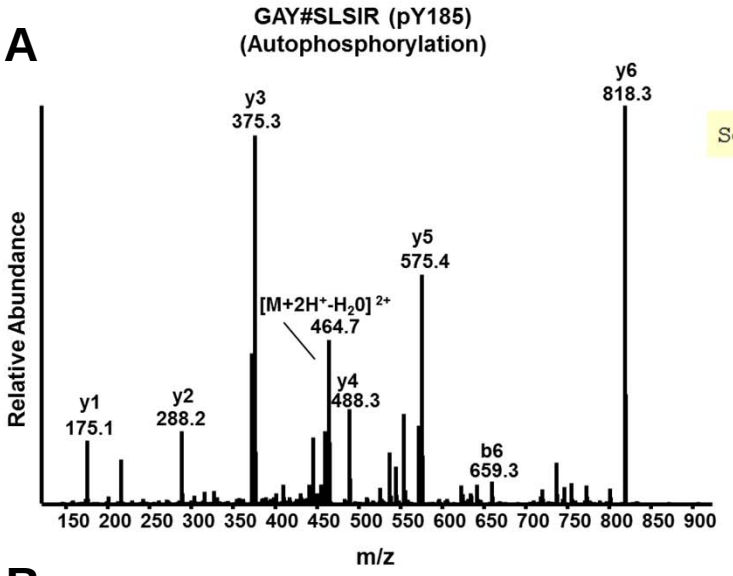
Supplementary Figure 1

A

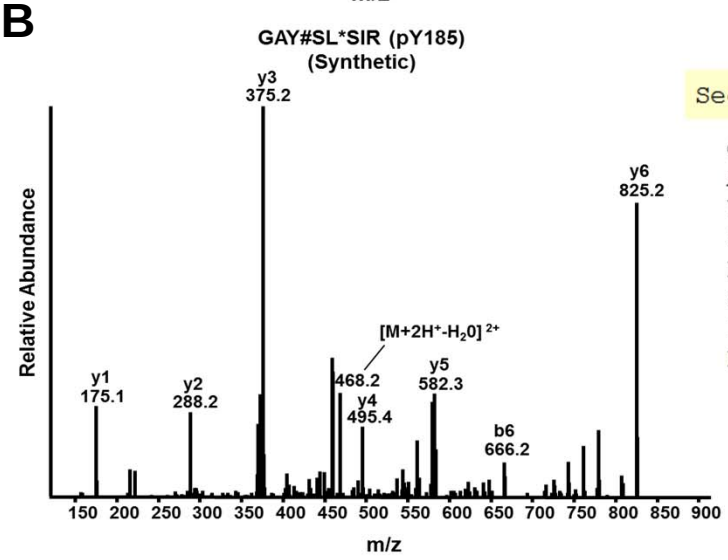


Fragment Ions *z*=1

Seq	#	b		y		# (+1)
G	1	58.02		946.43		8
A	2	129.06		889.41		7
Y#	3	372.09		818.38	*	6
S	4	459.12		575.35	*	5
L	5	572.21		488.31	*	4
S	6	659.24	*	375.23	*	3
I	7	772.32		288.20	*	2
R	8	928.42		175.11	*	1

$[M+2H^+]^{2+}$= 473.723

B

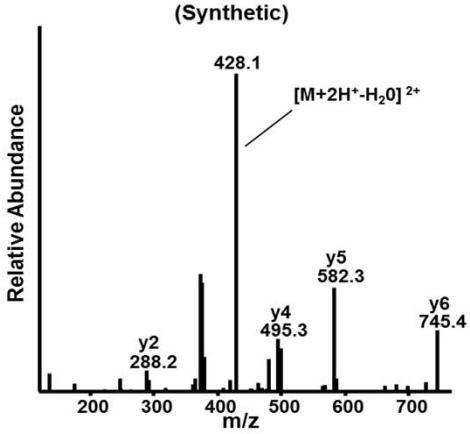


Fragment Ions *z*=1

Seq	#	b		y		# (+1)
G	1	58.02		953.45		8
A	2	129.06		896.43		7
Y#	3	372.09		825.39	*	6
S	4	459.12		582.36	*	5
L*	5	579.22		495.33	*	4
S	6	666.26	*	375.23	*	3
I	7	779.34		288.20	*	2
R	8	935.44		175.11	*	1

$[M+2H^+]^{2+}$= 477.232

C



Fragment Ions *z*=1

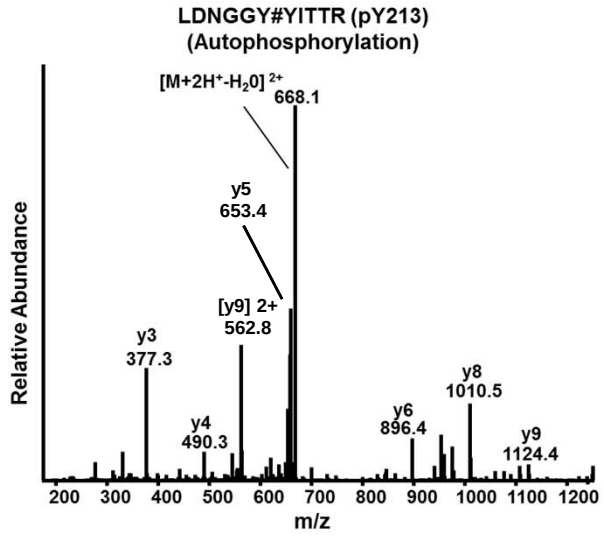
Seq	#	b		y		# (+1)
G	1	58.02		873.49		8
A	2	129.06		816.46		7
Y	3	292.12		745.43	*	6
S	4	379.16		582.36	*	5
L*	5	499.26		495.33	*	4
S	6	586.29		375.23		3
I	7	699.37		288.20	*	2
R	8	855.48		175.11		1

$[M+2H^+]^{2+}$= 437.249

Supplementary Figure 1. Low-energy collision-induced dissociation spectra for (A) the phosphopeptide immunoprecipitated from yeast, (B) the synthetic phosphopeptide and (C) the synthetic unphosphorylated peptide. and their predicted fragment ions. # denotes phosphophorylation, * in sequence denotes residue containing ^{13}C and ^{15}N. Red and blue stars indicate select fragment ions specifically labeled in spectra.

Supplementary Figure 2

A

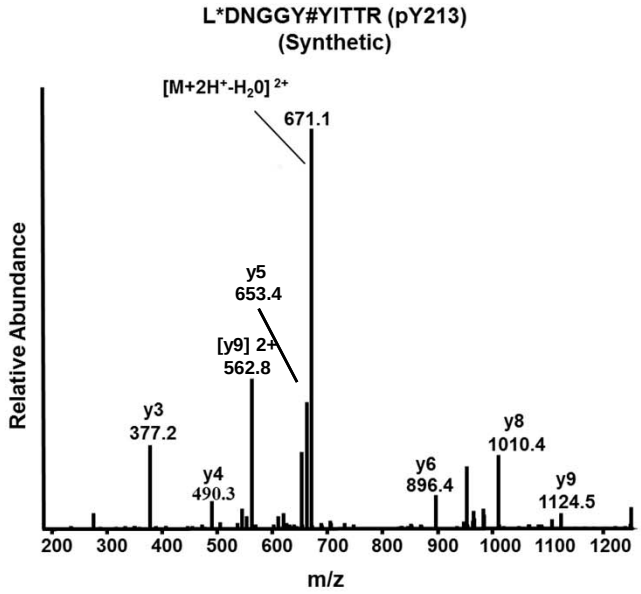


Fragment Ions *z*=1

Seq	#	b	y		# (+1)
L	1	114.09	1352.58		11
D	2	229.11	1239.50		10
N	3	343.16	1124.47	*	9
G	4	400.18	1010.43	*	8
G	5	457.20	953.41		7
Y#	6	700.23	896.39	*	6
Y	7	863.29	653.36	*	5
I	8	976.38	490.29	*	4
T	9	1077.42	377.21	*	3
T	10	1178.47	276.16		2
R	11	1334.57	175.11		1

$[M+2H^+]^{2+}$= 676.798

B

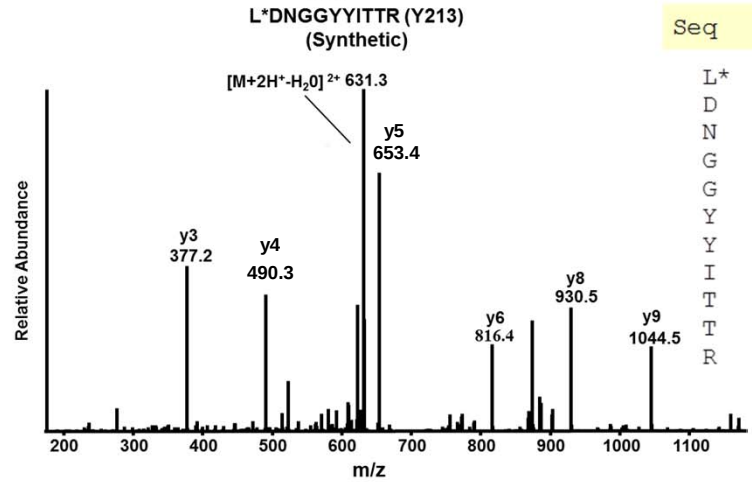


Fragment Ions *z*=1

Seq	#	b	y		# (+1)
L*	1	121.10	1359.60		11
D	2	236.13	1239.50		10
N	3	350.17	1124.47	*	9
G	4	407.20	1010.43	*	8
G	5	464.22	953.41		7
Y#	6	707.25	896.39	*	6
Y	7	870.31	653.36	*	5
I	8	983.39	490.29	*	4
T	9	1084.44	377.21	*	3
T	10	1185.49	276.16		2
R	11	1341.59	175.11		1

$[M+2H^+]^{2+}$= 680.306

C



Fragment Ions *z*=1

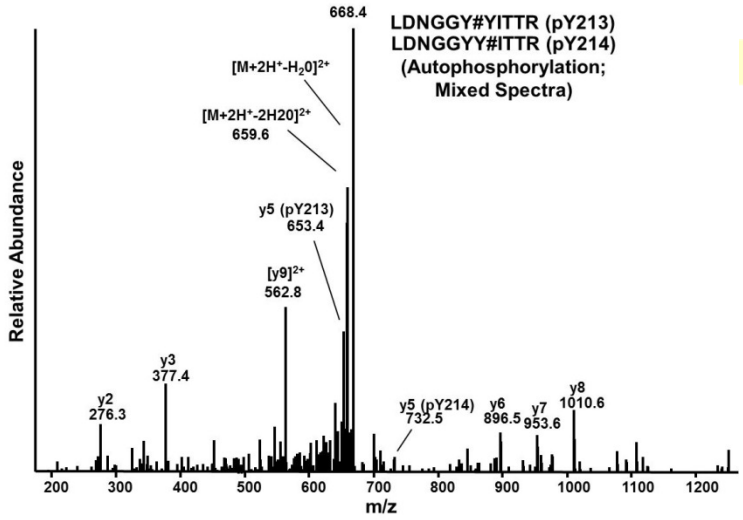
Seq	#	b	y		# (+1)
L*	1	121.10	1279.63		11
D	2	236.13	1159.53		10
N	3	350.17	1044.51	*	9
G	4	407.20	930.46	*	8
G	5	464.22	873.44		7
Y	6	627.28	816.42	*	6
Y	7	790.34	653.36	*	5
I	8	903.43	490.29	*	4
T	9	1004.48	377.21	*	3
T	10	1105.52	276.16		2
R	11	1261.62	175.11		1

$[M+2H^+]^{2+}$= 640.323

Supplementary Figure 2. Low-energy collision-induced dissociation spectra for (A) the phosphopeptide immunoprecipitated from yeast, (B) the synthetic phosphopeptide and (C) the synthetic unphosphorylated peptide. and their predicted fragment ions. # denotes phosphophorylation, * in sequence denotes residue containing ^{13}C and ^{15}N. Red and blue stars indicate select fragment ions specifically labeled in spectra.

Supplementary Figure 3

A

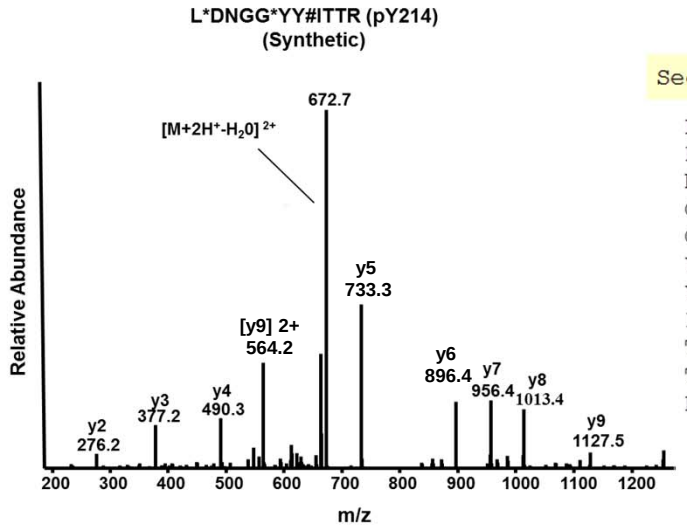


Fragment Ions *z*=1

#	b	y		# (+1)
1	114.09	1352.58		11
2	229.11	1239.50		10
3	343.16	1124.47		9
4	400.18	1010.43	*	8
5	457.20	953.41	*	7
6	620.26	896.39	*	6
7	863.29	733.32	*	5
8	976.38	490.29		4
9	1077.42	377.21	*	3
10	1178.47	276.16	*	2
11	1334.57	175.11		1

$[M+2H^+]^{2+}$= 676.798

B

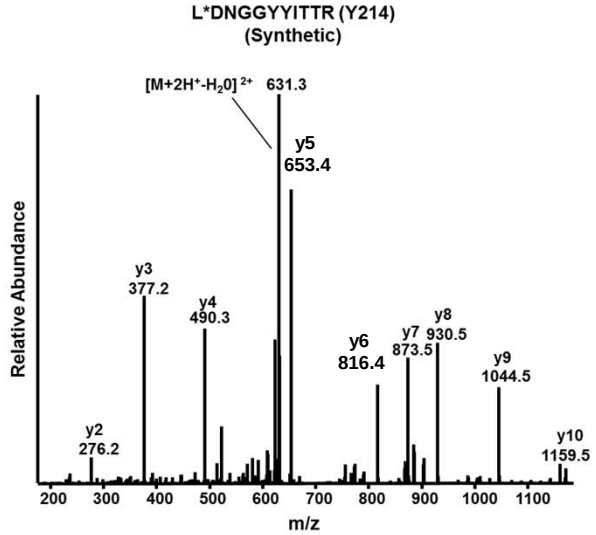


Fragment Ions *z*=1

Seq	#	b	y		# (+1)
L*	1	121.10	1362.60		11
D	2	236.13	1242.50		10
N	3	350.17	1127.48	*	9
G	4	407.20	1013.43	*	8
G*	5	467.22	956.41	*	7
Y	6	630.28	896.39	*	6
Y#	7	873.31	733.32	*	5
I	8	986.40	490.29	*	4
T	9	1087.45	377.21	*	3
T	10	1188.49	276.16	*	2
R	11	1344.59	175.11		1

$[M+2H^+]^{2+}$= 681.808

C



Fragment Ions *z*=1

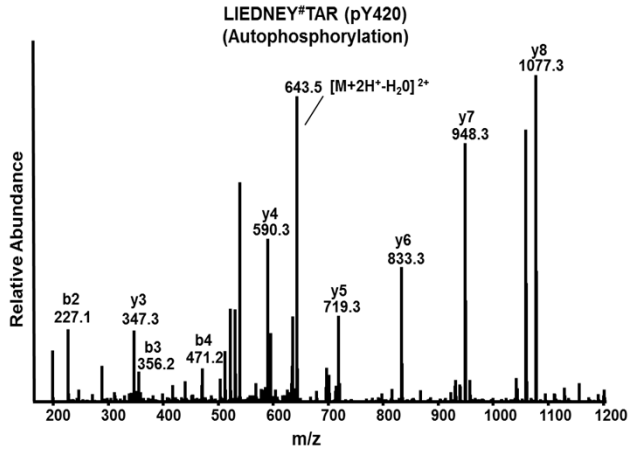
Seq	#	b	y		# (+1)
L*	1	121.10	1279.63		11
D	2	236.13	1159.53	*	10
N	3	350.17	1044.51	*	9
G	4	407.20	930.46	*	8
G	5	464.22	873.44	*	7
Y	6	627.28	816.42	*	6
Y	7	790.34	653.36	*	5
I	8	903.43	490.29	*	4
T	9	1004.48	377.21	*	3
T	10	1105.52	276.16	*	2
R	11	1261.62	175.11		1

$[M+2H^+]^{2+}$= 640.323

Supplementary Figure 3. Low-energy collision-induced dissociation spectra for (A) the co-eluting phosphopeptides immunoprecipitated from yeast, (B) the synthetic phosphopeptide and (C) the synthetic unphosphorylated peptide. and their predicted fragment ions. # denotes phosphophorylation, * in sequence denotes residue containing ^{13}C and ^{15}N. Red and blue stars indicate select fragment ions specifically labeled in spectra.

Supplementary Figure 4

A

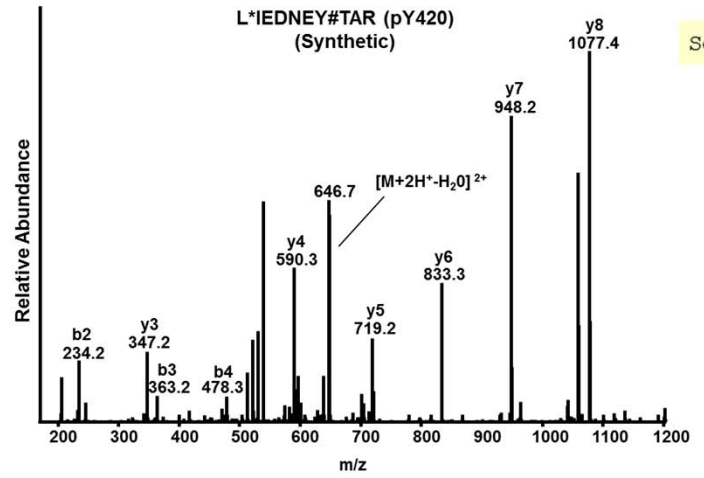


Fragment Ions *z*=1

Seq	#	b		y		# (+1)
L	1	114.09		1303.55		10
I	2	227.17	*	1190.47		9
E	3	356.21	*	1077.38	*	8
D	4	471.24	*	948.34	*	7
N	5	585.28		833.31	*	6
E	6	714.33		719.27	*	5
Y#	7	957.36		590.23	*	4
T	8	1058.40		347.20	*	3
A	9	1129.44		246.15		2
R	10	1285.54		175.11		1

$[M+2H^+]^{2+}$= 652.282

B

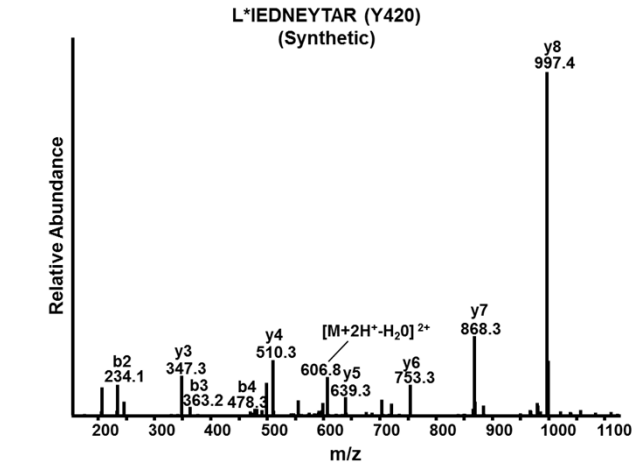


Fragment Ions *z*=1

Seq	#	b		y		# (+1)
L*	1	121.10		1310.57		10
I	2	234.19	*	1190.47		9
E	3	363.23	*	1077.38	*	8
D	4	478.26	*	948.34	*	7
N	5	592.30		833.31	*	6
E	6	721.34		719.27	*	5
Y#	7	964.37		590.23	*	4
T	8	1065.42		347.20	*	3
A	9	1136.46		246.15		2
R	10	1292.56		175.11		1

$[M+2H^+]^{2+}$= 655.791

C



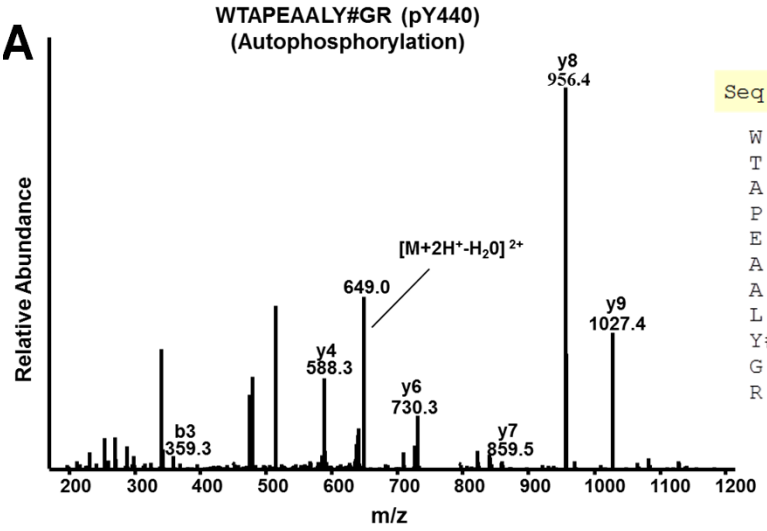
Fragment Ions *z*=1

Seq	#	b		y		# (+1)
L*	1	121.10		1230.60		10
I	2	234.19	*	1110.50		9
E	3	363.23	*	997.42	*	8
D	4	478.26	*	868.38	*	7
N	5	592.30		753.35	*	6
E	6	721.34		639.31	*	5
Y	7	884.41		510.26	*	4
T	8	985.45		347.20	*	3
A	9	1056.49		246.15		2
R	10	1212.59		175.11		1

$[M+2H^+]^{2+}$= 615.807

Supplementary Figure 4. Low-energy collision-induced dissociation spectra for (A) the phosphopeptide immunoprecipitated from yeast, (B) the synthetic phosphopeptide and (C) the synthetic unphosphorylated peptide. and their predicted fragment ions. # denotes phosphophorylation, * in sequence denotes residue containing ^{13}C and ^{15}N. Red and blue stars indicate select fragment ions specifically labeled in spectra.

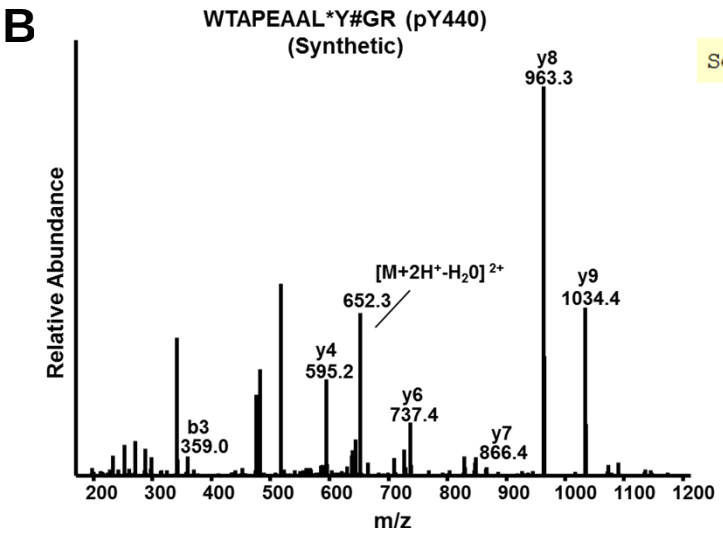
Supplementary Figure 5



Fragment Ions *z*=1

Seq	#	b		y		# (+1)
W	1	187.08		1314.58		11
T	2	288.13		1128.50		10
A	3	359.17	*	1027.46	*	9
P	4	456.22		956.42	*	8
E	5	585.26		859.37	*	7
A	6	656.30		730.32	*	6
A	7	727.34		659.29		5
L	8	840.42		588.25	*	4
Y#	9	1083.45		475.17		3
G	10	1140.47		232.14		2
R	11	1296.57		175.11		1

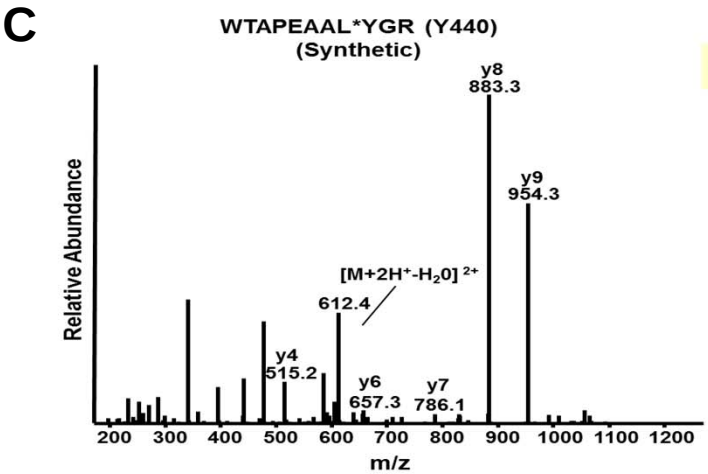
$[M+2H^+]^{2+}$= 657.7981



Fragment Ions *z*=1

Seq	#	b		y		# (+1)
W	1	187.08		1321.60		11
T	2	288.13		1135.52		10
A	3	359.17	*	1034.47	*	9
P	4	456.22		963.44	*	8
E	5	585.26		866.38	*	7
A	6	656.30		737.34	*	6
A	7	727.34		666.30		5
L*	8	847.44		595.27	*	4
Y#	9	1090.47		475.17		3
G	10	1147.49		232.14		2
R	11	1303.59		175.11		1

$[M+2H^+]^{2+}$= 661.3067



Fragment Ions *z*=1

Seq	#	b		y		# (+1)
W	1	187.08		1241.63		11
T	2	288.13		1055.55		10
A	3	359.17		954.51	*	9
P	4	456.22		883.47	*	8
E	5	585.26		786.42	*	7
A	6	656.30		657.37	*	6
A	7	727.34		586.34		5
L*	8	847.44		515.30	*	4
Y	9	1010.50		395.20		3
G	10	1067.52		232.14		2
R	11	1223.62		175.11		1

$[M+2H^+]^{2+}$= 621.323

Supplementary Figure 5. Low-energy collision-induced dissociation spectra for (A) the phosphopeptide immunoprecipitated from yeast, (B) the synthetic phosphopeptide and (C) the synthetic unphosphorylated peptide. and their predicted fragment ions. # denotes phosphophorylation, * in sequence denotes residue containing ^{13}C and ^{15}N. Red and blue stars indicate select fragment ions specifically labeled in spectra.

Weir *et al.* Supplementary Figure 6

A

```
Yes    GIFLVRESETTKGAYSLSIRDWDEIRGDNVKHYKIRKLDNGGYYITTRAQF
Src    GTFLVRESETTKGAYCLSVSDFDNAKGLNVKHYKIRKLDSGGFYITSRTQF
Fyn    GTFLIRESETTKGAYSLSIRDWDDMKGDHVKHYKIRKLDNGGYYITTRAQF
Fgr    GAFLIRESETTKGAYSLSIRDWDQTRGDHVKHYKIRKLDMGGYYITTRVQF

Lyn    GAFLIRESETLKGSFSLSVRDFDPVHGDVIKHYKIRSLDNGGYYISPRITF
Hck    GSFMIRDSETTKGSYSLSVRDYDPRQGDTVKHYKIRTLDNGGFYISPRSTF
Lck    GSFLIRESESTAGSFSLSVRDFDQNQGEVVKHYKIRNLDNGGFYISPRITF
Blk    GSFLIRESETNKGAFSLSVKDVT-TQGELIKHYKIRCLDEGGYYISPRITF

Frk    GSFLIRESESQKGEFSLSVLD-----GAVVKHYRIKRLDEGGFFLTRRRIF
Srm    GAFLIRPSESSLGGYSLSVR-----AQAKVCHYRVSMAADGSLYLQKGRLF
Brk    GAFLIRVSEKPSADYVLSVRD-----TQAVRHYKIWRRAGGRLHLNEAVSF
```

B

```
Hs_Fyn      GTFLIRESETTKGAYSLSIRDWDDM-KGDHVKHYKIRKLDN-GGYYITTRAQF
Mm_Fyn      GTFLIRESETTKGAYSLSIRDWDDM-KGDHVKHYKIRKLDN-GGYYITTRAQF
Rn_Fyn      GTFLIRESETTKGAYSLSIRDWDDM-KGDHVKHYKIRKLDN-GGYYITTRAQF
Gg_Fyn      GTFLIRESETTKGAYSLSIRDWDDM-KGDHVKHYKIRKLDN-GGYYITTRAQF
Xt_Fyn      GTYLIRESETTKGAYSLSIRDWDDM-KGDHVKHYKIRKLDN-GGYYITTRAQF
Dr_Fyn      GTFLIRESETTKGAYSLSIQDWDET-KGDHVKHYKIRKLDN-GGYYITTRAQF

Ce_Src1     GTFLIREREADTREFALTIRDTDDQRNGGTVKHYKIKRLDHDQGYFITTRRTF
Dm_Src64B   GTFLVRPSEHNPNGYSLSVKDWEDGR-GYHVKHYRIKPLDN-GGYYIATNQTF
Ce_Src2     GAFLVRDSESRQHDLSLSVRE------NDSVKHYRIRQLDH-GGYFIARRRPF
Dm_SRC42    GAFLIRDSESRHNDYSLSVRD------GDTVKHYRIRQLDE-GGFFIARRTTF
Sl_Srk1     GSFLIRDSETTPGDFSLSVKD------QDRVRHYRVRRLED-GSLFVTRRSTF
Sl_Srk4     GSFLIRDSDTTPGDFSLSVRD------IDRVRHYRIKKLEN-GTYFVTRRLTF
```

Supplementary Figure 6. Multiple sequence alignment of the region of the SH2 domain harboring the equivalents of Tyr185, Tyr213 and Tyr14 of human Fyn for (A) all human SFKs and (B) vertebrate Fyn orthologues (upper) and the SFKs found in worm (Ce), fly (Dm) and sponge (Sl). Hs=human, Mm=mouse, Rn=rat, Gg=chicken, Xt=frog and Dr=zebrafish. Blue highlight indicates the phosphotyrosine-interacting arginine. Red highlight indicates tyrosine residues and yellow highlight indicates non-tyrosine residues.

Weir *et al.* Supplementary Figure 7

A

```
Yes    DFGLARLIE-DNE--YTARQGAKFPIKWTAPEAALYGRFTIK
Src    DFGLARLIE-DNE--YTARQGAKFPIKWTAPEAALYGRFTIK
Fyn    DFGLARLIE-DNE--YTARQGAKFPIKWTAPEAALYGRFTIK
Fgr    DFGLARLIK-DDE--YNPCQGSKFPIKWTAPEAALFGRFTIK

Lyn    DFGLARVIE-DNE--YTAREGAKFPIKWTAPEAINFGCFTIK
Hck    DFGLARVIE-DNE--YTAREGAKFPIKWTAPEAINFGSFTIK
Lck    DFGLARLIE-DNE--YTAREGAKFPIKWTAPEAINYGTFTIK
Blk    DFGLARIID-S-E--YTAQEGAKFPIKWTAPEAIHFGVFTIK

Frk    DFGLARVFKVDNEDIYESRHEIKLPVKWTAPEAIRSNKFSIK
Srm    DFGLARLLK—--DDIYSPSSSSKIPVKWTAPEAANYRVFSQK
Brk    DFGLARLIK—--EDVY-LSHDHNIPYKWTAPEALSRGHYSTK
```

B

```
Hs_Fyn     DFGLARL-IEDNEYTARQGAKFPIKWTAPEAALYGRFTIK
Mm_Fyn     DFGLARL-IEDNEYTARQGAKFPIKWTAPEAALYGRFTIK
Rn_Fyn     DFGLARL-IEDNEYTARQGAKFPIKWTAPEAALYGRFTIK
Gg_Fyn     DFGLARL-IEDNEYTARQGAKFPIKWTAPEAALYGRFTIK
Xt_Fyn     DFGLARL-IEDNEYTARQGMKFPIKWTAPEAALYGRFTIK
Dr_Fyn     DFGLARL-IEDNEYTARQGAKFPIKWTAPEAALYGRFTIK

Ce_Src1    DFGLARKLMEEDIYEARTGAKFPIKWTAPEAATCGNFTVK
Dm_Src64B  DFGLARV-IADDEYCPKQGSRFPVKWTAPEAIIYGKFSIK
Ce_Src2    DFGLARILMKENEYEARTGARFPIKWTAPEAANYNRFTTK
Dm_SRC42   DFGLARV-IKEDEYEARVGARFPIKWTAPEAANYSKFSIK
Sl_Srk1    DFGLARV-IDEEIYEAHTGAKFPIKWTAPEAAMYNRFTIK
Sl_Srk4    DFGLARV-IDEEIYEAKLGAKFPIKWTAPEAAMYSRFTIK
```

Supplementary Figure 7. Multiple sequence alignment of the activation loop region of the kinase domain harboring the equivalent of Tyr420 (green highlight) of human Fyn for (A) all human SFKs and (B) vertebrate Fyn orthologues (upper) and the SFKs found in worm (Ce), fly (Dm) and sponge (Sl). Hs=human, Mm=mouse, Rn=rat, Gg=chicken, Xt=frog and Dr=zebrafish. Blue highlight indicates the conserved activation loop features. Red highlight indicates tyrosine residues and yellow highlight indicates non-tyrosine residues.