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Phafin2 modulates the structure and function of endosomes by a Rab5-dependent mechanism

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ABSTRACT

By regulating the amount of protein receptors on the cell membrane and the metabolisms of receptor-bound ligands, endocytosis represents one of the fundamental biological activities that regulate how cells respond to the environment. We report here that a Fab1–YotB–Vac1p–EEA1 (FYVE) domain-containing lipid associated protein, called Phafin2, is preferentially expressed in the human hepatocellular carcinoma (HCC) and is involved in the biogenesis of endosomes. Over-expression of Phafin2 or its FYVE domain results in the formation of enlarged endosomes that are still functional for endocytosis; the biogenesis of such abnormal organelles is mediated by phosphoinositide 3-kinases (PI3K) and Rab5 signaling. Using fluorescence resonance energy transfer measured by fluorescence lifetime imaging microscopy (FLIM-FRET), we further demonstrate in live cells that Phafin2 can directly activate Rab5. By modulating the receptor internalization/recycling and Rab5 activation, Phafin2 affects the density of membranous insulin receptors, and regulates the transcriptional activity of AP-1 that is downstream of the insulin signaling pathway. These results provide a vivid example that an endosome modulator, such as Phafin2, may control the cells' responses to the extracellular cues.

Introduction

The plasma membrane is the principle site where the cell senses the extracellular environment; this is achieved primarily through protein receptors residing on the cell surface. The composition of membrane proteins is therefore important not only in maintaining normal cell functions [1,2], but also in mediating pathological cellular processes [3], including carcinogenesis. The membrane proteins can be re-internalized by endocytosis [4]. The endocytosed molecules are delivered to the endosome for further sorting [5]; they may be recycled back to the cell surface or further transported, either facilitated by ubiquitination or not, to the lysosomal compartment for protein degradation. However, it is unclear as to what extent and by what type of regulation a cell may use such post-translational machineries to control the molecular composition on the cell surface, and to modulate the cell's response to the signals from the environment.

Proteins, lipids and various lipid-binding proteins all participate in the above-mentioned vesicular sorting and membrane trafficking processes [6,7]. Phosphoinositide phosphates (PIPs) and derivatives serve as intrinsic membrane signals for trafficking via their structural roles as membrane components or by engaging with protein regulators [8,9]. Rab proteins represent another important set of regulators of vesicle transportation [7]. Activity governed by Rab5 is essential for early endocytotic events [10]. The finding that Rab5 can activate hVPS34, a class III PI3-kinases, further couples Rab5 signaling to phosphatidylinositol-3-phosphate or PI(3)P production [11]. Besides Rab proteins, a group of proteins containing Fab1–YotB–Vac1p–EEA1 (FYVE) domain have been shown to bind to the membrane [12] and regulate vesicular trafficking [9]. In the human genome, there are 38 predicted gene products that contain the FYVE domain, but their functions vary [13]. Here we identify and characterize a FYVE domain-containing gene, called Phafin2 (also known as EAPF [14], FLJ13187, ZFYVE18 or PLEKHF2), that is differentially expressed in the liver cancer cell and regulates the structure and function of the endosomes through Rab5-dependent processes. By modulating the receptor density on the cell surface, Phafin2 can modulate the cell's response to the extracellular stimulation.

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Methods

Reagents and gene cloning. The human cDNA clones MGC: 19,473 (Phafin2) and MGC: 5048 (Rab5a) were obtained from VYMGC. The open reading frames (ORFs) of the MGC clones and their mutants were cloned as donor vectors using the PCR-based In-Fusion™ Kit (Clontech) (the primers listed in Supplemental Table 1). The ORF of interest was transferred from the donor vector into expression vectors containing *LoxP* site using *Cre* recombinase. The GDP-binding and GTP-binding mutants of Rab5a were generated by a QuikChange® Site-Directed Mutagenesis Kit (Stratagene).

Validated Stealth RNAi™ for Phafin2 was from Invitrogen and siCONTROL non-targeting siRNA #2 (Dharmacon, Inc.) was used as a control. Alexa Flour® 633-conjugated human transferrin was from Molecular Probes (Invitrogen). The antibodies used included EEA1 mAb, (BD Transduction Laboratory) and insulin receptor α-subunit mAb (83-7) (Chemicon).

Lipid-binding assay. Dot-blot experiments were carried out using PIP strips (Echelon Biosciences) according to the manufacturer's protocol. The strips were incubated with GST-Phafin2 and then blotted with anti-GST antibody followed by horseradish peroxidase-conjugated secondary antibody (Upstate). The blotting signal was detected with Hyperfilm (Amersham-Pharmacia).

Cell culture, transfection and staining. HEK 293T cells were grown in DMEM supplemented with 5% fetal bovine serum at 37 °C in the presence of 5% CO_2. Transient transfection of HEK 293T cells was carried out using Genejuice® (Novagen) following the manufacturer's instructions. For RNA interference, 12 μl of *Trans*IT-TKO® Transfection Reagent (Mirus Bio LLC) with 50 nM of indicated siRNA (Supplemental Table 1) duplexes were transfected into HEK 293T cells in 6-well cell culture dishes for 48 h. For immuno-fluorescence staining, cells were fixed with 4% paraformaldehyde, permeabilized with 0.04% Triton X-100, blocked with 5% fatty acid-free BSA at room temperature for 1 h, then incubated with primary antibody followed by fluorophore-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA) in PBS buffer at room temperature for 1 h each. For insulin receptor staining, primary antibodies were incubated with live cells before fixation. The stained samples were examined under a confocal microscope (TCS-SP5, Leica Microsystem, Germany). To label the endosomes formed by receptor-mediated endocytosis, HEK 293T cells were incubated with 5 μg/ml Alexa Flour® 633-conjugated human transferrin (Molecular Probes) at 37 °C for 15 min.

Flow cytometry. To measure the density of receptors on the cell surface, HEK 293T cells in suspension were first incubated with specific antibodies on ice for 15 min. The cells were washed twice with cold PBS, fixed and stained with fluorochrome-conjugated secondary antibodies, then subjected to flow cytometry scanning using FACSCalibur (BD, San Jose, CA). At least 30,000 GFP-positive cells (indicating successful Phafin transfection) were analyzed per sample.

Luciferase reporter assay. 293T cells were co-transfected with indicated expression plasmids, plasmids containing AP-1-driven firefly luciferase (Panomics) and TK-driven *Renilla* luciferase (Promega). To assay luciferase activities, cells were lysed using passive lysis buffer (Promega), and luciferase activities were determined following standard protocols. Firefly luciferase activity values were normalized to *Renilla* luciferase activity values as internal control of transfection efficiency.

Results

Characteristics of Phafin2

From the public database [15] and the results of our own microarray experiments, we identified around 200 genes including Phafin2, that were differentially expressed in the human hepatocellular carcinoma (HCC) but without clear functional annotations [16]. As shown in Fig. 1A, Phafin2 mRNA (ArrayExpress Access ID: E-TABM-36) were significantly higher in the transcriptome of HCC tumors than the normal liver (*t*-test, $p < 0.005$). Immuno-fluorescence staining showed that Phafin2 was present in HepG2 cells as punctate vesicles; some of them were also stained positively for the endosomal marker, early endosome antigen 1 or EEA1 (arrows, Fig. 1B and inset). Sequence analysis revealed that Phafin2 was mainly composed of a pleckstrin homology (PH) domain close to the N-terminal and a FYVE domain at the C-terminal [14]. Lipid-binding experiments (Fig. 1C) demonstrated that Phafin2 could bind strongly to PI(3)P, which was abundant in the endosomes [8]. As shown in Fig. 1D, we noted that over-expression of wild-type Phafin2 tagged with GFP (GFP-Phf2) in HEK 293T cells caused the formation of one or a few unusually large vesicular compartments (arrowheads) that were not normally observed in the control cells. All of the enlarged vesicles contained Phafin2 and stained positively for EEA1, suggesting that they represented "enlarged endosomes". Similar results were obtained using a FLAG tag instead of GFP to label Phafin2 or using HeLa cells, HuH7 or MCF7 cells instead of HEK 293T as the cell model (data not shown). We found little association of Phafin2 with the endoplasmic reticulum. Interestingly, the Phafin2 mutant devoid of the PH domain (Phf2-ΔNter that contains aa 132–249, Fig. 1E) was still capable of forming and binding to the enlarged endosomes, while the Phafin2 mutant missing the FYVE domain (Phf2-ΔCter that contains aa 1–144) was not, thus indicating that binding of Phafin2 to the endosomal membrane was mediated mainly by the FYVE domain. The enlarged endosomes formed by Phafin2 were still functional for fluid-phase endocytosis or receptor-mediated endocytosis, since they could engulf fluorescently labeled 3 kDa dextran (Supplemental Fig. 1A) or Cy5-conjugated transferrin (Supplemental Fig. 1B) added to the culture medium, respectively.

Phafin2's endosome swelling phenotype was mediated by Rab5 and PI3K

To investigate the signaling underlying the endosomal swelling phenotype, we first treated GFP-Phf2 cells with LY294002, an inhibitor of PI3K [17]. As shown in Fig. 2A and B, the drug treatment resulted in a progressive shrinkage of the enlarged endosomes caused by Phafin2 or Phafin2's FYVE domain (Phf2-ΔNter). In addition, we also addressed the role played by Rab5 signaling, which had been shown to modulate endocytosis and/or the biogenesis of the endosome [7], in mediating the vesicular swelling phenotype of Phafin2. As shown in Fig. 2C and quantified in Fig. 2D, neither the wild-type Rab5 (Rab-wt) nor its constitutively active mutant (Rab5Q79L or Rab5:GTP) significantly affected Phafin2-induced vesicular enlargement. On the other hand, co-expression of the dominant-negative Rab5 mutants (RAB5S34N or Rab5:GDP) in GFP-Phf2 cells abolished the endosomal swelling phenotype and caused Phafin2 to dissociate from the vesicle (Fig. 2C). Interestingly, co-expression of RAB5S34N had only minor effect on the vesicular swelling phenotype generated by the Phf2-ΔNter (Fig. 2D) (see Discussion).

Phafin2 activated Rab5 signaling

To examine whether Phafin2 triggered Rab5 signaling, we contrived a FLIM-FRET [18] technique to detect the binding between Rab5 and Rab5-binding fragments of Rabaptin5 (Rab5BD) and used such intermolecular interaction as an indicator for the activation of Rab5 pathway [19]. GFP-labeled wild-type Rab5 (GFP-Rab5wt) or constitutively active Rab5Q79L (GFP-Rab5Q79L) were used as the FRET donor, while RFP-labeled Rab5BD (Rab5BD-RFP) as the acceptor. Reduction of the donor (GFP-Rab5) fluorochrome's

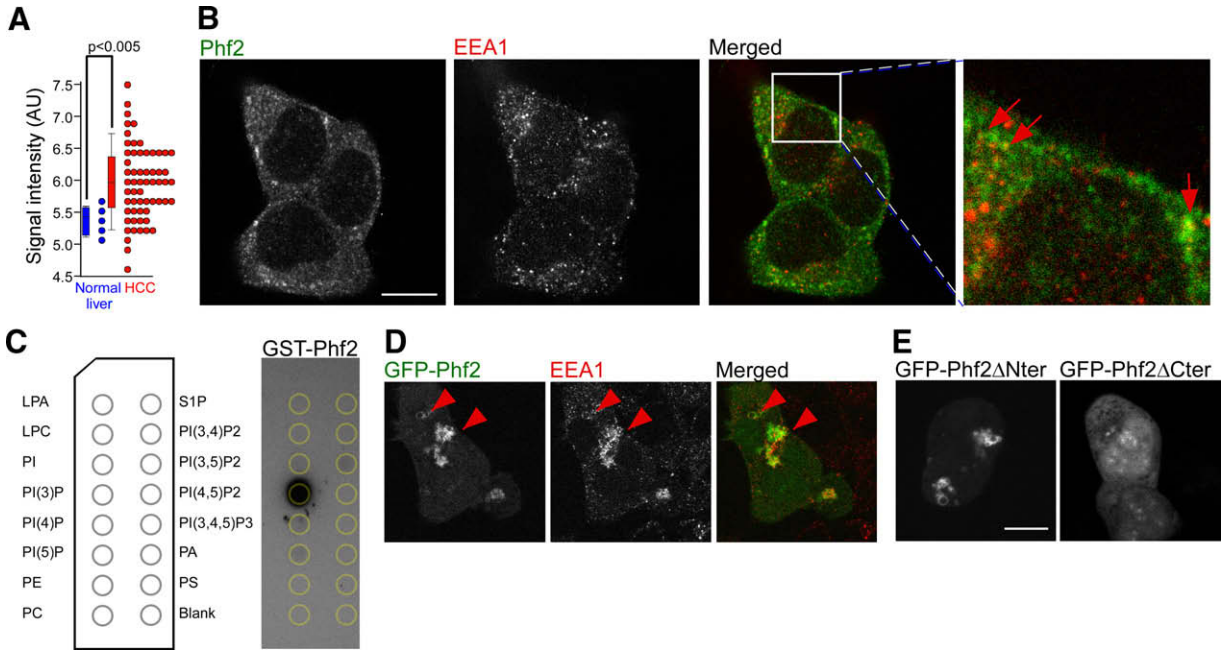

Fig. 1. Characteristics of Phafin2. (A) Box plot shows expression levels of Phafin2 in public HCC microarray dataset (ArrayExpress: E-TABM-36). Phafin2 mRNA levels of HCCs (red) are significantly higher than normal livers (blue) ($p < 0.005$, one-tailed t-test). (B) Immuno-fluorescence staining of Phafin2 (Phf2, stained green) and the early endosome marker EEA1 (stained red) in HepG2 cells. Phafin2 co-localizes with EEA1 (arrows, inset). (C) Preferential binding of Phafin2 to PI(3)P. The recombinant GST-Phafin2 proteins are incubated with the dotted strip of lipid and assayed by immuno-blotting. LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; S1P, sphingosine 1-phosphate; PA, phosphatidic acid; PS, phosphatidylserine. (D) Over-expression of GFP-tagged Phafin2 (GFP-Phf2) in HEK 293T cells resulted in the formation of enlarged vesicles (arrowheads), which stained positively for EEA1 (red). (E) Over-expression of the Phafin2 mutant devoid of the PH domain (GFP-Phf2-ΔNter) or the mutant devoid of the FYVE domain (GFP-Phf2-ΔCter) in HEK 293T cells. Only GFP-Phf2-ΔNter caused the endosome swelling phenotype. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

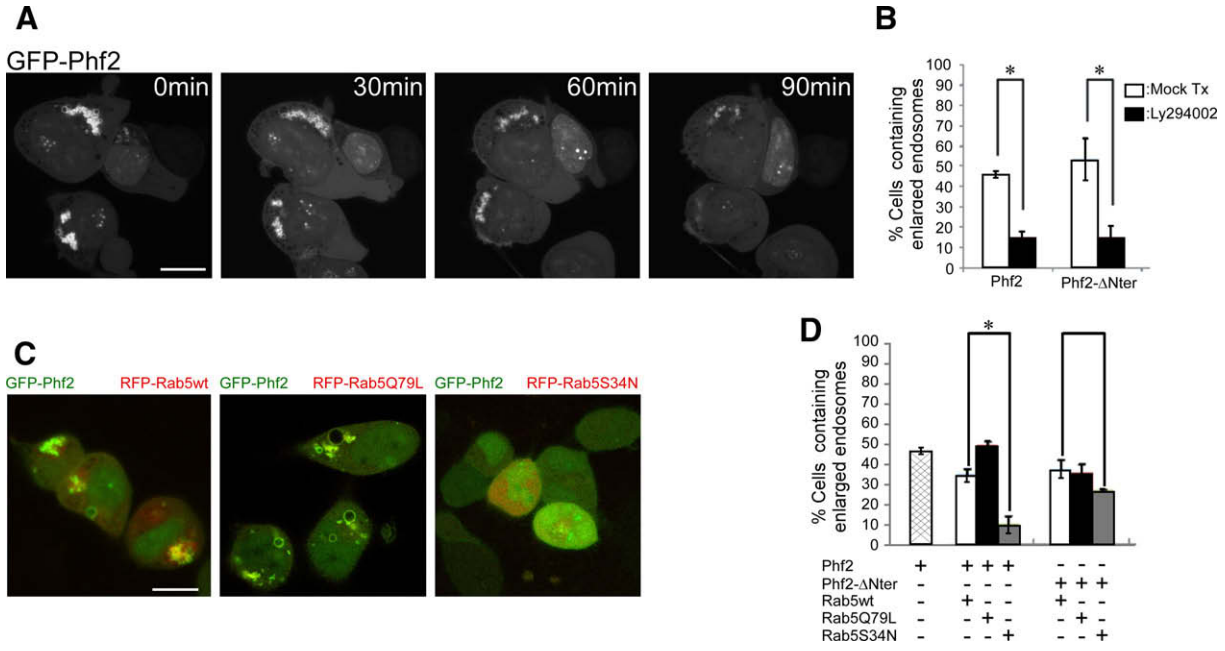

Fig. 2. Signaling involved in Phafin2-mediated endosomal enlargement. (A) HEK 293T cells transfected with GFP-Phf2 are treated with LY294002. Fluorescence imaging over time revealed progressive shrinkage of the enlarged vesicles following LY294002 treatment. (B) HEK 293T cells transfected with wild-type Phafin2 or Phafin2 missing the PH domain (Phf2-ΔNter) were treated with LY294002 for 2 h. The percentages of cells that still contained enlarged vesicles were calculated. Mean ± SD from >100 cells are shown. (C) HEK 293T cells transfected with GFP-Phf2 were co-transfected with RFP-labeled wild-type Rab5 (Rab5wt), or constitutively active Rab5Q79L mutant, or dominant-negative Rab5S34N mutant for 24 h. Co-expression of Rab5S34N not only abolished vesicular swelling, but also caused Phafin2 to dissociate from the endosome. (D) Vesicular enlargement induced by Phafin2 was inhibited by co-transfecting the cells with dominant-negative Rab5S34N. In contrast, endosomal enlargement caused by Phf2-ΔNter was less sensitive to Rab5S34N mutant. Bar = 10 μm. * indicates $p < 0.05$ by Student's t-test.

fluorescence lifetime in the presence of the acceptor (Rab5BD-RFP) fluorochrome is indicative of a very close intermolecular apposition (<10 nm) between Rab5 and RabBD, and thus demonstrates the activation of Rab5 signaling. We found that the cells containing GFP-Rab5wt alone (Fig. 3A) exhibited an average lifetime of 2350 pico-sec (ps) and the corresponding pseudo-colored lifetime

images were coded mostly in blue–green and some scattered yellow. Co-transfection of Rab5BD-RFP in the GFP-Rab5wt cells (Fig. 3B) did not significantly affect the lifetime of GFP-Rab5wt, suggesting that the background level of Rab5/Rab5BD binding was very low. Additional expression of Phafin2 in cells containing the FRET pair of GFP-Rab5/Rab5BD-RFP caused a significant lifetime reduction in GFP-Rab5, from the average lifetime of 2100 ps before, to <2000 ps after Phafin2 expression ($^{**}p < 0.05$, Fig. 3E), as the FRET acceptor Rab5BD-RFP bound to the FRET donor GFP-Rab5. The decrease of GFP-Rab5's lifetime was also evidenced by the appearance of orange–red pixels in the lifetime image (Fig. 3C), especially in the area surrounding the enlarged vesicles (arrows) where Phafin2 accumulated. This result demonstrated that Phafin2 was able to activate Rab5 signaling. In the cells that had FRET pair GFP-Rab5Q79L and Rab5BD-RFP, the FRET donor and acceptor were constitutively bound to each other; this also resulted in a reduction of average lifetime value to <2000 ps (Fig. 3E) and the color coding shifted to orange–red (Fig. 3D). Although Phafin2 could activate Rab5, GFP-Phf2 itself exhibited little FRET with RFP-Rab5wt (Fig. 3F), indicating that Phafin2 did not bind directly to Rab5.

Phafin2 modulated the presence of insulin receptor on the cell surface and the downstream AP-1 signaling

Given the roles played by Phafin2 in regulating the structure and function of endosomes, we asked whether Phafin2 could modulate the density of membrane receptors and thereby control the cells' responses to the extracellular cues. To this end, we conducted a series of experiments using flow cytometry to determine which and how membranous receptors were affected by Phafin2. Overexpression of Phafin2 increased membranous insulin receptor (InsR), uPAR, frizzled, interferon receptor (IFNR), IL-6 receptor, purinergic receptor P2Y5 and transforming growth factor-beta receptors (TGFβR), but did not change the amount of hepatocyte growth factor receptor (HGFR) and epidermal growth factor receptor (EGFR) on the cell surface (Fig. 4A and Supplemental Fig. 2). Notably, transfecting the cells with Phafin2 increased the amount of membranous InsR (Fig. 4A), whereas knocking-down Phafin2 expression decreased InsR (Fig. 4B). In Fig. 4C, the cells transfected with GFP-Phf2 were first treated with anti-InsR antibodies then with fluorescent secondary antibodies to trigger internalization of InsR. We noticed that the GFP-Ph2 cells contained much more InsR immuno-complexes on the plasma membrane (arrowheads) and much less internalized InsR (arrows) following antibody cross-linking-induced internalization, than the control (GFP-free) cells. This result suggests that high level of Phafin2, by decreasing InsR internalization, could increase the presence of InsR on the cell surface, and vice versa. The next question is whether Phafin2 could indeed affect the cell's response to the insulin stimulation. To address this, we monitored the AP-1 activity that has been known to be one of the InsR's downstream signaling pathways [20,21]. The AP-1-driven firefly luciferase (AP-1-Luc) reporter was used to measure AP-1 activity. As shown in Fig. 4D, we found that AP-1 activity was much higher in GFP-Phf2-expressing cells than the control cells that expressed only GFP (**). Treating the control GFP cells with 5 μg/ml insulin resulted in significant increase of AP-1 activity (*), but the already high or fully activated AP-1 activ-

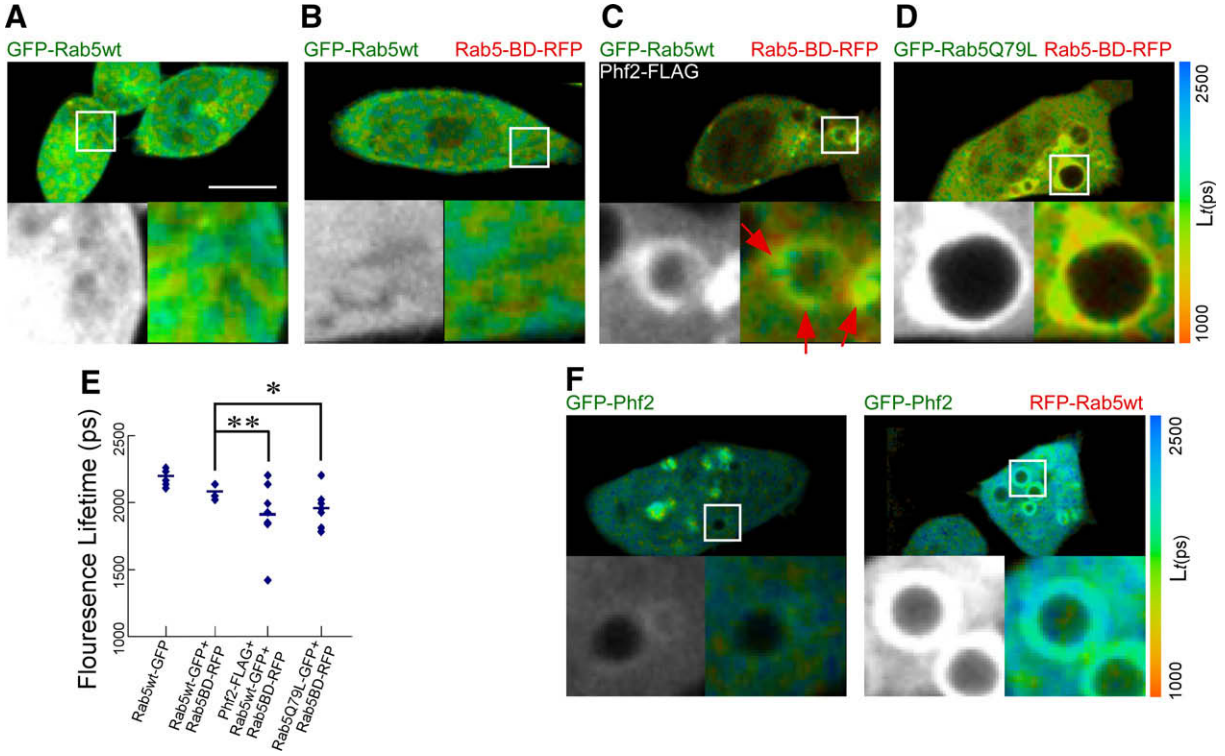

Fig. 3. Phafin2 activated Rab5 signaling. (A–E) Fluorescence lifetime images of the FRET donor in the cells co-transfected with the GFP-labeled gene construct as the FRET donor and RFP-labeled gene construct as the FRET acceptor. In the zoom-in inset, fluorescence intensity images are shown in gray-scale, while their corresponding lifetime images are displayed in pseudo-color. FRET was not detected in the cell containing FRET donor GFP-Rab5wt alone (A) or the FRET pair GFP-Rab5wt and Rab5BD-RFP (B), so most lifetime image pixels are shown in blue–green. FRET was detected when co-transfecting the FRET pair-containing cells with FLAG-tagged Phafin2 (FLAG-Phf2), evidenced here by the reduction of the FRET donor's fluorescence lifetime, or the appearance of orange–red image pixels (arrows, C). FRET between the constitutively active GFP-Rab5Q79L and Rab5BD-RFP without exogenous Phafin2 was also detected (D). (E) Quantification of FLIM-FRET experiments using the FRET pairs as indicated. The average lifetime value for each and every lifetime imaging experiment is shown (diamonds); the mean value from six experiments is indicated (bars). (F) No FRET was observed in the cells transfected with GFP-Phf2 alone, or with the FRET pair GFP-Phf2 and RFP-Rab5wt. Bar = 10 μm. * indicates $p < 0.05$ by Student's *t*-test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

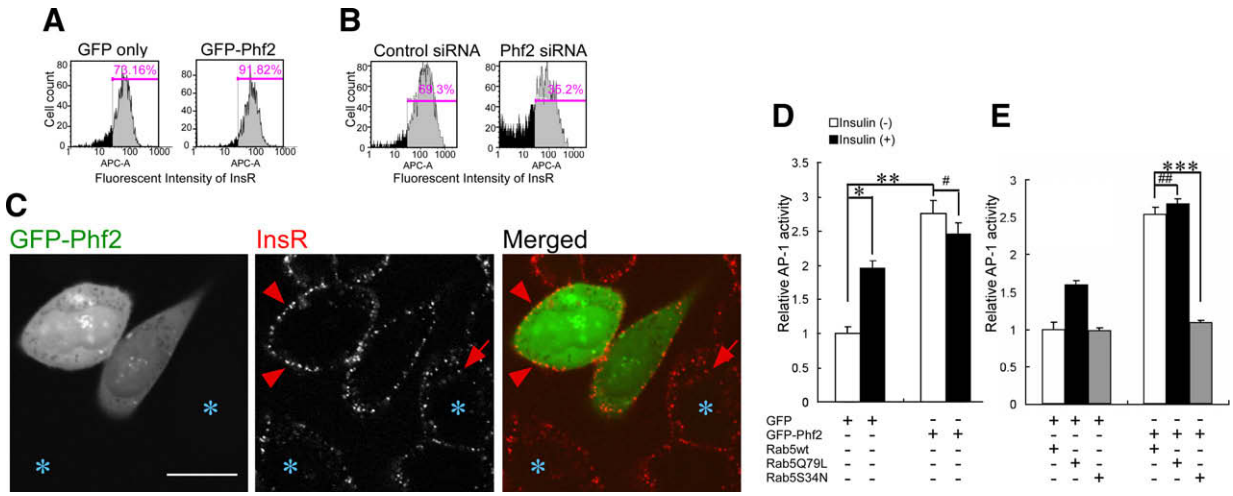

Fig. 4. Phafin2 regulates the presence of insulin receptor on the surface and its downstream signaling. (A and B) The contents of membranous insulin receptor (InsR) in HEK 293T cells transfected with GFP or GFP-Phf2 (A), or knocked down with the control or Phf2 siRNA (B), were quantified by flow cytometry. In (A), only cells stained positively for GFP were selected for analyses, whereas the entire cell population was counted in (B). Numbers (%) indicate the cells that contained InsR on their cell surfaces. (C) Increased Phafin2 inhibits internalization of InsR. Cells transfected with GFP-Phf2 were treated first with primary anti-InsR antibodies at 37 °C for 10 min, then with Alexa Fluor 643 (red)-labeled secondary antibody for 25 min to induce InsR uptake. In the cells containing GFP-Phf2, there were more immuno-complexes of InsR (arrowheads) on the cell surface, and much less present intracellularly (arrows), than the control cells (asterisks). (D and E) Phafin2 activates AP-1 activity that is Rab5-dependent. (D) HEK 293T cells containing GFP-Phf2 exhibited significantly higher AP-1 activity than the control GFP cells (**$p < 0.001$). Treating the cells with insulin ligands resulted in increase of AP-1 activity in the control cells (*$p < 0.001$) but not in GFP-Phf2 cells that already had high AP-1 activity (#). (E) The increased AP-1 activity in cells containing exogenous Phafin2 was reduced by co-transfecting the cells with dominant-negative Rab5S34N that inhibited Rab5 signaling (***$p < 0.001$), while co-expression of constitutively active Rab5R97Q did not further increase AP-1 activity (##). Data shown are mean ± SD from four separate experiments. Bar = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ity of the GFP-Phf2 cells could not be further increased by the insulin treatment (#). To find out whether the increase of AP-1 activity by Phafin2 was mediated by Rab5, we co-expressed dominant-negative Rab5S34N in GFP-Phf2 cells and found that the high level of AP-1 activity induced by the exogenous Phafin2 was effectively abolished by inhibiting Rab5 (***, Fig. 4E), whereas co-expressing Rab5S34N in the GFP control cells had no obvious effect. In contrast, co-expression of constitutively active Rab5Q79L increased AP-1 activity in the control cells, but did not further increase the already high AP-1 activity in GFP-Phf2 cells (##, Fig. 4E). Our data suggest that Phafin2 increases the cell's AP-1 response to insulin and such modulation is dependent on Rab5 signaling.

Discussion

As regulators for vesicular membrane trafficking, different FYVE-containing proteins may possess different functions. Our results demonstrate that Phafin2 is a novel endosome-associated protein whose functions affect not only the structure, but also the function of endosomes, as well as the endocytotic process. The endosomal function of Phafin2 reported here appears to reside mainly in its FYVE domain, rather than the PH domain. The binding of Phafin2 to the endosome, for example, is attributed to the FYVE domain's binding to the lipid PI(3)P which has been shown to be a major constituent of the endosomal membrane [22].

Most of the Phafin2's functions reported in this study are mediate by the small GTP-binding protein Rab5 and/or its downstream regulator PI3K [10,11]. For example, the endosomal swelling phenotype of Phafin2 is abolished by inhibiting the Rab5 signaling. We further demonstrate that elevated level of Phafin2 can directly activate the Rab5 pathway, which, in turn, causes the swelling of the endosomal compartments. A novel experimental approach is developed in this study using FLIM-FRET measurements to monitor the intermolecular binding between Rab5 and Rab5BD (Rab5-binding fragments of Rabaptin5) that serves as a reporter for Rab5 activation. This technique can be readily applied to live cells. Although Phafin2 can activate Rab5 signaling, our FLIM-FRET results do not reveal direct binding between Phafin2 and Rab5 (Fig. 3D). How then does Phafin2 activate Rab5 remains an open question. Note that the endosome swelling phenotype caused by Phafin2 deletion mutant devoid of the PH domain (Phf2-ΔNter) is much less dependent on Rab5 activity than the full length Phafin2 (Fig. 2D). Such results do suggest a role played by the PH domain in regulating Phafin2's interaction with its downstream effector Rab5 [23].

By regulating the dynamics of endocytosis and the sorting of endosomes that are either recycled back to the plasma membrane or transported to the lysosomal compartment, Phafin2 may actively modulate the molecular composition of the cell membrane, as well as the metabolisms of ligands bound to the membrane receptors. It is expected that the mechanisms of such internalization/recycling/degradation processes may affect different ligands or membrane proteins to individually different extents. In this study, we focus on insulin receptor InsR. Increased Phafin2 inhibited the internalization of InsR triggered by cross-linking, resulting in increased density of InsR on the plasma membrane, compared with the control cells or Phafin2 depleted cells. Increased presence of InsR on the cell surface could indeed increase the cell's response to insulin stimulation [24]. We report here that high expression of Phafin2, by activating Rab5, also causes an increase of AP-1 activity that is a downstream mediator of the insulin pathway. So, Phafin2 controls the cell's response to insulin not only by regulating the density of InsR on the cell surface, but also by directly modulating the InsR's downstream effectors.

The expression of Phafin2 is up-regulated in the liver cancer (Fig. 1A) and in the breast cancer [17]. The potential cancer promoting effects of Phafin2 can be multifold. By modulating the endosomal pathway, Phafin2 may work in concert with other FYVE-containing molecules and functions as a membrane organizer that controls (at a cellular or subcellular level) the space and time of how cells respond to the environmental cues, including growth factors or cytokines [7,25]. In addition, Phafin2's activation of Rab5 can also be carcinogenic, since activation of small GTP-binding proteins has been shown to contribute to various carcinogenetic mechanisms; for example, increased expression of RAB25

could promote cell proliferation and prevent apoptosis/anoikis [26]. Phafin2, via Rab5, could also regulate the presence of membranous P-glycoprotein, which confers multi-drug resistance to cancer therapy [27].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.12.016.

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