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PTPH1 Dephosphorylates and Cooperates with p38γ MAPK to Increase Ras Oncogenesis through PDZ-Mediated Interaction

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Abstract

Protein phosphatases are believed to coordinate with kinases to execute biological functions, but examples of such integrated activities, however, are still missing. In this report, we have identified protein tyrosine phosphatase H1 (PTPH1) as a specific phosphatase for p38γ mitogen-activated protein kinase (MAPK) and shown their cooperative oncogenic activity through direct binding. p38γ, a Ras effector known to act independent of its phosphorylation, was first shown to require its unique PDZ-binding motif to increase Ras transformation. Yeast two-hybrid screening and *in vitro* and *in vivo* analyses further identified PTPH1 as a specific p38γ phosphatase through PDZ-mediated binding. Additional experiments showed that PTPH1 itself plays a role in Ras-dependent malignant growth *in vitro* and/or in mice by a mechanism depending on its p38γ-binding activity. Moreover, Ras increases both p38γ and PTPH1 protein expression and there is a coupling of increased p38γ and PTPH1 protein expression in primary colon cancer tissues. These results reveal a coordinative oncogenic activity of a MAPK with its specific phosphatase and suggest that PDZ-mediated p38γ/PTPH1 complex may be a novel target for Ras-dependent malignancies. *Cancer Res; 70(7); 2901–10. ©2010 AACR.*

Introduction

Mitogen-activated protein kinases (MAPK) are major signaling pathways in regulating Ras oncogene activity, including extracellular signal–regulated kinase (ERK), Jun NH_2-terminal kinases (JNK), and p38s. Whereas the ERK pathway is generally required for Ras activity (1), a suppressive role has been proposed for p38 (2, 3). The Ras inhibitory activity of the p38 pathway was first shown by the fact that p38 activation leads to either an inhibition of Ras-dependent growth (4) or an induction of Ras-dependent cell death (5). This observation has been further consolidated by an increased Ras tumorigenesis through knocking out of p38 activating kinases MKK3/6 (6), p38α (7), or downstream p38-regulated/activated protein kinase (8). Studies of inhibitory p38 MAPK pathways may offer a great promise to control Ras oncogene activity.

The p38 family, however, consists of four proteins [p38α (also called p38), p38β, p38γ, and p38δ], and our recent studies suggest that p38γ is required for Ras oncogenesis (9–11). p38γ is a 43-kDa protein with a unique COOH-terminal sequence (-ETXL) that can dock with PDZ (PSD-95/Dlg/ZO-1 homology) domains of different proteins as substrates such as α_1-syntrophin (12), SAP90/PSD-95 (13), and SAP97 (14). Among p38 family proteins, p38γ is the only member whose expression is induced during cell differentiation (15) and Ras activation (9, 10). More interestingly, p38γ is dephosphorylated by Ras signaling inside cells by unknown mechanisms (9), and a nonphosphorylated p38γ has a greater potency in increasing Ras transformation (11). These results together indicate a potential involvement of protein phosphatases in the p38γ regulation of Ras transformation through its PDZ binding motif–mediated protein-protein interactions. In this report, we have identified protein tyrosine phosphatase H1 (PTPH1) as a p38γ-specific phosphatase and shown that p38γ and PTPH1 cooperate to promote Ras oncogenesis through direct binding.

Materials and Methods

Gene expression, gene silencing, and viral infection. Flag-tagged wild-type (WT) p38γ and p38γΔ4 or Δ13 were stably expressed in IEC-6 cells through G418 selection, and pooled resistant cells were infected with LZRS-K-Ras through a puromycin selection (9). PTPH1 and its mutant were similarly stably expressed in IEC-6/K-Ras cells. For gene silencing, the target sequence was cloned into a plenti6/Block-iT vector by including a sequence from luciferase gene as a control. To produce virus, retrovirus and lentivirus were transfected into

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their respective packaging cells and supernatants were collected, filtered, and used to infect target cells.

In vitro *binding and p38γ/α dephosphorylation experiments.* Flag-tagged p38γ and its Δ4/Δ13 mutants (together with p38α and its mutant) were expressed in 293T cells. Thereafter, cells were lysed, supernatants mixed with 8 μg of glutathione *S*-transferase (GST) or GST-PTPH1 proteins, the mixtures incubated with reduced glutathione beads overnight, and precipitates were analyzed by Western blot. For *in vitro* p38γ/α dephosphorylation experiment, Flag-p38γ/α was coexpressed with an active MKK6/2E in 293T cells and purified by an anti-Flag antibody (M2-conjugated agarose beads). Precipitates were then incubated with GST-PTPH1 in a reaction buffer [50 mmol/L Tris-HCl (pH 7.5), 3 mmol/L DTT, 30 mmol/L $MgCl_2$] at 37°C for 30 min, and mixtures were analyzed by Western blot for p38 phosphorylations.

Cell growth, soft-agar assays, and mouse experiments. Cell proliferation was estimated by thymidine incorporation assays after the peptide incubation (9). For soft-agar assays, cells were plated on growth medium containing 0.33% SeaPlaque agarose, and colonies photographed and counted about 2 wk later. For animal experiments, HCT116 cells were infected with Lenti-shPTPH1 or Lenti-shLuc (luciferase) and selected with blasticidin (15 μg/mL) for 7 d. Cells (2×10^6) in 0.1-mL PBS were then s.c. injected into athymic nude mice (Harlan) at both front flanks, and the tumor volume ($\pi abc/6$) was measured every 2 to 3 d. The animal experimental procedures were done in accordance with the Institutional Animal Care and Use Committee–approved protocol.

Human colon cancer specimens and immunohistochemistry. All human colon cancer tissues were collected by the Department of Pathology, Medical College of Wisconsin, with informed consent. Immunohistochemistry analyses were conducted in accordance with Institutional Review Board approval from the Medical College of Wisconsin. Briefly, sections of formalin-fixed and paraffin-embedded blocks were subjected to immunostaining as described (16). Anti-p38γ (1:1,200; R&D) and anti-PTPH1 (1:900; Santa Cruz) were used as primary antibodies. Staining results (intensity × percentage of positive cells) were examined independently by two observers as previously described (16, 17) and a consensus score was assigned to each case. The intensity was scored according to the following scale: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong), whereas the percentage was rated using the following categories: 0 (0%), 1 (<10%), 2 (11–50%), 3 (51–75%), and 4 (76–100%). The signal increases for PTPH1 in the malignant tissues after subtracting from the matched normal tissues were plotted against those from p38γ for their correlation.

Statistical analysis. Colony numbers, tumor volume, and tumor weight were analyzed by Student's *t* test or ANOVA for statistical difference. A Pearson's analysis was used to determine the correlation between increased p38γ and PTPH1 protein expression. Increased protein expression (p38γ and PTPH1) in colon cancer tissues versus matched normal tissues was analyzed by ANOVA.

Results

p38γ requires its PDZ-binding motif to increase Ras transformation and to bind PTPH1. To investigate the roles of p38γ PDZ-binding motif in Ras tumorigenesis, two Flag-tagged COOH-terminal truncated p38γ mutants lacking 4 (Δ4) and 13 (Δ13) amino acids were generated by PCR and stably expressed in rat intestinal epithelial IEC-6 cells through G418 selection together with a WT p38γ. These cells were further expressed with K-Ras oncogene via retroviral infection, and Ras oncogenic activity was assessed by anchorage-independent growth on soft agar (9). Results in Fig. 1A showed that expression of p38γ, but not its mutants, increases soft-agar growth. Because both p38γΔ4 and p38γΔ13 lack the PDZ motif ETPL, these results suggest that p38γ requires its PDZ protein-binding activity to increase Ras transformation.

To search for candidate PDZ proteins, we performed a yeast two-hybrid screen of a human adult colon cDNA library using human p38γ and p38γΔ13 as baits. This screen yielded 13 individual clones encoding residues 410–912 of PTPH1 when p38γ was used as bait, but no PTPH1 was detected in all five clones assayed with p38γΔ13 (Supplementary Fig. S1A). Because this region contains a single PDZ domain and only p38γ, but not p38γΔ13, binds PTPH1, these results indicate that p38γ interacts with PTPH1 through its PDZ motif ETPL. To confirm this speculation, p38γΔ4 (lacking ETPL only) was fused to GAL4 DNA binding domain; β-galactosidase (β-Gal) activity assay was done after retransformation with isolated PTPH1 by including GAL4-p38γ and GAL4-p38γΔ13 for comparison. Results in Fig. 1B (left) show that PTPH1 only interacts with p38γ but not with the Δ4 or Δ13 mutant, indicating a necessary role of the PDZ motif for the interaction (full results of this screening are given in Supplementary Fig. S1B). Previous two-hybrid screening revealed that rat p38γ interacts with PDZ protein α_1-syntrophin via the PDZ motif (ETAL), which was not observed in our analyses, likely as a result of their utilization of rat p38γ as a bait to screen a human brain cDNA library (12). These results together indicate that human p38γ requires the COOH-terminal PDZ binding motif to interact with PTPH1.

To further show PDZ-dependent p38γ-PTPH1 interaction, PTPH1 was expressed as a GST fusion protein that was incubated with 293T lysates containing transfected p38γ and its mutants, and GST pull-down assays were then done. To examine if the PDZ motif confers a binding activity toward PTPH1, the last COOH-terminal 13-amino-acid sequence of p38γ was fused to the corresponding portion of a human p38α, and this modified p38α (p38α+13) was also included in the analysis for comparison with WT p38α. Results in Fig. 1B (middle and right) and Supplementary Fig. S1C show that GST-PTPH1 binds p38γ and p38α+13, but not p38γΔ4, p38γΔ13, or WT p38α, indicating an important role of the PDZ motif in the p38α/γ interaction with PTPH1.

PTPH1 is a p38γ MAPK–specific phosphatase through PDZ-mediated interaction. To further show the role of the PDZ motif in the p38 interaction with PTPH1 *in vivo*, 293T cells were transiently expressed with indicated constructs

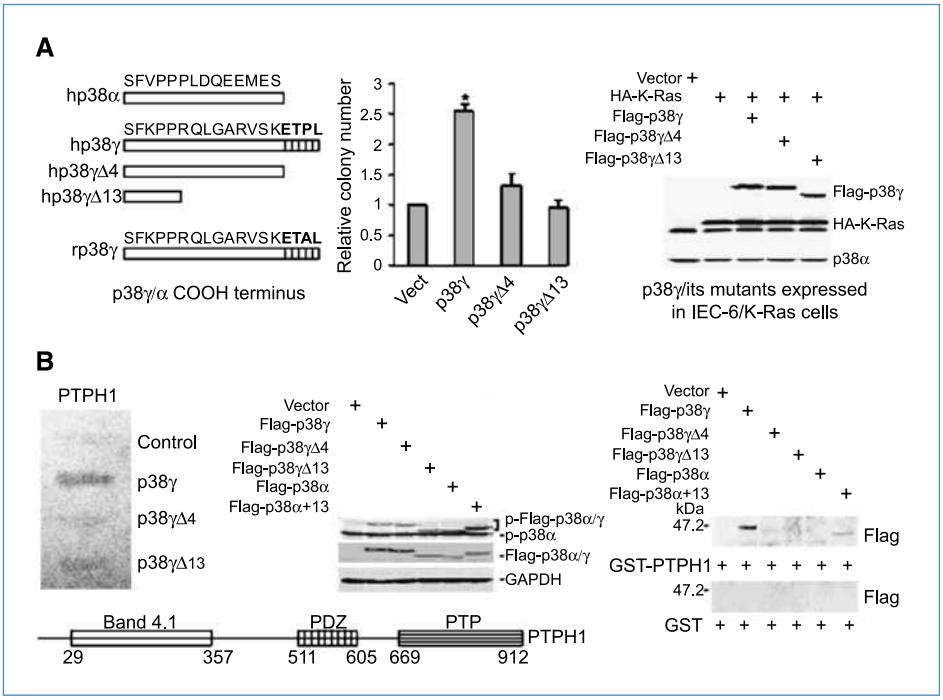

Figure 1. p38γ requires its COOH terminus to increase Ras transformation and to bind PTPH1. A, p38γ requires its COOH terminus to increase Ras transformation. IEC-6 cells were stably expressed with p38γ or its mutants (see left for structures) and then transduced with a retrovirus containing K-Ras and then were analyzed for soft-agar growth (middle) and protein expression (right). Colony numbers from 20 fields per 60-mm dish were counted and results shown are relative to vector controls (from three separate experiments, each in triplicate; *, $P < 0.05$ by ANOVA). B, yeast two-hybrid screening and GST pull-down reveal a PDZ-dependent interaction of p38γ with PTPH1. The detailed procedure and full results of the screening were described in Supplementary data and Supplementary Fig. S1A and B. The β-Gal activity assay with PTPH1 confirmed a positive reaction with p38γ (left) and the structure of human PTPH1 protein was depicted on the bottom (left). GST pull-down results were given on the right, with the input control in the middle (see Supplementary Fig. S1C for similar GST and GST-PTPH1 proteins used).

and analyzed by coimmunoprecipitations (IP) and Western blotting. Consistent with the GST pull-down results, analysis of Flag and HA precipitates showed an interaction of PTPH1 with both WT p38γ (but not its Δ4 and Δ13 mutants) and p38α+13 (Fig. 2A). Furthermore, PTPH1 binds p38γ and its kinase-dead mutant but not its nonphosphorable mutant p38γ/AGF, indicating a role of p38γ phosphorylation in this interaction (Fig. 2B and Supplementary Fig. S2A and data not shown). In addition, a PDZ domain–deleted PTPH1 mutant (PTPH1ΔPDZ) fails to bind p38γ, and stably transfected p38γ also requires the PDZ motif to bind endogenous PTPH1 protein in IEC-6/K-Ras cells (Fig. 2C and D). These results together indicate that it is the PDZ switch (the PDZ-binding motif of p38γ plus the PDZ domain of PTPH1) that controls their direct interaction.

PTPH1 is a 120-kDa protein that belongs to the nontransmembrane PTP superfamily (18). Previous studies have identified VCP (p97/CDC48; ref. 19) and T-cell receptor ζ subunit (20) as its substrates, but the mechanisms as well as the biological consequences of these reactions remain unknown. The demonstration of PDZ-dependent p38γ-PTPH1 interaction prompted us to explore if PTPH1 may be a novel p38γ phosphatase. In this regard, different amounts of GST-PTPH1 proteins were incubated with Flag precipitates containing MKK6-activated Flag-p38γ or Flag-p38α through cotransfection in 293T cells. *In vitro* phosphatase assay was done to assess p38 phosphorylation using a phosphor-specific p38 antibody that is reactive with all phosphorylated p38 (p-p38) family proteins. Results in Fig. 3A show that PTPH1 decreases p-p38γ without substantial effects on p-p38α. The cotransfection experiment further reveals that PTPH1 *in vivo* decreases phosphorylation of p38γ but not of p38γΔ4, p38γΔ13, or p38α (Fig. 3B; Supplementary Fig. S2B, left), indicating its specific p38γ phosphatase activity.

To further show if p38γ is a physiologic substrate of PTPH1, a substrate-trapping PTPH1 mutant (PTPH1/DA; ref. 19) was coexpressed with Flag-p38γ and their binding activity was analyzed in the absence and presence of phosphatase inhibitors (21). Results in Fig. 3C showed an increased p38γ-PTPH1/DA complex formation over PTPH1 transfection, consistent with an increased enzyme-substrate binding by PTP DA mutants (19, 21). The increased binding however was abolished in the presence of phosphatase inhibitors, which couples with increased p38γ phosphorylation as detected with either p-p38 or p-Tyr antibody, thus further confirming their direct interaction (22). Importantly, the p-p38γ signal is decreased after cotransfection with PTPH1 but not with its DA mutant. A stronger suppression of p-p38γ than p-p38γ-Tyr in Flag precipitates by PTPH1 (Fig. 3C) suggests that PTPH1 may specifically act on the tyrosine residue

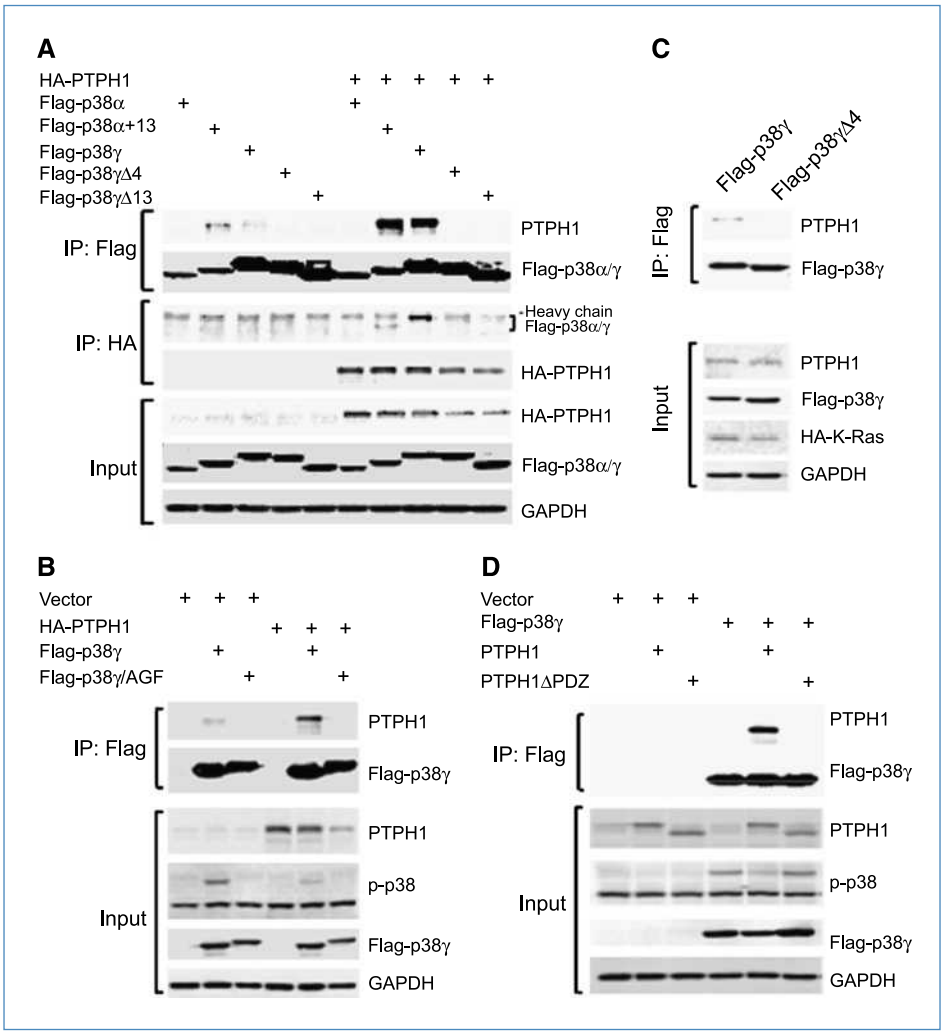

Figure 2. The PDZ motif dictates p38 interaction with PTPH1 *in vivo*. A, a role of the PDZ motif in the p38 interaction with PTPH1. Flag-tagged p38γ/α constructs were expressed with and without HA-PTPH1 in 293T cells and Flag/HA precipitates were examined by Western blot. Note that Flag p38γ and Flag p38α + 13 also bind endogenous PTPH1 in the absence of HA-PTPH1 (lanes 2 and 3 from left). B to D, p38γ depends on its phosphorylation and COOH terminus to bind the PDZ domain of PTPH1 protein. Expressed proteins in 293T cells were isolated and examined for p38γ-PTPH1 binding. Note that Flag-p38γ fails to bind the PTPH1ΔPDZ and the bound WT PTPH1 also leads to decreased p38γ phosphorylation from the input control (*B*/*D*). Results from C show that stably expressed Flag-p38γ (but not its Δ4 mutant) also binds endogenous PTPH1 protein in IEC-6/K-Ras cells.

within the activation loop of p38γ kinase, a finding consistent with the notion that dephosphorylation of the tyrosine residue is a determinant step for MAPK inactivation (23).

To investigate if PTPH1 regulates p38γ phosphorylation via PDZ binding under physiologic conditions, the last 13 amino acids of p38γ COOH terminus (p38γC13 or WT) and its mutant (p38γC13A or MT, with the last four amino acids changed to Alanine) were cloned into a pTAT-HA vector (24) and expressed as HA-tagged TAT-fusion peptides to regulate *in situ* p38γ-PTPH1 interaction. Results in Fig. 3D and Supplementary Fig. S2B (bottom and right) show that PTPH1-induced p38γ dephosphorylation is substantially inhibited by p38γC13 but minimally affected by its mutant, which couples with its activity to disrupt the p38γ-PTPH1 binding, indicating that p38γ is a physiologic substrate of PTPH1 through PDZ-mediated interaction.

PTPH1 signals downstream of Ras and p38γ to promote colon cancer growth. Previous genetic analysis suggested a tumor suppressor function of PTPH1 (also called *PTPN3*) along with its family members as a result of their somatic mutations in human colon cancer (25). Our results that p38γ requires its PTPH1-binding activity to increase Ras transformation, however, suggest that PTPH1 may play a positive role in Ras oncogenesis. To show if Ras signals to PTPH1, IEC-6/K-Ras and control cells were analyzed for protein expression by Western blotting. Results in Fig. 4A (left) show that p38γ and PTPH1 protein expression are both induced by K-Ras. Furthermore, transient expression of either oncogenic Ras or p38γ protein in normal IEC-6 cells also stimulates PTPH1 expression (Fig. 4A, middle and right), suggesting PTPH1 signaling downstream of both Ras and p38γ. Because p38γ phosphorylation is inhibited by Ras and p38γ promotes Ras transformation independent of phosphorylation (9), Ras-induced p38γ and PTPH1 proteins may cooperate to promote its oncogenesis through PDZ-mediated binding and resultant p38γ dephosphorylation.

To directly examine the roles of PTPH1 and p38γ in Ras-dependent growth, their gene expression was silenced by shRNA in Ras-activated HCT116 and SW480 human colon cancer cells and resultant effects on soft-agar growth were next determined. Results in Fig. 4B and C show that depletion of either PTPH1 or p38γ protein expression by two

separate shRNAs inhibits the malignant growth of both cell lines, indicating their growth-promoting roles in Ras-activated colon cancer. To further show the PTPH1 oncogenic activity, HCT116 cells were stably depleted of PTPH1 protein (Supplementary Figs. S3A–B and S4A), which were then injected into the right and left front flanks of nude mice and examined for their tumor-forming activities. Results in Figs. 5A and Supplementary Fig. S3A and B show that the intratumor PTPH1 depletion significantly inhibits tumor growth as revealed either by a lowered growth curve over a 2- to 3-week period or by a decreased tumor weight by the end of the experiment. Comparative analyses of tumor weight with PTPH1 protein expression from no. 1 and no. 5 mice further revealed a coupling of growth inhibition with PTPH1 depletion (Fig. 5A, inset; Supplementary Fig. S4B). These results indicate that both p38γ and PTPH1 are required for Ras-dependent colon cancer growth *in vitro* and/or in mice.

PTPH1 depends on its interaction with p38γ to increase malignant growth. We have shown that p38γ requires its PDZ-binding motif to increase Ras transformation (Fig. 1A). To show if PTPH1 depends on its p38γ binding activity to promote malignant growth, IEC-6/K-Ras cells were stably expressed with WT and PDZ-deleted PTPH1 proteins and their colony forming activities were then compared. Results in Fig. 5B and C show that the stable expression of PTPH1 but not its mutant increases soft-agar growth and similar results were also obtained with another p38γ binding–deficient PTPH1 mutant (data not shown). Because PTPH1ΔPDZ fails to bind/dephosphorylate p38γ (Fig. 2D),

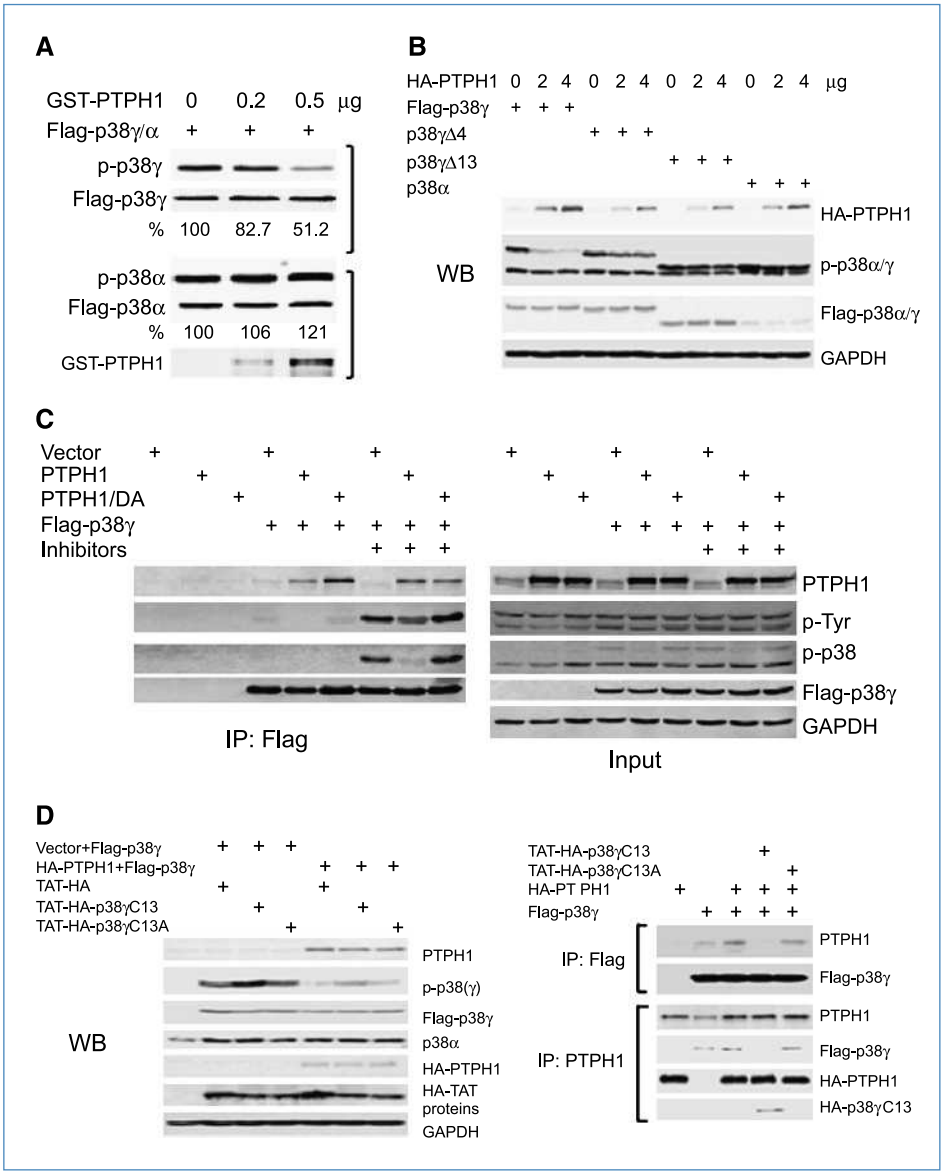

Figure 3. PTPH1 dephosphorylates p38γ but not p38α *in vitro* and *in vivo*. A, PTPH1 dephosphorylates p38γ but not p38α *in vitro*. Flag-tagged p38α/γ were coexpressed with MKK6 in 293T cells and activated p38s were isolated with a Flag antibody, which were examined for *in vitro* phosphorylation using a specific p-p38 antibody following incubation with GST-PTPH1. The percentage indicates the relative p-p38s (normalized to Flag-p38α/γ) over those in the absence of GST-PTPH1 (measured with ImageQuant 5.0 software). B, p38γ is dephosphorylated by PTPH1 *in vivo* dependent on its PDZ-binding motif. Different amounts of HA-PTPH1 were coexpressed with the indicated constructs in 293T cells and examined for p38γ phosphorylation. C, there is an increased complex formation between p38γ and PTPH1/DA. Flag-p38γ was transiently coexpressed with PTPH1 or PTPH1/DA and expressed proteins were isolated in the absence or presence of phosphatase inhibitors (1 mmol/L sodium vanadate, 20 mmol/L β-glycerophosphate, and 20 mmol/L *p*-nitrophenylphosphate) and analyzed by Western blot. D, PTPH1 binding and p38γ dephosphorylation are both inhibited by a peptide corresponding to the p38γ COOH terminus. p38γ and PTPH1 were expressed in 293T cells for 24 h, which were then subjected to peptide treatment (10 μmol/L, 5 h) and Flag IP and/or Western blot analysis (see Supplementary Fig. S2B for input control).

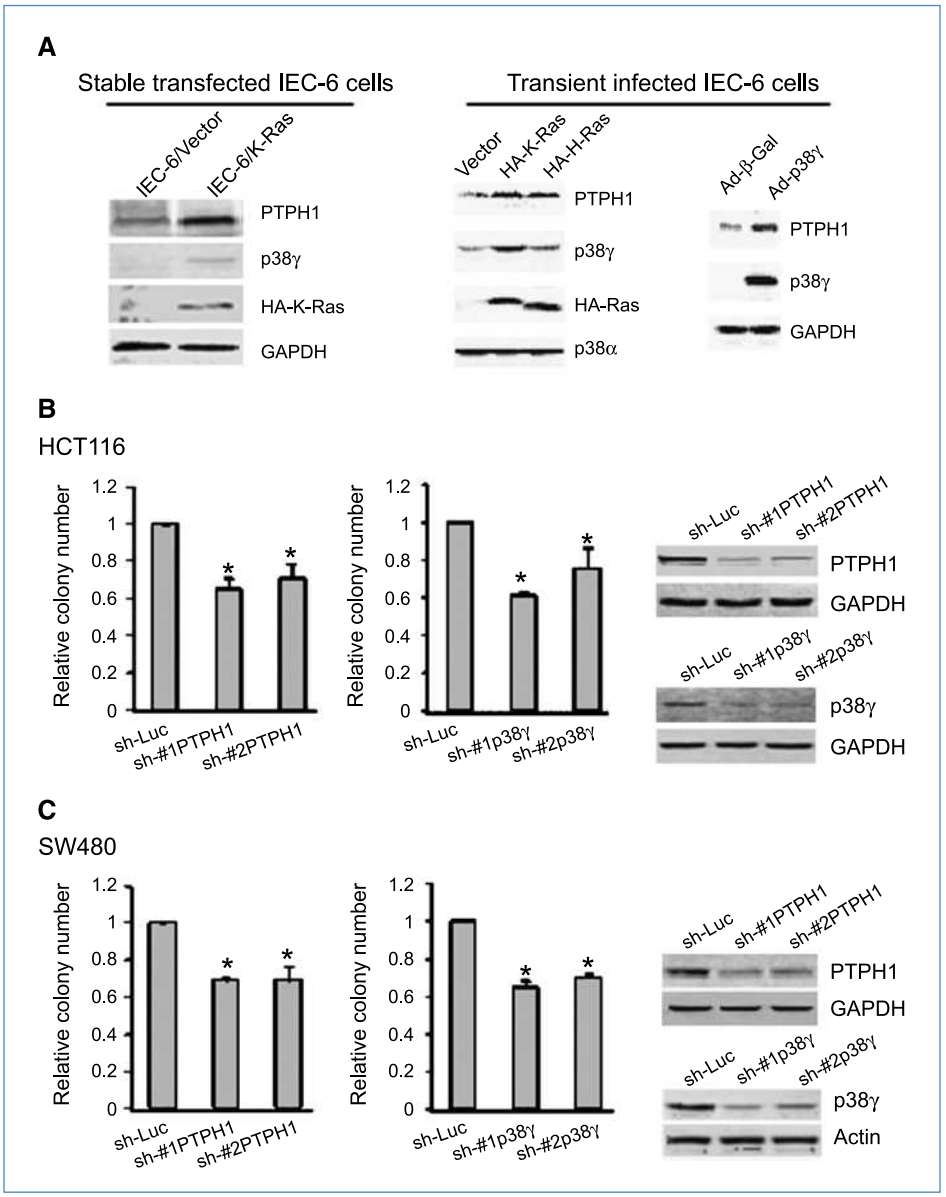

Figure 4. PTPH1 signals downstream of Ras and p38γ and is required for Ras-dependent colon cancer growth. A, Ras increases p38γ and PTPH1 protein expression. Ras-transformed IEC-6 cells were examined for protein expression (left). In addition, normal IEC-6 cells were transiently infected with LZRS retrovirus (expressing H-Ras or K-Ras) or adenovirus (p38γ) for 48 h and examined for protein expression (middle and right). B and C, depletion of PTPH1 or p38γ protein expression inhibits the soft-agar growth of HCT116 or SW480 human colon cancer cells. Cells were transiently infected with lentivirus (shLuc, #1 and #2 shRNA against PTPH1 or p38γ as indicated) and, 72 h later, assayed for soft-agar growth and Western blot analysis. The relative colony number is shown as in Fig. 1B from three separate experiments (*, $P < 0.05$, versus shLuc).

these results suggest that PTPH1 requires its p38γ binding/dephosphorylating activity to promote Ras tumorigenesis. Together with the role of the p38γ COOH terminus in Ras transformation, these results indicate that it may be the PDZ-mediated p38γ-PTPH1 complex that acts to promote malignant growth through resultant p38γ dephosphorylation.

To directly test this hypothesis, HCT116 cells were incubated with TAT-p38γC13 and its mutant TAT-p38γC13A peptide with TAT-green fluorescent protein (GFP) as a separate control, and cell proliferation was assessed by thymidine incorporation. Results in Fig. 5D show that the WT peptide significantly inhibits DNA synthesis as compared with the mutant and GFP control. Because this peptide blocks the p38γ/PTPH1 interaction as well as p38γ dephosphorylation as compared with its mutant (Fig. 3D), its cell growth–suppressive effect strongly indicates a required role of the p38γ/PTPH1 complex and resultant p38γ dephosphorylation in colon cancer growth. Because the mutant peptide sequence only differs from its WT counterpart at the ETPL, these results suggest that it is this four-amino-acid PDZ motif that integrates p38γ oncogenic activity with its phosphatase PTPH1.

There is a correlation of hyper-expressed p38γ with PTPH1 in primary colon cancer tissues and a coupling of decreased PTPH1 expression with increased p38γ phosphorylation. The cooperative oncogenic activity of p38γ and PTPH1 prompted us to examine if both proteins are overexpressed in primary colon cancer tissues. In this regard, 142 cases of human colon cancer specimens (invasive carcinoma) were analyzed by immunostaining for their

protein expression. Two slides of each specimen were processed for staining with a specific antibody against p38γ and PTPH1, respectively, and their signals in the tumor and the nearby matched normal tissues were independently scored. Results from Fig. 6A (left) and Supplementary Fig. S4C show that the levels of cytoplasmic p38γ and PTPH1 staining signals are both significantly increased in the malignant over the nearby normal tissues, and overall, increased p38γ and PTPH1 protein expression in the malignant tissues was observed in 81% and 75% of samples, respectively. Importantly, when signals from tumors are subtracted from signals from their matched normal tissues, there is a significant correlation between increased p38γ and PTPH1 protein expression (Fig. 6A, right). Because Ras induces both PTPH1 and p38γ expression and depletion of either of them or disruption of their binding inhibits malignant growth, a coupling of hyper-expressed p38γ with PTPH1 in primary tissues further suggests their cooperative oncogenic activity under pathophysiologic conditions.

Because PTPH1 was shown as a p38γ MAPK isoform–specific phosphatase, we wished to explore if levels of endogenous PTPH1 protein expression negatively correlate with intrinsic p38γ phosphorylation. Because p-p38γ proteins are undetectable by direct Western blot, total p-p38s were isolated by IP and examined for p38γ abundance by Western blot for its correlation with the residual PTPH1 after the shRNA-induced PTPH1 depletion. Results in Fig. 6B (left) show that the levels of decreased PTPH1 protein expression from the input control seem to be negatively correlated with increased p-p38γ phosphorylations from the precipitates. To further link this negative correlation with the growth regulatory activity of the p38γ/PTPH1 complex, protein samples were prepared from HCT116 tumors excised from no. 1 and no. 4 mice and further analyzed by p-p38 IP/Western blot. *In vivo* tumor growth assay showed that PTPH1 depletion exhibited greater tumor growth inhibition in no. 4 mouse (0.17/0.72; shPTPH1/shLuc) over the no. 1 counterpart (0.31/0.45; Fig. 5A, inset), which again couples with greater PTPH1 depletion and a more substantial p-p38γ elevation from p-p38 precipitates (Fig. 6B). These results thus provide further evidence to

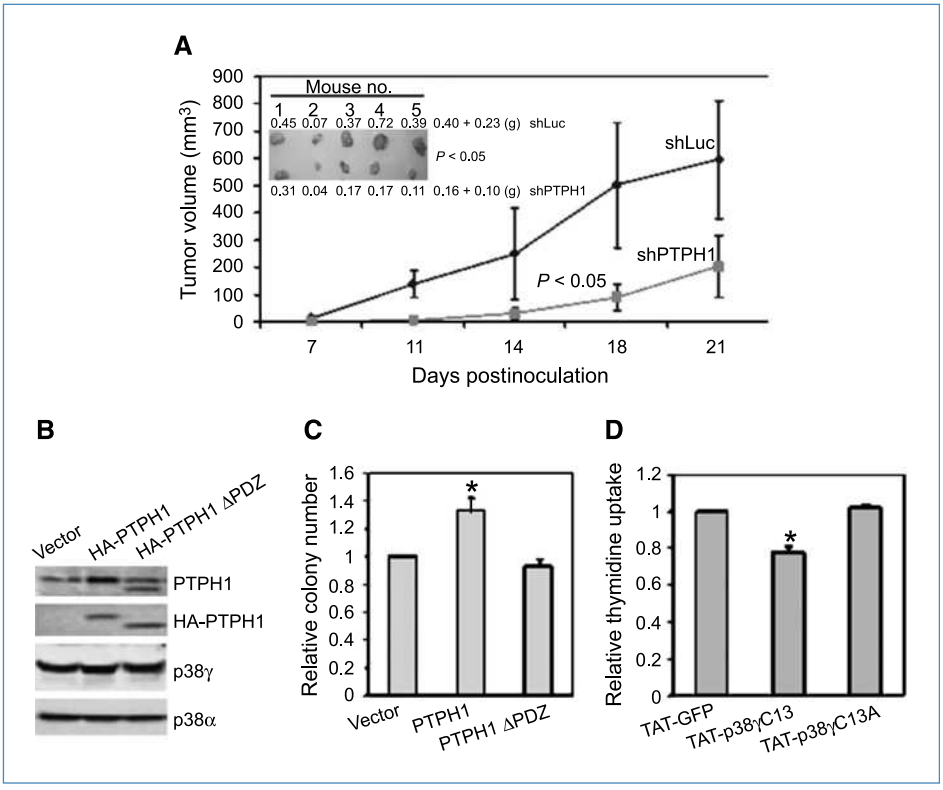

Figure 5. PTPH1 promotes colon cancer growth *in vivo* and requires its p38γ-binding activity to increase Ras-dependent growth *in vitro*. A, PTPH1 depletion inhibits tumor growth in mice. HCT116 cells were stably infected with Lenti-shLuc or Lenti-#1shPTPH1 in cell culture (see Supplementary Fig. S4A for Western blot) and 2×10^6 of these cells were s.c. injected into both front flanks of nude mice (right, shLuc; left, shPTPH1) and tumor growth was monitored. Moreover, tumors were excised, photographed, and weighed at the end of the experiment (inset; $P < 0.05$, between two groups for tumor weight or volume in all time points) and similar results were obtained from two additional experiments (Supplementary Fig. S3A and B). B and C, PTPH1 requires its PDZ domain to increase Ras transformation. IEC-6/K-Ras cells were stably expressed with PTPH1 or its PDZ-deleted mutant PTPH1ΔPDZ and subjected to Western blot (B) and soft-agar assays (C; mean of three separate experiments; *, $P < 0.05$). D, disruption of the p38γ-PTPH1 interaction inhibits colon cancer cell proliferation. HCT116 cells were incubated with WT and mutant peptides as described in Fig. 3D, with a TAT-GFP as a separate control, and cell growth was estimated by thymidine incorporation as previously described (10). Results shown (relative to TAT-GFP control) are mean of three separate experiments (*, $P < 0.05$, p38γC13 versus p38γC13A).

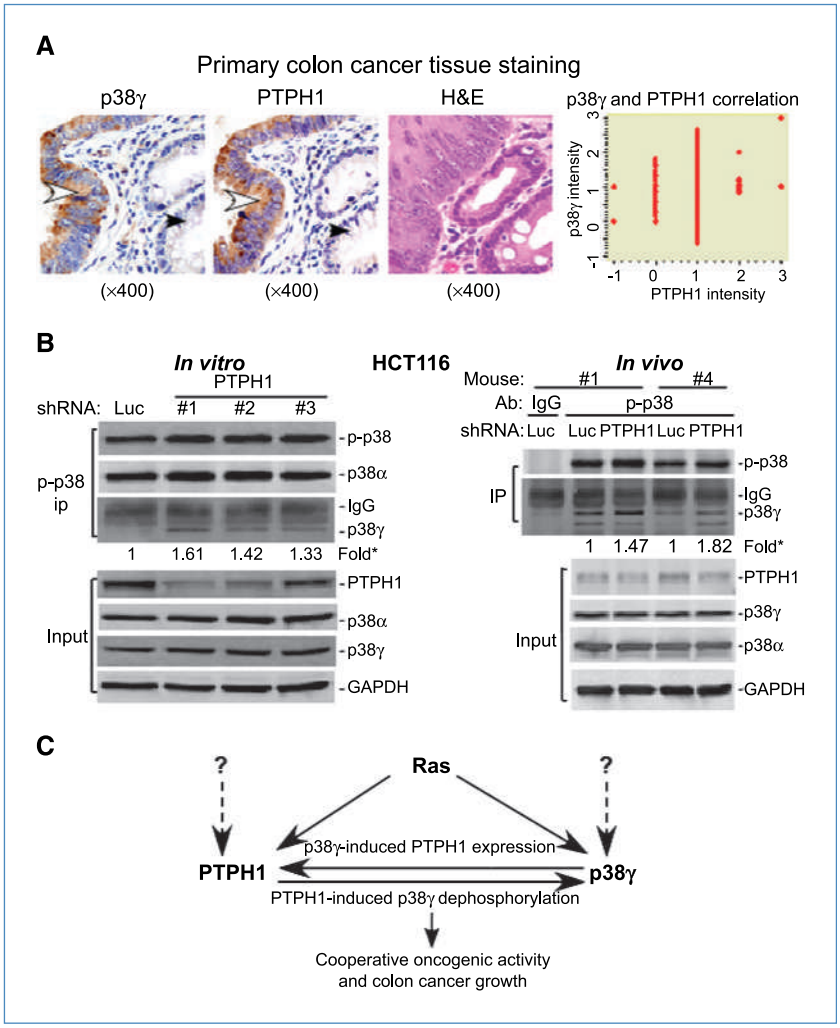

Figure 6. Roles of PTPH1 and p38γ in colon cancer. A, hyper-expressed p38γ correlates with increased PTPH1 expression in primary colon cancer tissues. p38γ and PTPH1 protein expressions in invasive colon carcinomas were analyzed by immunohistochemical staining. Representative pictures from the same patient were given in the two left images, showing increased positive brown signals for p38γ and PTPH1 in malignant tissues (indicated by an open arrowhead) over those in normal glands (marked with a closed arrowhead). A Pearson's correlation was reached between increased p38γ and PTPH1 protein expression in this group of specimens (142 cases) after subtracting signals of the tumors from those of matched normal tissues ($P < 0.05$, right). Additional results on p38γ/PTPH1 protein expression in colon cancer specimens are shown in Supplementary Fig. S4C and Supplementary Table S1. B, levels of endogenous PTPH1 protein expression inversely couple with intrinsic p38γ phosphorylation. Cells were depleted of PTPH1 protein and subjected to IP/Western blot analysis for increased p-p38γ proteins (IP; left). Right, protein samples were prepared from two pairs of tumors (#1 and #4 mice, Fig. 5A, inset) and subjected to IP/Western blotting analysis, which together with another set of experiments (Supplementary Fig. S3C) showed a coupling of decreased PTPH1 protein expression with increased p38γ phosphorylation. *, fold increase in p-p38γ over individual shLuc control. C, an experimental model shows a PDZ-mediated cooperative oncogenic activity of p38γ MAPK with its phosphatase PTPH1. Ras is shown to increase the protein expression of p38γ and PTPH1 in which PTPH1 is also induced by its substrate p38γ and acts to dephosphorylate p38γ through PDZ-mediated binding. Experimental evidence is presented to indicate that it is the PDZ-mediated p38γ/PTPH1 complex that possesses cooperative oncogenic activity leading to increased colon cancer growth. Dotted lines, p38γ and PTPH1 may be upregulated by Ras-independent proliferative signals.

indicate the cooperative oncogenic activity of p38γ with its phosphatase PTPH1 through resultant p38γ dephosphorylation (Fig. 6C).

Discussion

MAPKs function through coordinative phosphorylation and dephosphorylation to regulate dynamic cellular programs leading to various biological responses. Although extensive efforts have been made, there have been thus far no reports about a cooperative activity of a MAPK with its phosphatase (26). Our studies reported here first identified the p38γ MAPK isoform–specific phosphatase PTPH1 and then provide evidence to indicate that it is the p38γ/PTPH1 complex that possesses an oncogenic activity through PDZ-mediated direct binding and resultant p38γ dephosphorylation. This conclusion is supported by the following observations: (*a*) Removal of p38γ COOH-terminal PDZ-motif eliminates both

its oncogenic activity and its interaction with/dephosphorylation by PTPH1; (*b*) deletion of the PDZ domain of PTPH1 also leads to a loss of both its p38γ-binding/dephosphorylating activity and its promoting effect on Ras transformation; (*c*) depletion of p38γ or PTPH1 alone inhibits malignant growth, which can be mimicked by application of a specific peptide through inhibition of p38γ-PTPH1 interaction and resultant p38γ dephosphorylation; (*d*) Ras induces both p38γ and PTPH1 expression; and (*e*) there is a correlation of hyper-expressed p38γ with PTPH1 in primary colon cancer tissues and a coupling of decreased PTPH1 expression with increased intrinsic p38γ phosphorylation. These results together reveal a cooperative oncogenic activity of p38γ MAPK with its phosphatase PTPH1 through PDZ-mediated interaction (Fig. 6C).

MAPKs are best known to be inactivated by dual-specificity (Thr/Tyr) MAPK phosphatases, with most of MAPK phosphatases, however, acting on more than one MAPK member (27). Our results showed that p38γ, but not p38α, MAPK is a physiologic PTPH1 substrate, and this specificity seems to be determined by both of its COOH-terminal PDZ binding sequence ETPL and the conserved TGY motif within the kinase subdomain. This is because either removal of p38γ COOH terminus or mutation of the TGY disrupts its interaction with PTPH1, and fusion of the p38γ COOH-terminal fragment to p38α confers PTPH1-binding activity. Among the 10 classic and nonclassic MAPK family members, p38γ (also called ERK6 or MAPK12) is the only kinase that meets this dual requirement (26). This unique p38γ structure together with the fact that PTPH1 is the only classic PTP that contains a single PDZ domain (28) may be the foundation for their specificity. We are currently investigating if the PDZ binding enables PTPH1 as a p38γ substrate that may additionally contribute to their cooperative oncogenic activity.

In contrast to the proliferative effect shown in this study, PTPH1 transfection in mouse NIH3T3 cells was previously reported to inhibit cell cycle progression (19), which may be a tissue- and/or species-specific effect. Besides PTPH1, several PTPs are known to be oncogenic, including SHP2 (*PTPN11*; ref. 29), HePTP (30), and PTP1B (31), but none of these is shown to promote malignant growth through cooperation with its substrate. Our results, on the other hand, indicate that PTPH1 may cooperate with its substrate p38γ to increase malignant growth. This finding is further highlighted by the fact that Ras induces both p38γ/PTPH1 protein expression (Fig. 4A) and p38γ dephosphorylation (9), and nonphosphorylated p38γ is more potent in increasing Ras transformation (11). It remains unclear, however, if PTPH1 depends on its phosphatase activity to increase malignant growth and whether PTPH1 is required for Ras-induced p38γ dephosphorylation. Future studies toward these goals may reveal the p38γ/PTPH1 complex as a novel target in Ras-dependent malignancies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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