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THÈSE

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DOCTORAT

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Modena e Reggio Emilia**

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Direct activation of endogenous Calcineurin A: Biological impact of selective peptide aptamers

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ABBREVIATIONS

AI: Auto Inhibitory domain

CaM: Calmodulin

CaMBD: Calmodulin Binding Domain

CnA: Calcineurin A

CnB: Calcineurin B

CnBBD: Calcineurin B Binding Domain

CsA: Cyclosporine A

CSA: Cross Sectional Area

DMD: Duchenne Muscular Dystrophy

EDL: Extensor Digitorum Longus

IGF-1: Insulin like Growth Factor I

IL: Interleukin

MEF2: Myocyte Enhancer Factor 2

MRF: Myogenic Regulatory Factor

MyHC: Myosin Heavy Chain

NFAT: Nuclear Factor of Activated T-cell

NS5A-TP2: Non Structural 5A- Trans activated Protein 2

TGF: Transforming Growth Factor

Tnlf: Troponin I fast

Tnls: Troponin I slow

Trx: Thierodoxin

INTRODUCTION

SKELETAL MUSCLE

1. OVERVIEW

Skeletal muscle, also called striated muscle because of its striped appearance under the microscope, is a contractile tissue present in all animals. The activity of this tissue is under the control of somatic nervous system and for this reason it is also known as voluntary muscle. Two other major muscle types are known, cardiac and smooth muscle, both classified as involuntary muscles. Skeletal muscle is attached to the bone by bundles of collagen fibers known as tendons. Skeletal muscle contributes to approximately 40% of the human body weight and it is responsible for movement, postural behavior and breathing. Skeletal muscle is a highly vascularized tissue, blood vessels being essential to provide oxygen and nutrients for muscle functions.

2. MUSCLE FIBERS

Muscle fibers, the basic units of skeletal muscles, are among the largest cells in human body. Ranging from 10 to 100 μm in diameter, each muscle fiber runs the entire length of the muscle¹. Muscle cells exhibit multiple nuclei and constitute one of the few syncytia in the mammalian body, other examples being osteoclasts and some large macrophages (Bruusgaard et al., 2003). Muscle fibers are individually surrounded by a connective tissue layer, the endomysium, and organized into bundles. Bundles are surrounded by perimysium and together form muscle that is in turn enclosed in a sheath of epimysium. (Fig 1)

The plasma membrane of muscle cells is called sarcolemma and encloses the cytoplasm known as sarcoplasm. The multiple nuclei are positioned near the surface of the muscle cells

¹ The largest muscle in human body is the Sartorius, a long thin muscle that runs down the length of the thigh. It can reach 50 cm of length. However, the largest muscle known is the *longissimus* of the blue whale. Running from the neck to the tail of the animal it can reach up to 24 meters in length!

under the sarcolemma. In muscle fibers the smooth endoplasmic reticulum is absent but they contain a sarcoplasmic reticulum that holds a reserve of the calcium ions needed for muscle contraction. Periodically the sarcoplasmic reticulum presents dilated end sacs known as terminal cisternae. These cross the muscle fiber from one side to the other.

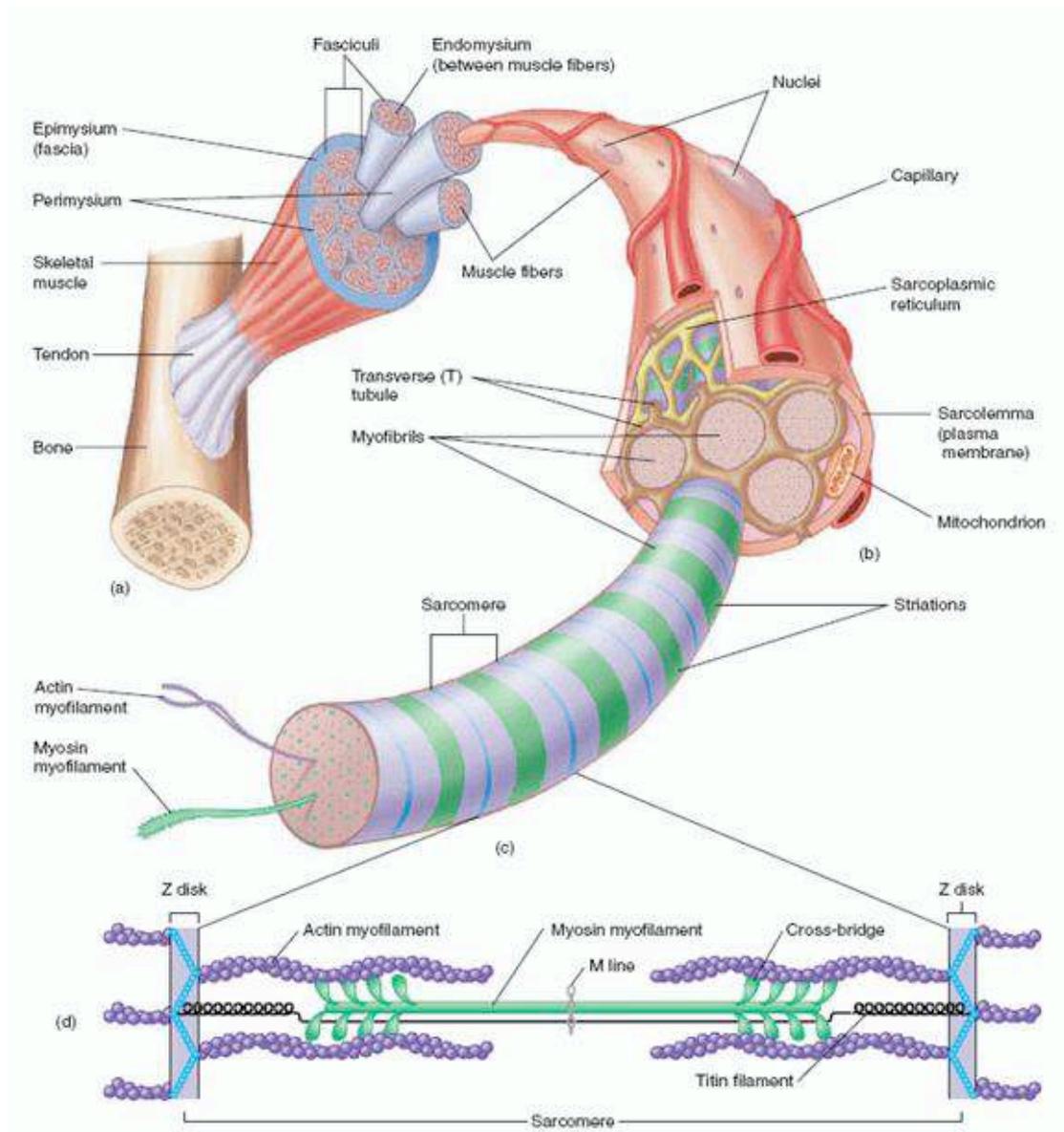


Figure 1 Overview of skeletal muscle structure

Between two terminal cisternae there are tubular sarcolemma invaginations called transverse tubule or T tubule. Together, two terminal cisternae and a transverse tubule form a structure called triad. Transverse tubules are responsible for the coupling of excitation and contraction. In more detail, electrical stimuli activate the calcium channels of T-tubules, which results also in activation of the calcium-release channels of the adjacent sarcoplasmic reticulum membrane. Release of calcium from both types of channels is essential for muscle contraction.

Muscle fibers are composed of myofibrils, made up of thick and thin filaments. Thick filaments are composed of myosin and thin filaments predominantly of Actin. Other proteins such as Nebulin and Titin extend along, respectively, thin and thick filament to ensure the right arrangement of Actin and Myosin. The filaments are organized into repeated subunits along the length of the myofibril. These subunits are called sarcomeres. (Fig. 2) The sarcomeric subunits of one myofibril are in perfect alignment with those of the myofibrils next to it. This alignment gives rise to the appearance of dark and light bands, which cause the cell looks like striped or striated.

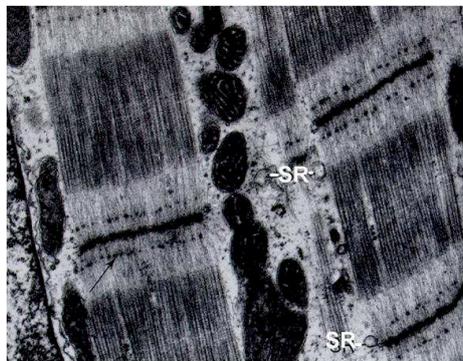


Figure 2. Sarcomere (Electronic microscopy). SR= sarcoplasmic reticulum

Myosin has a long, fibrous tail and a globular head, which binds to Actin. The myosin head also binds to ATP, which is the source of energy for muscle activity. Myosin can only bind to Actin when the binding sites on its surface are exposed by calcium ions. Contraction of skeletal muscle requires the sliding, after neuronal activation, of thick filaments over thin filaments resulting in a shortening of sarcomeres. The contraction of muscle fibers is then transformed into movement *via* the myo-tendinous junctions at their ends, the points of

attachment of muscle to the skeleton. The functional properties of a skeletal muscle depend thus on a correct “orchestration” amongst muscle fibers, motor neurons, connective tissue and blood vessels.

SKELETAL MUSCLE PLASTICITY

Despite the finely regulated organization that allows the skeletal muscle to carry out its functions, this tissue is constantly subject to modifications regarding mass and muscle fibers composition as response to different external signals (Schiaffino et al., 2007).

Thus, one of the most peculiar properties of skeletal muscle is its plasticity. Skeletal muscle has a striking ability to adapt to a wide range of stimuli, such as endurance exercise, electrical stimulation, hormonal factors, ageing etc. The ability of adaptation is most definitively due to the complexity of this tissue. A large variety of fiber phenotypes with a different distribution in each muscle makes this tissue unique for its architectural design (Schiaffino, 2010). The properties of each fiber are controlled by endogenous processes that can be modulated by various external influences making muscle a highly adaptive tissue.

1. MUSCLE FIBER TYPES

Over the last 50 years a large number of studies have been carried out to characterize the composition of the different fibers types. In a first time the prevailing paradigm was that there are two major types of skeletal muscles and corresponding fiber types: slow “red” muscles, with an oxidative metabolism and therefore composed of fibers rich in mitochondria and myoglobin and fast “white” muscles, with a glycolytic metabolism and therefore, composed of fibers poor in mitochondria and myoglobin (Dubowitz and Pearse, 1960).

The observation that fast and slow fibers differ in myosin ATP activity, which is correlated with the speed of muscle shortening (Barany, 1967) gave an important acceleration in the evolution of the notion of skeletal muscle fibers types. Henceforth, Myosin, and particularly Myosin Heavy Chain (MyHC) are used as a standard marker of muscle fiber type. Subsequent studies, based on electrophoretic separation of MyHC bands, observations on twitch property of motor units, histochemical staining and the generation of

monoclonal antibodies directed to the different MyHCs, demonstrated the existence of three intermediate populations of fast fibers (Schiaffino, 2010).

Taken together, these observations bring us to the last prevailing paradigm according to which there are four muscle fiber types, each of them with a specific myosin and metabolic enzyme composition: a type of slow fibers, named type 1, which express MyHC-slow and are oxidative and fatigue resistant, and three types of fast fibers, 2A, 2X and 2B, displaying a graded range of functional properties. 2A fibers are the slowest and more oxidative, whereas 2B fibers are the fastest and more glycolytic. 2X show intermediate properties. (Fig.3 and Table 1)

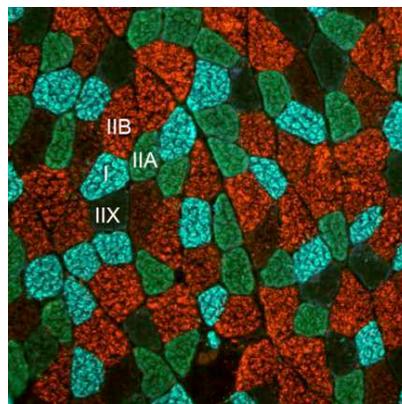


Figure 3. Fiber types in mouse skeletal muscle. Staining with different MyHC antibodies. In cyan the fibers type I, in green the type IIA, in red the type IIB. The unstained fibers are the type IIX. (from Stefano Cicilioti)

	Slow fiber	Fast fibers		
	Type 1	Type 2a	Type 2x	Type 2b
Contraction time	Slow	slow	fast	very fast
Mythochondria density	High	High	Medium	Low
Myoglobin density	High	-	-	-
Power produced	Low	Medium	High	Very high
Resistance to fatigue	High	Fairly high	Intermediate	Low
Oxydative capacity	High	High	Intermediate	Low
Glycolitic capacity	Low	High	High	High

Table 1. Main characteristics of muscle fiber types (Inspired from Fibres types in skeletal muscle: a personal account. Schiaffino, Acta Physiol 2010, 199, 451-463 (Schiaffino, 2010))

As the complexity of skeletal muscle composition was investigated, exploration of fiber type distribution was dissected in more and more detail unveiling interesting results. *In situ* hybridation analyses, performed by DeNardi in 1993 in the Schiaffino group (DeNardi et al., 1993), showed that rat muscles contain, in fact, a large range of fibers type, including hybrid fibers made up of combination of different MyHC. Using immune-histochemical and single fiber analysis it was possible to identify fibers with the following combinations: (1); (1/2A); (2A-2A/2x); (2X); (2X/2B); (2B). Hybrid fibers were found to be more frequent in ageing skeletal muscle and after endurance training, underlining the ability of the muscle to adapt to different stimuli modifying the expression of the MyHC isoforms and therefore its twitch properties.

Muscles are able to modulate their mass and function using Calcium as second messenger, linking nerve activity to regulation of gene expression. More precisely, in the case of frequent neuronal stimulation, slow fibers tend to accumulate more intracellular free Calcium than fast fibers. Increase in the intracellular concentration of Calcium leads to the activation of the protein phosphatase Calcineurin and subsequently, to the expression of various genes implicated in remodeling. Involvement of Calcineurin in modulating the nerve activity will be described in the sections below.

SKELETAL MUSCLE REGENERATION

Upon injury, skeletal muscles are able to regenerate in order to preserve the muscle mass. Skeletal muscle repair is a highly organized and finely regulated process (Charge and Rudnicki, 2004). A subpopulation of muscle cell progenitors, the satellite cells, is one of the key elements in this process. Several signaling pathways involving protein kinase and transcription factors are responsible for the activation and differentiation of these cells into myoblasts. Once activated and differentiated, satellite cells fuse together or with existing myofibers to start the process of muscle repair. Adult satellite cell activation, shares the same mechanism as embryonic myogenesis, being induced by the same myogenic regulatory factors.

Considering the similarity between the two processes, *i.e.* muscle regeneration and embryonic myogenesis, it seems to be opportune to introduce the main aspects of skeletal muscle development. Thus, the next section will offer an overview of the molecular factors involved in the differentiation of progenitor stem cells into contractile adult muscle fibers. We will then focus on the principal steps of muscle regeneration, going more in detail in the description of signaling pathways that regulate this process.

SKELETAL MUSCLE DEVELOPMENT: AN OVERVIEW

Skeletal muscle derives from mesodermal precursor cells originating from somites. After formation of the neurotube, each somite forms a dermomyotome, which give rise to skin and muscles and a sclerotome, which differentiates into vertebrae. (Fig.4) Cells from the edge of the dermomyotome will then delaminate to form the first skeletal muscle, the myotome, or migrate to more distant sites of myogenesis to develop in the muscles of the limbs (Relaix, 2006). The dorsal region of the dermomyotome, on the other hand, gives rise to the dermatome, which is the source of connective tissue of the skin precursor cells.

Determination of the fate of mesodermal precursor depends on different signals coming from surrounding tissues, such as Wnt and Shh signalling, and it is mainly under the control of several transcription factors, such as the member of Myogenic Regulatory Factor (MRF) family and Pax.

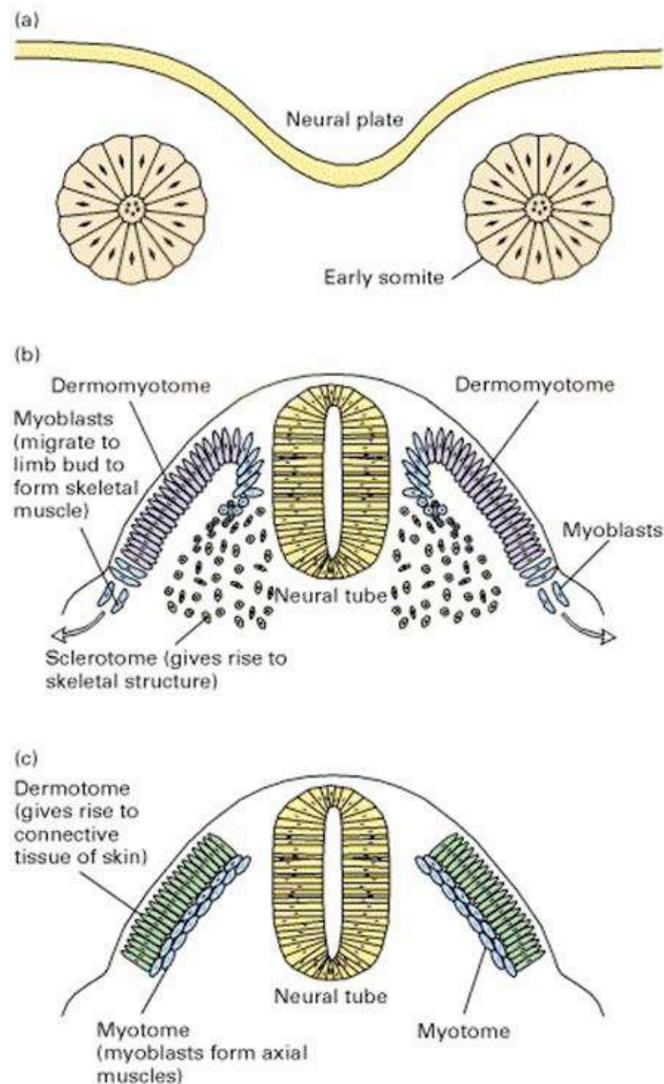


Figure 4- Stages of muscle development (a) Skeletal muscle is derived from embryonic structures called somites, which are blocks of mesodermal cells. (b) After formation of the neural tube, each somite forms a dermomyotome, which gives rise to skin and muscle, and a sclerotome, which develops into skeletal structures. Myoblasts form at each edge of a dermomyotome. Lateral myoblasts migrate to the limb bud. Axial myoblasts form the myotome. (c) The dermatome gives rise to skin elements (dermis), and the myotome to axial muscle. (Molecular Cell Biology. 4th edition. Lodish H, Berk A, Zipursky SL, et al. New York: W.H Freeman; 2000)

1. REGULATION OF MUSCLE DEVELOPMENT

a. MRF family

Specification of the myogenic lineage is controlled by the upregulation of MyoD and Myf5 (Rudnicki et al., 1993), which are helix-loop-helix transcription factors of the Myogenic Regulatory Factor (MRF) family. The genes encoding the MRFs are considered as master regulatory genes, whose expression initiates a cascade of events that lead to muscle cell differentiation. The first member of the MRFs family discovered was MyoD. It was identified, in 1987, as a factor able to convert fibroblasts into stable myoblasts (Davis et al., 1987).

Upon activation by MyoD and Myf5, mesodermic somite cells are committed to the myogenic lineage and thus convert in myoblasts. Later, with the up-regulation of other members of the MRF family, such as myogenin and MRF4, myoblasts differentiate into myocytes. Finally, mononucleated myocytes fuse to each other to give rise to multinucleated syncytia that differentiate into mature muscle fibers.

During this sequential process, a subpopulation of myoblasts fails to differentiate and remain associated with the surface of the developing muscle fiber as quiescent muscle satellite cells. They represent a source of myogenic precursor cells and they play a central role in skeletal muscle growth and regeneration. (see section Satellite cells)

b. Skeletal muscle progenitors and Pax genes

New insights in myogenesis have been offered by the characterization of the role of the transcription factors, Pax3 and Pax7 (paired box protein 3 and 7) in the regulation of the embryonic muscle development. Pax3 is first expressed in the presomitic mesoderm; however it is also detected in the myotome. Similar evidence was obtained for Pax7, which is expressed first in the dermomyotome and later co-localizes with Pax3 in the myotome. It is now well established that both Pax3 and Pax7 are indispensable for conferring the myogenic potential on the progenitor cells of the myotome. In the absence of both transcription factors myoblasts do not enter the myogenic process. Pax3 and 7 are thus considered as key upstream regulators of the development of skeletal muscle. More precisely, they have been shown to be responsible for the entry of the precursor cells in the myogenic lineage via the activation of the myogenic determination factor, MyoD (Buckingham and Relaix, 2007).

Recently, a new population of Pax3/Pax7 positive cells has been identified (Relaix et al., 2005). After the formation of the dermomyotome, cells that express Pax3 and Pax7 enter the myotome and differentiate into myoblasts, the precursors of skeletal muscle. This population is composed of proliferating cells which do not express skeletal muscle markers. However they maintain their ability to differentiate into myoblasts with the activation of Myf5 and MyoD, followed by their fusion in skeletal muscle fibers. (Fig.5) The crucial role of these cells during muscle development has been underlined by the important muscle deficit observed in embryos of Pax3/7 double knock-out mice. In these embryos only the early myotome and its derivatives are formed, while the population of precursor cells dies or are incorporated into other tissues. Taken together, these results indicate that Pax3 and 7 are major initiators of the myogenesis program but are also indispensable to ensure cell survival of muscle precursors.

Moreover, during the late fetal stages of myogenesis, the Pax3/7 positive population is located in proximity of muscle fibers, under the basal lamina, adopting then a satellite cell position. This population is thus also a source of quiescent muscle cell precursors involved in the post-natal muscle growth.

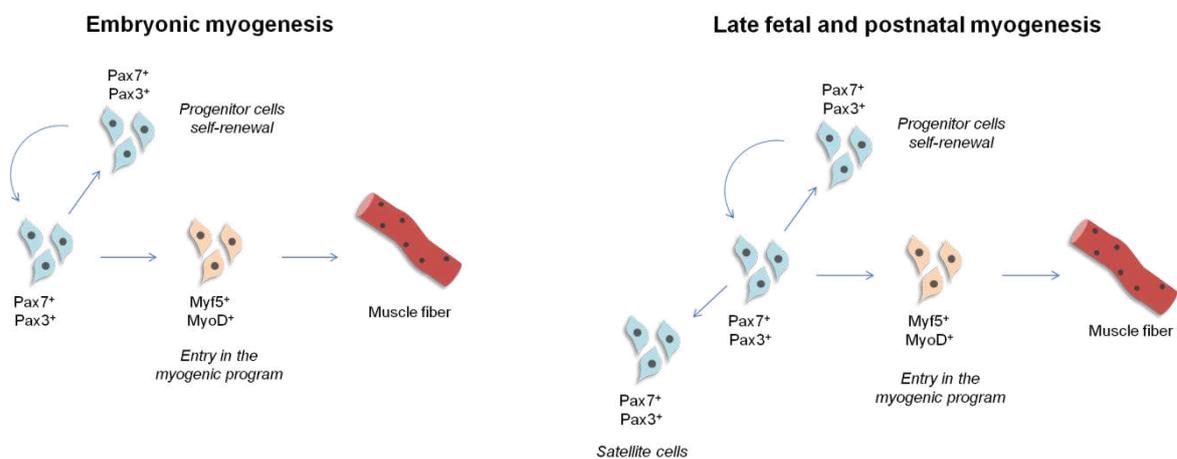


Figure 5. Schematic representation of progenitor cell self-renewal and myogenic differentiation in the embryo and the adult. (Adapted from Skeletal muscle progenitor cells and the role of Pax genes. M. Buckingham, C.R. *Biologies* 330 (2007) 530-533) (Buckingham, 2007)

2. MOLECULAR ASPECTS OF MUSCLE REGENERATION

The process of muscle repair is characterized by two main phases: a degenerative phase and a regenerative phase. Upon injury, muscle undergoes necrosis that is initiated by lesions in the sarcolemma. The disruption of the muscle fiber integrity triggers the outflow of muscle proteins. One of the main markers of muscle degeneration is the increase of serum level of a muscle-specific protein, the Creatine Kinase. This increase is observed whether the muscle injury is provoked by mechanical stress, such as extensive physical exercise, or the consequence of genetic muscle degenerative diseases.

Skeletal muscle regeneration is characterized by the activation of the inflammatory mononucleated cells. In the first phase of the inflammatory response activation, muscles are invaded by neutrophils and then by macrophages. The latter have a dual function in this process: they are responsible for the phagocytosis of cellular debris but also for the activation of myogenic factors. In this regard different populations of macrophages can be distinguished (Chazaud et al., 2009). In the first days after injury, muscles are invaded by a population of macrophages with a pro-inflammatory phenotype, expressing IFN- α (Interferon-alpha) and IL-1 (Interleukin-1). They are, as mentioned before, the principal cells responsible for the phagocytosis of cellular debris and the activation of immune-system cell proliferation. Within four-five days after injury, another population of macrophages appears, also identified as anti-inflammatory macrophages. The expression, in this phase, of TGF-beta (Transforming Growth Factor-beta), and different Interleukins, such as IL-4 and IL-10, stimulates the proliferation of satellite cells and their consequent differentiation and fusion resulting in the formation of new fibers. The environment of stem cells thus plays a crucial role in controlling their fate (Abou-Khalil et al., 2010).

In cross sections, newly formed fibers appear smaller than the pre-existing fibers and exhibit centrally located myonuclei. They are also characterized by the expression of embryonic or developmental form of MyHC, reflecting the formation of new fibers. Once fusion of myogenic cells is completed, new myofibers increase in size and the nuclei migrate to the periphery of the fiber (Fig.6). At the end of the process regenerated muscles are morphologically and functionally indistinguishable from undamaged muscle.

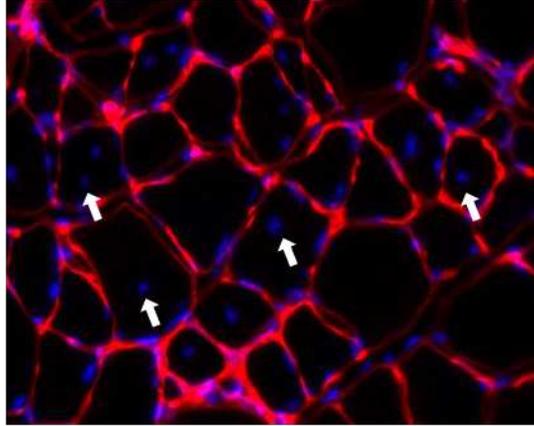


Figure 6. Fibers with centrally located nuclei. Membranes are stained with Rhodamine-conjugated Lectin and nuclei with DAPI. Some central nuclei are indicated by arrows.

3. SATELLITE CELLS

As mentioned before, the most typical morphological characteristic of regenerating muscles is the presence of fibers with centrally-located nuclei. This phenomenon, easily visible by microscopic investigation, has already been well described in the 19th century. At that time several hypotheses to explain the cellular mechanism of muscle regeneration were proposed. In a first instance it was thought that during the regeneration process, cytoplasm buds protruded from broken ends of fibers to close the gap formed by injury. This hypothesis however did not explain the presence of numerous nuclei in the undamaged region. It was then proposed that new myonuclei derived from amitosis or splitting of nuclei already existing in the muscle fibers.

Despite all the hypotheses proposed during almost 100 years after the observation of multinucleated cells in regenerating muscle, the most important insight in the understanding of the precise cellular mechanism of muscle regeneration was obtained by the discovery of A.Mauro, in 1961, of a population of cells located between the basal lamina and the plasma membrane of muscle fibers. On the basis of their peripheral localization, these cells were called “satellite cells” (Mauro, 1961). Mauro was the first to propose, in the same publication, that satellite cells could be responsible for muscle regeneration. He proposed that satellite cells represent a residue of embryonic muscle cell precursors and that they are able to fuse, recapitulating the developmental process, in order to generate larger fibers

when the muscle is injured. On this basis, they are considered the source of the new myonuclei observed during regeneration.

Since their discovery satellite cells have been at the center of a wide range of studies focusing on the characterization of this cell population. First observations have been done regarding their morphological aspects and later the main focus has been the understanding of the molecular regulation of their activation and fusion with the pre-existing muscle fibers.

Satellite cells are characterized by an increased nuclear/cytoplasm ratio and a reduced organelle content. The nucleus appears to be smaller than the fiber myonuclei and displays an increased amount of heterochromatin, reflecting the fact that satellite cells are mitotically quiescent and transcriptional less active than myonuclei (Snow, 1983). From a molecular point of view, satellite cells are characterized, as are the precursor cells involved in the development of muscle fibers, by the expression of the transcription factor Pax7, the marker of the myogenic fate of these cells (Zammit et al., 2006) (Seale et al., 2004).

a. Satellite cells in muscle repair

Involvement of satellite cells in muscle repair follows different steps. First satellite cells are activated, exiting quiescence to enter into the cell cycle and proliferate. After several rounds of division, a portion of the daughter cells commit to enter the myogenic lineage by the expression of Myf5 and MyoD. The next stage is the differentiation of these precursor cells into myofibers. Cells withdraw from the cell cycle and fuse either to existing fibers to repair a damaged region, or to each other to form new fibers. At this stage, cells are characterized by the expression of the myogenic regulatory factors, Myogenin and MRF4.

During the proliferative step a group of daughter cells, called reserve cells, dividing more slowly than those implicated in muscle repair, return to a state of quiescence and replenish the satellite cell pool. Satellite cell self-renewal is an extremely important process without which, recurrent muscle regeneration events would rapidly lead to the depletion of satellite cell pool. This process is finely regulated by the expression of specific proteins during the late differentiation, in order to make cells return to a quiescent state. Myostatin, a growth and differentiation factor (renamed GDF-8) has been proposed as a key regulator of satellite cells quiescence since its over-expression blocks muscle regeneration. Myostatin negatively regulates the G1 to S progression in cell cycle and is thus a potent negative

regulator of satellite cell activation maintaining the cells in quiescent state (McCroskery et al., 2003). Furthermore, Myostatin knock-out mice show a significant increase in muscle growth and regeneration (McPherron et al., 1997) (Fig.7)

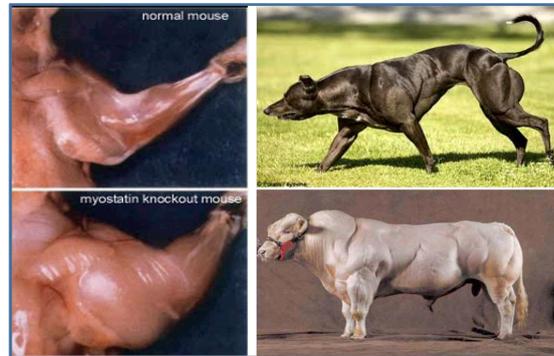


Figure 7. Examples of Myostatin knockout animals

b. Satellite cell activators

Activation of satellite cells requires the expression of many muscle transcription factors and muscle-specific genes. The activation process is regulated by cell-cell interaction and cell-matrix interaction and it is mainly controlled by secreted factors. Upon injury, muscle fibers release, in the extracellular space, soluble factors that are implicated in the activation of satellite cell proliferation and thus, differentiation. *In vitro* studies have underlined the involvement, in this process, of several trophic factors, such as HGF (Hepatocyte Growth Factor), FGF (Fibroblast Growth Factor) and IGF (Insulin-like Growth Factor)

HGF is one of the most important growth factors involved in organ regeneration. Its role in muscle regeneration is crucial in the early phase of the repair process. HGF has been demonstrated to play a role in the activation of quiescent satellite cells (Tatsumi et al., 1998). Moreover HGF plays a role in promoting cell migration to the site of injury. In a work of Suzuki in 2002 (Suzuki et al., 2002), it has been shown that, upon muscle injury, a rapid increase in HGF transcription is observed in the spleen. This means that HGF from other tissue may also have an important role in muscle regeneration, underlining that HGF can exert its role through an endocrine and paracrine mechanism. The action of HGF is mediated by its receptor, c-met, expressed by satellite cells. The binding of HGF to its receptor activates several signaling pathways including the MAPK (Mitogen-activated

Protein Kinase) pathway and the phosphatidyl Inositol 3 kinase, pathway that activate the transcription of genes important for cell growth and proliferation.

On the other hand, FGF and IGF do not seem to be implicated in the activation of quiescent cell proliferation (Johnson and Allen, 1995). However they play a role in promoting the proliferation of cells that have already exited from their quiescent state. For example, the role of IGF-1 and IGF-2 has already been well characterized. They are both able to enhance the MRFs expression resulting in the promotion of the proliferation and differentiation of myoblasts (Musaro et al., 1999, Wilson and Rotwein, 2006). In addition, IGF-1 and IGF-2 exert a role in both activation of satellite cells and increase of protein synthesis (Bark et al., 1998) within the existing fibers, resulting in a hypertrophic effect on muscle fibers.

These are some of the activators of the myogenic fate of satellite cells. It appears obvious that a wide range of other signaling pathways is involved in this process according to the fact that muscle regeneration can be activated by several different stimuli.

Several of these activators stimulate changes in intracellular concentration of Calcium, which has been demonstrated to play a critical role in differentiation, hypertrophy and fiber type determination in skeletal muscle. The serine/threonine protein phosphatase, Calcineurin, is the main mediator of calcium signaling in a wide range of cellular processes. *Via* the dephosphorylation of the transcription factor NFAT (Nuclear Factor of Activated T-cells), Calcineurin is involved in the activation of several regulatory genes resulting in the transduction of calcium signal in a cellular response.

The role of the Calcineurin–NFAT pathway in muscle repair, differentiation and fiber type determination has been widely investigated over the last 15 years, offering important insights into the understanding of muscle processes. Nevertheless, the complete picture of the role of Calcineurin in skeletal muscle remains to be determined, some aspects still being the focus of controversy.

The aim of this review is thus to offer an overview of the insights into the role of Calcineurin in muscle regeneration. We will first introduce the general aspects about structure and mechanism of action of the phosphatase and then we will go more in detail regarding its role in the skeletal muscle context.

CALCINEURIN

Calcineurin (Cn), also known as protein phosphatase 2B (PP2B), is a threonine and serine protein phosphatase conserved in all eukaryotes. It has been identified, by purification from bovine brain extract, in a work of Klee in 1979 as a calcium and Calmodulin binding protein of the nervous system (Klee et al., 1979). The name “Calci”-“neurin” was therefore proposed by the authors on the basis of Calcium binding property and the localization to the nervous system of this enzyme (Klee et al., 1979). Though most highly expressed in the brain and nervous system, later studies have demonstrated that this phosphatase is ubiquitously expressed, acting as the main mediator of cellular responses triggered by intracellular Calcium fluctuations.

Calcineurin is composed of two polypeptides chains: a catalytic subunit (CnA) of 61 kDa and a regulatory subunit (CnB) of 19 kDa. CnA includes different domains: a catalytic domain, ensuring the phosphatase activity, a CnB binding domain (CnBBD), a Calmodulin (CaM) binding domain (CaMBD) and an Auto-Inhibitory domain (AI), these latter three regions being responsible for the regulation of the Cn activity (Fig.8). CnB, the regulatory subunit of Calcineurin is tightly associated with the enzyme while CaM requires calcium activation for its binding. In the inactivated form of Calcineurin, the AI domain blocks the catalytic domain, preventing substrate access. Increase in intracellular Calcium activates Calmodulin, which binds to its specific domain, triggering a conformational change within the Calcineurin structure that displace the AI domain from the active site (Tokoyoda et al., 2000).

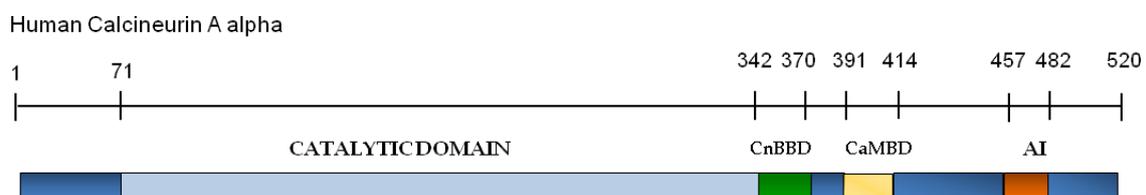


Figure 8. Schematic representation of CnA domains

Three Calcineurin A genes have been described, CnA-alpha and Cna-beta, ubiquitously expressed and CnA-gamma, restricted to brain and testis. Two Cna-beta isoforms, Cna-beta1 and Cna-beta2, which differ in their C-terminal region, are the result of alternative splicing

events (Guerini and Klee, 1989). The auto-inhibitory domain presents in CnA-beta2 and in the other isoforms is absent from Cna-beta1, in which a different C-terminal domain is generated by the translation of intronic sequences. This novel domain of Cna-beta1 is highly conserved especially in vertebrates, suggesting an evolutionarily conserved role for this Calcineurin variant.

1. CALMODULIN, A CALCIUM-DEPENDENT MODULATOR OF CALCINEURIN

Calmodulin (CaM; Contraction of **Calcium Modulated Protein**) is an “EF-hand” ubiquitous Ca^{2+} dependent-regulatory protein (Klee et al., 1980). EF-hand proteins exhibit helix-loop-helix domains, which looks like the spread thumb and forefinger of the human hand. The two α -helices are positioned perpendicular to one another and are linked by a short loop region (~12 aa) that binds Ca^{2+} .

CaM has two symmetrical domains separated by a flexible hinge region. CaM works as Calcium sensor and signal transducer for various proteins, including enzymes, transcription factors and cytoskeletal proteins. Binding of four Ca^{2+} ions via the EF-hands, induces a conformational change in the protein that results in the exposure of a hydrophobic region, which can, in turn, bind to Basic Amphiphilic Helices (BAA helices) on the target. (Fig.9)

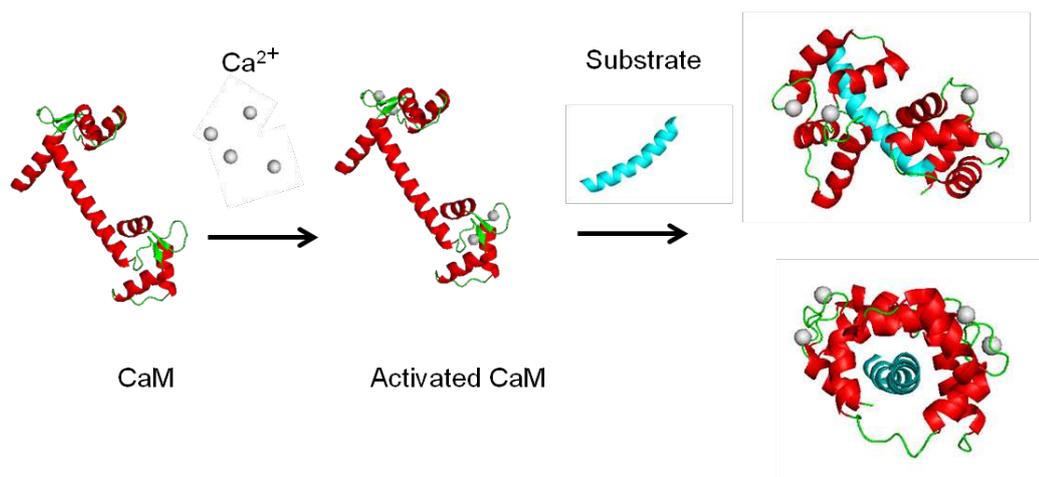


Figure 9. Representation of the CaM conformational changes induced by binding of Ca^{2+} ions. Once activated, CaM binds its target enfolding the binding region. PDB: 3CLN= CaM structure; PDB 2JZI= CaM complexed with the CaM Binding Domain of CnA.

The flexibility of the hinged region allows the protein to wrap around its target. This property makes CaM able to bind to a wide range of protein targets. The presence of the highly conserved BBA helices domain in the sequence of CnA, suggested that the activity of the phosphatase was indeed modulated *via* Calmodulin in a Calcium-dependent manner.

2. CALCINEURIN STRUCTURE

The crystal structure of CnA has been solved using the complex with its inhibitors FK506 and CyclosporinA (Huai et al., 2002) (Ke and Huai, 2003) (Fig.10). The catalytic domain consists of two β sheets flanked by a mixed α/β structure on one side and α helices on the other side. However, the structure of the regulatory region, located between the CnBBD and the AI domain remains to be determined.

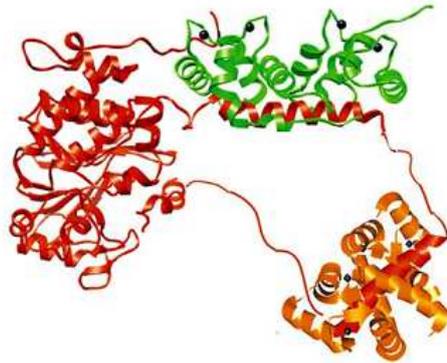


Figure 10. Crystal structure of Calcineurin with CnB and CaM bound to their respective binding site. The structure of the region between the CnBBD and CaMBD and the region between the CaMBD and the AI has not been determined. PDB entry: 1AUI

On the other hand the structure of CnB has been well characterized. This regulatory subunit presents four EF-hands, typical of the Ca^{2+} binding protein family. Being a member of the same family, CnB shows a high structural similarity with Calmodulin. Unlike CaM, however, the four binding sites do not have the same affinity for Calcium. More precisely, Ca^{2+} binding sites 1 and 2 in the N-terminal part of the protein have a low affinity to Calcium while the binding sites 3 and 4 in the C-terminal part bind Calcium with higher affinity. It has been proposed that the binding of Calcium at the site 3 and 4 is necessary for the stability of the heterodimer conformation, while the binding to the low affinity sites results in a partial

activation of the enzyme, but more importantly, allows Calmodulin binding to its site in a Calcium dependent manner (Yang and Klee, 2000).

New insights into the regulation of the phosphatase activity of Calcineurin have been proposed by Wang et al in 2008 (Wang et al., 2008). In this work, using several truncations of the C-terminal part of Calcineurin, the authors have shown that the CaMBD, rather than having only an activation role permitting the binding of CaM, it also has an inhibitory effect on the phosphatase activity of Calcineurin. In their revised model, the CaMBD binds to the CnBBD inducing a folding of the protein which results in the emplacement of the AI domain within the active site. Binding of Calcium ions to the low affinity sites of the CalcineurinB leads to the dissociation of the CaMBD (Wang et al., 2008). The subsequent binding of CaM to its specific site induces finally, the full activation of the enzyme with the displacement of the auto-inhibitory domain from the active site. In fig. 11 a model of this allosteric regulation is proposed.

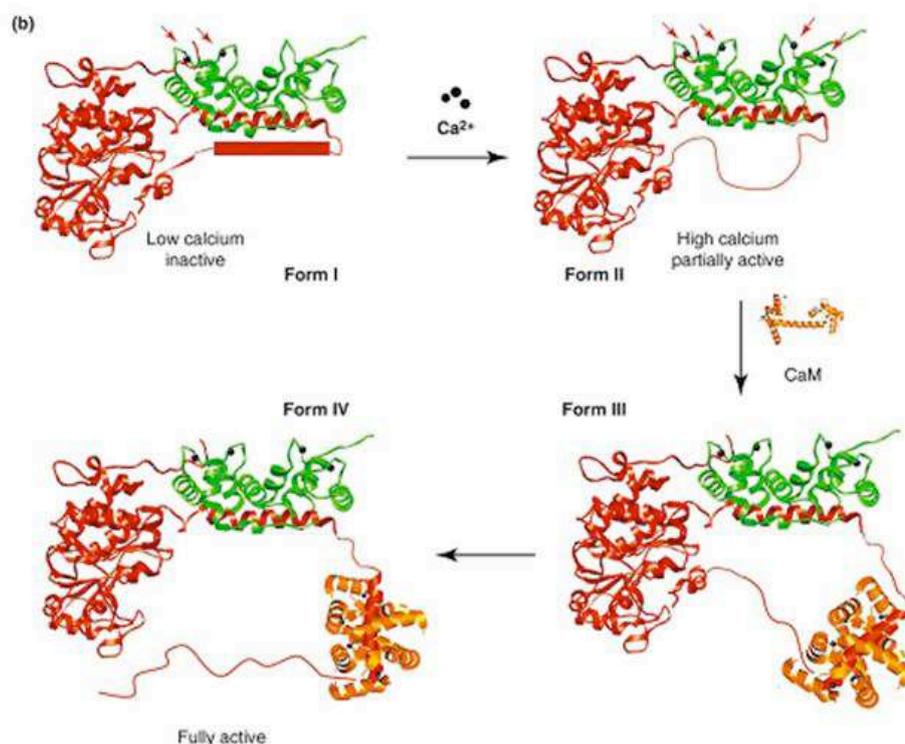


Figure 11. Proposed mechanism of activation of calcineurin. Ca^{2+} occupancy of the low affinity sites on calcineurin B causes dissociation of the calmodulin binding region of calcineurin A from the calcineurin B binding region and causes the transition from form I to form II, facilitating the subsequent binding of calmodulin (form III), which leads to displacement of the autoinhibitory peptide and full calcineurin activation (form IV) (From Interaction of Calcineurin with substrates and targeting protein Trends in Cell biology, Li and Hogan2011 (Li et al., 2011))

3. CALCINEURIN INHIBITORS REVEAL ITS' FUNCTION IN THE IMMUNE SYSTEM

Calcineurin is a target of two immunophilin-immunosuppressant complexes, Cyclophilin A-Cyclosporin A (CyPA-CsA) and FKBP-FK506. Despite their different structures, CsA and FK506 have highly similar biological properties. Both molecules interfere with the TCR (T-Cell Receptor)-mediated signaling pathway which results in the transcription of T-cell activation genes. Based on this observation, Liu et al (1991) supposed that the drug-immunophilin complexes shared a common target. Using CyPA-CsA and FKBP-FK506 affinity chromatography, this group identified Calcineurin as target of both immunosuppressants².(Liu et al., 1991)

Further studies on the crystal structure of the CnA-immunosuppressant complexes offered new insight, not only into the understanding of the molecular basis of the regulation of CnA by both drugs, but also into the structure and consequently into the mechanism of action of Calcineurin. The resolution of the structure of the complexes revealed that both, CsA and FK506, bind to the same region of CnA (Fig.12). The binding site was identified at the level of a composite region formed at the junction of CnA and CnB. The mechanism of action of the immunosuppressant drugs has been elucidated only in 2009 in a work of Rodriguez et al (Rodriguez et al., 2009), following the characterization of the docking site of NFAT, one of the principal substrate of CnA (see section below). The binding of CsA and FK506 to Calcineurin was demonstrated to inhibit the phosphatase activity of the enzyme in a competitive manner, interfering with the interaction of the substrate with its docking site.

Demonstration that Calcineurin is the target of two of the most employed immunosuppressant offered new insights into the role of the phosphatase, underling its participation in T-cell signaling pathways. The role of Calcineurin in the activation of the immune response was elucidated *via* several subsequent studies (Kincaid, 1995, Lyson et al., 1993) (Liu, 2009) (Panther et al., 2011)the essence of which is summarized below.

² Immunophilin are propyl isomerase, but this activity seems to be unrelated to immunosuppression. Immunophilins bind to Calcineurin only in the presence of immunosuppressants.

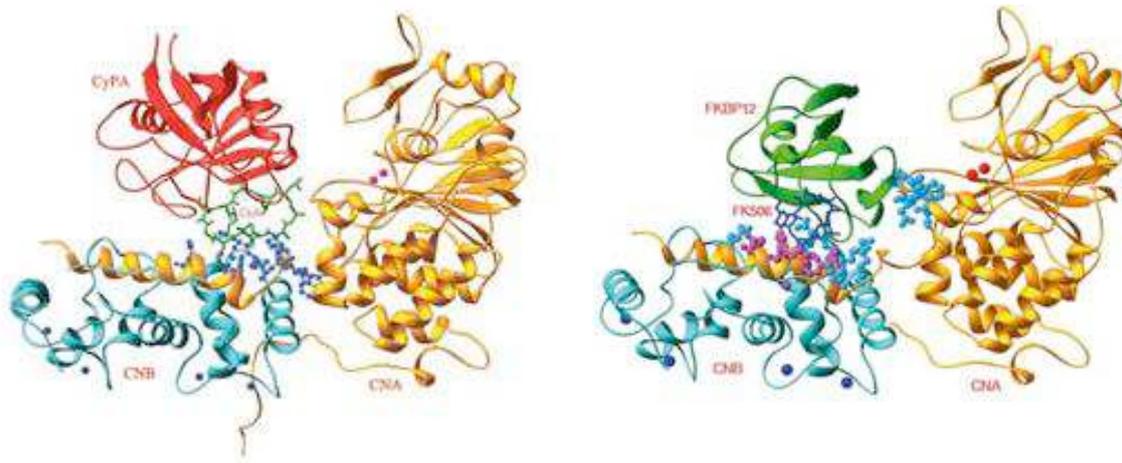


Figure 12. Crystal structure of the CnA-immunosuppressant complexes (Ke and Huai, 2003)

Activation of T-cells mediated by Calcineurin is achieved *via* the nuclear translocation of NFATc (Nuclear Factor of Activated T-cell cytoplasmic form). TCR (T-cell Receptor) activation initiates a protein kinase-mediated signal transduction cascade and simultaneously triggers an influx of Calcium in the cytoplasm. Activation of the protein kinase pathway results in *de novo* synthesis of *fos* and *jun*, whereas increase in intracellular Calcium level activates Calcineurin that in turn dephosphorylates NFAT. Dephosphorylation of NFAT leads to a conformational change resulting in the exposure of the NLS site of NFAT and subsequent translocation in the nucleus (Rao et al., 1997). Once in the nucleus NFAT oligomerizes with *fos* and *jun* to create a functional DNA binding-NFAT complex and activates the transcription of several target genes, such as IL-2, IL-4, TNF, which are responsible for T-cells activation. (Fig.13)

Evidence that NFAT is a substrate of Calcineurin was offered by the observation that CsA and FK506, which inhibit CnA activity, block the translocation of NFAT from the cytoplasm to the nucleus. Consistent with this observation, *in vitro* experiments, have indicated that NFAT is a direct substrate of Calcineurin being able to form a complex with the phosphatase.

Although the role of Calcineurin in the activation of the immune response is the first and the most well characterized, it represents just one of a wide range of pathways regulated by this phosphatase *via* NFAT. The Calcineurin-NFAT pathway transduces calcium signal into genes expression in diverse biological processes, depending on the cell type. Deregulation of Calcineurin-NFAT pathway results in pathologies including cardiac

hypertrophy (Wilkins and Molkenin, 2004), muscular dystrophy (Olson and Williams, 2000), osteoporosis (Winslow et al., 2006) (Yeo et al., 2006), Alzheimer disease (Gong et al., 1994) (Ermak et al., 2001) and Down syndrome (Fuentes et al., 2000).

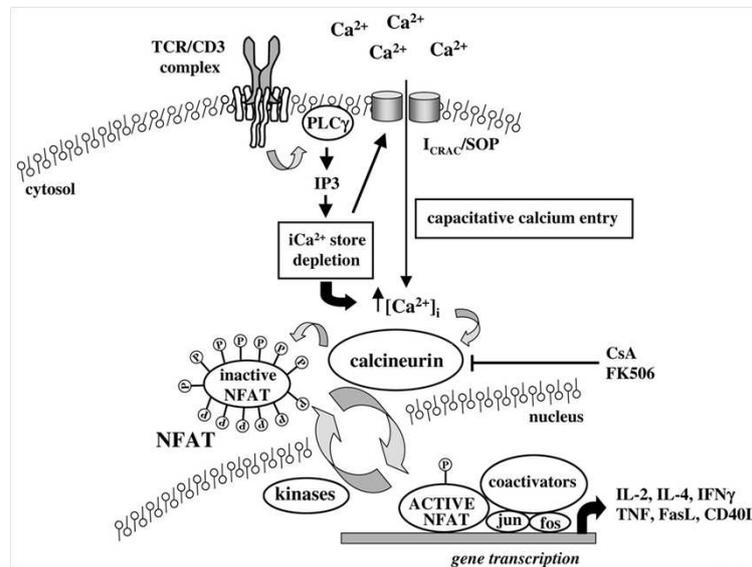


Figure 13. CNA-NFAT pathway in the activation of immune system response

4. NFAT AND CALCINEURIN BINDING

NFAT transcription factor family consists of four members: NFATc1, NFATc2, NFATc3 and NFATc4³. All the members of this family share the same structure and all are activated by Calcineurin in a Ca²⁺/CaM dependent manner. On the basis of the insight into the activation of the transcription factor *via* Calcineurin, NFAT has been used as model to define the sites of interaction of Calcineurin with its substrates. Two main binding sites for Calcineurin on NFAT have been identified: the PxlIT motif located near the N terminus of the regulatory region and the LxVP motif located near its C terminus.

³ The NFAT family includes also another member: NFAT5. Unlike the other four members of the family, this transcription factor is not regulated by Calcineurin and it is mainly implicated in the response of the osmotic stress in mammalian cells. In this review, references to NFAT will take into account exclusively NFAT1, 2, 3 and 4.

The PxlIT motif is conserved amongst the four members of the NFAT family and is further shared with other Calcineurin substrates and regulatory proteins from yeast to human. The crystal structure of the complex Calcineurin-NFAT binding site, obtained using a synthetic peptide, pVIVIT, illustrates then the mechanism by which Calcineurin interact with all its substrates. Thus, the binding site has been located along the edge of strand β 11 and β 14 of the catalytic domain.

On the other hand, the LxVP motif is less conserved and seems to have different binding affinities amongst the four members of NFAT family. For example, the LxVP motif of NFAT1 has a weaker affinity for Calcineurin than the other members of the family (Rodriguez et al., 2009). This difference, due to the absence in NFAT1 of an aromatic aminoacid before the L of the motif could be a source of differential regulation of NFAT isoforms activity within the cell.

The site of interaction of the LxVP motif with Calcineurin has been localized to a composite site formed by the CnA and the CnB, the same region that was been identified as a docking site for the immune-suppressants CsA and FK506. This observation underlines that immunosuppressant complexes and substrates of Calcineurin share the same docking site and consequently proves that CsA and FK506 inhibit Calcineurin in a competitive manner, interfering with the substrate recognition and interaction.

5. CALCINEURIN FUNCTIONS

Although Calcineurin has been identified initially, 40 years ago, as an important calcium signaling mediator in the nervous system, a multitude of subsequent studies has demonstrated that this phosphatase has far more extensive roles. The discovery that CnA is the principal target of the immunosuppressants CsA and FK506, underlined the involvement of the phosphatase in the activation of the immune system. Subsequently, the finding that CsA blocks the nuclear translocation of the transcription factor NFAT and that this protein is dephosphorylated by calcineurin, revealed that Calcineurin activity plays a central role in regulation of target gene transcription. Since then, CnA and NFAT have been shown to participate in signaling pathways that control the development and the function of the immune (Jain et al., 1993) (Gwack et al., 2007), nervous (Ermak et al., 2001, Gong et al.,

1994), cardiovascular (Wilkins and Molkenin, 2004) and musculoskeletal system (Chin et al., 1998) (Calabria et al., 2009) (Dunn et al., 1999) (Naya et al., 2000).

a. Calcineurin and skeletal muscle

Skeletal muscle is a tissue in perpetual remodeling. It is continuously submitted to internal and external stimuli and it must adapt to the stress of use or disuse. In case of overwork muscles tend to become larger and more efficient going toward hypertrophy; in case of disuse they became smaller and less efficient, which results in muscle atrophy. These changes are the response to the electrical stimuli coming from the motor neurons. According to the nature of signals received, skeletal muscle cells activate the expression of different proteins. This process is regulated by a complex signaling pathway that allows the muscle to finely adapt to the different stimuli received.

The principal messenger of the motor neuron is calcium and the oscillation in its cellular concentration is the code used to communicate with the nucleus to induce the transcription of the appropriate genes. In this process the activity of Calcineurin assumes an important role. Transducing calcium signaling through the activation of the transcription factor NFAT, this phosphatase is the principal propagator of motor neuron information.

Calcineurin is expressed in all muscle types and its role in skeletal muscle has been widely investigated. Given its role in modulating the adaptation of the muscle to the different physiological and environmental stimuli, Calcineurin could be considered, citing Robin Michel, as *a sensor of change*. However, as is it easily conceivable, Calcineurin does not act alone in modulating muscle processes, but it cooperates with other pathways. Although the involvement of this phosphatase in skeletal muscle growth, muscle regeneration and differentiation has already been demonstrated in a very large number of works, some aspects of its mechanism of action remained still to be determined. Particularly, given the discrepancy between the readout of different experimental systems, the role/impact of Calcineurin in skeletal muscle repair is still controversial.

In the next section a review of the role of Calcineurin in skeletal muscle remodeling will be proposed.

CELLULAR AND ANIMAL MODELS FOR STUDY OF MUSCLE REGENERATION

1. C2C12 CELLS

C2C12 cells were originally obtained by Yaffe and Saxel (Yaffe and Saxel, 1977) through selective serial passages of myoblasts cultured from the thigh muscle of C3H mice 70 h after a crush injury. The C2C12 cell line proliferates in culture and can be stimulated to differentiate by lowering serum level in the media. Within three days cells fuse, forming contractile myotubes and producing characteristic muscle proteins, such as MyHC, Troponin and MRFs. C2C12 cells are a useful model to study the differentiation of skeletal muscle cells, because they mimic many aspects of myoblast differentiation, facilitating the study of underlying signaling pathways. (Fig.14)

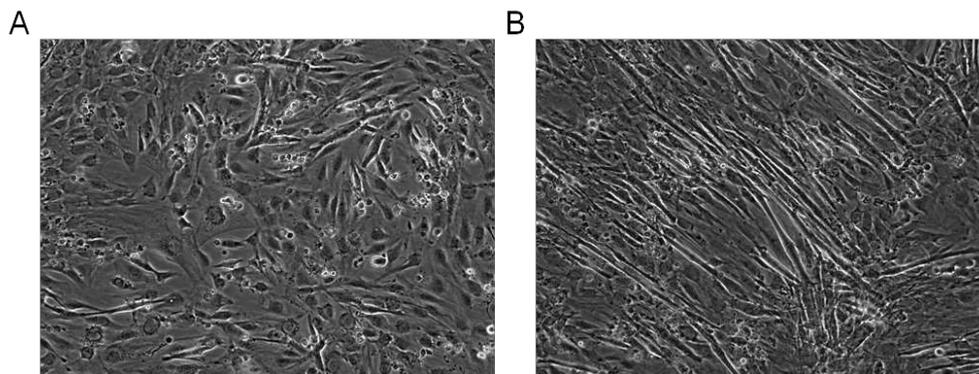


Figure 14.C2C12 cells (A) non differentiated myoblasts; (B) differentiated myotubes.

2. ANIMAL MODELS

Several different models are used to study muscle remodeling. These are based on insult, conferred by toxins, direct mechanical injury or denervation. The essence of these models is summarized below.

Injection of myotoxin, such as bupivacaine, cardiotoxin (CTX) and notexin (NTX) is commonly used to provoke muscle regeneration. They act either blocking the neuromuscular transmission (CTX) or inducing depolarization and contraction of myofiber resulting in the disruption of membrane organization (NTX), inducing massive muscle degeneration. 3-4 days after the injection of these toxins, muscles initiate a phase of

regeneration characterized by the occurrence of smaller fibers and numerous centrally located nuclei.

Muscle growth and regeneration can also be provoked by mechanical injury. One of the techniques used to induce muscle growth is the “over-loading”. When a fast muscle is subjected to an increase of workload by removing synergistic muscles, the muscle responds by increasing fiber size and muscle weight. A switch of fibers from fast to slow phenotype is also observed.

Denervation-induced atrophy is another model used to investigate the involvement of specific signaling pathways in muscle growth and regeneration. Denervation to the hind limbs by removal of a small portion of the sciatic nerve induces atrophy in skeletal muscle. Muscle fibers become smaller, resulting in the loss of 50% of muscle mass within 2 weeks. This model is relatively easy to implement, offering a means to study muscle atrophy (Tomanek and Lund, 1973) (Shavlakadze et al., 2005) as well as possible potential therapeutic approaches to prevent loss/restore muscle mass and, hopefully, function (Shavlakadze et al., 2005).

3. MDX MICE

A strain of mice arose from a spontaneous mutation (*mdx*) in inbred C57BL mice (Bulfield et al., 1984). This mutation is X chromosome-linked and produces viable homozygous animals that lack the muscle protein dystrophin. The human Duchenne Muscular Dystrophy (DMD) is provoked by the same mutation. Dystrophin is an important component of the dystrophin-glycoprotein complex that leads the anchor of the myofiber cytoskeleton to the extracellular matrix. The lack of this protein causes a fragility of the sarcolemma and induces an increase of the susceptibility to contraction induced injury. After 3-5 weeks postnatal, muscles of these mice show extensive signs of degeneration, followed by a phase of muscle regeneration leading to a transient muscle repair. The constant alternation of degenerative and regenerative phases, characterized by the occurrence of extensive necrotic areas, is typical of this model and is present during the entire lifespan of these animals. The regeneration process appears to be defective, however, since animals are weak and die earlier than wild-type mice. All these features make *mdx* mice a valid animal model of Duchenne muscular dystrophy.

THE CONTROVERSIAL ROLE OF CALCINEURIN IN SKELETAL MUSCLE

1. FIBER TYPE SPECIFICATION, HYPERTROPHY AND DIFFERENTIATION

a. Calcineurin and Muscle Fibers Specification

Muscles are able to modulate their mass and function using Calcium as second messenger linking nerve activity in change of gene expression. More precisely motor nerve activity has a central role in determining the fiber type composition of skeletal muscle.

Chin et al, (Chin et al., 1998) have shown that over-expression of a constitutively-active form of CnA in C2C12 cells leads to an increase of the transcriptional activity of myoglobin (Mb) and Troponin I slow (TnIS), whereas no effect is observed on the activity of MCK (Muscle Creatin Kinase) (Chin et al., 1998). Mb and TnIS are expressed selectively in slow muscle fibers, whereas MCK is expressed preferentially in fast muscle fibers.

Treatment with CsA, an inhibitor of CnA, blocks the up-regulation of Mb genes expression, thus underling the specificity of the response. In the same publication, the team showed that the effect of CnA on the up-regulation of slow fiber specific gene is mediated by NFAT activation. Consistent with the results obtained in C2C12 cells, treatment with CsA in adult rat resulted in a partial conversion of slow to fast fibers, underlining the importance of Calcineurin in the regulation of the program of gene expression. Moreover, in transgenic mice expressing a constitutively-activated Calcineurin, an increase in slow muscle fibers has been observed (Naya et al., 2000), suggesting that activation of Calcineurin *in vivo* is sufficient to activate the slow fiber gene expression program

NFAT collaborates with other transcription factors, such as MEF2, to transduce the Calcineurin generated signal. Binding sites for the transcription factor MEF2 are present in the promoter region of muscle specific genes. In 1999, Chin et al (Chin et al., 1998), proposed a first model to explain how motor neuron can modulate the expression of fiber type specific genes *via* the CnA-NFAT pathway. (Fig.15) Tonic motor nerve activity, characterized by low amplitude and long duration impulses, leads to an influx of calcium in the muscle fiber that is sufficient to activate Calcineurin. Once activated Calcineurin

dephosphorylates and promotes the nuclear translocation of NFAT, which can then in association with MEF2, control the transcription of slow fiber specific genes. On the contrary, in fast fibers, the phasic and high amplitude impulses are of insufficient duration to maintain calcineurin in an active state. In this case NFAT remains in the cytoplasm, resulting in a down-regulation of the slow fibers gene expression program versus an up-regulation of the fast fiber program.

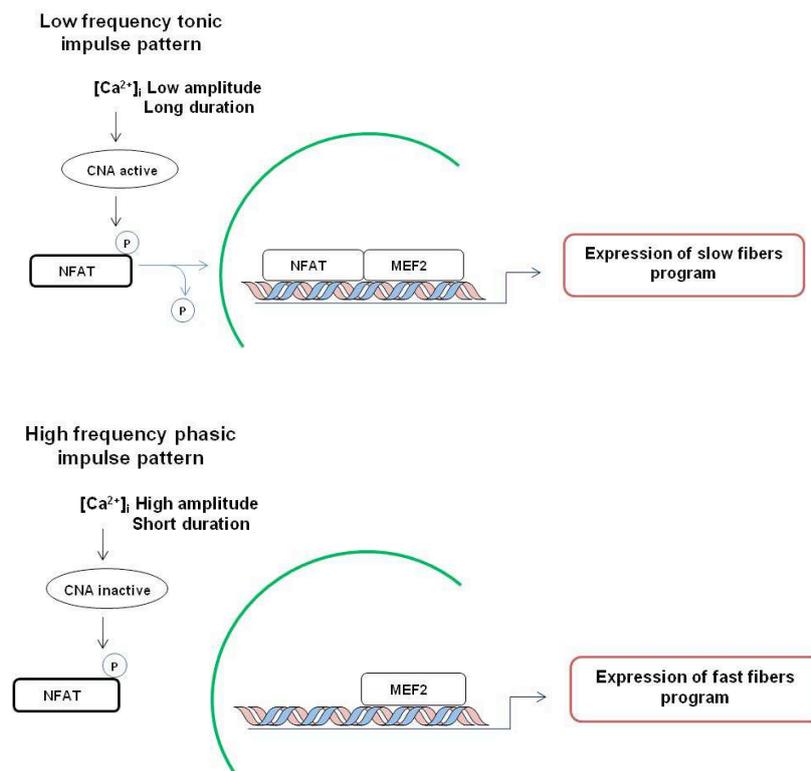


Figure 15. Model proposed by (Chin et al., 1998) for the regulation of the fiber-specific gene expression.

However, the model proposed here by (Chin et al., 1998) based only on the first insights into the mechanism of action of the Calcineurin-NFAT pathway in the regulation of the fiber type specification does not reflect the real picture of this process. Nevertheless this model offers the primordial representation of how the variations in intracellular calcium concentration can be transduced in a different expression pattern. The following studies, even if sometime strikingly opposite to each other, have allowed to understanding in more

detail the complex network of factors that regulate the fiber type determination in skeletal muscle.

Opposing view to the results presented by Chin et al, come from the work of Swoap and collaborators (Swoap et al., 2000). They have shown that activated CnA in C2C12 cells induces the transcription of both, fast- and slow-specific genes. In his work, Chin tested only the expression of MCK, in contrast to Swoap, who showed that other fast specific genes, such as SERCA 1, PGAM-M and IIB MHC, were over-expressed upon CNA activation. In adult soleus muscle (slow) activation of CNA (*via* intramuscular injection) results in inhibition of SERCA1 and MLC2 slow reporter, whereas in EDL (fast) reporter activity was unchanged. The contrasting observation obtained in previous studies on C2C12 cells, indicate a possible difference amongst the factors regulated by Calcineurin *in vitro* and *in vivo*.

Results obtained in this work are also opposed to those obtained after treatment with CsA. However it appears necessary to underline that, it has been shown that after 2 weeks of treatment with CsA, muscle mitochondrial respiration in mice was decreased due to the suppression of mitochondrial electron chain capacity (Mercier et al., 1995). The use of CsA to confirm the involvement of CnA in determining fiber type gene expression program is indeed questionable, according to the possible secondary effects related to mitochondrial respiration. Furthermore CnA is a protein widely expressed in all tissues and in particular high levels of this phosphatase are present in neurons. Thus, changes in fiber phenotype upon treatment with CsA might reflect principally the effect of CnA inhibition in neurons rather than its direct activity in muscle fibers.

The hypothesis proposed by Swoap was finally contested by Serrano and et al, (Serrano et al., 2001), who demonstrated that the effect of CsA is not linked to a change in nerve activity. In regenerating *soleus*, stimulation with a tonic (20Hz) pattern of impulse, similar to whom present in slow fiber, induce the expression of slow MyHC. This expression is inhibited by treatment with CsA (Serrano et al., 2001), proving that the effect of CsA is not linked to the nerve activity but is the result of an inhibitory effect on muscle fibers. The same readout was obtained with a specific inhibitor of CnA, such as cain/cabin1.

b. Role of NFATc1 in the regulation of the slow fiber type gene program

To confirm that the Calcineurin activity was mediated by its major target, NFAT, adult rats were transfected with a plasmid coding for VIVIT, an inhibitor peptide of NFAT activity. As expected VIVIT blocks the up-regulation of slow MyHC in regenerating *soleus* muscle and up-regulates fast MyHC 2X and to a minor degree MyHC 2B in adult and in regenerating *soleus* (Serrano et al., 2001).

Consistent with these results, the over-expression of a constitutively-active NFATc1 results in an up-regulation of the expression of slow MyHC, whereas the fast MyHC-2B promoter appears to be inhibited. This is the case for regenerating *soleus* and EDL. In contrast no up-regulation of slow MyHC was observed in adult EDL. Even if stimulated for a long period of time with slow-patterned electrical impulses, fast twitch muscles only exhibit an up-regulation of MyHC-2X and 2B versus a down-regulation of MyHC 2A, while no expression of slow MyHC is observed. Thus, this difference between adult and regenerating fast muscles can be explained considering that transformation from fast to slow fibers in adult muscle can take place only to a moderate extent.

In adult fast muscles, thus, the fast fiber program appears to be established by an efficient system of repression of slow fiber protein expression. In contrast, regenerating muscles seem to have a greater plasticity and they appear to be more sensitive to the slow patterned electrical stimuli than adult muscles. In this regard it is important to remember that the muscle regeneration process retraces the steps of muscle development. In the embryonic stage, both slow and fast genes are co-expressed in all muscle groups. Only in the later stage of development, once fibers are innervated and depolarized by specific motor neurons, the expression of slow and fast genes is confined to specific fibers (Rana et al., 2008). Consequently, the higher plasticity observed during regeneration could be explained by the fact that, in newly formed fibers, the fast twitch program is not yet definitively established and that promoters for slow fiber genes are still accessible to transcription factors, such as NFAT.

Important insights into the transduction of motor neuron activity in modification in contractile properties of skeletal muscle were obtained by investigating the involvement of *cis*- and *trans*- acting transcription regulatory elements in modulating the activity of specific transcription factors. Rana et al, (Rana et al., 2008) focused in their work, on the regulation

of Troponin I slow (TnIs) and Troponin I fast (TnIf) regulation, being the expression of this genes restricted respectively to slow and fast fibers in adult muscles.

NFATc1 can bind to the TnIf FIRE (Fast Intronic Regulatory Element) and act as a repressor of transcription in response to slow-patterned electrical stimuli in slow muscles (Rana et al., 2008). Consistent with the observation that NFATc1 plays a critical role in the determination of slow fiber gene expression program, is the evidence that the level of nuclear NFATc1 is much higher in slow muscle, such as *soleus*, than in fast muscles, such as EDL. The conclusion of this work is that in slow muscles, stimulated by a low frequency and tonic impulse pattern (see Fig.15), NFATc1 appears to have a dual function: it simultaneously promotes the expression of TnIs and represses the expression of TnIf (Rana et al., 2008) (Fig. 16).

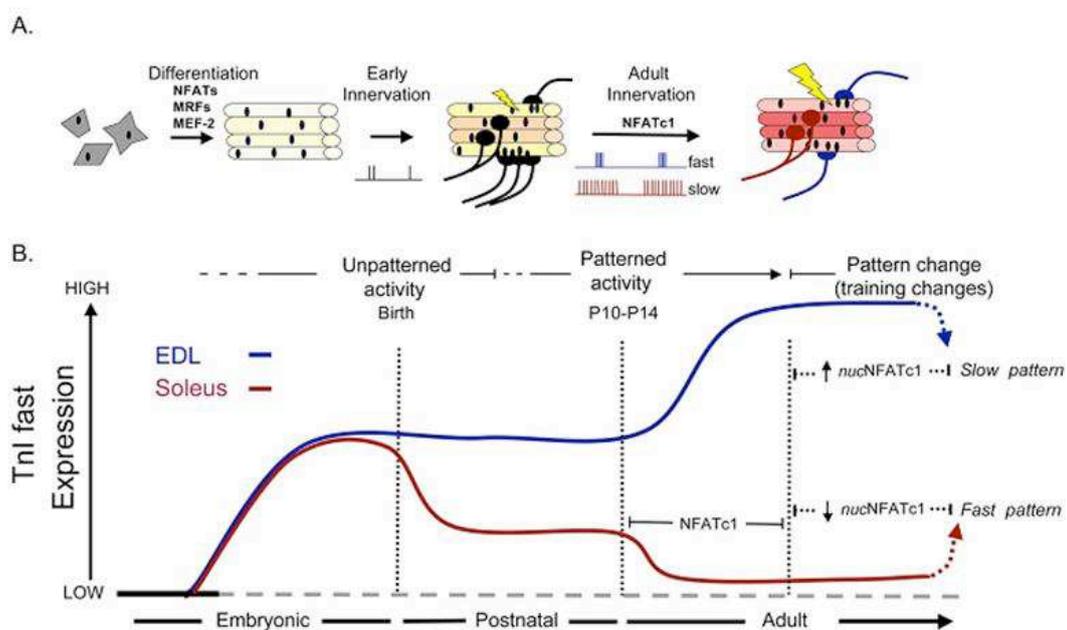


Figure 16. Model representing the developmental and activity-dependent regulation of TnIf in slow- and fast-twitch myofibers. (A) Depiction of the progressive stages of skeletal muscle differentiation, motoneuron innervation and retraction, and the role of motoneuron patterned activity. Some of the transcription factors implicated in these different stages of muscle development are shown above the arrows. While motoneuron and muscle diversification are evident during embryonic/fetal and perinatal development, further specialization of adult slow-and fast-twitch myofibers after P7 coincides with their stimulation by slow and fast patterns of motoneuron activity. The different color intensities of myofibers are to emphasize that slow and fast motoneuron activity enhances the differences between slow- and fast-twitch muscles, but yet adult muscles retain an adaptive capacity. (B) Illustration of the transcriptional regulation of TnIf in developing and maturing fast (blue) and slow (red) myofibers. The diagram is based on this work and prior studies on the

quail Tnlf gene. (Taken from: Activity-dependent repression of muscle genes by NFAT. Rana *et al.* 2008, PNAS (Rana et al., 2008))

How NFATc1 can exert this dual function remains to be determined. A possible explanation could be, that since in all signaling pathways, NFATs cooperate with other transcription factors to regulate gene expression, it is likely that this dual function is due to other proteins interacting with NFATc1.

c. NFAT isoforms and muscle fiber type specification

The four members of the NFATc family, *i.e.* NFATc1, c2, c3 and c4 have been demonstrated to have different roles in the activity-dependent muscle fiber specification (Calabria et al., 2009). It has been shown that the slow muscle-specific gene, MyHc, is under the control of the all four members of the NFAT family; the fast muscle specific genes, MyHC 2A and MyHC 2X depend on NFATc2 and c3, whereas MyHC 2B is mainly regulated by NFATc4. Furthermore the three fast specific genes, MyHC2A, 2X and 2B result to be independent from NFATc1 (Calabria et al., 2009). Taken together, these observations confirm that NFATc1 is a specific determinant of slow muscle fiber specification.

Consistent with these results, histological observation of the NFAT isoforms have underlined a different nuclear-cytoplasmic distribution of the members of the family in fast and slow muscles. NFATc1 has been shown to be localized in the nucleus in most fibers of the slow muscle *soleus*, whereas a very low nuclear localization was observed in EDL, a fast muscle. NFATc2 and c3 show a low nuclear localization in both *soleus* and EDL. On the other hand, NFATc4 is localized exclusively into the nucleus in both muscles. On the basis of these insights, it has been proposed that NFATc4 is responsible for the default specification of fast 2B fiber program and that a graded nuclear recruitment of the other NFAT isoforms can modulate the expression of the fiber type specific genes according to the motor neuron activity.

The fact that NFATc1 is an important determinant of slow genes expression could be explained by its weaker affinity for CnA (Rodriguez et al., 2009). Being less affine to CnA than the other isoforms, it needs a significant activation of Calcineurin to be maintained in an activated state. Thus, the low intensity and long duration calcium oscillation, typical of slow muscle fibers, seems to be the optimal condition for the activation of NFATc1. In contrast,

the short duration of calcium oscillations in fast fibers are not sufficient to maintain NFATc1 in an activated state.

These observations confirm the importance of a co-operation among the members of the NFAT family in the determination of the fiber-specific genes expression program. However the precise mechanism that leads to a sequential recruitment of the different NFAT isoforms in response to different activity patterns remains to be established.

2. ROLE OF CALCINEURIN IN SKELETAL MUSCLE HYPERTROPHY

a. Growth factor induction

IGF-1 is known to mediate the growth in muscle and other tissues. In muscle, IGF-1 has been shown to induce hypertrophy *via* the CnA-NFAT pathway (Musaro et al., 1999). Treatment with IGF-1 induces a mobilization of intracellular calcium that activates the calcium-calmodulin-dependent phosphatase Calcineurin that results in the nuclear translocation of the transcription factor NFATc1. In C2C12 cells, hypertrophy observed after over-expression of IGF-1 is suppressed by inhibitors of CnA, such as CsA and FK506.

Expression of an activated form of Calcineurin has been shown to mimic the effect of IGF-1 on skeletal muscle hypertrophy, proving the involvement of the CnA-NFAT pathway in the hypertrophic action of IGF-1 (Musaro et al., 1999). Moreover, expression of IGF-1 or activated Calcineurin has been shown to induce the expression of GATA-2, which is known as a marker of skeletal muscle hypertrophy. Once accumulated in myocytes nuclei, GATA-2 cooperates with NFATc1 to activate a gene expression program resulting in the hypertrophic response.

Interestingly, the effect of IGF-1 as well as of CNA inhibitors on C2C12 cells is restricted to a defined time window. Hypertrophy in C2C12 cells was observed only when recombinant IGF-1 was added before or during cell differentiation. In the same way, CsA blocked myotubes growth in cultures transfected with IGF-1, only if added before or at the time of differentiation but not later (Semsarian et al., 1999). This suggests that there is a critical timing for either activation or inhibition of muscle hypertrophy and that this window corresponds to the early period of transition from myoblasts (satellite cells) to differentiated myotubes.

According to these results, the hypertrophic process induced by CnA-NFAT pathway could, at a certain time, become resistant to the effect of CnA inhibitors. Calcineurin seems to be indeed crucial for promoting the early stage of myogenesis, however other signaling pathways, downstream of IGF-1 and independent to Calcineurin activity, are active during the later stage of differentiation. This could explain the failure of CnA inhibitors to suppress muscle growth in regenerating *soleus* when added 3 days after injury (Serrano et al., 2001). It is probable, at that time, the regenerative process has already taken place and it is thus insensitive to the effect of CnA-NFAT pathway inhibitors.

b. Contractile stimulus, Calcineurin and muscle growth

Skeletal muscle fibers adapt to higher contractile stimuli, increasing their size and promoting the expression of slow fiber-specific contractile protein. The higher motor neuronal activity stimulating overloaded muscles, triggers an increase in intracellular calcium concentration and an increase in IGF-1 release, resulting in the activation of Calcineurin

This response has been shown to be mediated by the Calcineurin pathway, since treatment with CsA and FK506 results in the inhibition of muscle hypertrophy and of conversion of fast fibers into slow fibers typically observed under conditions of overload (Dunn et al., 1999).

3. CALCINEURIN AND MUSCLE DIFFERENTIATION

Involvement of Calcineurin-NFAT pathway in muscle differentiation process has been underlined by the observation that one of the downstream targets of this phosphatase is the up-regulation of Myf5 gene expression. Myf 5, which is a member of the MRF family, has been shown to play a crucial role in the development and differentiation of skeletal muscle (Rudnicki et al., 1993). It is mainly involved in the activation of the myogenic program in satellite cells, which are the precursors of muscle fibers. Activation of the Calcineurin-NFAT pathway results in an increase of Myf5 mRNA level in myoblast cultures (Friday and Pavlath, 2001).

Once activated and thus committed to the myogenic lineage, myoblasts fuse to form a multinucleated myofiber. Abbott *et al.* (Abbott et al., 1998), have demonstrated that the members of the NFAT family exhibit a different pattern of nuclear localization during the

various steps of myogenesis. In more detail, NFATc3 appears to be nuclearly localized only in myoblasts, whereas NFATc1 and c2 are in the nucleus only in myotubes, with the nuclear translocation of NFATc2 restricted to newly formed myotubes (Abbott et al., 1998). This observation suggests that NFATc2 is involved in the last stage of myogenesis.

The crucial role of NFATc2 in regulating the growth of multinucleated fibers has been confirmed in a work of Horsley *et al.* (Horsley et al., 2001), in which they showed that NFATc2^{-/-} mice show a decrease of fibers size in both fast and slow muscles. The same observation has been done in regenerating muscle. In NFATc2^{-/-} regenerating muscles new myofibers are formed normally, but the growth of these fibers is blocked. A reduction in the number of myonuclei is also observed in these mutants (Horsley et al., 2001).

Taken together, these observations indicate that NFATc2 plays a crucial role in skeletal muscle growth of newly formed myotubes. NFATc2 has been shown to exert its role through the activation of the expression of the specific cytokines, such as Interleukin-4 (IL-4). IL-4 is known to induce macrophage fusion and, in addition, this cytokine has been demonstrated to promote the recruitment and fusion of myoblasts in order to increase myotube size (Horsley et al., 2003). First, a subset of differentiated myoblasts fuses to form a nascent myotube with a limited number of nuclei. In these newly formed myotubes, the activation of NFATc2 leads to the expression and the release in the extracellular matrix of the IL-4, which induces the second phase of fusion through IL-4R on myoblasts. This action leads to an increase in the number of nuclei within nascent myotubes and the formation of a larger mature myotube. (Fig.17)

Therefore, NFATc2, *via* the activation of IL-4, plays a crucial role in regulating the fusion of differentiated muscle cells and thus, in muscle cell growth.

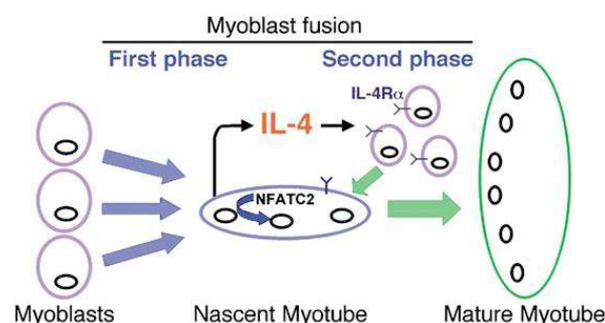


Figure 17. Model for the role of IL-4 in the recruitment of myoblast fusion during muscle growth (Horsley et al., 2003)

It appears now clear, that Calcineurin plays a crucial role in the skeletal muscle remodeling. This phosphatase is an important modulator of both skeletal muscle hypertrophy and specific-fiber type gene expression. According to the different stimuli, such as variation in motor neuron activity or contractile overload, Calcineurin can activate various downstream pathways that allow muscle to adapt to different conditions. A schematic representation of some of the key factors that co-operate with Calcineurin and are involved in the transduction of calcium signal in muscle differentiation, growth and fiber type specification is presented below.

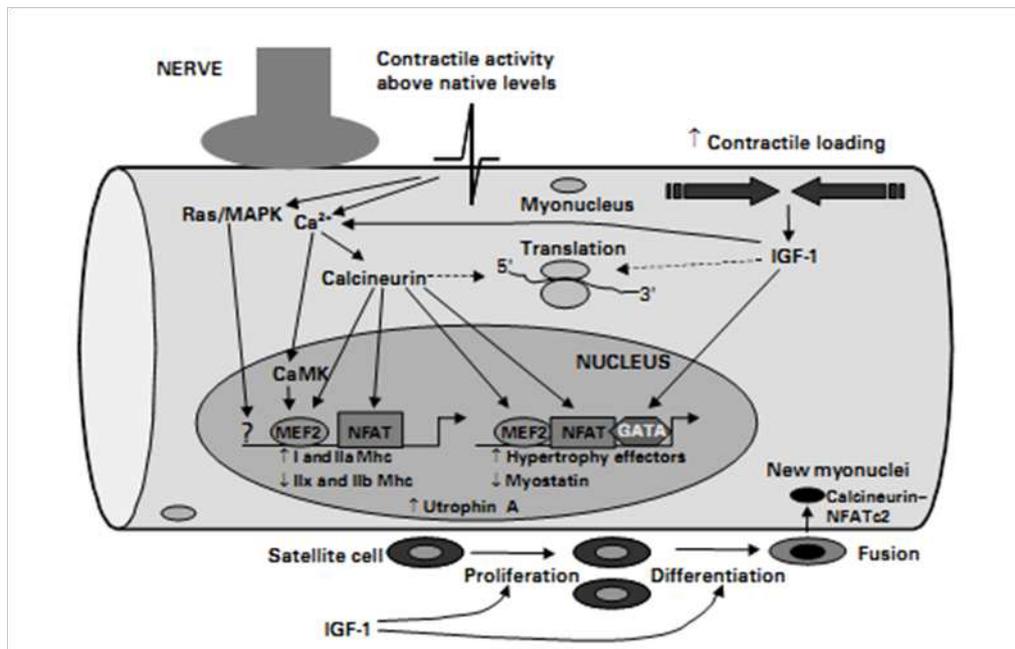


Figure 18. Overview of muscle signalling mechanisms. Neural activation and mechanical loading of the muscle activate numerous intracellular signals that result in hypertrophic growth of the muscle. Neural recruitment results in sarcolemmal membrane depolarizations and subsequent increases in intracellular Ca^{2+} levels, with the resultant intracellular Ca^{2+} concentration dependent on the stimulation frequency. This increase in intracellular Ca^{2+} can activate a number of transcriptional pathways including the Ca^{2+} /calmodulin-dependent phosphatase calcineurin and Ca^{2+} /calmodulin-dependent kinase (CaMK) pathways. Calcineurin and its downstream transcription factor nuclear factor of activated T cells (NFAT) play an important role in the activation of type I and IIa myosin heavy chain (Mhc), oxidative enzyme and utrophin A genes as well as in the hypertrophy response through decreases in myostatin expression and altered expression of other unknown targets. Calcineurin also acts through myocyte-enhancing factor 2 (MEF2)-dependent transcription. In parallel with the calcineurin–NFAT and calcineurin–MEF2 signals, other intracellular signals are activated to effect growth. These signals are triggered by membrane depolarizations, contractile loading or the release of growth factors, such as insulin-like growth factor 1 (IGF-1), which activate hypertrophic responses through Ras/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase and Akt/protein kinase B. One of the downstream effects of calcineurin and IGF-1 is activation of satellite cell proliferation and fusion with existing myofibres, which contributes, in part, to the resultant hypertrophy. (Michel et al., 2004)

The discrepancies that arose from the numerous studies on the role of the CnA/NFAT pathway in muscle remodeling (Table 2), are most likely due to the different experimental models and approaches used. Different dose regimens of the immunosuppressant CsA, for example, combined with different time windows of the treatment, can result in totally divergent readouts. Moreover, slow and fast muscles, characterized by different fiber type composition, respond differently to the same stimulus. Therefore the effect exerted by the activation of Calcineurin is highly dependent on a wide range of conditions.

Transgenic mice expressing a constitutively-activated form of Calcineurin (CnA*) have been widely used. While a pioneering tool for the study of the role of CnA in development and muscle remodeling, it is important to underline that in this model, the activated forms of the phosphatase are constitutively expressed from the first stages of the development. Therefore, the phenotype observed in these mice could be the result of the action of the transgene during the developmental stage and not in adult muscle. The same comment could be made for knockout mice. Developing muscles are more plastic than adult muscle and therefore they are more sensitive to the modulation of the gene programs generated from either constitutive activation or constitutive absence of Calcineurin. Therefore, care must be used when comparing the readouts obtained with transgenic or knockout mice with the effects obtained after over-expression of CnA* or inhibition of its activity in adult mice.

These results underline the difficulty to compare the readouts obtained from the published studies and raise the question of the choice of the approach to adopt for the investigation of a given signaling pathway. Finally, the cumulative results obtained from all the strategies, encompassing over-expression of activated enzyme, use of inhibitors, transgenic knock in and knockout mice, constitutive or inducible expression, used to characterize the role of Calcineurin in muscle remodeling, have validated it as a target of choice for modulating muscle development and performance. Unfortunately, until now, it has been impossible to investigate the effects of this signaling pathway by the direct activation of endogenous Calcineurin.

MODEL	MUSCLE	TREATMENT	EFFECT	REF
C2C12	NA	CnA* overexpression	Myoglobin, Troponin I slow upregulated Muscle Creatin Kinase downregulated	Chin <i>et al.</i> 1998
C2C12	NA	CnA* overexpression	Upregulation of both fast and slow fiber-specific genes (different from those monitored by Chin 1998)	Swoap <i>et al.</i> 2000
C2C12	NA	CsA 125 mM, 250 mM	Myoglobin downregulated	Chin <i>et al.</i> 1998
CD-1 mice (male) Overload	<i>plantaris</i> (Fast)	CsA 25mg/kg, s.c. # twice daily 1, 2, 4 wks.	After 4 wks of treatment, both CsA and FK506 block hypertrophy and switch from fast to slow fibers normally observed in overload model	Dunn <i>et al.</i> 1999
CD-1 mice (male) Overload	<i>plantaris</i> (Fast)	FK506 3-5mg/kg, s.c. twice daily 1, 2, 4 wks.		
CnA* Transgenic mice (2 month)	<i>gastrocnemius</i> (Fast) <i>soleus</i> (Slow)	NA	Increase in slow muscle fibers, no hypertrophy	Naya <i>et al.</i> 1999
Rat	<i>soleus</i> (Slow)	CsA 5mg/kg, <i>i.p.</i> once daily, 6 wks	Slow to fast fiber conversion	Chin <i>et al.</i> 1998
Rat	<i>Soleus</i> (Slow)	<i>i.m.</i> injection of plasmid expressing CnA*	Downregulation of fast and slow reporters	Swoap <i>et al.</i> 2000
Rat <i>Marcaffeine injection j-3</i>	Regenerating <i>soleus</i> (Slow)	<i>i.m.</i> injection of plasmid expressing NFATc2	Inhibition of MLC2 (Slow fiber marker)	
Wistar Rat (male) <i>Bupivacaine injection j-3</i>	Regenerating <i>soleus</i> (Slow)	CsA 5mg/kg, <i>i.p.</i> FK506 1 mg/kg, <i>i.p.</i> (Both, once daily, 1 wk) <i>i.m.</i> injection of plasmid expressing Cain/Cabin1	Inhibition of slow MyHC No effect on muscle growth	Serrano <i>et al.</i> 2001
Wistar Rat (male) <i>Denervation via sciatic nerve transection</i>	Denervation <i>soleus</i> (Slow)	CsA 5mg/kg, <i>i.p.</i> once daily, 1 wk, followed by electrostimulation (20Hz)	Inhibition of slow MyHC	
Rat	EDL (Fast)	<i>i.m.</i> injection of plasmid expressing CnA*	No change in fast and slow reporters	Swoap <i>et al.</i> 2000

#: s.c. = subcutaneous; *i.p.* = intraperitoneal; *i.m.* = intramuscular

∞ Table 2. Impact of CnA pathway modulation on muscle fiber specification in C2C12 cell and animal models.

Therefore, the identification of a molecule able to interact and directly activate the endogenous Calcineurin could represent a strategic tool to better understand the role of this phosphatase in the main processes related to skeletal muscle development, growth and regeneration. This, in turn, could allow development of a therapeutic strategy for treatment of certain muscle diseases or afflictions.

PROTEIN-PROTEIN INTERACTION NETWORKS

1. MODULATION OF NETWORK FUNCTION

Biological processes in a cell are principally governed by the activity of proteins, organized in an intricate and complex network of interactions. The dynamics of these interactions is tightly regulated and is crucial for the proper occurrence of cellular events. In this context, the correct sequence of the binding region, the expression level and the correct folding of each protein and the cellular availability of the partners are crucial factors for the control of biological processes. Disruption of a single protein-protein interaction can trigger a perturbation that affects the downstream pathways, leading, in some cases, to the manifestation of a pathological condition.

On the basis of the insights into the molecular mechanisms of human diseases, one approach for medical research is the development of tools enabling interference with the function of specific proteins implicated in the evolution of pathologies (del Sol et al., 2010). A better understanding of the biological function of proteins and of the molecular basis of cellular signaling pathways is thus necessary for therapeutic drug design.

Over the past decade, integrative approaches have been developed to identify all the protein interactions in the human proteome, aiming at achieving a precise map of the protein-protein interaction network (Chautard et al., 2009) (Elefsinioti et al., 2011). This kind of investigation enables the identification of the key actors of a given signaling pathway and therefore offers new insights for the choice of targets for therapeutic drugs.

An example of the intricate organization of a protein network is shown in Fig 19. An interaction map involving 401 human proteins linked *via* 911 interactions was obtained in a study reported by Stelzl (Stelzl et al., 2005). This picture offers thus an overview of the protein-protein interaction complexity and enables the identification of all the known partners of the selected proteins implicated in disease.

The question remains, how to know which interaction to target in a given pathology? Several approaches for probing protein function in regulatory networks have been developed and tried to date, including siRNA, intracellular antibody, dominant negative proteins and nucleotide and peptide aptamers (Baines and Colas, 2006).

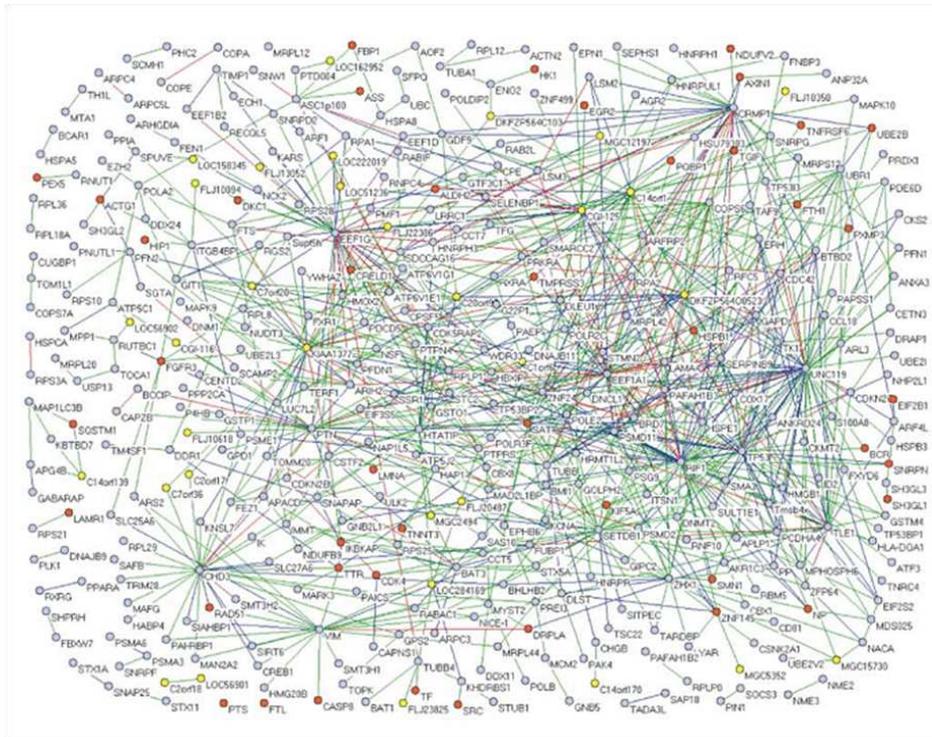


Figure 19. A graph of the High Confidence interaction network involving 401 proteins linked *via* 911 interactions. Orange: disease proteins (according to OMIM morbidmap, NCBI); light blue: proteins with GO (Gene Ontology) annotation; yellow: proteins without GO and disease annotation. Interactions connecting the nodes are represented by color-coded lines according to their confidence scores. Green: 3 quality points; blue: 4 quality points; red: 5 quality points; purple: 6 quality points. (Taken from (Stelzl et al., 2005))

PEPTIDE APTAMERS

Peptide aptamers are man-made combinatorial proteins composed of a variable loop constrained by a rigid protein platform. The variable loop confers the specificity for the target and it is generally composed of 10 to 20 amino acids, while the platform, also called scaffold, is believed to increase the stability of the conformation adopted by the variable loop. Similarly to intracellular antibodies, peptide aptamers can recognize and modulate the function of a given target *in vitro* and *in vivo*, without having their limitations *e.g.* in size and reduced cellular expression (Fig 20).

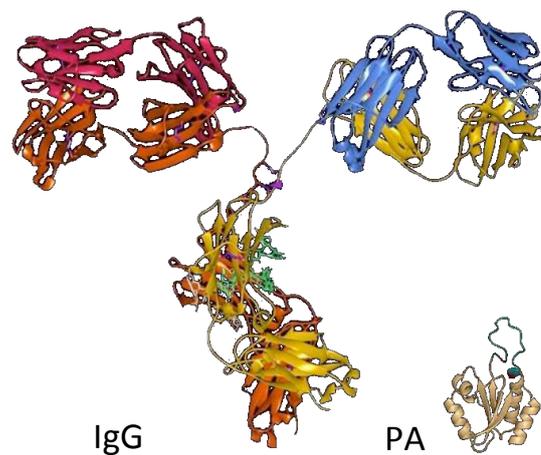


Figure 20. Comparison between immunoglobulin G (≈ 140 kDa) (IgG, left) and a peptide aptamer (≈ 12.5 kDa) (PA, right), showing their approximate size and complexity.

1. SCAFFOLD

The double constraint conferred by the continuity of the variable loop within a given scaffold has several advantages:

- Increased stability, with subsequent improvement of the binding affinity by decreasing the degrees of freedom, and therefore, the flexibility of the structure;
- Reduced exposure of the variable loop to the action of proteases compared to free peptides ;
- A rigid conformation in which facilitates determination of the binding structure in view of therapeutic drug design.

Different proteins have been used as scaffold by numerous groups, such as GFP (Abedi et al., 1998), Staphylococcus nuclease (Norman et al., 1999) and bacterial (LaVallie et al., 1993) or human Thioredoxin (TrxA)(de Chassey et al., 2007) (Fig.21)

However, TrxA is the original and most commonly used, in part thanks to the small size and the stability of the structure, but also due to the availability of random libraries. A comparative study performed in the Hopper-Seyler lab (Klevenz et al., 2002), testing the same variable loop within the different proteins scaffolds mentioned above, showed that TrxA is the most suitable scaffold for displaying the variable loop in a conformation that is effective for binding

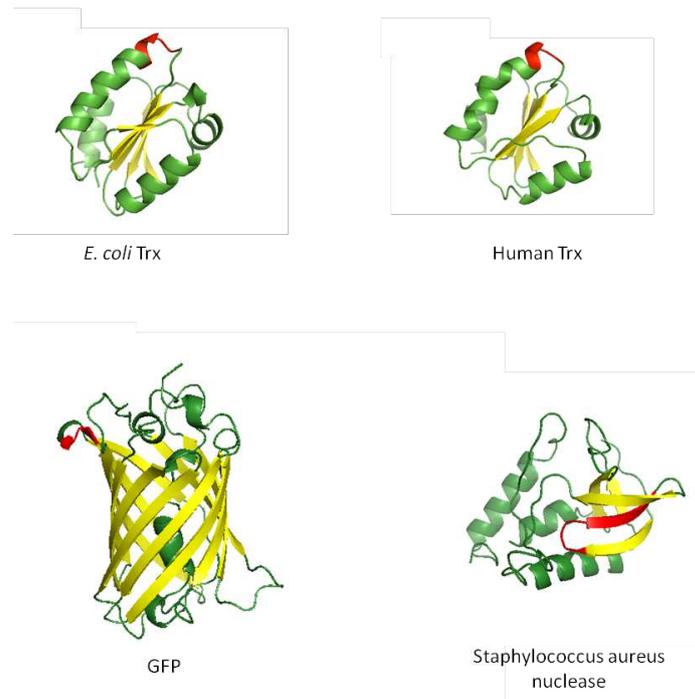


Figure 21 3D structure of platforms for peptide aptamers. All of them are wild type, the domain for insertion of a variable loop is indicated in red.

2. PEPTIDE APTAMERS SELECTION

Two main approaches are adopted for selection of peptide aptamers towards a target protein (Geyer and Brent, 2000): protein targeting and functional selection. In the first case, a library of random peptide aptamers is created and a screen is performed in order to identify peptide aptamers that specifically bind to a given protein. This strategy is most often performed *in vitro*, expressing the peptide aptamers in phage and “panning” of bacterial cultures expressing the library, or in yeast using the yeast two-hybrid approach. The main advantage of this method of selection is the size of the random libraries that can be generated. Generally, libraries of 10^{10} clones can easily be screened, providing a high probability that a specific binder of the protein of interest might be selected.

The yeast two-hybrid approach is implemented by expressing in yeast, the target protein fused to a DNA-binding domain and a library, encoding different peptide aptamers, to a transcriptional-activating domain. If a peptide aptamer binds to the target protein, the two hybrid proteins will interact and a functional transcription factor is formed (Colas et al.,

1996). This transcription factor is then able to activate the promoter of a marker gene that can be monitored by colorimetric enzymatic assays or by growth selection (Fig.22).

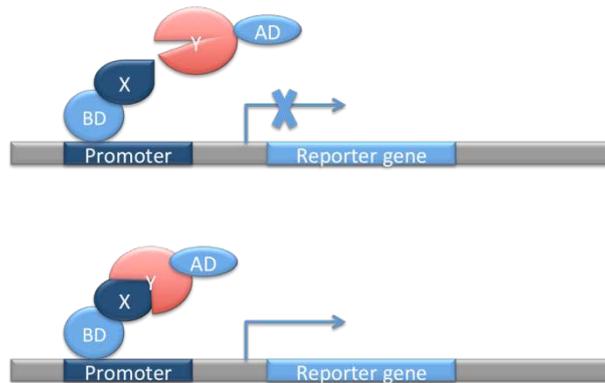


Figure 22. Two-hybrid selection of peptide aptamers against certain target proteins. As shown, a DNA-binding domain (BD) fused target protein (bait: X) interacts with an Activation-domain (AD)-fused partner peptide aptamers (prey: Y), either known or selected from a library. The interacting pair binds a specific sequence motif, activating transcription of at least two separate reporter genes.

Functional selection is usually performed in cultured cells (bacteria, yeast, mammalian). In this context, peptide aptamers are selected according to their ability to confer a particular cellular phenotype: cell proliferation arrest, inhibition of cell death, viral replication inhibition, response to growth factor, *etc.* Once the peptide aptamer is selected, the specific cellular target is identified by yeast two-hybrid screening of one or more cellular cDNA libraries. (Fig.23)

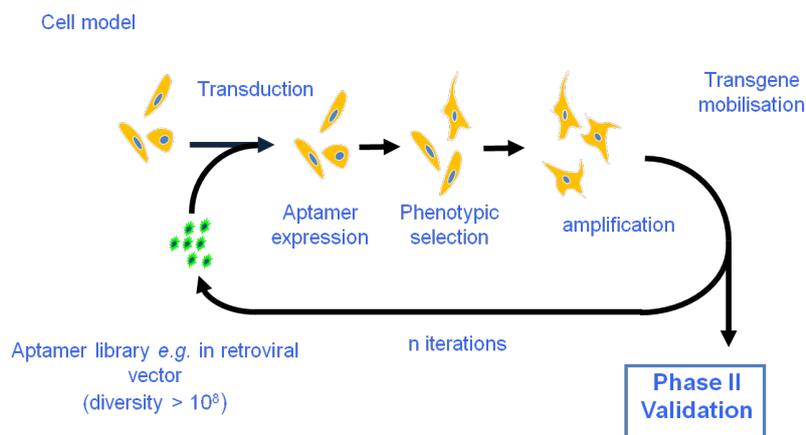


Figure 23. Scheme for functional selection of peptide aptamers in mammalian cells.

3. PEPTIDE APTAMERS AS PRECISION TOOLS FOR DISSECTING SIGNALLING PATHWAYS

One of the objectives of modern medical research is the leverage of the systems biology approach for development of drugs able to modulate the function of proteins implicated in the occurrence of pathological conditions (Hood, 2003) (Bousquet et al., 2011) Towards this goal, the technology of peptide aptamers offers a powerful tool either for the identification and validation of targets, or for the design of therapeutic drugs. For example, functional selection with a random library of peptide aptamers enables the identification of specific targets whose perturbations result in a given phenotype. Placement of the targets within established networks can allow linking to known functional modules, responsible for regulating key cellular processes. Knowledge thus gained, confer both a better understanding of the underlying mechanisms responsible for generating a given phenotype and the means to modulate it. Indeed, once their target has been identified, peptide aptamers can be used as guide for the design of peptides or small molecules that can bind the target at the same site and thereby induce the comparable biological effects.

The high specificity of their binding enables the discrimination even between different alleles of the same protein. E. Guida describes in her work the characterization of a specific peptide aptamer able to interact with and specifically inhibit the activity of a form of p53 carrying a single point mutation. Consequently, this peptide aptamer represents an important tool for the development of mutant p53-targeted cancer therapies (Guida et al., 2008). Similarly Xu *et al.*, (Xu and Luo, 2002) were able to select for peptide aptamers that preferentially bound to the oncogenic form of Ras carrying a single point mutation (H-RasV12), again, offering the potential for development of Ras-targeted cancer therapeutics.

The precision of peptide aptamers offers the possibility to go beyond the already exquisite insight generated through the use of siRNA, for target identification and validation. Fig. 24 elegantly illustrates this. A model network with two branches is depicted in (a). Use of different approaches to reduce the expression of a given protein (here the one in orange in the interface between the two branches) (b), offers information on its role in the signalling pathway. While extremely useful, the caveat is that the target interacts with four different proteins. Removing it affects therefore all of the interactions and the totality of the protein's

functions. Peptide aptamers, in contrast, are able to disrupt individual interactions with other proteins, thereby modulating one aspect of the target function (c).

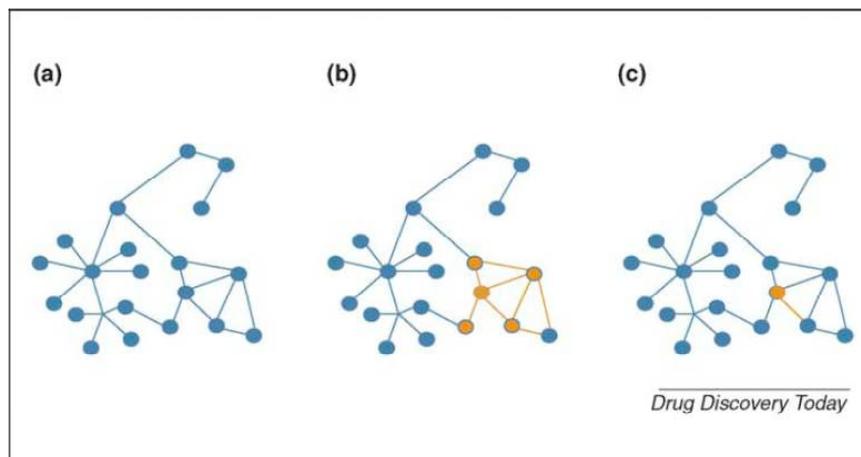


Figure 24. Perturbations on molecular regulatory networks. (a) An imaginary molecular regulatory network is depicted, where the balls represent proteins and the lines linking the balls represent protein interactions. (b) Perturbations exerted by popular methods that decrease or abolish protein expression. The target protein is colored in orange. As it interacts with, and acts upon, more than one protein (a common scenario), different molecular pathways, leading to different cellular responses, are affected. (c) Perturbation exerted by a peptide aptamer or a therapeutic molecule. The peptide aptamer or the molecule binds to a specific surface on its target protein, preventing the target protein from interacting with one of its partners or inhibiting a specific function, without affecting the 'hardware' of the regulatory network. Consequently, a more targeted perturbation is achieved. Taken from Baines and Colas (2006) (Baines and Colas, 2006)

This is the case, for example, of the peptide aptamers specific for Cdk2, characterized in (Colas et al., 1996) The peptide aptamers selected in this study are able to inhibit the activity of Cdk2 by binding to a region of the protein and blocking its interaction and enzymatic activity with one substrate, Histone H1, but not another (Retinoblastoma protein - Rb).

Another example of functional specificity of peptide aptamers within a complex signaling network is offered in (Kunz et al., 2006) A peptide aptamer (AII-7) was selected, specific for the intracellular domain outside of the ATP binding site of ErbB2, a receptor tyrosine kinase over-expressed in breast cancer and responsible for the insensitivity of this tumor toward chemotherapy. *Via* its interaction with the intracellular domain of ErbB2, the peptide aptamer strongly inhibits the activation of AKT kinase, resulting in restoration of sensitivity of breast cancer cells toward Taxol.

These are but a few examples of how peptide aptamers have proven to be powerful tools to dissect signaling pathways, and to modulate target function.

CONTEXT OF THESIS RESEARCH

An antiproliferative screening performed in our laboratory by De Chassey *et al.*, 2007, allowed the identification of a peptide aptamer, R5G42, that interacts with CnA and NS5A-TP2 (Hepatitis C virus Non-structural protein 5A-transactivated protein 2)(de Chassey *et al.*, 2007). In this study, a lentiviral peptide aptamer library was built to isolate peptide aptamers that inhibit cell proliferation (de Chassey *et al.*, 2007) and then a yeast two-hybrid screen was performed on a library on human cDNA, allowing the identification of CnA and NS5A-TP2 as targets of this peptide aptamer. Since very little was known about the biological function of NS5A-TP2 (Yang *et al.*, 2004), attention was turned to CnA, since it was an identified pharmacological target, albeit due to the therapeutic benefit of its inhibition. The effect of the peptide aptamer on the enzymatic activity of CnA was proven by an *in vitro* phosphatase assay using purified CnA and pNPP as substrate. The addition of GST-R5G42 activates CnA activity, demonstrating that the peptide aptamer is able to bind CnA and up-regulate its phosphatase activity. Consistent with the *in vitro* results, the transient expression of R5G42 in human cells leads to dephosphorylation of BAD on serine 136 which was totally reversed by FK506, a well known inhibitor of CnA.

While molecules able to *inhibit* the phosphatase activity of CnA, such as Cyclosporin A and FK506, have been extremely well characterized, the peptide aptamer R5G42 is, at the moment the *only* molecule reported able to *activate* endogenous CnA. The identification of this peptide aptamer offers, thus, a new and important tool to investigate the role of Calcineurin in different biological processes, as the development of the nervous system, of skeletal muscle tissue and of the cardiovascular system.

The focus of my PhD thesis research was to characterize the effect of CnA activation *via* the peptide aptamer R5G42 in models for skeletal muscle development and degeneration. Since this peptide aptamer binds to two target proteins, it was necessary to select for forms that were specific for one or the other. The peptide aptamer specific for CnA was then used in studies implementing C2C12 myoblast cultures and a mouse model for denervation-induced muscle atrophy and in aged *mdx* mice. The following pages describe the essence of this work.

RESULTS

In view of the opportunity offered by the discovery of R5G42, there is significant scientific interest to identify specific aptamers for one or the other target. This would allow a better understanding of the cellular responses generated by the aptamer R5G42 and would offer two powerful tools to investigate:

1. the impact of the activation of the endogenous Calcineurin in different signaling pathways;
2. the functional role of NS5A-TP2 in selected cellular processes and pathological responses.

The aim of this first paper is, therefore, the generation of a library of mutants for the identification of specific aptamers for CnA and NS5AT-P2.

The library was generated by random mutagenesis in the variable loop of R5G42 and the phenotype of interaction of the mutants obtained was determined by Two Hybrid assay in yeast.

1. MANUSCRIPT 1

Dissecting target specificity with peptide aptamers

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Dissecting target specificity with peptide aptamers

Running title : Dissecting target specificity with peptide aptamers

Abbreviations: Apta, peptide aptamer; CnA, Calcineurin A; NS5A-TP2, (Hepatitis C virus) Non-structural protein 5A-transduced protein 2; HDDC2, HD Domain Containing 2; Y2H, Yeast two hybrid; AI, auto-inhibitory domain (CnA); CaM, Calmodulin; CBD Calmodulin binding domain

Summary

We have previously identified a peptide aptamer (named “R5G42”) *via* functional selection, for its capacity to slow cell proliferation. A yeast two-hybrid screen of human cDNA libraries, using Apta R5G42 as “bait”, allowed identification of two binding proteins with very different functions: Calcineurin A, a protein phosphatase well characterized *e.g.* for its role in the immune response, and NS5A-TP2/HDDC2, a much less studied protein, induced subsequent to Hepatitis C virus Non-structural protein 5A expression in HepG2 hepatocellular carcinoma cells. Our objective, in the present study, was to dissect their specificity in order to have tools with which to be able to better characterize the actions of the peptide aptamers towards their individual targets. Our initial focus was on selectively targeting CnA, in view of its potential for the study of the effect of specific activation of the endogenous protein on selected physiological processes in certain models for human disease. This was achieved by the selection of random mutants of the variable loop, derived from R5G42, and evaluating their specificity towards CnA and NS5A-TP2. Based on analysis of the sequence and binding phenotype of these mutants, we selected two peptide aptamers mutated at the same position, differing in a single amino acid from R5G42: Apta-C8, F39I, specific for CnA, and Apta E1, F39Y, specific for NS5A-TP2. In view of the known potential therapeutic impact of activating CnA, Apta-C8 was further characterized. By dissecting target specificity from R5G42, we have generated novel tools with which to study each target individually. Apta-C8 is the first exogenous molecule reported, capable of directly activating CnA whereas Apta-E1 is the first molecule reported that will allow dissection of the function of this essentially unknown protein. For both CnA and NS5A-TP2, this represents a major step forward while serving as an example of the usefulness of peptide aptamer technology for investigating signalling pathways.

Introduction

Peptide aptamers are combinatorial proteins that consist of a variable loop constrained within a constant scaffold protein (Colas et al., 1996). They are conceived to selectively interact with specific targets and modulate their function. The high specificity of their binding to the target enables discrimination between closely-related members of a protein family (Colas et al., 1996) or proteins that differ by a single point mutations (Guida et al., 2008); (Xu et al., 1997). Thanks to their ability to influence the function of a given protein, or protein-protein interaction, they are used as precision tools for the dissection of signaling pathways (Hoppe-Seyler et al., 2004, Buerger and Groner, 2003). Moreover, peptide aptamer-target interactions can be used to identify small molecules that bind to the target and displace the peptide aptamer (Guida et al., 2008, Bardou et al., 2009). These, in turn, can become precursors for the development of leads for research purposes or for pre-clinical development (Seigneuric et al., 2011).

We have previously identified through functional selection, a peptide aptamer, R5G42, that slows cell proliferation in culture (de Chassey et al., 2007). Two proteins have been isolated as principal targets of this peptide aptamer, Calcineurin A (CnA) and a Hepatitis C virus NS5A-Transactivated Protein 2 (NS5A-TP2) (Yang et al., 2004). The present study aimed at understanding if one, or the other, or both interactions are necessary for the observed effect on cell proliferation. This involved development and characterization of peptide aptamers derived from R5G42 that are exclusively specific for one or the other of these targets.

NS5A-TP2 is also known as HDDC2 (HD Domain Containing 2). The HD domain defines a super family of proteins with a cation-dependent phosphodiesterase activity (Aravind and Koonin, 1998). This family includes proteins with different HD domain architecture, phylogenetic distribution and, above all, biological functions. Despite the heterogeneity of this family, they seem to be involved in the same type of process, such as, nucleic acid metabolism and signal transduction (Aravind and Koonin, 1998). Regarding NS5A-TP2, analysis of the transcriptome in various organisms including man, revealed that the expression of this protein is modulated under certain pathophysiological conditions. For example, it is down-regulated in pterygia, an affliction of the cornea (Tong et al., 2009), is a candidate gene for resistance to ISA (Infectious Salmon Anemia; the “hoof and mouth”

disease equivalent for salmon fisheries) (Li et al., 2011), belongs to a cluster of SNPs responsible of differences between individuals dependence on alcohol (Johnson et al., 2011), but so far little is known about the specific biological role of this protein.

CnA is a well characterized protein serine/threonine phosphatase that plays a key role in several signaling pathways. The most well described role of Calcineurin is its critical participation in immune response activation *via* the nuclear translocation of the transcription factor NFAT (Nuclear Factor of Activated T-cells). Furthermore, Calcineurin and NFAT have been shown to participate in signalling cascades that govern the development and function of the nervous system (Nguyen and Di Giovanni, 2008), skeletal muscle tissue (Michel et al., 2004) and cardiovascular system (Wilkins and Molkentin, 2004)

Calcineurin is a hetero-dimer composed of a catalytic subunit of 61 kDa, CnA, and a regulatory subunit of 19 kDa, CnB (Klee et al., 1979). The catalytic subunit contains several distinct functional regions: a catalytic domain (residues 71-342), a CnB binding domain (residues 346-370), a Calmodulin (CaM) binding domain (residues 391-414) and an Auto-Inhibitory (AI) domain (residues 457-482). In the inactive form of the enzyme, the auto-inhibitory domain blocks the active site, preventing access of the substrate. Increase in intracellular Calcium concentration, leads to the activation of Calmodulin, and through subsequent interaction with the CaM binding domain of CnA, triggers a conformational change that displace the AI domain. The catalytic site of the enzyme is thus available for the substrate (Tokoyoda et al., 2000).

Considering the importance of the CnA in the transduction of Ca^{2+} signaling in cell, modulation of its activity has been the focus of numerous studies over the years, with a view to developing therapeutic approaches for treatment of several human pathologies. CnA has been shown to be a target of two widely used immune-suppressive drugs, Cyclosporin and FK506 (Ke and Huai, 2003) (Huai et al., 2002). These drugs are used to inhibit the phosphatase activity of CnA and the subsequent activation of the immune response in order to prevent rejection of transplanted organs.

While molecules able to *inhibit* the Calcineurin activity have been well characterized, to our knowledge, at this writing, peptide aptamer R5G42 was the only reported molecule capable of direct *activation* of endogenous Calcineurin.

Several studies propose that activation of Calcineurin could be beneficial in the treatment of skeletal muscle disease (Stupka et al., 2006) and neurodegenerative disease (Gong et al., 1994) (Ermak et al., 2001). These conclusions were obtained, however, in an indirect manner, either through forced expression of constitutively-activated CnA or observation of the impact of the inhibitors described above. The peptide aptamer R5G42 can be considered as a guide for the development of a molecule that could be used for investigating the therapeutic potential of direct activation of endogenous CnA. However, its dual target specificity introduces additional factors that would make interpretation of the results more challenging. Thus, identification of peptide aptamers specific for one or the other would allow a more precise dissection of the cellular response.

In order to optimize the binding to CnA and abolish that to NS5A-TP2 (and the contrary), a library of peptide aptamers, derived from R5G42, has been generated *via* a low frequency region specific random mutagenesis of the variable loop and the selection of mutants exclusive for one or the other target binding. Moreover we have identified the minimal binding domain on CnA of a CnA exclusive peptide aptamer, and further discuss a possible model for its action on the activation of Calcineurin.

Experimental Procedures

Plasmids

For the Y2H assay, the peptide aptamers were cloned as bait in the plasmid pGilda, in fusion with the *E. coli* LexA repressor and under control of the galactose-inducible Gal4 promoter whereas CNA and NS5A-TP2, were used as prey in the pJG4-5 plasmid (Gyuris et al., 1993) (Clontech; Genbank Accession Number U89961) as previously described (de Chassey et al., 2007). The prey vector pWP2 coding for RG22, an anti-LexA peptide aptamer; and the pWP2-C5 vector coding for a non-relevant peptide aptamer were used, respectively, as positive and negative controls. The pSH18-34 plasmid was used as reporter vector, as bearing a Beta-galactosidase gene under the control of 4 overlapping LexA operators (Estojak et al., 1995).

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The truncation mutants of Calcineurin were constructed by amplification *via* PCR from on a full-length cDNA. Constructs were then cloned by homologous recombination in yeast into the prey plasmid pJG4-5.

For the co-immunoprecipitation, peptide aptamers were cloned into the mammalian pCI-HA plasmid (derived from Promega pCI) under the control of CMV promoter, while CNA was expressed using the pCMV-pSPORT vector (de Chassey et al., 2007).

Evaluation of the localization of the transcription factor NFATc1 was achieved using a plasmid containing a full length wild type NFATc1 linked to E-GFP (NFATc1-GFP) (Chin et al., 1998)

Random Mutagenesis

Specific random mutagenesis on the variable loop of the peptide aptamer R5G42 was performed using the following primers: Forward: 5'-AAACTTGTAGTAGTTGACTTCTCAGCCACG-3' and Reverse: 5'-AGAGAGGGAATGAAAGAAAGGCTTGATCAT- 3'. In order to generate low frequency mutations, PCR was performed with 0,25 mM of dATP and dGTP, and with 1.25 mM of dCTP and dTTP, using the taq polymerase (New England BioLabs). The PCR products were then cloned using homologous recombination in Yeast, in pEG202-hTrx opened in the active site of hTrx using RsrII digestion. A mutant library was then created by picking isolated clones, which were subsequently sequenced and characterized for CNA and/or NS5A-TP2 binding. The Y2H assay was performed using TB50-alpha and EYG42 yeast strains as previously described (Bickle et al., 2006) to verify the phenotype of interaction with each target

Cell culture

HeLa cells (*ATCC number*: CCL-2) were maintained in Dulbecco's modified Eagle's Medium (DMEM) (Invitrogen; France) supplemented with 10% (v/v) heat-inactivated foetal calf serum (Sigma; France) and 1% (v/v) penicillin/ streptomycin solution (Invitrogen) at 37°C in 5% CO₂. For immunofluorescence, cells were cultured on glass coverslips.

Immunoprecipitation

The day prior to transfection, 5×10^6 cells were seeded in a T75 flask. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were collected 48h after transfection. Culture medium was removed and cells washed with PBS at 4°C. Cells were then scraped in 10ml of 1X PBS and centrifuged at 4°C, 320xg (1500 rpm) for 10 min. The pellet was resuspended in 1 ml of RIPA buffer (50mM Tris-HCl pH 7,5; 150 mM NaCl; 1% NP40; 0,5% Deoxycholate; 0,1 % SDS) + inhibitors of proteases (complete EDTA-free protease inhibitor cocktail, Roche) and rotated end over end at 4°C on a rotor for 1h.

Immunoprecipitation was performed using Protein A-Sepharose 4-Fast Flow beads (GE Healthcare). A pre-clearing is performed with 50 µl of beads (50% slurry) to the cell lysate (1 mg total protein/ml) rotating end over end 1h at 4°C. The beads were collected for a WB analysis. 10 µg /ml of CnA-specific antibody (StressGene) was added to the pre-cleared cell lysate and put 2h on rotor at 4°C. 50 µl of beads were added to the lysate-antibody mix and put on a rotor at 4°C, overnight. Centrifugation 4°C at 19000xg (13000 rpm) for 30 seconds. Supernatant is collected to be analyzed on Western blot as a "flow through".

Samples were analyzed by Western blot. Proteins contained in beads and flow-through fractions were separated by SDS-PAGE, in a 12% polyacrylamide gel (29:1 acrylamide:bis acrylamide) and electro-blotted on a nitrocellulose membrane (Whatman Optitran BA-S 83, 0.2 µm). Peptide aptamers, co-immunoprecipitated with CnA, were detected using an anti-HA mouse antibody (clone A-7, Sigma ref H3663). The presence of CnA was confirmed with an antibody against CnA (Santa Cruz, ref C-20). Blot was revealed using the ECL system (GE Healthcare).

Confocal Microscopy

48 h after transfection with appropriate plasmids, cells were rinsed with ice cold PBS then fixed in 4% paraformaldehyde for 10min at 20°C and permeabilized with 0.3% Saponin for 30min at 20°C. Cells were labeled with HA-specific primary antibody (above) then with Donkey anti-mouse Alexa Fluor 555 (Invitrogen, Ref. A31570) to visualize the peptide aptamers, Trx and can*. Coverslips were rinsed in PBS, then dipped in Millipore water before

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mounting with vectashield containing DAPI (Vectashield DAPI – Vector Laboratories Ref.H-1200). Slides were observed using a Spectral Confocal Microscope TCS SP5 AOBS DM6000 (Leica), using a 40x objective. DAPI was excited with a 405 nm Diode laser; GFP with the Argon 458/488/514 nm laser; Alexa Fluor 555 with the Helium/Neon 633 nm laser. Image acquisition was achieved using the manufacturers software (Leica LAS AF SP5).

Results

Random mutagenesis

Peptide aptamer R5G42 recognizes both CnA and NS5A-TP2 (Fig. 1 and (de Chasseay et al., 2007)). In order to identify peptide aptamers specific for either CnA or NS5A-TP2, a library of mutants was generated by a random mutagenesis within the variable loop of R5G42. Four amino acids at the N-terminal and four at the C-terminal side of the variable loop were also included in the mutagenised region. Each mutant generated was cloned by homologous recombination into the hTrx platform. Approximately 1200 clones were obtained, of which 225 clones were tested in the Y2H mating assay versus CnA, NS5A-TP2 and relevant controls in a 15x15 matrix (Fig. 2). Among these mutants, 48 clones were selected and sequenced according to their interaction phenotype. The frequency of mutation of each residue (Fig. 3) shows that random mutagenesis followed by yeast gap repair created, as expected, mutants primarily within the targeted region (> 85% of the clones). Among the residues of the targeted domain, positions T38 and F39 were the most frequently mutated.

Identification of target-specific peptide aptamers

Of the 48 clones that were sequenced, there were 23 unique full-length clones of which 5 had greater than or equal to three mutations, 4 had two and the remainder, were single mutations. Fig. 4 offers a selection of single mutations of the peptide aptamer R5G42 arranged according to binding phenotype *i.e.* with specific selectivity towards CnA or NS5A-TP2.

Of particular import, F39 mutants had phenotypes with opposite specificity *i.e.* different modifications of this residue, enabled selection of peptide aptamers specific for one or the other target. Specifically, the mutation F39I generated a CnA-specific peptide aptamer, (Apta-C8), while the mutation F39Y created an NS5A-TP2-specific phenotype (Apta-E1). Thus, the F39 residue appears to be a key residue implicated in the dual target phenotype. Apta-C8 was therefore, retained as specific peptide aptamer for CnA, and then used for the further characterization in mammalian cells.

Peptide aptamer-Target interaction in HeLa cells

To confirm the interaction between the Apta-C8 and CnA in HeLa cells, we performed an Immunoprecipitation followed by a Western analysis. Cells were co-transfected with CnA plasmid and an HA-tagged construct of Apta-C8. After immunoprecipitation of CnA, interaction with the peptide aptamers was determined by Western Blotting using an antibody against HA. As shown in Fig. 5, Apta-C8 is immunoprecipitated with CnA but not with the negative control Trx.

Apta-C8 stimulates nuclear translocation of NFAT in HeLa cells

In order to validate whether this mutant is able, as the parent aptamer R5G42 to activate the endogenous CnA, we have evaluated if Apta-C8 is able to provoke the nuclear translocation of NFATc1 in HeLa cells. As shown in Fig. 6, over-expression of CnA* induce a translocation of NFATc1 into the nucleus, whereas in the negative control, Trx, NFATc1 is localized exclusively in the cytoplasm. In cells transfected with R5G42 or Apta-C8, characteristic green spots are visible in the nucleus, confirming the activation, even if weaker than CnA*, of NFAT.

Mapping of Apta-C8 binding site on CnA

An initial step towards elucidating the mechanism of action of the Apta-C8 on the activation of Calcineurin activity, entailed identification of the specific binding site of the peptide aptamer on the phosphatase. Several deletions of selected portions of the protein were performed. Each construct was conceived in order to keep the structure of the different domains of the protein. The truncated forms were then used as preys in a Y2H assay. As shown in Fig. 7, both R5G42 and Apta-C8 show a binding phenotype with the C-terminal part of Calcineurin in a region that includes the CaM binding domain and the AI domain. No interaction is detected when the domains are expressed individually. When the last 30 amino-acids are truncated from C-terminal region, binding to R5G42 is lost whereas Apta-C8 still interacts. Thus, the minimal region for interaction is from residues 385-490.

Discussion

We have previously identified a peptide aptamer, R5G42, that up-regulates CnA activity (de Chasse et al., 2007). R5G42 shows, however, a dual target specificity. It binds to CnA, a serine/threonine protein phosphatase (Klee et al., 1979) and NS5A-TP2, a protein trans-activated by the Hepatitis C virus Non-Structural protein 5A (Yang et al., 2004).

Random mutagenesis in the variable loop of the peptide aptamer R5G42 was performed in order to identify specific peptide aptamers that bind one or the other target. Generation of a library of mutants and the characterization of their phenotype of interaction, allowed the identification of the peptide aptamer Apta-C8, specific for CnA and Apta E1, specific for NS5A-TP2.

Amongst the 48 mutants selected from a library of 1200 mutants obtained with the random mutagenesis approach, we focused on the mutants showing single mutation in the variable loop. The analysis of the frequency of mutation for each residue showed that T38 and F39 were the most frequently mutated. In particular, mutations in F39 offered strategic insight, important for the selectivity of the binding with one or the other target. Notably, the mutation F39I confers the specificity for CnA (Apta-C8), whereas the mutation F39Y, confers the specificity for NS5A-TP2 and subsequent loss of binding with CnA (Apta-E1).

For the binding of the peptide aptamer to NS5A-TP2, the aromaticity of the amino acid 39 is decisive. Mutating the aromatic, apolar F39 to a non-aromatic, apolar amino acid leads to loss of the NS5A-TP2 binding phenotype. On the other hand, mutating F39 to Tyrosine, an aromatic, less apolar amino acid, still maintains the binding phenotype. This points to pi-stacking effects involved in either the direct binding interaction of F39 with NS5A-TP2, or the stabilization of a NS5A-TP2-specific conformation of peptide aptamer R5G42.

Regarding the binding of the peptide aptamer to CnA, the apolarity of residue 39 is crucial. Increasing hydrophobicity ensures the binding to CnA whereas the addition of a polar hydroxy-group (F39Y) causes a loss in the binding phenotype. We have demonstrated by mapping studies (see above) that the auto-inhibitory domain (AI) and the calmodulin-binding domain (CBD) both are needed to achieve the binding to CnA. This could mean that either both domains are involved in the interaction with the peptide aptamer, or the

presence of both domains is necessary for the region to adopt a conformation recognized by the peptide aptamer.

In principle, the peptide aptamers can be present in two different states, oxidized (with a disulfur bridge between C32 and C47), or reduced (with two thiol groups). Construct A13 in Fig. 4 (mutation C32S, where no di-sulfur bridge is possible since one of the cysteines has been mutated), indicates that the oxidized state is not required for the binding of the peptide aptamer to CnA. For the binding to NS5A-TP2, however, it is possible that the oxidized state is required.

Overall, this analysis offers insight into the binding of each target and sets the stage for future investigations into the structural basis for the interactions.

The dissection of the target specificity of R5G42 has allowed identification of these two peptide aptamers, which offer interesting potential for future studies. NS5A-TP2 is a protein whose function has not been characterized. The presence in its sequence of an HD domain suggest its potential involvement in nucleic acid metabolism and signal transduction (Aravind and Koonin, 1998). However, to date nothing is known about its biological function. The identification of a peptide aptamer that binds NS5A-TP2, modulating its activity, represents, therefore a crucial tool for dissecting its role in cellular signalling pathways. Further investigations of the impact of Apta-E1 on cellular response can thus offer important insight to determine the functional role of NS5A-TP2.

On the other hand, Calcineurin is a well-characterized protein phosphatase. CnA is ubiquitously expressed and is involved in the regulation of different biological processes, such as development and regulation of skeletal and cardiac muscle, nervous system and immune system. Upon Calcium activation, CnA modulates the expression of specific genes, *via* the dephosphorylation, and thus activation, of NFAT (Nuclear Factor of Activated T-cell). Although inhibitors of this phosphatase are already well known, such as Cyclosporin A and FK506, today no molecule able to directly *activate* Calcineurin has been identified.

Our peptide aptamer Apta-C8 is the first exogenous molecule reported that binds CnA, up-regulating its activity. It represents, thus, an important tool to study the impact of the

activation of the endogenous CnA in the regulation of different signaling pathways in immune system, muscle and nervous system.

Given the crucial role of Calcineurin in different signaling pathways and moreover, considering the impact of the modulation of its activity in numerous pathologies, such as muscle atrophy (Sakuma and Yamaguchi, 2010) (Olson and Williams, 2000) and dystrophy (Stupka et al., 2006) and neurodegenerative diseases (Ermak et al., 2001), we focused principally on the further characterization of Apta-C8.

The interaction between Apta-C8 and CnA observed by Yeast Two Hybrid, was confirmed in this work by co-immunoprecipitation in HeLa cells. Similarly to R5G42, Apta-C8 was able to stimulate the nuclear translocation of NFATc1, indicative of direct activation of CnA and subsequent dephosphorylation of NFAT. Moreover, using truncations of the protein we have established that the binding region of the Apta-C8 on the CnA sequence is restricted to the C-terminal part, in a region that encompassing the CaM binding Domain and the AI domain.

This peptide aptamer - minimal binding domain pair can be used for the identification of small molecules that displace the peptide aptamer from the target (Bardou *et al.* 2009). Such molecules could then be evaluated for their capacity to exert the same activating function as Apta-C8 on CnA. This could lead to the development of a novel therapeutic approach to alleviate the pathological effects of several diseases, such as Duchenne Muscular Dystrophy (DMD), Amyotrophic Lateral Sclerosis (ALS) and Alzheimer's disease.

In summary, the implementation of peptide aptamer technology described herein, has allowed the development of two precision tools for investigating the role of their respective targets. On the one hand, for a well known target, CnA, characterized therapeutically for its inhibition, Apta-C8 will allow investigation of the therapeutic potential for direct activation of endogenous CnA. On the other hand, an essentially unknown target – NS5A-TP2, for which Apta-E1 will be a unique resource for understanding its role in cellular processes and pathological situations.

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Dissecting target specificity with peptide aptamers

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Figures

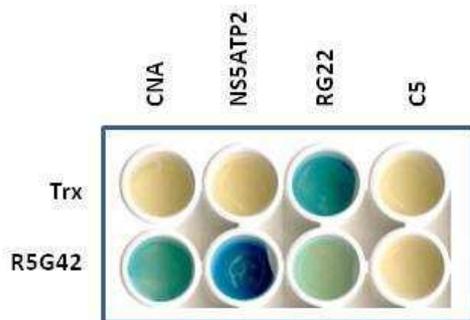


Figure 1. Specificity of the R5G42 peptide aptamer. Phenotype of interaction in the Yeast Two Hybrid assay, of peptide aptamer R5G42 (de Chasseay et al., 2007) versus CnA and NS5A-TP2. Blue indicates interaction between two proteins. RG22 is a peptide aptamer that binds to LexA and C5 is a non-relevant peptide aptamer, used as negative control. (*cf.* Experimental Procedures).

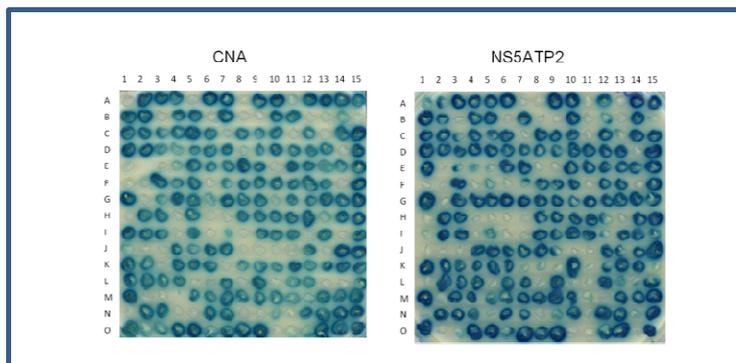


Figure 2. Example of arrays for analysis of binding specificity of each mutant. All mutants were tested on a 15x15 Y2H matrix vs CnA and NS5A-TP2 in order to identify specific peptide aptamers for each target. 48 clones were selected according to their behaviour in this assay (*cf.* Experimental Procedures).

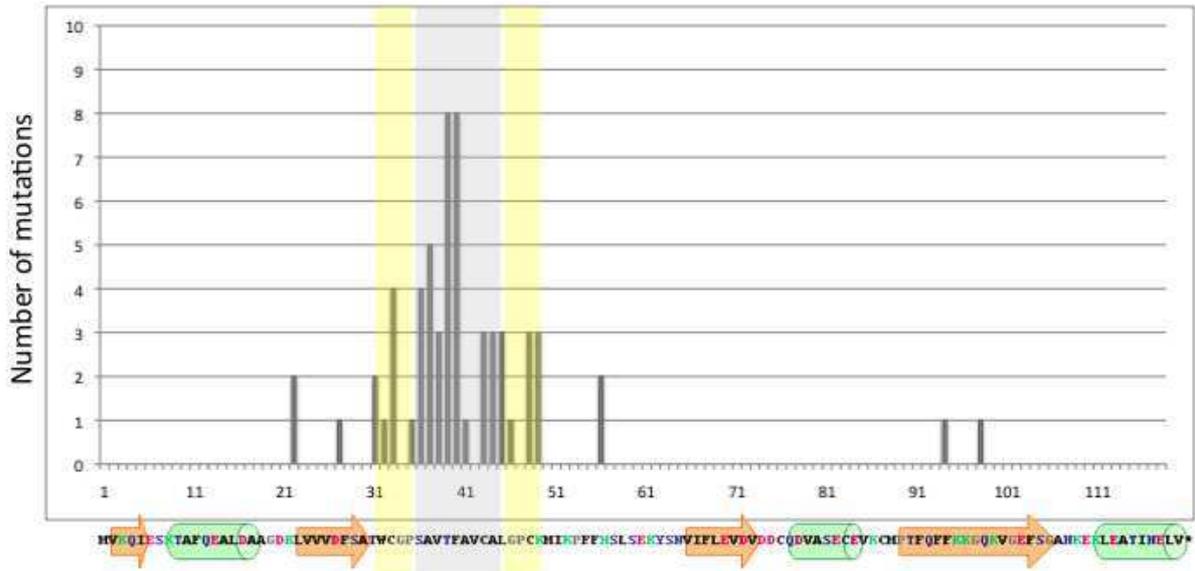


Figure 3. Overall frequency of mutations observed in the variable loop and scaffold. Representation of the frequency of mutations after random mutagenesis of the variable loop performed as described in Experimental Procedures. The sequence of the R5G42 peptide aptamer is indicated under the x-axis, with the relative position of the beta sheets (Orange arrows) and alpha helices (Green cylinders) derived from the NMR structure of Human Trx (PDB-4TRX). The gray zone encompasses the variable loop. The yellow zones indicate the 4 flanking amino acid residues.

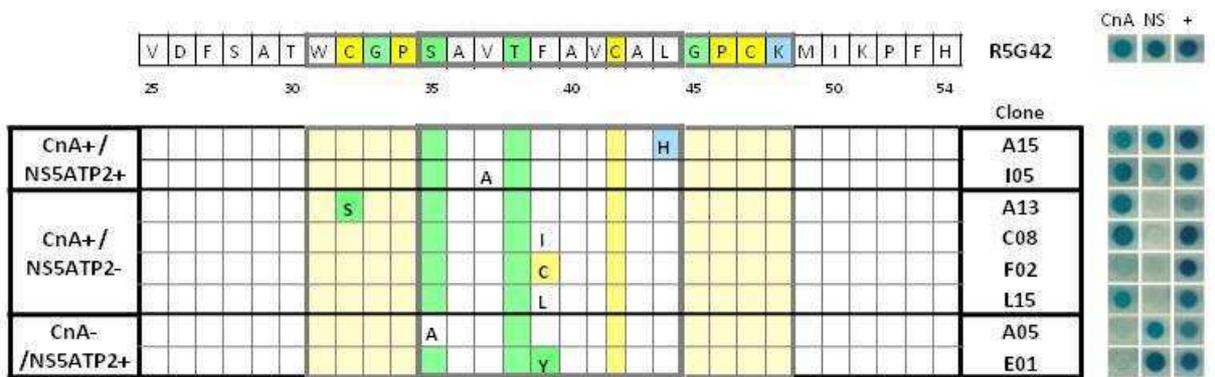


Figure 4. Position and binding properties of selected single mutations of R5G42. Single mutations in the region including the variable loop (S35 - L44) and four residues N- and C- terminal thereof, are represented. Physico-chemical characteristics are color-coded: Blue – Positive; Green – Polar; White – Apolar; Yellow – Special. The bold gray box indicates the variable loop while the pale yellow region indicates the flanking 4 residues. The results of a characteristic Y2H mating assay for each clone vs CnA, NS5A-TP2 (NS); or positive control (+). Blue indicates binding. Translucent indicates no binding. Clones C08 and E01 were used for subsequent tests.

Dissecting target specificity with peptide aptamers

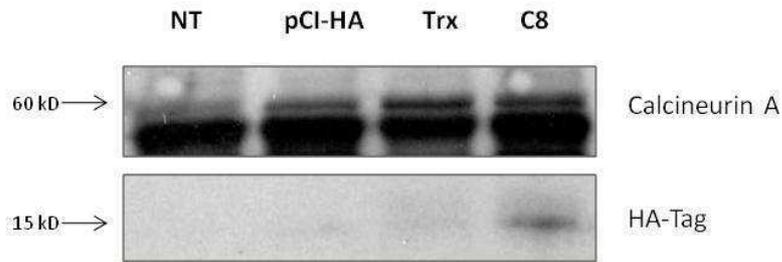


Figure 5. Validation of interaction in HeLa cells. Western blot showing the co-immunoprecipitation of Apta-C8 with CnA. Cells were co-transfected with the individual target and corresponding expression plasmid pCI-HA (empty vector), Trx, Apta-C8 or NT – non-transfected.

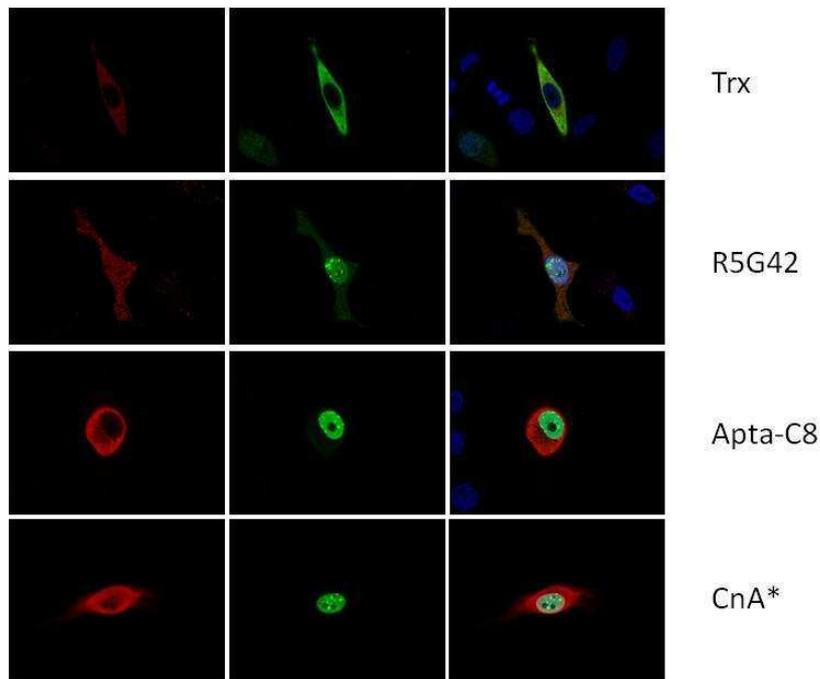


Figure 6. Nuclear translocation of NFATc1. Trx, R5G42, Apta-C8 and CnA* were evaluated for their capacity to provoke the nuclear translocation of NFATc1-GFP, an indication of activation of CnA and dephosphorylation of NFAT (*cf* Experimental Procedures). The pictures are a compilation from images generated during a characteristic experiment.

Dissecting target specificity with peptide aptamers

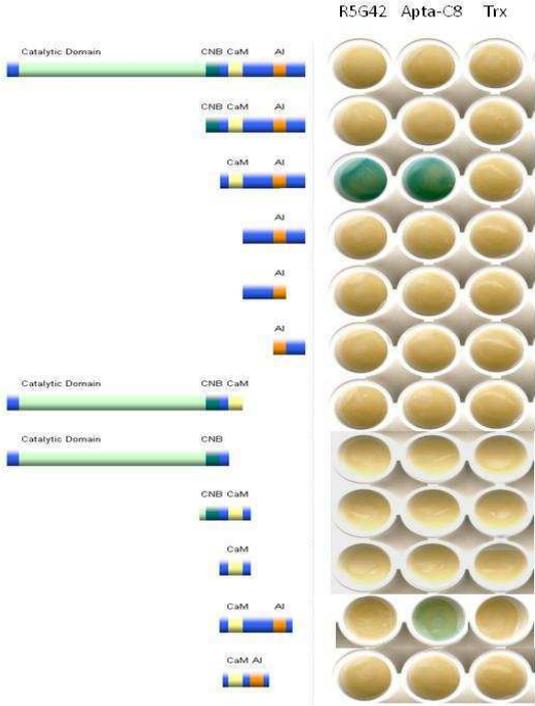


Figure 7. Identification of the binding domain of Apta-C8 on CnA. R5G42, Apta-C8 and Trx were evaluated by Y2H for their interaction with different constructs of CnA, truncated as described (*cf* Experimental Procedures). The pictures are a compilation from plates generated during a characteristic experiment.

The work performed in the first paper, allowed the identification of a peptide aptamer, named Apta-C8, that binds specifically to Calcineurin A.

The CnA-NFAT pathway has been demonstrated to play a critical role in key skeletal muscle processes, such as development, growth and regeneration. As indicated in the introduction, the observations obtained regarding the involvement of CnA in skeletal muscle have been obtained using specific inhibitors of this phosphatase, over-expression of a constitutively active form, CnA* or *via* the use of transgenic mice overexpressing CnA* in muscle.

Apta-C8 is the first molecule able to bind and activate the phosphatase activity of CnA. We, thus, aimed to test the effect of this aptamer in an *in vivo* model in order to evaluate the impact of the activation of the endogenous Calcineurin A in muscle remodeling.

Thanks to a collaboration with Schaeffer team, Neuromuscular Differentiation lab, within the same laboratory as ours (Laboratory of Molecular Biology of the Cell; UMR 5239 CNRS/ENS Lyon/Univ Lyon1) we had the possibility to integrate and master a mouse model for acute muscle atrophy. This allowed us to evaluate the impact of our peptide aptamers and notably Apta-C8. Apta-C8 was transfected in *tibialis anterior* by electroporation and atrophy was induced by removal of a portion of the sciatic nerve. The impact of Apta-C8 was evaluated on the Cross Sectional Area of muscle, number of fiber and resistance to fatigue.

We further analyzed the impact of Calcineurin activation *via* the peptide aptamer, in a more severe condition for muscle degeneration. In collaboration with Arnaud Ferry, Université Paris Descartes, Sorbonne Paris Cité, Paris, we had the possibility to test our peptide aptamer in aged (20 month old) *mdx* mice. The *mdx* mouse model is considered as pertinent for evaluation of mechanisms for attenuating muscle loss and enhancing muscle function.

The following manuscript relates the results of the first experiments performed *in vivo*, in a mammal using a peptide aptamer, Apta-C8.

2. MANUSCRIPT 2

CnA-activating peptide aptamer protects against muscle atrophy

Direct activation of endogenous Calcineurin A *in vivo* confers protection against denervation-induced muscle atrophy.

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Running title : CnA-activating peptide aptamer protects against muscle atrophy

Abbreviations: Apta, peptide aptamer; CnA, Calcineurin A; CSA, Cross-sectional area; CsA, Cyclosporin A; NFAT, Nuclear Factor of Activated T-Cell; TA, *tibialis anterior*; Trx, Thioredoxin

Summary

A peptide aptamer, called Apta-C8, which has been shown to bind to and activate Calcineurin A (CnA), *in vitro* and in mammalian cells, has been evaluated in mouse models for denervation-induced atrophy and muscular dystrophy. Apta-C8 is the first exogenous molecule capable of directly activating endogenous Calcineurin *in vivo*, resulting in promotion of muscle regeneration and in protection against contraction-induced muscle injury in aged (20 month old) *mdx* mice. Expression of Apta-C8 by injection and electroporation prior to transection of the sciatic nerve, results in protection against the characteristic decrease in overall muscle and fiber cross-sectional area while protecting against the loss of fiber number. The results presented herein offer the first proof of concept that direct activation of endogenous CnA, *in vivo*, can reverse muscle degeneration as measured morphologically and functionally. Studies in *mdx* mice further supported these observations. Expression of Apta-C8 conferred significant protection against force drop induced by eccentric contractions. This effect was comparable to the injection of activated CnA. These results open the way towards development of novel therapeutic approaches.

Introduction

Skeletal muscle is constantly subject to modification regarding mass and muscle fiber composition in response to different external stimuli. In case of injury, skeletal muscle undergoes a process of degeneration, characterized by a loss of muscle mass and reduction of contractile function, resulting in muscle atrophy. Muscle atrophy can be caused by different factors, such as, disuse, ageing and mechanical injury. The high plasticity of skeletal muscle, allows this tissue to adapt to different conditions and to respond to damage *via* the activation of different signaling pathways. After injury, for example, skeletal muscle is able to regenerate, *via* the activation of satellite cells, the precursors of muscle cells. Once activated, satellite cells proliferate and fuse to existing myofibers to form larger cells and repair the damaged region. (Charge and Rudnicki 2004)

The activation of key signaling pathways in skeletal muscle is under the control of motor neuronal stimuli, that regulate oscillations in intracellular calcium concentration. The increase of calcium influx activates a cellular signaling cascade involved in the skeletal muscle response, including the CnA-NFAT pathway (Schiaffino et al. 2007)

Calcineurin, a calcium-dependent serine and threonine protein phosphatase, is expressed in all fiber types. By modulating the expression of different genes, *via* the activation of the transcription factor, NFAT (Nuclear Factor of Activated T-cells), Calcineurin has been demonstrated to be involved in the main processes that regulate skeletal muscle remodeling (Michel et al. 2004).

The use of transgenic mice over-expressing an active form of Calcineurin, CnA*, suggests that this phosphatase plays a critical role in the determination of the slow fiber gene expression program (Naya et al. 2000). Treatment with specific-CnA inhibitors, such as CsA and FK506, has confirmed the involvement of CnA-NFAT in the up-regulation of the slow twitch fiber specific genes (Serrano et al. 2001)

Expression of an activated form of Calcineurin in the C2C12 model, has been shown to mimic the effect of IGF-1 on skeletal muscle hypertrophy, underlining the involvement of the CnA-NFAT pathway in the hypertrophic response activated by IGF-1 (Musaro et al. 1999).

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Treatment with CnA-specific inhibitors results in the inhibition of the hypertrophic response, confirming the involvement of CnA in muscle growth (Semsarian et al. 1999). In addition, CnA–NFAT pathways are involved in activation and differentiation of satellite cells, *via* the up-regulation of Myf5, a member of the MRF (Myogenic Regulator Factor) family. Moreover, in regenerating NFATc2^{-/-} mice, a decrease in fiber size and in number of myonuclei has been observed. During the process of regeneration, myofibers are formed normally, but their growth is blocked, suggesting a role for the CnA-NFAT pathway in the muscle growth of new myofibers. NFAT, *via* the up-regulation and the release of IL-4, promotes satellite cells fusion *in vivo* (Horsley et al. 2003).

The impact of the CnA-NFAT pathway has been evaluated in the degenerative process of muscle pathologic diseases, such as dystrophinopathies. In *mdx* mice, a model for Duchenne Muscular Dystrophy, CnA appears involved in the regulation of the regenerative process and in the reduction of contraction-induced muscle injury (Stupka et al. 2006b; Stupka et al. 2007)

Although there is significant evidence in support of involvement of Calcineurin in key processes of skeletal muscle regulation, some aspects remain elusive. Due to discrepancies of results obtained using different approaches, such as transgenic mice expressing constitutively-active CnA (CnA*), over-expression of CnA* and treatment with CnA-inhibitors such as Cyclosporin A (CsA) or FK506, the role of Calcineurin in skeletal muscle is still controversial.

Notably, some studies suggest that CnA is involved mainly in the regulation of slow fiber-specific gene expression with little or no impact on muscle growth (Naya et al. 2000; Serrano et al. 2001). Others indicate that, on the contrary, CnA is indeed necessary for muscle growth and hypertrophy (Dunn et al. 1999; Horsley et al. 2003)

Until now, it has been impossible to investigate the effects of this signaling pathway in skeletal muscle by the *direct* activation of the *endogenous* Calcineurin. The identification of a molecule able to activate Calcineurin could therefore, represent an important tool to dissect the functional role of the phosphatase and offer a possible means for treatment of certain muscle diseases (Sakuma and Yamaguchi 2010)

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Peptide aptamers are combinatorial proteins composed of a variable loop constrained in a rigid platform, designed in the image of antibodies (Colas et al. 1996). They are able to bind and modulate the function of a specific target. Because the high specificity of their binding they represent a strategic tool for dissecting signaling pathways. Once their target has been identified, peptide aptamers can be used as guide for the design of peptides or small molecules that can bind the target at the same site and thereby induce the comparable biological effects (Buerger and Groner 2003; Bardou et al. 2009).

We have identified a peptide aptamer, Apta-C8 that binds specifically to CnA and up-regulates its phosphatase activity *in vivo*. The study presented herein, describes the impact of the activating peptide aptamer in a mouse model for denervation-induced muscle atrophy, in order to evaluate the effect of the CnA activation during muscle degeneration. The impact of the activation of the endogenous CnA was also evaluated in aged (20 month old) *mdx* mice. In both models, Apta-C8 exerts a beneficial effect, improving the regeneration process in the former and inducing an increase of the resistance to physical exercise in the later.

The possibility to directly activate CnA *in vivo*, offers new and exciting perspectives for the development of novel therapeutic approaches for the treatment of muscular disorders.

Materials and Methods

In vivo experiments

In vivo experiments were carried out on 4 weeks old OF-1 female mice (Charles River Laboratories) and on 20 month old *mdx* mice (provided by L.Garcia, INSERM U974, UPMC University, Paris). Animals were weighed and anesthetized *via* intraperitoneal injection of a mixture of Ketamine (100mg/kg) (Panpharma, Ref. PF250211) and Xylazine hydrochloride (10mg/kg) (Sigma, Ref. X1251). The animals were provided with mouse chow and water *ad libitum* in a restricted access and specific pathogen-free animal care facility. All procedures were performed in accordance with French and European legislation on animal experimentation.

DNA electroporation

Expression vectors were injected into, both right and left, *tibialis anterior* (TA) muscles (7 µg total DNA in 30 µl 0.9% NaCl) Injection was followed by electroporation with the following setting: 8 pulses of 20 milliseconds each and 500 milliseconds of interval. The voltage was set according to the thickness of the muscle (200 Volts/cm).

Plasmids

Peptide aptamer, as well as, positive and negative controls were cloned in the expression vector pCI-HA (derived from pCI, Promega E1731) carrying an HA-tag located at the N-terminal part of the expressed gene.

The expression plasmids injected are the following:

Plasmid Name	Expressed Protein	Function
pCI-HA	None	Negative control
pCI-HA Trx	Inactivated human Trx	Non functional peptide aptamer
pCI-HA-AptaC8	Apta-C8	Lead peptide aptamer
pCI-HA-CnA*	CnA*	Constitutively-active CnA
pRNAT-GFP	GFP	Control of transfection

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The plasmid pCI-HA-CnA* was generated in the lab, cloning into the vector, a truncated form (1-390) of the full length CnA, obtained by PCR.

In order to evaluate the localization of the transcription factor NFATc1, muscles were co-electroporated with a plasmid containing a full length wild type NFATc1 linked to E-GFP (NFATc1-GFP) (Chin et al. 1998).

Denervation

Three days after electroporation, the left leg of each mouse was denervated (as described in (Tomanek and Lund 1973)) in order to induce muscle atrophy. To avoid re-innervation, approximately 5 mm sciatic nerve was excised and the skin was closed and sutured. Surgery was performed on mice anesthetized as described above. The contralateral leg was not denervated to allow normal activity of the animals.

Muscle immunohistochemistry, morphometric measurements, and imaging

Mice were sacrificed by cervical dislocation 3, 7 and 14 days after denervation and the TA (denervated and not) were collected, embedded in Tragacanth gum (Acros Organics, Ref. AC-421385000) and quickly frozen in isopentane cooled in liquid nitrogen. Transversal cross section of 10- μ m-thick were obtained from the middle portion of frozen muscles and processed for morphometric and immune-histochemical analysis.

For immunostaining, cross sections were fixed in formaldehyde 4% for 10 min and incubated 1 hour at RT on a permeabilizing/blocking buffer (PBS1X – 0,1% Triton – 5% FBS)

For fiber number and surface detection slides were stained with TRITC-conjugated Lectin (Sigma, Ref. L5266) or incubated with Dystrophin primary antibody (NCL-DYS2, Novocastra).

In order to enhance the weak fluorescent signal obtained after transfection of GFP-NFATc1 muscles are incubated with a mouse NFATc1 (H-110) (Santa Cruz, Ref. sc-13033) antibody.

For the proliferation assay, muscles were incubated with a rabbit p-Histone H3 (Ser 10) (Santa Cruz, Ref. sc-8656R) and a mouse Pax7 (Santa Cruz, Ref. sc-81975) primary antibody.

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A donkey anti-Mouse Alexa Fluor 488 (Invitrogen, Ref. A-21202) and a donkey anti-Rabbit Alexa Fluor 555 (Invitrogen, Ref. A-31572) were used as secondary antibodies. Sections were mounted with a mounting medium with DAPI (Vectashield Dapi – Vector Laboratories Ref.H-1200)

Measurement of contractile properties and susceptibility to eccentric contraction-induced muscle injury

Maximal force production and fragility was evaluated by measuring the in situ tibialis anterior (TA) muscle contraction in response to nerve stimulation, as described previously (Koo et al. 2011) (Mouisel et al. 2010). Mice were anesthetized using pentobarbital (60 mg/kg intraperitoneally). Body temperature was maintained at 37°C using radiant heat. The knee and foot were fixed with pins and clamps and the distal tendon of the muscle was attached to a lever arm of a servomotor system (305B, Dual-Mode Lever, Aurora Scientific) using a silk ligature. The sciatic nerve was proximally crushed and distally stimulated by a bipolar silver electrode using supramaximal square wave pulses of 0.1 msec duration. We measured the absolute maximal force that was generated during isometric contractions in response to electrical stimulation (frequency of 75–150 Hz, train of stimulation of 500 msec). Maximal force was determined at L0 (length at which maximal tension was obtained during the tetanus). Absolute maximal force was normalized to the muscle mass as an estimate of specific maximal force, i.e. force-generating capacity.

Fragility was estimated from the force drop resulting from eccentric contraction-induced injury. The sciatic nerve was stimulated for 700 msec (frequency of 150 Hz). A maximal isometric contraction of the TA muscle was initiated during the first 500 msec. Then, muscle lengthening (10% L0) at a velocity of 5.5 mm/sec was imposed during the last 200 msec. All isometric contractions were made at an initial length L0. Nine lengthening contractions of the TA muscles were performed, each separated by a 60-sec rest period. Maximal force was measured 1 min after each eccentric contraction and expressed as a percentage of the initial maximal force. After contractile measurements, the animals were killed cervical dislocation and muscles were weighed.

Results

Impact of CnA-activating peptide aptamers on Muscle Growth

In order to evaluate the impact of the activation of endogenous CnA on muscle growth and repair, *tibialis anterior* muscles were transfected by electroporation with a pCI-HA plasmid encoding for Apta-C8. Empty plasmid and the human inactivated Trx, were also injected and used as control. The cross sectional area (CSA) and the total number of fibers of each muscle were then evaluated 3, 7 and 14 days after denervation.

Cross Sectional Area

In non-denervated TA transfected with the empty plasmid and Trx no significant change in the cross sectional area was observed at days 3, 7 and 14, whereas a significant increase of 15% of the CSA compared to empty plasmid was observed in TA transfected with Apta-C8 at day 14. (Fig. 1.A)

As expected, after denervation an important loss of muscle area was observed in muscles transfected with the negative controls, *i.e.* empty plasmid and Trx. In contrast, Apta-C8 appears to reduce the impact of denervation-induced atrophy, resulting in a reduced loss of muscle mass. The protective effect of AptaC8, detectable already at day 7, appears to be stronger at day 14, resulting in a reduction of about 50% of CSA loss compared to empty plasmid. (Fig.1.B)

Number of fibers.

The number of fibers per muscle was also analyzed. As with the CSA, no significant changes were observed in non-denervated muscles, nor does Apta-C8 does show any significant effect on this parameter. In contrast, after denervation, a decrease in number of fibers was observed at day 7 and 14, particularly in muscles transfected with the empty plasmid. This loss of fibers could be explained considering that denervation initiates a process of muscle degeneration that results in necrosis of some fibers. Nevertheless, denervated muscles transfected with Apta-C8 are not subjected to this loss of fibers. The effect of Apta-C8 is more significant at day 14, when fiber number is comparable to non-denervated muscles (Fig. 2). Apta-C8 could, thus, have an effect on the activation of myoblast differentiation resulting in the formation of new myofibers.

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Individual fiber surface area

It is assumed that in case of denervation, muscle atrophy is associated with individual fiber CSA reduction. Since AptaC8 protects against acute muscle atrophy as evidenced by the preservation of the muscle global CSA, we investigated its impact on individual fiber CSA distribution.

As shown (Fig. 3), under control conditions (no denervation) in muscles transfected with Apta-C8 muscle fibers are larger than those that received the empty plasmid. The effect of Apta-C8 is even more pronounced in TA denervated. The distribution of the fiber CSA shows clearly that, in denervated muscles, Apta-C8 induces an increase in the individual CSA and in the number of fibers in regenerating muscles. Thus, AptaC8 seems to have an effect in the stimulation of the regenerative process.

Activation of CnA-NFAT pathway

In order to verify whether the effect observed with Apta-C8 is linked to the activation of CnA, we evaluated the effect of the peptide aptamer on the activation of NFAT, the main substrate of CnA. NFAT in the inactive form is localized in the cytoplasm. Once activated by dephosphorylation, NFAT goes into the nucleus to activate the transcription of several genes.

To evaluate the nuclear translocation of NFAT, muscles were co-transfected with a plasmid encoding for a GFP-fused NFATc1 and the corresponding peptide aptamer or control. A plasmid expressing an activated form of Calcineurin (CnA*) was also transfected in order to compare the effect of Apta-C8 with a positive control. In muscle transfected with Trx, NFATc1 exhibits a predominant cytoplasmic localization. In contrast, in TA transfected with Apta-C8, NFATc1 was localized predominantly into the nuclei, resulting, thus, in a massive activation of this transcription factor (Fig. 4). This observation indicates that the effects observed in TA with the peptide aptamer were indeed a downstream effect of the activation of CnA. The nuclear localization of NFATc1 was quantified as a ratio of positive nuclei over the total number of nuclei. The NFAT nuclear translocation obtained with Apta-C8 was even more pronounced than that obtained with the activated form of CnA (CnA*).

Satellite cell mobilization

The previous results suggest Apta-C8 appears to have an effect on muscle growth and regeneration. The muscle regeneration process requires the activation of satellite cells, resulting in the exit from a state of quiescence, followed by stimulation of proliferation and differentiation. During quiescence, satellite cells are blocked in the G₀ phase of the cell cycle. Once activated, they exit G₀ and start to proliferate. We tested whether Apta-C8 is involved in the activation of satellite cells. Denervated muscles (day 3 and 7) were labelled with an antibody directed toward pHis3. Histone 3 is phosphorylated at the moment of chromatin condensation occurring during mitosis (Hans and Dimitrov 2001) and therefore can be considered as a marker of cell proliferation.

The ratio of positive nuclei for pHis3 over the number of total nuclei was estimated on the cross sectional surface of each muscle. As expected, according to the hypertrophic effect underlined above, Apta-C8 activates the proliferation of satellite cells already at day 3 after denervation. (Fig. 5) The same profile was observed at day 7 (data not shown). The increase of the proliferating cell rate means that Apta-C8 acts as an enhancer of the regeneration process. The co-localization of the Pax7 and p-His-3 labeling signal confirms that the proliferating population identified with the antibody pHis3 was indeed composed of satellite cells. The result obtained in this experiment demonstrates, therefore, that AptaC8 induces satellite cells proliferation in degenerating muscles.

A possible paracrine effect of CnA activation.

The regenerative process induced by Apta-C8, results in an increase of centrally nucleated myofibers. The analysis of the distribution of the fiber type phenotypes reveals an interesting phenomenon. Muscle fibers transfected with the peptide aptamer, rarely present central nuclei. They are, however, surrounded by centrally nucleated myofibers (Fig. 6). This observation suggests that the effect of the Apta-C8 can be exerted *via* paracrine signaling, resulting in the stimulation of regenerative process in the neighboring cells

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Apta-C8 modulates maximal force and fatigue resistance

To determine the impact of Apta-C8-induced-CnA activation on the physiological function of the skeletal muscles and their resistance to the fatigue, we analyzed the contractile properties of TA upon electroporation of the peptide aptamers and controls.

The maximal force was measured in both denervated and non-denervated muscles. We observed no significant difference in maximal force of the non-denervated TA in the various conditions. In denervated muscles, whereas the TA transfected with CnA* show a ~20% increase in the specific maximal force (Fig.7A).

The fatigue resistance was also measured in non-denervated muscles. In this case TA transfected with Apta-C8 show a 20% increase in fatigue resistance (Fig. 7B).

Apta-C8 reduces contraction-induced injury to skeletal muscles of aged *mdx* dystrophic mice

The effect of Apta-C8 was also ascertained in *mdx* mice, a model of Duchenne Muscular Dystrophy. The lack of Dystrophin in these animals, causes fragility of the sarcolemma and leads to an increase of the susceptibility to contraction-induced muscle injury.

The effect of the Apta-C8, as well as of CnA* was evaluated in aged (20 month old) *mdx* mice. These mice are even more sensitive than young *mdx* mice to the injury induced by muscle contraction. In this model we have observed a significant increase of the resistance to the eccentric contractions in muscle transfected with CnA* and Apta-C8. The loss of force observed in muscle transfected with the negative control, visible already after 3 contractions is significantly reduced in the presence of both CnA* and Apta-C8 (Fig. 8).

Discussion

CnA has been shown to be involved in skeletal muscle growth (Horsley et al. 2001; Michel et al. 2004), regeneration (Charge and Rudnicki 2004) and hypertrophy (Dunn et al. 1999; Olson and Williams 2000). Despite the numerous studies performed to determine the impact of Calcineurin signaling in skeletal muscle, some aspects remain controversial. All the insights into the involvement of CnA activation in the main processes related to muscle remodeling, have been obtained using different approaches, encompassing over-expression of a constitutively-active forms of the protein, knock in and knock out transgenic mice or use of specific inhibitors. The identification of a molecule able to directly activate the endogenous Calcineurin represents, therefore, a strategic tool to confirm the role of this phosphatase in skeletal muscle development, growth and regeneration, while offering a potential means for development of novel therapeutic approaches.

Our lab has identified a peptide aptamer (Apta-C8) that binds specifically to Calcineurin and activates its phosphatase activity. The present study investigated the impact of expression of Apta-C8 on activation of endogenous Calcineurin and muscle degeneration in denervation-induced muscle atrophy. It further examined the effect on selected physiological parameters in this model and in aged dystrophic mice (*mdx*)

It has been demonstrated that denervation causes skeletal muscle atrophy, resulting from activation of proteolytic systems, with subsequent loss of contractile proteins (Furuno et al. 1990). Denervation results, thus, in a loss of muscle mass and in a decrease in contractile properties. Muscle atrophy and degeneration of muscle structures, induced by removal of neuronal stimulation, have been shown to be more severe in fast twitch muscles.

In the mouse model for denervation-induced atrophy, Apta-C8 appears to have a significant protective effect against the loss of muscle mass upon denervation in TA. While the negative control undergoes characteristic atrophy, evidenced by a decrease of muscle CSA, expression of Apta-C8 resulted in a reduced loss of muscle CSA in denervated TA.

Moreover, after denervation, a decrease in the number of fibers was observed at day 7 and 14, particularly in muscles transfected with the negative control. The reduction in

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muscle fiber number could be explained considering that muscle electroporation followed by denervation initiates a process of degeneration that can lead to cell death in some fibers (Borisov and Carlson 2000).

The decrease in fiber number, appears to be preserved by the action of Apta-C8, suggesting a role of this CnA-activating peptide aptamer in protecting muscle from degeneration, or possibly in the generation of new fibers that could balance the loss resulting from electroporation and denervation. In addition, we have shown that Apta-C8 has an effect on skeletal muscle growth in non-denervated muscles as well, resulting in a larger overall muscle surface area in healthy TA. Taken together, these observations suggest that the activation of the endogenous CnA *via* Apta-C8 plays a role in the promotion of muscle hypertrophy and in the protection against loss of muscle fibers in this model for muscle atrophy.

This appears to be consistent with the insights into the involvement of CnA pathway in mediating the hypertrophic response induced by IGF-1 (Musaro et al. 1999). Treatment with IGF-1 induces an increase in intracellular calcium concentration resulting in the activation of CnA. The phosphatase dephosphorylates NFATc1, resulting in the activation and nuclear translocation of the transcription factor. Co-operating with other transcription factors, such as GATA-2 and MEF-2, NFATc1 activates the transcription of genes implicated in muscle hypertrophy and regeneration.

The impact of Apta-C8 in the activation of the CnA-NFAT signalling has been observed, *via* monitoring the nuclear localization of NFATc1 and the subsequent cellular responses. The results presented herein provide the first evidence that Apta-C8 activates Calcineurin A *in vivo*, resulting in the, nuclear translocation of the transcription factor, NFATc1.

In this context, the effect of the peptide aptamer in non-denervated muscles fibers could be linked to the activation of the hypertrophic response triggered by the activation of CnA-NFAT signalling. On the other hand, the relative contribution of protection against the loss of fibers subsequent to denervation *vs* the activation of regeneration, resulting in the appearance of fibers with central nuclei, remains to be clarified.

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The regeneration process requires activation, proliferation, and differentiation of satellite cells. CnA has been demonstrated to be a key mediator of muscle development and the regeneration processes. The CnA-NFAT pathway is involved in the activation of the myogenic program in satellite cells, *via* the up-regulation of the expression of the MRF (Myogenic Regulator Factor) family members, such as Myf5. Once activated, satellite cells fuse, either with each other to form new myofibers, or with existing fibers to repair a damaged region. However, satellite cells are not susceptible to transfection through electroporation (Lagirand-Cantaloube et al. 2009). This infers that the activation observed in the experiments described herein, is due to the expression and action of Apta-C8 on CnA in the muscle fibers. The CnA-NFAT pathway is involved in satellite cell differentiation and fusion, *via* the up-regulation of the expression of interleukins, such as IL-4 (Horsley et al. 2003). Release by muscle fibers, of IL-4 into the extracellular matrix, promotes the recruitment of satellite cells and their fusion to the already existing myofibers, resulting in skeletal muscle growth.

In the denervation-induced muscle atrophy model, we observed an increase in the number of proliferating satellite cells in muscle transfected with Apta-C8, compared to the negative control, indicating that the regeneration process has been enhanced by the action of the peptide aptamer. An increase in fibers with central nuclei has also been observed, notably, in the direct vicinity of transfected fibers. This suggests that there is a direct communication between transfected cells and surrounding satellite cells. Thus, we have hypothesized that Apta-C8 could induce the activation of neighboring satellite cells *via* a paracrine effect, for example, through the expression and release of Interleukins.

In conclusion, the results presented herein, using a mouse model for denervation-induced atrophy, describe a way to, simultaneously, stimulate satellite cell activation while enhancing protection against atrophy, of transfected muscle fibers.

Another important aspect observed in our work is the increase of the resistance to fatigue in non-denervated muscles. This correlates with a slight increase in muscle mass, visible by observation of the mice, reinforced by the CSA measurements. It is important to ascertain the characteristics of these muscle fibers. One of the key roles of CnA activation is the modulation of the fiber type gene expression programme. Over-expression of CnA* has been shown to induce the switch of fast twitch fibers in slow twitch fibers, which are

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characterized by an oxidative metabolism and have a higher endurance during physical exercise. Activation of NFATc1, has been demonstrated to be crucial for the regulation of the slow fiber gene expression program (Calabria et al. 2009). The impact of Apta-C8 observed in non-denervated muscle, where the localization of NFATc1 is predominantly nuclear, is consistent with these observations. The analysis of the fiber type distribution will confirm the impact of Apta-C8 in the regulation of slow fiber-specific gene expression.

Taken together, the results presented herein, represent the first proof of concept of the benefit of direct activation of endogenous CnA for protecting muscle against atrophy and degeneration, while reinforcing function of non-denervated muscle. The question remained, whether any benefit could be achieved in a robust animal model for dystrophinopathy, *i.e.* *mdx* mice. It has been reported that constitutive expression of activated Calcineurin A in the muscle of *mdx* mice, improves the regeneration efficacy (Stupka et al. 2006a; Stupka et al. 2007) and protects against contraction-induced injury (Stupka et al. 2006b). To date, the impact of Calcineurin activity has been mainly monitored using 6 week – 10 month old transgenic *mdx* mice over-expressing a constitutively-active Calcineurin (CnA*) in the muscle. Working with aged *mdx* mice (20 months old), which are even more sensitive than young mice to degeneration and to contraction-induced injury, offered a significant test of the potential therapeutic benefit of the CnA-activating peptide aptamer. In the present study, transfection of CnA* reduces, significantly, the force drop occurring after eccentric contraction in aged *mdx* mice, offering the first such report. These results set the benchmark for evaluating the impact of Apta-C8, which, through activation of endogenous CnA, is able to induce the same effect in this model.

Our work, thus, offers the first insight into the involvement of CnA in conferring functional benefit in aged *mdx* upon transfection of CnA* and, above all, *via* the direct activation of endogenous CnA with a specific peptide aptamer. In this regard it is important to underline that the average life span for *mdx* mice is estimated to be around 22 months (Chamberlain et al. 2007). This means that Apta-C8 can exert a beneficial effect even under very challenging physiological conditions.

In conclusion we have identified and characterized the first molecule able to activate endogenous CnA *in vivo*. The impact of this specific peptide aptamer (Apta-C8) in muscle regeneration and hypertrophy has been shown for the first time in aged *mdx* mice, and in a

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mouse model for denervation-induced atrophy. In essence, direct activation of endogenous CnA has a quantifiable morphological and functional protective effect, offering important insight for the development of novel therapeutic approaches.

Acknowledgements

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FIGURES

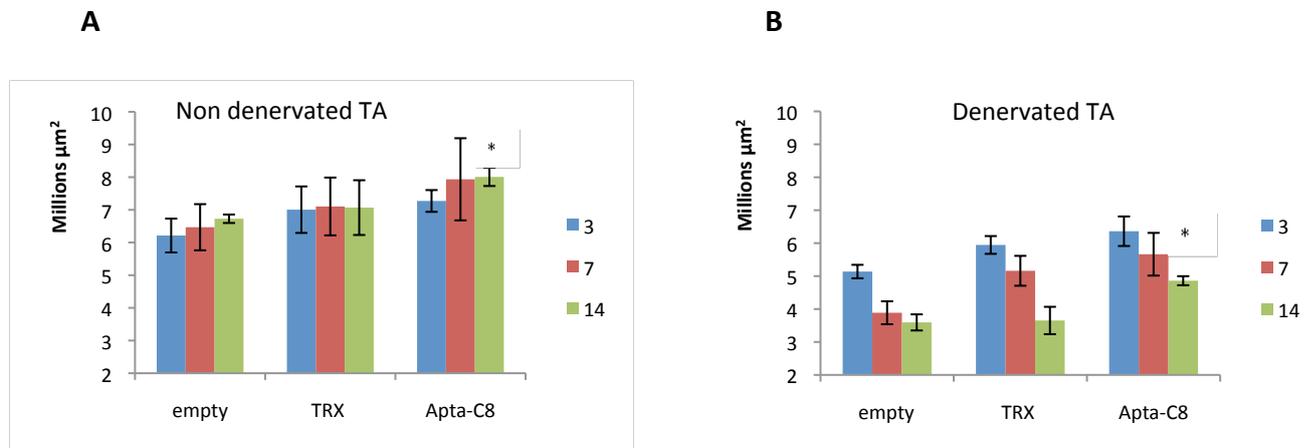


Figure 1. Apta C8 promotes muscle growth and protects muscle against denervation-induced atrophy. Mice TA were transfected with Apta-C8 and the controls, empty plasmid and Trx. 3 days after electroporation the left hind limb legs were denervated in order to induce muscle atrophy. Muscles were collected 3, 7 and 14 days after denervation and the cross sectional area of each muscle was measured. In non denervated muscles (A), Apta-C8 induces an increase of CSA (15% compared to empty plasmid). In denervated muscles (B) the loss of muscle mass observed at day 14 in muscles treated with the two controls, is reduced of 50% in presence of Apta-C8. Cross sectional Area was determined on slices taken at the muscle median using Metamorph™. Average +/- SEM of 3 animals per conditions. P-value < 0,003 for Apta C8 vs empty at day 14 for non denervated ; P-value < 0,004 for Apta C8 vs empty at day 14 for denervated

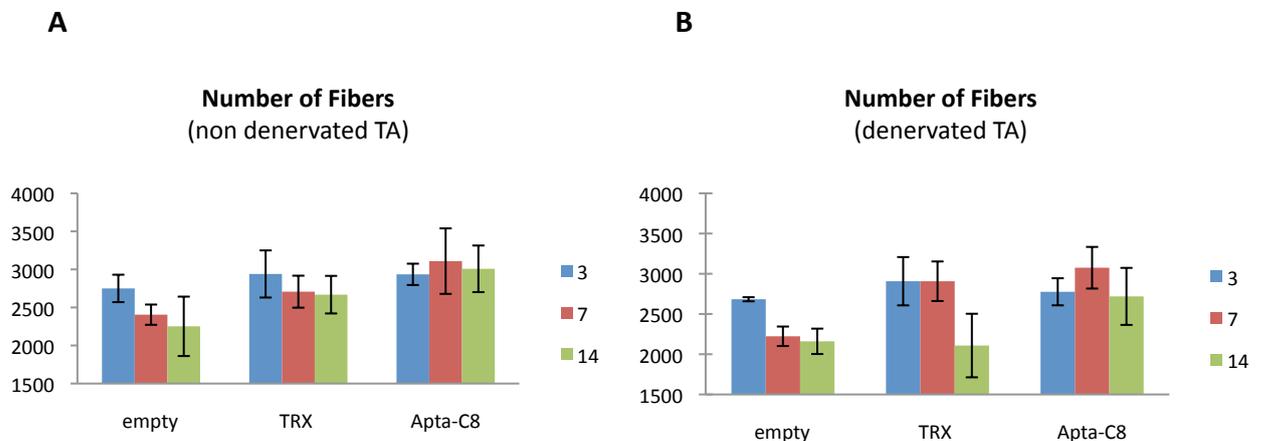
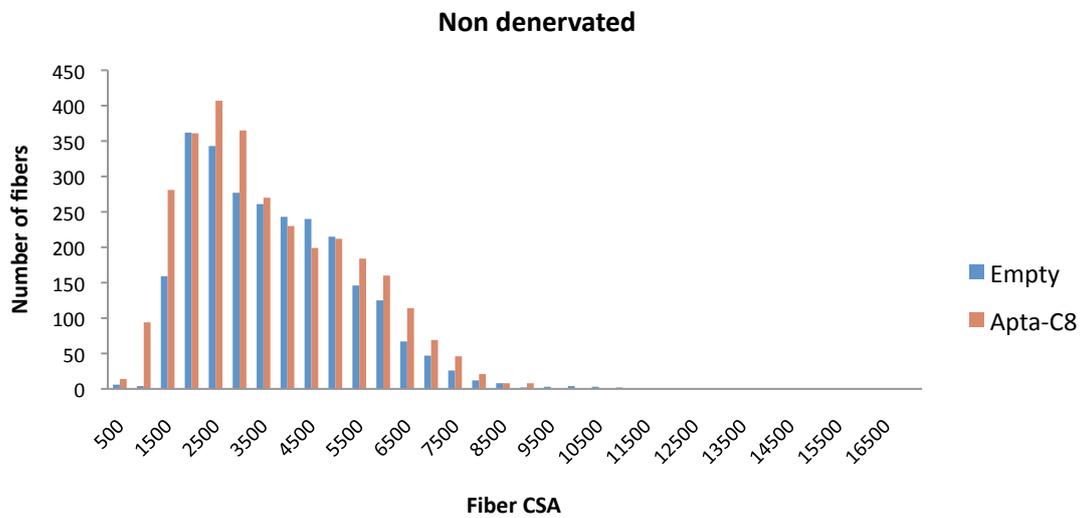


Figure 2. Apta-C8 reduces the loss of fiber number in denervated TA. In non denervated muscles no significant changes in the number of fibers were observed (A). In denervated muscles the loss of fibers observed in the negative controls, particularly at day 14 is preserved by Apta-C8. Fibers in *tibialis anterior* were manually counted using ImageJ. Average +/- SEM of 3 animals. p-value < 0,09 for C8 vs Empty plasmid at 14 days.

CnA-activating peptide aptamer protects against muscle atrophy

A



B

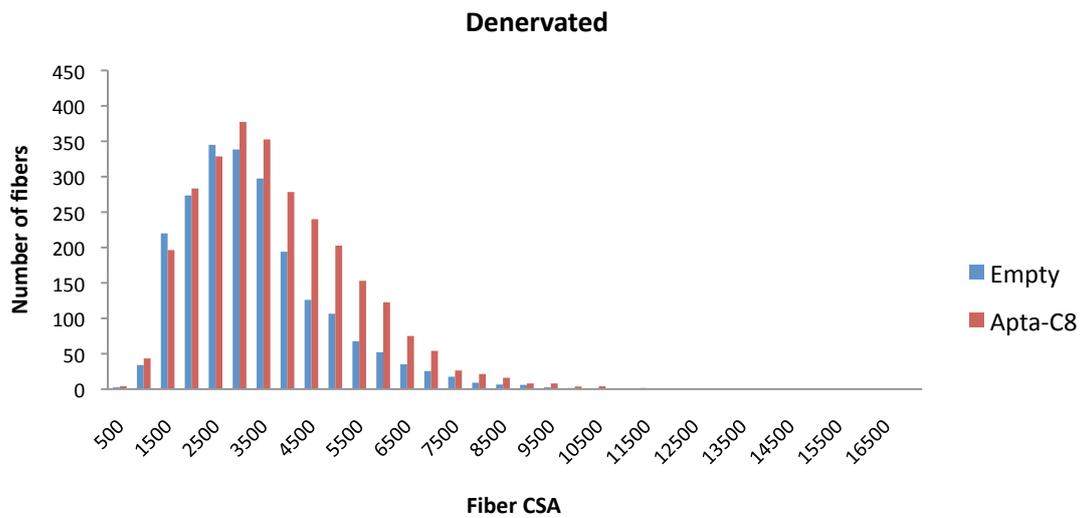


Figure 3. Fiber surface distribution. The graphs represent the distribution of the individual surface area of non denervated (A) and denervated (B) muscles transfected with the empty plasmid and the Apta-C8. Surface area of fibers in the denervated *tibialis anterior* was manually evaluated using ImageJ™. Results represent the average distribution of the muscle fibers of 3 animals for each condition.

CnA-activating peptide aptamer protects against muscle atrophy

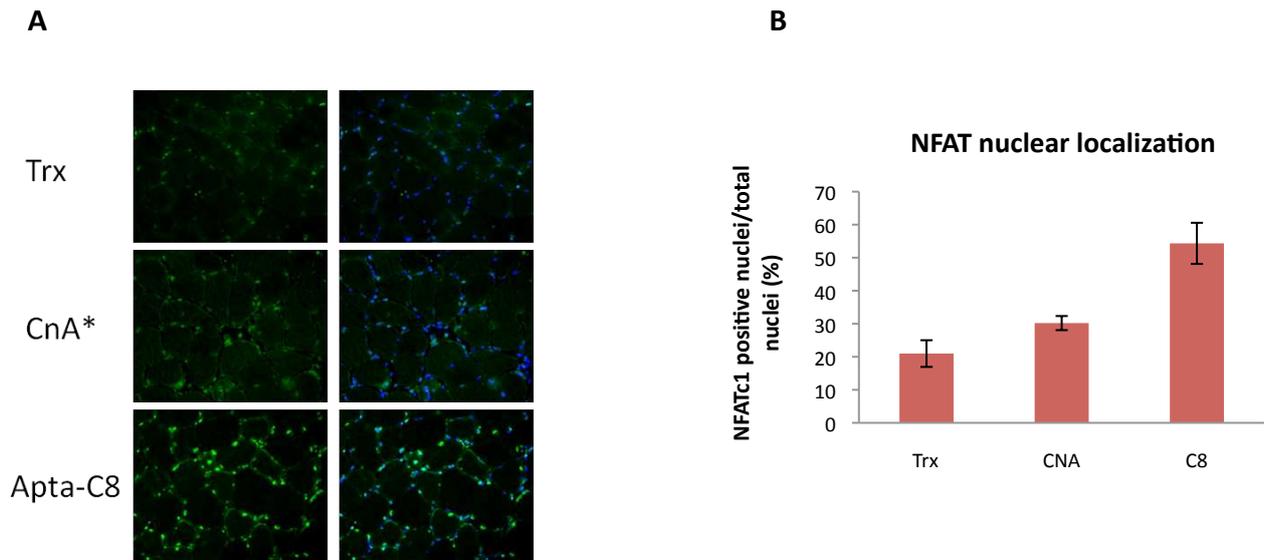


Figure 4. Nuclear shuttling of NFATc1 in *tibialis anterior*. Pictures correspond to muscles co-transfected with a plasmid expressing GFP-NFATc1 and respectively, Trx, CNA* and Apta-C8. 7 days after electroporation, a predominant NFATc1 nuclear localization is observed in the Apta-C8 transfected muscles (A). Quantification of NFATc1-positive nuclei vs total nuclei (B) (total of 300 cells counted on 3 muscles for each condition).

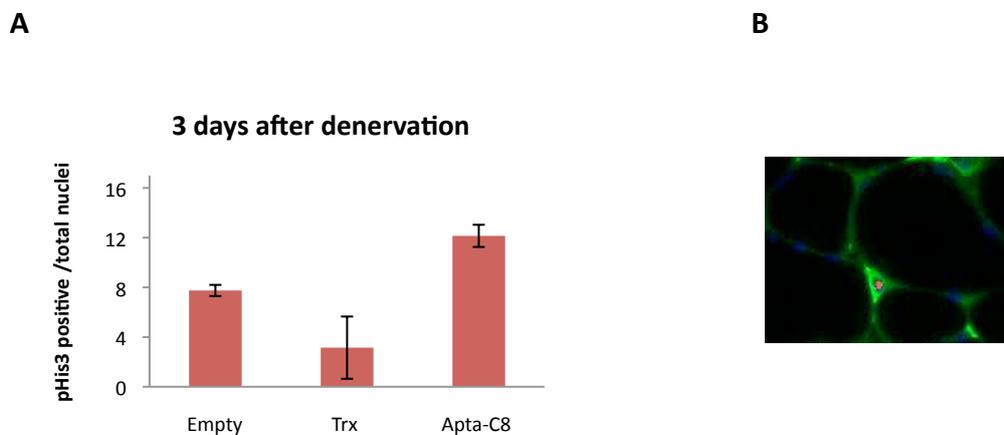


Figure 5. Activation of satellite cell proliferation in denervated TA. *Tibialis Anterior* cross-sections of denervated mice were labelled with anti P-His3 (marker of proliferation) and DAPI in order to evaluate the rate of proliferating cells (A). The graph represents the ratio (%) of P-His3-positive nuclei vs total nuclei. Images were analyzed using ImageJ software. Co-localization of p-His3 (red) and Pax7 (green) (B). Nuclei are stained with DAPI (blue).

CnA-activating peptide aptamer protects against muscle atrophy

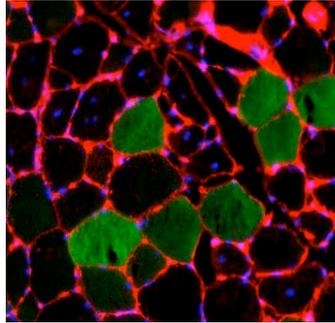
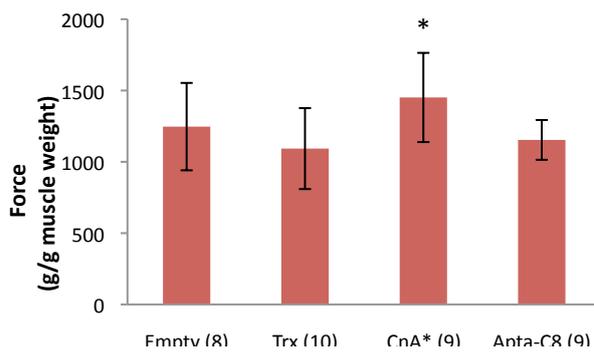


Figure 6. Distribution of multinucleated muscle fibers. Detail of a denervated TA co-transfected with Apta-C8 and GFP at day 14. The transfected myofibers (green) appear surrounded by centrally nucleated cells (blue).

A



B

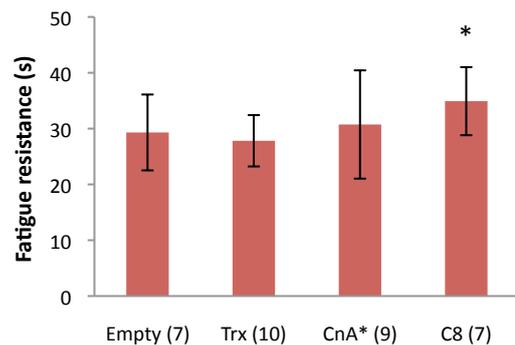


Figure 7. Specific maximal and fatigue resistance. Measurement of maximal force in denervated muscles show an increase of ~20% of the specific maximal force compared to Trx and 15 % compared to empty plasmid.(A). (CnA vs empty, $p=0,09$. CnA vs Trx, $p<0,01$). Fatigue resistance was measured in non denervated muscles. The time corresponding to a decrease of 50% of maximal force is increase of 20% compared to the negative controls in muscle transfected with Apta-C8 (B). (Apta-C8 vs empty $p=0,07$; Apta-C8 vs Trx $p=0,01$). The number of animals for each condition is shown in parenthesis in the graph. Statistical analysis were done using the Welch's T test

CnA-activating peptide aptamer protects against muscle atrophy

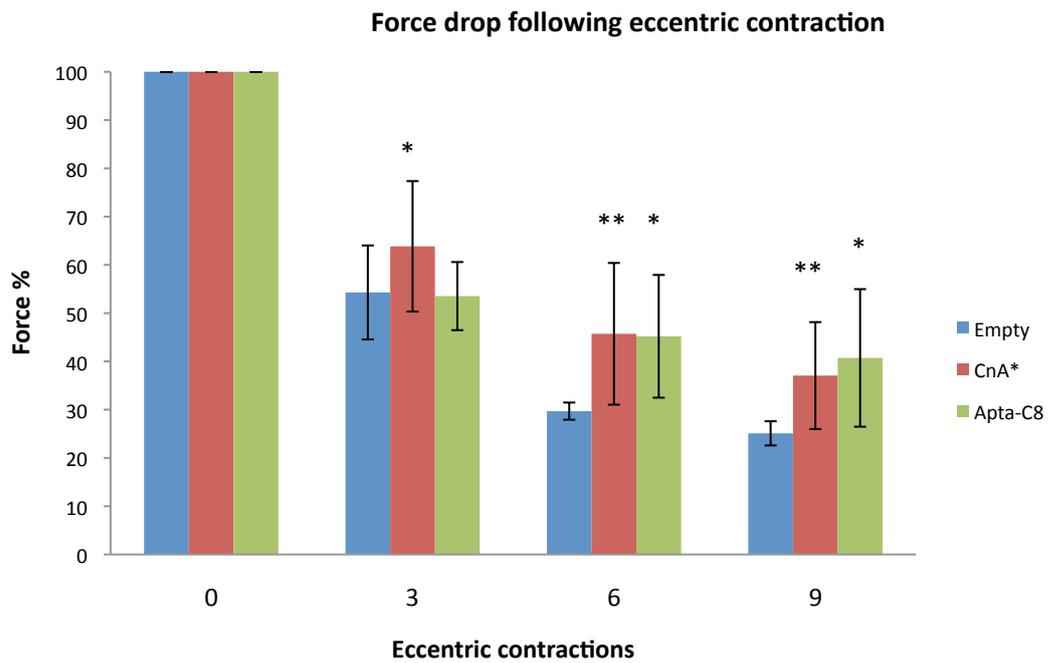


Figure 8. Susceptibility to eccentric contraction induced muscle injury. Force drop following eccentric contractions was measured in 20 month old *mdx* mice. While the negative control undergoes the expected loss of force induced by eccentric contractions, CnA* and Apta-C8 prevent significantly the force drop, with an effect visible already after 3 contractions in the case of CnA* and after 6 contractions in the case of Apta-C8. (* for $p < 0,01$; ** $p < 0,001$) Statistical analysis were done using the Welch's T test

IMPACT AND PERSPECTIVES

In the work presented in my thesis, we have identified two specific peptide aptamers, Apta-C8 and Apta-E1 that bind respectively, to Calcineurin A and NS5A-TP2. While the role of Calcineurin has been well characterized in different signaling pathways, little is known about the functional role of NS5A-TP2. In this work we decided to focus on developing the specific CnA peptide aptamer (Apta-C8) and to investigate its impact in the regulation of the CnA NFAT pathway in cells and *in vivo*.

Apta-C8 is, to date, the first exogenous molecule known able to activate endogenous CnA. The impact of this peptide aptamer was tested in mouse models for muscle degeneration. The results obtained show that Apta-C8 is able to activate the endogenous Calcineurin *in vivo*, resulting in the promotion of muscle regeneration in a mouse model for denervation-induced atrophy and in the protection against the contraction induced muscle injury in aged *mdx* mice.

In denervated TA muscles, previously-transfected with Apta-C8, expression of this peptide aptamer generates an increase in the CSA and protects against the loss of fiber number observed during muscle degeneration. The results obtained during the course of my PhD thesis research, offer the first proof of concept that *direct activation of endogenous CnA in vivo* can indeed impact upon and *reverse* muscle degeneration at a *morphological* and *functional* level. Furthermore, they show that *normal* muscle function can be *enhanced* through the same mechanism.

Finally, in aged (20 month old) *mdx* mice, Apta-C8 shows a significant protection against the force drop induced by eccentric contraction, comparable with the effect obtained with CnA*. These results are quite encouraging considering the difficulties working with old *mdx* mice, which are extremely sensitive to eccentric contractions. This is a widely accepted model for neuromuscular disease, wherein the degeneration is inherent to physiological genetic perturbation. That expression of Apta-C8 was able to confer a protection is a major step towards development of novel therapeutic approaches based thereupon.

PERSPECTIVES

My PhD thesis has allowed me to make significant observations regarding prevention of muscle degeneration. It has also raised many additional questions, some of which I was able to answer in the course of my research. However, many others remain outstanding, that were answers to be obtained, would allow advance on this and other fronts.

Analysis of mutations

The random mutagenesis performed on the variable loop of the parental peptide aptamer, R5G42, lead to the generation of a library of ~1200 mutants. Amongst these clones, 48 mutants were selected for their binding phenotype vs CnA and NS5A-TP2, and sequenced. Physical and chemical analysis of the gain or loss of interaction correlated to each mutation could lead, along with structural analysis, to a better understanding of the mechanism of action of Apta-C8, resulting in the activation of Calcineurin. This, in turn, would facilitate the conception of strategies to mimic the peptide aptamer using peptides or small molecules.

Calcineurin activation and muscle regeneration.

In this work we have shown that the expression of Apta-C8 has a protective effect against muscle atrophy, resulting in an increase of muscle mass and activation of satellite cells. We have confirmed that Calcineurin activation *via* Apta-C8, results in the nuclear translocation of NFATc1. This transcription factor normally co-operates with other factors, such as MEF-2 and GATA-2 in order to induce the expression of several genes involved in muscle growth and regeneration. Analysis of the gene expression *via* Real Time PCR of factors that are up-regulated during muscle regeneration, such as Myf5, MyoD and myogenin, could offer important information about the downstream actors of the regenerative process activated by Apta-C8. This, in turn would offer precious insight into the contribution of specific activation of CnA to the regeneration program in selected models.

Satellite cell activation

We have hypothesized that the activation of Calcineurin in transfected fibers could exert a paracrine effect on the surrounding cells *via* the release of interleukins in the extracellular matrix. NFATc2 has been demonstrated to play a critical role in the recruitment of satellite cells and in the promotion of their fusion to existing myofibers *via* the

intermediate action of IL-4. In order to obtain further understanding and explain the phenomena observed in our model, it would be worthwhile to monitor the activation of NFATc2 and quantify the expression of IL-4, as well as other cytokines specific to this process. An increase of this signal could help to reinforce the current observations and our hypothesis

***Mdx* mice**

Our pioneering study performed on *mdx* mice has offered very encouraging results. We have shown that Apta-C8, reduces as well as CnA*, the drop force induced by eccentric contraction. Over-expression of Utrophin, a cytoskeletal protein with a high affinity to Dystrophin is able to compensate the lack of dystrophin in DMD muscle (Deconinck et al., 1997). Increase of utrophin reduces overall disease pathology in *mdx* mice. Activation of Calcineurin has been shown to increase expression of utrophin in *mdx* mice resulting in enhancement of the pathological conditions of this model (Chakkalakal et al., 2003). Evaluation of the level of the expression of the utrophin upon transfection with our peptide aptamer could offer insight on the impact of the CnA-activating peptide aptamer in this pathological model.

Characterization of NS5A-TP2 function

The random mutagenesis allowed me to dissect the binding of R5G42 to CnA and NS5A-TP2, yielding in addition to Apta-C8, a peptide aptamer, Apta-E1, specific for NS5A-TP2. This protein belongs to the HD family and is trans-activated by the Hepatitis C-virus NS5A protein, but its biological function is still unknown. The peptide aptamer identified herein, could represent an important tool for the characterization of the functional role of this protein. Over-expression of Apta-E1 can be used to monitor the impact of NS5A-TP2 in different cellular process, such as cell proliferation, apoptosis or differentiation. It is expected that, these experiments could yield high returns in knowledge, in light of the potential role this protein may play in cellular signalling networks.

Concluding remark

Clearly, one of the most exciting aspects of this work is that it may prepare the way for the development of novel therapeutic approaches to treatment of muscle wasting diseases.

It allows conception of protocols for evaluation of direct activation of CnA on other models such as cancer-related cachexia, fasting, age-related reduction in muscle mass, ALS.

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