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CICLO XXI

ROLE OF THE TRANSCRIPTION FACTOR MRF4 IN ADULT SKELETAL MUSCLE

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RUOLO DEL FATTORE DI TRASCRIZIONE MRF4 NEL MUSCOLO SCHELETRICO ADULTO

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ABBREVIATIONS

ActRIIB	Activin type II B Receptor
bHLH	basic Helix Loop Helix
eIF-2B	eukaryotic Initiation Factor 2B
eIF-4E	eukaryotic Initiation Factor 4E
FKBP12	FK506 Binding Protein 12
FLRG	Follistatin-Related Gene
FoxO	Forkhead box O
GASP1	Growth and differentiation factor-Associated Serum Protein-1
GDF-8	Growth Differentiation Factor 8
GSK3 β	Glycogen-synthase kinase 3 β
HAT	Histone acetyltransferases
HDAC	Histone Deacetylases
hSGT	human Small Glutamine-rich Tetratricopeptide repeat-containing protein
IGFI	Insulin-like Growth Factor I
IGFR	IGFI Receptor
IRS 1	Insulin Receptor Substrate 1
MADS	MCM1, agamous, deficiens, serum response factor
MAPK	Mitogen-Activated Protein Kinase
MCK	Muscle Creatin Kinase
Mef2	Myocyte Enhancer Factor 2
MRF	Muscle Regulatory Factor
MuRF1	Muscle Ring-Finger protein 1
MyHC	Myosin Heavy Chain
NFAT	Nuclear Factor of activated T-cells
PDK1	Phosphoinositide-Dependent protein Kinase 1
PHAS1	Phosphorylated Heat- and Acid-Stable protein 1
PI3K	Phosphoinositide 3-kinases
PIP2	Phosphatidylinositol(4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PP2A	Protein Phosphatase 2A
PTEN	Phosphatase and Tensin homologous on chromosome 10
SHIP	SH2-domain-containing Inositol Phosphatase
TGF- β	Transforming Growth Factor- β
TnI	Troponin I
Tsc1-2	Tuberous Sclerosis Complex 1 and 2

ABSTRACT

Myogenesis is a dynamic process in which mononucleated undifferentiated myoblasts first proliferate, then withdraw from the cell cycle and finally differentiate and fuse to form the multinucleated mature muscle fibers. This process is controlled by members of a family of muscle-specific basic helix-loop-helix (bHLH) proteins that, in concert with members of the ubiquitous E2A and myocytes enhancers factor 2 (MEF2) families, activate the differentiation program by inducing transcription of regulatory and structural muscle specific gene. The MRF proteins contains one or two transactivation domain, a conserved basic DNA-binding domain essential for sequence-specific DNA binding, and an HLH motif required for heterodimerization.

MRFs specific knockout studies suggest that MyoD and Myf5 are required for commitment to the myogenic lineage, whereas myogenin plays a critical role in the expression of the terminal muscle phenotype; Mrf4 partly subserves both roles.

In the adult skeletal muscle, the expression of MRFs considerably changes: Myf5 is not expressed in adult fibers, MyoD and myogenin are expressed at low levels and respectively in fast muscle and in slow muscle, Mrf4 is the only muscle regulatory factors expresses at high levels in adult skeletal muscle, but its role is still unknown.

The aim of this work is study the expression and the physiological role of Mrf4 in the adult skeletal muscle.

We demonstrate that Mrf4 is similarly expressed at mRNA and protein levels in the slow soleus muscle and in the fast EDL muscle, but this transcription factor has a predominantly nuclear localization in soleus and a predominantly cytosolic localization in EDL. We also demonstrate that Mrf4 expression is activity dependent using two experimental models: elettrostimulation and denervation (inactivity condition). When EDL muscles were stimulates with a slow pattern, Mrf4 translocates to the nucleus, whereas with a fast pattern Mrf4 remains in the cytoplasm. Moreover, after denervation Mrf4 accumulates in to the nucleus. This observation suggests that MRF4 may undergo nucleo-cytoplasmic

shuttling, as in the case of other transcription factors, but not describes for the MRFs.

To get further insight about the function of Mrf4 in adult muscle we examined the effect of Mrf4 overexpression and knockdown using an in vivo transfection approach in adult rat skeletal muscles. We evaluated if Mrf4 is involved in the regulation of two features of muscle phenotype: the muscle growth and the fiber type specification.

Muscle growth: we demonstrate that Mrf4 silencing in adult and regenerating muscles induces hypertrophy. On the other hand, the overexpression of Mrf4 cDNA in regenerating muscles, but not in the adult muscles, causes a decrease of cross sectional area of transfected fibers. Moreover the MRF4 knockdown prevents denervation atrophy. This data suggest that Mrf4 acts as a negative regulator of muscle fiber growth.

Fiber type specification: we used two luciferase reporter under control of the MyHC slow (MyHC slow-Luc) and the MyHC 2B (MyHC 2B-Luc) promoter. We demonstrate that Mrf4 knockdown in adult skeletal muscle inhibits the activity of MyHC slow-Luc and induces the activity of MyHC 2B-Luc; on the other hand, this reporter is activated by Mrf4 overexpression. We have also study the effect of Mrf4 silencing on endogenous gene: we demonstrate that Mrf4 knockdown blocks the expression of endogenous MyHC slow. This data suggest that Mrf4 is involved in the induction and in the maintenance of slow gene program.

SOMMARIO

Lo sviluppo del muscolo scheletrico è controllato da una famiglia di fattori trascrizionali, chiamati Muscle Regulatory Factors (MRFs), i cui membri sono MyoD, Myf5, Mrf4 e miogenina. Questi fattori trascrizionali sono in grado di dare inizio al programma miogenico, convertendo cellule non muscolari in derivati miogenici.

Gli MRFs appartengono alla famiglia di proteine bHLH (basic helix-loop-helix) e presentano motivi strutturali caratteristici: uno o due domini di transattivazione, un dominio basico di legame al DNA molto conservato e la regione HLH, necessaria per l'eterodimerizzazione. È stato osservato in vitro ed in vivo che i fattori MRF sono in grado di eterodimerizzare con un'altra famiglia di proteine bHLH, le proteine E, e di legarsi al DNA su una sequenza consenso specifica, detta E box (CANNTG). Questo legame permette l'attivazione trascrizionale di specifici geni muscolari, come α -actina, MCK (Muscle Creatin Kinase) e troponina I.

L'analisi di diversi knockout degli MRFs ha permesso di definire ruoli diversi nello sviluppo muscolare per i vari membri della famiglia. In particolare, Myf5 e MyoD sono induttori del programma miogenico, mentre miogenina ha un'azione fondamentale nelle fasi successive del differenziamento dei mioblasti. Mrf4 è l'unico fattore ad essere coinvolto sia nella fase iniziale di induzione, che in stadi avanzati del differenziamento miogenico.

Nel muscolo scheletrico adulto l'espressione degli MRFs viene mantenuta, ad eccezione di Myf5. In particolare, MyoD e miogenina sono espressi a livelli bassi, e sono più abbondanti rispettivamente nelle fibre di tipo rapido e di tipo lento. Mrf4 è l'unico dei quattro fattori trascrizionali a mantenere livelli di espressione molto elevati nel muscolo scheletrico adulto, ma la sua distribuzione in diversi tipi di muscoli (rapidi e lenti) ed il suo ruolo fisiologico non sono stati ancora caratterizzati.

Questo progetto ha avuto come obiettivo principale quello di definire il profilo di espressione e il ruolo di Mrf4 nel muscolo scheletrico adulto.

Abbiamo pertanto analizzato l'espressione di Mrf4 in un muscolo tipicamente lento, il soleo, ed in un muscolo rapido, l'extensor digitorum

longus (EDL). I nostri risultati indicano che la sua espressione è paragonabile nei due tipi di muscoli, sia a livello di mRNA che a livello di proteina. Abbiamo invece messo in luce delle differenze nella localizzazione di Mrf4, che risulta essere prevalentemente nucleare nel soleo, mentre l'EDL presenta solo alcuni nuclei positivi ed una marcatura diffusa nel citoplasma. Per chiarire se l'espressione di Mrf4 fosse controllata dall'attività nervosa, ci siamo serviti di due modelli sperimentali in vivo su ratto: l'elettrostimolazione e la denervazione (condizione di inattività). Mrf4, in seguito a stimolazione di tipo lento, trasloca nei nuclei, mentre rimane nel citosol se stimolato con un pattern di tipo rapido. In seguito a denervazione Mrf4 sia accumula nei nuclei sia nel soleo che nell'EDL. Queste osservazioni suggeriscono che Mrf4 possa andare incontro ad un fenomeno di shuttling nucleo-citoplasmatico, fenomeno comune a vari fattori trascrizionali ma non descritto nel caso degli MRFs.

Per comprendere il suo ruolo fisiologico nel muscolo scheletrico adulto, abbiamo effettuato esperimenti di iperespressione e di silenziamento genico. Abbiamo valutato se Mrf4 potesse essere coinvolto nella regolazione di due aspetti del fenotipo muscolare: la crescita e la specificazione del tipo di fibre.

Regolazione della crescita muscolare: abbiamo dimostrato che il silenziamento genico di Mrf4 in muscoli adulti e rigeneranti induce ipertrofia delle fibre trasfettate; per contro l'iperespressione di Mrf4 in muscoli rigeneranti, ma non adulti, causa una diminuzione dell'area delle fibre trasfettate. Abbiamo inoltre dimostrato che Mrf4 previene l'atrofia indotta da denervazione. Questi dati suggeriscono che Mrf4 agisce come regolatore negativo della crescita.

Specificazione del tipo di fibra: abbiamo utilizzato due reporter luciferasi sotto il controllo dei promotori della catena pesante della miosina lenta (MyHC slow-Luc) e della miosina rapida 2B (MyHC 2B-Luc). Abbiamo dimostrato che il silenziamento genico di Mrf4 in muscolo scheletrico adulto inibisce l'attività del reporter MyHC slow-Luc mentre induce quella del reporter MyHC 2B-Luc. Al contrario l'iperespressione di Mrf4 con il promotore della miosina rapida induce diminuzione

dell'attività, mentre non modifica l'attività della miosina lenta. Abbiamo inoltre analizzato l'effetto del silenziamento di Mrf4 su geni endogeni in muscolo rigenerante e abbiamo dimostrato che il silenziamento genico di Mrf4 blocca l'espressione della miosina lenta indotta dal nervo. Questi esperimenti dimostrano quindi che Mrf4 attiva il programma genico lento e inibisce quello rapido, contribuendo ai meccanismi di induzione e di mantenimento dei programmi genici coinvolti nella specificazione del tipo di fibra.

INTRODUCTION

1. Skeletal muscle growth and hypertrophy

1.1. Muscle growth during development

Skeletal muscle is the most abundant tissue in the vertebrate body. Animals have evolved individual muscles specialized to perform different types of movements. Each muscle is comprised of a variable number of contracting fibers, formed by the fusion of a large number of myogenic progenitors and thus containing up to many thousands of nuclei. Fibers are highly heterogeneous for different anatomical, physiological and biochemical features. Most of vertebrate muscles are composed of variable proportions of different (fast or slow, glycolytic or oxidative) fiber types determining the appropriate force, speed and duration of contraction. These differences derives from the contribute of distinct classes of myogenic progenitors appear to be involved in muscular patterning and growth. All the skeletal muscles in vertebrate body, with the exception of some craniofacial muscles, derive from progenitors present in the somites (Christ and Ordahl, 1995). Somites are transient mesodermal units, which form at embrionic day 8.75 (E8.75) in the mouse in a cranio-caudal succession by segmentation of the paraxial mesoderm on both sides of the neural tube. Each newly formed somite rapidly differentiates into a ventral sclerotome and a dorsal dermomyotome from which myogenic precursors originate. These myogenic precursors cells give rise to terminally differentiated, mononucleated muscle cells (myocytes) of the primary myotome. Primary myotome formation is a multistep process in which precursors translocate from the dermomyotome to a ventrally located domain where they elongate along the axis of the embryo to span the entire somite length. Only a fraction of myogenic progenitors terminally differentiate during primary myotome formation.

Skeletal muscle is established in successive distinct, though overlapping steps involving different type of myoblasts (embryonic, fetal

myoblasts and satellite cells). The continued growth of muscles that occurs during late embryonic (E10.5– 12.5), fetal (E14.5–17.5) and postnatal life was recently attributed to a population of muscle progenitors already present at embryonic stage ((Kassar-Duchossoy et al., 2005; Relaix et al., 2005; Schienda et al., 2006). These skeletal muscle progenitor cells arise in the central part of the dermomyotome, co-express Pax3 and Pax7 and can differentiate into skeletal muscle fibers during embryogenesis or possibly remain as a reserve cell population within the growing muscle mass during peri- and postnatal stages (Fig.1). The analysis of the Pax3/Pax7 double knockout mice has demonstrated that all of the cells of the myogenic lineage (with the exception of myotomal cells) may be derived from a Pax3/Pax7 positive population of myogenic progenitors resident in the central part of the dermomyotome. At around E11 in the mouse, embryonic myoblasts invade the myotome and fuse into myotubes, probably incorporating the initially mononucleated myocytes of the early myotome, although this has not been formally demonstrated.

This embryonic phase appears to depend upon the myogenic factor Mrf4 since it is maintained in the Myf5 null embryo but is disrupted in the Myf5-Mrf4 double mutant embryo (Kassar-Duchossoy et al., 2004). During primary myogenesis muscles consist of small number of myotubes that progressively increase in size and get a characteristic round shape in transverse section.

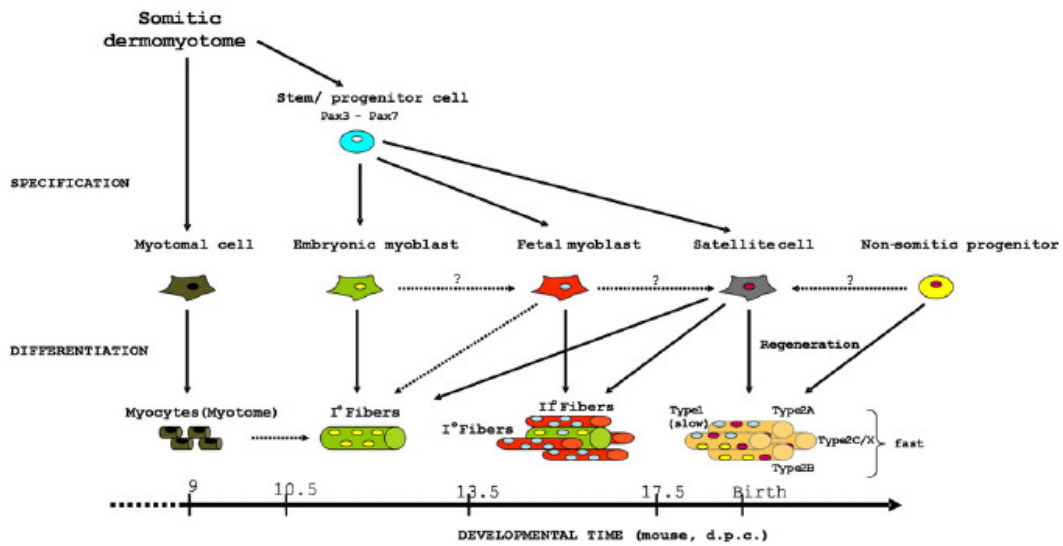


Fig.1 Proposed lineage scheme for skeletal muscle. The somitic dermomyotome is the origin of the myotomal cells, which differentiate into the myocytes of the early myotome. Pax3/Pax7 positive cells identified in the dermomyotome release muscle precursors during development (embryonic, fetal myoblasts and satellite cells). Embryonic and fetal myoblasts give rise to 1° and 2° fibers, respectively. Satellite cells appear at the end of gestation and are responsible for postnatal growth and regeneration. Other non-somatic progenitors are involved in muscle regeneration, although their role in non-pathological conditions remains largely unexplored. Abbreviations used: d.p.c. days post coitum. An indicative timing of murine development is depicted. Adapted from (Cossu and Biressi, 2005)

A new wave of myogenesis takes place between E14.5 and E17.5. This phase is called secondary myogenesis and involves fusion of fetal myoblasts either with each other to give rise to secondary fibers (originally smaller and surrounding primary fibers) or also with primary fibers (Dunlison et al., 1999; Evans et al., 1994, Duxson, 1989 #394). It is only at the end of this phase that satellite cells can be morphologically identified as mononucleated cells lying between the basal lamina and the fiber plasma membrane. During peri- and postnatal development, satellite cells divide at a slow rate and a large part of the progeny fuse with the adjacent fiber to contribute new nuclei to growing muscle fibers (whose nuclei cannot divide), so that the majority of the nuclei of a mature muscle are presumably derived from satellite cells. At the end of postnatal growth, satellite cells enter a phase of quiescence but can be activated if the muscle tissue is damaged or in response to further growth demands. In these cases satellite cells undergo a number of cells divisions producing fusion competent cells that can either fuse with damaged fibers or form new ones,

and other cells that return to quiescence, thus maintaining the progenitor pool.

Secondary fibers form initially at site of innervation of the primary fiber and are surrounded by the same basal lamina as the primary fiber on which they lie (Duxson et al., 1989). The secondary myotubes remain attached for a short period to primary fibers and subsequently elongate and become independent fibers, which can be distinguished from primary fibers by their relative small size (Kelly and Zacks, 1969). The innervation of muscles starts while fibers are still forming. Each muscle fiber is initially innervated by multiple axons, all but one of which are subsequently eliminated. Postnatally, all the muscle fibers that remain contacted by the axon branches of an individual motor neuron are of the same type. The mechanisms whereby nerves become associated with fast or slow muscle fibers are currently unknown, but it has been generally assumed that the nerve plays a role in generating fiber type diversity. In the absence of functional innervation the formation of muscle fibers is impaired, leading to a reduction in the total number of fibers, with primary fibers being in general less affected than secondary. Chronic denervation leads to eventual degeneration of both primary and secondary fibers (McLennan, 1994; Wigmore and Evans, 2002).

1.2.Hypertrophy in the adult skeletal muscle

At the end of embryonic development, skeletal muscles are completely formed, but their growth continues during the first few weeks after birth. However the number of fibers in a muscle is fixed at birth or soon after and the extensive muscle growth which occurs in young animals is due to hypertrophy of the fibers (Enesco and Puddy, 1964; Maggs et al., 2000; Moss, 1968). Skeletal muscle hypertrophy is defined as an increase muscle mass and experiments of DNA labeling have shown that this hypertrophy is due to the fusion of satellite cells daughter with the adjacent fiber (Moss and Leblond, 1971).

Once they have reached their final size in adult animals, skeletal muscle fibers maintain their diameter and length relatively constant in

normal loading conditions. Nevertheless, skeletal muscle is one of the more plastic tissue of the body and it is able to adapt to different working demands and to variations in motor neuron activity by performing radical changes in both its morphological and molecular properties. The nerve function is absolutely crucial in regulating the muscle phenotype since the activity of the muscle directly depend on the activity of the motor neuron. It is well known that in conditions of increased neuronal activity, as during exercise, functional overload and electrostimulation, muscle undergoes hypertrophy whereas in conditions of nerve-silencing, both physiological (rest, inactivity) or pathological (nerve-cuffing, spinal cord injury, neurodegenerative disease and denervation), muscle fibers undergo extensive and relatively rapid atrophy.

It is widely accepted that in adulthood muscle fiber size results from a balance between protein synthesis and protein degradation, and signalling pathways that control protein synthesis appear to play a major role in adult muscle hypertrophy.

1.2.1. Pathways mediating skeletal muscle hypertrophy

Hypertrophy via IGF-1 signaling

In the adult animal, hypertrophy comes as a result of an increase in the size of skeletal muscle fibers. The protein growth factor insulin-like growth factor 1 (IGF-I) has been demonstrated to be sufficient to induce skeletal muscle hypertrophy. In an *in vivo* model of compensatory muscle hypertrophy IGF-I transcription was shown to be increased defining a correlation between the activation of IGF-I expression and locally induced muscle growth *in vivo* (DeVol et al., 1990). Treatment of avian primary myotubes with IGF-I stimulate both cell hyperplasia and myofiber hypertrophy (mean myofiber diameter increased 71-98%). IGF-I induces hypertrophy in these culture cells resulted from long-term stimulation of total protein synthesis rates and inhibition of total protein degradation rates. Furthermore this hypertrophy is accompanied by an increase of nuclei per myofiber (Vandenburgh et al., 1991). Transgenic mice, in which local expression IGF-I was increased using two different muscle-specific

promoters (skeletal alpha-actin and Myosin Light Chain), show strong muscle hypertrophy (Coleman et al., 1995; Musaro et al., 2001)(Fig.2).

On the other hand, genetic truncations of the single murine IGF-I gene (Powell-Braxton et al., 1993) and of the type I IGF receptor (Liu et al., 1993) have provided direct evidence *in vivo* for the ascribed functions of IGF-I in skeletal muscle development. Powell-Braxton et al. reported that IGF-I mutant mice show severe muscular dystrophy and highly reduced myofibrillar organization in both heart and skeletal muscle.

Most of the biological effects of IGF-I are mediated by the IGF-I receptor (IGFR). It is a tyrosine kinase receptor structurally similar to the insulin receptor and it is mainly involved in the transduction of growth and differentiation types of signals. However, the role of the IGFR in the induction of skeletal muscle hypertrophy in adult mice following a chronic

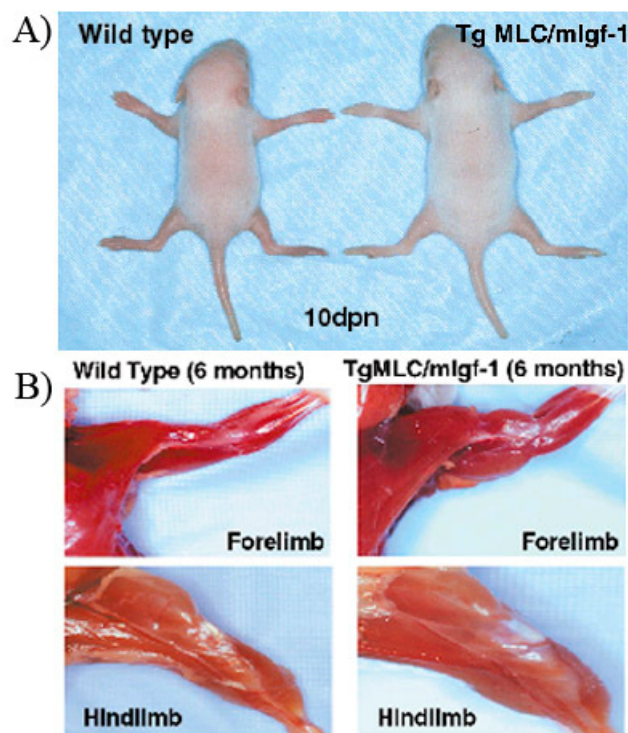


Fig.2. IGF1 transgenic mice under Myosin light chain promoter display pronounced muscle hypertrophy. A) Wild-type and MLC/mIgf-1 transgenic mouse pups at 10 days after birth. B) Skinned forelimb and hindlimb muscles of 6-month wild-type and MLC/mIgf-1 transgenic mice.

Adapted from (Musaro et al., 2001)

increase in mechanical loading is still controversial (Spangenburg et al., 2008). Binding of the IGF-I induces a conformational change in the IGF-I receptor tyrosine kinase resulting in its trans-phosphorylation and

subsequent phosphorylation of the insulin receptor substrate 1 (IRS-1). This event leads to the activation of the PI3K–Akt pathway (Moelling et al., 2002) and genetic activation of PI3K in mammalian muscles is shown to be sufficient to induce skeletal muscle hypertrophy (Murgia et al., 2000). Moreover, skeletal myotube hypertrophy induced by IGF-1 could be inhibited by wortmannin, a pharmacological inhibitor of PI3K (Rommel et al., 1999), showing that PI3K activity is necessary and sufficient to induce IGF-I mediated hypertrophy.

PI3K is a lipid kinase; it phosphorylates phosphatidylinositol(4,5)-bisphosphate, producing phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P₃] (Matsui et al., 2003; Vivanco and Sawyers, 2002). PtdIns(3,4,5)P₃ is a membrane-binding site for two kinases: Akt1 (also known as protein kinase B) and PDK1 (phosphoinositide-dependent protein kinase). Akt1 is phosphorylated by PDK1, and thereby activated upon translocation to the membrane (Alessi et al., 1997; Andjelkovic et al., 1997) (fig.3). Once activated, Akt1 phosphorylates a wide set of substrates, including proteins that block apoptosis, induce protein synthesis, gene transcription and cell proliferation (Matsui et al., 2003; Vivanco and Sawyers, 2002). Knockout mice that are Akt1^{-/-} are smaller than wildtype littermates, demonstrating that Akt1 is required for normal organ growth (Chen et al., 2001). Transgenic mice that express a constitutively active form of Akt1 in cardiac or skeletal muscle have hypertrophic hearts or muscles (Lai et al., 2004; Shioi et al., 2002). During skeletal muscle hypertrophy, endogenous Akt1 phosphorylation increases, as does the relative amount of Akt1 protein (Bodine et al., 2001; Pallafacchina et al., 2002). Expression of a dominant-negative mutant form of Akt1, which inhibits the endogenous activity of this protein, blocks IGF-1-mediated hypertrophy *in vitro* and muscle fiber hypertrophy in regenerating skeletal muscle *in vivo* (Rommel et al., 2001). These data demonstrate that Akt1 activity is required for IGF-I-mediated hypertrophy. The finding that Akt1 is activated subsequent to PI3K stimulation and that Akt1 can recapitulate the hypertrophic effects seen with PI3K, suggests that PI3K and Akt1 are members of a linear pathway. Akt1 can be modulated either by directly controlling its phosphorylation state or by altering the concentration of the

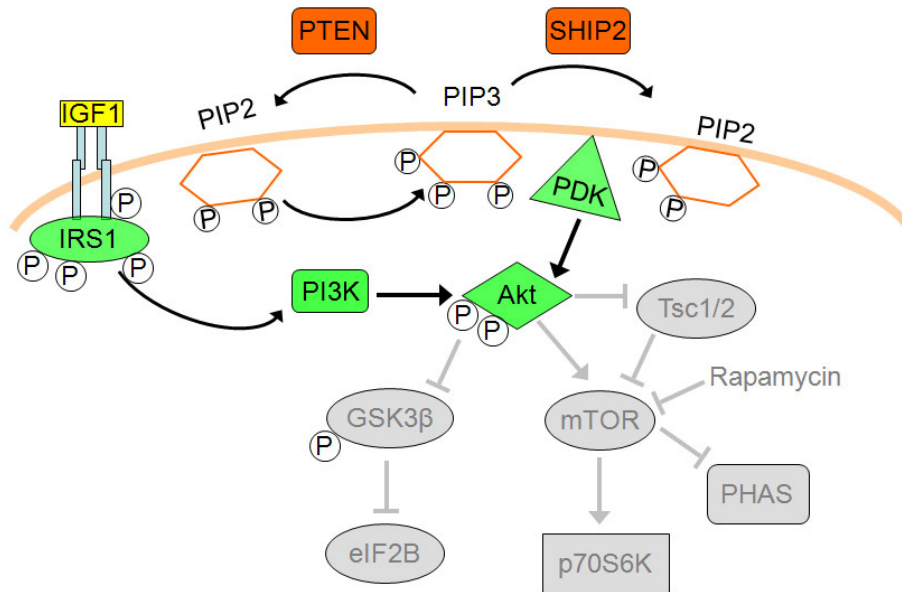


Fig.3. IGF1 signalling The IGF receptor phosphorylates substrate proteins, including the IRS1. Once phosphorylated, these proteins bind to PI3K, which generates the lipid product PI_{3,4,5}-P₃ (also known as PIP₃). PIP₃ in turn can bind to membrane and activate the enzyme PDK, which leads to a phosphorylation cascade that includes activation of Akt and other protein kinases. PI3K signaling can be blocked by two phosphatases : PTEN and SHIP2.

lipid that it binds at the plasma membrane, PtdIns(3,4,5)P₃ (Alessi et al., 1997).Akt1 activity depends on phosphorylation at two sites: Ser473 and Thr309 (Alessi et al., 1996) and the protein phosphatase 2A (PP2A) has been shown to dephosphorylate Akt1 (Andjelkovic et al., 1996; Pankov et al., 2003; Resjo et al., 2002).Two different phosphatase, SHIP2 and PTEN, can mediate lipid de-phosphorilation (Goberdhan et al., 1999; Rommel et al., 2001; Stambolic et al., 1998; Xu et al., 2002)

The Akt–mTOR pathways

Early experiments in *Drosophila* helped to define a particular pathway downstream of PI3K and Akt that can control cell size. Genetic loss or inhibition of either IRS-1 (Bohni et al., 1999), PI3K (Leever et al., 1996), TOR (target of rapamycin) (Zhang et al., 2000), or p70S6K (Montagne et al., 1999), all resulted in decreased cell size in the *Drosophila* wing. Although mTOR (mammalian target of rapamycin) can be activated by IGF-I via PI3K–Akt activation, also amino acids can activate mTOR directly, causing a subsequent stimulation of p70S6K activity (Burnett et al., 1998; Hara et al., 1998). mTOR has an important

and central function in integrating a variety of growth signals, from simple nutritional stimulation to activation by protein growth factors, resulting in protein synthesis. Akt phosphorylates mTOR, thereby activating it (Nave et al., 1999; Scott et al., 1998); both Akt phosphorylation (Bodine et al., 2001) and mTOR phosphorylation are increased during muscle hypertrophy (Reynolds et al., 2002). Rapamycin is a chemical compound that forms a complex with a protein called FK506-binding protein (FKBP12), disrupting activation of mTOR. When applied to myotube cultures *in vitro*, rapamycin blocks IGF-1 mediated muscle growth (Rommel et al., 2001) and pharmacological treatment blocks hypertrophy in regenerating innervated muscles *in vivo* (Pallafacchina et al., 2002). Moreover treatment with rapamycin during compensatory hypertrophy does not block activation of Akt1, demonstrating that Akt1 is upstream of mTOR, (Rommel et al., 2001)(Fig. 4). Thus, rapamycin provides pharmacological evidence for the activation of a linear Akt1–mTOR–p70S6K pathway during hypertrophy. Genetic support comes from experiments in mammalian cells (HEK293), showing that the introduction of an inhibitor of mTOR acting downstream of Akt1, the Tsc1–Tsc2 complex (Inoki et al., 2002), inhibits the activation of p70S6K (Fig.4).

In addition to stimulation p70S6 kinase-mediated protein translation, activation of mTOR points also to other factors, creating a complex regulatory network. In the context of trimeric complex, comprising mTOR, Raptor and PHAS1, mTOR can phosphorylates PHAS1. Phosphorylated PHAS1 is not able to inhibit the translation initiation factor eIF-4E, allowing an increase of the rate of protein synthesis(Choi et al., 2003; Hara et al., 2002; Kim et al., 2002).

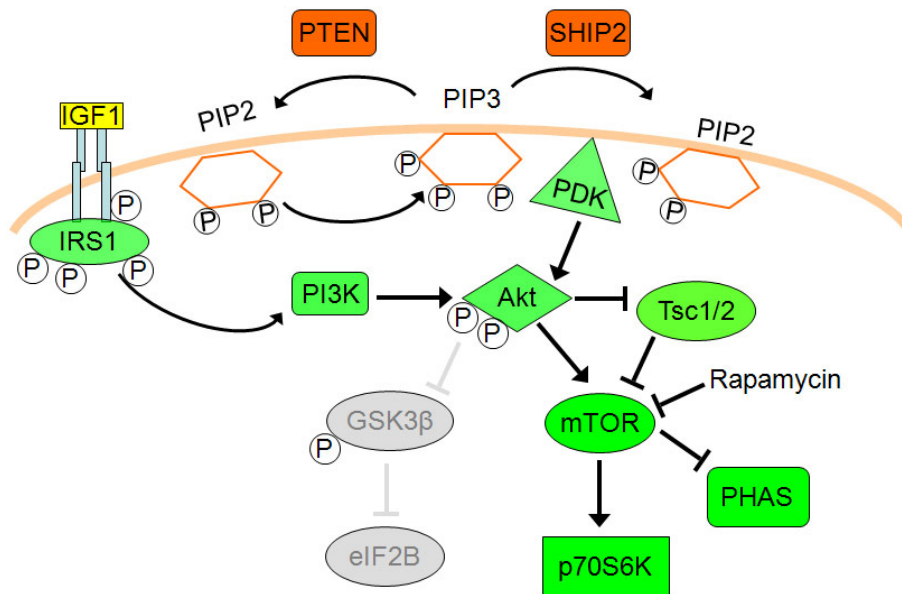


Fig.4. The Akt-mTOR pathway. mTOR can increase protein synthesis by modulating two distinct pathways, the p70S6K pathway and the PHAS-1 pathway

Thus, mTOR can modulate two distinct pathways, the p70S6K pathway and the Raptor–PHAS-1 pathway.

PI3K–Akt–GSK3 β pathway

GSK3 β (Glycogen-synthase kinase 3 β) is a distinct substrate of Akt1 involved in the modulation of hypertrophy. GSK3 β activity is inhibited by Akt1 phosphorylation (Cross et al., 1995)(Fig. 5). Expression of a dominant-negative, kinase-inactive form of GSK3 β induces dramatic hypertrophy in skeletal myotubes (Rommel et al., 2001). In cardiac hypertrophy, GSK3 β phosphorylation is also evident (Hardt and Sadoshima, 2002) and expression of a dominant-negative form of GSK3 β can induce cardiac hypertrophy (Hardt and Sadoshima, 2002). GSK3 β blocks protein translation initiated by the eIF-2B (eukaryotic initiation factor 2B) protein (Hardt and Sadoshima, 2002) (Fig.5). Therefore GSK3 β inhibition might induce hypertrophy by stimulating protein synthesis independent of the mTOR pathway.

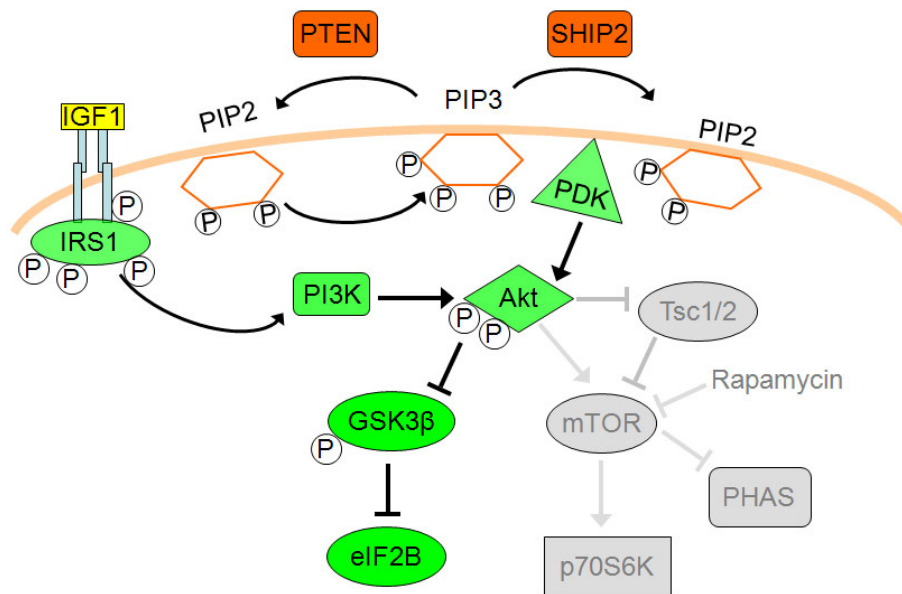


Fig.5. PI3K-Akt-GSK3 β pathway Activation of AKT, in turn, phosphorylates and inhibits GSK3, resulting in the dephosphorylation of substrates of GSK3, including eIF2B. This contributes to the insulin-induced stimulation of glycogen and protein synthesis.

Akt-FOXO pathway

The regulation of skeletal muscle mass results from an equilibrium between protein synthesis, under the control of the Akt/mTOR and Akt/GSK3 pathways, and protein degradation, controlled by the Akt/FoxO (Forkhead box O) pathway.

Many catabolic conditions can drive to skeletal muscle atrophy, characterized by a decrease in muscle mass and fiber size (Jackman and Kandarian, 2004; Jagoe and Goldberg, 2001). This atrophy is mediated by the ATP-dependent proteolytic ubiquitin-proteasome pathway (Jagoe and Goldberg, 2001). A screen for genetic markers of atrophy identified two genes that are up-regulated rapidly in multiple models of muscle atrophy *in vivo*: MuRF1 (muscle Ring Finger 1) and Atrogin1, both encoding ubiquitin ligases, that conjugates ubiquitin to protein substrates (Bodine et al., 2001; Gomes et al., 2001). The generation of null mice (Bodine et al., 2001), resistant to denervation atrophy, and the observation that overexpression of Atrogin1 in myotubes leads to atrophy demonstrates the importance of these gene products in atrophy processes. Furthermore different studies demonstrate that *in vitro* treatment of myotubes with dexamethasone induces atrophy, accompanied by the specific increased expression of Atrogin1 and MuRF1 (Sandri et al., 2004; Stitt et al., 2004).

This upregulation of Atrogin1 and MuRF1 was antagonized by simultaneous treatment with IGF-1 (Sacheck et al., 2004; Sandri et al., 2004; Stitt et al., 2004), acting through the PI3K/Akt pathway (Sandri et al., 2004; Stitt et al., 2004). The mechanism, by which Akt inhibited Atrogin1 and MuRF1, upregulation involves the FoxO family of transcription factors (Lee et al., 2004; Sandri et al., 2004; Stitt et al., 2004). In myotubes, FoxO transcription factors are excluded from the nucleus when phosphorylated by Akt, and translocate to the nucleus upon dephosphorylation. The translocation and activity of FoxO transcription factors is required for upregulation of MuRF1 and Atrogin1, and over-expression of FoxO in adult skeletal muscle results in atrophic phenotype (Kamei et al., 2004; Sandri et al., 2004). Thus, in conditions promoting growth, the positive balance is given both by enhanced synthesis and suppression of protein degradation.

Under anabolic conditions, general protein synthesis increases because AKT-induced phosphorylation activates mTOR, GSK3 and S6K, and inhibits FoxO factors, leaving them inactive in the cytosol. Together, these adaptations lead to protein accumulation and fiber hypertrophy (**Fig. 6** left). By contrast, in catabolic conditions, AKT is dephosphorylated and its activity reduced below control levels, leading to activation of FOXO and to transcription of Atrogin-1, MuRF1, and other genes that promote muscle wasting (Bodine et al., 2001). On the other hand, as a consequence of the dephosphorylation of GSK3 β , mTOR, and S6K, there is a reduced protein synthesis. All together, these adaptations lead to a dramatic decrease in cell protein content and myofiber size (Fig.6, right).

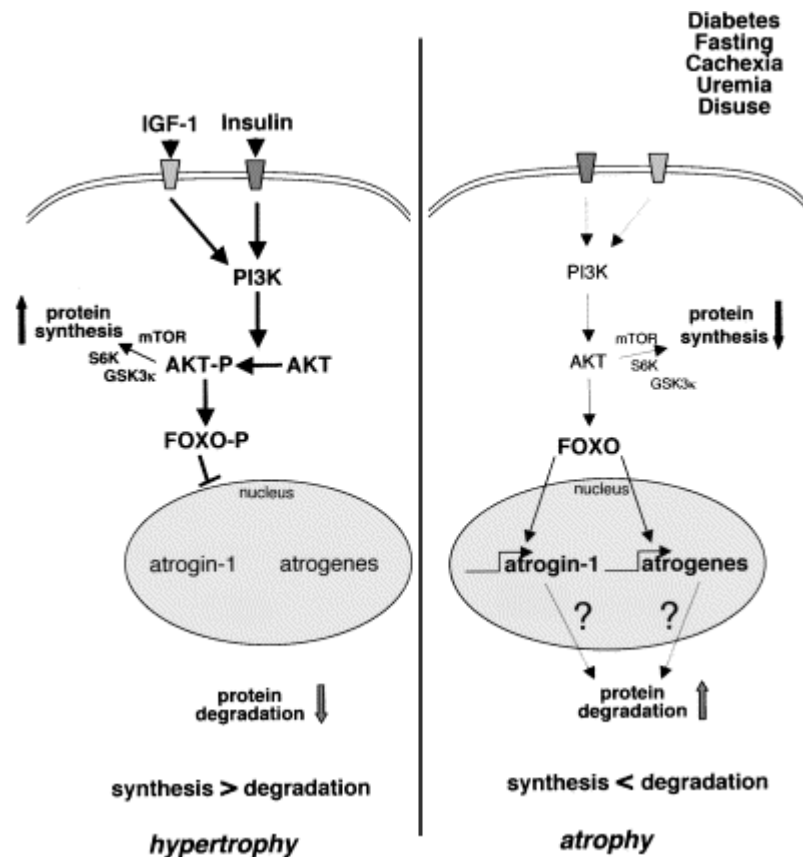


Fig.6. Akt-FOXO pathway. Right panel: growth factors, not only increases the synthesis through Akt mTOR pathway, but also suppresses proteolysis and the expression of atrophy-related genes through the phosphorylation of FOXO, which becomes inactive in the cytoplasm. Left panel: atrophic stimuli induces Akt dephosphorylation and the following dephosphorylation of FOXO, which translocates into the nucleus and activates the transcription of the atrogenes. Adapted from (Sandri et al., 2004)

Calcineurin pathway

Calcium acts as a second messenger in both cardiac and skeletal muscle, conveying extracellular stimuli into intracellular effects. However, the molecular mechanisms underlying contraction/relaxation mechanisms are different in the two tissues.

Calcineurin is one of the major mediators of Ca^{2+} signalling in different cell systems. It is a serin/threonin phosphatase consisting of a catalytic (Calcineurin A) and regulatory (Calcineurin B) subunits. It is well established that calcineurin is a crucial effector of cardiac hypertrophy: transgenic mice, that express activated forms of Calcineurin A or NFATc4 in the heart, develop cardiac hypertrophy (Molkentin et al., 1998), whereas calcineurin-A β deficient mice show an impaired cardiac hypertrophic response (Bueno et al., 2002). However, the role of calcineurin in skeletal

muscle hypertrophy has been a matter of debate. Skeletal muscles of transgenic mice overexpressing calcineurin do not develop skeletal muscle hypertrophy (Naya et al., 2000), and the use of pharmacological inhibitors of calcineurin activity gave controversial results, probably due to the different experimental settings (Dunn et al., 1999; Dunn et al., 2000). The discrepancies might depend on the dose of the drug, on the length of treatment, on the species investigated, on the type of muscle, on the model of muscle growth and even on the specific stage of growth. The best characterized substrate of calcineurin is the NFAT family of transcription factors NFATc1-c4 (Rao et al., 1997) that regulates the development and differentiation of several tissue types. Sustained elevation of intracellular calcium activates calcineurin, which in turn de-phosphorylates NFAT, allowing its translocation to the nucleus (Fig.7).

The analysis of adult NFATc2^{-/-} and NFATc3^{-/-} mice demonstrated that they are both involved in the control of muscle growth, even if at

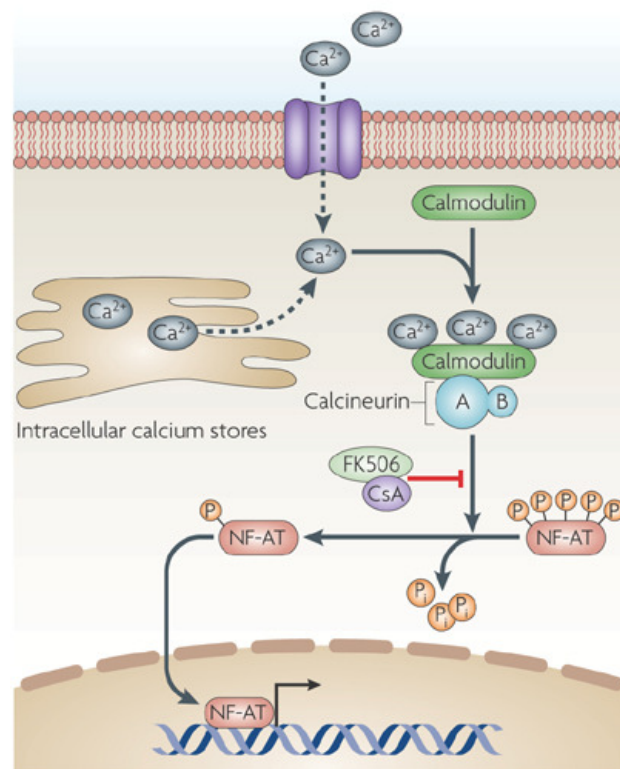


Fig.7. Calcineurin-NFAT pathway. The calcineurin catalytic subunit (A), when bound to the regulatory subunit (B) and calmodulin–Ca²⁺ complex, dephosphorylates nuclear factor of activated T-cells (NF-AT) in the cytoplasm, leading to nuclear migration of this transcription factor and the subsequent activation of various cellular processes. The dephosphorylation of NF-AT is inhibited by cyclosporine A (CsA) and FK506. Adapted from (Steinbach et al., 2007)

different stages. Both lines show reduced muscle mass: in NFAC2-null mice myofiber form normally but display impaired growth, as a result of a decreased myonuclear number (Horsley et al., 2001); in NFATc3-null mice there is a reduced number of fibers, as a consequence of a decreased total number of primary myofibers (Kegley et al., 2001)].

Myostatin pathway

Myostatin, also known as growth differentiation factor 8, or GDF-8, is secreted growth factor and a member of TGF- β (transforming growth factor- β) superfamily (McPherron et al., 1997). Myostatin is expressed in embryonic, fetal and adult skeletal muscle cells, where it acts as a potent negative regulator of skeletal muscle growth. Indeed lack of myostatin leads to increased muscle growth (Gonzalez-Cadavid et al., 1998; McPherron et al., 1997), while systemic injection of myostatin leads to muscle wasting (Zimmers et al., 2002). Two breeds of double-musled cattle, Belgian Blue and Piemontese, bring mutations, 11 nucleotide deletion or a missense mutation, in exon 3 of the myostatin coding sequence. Myostatin null mice exhibited a phenotype that was characterized by a marked hypertrophy and hyperplasia of skeletal mass and loss of fat mass (McPherron et al., 1997).

Like other members of the TGF- β family, myostatin is synthesized as a precursor protein of 376 aminoacids containing a signal sequence, a N-terminal propeptide domain with a hydrophobic core that functions as a secretory signal, and a C-terminal domain considered the active molecule (McPherron et al., 1997). Myostatin is proteolitically processed by a calcium-dependent serine protease called furin, which is highly concentrated in the trans-Golgi network (Lee et al., 2004; Molloy et al., 1994). The mature myostatin is generated as a high molecular weight protein in a latent form associated to various interacting proteins, that are able to modulate its activation, secretion or receptor binding (Fig.8). Titin cap and hSGT (human small glutamine-rich tetratricopeptide repeat-

containing protein) bind myostatin intracellularly, thus inhibiting its secretion and activation (Nicholas et al., 2002; Wang et al., 2003), Follistatin, FLRG (follistatin-related gene) and GASP1 (growth and differentiation factor-associated serum protein-1) bind to mature myostatin and inhibit myostatin receptor binding (Amthor et al., 2004; Hill et al., 2002).

Myostatin elicits its biological effects by binding to the activin type II B receptor (ActRIIB). This is a transmembrane serine/threonine kinase heterotetramer receptor, activation of which enhances receptor transphosphorylation, leading to stimulation of serine/threonine kinase activity. Lee and MacPherron (Lee and McPherron, 2001) have shown that the expression of a dominant negative form of ActRIIB in mice mimics myostatin gene knockout. The components involved in the downstream signal transduction of myostatin have not completely been identified, however it has recently been shown that myostatin negatively regulates the Akt/mTOR pathway. Overexpression of myostatin in rat tibialis muscles decreased muscle mass and phosphorylation of components of the Akt/mTOR pathway was attenuated (Amirouche et al., 2009). Myostatin signaling pathway requires the phosphorylation of Smad2 /3 and Smad4 proteins, whereas Smad7 is a negative regulator (Zhu et al., 2004) (Fig.8).

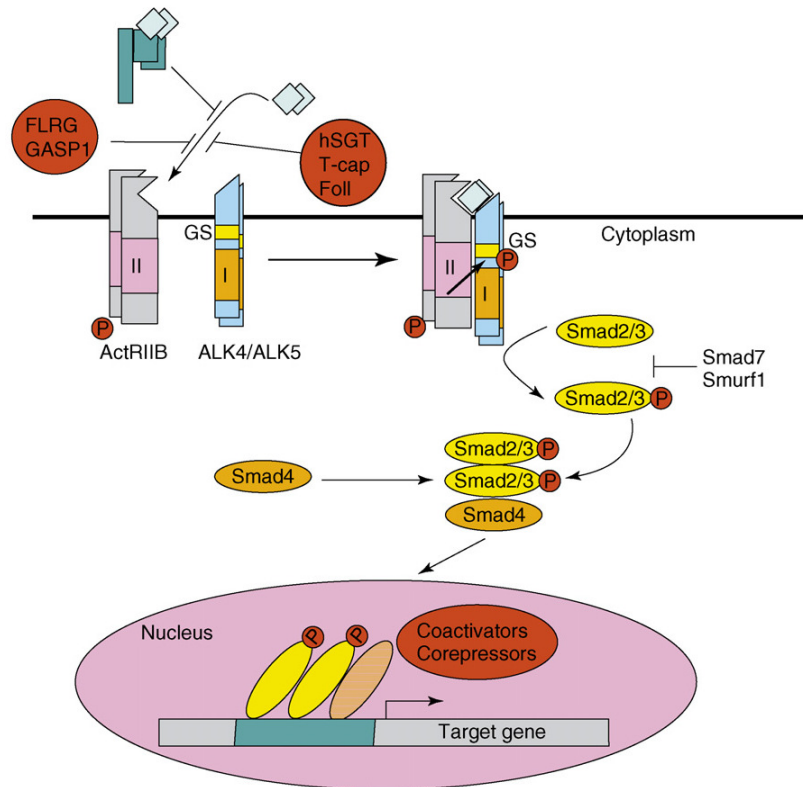


Fig 8. The myostatin pathway. Myostatin can be found in serum or locally in an inactive state when bound to FLRG, GASP-1, hSGT, T-cap, follistatin or the myostatin propeptide. The active myostatin dimer binds to the activine type II receptor (ActRIIB), which then recruits and activates by transphosphorylation the type I receptor (ALK4 or ALK5). Smad2 and Smad3 are subsequently activated: they form aggregates with Smad4 and are then translocated to the nucleus, activating target gene transcription. Two inhibitors of this signalling have been identified: Smad7 and Smurf1. Smad7 represses myostatin signal by the binding of its MH2 domain to activated receptors, thus preventing recruitment and activation of R-Smads. Smurf1 is an E3 ubiquitin ligase that mediates ubiquitination and consequent degradation of the R-Smads.ref Adapted from(Jouliia-Ekaza and Cabello, 2007)

2. Skeletal muscle fiber type

During postnatal development and regeneration, a default nerve activity-independent pathway of muscle fiber differentiation, which is controlled by thyroid hormone, leads to the activation of a fast gene program. In contrast, the postnatal induction and maintenance of the slow gene program is strictly dependent on slow motor neuron activity.

2.1. Role of the nerve in fiber type specification

During development primary and secondary fibers differ in the expression of myosin heavy chain (MyHC) isoforms. In mammals, primary fibers express the embryonic (fast) and MyHC β -slow and, shortly before the end of primary fiber formation, some (generally located on the superficial edge of the muscles) also express the perinatal/neonatal (fast) isoform. In contrast, secondary fibers express the fast embryonic and perinatal isoforms from their inception and (with the exception of the soleus muscle) do not express MyHC β -slow. Thus, in general, mammalian primary fibers (and embryonic myotubes *in vitro*) are programmed for a predominantly slow phenotype, whereas secondary fibers (and fetal myotubes *in vitro*) adopt a fast phenotype (Wigmore and Evans, 2002; Zhang and McLennan, 1998). During late fetal development, the Sox6 transcription factor acts as a repressor of slow fiber-type genes, allowing the differentiation of fast fibers. In Sox6-null mice all fetal muscle fibers have slow characteristics (Hagiwara et al., 2007). In turn Sox6 is under the transcriptional control of Prdm1, which plays a pivotal role in switching between alternative fiber types programmes. Prdm1 acts as a transcriptional repressor of Sox6, and, in this way, it activates slow-twitch-specific genes. Moreover it can bind directly to promoters directly interacts with the promoter regions of fast genes on which it act as a repressor (von Hofsten et al., 2008).

Adult muscle fibers are highly heterogeneous. The classification of adult muscle fibers can be based on their speed of contraction, which

depends mainly on the ATPase activity of the predominant myosin isoform, with fast and slow fibers containing isoforms with higher and lower ATPase activity, respectively. In rodents, a single slow MyHC gene has been identified which is subject to different post-translational modifications (Maggs et al., 2000) during pre- and postnatal life. In contrast, embryonic and perinatal MyHC isoforms are progressively replaced postnatally with the three adult fast MyHCs, 2A, 2X and 2B (Schiaffino and Reggiani, 1994). Adult rodent fibers can be divided into four major classes according to their speed of contraction and the predominant expression of a particular isoform of MyHC: Type I, Type 2A, Type 2X and Type 2B, with Type I being the slowest and Type 2B the fastest (Wigmore and Evans, 2002; Zhang and McLennan, 1998). Notably in humans, the MyHC 2B isoform is present in the genome (Weiss et al., 1999) but is not expressed (Smerdu et al., 1994). The boundaries between the different classes of adult fibers are not absolute and intermediate fibers co-expressing different MyHC isoforms are common. In addition to MyHCs, a large number of genes are also expressed at different levels in the different adult fiber types (Bottinelli and Reggiani, 2000).

Slow-twitch fibers express MyHC-slow, have an oxidative metabolism and are fatigue resistant; fast-twitch fibers, on the other hand, display a graded range of both functional properties and metabolic profiles according to the scheme $2A \leftrightarrow 2X \leftrightarrow 2B$; MyHC 2A has the slowest and 2B the fastest shortening velocity, and 2A is oxidative and fatigue resistant whereas 2B is glycolytic and easily fatigable.

Fiber-type switching can be induced in adult skeletal muscle by changes in nerve activity as shown by nerve cross-union and electrical stimulation studies. A slow-to-fast switch in the direction $1 \leftrightarrow 2A \leftrightarrow 2X \leftrightarrow 2B$ can be induced by phasic high-frequency electrical stimulation, resembling the firing pattern of fast motoneurons, whereas a fast-to-slow switch in the opposite direction $2B \leftrightarrow 2X \leftrightarrow 2A \leftrightarrow 1$ can be induced by tonic low-frequency electrical stimulation, resembling the firing pattern of slow motoneurons (Kirschbaum et al., 1989). Intrinsic differences between muscles and fiber types may limit the range of possible adaptations; thus fast muscles have the capacity to adapt in the range $2B \leftrightarrow 2X \leftrightarrow 2A$, while the $2A \leftrightarrow 1$ step

occurs only after a long delay (>2 month of slow stimulation); similarly, but in the opposite direction, slow muscles adapt in the range 1↔2A↔2X (Windisch et al., 1998). However, changes in the thyroid state can expand this range; for example MyHC 2B can be induced in slow muscles by the combined effect of hyperthyroidism and reduced activity consequent to mechanical unloading (Caiozzo et al., 1998), whereas MyHC β -slow can be induced in fast muscles by hypothyroidism combined with chronic low-frequency stimulation or overloading (Caiozzo et al., 2000; Kirschbaum et al., 1990). The time factor is also important in promoting fiber-type transitions by hyperactivity or inactivity. Low-frequency stimulation for 2 months does not lead to significant expression of MyHC β -slow in fast rat muscles (Ausoni et al., 1990; Termin et al., 1989), but a fast-to-slow switch has been detected after 4 months (Windisch et al., 1998). A complete disappearance of type I fibers has been demonstrated in human skeletal muscle after long-term spinal cord injury (Grimby et al., 1976) and in rat slow muscles 60–90 days following spinal cord isolation (Huey et al., 2001)

Developing and regenerating skeletal muscles appear to have a greater plasticity compared with adult muscle. For example, the compensatory hypertrophy of the extensor digitorum longus (EDL) muscle induced by ablation of the synergistic tibialis anterior in newborn rats is accompanied by an increase in type I fibers and a complete switch of the fast fibers from type 2B to 2A/2X, with correspondingly increased SDH staining (Schiaffino and Bormioli, 1973). In contrast, no significant change in EDL fiber-type profile was observed when the same experiment was performed in adult animals. These findings should be taken into account when the effects of perturbations of signalling pathways in transgenic mice are interpreted, since changes in fiber-type composition could be due to the effect of the transgenes during early developmental stages and not in the adult.

In the last years signaling pathway controlling fiber type specification have been characterized. Some studies point to a role of Ras–MAPK (mitogen-activated protein kinase) and calcineurin pathways in activity-dependent muscle fiber type specification. Evidence for a role of Ras–

MAPK is supported by the effect of Ras mutants in regenerating muscle (Murgia et al., 2000). Constitutively active Ras and a Ras double mutant, RasV12S35, that selectively activates the extracellular signal-regulated kinase (ERK) pathway, a major MAPK pathway, can mimic the effect of slow motor neurons by upregulating slow myosin and downregulating fast myosin genes. By contrast, the effect of slow motor neurons is inhibited by a dominant-negative Ras mutant.

As previously mentioned, calcineurin once activated by Ca^{2+} -calmodulin binding, affects gene expression by dephosphorylating specific substrates. Following dephosphorylation NFAT, the main target of calcineurin, translocates from the cytoplasm to the nucleus and regulates different genes in cooperation with other transcription factors (Crabtree and Olson, 2002).

Calcineurin activity is controlled by endogenous protein inhibitors, such as cain (also known as cabin-1) and MCIP1 [myocyte-enriched calcineurin interacting protein 1, the latter being particularly abundant in skeletal muscle (Fuentes et al., 2000; Rothermel et al., 2000)]. Calcineurin activity can be blocked *in vitro* or *in vivo* by the immunosuppressive drugs cyclosporin A (CsA) and FK506. CsA and FK506 form complexes with endogenous cyclophilin and FKBP12 (FK506 binding protein 12), respectively, and these complexes bind the catalytic subunit of calcineurin.

A role for calcineurin in the regulation of muscle fiber type was first suggested by the finding that constitutively active calcineurin selectively upregulates slow-fiber-specific gene promoters in cultured muscle cells, and administration of CsA to intact animals promotes slow-to-fast fiber transformation (Chin et al., 1998; Serrano et al., 2001). NFATc1 has been identified as a sensor selectively responsive to slow patterns of nerve electrical activity. It mediates an activity-dependent controls in the induction of the slow gene program during muscle regeneration and in the maintenance of the slow phenotype in adult skeletal muscle (McCullagh et al., 2004).

3. The muscle regulatory factors

Nearly 20 years ago, subtractive hybridization experiments were performed to identify and isolate myoblast specific transcripts that were capable of orchestrating myogenic conversion of 10T1/2 fibroblasts (Lassar et al., 1986; Pinney et al., 1988). This work led to the identification of a single cDNA, named MyoD, which was capable of converting a variety of cell types (e.g., fibroblasts, chondrocytes, neurons, amniocytes) to myoblasts, albeit with varying efficiency (Choi et al., 1990; Davis et al., 1987; Weintraub et al., 1989). MyoD belongs to a much larger class of DNA-binding proteins containing a basic helix-loop-helix (bHLH) domain. Soon after the discovery of MyoD, three closely related genes were identified: Myf5, myogenin, and Mrf4. *In vitro*, each MRF efficiently binds to consensus CANNTG sites (E boxes), which are present in the promoters and enhancers of muscle-specific genes (Blackwell and Weintraub, 1990; Lassar et al., 1989)

.In the two decades from their discovery, *in vivo* studies have elucidated the specific roles of MyoD and its relatives Myf5, myogenin, and Mrf4. Cell culture studies have uncovered the basic mechanisms by which they function in transcription. The MRFs, together with Mef2 family proteins and other general and muscle-specific factors, coordinate the activities of co-activators and co-repressors, resulting in tight control of gene expression during myogenesis.

The MRFs are class II (tissue-specific) bHLH transcription factors. The MRF protein contain one or two transactivation domains (at N- and C-termini), a conserved basic DNA binding domain essential for sequence specific DNA binding, and an HLH (Helix Loop Helix) motif required for heterodimerization (Davis et al., 1990; Olson and Klein, 1994) (Fig.9).

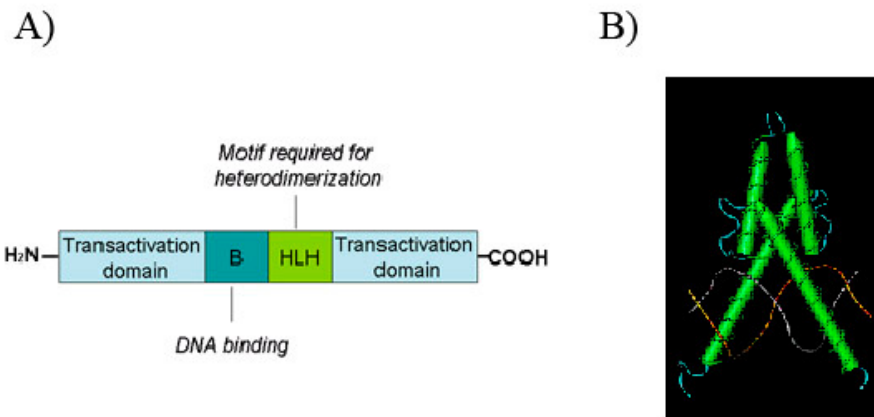


Fig. 9. A) Schematic representation of MRFs domains; B) MyoD dimer bound to DNA

Each of the MRFs has been shown to heterodimerize *in vitro* and *in vivo* with class I bHLH factors, such as the ubiquitous E proteins (like the E2A gene products E12 and E47), and to bind DNA in a sequence specific manner at sites known E-boxes (CANNTG) (Arnold and Winter, 1998; Lassar and Munsterberg, 1994). This leads to the transcriptional activation of muscle specific genes, such as α -actin, MCK (muscle creatin kinase), TnI (troponin I), α 7-integrin or desmin.

Since all four MRFs bind the same DNA sequence *in vitro*, it has been difficult to ascertain experimentally if they possess identical or distinct activities. Mrf4 and MyoD contain an N-terminal activation domains, yet Mrf4 is considered a weak inducer of the expression of many muscle specific genes despite its ability to bind E-box sequences (Braun et al., 1990; Mak et al., 1992; Moss et al., 1996; Schwarz et al., 1992). In this regard, whereas all myogenic factors bound with similar affinities to the MCK enhancer in the presence of the widely expressed HLH protein E12, only MyoD myogenin and Myf5 efficiently trans-activated the enhancer in transiently transfected 10T1/2 and 3T3 cells (Chakraborty et al., 1991). Moreover, when N-terminal MyoD and Mrf4 sequences were exchanged in transfection experiments, any construct containing the Mrf4 N-terminus was less capable of transactivating than those containing MyoD N-terminal sequences, demonstrating that the Mrf4 N-terminal transactivation domain is unique (Moss et al., 1996).

A number of proteins have been identified which act as myogenic antagonists by directly binding to E proteins and/or to MyoD family proteins, and blocking their ability to bind E boxes and/or activate transcription at muscle-specific promoters. Many of these inhibitors are themselves helix-loop-helix domain proteins, and include Id, Twist, MyoR and Mist-1. Id comprises a family of HLH proteins whose expression is upregulated under high-serum conditions. A high level of Id protein in the cell is inhibitory for MRF activity because Id is capable of efficiently heterodimerizing with E proteins, sequestering them and preventing their interaction with the MRFs (Benzra et al., 1990). Id proteins may also heterodimerize with MRFs, albeit with lower efficiency. Since Id lacks the basic region required for DNA-binding, MRF/Id heterodimers are thought to be devoid of transcriptional activity. Similarly to Id, Twist is also an HLH protein inhibits myogenic differentiation by dimerization with E proteins, sequestering them from MRFs in inactive complexes (Spicer et al., 1996). However, unlike Id, Twist possesses a basic region which functions not by binding to DNA, but by binding to the basic region in MRFs, thus preventing the interaction between MRFs and their cognate E boxes in muscle-specific promoters (Hamamori et al., 1997). Twist is also capable of inhibiting myogenesis via direct interaction with Mef2 proteins (Spicer et al., 1996). Also MyoR and Mist-1 are inhibitors of myogenesis. These factors contain basic regions and form dimers with the MRFs. MRF/MyoR and MRF/Mist-1 heterodimers are competent to bind E boxes; however, these dimers are unable to activate transcription when bound to DNA (Lemerrier et al., 1998; Lu et al., 1999). Mdf1 (formerly known as I-mfa) is a negative regulator of MRFs, it is a cytoplasmic protein which functions by binding to and sequestering MRFs within the cytoplasm, preventing their translocation into the nucleus (Chen et al., 1996).

The MRFs are assisted by Mef2 (myocyte enhancer factor 2) family of transcription factors in order to mediate expression of muscle-specific genes (Black and Olson, 1998). Mef2 proteins belong to the MADS (MCM1, agamous, deficiens, serum response factor) box-containing transcription factor family. The Mef2 family consists of four members, Mef2A-D, each of

them is encoded on a separate gene. While expression of MRFs is restricted to muscle, Mef2 genes are expressed widely during development. Mef2 proteins bind to an A/T-rich DNA sequence element (C/TTA(A/T)₄TAG/A) which is found in the promoters of many muscle-specific genes (Gossett et al., 1989). Mef2 does not possess the ability to recapitulate the myogenic differentiation program when expressed in cell lines *in vitro*, but seems that Mef2 has a critical role in assisting the MRFs. Mef2 and MyoD interact directly *in vitro* and synergistically activate transfected reporters driven by E boxes and Mef2 binding sites (Molkentin et al., 1995). In the promoters and enhancers of muscle-specific genes, E boxes and Mef2 binding sites are often located within close proximity to one another, providing further support for a model in which MyoD and Mef2 bind DNA and activate transcription in a cooperative fashion (Wasserman and Fickett, 1998).

A number of other factors have been shown to cooperate with MyoD family proteins to activate expression of muscle specific genes. For example, the muscle LIM protein (MLP) physically interacts with MRFs via the bHLH domain and is required for differentiation of C2C12 myoblasts in culture (Arber et al., 1994; Kong et al., 1997). In addition, muscle-specific gene expression often requires cooperation of ubiquitously expressed DNA-binding factors such as Sp1 and AP1 (Biesiada et al., 1999; Gustafson and Kedes, 1989).

Three classes of co-activators are known to cooperate with transcription factors to mediate specific and patterned gene expression: histone modifying proteins such as histone acetylases and methylases, SWI/SNF family chromatin remodeling factors, and proteins in the TRAP/Mediator family. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) both interact with MyoD and have opposing activities that might be critical to switch MyoD from a repressor to an activator at some loci. These mechanisms of DNA remodeling are influenced also by the subsequent recruitment of the SWI/SNF complexes. HAT proteins function by transferring acetyl groups from acetyl-coA to the lysines residues in histone proteins (namely, H3 and H4), and in some cases, to non-histone proteins. Histone acetylation promotes increases the

access of transcription factors to the DNA (Ito et al., 2000), and MyoD binding to specific promoters has been shown to occur concomitantly with histone acetylation (Bergstrom et al., 2002). HAT activity increases during the course of myogenic differentiation (Poleskaya et al., 2001), and the HAT protein p300/CBP is required for expression of muscle-specific genes (Puri et al., 1997).

3.1. Role of MRFs in skeletal muscle development

Gene targeting studies have revealed hierarchical relationships between Myf5, MyoD, Mrf4 and myogenin. In the developing mouse embryo, Myf5 expression is the first to be induced in the dorsal-medial somites (which later gives rise to trunk and intercostal muscles), and is followed by expression of MyoD in the dorsal-lateral somites (which later gives rise to body wall and limb muscles). The Wnt, Sonic hedgehog (Shh), and other signaling pathways have been shown to induce the expression of Myf5 and MyoD (Buckingham, 2001). Expression of both MyoD and Myf5 is a key step resulting in commitment of multipotential somite cells to the myogenic lineage, since disruption of both genes results in the absence of skeletal myoblasts (Rudnicki et al., 1993). Null mutations in either MyoD or Myf5 result in apparently normal muscle development, demonstrating an high degree of genetic redundancy between the MRFs (see Table 1). However, upon careful examination, mild defects in trunk skeletal muscle are observed in Myf5 null embryos, whereas early limb and branchial arch muscle development is delayed in MyoD null embryos, demonstrating that these genes control early specification steps (Kablar et al., 1998). Mice lacking myogenin have very poorly developed skeletal muscle tissue even though myoblasts are present (see Table 1), suggesting that myogenin plays a critical role in terminal differentiation of myoblasts, but is dispensable for establishing the myogenic lineage (Hasty et al., 1993; Nabeshima et al., 1993). Furthermore, myogenin cannot efficiently mediate myogenesis in the developing mouse embryo when substituted into the Myf5 locus, suggesting that the ability to establish the muscle lineage is not simply a matter of the timing of expression in the embryo,

but is an inherent property within the protein itself (Wang and Jaenisch, 1997). In support of this *in vivo* evidence, although each of the myogenic bHLH proteins can initiate myogenesis when expressed in non-muscle cells *in vitro*, myogenin is not nearly as efficient as MyoD or Myf5 in initiating expression of some muscle-specific genes. The ability of MyoD and Myf5 to initiate previously silent muscle-specific genes has been mapped to a C-terminal region of MyoD, which forms a putative α -helix (Bergstrom and Tapscott, 2001).

The specific role played by Mrf4 during myogenesis is somewhat more complex. Mrf4 is expressed transiently in the mouse myotome at embryonic day 9.0 (E9.0), immediately following Myf5 expression. Its expression tapers by E11.5, and is reinitiated at E16.0 in differentiating muscle fibers. Thus, its complex temporal expression pattern suggests potential roles in both muscle determination and terminal differentiation. In myogenin-null embryos myogenesis can be partially rescued by a myogenin promoter-Mrf4 transgene (Zhu and Miller, 1997), supporting a role for Mrf4 in terminal differentiation. Furthermore, in embryonic stem (ES) cells lacking myogenin, fully differentiated muscle fibers can be generated by overexpression of Mrf4 (Sumariwalla and Klein, 2001), but not MyoD (Myer et al., 2001). Analysis of the role played by Mrf4 *in vivo* has been complicated by the fact that Mrf4 and Myf5 are located in tandem and expression of each gene is not completely independent of the other. A sophisticated series of Myf5 mutants have been generated, some of which express Mrf4 in the absence of Myf5. In Myf5:MyoD double-null mice whose Mrf4 expression is unaffected, skeletal muscle is indeed present (Kassar-Duchossoy et al., 2004) (see Table 1); this work clearly demonstrates a role for Mrf4 in the early stages of myogenesis, in addition to its role in terminal differentiation. For the Mrf4 gene three different lines of knockout mice have been generated, but they will be described more in detail in chapter 3.3 .

Gene Knockout	Phenotype	References
MyoD	<ul style="list-style-type: none"> ▪ No skeletal muscle defect ▪ Twofold increase in Myf5 transcript levels 	(Rudnicki et al., 1992)
Myf5	<ul style="list-style-type: none"> ▪ No skeletal muscle defect ▪ Die at birth for absence of the distal parts of the ribs 	(Braun, et al.1992)
MyoD/Myf5	<ul style="list-style-type: none"> ▪ Lethal ▪ Myogenesis impaired ▪ Mrf4 expression is unaffected 	(Kassar-Duchossoy, et al 2004)
Myogenin	<ul style="list-style-type: none"> ▪ Lethal ▪ Normal myoblast population ▪ Terminal differentiation impaired ▪ Mrf4 expressed at very low levels 	(Hasty et al.1993)
MRF4	<ul style="list-style-type: none"> ▪ No apparent skeletal muscle defect ▪ Fourfold increase in myogenin transcript levels ▪ The mice exhibit multiple rib anomalies 	(Zhang, et al. 1995)

TABLE 1. Different phenotypes of MRFs knockout models

Taken together, these studies suggest that MyoD and Myf5 are required for commitment to the myogenic lineage, whereas myogenin plays a critical role in the expression of the terminal muscle phenotype previously established by MyoD and Myf5, and Mrf4 partly subserves both roles (Fig. 10).

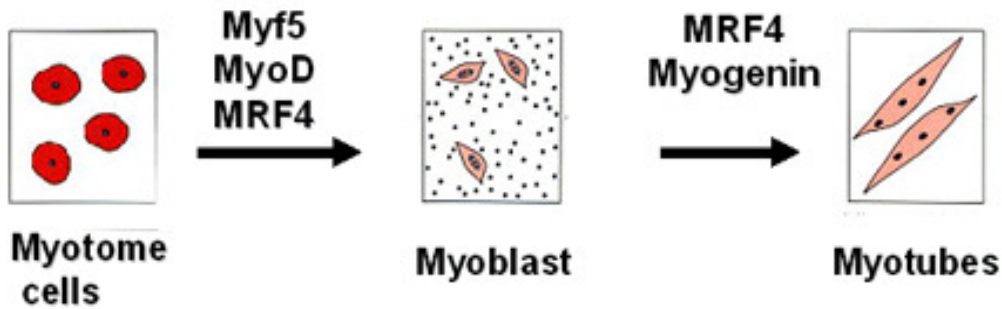


Fig. 10. Distinct and overlapping functions played by four MRFs. MyoD and Myf5 are “commitment” factors, whereas myogenin is a “differentiation” factor, and MRF4 has aspects of both functions.

3.2. The MRFs in adult muscle

Although the role of the myogenic regulatory factors during embryonic myogenesis has been established in numerous studies, their role in adult skeletal muscle is less known. A specific role of some of the myogenic factors in the terminal differentiation of fast fiber and slow fiber type has been suggested. Hughes et al observed preferential expression patterns of MyoD and myogenin in adult rat muscles. Myogenin mRNA was high in the soleus muscle, being composed mainly of slow-twitch fibers, while MyoD transcripts were found to be predominant in fast-twitch muscles (Hughes et al., 1993). Cross re-innervation of the soleus muscles with a fast nerve induced a slow to fast transformation and led to a reduction in myogenin mRNA (Hughes et al., 1993). High expression levels of MyoD in fast twitch muscles were documented also by studies on transgenic mice expressing the lacZ gene under the control of MyoD promoter. Moreover, the amount MyoD mRNA was shown to increase in rat soleus muscle under the influence of elevated thyroid hormone levels (Hughes et al., 1993). In 1997 Krauss and Pette quantified the transcript

levels of the myogenic regulatory factors MyoD, myogenin and Mrf4 by quantitative RT-PCR (reverse-transcriptase polymerase chain reaction) (Kraus and Pette, 1997). They confirmed previous data from Hughes, showing that myogenin and MyoD mRNAs were inversely distributed in slow and fast muscles, and they observed that there's a correlation between the levels of MyoD and MyHC 2B, the fastest MyHC isoform.

3.3. Mrf4

The Mrf4 gene is located only 8Kb 5' of Myf5 on mouse chromosome 10. The proximity of Mrf4 and Myf5 to each other on the locus raises the possibility of cis-regulatory interactions. The Mrf4/Myf5 locus has an extremely complicated enhancer organization, and is controlled by a multitude of interdigitated enhancers that activate gene expression at different times and in precisely defined progenitor cell populations. In the absence of the Myf5 minimal promoter, enhancers operating in the arches, the limb or the brain, engage the Mrf4 promoter; on the other hand deletion of the Mrf4 promoter affects transcriptional initiation at Myf5 promoter (Carvajal et al., 2008).

To study the role of Mrf4 three different knockout alleles were generated by the laboratories of Arnold, Olson and Wold, each designed by deleting part of the protein coding sequence and each mutation concomitantly inserted in the same PGK-neo selection cassette (Braun et al., 1994; Patapoutian et al., 1995; Zhang et al., 1995). Although all three alleles abolished Mrf4 expression, the Olson allele is the only mutation for which homozygotes survive to adulthood with high penetrance (see Table 2). These Mrf4-null animals have normal muscles, but display a five-fold elevation of myogenin RNA, and an even more important elevation of MyoD and Myf5 proteins, suggesting that other MRFs may compensate for the absence of Mrf4 (Thompson et al., 2005; Zhang et al., 1995).

In the embryo myotomal myogenesis is disrupted in homozygotes of the Arnold and Wold alleles. It is known that Myf5 homozygous nulls display a severe myotomal deficiency prior to E10.5 but later in development this deficiency is overcome by a compensation of MyoD and

myogenin and newborn mice have an almost normal muscle phenotype. Moreover this mice die at birth for the defects of rib (Braun et al., 1994). The Arnold *Mrf4* allele mimics the *Myf5*-null phenotype. The Wold *Mrf4* $^{-/-}$ allele during embryogenesis have a significant myotomal defects that corresponds spatiotemporally with the *Mrf4* expression in wild-type embryos; *Myf5* expressions in these animals begins normally at E8 but then falls during the period of co-expression with *Mrf4* (Patapoutian et al., 1995). Variable intensity rib defects characterize *Myf5* nulls, myogenin nulls, and all *Mrf4* alleles, and among the *Mrf4* alleles the severity of this phenotype appears to determine lethality due to respiratory insufficiency(see Table 2). *Myf5* is the earliest MRF to be expressed in the somite and its rib phenotype is by the far most severe, producing only short stubs (Braun et al., 1992) The *Mrf4* allele from Arnold group, showing a pronounced down-regulation of *Myf-5* transcription, gives the same phenotype with lack of distal rib structures (Braun et al., 1992) (see Table 2). The other two *Mrf4* alleles displays significant, though milder, rib pattern formation deficits, that include bifurcations and fusions of adjacent ribs. In homozygotes of the Olson *Mrf4* allele the join to the sternum is much more complete and they are viable and fertile(see Table 2). The rib anomalies associated with the *Mrf4* knockout are probably due to long-range *cis* effects of the neo cassette (Haldar et al., 2008; Kaul et al., 2000).

Although the role of *Mrf4* as commitment and differentiation factor during development is clear, not much is known about its role in adult skeletal muscle (Zhang et al., 1995).

It's well-known that *Mrf4* is the only MRF present at high levels in adult skeletal muscle, but there are contrasting opinions on its role in adult skeletal muscle.

As mentioned above, Hughes group suggested that the bHLH factors may regulate the specific patterns of isoform switching that accompany prenatal and postnatal muscle development (Hughes et al., 1993).

Genotype	<i>MRF4</i> ^{-/-}		
	Arnold	Wold	Olson
Lethality	Death at birth	Low penetrance survival to adulthood; death at birth	Viable
Major skeletal phenotypes	Rib stubs (<i>Myf5</i> phenocopy)	Malformed ribs; incomplete attachment to sternum	Malformed ribs; good attachment to sternum
Major newborn muscle phenotypes	Deep back muscle deficiency; decreased level of embryonic myosin heavy chain	Partial deficiency in intercostals; decreased level of embryonic myosin heavy chain	Grossly normal; decreased level of embryonic myosin heavy chain

TABLE2. Phenotype of MRF4 knockout.
Adapted from(Olson et al., 1996)

However the Olson *Mrf4*-null adult mice display normal expression of MyHC 2A, 2B and slow, thus indicating that *Mrf4* is not required for expression of muscle-specific genes at neonatal or adult stages (Zhang et al., 1995). However in situ hybridization studies have shown that in the gastrocnemius muscle, the *Mrf4* transcripts was preferentially expressed in slow fibers, present only in the deeper part of this muscle. In the soleus, which is composed almost exclusively of type I and type 2A fibers, there was no difference in expression between subpopulation of fibers (Walters et al., 2000). This study suggests again that *Mrf4* could play an important role in the regulation of fiber switching. More recently Hughes reported that in zebrafish muscle fibers *Mrf4* expression is pronounced in the region of slow muscle fibers during development (Hinits et al., 2007).

In contrast, previous studies demonstrated that *Mrf4* is expressed at high levels in adult skeletal muscles, but without any fiber-type specificity (Kraus and Pette, 1997). Quantitative RT-PCR and northern blot analyses have shown that *Mrf4* is expressed at relatively equivalent levels in several type of muscles, both slow and fast, and is the predominant MRF in the adult, supporting the hypothesis that *Mrf4* plays a crucial role in preserving the adult muscle phenotype (Kraus and Pette, 1997; Voytik et al., 1993). Moreover the expression of *Mrf4* is regulated by nerve activity: after 3 days of denervation by cutting of peroneal nerve, there is a significant and transient increase of *Mrf4* transcript level (about 12-fold)

accompanied by the accumulation of this transcription factor in the nucleus (Voytik et al., 1993; Weiss et al., 1999).

An interesting study on Mrf4-null mice comes from the group of Amy Thompson. They revealed a specific role of the Mrf4 factor in the regulation of the promoter of the voltage-sensitive channel Nav1.4. These channels underlie the propagation of regenerative action potentials in nerve and in muscle cells. In Mrf4 null mice the expression of the Na⁺ Nav 1.4 channels was substantially reduced (Thompson et al., 2005). Analysis of a Nav1.4 reporter gene have shown that Mrf4 is a positive regulator in C2C12 myotubes, and overexpression of Mrf4 can partially rescue the activity of the Nav1.4 reporter in Mrf4-null myotubes .

MATERIALS AND METHODS

1. Cell culture and transfection

HEK293 cells were maintained in culture in DMEM with 10% fetal bovine serum in a humidified incubator at 37 °C with 5% CO₂ and transfected using Lipofectamine2000 (Invitrogen) using the procedure recommended by the manufacturer.

2. Lysate preparation and Western blot analysis

HEK293 cells were lysed in RIPA buffer containing "complete" protease inhibitor cocktail (Roche, 1 tablet per 50 ml), 1 mM sodium orthovanadate, 50 mM sodium fluoride and sonicated for 5 seconds. For total muscle lysates, 25 slides (20 µm thick) of muscles were lysated in laemmli buffer (10% w/v glycerol, 5% w/v β-mercaptoethanol, 2,3% w/v SDS, 62,5 mM Tris-HCl containing "complete" protease inhibitor cocktail (Roche, 1 tablet per 1 ml). Homogenates were sonicated for 5 seconds. Muscle or cell lysates (30 µg) were heated in SDS sample buffer and subjected to SDS-PAGE and electrotransfer to nitrocellulose membranes. Membranes were then blocked in 50 mmol/l Tris·HCl, pH 7.5, 0.15 mol/l NaCl, and 0.1% Tween (TBST) containing 5% skimmed milk and probed for 16 h at 4°C in TBST, 5% skimmed milk. The MRF4, β-Tubulin and antibodies were diluted 1:5000; the GFP antibody was diluted 1:1000. Detection of proteins was performed using horseradish peroxidase-conjugated secondary antibodies (Biorad) and the enhanced chemiluminescence reagent.

3. RNAi mediated gene silencing

To induce RNA interference we used the pSUPER vector (from Oligoengine). All 19-mers were inserted in a backbone sequence GATCTCC-(sense19-mer)-TTCAAGAGA-(antisense 19-mer)-TTTTTGGAAA, to produce a 64-mer. This 64-mer was cloned into pSUPER vector, so that, once it is transcribed by the RNA polymerase III (pSUPER has a H1 promoter for RNA polymerase III), it forms a hairpin structure (Fig. 1), that is recognized by and cleaved by Dicer. The fragment produced is the 19-mer of double stranded RNA responsible for RNAi. To clone 64-mer into pSUPER, 2 μ l of 4 μ M oligos were incubated 30 min at 37°C in phosphorylation buffer, then were incubated 10 min at 70°C (heat inactivation on PNK). Then, 2 μ l of phosphorylated oligos were incubated in ligation buffer 1 hour at room temperature.

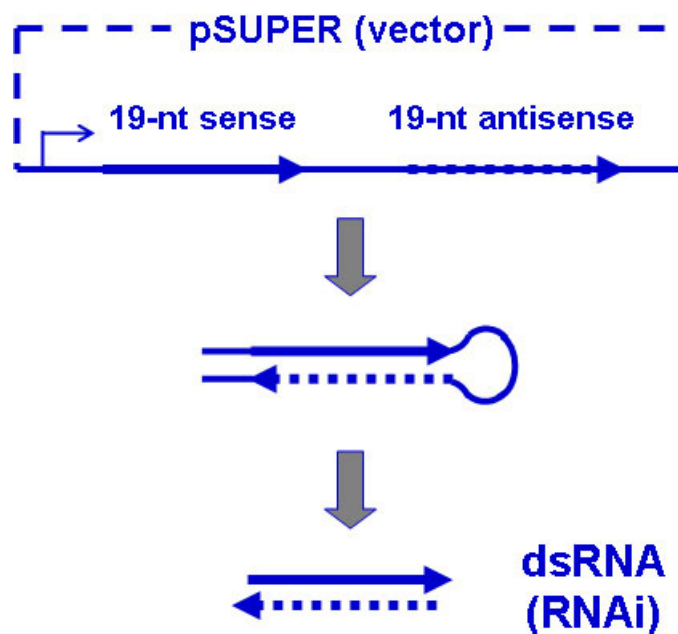


Fig.1. pSUPER induced RNAi.

The sequences of MRF4 gene have been retrieved and analysed. The target sequences have been selected from uncommon regions with the other MRFs. Target oligos were designed using the criteria defined by Reynolds et al., 2004 (Reynolds et al., 2004). We have selected 10 oligos on the basis of a specificity screening performed with BLAST analysis. The

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selected oligos have been cloned into the pSUPER vector or pSUPER-GFP vector. Evaluation of suppression efficiency has been performed in cell cultures (HEK293) and in adult rat muscles. We co-transfected Mrf4 cDNA together with each pSUPER. As a control, we used pSUPER constructs targeting the LacZ. We have selected at least two sequences with high silencing efficiency for each gene and performed every experiment with both of them.

4. *In vivo* transfection

For the transfection of adult muscles, Wistar rats (150-200g) were anaesthetized by intraperitoneal injection of a mixture of Zoletil 100® (a combination of Zolazepam and Tiletamine, 1:1, 10 mg/kg, Laboratoire Virbac) and Xilor® (Xilazine 2%, 0.06 ml/kg, BIO 98 Srl, Milan, Italy) or using an isoflurane vaporizer maintained 2% isoflurane, 2.5 liters/minute oxygen. Leg skin was opened in anaesthetized rats, *extensor digitorum longus* (EDL) and soleus muscles were exposed and injected with plasmid DNA (30 µg in saline). Injection was followed by electroporation with stainless steel electrodes connected to a ECM830 BTX porator (Genetronics, San Diego, CA) with the following settings: 5 pulses of 20 milliseconds each and 200 milliseconds interval, the voltage was adjusted according to the thickness of the muscle (220 V/cm). For the transfection of regenerating muscles, plasmid DNA (30 µg in 20% sucrose) was directly injected into the muscles at day 3 after bupivacaine treatment (Vitadello et al., 1994). Muscles were removed 7 or 14 days after transfection, frozen in liquid nitrogen cooled isopentane or processed for *in toto* fixation then stored at -80°C. Denervation was produced by cutting the sciatic nerve high in the thigh.

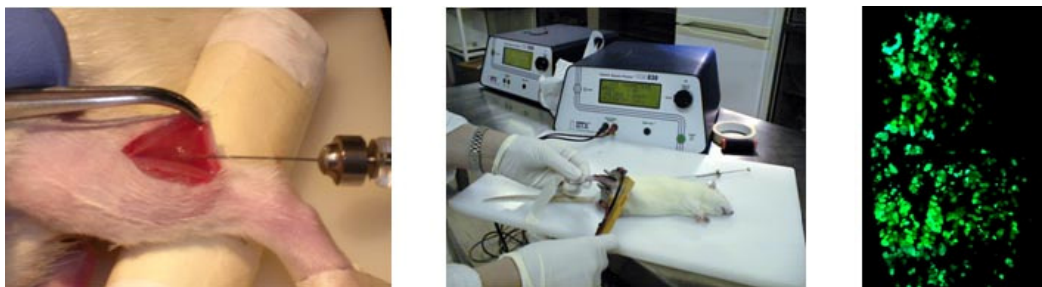


Fig.1. *In vivo* transfection of adult muscles. Injection of DNA in the soleus muscle (*left*) and electroporation (*centre*). Example of a muscle transfected with GFP (*right*), seven days after transfection.

5. Electrostimulations

Wistar rats (150-200g) were anaesthetized by intraperitoneal injection of a mixture of Zoletil 100® (a combination of Zolazepam and Tiletamine, 1:1, 10 mg/kg, Laboratoire Virbac) and Xilor® (Xilazine 2%, 0.06 ml/kg, BIO 98 Srl, Milan, Italy) or using an isoflurane vaporizer maintained 2% isoflurane, 2.5 liters/minute oxygen. Muscles are denervated by cutting a portion (about 10 mm) of the sciatic nerve high in the thigh, Teflon-covered stainless steel electrodes were implanted directly in the soleus muscles. Soleus and EDL muscles were then stimulated with a slow pattern (trains of 10-second duration and 20-Hz frequency given every 30 seconds) or fast pattern (trains of 0.6-second duration and 100-Hz frequency given every 60 seconds); (Tothova et al., 2006).

6. Muscle processing for *in toto* fixation

Muscles transfected with pSUPER.GFP vectors were removed 7 or 14 days after transfection and fixed *in toto* with PFA 2% for two hours. The muscles subsequently were treated with 5 to 20% sucrose gradient and frozen in liquid nitrogen cooled isopentane.

7. Histology immunohistochemistry and fiber size measurements

Muscle cryosections, 10 µm thick, were processed for immunostaining. For MRF4 staining, cryosections were fixed with methanol and incubated with the antibody at room temperature for 1 hour. The sections were treated with 0,2%Triton. Cy3-labeled second antibodies were purchased from Jackson Laboratories. Controls were performed without primary antibodies or by pre-incubating the primary antibody with the specific immunizing peptide at room temperature for 2 hours under gentle shaking. A mock incubation was performed in parallel. The antibody was then used to process cryosections under standard conditions.

For MyHC slow staining, sections were incubated with the primary antibody (BA-D5(Schiaffino et al., 1989)) for 1 hr at room temperature then washed 3 times with PBS buffer 5 min each, incubated with secondary

antibody for 1 h at room temperature, then washed 3 times with PBS 5 min each. Finally, sections are mounted with elvanol.

Images were collected with an epifluorescence Leica DMR microscope equipped with a Leica DFC300 digital charge-coupled device camera by using Leica DC Viewer software.

Fiber cross-sectional areas were measured by using Image J 1.33u software (National Institutes of Health). All data are expressed as the mean SEM (error bars). Comparisons were made by using *t* test, with $P < 0.05$ being considered statistically significant.

8. Antibodies

The following antibodies were purchased from Santa Cruz BioTechnology: Myf6 C-19 rabbit polyclonal (sc-301 X); β -Tubulin rabbit polyclonal (sc-9104), Green Fluorescent Protein rabbit polyclonal (sc-8334). The immunizing peptide for sc-301 was also from SCBT.

9. Quantitative real time PCR

For quantitative Real Time-PCR assays, total RNA was purified [SV Total RNA Isolation, Promega, Madison, WI] and characterized by electrophoresis [Agilent, Santa Clara, CA]. 400 ng of RNA was converted to cDNA using random hexamers and Superscript II [Invitrogen, Carlsbad, CA]. Amplification was carried out in triplicates with an IQ5 real time PCR system [Bio-Rad, Hercules, CA] using SYBR green chemistry [IQ SYBR Green Supermix, Bio-Rad, Hercules, CA] and a standard 2-step protocol. Each experiment was performed at least twice, on two individually prepared cDNAs.

The primers specific for MRF4 were designed and analyzed with Primer3 [freeware] and Vector NTI [Invitrogen, freeware]. Identity of the amplicons was confirmed by their dissociation profiles and gel analysis.

Quantitative PCR standard curves were constructed by using serial dilutions of pooled cDNAs of the analyzed samples, using at least 4 dilution points and the efficiency of all primer sets was between 94 and 105%.

The data were normalized against Tbp and GusB using the geNorm software (<http://medgen.ugent.be/genorm> Ghent University Hospital Center for Medical Genetics).

10. Luciferase assay

For luciferase assay was used Dual luciferase kit E1960, Promega Corp., Madison WI, U.S.A. Muscles were crushed with a pestle and mortar cooled with liquid nitrogen. Powder was weighted and added with 2.5 µl of lysis buffer each mg of tissue. Lysates were frozen with liquid nitrogen and thawed at 4°C (twice). Lysates were then centrifuged for 20 min at 13.000 rpm at 4°C, and supernatants were collected. 10 µl of supernatant were added to 100 µl of LarII buffer, and Firefly luciferase activity was measured. Stop and Glow buffer was then added, and Renilla luciferase measured.

11. Plasmids

The plasmids were prepared using the procedure recommended by the manufacturer of kit Quiagen maxiprep purchased from Invitrogen.

Plasmid	Vector	Promotor	Expressed gene	Reference
MyHC-slow 1145bp	pxp2	1145bp of the MyHC-slow promoter	Luciferase	(Hasegawa et al., 1997)
MyHC-2B	pGL3	MyHC-2B promoter	Luciferase	(Swoap, 1998)
Rat Mrf4	pEMSV	EMSV	Mrf4	(Sabourin and Rudnicki, 2000)
Mouse Mrf4	pCDNA3	CMV	Mrf4	(Miner and Wold, 1990)
Human Mrf4	pCMV6	CMV	Mrf4	Origene
pCDNA3	pCDNA3	CMV	-----	Invitrogen

Tk-Renilla	pGL4	RSV	Luciferase	(Gorman and Hermann, 1982)
pSUPER	pSUPER-basic	H1	shRNA	Ambion

12. Statistical analysis

All data in bar graphs are expressed as mean values. Error is always indicated as standard error (SE), calculated as the ratio between standard deviation and square root of number of data minus one. Student's t test was used to assess the significance of data ($p < 0.05$).

RESULTS

1. Expression of Mrf4 in adult skeletal muscle

1.1. Mrf4 is expressed at similar levels in slow and fast rat skeletal muscles

Since from previous works there are controversial indications about the levels of expression of Mrf4 in slow versus fast muscles (compare Walters Loughna 2000, Kraus Pette 1997), we examined the distribution of Mrf4 at the mRNA and protein levels in rat soleus and extensor digitorum longus (EDL), a slow and a fast twitch muscle, respectively. We used the highly sensitive technique of the real-time PCR to quantify the transcripts levels, and western-blot analysis for the proteins. As shown in Fig. 1A, the level of the Mrf4 mRNA was similar in adult fast and slow muscles and was much higher than in fetal (E18) thigh muscles. Mrf4 protein levels are also similar between fast and slow muscles (Fig. 1B)

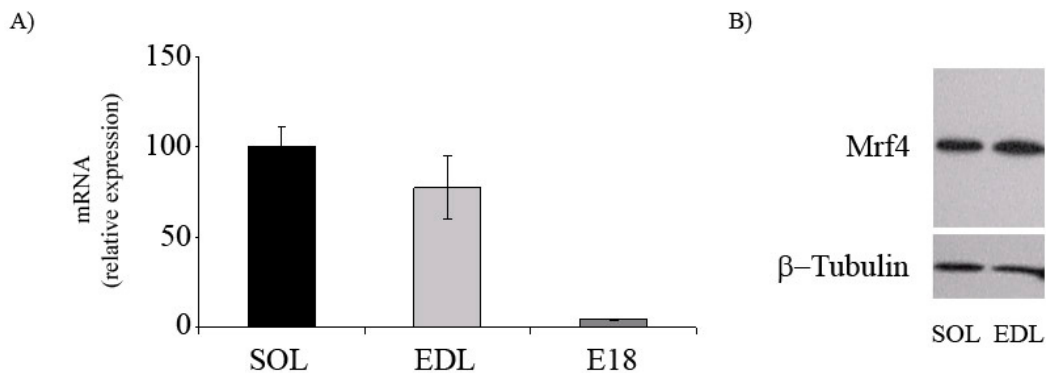


Fig. 1. Mrf4 expression in adult rat skeletal muscle. *A)* RT-PCR on RNA prepared from adult soleus, EDL and 18 days embryo muscles was performed and normalized to the expression of two housekeeping gene RNAs using the geNorm software (<http://medgen.ugent.be/genorm> Ghent University Hospital Center for Medical Genetics). The expression of Mrf4 is strongly upregulated in adult muscles as compared to the embryo, however there is no significant difference between soleus and EDL. Data are means; $n=8$ for each group. *B)* Western blot analysis on adult soleus and EDL whole protein extracts with an antibody specific for Mrf4 (sc-301) or for β -Tubulin (sc-9104) to normalize loading levels. Mrf4 protein levels are similar in adult soleus and EDL muscles.

1.2.Mrf4 has a different localization in slow and fast muscles

We verified specificity of the MRF4 antibody through the use of the specific peptide or a scramble peptide (Fig.2), then we analyzed the localization of Mrf4 in soleus and EDL muscles. Although it is expressed at comparable levels, Mrf4 display a different localization in slow and in fast muscles: in soleus Mrf4 is mainly localized in the nuclei, conversely in EDL it is mainly cytosolic, but not excluded from nuclei (Fig.3).

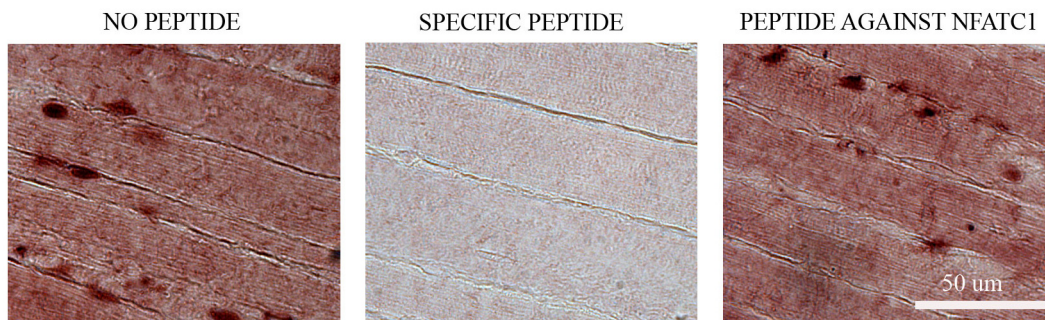


Fig.2. Mrf4 antibody specificity. Longitudinal section of EDL muscle. The primary antibody was pre-incubating with the specific immunizing peptide or with the peptide against NFATc1 (scramble). Only the specific peptide completely blocks the labeling of MRF4 antibody.

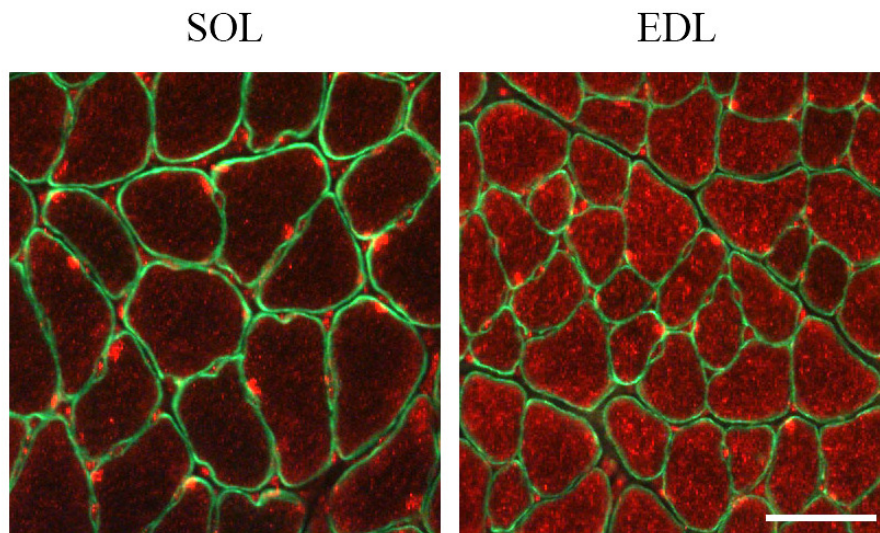


Fig.3. Mrf4 localization in adult rat skeletal muscle. Transversal sections of soleus and EDL muscles were stained with antibodies against Mrf4 (red) and dystrophin (green). Mrf4 localization is clearly nuclear in soleus muscle, while it is more cytosolic in edl muscle . Scale bar= 50µm.

1.3.Mrf4 localization is dependent on nerve activity

Previous works have demonstrated that Mrf4 transcript levels increase after denervation (Kraus and Pette, 1997; Voytik et al., 1993). To study Mrf4 mRNA expression after denervation, we performed a quantitative PCR analysis on the transcript from muscles collected at different time points (1, 3 and 7 days) after sciactomy. We show that Mrf4 mRNA increases until the third day after denervation and then it start to decrease (Fig.4), however the range of up-regulation of Mrf4 is greater in edl muscle (8 fold) than soleus muscle (2,5 fold).

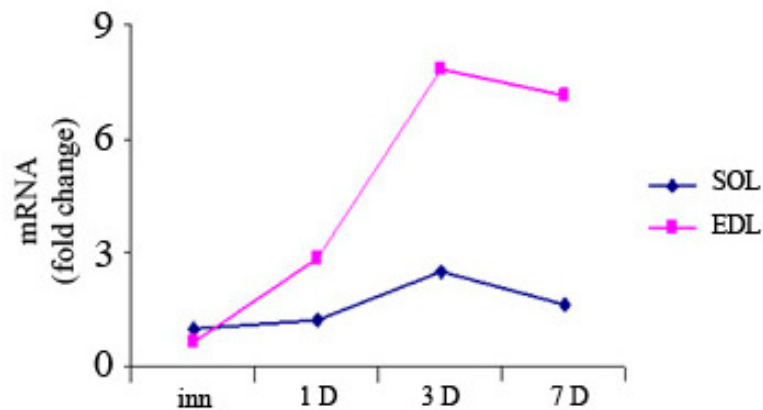


Fig.4. Time course of Mrf4 expression after denervation. RT-PCR on RNA prepared from adult soleus, EDL muscles was performed and normalized to the expression of the two housekeeping genes using the geNorm software (<http://medgen.ugent.be/genorm> Ghent University Hospital Center for Medical Genetics). The mRNA expression of Mrf4 is strongly up-regulated in EDL muscles denervated for 3 days as compared to soleus muscles. Data are means; $n=4$ for each group.

To understand if, similarly to other transcription factors, Mrf4 displays an activity dependent nucleo-cytoplasmic shuttling, we have studied the localization of this protein after three days of denervation, or after 2 hours of inactivity, electrostimulation for 2 hours with a slow (20 Hz) or a fast (100 Hz) pattern. The slow and fast patterns reproduce the trains of impulses characteristic of slow and fast motor units respectively (Hennig and Lomo, 1987). We observed that after 3 days of denervation there is also an accumulation of Mrf4 in muscle nuclei, that is more evident in edl muscle (Fig.5).

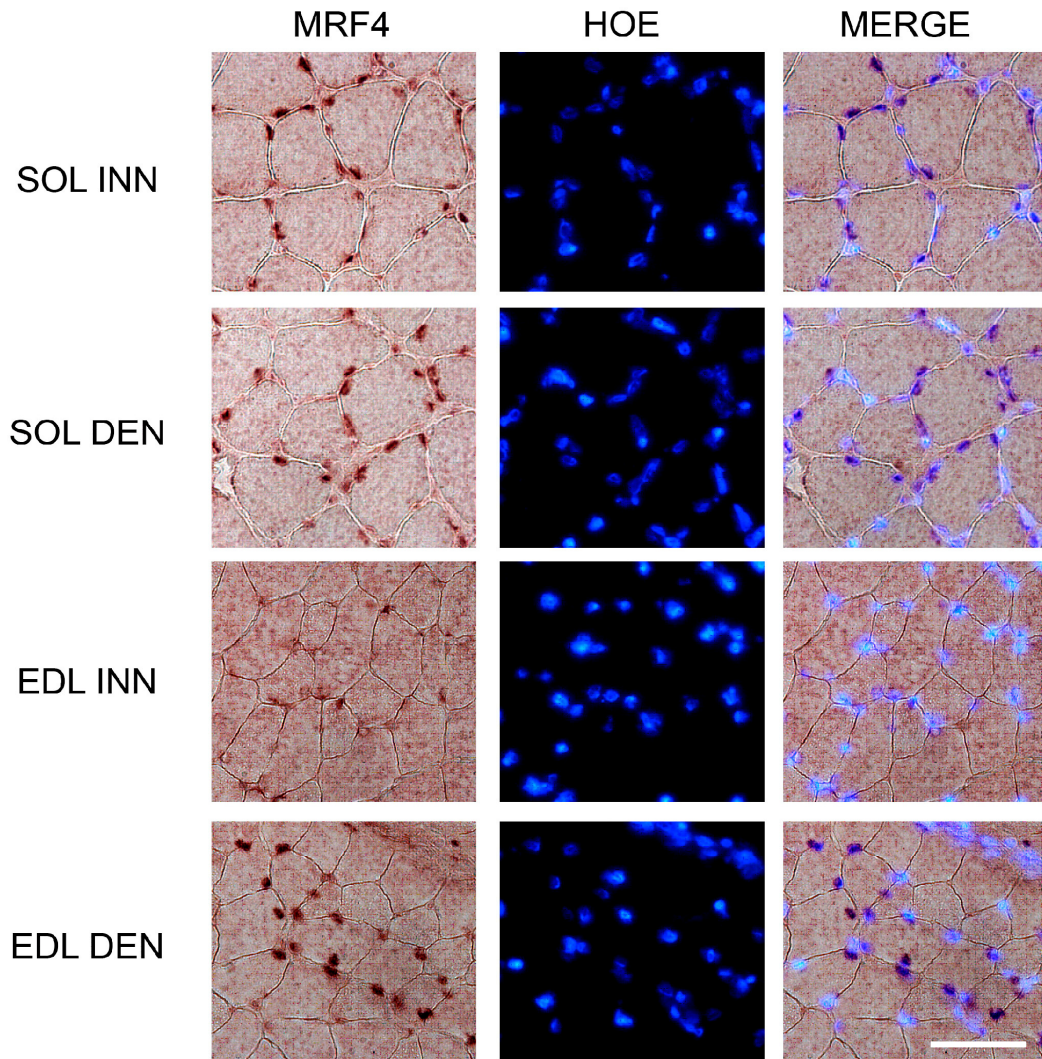


Fig.5. Mrf4 localization after 3 days of denervation. Transversal section of innervated and denervated soleus and EDL muscles. Mrf4 purple staining is nuclear in innervated soleus and largely cytoplasmic in innervated edl; after three days of denervation Mrf4 is nuclear both in soleus and edl muscles. Hoechst nuclear staining is blue. Scale bar = 50µm

The situation is different if the observations are done only after 2 hours of inactivity, slow or fast elettrostimulation, through the peroneal nerve. In EDL muscles after 2 hour of inactivity any relevant change in localization occurs as compared to innervated muscles, or to the fast/100 Hz condition. However the slow/20 Hz stimulation rapidly induces Mrf4 accumulation in the nuclei of EDLmuscles (Fig.6).

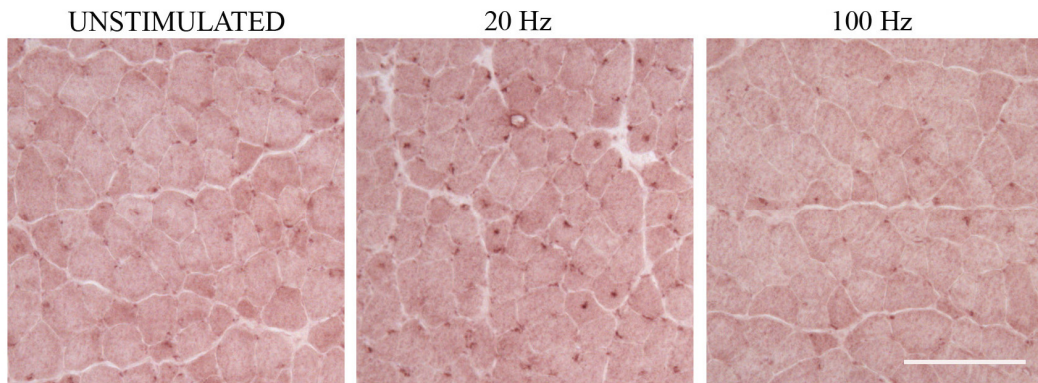


Fig.6. Mrf4 localization after 2 hours of slow or fast elettrostimulation. Croos-section of EDL electrically stimulated through the peroneal nerve with patterns of impulses at 20 Hz and 100 Hz. Contralateral EDL was used as unstimulated controls . In control muscle and in EDL muscle stilmulated with 100Hz pattern Mrf4 is largely cytoplasmic; Mrf4 translocates in the nuclei under 20Hz-slow stimulation. Scae bar= 100µm.

2. Mrf4 knockdown by RNAi: validation *in vitro* and *in vivo*

To study the function of Mrf4 in adult skeletal muscle we used an *in vivo* RNAi approach based on a shRNA expression vector. We designed at least 5 oligos on the basis of a theoretical score based on Reynolds criteria (Reynolds et al., 2004) and we performed a specificity screening with BLAST analysis. We cloned the selected oligos into pSUPER or pSUPER.GFP vectors (from Oligoengine). To evaluate the efficiency of silencing of different oligos, we co-transfected HEK293 cells with the rat Mrf4 cDNA and the pSUPER targeting Mrf4 or LacZ, used as a control. RNAi-mediated knockdown of Mrf4 was determined by immunoblotting; we selected two sequences (M1 and M2) with high silencing efficiency for further studies (Fig.7).

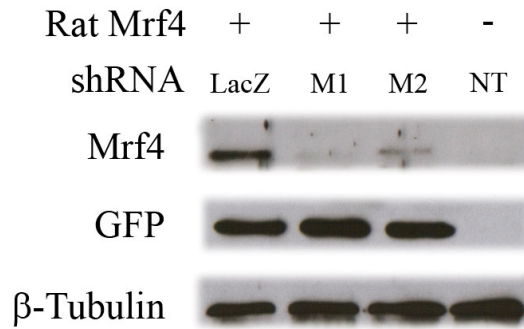


Fig.7. *In vitro* RNAi-mediated knockdown of Mrf4. HEK293 were co-transfected with the rat Mrf4 cDNA and the pSUPER-GFP constructs against LacZ, as control, or against Mrf4 (M1 and M2). NT is the negative control with untransfected cells. β -tubulin labelling shows that the proteins were loaded in equal manner. GFP labelling shows the efficiency of transfection. M1 is more efficient than M2.

The efficacy of these shRNAs in blocking the expression of endogenous Mrf4 was confirmed *in vivo* by co-transfection of adult soleus muscles with the corresponding pSUPER constructs and SNAP-GFP. As shown in Fig. 8, the characteristic staining of muscle fiber nuclei with anti-Mrf4 antibody in the non-transfected fibers (GFP negative) was almost completely abolished in transfected fibers (GFP positive) (Fig.8).

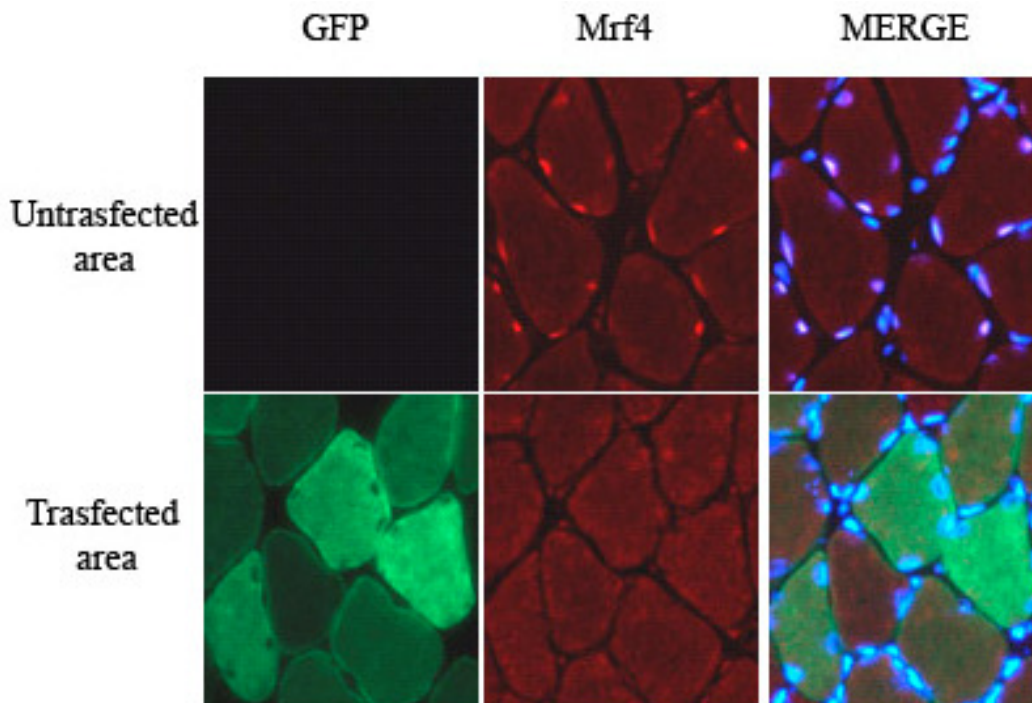


Fig.8. *In vivo* RNAi-mediated knockdown of Mrf4. Adult soleus muscles were co-transfected with SNAP-GFP and Mrf4 shRNAs. Immunostaining with an anti-Mrf4 antibody (red) shows that in the GFP positive fibers there is no expression of Mrf4.

3. Effect of Mrf4 knockdown and overexpression on fiber size

To determine the role of Mrf4 in the control of muscle fiber phenotype we have examined the effects of Mrf4 knockdown in adult and in regenerating rat muscles. At first we pointed our attention to changes in fiber.

3.1. Mrf4 silencing induces muscle hypertrophy in adult muscle

We co-transfected adult rat soleus muscle with the strongest shRNA targeting Mrf4 (M1) or LacZ, as a control, and the SNAP-GFP. Muscles were dissected after 7 and 14 days. Morphometric analyses of cross-sectional areas show that Mrf4 knockdown induces a significant increase (about 30%) in fiber size at day 7 after transfection (Fig. 9A) and an even greater hypertrophy, with a three-fold increase in cross-sectional area, at day 14 (Figs. 9 B,C).

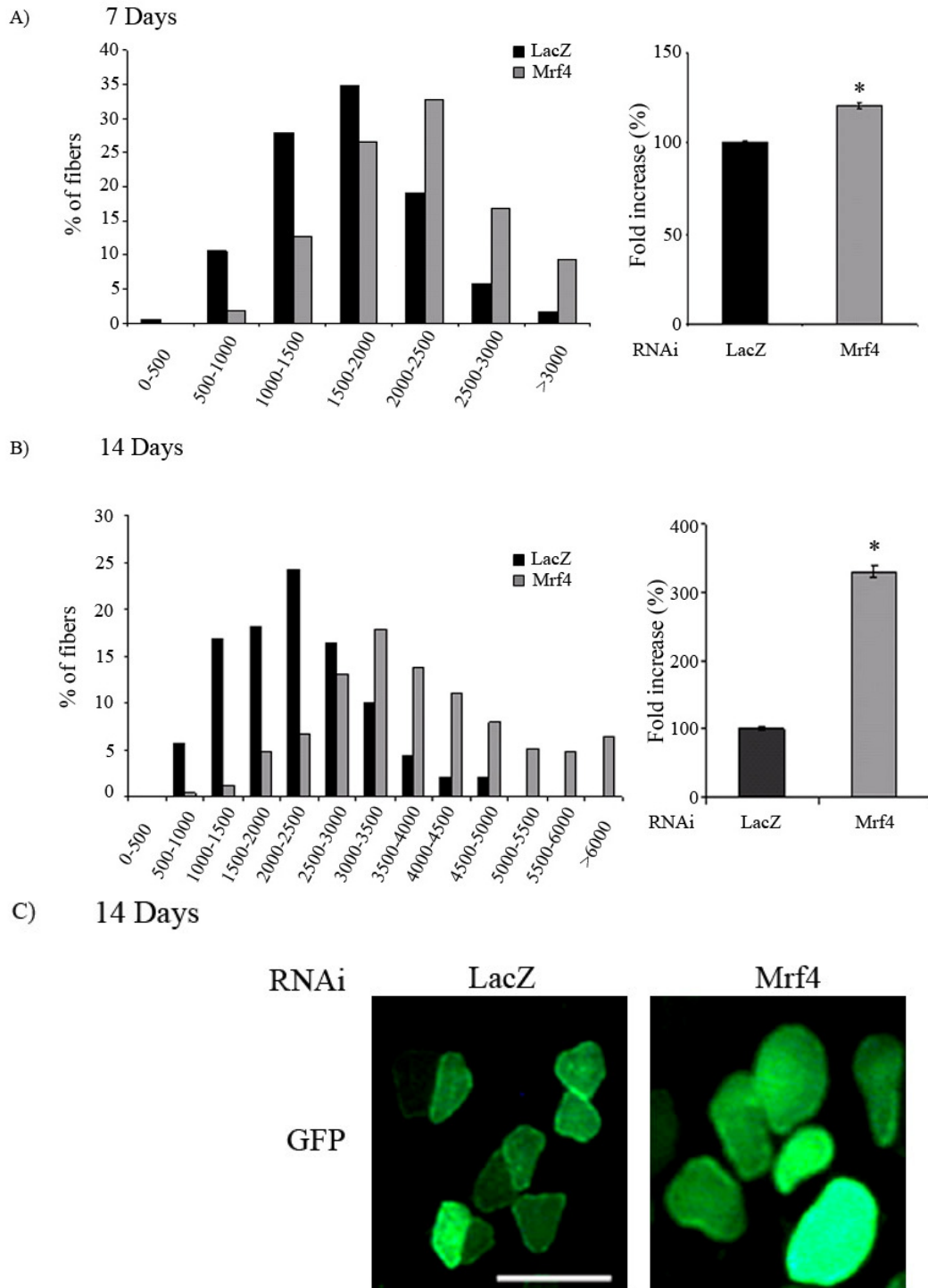


Fig.9. Mrf4 silencing induces fiber hypertrophy in adult muscle. A),B) Adult innervated rat soleus muscles were co-transfected with SNAP-GFP and shRNAs constructs. Muscles were analysed 7 (A) or 14 (B) days after transfection. In left panel histograms show the distribution of cross-sectional areas (μm^2) of fibers expressing shRNAs targeting Mrf4 (grey bars) or LacZ (black bars). In the graphs on the right changes in fiber size are expressed as mean \pm SEM % of fold increase. C) Transverse sections of adult soleus muscles co-transfected with SNAP-GFP and shRNAs for LacZ (left panel) or Mrf4 (right panel) were analysed for GFP fluorescence 14 days after transfection. Scale bar= 50 μm .

3.2. Mrf4 knockdown induces muscle hypertrophy in regenerating muscle

We also examined the effect of Mrf4 silencing in regenerating innervated soleus muscle, using a model in which muscle injury is induced by bupivacaine injection and plasmids are transfected at day 3 after injury without electroporation (Vitadello et al., 1994). Muscles were dissected after 10 days from injury. In this experimental model muscle growth was accelerated by Mrf4 knockdown, with a two-fold increase in cross-sectional area at day 7 after transfection (Fig. 10 A, B).

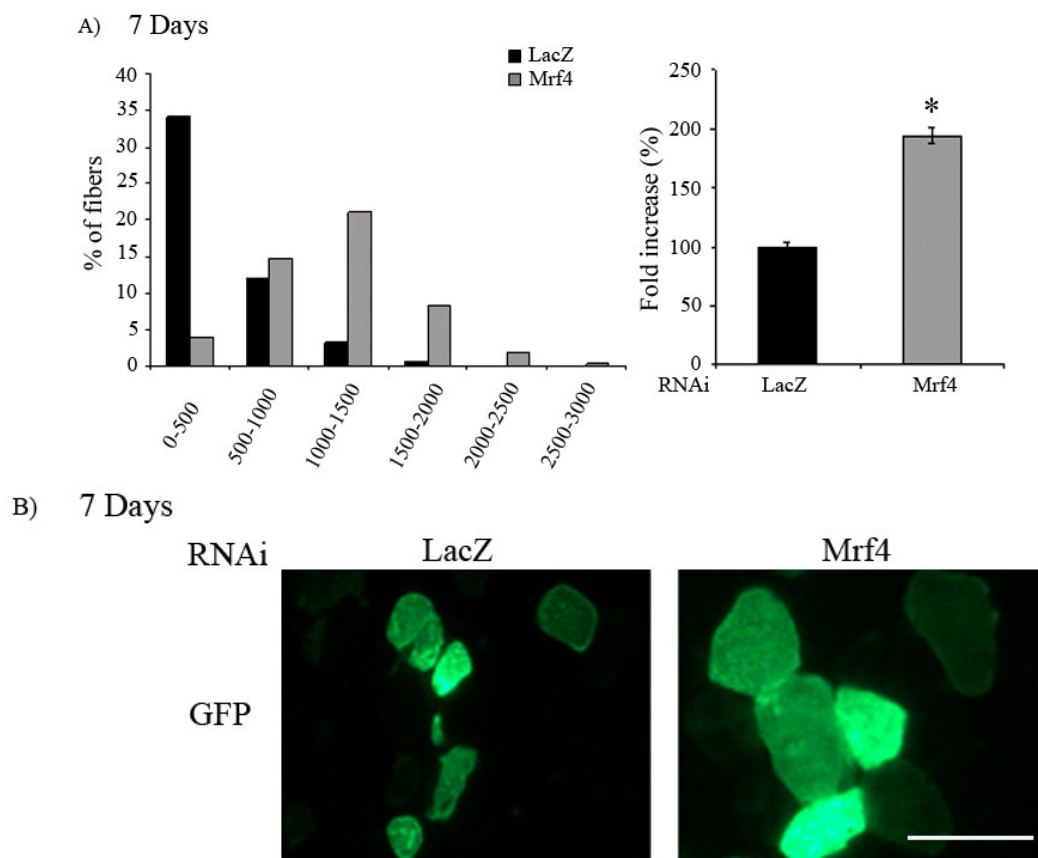


Fig.10. Mrf4 knockdown induces fiber hypertrophy in regenerating muscle Regenerating innervated rat soleus muscles were co-transfected with SNAP-GFP and shRNAs constructs. Muscles were analysed 7 days after trasfection (10 days from bupivacaine injection). A) In left panel histograms show the distribution of cross-sectional areas (μm^2) of fibers expressing shRNAs targeting Mrf4 (grey bars) or LacZ (black bars). In the graph on the right changes in fiber size are expressed as mean \pm SEM of % of fold increase. B) Transverse sections of regenerating soleus muscles co-transfected with SNAP-GFP and shRNAs for LacZ (left panel) or Mrf4 (right panel) were analysed for GFP fluorescence 7 days after trasfection. Scale bar= 50 μm .

3.3. Mrf4 knockdown prevents denervation atrophy

Next, we asked whether Mrf4 knockdown could contrast muscle atrophy induced by denervation in adult muscles. We performed morphometric analyses of denervated soleus and EDL muscles transfected with pSUP.GFP against Mrf4 or LacZ, as control, after 7 days from electroportation and denervation. As shown in Fig. 11, both in soleus (Fig.11A) and edl (Fig.11B) muscles, the degree of muscle atrophy was markedly reduced in transfected fibers with shRNAs targeting Mrf4 versus the fibers transfected with control plasmid or untransfected fibers. This effect was seen at 7 days after denervation and was especially prominent with the M1 construct, although it was significant also with M2 shRNA (Fig.11A-B).

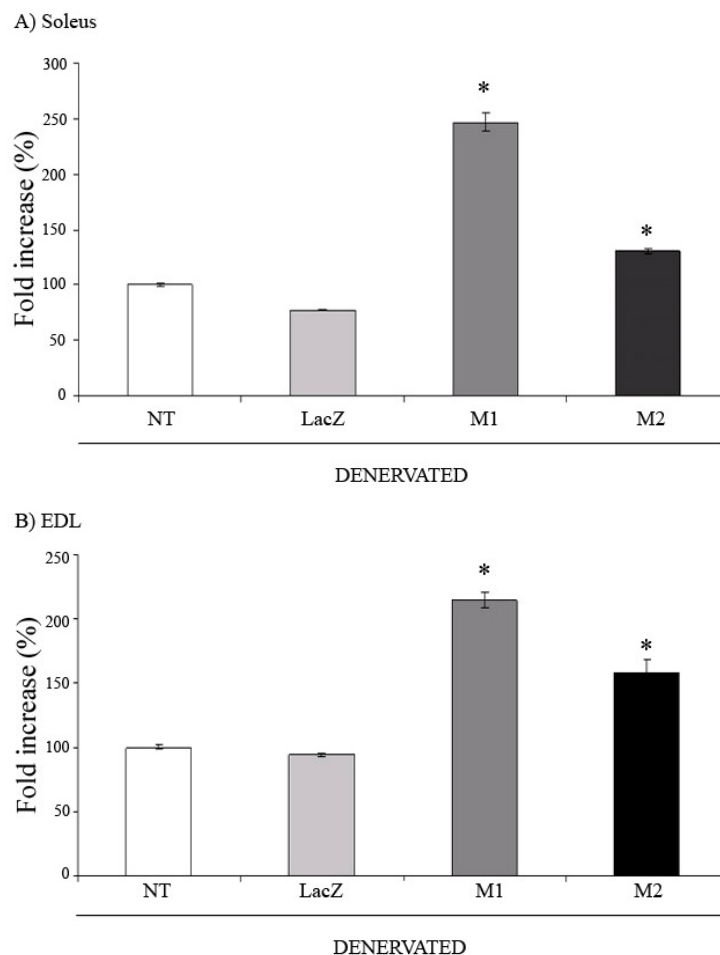


Fig.11. Mrf4 knockdown prevents denervation atrophy. A).B) Adult rat denervated soleus and edl muscles were transfected with the pSUPER.GFP vector targeting Mrf4 (M1 and M2) or LacZ, as a control. Transverse cross-sectional areas have been analysed 7 days after transfection and denervation. We compared the cross-sectional areas of untransfected fibers (NT), fibers expressing shRNA for LacZ, and fibers expressing the two shRNAs for Mrf4 (M1 and M2). The changes in fiber size are expressed as mean \pm SEM of % of fold increase.

We have also demonstrated that the silencing of *Mrf4* not only prevents denervation atrophy, but results in hypertrophy. Indeed the cross-sectional area of fibers transfected with M1 shRNA, after 7 days of denervation, is larger as compared to innervated untransfected fibers or denervated transfected with the control plasmid (Fig. 12 A). The hypertrophy is more important after 14 days of denervation (Fig.12A). Although to a lesser extent, also M2 shRNA leads to the same significant results (Fig 12B). It has to be considered that this construct has a lower efficiency of silencing.

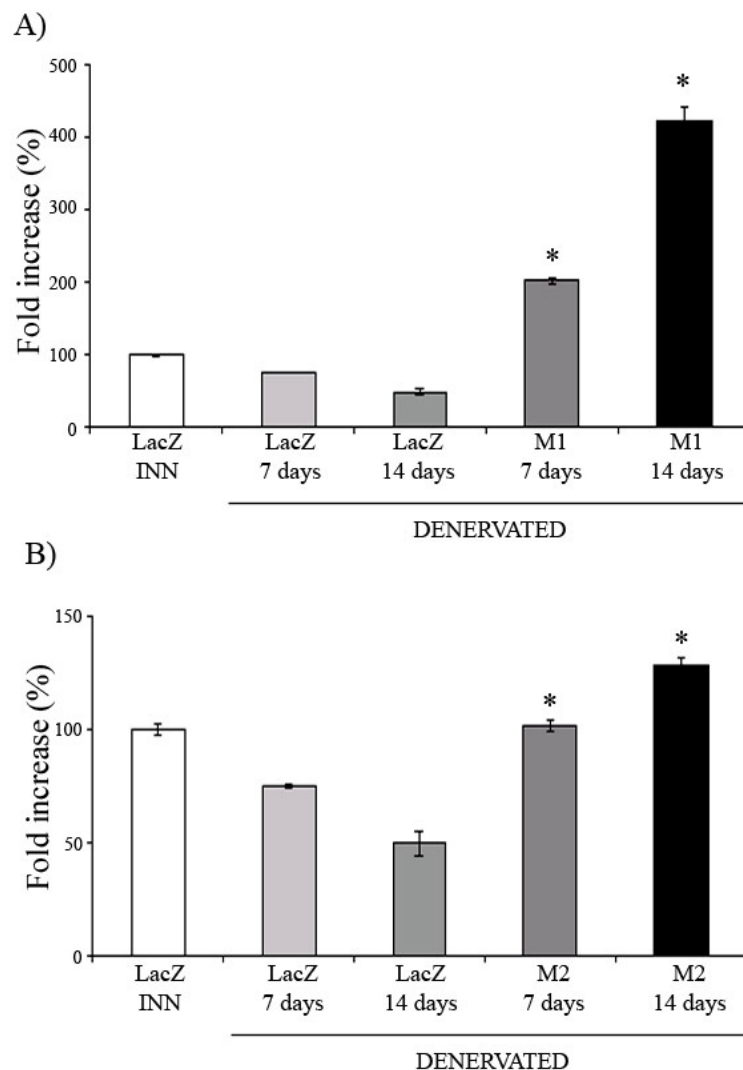


Fig.12. The silencing of *Mrf4* not only prevents denervation atrophy, but results in hypertrophy. Adult denervated soleus were transfected with the pSUPER.GFP -M1, -M2 or -LacZ constructs. Morphometric analyses were performed 7 days after denervation and transfection. We compared innervated fibers expressing the anti-LacZ shRNA with fibers from denervated muscles expressing the same control shRNA or the anti-*Mrf4* constructs. The changes in fiber size are expressed as mean \pm SEM of % of fold increase.

3.4. The effect of Mrf4 knockdown on muscle fiber size in denervated rat soleus is rescued by mouse or human Mrf4.

To exclude off-target effects of RNAi approach, we performed two different rescue experiment. First, we co-transfected, in HEK293 cells, the human Mrf4 with the rat and mouse specific shRNA M1, or the mouse Mrf4 cDNA and the rat specific shRNA M2. By immunoblotting analyses, we demonstrate the species specificity of the pSUPER construct (Fig. 13 A-B). In effect, the M1 shRNA fails to abolish the expression of the human Mrf4 and protein (Fig. 13 A), and the shRNA M2 fails to abolish the expression of mouse Mrf4 protein (Fig. 13 B)

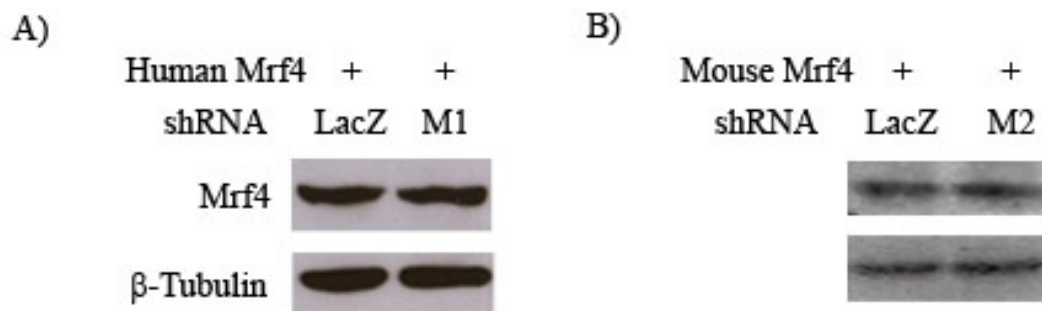


Fig.13. Species specificity of shRNA against Mrf4. A) HEK293 cells were co-transfected with the human Mrf4 cDNA and the shRNA M1. Western blot immunolabeling with anti-Mrf4 shows that the pSUPER-GFP-M1 fails to abolish the expression of the Mrf4 protein. B) HEK293 cells were co-transfected with the mouse Mrf4 cDNA and the shRNA M2. Western blot immunolabeling with anti-Mrf4 shows that the shRNA M2 fails to abolish the expression of the Mrf4 protein.

Then we used the mouse Mrf4 or human Mrf4 to rescue the hypertrophic effects of shRNA in rat muscles. We co-transfected adult denervated soleus with the M1 shRNA construct and the human Mrf4 cDNA or pcDNA3 vector, as control. Alternatively we co-transfected M2 construct with mouse Mrf4 cDNA. In all these experiments SNAP-GFP was co-transfected to identify transfected fibers. Analyses done 7 days of transfection show that the prevention of denervation atrophy mediated by Mrf4 knockdown, is abolished by overexpression of mouse or human Mrf4 (Fig. 14 A-B).

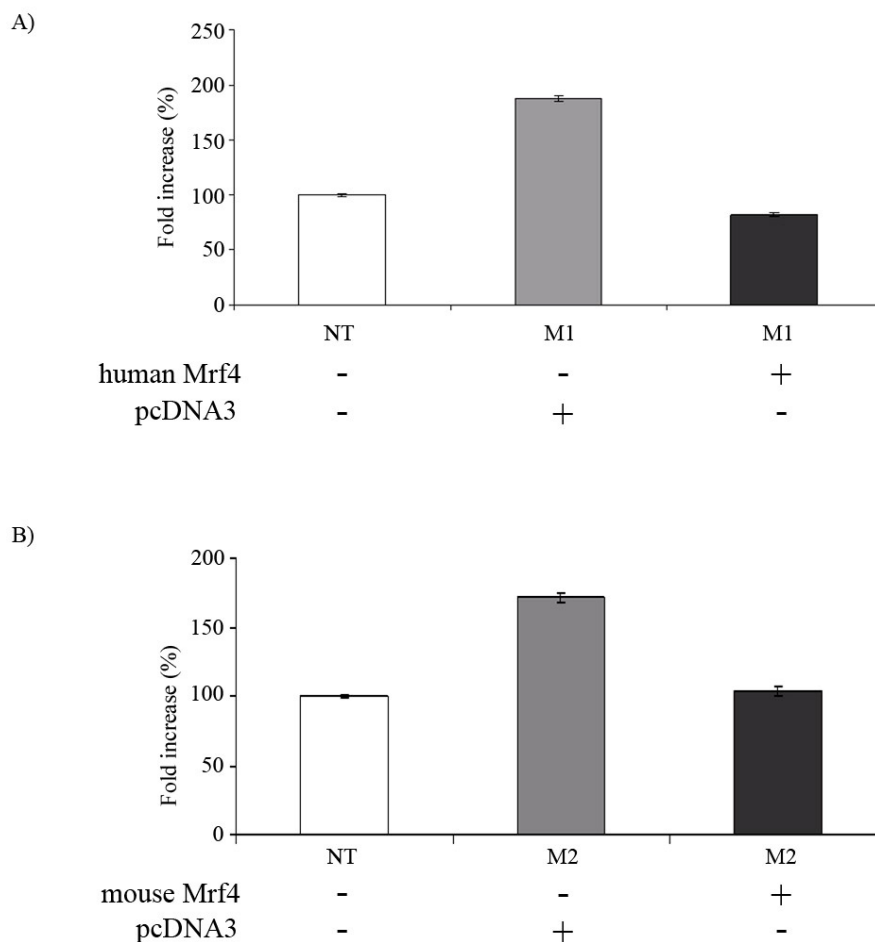


Fig.14. The effect of Mrf4 knockdown is rescued by mouse or human cDNA. A) Adult rat denervated soleus were co-transfected with the M1 pSUPER.GFP constructs with pcDNA3, as control, or with human Mrf4 cDNA. The overexpression of the human Mrf4, which is unaffected by the M1 shRNA construct, completely prevents the increase of fiber size induced by the knockdown of Mrf4. B) Adult rat denervated soleus were co-transfected with the M2 pSUPER.GFP constructs with pcDNA3, as control, or with mouse Mrf4 cDNA. The overexpression of the mouse Mrf4, which is unaffected by the M2 shRNA construct, completely prevents the increase of fiber size induced by the knockdown of Mrf4. A), B) We compared the cross-sectional area of transfected fibers with the area of untransfected fibers. Values are expressed as mean \pm SEM of % of fold increase.

3.5. Mrf4 overexpression does not change fiber size in adult muscle

To confirm the results obtained with RNAi we went to the opposite approach: we evaluated the effects of overexpression of MRF4 in adult and regenerating muscles.

We examined the effect of Mrf4 overexpression in adult soleus muscle by co-transfecting SNAP-GFP with Mrf4 cDNA or pcDNA3, as control. After 7 days, we measured the cross-sectional area of transfected fibers. As shown in Fig. 15, in adult muscles there are no differences between fibers overexpressing Mrf4 and the controls.

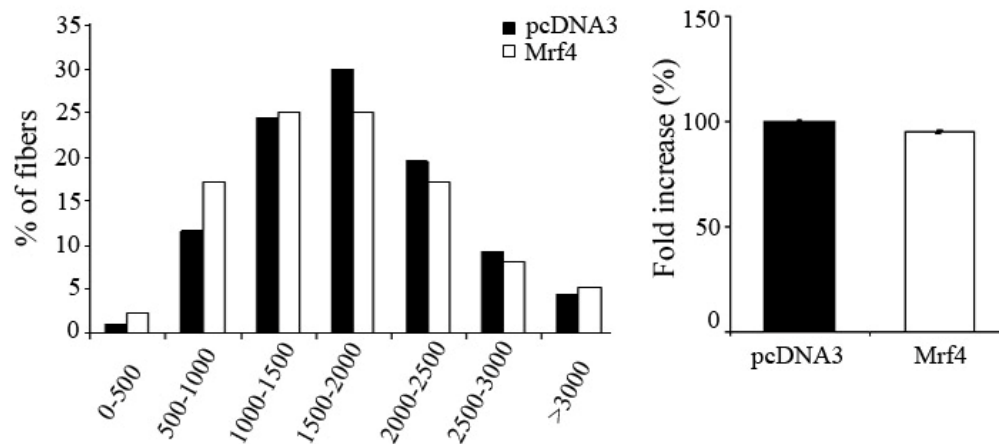


Fig.15. Mrf4 overexpression does not affect fiber size in adult muscle. Analyses at 7 days of cross-sectional areas of soleus muscle fibers transfected with Mrf4 expression vector or pcDNA3. In left panel histograms show the distribution of cross-sectional areas (μm^2) of fibers expressing Mrf4 (white bars) or pcDNA3 (black bars). In the graph on the right changes in fiber size are expressed as mean \pm SEM of % of fold increase.

3.6. Mrf4 overexpression decreases fiber size in regenerating muscle

Then we asked if Mrf4 overexpression could affect the growth of regenerating soleus muscles. We induced muscle injury with bupivacaine injection and after 3 days we transfected the muscles with Mrf4 cDNA or with an empty vector. As shown in Fig. 16 A-B regenerating fibers overexpressing Mrf4 are by 25% smaller than control fibers.

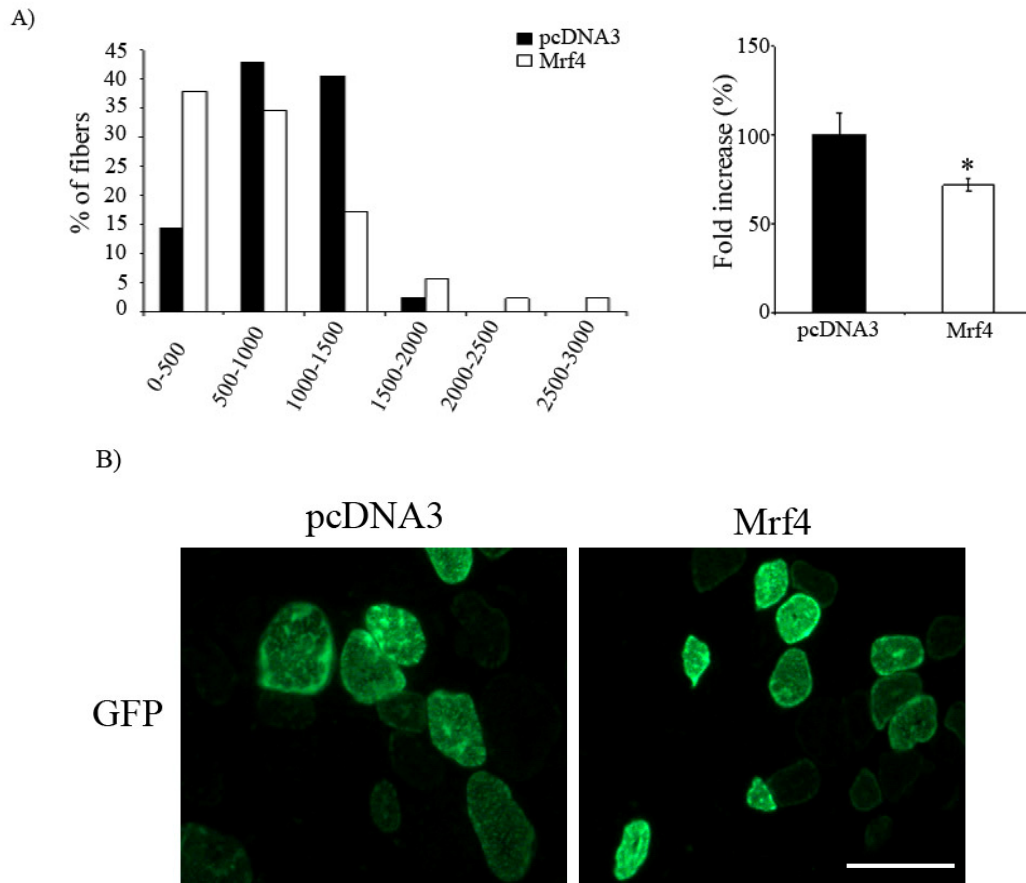


Fig.16. Mrf4 overexpression delays the growth of regenerating fibers. A),B)Regenerating soleus muscles were co-transfected with SNAP-GFP and Mrf4 cDNA or with an empty vector, as control. Muscles were dissected and analysed 7 days later. A) In left panel histograms show the distribution of cross-sectional areas (μm^2) of regenerating fibers expressing Mrf4 (white bars) or pcDNA3 (black bars). In the graph on the right changes in fiber size are expressed as mean \pm SEM of % of fold increase. B) Transverse sections of regenerating soleus muscles co-transfected with SNAP-GFP and pcDNA3 (left panel) or Mrf4 (right panel) were analysed for GFP fluorescence 7 days after transfection. Scale bar= 50 μm .

Therefore, collecting the data concerning Mrf4 knockdown and Mrf4 overexpression, we can conclude that Mrf4 acts as negative growth regulator.

4. Effect of Mrf4 knockdown and overexpression on fiber type

Previous studies have suggested a role of bHLH factors in the determination of fibr-type switching (Hughes et al., 1993)and more

recently the group of Loughna (Walters et al., 2000) demonstrated by in situ hybridization that Mrf4 shows display a fiber-type specific profile of expression in adult skeletal muscle, showing stronger RNA labelling in slow fibers. We have previously shown that the expression levels in slow and fast adult muscle are indistinguishable (see Chpt. 1.1), but to elucidate the role of Mrf4 in the determination and the maintenance of fiber-type switching, we used the RNAi and over-expression approaches .

4.1.Mrf4 silencing blocks the expression of endogenous MyHC slow in regenerating muscles

To evaluate if Mrf4 is involved in induction of the slow phenotype, we induced Mrf4 knockdown in regenerating muscle, which recapitulate the muscle embryogenesis. In this phase MyHCs are produced *ex novo*, allowing to analyze changes in the induction of endogenous genes. We co-transfected regenerating soleus with SNAP-GFP and the M1 construct or with pSUPER targeting LacZ, as control. After 7 days from the transfection, we observed a marked decrease of the expression of endogenous MyHC slow in fibers transfected with shRNA against Mrf4 (Fig.17). This results support the idea that Mrf4 promotes the induction of the slow phenotype in skeletal muscle.

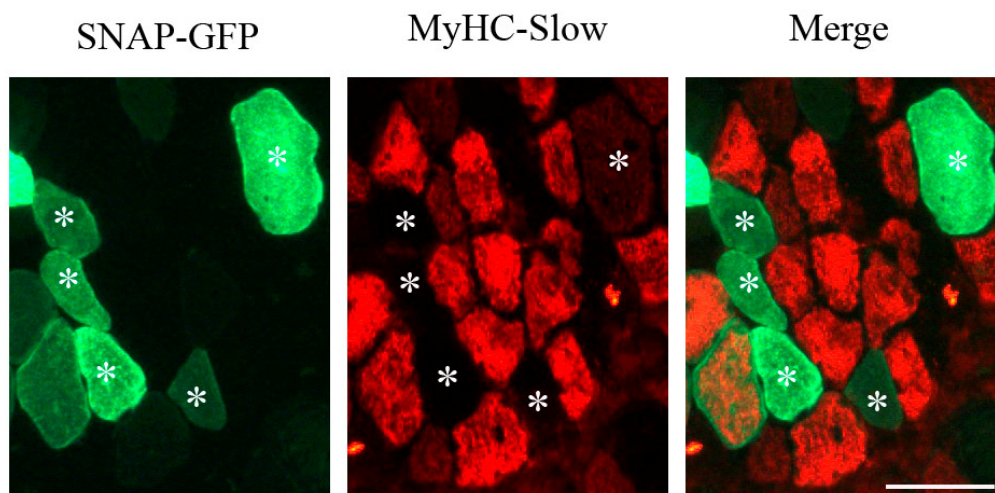


Fig.17. Mrf4 is involved in induction of slow phenotype. Regenerating soleus muscles were co-transfected with M1 construct and SNAP-GFP, to visualize the transfected area . Cryosections were labelled with an antibody specific for MyHC-slow (BA-D5). The transfected fibers (marked with the asterisk) show a decrease of endogenous MyHC slow . Scale bar=50µm.

4.2. Mrf4 knockdown blocks the activity of MyHC slow promoter and induces the activity of MyHC 2B promoter in adult skeletal muscle

To further investigate the role of Mrf4 is in fiber-type switching in adult muscles, we used two muscle specific reporters. They contain the firefly luciferase gene under control of a portion of the promoter regions of myosin heavy chains (1,1 kb for MyHC-slow and 2,5Kb for MyHC-2B) (Hasegawa et al., 1997; Swoap, 1998). To evaluate if Mrf4 silencing could perturbate the transcriptional activity of myosin heavy chain regulatory regions we co-transfected adult soleus muscles with MyHC β -slow promoter and with M1 or M2 shRNA constructs targeting Mrf4 or with pSUPER against LacZ, as control. Data were normalized for variations in efficiency of transfection with a plasmid coding for Renilla luciferase under the control of a minimal constitutive promoter (pRL-TK - Promega). Muscles were analysed 7 days after transfection. We demonstrate that Mrf4 knockdown with both shRNA constructs (M1 and M2) reduces the activity of MyHC β -slow promoter in adult soleus muscles (Fig.18). On the other hand in EDL muscles the activity of MyHC-2B promoter is significantly activated by MRF4 silencing (Fig. 19).

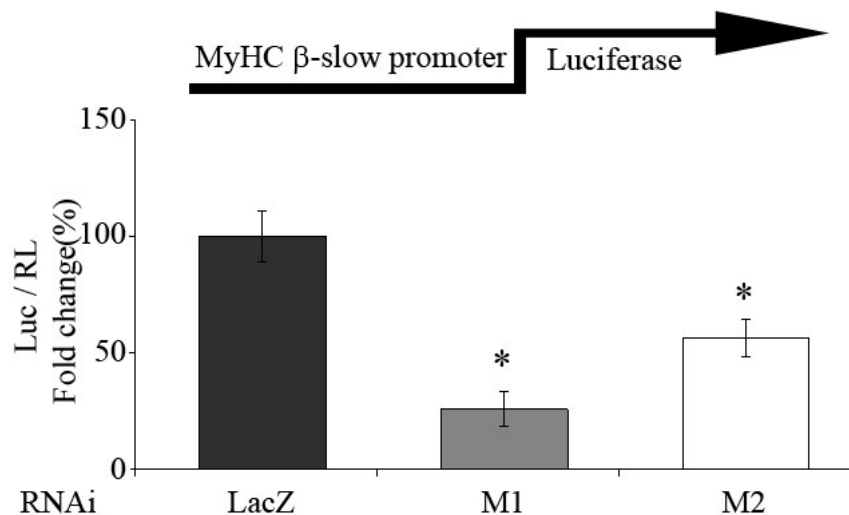


Fig.18. Mrf4 silencing reduces the activity of MyHC β -slow promoter. Adult soleus muscles were co-transfected with MyHC β -slow promoter and the pSUPERs against Mrf4 (M1 and M2) or pSUPER-LacZ, as control, and with Renilla plasmid to normalize for transfection efficiency. The luciferase activity is expressed as mean \pm SEM of fold change.of ratio Firefly/ Renilla. M1 is more efficient than M2 to block the slow promoter activity.

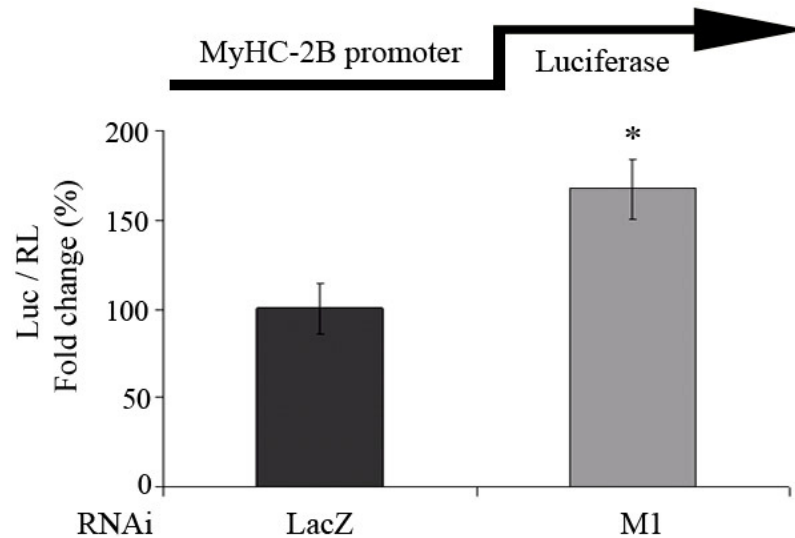


Fig.19. Mrf4 silencing induces the activity of MyHC-2B promoter. Adult EDL muscles were co-transfected with MyHC-2B promoter and the pSUPERS against Mrf4 (M1) or pSUPER-LacZ, as control, and with Renilla plasmid to normalize for transfection efficiency. The luciferase activity is expressed as mean±SEM of fold change (%) of ratio Firefly/ Renilla.

4.3. Mrf4 overexpression blocks the activity of MyHC 2B promoter and do not change the activity of MyHC slow promoter

To confirm the specificity of the phenomenon observed with the loss of function approach, we did overexpression experiments co-transfecting Mrf4 cDNA with MyHC β -slow promoter in adult soleus muscles or with MyHC-2B in fast EDL muscles. We used the empty vector pcDNA3 as control. Overexpression of Mrf4 in soleus muscle does not change the activity of MyHC β -slow promoter (Fig. 20A), but in EDL muscles it strongly inhibits the activity of the fast MyHC-2B promoter(Fig.20B).

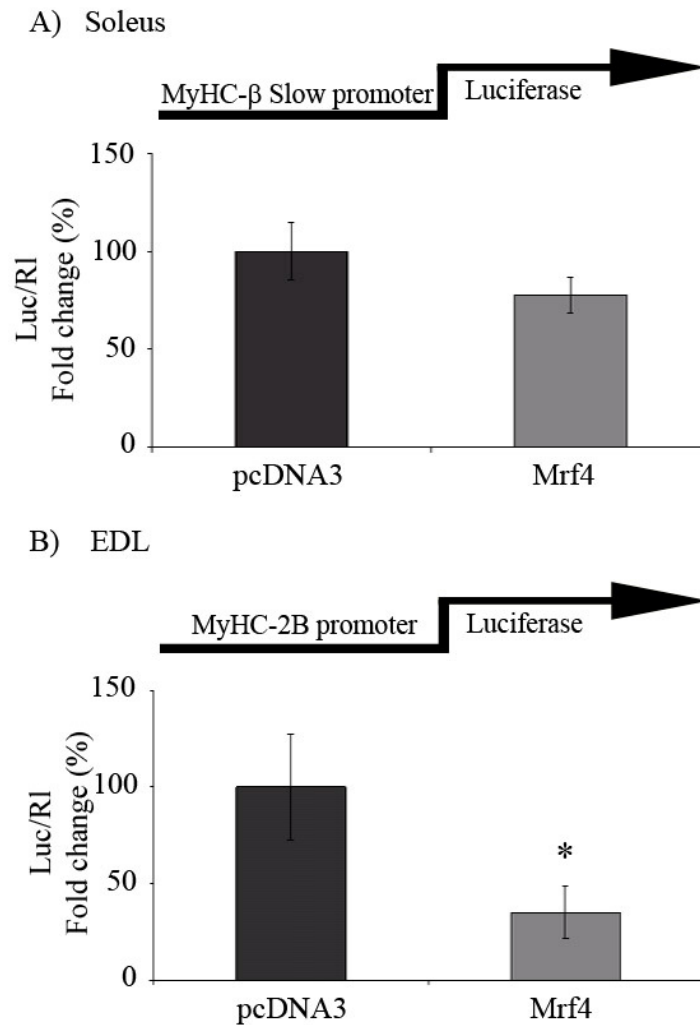


Fig.20. Mrf4 overexpression does not change the activity of MyHC β -slow promoter and inhibits the activity of MyHC-2B promoter. Adult soleus muscles were co-transfected with Mrf4 cDNA or pcDNA3 and MyHC β -slow (A) or MyHC-2B promoter (B). After 7 days of transfection, luciferase activity was measured. The data were normalized for efficiency of transfection with pTK-RL. Data are expressed as mean \pm SEM of fold change of the ratio Firefly/Renilla.

Data obtained so far, show that Mrf4 is an active element of the transcriptional machinery determining fiber-type switching: it is necessary to support the induction and maintenance of the slow phenotype in regenerating and adult muscles, respectively.

DISCUSSION

Four myogenic basic helix-loop-helix (bHLH) transcription factors (MyoD, Myf5, myogenin and Mrf4) specify the myogenic lineage during embryonic development and control muscle differentiation (Berkes and Tapscott, 2005). Gene targeting studies initially suggested that MyoD and Myf5 can be considered “commitment” factors, whereas myogenin is a “differentiation” factor. Mrf4 has a commitment functions, as demonstrated by Myf5:Myod double-null mice, where skeletal muscle is formed if Mrf4 expression is not compromised (Kassar-Duchossoy et al., 2004). Mrf4 has a biphasic expression profile in the developing mouse embryo: it is transiently expressed in mouse somites around embryonic day 9.0 (E9.0), then its expression decreases by E11.5 and is reinitiated at E16 in differentiating muscle fibers with expression continuing into adult stages(Bober et al., 1991; Sumariwalla and Klein, 2001). Mrf4 is the only member of the MyoD family expressed at high levels in adult skeletal muscle. While the early phase of Mrf4 expression is apparently related to its function as a determination factor, the role of Mrf4 at later developmental stages and in adult skeletal muscle is still largely unknown. Mice with targeted disruption of Mrf4 are viable and have normal skeletal muscles, but this lack of phenotype could be due to compensatory effects established during development. These mice display upregulation of myogenin, MyoD and Myf5 expression, which could potentially compensate for the absence of Mrf4(Thompson et al., 2005; Zhang et al., 1995). Indeed, myogenesis can be partially rescued in myogenin null embryos by a myogenin promoter-Mrf4 transgene (Zhu and Miller, 1997). In addition, myogenin and Mrf4 seem to have overlapping functions in ventral myotome differentiation and intercostal muscle morphogenesis(Vivian et al., 2000). Finally, a synergy between myogenin and Mrf4 is suggested by the finding that only Mrf4 restores myofiber formation in differentiated myogenin null ES cells (Sumariwalla and Klein, 2001).

Previous observations about Mrf4 fiber type specificity are quite contrasting, and the reason can be partially attributed to different

techniques used. In 1993 the group of Konieczny analysed the distribution of Mrf4 transcripts in different muscles by northern blot analysis, and they reported a constant level of expression among the analysed muscles (Voytik SL1993n pg 214) In 1997 Kraus analysed the levels of Mrf4 transcripts by quantitative PCR and he reported a two-fold higher expression in EDL as compared to soleus muscle (Kraus and Pette, 1997). More recently *in situ* hybridization analyses indicated that Mrf4 transcripts accumulate preferentially in slow fibers in adult rat muscles and in the developing zebrafish (Hinitz et al., 2007; Walters et al., 2000). Here we show that the transcripts (measured by real time PCR) and the protein levels of Mrf4 are similar in slow soleus muscle and in fast EDL muscle.

We demonstrate that Mrf4 has a different localization in slow or fast muscles: in soleus Mrf4 is accumulated in the nuclei and in EDL it is predominantly cytosolic. Moreover we demonstrate that Mrf4 is affected by nerve activity. When EDL muscles were stimulated with a slow pattern (20 Hz for 2 hr), Mrf4 translocates to the nucleus. If the same muscles were stimulated with a fast pattern (100 Hz for 2 hr), Mrf4 remains in the cytoplasm. These data suggest that Mrf4 may undergo to “nucleo-cytoplasmic” shuttling possible due to post-transcriptional events, such as phosphorylation. Indeed each of the MRFs exists as a phosphoprotein *in vivo*, and analysis of the amino acid sequence of the various MRFs reveals several potential sites of phosphorylation, including consensus sites for cyclic AMP-dependent protein kinase (PKA) (Li et al., 1992), protein kinase C (PKC) (Hardy et al., 1993), and casein kinase 2 (CKII) (Johnson et al., 1996). Moreover, it has been demonstrated that Mrf4 is phosphorylated *in vitro* and *in vivo* by p38 mitogen activated protein kinase on two serines (Ser31 and Ser42) located in the N-terminal transactivation domain. These phosphorylation repress the transcriptional activity of Mrf4 resulting in downregulation of specific muscle genes. Non-phosphorylatable Mrf4 mutants display increased transcriptional activity and are able to advance both myoblast fusion and differentiation (Suelves et al., 2004). It is possible that the phosphorylation state of Mrf4

determines the cellular localization of this muscle regulatory factor, as in the case of other transcription factors (e.g. NFATc1 (Tothova et al., 2006)).

In addition, after 3 days of denervation we observed a strong nuclear accumulation of the Mrf4 factor, at comparable levels in soleus and EDL muscles. This observation confirms previous results describing an increase of nuclear immunoreactivity for Mrf4 following denervation, with the highest proportion of immunopositive nuclei 2-3 days after denervation (Weis et al., 2000). Northern blot analyses show that the levels of Mrf4 transcripts increase rapidly after 8 hours of denervation (Eftimie et al., 1991) and became 10-20 fold after 2-3 days of denervation compared to the innervated controlateral control (Voytik et al., 1993). The transcripts of the other MRFs are increased after denervation, although with different kinetics (Eftimie et al., 1991; Voytik et al., 1993). These changes in MRF gene expression observed following denervation reflect the “reprogramming” of existing myofiber nuclei (Voytik et al., 1993). In our hands, quantification of Mrf4 mRNA by real-time PCR shows that Mrf4 transcripts peak at 3 days of denervation, with a stronger up-regulation in EDL muscle than soleus muscle, then its expression decreases. Similar results were presented by Konieczny using northern blot analyses (Voytik et al., 1993).

To study the function of Mrf4 we used two different approaches: a loss of function approach mediated by RNAi *in vivo*, and a gain of function approach mediated by overexpression of Mrf4 cDNA *in vivo*. The RNAi technique enables to knockdown specifically Mrf4 in adult animals. In this way, we can prevent the compensatory effects demonstrated in Mrf4 knockout mice, in which is evident an up-regulation of the other MRFs (Olson et al., 1995; Thompson et al., 2005). To exclude potential off-target effects, we studied the effects mediated by two different shRNA (M1 and M2, with M1 more efficient than M2 to silence the Mrf4 expression) and with both we performed different rescue experiment that restored the initial phenotype.

Our results demonstrate that Mrf4 is involved both in the control of muscle growth and in the regulation of fiber type phenotype.

In particular we show that Mrf4 acts as a negative regulator of growth. Indeed, Mrf4 knockdown induces a significant increase of fiber size which is more evident in regenerating muscles. This result is supported by the finding that over-expression of Mrf4 leads to fiber atrophy. The silencing effects are more evident during denervation, when Mrf4 knockdown is able to totally prevent denervation atrophy. It has to be noted that this occurs in a condition that enables the nuclear translocation and accumulation of this transcription factor. It remains to be verified whether Mrf4 targeting can contrast the loss of force that is associated with denervation atrophy. The observations reported here can account for the surprising finding that transgenic mice over-expressing Mrf4 under the control of a myogenin promoter display a transient growth retardation during regeneration after local freeze injury (Pavlath et al., 2003). In this transgenic model, regenerating tibialis anterior muscle fibers are smaller than wild type control at day 11 after injury, when myogenin promoter is active and drives high Mrf4 expression levels. Fiber size becomes identical to control at day 19 after injury, when regeneration is essentially complete, thus one expects that the myogenin promoter becomes inactive and that Mrf4 expression is decreased.

The mechanisms underlying inhibitory effect of Mrf4 on muscle growth remain to be established. One possibility is that Mrf4 may act as a transcriptional repressor of an unidentified growth stimulating factor. Previous studies suggested a role of Mrf4 as a transcriptional repressor. Both construct containing a multimerized E-box and the alpha-cardiac promoter, that are activated by MyoD, are instead inhibited by Mrf4 (Moss et al., 1996). By exchanging N- and C-terminal domains of Mrf4 and MyoD, the N terminus of MRF4 was identified as the mediator of repressive activity. This region contains two serine residues (Ser31 and Ser42) that are phosphorylated by p38 kinase and this phosphorylation mediates repression of specific myogenic genes (Suelves et al., 2004).

Mrf4, therefore, could act as negative regulator of pathways that mediate hypertrophy in adult skeletal muscle. The main pathway involved could be the IGF-I-PI3k-Akt pathway, known to enhance differentiation and to induce hypertrophy in different experimental models. However we

cannot exclude the hypothesis that Mrf4 controls the expression of some factors involved in myostatin pathway or myostatin itself. Finally Mrf4 could be acting through a still unidentified pathway. To get further insights on this aspect of the Mrf4 function we are going to evaluate changes in the transcriptional profile of expression of muscle transfected with shRNA targeting Mrf4 or LacZ.

Our results also suggest that Mrf4 is involved in the regulation of the fiber type. The finding that Mrf4 controls the transcriptional activity of MyHC slow and MyHC 2B promoters is not surprising, since E-boxes have been found in the promoter regions and enhancers of many muscle-specific genes including myosin light chain (Ceccarelli et al., 1999; Wentworth et al., 1991), MyHC-slow (Meissner et al., 2006) and MyHC-2B (Wheeler et al., 1999). Mrf4 knockdown blocks the MyHC slow promoter activity and induces the activity of the MyHC 2B promoter. Conversely overexpression of Mrf4 inhibits the MyHC 2B activity. The relevance of these data is supported by the finding that Mrf4 silencing in regenerating muscles blocks the expression of endogenous MyHC slow.

Together these data suggest that Mrf4 is involved in the induction and maintenance of slow gene program. Other transcription factors, such as the NFAT (and the Mef2 families, have been demonstrated to act in the regulation of slow genes (Chin et al., 1998; McCullagh et al., 2004). It remains to be established whether Mrf4 acts in these pathway.

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