A PAK5–DNPEP–USP4 axis dictates breast cancer growth and metastasis

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Although clinically associated with the progression of multiple cancers, the biological function of p21-activated kinase 5 (PAK5) in breast cancer remains largely unknown. Here, we reveal that the PAK5–aspartyl aminopeptidase (DNPEP)–ubiquitin-specific protease 4 (USP4) axis is involved in breast cancer progression. We show that PAK5 interacts with and phosphorylates DNPEP at serine 119. Functionally, we demonstrate that DNPEP overexpression suppresses breast cancer cell proliferation and invasion and restricts breast cancer growth and metastasis in mice. Furthermore, we identify USP4 as a downstream target of the PAK5–DNPEP pathway; DNPEP mediates USP4 downregulation. Importantly, we verify that DNPEP expression is frequently downregulated in breast cancer tissues and is negatively correlated with PAK5 and USP4 expression. PAK5 decreases DNPEP abundance via the ubiquitin–proteasome pathway. Consistently, analyses of clinical breast cancer specimens revealed significantly increased PAK5 and USP4 levels and an association between higher PAK5 and USP4 expression and worse breast cancer patient survival. These findings suggest a pivotal role for PAK5-elicited signaling in breast cancer progression.

Introduction

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death among females worldwide.1,2 There have been great advances in controlling breast cancer in recent years. However, metastasis, as the leading cause of breast cancer-related death, still remains poorly solved. Hence, exploration of crucial players that involved in breast cancer metastasis has received much interest among global researchers.

The p21-activated kinases (PAKs) are a class of conserved serine/threonine protein kinases that are considered modulators of cell cycle progression, gene transcription, cell motility and cell transformation. Six mammalian PAKs have been identified, and they can be categorized into two subgroups, Group I (PAK1–3) and Group II (PAK4–6), based on sequence and structure.3,4 Studies indicate that p21-activated kinase 5 (PAK5), the most recently identified member of the PAK family, plays an...
What’s new?
Increasing lines of evidence suggest that PAK5 is an oncogenic protein that is commonly overexpressed in many cancer tissues and plays multiple functions in cancer progression. However, the underlying molecular mechanisms and the biological significance of PAK5 in breast cancer remain to be fully explored. Here, the authors reveal that DNPEP links the upstream PAK5 kinase to the downstream target USP4 through a phosphorylation-based regulatory mechanism in malignant breast cancer. Moreover, they identify an association between elements of the PAK5–DNPEP–USP4 pathway and clinicopathological features in breast cancer patients, altogether providing potential prognostic markers and therapeutic targets for breast cancer intervention.

Molecular Cancer Biology

indispensable role in regulation of the cytoskeleton, microtubule stability and cell survival. Increasing lines of evidence suggest that PAK5 is an oncogenic protein that is commonly overexpressed in many cancer tissues and contributes to the progression of diverse cancers. Likewise, in human breast cancer, PAK5 has been shown to activate a PAK5–early growth response protein 1 (Egr1)–matrix metalloproteinase 2 (MMP2) pathway to control cell migration and invasion. PAK5-directed GATA1 phosphorylation has been reported to induce epithelial–mesenchymal transition in breast cancer cells. In addition, PAK5 facilitates breast cancer cell proliferation by phosphorylating NF-κB-p65. Although studies have suggested that PAK5 is a key regulator of breast cancer progression, the underlying molecular mechanisms remain incompletely characterized.

Aspartyl aminopeptidase (DNPEP) is an ~55 kDa metalloenzyme belonging to the M18 aminopeptidase family and exerts zinc-dependent enzymatic function. In most vertebrates, DNPEP is the only encoded M18 family peptidase, indicating its high degree of evolutionary conservation and indispensable biological functions.Conventionally, DNPEP is considered an essential regulator of hydroelectrolytic balance and blood pressure, owing to its preference for hydrolyzing an N-terminal aspartyl tail over glutamyl residues in peptides such as angiotensin II. There is now accumulating evidence of DNPEP involvement in various neoplastic disorders. Beyond these findings, little is known about whether and how DNPEP is related to breast cancer progression.

In the present study, we found that PAK5 phosphorylates DNPEP, which modulates the expression of ubiquitin-specific protease 4 (USP4) in breast cancer. Importantly, we investigated the biological function and clinical importance of the PAK5–DNPEP–USP4 pathway described here in breast cancer progression. Furthermore, we suggest a promising antimetastatic strategy for breast cancer treatment.

Materials and Methods

Cell lines and cell culture
The MCF7 (RRID: CVCL_0045) breast cancer cell line was a kind gift from Dr Minjie Wei, and was maintained in modified Eagle’s medium (MEM) with 10% FBS (Gibco), 10 μg/ml insulin (Sigma, St. Louis, MO), 100 μg/ml streptomycin, 100 U/ml penicillin and 1% l-glutamine (Invitrogen, Carlsbad, CA). HEK293 (RRID: CVCL_0045) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA), The MDA-MB-231(RRID: CVCL_0062) cell line was a gift from Dr Yujie Sun, and MDA-MB-231-luc (RRID:CVCL_JG53, Scienelight, Shanghai) breast cancer cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS (Gibco, Waltham, MA), 100 U/ml penicillin–streptomycin and 1% l-glutamine (Invitrogen, Carlsbad, CA). All cell lines described here were mycoplasma-free and have been authenticated using STR profiling within the last 3 years.

Plasmid construction
MyC-PAK5, Flag-PAK5, GFP-PAK5 (WT, KM), GST-PAK5 and Myc-ubiquitin constructs were previously generated in our laboratory. The His-USP4 construct was purchased from Public Protein/Plasmid Library (Nanjing, China). Flag-DNPEP (WT and S119A mutant), His-DNPEP (WT) and GST-DNPEP (WT and S119A mutant) constructs were generated using PCR amplification and subcloned into p3XFLAG-CMV (Sigma, St. Louis, MO) and pGEX-4T-2 (GE Healthcare, Chicago, IL) vectors. A QuikChange XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) was used to generate mutations in DNPEP. The indicated cells were transfected with the appropriate plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Lentiviral transduction and generation of stable cell lines
Lentiviruses harboring PAK5, DNPEP WT and DNPEP SA were purchased from GeneChem Company (Shanghai, China). To obtain stable cell lines, cells were infected with lentiviral supernatants for 24 hr and then selected with 1 μg/ml puromycin (Sigma, St. Louis, MO) for 48 hr. The infected cells were passaged before use after identification by Western blotting.

Immunoprecipitation, immunoblotting, GST pull-down assays and antibodies
Cells were lysed with 800 μl of lysis buffer containing protease and phosphatase inhibitors (Sigma) for 30 min at 4°C and centrifuged at 12,000g and 4°C for 20 min. Total protein in whole-cell extracts was measured using the Bradford method and equal amounts of lysate (2 mg) were used for immunoprecipitation (IP) with the indicated antibodies and protein A-Sepharose (GE Healthcare) for
DNPEP (L-DOPS) was purchased from Sigma. A selective hydrolase activity inhibitor for tau (see ref 18). Immunizing rabbits with the corresponding phosphopeptide (Bioss Inc., Woburn, MA); MG-132 and cycloheximide (CHX) were purchased from Merck KGaA (Darmstadt, Germany). PAK5 and Myc (Santa Cruz Bio-technology, Dallas, TX); GFP and His (GenScript, Nanjing, China); Flag M2 (Sigma, St Louis, MO); and GAPDH (loading control; Sigma, St. Louis, MO). A phospho-specific antibody recognizing Ser119-phosphorylated DNPEP (p-DNPEP) was generated by immunizing rabbits with the corresponding phosphopeptide (Bioss Inc., Woburn, MA); MG-132 and cycloheximide (CHX) were purchased from Sigma. A selective hydrolyase activity inhibitor for DNPEP (L-DOPS) was purchased from Sigma.

**PAK5 kinase assay**

GST-fusion proteins were purified in vitro and washed three times with kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl₂, 2 mM MnCl₂ and 0.2 mM DTT). PAK5 protein was purchased from Life Technologies, Carlsbad, CA. The PAK5 kinase domain was synthesized in cells and immunoprecipitated for use in the kinase assay; the reaction also contained 50 µl of kinase buffer, 10 µCi of [γ-³²P] ATP (5,000 Ci/mmol) and 2.5 µM cold ATP and was allowed to proceed for 30 min at 30°C. Reactions were stopped by addition of 6× SDS loading buffer. After all proteins were separated on 10% SDS-PAGE gels and transferred onto PVDF membranes, ³²P-labeled proteins were visualized via autoradiography with a Molecular Imager XR system (BIO-RAD, Hercules, CA). Myelin basic protein (MBP) was used as a positive control. The GST-fusion proteins were stained with Ponceau S.

**Migration and invasion assays**

Transwell migration and Matrigel invasion assays were performed in 24-well polyethylene terephthalate inserts (Falcon, 8.0-µm pore size). Stable MDA-MB-231 cells were seeded in each well and incubated in serum-free DMEM overnight. Then, 5 × 10⁴ cells were plated in transwell inserts (three replicates per sample). These cells were allowed to migrate toward DMEM containing 10% FBS (bottom chamber) for 16 or 32 hr at 37°C. Cells in the upper transwell chambers were removed with a cotton swab, and the migrated cells were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. Random fields were photographed using a 20× objective, and the number of migrated cells was determined. Each experiment was repeated independently at least three times.

**Wound healing assay**

A total of 1 × 10⁶ MDA-MB-231 cells stably transfected with the indicated constructs were seeded in six-well plates. When the cells reached nearly 90% confluence, the cell layer was scratched with a sterile plastic tip, washed three times with phosphate-buffered saline (PBS) and cultured for another 16 or 32 hr in low-serum medium (1% FBS). At different time points, photographs of the plates were obtained using a microscope, and data from multiple independent experiments were summarized.

**Mass spectrometry**

Stable Flag-PAK5-overexpressing MCF7 cells were harvested and lysed with lysis buffer. Then, the cell extracts were immunoprecipitated with an anti-Flag monoclonal antibody conjugated to M2 agarose beads (Sigma). The Flag peptide-eluted material was resolved on a 10% SDS–PAGE gel. The bands were excised from the gel and subjected to tryptic digestion and mass spectrometry (MS) analysis. Proteins were identified through a database search, and the peptide identifications were validated with PeptideProphet.

**Tumor xenograft analysis**

Female nude mice (nu/nu, 4–5 weeks old, Vital River Laboratories, Beijing, China) were injected subcutaneously in the flank region with 3 × 10⁶ stably transfected MDA-MB-231 cells. Tumor volume was measured every 3 days for 35 days and calculated using the formula tumor volume = π/6 × (L × W²). Mice were killed on Day 36, and the tumors were dissected and analyzed. The work described herein was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978).

**Immunohistochemistry**

Paraformaldehyde-fixed, paraffin-embedded tissues were sectioned, and slides were stained with hematoxylin and eosin (H&E) or the Dako Liquid 3,3’-diaminobenzidine tetrahydrochloride (DAB) + Substrate Chromogen System and counterstained with hematoxylin. Immunohistochemical staining of paraformaldehyde-fixed tumors was performed using appropriate primary and secondary antibodies and the ABC Elite immunoperoxidase kit according to the manufacturer’s instructions. The H-score (histologic score) was used to evaluate the staining results.²¹

**In vivo metastasis model**

Female 4- to 5-week-old nude mice were purchased from Vital River Laboratories (Beijing, China). MDA-MB-231-luc
cells stably expressing the indicated luciferase constructs (2 × 10^6 cells/mouse) were injected into the lateral tail vein of the mice. The animals were imaged weekly for 35 days using an IVIS Lumina II Imaging System (Xenogen). For bioluminescence in vivo imaging (BLI), mice first received an intraperitoneal injection of substrate and then anesthetized with...
isoflurane 5 min later; whole-body images were then acquired. After the final imaging session, the mice were sacrificed, and the organs (lungs, liver, kidneys and intestines) were examined for metastases. Tumor tissues were then fixed, embedded in paraffin and serially sectioned at a thickness of 4 μm. Finally, we performed H&E staining to confirm the presence of a tumor. The work described herein was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978).

Ubiquitination assay
MCF7 cells were transfected with Myc-ubiquitin constructs encoded in the indicated plasmids and treated with MG132. Forty-eight hours after transfection, the cells were harvested and sonicated in ubiquitination-lysis buffer with 250 ng/ml ubiquitin-aldehyde. Then, Western blot analysis was performed to evaluate protein degradation.

Patient tissues and specimens
Formalin-fixed, paraffin-embedded sections of paired tissue specimens and matched adjacent (5 cm distance) normal tissues collected between 2008 and 2009 were obtained from the Department of Breast Surgery in the First Affiliated Hospital of China Medical University. All of the tissues were collected at the time of surgical resection and immediately stored in liquid nitrogen until protein extraction for Western blotting. Patient clinical characteristics were collected and recorded. The work described herein was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Chip arrays
Purified DNPEP protein was purchased from Origene Corp and used for chip array screening performed by Wayen Biotechnologies Inc. (Shanghai, China).

Colonies formation assay
Cells were seeded and cultured in 6-cm dishes until visible cell colonies formed. Then, the cells were fixed and stained, and the number of colonies was determined under a microscope (Leica, Buffalo Grove, IL).

Statistical analysis
We used GraphPad Prism software for statistical analyses. Student’s t-test (two-tailed) was applied to compare differences between two groups in repeated studies. The significance of differences in specimen data was determined with a chi-square test. *p < 0.05 was considered statistically significant, and **p < 0.01 and ***p < 0.001 were considered highly significant. The Kaplan–Meier method was used to generate survival curves.

Data availability
All data supporting the findings of our study are available within the article and are available from the corresponding author on reasonable request.

Results
DNPEP is phosphorylated by PAK5 at S119
To gain mechanistic insight into the function of PAK5 in breast cancer, we initially employed IP experiments combined with MS-based proteomic analysis to identify potential PAK5-interacting proteins in the MCF7 cell line. Silver staining showed the comparative protein expression in MCF7 cells stably expressing Flag or Flag-PAK5 (Fig. 1a). MS analyses identified proteins that were specifically associated with PAK5 (Supporting Information Fig. S1A). Among the previously unidentified interactors, one protein immediately caught our attention, aspartyl aminopeptidase (GI: 156416028), also known as DNPEP, which has been identified as the sole mammalian member of the M18 metalloprotease family. Little is known about DNPEP involvement in breast cancer progression. Thus, we focused on DNPEP as a promising, previously unidentified PAK5-interacting protein. To this end, we performed a coimmunoprecipitation (coIP) assay to validate the PAK5–DNPEP interaction. HEK293 cells were transiently transfected with Myc-PAK5 and Flag-DNPEP, and IP with an anti-myc antibody was performed; as shown in Figure 1b, PAK5 coimmunoprecipitated with DNPEP. Similarly, the reciprocal IP with endogenous DNPEP antibody brought down PAK5 (Fig. 1c). Furthermore, a GST pull-down assay showed that PAK5 and DNPEP directly interacted in vitro (Figs. 1d and 1e). Given that PAK5 is a well-known serine/threonine protein kinase, we next sought to determine whether PAK5 phosphorylates DNPEP. As shown in Figure 1f, in vitro kinase assays confirmed that DNPEP can be phosphorylated by PAK5. Four serine residues with potential for phosphorylation by PAK5 were selected and individually mutated to alanine and the mutants were subjected to PAK5 kinase assays (Supporting Information Fig. S1B). These experiments revealed that mutation of S119 alone (lane 5), but not other sites (lanes 4, 6 and 7), resulted in dramatic elimination of DNPEP phosphorylation (Fig. 1g). Next, DNPEP phosphorylated at S119 (p-DNPEP) was detected in breast cancer cells (Fig. 1h). In addition, abrogation of PAK5 kinase activity significantly reduced DNPEP phosphorylation at S119 (Fig. 1i). Taken together, these data indicate that PAK5 binds to DNPEP and phosphorylates it at S119.

Phosphorylation of DNPEP at S119 inhibits breast cancer cell proliferation and invasion
To determine the biological function of DNPEP phosphorylation, we evaluated various features of cancer cells. Colony formation assays showed that cells stably expressing DNPEP WT formed significantly fewer colonies than control cells, whereas expression of the phosphorylation-disabled mutant (DNPEP SA) had little effect compared to the control (Fig. 2a).
Furthermore, cell migration experiments showed markedly suppressed migration by cells expressing DNPEP WT but not DNPEP SA (Fig. 2b). Transwell assays supported the antimigration and antiinvasion roles of DNPEP (Figs. 2c and 2d). Moreover, knockdown of DNPEP appeared to exert the opposite effects (Supporting Information Figs. S2A–S2D).

Figure 2. Legend on next page.
These results suggest that DNPEP phosphorylation is required for limiting the growth and metastasis of breast cancer cells.

**Phosphorylation of DNPEP at S119 suppresses breast cancer growth and metastasis in vivo**

Next, in vivo evidence supporting the connection between DNPEP phosphorylation and breast cancer progression was obtained from tumor xenograft studies. Cells expressing DNPEP WT grew xenografts at a significantly slower rate than those in the DNPEP SA and vector groups (Figs. 2e and 2f). Consistently, histopathologic analyses revealed significantly less Ki-67 staining in DNPEP WT xenografts, while DNPEP SA xenografts displayed strong Ki-67 staining (Fig. 2g). Moreover, MDA-MB-231-luc cells stably expressing control, DNPEP WT or DNPEP SA were injected into the tail vein of nude mice to determine the impact of DNPEP on tumor metastasis. As shown in Figure 2h, mice injected with DNPEP WT cells exhibited markedly less lung metastasis than the other two groups. We thus examined tumor metastasis to the lungs at the study endpoint via fluorescence imaging and observed consistent results (Fig. 2i). Additionally, H&E staining of metastatic lung sections further confirmed our findings (Fig. 2j). Collectively, these results demonstrate that phosphorylation of DNPEP suppresses breast tumor growth and metastasis in vivo.

**USP4 is downstream of the PAK5–DNPEP axis**

To further define the tumor-suppressive function of DNPEP, we screened the DNPEP interactome using protein chip assays (Fig. 3a). Based on previous studies and in silico analysis, five proteins were selected for further study. We next sought to determine the potential link between DNPEP and these candidates. As shown in Figure 3b, the addition of Flag-DNPEP dose-dependently downregulated USP4, an enzyme that specifically cleaves ubiquitin, but not the other selected proteins. A further analysis showed that the hydrolysis pathway contributes to DNPEP-mediated degradation of USP4 (Supporting Information Figs. S3A–S3F). The interaction between USP4 and DNPEP was confirmed by coIP assays (Figs. 3c and 3d). Moreover, we examined whether USP4 downregulation requires PAK5-mediated DNPEP phosphorylation. As expected, the results indicated that DNPEP-driven USP4 downregulation is dependent on DNPEP phosphorylation (Fig. 3e). Overall, these results suggest that USP4 is a downstream component of the PAK5–DNPEP axis.

**PAK5 promotes DNPEP degradation**

To investigate the significance of the PAK5–DNPEP–USP4 axis outlined above, we first compared the expression levels of PAK5, DNPEP and USP4 in fresh frozen clinical breast cancer tissues and paired adjacent nonneoplastic tissues from 103 patients with breast cancer. Remarkably, we found that breast cancer tissues had apparently higher PAK5 and USP4 expression but markedly lower DNPEP expression than paired adjacent nonneoplastic tissues (Figs. 4a and 4b). Supporting Information Table S1 shows the association of PAK5 with DNPEP and USP4. Supporting Information Table S2 shows a correlation between DNPEP and USP4. These findings prompted us to determine whether PAK5 directly modulates DNPEP abundance. Accordingly, using cells transiently expressing different levels of recombinant human Flag-PAK5, we verified that DNPEP levels were downregulated and that USP4 levels were upregulated in a dose-dependent manner in response to ectopic PAK5 expression, while PAK5 knockdown led to opposing effects (Figs. 4c and 4d). Because USP4 is a deubiquitylating enzyme, we then sought to investigate its role in PAK5 stability. The data suggested that USP4 is not a deubiquitinating enzyme (DUB) required for PAK5 stability (Supporting Information Fig. S4A). Furthermore, cells transiently overexpressing PAK5 WT or PAK5 KM were treated with CHX, a protein synthesis inhibitor, for various lengths of time; in these cells, DNPEP expression appeared to decrease over time upon ectopic PAK5 WT, but not ectopic PAK5 KM expression (Fig. 4e). Remarkably, we found that DNPEP WT but not DNPEP SA levels were reduced over time in response to forced PAK5

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**Figure 2.** DNPEP phosphorylation suppresses breast cancer cell growth and metastasis. (a) DNPEP phosphorylation inhibits colony formation. Colonies of cells stably expressing control, DNPEP WT or DNPEP S119A were monitored. Representative pictures of the colonies are shown. The data are presented as a histogram of the mean ± SEM of three independent experiments (Student’s t-test). (b–d) DNPEP phosphorylation restricts cell migration and invasion. MDA-MB-231 cells transfected with the same constructs as in (a) were subjected to wound healing and transwell assays. Cells were photographed at the indicated times, and the data are presented as the mean ± SEM of three independent experiments. (e) p-DNPEP inhibits xenograft tumor growth. MDA-MB-231 cells (6 × 10⁶ cells) stably expressing vector control, DNPEP WT or DNPEP S119A were injected subcutaneously into the right flank of nude mice. Mice were imaged at 35 days after injection. Tumor diameter was measured at the indicated time points, and tumor volume was calculated. The results are presented as the mean ± SEM of 6 mice per group per time point. (f) Tumors formed by cells described in (a) were extracted from mice and photographed. Tumor weight in mice from this experiment was measured upon autopsy at Day 35, and the results are presented as a histogram. (g) p-DNPEP S119 suppresses breast tumor cell proliferation in vivo. Tumor sections from xenografts were prepared for immunohistochemistry analysis. Representative images of H&E and Ki-67 staining of the indicated MDA-MB-231 xenograft tumor genotypes. Scale bar, 300 μm. (h) p-DNPEP S119 suppresses breast tumor metastasis in vivo. In vivo fluorescence images of the breast tumor metastasis nude mouse model (three groups) after tail vein injection of stable MDA-MB-231-luc breast cancer cells at the indicated time points (n = 7 mice/group). The colored region represents the GFP signal of MDA-MB-231-luc breast cancer cells in nude mice. The right picture shows the signal intensity scale. Quantitation of GFP emitted from primary tumors in the mice is presented. The histogram shows the lung metastasis ratio in the three groups. (i) After the final imaging session, the mice were sacrificed, and the indicated organs were excised. Bioluminescence images of select mouse organs on day 35 after inoculation. Abbreviations: LU, lung; LI, liver; IN, intestine; KI, kidney. (j) H&E staining shows lung metastases in tumor sections from the animals described in (i). *p < 0.05, **p < 0.01, ***p < 0.001. See also Supporting Information Figure S2. [Color figure can be viewed at wileyonlinelibrary.com]
Figure 3. USP4 is downstream of the PAK5–DNPEP axis. (a) DNPEP-associated proteins. A list of DNPEP-associated proteins identified by chip assays is provided. (b) Overexpression of DNPEP downregulates USP4. MCF7 and MDA-MB-231 cells transiently expressing different doses of Flag-DNPEP were analyzed with the indicated antibodies via Western blotting. USP4 levels were quantified in three independent experiments. Error bars represent the mean ± SEM. Asterisks denote significant changes from dose 0 values. *p < 0.05, **p < 0.01, ***p < 0.001. (c) DNPEP interacts with USP4 in cells. MCF7 cells were transfected with Flag-DNPEP, and cell lysates were subjected to IP with anti-Flag antibody. The immunoprecipitates were then blotted with the indicated antibodies. (d) The endogenous interaction between DNPEP and USP4 in MCF7 cells was examined via anti-DNPEP IP, and the immunoprecipitates were analyzed by Western blotting with the indicated antibodies. (e) DNPEP phosphorylation at S119 downregulates USP4. MCF7 cells transfected with the indicated constructs were lysed, and the cell lysates were blotted with the indicated antibodies. The data are shown as the mean ± SEM of triplicate experiments (*p < 0.05, **p < 0.01). See also Supporting Information Figure S3. [Color figure can be viewed at wileyonlinelibrary.com]

Int. J. Cancer: 146, 1139–1151 (2020) © 2019 The Authors. International Journal of Cancer published by John Wiley & Sons Ltd on behalf of UICC

| Names & ID     | Enlarged View | Names & ID     | Enlarged View |
|----------------|---------------|----------------|---------------|
| USP4           |               | MCM7           |               |
| NM_003363.2    |               | NM_005916.3    |               |
| TMOD1          |               | GAMT           |               |
| NM_003275.1    |               | NM_000156.4    |               |
| DIABLO         |               |                |               |
| NM_138930.2    |               |                |               |
Figure 4. Legend on next page.
expression (Supporting Information Figs. S4B and S4C). These results suggest that DNPEP phosphorylation at S119 is required for PAK5-mediated degradation. To this end, we exposed PAK5-overexpressing cells to MG132, a well-established inhibitor of the ubiquitin-proteasome pathway, for the indicated times. Consistent with our assumptions, DNPEP expression obviously accumulated (Fig. 4f). Moreover, DNPEP WT but not DNPEP SA, showed marked ubiquitin-linked degradation in the presence of MG132 (Fig. 4g). These data show that PAK5-mediated DNPEP phosphorylation facilitates its ubiquitination.

High PAK5 and USP4 expression contributes to breast cancer metastasis

We next sought to obtain further support for our findings in human breast cancer. Histopathologic analyses of 293 breast cancer specimens revealed that higher PAK5 and USP4 expression was significantly associated with distant tumor metastasis (Figs. 5a–5c, Supporting Information Tables S3 and S4). In support of our discoveries, Kaplan–Meier survival analysis based on PAK5 and USP4 expression showed that high PAK5 and USP4 levels were associated with poor overall survival (Figs. 5d and 5e, upper) and poor disease-free survival (Figs. 5d and 5e, lower) in breast cancer patients. Stratification of patients based on PAK5 and USP4 levels further improved the predictive capability (Fig. 5f). Taken together, these data stand the conclusions reached with the functions of PAK5, USP4 and DNPEP described in the current study and in previous reports and therefore provide strong support for our conclusions.

Discussion

In the present work, we report that DNPEP links the upstream PAK5 kinase to the downstream target USP4 through a phosphorylation-based regulatory mechanism in malignant breast cancer (Fig. 6). Importantly, we identified an association between related factors and clinicopathologic features in patients with breast cancer. Taken together, these findings help us better understand the molecular mechanism underlying PAK5-promoted malignant breast cancer progression.

Aminopeptidases catalyze the cleavage of N-terminal residues from peptide or protein substrates. Previous studies have shown that these enzymes play an important role in various physiologic and pathophysiologic processes involved in peptide signaling and have suggested the involvement of aminopeptidases in the progression of different cancer types, including non-small cell lung cancer, pancreatic carcinoma, hepatocellular carcinoma (HCC), colon cancer and breast cancer. Unlike other aminopeptidases, DNPEP is the only evolutionarily conserved mammalian member of the M18 family; DNPEP is expressed widely in human tissues and body fluids and is an indispensable modulator of biological processes. A previous report indicated that DNPEP is overexpressed in colorectal cancer and that its activity in tissue and plasma may be a promising prognostic marker in colorectal cancer, suggesting translational potential for DNPEP in solid tumors. To date, the clinical impact of DNPEP on breast cancer progression has yet to be defined. Thus, future investigations related to our current work are needed to delineate the underlying connection.

We identified DNPEP as a downstream substrate of PAK5, a protein kinase that primarily functions via kinase-dependent mechanisms. In this report, we demonstrated that DNPEP can be phosphorylated by PAK5 at S119 (Fig. 1g). To the best of our knowledge, this is the first report showing DNPEP phosphorylation; furthermore, we identified ubiquitination as another post-translational modification of DNPEP, which is mediated by PAK5-dependent phosphorylation. Notably, we failed to discover the E3 ligase engaged in this process; it is possible that PAK5-directed DNPEP ubiquitination is E3 ligase independent. Further studies are required to determine the detailed mechanisms, including to identify the precise ubiquitinated residues and uncover the mechanism of crosstalk between the two modifications. Nevertheless, the results of our study suggest a strong regulatory relationship between PAK5 and DNPEP.

USP4 was the first member of the USP subfamily to be identified in mammalian cells. Since its discovery, USP4 has been well characterized as a tumor promotor in multiple cancer types. For example, USP4 suppresses stress-induced cell apoptosis and...
facilitates tumor metastasis in melanoma.\textsuperscript{26} USP4 promotes the suppressive function of Treg cells by interacting with and stabilizing interferon regulatory factor 8 (IRF8).\textsuperscript{27} Furthermore, USP4 targets transforming growth factor-β-activated kinase 1 (TAK1) to regulate tumor necrosis factor-α (TNFα)-induced NF-κB activation.\textsuperscript{28} In HCC, USP4 facilitates tumor development via deubiquitylation and cyclophilin A stabilization.\textsuperscript{29} Studies also indicate that USP4 targets histone deacetylase 2 (HDAC2) to downregulate TNFα-induced NF-κB activation and suppresses p53.\textsuperscript{30} Consistently, in breast cancer, has been shown to be dispensable for AKT-induced breast cancer cell migration by linking AKT signaling to transforming growth factor-beta (TGF-β) signaling.\textsuperscript{31} Another study indicated that USP4 promotes breast cancer cell invasion through the Relaxin/TGF-β1/Smad2/MMP-9 axis.\textsuperscript{32} Furthermore, a TRPS1–USP4–HDAC2 axis that contributes to tumor growth was established.\textsuperscript{33}

Figure 5. High expression of both PAK5 and USP4 contributes to breast cancer metastasis. (a) Representative images of immunohistochemical staining showing PAK5 and USP4 protein expression. Original magnification, 200×. Comparison of PAK5 and USP4 expression levels with basic clinical data for the analyzed cases, as shown in Supporting Information Table S3 (**p < 0.001). (b) IHC staining was scored, and a Pearson correlation test was performed. Note that the scores of some samples overlap. (c) The subjects with breast cancer were divided into two groups based on nonmetastasis (n = 228) and metastasis (n = 65). The intensity values in (d) are expressed as the H-score. The middle, upper, and lower lines in the boxplot represent the mean and the upper and lower quartiles, respectively, of relative PAK5 (upper) and USP4 (lower) staining across all samples (**p < 0.001). A summary of PAK5 expression in the metastatic and nonmetastatic tissues in (a) is shown, and overall comparisons were determined, as shown in Supporting Information Table S4 (**p < 0.001). (d–f) Kaplan–Meier survival analysis (GraphPad) of the relationship between overall survival (upper) and disease-free survival (lower) in breast cancer cases and PAK5 and/or USP4 expression. The subjects were divided into different groups based on the indicated PAK5 and USP4 expression scores in the tumors: PAK5 low (n = 199) and PAK5 high (n = 94); USP4 low (n = 124) and USP4 high (n = 169); n = 108 for PAK5 low/USP4 low, n = 91 for PAK5 low/USP4 high, n = 16 for PAK5 high/USP4 low, n = 78 for PAK5 high/USP4 high (f). [Color figure can be viewed at wileyonlinelibrary.com]
In summary, our findings provide further evidence of the importance of PAK5 in dictating breast cancer malignancy, thereby supporting investigation of components of the PAK5 signaling pathway as targets for breast cancer intervention.

Acknowledgements

We would like to thank Yejun Wang (Shenzhen University, Health Science Center) and Haitao Li (Tsinghua University, Tsinghua-Peking Joint Center for Life Sciences) for technical support. This research was supported by Grants from the National Natural Science Foundation of China (Nos. 31371424, 31571457, 31771553, 81602564, 31000627, 81773163) and Ministry of Education PRC (IRT13101) and Basic Research Project of Education Department of Liaoning Province No. ZZ2013071.

Ethics approval and consent to participate

All animal experiments complied with ethical regulations and were approved by the Subcommittee on Research Animal Care of China Medical University. For patient’s tissues and specimens, all individuals provided written informed consent, and the study was approved by the Multicenter Ethics Committee.

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