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Retromer complex-based coated vesicular-tubular structures mediate retrograde transport of a vast array of transmembrane proteins of widely differing function from endosomes to the cell surface or the TGN (reviewed in refs. 1–4). At the heart of all retromer-based coats is a 1:1:1 core heterotrimer comprised of VPS35 (helical solenoid), VPS26 (arrestin-like fold) and VPS29 (inactive phosphatase fold), which can be identified across nearly all eukaryotic organisms5. Loss of retromer in mice is embryonically lethal6,7, and mutations in the core retromer components are linked to Alzheimer’s and Parkinson’s diseases (reviewed in ref. 8). In mammalian cells, retromer-based coats also contain other components including the P13P13P52-binding PX domain-containing sorting nexin (SNX) proteins (including SNX3, SNX-BAR proteins, and SNX27)9–12 and associate with regulatory proteins/protein complexes including WASH complex13,14, TBC1D515,16, REM517, EHD118 and Rabankyrin14,18.

We and others19,20 identified the 100 kDa, multi-domain, multifunctional endosomal protein VARP as a further regulatory/accessory factor for retromer-based coats. VARP variously binds and regulates the membrane-fusion activity of the SNARE (soluble N-ethylmaleimide attachment protein receptor) VAMP721,22; is a GEF for Rab21/524 (Fig. 1a). N-ethylmaleimide attachment protein receptor (VAMP)721,22; is a GEF for Rab21/524 (Fig. 1a). N-ethylmaleimide attachment protein receptor (VAMP)721,22; is a GEF for Rab21/524 (Fig. 1a). N-ethylmaleimide attachment protein receptor (VAMP)721,22; is a GEF for Rab21/524 (Fig. 1a).

VARP, where

Results and discussion

Characterisation of VPS29: VARP CHPLCxxCxxC interaction.

The two mammalian VARP CHPLCxxCxxC sequences differ only slightly in their core sequences but their 20–30 residue flanking sequences differ considerably (Fig. 1b) and are all predicted to be unstructured26. Constructs containing the CHPLCxxCxxC sequences and their linkers (residues 396–460 and 692–746) were created and a Surface Plasmon Resonance (SPR) assay was developed to study their binding to VPS29 under different conditions (Isothermal Titration Calorimetry (ITC) proved unsuitable primarily due to the constructs’ rapid precipitation on stirring).

Recombinant GST–692–746 and a similarly sized GST control fusion protein were bound to an anti-GST antibody, which had previously been covalently coupled to a sensor chip and untagged VPS29 passed over the resulting surface. The KD was measured using both equilibrium and kinetic analysis, both of which yielded a value of 2–3 μM at 12 °C. (Fig. 1c–f). Thermodynamic analysis over a range of temperatures between 8 °C and 25 °C shows that binding has favorable enthalpy and entropy changes (ΔH° ~ −20 kJ mol−1 and ΔS° ~ +36 J K−1 mol−1) and the reaction is exothermic. When the experiment was repeated using protein that had been dialyzed overnight against buffer lacking Zn++ but containing 10 mM EDTA in order to remove bound Zn++, and the assay carried out in buffer lacking Zn++ and containing 10 mM EDTA, the measured binding dropped ~20 fold to 45 μM (additional unsuitability of ITC resulted from EDTA stripping divalent metal ions from VPS29)25. A similar weaker KD (55 μM) was also obtained in the presence of Zn++ ions when all four Cys residues, which we believe coordinate a single Zn++, were mutated to serines (4C mutant, Fig. 1e). These data demonstrate a key role for the 4xCys/Zn++ cluster in mediating the interaction between VARP and VPS29. Although being more physically unstable and the data consequently of poorer quality, residues 369–460 displayed a similar KD to residues 692–746 of ~5 μM (Fig. 1e): further study of this sequence was not pursued.

VARP: VARP CHPLCxxCxxC complex structure.

The structure of the complex of VARP 692–746 with VPS29 was determined using a hybrid NMR/X-ray crystallographic approach. VPS29 and VARP residues 692–746 were produced in untagged forms as unlabelled, 15N-labelled or 15N,13C labelled versions. NMR signal assignments for the two free components (Supplementary Fig. 1a, b) and for the 1:1 complex were made using a suite of multinuclear 2D and 3D NMR experiments, in conjunction with samples having different isotopic labelling schemes. Structures were calculated using a combination of inter- and intra-molecular NOE-derived distance constraints for both molecules, J-coupling-derived χ1 angle constraints for VARP, and locally adjustable non-crystallographic symmetry (NCS) terms that maintain similarity to a fixed template structure of VPS29 derived from PDB 2R17 (Table 1). This approach is similar to that employed in a recent study of a protein-DNA complex28, and builds on previous related approaches for characterizing flexible multi-domain proteins and their complexes29,30; in this way we were able to incorporate direct knowledge of the previously known VPS29 domain crystal structure, while our NMR data provided the key information to determine the native of the interaction with VARP, where flexibility and weak binding pose problems for cryo-crystallography. Although an independent structure for VPS29 was not calculated during this study, analysis of secondary chemical shift and CSI (chemical shift index31) data showed that the crystal structure of VPS29 is maintained in solution and does not change appreciably upon the formation of the complex with VARP 692–746 (Supplementary Fig. 1c, d), which chemical shift perturbation analysis indicates binds to a patch on the central β-sheet of VPS29 (Fig. 2a, b).

The structure of the complex is shown in Fig. 2c, d. The only residues of VARP for which medium- or long-range restraints could be determined were 711 to 720 (CHPLCQQCPK) (Supplementary Fig. 1e, f). This region forms a tightly folded series of turns about a single Zn++ atom (Fig. 2c–e) in agreement with micro PIXE data19. The three intervening loops between the cysteines are highly conformationally restricted (Fig. 2e). The loop linking the first two cysteines adopts a specific conformation that presents its His Pro Leu residues (712–714) along with Gln716 to a largely hydrophobic patch formed by side chains from VPS29 Leu4, Leu25, Leu26, Lys30, Leu152, Tyr163 and Tyr165 on VPS29 (Fig. 3). There are also probably contributions to the interface from two well-conserved tyrosine residues on VPS2932, which hydrogen-bond to two backbone carbonyl oxygens on VARP: Tyr 165 OH (VPS29) – Pro 713O (VARP) and Tyr 163 OH (VPS29) – Cys 711O (VARP), the latter probably being water-mediated. Simultaneous mutation of the His and Leu residues from the loop in VARP to alanines abolishes binding to wt VPS29 in our SPR assay (Fig. 2e). We had previously postulated a role for VPS29 Leu152 in retromer
when it was fortuitously identified as forming a central part of a highly conserved surface-exposed hydrophobic patch of unknown function in mammalian VPS29, although in yeast Vps29p it appeared to be involved in interacting with a Vps5p/Vps17p dimer. The complex structure presented here provides a mechanistic basis for Leu152’s critical role in binding VARP and its mutation does indeed abolish binding (Fig. 3c, d). Mutations, L26S and Y165S, designed on the basis of the structure likewise abolish binding of VPS29 to VARP residues 692–746 without significantly affecting the folding of VPS29 (on the basis of circular dichroism and incorporation into retromer complexes in vivo—see later and Fig. 4).

The presence of the Zn++/4Cys constellation largely fixes the structure of the Zn-fingernail in solution, i.e., it is a conformationally restrained scaffold for displaying the His Pro Leu motif (Figs. 2e and 3c). This reduces the entropic penalty on binding to VPS29 that would occur if the interacting side chains were part of a mobile segment of polypeptide, explaining how the comparatively tight binding seen can be produced from a relatively small buried interaction surface of only ~600 Å² (Fig. 3b). In agreement with this, removal of the Zn++ or mutating all four cysteines, either of which actions would generate a structurally unconstrained peptide, causes a ~20-fold decrease in binding affinity (Fig. 1e).

**Fig. 1 The VARP:VPS29 interaction.** a Schematic representation of VARP with conserved cysteine motifs highlighted in yellow - designated Zn fingernails (see later). b Relevant sections of VARP with the conserved 4xCys motifs (yellow) and the His, Pro, Leu triplet motifs (red) highlighted. Residues identical between the two sequence sections are marked *. c K<sub>D</sub>s between short VPS29 and VARP residues 692–746 measured by SPR at five temperatures. d Van’t Hoff plot resulting from c of the interaction to estimate changes in binding enthalpy and entropy: the negative slope (ΔH < 0) demonstrates that the interaction is exothermic displaying favourable enthalpy and entropy changes (ΔH° ~ −20 kJ mol⁻¹ and ΔS° ~ +36 J K⁻¹ mol⁻¹). e, f Equilibrium analyses by SPR (e), and resulting K<sub>D</sub> (f), of short VPS29 binding to VARP immobilized on the sensor surface. In the absence of Zn+ either through mutation of cysteines or treatment with EDTA binding is reduced ~20 fold.
The Zn-fingernail. Zinc fingers are autonomously folding, molecular scaffold domains, in which zinc plays a structural role. They were first identified as DNA binding domains but are now known also to mediate interactions with DNA, RNA or protein. Zinc fingers can be subdivided into eight groups. The CHPLCxCxxC motifs resemble none of these to any significant degree: they are considerably smaller than any other reported zinc finger/zinc knuckle domain, being only ten residues in length; have no secondary structural elements; contain no hydrophobic core formed by residues between the cysteines; and are not buried or tightly associated with a protein surface but will instead protrude ~60 Å on ~20 residue unstructured linkers from the main body of VARP. We have therefore named this structure a Zn-fingernail.

Interrogation of the PDB found only a few autonomously folding microdomains of a similar size, none of which were metal-ion binding and none were of a similar structure. ALEPH software indicates that there are no regions of any known structure’s peptide backbone that have an r.m.s.d. less than 1.6 Å with the backbone of the Zn-fingernail and in those closest in structure to the Zn-fingernail, several of the side chains always point in very different directions and none bind metal ions. ALEPH also indicates that there are no structures in the PDB containing the pattern of four cysteines in CHPLCxCxxC that bear any structural resemblance to the Zn-fingernail. In bioinformatic searches of the existing non-redundant protein sequence database using CHPLCxCxxC, we did not detect any other meaningful sequence matches other than in VARP orthologues, although using the conserved pattern of cysteines alone revealed several hits corresponding to parts of larger multiple metal ion binding proteins including FeS centres and Metallothioneins. Hence, to the extent that primary sequence reflects secondary structure, it appears that the Zn-fingernail is itself unique and is unique to VARP.

The VPS29:VARP interaction in vivo. When transiently expressed in HeLa cells, VPS29-TagRFP harbouring mutations in the Zn-fingernail:VPS29 interface L26S and Y165S as well as L152E all colocalized with retromer (VPS35) on endosomes similarly to wt VPS29-TagRFP, indicating that they were correctly

### Table 1 NMR data collection and refinement statistics for the final set of 25 accepted structures.

| Structural restraints | Total | VPS29 | VARP |
|-----------------------|-------|-------|------|
| NOE-derived distance restraints | | | |
| Intraresidue | 33 | 39 |
| Sequential | 22 | 40 |
| Medium (2 ≤ |i−j| ≤4) | 24 | 17 |
| Long (|i−j| > 4) | 40 | 2 |
| Total | 119 | 98 |
| Intermolecular | 49 | | |
| Dihedral angle restraints | | None | 11 |
| Template non-crystallographic symmetry restraints for VPS29 (to pdb 2R17) | | | |
| Backbone | | | |
| Strong\(^a\) | Residues 7-19, 60-148 | | |
| Medium\(^b\) | Residues 1-6, 20-59, 149-181 | | |
| Sidechain | | | |
| Medium\(^b\) | Residues 1, 3-6, 20-24, 26, 28-29, 31-59, 149-151, 153, 155-160, 162, 164, 166-171, 173, 175-181 | | |
| Weak\(^c\) | Residues 2, 25, 27, 30, 152, 154, 161, 163, 165, 172, 174 | | |
| Structural statistics for accepted structures | | | |
| Number of accepted structures | 25 | | |
| Mean XPLOR-NIH energy terms (mean ± s.d., kcal.mol\(^{-1}\)) | | | |
| E\(^{\text{total}}\) | $-1694.0 ± 23.1$ | | |
| E\(^{\text{van der Waals}}\) | $175.8 ± 4.3$ | | |
| E\(^{\text{NCS}}\)\(^d\) | $153.8 ± 14.2$ | | |
| E\(^{\text{distance restraints}}\) | $39.8 ± 8.3$ | | |
| Restraint violations (mean ± s.d.) | | | |
| Distance ($\text{Å}$) | $0.081 ± 0.065$ | | |
| Dihedral angles ($^\circ$) | $0.99 ± 0.50$ | | |
| Max. distance restraint violation ($\text{Å}$) | $0.646$ | | |
| Max. dihedral angle violation ($^\circ$) | $2.00$ | | |
| RMS deviations from the ideal geometry | | | |
| Bond lengths ($\text{Å}$) | $0.0024$ | | |
| Bond angles ($^\circ$) | $0.68$ | | |
| Improper angles ($^\circ$) | $0.51$ | | |
| Average pairwise atomic rmsd (±s.d.) | | | |
| (N, C\(^\alpha\), C’ atoms) | $0.10 ± 0.03 \text{ Å}$ | $0.33 ± 0.12 \text{ Å}$ | |
| (All heavy atoms) | $0.21 ± 0.04 \text{ Å}$ | $0.69 ± 0.15 \text{ Å}$ | |

\(^a\)Force constant 100.0 kcal mol\(^{-1}\).
\(^b\)Force constant 2.0 kcal mol\(^{-1}\).
\(^c\)Force constant 0.1 kcal mol\(^{-1}\).
\(^d\)NCS energy terms are reported for the structures prior to addition of the unstructured tails of VPS29; no NCS terms are active during the final stage of the calculation when these tails are added.
\(^e\)Residues 1-181.
\(^f\)Residues 710-721.
folded (Supplementary Fig. 2a). However, all three of the mutations that abolished the interaction of VPS29 and VARP in vitro resulted in only cytosolic localization of VARP-GFP, which was lost upon cytosol extraction (by saponin treatment prior to fixation of the cells; Supplementary Fig. 2b). Trafficking of the transmembrane protein GLUT1 between endosomes and the cell surface can be used as a measure of retromer function19,20,37. Depleting VPS29 in HeLa cells resulted in increased colocalization of GLUT1 and the late endosomal/lysosomal membrane protein LAMP1 ((Fig. 4) and ref. 19), consistent with a block in recycling of GLUT1 from endosomal compartments to the plasma membrane. This could be rescued in transiently transfected HeLa cells expressing wtVPS29-tagRFP but not the L26S, Y165S and L152E mutant versions of VPS29-tagRFP (Fig. 4b).

The lack of rescue by the L152E mutant was consistent with our previous data18, but not with the subsequent observation by Jimenez-Orgaz et al.15, that expression of the L152E mutant was as effective in rescuing GLUT1 recycling as wild type protein in a VPS29 knockout cell line. In the course of these experiments, we noticed a subtle change in the pattern of some endosomal/lysosomal markers when expressing the L26S, Y165S and L152E mutant versions of VPS29-tagRFP but not wtVPS29-tagRFP (see LAMP1 in Fig. 4b, VPS35 and VARP-GFP in Supplementary Fig. 2b). A likely explanation is the inability of the VPS29 mutants to bind TBC1D5, since it has previously been observed that changes to endosome clustering and morphology occur when expressing the catalytically inactive TBC1D5 R169A/Q204A mutant (see Fig. 1 in ref. 38). We did not investigate further as the main focus of this work is not on TBC1D5.

The interaction of VARP with VPS29 is highly unusual, possibly unique, amongst transport coat protein networks, in that although mediated by a short linear motif with a folded domain as is normal (see ELM database elm.eu.org), the motif is actually part of a structurally defined fold (the Zn-fingernail) resulting in a decrease in entropy loss and hence relatively tight binding of 2–3 μM for the motif when compared with similar motifs such as Asp Pro Phe, Asp Pro Trp (binding AP2 α-adaptin appendage) and
Asn Pro Phe (binding EH domains) all of which consist of a proline flanked by a hydrophilic and a hydrophobic residues and have $K_D$s in the 100 μM range. Notably, conformational restraining of an Asn Pro Phe motif with a non-physiological disulphide link causes a ~10 fold reduction for its cognate EH domain in $K_D$ to ~10 μM. Similarly, removal of the Zn$^{++}$ ion destabilizes the VARP Zn-finger domain, thereby reducing its affinity to VPS29. However, it is possible to regain this affinity by adding back Zn$^{++}$ ions since in bio-layer interferometry it was shown that GST VARP 692–746 (finger nail 2) bound VPS29 prior to the EDTA treatment and following EDTA treatment and subsequently refolding into buffer containing Zn$^{++}$ with similar affinities (Supplementary Fig. 3a).

Comparison with other VPS29 binding partners. Whilst this study was underway, X-ray crystallography structures of VPS29 and VPS29/VPS35 C-terminal domain complexed with different ligands (Legionella infectivity factor RidL (PDB IDs 5WYH and 5OSI) and a small peptide fragment of the endosomal Rab7 GAP TBC1D5) were reported. Notably, these studies used a slightly longer isoform of VPS29 that has the N-terminal sequence MAGHRVLVL (referred to here as long VPS29) instead of MLVL; the latter was the originally reported isoform and is the one used in this work. Importantly, the affinities of either VPS29 isoforms for VARP were similar: $K_D$s were 1.3 and 2.7 μM respectively, with equivalently fast on-rates (Supplementary Fig. 3b).

Both RidL and TBC1D5 bind to the same site on VPS29 as do the VARP Zn-fingernails using proline and leucine/isoleucine residues, but neither possesses a Cys/Zn$^{++}$ cluster (Fig. 5a–c). Binding of the legionella infectivity factor RidL does not involve the additional MAGHR residues and has a comparatively high affinity ($K_D$ 200–400 nM) due to a large total interaction interface of 850–920 Å$^2$, consistent with its ability to outcompete VARP and TBC1D5 binding (Fig. 5b). The MAGHR residues however, are involved in binding of TBC1D5 to VPS29 (Fig. 5c). A short α-helix formed by residues upstream of the critical Asn Pro Leu motif in TBC1D5 provides a major part of the binding interface, packing against MAGHR residues. Using our SPR assay (Supplementary Fig. 3b), we measured a $K_D$ ~67 μM for the affinity of TBC1D5 (132–156) to the long VPS29, which is weaker than has been reported by ITC (residues 132–158: ~20 μM). The interaction of short VPS29 lacking MAGHR was too weak to assess reliably ($K_D > 300$ μM) likely due to factors including the interaction being mediated only by the Asn Pro Leu of TBC1D5.
(analogous to His Pro Leu in VARP (Fig. 5a)) contacting the Leu152-centred hydrophobic patch and the entropic penalty resulting from the absence of the conformationally restrictive 4Cys:Zn\(^{++}\) cluster present in VARP. However, the overall affinities of TBC1D5 for the retromer core complex containing either form of VPS29 will be enhanced by simultaneous binding to VPS35\(^{-42+-45}\) due to avidity effects. Tighter binding to VPS29 would likely render TBC1D5 constitutively attached to retromer, and this does not appear to be the case as some retromer positive tubes are TBC1D5 negative; VARP can readily compete with TBC1D5 for retromer binding (see next section); and Rab7:GTP-dependent retromer recruitment\(^{16}\) would be difficult as the GTP on the Rab7 would be immediately hydrolysed. Use of different relative amounts of the two versions of VPS29, for instance in different cell types, could bias the ratio of VARP to TBC1D5 in a given retromer coat, with various physiological outcomes, e.g., amount of Rab7 or VAMP7, resulting (see later).

**VARP and TBC1D5 binding in the context of retromer assemblies.** One Zn-fingernail can be accommodated on the surface of one Leu152-centred binding site on VPS29 (Fig. 3a, b). The recent cryo-electron tomography structures of Chaetomium thermophilum and Chlamydomonas reinhardtii retromer coats reveals the presence of VPS35 dimers forming ~100 Å high arches extending away from the membrane with two VPS29 molecules placed at each apex\(^{47}\) (Fig. 5d). The existence of arches has also been confirmed by SAXS and electron microscopy\(^{48-50}\). On a membrane, the ~80 Å separating the L152-centred binding sites on the VPS29s in a single arch (Fig. 5d) would easily allow simultaneous binding of the two Zn\(^{++}\)-fingernails protruding on their unstructured linkers from the starts of the two Ankyrin repeat domains of a single VARP molecule. Thus, by avidity effects, a VARP molecule may prefer to bind simultaneously to adjacent VPS29’s in a coat-assembled arch over isolated retromer VPS29/VPS35 heterotrimers. As well as binding to VPS29, VARP may also bind weakly to retromer via VPS35\(^{20}\) (the two possibilities are not mutually exclusive).

In line with this, using immunofluorescence microscopy VARP is seen on retromer and Rab7 positive endosomes and tubular structures protruding from them (Fig. 6a) and refs. \(^{16,19}\). Immunoelectron microscopy, which allows short, retromer- and VARP-positive tubules to be resolved from the endosomal body from which they emanate, suggests that the retromer and VARP are enriched on the tubular processes (Fig. 6b), where the retromer will be anchored through coincidence detection with cargo and SNX proteins\(^{51}\) and the VARP through binding to retromer arches. The presence of VARP in these retromer carriers will facilitate both the incorporation of VAMP7 into the carriers as well as the generation of Rab21:GTP on them for their subsequent docking and fusion to their target membranes\(^{21,22,24}\). The presence of VARP on the main bodies of endosomes (Fig. 6) can be accounted for by its ability to bind the late endosomal...
Fig. 5 Ligand binding to VPS29 in context of whole retromer complex. a Structure-based alignment of VPS29 binding sequences from vertebrate VARP fingernails, TBC1D5 and Legionella RidL (C to N direction as in peptide complex structures). The key binding residues are boxed in red and the cysteines in VARP boxed in yellow. b Superposition of RidL peptide ligand (green) with VARP Zn-fingernail (N pale cyan) to C (dark cyan) binding at the same hydrophobic site (PDB ID 5WYH). c Superposition of TBC1D5 peptide (PDB ID 5GTU) N (pale purple) to C (dark purple) bound to longer N-terminal isoform of VPS29 and VARP Zn-fingernail (N pale cyan to C dark cyan). The ligands bind to the same site on VPS29 with the Pro and Leu residues from both superimposing well. The extended VPS29 N-terminus including the important binding residues -3 to 0 pack against an α-helix from TBC1D5. d, e views of a retromer coat arch formed by two VPS26(green)/Vps29(red)/VPS35(gold) heterotrimers shown parallel to (d) and looking down towards (e) the endosomal membrane. The distance between VPS29 binding sites is ~80 Å allowing the Zn-fingernails from one VARP to easily bind to a single arch.

Fig. 6 VARP is enriched on endosomal tubular/vesicular carriers. a Immunofluorescence wide field microscopy images of HeLaM cells stably expressing VARP-GFP (green) showing colocalization with Rab7a (red) and VPS35 (blue) on puncta and tubules (arrow heads). Scale bar 20 μm. b Immunoelectron micrograph showing colocalization of VPS35 and VARP-GFP on tubules emanating from a multivesicular body in a HeLaM cell stably expressing VARP-GFP. Scale bar, 200 nm. c Immunofluorescence wide field microscopy images of a mixed population of HeLaM cells, some stably expressing VARP-GFP. Cells expressing VARP-GFP show a reduction in punctate labelling of TBC1D5 and increase in labelling of Rab7a. Scale bar 20 μm.
Rab family member, Rab32:GTP\(^{19,23}\), to free endosomal VAMP7 and likely also to ‘unpolymerised’ membrane-attached, VPS26/VPS29/VPS35 heterotrimers.

The Rab7 GAP TBC1D5 is also a ligand of retromer, but will have little preference for arch-assembled retromer over unpolymerised cytosolic or membrane-associated retromer as it binds to both VPS29 and VPS35 simultaneously in the same retromer heterotrimer\(^{49}\). By immunofluorescence microscopy TBC1D5 is found on retromer-positive endosomes and some of their tubular projections (Supplementary Fig. 3c)\(^{16,19}\). Mild ectopic over-expression of VARP-GFP such that the amount of VARP-GFP + VARP in the mixed stable cell population is <2 fold that of VARP alone in untransfected cells, causes TBC1D5 to be largely displaced from endosomal membranes with a concomitant increase in levels of membrane-attached Rab7 (Fig. 6c) due to removal of the TBC1D5’s Rab7 GAP activity, consistent with a competition between the two proteins for the VPS29-binding pocket (Fig. 5c). This competition occurs despite there being <50-fold excess of retromer core subunits over both VARP and TBC1D5\(^{52}\), which implies that there should be many VPS29 proteins that are ligand-free. One possible explanation is that the in vivo situation may thus be more complex than current models suggest: nevertheless, the data presented here show that VARP can compete with TBC1D5 in vivo for the pool of retromer that is assembled into a coat on endosomes. Regardless of precise mechanism, these data indicate that the ratio of bound VARP vs. TBC1D5 is finely balanced such that even a slight perturbation can cause an obvious in vivo effect. In this context, it is worth noting the differences amongst previous reports with regard to whether modulation of TBC1D5 has an effect on retromer-dependent recycling of plasma membrane proteins. Whereas both we\(^{18}\) and Jimenez-Orgez et al.\(^{15}\) found no effect of depleting TBC1D5 in mammalian cells on GLUT1 recycling, an effect on integrin recycling has been reported\(^{10}\) and the effect of modulating autophagy on GLUT1 recycling has been proposed to be mediated via redistribution/shuttling of TBC1D5\(^{53}\). In addition, both VPS29 and TBC1D5 have been implicated in synaptic vesicle recycling and synaptic transmission in Drosophila, an experimental model in which, as in our experiments, only wild type and not the L152E mutant of VPS29 was able to rescue the effect of VPS29 knockout\(^{54}\).

Access of the VPS9 ANKRD1 (Rab21GGEF) and the ANKRD2 domains of VARP to membrane-associated VAMP7 and Rab21 will be facilitated by the unstructured (lack of medium or long range restraints (Supplementary Fig. 1c, d) and a lack of predicted secondary structure\(^{26}\) links on the Zn-fingernails. Efficient VARP recruitment into retromer-based coats is easiest to envisage occurring as the retromer coat assembles: either if the VARP is already dynamically localized to the endosome surface through transient interactions with its membrane-associated binding partners and it ‘reaches up’ to the arches or if it docks from the cytosol and ‘reaches down’ to its partners (Fig. 7a).

**Molecular evolution of the VPS29 interaction platform.** VARP, VPS29 and TBC1D5 are all ancient proteins, present in the Last Eukaryotic Common Ancestor\(^{33,55,56}\). However, co-occurrence need not necessarily indicate interaction. Orthologues of each protein were sampled across the relevant taxonomic range (Fig. 7, Supplementary Fig. 4, Supplementary Table 1) and examined for the presence of the key biochemical determinants identified above, enabling an evolutionary reconstruction of the VPS29 interaction platform.

While VARP orthologues are present across eukaryotes, the Zn-fingernails and the stacked Ankyrin repeats, the latter binding VAMP7 and Rab32 are not\(^{56}\). Some fungal ‘VARP proteins’ possess a single set of ankyrin repeats but without the Zn-fingernail C-terminal to the VPS9 domain, suggesting that this domain organization existed in the VARP orthologue present in the ancestor of animals and fungi (i.e. the Opisthokonta) (Fig. 7, Supplementary Fig. 4). Nonetheless, VARP sequences from Salpingoeca rosetta and Captagora ovazuzuki possess the double Zn-fingernail-Ankyrin Domain architecture, suggesting that this was already present in the ancestor of the Holozoa (Fig. 7, Supplementary Fig. 4). In VPS29 proteins, the critical VARP and TBC1D5 binding residues Leu26 and Leu152, are highly conserved in diverse eukaryotes (Supplementary Fig. 4). Finally, in TBC1D5, the VPS29 binding Asn Pro Leu motif is also relatively well conserved (occasionally His Pro Leu or Asp Pro Leu (Supplementary Fig. 4) across eukaryotes, and so these three last biochemical determinants can be reconstructed as present in LECA.

We therefore propose an evolutionary model for the VARP-TBC1D5-Retromer regulatory network (Fig. 7b–e). In LECA (Fig. 7b), VPS29 likely interacted exclusively, albeit weakly, with TBC1D5 that acted as the Rab7GAP. Rab7 and VAMP7 were present in LECA\(^{57–59}\) – the former acting in late endosomal function and thus being inactivated by TBC1D5, and the latter likely acting to promote fusion of the tubule with the plasma membrane. A VARP-like protein would have acted as a GEF on Rab21, on internal membranes and the plasma membrane but likely independently from retromer\(^{60}\). In the opisthokont ancestor (Fig. 7c), however, a single set of Ankyrin repeats had been acquired in VARP, which allows for direct interaction with VAMP7, and potentially indirectly with retromer. Between the opisthokont and holozoan ancestor, the Zn-fingernail was acquired and the Zn-fingernail-Ankyrin domain unit most likely duplicates, establishing the modern holozoan and animal configuration of VARP (Fig. 7d).

By providing selective pressure to fix the relevant residues in VPS29, the VPS29:TBC1D5 interaction from the LECA onward essentially facilitated the much later emerging VPS29:VARP interaction at the Zn-fingernails, since only the relevant residues in VARP would need to evolve for the interaction to evolve. This sets up potential competitive binding between VARP and TBC1D5 demonstrated in vivo in the previous section, which in turn could influence retromer-based carrier generation dynamics and thus be a pre-adaptation for the development of complex endosomal recycling pathways in multicellular animals. In vertebrates, the binding of TBC1D5 is enhanced by the evolution of the long splice form of VPS29, potentially shifting the balance of binding to TBC1D5 again.

In summary, in mammalian cells, a VARP molecule can interact with two VPS29’s, likely on a single retromer arch through insertion of an [His Asn Asp]Pro Leu motif, whose presentation is defined by a unique 10 residue Zn\(^{++}\)-stabilised structure, into a hydrophobic pocket on VPS29. VARP and TBC1D5, which can compete for binding to the same site on VPS29, play key roles in coordinating endosomal recycling dynamics in animal cells by integrating Rab21 and Rab7 function to generate an appropriate Rab complement on retromer-coated tubes so as to prepare them for fusion with/integration into the plasma membrane or earlier endosomes\(^{61–63}\). VARP and TBC1D5 do this by binding to a common site on VPS29 that likely arose initially for TBC1D5 binding. Over the course of the lineage that gives rise to animals and fungi, and then animals themselves, VARP began interacting increasingly with members of the retromer regulatory network, reaching the highly integrated state observed today. VARP additionally affects trafficking within the endocytic system through being an effector for Rab32\(^{19,23,51}\) and ensures efficient recruitment of a SNARE, VAMP7, into

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retromer-based carriers\textsuperscript{21,22,25} that can drive their ultimate fusion with their target membranes. Understanding the molecular basis of VARP’s binding to retromer is key to understanding this protein’s role in membrane trafficking.

Methods

Protein expression and purification. All constructs used are summarised in Supplementary Table 1. Zn-fingernails were expressed as N-terminal, prescission-cleavable GST fusion protein from pGEX6P1 in BL21 (DE3) pLysS E. coli. Expression was carried out in media supplemented with 0.5 μM ZnCl\textsubscript{2} at 22 °C for

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**Fig. 7 Model and evolution of the VPS29/VARP/TBC1D5 interactions.** a Model depicting possible assemblage of VARP, TBC1D5 and their functional partners onto endosomal membrane-attached retromer arches. b–d Hypothesized cellular configuration of VARP, TBC1D5, and VPS29 and associated endosomal partners (coloured as in key) in three reconstructed ancestral nodes. Reading anticlockwise, in the LECA (b), Retromer interacts with Rab7 mediated by TBC1D5, while Varp interacts with Rab21 at the endosome and Plasma membrane. In the opisthokont ancestor (c), the addition of the ankyrin repeats allows for possible interaction of Varp with Vamp7. By the time of the holozoan ancestor (d), all of the relevant domains (including the two Zn-fingernails) had been added to VARP to compete for Vps29 with TBC1D5 using the same conserved motif providing for direct interaction with retromer.

e Proposed origin timing of the VPS29 Leu26, Leu152, and Tyr165 residues, the VARP Zn-fingernail, and the TBC1D5 AsnProLeu motif in eukaryotes. This schematic of eukaryotic relationships, with emphasis on the lineages leading to metazoan shows the acquisition points of proteins (bold) and motifs/residues at relevant nodes. See Supplementary Fig. 4 for supporting alignments.
16 h following incubation with 0.2 mM IPTG at OD_{600} of 0.8. For expression of isotopically labelled proteins, 2TY 10 ml starter cultures at OD_{600} of 0.8 were pelletted at 10000 g and resuspended in M9 minimal media (enriched only with 15NH_{4}Cl (0.5 g/L) or with both 15NH_{4}Cl (0.5 g/L) and 13C_{6} glucose (2 g/L)) (and, 0.5 μM ZnCl_{2} for Zn-fingered). Expression was carried out for 18–20 h at 28 °C following incubation of addition of 0.2 mM IPTG at OD_{600} of 0.8. For expression of proteins purified in 20 mM Tris, 100 mM NaCl, pH7.4, 0.2 mM βME, 0.2 mM AEBSS on GST-Sepharose, Precipitation cleavage was carried out overnight at 4°C with needed with 0.5 mg Precipitation Protease. Eluted proteins were further purified on 750 Superdex gel filtration in the same buffer. Samples for NMR were subsequently buffer exchanged into 20 mM Tris buffer pH 7.0, 200 mM NaCl, 1 mM H_{4} DT, either in a 95:5 H_{2}O:H_{2}O mixture or in H_{2}O, using a VivaSpin 3000 mwco spin filter. In the latter case, buffer exchange was repeated five times with a 3–5 fold dilution so as to reduce the level of H_{2}O from the original buffer to <0.5%. VPS29 was expressed from pGEX2T2 and purified as described above except that cleavage was carried out with thrombin.

**SPLAR analysis.** Experiments were performed on a Biacore T200 (GE Healthcare Life Sciences). In the first instance, goat anti-GST polyclonal antibody was coupled to Series S CM5 Sensor Chips (GE Healthcare Life Sciences, catalog no. BR-1003-49) by the amine coupling protocol specified in the Biacore T200 Control Software with reagents purchased from GE Healthcare Life Sciences. In brief, after the sensor surface was activated by applying a 1:1 mixture of 50 mM N-hydroxysuccinimide sodium acetate, pH 5.0 (GE Healthcare Life Sciences, catalog no. BR-1003-49) and injected with all flow cells and injected for 7 minutes. Every binding experiment consisted of three sequential binding experiments were therefore performed on each chip in 10 mM glycine-HCl pH 2.1 at 30 °C, the binding is therefore characterized by favorable enthalpy and entropy (∆H < 0.91). The equation assumes that the enthalpy is independent of temperature (∆H = constant-temperature). Although VPS29 binding in the presence of 10 mM EDTA was given similar binding properties and equilibrium constants.

For H_{2}O samples of 15N,13C-labelled free VSP29 the following datasets were acquired: 2D datasets: [15N-1H] HSQC, [13C-1H] HSQC (aliphatic region in 13C), [13C-1H] HSQC (aromatic region in 13C), constant-time [13C-1H] HSQC (aliphatic region in 13C), constant-time [13C-1H] HSQC (aromatic region in 13C), 3D datasets: CBCANH, CBCA(CO)NH, HNCA, HN(CO)CA, HBCA(NH), HBCA(CO)NH, [1H-15C] HHCCH-COSY, [1H-15C] HCCCH, [1H-15C] HCCCH, [1H-15C] HCCCH, [1H-13C] HCCCH, [1H-13C] HCCCH, [1H-13C] HCCCH. For 2H_{2}O samples of 15N,13C-labelled VSP29 the following datasets were acquired: 2D datasets: [1H-15C] HCCCH (full spectral width in 13C), constant-time [1H-15C] HSQC (aliphatic region in 13C), constant-time [1H-15C] HSQC (aromatic region in 13C), [1H-15C] NOESY-HSQC (r = 50 ms; and 120 ms; aromatic region in 13C), [1H-15C] HCCCH (aromatic region in 13C), constant-time [1H-15C] HSQC (aromatic region in 13C), [1H-15C] NOESY-HSQC (t = 50 ms), [13C-1H] HSQC (t = 150 ms; aromatic region in 13C). For H_{2}O samples of complex containing 1:1 15N,13C-labelled VSP29 and natural abundance VARP 692–746 the following datasets were acquired: 2D datasets: [15N-1H] HSQC, [13C-1H] HSQC (aliphatic region in 13C), [13C-1H] HSQC (aromatic region in 13C), constant-time [1H-15C] HSQC (aliphatic region in 13C), constant-time [1H-15C] HSQC (aromatic region in 13C), [1H-15C] NOESY-HSQC (r = 50 ms; and 120 ms; aromatic region in 13C), [1H-15C] NOESY-HSQC (r = 50 ms), [13C-1H] HSQC (t = 150 ms; aromatic region in 13C). For H_{2}O samples of complex containing 1:1 15N,13C-labelled VSP29 and natural abundance VARP 692–746 the following datasets were acquired: 2D datasets: [15N-1H] HSQC, [13C-1H] HSQC (aliphatic region in 13C), [13C-1H] HSQC (aromatic region in 13C), constant-time [1H-15C] HSQC (aliphatic region in 13C), constant-time [1H-15C] HSQC (aromatic region in 13C), [1H-15C] NOESY-HSQC (r = 50 ms; and 120 ms; aromatic region in 13C), [1H-15C] NOESY-HSQC (r = 50 ms).
containing 1:1 natural abundance VS29 and 13C,15N-labelled VARP 692–746, the following datasets were acquired: 2D datasets: [13C-1H] HSQC (aliphatic region in 13C), [13C-1H] HMQC, [13C,15N-1H] HMBC, and assignment spectra for N, Cα, and NCS; [13C-1H] HCCCH-TOSY, [13C] NOESY-HSQC (τm = 50 ms, 70 ms and 120 ms; aliphatic region in 13C), [13C] NOESY-HSQC (τm = 70 ms; aromatic region in 13C).

All of the NOESY datasets used for structure calculations (see below) were acquired using pulse sequences modified to ensure equal RF heating in each case, e.g., for 13C experiments, a period of 2N decoupling equal in length to the acquisition period was applied at the beginning of the inter-scan delay, and for 15N experiments an equivalent period of 12C decoupling was similarly applied. All spectra were processed using the program TOPSPIN versions 3.1 and 3.2 (Bruker GmbH, Karlsruhe) and analysed using the program CCPN analysis.

**Shift perturbation analysis.** Backbone amide group chemical shift perturbations were calculated for VPS29 and for VARP 692–746 using the assignments described above using the formula Δδ = (Δδ(1H))2 + (Δδ(15N))2/5.0.

**Structure calculations.** Structural models of the VPS29-VARP complex were generated using a hybrid NMR/X-ray crystallographic approach, using simulated annealing calculations run with the program XPLOR-NIH60. The conformation of the majority of the VPS29 component was restrained to a template conformation adapted from a previously published X-ray structure (see below), while the conformation of the VARP peptide and interfacial residues of VPS29 (selected on the basis of preliminary structures) were allowed to evolve under a combination of intermolecular and intra-peptide NOE-derived distance restraints, as well as limited NOE-derived restraints for the interfacial region of VPS29 and J-coupling-derived χ1 dihedral angle restraints for the interfacial region of the VARP peptide. The NOE restraints of VARP were classified into very strong (0–2.3 Å), strong (2.3–2.9 Å), medium (3.5–3.9 Å) and weak (5.0–6.0 Å) intensity categories. The upper distance bounds used for these categories were calibrated using assigned NOE cross-peaks in the unfiltered NOE spectra of samples in which the VPS29 component was labelled, specifically, the intensity corresponding to the very strong category (0–2.3 Å) was set using sequential δDNOE peaks in regions of regular anti-parallel β-sheet, that of the strong category (0–2.9 Å) was set using sequential δDNOE peaks in regions of regular α-helix, that of the medium category (0–3.5 Å) was set using (i, i + 3) δDNOE peaks in regions of regular α-helix, and those in the weakest category were set to be consistent with the expected approximate detectability limit for NOEs, set as 5 Å. This calibration was used for the filtered versions of the NOE-derived NOE restraints for these categories. For each sample, the upper bounds for these restraints were either tightened or loosened slightly; it was found that tightening them caused a significant increase in their violations, whereas loosening had relatively little effect since violations were already very few.

The VPS29 template structure was derived from residues 1–181 of the VPS29 component of the VPS29-VS29 complex in pdb 2R17,66 with all seleno-Met residues changed to Met and the 11–12, 40–41 and 91–92 peptide bonds set to the cis conformation; this structure was selected since it lacks the protruding conformation of helix 2 (95–108) see in pdb 1Z2X67 that makes a crystal contact to its cis conformation in a symmetry-related molecule and which is inconsistent with NOE data from the present study68. The Leu40-Cys41 peptide bond was modelled in the cis conformation because (i) in the highest resolution structure of VPS29 (pdb 5GTU) this peptide bond was found to be cis69, and (ii) modelling the trans conformation for Leu40-Cys41 led to consistent, severe outliers in the Ramachandran statistics for this region both in pdb 2R17 and in trial calculations for our system. The protein co-ordinates from 2R17 were adapted as follows: Hydrogen atoms were first added according to standard geometries, then all atoms except for the guanidinium protons of all arginines and all arginines within 5 Å of residues 40 and 41 were fixed and the structures were then subjected to Powell energy minimization (1000 steps) using rigid body oligonucleotide (DNAlike), increased (Dna-like), (Dna-like)*, non-targeted (NT) control (D-001810-01) and VPS29-1 (J-009764-09). For VPS29 ‘rescue experiments’ and colocalization studies, cells were seeded on glass coverslips and transfected with siRNA-resistant VPS29-TagRFP constructs 24 h after the second knock-down using FuGENE®6 Transfection Reagent (Roche) according to the manufacturer’s protocol. All cells were either fixed with 4% formaldehyde in PBS for 1 h, or incubated for 2 h in fresh cell culture medium prior to fixation (VPS29 ‘rescue experiments’). Knock-down of VPS29 (with actin as a control and over-expression of VARP-GFP were assessed by immunoblotting using primary antibodies to VPS29 (1:500 Ab108216, Abcam) and secondary antibodies to IgG (1:1000 anti-goat Ab96938, Abcam and anti-rabbit 926-32221, LI-COR Biosciences).

For immunolocalization confocal microscopy, cells were fixed with 4% paraformaldehyde in PBS for 10 min. Cells were then permeabilized by incubation with 0.1% (v/v) Triton X-100 in PBS for 5 min and incubated with blocking solution (5% BSA in PBS) for 30 min. This was followed by incubation with primary antibodies used at 1:250 dilution (VPS35, mAb B-5, Santa Cruz; GLUT1, Glut1).

**Mammalian cell culture, siRNA-mediated gene knock-down microcopy.** HeLa or HeLaM cells stably expressing VARP-GFP70 cultured under standard conditions in RPMI supplemented with 10% (vol/vol) FBS, 2 mM HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin. siRNA-mediated gene knock-down was performed as described before19. In short, cells were transfected (two transfections separated by 48 h) with 100 nM oligonucleotide using Oligo-Fectamine (Life Technologies), according to the manufacturer’s protocol. On- and off-target oligonucleotides (DNAlike, Dna-like, Dna-like*) were: non-targeting (NT) control (D-001810-01) and VPS29-1 (J-009764-09). For VPS29 ‘rescue experiments’ and colocalization studies, cells were seeded on glass coverslips and transfected with siRNA-resistant VPS29-TagRFP constructs 24 h after the second knock-down using FuGENE®6 Transfection Reagent (Roche) according to the manufacturer’s protocol. All cells were either fixed or incubated for 2 h in fresh cell culture medium prior to fixation (VPS29 ‘rescue experiments’). Knock-down of VPS29 (with actin as a control and over-expression of VARP-GFP were assessed by immunoblotting using primary antibodies to VPS29 (1:500 Ab10160, Abcam), actin (1:3000 Ab2866, SigmaAldrich), VARP (1:500 Ab108216, Abcam) and secondary antibodies to IgG (1:1000 anti-goat Ab96938, Abcam and anti-rabbit 926-32221, LI-COR Biosciences).

For immunolocalization confocal microscopy, cells were fixed with 4% paraformaldehyde in PBS for 10 min. Cells were then permeabilized by incubation with 0.1% (v/v) Triton X-100 in PBS for 5 min and incubated with blocking solution (5% BSA in PBS) for 30 min. This was followed by incubation with primary antibodies used at 1:250 dilution (VPS35, mAb B-5, Santa Cruz; GLUT1, Glut1).
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**Additional information**

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