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Lysosomal targeting of phafin1 mediated by Rab7 induces autophagosome formation

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ABSTRACT

Autophagy orchestrates programmed cell death via crossroads of complex vesicle trafficking including autophagosome and lysosome interaction. Phafin1, an endosome proteins composed of Pleckstrin homology (PH) and Fab1-YotB-Vac1p-EEA1 (FYVE) domain membrane-binding domains, is involved in caspase-independent apoptosis. We report here that the increased expression of phafin1 and its FYVE domain caused the formation of enlarged endosomes. Phafin1 also modulates the membrane density of certain receptors and participates in endocytosis and autophagy processes. The PH-domain of phafin1 is dispensable for lysosomal targeting. Moreover, the tail-domain of phafin1 provides lysosomal targeting signature and the ability to induce autophagy that is mediated by Rab7 signaling. The results suggest that in addition to its role in endosome transport, phafin1 is also involved in lysosomal targeting and autophagosome formation.

1. Introduction

Autophagy, a vital process in programmed cell death and development, is triggered by several signal pathways including growth factor signaling, energy level and nutritional status [1,2]. Autophagy is a well-documented cellular mechanism for lysosomal degradation of cytoplasmic content. Besides apoptosis, autophagy is another mechanism that modulates cell survival or death. Therefore, a proper understanding of the mechanism underlying the autophagic cargo delivery and interplay between autophagosome and lysosome is important not only in preventing cancer cell development, but also in host–pathogen interaction.

Cancer cell ought to possess a membrane protein profile distinct from the equivalent non-cancerous cell or other different types of cancer cells. This molecular signature is reflective of the origin of the cancer cell, as well as its capability to invade and to metastasize [3,4]. Despite ample information on how cancer cells control the synthesis and transport of membrane proteins to the plasma membrane are available, relatively less is known regarding the metabolism of membrane protein once they are made and targeted. Proteins, lipids and various lipid-binding proteins are involved in governing vesicular sorting and multiple trafficking processes [5,6]. Lipids are important regulators of vesicular trafficking [7,8]. Rab proteins are another crucial group of modulators for vesicle transportation [5]. Rab7 participates in several trafficking processes including endocytosis and enhancing fusion of autophagosomes and lysosome [1,9–11]. In addition to Rab proteins, the Fab1-YotB-Vac1p-EEA1 (FYVE) domain-containing proteins have been shown to interact with the membrane [12,13] and modulate endosomal functions [7]. Moreover, the pleckstrin homology (PH) domain is also known for its ability to bind to phosphoinositides [14]. Previous studies have shown that phafin1 (also known as PLEKHF1 or LAPF), composed of both PH and FYVE domains, recruits phosphorylated p53 to lysosomes and triggers caspase-independent apoptosis [15,16]. In this study, we found that phafin1 is involved in lysosomal sorting and induction of autophagosome formation via Rab7 signaling,

2. Methods

2.1. Gene cloning and mutagenesis

The human cDNA clones MGC: 4090 (phafin1) and MGC: 8453 (Rab7) were obtained from VYMGC. The open reading frames (ORFs) of the MGC clones and their mutants were PCR-amplified using the

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primers listed in Supplemental Table 1, cloned into pDNR-Dual and used as donor vectors for cloning using In-Fusion™ PCR Cloning method (Clontech). The ORF of interest was then transferred from the donor vector into expression vectors containing LoxP site using Cre Recombinase. The Rab7 mutants (GDP-binding and GTP-binding) were generated using a QuikChange® Site-Directed Mutagenesis Kit (Stratagene). Tetramethylrhodamine-labeled 3 kD dextran, LysoTracker Red DND-99, Alexa Flour® 633-conjugated human transferrin were purchased from Molecular Probes (Invitrogen). The antibody EEA1 mAb was used in this study (from BD Transduction Laboratory).

2.2. Lipid binding assay

Dot-blot experiments were carried out as previously described [17].

2.3. Cell culture, transfection and staining

HEK293T cells were cultured, transfected and stained as previously described [17].

2.4. Endocytosis assays and organelle staining

To label the endosomes formed by fluid-phase endocytosis, HEK 293T cells were incubated with 20 ng/ml 3 kD dextran (Labeled with Alexa Flour® 647 or tetramethylrhodamine, Molecular probes) at 37 °C for 1 h. To label the endosomes formed by transferrin 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. To label the lysosomes, cells were incubated with 1 μM LysoTracker Red DND-99 (Molecular probes) at 37 °C for 15 min.

2.5. Flow cytometry

The flow cytometry was performed as previously described [17].

3. Results

3.1. Over-expression of phafin1 caused the formation of enlarged vesicles

Phafin1 was mapped to the chromosome 19q12 region. This gene shares 56% sequence identity with a gene already mapped to chromosome 8q22.1 region, called phafin2 [15,17]. As shown in Fig. 1A, both phafin1 and phafin2 comprise a PH domain (Conserved domain database ID: cd01218) and a FYVE domain (CDD ID: cd00065). However, Phafin1 has an additional C-terminal tail domain (amino acids 253–279) that is absent in phafin2. Immunofluorescence staining showed exogenous phafin1 to be present in HEK 293T cells as punctate vesicles that were partially co-localized with the endosomal marker EEA1 (*arrows*, Fig. 1C). In addition to endosomal compartmentalization, phafin1 was also found in the lysosomes (see below and [15]).

Though Phafin1 does not contain a transmembrane domain, it may bind to the membrane through membrane insertion using either the PH or FYVE domain. In the protein-lipid overlay experiments shown in Fig. 1B, we found that the recombinant phafin1 protein was strongly bound to phosphatidylinositol(3)-phosphate or PI(3)P. Such a lipid-binding property agrees well with the preferential association of phafin with early endosomes [8]. Phafin1, but not phafin2, exhibits significant binding to PI(4)P and PI(5)P [17]. This difference in lipid-binding properties might account for some of the protein's unique localization properties, as well as the different functions exerted by the individual phafins that are described below.

HEK 293T cells expressing GFP-tagged phafin1 (GFP-Phf1) also displayed enlarged vesicles (Fig. 1C). Some of the vesicles stained positively for EEA1 (*arrows*), while others did not (*arrowheads*). In HEK 293T cells co-transfected with both GFP-Phf2 and DsRed-Phf1 (Fig. 1E), we noticed that some of the enlarged vesicles contained both phafin1 and phafin2 (*arrow*), while others contained only phafin2 (*arrowhead*).

To investigate whether the PH or FYVE domain are required for phafin1-mediated enlargement of vesicles, deletion mutants of phafin1 were examined for their subcellular distribution and the presence of vesicular swelling (Fig. 1D and F) As shown in Fig. 1D, the phafin1 construct without the PH domain (Phf1-ΔNter) was still capable of forming enlarged vesicles and the mutant protein could still bind to the enlarged vesicles. In contrast, the mutant lacking the FYVE domain (Phf1-ΔCter) was incapable of causing vesicular enlargement and the mutant protein was not able to bind to the vesicular compartment.

3.2. Phafin1 modulated the protein content of the plasma membrane and was involved in different endocytosis events

To further examine whether phafin1 might help control the density of membrane receptors, we measured changes in various membrane receptors by flow cytometry in response to overexpression of exogenous phafin1 (Data not shown). We found that over-expression of phafin1 in HEK 293T cells decreased the amount of E-cadherin and increased the presence of TGF-β receptors on the cell membrane, whereas previous study showed over-expression of phafin2 caused significant increases in uPAR and the insulin receptor (InsR) on the cell surface, as well as dysadherin, Frizzled, interferon receptor (IFNR), IL-6 receptor and the purinergic receptor P2Y5 [17]. Expression of phafin1 or phafin2 did not affect the level of transferrin receptor, HGF receptor or EGF receptor (data not shown).

Next, we examined the identity and function of the enlarged vesicles formed by over-expression of phafin1. The endosomes generated by fluid-phase endocytosis were marked by fluorescent-labeled small molecular weight (3 kD) dextran, which had been added to the culture medium and engulfed by the cells over 15 min of treatment. As shown in Supplemental Fig. 1A and quantified in Supplemental Fig. 1B, a great proportion (27%) of the enlarged vesicles caused by phafin1 did not have dextran marker (*arrowheads*, Supplemental Fig. 1A), which suggests that they might belong to a vesicular compartment system other than the endosomes. In another experiments, Cy5-conjugated transferrins added to the medium were used to identify the endosomes involved in the receptor-mediated endocytosis. As shown in Supplemental Fig. 1C and quantified in Supplemental Fig. 1D, most (>86%) of the enlarged vesicles caused by phafin1 were not involved in transferrin uptake (inset, *arrowheads*, Supplemental Fig. 1C). These results suggest that phafin1 participates in receptor-mediated endocytosis.

3.3. The lysosomal targeting of Phafin1, but not phafin2, was dependent on Rab7

The phafin1-containing vesicles were not endosomes evident from the result of the fluorescent dextran loading experiments (Supplemental Fig. 1A). To investigate whether phafin1 was targeted to the lysosome in the cell model employed here [15], we performed double staining experiment using GFP-tagged phafin1 and LysoTracker to mark the lysosomal compartments. The degrees of co-localization were quantified by Pearson's correlation (Fig. 2F);

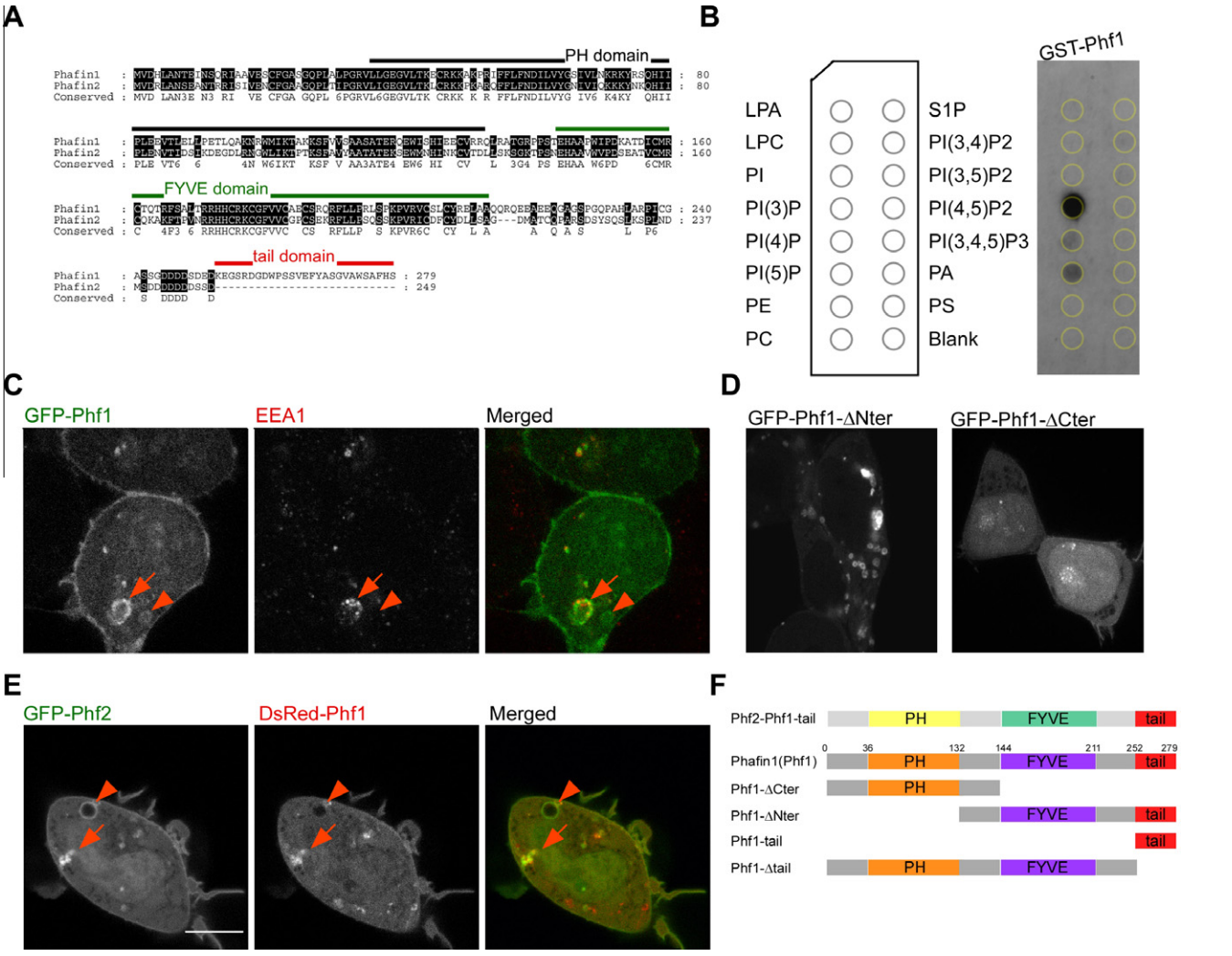

Fig. 1. Characteristics of phafin1. (A) Amino acid sequence comparison between phafin1 and phafin2. (B) Interaction of phafin1 with lipids. Recombinant GST-phafin1 protein was incubated with dotted strips of lipid; the bound proteins were visualized by immuno-blotting. LPA, lysophos-phatidic acid; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; S1P, sphingosine 1-phosphate; PA, phosphatidic acid; PS, phosphatidylserine. (C) HEK 293T cells transfected with phafin1 (GFP-Phf1) were stained for the endosomal marker EEA1. (D) Various mutant phafin1 genes (as depicted in F) were expressed in HEK 293T cells. Phafin1 mutants devoid of the PH domain (GFP-Phf1-ΔNter) were still capable of causing enlarged vesicles and were bound to the enlarged vesicles, while the constructs missing the FYVE domain (GFP-Phf1-ΔCter) lost the ability to cause enlarged vesicles. (E) HEK 293T cells were co-transfected with GFP-Phf2 and DsRed-Phf1. Some of the enlarged vesicles contained both phafins (*arrow*), but some had only phafin2 (*arrowhead*). Bar = 10 μm.

the closer Pearson's correlation value is to 1, the more double-stained image pixels are detected. We found that some, but not all, of the phafin1-containing vesicles were also stained positively for LysoTracker (*arrows*, Fig. 2A) and exhibited an average Pearson's correlation value of 0.26. In contrast, phafin2-containing vesicles were never associated with LysoTracker (*arrowheads*, Fig. 2B) and hence a negative Pearson's correlation was obtained.

We then speculated whether targeting of phafin1 to the lysosomes was dependent on Rab7. As shown in Fig. 2C and D and quantified in Fig. 2F, co-transfection of the dominant negative mutant Rab7T22N (or Rab7:GDP) profoundly inhibited phafin1's binding to the lysosomes (*arrowhead*), while co-expression of wild-type Rab7 had no effect (*arrow*). These results strongly suggested that binding of phafin1 to lysosomes was mediated by Rab7 signaling.

Next, we looked for the lysosomal targeting domain of phafin1. Various deletion mutants of phafin1 were generated (Fig. 1F), transfected into cells and their association with lysosomes were examined by co-staining with LysoTracker (Fig. 2E) and then quantified using Pearson's correlation (Fig. 2F). We noticed that deletion of phafin1's PH domain (Phf1-ΔNter) did not affect phafin1's lysosomal binding, while the phafin1 mutant devoid of the tail domain (Phf1-Δtail) was unable to bind to the lysosome. However, adding the phafin1 tail to the C-terminal of phafin2 (Phf2-Phf1tail) resulted in the chimera protein being able to target lysosomes (*double arrow*). These results strongly suggested that the phafin1 "tail" possessed a *bonafide* lysosomal targeting signal.

3.4. Phafin1 but not phafin2 participated in autophagy

Autophagy is a self-digesting pathway responsible for the removal of long-lived proteins and organelles by the lysosomal compartment [18]. Given the association of phafin1 with the lysosome, we wondered if phafin1 is also involved in autophagy. HEK293T cells transfected with DsRed-tagged human MAP1LC3A (DeRed-hLC3A) did not undergo autophagy; however, subjecting these cells to amino acid starvation was able to induce autophagy, which was characterized by the translocation of DsRed-hLC3A from the cytoplasm to accumulate in newly formed autophagosomes (data not shown, [19]). Co-transfecting hLC3A and phafin1 into cells was sufficient to induce autophagy without the need to starve the cells of amino acids (Fig. 3B). In such cells, DsRed-hLC3A was noted to associate with some (*arrow*) but not all (*arrowhead*) of

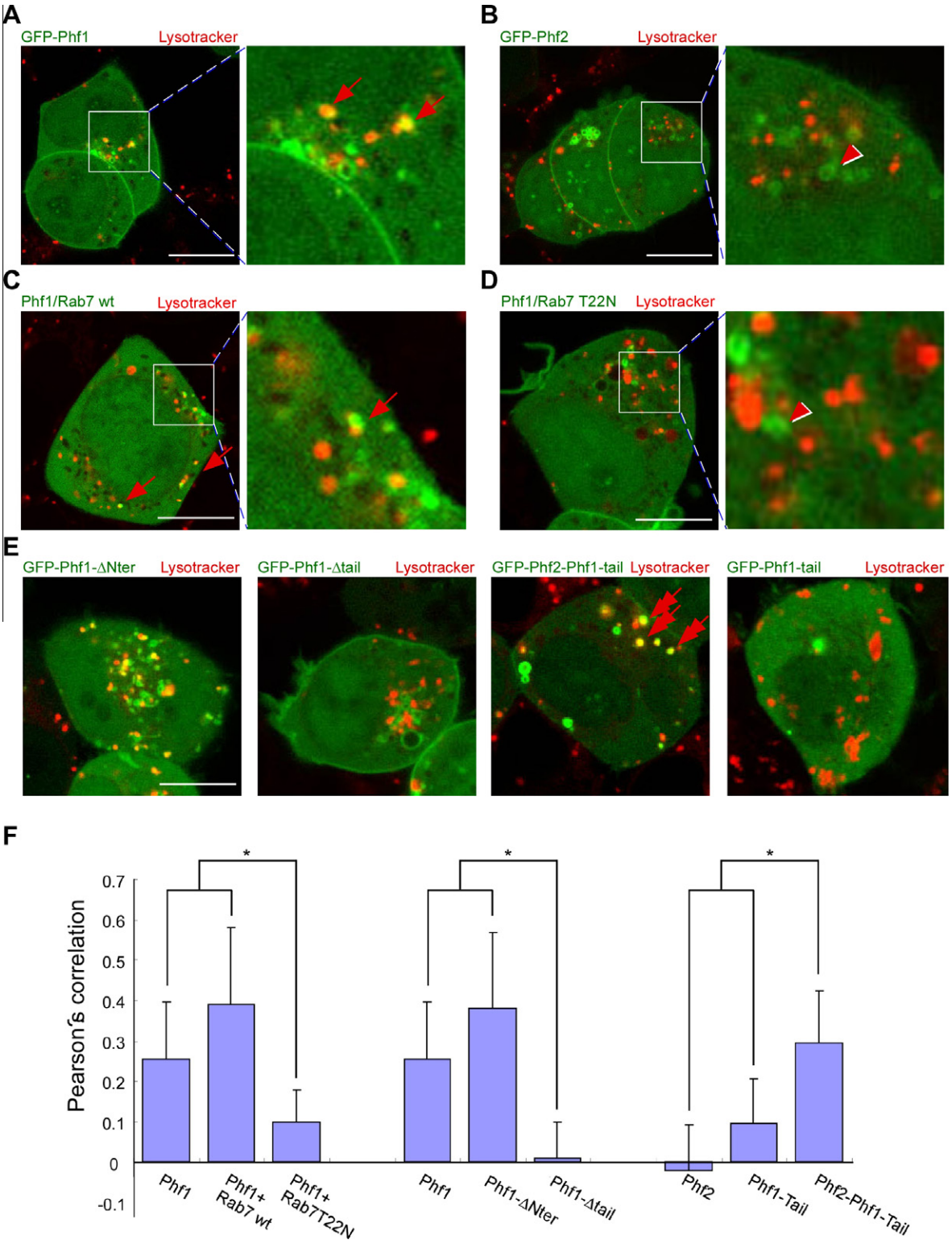

Fig. 2. Lysosomal targeting by phafin1. (A and B) LysoTracker staining in HEK 293T cells transfected with GFP-Phf1 or GFP-Phf2 indicated that some of the swollen vesicles in GFP-Phf1 cells belonged to the lysosomal compartment (*arrows*). On the other hand, all of the phafin2 vesicles stained negatively with LysoTracker (*arrowheads*). (C and D) HEK 293T cells were co-transfected with GFP-Phf1 (green channel) and YFP-Rab7 or YFP-Rab7T22N (not shown). The LysoTracker staining (red channel) revealed that co-expression of dominant negative Rab7T22N inhibited lysosomal targeting by phafin1 (*arrowhead*), while co-expression of Rab7 had no obvious effect (*arrow*). (E and F) HEK 293T cells were transfected with phafin1 mutants as indicated and labeled with LysoTracker. The degrees of co-localization by green and red fluorescence were quantified by Pearson's correlation algorithm of ImageJ; higher values indicate more signal co-localization. Bar = 10 μm. *Indicates $p < 0.05$ by Student's *T*-test. Mean ± S.D. from 50 independent experiments is shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the enlarged vesicles formed by excessive levels of phafin1. In contrast, co-transfecting hLC3A and phafin2 into cells did not induce autophagy. In these cells, DsRed-hLC3A was found distributed throughout the cytoplasm without any obvious association with

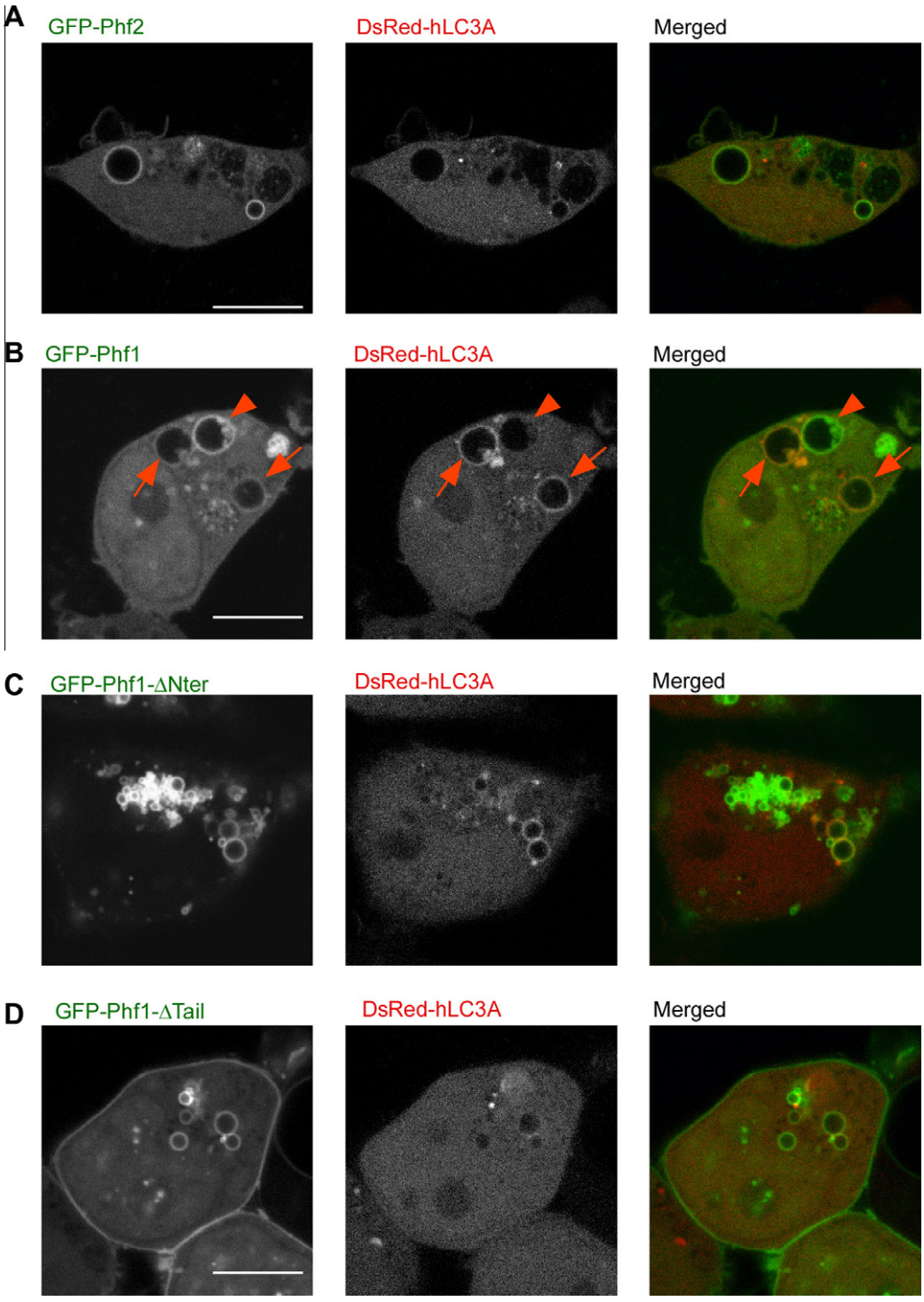

Fig. 3. Phafin1 activated autophagy. (A–D) HEK 293T cells containing DsRed-labeled hMAP1LC3A (Ds-Red hLC3A) were co-transfected with GFP-tagged phafins and phafin mutants as indicated. The association of hLC3A from the cytoplasm to the vesicles was indicative of activation of autophagy (*arrows*). Bar = 10 μm.

the enlarged vesicles formed by excessive levels of phafin2 or with any other vesicle (Fig. 3A). Nonetheless, following serum starvation, DsRed-hLC3A was noted to bind to autophagosomes but phafin2 did not co-localize with DsRed-hLC3A (data not shown).

The Phf1-ΔNter mutant, like the wild-type phafin1, was still able to induce autophagy in the absence of nutrient depletion, as shown by the accumulation of hLC3A into vesicles containing Phf1-ΔNter staining (Fig. 3C). In contrast, phafin1 devoid of the tail domain (Phf1-ΔTail) failed to induce autophagy (Fig. 3D). These results suggest strongly that the tail domain of phafin1 possesses both lysosomal targeting and autophagy induction signals. In support of this notion, we noticed that transfection of wild-type phafin1 for >72 h induced apoptosis, while transfection with Phf1-ΔTail did not (data not shown).

4. Discussion

Based on the experimental assays performed in previous and this study of phafin1 and phafin2, most of the functions appear

to center on the FYVE domain [17]. Both phafin1 and phafin2 are associated with the endosomes and caused abnormally enlarged vesicles in a variety of cell models; however, further analyses revealed that phafin1 and phafin2 may selectively regulate different steps along the endosomal and/or lysosomal pathways. While phafin2 is more functionally related to early endocytosis and early endosome recycling activity, the C-terminal tail domain of phafin1 possesses lysosomal targeting capability and contributes to the induction of autophagy and the formation of autophagosomes. Through regulating the uptake, recycling and lysosomal degradation of membrane receptors or internalization of extracellular ligands, the combined effects of phafin1 and phafin2 are able to actively participate in the control of cell signaling. Fig. 4 summarizes the experimental findings made in our previous and this study [17].

Extracellular ligands (*red dots*) and/or membrane receptors (*blue ovals*) may be internalized through the endocytosis process. At the sorting endosome, some of the endosomal contents are recycled back to the cell surface, while others are further transported to the late endosome and subjected to protein degradation in the lysosomal compartment. The small GTP-binding protein Rab5 and its downstream regulator PI3K play key roles in the early endocytosis events. Phafin2, by directly activating Rab5 signaling ❶ and phafin1, by modulating downstream PI3K ❷, regulate the formation and metabolism of early endosomes. The optimal levels of phafin1 or phafin2 are essential for maintaining normal endosomal activities. Overabundance of either phafin1 or phafin2 may lead to abnormally enlarged endosomal compartments ❸. Individual endocytosis-related functions (such as pinocytosis or fluid-phase endocytosis, receptor-mediated endocytosis, etc.) and any internalized content are individually affected to different extents by either phafin1 or phfin2. For example, uptake of transferrin by receptor-mediated endocytosis is more sensitive to phafin2 control than phafin1, and phafin2, by inhibiting internalization, increases the density of InsR on the cell membrane [17].

How different endosomal contents are sorted by the phafin1- or phafin2-mediated pathways is not yet known. It is noteworthy that the phafin-induced vesicular engorgement bears a strong resemblance to the phenotype caused by the FYVE domain-containing SARA and by endofin genes [20,21]. Given the fact that the FYVE domain tends to form dimers [22], it is possible that FYVE domain-mediated oligomerization may contrive to produce various complex protein–lipid and/or protein–protein interactions pathways [23] and that contribute to the sorting mechanisms at the endosomal compartments.

Unlike phafin2 whose functions center on the endosomal compartments, phafin1 possesses additional association with the late endosomes or lysosomal compartments and is also involved in the functions therein [15]. Indeed, some of the enlarged vesicles caused by overactive phafin1 contain a lysosomal marker ❺ (Fig. 4). The unique lysosomal targeting ability of phafin1 is attributable to its C-terminal tail domain and mediated by Rab7-mediated signaling

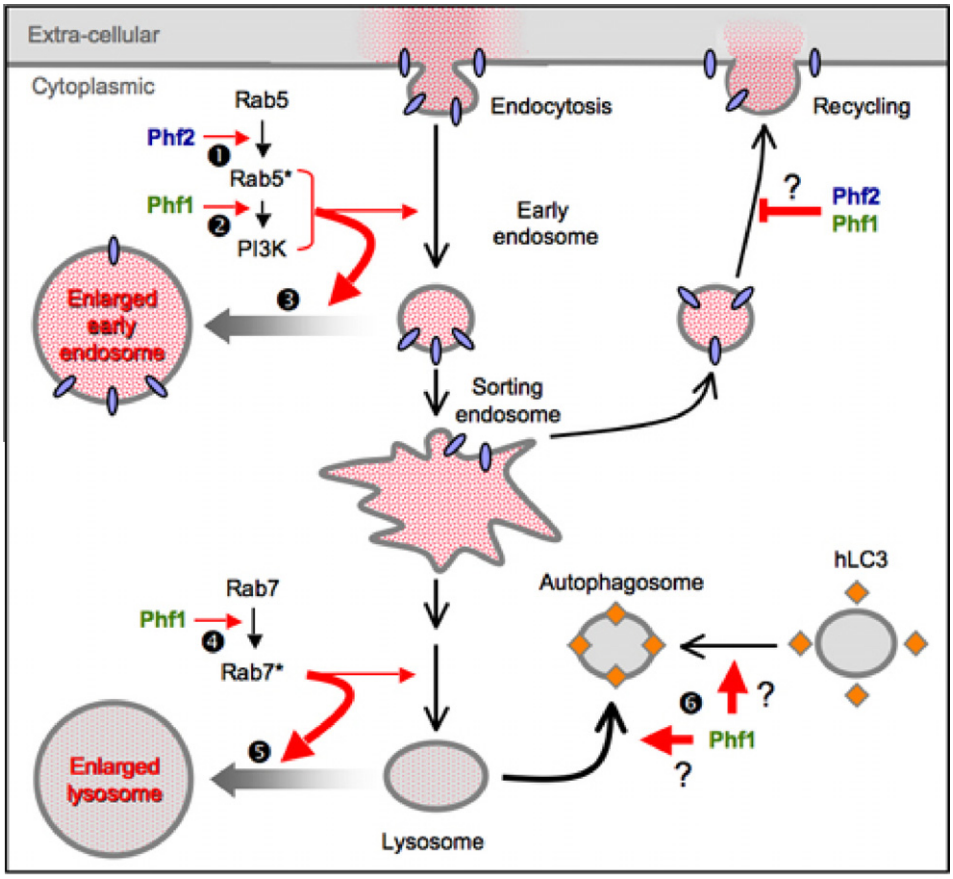

Fig. 4. The proposed model summarizes the roles played by phafin1 and phafin2 in modulating endocytosis and subsequent vesicular sorting events found in previous and this study. Extracellular ligands (*red dots*) and membrane receptors (*blue ovals*) are internalized through endocytosis. At the sorting endosome, some of the endosomal contents are recycled back to the cell surface, while others are further transported to the late endosome and/or the lysosomal compartment. Phafin2, by directly activating Rab5 signaling ❶, and phafin1, by modulating downstream PI3K ❷, both regulate the formation and metabolism of early endosomes. Increased phafin1 or phafin2 leads to abnormally enlarged endosomal compartments ❸. Phafin1, through Rab7-mediated signaling ❹, is involved in lysosomal sorting. Overactive phafin1 causes enlargement of lysosomal compartments ❺ and affects the formation of autophagosomes ❻. See texts for details. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

❹. Unlike relatively better-defined nucleus- or mitochondria-targeting signals, mechanisms underlying lysosomal sorting remain relatively obscure. For transmembrane proteins, several lines of evidence suggest that lysosomal translocations can be mediated by binding of the "targeting sequences" within the cytosolic domains to adaptor protein complexes [24]. Putative targeting sequences include YXXF for lysosome associated membrane proteins (lamps) [25] and mannose 6-phosphate receptors [26], GYEVM for CD63 [27] and the di-leucine-based motif for the CD3-g chain [28]. Lysosomal targeting for cytosolic proteins is even less understood. A single FYVE domain-containing protein in *Plasmodium falciparum*, FCP, was found to be localized to a lysosomal compartment by a C-terminal 44-amino acid peptide domain [29]. The 27-amino acid tail domain of phafin1 bears no homology to any of the above-mentioned lysosomal sorting sequences. The peptide stretch itself contains no membrane-binding motif and is unable to bind to a lipid bilayer as a peptide on its own. However, on adding phafin1's tail domain to phafin2, which is purely endosomal, the chimeric protein is translocated to the lysosome (Fig. 2E). This result suggests strongly that the tail domain of phafin1 possesses a novel lysosomal targeting signal.

Using hLC3A as an autophagosome marker [19], we found that phafin1 can promote biogenesis of autophagosomes and/or conjugation between the lysosomal compartment and autophagosomes ❻. Note that over-expression of phafin1 is sufficient to trigger autophagy, whereas nutrition starvation is required for initiating autophagy in the control or phafin2-expressing cells. Previous studies have demonstrated that both the Rab7 and PI3K (or hVps34) pathways play important roles in the formation of autophagosomes [30–32]. Given the findings that Rab7 activation and PI3K are downstream of phafin1, phafin1 may induce autophagy by directly activating the signaling processes involved, bypassing the amino acid deprivation triggering mechanisms. Note that both the lysosomal targeting and autophagy activation functions of phafin1 depend on its tail domain. Similar to this finding, in the malaria parasite, the C-terminal 44 amino acids peptide of FCP governs both lysosomal targeting and the cytoplasm to vacuole targeting (Cvt) pathway [29], which is analogous to autophagy as described in mammalian cells [18]. Phafin1 has previously been shown to induce caspase-independent apoptosis [15] and therefore we argue that some of the apoptotic activity induced by phafin1 may involve autophagy-related cell death.

Differential expression of FYVE-domain containing phafin2 gene has been reported in cancer tissue [33]. The potential cancer promoting effects of phafin1 or phafin2 can be multifold. Phafin1 is involved in autophagy and apoptosis, therefore it can modulate the cytoprotective functions during carcinogenesis [34].

By modulating the endosomal and/or lysosomal pathway, phafin1 and phafin2 may function as a membrane organizer that controls (at a cellular or subcellular level) the space and time of intracellular signal propagation [5,35]. Several reports have linked endocytosis to carcinogenesis, especially in cases like TGF-β signaling and other receptor tyrosine kinases. Under such a scenario, receptor down-regulation by rapid endocytosis and degradation in the lysosomes is essential to prevent the overgrowth of cells by terminating signal transduction [36,37]. Endocytosis depends on an extensive network of interacting proteins that execute a series of distinct sub-processes. Phafin1 and phafin2 are very good examples of such proteins.

Acknowledgments

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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.2011.11.043.

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