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EARLY SIGNALING EVENTS INVOLVED IN MUSCLE REMODELING AFTER EXERCISE

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RIASSUNTO

La plasticità muscolare dopo l'esercizio è ottenuta tramite diversi meccanismi molecolari che regolano la trascrizione dei geni incidendo sulla struttura della cromatina e sui fattori di trascrizione. L'esercizio fisico è in grado di indurre una risposta adattativa al fine di migliorare l'efficienza metabolica, la capacità ossidativa e l'attività contrattile alterando i profili di espressione genica e i livelli delle proteine. Tuttavia, i meccanismi molecolari alla base di questi cambiamenti non sono ancora ben caratterizzati. Lo scopo del nostro studio è di analizzare i segnali molecolare che stanno alla base del rimodellamento muscolare. Per rispondere a questa domanda abbiamo diviso il progetto in due parti.

In primo luogo, abbiamo focalizzato la nostra attenzione sulle variazioni del fosfoproteoma dopo l'esercizio e come questo può influenzare la trascrizione genica. Al fine di determinare i cambiamenti globali del fosfoproteoma dopo esercizio ad alta intensità abbiamo eseguito un'analisi quantitativa fosfoproteomica e abbiamo confrontato i muscoli stimolati (50 contrazioni eccentriche) ai muscoli controlaterali non stimolati. Abbiamo identificato una lista di peptidi in cui c'era un aumento di fosforilazione dopo contrazioni eccentriche e, in particolare, abbiamo individuato un aumento significativo in un sito di fosforilazione precedentemente non caratterizzato nel fattore di trascrizione *myocardin-related transcription factor 2* (MRTF-B), la serina 77. Le proteine della famiglia *myocardin / MKL* sono state descritte come potenti coattivatori del fattore di trascrizione *Serum Response Factor* (SRF), un importante *pathway* noto per controllare la crescita del muscolo scheletrico. E' stato riportato in vari sistemi cellulari che la localizzazione intracellulare di MRTF-B è regolata dalla fosforilazione, che determina il suo spostamento dal citoplasma al nucleo. Nel nostro studio abbiamo scoperto che, dopo le contrazioni eccentriche, vi è un accumulo nucleare di MRTF-B. Eseguendo una mutazione puntiforme, in cui la serina 77 è stata sostituita da un'alanina, l'accumulo nucleare del MRTF-B dopo contrazioni eccentriche viene completamente abolito. Per capire se l'accumulo nucleare di MRTF-B dopo contrazioni eccentriche fosse accompagnato da un aumento della trascrizione genica SRF-dipendente, abbiamo effettuato un'analisi *microarray* 24 ore dopo la stimolazione. Eseguendo un *gene set enrichment*

analyses (GSEA) sui geni differenzialmente regolati, abbiamo identificato SRF come uno dei principali fattori di trascrizione la cui attività è aumentata.

Nella seconda parte di questa tesi abbiamo analizzato come una singola ripetizione di esercizio fisico può influenzare le modificazioni degli istoni e modulare il paesaggio epigenetico, in modo da portare a un aumento dell'attività SRF-dipendente.

Un numero crescente di osservazioni indica che il rimodellamento della cromatina è uno dei meccanismi più importanti che influenza l'espressione genica. Recentemente sta emergendo come le modificazioni post-traduzionali degli istoni rappresentino un evento primario nel processo dinamico di rimodellamento della cromatina. Abbiamo concentrato la nostra attenzione sull'identificazione di modificazioni post-traduzionali degli istoni collegate ad un aumento della trascrizione genica, ed in particolare: H3-K4me3, H3-K9K14ac e H3S10ph. Abbiamo esaminato l'effetto di una singola ripetizione di esercizio fisico ad alta intensità (contrazioni eccentriche) sulle modificazioni delle code N-terminali degli istoni in muscoli scheletrici murini. Abbiamo osservato un significativo aumento nelle fosforilazione della serina 10 dell'istone H3 (H3S10ph) nei muscoli stimolati, rispetto ai muscoli di controllo in cui questa fosforilazione è completamente assente. Tramite analisi di immunofluorescenza abbiamo dimostrato che questa modificazione post-traduzionale si verifica in circa il 40% dei mionuclei. Abbiamo anche dimostrato che questa fosforilazione dell'istone H3 è seguita da un aumento dell'acetilazione della vicina lisina 14 e che queste modificazioni sono indicative dell'attivazione della trascrizione genica.

Successivamente, abbiamo lavorato sull'individuazione delle putative chinasi coinvolte nella fosforilazione dell'istone H3. E' stato dimostrato, che la famiglia delle MAP-chinasi (MAPK) è coinvolta nella fosforilazione H3S10. Nel nostro studio abbiamo osservato un aumento di fosforilazione di p38-MSK1 nei muscoli soggetti a esercizio rispetto a quelli di controllo. In numerosi tipi cellulari di mammifero, le proteine chinasi MSK1/2 sono state identificate come le chinasi responsabili della fosforilazione sulla serina 10 dell'istone 3. Utilizzando un siRNA contro MSK1 abbiamo scoperto che MSK1 è necessaria per la fosforilazione sulla serina 10 dell'istone 3 dopo contrazioni eccentriche nel

muscolo scheletrico. Infatti, le fibre elettroporate con il siRNA non mostrano colorazione nucleare dell'i stone 3 rispetto alle fibre circostanti non transfettate.

Infine, abbiamo iniziato ad analizzare il ruolo della fosforilazione dell'istone 3 nella variazione dell'espressione genica indotta da esercizio fisico. Per rispondere a questo interrogativo abbiamo effettuato un'immunoprecipitazione della cromatina (ChIP) seguita da PCR quantitativa (real time PCR). I risultati preliminari mostrano una marcata fosforilazione sul promotore di p21, c-fos, PGC-1α1 e PGC-1α4 e la conseguente espressione di questi geni. Attualmente stiamo lavorando sulla configurazione della Chip-Seq al fine di generare mappe ad alta risoluzione della distribuzione in tutto il genoma di H3S10ph.

Oltre ai nostri risultati nei topi, abbiamo studiato biopsie umane prelevate subito dopo l'attività fisica ad alta intensità e abbiamo osservato cambiamenti simili nei pathway a monte e nella fosforilazione dell'Istone 3.

In conclusione, in questa tesi abbiamo identificato due *signaling pathway* che si verificano dopo l'esercizio fisico ad alta intensità, vale a dire l'aumento della trascrizione genica dipendente da MRTF-B-SRF e la modificazione post-traduzionale degli istoni legata all'attivazione genica.

SUMMARY

Muscle plasticity after exercise is achieved by different molecular mechanisms that regulate gene transcription by impinging on chromatin structure and transcription factors. Exercise is able to induce adaptive response to improve metabolic efficiency, oxidative capacity, and contractile activity by altering gene expression profile and protein levels. Nonetheless, the molecular mechanisms at the basis of these changes remain not very well characterized. The aim of our study is to dissect the early signaling events linked to skeletal muscle remodeling. To address this question we decided to divided the project in two parts.

First, we focused our attention on changes in the phosphoproteome after exercise and how this can affect gene transcription. In order to determine the global changes in the phosphoproteome after high intensity exercise we performed a quantitative phosphoproteomic analysis and we compared the stimulated (50 eccentric contraction) to the non-stimulated controlateral muscle. We identified a list of peptides with the strongest increase in phophorylation after eccentric contractions and in particular we found a significant increase in a previously uncharacterized phosphorylation site of myocardin-related transcription factor 2 (MRTF-B), the serine 77. The myocardin/MKL family of proteins has been described as strong coactivators of the Serum Response Factor (SRF), an important signaling pathway known to control skeletal muscle growth. It has been reported in various cell systems that the intracellular localization of MRTF-B is regulated by phosphorylation, enabling it to shuttle from the cytoplasm to the nucleus. We found that after eccentric contractions there is a nuclear accumulation of MRTF-B. Performing a point mutation, in which Serine 77 is replaced by an alanine, the nuclear accumulation of MRTF-B after eccentric contractions is completely abrogated. In order to understand if the nuclear accumulation of MRTF-B after eccentric contractions is also accompanied by an increase in SRF-dependent gene transcription, we performed a microarray analyses 24 hours after stimulation. Performing a gene set enrichment analyses (GSEA) on the differentially regulated genes, we identified SRF activity as one of the major transcription factors whose activity is increased.

In the second part of this thesis we analyzed how one bout of exercise can influence histone modification and modify the epigenetic landscape, possibly leading to an increase in SRF-dependent activity.

Growing evidence indicates that chromatin remodelling is one of the most important mechanisms that might influence gene expression. Post-translational modifications occurring on histones is emerging as a primary event contributing to the dynamic process of chromatin remodelling. We focused our attention on identifying histone marks, which have been linked to gene transcription, i.e. H3-K4me3, H3-K9K14ac and H3S10ph. We have examined the effect of an acute bout of high-intensity exercise (eccentric contractions) on these histone modifications in mouse skeletal muscle. We have found that histone 3 is very rapidly phosphorylated at serine 10 (H3S10ph) after eccentric contractions, which is completely absent in normal muscles and in the controlateral non-stimulated muscles. Immunofluorescence co-staining for dystrophin showed that this change occurs in about 40% of myonuclei. We have also found that this histone phosphorylation is followed by an increase in the acetylation of the nearby lysine 14, suggestive of an activation of gene transcription.

Next we worked on the identification of the putative kinases involved. It has been shown by other groups that the mitogen-activated protein kinase (MAPK) family is involved in H3S10 phosphorylation. We observed an increase in p38-MSK1 phosphorylation in exercised muscle compared to the control ones. In numerous mammalian cells, the mitogen- and stress-activated protein kinases (MSK1/2) have been identified as the kinases responsible for H3S10ph. Using a siRNA against MSK1 we found that MSK1 is required for H3S10ph after eccentric contractions in skeletal muscle. Indeed we completely abolished nuclear histone 3 staining in fibers electroporated with the siRNA, compared with the surrounding non transfected fibers.

We have started to analyse whether histone phosphorylation plays a role in exercise-induced gene expression and to address this we performed chromatin immunoprecipitation (ChIP) and quantitative polymerase chain reaction (real time PCR). Preliminary results show a marked phosphorylation on the promotor of *p21*, c-*fos*, *Pgc-1α1* and *Pgc-1α4* and a consequent expression of these genes. We are currently working on setting up the Chip-seq in order to generate high-resolution maps for the genome-wide distribution of H3S10ph.

In addition to our results in mice, we have studied human biopsies taken immediately

after high intensity exercise and we have observed similar changes in signaling and histone phosphorylation.

In conclusion, we report two early signaling events which occur after high intensity exercise, namely an increase in MRTF-B-SRF dependent gene transcription and histone remodeling linked to gene activation.

1. INTRODUCTION

1.1 Skeletal muscle

1.1.1 Skeletal muscle biology and metabolism

Skeletal muscle represents 40% of total body mass in mammals and has an important role in the control blood glycemia and metabolic homeostasis, and is the predominant (80%) site of glucose disposal under insulin stimulated conditions (DeFronzo et al, 1981). Moreover skeletal muscle represents the largest glycogen storage which is 4-fold greater than the storage capacity of the liver.

Mammalian skeletal muscles are characterized by the heterogenic presence of different fiber types. Skeletal muscle fibers can be identified as slow- or fast-twitch, based on their contractile properties, like the speed of tension development and relaxation. This classification coincides with histochemical staining for myofibrillar (myosin) ATPase activity, i.e. type I (slow-twitch) and type II (fast-twitch, highest ATPase activity). A subclassification of type IIa and type IIx exists in humans, while type IIb is primarily found in rodents. While type I muscle fibers are classically red in appearance, type IIx and IIb fibers are white in appearance, and type IIa fibers have an intermediate color. This difference in color reflects both the amount of blood perfusion as well as the abundance of the oxygen transport protein myoglobin, which is closely related to mitochondrial density and the relative contribution of oxidative metabolism in the respective fiber types (each highest in type I fibers). Immunohistochemical staining and electrophoretic protein separation can independently determine myosin heavy chain (MHC) isoform protein expression, another key criterion of fiber type classification. Each isoform displays distinct contractile properties that parallel ATPase activity and twitch tension kinetics. Fiber type composition is generally determined during development, but the possible switch in fibers type is still hotly debated (Booth et al, 2012). After an appropriate training stimulus, the plasticity of muscle permits changes in metabolic potential and morphology irrespective of whether a transformation of muscle fiber type measured by change in MHC expression is observed (Leblanc et al, 2004; Phillips et al, 1996). In response to

changes in activity, skeletal muscle is able to modulate the amount of energy production, blood flow and substrate utilization. Locomotion is powered by actin-myosin crossbridge cycling according to the sliding filament theory of skeletal muscle contraction (Podolsky and Schoenberg, 1983). The myosin ATPase hydrolyze adenosine triphosphate (ATP) and provides the energy source for crossbridge cycling, but ATP is also consumed by the dynamics of both sodium-potassium-and calcium exchange necessary for contraction. Skeletal muscle is the principal contributor to exercise-induced changes in metabolism. Maximal exercise can induce a 20-fold increase in whole-body metabolic rate over resting values, whereas the ATP turnover rate within the working skeletal muscle can be more than 100-fold greater than at rest (Gaitanos et al, 1993). The four negative charges and the hydrophilic nature of the ATP does not permit its high intramuscular storage, but muscle contraction activates different metabolic pathways that drive ATP generation in order to maintain adequate intracellular levels. Skeletal muscle is richly endowed with mitochondria and heavily reliant on oxidative phosphorylation for energy production. During strenuous exercise, dramatic (>30-fold) increases in intramuscular oxygen consumption and local blood flow occur (Andersen & Saltin, 1985) (Gibala et al, 1997). Estimated TCA cycle flux increases by _70- to 100-fold under the same conditions (Gibala et al, 1997). Thus, skeletal muscle is the primary site for CHO and lipid metabolism for energy production. Importantly, the biochemical consequences of contractile bioenergetics can regulate molecular processes governing skeletal muscle adaptation.

1.2 Exercise-induced muscle remodelling

Skeletal muscle is a plastic tissue capable of altering the type and amount of protein in response to disruptions of cellular homeostasis. Skeletal muscle is a post-mitotic tissue, and unless the muscle is damaged there is little turnover of muscle fibers (Moss & Leblond, 1970; Moss & Leblond, 1971) (Stockdale & Holtzer, 1961). Indeed, it has been demonstrated that dramatic changes in gene expression, protein composition and physiological properties can occur in pre-existing fibres without de- or regeneration (Delp & Pette, 1994) (Gorza et al,

1988) (Mayne et al, 1993) (Windisch et al, 1998). The ability of skeletal muscle to change its mass or contractile properties in response to new conditions is not a recent notion. Training and detraining effects on skeletal muscles are known for centuries. In ancient Greece, the myth tells that Milo of Croton, who was five times a Periodonikes, that is, winner of wrestling at Olympic, Pythian, Isthmian, and Nemean Games, trained his muscles by lifting a bull-calf daily until it was fully grown, thus increasing his muscle strength in close proportion to the increase in weight of the calf.

Training-induced adaptations are reflected by changes in contractile protein and function (Widrick et al, 2002), mitochondrial function (Spina et al, 1996), metabolic regulation (Green et al, 1992), intracellular signaling (Benziane et al, 2008), and transcriptional responses (Pilegaard et al, 2003).

Moreover, many features of training adaptations are specific to the type of stimulus, such as the mode of exercise.

Generally, exercise can be divided into either endurance training (aerobic-based) or resistance training (anaerobic-based).

1.2.1 Endurance training

Endurance training is characterized by recruiting muscle with a high – frequency (repetition) and low-power output (load). Many types of sports involve endurance exercise, including long-distance running, cycling, and crosscountry skiing. Animals can also undergo endurance training, by performing treadmill running or swimming. Endurance training leads to cardiovascular and respiratory adaptations, but is also involved in the adaptation of skeletal muscles. It well known that mitochondrial biogenesis occurs in response to aerobic exercise training and is defined by an increase in muscle mitochondrial number and volume, as well as concomitant changes in organelle composition (Howald et al, 1985). After 6 weeks of exercise training, mitochondrial density increases by 50%–100% (Hood, 2001). It has been shown that these increases in mitochondrial content is linked to the expression level of a transcription coactivator, the peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α1)(Lin et al, 2005). Increases in cytosolic calcium and depletion of ATP, leading to the

activation of the ‘5 AMP activated protein kinase (AMPK), are common features during exercise and represent two possible signals that lead to the activation of PGC-1α1. PGC-1α1 and AMPK activate various programs of gene transcription required for muscle adaptation to exercise. Indeed it has been shown that activation of AMPK and PGC-1α1 in endurance exercise on one hand enhances glucose uptake (Ivy, 2004), and on the other hand mediates the metabolic shift and the increased lipid oxidation (Holloszy & Coyle, 1984). It is also well known that after endurance training there is an increase in angiogenesis (Wagner, 2011). The generation of a more developed microcirculation may be related to the response of perivascular cells (fibroblasts, macrophages, and pericytes) to altered activity, likely acting as a source for chemical signals modulating capillary growth such as vascular endothelial growth factor (VEGF). The importance of VEGF in muscle capillarity adaptaion after exercise is support by the finding that does not occurs in muscle-specific VEGF knockout mice (Olfert et al, 2010).

1.2.2 Resistance training

Resistance exercise elicits a range of morphological adaptation that contribute to changes in muscle function with respect to size, strength, and power, and are induced by performing exercise at low frequncies (few repetitions) and with a high load. This kind of training cannot be easily applied to animals, although studies of resistance training in rodents have been carried out either using electrical stimulation of selected muscle groups to contract against a computer driven ergometer system in anesthetized animals (Caiozzo et al, 1997) or ad hoc designed cages where the access to food is related to lifting weights (Nicastro et al, 2012). The major morphologial adaptation of resistance training is the increase in muscle mass, that is due to an increase in single muscle fiber cross-sectional area (hypertrophy), often preferntially occuring in type IIa fibers (Adams et al, 1993). Instead, the contribution due to an increase in fiber number (hyperplasia) is controversial (McCall et al, 1996). The resistance training results in an increased rate of myofibrillar and mitochondrial protein synthesis (Luthi et al, 1986) (Phillips et al, 1997). An important role in fiber hypertrophy is thought to be

played by satellite cells activation after resistance training. Indeed, it has been shown by Allen et al. (Allen et al, 1999), that the addition of new myonuclei is required for muscle hypertrophy, indeed destroying of satellite cells by irradiation prevents development of hypertrophy in rat EDL overloaded by TA removal. More recent studies, however, have questioned the need for satellite cells in the induction of muscle hypertrophy (Blaauw et al, 2009; McCarthy et al, 2011), with no clear consensus reached yet (Blaauw & Reggiani, 2014).

	Aerobic (Endurance)	Resistance (Strength)
Skeletal Muscle Morphology and Exercise Performance		
Muscle hypertrophy	↔	↑ ↑ ↑
Muscle strength and power	↔ ↓	↑ ↑ ↑
Muscle fiber size	↔ ↑	↑ ↑ ↑
Neural adaptations	↔ ↑	↑ ↑ ↑
Anaerobic capacity	↑	↑ ↑
Myofibrillar protein synthesis	↔ ↑	↑ ↑ ↑
Mitochondrial protein synthesis	↑ ↑	↔ ↑
Lactate tolerance	↑ ↑	↔ ↑
Capillarisation	↑ ↑	↔
Mitochondrial density and oxidative function	↑ ↑ ↑	↔ ↑
Endurance capacity	↑ ↑ ↑	↔ ↑
Whole-Body and Metabolic Health		
Bone mineral density	↑ ↑	↑ ↑
Body composition		
Percent body fat	↓ ↓	↓
Lean body mass	↔	↑ ↑
Glucose metabolism		
Resting insulin levels	↓	↓
Insulin response to glucose challenge	↓ ↓	↓ ↓
Insulin sensitivity	↑ ↑	↑ ↑
Inflammatory markers	↓ ↓	↓
Resting heart rate	↓ ↓	↔
Stroke volume, resting and maximal	↑ ↑	↔
Blood pressure at rest		
Systolic	↔ ↓	↔
Diastolic	↔ ↓	↔ ↓
Cardiovascular risk profile	↓ ↓ ↓	↓
Basal metabolic rate	↑	↑ ↑
Flexibility	↑	↑
Posture	↔	↑
Ability in activities of daily living	↔ ↑	↑ ↑

Fig.1 Adaptations and Health Benefits of Aerobic Compared to Resistance Exercise

Aerobic exercise training generally encompasses exercise durations of several

minutes up to several hours at various exercise intensities, incorporating repetitive, low-resistance exercise such as cycling, running, and swimming. Resistance training generally encompasses short-duration activity at high or maximal exercise intensities, and increases the capacity to perform high-intensity, high-resistance exercise of a single or relatively few repetitions such as Olympic weightlifting, bodybuilding,and throwing events. ↑, values increase; ↓, values decrease; ←, values remain unchanged; ↑ or ↓, small effect; ↑↑ or ↓↓ , medium effect; ↑↑↑ or ↓↓↓, large effect; ← ↑ or ← ↓, no change or slight change.
(Image taken from,Exercise Metabolism and the Molecular Regulation of Skeletal Muscle Adaptation,(Egan & Zierath, 2013)).

1.3 Activity dependent signaling pathways

Skeletal muscle contraction leads to a dramatic perturbation in mechanical strain, ATP turnover, calcium flux, redox balance, reactive oxygen species (ROS) production, and intracellular oxygen pressure, and all these changes have been implicated in the activation of signal transduction cascades regulating skeletal muscle plasticity. This homeostatic perturbation is transduced by various cellular sensors that can couple muscle contraction to changes in gene transcription. This provides a framework, known as excitation-transcription coupling, that integrates signaling events regulated by cellular bioenergetics and the expression of genes that dictate skeletal muscle phenotype.

1.3.1 Oxygen availability and hypoxia-inducible factors

The amount and types of nutrients available determine the substrates used and strongly influence the size and function of skeletal muscles. During physical activity, there is a shift of substrates used by skeletal muscles depending on the intensity of the exercise. When exercise is performed at maximal intensity, the main substrates are glucose and glycogen consumed by fast glycolytic fibers, while during moderate and prolonged exercise metabolism relies on oxidation of carbohydrates and, eventually, fatty acids mainly by slow oxidative fibers. The

major signal transduction pathway sensitive to the intracellular partial pressure of oxygen (P_iO_2) is regulated by the hypoxia-inducible factor (HIF). HIF is a heterodimeric transcription factor composed of two subunits, HIF-1α and HIF-1β. Under normoxic conditions, hydroxylation of HIF-1α occurs by prolyl hydroxylase (PHD) enzymes which act as sensors of cellular oxygen tension. This triggers the binding of the E3 ubiquitin ligase von Hippel Lindau tumor-suppressor protein (pVHL) to HIF-1α and targets it for proteasomal degradation (Maxwell et al, 1999). During hypoxia or conditions of reduced P_iO_2, the hydroxylase activity of PHD enzymes is inhibited, allowing stabilization of HIF-1α, which translocates to the nucleus to form an active complex with HIF-1β. Activation of HIF-1 induces transcription of target genes involved in erythropoiesis, angiogenesis, glycolysis, and energy metabolism (Taylor, 2008) in a manner analogous to exercise (Mahoney et al, 2005) (Schmutz et al, 2006). HIF-dependent transcriptional regulation augments survival during low O_2 tension either by increasing O_2 delivery and extraction or by enhancing the ability to obtain ATP from O_2 -independent pathways. HIF-1 activates transcription by binding to hypoxia response elements within target genes and recruiting coactivators such as p300 and CBP (CREB-binding protein) (Jiang et al, 1996). Although the P_iO_2 in resting skeletal muscle is about 1/5th of the oxygen pressure of inhaled air, acute exercise reduces P_iO_2 in contracting muscle to about 1/40th of that of inhaled air (Richardson et al, 1995). Unsurprisingly, HIF-1a protein abundance increased during acute exercise, accumulates in the nucleus, and shows enhanced DNA binding, coincident with a decrease in pVHL expression (Ameln et al, 2005). HIF-related processes are relevant to exercise-induced skeletal muscle metabolism and adaptation (Mason et al, 2004) (Formenti et al, 2010) and likely regulate the beneficial effects of simulated altitude training practiced by athletes (Vogt et al, 2001).

1.3.2 Cellular energy status

AMP-activated protein kinase (AMPK) is a serine/threonine kinase which forms a heterotrimeric complex composed of a catalytic subunit (AMPK-α) and two regulatory subunits (AMPK-β and AMPK-γ). AMPK modulates cellular

metabolism through phosphorylation of metabolic enzymes (Carling & Hardie, 1989) and, over time, via transcriptional regulation (Bergeron et al, 2001) (Jager et al, 2007). AMPK is activated by a cellular energy deficit, which is reflected by increases in the AMP/ATP and Cr/PCr ratios (Knab & Lightfoot, 2010). Intense exercise (Green et al, 1992; Howlett et al, 1998) that depletes ATP and glucose deprivation is the major stress that activates AMPK (Kahn et al, 2005). During exercise there is a high turnover of ATP, and so this alteration in the cellular energy status activates AMPK that acts as a transducer for metabolic adaptations. The increases in AMPK phosphorylation during acute exercise occur in an intensity-dependent manner (Wojtaszewski et al, 2000) (Egan et al, 2010) reflecting the intensity-dependent turnover of ATP and adenine nucleotide concentrations during exercise (Howlett et al, 1998). Overall, AMPK activation acts to conserve ATP by inhibiting biosynthetic pathways and anabolic pathways, while simultaneously stimulating catabolic pathways to restore cellular energy stores (Kahn et al, 2005). In skeletal muscle acute AMPK activation stimulates an increase in insulin-indipendent glucose transport, due to the translocation of GLUT4 to the membrane (Merrill et al, 1997). Several studies have shown that AMPK is a negative regulator of skeletal muscle growth, indeed, AMPK phosphorylates tuberous sclerosis complex 2 (TSC2) and also directly mTORC1, leading to an inhibition of translation initiation. Besides its role in repressing protein synthesis, AMPK activation also stimulates protein degradation through phosphorylation and activation of FoxO3a, stimulating the ubiquitin-proteasome and the autophagy-lysosome systems. Chronic AMPK activation alters metabolic gene expression and induces mitochondrial biogenesis (Bergeron et al, 2001), indeed AMPK phosphorylates PGC-1α1 on two distinct residues and requires PGC-1α1 for the transcriptional regulation of a great part of its target genes (Jager et al, 2007).

1.3.3 Calcium signalling

Muscle contraction leads to a strong increase in intracellular calcium levels, which is essential to allow for the interaction between myosin and actin and therefore

muscle contraction.

Moreover, calcium oscillations during contractions lead to the activation of signaling pathways that are involved in the determination of fiber type. In particular the Ca^{2+} -calmodulin complex is known to activate numerous downstream signaling kinases and phosphatases like CaMK and calcineurin (Cn). Calcineurin has a pivotal role in the the regulation of the slow gene program. Indeed, the overexpression of an activated form of Cn leads to small increase in slow fibers, while the genetic ablation of CnB1 in skeletal muscle prevents a fast-to-slow fibertype transformation during overload hypertrophy (Chin et al, 1998) (Parsons et al, 2004). Activated Cn dephosphorylates many substrates, including the nuclear factor of activated T cells (NFAT) and the myocyte enhancer factor 2 (MEF2) transcription factors (117). When NFAT is dephosphorylated by calcineurin it translocates into the nucleus an induces a slow gene program while inhibiting the fast program. Translocation of NFAT into the nucleus is a very rapid event (20-30min) but before it requires more time before it can become trascriptionally active. Indeed, in adult skeletal muscle fibers NFATc1 co-localizes with nuclear heterochromatin, suggesting other posttranslational modifications are needed. Interestingly, phosphorylation of ERK1/2, which is known to occur during muscle contraction (Raney & Turcotte, 2008) leads to an increased phosphorylation of the transcriptional coactivator p300 and the subsequent acetylation of NFATc1 which in turn stimulates its transcriptional activity (Meissner et al, 2011). Another important calcium-responsive transcription factor is Myocyte Enancher Factor 2 (MEF2) which together with various cofactors plays am important role in the regulation of fiber type. Its activity in muscle depends on the activation of CaMKII that leads to phosphorylation and nuclear exclusion of HDAC4, which relieves repression of MEF2 (Liu et al, 2005). This model couples contraction-induced calcium signaling to an increased rate of transcription of MEF2 target genes such as PGC-1α1 and GLUT4 (Chin, 2010).

1.3.4 Mechanical stress

Cytokines, growth factors and mechanical stress are all factors that have been

shown to trigger mitogen-activated protein kinase (MAPK) activation. The MAPK family of proteins is composed of four distinct signaling modules in skeletal muscle:

1) extracellular signal regulated kinases (ERK) 1 and 2 (ERK1/2)
2) p38 MAPK
3) c-Jun NH2-terminal kinases (JNK)
4) ERK5 or big MAPK

MAPKs contribute to the adaptive changes observed in skeletal muscle in response to exercise training. Indeed, after exercise three main cascades of MAPKs are activated: ERK1/2, p38, and JNK. The activation of these distinct MAPK pathway depend on type, duration and intensity of the exercise. MAPKs regulate transcriptional events by phosphorylation of diverse substrates localized in the cytoplasm or nucleus, including transcription factors and coactivators, and thereby regulate a variety of physiological processes, such as differentiation, hypertrophy, inflammation, and gene expression. Transcription factors such as Elk1, ATF2, AP1, CREB and MEF2C have been reported to be downstream of MAPKs (Widmann et al, 1999) (Zetser et al, 1999). For example, it has been reported that during contraction, p38 MAPK stimulates ATF2 and MEF2 leading to an increase in PGC-1α1 expression. Skeletal muscle-specific expression of a constitutively active activator of p38, resulted in enhanced PGC-1α1 and cytochrome oxidase IV protein expression suggesting that contractile activity-induced activation of the p38 MAPK pathway promotes mitochondrial adaptation in skeletal muscle. The mechanical and energetic perturbations associated with exercise result in immediate increases in ROS in skeletal muscle. Alterations in free radical concentrations are rapidly sensed, neutralized, and accompanied by adaptive increases in oxidant buffering capacity through upregulation of key antioxidant enzymes. MAPK activity may partially mediate this response as it has been shown that ROS activate MAPK signaling and the transcription factor nuclear factor-kB (Powers et al, 2010). Acute exercise activates JNK signaling in a ROS-dependent manner, as evidenced by attenuated JNK signaling during exercise with infusion of the antioxidant N-acetylcysteine (Petersen et al, 2012). JNk signaling also has a role in the control of IL-6 expression, which is an exercise-associated cytokine which has various metabolic effects. MAPK can also

modulate gene expression through chromatin remodelling. Histone phosphorylation in the nucleus can modify chromatin structure and is, therefore, an alternate means of transcriptional regulation. It has shown that after intense exercise in human there is a rapid serine phosphorylation of histone H3 by the activation of the MAPK pathway. Although not a direct substrate of MAPK, histone H3 is a downstream target of p90 ribosomal S6 kinase (He et al, 2003) and mitogen- and stress-activated kinase 1 (Kim et al, 2008), which are both activated by exercise (Long et al, 2004).

1.3.5 Redox balance

NAD+ is an electron carrier that plays a critical role in fuel metabolism. NAD+ couples ATP synthesis with the electron transport chain, through its reduction during glycolysis and reoxidation by lactate generation in the cytosol or mitochondrial shuttle activity, as well as reduction reactions of the TCA cycle. Of interest, the regulation of the sirtuin (SIRT) family of protein deacetylases is NAD+ dependent (Schwer & Verdin, 2008). The deacetylase activity of SIRT1 (cytoplasmic, nuclear) and SIRT3 (mitochondrial) is sensitive to elevations in [NAD+] and NAD+/NADH ratio. The subsequent deacetylation of lysine residues on transcriptional regulators (Nemoto et al, 2005) and mitochondrial enzymes (Hirschey et al, 2010) allows the coupling of alterations in the cellular redox state to the adaptive changes in gene expression and cellular metabolism (Lagouge et al, 2006) (Canto et al, 2009). Enhanced SIRT activity is associated with favorable adaptations in skeletal muscle metabolism, including enhanced mitochondrial function, exercise performance, and protection against obesogenic feeding (Lagouge et al, 2006) (Gerhart-Hines et al, 2007). Dynamic fluctuations in the NAD+/NADH ratio occur in response to stimuli such as acute exercise and fasting (Canto et al, 2009; Sahlin et al, 1987). With increasing exercise intensity, the cytosolic NAD+/NADH ratio declines as the lactate/pyruvate ratio increases (Sahlin et al, 1987) (Green et al, 1992). In rodents, elevations in SIRT1 activity peak around 3 hr into recovery (Canto et al, 2009), which coincides with a dramatic elevation in the NAD+/NADH ratio, predominantly due to an increase in [NAD+]. The aforementioned glucose sparing during postexercise recovery

coincides with an increase in b-oxidation and reoxidation of NADH to NAD+. SIRT activation is therefore in contrast to phosphorylation of various protein kinases that tend to be elevated immediately after exercise, and return rapidly to basal values. Conversely, short-term interval training increases SIRT1 expression in skeletal muscle (Little et al, 2010), while higher SIRT3 protein is reported in glucose transport (Merrill et al, 1997) and lipid metabolism (Winder & Hardie, 1996). Chronic AMPK activation alters metabolic gene expression and induces mitochondrial biogenesis (Bergeron et al, 2001), partly via AMPK-induced modulation of the DNA binding activity of transcription factors including NRF-1, MEF2, and HDACs (Bergeron et al, 2001) (McGee et al, 2009).

1.4 Molecular regulation of hypertrophy

1.4.1 Regulation of protein synthesis

Adult skeletal muscle mass is determined by a balance between protein synthesis and protein breakdown. In catabolic conditions, like denervation, starvation, or cancer cachexia, protein degradation increases leading to muscle atrophy, while during anabolic conditions, like exercise or increased growth hormone activity, increased protein synthesis leads to muscle hypertrophy.

Resistance training is known to be a strong stimulus for inducing muscle hypertrophy and it is strongly associated with the activation of the $p70^{S6K}$ phosphorylation (Baar & Esser, 1999) (Terzis et al, 2008). The activation of p70S6K during muscle contraction is dependent to on the kinase mammalian target of rapamycin (mTOR). mTOR is able to sense and integrate nutritional and environmental cues, including growth factors, energy levels, cellular stress, and amino acids in order to regulate cell growth and proliferation. The control of mTOR on protein synthesis operates at different levels (e.g. translation capacity, translation efficiency) modulating the translation of specific mRNAs, leading to skeletal muscle fiber enlargement. mTOR exists in two structurally and functionally distinct multiprotein complexes. In the rapamycin-sensitivem TOR complex 1 (mTORC1) mTOR is bound to GbL (also called mLST8), regulatory-associated protein of mTOR (raptor), and proline-rich Akt substrate of 40 kDa (PRAS40). mTORC1 is responsible for the phosphorylation of its downstream

targets S6K1 and 4E-BP1, which are important in the initiation of mRNA translation. In contrast, mTOR complex 2 (mTORC2) is, when treated for short periods (Lamming et al, 2012), rapamycin insensitive and composed of mTOR bound to GbL, rapamycin-insensitive companion of mTOR (rictor), and mammalian stress-activated protein kinase-interacting protein 1. mTORC2 has been implicated in the activation of Akt via phosphorylation at serine Ser473 (Laplante & Sabatini, 2012). Mechanical stretching of isolated muscles is known to lead to the activation of mTORC1. The importance of the activation of mTOR in load-induced muscle growth is also demonstrated by the attenuation of hypertrophic responses and protein synthesis by using the mTOR inhibitor, rapamycin (Bodine et al, 2001). In the past it was establish as paradigm that resistance exercise-dependent hypertrophy was due to autocrine production of factors impinging on mTORC1 function. Indeed it's known that exercise increases the release of insulin-like growth factor (IGF) and consequent activation of a signaling cascade through phosphatidylinositol 3-kinases (PI3K)-Akt-mTOR by IGF-I interaction with insulin and IGF receptors. Recently, instead, we observed a paradigm shift. It is now believed that, for both human and rodents, the hypertrophic response to resistance exercise occurs in a growth factor-independent manner through mechanosensory regulation.

1.4.2 Additional signaling pathways controlling muscle growth:Serum Response Factors (SRF)

An another important signaling pathway known to control skeletal muscle growth and potentially activated by mechanical overload, is the Serum Response Factor (SRF) signaling pathway. SRF is an ubiquitously expressed member of the MADS (MCM1, Agamous, Deficiens, SRF) box transcription factor family, sharing a highly conserved DNA-binding/dimerization domain, which binds the core sequence of SRE/CArG boxes [CC (A/T)6 GG] as homodimers (Treisman, 1987). SRF is required for the serum induction of immediate early genes such as c-fos and for the expression of many muscle specific genes. Indeed inductional CArG boxes have been found in the cis-regulatory regions of α-actin, muscle creatine kinase, dystrophin, tropomyosin, and myosin light chain genes. SRF

regulates different physiological processes, including cell cycle regulation, apoptosis, cell growth, cell differentiation and cytoskeletal organization (Pipes et al, 2006). Myocardin and the myocardin-related transcription factors (MRTFs) MRTF-A (MAL/MKL1) and MRTF-B (MKL2) comprise a family of transcriptional coactivators have been established as strong coactivators of SRF and as critical components for immediate early and muscle specific gene expression. Data obtained from mouse models with skeletal muscle-specific loss of Srf or MRTFs emphasize their crucial role in postnatal muscle growth (Charvet et al, 2006) (Li et al, 2005). In adults mices, SRF activity could also be important for the control of skeletal muscle growth, as suggested by increased SRF expression during overload-induced hypertrophy (Fluck et al, 1999). In cardiac muscle, SRF and MRTF-A are necessary in mediating cardiomyocyte hypertrophy (Kuwahara et al, 2010) (Parlakian et al, 2005). Using an inducible, muscle-specific knockout model, SRF was also found to be required for muscle hypertrophy. In this model, the effect of SRF is apparently mediated via release of interleukins 4 and 6 (IL-4 and IL-6), which act in a paracrine manner to induce satellite cell proliferationand fusion. Akt phosphorylation is apparently unchanged in control and SRF mutant muscles at various time points after synergist ablation. However, a previous study reported that SRF is able to activate the Akt pathway via a muscle-enriched microRNA, miR-486, that targets the phosphatase and tensin homolog PTEN, which negatively affects PI3K–Akt signaling (Small et al, 2010). In addition, as activation of mTOR and its downstream effectors were not examined in theprevious study (Guerci et al, 2012), it is possible that mTOR activity and protein synthesis are increased by SRF in muscles undergoing overload-induced hypertrophy, either via a transcriptional mechanism mediated by SRF itself or as a secondary consequence of incorporation of new myonuclei resulting from satellite cell activation and fusion. Taken together, these data point toward SRF as a good candidate transcription factor in the control of skeletal muscle mass during hypertrophy. Since SRF activity does not appear to be regulated by its direct modification, these coactivators provide a novel point for regulation of SRF target genes

1.4.3 Role of skeletal muscle satellite cells in adaptive hypertrophy

Skeletal muscles are complex structures composed of gigantic multinucleated cells and associated stem cells, satellite cells. Skeletal muscle satellite cells are quiescent cells located adjacent to muscle fibers and under the fiber basal lamina, which can be induced to divide during conditions of muscle damage. The role of satellite cell during regeneration is well established, but its role during muscle plasticity in adult skeletal muscle is debated. Recently, it has been shown that activation of satalite cells occurs also without muscle damage in response to different simuli, including exercise. After activation, satellite cells proliferate and fuse with existing fibers resulting in the addition of a myonucleus to the fiber syncytium. The proposed role of the satellite cells in muscle hypertrophy revolves around the concept of a myonuclear domain—a theoretical volume of cytoplasm associated with a single myonucleus—and each myofiber being composed of many myonuclear domains (Allen et al, 1999). For many years, it has been suggested that satellite cells provide a source for new myonuclei at a rate sufficient to maintain the myonuclear domain constant during muscle growth. Recently, different groups have tried to clarify the role of satellite cells in adult muscle homeostasis using different experimental models. Indeed, different studies in which there is an ablation of the satellite cells or a model in which myostiatin was inhibited, describe that muscle hypertrophy occurs without satellite cell incorporation (Wang & McPherron, 2012). For example, using a transgenic model in which there is depletion in 90% of satellite cells, overload- induced hypertrophy is normal, whereas regeneration from acute muscle injury is impaired. These results indicate a distinct requirement for satellite cells during these two different processes and also that skeletal muscle fibers are able to responde to overload with an significant hypertrophy that is independent of satellite cells activation. In contrast to these results, the human skeletal muscle hypertrophic response to resistance training has shown a strong interindividual variability that can explained with a different capacity to mobilize the satellite cells and add myonuclei to existing muscle fibers (Petrella et al, 2006) (Petrella et al, 2008). In human skeletal muscle, large ranges of interindividual variability in the magnitude of hypertrophic response to resistance exercise training are

explained by the relative ability to expand the SC pool, incorporate new nuclei, and achieve robust growth (Petrella et al, 2006) (Petrella et al, 2008) . In conclusion, satellite cells are clearly required for adult skeletal muscle regeneration but whether myonuclear addition is required during skeletal muscle hypertrophy is less clear.

1.5 Epigenetic control of gene expression

In eukaryotic cells, DNA is packaged into chromatin, and covalent modifications on the histone proteins of the chromatin, and modifications on the DNA itself can influence the expression level of genes. Epigenetics is defined as the study of mitotically and/or meiotically heritable changes in gene expression which occurs without modifications of the DNA sequence. In mammals the genome-wide patterns of DNA and chromatin modifications ('epigenomes') do not persist throughout life, but undergo precise, coordinated changes at defined stages of development. Indeed, even if the genetic code is the same for all cells in the organism, each cell has its own gene expression pattern, dictate by a specific epigenetic signature. Many epigenetic modifications become stabilized during development and present a pattern of epigenetic inheritance. However, a very fascinating point is the role of the environment in changing some specific epigenetic marks leading to the modification of the phenotype without impinging on the genetic sequence. Two major epigenetic mechanisms are DNA methylation and histone modifications.

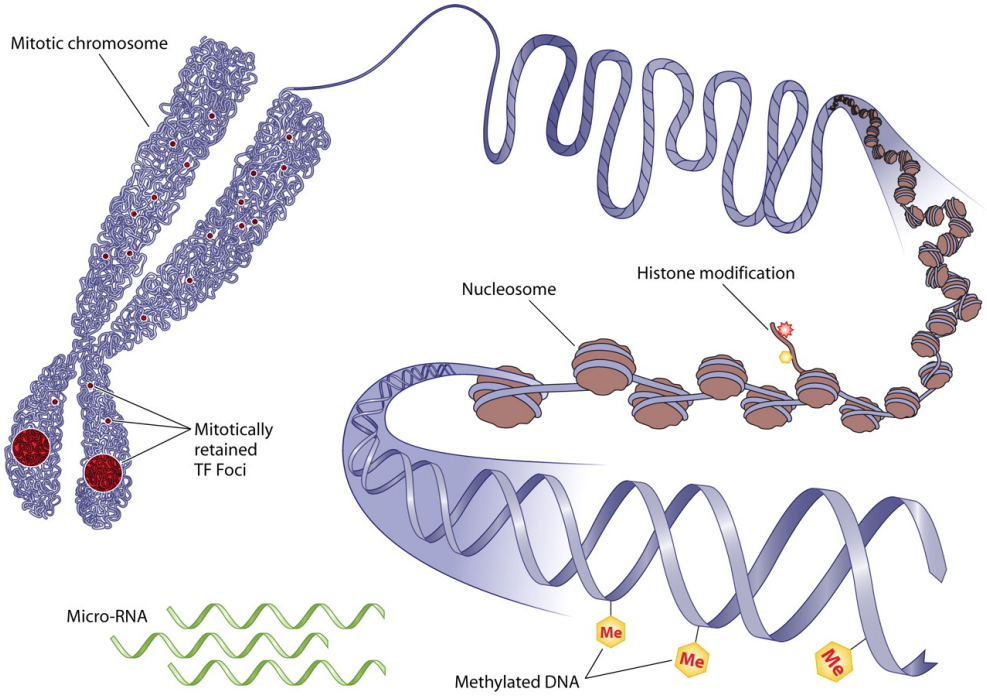


Fig.2 Epigenetic modification of DNA

Epigenetics is defined as the study of mitotically and/or meiotically heritable changes in gene expression which occurs without modifications of the DNA sequence. Two major epigenetic mechanisms are DNA methylation and histone modifications.

1.5.1 DNA methylation

DNA methylation is an epigenetic mechanism that occurs by the addition of a methyl (CH_3) group to DNA, thereby often modifying the function of the genes. The most widely characterized DNA methylation process is the covalent addition of the methyl group at the 5-carbon of the cytosine ring. In human DNA, 5-methylcytosine (5-mc) is found in approximately 5% of genomic DNA under physiological conditions (Lister et al, 2009). In somatic cells, cytosine methylation occurs almost exclusively in the sequence context of CpG dinucleotides, in which a cytosine nucleotide is located next to a guanidine nucleotide. An exception to this is seen in embryonic stem (ES) cells, where a substantial amount of 5-mC is also observed in non-CpG contexts. Generally, DNA methylation has been linked to repress gene expression. Therefore in

telomeres, centromeres, and inactive X-chromosomes high levels of DNA methylation are found. Methylation of the DNA is carried out by family of enzymes called DNA methyltransferases (DNMTs). In particular the establishment and maintenance of DNA methylation patterns occurs by three DNMTs (DNMT1, DNMT3a and DNMT3b). Dysfunction in the DNMTs has been associated with embryonic lethality suggesting an important role in cell development and differentiation. Similarly, changes in DNA methylation is associated with cancer development. Equally important in epigenetic reprogramming of genes and also directly involved in many important disease mechanisms, is DNA demethylation. Recently, TET enzymes were shown to play an important role in this process (He et al, 2011) (Ito et al, 2011). TET enzymes belong to the family of a-ketoglutarate-dependent oxygenases and oxidize 5-methylcytosine to 5-hydroxymethylcytosine. DNA methylation can occur at different position, including the promoter region and within the gene body introns or exons, but also in intergenic and non-translated regions. DNA methylation leads to gene silencing at the promoter of the genes, because the presence of methyl groups allosterically inhibits the binding of transcription factors or transcriptional enhancers (Nguyen et al, 2001). Conversely, the role of intragenic DNA methylation, compared with gene promoter methylation, is not fully understood and it seems to enhance the gene activation. For a long time different studies have addressed the role of DNA methylation in muscle development without considering that it could have a role in adult muscle plasticity. This view, however, has recently been challenged. Differential DNA methylation correlates with expression of angiogenic factor genes, mainly PECAM1, ARHGAP24 and AMOTL2. Moreover, DNA methylation patterns differ in promoters and gene bodies from normal individuals and patients with end-stage heart failure (Movassagh et al, 2010). In human adult skeletal muscle it was shown that acute exercise induces a significant decreased in genome methylation. This change in Dna methylation correlates with and acute gene activation of different genes such as PGC-1a, PDK4, and PPAR-d, suggesting that DNA hypomethylation is an early event in contraction-induced gene activation (Barres et al, 2012).

1.5.2 Posttranslational histone modifications

1.5.2.1 Histone structure and function

DNA is organized into chromatin, the proper function and maintenance of which is crucial for cell identity and survival. Histones are essential proteins of chromatin and facilitate the packaging of DNA into nucleosomes (Luger et al, 1997). Histone proteins can be subdivided into five major classes in humans (H1, H2A, H2B, H3, and H4), all of which possess tails that are subjected to a large variety of reversible covalent posttranslational modifications (PTMs) such as acetylation, methylation, phosphorylation, ADP-ribosylation, and ubiquitination (Li et al, 2007). PTMs of histones can not only alter the accessibility of the DNA that is wrapped around the histones but also affect the binding of a variety of effector proteins that mediate further biological events. These modifications can occur at various aminoacids and the effect on transcription are different and depend on the kind of modification (Koch et al, 2007). Therefore, histone modification can act either by silencing or activating gene transcription. All these changes create what is referred to as the histone code (Strahl & Allis, 2000). Chromatin modifications allow for the maintenance of established patterns of PTMs through cell divisions, enabling the inheritance of specific patterns of gene expression acquired during cell lineage specification and, thus, maintaining cellular identity (Schreiber & Bernstein, 2002). Nonetheless, some histone modifications are only transient to temporary modify the chromatin structure as intermediate stage of a multiphase process or as a consequence of adaptation to changes in extracellular microenvironment (Lee & Mahadevan, 2009).

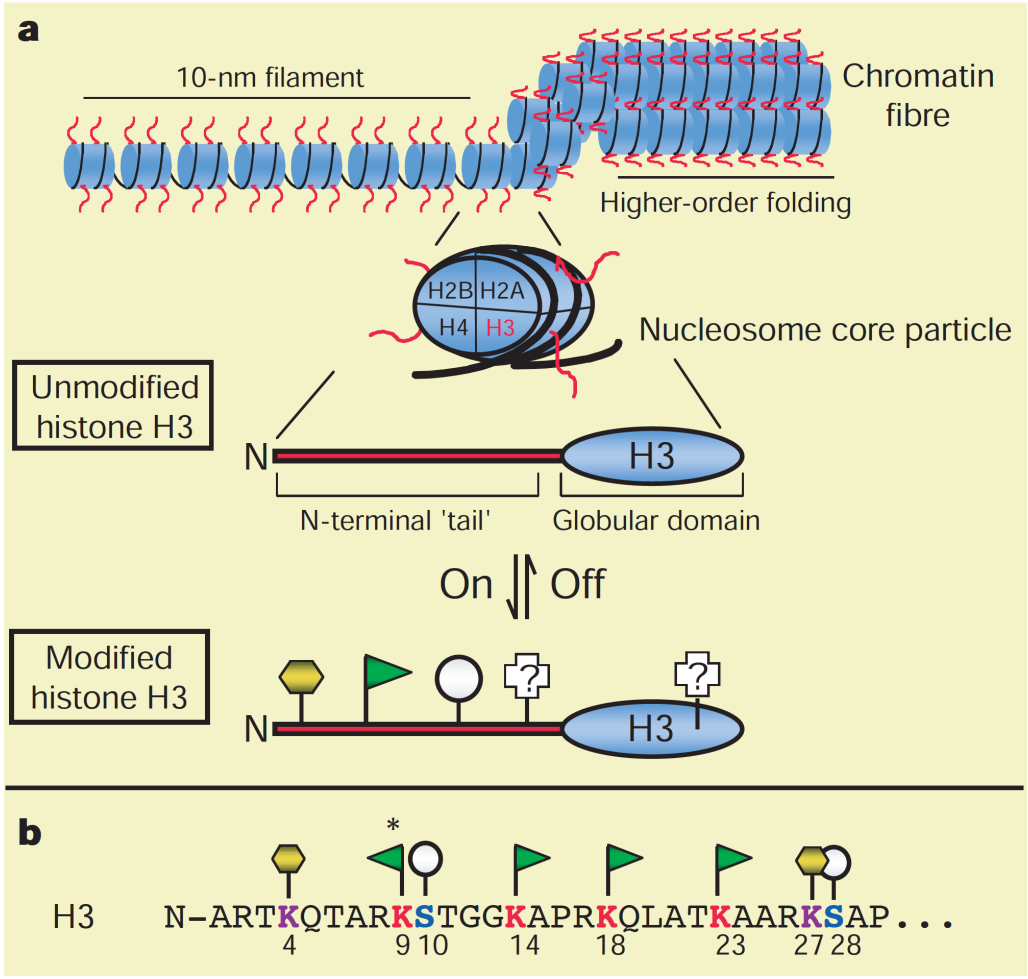


Fig.3 Chromatin organization and the tail of histone H3.

A.*General chromatin organization. Like other histone `tails', the N terminus of H3 (red) represents a highly conserved domain that is likely to be exposed or extend outwards from the chromatin. A number of distinct post-translational modications are known to occur at the N terminus of H3 including acetylation (green flag), phosphorylation (grey circle) and methylation (yellow hexagon). Other modifications are known and may also occur in the globular domain.* ***B***. *The N terminus of H3 is shown in single-letter amino-acid code.*

(Image taken from "The language of covalent histone modifications" (Strahl & Allis, 2000)).

1.5.2.2 Histone modifications

Growing evidence indicates post-translational modifications on histone tails play

an important role in orchestrating DNA transcription. Whether the modification results in transcriptional activation or inactivation depends on the type of modification. Moreover, each modification can influence another amino acid, either on the same histone tail, or even across different histones leading to an exponential increase in the complexity of the code. For example, methylation at lysine 4 of class H3 histones is associated with transcriptional activation (McManus & Hendzel, 2006), whereas methylation at lysine 36 of the same histone class results in transcriptional repression. Conversely, acetylation of histone lysines, due to the reduction of the positive charge of lysine, leads to a reduction in the histone-DNA contact and therefore a more accessible chromatin, correlated to gene transcription activation (Li et al, 2007). Histone lysine acetylation is highly dynamic and regulated by two opposing enzyme families. Histone lysine acetyltransferases (KATs) modify histones by adding acetyl residues, whereas histone deacetylases (HDACs) remove acetyl groups from lysine residues. Recent evidence suggests that HDACs play a crucial role in skeletal muscle physiology and may contribute to the development of metabolic diseases during physical inactivity (McGee et al, 2006). Histone modification and DNA methylation often function synergistically, as demonstrated by the acute effect of exercise-induced myocyte enhancer factor-2 (MEF2) activation (McGee et al, 2006; Yu et al, 2001).

1.5.2.3 Histone H3 phosphorylation

The N-terminal tails of the four core histones emanate in all directions from the nucleosome, with the H3 Nterminal tail being the longest (Richmond et al, 1984) . The length of the H3 tails and their points of exit from the nucleosome positioned to make extensive interactions with the linker DNA are key features enabling the H3 tails to contribute to the three-dimensional structure of chromatin (Zlatanova et al, 1998). The N-terminal tail of histone H3, present several position-specific modifications that are associated with distinct chromatin-based outputs, such as transcriptional regulation (Lys9/Lys14 acetylation, Ser10 phosphorylation), transcriptional silencing (Lys9 methylation), histone deposition (Lys9 acetylation), and chromosome condensation/segregation (Ser10/ Ser28

phosphorylation) (Jenuwein & Allis, 2001). Among these modifications, phosphorylation on serine 10 of histone H3 (H3S10ph) offers the best example of a direct link between signal transduction and histone modification (Cheung et al, 2000a). Early studies in PC12 cells treated with epidermal growth factor (EGF) provided the first indication that H3 could be a substrate for a signalling kinase (Halegoua & Patrick, 1980). Further evidence supporting this hypothesis arose from identification of the nucleosomal response. H3S10ph has been linked with transcriptional activation after mitogen or stress stimuli. Mahadevan and colleagues showed that after treatment of fibroblast with various growth factors or protein synthesis inhibitors there is a rapid and transient phosphorylation of histone H3 (Mahadevan et al, 1991), which has been associated with the transient expression of immediate-early genes (IEGs) (Cheung et al, 2000b; Nowak & Corces, 2000). Next, studies using the kinase inhibitor H89 demonstrated that the nucleosomal response is mediated, depending on the stimulus, either via the extracellular signal e regulated kinase (ERK) or p38 MAP kinase cascades, but not the c-Jun amino-terminal kinase/stress-activated kinase (JNK/ SAPK) pathway (Thomson et al, 1999). H3S10ph has an interesting feature that distinguishes it from all other PTMs is that it regulates two opposite processes. Indeed, it is observed in the metaphase as a hallmark for condensated chromatin during mitosis. In mammals, all H3 molecules are phosphorylated during mitosis by a kinase of the Aurora family (Crosio et al, 2002). By contrast, during interphase, mitogenic signals induce a rapid, transient phosphorylation of only a small subset of H3 molecules at the same Ser10 residue (Sassone-Corsi et al, 1999).

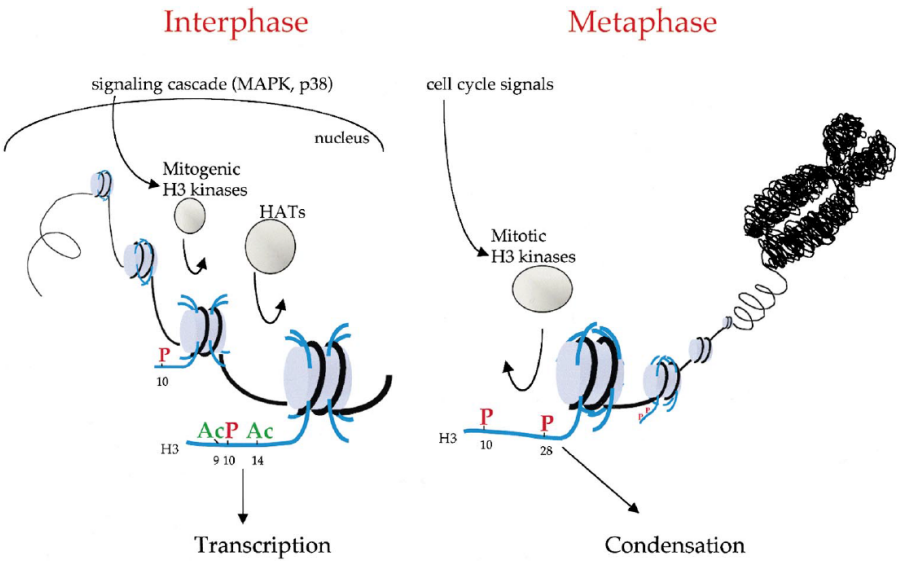


Fig.4 Duality of Histone H3 Phosphorylation in Interphase and Metaphase Cells

H3 regulates two apparently opposite processes: condensation of mitotic chromosomes associated with transcriptionally repressive state and signal induced gene activation during interphase.

(Image taken from, Signaling to Chromatin through Review Histon Modifications, (Cheung et al, 2000a)).

1.5.2.3.1 Effector kinases of Histone H3

Considering that the motif containing H3S10ph does not look like the consensus sequence for ERK and p38, the putative candidate for targeting histone H3 are the effector kinases downstream of MAPK activation. Thanks to their nuclear localization upon signalling and their ability to phosphorylate histone H3 peptide in vitro, the strongest candidates are mitogen- and stress-activated kinase 1 (MSK1) and ribosomal subunit protein S6 kinase 2 (RSK2). In particular *in vitro* kinase assay showed 4-fold higher efficiency of MSK1 to phosphorylate H3 compare to either RSK1 or RSK2. Moreover MSK1 but not RSK2 was sensitive to H89 in concentration that inhibited nucleosomal response in vivo, leading to the conclusion that MSK1 is potential kinase implicated in phosphorylating histone H3 (Thomson et al, 1999). To better understand which is the mitogen-

stimulated H3 kinase, Soloaga et al. performed an analyses (Sassone-Corsi et al, 1999) in fibroblasts from Coffin-Lowry syndrome (CLS), a disease associated with mutations in RSK2 gene. In contrast to the previous study, they found that after cellular stress or mitogenic stimulation in CLS, H3 phosphorylation was not affected. Indeed, after the analyses of mouse embryonic fibroblasts (MEFs) lacking MSK1, MSK2, or both enzymes, the authors found an impaired H3 phosphorylation upon anisomycin and 12-O tetradecanoylphorbol-13-acetate (TPA) stimulation, accompanied by reduced activation of immediate early genes (Soloaga et al, 2003). Interestingly, MSK1 deficient fibroblasts shown a slight reduction on H3S10ph phosphorylation after anisomycin stimulation, but this response was completely diminished in MSK2 and MSK1/2 double-knockout fibroblasts, suggesting that MSK2 was the required H3 kinase in response to stress inducer. Although MSK1/2 are the best characterised, they are not the sole kinases involved in H3S10ph phosphorylation. It was shown that IKK-α mediates the cytokine-induced H3S10 phosphorylation at the promoter of NF-kβ responsive genes in mouse fibroblast (Yamamoto et al, 2003), showing a potential role for histone phosphorylation in stimulating gene transcription in the immune system. Surprisingly, the common environmental carcinogen arsenite induces H3S10ph in JB6 Cl 41 cells mediated by Akt1 (He et al, 2003). Moreovere, it was been shown that PIM1 kinase induces H3S10ph at MYC-target genes and therby contributing to their transcriptional activation and inducing cellular transformation (Zippo et al, 2007).

	Substrates Modified		
Kinases that Modify Histones and/or Histone Modifiers	Histones	Transcription Factors and Histone Modifiers	Functions/Downstream Consequences
PIM1	H3S10	p21-T145	Txn Activation (H)/Apoptosis (TF)
IKKα	H3S10	CBP-S1382	Txn Activation (H/TF)
Rsk2	H3S10	IκBα-S32, p53-S15	Txn Activation (H/TF)
PKB/Akt	H3S10	EZH2-S21, p300-S1834	Txn Activation (H/TF)
Aurora B	H3S10, H3S28	MYBBP1A-S1303	Chromatin Condensation (H/TF)
MSK1/2	H3S10, H3S28	CREB-S133, p65-S276, STAT3-S727	Txn Activation (H/TF)

Fig.5 Regulators of histone H3 phosphorylation

1.5.2.3.2 Function of histone H3 phosphorylation

The mechanism by which histone phosphorylation contributes to transcriptional activation involves the generation of negatively charged phosphate groups that neutralize basic charges on the histone tails. Strikingly, histone H3 phosphorylation was demonstrated to accompany ERK/MAPK pathway mediated activation of c-fos by agonists of dopamine (DA), muscarinic acetylcholine (mACh) and glutamate (GLU) receptor in hippocampal neurons, suggesting an important role of nucleosomal response in memory formation (Crosio et al, 2003) Supporting this hypothesis, MSK1 deficient mice, despite no apparent changes in overall brain morphology, displayed numerous behavioral defects, including impaired Pavlovian fear conditioning and spatial learning, what was attributed to the failure in inducing histone H3 and c-AMP response-element-binding protein (CREB) phosphorylation downstream ERK/MAPK pathway in hippocampal neurons (Chwang et al, 2007; Heffron & Mandell, 2005) . In addition, mice lacking MSK1 showed decreased locomotor sensitization in response to repeated cocaine administration due to the failure to induce c-fos and dynorphin in their striatal neurons [54]. In conclusion, MSK1 emerges as a crucial regulator of neuronal functions upon stimulation with extracellular signals as its deficiency results in severe behavioral abnormalities. It remains a major challenge to determine to which extent histone H3 phosphorylation contributes to this regulation. Recently, a specific nuclear isoform of calcium-dependent protein kinase II (CaMKIIδB) was shown to bind and phosphorylate histone H3 at serine-10. Increased H3 phosphorylation is detected in ventricular myocytes during cardiac hypertrophy and at hypertrophic gene loci. This phosphorylation event increases chromatin accessibility and is required for chromatin-mediated transcription of the MEF2 transcription factor (Awad et al, 2015).

1.5.2.3.3 Additional histone H3 modifications

In addition to Ser10, Ser28 can be targeted by MSKs (Zhong et al, 2001). Indeed fibroblast derived by MSK1/MSK2 deficient mice showed an impaired phosphorylation on serine 28, even though less than the reduction seen in H3S10 phosphorylation. H3Ser28 phosphorylation is also associated with IE gene

induction. An important characteristic of histone modification is that they can occur in concert on the same histone tail or on tails of different histones. All these possible combination of histone modification indicate the presence of an histone code, in which different combinations or modifications can correspond to a specific output of chromatin remodeling. Allis and colleague poposed the idea that one histone modification may prime the histone for a secondary modification. (Jenuwein & Allis, 2001) (Strahl & Allis, 2000). In particular, it has been shown in different studies that H3S10 phosphorylation preceded, and facilitates Lys14 Acetylation by the recruitment of histone acetyltransferase (HATs). H3S10 phosphorylation can bind 14-3-3 proteins which are stabilized by the additional acetylation on Lys14, and this interaction protects the phosphorylation mark from PP2A phosphatase, that was shown to dephosphorylate H3S10ph.

1.6 AIM

The aim of our study is to dissect the early signaling events linked to skeletal muscle remodeling. For this purpose we have divided the project in two parts. The first one focusing on changes in the phosphoproteome after exercise and how this can affect gene transcription. In the second part we analyze how one bout of exercise can influence histone modification and modify the epigenetic landscape.

2. MATERIALS AND METHODS

2.1 Electrical stimulation

The animals were anesthetized by an intraperitoneal injection of xylazine (Xilor) (20 mg/Kg) and Zoletil (10 mg/Kg). Muscle lengthening was achieved by moving the foot backward at a velocity of 40 mm/s while the tibialis anterior (TA) was stimulated with a frequency sufficient to induce full tetanic fusion (100 Hz). The footplate was moved 200 ms after initiation of stimulation train, thus eccentric pull occurs during the isometric plateau of the tetanus. The range of movement during the pull was calculated to be 308, clearly inside physiological limits of movement for the foot. Total duration of tetanic stimulation was limited to 600 ms, assuring no sag of force. This protocol was repeated 50 times.

2.2 Exercise protocol

For acute exercise, 12-week-old wild type mice (C57BL/6) were subjected to eccentric training protocol consisting of 3 days running to exhaustion, with a 10 degree decline, at increasing velocity, according to the protocol of exercise previously described (He et al., 2012). Briefly, exercise consists in 17 cm/sec for 40 minutes, 18 cm/sec for 10 min, 20 cm/sec for 10 min, 22 cm/sec for 10 min, and then increasing velocity of 1cm/sec and or 2 cm/sec alternatively every 5 minutes, until they were exhausted. Exhaustion was defined as the point at which mice spent more than 5 s on the electric shocker without attempting to resume running.

2.3 AICAR treatment

In order to investigate the effects of metabolic stress on histone modifications we performed a single IP injections of AICAR (Toronto Research Chemicals Inc.) The AICAR-treated group was injected with AICAR (0.65 mg/g body wt) and the

control groups were injected with an equal volume of vehicle (saline). AICAR was always injected at 9:00 am and 30 minutes after injection animals were sacrified for muscle extraction by cervical dislocation.

2.4 In vivo skeletal muscle electroporation

Experiments were performed 12-week-old wild type mice (C57BL/6) tibialis anterior (TA). The animals were anesthetized by an intraperitoneal injection of xylazine (Xilor) (20 mg/Kg) and Zoletil (10 mg/Kg). Tibialis anterior (TA) muscle was isolated through a small hindlimb incision, and DNA was injected along the muscle length. Electric pulses were then applied by two stainless steel spatula electrodes placed on each side of the isolated muscle belly (50 Volts/cm, 5 pulses, 200 ms intervals). Muscles were analyzed 14 days later. No evidence of necrosis or inflammation as a result of the transfection procedure were observed (Sandri et al., 2004 and Donà et al., 2003).

Tibialis Anterior muscle were transfected with different plasmids:

- MRTF-B-GFP
- MRTF-B-(S66A)GFP
- Histone 2B-RFP
- pZacf-U6-luc-ZsGreen-MSK1
- shLuc pRRL

2.5 Cryosections

Muscles were collected and then immediately frozen in precooled isopentane in liquid nitrogen. Samples were stored at −80ºC until the time of sectioning. Immediately prior to sectioning, muscles were equilibrated to −20ºC, and cut in the cryostat. Serial sections of 8 to 10um thickness were cut and attached to positively charged slides (SuperFrost Plus, ThermoFisher). Slides were freshly prepared before use and not stored at −20ºC.

2.6 Immunofluorescence analyses

2.6.1 MRTF-B-GFP staining

Cryosections were fixed with PFA 4%, treated with 0,1% Triton for 5 minutes and incubated in blocking solution (0.5% BSA, 10% mouse serum in PBS) at RT for 30 minutes. After that samples were incubated with the primary antibody anti-GFP (, Molecular Probes,final dilution 1:200) at 4°C over-night. Sections were then washed with PBS three times for 5 minutes and incubated with the Alexa-Fluor 488 conjugated AffiniPure Goat Anti-Rabbit IgG secondary antibodies (dilution 1:200) at 37°C for 1hour (Jackson). After the washes and incubation with DAPI, slides were mounted with mounting medium (Dako).

2.6.2 Phospho-Histone H3 (Ser10) – Dystrofin staining

Cryosections were fixed with PFA 4% for 10 minutes, treated with 0,1% Triton for 10 minutes and incubated with M.O.M blocking reagent (Vector laboratories) to avoid nonspecific binding of the at RT for 40 minutes. After that samples were incubated with the primary antibodies against Phospho-Histone H3 (Ser10)(Rabbit mAb, Cell Signaling; final dilution 1:1000) and Dystrofin (MANDRA1(7A10;DSHB), final dilution 1:300), at 4°C over-night. Sections were then washed with PBS three times for 5 minutes and incubated with the the Alexa-Fluor 594 conjugated AffiniPure Goat Anti-Rabbit IgG secondary antibodies (dilution 1:200) and Alexa-Fluor 488 conjugated AffiniPure Goat Anti-Mouse secondary antibodies (dilution 1:200) at 37°C for 1hour (Jackson). After the washes and incubation with DAPI, slides were mounted with mounting medium (Dako).

2.7 Imaging

Images were captured with an epifluorescence microscope coupled with a CCD camera. Different excitation wavelengths and filters were used depending on the fluorophore conjugated to the secondary antibody. The software used for the image acquisition was the Leica Application Suite (LAS).

2.8 Immunoblotting

Cryosections of 20 µm of TA muscles were lysed with 100 µl of a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl2, 0.5 mM DTT, 1 mM EDTA, 10% glycerol, 2% SDS, 1% Triton X-100, Roche Complete Protease Inhibitor Cocktail and Sigma Protease Inhibitor Cocktail. After incubation at 70°C for 10 minutes and centrifugation at 11000 g for 10 minutes at 4°C, the surnatant protein concentration was measured using BCA protein assay kit (Pierce) following the manufacturer's instructions.

2.8.1 Protein gel Electrophoresis

The extracted proteins from TA muscle were solubilized in Loading buffer composed by 5µl of 4X NuPAGE® LDS Sample Buffer (Life Technologies) and 1µl of 20X DTT (Life Technologies). The volume of each sample was brought to 20µl with TBS 1X. The samples were denaturated at 70°C for 10 minutes. Samples were loaded on SDS 4-12% precast polyacrylamide gels (NuPAGE Novex-Bis-tris-gels) or in SDS 3-8% depending on the protein to be analyzed (Life Technologies). The electrophoresis was run in 1X MES Running buffer or 1X Tris-Acetate Running buffer respectively (Life Technologies) for 1 hour and 30 minutes at 150V constant

2.8.2 Transfer of the proteins to the Nitrocellulose membrane

After the electrophoretic run, proteins were transferred from gels to nitrocellulose membranes. The gel and the membrane were equilibrated in Transfer Buffer. The Transfer Buffer was prepared as follows: 50 ml of 20X NuPAGE® Transfer buffer (Life Technologies), 1 ml of 10X NuPAGE® Antioxidant (Life Technologies), 200 ml of 20% Methanol (Sigma-Aldrich). The volume was brought to 1l with distilled water. The blotting was obtained by applying a current of 400mA for two hours at 4°C. To evaluate the efficiency of the transfer, proteins were stained with Red Ponceau 1x (Sigma-Aldrich). The staining was easily reversed by washing with distilled water.

2.8.3 Incubation of the membrane with antibodies

Once the proteins were transferred on nitrocellulose membranes, the membranes were saturated with Blocking Buffer (5% no fat milk powder solubilized in TBS 1X with 0.1% TWEEN) for 1 hour at room temperature and were incubated overnight with various primary antibodies at 4°C . Membranes were then washed 3 times with TBS 1X with 0.1% TWEEN at RT and incubated with secondary antibody-HRPConjugate (Bio-Rad). Secondary incubation was conducted at a dilution of 1:2000 in 5% (w/v) milk/TBST for 1h at room temperature (Anti-mouse, Biorad 170-6516; Anti-rabbit, Biorad 170-6516;). Immunoreaction was revealed by SuperSignal West Pico Chemiluminescent substrate (Pierce) and followed by exposure to Xray film (KODAK Sigma-Aldrich).

Antibody	Customer	Concentration	Incubation	Secondary Ab. HRP-Conjugated
Phospho-Histone H3 (Ser10)	Cell Signaling #3377	1:1000	O.N. 4°C	Anti-Rabbit
Acetyl-Histone H3 (Lys9/lys14)	Cell Signaling #9677	1:1000	O.N. 4°C	Anti-Rabbit
Anti-Histone H3 (tri methyl K4)	Abcam Ab71998	1:200	O.N. 4°C	Anti-Rabbit
Histone HJ3	Cell Signaling #9715	1:5000	O.N. 4°C	Anti-Rabbit
Phospho-p38 MAPK (Thr180 /Tyr182)	Cell Signaling #9211	1:1000	O.N. 4°C	Anti-Rabbit
p38 MAPK	Cell Signaling #9212	1:1000	O.N. 4°C	Anti-Rabbit
Phospho-MSK1	Cell Signaling #9595	1:1000	O.N. 4°C	Anti-Rabbit
MSK1	Cell Signaling	1:1000	O.N. 4°C	Anti-Rabbit
Phospho-AMPKα (Thr172)	Cell Signaling #2531	1:1000	O.N. 4°C	Anti-Rabbit
Phospho-Acetyl-	Cell Signaling	1:1000	O.N. 4°C	Anti-Rabbit

CoA Carboxylase (Ser79)	#3661			
Anti-c-myc	Roche 11667149001	1:1000	O.N. 4°C	Anti-Mouse
Anti -GFP	Life technologie A11122	1:1000	O.N. 4°C	Anti-Rabbit
GAPDH	Abcam-Aldrich AB8245	1:5000	O.N. 4°C	Anti-Mouse

Tab. 1: Antibodies used for western blot analyses.

All the peroxidase-conjugated secondary antibodies were from Bio-Rad. Blots were stripped using Stripping Solution (Restore PLUS Western Blot Stripping Buffer, Thermo Scientific).

2.9 Gene expression analysis

Quantitative Real-time PCR was performed with SYBR Green chemistry (Applied Biosystems). SYBR green is a fluorescent dye that intercalates into double-stranded DNA and produces a fluorescent signal. The Real-Time PCR Instrument allows real time detection of PCR products as they accumulate during PCR cycles and create an amplification plot, which is the plot of fluorescence signal versus cycle number. In the initial cycles of PCR, there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR products. A fixed fluorescence threshold can be set above the baseline. The parameter Ct (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. So the higher the initial amount of the sample, the sooner the accumulated product is detected in the PCR process as a significant increase in fluorescence, and the lower is the Ct value.

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

A relative quantification method were used to evaluate the differences in gene expression, as described by Pfaffl (Pfaffl, 2001). In this method, the expression of a gene is determined by the ratio between a test sample and a housekeeping gene. The relative expression ratio of a target gene is calculated based on the PCR efficiency (E) and the threshold cycle deviation (ΔCt) of unknown samples versus a control, and expressed in comparison to a reference gene.

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

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

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

2.9.2 Primer pairs design

Gene-specific primer pairs were selected with Primer Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer pairs were selected in a region close to the 3'-end of the transcript, and amplified fragments of 150-250bp in length. To avoid the amplification of contaminant genomic DNA, the target sequences were chosen on distinct exons, separated by a long (more than 1000bp) intron. The melting temperature was chosen to be of about 58-60° C. The sequences of the primer pairs are listed in Table 2.

qRT-PCR primer	Oligo sequence (5' – 3')
m-cFos	Fw: AAGGACTTGCAAGCATCCAC Rev: TCCAGCACCAGGTTAATTCC
m- Pgc-1α1	Fw: GGA ATG CAC CGT AAA TCT GC Rev: TTC TCA AGA GCA GCG AAA GC
m- Pgc-1α4	Fw: TCACACCAAACCCACAGAAA Rev: CTGGAAGATATGGCACAT
m-Pan-Actin	Fw: TGCACCACCAACTGCTTAGC Rev: GGCATGGACTGTGGTCATG
m-Gapdh	Fw: TCAGAACAAGGTGAACGCCCAGC Rev: GTGAGCGCAGATTCCGGGCA

Tab. 2: Sequence of primers used in q-RT-PCR analyses.

2.9.3 Extraction of total RNA

Total RNA was isolated from TA using Trizol (Life Technologies) following the manufacturer's instructions.

2.9.4 Synthesis of the first strand of cDNA

400ng of total RNA was reversly transcribed with SuperScriptTM III (Life Technologies) in the following reaction mix:
Random primer hexamers (50ng/µl random): 1µl
dNTPs 10 mM: 1µl
H2O Rnase-free: 8.5µl

The samples were mixed and briefly centrifuged and denaturised by incubation for 5 minutes at 65°C to prevent secondary structures of RNA.

Samples were incubated on ice for 2 minutes to allow the primers to align to the RNA, and the following components were added sequentially:
First strand buffer 5X (Life Technologies): 5µl
DTT 100mM: 2µl
RNase Out (Life Technologies): 1µl
SuperScriptTM III (Life Technologies): 0.5µl
The volume was adjusted to 20ul with RNase free water.
The used reaction program was:
step1: 25°C for 10 minutes
step2: 42°C for 50 minutes
step3: 70°C for 15 minutes

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

2.9.5 Real-time PCR reaction

1µl of diluted cDNAs was amplified in 10µl PCR reactions in a ABI Prism 7000 (Applied Biosystem) thermocycler, coupled with a ABI Prism 7000 Sequence Detection System (Applied Biosystems) in 96-wells plates (Micro Amp Optical, Applied Biosystems). In each well 5ul Sample mix and 5ul reaction mix were mixed.
Sample mix was prepared as follows for 5 µl total volume:
Template cDNA: 1 µl
H2O Rnase-free: 4 µl
The SYBR Green qPCR (Applied Biosystem) was used for the Real-Time PCR reaction as follows:
SYBR Green qPCR (Applied Biosystem): 4.8µl
Mix Primer forward /reverse 50mM: 0.2µl
The PCR cycle used for the Real-Time PCR was:
step 1: 95° C for 15 minutes
step2: 95° C for 25 seconds
step3: 58° C for 1 minute

step4: go to step 2 for 40 times

2.9.6 Microarray

For gene expression profiling, 250ng of RNA from tibialis anterior or was hybridized to Mouse Gene 1.0 ST Arrays (Affymetrix) Three biological replicates were used per group for each time point. Gene expression data was normalized and summarized with Robust Multichip Average (RMA) using custom Chip Description File (CDF) files (Version 14.1.0; EntrezG) to remap the probes on the arrays to recent genome and transcriptome library data (Dai et al., 2005). Differentially expressed genes were determined using a default R2 threshold of 0.6 and a cubic regression model in maSigPro (microarray Significant Profiles), a two−step regression−based method to identify genes with significant temporal expression changes and significant differences between experimental groups in time series microarray experiments (Conesa et al., 2006). Enrichment was assessed using Gene set enrichment analysis (GSEA) (Subramanian, Tamayo et al., 2005) using gene sets from the TRANSFAC and Biocarta databases. Using default settings, gene sets were called significant with a $p<0.05$ and False Discovery Rate (FDR) <0.25.

2.10 In vivo MSK1 RNAi

Oligos were cloned into the pSUPER vector (Brummelkamp et al., 2002). For validation of shRNA constructs, HEK cells were maintained in DMEM/10%FBS and transfected with shRNA constructs using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Cells were lysed 24 hours later, and immunoblotting was performed as described below. The sequences of the Oligos Used for siRNA production are listed in the Table 1:

shRNA oligos	Oligo sequence (5'-3')
MSK1-oligo 1	Fw:gatctccCCCAAGAAGCGATTGGGATttcaagagaATCCCAATCGCTTCTTGGGtttttggaaa Rev:agcttttccaaaaaCCCAAGAAGCGATTGGGATtctcttgaaATCCCAATCGCTTCTTGGGgga
MSK1-oligo 2	Fw:gatctccGGACAAACCTTTGGGAGAAttcaagagaTTCTCCCAAAGGTTTGTCCtttttggaaa Rev:agcttttccaaaaaGGACAAACCTTTGGGAGAAtctcttgaaTTCTCCCAAAGGTTTGTCCgga

Tab. 3: Sequence shRNA used for in vivo transfection

2.11 Chromatin immunoprecipitation (ChIP) assay

ChIP-qPCR of mouse skeletal muscle was performed with sonicated nuclear extract prepared from formaldehyde-crosslinked tissue according to Nelson et al (Nature Protocols, Vol.1 No.1; 2006) using anti-H3S10ph antibody (ChIPAb + Phospho-Histone H3 (Ser10) clone CMA312) or rabbit IgG (Santa Cruz 2027x). Immunoprecipitated DNA was decrosslinked, purified and used for quantitative real-time PCR.

2.12 Exercise protocol human

All exercise-habituation sessions and the single-bout exercise session conducted on the single-bout trial day were conducted in an isokinetic dynamometer (Humac Norm, CSMiMedical Solutions, Stoughton, MA,USA), as six sets of 10 maximal eccentric or concentric repetitions at 30◦deg s−1 angular velocity, with 1min of recovery between sets. All sets were completed using the same leg before switching to the other leg, with the eccentric and concentric exercises conducted in randomized order. The overall protocol of the single-bout trial is represented in Fig. 1B. Subjects had been instructed to abstain from physical activity in the days prior to the single-bout trial and arrived after an overnight fast at 08.00 h on the day of the single-bout trial. Prior to the exercise session, the subjects rested in the

supine position for approximately 30min. At 08.30 h, subjects commenced the exercise session; the same protocol was used for the exercise-habituation sessions. Immediately after completion of the exercise session, the subjects ingested a whey protein supplement (see below). The subjects then continued fasting for another 5 h post exercise (except for water provided ad libitum), and biopsies were taken from each leg at time points corresponding to 1, 3 and 5 h after exercise. To control for potential effects of circadian rhythm, the absolute daily time points and time resolution of the protocol were strictly adhered to, as far as possible, for all subjects.

2.13 Statistical analyses

Comparisons were made by using t test, with $p<0.05$ being considered statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Values are indicated in the graphs by mean +/- standard error.

3. RESULTS

PART I

3.1 Quantitative changes in phosphoproteome after high intensity exercise

A single bout of resistance exercise is known to lead to increases in muscle protein synthesis. However, while some of the signaling cascades, which are activated after a single bout of exercise, are known, a more global analyses is needed to describe the effects on muscle plasticity. The major posttranslational modification leading to rapid changes in protein function is protein phosphorylation. In order to determine the global changes in the phosphoproteome after high intensity exercise we decided to perform a quantitative phosphoproteomic analysis using multiplexed isobaric labelling and phosphopeptide enrichment coupled to tandem mass spectrometry (MS) (Figure1).

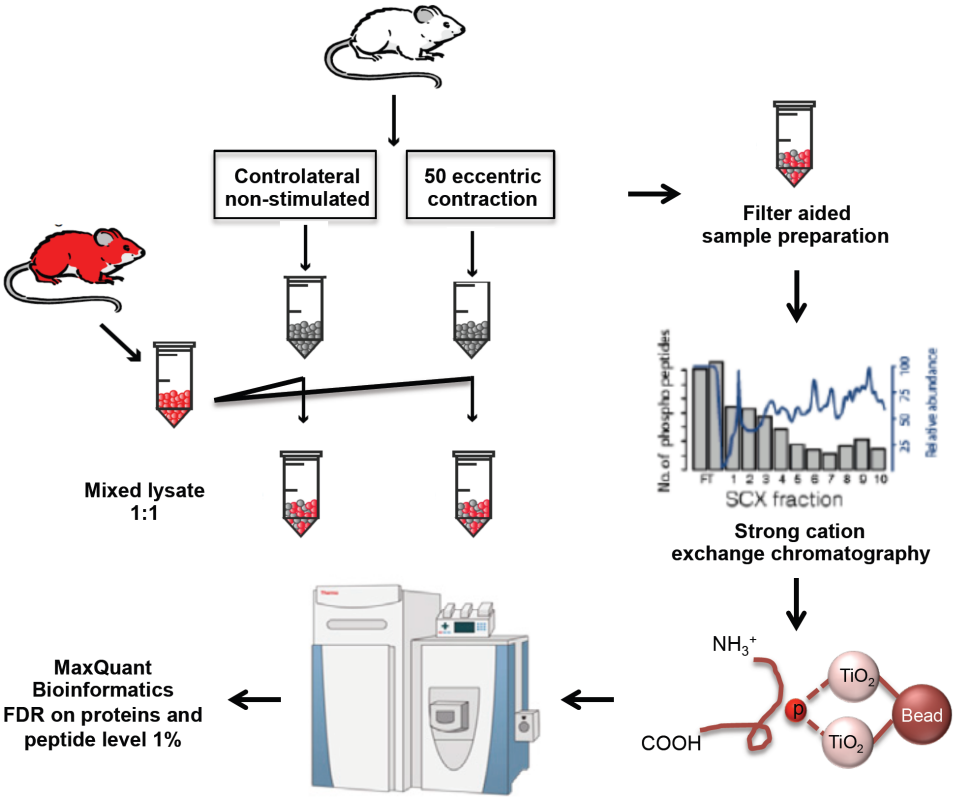


Fig.1 Schematic representation of the quantitative phosphoproteomics as performed in muscles taken after isometric and eccentric contractions. *Experimental setup to investigate phosphorylation changes after isometric and eccentric contractions. Enrichment of phosphorylation sites using a combination of a strong cation exchange chromatography approach and TiO2 beads extraction. Peptides were analyzed by LC-MS/MS and raw spectra were processed by MaxQuant at a FDR on protein and peptide level of 1 %. SILAC mouse serves as an internal standard for relative quantification. Reproducibility was determined by Pearson correlation coefficient between biological replicates.*

Since resistance training cannot be easily applied to animals, we used unilateral electrical stimulation in anesthetized animals as an *in vivo* model of high-intensity exercise. We used a protocol of 50 isometric and eccentric contractions, as described in the materials and Methods section, comparing the stimulated gastrocnemius muscle to the contralateral unstimulated one. The main difference between the two stimulation conditions, is the loading placed on the muscle. In the case of eccentric contractions, muscles were lengthened using a footplate at

40mm/s, leading significant reduction in isometric force of 23±2% of initial force (figure 2A). When performing the same 50 contractions, but without performing the forced lengthening of the muscle, we observed a reduction of 9±1% of initial force (figure 2B). Immediately after stimulation, muscles were taken out and frozen in liquid nitrogen.

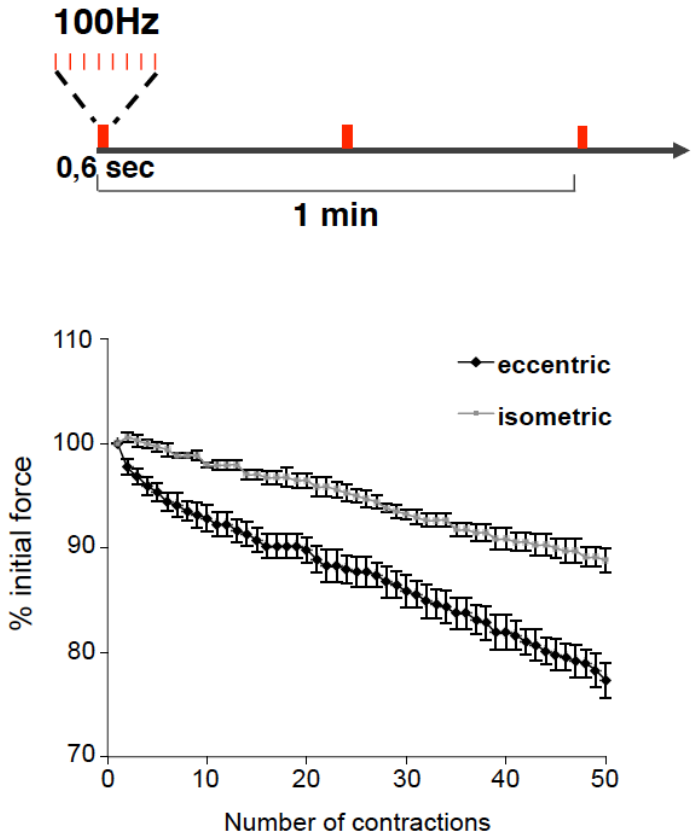


Fig.2 High intensity electrical stimulation of the gastrocnemius muscle reduces muscle tension

A*. TA muscle was electrially stimulated with a pattern similar to those observed in fast EDL muscles (trains of 0.6-second duration and 100-Hz frequency given every 60 seconds).* ***B****. Isometric and eccentric contractions repeated 50 times leads to a more pronounced force reduction in the eccentric group compared to the isometric group.*

In order to get a quantitative indication of the changes occurring in the phosphoproteome, we compared the phosphopeptides in stimulated and control

muscles to those found from muscles taken from a SILAC mouse (Kruger et al, 2008). Stable isotope labeling by amino acids in cell culture (SILAC) is a simple and straightforward approach for in vivo incorporation of a label into proteins for mass spectrometry (MS)-based quantitative proteomics. SILAC relies on metabolic incorporation of a given 'light' or 'heavy' form of the amino acid into the proteins. The method relies on the incorporation of amino acids with substituted stable isotopic nuclei (e.g. deuterium, 13C, 15N). In the SILAC mouse, the whole proteome is labelled by incorporation of (13)C(6)-Lysine (Lys6), making it possible to quantitatively compare the peptides obtained from this mouse with those obtained from a treated sample. When performing a quantitative analyses of the phosphoproteome in vivo in skeletal muscle after exercise, we found a total of 4000 different phosphorylated peptides. Many of these peptides belonged to structural muscle proteins, like titin. A list showing the peptides with the strongest increase in phophorylation after eccentric contractions is shown in figure 3.

PROTEIN NAME	Amino Acid	Positions	ratio_eccentric	ratio_isometric
Dedicator of cytokinesis protein 2	S	1704	28,72	1,06
IWS1-like protein	S	187	22,02	#NUM!
MKL/myocardin-like protein 2	S	77	16,34	6,99
Adenylosuccinate synthetase isozyme 1	T	14	15,74	8,59
AICAR transformylase	T	566	14,95	#NUM!
Chloride channel protein 1	S	682	13,47	0,06
Nebulin	S	84	13,37	1,40
E3 ubiquitin-protein ligase RNF123	S	14	12,83	1,20
C-protein, skeletal muscle fast isoform	T	942	12,51	#NUM!
Cardiomyopathy-associated protein 5	S	1419	10,73	4,43
THUMP domain-containing protein 1	S	88	9,40	#NUM!
Heat shock 70 kDa protein 4	T	540	9,01	5,51
Alpha-NAC, muscle-specific form	S	1744	8,54	5,28
Connectin/titin N2A-PEVK	T	5691;6585	8,30	#NUM!
Connectin/titin N2A-PEVK	S	9688;10579	8,30	2,77
Cofilin-1	S	8	8,15	#NUM!
Alpha-NAC, muscle-specific form	S	1869	8,15	4,07
SAFB-like transcription modulator	S	199	7,86	0,89
Connectin/titin N2A-PEVK	T	850;804;850	7,37	#NUM!

Fig.3 Relative increases in phosphorylation after electrical stimulation when compared to the non-stimulated controlateral muscles. *Phosphorylation sites are rankes for their intensity after eccentric contractions.*

Interestingly, we found a significant increase in a previously uncharacterized phosphorylation site of myocardin-like protein 2 (MKL2 or MRTF-B), the serine 77. The myocardin/MKL family of proteins have been described as strong coactivators of SRF, an important signaling pathway known to control skeletal muscle growth. Since SRF activity does not appear to be regulated by its direct modification, these coactivators provide a novel point for regulation of SRF target genes. Thus, the identification of new phosphorylation site of MRTF-B is an important step to dissect this pathway.

3.2 Eccentric contractions induce nuclear translocation of MRTF-B-GFP

It has been reported in various cell systems that the intracellular localization of MRTF-B is regulated by phosphorylation, as it is known to shuttle from the cytoplasm to the nucleus. In addition, increases in mechanical stress, as occurs during high intensity exercise, is known to lead to the nuclear accumulation of MRTF (Varney et al, 2016)(Varney, JCS 2016). In order to understand if high intensity exercise can lead to a nuclear accumulation of MRTF-B, we electroporated TA muscles with a construct coding for MRTF-B-GFP. After two weeks we performed electrical stimulation in one TA muscle comparing it to the non-stimulated controlateral muscle. As depicted in figure 4, the controlateral non stimulated muscle shows a cytoplasmic localization of MRTF-B-GFP (A.). Interestingly, immediately after 50 eccentric contractions we observed a significant nuclear localization of MRTF-B-GFP (B.). In order to quantify this effect, we counted the number of transfected fibers, which showed a nuclear localization of MRTF-B-GFP. As can be seen in figure 3B, eccentric contractions lead to a nuclear accumulation of MRTF-B in 42±11% of transfected fibers, compared to only 8±2% in non-stimulated fibers. The shuttling of MRTF-B from the cytoplasm to the nucleus suggests a possible activation of MRTF-B after eccentric contractions.

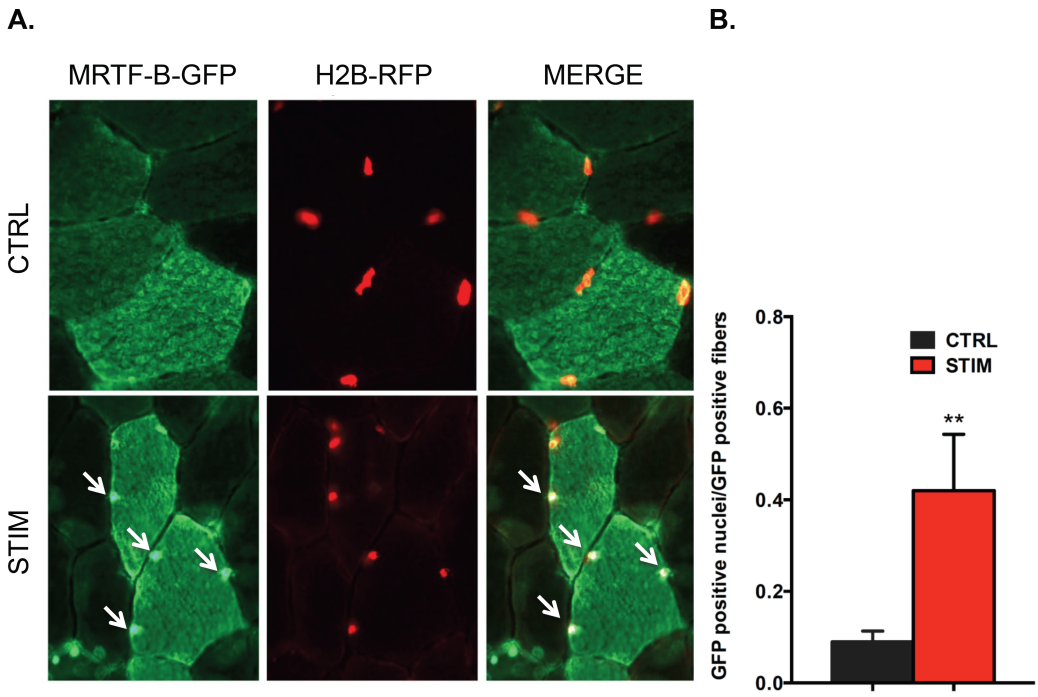


Fig. 4 Eccentric exercise leads to nuclear traslocation of MRTF-B

A-B*. MRTF-B-GFP localization after 30 minutes of fast electrostimulation is shown in transverse sections of transfected tibialis anterior (TA) muscle. Trasversal section of the contralateral TA, used as an unstimulated control, is shown in top left panel. As can be seen in both the images and the quantification, eccentric contractions lead to a pronounced and significant nuclear increase of MRTF-B-GFP. (n=5 muscles per group, more than 30 transfected fibers per muscle. p<0,05)*

In order to understand if the newly identified phosphorylation on serine 77 in MRTF-B can be linked to the intracellular localization of the protein, we performed a point mutation, in which serine 77 is replaced by an alanine (MRTF-B(S77A)-GFP). We transfected the TA muscle with the plasmid coding for MRTF-B(S77A)-GFP and after two weeks we performed electrical stimulation in one TA muscle and maintain the other one like a control. As shown in the figure 5, muscles transfected with MRTF-B(S77A)-GFP showed no nuclear accumulation of MRTF-B-GFP. Quantification showed that 1±1% of fibers transfected with the mutant MRTF-B(S77A)-GFP showed a nuclear staining.

Importantly, 50 eccentric contractions failed to significantly increase the number of fibers showing a nuclear staining after transfection with the mutant construct, showing that the phosphorylation on serine77 is required for the nuclear accumulation of MRTF-B after eccentric contractions.

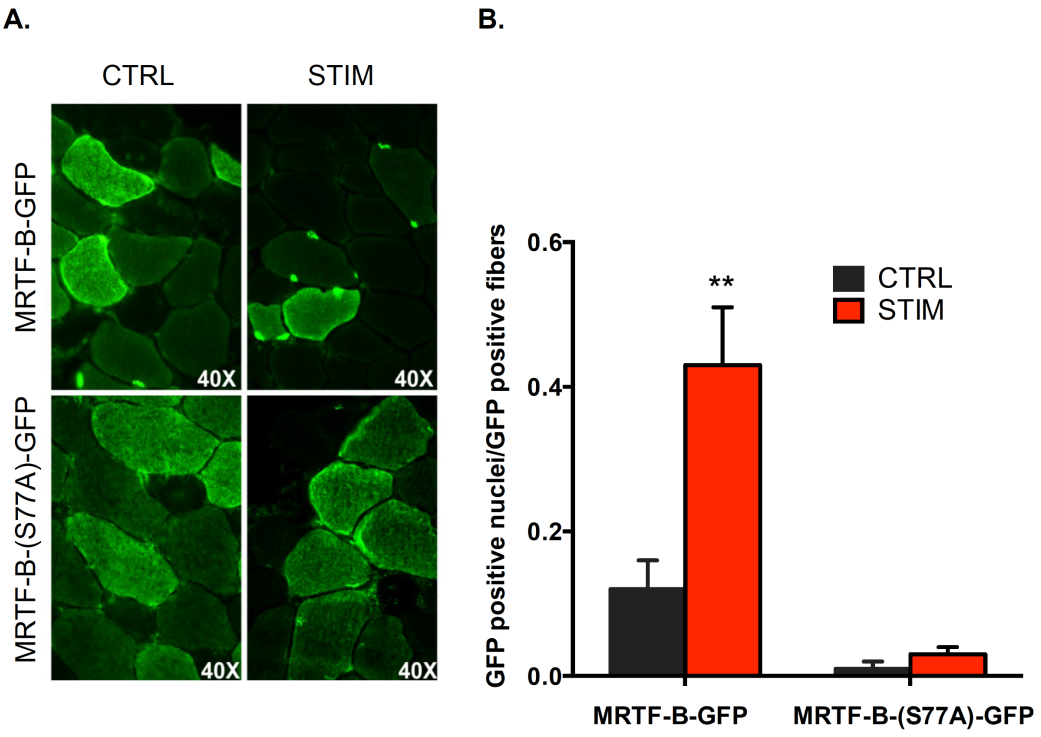
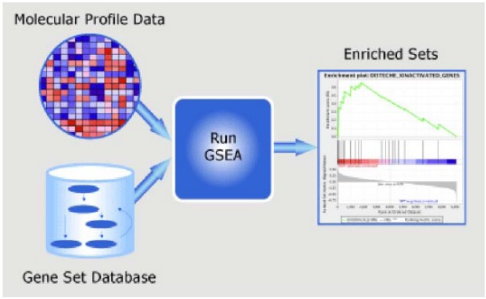


Fig. 5 Phosphorylation on serine 77 is required for nuclear translocation of MRTF-B

A. *MRTF-B-GFP and MRTF-B(S77A)-GFP localization after 30 minutes of fast electrostimulation. Trasversal sections of TA electrically stimulated through the peroneal nerve with patterns of 100 Hz (right panels). Contralateral TA was used as unstimulated controls (left panels). Nuclear translocation of MRTF-B(S77A)-GFP after stimulation is abolished when serine 77 is substituted for alanine.* ***B.*** *Nucleocytoplasmic shuttling of MRTF-B-GFP and MRTF-B(S77A)-GFP was quantified as the ratio of transfected fibers showing nuclear staining of MRTF-B-GFP (+/- SEM). (n=6 muscles with >100 transfected fibers analyzed for each control and stimulated muscle).*

3.3 Transfac analyses reveal an increase in SRF-dependent gene transcription after eccentric contractions

In order to understand if the nuclear accumulation of MRTF-B after eccentric contractions is also accompanied by an increase in SRF-dependent gene transcription, we performed Gene Set Enrichment Analysis (Subramanian et al, 2005), interrogating genesets from the TRANSFAC database with genes found to be differentially expressed 24 hours after eletrical stimulation. The TRANSFAC geneset collection calculates enrichment scores for groups of genes that share specific transcription factor binding motifs within promoter regions, +/-2 kb from their transcription start sites. We identified that SRF is one of the major transcription factors significantly increased 24 hours after eccentric contractions. These data show that eccentric contractions lead to the nuclear accumulation of the SRF transcriptional coactivator MRTF-B, which correlates to the increase in SRF-dependent gene transcription 24 hours after electrical stimulation (Figure 6).



	GS follow link to MSigDB	GS DETAILS	SIZE	ES	NES	NOM p-val	FDR q-val	FWER p-val	RANK AT MAX	LEADING EDGE
1	CGGAARNGGCNG_UNKNOWN	Details	30	-0.63	-2.00	0.000	0.004	0.004	1286	tags=37%, list=8%, signal=40%
2	VSSRF_Q5_01	Details	178	-0.46	-1.97	0.000	0.003	0.006	2350	tags=39%, list=15%, signal=39%
3	CCAWWNAAGG_VSSRF_Q4	Details	67	-0.52	-1.95	0.000	0.003	0.009	2288	tags=37%, list=15%, signal=44%
4	VSSRF_Q4	Details	179	-0.45	-1.92	0.000	0.003	0.012	2350	tags=31%, list=15%, signal=36%
5	VSSRF_C	Details	166	-0.46	-1.91	0.000	0.003	0.012	2234	tags=34%, list=15%, signal=40%
6	VSSRF_01	Details	46	-0.53	-1.86	0.000	0.006	0.033	1538	tags=37%, list=10%, signal=41%

Fig.6 Gene Set Enrichment Analyses

Gene Set Enrichment Analyses of microarrays performed 24 hours after eccentric contractions show a strong increase in SRF-dependent gene transcription. A comparison was made between 4 microarrays performed in resting EDL muscles compared to 4 EDL muscles 24 hours after eccentric contractions.

PART II

3.4 High intensity exercise leads to histone modifications

It is known that exercise induces significant alterations in gene expression and protein levels, but the effect of exercise on the epigenetic modulation of gene transcription is not well established. In order to understand the effect of acute exercise on histone modifications we started by analyzing three different modifications, i.e. H3K4me3, H3-K9K14ac and H3S10ph, comparing stimulated to non-stimulated muscles (Figure 7). Immediately after stimulation, no significant changes were found in H3K4me3 and H3-K9K14ac. However, we observed that electrical stimulation induces a strong 4 fold increase in H3S10ph, compared to the very low basal level of phosphorylation on H3 in controlateral non stimulated muscle.

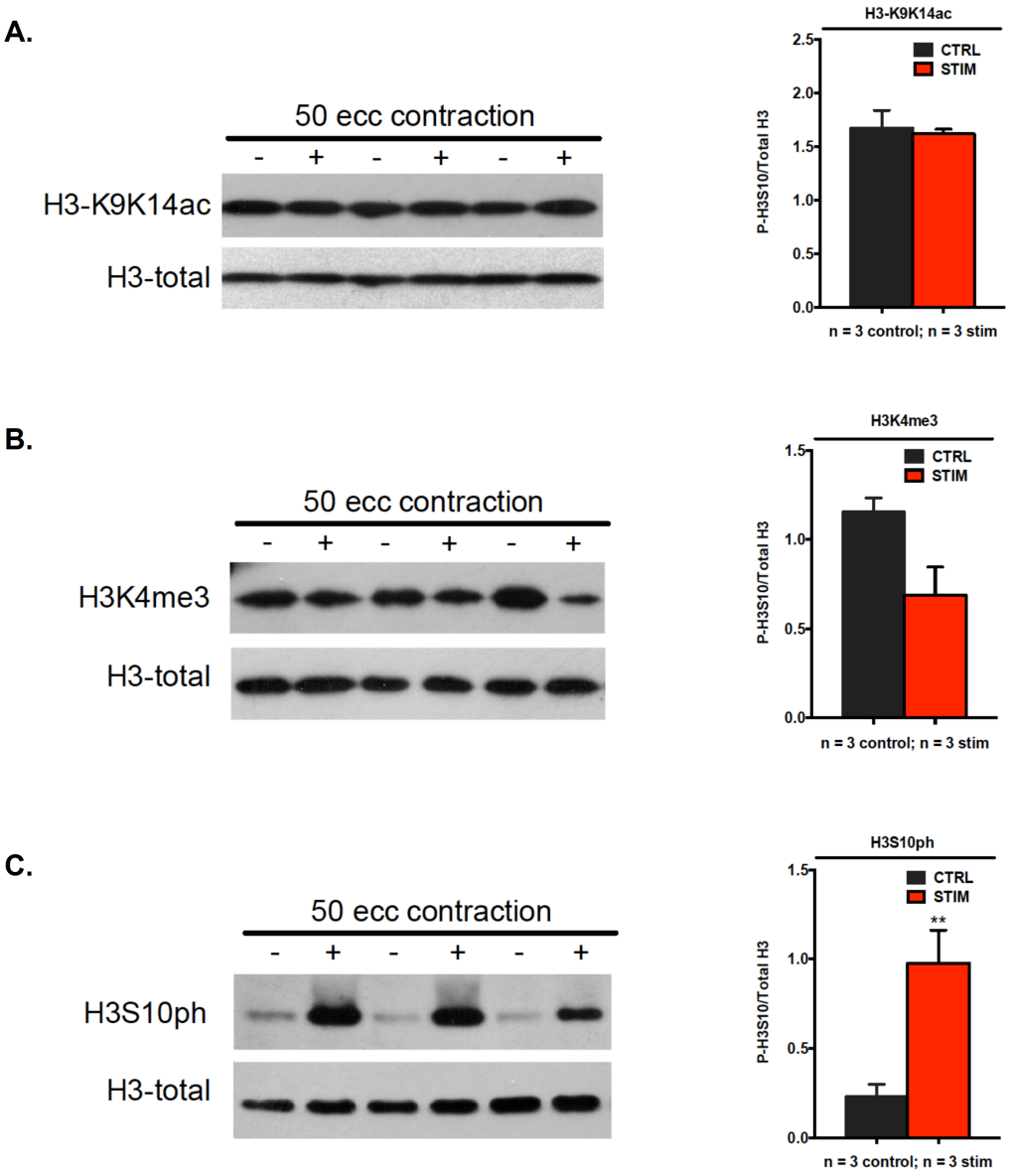


Fig.7 Electrical stimulation induces histone modifications.

Western blot analysis are performed on total protein extracts after 30 minutes of electrostimulation. The contralateral TA was used as an unstimulated control. The control muscle is indicated with the symbol (–), instead, the electrically stimulated TA is indicated with the symbol (+). ***A,B,C****. Representative immunoblots of H3K4me3, H3-K9K14ac and H3S10ph and total H3 levels after stimulation are shown. Western blot do not show changes in H3K4me3 and H3-K9K14ac upon stimulation, but show a significant increase in H3S10ph in stimulated muscle. Quantification analyses are reported on the right (n>3 for each group **p<0.01).*

Next, in order to determine the kinetics of this increase in H3S10ph, we analysed H3S10ph at various time points after stimulation (Figure 8). As can be seen in figure 6, the strong increase in H3S10ph found immediately at the end of stimulation, decreases steadily over time, returning to basal levels 3 hours after stimulation. These results show that ph is a strong and rapid event which decreases within a couple of hours.

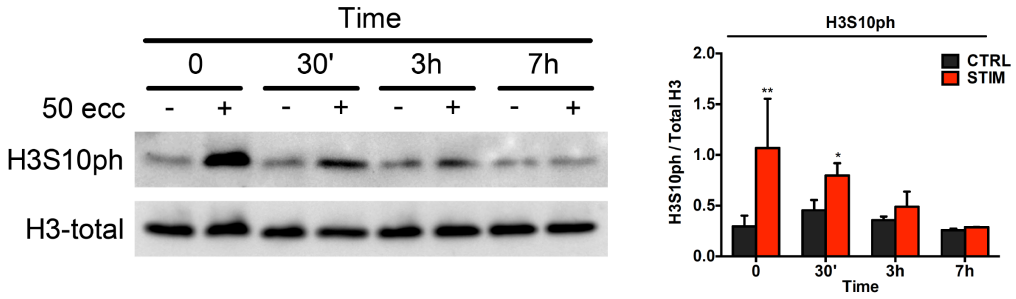


Fig.8 H3S10ph is a rapid event that decreases over time

Western blot analysis on total protein extracts obtained from mice sacrificed immidiatly after, 30' minutes, 3 hours or 7 hours after electrical stimulation. Contralateral TA that was used as unstimulated controls, was indicated with the symbol (–), instead, TA electrically stimulated through the peroneal nerve with patterns of impulses 100 Hz, was indicated with the symbol (+). ***A.*** *Phosphorylation of histone H3 decreases over time and returns to basal levels 3 hours after the stimulation.* ***B.*** *Quantification analyses are reported on the right (n=3 for each group **p<0.01).*

In order to better understand if this phosphorylation occurs within the muscle fibers and not in the interstitial cells, we counted the percentage of positive nuclei for H3S10ph inside the dystrophin staining and normalized them for the total amount of myonuclei. As can be seen in figure 9, 40% of myonuclei are positive for this histone modification, suggesting that it is an important event that occurs primarily within the muscle fibers.

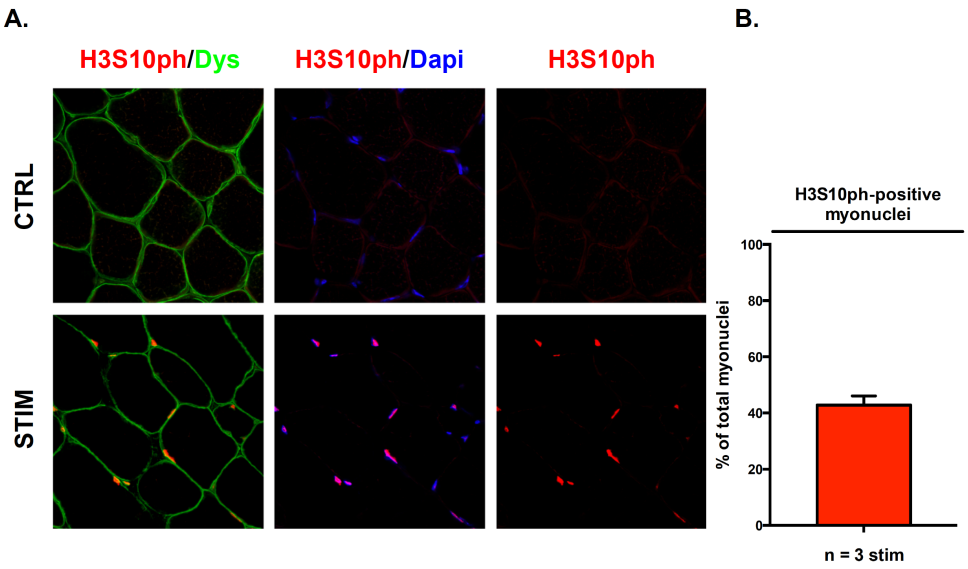


Fig. 9 H3S10ph primarly occurs in myonuclei

A. *Representative confocal images of trasversal section of control and stimulated TA muscle. Nuclei are shown in blue with 40,6-diamidino-2-phenylindole (DAPI) staining, H3S10ph is in red. The muscle fiber perimeter is stained with a-Anti-Dystrophin antibody in green. Merged images showing no detectable histone H3 phosphorylation in control muscle,and postive H3S10ph staining in stimulated ones are shown.* ***B****. Percentage of myonuclei positive for H3S10ph are presented.*

3.5 Histone phosphorylation is increased after endurance exercise and energy stress

In order to address the question if H3S10ph only occurs after high intensity exercise, we performed downhill running exercise until exhaustion, as a model for endurance exercise. (He et al, 2012). We observed a clear H3S10ph after downhill running, even though less intense than the phosphorylation seen after electrical stimulation (Figure 10). These results suggest that physical activity leads to a rapid change in H3S10ph, with the intensity of the change corresponding to the intensity of the exercise. It is known that after intensive exercise, there is a decrease in ATP content and a consequent increase in AMP levels. The major

molecular sensor for AMP levels in cells is the AMP- activated protein kinase (AMPK), an enzyme that is activated by ATP depletion.

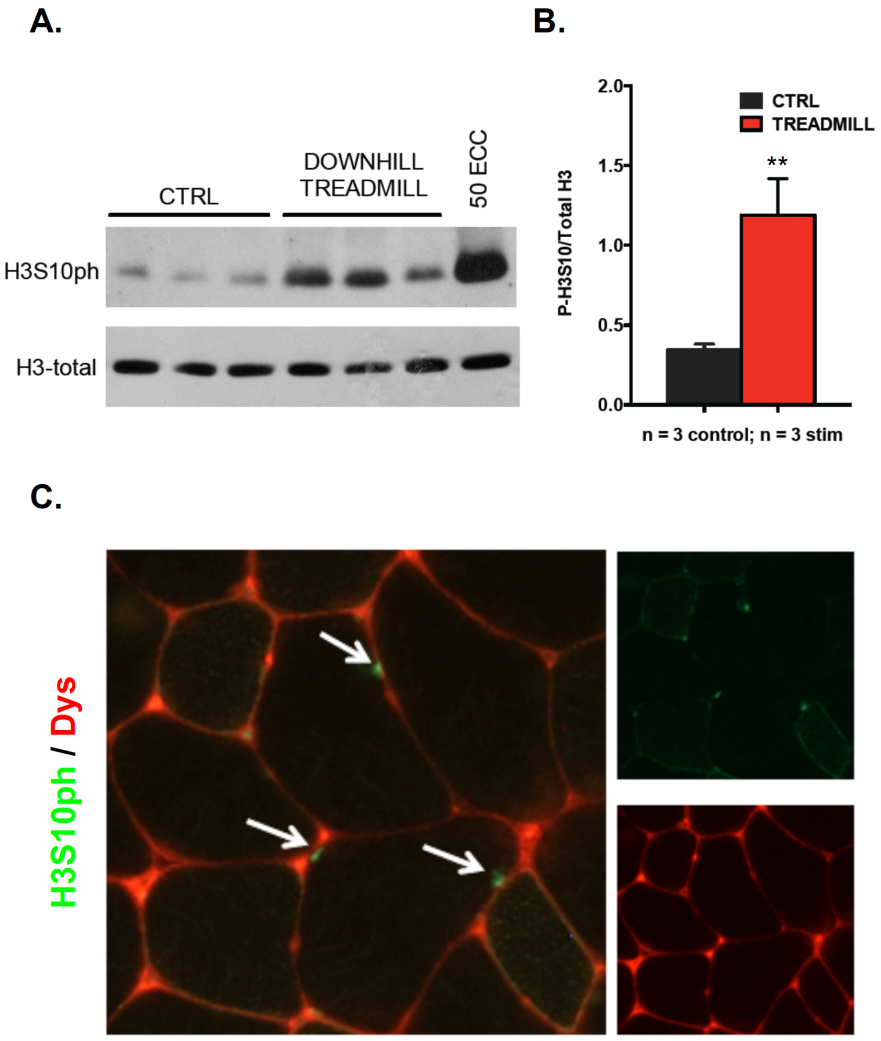


Fig. 10 Treadmill running induce H3S10ph

A. *Representative western blot showing a significant increase in H3S10ph upon exercise compared to the control non-exercised mice.* ***B.*** *Quantification analyses are reported on the right (n=3 for each group, **p<0.01).* ***C.*** *Representative trasversal section of exercised TA muscle are shown. H3S10ph is stained in green. Muscle fibers perimeter is stained with a- Anti-Dystrophin antibody in red. Merged images showing postive H3S10ph staining in muscle after exercise are presented.*

In order to understand if metabolic stress can induce H3S10ph we decided to use AICAR (5-aminoimidazole-4-carboxamide ribonucleoside), a pharmacological activator of AMPK and an exercise mimetic, to understand if metabolic stress induced by exercise is able to lead to an increase in H3S10ph (Figure 11). By western blot analysis we confirm the activation of AMPK and its downstream target ACC, 30 minutes after AICAR injection. Interestingly, the activation of AMPK corresponds to a significant increase in H3S10ph compared to the muscle treated with NaCl 0,9% (A,B.). We also performed an immunohistochemistry analyses for H3S10 phosphorylation showing that this occurs predominantly inside the myonuclei (C.). The results suggest that in addition to mechanical stress, also metabolic stress can lead to histone phosphorylation.

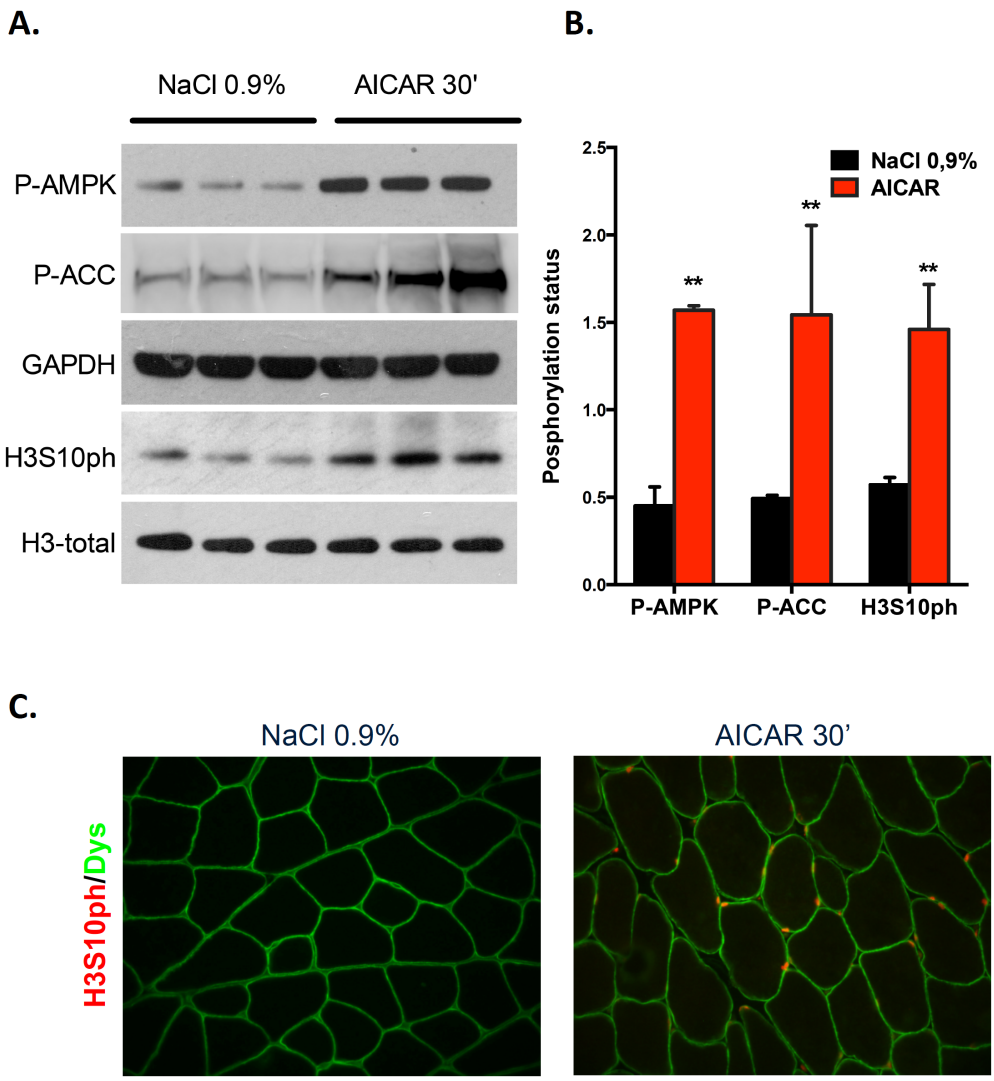


Fig.11 Metabolic stress induces H3S10ph

Muscle were collected 30 minutes after injection of NaCl 0,9% (control mice) and after injection of AICAR (conc). ***A.*** *Representative immunoblots of P-AMPK, P-ACC ,GAPDH, H3S10ph and H3 total are shown.* ***B.*** *Quantification analyses are reported on the right (n=3 for each group **p<0.01).* ***C.*** *Representative trasversal section of control and AICAR-treated mice are shown . H3S10ph is stained in red. Muscle fibers perimeter is stained with a- Anti-Dystrophin antibody in green. Merge images showing postive H3S10ph staining in muscle after AICAR-treatment are presented.*

3.6 Histone phosphorylation after exercise requires MSK1

The p38 MAP kinase cascade is activated in skeletal muscle in response to exercise and, moreover, the MAPK signaling pathway plays a central role in H3 phosphorylation. For this reason we checked by western blot analysis the activation of this pathway immediately after 50 eccentric contractions. p38 MAPK as well as MKK3, that is one of the upstream kinases of p38 MAPK, are highly phosphorylated and therefore activated after electrical stimulation (Figure 12). Considering that the motif containing H3S10 does not look like the consensus sequence for p38, the putative candidate for targeting histone H3 are the effector kinases downstream of MAPK activation. In particular, the strongest candidate is mitogen- and stress-activated kinase 1 (MSK1) that has been shown in many cell types to be responsible for the phosphorylation of histone 3. We found that MSK1 is strongly activated by p38 stress kinase pathways in response to electrical stimulation, and the activation of this pathway is parallel by the induction of H3 phosphorylation.

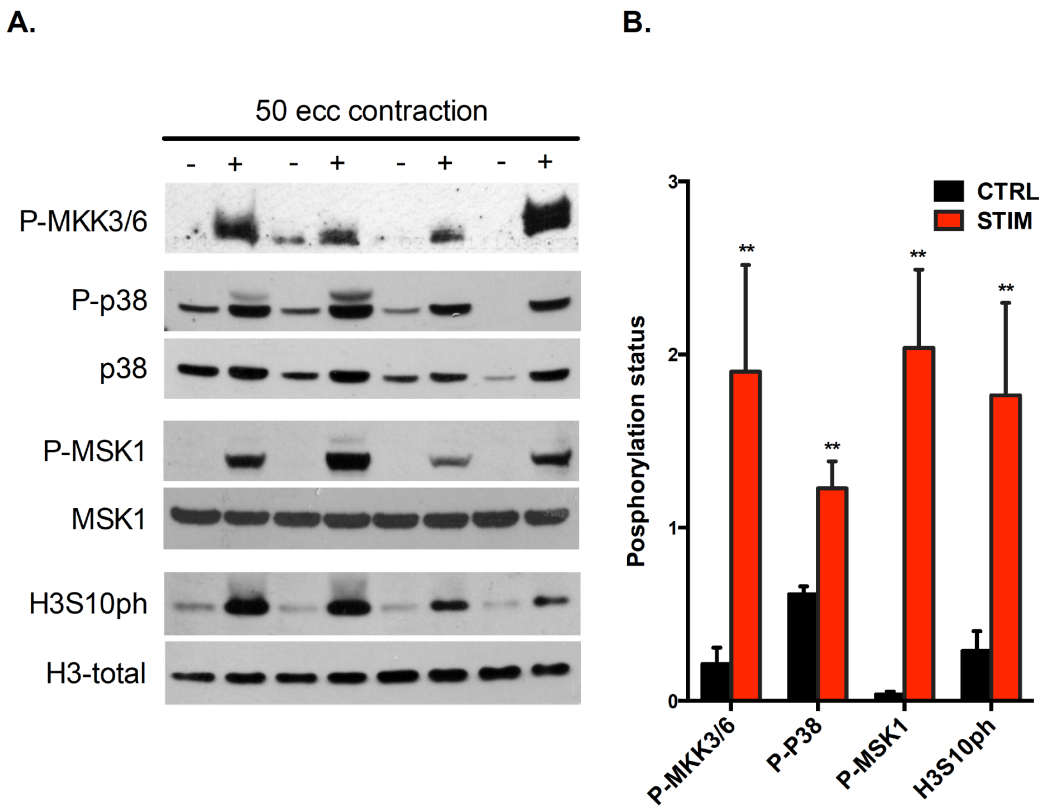


Fig. 12 Electrical stimulation induces activation of the MAPK pathway and MSK1 kinase.

Western blot analysis are performed on total protein extracts after 30 minutes of electrostimulation. ***A.*** *Representative immunoblots of P-MKK3/6, P-p38, p38, P-MSK1, MSK1 H3S10ph and total H3 levels after stimulation are shown.* ***B.*** *Quantification analyses are reported on the right (n=3 for each group **p<0.01).*

The next step was establishing a causal link between MSK1 and H3 phosphorylation after high intensity exercise. To address this point we performed a loss of function approach for MSK1 in adult muscles of wild type mice (Figure 13). Two different shRNAs were tested to specifically reduce MSK1 protein levels. HEK cells were transfected for 24 hours with a myc-tagged MSK1 and two different oligos against MSK1, followed by a western blot using the HEK protein extracts. One of the oligos efficiently knocked down MSK1 as can be seen in figure 13 (A).

For *in vivo* transfection experiments we used bicistronic vectors that

simultaneously encode shRNAs and GFP. Therefore, detection of GFP fluorescence allows us to identify the transfected fibres. Adult TA muscles were electroporated with either the siRNA MSK1-GFP or with siScramble-GFP. In order to evaluate the effect of MSK1 on exercise-induced H3 phosphorylation, we performed electrical stimulation on electroporated muscles and examined histone phosphorylation by immunohistochemistry analyses. Electroporation with siScramble-GFP did not affect nuclear staining of H3S10ph after stimulation. Interestingly, after 50 eccentric contraction in electroporated fibers, in which there was a strong downregulation of MSK1, we found an almost complete prevention of H3S10ph, when compared to the surrounding non-transfected fibers, in which we observed a clear H3S10ph (B,C.). These results show that MSK1 is required for histone phosphorylation after exercise.

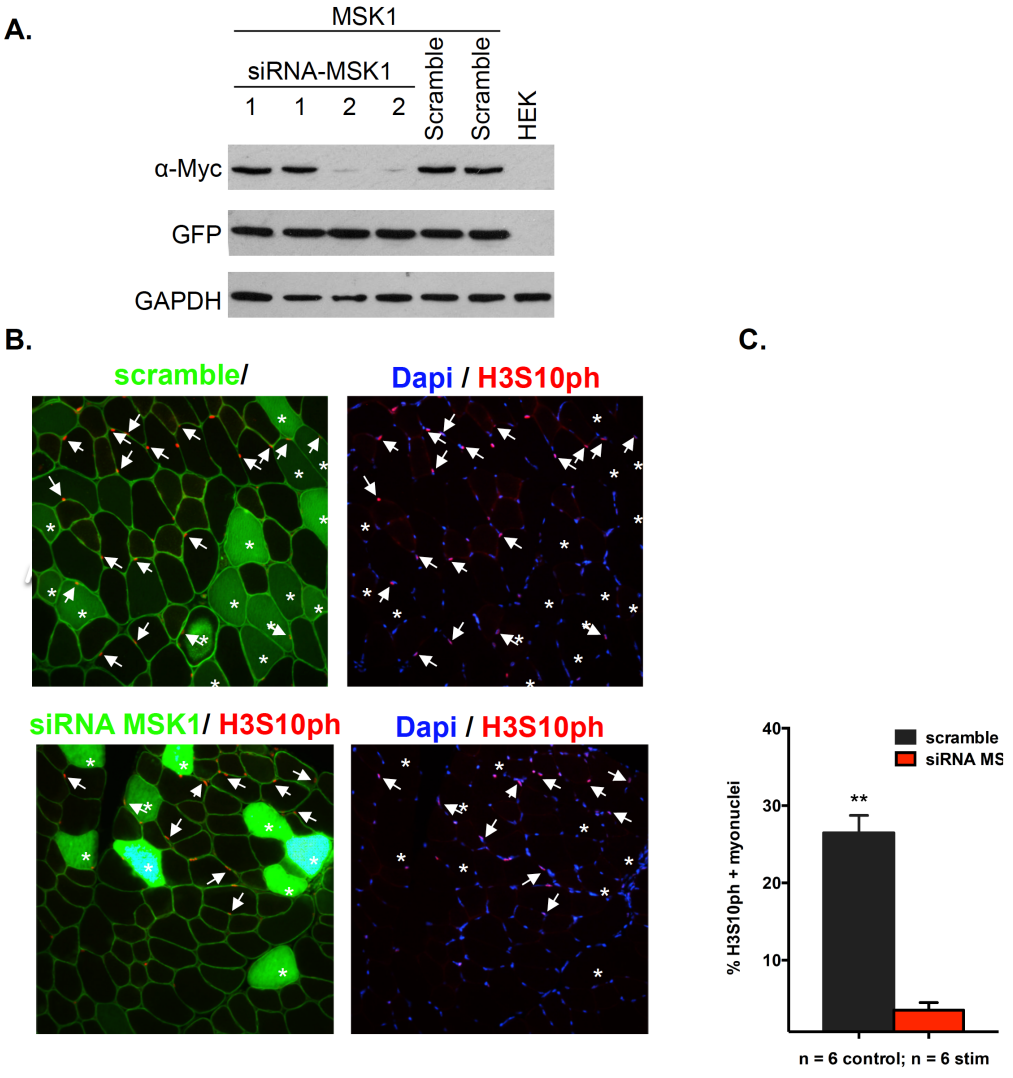


Fig.13 MSK1 is required for H3S10ph

siRNAs against MSK1 were tested in HEK cells overexpressing mMSK1. ***A.*** *Western blot of HEK protein extract revealed that shRNA 2 successfully blocked MSK1 expression.* ***B.*** *Electrostimulation was performed 2 weeks after in vivo transfection of TA muscle with control siRNA (scrable-GFP plasmid) and siRNA against MSK1 (siRNA MSK1). Representative transversal sections of muscles treated as described above were immunostained for H3S10ph in red. Left panels show GFP-positive transfected fibers in control muscle with scrambled (top left) and in muscle transfected with siRNA against MSK1 (bottom left). Right panels show merge between dapi and H3S10ph in muscle treated as described above. Arrows point to the myonuclei positive for H3 Ser-10 and asterisks indicate to the transfected fibers.* ***C.*** *Quantification analyses are reported on the right (n=3 for each group **p<0.01).*

3.7 H3S10ph is coupled to acetylation at the nearby Lys14 residue

Using different experimental systems, H3S10ph has been shown to be a priming step which facilitates Lys14 acetylation (Cheung et al, 2000b; Lo et al, 2000), an event that has been linked to local opening of chromatin structure. We used an antibody that recognize the histone H3 tail when it is phosphorylated on both ser10 and acetylated on Lys14 (H3S10phK14ac). Interestingly, we found an important induction on H3S10phK14ac after electrical stimulation, suggesting that exercise is able to induce, in differentiated non-dividing somatic cells, histone modification linked to gene activation (Figure 14).

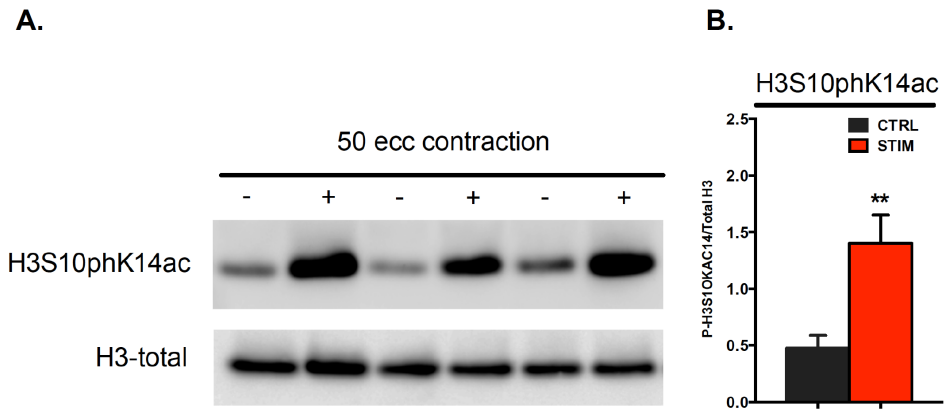


Fig. 14 H3S10ph is followed by acetylation on the nearby Lys14 residue.
Western blot analysis are performed on total protein extracts after 30 minutes of fast electrostimulation ***A****. Representative immunoblots of H3S10phK14ac and total H3 levels after stimulation are shown . Western blots showing a significant increase in H3S10phK14ac in stimulated muscle.* ***B.*** *Quantification analyses are reported on the right (n>3 for each group **p<0.01).*

3.8 Increased histone H3S10ph at the promoters of genes involved in structural and metabolic remodelling of skeletal muscle

In order to understand where this histone modification occurs we performed chromatin immunoprecipitation (ChIP) followed by qPCR in control and stimulated muscles. Chromatin immunoprecipitation is a powerful tool for the characterization of covalent histone modifications and DNA. We assessed H3 phosphorylation at the promoter region of different of genes that are involved in the structural and metabolic remodeling of skeletal muscle. In particular evidences in different biological systems show that H3S10 phosphorylation is an essential prerequisite for the activation of IEG transcription (Cheung et al., 2000a; Berger and Felsenfeld, 2001), and indeed we found and significant increase in the H3S10 phosphorylation at c-fos promoter in stimulated muscle compare to the control. We also found an significant increase in the H3S10 at the promoter region of other genes such *Pgc-1α1* and *Pgc-1α4* (Figure 15 A,B,C).

Next, in order to establish a direct link between H3 phosphorylation and gene expression, we used qRT-PCR to analyse the expression of c-*fos*, *Pgc-1α1* and

Pgc-1α4 after stimulation We evaluated the expression of *c-fos*, *Pgc-1α1* and *Pgc-1α4* at different time points after the electrical stimulation. *c-fos* expression is already significantly increased immediately after the end of electrical stimulation (time 0), 3 hours after we found the peak of the expression that decreased overtime and returns to basal levels 12 hours after stimulation. *Pgc-1α1* and *Pgc-1α4* is less induced but 24 hours after stimulation we found and significant increase in the expression (Figure 15 D,E,F).

The results indicate that nucleosomal changes associated with histone H3 modifications could play an important role in mediating transcriptional activation of *c-fos*, *Pgc-1α1* and *Pgc-1α4* genes.

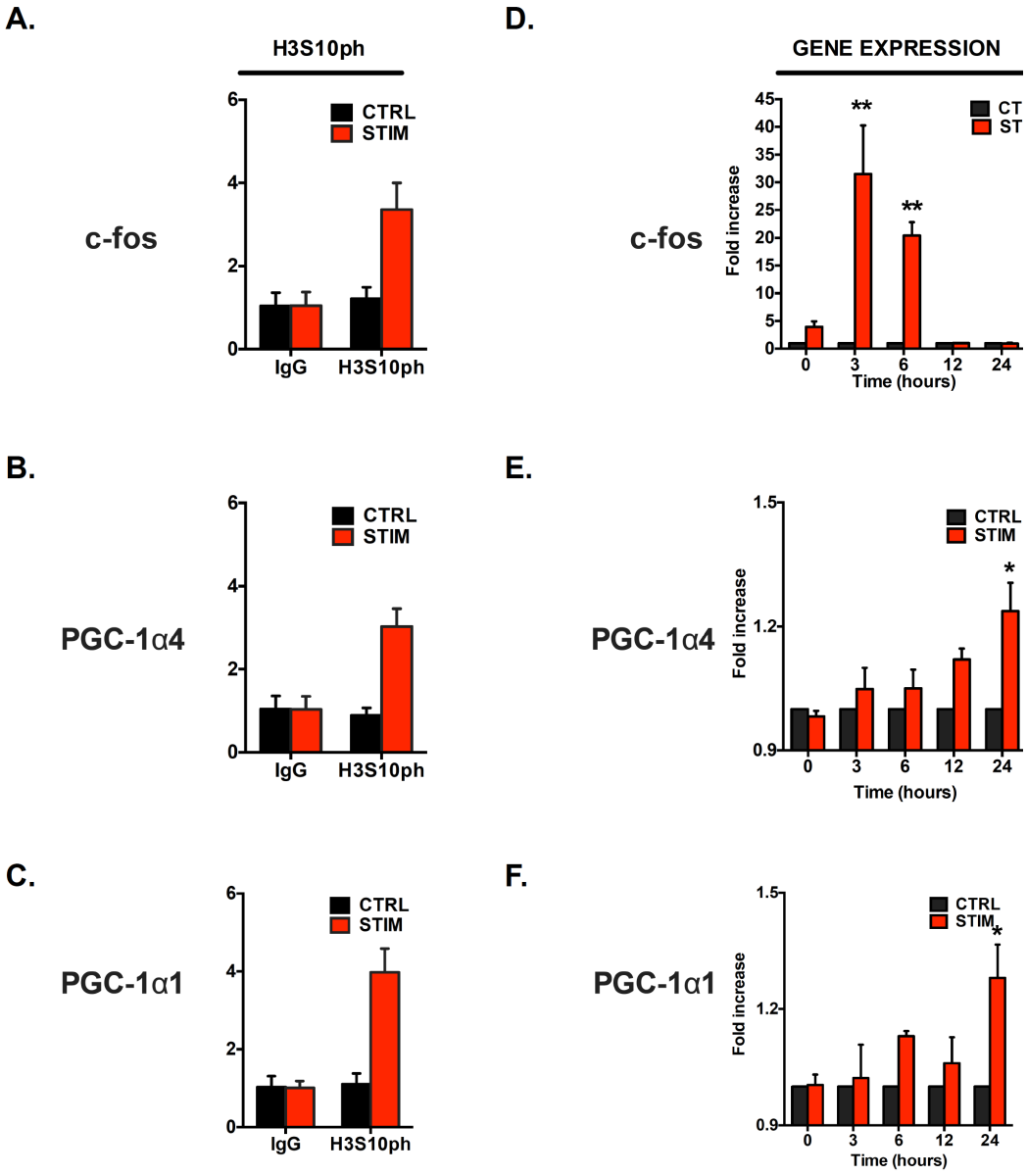


Fig.15 Increased histone H3 Ser-10 at the promoters of genes involved in the structural and metabolic remodelling of skeletal muscle*. **A,B,C**. ChIP–Q-PCR assay using H3S10ph antibody to precipitate chromatin from a pool of 4 muscles obtained after 30 minutes of elettrostimulation. Primer sets are specific for c-fos (**A**), Pgc-1α1 (**B**) and Pgc-1α4 (**C**) and were designed around the transcription start site (TSS). Results are expressed as % input over basal condition. **D.E.F.** Real time PCR was performed from RNA obtained from mice immidiatly after (0), 3, 6 12, and 24 hours after electrical stimulation. Contralateral TA muscles were*

*used as unstimulated controls. Expression levels of c-fos (**D**), Pgc-1α1 (**E**) and Pgc-1α4 (**F**) are shown. (n=4 for each group,*p<0.05, **p<0.01).*

3.9 MAPK pathway activation and H3S10ph occurs in humans after high intensity exercise.

Eventually, to understand if also in humans histone 3 is phosphorylated, we analyzed skeletal muscle biopsies after resistance exercise. Skeletal muscle biopsies were obtained pre and at 1, 3 and 5 hours after sessions of six sets of 10 maximal eccentric concentric repetitions, as described in the materials and Methods section. We found that 1 hour after the eccentric contractions there is a peak of H3S10ph that decreases over time. We also check the activation of the MAPK pathway, and also in humans we find a remarkable phosphorylation and activation of p38 and MSK1. This observation suggests that also in human muscle acute exercise induces chromatin remodelling potentially linked to enhanced gene transcription. (Figure 16).

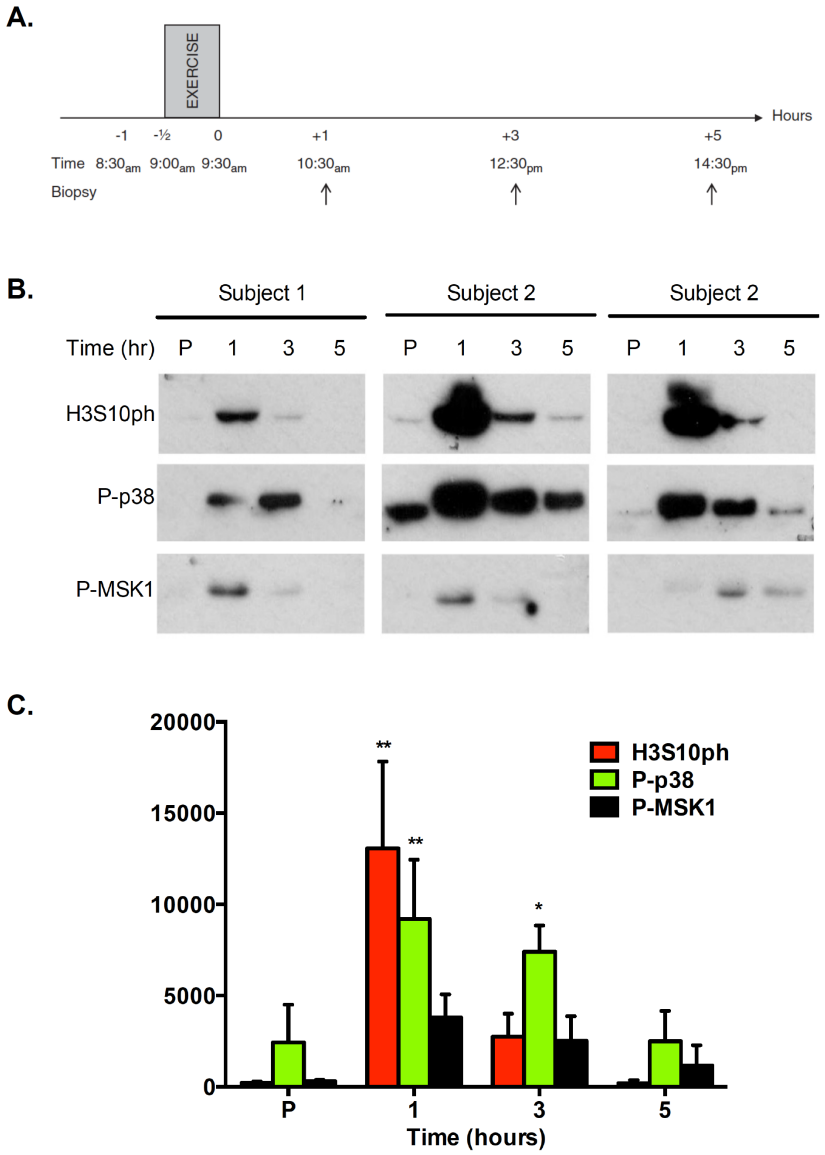


Fig.16 MAPK pathway activation and H3S10ph occur in humans after high intensity exercise.

A. *Human skeletal muscle biopsies were obtained from 3 healthy donors, pre and at 1, 3 and 5 hours after session of six sets of 10 maximal eccentric concentric repetitions.* ***B.*** *Representative immunoblots of H3S10ph, P-p38 and P-MSK1 are shown.* ***C.*** *Quantification analyses are reported on the bottom (**p<0.01).*

4. DISCUSSION

PART I

Our study represents an important advance in order to understand the global changes occurring after high intensity exercise and how the signalling pathway governing the muscle plasticity is regulated. Through the phosphoproteomics analyse we found a total of 4000 different phosphorylated peptides, and in particular we identified a new phosphorylation site of MRTF-B. MRTF is a transcriptional co-activator responsible for the SRF binding to SRE-binding sites. It has been shown that MRTF is an actin-monomer-binding protein that translocates from the cytoplasm to the nucleus as a result of its dissociation from actin monomers (Miralles et al, 2003). It exits in two forms: MRTF-A and MRTF-B, which exhibit redundant functions in response to serum and RhoA induction of reporter genes (Cen et al, 2003). However, interestingly for our observations, in force-induced actin assembly leading to regulation of genes regulated by SRF, MRTF-A and MRTF-B exhibit distinctly different regulatory roles. Specifically, it has been shown that force application induced nuclear translocation of the transcriptional co-activator MRTF-A but not MRTF-B, being this translocation dependent Rho kinase and intact actin filaments (Zhao et al, 2007). However, how MRTF-B was instead regulated, whether kinase and phosphorylation were responsible for nuclear translocation of MRTF-B was not known. Mice lacking MRTF-B results in embryonic lethality owing to cardiovascular defects, including abnormal patterning of the branchial arch arteries, a double-outlet right ventricle, ventricular septal defects and a thin-walled myocardium (Oh et al, 2005). We demonstrated that after eccentric contraction there is traslocation of MRTF-B from cytoplasm to nucleus suggesting a possible activation. In the paper of Interestingly, Sheetz's group observe that MRTF-A translocation from the cytosol to the nucleus is subject to momentum by continuing even after suspending the stretch protocol was and only slowly returning to the initial low nuclear levels in a matter of hours (Cui et al, 2015). Eventually, the activation and nuclear translocation of MRTF-A induces growth. We could speculate that a similar mechanism governs MRTF-B activation and its nuclear translocation. Indeed, in our project we were able to identify that MRTF-B translocation to the nucleus is

dependent on the phosphorylation on ser 77. Eventually, MRTF-B translocation to the nucleus could promote its interaction with the transcription factor SRF and SRF-mediated muscle growth. Noteworthy, it has been shown that in adults, SRFactivity could also be important for the control of skeletal muscle growth, as suggested by increased SRF expression during overload-induced hypertrophy (Fluck et al, 1999). In cardiac muscle, SRFand MRTF-A are necessary for mediating cardiomyocyte hypertrophy (Kuwahara et al, 2010) (Parlakian et al, 2005). Using an inducible, muscle-specific knockout model, SRF was also found to be required for muscle hypertrophy. In this model, the effect of SRF is apparently mediated via release of interleukins 4 and 6 (IL-4 and IL-6), which act in a paracrine manner to induce satellite cell proliferationand fusion.

PART II

High intensity exercise is able to induce histone modifications

Skeletal muscle is a very plastic tissue able to adapt its structural and metabolic properties in response to environmental stressors. Exercise is usually divided in two categories: endurance training (aerobic-based), which involves low-resistance activity of longer duration, and resistance training, which requires more powerful movements of shorter duration. Exercise is able to induces adaptive response to improve metabolic efficiency, oxidative capacity, and contractile activity by altering gene expression profile and protein levels (Coffey & Hawley, 2007). While the global phenotypical changes in muscle properties are well-established, the molecular mechanisms at the basis of these changes are not very well characterized. Here we show that exercise induces a rapid increase in histone phosphorylation which depends on the activation of the MAPK signaling pathway. Furthermore, histone phosphorylation occurs close to the TSS of important genes for muscle plasticity, like *Pgc-1α1* and *Pgc-1α4*.

While little is known about the role of chromatin remodeling in adult skeletal muscle, some studies suggest an important role in muscle plasticity. It was shown that another histone marker linked to gene activation, H3K4me3, is significantly enriched at the promotor of type 1 myosin in slow muscle, and is associated with type 2 myosin in fast muscle REF. Interestingly, when performing hindlimb

suspension, a model known to lead to atrophy and a shift to a fast MHC isoform, a significant increase in H3K4me3 occurs on the promotors of type IIx and IIb MHC in the slow soleus muscle. In addition to disuse, also the increased recruitment of muscles as occurs during exercise is linked to the activation of transcription factors important for muscle plasticity REF. Acetylation of histone 3 (on K36), which attenuates the interaction of histones with DNA, occurs immediately after an acute cycling bout (McGee et al, 2009). Class II histone deacetylases (HDACs) 4 and 5 were shown to translocate from the nucleus to the cytoplasm in response to exercise. CaMK and AMPK were shown to phosphorylate HDACs, allowing transcription factors to access promoters, notably MEF2, which in turn initiates transcriptional activity of exercise-responsive genes including myogenin, muscle creatine kinase (MCK) and glucose transporter 4 (GLUT4). (McKinsey et al, 2000) (Smith et al, 2008).

With regards to H3S10ph and its role in skeletal muscle not much is known. It was shown that exercise in human muscles leads to a small, but significant increase in histone phopsphorylation which occurs concomittantly with the activation of MAPK signaling, simlar to what we observe in mouse muscle. This is in contrast with what has been shown in cardiac muscle, where histone phosphorylation occurs through a CaMKII-dependent mechanism REF. Interestingly, in this paper it was shown that histone phosphorylation is required for 14-3-3 binding and subsequent MEF2 transcription. Using a mutant version of histone 3, in which serine 10 is substituted by an alanine, no binding of 14-3-3 occurs and also MEF2 transcription is blunted. This necessity of H3S10ph to increase 14-3-3 binding has been reported previously REF. Indeed, it has been shown that H3S10ph precedes the acetylation of lysine 14 on the same histone tail. Not only has this acetylation been linked to local opening of chromatin structure (Cheung et al, 2000b; Crosio et al, 2003; Lo et al, 2000), it also leads to the recruitment of 14-3-3 and stabilization of the phosphorylation by protecting it from phosphatases. This stabilization of the histone modifications makes it more likely for these histone modifications to have a more long-lasting effect on gene transcription. Interestingly, we find that immediately after eccentric contractions there is a strong increase in the double phosphoacetylation on histone 3. The best known histone acetyltransferase (HAT) of lysine 14 in histone 3 are the HATs

Hbo1 and Brd1. While Brd1 has been linked to increased neural activity (Fryland et al, 2012), and is suggestive of activity-dependent regulation, nothing is known about their roles in muscle, much less after exercise. While not much is known about the HATs, it has been reported that histone deacetylases (HDACs) translocate out of the nucleus after exercise, potentially increasing the effect of nuclear HATs on lysine 14. Indeed, HDAC-mediated repression of gene transcription leads to the reduction of MEF2 activity, a known mediator of activity-induced muscle plasiticity (Lu et al, 2000). In support of a further role for histone acetylation in muscle plasticity, is the fact that overexpression of HDAC5 is sufficient to prevent the exercise-induced change in muscle fibertype (Potthoff et al, 2007). While it was not addressed if this effect of HDACs is through its deacetylation of histone, or through direct modifications of transcription factors, it is suggestive of an important role for histone modifications in muscle plasticity.

As stated previously, not much is known about exercise-induced histone modifications. However, another model of activity-induced plasticity, namely neural activity in the hippocampus, has reported an involvement of H3S10ph. Indeed, H3S10ph was demonstrated to accompany ERK/MAPK pathway mediated activation of *c-fos* by agonists of dopamine (DA), muscarinic acetylcholine (mACh) and glutamate (GLU) receptor in hippocampal neurons, suggesting an important role of nucleosomal response in memory formation REF. Furthermore, it was recently shown that inhibition of histone phosphorylation prevents the formation of a persistent long-term potentiation in the hippocampus after noradrenaline administration (Maity et al, 2015). These results suggest a strong functional role for H3S10ph in neuronal memory formation, and makes it tempting to imagine a similar role in muscle remodeling during exercise.

While we show that multiple signals lead to H3S10ph, the intensity of the signals seems to depend on the intensity of the mechanical component. Increased loading on the muscle, as occurs during eccentric contractions, leads to a more pronounced increase than treadmill running, even though the total muscle recruitment is more in the latter. This difference can be explained by the fact that p38-signaling is more strongly activated by increased mechanical stress (Egan et al, 2010). Further supporting the hypothesis that H3S10ph in skeletal muscle reflects the activity levels of p38-signaling is the fact that AICAR leads to a mild

activation of p38 accompanied by a mild increase in H3S10ph. Also in other cell types AMPK activation has shown to promote p38 MAPK activation (Quan et al, 2015).

As mentioned, H3 phosphorylation is closely related to the activation of gene transcription. Generally, DNA that is upstream from the TSS lacks contact with nucleosomes, which enables transcriptional factors and Pol II binding and the onset of transcription (11, 29, 39). Nucleosomes that are downstream of the TSS play an important role in gene transcription, with histone modification such as histone H3 phosphorylation allowing for transcription to occur. Through ChIP-qPCR we found that H3 phosphorylation is significant increased in the TSS of *c-fos*, *Pgc-1α1* and *Pgc-1α4* genes after high intensity exercise. Moreover we found an significant increase in the expression levels of these genes after stimulation. Immediate-early genes, like *c-Fos*, have been linked to H3S10ph in multiple tissues (Mahadevan et al, 1991) (Chadee et al, 1999). In muscle, *c-fos* is an interesting candidate gene for muscle plasticity, as it has been linked to muscle hypertrophy (McBride, 2003). Furthermore, PGC-1α4 (Ruas et al, 2012) is one of the few transcriptional coactivators linked to muscle hypertrophy in response to exercise, while PGC-1α1 plays an important role in regulation of mithocondrial function and fuel usage

H3S10ph and SRF-dependent gene transcription

In this thesis we report two apparently distinct events which occur after high intensity exercise, namely an increase in MRTF-B-SRF dependent gene transcription and histone remodeling linked to gene activation. However, it has been reported that the immediate early genes, which are known to depend on H3S10ph, like c-fos and Jun, are also dependent on SRF transcription (Lee et al, 2010). Indeed, inducible deletion of SRF in the hippocampus of adult mice completely prevents the transcription of many immediate early genes after kainic acid administration (Kuzniewska et al, 2015), a well established stimulus leading to H3S10ph (Crosio et al, 2003). Currently, experiments are ongoing to address if the two stories as outlined in these thesis are indeed involved in the same genetic reprogramming (ChIP-seq for H3S10ph, and induction of SRF-dependent genes after exercise in MSK1/2 k.o. mice). If confirmed, we would propose a

mechanism by which high-intensity exercise leads to the phosphorylation of MRTF-B and H3S10, which together would induce an increase in SRF-dependent gene transcription, a major player in skeletal muscle plasticity.

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