Supporting Information

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SI Materials and Methods

Cell Lines. All MM cell lines were maintained in RPMI 1640 (Cellgro, Mediatch) supplemented with 10% (vol/vol) FBS, 100 units/mL penicillin, and 100 units/mL streptomycin. H929 cells were obtained from Kenneth Anderson (Dana–Farber Cancer Institute, Boston, MA, ATCC, DSMZ) and Alex Almasan (Taussig Cancer Institute, Cleveland, OH); MOLP-8 (DSMZ), RPMI-8226 (ATCC), U266, OCI-Myc, KMS 12BM, IIN3, and XG7 MM cells were obtained from Wenyi Wei [Beth Israel Deaconess Medical Center (BIDMC)]; K562 CML cells were obtained from Pier Paolo Pandolfi (BIDMC); and 293T cells were obtained from Lewis Cantley (BIDMC). K562 and 293T were maintained in DMEM supplemented with 10% (vol/vol) FCS, 100 units/mL penicillin, and 100 units/mL streptomycin.

Inhibitor Drugs. Imatinib was purchased from Tocris Bioscience. BKM-120 was purchased from Active Biochemical. BEZ-235, bortezomib, and rapamycin were purchased from LC Laboratories. UO126 was purchased from Promega. Sodium orthovanadate (Na_3VO_4) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Ly294002 was purchased from Calbiochem. Picropodophillin (PPP) was purchased from Biomol International. PD98095 was purchased from Cell Signaling Technologies. In most cases, cells in normal growth conditions were treated with inhibitors for 1 h before lysis using DMSO as vehicle.

Growth Factor Stimulation. Cells were starved under serum free conditions for 16 h and stimulated by adding insulin (Sigma-Aldrich), EGF (PeproTech), or IGF1 (Austral Biologicals) for 90 min before lysis.

Immunoprecipitation. Cells were grown in up to five 15-cm^2 dishes to 80% confluence, washed with PBS, and lysed in a 0.5% Nonidet P-40-containing lysis buffer. Lysates were centrifuged at 16,000 × *g* for 5 min at 4 °C. The supernatant was used for subsequent procedures. Co-IPs were done by incubating 1 mg (Western blotting)/10 mg (LC-MS/MS) of the cell lysate with 1 μg (Western blotting)/10 μg (LC-MS/MS) of antibody overnight at 4 °C. Protein A/G Sepharose beads (GE Healthcare) were added to the lysate antibody solution for the last 2 h of incubation. After incubation, beads were washed three times with 1 mL of lysis buffer, and boiled in 1× SDS sample buffer containing β-mercaptoethanol.

Western Blot Analysis. Western blot analyses were conducted after separation by SDS/PAGE and transfer to a nitrocellulose membrane. Antibodies against BCR (rabbit, polyclonal), phospho-p44/42 ERK1/2 (rabbit polyclonal), phospho-tyrosine (mouse monoclonal), phospho-p70 S6 Kinase (mouse, monoclonal) and phospho-AKT (rabbit, monoclonal), p70 S6 Kinase (rabbit monoclonal), p44/42 ERK1/2 (rabbit, polyclonal), AKT1 (rabbit monoclonal), c-ABL (rabbit polyclonal), and actin (mouse, monoclonal) were purchased from Cell Signaling, Technologies. Antibody against GRB2 (rabbit polyclonal) was purchased from Santa Cruz Biotechnology. P85 (rabbit, polyclonal) antibody was purchased from Millipore. All antibodies were used per manufacturer's instructions. Antibody binding was detected using enhanced chemiluminescence (PerkinElmer).

pY enrichment. Cells were lysed in 6 M urea (Sigma-Aldrich) containing buffer. Lysates were centrifuged at 16,000 × *g* for 5 min at 4 °C. The supernatant was reduced with DTT (Fluka), alkylated with iodoacetamide (Sigma-Aldrich), and digested overnight with TPCK modified sequencing grade trypsin (Worthington). After purification with 6-cc SepPak Vac (500 mg) tC_{18} cartridges (Waters), the phosphopeptides were enriched for 4 h at 4 °C either with antibodies separate or as a mixture of antibodies against pY residues [bead conjugated, monoclonal pY-100 antibody (Cell Signaling Technologies] and bead conjugated, monoclonal pY antibody from Sigma-Aldrich), eluted with 0.15% TFA and concentrated to 10 μL using a SpeedVac with no heat.

PathScan RTK Antibody Array Kit. The PathScan RTK signaling array kit containing 39 fixed antibodies against phosphorylated forms of kinases and key signaling proteins by the chemiluminescent sandwich ELISA format was used per manufacturer's direction (Cell Signaling Technologies). Images were analyzed with ImageJ (http://rsbweb.nih.gov/ij/) by loading the image as a gray scale picture. Each kinase array dot was manually selected, and an average intensity for each kinase was calculated. Normalization within one stimulation experiment was done by subtracting the intensity of the negative control dot from each value. For comparison of different stimulation conditions, sets were normalized so that the positive controls had equal intensities.

Cell Viability Assays. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid] (Sigma-Aldrich) solutions were prepared at 5 mg/mL concentrations in PBS and filtered through a 0.2-μm filter. Then, 60 μL of MTT solution were added into 48 wells containing 500 mL of medium and cells, and a cell-free blank well. Cells were incubated for 3 h at 37 °C with 5% CO_2, 95% air and complete humidity. After 3 h, 100 μL of MTT stop-solution (10%, vol/vol SDS/5%, vol/vol isobutanol/0.01 M HCl) was added to each well. The plate was further incubated overnight at 37 °C with 5% CO_2, 95% air and complete humidity, and the optical density (OD) of the wells was determined using a plate reader at a test wavelength of 570 nm and a reference wavelength of 630 nm. The values for the drug-treated cells were normalized to the value of untreated cells and plotted relative to the day 1 time point (100%).

Histopathology Analyses. ***Immunoperoxidase (IMPOX).*** IMPOX staining was performed by suspending cells in growth medium were washed in 1× PBS and fixed in 10% (vol/vol) buffered formalin at a 1:1 ratio for 6 h. Fixed cells were centrifuged at 400 × *g* for 10 min, resuspended to a slurry in PBS, and added to 1 vol of melted 3% low-melting-point agarose, all steps being carried out at 40 °C. After agarose gelling, pellets were dislodged, split, embedded in paraffin, and microtome-sectioned at 5 μm. Sections were placed on charged glass slides and stained with hematoxylin and eosin, or used for immunohistochemistry (ICH). ICH was performed using well characterized monoclonal antibodies that were routinely used in a clinical diagnostic ICH laboratory. Antibody detection was effected using EnVisionTM+, a secondary antibody/polymer/peroxidase conjugate (Dako) and diaminobenzidine (DAB) as chromogenic substrate. Reagent concentration and reaction times were those routinely used in clinical ICH.

FISH. FISH was performed for testing chromosomal *t*(9;22) rearrangement (BCR–ABL). Fixed cell suspensions were dropped onto glass slides and aged for ~45 min at 60 °C, after which probe (BCR–ABL double fusion probe; Abbott Molecular) was added to cell area of slide. After coverslipping and sealing with rubber cement, cells and probes were codenatured on a programmable temperature controlled slide system (Thermobrite) and hybridized overnight at 37 °C. Posthybridization processing was performed per probe following manufacturer's instructions.

RT-PCR and DNA Sequencing. RNA was extracted from 8 × 10E6 cells of H929 and K562 with the RNeasy MiniKit from Qiagen (catalog no. 74104) following the manufacturer's instruction for animal cells. One microgram of total RNA was transcribed to cDNA with High Capacity RNA-to-cDNA Master Mix from Applied Biosystems (catalog no. 4390776) following the manufacturer's instruction. The PCR was performed with the cDNA, dNTPs (Denville Scientific, catalog no. CB4421-6), Platinum Taq DNA polymerase (Invitrogen catalog no. 10966–018) and different combinations of primers (forward primers: BCR1 GGAGCTGCAGATGCTGACCAAC, BCR2 CAGATCTGGCCCAACGATG; reverse primers: ABL1 CCATTTTTGGTTTGGGCTTCACACCATTCC, ABL2 CGGCTCTCGGAGGAGACGTAGA) in the AB 2720 Thermal Cycler. After the reaction, the samples were loaded on a 1.5% agarose gel and were run until the marker was separated. After staining the gel with ethidiumbromid, the bands of interest were cut out and the DNA was extracted and purified with the kit from GE Healthcare (catalog no. 28-9034-71) following the manufacturer's instructions. Twenty nanograms of DNA was mixed with 5 μM BCR1 forward primer and sent out to Genewiz for DNA sequencing. The obtained sequences were blasted against the National Center for Biotechnology Information (NCBI) human database to identify the exact BCR–ABL transcript.

IP–LC-MS/MS. Immunoprecipitations for LC-MS/MS PPI analyses were separated by SDS/PAGE until the 55-kDa prestained marker was separated from the band above and below (short gel run, ~1/6 distance of mini gel lane). After staining and destaining with Coomassie blue stain, gel sections were excised above and below the IgG heavy chain band to avoid antibody contamination and peptide signal suppression. IPs for BCR–ABL LC-MS/MS sequence analysis were separated by SDS/PAGE until the dye front reached the bottom of the gel, and stained and destained with Coomassie blue; only the ~210-kDa region of the BCR–ABL fusion protein band was excised. Gel sections were reduced with 55 mM DTT, alkylated with 10 mM iodoacetamide (Sigma-Aldrich), and digested overnight with TPCK modified trypsin (Promega) and chymotrypsin (Princeton Separations) separately at pH 8.3. Peptides were extracted, concentrated to 10 μL using a SpeedVac with no heat, and analyzed by positive ion mode LC-MS/MS using a hybrid LTQ-Orbitrap XL-ETD mass spectrometer (Thermo Fisher Scientific) via CID with data-dependent analysis (DDA). Peptides were delivered and separated using an EASY-nLC nanoflow HPLC (Thermo Fisher Scientific) at 300 nL/min using self-packed 15 cm length × 75 μm i.d. C_{18} fritted microcapillary columns. Solvent gradient conditions were 140 min from 3% B buffer to 38% B (B buffer: 100% acetonitrile; A buffer: 0.1% formic acid/99.9% water). MS/MS spectra were analyzed using the Sequest algorithm in Proteomics Browser Software (PBS; W. S. Lane, Harvard University, Cambridge, MA) by searching the reversed and concatenated Swiss-Prot protein database (version 2011_9 containing 53,214 protein sequence entries; www.ebi.ac.uk/uniprot/database/download.html) with a parent ion tolerance of 40 ppm and fragment ion tolerance of 0.80 Da. Carbamidomethylation of cysteine (+57.0293 Da) was specified in Sequest as a fixed modification, and oxidation of Methionine (+15.9949) and phosphorylation of Serine/Threonine/Tyrosine (+79.97) were specified as variable modifications. Results were imported into Scaffold 3.5 software (Proteome Software) with a peptide threshold of ~85%, protein threshold of 95%, resulting in a peptide false discovery rate (FDR) of ~1%. Known contaminants such as keratins, caseins, trypsin, and BSA were removed from the analysis. ScaffoldPTM 2.0 (Proteome Software) was used to organize and export the pY peptide data. pY peptides were accepted with 75% Ascore site probability threshold.

PPI Network. Protein reports were exported from Scaffold 3.5 software and imported into Cytoscape version 2.6 software (www.cytoscape.org) to create a PPI landscape for GRB2, p85, and pY IPs.

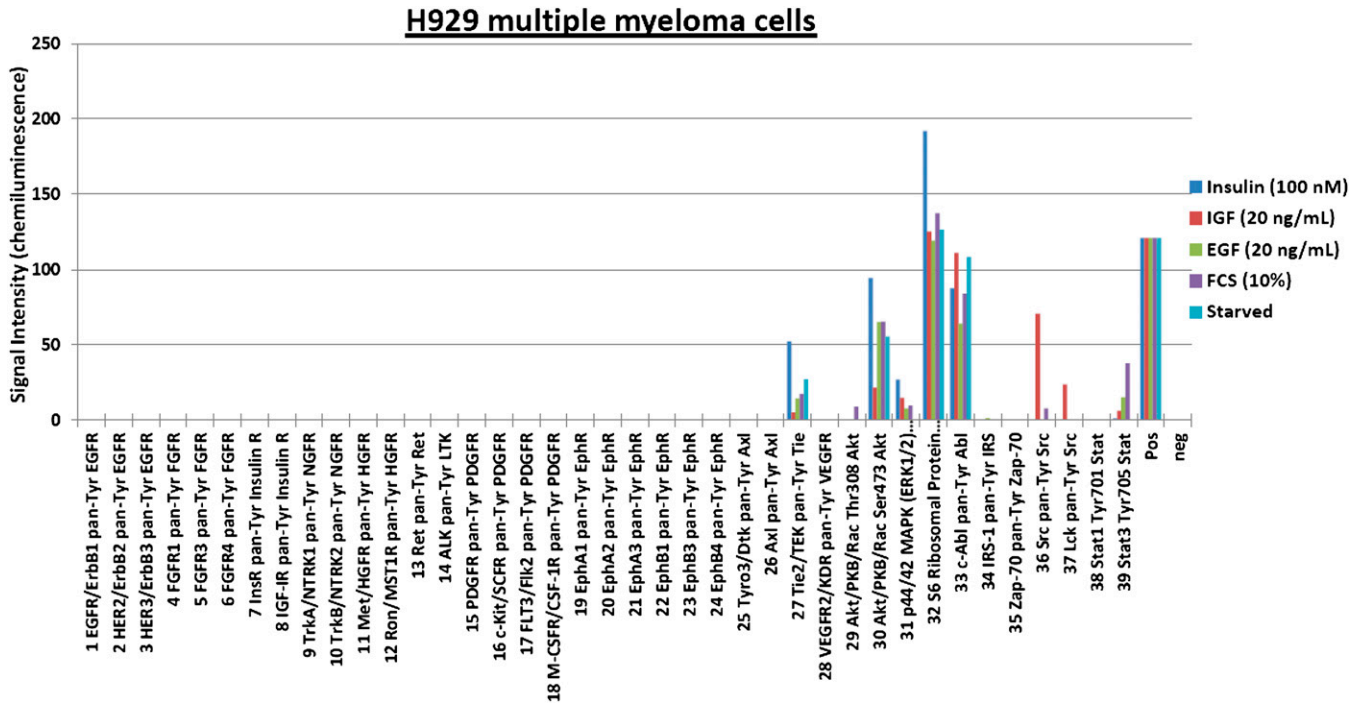

Fig. S1. Complete PathScan RTK Signaling antibody array results with phosphorylated signaling from 39 different kinases and signaling nodes. After serum starvation overnight, H929 MM cells were stimulated for 90 min with insulin, insulin growth factor (IGF), epidermal growth factor (EGF), and 10% FCS.

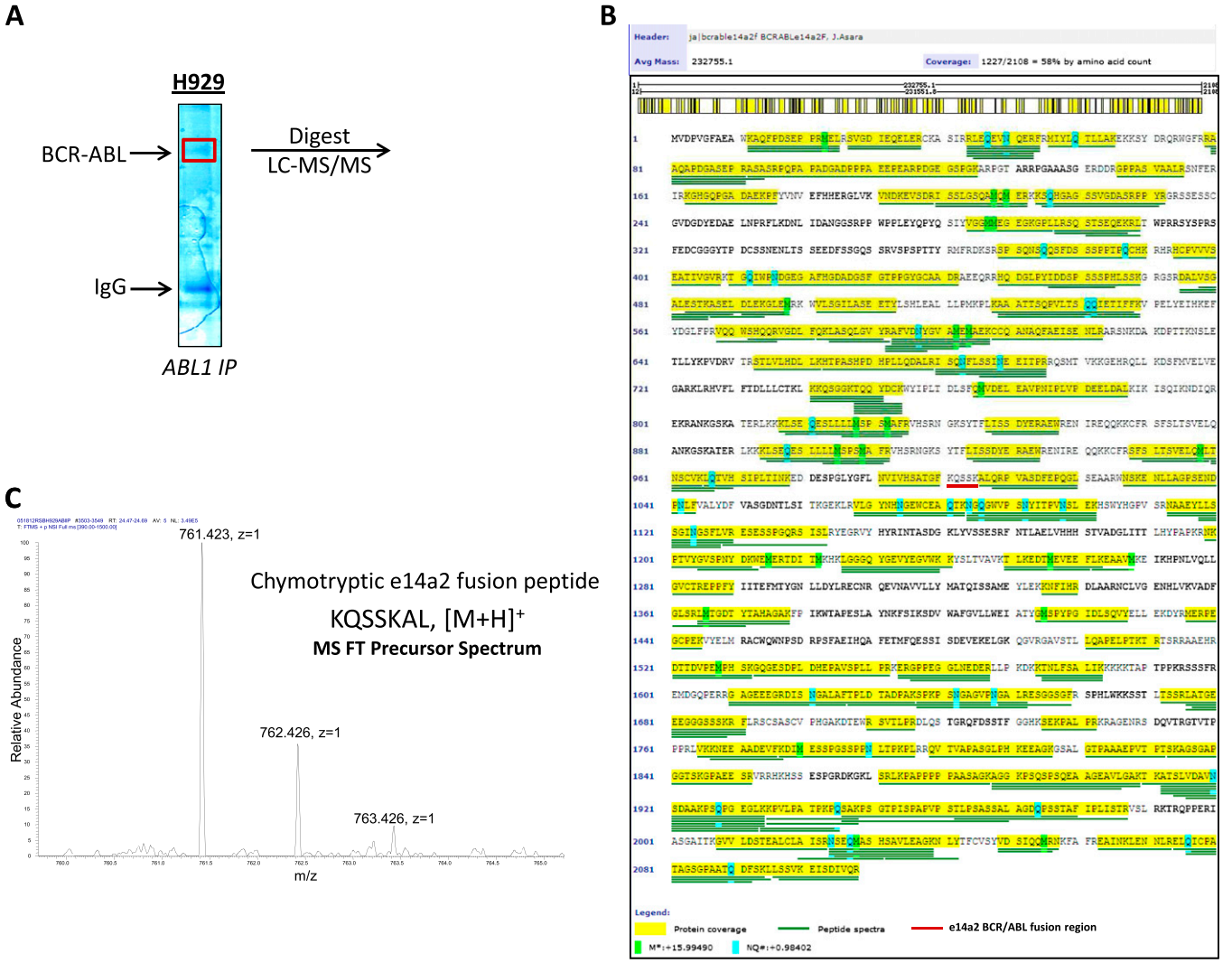

Fig. S2. LC-MS/MS sequencing of BCR–ABL fusion protein. (*A*) Picture of a Coomassie-stained SDS/PAGE gel showing the BCR–ABL protein band region excised for LC-MS/MS. (*B*) LC-MS/MS shotgun sequencing of a tryptic and chymotryptic digestion (combined data) of the gel region at ~210 kDa, which represents the BCR–ABL fusion protein in H929 MM cells showing 58% amino acid coverage. (*C*) The high-resolution precursor MS-FT spectrum for a potential singly charged chymotryptic peptide at *m/z* 761.423 representing the fusion sequence KQSSKAL.

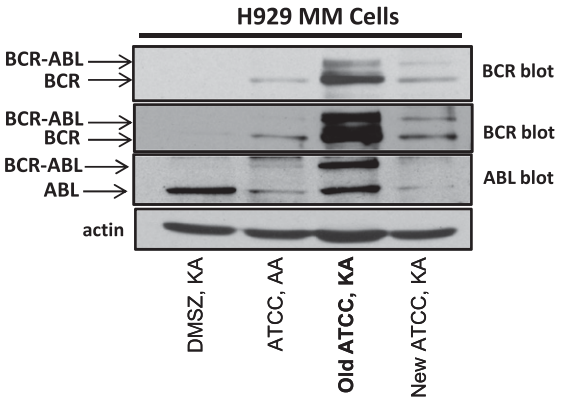

Fig. S3. Immunoblot of different lots of H929 MM cell lines from different vendors (DSMZ and ATCC) and academic laboratories (Kenneth Anderson, Dana–Farber Cancer Institute; and Alex Almasan, Taussig Cancer Institute). All lots of H929 showed some level of BCR–ABL fusion.

Table S1. Tyrosine-phosphorylated tryptic peptides immunopurified from H929 MM cells

Protein	Tyr-phosphorylated peptide sequence	Peptide Probability	*m/z*	Charge	Phosphosite (localization probability)	
ABL1	LGGGQyGEVyEGVWK	0.97	900.85	2	Y253 (1)	Y257 (1)
ABL1	NAAEyLLSSGINGSFLVR	1.00	994.98	2	Y139 (1)	
ABL1	VLGyNHNGEWCEAQTK	0.94	992.41	2	Y93 (1)	
BCR	AFVDNyGVAMEMAEK	1.00	876.85	2	Y598 (1)	
BCR	KGHGQPGADAEKPFyVNVEFHHER	0.99	707.07	4	Y177 (1)	
BCR	NSLETLLyKPVDRVTR	1.00	991.51	2	Y644 (1)	
BTK	VVALyDYMPMNANDLQLR	1.00	1,102.50	2	Y223 (1)	
CALM	VFDKDGNGyISAAELR	1.00	916.92	2	Y100 (1)	
CAPZB	STLNEIyFGK	0.99	625.28	2	Y232 (1)	
CASL	LyQVPNPQAAPR	0.99	716.84	2	Y92 (1)	
CASS4	GPVVLKEPEKQQLyDIPASPK	1.00	832.07	3	Y195 (1)	
CASS4	SEWIyDTPVSPGK	0.98	778.84	2	Y244 (0.97)	
CBL	IKPSSSANAIySLAARPLPVPK	0.93	813.41	3	Y674 (0.92)	
CDCP1	GPAVGIyNGNINTEMPR	1.00	949.41	2	Y6 (1)	
CDK1	IGEGtyGVVYK	1.00	672.27	2	Y15 (1)	
CRKL	IHyLDTTTLIEPAPR	1.00	909.45	2	Y92 (1)	
CRKL	RVPCAyDKTALALEVGDIVK	1.00	765.72	3	Y251 (1)	
CTND1	LNGPQDHSHLLySTIPR	1.00	675.98	3	Y96 (0.97)	
DDX3X	KGADSLEDFLyHEGYACTSIHGDR	1.00	940.06	3	Y462 (0.99)	
DOK1	LPSPPGPQELLDSPPALyAEPLDSLR	1.00	950.80	3	Y296 (0.97)	
DOK1	LTDPKEDPIyDEPEGLAPVPPQGLYDLPREPK	1.00	1,222.92	3	Y362 (0.82)	
DOK1	VKEEGyELPYNPATDDYAVPPPR	1.00	899.74	3	Y398 (0.89)	
DOK2	GQEGEyAVPFDAVAR	0.97	843.86	2	Y299 (1)	
DYR1A	KVYNDGYDDDNyDYIVK	1.00	1,089.43	2	Y145 (0.83)	
EF1A1	EHALLAyTLGVK	0.93	696.85	2	Y141 (1)	
EIF3L	GDPQVYEELFSySCPK	1.00	998.91	2	Y415 (0.95)	
ELAV1	NVALLSQLyHSPAR	1.00	823.91	2	Y200 (0.82)	
ENOA	AAVPSGASTGIyEALELR	1.00	941.95	2	Y44 (1)	
FLNB	MDGTyACSYTPVK	0.98	785.80	2	Y704 (0.91)	
FYN	LIEDNEyTAR	0.99	651.27	2	Y420 (1)	
G6PD	NSYVAGQyDDAASYQR	1.00	943.37	2	Y112 (1)	
GAB2	EFGDLLVDNMDVPATPLSAyQIPR	1.00	1,378.63	2	Y324 (1)	
GAB2	MSGDPDVLEyYK	1.00	755.79	2	Y48 (1)	
GSK3A	GEPNVSyICSR	0.88	680.77	2	Y279 (0.93)	
GSTP1	FQDGDLTLyQSNTILR	0.94	981.45	2	Y64 (1)	
HS90B	SIyYITGESK	0.98	619.77	2	Y484 (1)	
HSP74	KEDIyAVEIVGGATR	0.99	849.91	2	Y336 (1)	
ITSN2	REEPEALyAAVNK	0.99	784.36	2	Y968 (1)	
KPCD	SDSASSEPVGIyQGFEK	1.00	939.89	2	Y313 (1)	
LDHB	MVVESAyEVIK	0.98	681.31	2	Y240 (1)	
LYN	SLDNGGYyISPR	1.00	710.30	2	Y194 (0.94)	
LYN	VIEDNEyTAR	0.88	644.27	2	Y397 (1)	
MPZL1	SESVVyADIR	1.00	608.77	2	Y263 (1)	
MYH9	ALELDSNLyR	0.98	636.29	2	Y754 (1)	
NOA1	FLFPEyILDPEPQPTR	0.96	1,020.97	2	Y5 (1)	
P85B	SREYDQLyEEYTR	0.87	915.37	2	Y464 (0.99)	
PRI2	IILSNPPSQGDyHGCPFR	0.98	1,068.47	2	Y381 (0.99)	
PRP4B	LCDFGSASHVADNDITPyLVSR	1.00	1,258.54	2	Y849 (0.96)	
PSA2	HIGLVySGMGPDYR	1.00	829.86	2	Y76 (1)	
PSA2	LAQQYYLVyQEPIPTAQLVQR	0.99	1,300.64	2	Y101 (1)	
PSA7L	LYQTDPSGTyHAWK	0.99	581.92	3	Y161 (0.9)	
PSB4	VNNSTMLGASGDyADFQYLK	0.99	1,144.48	2	Y102 (0.93)	
PTCD3	DIAEPHIPCLMPEyFEPQIK	0.99	835.71	3	Y144 (1)	
PTN11	IQNTGDyYDLYGGEK	0.95	907.37	2	Y62 (0.99)	
PTPRA	VVQEYIDAFSDyANFK	1.00	993.93	2	Y798 (1)	
RHG35	NEEENIySVPHDSTQGK	0.94	1,013.41	2	Y1105 (0.99)	
ROA1	SSGPYGGGGQyFAKPR	0.93	853.87	2	Y347 (0.99)	
RS10	IAIyELLFK	1.00	594.31	2	Y12 (1)	
RS27L	LVQSPNSyFMDVK	1.00	811.84	2	Y31 (0.97)	
SDCB1	LYPELSQyMGLSLNEEEIR	1.00	793.02	3	Y56 (0.95)	
SEPT7	NLEGyVGFANLPNQVYR	1.00	1,016.96	2	Y30 (1)	
SET	IDFyFDENPyFENK	1.00	960.37	2	Y140 (1)	Y146 (1)
SHC1	ELFDDPSyVNVQNLDK	1.00	987.43	2	Y427 (1)	

Table S1. Cont.

Protein	Tyr-phosphorylated peptide sequence	Peptide Probability	*m/z*	Charge	Phosphosite (localization probability)
SHIP2	NSFNNPAyYVLEGVPHQLLPPEPPSPAR	0.86	1,061.17	3	Y986 (0.98)
SHIP2	TLSEVDyAPAGPAR	1.00	762.84	2	Y1135 (0.99)
SSBP	SGDSEVyQLGDVSQK	1.00	845.35	2	Y73 (1)
TBB4B	NSSyFVEWIPNNVK	1.00	887.90	2	Y340 (0.82)
TKT	NMAEQIIQEIySQIQSK	0.92	706.32	3	Y275 (0.97)
TM192	AKPEPDILEEEKIyAYPSNITSETGFR	1.00	1,059.16	3	Y213 (0.95)
VASP	VQIyHNPTANSFR	1.00	812.87	2	Y39 (1)
VIME	SLyASSPGGVYATR	1.00	753.84	2	Y61 (0.9)
WDR1	AHDGGIyAISWSPDSTHLLSASGDKTSK	1.00	993.45	3	Y238 (0.78)
YES	LIEDNEyTAR	0.99	651.27	2	Y426 (1)
ZO2	IEIAQKHPDIyAVPIK	0.97	957.49	2	Y1118 (1)

List of tyrosine-phosphorylated tryptic peptides immunopurified from H929 MM cells with pY antibodies and identified using LC-MS/MS with a 1% peptide FDR and 75% phosphosite localization probability threshold by Scaffold PTM software.

Other Supporting Information Files

Dataset S1 (XLSX)
Dataset S2 (XLSX)
Dataset S3 (XLSX)
Dataset S4 (XLSX)