

Supplementary figure legends

Figure S1 Combination of different fiber types in mouse soleus and EDL

A Cross-section of mouse soleus and EDL muscles stained with three antibodies specific for MyHC-1, -2A and -2B, respectively, as described (Dyar et al., 2014). Type 2X fibers are unstained and appear black. Representative fibers from each fiber type are marked by arrows as indicated. Pseudocolors were defined to match the color coding used for each fiber type throughout the paper. The percentage of each fiber type in the two different muscles, as determined by immunofluorescence, is reported on the right. These values are just indicative and do not take into consideration the existence of many intermediate fiber types. In particular, many fibers assigned to the type 2B fiber type group are in fact hybrid fibers, containing variable proportions of 2B and 2X MyHCs.

B Section of a tissue block containing mouse fast EDL and slow soleus muscles stained for succinate dehydrogenase (SDH) activity. Note the wide variation in staining intensity in different muscle fibers. This is especially evident in EDL, in which SDH staining is correlated with fiber size: the smallest fibers, which are type 2A or 2X (see panel A) show the strongest staining, the largest 2B fibers show the weakest staining, and a whole spectrum of intermediate-size fibers, corresponding to mixed 2B-2X fibers, show intermediate staining.

C Myh isoform analysis of each fiber type that was used as a basis to define pure fiber subsets. Fibers were ranked according to their majority Myh isoform (see **Supplementary experimental procedures**).

Figure S2. Main features of skeletal muscle proteome

A Subdivision of the 100 most abundant proteins according to their general function

B Features of the skeletal muscle proteome described by 1D annotation enrichment (Cox and Mann, 2012). The bar graph shows the 20 most highly enriched Uniprot keywords annotations in the muscle proteome. Color-coding corresponds to that in panel A.

Figure S3 Unbiased MS-based fiber type assignment is solid and accurate.

A Reproducibility of fiber type assignment shown in technical duplicates consisting of half fiber lysates processed separately and of the same peptide preparation subdivided into two MS runs, as schematically represented.

B Correspondence of relative and absolute quantification of Myh isoforms in single fibers from Soleus (S) and EDL (E). Fibers were lysed and spiked-in with a defined amount of heavy-labelled PrESTs specific for the four different isoforms of Myh (see supplemental experimental procedures). Two different PrESTs sequences were used for each isoforms. Relative and absolute quantifications shown were carried out in the same fiber as indicated. Different amounts of Myh in the fibers likely reflect different sizes as a result of sampling heterogeneity during fiber isolation.

C Unsupervised hierarchical clustering of pure and mixed-type single fibers separates type 1 and 2B fibers from intermediate groups of 1/2A and 2X/2B fibers respectively. A cluster highly enriched in mitochondrial annotations spans oxidative fibers types, 1, 2A and 2X. 2B fibers are enriched in sarcoplasmic reticulum, calcium channels and glycolytic enzymes. Type 1 and 2A fibers also define a cluster enriched in cytosol and phosphoprotein annotations. Enrichment is based on Fischer's exact test of Keyword annotations at FDR=0.04. Fibers that had a similar number of detected proteins (between 1700 and 1100) were selected for this analysis. Data were filtered for 50% valid values, corresponding to 1011 proteins.

D Myh isoform composition of 9 fiber pairs analysed. The bottom line shows the percent difference in the expression of the most abundant isoform between each fiber pair. All fiber pairs were isolated from EDL and thus express predominantly predominantly My4 and Myh1 (see Fig.S1).

Figure S4. Fiber type resolved analysis of structural features of muscle fibers

A Heat-map distribution of sarcomeric protein isoforms. For each protein, boxes represent the values of each fiber type, always following the order type 1/2A/2X/2B from left to right as indicated on top. Boxes are, color-coded based on Z score as indicated. For each protein, the numbers represent normalized expression values (intensity normalised by Acta1) as in Supplemental table S2.

B Validation of MS results by western blot. Expression of the titin-binding protein Tcap/telethonin in soleus (S) and in two predominantly fast muscles, EDL (E) and quadriceps (Q).

C Heat-map representation of triadic junctions in different fiber types. Each protein is schematically placed in its known cellular location and represented as a bar composed of four squares, showing the Z score of the corresponding fiber type (color/coded at the bottom of the bars). Quantification and color-coding were carried out as described in panel A.

Figure S5. Expression profiles of proteins showing higher expression in a specific fiber type or in a defined subset of fiber types

Profiles were derived from an ideal profile (dashed black line) by correlation. The ten most closely correlated profiles are shown, with the corresponding gene names on the right. Examples of proteins

that have essentially the same expression level in all fiber types are shown bottom right. Sarcomeric proteins and proteins with less than 3 valid values per fiber type were excluded.

Figure S6. Type 2A fibers display stronger mitochondrial staining than type 1 fibers in soleus.

A Immunofluorescence (left panel), merge of two sections stained for type 2A fibers (Myh2, green) and type 1 fibers (Myh7, red). Enzyme histochemistry (right panel), section stained for succinate dehydrogenase activity. Red and green arrows in the two serial sections point to the same type 1 and 2A fibers, respectively. Note that type 2A muscle fibers have higher succinate dehydrogenase activity than type 1 fibers.

Supplementary table legends

Table S1. All fibers used in this study are listed with corresponding name, muscle of origin, number of proteins quantified and fiber type based on the prevalent isoform of Myh, as described in figure 2. The corresponding raw files were processed together using MaxQuant (see Methods), except Sol1-5 and EDL9 which were processed in parallel due to a difference in the elution gradient.

Table S2. Fiber type-resolved proteome. Numbers represent protein intensity normalized by skeletal actin (Acta) and multiplied times 10^5 to obtain integers. These values are referred to as relative abundance in the figures and represent an arbitrary but linear function of protein abundance. Acta is highlighted in yellow, with a value of 10^5 corresponding to the multiplier. The table shows the median value of pure fibers for each subtype. Standard error of the mean values are also reported in the table. The valid values columns show the number of fibers in which every protein was quantified. Only proteins with over 10 identifications (valid values) were included. Proteins with significantly different abundance in type 1 compared to type 2B were searched for by performing a two-sample test. Significant hits are marked with (+) and P values are reported. ND, not detected; NA, not applicable.

Table S3 Comparisons of selected mitochondrial proteins in different fiber groups showing metabolic specialization. Two sample tests (Welch) were performed between the indicated groups; the corresponding P values are listed, red color indicates non-significant. The comparison between type 1 and 2A fibers was performed after normalization for mitochondrial number, using both cytochrome C (Cyts) and succinate dehydrogenase (SDHA) a proxy.

Table S4 2X 2B Mitochondrial proteins significantly higher in type2B than 2X fibers after normalization for mitochondrial number, using both cytochrome C (Cyts) and succinate dehydrogenase (SDHA) a proxy.

Supplementary methods

in-StageTip method for single fibers sample processing

Lysis. Fibers were lysed in by adding 35 μ l of a solution containing 6M of the chaotropic agent guanidinium chloride (GCl), 10 mM of the reducing agent tris(2-carboxyethyl)phosphine (TCEP) and 40 mM of the alkylating agent 2-chloroacetamide (CAA), an optimized reaction mix that allows reduction and alkylation to be performed as a single step (Kulak et al., 2014) . The fiber lysates were boiled for 5 min and then sonicated in a water-bath sonicator equipped with an adaptor for Eppendorf tubes (Bioruptor, model UCD-200, Diagenode) for 15 min at level 5. The lysate was then transferred to the original tube and 2 volumes (70 μ l) of a digestion buffer containing 20mM Tris-HCl, pH 8.5, and 10% acetonitrile, were added to the fiber lysate, bringing the GCl concentration to 2M.

LysC digestion. The endoproteinase LysC, 500 ng per sample, was added to the mixture and digestion was carried out at 37°C for 3 hs under continuous stirring. The sample was sonicated again for 5 minutes under the conditions described above.

Trypsin digestion and peptide elution. Digestion chambers consisted of standard Eppendorf pipet tips containing gauge-18 plugs made of 3 layers of poly(styrenedivinylbenzene) reverse phase sulfonate (SDB-RPS). The bottom of the tip was heat-sealed to prevent leakage. The lysates were transferred directly into the chamber and further diluted with digestion buffer to a final GCl concentration of 0.6M. Trypsin (400 ng per sample) was added and the digestion was carried out at 37°C overnight. Samples were acidified by addition of 1% TFA and peptides were eluted from the SDB-RPS matrix with 60 μ l of a solution containing 80% acetonitrile and 5% NH_3 . Eluates were collected in autosampler tubes and dried using a SpeedVac centrifuge at room temperature. Peptides were resuspended in a solution containing 2% acetonitrile and 0.1% TFA and were briefly sonicated to ensure solubilization.

Myh isoform identification by SDS-PAGE

SDS-PAGE was carried out as described (Talmadge and Roy, 1993) using 4% stacking (4% polyacrylamide 50:1, 30% glycerol, 70 mM Tris pH 6.7, 4 mM EDTA and 0.4% SDS) and 8% resolving gels (8% polyacrylamide 50:1, 30% glycerol, 0.4% SDS, 0.2 M Tris, and 0.1 M glycine). Electrophoresis was carried out at 4°C at 100 V for 3 hs and at 230V for another 40 hs. Protein staining was carried out using a Bio-Rad Silver stain kit. Myh protein bands were identified according to their mobility compared to molecular weight standards, using whole muscle lysates as positive controls.

Assignment of fiber type

Myh isoforms were quantified based only on unique peptides to overcome the problem of high homology. We considered the summed intensity of the four adult isoforms (Myh7, Myh2, Myh1, Myh4) as 100% and calculated their relative amount for each fiber. All fibers were initially sorted into four groups based on the highest expressed Myh isoform regardless of coexpression of other isoforms and of total number of identifications; these latter criteria lead to the exclusion of some fibers from the main analysis. For each group we ranked the abundance of the specific Myh isoform and calculated the average and standard deviation. We obtained 83+/-18 for type 1, 71+/-24 for type 2A, 62+/-11 for type 2X and 88+/-11 for type 2B. We approximated these values to set cut-off. This procedure yielded fiber groups characterized by over 80% Myh7 and Myh4 for type 1 and 2B fibers and 60% Myh2 and Myh1 for 2A and 2X fibers (see figure S1c).

In-gel protein digestion for mass spectrometry

The lysates corresponding to half fibers were loaded onto 4–12% gradient polyacrylamide precast gels (NuPAGE, Invitrogen) and stained with colloidal Coomassie (Invitrogen). Whole gel lanes were cut and subjected to in-gel tryptic digestion. Following complete destaining, gel slices were minced, washed with 50 mM ammonium bicarbonate and dried with ethanol. Reduction/alkylation of proteins was performed with 10 mM DTT and 55 mM iodoacetamide. After washing the gel was dried with ethanol and incubated with 12.5 ng/μl trypsin (Promega) in 50 mM ammonium bicarbonate at 4 °C for 15 min. The supernatant was discarded and replaced with 50 mM ammonium bicarbonate; digestion was carried out overnight at 37 °C. The reaction was stopped by adding 1% TFA and the supernatant was recovered. Additional peptide extraction steps were performed with 30% acetonitrile and 100% acetonitrile. Supernatants were concentrated and processed for MS analysis.

Preparation of whole muscle lysate and peptide fractionation

Muscles were crushed in liquid nitrogen and resuspended in a lysis buffer containing 100 mM Tris-HCl pH8, 4mM SDS and 100 mM DTT. Lysates were subsequently diluted in 8 M urea in 100m M Tris-HCl pH 8.5 followed by protein digestion with trypsin using to the FASP protocol (Wisniewski et al., 2009). 100 μg of peptides were separated into 12 fractions by isoelectric focusing on an OffGel fractionator (Agilent) as previously described (Hubner et al., 2008). Peptides from each fraction were purified on C18 StageTips.

Absolute Myh quantification using PrESTs

Unique sequences for each Myh isoforms were selected to be optimal for mass spectrometric analysis, specifically for yielding many peptides that could allow us to distinguish isoforms. Two different PrESTs were used for each Myh isoform. The synthetic genes of the selected protein standards were fused to Albumin Binding Protein (ABP). The heavy SILAC (Stable isotope labeling by amino acids in cell culture) standards were produced using an auxotrophic *E. coli* (Ong et al., 2002) strain in the presence of heavy arginine (13C615N4) and heavy lysine (13C615N2). The recombinant His-tag containing proteins were purified on Ni-NTA columns and quantified by MS comparing to a light ABP preparation where concentration had been determined by amino acid analysis (Zeiler et al., 2012, see main references). PrESTs with known concentration were spiked-in the fiber samples and LyC digestion was carried out overnight. Peptides were recovered as described above. Protein amount in fiber lysate was measured using standard methods and used as a reference to calculate protein content and copy number per fiber.

Western blot

Muscles were crushed in liquid nitrogen and dissolved in a lysis buffer containing 4% SDS, 100 mM DTT, pH 8, additioned with protease inhibitors (Complete™, Roche) and PhosSTOP (Roche). 20 ug of protein lysate were loaded in each lane. The Telethonin (G-11) monoclonal antibody was purchased from Santa Cruz Biotechnology.

Supplementary references

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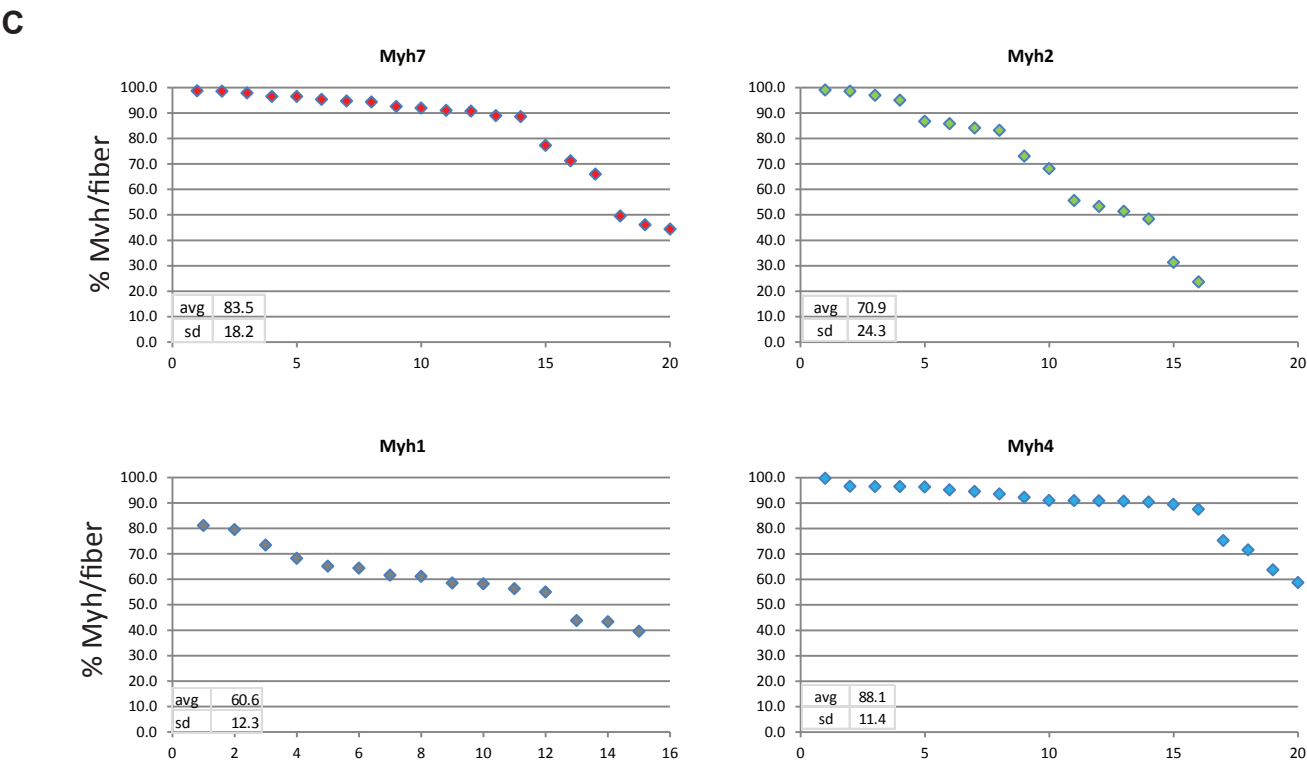
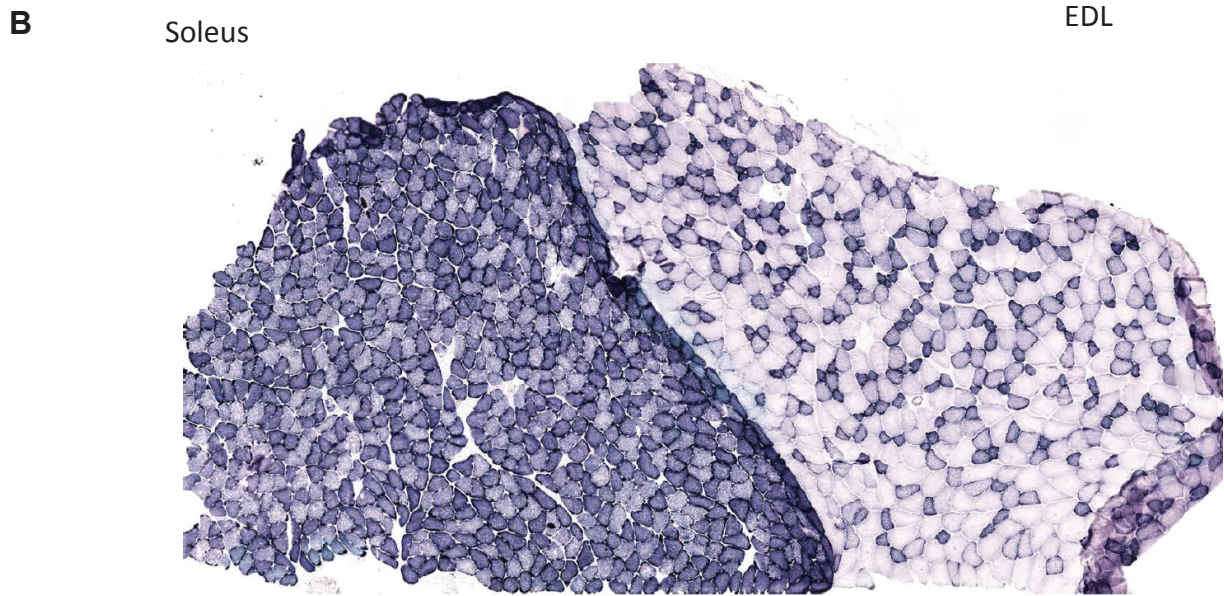
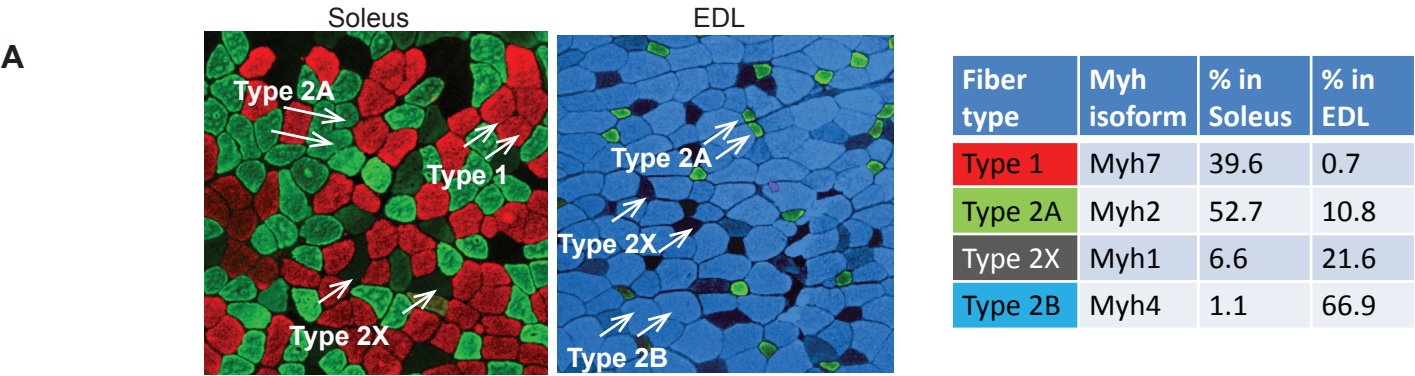
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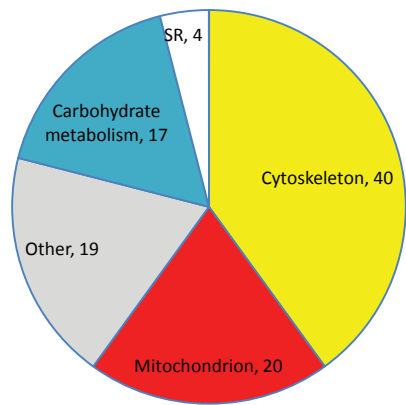
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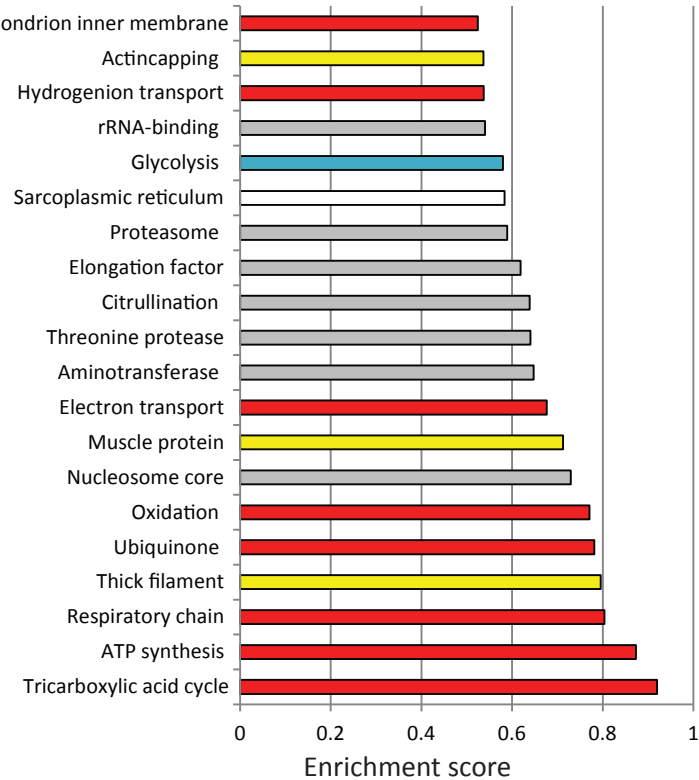


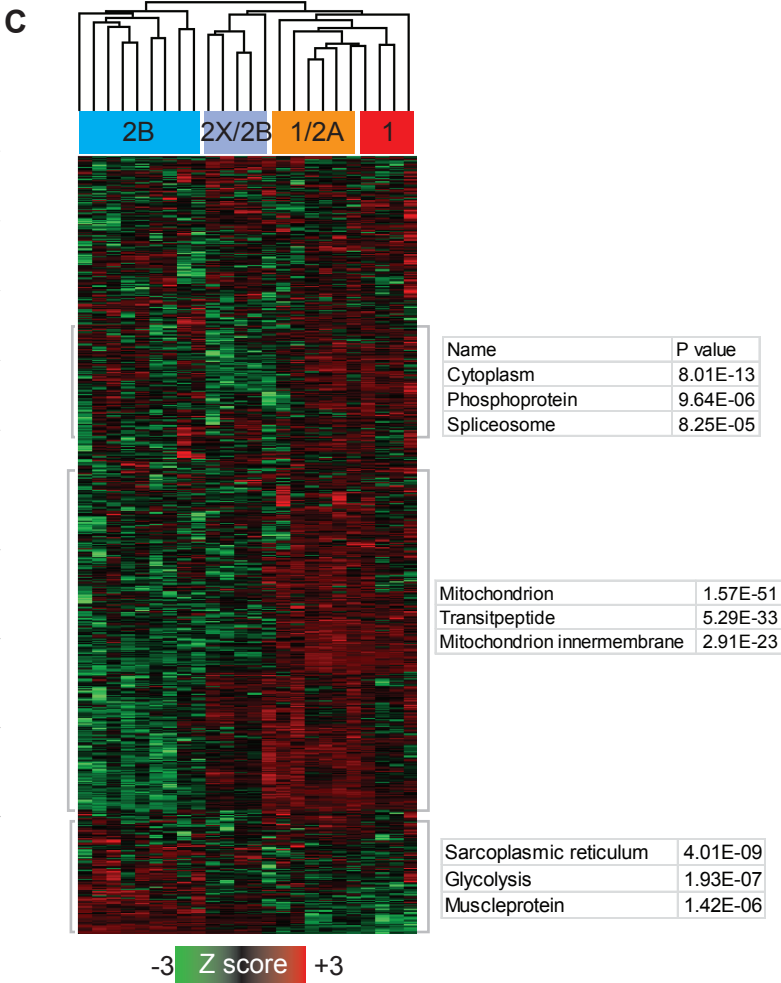
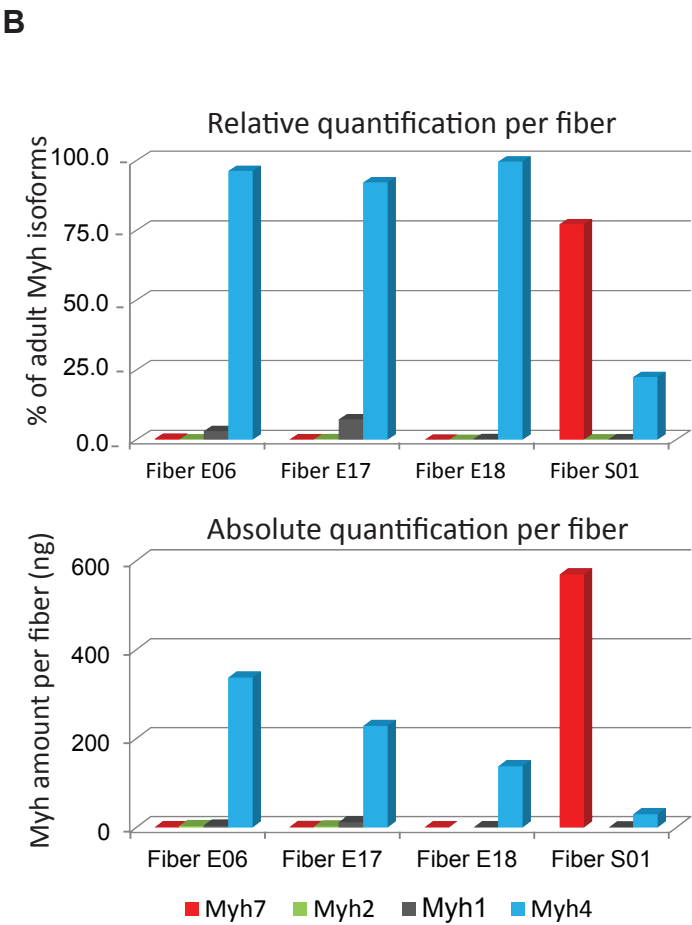
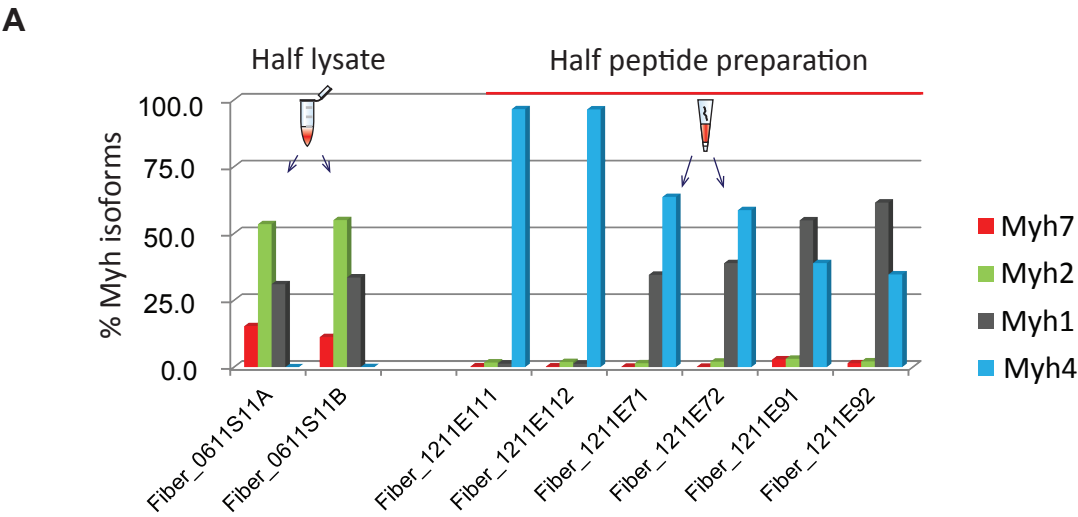
Murgia et al., Figure S2

A



B





D

Fiber pair	1A	1B	2A	2B	3A	3B	5A	5B	7A	7B	8A	8B	9A	9B	11A	11B	12A	12B
Myh7	1.8	0.5	0.8	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0
Myh2	5.8	0.6	1.5	0.7	0.2	0.2	0.2	0.4	2.4	0.2	0.1	0.0	0.1	0.3	0.2	0.1	4.5	0.0
Myh1	70.4	1.1	72.8	77.3	1.4	6.3	33.1	72.6	2.6	2.2	0.1	28.7	21.4	16.2	0.1	0.1	16.7	29.4
Myh4	22.1	97.8	24.9	21.8	98.4	93.5	66.7	27.0	95.0	97.6	99.8	71.3	78.5	83.6	99.7	99.8	76.9	70.6
% difference	75.7		4.5		4.9		39.7		2.7		28.5		5.1		0.1		6.3	

