Mitsugumin 53 attenuates sarcoplasmic reticulum Ca^{2+}-ATPase 1a (SERCA1a) in skeletal muscle

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Supplementary Material 1

PCR primers for the cloning of GST-fused MG53 proteins

	PCR primers	
GST-TRIM	forward	5’ – GCGTCGACTCATGTCGGCTGCACCCGG –3’
	backward	5’– TAAAGCGGCCGCTCACAGAGCCCGGAACATC –3’
GST-PRY	forward	5’ – GCGTCGACCTATGCCAGCGCTGGAGGAAC –3’
	backward	5’– TAAAGCGGCCGCTCACTGTGACAGCAGCTGCTG –3’
GST-SPRY	forward	5’ – GCGTCGACTCATGGGCGAGCACTATTGGGAG –3’
	backward	5’ – TAAAGCGGCCGCTCAGGCCTGTTCCTGCTCC –3’
GST-PRY-SPRY	forward	5’ – GCGTCGACCTATGCCAGCGCTGGAGGAAC –3’
	backward	5’ – TAAAGCGGCCGCTCAGGCCTGTTCCTGCTCC –3’
GST-MG53	forward	5’ – GCGTCGACTCATGTCGGCTGCACCCGG –3’
	backward	5’ – TAAAGCGGCCGCTCAGGCCTGTTCCTGCTCC –3’

Supplementary Material 2

Materials and methods

(A) Binding assay of GST-fused MG53 proteins with triad proteins

Briefly, affinity beads were prepared by immobilizing each GST-fused MG53 protein on GST beads (Amersham Biosciences, Pittsburgh, PA). The affinity beads were then incubated with 150 μg of the solubilized triad vesicle sample for 8 h at 4 °C. The proteins that were bound to the affinity beads were separated on SDS-PAGE gels and the gels were stained with Coomassie Brilliant Blue in order to obtain the protein bands.

(B) Protein identifications by qTOF MS and database searches

The digested peptide solution by in-gel digestion was desalted and concentrated, and was eluted using a home-made C18 nano-column (100-300 nl with trypsin of POROS reverse-phase R2 material (20–30 μm in bead size, PerSeptive Biosystems, Foster City, CA)) and 1.5 ul of 50% MeOH, 49% H_2O, and 1% HCO_2H. qTOF MS of the eluted peptides was performed by Hybrid Quadrupole-TOF LC/MS/MS Mass Spectrometer (AB Sciex Instruments, Framingham, MA) equipped with an electrospray ionization (ESI) source. Source temperature, room temperature; potential, 1 kV; flow rate, 10–30 nl/min; cone voltage, 40 V; collision gas and energy, Ar at a pressure of 6^{-7} x 10^{-5} mbar and 25–40 V. The quadrupole analyzer was used to select precursor ions for fragmentation in the hexapole collision cell. The produced ions were analyzed using an orthogonal TOF analyzer and fitted with a reflector, a micro-channel plate detector, and a time-to-digital converter. Mode, ESI+; MS parameter and scan type, positive TOF MS; intensity threshold, 1 count; MCA No., GS1: 2.00 and GS2: 0.00; CUR, 30.00; IS, 1000.00; CID, 50~55; scan range and rate, m/z 50-2000 and 2 sec/scan.

Database searches were conducted using Mascot (http://www.matrixscience.com/). Type of search, MS/MS ion search; enzyme, trypsin; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M); mass values, monoisotopic; protein mass, unrestricted; peptide mass tolerance, ±1 Da; fragment mass tolerance, ±0.6 Da; max missed cleavages, 1; instrument type, default; number of queries, 1.

Supplementary Material 2 (continued)

(C) Co-immunoprecipitation, immunoblot assay, and immunocytochemistry

The solubilized lysate (800 μg of total protein) was incubated with anti-MG53 antibody (10 μg/ml, Sigma, St. Louis, MO) overnight at 4°C for co-immunoprecipitation. Anti-MG53 (1:1000) or anti-SERCA1a antibody (1:1000, Affinity BioReagents, Rockford, IL) was used for immunoblot assay. For immunocytochemistry, anti-MG53 (1:500), anti-SERCA1a antibodies (1:500), Cy-3-conjugated anti-mouse (1:500, Jackson ImmunoResearch, West Grove, PA), and FITC-conjugated anti-rabbit secondary antibody (1:500, Sigma) were used.

(D) Preparation of myotube homogenate and the oxalate-supported $^{45}Ca^{2+}$-uptake experiment

The MG53 knockdown myotubes were homogenated in a buffer (50 mM KH_2PO_4, 10 mM NaF, 1 mM EDTA, 0.3 M sucrose, protease inhibitor cocktail, and 0.5 mM DTT at pH 7.0) with a homogenizer for 15 sec at speed 5 (IKA T10basic Ultra-turrax, Wilmington, NC). 250 μg of the myotube homogenate was subjected to the oxalate-supported $^{45}Ca^{2+}$-uptake experiment. Briefly, the reaction buffer was composed of 40 mM imidazole, 100 mM KCl, 5 mM $MgCl_2$, 5 mM NaN_3, and 0.5 mM EGTA at pH 7.0. The washing buffer was composed of 100 mM KCl and 20 mM MOPS at pH 7.0. The uptake reaction was begun by the rapid sequential addition of 5 mM MgATP, 5 mM K-oxalate, and 70 nM or 1 μM of free $^{45}Ca^{2+}$ (PerkinElmer, Waltham, MA). The rate of $^{45}Ca^{2+}$-uptake was calculated from the linear regression of $^{45}Ca^{2+}$-uptake at 0, 1, 2, 3, and 4 min.

Supplementary Material 3

Sequences of siRNAs used for knocking down MG53 and qPCR primers

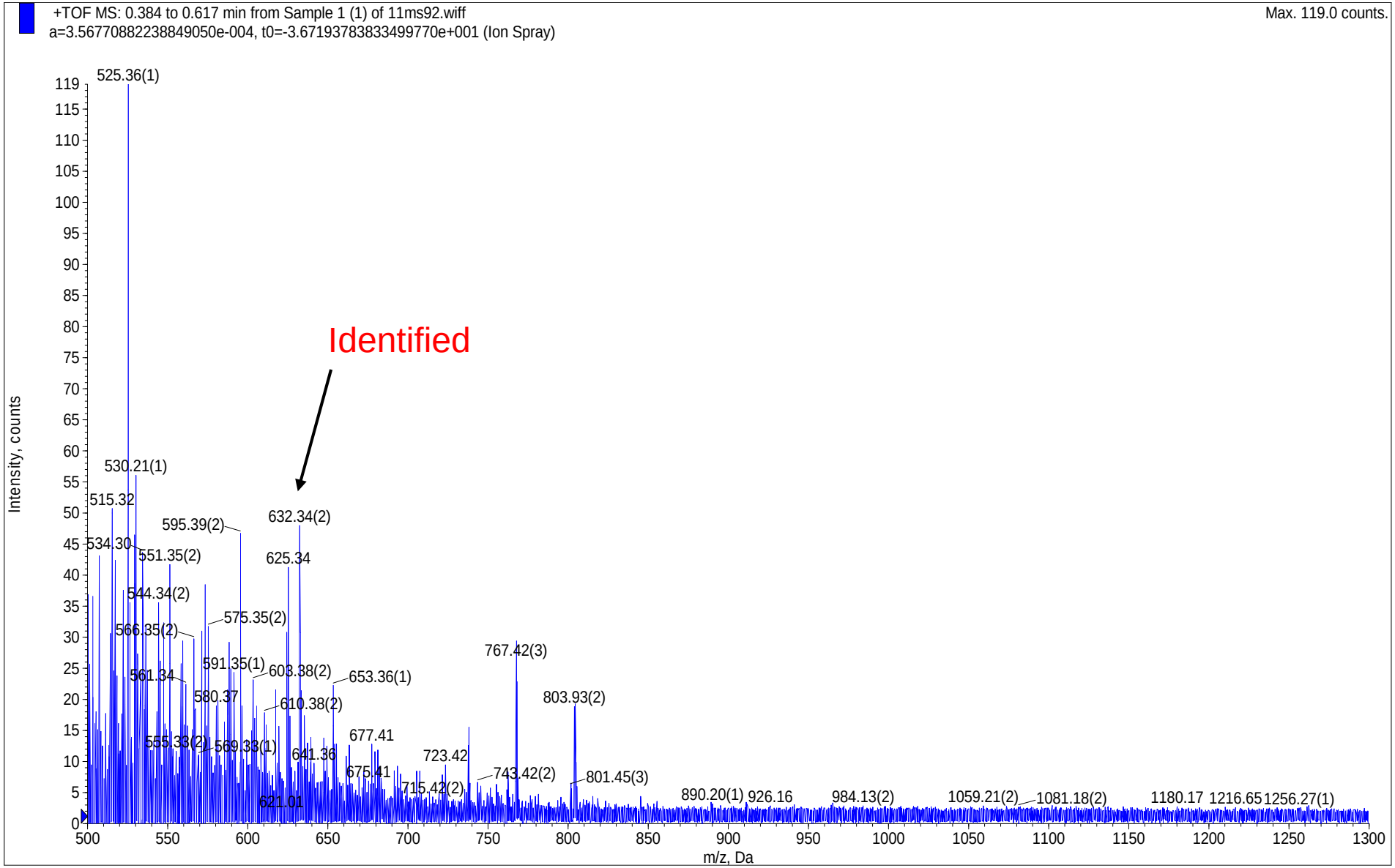
siRNA	Sense	Antisense
Scrambled siRNA	5’ ACGUGACACGUUCGGAGAAUU 3’	5’ UUCUCCGAACGUGUCACGUUU 3’
siRNA #1	5’ CCGCAGGCUCUAAGCACUAUU 3’	5’ UAGUGCUUAGAGCCUGCGGUU 3’
siRNA #2	5’ CUGUCAAGCCUGAACUCUUUU 3’	5’ AAGAGUUCAGGCUUGACAGUU 3’

qPCR primers	Forward	Backward
	5’ AACACCTGGATCCACTGAGC 3’	5’ TCTGCTGTGGAAGCTGTGTC 3’
	(expected size of PCR product: 147 bp)	

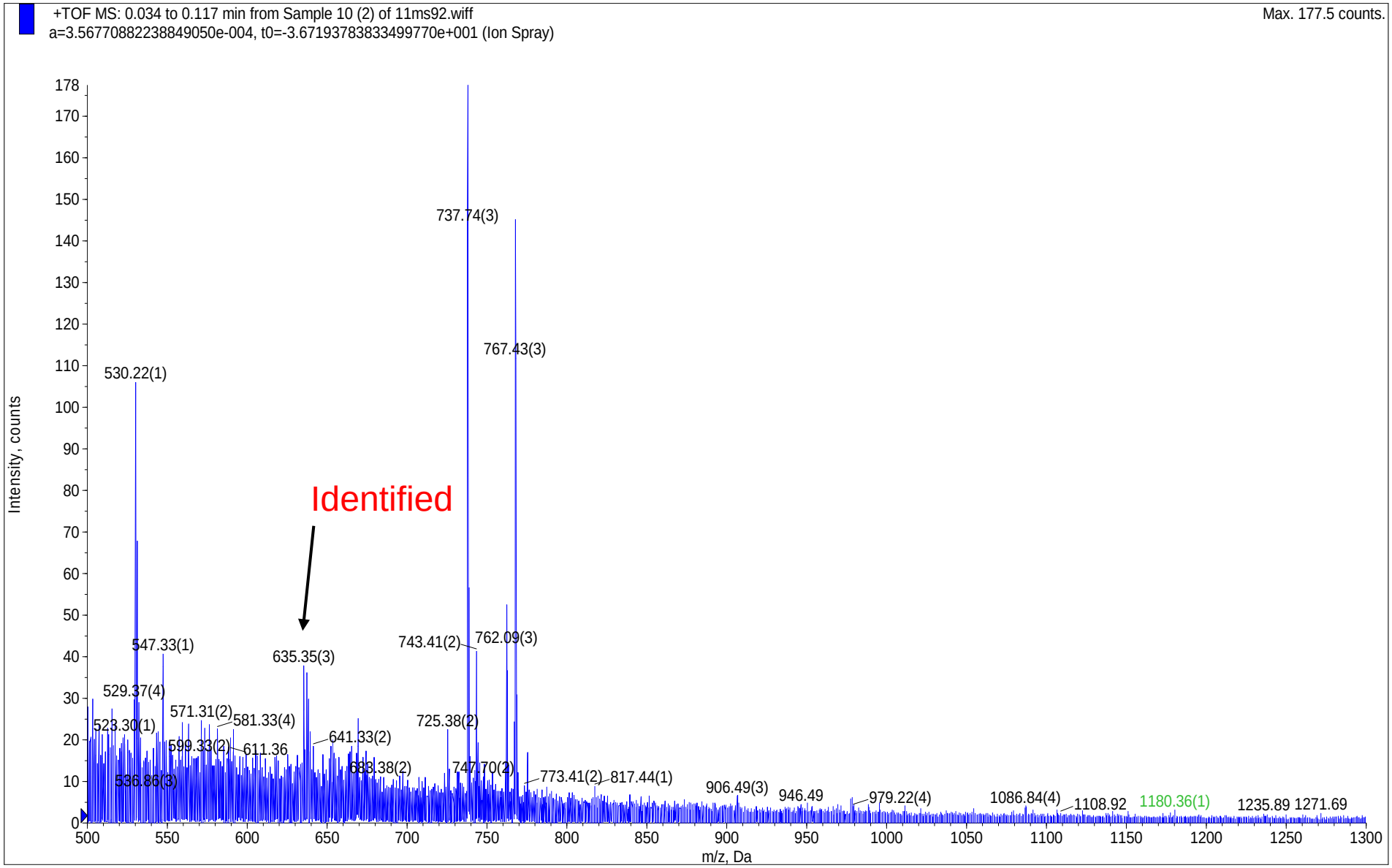
Supplementary Material 4

MS spectrums for bands 1 to 6 and 9 in Figure 1C

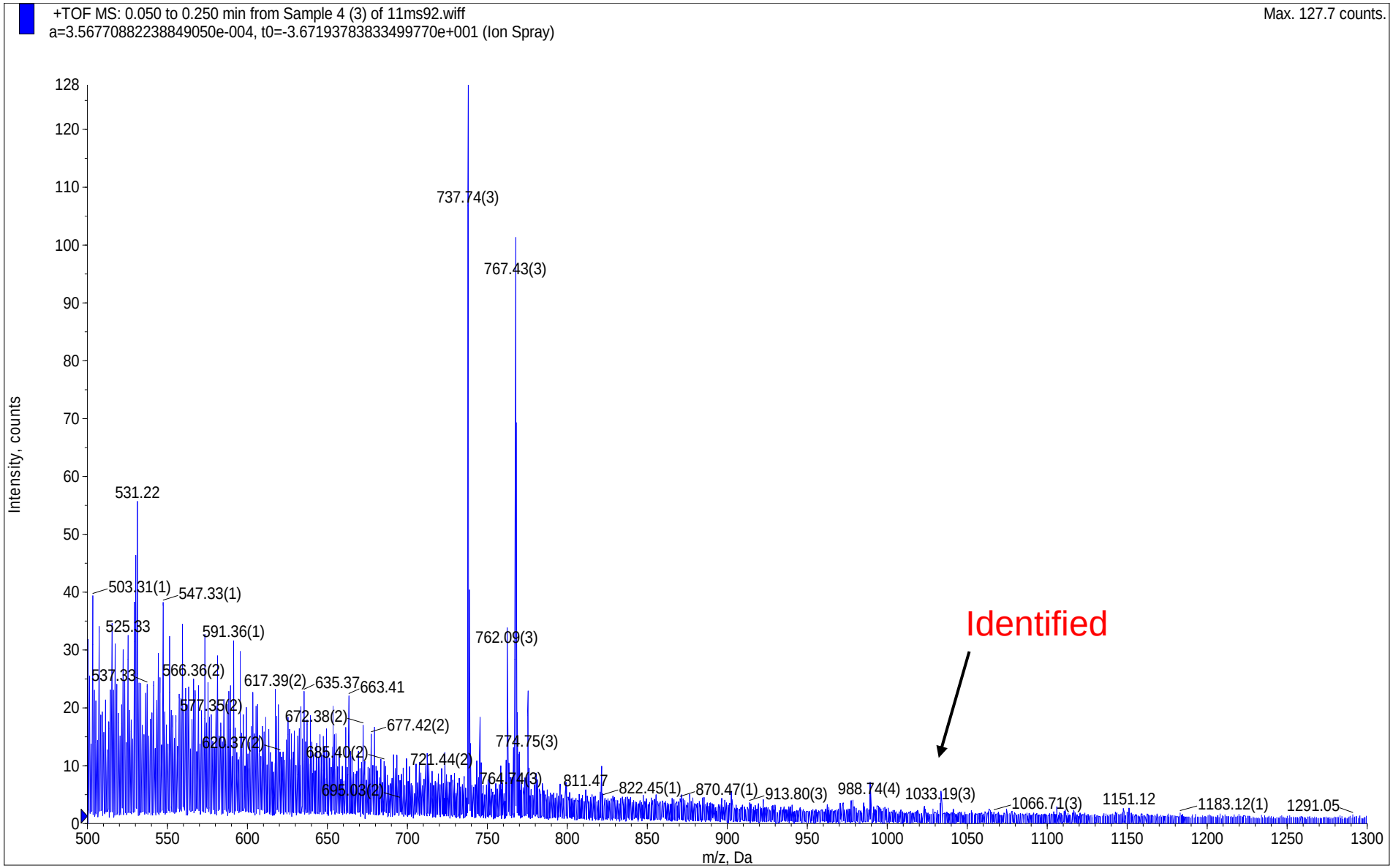
(A) MS spectrum of the band 1 in Figure 1C



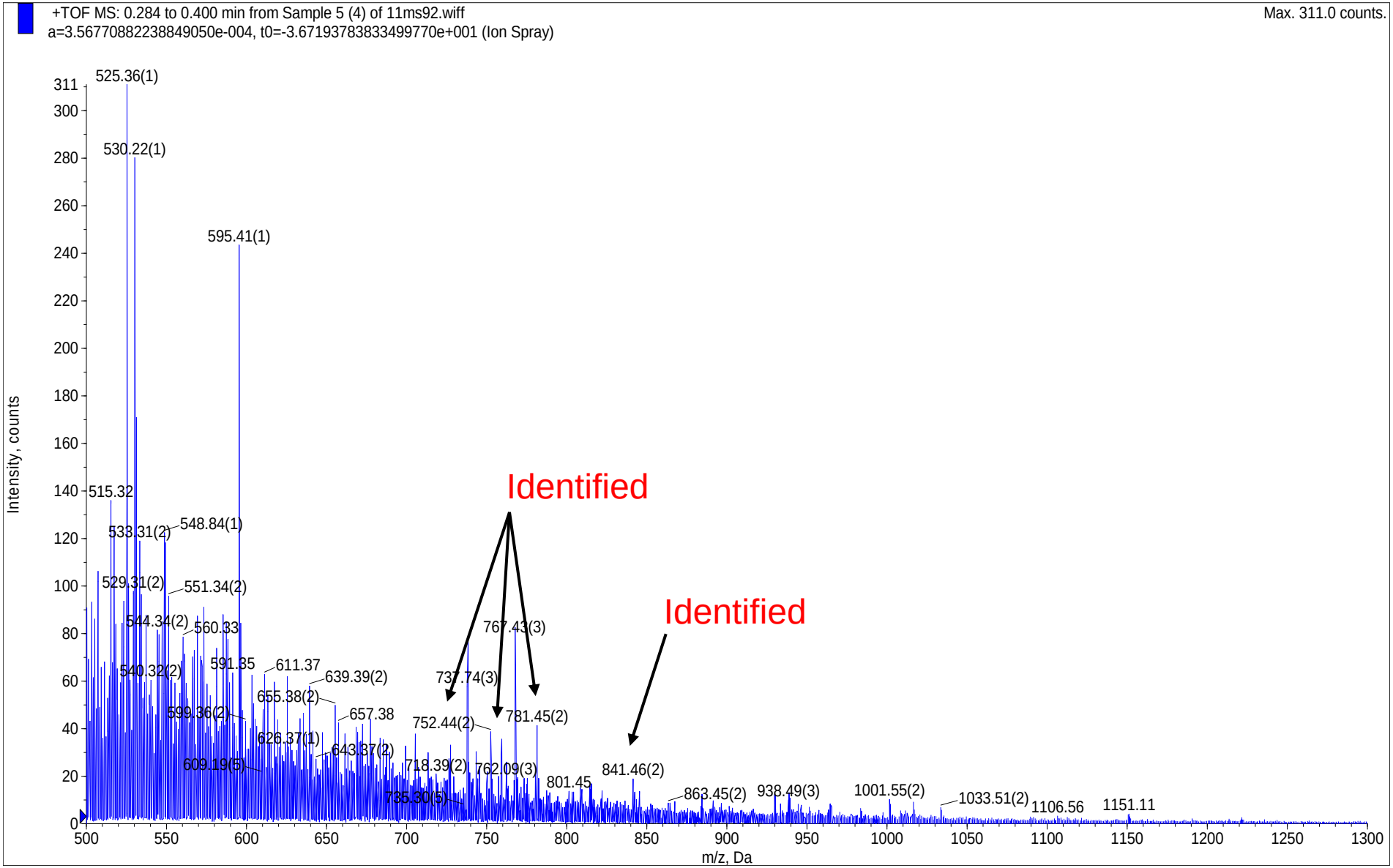
(B) MS spectrum of the band 2 in Figure 1C



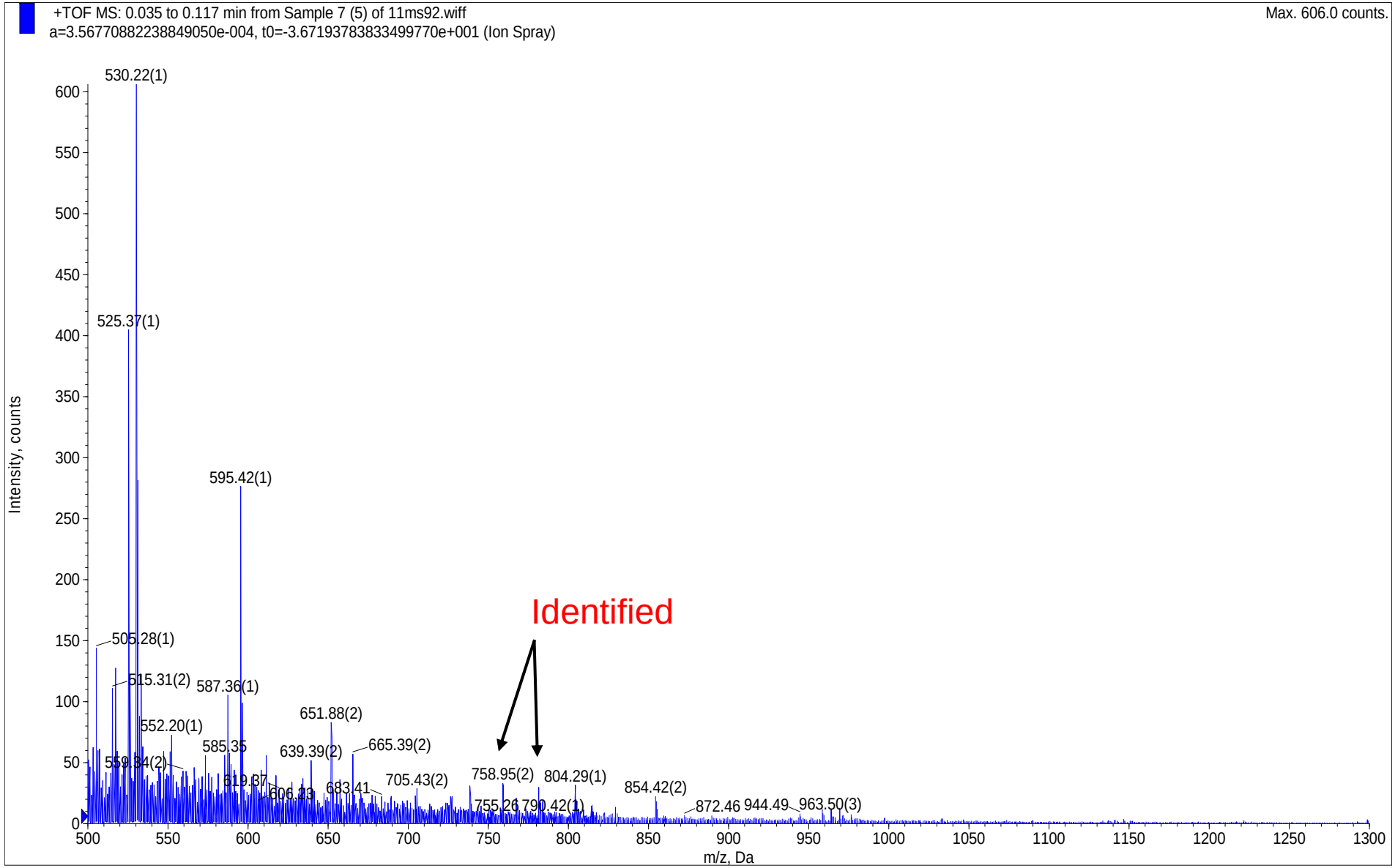
(C) MS spectrum of the band 3 in Figure 1C



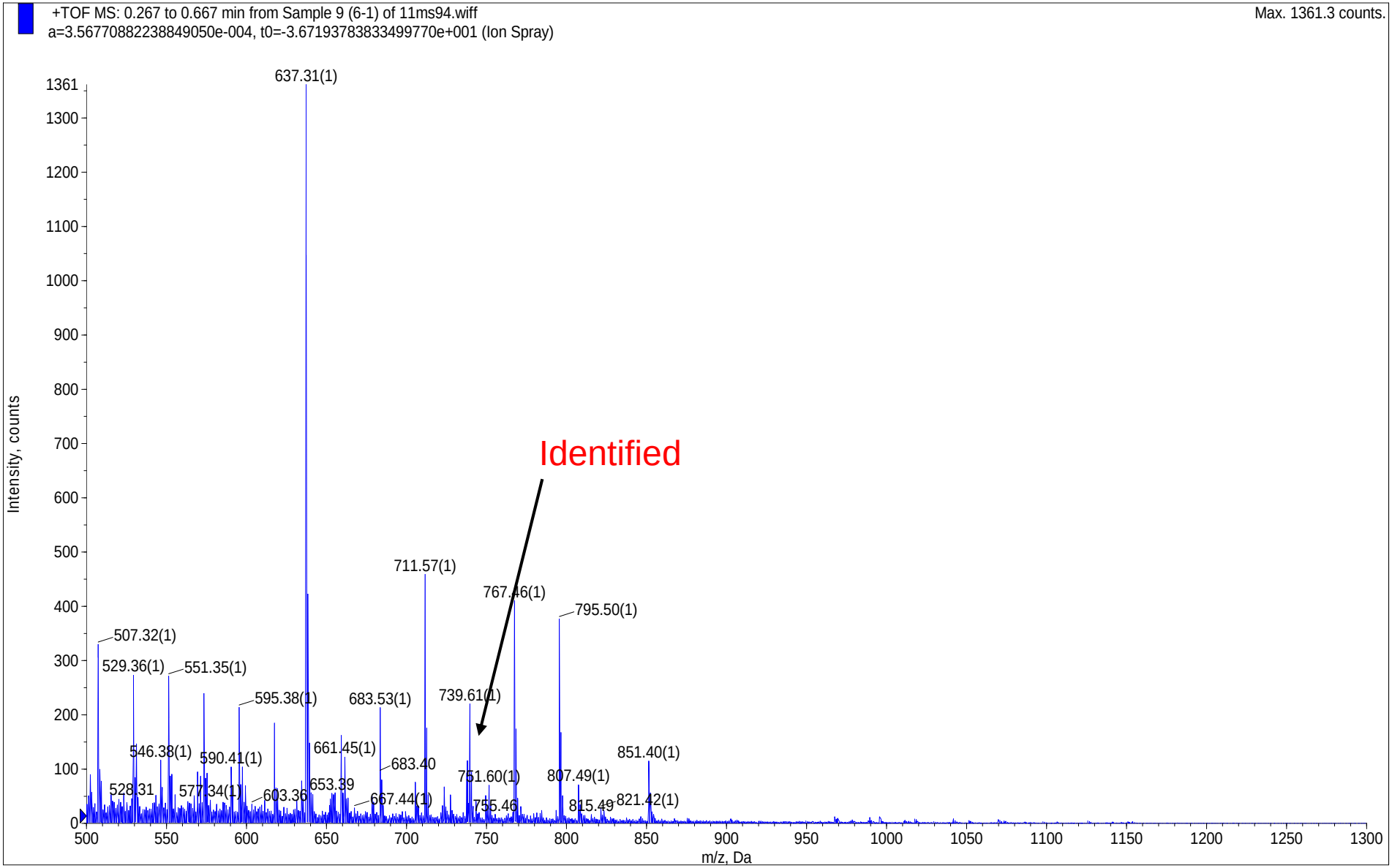
(D) MS spectrum of the band 4 in Figure 1C



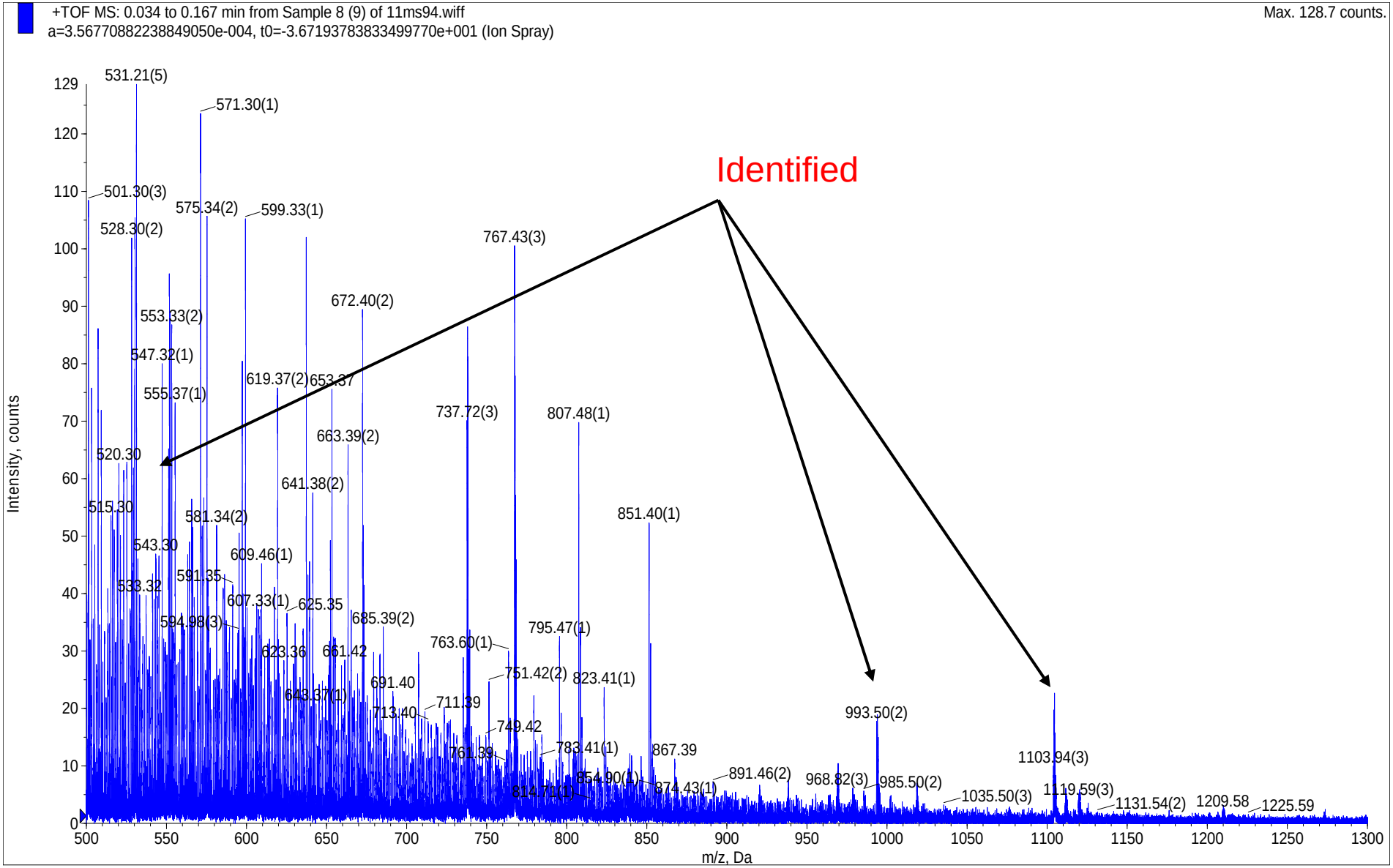
(E) MS spectrum of the band 5 in Figure 1C



(F) MS spectrum of the band 6 in Figure 1C



(G) MS spectrum of the band 9 in Figure 1C



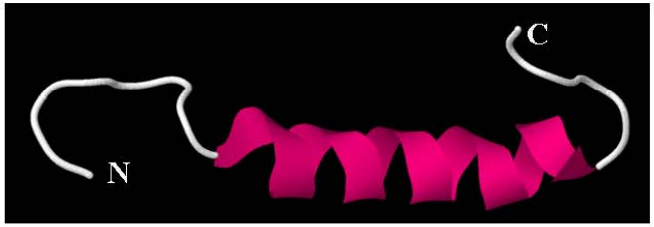
Supplementary Material 5

Structure prediction for the TRIM and PRY domains of MG53.

(A) The 3D structure of mouse sarcolipin (PDB ID: 1JDM) is presented (upper panel). Secondary structures of mouse sarcolipin (A) or the TRIM domain of mouse MG53 (B) were predicted. H (red), E (yellow), or short dash (-) represents alpha-helix, beta-strand, or random coil. Confidence level ranged from 0 to 9, low to high, respectively, and from 7 to 9 colored in green indicates very high confidence. Numbers indicate the sequence of amino acids. (C) The PRY and SPRY domains of human MG53 (PDB ID: 3kb5) is presented as a ribbon diagram with side chains. Beta-strands and connecting loops are presented by arrows or random coils. The PRY domain is colored in green. The prominent binding pocket in the PRY and SPRY complex is presented by a transparent red circle. The phosphorable serine at 306 in the PRY domain is emphasized by a space-filling model. Small red dots indicate water molecules surrounding the complex. N and C means N- and C-terminus, respectively. The 3D structures of proteins were presented using an Open Astex Viewer (3.0 by Mike Hartshorn Whilst at Astex Therapeutics, Cambridge, United Kingdom) and STRAPS (by Christoph Gille at Institut für Biochemie, Berlin, Germany).

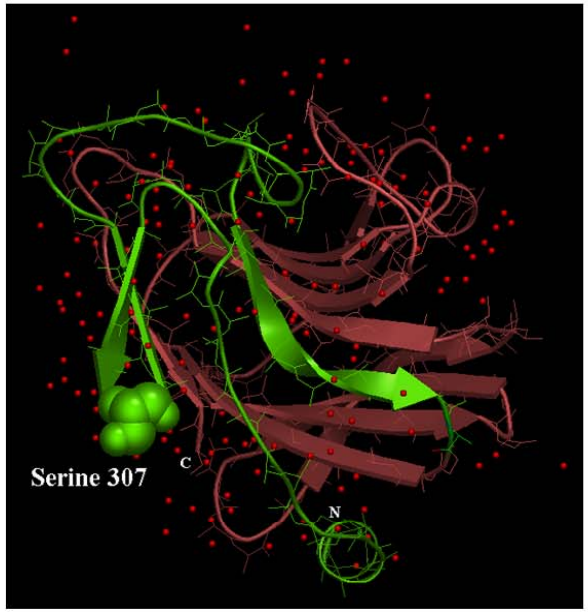
Supplementary Material 5 (continued)

A Sarcolipin (1-31 amino acids)




```
                1---------11--------21--------31
Sequence :      MERSTQELFINFTVVLITVLLMWLLVRSYQY
2nd structure : ----HHHHHHHHHHHHHHHHHHHHHH----
confidence score : 99956899999999999999999862389
```

C



B The TRIM domain of mouse MG53 (1-284 amino acids)

```
                1---------11--------21--------31--------41--------51--------61--------71--------81--------91--------
Sequence :      MSAAPGLLRQELSCPLCLQLFDAPVTAECGHSFCRACLIRVAGEPAADGTVACPCCQAPTRPQALSTNLQLSRLVEGLAQVPQGHCEEHLDPLSIYCEQD
2nd structure : -------HHHHH---H-------------------HHHHHHHHHH----------------------------------------------------E-----
confidence score : 998762004400365000002565312577766517899999000267777777776300467777777777766212000000000157770002114

101-------111-------121-------131-------141-------151-------161-------171-------181-------191-------
RTLVCGVCASLGSHRGHRLLPAAEAQARLKTQLPQQKMQLQEACMRKEKTVAVLEHQLVEVEETVRQFRGAVGEQLGKMRMFLAALESSLDREAERVRGD
--EEEEE---------------HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH
60002210123555677000014777752001138999999999999999999999998743212799999999999999999999999999999999

201-------211-------221-------231-------241-------251-------261-------271-------281--
AGVALRRELSSLNSYLEQLRQMEKVLEEVADKPQTEFLMKFCLVTSRLQKILSESPPPARLDIQLPVISDDFKFQVWKKMFRALM
HHHHHHHHHHHHHHHHHHHHHHHHHHHHHH----------HHHHHHHH--------------------------------HHHHH-
99999999999999999999999999998605578000000899900000467887776531002446763000000000000000
```

Supplementary Material 6

Sequence alignment of various proteins containing a PRY domain

Amino acids corresponding to the position of serine 306 in mouse MG53 are highlighted by a pink column. Most of the other proteins contain aspartic acid (D) in the position instead of serine (S). Numbers indicate the amino acid sequence of mouse MG53.