Scribble-mediated membrane targeting of PHLPP1 is required for its negative regulation of Akt

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PHLPP1 (PH domain leucine-rich-repeats protein phosphatase) is a Ser/Thr protein phosphatase that acts as a tumour suppressor by negatively regulating Akt. Here, we show that PHLPP1 is recruited to the cell membrane by binding to a scaffolding protein: Scribble. Knockdown of Scribble (Scrib) results in redistribution of PHLPP1 from the membrane to the cytoplasm and an increase in Akt phosphorylation, whereas overexpression of Scrib has the opposite effect. Furthermore, PHLPP1-dependent inhibition of cell proliferation is facilitated by the formation of a Scrib, PHLPP1 and Akt trimeric complex. Thus, our findings identify a functional interaction between PHLPP1 and Scrib in negatively regulating Akt signalling.

Keywords: PHLPP; Scribble; Akt phosphorylation; scaffolding protein

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INTRODUCTION

PHLPP (PH domain leucine-rich-repeats protein phosphatase) belongs to a new family of Ser/Thr protein phosphatases that have an important role in maintaining the balance in cell signalling (Brognard & Newton, 2008). Akt has been identified as a main substrate of PHLPP, and PHLPP-mediated dephosphorylation results in an increase in apoptosis and a decrease in proliferation in various cancer cells (Gao *et al*, 2005; Brognard *et al*, 2007; Liu *et al*, 2009). The activation of Akt involves phosphorylation at the activation loop (Thr308) within the kinase domain and the hydrophobic motif (Ser473) in the carboxy-terminus (Manning & Cantley, 2007). As the activity of Akt is tightly controlled by phosphorylation, PHLPP exerts its tumour suppressor function by dephosphorylating and inactivating the kinase directly (Gao *et al*, 2005; Brognard *et al*, 2007). Moreover, our recent studies have

demonstrated that downregulation of PHLPP occurs at high frequency in colorectal cancer specimens, and re-expression of PHLPP in colon cancer cells inhibits cell proliferation and tumour growth (Liu *et al*, 2009). Given the role of PHLPP in inhibiting tumorigenesis, it is important to understand the regulation of PHLPP.

Scribble (Scrib) is one component of the Scrib/Discs large (Dlg)/Lethal giant larvae (Lgl) polarity complex, localized at the basolateral side of the epithelial cell membrane (Bilder & Perrimon, 2000; Navarro *et al*, 2005). Scrib is important for maintaining proper segregation of membrane proteins, as deletion of the *Scrib* gene causes disruption of embryonic epithelial polarity in *Drosophila* (Bilder & Perrimon, 2000). In addition, loss of Scrib expression results in cancerous overgrowth of imaginal discs in *Drosophila* larvae, suggesting a tumour suppressor role for Scrib (Bilder & Perrimon, 2000; Bilder, 2004; Wodarz & Nathke, 2007). However, the mechanism underlying the tumour suppressor function of Scrib in mammalian systems is less clear. Scrib contains 16 leucine-rich repeats (LRR) in its amino-terminus and four PDZ domains in its C-terminus. Both the LRR and PDZ domains in Scrib are protein–protein interaction modules that might have an important role in facilitating cell signalling by acting as a scaffold (Assemet *et al*, 2008).

In this study, we report the identification of PHLPP1 as a membrane-bound protein through its interaction with Scrib. We show that Scrib-mediated membrane targeting of PHLPP1 is required for dephosphorylation of Akt and inhibition of cell growth.

RESULTS AND DISCUSSION

PHLPP1 interacts with Scrib

To identify PHLPP1 regulatory proteins, we tested whether a group of PDZ-domain-containing scaffolding proteins—including Scrib, Dlg1, Dlg4, Cypher and MAGI-2—can interact with PHLPP1 by using coimmunoprecipitation experiments. We found that PHLPP1 specifically interacted with Scrib (Fig 1A), and that the LRR domain, but not the PDZ domains, of Scrib was required for this interaction (Fig 1B). Deletion of the PH domain (P1-ΔPH) or the PDZ-binding motif (P1-ΔC) in the extreme C-terminus of PHLPP1 had no effect on binding to Scrib, whereas deletion of the entire C-terminus of PHLPP1 (P1-Δ910) abolished the interaction (Fig 1C). PHLPP2, a closely related isoform of PHLPP1, also had

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Fig 1 | PHLPP1 interacts with Scribble. (A) Cells (293T) were co-transfected with HA-PHLPP1 together with one of the following plasmids: vector, Flag-Scrib, Flag-DLG1, Flag-DLG4, Flag-Cypher or Myc-MAGI-2 (lanes 1–6, respectively). The cell lysates were immunoprecipitated with the anti-Flag agarose (lanes 1–5) or the Myc mAb coupled to protein A/G agarose (lane 6). PHLPP1 was detected using the PHLPP1 (P1) antibody. (B) 293T cells were co-transfected with HA-PHLPP1 together with vector, Flag-Scrib, Flag-LRR, Flag-Linker or Flag-PDZ(I–IV) (lanes 1–5, respectively). The cell lysates were immunoprecipitated with the anti-Flag agarose. Different Flag-tagged proteins were detected using the Flag mAb. (C) Cells (293T) were co-transfected with Flag-Scrib and one of the following plasmids: vector, HA-PHLPP1, HA-P1-ΔPH, HA-P1-ΔC, HA-P1-Δ910 and HA-PHLPP2 (lanes 1–6, respectively). The cell lysates were immunoprecipitated with the anti-HA agarose. Scrib was detected using the Flag mAb. The HA-tagged proteins were detected using the HA mAb. (D) Cells (293T) were co-transfected with Flag-Scrib and one of the following plasmids: vector, HA-PHLPP1, HA-P1-Δ1088, HA-P1-Δ1010, HA-P1-Δ940 and HA-P1-Δ910 (lanes 1–6, respectively). The cell lysates were immunoprecipitated with the anti-HA agarose. Scrib was detected using the Flag mAb. The wild-type and mutant PHLPP1 proteins were detected using the P1-PP2C antibody. (E) Equal amounts of DLD1 cell lysates were incubated with the protein A/G beads alone or with the beads and the Scrib antibody (lanes 1 and 2, respectively). PHLPP1 and Scrib were detected with the PHLPP1 (P1) and Scrib antibodies, respectively. Dlg, Discs large; HA, haemagglutinin; IP, immunoprecipitation; LRR, leucine-rich repeat; mAb, monoclonal antibody; MAGI-2, membrane-associated guanylate kinase WW and PDZ domain-containing 2; PHLPP1, PH domain leucine-rich-repeats protein phosphatase; PDZ, PSD95/Dlg/ZO-1; Scrib, Scribble.

a reduced affinity for Scrib (Fig 1C). Additional truncation mutants in the C-terminus of PHLPP1 were used to coimmunoprecipitate Scrib, and a section of 30 amino-acid residues between 910 and 940 in the C-terminus of PHLPP1 was necessary for binding Scrib (Fig 1D). Furthermore, the interaction between endogenous PHLPP1 and Scrib was readily detected in colon cancer DLD1 cells (Fig 1E).

Collectively, our data show that Scrib uses its LRR domain to bind to the C-terminus of PHLPP1. Although PHLPP1 and PHLPP2 are highly homologous, the C-terminal region required for binding to Scrib in PHLPP1 contains more Pro residues than the same region in PHLPP2. Our finding that Scrib differentially associates with PHLPP isoforms suggests that PHLPP1 and PHLPP2 might

regulate a distinct pool of substrates on binding to different scaffolding proteins in cells. Further studies are needed to define the specificity of PHLPP, by identifying more docking proteins for individual PHLPP isoforms.

PHLPP1 is a membrane-bound protein

Next, we performed immunofluorescence-staining experiments to determine the localization of PHLPP1 and Scrib in DLD1 cells. Confocal images showed almost identical staining patterns of PHLPP1 and Scrib at the basolateral membrane along cell–cell junctions in control cells. By contrast, the localization of PHLPP1 became diffuse in Scrib knockdown cells (Fig 2A). In addition,

Fig 2 | Knockdown of endogenous Scribble results in a decrease in membrane-localized PHLPP1. (A) Stable control (Sh-Con) and Scrib knockdown (Sh-Scrib) DLD1 cells were costained with antibodies for PHLPP1 (red) and Scrib (green). The inserts in the merged images are confocal XZ images. Scale bars, 10 μ m. (B) Cell-fractionation analysis of Sh-Con and Sh-Scrib DLD1 cells. PHLPP1 (P1), PHLPP2 (P2) and Scrib in the membrane (M) and cytosol (C) fractions were detected by immunoblotting. E-cadherin and ERK were used as protein markers of membrane and cytosol fractions, respectively. (C) The percentage of PHLPP1 localized in the membrane fraction was quantified by normalizing the level of PHLPP1 in the membrane fraction to the total PHLPP1 detected in both membrane and cytosol fractions. Data represent the mean $\pm$ s.e.m. ($n=3$, * indicates $P<0.05$ by Student's t-test). (D) The cell lysates prepared from Sh-Con and Sh-P1 (PHLPP1 knockdown) DLD1 cells were immunoprecipitated with the Akt antibody. (E) The cell lysates prepared from Sh-Con and Sh-Scrib DLD1 cells were immunoprecipitated with the Akt1 antibody. PHLPP1 and Scrib were detected using the PHLPP1 and Scrib antibodies, respectively. Note that only Akt1 was detected in the complex with PHLPP1 and Scrib, as the expression of other Akt isoforms was low in DLD1 cells (supplementary Fig S4 online). ERK, extracellular signal-regulated kinase; IP, immunoprecipitation; LRR, leucine-rich repeat; PHLPP1, PH domain leucine-rich-repeats protein phosphatase; Scrib, Scribble.

both PHLPP1 and Scrib were found to colocalize with E-cadherin, but not with ZO-1, at the basolateral membrane in DLD1 cells (supplementary Fig S1 online). The basolateral expression of Scrib and its colocalization with E-cadherin has also been observed in other mammalian cells (Navarro *et al*, 2005; Kallay *et al*, 2006). Cell-fractionation analysis showed that approximately 45% of

PHLPP1 was found in the membrane fraction in control cells, whereas the amount of PHLPP1 in the membrane fraction was decreased to approximately 20% in Scrib knockdown cells (Fig 2B,C). Similar results were obtained in another colon cancer cell line, Caco2, and an additional Scrib-knockdown DLD1 cell line (supplementary Fig S2 online). Consistent with our finding

Fig 3 | Membrane localization of PHLPP1 relies on interaction with Scribble. (A) Confocal images from HCT-P1 cells costained with the PHLPP1 (P1; red) and Scrib (green) antibodies. Scale bar, 10 μ m. (B) Cell-fractionation analysis of HCT-P1 cells transfected with vector (lanes 1 and 2), Flag-Scrib (lanes 3 and 4) or Flag-LRR (lanes 5 and 6), respectively in the membrane (M) and cytosol (C) fractions. (C) The percentage of PHLPP1 (P1) localized in the membrane fraction was quantified by normalizing the level of PHLPP1 in the membrane fraction to the total level of PHLPP1 detected in both membrane and cytosol fractions. Data represent the mean $\pm$ s.e.m. ($n = 3$). (D) HCT116 cells co-transfected with HA-P1- Δ 910 and vector (lanes 1 and 2) or Flag-Scrib (lanes 3 and 4) were fractionated into membrane and cytosol fractions. HA-P1- Δ 910 was detected using the P1-PP2C antibody. (E) HCT-Con (lanes 1–3) and HCT-P1 (lanes 4–6) cells transfected with vector, Flag-Scrib or Flag-LRR were analysed by immunoblotting. The amount of Akt phosphorylation is indicated below the pS473 panel. (F) Graph showing the relative rate of cell proliferation in HCT-Con and HCT-P1 cells. The rate of cell proliferation was set to 100% for HCT-Con cells transfected with the vector, and the cells in other settings were normalized accordingly. Each experimental point was performed in duplicate and the experiments were repeated three times. Data represent the mean $\pm$ s.e.m. ($n = 3$). Con, control; ERK, extracellular signal-regulated kinase; LRR, leucine-rich repeat; PHLPP1, PH domain leucine-rich-repeats protein phosphatase; Scrib, Scribble.

that Scrib binds to PHLPP2 with lower affinity, knockdown of Scrib had little effect on the amount of PHLPP2 associated with the membrane (Fig 2B).

We have previously shown that PHLPP1 binds to its substrate Akt (Gao *et al*, 2005). To determine whether Akt can be incorporated into the PHLPP1–Scrib complex, coimmunoprecipitation experiments were performed using control, PHLPP1 and Scrib-knockdown cells. In control cells, both PHLPP1 and Scrib formed a complex with Akt. However, Scrib was unable to bind to Akt in the absence of PHLPP1 (Fig 2D), whereas PHLPP1 failed to interact with Akt in the absence of Scrib (Fig 2E). These results

indicate that Akt, PHLPP1 and Scrib form a hetero-trimeric complex in cells, and incorporation of Akt into the complex depends on the integrity of the PHLPP1–Scrib interaction.

Scrib increases membrane association of PHLPP1

To confirm our finding that membrane localization of PHLPP1 depends on Scrib, we screened several colon-cancer cell lines for Scrib expression (supplementary Fig S3 online). In contrast to DLD1 cells, we found that the expression of Scrib was difficult to detect and diffuse in HCT116 and SW480 cells (Fig 3A; supplementary Fig S3 online). Concomitant with the lack of Scrib

Fig 4 | PHLPP1-dependent downregulation of Akt signalling is facilitated by Scribble. **(A)** Stable Sh-Con (lanes 1–2) and Sh-Scrib (lanes 3–4) DLD1 cells were serum-starved overnight and treated with insulin (0.1 μ M) for 15 min. The cell lysates were analysed by immunoblotting. The amount of relative Akt phosphorylation is indicated below the pS473 panel. **(B)** Stable Sh-Con (lanes 1–12) and Sh-Scrib (lanes 13–24) DLD1 cells were infected with retrovirus-encoding vector, WT PHLPP1, membrane-targeted PHLPP1 (MP-P1) or P1- Δ 910. The cells were serum-starved overnight and treated with insulin (0.1 μ M) for 0–15 min. The expression of PHLPP1 was detected using the P1-PP2C antibody. **(C)** Graphical representation of data shown in **(B)**. The relative Akt phosphorylation was obtained by normalizing the amount of pS473 to total Akt, and for the untreated cells this number was set to 1 at the time point 0. **(D)** Stable Sh-Con and Sh-Scrib DLD1 cells were maintained in regular growth medium containing 10% fetal bovine serum (FBS) or serum-starved for 24 h. The amount of apoptosis was measured using a DNA-fragmentation-based enzyme-linked immunosorbent assay and is expressed as colorimetric readings on the graphs. Each experimental point was performed in duplicate and the experiment was repeated three times. Data represent the mean $\pm$ s.e.m. ($n = 3$, * indicates $P < 0.0005$ by Student's t -test). **(E)** Sh-Scrib DLD1 cells were infected with retrovirus-encoding vector, WT, MP-P1 or P1- Δ 910. Two days post-infection, one set of the cells were serum-starved (Serum (–)) for 24 h before the assay and the other set of cells were maintained in regular growth medium containing 10% FBS (Serum (+)). Data represent the mean $\pm$ s.e.m. ($n = 3$, * indicates $P < 0.01$ by Student's t -test). Con, control; GSK, glycogen synthase kinase; LRR, leucine-rich repeat; PHLPP1, PH domain leucine-rich-repeats protein phosphatase; Scrib, Scribble; WT, wild type.

expression at the membrane, PHLPP1 showed a diffuse cytosolic expression pattern in HCT-P1 cells, a HCT116 cell line stably expressing low levels of haemagglutinin-tagged PHLPP1 (Liu et al, 2009; Fig 3A). Cell-fractionation analysis indicated that only a small percentage of PHLPP1 (approximately 10%) was found in the membrane fraction. Similarly, most PHLPP1 was cytoplasmically localized in SW480 cells (supplementary Fig S3 online). Re-expression of wild-type or LRR domain of Scrib resulted in a two- to threefold increase in the amount of PHLPP1 associated with the membrane in HCT-P1 cells (Fig 3B,C). By contrast, overexpression

of Scrib failed to target Δ 910—a Scrib-binding-deficient mutant of PHLPP1—to the membrane (Fig 3D). Thus, the level of Scrib expression controls PHLPP1 localization at the membrane.

To further assess the effect of Scrib on Akt signalling, we expressed wild-type or LRR domain of Scrib in control HCT116 (HCT-Con) and HCT-P1 cells and examined the status of Akt phosphorylation. Overexpression of Scrib or LRR had no effect on the level of Akt phosphorylation in HCT-Con cells (Fig 3E). Expression of PHLPP1 alone resulted in a decrease in Akt phosphorylation, and this inhibition of Akt was further enhanced

Fig 5 | Membrane localization of PHLPP1 is not regulated by Dlg1. (A) Stable Sh-Con and Sh-Dlg1 DLD1 cells were costained with antibodies for PHLPP1 (P1; red) and Dlg1 (green). The inserts in the merged images are confocal XZ images. Scale bars, 10 μ m. (B) Sh-Con and Sh-Dlg1 cells were serum-starved overnight and treated with insulin (0.1 μ M) for 15 min. Cell lysates were analysed by immunoblotting. Con, control; Dlg, Discs large; PHLPP1, PH domain leucine-rich-repeats protein phosphatase.

by Scrib or LRR in HCT-P1 cells (Fig 3E). Functionally, the growth inhibitory effect of PHLPP1 was potentiated in the presence of Scrib or LRR (40% inhibition with PHLPP1 compared with 70% inhibition with combinations; Fig 3F). The rate of proliferation was slightly decreased in HCT-Con cells when transfected with Scrib or LRR (Fig 3F). This is probably due to the low level of endogenous PHLPP1 expression in HCT116 cells (Gao *et al*, 2008; Liu *et al*, 2009), and suggests that Scrib regulates Akt signalling in a PHLPP1-dependent manner.

Dephosphorylation of Akt by membrane-targeted PHLPP1

The importance of scaffolding proteins in controlling the spatial-temporal propagation of cell signalling has been well documented in recent studies. Given the notion that activation of Akt occurs at the cell membrane (Alessi & Cohen, 1998; Yang *et al*, 2002), we postulated that dissociation of PHLPP1 from the membrane, as seen in Sh-Scrib cells, impairs its ability to dephosphorylate Akt. Indeed, both the basal and insulin-stimulated phosphorylation of Akt at Ser473 was increased in Scrib-knockdown cells, whereas phosphorylation at Thr308 was unaffected (Fig 4A). This is consistent with previous reports that PHLPP selectively dephosphorylates the hydrophobic motif of Akt (Gao *et al*, 2005; Brognard *et al*, 2007). The phosphorylation of glycogen synthase kinase 3 β (GSK3 β) (at Ser9), a substrate of Akt, was also increased, indicating higher Akt activity (Fig 4A). In addition, we found that the phosphorylation of both Akt1 and Akt2 isoforms was elevated on insulin stimulation in Sh-Scrib cells, although Akt1 was the main Akt isoform expressed (supplementary Fig S4 online).

Next, we determined whether loss of Scrib-induced activation of Akt can be reversed by overexpressing a membrane-targeted PHLPP1 (MP-P1; PHLPP1 tagged with a myristoylation and palmitoylation sequence at the amino-terminus). Overexpression of either wild-type or MP-P1 suppressed insulin-induced phosphorylation of Akt in the control cells, whereas a Δ 910 mutant failed to do so (Fig 4B,C). By contrast, overexpression of wild-type PHLPP1

was unable to inhibit insulin-stimulated phosphorylation of Akt in Sh-Scrib cells, suggesting that wild-type PHLPP1 became less effective against Akt in the absence of Scrib. However, MP-P1 maintained its ability to suppress Akt phosphorylation in Sh-Scrib cells (Fig 4B,C). Consistent with increased Akt phosphorylation, knockdown of Scrib made the cells resistant to apoptosis both basally and in the serum-starvation condition (Fig 4D). Overexpression of MP-P1, but not wild-type or Δ 910 PHLPP1, resensitized Sh-Scrib cells to serum-starvation-induced apoptosis (Fig 4E).

We have previously shown that deletion of the PDZ-binding motif in PHLPP1 decreases its ability to dephosphorylate Akt in 293T cells (Gao *et al*, 2005). Intriguingly, the PDZ-binding motif was dispensable for regulating Akt in epithelial cells expressing Scrib, such as DLD1, as mutant P1- Δ C functioned similarly to wild-type PHLPP1 in both Sh-Con and Sh-Scrib cells (supplementary Fig S4 online). By contrast, membrane-targeted mutant Δ 910 (MP- Δ 910) was able to dephosphorylate Akt regardless of Scrib expression (supplementary Fig S4 online), further confirming the importance of membrane localization of PHLPP1 in inhibiting Akt. Both MP-P1 and MP- Δ 910 were localized to the membrane when expressed in cells (supplementary Fig S4 online). Collectively, our findings indicate that PHLPP1-mediated dephosphorylation of Akt and subsequent apoptosis require Scrib-dependent membrane targeting of PHLPP1. However, the domains in PHLPP1 required for regulating Akt might be determined by the expression of different scaffolding proteins in a cell-type-specific manner.

Membrane targeting of PHLPP1 is mediated by Scrib

As deletion of Scrib results in the disruption of epithelial cell polarity in *Drosophila* embryo (Bilder & Perrimon, 2000), we examined whether loss of PHLPP1 membrane localization is related to potential alterations of cell polarity in Scrib-knockdown cells. It has been shown that Dlg1, another component of the Scrib/Dlg/Lgl polarity complex, functions together with Scrib to maintain cell polarity (Bilder *et al*, 2000). As PHLPP1 does not

interact with Dlg1 (Fig 1), we determined the localization of PHLPP1 in Dlg1-knockdown cells, to control for specificity. Although both PHLPP1 and Dlg1 localized to similar membrane domains at the cell–cell junction in DLD1 control cells, the membrane localization of PHLPP1 remained mostly unchanged in cells depleted of Dlg1 (Fig 5A). Furthermore, knockdown of Dlg1 had no effect on the insulin-stimulated phosphorylation of Akt (Fig 5B), thus confirming the specificity of Scrib-mediated membrane targeting of PHLPP1 in suppressing Akt signalling. Curiously, the morphology of Sh-Scrib and Sh-Dlg1 cells was slightly altered, as indicated by disorganized and diffused E-cadherin staining. However, the relative positions of ZO-1 and E-cadherin along the cell–cell junction was not affected, as shown in the XZ images (supplementary Fig S5 online). This lack of an effect on cell polarity might be explained by the fact that DLD1 cells are derived from a late-stage colon cancer patient and are no longer able to polarize fully. Therefore, loss of PHLPP1 membrane localization in Sh-Scrib cells is unlikely to be due to a general alteration of cell polarity. Several recent studies have indicated that loss of Scrib expression or mislocalization occurs in human cancers (Nakagawa *et al*, 2004; Gardiol *et al*, 2006). It is likely that downregulation of Scrib during tumorigenesis leads to dissociation of PHLPP1 from the membrane and subsequent elevation of Akt phosphorylation as a result of disruption of epithelial-cell polarity. Further studies are needed to identify the potential correlation between the expression of Scrib and PHLPP1 in human cancers.

Functional implications of Scrib interaction

The tumour suppressor function of PHLPP1 and Scrib has been established previously (Humbert *et al*, 2008; Liu *et al*, 2009). Our results here indicate that Scrib and PHLPP1 might function together to antagonize Akt signalling in a mutually dependent manner. As the ability of PHLPP1 to regulate Akt depends on Scrib, and the effect of Scrib on Akt phosphorylation is reversed by a membrane-targeted PHLPP1, we propose a model in which Scrib, PHLPP1 and Akt form a ternary complex to ensure rapid inactivation of Akt signalling. Further understanding of the way in which loss of Scrib and PHLPP1 contribute to tumour progression will help to develop new diagnostic and therapeutic strategies.

METHODS

Cell culture, antibodies and expression constructs. Details of cell culture conditions and sources of antibodies, as well as all plasmids used, are in the supplementary information online.

Immunofluorescence staining. Cells were fixed in 4% paraformaldehyde, followed by incubation with labelling buffer (1% bovine serum albumin in PBS, pH 7.4) to block nonspecific binding. Primary antibodies were diluted in labelling buffer and incubated with cells for 1–2 h at 22 °C. The Alexa 594-conjugated anti-rabbit and Alexa 488-conjugated anti-mouse IgG (Invitrogen) were used subsequently. The cellular distributions of endogenous proteins were visualized using an Olympus FlowView FV1000 confocal laser-scanning microscope.

Cell fractionation. To examine cellular localization of PHLPP1, cells were collected and resuspended in fractionation buffer (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol, 200 μM benzamidine, 40 μg ml⁻¹ leupeptin and 200 μM phenylmethylsulphonyl fluoride). The cells were lysed by passing through 25G

needles 12 times, and the cell lysates were subjected to centrifugation at 2,600 r.p.m. for 1 min at 4 °C. The resulting supernatant was subjected to a second centrifugation at 50,000 r.p.m. for 20 min at 4 °C. The supernatant after the second centrifugation is defined as cytosol, whereas the pellet is defined as the membrane fraction.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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