

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

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### 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)P<sub>2</sub>] to phosphatidylinositol 3,4,5-triphos-  
26 phate [PtdIns(3,4,5)P<sub>3</sub>] 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<sub>A</sub> 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 $\alpha$  subunit, and  
in *PTEN*, the gene encoding the phosphatase that degrades  
PIP<sub>3</sub>, 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<sub>A</sub> 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.

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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 $\alpha$ 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	<b>Materials and Methods</b>		
84	<b>Cell lines and reagents</b>	<b>Scrambled siRNA transfection and E545K expression</b>	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 $\alpha$ 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 $\mu$ 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 $\mu$ 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	<b>Xenograft studies</b>	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 $5 \times 10^6$ 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 <sup>3</sup> , 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 <sup>3</sup> .	163
112	Custom Chemical and used at 1 $\mu$ mol/L. TAE-684 was pur-		
113	chased from Selleck and was used at 100 nmol/L. Rapamycin	<b>Mass spectrometry</b>	164
114	was purchased from Sigma and used at 50 nmol/L. Cells were	<b>Targeted Ion MS/MS.</b> 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	<b>Immunoprecipitation and Western blotting</b>	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 $\mu$ 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

XL mass spectrometer (Thermo Fisher Scientific). Peptides were delivered and separated using an EASY-nLC nanoflow HPLC (Proxeon Biosystems) at 300 nL/min using self-packed 15 cm length  $\times$  75  $\mu$ m i.d. C<sub>18</sub> fritted microcapillary columns. Solvent gradient conditions were 50 minutes from 3% B buffer to 38% B (B buffer: 100% acetonitrile; A buffer: 0.1% formic acid/99.9% water). Peptide precursor *m/z* ratios representing p85 binding proteins (Table 1) were targeted in the ion trap portion of the LTQ-Orbitrap XL for MS/MS via collisionally induced dissociation (CID) using Xcalibur software (Thermo Fisher Scientific) across the entire chromatogram. For targeted ion MS/MS (TIMM) experiments, approximately 5 to 9 sequencing events were acquired per peptide sequence for average total ion current (TIC) calculations, depending upon sample abundance. TIMM cycle time for 16 IT MS/MS scans including 1 FT MS scan was approximately 2.4 seconds using a MS<sup>2</sup> max inject time of 100 m per second. MS/MS spectra were analyzed using Sequest in Proteomics Browser Software (PBS; W.S. Lane, Harvard University) by searching the reversed and concatenated Swiss-Prot protein database (version 57.5: 470,369 entries) with a parent ion tolerance of 50 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) as a variable modification. Targeted peptide sequences were initially accepted if they matched the targeted protein from the forward database and met the following PBS scoring thresholds for 2+ ions: Xcorr  $\geq$  1.9, Sf  $\geq$  0.4, P  $\geq$  5,  $\Delta$ mass < 10 ppm. 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. Validated 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 $\alpha$ / $\beta$  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 triplicates were done. Coefficient of variation values were calculated and used in error bars on plots.

## Results

To determine how Class I<sub>A</sub> 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 $\alpha$  and p85 $\beta$  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 complexes 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 unreliable 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 fragmentation 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] <sup>2+</sup>
p85 $\alpha$	TWNVGSSNR	510.747
p85 $\beta$	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	SSPAELSSSQHLLR	799.910
	SAESM <sub>sx</sub> SDGVGSFLPGK	792.864
ERBB3	ESGPGIAPGPEPHGLTNK	879.444
	GESIEPLDPSEK	650.817
	VLGSGVFGTVHK	600.840
PDGFR	LAEPDLLLEK	563.821
	VVEGTAYGLSR	576.306
	ATSELDLEM <sub>sx</sub> EALK	725.361

NOTE: Peptide sequences containing methionine sulfoxide (M<sub>sx</sub>) were fully oxidized.

265 tryptic digestion of a gel purified p85 complex, we targeted and  
 266 quantified only 16 peptides representing p85 $\alpha$ , p85 $\beta$ , and 6  
 267 proteins (IRS1, IRS2, GAB1, GAB2, PDGFR, and ERBB3) known  
 268 to bind directly to p85 $\alpha$  and p85 $\beta$  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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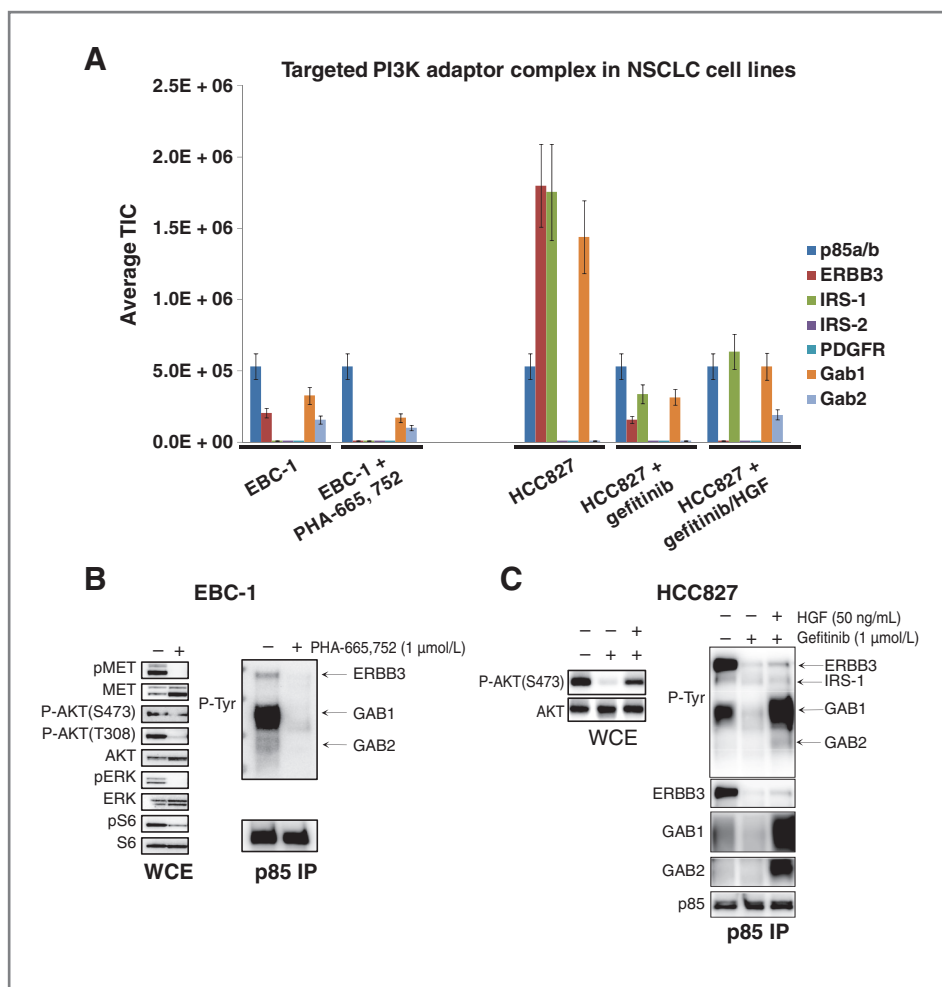


Figure 1. A, the quantitative output of the TIMM approach in EBC-1 and HCC827 cells subjected to the indicated conditions. Cells were treated with the indicated drugs and ligands for 6 hours before lysis. The relative signal level of each detected adaptor (normalized for p85 levels) is shown. B, EBC-1 cells were treated in the absence or presence of the MET inhibitor, PHA-665,752 (1  $\mu$ mol/L) for 6 hours and then subjected to lysis. C, HCC827 cells were treated in the absence or presence of gefitinib (1  $\mu$ 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.

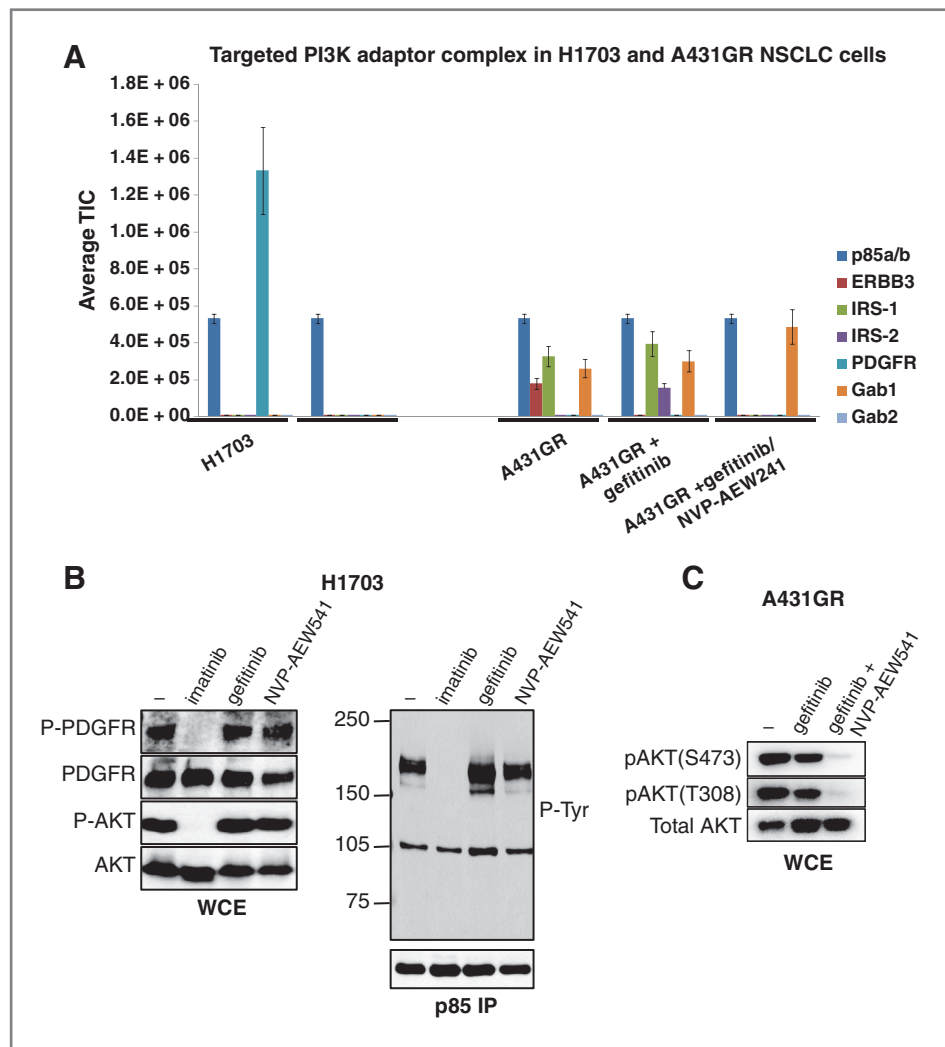
312 to a loss of AKT phosphorylation and a marked reduction in  
 313 the binding of several phosphotyrosine proteins to PI3K that  
 314 correspond to ERBB3, GAB1, and GAB2 (Fig. 1B). These results  
 315 were recapitulated independently by the TIMM approach  
 316 (Fig. 1A). Please note that the quantitative 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 phos-  
 325 photyrosine adaptor proteins (14–17). Therefore, in this cell  
 326 line, we compared the p85 complexes in the absence and  
 327 presence of the EGFR kinase inhibitor, gefitinib. In *EGFR*  
 328 mutant NSCLCs, we recently determined that HGF causes  
 329 resistance to EGFR inhibitors by rescuing PI3K signaling via a  
 330 mechanism independent of ERBB3 via GAB1 (16). As shown in  
 331 Figure 1A, the TIMM method shows that treatment of HCC827

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

337 The encouraging results from the EBC-1 and HCC827 cells  
 338 prompted us to test this targeted MS-based methodology in  
 339 other NSCLC models. We examined H1703 cells, and inter-  
 340 estingly, found that platelet-derived growth factor receptor  
 341 (PDGFR) was the only adaptor bound to PI3K (Fig. 2A). This  
 342 interaction was obliterated by the PDGFR inhibitor, imatinib.  
 343 Using Western blots, we confirmed that the association  
 344 between PI3K and a 160 kDa phosphotyrosine protein (con-  
 345 sistent with PDGFR) was disrupted only by imatinib and not  
 346 by gefitinib or the by IGF-IR/InsR inhibitor, NVP-AEW541  
 347 (Fig. 2B). Accordingly, only imatinib led to loss of AKT  
 348 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.



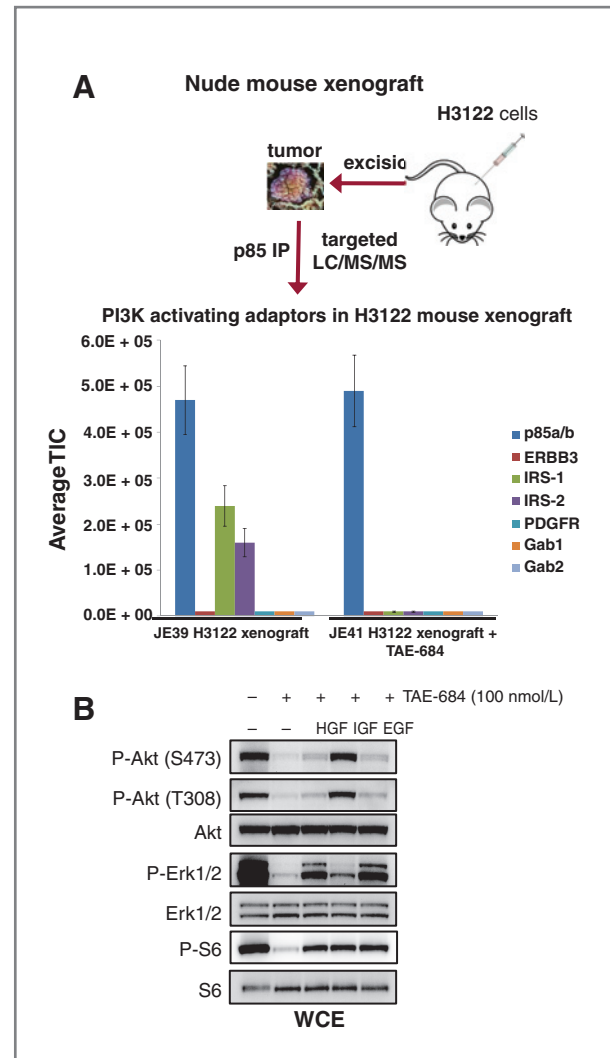
355 resistant to gefitinib, A431GR. We previously showed that  
 356 these cells are resistant to EGFR inhibitors through activation  
 357 of IGF1-R that leads to persistent PI3K signaling despite EGFR  
 358 inhibition (17). When these cells were analyzed by TIMM, we  
 359 observed that treatment 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 mm<sup>3</sup>). 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**

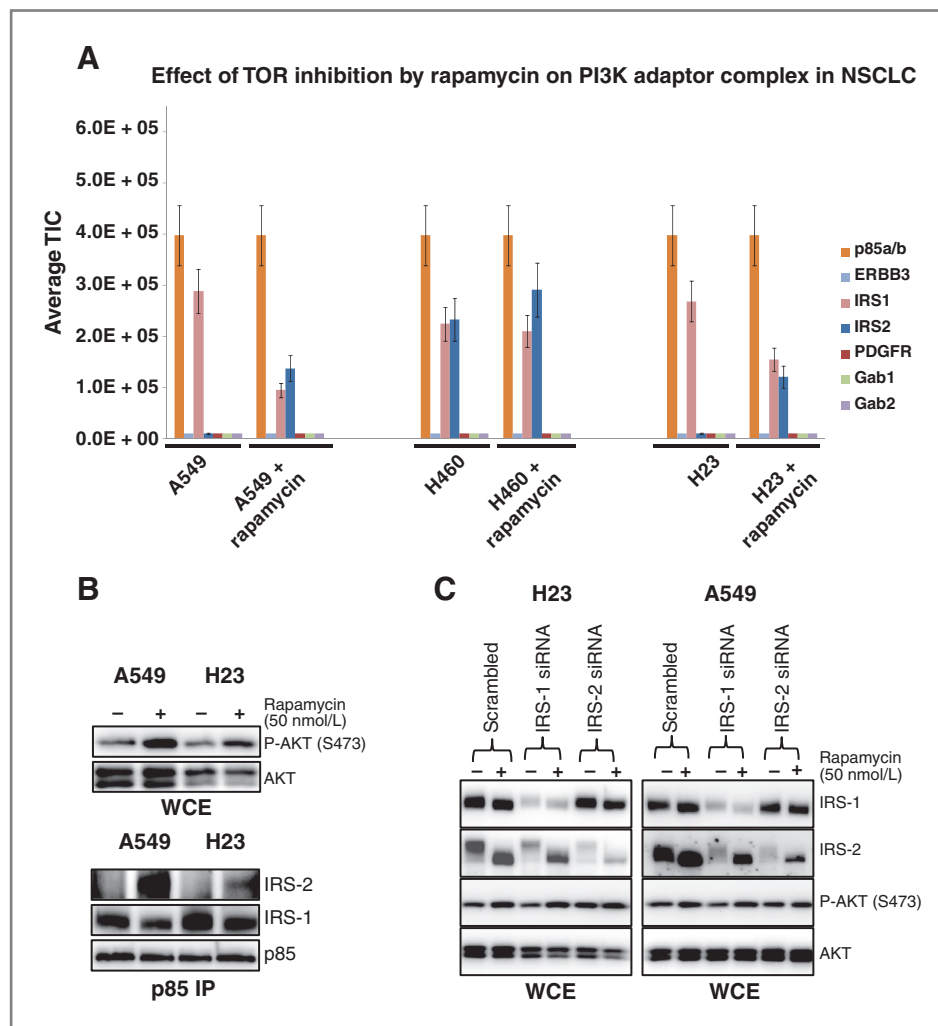
392 Previous studies have shown that inhibition of TORC1 with  
 393 rapamycin induces PI3K-AKT signaling by derepressing a  
 394 negative feedback. In some cancer types, this is mediated  
 395 by derepression of IRS1 and activation of IGF-IR signaling (33).  
 396 Thus, we utilized the TIMM approach 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 $\alpha$ , 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 $\alpha$  WT breast  
441 cancer cell lines, SKBR3 and BT474 that were infected with  
442 retroviruses encoding E545K p110 $\alpha$  mutant. The total level of  
443 the WT E545 p110 $\alpha$  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 $\alpha$  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 $\alpha$  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

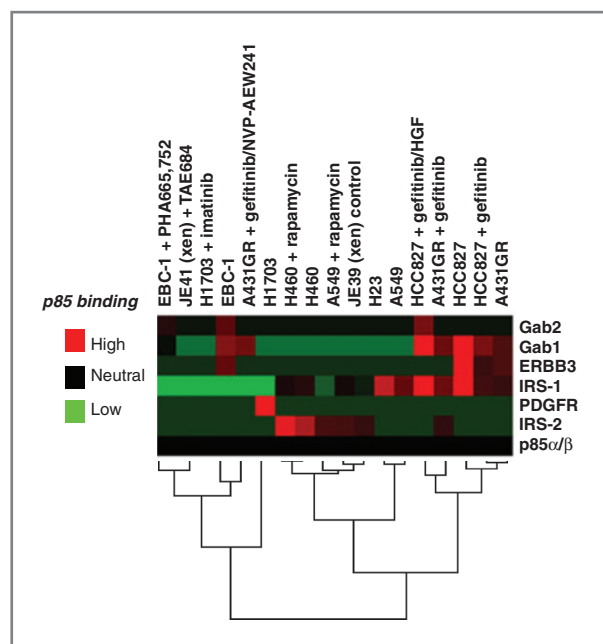


Figure 5. Unsupervised hierarchical clustering heat map for the targeted p85 IP mass spectrometry results done in Figures 1–4. TIC was normalized to p85 $\alpha$ /p85 $\beta$  across all experiments.

469 utilized the H460 NSCLC cells that harbor the E545K p110 $\alpha$   
 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 $\alpha$  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 mechan-  
 isms 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



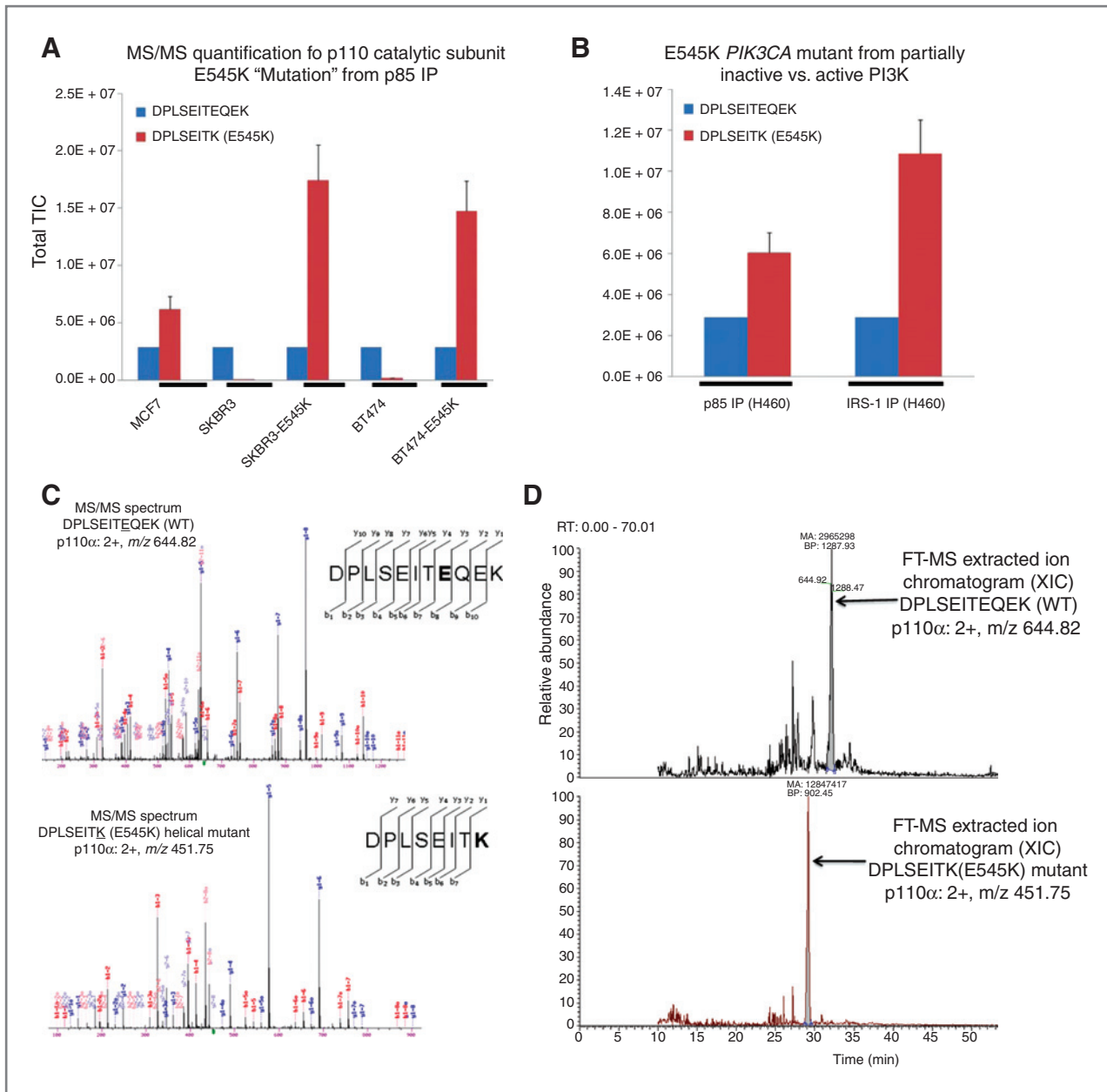


Figure 6. A, quantitative analyses of E545K peptide targeted by mass spectrometry from the MCF7 cells and from SKBR3 and BT-474 cells engineered to express the WT p110 $\alpha$  or the E545K mutant. B, H460 cells were lysed and immunoprecipitated with an anti-p85 antibody or an anti-IRS1 antibody. The 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 $\alpha$  WT and E545K somatic mutant peptide quantified from H460 cells. D, the extracted ion chromatograms for the WT and E545K mutant p110 $\alpha$  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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## References

- 588 1. Engelman JA. Targeting PI3K signaling in cancer: opportunities,  
589 challenges and limitations. *Nat Rev Cancer* 2009;9:550–62.
- 590 2. Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol  
591 3-kinases as regulators of growth and metabolism. *Nat Rev  
592 Genet* 2006;7:606–19.
- 593 3. Samuels Y, Ericson K. Oncogenic PI3K and its role in cancer. *Curr  
594 Opin Oncol* 2006;18:77–82.
- 595 4. Engelman JA, Chen L, Tan X, Crosby K, Guimaraes AR, Upadhyay R,  
596 et al. Effective use of PI3K and MEK inhibitors to treat mutant Kras  
597 G12D and PIK3CA H1047R murine lung cancers. *Nat Med*  
598 2008;14:1351–6.
- 599 5. Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM,  
600 Beelen K, et al. A functional genetic approach identifies the PI3K  
601 pathway as a major determinant of trastuzumab resistance in breast  
602 cancer. *Cancer Cell* 2007;12:395–402.
- 603 6. Engelman JA, Mukohara T, Zejnullahu K, Lifshits E, Borrás AM, Gale  
604 CM, et al. Allelic dilution obscures detection of a biologically sig-  
605 nificant resistance mutation in EGFR-amplified lung cancer. *J Clin  
606 Invest* 2006;116:2695–706.
- 607 7. Sos ML, Fischer S, Ullrich R, Peifer M, Heuckmann JM, Koker M, et al.  
608 Identifying genotype-dependent efficacy of single and combined  
609 PI3K- and MAPK-pathway inhibition in cancer. *Proc Natl Acad Sci  
610 U S A* 2009;106:18351–6.
- 611 8. Faber AC, Li D, Song Y, Liang MC, Yeap BY, Bronson RT, et al.  
612 Differential induction of apoptosis in HER2 and EGFR addicted can-  
613 cers following PI3K inhibition. *Proc Natl Acad Sci U S A*  
614 2009;106:19503–8.
- 615 9. Gupta S, Ramjaun AR, Haiko P, Wang Y, Warne PH, Nicke B, et al.  
616 Binding of ras to phosphoinositide 3-kinase p110alpha is required for  
617 ras-driven tumorigenesis in mice. *Cell* 2007;129:957–68.
- 618 10. Shekar SC, Wu H, Fu Z, Yip SC, Nagajothi , Cahill SM, et al.  
619 Mechanism of constitutive phosphoinositide 3-kinase activation by  
620 oncogenic mutants of the p85 regulatory subunit. *J Biol Chem*  
621 2005;280:27850–5.
- 622 11. Miled N, Yan Y, Hon WC, Perisic O, Zvebil M, Inbar Y, et al.  
623 Mechanism of two classes of cancer mutations in the phosphoinosi-  
624 tide 3-kinase catalytic subunit. *Science* 2007;317:239–42.
- 625 12. Huang CH, Mandelker D, Schmidt-Kittler O, Samuels Y, Velculescu  
626 VE, Kinzler KW, et al. The structure of a human p110alpha/p85alpha  
627 complex elucidates the effects of oncogenic PI3Kalpha mutations.  
628 *Science* 2007;318:1744–8.
- 629 13. Engelman JA, Jänne PA. Mechanisms of acquired resistance to  
630 epidermal growth factor receptor tyrosine kinase inhibitors in non-  
631 small cell lung cancer. *Clin Cancer Res* 2008;14:2895–9.
- 632 14. Engelman JA, Jänne PA, Mermel C, Pearlberg J, Mukohara T, Fleet C,  
633 et al. ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-  
634 sensitive non small cell lung cancer cell lines. *Proc Natl Acad Sci U S A*  
635 2005;102:3788–93.
- 636 15. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO,  
637 et al. MET amplification leads to gefitinib resistance in lung cancer by  
638 activating ERBB3 signaling. *Science* 2007;316:1039–43.
- 639 16. Turke AB, Zejnullahu K, Wu YL, Song Y, Dias-Santagata D, Lifshits E,  
640 et al. Preexistence and clonal selection of MET amplification in EGFR  
641 mutant NSCLC. *Cancer Cell* 2010;17:77–88.
- 642 17. Guix M, Faber AC, Wang SE, Olivares MG, Song Y, Qu S, et al.  
643 Acquired resistance to EGFR tyrosine kinase inhibitors in cancer cells  
644 is mediated by loss of IGF-binding proteins. *J Clin Invest*  
645 2008;118:2609–19.
- 646 18. Stommel JM, Kimmelman AC, Ying H, Nabioullin R, Ponugoti AH,  
647 Wiedemeyer R, et al. Coactivation of receptor tyrosine kinases affects  
648 the response of tumor cells to targeted therapies. *Science* 2007;318:  
649 287–90.
- 650 19. Wang Q, Chaerkady R, Wu J, Hwang HJ, Papadopoulos N, Kopelo-  
651 vich L, et al. Mutant proteins as cancer-specific biomarkers. *Proc Natl  
652 Acad Sci U S A* 2011;108:2444–9.
- 653 20. Zhang G, Fang B, Liu RZ, Lin H, Kinose F, Bai Y, et al. Mass  
654 spectrometry mapping of epidermal growth factor receptor phosphor-  
655 ylation related to oncogenic mutations and tyrosine kinase inhibitor  
656 sensitivity. *J Proteome Res* 2011;10:305–19.
- 657 21. ten Have S, Boulon S, Ahmad Y, Lamond AI. Mass spectrometry-  
658 based immuno-precipitation proteomics—The user's guide. *Proteom-  
659 ics* 2011;11:1153–9.
- 660 22. Koivunen JP, Mermel C, Zejnullahu K, Murphy C, Lifshits E, Holmes  
661 AJ, et al. EML4-ALK fusion gene and efficacy of an ALK kinase  
662 inhibitor in lung cancer. *Clin Cancer Res* 2008;14:4275–83.
- 663 23. McDermott U, Ames RY, Iafrate AJ, Maheswaran S, Stubbs H,  
664 Greninger P, et al. Ligand-dependent platelet-derived growth factor  
665 receptor (PDGFR)-alpha activation sensitizes rare lung cancer and  
666 sarcoma cells to PDGFR kinase inhibitors. *Cancer Res* 2009;69:3937–  
667 46.
- 668 24. Yang X, Friedman A, Nagpal S, Perrimon N, Asara JM. Use of a label-  
669 free quantitative platform based on MS/MS average TIC to calculate  
670 dynamics of protein complexes in insulin signaling. *J Biomol Tech*  
671 2009;20:272–7.
- 672 25. Asara JM, Christofk HR, Freemark LM, Cantley LC. A label-free  
673 quantification method by MS/MS TIC compared to SILAC and spec-  
674 tral counting in a proteomics screen. *Proteomics* 2008;8:994–9.
- 675 26. Jiang X, Chen S, Asara JM, Balk SP. Phosphoinositide 3-kinase  
676 pathway activation in phosphate and tensin homolog (PTEN) deficient  
677 prostate cancer cells is independent of receptor tyrosine kinases and  
678 mediated by the p110beta and p110delta catalytic subunits. *J Biol  
679 Chem* 2010;285:14980–9.
- 680 27. Clements RT, Smejkal G, Sodha NR, Ivanov AR, Asara JM, Feng J,  
681 et al. Pilot proteomic profile of differentially regulated proteins in right  
682 atrial appendage before and after cardiac surgery using cardioplegia  
683 and cardiopulmonary bypass. *Circulation* 2008;118:S24–31.
- 684 28. Kesavan K, Rattliff J, Johnson EW, Dahlberg W, Asara JM, Misra P,  
685 et al. Annexin A2 is a molecular target for TM601, a peptide with  
686 tumor-targeting and anti-angiogenic effects. *J Biol Chem*  
687 2010;285:4366–74.
- 688 29. Yang L, Zhang H, Bruce JE. Optimizing the detergent concentration  
689 conditions for immunoprecipitation (IP) coupled with LC-MS/MS  
690 identification of interacting proteins. *Analyst* 2009;134:755–62.
- 691 30. Malovannaya A, Li Y, Bulynko Y, Jung SY, Wang Y, Lanz RB, et al.  
692 Streamlined analysis schema for high-throughput identification of  
693 endogenous protein complexes. *Proc Natl Acad Sci U S A*  
694 2010;107:2431–6.
- 695 31. Slupianek A, Nieborowska-Skorska M, Hoser G, Morrione A,  
696 Majewski M, Xue L, et al. Role of phosphatidylinositol 3-kinase-  
697 AKT pathway in nucleophosmin/anaplastic lymphoma kinase-  
698 mediated lymphomagenesis. *Cancer Res* 2001;61:2194–9.
- 699 32. Marzec M, Kasprzycka M, Liu X, El-Salem M, Halasa K, Raghunath  
700 PN, et al. Oncogenic tyrosine kinase NPM/ALK induces activation of  
701 the rapamycin-sensitive mTOR signaling pathway. *Oncogene*  
702 2007;26:5606–14.
- 703 33. O'Reilly KE, Rojo F, She QB, Solit D, Mills GB, Smith D, et al. mTOR  
704 inhibition induces upstream receptor tyrosine kinase signaling and  
705 activates AKT. *Cancer Res* 2006;66:1500–8.
- 706 34. Shah OJ, Hunter T. Turnover of the Active Fraction of IRS1 Involves  
707 Raptor-mTOR and S6K1-Dependent Serine Phosphorylation in Cell  
708 Culture Models of Tuberous Sclerosis. *Mol Cell Biol* 2006;26:6425–34.
- 709 35. Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, et al. High  
710 frequency of mutations of the PIK3CA gene in human cancers.  
711 *Science* 2004;304:554.
- 712

- 715 36. Kang S, Bader AG, Vogt PK. Phosphatidylinositol 3-kinase mutations  
716 identified in human cancer are oncogenic. *Proc Natl Acad Sci U S A*  
717 2005;102:802–7.
- 718 37. Ikenoue T, Kanai F, Hikiba Y, Obata T, Tanaka Y, Imamura J, et al.  
719 Functional analysis of PIK3CA gene mutations in human colorectal  
720 cancer. *Cancer Res* 2005;65:4562–7.
- 721 38. Samuels Y, Velculescu VE. Oncogenic mutations of PIK3CA in human  
722 cancers. *Cell Cycle* 2004;3:1221–4.
- 723 39. Isakoff SJ, Engelman JA, Irie HY, Luo J, Brachmann SM, Pearline RV,  
724 et al. Breast cancer-associated PIK3CA mutations are oncogenic in  
725 mammary epithelial cells. *Cancer Res* 2005;65:10992–1000.
- 726 40. Hollestelle A, Elstrodt F, Nagel JH, Kallemeijn WW, Schutte M.  
727 Phosphatidylinositol-3-OH kinase or RAS pathway mutations in  
728 human breast cancer cell lines. *Mol Cancer Res* 2007;5:195–201.
- 729 41. Saal LH, Holm K, Maurer M, Memeo L, Su T, Wang X, et al. PIK3CA  
730 mutations correlate with hormone receptors, node metastasis, and  
731 ERBB2, and are mutually exclusive with PTEN loss in human breast  
732 carcinoma. *Cancer Res* 2005;65:2554–9.
- 733 42. Dibble CC, Asara JM, Manning BD. Characterization of Rictor phos-  
734 phosphorylation sites reveals direct regulation of mTOR complex 2 by  
735 S6K1. *Mol Cell Biol* 2009;29:5657–70.
43. Zheng B, Jeong JH, Asara JM, Yuan YY, Granter SR, Chin L, et al. 737  
Oncogenic B-RAF negatively regulates the tumor suppressor LKB1 to 738  
promote melanoma cell proliferation. *Mol Cell* 2009;33:237–47. 739
44. Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, Mair 740  
W, et al. Phosphorylation of ULK1 (hATG1) by AMP-activated protein 741  
kinase connects energy sensing to mitophagy. *Science* 742  
2011;331:456–61. 743
45. Ong SE, Foster LJ, Mann M. Mass spectrometric-based approaches 744  
in quantitative proteomics. *Methods* 2003;29:124–30. 745
46. Chen X, Sun L, Yu Y, Xue Y, Yang P. Amino acid-coded tagging 746  
approaches in quantitative proteomics. *Expert Rev Proteomics* 747  
2007;4:25–37. 748
47. Lundgren DH, Hwang SI, Wu L, Han DK. Role of spectral counting in 749  
quantitative proteomics. *Expert Rev Proteomics* 2010;7:39–53. 750
48. Lange V, Picotti P, Domon B, Aebersold R. Selected reaction monitor- 751  
ing for quantitative proteomics: a tutorial. *Mol Syst Biol* 2008;4:222. 752
49. Quaglia M, Pritchard C, Hall Z, O'Connor G. Amine-reactive isobaric 753  
tagging reagents: requirements for absolute quantification of proteins 754  
and peptides. *Anal Biochem* 2008;379:164–9. 755
50. Li HF, Kim JS, Waldman T. Radiation-induced Akt activation modulates 756  
radioresistance in human glioblastoma cells. *Radiat Oncol* 2009;4:43. 757

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