G-proteins, which are released upon stimulation of GPCRs [2,3,21]. This dual mode of regulation makes P-Rex family GEFs ideal coincidence detectors for the concomitant activation of PI3K and GPCRs. Synergy of $PI(3,4,5)P_3$ and $G\beta\gamma$ subunits is also required for P-Rex1 translocation to the plasma membrane [22], binds

with HisÐP-Rex2-(983Ð1187), equimolar amounts of GST and GSTÐPP1 were preincubated for 1 h at 10°C with glutathione agarose prior to the addition of HisÐP-Rex2-(983Ð1187) for 1 h and analysis of precipitates by SDS/PAGE and Western blotting. For binding assays with native P-Rex1, 50 pmol of GST or GSTÐPP1 were incubated with 8 pmol of native pig-neutrophil-derived puriÞed P-Rex1 for 1.5 h at 4°C, before pull down with glutathioneÐSepharose, washing (in PBS, 1% Triton X-100, 5 mM EGTA, 1 mM EDTA, 25 mM NaF and 20 mM 2-glycerophosphate) and analysis by SDS/PAGE (8% gel) and Western blotting.

Co-immunoprecipitation assays

Figure 1 P-Rex1 and P-Rex2 are PP1-binding proteins

(A) Dose-dependent inhibition of the phosphorylase phosphatase activity of puri ed rabbit PP1 by puri ed Sf9-cell-derived full-length human EE–P-Rex1, GST–P-Rex2 and GST–P-Rex2 (*n* = 3). (B) Schematic representation of the RVxF motif in P-Rex1 and P-Rex2 and its mutation to AVxF. (C) Binding of native P-Rex1 to GST–PP1 GST were incubated with puri ed native pig P-Rex1 prior to pull down with glutathione–Sepharose, SDS/PAGE and Western blotting (WB) with anti-P-Rex1 and -GST an markers are given in kDa on the right-hand side. (D) Far-Western: immunoprecipitated eGFP–P-Rex1 (full-length) was subjected to SDS/PAGE and Western transfer. The membrane which were incubated separately with GST–PP1 in the presence or absence of excess RVxF peptide, or with GST, as indicated, and then together with anti-GST antibody. The stri panel was incubated with anti-GFP antibody. (E) P-Rex1 forms a complex with PP1 in vivo, which is disrupted by mutation of the RVxF motif. Lysates of COS-7 cells expressing EE–P-Re EE–P-Rex1 VAFA and/or eGFP–PP1 were subjected to immunoprecipitation with an anti-GFP antibody. Western blot analysis of precipitates and total lysates (TL) was performed wi anti-PP1 antibodies. Blots shown are from one experiment and representative of three. Molecular mass markers are given in kDa on the right-hand side. (F) Endogenous P-Rex1 forms a complex with endogenous PP1 in vivo. HEK-293 cell lysates were subjected to immunoprecipitation with anti-PP1 antibody. Western blots of precipitates and total lysates (TL) were performed us anti-P-Rex1 and -PP1 antibodies. Blots shown are from one experiment and representative of four. IP, immunoprecipitation.

at http://www.BiochemJ.org/bj/443/bj4430173add.htm). It bound PP1 activity to a similar degree as did eIF-2(1–144), a known PP1 interactor, unlike eIF-2(1–144) with a mutated RVxF motif (mut) which did not (Supplementary Figure S1B). As most PP1-interacting proteins inhibit the constitutive catalytic activity of the phosphatase [32], we tested whether this was also the case for P-Rex2. Indeed, the P-Rex2 fragment inhibited PP1 activity in vitro to a similar degree as eIF-2(1–114) (IC_{50} 500 nM); full-length P-Rex2 was even more effective than the fragment (IC_{50} 200 nM), whereas eIF-2(1–114) (mut) again had no effect (Figure 1A and Supplementary Figure S1C).

The RVxF-type PP1-docking motif is highly evolutionarily conserved between the full-length members of the P-Rex family (Figure 1B and Supplementary Figure S1D). Hence it seemed plausible that the interaction with PP1 is conserved between P-Rex1 and P-Rex2. Indeed, full-length P-Rex1 also inhibited PP1 activity, to a slightly greater extent than P-Rex2 (IC_{50} nM) (Figure 1A). The known mechanisms of regulation are very similar between different members of the P-Rex family, but most previous characterization work has been done on P-Rex1, so we focused on the interaction of P-Rex1 with PP1 from this point onwards. We tested binding of native P-Rex1 to PP1. Puri ed native P-Rex1 from pig neutrophils bound to puri ed recombinant bacterial GST–PP1, but not GST, suggesting that the interaction between P-Rex1 and PP1 is direct and independent of additional proteins (Figure 1C). We con rmed this by far-Western analysis of eGFP (enhanced GFP)–P-Rex1 binding to GST–PP1. P-Rex1 bound to GST–PP1, but not GST, and could be competed off with a synthetic RVxF-containing decapeptide, indicating that direct PP1 binding to P-Rex1 is mediated through the RVxF motif (Figure 1D).

a n d x 1 c o - h e b l o t P w h e t h e r x 1 c o - i m m u 5 2 T

To investigate whether P-Rex1 and PP1 interact in vivo, we overexpressed eGFP–PP1 and EE–P-Rex1 in COS-7 cells and analysed anti-GFP immunoprecipitates by Western blotting. EE–P-Rex1 co-immunoprecipitated with eGFP–PP1, suggesting that P-Rex1 and P

a n d 0 4 5 i h 0 . 0 4 3 (b) 2 n m . 3 6 0

Figure 2 PP1 stimulates P-Rex1 Rac-GEF activity *in vitro*

(A) Native rabbit PP1 activates P-Rex1. The Rac2-GEF activity of Sf9-cell-derived puri ed human EE–P-Rex1 was assayed with the indicated concentrations of stearoyl-ar
and/or $G_{1\ 2}$ after a 30 min pre-incubation of P-Rex1 in the presence or absence of 100 nM native rabbit PP1. Results are means

Figure 3 PP1 stimulates P-Rex1 Rac-GEF activity *in vivo*

(A) PP1 stimulates P-Rex1 WT, but not P-Rex1 VAFA, Rac-GEF activity. GST–Pak1-CRIB pull-down assay for endogenous Rac1 activity in serum-starved HEK-293 cells expressing My
WT, Myc–P-Rex1 VAFA and/or eGFP. Stimulated with (right-hand panel, dark grey bars) or without (right-hand panel, light grey bars) 50 nM LPA for 1 min. The GTP-loading of
panel, Rac1 activity), and the expression of Rac1 (2% of the total loaded), P-Rex1 and PP1 by immunoblotting. The left-hand panel shows blots from one experiment
representative of four. The right-hand panel shows densitometric analysis of four experiments (two for untransfected cells). Data are means
determined using Tukey's Honestly Signi cant Differences test. (B) Protein phosphatase-dead PP1 does not stimulate P-Rex1 Rac-GEF activity. GST–Pak-CRIB pull-down assays, measuring
P-Rex1 Rac-GEF activity in serum-starved HEK-293 cells upon co-expression with either phosphatase-dead eGFP–PP1 or eGFP. (C) PP2A1 and PP1 can both stimulate P-Rex1
Rac-GEF activity *in vivo*. Pak-CRIB pull-down assays, measuring P-Rex1 Rac-GEF activity in serum-starved HEK-293 cells upon co-expression

higher phosphorylation levels (Figure 5B). A similar pattern was observed in phosphoserine Western blots (Figure 5B). Hence, although the ability of P-Rex1 to bind PP1 does affect gel migration, it has no major impact on global P-Rex1 phosphoserine levels, suggesting that PP1 target sites represent a minority of all P-Rex1 phosphorylation sites.

To identify the site(s) of PP1-dependent dephosphorylation, we analysed PAE-cell-derived P-Rex1 WT and P-Rex1 VAFA by MS, reasoning that PP1 target residues would be more highly phosphorylated in P-Rex1 VAFA. Importantly, we did not overexpress PP1 in these experiments, but relied solely on endogenous PP1 in order to reveal only physiologically

le55(xperiments,)-47d [(T)79

Figure 5 Ser^{1165} is a PP1 target dephosphorylation site on P-Rex1 important in the regulation of P-Rex1 activity

(A) Phosphoserine content of puri ed recombinant Sf9-cell-derived P-Rex1 (25 nM) after incubation with or without recombinant PP1 (50 nM) for 30 min. Shown is a Western blot analysis from one experiment that is representative of two. (B) Gel-migration properties of P-Rex1 WT and P-Rex1 VAFA. Lysates of PAE cells expressing EE–P-Rex1 WT or EE–P-Rex1 VAFA together with eGFP–PP1 were subjected to anti-EE immunoprecipitation and precipitates were analysed by anti-P-Rex1 or phosphoserine Western blots. (C) MS-mapped phosphorylation sites in P-Rex1 WT (grey bars) and P-Rex1 VAFA (black bars). EE–P-Rex1 WT and EE–P-Rex1 VAFA were expressed in PAE cells (without exogenous PP1) and anti-EE immunoprecipitates were subjected to SDS/PAGE. P-Rex1 bands were isolated, digested either with trypsin, chymotrypsin or AspN, and subjected to LC–MS/MS. This achieved 96 % coverage of P-Rex1 and revealed ten phosphopeptides on to which ten serine phosphorylation sites were mapped. Their level of phosphorylation (compared with the total peptide) was measured by MS, and was suf ciently high for analysis in the eight indicated sites. (D) Rac1 activity in P–Rex1 WT, P–Rex1 Ser1165A or P-Rex1 'cluster' mutant were expressed in HEK-293 cells with or without eGFP–PP1 serum-starved and subjected to a Pak-CRIB pull-down assay to measure endogenous Rac1 activity. Results are means + S.E.M. for four independent experiments. Signi cance was determined using a Student's *t* test.

HEK-293 and PAE cells showed no obvious changes in the largely cytosolic subcellular localizations of P-Rex1 and PP1 upon their co-expression (results not shown).

We next compared the gel-migration properties of EE–P-Rex1 WT and EE–P-Rex1 VAFA upon co-expression with PP1 in basal PAE cells. Immunoprecipitated P-Rex1 WT migrated as a doublet like Sf9-cell-derived P-Rex1, whereas P-Rex1 VAFA lacked the lower band and gained a higher band, indicative of

in vivo Rac-GEF activity levels is not straightforward and should be addressed in vitro in the future by assessing the Rac-GEF activities of puriÞed recombinant P-Rex1 proteins with the relevant phospho-deÞcient and phospho-mimetic point mutations. Future analysis of Ser^{834} and Ser^{1001}, as well as individual serine residues in the P-Rex1 cluster mutant, is likely to reveal further PP1 -dependent sites. Of the seven other phosphoserine residue sites identiÞed, two more (Ser^{1191} and Ser^{1200}) may be PP1

17 Montero, J. C., Seoane, S., Ocana, A. and Pandiella, A. (2010) P-Rex1 participates in Neuregulin-ErbB signal transduction and its expression correlates with patient outcome in breast cancer. Oncogene, 30, 1059–1071
18 Qin, J., Xie, Y., Wang, B., Hoshino, M., Wolff, D. W., Zhao, J., Sco eld, M. A., Dowd, F. J., Lin, M. F. and Tu, Y. (2009) Upregulation of PIP3-dependent Rac exchanger 1 (P-Rex1) promotes prostate cancer metastasis. Oncogene, 28, 1853–1863
19 Sosa, M. S., Lopez-Haber, C., Yang, C., Wang, H., Lemmon, M. A., Busillo, J. M., Luo, J., Benovic, J. L., Klein-Szanto, A., Yagi, H. et al. (2010) Identi cation of the Rac-GEF P-Rex1 as an essential mediator of ErbB signaling in breast cancer. Mol. Cell, 40, 877–892
20 Fine, B., Hodakoski, C., Koujak, S., Su, T., Saal, L. H., Maurer, M., Hopkins, B., Keniry, M., Sulis, M. L., Mense, S. et al. (2009) Activation of the PI3K pathway in cancer through inhibition of PTEN by exchange factor P-Rex2a. Science, 325, 1261–1265
21 Hill, K., Krugmann, S., Andrews, S. R., Coadwell, W. J., Finan, P., Welch, H. C., Hawkins, P. T. and Stephens, L. R. (2005) Regulation of P-Rex1 by phosphatidylinositol (3,4,5)-trisphosphate and Gβ subunits. J. Biol. Chem., 280, 4166–4173
22 Barber, M. A., Donald, S., Thelen, S., Anderson, K. E., Thelen, M. and Welch, H.C.E. (2007) Membrane translocation of P-Rex1 is mediated by G protein and

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online data

SUPPLEMENTARY ONLINE DATA

The guanine-nucleotide-exchange factor P-Rex1 is activated by protein phosphatase 1

[redacted]

Figure S2 Effects of P-Rex1 and PP1 expression levels on endogenous Rac1 activity

(A) Endogenous Rac1 activity is not signi cantly affected by 5-fold variations in P-Rex1 overexpression levels. Myc–P-Rex1 WT levels were titrated over an approximately 5-fold range by varying the amount of plasmid used for transfection of HEK-293 cells. Cells

Figure S3 MS analysis of P-Rex1

EE–P-Rex1 WT and EE–P-Rex1 VAFA were expressed in PAE cells (without exogenous PP1) and immunoprecipitates subjected to SDS/PAGE. P-Rex1 bands were isolated, digeste with trypsin, chymotrypsin or AspN, and subjected to LC–MS/MS. (A) Phosphopeptides and serine phosphorylation sites (bold red) identi ed in P-Rex1 WT and P-Rex1 V sulfoxide. (B) A 96 % coverage (residues in red) was achieved and revealed ten phosphoserine residues (highlighted in yellow) both in P-Rex1 WT and P-Rex1 VAFA. The R green. (C) Conservation of the ten identi ed phosphoserine sites throughout P-Rex1 evolution. The alignment was performed with ClustalX. Fully conserved residues; green, residues conserved by structural similarity; black, divergent residues.

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