

# Evidence for a Novel Mechanism of the PAK1 Interaction with the Rho-GTPases Cdc42 and Rac

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## Abstract

P21-activated kinase 1 (PAK1) is activated by binding to GTP-bound Rho GTPases Cdc42 and Rac via its CRIB domain. Here, we provide evidence that S79 in the CRIB domain of PAK1 is not directly involved in this binding but is crucial for PAK1 activation. S79A mutation reduces the binding affinity of PAK1 for the GTPases and inhibits autophosphorylation and kinase activity of PAK1. Thus, this mutation abrogates the ability of PAK1 to induce changes in cell morphology and motility and to promote malignant transformation of prostate epithelial cells. We also show that growth of the prostate cancer cell line PC3 is inhibited by the treatment of a PAK1-inhibiting peptide comprising 19 amino acids centered on S79, but not by the PAK1 peptide containing the S79A mutation, and that this growth inhibition is correlated with reduced autophosphorylation activity of PAK1. Together, these findings demonstrate a significant role of S79 in PAK1 activation and provide evidence for a novel mechanism of the CRIB-mediated interaction of PAK1 with Cdc42 and Rac.

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## Introduction

PAK1 is a major downstream effector of the Rho-GTPases Cdc42 and Rac, which act as molecular switches that transduce various extracellular signals into intracellular responses [1]. PAK1, the best-characterized member of the PAK family, forms a *trans*-inhibited dimer in its inactive state, in which the catalytic domain of one PAK1 monomer is blocked by the autoinhibitory domain (AID) of the other [2,3]. This autoinhibitory conformation is disrupted by binding of the GTP-bound Cdc42 and Rac to the CRIB (Cdc42/Rac-interactive binding region) domain [4,5,6], leading to autophosphorylation at specific sites including T423 within the activation loop and consequent activation of PAK1 [7,8]. Efficient activation of PAK1 requires its membrane targeting. PAK1 is recruited to the plasma membrane via the SH3-containing proteins Nck and Grb2 [9,10,11], where it may be activated by signaling molecules such as PDK1 kinase [12], sphingosine [13,14] and PIP2 [15] in a manner independent of the GTPases.

PAK1 is frequently overexpressed and hyperactivated by dysregulation of a number of signaling pathways in human cancer cells that are stimulated by growth factor receptors such as EGFR, PDGFR, and VEGFR [16]. The activated PAK1 in turn promotes cancer cell invasion and metastasis by phosphorylating key regulators involved in cytoskeleton reorganization, such as Lim kinase (LIMK) [17,18] and the P41-ARC subunit of the ARP2/3 [19]. PAK1 activation also stimulates anti-apoptotic pathways, such as the Pak-Raf1-Bad [20,21] and NFκB [20] pathways, rendering PAK1 attractive as a cancer therapeutic target [22]. There has been a rapid expansion in the development of peptides as potential drugs for cancer therapy over the last decade [23].

HIV-1 TAT protein transduction domain-mediated delivery of macromolecules has emerged as an alternative approach for the internalization of proteins into the cell from the external environment [24]. PAK peptides have been also examined by two groups in different methods; 1) treatment of the PAK peptide (aa 11–23) that interacts with NCK [25]; 2) expression of PAK1 inhibitory domain (aa 83–149) [26].

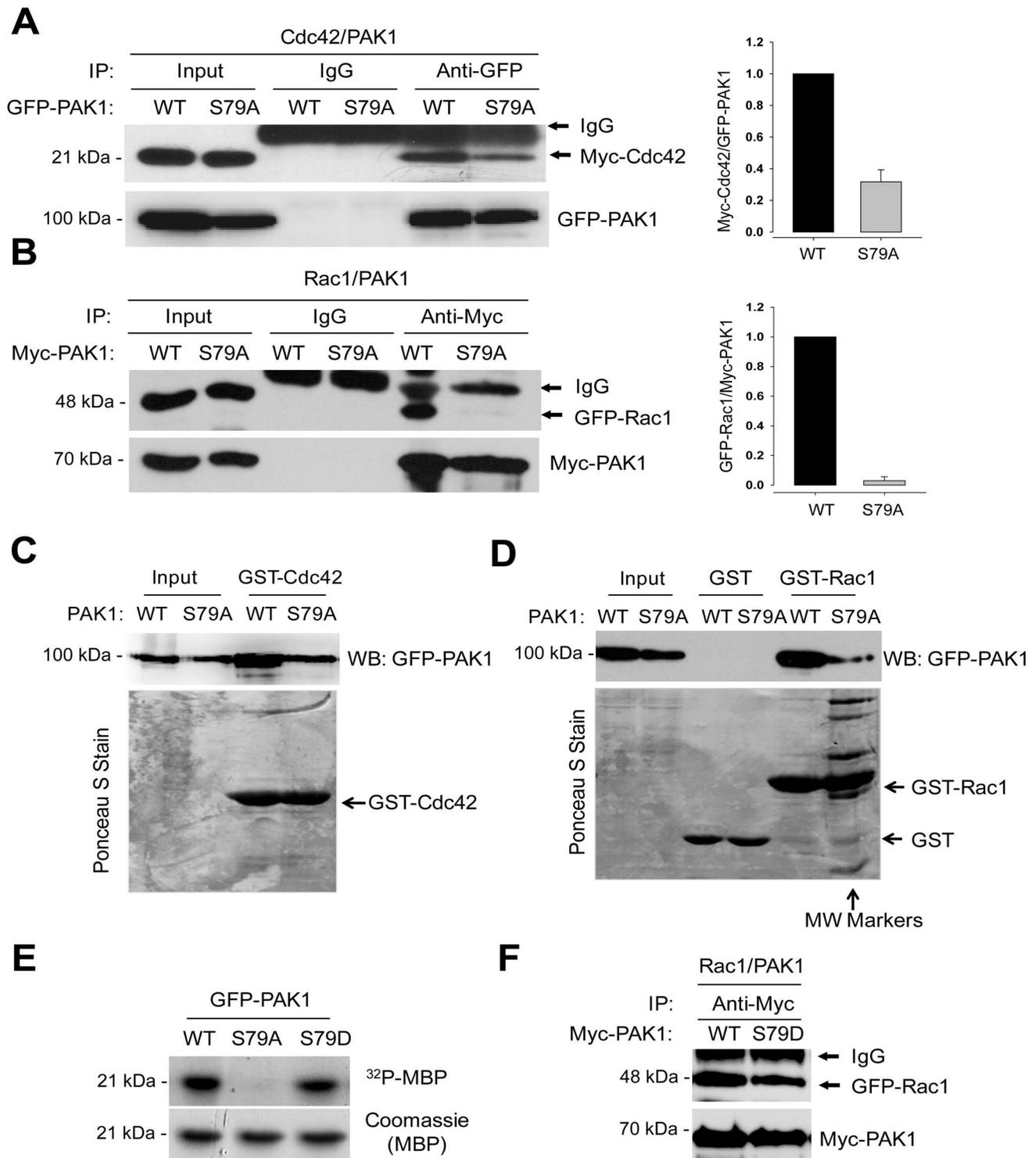
The crystal structure of the Cdc42-PAK1 complex revealed that the CRIB domain of PAK interacts with Cdc42 by forming an intermolecular β-sheet between residues Y40-I46 of Cdc42 and I76-H83 of PAK but that this interaction seems to be disrupted by the presence of a β-bulge in PAK formed by the sequence <sup>79</sup>SDF<sub>81</sub> [4]. To get insights into the role of this sequence, here we investigated the effect of the mutation at S79, one of the three residues of the <sup>79</sup>SDF<sub>81</sub> sequence on the regulation of PAK1 activity. Our biochemical and cell biological studies have demonstrated that S79 plays a crucial role in the PAK1 interaction with Cdc42 and Rac1 and is required for PAK1-mediated malignant transformation of prostate epithelial cells. Thus, this study uncovers a previously unappreciated role of S79 in the regulation of PAK1 activity and demonstrates a novel concept for the activation of PAK1 by the GTPases.

## Results and Discussion

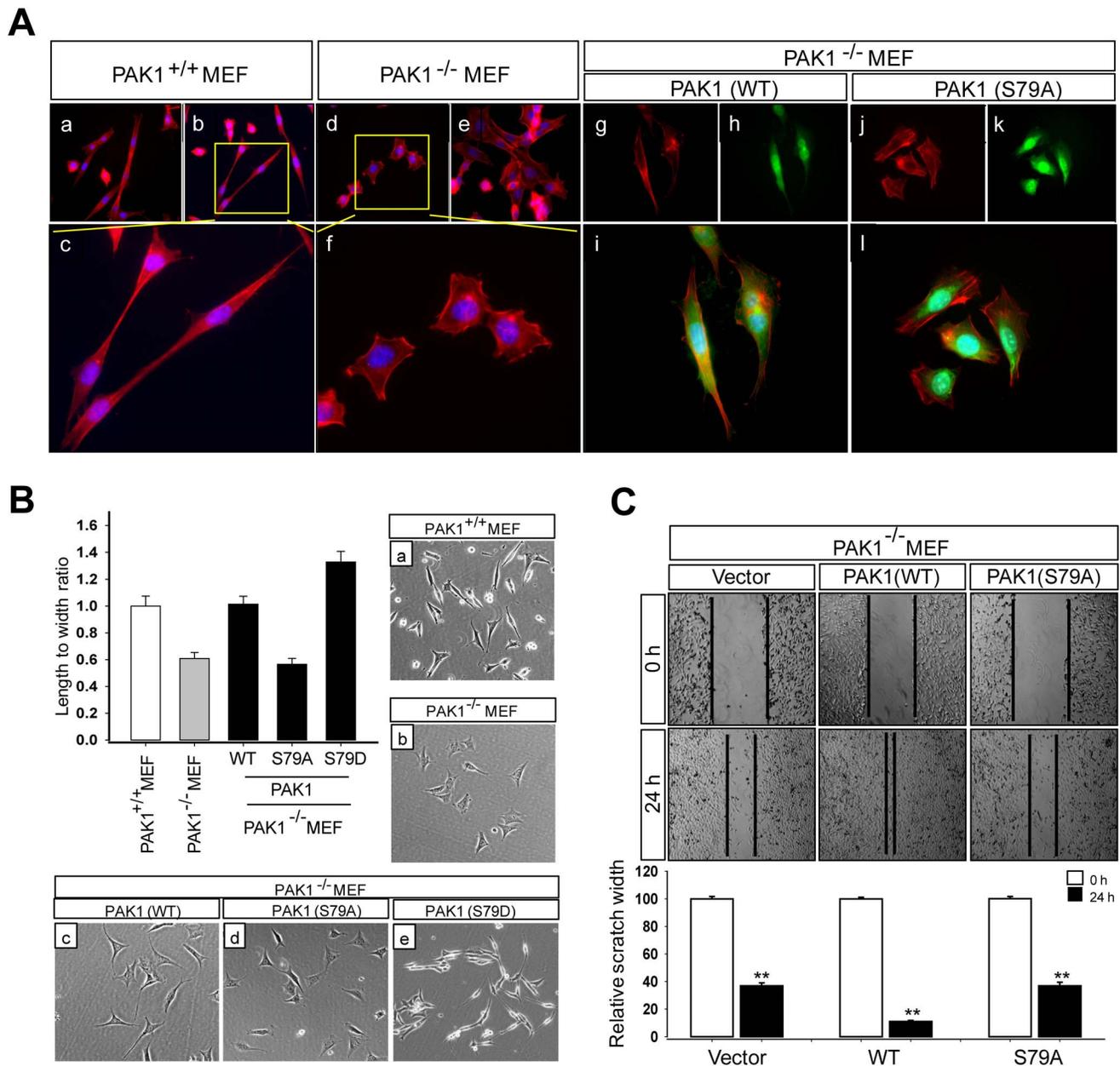
### PAK1<sup>S79</sup> Plays an Important Role in Autophosphorylation and Kinase Activities of PAK1

PAK1 interacts with Cdc42 and Rac via the CRIB domain (amino acid residues 74–88) [1]. A study indicated that <sup>79</sup>SDF<sub>81</sub> motif is positioned near the center of the CRIB domain and appears to disrupt the intermolecular β-sheet interaction between





**Figure 2. S79A mutation impairs the interaction of PAK1 with Cdc42 and Rac1.** (A) Interaction between Myc-Cdc42 and GFP-PAK1. GFP-PAK1<sup>WT</sup> or GFP-PAK1<sup>S79A</sup> was coexpressed with Myc-Cdc42 in 293T cells. Cell extracts were immunoprecipitated with anti-GFP antibody (IP) and then immunoblotted with anti-GFP or anti-Myc antibody (left). (B) Interaction between GFP-Rac1 and Myc-PAK1. Cell extracts were immunoprecipitated with anti-Myc antibody (IP) and then immunoblotted with anti-Myc or anti-GFP antibody. (C and D) Cell lysates prepared from 293T cells expressing GFP-PAK1<sup>WT</sup> or GFP-PAK1<sup>S79A</sup> were incubated with GST-Cdc42 (C) or GST-Rac1 (D) bound to GST-beads. Upper panels, Western blot analysis of the eluates from the beads using anti-GFP antibody; Lower panels, the blots were stained with Ponceau S Stain. MW markers; molecular weight markers. doi:10.1371/journal.pone.0071495.g002



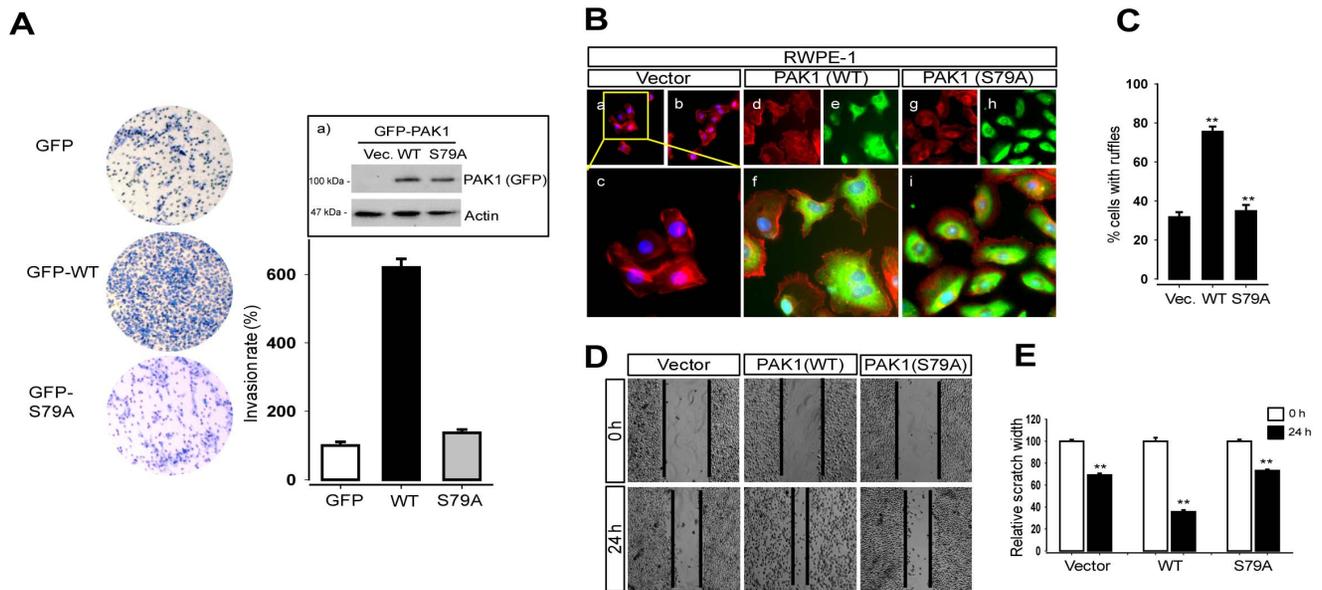
**Figure 3. The S79A mutation impairs the ability of PAK1 to regulate cell morphology and motility.** (A) Wild type (a–c) and PAK1<sup>-/-</sup> (d–f) MEF cells were stained with the high affinity F-actin probe Phalloidin (red) and DAPI (blue). PAK1<sup>-/-</sup> MEF cells expressing GFP-PAK1<sup>WT</sup> (WT, g–i) and GFP-PAK1<sup>S79A</sup> (S79A, j–l) were stained with Phalloidin (red) and DAPI (blue) and visualized by GFP fluorescence (green). (B) Quantification of the length and width (L/W) ratio of MEF cells was obtained as described previously [44]. (C) Wound healing migration assays of PAK1<sup>-/-</sup> MEF cells infected with lentivirus expressing the vector control, GFP-PAK1<sup>WT</sup>, or GFP-PAK1<sup>S79A</sup>. Results were expressed as the percentage of the remaining area determined by normalizing the area of wound after 24 h to the initial wound area at 0 h (set to 100%). Each bar represents the mean  $\pm$  S.D of five fields measured.

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### The S79A Mutation Impairs the Ability of PAK1 to Induce Changes in Cell Morphology and Motility

PAK1 is translocated to the focal adhesions and membrane ruffles [27,28] and the sites of cortical actin remodeling [29] in stimulated cells. We examined the functional importance of PAK1<sup>S79</sup> by comparing the morphology and motility of PAK1<sup>-/-</sup> MEF (mouse embryonic fibroblast) cells expressing GFP-PAK1 and GFP-PAK1<sup>S79A</sup> (Fig. 3A). Wild type MEF cells (PAK1<sup>+/+</sup>) exhibited a bipolar fusiform shape (Fig. 3A, a–c), whereas PAK1<sup>-/-</sup>

MEF cells displayed a more rounded morphology (Fig. 3A, d–f). Expression of GFP-PAK1 in PAK1<sup>-/-</sup> MEF cells restored the wild type cell shape (Fig. 3A, g–i), whereas GFP-PAK1<sup>S79A</sup> expression did not rescue this defect (Fig. 3A, j–l). F-actin was colocalized with PAK1, as observed previously in Swiss 3T3 cells [28]; however, this colocalization was significantly reduced in MEF cells expressing PAK1<sup>S79A</sup> (Fig. 3A and Fig. S1). MEF cells expressing GFP-PAK1<sup>S79A</sup> exhibited 1.5–2 fold decrease in the ratio of length to width (L/W), compared with MEF cells expressing GFP-PAK1, whereas those cells expressing GFP-



**Figure 4. S79A mutation abolishes the migration activity of PAK1.** The benign prostate RWPE-1 cells infected with lentivirus expressing the vector plasmid, GFP-PAK1<sup>WT</sup> (WT), or GFP-PAK1<sup>S79A</sup> (S79A) were used for experiments (A) to (E). (A) The invasion rate was determined by counting the cells that migrated through BME-coated inserts in the Transwell Boyden chamber and expressed as the percentage relative to control (vector). Each bar represents the mean  $\pm$  S.D. of five fields counted. (B) Western blot analysis of PAK1 expression using anti-GFP antibody. Actin was used as an internal control. (C) The S79A mutation impairs the ability of PAK1 to regulate formation of membrane ruffles of cells. RWPE-1 cells infected with lentivirus expressing the vector plasmid (a–c), GFP-PAK1<sup>WT</sup> (WT, d–f) or GFP-PAK1<sup>S79A</sup> (S79A, g–i) were stained with Phalloidin (red) and DAPI (blue) and visualized by GFP fluorescence (green). (D) Quantification of the cells with membrane ruffles (B). (E) Wound healing migration assays of RWPE-1 cells expressing GFP-PAK1 or GFP-PAK1<sup>S79A</sup>. (F) Each bar represents the mean  $\pm$  S.D. of five fields measured in (D). doi:10.1371/journal.pone.0071495.g004

PAK1<sup>S79D</sup> displayed  $\sim 1.5$  fold decrease in the ratio of L to W (Fig. 3B). Wound healing migration assays showed that impaired ability of PAK1<sup>-/-</sup> MEF cells to migrate into, and close to, the wound was restored by expression of GFP-PAK1 but not of GFP-PAK1<sup>S79A</sup> (Fig. 3C).

Next, we compared the ability of wild type and mutant (S79A) PAK1 proteins to confer a migration phenotype on the benign prostate RWPE-1 cells. To this end, RWPE-1 cells were infected with lentivirus expressing the vector plasmid, GFP-PAK1, or GFP-PAK1<sup>S79A</sup> (S79A). Western blot analysis indicated no significant difference in the expression of GFP-PAK1 and GFP-PAK1<sup>S79A</sup> in RWPE-1 cells (Fig. 4A<sup>a</sup>). Cell invasion assay showed that expression of GFP-PAK1, but not of GFP-PAK1<sup>S79A</sup>, confers an invasive phenotype to RWPE-1 cells (Fig. 4A). We also examined cell morphology of RWPE-1 cells expressing PAK1 or PAK1<sup>S79A</sup>. The formation of membrane ruffles was increased by  $\sim 2.5$ -fold (Fig. 4C) in RWPE-1 cells expressing GFP-PAK1 (Fig. 4B, d–f), compared with cells expressing GFP-PAK1<sup>S79A</sup> (Fig. 4B, g–i) or control cells (Fig. 4B, a–c). These results are consistent with previous observations that PAK1 regulates the formation of membrane ruffles of Swiss 3T3 cells [28] and breast cancer cells [30]. We also found that RWPE-1 cells fill  $\sim 30\%$  and  $\sim 60\%$  of the wounded areas by expression of PAK1 and PAK1<sup>S79A</sup>, respectively, at 24 h after scratching (Fig. 4D and Fig. 4E). These observations demonstrate that S79 is crucial for PAK1-mediated cell migration.

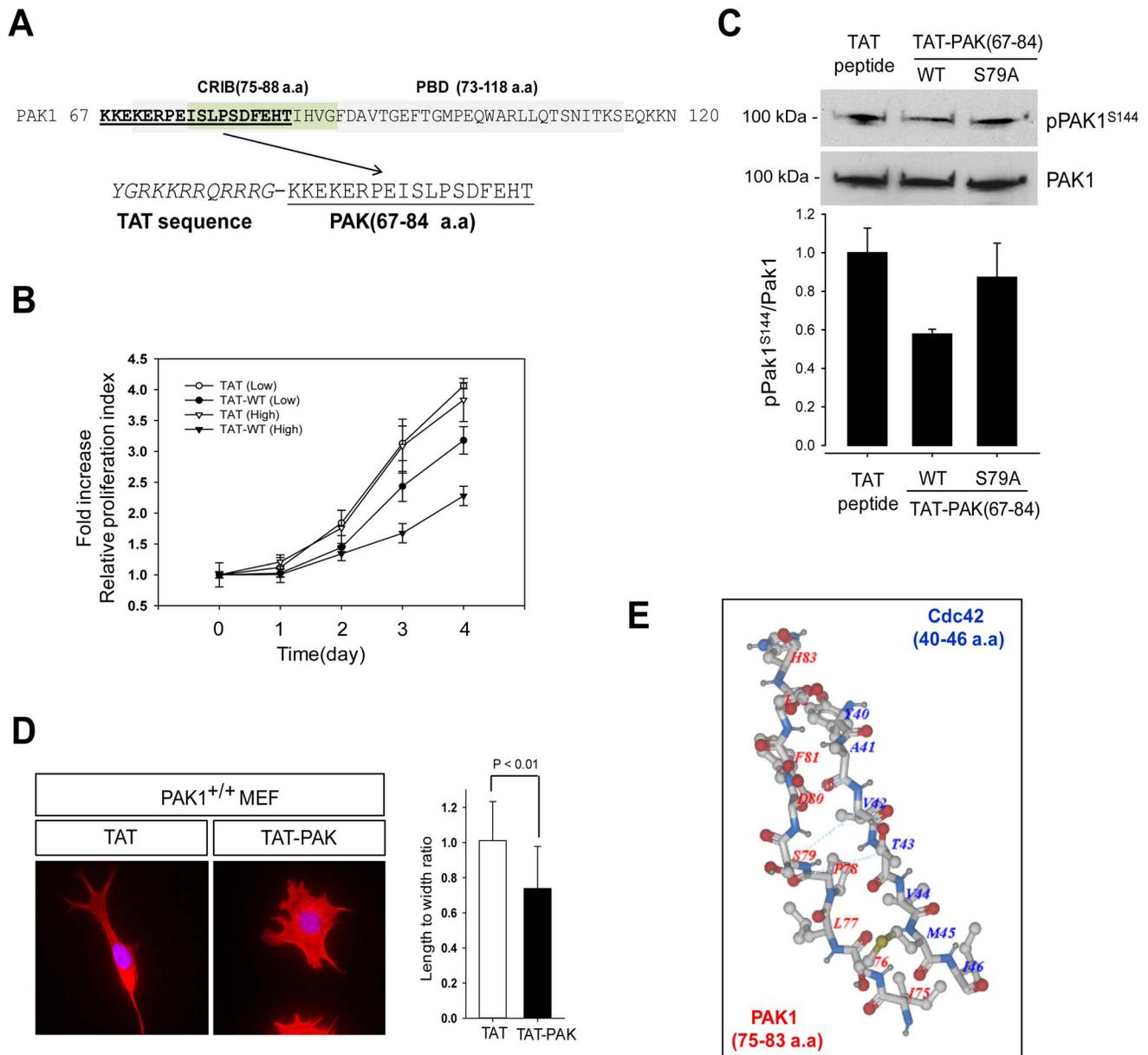
#### A PAK1-inhibiting Peptide (TAT-PAK1<sub>67–84</sub>) Blocks the Growth of the Prostate Cancer Cell Line PC3

Given that PAK1 activation is triggered by interaction with Cdc42 and Rac1, we examined whether PAK1 activity is reduced by a PAK1-inhibiting peptide. Towards this aim, a peptide

containing the CRIB (PAK1<sub>67–84</sub>) was chemically synthesized (Fig. 5A) and fused to the C-terminus of the TAT protein polybasic sequence to facilitate entry into cells as a protein transduction domain [31,32]. PC3 cells were treated with the peptide at low (2  $\mu\text{g/ml}$ ) and high (20  $\mu\text{g/ml}$ ) concentrations and were examined for cell growth by MTT assay. We found that treatment of the PAK1 peptide has  $\sim 2$ -fold inhibitory effect at high dose used (Fig. 5B). To confirm the significance of S79 in the activation process of PAK1 as described above, we also generated a TAT-PAK1<sub>67–84</sub> peptide with S79A mutation and tested its ability to inhibit PAK1 activation. Western blot analysis indicated that S144 phosphorylation is reduced by  $\sim 40\%$  by the treatment of the PAK1 peptide but is not affected by the treatment of the PAK1 (S79A) peptide (Fig. 5C). We also examined the inhibitory effect of the PAK1-inhibiting peptide on the morphology PAK1<sup>+/+</sup> MEF cells (Fig. 5D). The bipolar fusiform shape of MEF cells (PAK1<sup>+/+</sup>) was changed to a more rounded morphology by treatment of the PAK1-inhibiting peptide, whereas the treatment of the PAK1 (S79A) peptide has little effect on the morphology of MEF cells (Fig. S2).

#### Conclusion

The CRIB proteins such as PAK1-3 kinases [28], ACK tyrosine kinases [33,34] and the Wiscott-Aldrich-syndrom proteins (WASPs) [35,36] are activated by direct binding to Cdc42 [37]. While PAK binds to both Cdc42 and Rac, ACK and WASP do not bind to Rac [4]. Structural analyses indicate that the CRIB motif of all the three proteins make an intermolecular  $\beta$ -sheet interaction with the  $\beta 2$  strand of Cdc42 [4,38,39]. Thus, mutations of the amino acid residues that are evolutionarily conserved from human to Drosophila such as I75, S76 and P78 (for sequence alignment, see Fig. 1B) dramatically reduces the



**Figure 5. A PAK1-inhibiting peptide blocks the growth of the prostate cancer cell PC3. (A)** Schematic design of a PAK1-inhibiting peptide (TAT-PAK1 peptide; PAK peptide, aa 67–84). The CRIB domain is underlined. **(B)** PC3 cells were treated with 2  $\mu\text{g/ml}$  or 20  $\mu\text{g/ml}$  of each peptide and examined by MTT assay for 4 days. Cell viability over time was expressed as fold increase, compared with the initial absorbance at day 0. Error bars represent mean  $\pm$  S.D. ( $n=5$ ). **(C)** Western blot analysis of S144 phosphorylation of PAK1 in PC3 cells treated with TAT peptide, TAT-PAK1 peptide (WT) or TAT-PAK1 (S79A). **(D)** MEF cells were stained with the high affinity F-actin probe Phalloidin (red) and DAPI (blue) after treatment with with TAT peptide, TAT-PAK1 peptide (WT) or TAT-PAK1 (S79A) (left). Quantification of the length and width (L/W) ratio of MEF cells was obtained as described previously (right). **(E)** A structural overview of CRIP domain of PAK1 (red, 75–83 a.a) with bound Cdc42 (blue, 40–46 a.a). Data Bank ID number 1E0A and visualized using Molsoft ICM Browser (Molsoft, L.L.C., San Diego, CA, USA). doi:10.1371/journal.pone.0071495.g005

binding affinity of PAK for Cdc42 [40]. S79 is conserved only in higher eukaryotes and one of three residues ( $_{79}\text{SDF}_{81}$ ) forming a  $\beta$ -bulge that disrupts this interaction [4]. The structure of the Cdc42-PAK complex infers that S79 might interact with the V42 of Cdc42 [4] (Fig. 5E), whose mutation does not significantly affect the interaction of Cdc42 with PAK [39]. Hence, S79 is not essential for the PAK interaction with Cdc42. This may be in line with our finding that S79A mutation has little effect on the PAK1-Cdc42 interaction (Fig. 2A–Fig. 2D). However, this mutation is shown to abolish the binding of PAK1 to Rac1, suggesting that

PAK1 may bind to Cdc42 and Rac1 by different mechanisms and that S79 may play a key role in enabling PAK1 to distinguish Cdc42 and Rac1. This view is reinforced by the previous work that PAKs bind Rac1 with higher affinity than Cdc42. [41]. The crystal structure of the PAK1-Rac1 complex will help to elucidate the role of S79 in the interaction with and activation by Rac1.

## Materials and Methods

### Reagents and Antibodies

Anti-Pak1, Pak1-pT423, Pak1-pS119 and Pak1-pS144 antibodies were from Cell Signaling. Normal mouse *Protein A/G PLUS-Agarose beads*, IgG, anti-actin, anti-c-Myc, and anti-GFP antibodies were from Santa Cruz Biotechnology. Recombinant EGF was from Millipore. Alexa Fluor 568 conjugated-phalloidin was from Invitrogen.

### Cell Culture

The RWPE-1 cells were grown in keratinocyte serum-free medium (K-SFM) containing bovine pituitary extract and epidermal growth factor, as described previously [42]. MEF, PC3, and 293T cells were cultured in RPMI 1640 or DMEM containing 10% FBS and penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. PAK1 wild type and PAK1<sup>-/-</sup> murine embryonic fibroblasts (MEFs) were kindly provided by Dr. Rakesh Kumar [43]. MEFs were isolated from day 13.5 wild-type or Pak1<sup>-/-</sup> embryos. Wild-type and Pak1<sup>-/-</sup> MEFs were immortalized with SV40 T antigen and were maintained in DME supplemented with 12% FBS. RWPE-1, PC3, and 293T cell lines used in this study were obtained from the American Type Culture Collection (ATCC; Rockville, MD).

### Lentiviral Infection

Lentiviral expression vectors for wild-type GFP-PAK1<sup>WT</sup> and mutant GFP-PAK1<sup>S79A</sup> were constructed by subcloning corresponding cDNAs into pLV-puro lentiviral vector, as described previously [42]. For viral production, 293T cells were cotransfected with pLV-GFP, pLV-GFP-PAK1<sup>WT</sup>, or pLV-GFP-PAK1<sup>S79A</sup>, and packaging plasmids (psPAX2 and pCMV-VSV-G) using CalPhos Mammalian Transfection Kit (Clontech).

### Co-immunoprecipitation (IP) and Western Blot Analysis

Cells expressing Pak1, Cdc42, or Rac1 were lysed in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X100) containing phosphatase inhibitors (10 mM Na-pyrophosphate, 200 μM Na-orthovanadate, 50 mM Na-fluoride). The cell lysates were incubated with appropriate antibodies overnight at 4°C. The bound proteins were eluted by

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boiling the beads in sodium dodecyl sulfate (SDS) sample buffer for 5 min and were resolved in SDS-polyacrylamide gels. Western blot analysis was performed, as described previously [42].

### Cell Invasion and Migration Assays

Cell invasion assay was performed using the cell invasion kit (Transwell Boyden's chamber with Transwell® Permeable Support Inserts Coated with Cultrex® BME (basement membrane extract) Corning Costar) according to the manufacture's instruction. For the cell migration assay, confluent RWPE-1 or MEF cells were scratched with a P-200 pipette tip to cause wounding and subjected to the wound healing assay as described previously [42].

### MTT Cell Proliferation Assays

For MTT cell proliferation assay, PC3 cells were cultured in 96-well microplate. Cell growth was evaluated by replacing the culture media with 200 μl of 0.5 mg/ml MTT-media solution after incubation for 1–4 days. The absorbance was determined at 595 nm using a microplate reader (Bio-Rad Laboratories, iMark).

In all experiments, statistical significance was defined by P-values: \**P* < 0.05, \*\**p* < 0.005, \*\*\**p* < 0.001 (as compared with control).

## Supporting Information

**Figure S1 PAK1 S79A mutation impairs the ability of Pak1 to change cell morphology.** PAK1<sup>-/-</sup> MEF cells expressing GFP-PAK1<sup>WT</sup> (WT) and GFP-PAK1<sup>S79A</sup> (S79A) were stained with Phalloidin (red) and DAPI (blue) or visualized by GFP fluorescence (green).

(TIF)

**Figure S2** MEF cells were stained with the high affinity F-actin probe Phalloidin (red) after treatment with TAT (a–f) or TAT-PAK1 peptide (g–l) [43].

(TIF)

## Author Contributions

Conceived and designed the experiments: YJS JHK. Performed the experiments: YJS EK AR. Analyzed the data: YJS JHK. Contributed reagents/materials/analysis tools: YJS JHK. Wrote the paper: YJS JHK.

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