Activation of diverse signalling pathways by oncogenic PIK3CA mutations

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The PIK3CA gene is frequently mutated in human cancers. Here we carry out a SILAC-based quantitative phosphoproteomic analysis using isogenic knockin cell lines containing ‘driver’ oncogenic mutations of PIK3CA to dissect the signalling mechanisms responsible for oncogenic phenotypes induced by mutant PIK3CA. From 8,075 unique phosphopeptides identified, we observe that aberrant activation of PI3K pathway leads to increased phosphorylation of a surprisingly wide variety of kinases and downstream signalling networks. Here, by integrating phosphoproteomic data with human protein microarray-based AKT1 kinase assays, we discover and validate six novel AKT1 substrates, including cortactin. Through mutagenesis studies, we demonstrate that phosphorylation of cortactin by AKT1 is important for mutant PI3K-enhanced cell migration and invasion. Our study describes a quantitative and global approach for identifying mutation-specific signalling events and for discovering novel signalling molecules as readouts of pathway activation or potential therapeutic targets.
The p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3K) is encoded by PIK3CA, one of the most frequently mutated genes in human cancers. Recent studies have shown that three activating mutations, E542K and E545K in the helical domain and H1047R in the kinase domain, can lead to downstream activation of PDK1 and/or AKT to promote carcinogenesis and metastasis1–3. Studies have also suggested that mutations in the kinase or helical domains have distinct effects on PI3K downstream signalling events. Zhao and Vogt4 showed that binding to p85, the regulatory subunit of PI3K, is required for activation of wild-type PIK3CA or PIK3CA with kinase domain mutations5. Clinical studies have shown that tumours with H1047R mutation exhibit a better response to PI3K/mammalian target of rapamycin (mTOR) inhibitors in comparison with those carrying helical domain mutations6,7.

To dissect the signalling mechanisms underlying the mutant PIK3CA-induced transformation, through genetic engineering, we have developed a series of human cell lines that differ only in their PIK3CA allele status, containing either the wild-type (wt) or mutant forms of PIK3CA at codon 545 or 1,047 (refs 8,9). Both of these PIK3CA mutations can activate multiple downstream pathways, which confer the ability for growth factor-independent proliferation in vitro and metastatic capability in vivo8,9. We have also previously developed J124 as a novel and specific inhibitor of PIK3CA activity10. Treatment with this inhibitor can dramatically reduce AKT activity and inhibit metastasis of cancer cells bearing PIK3CA mutations.

In this study, we performed phosphoproteomic analysis of a spontaneously immortalized non-tumorigenic breast epithelial cell line MCF10A along with two isogenic derivatives generated by knock-in of mutant alleles—one bearing the E545K mutation located in the helical domain in exon 9 and the other bearing the H1047R mutation located in the kinase domain in exon 20 of the PIK3CA gene (hereafter referred to as Ex9-KI and Ex20-KI)8. Mass spectrometry-based phosphoproteomics has become a powerful tool for studying signalling networks in a global manner, especially in conjunction with stable isotope labelling by amino acids in cell culture (SILAC) for a precise quantitative readout11–15.

Here we identify and quantify 8,075 phosphopeptides, of which 1,142 are more phosphorylated in PIK3CA mutant cells and undergo a decrease in their phosphorylation status when treated with J124, a specific inhibitor of PIK3CA gene product. We use protein microarrays as a complementary platform to validate direct AKT1 substrates in vitro. Integration of the data from phosphoproteomic analysis with that from protein microarrays led to identification of a number of previously uncharacterized signalling molecules that appear to be involved in oncogenic signalling mediated through mutation of PIK3CA. Most notably, our studies identify cortactin as a novel AKT1 substrate whose phosphorylation enhances migration and invasion, key downstream events of the PIK3CA and AKT1 activation.

Results
Phosphoproteomic analysis of mutant PIK3CA knockin cells. Although many studies have associated PIK3CA mutations with features of transformation, a global and quantitative study of how mutant PIK3CA impacts the signalling networks and consequently transforms epithelial cells has not yet been described. The isogenic knockin system along with a novel PIK3CA-specific inhibitor and high-resolution mass spectrometry-based quantitative phosphoproteomics provides the tools to map the signal transduction pathways that are specifically modulated by PIK3CA mutations in a comprehensive manner. To validate this system, we first examined the phosphorylation levels of AKT1 and mitogen-activated protein kinase (MAPK) in the mutant cells where we found phospho-AKT and phospho-MAPK levels to be dramatically elevated in both cells and substantially suppressed by J124 treatment (Fig. 1a).

To interrogate the aberrant signalling triggered by the mutations in PIK3CA, we combined SILAC and TiO2-based phosphopeptide enrichment followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. Lysates of MCF10A parental cells were mixed with Ex9-KI cells that were treated with vehicle or J124 in a 3-plex SILAC experiment (Fig. 1b). The same experimental strategy was employed for the analysis of Ex20-KI cells in a separate 3-plex SILAC experiment (Supplementary Fig. 1b). After enrichment of phosphopeptides with TiO2 beads, the samples were desalted and analysed on a high-resolution Fourier transform mass spectrometer. We also carried out replicate experiments in which the SILAC labels were swapped. In all, we identified 8,075 unique phosphopeptides derived from 2,016 proteins (Supplementary Data 1 and 2). Of these, 7,199 phosphopeptides harboured serine phosphorylation and 168 phosphopeptides harboured tyrosine phosphorylation, and most of the phosphopeptides were singly or doubly phosphorylated (Supplementary Fig. 1cd).

The SILAC ratios (KI cells versus MCF10A) of phosphopeptides obtained from the two replicate experiments of both PIK3CA knockin cells showed a strong positive correlation (the correlation coefficient R = 0.86 for Ex9-KI group and R = 0.87 for the Ex20-KI group) for two independent biological replicates (Fig. 1c,d). There were 2,469 phosphopeptides that were detected in common in the Ex9-KI and Ex20-KI experimental groups, and the SILAC ratios (Ex9-KI or Ex20-KI cells versus MCF10A) of these phosphopeptides in the two cells with PIK3CA mutations were also quite correlated (R = 0.70) (Fig. 1e). Of the 2,469 phosphopeptides detected in common, 826 peptides derived from 338 proteins demonstrated the same increased or decreased phosphorylation pattern (>1.5-fold change in phosphopeptide intensity for both Ex9-KI and Ex20-KI cells) when compared with MCF10A cells (Supplementary Data 3). However, we also found that 417 peptides from 243 proteins were highly phosphorylated (>1.5-fold change) only in Ex9-KI (Supplementary Data 4) or Ex20-KI (Supplementary Data 5) cells compared with MCF10A cells, but not in both. These changes in phosphorylation patterns suggest that although downstream signalling effects are largely similar for these two particular mutant forms of PIK3CA, there are also some that are unique to the individual PIK3CA mutations. For instance, we found three kinases, PAK2, PAK4 and SLK, that were highly phosphorylated only in Ex9-KI cells and have been reported to be activated by PI3K-AKT pathway to promote cell migration/invasion16–18. It has been shown that breast cancer cells expressing PIK3CA with helical domain (Ex9) mutation are more invasive than the cells expressing PIK3CA with kinase domain (Ex20) mutations19, which is consistent with the data from this isogenic knockin system. The evidence of increased phosphorylation of these kinases specific to each knockin mutant cell could shed new light on some of the mechanisms underlying the phenotypic differences induced by PIK3CA Ex9 or Ex20 mutants.
Global elevation of protein phosphorylation by mutant PIK3CA. Overall, we observed that introduction of a single oncogenic amino acid change (E545K or H1047R) in PIK3CA can substantially elevate protein phosphorylation levels. In both Ex9-KI and Ex20-KI experimental groups, four major regulation patterns were observed. The first pattern included phosphopeptides identified in the Ex9-KI experimental group and in Ex20-KI group whose phosphorylation levels were 1.5-fold higher in mutant cells than parental cells and exhibited at least a 33% reduction in Ex9-KI/Ex20-KI cells on treatment with J124 (Fig. 2a and Supplementary Fig. 2a). A second pattern included peptides that were highly phosphorylated in Ex9-KI/Ex20-KI but were not substantially altered on treatment with J124 (Fig. 2b and Supplementary Fig. 2b). A third pattern was of peptides that were less phosphorylated in Ex9-KI/Ex20-KI but were not substantially altered on treatment with J124 (Fig. 2c and Supplementary Fig. 2c). The fourth pattern included a set of phosphopeptides whose phosphorylation levels were unaltered in knockin cells but underwent suppression on J124 treatment (Fig. 2d and Supplementary Fig. 2d).

Overall, as compared with MCF10A cells, we observed increased phosphorylation of about 47% and 33% of peptides in Ex9-KI and Ex20-KI cells, respectively. To examine the effects of J124 on global protein phosphorylation, we generated an intensity plot depicting the distribution of log2-transformed intensity ratios of highly phosphorylated peptides in Ex9-KI cells versus MCF10A cells, or J124-treated Ex9-KI cells versus MCF10A cells (Fig. 2e). We observed that J124 treatment resulted in a significantly global shift of phosphorylation pattern in Ex9-KI cells and significantly reduced the phosphorylation levels of highly phosphorylated peptides identified in this study. A similar trend was observed in the case of Ex20-KI cells.

Figure 1 | Phosphoproteomic analysis of MCF10A cells with PIK3CA mutations. (a) Western blot analysis of phosphorylated AKT (pT308), total AKT, phosphorylated p42/44 MAPK (pThr202/Tyr204) and total p42/44 MAPK in MCF10A parental cells, Ex9-KI and Ex20-KI cells with or without J124 treatment. (b) A schematic depicting the strategy used for quantitative phosphoproteomic profiling of PIK3CA Ex9 knockin mutant cells. (c,d) Density scatter plot of log₂-transformed phosphopeptide ratios (Ex9-KI or Ex20-KI versus MCF10A) from two SILAC reverse-labelled biological replicates. (e) Density scatter plot of log₂-transformed phosphopeptide ratios (x axis: Ex9-KI versus MCF10A and y axis: Ex20-KI versus MCF10A). Pearson coefficient correlation (R) is indicated.
was confirmed by western blot analysis using phospho-specific antibodies targeting some of the well-known key molecules downstream of the PI3K signalling pathway, including AKT1 and GSK3β and recently identified AKT1 substrates, ATP citrate lyase (ACLY) and EPHA2 (ref. 22), which were elevated in knockin cells and were efficiently suppressed by J124 treatment (Fig. 2f). These results clearly demonstrate that activation of mutated PIK3CA leads to a global increase in protein phosphorylation and profoundly affects signalling networks.

**Activation of multiple signalling pathways by mutant PIK3CA.**

To better understand the global phosphorylation alterations induced by oncogenic PIK3CA mutations, we performed a Kyoto Encyclopedia of Genes and Genomes pathway analysis using an integrated online functional annotation tool, DAVID, for the proteins with increased phosphorylation in Ex9-KI and Ex20-KI cells. Representative signalling pathways that were significantly enriched (P < 0.05) in PIK3CA mutant knockin cells and involved in the biological processes, including cytoskeleton and migration, kinase-regulated signalling and cell cycle regulation are shown in Fig. 2g. In agreement with our previously reported observations, our global phosphoproteomic study revealed that multiple oncogenic kinase-regulated signalling pathways such as MAPK, mTOR and ErbB were highly phosphorylated and enriched in PIK3CA mutant knockin cells. We also observed that multiple cell
cycle-related pathways were enriched in mutant knockin cells, which has been previously suggested to provide a proliferative advantage in basal cell culture medium\(^1\). Notably, one of the pathways among the cell cycle- and cell proliferation-related pathways pertains to pyrimidine metabolism. The link between activation of PI3K-AKT pathway and regulation of pyrimidine metabolism has been described in two recent studies\(^{24,25}\). These studies demonstrated that mTOR signalling downstream of PI3K-AKT module could enhance the de novo pyrimidine synthesis through phosphorylation of Ser1859 on CAD (carbamoylphosphate synthetase 2, aspartate transcarbamylase and dihydroorotase), a site that was also detected as highly phosphorylated in our experiments. We also observed increased phosphorylation of four other key enzymes (CTPS, RRM2, TK1 and DUT), which are involved in pyrimidine metabolism (Supplementary Fig. 4a). Finally, pathways regulating cell migration and invasion, such as those mediating actin rearrangements, cell adhesion and tight junction networks, were also found to be enriched (Fig. 2g and Supplementary Fig. 2e).

**Widespread modulation of phosphorylation of the kinome.** Of the 972 proteins that were found to be highly phosphorylated by one or both PIK3CA mutants, 46 were protein kinases. Of these protein kinases, 39 and 30 kinases were highly phosphorylated at serine/threonine residues in Ex9-KI or Ex20-KI cells, respectively (22 in common), suggesting that PI3K has a broad role in regulating cellular protein kinase activity. To obtain a systematic view of these differentially phosphorylated kinases, we mapped them and the corresponding phosphorylation sites onto a phylogenetic tree of the human kinome (Fig. 3a and Supplementary Fig. 3). Many of these modulated kinases have been shown to be associated with oncogenic transformation or metastasis in diverse cancers. Of the 39 highly phosphorylated kinases detected in Ex9-KI cells, elevation in the phosphorylation level of 25 kinases could be reversed by a short treatment with J124, suggesting that these kinases are likely to be directly regulated by AKT and/or PDK. Consistent with this suggestion, increased phosphorylation of S21 on GSK3A, S9 on GSK3B and S897 on EPHA2 is already known to be directly phosphorylated by AKT\(^{22,26,27}\) and phosphorylation of these sites is critical in regulation of their kinase activities\(^{19,22,23}\). For instance, AKT has been recently shown to phosphorylate EPHA2 to induce ligand-independent activation of EPHA2 and to promote cell migration and invasion\(^{25}\). In addition to increased phosphorylation of receptor tyrosine kinases, such as EGFR (T693) and EPHA2 (S897), we also observed increased phosphorylation of a non-receptor tyrosine kinase, PTK2 (S932) also known as FAK1.

**Phosphorylation motifs enriched in mutant PIK3CA cells.** The preference of amino acid motifs surrounding the phosphorylation
sites is one of the major mechanisms that contribute to kinase specificity\textsuperscript{28}. Identification of overrepresented motifs could help pinpoint upstream kinases activated by mutant PIK3CA. To determine which linear motifs were overrepresented in our data set, we used the motif-X algorithm (Fig. 3b,c). The peptides whose phosphorylation was increased in Ex9-KI and Ex20-KI cells and reduced on treatment with J124 were selected for this analysis. We identified 15 significantly enriched pS motifs (Fig. 3b) and the sequence logos of top four enriched motifs are depicted in Fig. 3c. Among these enriched motifs, eight were basic-rich motifs while three resembled the minimal AKT substrate motifs (RxRxxpS or RxxpS), which were ranked as the top two enriched phosphorylation motifs by motif-X analysis and Fig. 3c. Prediction of the kinases upstream of these regulated phosphopeptides using the NetworKIN algorithm led to identification of 34 kinases. The fraction of phosphopeptides corresponding to substrate motifs of each kinase was calculated and plotted in a heat map (Fig. 3b). In agreement with the results of analysis with motif-X, the AKT1 kinase family was predicted to target the basic-rich motifs that were enriched in our data set (Fig. 3b). In the integrated heat map combining motif-X and NetworKIN analysis, we observed that a large number of phosphopeptides contained sequence motifs that could be phosphorylated by RPS6K, a kinase downstream of mTOR, indicating activation of the canonical pathway from PI3K to AKT–mTOR–RPS6K. The activation of RPS6K is also supported by the fact that several phosphorylation-regulated sites known to be specifically phosphorylated by RPS6K were identified to be highly phosphorylated in PIK3CA mutant knockin cells. They are RPS6 S235/S240, EIF4B S442, HSPB1 S78/S82 and NCBP1 S22/T21. We also observed that CDK and MAPK kinases were predicted as the activated upstream kinases phosphorylating the peptides with the PxxSP substrate motif, (Fig. 3b,c), consistent with our previous report\textsuperscript{8} that MAPK1 and MAPK3 were highly phosphorylated and activated in mutant PIK3CA knockin cells (Fig. 1a). More importantly, of these upstream kinases, 11 (MAP4K4, MAPK1, PAK2, PAK4, PRKAA1, PRKCD, RPS6KA1, CDK2, CDK3, CSNK1A1 and AKT1) were indeed found to be more phosphorylated in PIK3CA mutant cells and were downregulated upon J124 treatment (Supplementary Data 1). In addition to the activation of canonical kinases (such as AKT1, RPS6K1 and MAPK1) by oncogenic PIK3CA mutations, identification of a broader spectrum of PI3K-modulated kinases, including PAK2/4, CASNK1A1, MAP4K4 and PRKCD, could bring new insights into the understanding of the mechanisms of oncogenic transformation induced by mutant PIK3CA.

**Interactome analysis to identify activated kinase pathways.** Our motif analysis indicated that kinases, including AKT, MAPKs and CDKs, were activated in PIK3CA mutant knockin cells. To understand the signalling networks more fully, we decided to study the kinase–substrate relationships and protein–protein interactions among the identified phosphoproteins. To do so, we first generated a database by integrating three kinase–substrate interaction databases, HPRD\textsuperscript{29}, PhosphoSitePlus\textsuperscript{30} and Phospho.ELM\textsuperscript{31}, with our previous report\textsuperscript{8} that MAPK1 and MAPK3 were highly phosphorylated and activated in mutant PIK3CA knockin cells (Fig. 1a). More importantly, of these upstream kinases, 11 (MAP4K4, MAPK1, PAK2, PAK4, PRKAA1, PRKCD, RPS6KA1, CDK2, CDK3, CSNK1A1 and AKT1) were indeed found to be more phosphorylated in PIK3CA mutant cells and were downregulated upon J124 treatment (Supplementary Data 1). In addition to the activation of canonical kinases (such as AKT1, RPS6K1 and MAPK1) by oncogenic PIK3CA mutations, identification of a broader spectrum of PI3K-modulated kinases, including PAK2/4, CASNK1A1, MAP4K4 and PRKCD, could bring new insights into the understanding of the mechanisms of oncogenic transformation induced by mutant PIK3CA.
kinases, four are non-receptor tyrosine kinases belonging to TEC tyrosine kinase family, namely TEC, TIK, BMX and BTK. Recent studies demonstrated that mutant PIK3CA could indeed activate BMX and directly phosphorylate STAT3 on Y705 (refs 42,43). In our study, we observed increased phosphorylation on S727 of STAT3 in our mutant Ex9-KI cells (Supplementary Data 1) and treatment with J124 could reduce this phosphorylation. Besides these four tyrosine kinases, there are two serine/threonine kinases, TRIO and KALRN, which can also bind PIP3 through pleckstrin homology domains. However, exactly how these two kinases are regulated by and involved in PI3K signalling remains unknown. In this regard, it is important to note that we found that TRIO phosphorylation on S2455, S2476 and S2477 was closely associated with the activity of PI3K in mutant PIK3CA Ex9-KI cells. Further studies on these non-canonical PIP3-regulated kinases are necessary to interpret the profound alterations induced by mutant PIK3CA. The abundance of these novel PI3K-modulated phosphorylation events also indicates that our knowledge of this serine/threonine kinase-centred signalling cascade is still far from complete.

Identification of novel AKT substrates. Using the mass spectrometry-based phosphoproteomic approach, we identified 1,142 phosphopeptides (derived from 474 proteins) that were stringently correlated with PIK3CA and AKT activities (that is, more phosphorylated in knockin cells and less phosphorylated on J124 treatment). Of these, 358 phosphopeptides were derived from 204 proteins matching a minimal AKT substrate motif, R/KxxpS/T. When compared with the kinase substrate databases described above, 16 proteins were known AKT substrates (Supplementary Data 7), including well-studied molecules in AKT signalling such as GSK3A, GSK3B, AKT1S1, EPHA2 and PFKFB2. However, ~92% (188 of the 204 proteins) were not previously reported as AKT substrates. It is indeed possible that although they contain the AKT substrate motif, other kinases in addition to AKT may also be able to phosphorylate these proteins on the same residues in vivo. A definitive way to assess this possibility is to use in vitro phosphorylation reactions to capture the direct phosphorylation targets of AKT. We employed a human protein microarray with 4,191 unique, full-length human proteins belonging to 12 major protein families to perform phosphorylation reactions with recombinant human AKT1 proteins in the presence of [γ-32P]-ATP (ref. 44). In comparison with a negative control reaction in which AKT1 was omitted, we identified 316 proteins (Supplementary Data 8) that could be directly phosphorylated by AKT1 in vitro.
Figure 5 | Integrative analysis for identification of novel AKT substrates. (a) In vitro AKT1 kinase assays with indicated GST fusion proteins. Right panels: autoradiograph of 32P-phosphorylated AKT1 substrates; left panels: Memcode staining of GST fusion proteins. (b) Matrigel migration/invasion assays for MCF10A, Ex9-KI and Ex20-KI cells treated with J124, AKT inhibitor IV or DMSO. Data are shown as mean ± s.e.m. Mann–Whitney test was carried out to determine the statistical significance. The experiments were repeated twice. (c) Relative phosphorylation levels of cortactin in DLD1-wt and DLD1-mt cells (left), and in BT20 cells treated with or without J124 and parental MCF10A cells. (d) Domain structure of cortactin with phosphosites identified in this study. (e) Representative MS/MS spectra confirming phosphorylation of cortactin at T401 and S417 in in vitro AKT kinase-substrate assays followed by LC-MS/MS analysis. (f) Confocal immunofluorescence images of subcellular localization of cortactin (red) and pAKT(green) in MCF10A, Ex9-KI and Ex20-KI cells. Nuclei stained with DAPI. White arrows indicate the co-localization of cortactin and pAKT at peripheral region of lamellipodia. Scale bar, 20 μm. (g) Relative phosphorylation levels of cortactin in DLD1-wt and DLD1-mt cells (left), and in BT20 cells treated with or without J124 or AKT inhibitor IV (right).

To maximize the advantages of the two high-throughput proteomic approaches and to increase the confidence of identification of novel AKT substrates, we overlaid our mass spectrometry-based phosphoproteomic data with protein microarray-based data set and found six novel substrates that were regulated by PI3K in our PIK3CA mutation knockin cell line model and could also be directly phosphorylated in vitro by AKT1 in the protein microarray experiment (Supplementary Fig. 5a). To test whether these proteins were direct substrates of AKT1, we performed in vitro kinase reactions by mixing recombinant AKT1 with each of these six proteins (cortactin, TRIP10, PRKCD, PPP1R13L, EIF4B and C19ORF21) purified as glutathione S-transferase (GST) fusion proteins. All six proteins were phosphorylated by AKT1 (Fig. 5a), which provides solid evidence that these six proteins are bona fide AKT substrates. Interestingly, we identified a highly interconnected cluster containing PRKCD as a hub in our kinase–substrate and protein–protein interaction network analysis (Fig. 4c). This integrative analysis allowed us to confirm that the enrichment of PRKCD-centred network is probably the result of elevated phosphorylation of PRKCD by AKT1.

Cortactin is a novel AKT substrate regulating cell invasion. We have previously demonstrated that PIK3CA mutations can
enhance cell migration and invasion of cancer cells. Analysis of the phosphorylation data revealed enrichment of signalling pathways regulating cell migration and cytoskeletal rearrangement in PIK3CA mutant knockin cells (Fig. 2g and Supplementary Fig. 2e). To investigate the effects of these phosphorylation-increased pathways in non-tumorigenic breast epithelial cells containing mutant alleles of PIK3CA, we employed matrigel-coated Boyden chamber assays to evaluate their invasive abilities. The results revealed that Ex9-KI and Ex20-KI cells could indeed penetrate matrigel to a much greater extent than the parental MCF10A cells. Moreover, the invasive ability enhanced by activation of PIK3CA could be dramatically attenuated by treatment with the PIK3CA inhibitor, J124, as well as by the AKT inhibitor IV (Fig. 5b).

Of the six confirmed novel direct substrates of AKT substrates, cortactin (CTTN) was of special interest because it is a component of the enriched pathways related to cell motility and cytoskeleton (Fig. 2g and Supplementary Fig. 2e). Cortactin is a key branched actin regulator that regulates cell motility and transduces signals from the cell membrane to cytoskeletal proteins. It is frequently overexpressed in advanced cancers and enhances cancer cell migration and invasion, and ectopic overexpression of cortactin in head and neck squamous cell carcinoma cell lines has been reported to increase AKT activity. The results revealed that Ex9-KI and Ex20-KI cells could be dramatically attenuated by treatment with the PIK3CA inhibitor, J124, as well as by the AKT inhibitor IV (Fig. 5b).

To further characterize the role of cortactin in promoting migration/invasion of PIK3CA mutant cells, we first employed small interfering RNA (siRNA) to knockdown cortactin expression in MCF10A, Ex9-KI and Ex20-KI cells (Fig. 6a top panel). We found that knockdown of cortactin significantly reduced the invasive ability of PIK3CA mutant knockin cells (Fig. 6a). A similar suppression of invasion was observed in DLD1-mt and BT20 cells after knockdown of cortactin expression (Fig. 6b,c). We next wanted to determine the phosphorylation effect of cortactin on its function. Cortactin has been shown to possess three alternatively spliced isoforms encoding three different proteins that are 550, 513 or 634 amino acids long. To identify the predominant isoform(s) expressed in MCF10A cells, we conducted a database search against transcript databases in addition to carrying out reverse transcriptase–PCR using primers that could distinguish specific isoforms (Supplementary Fig. 6d). We found that transcripts of isoforms A and B, but not C, were detectable in MCF10A and mutant PIK3CA knockin cells. Thus, we cloned both of these isoforms into a retroviral expression vector and carried out site-directed mutagenesis to alter all four detected AKT phosphorylation sites (S405A, T401A, S417A and S418A). Wild-type and mutant versions of both cortactin isoforms (A and B) were stably expressed in parental MCF10A, Ex9-KI and Ex20-KI cells, as confirmed by western blot analysis (Fig. 6d). Boyden chamber assays were employed to examine the effects of cortactin phosphorylation on cell migration and invasion where we found that overexpression of wt isoforms A and B promoted cell migration and invasion (Fig. 6d), and this enhanced invasive capacity could be reduced by the treatment of AKT inhibitor (Supplementary Fig. 6e), suggesting that AKT-mediated phosphorylation of cortactin contributes to the invasive ability induced by overexpression of cortactin. In contrast, overexpression of the mutant isoforms that could not be phosphorylated by AKT1 did not enhance, and perhaps even suppressed the migration and invasive ability of the cells (Fig. 6d).

Discussion

Oncogenic mutations in PIK3CA gene have been reported in many human cancer types. Using a gene targeting approach to knockout either wt or mutant PIK3CA alleles in colorectal cancer cell lines, Samuels et al. have previously demonstrated that mutant PIK3CA selectively regulated the phosphorylation level of AKT and its downstream transcription factors FKHR and FKHRL1. However, a comprehensive and quantitative analysis of how PIK3CA mutants globally impact signalling networks and consequently transform epithelial cells has not yet been reported.
In this study, we employed an isogenic model system to characterize the signalling alterations induced by the knockin of two hotspot oncogenic PIK3CA mutations (E545K or H1047R) in a spontaneously immortalized non-tumorigenic breast epithelial cell line, MCF10A. This system can model breast epithelial cell malignancy induced by PIK3CA mutations. Using this unique model system, we applied a comprehensive phosphoproteomic analysis to discern and quantify global activation of phosphorylation-mediated signalling networks caused by these two PIK3CA mutations. Based on our phosphoproteomic analysis, it is possible that the elevation of the phosphorylation of some proteins resulted from the increased protein expression or the accumulation of both increased protein abundance and phosphorylation level. More importantly, we also observed phosphorylation of more than a thousand peptides from 474 proteins to be increased in PIK3CA mutant knockin cells and reduced on the short-term treatment with the PIK3CA inhibitor, J124. These changes are probably regulated by phosphorylation.

**Figure 6 | AKT1-mediated phosphorylation on cortactin is important for migration/invasion induced by activation of PI3K.** (a) Top panel: western blotting with cortactin antibody to assess the knockdown efficiency of siRNA targeting cortactin in MCF10A, Ex9-KI and Ex20-KI cells. β-Actin served as loading control. Bottom panel: matrigel-coated Boyden chamber assays for the assessment of the migration/invasion ability of cells with indicated siRNA knockdown. (b,c) siRNA knockdown of cortactin in DLD1-mt (b) and BT20 cells (c). Top panels: western blotting of cortactin and β-actin; bottom panels: Boyden chamber assays of indicated cell lines. (d,e) Migration/invasion assays for MCF10A, Ex9-KI, Ex20-KI and BT20 cells overexpressing wild-type cortactin isoform A and B (cortactin-wt) and phosphosite-mutated cortactin isoform A and B (cortactin-mut). Top panels: western blotting of overexpressed cortactin; bottom panels: migration/invasion assays of indicated cells. (f) A proposed model of enhancing invasiveness by oncogenic activation of PI3K-AKT signalling cascades and phosphorylation of cortactin. (a-e) Data are shown as mean ± s.e.m. Mann–Whitney test was carried out to determine the statistical significance. These experiments were repeated at least twice.

In this study, we employed an isogenic model system to characterize the signalling alterations induced by the knockin of two hotspot oncogenic PIK3CA mutations (E545K or H1047R) in a spontaneously immortalized non-tumorigenic breast epithelial cell line, MCF10A. This system can model breast epithelial cell malignancy induced by PIK3CA mutations. Using this unique model system, we applied a comprehensive phosphoproteomic analysis to discern and quantify global activation of phosphorylation-mediated signalling networks caused by these two PIK3CA mutations. Based on our phosphoproteomic analysis, it is possible that the elevation of the phosphorylation of some proteins resulted from the increased protein expression or the accumulation of both increased protein abundance and phosphorylation level. More importantly, we also observed phosphorylation of more than a thousand peptides from 474 proteins to be increased in PIK3CA mutant knockin cells and reduced on the short-term treatment with the PIK3CA inhibitor, J124. These changes are probably regulated by phosphorylation.
induced by mutant PIK3CA and not through the increase of protein abundance. Among these phosphorylation-increased proteins, only a fraction (208/474) has been reported to be involved in signalling networks related to the canonical PI3K-AKT signalling pathway. To our knowledge, this study provides the most comprehensive survey of quantified signalling perturbations in phosphorylation resulting from oncogenic activation of mutant PIK3CA. These newly identified signalling events should increase our understanding of the oncogenic effects resulting from mutations in PIK3CA gene, especially for development of novel therapeutic strategies for cancers with PIK3CA mutations.

From this data set, we were able to demonstrate increased phosphorylation of many key enzymes involved in important signalling networks and cellular processes in this predominantly serine/threonine kinase-driven signalling network. For instance, we have demonstrated increased phosphorylation of several key tyrosine kinases (Fig. 3a). In addition, we identified modulation of phosphorylation of an AKT substrate, ACLY, which is the primary enzyme synthesizing cytosolic acetyl CoA. Acetyl CoA is the essential precursor for fatty acids, mevalonate synthesis and a major source for protein acetylation reactions, including histone acetylation. Phosphorylation-induced activation of ACLY by oncogenic mutation of PIK3CA could have the potential to enhance de novo fatty acid synthesis and also to globally regulate chromatin architecture and gene transcription. We also observed increased phosphorylation of ubiquitin protein E3 ligase (UBR4, ubiquitin protein ligase E3 component n-recogin 4) and several ubiquitin–specific peptides (USP10, USP24 and USP43) in mutant PIK3CA knockin cells and the increase phosphorylation was diminished on J124 treatment. Some of these (UBR4, USP10 and USP24) were reported to be involved in oncogenic transformation of epithelial cells. These data suggest that PIK3CA oncogenic mutations not only globally modulate protein phosphorylation but can also potentially regulate multiple other post-translational modifications via the cross-talk between kinases and other enzymes.

Our integrated approach to identify kinase substrates by combining two high-throughput proteomic platforms—mass spectrometry–based phosphoproteomics and protein microarray-based kinase assays—enabled us to identify six novel AKT substrates. Functional studies confirmed that phosphorylation of one novel substrate, cortactin, is critical for migration/invasion induced by oncogenic activation of PI3K. Similar approaches employing high-throughput proteomic technology-based strategies can be applied to understand other cancer signalling pathways in a systematic manner. In summary, mutant PI3K-induced signalling events uncovered by our phosphoproteomic approaches along with the newly identified AKT1 substrates should be invaluable for research as well as clinical studies involving development of novel targeted therapies.

Methods

Cell culture and reagents. Cell lines were grown in 5% CO2 at 37°C. The breast epithelial cell line MCF-10A was purchased from American Type Culture Collection, and its PIK3CA mutant knockin cell lines, Ex9-Ki and Ex20-Ki were previously generated using gene targeting method. The cells were cultured in DMEM/F12 (1:1) supplemented with 5% horse serum, 20 mg ml−1 epidermal growth factor (EGF) for MCF10A parental cells and 0.2 mg ml−1 EGF for knockin cells. In addition, 10 μg ml−1 insulin (Roche), 0.5 μg ml−1 hydrocortisone (Sigma) and 100 ng ml−1 cholera toxin (Sigma) were supplemented for all cells. To label proteins, only a fraction (208/474) has been reported to be involved in signalling networks related to the canonical PI3K-AKT signalling pathway. To our knowledge, this study provides the most comprehensive survey of quantified signalling perturbations in phosphorylation resulting from oncogenic activation of mutant PIK3CA. Newly identified signalling events should increase our understanding of the oncogenic effects resulting from mutations in PIK3CA gene, especially for development of novel therapeutic strategies for cancers with PIK3CA mutations.

Liquid chromatography–tandem mass spectrometry. LC–MS/MS analysis of enriched phosphopeptides was carried out using a reverse-phase LC system interfaced with an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). The peptides were loaded onto an analytical column (10 cm × 75 μm, Magic C18 AQ, 5 μm, 120 Å) at 0.1% formic acid and eluted with a linear gradient from 5% to 60% ACN in 90 min. Precursor scans of MS scans were acquired in the range of 350–1,700 m/z at 60,000 resolution at 400 m/z on an Orbitrap analyser. Ten most abundant precursor ions from a survey scan were selected for Higher-energy collisional dissociation (HCD) fragmentation with a normalized collision energy of 35% R for heavy state and 40% for light state. Full scans of MS2 were acquired at 15,000 resolution. Intensities of precursors were used to ensure the proper confluence and healthy status. The phase-contrast images of cells before harvesting were shown in Supplementary Fig. 1a.

Immunoblotting and siRNA knockdown. Cells were grown and lysed in modified RIPA buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate and 1 mM sodium orthovanadate in the presence of protease inhibitors). Whole-cell protein extracts were denatured and separated in NuPAGE gels (Invitrogen), transferred to nitrocellulose membranes and probed with primary and horseradish peroxidase-conjugated secondary antibodies. Anti-p44/p42 MAPK (9102: 1:1,000), anti-phospho-p44/p42 MAPK-Thr202/Tyr204 (9106: 1:1,000), anti-AKT (9272: 1:1,000), anti-p-AKT-Ser473/Thr308 (9271, 2965: 1:1,000), anti-ACLY (4332: 1:1,000), anti-pACLY-Ser544 (4331: 1:1,000), anti-pGSK3β-Ser9 (9382:1:1,000) and anti-α- tubulin (2014:1:10000) were purchased from Cell Signaling Technology. Other antibodies used are anti-α-TUBULIN (2014:1:10000) from Epitomics and anti-β-actin (A35161:5,000) from Sigma. Full scans of western blotting results are provided in Supplementary Fig. 7. siRNA (50 nm) (5'-CACCCAGGAGCAUAUCAACAUAU-3') targeting cortactin (Qiaegen) were used for transfections with RNAiMax (Invitrogen). Cells were harvested 48 h post transfection for assessing knockdown efficiency or other follow-up experiments.

Site-directed mutagenesis and stable overexpression. Full-length cortactin CDS plasmids for isoform A and B were purchased from DNA Resource Core (Harvard) and subcloned into pBABE-puro vector. Site-directed mutagenesis for cortactin mutants was performed with QuickChange Site-Directed Mutagenesis Kit (Agilent). HEK293T cells were used for retroviral package. Briefly, pBABE-cortactin and mutant expression plasmids or control empty vectors were co-transfected with pCL-Ampho (Imagenex) packing virus. Virus supernatants were collected and used for infection of MCF10A and knockin cells. Infected cells were then selected by 0.5 μg ml−1 puromycin to obtain cells stably expressing cortactin proteins.

Trypsin digestion. Cell lysates were prepared in urea lysis buffer containing 20 mM HEPES pH 8.0, 9 mM urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate and 5 mM sodium fluoride. The lysates were sonicated and cleared by centrifugation at 3,000 × g at 4°C for 10 min. Protein extraction was carried out using BCA protein assay kits. Equal amount of protein from three SILAC-labelled states were mixed, reduced with 5 mM dithiothreitol and alkylated with 10 mM iodoacetamide. Lysates were diluted to <2 μg protein concentration in 20 mM HEPES (pH 8.0) and incubated with L-1-lysino-l-1-proline (L-1-lysino-l-proline, 1-lysino-1-proline) treated trypsin at 25°C overnight. The reaction was quenched using 1% trifluoroacetic acid. The protein digest was desalted using C18 reverse phase column (Waters, UK) and eluted peptides were lyophilized and subjected to phosphopeptide enrichment.
Mass spectrometry data analysis. Proteome Discoverer (v 1.3; Thermo Fisher Scientific) suite was used for quantitation and database searches. The MS/MS data were searched using Mascot (Version 2.2.0) and SEQUEST search algorithms against a Human ReSeq database (v 46 containing 33,249 entries) supplemented with frequently observed contaminants. For both algorithms, the search parameters included the following: (i) a maximum of one missed cleavage; (ii) carbamidomethylation of cysteine as a fixed modification; (iii) amino-terminal acetylation; (iv) oxidation at methionine; (v) phosphorylation at serine, threonine, and tyrosine; and (v) SILAC labelling $^{13}$C$_6$N$_2$-lysine, $^2$H$_4$-lysine, $^{13}$C$_6$-arginine and $^{13}$C$_2$N$_2$-arginine as variable modifications. The MS tolerance was set to 10 p.p.m. and MS/MS tolerance to 0.1 Da. Score cutoff value was set to 0.01 false discovery rate at 5% and the probability of a rabbit anti-rabbit secondary antibody (A1034, 1:1,000) from Invitrogen. The nuclei were stained with SlowFade Gold Antifade Mountant with DAPI (S3963) from Life Technologies. IF analysis was carried out using an LSM710 confocal laser scanning microscope (Carl Zeiss).

Immunoprecipitation. SILAC-labelled DLD1-wt (light) and DLD1-mt (heavy) cells and SILAC-labelled BT20 cells treated with DMSO (light) or J124 (heavy) were lysed with modified RIPA buffer. Equal amount of the protein lysates from DLD1-wt (light) and DLD1-mt (heavy), or BT20-RTO2-treated (light) and BT20-J124-treated (heavy) cells were mixed and followed by immunoprecipitation with cortactin antibody 4F11 (05–180, Millipore). The precipitated proteins were subjected to SDS–PAGE and stained with Coomassie brilliant blue R250. The cortactin band was excised for MS analysis.

Reverse transcriptase-PCR. Total RNA was extracted with TRIZOL reagent (Invitrogen) and complementary DNA synthesized using SuperScript II Reverse Transcriptase kit (Invitrogen) according to the manufacturer’s instructions. Forward: 5'-GAGGGCAGGTGCCTGAGCT-3' and reverse: 5'-CTCCGCTTCGGCCGCTTCTCC-3' primers were used to amplify isoform-specific region to distinguish isoform A from isoform B and C. Forward: 5'-GGGCCACATTGCCAGAGACG-3' and reverse: 5'-GACTGCACTTGAAGGAGAGGAG-3' primers were used to amplify a specific region to distinguish isoform C from isoforms A and B (Supplementary Fig. 6d).

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Author contributions
X.-W., B.V., B.H.P. and A.P. designed the research; X.W., S.R., N.A.S., M.S.Z., R.C., M.-S.K., M.M., J.Z., J.Y., J.N., J.-S.J. and R.N. performed the research; H.Z., J.Q. and B.V. contributed new reagents; X.W., S.R., H.Z., J.Q., B.V., B.H.P. and A.P. analysed the data; E.G., S.S., J.Z., J.Y., J.N. and R.N. prepared the figures and tables; M.S.Z., S.R., N.A.S., B.H.P. and A.P. wrote the manuscript. All authors have read and approved the manuscript.

Additional information
Accession codes: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the data set identifier PXD000599.

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