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A likely role for a novel PH-domain containing protein, PEPP2, in connecting membrane and cytoskeleton

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ABSTRACT: PH domains (pleckstrin homology) are well known to bind membrane phosphoinositides with different specificities and direct PH domain-containing proteins to discrete subcellular apartments with assistances of alternative binding partners. PH domain containing proteins are found to be involved in a wide range of cellular events, including signalling, cytoskeleton rearrangement and vesicular trafficking. Here we showed that a novel PH domain-containing protein, PEPP2, displayed moderate phosphoinositide binding specificity. Full length PEPP2 associated with both plasma membrane and microtubules. The membrane-associated PEPP2 nucleated at cell-cell contacts and the leading edge of migrating cells. Overexpression of PEPP2 increased membrane microviscosity, indicating a potential role of PEPP2 in regulating function of membrane and microtubules.

Introduction

Phosphatidylinositols are intrinsic components of membrane lipids on the cytoplasmic surface of membrane. The different combination of phosphorylation on the inositol rings gives rise to different phosphoinositides and their synthesis is temporally and spatially controlled by kinases and phosphatases. The difference in spatial distribution phosphoinositides decides their involvement in different aspects of membrane events. Phosphoinositides (PI) function through their cytoplasmic effector proteins that contain PI-binding domains by recruiting them onto the location where membrane events take place or assist protein-membrane association via electrostatic interactions.

The first PI-binding domain described was the PH domain in pleckstrin, a major protein kinase C substrate of platelets (Tyers *et al*., 1988). The PH domain is the 11th most common domain and is shared by various proteins, including signaling proteins, trafficking proteins and cytoskeletal proteins and consists of a conserved region of 100-120 amino acids (Lemmon *et al*., 2002). PH domains have been found in proteins that have binding specificity for all different phosphoinositides and, as such, are involved in many different cellular processes. For example, the activity of the Bbl family of guanine nucleotide exchange factors (GEFs), which couple receptor signaling with actin organization, are regulated by their interaction with PI(4,5)P_2 through their PH domains (Russo *et al*., 2000). PEPP2 is a novel PH-domain-containing protein with uncharacterized function. PEPP2 expressed ubiquitously while its two related isoforms, PEPP1 and PEPP3, displayed very restricted expression at very low levels (Dowler *et al*., 2000). Three proteins shared high homology in their PH domains, especially in the putative phosphotidylinositol-

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binding motif (PPBM) and were proposed to bind phosphotidylinositol-3-phosphate (PI3P).

In this study, we determined the lipid-binding specificity of PEPP2 and its subcellular distribution. Our results showed that unlike PEPP1, the PH domain of PEPP2 actually exhibited less specificity in lipid binding and displayed moderate to strong interactions with PI(5)P, PI (3)P and PI(3,5)P2 *in vitro*. However, PEPP2-PH was unlikely to bind PI-3-phosphate derivatives *in vivo* due to the unaffected membrane-association when treated with a PI3-kinase inhibitor, wortmannin. Full length PEPP2 displayed both plasma membrane-associated and microtubule-associated distribution, suggesting a potential role of PEPP2 in directing events between these two cellular compartments.

Material and Methods

Miscellaneous enzymes, chemicals and constructs

Restriction endonucleases were purchased from New England Biolabs (Genesearch Pty Ltd, Arundel, Queensland), Klenow fragment from GeneWorks Pty Ltd (Thebarton, South Australia), and both T4 ligase and T4 DNA polymerase from Roche Diagnostics Australia (Castle Hill, New South Wales). The fluorescence stain 4',6-diamidine-2-phenylindole' dihydrochloride (DAPI) was obtained from Sigma-Aldrich (Castle Hill, New South Wales). Full length PEPP2 in TOPO pCR2.1 was a generous gift from Dario R Alessi, MRC protein phosphorylation Unit, University of Dundee, Scotland UK. PEPP2 ORF was subcloned into pEGFP-C2 (Clontech). COS-1 cells and HEK-293T cells used in immunofluorescence were transfected with FuGene transfection reagent (Roche Diagnostic, Australia) using recommended protocol.

Immunofluorescence analysis

Preparations of the various GFP- tagged expression constructs were made using the Qiagen Midi kit (Qiagen, Clifton Hill, Victoria). Two picomoles (approximately 1 microgram) of each construct were transfected into cultured COS1 cells on 6-well plates using FuGene transfection reagent (Roche Diagnostics Australia). Transfected cells were grown on coverslips in DMEM plus 10% FBS and fixed 24 hours post-transfection as previously described. Cells were then incubated with an anti-α tubulin (Sigma) or an anti-EEA1 (BD) antibody, followed by incubation with an anti-mouse Texas Red-conjugated secondary antibody (Jackson Laboratories, Bar Harbor, Maine). In all cases, nuclei were stained using the DNA-specific stain, DAPI. GFP and Texas Red fluorescence were visualised under appropriate wavelength light on an Olympus AX70 microscope. Images were captured using a Photometrics CE200A Camera Electronics Unit and processed using Photoshop 6.01 software (Adobe Systems Incorporated, San Jose, California).

Wortmannin treatment

Wortmannin was added to cell culture media (DMEM+10%FCS) at a final concentration of 100 nM (Simonsen *et al*., 1998). Following addition, cells were cultured for a further 30 minutes before immunofluorescence analysis.

FRAP analysis for membrane microviscosity

Cells were grown on 90 mm dishes, which were modified with 15 mm diameter holes drilled at the centre of the base and sealed with coverslips. Plasma membrane was labelled with 8.3 µg/ml $DiIC_{16}$ at 4º for 15 min and medium replaced with serum-free DMEM plus 25 mM HEPES before labelling (Ghosh *et al*., 2002). Photobleaching of approximately a micron diameter region on the plasma membrane were made with short pulses from a 543nm HeNe laser beam at 100% power and the fluorescence recovery was monitored by scanning the bleached area with an attenuated beam of 20% power using a BioRad Radiance 2100 confocal microscope.

GST fusion protein expression and purification

An overnight culture of BL21 cells carrying the GST fusion protein expressing construct was diluted 100 fold into fresh L-broth +ampicillin and incubated at 37º with vigorous shaking until the A_{600} reached 1.0. Cells were then induced with 0.2mM IPTG at 37º for additional 3 hours, after which they were pelleted by centrifugation at 7,700 X g for 10 min and resuspended in 1/100 volume of STE plus 5mM DTT and 1.5% Sarkosyl. Collected cells were subsequently lysed on ice by sonication in short bursts. The insoluble fraction was separated by centrifugation at 12,000 X g for 5 min. 1/50 volume 50% slurry of Glutathione Sepharose 4B was then added to the remaining supernatant and incubated with gentle agitation at room temperature for 30 min. The Glutathione Sepharose 4B matrix was then

washed three times with 1 X PBS. The bound GST or GST fusion protein was finally eluted with an equal volume of Glutathione Elution Buffer.

Results

The lipid binding specificity of the PH domain of PEPP2

The PH-domain containing proteins function through their interaction with phosphoinositides. Although no experimental data has shown that PEPP2 interacts with phosphoinositide, the PH domain of a closely related isoform, PEPP1, demonstrated PI3P binding specificity in an *in vitro* lipid binding assay. The coding sequence of PH domain of PEPP2 was cloned into pGEX-4T-2 vector. The GST-PH (from aa130 to aa289) was expressed in BL21 cells and purified from cell lysates using Glutathione Sepharose 4B. Approximately 0.5ug/ml GST or GST-PEPP2PH were used in the protein-lipid overlay assay using the PIP strips with the indicated phosphoinositides of 100 pmol each. The strongest interactions were observed between the PH domain of PEPP2 and PI(5)P, PI(3)P and PI(3,5)P2, while weak interactions were also observed with the PI(4)P, phosphatidic acid (PA) and phosphatidylserine (PS) (Fig. 1). Control GST protein didn't bind any of these lipids (not shown).

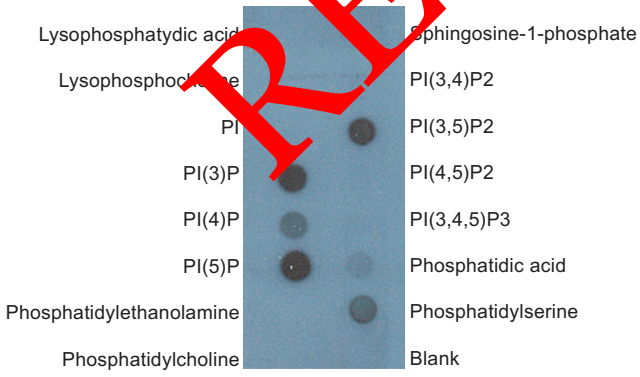

FIGURE 1. Lipid-binding specificity of the PH domain of PEPP2.About 0.5 µg/ml GST and GST-PEPP2PH were used in a protein-lipid overlay assay. The interaction between GST-PEPP2PH and immobilized lipids on the PIP strip was detected by western blot using a monoclonal anti-GST antibody. Strong interactions were observed between GST-PEPP2PH and PI(3,5)P2, PI3P, PI4P and PI5P, respectively. No specific binding was seen with GST only (not shown).

To verify its lipid binding ability *in vivo*, the PH domain was also expressed as a GFP-fusion protein in COS-1 cells. Despite of the diffusive distribution in cytoplasm and in nuclei, a significant portion of GFP-PH localize on plasma membrane (Fig. 2). Furthermore, this plasma membrane association of GFP-PH was not affected after wortmannin treatment (a PI3-kinase inhibitor), while the distribution of EEA1 (recruited onto the membrane of early endosome through association with PI3P) was changed after the same treatment (Fig. 2). It indicated that the PH domain of PEPP2 was unlikely to have PI3P or PI(3,5)P2 as its membrane binding partner *in vivo*.

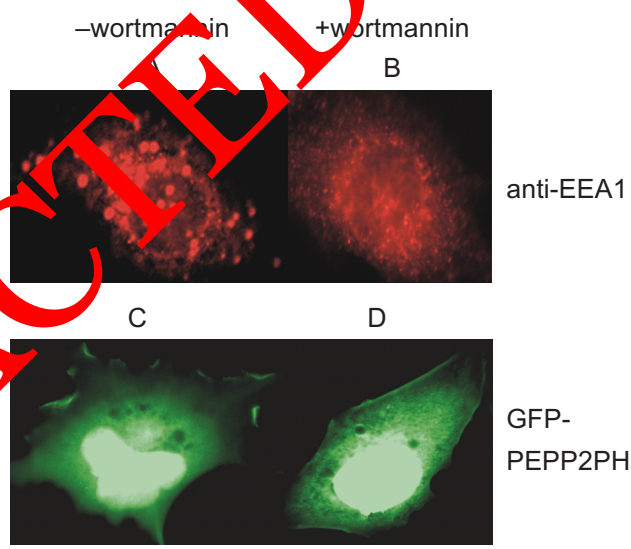

FIGURE 2. The membrane-association of the PH domain of PEPP2.The PH domain of PEPP2, expressed as a GFP fusion protein in COS1 cells (C and D), was observed on the plasma membrane as well as diffusely in both the cytoplasm and in the nucleus. The plasma membrane-association of GFP-PEPP2PH was unlikely to be mediated by PI3K product since the membrane-association remained unaffected after the treatmentwith wortmannin (D). Wortmannin inhibits PI3-kinase activity and reduces PI3K products, such as PI(3)P and PI(3,4,5)P3. Early endosome antigen 1 (EEA1), which associates with early endosomes (A) through interaction with PI(3)P on the early endosomal membrane, was redistributed into the cytoplasm (B) post wortmannin treatment.

The cellular localization of PEPP2

To check that if the membrane association is bona fide with full length PEPP2, PEPP2 was also expressed as a GFP fusion protein in COS1 cells. GFP-PEPP2 was predominantly distributed on plasma membranes or microtubules (Fig. 3). Notably, a significantly increased

amount of fluorescence of membrane associated GFP-PEPP2 was observed at cell-cell contacts (in both mesenchymal COS1 cells and polarized MDCK cells) and at the actin-polymerization site at the leading edge of the migrating HEK-293T cells (Fig. 4). The migration of HEK-293T cells was induced by 'scratching' confluent cells that were transiently overexpressing GFP-PEPP2 and cell migration was consequently promoted for 'wound' healing.

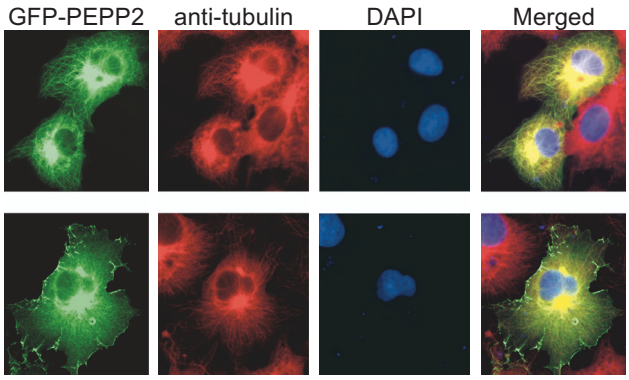

FIGURE 3. The microtubule- and plasma membrane-associated PEPP2.Cellular localization of overexpressed GFP-PEPP2 fusion protein in COS1 cells. Microtubule-association (top panel) and dual microtubule-/plasma membrane- association (bottom panel) of GFP-PEPP2 was observed. Microtubules were stained with monoclonal anti-α-tubulin and a Texas-red-conjugated secondary antibody. Nuclei were stained with DAPI (blue). Enriched GFP-PEPP2 in the perinuclear region, in the proximity of the microtubule organization center, was observed with both microtubule and membrane-associated distributions.

PEPP2 affects membrane microviscosity

Given that a significant proportion of PEPP2 is located on the plasma membrane, the effect of PEPP2 on a general plasma membrane property, membrane microviscosity, was investigated by fluorescence recovery after photo-bleaching (FRAP). FRAP has been shown to be easy and effective for measuring the lateral diffusion of membranes, which is inversely proportional to membrane viscosity (Ghosh *et al*., 2002; Vereb *et al*., 2003). Furthermore, the diffusion rate in a lipid bilayer is expected to be independent of the size of the bleached area (Vereb *et al*., 2003). The plasma membrane of live COS1 cells or COS1 cells overexpressing GFP-PEPP2 was initially fluorescently labeled using $DiIC_{16}(3)$. A small defined area (μm range) was then photo-bleached and the recovery of fluorescence in this spot was subsequently monitored and imaged by scanning with a confocal microscope (Fig. 5a). The fluorescence recovery was monitored every second for 10 seconds post photobleaching. Comparing with the control COS1 cells, the fluorescence recovery was enhanced in COS1 cells overexpressing PEPP2, indicating a potential role for PEPP2 in regulating membrane function (Fig. 5b).

Discussion

The physical characteristics of microtubules and their interaction with other microfilaments and intracellular membranes through cross-linking proteins inextricably link this cytoskeletal network to a vast array of cellular processes. Cross-talk between membranes and

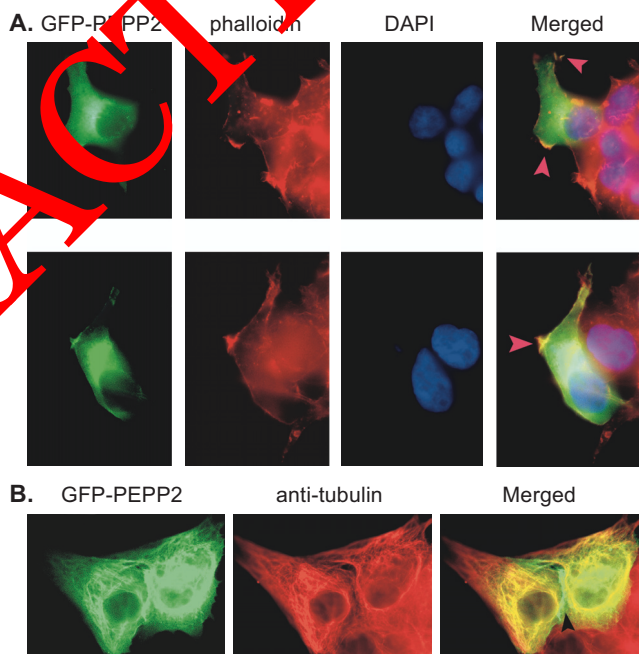

FIGURE 4. The non-uniform distribution of GFP-PEPP2 on the plasma membrane. **A.** In confluent HEK-293T cells transiently expressing GFP-PEPP2, scratches were introduced to promote cell migration in wound healing. The actin filaments were stained with phalloidin (red). A non-uniform distribution of GFP-PEPP2 on the plasma membrane at the leading edge of migrating cells was observed using direct immuno-fluorescence. The membrane-associated GFP-PEPP2 colocalized with the actin polymerization site (yellow), indicated by arrowhead on merged images. Nuclei in these cells were stained with DAPI (blue). **B.** Microtubule-associated GFP-PEPP2 and membrane-associated GFP-PEPP2 enriched at site of cell-cell contact (indicated by arrowhead) were observed in epithelial MDCK cells. Microtubules were stained with anti-α-tubulin and Texas-red-conjugated secondary antibody.

the microtubule cytoskeleton is critical for signal transduction, intracellular trafficking and organelle positioning (Mayer and Jürgens, 2002). The membrane-cytoskeleton linkages are established via both protein-protein and protein-lipid interactions. Crosslinking proteins including proteins of CLIP family, microtubule motors, enzymes and MAPs, which establish the connection between microtubules and subcellular membranes, can serve as multiple docking sites for both organelle localization and vesicle trafficking (Russo *et al*., 2000). A large number of cross-linking proteins utilize protein-lipids interactions to help them to anchor on membranes (Russo *et al*., 2000).

PEPP2 is a novel PH domain-containing protein, consisting of 1060 amino acids and having two WW domains at its N-terminal. The plasma membrane-association of PEPP2 was likely to be directed by the protein-lipid interaction between the PH domain and phosphoinositides since the overexpressed GFP fusion protein of the PH domain in COS1 cells displayed association with the plasma membrane. Although interactions have been noted between the PEPP2 PH domain and a number of different phosphoinositides (PI5P, PI(3,5)P2 and PI4P) in the *in vitro* lipid interaction assay, the binding of PI-3-phosphate derivatives *in vivo* is unlikely since the membrane associated PH domain of PEPP2 remained unaffected following wortamannin (a PI3K inhibitor) treatment.

A non-uniform distribution of PEPP2 was observed on plasma membrane ruffles, where actin polymerization occurs. Actin polymerization and microtubule protrusion at the leading edge of motile cells is required for directed cell migration (Nabi, 1999). Different phosphoinositides, including PI(3,4,5)P_3, PI(3,4)P_2 and PI(4,5)P_2, have been indicated in regulating actin dynamics (Insall and Weiner, 2001). For instance, PI(3,4,5)P3 serves as an instructive signal for stimulus-induced actin polymerization via activation of the guanine nucleotide exchange factors (GEFs) of the Rho family (Insall and Weiner, 2001). Clustering of PI(4,5)P_2 at the actin nucleation site on the plasma membrane has also been shown (Tall *et al*., 2000).

PI(4,5)P_2 can be generated by PI(4)P-5-kinases or PI(5)-4-kinases from both PI(4)P and PI(5)P, which have been shown to be able to interact with the PH domain of PEPP2. Although the PH domain of PEPP2 showed much higher affinity with PI5P than with PI4P in *in vitro* lipid binding assay, the local concentration of each phosphoinositide and the interaction of PEPP2 with other proteins may be as, or more, important than the lipid binding affinity in directing the protein-lipid interaction under physiological conditions. For example,

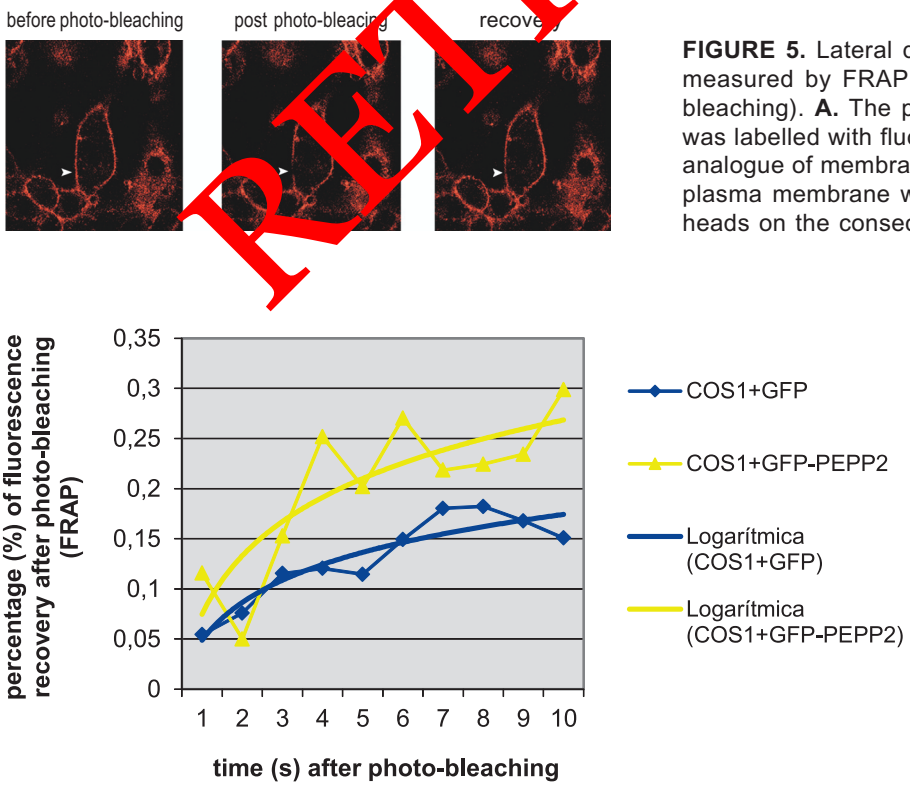

FIGURE 5. Lateral diffusion of the plasma membrane as measured by FRAP (fluorescence recovery after photo-bleaching). **A.** The plasma membrane of live COS1 cells was labelled with fluorescent DiIC16 (3) (red), which is an analogue of membrane lipids. A well defined region on the plasma membrane was bleached, as indicated by arrow-heads on the consecutive images above. The intensity of fluorescence recovery in the bleached area, which represented the lateral diffusion of membrane, was monitored and imaged over the first 10 seconds post bleaching. **B.** Plasma membrane microviscosity of COS1 cells is affected by PEPP2. The average recovery of fluorescence intensity (%) obtained from individual cells is presented in the chart (n=8). Trend lines are also shown. Increased membrane microviscosity has been seen with COS1 cells overexpressing PEPP2.

it is possible that the interaction between other proteins and the WW-domain or other uncharacterized regions of PEPP2 also helps to specify the membrane targeting and hence the cellular function of PEPP2 (Chang *et al*., 2010). Such a mechanism is generally used by PH-domain-containing proteins with no clear ligand specificity and low binding affinity (Lemmon *et al*., 2002).

The observation that PEPP2 concentrated at the actin nucleation site raises the possibility of its influence on actin polymerization. The polymerization of actin and microtubules are coordinated by Rho family GTPases. The ruffled leading lamellipodium of migrating cells reflects the underlying polarized organization of the cytoskeleton (Rodriguez *et al*., 2003). The structural linkage between microtubules and actin also orients the plus end of microtubules towards the leading edge along moving actin bundles (Gundersen and Bretscher, 2003). Therefore, PEPP2 may be involved in coupling the reorganization of microtubules with actin polymerization in directing cell migration.

Both phospholipids and the cytoskeleton (as well as cholesterol and integral membrane proteins) affect the viscosity of membranes (viscosity being the reciprocal of membrane fluidity) (Vereb *et al*., 2003). Given the membrane-association of PEPP2 and its possible role in regulating actin polymerization, the potential influence of PEPP2 on membrane microviscosity was investigated using FRAP. A notable increase in the lateral movement of the plasma membrane was observed in cells overexpressing GFP-PEPP2. The relationship between membrane microviscosity and cell motility is biphasic. Increased cell motility has been seen with increased microviscosity to a threshold, beyond which resulted in significantly decreased motility (Ghosh *et al*., 2002). The potential impact of PEPP2 on cell motility due to the altered membrane viscosity needs further investigation.

We have shown here the lipid binding specificity of PEPP2 PH domain as well as that of full length PEPP2. Overexpressed wild type PEPP2 displayed dual subcellular localization, predominantly on plasma membrane or on microtubules. The pattern of cellular distribution of PEPP2 suggesting a likely role for this novel PH domain containing protein in participating in cellular events, such as cell migration and membrane trafficking, involving these two distinct cellular compartments.

Acknowledgements

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