Electronic supplementary material

Methods

Preparation of samples for MS analysis Briefly, each slice corresponding to either APPL1 or actin was cut into 1 mm cubes prior to digestion. Gel pieces were placed in 0.6 ml tubes containing 400 µl water. Pieces were destained twice with 50% acetonitrile per 40 mmol/l bicarbonate solution, then dehydrated with 100% acetonitrile for 15 min. Acetonitrile was removed by aspiration, upon which the gel pieces were dried for 30 min in a centrifuge vacuum (22,000 *g*) at 60°C. Next 20 µl 40 mmol/l bicarbonate containing trypsin (1 µg; Sigma Chemical) was added and samples were maintained at 4°C for 15 min. Bicarbonate (50 µl) containing 10 fmol/µl digestion standards (Bradykinin fragment 2-9; β-Sheet breaker peptide; and anaphylatoxin C3a fragment; Sigma) was added to samples and incubated overnight at 30°C. After incubation, the reaction was stopped by adding 5% formic acid. Samples were then incubated for 30 min at 37°C, centrifuged (22,000 *g*) for 1 min, transferred to clean polypropylene tubes and speed-vacuum dried to ~5 µl. Formic acid, 0.5% (40 µl), was added to the gel pieces, which were then incubated for 30 min at 37°C, centrifuged (22,000 x *g*) for 1 min and added to the 5 µl extraction tube. This combined extraction volume were then speed-vacuum dried to ~5 µl. This peptide mixture was purified by solid-phase extraction (C18 ZipTip; Millipore, Billerica, MA, USA) after sample loading in 0.05% heptafluorobutyric acid: 5% formic acid and elution with 50% acetonitrile:1% formic acid. Samples were then dried by speed vacuum, followed by addition of 4 µl 0.1% formic acid: 2% acetonitrile containing 125 fmol/µl MS peptides (angiotensin 2, [Ile7]-angiotensin III and influenza haemagglutinin (HA) peptide; Sigma; St Louis, MO).

APPL1 identification To determine whether APPL1 was present in human skeletal muscle, protein was immunoprecipitated with the anti-APPL1 antibody linked to protein A sepharose beads. Immunoprecipitates were separated by SDS-PAGE electrophoresis and blotted against

corresponding antibodies. Western blots showed the presence of APPL1 in immunoprecipitated human skeletal muscle (ESM Fig. 1a). To validate the presence of APPL1 in human skeletal muscle, homogenates were first fractionated by one-dimensional gel electrophoresis (ESM Fig. 1B) and then subjected to HPLC-ESI-MS analysis. A 'top 10' approach was used to identify APPL1 peptides and obtain HPLC retention times. The 56% sequence coverage of APPL1 tryptic peptides unambiguously detected the presence of APPL1 in human skeletal muscle (ESM Fig. 1c).

Validation of APPL1:β-actin ratio for quantification of APPL1 protein We assessed the linearity of ß-actin:APPL1 normalisation quantification by first testing three different protein amounts on a gel, running 50, 75 and 100 µg of the same whole-muscle homogenate on three different lanes on the same 10% SDS-PAGE gel. Bands corresponding to APPL1 and ß-actin were excised, trypsinised and desalted, and the resulting peptides analysed by targeted HPLC-ESI-MS. Peptides from β-actin and APPL1 were added to the target list and used for quantification. The average peak areas of APPL1 and β-actin were plotted separately; the linearity is shown in ESM Fig 2 . After determining that β-actin and APPL1 show linear regression in a similar manner, the ratios of APPL1:β-actin were calculated from the average peak area of the peptides targeted. Results show a linear regression of APPL1 (R^2=0.91) and β-actin (R^2=0.92). Based on these ratios (0.000639, 0.000712 and 0.000719 for 50, 75 and 100 µg, respectively) the coefficient of variance was determined to be 6.59%, indicating that use of β-actin for normalisation of protein loading was valid and accurate.