Supplementary Information

Crystal Structure of Cwc2 Reveals a Novel Architecture of a Multipartite RNA-binding Protein

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Supplementary materials and methods

Limited proteolysis

A stable fragment resulting from digestion with GluC was identified as Cwc2$^{1-240}$ by tryptic peptide mass fingerprinting. For preparative digestion, 40 mg of full-length Cwc2 were mixed with 0.040 mg GluC and incubated for 2 h at 4 °C. The reaction was stopped by the addition of PMSF to 1 mM and EDTA free protease inhibitors (Roche). The Cwc2$^{1-240}$ stable fragment was purified by size-exclusion chromatography on Superdex S75 (GE Healthcare) equilibrated with 10 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 2 mM DTT and 5% glycerol. The peak fractions were collected and concentrated to 9 mg/ml.

Cloning, expression and mutagenesis

The full-length *CWC2* gene and a deletion mutant coding for its RRM (Cwc2$^{124-234}$) were PCR-amplified from genomic DNA of *S. cerevisiae* (Strain W303A) cleaved with BsaI /NotI or NcoI/NotI and ligated in frame into the vector pETM11 (EMBL Protein Expression and Purification Facility) that allows expression of the fusion proteins with an N-terminal hexahistidine tag, cleavable with a tobacco etch virus (TEV) protease. The gene coding for the deletion mutant Cwc2$^{1-234}$ was cloned into pET22b (Novagen) in frame with a C-terminal 6xHis tag. Point mutations were introduced into the full-length *CWC2* gene cloned in the pETM11 by the QuickChange Site-Directed Mutagenesis strategy (Stratagene). The sequences of all primers used for PCR are available upon request. The verified constructs were transformed into *E. coli* strain Rossetta II (DE3) (Novagen). Cultures were grown in 2xYT (induced with 0.4 IPTG) or using autoinducing media (Studier, 2005), incubated at 17°C for 20 or 48 h, respectively. Cells were harvested by centrifugation, washed and the pellets stored at –80°C.

Protein purification

Purification of the full-length Cwc2 protein was performed at 4 °C. The cell pellets were resuspended by vortexing in 5 ml lysis buffer (50 mM HEPES-NaOH pH 7.5, 600 mM NaCl, 2 mM β-mercaptoethanol (β-ME), 20 mM imidazole and 10% glycerol) supplemented with a mix of EDTA-free protease inhibitors (Pefabloc, Roche) per 1 g cells and lysed with a fluidiser system at 80 p.s.i. 6 times (Microfluidics). Insoluble material was removed by centrifugation for 40 min at 10,000 rpm (Sorval SS-34 rotor). The supernatant containing His-tagged proteins was applied to a HisTrap HP FF column (GE Healthcare), pre-equilibrated with lysis buffer, using the Äkta Prime system (GE Healthcare). Unspecifically bound proteins were removed by washing with 10 column volumes (CV) lysis buffer followed by 2x CV of washing buffer (20 mM HEPES-NaOH pH 7.5, 2 M LiCl, 5% glycerol) and further by 2x CV of lysis buffer, 3x CV of 5% of elution buffer (50 mM HEPES-NaOH pH 7.5, 600 mM NaCl, 2 mM β-ME, 250 mM imidazole and 10% glycerol) followed by 2x CV of 10%, 15% and 20% of elution buffer. Bound proteins were eluted with a 20x CV gradient of 20–100% elution buffer. The buffer was exchanged to lysis buffer containing 10 mM imidazole using a desalting column 26/10 (GE Healthcare). The 6xHis tag was cleaved with TEV protease (made in house) using 1 mg protease to 100 mg His-tagged protein and incubating overnight at 4 °C. The proteins were then applied again to a NiNTA column, where the cleaved tag and protease were bound and purified proteins without tag were collected in the flow-through. Proteins were concentrated using Centricon concentrators (Millipore) and further purified by size-exclusion chromatography (GE Healthcare) using buffer containing 10 mM HEPES-NaOH pH 7.5, 100 mM NaCl, 2 mM DTT and 5% glycerol. The purified proteins were analysed by SDS-PAGE (Fig. S4). Fractions with 95% or higher purity (estimated by Coomassie Blue staining) were flash frozen in liquid nitrogen and stored at –80 °C. The concentration of the proteins was determined by the Bradford assay. Cwc2

mutants were purified from Rosetta II (DE3) cells in batch using 5 ml NiNTA beads per 10 g cells.

Crystallographic procedures

Crystallisation was carried out at 20 ºC using the sitting-drop vapour-diffusion method, by mixing 2 µl of protein solution with 1 µl reservoir solution containing 30% PEG 5000 MME (monomethyl ether), 0.1 M MES pH 6.5 and 0.2 M NH_4SO_4. Single crystals appeared within a few days. The crystals were cryoprotected by transfer into 90% reservoir solution, plus 10% glycerol and flash-frozen in a nitrogen cryostream. The best crystals diffracted to a resolution of 2.0 Å at the beamline PXII (SLS, Villigen, Switzerland). Diffraction data were processed with XDS (Kabsch).

Structure solution and refinement

Datasets complete to 2.5 Å resolution were collected with a native crystal at the peak wavelengths of the Zn K-edge. Phasing and solvent flattening were performed with Sharp (Vonrhein et al, 2007). The majority of the structure was built automatically using Arp-wArp (Perrakis et al, 1999) and the model was completed manually using COOT (Emsley & Cowtan, 2004). Refinement proceeded against a 2.4Å native dataset *via* alternating runs of REFMAC5 (Murshudov et al, 1997) and manual inspection with COOT (Table 1).

Analysis of the structure

Automatic searches for homologous proteins were conducted with the DALI server (Holm & Sander, 1995). Structural superimpositions were carried out with the SSM tool (Krissinel & Henrick, 2004) implemented in COOT. Secondary structure elements of scCwc2$^{1-227}$were extracted with PROMOTIF (Hutchinson & Thornton, 1996). Interfaces between structural

modules, including surface areas and chemical composition of surfaces, were analyzed with the PISA Server http://www.ebi.ac.uk/pdbe/prot_int/pistart.html) (Krissinel & Henrick, 2007)). All 3D structure figures were produced with PyMOL (http://www.pymol.org). Sequences were aligned with ClustalW (Chenna et al, 2003) and colored by conservation with BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html).

Electrophoretic mobility shift assay

20 µl binding reactions were assembled in a buffer containing 20 mM HEPES-NaOH, pH 7.5, 0.2 M NaCl, 2 mM $MgCl_2$, 15 µM $ZnCl_2$, 5 mM DTT, 8% glycerol, 0.1 g/l tRNA (*E. coli*), and 0.5 g/l BSA. 100 fmol of [^{32}P]-5'-labeled, *in vitro* transcribed yeast U6 snRNA was incubated for 30 min on ice with increasing amounts of recombinant Cwc2 full-length protein or fragments thereof as indicated in Figure 1C. RNA and RNA-protein complexes were resolved on a 6% (37.5:1 acrylamide:bisacrylamide) native polyacrylamide gel in 0.5x TBE buffer by running at 7W in a cold room. Visualization was performed using a Typhoon Phosphoimager (Amersham).

Supplementary Table 1

peptide	aa position	RNA	experimental mass		calculated mass		
			m/z	m	crosslink	peptide	RNA
FVSPFALQPQLHSGK	F47-K61	U –H_2O	981.4640	1960.9124	1960.9083	1654.8830	306.0253
CEYLHHIPDEEDIGK	C87-K101	AU + 152	868.3060	2601.8946	2601.8923	1796.8039	805.0884
FADYREDMGGIGSFR	F117-R131	U	1022.9128	2043.8100	2043.8033	1719.7674	324.0359
TLYVGGIDGALNSK	T136-K149	AAU	797.3030	2388.8856	2388.8814	1406.7405	982.1409
HLKPAQIESR	H150-R159	U –H_2O	495.5687	1483.6827	1483.6820	1177.6567	306.0253
NCGFVK	N180-K185	AU + 152	736.7095	1471.4034	1471.4043	666.3159	805.0884

Supplementary Table 1: Experimental and calculated masses of Cwc2 peptides found to be crosslinked to U6 snRNA and shown in Supplementary figures 1-6. Cwc2 bound to U6 snRNA was UV irradiated at 254 nm and crosslinked peptide-oligonucleotide heteroconjugates were

purified and analyzed by LC-MSMS as described (Kramer et al, 2011). The crosslinked RNA moiety was calculated from the experimental precursor mass (mass of the peptide-RNA oligonucleotide) minus the calculated mass of the crosslinked peptide moiety as identified by tandem MS (MSMS, see Supplementary figures 1-6). Tandem MS also revealed the crosslinked amino acid (see Supplementary figures 1-6). Ox = Oxidation of Methionine.

Supplementary Table 2

		crosslinked RNA	U6				U4			ISL
		irradiation time (min)	10			2	10		2	10
domain	**aa position**	**peptide sequence** / **RNA sequence**	a	b	c	d	e	f	g	h
Torus	W37-K61	WSQGFAGNTRF[47]VSPFALQPQLHSGK	UU	+	(+)	(+)	-	-	-	-
			AUU	+	-	-	-	-	-	-	-
	F47-K61	F[47]VSPFALQPQLHSGK	U –H_2O	+	+	+	+	-	(+)	(+)	-
			U	+	+	-	(+)	(+)	-	-	-
			UU	-	+	+	(+)	(+)	(+)	-	-
zinc finger	G79-K101	GM(Ox)CCLGPKC[87]EYLHHIPDEEDIGK	U + 152 – H_2O	+	-	-	-	-	-	-	(+)
	C87-K101	C[87]EYLHHIPDEEDIGK	U	-	-	+	(+)	-	(+)	(+)	-
			U + 152	+	+	+	+	(+)	+	(+)	-
			AU + 152	+	(+)	+	+	(+)	(+)	-	(+)
			AAU + 152	+	-	-	-	-	-	-	-
			GU + 152	+	(+)	(+)	(+)	(+)	(+)	(+)	-
ce	F117-R131	FADY[120]REDMGGIGSFR	U	+	+	+	+	+	(+)	(+)	(+)
			AU	(+)	(+)	+	(+)	(+)	(+)	(+)	(+)
RRM/RNP2	T136-K149	TLY[138]VGGIDGALNSK	U	+	+	+	+	+	+	+	+
			AU	+	+	+	+	+	+	+	+
			AAU	+	+	+	+	+	(+)	+	(+)
			AUU	(+)	(+)	+	(+)	-	-	(+)	-
			GU	+	+	+	+	+	+	+	+
			GGU	-	-	+	(+)	-	-	-	-
			AGU	-	(+)	+	(+)	(+)	(+)	(+)	+
			UU	(+)	+	+	+	+	(+)	+	+
			CU	(+)	(+)	+	+	-	-	+	+
			ACU	(+)	(+)	+	(+)	(+)	(+)	(+)	-
			CGU	-	+	(+)	(+)	(+)	(+)	(+)	-
RRM	H150-R159	HLK[152]PAQIESR	U –H_2O	+	(+)	+	(+)	-	(+)	(+)	+
			AU –H_2O	+	-	-	-	-	-	-	-
RRM/RNP1	N180-K185	NC[181]GFVK	U	+	+	+	+	+	+	+	+
			U + 152	+	+	+	+	+	+	+	(+)
			AU	+	+	+	+	+	(+)	+	+
			AU + 152	+	+	+	+	+	+	+	+
			AAU + 152	+	+	+	+	-	+	(+)	-
			AUU + 152	(+)	+	+	+	(+)	(+)	(+)	-
			GU	-	+	(+)	(+)	+	(+)	(+)	-
			GU + 152	+	+	+	+	+	+	+	(+)
			AGU + 152	(+)	-	+	+	-	(+)	(+)	(+)
			UU	-	+	(+)	(+)	(+)	(+)	+	-
			UU + 152	+	+	+	+	+	+	+	(+)

Supplementary Table 2: Overview of identified crosslinks of Cwc2 to U6 snRNA (columns a-d), U4 snRNA (columns e-g), and U6 internal stem loop (ISL, column h). The respective complex was UV irradiated for 10 min (columns a, b, c, e, f, h) or 2 min (columns d, g). 10 min UV irradiation of Cwc2 and

U6 snRNA was done in three replicates (columns a-c), Cwc2 and U4 snRNA in two replicates (columns e,f). Of note, the crosslinks described in this study are reproducibly identified after 2 and 10 min of UV irradiation. The protein domain, amino acid (aa) position, the peptide and RNA sequence for each identified crosslink are listed. The crosslinked aa is highlighted in red. Crosslinks identified and validated by a MSMS fragment spectrum are highlighted in green. (+) highlighted in yellow indicate that a species with the respective precursor mass was detected in the MS analysis only, but was not selected for MSMS fragmentation owing to the low intensity of the MS peak. Thus, the species cannot be unambiguously identified because of its lower abundance.

Supplementary figure legends

S1 SDS-PAGE analysis of limited proteolysis of the full-length Cwc2 with the protease GluC. The stable fragment corresponds to the Cwc2 folding unit (residues 1-240, right).

S2 Crystal contacts between the connector (orange) Cwc2 molecule and the RRM domain (green) of the symmetry related molecule. The residues are shown as sticks and labeled.

S3 Cwc2 surface charge distribution. The molecule is rotated 90° about the y axis compared to the orientation shown in Figure 2A. The residues cross-linked to RNA are outlined with dashed lines and labeled.

S4 SDS-PAGE analysis of purified recombinant full-length and deletion mutants of Cwc2, and point mutants thereof used for EMSA and for *in vitro* splicing complementation assays.

S5 (A-C) Gel filtration analysis of Cwc2-U6 snRNA interactions. (A) Cwc2 migrates as a homodimer. (B) Complex of Cwc2 and U6 snRNA elutes as one defined peak (C) Elution of U6 snRNA. SDS PAGE (15%) analysis of eluted fractions (indicated at the top). The gels were stained with silver. The protein, U6 snRNA, and the complexes are denoted on the right.

Numbers on the left indicate the molecular mass of protein standards in kilodaltons. (Right) Elution profiles. Elution volumes are given at the bottom. Molecular weight standards are denoted at the top of the Cwc2 elution profile. Fractions analyzed by SDS PAGE are indicated at the bottom and by the defined areas. Size exclusion chromatography using Superdex S200 (10/300) column was performed in buffer containing 20 mM HEPES-NaOH pH7.5, 100 mM NaCl and 2 mM DTT. U6 snRNA was denaturated at 65°C for 3 min and chilled on ice. Full-length Cwc2 and U6 snRNA were mixed in a molar ratio of 10:1 in the running buffer, centrifuged, incubated for 30 min at 4°C and applied to the column.

S6 Electrophoretic mobility shift analysis of the interaction of recombinant Cwc2 mutants with yeast U6 snRNA. Description as in Figure 1C. The amino acids mutated are shown above the gel.

S7 Product ion spectrum (fragment spectrum) of CWC2 peptide FVSPFALQPQLHSGK (F47-K61) cross-linked to U with loss of H_2O. y and b-type fragment ions that unambiguously reveal the sequence of the cross-linked peptide are assigned. The a2- and b2- ions show a mass shift of 94 a.m.u. that is indicative of cross-linked U $-H_2O$ (Kramer et al, 2011) and importantly, the immonium ion (IM) of F also exhibits an additional mass of 94 a.m.u., thus strongly suggesting that F47 is the actual cross-linked amino acid (all 94 Da mass shifts in red). Identified peptide fragments, peptide sequence and cross-linked nucleotides are indicated at the upper right. The insert shows the precursor ion spectrum. Asterisk denotes neutral loss of ammonium. a.m.u. = atomic mass units.

S8 Product ion spectrum (fragment spectrum) of CWC2 peptide CEYLHHIPDEEDIGK (C87-K101) cross-linked to AU with an additional mass of 152. y-type fragment ions that unambiguously reveal the sequence of the cross-linked peptide are assigned. *m/z* regime from 137 on is magnified five-fold. The additional mass of 152 is indicative of cysteine being the actual

cross-linked amino acid. Marker ion for A in the cross-linked RNA moiety is shown in green and A' is the nucleic acid base. Identified peptide fragments, peptide sequence and cross-linked RNA moiety are indicated above the spectrum. The insert shows the precursor ion spectrum.

S9 Product ion spectrum (fragment spectrum) of CWC2 peptide FADYREDMGGIGSFR (F117-R131) cross-linked to U. y and b-type fragment ions that unambiguously reveal the sequence of the cross-linked peptide are assigned. The y12 ion (in red) - but not the y10 to y1 ions - shows the additional mass of U, thus demonstrating that Y120 or R121 is the cross-linked amino acid. Peptide sequence with identified fragments and cross-linked nucleotide as well as an insert with the precursor ion spectrum are given in the upper left. IM: immonium ion; asterisk: neutral loss of ammonia, ^{0}: neutral loss of water.

S10 Product ion spectrum (fragment spectrum) of CWC2 peptide TLYVGGIDGALNSK (T136-K149) cross-linked to AUU. y and b-type fragment ions that unambiguously reveal the sequence of the cross-linked peptide are assigned. Of note, sequence information is only obtained up to b2 and y11. b3 and importantly y12 fragment ions cannot be observed, which strongly suggests Y138 as the cross-linked amino acid. Marker ions for A in the cross-linked RNA moiety are shown in green, A' denotes the nucleic acid base. Peptide sequence with identified fragments and cross-linked nucleotides are given above the spectrum, the insert on the right shows the precursor ion spectrum.

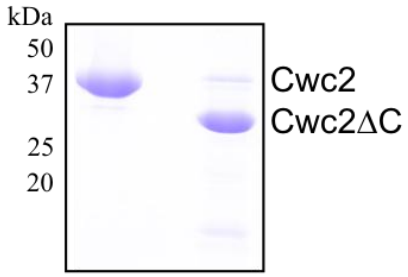
S11 Product ion spectrum (fragment spectrum) of CWC2 peptide HLKPAQIESR (H150-R159) cross-linked to U via a loss of H_2O. y and b-type fragment ions that unambiguously reveal the sequence of the cross-linked peptide are assigned. Of note, the a3, b3, a5, b5, a6, b6 and the y8 ion in the fragment spectrum show a mass shift of 94 a.m.u. (in red) that demonstrates that K152 is cross-linked to U (Kramer et al, 2011). Peptide sequence with assigned fragments and cross-

linked RNA moiety, as well as an insert with the precursor mass spectrum are shown in the upper right corner. IM: immonium ion.

S12 Product ion spectrum (fragment spectrum) of CWC2 peptide NCGFVK (N180-K185) cross-linked to an AU dinucleotide plus 152. y-type fragment ions that unambiguously reveal the sequence of the cross-linked peptide are assigned. *m/z* regime above 140 is magnified five-fold. Marker ions for the cross-linked RNA moiety are shown in green, A' being the nucleic acid base. The additional mass of 152 is indicative of cysteine being the actual cross-linked amino acid. Peptide sequence with identified y-ions and cross-linked RNA and an insert with the precursor mass spectrum are shown in the upper right.

S13 The close-up view highlighting inter-domain contacts mediated by conserved residues from the RRM, ZnF and the Torus domain (left). Equivalent contact residues from the RRM domain of Rbm22 (right, pdb 2YTC).

S1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25
20
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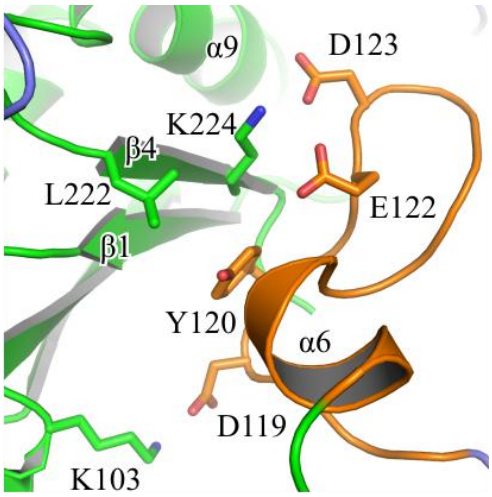
Cwc2ΔC

S2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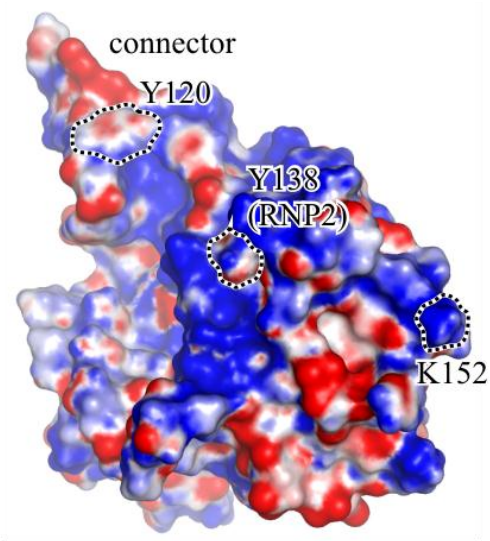
S3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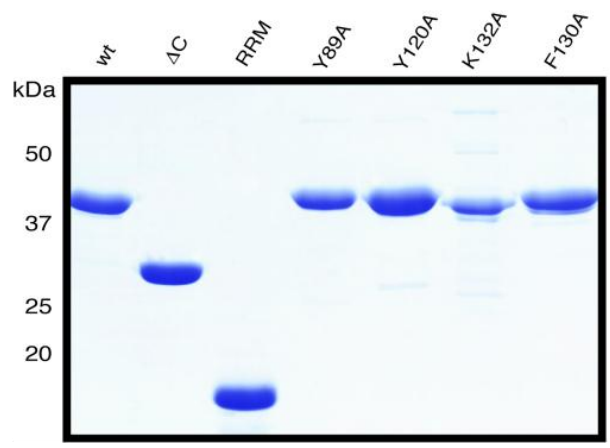
S4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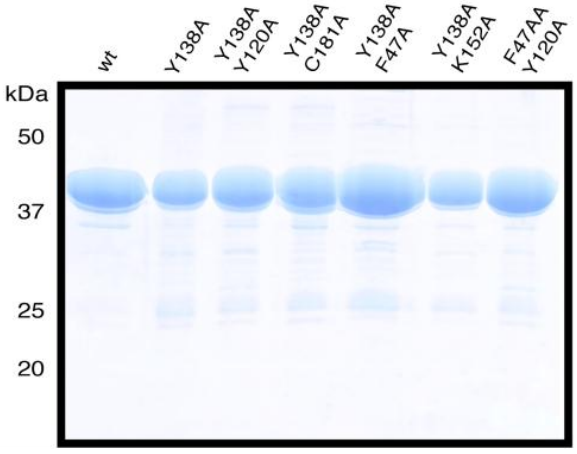
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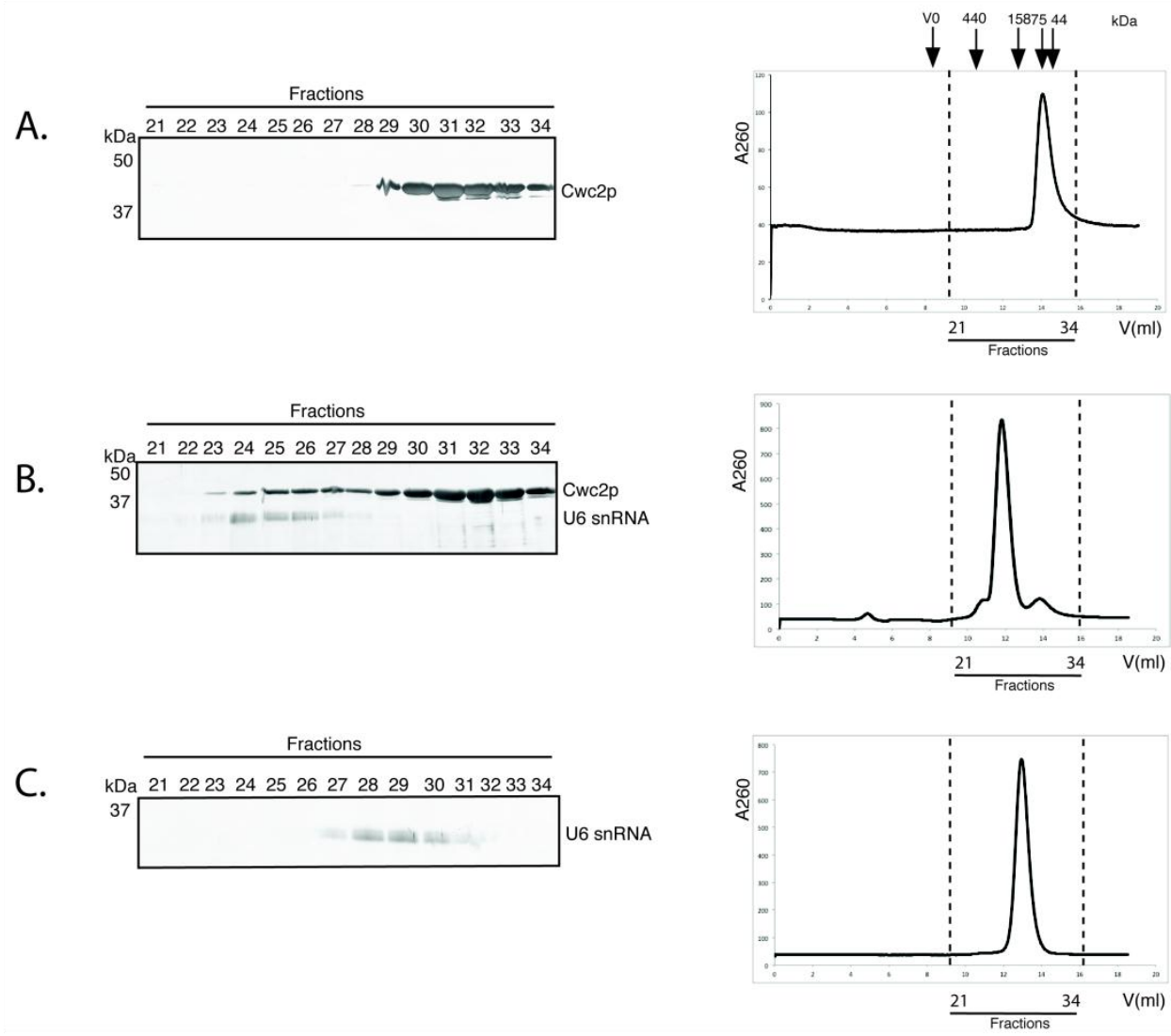
20

S5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21
34
V(ml)
Fractions
B.
Fractions
kDa
21 22 23 24 25 26 27 28 29 30 31 32 33 34
50
37
Cwc2p
U6 snRNA
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21
34
V(ml)
Fractions
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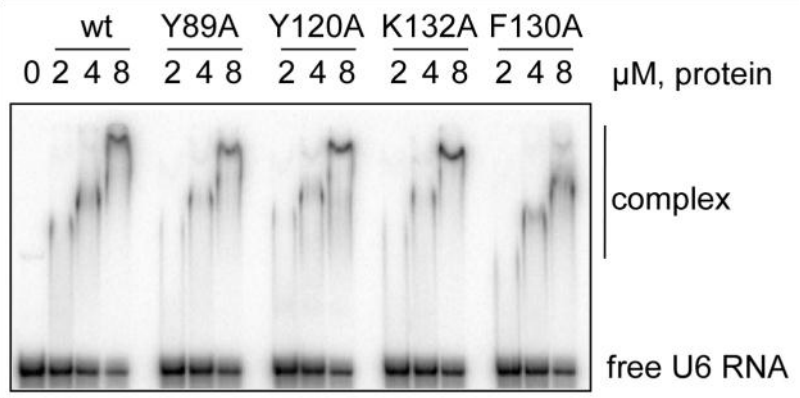
S6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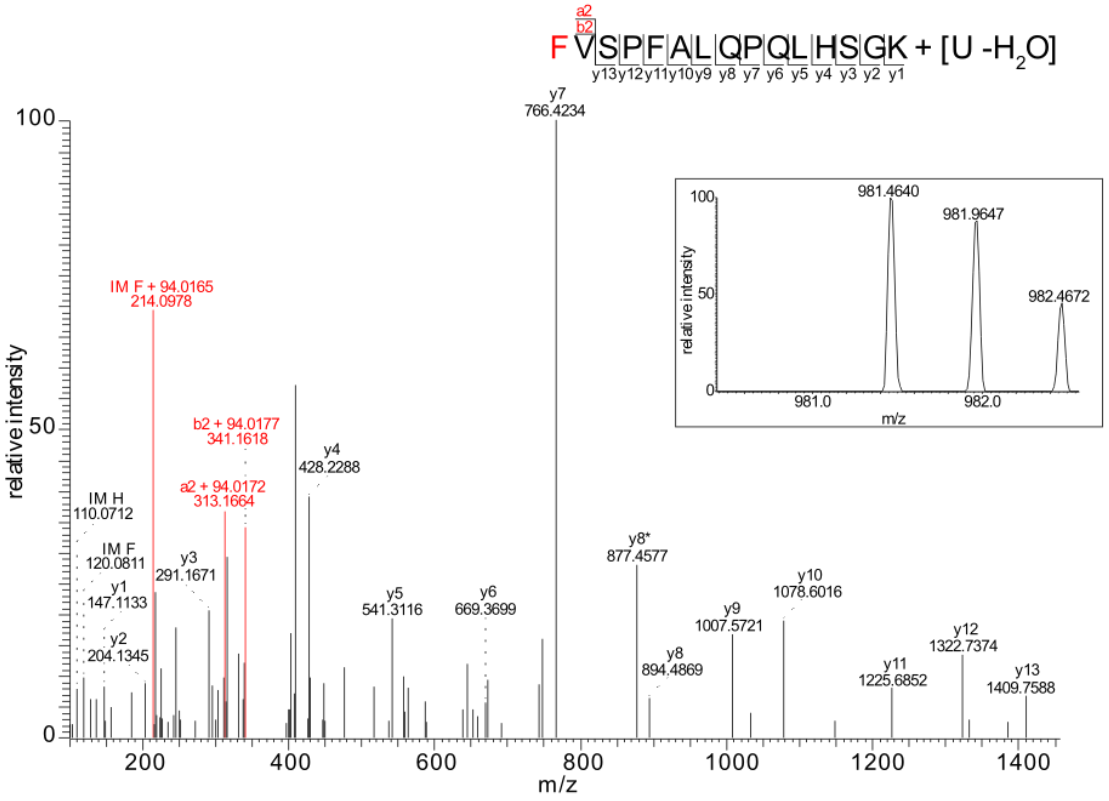
S7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b2 + 94.0177
341.1618
a2 + 94.0172
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y4
428.2288
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IM F
120.0811
y3
291.1671
y1
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y2
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y5
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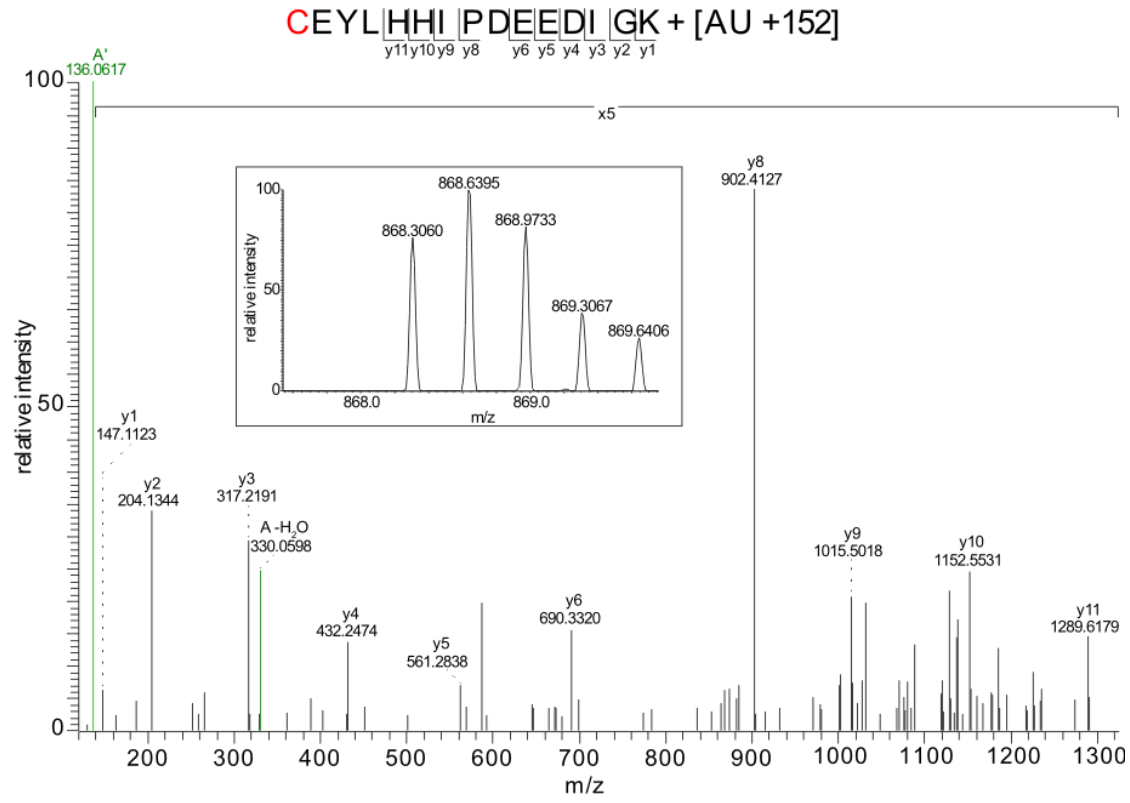
S8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y2
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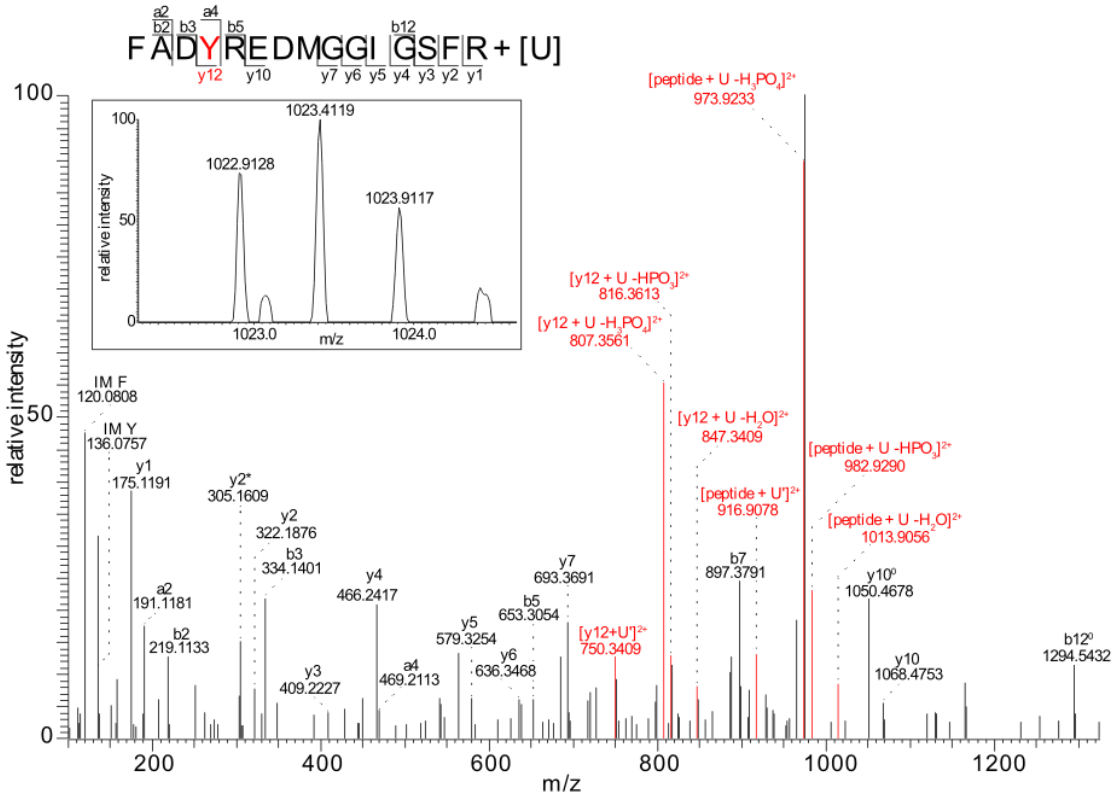
m/z

S9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y10
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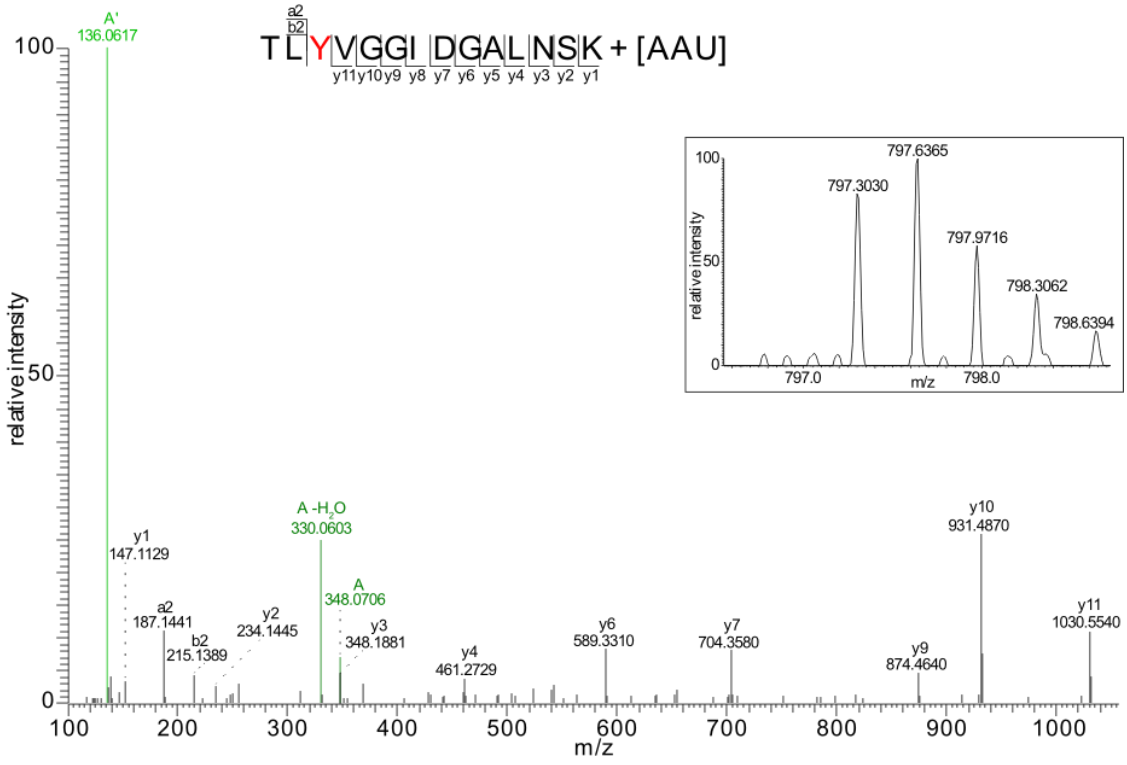
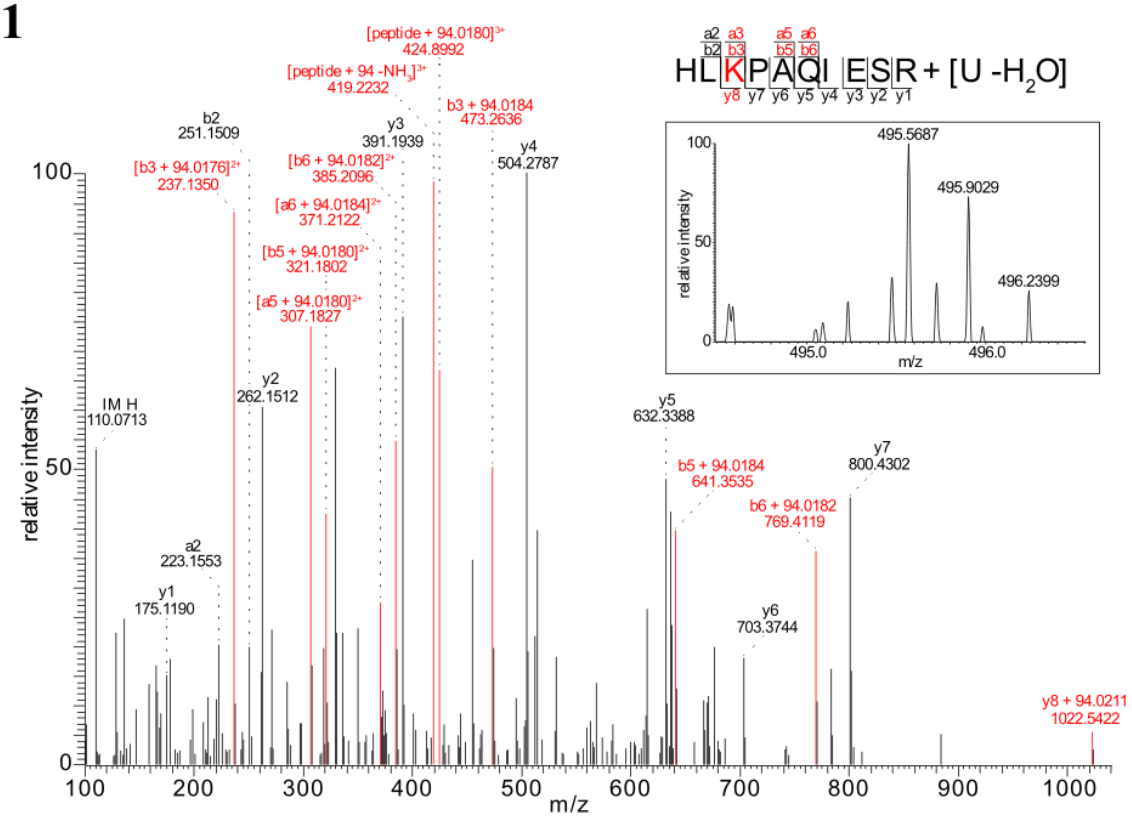
b12°
1294.5432

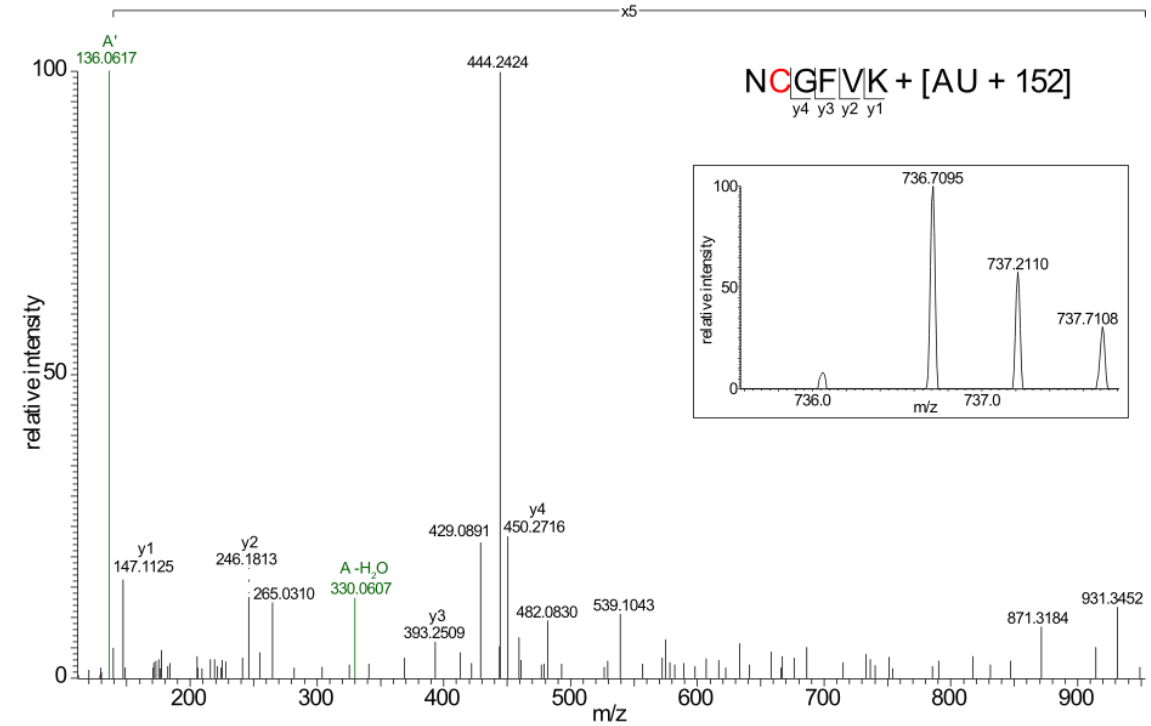
S10


TLYVGGIDGALNSK + [AAU]
relative intensity
m/z
A'
136.0617
797.6365
797.3030
797.9716
798.3062
798.6394
A -H2O
330.0603
A
348.0706
y1
147.1129
a2
187.1441
b2
215.1389
y2
234.1445
y3
348.1881
y4
461.2729
y6
589.3310
y7
704.3580
y9
874.4640
y10
931.4870
y11
1030.5540

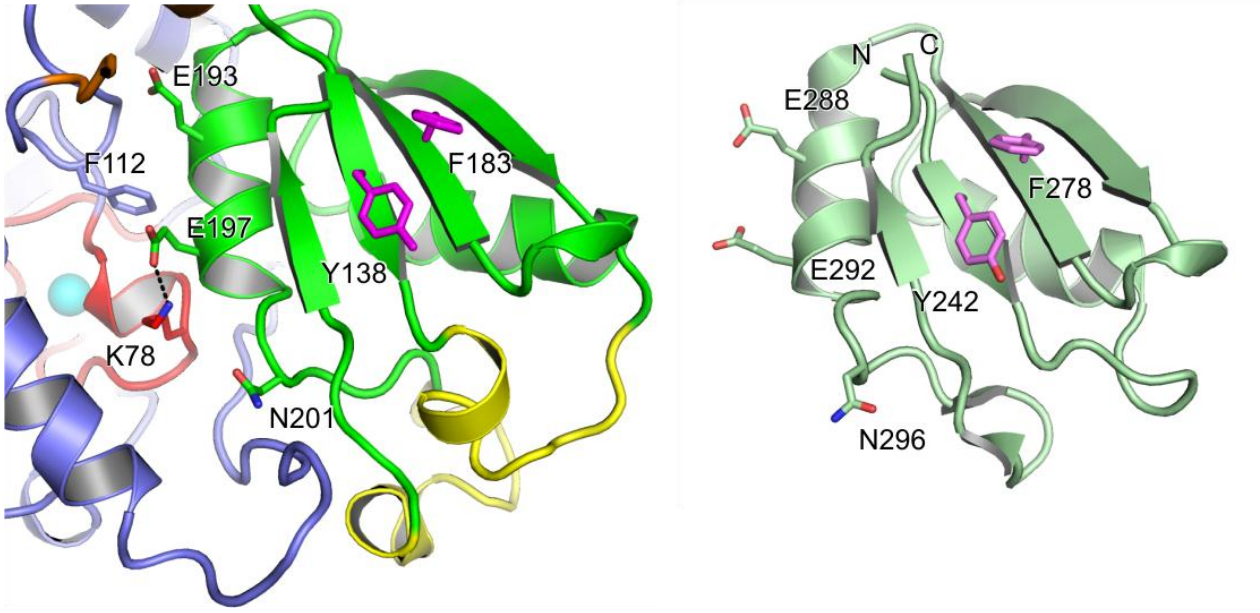
S11


HLKPAQIESR + [U -H2O]
a2 a3 a5 a6
b2 b3 b5 b6
y8 y7 y6 y5 y4 y3 y2 y1
[peptide + 94.0180]3+ 424.8992
[peptide + 94 -NH3]3+ 419.2232
b3 + 94.0184 473.2636
b2 251.1509
y3 391.1939
y4 504.2787
[b3 + 94.0176]2+ 237.1350
[b6 + 94.0182]2+ 385.2096
[a6 + 94.0184]2+ 371.2122
[b5 + 94.0180]2+ 321.1802
[a5 + 94.0180]2+ 307.1827
y2 262.1512
IM H 110.0713
y5 632.3388
y7 800.4302
b5 + 94.0184 641.3535
b6 + 94.0182 769.4119
a2 223.1553
y1 175.1190
y6 703.3744
y8 + 94.0211 1022.5422
relative intensity
m/z
495.5687
495.9029
496.2399

S12


x5
NCGFVK + [AU + 152]
y4 y3 y2 y1
A' 136.0617
444.2424
y1 147.1125
y2 246.1813
265.0310
A -H2O 330.0607
429.0891
y4 450.2716
y3 393.2509
482.0830
539.1043
871.3184
931.3452
736.7095
737.2110
737.7108
relative intensity
m/z

S13



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