p70S6K1 (S6K1)-Mediated Phosphorylation Regulates Phosphatidylinositol 4-Phosphate 5-Kinase Type Iγ Degradation and Cell Invasion

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Phosphatidylinositol 4-phosphate 5-kinase type I γ (PIPKIγ90) ubiquitination and subsequent degradation regulate focal adhesion assembly, cell migration, and invasion. However, it is unknown how upstream signals control PIPKIγ90 ubiquitination or degradation. Here we show that p70S6K1 (S6K1), a downstream target of mechanistic target of rapamycin (mTOR), phosphorylates PIPKIγ90 at Thr-553 and Ser-555 and that S6K1-mediated PIPKIγ90 phosphorylation is essential for cell migration and invasion. Moreover, PIPKIγ90 phosphorylation is required for the development of focal adhesions and invadopodia, key machineries for cell migration and invasion. Surprisingly, substitution of Thr-553 and Ser-555 with Ala promoted PIPKIγ90 ubiquitination but enhanced the stability of PIPKIγ90, and depletion of S6K1 also enhanced the stability of PIPKIγ90, indicating that PIPKIγ90 ubiquitination alone is insufficient for its degradation. These data suggest that S6K1-mediated PIPKIγ90 phosphorylation regulates cell migration and invasion by controlling PIPKIγ90 degradation.

In addition, PIP₃ is a precursor of several lipid second messengers, such as phosphatidylinositol 3,4,5-triphosphate (PIP₃), inositol 1,4,5-triphosphate, and diacylglycerol. We have shown that depletion of PIPKIγ90 completely abolishes PIP₃ production in HCT119 human colon cancer cells (11), indicating a critical role of PIPKIγ90 in lipid signaling. PIPKIγ90 is necessary for epithelial cell adherens junction assembly and progression through the E-cadherin–β-catenin signal pathway (12). PIPKIγ90 depletion inhibits cell proliferation, MMP9 secretion, and cell motility (13, 14).

PIPKIγ90 is essential for cell migration, invasion, and metastasis. It is required for focal adhesion assembly and disassembly, key steps in cell migration (11). Depletion of PIPKIγ90 inhibits growth factor-stimulated cell migration in MDA-MB-231 breast cancer cells and HeLa cervical cancer cells (14, 15). PIPKIγ90 knockdown also blocks the invasion of breast cancer and colon cancer cells (11, 16). Furthermore, PIPKIγ90-depleted 4T1 breast cancer cells show significant reduction in tumor progression and metastasis (13). PIPKIγ90 also regulates neutrophil migration by controlling cell polarity as well as rear retraction (17–19). PIPKIγ90 is a substrate for Src, which phosphorylates PIPKIγ90 at Tyr-644, enhancing its binding to talin and reducing talin-β integrin interaction (20). Talin, in turn, activates integrins and initiates FA assembly to regulate cell migration and invasion. In addition, phosphorylation of PIPKIγ90 at Tyr-639 by epidermal growth factor (EGF) receptor influences tumor cell migration and metastasis (13).

It has been demonstrated that the ubiquitin proteasome pathway regulates FA assembly and disassembly and, consequently, cell migration and invasion through ubiquitinating FA proteins (16, 21–26), and our research indicates that PIPKIγ90 is a key molecule that mediates the role of the ubiquitin proteasome pathway in this regard. Our published data indicate that PIPKIγ90 functions to regulate focal adhesion assembly and disassembly (11). We also demonstrated that PIPKIγ90 ubiquitination at Lys-97 by HECTD1, an E3 ubiquitin ligase that regulates cell migration, results in PIPKIγ90 degradation, thus controlling dynamic PIP₃ production to mediate FA assembly/disassembly, cell migration, invasion, and metastasis (16). However, it is not clear how upstream signaling pathways control PIPKIγ90 ubiquitination or degradation during cell migration and invasion.
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Ribosomal protein S6 kinase β 1 (also called p70S6K or S6K1), a serine-threonine kinase, is one of the mTOR pathway effectors. It is well known that S6K1 regulates cancer cell growth, survival, and metabolism (27–31). Recent evidence indicates that it also regulates cancer cell invasion and metastasis (32, 33), but the molecular mechanisms behind these processes are less defined. In this study, we demonstrate that S6K1 phosphorylates PIPKιγ90 at Thr-553 and Ser-555 and that S6K1-mediated phosphorylation controls PIPKιγ90 degradation to regulate the development of FAs and invadopodia and, consequently, cell migration and invasion.

Results

The residues Thr-553 and Ser-555 of human PIPKιγ90 are consensus sites for Akt and S6K1 (Fig. 1A). To learn whether Akt1 and S6K1 phosphorylate PIPKιγ90, FLAG-PIP1κγ90 was co-transfected with an empty vector, constitutively active Akt1, and S6K1 (Myr-Akt1 and S6K1-F5A) into CHO-K1 cells (Figs. 2A and 3A). Active S6K1 phosphorylated PIPKιγ90 was detected with an anti-RpS/T motif antibody. Data are presented as mean ± S.E. of four independent experiments. *, p < 0.05; **, p < 0.001 versus Wt, Ctrl, EGF, and HGF stimulated PIPKιγ90 phosphorylation. MDA-MB-231 cells stably expressing FLAG-PIP1κγ90 were serum-starved and then stimulated with EGF (20 ng/ml), HGF (50 ng/ml), SCF (20 ng/ml), and PDGF (20 ng/ml) for 20 min. FLAG-PIP1κγ90 was immunoprecipitated (IP), and phosphorylation was detected with an anti-RpXpS/T motif antibody. Data are presented as mean ± S.E. of three independent experiments. *, p < 0.05; **, p < 0.001 versus Wt, Ctrl, EGF, and HGF stimulated PIPKιγ90 phosphorylation.
phorylation was detected with an anti-RXRXXpS/T motif antibody. Both Myr-Akt1 and S6K1-F5A-E389-R3A promoted PIPK1γ90 phosphorylation (Fig. 1B). To examine whether S6K1 phosphorylates PIPK1γ90 at Thr-553 and Ser-555 in vitro, HA-S6K1-F5A-E389-R3A was immunoprecipitated from CHO-K1 cells and incubated with purified recombinant GST-PIPK1γ90T553A/S555A, -PIPK1γ90T553E/S555E, and -PIPK1γ90T553E,S555E in a kinase reaction buffer containing ATP. The phosphorylation of these recombinant proteins was detected as described in Fig. 1B. Mutation at Thr-553 or Ser-555 caused a decrease in PIPK1γ90 phosphorylation, whereas mutation at both Thr-553 and Ser-555 abolished its phosphorylation (Fig. 1C). To determine whether S6K1 phosphophorylates PIPK1γ90 at the same sites in cells, HA-S6K1-F5A-E389-R3A was co-transfected with FLAG-PIPK1γ90, PIPK1γ90T553A/S555A, and PIPK1γ90T553A,S555A into CHO-K1 cells. The phosphorylation of FLAG-PIPK1γ90 and the mutants was determined as described in Fig. 1B. Substitution of Thr-553 with Ala caused a significant reduction in PIPK1γ90 phosphorylation, and substitution of Ser-555 with Ala dramatically inhibited the phosphorylation, whereas substitution of both Thr-553 and Ser-555 completely abolished PIPK1γ90 phosphorylation (Fig. 1D). These data suggest that S6K1 phosphorylates PIPK1γ90 at residues Thr-553 and Ser-555.

To find out whether EGF or HGF stimulates PIPK1γ90 phosphorylation at residues Thr-553 and Ser-555, MDA-MB-231 cells stably expressing FLAG-PIPK1γ90 were serum-starved and stimulated with EGF, HGF, SCF, and PDGF. FLAG-PIPK1γ90 was immunoprecipitated with anti-FLAG-agarose beads, and PIPK1γ90 phosphorylation was detected with an anti-RXRXXpS/T motif antibody. EGF and HGF stimulated PIPK1γ90 phosphorylation, whereas SCF and PDGF did not (Fig. 1E). Similar results were observed in MDA-MB-468 cells (supplemental Fig. S1A). HGF and EGF stimulated Akt and S6K1 activation in a time-dependent manner, whereas SCF and PDGF had no obvious effects (Fig. 1E and supplemental Fig. S1, B and C) Because both S6K1 and Akt were activated by HGF or EGF in MDA-MB-231 cells, we tested whether S6K1 or Akt mediate PIPK1γ90 phosphorylation. MDA-MB-231 cells that stably express FLAG-PIPK1γ90 were treated with Akt inhibitor VIII or the S6K1 inhibitors DG2 and PF4708671 and then challenged with HGF. Akt inhibitor VIII inhibited HGF-stimulated Akt, S6K1, and PIPK1γ90 phosphorylation. The S6K1 inhibitors DG2 and PF4708671 did not influence Akt and S6K1 activation but inhibited S6K1 activity (as indicated by the reduction in ribosomal protein S6 phosphorylation) and PIPK1γ90 phosphorylation (Fig. 1F). To further examine whether S6K1 phosphorylates PIPK1γ90 in cells, MDA-MB-231 cells that stably express FLAG-PIPK1γ90 were infected with lentiviruses expressing codon-modified ZZ-PIPK1γ90, ZZ-PIPK1γ90T553A/S555A, or ZZ-PIPK1γ90T553E,S555E were measured. Re-expression of PIPK1γ90WT in PIPK1γ90-depleted cells restored cell invasion to an extent comparable with the invasion of cells expressing empty pLKO.1 vector, and that of PIPK1γ90T553E, S555E partially rescued cell invasion. In contrast, re-expression of PIPK1γ90T553A, S555A only slightly enhanced cell invasion (Fig. 3, A and B). Similar results were observed when PIPK1γ90 and the mutants were expressed in parental MDA-MB-231 cells (supplemental Fig. S2), suggesting a dominant negative function of PIPK1γ90T553A,S555A. To explore the role of S6K1 in cell invasion, we examined the effect of the S6K1 inhibitor DG2 on the invasion of MDA-MB-231 cells. We found that S6K1 inhibition impaired invasion of the cells (Fig. 3C). In particular, 10 μM S6K1 inhibitor DG2 significantly decreased the invasive potential of the cells by ~90% (in the absence of HGF) and 80% (in the presence of HGF). To further examine the requirement for S6K1 in cell invasion, this kinase was depleted in MDA-MB-231 cells using S6K1 shRNA (Fig. 3D). Cells transfected with S6K1 shRNA could not invade efficiently compared with cells expressing shRNA control (Fig. 3E). S6K1-depleted cells, even in the presence of HGF, could not invade normally compared with cells expressing shRNA control. Akt1, another protein kinase that potentially phosphorylates PIPK1γ90, was also depleted in MDA-MB-231 cells by using two different shRNAs. Depletion of Akt1 caused a slight reduction in the phosphorylation of S6K1 and S6 ribosomal protein (Fig. 3F). Depletion of Akt1 in MDA-MB-231 cells did not exhibit a significant reduction in invasive ability. As shown
in Fig. 3G, depletion of Akt1 slightly reduced HGF-induced invasion of MDA-MB-231 cells. However, in the absence of HGF, cells expressing Akt1 shRNAs had higher number of invaded cells compared with cells with shRNA control, implying that Akt1 is not mandatory for the invasion of MDA-MB-231 cells. To further examine the role of S6K1-mediated PIPK1γ90 phosphorylation in cell invasion, the effects of the S6K1 inhibitor DG2 on the invasion of PIPK1γ90-depleted cells that express ZZ-PIPK1γ90, -PIPK1γ90T553A,S555A, or -PIPK1γ90T553E,S555E were examined. DG2 significantly inhibited the invasion of cells expressing PIPK1γ90 but had only marginal effects on the invasion of cells expressing PIPK1γ90T553A,S555A or -PIPK1γ90T553E,S555E (Fig. 3H). These results indicate that S6K1-mediated PIPK1γ90 phosphorylation regulates cell invasion.

Because of the crucial role of matrix metalloproteinase-mediated matrix degradation in cell invasion (36–38), we set out to determine whether the S6K1-PIPK1γ90 pathway regulates
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matrix degradation. To examine whether the phosphorylation-deficient mutants of PIPKIγ90 influence matrix degradation, we examined the gelatin degradation activity of PIPKIγ90-depleted MDA-MB-231 cells that were rescued with PIPKIγ90$^{WT}$, PIPKIγ90$^{T553A,S555A}$, and PIPKIγ90$^{T553E,S555E}$. Glass-bottom dishes were coated with Alexa 488-conjugated gelatin. The coated dishes were then dried, fixed with glutaraldehyde, and reduced with sodium borohydride. The cells were plated on dishes and treated with HGF. The cells were fixed and stained with cortactin, an invadopodium marker. Matrix degradation was examined by TIRF microscopy. Cells expressing PIPKIγ90$^{WT}$ had similar matrix degradation activity compared with cells expressing shRNA control. However, cells with PIPKIγ90$^{T553A,S555A}$ showed a slight reduction in degraded areas (Fig. 4C, A and B). To further corroborate these findings, we tested the effect of S6K1 inhibition on matrix degradation. Similar to invasion, S6K1 inhibition affected this function and considerably decreased the gelatin degradation (Fig. 4C). These data suggest that...
S6K1-mediated PIPKι90 phosphorylation regulates matrix degradation.

To examine the possible association of the S6K1 pathway with cancer metastasis, human breast cancer tissue array slides, including primary tumors and the matched metastatic tumors of lymph node tissues (US Biomax), were stained for phospho-S6 ribosomal protein (Ser(P)-235/236), a substrate of S6K1. Among the tissues from 50 subjects analyzed, phospho-S6 staining was positive in 20 cases of metastatic tumors (40%) and in six cases of the matched primary tumors (12%) (Fig. 5, A and B). Also, phospho-S6 staining in 15 cases of metastatic tumors (30%) was significantly higher than the staining in the matched primary tumors; one case was lower (2%), and 34 cases were unchanged (68%). These data suggest that activation of the S6K1 pathway positively correlates with human breast cancer metastasis (p < 0.001).

To measure the kinase activity of PIPKIγ90, ZZ-PIPKIγ90 was transfected into CHO-K1 cells and immunoprecipitated with ZZ-PIPKIγ90K188,200R, a kinase-deficient mutant, as a negative control. The activities of PIPKIγ90 and mutants were measured by PIP2 production using PIP and [γ-32P]ATP as substrates. PI(4,5)P2 was separated by thin layer chromatography, imaged by autoradiography, and quantified by liquid scintillation counting. The kinase activity was detected in IgG-agarose beads that were incubated with ZZ-PIPKIγ90-transfected lysates but not in protein A-agarose beads incubated with the

![Image](image-url)
same lysate; very low activity was observed in IgG-agarose beads that were incubated with ZZ-PIPKI90, ZZ-PIPKI90 WT, -PIPKI90 T553A, S555A, and -PIPKI90 T553E, S555E were transfected into CHO-K1 cells and immunoprecipitated with IgG-agarose beads. The activities of PIPKI90 and mutants were measured using the same method. Substitution of Thr-553 and Ser-555 with alanine and glutamate did not affect PIPKI90 activity in vitro (supplemental Fig. S3A).

To determine whether PIPKI90 phosphorylation regulates its degradation, CHO-K1 cells were transfected with FLAG-PIPKI90 WT, FLAG-PIPKI90 T553A, S555A, and FLAG-PIPKI90 T553E, S555E and treated with DMSO and carfilzomib, a specific proteasome inhibitor. As shown in Fig. 6A, PIPKI90 T553A, S555A was not efficiently degraded and was more resistant to degradation than PIPKI90 WT and PIPKI90 T553E, S555E. To further confirm the stability of the T553A, S555A mutant, we determined the time course of PIPKI90 degradation. Avi-tagged PIPKI90 WT and mutants were transfected into CHO-K1 cells with stable expression of BirA, and then labeled with biotin. Then, biotin was washed away and cells were split into dishes with media containing avidin. PIPKI90 and mutants were detected using Dylight 680 streptavidin by harvesting the cells at different time points. PIPKI90 T553A, S555A was more resistant to degradation in comparison to WT and PIPKI90 T553E, S555E mutant (Fig. 6B) and had a significantly longer half-life than the WT and PIPKI90 T553E, S555E (Fig. 6C).

To further demonstrate the role of S6K1-mediated PIPKI90 phosphorylation in PIPKI90 degradation, CHO-K1 cells were transfected with Dendra2-PIPKI90, -PIPKI90 T553A, S555A, and -PIPKI90 T553E, S555E and plated on fibronectin-coated glass-bottom dishes. The cells were irradiated by a 408-nm laser to convert the Dendra2 fusion protein into its red fluorescence form. The red fluorescence protein degradation was recorded by time-lapse imaging at 10-min intervals. Dendra2-PIPKI90 T553A, S555A was more stable/resistant to degradation, with a half-life of >4 h, in comparison with the WT and T553E, S555E mutant of PIPKI90, which both showed a relatively higher rate of degradation, with half-lives of 2.5 and 3 h, respectively (Fig. 6, D and E). To examine the role of S6K1 in regulating PIPKI90 degradation, CHO-K1 cells that expressed Dendra2-PIPKI90 were treated with the S6K1 inhibitors DG2 (10 μM) or PF4708671 (10 μM), and the degradation of Dendra2-PIPKI90 was analyzed. As shown in Fig. 6F, S6K1 inhibition caused a significant increase in the stability of Dendra2-PIPKI90 WT compared with the control. However, DG2 had no effect on the degradation of Dendra2-PIPKI90 T553E, S555E (Fig. 6G). These results further support the concept that S6K1-mediated phosphorylation of PIPKI90 facilitates its degradation.

This prompted us to examine the ubiquitination of PIPKI90 and these mutants. To this end, Avi-ubiquitin was co-transfected with ZZ-PIPKI90, -PIPKI90 T553A, S555A, or -PIPKI90 T553E, S555E into CHO-K1 cells expressing BirA, labeled with biotin, and immunoprecipitated with IgG-agarose. Ubiquitination was detected with Dylight 680 streptavidin. Substitution of Thr-553 and Ser-555 with Ala caused an increase in PIPKI90 ubiquitination, whereas substitution with Glu had no significant change compared with the WT protein (Fig. 7A), indicating that PIPKI90 ubiquitination is not sufficient for its degradation.

To compare the roles of S6K1 and Akt1 in PIPKI90 degradation, we examined the steady-state levels of PIPKI90 in S6K1-depleted MDA-MB-231 cells. The level of PIPKI90 in S6K1-depleted cells was significantly higher than that in cells expressing a control shRNA (Fig. 7B). Treatment with carfilzomib resulted in a significant increase in PIPKI90 level in cells expressing control shRNA but not in S6K1-depleted cells. However, depletion of Akt1 by expressing its shRNA had no significant effect on the steady-state levels of PIPKI90 (Fig. 7C). These results suggest that S6K1-mediated phosphorylation facilitates PIPKI90 degradation.

Our previous published results indicate that PIPKI90 ubiquitination at lysine 97 and subsequent degradation are necessary for breast cancer cell invasion (16). To examine the role of PIPKI90 degradation in matrix degradation, we compared the matrix degradation activities of PIPKI90-depleted MDA-MB-231 cells that express codon-modified ZZ-PIPKI90 or ZZ-PIPKI90K97R using normal and PIPKI90-depleted MDA-MB-231 cells as controls (Fig. 7D). PIPKI90 K97R is an ubiquitination- and degradation-resistant mutant. Depletion of PIPKI90 inhibited matrix degradation, and re-expression of PIPKI90 restored matrix degradation in PIPKI90-depleted cells whereas that of PIPKI90 K97R did not (Fig. 7, E and F), further supporting the hypothesis that dynamic PIPKI90 degradation is essential for extracellular matrix degradation.

Discussion

The ubiquitin proteasome pathway regulates FA assembly and disassembly and, consequently, cell migration and invasion by ubiquitinating FA proteins (16, 21–26), and we recently demonstrated that PIPKI90 ubiquitination and subsequent degradation control FA dynamics to regulate cell migration and invasion (16). In this study, we demonstrated that S6K1-mediated PIPKI90 phosphorylation regulates PIPKI90 degradation to control the development of FAs and invadopodia and, consequently, cell migration and invasion.

We demonstrated that PIPKI90 is a substrate for S6K1. We showed that S6K1 phosphorylated PIPKI90 when they were co-transfected into CHO-K1 cells (Fig. 1B) and that substitution of the Thr-553 and Ser-555 sites with alanine abolished PIPKI90 phosphorylation by S6K1 in vitro and in cells (Fig. 1, C and D). We also revealed that PIPKI90 phosphorylation was stimulated by HGF and EGF and that HGF-stimulated phosphorylation was inhibited by the S6K1 inhibitors DG2 and PF4708671, Akt inhibitor VIII, as well as S6K1 knockdown (Fig. 1, E–G). The S6K1 inhibitors DG2 and PF4708671 caused 68% and 45% reduction in PIPKI90 phosphorylation in HGF-stimulated MDA-MB-231 cells, respectively. Akt inhibitor VIII suppressed 85% of PIPKI90 phosphorylation. The related higher efficiency of Akt inhibitor is probably due to its inhibition of both Akt and S6K1 activation. Thus, we estimated that S6K1 mediated approximately 50–70% of Thr-553 and Ser-555 phosphorylation in HGF-stimulated MDA-MB-231 cells. Endogenous PIPKI90 phosphorylation has not been examined because of reagent limitation. Nevertheless, these results indi-
FIGURE 6. S6K1-mediated phosphorylation regulates PIPKIγ degradation. A, the steady-state levels of PIPKIγWT, PIPKIγT553A,S555A and PIPKIγT553E,S555E in CHO-K1 cells that were transiently transfected with FLAG-PIPKIγ WT, -PIPKIγT553A,S555A and -PIPKIγT553E,S555E, respectively, and treated with DMSO or carfilzomib (1 μM). B, substitution of Thr-553 and Ser-555 with Ala, but not Glu, inhibited degradation of PIPKIγ. CHO-K1 cells expressing BirA were transfected with Avi-PIPKIγ WT, -PIPKIγT553A,S555A, and -PIPKIγT553E,S555E and labeled with biotin. The levels of PIPKIγ were detected by Western blotting using Dylight 680-streptavidin. C, time course of degradation of PIPKIγWT, PIPKIγT553A,S555A, and PIPKIγT553E,S555E in CHO-K1 cells. Data represent mean ± S.E. of three experiments. **, p < 0.01; ***, p < 0.001. D, CHO-K1 cells were transiently transfected with Dendra2-PIPKIγ90WT, -PIPKIγ90T553A,S555A, and -PIPKIγ90T553E,S555E and plated on fibronectin. The cells were irradiated for 2 min by a 408-nm laser to convert the Dendra2 fusion protein into red Dendra2 fusion protein. The intensities of the red fluorescence were recorded using time-lapse imaging. Scale bar = 20 μm. E, quantification of the degradation of Dendra2-PIPKIγ90WT, -PIPKIγ90T553A,S555A, and -PIPKIγ90T553E,S555E and platted on fibronectin. Data are presented as mean ± S.E. of four independent experiments. F, the S6K1 inhibitor DG2 or PF4708671 stabilizes PIPKIγ90WT. CHO-K1 cells were transfected with Dendra2-PIPKIγ90WT. 24 h post-transfection, cells were treated with DG2 (10 μM) or PF4708671 (10 μM) for 30 min and then irradiated for 2 min using a 408-nm laser. Data are presented as mean ± S.E. of three experiments. G, the S6K1 inhibitor DG2 (10 μM) had little effect on the degradation of Dendra2-PIPKIγ90T553A,S555E. Data are presented as mean ± S.E. of three experiments.
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The ubiquitination of PIPKIγ90 is a substrate for S6K1 in the system we used. When we started writing this manuscript, Le et al. (39) reported that Akt1 phosphorylated PIPKIγ90 at Ser-555. Indeed, PIPKIγ90 was phosphorylated when it was co-transfected with Akt1 (Fig. 1B), and HGF-stimulated PIPKIγ90 phosphorylation was inhibited by Akt inhibitor VIII (Fig. 1F), suggesting that Akt1 is also a potential protein kinase that phosphorylates PIPKIγ90. However, depletion of Akt1 did not significantly inhibit the invasion of MDA-MB-231 cells (Fig. 3G). This result is consistent with previous reports showing that Akt activation potentially blocks carcinoma motility, including migration and invasion in breast cancer cells (40–43). Therefore, although both S6K1 and Akt1 phosphorylate PIPKIγ90, S6K1 is functionally more relevant than Akt1 in regulating PIPKIγ90 phosphorylation and cell invasion in breast cancer cells.

It is generally believed that protein polyubiquitination is sufficient for protein degradation (44, 45), but our findings indicate that PIPKIγ90 ubiquitination alone is insufficient for its degradation. The phosphorylation-deficient mutant PIPKIγ90*T553A,S555A cannot be degraded efficiently compared with the WT and T553A,S555A mutant (Fig. 6, B–E). Moreover, the S6K inhibitors DG2 and PF4708671 inhibited the degradation of PIPKIγ90 but not that of PIPKIγ90*T553A,S555A. However, substitution of Thr-553 and Ser-555 with alanine did not sup-

FIGURE 7. PIPKIγ90 degradation is required for cancer cell-mediated matrix degradation. A, ubiquitination of PIPKIγ90, PIPKIγ90T553A,S555A, and -PIPKIγ90T553E,S555E. Avi-ubiquitin (Avi-Ub) was co-transfected with ZZ-PIPKIγ90, -PIPKIγ90T553A,S555A, and -PIPKIγ90T553E,S555E into CHO-K1 cells expressing BirA. The cells were labeled with biotin, and the ZZ-tagged proteins were immunoprecipitated with IgG-agarose. The ubiquitination was detected using Dylight 680-streptavidin. Data are representative of two independent experiments. B, the steady-state levels of PIPKIγ90 in MDA-MB-231 cells that express empty pLKO.1 vector or S6K1 shRNAs, treated with DMSO or carfilzomib (Carf, 5 μM). Data are presented as mean ± S.E. of three independent experiments. *, p < 0.05. Ctrl, control. C, the steady-state levels of PIPKIγ90 in MDA-MB-231 cells that express empty pLKO.1 vector or Akt1 shRNA, treated with DMSO or bortezomib/carfilzomib (B+C, 1 μM each). Data are presented as mean ± S.E. of three independent experiments. D, the expression levels of PIPKIγ90 in MDA-MB-231 cells expressing a control shRNA or PIPKIγ90 shRNA A1 and the PIPKIγ90-depleted cells that stably express ZZ-PIPKIγ90 and -PIPKIγ90K97R. E, PIPKIγ90WT restored gelatin degradation in PIPKIγ90-depleted MDA-MB-231 cells but PIPKIγ90K97R, a ubiquitination-deficient mutant, did not. Scale bar = 20 μm. F, quantification of the experiment in E. Data are mean ± S.E. of three independent experiments. *, p < 0.05. AU, arbitrary unit.
press but, instead, enhanced PIPKIγ90 ubiquitination (Fig. 7A). Our data show that PIPKIγ90 binds to 14–3–3 proteins, a family of adaptor proteins that regulate protein degradation (46–48), in a phosphorylation-dependent manner.3 However, although a role for this interaction with 14–3–3 proteins may be involved, it remains unknown how S6K1-mediated phosphorylation regulates PIPKIγ90 degradation.

The suppressive role of the phosphorylation-deficient mutant PIPKIγ90T553A,S555A in cell migration provides a new evidence for the role of PIPKIγ90 degradation in cell migration. Previous studies have demonstrated the essential role of PIPKIγ90 in the regulation of cell migration (14–16). Our recent study indicates that PIPKIγ90 ubiquitination by HECTD1 and subsequent degradation control FA dynamics and cell migration. Here we show that the phosphorylation-deficient mutant PIPKIγ90T553A,S555A was resistant to degradation and inhibited migration behavior by suppressing directionality and net distance from origin in comparison with the WT or PIPKIγ90T553E,S555E mutant had similar abilities to digest gelatin. In contrast, cells expressing the WT or PIPKIγ90T553E,S555E mutant had similar abilities to digest gelatin (Fig. 4A and B). Moreover, PIPKIγ90K97R, which is an ubiquitination site mutant and is resistant to proteasome degradation, was unable to restore the matrix degradation in PIPKIγ90-depleted cells (Fig. 7, E and F). Furthermore, depletion of S6K1 by shRNA enhanced the stability of PIPKIγ90 (Fig. 7B) but significantly reduced the cellular capability to degrade the gelatin matrix (Fig. 4C). These data suggest that the S6K1-PIPKIγ90 pathway controls PIPKIγ90 degradation to regulate matrix degradation and cell invasion, probably through modulating the secretion of matrix metalloproteinases (13).

Spatial and temporary production of PIP2 is crucial for cell migration and invasion. This highly regulated PIP2 production is controlled by PIPKIγ90 ubiquitination and subsequent degradation. However, PIPKIγ90 ubiquitination alone is insufficient for its degradation; instead, the new data presented here show that S6K1-mediated PIPKIγ90 phosphorylation is also necessary for the degradation of ubiquitinated PIPKIγ90. S6K1 phosphorylates PIPKIγ90 at Thr-553 and Ser-555 to mediate the dynamic degradation of PIPKIγ90, thus controlling FA dynamics and matrix degradation and, consequently, cell migration and invasion. Our findings uncover a new paradigm for control of protein degradation, implying that a similar mechanism may also occur in other systems and processes.

### Experimental Procedures

**Reagents**—IgG-agarose was described previously (50). The S6K1 inhibitor DG2 and anti-paxillin antibody (clone 5H11) were from Millipore. The S6K1 inhibitor PF4708671 was from ApexBio (Houston, TX). Akt inhibitor VIII was from Cayman Chemical Co. The anti-RXRXXpS/T motif antibody (23C8D2), anti-p70 S6 kinase antibody (49D7), anti-phospho-p70 S6 kinase (Thr(P)-389) antibody (9205), anti-S6 ribosomal protein antibody (5G10), anti-phospho-S6 ribosomal protein (Ser(P)-235/236) antibody (D57.2.2E), anti-Rsk2 antibody, and anti-phospho-Rsk2 and (Ser(P)-227) antibody were purchased from Cell Signaling Technology. The anti-PIPKIγ90 polyclonal antibody (MAO-R1), anti-Akt1 antibody (Tyr-89), and anti-phospho-Akt (Ser(P)-473) antibody (EP2109Y) were from Abcam. Anti-FLAG M2-agarose beads, anti-tubulin antibody, and pLK01 lentivirus shRNAs that target PIPKIγ90, S6K1, and Akt1, respectively, were from Sigma. The PIPKIγ90 shRNA clone was TRCN0000037668 (A1). The S6K1 shRNA clones were TRCN0000003158 and TRCN0000003159. The Akt1 shRNA clones were TRCN0000010174 and TRCN0000039793. pBabe-Puro-Myr-FLAG-AKT1 was a gift from William Hahn (Addgene plasmid 15294). pRK7-HA-S6K1-F5A-E389 was a gift from John Blenis (Addgene plasmid 8988). DyLight 549-phalloidin were from Life Technologies. Fibronecin was from Akorn Biotech. HGF, EGF, PDGF, and SCF were from Prospec.

Matrix metalloproteinases-mediated matrix degradation is critical for cell invasion (36–38). However, the molecular mechanisms that regulate this process are not entirely understood. Our data show that PIPKIγ90T553A,S555A, a degradation-resistant mutant, had a significantly limited cellular ability to mediate gelatin degradation. In contrast, cells expressing the WT or PIPKIγ90T553E,S555E mutant had similar abilities to digest gelatin (Fig. 4A and B).

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S6K1 Regulates PIPKIγ90 Degradation and Cell Invasion

Inc. Growth factor-reduced Matrigel was from BD Biosciences. Pfu Ultra was from Agilent Technologies. The Safectine RU50 transfection kit was purchased from Syd Labs (Malden, MA). DNA primers were synthesized by Integrated DNA Technologies.

**Plasmid Construction**—pZZ-PIPKI90 and the codon-modified plasmids pZZ-PIPKI90 and pBabe-ZZ PIPKI90 were described previously (16, 50). The codon-modified plasmids pZZ-PIPKI90 and pBabe-PIPKI90 were generated by Pfu Ultra-based PCR using the codon-modified pZZ-PIPKI90 as a template and 5′-cgg tag acc cgg cgg gca gag gct gga gat ggc agg 3′-5′-cct gcc atc tgt ccc cag acc cgg cgg cct gta cca cgg-3′ and 5′-cgg tag acc cgg cgg gca gag gct gga gat ggc agg 3′-5′-cct gcc atc tgt ccc cag acc cgg cgg cct gta cca cgg-3′ as primers, respectively. The codon-modified pBabe-ZZ PIPKI90 was generated by sequential digesting the codon-modified pZZ-PIPKI90 with AgeI, blunting with Klenow, and digesting with SalI. The smaller fragments were subcloned into the pBabe-neo vector that had been treated with BamH1, Klenow, and SalI. pFLAG-PIPKI90 was generated by PCR amplifying PIPKI90 using pEGFP-PIPKI90 as a template and 5′-aatt tag ata cta gat cta gga ctc ggc gag gga 3′-5′-aatt tag ata cta gat cta gga ctc ggc gag gga-3′ as primers. The PCR products were digested with BglII and EcoRI and inserted into the pFLAG-C1 vector cut with the same enzymes.

**Preparation of Viruses and Cell Infection**—CHO-K1 cells were transiently transfected with Dendra2-PIPKI90WT, -PIPKI90T553E,S555E, and -PIPKI90T553A,S555A and cultured in fibronectin-coated glass-bottom dishes. Time-lapse live cell imaging was conducted on a Nikon A1 R microscope. Before excitation, there should not be any red Dendra2-emission signal visible. Photoconversion was performed at ×100 magnification with near-UV irradiation (408 nm) for 120 s.

**Ubiquitination Assays**—Avi-ubiquitin was co-transfected into CHO-K1 cells stably expressing EGFP-BirA (50). 24 h post-transfection, cells were incubated with 500 μM biotin for 2 h, washed with PBS, and cultured in normal culture medium containing 200 μg/ml Avidin. The cells were lysed at different time points, and the levels of ubiquitin-labeled PIPKI90 (or mutants) were detected by Dylight 680-streptavidin.

**Degradation**—CHO-K1 cells stably expressing BirA were co-transfected with Avi-PIPKI90, Avi-PIPKI90T553A,S555A, and Avi-PIPKI90T553E,S555E. The cells were incubated with 500 μM biotin for 2 h, washed with PBS, and cultured in normal culture medium containing 200 μg/ml Avidin. Green-to-red photoconversion was monitored in real time using a 561-nm channel. Images were captured at 20-min intervals and analyzed using NIS-Elements software.

**Ubiquitination Assays**—Avi-ubiquitin was co-transfected with ZZ-PIPKI90, -PIPKI90T553A,S555A, and -PIPKI90T553E,S555E and co-transfected with an ubiquitin ligase or an empty vector into CHO-K1 cells stably expressing EGFP-BirA (50). 24 h post-transfection, cells were incubated with 500 μM biotin, 1 μM bortezomib, and 1 μM carfilzomib for 6 h and then scraped in PBS. The cells were spun down, lysed with 150 μl of 1× SDS sample buffer (without 2-mercaptoethanol) containing protease inhibitor mixture and bortezomib/carfilzomib and boiled immediately. The lysates were cleared, diluted to 1 ml, and incubated with rabbit IgG-Sepharose beads at 4 °C for 2 h to precipitate ZZ-tagged PIPKI90 (or the mutants). The beads were washed and analyzed by SDS-PAGE and Western blotting as above. The ubiquitination of the ZZ domain fusion protein was detected with Dylight 680-Streptavidin, whereas the

**Preparation of Viruses and Cell Infection**—CHO-K1 cells were transiently transfected with pBabe retroviral or pLKO1 lentiviral systems using Safectine RU50 transfection reagent according to the protocol of the manufacturer. The virus particles were applied to overnight cultures of breast cancer cells for infection. Cells that stably express pLKO1 lentiviral shRNAs were obtained by selecting the infected cells with 1 μg/ml puromycin, and cells that were infected with pBabe retroviruses were stabilized by growing infected cells in the presence of 0.7 mg/ml neomycin for 10 days.

**Phosphorylation**—FLAG-PIPKI90 (or mutants) was co-transfected with an empty vector or a plasmid expressing active kinase into CHO-K1 cells. The cells were lysed by radioimmune precipitation assay buffer (50 mm Tris-HCl (pH 7.5), 150 mM NaCl, 1% IGEPAL, and 5 mM EDTA) containing protease inhibitor mixture and phosphatase inhibitor mixture. FLAG-PIPKI90 was immunoprecipitated with anti-FLAG-agarose beads. The immune complexes were analyzed by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. PIPKIγ90 phosphorylation was detected with an anti-RRXXpS/T motif antibody. To detect PIPKI90 phosphorylation in breast cancer cells, cells stably expressing FLAG-PIPKI90 were treated with Akt or S6K1 inhibitor and then stimulated with growth factors. FLAG-PIPKI90 was immunoprecipitated, and PIPKI90 phosphorylation was detected as described above.

**Cell Culture and Transfection**—CHO-K1 cells, MDA-MB-231 and MDA-MB-468 human breast cancer cells, and 293T human embryonic kidney cells were from the American Type Culture Collection and were maintained in DMEM (Sigma) containing 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). CHO-K1 and 293T cells were transfected with Safectine RU50 according to the protocol of the manufacturer.
expression of the ZZ domain fusion protein was probed with Dylight 680-rabbit IgG.

**In Vitro PIPK1γ90 Activity Assays**—PIPK1γ90 activity was measured as described previously (11). Briefly, pZZ-PIPK1γ90, pZZ-PIPK1γ90K188,200R, pZZ-PIPK1γ90T553A,S555A, and pZZ-PIPK1γ90T553E,S555E were transiently expressed in CHO-K1 cells and immunoprecipitated with IgG-agarose beads (50). The beads were washed and incubated with 100 µl of a kinase buffer containing 100 µM Pi(4)P for 30 min at 37 °C. PIP2 formed in these assays was extracted as described previously (51) and separated by silicon TLC. PIP2 was visualized by autoradiography and quantitated by a Beckman liquid scintillation counter.

**Cell Migration Assays**—Cells were treated with trypsin and resuspended in DMEM containing 1% FBS and 10 ng/ml EGF, plated at low densities on glass-bottom dishes (Cellvis) coated with 5 µg/ml fibronectin, and cultured for 3 h in a CO2 incubator. Cell motility was measured with a Nikon Biostation IMQ. Cell migration was tracked for 6 h. Images were recorded every 10 min. The movement of individual cells was analyzed with NIS-Elements AR (Nikon) as described previously (16).

**Focal Adhesion Staining**—MDA-MB-231 cells were infected with lentiviruses that express PIPK1γ shRNA (A1) to deplete endogenous PIPK1γ, infected with retroviruses that express pBabe-FLAG-PIPK1γ90WT or FLAG-PIPK1γ90T553A,S555A, and selected with neomycin (0.7 mg/ml). The cells were trypsinized and plated on glass-bottom dishes that had been precoated with fibronectin (5 µg/ml). The cells were cultured for 4 h. The cells were fixed with 4% paraformaldehyde for 15 min, permeabilized for 15 min with 0.5% Triton X-100, and then blocked with 5% BSA in PBS for 1 h. The cells were then incubated with a rabbit polyclonal anti-PIPK1γ antibody and a mouse monoclonal anti-paxillin antibody, washed with PBS, and then incubated with a Dylight480-labeled goat anti-rabbit and a Dylight 680-rabbit IgG. Expression of the ZZ domain fusion protein was probed with a Dylight480-labeled goat anti-rabbit and a Dylight 648-labeled goat anti-mouse secondary antibody. After washing with PBS, the images of PIPK1γ and paxillin were acquired with a Nikon Eclipse Ti TIRF microscope equipped with a x60, 1.45 numerical aperture objective, CoolSNAP HQ2 charge-coupled device camera (Roper Scientific). Focal adhesion area distribution was analyzed with Nis-Elements.

**Invagination Assays**—One hundred microliters of Matrigel (1:30 dilution in serum-free DMEM) was added to each Transwell polycarbonate filter (6-mm diameter, 8-µm pore size, Costar) and incubated with the filters at 37 °C for 4 h. Breast cancer cells were trypsinized and washed three times with DMEM containing 1% FBS. The cells were resuspended in DMEM containing 1% FBS at a density of 5 × 105 cells/ml. The cell suspensions (100 µl) were seeded into the upper chambers, and 600 µl of DMEM containing 50 ng/ml HGF were added to the lower chambers. The cells were allowed to invade for 12 h (or as indicated) in a CO2 incubator, fixed, stained, and quantitated as described previously (11).

**Gelatin Degradation Assays**—Gelatin degradation assays were performed as described previously (52). Briefly, glass-bottom dishes were coated with warm Alexa 488-conjugated gelatin (0.2 mg/ml) in PBS containing 2% sucrose. The coated dishes were dried, fixed with prechilled glutaraldehyde solution (0.5%), washed with PBS, and then reduced with 5 mg/ml of sodium borohydride in PBS. The dishes were washed extensively with PBS and then incubated with DMEM containing 10% FBS and antibiotics for 1 h. Cells were plated at low density to the dishes and cultured for 12 h, fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and stained with cortactin or Alexa 647 phalloidin. Images were acquired using a TIRF microscope and analyzed with NIS Elements software.

**Gel Data Quantification**—Gel data were quantified by analyzing inverted images using ImageJ as described previously (21). Data from different experiments were normalized to controls. If values from different experiments had a high variation, then datasets were further normalized by dividing the numbers in a dataset with a factor (e.g. 2) so that the biggest values from different experiments were similar.

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**References**

Supplementary Fig. S1. HGF and EGF stimulate PIPKI90 phosphorylation. A. MDA-MB-468 cells that stably express Flag-PIPKIγ90 were serum-starved and stimulated with EGF (20 ng/ml), HGF (50 ng/ml), SCF (20 ng/ml) and PDGF (20 ng/ml) for 20 min, respectively. Flag-PIPKIγ90 was immunoprecipitated and the phosphorylation was detected with an anti-RXRXXpS/T motif antibody. B. Time-course of Akt, S6K1 and Rsk2 activation in EGF-stimulated MDA-MB-231 cells. C. Time-course of Akt and S6K1 activation in HGF-stimulated MDA-MB-231 cells.

Supplementary Fig. S2. Overexpression of PIPKIγ90T553A,S555A in MDA-MB-231 cells suppressed cell invasion. A. Expression of ZZ-PIPKIγ, -PIPKIγT553A,S555A, or -PIPKIγT553E,S555E in parental MDA-
MB-231 cells. MDA-MB-231 cells were infected with retroviruses that express ZZ-PIPKIγ90, -PIPKIγ90T553A,S555A, or PIPKIγ90T553E,S555E, and then selected with neomycin. **B.** MDA-MB-231 cells that express ZZ-PIPKIγ90, ZZ-PIPKIγ90T553A,S555A, and ZZ-PIPKIγ90T553E,S555E, respectively, were examined for their Matrigel invasive activities in the absence and presence of HGF. **C.** Quantification of Experiment "B". White bar, without HGF, grey bar, 20 ng/ml HGF. Data are presented as mean±SEM, n=3. *P<0.05.

**Supplementary Fig. S3.** Mutation at T553 and S555 did not affect PIPKIγ activity in vitro. **A.** ZZ-PIPKIγ90WT and ZZ-PIPKIγ90K188,200R were transfected into CHO-K1 cells, respectively, and immunoprecipitated with IgG-conjugated-agarose beads or protein A agarose. The activities of PIPKIγ and the mutants were determined using PI(4)P and [γ-32P]ATP as substrates. **B.** ZZ-PIPKIγ, -PIPKIγT553A,S555A, and -PIPKIγT553E,S555E were immunoprecipitated using IgG-Agarose beads from CHO-K1 cells. The activities of PIPKIγ90 and mutants were determined as described above.
p70S6K1 (S6K1)-mediated Phosphorylation Regulates Phosphatidylinositol 4-Phosphate 5-Kinase Type I γ Degradation and Cell Invasion

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