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Phosphatome profiling reveals PTPN2, PTPRJ and PTEN as potent negative regulators of PKB/Akt activation in Ras mutated cancer cells

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SYNOPSIS

Oncogenic Ras mutations render the protein constitutively active and promote tumourigenisis via chronic stimulation of effector pathways. In A549 lung adenocarcinoma approximately 50% of the total Ras population is constitutively active yet these cells display only weak activation of the effectors: ERK1/2 and Akt. In order to identify key negative regulators of oncogenic Ras signalling we performed a phosphatome RNAi screen in A549 cells and ranked their effects on phosphorylation of Ser473 of Akt. As expected, the tumour suppressor PTEN emerged as a leading hit – knockdown elevated Akt activation to 70% of maximal generated by acute EGF stimulation. Importantly, we identified other phosphatases with similar potencies including PTPN2 (TC-PTP) and PTPRJ (DEP-1/CD148). Potentiation of Akt phosphorylation by knockdown of PTEN or PTPRJ was contingent on the presence of oncogenic K-Ras. Our data reveal a synergy between oncogene function and the loss of a tumour suppressor within the same pathway that was necessary for full effector activation since each alone failed to elicit significant Akt phosphorylation. Together, these data reveal potent regulators of Akt signalling that contribute to ameliorating the consequences of oncogenic K-Ras activity.

Keywords

Phosphatase; siRNA; KRAS; PTEN; signalling

INTRODUCTION

Ras proteins are molecular switches cycling between the inactive GDP-bound state and active GTP-bound state. Upon stimulation by growth factor receptor tyrosine kinases (RTKs) they activate an array of downstream cell signaling pathways to promote cell proliferation and survival [1]. Whilst Ras proteins control a network of pathways, the best studied and most important from a human health perspective are the Raf-MEK-ERK and PtdIns 3-kinase-Akt pathways. Dysregulation of the RTK-Ras pathway has been implicated in the majority of cancer cases whilst oncogenic mutations of Ras directly contribute to 15% of human cancers [2]. Constitutive activation of Ras by these oncogenic point mutations results in an isoform that is effectively permanently switched on and a basic assumption might be that this will lead to hyperactivation of effectors. However, we and others have shown that constitutive activation of endogenous Ras often results in comparatively low levels of effector activation [3-8]. Similarly, predictions of signalling pathway activation are

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unreliably correlated to oncogene mutation or expression status in cancer cells compared to gene expression signatures [9].

These studies raise the question of what oncogenic Ras signalling really looks like particularly since most analysis of these mutants has been performed *in vitro* following transient transfections – conditions that maximize outputs over sustained periods. *In vivo*, is it necessary for oncogenic Ras to overcome negative regulation of outputs to continue to promote tumourigenesis or are low but sustained levels of effector activation sufficient? There is compelling evidence using a mouse model with inducible K-Ras^{G12D} expressed at endogenous levels that despite low levels of effector activation, tumourigenesis can be initiated and sustained [7]. However, whilst mutant K-Ras could generate early stage lung cancer lesions, progression to late stage disease required additional co-operating mutations [10]. Significantly, this progression correlated with a conversion of Ras output from low to high levels of MAP kinase activation. Therefore, the co-incident down-modulation of Ras negative feedback pathways can be a critical event in cancer progression. Identifying these negative regulators provides important prognostic information and possible targets for therapeutic intervention.

We have established a model approach that enables negative regulators of oncogenic Ras signalling to be identified. Using cell lines expressing endogenous oncogenic K-Ras but exhibiting suppressed effector activation we performed an RNAi screen of potential negative regulators and ranked them according to their ability to relieve the inhibition of K-Ras output. The screen focused on phosphatases due to their broad regulatory functions in signalling pathways. We identified many significant regulators of Akt and ERK activation and importantly, ranked their relative potencies at modulating these key pathways. Amongst these we discovered that the phosphatases PTEN and PTPRJ are specific inhibitors of oncogenic K-Ras activation of Akt. Our findings highlight the complex adaptive changes within cells to mitigate the effects of aberrant signalling.

EXPERIMENTAL

Cell culture and plasmids

Prior to the study commencing A549 lung adenocarcinoma and Panc-08-13 pancreatic ductal carcinoma cells were purchased from ECACC (Salisbury, UK). GST-RBD K85A was a generous gift of Tony Burgess, Ludwig Inst., Melbourne [11].

Antibodies and other reagents

Monoclonal anti-c-K-Ras (clone 234-4.2) and monoclonal anti-tubulin were from Sigma. Polyclonal anti-H-Ras (C20) and monoclonal anti-N-Ras (F155) both Santa Cruz Biotechnology. Polyclonal anti-Pan-Ras (#3965), monoclonal anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) (E10), polyclonal anti-p44/42 MAP kinase, polyclonal anti-phospho-MEK1/2 (Ser217/221) (9427), monoclonal anti-phospho-Akt (Ser473) (587F11) and polyclonal anti-Akt (9272) were all from Cell Signalling Technology. Monoclonal anti-PTEN (sc-7974) and monoclonal anti-DEP-1/PTPRJ (sc-21761) were from Santa Cruz. Monoclonal anti-PTPN2 (MAB1930) was from R&D Systems and polyclonal anti-PPAP2A (ab-58745) was from Abcam. Purified mouse EGF was obtained from John Smith, Liverpool University.

The following ON-Target plus K-Ras-specific duplexes were purchased from Dharmacon, USA: GGAGGGCUUUCUUUGUGUAUU and GAAGUUAUGGAAUUCCUUUUU [5]. A custom Phosphatome siRNA library was purchased from Qiagen consisting of 204 pools of 4 oligos and 40 single oligos specific for 20 additional phosphatases (see Supplementary Table 1 for details). For deconvolution experiments, batches of four single oligos

corresponding to the original pooled oligos were used: PTEN-1: ACGGGAAGACAAGUUCAUGUA, PTEN-2: AUCGAUAGCAUUUGCAGUAUA, PTEN-3: UCGACUUAGACUUGACCUAUA, PTEN-4: AAGGCGUAUACAGGAACAAUA; PTPN2-1: AUAAAGGGAGAUUCUAGUAUA, PTPN2-2: CACAGUCGUGUUAAACUGCAA, PTPN2-3: CCGCUGUACUUGGAAAUUCGA, PTPN2-4: CACAAAGGAGUUACAUCUUAA; PTPRJ-1: ACCCGUAUCUUCUACAAUCAA, PTPRJ-2: ACGAGUCGUCAUCUAACUAUA, PTPRJ-3: UCGGGUAGAAAUAACCACCAA, PTPRJ-4: UCCGAGUAUGUCUACCAUUUA.

RFLP analysis of oncogenic K-Ras mutations

Restriction fragment length polymorphism (RFLP) was used to distinguish between wild type and mutated codon 12 sequences in K-Ras [12]. Briefly, genomic DNA was extracted using a NucleoSpin Tissue kit (Macherey Nagel) and exon 1 of K-Ras amplified by PCR using the forward primer 5′-CTGAATATAAACTTGTGGTCCATGGAGCT-3′, and reverse primer: 5′-TTTACCATATTGTTGGATCATATTC-3′. The forward primer together with the first two nucleotides (GG) of Gly at codon 12 of wild type K-Ras introduce a second BstX1 site resulting in a shorter 76 bp restriction digest product compared to 103 bp mutant K-Ras.

Depletion of phosphatases and Ras by RNA interference

For phosphatome analysis A549 cells were transfected with 40 nmoles (per 1×10^5 cells) of each siRNA pool using oligofectamine (Invitrogen). 24 hours post-transfection the cells were re-seeded and two days post-transfection, cells were re-transfected with siRNA; 24 h later (72 hours total knockdown time) the cells were lysed for Western blot analysis. Cells were grown throughout in the presence of 10% FCS unless otherwise stated. The large-scale screen was performed once in A549 cells with the top twenty hits chosen for comparative analysis in Panc-08-13 cells. All RNAi experiments with the leading hits (single or combined knockdowns with K-Ras) were repeated at least four times with 40 nmoles of each duplex, identical results were observed for each pair of oligonucleotides. Analysis of specific phosphatase knockdown and effector pathway activation was confirmed by Western blotting lysates resolved on NuPAGE 4-12% Bis-Tris gels (Invitrogen) and measured by quantitative analysis of Western blots using the Odyssey (LI-COR Biosciences) digital imaging system.

Where indicated, control and RNAi treated cells were serum-starved for 6 hours before being stimulated for 10 minutes with 100ng/ml of EGF, washed once with ice-cold PBS and lysed for 5-10 min on ice in a lysis buffer containing 0.5% NP-40, 25mM Tris pH 7.5, 100mM NaCl, 50mM NaF, supplemented with mammalian protease inhibitor cocktail and mammalian phosphatase inhibitor cocktail II (Sigma). Lysates were probed for effector activation using quantitative Western blotting.

Ras activation assay

Sub-confluent cells were serum-starved over-night before being stimulated by 100ng/ml of EGF for 10 minutes. The cells were then washed with ice-cold PBS and lysed on ice in 50 mM Tris-HCl, pH 7.2, 500mM NaCl, 10mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 1:250 protease-inhibitor cocktail (Sigma). 15μg of GST-RBD K85A fusion protein linked to Glutathione Sepharose(4B) was used to affinity purify activated Ras from 200μg of each lysate as described [11]. Pull downs were immuno-blotted for Ras isoforms and pan-Ras.

RESULTS

Oncogenic K-Ras is uncoupled from effector activation

A549 and Panc-08-13 cells harbour K-Ras that is homozygously mutated at the G12 position (Figure 1A); mutation at this position is known to render Ras constitutively active and transforming [2, 13]. Previous analysis of Ras isoform abundance indicated that K-Ras represents 50% of the total Ras pool in A549 cells [5]. As expected, A549 cells harbouring mutant K-Ras displayed equivalent levels of significant K-Ras activation regardless of the presence or absence of serum overnight or acute saturating 100 ng/ml EGF stimulation (Figure 1B). In contrast, the activity of endogenous wild type K-Ras in HeLa cells and the other Ras isoforms in both cell lines significantly increases in response to acute EGF stimulation. Pan-Ras activation in A549 cells is dominated by K-Ras and consequently barely increases in response to EGF. Taken together with the data on K-Ras abundance and mutation status this means that in A549 cells 50% of the total Ras pool is activated to a level that is 90-100% of that seen with maximal acute EGF stimulation (Figure 1B).

Given the presence of a significant pool of active Ras in A549 and Panc-08-13 cells there is an unexpected lack of robust activation of the main Ras effector pathways: Raf-MEK-ERK and PtdIns-3-kinase-Akt (Figure 1C). We observe reduced or minimal pMEK, pERK and pAkt under continuous culture conditions in the presence of FBS, however these pathways are still able to robustly respond to acute growth factor stimulation. In A549 and HeLa cells we see an equivalent increase in pAkt and pERK labeling with acute EGF stimulation. This indicates that the low outputs seen under steady state culture conditions are due to specific uncoupling of the signal from oncogenic K-Ras rather than a complete down-regulation of these pathways.

Phosphatome screening for oncogenic Ras regulators

Ras-dependent activation of ERK and Akt involves a series of intermediary kinases and accessory proteins regulated by phosphorylation. We reasoned that the dampened Ras output in A549 cells was likely to be at least partially dependent on the up-regulation of expression or specific activity of one or more phosphatases. To identify these potential regulators we performed a phosphatome RNAi screen incorporating approximately 85% of all tyrosine, serine and threonine phosphatases (Supplementary Table 1). These included all 37 classical protein tyrosine phosphatases (PTPs), 48/66 Dual-specificity or VH1-like phosphatases (DUSPs), 27/31 Ser/Thr phosphatases and 40/51 regulatory subunits of Ser/Thr phosphatases [14]. Any phosphatases directly or indirectly negatively regulating Ras outputs would be expected to cause an increase in pAkt or pERK when knocked down.

The screen was carried out under steady state tissue culture conditions containing FBS; the result of each phosphatase knockdown was normalized to tubulin to control for loading and then compared to acute EGF stimulated positive controls loaded on the same gels. The scores from all 244 knockdowns were placed in rank order; for pERK labelling the leading hits generated 20-25% of the maximal signal observed following acute EGF stimulation (Figure 2). In comparison, control mock-transfected A549 cells that were not incubated with EGF generated an average of 12.6% of maximal pERK labelling. This value is at the upper end of the range of the standard deviation from the mean indicating that compared to an unstimulated mock transfected control the majority of the phosphatase knockdowns had a relative inhibitory effect on pERK production.

Parallel analysis of Akt phosphorylation revealed a far more significant impact of phosphatase knockdown on this pathway compared to MAPK activation with leading hits restoring Akt activation to 60-70% of that seen with acute EGF stimulation (Figure 3A). The

mock-transfected, unstimulated controls produced an average of 32.4% of maximal pAkt labeling – close to the mean value for this dataset.

A necessary step in Akt activation is recruitment to the cell surface via interaction of its PH-domain with phosphatidylinositol-3,4,5-trisphosphate (PtdIns*P*3) produced by PtdIns-3-kinase. One of our leading hits – PTEN is a PtdIns*P*3 3-phosphatase that has been well characterized as a critical and ubiquitous negative regulator of the PtdIns-3-kinase function [15]. Similarly, although the ubiquitously expressed inositol-5-phosphatase SHIP2 was not in our screen, knockdown of a related family member - INPP5E significantly derepressed Akt activation. Importantly, our unbiased screen reveals several other phosphatases with similarly potent effects to PTEN at regulating Akt phosphorylation. These included PTPN2 and PTPRJ known regulators of receptor tyrosine kinase activity; and PPAP2A a phosphatidic acid phosphatase whose knockdown would result in increased cellular phosphatidic acid - a known facilitator of Ras activation [16].

Identification of potent regulators of Akt phosphorylation

Due to the robustness of the response we decided to focus on leading phosphatases modulating Akt phosphorylation. The large scale of the phosphatome screen precludes direct validation of every data point. Therefore, we performed a limited phosphatome RNAi screen in Panc-08-13 cells focusing on the top 20 pAkt hits plus five phosphatases that had average responses in A549 cells (Supplementary Table 1). Seven phosphatases were chosen for detailed follow-up based on best aggregate performance in the screens with the two cell lines.

The phosphatome screen used a pool of four oligos specific to each phosphatase – we deconvolved these four oligos and looked for at least 3 of the 4 to re-confirm the phenotype in A549 cells to increase confidence in the data (Figure 3B). PPAP2A, PTEN, PTPN2 and PTPRJ all met this criterion. Whilst RNAi typically produced a 75-90% knockdown of each phosphatase, in the case of PPAP2A we observed no evidence of specific knockdown (Figure 3C). This was despite using two separate antibodies that could recognize all isoforms of PPAP2A (data not shown); therefore, the results for PPAP2A are likely to represent an off-target effect.

Our screen is performed under tissue culture conditions where serum is present. This has the potential to reveal general regulators of RTK-Akt activation that may be independent of oncogenic Ras. In order to investigate the Ras-dependence we performed combined phosphatase-Ras knock-downs. If the phosphatases are contingent on oncogenic Ras then the increased Akt phosphorylation seen with phosphatase knockdown should be significantly reduced when K-Ras expression is also lost. This is exactly what we observe with PTEN/K-Ras and PTPRJ/K-Ras double knockdowns implicating these phosphatases as the two main negative regulators of oncogenic Ras-dependent activation of Akt in these cells (Figure 4). In contrast, PTPN2 knockdown significantly increases Akt phosphorylation but shows no coupling to K-Ras (Figure 4).

DISCUSSION

Our screen revealed many phosphatases whose knockdown potentiated Akt and ERK phosphorylation, several of which have not previously been implicated in regulating these pathways. We ranked the relative contributions of phosphatases in regulating the main Ras effector pathways and revealed specific antagonists of oncogenic Ras signaling. We also showed that co-operating mutations within the same pathway are needed to overcome cellular regulation and achieve full signaling potential. Each of these points is discussed below.

Identification of negative regulators of Ras pathways

We focused on Ras activation of Akt and identified PTPN2, PTPRJ and PTEN as potent regulators of this pathway. PTEN is the archetypal negative regulator of PtdIns-3-kinase signalling and is frequently mutated in cancer [17]. Whilst it is perhaps no surprise to see PTEN as one of our leading hits it is significant that we have identified other phosphatases that are similarly potent regulators of this important signaling pathway.

The other two leading hits – PTPN2 and PTPRJ, are known negative regulators of RTKs. PTPN2 is a ubiquitously expressed, nuclear localised phosphatase with several RTKs as substrates [18-21]. In response to EGF stimulation, PTPN2 translocates to the plasma membrane and dephosphorylates EGFR thereby decreasing PtdIns-3-kinase/Akt signalling [21]. Modulation of EGFR signaling was specific for PtdIns-3-kinase/Akt and had no inhibitory effect on ERK activation [22], concordant with our screen. PTPRJ negatively regulates several RTKs including: EGFR, Met, PDGFR [23-26]; and is a known tumour suppressor mutated or lost in several cancer types [27, 28]. PTPRJ was a leading hit in both our pERK and pAkt screens however only 1 of the 4 oligos reproduced this pERK result whilst all 4 resulted in potentiation of Akt phosphorylation. Whilst our data indicated an off-target effect on pERK labelling another group recently showed that PTPRJ directly dephosphorylates ERK1/2 [29].

Since the function of these phosphatases is linked to receptors upstream of Ras, their presence as leading hits may reflect an important general role in modulating RTK activation of PtdIns-3-kinase/Akt. In this case, loss of the phosphatase will result in enhanced RTK phosphorylation and consequent activation of wild type Ras and PtdIns-3-kinase. This stimulatory effect on PtdIns 3-kinase/Akt would be independent of oncogenic K-Ras activity. For PTPN2 at least this seems to be the case since knocking down K-Ras did not significantly alter the response (Figure 4), indicating no requirement for oncogenic Ras. In contrast, PTPRJ shows significant coupling to oncogenic K-Ras indicating either co-operativity or upstream regulation of PTPRJ by K-Ras. The basis for this may be due to the role of PTPRJ in dephosphorylating residues within the inhibitory domain of the p85 subunit of PtdIns-3-kinase resulting in attenuated PtdIns-3-kinase activation [30]. Both tyrosine phosphorylation and Ras binding to the p85 subunit result in a synergistic activation of PtdIns-3-kinase [31] and the identification of PTPRJ as one of our most potent Akt activation hits may be a consequence of this synergism with oncogenic K-Ras. Alternatively PTPRJ may be in a feedback loop regulated by K-Ras – further work is required to differentiate between these possibilities.

Whilst we identified many phosphatases for which knockdown significantly modulated Akt and ERK phosphorylation none of our top hits were known direct inhibitory modulators of Raf-MEK-ERK or Akt such as PP5, PHLPP, PP2A and DUSPs 6, 7 and 9 [32-34]. One explanation for these direct regulators not featuring in our leading lists is that we do not provide a pulse of growth factor stimulation. Our assay is primarily limited to oncogenic Ras driving signalling whereas acute application of growth factors will concurrently activate a network of Ras-independent effectors that may transiently stimulate phosphatases directly regulating pathways leading to Akt and ERK activation.

Co-operation between oncogenes and tumour suppressors

The phosphatases that we have identified are either directly downstream of Ras function or are involved in down-regulating signal flow from RTKs and PtdIns-3-kinase. They appear to be capable of functioning in the absence of acute growth factor stimulation and may therefore represent important constitutive regulators of the basal state of the RTK-Ras-Akt pathway.

As discussed earlier, mutation of an oncogene is not necessarily sufficient for full tumour progression. Co-operating genetic lesions are needed to achieve full signaling potential and drive the tumourigenic process. This effect was clearly seen in our combined analysis of K-Ras and PTEN. Mutant K-Ras alone or loss of PTEN in the absence of mutant K-Ras could not maximize phospho-Akt labelling – however when combined we observed a synergy that generated significant Akt phosphorylation (Figure 4). This co-operation may explain the *in vivo* observation that inactivation of PTEN significantly accelerates K-Ras initiated lung cancer compared to controls harbouring either one of these mutations [35]. Similarly, co-incident combinations of K-Ras, PtdIns-3-kinase p110α (PIK3CA) and PTEN mutations or disruption have been observed in endometrial, breast and colorectal cancers [36].

Recent work using mouse models expressing oncogenic K-Ras^{G12D} at endogenous levels in thyroid tissue revealed that Ras activation alone was not sufficient to induce visible neoplasia [37]. However, PTEN loss in combination with oncogenic Ras synergized to kill the entire cohort within 4 months of birth. Therefore, analogous to our study, oncogenic Ras displayed attenuated output that needed the removal of a key negative regulator to achieve its full oncogenic potential. This was achieved by generating constitutive PtdIns-3-kinase signalling that was also necessary for relieving inhibition of MAPK activation. Therefore understanding the tumour-specific interplay between oncogenic Ras, effector proteins and negative regulators has implications for therapeutic design and treatment. For example, drugs such as Iressa and Herceptin that target receptors upstream of PtdIns-3-kinase and Ras may be ineffective in the context of cancer cells that already down-regulate mutant Ras output. Similarly, whilst many Raf driven tumours respond to MEK inhibitors, Ras mutant cell lines are typically less sensitive – in part dependent on the extent of cross talk between the PtdIns-3-kinase and MAPK pathways mediated by co-incident mutations in PIK3CA or PTEN [38, 39].

In summary, our work has provided clear examples where negative regulation is keeping in check the output of major fractions of total cellular Ras that are constitutively active. We have also identified many potential positive and negative regulators of the pathways leading to Akt and ERK activation and importantly, ranked their relative potencies at modulating these key signalling pathways. Amongst these, we have identified a group of phosphatases that rival PTEN for potency in regulating RTK-PtdIns-3-kinase-Akt signalling and revealed the synergy between oncogenic genetic perturbations within a pathway that are required to achieve full signalling potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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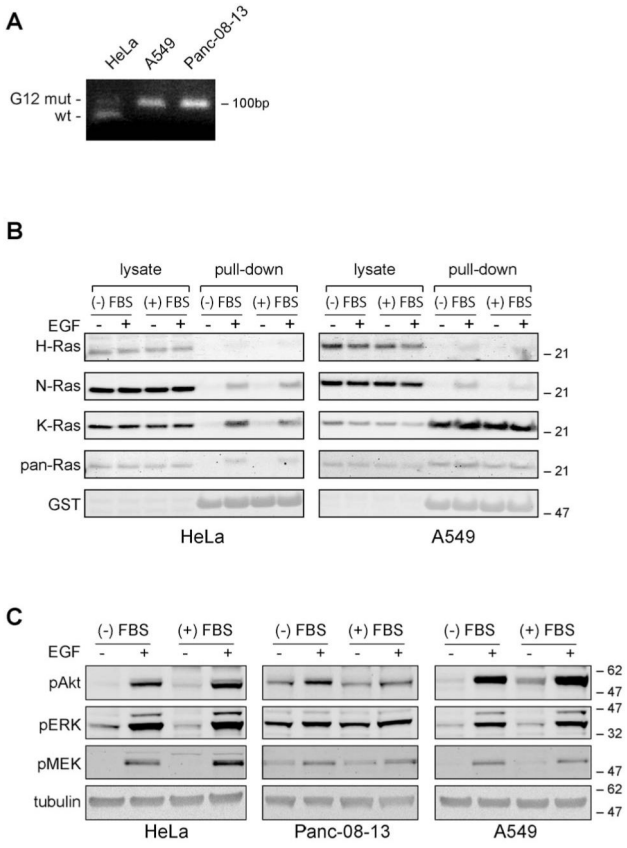

FIGURE 1. Oncogenic Ras does not necessarily result in hyperactivation of effectors

Restriction fragment length polymorphism (RFLP) confirms that K-Ras is homozygously mutated at the G12 position in A549 and Panc-08-13 cells and wild type in HeLa cells (A). Pull-down of activated Ras reveals that under all conditions in A549 and Panc-08-13 cells K-Ras is activated to a level only observed in HeLa cells following EGF stimulation (B). Despite the presence of a significant pool of activated K-Ras, strong Akt and ERK phosphorylation is only seen following 10 minutes of 100 ng/ml EGF stimulation (C).

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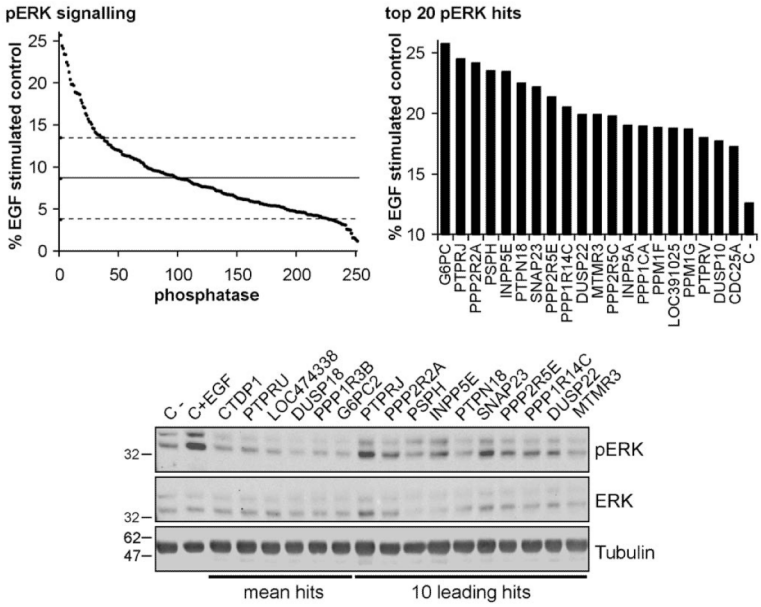

FIGURE 2. A phosphatome RNAi screen reveals significant regulators of ERK activation

Following RNAi of individual phosphatases in A549 cells, pERK bands normalised to tubulin loading controls were compared to 10 minute 100 ng/ml EGF positive controls (C +EGF) on each blot and the data ranked (phosphatome mean: solid line, standard deviation: dotted line). The top 10 most significant hits produced approximately 20-25% of the signal generated by acute EGF stimulation and as illustrated in the blot pERK labelling is clearly stronger when they are knocked down compared to a selection of hits representing the phosphatome mean response and the unstimulated A549 control (C-).

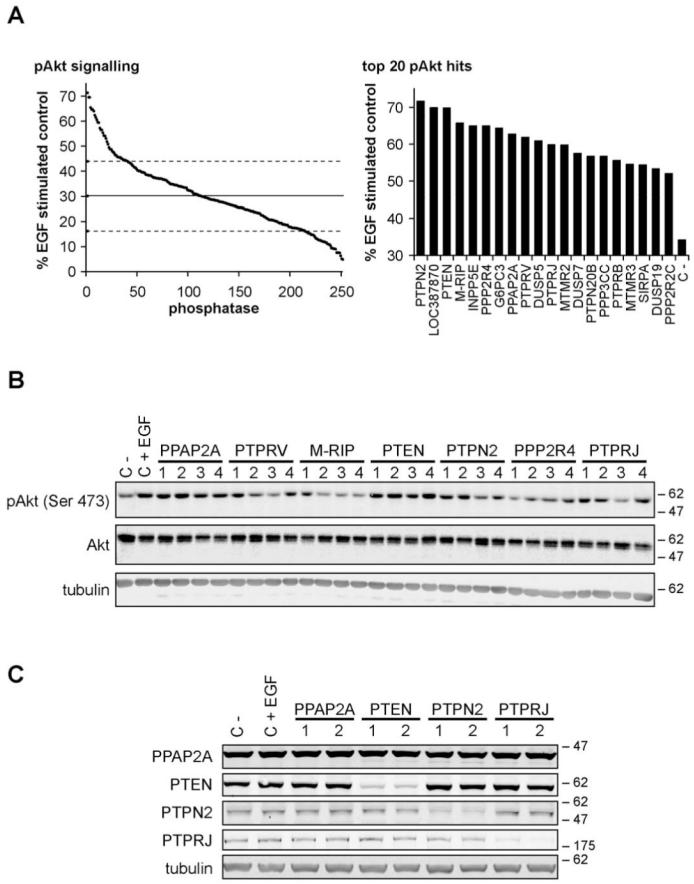

FIGURE 3. RNAi phosphatome screening reveals regulators of Akt activation with similar potency to PTEN

A. Following RNAi of individual phosphatases in A549 cells, pAkt bands normalised to tubulin loading controls were compared to 10 minute 100 ng/ml EGF positive controls (C +EGF) on each blot and the data ranked (phosphatome mean: solid line, standard deviation: dotted line). Deconvolution of selected leading hits in A549 cells reveals PPAP2A, PTEN, PTPN2 and PTPRJ siRNA oligos are reproducible inhibitors of Akt activation (B). Under these experimental conditions, significant knock-down of PTEN, PTPN2 and PTPRJ is seen (C). Representative blots from at least three independent experiments are shown.

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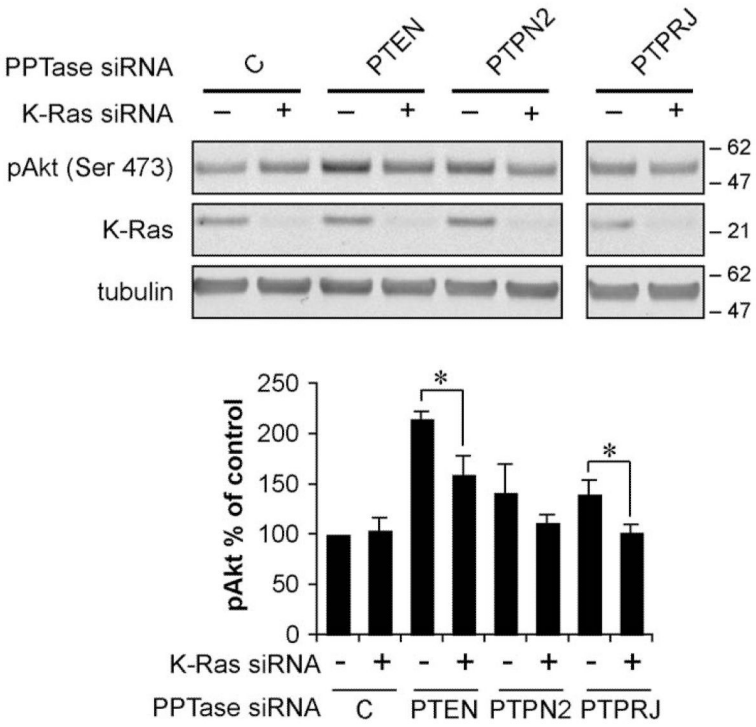

FIGURE 4. PTEN and PTPRJ down-regulation of Akt phosphorylation is contingent upon oncogenic K-Ras

Knockdown of each phosphatase in the presence of K-Ras expression in A549 cells results in increased pAkt phosphorylation compared to the phosphatase mock transfected negative control (C). Knocking down K-Ras (+ lanes) specifically and significantly reduces the stimulatory effect of knocking down PTEN and PTPRJ (means +/− S.E.M., n=4; asterisk indicates $p < 0.05$; unpaired Student's t-test). In contrast, the stimulatory effect on Akt phosphorylation produced by loss of PTPN2 is not specifically coupled to K-Ras. Representative blots are shown; quantitation shows mean values from four independent experiments.

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