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Prohibitin is involved in the activated internalization and degradation of protease-activated receptor 1

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ABSTRACT

The protease-activated receptor 1 (PAR1) is a G-protein-coupled receptor that is irreversibly activated by either thrombin or metalloprotease 1. Due this irrevocable activation, activated internalization and degradation are critical for PAR1 signaling termination. Prohibitin (PHB) is an evolutionarily conserved, ubiquitously expressed, pleiotropic protein and belongs to the stomatin/prohibitin/flotillin/HflK/C (SPFH) domain family. In a previous study, we found that PHB localized on the platelet membrane and participated in PAR1-mediated human platelet aggregation, suggesting that PHB likely regulates the signaling of PAR1. Unfortunately, PHB's exact function in PAR1 internalization and degradation is unclear. In the current study, flow cytometry revealed that PHB expressed on the surface of endothelial cells (HUVECs) but not cancer cells (MDA-MB-231). Further confocal microscopy revealed that PHB dynamically associates with PAR1 in a time-dependent manner following induction with PAR1-activated peptide (PAR1-AP), though differently between HUVECs and MDA-MB-231 cells. Depletion of PHB by RNA interference significantly inhibited PAR1 activated internalization and led to sustained Erk1/2 phosphorylation in the HUVECs; however, a similar effect was not observed in MDA-MB-231 cells. For both the endothelial and cancel cells, PHB repressed PAR1 degradation, while knockdown of PHB led to increased PAR1 degradation, and PHB overexpression inhibited PAR1 degradation. These results suggest that persistent PAR1 signaling due to the absence of membrane PHB and decreased PAR1 degradation caused by the upregulation of intracellular PHB in cancer cells (such as MDA-MB-231 cells) may render cells highly invasive. As such, PHB may be a novel target in future anti-cancer therapeutics, or in more refined cancer malignancy diagnostics.

1. Introduction

Prohibitin (PHB or PHB1), a 32 kDa protein, is ubiquitously expressed and evolutionary conserved across organisms from yeast to humans, playing roles in the regulation and maintenance of mitochondrial functions [1]. Nuclear PHB meanwhile is engaged in several important transcriptional regulations, mainly being associated with cell-cycle progression and apoptosis [2]. A number of reports over the past few years have highlighted the function of plasma membrane PHB. In human intestinal epithelial cells, for example, the complex formed by PHBs functions as a binding site for the Vi capsular polysaccharide of *Salmonella typhi*, the causative agent of typhoid fever in humans [3]. Other studies report that the membrane PHB may facilitate the entry of DENV-2 (the causative agent of the most common mosquito-borne viral disease in human) into insect cells [4]. Meanwhile, PHB engaged in cell surface Raf-MEK-ERK signaling and human platelet PAR1 signaling [5,6]. The relationship between PHB and cancer has gained increased scrutiny among researchers in recent years. Several studies on various cancer cells have found elevated protein levels of PHB [7,8]; however, the role of PHB in cancer cell remains controversial.

PAR1, the protease activated receptor 1, also known as the thrombin receptor, which belongs to the G-protein-coupled receptor family is highly expressed in a variety of cell types, including endothelial cells, platelets, monocytes, neurons and cancer cells. Consequently, PAR1 plays important roles in thrombosis, angiogenesis, inflammation and metastasis [9–11]. PAR1 is activated in a unique proteolytic manner, wherein thrombin binds to and cleaves the extracellular N-terminal

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domain of the receptor, resulting in a tethered ligand that activates PAR1 [12]. Similarly, PAR1 can be activated directly via the six-residue peptide (S/TFLLRN) that corresponds to the tethered ligand of PAR1 [13]. At this juncture, the tethered ligand cannot diffuse away, leading to activated PAR1 becoming internalized and sorted into the lysosome, resulting in its degradation. Recent investigations have demonstrated that irreversibly and proteolytically-activated PAR1 was internalized through a clathrin-and dynamin-dependent pathway and sorted to lysosome for degradation [14]. Meanwhile, experimental evidences have shown that several functional molecules participate in the regulation of PAR1 internalization and degradation, including the clathrin adaptor AP-2 [15], epsin-1 [16], ALIX [17] and sorting nexin 1 (SNX1) [18]. Additionally, constitutive internalization also plays an important role in PAR1-related functions. Unactivated PAR1 constantly cycles between the cell surface and the intracellular stores, which provides a pool that replenishes the cell surface after PAR1 activation and leads to the rapid resensitization of PAR1 signaling independent of *de novo* receptor synthesis [19].

The precise regulatory mechanisms underlying PAR1 signal termination—including internalization and degradation—are critical in the PAR1 response present in many physiological and pathological processes. Unfortunately, some aspects of the internalization and degradation of PAR1 remain unclear, especially regarding the relationship between PHB and PAR1 internalization and degradation. In a recent study, we found that PHB is localized on the platelet membrane and also involved in PAR1-mediated human platelet aggregation, indicating that PHB is a previously unknown cofactor of the PAR1-related signaling pathway [5]. For platelets which were anuclear cells, the activation of PAR1 only occurred a single time during the lifespan of platelets, suggesting that many other important events of PAR1, *e.g.*, internalization and degradation are not carried out in platelets. Previous evidence, however, found that PAR1 mainly expressed in primary cells and cancer cells [20], and likewise that PHB or PAR1 is involved the proliferation and metastasis of carcinoma cells [21]. However, there is a lack of clear evidence highlighting the different relationship between cancerous cells and either PHB or PAR1, and such information may prove useful in finding ways to overcome cancerous cell growth.

In this study, we selected two nuclear cell lines to serve as models: normal endothelial cells (HUVECs) and breast cancer cells (MDA-MB-231 cells). Our analyses of these cells showed that PHB participated in PAR1-activated internalization, Erk1/2 phosphorylation and PAR1 degradation induced by PAR1-AP in HUVECs. Meanwhile, the regulation of these processes was aberrant in MDA-MB-231 cells; showing that PHB did not regulate PAR1 activated internalization or Erk1/2 phosphorylation, but that the increased expression of PHB in cancer cells inhibited PAR1 degradation. Together, these differing properties may be responsible for the invasive capacity of different types of cancer cells, making them key targets for further research into the activities and characterization of cancerous cells.

2. Materials and methods

2.1. Materials

The PAR1-activating peptide PAR1-AP (TFLLRN) was synthesized by GL Biochem (Shanghai, China). The anti-PAR1 monoclonal antibody (ATAP2), mitochondrial marker antibody, anti-COXIV and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while the anti-PHB polyclonal antibodies were purchased from R&D Systems (AF3470) (Minneapolis, MN, USA) and Santa Cruz Biotechnology (H-80) (Santa Cruz, CA, USA), and the monoclonal antibody was obtained from Neomarkers (Fremont, CA, USA). The anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody and the anti-p44/42 MAPK (Erk1/2) antibody were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The anti-EEA1, anti-LAMP1, anti-Histone H3 rabbit polyclonal antibodies and the mouse monoclonal M2 anti-Flag antibody were purchased from Sigma (St. Louis, MO, USA). The Alexa 488-, Alexa 594- and Alexa 647-conjugated goat anti-rabbit, goat anti-mouse and donkey anti-goat antibodies were respectively obtained from Invitrogen (Carlsbad, CA, USA). Cell mitochondria isolation and nuclear protein extraction kits were purchased from the Beyotime Institute of Biotechnology (Wuhan, Hubei, China) and the Matrigel Basement Membrane Matrix was purchased from Becton Dickinson (BD) Biosciences (San Jose, CA, USA). All other reagents were purchased from Sigma (St. Louis, MO, USA). Protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA, USA) with BSA as a standard.

2.2. Calcium mobilization

To measure cytoplasmic Ca^{2+}, cells were dissociated using enzyme-free cell dissociation buffer (Invitrogen) and then incubated for 30 min with 5 μM fluo-3 AM in a buffer containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose and 1% BSA (PH 7.4, adjust by NaOH at 37 °C). Fluo-3 fluorescence was measured at 37 °C in a Perkin-Elmer LS-5 fluorimeter at 505 nm excitation and 530 nm emission. For the cell inhibition assays, cells were pretreated with a monoclonal antibody (IV.3) to block anti-FcγRIIA (CD32) and prevent the nonspecific actions of the anti-PHB antibody.

2.3. Internalization assay

PAR1 internalization was assessed using the method previously described by Chen et al. [16]. In brief, cells were plated in 96-well dishes at a density of 5×10^3 cells per well and grown overnight. The cells were washed with PBS, and then incubated in DMEM containing 1 mg/ml BSA (pH 7.4), and subsequently either treated or not treated with 75 μM PAR1-AP for various times at 37 °C. Cells were then fixed with 4% paraformaldehyde for 5 min at 4 °C and subsequently incubated with an anti-PAR1 antibody for 1 h at 25 °C. The cells were then washed, and incubated with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody for 1 h at 25 °C. Next, the cells were washed and incubated with the horseradish peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB) for 10 min, and equal aliquots were removed to new 96-well dishes. Optical density of the cells was determined at 450 nm using an Infinite M200 PRO Microplate Reader (TECAN Company, Switzerland).

2.4. Flow cytometry

The flow cytometry methods used in the present study are similar to those mentioned in our previous report [5]. In brief, to detect the surface expression of PAR1 and PHB using immunofluorescence staining, human umbilical vein epithelial cells (HUVECs) and MDA-MB-231 cells were dissociated using enzyme-free cell dissociation buffer (Invitrogen), and subsequently incubated with the appropriate primary and secondary antibodies. After washing three times, all samples were analyzed using a flow cytometer (FACSVantage SE, Becton Dickinson, NJ, USA).

2.5. Confocal microscopy

Confocal microscopy analysis was performed according to the method described by Booden et al. [22]. Both HUVECs and MDA-MB-231 cells were grown on cover slips in a 24-well tissue culture plate. The cells were then washed with PBS and incubated with an anti-PAR1 antibody for 1 h at 4 °C. Cells then were washed three times with PBS and incubated, either with or without the PAR1 agonist at 37 °C for various times. Finally, cells were fixed, permeabilized, and immunostained with the appropriate primary and secondary antibodies, and the slides were observed using a confocal microscope (Olympus FV1000, Olympus Corporation, Tokyo, Japan).

2.6. Co-immunoprecipitation

Washed cells were incubated with the agonist, PAR1-AP, for the indicated times and then washed and lysed with NP-40 buffer (50 mM Tris, 150 mM NaCl, 0.5% NP-40, 2% BSA and complete protease inhibitor cocktail, pH 7.4). The lysates were then immunoprecipitated with the respective primary antibodies, as described in our previous report [5].

2.7. Cell culture and transfection

The breast carcinoma MDA-MB-231 cell line was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and maintained in growth media as recommended by the ATCC. HUVECs were also obtained from the ATCC and cultured in growth media as recommended by the ATCC. The full open-reading frame of the mature human PHB cDNA, obtained from cDNAs prepared from human placenta, was subcloned into the pCMV-Myc vector at the EcoRI and HindIII sites; a Flag-tag was included at the N-terminus of the protein. For transient transfection, cells were placed in 24-well tissue culture plates and then transfected with 4 μg of plasmids (pCMV-Myc vector or pCMV-Myc-Flag-PHB) for 48 h, using either the HiPerFect transfection reagent (Qiagen, Valencia, CA, USA) or FuGENE 6 transfect reagent (Promega, Madison, WI, USA). For RNA interference (RNAi) experiments, cells were cultured to 40–50% confluence prior to transfection. Small interfering RNAs targeted against PHB (siPHB) or a negative control (siCtrl) were transfected into cells using the Lipofectamine RNAiMax Transfection reagent (Invitrogen, Carlsbad CA, USA). The siPHB (5′-CAGAAAUC ACUGUGAAAUUTT-3′) and the negative control siRNA (siCtrl, 4390843) were both obtained from Qiagen (Valencia, CA, USA).

2.8. Cell invasion and migration assays

In vitro cell invasion assays were carried out using a Transwell chamber of 6.5 mm diameter and 8.0 μm pore size polycarbonate membrane (Corning Costar, NY, USA) coated with Matrigel (BD Biosciences, San Jose, CA, USA). MDA-MB-231 cells were transfected with either Flag-PHB1 or empty vector (pCMV-myc). After being starved for 12 h with a serum free medium, cells were dissociated and added into each upper chamber of the Transwell chamber, while the bottom chamber contained a medium with 20 μM PAR1-AP incubated for 24 h at 37 °C. The invaded cells were dyed with crystal violet and counted microscopically. Three fields were counted for each assay.

To measure cell migration, after transfection and starvation, cells were seeded onto Transwell Chambers (8.0 μm pores) (Corning Costar, NY, USA) coated with collagen type I. After 30 min, cells were stimulated with 20 μM PAR1-AP and incubated for 16 h at 37 °C. Migrated cells were then dyed with crystal violet. Bound crystal violet was eluted with 1 ml 10% acetic acid and the migration activity was expressed as the value monitored at 586 nm of extraction.

2.9. Erk1/2 phosphorylation assay

The Erk1/2 phosphorylation assay was conducted as described previously [23]. For the internalization inhibitory assay, after HUVECs

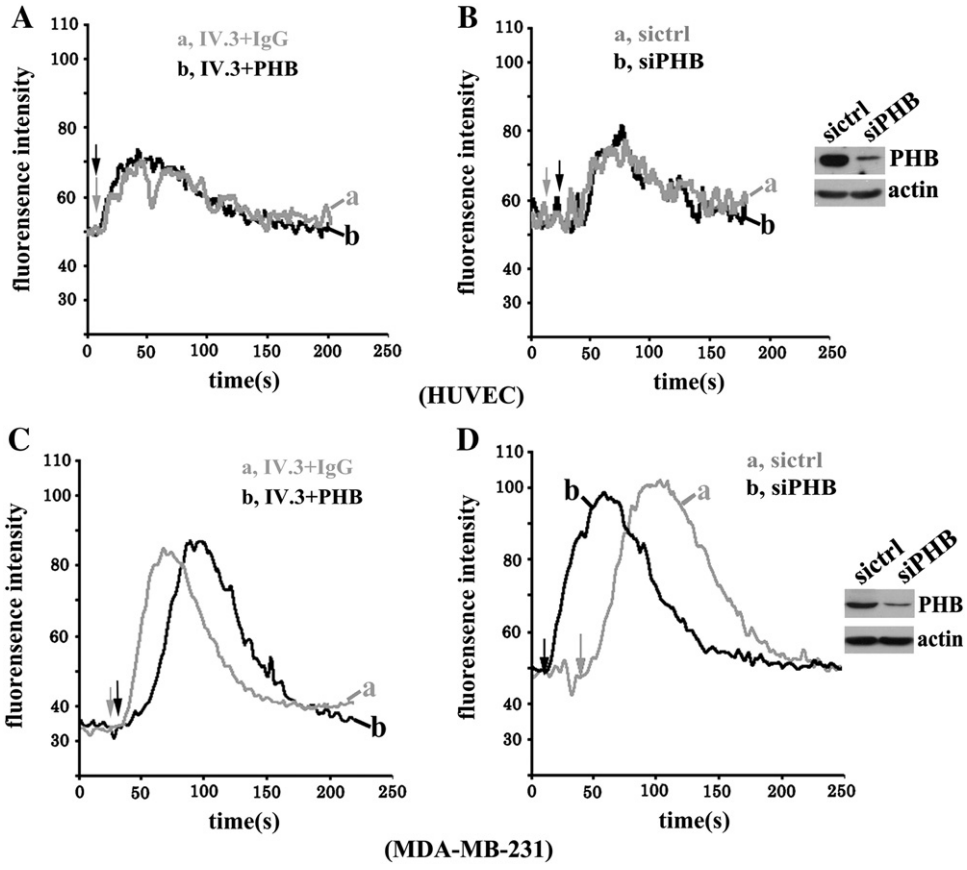
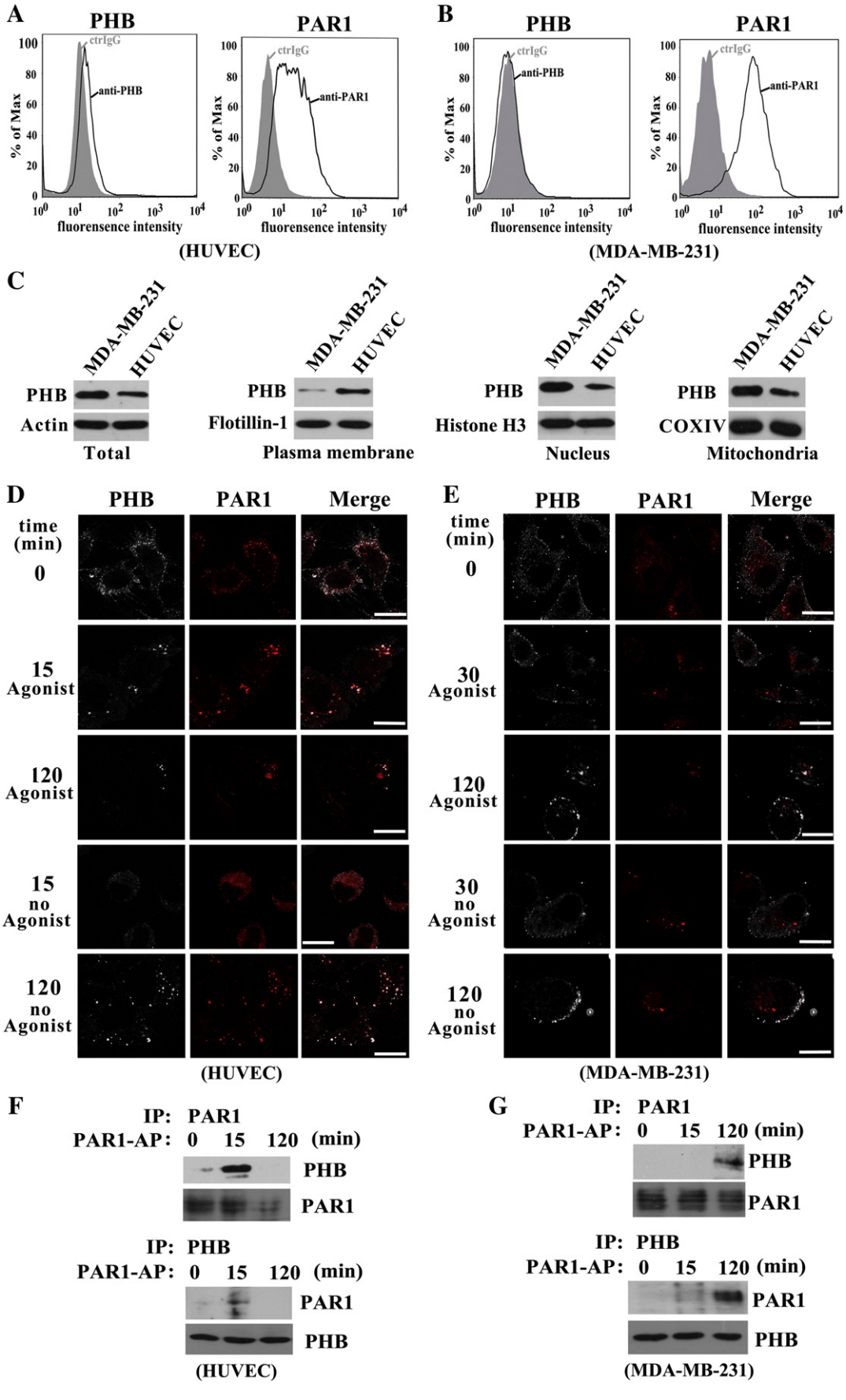


Fig. 1. PHB is not involved in PAR1-mediated Ca^{2+} mobilization in endothelial or cancer cells. Fluo 3-AM-loaded cells were pretreated with IV.3 (10 μg/ml) and were incubated with anti-PHB antibodies or control IgGs (10 μg/ml). Ca^{2+} mobilization downstream of PAR1 activation in human endothelial cells (A) and MDA-MB-231 cells (C) were not affected by the blockade of PHB using anti-PHB antibody. HUVECs and MDA-MB-231 cells were transfected with siRNA targeted against PHB, and 48 h after RNAi, Western blot showed that PHB expression was significantly decreased. Knockdown of PHB did not affect the Ca^{2+} mobilization induced by PAR1-APin the HUVECs (B) or MDA-MB-231 cells (D). PAR1-AP (20 μM) was added at the times indicated by the arrows, and the fluorescence was monitored using a fluorescence spectrophotometer.

were starved for 12 h with a serum-free M199 medium, cells were incubated for 4 h, either with or without 0.4 M sucrose, and then stimulated with 75 μM PAR1-AP for the indicated times at 37 °C.



2.10. Analysis of the sub-cellular location of PHB in MDA-MB-231 cells and HUVECs by Western blotting

The isolation of plasma membranes was done according to a modification to the method described by Wahn Soo Choi et al. [24]. Cells (2×10^7) were homogenated for 30 cycles in 0.25 M sucrose in 20 mM Tris-Hepes (pH 7.4) and centrifuged (3000 ×*g*, 10 min). A stepwise sucrose gradient was created by the sequential addition to centrifuge tubes of 1.6 ml of 40%, 0.8 ml of 15%, and 1.3 ml of 11.5% (*w*/*v*) sucrose solution, and finally the homogenated cell lysate. After ultracentrifugation at 27,000 *g* for 3 h at 4 °C, the middle opaque fractions (plasma membrane fraction) were collected. The isolations of mitochondria and nucleus were done in accordance with the isolation kit protocols. After isolation of the cells' plasma membrane, mitochondria and nucleus, protein concentrations were determined using a protein assay. The cell fractions (30 μg of protein) were loaded on an SDS-PAGE gel, and following electrophoresis, electrotransferred onto a PVDF membrane that was subsequently blocked with 3% BSA and incubated with the appropriate primary and secondary antibodies. Resulting protein bands were visualized using an enhanced chemiluminescence reagent (Thermo-Fisher).

2.11. Statistical analysis

Each experiment included three to four replicates, and the results were analyzed using Prism 5.0. Statistical analysis included two-way ANOVA with Bonferroni correction and Student's *t*-tests. All experimental values are expressed as the means ± SD, with $P < 0.05$ being considered statistically significant.

3. Results

3.1. PHB does not affect PAR1-mediated Ca^{2+} mobilization in endothelial or cancer cells.

In our previous work, we found that PHB was a novel cofactor in PAR1-related signaling activation inhuman platelets. Unfortunately, these findings were somewhat limited because of anucleate human platelets. So we examined the specific role of PHB in the PAR1 signaling pathway in nucleated cells, including human endothelial cells (HUVECs) and highly invasive cancer cells (MDA-MB-231), that express endogenous PHB and PAR1. Accordingly, we investigated the effects of PHB on Ca^{2+} mobilization stimulated by PAR1-AP in these two cell types. Blocking PHB with an anti-PHB antibody or depletion PHB by RNAi did not affect the Ca^{2+} mobilization induced by 20 μM PAR1-AP in either HUVECs (Fig. 1A, B) or MDA-MB-231 cells (Fig. 1C, D). Likewise, treatment of cells with selective siRNA against PHB significantly decreased the expression of PHB in both HUVECs and MDA-MB-231 cells, as determined by Western blotting (Fig. 1B, D). These results indicate that PHB is not involved in PAR1-mediated Ca^{2+} mobilization in endothelial and cancer cells, indicating that PHB plays a different role here than it does with human platelets.

3.2. Colocalization of PHB and PAR1 is dynamic and is dependent on the duration of agonist activation in endothelial and cancer cells

While the cellular localization of PHB is very important for its function, for example, membrane PHB could be a receptor or a cofactor to regulate the cell signaling pathway [4], and the function of PHB in the regulation of PAR1 is unclear in HUVECs and MDA-MB-231 cells. In the present study, flow cytometry showed that PHB and PAR1 were expressed on the surface of HUVECs (Fig. 2A), but in MDA-MB-231 cells, PAR1 was expressed on the cell membrane and PHB was not localized on the cell surface (Fig. 2B). Western blot also showed that PHB was presented on the plasma membrane of HUVECs but was hardly found in MDA-MB-231 cells. Total PHB expression in MDA-MB-231 was likewise higher than that in HUVEC, especially in mitochondria and nuclear (Fig. 2C). Confocal microscopy revealed that PHB colocalized with PAR1 in the rest (0 min) HUVECs. This colocalization was strengthened when the cells were activated with PAR1-AP for 15 min but weakened after 120 min (Fig. 2D). However, in the MDA-MB-231 cells, PHB and PAR1 colocalized with each other in cells that were activated with PAR1-AP for 120 min, but not in unactivated or short-term activated cells (Fig. 2E). There was no difference in cells incubated with no agonist (Fig. 2D, E).

Co-immunoprecipitation assays yielded the same results. PHB and PAR1 could interact with each other when cells had been activated with PAR1 activated at 15 min (HUVECs) or 120 min (MDA-MB-231 cells) (Fig. 2F, G). These results revealed that PHB and PAR1 colocalized in a dynamic process dependent on the length of time following activation with PAR1-AP. Additionally, the colocalization of PHB and PAR1 differs between endothelial and cancer cells, suggesting that PHB may regulate PAR1 function differently among these different types of cell.

3.3. PHB participates in PAR1 activated internalization and Erk1/2 signaling, but not constitutive internalization, in endothelial cells

To determine the function of PHB in PAR1 trafficking, we depleted endogenous PHB in human endothelial cells (HUVECs) using a specific siRNA (siPHB). Confocal microscopy showed that constitutive internalization of PAR1 was not affected in the siPHB-transfected HUVECs (Fig. 3A), and a further ELISA showed that there was no difference in PAR1 constitutive internalization between the cells transfected with the control siRNA or the PHB siRNA in HUVECs (Fig. S1). Interestingly though, PAR1 activated internalization was significantly decreased when PHB was knocked down by siRNAs against PHB as compared with HUVECs transfected with control siRNAs. Confocal microscopy revealed that the colocalization of PAR1 and EEA1 (Early Endosome Antigen 1), an early endosome marker [25], was significantly decreased, and a fraction of the activated PAR1 did not internalize and sort to the early endosome in PHB knockdown cells (Fig. 3B, lower panel). ELISA showed that knockdown of PHB inhibited PAR1-AP-induced internalization of activated PAR1, which was highly significant at 15 min (inhibited 55.6% compared control) (Fig. 3C). Additionally, in the HUVECs, PAR1-AP-induced phosphorylation of Erk1/2 was affected by PHB depletion. Erk1/2 phosphorylation was sustained for 15 min in endothelial cells transfected with PHB siRNA, whereas Erk1/2 phosphorylation in the cells transfected with control siRNA was only sustained for 5 min following PAR1-AP induction (Fig. 3D).

3.4. PHB does not regulate PAR1 activated internalization or Erk1/2 signaling in breast cancer cells

MDA-MB-231 cells were incubated with an anti-PAR1 antibody for 1 h at 4 °C, then were washed and treated with PAR1-AP for 30 min at 37 °C, processed, and finally examined using a confocal microscope. After stimulation with PAR1-AP for 30 min, a fraction of the activated

Fig. 2. Colocalization of PHB and PAR1 is a dynamic process dependent on the duration of agonist (PAR1-AP) activation. (A) Based on flow cytometry analysis, both PHB and PAR1 were expressed on the cell surface in HUVECs. (B) In MDA-MB-231 cells, only PAR1 was expressed on the cell surface but not PHB. (C) Total cell lysates and cell fractions were blotted with anti-PHB, anti-actin, anti-flotillin (a membrane marker), anti-Histone H3 (a nuclear marker) and anti-COXIV (a mitochondria marker). (D) In the rest HUVECs, PHB and PAR1 colocalized. The colocalization strengthened at 15 min and weakened at 120 min when activated with agonist. However, there was no difference within cells incubated with no agonist. (E) In the MDA-MB-231 cells, PHB and PAR1 colocalized at 120 min after induction with PAR1-AP, and no colocalization in cells incubated with on agonist. (F, G) Dynamic interactions between PAR1 and PHB in HUVECs and MDA-MB-231 cells were confirmed by co-immunoprecipitation. Bar, 15 μm.

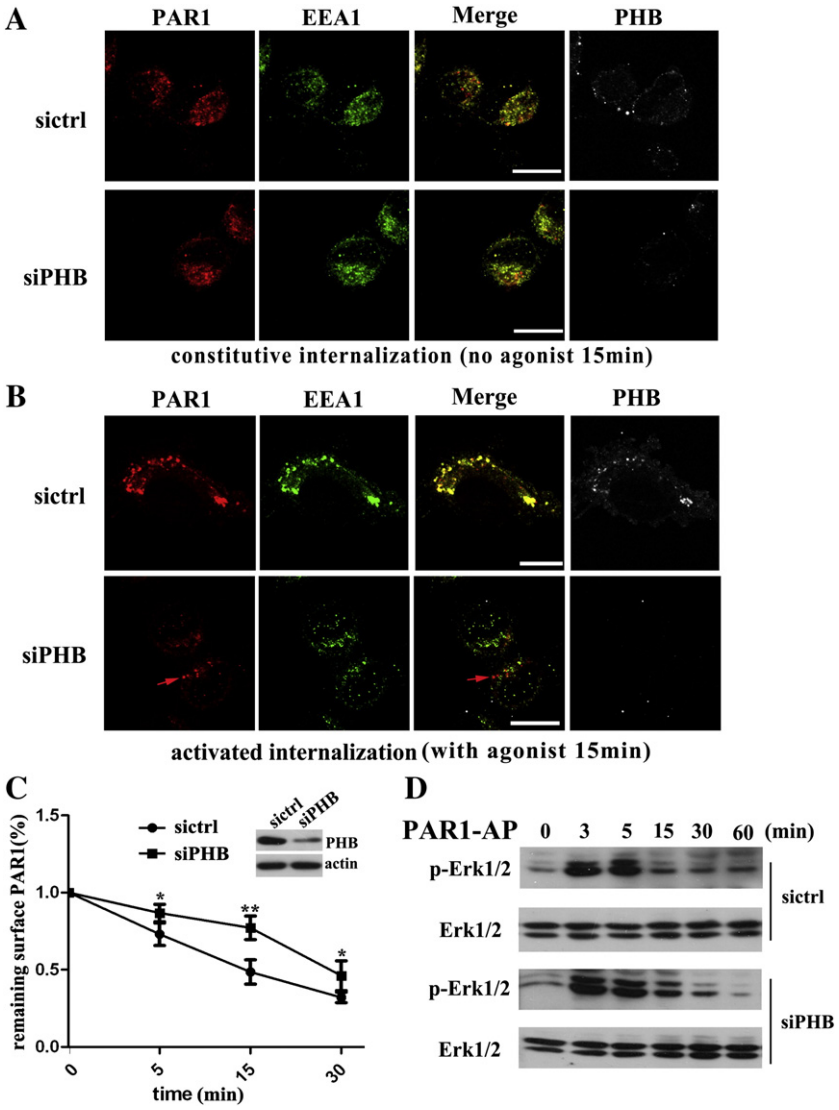


Fig. 3. PHB regulated Erk1/2 signaling and PAR1 activated internalization but not PAR1 constitutive internalization in HUVECs. (A) Constitutive PAR1 internalization was not affected by PHB knockdown. PAR1 (red), EEA1 (green) and PHB (gray). The colocalization of PAR1 and EEA1 is shown in yellow. (B) Knockdown of PHB inhibited activated PAR1 internalization according to confocal microscopy analysis. PAR1 (red), EEA1 (green) and PHB (gray).The colocalization of PAR1 and EEA1 is shown in yellow. When the cells were transfected with PHB siRNA, a fraction of the PAR1 protein did not colocalize with EEA1, as shown by the red arrow (lower panel). (C) Knockdown of PHB inhibited PAR1 activated internalization as shown by ELISA. (D) Depletion of PHB prolonged the phosphorylation of Erk1/2 in HUVECs induced with PAR1-AP. The total Erk1/2 served as a loading control. Bar, 15 μm.

PAR1 did not internalize to endocytic vesicles and colocalize with EEA1, nor did PHB colocalize with PAR1 (Fig. 4A). To further characterize the potential role of PHB in the PAR1 activated internalization in the MDA-MB-231 cells, we transfected these cells with PHB siRNA and using an ELISA found that depletion of PHB did not affect the activated internalization of PAR1 induced by PAR1-AP (Fig. 4B). By contrast, when we overexpressed Flag-tagged PHB in the MDA-MB-231 cells, PAR1 activated internalization was increased 33.1% as compared with cells transfected with the pCMV-Myc vector, the expression of Flag-tagged PHB was determined by Western blotting (Fig. 4D) and detection of the surface expression of PHB was determined by flow cytometry (Fig. S2). Further testing of Erk1/2 phosphorylation in MDA-MB-231 cells showed that depletion of PHB did not affect Erk1/2 phosphorylation (Fig. 4C), and that overexpresion of Flag-tagged PHB shortened the duration of Erk1/2 phosphorylation to 30 min, nearly half the time observed in the cells transfected with the pCMV-Myc vector (60 min) (Fig. 4E). Interestingly, overexpression of Flag-tagged PHB could reduce both cell invasion (26.86%) and cell migration (19.27%) stimulated by PAR1-AP as compared with the cell transfected with the empty vector (Fig. 4F).

3.5. PHB negatively regulates the degradation of PAR1 in endothelial and cancer cells

To examine the effects of PHB on PAR1 degradation, cells were incubated with an anti-PAR1 antibody for 1 h at 4 °C, then washed and treated with PAR1-AP for 120 min at 37 °C, processed, and examined using a confocal microscope. When endothelial cells were treated with PAR1-AP for 120 min, surface PAR1 was divided into two fractions, one fraction colocalized with PHB, whereas the other colocalized with LAMP1, a late endosome and lysosome marker. The second fraction of PAR1 was sorted into the lysosome and degraded (Fig. 5A, upper panel). Endothelial cells transfected with PHB siRNA showed higher levels of PAR1 degradation than cells transfected with control siRNA (Fig. 5B). Similarly, PAR1 degradation level was decreased in endothelial cells overexpressing Flag-tagged PHB as compared to cells transfected with the pCMV-Myc vector (Fig. 5C). These results indicate that PHB negatively regulates PAR1 degradation in endothelial cells. In breast cancer cells, we found that PHB has a similar function. When MDA-MB-231 cells were treated with PAR1-AP for 120 min, PAR1 colocalized with PHB but not with LAMP1, indicating that PAR1 was not sorted to the

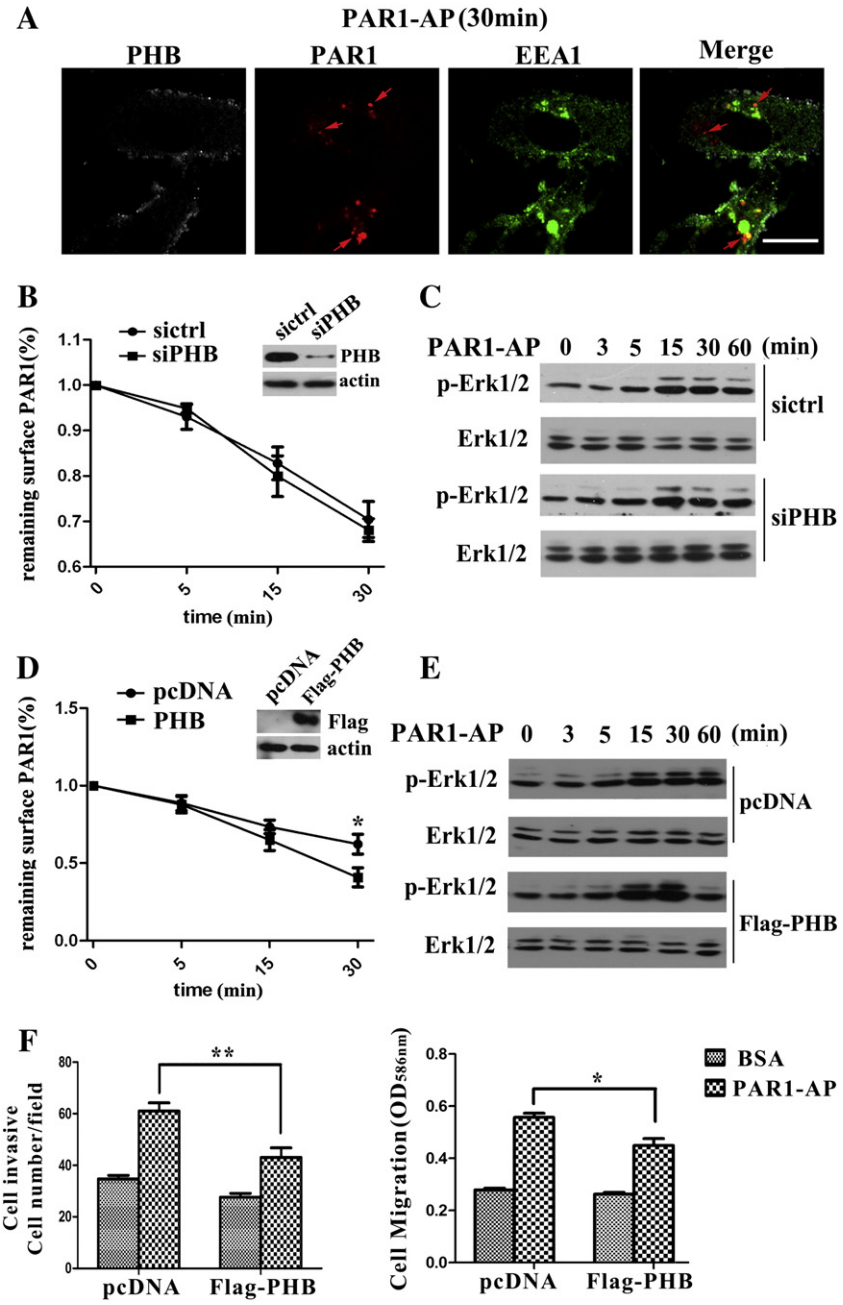


Fig. 4. PHB loses the ability to regulate PAR1 activated internalization and Erk1/2 phosphorylation in MDA-MB-231 cells while the overexpression of PHB restores this function. (A) Activated PAR1 did not completely internalize to the early endosome as determined by confocal microscopy. PAR1 (red), EEA1 (green) and PHB (gray).The colocalization of PAR1 and EEA1 is shown in yellow, and the fraction of PAR1 did not colocalize with EEA1 shown in red and indicated by the red arrows. Bar, 15 μm. (B) Knockdown of PHB did not affect the internalization of activated PAR1 as determined by an ELISA. (C) Depletion of PHB did not affect the PAR1-AP-induced phosphorylation of Erk1/2.(D) Overexpression of PHB increased activated PAR1 internalization. Western blot using anti-Flag antibodyrevealed the expression level of Flag-PHB. (E) Overexpression of PHB shortened the duration of PAR1-AP-induced Erk1/2 phosphorylation. (F) Overexpression of PHB reduced the cell invasion (left) and cell migration (right) induced by PAR1-AP.

lysosome or degraded in these cells (Fig. 5A, lower panel). Higher levels of PAR1 degradation were also observed in cells transfected with PHB siRNA as compared with cells transfected with control siRNA (Fig. 5D). However, overexpression of PHB had no effect on PAR1 degradation in MDA-MB-231 cells (Fig. 5E). These results indicate that PHB negatively regulates PAR1 degradation in both endothelial cells and cancer cells.

4. Discussion

In our present study, we first defined a previously unknown role for PHB in the regulation of PAR1 activated internalization and degradation in endothelial cells and cancer cells. PHB was necessary for PAR1 activated internalization and Erk1/2 phosphorylation, and PHB negatively regulated PAR1 degradation. Furthermore, our findings showed that PHB membrane absence contributes to the high invasivity in highly malignant breast carcinoma MDA-MB-231 cells. It is universally accepted that a sustained PAR1 signal could be observed in carcinoma cells, and our findings proved that membrane PHB absence leads to a null PAR1 activated internalization then induces the sustained PAR1 signal in MDA-MB-231 cells and it is consistent with the previous report [22]. We hypothesize that PHB acts as a chaperone in this process to regulate PAR1 cellular trafficking and sorting; during PAR1 degradation, PAR1 dissociates from PHB and is sorted to the lysosome, where it is degraded. PHB is a member of the SPFH family, a family of proteins that act

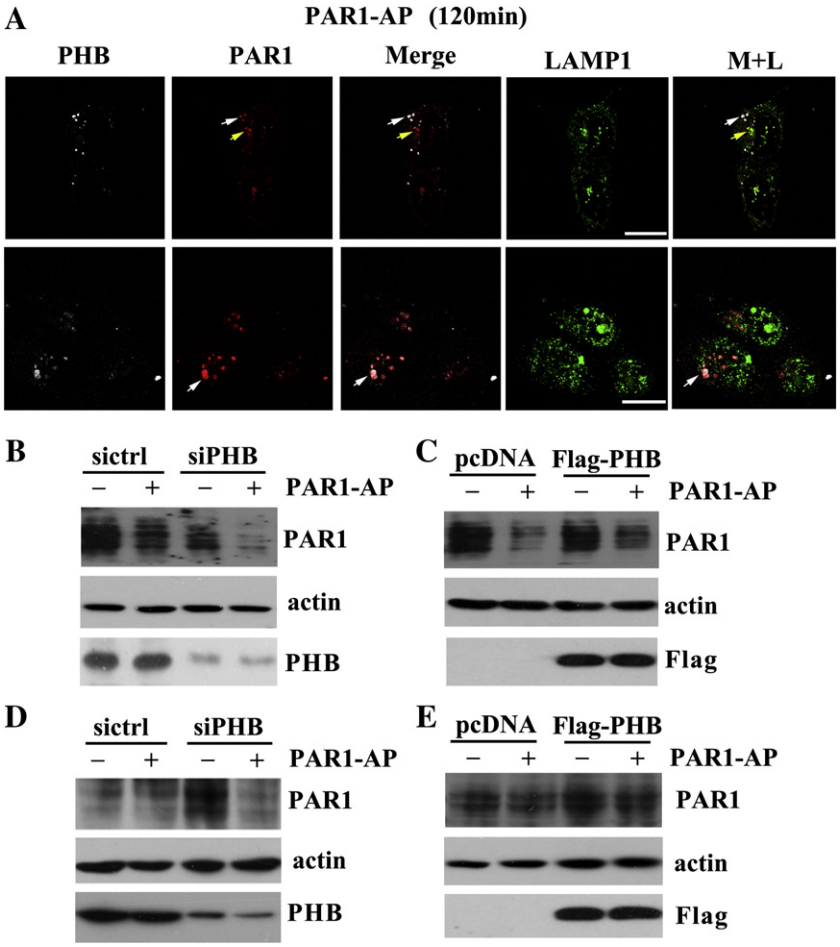


Fig. 5. PHB negatively regulates the degradation of PAR1 in HUVECs and MDA-MB-231 cells. (A) PAR1 degradation in HUVECs (upper panel) and MDA-MB-231 cells (lower panel). PAR1 (red), LAMP1 (green) and PHB (gray). The colocalization of PAR1 and LAMP1 is shown in yellow and is highlighted by the yellow arrow (upper panel). The colocalization of PAR1 and PHB is shown in gray and is highlighted by the gray arrow (upper and lower panel). (B) Knockdown of PHB increased PAR1 degradation in HUVECs. Cells were incubated in the absence (−) or presence (+) of 75 μM PAR1-AP for 120 min at 37 °C. (C) Overexpression of PHB decreased the level of PAR1 degradation in HUVECs. (D) Knockdown of PHB increased the level of PAR1 degradation in MDA-MB-231 cells. (E) Overexpression of PHB had no effect on PAR1 degradation in MDA-MB-231 cells.

as membrane chaperones and regulate protein trafficking [26–28]; therefore, our findings are consistent with these features of the PHB family.

The relationship between the protein PHB and cancer has been extensively studied, and the extant evidence indicates that the PHB gene is likely a tumor suppressor, since its 3′-UTR was previously shown to inhibit cell cycle progression by blocking G1–S transition in breast and other cancers [29]. PHB levels are elevated in cervical, esophageal, stomach, breast, lung, bladder, thyroid, ovarian, and prostate cancers [7,8]. However, the role of PHB in cell proliferation or tumor suppression remains controversial. Previous evidence showed that the subcellular localization of PHB affects cell fate, and as such, the role of PHB in tumorigenesis may be explained by its subcellular localization. In the present study, we found that PHB is not expressed on the surface of the highly invasive MDA-MB-231 breast cancer cells; however, it is expressed on the surface of primary HUVECs and the lowly invasive MCF-7 breast cancer cells (Fig. S3). In higher invasive cancer cells, the loss of PHB membrane localization may have resulted in the loss of PHB-dependent regulation of PAR1 activated internalization and Erk1/2 phosphorylation. Therefore, we deduced that membrane PHB exerted this function and the detection of plasma membrane PHB after PHB overexpression confirmed this hypothesis. Likewise, because PHB may have lost its regulatory functions in cancer cells, PAR1 should not internalize and Erk1/2 phosphorylation should be sustained when activated by PAR1-AP; and these mechanisms may, at some level, be responsible for the highly invasive nature of these cancer cells.

Our results also showed that PHB negatively regulated PAR1 degradation. The role that PHB plays in this function is due to its association with PAR1 when cells have been activated by PAR1-AP for long time. In effect, once PHB dissociates from PAR1, PAR1 can be degraded. Previous work has shown that PAR1 is highly expressed in breast carcinoma cells and increases in PAR1 expression have been correlated with carcinoma cell invasiveness [30]. Additionally, recent research revealed that deregulated PAR1 trafficking leads to constitutive signaling and promotes cellular invasion [22]. MDA-MB-231 is a type of a highly invasive breast cancer cell with a high level of endogenous PAR1 and PHB expression. PAR1 activation and signaling are normally observed in this cell type, and PHB did not affect the Ca^{2+} mobilization mediated by PAR1 (Fig.1C, D). PHB is highly expressed in this cell line; however, it is not expressed on the cell surface. Therefore, we believe that the majority of PHB is intracellular. When the cancer cells were activated with the agonist for long time (120 min), the tight interaction between PHB and PAR1 inhibited PAR1 degradation. Overall, our results indicated that high intracellular levels of PHB inhibited PAR1 degradation in MDA-MB-231 cells, leading to persistent, activated PAR1 signaling. This signaling may be responsible for cancer cell metastasis and invasion.

Our results also showed that when PAR1 activated internalization was reduced, Erk1/2 phosphorylation mediated by PAR1 was sustained, and vice versa. Gourlaouen et al. [31] showed that VEGF2R internalization was necessary for Erk1/2 phosphorylation. And a mutation in the YRRL motif of the thrombopoietin receptor greatly reduced receptor

internalization and prolonged Erk1/2 phosphorylation [32]. Taken together, these results indicate that receptor activated internalization is related to Erk1/2 signaling, and we speculate that PAR1 activated internalization is related to the duration of Erk1/2 activation. In our study, we found that inhibition of clathrin-mediated endocytosis by sucrose (0.4 M) [33] resulted in a prolonged duration of Erk1/2 phosphorylation as compared to control (Fig. S4).

In conclusion, results of our analyses characterized for the first time how PHB is involved in PAR1-related activated internalization, Erk1/2 phosphorylation and PAR1 degradation in both human endothelial and cancer cells. Our results also suggest that the absence of membrane PHB indeed contributes to the observed high malignancy in breast carcinoma cells. These interesting and fascinating findings provide clues and evidences for cancer malignant degree classification and diagnosis. These findings provide novel clues to some of the underlying mechanisms at play in certain cancerous cells invasiveness and proliferation, and likewise offer evidence that may be useful in developing future refined classifications and diagnostics of cancer malignancy.

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