

## **STUDY OF THE POSSIBLE INTERACTIONS OF TUBULIN, MICROTUBULAR SYSTEM AND PROTEIN STOP WITH MITOCHONDRIA IN MUSCLES CELLS**

Karen Guerrero<sup>1</sup>, Annie Andrieux<sup>2</sup>, Ulo Puurand<sup>3</sup>, Marko Vendelin<sup>4</sup>, Tiia Anmann<sup>5</sup>, Jose Olivares<sup>1</sup>, Didier Job<sup>2</sup>, Enn Seppet<sup>3</sup> and Valdur Saks<sup>1,5</sup>

<sup>1</sup> Laboratory of Fundamental and Applied Bioenergetics,  
INSERM E221, Joseph Fourier University, Grenoble, France;

<sup>2</sup> CEA, France

<sup>3</sup> Departments of Pathophysiology, University of Tartu, Tartu, Estonia;

<sup>4</sup> Institute of Cybernetics, Tallinn, Estonia

<sup>5</sup> Laboratory of Bioenergetics,  
National Institute of Chemical Physics and Biophysics, Tallinn, Estonia

Address for Correspondence : V.A.Saks  
Laboratory of Bioenergetics  
Joseph Fourier University  
2280, Rue de la Piscine  
BP53X – 38041  
Grenoble Cedex 9, France  
Tel : +33-4-76-63-56-27  
Fax : +33-4-76-51-42-18  
e-mail : [Valdur.Saks@ujf-grenoble.fr](mailto:Valdur.Saks@ujf-grenoble.fr)

## ABSTRACT

The aim of this work was to study possible connection of tubulin, microtubular system and a microtubular network stabilizing STOP protein with mitochondria in rat and mouse cardiac and skeletal muscles by immunofluorescence confocal microscopy and oxygraphy. Selective treatment of permeabilized fibers with trypsin caused abrupt changes in the mitochondrial arrangement and shape of isolated permeabilized cardiomyocytes, and is known to decrease apparent affinity of mitochondrial respiration for exogenous ADP in permeabilized muscle fibers with degradation of tubulin and several other cytoskeletal proteins (Appaix et al. *Exp. Physiol.* 2003). Intracellular localization and content of tubulin was found to be tissue specific, high in oxidative muscles and low in fast glycolytic skeletal muscle. Dissociation of microtubular system by colchicine (1-10  $\mu\text{M}$ ) only slightly decreased apparent  $K_m$  (ADP) with appearance of population of cells with low apparent  $K_m$  (ADP). Similar effect had a knock-out of STOP protein which localisation and content in muscle cells was also tissue specific. To evaluate the differences between muscles at the level of gene expression, total RNA pools from mouse tissues of interest: from slow-twitch oxidative heart (H) and *m. soleus* (S) as well as fast-twitch glycolytic *m. vastus lateralis* (VE) were isolated. Thereafter the total cellular RNA pools were reverse-transcribed into double-stranded (ds) full-length cDNAs and the electrophoresis technique was applied for DNA analysis. The results showed that in adult mice the mouse  $\beta$ -tubulin gene M-beta-4 is muscle fiber-type specific. It is concluded that the role of tubulin may be significant in regulation of mitochondrial outer membrane permeability in the cells *in vivo*, but the organisation of tubulin into the microtubular network has a minor significance.

*Key words:* cytoskeleton, mitochondria, respiration, tubulin, microtubular network, STOP protein.

## INTRODUCTION

In muscle cells mitochondria function within highly organized intracellular structures, their intracellular arrangement is very regular and follows the crystal-like pattern, in accordance with the hypothesis of their organisation into functional complexes with sarcoplasmic reticulum and myofibrils, intracellular energetic units, ICEUs (Vendelin et al, 2005). This specific arrangement can be changed by rather selective proteolytic treatment of the permeabilized cells which results also in the alteration of the parameters of mitochondrial respiration regulation, decreasing the value of apparent  $K_m$  for exogenous ADP (Saks et al., 2003). It is known that microtubules and molecular motors like dyneins and kinesins are responsible for the intracellular localization of organelles in many types of the cells, but their connection to mitochondria and role in regulation of mitochondrial function in muscle cells is still unknown. Microtubules are dynamic structures which depend on many factors, such as temperature and ionic force. They are stable at physiological temperature (37°C) and even at 25°C but undergo a spontaneous cold-induced depolymerisation at 4°C. Microtubular network can be stabilized at cold temperatures in the presence of a MAP (Microtubule Associated Protein) called STOP for Stabilizing Tubule Only Polypeptide. This protein of 145 kDa has been identified from the 80's (Pabion et al., 1984). It is present in many tissue such as brain, heart, muscles, lung, testis (Aguzzoul et al., 2003). The cytoskeleton of cardiac myocytes consists of actin, the intermediate filament desmin and of tubulin that form the microtubules by polymerisation. Microtubular associated proteins (MAPs) bind to tubulin and play a significant role in stabilizing microtubules and enabling an interaction with other cellular organelles (Hein *et. al.*, 2000). The tubulin molecule is a heterodimer of an  $\alpha$  and  $\beta$ -isoform with a molecular weight of 55 kDa per monomer. Tubulin occurs in cells as organized microtubules with a diameter of 25 nm. A constant turnover of microtubules by

polymerization and depolymerization takes place. In cardiomyocytes, only 30% of total tubulin is present in the polymerized form as microtubules whereas 70% occurs as non-polymerized cytosolic protein (Tagawa, *et al.*, 1998).

Lewis *et al.* have cloned described five mouse tubulin cDNAs, two (M alpha 1 and M alpha 2) that encode alpha-tubulin and three (M beta 2, M beta 4, and M beta 5) that encode beta-tubulin. The sequence of these clones reveals that each represents a distinct gene product. Two of the beta-tubulin isotypes defined by the cloned sequences are absolutely conserved between mouse and human, and all three beta-tubulin isotypes are conserved between mouse and rat. M alpha 1 and M beta 2 are expressed in an approximately coordinate fashion, and their transcripts are most abundant in brain and lung. M alpha 2 and M beta 5 are ubiquitously expressed and to a similar extent in each tissue, with the greatest abundance in spleen, thymus, and immature brain. In contrast, M beta 4 is expressed exclusively in brain. Whereas the expression of the latter isotype increases dramatically during postnatal development, transcripts from all four other tubulin genes decline from maximum levels at or before birth.

A major interest in our laboratory is the study of molecular mechanisms of interactions of mitochondria with cytoskeletal proteins in cardiac and skeletal muscle cells. Our previous studies (Kuznetsov, 1996; Voloshchuk, 1998) demonstrate the low apparent affinity of mitochondrial respiration to exogenously added ADP in oxidative (myocardium, *m. soleus*) versus glycolytic muscles (*m. extensor digitorum longus*, *vastus lateralis*). Selective proteolysis resulted in increased affinity of mitochondria to ADP in oxidative muscles but exerted no effect to that parameter in glycolytic ones (Kuznetsov, 1996; Voloshchuk, 1998). It was hypothesized that both oxidative muscles, myocardium and *m. soleus*, likely express some identical proteins capable of exerting intracellular control over mitochondrial respiration, whereas the glycolytic muscles lack such proteinic compounds. In oxidative muscle cells, these proteins may participate in the organization of the intracellular energetic

units, representing the functional and structural complexes of mitochondria with adjacent ATPases. This type of organization likely confers specific regulatory properties of respiration to these muscles (Saks, 2001; Seppet, 2001). To define the nature of these proteins, we isolated genes that are expressed in common between both types of oxidative muscles but not expressed in glycolytic muscle (*m. vastus lateralis*). The aim of this present work was to investigate, in a first stage, the possible role of microtubules in the regulation of mitochondrial respiration in cardiac and skeletal muscles. In a second stage, implication of the microtubule associated protein STOP in muscle cellular energetics was also studied.

## **MATERIALS AND METHODS**

### **Animals**

Wistar rats were used in experiments. Control mice and STOP-deficient mice were also used in experiments. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the National Institute of Health (NIH Publication No 85-23, revised 1985).

### **Isolation of adult rat cardiac myocytes**

Calcium-tolerant myocytes were isolated by perfusion with a collagenase-medium as described earlier by Kay et al. (1997b).

### **Preparation of skinned muscle fibers**

Skinned (permeabilized) muscle fibers were prepared from cardiac and skeletal muscles (soleus, EDL) according to the method described earlier (Saks et al, 1998b).

### **Colchicine treatment**

Once permeabilized fibers prepared, they were incubated for 2h at 4°C in solution B in the presence of 1 or 10  $\mu\text{M}$  colchicine (Sigma). Then they were washed 3 times in solution B before experiments.

### **Determination of the rate of mitochondrial respiration in skinned fibers**

The rates of oxygen uptake were recorded by using a two-channel respirometer (Oroboros oxygraph, Paar KG, Graz, Austria) in solution B, containing respiratory substrates (see below) and 2  $\text{mg}\cdot\text{ml}^{-1}$  of bovine serum albumine (BSA). Determinations were carried out at 25°C and the solubility of oxygen was taken as 215  $\text{nmol}\cdot\text{ml}^{-1}$  (Kuznetsov et al, 1996)

Confocal microscopy: immunofluorescence labelling of  $\beta$ -tubulin, STOP protein and desmin and mitochondrial imaging by autofluorescence of flavoproteins.

Labelling of cytoskeletal proteins and mitochondrial imaging was performed on permeabilized mice fibers or rat cardiomyocytes in suspension. Cells were fixed for immunofluorescence labelling with 4% paraformaldehyde (PFA) for 20 min at room temperature under mild stirring. Cardiomyocytes or fibers were washed with PBS containing (mM): NaCl 56,  $\text{KH}_2\text{PO}_4$  1.5, KCl 2.7 and  $\text{Na}_2\text{HPO}_4$  8 (Biomedica) and incubated in PBS/BSA 2% with primary antibodies of cytoskeletal proteins overnight at 4°C. Monoclonal anti- $\beta$ -tubulin (mouse IgG1 isotype) antibody (Sigma) at 1/200, polyclonal anti-STOP (rabbit) 23C and 23N antibodies 1/400 (Guillaud et al., 1998) and polyclonal anti-desmin (rabbit) antibody 1/200 (gift of L. Rappaport, INSERM Unit 127, Paris) were used. Monoclonal anti- $\beta$ -tubulin was immunospecific for tubulin as determined by indirect immunofluorescence staining and immunoblotting procedures. After three washed in PBS, cells were incubated for 4h in PBS/BSA 2% with secondary antibody rhodamine (TRITC)-conjugated AffiniPure F(ab')<sub>2</sub> fragment Donkey Anti-Mouse IgG or anti-rabbit IgG (excitation 503 nm, emission 530 nm) at 1/50 (Interchim). This time, cardiomyocytes or fibers were washed 3 times in PBS and the n3 times in distilled water. The labelled cells were deposited on glass coverslips and mounted in a mixture of Mowiol and glycerol to which 1.4-diazobicyclo-[2,2,2]-octane (Acros Organics) was added to delay photobleaching. Samples were observed by confocal microscopy (DME IRE2, Leica) with x40 oil immersion, NA 1.4, objective lens.

Autofluorescence of mitochondrial flavoproteins

Video

Western blot analysis.

For western blot analysis the proteins were extracted after powdering the frozen tissue samples in the liquid nitrogen. The protein extraction was carried out in .....

Reagents.



## **RESULTS**

The results show that tubulin and STOP protein contents and microtubular organisation are tissue specific. Both tubulin and STOP protein are highly expressed in heart and oxidative muscle cells and almost absent in fast glycolytic skeletal muscles. Microtubular network can be dissociated by colchicine with minor changes in the apparent  $K_m$  values for exogenous ADP. Similar changes were observed in mice with knocked-out STOP protein. It is concluded that organisation of microtubular network has only a minor role in regulation of mitochondrial function, but the connection of tubulin and other associated proteins to mitochondria may be important in the regulation of the VDAC channels permeability for adenine nucleotides.

1. The dynamics of trypsin effect on the mitochondrial arrangement and shape of permeabilized cardiomyocytes.

The “trypsin movie” shows the rather dramatic changes induced by short – time treatment of isolated permeabilized cardiomyocytes by low concentration of trypsin. The dynamics of these changes in time is shown by supplementary online video recordings. In isolated permeabilized cardiomyocytes one can see very regular intracellular arrangement of mitochondria, with characteristic crystal-like pattern (Vendelin et al., 2005). This regular arrangement is changed by trypsin treatment. Observation of nonfixed cells in confocal microscopy showed very interesting abrupt changes in the shape of cardiomyocytes: after some lag-period (see online video), one observes rapid contraction of the cells resulting in decrease of cell volume by factor of about three , and subsequent relaxation and restoration of initial cell volume, but with completely disordered mitochondrial arrangement. Remarkably, disorganized mitochondria stay usually still attached to some intracellular structures after treatment with trypsin in low concentration. Increase of the trypsin concentration up to 5  $\mu\text{M}$

resulted in dissociation of mitochondria from cell due to complete digestion of cytoskeletal structures (results not shown).

This interesting dynamics means that in the intact cardiomyocytes there is a complex equilibrium of elastic forces within the cytoskeletal networks which is changed by successive digestion of the cytoskeletal components by trypsin. Since one of the major cytoskeletal components is the microtubular network, and tubulin has been sensitive to the trypsin treatment (Appaix et al., 2003), in this work we decided to investigate in details the possible roles of both tubulin and microtubular network in regulation of mitochondrial function in muscle cells *in situ*.

## 2. Microtubular network and tubulin localization in muscle cells.

Both rat cardiomyocytes and permeabilized fibers from heart and skeletal muscle of mice were used to study the roles of microtubular network, STOP protein and tubulin in regulation of mitochondrial function. The latter was characterized by apparent  $K_m$  for exogenous ADP and  $V_{max}$  of respiration.

Fig. 1 shows the results of immunolabeling of  $\beta$ -tubulin in different permeabilized muscle fibers of mouse when tissue fixation was carried out at 4 C. In spite of low temperature, the tubulin is organized into the microtubular network in mouse cardiac muscle fibers in the same way as in rat cardiomyocytes (see below). Since the localisation of STOP protein shows similar network organisation (Fig. 2), the presence of microtubular network at 4 C in heart cells is most probably due to stabilizing action of STOP protein. Indeed, in the STOP-protein knock-out mice the network organisation disappears in cardiomyocytes and tubulin is localised diffusely, in some parts co-localized with mitochondria. For soleus muscle the microtubular network was not seen neither before nor after knockout of STOP protein (Fig. 1), and in both cases  $\beta$ -tubulin was colocalized with mitochondria (Figure 3), which in skeletal muscle cells are localized mostly close to the Z-line, while in heart cells mitochondria are

localised at the level of A-band of sarcomeres between Z-lines (Ogata et al. 1997; Vendelin et al. 2005). Both tubulin and STOP protein were practically absent in the glycolytic fast twitch skeletal muscle (Figs. 1C and F, 5 and 6). Gastrocnemius muscle showed different content of tubulin due to mixed types of fibers (Figs. 7 and 8).

The observed tissue-specificity of the expression of tubulin in muscle cells was confirmed by western-blot analysis of muscle samples (Fig. 9).

### 3. Functional analysis of mitochondria in mouse muscle fibers with and without STOP protein.

Fig. 10 shows the values of apparent  $K_m$  for exogenous ADP in regulation of mitochondrial respiration in the permeabilized fibers of heart muscle, soleus, white gastrocnemius and EDL. As it has been described before for different rat and pig muscle fibers (Veksler et al. 1995; Kuznetsov et al. 1996; Gueguen et al. 2004), the apparent  $K_m$  for exogenous ADP is tissue specific. In spite of smaller diameters of heart and soleus muscle fibers as compared to the gastrocnemius and EDL, these oxidative muscles have a very high apparent  $K_m$  (ADP) which exceeds that for isolated mitochondria *in vitro* by more than order of magnitude, while the fast glycolytic muscle fibers the apparent  $K_m$  (ADP) is close to that in mitochondria *in vitro* (Fig. 10). The thinnest oxidative muscle studied until now is the heart muscle of ... with cell diameter of 5  $\mu$ m, but with high apparent  $K_m$ (ADP) (Rikke, ). These data confirm the earlier conclusion that the diffusion distance in bulk water phase is not a parameter upon which depends the value of the apparent  $K_m$  for exogenous ADP (Seppet et al., 2004), and the latter parameter is related to the peculiarities of the intracellular organisation and mitochondrial arrangement and their interaction with cytoskeletal proteins (Andrienko et al., 2003).

The tissue specificity of the apparent  $K_m$  for exogenous ADP is observed also in the case of muscle fibers from STOP-protein knock-out mice (Fig. 10), the values of the parameter being lower by about 30 % than in controls for heart and soleus muscles, with no observable changes in their values for fast glycolytic muscles of gastrocnemius and EDL. No changes in  $V_{max}$  of respiration were seen in heart and soleus muscles (Fig. 11).

4. The STOP protein and the effect of microtubular dissociation in rat cardiomyocytes by colchicine on mitochondrial respiration.

In rat cardiomyocytes the localisation of STOP protein at 4 C was very similar to that in the mouse heart, showing the network pattern with regular striations inside (Figs. 2 and 12). Fig. 12 shows that the striated pattern results from its connection to the Z-line structures immunolabelled by anti-desmin antibody (Fig. 12).

At 4 C, the microtubular network is similar to that for STOP protein, but without connection to the Z-lines (Fig. 13A). This microtubular network is dissociated by colchicines partially at its 1  $\mu$ M (Fig. 13B) concentration and totally at 10  $\mu$ M concentration (Fig. 13C).

Figure 14 shows that in ghost cardiomyocytes obtained by extraction of permeabilised cells with 800 mM KCl solution the tubulin distribution is changed and gives the somehow ordered striated pattern earlier observed for mitochondrial distribution (Andrienko et al. 2003). This distribution was not changed by colchicine.

Figure 15 shows that complete dissociation of microtubular network results in appearance of new population of mitochondria with low apparent  $K_m$  for exogenous ADP, but the main population (60 % of total) is still characterised by high apparent  $K_m$  (ADP).

5. Tissue-specificity of the gene expression of muscle proteins.

Figure 16 shows the experimental approach to evaluation of differences between muscles at the level of gene expression. Total RNA pools from mouse tissues of interest: from slow-twitch oxidative heart (H) and *m. soleus* (S) as well as fast-twitch glycolytic *m. extensor*

*digitorum longus* (E) were isolated. Thereafter the total cellular RNA pools were reverse-transcribed into double-stranded (ds) full-length cDNAs. The electrophoresis technique was applied for DNA analysis. Figure 17 shows fragments three total cDNA pools characteristic in *m. soleus* and heart (both oxidative) and *m. extensor digitorum longus* (glycolytic). After that cDNA libraries for *m. soleus* (S), ventricular myocardium (H) and *m. extensor digitorum longus* (E) were generated, and H was subtracted against E resulting in the cDNA population of heart-specific genes (HE). Next step was kindred DNA amplification by novel recently described method (Puurand *et al.* 2003) to obtain common cDNA fragments from HE and S. To identify the shared genes (SHE) the fragments of SHE cDNAs were inserted into the plasmid vector. Approximately one hundred inserts were amplified for 22 cycles in PCR reaction. Full-length denatured cDNAs H, S and E were labeled with Digoxigenin-11-dUTP and hybridized to 54 clones of the genes of common pool SHE, dot blotted in a piece of nylon membrane. The results are shown in Figure 18. The binding occurred with each clone of 54 when all cDNAs of *m. soleus* (S) or heart (H) were hybridized. Very rare pattern of binding was observable in case of E indicating that large majority of genes, specific for *m. extensor digitorum longus* (E), were eliminated from common pool of SHE.

Nucleotide sequence of cloned slow-twitch muscle specific cDNA (ID=50) as revealed by Southern hybridization is shown below.

The clones containing cDNA inserts were sequenced and submitted to a similarity search in NCBI database using BLASTn algorithm (Altschuld *et al.*, 1990).

We report here the mouse cDNA nucleotide sequence that we have found indicating the homology with mouse beta-tubulin gene M-beta-4.

```
GCAAATGCACAGTGGACATGGCTAGCAGACAGGCTGTGAATGAATAAAGAGTTC  
ACACTGCCCCCATGCTTTAGTGACTAAGACTGCTCTAAGCCA
```

similar to gb:X79535 TUBULIN BETA-2 CHAIN (HUMAN);

gb:M28730 Mouse beta-tubulin gene M-beta-4, 3' end

(MOUSE);

Score = 174 bits (88), Expect = 4e-42

Identities = 94/96 (97%)

Strand = Plus / Plus

Our results show that beta-tubulin cDNA is not ubiquitously expressed. The cDNA is present in mouse myocardium and oxidative *m. soleus* but is absent in *m. extensor digitorum longus*.

## DISCUSSION

The first interesting and novel observation in this work is the localisation of STOP protein and its connection both to the microtubular network and Z-line structures in the cardiomyocytes. The presence of this protein may explain why the microtubular system in cardiomyocytes *in situ* is stable at low temperatures. Indeed, in the heart muscle of mice deficient in STOP protein the microtubular network was absent, and tubulin distribution was completely diffuse in these cells (Fig. 1). In soleus muscle both STOP protein and tubulin were found in close connection to the Z-line area, where the mitochondria are also localized. Interestingly, both proteins were almost absent in the fast-twitch skeletal muscle (Figs. 1, 5 and 6).

The tissue-specificity of tubulin expression and organisation into microtubular network is another novel observation. Indeed, we have shown different patterns of organization depending of the fiber type. Free tubulin (non polymerized, in heart cells after colchicine treatment or in STOP K/O mice) is soluble in the cytoplasm and as we work with permeabilized cells, the presence of this protein is indicating that it is attached to some unknown intracellular structures.

We observed parallel changes in tubulin expression and localisation with alteration of the apparent  $K_m(\text{ADP})$  values between heart, soleus, gastrocnemius and EDL muscles. The intriguing question is whether we can explain the high values of apparent  $K_m(\text{ADP})$  in oxidative muscles by these observations.

The treatment with colchicine lead to a resistant population of mitochondria (with a high apparent  $K_m$  for ADP). These experimental data are in agreement with the resistant population of microtubules. Colchicine is a drug known to induce a human myopathy (Choi and al., 1999, Wilbur and Makowsky., 2004). This myopathy is accompanied with an increase

in CK levels as well as a disorganization in myofibrills and the presence of vacuoles containing lysosomes (Choi et al., 1999).

In STOP (-/-) mice, apparent  $K_m$  for exogenous ADP is strongly decreased by 30 and 40 %, respectively. STOP protein is hence indirectly involved in the regulation of mitochondrial function. In the glycolytic muscles, no modification was observed. In the oxidative muscle cells this increase in the apparent affinity for exogenous ADP can be correlated with disorganization of tubulin that could decrease the restrictions of ADP diffusion in the neighbourhood of mitochondria. One can imagine that the polymerized form of tubulin could create spatial constraints in the cytoplasm preventing an homogenous diffusion of adenylic nucleotides *in vivo*. Nevertheless, disruption of microtubular network is not accompanied with a decrease of the apparent  $K_m$  as high as a proteolytic treatment for example. This can be explained by an indirect interaction of microtubules with mitochondria via microtubule associated proteins : MAPs.

Linden and Karlsson in 1996 have shown that (Linden et Karlsson., 1996) MAP-2 directly interacts with VDAC at the external mitochondrial membrane.

Moreover, presence or absence of tubulin is tissue specific as the high value or the low value of the apparent  $K_m$  for exogenous ADP in muscle cells. It is possible that probably only a mitochondria-MAP-tubulin interaction is important for the regulation of the external mitochondrial membrane permeability without a mitochondria-MAP-microtubule interaction. This hypothesis can be in agreement with the data from Carré and al., (Carré and al., 2002). They have shown by electronic microscopy the presence of tubulin in mitochondrial membranes from human cancerous or not cells. They have also analysed different isotypes of tubulin present in these membranes. Mitochondrial tubulin is enriched in acetylated and tyrosinated  $\alpha$ -tubulin and in  $\beta$ -tubulin 3 but contains a few of  $\beta$ -tubulin 4 in comparison with cellular tubulin. These results underline that the isotype  $\beta$ -tubulin 4 is mainly expressed in the cytoplasm that is in agreement with our study concerning its tissue specificity. Mitochondrial tubulin is probably organised in hétérodimères  $\alpha/\beta$  (approximately 2% of cellular tubulin).

Immunoprecipitation experiments showed that mitochondrial tubulin is specifically associated with the mitochondrial VDAC (Carré and al., 2002). One can submit the hypothesis that there is a transmembranar signal able to modify the VDAC permeability, thus to regulate mitochondrial respiration according to the cytoplasmic interaction of  $\beta$ -tubulin 4, tissue specific, with MAP and mitochondria.



The results of this study show that the microtubular network and STOP-proteins are organized in muscle cells in tissue specific manner. In adult mice the mouse  $\beta$ -tubulin gene M-beta-4 is muscle fiber-type specific. Gene expression analysis revealed that mouse  $\beta$ -tubulin gene M-beta-4 is expressed not ubiquitously and not exclusively in (developing) brain as reported by Lewis group (Lewis *et al.*, 1985) but homological cDNA sequences have co-expressed in mouse myocardium and oxidative skeletal muscle *m. soleus*. The results show also that the presence of tubulin with associated proteins, but not its organization into microtubular network may be important for regulation of mitochondrial affinity for exogenous ADP in muscle cells.

### **LEGENDS TO FIGURES**

Figure 1. Localisation of beta-tubulin in different types of wild type and STOP-deficient mice muscles. In wild mice muscles, microtubular network is present in heart (A), while there are regular striations in soleus (B) and a few staining at the subsarcolemmale level in EDL (C). In STOP-deficient mice muscles, only in heart microtubular network is destroyed (D) but tubulin is still present in the cells, for soleus and EDL, (E) and (F) respectively, there are no change in the distribution of beta-tubulin.

Figure 2. Localisation of STOP protein in different wild mice muscles at 4°C. In heart fibers (A), soleus (B) and EDL (C).

Figure 3. Immunostaining of beta-tubulin and mitochondrial flavoproteins autofluorescence in wild type mice soleus permeabilized fibers. Mitochondria are very regularly arranged (A)

across the fiber as well as beta-tubulin (B). The superposition of the two pictures reveals a complete colocalisation of both mitochondria and beta-tubulin in oxidative permeabilized fibers (C).

Figure 4. Immunostaining of beta-tubulin (A) and mitochondrial flavoproteins autofluorescence (B) in STOP-deficient mice soleus permeabilized fibers. The superposition of the two pictures reveals a complete colocalisation of both mitochondria and beta-tubulin in oxidative permeabilized fibers (C).

Figure 5. Immunostaining of mitochondrial flavoproteins autofluorescence (A) and beta-tubulin (B) in wild type mice EDL permeabilized fibers. Mitochondria are precisely arranged while there is no immunofluorescent signal for beta-tubulin in glycolytic muscle fibers.

Figure 6. Immunostaining of mitochondrial flavoproteins autofluorescence (A) and beta-tubulin (B) in STOP-deficient mice EDL permeabilized fibers. Mitochondria are precisely arranged while there is no immunofluorescent signal for beta-tubulin, as in the wild type EDL permeabilized fibers.

Figure 7. Immunostaining of beta-tubulin and mitochondrial flavoproteins autofluorescence in wild type mice white gastrocnemius permeabilized fibers. Mitochondria are arranged predominantly at the subsarcolemmal level (A) as well as beta-tubulin (B). The superposition of the two pictures reveals a complete colocalisation of both mitochondria and beta-tubulin in a mixed muscle permeabilized fibers (C).

Figure 8. Immunostaining of beta-tubulin and mitochondrial flavoproteins autofluorescence in wild type mice white gastrocnemius permeabilized fibers. This figure demonstrates another organisation of mitochondria (A, C) and tubulin (B, D) in gastrocnemius muscle (A, B, C). While the pictures (D, E, F) show two neighbour fibers. The upper one where mitochondria are regularly arranged across the fiber and the lower one where they are predominantly at the subsarcolemmal level as well as beta-tubulin. The superposition of the two pictures reveals a complete colocalisation of both mitochondria and beta-tubulin in a mixed muscle permeabilized fibers (C and F).

Figure 9. Western blot analysis of alpha-tubulin (A), beta-tubulin (B) and STOP protein (C) in rat heart (C), soleus(S) and vastus lateralis (VE). Alpha tubulin is only present in heart, while beta-tubulin is present in heart and soleus as well as STOP protein. None of these three proteins were found in VE. 3T3 cells served as controls.

Figure 10. Apparent Km for exogenous ADP in regulation of mitochondrial respiration in the permeabilized fibers of heart muscle, soleus, white gastrocnemius and EDL of control and transgenic mice.

Figure 11. Rates of mitochondrial respiration in the permeabilized fibers of heart muscle, soleus, white gastrocnemius and EDL of control and transgenic mice.

Figure 12. Immunostaining of STOP /desmin in rat cardiomyocytes at 4°C. Both proteins are revealed with the same secondary TRITC-antibody. These pictures show a mixed pattern of desmin (striations at the Z lines) and of STOP protein (colocalized with tubulin).

Figure 13. Immunostaining of STOP /desmin in rat cardiomyocytes at 25°C. Both proteins are revealed with the same secondary TRITC-antibody. These pictures show that desmin and STOP have the same organized pattern (striations at the Z lines).

Figure 14. Immunofluorescence of beta-tubulin in rat permeabilized cardiomyocytes (A). In the control, one can see the microtubular network. (B). incubated, 2h, 4°C, with 1 µM colchicines, there is an incomplete destruction of the microtubules. (C). incubated, 2h, 4°C, with 10 µM colchicines. The collapse of the network seems complete, free tubulin is still present uniformly in the cells .

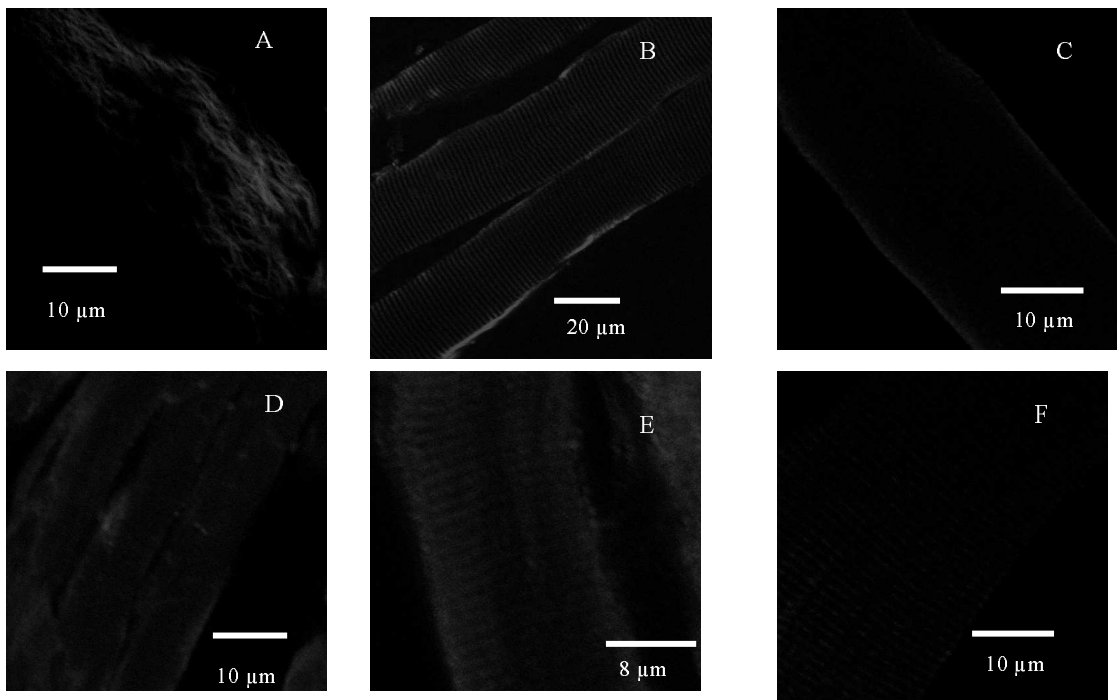
Figure 15. Immunostaining of tubulin in rat ghost cardiomyocytes (A) control. (B) treated with colchicine 10 µM.

Figure 16. Effect of colchicine 10 µM on the apparent Km for exogenous ADP in skinned and ghost rat fibers.

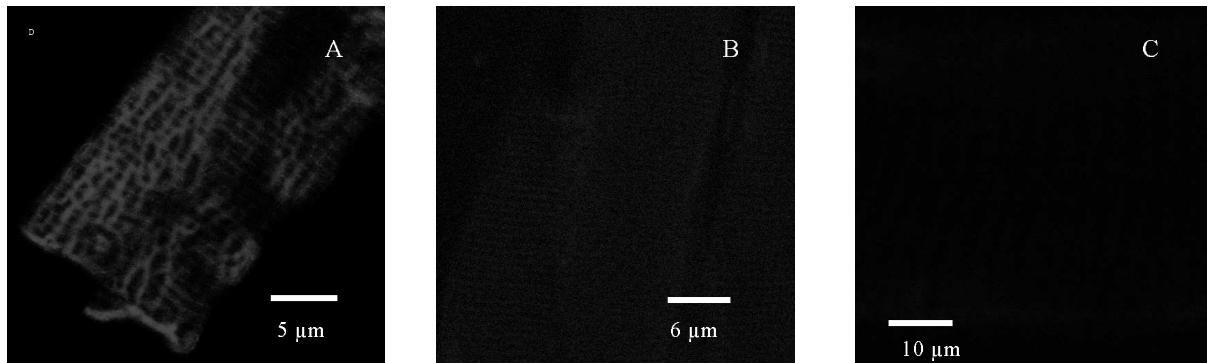
Fig. 17. Our experimental course to identify the genes that are expressed exclusively in oxidative muscles.

Fig. 18. Agarose gel electrophoresis of full-length total cDNAs from different muscle tissues of mouse.

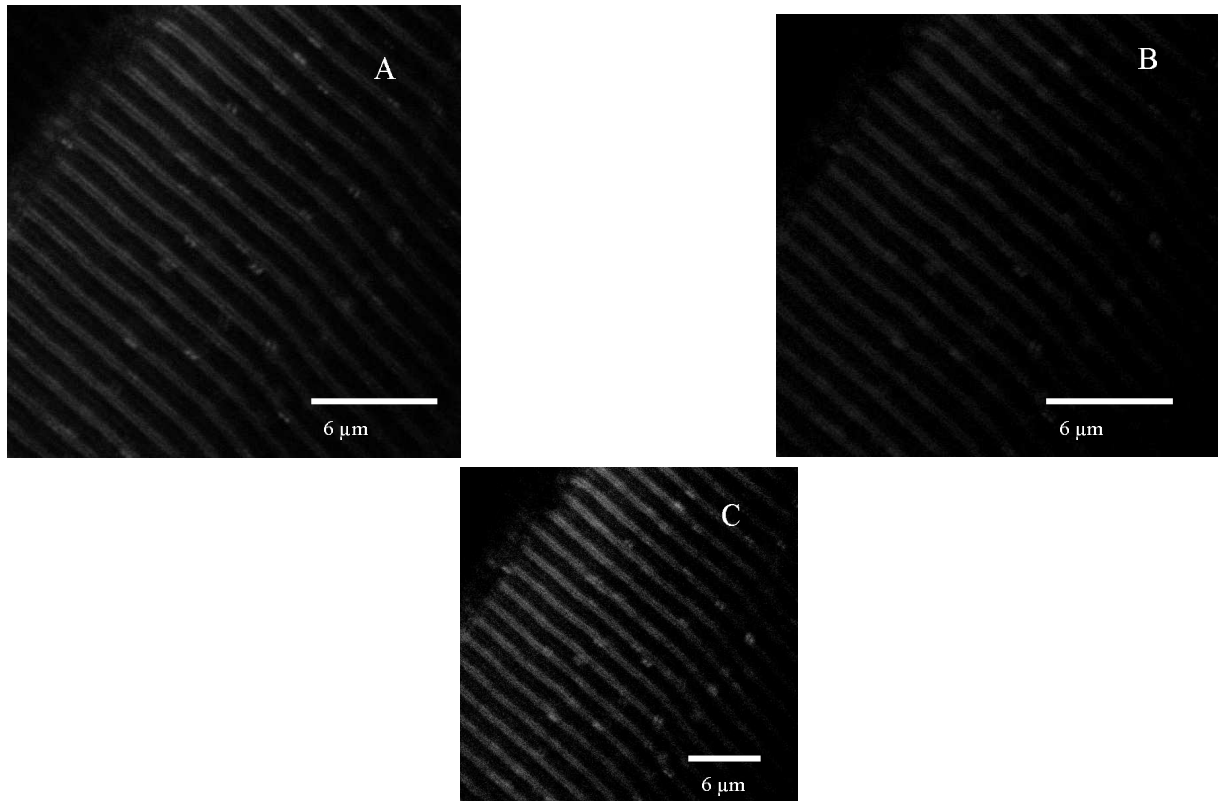
**Fig.1**



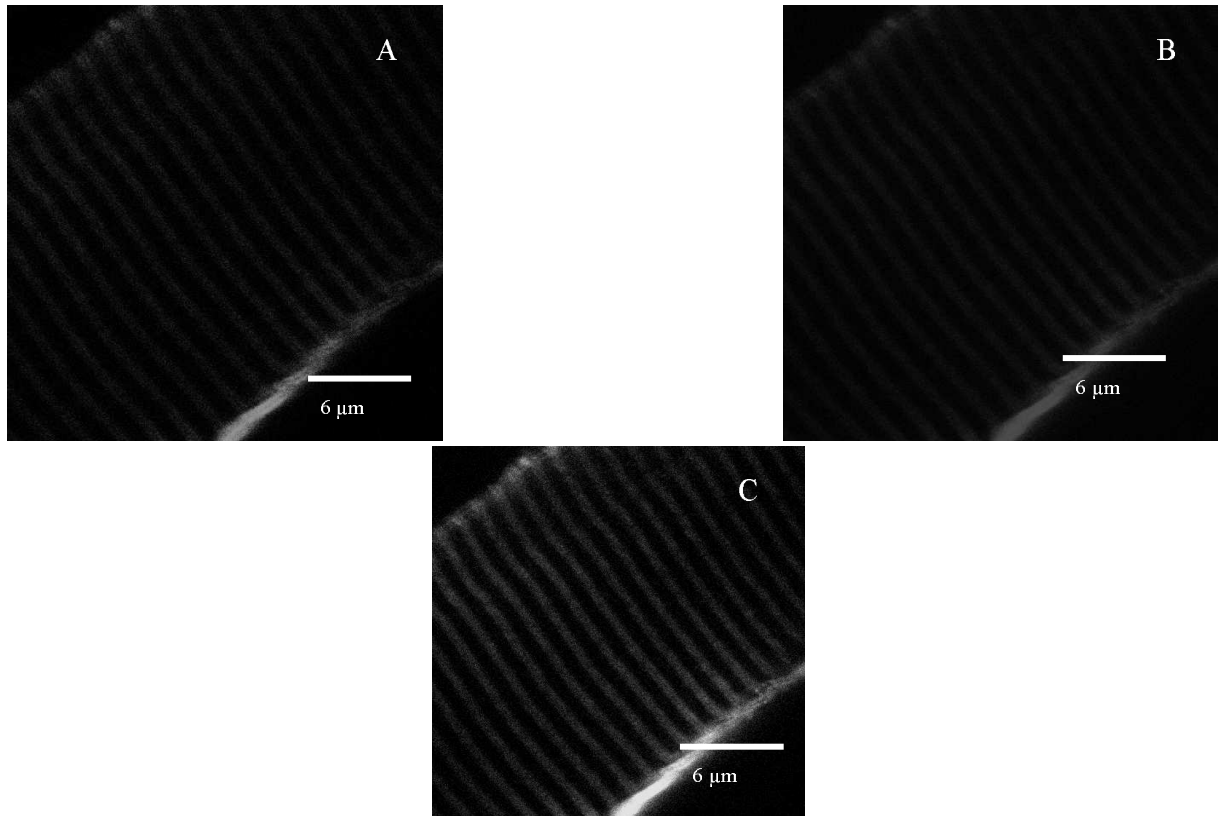
**Fig.2**



**Fig.3**

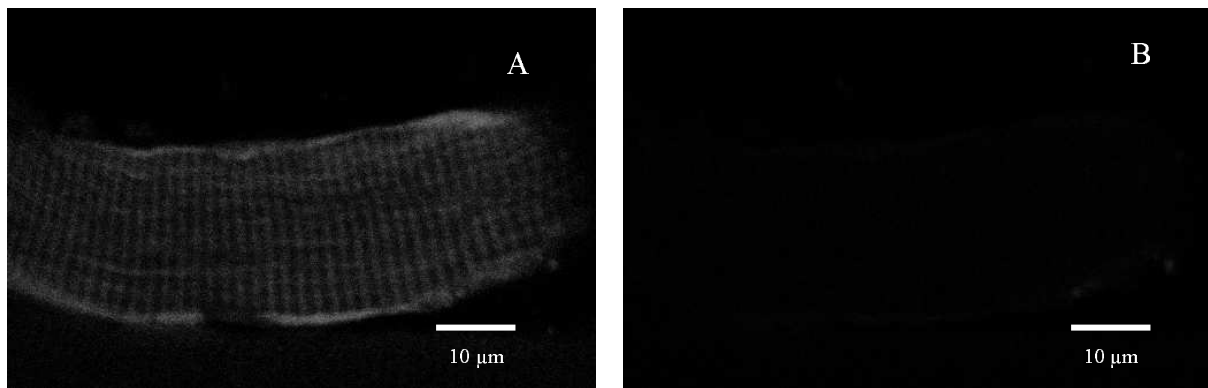


**Fig.4**

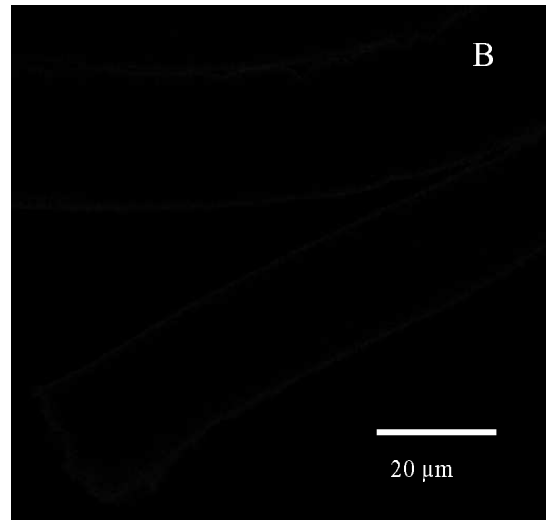
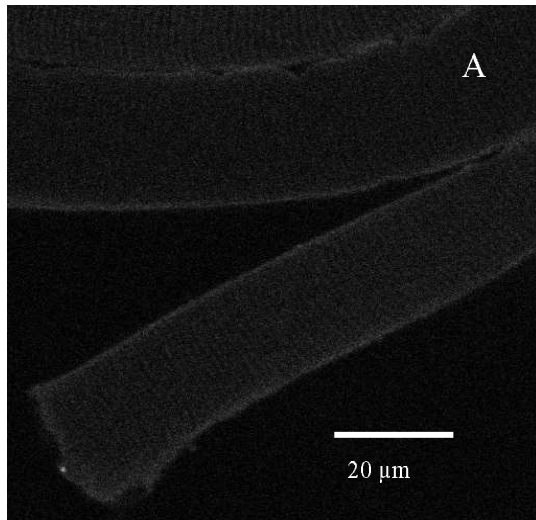




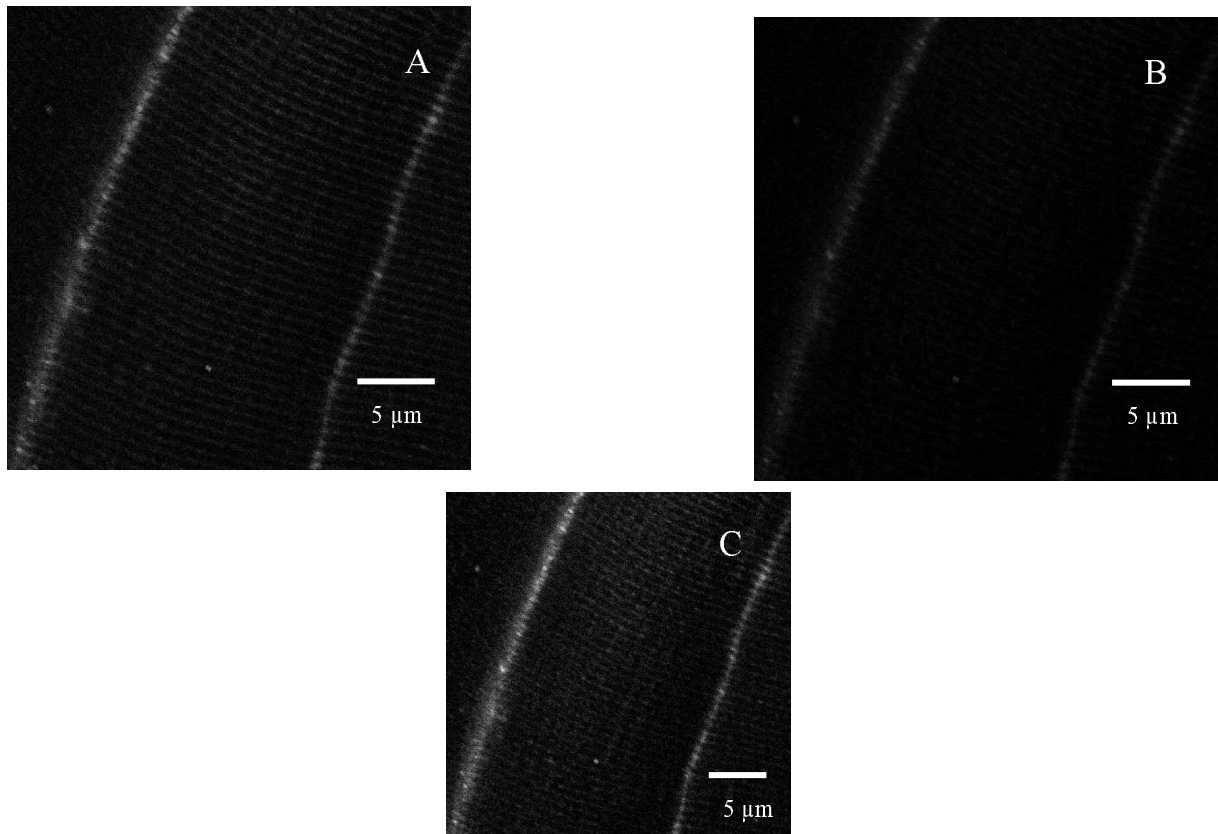
**Fig.5**



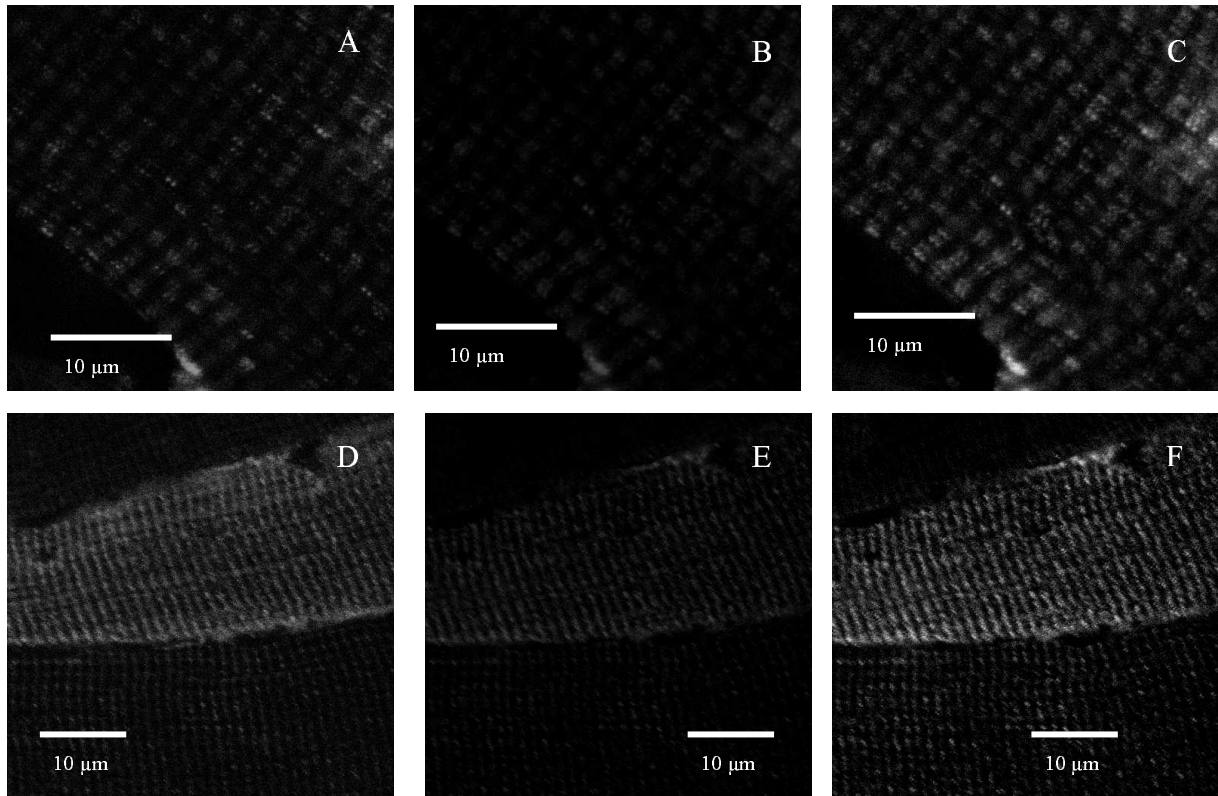
**Fig.6**



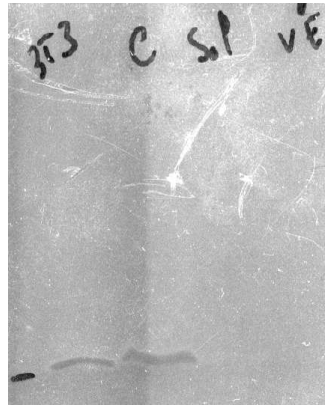
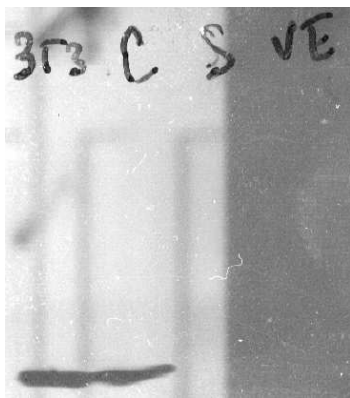
**Fig.7**



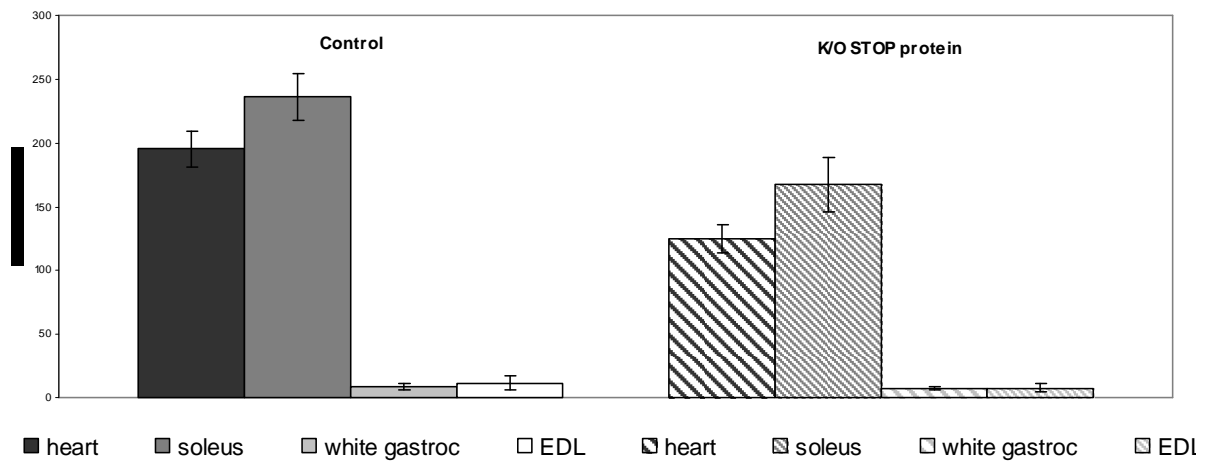
**Fig.8**



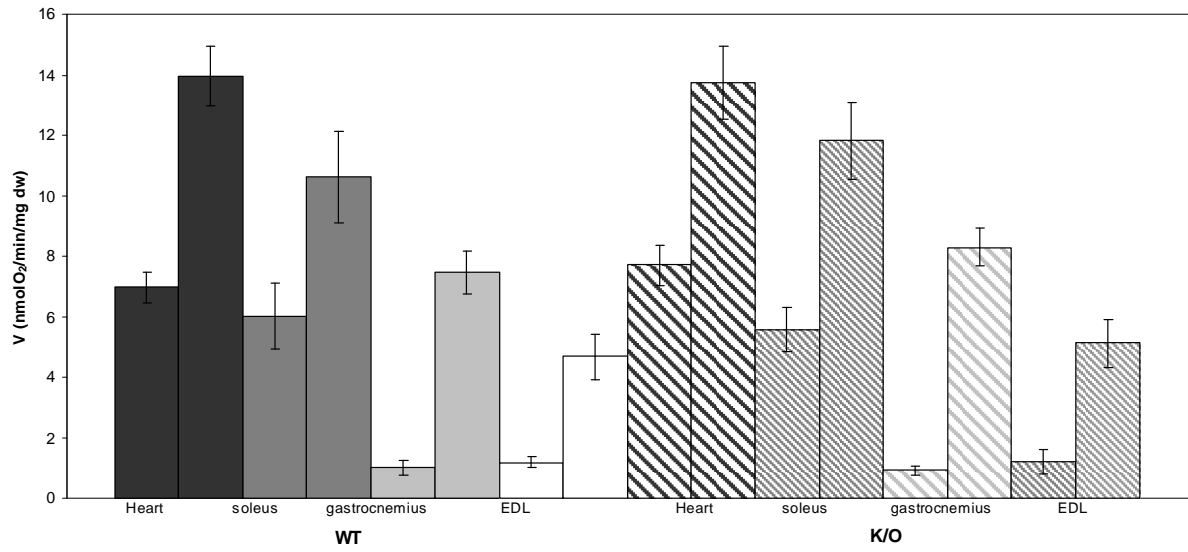
**Fig.9**



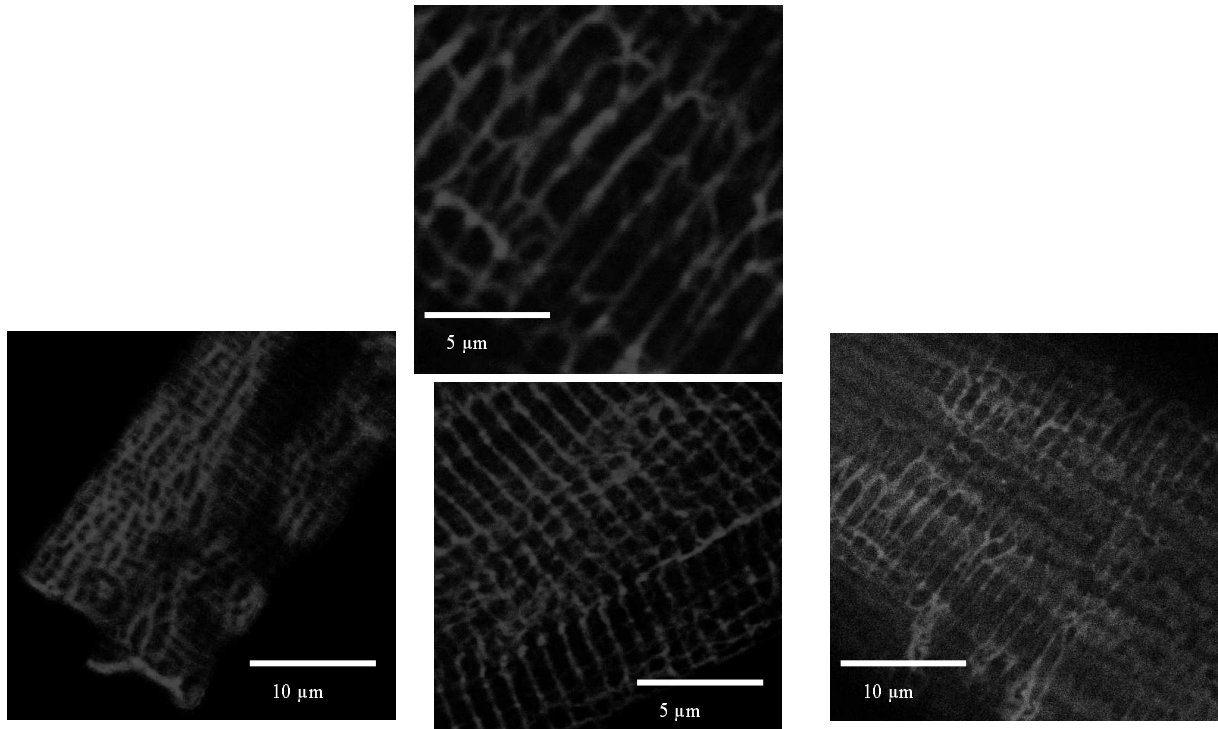
**Fig.10**



**Fig.11**

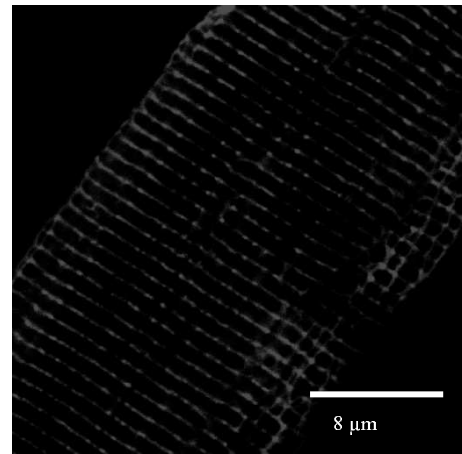
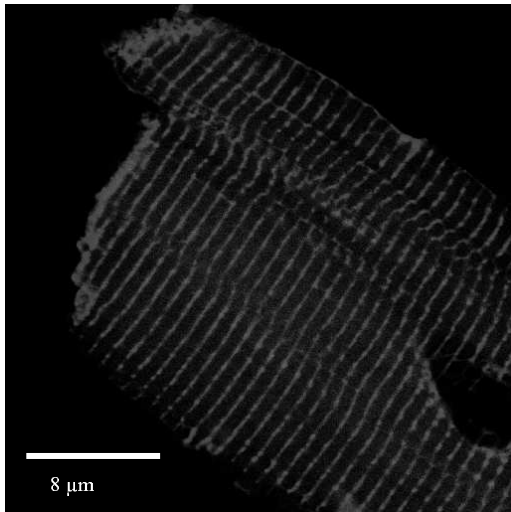


**Fig.12**

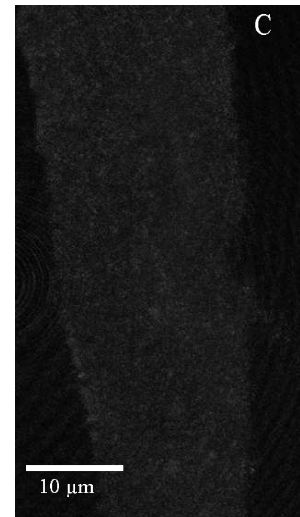
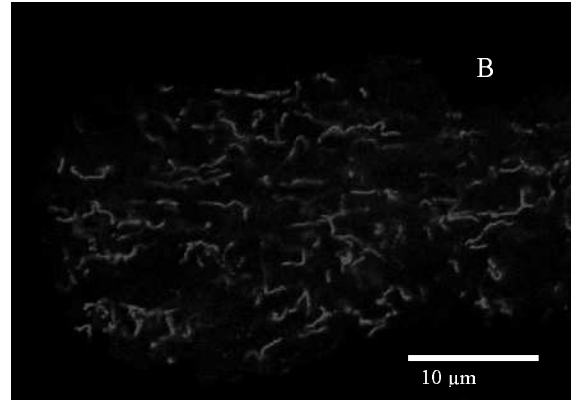
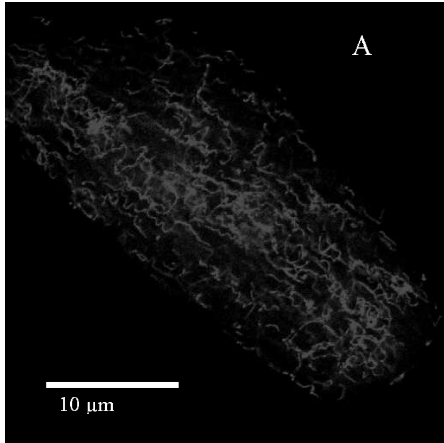




**Fig.13**



**Fig.14**



**Fig.15**

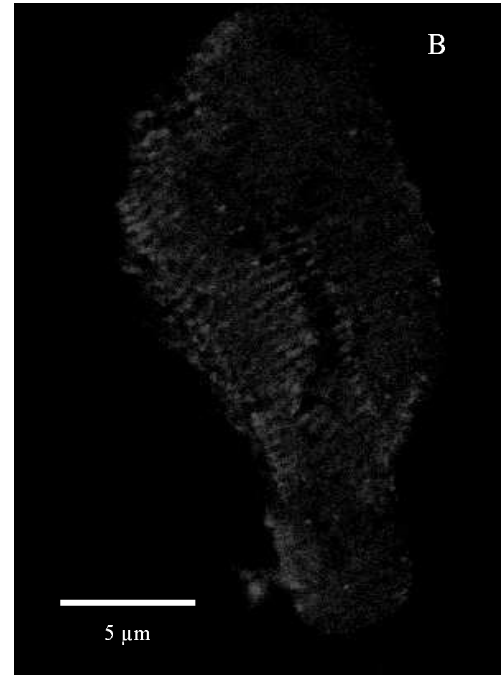
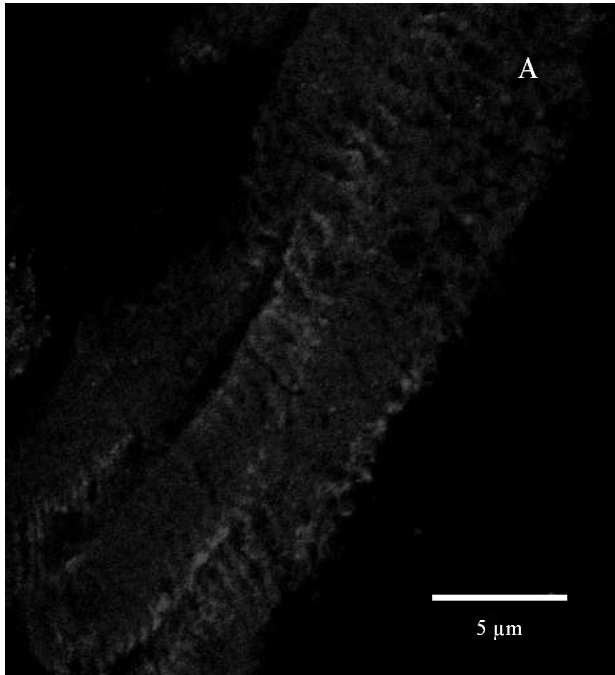


Fig.16

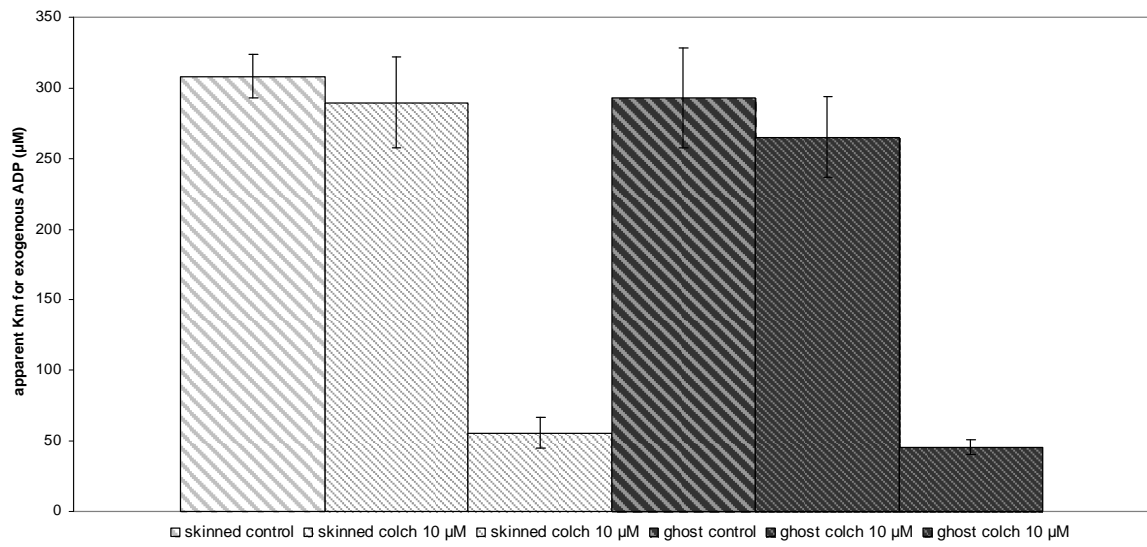
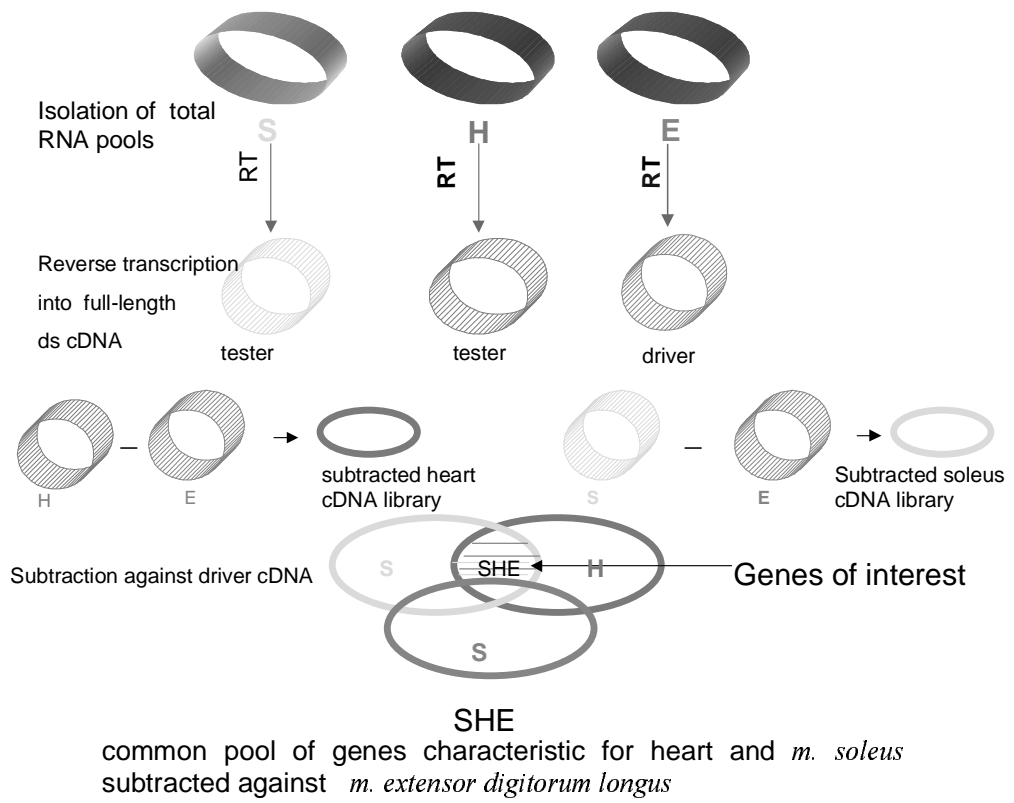
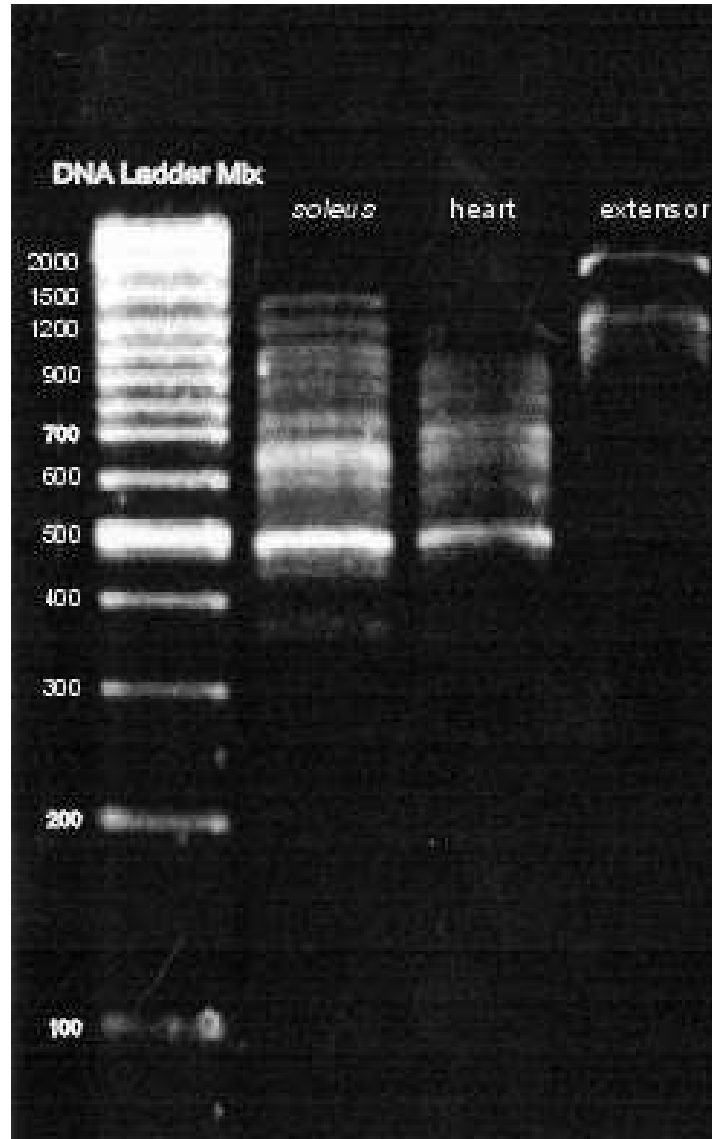


Fig.17



**Fig.18**



## REFERENCES

Kuznetsov AV, Tiivel T, Sikk P, Kaambre T, Kay L, Daneshrad Z, Rossi A, Kadaja L, Peet N, Seppet E, Saks VA. Striking differences between the kinetics of regulation of respiration by ADP in slow-twitch and fast-twitch muscles in vivo. *Eur J Biochem*. 1996 Nov 1;241(3):909-15.

Saks VA, Kaambre T, Sikk P, Eimre M, Orlova E, Paju K, Piirsoo A, Appaix F, Kay L, Regitz-Zagrosek V, Fleck E, Seppet E. Intracellular energetic units in red muscle cells. *Biochem J*. 2001 Jun 1;356(Pt 2):643-57.

Seppet EK, Kaambre T, Sikk P, Tiivel T, Vija H, Tonkonogi M, Sahlin K, Kay L, Appaix F, Braun U, Eimre M, Saks VA. Functional complexes of mitochondria with Ca,MgATPases of myofibrils and sarcoplasmic reticulum in muscle cells. *Biochim Biophys Acta*. 2001 Apr 2;1504(2-3):379-95.

Saks VA, Kuznetsov AV, Vendelin M, Guerrero K, Kay L, Seppet EK. Functional coupling as a basic mechanism of feedback regulation of cardiac energy metabolism. *Mol Cell Biochem*. 2004 Jan-Feb;256-257(1-2):185-99.

Saks V, Kuznetsov A, Andrienko T, Usson Y, Appaix F, Guerrero K, Kaambre T, Sikk P, Lemba M, Vendelin M. Heterogeneity of ADP diffusion and regulation of respiration in cardiac cells. *Biophys J*. 2003 May;84(5):3436-56.

M. Vendelin, N.Béraud\*, K. Guerrero\*, T. Andrienko, A. Kuznetsov, J. Olivares, L. Kay and V. Saks (\*equal contribution). (2005). Mitochondrial regular arrangement in muscle cells : a « crystal-like » pattern. *Am J Physiol Cell Physiol* (submitted)

Appaix F, Kuznetsov AV, Usson Y, Kay L, Andrienko T, Olivares J, Kaambre T, Sikk P, Margreiter R, Saks V. Possible role of cytoskeleton in intracellular arrangement and regulation of mitochondria. *Exp Physiol*. 2003 Jan;88(1):175-90.

Andrienko T, Kuznetsov AV, Kaambre T, Usson Y, Orosco A, Appaix F, Tiivel T, Sikk P, Vendelin M, Margreiter R, Saks VA. Metabolic consequences of functional complexes of mitochondria, myofibrils and sarcoplasmic reticulum in muscle cells. *J Exp Biol.* 2003 Jun;206(Pt 12):2059-72.

Capetanaki Y. Desmin cytoskeleton: a potential regulator of muscle mitochondrial behavior and function. *Trends Cardiovasc Med.* 2002 Nov;12(8):339-48.

Schroder R, Goudeau B, Simon MC, Fischer D, Eggermann T, Clemen CS, Li Z, Reimann J, Xue Z, Rudnik-Schoneborn S, Zerres K, van der Ven PF, Furst DO, Kunz WS, Vicart P. On noxious desmin: functional effects of a novel heterozygous desmin insertion mutation on the extrasarcomeric desmin cytoskeleton and mitochondria. *Hum Mol Genet.* 2003 Mar 15;12(6):657-69.

Ogata T, Yamasaki Y. Ultra-high-resolution scanning electron microscopy of mitochondria and sarcoplasmic reticulum arrangement in human red, white, and intermediate muscle fibers. *Anat Rec.* 1997 Jun;248(2):214-23.

Ogata T, Yamasaki Y. Scanning electron-microscopic studies on the three-dimensional structure of sarcoplasmic reticulum in the mammalian red, white and intermediate muscle fibers. *Cell Tissue Res.* 1985;242(3):461-7.

Gueguen N, Lefaucheur L, Fillaut M, Vincent A, Herpin P. Control of skeletal muscle mitochondria respiration by adenine nucleotides: differential effect of ADP and ATP according to muscle contractile type in pigs. *Comp Biochem Physiol B Biochem Mol Biol.* 2005 Feb;140(2):287-97.

Segretain D, Rambourg A, Clermont Y. Three dimensional arrangement of mitochondria and endoplasmic reticulum in the heart muscle fiber of the rat. *Anat Rec.* 1981 Jun;200(2):139-51.



Kay L, Li Z, Mericskay M, Olivares J, Tranqui L, Fontaine E, Tiivel T, Sikk P, Kaambre T, Samuel JL, Rappaport L, Usson Y, Leverve X, Paulin D, Saks VA. Study of regulation of mitochondrial respiration in vivo. An analysis of influence of ADP diffusion and possible role of cytoskeleton. *Biochim Biophys Acta*. 1997 Nov 10;1322(1):41-59.

Bereiter-Hahn J, Voth M. Dynamics of mitochondria in living cells: shape changes, dislocations, fusion, and fission of mitochondria. *Microsc Res Tech*. 1994 Feb 15;27(3):198-219.

Pabion M, Job D, Margolis RL. Sliding of STOP proteins on microtubules. *Biochemistry*. 1984 Dec 18;23(26):6642-8.

Aguezzoul M, Andrieux A, Denarier E. Overlap of promoter and coding sequences in the mouse STOP gene (Mtap6). *Genomics*. 2003 Jun;81(6):623-7.

Lewis, S A., Lee, M. G., Gowan, N. J. Five mouse tubulin isotypes and their regulated expression during development. *J Cell Biol* 1985 Sep 101:3 852-61.

+ others (cited above, referred in KDA paper + related methods (described in KDA paper+ speculative hypothesis by Ylo Puurand.

Hein S, Kostin S, Heling A, Maeno Y, Schaper J. The role of the cytoskeleton in heart failure. *Cardiovasc Res*. 2000 Jan 14;45(2):273-8.

Tagawa H., Koide M., Sato H., Zile M.R., Carabello B.A., Cooper G. 4th. 1998. Cytoskeletal role in the transition from compensated to decompensated hypertrophy during adult canine left ventricular pressure overloading. *Circ Res* 82:751-61.

Voloshchuk S.G., Belikova Y.O., Klyushnik T.P., Benevolensky D.S., Saks V.A. 1998. Comparative study of respiration kinetics and protein composition of skinned fibers from various types of rat muscle. *Biochemistry* 63:155-8.

Seppet E.K., Eimre M., Andrienko T., Kaambre T., Sikk P., Kuznetsov A.V., Saks V. 2004. Studies of mitochondrial respiration in muscle cells in situ: use and misuse of experimental evidence in mathematical modelling. *Mol Cell Biochem* 256-257:219-27.

Carre M., Andre N., Carles G., Borghi H., Brichese L., Briand C., Braguer D. 2002. Tubulin is an inherent component of mitochondrial membranes that interacts with the voltage-dependent anion channel. *J Biol Chem* 277:33664-9.

Linden M., Karlsson G. 1996. Identification of porin as a binding site for MAP2. *Biochem Biophys Res Commun* 218:833-6.

Choi S.S., Chan K.F., Ng H.K., Mak W.P. 1999. Colchicine-induced myopathy and neuropathy. *Hong Kong Med J* 5:204-207.

Wilbur K., Makowsky M. 2004. Colchicine myotoxicity: case reports and literature review. *Pharmacotherapy* 24:1784-92.