Crystal Structure of Human Vacuolar Protein Sorting Protein 29 Reveals a Phosphodiesterase/Nuclease-like Fold and Two Protein-Protein Interaction Sites*

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Vacular protein sorting protein 29 (Vps29p), which is involved in retrograde trafficking from prevacuolar endosomes to the trans-Golgi network, performs its biological functions by participating in the formation of a “retromer complex.” In human cells, this complex comprises four conserved proteins: hVps35p, hVps29p, hVps26p, and sorting nexin 1 protein (SNX1). Here, we report the crystal structure of hVps29p at 2.1 Å resolution, the first three-dimensional structure of the retromer subunits. This novel structure adopts a four-layered α-β-β-α sandwich fold. hVps29p contains a metal-binding site that is very similar to the active sites of some proteins of the phosphodiesterase/nuclease protein family, indicating that hVps29p may carry out chemically similar functions. Structure and sequence conservation analysis suggests that hVps29p contains two protein-protein interaction sites. One site, which potentially serves as the interface between hVps29p and hVps35p, comprises 5 conserved hydrophobic and 8 hydrophilic residues. The other site is relatively more hydrophilic and may serve as a binding interface with hVps26p, SNX1, or other target proteins.

To function properly for the organelles in eukaryotic cells, it is necessary that specific sets of proteins and lipids are sorted and delivered to various intracellular compartments efficiently. The trafficking of proteins and lipids between intracellular organelles requires a series of proteins that are involved in the membrane transport pathways (1–3). Genetic screens in cells, the retromer complex comprises Vps26p, Vps29p, Vps35p, Vps5p, and Vps17p provides structural components to complete the retromer complex and mechanical force for vesicle budding (10, 14, 15). The retromer complex is conserved and has homologs in mammalian cells. In human cells, the retromer complex comprises hVps26p, hVps29p, hVps35p, and SNX1 (the mammalian homolog of Vps5p) (16, 17). hVps35p serves as the core of this multimeric complex and binds directly to the other retromer subunits. In addition, there are some potential interactions that may stabilize the complex between hVps26p and hVps29p, hVps29p and SNX1, as well as hVps26p and SNX1 (17).

Vps35p, a receptor for cargo proteins, is directly associated with sorting signaling in the cytosolic domains of many cargo proteins, including Vps10p and A-ALP, a model trans-Golgi network resident protein (6, 13, 18). As a necessary component of the retromer complex, Vps29p directly binds to Vps35p but not to cargo proteins. Vps29p possibly assists Vps35p to bind to cargo proteins by enhancing its association with membranes and/or with other retromer proteins (10). In the absence of Vps29p, Vps35p cannot bind to Vps26p in solution (17). Furthermore, when both Vps29 and Vps26 are mutated or knocked out, Vps35p will be very unstable (10, 19). Vps35p and Vps29p

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§The abbreviations used are: Vps, vacuolar protein sorting; Vps29p, these Vps genes are involved directly or indirectly in protein sorting or trafficking between the late Golgi and the vacuoles (4–6). Disruption of Vps genes can cause disturbance and mis-sorting of many proteins, such as CPY. As a vacuolar hydrolase, CPY is recognized by Vps10p in the late Golgi. Vps10p binds to CPY and conveys it via vesicle to the prevacuolar compartment where Vps10p releases it. After that, Vps10p returns to the Golgi for further rounds of transportation. Some yeast Vps gene products, Vps26p, Vps29p, Vps35p, Vps5p, and Vps17p, are necessary for the retrograde retrieval process of Vps10p from endosomes back to the trans-Golgi network (7–10). By forming a complex named “retromer complex,” the five proteins are also involved in the proper sorting and transportation of dipetidyl aminopeptidase A, Kex2 protease (Kex2p), polymeric immunoglobulin receptor, and mannose 6-phosphate receptor (9–12). This complex consists of two subcomplexes. One subcomplex, consisting of Vps35p, Vps29p, and Vps26p, is responsible for selecting cargoes retrieved from the prevacuolar compartment (10, 13). The other subcomplex of Vps5p and Vps17p provides structural components to complete the retromer complex and mechanical force for vesicle budding (10, 14, 15). The retromer complex is conserved and has homologs in mammalian cells. In human cells, the retromer complex comprises hVps26p, hVps29p, hVps35p, and SNX1 (the mammalian homolog of Vps5p) (16, 17). hVps35p serves as the core of this multimeric complex and binds directly to the other retromer subunits. In addition, there are some potential interactions that may stabilize the complex between hVps26p and hVps29p, hVps29p and SNX1, as well as hVps26p and SNX1 (17).

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vacular protein sorting protein 29; hVps29p, human Vps29p; SNX1, sorting nexin 1 protein; DPAP A, dipetidyl amino peptidase; pIgR, polymeric immunoglobulin receptor; SeMet, Seleno-L-methionine; MAD, multiple wavelength anomalous dispersion; r.m.s.d., root mean square deviations; PDB, Protein Data Bank.
assemble into a stable subcomplex that is fit together with Vps5p/Vps17p through the interactions in which Vps26p is required to participate (7, 8, 10, 20). SNX1 contains a Phox homology domain that can bind to phosphatidylinositol-3-phosphate, phosphatidylinositol 3,5-bisphosphate, or phosphatidylinositol 3,4,5-bisphosphate. These phosphoinositides may serve as direct, local regulators and recruiters of protein machinery.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification**—The complete cDNA encoding hVps29p (accession number NM_016226) was subcloned from a human brain cDNA library (BD Biosciences) into pET-22b vector (Novagen) with the NdeI and XhoI restriction sites. hVps29p was expressed in E. coli strain B834 (Novagen) using M9 medium supplemented with SeMet and 6 amino acids, including leucine, isoleucine, valine, phenylalanine, lysine, and threonine. The SeMet-derivative protein was purified by similar procedure to the wild-type protein.

**Crystallization and Data Collection**—Crystals of both the wild-type and the SeMet-derivative proteins of hVps29p were grown using the hanging drop vapor diffusion method at room temperature. The initial crystallization conditions were screened by the sparse matrix sampling method using Crystal Screen I and II (Hampton Research). The crystals suitable for x-ray diffraction were grown in 25% polyethylene glycol 4000, 10% ethylene glycol, and 100 mM Hepes, pH 7.5, with a protein concentration of 3 mg/ml in 50 mM NaCl, 5 mM Tris·HCl, pH 7.5.

A MAD data set was collected from a single crystal of SeMet-derivative protein at 100 K without additional cryoprotectant on beamline 3W1A of the Beijing Synchrotron Radiation Facility at the Institute of High Energy Physics, Chinese Academy of Sciences. The data were collected at two wavelengths (\(\lambda_{\text{low}} = 0.9814 \text{ Å} \) and \(\lambda_{\text{high}} = 0.9817 \text{ Å} \)). The MAD data were processed using MOSFILM (25) and Scala in CCP4 suite (26). In addition, by using imaging plates (Mar Research) and a rotating anode source (Rigaku), a native data set with 2.1 Å resolution was obtained from a wild-type protein crystal at 100 K. The native data set was processed by Automar (marresearch GmbH).

The MAD and the SeMet-derivative crystals are in space group p4_2_2 with similar cell parameters. Data collection statistics are presented in Table I.

**Structure Determination and Refinement**—Two of the three expected selenium positions were determined by SHELX (27) using Bijvoet differences of the MAD data prepared by XPREP (Bruker Analytical X-ray Systems). The initial phases were calculated by SHARP with the resolution ranging from 25 to 2.7 Å (28). The resulting phases were extended to 2.3 Å and improved through solvent flattening and histogram matching using DM and SOLOMON (29). An initial model (112 residues) was built by DM, and the model was further built and refined at 2.3 Å resolution using Refmac 5.0 (31) and O (32) by manual model correction. After the model reached a reasonable quality, refinement was continued with the 2.1 Å data collected from native protein crystal. Further cycles of refinement and model building were carried out until the crystallography R-factor and free R-factor converged to 21.8 and 24.4%, respectively (33). The stereochemistry of the structure was checked by PROCHECK (34). Figures were prepared using PyMOL and GRASP (35). MAD phasing and model refinement statistics are shown in Table I.

**RESULTS**

**Structure Solution and Model Quality**—The hVps29p crystal structure was phased by MAD with SeMet-substituted protein and refined against the diffraction data of native protein crystal. The current model consists of 180 amino acids (residues 1–92 and 95–182) and 48 water molecules. Due to conformational disorder, Trp-93 and Gly-94 are invisible in the electron density.

![Structure of hVps29p](image)

**FIG. 1.** Sequence alignment of seven hVps29p homologs of eukaryotic species, including hVps29p of *H. sapiens* (accession number NM_016226), DrVps29 of *Danio rerio* (accession number NP_955331), DmVps29 of *Drosophila melanogaster* (accession number NP. 608575), CeVps29 of *C. elegans* (accession number CAA87426), NeVps29 of *Neurospora crassa* (accession number XP_328261), EgVps29 of *Eremothecium gossypii* (accession number NP_985558), ScVps29 of *S. cerevisiae* (accession number P38759). Identical and similar residues are shown in red and yellow, respectively. Secondary structure elements of hVps29p are shown above the sequences.
Structure of hVps29p

Table 1

| Data set          | Native | Peak | Edge          |
|-------------------|--------|------|---------------|
| Wavelength (Å)    | 1.5418 | 0.9814 | 0.9817       |
| Resolution range  | 30–2.1 | 25–2.3 | 25–2.7       |
| (outer shell)     | (2.18–2.1) | (2.42–2.3) | (2.85–2.7) |
| Total reflections | 54013  | 110866 | 56484        |
| Unique reflections| 10856  | 8739  | 3879         |

- Overall (outer shell) for the native data set gives Rmerge = 96.5% (95.5%) and completeness = 99.92% (100%).
- Resolution for native data set is 15–2.1 Å.
- Percent completeness for the native data set is 99.92% (100%).
- Rmerge for native data set is 7.0% (35.4%)
- Redundancy for native data set is 4.97 (5.05)
- Rvalues for native data set are 1.67/1.06
- FOM fatigue factor is calculated using a randomly selected 5% of the reflections set aside throughout the refinement. Figure of merit (FOM) is as defined in SHARP (28).

**Structure overview—hVps29p**: an approximately spherical molecule with dimensions of 36 × 42 × 44 Å comprises two β sheets (five-stranded and six-stranded) and three α helices (α1, α2, and α3) adopting a four-layered α-β-β-α sandwich fold (Fig. 2A). The five-stranded β sheet that is flanked by one helix (α3) consists of strands β4-β8, and β4 and β7 are antiparallel to the other three strands. In a similar manner, the six-stranded β sheet, flanked by two α helices (α1 and α2), consists of strands β1-β3 and β9-β11, with β9 and β11 antiparallel to the other four strands. The interfaces between layers in the α-β-β-α sandwich are mainly formed by tightly packed hydrophobic side chains.

There is only one hVps29p molecule in the asymmetric unit, and the crystallographic contact between molecules involves very limited interactions, which seem unlikely to be physiologically significant. Size exclusion chromatography and dynamic light scattering experiments confirmed the presence of hVps29p as a monomer in solution (data not shown). These data imply that the monomer of hVps29p may be a stable and functional unit in solution.

The electrostatic potential surface of hVps29p displays prominent asymmetry. As shown in Fig. 2B, a negatively charged groove stretches along one side of the molecule. The 11 amino acids at the groove, including Asp-8, Glu-44, Asp-62, Asp-64, Glu-65, Glu-71, Asp-95, Glu-120, Glu-143, Asp-170, and Asp-171, contribute the negative charge of the surface. Most of these amino acids are highly conserved (Fig. 1), suggesting that the negative groove may be a feature of Vps29p homologs.

**Comparison of Sequence and Structural Homologs**—A Pfam (36) annotation suggests that hVps29p belongs to a family of density and therefore not included in the final model. Moreover, side chains of 5 residues, i.e., Lys-21, Asp-47, Ile-91, Lys-118, and Lys-180, show weak electron density. The final model was refined to 2.1 Å with free R-factor of 24.4% and crystallographic R-factor of 21.8%. The r.m.s.d. from ideal values of bond lengths and bond angles were 0.009 Å and 1.774°, respectively. Of all the residues in the final refined model, 87.3% have main chain torsion angles in the most favored regions and only one residue, His-115, which can be well defined in the experimental electron density, is in a disallowed region. Data collection and refinement statistics are summarized in Table I.

**Structure of hVps29p**: a, a stereo view of hVps29p. The secondary structure elements are numbered correspondingly. The purple arrow represents the negatively charged groove. Sticks represent the hydrophilic amino acids (Asp-8, His-10, Arg-14, Asn-39, Asp-62, His-86, His-115, His-117, and Asn-140) that are possibly involved in binding metal ions. B, the electrostatic potential surface of hVps29p. Saturating red indicates φ < -10 kiloteslas, and saturating blue indicates φ > 10 kiloteslas, T = 293 K (38). The purple arrow represents the negatively charged groove.

Calcineurin-like related phosphoesterase. This family contains many kinds of phosphoesterases, such as phosphoserine phosphohydrolases, nucleotidases, sphingomyelin phosphodiesterases, 2′-3′-cyclic AMP phosphodiesterases, and nucleases. A BLAST search reveals a large number of homologs of hVps29p. Besides the proteins from C. elegans, Saccharomyces cerevisiae, plants, insects, and mammalians (Fig. 1), the homologs of Vps29p are also found in one thermophilic bacterium (Thermatoga maritima) and several species of Archaea (Methanococcus jannaschii, Methanobacterium thermoautotrophicum, Pyrococcus abyssi, and Pyrococcus horikoshii), suggesting that they are
ancient proteins and perform essential biological functions (17).

Despite the low sequence identity, hVps29p shows structural similarity to many previously determined structures in the Protein Data Bank (PDB). The most similar proteins found by the Dali server (37) in the PDB are phosphodiesterase MJ0936 of M. jannaschii (PDB code 1S3N, r.m.s.d. = 2.5 over 150 Ca atoms) (38), hypothetical protein of Pyrococcus furiosus (PDB code 1NNW, r.m.s.d. = 3.1 over 159 Ca atoms), purple acid phosphatase of Sus scrofa and Phaeosphaeria vulgaris (PDB code 1UTE, r.m.s.d. = 3.7 over 168 Ca atoms; PDB code 4KBP, r.m.s.d. = 3.5 over 167 Ca atoms) (39, 40), mre11 nuclease of Pyrococcus furiosus (PDB code 1I17, r.m.s.d. = 3.2 over 149 Ca atoms) (41), hypothetical protein of Thermus thermophilus (PDB code 1UF3, r.m.s.d. = 3.2 over 140 Ca atoms), serine/threonine phosphatase 2B of Homo sapiens and Bacteriophage lambda, respectively (PDB code 1AU1, r.m.s.d. = 3.1 over 151 Ca atoms; PDB code 1G5B, r.m.s.d. = 3.2 over 131 Ca atoms) (42, 43), and 5’-nucleotidase of E. coli (PDB code 1USH, r.m.s.d. = 3.3 over 150 Ca atoms) (44). Except for the two hypothetical proteins, 1NNW and 1UF3, the other proteins perform molecular functions that are chemically similar (phosphodiesterase, phosphatase, nuclease, or nucleotidase). These enzymes adopt the common sandwich fold, and the metal-chelating residues involved in enzymatic activity are also conserved.

The Metal-binding Site of hVps29p—Based on the structural comparison with these enzymes, we determined that the presumed metal ion-binding site of hVps29p is located at the highly negatively charged groove and formed by a cluster of turn/loop regions among β1/α1, β2/α2, β3/β4, β5/α5, β6/β7, and β8/β9 (Fig. 2, A and B). The residues, which are proposed to be involved in metal binding, include Asp-8, His-10, Arg-14, Asn-39, Asp-62, His-86, His-115, His-117, and Asn-140.

hVps29p shares similar fold and overall structure with MJ0936 (Fig. 3A), a kind of phosphodiesterase of M. jannaschii (41). Some ions, such as Mn$^{2+}$, are necessary for the enzyme activity of MJ0936. The di-Mn$^{2+}$-binding sites of MJ0936 show great resemblance to the presumed metal-binding site of hVps29p. The corresponding residues at the metal-binding site (hVps29p:MJ0936) are: Asp-8:Asp-8, His-10:His-10, Asn-39:Asp-36, Asp-62:Asn-59, Phe-63:Gln-60, His-86:His-97, His-115:His-120 and His-117:His-122, respectively (Fig. 3B). In addition, Arg-14 and Asn-140 of hVps29p are highly hydrophilic and may also be involved in metal binding. Although Phe-63 of hVps29p locates at a similar position to Gln-60 of MJ0936, the phenylalanine apparently cannot bind to metal ions. Residues of the presumed metal-binding site of hVps29p also show similar architecture to those of purple acid phosphatase (PDB code 1UTE) and mre11 nuclease (PDB code 1I17) (42, 44). To carry out the enzymatic function, one Fe$^{3+}$ and one Fe$^{2+}$ are necessary for purple acid phosphatase, and two Mn$^{2+}$ are necessary for mre11 nuclease. Thus, it is proposed that the nine hydrophilic residues of hVps29p may harbor suitable metal ion(s) at this site. In fact, we found that hVps29p can bind to some metal ions (such as Fe$^{3+}$ and Ni$^{2+}$) in solution (data not shown). However, the experimental electron density map does not indicate the presence of any metal ion in the crystal structure of hVps29p, although many kinds of metal ions were soaked and co-crystallized. The crystallization environment may interfere with the incorporation of metal ions.

Two Possible Protein-Protein Interaction Sites of hVps29p—As a key subunit of the retromer complex, hVps29p strongly binds to hVps35p, which is crucial to the assembly of this molecular machine (18). In addition, some weak interactions between hVps29p and other proteins, such as hVps26p, SNX1, etc., may exist (10, 11, 18). Similar to many other membrane trafficking machineries, the sequences and functions of the retromer proteins, including Vps35p, Vps29p, Vps26p, and SNX1/Vps5p, were conserved during evolution (17, 18). Although only the Vps29p proteins of human and yeast have been characterized, their homologs of other eukaryotes may play a similar role in the retromer complex (10, 11, 18). Accordingly, it is deduced that the amino acids at the protein-protein interaction sites of hVps29p are possibly conserved.

Seven homologs of eukaryotes, with identity from 96 to 40%, were selected to delineate the conservative sites of Vps29 proteins. As shown in Fig. 1, there are about 80 identical or similar residues colored in red and purple, respectively. The conserved amino acids can be classified as exterior and interior residues. The interior conserved residues may mainly serve as structural components sustaining the stability of structure, whereas the exterior ones are probably involved in protein functions, such as protein-protein interaction, enzymatic activity, etc. The exterior conserved residues mainly cluster together at two regions (regions I and II), which are candidates for protein-protein interaction interfaces (Fig. 4, A and C). Other conserved exterior residues (such as Pro-12, Arg-14, Asp-55, Arg-60, Phe-65, Pro-70, Asp-95, Tyr-139, Pro-148, etc.) are located separately in space or near the metal-binding site that possibly correlates with the biochemical activity (Fig. 4B).

As shown in Fig. 4A, at region I, which is formed by the residues in β11, β10, β9, β1, and α1, there are 13 conserved residues, including 5 charged, 2 hydrophilic, and 6 hydrophobic residues. Similar to many protein-protein interfaces (45), this region has a hydrophobic center formed by 6 conserved hydrophobic residues, i.e., Leu-2, Leu-4, Leu-25, Leu-152, Val-172, and Val-174, and surrounded by 7 charged or hydrophilic residues, i.e., Lys-21, Lys-23, Lys-25, Lys-30, Asp-154, Tyr-163, and Tyr-165 (Fig. 4A). At region II, which is on the opposite
side of region I, there are 7 conserved exterior residues, including 4 charged residues, i.e., Arg-104, Asp-107, Asp-109, and Lys-127, 2 hydrophilic residues, i.e., Gln-79 and Gln-105, and one hydrophobic residue, Val-74 (Fig. 4C). These exterior residues, located at β4, β6, β8, and α3, encompass a smaller but more hydrophilic surface area as compared with region I.

**DISCUSSION**

**Interaction of hVps29p with hVps35p and Other Target Proteins**—Previous research suggests that hydrophobic interactions serve as not only the major sources of protein folding and protein stabilization (46–48) but also the predominant driving force in the formation of biologically relevant complexes (45, 49). Hydrophilic interactions participate in stabilizing protein complexes by providing strong interactions and shielding the hydrophobic core from hydrophilic environments (50, 51). Both the hydrophobic and the hydrophilic components are essential to molecular recognition (52), and they often work cooperatively to stabilize favorable protein-protein interaction (50).

As a subunit of the retromer complex, hVps29p can bind to hVps35p directly and may also interact with other retromer proteins, hVps26p and SNX1 (17). The C-terminal (residues 307–796) of full-length hVps35p can strongly bind to hVps29p. However, some fragments of hVps35p show little interaction with hVps29p, for instance, residues 1–603, 307–403, 307–500, 307–603, 307–754, and 603–796 (17). These data imply that the C-terminal of hVps35p (residues 307–796) may fold into either one large domain or multiple domains that interact with hVps29p solely or cooperatively. Additionally, it is observed that large protein-protein complexes have large interfaces consisting of many hydrophobic residues, whereas polar amino acids are common in small interfaces (45, 49). Thus, it is possible that hVps29p binds to hVps35p by a large hydrophobic surface area. As compared with region II, region I contains more conserved hydrophobic amino acids, encompasses a larger hydrophobic surface area, and thus potentially provides stronger interaction with target proteins. Taken together, these results imply that region I serves as the interface between hVps29p and hVps35p. With hydrophobic residues surrounded by several charged and hydrophilic ones, the interaction between hVps29p and hVps35p would be cooperatively stabilized by both kinds of interactions.

Vps29p binds to Vps35p directly and possibly assists the interaction of Vps35p with cargo proteins (10). Without Vps29p, Vps35p cannot bind to Vps26p in *vitro* (17) and becomes very unstable when both Vps29p and Vps26 are knocked out or mutated (10, 19). Vps35p and Vps29p assemble into a stable subcomplex that is complexed with Vps5p/Vps17p through the interaction with Vps26p (17). Thus, Vps29p, together with Vps35p, may act as a fundamental scaffold for trafficking protein machines and interact with various protein partners. The sequence conservation analysis reveals that besides the large surface region I that potentially interacts with Vps35p, a relative small and hydrophilic region II is also conserved among the eukaryote homologs. On the spherical molecule of hVps29p, this region resides at a flank of region I (Fig. 4). Along with the association with hVps35p, region II of hVps29p possibly provides the interface for the interaction with other target proteins, such as, SNX1 and hVps26p. Alternatively, by binding to hVps35p through region I, the nearby region II of hVps29p may get close to hVps35p and form a complete interaction site with regions in hVps35p for other partners. However, unraveling the detailed binding map of hVps29p to target proteins requires further studies.

**Putative Active Site for Phosphate Group-containing ligand**—As an ancient protein, Vps29p possibly plays some other biological roles in addition to serving as a subunit of the retromer complex (17). As shown in Fig. 2, A and B, the most prominent feature of hVps29p structure is the distinctive groove that is negatively charged and about 18 Å long and 8 Å wide. The presumed metal-binding site of hVps29p is located at the center of this groove. This cleft is free from crystal packing and entirely exposed to the solvent tunnel in the crystal structure. The architecture of the metal-chelating residues is similar to those of many enzymes with α/β four-layered sandwich structures and binding di-metal ions, such as MJ0936, purple acid phosphatase, and mre11 nuclease (38, 39, 41). This similarity suggests that hVps29p not only acts as a subunit of the retromer complex but may also function as an enzyme with substrates containing phosphate groups or bases, chemically similar to MJ0936, purple acid phosphatase, and mre11 nuclease. The higher expression of hVps29 relative to its partners of the retromer complex in some tissues indicates the multiple functions of hVps29p beyond a subunit of the retromer complex (17). We speculate the possible enzymatic activity, as well as the interactions with other target proteins, to be a candidate for the additional functions of hVps29p. Interestingly, the location of this possible active site in hVps29p is flanked by the conserved surface regions I and II (Fig. 4). The spatial separation of these three sites implies that they can function individually or cooperatively. However, there is no evidence that hVps29p can catalyze any phosphodiesterase-like reactions. Consequently, there is still another possibility that this negatively charged groove is utilized to bind the phosphate groups or bases containing ligand, instead of catalyzing it. Similarly,
SNX1 contains Phox homology domain that binds phosphatidylinositol-3-phosphate, phosphatidylinositol 3,5-bisphosphate, or phosphatidylinositol 3,4,5-bisphosphate and may serve as directly local regulators or recruiters of protein machines that control the membrane trafficking in some pathway (20–23). Further investigations are needed to determine the exact role of the negatively charged groove of hVps29p.

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