A non-canonical GTPase interaction enables ORP1L-Rab7-RILP complex formation and late endosome positioning

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Endosomal transport represents the primary mode for intracellular trafficking and signaling transduction and thus has to be tightly controlled. The molecular processes controlling the endosomal positioning utilize several large protein complexes, one of which contains the small GTPase Rab7, Rab-interacting lysosomal protein (RILP), and oxysterol-binding protein–related protein 1 (ORP1L). Rab7 is known to interact with RILP through a canonical binding site termed the effector-interacting switch region, but it is not clear how Rab7 interacts with ORP1L, limiting our understanding of the overall process. Here, we report structural and biochemical investigation of the Rab7-ORP1L interaction. We found that, contrary to prior studies, the interaction between Rab7 and the N-terminal ankyrin repeat domain (ARDN) of ORP1L is independent of Rab7’s GTP- or GDP-binding protein–related protein 1 (ORP1L). Rab7 is known to interact with RILP through a canonical binding site termed the effector-interacting switch region, but it is not clear how Rab7 interacts with ORP1L, limiting our understanding of the overall process. Here, we report structural and biochemical investigation of the Rab7-ORP1L interaction. We found that, contrary to prior studies, the interaction between Rab7 and the N-terminal ankyrin repeat domain (ARDN) of ORP1L is independent of Rab7’s GTP- or GDP-binding state. Moreover, we show that Rab7 interacts with ORP1L ARDN via a unique region consisting of helix3 (α3) and 3_10-helix 2 (η2). This architecture leaves the canonical effector-interacting switch regions available for RILP binding and thus allows formation of the ORP1L-Rab7-RILP tripartite complex. Mutational disruption of the interacting interface between ORP1L and Rab7 compromised the ability of ORP1L-Rab7-RILP to regulate the late endosome positioning. Collectively, our results again manifested the versatility in the interaction between GTPase and its effector.

The protein family of Rab small GTPase contains key regulators of intracellular trafficking processes (1). For example, Rab7 plays a central role in early-to-late endosome conversion, late endosome-lysosome fusion, and transport of late endosomes (LEs) along microtubules (2–6). Like various other Rabs, Rab7 cycles between a GTP-bound active state (GTP-Rab7) and a GDP-bound inactive state (GDP-Rab7). This cycle is controlled by a guanine nucleotide exchange factor complex and a GTPase-activating protein. GTP-Rab7 localizes to late endosomes where it interacts with distinct Rab7 effectors to control the late endosome–related events. For example, GTP-Rab7 interacts with Rab-interacting lysosomal protein (RILP), which forms a complex with dynein-dynactin motor and thus drives movement of late endosomes toward microtubule minus ends (2, 3, 7, 8). Alternatively, GTP-Rab7 interacts with FYVE and coiled-coil domain–containing protein 1 to drive transport of late endosomes toward microtubule plus ends (4).

Recent reports revealed that late endosomal positioning is regulated by the cholesterol levels in the late endosomes. The cholesterol level in the late endosomes is sensed by oxysterol-binding protein–related protein 1 (ORP1L) (9). ORP1L contains an N-terminal ankyrin repeat domain (ARD), a pleckstrin homology domain (PHD), a FFAT motif (two phenylalanines (FF) in an acidic tract), and a C-terminal oxysterol-binding protein (OSBP)-related domain (ORD). When cholesterol molecules in the late endosomes are present at high levels, ORP1L binds to cholesterol through the ORD and adopts a conformation that enables the interaction of ARD in ORP1L with the Rab7-RILP complex. The latter further recruits the dynein-dynactin complex, resulting in a clustering of late endosomes at the microtubule-organizing center. When cholesterol molecules are present at low levels, ORP1L fails to bind cholesterol, thus allowing exposure of the FFAT motif, which interacts with the endoplasmic reticulum (ER)–resident vesicle-associated membrane protein (VAMP)-associated protein (VAP). As a consequence, the dynein-dynactin complex dissociates from Rab7-RILP, facilitating the

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This article contains Figs. S1–S4.

The atomic coordinates and structure factors (codes 5Z2N and 5Z2M) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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4 The abbreviations used are: LE, late endosome; ER, endoplasmic reticulum; RILP, Rab-interacting lysosomal protein; ORP1L, oxysterol-binding protein; ARD, ankyrin repeat domain; mARD, mouse ARD; ORD, oxysterol-binding protein (OSBP)-related domain; PHD, pleckstrin homology domain; FFAT, two phenylalanines (FF) in an acidic tract; VAP, vesicle-associated membrane protein (VAMP)-associated protein; hORP1L, human ORP1L; WDR91, WD repeat domain 91; HEK, human embryonic kidney; ITC, isothermal titration calorimetry; mORP1L, mouse ORP1L; ANK, ankyrin; GST, GSH S-transferase; P14KIIβ, phosphatidylinositol 4-kinase; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
formation of ER–LE membrane contact sites and distribution of LEs at the cell periphery (9).

The interaction between Rab7 and RILP has previously been studied using X-ray crystallography. Analysis of the Rab7-RILP structure at high resolution showed that GTP-bound Rab7 interacts with RILP using its “effector-interacting switch region” and Rab subfamily motifs 1 and 4 (10). These effector-interacting switch regions represent canonical binding sites recognized by most Rab effectors (11–13). However, little structural detail is established as to how ORP1L and RILP simultaneously interact with Rab7 during cholesterol-dependent positioning of late endosomes. Here, we set out to identify the molecular details of this mechanism by performing both structural and biochemical analyses of ORP1L-Rab7-RILP complex. Our structural data clearly show that ORP1L ARD\textsubscript{N} binds to a noncanonical region of Rab7, which is positioned away from the effector-interacting switch region, thus enabling Rab7 to bind both ORP1L and RILP in a simultaneous manner.

Results

**Human ORP1L (hORP1L) ARD\textsubscript{N} binds to both GTP- and GDP-bound Rab7**

GTP is crucial for the interactions between Rab7 and its downstream effectors. ORP1L was reported to preferentially bind to GTP-Rab7 in mammalian two-hybrid assays (8). To test whether GTP is required for the interaction between hORP1L and Rab7, we used dominant active mutant and dominant negative mutant of Rab7 and performed coimmunoprecipitation assays. According to previous reports, the dominant active mutant, Q67L, specifically binds to GTP and exhibits a much slower rate of GTP hydrolysis compared with the WT form. In contrast, the dominant negative mutant, T22N, exhibits higher binding affinity for GDP than GTP (2, 14, 15). For example, the interaction between T22N and WD repeat domain 91 (WDR91) or Rubicon was barely detectable (5, 16). However, we found that full-length hORP1L coprecipitated with both Q67L and T22N in HEK 293 cells (Fig. 1A), although the amount of the hORP1L protein precipitated by Q67L was higher than that precipitated by T22N.

We next defined the core regions in hORP1L necessary for its interaction with Rab7. It was reported that the ARD of hORP1L binds to a noncanonical region of Rab7, which is positioned away from the effector-interacting switch region, thus enabling Rab7 to bind both ORP1L and RILP in a simultaneous manner.
we separated ARD into two regions, ARDN (ORP1L(1–136)) and ARDC (ORP1L(141–237)), and tested their interactions with Rab7. Our pulldown assay results clearly showed that ARDN, but not ARDC, is required for the interaction between hORP1L and Rab7 (Fig. 1B). As shown in Fig. 1C, the in vitro interaction between recombinant ARDN and Rab7 is independent of the GTP- or GDP-binding states of Rab7. To determine the binding affinity between ARDN and Rab7, we performed an isothermal titration calorimetry (ITC) assay. As shown in Fig. 1D, the dissociation constants of ARDN binding to Rab7-WT-GTP, Rab7-Q67L-GTP, and Rab7-T22N-GDP were calculated as 3.2 \( \pm \) 0.3, 3.5 \( \pm \) 0.6, and 2.5 \( \pm \) 0.3 \( \mu \)M, respectively. These in vitro results showed that hORP1L binds to Rab7 using ARDN and that their interaction is independent of the nucleotide-binding states of Rab7.

**Structure of mouse ORP1L (mORP1L)-ARDn and its complex with Rab7**

To determine the structure of ARDN and Rab7 at high resolution, we expressed and purified the ARDN of mORP1L and N-terminal residues 1–176 of dominant active mutant Rab7-Q67L, which binds to ORP1L. The identity of mORP1L and hORP1L in the ARDN region is 86.76%, suggesting that the structures of hORP1L and mORP1L are identical. The elution volumes of the single proteins and their complex in a Superdex 75 gel filtration column suggest that the monomeric ARDN and Rab7 form heterodimers in solution (Fig. 2A).

Both ARDN and the ARDN-Rab7 complex were crystallized. The crystal structure of ARDN was determined at 2.14 Å using the molecular replacement method (Fig. 2B). Comparable with canonical ankyrin repeat domain structures, the structure of ARDN is composed of four tandem ankyrin repeats (ANK 1–4). Each ANK repeat contains a pair of antiparallel \( \alpha \)-helices. The ANK repeats pack side by side and constitute a concave structure with the first helices of the ANK repeats (H1, H3, H5, and H7) in the inner side. The loops between ANK repeats fold into \( \beta \)-hairpin structures, known as Fingers 1–3 in this structure (19). The fingers stretch out perpendicularly to the inner side of the concave structure.

In addition, we determined the structure of the ARDN-Rab7 complex at 2.14 Å (Fig. 2C). Different from the reported ARD of the VPS9 domain ankyrin repeat protein, which uses its convex side to bind the switch regions of Rab32 (20), the ARDN of ORP1L binds to Rab7 using the inner side of its concave structure, involving Finger 1 and the four inner \( \alpha \)-helices (H1, H3, H5, and H7). The ARDN-binding site in Rab7 consists of \( \alpha \)3, \( \eta \)2,
and the loop between them, which is located away from the conventional effector-binding switch regions (Fig. 2C). As shown in Fig. S2, we found a conformation change of Finger 1 in ARDN through comparison of the structures of ARDN alone and ARDN-Rab7. The buried interface area between ARDN and Rab7 is approximately 680 Å² as calculated by PISA (Proteins, Interfaces, Structures and Assemblies) (21). As the ARDN-binding site in Rab7 is located away from its nucleotide-binding pocket (Fig. 2C), this structure is consistent with our hypothesis that ARDN-Rab7 interaction is nucleotide-independent.

To verify the interaction between ARDN-Rab7 and RILP, we performed pulldown assays using purified GSH S-transferase (GST)-tagged ARDN (GST-ORP1L ARDN), His-tagged RILP(241–320), and His-tagged Rab7. As shown in Fig. 2D, in the presence of GTP-bound Rab7, RILP binds to ARDN. To build a model for the ORP1L-Rab7-RILP ternary complex, we superposed the structures of ARDN-Rab7 with Rab7-RILP (Protein Data Bank (PDB) code 1YHN) (10) based on the structure of Rab7 (Fig. 2E). In this model, two ARDN-Rab7 heterodimers were bridged by an RILP homodimer, forming an X-shaped structure with C2 symmetry. Because ARDN and RILP interact with Rab7 at different positions, this model provides structural explanations for the fact that ORP1L and RILP simultaneously interact with Rab7 (3).

**The interaction between ARDN and Rab7**

To study the amino acids required for ARDN-Rab7 heterodimer formation, we analyzed the structure of ARDN-Rab7 complex, mutated the crucial amino acids in the protein-protein interface, and performed GST pulldown assays. As shown in Fig. 3, A and B, Trp-49 (Finger 1), Tyr-57 (H3), Phe-58 (H3), and Phe-90 (H5) in ARDN constitute a hydrophobic region in the inner face of the concave structure. The aromatic groups in these residues interact with the side chains of Ile-108 and Pro-115 in Rab7. In addition, the positive-charged side chains of Arg-14, Lys-44, and Arg-93 in ARDN interact with the negative-charged side chains of Asp-104, Glu-105, and Glu-116 in Rab7, respectively (Fig. 3A). As shown in Fig. 3A, hydrogen bonds formed between Phe-90, Arg-87, and Tyr-57 in ARDN and Arg-113, Ser-111, and Pro-112 in Rab7. In addition, we performed sequence alignments for Rab7 and other Rab GTPases family members. As shown in Fig. 3C, the ARD-binding sites in Rab7 are not conserved in the Rab family, suggesting that ORP1L specifically binds Rab7 but not any other Rab proteins. This result is consistent with a previous study (22). Compared with the WT ARDN, the binding abilities of ARDN mutants W49A, Y57A/F58A, and F90A with Rab7 were greatly reduced (Fig. 3D). In agreement with this result, mutations at Ile-108 and Pro-115 in Rab7 greatly reduced the interaction between Rab7 and ARDN (Fig. 3F). In addition, the interaction between Rab7 and ARDN was also abolished by mutants affecting the electrostatic interactions and hydrogen bond formation, including R14A, R87A, and R93A in ARDN and D104A, E105A, E116A, S111A, and R113A in Rab7 (Fig. 3, E–G). In contrast, the mutants L98D, E65K/D66K, and Y103A, whose mutation sites are far from the ARDN-Rab7 interface, preserved their binding
abilities (Fig. S3A). Although failing to bind ARD\textsubscript{D}, Rab7 mutants D104A and S111A still interacted with RILP (Fig. S3B), indicating that the structural integrity of Rab7 is not destabilized by these mutations.

To examine the interaction between ORP1L and Rab7 in vivo, we performed coimmunoprecipitation assays. As shown in Fig. 4A, full-length ORP1L-Y57A/F58A mutant failed to coprecipitate with Rab7, and Rab7-S111A mutant did not associate with ORP1L (see also Fig. 1A). In agreement with this result, ORP1L-Y57A/F58A mutant failed to colocalize with Rab7 and was distributed evenly in the cytosol (Fig. 4, B and C).

In contrast, the WT ORP1L proteins are recruited to late endosomes, indicated by CD63, an independent endosome marker (23). Moreover, siRNA knockdown of Rab7 abolished the endosomal localization of ORP1L, which was rescued by expression of an siRNA-resistant Rab7. However, expression of siRNA-resistant Rab7-S111A mutant failed to recruit ORP1L to late endosomes (Fig. 4, D and E). Taken together, these results confirmed that ORP1L binds to the \(\alpha_3-\eta_2\) region but not the switch regions of Rab7.
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**ORP1L-Rab7 interaction is indispensable for ORP1L-mediated LE-ER contact site formation and LE positioning**

Endosomal ORP1L facilitates the formation of ER-endosome contact sites by sensing cholesterol embedded in late endosomal membranes, which is essential for properly directing and docking of late endosomes to the ER (9). To determine how important ORP1L-Rab7 interaction is for endosomal transport to the ER, we designed ORP1L(1–514) and Rab7 interaction–defective mutants of ORP1L. Because the C-terminal ORD of oxysterol-binding protein–related protein down-regulates its membrane–tethering ability (24), we proposed that ORP1L(1–514), a truncated form of ORP1L without the ORD (residues 548–940), should display a heightened ability to bind to Rab7 and VAP-A when compared with its full-length version. As shown in Fig. 5A, overexpression of ORP1L(1–514) rather than the full-length protein significantly stimulated the formation of LE-ER contact sites in HeLa cell as indicated by a strong increase in colocalization of Rab7 with VAP-A (Fig. 5A). However, expression of ORP1L(1–514) with Y57A/F58A mutation failed to induce LE-ER contacts, suggesting that the interaction with Rab7 is essential for ORP1L to promote LE-ER contact site formation (Fig. 5A). As shown in Fig. 5B, overexpression of RILP resulted in clustering of LEs around the perinuclear region in all HeLa cells because RILP drives microtubule minus-end transport of LEs. Coexpression of full-length ORP1L enhanced RILP-induced perinuclear clustering of LEs. However, coexpression of ORP1L(1–514) abolished RILP-induced perinuclear clustering of LEs. In comparison, coexpression of another ORP1L(1–514) mutant containing Y57A/F58A failed to abolish such clustering (Fig. 5B). This finding suggests that an ORP1L(1–514)-induced increase in LE-ER contacts antagonizes RILP-mediated microtubule minus-end transport of LEs and that the interaction of ORP1L with Rab7 is critical for the positioning of LEs.

**Discussion**

In this study, we found that ARD_N (ORP1L(1–136)) is sufficient for ORP1L to interact with Rab7. Further crystal structure analysis of ARD_N-Rab7 complex revealed that the α3–η2 region on Rab7 is required for ORP1L binding. This unique Rab effector-binding mode explained our finding that ORP1L ARD_N-Rab7 binding is GTP-independent and how ORP1L-Rab7-RILP ternary complex is formed. The structure was further verified by mutagenesis, in vitro pulldowns, and colocalization analysis in cells. ORP1L was first discovered as a Rab7 effector that preferentially binds to GTP-bound Rab7 in mammalian two-hybrid assays (8). In agreement, our coimmunoprecipitation assays revealed that the full-length ORP1L interacts with GTP-Rab7 more strongly than GDP-Rab7. In contrast, ORP1L ARD_N binds equally well to both GTP- and GDP-bound Rab7 in vitro, and the Rab7-S111A mutant failed to interact with either ARD_N or the full-length ORP1L, suggesting that Rab7 binds to ARD_N independently of Rab7 nucleotide-loaded states. We reason that the discrepancy in binding preference of Rab7 with ARD_N and full-length ORP1L might result from the regulatory effects of the C-terminal domains within ORP1L. The regulatory effects of Rab7 effectors has been previously reported. For example, C-terminal residues 392–747 of WDR91, another Rab7 effector, interact with both GTP- and GDP-Rab7 equally; nevertheless, the full-length WDR91 preferentially binds to GTP-Rab7 (16), suggesting a regulatory function of its N terminus. In addition, it is possible that a third protein or some unknown indirect interactions are involved in the in vivo situation. For instance, it was shown that addition of RILP stabilized and increased ORP1L binding to immobilized GST-Rab7 (3).

Previous studies suggested that Rab GTPases use the switch regions to interact with more than one effector. For example, in the tripartite complex Rab11–phosphatidylinositol 4-kinase (PtdKIIIβ)-FIP3, PtdKIIIβ makes contacts with Rab11 on its switch I region, leaving most of the switch regions available for FIP3 to join the complex (25). In the Rab11–Rabin8–FIP3 complex, Rabin8 and FIP3 specifically interact with GTP-Rab11 and share the same switch regions on Rab11 (26). Before this study, it was known that Rab7 forms a tripartite complex with ORP1L and RILP to mediate cholesterol-dependent LE transport and positioning (3, 9). In this tripartite complex, Rab7 interacts with RILP using the switch and interswitch regions, the canonical Rab GTPase effector-binding sites (10–13). However, in contrast to the Rab11–Rabin8–FIP3 model, our structure reveals that the ARD_N of ORP1L makes contacts with Rab7 on its α3–η2 region, which is far away from the switch regions. Thus, the interaction of ORP1L ARD_N with Rab7 is not affected by the GTP- or GDP-binding state of Rab7.

Our findings that ORP1L and RILP simultaneously interact with Rab7 by binding to distinct regions explain the specificity of ORP1L-Rab7 interaction and provide a molecular basis for ORP1L-mediated LE-ER contact site formation and LE positioning. Our structure provides an additional interaction mode as to how one Rab GTPase binds two effectors at the same time, which will increase the understanding of their regulatory mechanisms.

**Experimental procedures**

**Genes and plasmids**

The genes for either human or mouse source ORP1L, Rab7, and RILP were obtained using reverse transcription and PCR technologies. The genes of all recombinant proteins used in crystallization, pulldown, and ITC assays, including WT and mutated ARD_N (ORP1L(1–136)), Rab7 (Rab7(1–176)), and RILP (RILP(241–320)), were cloned into pGEX-6P-1 (GE Healthcare) or pRSFduet-1 (Novagen) vector. The genes of ORP1L, Rab7, RILP, CD63, and VAP used in immunoprecipitation, confocal imaging, and ER-LE contact and LE positioning assays were cloned into pCMV-Tag2B (FLAG tag), pEGFP-c1 (GFP tag), pCMV-BFP (blue fluorescent protein tag), pmCherry-c1 (mCherry tag), and pCMV-HA, respectively. The mutations were produced using a QuickMutation™ kit (Beyotime). All constructs were verified by sequencing (BGI).

**Protein expression and purification**

The gene of mouse ARD_N (ORP1L(1–136)) was inserted into the pRSFduet-1 vector and expressed as an N-terminal His-tagged protein in the Escherichia coli strain BL21 (DE3). Cells were cultured in Luria-Bertani (LB) medium with 100 mg/liter ampicillin at 37 °C until the A_600 of the culture reached 0.8–1.0.
mARDN protein expression was induced by 0.25 mM isopropyl β-D-thiogalactopyranoside (Sigma) for 20 h at 16 °C. The cells were harvested by centrifugation at 4000 rpm. The pellet was resuspended with lysis buffer (20 mM Tris-HCl, pH 7.5, 400 mM NaCl, 30 mM imidazole) and lysed by sonication. The lysate was centrifuged at 16,000 rpm for 30 min, and the supernatant was loaded to a nickel-nitrilotriacetic acid column (Novagen). After extensive washing with lysis buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 500 mM imidazole) and further purified by anion

Figure 5. ORP1L-Rab7 interaction affects ER-LE contact and LE positioning. A, effects of Rab7 interaction on ORP1L(1–514)-induced ER-LE membrane contact site. HeLa cells were transfected with the indicated expression vectors and stained with Rab7 and HA antibodies 12 h post-transfection. Quantifications are Pearson’s coefficients between Rab7 and HA-VAP in ≥50 cells. Scale bars, 5 μm. B, effects of Rab7 interaction on ORP1L(1–514)-mediated LE positioning in the presence of RILP. HeLa cells were transfected with the indicated expression vectors and observed 12 h post-transfection. Quantifications are Pearson’s coefficients between GFP-ORP1L and mCherry-RILP in ≥50 cells. Scale bars, 5 μm. Error bars represent S.E. Significance in this figure was tested by one-way analysis of variance: ***, p < 0.001; NS, not significant. BFP, blue fluorescent protein.
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exchange chromatography using a HiTrap Q HP column (GE Healthcare). Flow-through fractions containing target proteins were further purified using a HiLoad 16/60 Superdex 75 gel filtration column (GE Healthcare) in buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 10 mM dithiothreitol (DTT) (Sigma). Other His-tagged proteins were purified in a similar fashion, including His-Rab7-WT, His-Rab7-Q67L, His-Rab7-T22N, His-RILP, and His-RILP (241–320).

The human Rab7 (Rab7(1–176)) with Q67L mutation, Rab7-Q67L, used in crystallization was inserted into the pGEX-6P-1 vector and expressed using the same protocol as for mARDN mentioned above. The harvested cells were resuspended using lysis buffer (20 mM Tris-HCl, pH 7.5, and 200 mM NaCl) and lysed by sonication. The lysate was centrifuged at 16,000 rpm for 30 min, and the supernatant was loaded to a GSH-Sepharose column (GE Healthcare). Contamination was removed by extensive washing using lysis buffer. The GST tag of the target protein can be kept or removed by on-column cleavage using PreScission protease (homemade). The cleavage was conducted at 4 °C overnight. The eluted target protein was further purified using a HiTrap Q HP column (GE Healthcare). Other GST-tagged proteins, including ORP1L truncated protein, the WT and mutated ARDN (ORP1L(1–136)), and Rab7 (Rab7(1–176)) used in pulldown and ITC assays, were purified following a similar procedure.

**Protein crystallization and structure determination**

Purified mARDN was concentrated to 10 mg/ml and subjected to crystallization screens by the hanging-drop vapor diffusion method at 16 °C. To set up trials for crystallization, the protein was mixed with precipitant (Hampton Research) at a ratio of 1:1. The best crystal diffracted at 2.14 Å and was obtained in conditions with 200 mM NaCl, 100 mM Bis-Tris, pH 6.5, and 25% PEG 3350. The diffraction data were collected at the Swiss Light Source PX-II beamline at a wavelength of 1.00 Å and processed with XDS (27). The structure of mORP1L ARDN was solved by molecular replacement with the program Phaser (28) using the structure of a designed ankyrin repeat protein (PDB code 4K5B, chain A) as the search model. The initial model was automatically built by Buccaneer (29–31). After that, the structure was manually modified using Coot (32) and refined with Phenix.Refine (33). The final model has an \( R_{work} \) of 0.238 and an \( R_{free} \) of 0.240. Data scaling, refinement, and validation statistics are listed in Table 1. All figures were prepared using PyMOL. The coordinates and structure factors have been deposited in the RCSB Protein Data Bank (PDB codes 5Z2N and 5Z2M).

**Western blotting**

Western blotting was performed using a standard protocol as described previously (34). The Rab7 rabbit mAb was purchased from Cell Signaling Technology (catalogue number 9367) and used at 1:1000 dilution. Anti-His antibody was from Sigma-Aldrich (catalogue number AB102) and used at 1:1000 dilution. Anti-FLAG antibody was from Sigma-Aldrich (catalogue number F1804) and used at 1:1000 dilution. GFP polyclonal antibody was from Cell Signaling Technology (catalogue number 9362) and used at 1:1000 dilution. Anti-GFP antibody was from Biorad (catalogue number 2018-1502) and used at 1:1000 dilution. The results are shown in Figs. 1A–H and 2D and 3, D and E. Results shown in Figs. 1C and 3, F and G, were detected using GST-tagged Rab7 and His-tagged ORP1L. The results from an independent replicate are shown in Fig. S4.

**Isothermal titration calorimetric analysis**

The binding affinity of ARDN and Rab7-WT-GTP, Rab7-Q67L-GTP, or Rab7-T22N-GDP was determined using ITC200 isothermal titration calorimetry at 20 °C (MicroCal, GE Healthcare).
Healthcare). ARDN was expressed and purified using a His tag. All the Rab7 proteins were expressed as GST fusion proteins, and the GST tag was removed during the purification process. The proteins were maintained in ITC assay buffer (20 mM Tris-HCl, pH 7.5, and 150 mM NaCl). The exothermic heat of the reaction was measured by 20 sequential 1.5-μl injections of ARDN (450 μM) into 200 μl of Rab7 (25 μM), spaced at intervals of 120 s. The heat of dilution was obtained by injecting ITC assay buffer into Rab7 and was subtracted from the heat of reaction before the fitting process. Binding curves were analyzed by nonlinear least-squares fitting of the data using MicroCal Origin software.

**Mammalian cell culture and small RNAi**

HeLa or HEK 293 cells were cultured at 37 °C with 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone, Novato, CA), 100 units/ml penicillin, and 100 mg/ml streptomycin. Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. siRNA was achieved per transfection of the following oligonucleotides: siRNA to the manufacturer’s instructions. siRNA was used: UUCUCCGAACGUGUCACGUTT. Cells were observed under microscopy or harvested for Western blotting 24 h post-transfection.

**Immunoprecipitation**

Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1 mM phenylmethanesulfonyl fluoride). Immunoprecipitations were then performed as described before (35) using individual antibodies. Precipitated proteins were resolved by SDS-PAGE and detected using the different antibodies listed above.

**Immunostaining and imaging of mammalian cells**

Cells grown on coverslips were fixed in 4% paraformaldehyde followed by permeabilization with 0.2% saponin for 8 min. After extensive washing with phosphate-buffered saline (PBS), coverslips were incubated in blocking buffer 1 (5% BSA and PBS) for 1 h at room temperature and then incubated with primary antibodies in the same buffer at 4 °C overnight. Cells were washed extensively again and incubated with Cy3- or FITC-conjugated secondary antibodies for 1 h at room temperature. Following another round of thorough washing, cells were sealed with VECTASHIELD mounting medium (Vector Laboratories) for confocal microscopy. Fluorescence images were obtained with an inverted Olympus FV1000 confocal microscope system (IX81) using a 60 × 1.42 numerical aperture oil objective. Excitation was achieved using solid-state 488 nm and gas-state 595 nm lasers. All images were taken at 25 °C. Colocalization between two channels was calculated using Pearson’s coefficient with FV10-ASW 4.0a Viewer software (Olympus).

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**References**

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