Supplementary information

Group I and II mammalian PAKs have different modes of activation by Cdc42

Supplementary Figure 1 (as related to data in Fig. 1)

(A) Triton soluble cellular lysate (40 μg) from cell lines as in Fig. 1C were subjected to Western analysis using anti-PAK4 and anti-pS474 from Cell Signaling (CS). The lower panels show in-house antibodies (see methods). Note the weak signal seen with the anti-pS474 (upper panel) and non-specific bands in some lysates, versus the lower panel. (B) Kinase dead (KD) and wild-type (WT) Flag-PAK4 constructs were expressed in COS7 cells, immunoprecipitated and probed with anti-pS474 and anti-Flag antibodies. (C) U2OS cells were treated with calyculin (calyc. 1 μM, 10 min) or staurosporine (0.1 μM, for 1h) in the presence of serum (10%). Triton-X-100 soluble cell lysates (40μg, lane) were probed for anti-PAK4 or pS474 using antibodies shown in the lower panel of Fig. S1A.

Supplementary Figure 2 (as related to data in Fig. 2)

(A) Molecular model of the PAK1 AID (red) interaction with the catalytic domain (with contact residues in the α-G helix shaded grey) as seen in the structure 1F3M. The activation loop (incomplete) is displaced and highlighted in yellow.

(B) Molecular model of PAK4 catalytic domain (2Q0N) with a substrate peptide highlighted in blue, and the activation loop in yellow. Residues equivalent to those in PAK1 that provide critical contacts with the AID (cf. PAK4 L521, K522 and K525) are also shown in grey. (C) Alignment of the PAK1 and PAK4 αG-helix residues. PAK4 residues previously mutated are marked (indicated in panel B). (D) Flag-PAK4

constructs were expressed in COS7 cells and subjected to immunoprecipitation and radioactive KA. Samples were analysed for radioactivity, stained and immunoblotted for PAK4 levels with anti-Flag antibody.

Supplementary Figure 3

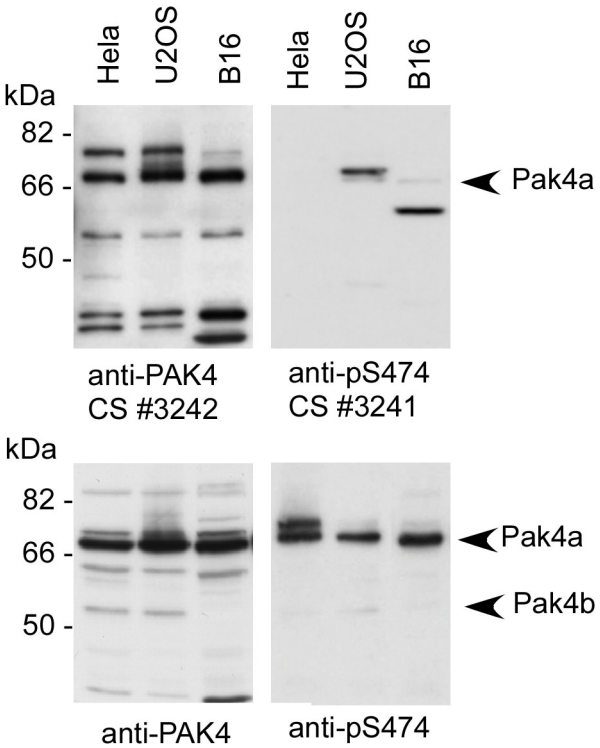
(A) Data relating to samples similar to those in Figure 3A, showing substrate and kinase auto-phosphorylation (top panel). The PAK4 was expressed in COS7 cells and PAK4 captured on anti-Flag Sepharose directly, or via Flag-Cdc42(G12V). The chimaera Cdc42-G6-PAK4 is illustrated in Fig. 3B. *In vitro* kinase assays were performed as described in methods using 10 μM [γ32P]ATP and 20 μg GST-Raf13 substrate. (B) Bacterial expressed GST-PAK1 or 6His-PAK4 variants were purified and stored at -80C in 5% glycerol. Each kinase assay (25 μl) contained 1.0 μg kinase, 20 μg substrate (GST-Raf13p or GST-FL mouse BAD) and 10 μM [γ32P] ATP (incubated 30 min at 30°C). Half of each reaction was subjected to SDS-PAGE, stained with Coomassie blue (as shown), dried and placed to film for 10 min.

Supplementary Figure 4.

(A) COS7 cell expressed Flag-PAK4 as indicated were subjected to *in vitro* kinase assays as for Fig S3. The proteins were separated by SDS-PAGE and the dried gel placed to X-ray film. (B) Commercial anti-PAK4 antibody from Cell Signaling #3242 (5.0 μg) was used for immuno-precipitation of endogenous PAK4 from 2 mg LNCaP lysate. Samples from serum starved or treated cells were compared with equivalent amounts of 6His-PAK4 purified from *E.coli* and back probed with anti-PAK4 and anti-pS474 antibodies. The right panel shows the Coomassie blue stain of excess 6His-PAK4. (C) LNCaP lysates of cells treated with 0.4M sorbitol or 10% serum with

or without indicated inhibitors, were subjected to anti-PAK4 immuno-precipitation and probed for anti-PAK4(#3242) or anti-pS474. Total lysate was immunoblotted with antibodies to p38 or phospho-p38. WT, wild type; KD, kinase dead; Δ50, PAK4(51-591); 41D, PAK4(S41D); 46D, PAK4(S41D); 60E, PAK4(T60E); HC, heavy-chain; LC, light-chain; U, untreated; SB, SB203580; LY, LY294002.

A



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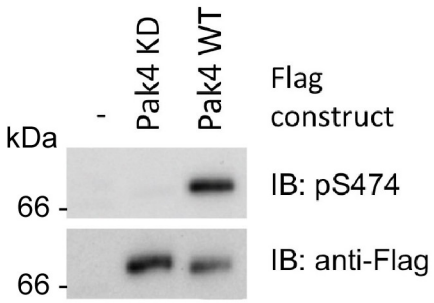

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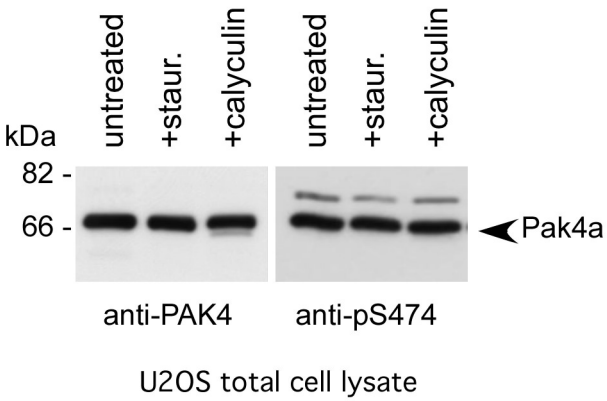
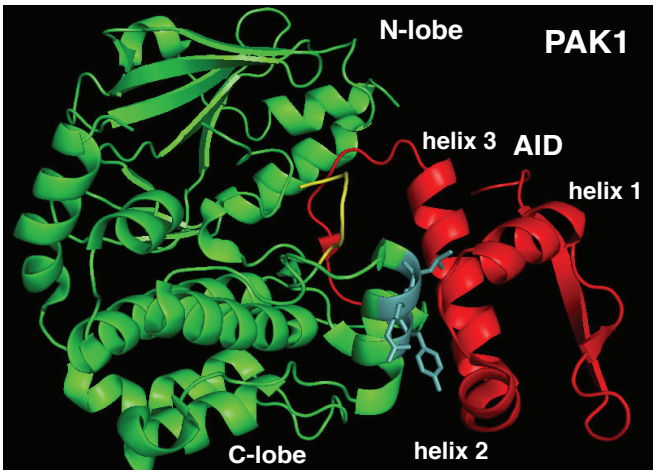


Supplementary Figure 1

A



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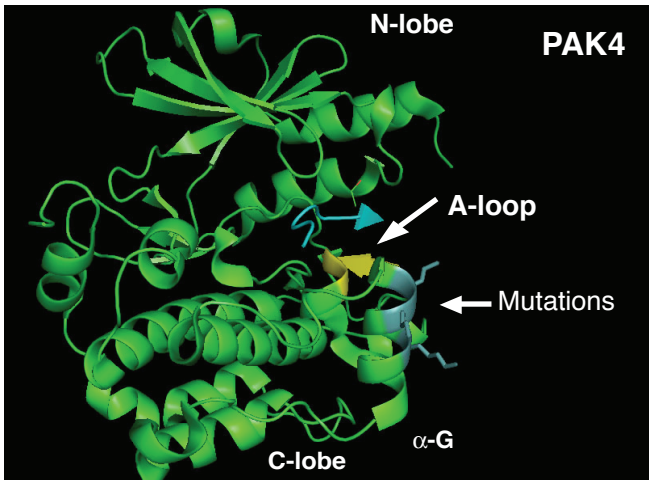

C

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PAK1   469 PLRALYLIAT 478
PAK4   520 PLKAMKMIRD 529
           mutations
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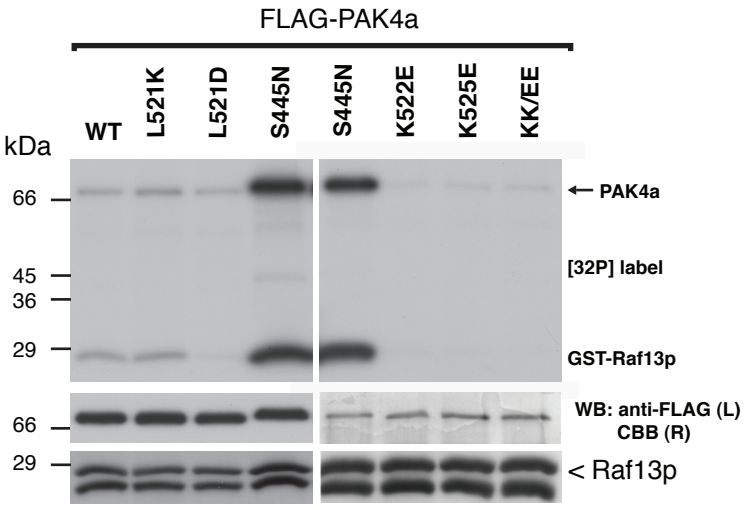
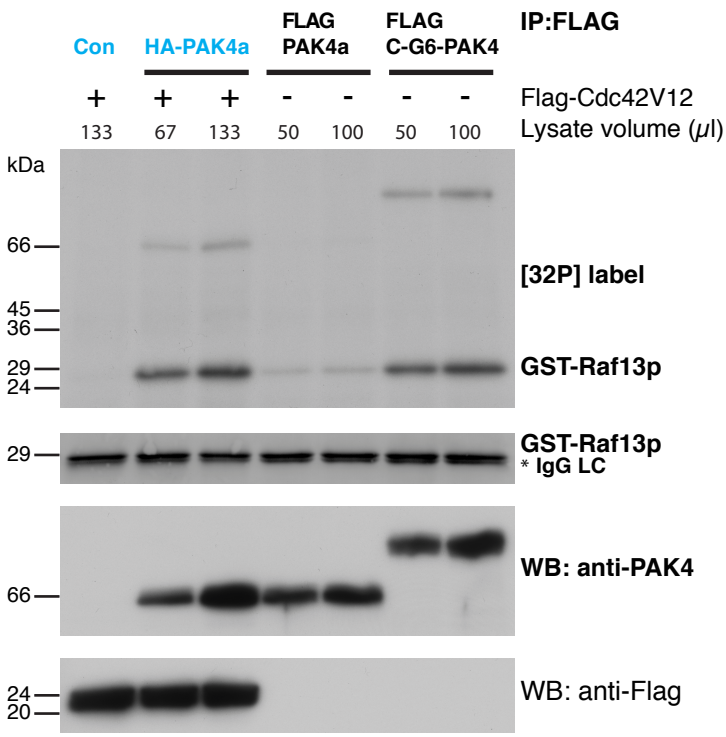
D



IP: anti-Flag-PAK4
in vitro kinase assay

Supplemenatary Figure 2

A



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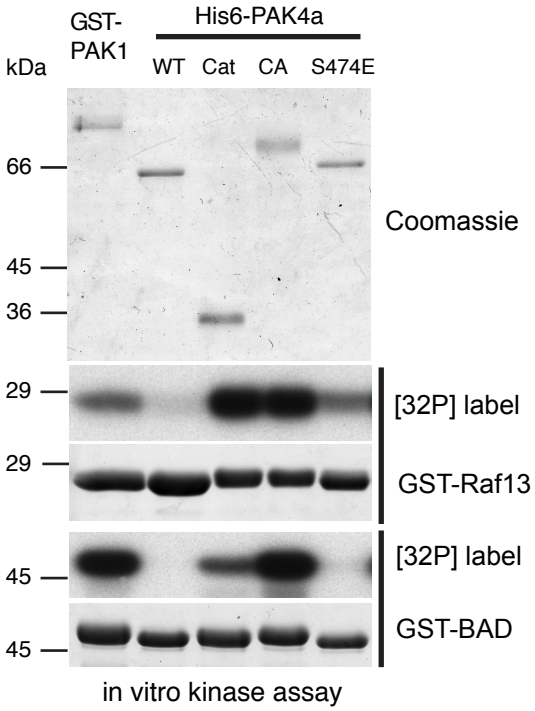
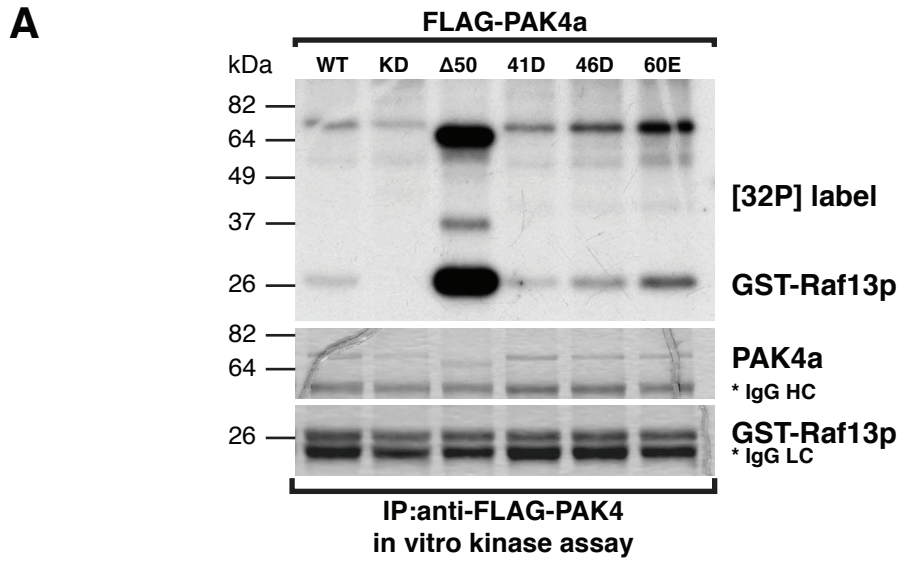
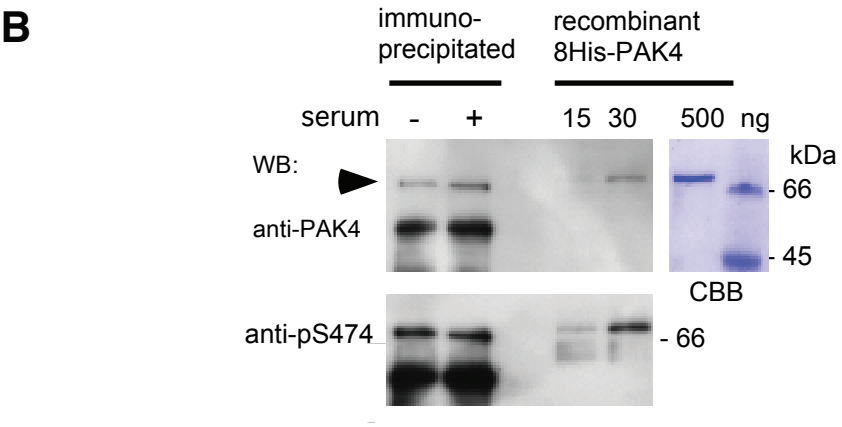
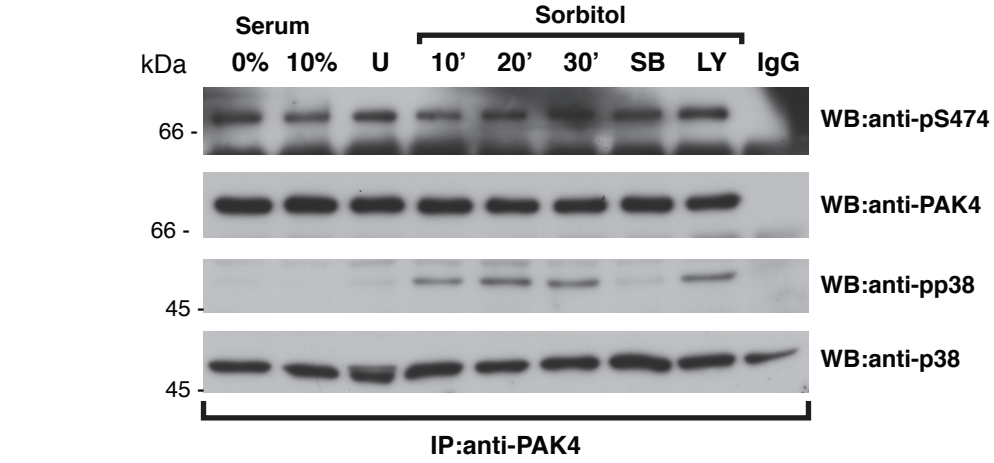


Supplementary Figure 3







Supplementary Figure 4

Materials and methods

Antibodies. Mouse M2 and rabbit anti-Flag antibodies, and M2-Sepharose and protein A-Sepharose were from Sigma-Aldrich. Mouse anti-HA (F7) and mouse anti-GFP were from Santa Cruz. (Sigma-Aldrich). The p38 MAP kinase inhibitor (SB203580) and PI-3 kinase inhibitor (LY294002) were purchased from Ascent Scientific.

Cloning and mutagenesis Full length human PAK4a was amplified from the pSRα3 PAK4 vector (provided by Dr. Audrey Minden, Columbia University, New York, NY), the PAK4b was amplified from a pBluescript II SK plus vector (provided by Dr. Staffan Strömblad, Karolinska Institutet) and the PAK4c was derived from the relevant EST (Accession No. AAH02921.1). Mammalian pXJ40 based vectors (Manser *et al.*, Mol. Cell. Biol. 17 1997) contain N-terminal Flag-, HA- or GFP fusion tags. Truncation mutants were generated by PCR and point substitutions were generated by QuikChange (Stratagene) mutagenesis.

The PAK4(s) construct was generated by ligation of DNA fragments encoding residues 1-68 (BamHI/EcoRI fragment) to the residues 278-591 (EcoRI/XhoI fragment), and includes a 2 residue Glu-Phe linker. The Cdc42-Gly6-PAK4 construct was constructed using a Cdc42V12(1-184)-Gly5 sequence (cloned EcoRI/BamHI) linked to full-length PAK4 construct (cloned in-frame BamHI/XhoI): the linker therefore encodes GGGGGGS. Complementary DNA was generated from the specified cell-lines using RNA purification and M-MuLV Reverse Transcriptase kit (Promega). The PAK4 primers matched both human and mouse sequences: F1= 5'-GAAGAAGCGGGTGGAGATCTC, R1= 5'-CAGCCCGGAACTGCTCATGG, and

R2= 5'-TCCATCTTCTTGACGGCCACCAG.

Expression and purification of recombinant protein For bacteria expression, pGEX4T1 (GE Healthcare) and pET28a (Novagen) vectors were used. For the cloning of PAKs, *Escherichia coli* JM109 (grown at 30°C) were induced (1 mM IPTG) 16 hours at 4°C, and proteins were purified according to manufacturers conditions. Elution buffers (50 mM Tris HCl, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol) were supplemented with 1mM MgCl2 for protein kinases. Protein purity was assessed to be greater than 90% by SDS-PAGE.

Cell culture, transfection and lysis COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4500 mg/l glucose and LNCaP cells were grown in RPMI media with 10% bovine calf Serum (Hyclone). Transient transfections were performed with Lipofectamine 2000 using 5.0 μg plasmid DNA per 60 mm dish. After 16 hours cells were harvested in 500 μl ice-cold buffer (25 mM Hepes pH 7.3, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% Triton X-100, 4% glycerol, 5 mM DTT, 20 mM β-glycerol phosphate, and protease inhibitor mix without EDTA, Roche). Typically 200 μl of cell lysate was added to 20 μl M2 anti-Flag sepharose (Sigma-Aldrich), and rolled on ice, before washing (400 μl lysis buffer twice, 10 min each). Sepharose was incubated with SDS sample buffer at 50C to release proteins (10 min). The p38 MAP kinase inhibitor SB203580 (20 μM) or PI-3 kinase inhibitor LY294002 (20 μM) were added 30 min before analysis. Osmotic stress was induced with 0.4 M sorbitol; cells pre-incubated in 1% serum for 90 min. Prior to 10% serum treatment, LNCaP cells were starved for 4 hrs.

In vitro kinase assays Mammalian constructs were immunoprecipitated with 20 µl of mouse M2 anti-Flag agarose beads (Sigma-Aldrich). Endogenous PAK4 was immunoprecipitated with 5.0 µg Protein A-Sepharose bound antibodies (Cell Signaling #3242). PAK4 kinase assays were performed in 50 µl of 50 mM Hepes pH 7.3, 50 mM KCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 2 mM DTT, 10 mM β-glycerol phosphate, 2 µCi ^{32}P γATP and 10 µM ATP with 20 µl sepharose-bound or recombinant kinase. Each reaction contained 20 µg of recombinant GST-Raf1(332-344) or GST-mBad(1-204) fusion protein and were incubated 30 min at 30°C , and terminated by adding SDS sample buffer. Samples were separated by SDS-PAGE, transferred onto Polyvinylidene fluoride membrane (PVDF , GE healthcare), and processed for Western blotting or exposed to SuperRX (Fujifilm) X-ray film.

Western blotting Proteins were transferred from acrylamide gels to PVDF membranes (Immobilon P). These were blocked with 5% skimmed milk in PBS for 1h, and incubated with primary antibody (0.25-1.0 µg/ml) in 1% BSA/PBS for 2 hours at RT or overnight at 4°C. Appropriate horseradish peroxidase (HRP) linked antibodies (DAKO) were visualised using luminol (Amersham) and detected by SuperRX film (Fujifilm).