The protein kinase Akt acts as a coat adaptor in endocytic recycling

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Coat proteins have a central role in vesicular transport by binding to cargoes for their sorting into intracellular pathways. Cargo recognition is mediated by components of the coat complex known as adaptor proteins1–3. We previously showed that Arf-GAP with coil-coil, ANK repeat and PH domain-containing protein 1 (ACAP1) functions as an adaptor for a clathrin coat complex that has a function in endocytic recycling4–6. Here, we show that the protein kinase Akt acts as a co-adaptor in this complex, and is needed in conjunction with ACAP1 to bind to cargo proteins to promote their recycling. In addition to advancing the understanding of endocytic recycling, we uncover a fundamentally different function in which a kinase acts, as Akt in this case is an effector rather than a regulator in a cellular event.

Endocytic recycling is needed for a variety of cellular events, including cell motility and polarity, nutrient uptake and signal transduction7–12. Recycling can occur either constitutively, for which the transferrin receptor (TfR) has been used as a model cargo, or in a stimulation-dependent manner, for which integrin has been used as a model cargo11. Previously, we found that ACAP1 acts as a coat adaptor for both types of recycling, transporting cargo from the recycling endosome to the plasma membrane11. For integrin recycling, we further found that ACAP1 binds to the β1 subunit of the α5β1 integrin heterodimer11. In considering whether another adaptor binds to the α5 subunit, we noted that the stimulation-dependent recycling of integrin requires the Ser554 residue in ACAP1 to be phosphorylated by Akt11. However, whereas phosphorylation typically involves kinases associating transiently with their substrates, Akt associates more stably with ACAP1 for integrin recycling4. We therefore examined the possibility that Akt has a coat function.

We initially performed pull-down analysis followed by immunoblotting, which revealed that ACAP1 does not bind directly to the cytoplasmic tail of multiple integrin α subunits that heterodimerize with β1 (Fig. 1a, Extended Data Fig. 1a). By contrast, direct binding was observed between Akt and the integrin α tails (Fig. 1b, Extended Data Fig. 1b). Moreover, the presence of Akt enables ACAP1 to associate with α tails (Fig. 1c, Extended Data Fig. 1c). We next assessed interactions using Coomassie staining, which revealed that Akt, either alone (Extended Data Fig. 1d) or together with ACAP1 (Extended Data Fig. 1e), binds efficiently to the α5 tail. The three components can also assemble efficiently into a complex in solution (Extended Data Fig. 1f).

Akt has two major domains—a pleckstrin homology (PH) and a kinase domain. We found that only the kinase domain binds to the α5 tail (Fig. 1d, Extended Data Fig. 1a,g) and ACAP1 (Fig. 1e, Extended Data Fig. 1h). For the Akt–ACAP1 interaction, Akt binds to the carboxyl portion of ACAP1, which contains the GTPase-activating protein (GAP) and ankyrin-repeat (ANK) domains of ACAP1 (Fig. 1f, Extended Data Fig. 1i). We also quantified the binding of the Akt kinase domain to the α5 tail (Fig. 1g, Extended Data Fig. 1j) and to ACAP1 (Fig. 1h, Extended Data Fig. 1j). Thus, the collective results suggest a model for how Akt acts as a coadaptor with ACAP1 in cargo binding (Extended Data Fig. 1i).

We next examined integrin recycling in cells. We had previously shown that mutating Ser554 in ACAP1 to aspartate (S554D), which mimics its constitutive phosphorylation by Akt, renders integrin recycling a constitutive process13. Thus, as the Akt kinase activity is no longer needed for integrin recycling in this situation13, we revisited the ACAP1S554D-expressing cells to examine whether Akt has a non-kinase role in integrin recycling. After treatment with short interfering RNA (siRNA) against AKT1 and AKT2 (Extended Data Fig. 2a), we found that integrin recycling in these cells is inhibited (Fig. 1i, Extended Data Fig. 2b). Moreover, this inhibition is rescued by not only the wild-type, but also the kinase-dead form of Akt (Fig. 1j, Extended Data Fig. 2b). As integrin recycling mediates cell migration14, we also found that siRNA against AKT1 and AKT2 inhibits cell motility, and this inhibition is also rescued by both wild-type and kinase-dead Akt (Extended Data Fig. 3a). Furthermore, treatment with an Akt kinase inhibitor, GDC0068, did not inhibit integrin recycling in these cells (Extended Data Fig. 3a). We also detected an interaction between Akt and ACAP1 in cells (Fig. 1j). Moreover, siRNA against AKT1 and AKT2 prevents endosomal integrin β1 from interacting with Akt and ACAP1 in the ACAP1S554D-expressing cells, and this disruption is also rescued by both wild-type and kinase-dead Akt (Fig. 1k). Thus, in addition to the previously described kinase role13, Akt also has a non-kinase role in integrin recycling.

Coat adaptors recognize specific sequences in cargoes, known as sorting signals, and mutating these sequences inhibits cargo transport in the pathway that is governed by the coat complex14. As such, we next performed truncations followed by point mutagenesis to identify key residues in the α5 tail that are required for its direct interaction with Akt (Fig. 2a,b). We then mutated these residues (Fig. 2c) in the full-length α5, and replaced endogenous α5 with the mutant ACAP1S554D.
α5 in cells. By examining integrin recycling (Fig. 2d, Extended Data Fig. 4a) and cell migration (Extended Data Fig. 4b), we found that both are inhibited. Consistent with these findings, ACAP1 does not associate with the β1 integrin in cells that express the mutant α5, rather than the wild type (Fig. 2e). We also confirmed that the mutant α5 heterodimerizes with the wild-type β1 in cells (Fig. 2f). Moreover, the resulting mutant integrin localizes to the recycling endosome in unstimulated cells (Fig. 2g, Extended Data Fig. 4c). Thus, the collective results reveal the importance of the interaction between Akt and the α5 tail for recycling the α5β1 integrin.

To test the broader relevance of our results described above, we next examined TIR recycling, as it has been used as a general model for studies on endocytic recycling. After siRNA treatment against AKT1 and AKT2 in HeLa cells, we found that TIR recycling is also inhibited, and was again rescued by both wild-type and catalytic-dead Akt (Fig. 3a, Extended Data Fig. 5a). Similar results were also observed for TIR recycling in human embryonic kidney (HEK293) cells (Extended Data Fig. 5b). We also found that the siRNA treatment did not affect the internalization of surface TIR to the early endosome (Extended Data Fig. 6a). Thus, the inhibition in TIR recycling cannot be attributed to an indirect effect of having affected TIR internalization.

The Akt kinase activity is stimulated by phosphorylation at two sites, Thr308 and Ser473 (ref. 13). Akt phosphorylation mutants have been generated that mimic the constitutive phosphorylation (T308D/S473D) and dephosphorylation (T308A/S473A) at these
We found that the inhibition of TIR recycling induced by siRNA against AKTI and AKT2 is rescued similarly by either Akt phosphorylation mutant (Extended Data Fig 6b). Treatment with the Akt kinase inhibitor GDC0068 also does not affect TIR recycling (Extended Data Fig 7a). These results therefore further support a non-kinase role for Akt in TIR recycling. As we had thus far been using an siRNA oligonucleotide that targets a common sequence in AKTI and AKT2, we also assessed the relative contribution of the two Akt isoforms in TIR recycling. By treating cells with siRNA specifically targeting either AKTI or AKT2, we found that both treatments partially reduced the total level of Akt (Extended Data Fig 7b), and this correlates with partial inhibition of TIR recycling (Extended Data Fig 7c,d), suggesting that both Akt isoforms participate in TIR recycling.

We next found that Akt also binds directly to the cytoplasmic domain of TIR (Fig. 3b). Moreover, an α5 cargo peptide competes with this binding (Fig. 3c), suggesting that Akt has a common binding site for both TIR and α5. To further test whether Akt has a function in the cargo sorting of TIR for its recycling, we next determined whether the sequence in TIR that is recognized by Akt functions as a recycling sorting signal. We previously found that ACAP1 binds to the identified residues are the same as the residues that we previously found to participate in TfR recycling. Pursuing a reconciliation explanation, we next titrated an increasing level of ACAP1 for incubation with either the N19 construct or the NΔ19 TIR construct in pull-down studies, and found that Akt binds preferentially to the N19 construct (Fig. 3g), whereas ACAP1 binds preferentially to the NΔ19 construct (Fig. 3h). We next generated fusion proteins that contain the cargo-binding portions of ACAP1 (GAP–ANK domains) or Akt (kinase domain), having homodimers of either the ACAP1 or Akt domains, or a heterodimer containing both Akt and ACAP1 domains. Performing pull-down studies, we found that the heterodimer exhibited greater affinity to the TIR cytoplasmic domain than that of either homodimer (Extended Data Fig. 8b). We also found that siRNA against AKTI and AKT2 disrupted the association between ACAP1 and endosomal TIR in cells (Extended Data Fig. 8c). Similarly, siRNA against ACAP1 disrupted the association between Akt and endosomal TIR in cells (Extended Data Fig. 8d). Thus, the collective results confirmed that ACAP1 and Akt act cooperatively in binding to TIR.

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In addition to binding to cargoes, coat adaptors also link clathrin to cargoes in coat complexes. To examine whether Akt also acts in this manner, we initially identified that the kinase domain of Akt, but not the PH domain, binds directly to clathrin (Fig. 4a). We then found that, whereas neither the α5 nor the TIR cytoplasmic domain can bind directly to clathrin, the addition of the Akt kinase domain enables clathrin to form complexes with either cargo (Fig. 4b). We also found that the additional presence of ACAP1 enables clathrin to associate with the TIR cytoplasmic domain more efficiently (Fig. 4c). Moreover, co-precipitation studies revealed that siRNA against AKT1 and AKT2 disrupts the association between ACAP1 and clathrin in cells (Fig. 4d), whereas siRNA against ACAP1 disrupts the association between Akt and clathrin (Fig. 4e). Thus, Akt also behaves as a co-adaptor with ACAP1 in linking clathrin to cargoes.

We previously found that the small GTPase ADP-ribosylation factor 6 (ARF6) regulates the recruitment of ACAP1 to the recycling endosome. To determine whether ARF6 regulates the recruitment of Akt similarly, we isolated a membrane fraction enriched for the recycling endosome (Extended Data Fig. 9a). Initially, we found that Akt alone cannot be recruited to this membrane (Extended Data Fig. 9b). However, ARF6 in its activated form can be recruited to this membrane (Extended Data Fig. 9c). We next found that activated ARF6 is needed to recruit Akt (Fig. 4f) and ACAP1 (Fig. 4g) to this membrane. Moreover, activated ARF6 alone cannot recruit clathrin to this membrane (Fig. 4h), but requires the presence of Akt and ACAP1 (Fig. 4h, Extended Data Fig. 9d).

Thus, Akt and ACAP1 also act as co-adaptors in recruiting clathrin to the endosomal membrane. The recruitment of all of the coat components onto the membrane can also be reconstituted using liposomes, which revealed that the coat components assemble on membrane at nearly stoichiometric levels (Extended Data Fig. 9e).

We next pursued cell-based studies. Initially, we found that endogenous Akt exhibits a diffuse distribution, having only a minimally detectable pool at the recycling endosome (Fig. 5a). This finding was confirmed by staining using another antibody against Akt (Extended Data Fig. 10a). As one explanation, we found using cell fractionation that Akt exists mostly in the cytosol (Fig. 5b). We then elucidated a complementary explanation. Pursuing a more denaturing condition of cell fixation, we found that Akt at the recycling endosome can be detected more efficiently (Fig. 5c). Moreover, as the harsher fixation protocol did not affect the detection of clathrin (Fig. 5d), our collective observations suggested that Akt at the recycling endosome is mostly assembled into complexes that hinder its detection by antibodies.

To provide additional support for this conclusion, we analysed the ARF6-dependent recruitment of Akt, ACAP1 and clathrin onto the endosomal membrane. We added anti-Akt antibodies after the recruitment using two methods, either before or after membrane solubilization with detergent. After isolating the anti-Akt antibodies, we detected higher levels of Akt in the latter condition (Fig. 5d). By contrast, when only Akt is recruited to the endosomal membrane by ARF6, the anti-Akt antibodies detected similar levels.
of membrane-bound Akt, regardless of whether the antibodies were added before or after membrane solubilization with detergent (Fig. 5e). Thus, these results further support that Akt at the recycling endosome exists mostly in assembled complexes that impair the antibody detection of Akt.

We then noted that ARF nucleotide binding site opener (ARNO) has been shown to act as an ARF6 guanine nucleotide exchange factor in endocytic recycling\(^1\). After ARNO overexpression, we observed enhanced recruitment of Akt to the recycling endosome, as reflected by its enhanced detection through antibody staining (Fig. 5f). The catalytic activity of ARNO is needed for this enhanced recruitment (Fig. 5f). We also found that Akt staining is reduced by the overexpression of mutant ARNO (Fig. 5f), which is explained by the ability of mutant ARNO to localize similarly to the recycling endosome as the wild-type form (Extended Data Fig. 10c), resulting in a dominant-negative effect on Akt recruitment to the recycling endosome. We further discovered that cell stimulation does not have an appreciable effect on the pool of Akt at the recycling endosome (Extended Data Fig. 10d). Thus—in contrast to Akt recruitment to the plasma membrane, which is regulated by growth factors\(^1\) and which has been shown to induce Akt into clathrin complexes at the cell surface that act in endocytosis\(^1\)—Akt recruitment to the recycling endosome is regulated differently, modulated instead by ARF6 and its guanine nucleotide exchange factor, which results in Akt assembly into clathrin complexes on the endosomal membrane that act in recycling.

In summary, we have uncovered a non-kinase role of Akt, whereby Akt functions as a coat adaptor in endocytic recycling. An interesting implication also arises from this discovery. Many kinases have previously been identified to act in different intracellular transport pathways. At present, these kinases are known to act only as regulators, which involves the phosphorylation of downstream targets to modulate function through their kinase activity\(^16\). Our finding that Akt acts as a coat adaptor in endocytic recycling raises the prospect that this fundamentally different role—acting as an effector rather than as a regulator—may be more widespread across the intracellular pathways than appreciated at present.

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Fig. 5 | Akt at the recycling endosome exists mostly within assembled complexes. a, Confocal microscopy analysis of the colocalization of endogenous Akt with the endosomal pool of Tf in HeLa cells; n = 3 independent experiments; 10 cells were examined for each experiment. Primary images along with the line scan are shown; Akt is shown in red and Tf is shown in green. Scale bar, 10 μm. b, Subcellular fractionation of HeLa cells, assessing the relative distribution of endogenous Akt in the total membrane (P, pellet) versus the cytosol (S, supernatant) fraction; n = 2 independent experiments. Actin tracks the cytosol and cellubrevin tracks the endosomal membrane. c, Confocal microscopy analysis of the effect of a more-denaturing fixative, containing methanol and acetone (Me/Ac), in detecting endogenous Akt at the recycling endosome in HeLa cells; n cells, assessing the relative distribution of endogenous Akt in the total membrane (P, pellet) versus the cytosol (S, supernatant) fraction; *t-test; *P = 1.18 × 10−22. d–f, An antibody-based assay to detect Akt assembled on the endosomal membrane; n = 2 independent experiments. Akt along with ACAP1 and clathrin (d) or Akt alone (e) is recruited to the endosomal membrane in an ARF6-dependent manner. Anti-Akt antibodies (Abs) were then incubated with the membrane, either before or after membrane solubilization with detergent, and the antibodies were then isolated to detect associated Akt. TX-100, Triton X-100. f, Confocal microscopy analysis of the effect of overexpressing either wild-type or catalytic-dead (E156K) ARNO on the ability to detect endogenous Akt at the recycling endosome in HeLa cells; n = 3 independent experiments; 10 cells were examined for each experiment. Primary images along with the line scan are shown on the left for the colocalization of Akt with endosomal Tf; Akt is shown in red and Tf is shown in green. Scale bar, 10 μm; inset shows boxed area with further 2.5x magnification. Quantification is shown on the right. Data are mean ± s.d.; statistical analysis was performed using a paired two-tailed Student’s t-test; *P = 1.57 × 10−25 (no ARNO versus ARNO); *P = 1.56 × 10−33 (ARNO WT versus ARNO E156K). Source data are available online.

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Methods

Results and discussion

Cytoskeletal organization and reorganization

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the cell surface and in endosomal compartments. Cells were then washed with an acidic buffer (0.5 M NaCl and 0.5% acetic acid) to release biotin–TF bound to TIR at the cell surface. Cells were then lysed and then incubated with streptavidin agarose beads at 4 °C for 2 h. The beads were next washed three times with lysis buffer and then analysed using SDS-PAGE followed by western blotting.

For immunoprecipitation of Myc-tagged ACAP1, HeLa cells were transfected with the ACAP1-Myc construct and then disrupted in lysis buffer (PBS with 0.5% Triton X-100 and protease inhibitors). Lysates were cleared by centrifugation at 16,000g for 15 min at 4 °C, and were then incubated with anti-Myc antibodies for 2 h at 4 °C followed by incubation with protein A beads. The beads were then washed three times with lysis buffer and then analysed using western blotting.

**Transport assays.** The TIR endocytosis and recycling assays have been described previously\(^1\). In brief, for TIR endocytosis, cells were incubated with Alexa-546-conjugated TF (5 μg ml\(^{-1}\) in DMEM) for 1 h at 4 °C. Cells were then washed to clear unbound TF, followed by shifting to 37 °C for different time points. Cells were stained for EEA1, and then analysed using confocal microscopy to assess the arrival of TF to the early endosome.

For TIR recycling, cells were incubated with Alexa-546-conjugated TF (5 μg ml\(^{-1}\) in DMEM without serum) at 37 °C for 2 h to label TIR in the endosomal compartments. Cells were then incubated with complete medium (which contains unlabelled TF) at 37 °C for the different time points. After staining for a recycling endosome (RE) marker (Rab11 or cellubrevin), cells were analysed using confocal microscopy to quantify the colocalization of labelled TIR with RE marker at different time points.

The integrin-recycling assay to track the stimulation-dependent recycling of integrin β1 in HeLa cells has been described previously\(^2\). In brief, serum-starved cells were incubated with anti-β1 antibodies at 4 °C for 1 h to bind to the surface pool of integrin β1. After washing away unbound antibodies, cells were incubated at 37 °C for 2 h to enable the antibody-bound pool of surface β1 to accumulate at the recycling endosome. After serum stimulation, cells were then analysed using confocal microscopy to quantify the colocalization of endosomal integrin β1 with RE marker at different time points.

The integrin-recycling assay to track the constitutive recycling of integrin β1 in cells expressing the mutant ACAP1\(^{546,40}\) was described previously\(^1\). In brief, cells were incubated with anti-β1 antibodies at 4 °C for 1 h to bind to the surface pool of integrin β1. After washing away the unbound antibodies, cells were incubated at 37 °C for 2 h in the presence of primaquine to enable the antibody-bound pool of surface β1 to accumulate at the recycling endosome. After washout, cells were then analysed using confocal microscopy to quantify the colocalization of endosomal integrin β1 with RE marker at different time points.

Note that integrin recycling exhibits a V-shaped curve in the quantitative assay, because stimulation induces endosomal integrin (which had been gathered at the RE in the serum-starved condition) to cycle between the RE and the plasma membrane. Thus, as the antibody remains bound to the integrin, the assay tracks the entire cycling itinerary of the endocytic integrin\(^5\). By contrast, TIR recycling exhibits only a downward slope in the quantitative assay, because TF is released from TIR when endosomal TIR reaches the plasma membrane, for which a more detailed explanation has been provided previously\(^2\).

For both integrin and TIR recycling, GDC0068 was added at 200 nM for 1 h to assess the role of the Akt kinase activity.

**Confocal microscopy.** Colocalization studies were performed using a Zeiss system equipped with the Axios Observer Z1 Inverted Microscope, with a Plan-Apochromat 63× plan objective coupled to a LSM 800 confocal system with Airyscan package, and the Zen 2.3 blue edition confocal acquisition software.

To quantify colocalization, 10 fields of cells were examined; each field typically had five cells. Images were imported into NIH Image J (v1.50i) with the colocalization plugin (https://imagej.net/Coloc_2) for analysis. The threshold values were chosen automatically by the program on the basis of the Costes method. Manders’ coefficients were then calculated and expressed as the fraction of protein of interest (cargo protein or Akt) colocalized with a compartmental marker.

**Cell-migration assay.** Cell migration using a Transwell chamber (Corning, 3422) was described previously\(^1\). In brief, HeLa cells were plated onto the fibronectin-coated membrane in the Transwell chamber, and were then allowed to migrate for 4 h at 37 °C. To detect cells that migrated across the membrane, cells were stained with 4,6-diamidino-2-phenylindole and then quantified using fluorescence microscopy.

**Statistics and reproducibility.** Quantitative data are shown as mean ± s.d. Statistical significance was determined using paired two-tailed Student’s t-tests in Prism (v7.04) or Excel (Office 2016). The sample number (n) indicates the number of independent biological experiments. For quantitative colocalization studies that involve n = 3, the mean for each experiment was first determined by examining 10 cells per experiment. Subsequently, these values were used to determine the mean ± s.d. for the quantitative data shown in figures. The following figures were generated on the basis of n = 2 independent biological experiments: Figs. 1a–h, k, 2a, b, e, f, 3b–h and 4a–e. All of the other figures, except for illustrations (Fig. 2c and Extended Data Figs. 1f and 8a), were generated on the basis of n = 3 independent biological experiments.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The data that support the findings of this study are available from the corresponding authors on reasonable request. Source data are provided for Figs. 1–5 and Extended Data Figs. 1–10.

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**Author contributions**

J.-W.H., M.B., J.-L.K., J.-Y.S. and N.C. performed experiments. J.-W.H., M.B., J.-L., J.-S.Y., N.C., P.A.C., M.J.E. and V.W.H. participated in designing experiments and analysing the results. V.W.H. supervised the research with help from J.-L. V.W.H. wrote the manuscript with help from J.-W.H.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Extended Data Fig. 1 | Further characterizing cargo binding interactions. a, b, c, Pulldown interactions from 2 independent experiments are quantified for studies shown in Fig. 1a (a), in Fig. 1b (b), and in Fig. 1c (c). Data available in Source Data Extended Data Fig. 1a–c. d, e, Pulldown studies followed by Coomassie staining to detect soluble forms Akt bound to GST-α5 on beads (d) or soluble Akt and ACAP1 bound to GST-α5 on beads (e); n = 2 independent experiments. Input shows 10% of soluble components used in the incubation. Data available in Unprocessed Blots Extended Data Fig. 1d, e. f, A soluble complex containing GST-α5, Akt, and ACAP1 is detected by incubating the three components in solution, followed by isolation using glutathione beads, and then Coomassie staining to assess complex formation; n = 2 independent experiments. Input shows 10% of soluble components used for the incubation. Data available in Unprocessed Blots Extended Data Fig. 1f. g, h, i, Pulldown interactions from 2 independent experiments are quantified for studies shown in Fig. 1d (g), in Fig. 1e (h), and in Fig. 1f (i). Data available in Source Data Extended Data Fig. 1g–i. j, k, Primary data for biolayer interferometry measurements that quantify the interaction between GST-α5 and the Akt kinase domain shown in Fig. 1g (j), and between the Akt kinase domain and the carboxyl portion of ACAP1 shown in Fig. 1h (k); n = 2 independent experiments. l, A model for how Akt and ACAP1 act as co-adaptors in binding to the α5β1 integrin heterodimer.
Extended Data Fig. 2 | Akt having a non-kinase role in integrin recycling. 

**a.** Efficiency of siRNA against Akt and its rescues in HeLa cells that express the ACAP1 mutant (S554D), as detected by immunoblotting of whole cell lysates; n = 2 independent experiments. Data available in Unprocessed Blots Extended Data Fig. 2a. 

**b.** Representative primary images from the integrin recycling assay shown in Fig. 1i; n = 3 independent experiments. The colocalization of endosomal β1 with Rab11, a marker of the recycling endosome, is assessed; β1 (red), Rab11 (green), bar = 10 um.
Extended Data Fig. 3 | Further supporting a non-kinase role of Akt in integrin recycling.

**a** Cell migration of the S554D-expressing HeLa cells as assessed through the transwell-based assay; n = 3 independent experiments, with each experiment examining three fields of transwell membranes. Primary images are shown on left, bar = 200 μm. Quantitation is shown on right, mean ± SD, *p = 1.57 \times 10^{-20}, \text{NS} p = 0.125, \text{paired two-tailed student's t-test. Data available in Source Data Extended Data Fig. 3a.}

**b, c** Integrin recycling assay assessing the effect of treating the S554D-expressing HeLa cells (b), or control HeLa cells (c), with the Akt kinase inhibitor GDC0068; n = 3 independent experiments. Quantitation is shown on left, mean ± SD, with statistics performed for the 5-minute time point, \text{NS} p = 0.248 (for analysis in b), *p = 1.54 \times 10^{-25} (for analysis in c), \text{paired two-tailed student's t-test. Primary images, assessing the colocalization of endosomal } \beta 1 \text{ with cellubrevin (a marker of the recycling endosome), are shown on right, } \beta 1 \text{ (red), Cbv (green), bar = 10 μm. Data available in Source Data Extended Data Fig. 3b, c.}
Extended Data Fig. 4 | Further characterizing the effect of expressing a mutant α5 integrin. **a**, Primary images from the integrin recycling assay shown in Fig. 2d; n = 3 independent experiments. The colocalization of endosomal β1 with Rab11 is assessed; β1 (red), Rab11 (green), bar = 10 μm.

**b**, Cell migration of HeLa cells that express different α5 forms as assessed through the transwell-based assay; n = 3 independent experiments, with each experiment examining three fields of transwell membranes. Primary images are shown on left, bar = 200 μm. Quantitation is shown on right, mean ± SD, *p = 2.08 × 10^{-24}, paired two-tailed student’s t-test. Data available in Source Data Extended Data Fig. 4b.

**c**, Representative primary images for the colocalization study shown in Fig. 2g; n = 3 independent experiments. The colocalization of endosomal β1 with Rab11 is assessed; β1 (red), Rab11 (green), bar = 10 μm.
Extended Data Fig. 5 | Further supporting a non-kinase role of Akt in Tfr recycling. 

**a**, Primary images from the Tfr recycling assay shown in Fig. 3a; n = 3 independent experiments. The colocalization of endosomal Tfr with Rab11 is assessed; Tfr (red), Rab11 (green), bar = 10 μm. 

**b**, TIR recycling assay examining the effect of siRNA against Akt, and also rescue using wild-type (WT) or kinase-dead (K179M) Akt in HEK293 cells; n = 3 independent experiments, with each experiment examining 10 cells. Quantitation is shown above, mean ± SD, with statistics performed on the 25-minute time point, *p = 8.86 × 10^{-27}, NS p = 0.557*, paired two-tailed student’s t-test. Primary images along with line scans are shown below, Tfr (red), cellubrevin (green), bar = 10 μm. Data available in Source Data Extended Data Fig. 5b.
Extended Data Fig. 6 | Further characterizing how Akt acts in the endocytic transport of Tfr. **a**, Tfr internalization assay examining the effect of siRNA against Akt, n = 3 independent experiments, with each experiment examining 10 cells. Quantitation is shown above, mean ± SD, with statistics performed for the 5-minute time point, NS p = 0.538, paired two-tailed student’s t-test. Primary images are shown below, Tfr (red), EEA1 (a marker of the early endosome, green), bar = 10 μm. Data available in Source Data Extended Data Fig. 6a. **b**, Tfr recycling assay examining the effect of siRNA against Akt, and also rescues using various forms of Akt in HeLa cells; n = 3 independent experiments, with each experiment examining 10 cells. Quantitation is shown above, mean ± SD, with statistics performed on the 25-minute time point, *p = 2.68 × 10^{-27}, NS p = 0.303, paired two-tailed student’s t-test. Primary images along with line scans are shown below, Tfr (red), Rab11 (green), bar = 10 μm. Data available in Source Data Extended Data Fig. 6b.
Extended Data Fig. 7 | Further characterizing how Akt acts in TfR recycling. a, TfR recycling assay examining the effect of treating HeLa cells with the Akt kinase inhibitor GDC0068; n = 3 independent experiments, with each experiment examining 10 cells. Quantitation is shown on left, mean ± SD with statistics performed on the 30-minute time point, NS p = 0.222, paired two-tailed student’s t-test. Primary images along with line scans are shown on right; Tf (red), cellubrevin (green), bar = 10 um. Data available in Source Data Extended Data Fig. 7a. b, Efficiency of siRNA against Akt1 and siRNA against Akt2 in HeLa cells, as assessed by immunoblotting of whole cell lysates; n = 2 independent experiments. Actin level confirms similar levels of cells examined. Data available in Unprocessed Blots Extended Data Fig. 7b. c, d, TfR recycling assay examining the effect of siRNA against different isoforms of Akt in HeLa cells; n = 3 independent experiments, with each experiment examining 10 cells. Quantitation is shown in (c), mean ± SD, with statistics performed on the 25-minute time point, *p = 1.72 x 10^{-22} (control vs si-Akt1), p = 1.53 x 10^{-20} (control vs si-Akt2), *p = 1.78 x 10^{-43} (control vs si-Akt1/ si-Akt2), paired two-tailed student’s t-test. Data available in Source Data Extended Data Fig. 7c. Primary images along with line scans are shown in (d), Tf (red), Rab11 (green), bar = 10 um.
Extended Data Fig. 8 | Further supporting Akt acts as a co-adaptor with ACAP1 in cargo binding. a, Schematic showing the sequence of the TfR cytoplasmic domain, and the portions covered by the N19 and the NΔ19 constructs. b, Pulldown studies titrating increasing level of different fusion proteins of Akt and ACAP1 for their binding to the TfR cytoplasmic domain; n = 2 independent experiments. Left panel compares binding by Akt-ACAP1 heterodimer and Akt homodimer. Right panel compares binding by Akt-ACAP1 heterodimer and ACAP1 homodimer. Data available in Unprocessed Blots Extended Data Fig. 8b. c, d, Co-precipitation studies examining the effect of siRNA against Akt on the association of endosomal TfR with ACAP1 and Akt in HeLa cells (c), and siRNA against ACAP1 on the association of endosomal TfR with Akt and ACAP1 in HeLa cells (d); n = 2 independent experiments. Biotin-labeled Tf was internalized for 2 hours to label the endosomal pool of TfR. Immunoblotting of whole cell lysates confirms the efficiency of siRNA treatment. Data available in Unprocessed Blots Extended Data Fig. 8c, d.
Extended Data Fig. 9 | Further characterizing membrane recruitment of Akt. a, Isolating a membrane fraction from HeLa cells enriched for the recycling endosome using a sucrose gradient established through equilibrium centrifugation; n = 2 independent experiments. Fractions enriched for the recycling endosome were identified by tracking cellubrevin and internalized Tf (which bound to endosomal TfR), and not surface Tf (which bound to surface TfR). Data available in Unprocessed Blots Extended Data Fig. 9a. b, c, d, Recruitment studies showing that Akt alone cannot be recruited to endosomal membrane (b), while ARF6 alone in its activate form can be recruited to endosomal membrane (c), and clathrin recruitment to endosomal membrane requires ARF6 with either Akt or ACAP1 (d); n = 2 independent experiments. Cellubrevin tracks endosomal membrane. Data available in Unprocessed Blots Extended Data Fig. 9b–d. e, Recruitment study examining the relative levels of ARF6, Akt, ACAP1, and clathrin recruited to liposomes; n = 2 independent experiments. Input shows the total amount of each component added for the incubation. Data available in Unprocessed Blots Extended Data Fig. 9e.
Extended Data Fig. 10 | Further characterizing endogenous Akt at the recycling endosome. a, Confocal microscopy examining the colocalization of endogenous Akt with endosomal Tf in HeLa cells; n = 3 independent experiments, with each experiment examining 10 cells. Primary images are shown, Akt (green), Tf (red), bar = 10 um. b, Confocal microscopy examining the effect of a more denaturing fixative (containing methanol and acetone) on the ability to detect endogenous clathrin at the recycling endosome in HeLa cells; n = 3 independent experiments, with each experiment examining 10 cells. Primary images along with line scan are shown on left for the colocalization of clathrin with endosomal Tf, clathrin (green), Tf (red), bar = 10 um. Quantitation is shown on right, mean ± SD, NS p = 0.106, paired two-tailed student’s t-test. Data available in Source Data Extended Data Fig. 10b. c, Confocal microscopy examining the colocalization of different forms of ARNO with endosomal Tf in HeLa cells; n = 3 independent experiments, with each experiment examining 10 cells. Primary images along with line scan are shown on left for the colocalization of endogenous Akt with endosomal Tf, ARNO (green), Tf (red), bar = 10 um. Quantitation is shown on right, mean ± SD, NS p = 0.686, paired two-tailed student’s t-test. Data available in Source Data Extended Data Fig. 10c. d, Confocal microscopy examining the effect of serum stimulation on Akt localization at the recycling endosome in HeLa cells; n = 3 independent experiments, with each experiment examining 10 cells. Primary images along with line scan are shown above for the colocalization of endogenous Akt with endosomal Tf, Akt (red), Tf (green), bar = 10 um. Quantitation is shown below, mean ± SD, NS p = 0.325, paired two-tailed student’s t-test. Data available in Source Data Extended Data Fig. 10d.
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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

Colocalization studies were performed using the Zeiss confocal acquisition software (version 2.3 blue edition).

Data analysis

Quantification of colocalization used Image J [version 1.50]. Statistical analysis used Prism [version v7.04] or Excel (version Office 2016).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

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The data that support the findings of this study are available from the corresponding author upon reasonable request. The following figures have associated raw data: Figures 1a, 1b, 1c, 1d, 1e, 1f, 1g, 1h, 1i, 1j, 1k, 1l, 1m, 1n, 1o, 1p, 1q, 1r, 1s, 1t, 1u, 1v, 1w, 1x, 1y, 1z, 2a, 2b, 2c, 2d, 2e, 2f, 2g, 2h, 2i, 2j, 2k, 2l, 2m, 2n, 2o, 2p, 2q, 2r, 2s, 2t, 2u, 2v, 2w, 2x, 2y, 2z, 3a, 3b, 3c, 3d, 3e, 3f, 3g, 3h, 3i, 3j, 3k, 3l, 3m, 3n, 3o, 3p, 3q, 3r, 3s, 3t, 3u, 3v, 3w, 3x, 3y, 3z, 4a, 4b, 4c, 4d, 4e, 4f, 4g, 4h, 4i, 4j, 4k, 4l, 4m, 4n, 4o, 4p, 4q, 4r, 4s, 4t, 4u, 4v, 4w, 4x, 4y, 4z, 5a, 5b, 5c, 5d, 5e, 5f, and Extended Data Figures 1a, 1b, 1c, 1d, 1e, 1f, 1g, 2h, 1i, 1j, 1k, 2a, 2b, 3a, 3b, 3c, 4b, 5b, 6a, 6b, 7a, 7b, 7c, 8a, 8b, 8c, 8d, 9a, 9b, 9c, 9d, 9e, 10b, 10c, 10d.
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- Sample size: Sample size was determined based on prior experience for a particular assay in achieving reproducible results.
- Data exclusions: No data were excluded.
- Replication: Microscopy studies were performed at least three times. Biochemical studies were performed at least twice. All attempts at replication were successful.
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- Blinding: The investigators were not blinded to group allocation during data collection or subsequent analysis, which is considered standard for biochemical and microscopy experiments done in these studies.

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| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a | Involved in the study | n/a | Involved in the study |
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| ☒ | Eukaryotic cell lines | ☒ | Flow cytometry |
| ☒ | Palaeontology | ☒ | MRI-based neuroimaging |
| ☒ | Animals and other organisms | | |
| ☒ | Human research participants | | |
| ☒ | Clinical data | | |

Antibodies

The following antibodies were generated by us, and have been described previously [Dai, J. et al. 2004, Dev Cell 7, 771-776, Li et al. 2007, J Cell Biol 178, 453-464, and Bai, M. et al. 2011, Nat Cell Biol 13, 559-567]: rabbit antibodies against ACAP1 (WB: 1:500), ARF6 (WB: 1:500), and cellubrevin (IF/WB: 1:500). The following antibodies were obtained from other investigators mouse T52/16 against integrin beta1 [L. Klickstein, Brigham and Women’s Hospital, USA; IF/IP/WB 1:5] and mouse 9E10 against the Myc epitope [R. Klaunser, National Institutes of Health, USA; IF/WB 1:500]. The following antibodies were obtained from commercial vendors. Mouse antibodies include: GST (Santa Cruz, sc-138, 1:200), Akt (Cell Signaling, 2920, IF 1:1000), beta-actin (Ambion, AM4302, WB 1:1000), Rab11 [BD Biosciences, 610656, IF 1:100], and clathrin antibodies [ATCC, TD-1, WB 1:5; and ATCC, X22, IF 1:5]. Rabbit antibodies include: 6xHis (Santa Cruz, sc-803, WB 1:200), and Akt (Cell signaling, 9272, IF/WB 1:1000). Conjugated secondary antibodies were obtained from Jackson ImmunoResearch: horsedarish peroxidase-conjugated donkey antibodies against mouse IgG (715-035-150, WB 1:10,000) and against rabbit IgG (711-035-152, WB 1:10,000). Cy2 donkey antibodies against mouse IgG (715-225-151, IF 1:200) and against rabbit IgG (711-225-152, IF 1:200), Cy3 goat antibody against mouse IgG (115-165-062, IF 1:200), and Cy3 donkey antibody against rabbit IgG (711-165-153, IF 1:200).

Validation

For antibodies generated by us or obtained from other investigators we validated them through knockdown experiments followed by immunofluorescence (IF), immunoprecipitation (IP), or western blotting (WB) at dilution as indicated above. These experiments were done on human and mouse cells. For antibodies obtained from commercial vendors, validation information is posted online by the vendors.

Eukaryotic cell lines

Policy information about: cell lines

Cell line source(s) HeLa, HEK293, and SF9 cells were obtained from ATCC.
| Authentication                                      | Cells were authenticated by ATCC using DNA profiling. |
|----------------------------------------------------|--------------------------------------------------------|
| Mycoplasma contamination                           | Cells were documented by ATCC to be free of mycoplasma contamination. |
| Commonly misidentified lines (See UACC register)   | No commonly misidentified cell lines were used.        |