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1 **O-GlcNAcylation site mapping by (azide-alkyne) click chemistry**  
2 **and mass spectrometry following intensive fractionation of**  
3 **skeletal muscle cells proteins**

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29 **KEYWORDS**

30 O-GlcNAcylation; click chemistry; mass spectrometry; post-translational modifications; sites  
31 localization; skeletal muscle cells; fractionation.

## 1     **ABSTRACT**

2     The O-linked-N-acetyl-D-glucosaminylation (O-GlcNAcylation) modulates numerous aspects of  
3 cellular processes. Akin to phosphorylation, O-GlcNAcylation is highly dynamic and reversible, and  
4 responds rapidly to extracellular demand. Despite the absolute necessity to determine post-  
5 translational sites to fully understand the role of O-GlcNAcylation, it remains a high challenge for the  
6 major reason that unmodified proteins are in excess comparing to the O-GlcNAcylated ones. Based on  
7 a click chemistry approach, O-GlcNAcylated proteins were labelled with azido-GalNAc and coupled  
8 to agarose beads. The proteome extracted from C2C12 myotubes was submitted to an intensive  
9 fractionation prior to azide-alkyne click chemistry. This combination of fractionation and click  
10 chemistry is a powerful methodology to map O-GlcNAc sites; indeed, 342 proteins were identified  
11 through the sequencing of 620 peptides containing one or more O-GlcNAc sites. We localized O-  
12 GlcNAc sites on proteins involved in signalling pathways or in protein modification, as well as  
13 structural proteins. Considering the recent role of O-GlcNAcylation in the modulation of sarcomere  
14 morphometry and interaction between key structural protein, we focused on proteins involved in the  
15 cytoarchitecture of skeletal muscle cells. In particular, several O-GlcNAc sites were located into  
16 protein-protein interaction domain, suggesting that O-GlcNAcylation could be strongly involved in the  
17 organisation and reorganisation of sarcomere and myofibrils.

## 18 19 20     **SIGNIFICANCE**

21     O-GlcNAcylation is an atypical glycosylation involved in the regulation of almost all if not all  
22 cellular processes, but its precise role remains sometimes obscure because of the ignorance of the O-  
23 GlcNAc site localization; thus, it remains indispensable to precisely map the O-GlcNAcylated sites to  
24 fully understand its role on a given protein. For this purpose, we combined extensive fractionation of  
25 skeletal muscle cells proteome with click chemistry to map O-GlcNAc sites without an a priori  
26 consideration. A total of 620 peptides containing one or more O-GlcNAc sites were sequenced;  
27 interestingly, several of them belong to low expressed proteins, in particular proteins involved in  
28 signalling pathways. We also focused on structural proteins in view of recent data supporting the role  
29 of O-GlcNAcylation in the modulation of sarcomere cytoarchitecture; importantly, some of the O-  
30 GlcNAc sites were mapped into protein-protein interaction domain, reinforcing the involvement of O-  
31 GlcNAcylation in the organisation and reorganisation of sarcomere, and in larger extent, of myofibrils.

# 1 INTRODUCTION

2 The O-N-acetyl- $\beta$ -D-glucosaminylation, termed O-GlcNAcylation, is an atypical glycosylation  
3 corresponding to the transfer of a unique monosaccharide, the N-acetyl- $\beta$ -D-glucosamine, on the  
4 hydroxyl group of serine and threonine amino acids of nuclear, cytosolic and mitochondrial proteins  
5 (1, 2). The O-GlcNAcylation has emerged as a key regulator of several cellular processes such as  
6 transcription, translation, regulation of signalling pathways, degradative processes, subcellular  
7 localization of targets, and so on (1, 3-7). Because of its involvement in nearly all if not all cellular  
8 processes, O-GlcNAcylation is nowadays clearly associated with the aetiology of several acquired  
9 diseases, in particular diabetes, neuro-degenerative disorders, cardiovascular diseases or cancer (8).

10 The O-GlcNAcylated proteins bear similarities with the phosphorylated ones, in particular the  
11 reversibility of both processes since the phosphate and the GlcNAc moieties could be added and  
12 removed several times along the protein lifetime, and their turn-over is shorter than the protein  
13 backbone turn-over (9). The O-GlcNAcylation rapidly emerged as a major cellular mechanism which  
14 could compete with phosphorylation in terms of modified proteins and their importance in cellular  
15 physiology. But in contrast of the plethora of kinases and phosphatases responsible of the  
16 phosphorylation/ dephosphorylation process on specific proteins, a unique couple of antagonist  
17 enzymes (OGT/OGA) is involved in the O-GlcNAcylation process. While kinases recognize a  
18 consensus sequence, phosphorylation sites are easily predictable from primary sequence of a protein.  
19 However, no consensus sequence was clearly defined for OGT, but it appears that peptidic sequences  
20 modified by O-GlcNAcylation are enriched in small amino acids, with a proximal proline residue;  
21 these sequences also present preferential secondary structures such as loop and disorganized regions  
22 instead of  $\alpha$ -helix and  $\beta$ -strand (10-15). The O-GlcNAcylated sites could also correspond to  
23 phosphorylated ones; thus, many proteins are modified by both O-GlcNAc and phosphates groups, and  
24 these two post-translational modifications could compete to the same or to neighbouring sites (4, 16).

25 Despite the crucial role of O-GlcNAcylation in numerous cellular processes, the precise  
26 localization of O-GlcNAcylated sites remains an indispensable prerequisite for the fine understanding  
27 of its biological function. However, mapping the O-GlcNAcylated sites remains laborious but  
28 challenging, because of (i) the low stoichiometry of O-GlcNAcylation; (ii) the ion suppression of the  
29 modified peptide by the unmodified peptides present in large excess and (iii) the labile  $\beta$  bond between  
30 serine or threonine and the O-GlcNAc moiety which is broken during the CID (Collision-Induced  
31 Dissociation) fragmentation process, leading to loss of site information during mass spectrometry  
32 analysis (17, 18).

33 Therefore, new strategies such as enrichment of O-GlcNAc modified proteins and the use of other  
34 fragmentation processes like ECD (Electron Capture Dissociation), ETD (Electron Transfer  
35 Dissociation), or HCD (High-energy Collisional Dissociation), able to limit the O-GlcNAc loss during  
36 the fragmentation, have been developed (19-21). Thus, enrichment step of O-GlcNAcylated proteins

1 or tryptic peptides by immuno-affinity or affinity chromatography (in particular LWAC (lectin weak  
2 affinity chromatography)) were extensively used to identify O-GlcNAc sites (21-28), or combination  
3 of both approaches (29). Moreover, chemical-based enrichment using BEMAD ( $\beta$ -Elimination  
4 followed by Michael Addition of Dithiothreitol (DTT)) was applied to O-GlcNAcylated proteins to  
5 overcome the O-GlcNAc loss during fragmentation, since labile peptide-GlcNAc bonds were  
6 substituted by no-labile peptide-DTT bonds (30-32). New strategies based on a chemoenzymatic label  
7 with galactosyltransferase (GalT<sup>Y289L</sup>) appeared during the last decade. Azido-modified N-acetyl-  
8 galactosamine is transferred on the hydroxyl moiety of a GlcNAc acceptor allowing the fixation of  
9 aminoxy-biotin, and in consequence, the purification of O-GlcNAc proteins or peptides with  
10 streptavidin beads (33-36). Nevertheless, biotin fragmentation occurs during MS/MS fragmentation,  
11 leading to spectral analysis difficulties despite the stable GlcNAc (or more specifically LacNAc-  
12 biotin)-peptide bond (17). Moreover, the hard condition of elution, necessary to cut the biotin-  
13 streptavidin bond, can lead to peptide modifications and to low yield of the O-GlcNAc-  
14 proteins/peptides purification (37). To overcome these problems, UV cleavable biotin can be used, this  
15 approach presenting the advantage to obtain positive charged peptides, leading to enhanced ETD  
16 fragmentation and O-GlcNAc site localization (38-41). It is worth to note that BEMAD and  
17 purification of biotin-labelled O-LacNAc peptides could be paired to localize O-GlcNAc sites (42-46).

18 We previously demonstrated that O-GlcNAcylation is an original and important regulator of  
19 skeletal muscle physiology (47, 48), in particular in the modulation of contractile activity (49-52) as  
20 well as the morphometry of the sarcomere (53), supported by the fact that numerous contractile and  
21 structural proteins are O-GlcNAcylated (50, 54). Therefore, we developed an alternative strategy,  
22 specific, efficient and allowing purification of O-GlcNAc bearing proteins from skeletal muscle cells  
23 by the use, with minor changes, of the click chemistry methodology developed by Hahne and  
24 collaborators (55). Briefly, O-GlcNAc bearing proteins were linked to agarose beads through azide-  
25 alkyne chemistry to enhance the enrichment of O-GlcNAcylated proteins. Proteolytic digestion of  
26 proteins linked to agarose beads and mass spectrometry analysis of resulting peptides provided the  
27 global identification of O-GlcNAcylated proteins. Analysis of released O-LacNAc peptides from  
28 agarose beads, resulting from a chemical cut-off of glycosidic bond, led to localization of the modified  
29 amino acids. To extensively map O-GlcNAc sites on proteins, we proposed herein an intensive  
30 fractionation of the muscle cell proteome according to solubility, hydrophobicity and isoelectric point  
31 of proteins prior to the click chemistry. Thus, the method of click chemistry was achieved (i) on whole  
32 proteome extracted from C2C12 differentiated myotubes, (ii) on a subproteome, the cytosol-enriched  
33 extract, and (iii) on the cytosol-enriched extract extensively fractionated. The non-glycosylated  
34 peptides, and the glycosylated peptides released by beta-elimination, were analysed on mass  
35 spectrometry. Through the analysis of peptides retained on agarose beads, we identified 342 O-  
36 GlcNAcylated proteins in the fractionated subproteome, corresponding to a 2-fold increase of the  
37 number of identified proteins from the whole extract, or a 3.5-fold increase of identified proteins from

1 the non-fractionated subproteome, which reinforce the strength of the fractionation. Among these O-  
2 GlcNAcylated proteins, we also sequenced 620 peptides containing one or several dehydrated serine  
3 or threonine amino acids, corresponding so to O-GlcNAcylated sites.

# 1      **EXPERIMENTAL SECTION**

## 2      **Materials**

3      Heat-inactivated horse serum (HI-HS), foetal bovine serum (FBS) and Dulbecco's Modified  
4      Eagle's Medium (DMEM) were purchased from GIBCO; antibiotic-antimycotic, GlycoProfile  $\beta$ -  
5      elimination kit and all analytical grade reagents from Sigma-Aldrich; anti-proteases (Complete EDTA-  
6      free) and anti-phosphatases (Phos-Stop) from Roche Diagnostic; Bradford and RC DC™ reagents  
7      protein assays, dithiothreitol, iodoacetamide, and stain-free gels from Bio-Rad; Zeba spin column from  
8      Thermo Fisher Scientific; IPG buffer from GE Healthcare; Click-It™ reagents from Molecular Probes;  
9      trypsin/Lys-C mix from Promega;  $\lambda$  phosphatase and calf intestine phosphatase from New England  
10     Biolabs; C18 reversed-phase columns from Grace.

## 12     **Cell culture**

13     Mouse C2C12 skeletal myoblasts were obtained from ATCC (American Type Culture Collection).  
14     Myoblasts were grown on 100 mm Petri Dishes in proliferation medium (DMEM supplemented with  
15     10% FBS and 1% antibiotic-antimycotic) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> still  
16     reaching 90-95% confluence. They were then induced to differentiate into myotubes by switching to  
17     differentiation medium (DMEM containing 2% HI-HS and 1% antibiotic-antimycotic). Medium was  
18     changed every two-days, and myotubes were maintained for 5 days until they were mature.

## 20     **Cell harvesting and protein extraction**

21     C2C12 myotubes were rinsed three times with cold PBS and scraped with 2x200  $\mu$ l of cold lysis  
22     RipA buffer (10 mM Tris/HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% TritonX-100; 0.5% sodium  
23     deoxycholate; 0.1% SDS) for whole extraction, or 2x150  $\mu$ l of cold CMM buffer (50 mM Tris/HCl,  
24     pH 7.4; 2 mM EDTA; 5 mM EGTA; 5mM DTT; 0.05% saponin (w/v)) to get cytosol-enriched  
25     fraction, as previously described (56). Both lysis buffers were supplemented with 50 $\mu$ M PUGNAc (O-  
26     (2-acetamido-2-deoxy-D-glucopyranosilidene)amino-N-phenyl-carbamate), and proteases and  
27     phosphatases inhibitors. Collected materials were sonicated using Ultra-Sonic Cell Disruptor (Vibra  
28     Cell, 130 W ultrasonic processor); three pulses were performed at 70% of amplitude. Samples were  
29     then homogenized with gentle agitation for 1 h at 4 °C for RipA buffer in order to obtain the whole  
30     extract (WE), or centrifuged (14,000 g, 4°C, 30 min) after lysis with CMM buffer in order to obtain  
31     the enriched-cytosolic fraction in the supernatant. The residual pellet was then resuspended in the  
32     CMM buffer added with 1% Triton X-100, homogenised, centrifuged; the supernatant, corresponding  
33     to membrane-enriched fraction, was kept. The Triton-insoluble pellet, corresponding to the  
34     myofilament-enriched fraction, was resuspended in RipA buffer. The protein content of all fractions

1 (WE, cytosolic, membrane and myofilament fractions) was assayed using Bradford's method.

2

### 3 **Fractionation of the cytosolic extract**

4 ***Ammonium sulphate precipitation.*** One hundred milligrams of the protein extract were fractionated  
5 through 3-steps of ammonium sulphate (AS) precipitation. Briefly, extract was salted out with AS at  
6 25 % saturation for 2 h at 4°C and centrifuged (10000 g, 4°C, 15 min). The pellet, which corresponds  
7 to the fraction AS25, was stored, while the supernatant was salted out with 50 % saturation in AS. The  
8 third step corresponded to a precipitation with AS at 75% saturation. The three fractions obtained  
9 corresponded to AS25, AS50 and AS75 fractions, respectively. The pellets were solubilized by UTCD  
10 buffer (4M urea; 2M thiourea; 2% CHAPS (w/v); 5mM DTT), desalted with Zeba Spin columns, and  
11 assayed using reducing agent and detergent compatible protein assay (RC DC™ protein assay).

12

13 ***Liquid phase IEF.*** Each fraction resulting from ammonium sulphate precipitation was submitted to  
14 liquid phase IEF using the MicroRotor cell equipped with the PowerPac HV power supply,  
15 according to manufacturer (Bio-Rad). Protein samples (36 mg in total by fraction separated in 12  
16 samples of 3 mg) were diluted at 1 mg/ml in UTCD buffer added with 3% ampholyte-IPG buffer pH  
17 4-7, and loaded to pre-assembled focusing chamber of the MicroRotor cell. The ion exchange  
18 membranes, separating the electrode reservoirs and the focusing chamber, were equilibrated in 0.1 M  
19 H<sub>3</sub>PO<sub>4</sub> and 0.1 M NaOH for cation exchange membrane and anion exchange membrane, respectively.  
20 The focusing run was performed with constant power at 1W at 10°C. The separation typically  
21 occurred for 2.5 h and was terminated 30 minutes after the voltage stabilizes. After focusing, 10  
22 fractions of 200 µl (annotated F1 to F10 from the more acidic fraction to the more basic fraction) were  
23 quickly collected to avoid diffusion with the use of a vacuum. The pH of the individual fractions was  
24 measured with a pH meter, and fractions were kept at -20°C before further analysis.

25

### 26 **One-dimensional polyacrylamide gel electrophoresis (1D-PAGE)**

27 Twenty micrograms of each fraction (of 10 µl in case of IEF fractionation) were boiled in Laemmli  
28 buffer (62.5 mM Tris/HCl, pH 6.8; 10% glycerol; 2% SDS; 5% β-mercaptoethanol; 0.02%  
29 bromophenol blue) and separated electrophoretically on 7.5% or on any kD Mini-PROTEAN TGX  
30 Stain-Free™ (SF) Precast Gels (25 min, 300 V). The SF imaging was performed with ChemiDoc MP  
31 Imager and Image Lab 4.0.1 software (Bio-Rad); a 5-min activation time was used for the whole  
32 protein pattern imaging.

33

### 34 **O-GlcNAc proteins enrichment**

35 Click chemistry (azide/alkyne click reaction and enrichment) was performed on whole extract as

1 well as on cytosol-enriched extract using the Click-iT™ O-GlcNAc Enzymatic Labelling System and  
2 the Click-iT™ Protein Enrichment Kit according to the manufacturer's instructions and to the protocol  
3 described by Hahne and coworkers (55). The click chemistry protocol was also applied on each  
4 fraction issued from the MicroRotor runs (themselves issued from ammonium sulphate precipitation  
5 of cytosol-enriched proteins); a total of 30 fractions were labelled. It is worth to note that for each  
6 sample, the same protein quantity (*i.e.* 2 mg) was used for the click chemistry-based enrichment.

7 After chloroform/methanol precipitation, performed at room temperature, the O-GlcNAc proteins  
8 were labelled overnight at 4°C with the Click-iT™ O-GlcNAc Enzymatic Labelling System. Briefly,  
9 Gal-T1 (Y289L) was incubated with proteins in labelling buffer (20 mM HEPES, pH 7.9; 50 mM  
10 NaCl; 2% NP-40; 5.5 mM MnCl<sub>2</sub>; 25 μM UDP-GalNAz), according to manufacturer's  
11 recommendations. All reagents were provided in the kit, but the volume of each reagent was adjusted  
12 according to protein quantities. Reaction was performed at 4°C under gentle agitation for 20h, and  
13 then azide-labelled proteins were chloroform/methanol precipitated.

14 The azide-labelled proteins were then resuspended in urea lysis buffer, according to manufacturer's  
15 recommendations, following by an extensive homogenization. The proteins samples were then  
16 centrifuged at 10 000 rcf for 5 min in order to discard the insoluble material. The LacNAz-proteins  
17 were then precleared with agarose resin for 1 h, and the supernatant was then added with catalyst  
18 solution provided into the Click-iT™ Protein Enrichment Kit, containing copper (II) sulphate for a  
19 final concentration of 50 mM. The azide-labelled proteins were then incubated on an end-over-end  
20 rotator at room temperature for 18 h with 200 μL slurry of alkyne agarose resin according to the Click-  
21 iT™ Protein Enrichment Kit. After overnight click reaction, the supernatant was discarded and the  
22 resin was subjected to washes with 3 x 1.5 mL 10 mM diethylene triamine pentaacetic acid (DPTA)  
23 and 2 x 1.5 mL of MS grade water. Following reduction (10 mM dithiothreitol [DTT], 30 min, 55 °C)  
24 and alkylation (50 mM iodoacetamide [IAA], 60 min, RT), the resin was subjected to an extensive  
25 washing procedure performed in column as follow: 5 x 2 mL SDS wash buffer (100 mM Tris/HCl, pH  
26 8; 1% SDS; 250 mM NaCl; 5 mM EDTA); 5 x 2 mL urea buffer (8 M urea; 100 mM Tris/HCl, pH 8);  
27 10 x 2 mL 20% acetonitrile; 2 x 1 mL digestion buffer (100 mM Tris/HCl, pH 8.2; 2 mM CaCl<sub>2</sub>; 10%  
28 ACN). Digestion of resin-bound proteins was performed o/n in 200 μL digestion buffer containing 1  
29 μg of trypsin/Lys-C mix. Following the on-resin digestion, the remaining solution was discarded, and  
30 the resin washed with 500 μL of digestion buffer; both solutions, corresponding to non-retained  
31 peptides (NR peptides, *i.e.* the non-linked peptides), were pooled together and stored before desalting.  
32 The resin was then washed twice with 1.5 mL of MS grade water, following by 2 x 1.5 mL washes  
33 with dephosphorylation buffer (50 mM Tris/HCl, pH 7.6; 100 mM NaCl; 1 mM DTT; 10 mM MgCl<sub>2</sub>;  
34 1 mM MnCl<sub>2</sub>).

35 Peptides-linked to agarose beads were submitted to dephosphorylation at 37 °C for 6 h in 400 μL of  
36 dephosphorylation buffer using 800 U λ phosphatase and 20 U calf intestine phosphatase. Following  
37 dephosphorylation, the resin was washed twice with 1.8 mL of H<sub>2</sub>O, and the slurry volume was

1 adjusted to 300  $\mu\text{L}$  with  $\text{H}_2\text{O}$  before  $\beta$ -elimination with the GlycoProfile  $\beta$ -elimination kit. The  $\beta$ -  
2 elimination reaction was incubated on an end-over-end shaker with extensive mixing at 4  $^\circ\text{C}$  and  
3 quenched after 24 h with 1% TFA until pH6-8. Agarose beads were discarded, and the resulting  
4 solution contained the  $\beta$ -eliminated peptides which correspond to the initially O-GlcNAcylated  
5 peptides. The non-retained and the  $\beta$ -eliminated peptides were desalted with C18 reversed-phase  
6 columns and drained by vacuum concentrator before mass spectrometry analysis.

7

## 8 **Mass spectrometry**

9 Samples were reconstituted with 0.1% trifluoroacetic acid in 4% acetonitrile and analysed by liquid  
10 chromatography (LC)-tandem mass spectrometry (MS/MS) using a nanoLC Ultimate 3000  
11 chromatography system (Dionex, Sunnyvale, CA) coupled to an LTQ-Orbitrap-Velos or a Q-Exactive  
12 mass spectrometer (Thermo Electron, Bremen, Germany). Peptides were separated on a Dionex  
13 Acclaim PepMap RSLC C18 column. First, peptides were concentrated and purified on a pre-column  
14 from Dionex (C18 PepMap100, 2 cm x 100  $\mu\text{m}$  I.D, 100  $\text{\AA}$  pore size, 5  $\mu\text{m}$  particle size) in solvent A  
15 (0.1% formic acid in 2% acetonitrile). In the second step, peptides were separated on a reverse phase  
16 column from Dionex (C18 PepMap100, 15 cm x 75  $\mu\text{m}$  I.D, 100  $\text{\AA}$  pore size, 2  $\mu\text{m}$  particle size) at  
17 300 nL/min flow rate. After column equilibration using 4% of solvent B (20% water - 80% acetonitrile  
18 - 0.1% formic acid), peptides were eluted from the analytical column by a two-steps linear gradient (4-  
19 20% acetonitrile/ $\text{H}_2\text{O}$ ; 0.1 % formic acid for 90 min and 20-45% acetonitrile/ $\text{H}_2\text{O}$ ; 0.1 % formic acid  
20 during 30 min for the LTQ-Orbitrap Velos and 4-25% acetonitrile/ $\text{H}_2\text{O}$ ; 0.1% formic acid for 40 min  
21 and 25-50% acetonitrile/ $\text{H}_2\text{O}$ ; 0.1% formic acid during 10 min for the Q-Exactive). For peptide  
22 ionisation in the nanospray source, spray voltage was set between 1.4 and 1.5 kV and the capillary  
23 temperature at 275  $^\circ\text{C}$ . The LTQ-Orbitrap-Velos was set up in data dependent mode to switch  
24 consistently between MS and MS/MS. MS spectra were acquired with the Orbitrap in the range of m/z  
25 400-1700 at a FWHM resolution of 60 000 measured at 400 m/z and an automatic gain control (AGC)  
26 target value of 1e6. For internal mass calibration the 445.120025 ion was used as lock mass. The 20  
27 abundant precursor ions were selected and collision-induced dissociation fragmentation with 35%  
28 normalized collision energy (NCE), and an AGC target value of 1e4 was performed in the ion trap on  
29 the 20 most intense precursor ions measured to have maximum sensitivity and yield a maximum  
30 amount of MS/MS data. The signal threshold for an MS/MS event was set to 500 counts. Charge state  
31 screening was enabled to exclude precursors with 0 and 1 charge states. Dynamic exclusion was  
32 enabled with a repeat count of 1, exclusion list size 500 and exclusion duration of 30 s. For Q-Exactive  
33 runs, survey MS scan were acquired in the Orbitrap on the 300-1700 m/z range with a resolution of 70  
34 000 (AGC target at 1e6), the 12 most intense ions per survey scan (Intensity threshold 1e5) were  
35 selected for HCD fragmentation (AGC target 5e5, NCE 25%) and resulting fragments were analysed  
36 at a resolution of 17500 in the Orbitrap. Fragmented precursor ions were dynamically excluded for 25

1 s.

2

### 3 **Protein identification**

4 The acquired raw LC Orbitrap MS data were processed using Proteome Discoverer 1.4.1.14  
5 (Thermo Fisher Scientific). This software was used to search data *via* in-house Mascot server (version  
6 2.3.0; Matrix Science, London, U.K.) and SEQUEST HT against the Uniprot mouse reference  
7 proteome database (version 09.08.2013, 50,850 entries). The following parameters were used for  
8 searches: (i) trypsin; (ii) two missed cleavages were allowed; (iii) monoisotopic precursor tolerance of  
9 8 ppm, followed by 0.8 Da for fragment ions from MS/MS; and (iv) cysteine carbamidomethylation  
10 (+57.0215) and methionine oxidation (+15.995) as variable modifications. The false discovery rate  
11 (FDR) was processed the built-in Percolator and was set to 5% (57). Protein identification from  $\beta$ -  
12 elimination experiments was performed similarly as above for data processing with the exception of  
13 the following modifications. Dehydration of Ser and Thr (-18.011) as well as  $\beta$ -elimination of Cys (-  
14 33.988 Da) was added as variable modification. The mass spectrometry proteomics data have been  
15 deposited to the ProteomeXchange Consortium via the PRIDE (58) partner repository with the dataset  
16 identifier PXD004860.

17

### 18 **Bioinformatics resources and protein lists**

19 Protein lists were compared using nwCompare software (59). All identified proteins were  
20 converted into gene names and classified into families and subfamilies of shared function, which were  
21 then categorized by molecular function and biological process ontology terms in the Protein Analysis  
22 Through Evolutionary Relationships (PANTHER) classification system ([www.pantherdb.org](http://www.pantherdb.org)) (60-62).

23

24

# 1     **RESULTS**

## 2 3     **Fractionation of cytosol-enriched fraction from C2C12 myotubes**

4         The workflow applied in our study was presented on Fig.1. Thus, whole proteins were extracted  
5 from C2C12 differentiated myotubes (Whole Extract, WE), or submitted to successive fractionation  
6 steps as described on Fig.1; the protein profiles of the resulting fractions were shown on Fig.2. Briefly,  
7 a differential protocol of extraction was applied to recover a cytosol-enriched fraction (CYT), a  
8 membrane-enriched fraction (MB) and a myofilament-enriched fraction (MYO); these fractions  
9 contained 64.2%, 18.9% and 16.9% of WE proteins, respectively (Fig.2A). It is noteworthy that the  
10 profile of cytosol-enriched fraction was quite similar than those corresponding to whole extract  
11 (Fig.2A). This fraction was then submitted to two successive fractionation protocols, while the  
12 membrane- and the myofilament-enriched fractions were discarded from the following analysis for the  
13 main reason that these fractions were poorly resolubilized following the chloroform/methanol  
14 precipitation.

15         The cytosol-enriched fraction was firstly partitioned through ammonium sulphate (AS)  
16 precipitation. Four fractions were obtained, annotated as AS25, AS50, AS75 and AS100 according to  
17 the final percentage of ammonium sulphate, as indicated on Fig.2B. The protein profile of each  
18 fraction was shown on Fig.2B; 37.9%, 43.9%, 17.4% and 0.8% of the non-fractionated CYT proteins  
19 were obtained for AS25, AS50, AS75 and AS100 fractions, respectively. Because of the poor protein  
20 content on AS100 fraction, it was discarded from the resting analysis. A second fractionation was done  
21 on the AS25, AS50 and AS75 fractions, based on the separation of proteins according to their  
22 isoelectric point, using the MicroRotofor apparatus. Ten fractions were obtained in each case,  
23 containing on average 1.7%, 3.6%, 4.7%, 5%, 7%, 10.6%, 9.9%, 9.8%, 16.5% and 31.2% from F1 (the  
24 more acidic fraction) to F10 (the more basic fraction). The corresponding pH for each fraction was  
25 indicated on supplemented Table I. As shown on Fig.2C, the fractions F1 to F3 (pH 4.42 to 5.19)  
26 contained very few proteins comparing with the following fractions. In contrast, the neutral fractions  
27 (F7 to F10, pH 6.18 to 8.01) contained together approximately 59.7%, 68.5% and 73.8% of the AS25,  
28 AS50 and AS75 fractions, respectively.

29         At least, a total of 30 fractions were obtained for cytosol-enriched fractions (fCYT). Each of them,  
30 as well as non-fractionated cytosol-enriched fraction (CYT) and whole extract (WE), were submitted  
31 to click chemistry protocol in order to map the O-GlcNAc sites. These fractions, submitted to  
32 labelling-coupling protocol and analysed on mass spectrometry, were indicated in bold italic on Fig.1.

## 33 34     **Efficiency of the O-GlcNAcylation mapping after extensive fractionation**

1 The improvement of the fractionation on O-GlcNAcylation mapping was demonstrated in table I,  
2 which indicated the number of the sequenced peptides and the resulting identified proteins. Data were  
3 expressed for the peptides which were not linked to agarose beads (corresponding to non-O-  
4 GlcNAcylated peptides, but which belong to the O-GlcNAcylated proteins coupled to agarose beads),  
5 as well as for peptides which were linked to agarose beads (corresponding to O-GlcNAcylated  
6 peptides covalently linked to agarose beads and released from beads by beta-elimination). We also  
7 indicated the percentage of peptides containing one or more dehydrated serine or threonine  
8 (corresponding so to peptides containing one or more O-GlcNAc sites). Data were presented for the  
9 non-fractionated C2C12 extract (WE, whole extract), the non-fractionated cytosol-enriched extract  
10 (CYT), and the fractionated cytosol-enriched extract (CYT-AS-IEF combined fractions, corresponding  
11 to fCYT fraction). Based on these data, we determined the efficiency factor as the ratio of identified  
12 peptides/proteins after extensive fractionation (fCYT fraction) comparing with the non-fractionated  
13 cytosol-enriched extract (CYT).

14 We firstly analysed the non-linked peptides to order to identify the O-GlcNAcylated proteins linked  
15 to agarose beads, independently of their O-GlcNAc sites. In the non-fractionated whole extract, 554  
16 proteins were identified, while 449 were identified in the cytosol-enriched fraction. Once the  
17 fractionation protocol applied, the number of identifications was increased by a factor 3 comparing  
18 with CYT fraction since 1362 proteins were identified in fCYT fraction. It is worth to note that 14573  
19 peptides (containing 9.3% of dehydrated peptides) led to the identification of these 1362 O-  
20 GlcNAcylated proteins, while “only” 5540 peptides (containing 6.2% of dehydrated peptides) were  
21 identified in the non-fractionated cytosol-enriched fraction. Thus, three times more peptides were  
22 identified when extensive fractionation was applied on CYT fraction. Interestingly, we identified 620  
23 contained one or more dehydrated serine or threonine (corresponding so to O-GlcNAc sites) derived  
24 from the beta-elimination (BE) of peptides covalently linked to agarose beads; in parallel, 311 and 142  
25 dehydrated peptides were identified in WE and CYT fractions. Thus, extensive fractionation of cytosol  
26 enriched-fraction increased the number of identified peptides bearing one or more O-GlcNAc site(s)  
27 by a factor 4.4 compared with non-fractionated cytosol-enriched fraction. It should be mentioned that  
28 among the peptides identified in the beta-eliminated peptides fractions, some of them were not  
29 dehydrated, suggesting that some peptides remained aspecifically retained on agarose beads despite  
30 the pre-clearing step with agarose beads to avoid the non-specific retention of proteins on agarose  
31 beads, and despite the extensive washing steps applied to abrogate protein-protein interaction and so to  
32 eliminate the proteins which were not covalently linked to agarose beads.

33 All data files corresponding to the identification of proteins and the peptides sequenced were  
34 presented as supplemental data (Supplemented Table II to VII, for WE\_BE, WE\_NR, CYT\_BE,  
35 CYT\_NR, fCYT\_BE and fCYT\_NR, respectively); all mass spectrometry data were deposited to the  
36 ProteomeXchange Consortium *via* the PRIDE partner repository (58, 63) with the dataset identifier  
37 PXD004860.

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**Global analysis of O-GlcNAcylated proteins and focus on particular protein classes**

As indicated in Table I, 342 proteins were identified consecutively to the sequencing of peptides released from agarose beads by beta-elimination, *i.e.* threefold than those identified in non-fractionated CYT fractions. We classified these proteins using the PANTHER classification system (Protein Analysis THrough Evolutionary Relationships, <http://www.pantherdb.org/>) (64, 65). Thus, proteins were classified according to their molecular function (Fig.3A) or the protein class (Fig.3B). Among the classified proteins according to their molecular function, 44.1% had a binding activity, 27.8% a catalytic activity, and 16.8% a structural molecule activity; the other proteins, represented less than 5%, had transporter, translation regulator, channel regulator, receptor, signal transducer or antioxidant activities. Proteins were also classified according to the class they belong: nucleic acid metabolism (32.5%), enzymatic activities (27.7%), cytoskeletal proteins and chaperones (14.5%), signalling proteins (7.3%), structural proteins (5.7%), transporter and binding proteins (5.3%), or cell adhesion molecule (4.4%); 2.6% of proteins (others) were not assigned to the classes of proteins described just above.

We focused on proteins identified through the sequencing of beta-eliminated peptides containing one or several dehydrated serine and threonine. We mapped the O-GlcNAcylation site(s) (indicated in bold and underlined in peptide sequence) within different classes of proteins, in particular those involved in cytoskeleton and sarcomeric organization (Table II) or involved in signalling pathways, in protein degradation and in protein post-translational modification (Table III). Table II combined (i) cytoskeleton proteins and proteins involved in cytoskeleton organization; (ii) actin cytoskeleton organization and reorganization; (iii) proteins involved in sarcomeric organization, motor activity and contractile fibers proteins; (iv) microtubules cytoskeleton organization and reorganization; (v) chaperones; (vi) intermediate filament proteins; and (vii) cell adhesion molecules. Table III combined (i) proteins involved in signalling or apoptotic pathways; (ii) proteins involved in protein modification (such as phosphorylation, ubiquitination, SUMOylation); and (iii) proteins involved in protein degradation or autophagy. Classification was done according to gene ontology annotation indicated in UniProtKB (<http://www.uniprot.org>) or GeneCards database (<http://www.genecards.org/>). We indicated the fraction (WE, CYT, fCYT) in which the peptides were identified. As shown on these tables, some peptides were identified in all fractions; in contrast, a large number of them were only identified when cytosolic-enriched extract was extensively fractionated. We also observed that in some case, within the same peptide, serine or threonine residues were differently dehydrated (for example, this was particularly observed for the Nascent polypeptide-associated complex subunit alpha, in Table III).

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## 1 DISCUSSION

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3 Mapping O-GlcNAc sites is a crucial point to characterize the role of O-GlcNAcylation on a given  
4 protein; indeed, the identification of the O-GlcNAc site within a specific domain of a protein could  
5 bring arguments to make hypothesis and determine strategies to clearly demonstrate and understand  
6 the role of the monosaccharide in the modulation of the function of a protein of interest. Strategies  
7 based on copper-catalysed azide/alkyne click chemistry emerged during the last decade; Hahne and  
8 collaborators improve and validate this method permitting the linkage of O-GlcNAcylated proteins to  
9 agarose beads to enrich the modified proteins, to identify them, and to map the O-GlcNAc sites (55).  
10 Beside the method, we opted for the enzymatic labelling of O-GlcNAcylated proteins rather than  
11 metabolic labelling since GlcNAz incorporation preferentially occurs in complex glycans (66). We  
12 added several steps of fractionation prior to the click chemistry; from the analysis of linked peptides  
13 on agarose beads and released from beads by beta-elimination, we identified 342 proteins in the  
14 fractionated subproteome, corresponding to a 3.5-fold increase of identified proteins compared with a  
15 non-fractionated cytosol-enriched fraction. Indeed, through the fractionation protocol, based on the  
16 physicochemical properties of proteins, the sample is made less complex. Thus, there is an enrichment  
17 of certain proteins that could not have been identified in a complex mixture. As consequence, the  
18 number of identified peptides/proteins increased after fractionation. We also sequenced 620 peptides  
19 containing one or several O-GlcNAcylated sites. Over increasing the number of identified  
20 peptides/proteins, the fractionation protocol performed prior to click chemistry led to the mapping of  
21 O-GlcNAc sites on numerous signalling proteins, such as proteins involved in MAPK pathway,  
22 including the TGF-beta pathway. In addition, we identified several proteins involved in the  
23 ubiquitination process, in particular several E3-ubiquitin ligases, as well as proteins responsible of  
24 deubiquitination. Thus, our data suggest that modulation of ubiquitination through O-GlcNAcylation  
25 could be involved in the modulation of degradative process (and so in the regulation of protein  
26 homeostasis essential for muscle healthcare), as well as intracellular processes modulated by  
27 ubiquitination. It is worth to note that we opted for fractionation according to ionic force or isoelectric  
28 point; of course, it could be envisaged to apply other protocols of fractionation to yield complementary  
29 data to complete the non-exhaustive list of O-GlcNAcylated peptides.

30 About fifteen years ago, we attempted to cartography the O-GlcNAcylated proteins in skeletal  
31 muscle and we identified structural proteins, proteins involved in signalling pathways and contractile  
32 proteins as being O-GlcNAcylated (54). Five O-GlcNAc sites were mapped on actin and myosin using  
33 a BEMAD approach, and interestingly, some of them were located into or close to protein-protein  
34 interaction domain, suggesting that O-GlcNAcylation could play an important role in the modulation  
35 of sarcomeric protein interaction (32). In this way, we recently demonstrated that O-GlcNAcylation  
36 was a key modulator of sarcomere morphometry, in particular through the modulation of protein-

1 protein interaction within multiprotein complexes including key structural proteins such as desmin,  
2  $\alpha$ B-crystallin,  $\alpha$ -actinin, filamin-C and moesin (53). In addition, we showed that, consecutively to  
3 global changes of O-GlcNAcylation level, the interaction between desmin and  $\alpha$ B-crystallin was  
4 modulated; in this paper, we localized the O-GlcNAc sites of these two proteins. For desmin, a protein  
5 of intermediate filament essential for striated muscle cells integrity, GlcNAcylation site was localized  
6 on the serine 459, in the C-terminal domain or tail domain (67), suggesting a key role of this O-  
7 GlcNAcylation on the function of the protein. Indeed, this domain is involved in the head to tail  
8 longitudinal assembly of desmin tetramers, and controls the lateral packing and the elongation and  
9 stabilization of mature desmin filaments (68). In addition, this domain is also involved in the  
10 interaction between desmin and cytoskeletal protein partners, leading to the formation of a highly  
11 complex network of intermediate filaments (69). Moreover, the mutation of this site (corresponding to  
12 serine 460 for human desmin) leads to particular myofibrillar myopathies, *i.e.* desminopathies,  
13 characterized by the disintegration of desmin filament, the accumulation of insoluble protein  
14 aggregates (including some of the protein partners of desmin such as  $\alpha$ B-crystallin), and at terms, to  
15 the destructuration of myofibrils (70). In transfected SW13 human cells and C2C12 murine cells, the  
16 mutation S460I led to short desmin filaments, with irregular diameter, and numerous aggregates  
17 randomly distributed in cytoplasm (70).

18 Desmin integrity is regulated by chaperones; among them, the  $\alpha$ B-crystallin plays a crucial role in  
19 the localization or stabilization of cytoskeleton element (71), and in particular, desmin filaments (72).  
20 Thus, the  $\alpha$ B-crystallin modulates the polymerization of desmin filaments and prevent their  
21 aggregation (73, 74); in addition, it decreases the desmin interfilament interaction *in vitro* (75). The  
22 interaction between desmin and its molecular chaperone involved three domains on  $\alpha$ B-crystallin : the  
23  $\beta$ 3 and  $\beta$ 8 sheets, and the 155-165 C-terminal residues (76). We have localized the O-GlcNAc moiety  
24 on the threonine 162, *i.e.* in one of the interaction domain of  $\alpha$ B-crystallin with desmin. Interestingly,  
25 the mutation of the C-terminal domain of the chaperone is closely associated to an increase of  
26 association and aggregation of desmin filaments (76). It is worth to note that on  $\alpha$ A-crystallin and on  
27 HSPB1, O-GlcNAc sites were also localized on the C-terminal domain, which corresponds to a  
28 flexible conserved domain common to sHSPs, essential to maintain sHSP solubility, stability and  
29 chaperone activity (77). All together, these data strongly support that O-GlcNAcylation may be a key  
30 regulator of desmin- $\alpha$ B-crystallin interaction. Interestingly, it was demonstrated that stress induced  
31 changes of O-GlcNAcylation level of  $\alpha$ B-crystallin, associated to translocation of  $\alpha$ B-crystallin to  
32 cytoskeleton (71).

33 The role of O-GlcNAcylation in the maintenance of sarcomere cytoarchitecture and in modulation  
34 of interactions between structural and/or sarcomeric proteins was also supported by other data gained  
35 from this study. Indeed, on another chaperone involved in the maintenance of sarcomere integrity and  
36 in the turn-over of Z-line proteins (78, 79), BAG3 (Bcl-2-associated athanogene 3), two O-

1 GlcNAcylated sites out of the four are located into the PxxP domain, which is known to interact with  
2 SH3-containing proteins (80). Titin, essential for assembling and maintenance of sarcomere, providing  
3 to myofibril their property of elasticity (81), is O-GlcNAcylated on T24950 and S24954, localized in  
4 the mouse domain Kelch-12 (corresponding to the human domain Ig-like 123), which is the interaction  
5 domain with protein partners such as myosin or My-BPC (82). Plectin, a large intermediate filament-  
6 associated protein acting as cytoskeletal crosslinker and signalling scaffold, is O-GlcNAcylated into  
7 the plakin domain repeat B5 (83, 84), located at the C-terminal domain of plectin and described to  
8 bind to various intermediate filament proteins (85).

9 To sum, while our data demonstrated that the combination of fractionation and click chemistry was  
10 a powerful methodology to map O-GlcNAc sites on several proteins involved in signalling pathways  
11 or in protein modification (in particular ubiquitination), as well as structural proteins, it brought strong  
12 arguments in favour of the essential role of O-GlcNAcylation for the sarcomeric cytoarchitecture of  
13 skeletal muscle cells. Thus, we identified herein several key structural proteins involved in the  
14 maintenance of filament networks permitting force development and providing muscle cells  
15 mechanical strength. Moreover, we localized numerous O-GlcNAcylated sites in protein-protein  
16 interaction domains. In addition, key structural proteins such as desmin,  $\alpha$ B-crystallin, plectin, or  
17 BAG3 among others are O-GlcNAcylated; importantly, mutation that can occur at the O-  
18 GlcNAcylated sites of these proteins led to the sarcomere disorganisation and myofibrils  
19 destructuration because of the loss of protein-protein interactions; as consequence, proteins aggregate,  
20 resulting at terms to myofibrillar myopathies.

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1     **CONFLICT OF INTEREST**

2     The authors declare that they have no conflict of interest.

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16    proteomics data, including search result files have been deposited to the ProteomeXchange  
17    Consortium ([www.proteomexchange.org](http://www.proteomexchange.org)) via the PRIDE partner repository with the dataset identifier  
18    PXD004860.

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32

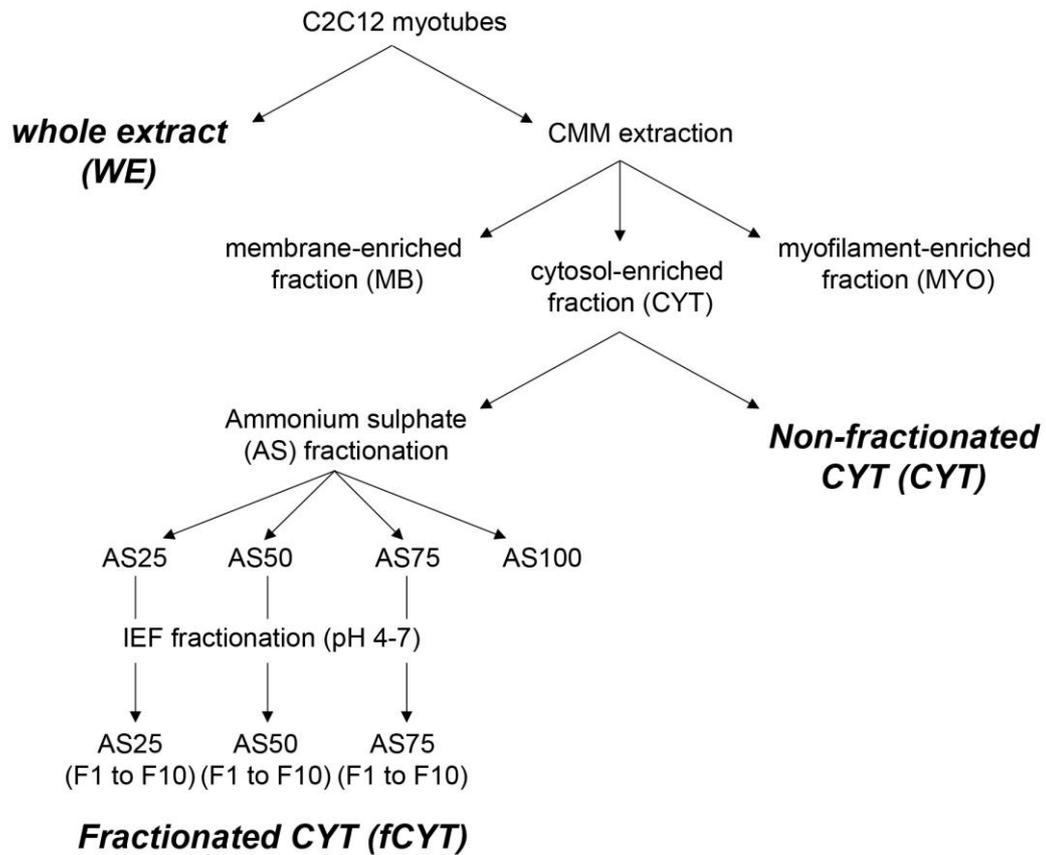
## FIGURE LEGENDS

**Table I: Overall evaluation of the O-GlcNAc mapping efficiency after extensive fractionation.**

The non-linked peptides on agarose beads and the peptides resulting from beta-elimination of peptides covalently linked to agarose beads were sequenced using mass spectrometry. The number of sequenced peptides (and the percentage of peptides containing dehydrated serine and threonine) and the corresponding number of identified proteins were indicated for each fraction (whole extract, WE; non-fractionated cytosol-enriched extract, CYT; fractionated cytosol-enriched extract, fCYT). The efficiency factor was determined as the ratio of sequenced peptides or identified proteins in fCYT fraction comparing with the WE or the CYT fractions.

		<b>Non fractionated whole extract (WE)</b>	<b>Non-fractionated cytosol-enriched extract (CYT)</b>	<b>Fractionated cytosol-enriched extract (fCYT)</b>	<b>Efficiency factor: fCYT vs CYT</b>
<b>Non-linked peptides on agarose beads (NR peptides)</b>	<b>Number of peptides (% dehydrated peptides)</b>	5340 (7.1%)	5540 (6.2%)	14573 (9.3%)	<b>x 2.6</b>
	<b>Number of identified proteins</b>	554	449	1362	<b>x 3</b>
<b>Linked peptides on agarose beads ; beta-eliminated</b>	<b>Number of dehydrated peptides</b>	311	142	620	<b>x 4.4</b>
	<b>Number of O-GlcNAcylated proteins</b>	180	97	342	<b>x 3.5</b>

**Figure 1: Representative scheme of the workflow applied prior to the click chemistry.** The protein fractions indicated in bold italic on figure 1 correspond to fraction submitted to azide-alkyne click chemistry and analysed on mass spectrometry.

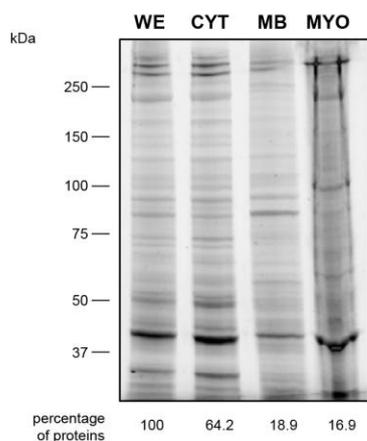


**Figure 1**

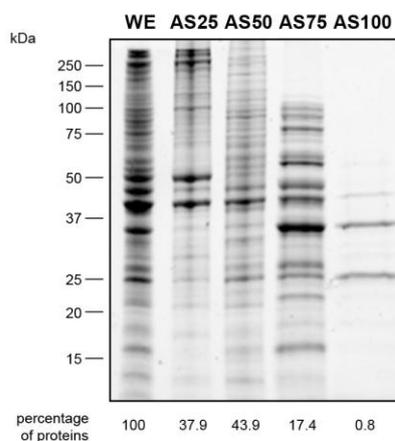
1  
 2 **Figure 2: Efficiency of the protein fractionation.** (A) Protein profiles of the differential extraction.  
 3 Twenty  $\mu\text{g}$  of proteins corresponding to whole extract (WE), cytosol-enriched fraction (CYT),  
 4 membrane-enriched fraction (MB) and myofilament-enriched fraction (MYO) were separated on 7.5%  
 5 Mini-PROTEAN TGX SF Precast Gels and visualized according to Stain-free technology. (B) Protein  
 6 profiles resulting from ammonium sulphate (AS) precipitation of cytosol-enriched extract. Twenty  $\mu\text{g}$   
 7 of proteins corresponding to whole extract (WE), AS25, AS50, AS75 and AS100 (corresponding to

1 final concentration of ammonium sulphate (AS)) were separated on AnykD Mini-PROTEAN TGX SF  
2 Precast Gels and visualized according to Stain-free technology. (C) Protein profiles of the 10 fractions  
3 resulting from MicroRotofor separation of proteins from AS25, AS50 and AS75 fractions, according to  
4 their isoelectric point on pH range of 4 to 7. Ten  $\mu$ l of each fraction were separated on AnykD Mini-  
5 PROTEAN TGX SF Precast Gels and visualized according to Stain-free technology. The percentage of  
6 proteins compared with the non-fractionated extract were indicated above each gel images. The pH of  
7 each fraction was indicated on supplemented table I.

### A Fractionated protein extraction

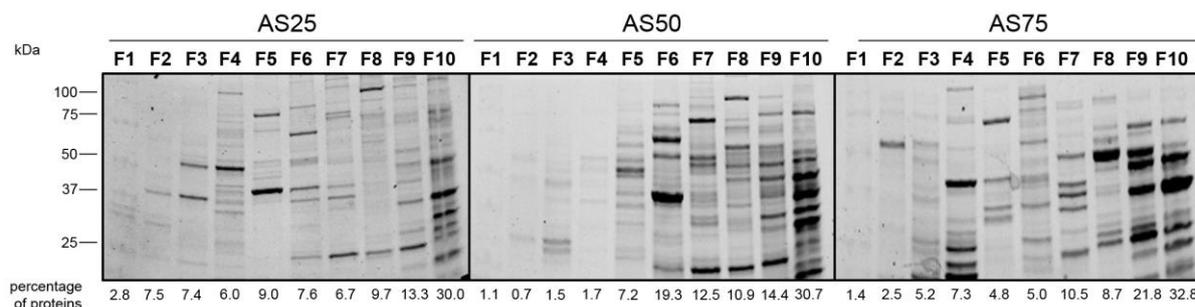


### B Ammonium sulphate precipitation (AS) of cytosol-enriched fraction



### C

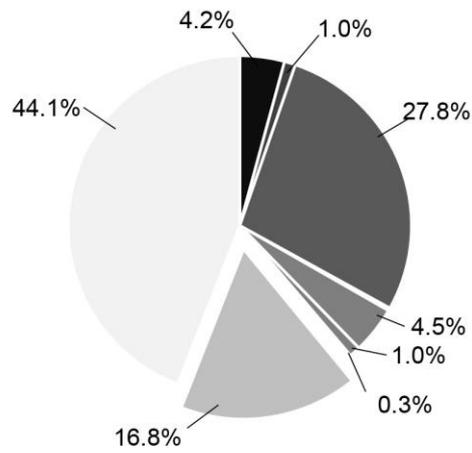
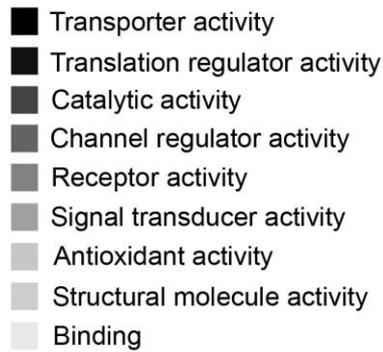
#### IEF fractionation (pH 4-7) of AS25, A50 and AS75



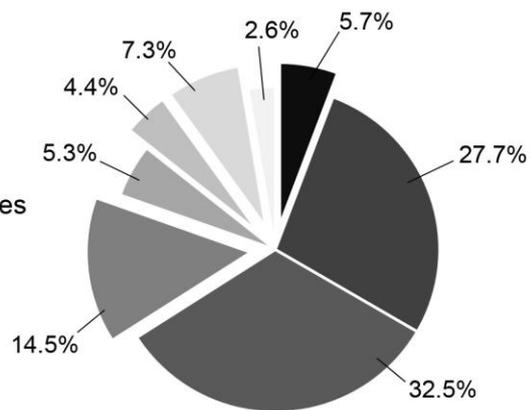
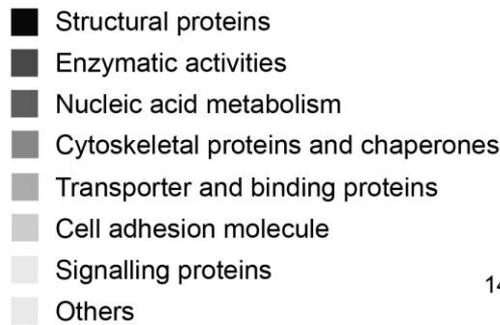
**Figure 2**

1  
2 **Figure 3: Classification of the 342 proteins identified from peptides released from agarose beads**  
3 **by beta-elimination.** The classification was done using the PANTHER classification system (Protein  
4 Analysis THrough Evolutionary Relationships, <http://www.pantherdb.org/>). (A) Classification of  
5 proteins according to their molecular function. (B) Classification of proteins according to the protein  
6 classes.  
7

## A Molecular Function



## B Protein Class



**Figure 3**

1

2 **SUPPLEMENTED TABLES**

3

4 **Table I:** Measurement of pH of fractions resulting from isoelectric separation using  
 5 **MicroRotofor cell on pH 4-7 range.** The pH of each fraction is indicated as value  $\pm$  SD (standard  
 6 deviation). The fraction F1 corresponds to the more acidic fraction, while the fraction F10 corresponds

1 to the more basic one.

2

3 **Table II, IV and VI: Data files corresponding to the identification of proteins from sequencing of**  
4 **peptides released from agarose beads by beta-elimination.** These peptides correspond to O-  
5 GlcNAcylated peptides (BE, beta-eliminated peptides). Table II corresponds to proteins identified  
6 from whole extract (WE), table IV to proteins identified from cytosol-enriched extract (CYT), and  
7 table VI to proteins identified from fractionated cytosol-enriched extract (fCYT).

8

9 **Table III, V and VII: Data files corresponding to the identification of proteins from sequencing**  
10 **of peptides released from agarose beads by trypsin.** These peptides correspond to non-O-  
11 GlcNAcylated peptides (NR, non-retained peptides), but they belong O-GlcNAcylated proteins  
12 covalently linked to agarose beads. Table III corresponds to proteins identified from whole extract  
13 (WE), table V to proteins identified from cytosol-enriched extract (CYT), and table VII to proteins  
14 identified from fractionated cytosol-enriched extract (fCYT).

15

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