Effects of SMPX on skeletal muscle in adult mice

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2009

Preface

This thesis is based on studies performed between 2008 and 2009 at the Department of Molecular Biosciences, University of Oslo under the supervision of Professor Kristian Gundersen.

Firstly, I would like to thank Kristian. He has always been helpful in discussing difficult matters I have encountered throughout this masters project, and he has been especially helpful in the writing process. I admire him for always saying what he means, and for always giving constructive feedback.

Secondly, I would like to thank Dr. Jo C. Bruusgaard for being an enthusiastic co-supervisor and a great help in dealing with technical difficulties both in the laboratory and with the processing of the results.

Thirdly, I would like to thank Dr. Zaheer A. Rana for his much needed help with the troublesome molecular methods, and Siobhan Anton for being very helpful in the cell culture lab.

I would also like to thank Julie, Ingrid and the whole group for making my time here valuable, both scientifically and socially.

Oslo, Juni 2009

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Abstract

Mechanical factors are important in the regulation of muscle phenotype. Adaptation of structural and mechanical proteins to withstand increased load and cope with increased work is needed in a muscle exposed to mechanical stimuli such as stretch.

Small muscle protein X-chromosome (SMPX) is a protein upregulated in stretched skeletal muscle, and could therefore work as a structural protein, a signalling factor in a mechanotransduction pathway, or a combination of both.

In my project I have studied the *in vivo* and *ex vivo* localization of SMPX in skeletal muscle, as well as the effects of overexpressing SMPX in the fast-twitch muscle *extensor digitorum longus* (EDL) and the slow-twitch muscle *soleus* in adult mice.

In vivo and *ex* vivo visualization of a SMPX-EGFP fusion protein suggests that the majority of SMPX is localized to the sarcomeric H-zone, with a minor portion possibly localized as a thin band to the middle of the Z-disc in adult skeletal muscle.

Overexpression of SMPX for 14 days gave no significant changes in fibre type distribution or cross sectional area in EDL. In *soleus* the results were variable, but there was a shift towards a faster fibre type and a less pronounced increase in cross sectional area.

Our experiments do not support the idea that SMPX works as a major regulatory protein or a signalling molecule related to force transduction in the I-band. If it serves as a regulatory protein, our results show that this could be true for *soleus*, but not for EDL.

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1. Introduction

Skeletal muscle is one of the most adaptive of all tissue types, and can change its phenotype and adapt to a variety of different conditions without cell death and regeneration. Adult skeletal muscles can modulate the expression of muscle specific genes and proteins in response to mechanical stimuli, hormones and the activity pattern they are exposed to. In response to these external stimuli, both individual muscles and muscle fibres are adapted to highly variable demands to strength, contraction speed and endurance. The pathway between external stimuli and gene expression is important in understanding these differences, and mechanical factors and their possible role in signalling is not well understood.

1.1 Mechanotransduction

The sarcomere is the specialized contractile unit of a skeletal muscle. In addition to its force generating structure; essential mechanical, structural and signalling proteins are also associated with regions of the sarcomere that do not directly participate in the actin-myosin interaction. The sarcomeric Z-disc, I-band, A-band, H-zone and M-line (see fig. 1.1) are all areas where proteins with one or more of these functions interact (e.g. review; Hoshijima, 2006; Kruger & Linke, 2009).

The giant elastic titin molecule with its spring-like structure running all the way from the Z-disc to the M-line, develops passive force in a nonlinear and highly isoform-dependent manner when stretched by external force (e.g. review Fukuda *et al.*, 2008). It is now increasingly recognized as a key area for protein-protein interactions and also a putative mediator of mechanosensory processes. Titin has so far been demonstrated to be an interacting partner for about 20 proteins, linking titin to multiple stress signalling pathways (Kruger and Linke 2009). Three titin regions are particularly engaged in the protein-protein interactions, the NH_2-terminal segment at the Z-disc, the elastic I-band part, and the COOH-terminal segment within and adjacent to the M-band (Kruger and Linke 2009).

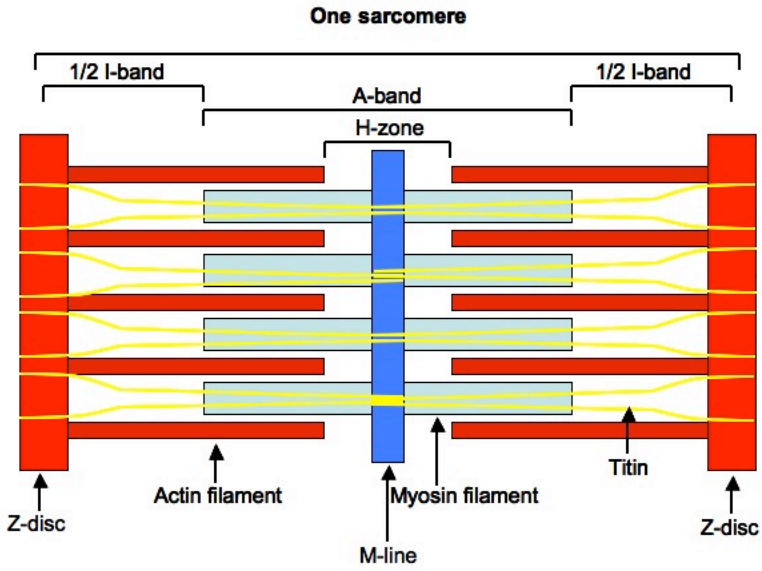

Figure 1.1 Schematic drawing of a skeletal muscle sarcomere
Only a small selection of the sarcomeric proteins is shown. Z-disc and actin filaments are shown in red, myosin filaments in turquoise and M-line in blue. Titin (yellow) is spanning from the Z-disc to the M-line, where it overlaps with titin from the opposite Z-disc.

In a structure like skeletal muscle, a force transmitting system from the contractile apparatus to the bone is needed. In addition to the myotendinous junction, exerting longitudinal force transmission, a transversal force transmission system has been implicated. Costameres are described as subsarcolemmal sites of cytoskeletal-membrane adhesion complexes, which connect the contractile apparatus with the cell membrane. They span over the Z-disc, M-line, and in some muscles parallel to the longitudinal axis of the muscle (L-line) (Pardo *et al.*, 1983; Bloch & Gonzalez-Serratos, 2003). Costameres are thought to reinforce the membrane and function as the link between the contractile elements and the extracellular matrix (ECM) (for all future abbreviations, see appendix B), exerting lateral force transmission (Bloch & Gonzalez-Serratos, 2003). The number of cytoplasmic costameric proteins is large and still growing (Bloch *et al.*, 2002). Recent studies suggest that this elaborate protein network acts as a structural and signalling center for striated muscle, and that it also contains factors that can function as mechanosensors (Bloch *et al.*, 2002; Hoshijima, 2006).

1.1.1 Muscle gene regulatory proteins

A number of gene regulatory proteins are important for the differentiation and function of a skeletal muscle. A brief introduction to the ones that have been discussed in connection with SMPX follows.

The four myogenic regulatory factors (MRFs) MyoD, myogenin, Myf-5 and MRF4 form a subclass of a large group of transcription factors characterized by a common basic helix-loop-helix (bHLH) domain. They are exclusively expressed in skeletal muscle, and their immediate precursors are found to be important for expression of muscle specific genes during development (Krempler & Brenig, 1999). The MRFs all share a common DNA binding domain, which is central for its binding specificity to E-box DNA sequences. The MRFs function as heterodimers with a second bHLH protein from the E protein family, such as E47 (Murre *et al.*, 1989). The MRFs are expressed at different levels and times during development, with Myf-5 and MyoD highly expressed in the transformation phase from somite cells to myoblasts, and myogenin and MRF4 expression peaking during transformation of myoblasts into myotubes (Buckingham, 1994).

The expression of MRFs continues in the adult animal. MRF4 is present at relatively high levels compared to the other three transcription factors, with no distinct distribution pattern when comparing fast and slow muscles (Rhodes & Konieczny, 1989; Voytik *et al.*, 1993). Myf-5 is expressed at relatively low levels in adult skeletal muscles. MyoD and myogenin are differentially expressed; with highest levels of MyoD in fast muscle, and of myogenin in slow muscle, respectively (Hughes *et al.*, 1993; Voytik *et al.*, 1993). MyoD can induce fast MyHC (Ekmark *et al.*, 2007), while myogenin can induce higher oxidative capacity in adult skeletal muscle (Ekmark *et al.*, 2003).

Myocyte enhancer factor-2 (MEF2) proteins are another family of transcription factors that play a critical role in embryonic development (Potthoff & Olson, 2007). In adult skeletal muscle they can be a regulator of the stress-response during cardiac hypertrophy (Zhang *et al.*, 2002), and function in tissue remodelling in cardiac and

skeletal muscle, including formation of slow-twitch myofibres in skeletal muscle (Potthoff *et al.*, 2007).

The Nuclear factor of activated T cells (NFAT) proteins are a family of transcription factors that are activated by slow stimulation and deactivated by muscle inactivation. A constitutively active form of NFAT can induce slow MyHC and inhibit MyHC type 2b expression in adult fast muscles (Chin *et al.*, 1998; McCullagh *et al.*, 2004).

1.2 Smpx background

In a reciprocal probing screen in the search for unknown genes, Patzak *et al.* (1999) were the first to identify and characterize the *small muscle protein X-chromosome* (*Smpx*) gene, which is localized near the telomere on the X-chromosome. It is predominantly expressed in skeletal and heart muscle, and has been studied by several groups to try and deduce its *in vivo* function (Patzak *et al.*, 1999; Kemp *et al.*, 2001; Christoffels *et al.*, 2004; Schindeler *et al.*, 2005; Ren *et al.*, 2006; Larsen, 2007).

1.2.1 Structure

SMPX consists of 85 amino acids in mouse and 86 amino acids in human and pig, with no paralogs. (Patzak *et al.*, 1999; Kemp *et al.*, 2001; Palmer *et al.*, 2001). Both the gene- and protein sequence is highly conserved between the orthologs in vertebrates tested (frog, mouse, rat, pig and human) (Patzak *et al.*, 1999; Kemp *et al.*, 2001; Palmer *et al.*, 2001; Ren *et al.*, 2006).

Based on *in silico* methods, Patzak *et al.* (1999) suggested that SMPX is strongly coiled, with a 23 amino acid (aa) N-terminal hydrophobic domain. This could possibly act as a membrane-anchoring domain. The N-terminal part of SMPX shows the highest conservation between species, and this points to an important function of this part of SMPX. Furthermore, sequence comparison between human and mouse 5' upstream gene sequences of *Smpx* showed 87% identity; even higher than that seen in

the mouse and human coding sequence comparison (Kemp *et al.*, 2001). This suggests functional conservation within the regulatory region.

1.2.2 Tissue distribution and expression level

In addition to its primary expression in heart and skeletal muscle, SMPX has also been found at lower levels in liver, testis, kidney, lung, large intestine and brain in mice (Patzak *et al.*, 1999; Kemp *et al.*, 2001; Palmer *et al.*, 2001), and in stomach and backfat in pigs (Ren *et al.*, 2006).

Interestingly, in skeletal muscle, SMPX expression levels were highest in slow type I fibres, intermediate in fast type IIa and lowest in fast type IIb fibres (Palmer *et al.*, 2001).

Analyses of SMPX distribution also demonstrated that it has a higher expression level in developing heart and skeletal muscle of neonatal and postnatal mice when compared with corresponding adult tissue. (Palmer *et al.*, 2001). Later studies of the developing heart first detected SMPX in 8.25 day old mouse embryos located at the ventral surface of the heart progenitor region undergoing active fusion; and later in the outer curvature of the ventricles and in the atrial appendages (Palmer *et al.*, 2001; Christoffels *et al.*, 2004).

A SMPX knockout mouse was made to assess the function of SMPX in muscle development, but no obvious developmental or structural deficits in heart or skeletal muscle were discovered (Palmer *et al.*, 2001).

1.2.3 Predicted SMPX motifs and their possible functions

In the predicted 9.25kDa mouse and human SMPX a putative pat 7 nuclear localization motif, two overlapping casein kinase II (CKII) phosphorylation sites and a PEST sequence were found (Kemp *et al.*, 2001). A PEST sequence indicates instability (Rogers *et al.*, 1986), and when compared with other proteins known to undergo rapid degradation such as the transcription factors c-fos and c-myc; the SMPX PEST sequence was predicted to be even more unstable (Kemp *et al.*, 2001).

In the predicted 9.3kDa pig SMPX, the CKII sites and PEST sequence were also conserved (Ren *et al.*, 2006).

CKII is a protein kinase, and its expression has been detected both in the nucleus and in the cytoplasm of cells (Stigare *et al.*, 1993). CKII has been implicated in the regulation of the MRFs MyoD and MRF4, independent of their own CKII phosphorylation sites (Johnson *et al.*, 1996). This suggested that CKII may act indirectly on MRFs via regulation by other proteins. Kemp *et al.* (2001) proposed SMPX as a candidate for this intermediate regulation of MRFs. Kemp *et al.* (2001) also suggested that phosphorylation of SMPX may regulate its function and cellular localization. CKII activity also increased as myoblasts differentiated and fused to form myotubes (Johnson *et al.*, 1996).

1.2.4 What can regulate SMPX in skeletal muscle?

Putative binding sites for several muscle-specific transcription factors were found in the promoter region located immediately 5' to the *Smpx* gene; including MyoD, MEF2 and Nkx2-5 (Patzak *et al.*, 1999; Kemp *et al.*, 2001; Palmer *et al.*, 2001). This is in agreement with the high expression level of SMPX in muscle, and suggests that these transcription factors can be regulators of SMPX.

Nkx2-5 is the human homologue of the *Drosophila* gene *tinman*, which has been identified as a candidate for the determination of the cardiac cell lineage in Zebrafish (Chen & Fishman, 1996). Palmer *et al.* (2001) found SMPX downregulated by more than 20 times in the heart of an Nkx2-5 null-mutant. Nkx2-5 expression has also been shown to increase during adrenergic (Saadane *et al.*, 1999) and pressure induced (Thompson *et al.*, 1998) cardiac hypertrophy. These results suggest a connection between Nkx2-5, SMPX and cardiac muscle development and hypertrophy.

In 2003, Christoffels *et al.* discovered that the temporal and spatial pattern of the T-box transcription factor Tbx2 repressed differentiation and formation of the cardiac chambers in mice; and expression patterns of Tbx2 and SMPX were mutually exclusive at all stages in myocardium development. Constitutively expressed Tbx2 in

the heart chambers suppressed SMPX and other chamber specific gene expression, and the hearts were smaller, unlooped and had the appearance of large linear heart tubes. Their results make SMPX a candidate for regulation of chamber differentiation, growth and looping (Christoffels *et al.*, 2004).

Passive stretch induces muscle fibre hypertrophy (Moss & Leblond, 1971; Tabary *et al.*, 1972). Kemp *et al.* (2001) found SMPX upregulated in the *tibialis anterior* muscle in response to 7 days of passive stretch. Kemp *et al.* (2001) therefore suggested that SMPX may induce hypertrophy in adult skeletal muscle as a response to stretch. The process by which mechanical stimulation is detected by cells, and the translation of these stimuli into biological signals leading to hypertrophy is poorly understood. Only a few key regulators that can lead to hypertrophy have been identified. Insulin-like growth factor-1 (IGF-1), mechanogrowth factor (MGF) (Yang *et al.*, 1996; Yang *et al.*, 1997; Barton-Davis *et al.*, 1998) and Prostaglandin $F_2\alpha$ (Vandenburgh *et al.*, 1995) have been proven to induce skeletal muscle hypertrophy in the absence of other stimuli.

1.2.5 Intracellular localization of SMPX in skeletal muscle in mice

In early fetal skeletal and heart muscle SMPX was evenly distributed in the cells. In late fetal and adult skeletal muscle SMPX was immunolocalized to repetitive double stripes at the level of the I-band, flanking the Z-disc (Palmer *et al.*, 2001). Palmer *et al.* (2001) suggested the localization of SMPX to be at costameres (Berthier & Blaineau, 1997), supported with the finding that it co-localized with talin both in costameres and in myotendinous junctions. Talin is a large protein with actin-, vinculin-, integrin- and dystrophin-binding activities enriched in costameres. Costameres have a substructure consisting of densely clustered patches of vinculin, which are segregated into two rows which flank the Z line and span the I band of the underlying sarcomere (Pardo *et al.*, 1983).

Palmer *et al.* (2001) further compared the localization of SMPX with desmin, and pointed out the striking resemblance in the localization of these two surrounding the Z-disc. Desmin is an intermediate filament protein precisely surrounding the Z-disc of

myofibrils in a cage-like manner in continuity with costameres at the membrane (Granger & Lazarides, 1979). Linking adjacent Z-discs together, desmin is a part of the force transmission system (Shah *et al.*, 2002).

In skeletal muscle, a weak single stripe of SMPX was also seen at the level of the M-line (Palmer *et al.*, 2001). In fibre cross sections of fetal myotubes Palmer *et al.* (2001) also found that SMPX was located predominantly surrounding or in between myofibrils, unlike α-actinin, which was located throughout the myofibril at the level of the Z-disc. The same localization pattern of SMPX was also seen in cardiac muscles; however here the M-line signal was more prominent than in skeletal muscles (Palmer *et al.*, 2001). The M-line signal was not further discussed in Palmer *et al.* (2001).

1.2.6 Intracellular localization of SMPX in cell culture

Kemp *et al.* (2001) immunolocalized SMPX at varying levels to the nuclei of mononucleate C2C12 myoblasts, while SMPX was absent from all nuclei in differentiated multinucleate myotubes (Kemp *et al.*, 2001). In contrast, Palmer *et al.* (2001) found endogenous SMPX immunolocalized primarily to the cytoplasm in both C2C12 myoblasts and myotubes.

In myoblasts, overexpressed FLAG- SMPX was localized to the leading end of lamellipodia and focal adhesions. Two myc-SMPX cell lines under the control of different promoters and a GFP-SMPX fusion protein, with GFP linked to the N-terminal side of SMPX, confirmed these results (Palmer *et al.*, 2001).

In 2005, Schindeler *et al.* presented additional results on the localization of SMPX in C2C12 cell culture, based on the results published by Palmer *et al.* (2001). When studying C2C12 myoblasts, Schindeler *et al.* (2005) found that MYC epitope-tagged SMPX co-localized with actin networks at peripheral membranes and in perinuclear compartments; and with the focal adhesion proteins vinculin, paxillin, integrin β1, and the small GTPase Rac1. In both C2C12 cells and native muscle, SMPX could be co-immunoprecipitated with vinculin. Rac1 is activated and recruited to sites of actin

reorganization by growth factors, integrin-mediated cell adhesion to the ECM and by biomechanical stress (Clark *et al.*, 1998; Price *et al.*, 1998; Del Pozo *et al.*, 2002; Putnam *et al.*, 2003).

1.2.7 Effects of overexpressing SMPX in cell culture

Palmer *et al.* (2001) did overexpression of SMPX in C2C12 muscle cell culture, and examined effects on myoblasts and myocyte formation in the presence and absence of IGF-1. FLAG-SMPX overexpressing myoblasts in the presence of IGF-1 induced a further increase in the activity of the transcription factors NFAT and MEF2 when compared to IGF-1 only controls (Palmer *et al.*, 2001). Both NFAT and MEF2 regulate the myoglobin promoter, which is selectively expressed in slow fibres (Chin *et al.*, 1998), and since SMPX itself is enriched in slow fibres, Palmer *et al.* (2001) suggested that SMPX may promote a slow fibre phenotype in C2C12 cells.

In FLAG- SMPX overexpressing myoblasts, Palmer *et al.* (2001) also found prominent lamellipodia formation accompanied by membrane ruffling and increased cell area solely due to cell spreading in the culture dish. Surprisingly, these cells migrated more slowly than control cells. Cell division and changes in cell shape and behavior, including formation of lamellipodia and membrane ruffling during cell migration, are mediated by actomyosin cytoskeletal dynamics and require the Rho family of small GTPases (Nobes & Hall, 1995; Kaibuchi *et al.*, 1999; Bishop & Hall, 2000). The findings of Palmer *et al.* (2001) suggest that ectopically expressed SMPX may engage up- or downstream elements of Rho/Rac-signalling cascades and thus dysregulate actomyosin dynamics. It may also function in stabilizing trailing edge focal adhesions in C2C12 cells (Palmer *et al.*, 2001).

Furthermore, when adding IGF-1 to FLAG- SMPX differentiating cells, large hypertrophied myotubes termed myosacs (Yotov & St-Arnaud, 1996) appeared. The myosac formation was not due to increased anabolic metabolism, but solely due to the increased fusion of myoblasts. Palmer *et al.* (2001) also suggested on basis of their findings that myosac formation when overexpressing SMPX may augment and dysregulate myosac fusion through perturbation of regulated cytoskeletal dynamics.

Schindeler *et al.* (2005) found that overexpression of the MYC epitope-tagged SMPX induced cell spreading (lamellipodia) at the expense of filipodia in a Rac1- and p38 kinase dependent manner, suggesting that SMPX may participate in regulation of cytoskeletal dynamics through the Rac1-p38 pathway (Schindeler *et al.*, 2005). They also suggested that SMPX may augment the adhesive function of integrins, since MYC- SMPX expression slowed actin turnover at steady state (Huttenlocher *et al.*, 1995; DeMali *et al.*, 2002; Brakebusch & Fassler, 2003; Nayal *et al.*, 2004).

1.3 Aims of the study

SMPX has been shown to be upregulated in stretched skeletal muscle (Kemp *et al.*, 2001). SMPX has also been shown to enhance fusion of myoblasts in cell culture, leading to large hypertrophied myosacs (Palmer *et al.*, 2001). These results suggest SMPX as a candidate for a mechanotransduction pathway leading to hypertrophy.

SMPX expression was higher in slow-twitch than in fast-twitch fibres (Palmer *et al.*, 2001), suggesting that SMPX can promote a slow-twitch fibre type. NFAT and MEF2 activity increased in an IGF-1 dependent manner in SMPX overexpressing C2C12 cells (Palmer *et al.*, 2001). Since both MEF2 (Potthoff *et al.*, 2007) and NFAT (Chin *et al.*, 1998; McCullagh *et al.*, 2004) has been shown to induce a slow fibre type in skeletal muscle, this further supports that SMPX may promote a slow fibre-type.

Previous studies by others have indicated that SMPX can have a function both as a structural protein and in the regulation of phenotype in muscle, but the *in vivo* function of SMPX is as yet unknown.

The aims of my study were to:

1. Investigate the *in vivo* localization of SMPX in skeletal muscle in adult mice.
2. Investigate if overexpression of SMPX *in vivo* has an effect on fibre type distribution and cross sectional area in skeletal muscle in adult mice.

2. Materials and methods

2.1 Animal experiments

All animal experiments were performed on female NMRI mice (20-40g), held in standard cages in the animal facility at the Department of IMBV, UIO, at a temperature of 20ºC, and humidity of 50-60%. Food and water were given *ad libitum*. The animal experiments were approved by the Norwegian Animal Research Authority, and were conducted in accordance with the Norwegian Animal Welfare Act of 20th December 1974 (no. 73, chapter VI, sections 20-22), and the Regulation on Animal Experimentation of 15th January 1996.

2.1.1 Anaesthetization

Prior to all non-terminal experiments, the mice were anaesthetized with isoflurane (Forene, Abbott Laboratories, Abbott Park, IL, USA). The anaesthesia was induced by placing the mice in an induction chamber with supply of 2.75% isoflurane. Pinching the metatarsus region to ensure that the retraction reflex was absent confirmed deep anaesthesia. The mice were then transferred to a mask with airflow of 600-700 CC/minute, containing between 1.7-2.4% isoflurane. Changes in the respiration frequency were used to regulate the anaesthesia dosage.

For terminal experiments, the mice were anaesthetized with an intraperitoneal injection of $5^{\mu l}/_{g}$ bodyweight of Equithesin (Ullevål Sykehus, Oslo, Norway). Pinching the metatarsus region monitored the depth of anaesthesia, and smaller doses were given to sustain deep anaesthesia throughout the experiment. After the experiments, the mice were sacrificed by neck dislocation while still under deep anaesthesia.

2.1.2 Animal operation procedures

For all animal operations, the mice were initially placed on a heated platform that was designed to keep the body temperature stable throughout the experiment. The fur was removed by shaving and application of hair removal cream (Nair, Carter Products

Inc., Folkstone, Kent, England). The hairless area was then washed with 70% ethanol. The leg was fastened to a platform, and the muscles were surgically exposed. Pulling the overlaying muscles to the sides exposed the EDL and/or *soleus* muscle. The exposed muscles were covered with ringer-acetate solution (Fresenius Kabi Norway A/S, N-1753 Halden, Norway). The muscles were then electroporated, made ready for imaging, or fixed, as described later.

2.2 Expression vectors

It has been demonstrated that when transfecting with two different plasmids, both will be transcribed in over 80% of the transfected cells (Utvik *et al.*, 1999). However, Rana, *et al.* concluded that the co-expression can be close to 100% because of localized expression and varying degree of sensitivity in the detection (Rana *et al.*, 2004).

2.2.1 Fibre type and cross sectional area (CSA) experiments

To induce overexpression of SMPX in muscle fibres, the plasmid pCMS-EGFP-SMPX was used (Larsen, 2007) (fig.2.1, C). pCMS-EGFP (Clonetech Laboratories Inc., Mountain View, CA, USA) (fig.2.1, B) without *Smpx* was used as a control. For identification of transfected fibres, the muscles were co-transfected with the reporter-plasmid pAP-lacZ (gift from N. Gautam) (fig.2.1, A). This plasmid has an *Escherichia coli* lacZ-sequence, and expresses the protein β-galactosidase (β-gal), which is detected in a colour-reaction where the muscle fibres that express β-gal are stained blue.

2.2.2 Intracellular localization experiments

In order to study the localization of SMPX *in vivo*, a fusion-protein was manufactured by placing *Smpx* in at the N-terminal end, in frame with EGFP in the expression-vector pEGFP-N1 (Clonetech Laboratories Inc., Mountain View, CA, USA) (fig.2.1-D). This was done because of the putative important area close to the N-terminal end of *SMPX* discussed in the introduction, and the possibility of EGFP affecting this

region if flanking the N-terminal end of SMPX. *Smpx* was isolated from pCMS-EGFP-*Smpx* (Larsen, 2007) with the restriction enzymes Nhe1 and Sal1, and the multiple cloning site (MCS) of pEGFP-N1 was opened using the same restriction enzymes. *Smpx* was then ligated into the MCS of pEGFP-N1. The new plasmid, hereby called pEGFP-N1-*Smpx* (fig.2.1-E) was modified by removing the kozak sequence from the EGFP gene, adding a kozak sequence to *Smpx*, and removing the stop-codon from *Smpx* using site-directed mutagenesis (QuickChange, Stratagene, CA, USA). This was done in order to optimize translation of the fusion protein, hereby called SMPX-EGFP. All mutations were verified by sequence analysis, and a western blot was performed to verify expression of SMPX-EGFP in HEK-293 cells (see results).

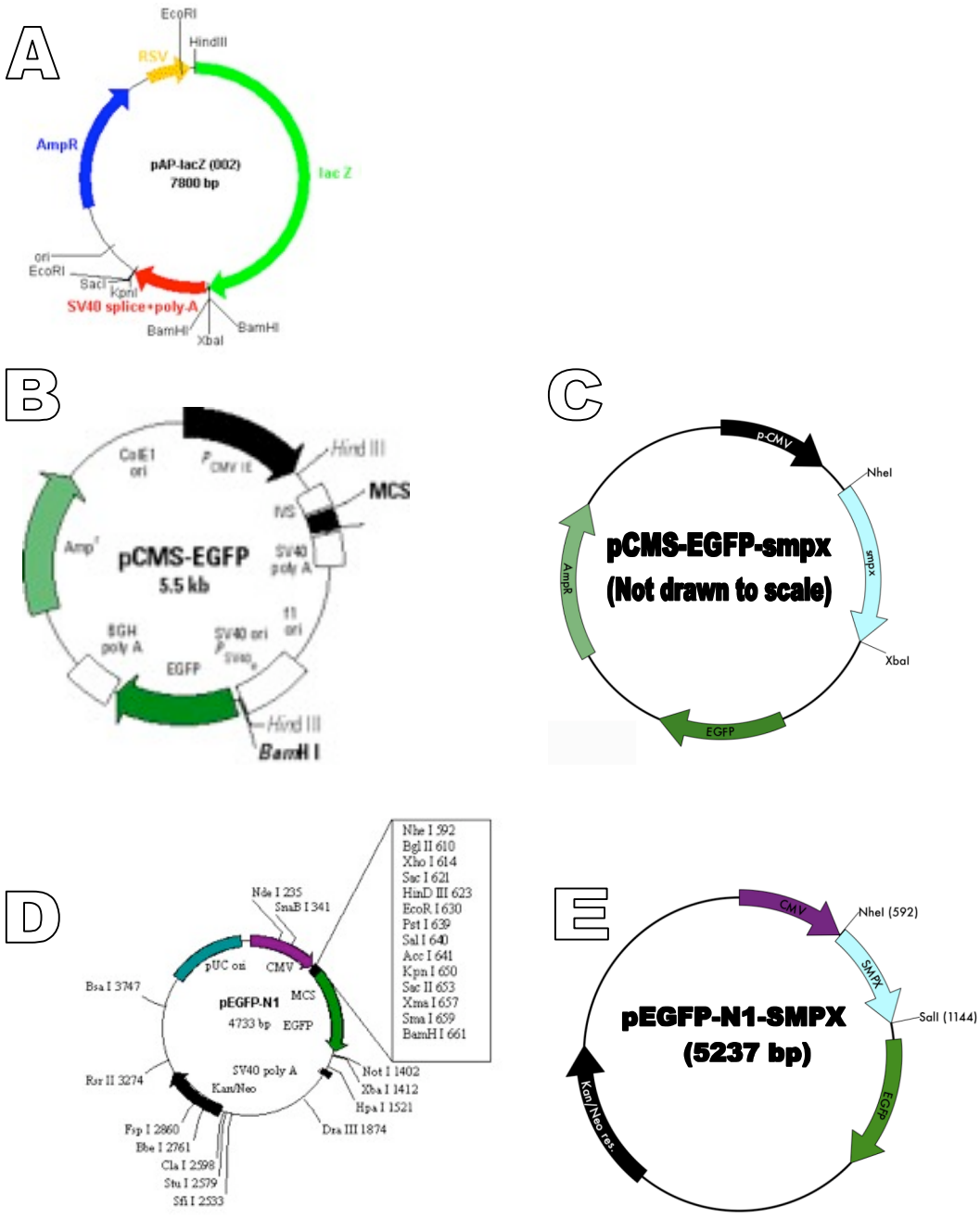

Figure 2.1 Expression vectors
A) pAP-LacZ (gift from N. Gautam) with the Rous sarcoma virus (RSV) promoter for enhanced expression of the *LacZ* gene in mammalian cells. B) pCMS-EGFP (Clonetech). This contains an ampicillin-resistance gene for bacterial selection, a Cytomegalovirus (CMV) promoter for enhanced mammalian expression and an *EGFP* gene for identification of transfection in cell culture and in vivo. Following the promoter region is the multiple cloning site (MCS) for insertion of genes to be overexpressed. C) pCMS-EGFP-*Smpx* (Larsen, 2007) with *Smpx* inserted into the MCS of pCMS-EGFP, using the restriction enzymes Nhe1 and Xba1 D) pEGFP-N1 used in manufacturing pEGFP-N1-*Smpx*. It contains a CMV promoter, an *EGFP* gene and a MCS between the two for insertion of *Smpx*. When *Smpx* is inserted, it will be transcribed in frame with *EGFP*. It also contains a Kanamycin/Neomycin resistance gene for bacterial selection. E) pEGFP-N1-*Smpx* with *Smpx* inserted into the MCS of pEGFP-N1 in frame with *EGFP*, using the restriction enzymes Nhe1 and Sal1.

2.3 Cell culture

HEK-293 (ATCC: CRL-1573), C2C12 (ATCC: CRL-1772) and NIH/3T3 (ATCC: CRL-1658) cells (gift from Jørgen Sikkeland) were used in the experiments. The cells were grown in Dulbecco´s modified Eagles medium (DMEM) (GIBCO, Invitrogen, Raisley, PA49RF, United Kingdom) containing appropriate amounts of fetal calf serum (Bio Whittaker, Lonza, MD, USA) for the respective cell cultures and 100 U/ml penicillin and streptomycin. All cells were grown in 75cm^2 nunclon flasks (Nunc A/S, Denmark) in a cell incubator (Farma Scientific Inc, Box 649, Morjetto, OHIO, USA) with a set temperature of 37°C, a CO_2 level of 5% and humidity of 100%. All cells were subcultured using 0.1% trypsin EDTA (Bio Whittaker, Lonza, Belgium) as previously described (Jainchill *et al.*, 1969), and split approximately 1:10 every fourth to sixth day, depending on desired cell density.

Transfections were performed with FuGENE 6 (Roche diagnostics GmbH, Mannheim, Germany) according to manufacturers instructions. Cells were plated on 6-well plastic transfection plates (Nunc A/S, Denmark) for the transfection experiments. As a sham control, one well was always transfected with the pCMS-EGFP plasmid without *Smpx* inserted. Transfection without any plasmid was used as a negative control. All cell culture images shown were obtained 1 day after transfection.

C2C12 cells (Yaffe & Saxel, 1977) were initially used to validate expression of SMPX-EGFP prior to animal experiments. The cells were grown in DMEM with 20% fetal calf serum to maintain the cells in an undifferentiated myoblast stage, and the media was changed to DMEM with 2% fetal calf serum to induce differentiation into myotubes.

HEK-293 cells are a stable embryonic cell line of human kidney cells. They were grown in DMEM with 10% calf serum. NIH/3T3 is a fibroblast cell line (Todaro & Green, 1963), and they were grown as the HEK-293 cells.

2.4 *In vivo* electroporation

It has been shown that muscle cells can take up DNA from the interstitium (Wolff et al., 1990). Nevertheless, this kind of transfection has an efficiency of about 1%. Electrical stimulation can increase the permeability of the cell membrane, and transfection efficiency can be increased to over 10% (Mathiesen, 1999).

Transfection with DNA was performed essentially as described previously (Mathiesen, 1999), with some modifications. The muscles were bathed in 10µl DNA solution ($1^{\mu g}/_{\mu l}$ in 0.9% NaCl) (appendix A) for expression in superficial fibres in the localization study. For the fibre typing and cross-sectional area (CSA) studies, a syringe (701, Hamilton Company, Reno, Nv, USA) was inserted into the belly of the muscle, and 10µl DNA solution ($1^{\mu g}/_{\mu l}$ in 0.9% NaCl) (appendix A) was injected with a pressure high enough to inflate the muscle and surround the fibres with DNA solution. In this way the electroporation efficiency can increase. An electrical field was then applied to the muscle using a pulse-generator (Pulsar 6bp-a/s, Fredrick Haer, Bowdoinham, ME, USA). Two silver electrodes (1cm long and 2 mm thick) were placed on both sides of the muscle, with a distance of approximately 2-3mm between them, so that the electrical current was conducted perpendicular to the length of the muscle. Five pulse-trains with 1-second intermission were conducted on the muscle, and the electrodes were moved length-wise along the muscle between each pulse-train. Each pulse-train consisted of 1000 symmetrical bipolar square pulses lasting 200µs in each polarity direction, with total amplitude of 20V. The voltage was measured using an analogue oscilloscope (OS245A, Gould Advance, Hainault, Great Britain).

2.5 Fibre type distribution and CSA experiments

2.5.1 Preparation of muscle serial sections

14 days after electroporation, muscles were dissected out and pinned onto a rubber form. The forms were filled with tissue-tek (Sakura Finetek, Zoeterwoude, Holland) and lowered into isopentane cooled to near freezing point (-160°C) in liquid nitrogen. This ensured rapid freezing, minimizing freeze-damage. The muscles were stored at -80°C. The muscles were cryo-sectioned in series of 10 μm sections (HM 560M, Microm, Bicester, Oxom, Great Britain). The knife held a temperature of -18°C and the preparation -24°C during sectioning. The sections were placed on slides (Super Frost Plus, Menzel Gläser, Braunschweig, Germany), air dried and stored at -80°C.

2.5.2 Staining for β-galactosidase-activity

The reporter gene lacZ expresses the protein β-galactosidase (β-gal). β-gal is detected in a staining-reaction (appendix A) where the substrate x-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) is hydrolysed by β-gal, yealding galactose and the colourless product indoxyl (5-bromo-4-chloro-3-hydroxyindole). Indoxyl is then dimerized/oxidised, forming insoluble blue crystals (5,5-dibromo-4,4-dichloro-indigo). Fibres that stained for β-gal were counted as transfected fibres. After mounting of the stained muscle sections in glycerine gel (appendix A), the staining was permanent. The control reaction without x-gal gave no staining (results not shown).

2.5.3 Fibre typing experiments

The fibre type was determined using monoclonal antibodies against the four muscle-specific myosin heavy-chains (MyHC) (appendix A, 5.3). The primary antibody (ab) binds via the antigen-binding site to a MyHC-epitope that is specific to one or several MyHCs. The primary ab is then bound via the FC-region to a secondary ab that is conjugated to a fluorescent chromophore. Comparing the different ab stains then

allowed identification of the different fibre types. Staining without primary ab was used as a negative control. No fluorescence was detected in this experiment.

2.5.4 CSA experiments

Images from the β-gal sections were used in measuring CSA on muscle fibres. Negatively stained fibres were chosen at random from all the transfected muscles and used as an internal negative control. Photoshop CS3 extended (Adobe Systems, San Jose, CA, USA) were used to encircle the fibres and calculate the pixel value. Conversion to μm^2 was performed on basis of a micrometer-scale image with the same microscope settings.

CSA in all muscles was normalized by dividing all single fibre values by the average of the normal fibres in the same muscle. This was done in order to adjust for possible differences in the degree of stretch in the isolated muscles, as there is a linear relationship between the degree of stretch and CSA. It also adjusts for the natural differences in fibre size between individuals.

2.6 Fibre type distribution, CSA and cell culture imaging

2.6.1 Fluorescence microscopy

Fluorescence images from the fibre type, CSA and cell culture experiments were taken with a light sensitive SIT video camera (C2400-08, Hamamatsu Photonics, Hamamatsu, Japan) in a dark room. The camera was connected to an upright fluorescence microscope (BX 50WI, Olympus, Norwood, MA, USA) with a 10x, 20x 40x or 60x water immersion objective (UMPlan, FI, Olympus, Tokyo, Japan). An adjustable 12 V, 100 W halogen lamp was used for epi-illumination, and precautions were taken to minimize the time and intensity of light exposure in order to avoid phototoxicity. The filter cube XF33, XF22 and XF11 (Omega Optical Inc., Brattleboro, VM, USA) was used to illuminate the samples with the correct wavelength. The pictures were digitalized using an Argus 20 picture processor (Hamamatsu photonics, Hamamatsu, Japan), transferred to a Power Macintosh G3

and further processed in Photoshop CS3 extended (Adobe Systems, San Jose, CA, USA).

2.6.2 Light microscopy

Sections stained for β-gal activity were photographed with a CCD videocamera (C2400, Hamamatsu Photonics, Hamamatsu, Japan) connected to a microscope (BX 50WI, Olympus, Tokyo, Japan) with a 10x water immersion objective (UMPlan, FI, Olympus, Tokyo, Japan). Images were digitalized and processed in the same way as the fluorescence images.

2.7 Intracellular localization experiments

2.7.1 Preparation of fixed muscles for confocal imaging

For the imaging experiments, 1-2 days after electroporation of superficial fibres in EDL and *soleus*, the muscles were fixed in 4% paraformaldehyde diluted in relaxing solution (FIX) (137mM NaCl, 5.4mM KCl, 5mM $MgCl_2$, 4mM EGTA, 5mM HEPES, pH7.0) (Wada *et al.*, 2002) by heart perfusion (Slezak & Geller, 1979). After anaesthetizing the mice, the feet were pinned up and stretched on a polystyrene plate. The thorax was opened, and the heart located. The right atrium was cut open, and from the apex of the left ventricle the heart was perforated with a 20ml syringe (Beckton Dickinson, Drogheda, Ireland). 20ml FIX was then injected with high pressure to replace all the blood and interstitial fluids with FIX, thereby fixating the muscles. The whole procedure was performed in a timeframe maximum of 5 minutes to ensure that the muscle protein was not degraded. EDL and *soleus* were isolated and directly bathed in FIX for another 10 minutes to ensure thorough fixation of the superficial fibres. The muscles were washed three times in PBS (pH=7.4) (appendix A) and stored in PBS in the dark at 4°C.

2.7.2 Immunohistochemistry on single fibres

Fibres positive for EGFP were located using a fluorescence microscope (BX 50WI, Olympus, Tokyo, Japan), and mechanically isolated from the fixed muscles using

tweezers and pins under a binocular microscope. The isolated fibres were transferred to a glass slide (Super Frost Plus, Menzel Gläser, Braunschweig, Germany) and air-dried for 10 minutes to allow attachment to the slide. The fibres were permeabilized with 0.5% Triton X-100 (Sigma Chemical) in PBS (pH=7.4) for 10 minutes, then blocked with 3% BSA (Sigma Chemical), 0.1% Triton X-100 in PBS for 15 min. Primary antibodies (appendix A) were diluted in PBS with 1% BSA and applied to the slides for 60 min at room temperature. Following three washing steps with PBS containing 0.1% Triton X-100, the slides were incubated at room temperature for 45 min with secondary ab (appendix A) diluted in PBS containing 1% BSA. After washing the fibres twice with 0.1% Triton X-100 in PBS and once with PBS, the fibres were mounted with ProLong Gold with DAPI (Invitrogen, Oregon, USA). Non-fluorescent fibres were used as negative controls. (Modified protocol from (Averbeck *et al.*, 2007).

2.7.3 Confocal microscopy and imaging

Imaging with a confocal microscope (Olympus BX61WI, Tokyo, Japan) connected to an imaging system (Olympus fluoview FV1000, Olympus, Europe GmBH) was performed on all the fibres from the *in vivo* experiments and on the isolated fibres from the SMPX-EGFP fusion protein experiments. An argon ion laser (Melles Griot, Stockholm, Sweden) was used as the light source. The fibres were scanned sequentially with the different wavelengths to hinder false imaging by the partly overlapping emitted light, and each sample were scanned ≥ 3 consecutive times (Kalman mode) to remove background noise from the sample. All adjustments were performed through the software-program FV10-ASW version 1.7 (Olympus), which was connected to the microscope. All images were taken with a 100X oil objective (UPlanApo) with numerical aperture (n.a.) 1.35. The images were further processed in FV10-ASW version 1.7 and Photoshop CS3 extended (Adobe Systems, San Jose, CA, USA).

2.8 Statistical analyses

All statistical calculations were done in Prism4 (Graph Pad Software Inc., San Diego, CA, USA), with a level of significance set to 5%. The fibre type distribution in normal control, sham control and SMPX muscle fibres were compared using a chi-square test. The CSA were compared using a Oneway ANOVA with a Bonferroni post test. ANOVA is a parametric test that assumes a normalized distribution. In all graphs presented in the results the P values are expressed with the following symbols: $P<0.001$= ***, $0.001 \leq P \leq 0.01$= **, $0.01<P \leq 0.05$ = *, $P>0.05$= n.s. (non significant).

3. Results

3.1 Intracellular localization of SMPX

3.1.1 Verification of protein expression

A western blot analysis was performed on cell lysates from HEK-293 cells to verify SMPX-EGFP fusion protein expression. The blot confirmed expression of SMPX-EGFP from 4 independent pEGFP-N1-*Smpx* transfected cultures, with a band at approximately 40kDa (fig.3.1, lane 3-6). In the control, transfected with pCMS-EGFP-*Smpx*, a band at approximately 27kDa confirmed EGFP expression. The negative control without transfection did not show any specific bands at the size of either SMPX-EGFP or EGFP. A weak EGFP band could also be seen in lane 3-6, where only SMPX-EGFP should be expressed. This shows that transcription and translation of EGFP alone also takes place. Still, the signal is much stronger for the SMPX-EGFP band, confirming better transcription and/or translation of SMPX-EGFP. The weak EGFP band is considered not to affect the localization results. HEK-293 cells transfected with pCMS-EGFP-*Smpx* has previously verified SMPX overexpression with northern blotting (Larsen, 2007).

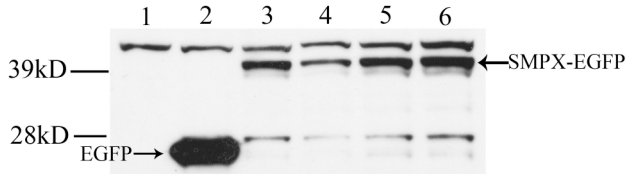

Figure 3.1 Western blot of SMPX-EGFP expression in HEK-293 cells
Stained with an ab against EGFP. Lane nr: 1) Negative control. 2) EGFP only. 3-6) SMPX-EGFP.

3.1.2 SMPX-EGFP distribution in cell culture

Cells transfected with pEGFP-N1-*Smpx* had a distinct localization of the SMPX-EGFP fusion protein, depending on the cell culture, and it was invariably excluded from the nuclei (fig. 3.2, left hand column). SMPX-EGFP was localized to the membrane in the HEK-293 cells (fig. 3.2, A). SMPX-EGFP showed a patchy distribution in the cytoplasm of both the NIH/3T3 fibroblasts (fig.3.2, C) and in the

C2C12 myoblasts (fig.3.2, E). SMPX-EGFP was denser towards the periphery in the myoblasts, while it was denser in the perinuclear areas in the fibroblasts. Cells transfected with pCMS-EGFP-*Smpx*, expressing EGFP and SMPX separately, had a uniform distribution of EGFP throughout the cell (fig. 3.2, right hand column).

Myotubes had a patchy distribution of SMPX-EGFP in the cytoplasm, as seen in the myoblasts and fibroblasts. Due to a degradation process in the cells expressing SMPX-EGFP and difficulties in transfecting myotubes, no good images on the localization of SMPX-EGFP were obtained from myotubes at early stages after transfection (see discussion).

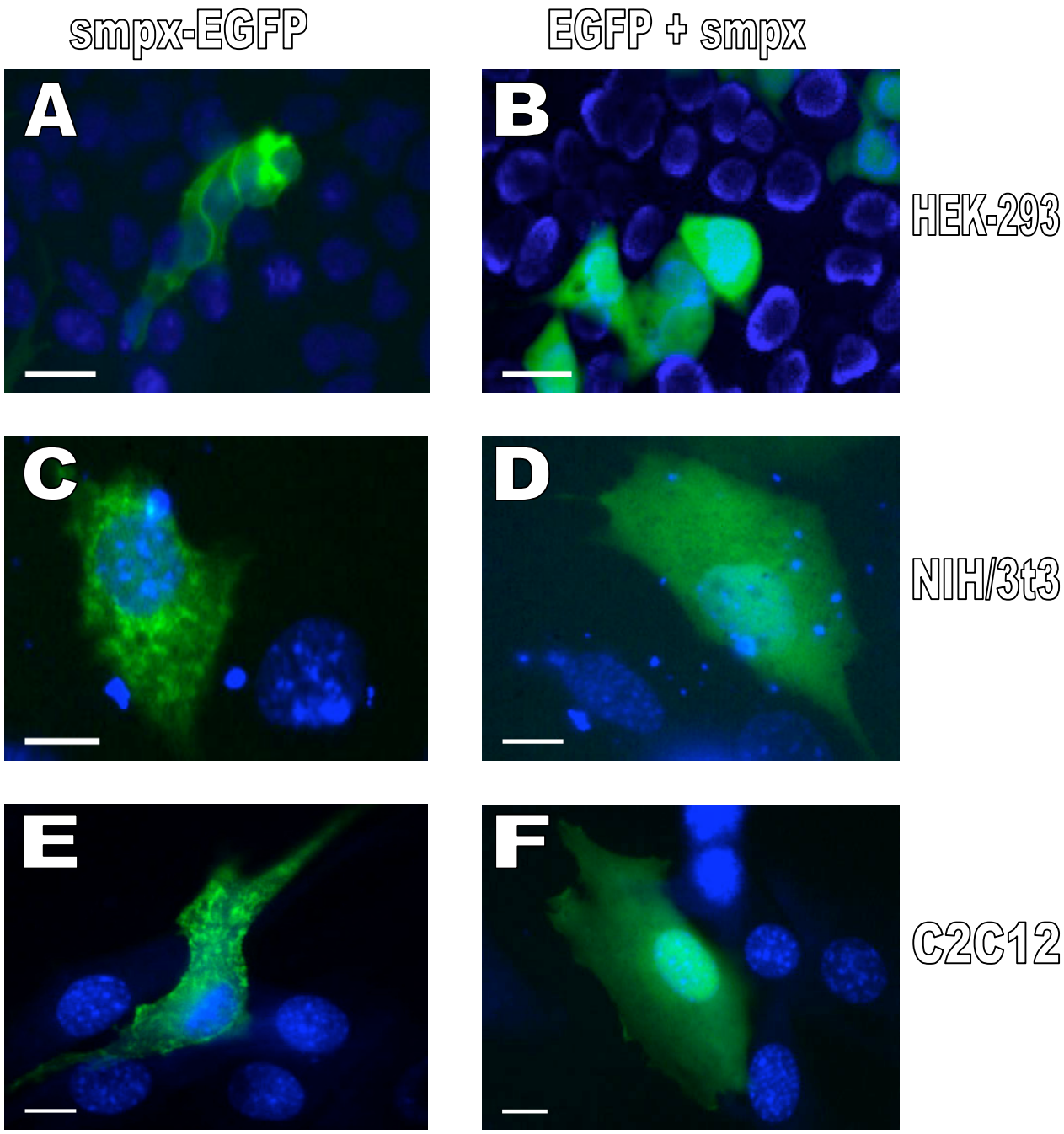

Figure 3.2 SMPX-EGFP intracellular localization in cell culture
A-B) HEK-293 cells. C-D) NIH/3T3 fibroblast cells. E-F) C2C12 myoblast cells. Green fluorescence represents the SMPX-EGFP fusion protein in the left column and EGFP in the right column. Blue fluorescence represents nuclei, stained with Hoechst-33342. Images were taken with a SIT camera on an upright fluorescence microscope. Scale bars: 10μm.

3.1.3 In vivo localization of SMPX-EGFP

Live, anaesthetized mice were placed under an upright confocal microscope in order to look at the *in vivo* intracellular localization of the SMPX-EGFP fusion protein in single fibres. A clear localization of SMPX-EGFP could be seen in repetitive cross striations (fig. 3.3, A). The control fibres, expressing EGFP and SMPX separately, had a uniform distribution throughout the cytoplasm (fig. 3.3, B).

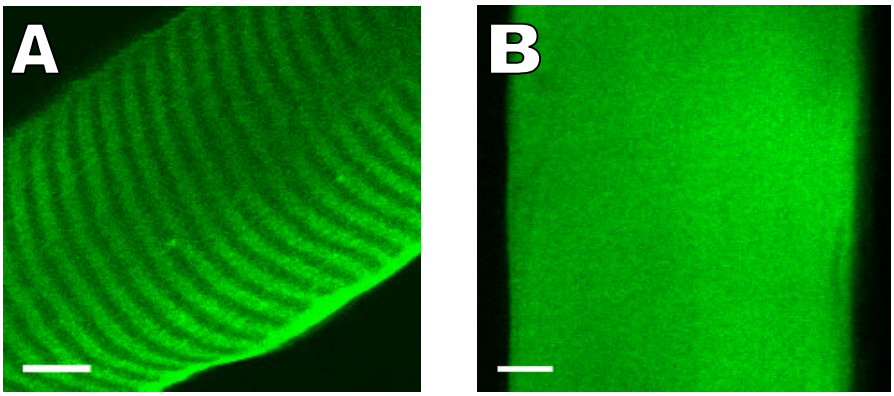

Figure 3.3 SMPX-EGFP localization in live fibres in anaesthetized mice
A) Fibre electroporated with pEGFP-N1-*Smpx*, expressing SMPX-EGFP fusion protein in green. B) Fibre electroporated with pCMS-EGFP-*Smpx*, expressing EGFP (green) and SMPX separately (control). Images were taken with an upright confocal microscope. Scale bars: 10μm.

3.1.4 SMPX-EGFP localization in isolated fibres

To further deduce the SMPX-EGFP localization in relation to other structural elements in the muscle fibre, fixation and mechanical separation of single fibres was performed. A toxin binding filamentous actin (phalloidin) was used (fig.3.4). The phalloidin first binds at both ends of the actin filaments and at the Z-disc, and the areas in between are stained in a time dependent manner (Balnave *et al.*, 1997). Due to the harsh treatment of the fibres, including mechanical separation, permeabilization and repeated staining and washing procedures, the SMPX-EGFP signal became weaker. The EGFP ab was therefore used to reinforce the initial SMPX-EGFP fluorescence signal (fig. 3.4).

SMPX-EGFP was localized to the H-zone at the level of the myosin filaments, with little or no overlap with actin filaments (fig.3.4, A-C). A very weak band of SMPX-

EGFP could also be seen at the level of the Z-disc (fig. 3.4, B, dark blue arrow). This band is not visible on the overlay (fig. 3.4, C) image because of the much stronger signal from the phalloidin staining. The normal control fibres (fig. 3.4, D-F) had no intracellular staining with the EGFP ab, but some staining at the membrane was evident. I attribute this to non-specific binding or autofluorescence due to one or more of the steps in the treatment of the isolated fibres.

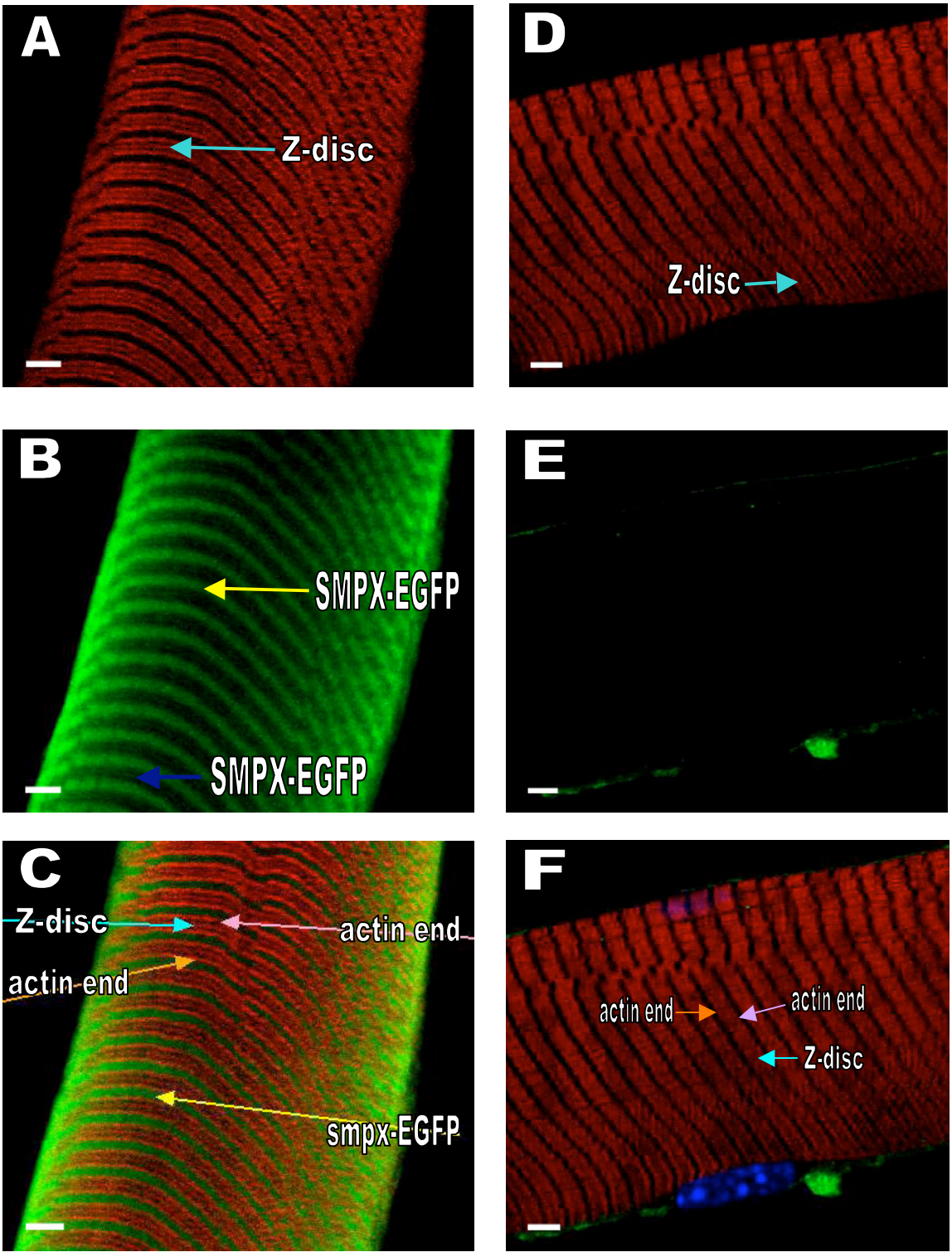

Figure 3.4 SMPX-EGFP localization in single isolated fibres
Triple immunofluorescence confocal images of mechanically isolated and fixed skeletal muscle fibres, showing GFP ab in green, actin stained with phalloidin in red and nuclei stained with Hoechst-33342 in blue. A-C) SMPX-EGFP expressing fibre. D-F) A normal control fibre. The bottom row is an overlay of the top two rows, in addition to a Hoechst-33342 image. Scale bars: 5μm.

In order to verify the localization observed with the phalloidin staining, double staining with antibodies against MyHC and phalloidin toxin was performed on normal fibres (fig. 3.5). The images indicate a localization of SMPX-EGFP to the H-zone. This part of the myosin filaments does not participate in the actin-myosin crossbridge cycling, but it holds many other structural and signalling proteins, many with an unknown function, and still more progressively being discovered (Kruger and Linke 2009). The SMPX-EGFP band was much narrower than the MyHC band (compare 3.4, B and 3.5, A), supporting a localization of SMPX-EGFP to the middle part of the myosin filament. The MyHC band was overlapping quite extensively with the ends of the actin band (yellow in fig. 3.5, C). SMPX-EGFP was not overlapping with the ends of the actin band (fig. 3.4, B-C), except from the very weak band of SMPX-EGFP at the Z-disc (fig. 3.4, B).

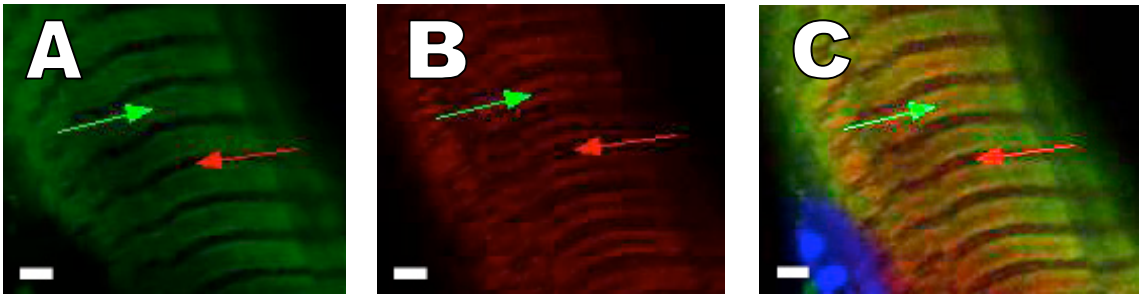

Figure 3.5 Actin filaments and MyHC staining in a normal fibre
Triple immunofluorescence confocal images of a mechanically isolated and fixed skeletal muscle fibre, showing myosin stained with a MyHC ab (my-32) in green, actin stained with phalloidin toxin in red and nuclei stained with Hoechst-33342 in blue. A) A green arrow indicates the middle of an A-band, and green indicates the myosin filaments. B) A red arrow indicates a Z-disc, and red indicates the actin filaments. C) The overlay of A and B shows that the myosin and actin filaments are partly overlapping (yellow). Blue represents Hoechst-33342, marking nuclei. Scale bars: 2.5μm.

3.2 Effects of SMPX on fibre type distribution

3.2.1 Fibre type distribution EDL

The fibre type distribution was not significantly different between the sham and SMPX transfected groups ($p>0.05$) (fig. 3.6 and table 3.1). There was a general shift towards a faster fibre type in the sham and SMPX transfected groups when compared with the normal group. An increase in type 2b fibres at the expense of type 2a and 2x fibres was evident. The fibre type distribution in both the sham and SMPX transfected groups were significantly different from the normal control group ($p<0.0001$). There was a 37% increase in type 2b fibres in the sham group compared with the normal group. There was a 26% increase in type 2b fibres in the SMPX group compared with the normal group.

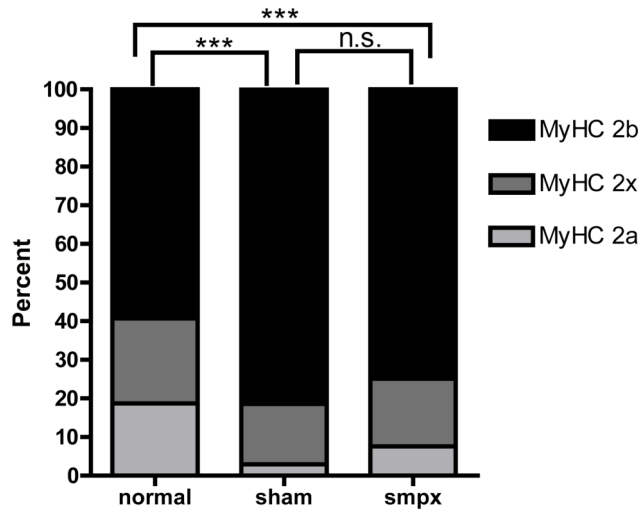

Figure 3.6 Fibre type distribution EDL
*** = $p<0.001$, n.s. = non significant. For further information, see table 3.1.

Table 3.1 Fibre type distribution EDL

	Normal	Sham	SMPX
Fibre type 2a (%)	18.7	2.9	7.6
Fibre type 2x (%)	22.0	15.7	17.5
Fibre type 2b (%)	59.3	81.4	74.9
Number of fibres	2787	172	251
Number of muscles	4	3	5

3.2.2 Fibre type distribution *soleus*

The fibre type distribution was significantly different between the sham and SMPX transfected groups (p=0.0474). There was a 23% decrease in type 1 fibres in the SMPX group when compared with the sham group (fig. 3.7 and table 3.2). A shift towards a faster fibre type in the sham and SMPX transfected groups was seen when compared with the normal group. There was also a shift towards a faster fibre type in the SMPX group when compared with the sham group. An increase in type 1/2a and 2a fibres at the expense of type 1 fibres was evident. The fibre type distribution in the sham group was significantly different from the normal control group (p=0.0053). There was a 14% decrease in type 1 fibres in the sham group when compared with the normal group. The fibre type distribution in the SMPX group was significantly different from the normal control group (p<0.0001). There was a 33% decrease in type 1 fibres in the SMPX group when compared with the normal group.

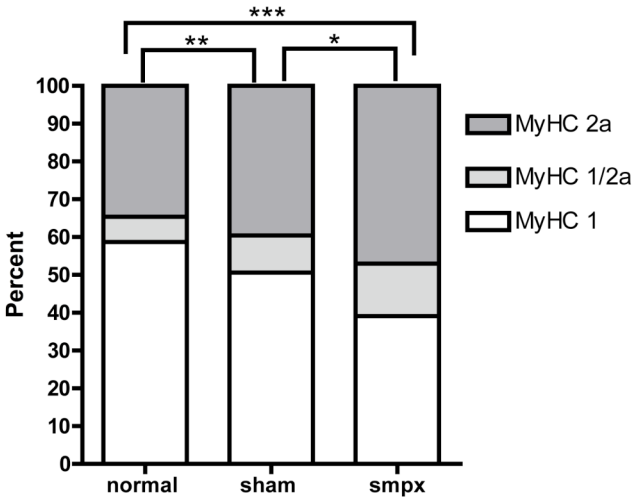

Figure 3.7 Fibre type distribution *soleus*
*** = p<0.001 ** = p<0.01 * = p<0.05. For further information, see table 3.2.

Table 3.2 Fibre type distribution Soleus

	Normal	Sham	SMPX
Fibre type 1 (%)	58.7	50.6	39.1
Fibre type 1/2a (%)	6.7	9.8	13.9
Fibre type 2a (%)	34.6	39.6	47.0
Number of fibres	1959	399	153
Number of muscles	3	6	6

3.3 Effects of SMPX on CSA

3.3.1 CSA EDL

There was no significant change in CSA in the SMPX group when compared with the sham group ($p>0.05$). There was a small, but still significant increase in CSA by 5% in the SMPX group when compared with the normal group ($p<0.05$). There was also a small, but still significant increase in CSA by 7% in the sham group when compared with the normal group ($p<0.01$).

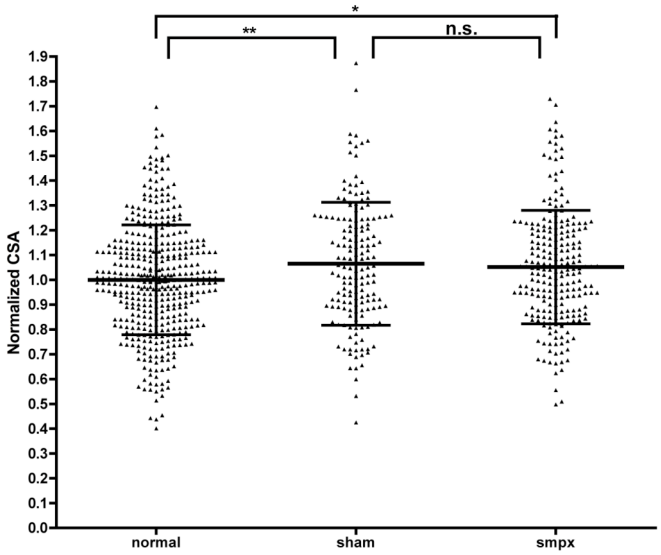

Figure 3.8 CSA distribution EDL
Each dot represents one fibre CSA value. Mean with standard deviations are shown. ** = $p<0.01$, * = $p<0.05$, n.s. = non significant. For further information, see table 3.3.

Table 3.3 CSA distribution EDL

	Normal	Sham	SMPX
Mean	1.000	1.065	1.052
Std. deviation	0.221	0.248	0.229
SEM	0.011	0.019	0.015
Number of fibres	423	164	232
Number of muscles	4	3	5
Normality test	p>0.10	p>0.10	p>0.10

3.3.2 CSA *soleus*

There was a significant increase in fibre CSA of 11% in the SMPX group when compared with the sham group ($p<0.01$). There was no significant change in the SMPX group when compared with the normal group ($p>0.05$). Fibre CSA was decreased by 12% in the sham group when compared with the normal group ($p<0.001$).

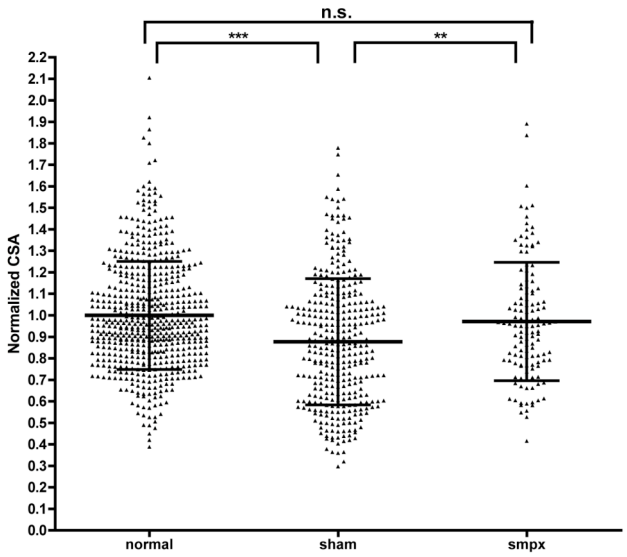

Figure 3.9 CSA distribution *soleus*
Each dot represents one fibre CSA value. Mean with standard deviations are shown. *** = $p<0.001$, ** =$p<0.01$ n.s. = non significant. For further information, see table 3.4.

Table 3.4 CSA distribution soleus

	Normal	Sham	SMPX
Mean	1.000	0.877	0.971
Std. deviation	0.251	0.294	0.275
SEM	0.010	0.016	0.024
Number of fibres	583	347	128
Number of muscles	3	6	6
Normality test	p = 0.0009	p>0.10	p>0.10

4. Discussion

4.1 Subcellular localization of SMPX

Our results show that SMPX-EGFP was absent from all nuclei both *in vivo* and in cell culture, indicating that SMPX did not work as a transcription factor under our experimental conditions. Our results are consistent with the cell culture immunolocalization results of endogenous SMPX from Palmer *et al.* (2001), but in disagreement with Kemp *et al.* (2001), who found SMPX immunolocalized to both nuclei and cytoplasm.

SMPX-EGFP had a patchy localization in the cytoplasm of the myoblasts, myotubes and fibroblasts in our experiments. This is in contrast to Palmer *et al.* (2001), who localized a GFP-SMPX fusion protein to the leading end of lamellipodia and focal adhesions. Localization of SMPX-EGFP to the membrane in the HEK-293 cells is difficult to interpret. Cells that do not express endogenous SMPX could misinterpret a localization signal.

Cell cultures with SMPX-EGFP lacked both costameres and a contractile apparatus, and the morphology and physiology of these cells were different from that of muscle fibres *in vivo*. An interpretation of our cell culture localization other than excluding SMPX-EGFP from the nuclei is therefore troublesome.

Our *in vivo* results indicate that the major portion of SMPX is localized to the H-zone, where myosin filaments do not overlap with actin filaments (fig. 4.1). With a localization to the H-zone, SMPX would join a series of proteins that have been localized to this part of the sarcomere; such as *muscle specific RING fingers* (MURFs) and the zink finger proteins *neighbor-of-BRCA1-gene-1* (Nbr1) and p62 ((Hoshijima, 2006; Kruger & Linke, 2009). Our results are in contrast to Palmer *et al.* (2001), who implied that the major portion of SMPX is localized to the I-band, flaking the Z-disc. However, Palmer *et al.* (2001) also found SMPX immunolocalized to the level of the M-line, with a stronger signal in heart muscle than in skeletal

muscle. They do not speculate whether this can be at the level of the contractile apparatus or not.

Our experiments show that a minor portion of SMPX may be localized to the Z-disc (fig. 4.1). The Z-disc SMPX-EGFP signal was only detected with a GFP antibody in isolated fibres, but not in the *in vivo* experiments. This could be an issue of sensitivity, considering that SMPX-EGFP had a weaker fluorescence signal than the EGFP antibody. SMPX localization to the Z-disc has not been reported in previous studies.

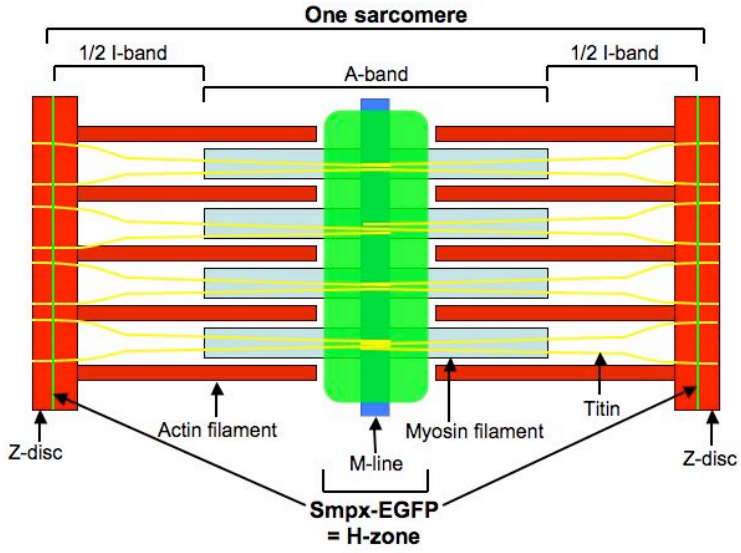

Figure 4.1 Schematic drawing of a skeletal muscle sarcomere with postulated SMPX localization
Modified from fig. 1.1 in the introduction; with localization of SMPX-EGFP (green) to the H-zone and the middle of the Z-discs added to the drawing.

In localization studies, a GFP fusion protein can be superior to antibodies because it is possible to study the *in vivo* distribution. In addition, non-specific binding of an antibody is not an issue. Indeed, Palmer *et al.* (2001) pointed out that the SMPX antibody cross-reacted with desmin, and that the pattern of SMPX in transverse sections of fetal myotubes resembled that of desmin. They try avoiding the problem by pre-immunoabsorption of the SMPX antibody against desmin. Kemp *et al.* (2001) also reported non-specific binding of their SMPX antibody. On the other hand, there

could also be problems linked to the use of a fusion protein. A protein tag could alter the endogenous localization e.g. by covering the localization signal.

Palmer *et al.* (2001) indicated costameric localization of SMPX in adult skeletal muscle. Yet, the only images showing SMPX surrounding myofibrils were of 17.5 day old embryos. Data from adult tissue was not shown. Further *in vivo* imaging studies with better sensitivity and resolution could confirm our Z-disc localization and rule out the possibility of non-specific GFP antibody staining and other *ex vivo* artefacts. Whether SMPX is found throughout the myofibril or surrounding it, and which proteins it interacts with, can be investigated with a three dimensional reconstruction of high-resolution confocal images or with electron microscopy.

4.2 Degradation of cells expressing SMPX-EGFP

In both cell culture and *in vivo* experiments, a degradation process leading to cell death started at day 1-3 after pEGFP-N1-*Smpx* transfection, with cells expressing more SMPX-EGFP degrading at an earlier time point. All images from our localization study were therefore obtained at an early time point, when the cells and fibres displayed normal morphology. The *in vivo* images showed a more patchy localization of SMPX-EGFP late in the degradation process, resembling the cell culture patchy localization.

One can attribute the cell death to cytotoxicity, which has been reported previously with overexpression experiments e.g. with a GFP fusion protein (Triplett & Pavalko, 2006; Wang *et al.*, 2006; Wang & Monteiro, 2007). Overexpression can lead to cytotoxicity e.g. by forming protein aggregates. Poly-glutamine and poly-alanine are examples of domains that can lead to aggregation of an overexpressed fusion protein (Wang *et al.*, 2006; Wang & Monteiro, 2007). A GFP fusion protein can then be seen with a patchy distribution in the cytoplasm (Wang, Lim *et al.* 2006). The FuGENE 6 protocol (Roche diagnostics GmbH, Mannheim, Germany) also reports that high expression levels of certain intracellular proteins (e.g. GFP) may be cytotoxic to some cell types.

Preliminary results from cell culture (results not shown) indicated that HEK-293 cells overexpressing SMPX-EGFP had a better survival rate than fibroblasts, myoblasts and *in vivo* muscle fibres. This can be attributed to the HEK-293 cells being more resistant to cytotoxicity. In my experiments, the fibroblasts and myoblasts had a shorter generation time than the HEK-293 cells (results not shown). A shorter generation time demands a higher metabolism, and thereby also a higher protein turnover. The higher protein turnover may lead to SMPX-EGFP reaching cytotoxic levels at an earlier time point.

Another explanation for the cell death can be that SMPX-EGFP works as a dominant negative, quenching the function of the endogenous SMPX (Ilkovski *et al.*, 2004; Wang *et al.*, 2006; Zhang *et al.*, 2006). The superior survival rate in the HEK-293 cells can then be explained by SMPX having a less critical function in these cells.

Whether SMPX-EGFP is cytotoxic or working as a dominant negative can be investigated with a study of competition between overexpressed endogenous SMPX and SMPX-EGFP, monitoring the cell death occurrence with an apoptosis kit.

4.3 Effects of SMPX on fibre type distribution and CSA

Our experiments showed that overexpression of SMPX for 14 days did not significantly alter the fibre type distribution or CSA in EDL. On the other hand, the *soleus* experiments had a shift towards a faster fibre type, with a mean decrease of 23% in type 1 fibres in the SMPX group compared with the sham group. However, the differences between the sham and SMPX group were only borderline significant: $p=0.05$. CSA was also significantly different between the sham and SMPX group in *soleus*, with a minor increase of 11% in the SMPX group compared with the sham group.

When comparing the sham and SMPX group in the same individual, the changes were not consistent. Some of the individuals even showed a change towards a slower fibre type and decreased CSA. There were also individual differences, and the number of fibres transfected in each muscle were highly variable. The overall

changes seen in the *soleus* could therefore be a result of chance. Experiments with a larger number of animals are needed to see if the effects seen in *soleus* are reproducible.

4.3.1 Selective transfection in electroporated mice muscle

The use of a sham control group is important to exclude possible effects following the electroporation procedure and uptake of foreign DNA. In both EDL and *soleus*, both the sham and SMPX transfected fibres displayed a faster fibre type distribution than the normal fibres from the same muscles. We attribute this to a selective transfection of fibres. The reason for this is at the present time not known. This selectivity has been confirmed by several others in our research group, and has been observed in mice, but not in rats (results not shown). The difference in size between rat and mice seen both at the macroscopic and cellular level may lead to important differences in geometry and strength of the electrical field during electroporation. We therefore find the comparison between the sham and SMPX groups to be the most reliable.

4.4 Possible functions of SMPX in skeletal muscle

In earlier studies of SMPX (Patzak *et al.*, 1999; Kemp *et al.*, 2001; Palmer *et al.*, 2001) it has been suggested that it could function both as a structural protein and a gene regulatory protein. Our effects of SMPX on fibre type distribution and CSA were relatively small, and one can question if they are genuine. Much of the experiments on SMPX have had a main focus on cell culture experiments. Our work has had a main focus on *in vivo* experiments, and it showed that SMPX generally does not promote a slow fibre type. Our results rather suggest a small shift in the fast direction. In contrast, Palmer *et al.* (2001) found increased MEF2 and NFAT activity in cell culture when overexpressing SMPX, and they speculated that SMPX may promote a slow fibre type.

Our results suggest that SMPX does not induce hypertrophy in EDL in adult skeletal muscle. Further studies are needed to confirm the changes seen in *soleus*, and a possible effect does not necessarily contradict SMPX being a structural protein. In

contrast, Kemp *et al.* (2001) found SMPX upregulated in the tibialis anterior muscle in response to 7 days of passive stretch. Kemp *et al.* (2001) therefore suggested that SMPX may induce hypertrophy in adult skeletal muscle.

That SMPX-EGFP is excluded from the nuclei at all times in our experiments supports a theory that SMPX is not working as a gene regulatory protein. Localization to the H-zone can then suggest a function as a structural protein with a possible link to a mechanical function in stretch and/or contraction. A structural protein could still indirectly affect the phenotype by being present in this force generating area; e.g. by changing the flexibility of the muscle, or by indirectly influencing a stretch-response or the contraction coupling. This is supported by there being highest levels of SMPX in *soleus*, and it can possibly have a more important structural role there than in EDL. This could explain the changes in fibre type distribution and CSA seen in *soleus* but not in EDL.

We have found that SMPX probably does not co-localize with actin filaments *in vivo*. In contrast, Schindeler *et al.* (2005) found that MYC epitope-tagged SMPX co-localized with actin networks at peripheral membranes and in perinuclear compartments *in vitro*.

The localization to the H-zone of the sarcomere, with a uniform signal throughout the H-zone, makes it intriguing to suggest that SMPX is bound to the central parts of the MyHC or the central parts of titin. It could also be a structural protein linking the two together. This is a plausible explanation, since both MyHC and titin are abundant and evenly distributed at the level of the H-zone throughout the sarcomere. This is in aggrement with our postulated even distribution of SMPX in this area. The titin COOH-terminal segment within and adjacent to the M-band has been shown to take part in several protein-protein interactions (Kruger and Linke 2009). This further supports the idea that SMPX could be linked to titin.

4.5 Future experiments

Taken that our results on fibre type distribution and CSA in *soleus* are somewhat unclear, new experiments with a larger number of animals should be performed to see if the results are reproducible.

A three dimensional reconstruction of *in vivo* confocal images with SMPX-EGFP can be performed to further deduce its localization. Electron microscopy could in this context also be applied in order to obtain high-resolution images. Whether SMPX-EGFP localizes to the H-zone inside myofibrils, at costameres or both can be investigated with these methods. There is also a possibility that SMPX is localized both inside and surrounding myofibrils.

4.6 Conclusions

- SMPX-EGFP was excluded from nuclei under our experimental conditions.
- SMPX-EGFP localized to the H-zone, with a minor portion possibly localized to the middle of the Z-disc in adult skeletal muscle.
- SMPX overexpression had no effect on fibre type distribution or CSA in EDL.
- SMPX overexpression could have changed the fibre type distribution in the fast direction and increased fibre CSA in *soleus*.

Our experiments do not support the idea that SMPX is a gene regulatory protein or a major signalling molecule related to force transduction in the I-band.

It remains unclear if SMPX has an effect on muscle phenotype, directly or indirectly.

Our results suggest that SMPX works mainly as a structural protein, with a possible connection with mechanical proteins like titin and myosin in the sarcomere in skeletal muscle.

5. Appendix A

5.1 DNA electroporation solution (1µg/µl)

4M NaCl	4µl
dH_2O	46µl
Reporter DNA (pAP-lacZ, $2^{\mu g}/_{\mu l}$) (gift from N. Gautam)	25µl
Fibre type and CSA experimental DNA (pCMS-EGFP-*Smpx*) or control DNA (pCMS-EGFP) ($2^{\mu g}/_{\mu l}$)	25µl
Localization experimental DNA (pEGFP-N1-*Smpx*) or control DNA (pEGFP-N1) ($2^{\mu g}/_{\mu l}$)	25µl
TOTAL	100µl

5.2 β-galactosidase staining

Fix solution:

Formaldehyde	2g
Glutaraldehyde	400µl
10xPBS	10ml
dH_2O	90ml

1. Dissolve formaldehyde in 70ml dH_2O heated to 60°C.
2. Add in PBS.
3. Cool off and add glutaraldehyde.
4. Adjust the pH to 7.1 with phenol red.

Staining solution:

10xPBS	150µl
0.2M potassium-ferrocyanide	30µl
0.2M potassium-ferricyanide	30µl
1M $MgCl_2$	3µl
dH_2O	1260µl
X-gal (50g in DMSO, Promega)	30µl

1. Draw a ring around the sections with a hydrophobic marker (H-4000, Vector Laboratories, Burlingame, CA, USA).
2. Fix with fixation solution at 4°C for 20 minutes.
3. Rinse 3x in PBS (pH 7.4).
4. Stain over night at 37°C.
5. Rinse 3x in PBS (pH 7.4).
6. Mount in glycerine gel:

Glycerine	15.0g
Glycerol	100ml
dH_2O	100ml

5.3 Antibody staining of MyHC

MyHC	**Primary ab**	**Secondary ab**
Type I	BA-D5	Rabbit anti-mouse IgG (FITC-conjugated, Sigma, F-9137)
All type II	My-32	Rabbit anti-mouse IgG (FITC-conjugated, Sigma, F-9137)
Type IIa	Sc-71	Rabbit anti-mouse IgG (FITC-conjugated, Sigma, F-9137)
All non IIx	BF-D5	Rabbit anti-mouse IgG (FITC-conjugated, Sigma, F-9137)
Type IIb	BF-F3	Anti-mouse IgM (Cy3.conjugated, J 115-165-020, Jackson ImmunoResearch Laboratories, West Grove, PE, USA)

The antibodies BA-D5, Sc-71 and BF-F3 were a gift from Stefano Schiaffino. My-32 (Molecular probes, Invitrogen Corp., Carlsbad, CA, USA).

Antibody staining of MyHC type I, type IIa, all type II and all non IIx:

1. Draw a ring around the sections with a hydrophobic marker (H-4000, Vector Laboratories, Burlingame, CA, USA).
2. Dilute primary ab 1:1000 in 1% BSA in PBS (pH 7.4).
3. Incubate the sections with primary ab for 60 minutes at room temperature.
4. Wash 3x 5 minutes in PBS (pH 7.4).
5. Dilute secondary ab 1:200 in 0.5% BSA in PBS (pH 7.4).

6. Incubate the sections with secondary ab for 30 minutes at 37°C.
7. Wash 3x 5 minutes in PBS (pH 7.4).

Ab staining of MyHC IIb:

1. Draw a ring around the sections with a hydrophobic marker (H-4000, Vector Laboratories, Burlingame, CA, USA).
2. Preincubate with 1% BSA in PBS (pH 7.4) for 60 minutes at 4°C.
3. Dilute primary ab 1:1000 in 0.5% BSA in PBS (pH 7.4).
4. Incubate the sections with primary ab for 45 minutes at 37°C.
5. Rinse 3x 5 minutes in PBS (pH 7.4).
6. Dilute secondary ab 1:200 in 0.5% BSA in PBS (pH 7.4).
7. Incubate the sections with secondary ab for 45 minutes at 37°C.
8. Wash 3x 5 minutes in PBS (pH 7.4).

5.4 Immunohistochemistry on single fibres

Binds with:	**Primary ab:**	**Dilution:**	**Secondary ab:**
MyHC	My-32	1:2000	Rabbit anti-mouse IgG (FITC-conjugated, Sigma, F-9137)
Filamentous actin	Phalloidin-TRITC (toxin)	1:2000	Directly linked to TRITC (Sigma Life Science, 1951)
EGFP	Mouse anti-GFP	1:2000	Rabbit anti-mouse IgG (FITC-conjugated, Sigma, F-9137)

5.5 10x PBS solution pH 6.9→1x pH 7.4

NaCl	80g
KCl	2g
Na_2HPO_4 x $2H_2O$	14.4g
KH_2PO_4	2g

6. Appendix B- Abbreviations

Aa = amino acid

Ab = antibody

bHLH = basic helix-loop-helix

β-gal = β-galactosidase

BSA = bovine serum albumin

CMV = cytomegalovirus

CSA = cross-sectional area

CsCl = cesium chloride

DAPI = 4,6-diamidino-2-phenylindole

DMEM = Dulbeccos modified Eagles medium

DMSO = Dimethyl sulfoxide

ECM = extracellular matrix

EDTA = ethylenediaminetetraacetic acid

EGFP = enhanced green fluorescent protein

EGTA = ethylene glycol tetraacetic acid

EDL = *extensor digitorum longus*

IGF-1 = insulin-like growth factor-1

kDa = kilodalton

MCS = multiple cloning site

MEF2 = myocyte-specific enhancer factor 2

MGF = mechanogrowth factor

MRF = myogenic regulatory factor

MURF = muscle-specific RING finger

MyHC = myosin heavy chain

MyLC = myosin light chain

neighbor-of-BRCA1-gene-1 = Nbr1

NFAT = nuclear factor of activated T-cells

PBS = phosphate buffered saline

RSV = rous sarcoma virus

Smpx = small muscle protein X-chromosome (gene)

SMPX = small muscle protein X-chromosome (protein)

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