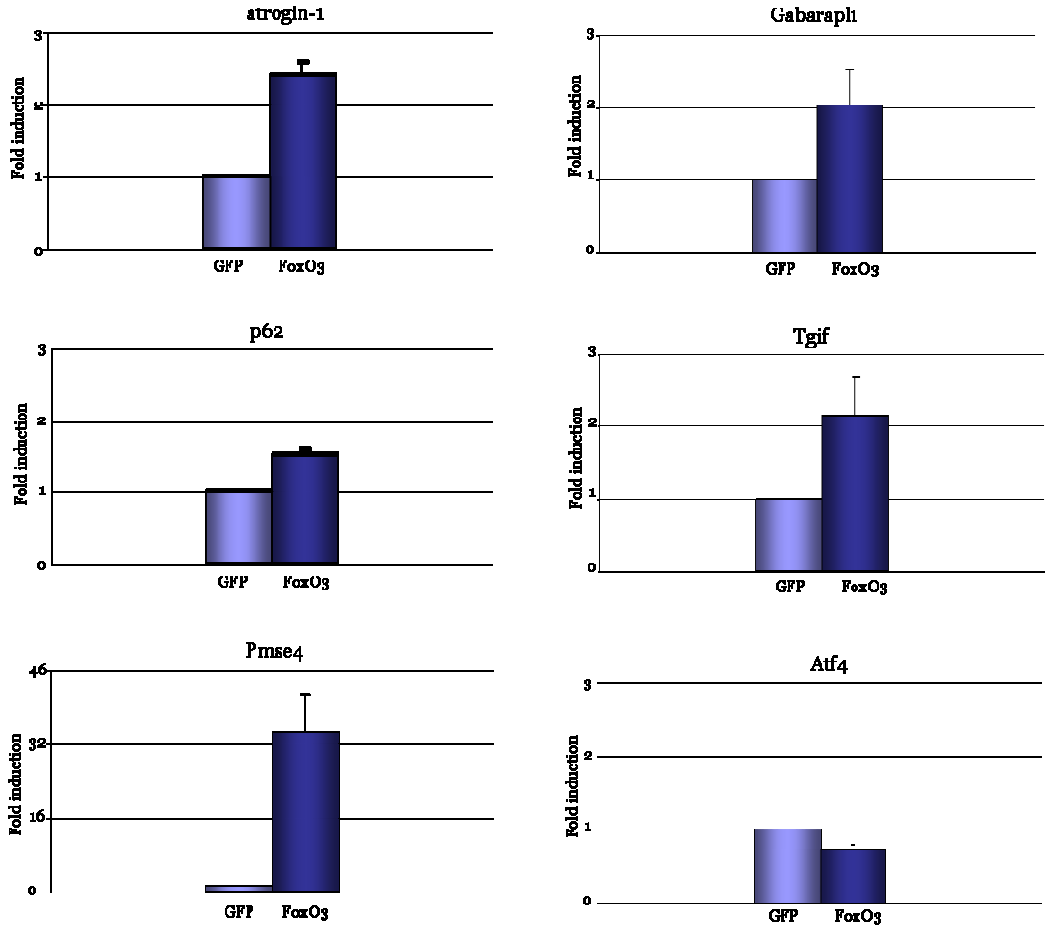


Figure 37: FoxO3 recruitment affect the expression of several atrogenes. The mRNA was extracted from C2C12 myotubes infected with pAdtrack/GFP (control) and pAdtrack-caFoxO3/GFP. The graphs show the quantitative real-time PCR (Materials and Methods) of different atrogenes which have been found by ChIP on Chip approach to be potential under FoxO3 regulation (**table primers real time**). The data were normalized to β-actin. The vertical bars indicates the standard error.

DISCUSSION:

Muscle loss occurs in many diseases including immobilization, disuse, denervation, sepsis, burn injury, cancer, AIDS, diabetes, renal or cardiac failure, and microgravity. All these different conditions from ageing sarcopenia to cancer cachexia, diabetes, denervation, immobilization and microgravity atrophy have in common an increased activation of protein degradation systems (McKinnell and Rudnicki, 2004). However the mechanisms of such protein loss was unclear until the beginning of this century. The critical contribution in understanding muscle atrophy comes from the pioneering studies on gene expression profiling performed independently by groups of AL Goldberg and DJ Glass (Bodine et al., 2001a; LECKER et al., 2004). The idea to compare gene expression in different models of muscle atrophy leads to the identification of a subset of genes that are commonly up- or down-regulated in atrophying muscle. Since all the diseases used for the microarray experiments (i.e. Diabetes, Cancer cachexia, chronic renal failure, fasting and denervation) have muscle atrophy in common, the commonly up- or down-genes are believed to regulate the loss of muscle components and are called atrophy-related genes or atrogenes. Together these findings indicate that muscle atrophy is an active process controlled by specific signaling pathways and transcriptional programs. Furthermore the two most induced genes are two novel muscle-specific ubiquitin ligases, atrogin-1/MAFbx and MuRF1, that are up-regulated in different models of muscle atrophy and are responsible for the increased protein degradation through the ubiquitin-proteasome system. In fact, knockout mice for either atrogin-1/MAFbx or MuRF1 are partially resistant to denervation atrophy. Thus, up to now these two genes are actually the best markers for muscle atrophy and could be considered as master genes for muscle wasting. A subsequent crucial step was the identification of the signaling pathways which regulate the expression of these two ubiquitin ligases. Our and other studies define a role of Akt-FoxO pathway (Sandri et al., 2004) (Sandri et al., 2004; Stitt et al., 2004). Akt phosphorylates FoxOs, promoting the export of FoxOs from the nucleus to the cytoplasm. As predicted, the reduction in the activity of the Akt pathway observed in different models of muscle atrophy results in decreased levels of phosphorylated FoxO in the cytoplasm and a marked

increase of nuclear FoxO protein. The translocation and activity of FoxO members is required for the up-regulation of atrogin-1/ MAFbx and MuRF1, and FoxO3 was found to be sufficient to promote atrogin-1/MAFbx expression and muscle atrophy when transfected in skeletal muscles *in vivo.* Accordingly, FoxO1 transgenic mice showed markedly reduced muscle mass and fiber atrophy, further supporting the notion that FoxO is sufficient to promote muscle loss (Kamei et al., 2004). Simultaneously to the FoxO story another regulatory mechanism has been published. The IKK-NFkB pathway was found to be sufficient to control MuRF1 expression, but not atrogin-1 expression, and partially required for denervation-induced muscle atrophy. However from 2001, the year of the discovery of atrogin-1 and MurF1, and from 2004, the year of publication of FoxO and NFkB regulatory mechanisms, no major advances in atrophy-related regulators appeared in literature. It is important to notice that several other genes among the atrophy-related genes are of potential interest including genes coding for lysosomal protease, transcription factors, regulators of protein synthesis and enzymes of metabolic pathways, but their particular role in muscle wasting is still unknown. Furthermore it is unlikely that just two ubiquitin ligases are in charge for the total protein degradation that occurs during muscle loss. We have recently found that also autophagy-lysosome system can contribute to organelle and protein removal (Mammucari et al, 2007). However we need to search new players and define both the role and the regulation of the still unknown and already published atrophy-related genes to get an almost final picture of the changes occurring during muscle loss. Moreover all FoxOs recognize and bind the same DNA sequence, thus those factors should control the same genes. However our functional promoter assay and results from FoxO1 transgenic mice (Kamei et al., 2004) show that FoxO1 is not a major regulator of atrogin-1 expression while FoxO3 is critical for expression of this ubiquitin ligase. For these seasons we decided to apply ChIP technology to our in vivo models of muscle atrophy in order to dissect which regulatory regions of atrogenes recruit a certain transcription factor and to understand whether this binding is functional for chromatin remodelling and for transcription activation (Orlando, 2000). Many technical problems have been solved and starting form in vitro system we succeed to perform ChIP in an in vivo contest. Our experimental conditions are able to detect both FoxO1 and FoxO3

binding on a commonly regulated genes like GADD45α. We could identify which of the 14 potential binding sites on atrogin-1 promoter were functional relevant for FoxO3 recruitment and for promoter activation. Indeed during starvation, FoxO3 binds only three sites. These three sites show a transcriptional active conformation of chromatin since histones are hyper-acetylated. Moreover the promoter activity assay confirmed that atrogin-1 is specifically regulated by FoxO3 and not by FoxO1. It is intriguing the fact that FoxO1 is not or is poorly recruited on the different promoters, studied so far, of atrophy-related genes. However these findings strongly suggest that different FoxOs can regulate different set of genes. Therefore there have to be a regulatory mechanism which discriminate the recruitment of a specific FoxO on a promoter and this is not caused by the nucleotide sequence which is the same for all the FoxO family members. To support this hypothesis and to define the genes regulated by each FoxO we decided to extend the ChIP approach to a genome wide analysis. Since the strategy to start the experiments using cell culture system to set up important and crucial steps of the methodology was successful we performed the genome-wide analysis on FoxO3 infected myotubes. This technique is based on the combination of ChIP and whole genome microarray analysis (ChIP on Chip). ChIP on Chip strategy have been applied to study both the binding of specific transcription factors to genome and the structural components of chromatin, including histone acetylation, methylation, ubiquitination and pCG enrich island. In our model we found that FoxO3 can interact with many regions of genome which include promoters of 9 atrophy related genes. These genes are implicated in different proteolytic systems and in transcriptional regulation. Beside atrogin1, the microarray results indicate that FoxO3 binds two other genes of ubiqitin proteasome system, Pmse4 and UBB. The first one, named also PA200, is a nuclear component of proteasome. Psme4 activates proteolysis by binding the 20S proteasome and is also involved in DNA double-strand breaks repair (DSBs) (Ustrell et al., 2002). Interestingly GADD45 another enzyme of DNA repairing system is also controlled by FoxOs (Ramaswamy et al., 2002; Tran et al., 2002a) suggesting that a certain amount of DNA damage can occur during muscle loss. FoxO3 binds the promoter of the a polyubiquitin B gene which at is strongly induce during skeletal muscle atrophy, cardiac atrophy, hypertrophy and

hypoxemia.(UBB) (Razeghi et al., 2006). Mutation that causes loos of function of UBB induces accumulation of the mutated UBB (UBB+1) in protein aggregates. A recent study has showed that p62 co-localizes with UBB+1 in the protein aggregates of myofibrillar myophaties Olive, van Leeuwen et al. 2008). P62 is a scaffold protein that is able to bind polyubiquitin chains and is under FoxO3 control. This protein can interact with LC3, and co-localized into autophagosomes. Another FoxO3 target gene is Gabarapl-1, which is a mammalian Atg8 homolog like GATE 16 and LC3 (Nemos, et al, 2003). Thus UBB, p62 and Gabarapl1 are all FoxO3 target genes, therefore it is possible that FoxO3 is implicated in coordinating the autophagy system to clear protein aggregates. Interestingly we did not find FoxO3 interactions with LC3 and Bnip3, which in vivo have been found to be under FoxO3 control (Mammucalri et al, 2007; Zhao et al. 2007). The lack of any significant binding may be caused by the cell culture system. In fact C2C12 myotubes can reach a level of differentiation similar to a newborn muscle and therefore the chromatin conformation on Bnip3 and LC3 promoter regions can be different from that one present in an adult muscle. A compact chromatin may be sufficient to inhibit FoxO3 binding even in condition of overxpression. For this reason we have now started to repeat these studies in vivo by sequencing the chromatin which immunoprecipitates with endogenous FoxOs (ChIP sequencing). This approach has several advantages on ChIP on Chip which include a more sensibility. In fact one of the major problem of the in vivo experiments is that the amount of fragmented chromatin obtained form adult muscles is too low for a successful hybridization on promoter arrays.

There are few transcription factors among the atrophy-related genes potentially regulated by FoxO3 which are Mdx, Tgif and Atf4. The first one is called Max demonization protein and it acts as transcriptional repressor antagonizing the c-Myc function. Interestingly Aurelio and co-worker have found that FoxO binds myc promoter and negatively regulates its expression.(Cell Metab 2008). Tgif (Trasforming growth factor β-induced factor) is a negative regulator of TGF-β signaling acting as a repressor of Smads transcription factors (LECKER et al., 2004). The reported Tgif action is similar to Ski, another Smad repressor. Interestingly Ski transgenic mice show hypertrophic muscles. Atf4 is related to unfolded protein response and it regulates the expression of genes involved in

oxidative stress, amino acid synthesis, differentiation and angiogenesis Although ATF4 belongs to the list of the up-regulated atrogenes, our RT-PCR results show that FoxO3 does not increase and seems to repress Atf4 expression.

In conclusion we have set up an approach which have been used to characterized the FoxO1 and 3 binding on different atrophy-related genes and to better understand the mechanisms of muscle atrophy. We have further extended this technique to the whole genome analysis which will enable us to identify which genes are controlled by specific transcription factors and to find new critical pathways involved in the control of muscle mass. These applications will be crucial to identify new potential drug target and to set up alternative therapies to combat muscle loss.

LEGEND OF ABBREVIATIONS:

bp = base pair;

BSA = albumin bovine serum;

°C = centigrade;

cDNA = DNA complementary to RNA;

DMEM = media for cell culture;

dNTPs = deoxyribonucleoside triphosphates;

EGTA= ethylene glycol tetraacetic acid)

EDTA= ethylenediaminetetraacetic acid

FBS = fetal bovine serum;

g = grams;

HCl = hydrochloric acid;

KCl = potassium chloride;

HEPES =4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HS = horse serum;

M = molar;

min = minutes;

ml = milliliters;

ms = milli seconds;

NaAc = sodium acetate;

NaCl= sodium chloride;

NaOH = sodium hydroxide;

NaHCO3 = sodium bicarbonate;

PCR = polymerase chain reaction;

PBS = Phosphate buffered saline

rpm = revolutions per minute;

r. t. = room temperature;

SDS = sodium dodecyl sulfate;

sec = seconds;

TA = tibialis anterior;

TBS = Tris Buffered Saline

µl = microliters

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