

2 Using Tandem Mass Spectrometry in Targeted Mode 3 to Identify Activators of Class IA PI3K in Cancer

4 AU Xuemei Yang¹, Alexa B. Turke², Jie Qi², Youngchul Song², Brent N. Rexer⁶, Todd W. Miller⁶,
5 Pasi A. Jänne³, Carlos L. Arteaga⁶, Lewis C. Cantley^{1,4}, Jeffrey A. Engelman^{2,5}, and John M. Asara^{1,5}

Abstract

6 Phosphatidylinositol-3-kinase (PI3K) is activated in some cancers by direct mutation, but it is activated
7 more commonly in cancer by mutation of upstream acting receptor tyrosine kinases (TK). At present, there is no
8 systematic method to determine which TK signaling cascades activate PI3K in certain cancers, despite the likely
9 utility of such information to help guide selection of tyrosine kinase inhibitor (TKI) drug strategies for
10 personalized therapy. Here, we present a quantitative tandem mass spectrometry (LC/MS/MS) approach that
11 identifies upstream activators of PI3K both *in vitro* and *in vivo*. Using non-small cell lung carcinoma to illustrate
12 this approach, we show a correct identification of the mechanism of PI3K activation in several models, thereby
13 identifying the most appropriate TKI to downregulate PI3K signaling. This approach also determined the
14 molecular mechanisms and adaptors required for PI3K activation following inhibition of the mTOR kinase
15 TORC1. We further validated the approach in breast cancer cells with mutational activation of *PIK3CA*, where
16 tandem mass spectrometry detected and quantitatively measured the abundance of a helical domain mutant
17 (E545K) of *PIK3CA* connected to PI3K activation. Overall, our findings establish a mass spectrometric approach
18 to identify functional interactions that govern PI3K regulation in cancer cells. Using this technique to define the
19 pathways which activate PI3K signaling in a given tumor could help inform clinical decision making by helping
20 guide personalized therapeutic strategies for different patients. *Cancer Res*; 71(00); 1–11. ©2011 AACR.

21 Introduction

22 The phosphatidylinositol-3-kinase (PI3K) signaling path-
23 way is central to growth and survival of many cancers. PI3K is
24 a lipid kinase that converts phosphatidylinositol 4,5-bispho-
25 sphate [PtdIns(4,5)P₂] to phosphatidylinositol 3,4,5-triphos-
26 phate [PtdIns(3,4,5)P₃] leading to membrane recruitment
27 and activation of specific proteins with pleckstrin homology
28 (PH) domains, including AKT. Although there are several
29 classes of PI3K, class I_A PI3Ks are the most intimately linked
30 to cancer cell growth and survival (1). These enzymes are
31 heterodimers, consisting of a regulatory subunit, p85, and a
32 catalytic subunit, p110 (2). Recent cancer genome sequencing

efforts have revealed that PI3K signaling can be directly
activated by genetic mutations. Most commonly, these muta-
tions are in *PIK3CA*, the gene encoding the p110 α subunit, and
in *PTEN*, the gene encoding the phosphatase that degrades
PIP₃, the lipid product of PI3K (3). Moreover, several studies
have shown that PI3K signaling is critical for tumorigenesis
and tumor maintenance, even in cancers that lack *PIK3CA* or
PTEN mutations (4–9). In many of these cancers, class I_A PI3K
is activated upon direct binding to receptor tyrosine kinases
(RTK) and/or adaptor proteins. The p85 regulatory subunit
binds to tyrosine phosphorylated proteins via 2 SH2 domains.
The engagement of the p85 SH2 domains with tyrosine-
phosphorylated receptors and adaptors recruits PI3K to the
membrane where its lipid substrate resides (10–12). Currently,
there is no validated method to determine how PI3K is
activated in different cancers, although this information
would provide insights into potential therapeutic strategies.

Recent work has shown that when RTK inhibitors are
effective against a particular cancer, inhibition of the RTK
invariably leads to downregulation of PI3K signaling (13).
Thus, when a cancer is "oncogene addicted" to an RTK,
PI3K is under the sole regulation of that RTK, and the
corresponding tyrosine kinase inhibitor (TKI) leads to sup-
pression of PI3K signaling. Furthermore, cancers develop
resistance to kinase inhibitors when secondary events restore
PI3K signaling in the presence of the TKI (13). Thus, detailed
understanding of the regulation of PI3K signaling is important
for determining both sensitivity and resistance to targeted
therapies.

Authors' Affiliations: ¹Beth Israel Deaconess Medical Center, Division of Signal Transduction; ²Massachusetts General Hospital, Center for Thoracic Cancers; ³Dana Farber Cancer Institute, Department of Medical Oncology; ⁴Harvard Medical School, Department of Systems Biology; ⁵Harvard Medical School, Department of Medicine, Boston, Massachusetts; and ⁶Vanderbilt University, Vanderbilt-Ingram Cancer Center, Nashville, Tennessee

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Corresponding Author: John M. Asara, Beth Israel Deaconess Medical Center, Division of Signal Transduction, Boston, MA 02115. Phone: 617-735-2651; Fax: 617-735-2624; E-mail: jasara@bidmc.harvard.edu or Jeffrey A. Engelman, Massachusetts General Hospital, Center for Thoracic Cancers, Boston, MA 02114. Phone: 617-724-7298; Fax: 617-724-9648; E-mail: jengelma@partners.org

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65	Over the past few years, we and others have utilized	4°C. The supernatant was used for subsequent procedures.	121
66	immunoprecipitations (IP) of PI3K to identify the associated	Coimmunoprecipitations were done by incubating 10 mg of	122
67	phosphotyrosine proteins, and thus the pathways directly	the cell lysate with the p85 α rabbit polyclonal antibody	123
68	activating PI3K (14–18). Currently, this is primarily accom-	(Millipore) and protein A sepharose beads (GE Healthcare)	124
69	plished through biochemical approaches using multiple p85	overnight at 4°C. Beads were precipitated, washed with lysis	125
70	IPs assessed by Western blot analyses and is only effective	buffer, and boiled in sample buffer containing beta mercap-	126
71	when sensitive and specific antibodies are available. To date,	toethanol. Western blot analyses were conducted after separa-	127
72	these approaches have been time consuming with low yield. In	tion by SDS-PAGE and transfer to nitrocellulose or	128
73	recent years, there has been a growing trend of using IP	polyvinylidene difluoride membranes. Antibodies against	129
74	combined with mass spectrometry (MS; refs. 19–21). In this	ERBB3 and AKT were purchased from Santa Cruz Biotech-	130
75	study, we evaluated the efficacy of tandem MS using a targeted	nology and antibodies against GAB1, GAB2, IRS1, and pTyr	131
76	approach to quantify and assess the association of adaptors	were purchased from Cell Signaling Technologies. All were	132
77	and RTKs with PI3K in several different cancer models and	used per manufacturer's directions. Antibody binding was	133
78	paradigms. We also test the mechanistic role of activating	detected using enhanced chemiluminescence (PerkinElmer).	134
79	<i>PIK3CA</i> mutations in cells that harbor these mutations. These	Western blot images were captured using GeneSnap image	135
80	results show that MS can identify the mechanisms of PI3K	acquisition software.	136
81	activation in cancers and can successfully point to the appro-		
82	appropriate RTK inhibitor(s) that will lead to PI3K suppression.		
83	Materials and Methods		
84	Cell lines and reagents	Scrambled siRNA transfection and E545K expression	137
85	The <i>EGFR</i> mutant non-small cell lung carcinoma (NSCLC)	IRS and scrambled siRNA was purchased from Dharmacon	138
86	cell line HCC827 (del E746_A750) has been extensively char-	and transfection was done by using Qiagen HiPerFect Tran-	139
87	acterized (15, 16). HCC827 cells were maintained in RPMI 1640	sfection Reagent. HA-tagged WT or E545K p110 α coding	140
88	(Cellgro; Mediatech Inc.) supplemented with 5% FBS. The	sequences were excised from the JP1520 retroviral vector	141
89	<i>EGFR</i> wild-type (WT) squamous cancer cell line A431 was	and cloned into the LZRS-Neo retrovirus (Gary Nolan labora-	142
90	made resistant to 1 μ mol/L gefitinib by Jeff Engelman (MGH)	tory, Stanford University). BT474 and SKBR3 cells were	143
91	as described previously (17) and named A431Gefitinib Resis-	infected with viral supernatants produced by Phoenix cells	144
92	tant (A431GR). A431GR cells were maintained in RPMI 1640	and transfected with the LZRS-p110a retroviral construct.	145
93	supplemented with 10% FBS and 1 μ mol/L gefitinib. EBC-1	After transfection, cells were selected for 10 to 14 days in G418.	146
94	cells containing <i>MET</i> amplification (15) were obtained from		
95	Jeff Settleman (MGH), H3122 containing an echinoderm	Xenograft studies	147
96	microtubule-associated protein-like 4-anaplastic lymphoma	Nude mice (<i>nu/nu</i> ; 6-8weeks old; Charles River Labora-	148
97	kinase (EML4-ALK) gene translocation (22) were obtained	tories) were used for <i>in vivo</i> studies and were cared for in	149
98	from Pasi Jänne (DFCI) and H1703 NSCLC cells (23) were	accordance with the standards of the Institutional Animal	150
99	obtained from ATCC and all were grown in RPMI-1640 media	Care and Use Committee. A suspension of 5×10^6 H3122 lung	151
100	with 10% FBS. ER positive MCF7, and <i>HER2</i> -amplified SKBR3	cancer cells in 0.2 mL PBS were inoculated s.c. into the lower	152
101	and BT474 breast cancer cell lines were grown in Dulbecco's	right quadrant of the flank of each mouse. When tumors	153
102	modified Eagle's medium media with 10% FBS. Three <i>KRAS</i> -	reached approximately 400 mm ³ , tumors were treated with	154
103	mutant NSCLC cells lines (A549, H460, and H23) were cultured	TAE-684 administered at 25 mg/kg/d or vehicle alone via	155
104	in RPMI 1640 media supplemented with 10% FBS. All growth	orogastric gavage as described previously (22). After 2 days of	156
105	media was supplemented with 100 units/mL penicillin, 100	treatment, tumors were excised and approximately 130 mg	157
106	units/mL streptomycin, and 2 mmol/L glutamine. Growth	was homogenized and lysed. A total of 12 mg of lysate was	158
107	factors (EGF, recombinant HGF and IGF) were purchased	used for p85 IP. Tumors were measured twice weekly and mice	159
108	from R&D Systems and used at 50 ng/mL. PHA-665752 was	were monitored daily for body weight and general condition.	160
109	purchased from Tocris. The stock solutions were prepared in	The experiment was terminated when the mean tumor	161
110	dimethyl sulfoxide (DMSO) and stored at -20°C. Imatinib,	volume of either the treated or control groups reached	162
111	gefitinib, and NVP-AEW541 were obtained from American	2,000 mm ³ .	163
112	Custom Chemical and used at 1 μ mol/L. TAE-684 was pur-		
113	chased from Selleck and was used at 100 nmol/L. Rapamycin	Mass spectrometry	164
114	was purchased from Sigma and used at 50 nmol/L. Cells were	Targeted Ion MS/MS. Immunoprecipitations for the p85	165
115	treated with TKIs in 10% FBS for 6 hours except for rapamycin	complex were separated by SDS-PAGE until the 52 kDa marker	166
116	which was used for 16 hours.	was observed (short gel run, ~1/6 distance of mini gel lane).	167
117	Immunoprecipitation and Western blotting	Gel sections were excised above the 55 kDa band (IgG heavy	168
118	Cells were lysed in a 1% NP-40 containing lysis buffer.	chain) to avoid antibody contamination and peptide signal	169
119	Lysates were centrifuged at 16,000 $\times g$ for 5 minutes at	suppression. Gel sections were reduced with DTT, alkylated	170
		with iodoacetamide and digested overnight with TPCK mod-	171
		ified trypsin (Promega Corp.). Peptides were extracted, con-	172
		centrated to 10 μ L using a SpeedVac and analyzed by positive	173
		ion mode reversed-phase liquid chromatography tandem	174
		mass spectrometry (LC/MS/MS) using a hybrid LTQ-Orbitrap	175

178 XL mass spectrometer (Thermo Fisher Scientific). Peptides
 179 were delivered and separated using an EASY-nLC nanoflow
 180 HPLC (Proxeon Biosystems) at 300 nL/min using self-packed
 181 15 cm length \times 75 μ m i.d. C₁₈ fritted microcapillary columns.
 182 Solvent gradient conditions were 50 minutes from 3% B buffer
 183 to 38% B (B buffer: 100% acetonitrile; A buffer: 0.1% formic
 184 acid/99.9% water). Peptide precursor *m/z* ratios representing
 185 p85 binding proteins (Table 1) were targeted in the ion trap
 186 portion of the LTQ-Orbitrap XL for MS/MS via collisionally
 187 induced dissociation (CID) using Xcalibur software (Thermo
 188 Fisher Scientific) across the entire chromatogram. For tar-
 189 geted ion MS/MS (TIMM) experiments, approximately 5 to 9
 190 sequencing events were acquired per peptide sequence for
 191 average total ion current (TIC) calculations, depending upon
 192 sample abundance. TIMM cycle time for 16 IT MS/MS scans
 193 including 1 FT MS scan was approximately 2.4 seconds using a
 194 MS² max inject time of 100 m per second. MS/MS spectra were
 195 analyzed using Sequest in Proteomics Browser Software (PBS;
 196 W.S. Lane, Harvard University) by searching the reversed and
 197 concatenated Swiss-Prot protein database (version 57.5:
 198 470,369 entries) with a parent ion tolerance of 50 ppm and
 199 fragment ion tolerance of 0.80 Da. Carbamidomethylation of
 200 cysteine (+57.0293 Da) was specified in Sequest as a fixed
 201 modification and oxidation of methionine (+15.9949) as a
 202 variable modification. Targeted peptide sequences were ini-
 203 tially accepted if they matched the targeted protein from the
 204 forward database and met the following PBS scoring thresh-
 205 olds for 2+ ions: Xcorr \geq 1.9, Sf \geq 0.4, P \geq 5, Δ mass < 10 ppm.
 206 After passing the scoring thresholds, all MS/MS were then

manually inspected to be sure that *b*- and *y*- fragment ions
 aligned with the assigned sequence. False discovery rates for
 peptide identifications were calculated to be less than 0.5%.

Relative quantification via average TIC. The TIC from
 each identified MS/MS spectrum was recorded in PBS. Vali-
 dated data files were imported as .txt into in-house developed
 NakedQuant (v1.1, Beth Israel Deaconess Medical Center)
 software for MS/MS based quantification (24). NakedQuant
 was programmed in MatLab and designed to conduct as a
 platform for quantifying proteins across biological conditions
 according to average TIC, spectral counts and sum TIC. It
 contains a protein grouping algorithm and normalization
 calculations based on median TIC signal or a single protein
 (bait) and calculates ratio changes between selected samples.
 Here, the level of p85 α / β was normalized across biological
 conditions. Relative quantities of each protein were calculated
 by averaging the TIC values from all targeted peptide MS/MS
 spectra per protein and compared across sample conditions.
 After NakedQuant conducted quantitative calculations, data
 were exported to Excel for plotting. Note that Scaffold 3.1
 software (Proteome Software) can also be used for MS/MS TIC
 calculations. Biological and/or technical replicates were done
 for all targeted experiments, including xenografts where 2
 mouse tumors were used and HCC827 where biological tri-
 plicates were done. Coefficient of variation values were cal-
 culated and used in error bars on plots.

Results

To determine how Class I_A PI3K is activated in various
 cancer models, we conducted label-free quantitative mass
 spectrometry on p85 IPs from cell lysates using antibodies
 that recognize both the p85 α and p85 β regulatory subunits.
 The complex of p85-associated proteins was purified via SDS-
 PAGE, excised above 55 kDa to avoid antibody contamination,
 digested with trypsin and analyzed by LC/MS/MS. The known
 activators of PI3K that bind directly to p85 all have MWs
 greater than 55kDa. Initially, we assessed these PI3K com-
 plexes using a nontargeted or "shotgun" data-dependent LC/
 MS/MS label-free method termed spectral TIC or "average
 TIC" that averages the MS/MS TIC values across all identified
 peptides per protein as we previously described (24–28).
 However, the shotgun results suffered from high levels of
 nonspecific protein associations, a common difficulty for
 antibody-based IP-MS experiments (29, 30). This led to unreli-
 able detection of the critical p85 binding proteins that we were
 attempting to quantify likely due to MS signal suppression of
 adaptor peptides.

Targeted MS/MS of PI3K interactions

Because most of the major PI3K-activating proteins are well
 known, we targeted specific tryptic peptide precursor ions
 from the known PI3K activating proteins for MS/MS fragmen-
 tation via CID over their chromatographic elution, a label-free
 quantitative experiment referred to as "Targeted Ion MS/MS"
 (TIMM). Supplementary Figure S1 shows the schematic of the
 TIMM approach using hybrid linear ion trap/orbitrap mass
 spectrometry technology. Using a single LC/MS/MS run from a

Table 1. List of peptide sequences, MS charge states and peptide ion mass/charge (*m/z*) ratios for the PI3K adaptor and RTK proteins used for targeted ion MS/MS (TIMM) experiments

Targeted protein	Peptide sequence	<i>m/z</i> ratios [M+2H] ²⁺
p85 α	TWNVGSSNR	510.747
p85 β	AALQALGVAEGGER	671.360
IRS1	HTQRPGEPEEGAR	732.353
	AAWQESTGVEMR	711.328
IRS2	PVSVAGSPLSPGPVR	710.401
	SNTPESIAETPPAR	735.365
GAB1	LTGPDVLEYK	706.851
	APSASVDSSLYNLPR	788.902
GAB2	SSPAELSSSSQHLLR	799.910
	SAESM _{sx} SDGVGSFLPGK	792.864
ERBB3	ESGPGIAPGPEPHGLTNK	879.444
	GESIEPLDPSEK	650.817
	VLGSGVFGTVHK	600.840
PDGFR	LAEPDLEK	563.821
	VVEGTAYGLSR	576.306
	ATSELDLEM _{sx} EALK	725.361

NOTE: Peptide sequences containing methionine sulfoxide (M_{sx}) were fully oxidized.

265 tryptic digestion of a gel purified p85 complex, we targeted and
 266 quantified only 16 peptides representing p85 α , p85 β , and 6
 267 proteins (IRS1, IRS2, GAB1, GAB2, PDGFR, and ERBB3) known
 268 to bind directly to p85 α and p85 β due to their multiple pYXXM
 269 motifs binding to SH2 domains on p85. The peptides were
 270 chosen because they represent the most consistent and abundant
 271 peptide signals from tryptic digestions and shotgun LC/
 272 MS/MS done in our laboratory and are from regions where
 273 phosphorylation or other posttranslational modifications were
 274 not significantly detected. The individual peptides were iso-
 275 lated, fragmented, identified via database searching, and quan-
 276 tified by averaging the TIC values across all identified MS/MS
 277 spectra per protein. Table 1 lists the peptides used for the
 278 TIMM experiment. Of note, 4 ERBB3 peptides were used
 279 whereas other adaptor proteins were represented by only 1
 280 or 2 peptides. More ERBB3 peptides were used because it was
 281 more difficult to identify this adaptor and the quantitative
 282 information was more robust with multiple peptides. Adding
 283 peptides to other proteins did not significantly improve sensi-
 284 tivity. Because the number of total peptides for targeting was
 285 low, chromatographic scheduling, a frequently used feature for
 286 multiple reaction monitoring (MRM) experiments, was not

required. Nonscheduled targeted runs allow for potential shifts
 in chromatographic elution that could otherwise result in
 missed information and allows for the technology transfer
 across different chromatographic platforms. It is important
 to note that average TIC values are dependent upon MS
 ionization and fragmentation efficiency and can be reliably
 assessed relative to other samples in a quantitative manner
 because TIMM data do not represent absolute concentrations
 of tryptic peptides and their protein precursors in the absence
 of isotope labeled standards. However, by normalizing the TIC
 values for each adaptor protein to the values for p85 from the
 same immunoprecipitate, it is possible to compare relative
 amounts of p85-associated proteins in different cells or com-
 pare changes in these ratios in response to growth factors or
 drugs.

This targeted approach was examined initially in the NSCLC
 EBC-1 and HCC827 cell lines (Fig. 1A). EBC-1 cells have
 amplification of the *MET* (HGF receptor) tyrosine kinase,
 and treatment with a MET TKI leads to suppression of
 PI3K signaling (15). EBC-1 cells were assessed in the absence
 and presence of the MET inhibitor PHA-665,752. As shown by
 Western blot analyses, treatment with the MET inhibitor led

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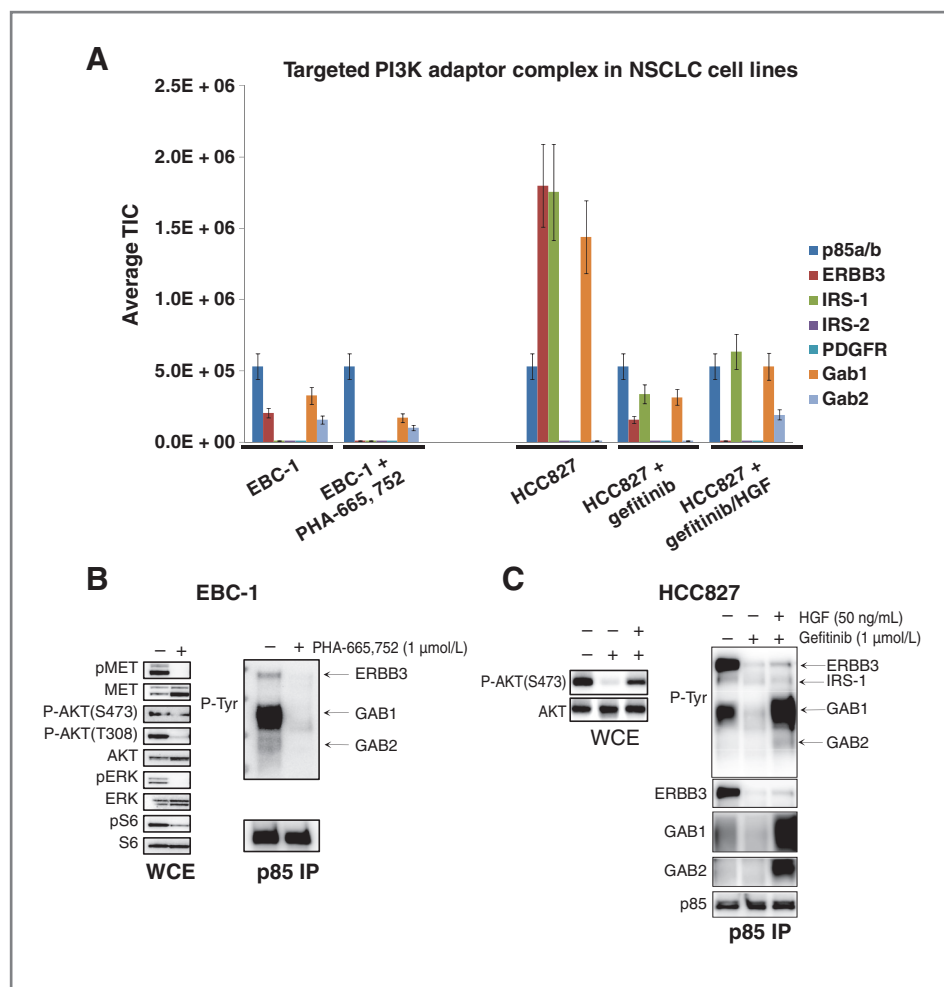


Figure 1. A, the quantitative output of the TIMM approach in EBC-1 and HCC827 cells subjected to the indicated conditions. Cells were treated with the indicated drugs and ligands for 6 hours before lysis. The relative signal level of each detected adaptor (normalized for p85 levels) is shown. B, EBC-1 cells were treated in the absence or presence of the MET inhibitor, PHA-665,752 (1 μ mol/L) for 6 hours and then subjected to lysis. C, HCC827 cells were treated in the absence or presence of gefitinib (1 μ mol/L) and HGF (50 ng/mL) for 6 hours. Left, whole cell extracts were probed with the indicated antibodies. Right, extracts were subjected to a p85 IP, and the IP was probed with an anti-PTyr and anti-p85 antibodies.

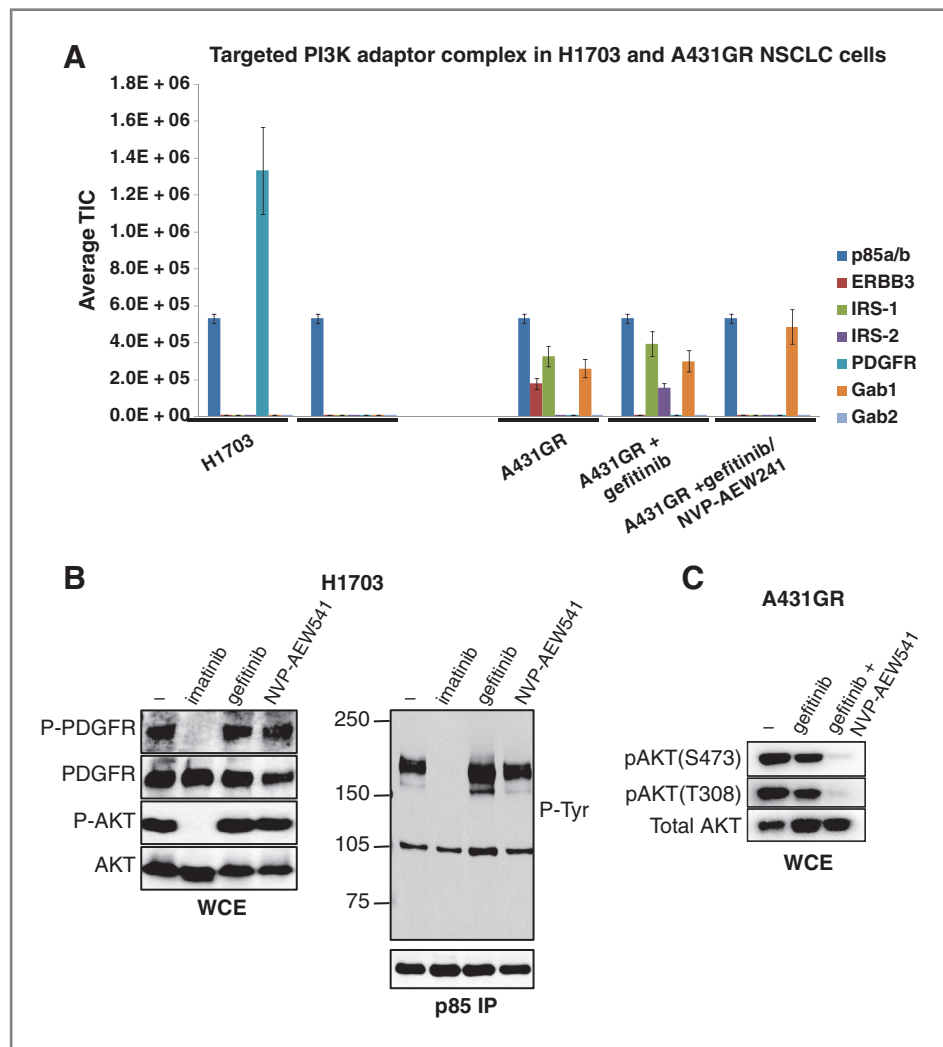
312 to a loss of AKT phosphorylation and a marked reduction in
 313 the binding of several phosphotyrosine proteins to PI3K that
 314 correspond to ERBB3, GAB1, and GAB2 (Fig. 1B). These results
 315 were recapitulated independently by the TIMM approach
 316 (Fig. 1A). Please note that the quantitative changes observed
 317 by Western and TIMM analyses were slightly different for
 318 HCC827 cells. This is partly due to the fact that the TIMM and
 319 Western experimenters were prepared from different cell
 320 preparations.

321 An *EGFR* mutant NSCLC cell line, HCC827 is highly sensitive
 322 to epidermal growth factor receptor (EGFR) TKIs. Treatment
 323 with an EGFR inhibitor leads to profound loss of PI3K-AKT
 324 signaling and decreased association between PI3K and phosphotyrosine adaptor proteins (14–17). Therefore, in this cell line, we compared the p85 complexes in the absence and presence of the EGFR kinase inhibitor, gefitinib. In *EGFR* mutant NSCLCs, we recently determined that HGF causes resistance to EGFR inhibitors by rescuing PI3K signaling via a mechanism independent of ERBB3 via GAB1 (16). As shown in Figure 1A, the TIMM method shows that treatment of HCC827

333 cells with gefitinib decreases the binding of p85 to ERBB3,
 334 IRS1, GAB1, and GAB2, and that HGF partially restores binding
 335 to the phosphotyrosine proteins except for ERBB3. These
 336 results are in agreement with the Western blot studies
 337 (Fig. 1C).

338 The encouraging results from the EBC-1 and HCC827 cells
 339 prompted us to test this targeted MS-based methodology in
 340 other NSCLC models. We examined H1703 cells, and interestingly,
 341 found that platelet-derived growth factor receptor (PDGFR)
 342 was the only adaptor bound to PI3K (Fig. 2A). This interaction
 343 was obliterated by the PDGFR inhibitor, imatinib. Using Western
 344 blots, we confirmed that the association between PI3K and a 160 kDa
 345 phosphotyrosine protein (consistent with PDGFR) was disrupted
 346 only by imatinib and not by gefitinib or the IGF-IR/InsR inhibitor,
 347 NVP-AEW541 (Fig. 2B). Accordingly, only imatinib led to loss of
 348 AKT phosphorylation (Fig. 2B). These results are in agreement
 349 with a recent report showing that this cell line has high
 350 activation of PDGFR and is sensitive to imatinib *in vitro*
 351 (23). In addition, we assessed a cell line that was made
 352

Figure 2. A, H1703 and A431 GR (gefitinib resistant) cells were treated with the indicated inhibitors and p85 adaptors were quantified using TIMM as in Figure 1. B, H1703 cells were treated with imatinib (1 μmol/L), gefitinib (1 μmol/L), or NVP-AEW541 (1 μmol/L) for the 6 hours. Left, whole cell extracts were probed with the indicated antibodies. Right, extracts were subjected to a p85 IP, and the IP was probed with an anti-PTyr and anti-p85 antibodies. C, A431 GR cells were treated with gefitinib (1 μmol/L) or gefitinib + NVP-AEW541 (1 μmol/L) for 6 hours. Extracts were probed with the indicated antibodies.



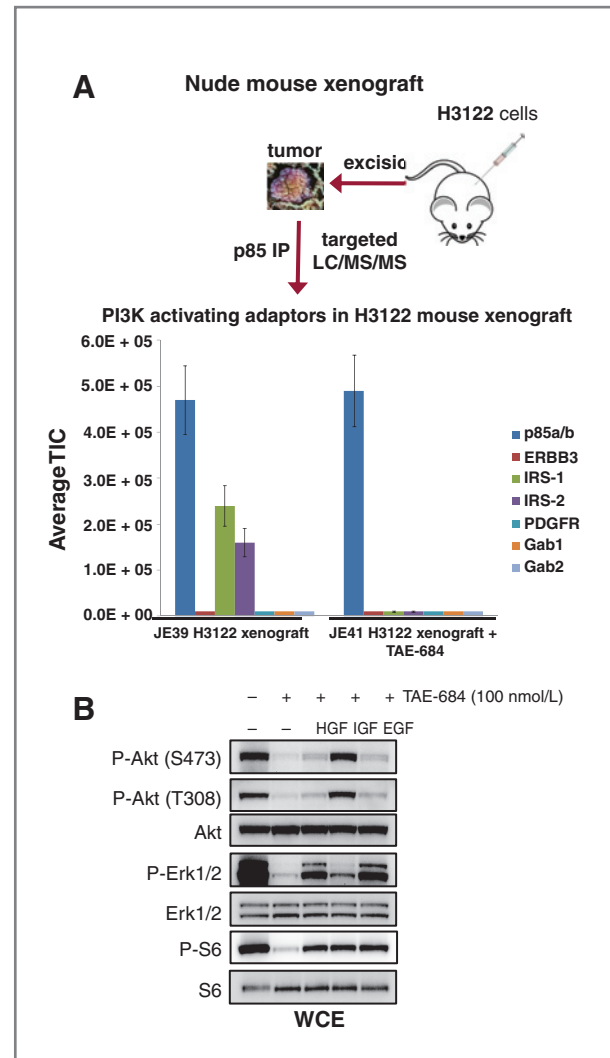
355 resistant to gefitinib, A431GR. We previously showed that
 356 these cells are resistant to EGFR inhibitors through activation
 357 of IGF1-R that leads to persistent PI3K signaling despite EGFR
 358 inhibition (17). When these cells were analyzed by TIMM, we
 359 observed that treatment with gefitinib led to loss of ERBB3
 360 binding to p85, but combined treatment with both an EGFR
 361 and IGF1-R/InsR inhibitor (NVP-AEW541) abrogated the
 362 interaction between p85 and both IRS proteins and ERBB3,
 363 consistent with our previously published Western blot analy-
 364 ses (17). In agreement with the TIMM data, concomitant
 365 EGFR and IGF1-R/InsR inhibition was required to suppress
 366 AKT phosphorylation in A431GR cells (Fig. 2C).

367 **Mouse xenograft model**

368 The encouraging results using cell lines led us to ask
 369 whether we could utilize this methodology to determine
 370 PI3K activators *in vivo*. For this study, immunodeficient mice
 371 were injected with H3122 NSCLC cells and tumors were
 372 allowed to develop (~400 mm³). H3122 tumors harbor an
 373 EML4-ALK translocation and are sensitive to ALK inhibitors *in*
 374 *in vivo* and *in vitro* (22). Mice-bearing tumors were treated
 375 with an ALK inhibitor, TAE-684, or vehicle (DMSO) control for 2
 376 days. Tumors (~130 mg) were excised and used to prepare
 377 approximately 12 mg of protein lysate for p85 IP and TIMM
 378 analysis. Figure 3A shows that the primary adaptors for PI3K
 379 activation are IRS1 and IRS2 in H3122 mouse xenografts.
 380 Interestingly, treatment with TAE-684 eliminates these inter-
 381 actions, suggesting that ALK signals to PI3K via IRS2 and IRS1.
 382 This is consistent with previous reports suggesting that NPM-
 383 ALK signals to PI3K via IRS proteins (31, 32). The finding that
 384 IRS proteins were utilized to activate PI3K suggested that
 385 these cells might activate PI3K in response to IGF1. Indeed,
 386 treatment of H3122 cells with IGF1, but not EGF or HGF,
 387 rescued PI3K-AKT signaling with TAE-684 treatment (Fig. 3B).
 388 Of note, EGF and HGF rescued ERK signaling showing that
 389 these cells do respond to those ligands, but that they are
 390 unable to rescue PI3K-AKT signaling.

391 **Effect of rapamycin on the PI3K complex**

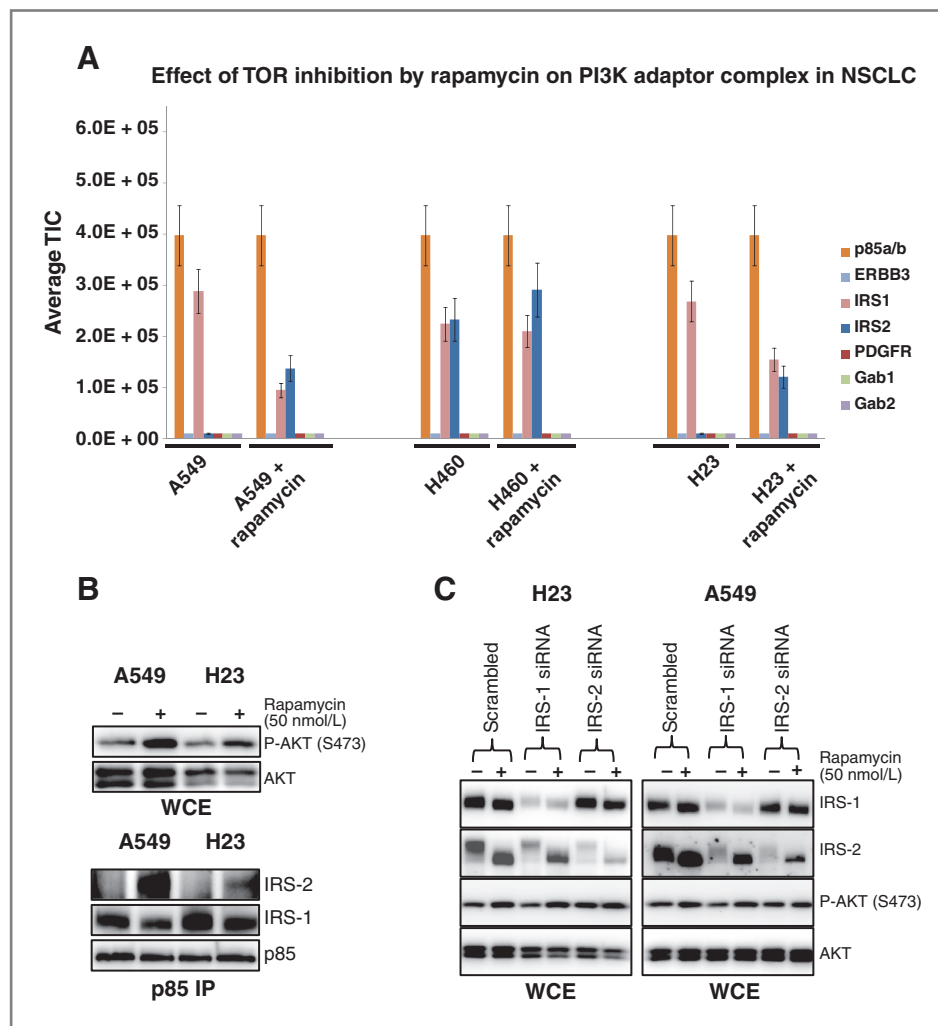
392 Previous studies have shown that inhibition of TORC1 with
 393 rapamycin induces PI3K-AKT signaling by derepressing a
 394 negative feedback. In some cancer types, this is mediated
 395 by derepression of IRS1 and activation of IGF-IR signaling (33).
 396 Thus, we utilized the TIMM approach to determine the
 397 molecular mechanism of this feedback. Several NSCLC cell
 398 lines were serum starved then treated with vehicle or rapa-
 399 mycin for 16 hours, lysed, subjected to immunoprecipitation
 400 with anti-p85 antibodies and analyzed by mass spectrometry.
 401 As shown in Figure 4A, in the *KRAS* mutant H23, A549, and
 402 H460 cells rapamycin induced an interaction of p85 with IRS2
 403 but decreased the interaction with IRS1. As shown in Figure
 404 4B, Western blot analysis of the A549 and H23 cells confirms
 405 that rapamycin enhanced the interaction between p85 and
 406 IRS2 but impaired the interaction between p85 and IRS1,
 407 thereby verifying the mass spectrometry data. Furthermore,
 408 knockdown of IRS2, but not IRS1, abrogated the capacity of
 409 rapamycin to induce AKT phosphorylation in these cells
 410 (Fig. 4C). The reason why p85 switches from IRS1 to IRS2



412 **Figure 3.** A, H3122 xenografts harboring the EML4-ALK translocation
 413 were treated with control vehicle or the ALK inhibitor, TAE-684, for 2 days;
 414 the tumors were excised and lysates were prepared. The TIMM results
 415 for the control and treated animals are shown. B, H3122 cells were treated
 416 in the presence or absence of TAE-684 (100 nmol/L) for 6 hours in the
 417 presence or absence of the indicated ligands [EGF (50 ng/mL), IGF1
 418 (50 ng/mL), and HGF (50 ng/mL)]. Extracts were probed with the indicated
 419 antibodies.

412 in response to rapamycin treatment of these *KRAS* trans-
 413 formed cell lines is not clear. Apparently, the IRS2-PI3K
 414 complex is more critical than the IRS1-PI3K complex for
 415 AKT activation. IRS protein band shifts are due to loss of
 416 previously characterized phosphoserine and phosphothreo-
 417 nine sites (34). Figure 5 shows an unsupervised hierarchical
 418 clustering heat map for all of the p85 IP-TIMM experiments in
 419 this study. The data generally cluster according to treatment
 420 and cell line. The expansion of this heat map with data from
 421 other cell lines, xenograft models and, eventually, human
 422 cancer specimens may ultimately provide a reference that
 423 will predict mechanisms of PI3K activation in different cancer
 424 types. For example, the heat map clearly indicates that PI3K in

Figure 4. A, the TIMM results from the p85 adaptor complexes in KRAS mutated A549, H460, and H23 NSCLC cells in the absence or presence of rapamycin (50 nmol/L) for 16 hours. Results were quantified as in Figure 2. B, extracts from the A549 and H23 treated as in (A) were probed with the indicated antibodies. C, H23 and A549 cells were transiently transfected with scramble, IRS1, or IRS2 siRNA. Cells were treated in the absence or presence of rapamycin (50 nmol/L) for 16 hours. Cells were lysed and probed with the indicated antibodies.



427 H1703 cells is driven solely by PDGFR, a situation where
428 imatinib was the effective treatment.

429 Quantifying the E545K PIK3CA mutation

430 In addition to determining the mechanism of PI3K activa-
431 tion, we tested whether the same p85 IPs could quantify the
432 amount of mutant p110 in those cancers harboring a somatic
433 mutation. *PIK3CA*, the gene encoding p110 α , is mutated in
434 some cancers. These mutations are usually located in one of
435 the 2 "hotspots" in the gene (35–37). One exists in the helical
436 domain (E545K) and the other in the kinase domain (H1047R).
437 Both have been linked to aberrant PI3K-AKT activation (36, 38,
438 39). We obtained several cancer cell lines known to possess the
439 E545K mutation including breast carcinoma MCF7 and
440 NSCLC H460. As controls, we included 2 p110 α WT breast
441 cancer cell lines, SKBR3 and BT474 that were infected with
442 retroviruses encoding E545K p110 α mutant. The total level of
443 the WT E545 p110 α peptide DPLSEITEQEK (2+, *m/z* 644.82)
444 was normalized across all samples. Because only a single
445 peptide was quantified across conditions, sum TIC was used

rather than average TIC. The WT and mutated [DPLSEITK
(2+, *m/z* 451.75)] tryptic peptides are different lengths and
contain amino acid differences, so it is possible that the
ionization efficiency of each peptide varies. However, a dif-
ference in the TIC value ratio of the E545 mutant to the WT
p110 α between cell types or in different immunoprecipitates
from the same cell provides useful information. As shown in
Figure 6A, the TIMM method correctly identified the presence
of the E545K mutation in MCF7 cells (40) and the lack of this
mutant protein in p85 immunoprecipitates from WT SKBR3
and BT474 cells (41). The TIMM method also detected the
E545K mutant in SKBR3 and BT474 cells that were engineered
to express this protein, and the relative TIC values indicate
that the ratio of mutant to WT p110 α is considerably higher
(nearly 10-fold) in the engineered cells, although this increase
may be inflated because the total level of p110 is higher in the
overexpressed cells.

In addition, we took advantage of the quantitative nature of
this assessment to determine whether the E545K mutant is
preferentially recruited to phosphotyrosine adaptors. We

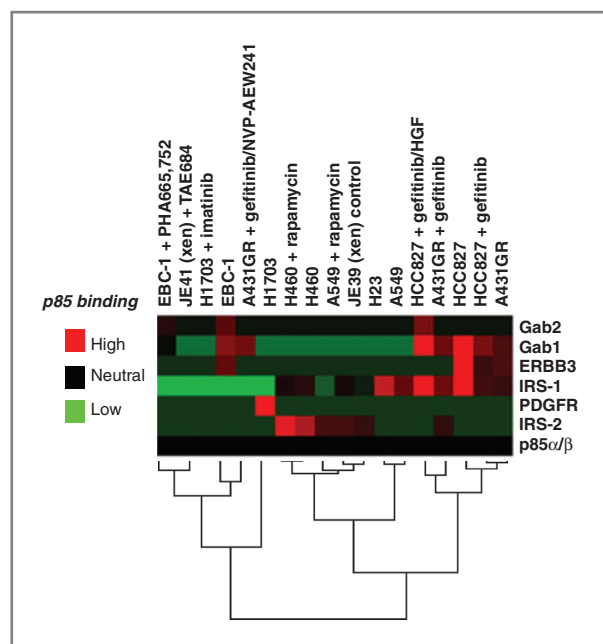


Figure 5. Unsupervised hierarchical clustering heat map for the targeted p85 IP mass spectrometry results done in Figures 1–4. TIC was normalized to p85 α /p85 β across all experiments.

469 utilized the H460 NSCLC cells that harbor the E545K p110 α
 470 catalytic site mutation and use IRS proteins to associate with
 471 p85 for activation of PI3K/AKT (Fig. 4A). We compared the
 472 ratio of the mutant PI3K in p85 IPs (holoenzyme bound to
 473 adaptor and free) versus IRS1 IPs (PI3K bound to adaptor in
 474 active state). We observed that the relative amount of E545K is
 475 greater than 2-fold higher in the IRS1 IPs (fully active form;
 476 Fig. 6B). This result is consistent with structural analyses of
 477 E545K suggesting that the mutation abrogates an intermole-
 478 cular interaction with an SH2 domain in p85 (11, 12). There-
 479 fore, in the PI3K holoenzymes containing E545K, the
 480 untethered SH2 domain could be more available to bind to
 481 phosphotyrosine adaptors. The MS/MS fragmentation spectra
 482 and the extracted ion chromatograms for the WT and mutant
 483 peptides are shown in Figure 6C and D, respectively. We also
 484 attempted to target the H1047R p110 α mutation from BT474
 485 engineered breast cancer cells; however, this mutation
 486 resulted in a tryptic peptide (QMNDAR) of weak signal in
 487 MS and its MW was too small for reliable MS/MS identifica-
 488 tion. Alternative proteolytic enzymes and/or MRM may be
 489 better suited for quantifying H1047R by MS.

490 Discussion

491 The central role of PI3K in the sensitivity and resistance to
 492 targeted therapies has increased the need for understanding
 493 the molecular regulation of this enzyme in cancer cells. In this
 494 study, we utilized a targeted MS approach. This approach
 495 effectively identifies the activators of PI3K and holds promise
 496 for understanding how various cancers regulate PI3K in a
 497 dynamic manner.

Initially, we utilized a nontargeted tandem MS (shotgun)
 approach to determine the adaptor proteins bound to p85 in
 cancer cells. Although this method effectively identified sev-
 eral PI3K-activating proteins, it was not sufficiently sensitive
 or quantitative to specifically determine the phosphotyrosine
 proteins activating PI3K across NSCLC cell lines. Thus, we
 switched to a targeted approach to quantitatively measure the
 association of PI3K with key adaptors and RTKs that are the
 most common activators of PI3K. Although there are several
 methods for conducting quantitative analyses of protein
 interactions by MS, we utilized hybrid ion trap-orbitrap
 technology whereby we targeted peptide ions for isolation
 and fragmentation in the ion trap component. The trap fills
 with product ions of each peptide precursor ion across the
 chromatogram and generates sufficient and quantitative sig-
 nal when the peptide of interest elutes from the column. We
 have used a similar technique to quantify phosphopeptide
 signals from various signaling proteins (42–44). Alternatively,
 one can use stable isotope labeling approaches such as SILAC
 (45) or chemical tagging approaches such as iTRAQ (46) in
 addition to label-free quantitative methodologies including
 spectral counting (47) and MRM (48). SILAC is a metabolic
 labeling approach that is useful in cell culture but is not readily
 adapted to *in vivo* tissue sources while iTRAQ requires cleanup
 and a chemical labeling step that can be affected by the
 sample matrix (49). In this study, we observed that hybrid
 linear ion trap–orbitrap mass spectrometers can be used for
 successful label-free quantification in targeted mode if the
 total number of peptides is kept low because the cycle time is
 slower than that for triple quadrupole mass spectrometers in
 MRM mode.

Currently, there are several methods for assessing the status
 of PI3K signaling pathway in cancers including genetic ana-
 lyses and assessment of the abundance of downstream signal-
 ing events such as phospho-AKT. However, there have not
 been any validated methods for determining how PI3K is
 activated in cancers, especially those without *PIK3CA* or *PTEN*
 mutations. This study validates a simple mass spectrometry
 method to make such determinations. Although we deter-
 mined how PI3K was activated in a variety of cancer para-
 digms in this study, there are clearly other applications. For
 example, MEK inhibitors (8) and other cellular stresses such as
 radiation (50) can lead to activation of PI3K signaling, and this
 method could be used to determine the molecular mecha-
 nisms of activation. Indeed, this approach could identify poten-
 tial therapeutic targets that would prevent PI3K activation in
 response to these stresses. In addition, one can include hot
 spot mutations in oncogenes such as *PIK3CA* in the targeted
 approach to correlate mutation status with adaptor activa-
 tion. We also evaluated other RTKs for PI3K activation such as
 EGFR and MET receptor but did not find evidence of direct
 p85 binding because these receptors lack multiple pYXXM
 motifs, although they associated with known adaptors at low
 levels in some p85 IPs.

Herein, we showed that mass spectrometry in combination
 with immunopurification of signaling complexes can be used
 to identify oncogenic pathways driving tumor growth using
 milligram quantities of tumor tissue. As these MS results are

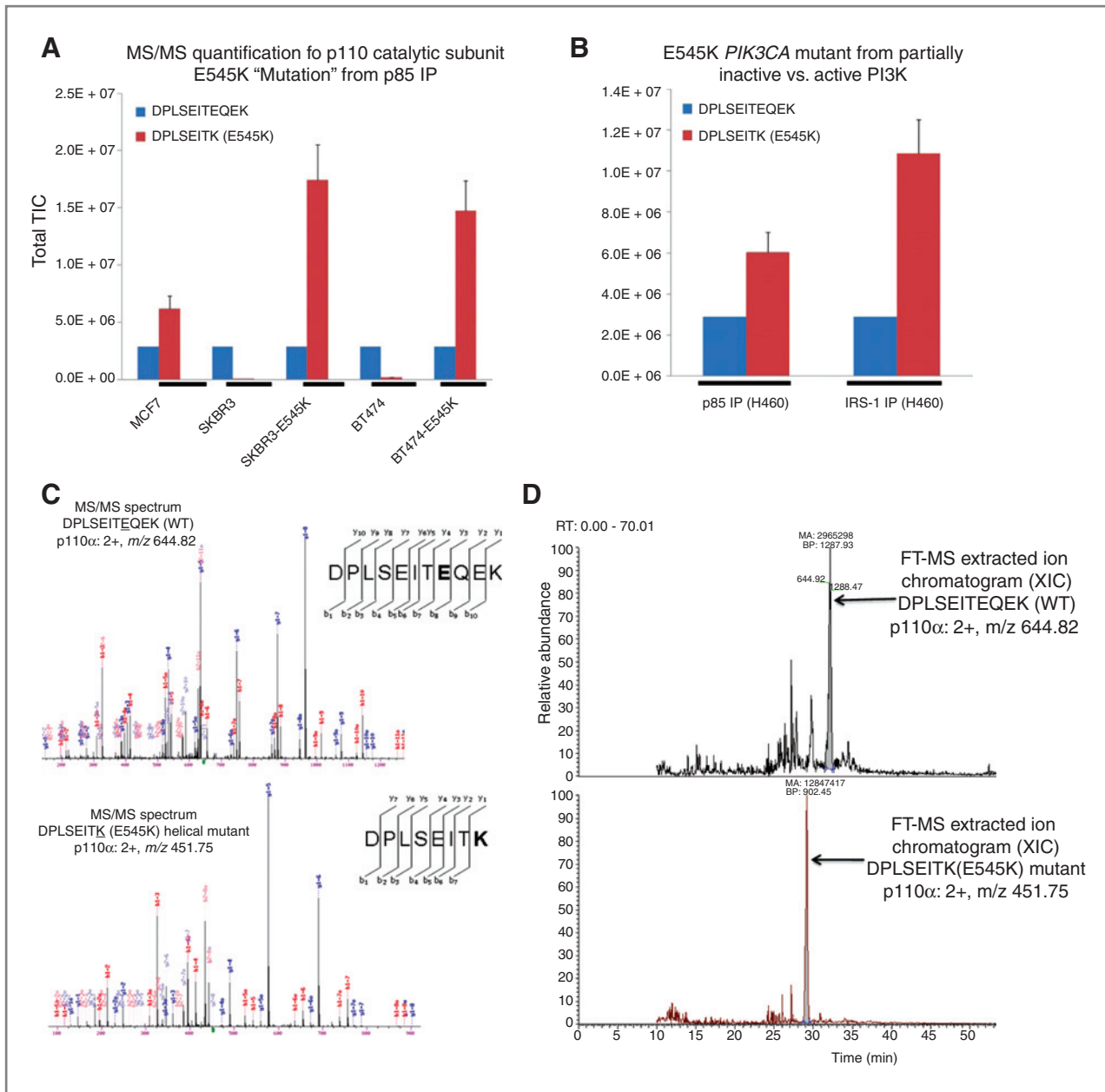


Figure 6. A, quantitative analyses of E545K peptide targeted by mass spectrometry from the MCF7 cells and from SKBR3 and BT-474 cells engineered to express the WT p110 α or the E545K mutant. B, H460 cells were lysed and immunoprecipitated with an anti-p85 antibody or an anti-IRS1 antibody. The TIMM data show that E545K is enriched more than 2-fold in the IRS1 complex. C, the MS/MS fragmentation spectra via CID identifying the p110 α WT and E545K somatic mutant peptide quantified from H460 cells. D, the extracted ion chromatograms for the WT and E545K mutant p110 α peptides. These represent the typical TIMM peak elution profile for targeted tryptic peptides using hybrid linear ion trap-orbitrap technology via LC/MS/MS.

559 coupled with comprehensive genetic analyses of cancers, the
 560 mechanisms of PI3K activation may be predicted by the
 561 genetic abnormalities harbored by a particular cancer, thus
 562 leading to personalized therapeutic strategies that block PI3K
 563 activation.

564 Q2 **Disclosure of Potential Conflicts of Interest**

565 No potential conflicts of interest were disclosed.

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