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Original Article

Protein tyrosine phosphatase PTPH1 potentiates receptor tyrosine kinase HER2 oncogenesis via a PDZ-coupled and phosphorylation-driven scaffold

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Abstract: Cancer cell overexpresses numerus proteins, however, how these up-regulated proteins, especially those enzymatically opposite kinases and phosphatases, act together to promote oncogenesis is unknown. Here, we reported that protein tyrosine phosphatase H1 (PTPH1) is a scaffold protein for receptor tyrosine kinase (HER2) to potentiate breast tumorigenesis. PTPH1 utilizes its PDZ domain to bind HER2, p38γ, PBK, and YAP1 and to increase HER2 nuclear translocation, stemness, and oncogenesis. PTPH1 de-phosphorylates HER2 and reciprocally increases HER2 protein expression dependent on cellular content. PTPH1 itself can be phosphorylated at S459 by redundant kinases p38γ and/or PBK, thereby distinctively regulating expression and/or turnover of scaffold proteins. Moreover, PTPH1 and HER2 cooperate to increase PBK and Yap1 transcription thus acting as an additional mechanism to activate the scaffold. PTPH1 protein levels are higher in HER2$^+$ breast cancer in which their phosphorylated forms are inversely correlated, indicating an integrated oncogenic activity through coordinated PTPH1 phosphorylation and HER2 de-phosphorylation. Combinational, but not individual, application of scaffold-kinases' inhibitors suppresses xenograft growth in mice. Thus, a PDZ-coupled and phosphorylation-driven scaffold can integrate proliferative signaling of enzymatically distinct proteins as a super-oncogene and as a target for combination therapy.

Keywords: PDZ-scaffold, phosphatase/kinase crosstalk, reciprocal regulation, protein tyrosine phosphatase H1 (PTPH1), HER2 tumorigenesis, breast cancer

Introduction

Deregulated receptor tyrosine kinases (RTKs) are implicated in nearly all types of human cancers and have been main targets for cancer therapy. Among this family of proteins, human epidermal growth factor receptor 2 (HER2, Neu or ERBB2) is particularly notable as it is over expressed in up to 30% of human breast cancers [1]. Elucidation of mechanisms by which HER2 proteins are overexpressed in cancer may be fundamentally important to understand and intervene HER2-dependent malignancies such as breast cancer [2].

Protein-protein interactions dictate integrated cellular outcomes [3]. Binding of HER2 to a PDZ (PSD95/DLG/ZO-1) domain of an Erbb2 interacting protein (Erbin, a tumor suppressor) increases HER2 stability and oncogenesis with mechanisms involved however unknown [4, 5]. PDZ is an 80-90 amino acid structural domain

that has a central role in organizing and assembling diverse cell signaling proteins. PDZ domain proteins specifically interact with proteins containing C-terminal peptide PDZ motifs, but also recognize internal peptide sequences. Protein tyrosine phosphatase H1 (PTPH1 with a gene name: *PTPN3*) [6] is a PDZ-domain protein with oncogenic properties in breast and colon cancer. PTPH1 depends on PDZ-domain to interact with and to specifically dephosphorylate PDZ-motif p38γ MAPK. Of interest, PTPH1 is paradoxically phosphorylated and activated by p38γ, which can potentiate both their oncogenic activity [7, 8] by an unknown mechanism. PTPH1 can also bind nuclear vitamin D receptor (VDR), estrogen receptor (ER), and the membrane epidermal growth factor receptor (EGFR) in breast cancer cells to drive a proliferative response [9-12]. The binding capability of HER2 to the PDZ domain protein of Erbin suggests it may be able to interact with PTPH1 to regulate its activity. Studies of PTPH1-HER2 interaction may reveal a novel HER2 de-phosphorylation pathway that may contribute to HER2 oncogene signaling.

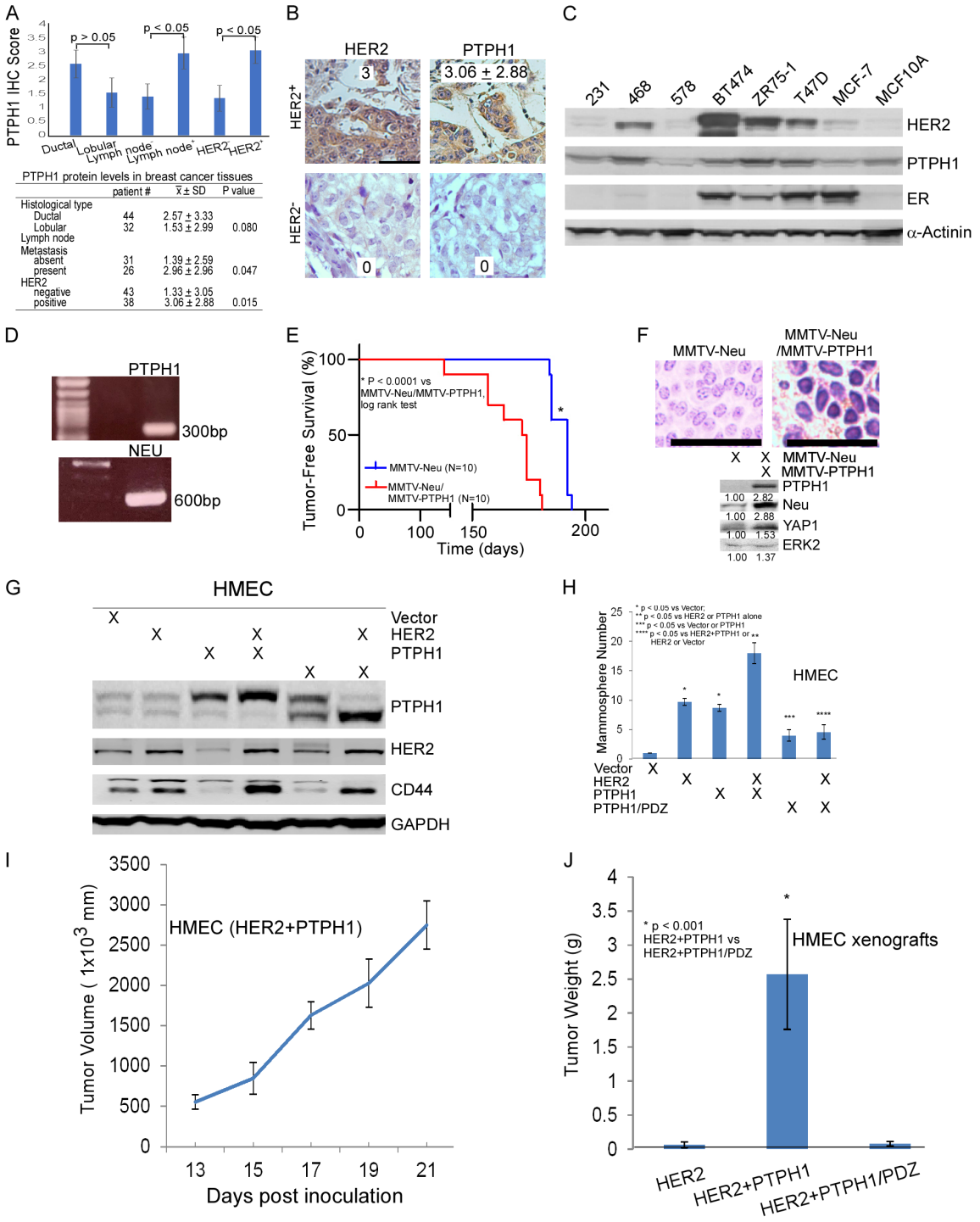
HER2 oncogenic signaling is regulated by multiple pathways [13]. HER2 dimerizes with itself or other epidermal growth factor receptors such as EGFR to induce receptor auto-phosphorylation and to activate downstream signal transduction pathways [14]. HER2 kinase activity is suppressed by small molecular tyrosine kinase inhibitors (TKI) such as lapatinib (Lap) through binding to the ATP-binding pocket of the cytoplasmic tyrosine kinase domain [15]. Lap has an established activity in the treatment of HER$^+$ breast cancer with resistance as a major limiting factor [16]. Of interest, Lap decreases HER2 phosphorylation but stabilizes HER2 protein, and cooperates with HER2 antibody trastuzumab to inhibit breast cancer growth [17], indicating an intrinsic link between decreased HER2 phosphorylation and increased HER2 protein stability. This notion is further supported by the fact that HER2 overexpression does not induce receptor auto-phosphorylation but increases its protein stability and sustains its proliferative signaling [18]. Recent studies also showed that breast cancer patients with a clinical classic HER2 negative phenotype such as detectable HER2 (HER2^{+1}) respond similarly to anti-HER2 therapy as those with HER2 positive (HER2^{+2}) [19], indicating that expression level of HER2 protein is a major determinant of HER2 small molecular inhibitors.

Our results showed that PTPH1 protein levels are higher in HER2 positive breast cancer and that PTPH1 promotes HER2 de-phosphorylation, stimulates HER2 protein expression, and increases HER2 tumorigenesis in transgenic mice and xenograft model. Further, the PDZ domain of PTPH1 is essential for binding HER2, PDZ-binding kinase (PBK), p38γ, and yes-associated protein 1 (YAP1) and to increase HER2 nuclear accumulation, HER2 stemness and oncogenesis. Moreover, combined application of small molecular inhibitors of scaffold kinases PBK or p38γ and HER2 inhibits breast cancer xenograft growth. Our results reveal that cancer cells can integrate and propagate oncogenic activities of enzymatically opposite proteins by forming a PDZ-coupled and phosphorylation-driven scaffold that can act as a super-oncogene and as a combination therapy target.

Results

PTPH1 is upregulated in HER2$^+$ breast cancer, increases HER2 tumorigenesis in transgenic mice, and depends on PDZ to increase HER2 stemness and transformation

To test if PTPH1 is implicated in HER2 pathogenesis, we assessed its protein expression in human breast cancer tissues by immunohistochemistry (IHC) [10]. **Figure 1A** and **1B** showed a higher level of PTPH1 proteins in HER2 positive (HER2$^+$: clinical IHC score; 1, 2, and 3) than in HER2 negative breast cancer tissues (HER2$^-$: the clinical IHC score; 0), indicating a potential role of PTPH1 in HER2$^+$ breast cancer. The clinical classic HER2 negative (HER2^{+1}) was considered HER2 positive [20] in our study because one recent study showed a similar therapeutic response for HER^{+1} and HER2^{+2} patients treated with anti-HER2 therapy [19]. Consistent with progressive nature of HER2$^+$ breast cancer, PTPH1 protein levels were also higher in lymph node metastatic breast cancer (**Figure 1A**). In addition, PTPH1 was detected in 5 of 5 HER2$^+$ human breast cancer cell lines (**Figure 1C**), indicating that upregulated PTPH1 may contribute to malignant development and progression of HER2$^+$ breast cancer.



PTPH1 protein levels in breast cancer tissues

	patient #	x̄ ± SD	P value
Histological type			
Ductal	44	2.57 ± 3.33	
Lobular	32	1.53 ± 2.99	0.080
Lymph node			
Metastasis			
absent	31	1.39 ± 2.59	
present	26	2.96 ± 2.96	0.047
HER2			
negative	43	1.33 ± 3.05	
positive	38	3.06 ± 2.88	0.015

Figure 1. PTPH1 potentiates HER2 tumorigenesis. (A-C) PTPH1 protein level is higher in HER2$^+$ breast cancer tissues and cells. PTPH1 and HER2 proteins in breast cancer tissues were assessed by IHC (A, top bar graph derived from the bottom table from which *p* values were calculated by Kruskal-Wallis test, n = 81). HER2 IHC scores and clinical information were obtained from the data base of Department of Pathology and PTPH1 staining results were reorganized from those previously published [10] according to the HER2 status. Representative HER2 and PTPH1 staining was shown (B, scale bar, 50 μM for all unless specified) [10], and PTPH1 and HER2 protein expression in breast cancer cell lines were analyzed by Western Blot (WB, C, similar results were obtained in a separate WB). (D-F) Transgenic PTPH1 potentiates HER2 mammary tumorigenesis in mice. Genotypes of MMTV-HA-PTPH1 and MMTV-Neu mice were shown in (D). Kaplan curves of tumor-free survival for *MMTV-Neu* (Jackson) and *MMTV-Neu/*

MMTV-PTPH1 mice were shown (E, n = 10 mice, P < 0.0001, log rank test), whereas representative H&E staining of primary breast tumors (top) and WBs showing protein expression of breast tumors were given in (F, ERK2 as a loading control, scale bar: 50 μM). (G-J) PTPH1 depends on PDZ to potentiate HER2 stemness *in vitro* and HER2 transformation in mice. HMEC cells co-expressed with HER2 and PTPH1 or its PDZ-deleted mutant (PTPH1/PDZ) were assessed for protein expression by WB (G). These cells were also analyzed for tumor-sphere formation (H) as described [24] and results are mean of three separate experiments with only data from the 1st tumor-sphere shown, which were largely reproduced in MCF10A cells (Figure S1G, S1H). For xenograft studies, 5×10^6 HMEC cells in 100 μl of 50% Matrigel solution were s.c. injected into nude mice at both sites as indicated (Figure S2C). Resultant tumors in HER2 and HER2 + PTPH1/PDZ groups are too small for measurements (Figure S2C, S2D) and only the growth curve from HER2 + PTPH1 cells was shown in (I) (means ± SD, n = 5 mice), but tumor weights in all palpable groups were given in (J) (means of 5 mice, ± SD).

To demonstrate this possibility in a genetic mouse model, we generated a MMTV-HA-PTPH1 transgenic mouse in an FVB background through a contract with Cyagen Biosciences, which was then bred it with MMTV-Neu mouse expressing activated c-neu (Jackson Lab) [21] (**Figure 1D**). While there was no spontaneous mammary tumor formation in MMTV-HA-PTPH1 mice (data not shown), tumor-free survival time of double transgenic MMTV-Neu/MMTV-PTPH1 mice was significantly decreased as compared with MMTV-Neu mice that had a medium tumor-free survival T50 similar to the previously reported in this model [22] (**Figure 1E**). Moreover, breast tumors of double transgenic mice had elevated HER2, PTPH1, and YAP1 proteins (**Figure 1F**), indicating that PTPH1 transgene increases HER2-driven mammary tumorigenesis in mice by a mechanism associated with their cooperative upregulation of YAP1 in tumor tissues. Moreover, HER2 and PTPH1 co-transfection in human breast cancer 231 and T47D cells also stimulated each other protein expression as observed in transgenic mice (**Figures 1F** and S1A, S1B). These results together demonstrate that PTPH1 and HER2 are co-overexpressed in breast cancer tissues, cooperate in mammary tumorigenesis in mice, and increase each other protein expression in breast cancer cells.

We next tested how PTPH1 potentiates HER2 tumorigenesis. In primary mouse breast tumor cells (NEU) isolated from MMTV-Neu mice [21], PTPH1 overexpression increased colony formation and upregulated HER2 proteins, further confirming that PTPH1 overexpression increases oncogenesis by elevating HER2 proteins as observed in mice (Figure S1C, S1D). PTPH1 silencing in the same cells, however, decreased colony formation without reducing HER2 protein levels, indicating that endogenous PTPH1 is oncogenic independent of HER2 (Figure S1C, S1D). In $HER2^+$ BT474 cells, however, HER2 depletion decreases PTPH1 proteins and colony formation, both of which were rescued by PTPH1-re-expression (Figure S1E, S1F). These results indicate that HER2 is required for PTPH1 expression and for PTPH1 oncogenic activity and that PTPH1-forced expression in HER2 depleted (or negative) cells is sufficient to increase HER2 expression and cell growth. Thus, PTPH1 signals downstream for HER2 in breast cancer cells and may cooperate with HER2 to promote breast oncogenesis.

Cancer-like stem cells (CSC) represent a small portion of more proliferative cells that are responsible for tumorigenesis and resistant to therapy, whereas HER2 has an established role in stimulation of CSC expansion [23]. Because PTPH1 has a functional PDZ domain [9] and PDZ binding is important for HER2 oncogenesis [5], we explored whether PTPH1 depends on PDZ to potentiate HER2 stemness, which may serve as a key mechanism for their cooperative oncogenic activity. PTPH1 and its PDZ-deleted mutant (PTPH1/PDZ) were stably co-expressed with HER2 in two normal human mammary epithelial cell lines (HMEC and MCF10A) by retrovirus and their effects on tumor-sphere formation (an indicator of CSC phenotype) [24] were assessed. Figure S1G and S1H showed that PTPH1 expression alone in MCF10A cells was sufficient to increase tumor-sphere formation and that its co-expression with HER2 further increased tumor-sphere numbers and elevated HER2 and PTPH1 protein expression. This potentiation effect however is significantly diminished in HER2 cells co-expressed with PTPH1/PDZ. Similar results were obtained in HMEC cells [25] with a significant induction of the CSC marker CD44 in PTPH1 and HER2 co-expressed cells (**Figure 1G** and **1H**). Transformed HMEC cells can form xenografts [25] and we determined if PTPH1 depends on PDZ

to increase HER2-induced xenograft growth. The same number of HER2 transformed HMEC cells stably co-expressed with PTPH1 or PTPH1/PDZ were injected s.c. into female nude mice for their tumor forming activity. Results (**Figures 1I**, **1J** and S2C-E) showed that PTPH1/HER2 co-expression dramatically increased xenograft formation as compared with those expressed with either alone, whereas HER2/PTPH1/PDZ co-expressed cells lacked such effect. IHC analysis of primary HER2 tumors showed that increased tumorigenesis is associated with elevation of another CSC marker CD133 and decreased Casp3 (likely as a result of increased tumor growth) in HER2/PTPH1 co-expressed tumor in which proliferation marker Ki67 is almost undetectable in both tumors (Figure S2A, S2B). These results together demonstrate that PDZ is essential for PTPH1 to increase HER2 stemness *in vitro* and HER2-induced xenograft growth in mice, which may be the key mechanism for PTPH1 potentiating HER2 tumorigenesis.

PTPH1 depends on its activity and cellular content to decrease HER2 phosphorylation and to reciprocally increase HER2 expression and PTPH1 depends on PDZ and S459 to regulate HER2 protein stability

HER2 phosphorylation and overexpression are both important for its oncogenic activity [26] and we next determined if PTPH1 regulates HER2 tyrosine phosphorylation and protein expression by co-transfection. WB analysis of 293T cells transfected with HER2 and PTPH1 or its phosphatase-deficient mutant PTPH1/S459A [7] showed that PTPH1 decreased HER2 phosphorylation and increased HER2 protein expression and both of these effects were attenuated in PTPH1/S459A co-transfected cells as compared to HER2 transfection alone, it did not further elevate PTPH1 in HER2/PTPH1 co-expressed cells (**Figure 2A**). These results indicate that PTPH1 depends on its activity to decrease HER2 phosphorylation and to increase HER2 protein expression. Similar effects were demonstrated with another phosphatase substrate-trapping mutant PTPH1/DA [27] in which the coupling of decreased p-HER2 and increased HER2 protein levels by PTPH1 however was demonstrated at 72 h but DA mutant was less efficient in decreasing p-HER2/Y877 at both 48 h and 72 h after transfection (**Figure 2B**). Thus, a certain threshold of p-HER2/HER2 as well as of PTPH1/HER2 in cells may be needed for the coupling effect of PTPH1 in decreasing HER2 phosphorylation and increasing HER2 protein expression. These results indicate that PTPH1 depends on phosphatase activity and cellular contents (i.e., p-HER2/HER2 and PTPH1/HER2 ratio) to regulate HER2 phosphorylation and expression.

To test whether PTPH1 depends on its protein level to increase endogenous HER2 expression in breast cancer cells, PTPH1 inducible expression system (Tet-on) was used. Twelve h after Tet addition, PTPH1 was induced about 10-fold leading to an elevation of HER2 protein by 1.5-fold ($P < 0.05$ vs No Tet in both cases (**Figure 2C**). After a further incubation with and without Tet for 24 and 48 h, induced PTPH1 proteins became further gradually attenuated, whereas induced HER2 protein was slowly back to normal (**Figure 2C**), indicating that additional proteins may be needed to maintain upregulated HER2. Stable PTPH1 and/or HER2 expression in 468 breast cancer cells also decreased p-HER2 and increased each other protein expression (**Figure 2D**), indicating a reciprocal stimulation of PTPH1 and HER2 protein expression and a decrease of p-HER2 by PTPH1. Together, these experiments together demonstrate that PTPH1 depends on its phosphatase activity and cellular content to decrease HER2 phosphorylation and to reciprocally increase HER2 protein expression.

The reciprocal stimulation of protein expression by PTPH1 and HER2 may be critical for their cooperative oncogenic activity through a scaffold [28]. We next examined if tyrosine phosphorylation of HER2 is also important for its reciprocal regulatory activity with PTPH1. HER2$^-$ 231 cells were stably expressed with HER2 or its dominant negative mutant HER2/Y877F, which were further transfected with PTPH1 or control vector for their reciprocal regulation. A reciprocal increase of HER2 and PTPH1 protein expression was again observed in HER2/PTPH1 co-expressed 231 cells (**Figure 2E**). In HER2/Y877F alone transfected cells, p-HER/Y877 signals were higher than those in Vector cells, likely as a reactive response from the intrinsic HER2. PTPH1 co-expression led to decreased PTPH1 and increased HER2 as compared to cell co-transfected with HER2 (**Figure 2E**). Together, these results indicate that tyrosine

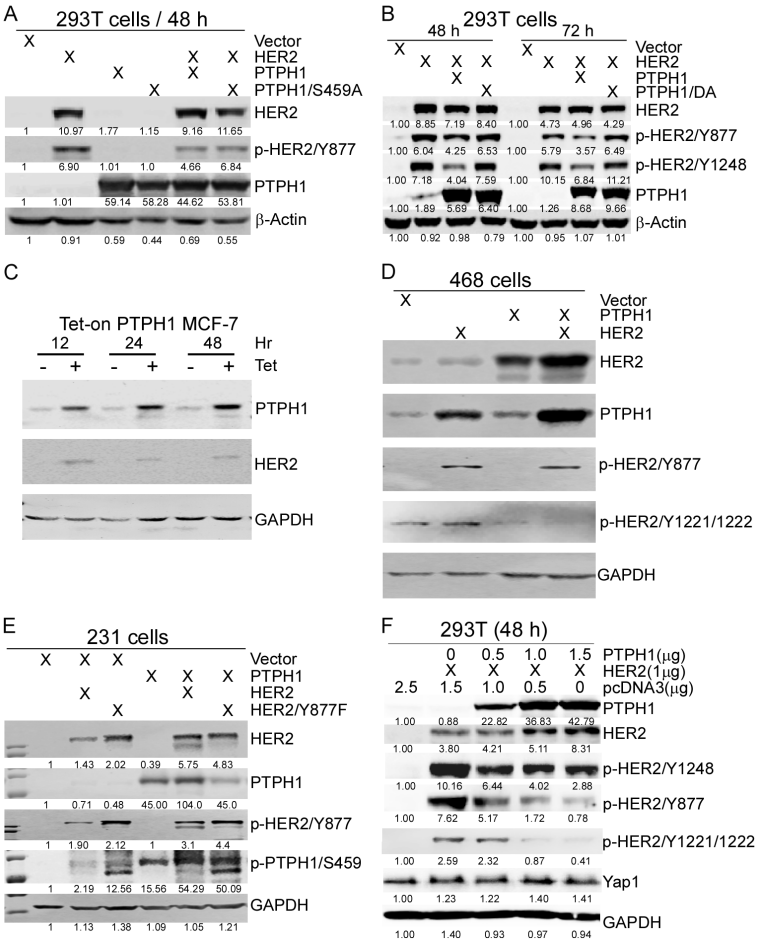


Figure 2. PTPH1 decreases HER2 phosphorylation and increases HER2 protein expression dependent on phosphatase activity, HER2/Y877 phosphorylation, and their cellular contents. (A, B) Phosphatase-deficient mutants PTPH1/S459A (A) and PTPH1/DA (B) have decreased activity in decreasing HER2 phosphorylation and in increasing HER2 protein expression in 293T cells by co-transfection. Cells were transfected with indicated constructs for 48 or 72 h before analyzed by WB. These experiments were repeated two to four times and their quantitative results (using the Image J software) were given. (C) Inducible PTPH1 expression transiently increases endogenous HER2 protein expression in breast cancer cells. PTPH1 inducible (Tet-on) MCF-7 cells were cultured with and without tetracycline for indicated time and cells were analyzed by WB (p-HER2 undetectable). These experiments were repeated once. (D) PTPH1-induced decrease in p-HER2 couples with an increase in HER2 and PTPH1 proteins in breast cancer cells. Cells (468) were stably expressed with PTPH1 and/or HER2 via retroviral infection and analyzed by WB. Similar results were obtained in a separate experiment. (E) Y877 phosphorylation is required for HER2 increases of PTPH1 protein expression in breast cancer cells. Indicated constructs were stably co-expressed with retrovirus vector through antibiotic selection and resistant cells were analyzed by WB. These experiments were performed three times. (F) Different amounts of PTPH1 constructs were co-transfected with HER2 (1 μg) in 293T cells, which were analyzed by WB 48 h later. These experiments were performed three times.

phosphorylation of HER2, at least at Y877, is required for HER2 increasing PTPH1 expression.

To further test if cellular PTPH1/HER2 ratio impacts HER2 de-phosphorylation and/or their reciprocal stimulation of protein expression, we transfected different amounts of PTPH1 plasmid together with a fixed amount of HER2 construct (1 μg) for 48 h and analyzed protein expression and phosphorylation by WB. **Figure 2F** showed that PTPH1 dose-dependently increased HER2 protein levels and decreased HER2 phosphorylation. Similar regulatory effects of PTPH1 on p-HER2 levels at Y1248 and/or Y1221/1222 were also observed in **Figure 2B**, **2D**, and **2F**, indicating an active role of PTPH1 in dephosphorylation of HER2 at multiple residues. The increased HER2 protein is not a consequence of increased proliferative response, as a dose-dependent elevation of protein expression was not observed for proliferative YAP1, a transcription co-activator. Together these results demonstrate that PTPH1 decreases HER2 phosphorylation and reciprocally increases HER2 protein expression dependent of cellular contents. This property may be fundamentally important for PTPH1/HER2 cooperative oncogenic activity via scaffolding (**Figures 1A-J** and S1, S2).

Protein phosphorylation is frequently associated with ubiquitination. To determine if PTPH1 increases HER2 protein levels by regulating its ubiquitination, HER2 was co-transfected with PTPH1 together with an ubiquitin-expressing construct in 293T cells and HER2 precipitates were analyzed. Results showed that PTPH1 transfection reduced HER2 ubiquitination in association with decreased

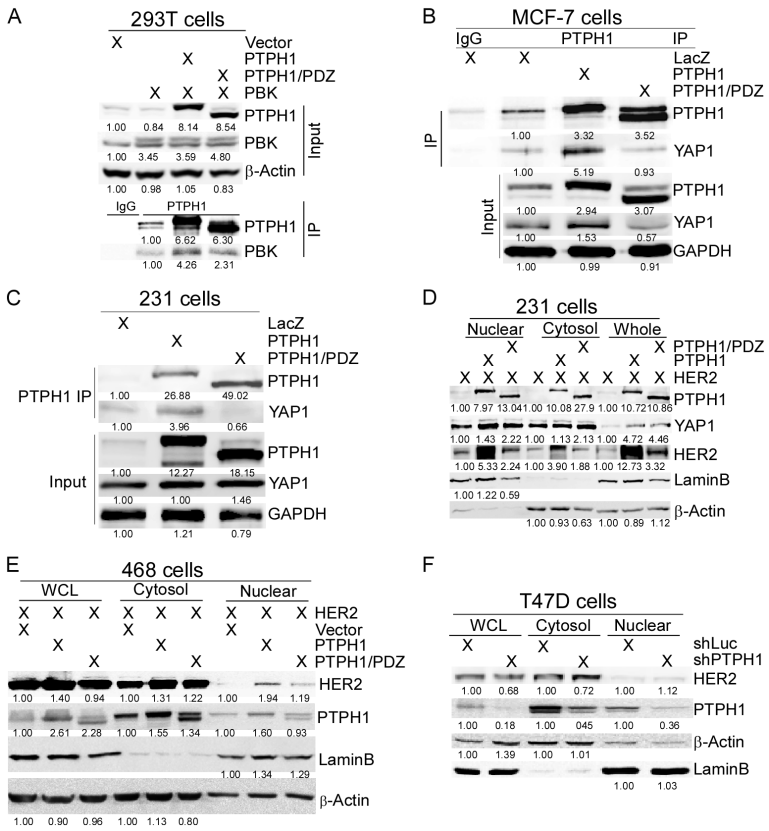


Figure 3. PTPH1 depends on PDZ to bind PBK and YAP1 and to promote HER2 nuclear accumulation in breast cancer cells. (A) PTPH1 depends on PDZ to bind PBK in 293T cells. Cells were transiently expressed with the indicated constructs, and PTPH1 precipitates were analyzed by WB and similar results were obtained in a separate experiment. (B, C) PTPH1 depends on PDZ to bind YAP1 in breast cancer cells. MCF-7 and 231 breast cancer cells were stably expressed with indicated constructs in retro-viral vector, which were analyzed by PTPH1 IP/WB with a portion of whole cell lysate as input control. (D, E) PTPH1 depends on PDZ to increase nuclear accumulation of co-transfected HER2. Breast cancer 231 (D) and 468 (E) cells were stably co-expressed with HER2 and PTPH1 or PTPH1/PDZ by retroviral infection and antibiotic selection. Cells were analyzed for cell fractionation as described [12]. Similar results in 231 cells were obtained in a separate experiment. (F) Knockdown (KD) of endogenous PTPH1 has no effect on nuclear HER2 protein concentration. T47D cells with (shPTPH1) and without PTPH1 KD (shLuc) cells were analyzed by cell fractionation for endogenous HER2 distributions. Similar results were obtained in a separate WB.

HER2 phosphorylation in HER2 precipitates (Figure S3A), indicating a potential role of PTPH1 in regulating HER2 ubiquitination through de-phosphorylation. Because PTPH1/S459 is phosphorylated by p38γ [7] and its mutant PTPH1/S459A has decreased activity in decreasing HER2 phosphorylation and increasing HER2 protein level (**Figure 3A**), we tested if expressing the S459A mutant in breast cancer cells affects p38γ and HER2 protein expression and stability. Figure S3B showed that PTPH1 transfection in T47D cells increased endogenous HER2 by about 2-fold over Vector cells and cells expressing PTPH1/S459A (at 0 h, *the number in italic*) but elevated p38γ and itself similarly as the mutant. These results indicate that PTPH1 expression depends on S459 to increase HER2 expression but stimulates its kinase p38γ and itself expression independent of S459.

Following incubation with cycloheximide (CHX, a protein synthesis inhibitor, 100 μg/ml), however, p38γ and its substrate PTPH1 became more stable in PTPH1/S459A cells than those expressing PTPH1 with HER2 degraded similarly in both groups. These results indicate that inhibition of PTPH1/S459 phosphorylation stabilizes p38γ as well as PTPH1 but has no effect on HER2 stability (Figure S3B). Thus, S459 phosphorylation can distinctively impact protein expression and/or turnover of PTPH1, its kinase, and its substrate within the scaffold, which may fine-turn their local concentrations via a coordinative phosphorylation and de-phosphorylation for their cooperative oncogenic activity.

To determine if PTPH1 depends on PDZ to reciprocally regulate HER2 protein stability, HER2 was transiently co-transfected with PTPH1 or PTPH1/PDZ in 293T cells, and their stability was examined. Consistent with the reciprocal stimulation of protein expression in several cell lines (**Figures 2D-F** and S1A, S1B), HER2 transfection stabilizes PTPH1 protein more effectively than PTPH1/PDZ, whereas HER2 is also more stable in cells co-transfected with PTPH1 (Figure S3C). These results illustrate a PDZ-dependent stabilizing phosphatase/substrate

complex, which may physically tether additional proteins to form a functional scaffold to promote oncogenesis.

PTPH1 depends on PDZ to bind PBK and YAP1, and to increase HER2 nuclear accumulation, whereas PBK promotes p-PTPH1/S459, PTPH1, HER2 and YAP1 expression in cells

PBK [29] and YAP1 [30] are oncogenic PDZ-binding proteins and were next examined for their interaction with PTPH1 for potential roles in scaffolding. Indeed, PTPH1 bound more co-transfected PBK than the PTPH1/PDZ mutant in 293T cells even though without impacts on PBK protein expression (**Figure 3A**). Moreover, stably transfected PTPH1 binds endogenous YAP1 in MCF-7 and 231 cells, whereas PTPH1/PDZ mutant completely lacks this activity in both lines. Of interest, transfected PTPH1/PDZ only decreased endogenous YAP1 expression in MCF7 but not in 231 cells (**Figure 3B**, **3C**), indicating that inhibition of PDZ binding by this mutant can suppress expression of a scaffold protein dependent of cellular contents.

PTPH1 is phosphorylated at S459 by serine/threonine kinase p38γ [7] and we next tested if serine/threonine kinase PBK may also be involved in PTPH1/S459 phosphorylation as a second kinase to activate PTPH1 in the scaffold. IP/WB analyses showed that p-PTPH1/S459 signals [7] in PTPH1 precipitates from PBK/PTPH1 co-transfected cells were higher than those from PBK cells co-transfected both with PTPH1/S459A and Vector, indicating that PBK may contribute to PTPH1 phosphorylation at S459 in cells (Figure S4A). Moreover, a separate co-transfection experiment showed that p-PTPH1/S459 was only detectable in PTPH1 precipitates from cells expressing PTPH1 but not its PDZ mutant (Figure S4B), indicating an essential role of PDZ in PTPH1 phosphorylation by co-transfected PBK. Because PBK and PTPH1 are upregulated in clinical breast cancer [10, 31], we next assessed if PBK may contribute to p-PTPH1/459 signals in breast cancer specimens. Figure S4C showed that increased PBK expression in a cohort of clinical breast cancer specimens was correlated with elevated p-PTPH1, indicating that PBK may contribute to PTPH1/S459 phosphorylation in clinical breast cancer. Furthermore, depletion of PBK in HER2^{+} BT474 cells decreased HER2, PTPH1, p-PTPH1, and YAP1 proteins in which HER2 is required for PBK, PTPH1 and p38γ expression and p38γ overexpression also increases PTPH1 and HER2 protein levels (Figure S4D-H). These results, together with those in **Figures 1**-**3** and S2, S3, S4, S5, indicate that PTPH1, HER2, p38γ, PBK, and YAP1 may form a PDZ-coupled scaffold resulting in elevation of their local concentrations that may cooperatively stimulate malignant process through coordinated phosphorylation and de-phosphorylation.

Scaffold often changes protein cellular localization [28] while nuclear translocation is important for HER2 oncogenic activity [32]. We next explored whether PTPH1 increases HER2 nuclear accumulation via PDZ binding. **Figure 3D** showed that PTPH1 transfection in 231 cells increased nuclear concentrations of co-transfected HER2, whereas PTPH1/PDZ had no such effect, indicating that PTPH1 depends on PDZ to increase HER2 nuclear accumulation. Similar effect was also demonstrated in 468 breast cancer cells (**Figure 3E**). To test if endogenous PTPH1 also impacts HER2 cellular distribution, T47D cells were stably infected with a viral vector expressing shPTPH1 or shLuc for cell fractionation analysis. **Figure 3F** showed that PTPH1 depletion moderately decreased HER2 proteins in whole cell lysates (WCL) and had no effect on nuclear HER2. These results demonstrate that only overexpressed, but not endogenous, PTPH1 drives HER2 nuclear accumulation via PDZ binding, which is consistent with PTPH1/HER2 co-overexpression in clinical breast cancer and with increased tumorigenesis in HER2/PTPH1 double transgenic mice (**Figure 1**).

PTPH1 and HER2 cooperate to stimulate PBK transcription and PTPH1 depends on PDZ to cooperate with PBK and YAP1 to stimulate AP-1 activity and to cooperate with HER2 to activate CSC transcriptional profile

PTPH1 increases HER2 nuclear accumulation (**Figure 3D**, **3E**) and we next examined if transcriptional regulations are involved in their cooperative oncogenic activity. PTPH1 was stably expressed with and without HER2 in MCF10A cells from which RNAs were prepared for RNA seq. analysis. Results showed that more than 1000 genes in 20 major pathways were upregulated in PTPH1/HER2-co-expressed cells as compared with those expressing either alone (**Figures 4A** and S4I). One of the

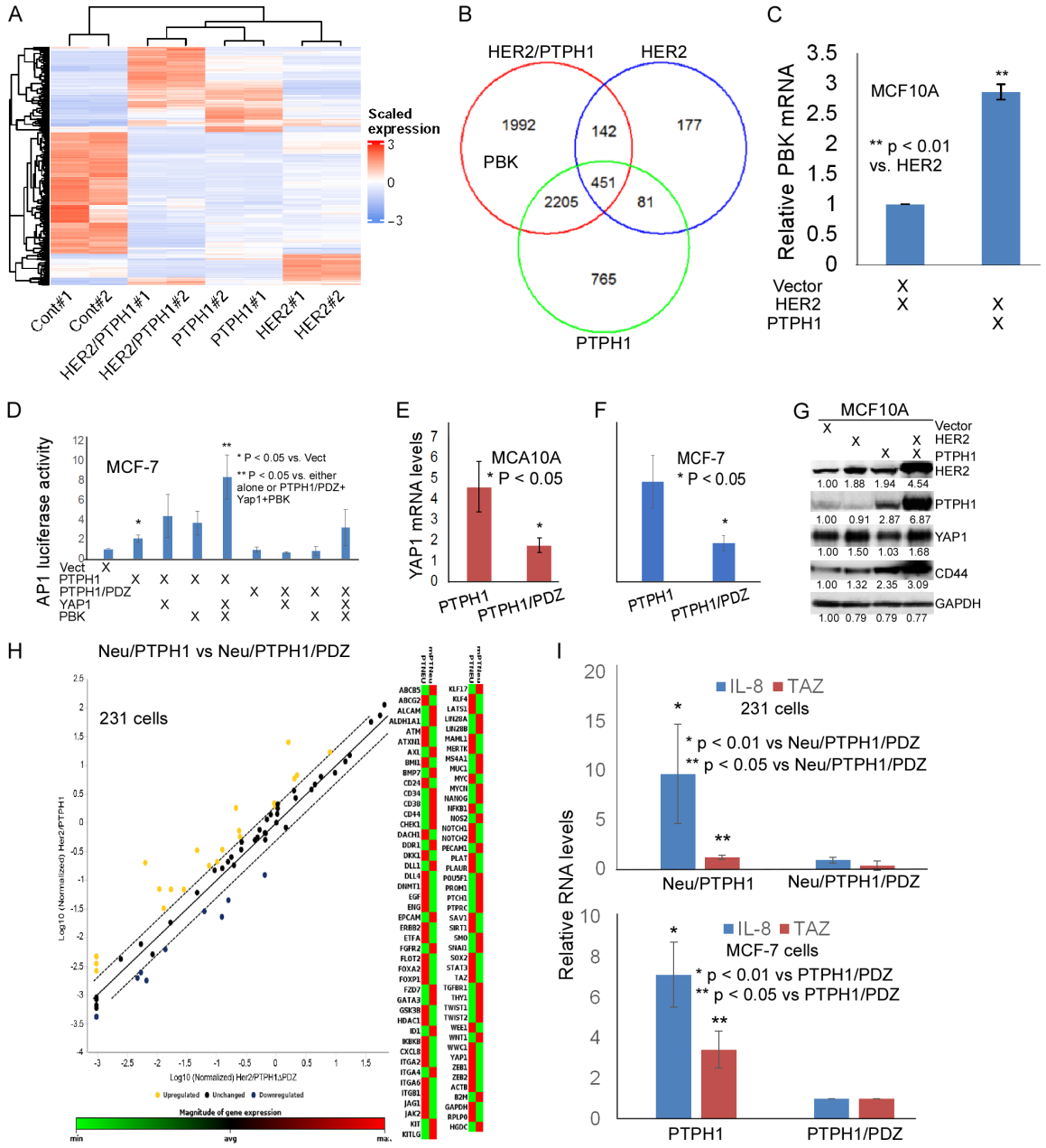


Figure 4. PTPH1 and HER2 collaborate to increase PBK transcription, whereas PTPH1 depends on PDZ to collaborate with YAP1 and PBK to activate AP-1, and with HER2 to stimulate CSC transcriptional profiles. (A, B) Heat map showed distinct patterns of gene expression in MCF10A cells overexpressed with HER2 ± PTPH1 as compared with those transfected with a vector (Cont.). RNA samples were prepared from indicated cells (in duplicate) and analyzed by RNA seq for different gene expression (A). A Venn diagram of genes showed numbers of distinct and common regulated genes in PTPH1 ± HER2 expressed MCF10A cells as compared with control cells in which PBK is one of most upregulated genes in PTPH1 + HER2 cells (B, $P < 0.001$). (C) PTPH1 potentiates HER2-induced elevation of PBK RNA expression. Indicated cells were analyzed for PBK RNA expression by qRT-PCR (means ± SD, n = 3). (D-F) PTPH1 depends on PDZ to collaborate with PBK and YAP1 to increase AP-1 activity (D) and to stimulate YAP-1 RNA expression in HER2-transfected MCF10A (E) and MCF-7 cells (F) (means ± SD, n = 3). (G) PTPH1 and HER2 increase each other protein expression and cooperate to increase CD44/YAP1 expression in MCF10A cells. Similar results were obtained in a separate WB. (H, I) PTPH1 depends on PDZ to cooperate with HER2 to stimulate CSC gene signature in breast cancer cells. PCR array was performed by using a Qiagen kit (H, PT, PTPH1; mPTPH1, PTPH1/PDZ; NEU, HER2). Elevated IL-8 and TAZ RNAs in 231 cells by HER2 and PTPH1 co-expression were further demonstrated by qRT-PCR as compared with those co-expressed with HER2 and PTPH1/PDZ (I, top, means ± SD, n = 3). Similar results were also obtained in MCF-7 cells stably expressed with PTPH1 as compared those expressed with PTPH1/PDZ (I, bottom, means ± SD, n = 3).

most up-regulated genes in the combination group is PBK, which was further confirmed by qRT-PCR analysis (**Figure 4B**, **4C**). Since PBK is involved in cellular PTPH1 phosphorylation, is required for PTPH1/HER2/YAP1 expression (Figure S4A-E), and binds PTPH1 and/or YAP1 via PDZ (**Figure 3A-C**), these results suggest that scaffold can be regulated and activated by a positive feedback loop through posttranslational and transcriptional mechanisms.

YAP1 plays a role in driving cancer-like stem cell (CSC) expansion [33], whereas activator protein-1 (AP-1) transcription factor is important for YAP1 activity [33] and for p38γ-induced CSC expansion [24]. The elevated YAP1 in tumor tissues of HER2/PTPH1 double transgenic mice (**Figure 1F**) and its presence in PDZ-dependent PTPH1 complex (**Figure 3B**, **3C**) suggest its active role in the cooperative oncogenic activity of PTPH1 and HER2. We examined if PDZ is important for PTPH1 to cooperate with YAP1 and PBK to stimulate AP-1 transcription. Transient co-transfection experiments showed that PTPH1 depends on PDZ to cooperate with PBK and YAP1 to stimulate AP-1 activity in MCF-7 cells (**Figure 4D**). qRT-PCR analyses further showed that there was a decrease in YAP1 RNAs in two PTPH1/PDZ expressed cell lines as compared with their sublines expressing wild-type PTPH1 (**Figure 4E**, **4F**), demonstrating a PDZ-dependent role of PTPH1 in stimulating YAP1 transcription.

Further experiments showed that PTPH1 transfection increased HER2 and CD44 whereas HER2 transfection elevated YAP1 proteins, with their co-transfection leading to elevated PTPH1, HER2, YAP1, and CD44 proteins in MCF10A cells (**Figure 4G**). These results, together with those in **Figures 1G**, **1H** and S1A, S1B, indicate that PTPH1 cooperates with HER2 to stimulate CSC population expansion. Indeed, there was an up-regulation of multiple CSC genes [24] in 231 cells co-expressed with HER2 and PTPH1 as compared with those expressing PTPH1/PDZ in which elevated IL-8 and TAZ (a highly YAP1-related transcriptional regulator) [34] were further demonstrated by qRT-PCR analysis (**Figure 4H**, **4I**). Similar results were obtained in MCF-7 cells transfected with PTPH1 as compared with cells expressed with PTPH1/PDZ (**Figure 4I**). These results demonstrate that PTPH1 and HER2 cooperate to stimulate PBK transcription and that PTPH1 depends on PDZ to cooperate with PBK and YAP1 to increase AP-1, to stimulate YAP1 expression, and to cooperate with HER2 in stimulating CSC programming. These results, together with the p38γ and PTPH1 reciprocal regulation of expression and phosphorylation or de-phosphorylation [7, 8], show that the PDZ-coupled scaffold proteins can utilize posttranslational and transcriptional mechanisms to maximize their local concentrations to program CSC for their cooperative oncogenic activity.

p38γ transformation increases PTPH1 and HER2 expression and p38γ inhibitor decreases p-PTPH1 and increases p-HER2 and cooperates with a HER2 inhibitor to inhibit breast cancer growth

Our previous studies identified that PTPH1 is a specific phosphatase for p38γ (*MAPK12*), whereas p38γ is also a kinase for PTPH1/S459 [7, 8]. PTPH1 and p38γ have a cooperative oncogenic activity and can reciprocally and allosterically regulate each other through PDZ-binding [7, 8, 35, 36]. Because p38γ can induce breast cancer transformation by stimulating CSC expansion [24, 37, 38], we examined if p38γ transformation regulates HER2 and PTPH1 expression thereby contributing to scaffold formation. **Figure 5A** showed that p38γ overexpression in MCF10A cells stimulates HER2 and PTPH1 protein expression as observed in BT474 cells (Figure S4F), indicating that p38γ alone is sufficient to activate the scaffold. This mechanism may not apply in 293T cells as p38γ transfection did not elevate HER2 nor PTPH1 protein but instead increased HER2/PTPH1 binding (**Figure 5B**), which likely acts an additional mechanism for the scaffold activation. Because p38γ directly phosphorylates PTPH1/S459 [7], the scaffold may be activated by p38γ and/or PBK-induced PTPH1 phosphorylation, which is stabilized by increased HER2/PTPH1 protein expression and their enhanced binding activity (**Figure 3**) and further sustained by transcriptionally activated PBK and YAP1 (**Figure 4**).

To further demonstrate this mechanism in $HER2^+$ BT474 breast cancer cells without transfection, we incubated cells with pirfenidone (PFD), a selective p38γ inhibitor [39] and examined HER2 precipitates for phosphorylation and interactions. **Figure 5C** (Exp. 1) showed that incubation with PFD resulted in a decrease

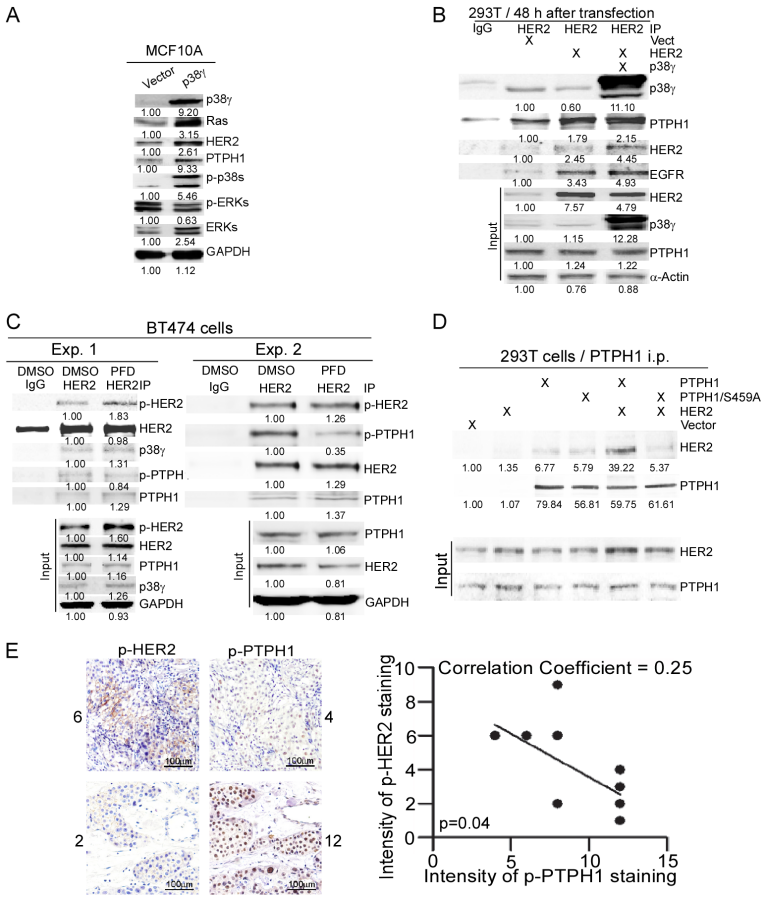


Figure 5. p38γ transformation increases PTPH1 and HER2 expression and p38γ inhibitor pirfenidone decreases p-PTPH1, increases p-HER2, and cooperates with HER2 inhibitor Laptinib to inhibit breast cancer growth. A. p38γ-transformation in MCF10A cells is sufficient to increase PTPH1 and HER2 protein expression. The indicated engineered cells by viral infection were analyzed by WB. Increased HER2 and PTPH1 expression was also observed in the positive control RAS-transformed MCF10A cells. B. p38γ overexpression in 293T cells promoted HER2 interaction with PTPH1. Cells were transiently expressed with indicated constructs for 48 h and HER2 precipitates were analyzed by WB with a portion of cells as input control. C. Incubation of BT474 cells with p38γ inhibitor pirfenidone (PFD) decreased p-PTPH1 and increased p-HER2 in HER2 precipitates. Cells were incubated with PFD (100 μg/ml for 16 h) and HER2 precipitates were analyzed for protein phosphorylation and interactions. Results of two separate experiments (Exp. 1 and Exp. 2) were shown in which p-HER2 was detected in both HER2 precipitates but was undetectable in input of Exp. 2. D. PTPH1/S459A is defective in HER2 binding in 293T cells. Indicated constructs were transiently transfected into 293T cells and PTPH1 precipitates were analyzed for its interaction with co-transfected HER2. E. Elevated p-PTPH1 is associated with decreased p-HER2 (Y877) in HER2$^+$ breast cancer specimens (16 cases) by IHC analysis with individual IHC score given for each panel. Please see Supplementary Table 1 for detail about (E).

in p-PTPH1 and a consequent increase in p-HER2 in HER2 precipitates. Similar results were obtained in a separate experiment (Exp. 2). These results indicate that PFD can suppress intrinsic p38γ activity in HER$^+$ breast cancer cells to decrease p-PTPH1 leading to increased p-HER2 in HER2 precipitates. To test if PTPH1/S459 is required for PTPH1 interaction with HER2, we performed transient transfection and IP/WB analyses in 293T cells. **Figure 5D** showed that PTPH1 bound co-transfected HER2, and this activity was completely lost when PTPH1/S459A was co-transfected instead, indicating an essential role of S459 in PTPH1/FER2 binding and in activating the scaffold. Thus, PTPH/S459 phosphorylation may play a key role in activating and maintaining a functional scaffold by linking two activating kinases p38γ and PBK with its substrate HER2.

PTPH1/S459 is important for its phosphatase activity [7] and is required for decreasing p-HER2 and increasing HER2 expression (**Figure 2A**). We next determined if this relationship exists in breast cancer tissues. Indeed, **Figure 5E** showed that there is an inversed correlation between p-HER2 and p-PTPH1 staining intensity in a group of HER2$^+$ breast cancer specimens, indicating their functional cooperative role in clinical breast cancer. HER2 inhibitor lapatinib (Lap) binds the tyrosine kinases domains of HER2 and EGFR and inhibits their phosphorylation. Although Lap is an established dual HER2/EGFR inhibitor, studies showed that it can inhibit breast cancer growth dependent of HER2 but independent of EGFR by unknown mechanisms [17, 40]. p-HER2 is the target for Lap and its elevation by PFD through inhibiting PTPH1 phosphorylation may increase growth-inhibitory activity of Lap via scaffolding mechanism. As expected, Figure S5A showed that Lap-induced

growth inhibition was increased by co-incubation with PFD in three breast cancer cell lines. Because p38γ increases p-PTPH1, stimulates PTPH1 and HER2 expression and interaction (**Figure 5B**, **5C**), these results indicate that combinational targeting p38γ and HER2 would be an effective strategy to disrupt the oncogenic scaffold.

PDZ-coupled and S459-driven PTPH1/HER2 scaffold may be a sustained therapeutic target for combination therapy

PBK co-transfection with PTPH1 also increases p-PTPH1 levels (Figure S5B). We tested if PBK inhibitor HI-TOPK-032 (032) [41] decreases p-PTPH1 and increases Lap-induced growth inhibition through the PDZ-coupled scaffold. Indeed, incubation with 032 decreased endogenous p-PTPH1 and increased the growth-inhibitory activity of Lap in T47D cells, which was however attenuated by PTPH1 depletion (Figure S5B). On the contrary, incubation of PTPH1-overexpressed HMEC/HER2 cells with 032 in combination with Lap led to a greater growth inhibition and more substantial p-HER2 and/or p-PTPH1 depletion than control cells (Figure S5C). These results indicate that high levels of PTPH1 in breast cancer cells can increase 032-induced p-PTPH1 downregulation and Lap-induced p-HER2 depletion by targeting the scaffold (**Figures 2**, **3A**, **4** and S3A, S3B). In support of this possibility, a cooperative growth inhibition and cooperative depletion of p-PTPH1 and p-HER2 by Lap and 032 were further demonstrated in 231 cells co-transfected with HER2 and PTPH1 (Figure S5D). These results indicate that PTPH1 overexpression in breast cancer cells increases sensitivity to 032 and/or Lap through PBK-phosphorylated PTPH1 in scaffold.

To further demonstrate if the PDZ-coupled scaffold is a combination therapeutic target in mice, $HER2^+$ ZR75-1 breast cancer cells intrinsically expressing HER2, PTPH1, PBK and p38γ proteins (**Figure 6A**) were s.c. inoculated into female nude mice [42] and the growth-inhibitory activity of Lap in combination with 032 or PFD was assessed. We chose a low dose of inhibitors [2, 17, 43] to show a proof of concept that only combined targeting of druggable kinases (HER2, p38γ or PBK) of scaffold instead of individual molecules is effective in inhibition of tumor growth in mice. Although all inhibitors alone failed to inhibit tumor growth and failed to decrease p-HER2 in tumors, both PFD and 032 significantly increased Lap-induced growth inhibition and HER2/p-HER2 depletion without noticeable effect on mouse body weight (**Figure 6B-D**, **6F**). Consistent with the theory that this PDZ scaffold may promote breast oncogenesis by stimulating CSC expansion (**Figure 1**), combination targeting of this complex by Lap with PFD or 032 significantly decreases protein levels of two CSC markers (Sox2 and Vimentin) in breast tumor xenograft (Figure S6B and S6C). These results indicate that p38γ/PBK-activated and PTPH1/PDZ-coupled scaffold may be a target for combination therapy. Because PTPH1 increases HER2 stemness and tumorigenesis, and depends on PDZ to bind YAP1, this scaffold may also function as a super-oncogene (**Figure 6E**).

Discussion

Protein scaffolds organize functional complexes and modulate kinase activities thereby contributing to the accurate coordination of specific signaling pathways [28]. By physically tethering proteins, scaffolds locally concentrate, compartmentalize, and position receptors or enzymes, and/or transcription factors in close vicinity of their substrates or regulatory proteins to maximize their cooperative functions [28]. Our results presented here reveal a PDZ-coupled and phosphorylation-driven scaffold [44] that integrates the oncogenic activity of phosphatase PTPH1 with several proliferative PDZ-binding kinases together with a transcription co-activator to promote oncogenesis. The super-oncogenic activity of scaffold is demonstrated by PTPH1 potentiating HER2 tumorigenesis in transgenic mice and increasing HER2 stemness and xenograft growth. Moreover, small molecular inhibitors of scaffold kinases suppress xenograft growth of $HER2^+$ human breast cancer cells when administered in combination (**Figure 6E**), suggesting the potential role of scaffold as a target for combination therapy. Therefore, distinct, overexpressed, and even enzymatically opposite proteins can be locally concentrated through a scaffold through PDZ binding to act as a super-oncogene and as a target for therapeutic intervention.

Our results, however, do have several limitations. First, current studies heavily depend on overexpression experiments, both in cells and in mammary tissues and whether endogenous PTPH1 collaborates with HER2 and other PDZ-

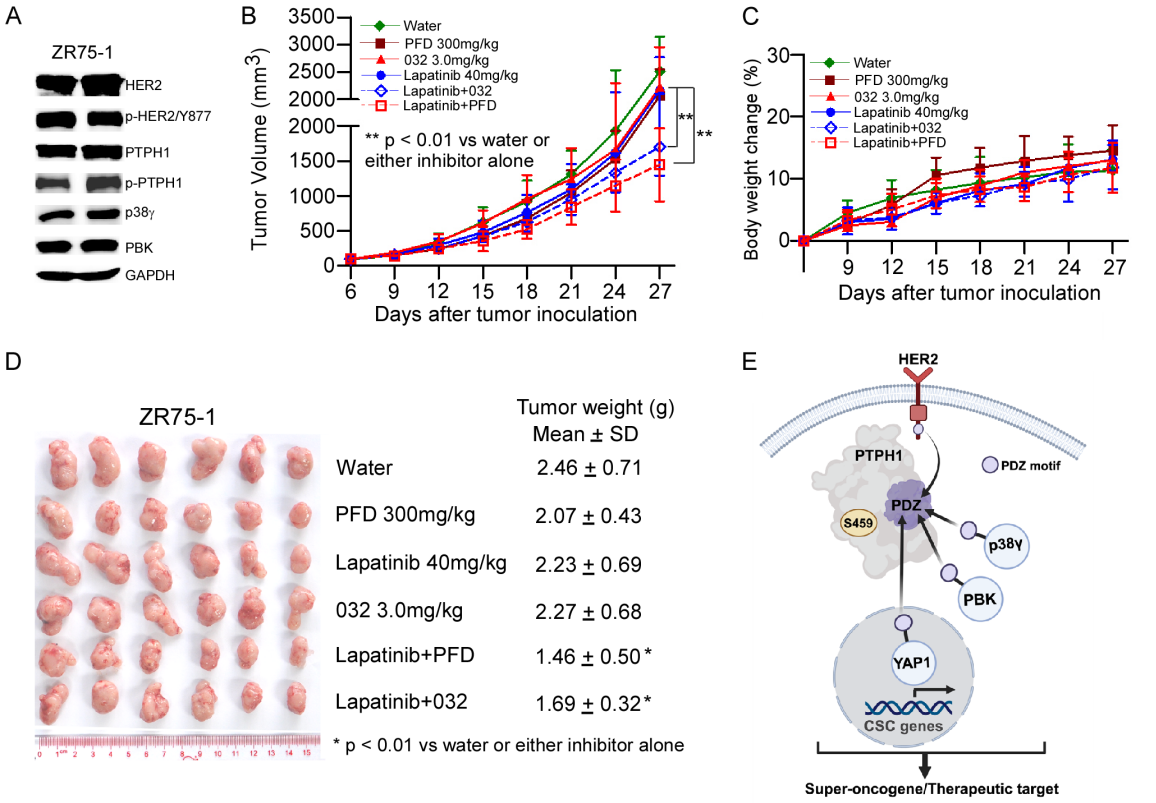


Figure 6. p38γ and PBK inhibitors cooperate with HER2 inhibitor to suppress xenograft growth and to decrease p-HER2, HER2, and p-PTPH1 expression in breast cancer tissues. (A) HER2, p-HER2, p-PTPH1, PTPH1, PBK, and p38γ are abundantly expressed in ZR75-1 cells as analyzed by WB in duplicate. Similar results were obtained in a separate WB. (B-D) PFD and 032 cooperate with Lap to inhibit xenograft growth of human breast cancer. ZR75-1 breast cancer cells were s.c. inoculated into female nude mice and targeted therapies with indicated inhibitors were initiated 6 days later. DMSO was used as a vehicle control to dissolve inhibitors, whereas working solutions were diluted in water for administration. Tumor volume was measured (B) whereas the mouse body weight changes were also recorded (C) with tumors weighed shown in (D) (means ± SD, n = 6). (E) An experimental model indicates that the PDZ-coupled and S459-activated PTPH1-HER2 scaffold integrates oncogenic signaling of several oncogenic proteins as a super-oncogene and therapeutic target. In this scaffold, PTPH1 binds four proteins via its PDZ domain and de-phosphorylates and reciprocally increases HER2 expression leading to increased tumorigenesis, whereas p38γ and PBK may phosphorylate PTPH1/S459 thus activating the scaffold and YAP1 may transcriptionally activate stemness, which together acts as a super-oncogene. Because combination of Lap with either 032 or PFD, not either alone, suppresses xenograft growth, this scaffold may also be a target for combination therapy. (F) A cooperative inhibition of p-HER2/HER2 signaling by Lap and 032 or PFD. Tumor tissues from (D) were analyzed by IHC with indicated antibodies and representative results from each group were shown, whereas their quantitative results were given in Figure S6A.

motif oncoproteins to induce mammary tumorigenesis remains to be established. Moreover, kinase-phosphatase interaction is frequently bidirectional and transient and both phosphorylation and dephosphorylation are involved in a biological output [36], whereas our experimentations can only approach this question under certain conditions. Furthermore, while our model suggests that PDZ binding integrates five oncoproteins as a super-oncogene to drive breast oncogenesis (**Figure 6E**), most of transformation studies in cell culture and in mice are focused on PTPH1 and HER2. Future studies may be warranted to investigate the role of the second kinase PBK and the transcription cofactor YAP1 in this oncogenic complex.

HER2 de-phosphorylation by a protein tyrosine phosphatase (PTP) has different effects on HER2 oncogenic activity. PTPN18 de-phosphorylates HER2 and promotes HER2 ubiquitination and degradation and inhibits HER2 oncogenic activity [45]. PTPN13, on the other hand, dephosphorylates HER2 and increases HER2 invasive activity [46]. Further, PTPRO dephosphorylates HER2 but inhibits HER2-induced mammary tumorigenesis in MMTV-*Neu* mice [47], whereas PTPα contributes to HER2 oncogenesis but regulates AKT pathway activities [48]. Thus, cellular outcome of PTP dephosphorylating HER2 may be either promoting or inhibiting HER2 oncogene activity dependent of PTP. Our results that a coupling of PTPH1-induced HER2 de-phosphorylation with its reciprocal increases of HER2 protein expression may be unique for their cooperative oncogenic activity. Through PDZ-integrated scaffolding, PTPH1-induced HER2 de-phosphorylation may play a key role in increasing local HER2 protein concentrations through coordinated PTPH1 phosphorylation and HER2 de-phosphorylation to promote oncogenesis.

Our results suggest that PTPH1-activated oncogenic scaffold may be functional only when scaffold proteins are overexpressed in $HER2^+$ breast cancer. For example, PTPH1 and HER2 reciprocally increase protein expression by overexpression and there is an increased breast tumorigenesis in their double transgenic mice. PTPH1 depletion, however, has no effect on HER2 protein expression even though it inhibits breast cancer growth (Figure S1C, S1D). Moreover, PTPH1 protein level is increased in $HER2^+$ breast cancer issues (**Figure 1A**, **1B**), and PTPH1 and HER2 cooperatively increase PBK and YAP1 transcription (**Figure 4A-G**). Furthermore, p38γ-forced expression can also increase HER2 and PTPH1 expression. Thus, scaffold proteins may locally stimulate each other expression by several mechanisms, which may be the basis for scaffold as a super-oncogene and as a target for combination therapy. Because PTPH1 can be a tumor-suppressor in certain cells and/or tissues [49], it would be important to investigate further if PTPH1 only forms a scaffold when it is oncogenic and if the tumor-suppressive PTPH1 lacks the scaffolding activity. Such results would indicate that scaffold may function to convert a tumor-suppressor role of PTPH1 into a super-oncogene via PDZ binding by integrating and propagating proliferative signaling of a group of oncogenic proteins.

Of interest, although PTPH1 is a non-membrane PTP [6], it inhibits nuclear accumulation of tumor suppressor vitamin D receptor VDR [10] but increases oncogenic estrogen receptor ER nuclear concentration to promote breast cancer growth [11]. These results suggest that regulation of nuclear concentrations of receptors by PTPH1 may be important for its oncogenic activity in breast cancer. It would be of interest to further test if PTPH1 depends on PDZ to bind ER and VDR for their integrated roles in HER^- and HER^+ breast cancer growth. Because phosphorylation is a key mechanism for a PDZ scaffold [28], it would be also important to determine whether PTPH1 depends on S459 to alter cellular distribution of these receptors, which may reveal a unique mechanistic link between membrane and nuclear receptors through PTPH1-induced HER2 dephosphorylation and nuclear translocation. Such results will indicate that PTPH1 phosphorylation may recruit more receptors into the scaffold and thereby link cell surface receptor HER2 signaling to nuclear transcription machinery and CSC activation to promote malignant development and progression [28].

Materials and methods

Animal studies

Following experiments in mice were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee (#AUA0042). MMTV-HA-PTPH1 mice (FVB) were generated by Cyagen (Cyagen Bioscience, CA) and were genotyped using the following prim-

ers: FW, TGCACCATCGCCTATGTGTC, RE, AGTCAGGCACGTCATAAGGA. Female MMTV-Neu transgenic mice were previously described [21] originally purchased from Jackson Laboratory (Bar Harbor, ME). MMTV-HA-PTPH1 was bred with MMTV-Neu to generate the double transgenic MMTV-Neu/MMTV-PTPH1 mouse. Tumor formation in female mice was examined by palpitation daily and tumor-free survival time was recorded. Littermates or sisters were kept as virgins for experiments. Tumor tissues were removed at the end of experiments for pathological analysis. For HMEC xenograft studies, female Balb/c nude mice (6 week-old) were purchased from Harlan for breast cancer xenograft studies with transformed HMEC cells. To assess tumor-forming activity *in vivo*, HMEC cells stably co-expressed with HER2 and/or with PTPH1 or PTPH1/PDZ by retrovirus were re-suspended in Matrigel and 5 × 0^6 of these cells in 100 µl was s.c. injected into both flanks of mice as indicated. Tumor volumes were measured, photographed, and processed for WB or histology whenever possible.

The Breast cancer xenograft studies were conducted in Sun Yat-sen University, Guangzhou, Guangdong, China. All animal experiments in mouse xenograft with inhibitors were performed in accordance with institutional guidelines and were approved by the Animal Ethics Committee at Sun Yat-sen University Cancer Center. ZR-75-1 xenografts were performed in immune deficient estrogenized nude female mice (5-6 weeks old, 17β-estrogen pellets) (0.72 mg/90 days release; IRA, Sarasota, FL, USA) were described previously [11]. Briefly, nude mice implanted with 17β-estradiol pellets were injected subcutaneously with 1 × 10^7 ZR-75-1 cells into the right dorsal flanks of mice. Tumor volume (T_{vol}) was calculated using the formula: T_{vol} = ½(length × width^2). When tumors reached an average size of 50-100 mm^3, mice were randomly assigned to 6 groups and treated with: (a) vehicle; (b) PFD (pirfenidone, 300 mg/kg, p.o., qd); (c) Lap (lapatinib, 40 mg/kg, p.o., twice a week); (d) 032 (HI-TOPK-032, 3.0 mg/kg, i.p., twice a week); (e) lapatinib plus pirfenidone; (f) lapatinib plus 032. Pirespa (pirfenidone, Hong Kong, China) was administrated in 100 µl of water (300 mg/kg) by gavage for xenograft experiments. All other inhibitors were dissolved in DMSO as stock solutions and stored at -20°C, which will be diluted in distilled water as a working solution for administration with distilled water as a solvent control. Tumor masses and body weight were monitored and measured every 3 days, and mice were sacrificed when the mean of tumor weight was over 1 g in control group.

Cell culture, reagents, and cell culture

MCF10A, 293T, and other human breast cancer cells were purchased from ATCC and maintained under standard culture conditions [24]. HMEC cells were kindly provided by Dr. Robert Weinberg [25]. Mouse Neu primary breast cancer cells were previously described [21]. Flag-tagged p38γ was provided by Dr. Jiahuai Han and cloned into a retroviral pLHCX vector [37]. Wild-type HA-PTPH1 and phosphatase-deficient PTPH1/DA mutant were provided by Nicholas Tonks [50]. Retroviral constructs expressing PTPH1, PTPH1/PDZ, and PTPH1/S459A were previously described [7, 8]. PBK shRNA retroviral constructs and PBK expression plasmid were provided by Dr. Z. Dong [41, 51], whereas YAP1 expression plasmid was provided by Dr. W. Hahn [52]. Pirfenidone (PFD) was purchased from Sigma for cell culture studies, whereas its powder was bought from Pirespa (Hong Kong, China) and was administrated in 100 µl of water (300 mg/kg) by gavage for xenograft experiments. Lapatinib (Lap) and 032 were obtained from Selleck and dissolved in DMSO as a stock solution and stored at -20°C until use.

Tumor-sphere formation, soft-agar, colony formation, and cell proliferation assays

Tumor sphere formation was performed as described previously with minor modifications [53]. For breast cancer cells, we used MEM medium containing 5 lg/ml insulin, 5 lg/ml hydrocortisone, 20 ng/ml EGF, and 0.5% fetal bovine serum. For MCF10A cells, cells were plated for sphere formation in medium containing 0.5% of horse serum. To assess sphere formation, cells (2.5 × 10^4 cells per well/2 ml for breast cancer and HER2 transformed mammary epithelial cells) were cultured as single-cell suspension in ultralow attachment plate (BioExpress and Costar) for about 10 days, with 0.2 ml of fresh medium added every 2-3 days. Mammospheres formed were then photographed and counted as described previously [7]. For in vitro transformation assays, cells at early passages were plated in growth media containing 0.33% Sea-plaque-agarose [54]. Two weeks later, colonies formed were visualized by staining, which were photographed and

counted [7, 8]. For colony formation assays, cells were seeded in 6-well plate (500 cells per well) to which various inhibitors or solvent were added for approximately two weeks. Colonies formed were stained and manually counted [55]. For cell proliferation assay, 1500 cells seeded per well in a 96-well plate in the presence of inhibitors or solvent control and cell growth was assessed after 48 h incubation using the cyquant NF cell proliferation kit [10] according to the manufactures' instructions. Resultant fluorescence signals were measured in triplicate in a plate reader (Invitrogen, Sunnyvale, CA) with excitation at 485 nM and emission detection at 530 nM. Results were expressed as relative growth of the respective solvent control (%).

Statistical analysis

Results were compared using student's t test, unless otherwise indicated. Sample size was estimated based on literature and lab experiences and the same numbers of age-matched female mice were allocated for control and experimental groups. *P* values less than 0.05 were considered significant. Survival was measured using the Kaplan-Meier method and statistical significance was determined by log-rank using GraphPad Prism software (Version 5). Detailed Materials and Methods are given in Supplementary Materials.

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Disclosure of conflict of interest

None.

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Materials and methods

Constructs, shRNAs, and primer information for qRT-PCR

HER2 and HER2/Y877F expression retroviral constructs were provided by Dr. D. Yu [8]. shRNAs for luciferase, PTPH1 and p38γ were described previously [1, 2]. Block-itTM Lentiviral RNAi expression system, Cat: K4943-00, Invitrogen) was used to clone shRNAs sequences. The target sequences for human HER2 shRNAs are given below.

HER2 shRNA#6, CACCgacattgacgagacagagtacCGAAgtactctgtctcgtcaatgtc; reversed, AAAAgacattgacgagacagagtacTTCGgtactctgtctcgtcaatgtc; HER2 shRNA#8, CACCgaatatgtgaaccagccagatCGAAatctggctggttcacatattc; AAAAgaatatgtgaaccagccagatTTCGatctggctggttcacatattc.

The primers for qRT-PCR are given below: Human PBK forward: AGACCCTAAAGATCGTCCTTCTG and reverse: GTGTTTTAAGTCAGCATGAGCAG. Human IL-8 forward: CCACCGGAGCACTCCATAAG and reverse: GATGGTTCCTTCCGGTGGTT. Human TAZ forward: ATCCCCAACAGACCCGTTTC and reverse: GAACGCAGGCTTGCAGAAAA. Human YAP1 forward: CGCTCTTCAACGCCGTCA and reverse: AGTACTGGCCTGTCGGGAGT.

Antibodies, inhibitors, and others

Antibody against pan Ras was purchased from Oncogene Sciences. Phospho-PTPH1 antibody (PTPH1/S459) was generated by Phospho-Solution Inc. (Boston, MA) as described [2]. Antibody against PTPH1 (mouse) was kindly provided by Dr. N. K. Tonks. Other antibodies used in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). These include GAPDH (sc-47724), EGFR (sc-03), HER2 (sc-7301, mouse, sc-284, rabbit), p-HER2 Tyr1248 (rabbit, sc-12352), p-HER2 Tyr 877 (sc-101695, rabbit), which was used to detect all phosphorylated HER2 (p-HER2) unless specified, PTPH1 (sc-515181, mouse), PBK (sc-293028, mouse), α-Actinin (sc-17829), anti-β-actin (sc-47778), anti-ubiquitin (sc-8017), anti-Lamin B (sc-6217), anti-α-Actinin (sc-17829), and anti-α-Tubulin (sc-6199). YAP1 antibody was purchased from Sigma (Y4770, rabbit). Antibody for p-p38 was Cell Signaling, for HA from Immunology Consultants Lab (Oregon), and p38γ from R&D Systems.

IHC and immunoreactivity scoring

In brief, slides with paraffin sections were deparaffinized by xylene, rehydrated with graded ethanol (100, 95, 75, and 50%), and rinsed with deionized water. Endogenous peroxidase activity was blocked by hydrogen peroxide (3%) for 10 min. Antigen retrieval was performed by boiling slides in EDTA solution (pH9.0, #ZLI-9069, ZSGB-Bio). Slides were blocked with 5% BSA and incubated with primary antibody at 4°C overnight, followed by incubation with enzyme-conjugated secondary antibodies at room temperature for 1 h (#PV6001, ZSGB-Bio). Immunoreactivity was visualized with 3,3-diaminobenzidine (DAB, #ZLI-9017, ZSGB-Bio). The primary antibodies used were: PBK (#16110-1-AP, Proteintech; 1:150 dilution), phospho HER2 (Y877) (#ab108371, Abcam; 1:50 dilution), HER2 (#18299-1-AP, Proteintech; 1:100 dilution). Details for IHC of human breast cancer tissues were the same as previously described [9]. The percentage of positive cells was scored as follows: 0, < 5%; 1, 5 to 25%; 2, 25 to 50%; 3, 50 to 75%; 4, 75 to 100%. The intensity of staining was scored as follows: 0, negative; 1, weak; 2, moderate; 3, strong. The staining results (staining index = positive percentage × intensity) were examined independently by two observers and a consensus score was assigned to each sample. For tissue staining, sections of formalin-fixed and paraffin-embedded human breast cancer tissues were prepared by the Histology Core of Medical College of Wisconsin. Immunohistology (IHC), pathological analyses, and scoring were performed as previously described [9]. To quantitate IHC scores, slides were scanned on the Vectra Polaris at 20× magnification (Akoya Biosciences), and analyzes with HALO software (Halo imaging software, Indica Labs, NM, USA) using the CytoNuclear v3.6 analysis mode. The percentage of positive cells was quantified. Data represent the mean ± SEM for n = 6 independent tumors and analyzed using a one-way ANOVA with Turkey' post-test. All experiments in human breast cancer tissues were approved by Institutional Review Board (IRB #2820 for Medical College of Wisconsin; IRB #GZR2020-343 for Sun Yat-Sen University Cancer Center).

Immune-precipitation, immune-blotting, and cell fractionation

Cells were lysed in RIPA buffer containing phosphatase and protease inhibitors as described [1] and lysates were then incubated with an antibody (or corresponding IgG) together with protein A or G agarose beads overnight. The immune complexes were then washed with RIPA buffer and pellets were resuspended in 1× loading buffer, followed by boiling at 100°C for 5 min. Protein samples from total cell lysates (input) or from immune-precipitates (IP) were resolved by SDS-PAGE, which were then transferred to a nitrocellulose membrane. After being blocked in 5% nonfat milk for 45 min, the membrane was blotted with an appropriate antibody. The remaining procedures were the same as previously described [1, 6]. Cell fractionation was performed as described [3, 5]. Protein density in WB was measured by Image J software, which was then normalized to loading controls and expressed as relative to Vector group. Precipitated proteins of various groups were expressed as relative to Vector or solvent control, whereas phosphorylated proteins were further expressed as relative to total proteins.

Transfection, infection, and siRNA knockdown

Transient transfection was performed by calcium phosphate in 293T cells. The tetracycline inducible system (Tet-on) was used to express HA-PTPH1 proteins in MCF-7 cells as described previously [4-6]. Adenoviral infection was performed as previously described [5]. For retroviral infection, constructs (pLHCX and pLHCX-p38γ, pLenti6 shLuc and pLenti6 shp38γ, plenti6 PTPH1#1 and plentiPTPH1#2, plenti shHER2#6, and plenti shHER2#8) were first transfected into package cells and supernatants were collected 72 h later for infecting target cells, followed by an antibiotic selection [1].

RNA seq analysis and qRT-PCR

Total RNA was extracted using Trizol (Invitrogen, 15596018) and purified according to the manufacture's guidelines (RNAeasy Mini Kit Qiagen, 74104). One microgram of RNA was added as template for reverse transcription with EasyScript™ cDNA Synthesis Kit (LAMDA BIOTECH, G234). RNAs were analyzed on Affymetrix arrays at the Genomics Core Facility at Medical College of Wisconsin and. Microarray data were exposed from Affymetrix GCOS software as CEL files.

Quantitative real-time PCR was performed according to the manufacturer's protocol using the iTag Universal SYBR Green Supermix (BIO-RAD, 172-5121). Total RNAs were prepared from cells and analyzed for RNA levels of PBK and others by qRT-PCR. Results are mean of three separate tumors (± SD). Quantitative RT-PCR (qRT-PCR)-Total RNA was extracted with Trizol Reagent (Invitrogen), and qRT-PCR was performed using the iScriptTM One-Step RT-PCR Kit with SYBR Green (170-8893, Bio-Rad). Samples were analyzed by ΔΔCt method for fold changes in expression and for the ratio of target genes over Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as previously described 4. All experiments were repeated at least three times.

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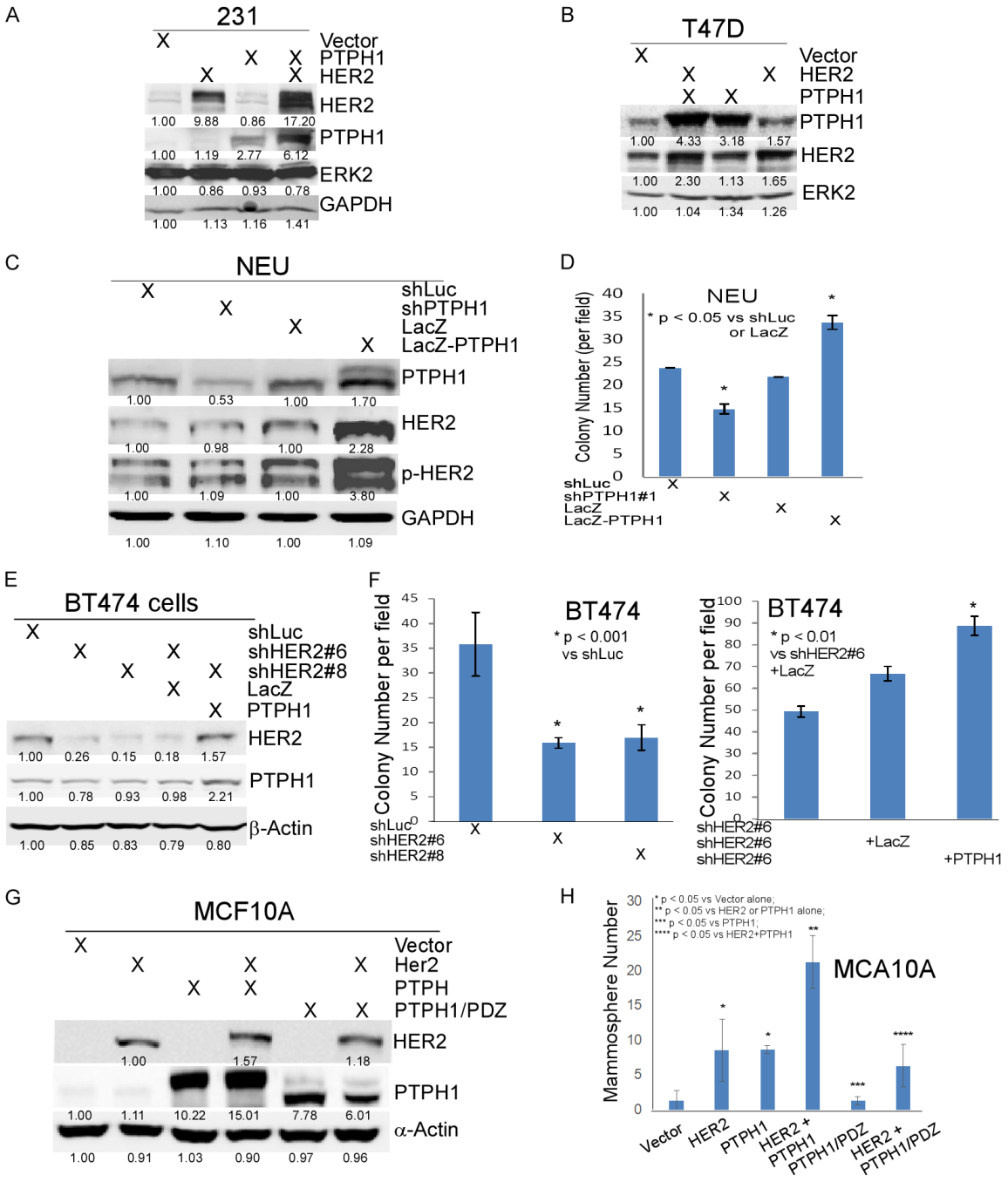


Figure S1. PTPH1 and HER2 reciprocally stimulate protein expression and PTPH1 signals downstream of HER2 to promote breast cancer growth. (A, B) PTPH1 and HER2 stimulate each other protein expression. Cells were stably expressed with indicated constructs and assessed for protein expression. (C, D) PTPH1 silencing inhibits colony formation without affecting HER2 protein expression, whereas PTPH1 over-expression elevates HER2 proteins (C) and increases the growth in mouse primary mammary tumor (Neu) cells (D). Similar results were obtained from a separate WB. Colony formation results (D, soft-agar growth) are means from 20 fields (± SD). (E, F) PTPH1 expression rescues HER2 protein expression and growth inhibition in HER2 silenced human breast cancer cells. HER2 silenced BT474 cells were re-expressed with PTPH1 for rescued effects on protein expression (E) and colony formation (F). Similar results were obtained in a separate WB. Colony numbers are means of 20 representative fields (± SD). (G, H) HER2 and PTPH1 or its PDZ-deleted mutant (PTPH1/PDZ) were stably co-expressed in MCF10A cells by retroviral infection, which were analyzed for protein expression (G) and tumor-sphere formation (H, only data from the 1st tumor- sphere shown, mean ± SD, n = 3).

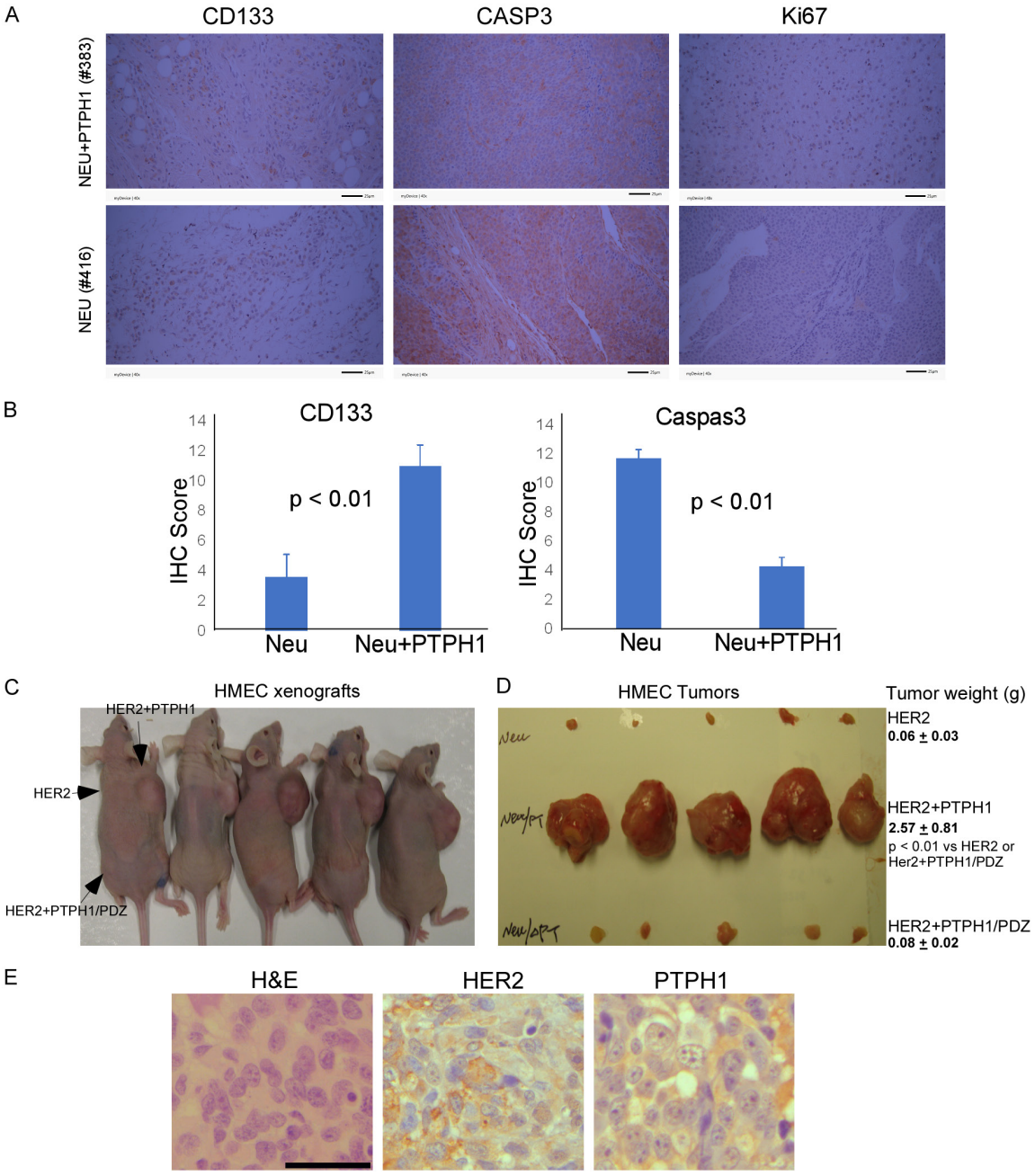


Figure S2. PTPH1 potentiates HER2 tumorigenesis in association with increased CD133 and decreased caspase 3 expression in tumor tissues and PTPH1 depends on PDZ to potentiate HER2-induced xenograft growth in mice. (A, B) Primary tumor tissues from **Figure 1F** were analyzed by IHC with indicated antibodies and summarized IHC scores from 3 tumors were given in (B) (mean ± SD, n = 3). (C-E) PTPH1 depends on PDZ domain to increase HER2-dependent xenograft growth. The same number (5 × 10^6 cells) of HER2-transformed HMEC cells with and without PTPH1 or PTPH1/PDZ co-expression were s.c. inoculated into female nude mice (C) and xenografts formed (at 21 days) were weighed and photographed (D). Tumor weights and volumes were also plotted in **Figure 1J** (mean ± SD, n = 5). Xenograft tumor tissues (HER2 ± PTPH1) were stained for H&E and for HER2 or PTPH1 protein expression by IHC (E, scale bar, 50 μM).

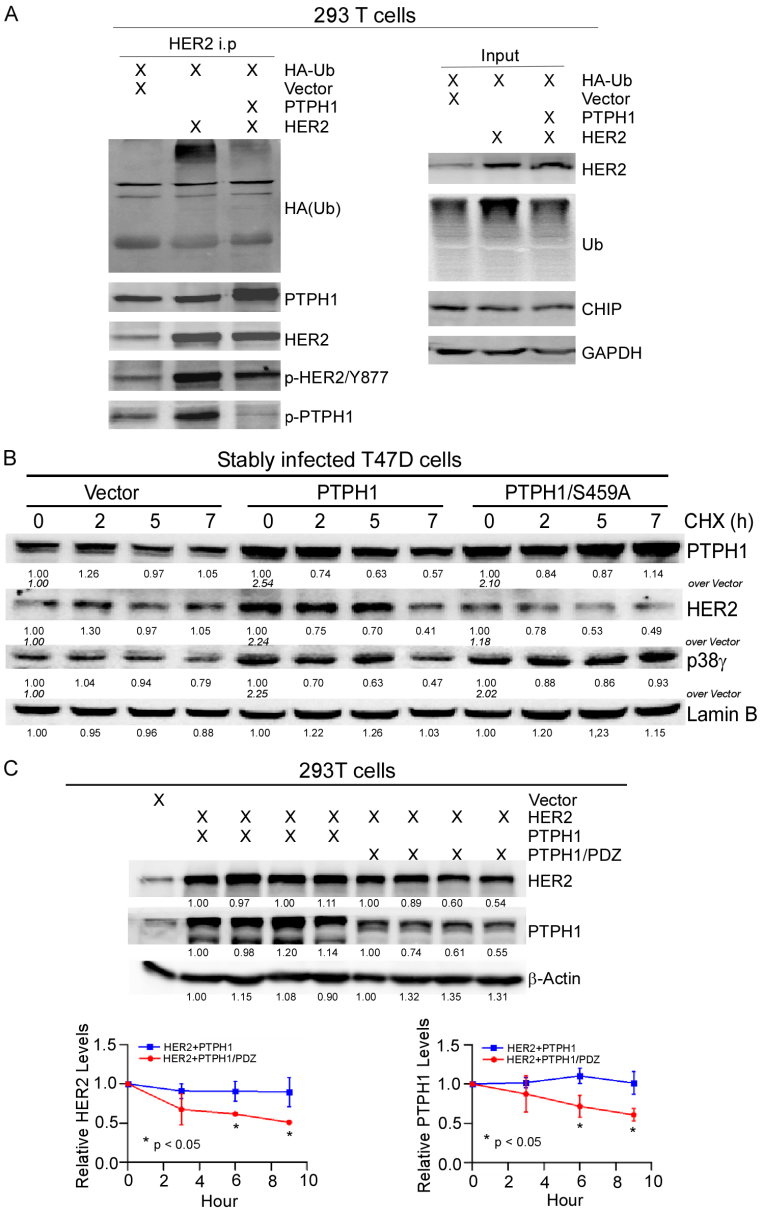


Figure S3. PTPH1 inhibits HER2 ubiquitination, whereas PTPH1/S459 phosphorylation plays a distinct role in PTPH1, HER2, and p38γ protein expression and turnover, whereas PTPH1/PDZ is required for reciprocal stabilization of PTPH1 and HER2. A. The indicated constructs were transiently transfected into 293T cells together with HA-Ub, which were analyzed by HER2 IP (left) and WB (right, input) 48 h later. B. T47D cells stably expressed with PTPH1 or PTPH1/S459A were analyzed for protein expression after incubation with a protein synthesis inhibitor CHX (100 μg/ml) for the indicated time. Protein levels of PTPH1, HER2, and p38γ were also quantitated in PTPH1 or PTPH1/S459A transfected cells at 0 h (shown as relative to Vector cells, *number in italic*) to assess their effects on protein expression. C. PTPH1 depends on PDZ to reciprocally increase HER2 stability. Indicated constructs were transiently expressed in 293T cells, which were analyzed for protein expression after incubation with CHX for the indicated times. Normalized HER2 or PTPH1 band intensity (to β-Actin, bottom) against time was plotted (bottom, mean ± SD, n = 3). Similar results were obtained in stably transfected 468 cells.

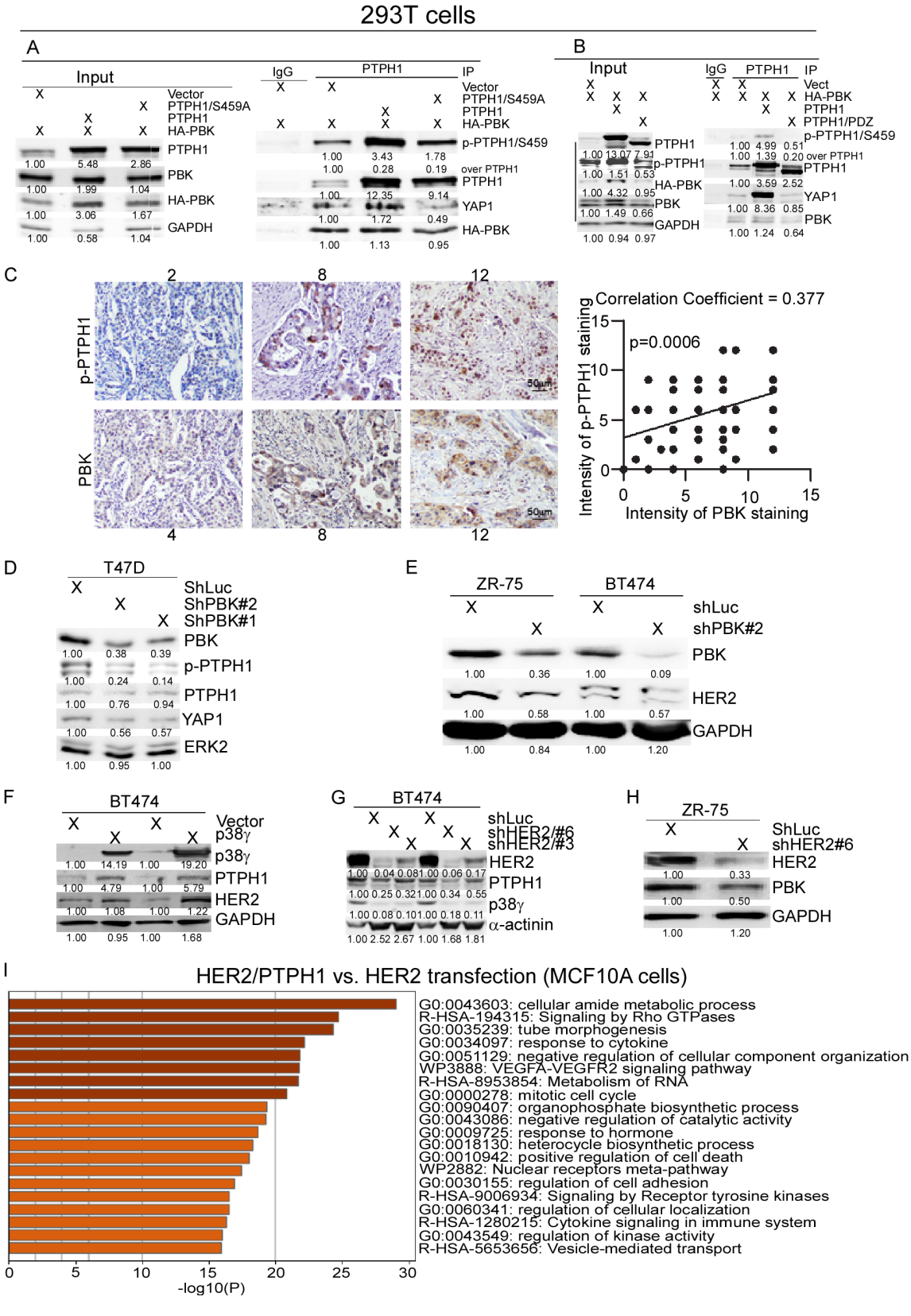
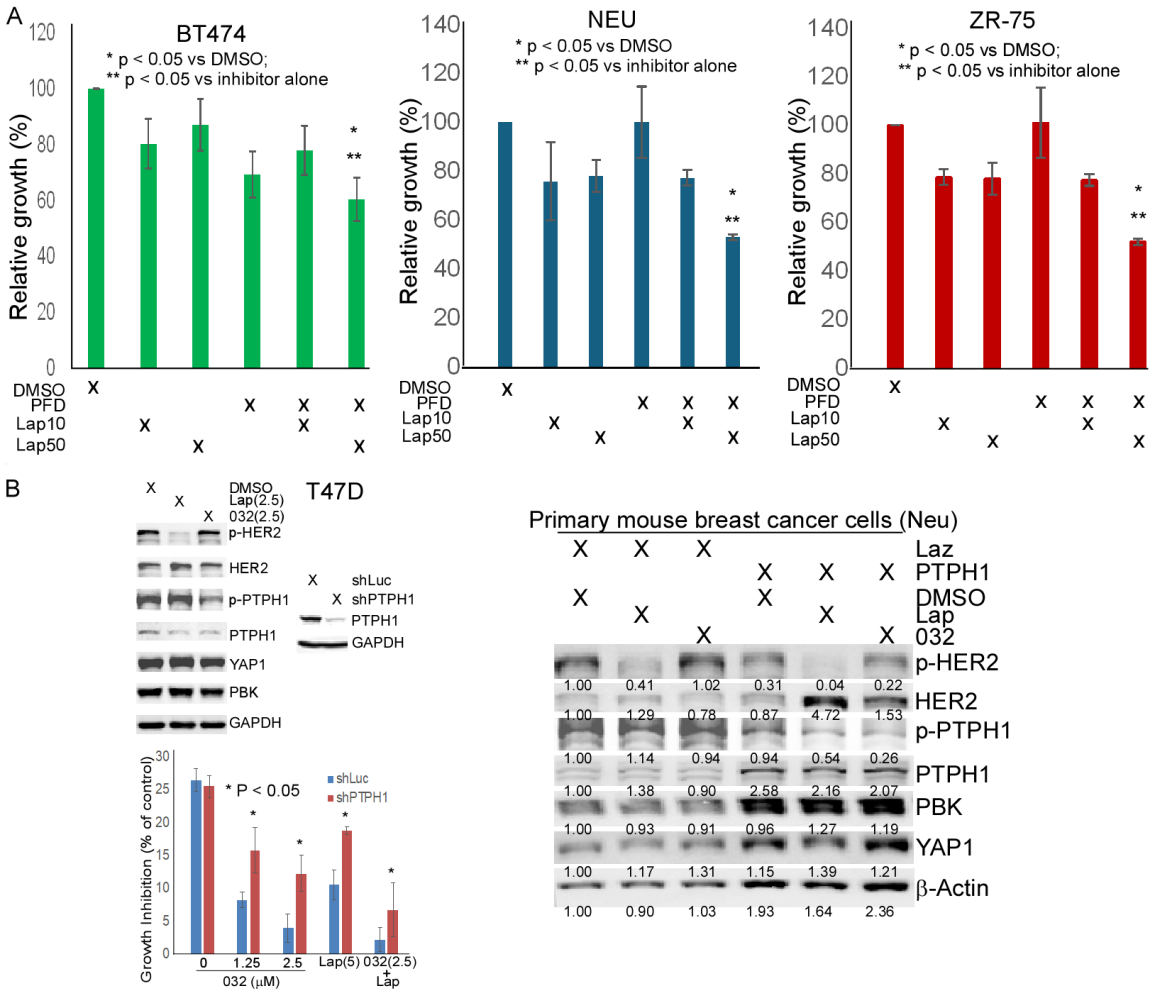

293T cells
A
Input
IgG
PTPH1
IP
Vector
PTPH1/S459A
PTPH1
HA-PBK
PTPH1
PBK
HA-PBK
GAPDH
p-PTPH1/S459
over PTPH1
YAP1
B
Vect
PTPH1/PDZ
p-PTPH1/S459
p-PTPH1
C
p-PTPH1
PBK
50μm
Correlation Coefficient = 0.377
p=0.0006
Intensity of p-PTPH1 staining
Intensity of PBK staining
D
T47D
ShLuc
ShPBK#2
ShPBK#1
p-PTPH1
ERK2
E
ZR-75
BT474
shLuc
shPBK#2
HER2
F
p38γ
G
shHER2/#6
shHER2/#3
α-actinin
H
ShLuc
shHER2#6
I
HER2/PTPH1 vs. HER2 transfection (MCF10A cells)
-log10(P)
G0:0043603: cellular amide metabolic process
R-HSA-194315: Signaling by Rho GTPases
G0:0035239: tube morphogenesis
G0:0034097: response to cytokine
G0:0051129: negative regulation of cellular component organization
WP3888: VEGFA-VEGFR2 signaling pathway
R-HSA-8953854: Metabolism of RNA
G0:0000278: mitotic cell cycle
G0:0090407: organophosphate biosynthetic process
G0:0043086: negative regulation of catalytic activity
G0:0009725: response to hormone
G0:0018130: heterocycle biosynthetic process
G0:0010942: positive regulation of cell death
WP2882: Nuclear receptors meta-pathway
G0:0030155: regulation of cell adhesion
R-HSA-9006934: Signaling by Receptor tyrosine kinases
G0:0060341: regulation of cellular localization
R-HSA-1280215: Cytokine signaling in immune system
G0:0043549: regulation of kinase activity
R-HSA-5653656: Vesicle-mediated transport

Figure S4. PBK co-transfection depends on S459 and PDZ to increase PTPH1/S459 phosphorylation and PBK protein level correlates with p-PTPH1/S459 in breast cancer tissues, and PBK is required for PTPH1, YAP1, and HER2 expression in HER$^+$ breast cancer cells. (A) Co-transfected PBK in 293T cells more significantly increased PTPH1/S459 phosphorylation in PTPH1 precipitates from cells transfected with HER2 plus PTPH1 than those transfected with HER2 plus PTPH1/S459A. Indicated cells were transiently expressed with indicated plasmids and analyzed by PTPH1 IP and WB 48 h later. (B) PBK expression increases PTPH1/S459 phosphorylation in PTPH1 precipitates from cells co-transfected with HER2 plus PTPH1 but not from cells co-transfected with HER2 plus PTPH1/PDZ. Indicated constructs were transfected in 293T cells, which were analyzed by IP/WB as in (A). (C) Increased PBK protein expression in human breast cancer tissues correlates with elevated p-PTPH1 [2]. A group of HER$^+$ breast cancer specimens were assessed for protein expression by IHC and representative images and associated IHC scores were shown at left and linear correlation analysis by using correlation coefficient analyses was displayed at right as previously described [7]. Pearson correlation analysis was performed to evaluate association between the expression levels of p-PTPH1 and PBK. The Pearson correlation coefficient (r) for these two variables was r = 0.377, which showed moderate correlation with statistical significance (P = 0.0006). (D, E) Endogenous PBK is required for PTPH1, YAP1, and HER2 protein expression. Indicated cells were stably knocked down of PBK by shRNA and analyzed for protein expression by WB. (F-H) p38γ overexpression increases PTPH1 and HER2 protein expression, whereas HER2 silencing decreases PTPH1, p38γ and PBK protein expression in HER2$^+$ human breast cancer cells. (I) PTPH1 and HER2 cooperate to regulate multiple signal transduction pathways. RNA seq. data from **Figure 4A** were analyzed for pathway changes.



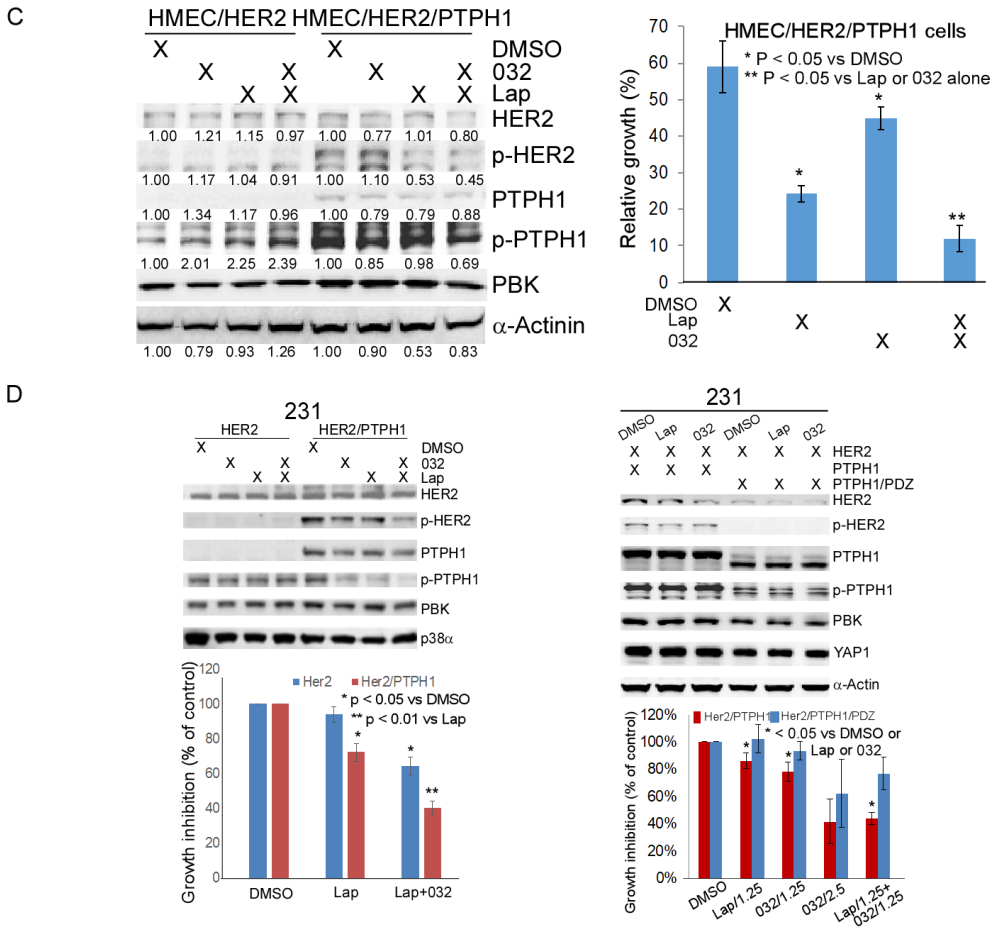


Figure S5. PTPH1 depends on PDZ to sensitize breast cancer cells to Lap and/or 032 induced p-HER2 down-regulation and growth inhibition. (A) PFD cooperates with Lap to inhibit breast cancer cell growth. Indicated cells were incubated with PFD (100 μg/ml) ± Lap (10 or 50 μM) for 48 h and assessed for cell growth by the cyquant NF cell proliferation assay as described [9] (over DMSO control, mean ± SD, n = 3). (B) Differential regulation of p-PTPH1 and p-HER2 by 032 and Lap, which is dependent on endogenous PTPH1 protein. T47D breast cancer cells were treated with the indicated inhibitors (2.5 μM for 4 h) for protein expression (top, left). PTPH1 knockdown was shown (top, middle), whereas resultant cell growth inhibition was given (bottom left) after incubation with and without 032 ± Lap (for 48 h). Primary mouse cancer cells (Neu) with and without PTPH1 expression were also cultured with 2.5 μM of Lap and 2.0 μM of 032 for 4 h for WB analysis (B, right). (C) PTPH1 co-expression with HER2 in HMEC cells increases depletion of p-PTPH1 and p-HER2 by 032 and Lap in association with greater growth inhibition. HMEC cells stably expressed with HER2 ± PTPH1 were analyzed for protein expression 4 h after incubation with Lap (2.5 μM) ± 032 (2.0 μM) and for cell growth (mean ± SD, n = 3) after 72 h incubation for cell proliferation using the cyquant NF cell proliferation assay kit as described [9]. (D) PTPH1 depends on PDZ to cooperate with HER2 as a therapeutic target for Lap, 032 and their combination in 231 breast cancer cells. Indicated engineered cells were incubated with Lap (5 μM) ± 032 (5 μM) for 4 h for WB and for 72 h for the growth inhibition (mean ± SD, n = 3).

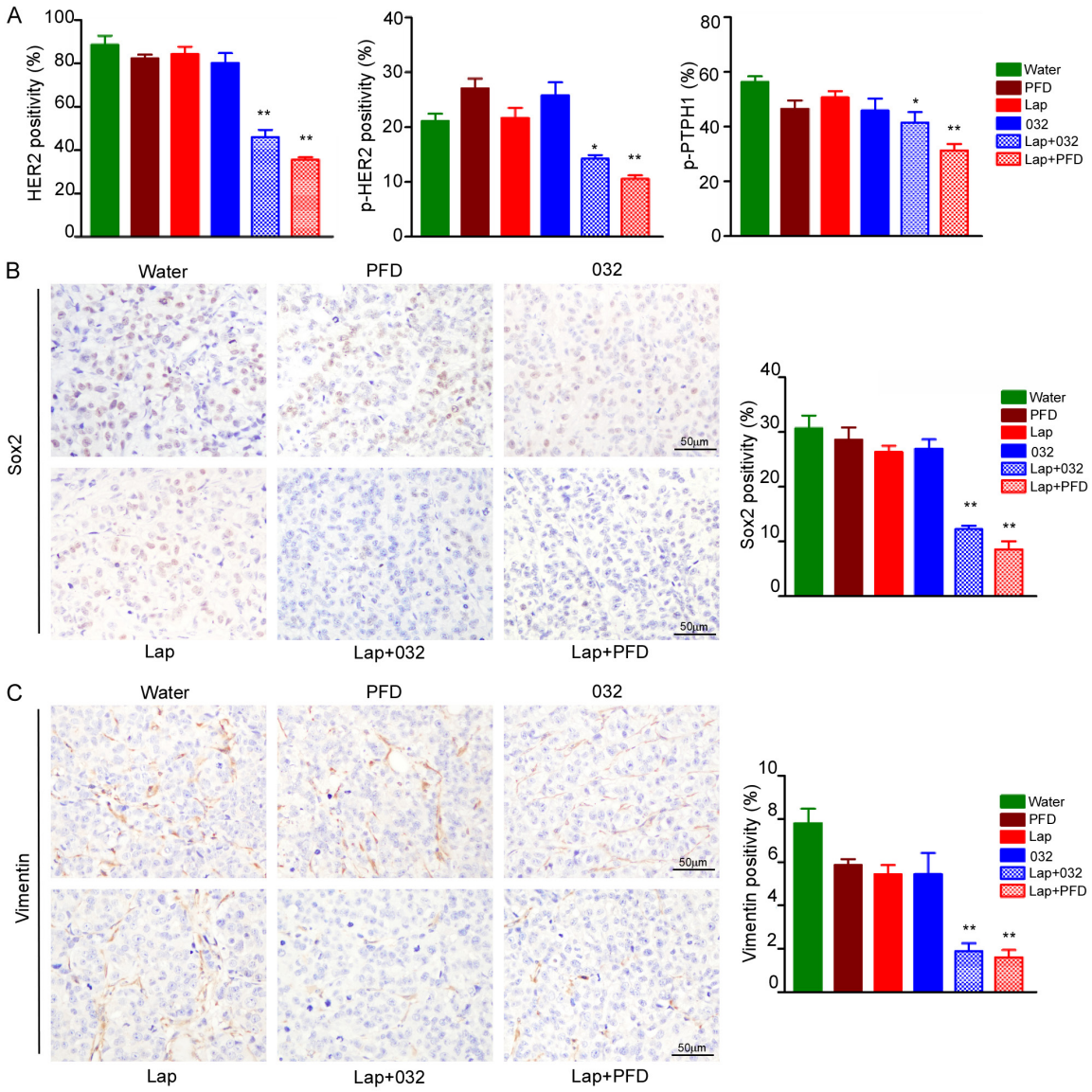


Figure S6. A. PFD or 032 cooperate to decrease protein levels of HER2, p-HER2, and p-PTPH1 in xenografts (from **Figure 6F**). Stained slides were scanned and results from 3 mice were shown (mean ± SD). B, C. Slides were stained with indicated antibodies, which were scanned for Sox2 and Vimentin expression. Please note that Vimentin positive staining appears mainly localized in tumor stromal cells (n = 6).

Supplementary Table 1 for Clinical samples

Total 102 HER2-positive breast cancer patients receiving trastuzumab treatment (at least 6 cycles) were enrolled in the study. All eligible patients were diagnosed with invasive breast cancer and treated at Sun Yat-sen University Cancer Center (Guangzhou, China) between 2005 and 2016. All studies have been approved by Institutional Review Boards (IRB 2020-343). The clinicopathological parameters of these patients were summarized in Table S1. Spearman's rank correlation analysis was used to analyze the correlation between p-PTPH1 level and PBK expression. The disease-free survival (DFS) was defined as the time from treatment to the first recurrence or death.

A cohort of all HER2-positive breast cancer patients who primarily underwent surgery between 2001 and 2008 (n = 74) was added for Her2 phospho-Tyr-877 analysis. All patients received standard chemotherapy regimen (CMF, CAF, TAC or AC-T) followed by surgery.

Abbreviations: CMF, cyclophosphamide, methotrexate, and fluorouracil; CAF, cyclophosphamide, doxorubicin, and fluorouracil; TAC, docetaxel, doxorubicin, and cyclophosphamide; AC-T, doxorubicin and cyclophosphamide followed by paclitaxel.

Table S1. Patient information and pathological characteristics of breast cancer specimens

A. Baseline clinical and pathologic characteristics of patients (n=102)	
Parameter	N (%) or Median (IQR)
Age (years)	49 (28, 73)
Ki67 (%)	30 (5, 90)
ER status	
Positive	61 (59.8)
Negative	41 (40.2)
PR status	
Positive	52 (51.0)
Negative	50 (49.0)
Tumor grade	
1	1 (1)
2	62 (60.8)
3	39 (38.2)
Lymph node status	
Positive	70 (68.6)
Negative	32 (31.4)
Tumor stage	
1	36 (35.3)
2	54 (52.9)
3	5 (4.9)
4	7 (6.9)
p-PTPH1 IHC	
Low (IRS 0-3)	11 (10.8)
High (IRS 4-12)	91 (89.2)
PBK IHC	
Low (IRS 0-3)	23 (22.5)
High (IRS 4-12)	79 (77.5)
p-HER2/877 IHC	
Positive	11 (10.8)
Negative	91 (89.2)

5-year disease free survival	
Yes	75 (73.5)
No	27 (26.5)

B. Baseline clinical and pathologic characteristics of patients (Supplementary, n=74)

Parameter	n (%) or Median (IQR)
Age (years)	50 (29, 72)
ER status	
Positive	45 (60.8)
Negative	29 (39.2)
PR status	
Positive	45 (60.8)
Negative	29 (39.2)
Lymph node status	
Positive	37 (50.0)
Negative	37 (50.0)
Tumor stage	
1	19 (25.7)
2	41 (55.4)
3	11 (14.9)
4	3 (4.0)
p-PTPH1 IHC	
Low (IRS 0-3)	6 (8.1)
High (IRS 4-12)	68 (91.9)
p-HER2/877 IHC	
Positive	5 (6.8)
Negative	69 (93.2)
5-year disease free survival	
Yes	49 (66.2)
No	25 (33.8)

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; IQR, interquartile range; IRS, immunoreactive score.