Manuscript JCI #40582: p90 ribosomal S6 kinase 2 promotes head and neck cancer cell invasion and tumor metastasis

Supplemental Experimental Procedures

Phospho-Antibody Array Data Analysis (PANDA). For the data analysis, background signals were removed from all measurements. Next, for each antibody, its respective negative control value was removed from each measurement. The results from the 6 replicate samples are averaged, after potential outliers that are statistically inconsistent with other replicates (possibly due to an experiment error) are discarded. More specifically, the outlier measurement is detected using the Grubbs' test or ESD method (extreme studentized deviate). The test calculates a ratio Z as the difference between the potential outlier value y_i and the mean of the replicates y_{mean} divided by the standard deviation SD.

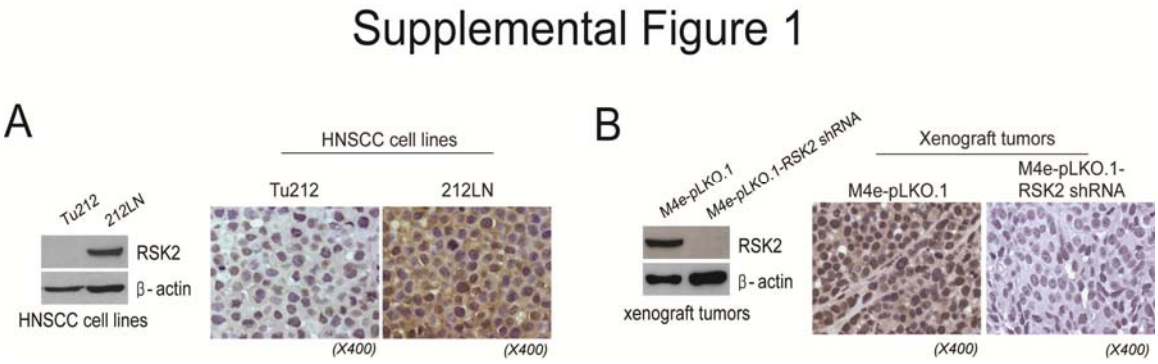
$$Z = \frac{| y_{mean} - y_i |}{SD}$$

The ratio is then compared to a critical value determined by the sample size and the desired significance level. In our case, the sample size is 6 and the significance level with alpha = 0.05 is used. Finally, the adjusted mean is computed after the outliers are removed. A ratio computation was used to measure the extent of protein phosphorylation. For each antibody (that has phosphorylated and matching unphosphorylated values denoted by phospho and unphospho in both the control data and experiment data), we computed the following phosphorylation ratio:

$$phophorylation\_ratio = \frac{phospho_{experiment}}{unphospho_{experiment}} \Big/ \frac{phospho_{control}}{unphospho_{control}}$$

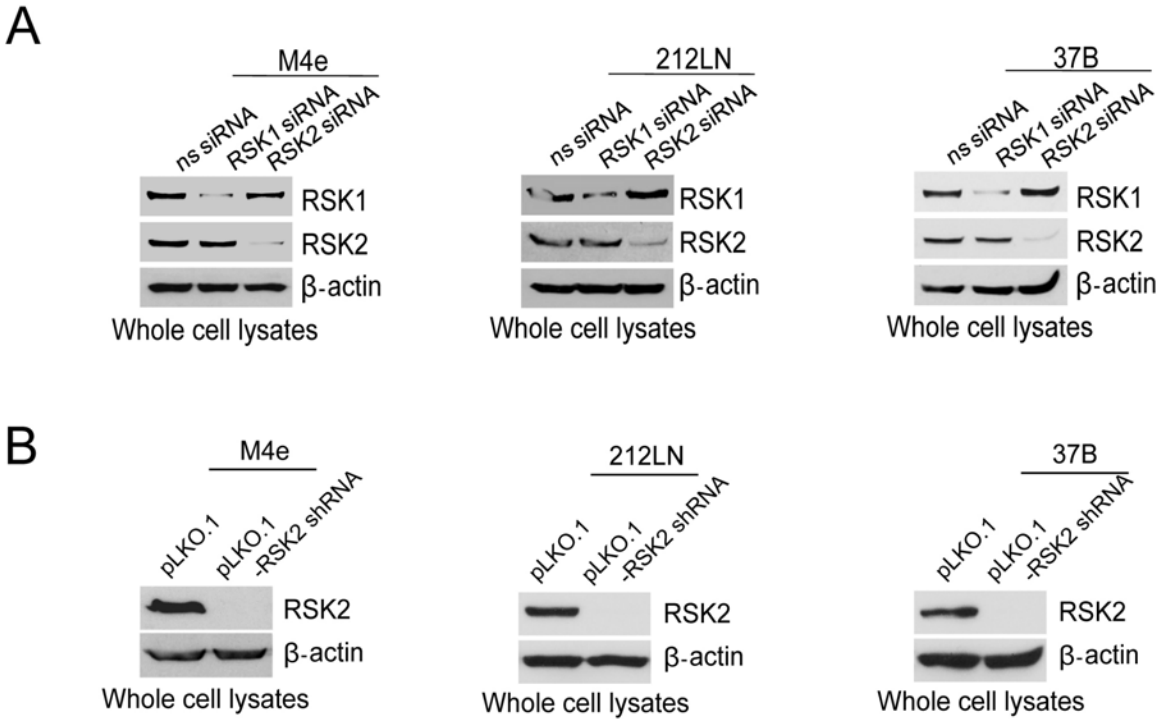
A 95% confidence interval was used to quantify the precision of the phosphorylation ratio based on the analysis of the replicates. A web-based program for conducting the data analysis and generating the result table is available at http://www.mathcs.emory.edu/panda.

Supplemental Figures

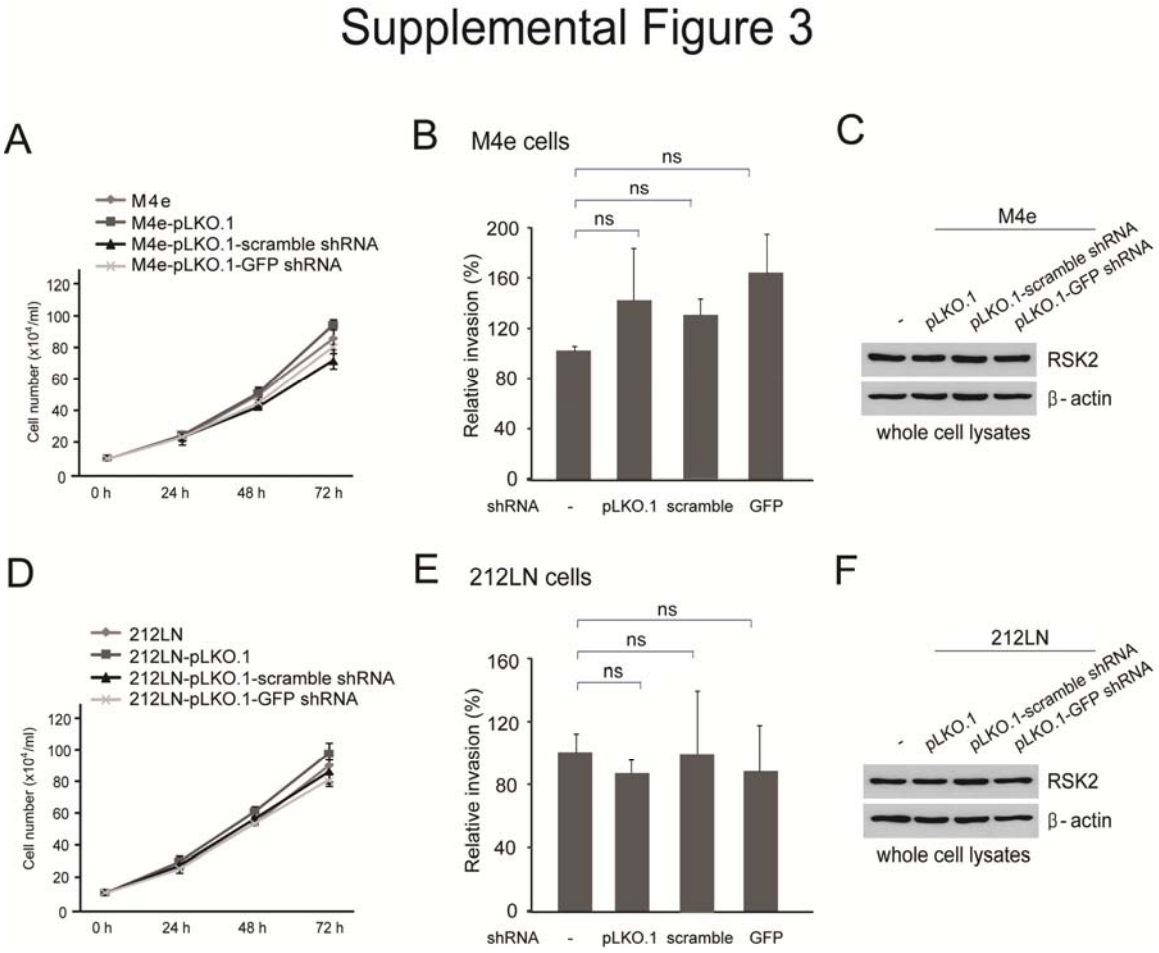


Supplemental Figure 1
Characterization of RSK2 antibody in diverse IHC experiments using samples from (**A**) embedded Tu212 and 212LN cell pellets, and (**B**) tumors harvested from xenograft mice injected with indicated cells.

Supplemental Figure 2

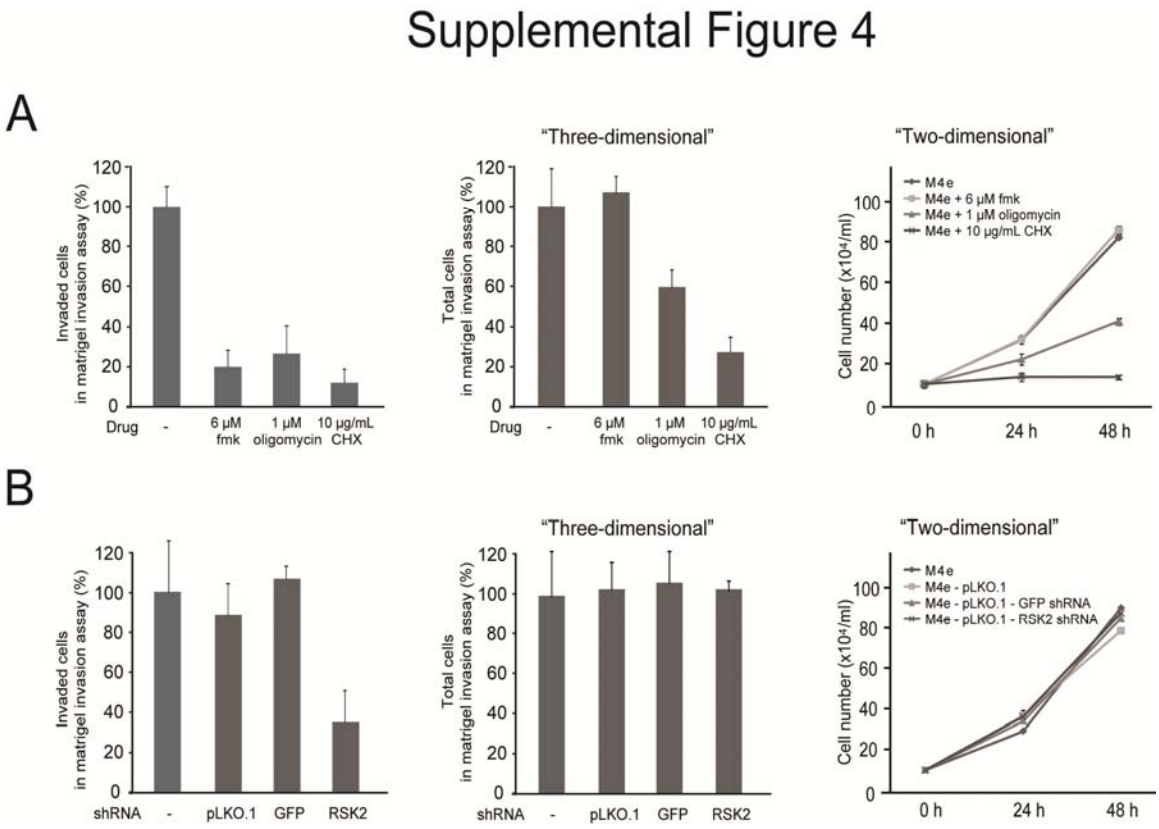


Supplemental Figure 2
Control Western blot experiments show RNAi-mediated transient (**A**) or stable (**B**) knockdown of RSK2 in M4e, 212LN and 37B in the cell proliferation experiments (Figure 3C and 3F, respectively).



Supplemental Figure 3

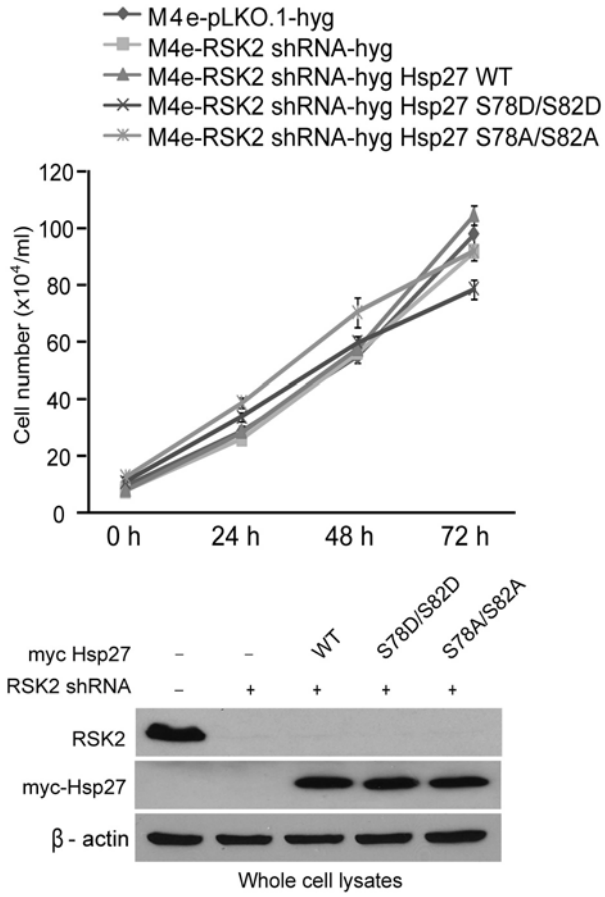
Stable transduction of M4e and 212LN cells with lentiviral vectors harboring scramble shRNA or shRNA targeting GFP does not significantly affect proliferation rate or invasive ability of M4e and 212LN cells. (**A**) Cell proliferation assay using M4e-pLKO.1, m4e-pLKO.1-scramble shRNA, m4e-pLKO.1-GFP shRNA and parental M4e cells. (**B**) In vitro Matrigel cell invasion assay using M4e-pLKO.1, M4e-pLKO.1-scramble shRNA, M4e-pLKO.1-GFP shRNA and parental M4e cells. (**C**) Protein expression levels of RSK2 or β-actin were not altered in M4e cells by scramble shRNA or GFP shRNA. (**D**) Cell proliferation assay using 212LN-pLKO.1, 212LN-pLKO.1-scramble shRNA, 212LN-pLKO.1-GFP shRNA and parental 212LN cells. (**E**) In vitro Matrigel cell invasion assay using 212LN-pLKO.1, 212LN -pLKO.1-scramble shRNA, 212LN-pLKO.1-GFP shRNA and parental 212LN cells. (**F**) Protein expression levels of RSK2 or β-actin were not altered in 212LN cells by scramble shRNA or GFP shRNA.



Supplemental Figure 4

Targeting RSK2 by small molecule inhibitors or shRNA attenuates invasive potential of HNSCC M4e cells but does not significantly affect cell proliferation in both "three-dimensional" and "two-dimensional" systems. (**A**) Effects of treatment with RSK specific inhibitor, RSKI-fmk on cell invasive ability and proliferation rate of HNSCC M4e cells. Relative cell invasive ability was obtained by in vitro Matrigel cell invasion assay (left panel). Cell proliferation rates were obtained by counting the remaining non-invasive cells in the chamber, plus the invaded cells ("three-dimensional system"), at the same time point when the invaded cells were counted (middle panel). An additional cell proliferation assay was performed in the "two-dimensional system" as described in our manuscript, and relative cell proliferation rates at each experimental time point were determined by using the Celltiter96AQ$_{ueous}$ proliferation kit (right panel). (**B**) Effects of stable transduction with lentiviral vector harboring shRNA targeting RSK2 on cell invasive ability, as well as proliferation rate of HNSCC M4e cells using both "three-dimensional" and "two-dimensional" systems. Stable cells harboring empty lentiviral vector or shRNA targeting GFP were included as negative controls.

Supplemental Figure 5



Supplemental Figure 5
RNAi-mediated stable knockdown of RSK2 or stable expression of Hsp27 WT, S78D/S82D or S78A/S82A variants did not significantly affect the proliferation rate of M4e cells.

Supplemental Table 1

Phosphorylation Sites	Control phospho	Control unphospho	treatment phospho	treatment unphospho	ratio	95% CI
IRS-1(Phospho-Ser639)	866	3970	677	4642	0.67	(0.56 - 0.78)
Histone H2A.X(Phospho-Ser139)	1879	1776	1662	2066	0.76	(0.71 - 0.81)
c-Jun(Phospho-Thr93)	1083	267	798	258	0.76	(0.73 - 0.79)
c-Jun(Phospho-Ser73)	1224	4721	957	4786	0.77	(0.72 - 0.82)
FAK(Phospho-Tyr861)	1156	643	868	616	0.78	(0.72 - 0.85)
Tau(Phospho-Ser396)	1116	274	858	269	0.78	(0.76 - 0.81)
CREB(Phospho-Ser133)	1177	351	852	317	0.80	(0.77 - 0.84)
HSP27(Phospho-Ser78)	889	174	755	181	0.81	(0.77 - 0.86)
Tau(Phospho-Thr212)	1075	219	867	216	0.82	(0.77 - 0.86)
CREB(Phospho-Ser129)	958	307	762	298	0.82	(0.79 - 0.85)
Tau(Phospho-Thr181)	649	270	531	269	0.82	(0.78 - 0.86)
FAK(Phospho-Tyr925)	717	364	559	346	0.82	(0.78 - 0.86)
JunB(Phospho-Ser79)	1003	379	780	359	0.82	(0.77 - 0.87)
Tau(Phospho-Ser404)	961	583	732	535	0.83	(0.79 - 0.87)
c-Jun(Phospho-Thr91)	615	209	482	197	0.83	(0.79 - 0.87)
Tau(Phospho-Ser214)	816	701	633	651	0.83	(0.78 - 0.89)
c-MET(Phospho-Tyr1234)	1196	855	884	756	0.84	(0.77 - 0.91)
Stathmin 1(Phospho-Ser24)	813	390	638	364	0.84	(0.75 - 0.93)
p44/42 MAP Kinase(Phospho-Tyr204)	883	356	712	341	0.84	(0.79 - 0.89)
Estrogen Receptor-alpha (Phospho-Ser118)	1045	351	823	328	0.84	(0.79 - 0.90)
SAPK/JNK(Phospho-Tyr185)	924	288	751	277	0.84	(0.79 - 0.90)
Elk1(Phospho-Thr417)	988	408	763	370	0.85	(0.79 - 0.92)
Estrogen Receptor-alpha (Phospho-Ser167)	1107	639	893	604	0.85	(0.78 - 0.92)
p53(Phospho-Ser6)	1011	342	890	352	0.86	(0.78 - 0.93)
4E-BP1(Phospho-Thr36)	1017	335	819	312	0.86	(0.83 - 0.90)
Tau(Phospho-Thr231)	938	735	718	650	0.87	(0.83 - 0.90)
Tau(Phospho-Ser262)	578	613	455	556	0.87	(0.83 - 0.90)
HSF1(Phospho-Ser303)	861	238	710	226	0.87	(0.80 - 0.93)
Tau(Phospho-Ser422)	943	705	741	635	0.87	(0.84 - 0.90)
JunB(Phospho-Ser259)	1022	769	814	701	0.87	(0.82 - 0.93)
Stathmin 1(Phospho-Ser37)	964	323	774	296	0.88	(0.83 - 0.92)
Elk-1(Phospho-Ser383)	1379	638	1000	527	0.88	(0.82 - 0.93)
IRS-1(Phospho-Ser636)	828	372	662	339	0.88	(0.83 - 0.92)
MEK1(Phospho-Thr291)	942	327	773	300	0.89	(0.86 - 0.93)
MEF2A(Phospho-Thr312)	675	933	533	825	0.89	(0.82 - 0.96)
HSP27(Phospho-Ser82)	735	653	609	603	0.90	(0.85 - 0.95)
JunD(Phospho-Ser255)	1026	416	812	367	0.90	(0.83 - 0.97)
ATF4(Phospho-Ser245)	1080	631	791	514	0.90	(0.83 - 0.97)
p53(Phospho-Thr18)	992	214	876	210	0.90	(0.84 - 0.96)
ATF2(Phospho-Ser62 or 44)	833	619	658	541	0.90	(0.83 - 0.98)
ASK1(Phospho-Ser966)	644	640	535	587	0.91	(0.86 - 0.95)
IRS-1(Phospho-Ser312)	845	458	692	414	0.91	(0.83 - 0.98)
SAPK/JNK(Phospho-Thr183)	892	552	752	511	0.91	(0.84 - 0.98)
ATF2(Phospho-Thr71 or 53)	541	354	458	328	0.91	(0.85 - 0.98)
p44/42 MAP Kinase(Phospho-Thr202)	806	421	696	397	0.91	(0.84 - 0.99)
Stathmin 1(Phospho-Ser15)	793	483	665	442	0.92	(0.87 - 0.96)
p53(Phospho-Ser15)	848	244	763	239	0.92	(0.84 - 0.99)
c-Jun(Phospho-Ser243)	968	762	752	646	0.92	(0.88 - 0.96)
MEF2A(Phospho-Thr319)	988	667	724	533	0.92	(0.86 - 0.98)
SEK1/MKK4(Phospho-Thr261)	691	470	585	430	0.93	(0.87 - 0.98)
Pyk2(Phospho-Tyr402)	986	479	803	420	0.93	(0.88 - 0.98)
elF4E(Phospho-Ser209)	747	445	660	422	0.93	(0.87 - 0.99)
Estrogen Receptor-alpha (Phospho-Ser106)	1131	3320	897	2827	0.93	(0.88 - 0.98)
MKK3(Phospho-Ser189)	614	447	514	399	0.94	(0.89 - 0.98)
MEK-2(Phospho-Thr394)	1193	963	870	745	0.94	(0.88 - 1.00)
p53(Phospho-Ser46)	909	368	824	354	0.94	(0.89 - 0.99)
c-Jun(Phospho-Tyr170)	931	638	737	536	0.94	(0.87 - 1.02)

P38 MAPK(Phospho-Tyr182)	778	326	711	313	0.95	(0.87 - 1.03)
Estrogen Receptor-alpha (Phospho-Ser104)	1016	1120	786	908	0.95	(0.89 - 1.02)
MEK1(Phospho-Ser217)	643	599	528	515	0.96	(0.91 - 1.00)
c-Jun(Phospho-Ser63)	856	578	701	492	0.96	(0.89 - 1.03)
c-Kit(Phospho-Tyr721)	996	533	872	484	0.96	(0.90 - 1.03)
Src(Phospho-Tyr529)	903	990	728	825	0.97	(0.92 - 1.01)
Elk1(Phospho-Ser389)	817	751	652	620	0.97	(0.90 - 1.03)
HSP27(Phospho-Ser15)	789	707	651	599	0.97	(0.91 - 1.03)
Src(Phospho-Tyr418)	758	557	665	498	0.98	(0.92 - 1.04)
Raf1(Phospho-Ser338)	1405	1138	1128	929	0.98	(0.94 - 1.03)
IRS-1(Phospho-Ser307)	511	421	462	384	0.99	(0.94 - 1.04)
4E-BP1(Phospho-Thr45)	316	478	282	430	0.99	(0.96 - 1.03)
Raf1(Phospho-Ser259)	1183	853	886	643	0.99	(0.93 - 1.05)
Met(Phospho-Tyr1349)	139	809	132	773	0.99	(0.95 - 1.04)
p53(Phospho-Ser33)	794	563	698	494	1.00	(0.94 - 1.06)
ATF2(Phospho-Thr73 or 55)	520	547	465	483	1.01	(0.95 - 1.07)
Tau(Phospho-Thr205)	841	925	694	753	1.01	(0.94 - 1.08)
p53(Phospho-Ser315)	623	549	585	508	1.01	(0.97 - 1.06)
MEK1(Phospho-Ser221)	776	516	697	455	1.02	(0.89 - 1.14)
p53(Phospho-Ser9)	578	532	520	463	1.03	(0.96 - 1.11)
Tau(Phospho-Ser235)	2364	856	2303	786	1.06	(0.99 - 1.13)
Myc(Phospho-Thr358)	522	1187	497	1051	1.07	(1.02 - 1.13)
ASK1(Phospho-Ser83)	636	805	580	679	1.08	(1.01 - 1.16)
p53(Phospho-Ser37)	666	844	589	689	1.08	(1.05 - 1.11)
Histone H3.1(Phospho-Ser10)	509	773	460	642	1.09	(1.03 - 1.15)
Myc(Phospho-Thr58)	712	736	656	616	1.10	(1.04 - 1.17)
Myc(Phospho-Ser373)	493	616	459	515	1.11	(1.04 - 1.19)
ATF2(Phospho-Ser112 or 94)	704	907	621	714	1.12	(1.01 - 1.23)
Tau(Phospho-Ser356)	461	618	450	532	1.13	(1.00 - 1.26)
ATF2(Phospho-Thr69 or 51)	731	513	719	442	1.14	(0.89 - 1.40)