– Supporting Information –

Reproducible Automated Phosphopeptide Enrichment using Magnetic TiO_2 and Ti-IMAC

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ABSTRACT: Reproducible, comprehensive phosphopeptide enrichment is essential to study phosphorylation-regulated processes. Here, we describe the application of hyper-porous magnetic TiO2 and Ti-IMAC for uniform automated phosphopeptide enrichment. Combining hyper-porous magnetic microspheres with a magnetic particle-handling robot enables rapid (45 minutes), reproducible ($r2 \geq 0.80$) and high-fidelity (> 90% purity) phosphopeptide purification in a 96-well format. Automated phosphopeptide enrichment demonstrates reproducible synthetic phosphopeptide recovery across two-orders of magnitude, "well-to-well" quantitative reproducibility indistinguishable to internal SILAC standards and robust "plate-to-plate" reproducibility across 5-days of independent enrichments. As a result, automated phosphopeptide enrichment enables statistical analysis of label-free phosphoproteomic samples in a high-throughput manner. This technique uses commercially available, off-the-shelf components and can be easily adopted by any laboratory interested in phosphoproteomic analysis. We provide a free downloadable automated phosphopeptide enrichment program to facilitate uniform inter-laboratory collaboration and exchange of phosphoproteomic data sets.

SUPPLEMENTAL EXPERIMENTAL SECTION

Reagents. Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich. TiO2 (MR-TID010) and Ti-IMAC (MR-TIM010) hyper-porous magnetic microspheres were purchased from ReSyn Biosciences. Custom synthesized ZrO2 magnetic microspheres were a kind gift from Dr. Isak Gerber and Dr. Justin Jordaan (ReSyn Biosciences). Titansphere™ microspheres were purchased from GL Sciences (5020) and MagSeph TiO2 microspheres were purchased from GE Healthcare (28-9440-10). All synthetic phosphopeptides were obtained as crude synthetics from INTRAVIS.

Peptide Sample Preparation. Pdx1-Cre; KRAS G12D/+; p53R172H/+ (KPC) pancreatic cancer cells (a kind gift from Dr. Owen Sansom, Beatson Institute, Glasgow) and HEK293 cells were maintained in DMEM (Gibco 41966052) +10% (v/v) FBS at 37ºC, 5% CO2. For "Heavy" SILAC isotopic labeling, KPC cells were grown in K/R-free DMEM (Caisson DMP49) supplemented with 10% dialysed FBS, 2.5 mM +8 Da *L*-lysine (Sigma Isotec 608041) and 2.5 mM +10 Da *L*-Arginine (Sigma Isotec 608033). "Light" KPC cells were grown to confluency in a 15cm dish, treated for 5 minutes with 100 ng/mL EGF (R&D Systems 236-EG), lysed in 6M urea buffer (6 M urea, 10 mM NaPPi, 20 mM HEPES pH 8), sonicated, centrifuged to clear cell debris and protein concentration was determined by BCA (Pierce 23225). "Heavy" KPC cells were treated identically but without EGF stimulation. 10 mg of each protein lysate was digested with 100 µg Lys-C (Wako 125-05061) (24 hours) and 100 µg Trypsin (Worthington) (24 hours) using the FASP method [1, 2] (Sartorious VS02H21). Note: each figure displayed in this manuscript uses a unique FASP tryptic digest.

Three separate isolations of iKRAS PDA cells [3] (a kind gift from Dr. Haoqiang Ying and Dr. Ronald A. DePinho, MD Anderson Cancer Center) were maintained in DMEM (Gibco 41966052) +10% (v/v) FBS +1 µg/mL doxycycline at 37ºC, 5% CO2. For multivariate phosphoproteomic analysis, each iKRAS cell line was cultured without doxycycline for one full passage (i.e. KRAS-WT), seeded in 10 cm dishes and then cultured +/- 1 µg/mL doxycycline (i.e. KRAS-G12D). After 24 hours cells were lysed in urea buffer. Lysates were Li-Cor fluorescent immuno-blotted for RAS(G12D) (NewEast Bioscience 26036), RAS (Abcam ab52939), ERK1/2 (pT183 pY185) (Sigma M8159), ERK1/2 (CST 4695), and β-Actin (Abcam ab8227). Phosphopeptides were automatically enriched as above (200 µg lysate / biological replicate).

Manual Phosphopeptide Enrichment. For the manual phosphopeptide enrichment detailed in Supplemental Fig. 1, 450 µg "cold" HEK293 tryptic digest was combined with 50 µg 32P-labeled digest (~40,000 cpm / vial) in 80% MeCN, 5% TFA, 1M glycolic acid [4]. 500 µg peptide mix was incubated with 1 mg microspheres for 20 minutes under agitation. Microspheres were resolved (either by magnet or centrifugation) and washed with 80% MeCN, 1% TFA (3 x 2 mins). Phosphopeptides were eluted in 1% NH4OH for 15 minutes (under agitation), acidified with FA and lyophilised. The location of phosphorylated material was calculated using a scintillation counter. For all 32P-labelled experiments, an identical "cold" cold experiment was performed for LC-MS/MS analysis. For the manual enrichment in Fig. 2a, identical buffers and incubation times were used to the automated method (see below).

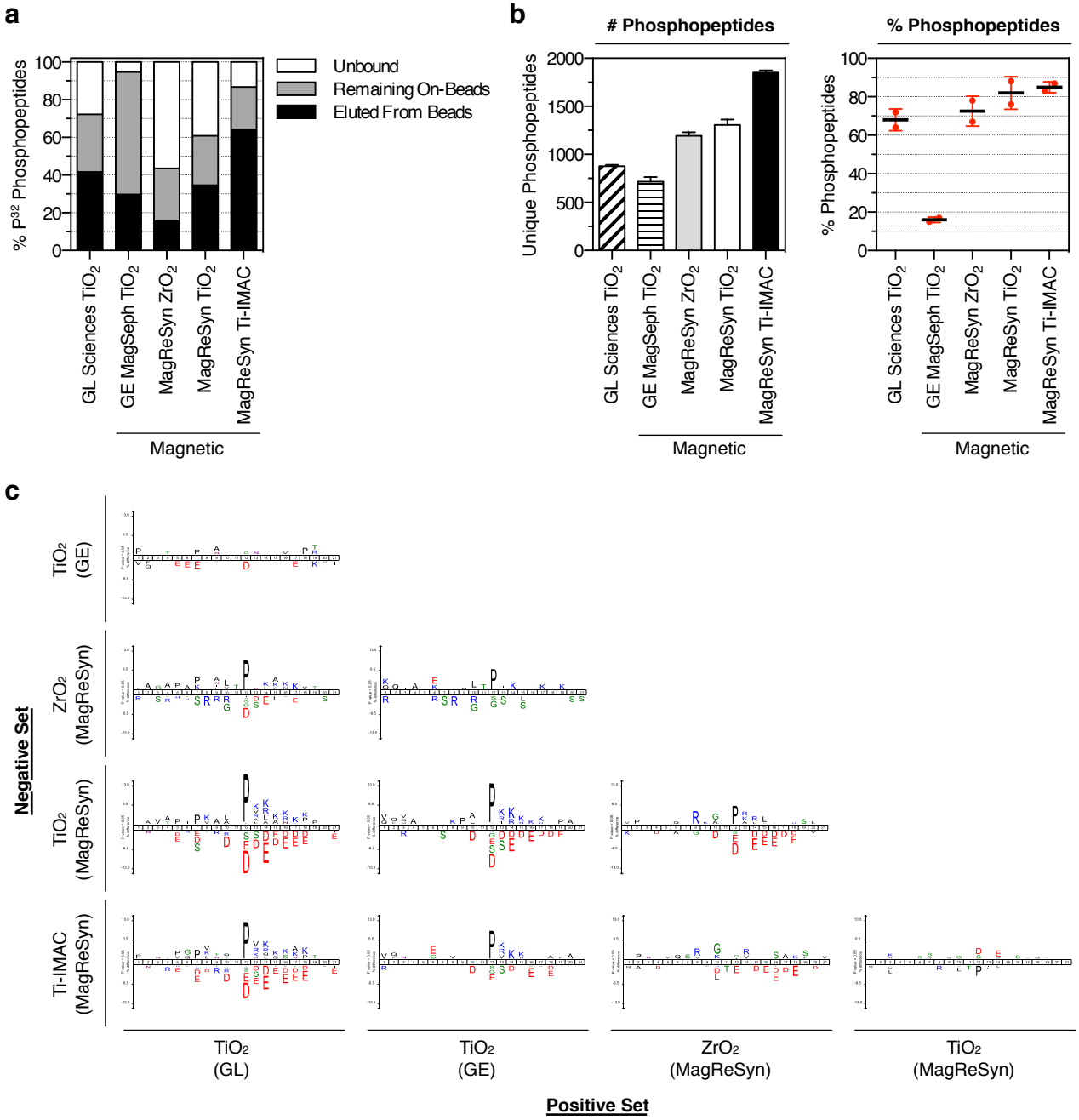
Data-Dependent Acquisition (DDA) LC-MS/MS (Orbitrap Velos). Samples shown in Figures 1-4 were subjected to data dependent analysis (DDA) on a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) coupled to a NanoLC-Ultra 2D (Eksigent). Reversed-phase chromatographic separation was carried out on an Acclaim PepMap100 C18 Nano-Trap Column (100 µm i.d. x 2 cm packed with C18, 5 µm bead size, 100 Å) (Thermo Scientific), and a 75 µm i.d. x 30cm column packed in house with C18 (5 µm bead size, Reprosil-Gold, Dr Maisch) using a 120 minute gradient of 3-40% solvent B (MeCN 100% + 0.1% FA) against solvent A (H2O 100% + 0.1% FA) with a flow rate of 300 nL/min. The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap MS and MS/MS acquisition. Survey full scan MS spectra (from m/z 375-2000) were acquired in the Orbitrap with a resolution of 60,000 at m/z 400 and FT target value of 1 x 10^{6} ions. The 10 most abundant ions were selected for fragmentation using the HCD and scanned in the Orbitrap at a resolution of 7,500 at m/z 400. Selected ions were dynamically excluded for 8 seconds. For accurate mass measurement, the lock mass option was enabled using the polydimethylcyclosiloxane ion (m/z 445.120025) as an internal calibrant.

DDA LC-MS/MS (Q-Exactive Plus). Samples shown in Figure 5 were run on a Q-Exactive Plus mass spectrometer (Thermo Scientific) coupled to a Dionex Ultimate 3000 RSLC nano system (Thermo Scientific). Reversed-phase chromatographic separation was performed on a C18 PepMap 300 Å trap cartridge (0.3 mm i.d. x 5 mm, 5 µm bead size; loaded in a bi-directional manner), a 75 µm i.d. x 50 cm column (5 µm bead size) using a 120 minute linear gradient of 0-50% solvent B (MeCN 100% + 0.1% formic acid (FA)) against solvent A (H2O 100% + 0.1% FA) with a flow rate of 300 nL/min. The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap MS and MS/MS acquisition. Survey full scan MS spectra (from m/z 400-2000) were acquired in the Orbitrap with a resolution of 70,000 at m/z 400 and FT target value of 1 x 10^6 ions. The 15 most abundant ions were selected for fragmentation using higher-energy collisional dissociation (HCD) and dynamically excluded for 30 seconds. Fragmented ions were scanned in the Orbitrap at a resolution of 17,500 at m/z 400. For accurate mass measurement, the lock mass option was enabled using the polydimethylcyclosiloxane ion (m/z 445.120025) as an internal calibrant.

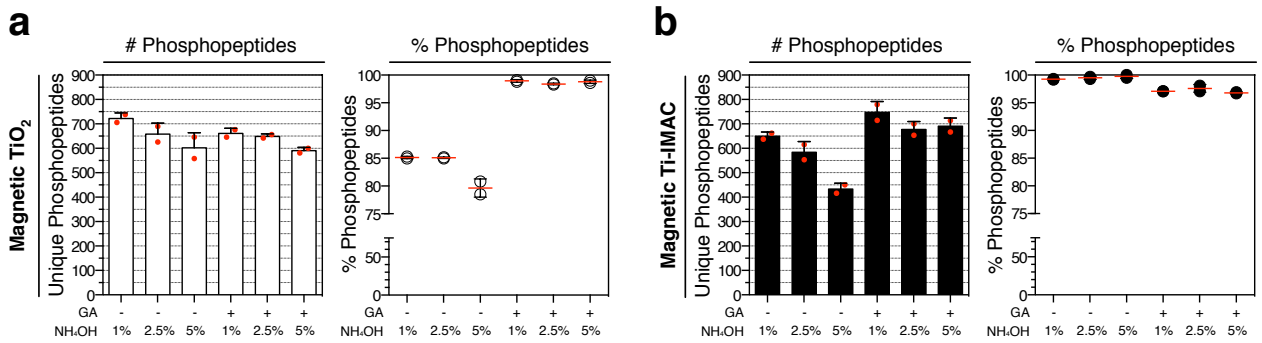
LC-MS/MS Data Processing. For peptide identification, raw data files produced in Xcalibur 2.1 (Thermo Scientific) were processed in Proteome Discoverer 1.4 (Thermo Scientific) and searched against either the SwissProt mouse database (2011_03 release, 15,082,690 entries) (KPC samples) or SwissProt human (20012_02 release, 20,662,136 entries) (HEK293 samples) using Mascot 2.2 (Matrix Science). Searches were performed with a precursor mass tolerance set to 10 ppm, fragment mass tolerance set to 0.05 Da and a maximum number of missed cleavages set to 2. Static modifications were limited to carbamidomethylation of cysteine, and variable modifications used were oxidation of methionine, deamidation of asparagine / glutamine, isotopomeric labeled lysine and arginine (+8/+10 Da) and phosphorylation of serine, threonine and tyrosine residues. Peptides were further filtered using a mascot significance threshold ≤0.05, a peptide ion Score ≥20 and a FDR ≤0.01 (evaluated by Percolator [5]). Localization probabilities for the phosphorylated residue(s) were calculated with phospho*RS* 3.1 [6]. Only phospho*RS* localization probabilities ≥75% were assigned. Phosphopeptide alignments were generated using ProteinModificationToolkit (http://ms.imp.ac.at/?goto=pmt) such that the phosphorylation is centred at position #11 +/- 10 local residues. Comparative amino acid motifs were produced using iceLogo 1.2 [7]. A complete comparison matrix of all affinity reagents was produced by assigning each aligned dataset as either a 'Positive' or 'Negative' set. Precursor area quantification was performed in Proteome Discoverer 1.4. All statistics were performed in GraphPad Prism 6. Principle Component Analysis (PCA) was performed in R using the 'prcomp' function.

The #205 .raw DDA files described in this manuscript have been deposited to the ProteomeXchange Consortium (http://www.proteomexchange.org) via the PRIDE partner repository [8] with the dataset identifier PXD000892.

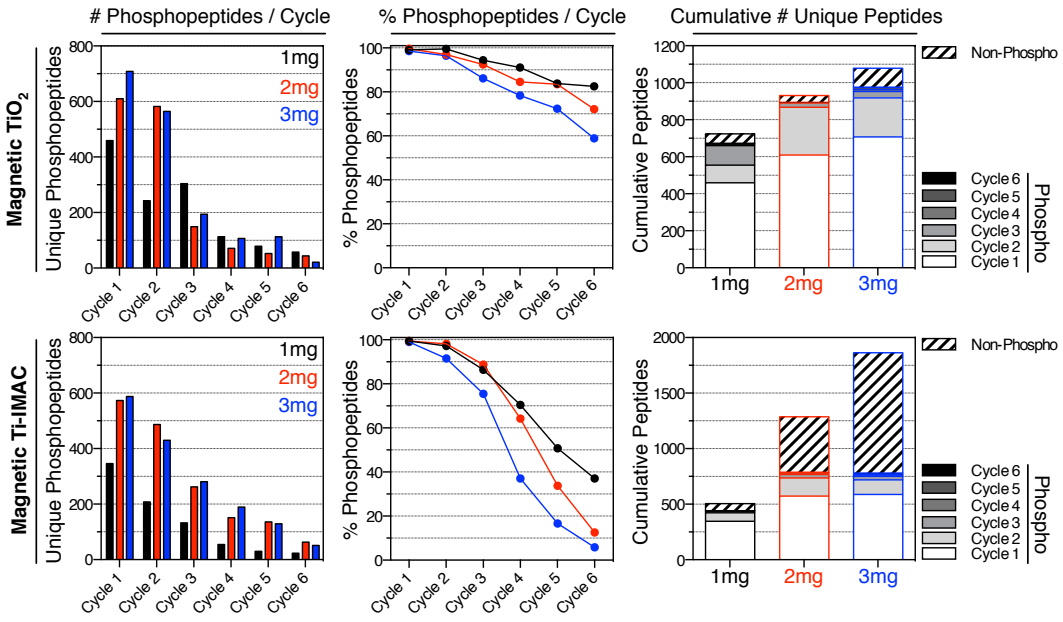
Selected Reaction Monitoring (SRM) LC-MS/MS. All synthetic phosphopeptide samples were analysed on a TSQ Vantage triple quadruple mass spectrometer (Thermo Scientific) coupled to a NanoLC-Ultra 1D (Eksigent). Reversed-phase chromatographic separation was performed on an Acclaim PepMap100 C18 Nano-Trap Column (100 µm i.d. x 2 cm packed with C18, 5 µm bead size, 100 Å) (Thermo Scientific) and a NTCC-360 packed tip column (75 µm i.d. 15cm, 3 µm particle size) (Nikkyo Technos) with a 30 min linear gradient of 5-50% solvent B (MeCN 100% + 0.1% FA). The TSQ Vantage was operated with a Q1 unit resolution of 0.7 FWHM and a Q3 of 0.7 FWHM, an ion spray voltage of 2200V and a capillary inlet temperature of 270°C. Peptide fragmentation carried out in Q2 at 1.5 mTorr and collision energies for each peptide were predicted [9]. Each SRM transition has a minimum dwell time of 20 ms, with cycle times of 1.2 s. The raw data files were produced in Xcalibur 2.1 (Thermo Scientific) and all data was processed using Skyline 2.1 [10]. Representative chromatograms of the synthetic phosphopeptide used in this study can be found in Supplementary Fig. 5. Mass values for quantified transitions can be found in Supplementary Table 1. All SRM files have been uploaded to PeptideAtlas with the dataset identifier PASS00472.



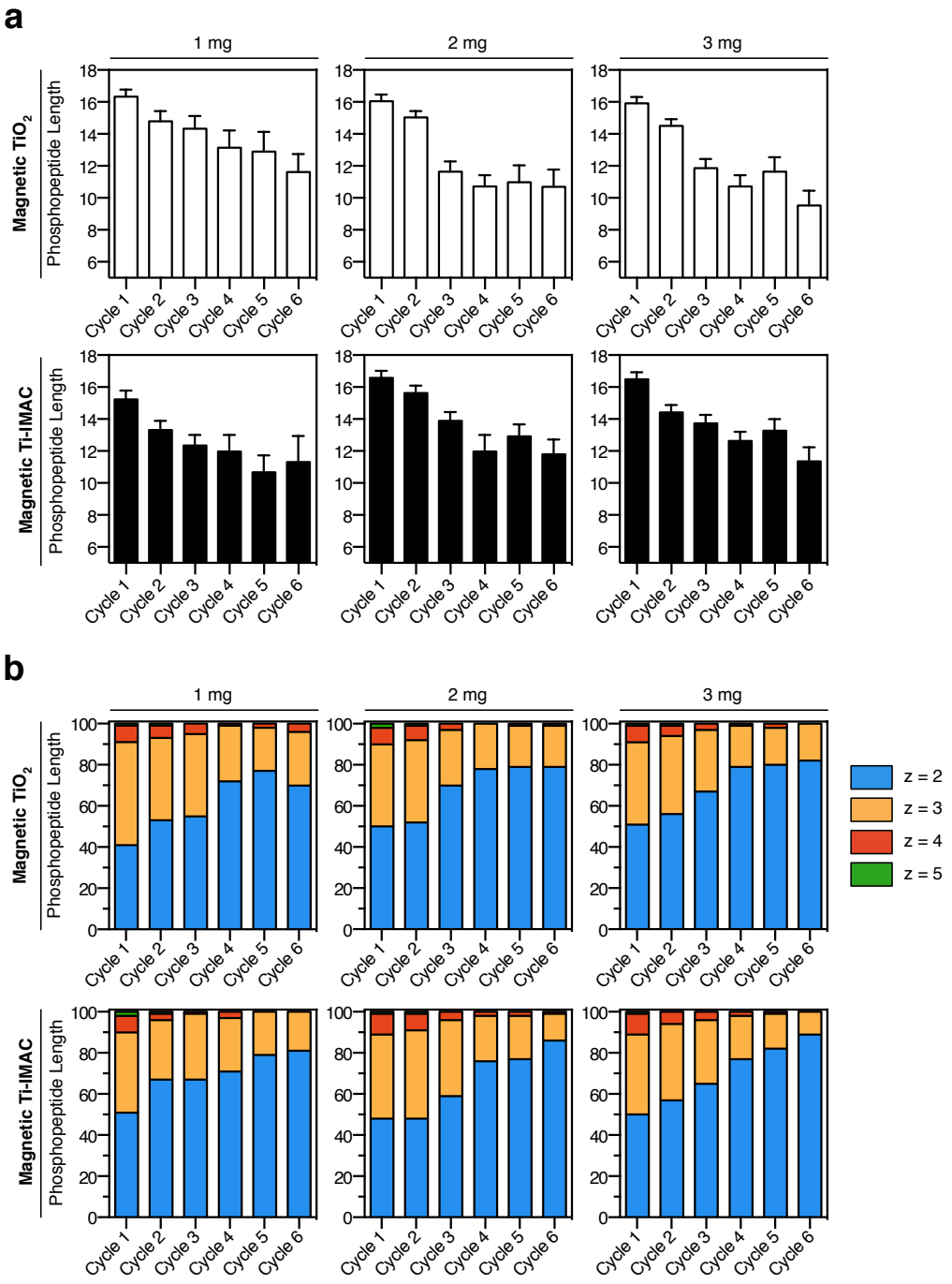
Supplementary Figure 1 – Initial Manual Batch-Mode Microsphere Comparison. a) 500 µg 32P-lablled human tryptic digests were manually phospho-enriched using various affinity matrices. The recovery of phosphorylated material was calculated using a scintillation counter. **b)** LC-MS/MS analysis of samples processed as in a). MagReSyn TiO2 and Ti-IMAC hyper-porous microspheres enrich the highest number of unique phosphopeptides. **c)** Comparison matrix of phosphopeptide iceLogo motifs enriched by each microsphere material. Aligned phosphopeptides were generated using ProteinModificationToolkit (http://ms.imp.ac.at/?goto=pmt) from manual enrichments described in b). Variation scale +/- 15% (p = 0.05; -1.96 σ; 1.96 σ). Phosphorylation is centred at position #11 +/- 10 local residues. When compared to all MagReSyn hyper-porous microspheres, both GL TiO2 and GE TiO2 demonstrate a preference to proline at position +1 and basic residues C-terminal to the phosphorylation. This suggests the MagReSyn hyper-porous matrix framework influences phosphopeptide enrichment bias beyond the coupled affinity chemistry.



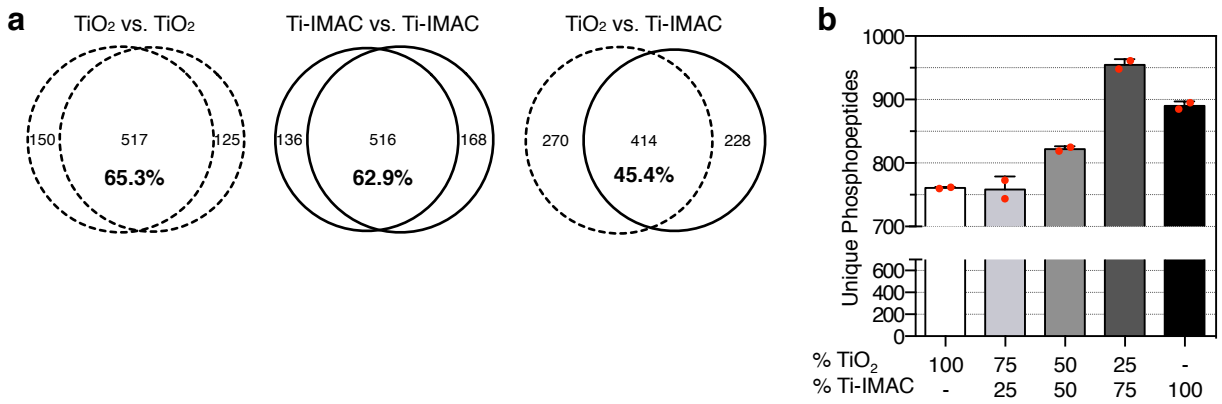
Supplementary Figure 2 – Automated Phosphopeptide Enrichment Buffer Conditions. 100 µg trypsin digested cell lysate was re-suspended in 80% MeCN, 5% TFA, +/- 1 M glycolic acid (GA) and automated phosphopeptide enrichment was performed using 1mg of either TiO2 **a)** or Ti-IMAC **b)** magnetic microspheres. Phosphopeptides were eluted using 1%, 2.5% or 5% ammonia solution (NH4OH). Each red dot represents data from an individual phosphopeptide enrichment well. LC-MS/MS DDA runs n = 24.



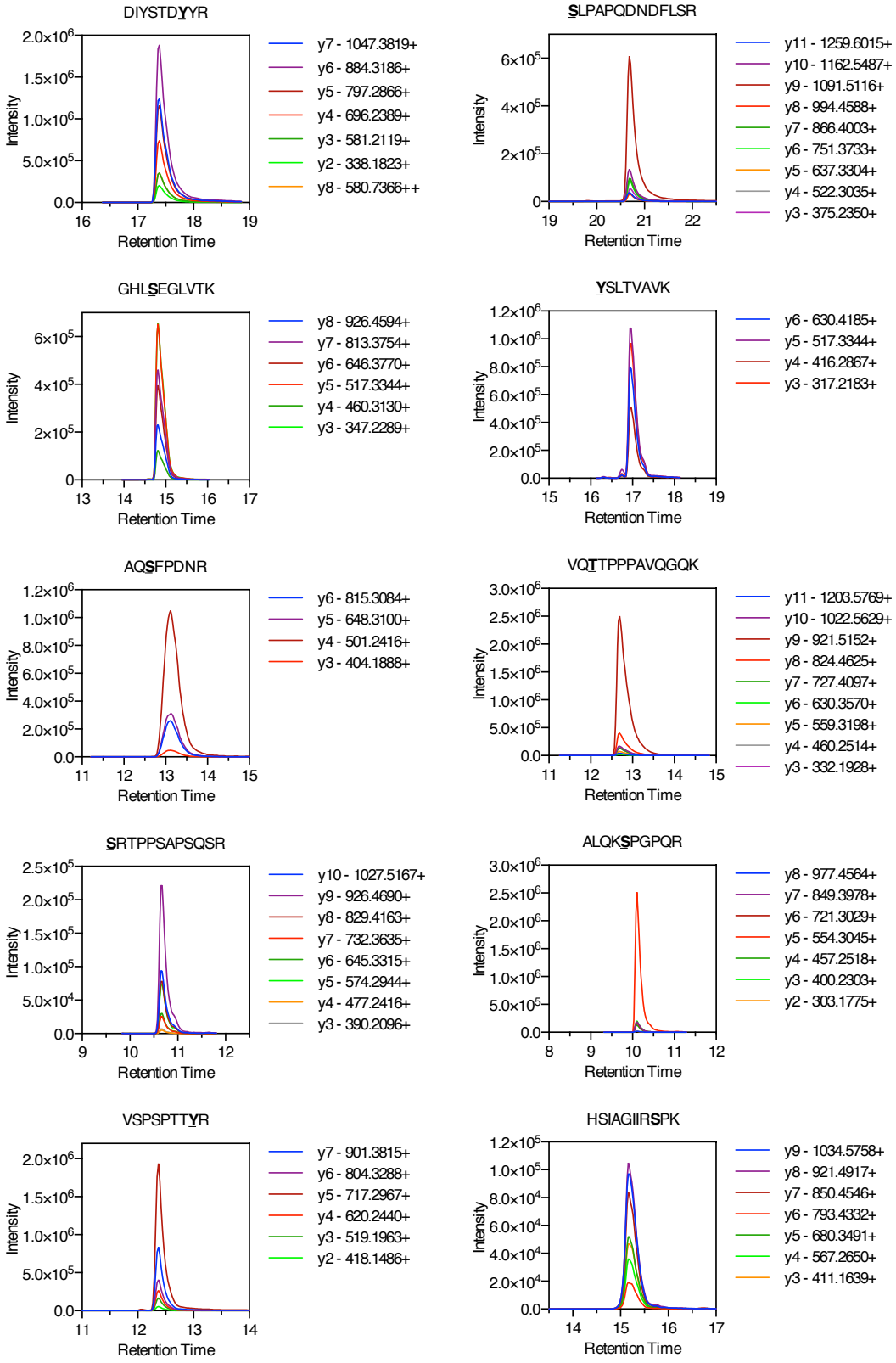
Supplementary Figure 3 – Successive Automated Phosphopeptide Enrichment Cycles. Successive phosphopeptide enrichment cycles were performed on 100 µg tryptic digests using different amounts of magnetic microspheres. Unique numbers of phosphopeptides per cycle, percentage of phosphopeptides per cycle and cumulative numbers of unique peptides are shown. LC-MS/MS DDA runs n = 36.



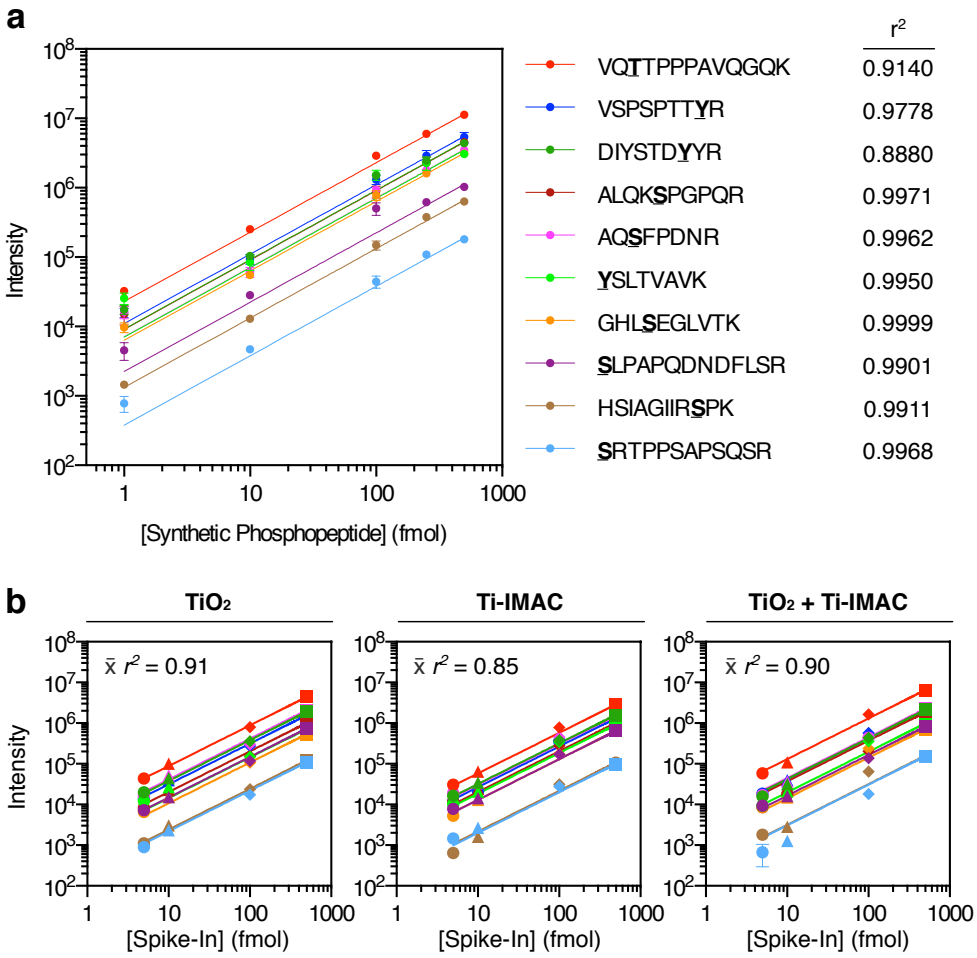
Supplementary Figure 4 – Phosphopeptide Lengths and Charges Across Successive Enrichment Cycles. a) Unique phosphopeptide length (# amino acids) across 6 successive enrichment cycles. Additional automated enrichment cycles with both TiO2 and Ti-IMAC magnetic microspheres enrich smaller phosphopeptides. Error bars = 95% CI. **b)** Unique phosphopeptide charges (z) across 6 successive enrichment cycles. Additional automated enrichment cycles with both TiO2 and Ti-IMAC magnetic microspheres trend towards enriching lower charge state phosphopeptides.



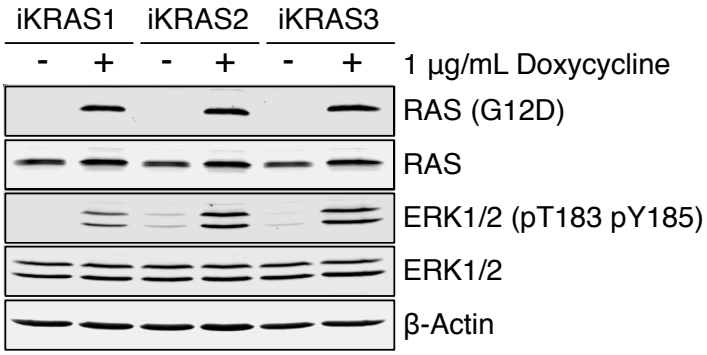
Supplementary Figure 5 – Combining TiO2 and Ti-IMAC. a) Unique phosphopeptide overlap between TiO2 and Ti-IMAC automated phosphopeptide enrichment samples (+1M GA, 1% NH4OH, from Supplementary Fig. 2). **b)** TiO2 and Ti-IMAC magnetic microspheres were combined at different ratios (1 mg total) and used to enrich phosphopeptides from 100 µg tryptic digests. Each red dot represents individual phosphopeptide enrichments. LC-MS/MS DDA runs *n* = 10.



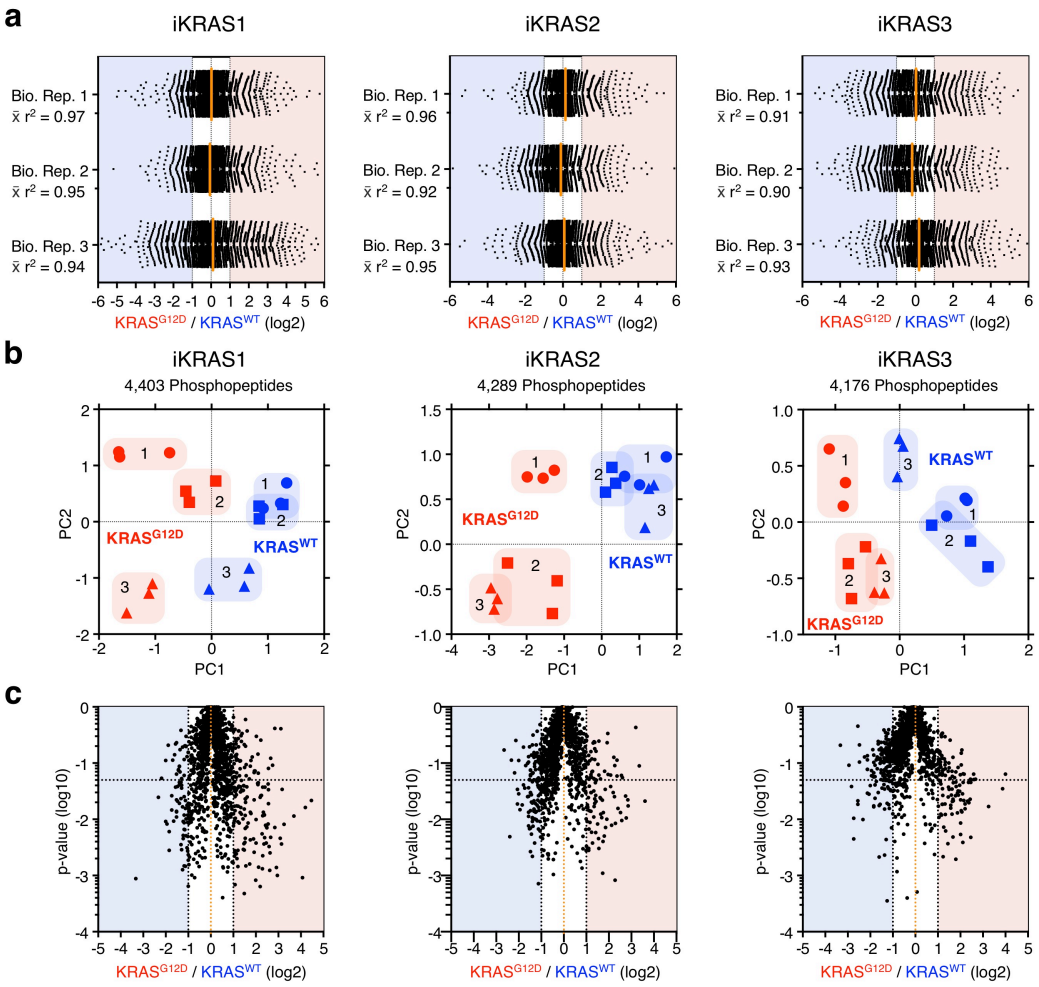
Supplementary Figure 6 – Synthetic Phosphopeptide Selected Reaction Monitoring (SRM). Representative SRM transitions for 10 synthetic phosphopeptides used to investigate phosphopeptide enrichment recovery (500 fmol injection). Phosphorylated residues are underlined in bold. The top two transitions for each phosphopeptide were used for quantitative analysis.



Supplementary Figure 7 – Synthetic Phosphopeptide Linearity. a) Human synthetic phosphopeptides (*n* = 10) were spiked into a SILAC "Heavy" (K +8 Da; R +10 Da) mouse cell-lysate matrix after automated phosphopeptide enrichment and analysed by selected reaction monitoring (SRM). Standard curves were produced by correlating transition intensity to spiked phosphopeptide concentration. Phosphorylated residues are underlined in bold. Error bars = SD (technical *n* = 3 / phosphopeptide). LC-MS/MS SRM runs *n* = 15. **b)** Recovered synthetic phosphopeptide recovery (n = 10) following automated phosphopeptide enrichment. Error bars = SD (technical *n* = 3 / phosphopeptide.) LC-MS/MS SRM runs *n* = 51.



Supplementary Figure 8 – Pancreatic ductal adenocarcinoma (PDA) inducible oncogenic KRAS (iKRAS) cells. Each iKRAS cell line was cultured without doxycycline for one full passage (i.e. KRAS-WT). Cells were then cultured +/- 1 µg/mL doxycycline (i.e. KRAS-G12D) for 24 hours. Immuno-blot analysis demonstrates KRAS-G12D induction and downstream activation of ERK1/2.



Supplementary Figure 9 – Multivariate label-free phosphoproteomic analysis of oncogenic KRAS. a) MS1 label-free phosphopeptide G12D/WT area ratios (log2) of biological replicates from iKRAS cell isolations (no normalization). Mean = orange line. Technical replicate mean Pearson correlation values are shown for each biological replicate. **b)** Principle component analysis (PCA) of MS1 phosphopeptide areas. Replicates cluster in PC space. **c)** Statistically significant regulated phosphopeptides (biological replicates) for each individual cell line (two-tailed *t*-test <0.05).

Sequence	Precursor Mass	Product Ion	Product Mass
SLPAPQDNDFLSR	770.345595	y10	1162.548727
		y9	1091.511613
DIYSTD**Y**YR	638.250101	y7	1047.381918
		y6	884.31859
YSLTVAVK	480.743726	y5	517.334424
		y3	317.218332
GHL**S**EGLVTK	560.773546	y5	517.334424
		y3	347.228896
VQ**T**TPPPAVQGQK	715.855599	y9	921.515242
		y8	824.462478
AQ**S**FPDNR	507.705663	y5	648.31000
		y4	501.241586
ALQK**S**PGPQR	581.292437	y7	849.397843
		y5	554.304521
SRTPPSAPSQSR	675.811723	y10	1027.516699
		y5	574.29435
HSIAGIIR**S**PK	629.837012	y9	1034.575808
		y8	921.491744
VSPSPTT**Y**R	544.244621	y7	901.381525
		y5	717.296732

Supplementary Table 1 – Synthetic Phosphopeptide Transitions. Precursor and product ion masses used for SRM quantification of synthetic phosphopeptides. Phosphorylated residues underlined in bold. All precursors ions are doubly charged and all product ions are singly charged.

	Standard Curve						Enriched														
							TiO2					Ti-IMAC					Mix				
	1	10	100	250	500	Mean	5	10	100	500	Mean	5	10	100	500	Mean	5	10	100	500	Mean
SLPAPQDNDFLSR	49	12	36	19	18	27	22	8	21	17	17	14	2	14	12	11	14	8	16	13	13
DIYSTD**Y**YR	26	11	23	11	20	18	14	5	30	31	20	18	13	8	32	18	15	22	8	19	16
YSLTVAVK	33	42	29	38	23	33	32	31	30	34	32	30	37	25	57	37	46	33	18	60	39
GHL**S**EGLVTK	28	24	28	23	22	25	18	23	22	28	23	22	24	21	40	27	21	20	8	40	22
VQ**T**TPPPAVQGQK	4	13	18	9	13	11	14	29	31	22	24	11	12	30	17	17	19	14	9	16	14
AQ**S**FPDNR	35	29	22	22	11	24	42	15	31	30	29	7	18	37	40	26	44	27	44	44	40
ALQK**S**PGPQR	52	7	34	37	10	28	36	65	51	32	46	31	39	22	42	34	55	52	20	41	42
SRTPPSAPSQSR	45	13	34	18	18	26	96	42	12	21	43	36	33	18	47	33	32	49	27	25	33
HSIAGIIR**S**PK	15	19	26	17	10	18	35	48	21	30	34	65	54	33	25	44	43	11	10	15	20
VSPSPTT**Y**R	26	28	26	33	30	29	31	34	26	34	31	8	23	35	63	32	30	33	20	36	30
						24					30					28					27

Supplementary Table 2 – Synthetic Phosphopeptide Coefficient of Variation (*Cv*). Technical *Cv* for all synthetic phosphopeptides used in SRM analysis. All *Cv* are calculated from raw intensity data (top two transitions per peptide). Phosphorylated residues underlined in bold.

		Enriched		
	Standard	TiO2	Ti-IMAC	Mix
SLPAPQDNDFLSR	0.99	0.97	0.98	0.98
DIYSTD**Y**YR	0.89	0.91	0.90	0.96
YSLTVAVK	1.00	0.85	0.72	0.73
GHL**S**EGLVTK	1.00	0.89	0.85	0.86
VQ**T**TPPPAVQGQK	0.91	0.95	0.96	0.97
AQ**S**FPDNR	1.00	0.92	0.84	0.83
ALQK**S**PGPQR	1.00	0.90	0.84	0.86
SRTPPSAPSQSR	1.00	0.95	0.81	0.94
HSIAGIIR**S**PK	0.99	0.86	0.92	0.98
VSPSPTT**Y**R	0.98	0.88	0.71	0.88

Supplementary Table 3 – Synthetic Phosphopeptide Correlations. Recovered synthetic phosphopeptide Pearson *r2* values following automated phosphopeptide enrichment (technical $n = 3$). Plotted curves can be observed in Supplementary Fig. 7. Phosphorylated residues underlined in bold.

				IKRAS1		IKRAS2		IKRAS3		Total	
Accession	Name	Peptide	Phosphosite	G12D / WT	*p*-value	G12D / WT	*p*-value	G12D / WT	*p*-value	G12D / WT	*p*-value
P11157	Rrm2	TPLATIADQQQLQLSPLK	S20	6.33	0.0048	2.08	0.0181	9.51	0.0169	5.97	0.0133
Q8CJF7	Ahctf1	EREVSVSSVTEEPK	S1928	5.22	0.0118	5.97	0.0096	5.22	0.0198	5.47	0.0137
Q01320	Top2a	FTVDLDSDEDFSGLDEK	S1328;S1333	9.58	0.0082	1.50	0.1103	4.02	0.0054	5.04	0.0413
P10923	Spp1	ISHELESSSSEVN	S283;S289	8.81	0.0036	1.83	0.0153	4.05	0.0223	4.90	0.0137
Q6PAM1	Txlna	EQGVESPGAQPASSPR	S522	6.21	0.0025	3.39	0.0132	5.00	0.0211	4.87	0.0123
Q69ZX6	Morc2a	KRSLAVSDEEEAEEEAEK	S737;S741	3.90	0.0725	4.78	0.0254	5.59	0.0506	4.76	0.0495
Q3UYV9	Ncbp1	TSDANETEDHLESLICK	S22	4.31	0.0450	4.85	0.0008	4.29	0.0286	4.48	0.0248
P09450	Junb	SRDATPPVSPINMEDQER	T252;S256	5.21	0.0121	5.29	0.0157	2.72	0.0037	4.41	0.0105
P58871	Tnks1bp1	DDGESQPRSPALLPSTVEGPPGAPLLQAK	S568	4.29	0.0413	4.36	0.0079	4.34	0.0039	4.33	0.0177
Q99LD4	Gps1	SPPREGSQGELTPANSQSR	S454	6.17	0.0012	3.63	0.0072	3.12	0.0586	4.31	0.0224
Q5F2E7	Nufip2	DYEIENQNPLASPTNTLLGSAK	S626	5.54	0.0038	2.69	0.0209	4.49	0.0637	4.24	0.0295
P35601	Rfc1	ARKDSEEGEESFSSVQDDLSK	S244	7.53	0.0014	2.36	0.0059	2.83	0.0340	4.24	0.0138
P63085	Mapk1	VADPDHDHTGFLTEYVATR	T185;Y187	4.69	0.0263	2.15	0.0030	5.51	0.0048	4.12	0.0114
P19001	Krt19	SLLEGQEAHYNNLPTPK	T399	5.83	0.0082	2.42	0.0716	3.91	0.0315	4.05	0.0371
P26645	Marcks	EAAEAEPAEPSSPAAEAEGASASSTSSPK	S113	4.59	0.0006	3.76	0.0100	3.50	0.0458	3.95	0.0188
Q8BGD9	Eif4b	SPPYTAFLGNLPYDVTEDSIK	S93	5.60	0.0074	1.74	0.0428	4.47	0.0217	3.94	0.0240
Q8C079	Strip1	KDSEGYSESPDLEFEYADTDK	S59	2.90	0.0069	3.32	0.0011	5.48	0.0522	3.90	0.0201
Q9CXF4	Tbc1d15	DDSPTQTLASPNACR	S662	5.28	0.0047	2.91	0.0045	3.36	0.0552	3.85	0.0215
Q8BHL4	Gprc5a	AQAPASPYNDYEGRK	S344	4.38	0.0014	2.32	0.0140	3.11	0.0716	3.27	0.0290
Q8JZQ9	Eif3b	GHPSAGAEEEGGSDGSAAEAEPR	S120	3.79	0.0026	3.22	0.0108	2.51	0.1053	3.17	0.0396
Q7TQH0	Atxn2l	GPPQSPVFEGVYNNSR	S109	4.96	0.0022	1.88	0.0820	2.32	0.0185	3.05	0.0342
Q8BHL4	Gprc5a	AQAPASPYNDYEGR	S344	3.72	0.0045	2.53	0.0318	2.49	0.0928	2.92	0.0431
P37913	Lig1	ERNQVVPESDSPVK	S51	2.73	0.0029	1.61	0.0547	4.36	0.0650	2.90	0.0409
O35130	Emg1	RFSVQEQDWETTPPK	S16	2.58	0.0495	2.15	0.0145	3.73	0.0320	2.82	0.0320
Q8K310	Matr3	RDSFDDRGPSLNPVLDYDHGSR	S188	2.48	0.0133	2.31	0.0298	2.32	0.0653	2.37	0.0361
Q8BT14	Cnot4	ELSVQDQPSLSPTSLQNASSHTTTAK	S432	1.43	0.1008	2.13	0.0243	3.47	0.0019	2.34	0.0423
P58871	Tnks1bp1	LDSPPPSPITEASEAAEAAEADSWAVSGR	S496;S500	2.78	0.0037	1.81	0.0050	1.77	0.0415	2.12	0.0167
Q7TPV4	Mybbp1a	SPAPSNPTLSPSTPAK	S1253	2.94	0.0015	1.51	0.0097	1.81	0.1336	2.09	0.0482
Q7TQH0	Atxn2l	EVDGLLTSDPMGSPVSSK	S600	2.50	0.0308	1.80	0.0422	1.71	0.0471	2.00	0.0400
Q62073	Map3k7	RRSIQDLTVTGTEPGQVSSR	S412	0.48	0.0179	0.51	0.0029	0.46	0.0883	0.48	0.0363
O54774	Ap3d1	HSSLPTESDEDIAPAQR	S760	0.59	0.0019	0.41	0.0101	0.45	0.0474	0.48	0.0198
Q8BI29	Sarg	AGSYSLPR	S132	0.73	0.0163	0.31	0.0001	0.37	0.1048	0.47	0.0404
Q62130	Ptpn14	ICTEQSNSPPPIR	S314	0.44	0.0015	0.40	0.0140	0.52	0.0259	0.45	0.0138
Q3TJ91	Llgl2	VAVGCRLSNGEAE	S1022	0.51	0.0086	0.42	0.1060	0.33	0.0177	0.42	0.0441
Q8K3X4	Irf2bpl	NSSSPVSPASVPGQR	S636;S638	0.50	0.0023	0.36	0.0070	0.29	0.0409	0.38	0.0167
Q80U72	Scrib	TTEAPCSPGSQQPPSPDELPANVK	S1292	0.30	0.0148	0.45	0.0407	0.39	0.0023	0.38	0.0193
P35486	Pdha1	YHGHSMSDPGVSYR	S293;S300	0.44	0.0120	0.25	0.0285	0.35	0.0676	0.35	0.0360
Q61687	Atrx	RQNYSESSNYDSELER	S801	0.52	0.0021	0.24	0.0343	0.20	0.0099	0.32	0.0155
P58871	Tnks1bp1	RFSEGVLQPPSQDQEK	S429	0.10	0.0009	0.24	0.0187	0.17	0.0213	0.17	0.0136

Supplementary Table 4 – Significantly Regulated Phosphopeptides by KRAS-G12D in PDA. Combined statistical analysis across all three iKRAS cell isolates (two-tail *t*-test). Uniprot protein accession and gene names are shown.

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