**Human and mouse NMJ TMT Analysis Methodology Data share:**


**Ethics**

Use of anonymous human tissue was granted by the Lothian NRS BioResource (SR719, 15/ES/0094); prospective tissue collection was approved by the Lothian Ethics Committee (REC 2002/1/22, 2002/R/OST/02) following internal (University of Edinburgh) and independent/external ethical review.

**Proteomics: tissue sampling**

Human muscle samples were obtained from patients following lower limb amputation surgery (see above for ethical/institutional approvals). Muscle samples were obtained from the amputation specimen in the operating theatre immediately after disconnection of the limb. Tissue was harvested from the proximal end of the specimen, close to the line of surgical incision, in areas demonstrating good back bleeding and muscle fasciculation, and away from areas of necrosis and infection. Any muscle that appeared obviously devitalized on gross inspection was not sampled. Small blocks of tissue, containing full-length muscle fibres from origin to insertion (approx. 2cm in length) were removed from each of the muscles selected and placed on wet ice. All specimens were then transferred from theatre to laboratory for immediate processing. A subset of PL muscle fibres was selected from four human cases.

To allow direct comparison with human NMJs, equivalent muscles were dissected from both sides of three CD1 littermate mice (adult, ~12 weeks old). Animals were euthanized with isoflurane, and the muscles dissected out within 30 minutes post-mortem. All animal
experiments were performed under the appropriate project and personal licenses granted by the UK Home Office.

**Proteomics: protein extraction**

Dissected tissue samples were pooled in M tubes (gentle MACS Miltenyi Biotec). Samples were homogenized in label-free extraction buffer [100 mM Tris-HCl (pH7.6) 4% (w/v) SDS] containing 1% protease cocktail inhibitor (Thermo Fisher, UK) using gentle MACS dissociator (Miltenyi Biotec) run on the M tube protein cycle. Post homogenization samples were spun at 300 x g for 2 minutes and left on ice for 20 minutes. Homogenates were transferred to Lo-Bind 1.5ml tubes (Sigma Aldrich) and centrifuged at 20,000 x g for 20 minutes at 4°C with the soluble fraction of each sample then transferred to new Lo-Bind tubes. Protein determination using the Bicinchoninic acid assay (Pierce, UK) was carried out according to manufacturer’s guidelines.

**Proteomics: mass spectrometry**

Preparation of samples, quantification and bioinformatics was performed according to a standardized protocol (Hughes et al., 2014). Samples were measured out to achieve 200ug/ul protein. DTT was added to give a 10mM final concentration and samples were left on a shaker at room temperature for 30 minutes, followed by incubation with IAA (50mM final concentration) in the dark for 30 minutes. 2ul DTT (1M) was added before samples were frozen.

Hydrophobic and hydrophilic beads (GE HealthCare) were mixed together and washed with MQ water. Beads were transferred to 4 tubes. Samples were then gently added to the beads
and mixed/shaken for 2 min. 1% FA was then added to each sample (2 min mix), followed by 50% ACN (8 min mix); samples were then transferred to a magnetic rack and the supernatant was removed. Next, 70% EtOH was added (1 min mix), followed by removal of the supernatant. Finally, ACN was added (1 min mix) and the supernatant again removed. Proteins were eluted with 100mM TEAB on a shaker for 10 min. Trypsin in 1mM HCl (1ug/ul) was added to each sample and incubated on a shaker at 30°C for 4-6h. A further portion of Trypsin was added and the samples were incubated overnight at 30°C. The tryptic peptides were then labelled with 6-plex TMT reagents (Thermo Fisher Scientific) using a protocol supplied by the manufacturer [TMT6plex-Nter126 & -Lys126 – Human +, TMT6plex-Nter127 & -Lys127 – Human -, TMT6plex-Nter128 & -Lys128 – Mouse +, TMT6plex-Nter129 & -Lys129 – Mouse -]. The labelled peptides were quenched then mixed together. The mixed sample was fractionated into 22 fractions using High pH reverse phase chromatography (Ultimate 3000 from Dionex). HPLC buffer A was 10mM ammonium formate in water (pH=10); HPLC buffer B was 10mM ammonium formate in 90% CH3CN (pH=10). An XBridge peptide BEH column (130Å, 3.5 μm 2.1 X 150 mm from Waters) was used to separate peptides, with the column temperature set to 20°C. Peptides were eluted from the column using a flow rate of 200ul/min and a linear gradient of 5% to 60% buffer B in 60min. 40 fractions were collected and concatenated into 22 fractions based upon UV signal. All fractions were vacuumed dried and re-suspended in 50 μl 1%FA acid prior to MS analysis. Fractions were analyzed on a Q-exactive-HF mass spectrometer (Thermo Scientific) equipped with Dionex Ultimate 3000 RS and Easyspray column (75 μm x 50 cm, PepMap RSLC C18 column, 2 μm, 100 Å). LC buffer A was 0.1% formic acid in MS grade Milli-Q water; LC buffer B was 80% acetonitrile and 0.08% formic acid in MS grade Milli-Q water. The peptides were eluted from the column using a flow rate of 300nl/min and a linear gradient of 5% to 40% buffer B in 122 min. The column temperature was set to 50°C.
Qexactive HF was performed in data dependent mode: an MS survey scan followed by 15 sequential dependent MS2 scans, with the 15 most intense precursor ions selected to be fragmented by Higher Energy Collisional Dissociation (HCD), with the isolation window at 0.4 da. The resolution of the MS1 and MS2 was set at 120,000 and 60,000 respectively. The maximum ion injection time for MS1 and MS2 was 50ms and 200ms respectively.

**Proteomics: quantification and bioinformatics analysis**

The raw mass spec data files obtained for each experiment were collated into a single quantitated dataset using MaxQuant (Cox and Mann, 2008) and Andromeda search engine software (Cox et al., 2011). Enzyme specificity was set to that of trypsin, allowing for cleavage of N-terminal to proline residues and between aspartic acid and proline residues. Other parameters used were: (i) variable modifications, deamidation (NQ), oxidation (M), protein N-acetylation, gln → pyro-glu; (ii) fixed modifications, carbamidomethylation (C); (iii) database: uniprot-reviewed_Mus_A10090_160916 & uniprot-Human-up5640_160516; (iv) Reporter ion MS2 – 4 TMT labels: TMT6plex-Nter126 to 129 & TMT6plex-Lys126 to 129; (v) MS/MS tolerance: FTMS- 10ppm, ITMS- 0.02 Da; (vi) maximum peptide length, 6; (vii) maximum missed cleavages, 2; (viii) maximum of labelled amino acids, 3; and (ix) false discovery rate, 1%. Peptide ratios were calculated using ‘Reporter Intensity’ for Mouse +/- samples, Human +/- samples and Human/Mouse +/- samples. Data was normalised using 1/median ratio value for each identified protein group per labelled sample. Proteins were filtered to include candidates identified by >1 unique peptide and demonstrating a >20% change between species. The outputs of this analysis are associated with this deposit. A single excel file containing tabs from a combined human/mouse database search (see iii above) – the protein ID summary and peptide information.