Supporting Information

G protein-coupled receptor kinase 2 (GRK2) and 5 (GRK5) exhibit selective phosphorylation of the neurotensin receptor *in vitro*

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Methods

Preparation of MSP1E3D1. The expression plasmid pMSP1E3D1 (#20066, Addgene, MA, USA) is a derivative of pET28a that codes for the extended MSP1D1: a deletion mutant (Δ1-54) of human apolipoprotein A-I that contains an insertion of three extra 22-mer amphipathic helices after Gln122. The protein has an N-terminal heptahistidine (H7) tag, a spacer sequence and a TEV protease recognition site (H7-MSP1E3D1)[1]. Expression was carried out as described[2], with modifications. BL21Gold(DE3) cells (Stratagene, CA, USA) harboring pMSP1E3D1 were grown at 37 °C to an OD_{600} of 4.0 in double strength YT medium containing 50 μg/ml kanamycin. Following induction with 1 mM isopropyl-β-D-thiogalactopyranoside, the temperature was decreased to 28 °C. The cells were harvested 4 hr later, frozen in liquid nitrogen and stored at –80 °C until further use.

The standard MSP purification method incorporates Triton X-100 and cholate to help improve its solubility[3], but because Triton X-100 denatures the neurotensin receptor 1 (NTSR1)[4], we purified MSP in the absence of any detergent[5]. Briefly, cells (20 g) were resuspended in 50 ml of buffer A (50 mM Tris, pH 7.4, 200 mM NaCl), and then passed twice through a French Press. Cell debris was removed by centrifugation at 55,000 rpm for 45 min and 4 °C in a Type 70 Ti rotor on a Beckman Coulter Optima L-90K Ultracentrifuge, and then imidazole was added to the supernatant to a final concentration of 25 mM. The sample was passed through a 0.2 μm filter (Stericup, Millipore, CA, USA) and loaded onto a Ni-NTA column (8 ml bed volume, Qiagen, CA, USA), equilibrated with buffer B (buffer A containing 25 mM imidazole). The resin was washed with buffer B and H7-MSP1E3D1 was eluted with buffer C (buffer A containing 280 mM imidazole).

Fractions containing H7-MSP1E3D1 were identified by SDS-PAGE (NuPAGE 4-12% Bis-Tris gel, 1xMES running buffer, Invitrogen, CA, USA) and a NanoDrop 1000 spectrophotometer (version 3.6.0, Thermo Scientific). Pooled fractions containing H7-MSP1E3D1 were concentrated to 9 mg/ml using a Centriprep Centrifugal Filter Unit with Ultracel-10 membrane (Millipore) and the imidazole was removed by passage over a PD10 column (GE Healthcare) equilibrated with buffer A. Purified H7-MSP1E3D1 (~7.0 mg/ml) was frozen in liquid nitrogen and stored at –80 °C until further use. The protein concentration was determined by measuring the absorbance at 280 nm using a calculated extinction coefficient of 29,910 $M^{-1}cm^{-1}$ and a calculated molar mass of 32,731 Da (http://web.expasy.org/protparam/). The protein content was also determined by the method of Schaffner and Weissmann with bovine serum albumin as the standard[6].

Prior to reconstitution, the H7-tag was removed by incubation with TEV protease [150:1 (mol/mol) H7-MSP1E3D1/TEV protease, in the absence of dithiothreitol (DTT)] at room temperature for 3 hr and subsequent incubation with Talon resin (1 ml resin per 5 mg of H7-MSP1E3D1, Clontech Laboratories) equilibrated with buffer D (buffer A containing 20 mM imidazole) for 1 hr at 4 °C. MSP1E3D1 was recovered from the flow-through and the subsequent wash with buffer D, concentrated in the presence of 2.5 mM cholate to 20-25 mg/ml using an Amicon Ultra-0.5 Ultracel-30 concentrator (Millipore) and used for reconstitution. The protein concentration was determined by measuring the absorbance at 280 nm using a calculated extinction coefficient of 26,930 $M^{-1}cm^{-1}$ and a calculated molar mass of 29,982 Da.

NTSR1$_f$ mutant preparation. NTSR1$_f$ mutants were created by PCR-based site-directed mutagenesis using the NTSR1$_f$ WT *E. coli* expression plasmid as a template and following the QuikChange Site-Directed Mutagenesis strategy (Agilent Technologies, CA, USA). Individual clones were fully sequenced in the NTSR1 coding region to ensure that only the desired mutations were introduced. DNA sequencing was performed by DNA sequencing facilities (National Institute of Neurological Disorders and Stroke, National Institutes of Health, and Macrogen Corp., MD, USA). The expression, purification, and nanodisc reconstitution of NTSR1$_f$ mutants were conducted in a manner similar to that for NTSR1$_f$ WT[2].

Reconstitution and purification of NTSR1$_f$-nanodiscs. Reconstitution was conducted at 4 °C or on ice to preserve NTSR1 activity, requiring the use of POPC and POPG, both of which have phase transition temperatures below 0 °C. A detergent solubilized lipid solution was prepared as follows: chloroform dissolved phospholipid was dried in a vacuum desiccator overnight, protected from light. Dried phospholipids were solubilized in buffer A containing 50 mM cholate (the molar ratio of phospholipid to cholate was 3:1), sonicated for 20-30 min at 4 °C and then stored on ice. The phospholipid/cholate solution was freshly prepared for each reconstitution experiment.

The reconstitutions of NTSR1-nanodiscs were performed as described[5] with modifications. Purified NTSR1$_f$ was concentrated to ~ 200 μl with an Amicon Ultra-0.5 mL Centrifugal Filter (Millipore) on Microcentrifuge 5417R (Eppendorf, NY, USA) at 10,000 rpm and 4 °C. The phospholipid/cholate solution, ice-cold buffer A and concentrated NTSR1$_f$ were combined, and then concentrated MSP1E3D1 was added such that the final concentrations of

the components were: 9.5 mM lipids, 3-4 μM $NTSR1_f$, and 100 μM MSP1E3D1 in a total volume of 1 ml. The mixture was incubated for 1 hr at 4°C and the detergent was removed by addition of Bio-Beads (50-fold the weight in detergent). After mixing gently for 3.5 hr at 4°C, the Bio-Beads were removed by centrifugation on Microcentrifuge 5417R at 14,000 rpm and 4 °C for 3 min. This procedure generated a mixture of $NTSR1_f$-nanodiscs and nanodiscs devoid of receptor. To enrich for the former, the mixture was incubated in batch mode for 1 hr at 4°C with 0.3 ml Talon resin (Clontech Laboratories, CA, USA), equilibrated with buffer D, transferred into a small column and washed with 4 bed volumes of buffer D. $NTSR1_f$-nanodiscs were eluted from the resin in 0.3 ml steps using buffer E (buffer A with 200 mM imidazole). The fractions were analyzed for protein by measuring the absorbance at 280 nm with a NanoDrop 1000 spectrophotometer using a calculated extinction coefficient of 192,520 $M^{-1}cm^{-1}$ and a calculated combined molar mass of 163 kDa, assuming the presence of 2 MSP1E3D1 and 1 $NTSR1_f$ per nanodisc. The protein content was also determined by the method of Schaffner and Weissmann with bovine serum albumin as the standard[6]. The fraction with the highest $NTSR1_f$-nanodisc concentration was used for further experiments. These $NTSR1_f$-nanodiscs were characterized by dynamic light scattering (data not shown) and sedimentation velocity analytical ultracentrifugation. Prior to their pharmacological characterization, the $NTSR1_f$-nanodiscs were treated with a stoichiometric amount of TEV protease for 2 hr on ice to remove the N-terminal maltose-binding protein and C-terminal *E. coli* thioredoxin-decahistidine tail. The completeness of the digest was confirmed by SDS-PAGE.

For the preparation of empty nanodiscs, the respective lipid/cholate solution, concentrated MSP1E3D1, and buffer A were mixed to a final concentration of 12 mM lipid and 100 μM MSP1E3D1 in a volume of 1 ml. The mixture was incubated for 1 hr at 4 °C and Bio-Beads (50-fold of the weight of cholate present in the mixture) were added for detergent removal. After mixing for 3.5 hr at 4 °C, the Bio-Beads were removed by centrifugation on Microcentrifuge 5417R at 14,000 rpm and 4 °C for 3 min.

Sedimentation velocity analytical ultracentrifugation. Sedimentation velocity experiments were conducted at 10 °C and 40,000 rpm in a Beckman Coulter An50 Ti rotor on a Beckman Coulter ProteomeLab XL-I analytical ultracentrifuge, as described[2]. 400 μl of each nanodisc sample were loaded in 2-channel centerpiece cells and scans were collected using both the absorbance (280 nm) and Rayleigh interference optical detection systems. Empty nanodiscs were studied at loading A_{280} of 1.0 – 1.2, whereas receptor nanodiscs were studied at loading A_{280} of 0.4 – 0.6. Absorbance and interference data were time-corrected [7] and analyzed in SEDFIT 14.4f[8] in terms a continuous c(*s*) distribution of Lamm equation solutions with a resolution of 0.05 S (empty nanodisc) or 0.1 S (receptor nanodisc) and a confidence level of 0.68. In all cases, excellent fits were obtained with absorbance and interference r.m.s.d. values ranging from 0.0041 – 0.0091 A_{280} and 0.0050 – 0.014 fringes. Solution densities ρ and viscosities η were measured on a Mettler Toledo DE51 density meter and Anton Paar AMVn rolling ball viscometer at 20.00 °C and corrected to 10 °C. Protein partial specific volumes were calculated in SEDNTERP 1.09[9] at 10.0 °C and 20.0 °C. Partial specific volumes of 0.981 and 0.968 cm^3g^{-1} were used for POPC and POPG at 10.0 °C, respectively[2]. Protein extinction coefficients at 280 nm (ε_{280})

were calculated in SEDNTERP 1.09, whereas the interference signal increments (ε_J) were calculated as described[2]. Sedimentation coefficients *s* were corrected to $s_{20,w}$ using partial specific volumes based on the expected stoichiometries of 2:240 MSP1E3D1:lipid and 2:1:190 MSP1E3D1:$NTSR1_f$:lipid for the empty and receptor nanodiscs, respectively.

NTSR1 phosphorylation by GRK2 in the presence of $G\beta_1\gamma_1$. NTSR1 in 75% POPC/25% POPG nanodisc was phosphorylated in 20 mM Tris, pH 7.4, 2 mM $MgCl_2$, 0.4 mM DTT, 40 mM NaCl and 40 mM imidazole (final concentration) for 10 min at 30 °C. Analytical reactions were carried out using 95 nM receptor, 310 nM of G protein-coupled receptor kinase 2 (GRK2) with various ratios of $G\beta_1\gamma_1$ in 20 µl with [γ-^{32}P]ATP (final specific activity ~500 cpm/pmol) in the presence of NTS (60 nM), stopped by the addition of 7 µl of NuPage 4xLDS Sample buffer (Invitrogen), and resolved on SDS-PAGE (NuPAGE 4-12% Bis-Tris gel, 1xMES running buffer, Invitrogen). Gels were stained with SimplyBlue™ SafeStain (Invitrogen), dried, and exposed to X-ray film (Kodak® Bio® XAR® Film, Kodak, NY, USA) for 14-17 hr. NTSR1 bands were then excised, and the radioactivity was quantified in a liquid scintillation counter. Means ± S.D. (error bars) from three experiments performed in duplicate are shown.

Tables and Figures

Table S1. Characterization of empty-nanodisc complexes by sedimentation velocity

Lipid compositions[a]	$s_{20,w}$ (S)	M_{exp} (kDa)	% load, c (μM)[b]	f/f_o[c]	Lipid[d] ($\varepsilon_J/\varepsilon_{280}$)	R_h[e] (nm)
POPC (3)	3.19 ± 0.02	287 ± 20	93 (17)	1.26	217	5.0
25% POPG (4)	3.48 ± 0.07	281 ± 22	92 (17)	1.18	190	4.8

[a] Experimental sedimentation coefficients and molar masses represent average values for the major species observed in the c(*s*) distribution. Averages are obtained from independent experiments (numbers shown in parentheses) and are based on both the absorbance and interference data.

[b] Percent of the loading absorbance that represents the major species of interest. The corresponding concentration of this species is indicated in parentheses.

[c] Best-fit frictional ratios from the continuous c(*s*) distribution in SEDFIT.

[d] Lipid stoichiometries per single nanodisc based on the presence of 2 MSP1E3D1 molecules. Data are based on signal contributions of the major species to the absorbance (protein alone) and interference (protein and lipid) data.

Table S2. Summary of phosphorylated sites on NTSR1 analyzed by mass spectrometry.

Phosphorylation Sites[a]	Obs m/z	Peptide[b]	Ascore 1[c]	Ascore 2[c]	Ascore 3[c]
WT, GRK2					
S415	931.7193	R.KPNSM*SSNHAFS#TSAT#RENLYFQ.-	22.1	14.9	
S407	958.3752	R.KPNS#M*SSNHAFS#TS#ATRENLYFQ.-	24.1	0.0	
WT, GRK5					
T279, T282	911.7137	R.VCT#VGT#HNGLEHSTFNM*TIEPGR.V	23.0	22.2	0.0
T290	933.0376	R.VCT#VGT#HNGLEHST#FNMTIEPGR.V	9.4	11.4	31.5
T294	712.8350	F.NM*T#IEPGRVQAL.R	1000.0	0.0	0.0
S402	422.1971	K.RPTFS#R.K	18.9	0.0	0.0
S407, T416, S417	958.3750	R.KPNS#M*SSNHAFST#S#ATRENLYFQ.-	33.5	20.6	24.1
S409	926.3892	R.KPNS#MS#SNHAFSTSATRENLYFQ.-	0.0	15.0	0.0
S410	958.3753	R.KPNSM*SS#NHAFS#TSAT#RENLYFQ.-	22.2	7.0	7.9
S410, **S415**, **T416**	693.5873	R.KPNSM*SS#NHAFS#T#SATR.E	17.2	21.4	19.0
T419	931.7188	R.KPNSM*SSNHAFS#TSAT#RENLYFQ.-	0.0	17.6	0.0

[a] Residues with certainty of > 99% (bold) and > 90% for correct phosphorylation sites based on Ascores were shown (see also [c]). Representative MS/MS mass spectra are shown in Figure S3.

[b] The symbols * and # represent methionine oxidation and amino acid phosphorylation, respectively.

[c] An Ascore ≥ 19 indicates a certainty of > 99% for correct phosphorylation site identification regardless of the data set; Ascores of 15-19 indicate > 90% certainty for correct phosphorylation site localization. An Ascore of 1000 indicates an unequivocal localization because there are no other possible sites to place the phosphate[10]. If there are two or more sites in a phosphopeptide, Ascore 1 refers to the most N-terminal site and Ascore 2 to the next site moving toward the C-terminus.

Figure S1.

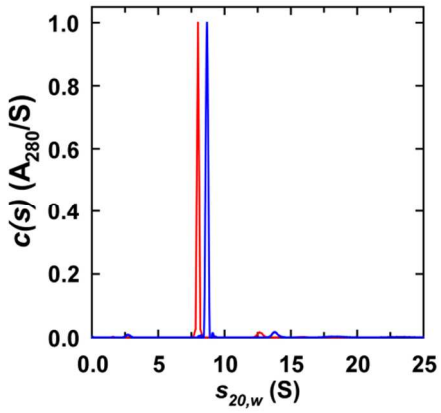


Figure S1. Sedimentation velocity-analytical ultracentrifugation of empty-nanodiscs. Absorbance sedimentation velocity c(*s*) distributions obtained for empty-nanodiscs reconstituted with POPC (red) and 75% POPC/25% POPG (blue). Similar profiles were obtained using interference data. In all cases, data were collected at 10 ˚C and 40,000 rpm in a Beckman Coulter An50 Ti rotor on a Beckman Coulter ProteomeLab XL-I analytical ultracentrifuge.

Figure S2.

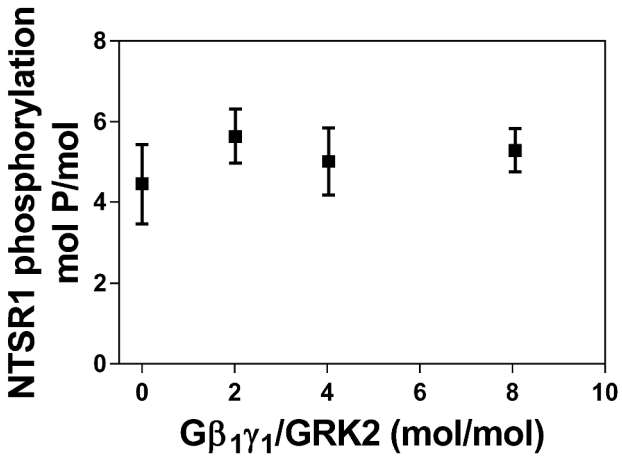
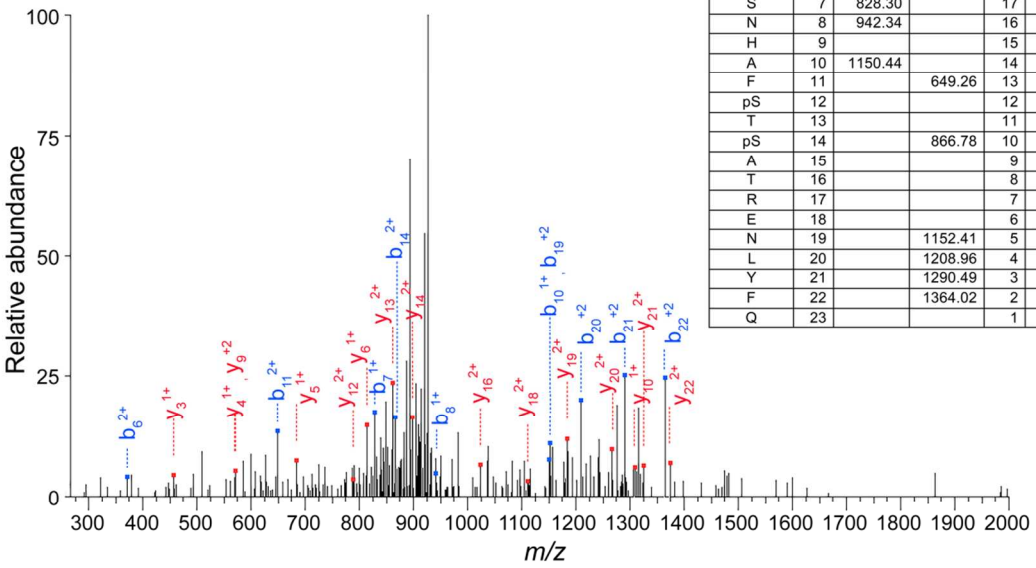


Figure S2. NTSR1 phosphorylation by GRK2 in the presence of $G\beta_1\gamma_1$. NTS-bound NTSR1 WT in nanodiscs prepared using 75% POPC/25% POPG was phosphorylated by GRK2 in the presence of $G\beta_1\gamma_1$ (mol/mol), and the stoichiometry of phosphorylation was determined. Means ± S.D. from three experiments performed in duplicate are shown.

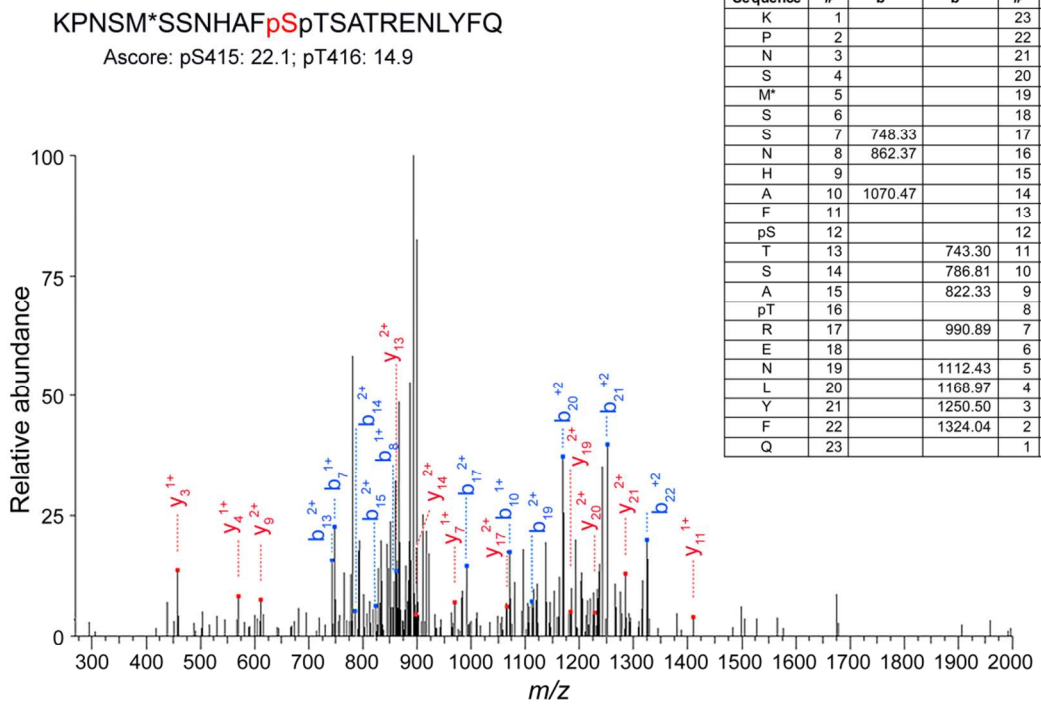
Figure S3.

(1) NTSR1 WT, GRK2

C-terminus (S407, S415)



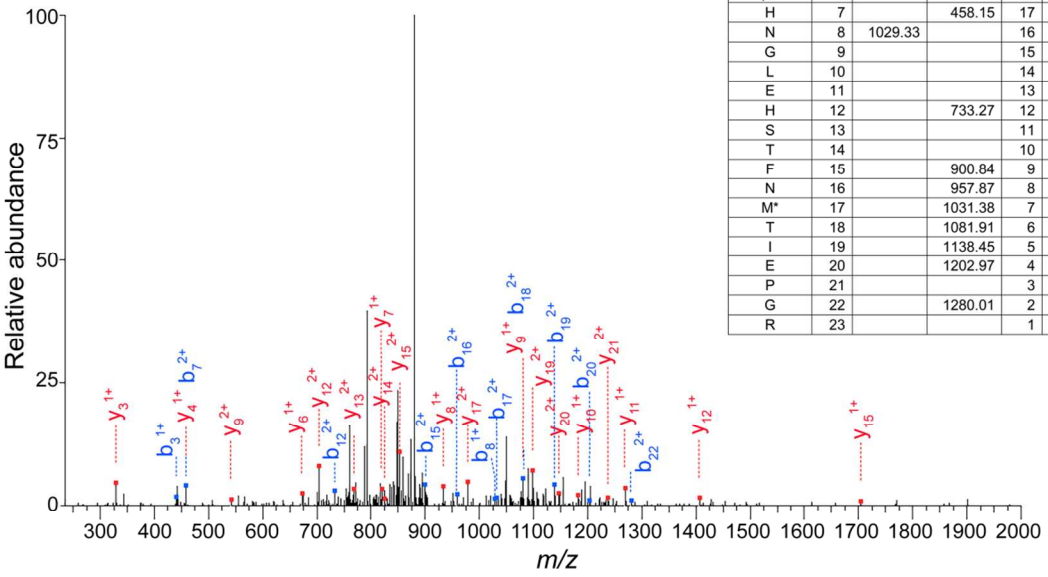
	b-ions			y-ions		
Sequence	#	b^{1+}	b^{2+}	#	y^{1+}	y^{2+}
K	1			23		---
P	2			22		1373.01
N	3			21		1324.48
pS	4			20		1267.46
M*	5			19		1183.96
S	6		371.14	18		1110.44
S	7	828.30		17		
N	8	942.34		16		1023.41
H	9			15		
A	10	1150.44		14		897.86
F	11		649.26	13		862.34
pS	12			12		788.81
T	13			11		
pS	14		866.78	10	1308.56	
A	15			9		571.29
T	16			8		
R	17			7		
E	18			6	813.38	
N	19		1152.41	5	684.34	
L	20		1208.96	4	570.29	
Y	21		1290.49	3	457.21	
F	22		1364.02	2		
Q	23			1		



	b-ions			y-ions		
Sequence	#	b^{1+}	b^{2+}	#	y^{1+}	y^{2+}
K	1			23		
P	2			22		
N	3			21		1284.50
S	4			20		1227.48
M*	5			19		1183.96
S	6			18		
S	7	748.33		17		1066.93
N	8	862.37		16		
H	9			15		
A	10	1070.47		14		897.86
F	11			13		862.34
pS	12			12		
T	13		743.30	11	1409.61	
S	14		786.81	10		
A	15		822.33	9		611.27
pT	16			8		
R	17		990.89	7	969.48	
E	18			6		
N	19		1112.43	5		
L	20		1168.97	4	570.29	
Y	21		1250.50	3	457.21	
F	22		1324.04	2		
Q	23			1		

(2) NTSR1 WT, GRK5

IL3 (T279, T282, T290, T294)



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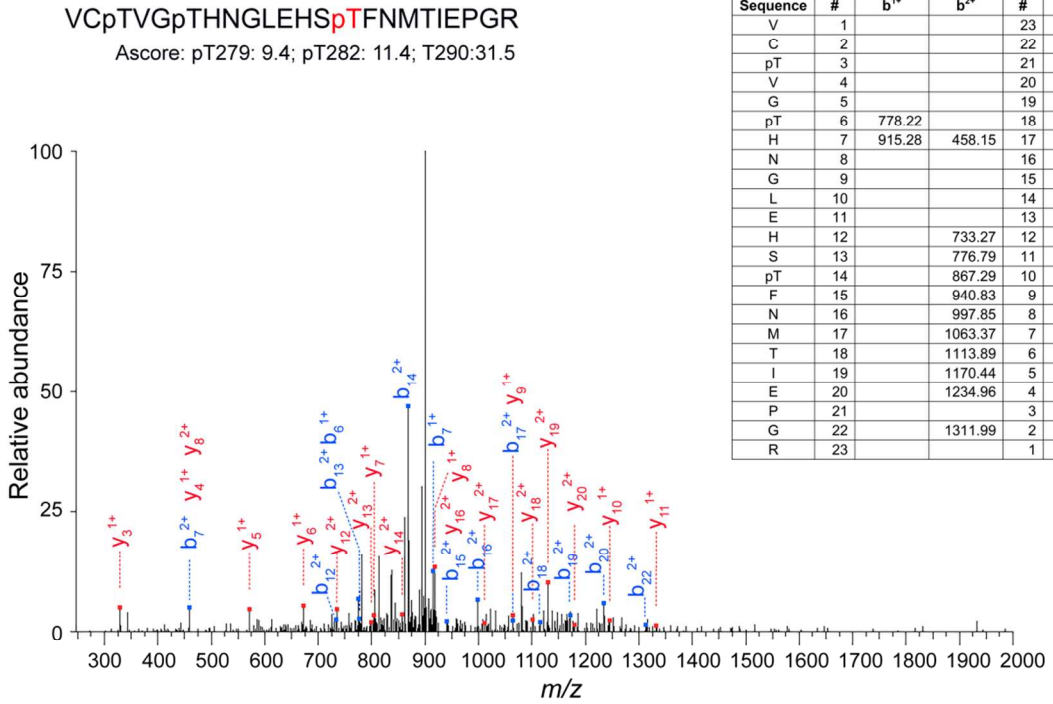
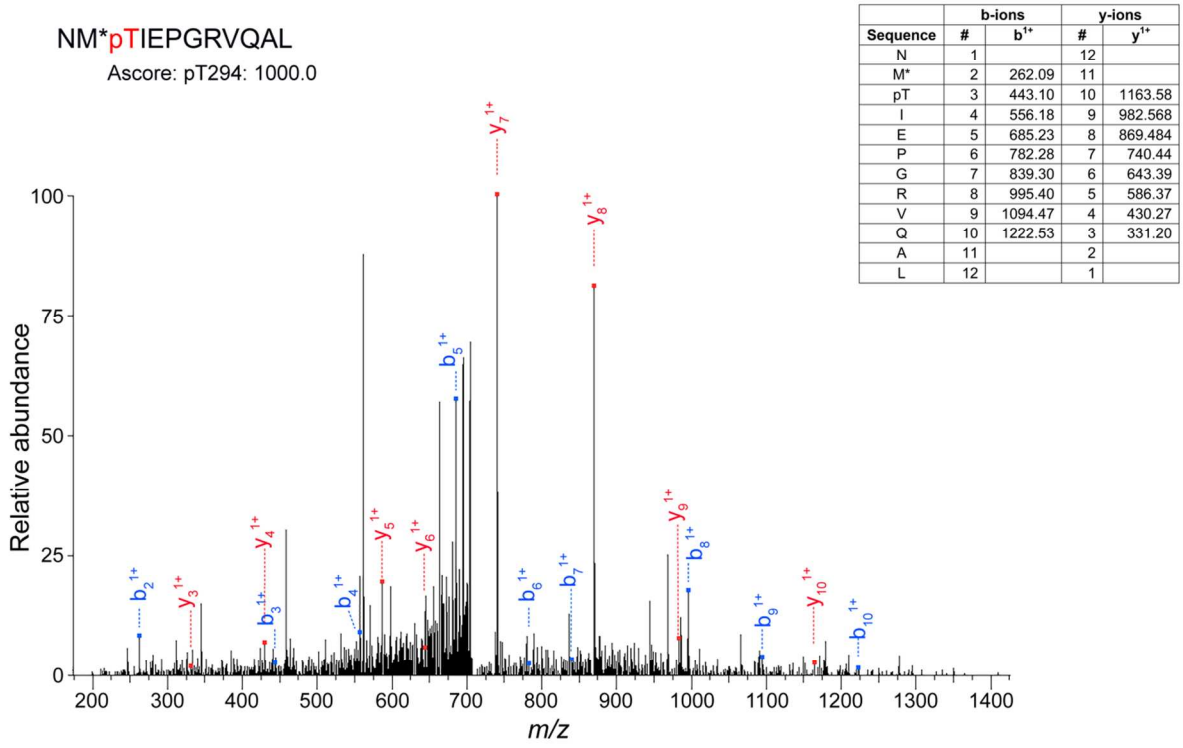
V	1			23		
C	2			22		
pT	3	441.12		21		1237.51
V	4			20		1147.01
G	5			19		1097.47
pT	6			18		
H	7		458.15	17		978.46
N	8	1029.33		16		
G	9			15	1704.80	852.90
L	10			14		824.39
E	11			13		767.85
H	12		733.27	12	1405.65	703.33
S	13			11	1268.59	
T	14			10	1181.56	
F	15		900.84	9	1080.51	540.76
N	16		957.87	8	933.45	
M*	17		1031.38	7	819.40	
T	18		1081.91	6	672.37	
I	19		1138.45	5		
E	20		1202.97	4	458.24	
P	21			3	329.19	
G	22		1280.01	2		
R	23			1		



	b-ions			y-ions		
Sequence	#	b^{1+}	b^{2+}	#	y^{1+}	y^{2+}
V	1			23		
C	2			22		
pT	3			21		
V	4			20		1178.99
G	5			19		1129.46
pT	6	778.22		18		1100.95
H	7	915.28	458.15	17		1010.44
N	8			16		941.91
G	9			15		
L	10			14		856.38
E	11			13	1598.67	799.84
H	12		733.27	12	1469.62	735.32
S	13		776.79	11	1332.57	
pT	14		867.29	10	1245.53	
F	15		940.83	9	1064.52	
N	16		997.85	8	917.45	459.229
M	17		1063.37	7	803.41	
T	18		1113.89	6	672.37	
I	19		1170.44	5	571.32	
E	20		1234.96	4	458.24	
P	21			3	329.19	
G	22		1311.99	2		
R	23			1		



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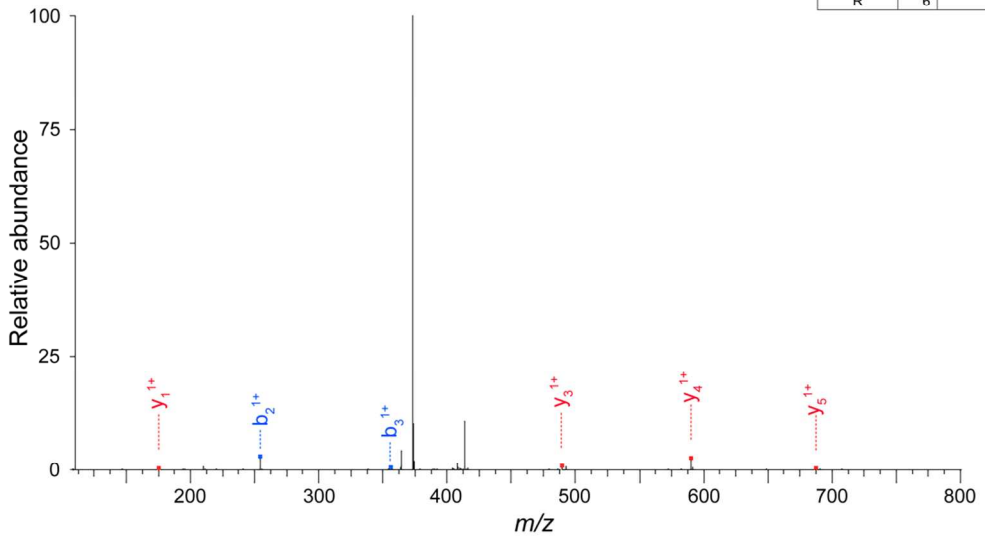
M*	2	262.09	11	
pT	3	443.10	10	1163.58
I	4	556.18	9	982.568
E	5	685.23	8	869.484
P	6	782.28	7	740.44
G	7	839.30	6	643.39
R	8	995.40	5	586.37
V	9	1094.47	4	430.27
Q	10	1222.53	3	331.20
A	11		2	
L	12		1	

C-terminus (S402, S407, S409, S410, S415, T416, S417, T419)



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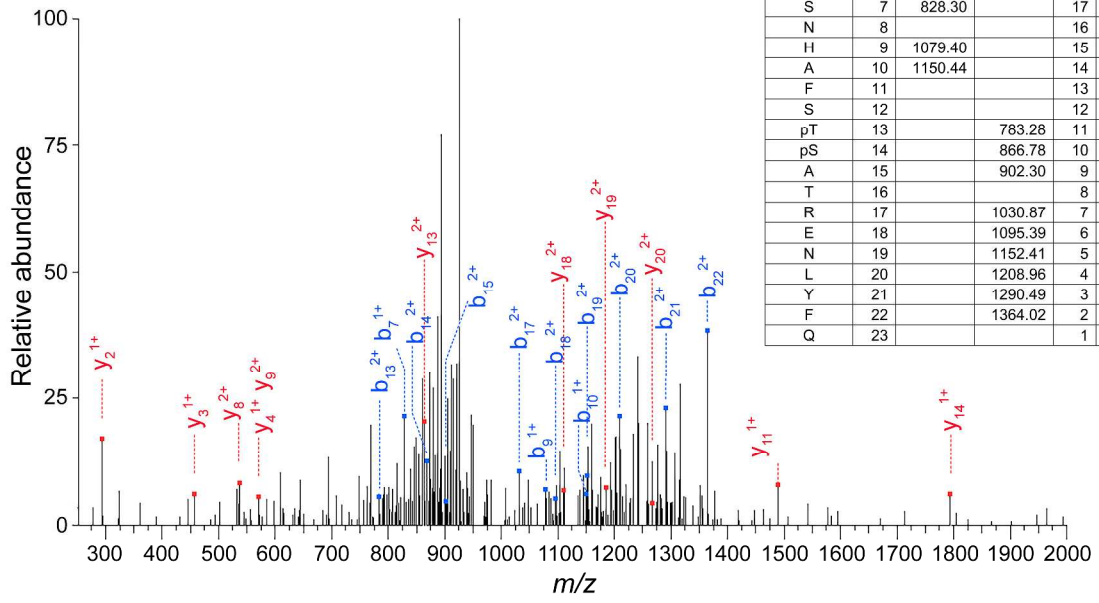
R	1		6	
P	2	254.16	5	687.29
T	3	355.21	4	590.23
F	4		3	489.19
pS	5		2	
R	6		1	1029.59

KPNpSM*SSNHAFSpTpSATRENLYFQ

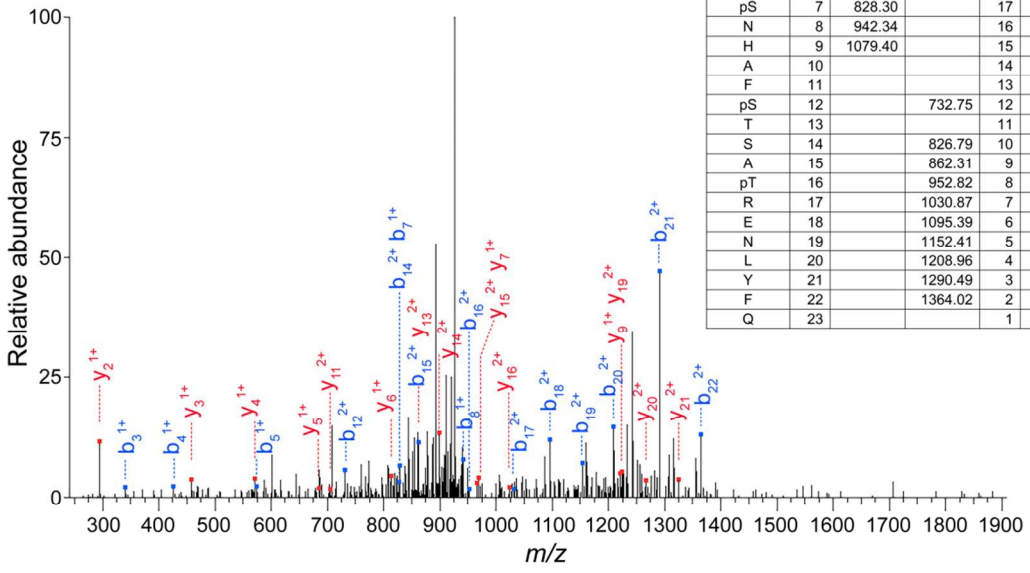
Ascore: pS407: 33.5; pT416: 20.6; pS417: 24.1



	b-ions			y-ions		
Sequence	#	b^{1+}	b^{2+}	#	y^{1+}	y^{2+}
K	1			23		
P	2			22		
N	3			21		
pS	4			20		1267.46
M*	5			19		1183.96
S	6			18		1110.44
S	7	828.30		17		
N	8			16		
H	9	1079.40		15		
A	10	1150.44		14	1794.71	
F	11			13		862.34
S	12			12		
pT	13		783.28	11	1489.58	
pS	14		866.78	10		
A	15		902.30	9		571.29
T	16			8		535.767
R	17		1030.87	7		
E	18		1095.39	6		
N	19		1152.41	5		
L	20		1208.96	4	570.29	
Y	21		1290.49	3	457.21	
F	22		1364.02	2	294.15	
Q	23			1		

KPNSM*SpSNHAFpSTSApTRENLYFQ

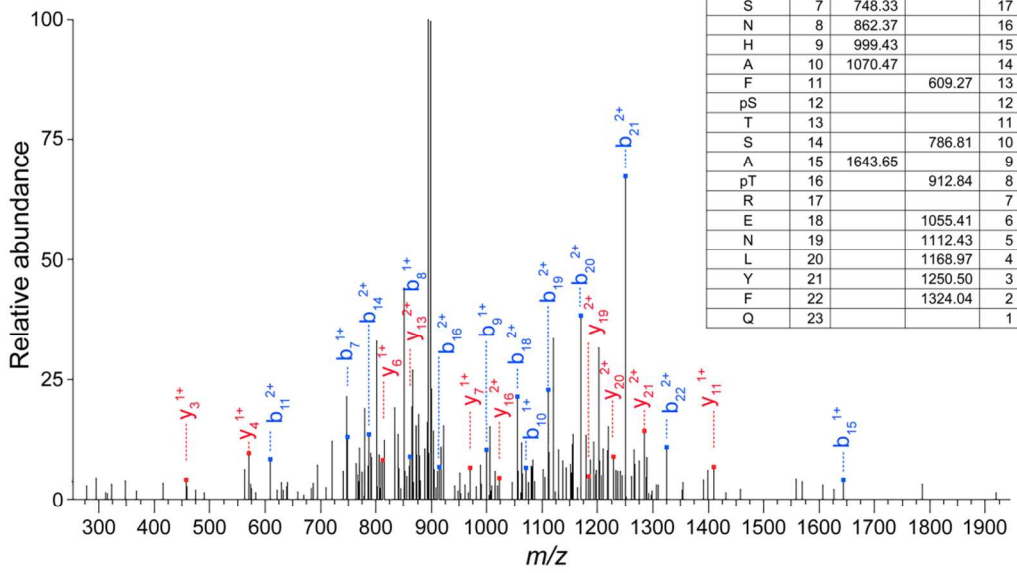
Ascore: pS410: 22.2; pS415: 7.0; pT419: 7.9



	b-ions			y-ions		
Sequence	#	b^{1+}	b^{2+}	#	y^{1+}	y^{2+}
K	1			23		
P	2			22		
N	3	340.20		21		1324.48
S	4	427.23		20		1267.46
M*	5	574.27		19		1223.94
S	6			18		
pS	7	828.30		17		
N	8	942.34		16		1023.41
H	9	1079.40		15		966.39
A	10			14		897.86
F	11			13		862.34
pS	12		732.75	12		
T	13			11		705.31
S	14		826.79	10		
A	15		862.31	9	1221.53	
pT	16		952.82	8		
R	17		1030.87	7	969.48	
E	18		1095.39	6	813.38	
N	19		1152.41	5	684.34	
L	20		1208.96	4	570.29	
Y	21		1290.49	3	457.21	
F	22		1364.02	2	294.15	
Q	23			1		

KPNSM*SSNHAFpSTSApTRENLYFQ

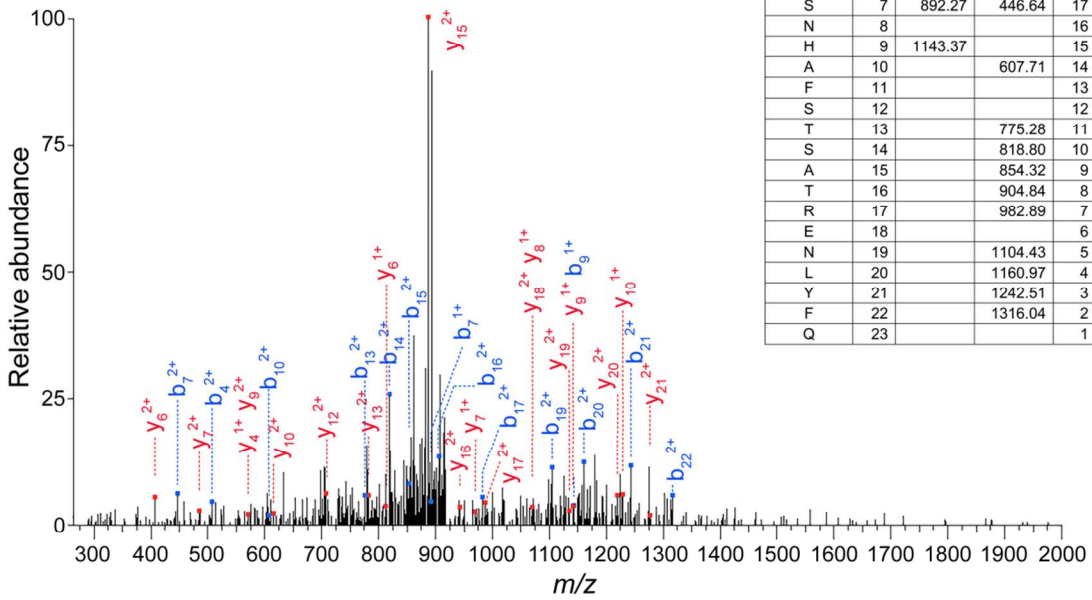
Ascore: pS415: 0.0; pT419: 17.6



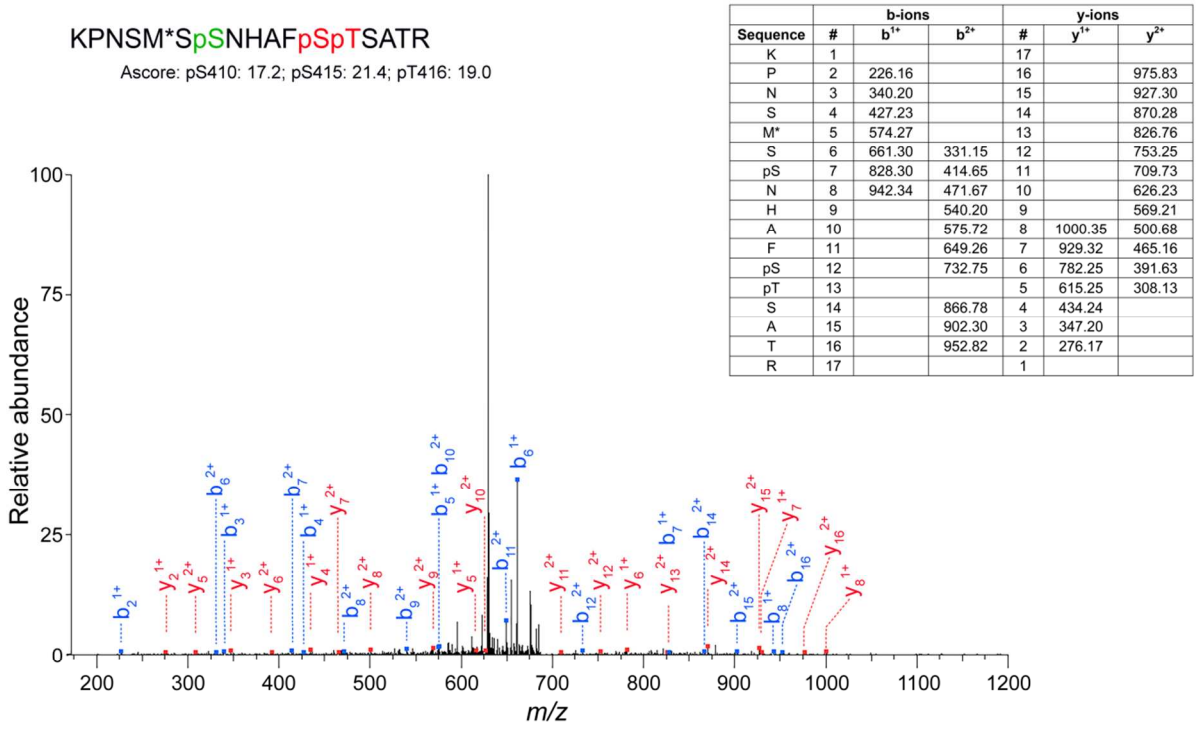
	b-ions			y-ions		
Sequence	#	b^{1+}	b^{2+}	#	y^{1+}	y^{2+}
K	1			23		
P	2			22		
N	3			21		1284.50
S	4			20		1227.48
M*	5			19		1183.96
S	6			18		
S	7	748.33		17		
N	8	862.37		16		1023.41
H	9	999.43		15		
A	10	1070.47		14		
F	11		609.27	13		862.34
pS	12			12		
T	13			11	1409.61	
S	14		786.81	10		
A	15	1643.65		9		
pT	16		912.84	8		
R	17			7	969.48	
E	18		1055.41	6	813.38	
N	19		1112.43	5		
L	20		1168.97	4	570.29	
Y	21		1250.50	3	457.21	
F	22		1324.04	2		
Q	23			1		

KPNpSMpSSNHAFSTSATRENLYFQ

Ascore: pS407: 0.0; pS409:15.0



	b-ions			y-ions		
Sequence	#	b^{1+}	b^{2+}	#	y^{1+}	y^{2+}
K	1			23		
P	2			22		
N	3			21		1276.50
pS	4	507.20		20		1219.48
M	5			19		1135.98
pS	6			18		1070.46
S	7	892.27	446.64	17		986.96
N	8			16		943.45
H	9	1143.37		15		886.42
A	10		607.71	14		
F	11			13		782.38
S	12			12		708.84
T	13		775.28	11		
S	14		818.80	10	1228.60	614.80
A	15		854.32	9	1141.56	571.29
T	16		904.84	8	1070.53	
R	17		982.89	7	969.48	485.243
E	18			6	813.38	407.193
N	19		1104.43	5		
L	20		1160.97	4	570.29	
Y	21		1242.51	3		
F	22		1316.04	2		
Q	23			1		



	b-ions			y-ions		
Sequence	#	b^{1+}	b^{2+}	#	y^{1+}	y^{2+}
K	1			17		
P	2	226.16		16		975.83
N	3	340.20		15		927.30
S	4	427.23		14		870.28
M*	5	574.27		13		826.76
S	6	661.30	331.15	12		753.25
pS	7	828.30	414.65	11		709.73
N	8	942.34	471.67	10		626.23
H	9		540.20	9		569.21
A	10		575.72	8	1000.35	500.68
F	11		649.26	7	929.32	465.16
pS	12		732.75	6	782.25	391.63
pT	13			5	615.25	308.13
S	14		866.78	4	434.24	
A	15		902.30	3	347.20	
T	16		952.82	2	276.17	
R	17			1		

Figure S3. Phosphorylation sites on NTSR1 as detected by LC/MS/MS. Representative mass spectra and associated fragmentation tables are shown. The peptide sequence is shown at the top of the MS/MS spectrum along with the phosphorylated residues (pS and pT). Those with an Ascore greater than or equal to 19 (> 99% certainty) are indicated in red, whereas those with an Ascore of 15-19 (>90% success rate) are highlighted in green. The identified fragmentation b (blue) and y (red) ions are also indicated.

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