Supplemental Figure S1. Diagram of combined SILAC and 14-3-3 affinity purification method.

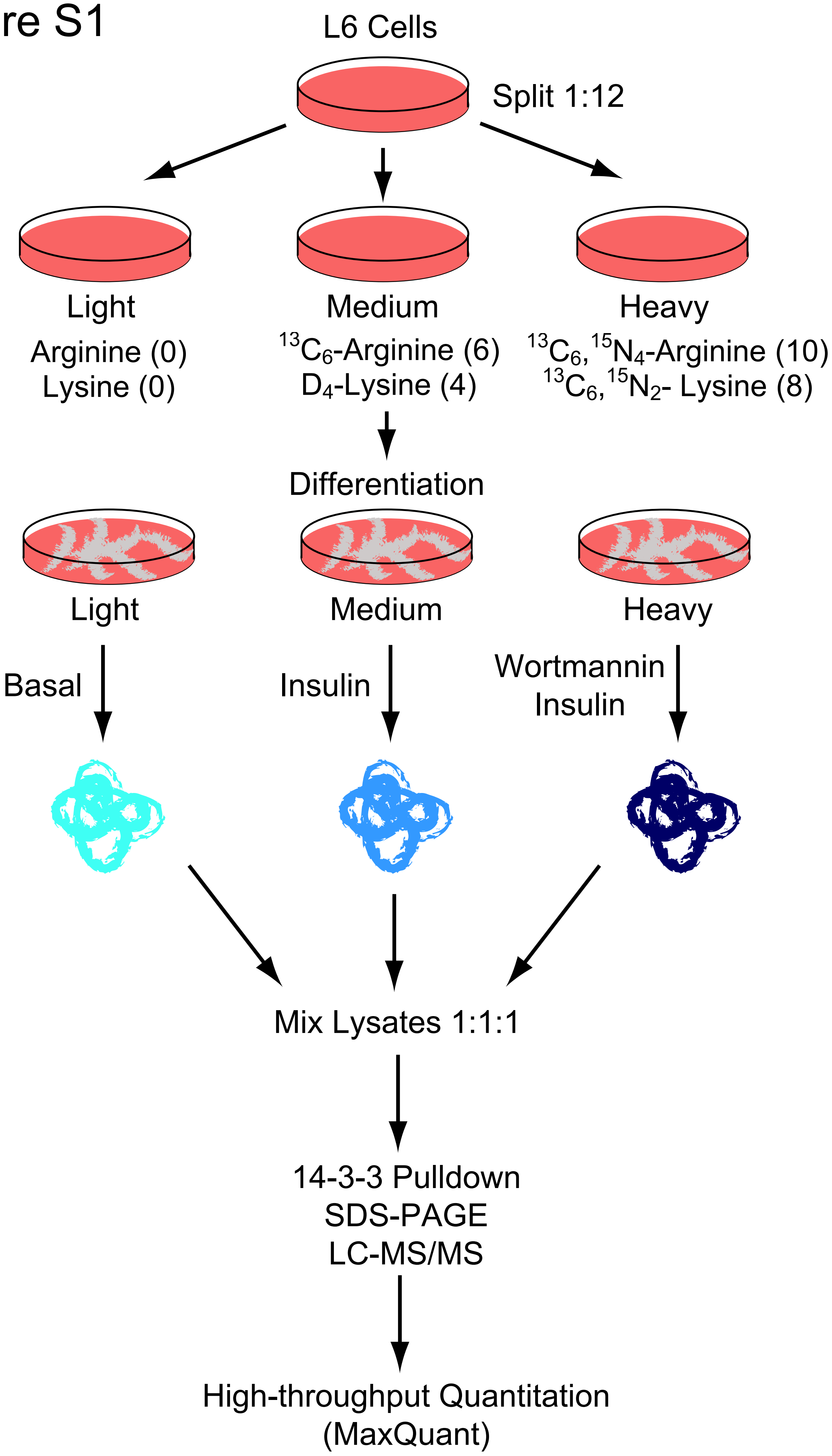
Supplemental Figure S2. Annotated MS/MS spectra of Edc3 phosphopeptides identified in this study using Mascot Server. A, Mascot annotated MS/MS spectrum and fragment ion table of the tryptic phosphopeptide HNpSWSSSSR containing pS161. Predicted fragment ions which were observed in the spectrum are highlighted within the table in bold red. The precursor ion and the y7 ion after neutral loss of phosphate are indicated in the lower MS/MS spectrum. The entire y-ion series of the intact phosphopeptide is also indicated in the lower spectrum. Mascot annotated MS/MS spectra and fragment ion tables for pS131 (B), pS109 (C), pT173 (D), and pY225 (E) are also shown. Predicted fragment ions which were observed in the spectra are highlighted within the table in bold red.

Supplemental Figure S3. Characterisation of the Edc3 pS161 antibody (**a**), Immunoblotting of immunoprecipitated TagRFP-FLAG-Edc3 constructs indicated from transiently transfected CHO IR/IRS-1 cells. Cells were stimulated with 100 nM insulin for 30 min. (**b**), Immunoblotting of endogenous Edc3 immunoprecipitated from NIH-3T3 cells with the Edc3 pS161 antibody. Cells were stimulated with 1 μM insulin for 30 min. Immunoprecipitations were performed in the presence of a 10-fold molar excess of either the pS161 peptide used to generate the antibody (P1+P2) or the cognate non-phosphorylated peptide (NP). A SYPRO ruby stain of the immunoprecipitated protein is shown to show equal protein binding. Immunoblots are from a representative experiment (n = 3).

Supplemental Figure S4. Co-localisation of TagRFP-FLAG-Edc3 constructs and endogenous p-body markers in NIH-3T3 cells. The TagRFP-FLAG-Edc3 constructs indicated were transiently transfected into NIH-3T3 cells and imaged 96 hours post-transfection. Cells were either left unstimulated or stimulated with 1 μM insulin for 30 min. Cells were then stained with antibodies specific for DDX6 and Hedls. Antibody labeling as well as TagRFP fluorescence was imaged by epifluorescence microscopy. White scale bar in top right panel indicates 10 μm. Images are from a representative experiment (n = 3).

Supplemental Figure S5. Diagram of experimental design used in the SILAC-based Edc3 Interaction partner analysis. Non-specific binding was assessed by the inclusion of a vector only control (light SILAC label) and specific binding to either S161A-R18 (medium SILAC label) or Edc3 S161A-R18-KK (heavy SILAC label) was assessed by transient transfection of each of these constructs. Experimental or environmental contaminant proteins which bind non-specifically to the beads or the antibody typically have ratios equal to or less than 1:1:1.

Figure S1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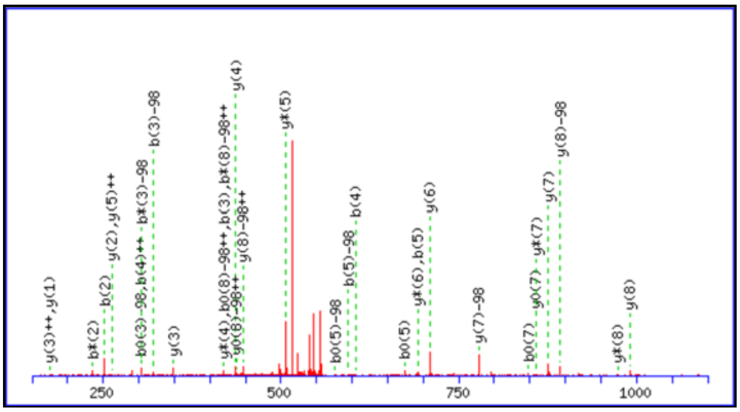
Arginine (0)
Lysine (0)
Medium
$^{13}C_6$-Arginine (6)
D_4-Lysine (4)
Heavy
$^{13}C_6$, $^{15}N_4$-Arginine (10)
$^{13}C_6$, $^{15}N_2$- Lysine (8)
Differentiation
Light
Medium
Heavy
Basal
Insulin
Wortmannin
Insulin
Mix Lysates 1:1:1
14-3-3 Pulldown
SDS-PAGE
LC-MS/MS
High-throughput Quantitation
(MaxQuant)

Figure S2

A

pS161
HNpSWSSSSR



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Monoisotopic mass of neutral peptide Mr(calc): 1126.419281
Variable modifications:
S3     : Phospho (ST), with neutral losses 97.976896(shown in table), 0.000000
Matches (Bold Red): 34/136 fragment ions using 53 most intense peaks
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#	b	b^{++}	b^{*}	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y^{*}	y^{*++}	y^{0}	y^{0++}	#
1	138.066188	69.536732					H							9
2	**252.109115**	126.558195	**235.082566**	118.044921			N	**892.390760**	**446.699018**	875.364211	438.185743	874.380195	**437.693735**	8
3	**321.130571**	161.068923	**304.104022**	152.555649	**303.120006**	152.063641	S	**778.347833**	389.677554	761.321284	381.164280	760.337268	380.672272	7
4	507.209884	254.108580	490.183335	245.595305	489.199319	245.103297	W	**709.326377**	355.166826	**692.299828**	346.653552	691.315812	346.161544	6
5	**594.241912**	297.624594	577.215363	289.111319	**576.231347**	288.619311	S	523.247064	**262.127170**	**506.220515**	253.613895	505.236499	253.121887	5
6	681.273940	341.140608	664.247391	332.627333	663.263375	332.135325	S	**436.215036**	218.611156	**419.188487**	210.097881	418.204471	209.605873	4
7	768.305968	384.656622	751.279419	376.143347	750.295403	375.651339	S	**349.183008**	**175.095142**	332.156459	166.581867	331.172443	166.089859	3
8	855.337996	428.172636	838.311447	**419.659361**	837.327431	**419.167353**	S	**262.150980**	131.579128	245.124431	123.065853	244.140415	122.573845	2
9							R	**175.118952**	88.063114	158.092403	79.549839			1

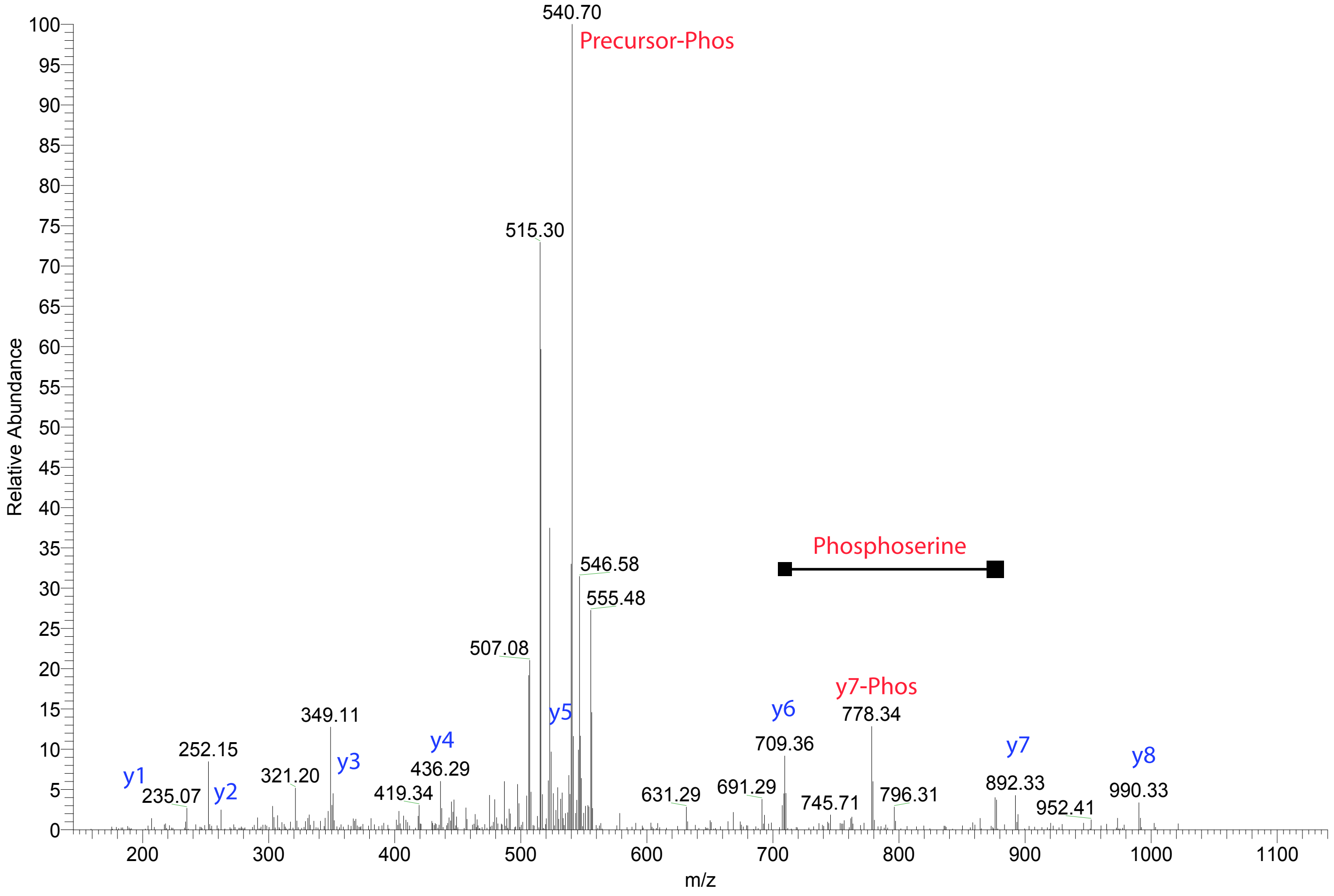
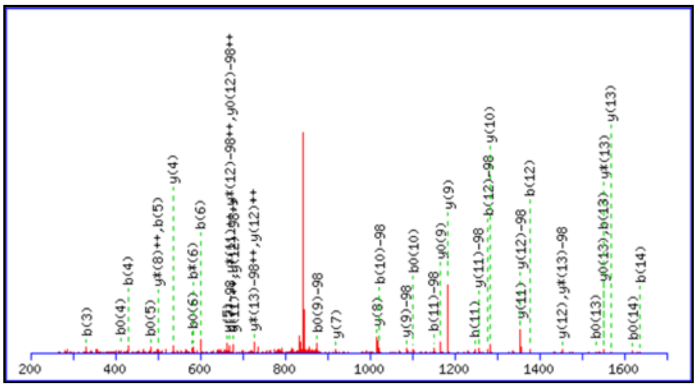


Figure S2

B

pS131
SQDVAVpSPQQQQCSK



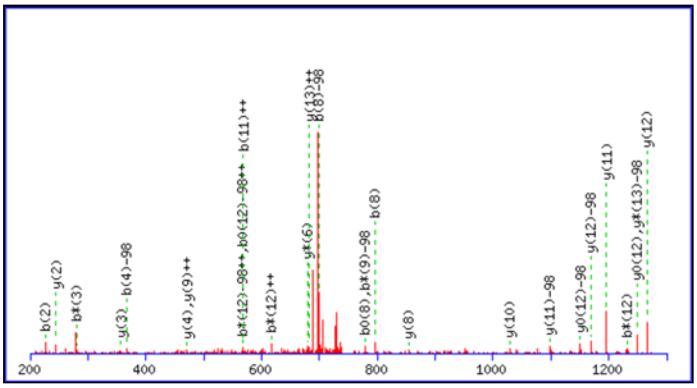
Monoisotopic mass of neutral peptide Mr(calc): 1782.760773
Variable modifications:
S7 : Phospho (ST), with neutral losses 0.000000(shown in table), 97.976896
C13 : Propionamide (C)
Matches (Bold Red): 44/248 fragment ions using 58 most intense peaks

#	b	b^{++}	b*	b^{*++}	b^0	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^0	y^{0++}	#
1	88.039304	44.523290			70.028739	35.518008	S							15
2	216.097882	108.552579	199.071333	100.039305	198.087317	99.547296	Q	1696.736017	848.871647	1679.709468	840.358372	1678.725452	839.866364	14
3	**331.124825**	166.066050	314.098276	157.552776	313.114260	157.060768	D	**1568.677439**	784.842358	**1551.650890**	776.329083	**1550.666874**	775.837075	13
4	**430.193239**	215.600257	413.166690	207.086983	**412.182674**	206.594975	V	**1453.650496**	**727.328886**	1436.623947	718.815612	1435.639931	718.323604	12
5	**501.230353**	251.118814	484.203804	242.605540	**483.219788**	242.113532	A	**1354.582082**	**677.794679**	1337.555533	**669.281405**	1336.571517	668.789397	11
6	**600.298767**	300.653022	**583.272218**	292.139747	**582.288202**	291.647739	V	**1283.544968**	642.276122	1266.518419	633.762848	1265.534403	633.270840	10
7	767.297119	384.152197	750.270570	375.638923	749.286554	375.146915	S	**1184.476554**	592.741915	1167.450005	584.228641	**1166.465989**	583.736633	9
8	864.349883	432.678580	847.323334	424.165305	846.339318	423.673297	P	**1017.478202**	509.242739	1000.451653	**500.729465**	999.467637	500.237457	8
9	992.408461	496.707869	975.381912	488.194594	974.397896	487.702586	Q	**920.425438**	460.716357	903.398889	452.203083	902.414873	451.711075	7
10	1120.467039	560.737157	1103.440490	552.223883	**1102.456474**	551.731875	Q	792.366860	396.687068	775.340311	388.173794	774.356295	387.681786	6
11	**1248.525617**	624.766447	1231.499068	616.253172	1230.515052	615.761164	Q	**664.308282**	332.657779	647.281733	324.144505	646.297717	323.652497	5
12	**1376.584195**	688.795736	1359.557646	680.282461	1358.573630	679.790453	Q	**536.249704**	268.628490	519.223155	260.115216	518.239139	259.623208	4
13	**1550.630489**	775.818883	1533.603940	767.305608	**1532.619924**	766.813600	C	408.191126	204.599201	391.164577	196.085927	390.180561	195.593919	3
14	**1637.662517**	819.334897	1620.635968	810.821622	**1619.651952**	810.329614	S	234.144832	117.576054	217.118283	109.062780	216.134267	108.570772	2
15							K	147.112804	74.060040	130.086255	65.546765			1

Figure S2

C

pS109
KPApSSSSAPQNIPK



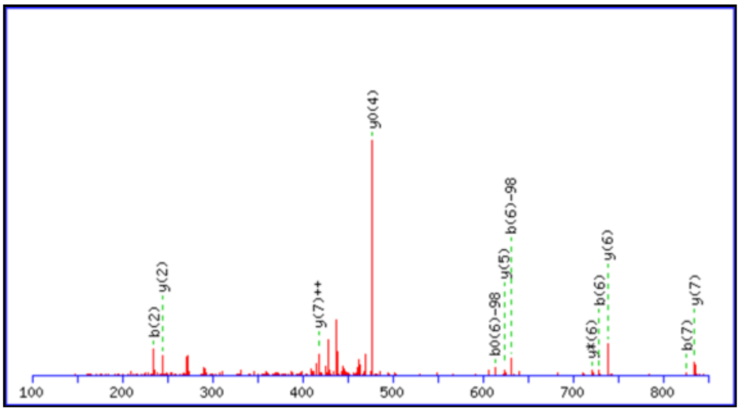
Monoisotopic mass of neutral peptide Mr(calc): 1490.712982
Variable modifications:
S4 : Phospho (ST), with neutral losses 97.976896(shown in table), 0.000000
Matches (Bold Red): 29/214 fragment ions using 49 most intense peaks

#	b	b^{++}	b^*	b^{*++}	b^0	b^{0++}	Seq.	y	y^{++}	y^*	y^{*++}	y^0	y^{0++}	#
1	129.102239	65.054757	112.075690	56.541483			K							14
2	**226.155003**	113.581139	209.128454	105.067865			P	1265.648433	633.327855	**1248.621884**	624.814580	1247.637868	624.322572	13
3	297.192117	149.099696	**280.165568**	140.586422			A	**1168.595669**	584.801473	1151.569120	576.288198	**1150.585104**	575.796190	12
4	**366.213573**	183.610424	349.187024	175.097150	348.203008	174.605142	S	**1097.558555**	549.282916	1080.532006	540.769641	1079.547990	540.277633	11
5	453.245601	227.126438	436.219052	218.613164	435.235036	218.121156	S	**1028.537099**	514.772188	1011.510550	506.258913	1010.526534	505.766905	10
6	540.277629	270.642452	523.251080	262.129178	522.267064	261.637170	S	941.505071	**471.256174**	924.478522	462.742899	923.494506	462.250891	9
7	627.309657	314.158466	610.283108	305.645192	609.299092	305.153184	S	**854.473043**	427.740160	837.446494	419.226885	836.462478	418.734877	8
8	**698.346771**	349.677023	681.320222	341.163749	680.336206	340.671741	A	767.441015	384.224146	750.414466	375.710871			7
9	795.399535	398.203405	**778.372986**	389.690131	777.388970	389.198123	P	696.403901	348.705589	**679.377352**	340.192314			6
10	923.458113	462.232694	906.431564	453.719420	905.447548	453.227412	Q	599.351137	300.179207	582.324588	291.665932			5
11	1037.501040	519.254158	1020.474491	510.740884	1019.490475	510.248876	N	**471.292559**	236.149917	454.266010	227.636643			4
12	**1150.585104**	575.796190	1133.558555	**567.282916**	1132.574539	**566.790908**	I	**357.249632**	179.128454	340.223083	170.615179			3
13	1247.637868	624.322572	1230.611319	615.809298	1229.627303	615.317290	P	**244.165568**	122.586422	227.139019	114.073148			2
14							K	147.112804	74.060040	130.086255	65.546765			1

Figure S2

D

pT173
HPNQApTPK



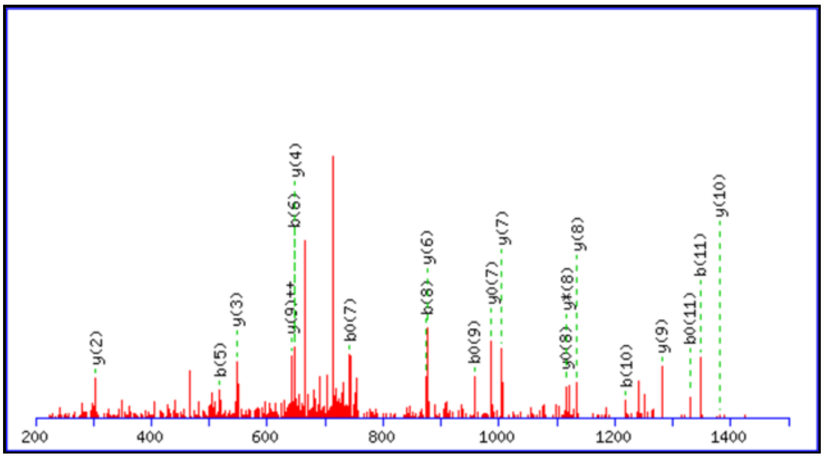
Monoisotopic mass of neutral peptide Mr(calc): 971.422577
Variable modifications:
T6 : Phospho (ST), with neutral losses 0.000000(shown in table), 97.976896
Matches (Bold Red): 12/108 fragment ions using 24 most intense peaks

#	b	b^{++}	b^{*}	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y^{*}	y^{*++}	y^{0}	y^{0++}	#
1	138.066188	69.536732					H							8
2	**235.118952**	118.063114					P	**835.370954**	**418.189115**	818.344405	409.675841	817.360389	409.183833	7
3	349.161879	175.084577	332.135330	166.571303			N	**738.318190**	369.662733	**721.291641**	361.149459	720.307625	360.657451	6
4	477.220457	239.113867	460.193908	230.600592			Q	**624.275263**	312.641270	607.248714	304.127995	606.264698	303.635987	5
5	548.257571	274.632424	531.231022	266.119149			A	496.216685	248.611981	479.190136	240.098706	**478.206120**	239.606698	4
6	**729.271574**	365.139425	712.245025	356.626151	711.261009	356.134143	T	425.179571	213.093424	408.153022	204.580149	407.169006	204.088141	3
7	**826.324338**	413.665807	809.297789	405.152533	808.313773	404.660525	P	**244.165568**	122.586422	227.139019	114.073148			2
8							K	147.112804	74.060040	130.086255	65.546765			1

Figure S2

E

pY225
AAVFEEIDTpYER



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Variable modifications:
Y10 : Phospho (Y)
Matches (Bold Red): 20/100 fragment ions using 44 most intense peaks

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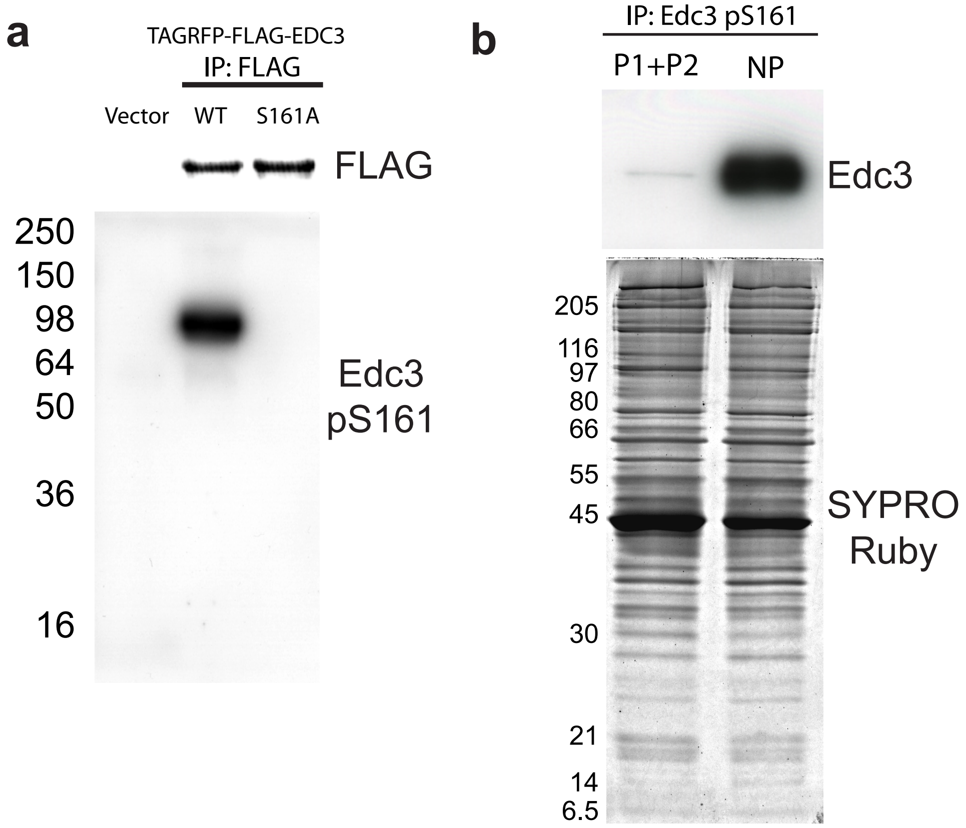
1	72.044390	36.525833			A							12
2	143.081504	72.044390			A	1451.609012	726.308144	1434.582463	717.794870	1433.598447	717.302861	11
3	242.149918	121.578597			V	**1380.571898**	690.789587	1363.545349	682.276313	1362.561333	681.784304	10
4	389.218332	195.112804			F	**1281.503484**	**641.255380**	1264.476935	632.742105	1263.492919	632.250097	9
5	**518.260925**	259.634101	500.250360	250.628818	E	**1134.435070**	567.721173	**1117.408521**	559.207898	**1116.424505**	558.715890	8
6	**647.303518**	324.155397	629.292953	315.150115	E	**1005.392477**	503.199876	988.365928	494.686602	**987.381912**	494.194594	7
7	760.387582	380.697429	**742.377017**	371.692147	I	**876.349884**	438.678580	859.323335	430.165305	858.339319	429.673297	6
8	**875.414525**	438.210901	857.403960	429.205618	D	763.265820	382.136548	746.239271	373.623273	745.255255	373.131265	5
9	976.462204	488.734740	**958.451639**	479.729458	T	**648.238877**	324.623076	631.212328	316.109802	630.228312	315.617794	4
10	**1219.491857**	610.249567	1201.481292	601.244284	Y	**547.191198**	274.099237	530.164649	265.585962	529.180633	265.093954	3
11	**1348.534450**	674.770863	**1330.523885**	665.765580	E	**304.161545**	152.584410	287.134996	144.071136	286.150980	143.579128	2
12					R	175.118952	88.063114	158.092403	79.549839			1

Figure S3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b
IP: Edc3 pS161
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NP
Edc3
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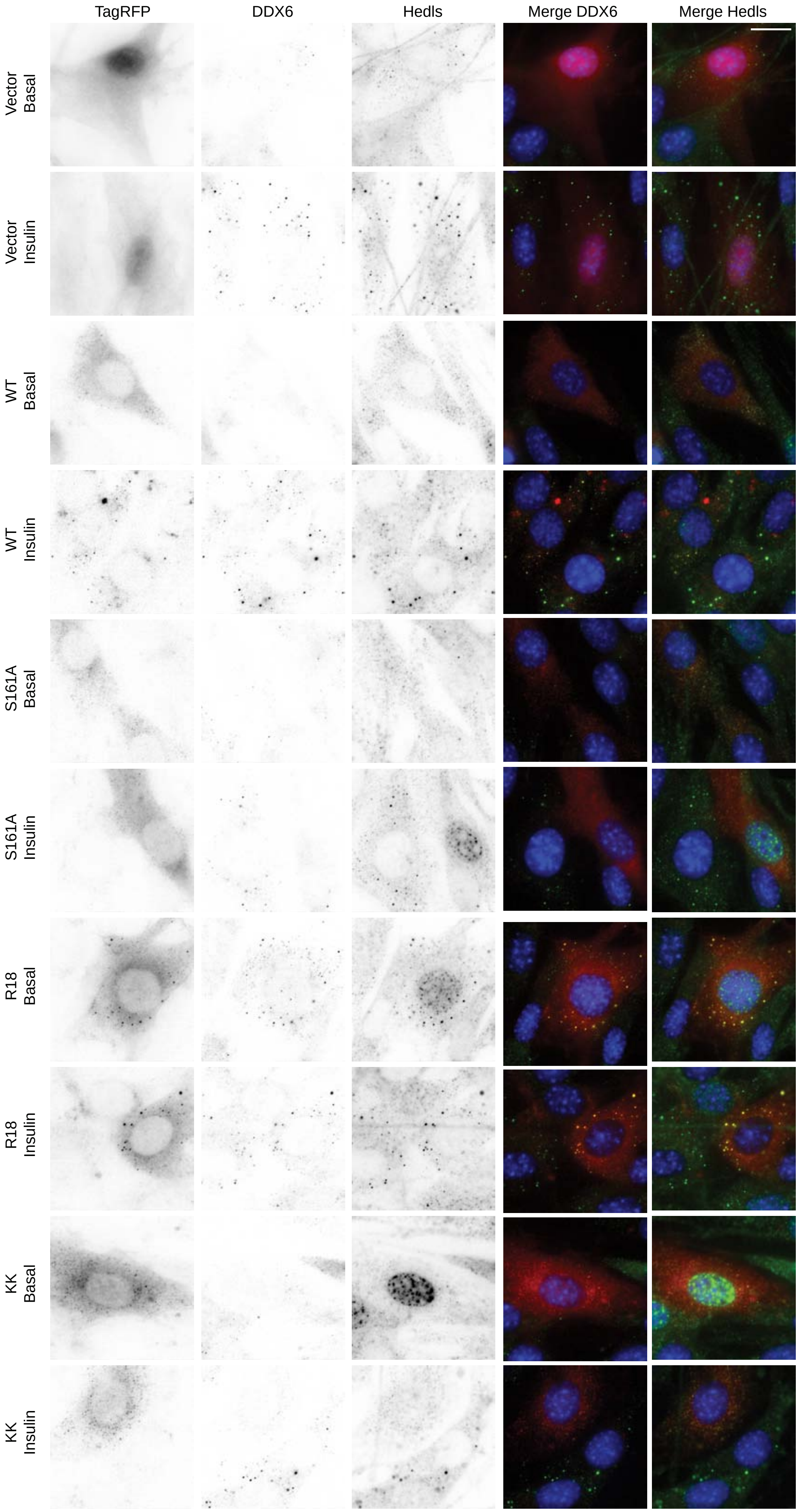
116
97
80
66
55
45
30
21
14
6.5
SYPRO
Ruby

Figure S4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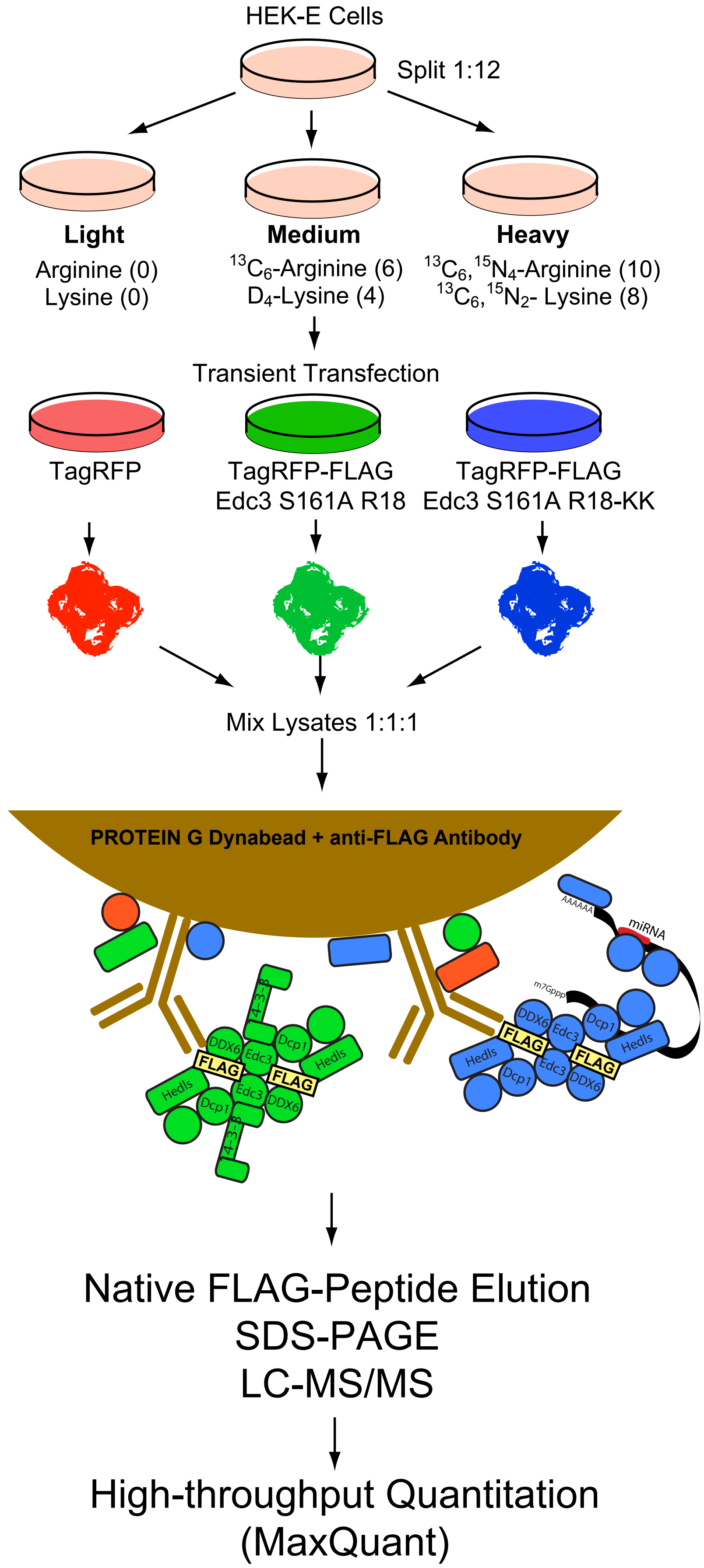
R18 Insulin
KK Basal
KK Insulin

Figure S5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D_4-Lysine (4)
Heavy
$^{13}C_6$, $^{15}N_4$-Arginine (10)
$^{13}C_6$, $^{15}N_2$- Lysine (8)
Transient Transfection
TagRFP
TagRFP-FLAG
Edc3 S161A R18
TagRFP-FLAG
Edc3 S161A R18-KK
Mix Lysates 1:1:1
PROTEIN G Dynabead + anti-FLAG Antibody
14-3-3β
DDX6
Edc3
Dcp1
Hedls
FLAG
AAAAAA
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SDS-PAGE
LC-MS/MS
High-throughput Quantitation
(MaxQuant)