Regulation of adult muscle phenotype by Ets-2

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Ivan Myhre Winje

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Now I know a lot of stuff about muscles, it is time to start using them…

Ivan Myhre Winje

Abstract

A unique characteristic of skeletal muscle is its heterogeneous nature and ability to acquire new functional characteristics to keep up with changing demands. These plastic properties are mainly regulated by activity. Distinct patterns of electrical stimuli, exerted by motorneurons, are sufficient to activate separate transcriptional programs that affect muscle strength, speed and endurance. Elucidating the molecular pathways linking electrical stimulation to gene expression is necessary to explain the effects of training and neuromuscular disorders.

This work presents *erythroblastosis virus E26 oncogene homolog 2* (Ets-2) as a possible mediator in the signaling network coupling electrical signals to transcriptional responses. The Ets-2 protein has been shown to be more abundant in fast muscle, where it selectively translocates and binds an enhancer element necessary for transcription of the troponin I fast (TnIf) gene. The purpose of this work was to determine the wild type expression pattern of the two skeletal troponin I (TnI) genes, and investigate the possible effects of Ets-2 overexpression and knockdown on TnI isoform distribution.

An expression vector encoding Ets-2 was transfected into the "slow" *soleus* muscle in rats by *in vivo* electroporation. Myosin heavy chain (MyHC) fiber type distribution and TnI isoform expression among the transfected fibers were analyzed, and compared to sham transfected and normal controls. In a separate series of experiments, an expression vector encoding siRNA against Ets-2 was transfected into the "fast" *extensor digitorum longus* (EDL). Fiber type and TnI distribution among the transfected fibers were analyzed. To investigate a possible effect on TnI expression, the fluorescent intensity of the siRNA-transfected fibers were measured and compared against normal scramble transfected and normal control fibers.

Mapping of TnI expression showed the slow TnI isoform (TnIs) is confined to slow-twitch fibers and TnIf to fast-twitch fibers, however fast-twitch fibers in *soleus* were shown to co-express both isoforms. Overexpression of Ets-2 had no effect on TnIf expression or MyHC composition. Knockdown of Ets-2 resulted in a 16% reduction of MyHC 2b fibers accompanied by 12% increase in 2a fibers. Measurements of TnIf fluorescence showed a minor reduction in TnIf staining intensity, however a larger sample material is needed to make any certain conclusions.

Abbreviations

ATP	Adenosine tri phosphate
CaM	Calmodulin
CaMK	Calmodulin-dependent protein kinase
Cn	Calcineurin
DMEM	Dulbecco's Modified Eagle Medium
EDL	*Extensor digitorum longus*
EMSA	Electrophoretic mobility shift assay
FIRE	Fast Intronic Regulatory Element
GLUT4	Glucose transporter 4
GTF3	General transcription factor 3
HDAC	Histone deacetylases
HEK-293	Human embryonic kidney cells
IRE	Internal regulatory element
mATPase	Myosin ATPase
MEF2	Myocyte enhancer factor 2
MRFs	Myogenic regulatory factors
MyHC	Myosin heavy chain
Native PAGE	Native polyacrylamide gel electrophoresis
NFAT	Nuclear factor of activated T-Cells
ORF	Open reading frame
PBS	Phosphate buffered saline
PNT domain	Pointed domain
RISC	RNA induced silencing complex
RNAi	RNA interference
SERCA	Sarco/Endoplasmatic reticulum Ca^{2+} ATPase
SiRNA	Small interfering RNA
SR	Sarcoplamatic Reticulum
SURE	Slow Upstream Regulatory Element
T115	Threonine 115
TBS	Tris-Buffered Saline
TBS-T	Tris-Buffered Saline Tween
Tm	Tropomyosin
Tn	Troponin complex
TnC	Troponin C
TnI	Troponin I
TnT	Troponin T
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

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1 Introduction

1.1 Fiber types

Since the middle of the 19th century scientists have distinguished skeletal muscles on the basis of their color and contractile properties (Ranvier, 1873). Based on these distinctions, two model muscles, one on each side of the spectrum has been established; The fast *extensor digitorum longus* (EDL) is described as a typical white and *soleus* as a typical red and slow contracting muscle (Lee, 1916).

Within the last 40 years our knowledge about muscle and muscle fiber diversity has rapidly expanded. Early work on the dynamic properties of muscle was based on the assumption that they were made up of a uniform population of fibers with similar mechanical properties. Modern classification arose with the identification of three distinct motor units, characterized by fiber type populations with different isometric contraction speed (Close, 1967). With the notion that fibers within the same motor unit were constrained to the same pattern of enzymatic activity, the correlation between fatigue resistance, twitch-speed and oxidative capacity was recognized (Edstrom and Kugelberg, 1968; Barnard et al., 1971).

The functional relationship between mechanical and biochemical properties was established in 1967, when Barany et al. (1967) provided evidence for the linear relationship between shortening velocity and the muscles' ability to hydrolyze adenosine triphosphate (ATP) via its myosin ATPase domain (mATPase). The same histochemical approach on serial sections led to the establishment of three main fiber types, designated type 1, type 2a, and type 2b (Brooke and Kaiser, 1970). The variations in mATPase activity was later shown to correlate with the expression of a specific myosin heavy chain (MyHC) isoforms (Reiser et al., 1985). With the subsequent development of immunohistochemical techniques and monoclonal antibodies, a fourth rodent MyHC isoform, 2x, was discovered (Schiaffino et al., 1989). According to its phenotypic features, this fiber was classified as an intermediate between 2a and 2b. This led to the current classification of skeletal muscle fibers, based on their individual expression of MyHC isoforms (Spangenburg and Booth, 2003).

Fibers expressing MyHC1 are commonly referred to as slow-twitch, or type 1 fibers. Being rich in myoglobin and oxidative enzymes, this sub-group of fibers produces ATP by oxidative phosphorylation, ensuring a steady supply of ATP for prolonged activity, hence their relative abundance in postural muscles (Schiaffino and Reggiani, 2011). Fibers expressing MyHC 2a, 2x, and 2b are commonly referred to as fast-twitch fibers. In the listed order they show an increased contraction speed, accompanied by a decrease in their mitochondrial content (Ingjer, 1979). As a result, fast-twitch fibers contain less oxidative enzymes and rely on anaerobic glycolysis for their ATP production. Some of the properties of different fiber types are listed in table 1.1.

Table 1. Overview of MyHC expression and physical properties of different fiber types in skeletal muscles of adult rodents

Fiber type:	MyHC isoform:	Shortening velocity:	Metabolic profile:	Endurance:
I	MyHC 1	Slow	Oxidative	Good
IIa	MyHC 2a	Fast	Oxidative-glycolytic	Good-medium
IIx	MyHC 2x	Faster	Glycolytic-oxidative	Medium-poor
IIb	MyHC 2b	Fastest	Glycolytic	Poor

Fiber type is classified according to MyHC expression, which is a major determinant of the shortening velocity. The metabolic profile reflects the endurance.

Twitch speed, i.e., the time course of tension development and decline in response to a single electrical stimulus, is not only dependent on the expression of a specific MyHC isoform, but is strongly related to expression of fiber type specific proteins involved in handling Ca^{2+} kinetics. Such proteins include Sarco/Endoplasmatic reticulum Ca^{2+} ATPase (SERCA) and the different subunits of the troponin complex (Tn). During muscle relaxation the active calcium transport mechanism into Sarcoplamatic Reticulum (SR) is based on SERCA pumps (Gundersen et al., 1988). In adult rodents there are two main isoforms expressed; SERCA1 is predominantly expressed in fast muscle fibers and SERCA2 in slow skeletal muscle, cardiac muscle, and smooth muscle (Periasamy, 2007). The activity of SERCA1 is greater than SERCA2, providing a faster uptake of calcium and faster relaxation time. Differential expression of subunits of the troponin complex affects the fibers' sensitivity towards Ca^{2+} (Perry, 1998) (further explained in section 1.4).

1.2 Plasticity of fiber type

Although adult skeletal muscle is composed of fully differentiated cells, they are highly susceptible to phenotypic alterations. Transition of phenotype is observed during aging (Larsson and Ansved, 1995), physical exercise (Thompson, 1994), hormonal influence (d'Albis et al., 1990) or experimentally by artificial stimulation of motorneurons or the muscle directly (Vrbova, 1963; Gundersen, 1985). This phenotypic plasticity is a result of the coordinated activation/repression of genes encoding different isoforms of contractile and metabolic proteins (Schiaffino and Reggiani, 1996). Changes in the pattern of neural influence are shown to have the most profound effect on phenotypic gene expression (Pette and Vrbova, 1985). This has been demonstrated in a series of classical cross-innervation studies were the fast EDL becomes slow when innervated by a "slow-nerve", similarly, the slow *soleus* adopt fast-twitch properties when innervated by a "fast-nerve" (Buller et al., 1960; Close, 1965). How the nerve managed to conduct these changes was elucidated by artificial stimulation of denervated *soleus* with a fast frequency pattern mimicking endogenous nerve activity (Lomo et al., 1974). This demonstrated that the electrical signal alone is sufficient to induce transformation in phenotype. Generally, slow-twitch motor units receive a high amount of impulses in low-frequency trains, while fast-twitch motor units seems to receive short bursts of high-frequent activity (Hennig and Lomo, 1985). The amount of activity seems to be important for the regulation of MyHC (Gundersen and Eken, 1992), while the frequency patterns seem to be important in determining the twitch duration (Westgaard and Lomo, 1988).

Denervation (interruption of the nerve connection) causes a gradual loss in fiber specificity; slow muscles become faster and fast muscles become slower (Gutmann et al., 1972; Gundersen, 1985; Schiaffino et al., 1988). Transitions of fiber type tend to follow a sequential and reversible scheme, from fast to slow and slow to fast: 1↔2a ↔2x ↔2b (Windisch et al., 1998). During transitional stages, hybrid fibers expressing two or more MyHC isoforms are common (Pette & Staron, 2000).

Muscle inactivity or decreased activity is associated with a shift of MyHC expression in a fast direction, accompanied by a reduction in fiber cross-sectional area (atrophy) (Gardetto et al., 1989; Pette and Staron, 2000). Endurance training is characterized by prolonged low-frequency activity, associated with an increase in the oxidative capacity of the muscle (Dudley et al., 1982; Carter, 2001). Depending on the duration and intensity, endurance training has been

reported to induce fast to slow MyHC transition (Sullivan et al., 1995; Allen et al., 2001). Resistance training is characterized by phasic high-frequency activity, commonly associated with an increase in fiber cross-sectional area (McCall and Byrnes, 1996).

In rodents, skeletal muscles are still immature after birth. Several important changes take place during the postnatal development, such as the elimination of the polyneuronal innervation (Brown et al., 1976), disappearance of embryonic and neonatal MyHC-isoforms (Schiaffino et al., 1988) and up-regulation of fast MyHC isoforms in prospective fast fibers (Russell et al., 1988; DeNardi et al., 1993). In rat *soleus*, an increase in the proportion of type I fibers is observed during postnatal development, a progress that continues throughout adulthood (Kugelberg, 1976). As the animal gets older, a progressive decline in shortening velocity is observed in EDL and the opposite is noted in *soleus* (Close, 1964).

Another factor involved in fiber type differentiation is the circulating level of thyroid hormones, which is low after birth and increase during postnatal-development until it reaches its maximum value at three weeks after birth (Gambke et al., 1983). Thyroid hormones have been implicated in the induction of fast MyHC expression during postnatal development. The transition from neonatal to adult MyHC is accelerated by hyperthyroidism and decelerated by hypothyroidism (d'Albis et al., 1990). The effect of thyroid hormone seems to be independent of the nerve, as daily injections of thyroid hormone in newborn rats causes a precocious increase in fast MyHC mRNA (Russell et al., 1988).

1.3 Signaling pathways regulating muscle plasticity

In eukaryotic cells, gene expression is mainly regulated at the level of transcription (Alberts et al., 2008). Cis-acting elements are short DNA sequences in the vicinity of the core-promoter, containing DNA consensus sequences that bind specific proteins (trans-acting factors), that can either repress or promote transcription (Buchwalter et al., 2004). As mentioned in the last section, the nerve is an important mediator of gene expression. How the nerve manages to conduct these changes is less clear. A reasonable assumption may be that there are some sensors that register the signal, which in turn activate/repress trans-acting factors resulting in phenotypic alterations.

In muscles, a lot of attention has been paid to the regulatory mechanisms of the calcium binding protein calmodulin (CaM). CaM has the ability to decode information carried by Ca^{2+} transients through activating a set of downstream proteins, including the calcium-dependent phosphatase calcineurin (Cn) and the calmodulin-dependent protein kinase (CaMK). CaM activates its substrates by allosteric regulation (Walsh, 1983). During the slow tonic-activity associated with slow-twitch fibers, sustained elevations in $[Ca^{2+}]_i$ facilitate the binding of CaM to Cn (Crabtree and Olson, 2002). Upon activation, Cn dephosphorylates multiple substrates; among them the nuclear factor of activated T-Cells (NFAT). Dephosphorylated NFAT translocates to the nucleus where it forms complexes with other transcription factors and participates in regulation of calcium-responsive target genes (Rao et al., 1997). *In vivo* overexpression of constitutively active NFATc1 in denervated EDL has been shown to induce the expression of MyHC I (McCullagh et al., 2004). Injections of Cyclosporine A, an inhibitor of Cn; has shown to increase the amount of fast MyHC isoforms in adult *soleus* (Chin et al., 1998). These findings demonstrate the importance of NFAT in the maintenance of a slow phenotype.

The transcriptional activity of NFAT is known to require the help of other transcription factors, among them the myocyte enhancer factor 2 (MEF2) (Wu et al., 2000). MEF2 cis-elements are found in promoters/enhancers of a number of muscle specific genes (Blaeser et al., 2000). Transgenic mice harboring MEF2 reporter constructs show that MEF2 activity is high during development and after endurance training (Wu et al., 2001). Treatment with Cyclosporine A results in decreased MEF2 reporter activity, suggesting that MEF2 is under the control of Cn (Wu et al., 2000). Several studies have demonstrated that MEF2 activity is controlled through association with class II Histone deacetylases (HDACs) (Lu et al., 2000; McKinsey et al., 2000), which bind and repress expression of MEF2-dependent genes by promoting the formation of heterochromatin (McKinsey et al., 2000). *In vitro* studies have shown that signaling by CaMK results in phosphorylation of class II HDACs, promoting shuttling of HDACs from the nucleus to the cytoplasm resulting in activation of MEF2-responsive genes (Lu et al., 2000).

Differential expression of the myogenic regulatory factors (MRFs), MyoD and myogenin, provides a possible link between phenotype and electrical activity (Ekmark et al., 2003). Being expressed in fast- and slow-fibers respectively; slow-patterned stimulation of denervated EDL result in an increased expression of myogenin (Hughes et al., 1993). Cross-innervation of a

fast-nerve to *soleus* results in increased MyoD expression followed by increased expression of MyHC 2 isoforms (Ekmark et al., 2007). MyoD is under the regulation of post-translational modification. Overexpression in denervated *soleus* results in slow-to-fast transition (Ekmark et al., 2007). When being overexpressed in innervated *soleus* no significant change is observed, due to the presence of a phosphorylated threonine (T115), which inhibits the transactivating ability of MyoD (Ekmark et al., 2007).

1.4 The troponin complex

The troponin complex (Tn) is at the center of Ca^{2+} mediated muscle contraction. Being located in the groove between the thin and thick filaments it prevents muscle contraction by inhibiting the initiation of the cross bridge cycle. In a relaxed muscle, the Tn associated protein tropomyosin (Tm) blocks the attachment-site for myosin on actin. Following depolarization, elevation in Ca^{2+} causes conformational changes in the complex that moves Tm out of the way, exposing the binding site on actin. Being a heteromeric protein, Tn is composed of three different subunits - Troponin T (TnT), Troponin C (TnC) and Troponin I (TnI).

TnT is the largest subunit of Tn, and derives its name based on its ability to form specific interactions with tropomyosin (Cabral-Lilly et al., 1997). It is the largest subunit of the complex, and is of particular importance in the structural positioning and stability of Tn along the myofibrils (Perry, 1998). In the mammalian genome, TnT has diverged into three isoforms corresponding to the cardiac, slow- and fast-skeletal fiber types (Schiaffino & Reggiani, 1996).

TnC is the subunit responsible of binding calcium and has diverged into two separate isoforms, each with a different sensitivity towards Ca^{2+} (Parmacek and Leiden, 1989). One isoform is expressed in fast skeletal muscle fibers and another in cardiac and slow skeletal fibers (Parmacek and Leiden, 1989). The differential sensitivity towards Ca^{2+} is related to the existence of a high affinity-binding site in the cardiac/slow isoform, whereas the TnC of fast fibers has two high affinity binding sites. As a result, slow fibers develop tension at lower $[Ca^{2+}]_i$ than fast fibers (Genchev et al., 2013).

TnI occupies a central position in the control of the contractile apparatus, where it acts as a link between Tn and myosin (Perry, 1999). Conformational changes resulting from the binding of calcium to TnC are conveyed through TnI to the mATPase domain of myosin where it

regulates sensitization of mATPase (Farah et al., 1994). *In vitro* studies using purified TnI have shown TnI-facilitated mATPase inhibition in the absence of Tm (Perry, 1998). In mammalian skeletal muscle two TnI isoforms are present, corresponding to fast and slow fiber-types (Hallauer et al., 1988). Interestingly, the expression of a respective TnI isoform is always coupled to the expression of the corresponding fast-/slow-twitch TnT isoform, suggesting a rather tight transcriptional regulation (Brotto et al., 2006).

1.5 Transcriptional regulation of the Troponin I genes

Studies investigating the transcriptional regulation of the troponin I genes were initially performed in cell culture, where the transcriptional regulation of rat troponin I slow (TnIs) and quail Troponin I fast (TnIf) could be analyzed during myoblast differentiation (Konieczny and Emerson, 1987; Banerjee-Basu and Buonanno, 1993). By using reporter constructs it was demonstrated that the 5´ flanking segments of the TnIf gene could be removed without having any notable effect on the normal expression pattern (Konieczny and Emerson, 1987). Instead, it was demonstrated that the developmental-specific transcription of TnIf is dependent upon an internal regulatory element (IRE) confined to the first intron (Yutzey et al., 1989). Transcriptional activation of TnIs was delineated using reporter constructs containing the 5´flanking sequence of TnIs, showing that the 200bp upstream of the translation initiation site was sufficient to confer the differentiation-specific expression associated with TnIs (Banerjee-Basu and Buonanno, 1993). Subsequent studies performing systematic deletions within the Intronic and the upstream elements led to the identification of the 144 bp Fast Intronic Regulatory Element (FIRE) and the 128 bp Slow Upstream Regulatory Element (SURE) (Nakayama et al., 1996). These elements contain series of regulatory cis-elements conferring general muscle and fiber specific expression (Calvo et al., 1996; Nakayama et al., 1996; Calvo et al., 1999). The relative order of the cis-acting elements is highly conserved (Fig. 1), and the interactions between the different trans-acting factors may therefore be important in determining the phenotypic outcome.

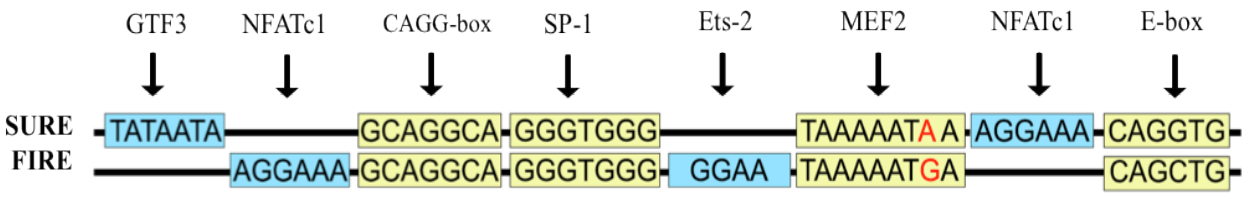

Figure 1. Regulatory elements in the Slow Upstream Regulatory Element (SURE) and the Fast Intronic Regulatory Element (FIRE). Simplified figure representing conserved sequence blocks conferring general muscle specificity (yellow) and cis-elements accountable for fiber specific expression (blue). Indicated by arrows are the target recognition sequences for trans-acting factors, which under the right circumstances may bind and

facilitate transcription/repression of the genes. The relative order of the cis-acting elements is highly conserved; interactions between various trans-acting factors may therefore be important in determining the phenotypic outcome. The figure is based upon data from (Hallauer et al., 1988; Yutzey et al., 1989; Banerjee-Basu and Buonanno, 1993; Calvo et al., 1996; Nakayama et al., 1996; Calvo et al., 1999; Calvo et al., 2001; Rana. et al., 2008; Rana. et al., 2011).

The functional influence of NFAT on TnI expression is diverse, with NFAT binding sites found in both SURE and FIRE (Fig. 1). *In vitro* studies show SURE activation in response to constitutively active Cn. By mutating the NFAT-element the effect was eliminated (Wu et al., 2000). NFAT has been proven necessary to inhibit FIRE expression during slow patterned activity (Rana. et al., 2008). siRNA mediated knockdown of NFATc1 and introduction of mutations in the NFAT binding-site have both shown to activate FIRE expression in adult *soleus*, without affecting the fast enhancer activity in EDL (Rana. et al., 2008). Together, these findings suggest a dual mechanism for transcriptional enhancement, where NFATc1 acts as a transcriptional activator and repressor in slow and fast-twitch fibers, respectively.

MEF2 is necessary for transcriptional activation of both TnI enhancers (Nakayama et al., 1996; Calvo et al., 1999). *In vitro* overexpression of Cn shows selective upregulation of reporter constructs harboring SURE (Wu et al., 2000), and when the SURE MEF2 consensus is mutated into the corresponding FIRE consensus (Fig. 1) the effect is abolished (Wu et al., 2000). In the same study it was demonstrated that the FIRE consensus provides a weaker binding site for MEF2, suggesting that MEF2 binds synergistically with NFAT in the activation of the SURE enhancer (Chin et al., 1998).

The general transcription factor 3 (GTF3) binds specifically to a bicoid like motif in the upstream region of SURE and represses activation of TnIs in developing and regenerating fibers (Calvo et al., 1999). During fetal development, the loss of TnIs expression in prospective fast fibers is associated with a period of high GTF3 expression (Vullhorst and Buonanno, 2003). *In vivo* overexpression of GTF3 has been shown to reduce transcriptional activation of reporter constructs harboring SURE, implicating GTF3 potential role in maintenance of phenotype in fast-twitch fibers (Calvo et al., 2001).

MRFs bind the E-box sequences found in the two TnI enhancers (Yutzey and Konieczny, 1992). Introductions of mutations (Calvo et al., 1999) or removal of the E-box sequence (Nakayama et al., 1996) abolishes the transcriptional activity of both elements. Since MRFs are known to form complexes with other trans-acting factors, including MEF2 (Molkentin et al.,

1995), they are believed to provide a scaffold for the recruitment and assembly of transcriptional complexes at the core promoter (Londhe and Davie, 2011). Trans-acting factors targeting the CAGG-box have not yet been identified, although their presence is essential for SURE and FIRE activation (Calvo et al., 1996; Calvo et al., 1999). In similar manner, the CACC-box, which binds SP-1 is necessary for activation of both enhancers (Bassel-Duby et al., 1994; Nakayama et al., 1996). An interaction between SP-1, myogenin and MEF2 is required for the activation of the cardiac actin gene (Biesiada et al., 1999), indicating that the formation of a multi-protein transcriptional complex and interaction between various transcription factors might be an important mechanism in the regulation of TnI.

In rodents, expression of TnI proceeds in two distinct stages according to the fetal and post-natal expression pattern. During fetal development, there is no confined expression pattern towards either fiber type, with TnIs and TnIf transcripts being widely expressed in all fibers (Calvo et al., 2001). As development continues, the uniform distribution segregates into prospective slow and fast fibers. Following birth, expression is highly upregulated in a motorneuron dependent fashion where TnIs and TnIf transcripts are confined to either slow- or fast-twitch fibers, respectively (Fig. 2) (Koppe et al., 1989; Calvo et al., 1996). Denervation causes a decrease in fiber-type specific transcription, resulting in expression levels similar to postnatal values (Rana et al., 2005). At protein level this expression is reflected as an increase in the population of hybrid fibers (Dhoot and Perry, 1981).

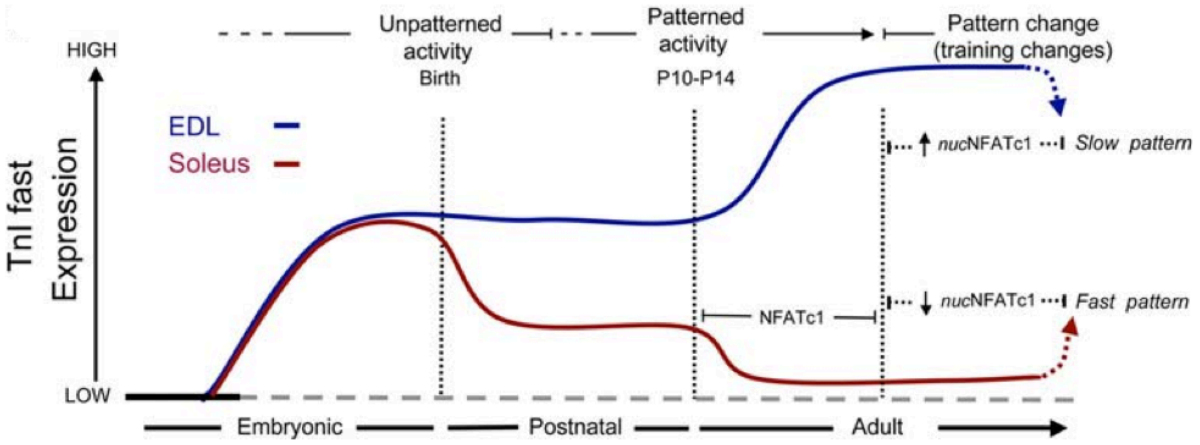

Figure 2. Model representing the regulation of Troponin I fast in maturing fast (blue) and slow (red) muscle fibers. The relative expression of TnIf varies during development and may be divided into three different stages according to the embryonic, postnatal and adult expression pattern. During prenatal development the expression is broad with no confined expression to either fiber type. After birth, emergence of patterned activity separates TnI expression into prospective fibers. (3) In fully developed fibers, trans-acting factors repress and maintain expression fast isoform in fast fibers and vice versa for slow fibers. This panel is from Rana. et al., (2008).

1.6 Ets Family of transcription Factors

The founding member of the Ets family, *v-ets* was first discovered as a fusion protein being expressed by the avian retrovirus E26 capable of inducing leukemia in infected chickens (Leprince et al., 1983). Cloning of cellular homologues (Watson et al., 1985) and through the observations that they were binding to similar DNA sequences, led to the establishment of ets-proteins as a new family of eukaryotic transcription factors (Karim et al., 1990). To date, at least 26 members have been identified in humans (Shaikhibrahim and Wernert, 2012) with family members ranging from primitive metazoans to mammals (Degnan et al., 1993) The common feature of the family is their highly conserved DNA binding domain, which binds to the purine rich sequence GGAA/T found in the promoter of multiple genes (Fig. 1) (Sharrocks et al., 1997). Most Ets-domain proteins are targets of signal transduction pathways and are directed to promoters through specific DNA-protein and protein-protein interactions (Wasylyk et al., 1998; Jayaraman et al., 1999). Ets proteins have been implicated in an array of different cellular functions and have roles in development, transformation and cellular proliferation (Maroulakou and Bowe, 2000; Oikawa and Yamada, 2003).

1.7 Ets-2

Although its function in human skeletal muscle is poorly understood, Ets-2 has been an intense field of study due to its high expression in a number of human malignancies (Galang et al., 2004; Seth and Watson, 2005; Xu et al., 2008). The ETS-domain of Ets-2 is located at its C-terminus, and the N-terminal portion of the protein contains two transactivation domains and a highly conserved pointed (PNT) domain (Wasylyk et al., 1998). Post-translational modification of the PNT-domain directly affects its function and subcellular compartmentalization (Foulds et al., 2004). In myoblasts, overexpression of Ets-2 increase the expression of Ctpr5 (Park and Kim, 2012), a protein involved in translocation of the glucose transporter 4 (GLUT4) and activator of AMP kinase. This proposes a putative involvement in regulation of glucose uptake and fatty acid oxidation (Lee et al., 2010). Ets-2 is a target of the MAP kinase pathway and CaMKII (Foulds et al., 2004; Yu et al., 2009), and is shown to couple extracellular signals to transcriptional response (Okamura et al., 2009) Binding of Ets-2 to the canonical (5´-GGAA-3´) binding site in the FIRE enhancer has recently been demonstrated by using electrophoretic mobility shift assay (EMSA) (Rana. et al., 2011) This binding site is located between the SP1 and MEF2 elements, factors which are known to form ternary complexes with Ets-domain proteins (Buchwalter et al., 2004). Microarray profiling has revealed selective upregulation in

denervated *soleus* when stimulated with fast patterned activity (Rana. et al., 2011). High frequency stimulation of denervated muscles transfected with GFP-tagged Ets-2 show an increase in the nuclear/cytoplasmic ratio of Ets-2 (Rana. et al., 2011), indicating that fast-patterned electrical activity may be an important mechanism for maintenance of nuclear Ets-2 in fast fibers (Fig. 2). Like NFATc1, that can either stimulate (Chin et al., 1998) or repress (Rana. et al., 2008) the activity of TnIs, Ets-2 might represent an equally important factor in maintaining a fast phenotype. The fact that Ets-2 accumulates in the nuclei in response to patterned activity allows for a dynamic regulation of its target genes.

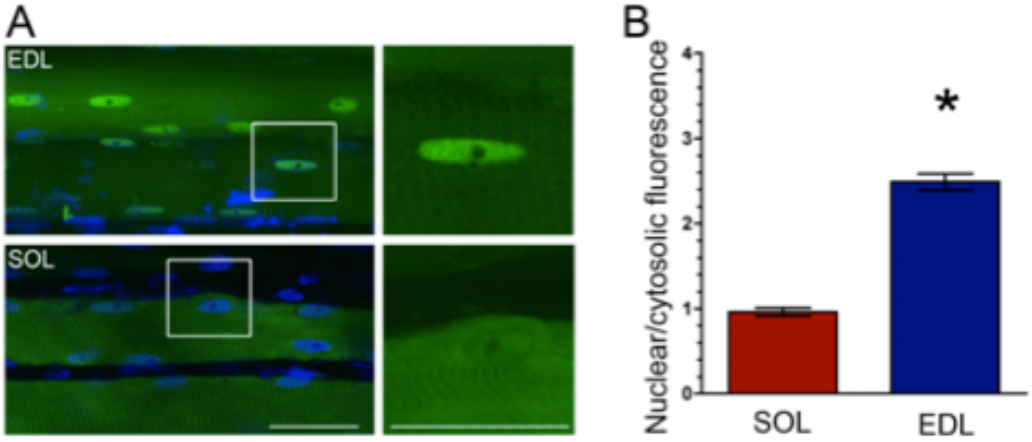

Figure 3. Nuclear shuttling of Ets-2. (A) Image is showing the intensity projections in unstimulated muscles transfected with a vector encoding GFP tagged Ets-2. In EDL Ets-2-eGFP selectively accumulates in the nucleus in response to fast patterned activity. In *soleus*, Ets-2-eGFP is uniformly distributed between nucleus and cytosol. Hoechst dye (blue) marks all nuclei (Bars= 20μm). B) Quantification of nuclear/cytoplasmic fluorescence intensity ratios. Each point represents the average of 3-10 nuclei/fiber from 8 rats. Graph shows that there is a significant accumulation of Ets2-eGFP in fast fibers of EDL. This figure is from Rana et al., 2009.

1.8 Aims of the study

What is the normal distribution of Troponin I isoforms in EDL and *soleus*?

To answer this question, normal muscles were excised, sectioned and stained for MyHC and TnI isoforms.

Will overexpression of Ets-2 change the phenotypic composition of adult *soleus* in a fast direction?

To answer this question, an expression vector encoding Ets-2 was transfected into adult muscle fibers of rat *soleus*. MyHC and TnI isoform expression in transfected fibers was analyzed after fourteen days and compared to normal non-transfected and sham transfected fibers.

Will knockdown of Ets-2 change the phenotypic composition of EDL in a slow direction?

To address this question, siRNAs targeting Ets-2 were transfected into muscle fibers of adult rat EDL. MyHC isoform distribution and TnI expression were analyzed and compared against fibers transfected with a scrambled siRNA sequence and normal control fibers.

2 Materials and Methods

2.1 Overview

In the first part of the study, the normal fiber type specific expression pattern of TnI in adult rats was determined. Untreated EDL and *soleus* muscles from 16 weeks old animals were sectioned and histochemically stained for TnI- and MyHC isoform expression.

In the second part of this study, *soleus* muscles of 10 adult rats were transfected with three plasmids; one expression vector encoding wild type Ets-2 (pEts-2), one sham control (pSham) and one plasmid encoding the reporter protein β-galactosidase (pLacZ), which were used to identify transfected fibers. The right *soleus* was transfected with pEts-2 and pLacZ and served as the experimental leg, while the left *soleus* was transfected with pSham and pLacZ, serving as a sham control. The transfer of DNA into the fibers was facilitated by *in vivo* electroporation. Fourteen days after surgery animals were anesthetized and muscles were removed and frozen in liquid isopentane (-160°C). Muscles were stored at -80°C and were later cryosectioned and histochemically stained for β-galactosidase activity. Fiber types from the different experimental groups were determined using various monoclonal antibodies against MyHC, and polyclonal antibodies against the troponin I isoforms.

In the third part of the study, EDL muscles of 12 adult rats were transfected with RNAi expression vectors encoding siRNA against Ets-2. The rats were divided into two groups; each group consisting of six rats transfected with a siRNA targeting different sites at the Ets-2 mRNA. In both groups the right leg served as experimental and the left as a sham control. All muscles were co-transfected with pLacZ. The procedures for analysis follow the same regime as the overexpression part of the study.

2.2 Animal experiments

2.2.1 Animals

All animal experiments were approved by the Norwegian Research Authority, and conducted in accordance to the Norwegian Animal Welfare Act of December 20th, 1974, no.37, chapter VI sections 20-22, and the Regulation of Animal Experimentation of January 15th 1996. The animal experiments were performed on 14 weeks old male Wistar rats, kept in the animal facility at Kristine Bonnevies Hus, University of Oslo. Groups of four to five animals were kept in cages in rooms regulated with a constant temperature of 22°C, air humidity at 50-60% and with regulated light cycles at 12h/12h intervals. Food and water were given *ad libitum*.

2.2.2 Anesthesia

Preceding surgical procedures, each animal was anesthetized with 2.0-2.5% isoflurane gas (506949, Forene, Abbot) with airflow of 500-700cc/min. The degree of anesthetization was determined by pinching the metatarsus region of the foot while observing the withdrawal reflex of the leg. Absence of reflex confirms that the animal is in deep anesthesia. Further anesthetics were administered if necessary.

2.2.3 Surgical procedures

When deep anesthesia was induced, hair from the leg was removed with an electric shaver followed by application of hair removal cream (Veet). EDL was surgically exposed by making an incision of 1cm at the lateral side of the leg; the distal tendon of EDL lies parallel with the distal tendon of *Tibialis Anterior*, which is easily identified as it lies on the lateral side of Tibia. *Soleus* was exposed by making an incision along the soleal line, a prominent ridge at the posterior side of tibia. During surgery the muscle and the surrounding area were continuously kept wet by applying ringer-acetate solution (131323, B. Braun Petzold). Following electroporation (below) the wound was closed with sutures (Softsilk, S-1172, 12 mm, Syneture).

2.2.4 *In vivo* electroporation

Uptake of plasmid DNA by electroporation/electropermeabilization is though to be facilitated by two separate events; permeabilization of the cell by the formation of small pores in the plasma membrane, and by electrophoresis that moves DNA into the cell (Somiari et al., 2000; Lu et al., 2003). There are several factors determining transfection efficiency, one of them being the quality of transfected DNA. DNA isolated with commercially available kits may contain large amounts of endotoxins, which have to be removed to avoid undesired inflammatory responses. Therefore, all vectors used in this study were purified by an endotoxin removal step and by isolation in a CsCl gradient (Engebrecht et al., 2001). Pulse pattern and field strength contributes to overcome the trans-membrane threshold of the fiber, with increasing diameter, a higher field strength is required to reach the trans membrane threshold (Lu et al., 2003).

In vivo transfection/electropermeabilization of plasmid DNA was performed as described by Mathiesen (1999). Following surgical exposure, an insulin syringe (0.3ml, BD Micro-Fine™, VWR) was used to inject 50µl of a 0.5µg/µl DNA solution (see appendix) into the belly of EDL or *soleus*. After injection, two silver electrodes (1cm long and 1mm thick) placed 2-3 mm apart were moved along the muscle. The electrical field was applied using a pulse generator (Pulsar 6bp, Fredrick Haer & Co). Five trains with 1 second intermission were applied over the muscle, each consisting of 1000 bipolar pulses with amplitude of 150V/cm, 200µs in each direction. The electrical charge was registered by an analogue oscilloscope (03245A, Gould Advance).

2.3 DNA constructs

2.3.1 Ets-2 expression vector

The vector used in this study was a kind gift from Professor H. Sugimoto (Dokkyo Medical University School of Medicine, Japan). It encodes an Ets-2 fusion protein covalently bound to enhanced green fluorescent protein (eGFP) (Fig. 4b). This vector has previously been used in studies concerning cytosolic-nuclear shuttling of Ets-2 (Okamura et al., 2009; Rana. et al., 2011). In the present study the aim was to investigate Ets-2 as a transcriptional activator. Expression of eukaryotic genes relies on the assembly of a pre-initiation complex at the core promoter, and because eGFP might interrupt this process, the wild type protein was restored by conventional cloning.

The full vector sequence was obtained by sequencing, (ABI 3730, high-throughput capillary electrophoresis sequencer). Sequences were aligned using Jalview V2 (Waterhouse et al., 2009), and imported into CLC Genomic Workbench 6 for the identification of unique restriction sites and open reading frame (ORF) analysis. Using site-directed mutagenesis (Quick-change, Stratagene, CA, USA), the 5´ HindIII recognition site located between the eGFP and Ets-2 reading frame were changed to AgeI (Fig. 4c). The experimental vector, pEts-2 (Fig. 4d) could now be generated by excision of eGFP via an AgeI restriction digest. The sham vector (pSHAM) (Fig 4e) was made by digesting pEts-2 with BamHI. ORF analysis was performed in CLC and all mutations were verified by sequence analysis. The vectors were transfected in human embryonic kidney (HEK-293) cells and western blots were run to verify protein expression (section 2.3.3). In addition, a reporter plasmid, pAP-lacZ (Fig. 4a), was used to identify transfected fibers. The 7.8 kb plasmid encodes β-galactosidase driven by a Rous sarcoma virus (RSV) (Mathiesen, 1999). This plasmid was a kind gift from Professor N. Gautam.

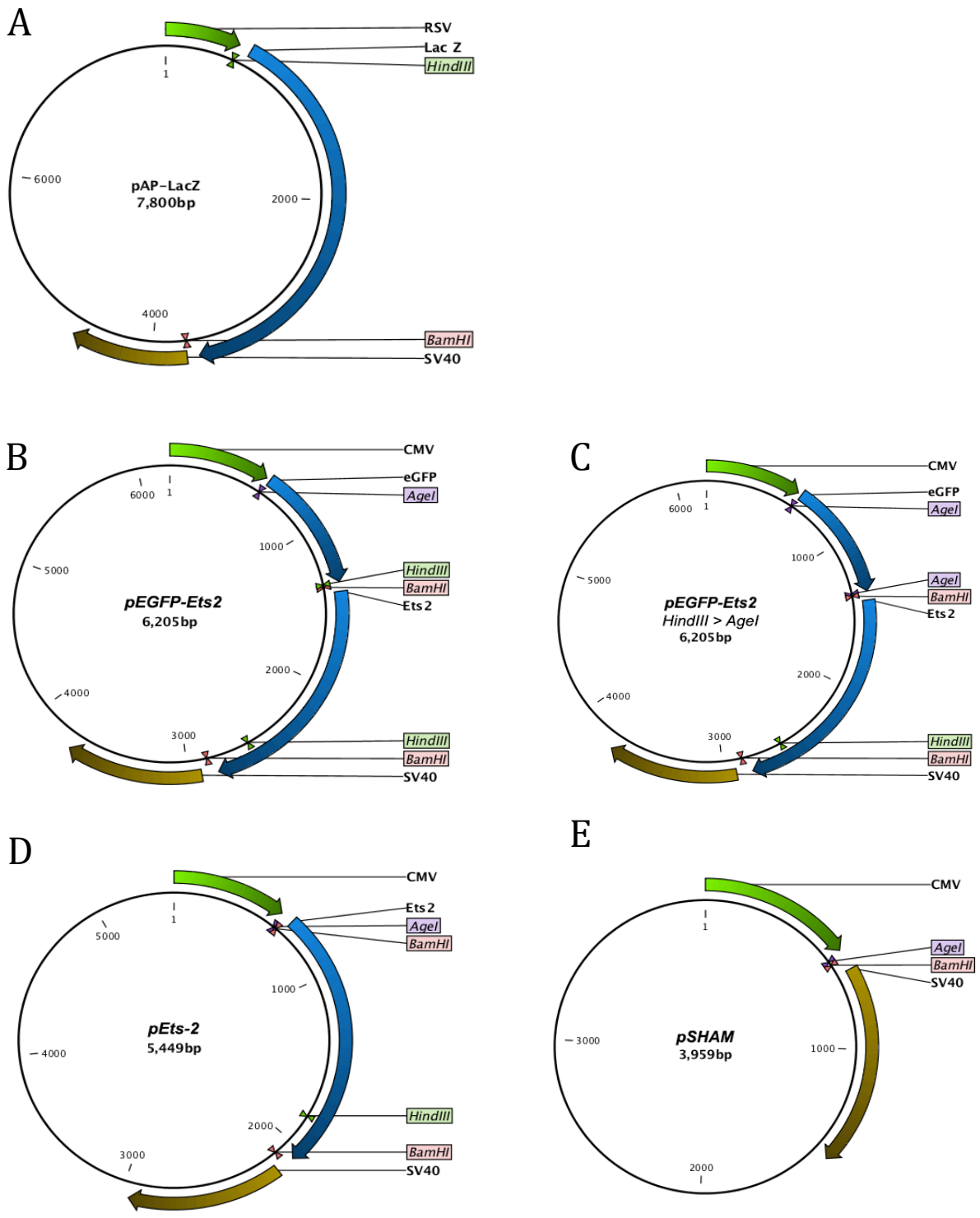

Figure 4. Expression vectors. (A) pAP-LacZ (gift from N. Gautam) with the Roussarcoma virus (RSV) promoter for enhanced expression of the LacZ gene in mammalian cells. A SV40 polyadenylation signals downstream of the LacZ gene direct proper processing of the 3' end of the LacZ mRNA. B) pEGFP-Ets2, a gift from professor Professor H. Sugimoto. Contained within the vector is a Cytomegalovirus (CMV) promoter for enhanced mammalian expression and a EGFP gene for identification of Ets-2. C) pEGFP-Ets2 after site directed mutagenesis, HindIII restriction site has been changed to AgeI. D) Experimental plasmid, Ets2 only, generated by excision of EGFP using AgeI restriction enzyme. E) Sham plasmid, generated by excision of Ets2 using BamHI.

2.3.2 siRNA expression vectors

During the last decade RNA interference (RNAi) has become an important tool in the field of functional genomics. Since its discovery, its ability to enable loss of function screens through degradation of specific mRNA molecules has shown to be a valuable resource in determining protein interaction and function (Moffat and Sabatini, 2006).

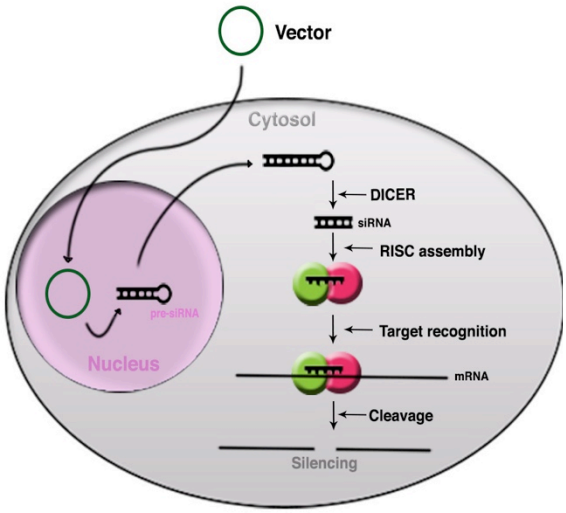

Figure 5. Mechanisms of RNA interference. An expression vector encoding the target sequence is electroporated into the cell. The primary transcript contains a hinge region followed by the reversed complimentary transcript, which facilitates formation of a hairpin loop. Presence of dsRNA activates Dicer, which cleaves the fragment into pieces of 18-24nt. Fragments are incorporated into the RNA induced silencing complex (RISC). One of the strands is degraded while the remaining strand facilitates recognition of target mRNA. Argonaute proteins confined within RISC catalyze cleavage of bound mRNA (Moffat and Sabatini, 2006).

GenScript is a commercial company, which provides siRNA vectors from submitted DNA sequences. Using a web-based computational algorithm an optimal target sequence is identified according to favorable thermo dynamical properties and GC-content, properties that affect the stability of the transcript. The program performs a BLAST search within the target organism to avoid overlap between endogenously produced mRNA transcripts and target siRNAs. By submitting the Ets-2 cDNA sequence from mouse (NM_011809.3) and rat (NM_001107107.1), two siRNA were predicted to knockdown expression of murine Ets2. The siRNAs sequences were cloned into a pRNA-CMV3.1/Neo expression vector allowing sustained expression of siRNA. Transcription of the siRNA gene is driven by a Cytomegalovirus promoter (CMV). An ampicillin resistance gene confined within the vector provides selection during transformation. In order to test desired knockdown of Ets-2, the RNAi vectors and Ets-2 expression vectors were co-transfected into HEK-293 cells and analyzed by western blotting.

2.4 Testing of DNA constructs

2.4.1 Transfection in cell culture

All expression vectors were transfected into HEK-293 cells. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, Invitrogen, Raisley, PA49RF, United Kingdom) at 37°C in an atmosphere of 5% CO_2. The cells were split every 4-5th day with trypsin EDTA 1:4, dependent on the confluence. Transfection of pEts-2 and pSham was carried out in six well plates in accordance with the Lipofectamine™ 2000 kit (11668, Invitrogen). RNAi vectors and pEts-2 were co-transfected into HEK-293 cells according to the Lipofectamine™ 2000 guidelines for co-transfection of RNAi and protein expression vectors (MAN0000995, Invitrogen).

2.4.2 Protein isolation and measurements

48 hours after transfection, the medium was removed and the cells put on ice before being washed twice in 1 ml cold PBS (70011-044, GIBCO). The cells were then lysed in 500 µl lysis buffer (see appendix). Cellular lysate was centrifuged at 13000 rpm for 20 minutes at 4°C. The supernatant was aliquoted and stored at -80°C until protein analysis. Protein concentration of cell lysates were measured according to the Bio-Rad Protein Assay Protocol (500-0006, Bio-Rad) and read at 595nm by a micro plate reader (Victor2 1420, Perkin Elmer).

2.4.3 Native PAGE and Western Blotting

To verify relative protein expression, lysates from transfected cells were run on a gel and immunoblotted with an antibody against Ets-2 (Sc-22803, Santa-Cruz Biotechnology). Native polyacrylamide gel electrophoresis (Native PAGE) was performed according to the NuPAGE Technical Guide (IM-1001, Invitrogen Instruction Manual (2003)). The gels were run with 20 µg protein sample on a 10 well NuPAGE® Novex 4-12% Bis-Tris Gels (NP0321, Invitrogen) at 150V. The size of the protein bands were determined using a protein ladder (See Blue Plus2 Prestained Standard, LC5925, Invitrogen). Western blotting was performed according to the Trans-Blot Electrophoretic Transfer Cell Instruction Manual (170-3939, Bio-Rad). The gels were blotted onto PVDF membranes for 4 hours at 1000mA/50V. The membranes were blocked in 5% skimmed milk powder (70166, Fluka) in TBS-T (see appendix) for 1 hour at room temperature before applying primary and secondary antibodies. Antibodies (Table 2) were diluted in 5% skimmed milk powder solution in TBS-T, and incubated for 1 hour at room temperature. After incubation the membranes were washed for 10min in TBS-T and rinsed

with TBS (see appendix). To ensure even loading on the gels, staining with a mouse monoclonal anti-Vinculin antibody (V9131, Sigma-Aldrich), followed by ECL Peroxidase labeled anti-mouse antibody (NA931VS, Amersham) was performed. Immunostaining was followed by visualization on film (28906837, Amersham) using the ECL Western Blotting Analysis System (RPN2109, Amersham).

Table 2. List of primary and secondary antibodies used in western blotting.

Protein:	Primary antibody:	Concentration:	Secondary:	Concentration
Ets-2	sc-22803, Santa-Cruz Biotechnology	1:1000	NA934V, Amersham (anti-rabbit)	1:2000
Vinculin	V9131, Sigma-Aldrich	1:40 000	NA931VS, Amersham (anti-mouse)	1:2000

2.5 Histochemistry

2.5.1 Freezing of muscles

After surgical removal, the muscles were slightly stretched between two pins on a home made form, and submerged in isopentane cooled to its freezing point (-160°C) with liquid nitrogen. The muscles were transferred to cryotubes (Microtube 2ml, PP, Sarstedt) and stored at -80°C until further processing.

2.5.2 Preparation of muscle sections

Muscles were cryosectioned in series of 10µm in a cryostat (CM1950, Leica Biosystems). During sectioning the sample held a temperature of -21°C and the knife -19°C. Serial sections from 10 - 12 muscles (right and left hind limb from the same animal) were placed on the same slide (Super-frost plus 2, Thermo Fisher), to achieve standardized conditions and exposure of all sections to near identical conditions during the following processing.

2.5.3 Staining for β-galactosidase activity

As previously mentioned, β-galactosidase was used as a reporter protein in the experiments to identify transfected fibers. The lacZ gene within pAP-LacZ encodes the enzyme β-galactosidase that breaks down β-galactosides. One of its substrates is X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside), which is broken down to indoxyl and galactose. Two monomers of indoxyl dimereize to form an insoluble blue compound. Therefore, transfected fibers will appear blue (Mathiesen, 1999).

2.5.4 Staining for MyHC isoforms

Monoclonal antibodies against MyHC-isoforms were used on serial sections to determine muscle fiber type. Secondary antibodies conjugated to FITC were used to visualize binding of the primary antibody to individual fibers (Table 3). When FITC is illuminated with blue-green light (488nm), it emits photons in the green region of the spectrum (495-519nm).

Table 3. Primary and secondary antibodies for detection of MyHC isoforms

MyHC:	Primary antibody:	Secondary antibody:
1	BA-D5	Rabbit Anti-mouse IgG FITC conjugated (F-9131, Sigma)
2a	SC-71	Rabbit Anti-mouse IgG FITC conjugated (F-9131, Sigma)
2x	6H1	Goat Anti-mouse IgM FITC conjugated (F-9259, Sigma)
2b	BF-F3	Goat Anti-mouse IgM FITC conjugated (F-9259, Sigma)

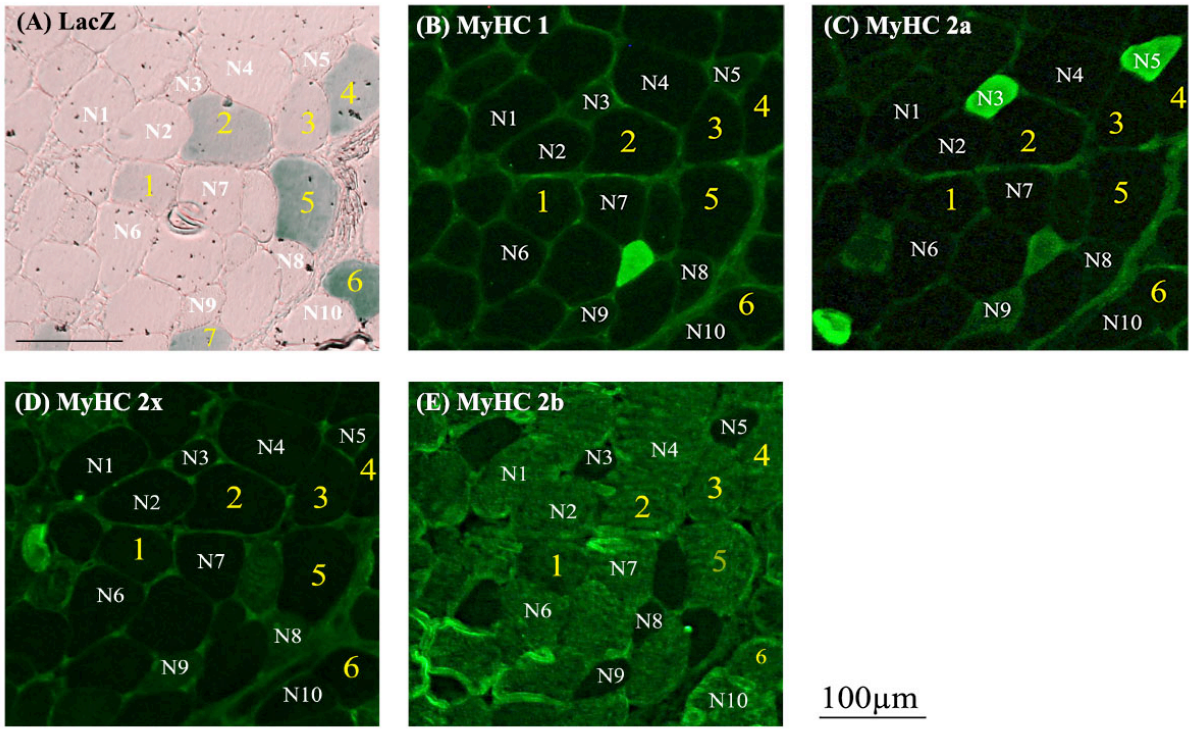

Figure 6. Example of serial sections of an EDL muscle stained for myosin heavy chain and β-galactosidase activity. Serial cross sections stained for (A) β-galactosidase activity, (B) MyHC 1, (C) MyHC 2a, (D) MyHC 2x, and (E) MyHC 2b. Fibers stained for β-galactosidase appear blue (numbered 1-7 in yellow), while non-transfected normal fibers for control appear bright (numbered N1-N10). Positively stained fibers for the respective MyHC isoforms appear bright green, while negative fibers appear dark. Scale bar = 100µm.

2.5.5 Staining for Troponin I isoforms

To determine the isoform-specific expression of troponin I, polyclonal antibodies against TnIf and TnIs were used on neighboring sections. Secondary antibodies conjugated to FITC or Alexa were used to visualize binding of the primary antibody.

Table 4. Primary and secondary antibodies used for detection of troponin

Isoform:	Primary antibody:	Secondary antibody:
TnI fast	Ab134838	Goat Anti-rabbit IgG FITC conjugated (T6778, Sigma)/ Goat Anti-Rabbit IgG TRITC conjugated (F987, Sigma)
TnI slow	Ab97711	Donkey Anti-sheep, Alexa Fluor® 488 (A11015, Invitrogen)

2.6 Imaging

2.6.1 Bright-field imaging

Bright field images of muscles stained for β-galactosidase activity were taken using a single-lens camera (Canon 60D) connected to a microscope (BX50W1, Olympus). The sections were photographed with water immersion at 10x magnification. Images were imported into Photoshop CS6 for further processing.

2.6.2 Fluorescent microscopy

Serial sections stained with fluorophore conjugated secondary antibodies were photographed in a dark room with the same microscope setup as with bright-field imaging. A blue-green filter (XF22) was used to illuminate the sections with the FITC and Alexa Fluor® 488 conjugated secondary antibody; while a green filter (XF53) was used to illuminate the sections with TRITC conjugated antibodies. Further processing was performed as with bright-field imaging.

2.6.3 Quantification of TnIf expression

TnIf expression was measured in muscle fibers transfected with siRNA, scrambled sequences and in randomly selected normal fibers within the same area. High-resolution images (40x) were captured of transfected fibers. Images was converted to 8bit, where the gray value is presented on a scale ranging for 0 (black) to 255 (white). TnIf intensity was quantified by the measurement of grey tone of manually encircled muscle fibers in ImageJ 1.46 (NIH, USA). In order to correct for photo bleaching, the mean gray value of normal MyHC IIb fibers in the same image was set to 1, and intensity of other fibers was normalized in relation to this value. The intensity of analyzed fibers was thus presented as a relative value in order to account for the potential differences in staining or bleaching of individual sections.

2.7 Statistical procedures

The films of the western blots were scanned and the band intensities measured with ImageJ 1.46 (NIH, USA). For statistical comparison of the relative protein expression (i.e. band intensities) in gels loaded with cell-lysate from the siRNA experiment, a one-way ANOVA with a Newman-Keuls post-test was performed. The level of significance was set to 0.05. For statistical comparison of fiber type distribution a Chi-square test was performed. The level of significance was set to 0.05. For statistical comparison of the fluorescence intensity measurements, a one-way ANOVA with a Newman-Keuls post-test was performed. The level of significance was set to 0.05. All statistical calculations were done in GraphPad Prism 6.

3 Results

3.1 Qualitative assessment of TnI expression.

Histochemical staining for Troponin I isoforms was performed on muscle cross-sections from normal non-treated rats in order to visualize potential differences in the expression patterns between the typical slow leg muscle, *soleus*, and the typical fast leg muscle, EDL. Expression patterns of TnI were compared to MyHC expression by histochemical staining of serial cross sections to determine whether the fibrillar expression of fast and slow TnI correlates with the expression patterns of fast and slow MyHC, respectively.

3.1.1 TnI expression pattern in EDL.

In EDL (Fig. 7), TnIs expression is restricted to MyHC 1 positive fibers. Expression of TnIf was confined to fibers expressing MyHC 2. These findings show that TnI expression in EDL follows the "normal" pattern, with TnIs confined to slow-twitch muscles and TnIf to fast-twitch fibers (Perry, 1999).

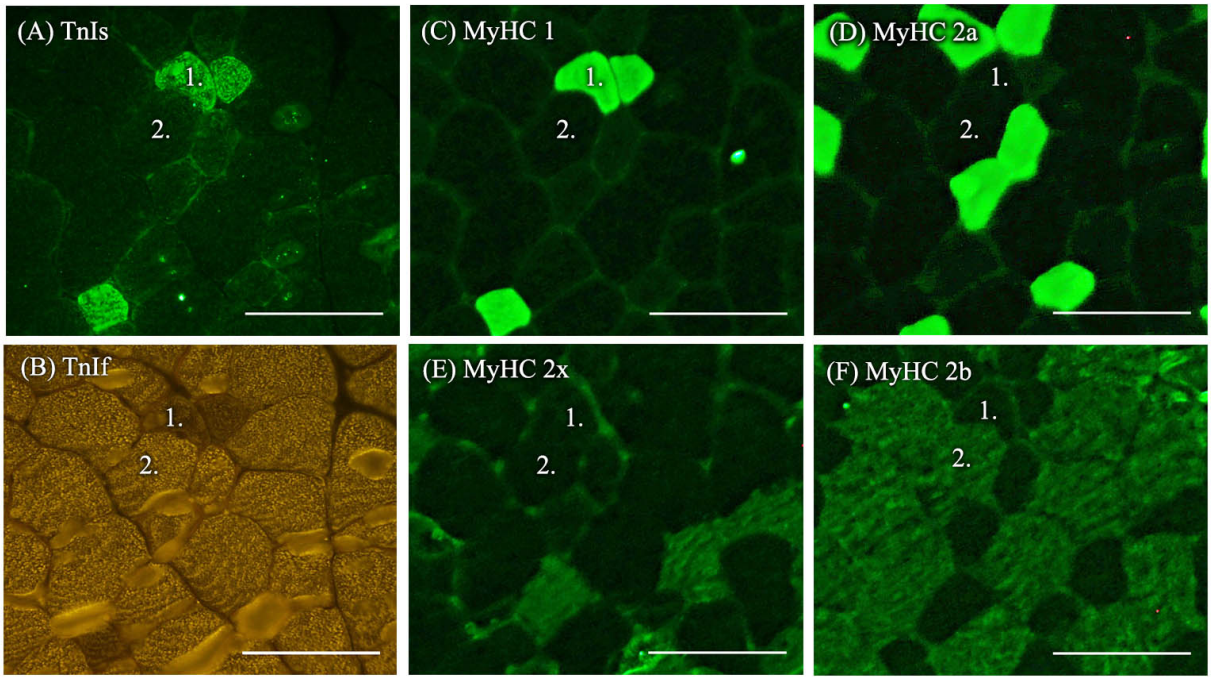

Figure 7. TnI and MyHC isoform expression in normal EDL. Serial cross sections stained with antibodies against (A) TnIs, (B) TnIf, (C) MyHC 1, (D) MyHC 2a, (E) MyHC 2x, and (F) MyHC 2b. The findings indicate that TnI follows the "normal" expression pattern in EDL, were TnIs are being expressed in MyHC 1 positive fibers and TnIf in fibers positive for type 2 MyHC. Examples of a MyHC 1 and 2a positive fiber are denoted as (1.) and (2.), respectively. Negative control sections stained only with secondary antibodies gave no fluorescence for any of the antibodies used (data not shown). Scale bar = 100µm.

3.1.2 TnI expression in hybrid fibers of EDL.

Hybrid MyHC 1/2a fibers in EDL express both TnI isoforms. Hybrid fibers are shown with white arrows (Fig. 8).

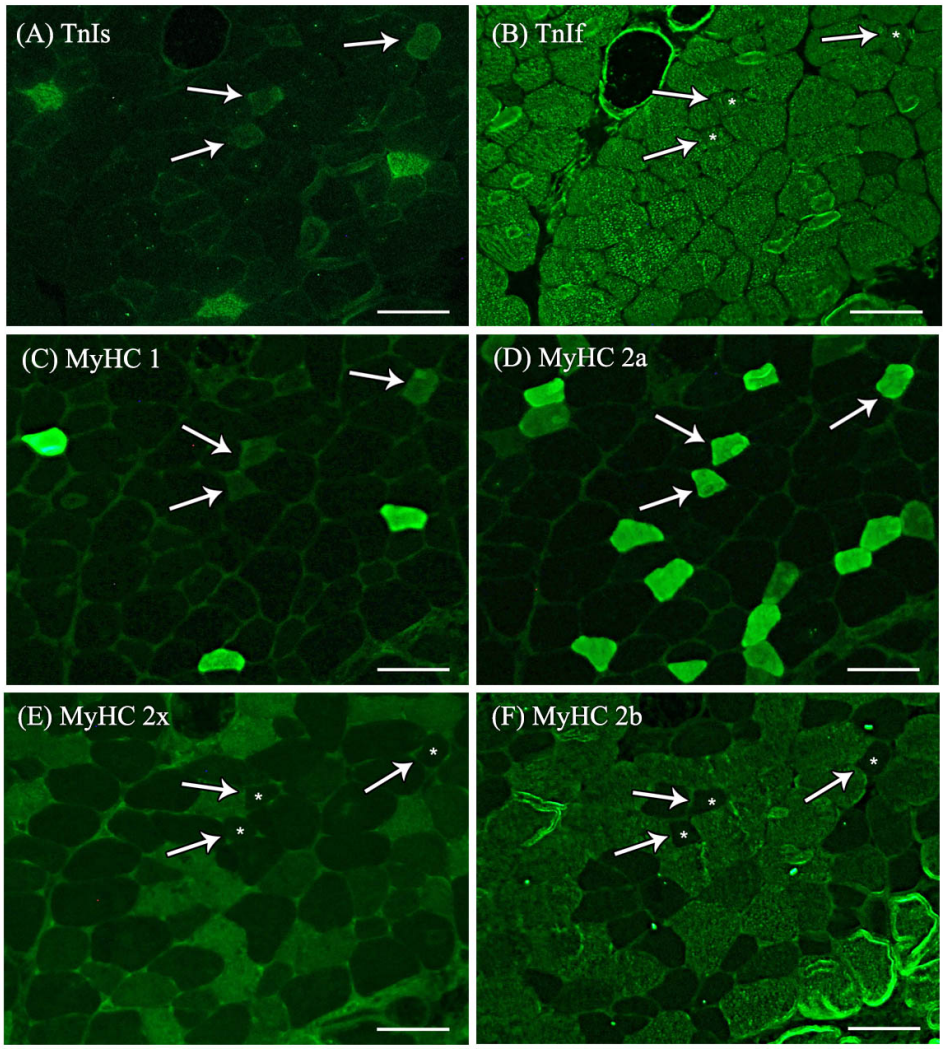

Figure 8. Hybrid fibers in normal EDL. Figure show cross sections staining with antibodies against (A) TnIs, (B) TnIf, (C) MyHC 1, (D) MyHC 2a, (E) MyHC 2x, and (F) MyHC 2b. White arrows indicate hybrid fibers that are positive for MyHC 1 and MyHC 2a. In figure B, E and F, hybrid fibers are indicated by an additional asterisks. Negative control sections stained only with secondary antibodies gave no fluorescence for any of the antibodies used (data not shown). Scale bar = 100μm.

3.1.3 TnI expression pattern in *soleus*.

In *soleus* (Fig. 9), MyHC 1 expression was found to be co-expressed with TnIs. MyHC 2a fibers were shown to stain positive for both TnI isoforms, reveling that fast fibers in *soleus* appear hybrid with respect to TnI expression.

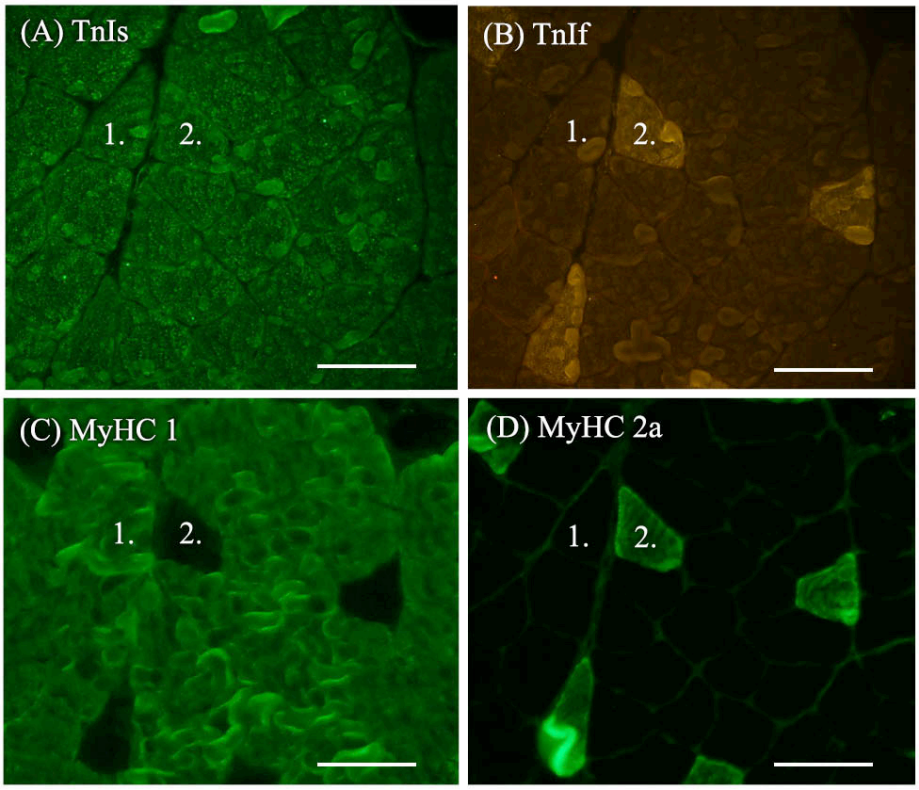

Figure 9. TnI and MyHC isoform expression in normal *soleus*. Serial cross sections of rat *soleus* stained with antibodies against (A) TnIs, (B) TnIf, (C) MyHC 1, and (D) MyHC 2a. Examples of a MyHC 1 and 2a positive fiber are denoted as (1.) and (2.), respectively. Note that MyHC 2a fibers are positive for both TnIs and TnIf. Negative control sections stained only with secondary antibodies gave no fluorescence for any of the antibodies used (data not shown). Scale bar = 100μm.

3.2 Overexpression of Ets-2 in *soleus*

3.2.1 Verification of Ets-2 expression in cell culture.

Expression and translation of the Ets-2 gene was tested in cell culture. HEK-293 cells were transfected with the experimental plasmid pEts-2 (Fig. 4d), and a sham group was transfected with an empty plasmid, pSHAM (Fig. 4e). Following protein extraction and native-PAGE, a western blot was run. The resulting blot was immunostained with an antibody against Ets-2, revealing a band of about 49 kDa in the lane transfected with pEts-2 (Fig. 10), a size consistent with the weight of the Ets-2 protein (Yu et al., 2009). No band was detected in the lane transfected with the sham plasmid.

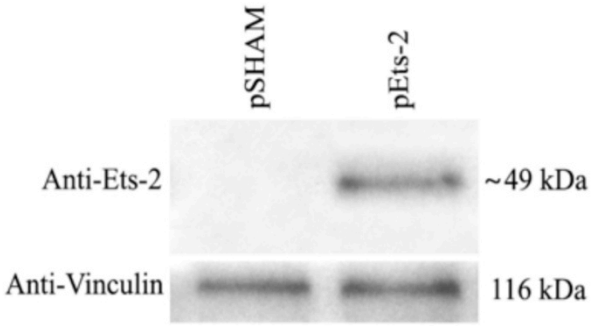

Figure 10. Expression of Ets-2 in tissue culture. Western blot of protein extracts from HEK-293 cells transfected with the Ets-2 expression vector pEts-2 or the sham plasmid pSham. The 49 kDa band represent the Ets-2 protein, visualized with an Ets-2 specific antibody. Staining with anti-Vinculin served as loading control.

3.2.2 Fiber type and isoform distribution in *soleus* overexpressing Ets-2.

Fourteen days after transfection, a total number of 452 fibers in three groups (normal control, sham and Ets-2) from 7 male Wistar rats were analyzed (Table 5). No change in fiber type distribution was significant when comparing the Ets-2 transfected, sham transfected and the normal control group. No semi-quantitative measurements of TnIf expression in Ets-2 transfected fibers were performed, because the quality of the samples precluded quantification.

Table 5. MyHC distribution in normal control, sham transfected and Ets-2 transfected *soleus*

Fiber type:	Normal control		Sham transfected		Ets-2 transfected	
	n:	%	n:	%	n:	%
1	287	95.7	22	100	122	93.8
2a	13	4.3	0	0	8	6.2
Total	300	100	22	100	130	100

There was no staining for 1/2a, 2x or 2b MyHC so these are excluded from the table

3.3 Effects of siRNA mediated knock-down in EDL

3.3.1 Verification of siRNA knockdown of Ets-2 in cell culture.

In this experiment, four different RNAi vectors were co-transfected with pEts-2 into HEK-293 cells. Two of the RNAi vectors were encoding siRNA targeting Ets-2 mRNA; the remaining two were encoding scrambled siRNA sequences, serving as negative controls. Cell lysates were run on gels and stained with an anti-Ets-2 antibody (Fig. 11a). The intensity values of the different bands were measured and compared with cells transfected with pEts-2 only (Fig. 11b). The degree of knockdown was measured as 57% and 56% for siRNA 1 and siRNA 2, respectively (Fig. 11b).

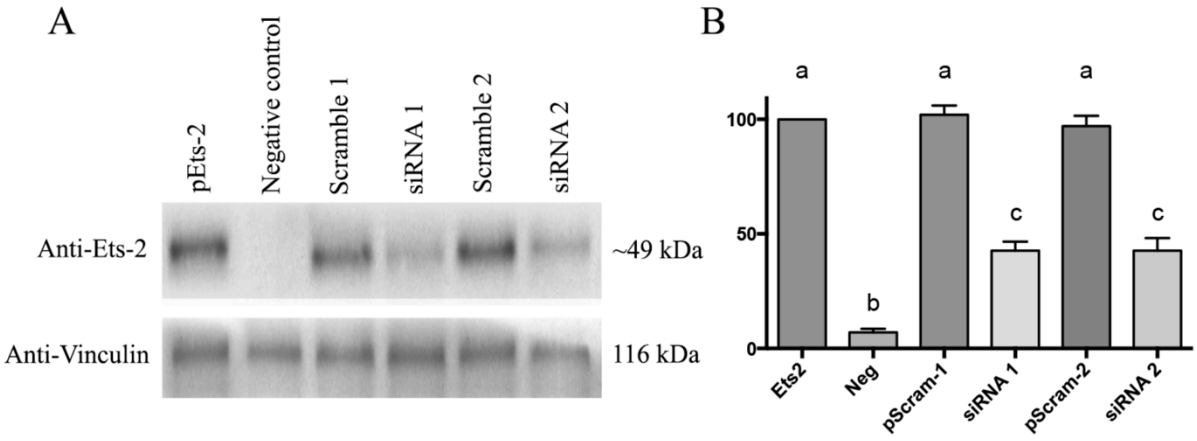

Figure 11. Ets-2 knockdown in HEK293-cells.
(A) Image of western blot. Upper row represents wells co-transfected with pEts-2 and RNAi vectors. Non-transfected HEK-293 cells were used as negative control, while the most left lane (pEts-2) served as positive control. Bands were visualized with an anti-Ets-2 antibody. Bottom row served as a loading control. Bands were visualized using an antibody against Vinculin. (B) A semi-quantitative assessment showing the relative amount of Ets-2 protein. n=3, SEM. All band intensities were normalized against the positive control. Intensity of the Ets-2 bands was normalized against the intensity of the vinculin band in the same well. Statistical differences are illustrated by dissimilar letters ($p<0.05$).

A significant difference was found when comparing the positive control lane (Ets-2 only) with lanes co-transfected with Ets-2 and siRNA 1 or siRNA 2. The CMV promoter located within pEts-2 is optimized for high expression in mammalian cells (GenBank Accession #: U57606), and considering the low amount of Ets-2 transcripts present in muscle (Rana. et al., 2011), the siRNA vectors might be sufficient to knockdown Ets-2, *in vivo*.

3.3.2 Effects of siRNA knockdown of Ets-2 on fiber type distribution in EDL.

A total number of 438 fibers in three groups (normal control, scramble, and RNAi transfected fibers) from 4 male Wistar rats were analyzed (Table 6). When comparing RNAi transfected fibers against normal and scramble control no significant differences were observed; however, a tendency towards a shift in the slow direction is seen in the siRNA transfected group. No significant change was found either when comparing fiber type populations in the normal against scramble control.

Table 6. MyHC distribution in normal control, scramble transfected and siRNA transfected EDL

Fiber type:	Normal Control:		Scramble transfected:		SiRNA transfected:	
	n:	%:	n:	%:	n:	%:
1	7	2.8	10	3.6	0	0.0
1/2a	1	0.4	4	1.4	0	0.0
2a	33	13.4	39	13.9	3	25.0
2a/2x	21	8.5	19	6.8	2	16.7
2x	55	22.4	63	22.5	2	16.7
2x/2b	28	11.4	40	14.3	2	16.7
2b	101	41.1	105	37.5	3	25.0
Total	246	100.0	280	100.0	12	100.0

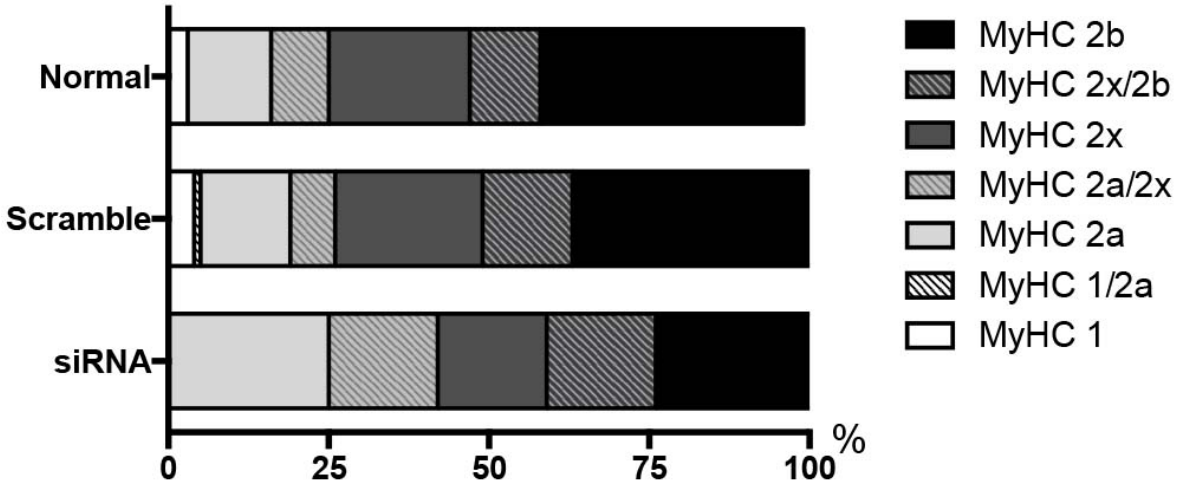

Figure 12. Fiber type distribution in EDL. EDL after RNAi and scramble transfection, and in normal controls. Total n is 438 fibers from 4 different animals. Hybrid fibers are shown in shaded boxes. No significant differences were found when comparing RNAi to normal or scramble control fibers, nor when comparing scramble and normal control fibers.

3.3.3 Semi-quantitative assessment of TnIf expression in Ets-2 siRNA transfected fibers in EDL.

The fluorescent intensity of siRNA and scramble transfected fibers was measured and compared against non-transfected control fibers. All siRNA- and scramble transfected fibers were of type 2 MyHC and were hence pooled (Fig. 13). A significant difference was found between the normal MyHC 1 and siRNA, scramble, and the normal MyHC 2 group. No significant differences were found between siRNA, scramble transfected and normal MyHC 2 control fibers. A small reduction in intensity is seen in the siRNA transfected group, but no preferential reduction was seen in any of the MyHC 2 subtypes.

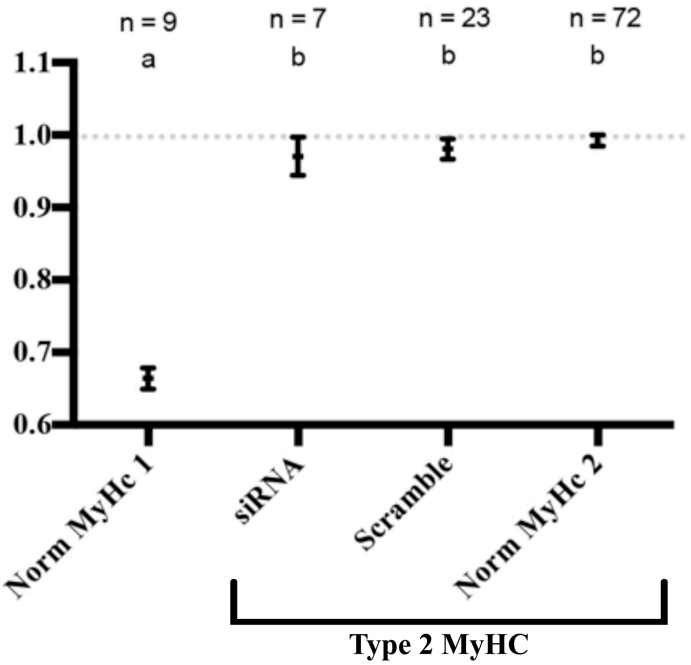

Figure 13. Relative intensity of TnIf. Figure represents the relative fluorescence intensity of fiber populations transfected with siRNA and scrambled siRNA sequences, compared with normal non-transfected control fibers. All siRNA and scramble fibers were of type 2 MyHC and were hence pooled. A significant difference in intensity was found when comparing normal MyHC 1 against the other populations. No significant differences were found when comparing siRNA, scramble and normal MyHC 2. Mean± SEM. Statistical differences are illustrated by dissimilar letters ($p<0.05$).

4 Discussion

4.1 Expression patterns of Troponin I

In the current study we demonstrate that the fibrillar expression pattern of the two troponin I isoforms is muscle type-specific. This study shows that MyHC 2a fibers in the slow *soleus* express both TnI isoforms, while MyHC 2a fibers in EDL express only TnIf. The finding is in agreement with Dhoot and Perry et al. (1979), who reported that a progressive increase in TnI hybrids is observed in aging *soleus*. In 12 week old animals, one third of the type 2 positive cells were reported to be TnI hybrids, whereas in animals older then 20 weeks, less than 10 % of the type 2 fibers stained for the slow isoform (Dhoot and Perry, 1982). In the present study, animals were sacrificed at 16 weeks of age, though all MyHC 2a fibers investigated were TnI hybrids.

On mRNA level, a study utilizing *in situ* hybridization with probes against the two TnI isoforms demonstrates that the labeling density of TnIs probes on type 2 fibers in *soleus* is higher when compared to fast fatigue resistant fibers in the fast *plantaris* muscle (Koppe et al., 1989). These findings may explain the muscle type-specific expression pattern observed in the current study.

It is well established that phenotypic variations may occur between the equal fiber types occurring in different muscles. (Larsson and Edstrom, 1986; Nakatani et al., 1999; Jouffroy et al., 2003). When comparing *soleus* and EDL, MyHC 2a fibers in *soleus* have a considerably larger cross sectional area than their counterparts in EDL (Larsson and Edstrom, 1986), demonstrating inter-muscular variation in MyHC 2a fibers of *soleus* and EDL. Thyroid hormone, T3, has been reported to influence MyHC 2a expression in a muscle type-specific manner (Izumo et al., 1986). Thyroid hormone causes an upregulation of MyHC 2a expression in *soleus*, but causes down regulation of the MyHC 2a gene with a different degree of sensitivity in fast-twitch muscles (Izumo et al., 1986). Denervation causes an increase in slow fibers expressing TnIf (Dhoot and Perry, 1982), this effect is counteracted by thyroidectomy (Dhoot and Perry, 1981). This indicates that T3 contributes to the differential regulation of the TnIf gene.

Due to the fact that *soleus* is a postural muscle, it would possibly be beneficial and more energy conserving if MyHC 2a fibers expressed both TnI isoforms, decreasing the twitch speed. In a study by R. Close (1967), measurements of the isometric twitch contraction times of single motor units demonstrated that the mean contraction time of fast fibers in EDL was 11 msec[*], compared to 38 and 17 msec for slow[†] and fast[‡] motor units in *soleus*, respectively. These findings propose that MyHC 2a fibers in *soleus* are slower than their equivalents in EDL, a difference that might be explained by the presence of TnIs in fast-twitch *soleus* fibers.

4.2 Trans activating abilities of Ets2 in slow fibers.

In the current study, *in vivo* overexpression of Ets2 in *soleus* had no significant effect on either MyHC distribution or TnI expression in adult slow muscles. Since fast patterned activity is a feature associated with fast-twitch fibers (Hennig and Lomo, 1985), no selective translocation of Ets-2 occurs in type 1 fibers (Rana. et al., 2011), possibly attenuating its trans-activating ability. If we were to assume that Ets-2 binds the TnIf FIRE enhancer under such circumstances, activated NFATc1 might repress a putative effect of Ets-2 on TnIf transcription. NFAT has been shown to reduce transcription of reporter constructs harboring FIRE during slow patterned activity (Rana. et al., 2008), so it is therefore reasonable to assume that activated NFAT may repress transcription of the TnIf gene *in vivo*.

Considering the fact that Ets-2 is naturally present in slow-twitch fibers (Rana. et al., 2011) (Fig. 3), one may assume that the trans-activational abilities of Ets-2 is under the regulation of post-translational modification, similar to MyoD (Ekmark et al., 2007). In cultured fibroblasts it has been demonstrated that stress induced elevation in $[Ca^{2+}]_i$ induces CaMKII mediated phosphorylation of Ets-2, directly affecting its subcellular compartmentalization and trans-activational abilities (Yu et al., 2009).

[*], n = 48. 10.8 msec ±1.07 (± standard deviation)
[†], n = 34. 38.0 msec ± 3.70
[‡], n = 4. 17.4 msec ± 1.70

4.3 Knockdown of Ets-2 in EDL

Due to low transfection efficiency, we were not able to determine whether knockdown of Ets-2 had an effect on fiber type distribution. However, a small increase in MyHC 2a fibers at the expense of 2x fibers was observed. Even though the sample size is small, the transition is in the slow direction, consistent with the hypothesis that Ets-2 maintains fast gene expression.

Whether knockdown of Ets-2 had an effect on TnIf expression in fast fibers is hard to interpret from these results; no significant decline in TnIf intensity was observed in type 2 MyHC fibers transfected with siRNA. In EDL, it has been demonstrated that the residual expression of TnIf remains around 50% two weeks after denervation (Rana et al., 2005). Under these circumstances, lack of fast patterned activity would cause a decline in the nuclear/cytoplasmic ratio of Ets-2 (Rana. et al., 2011). Rana et al. (2011) demonstrated that siRNA mediated knockdown of Ets-2 caused an approximately 50% decrease in the activity of reporter constructs harboring FIRE. These findings suggest that Ets-2 is necessary for high-level expression in fast-twitch fibers, consistent with our results. However, a reduction in transcription does not necessarily reflect a reduction in protein concentration (Lunde et al., 2011). Based on these findings, knockdown might cause a reduced transcription that we were not able to detect under the experimental setup.

4.4 Future prospects

Since the material in the knockdown was small due to variable transfection efficiency, future studies should focus on increasing the sample size. In the last decade, vector based recombinant adeno-associated viruses has emerged as a tool for exogenous gene transfer in skeletal muscle, providing high transduction efficiency (Blankinship et al., 2004). Commercial companies such as GenScript may provide viral vectors designed for overexpression or knockdown of desired genes. With a higher transfection (or transduction) efficiency it would have been interesting to quantify TnIf and Ets-2 transcripts in siRNA transfected fibers (Wacker et al., 2008), such investigations could further supported our findings. It would have been interesting to use mass spectrometry to investigate possible modifications of the Ets-2 protein, isolated from fast-twitch and slow-twitch single fibers (Yu et al., 2009; Bantscheff et al., 2012).

4.5 Conclusions

- Fast MyHC 2a fibers in *soleus* express TnIs and TnIf, while MyHC 2a fibers in EDL express only TnIf
- Overexpression of Ets-2 has no effect on MyHC fiber type distribution in *soleus*.
- Knockdown of Ets-2 has no significant effect on MyHC fiber type distribution or TnIf expression in EDL.

5 Appendices

5.1 DNA electroporation solutions

5.1.1 pEts-2 and pAP-LacZ solution (200 µl)

Solution:	Amount:
pEts-2 in TE buffer (2 µg/µl)	25 µl
pAP-lacZ in TE buffer (2 µg/ul)	25 µl
4 M NaCl	8 µl
dH_2O	142 µl

5.1.2 pSHAM and pAP-LacZ solution (200 µl)

Solution:	Amount:
pSHAM in TE buffer (2 µg/µl)	25 µl
pAP-lacZ in TE buffer (2 µg/µl)	25 µl
4 M NaCl	8 µl
dH_2O	142 µl

5.1.3 RNAi vector and pAP-LacZ solution (200 µl)

Solution:	Amount:
RNAi vector in TE buffer (2 µg/µl)	25 µl
pAP-lacZ in TE buffer (2 µg/ul)	25 µl
4 M NaCl	8 µl
dH_2O	142 µl

5.2 siRNA inserts

RNAi vector:	siRNA sequence:
siRNA 1	5´- AACGAGTTCAGCCTGGTGAAT - 3
scramble 1	5´- GTATGACGACGAACTCGTGTA - 3
siRNA 2	5´- AACGGCCAGATGCTGTGTAAC - 3
scramble 2	5´- GGACTTGCTCGATACACGGAA - 3

Sequences were cloned into the vector pRNA-CMV3.1/Neo

5.3 Cell culture

5.3.1 Cell lysis buffer

Solution:	Amount:
50 mM Trisacetate pH 7 12 g	12.00 g
0,27 M Sucrose 184.4 g	184.40 g
1 mM EDTA 0.75 g	0.75 g
1 mM EGTA 0.76 g	0.76 g
1 mM Sodium Orthovanadate	20 ml stock
10 mM B-glycerophosphate	6.30 g
50 mM Sodium Fluoride	4.20 g
5 mM Sodium Pyrophosphate	4.46 g
1 % Triton X-100	20.00 ml

- Make up to 2.0 l with distilled water.
- Before use (per 50 ml buffer), add 50 µl of protease inhibitor phenylmethane-sulphonylfluoride (PMSF), 4mM Benzamidine and β-mercaptoethanol.

5.3.2 10% FCS DMEM (Dulbecco's Modified Eagle Medium)

Solution:	Amount:
DMEM (GIBCO)	500 ml
Fetal calf serum (Bio Whittaker)	50 ml
Penicillin/Streptomycin (Bio Whittaker)	5 ml

5.4 Western blotting

5.4.1 10X transfer buffer solution

Solution:	Amount:
Tris-(hydroxymetyl)aminomethane	60 g
Glycine	288 g

- Dissolve Tris and Glycine in 800 ml dH_20 before adjusting to 1.0 l.
- To make 1X transfer buffer, take 100 ml 10X transfer buffer, 100ml methanol and 800 ml dH_20.

5.4.2 10X TBS solution (Tris-buffer saline) and TBS-T

Solution:	Amount:
Tris-(hydroxymetyl)aminomethane	24.25 g
NaCl	292.20 g

- Dissolve NaCl and Tris in 800 ml dH_20 before adjusting to 1.0 l.
- To make TBS-T, take 100 ml of 10X TBS and 900 ml dH_20, then add 1ml Tween20 (P1379, Sigma-Aldrich), mix well.

5.5 Histochemistry

5.5.1 10X PBS (phosphate-buffered saline) solution

Solution:	Amount:
NaCl	80.0 g
KCl	2.0 g
Na_2HPO_4 x $2H_20$	14.4 g
KH_4PO_4	2.0 g

- Dissolve salts in 800 ml of dH_20
- Adjust volume to 1.0 l, and then the pH to 6.8

5.5.2 Staining for MyHC isoforms

- Remove sections from -80°C freezer and let the sections thaw for 30 min before removing the surrounding foil.
- Apply antibodies for the different MyHC isoforms.

MyHC antibodies and staining conditions

MyHC:	Primary antibody:	Concentration:	Incubation:
1	BA-D5	1:50 in 1% BSA in PBS	1 hour RT
2a	SC-71	1:50 in 1% BSA in PBS	1 hour RT
2x	6H1	100%	Overnight, 4°C
2b	BF-F3	100%	45 min 37°C

- Wash sections 3 x 5 min in PBS (pH 7.2).
- Use secondary antibody Rabbit anti-mouse IgG FITC conjugated (F-9137, Sigma) on primary antibodies BA-D5 and SC-71, in a 1:200 dilution in 1% BSA in PBS. Incubate for 1 hour at 37°C in a moist chamber.
- Use secondary antibody Goat Anti-mouse IgM FITC conjugated (F-9259 SIGMA) on primary antibodies 6H1 and BF-F3, in a 1:200 in 1% BSA in PBS. Incubate for 1 hour in a 37°C moist chamber.
- Wash sections 3 x 5 min in PBS (pH 7.2).

5.5.3 Staining for Troponin isoforms

- Remove sections from -80^{o}C freezer and let the sections thaw for 30 min before removing the surrounding foil.
- Use a hydrophobic slide marker (H-400, Vector) to encircle the groups of sections.
- Hydrate the sections in 5% BSA in PBS for 2 hours at room temperature.
- Apply antibodies for the different troponin isoforms.

Troponin I antibodies and staining conditions

TnI isoform:	Primary antibody:	Concentration:	Incubation:
Troponin I fast	Ab134838	1:1000 in 2% BSA in PBS	Over night, 4^{o}C
Troponin I slow	Ab97711	1:1000 in 2% BSA in PBS	Over night, 4^{o}C

- Wash sections 3 x 10 min in PBS (pH 7.2).
- Use secondary antibody Donkey Anti-sheep, Alexa Fluor® 488 (A11015, Invitrogen) on primary antibody Ab134838, in a 1:200 dilution in 1% BSA in PBS. Incubate for 1 hour at 37^{o}C in a moist chamber.
- Use secondary antibody Goat Anti-rabbit IgG FITC/TRITC conjugated (T6778 Sigma or F987, Sigma) on primary antibody Ab97711, in a 1:200 in 1% BSA in PBS. Incubate for 1 hour in a 37^{o}C moist chamber.
- Wash sections 3 x 5 min in PBS (pH 7.2).

5.5.4 Staining for β -galactosidase activity

- Remove sections from -80°C freezer and let the sections thaw for 30 min before removing the surrounding foil.
- Use a hydrophobic slide marker (H-400, Vector) to encircle the groups of sections.
- Make X-gal fix solution.

X-gal fixative solution

Solution:	Amount:
Formaldehyde 36% (Rectapur)	4.0 ml
Glutaraldehyde 50% (EMS)	0.5 ml
1X PBS, pH 7.2	196.0 ml

- Add a few drops of ice-cold fix solution for 4 min in a fume hood.
- Wash slides thoroughly 3 x 10 min in PBS.
- Make X-gal staining solution.

X-Gal staining solution

Solution:	Amount:
0.2 M Potassium ferricyanide	30 µl
0.2 M Potassium ferrocyanide	30 µl
1.0 M Magnesium cloride	3 µl
10X PBS (pH 6.8)	150 ml
dH20	1260 µl
X-gal DMSO (50µg/µl) (Promega)	30 µl

- Stain overnight at 32°C in a moist chamber.
- Wash sections 3 x 5 min in PBS.

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