

# An ACAP1-containing clathrin coat complex for endocytic recycling

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Whether coat proteins play a widespread role in endocytic recycling remains unclear. We find that ACAP1, a GTPase-activating protein (GAP) for ADP-ribosylation factor (ARF) 6, is part of a novel clathrin coat complex that is regulated by ARF6 for endocytic recycling in two key physiological settings, stimulation-dependent recycling of integrin that is critical for

cell migration and insulin-stimulated recycling of glucose transporter type 4 (Glut4), which is required for glucose homeostasis. These findings not only advance a basic understanding of an early mechanistic step in endocytic recycling but also shed key mechanistic insights into major physiological events for which this transport plays a critical role.

## Introduction

Coat proteins represent the core machinery by which transport from different intracellular membrane compartments is initiated. Coat proteins have two well-characterized functions: (1) deformation of compartmental membrane in forming transport vesicles and (2) cargo sorting that entails direct interaction with cargo proteins. Currently, three major coat complexes have been well characterized. The clathrin coat complex is composed of heavy and light chains that form a triskelion, which is coupled to different adaptors for transport from the plasma membrane and the trans-Golgi network. Coat protein I (COPI) and COPII complexes form vesicles that shuttle in the early secretory system that includes the ER and the Golgi complex (Kirchhausen, 2000; Bonifacino and Glick, 2004).

The ADP-ribosylation factor (ARF) family of small GTPases regulates the recruitment of coat proteins from cytosol to membrane in instigating vesicle formation. Their GTPase cycle is regulated by guanine nucleotide exchange factors that activate ARFs and GTPase-activating proteins (GAPs) that deactivate ARFs (Donaldson and Jackson, 2000; Nie et al., 2003). The better-characterized GAPs for ARF-related small GTPases,

such as ARFGAP1 for ARF1 and Sec23p for Sar1p, function not only as key negative regulators of their small GTPases (Yoshihisa et al., 1993; Cukierman et al., 1995) but also as their effectors by being core components of coat complexes (Barlowe et al., 1994; Yang et al., 2002). Exploring whether other GAPs for ARF members may exhibit a similar behavior, we previously identified ACAP1 (ARFGAP with coiled coil, ANK repeat, and pleckstrin homology domains), a GAP for ARF6 (Jackson et al., 2000), to possess a novel function in cargo sorting by recognizing sorting signals in the cytoplasmic domain of the transferrin receptor (TfR) for its endocytic recycling (Dai et al., 2004). Extending this finding, we have shown more recently that ACAP1 also functions in the cargo sorting of recycling integrin as an example of regulated recycling (Li et al., 2005). However, whether these elucidated roles of ACAP1 reflect its function as part of a coat complex remains unknown.

On a broader note, whether cargo sorting by ACAP1 represents an important mechanism of endocytic recycling also needs to be clarified. One notable view has been that the conventional mechanism of cargo sorting does not play a substantial role in endocytic recycling (Gruenberg, 2001; Maxfield and McGraw, 2004). Instead, early endosomes have been proposed to use mainly lipid-based mechanisms along with compartmental retention for the selective recycling of proteins to the plasma membrane (Maxfield and McGraw, 2004). This view has been propagated to a large extent by investigations into TfR recycling in nonpolarized cells, for which evidence for a recycling sorting

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Abbreviations used in this paper: ACAP, ARFGAP with coiled coil, ANK repeat, and pleckstrin homology domains; AP, adaptor protein; ARF, ADP-ribosylation factor; CHC, clathrin heavy chain; COPI, coat protein I; EGFR, EGF receptor; GAP, GTPase-activating protein; Glut4, glucose transporter type 4; TfR, transferrin receptor; VSVG, vesicular stomatitis virus G protein.

The online version of this article contains supplemental material.

signal had been lacking (Maxfield and McGraw, 2004) until recently (Dai et al., 2004). However, it should be noted that studies in polarized cells have identified a variant of the clathrin adaptor protein 1 (AP1), which contains the  $\mu$ 1B subunit (Folsch et al., 1999), to mediate polarized TfR recycling to the basolateral surface of the plasma membrane (Rodriguez-Boulan et al., 2004). Nevertheless, because other well-characterized examples of endocytic recycling have not revealed a role for the conventional mechanism of cargo sorting by coat proteins (Maxfield and McGraw, 2004), the extent that this mechanism is relevant for endocytic recycling remains to be defined.

Another well-characterized example of endocytic recycling has been the insulin-stimulated recycling of glucose transporter type 4 (Glut4; Bryant et al., 2002; Ishiki and Klip, 2005; Watson and Pessin, 2006). Myo1c, which binds to the actin filament, has been proposed to shuttle Glut4-containing transport vesicles to the plasma membrane along a cytoskeletal track (Bose et al., 2002). Exo70, which is a component of the tethering complex, has been proposed to function in the docking of Glut4 vesicles at the plasma membrane (Inoue et al., 2003). VAMP2 and syntaxin 4 have been identified as SNAREs for Glut4 recycling, which mediate the final fusion step (Cheatham

et al., 1996; Volchuk et al., 1996). However, notably absent has been the identification of a coat complex for the early mechanistic steps of this recycling. Instead, only compartmental retention has been suggested by the identification of Tether, containing a UBX domain, for Glut4 (TUG), which has been shown to bind selectively to Glut4 and retain it at internal endosomal compartments until insulin stimulation disrupts this binding for Glut4 recycling to the plasma membrane (Bogan et al., 2003).

We now show that ACAP1 is part of a novel clathrin coat complex that mediates the stimulation-dependent recycling of integrin and insulin-stimulated recycling of Glut4. Our findings not only advance a basic understanding of how transport is accomplished in endocytic recycling but also provide mechanistic insights into these key physiological events for which endocytic recycling plays a critical role.

## Results

### ACAP1 overexpression inhibits endocytic recycling by locking a coat onto membranes

As we had previously found that knocking down ACAP1 by siRNA inhibited endocytic recycling of both TfR (Dai et al., 2004)

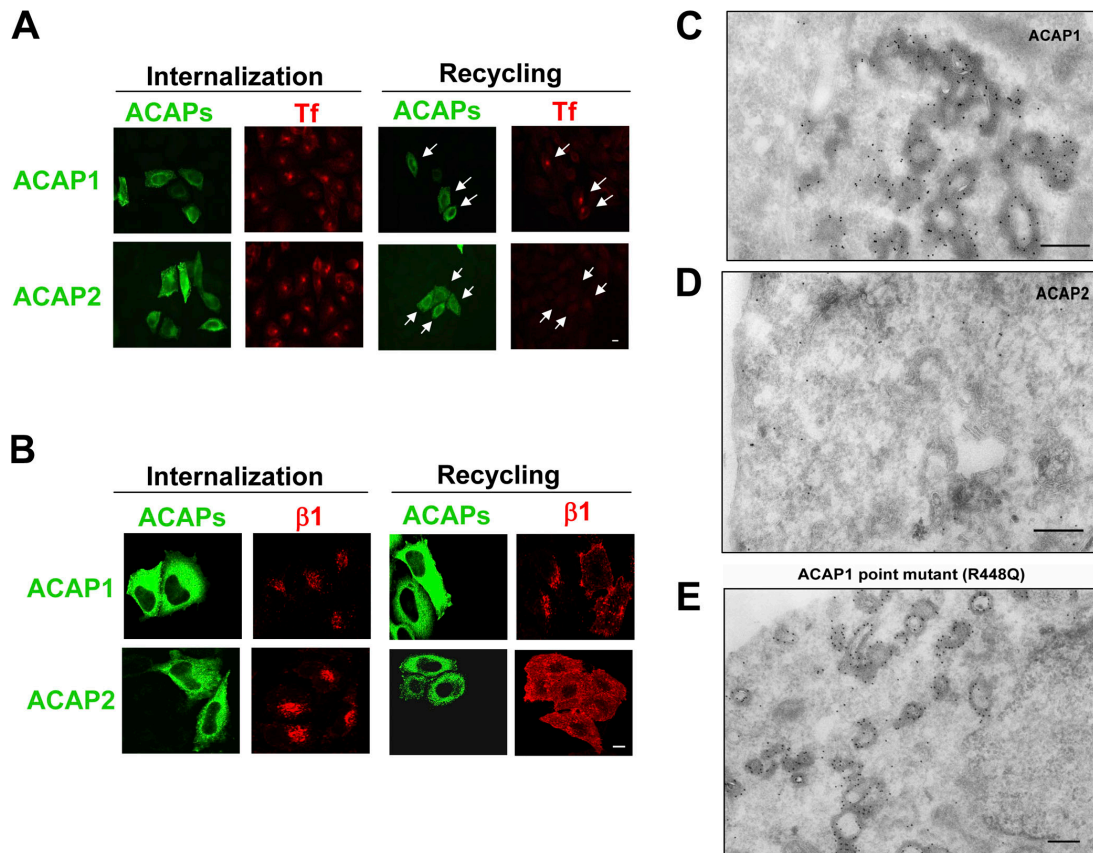


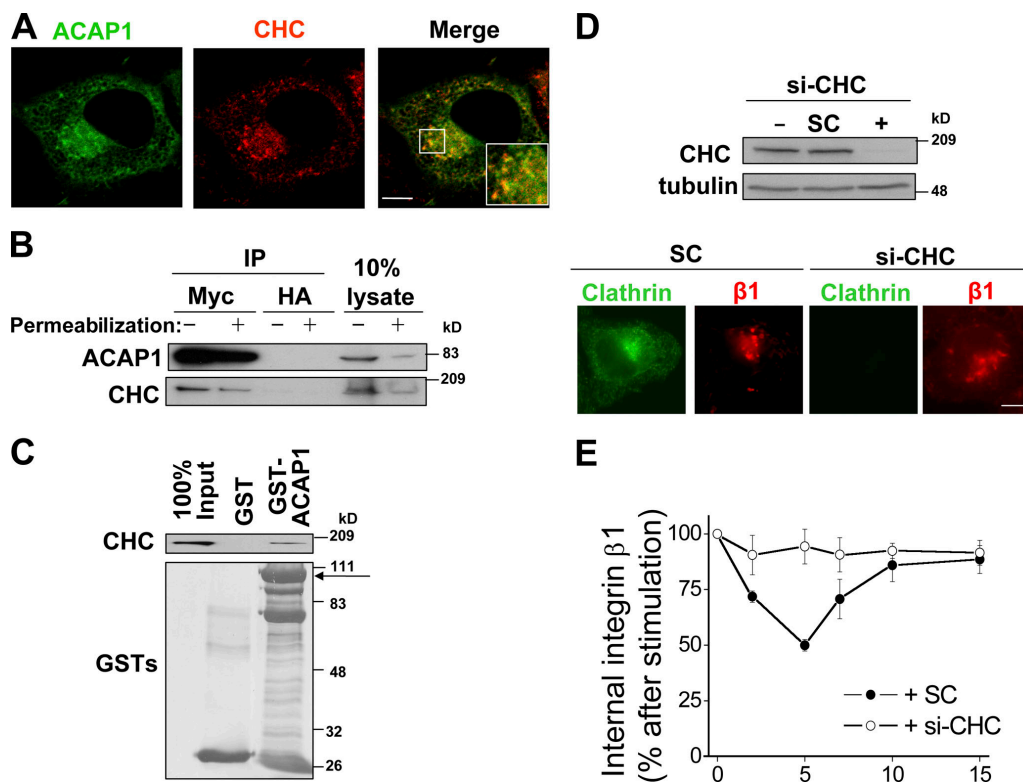
Figure 1. **Overexpression of ACAP1 inhibits endocytic recycling and induces membrane coating.** (A) Overexpression of ACAP1 but not ACAP2 inhibits TfR recycling from the pericentriolar recycling endosome but not its internalization to this compartment. TRVb-1 cells were transiently transfected with either Flag-ACAP1 or Flag-ACAP2 and assessed for the uptake and recycling of Alexa 594-conjugated transferrin by immunofluorescence microscopy. ACAPs are shown in green and transferrin in red. Bar, 15  $\mu$ m. Arrows indicate cells with high expression of transfected construct. (B) Integrin recycling is similarly affected by ACAP1 overexpression. HeLa cells were transiently transfected with Flag-ACAP1 or Flag-ACAP2 and then antibody-bound surface integrin and tracked for its internalization followed by recycling by immunofluorescence microscopy. ACAPs are shown in green and integrin in red. Bar, 10  $\mu$ m. (C–E) Overexpression of ACAP1 or its catalytic-dead mutant induces coated membrane structures. HeLa cells were transiently transfected with Flag-tagged ACAP1 (C), ACAP2 (D), or catalytic-dead mutant of ACAP1 (E) and fixed for immunogold EM using anti-Flag antibody. Bars, 200 nm.

and integrin (Li et al., 2005), we were surprised to find initially that overexpression of ACAP1 also inhibited the recycling of both TfR (Fig. 1 A) and integrin (Fig. 1 B). Inhibition of integrin recycling was further confirmed by a quantitative biochemical recycling assay (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200608033/DC1>), as we had done previously (Powelka et al., 2004; Li et al., 2005). The observed inhibitions appeared specific, as overexpression of other GAPs for ARF6, such as ACAP2, that had the greatest sequence similarity to ACAP1 (Jackson et al., 2000), did not have similar effects on either TfR (Fig. 1 A) or integrin (Fig. 1 B and Fig. S1 A) recycling. Moreover, internalization of surface TfR (Fig. 1 A) and integrin (Fig. 1 B) to the internal perinuclear recycling endosome was unaffected. ACAP1 overexpression also did not affect other major intracellular transport pathways, such as the secretory pathway, as assessed by a temperature-sensitive mutant of the vesicular stomatitis virus G protein (VSVG-ts045; Fig. S1 B), and endocytic transport to the lysosome, by examining the EGF receptor (EGFR) upon ligand binding at the cell surface (Fig. S1 C).

In considering how overexpressed ACAP1 achieved an apparent specific inhibition on endocytic recycling, we initially entertained the possibility that its GAP activity, which had been

shown to act on ARF6 (Jackson et al., 2000), might be enhanced, which might then inhibit ARF6-regulated endocytic recycling. A key prediction of this explanation was that overexpression of the catalytic-dead point mutant of ACAP1, previously generated by mutating residue 448 from arginine to glutamine (R448Q; Jackson et al., 2000), should abrogate the observed inhibition induced by the wild-type form. However, its overexpression also inhibited both TfR (Fig. S1 D) and integrin (Fig. S1 E) recycling.

In search of an alternate explanation, we noted that the GAPs for ARF family members in the better-characterized intracellular transport pathway acted not only upstream of ARFs as their negative regulators but also as their effectors by being components of coat complexes (Barlowe et al., 1994; Yang et al., 2002). Consistent with this possibility, we detected ACAP1 mainly on membranes that had an electron-dense coating by immunogold EM (Fig. 1 C). As control, overexpression of other GAPs for ARF6 did not exhibit a similar effect (for example, see ACAP2 overexpression in Fig. 1 D). Moreover, overexpression of the catalytic-dead mutant of ACAP1 induced a similar membrane coating (Fig. 1 E). Thus, as coat complexes need to cycle dynamically on and off their target membrane to accomplish



**Figure 2. Endosomal clathrin associates with ACAP1 and is required for integrin recycling.** (A) Endogenous CHC shows partial colocalization with transfected ACAP1. HeLa cells were transiently transfected with GFP-ACAP1 and examined by immunofluorescence microscopy for ACAP1 (green) and CHC (red). Bar, 10  $\mu$ m. (B) Clathrin interacts with ACAP1. HeLa cells were transiently transfected with ACAP1-Myc, either permeabilized (to release cytosol) or not, and lysed for immunoprecipitation followed by blotting proteins as indicated. (C) Clathrin can interact directly with ACAP1. ACAP1 as a GST fusion protein was bound to beads and incubated with soluble clathrin triskelia for a pull-down experiment. CHC was detected by immunoblotting for CHC, whereas GST proteins were detected by Coomassie staining. Arrow indicates the position of full-length GST-ACAP1. (D) Knocking down CHC does not prevent the internal accumulation of surface integrin  $\beta 1$ . HeLa cells were treated with siRNA against CHC. (top) Cell lysates from different conditions of siRNA treatment (SC denotes scrambled siRNA) were blotted for proteins as indicated. (bottom) Antibody-bound integrin was allowed to accumulate internally and then examined by immunofluorescence microscopy. Integrin is shown in red and clathrin in green. Bar, 10  $\mu$ m. (E) Knocking down CHC inhibits integrin recycling. A biochemical assay for integrin recycling was performed, with the graph showing the level of recycling integrin remaining internal at the times indicated. The mean from three independent experiments is shown with standard error.

a round of transport (Bonifacino and Glick, 2004), we considered the possibility that overexpressed ACAP1 locked a coat complex onto endosomal membrane to prevent endocytic recycling.

### Both ACAP1 and clathrin participate in integrin recycling

As further evidence in favor of this possibility, we initially examined the ACAP1-induced coating in more detail by immunogold EM. Using antibodies against different components of the currently known coat proteins, we detected no substantial labeling for subunits of AP1, AP2, AP3, AP4, COPI, COPII, and GGAs (Golgi-localized,  $\gamma$ -ear-containing, ARF-binding protein); however, we detected labeling for the clathrin heavy chain (CHC; unpublished data). The CHC couples with the light chain to form a triskelion, which is known to couple to distinct adaptors, resulting in different types of clathrin coat complexes (Owen et al., 2004). Consistent with this generalization, confocal microscopy revealed that overexpressed ACAP1 only showed a partial colocalization with endogenous CHC (Fig. 2 A). Thus, an intriguing implication was that ACAP1 functioned as part of a novel clathrin coat complex for endocytic recycling.

In favor of this possibility, we found that overexpressed ACAP1 coprecipitated with CHC using lysates derived from cells that overexpressed ACAP1, and in support of our hypothesis that overexpressed ACAP1 locked a coat onto membrane to inhibit endocytic recycling, we found that ACAP1 could be coprecipitated with CHC even when cells were first permeabilized to allow leakage of their cytosol before being subjected to the coprecipitation procedure (Fig. 2 B). Moreover, ACAP1 and CHC could interact directly, as assessed by a pull-down approach in which ACAP1 as a GST fusion protein was bound to beads and incubated with purified soluble clathrin triskelia (Fig. 2 C).

To assess whether ACAP1 acted in conjunction with the clathrin triskelion for endocytic recycling, we initially examined whether the recycling of a cargo protein known to be dependent on ACAP1 would be inhibited upon siRNA against CHC. Although we previously defined ACAP1 to be involved in TfR recycling (Dai et al., 2004), knocking down CHC is known to inhibit TfR internalization (Motley et al., 2003). Thus, as recycling could only occur after internalization from the plasma membrane, we overcame this confounding experimental hurdle

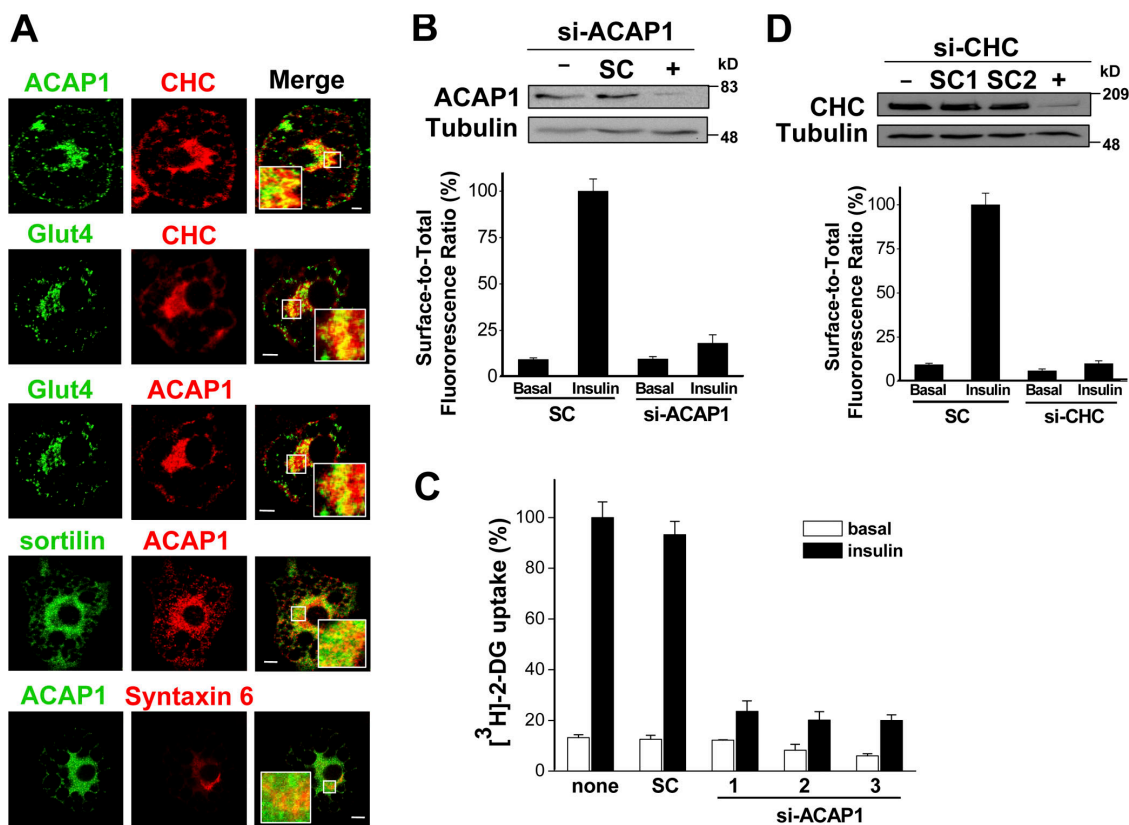


Figure 3. **Both ACAP1 and clathrin participate in Glut4 recycling in adipocytes.** (A) Localization of different proteins in adipocytes. Differentiated 3T3-L1 cells at the basal condition were examined by confocal microscopy for (top row) ACAP1 (green) and CHC (red), (second row) HA-Glut4-GFP (green) and CHC (red), (third row) HA-Glut4-GFP (green) and ACAP1 (pseudocolor), (fourth row) sortilin- $\delta$ xHis (green) and ACAP1 (red), and (bottom row) ACAP1 (green) and syntaxin 6 (red). Bars, 10  $\mu$ m. Note that staining for ACAP1, CHC, and syntaxin 6 involved endogenous proteins. (B) Knocking down ACAP1 inhibits insulin-stimulated redistribution of Glut4. Differentiated 3T3-L1 cells stably expressing HA-Glut4-GFP were treated with siRNA conditions as indicated. (top) Cell lysates were immunoblotted for proteins as indicated. (bottom) Cells were assayed for Glut4 translocation by measuring the level of surface Glut4 (tracked by HA antibody binding to unpermeabilized cells) when normalized to total Glut4 (indicated by GFP intensity), comparing basal versus stimulated condition. The mean from examination of 10 randomly selected cells is shown with standard error. (C) Knocking down ACAP1 also inhibits glucose uptake. Differentiated 3T3-L1 cells were treated with distinct siRNAs against ACAP1 and assessed by the cellular glucose uptake assay. The mean from three independent experiments is shown with standard error. (D) Knocking down CHC also inhibits insulin-stimulated redistribution of Glut4. The same experiment as described in B was performed, except cells were treated with siRNA against CHC.

by examining integrin recycling, as its internalization to the perinuclear recycling endosome was largely unaffected by siRNA against CHC (Fig. 2 D). In contrast, we found that integrin recycling was inhibited (Fig. 2 E).

### Both ACAP1 and clathrin also participate in Glut4 recycling

We next sought to confirm these findings using additional approaches that would examine ACAP1 under more physiological conditions, specifically, when ACAP1 was not overexpressed. As one clue, we noted the precedence that some coat complexes could be better observed on intracellular membrane in certain cell types whose physiological function involved its extensive use, such as COPII for transport from the ER in hepatocytes because it is needed for a hyperactive secretory pathway (Zeuschner et al., 2006). Pursuing this possibility, we eventually found that endogenous ACAP1 could be readily visualized in differentiated 3T3-L1 adipocytes and showed considerable colocalization with endogenous CHC (Fig. 3 A).

The 3T3-L1 adipocytes have been a model system to study insulin-stimulated Glut4 recycling, and Glut4 has been shown to reside in an internal endosomal compartment at the basal

condition, when no insulin stimulation is applied (Bryant et al., 2002; Ishiki and Klip, 2005; Watson and Pessin, 2006). A chimeric Glut4 construct, such as HA-Glut4-GFP (Zeigerer et al., 2002), has been used extensively to study Glut4 recycling, because the position of the two tags enabled a quantitative assessment of Glut4 recycling by fluorescence microscopy. When this construct was stably expressed in adipocytes, we found that both endogenous ACAP1 and CHC showed considerable colocalization with its internal pool under the basal (no insulin) condition (Fig. 3 A). The specificity of the observed staining for endogenous ACAP1 was confirmed by siRNA against ACAP1 (Fig. S2 A, available at <http://www.jcb.org/cgi/content/full/jcb.200608033/DC1>). As both sortilin (Shi and Kandror, 2005) and syntaxin 6 (Shewan et al., 2003) had been shown to have colocalization with internal Glut4, we also examined these two proteins. ACAP1 showed considerable colocalization with sortilin and, to a lesser extent, with syntaxin 6 (Fig. 3 A). Moreover, providing further support for our hypothesis that ACAP1 overexpression inhibited endocytic recycling by locking a coat onto membrane, we found that endogenous ACAP1 and CHC could no longer be detected in permeabilized adipocytes (Fig. S2 B). In contrast, permeabilization of HeLa cells with overexpressed

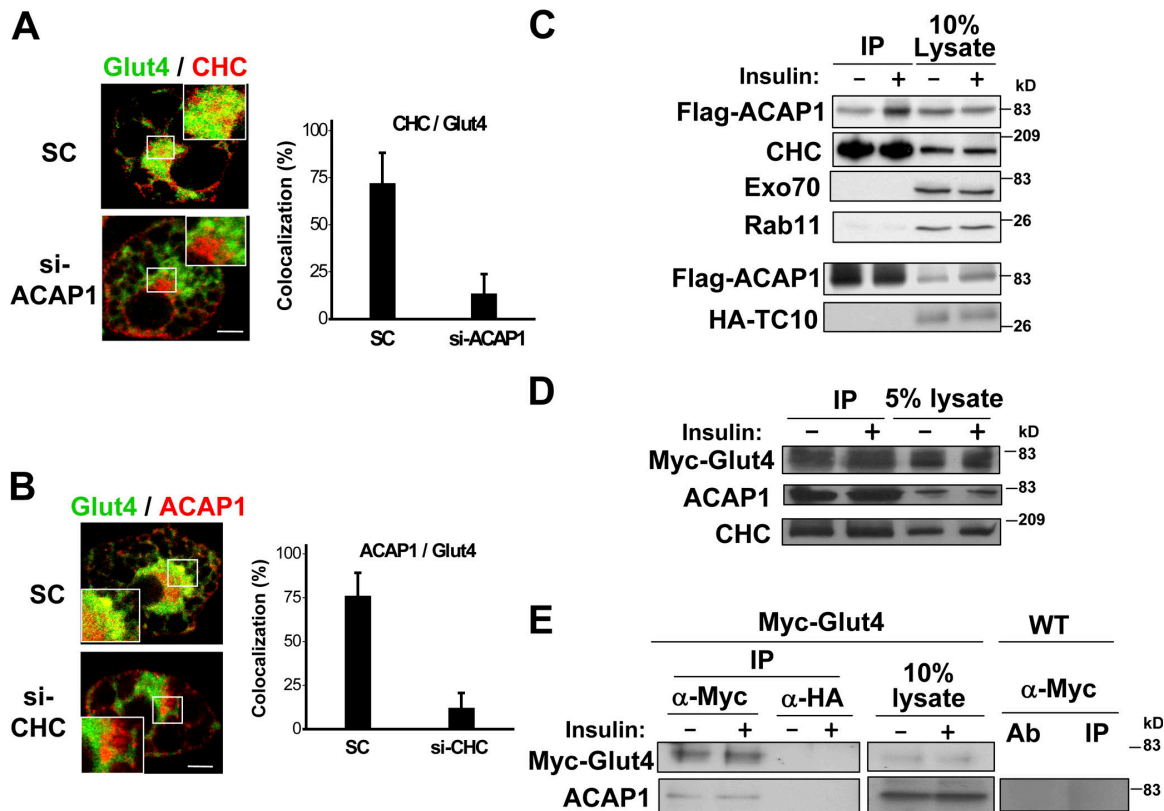
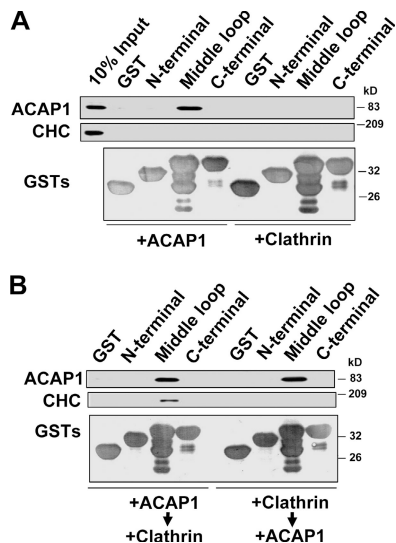


Figure 4. **Interaction between ACAP1 and CHC.** (A) Knocking down ACAP1 reduces the colocalization of CHC with internal Glut4. Differentiated 3T3-L1 cells stably expressing HA-Glut4-GFP were treated with siRNA against ACAP1 and examined at the basal condition by confocal microscopy for Glut4 (green) and CHC (red). Bar, 10  $\mu$ m. 10 cells were also randomly selected and quantified for the fraction of Glut4 that colocalized with CHC. The mean with standard error is shown. (B) Knocking down CHC also reduces the colocalization of ACAP1 with internal Glut4. The same experiment as described in A was performed, except that cells treated with siRNA against CHC and the degree of colocalization between Glut4 (green) and ACAP1 (red) was assessed. (C) ACAP1 interacts with CHC. Differentiated 3T3-L1 cells transfected with Flag-ACAP1 and treated with conditions of stimulation as noted were lysed followed by immunoprecipitation using an anti-Flag antibody and immunoblotting for proteins as indicated. TC10 was detected by transfecting cells with HA-TC10. (D) Both ACAP1 and CHC are in a complex with Glut4. Differentiated 3T3-L1 cells stably expressing Myc-Glut4 were analyzed in a co-precipitation experiment as described in C. (E) Specificity controls for immunoprecipitating Myc-Glut4. Experiment described in D was expanded to include additional controls as indicated.



**Figure 5. Determining how ACAP1 and CHC interact with Glut4.** (A) The middle cytoplasmic loop of Glut4 interacts directly with ACAP1. The different cytoplasmic domains of Glut4 as indicated were bound to beads as GST fusion proteins and incubated with either soluble ACAP1 or clathrin triskelium. Bound soluble components were detected by immunoblotting, whereas GST proteins were detected by Coomassie staining. (B) ACAP1 bridges the binding of clathrin triskelium to Glut4. The same pull-down experiment was performed as in A, except that soluble proteins were incubated sequentially as indicated. Note that unbound soluble proteins after the first incubation were removed before the second incubation.

ACAP1 retained a compact perinuclear distribution (Fig. S2 C), as previously described (Dai et al., 2004).

Intriguingly, we also found that endogenous ACAP1 detected on endosomal membrane was dependent on the differentiation state of the 3T3-L1 cells. Although it was readily visualized in the differentiated (adipocyte) state (Fig. 3 A), this staining was difficult to detect in the undifferentiated (fibroblast) state (Fig. S2 D). In contrast, staining for sortilin was not similarly affected (Fig. S2 E). The observed difference in ACAP1 staining could not be explained at the level of protein expression, as endogenous ACAP1 could be detected in either state by Western blotting (Fig. S2 F), implying a more subtle explanation for the observed phenomenon. Nevertheless, as our findings thus far on 3T3-L1 cells suggested the possibility that insulin-stimulated Glut4 recycling is an example of extensive physiological usage of ACAP1, we sought to further define the mechanistic relationship between ACAP1 and the clathrin triskelion in these cells, using Glut4 recycling as the context.

Initially, using quantitative microscopy to assess chimeric Glut4 recycling as previously described (Zeigerer et al., 2002), we found that siRNA against ACAP1 substantially inhibited the insulin-induced redistribution of internal Glut4 to the cell surface (Fig. 3 B). This result was further confirmed by an assay for the cellular uptake of glucose (Fig. 3 C), which had been shown to reflect Glut4 recycling (Bogan et al., 2003; Shi and Kandror, 2005). Using the same assay, we also demonstrated the specificity of the siRNA against ACAP1, as three distinct targeting sequences all led to similar levels in the inhibition of glucose uptake (Fig. 3 C). Further specificity for the siRNA approach was reflected by the silencing of ACAP1 not having a

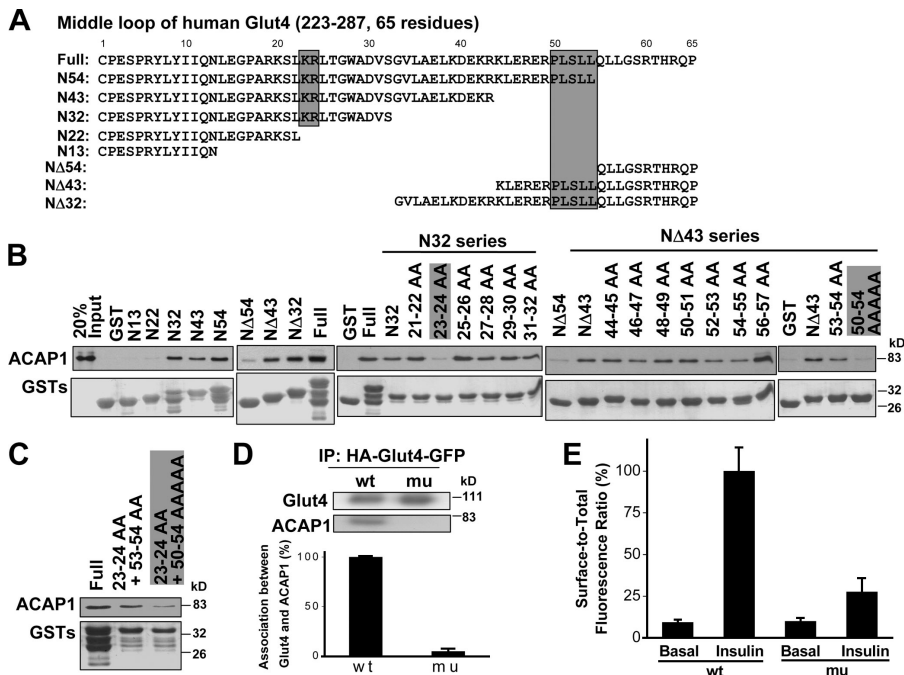
considerable effect on the level of CHC, ARF6, and select signaling proteins (Fig. S3 A, available at <http://www.jcb.org/cgi/content/full/jcb.200608033/DC1>). Moreover, we found that the internalization of Glut4 to its internal perinuclear location at the basal condition was not substantially affected (Fig. S3 B). Silencing CHC, we found that Glut4 recycling was also markedly inhibited (Fig. 3 D). Notably, siRNA against CHC did not prevent the accumulation of internal Glut4 in the basal condition (Fig. S3 C), which was consistent with surface Glut4 previously shown to internalize by both clathrin and nonclathrin means (Blot and McGraw, 2006).

#### Evidence that ACAP1 is part of a novel clathrin coat complex

To provide more direct evidence that ACAP1 acted in conjunction with CHC for Glut4 recycling, we took multiple approaches. First, we assessed whether ACAP1 and clathrin required each other for localization to the Glut4 compartment, by examining whether the localization of one was affected upon silencing the other. In differentiated 3T3-L1 cells treated with siRNA against ACAP1, we found that endogenous CHC had reduced colocalization with Glut4, with quantitation revealing about a fivefold reduction (Fig. 4 A). In cells treated with siRNA against CHC, we found that endogenous ACAP1 also had reduced colocalization with Glut4, with quantitation again revealing about a fivefold reduction (Fig. 4 B). In contrast, siRNA against neither ACAP1 nor CHC had a considerable effect on the colocalization of Glut4 with sortilin (Fig. S4 A, available at <http://www.jcb.org/content/full/jcb.200608033/DC1>). Similarly, the colocalization of Glut4 and syntaxin 6 was not affected considerably by either siRNA treatment (Fig. S4 B).

Second, we found that ACAP1 associated with CHC in differentiated adipocytes through a coimmunoprecipitation approach (Fig. 4 C). As specificity, other transport factors known to participate in Glut4 recycling, such as Exo70 (Inoue et al., 2003) and TC10 (Chiang et al., 2001), did not associate with this complex, and Rab11 (Zeigerer et al., 2002) showed very weak association (Fig. 4 C). In contrast, we detected a complex of Glut4 with both ACAP1 and CHC (Fig. 4 D), for which the specificity of the immunoprecipitating antibody was verified (Fig. 4 E). Notably however, although we had found that the association of ACAP1 with integrin  $\beta$ 1 was stimulation dependent (Li et al., 2005), insulin stimulation did not enhance the association of Glut4 with either ACAP1 or CHC (Fig. 4, D and E).

Third, because cargo sorting by coat proteins involves their direct interaction with cargo proteins (Bonifacino and Glick, 2004), we examined whether, and potentially how, the ACAP1-containing clathrin coat complex interacted with Glut4. Glut4 is a multispanning transmembrane protein that contains three prominent cytoplasmic domains, at the N terminus and at the C terminus, and also having a middle cytoplasmic loop (Watson and Pessin, 2006). Thus, we appended each domain to GST and initially examined which domains bound directly to soluble ACAP1 in a pull-down assay, as previously done (Dai et al., 2004; Li et al., 2005). ACAP1 was found to interact directly with the middle cytoplasmic loop, whereas the clathrin triskelion showed no substantial binding to any of the domains examined



**Figure 6. Key residues in Glut4 that mediate its direct binding to ACAP1 define recycling sorting signals.** (A) A scheme showing the sequence of the middle domain of Glut4, with truncation constructs as noted. Critical residues that define recycling sorting signals are boxed. (B) Systematic mutagenesis of the middle domain to identify key residues within two distinct regions that mediate binding to ACAP1. Different constructs as noted were generated as GST fusions and incubated with ACAP1 in pull-down experiments. Truncation mutants were initially screened (left), followed by alanine-scanning mutagenesis of two regions identified to bind ACAP1 (middle and right). ACAP1 was detected by immunoblotting, whereas GST fusions were detected by Coomassie staining. (C) Mutation of key residues within two distinct regions of the entire middle domain reduces its binding to ACAP1. Experiments similar to that described in B were performed using different GST fusions as noted. (D) Mutation of key residues in Glut4 prevents its interaction with ACAP1 in vivo. Wild-type or a mutant form of HA-Glut4-GFP (generated by replacing cytoplasmic domain, as defined in A, to alanines) was transfected into differentiated 3T3-L1 cells. Cell lysates were immunoprecipitated for the

chimeric Glut4 followed by immunoblotting for ACAP1. The mean from three experiments with standard error is also shown. (E) Key residues in Glut4 that mediate its binding to ACAP1 represent recycling sorting signals. Wild-type or mutant form of HA-Glut4-GFP (as described in D) were transfected into differentiated 3T3-L1 cells and assayed for their translocation by measuring their surface level (tracked by HA antibody binding to unpermeabilized cells) when normalized to their total (indicated by GFP intensity), comparing basal versus stimulated condition. The mean from examination of 10 randomly selected cells is shown with standard error.

(Fig. 5 A). We also assessed the relationship between ACAP1 and the CHC in binding to the middle domain of Glut4. Although the sequential incubation of ACAP1 followed by the clathrin triskelion resulted in both being recruited to the Glut4 fusion protein, the converse sequential incubation prevented the recruitment of CHC (Fig. 5 B). Thus, these results suggested that ACAP1 acted like AP adaptors in bridging an interaction between cargo tails and clathrin triskelion.

We also determined residues in the middle domain of Glut4 critical for its binding to ACAP1. A systematic mutagenesis approach was undertaken that initially involved truncation mutants of this domain (Fig. 6 A). These constructs were expressed as GST fusion proteins on beads and then analyzed for binding to soluble ACAP1. Remarkably, similar to our previous finding for TfR (Dai et al., 2004), we also found that ACAP1 bound to two distinct regions in the middle domain of Glut4 (Fig. 6 B). Subsequently, a more detailed analysis of these regions by alanine scanning mutagenesis revealed a requirement for two basic residues (KR) in one region (Fig. 6 B). For the other region, however, the approach of systematically mutating two tandem residues at a time only led to a mild reduction in binding to ACAP1 (Fig. 6 B). Focusing on residues where these mild reductions were detected, we found that a more extensive replacement with alanines at this subregion that consisted mostly of hydrophobic residues (PLSLL) resulted in a more dramatic reduction in the binding of ACAP1 to this second region in the middle domain of Glut4 (Fig. 6 B). When all these mutations were introduced into the entire middle domain of Glut4, we found that its binding to ACAP1 became reduced (Fig. 6 C). Confirming this result in the context of adipocytes using a

coprecipitation approach, we also found that mutations introduced in the context of the entire chimeric Glut4 construct also led to reduced association with ACAP1 (Fig. 6 D). Importantly, this mutant construct also exhibited reduced recycling in response to insulin (Fig. 6 E). Thus, key residues in Glut4 needed for its binding to ACAP1 represented recycling sorting signals.

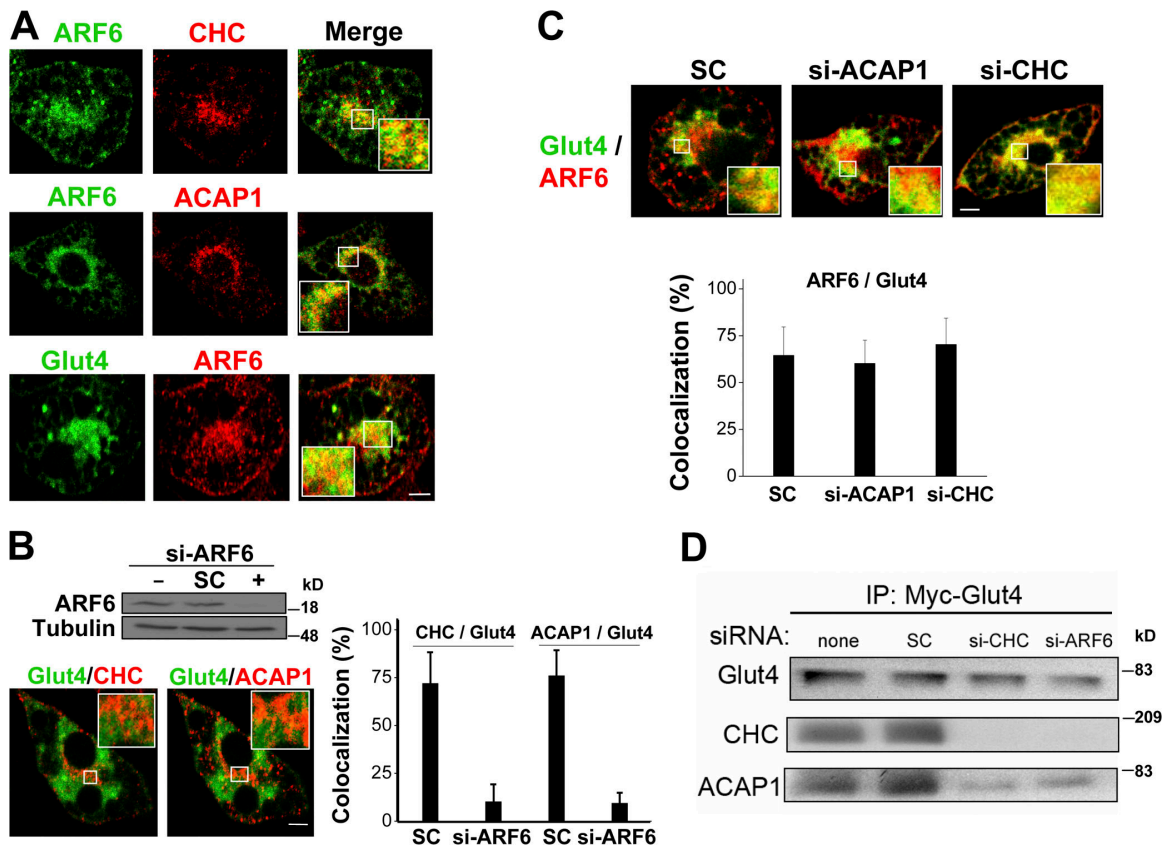
#### Evidence that ARF6 regulates the novel ACAP1-containing clathrin coat complex

As ACAP1 has been shown to be a GAP for ARF6 (Jackson et al., 2000), a prediction was that the novel clathrin coat complex would be regulated by ARF6. Consistent with this prediction, we found that endogenous ARF6 in differentiated 3T3-L1 cells showed considerable colocalization with ACAP1, CHC, and internal Glut4 at the basal condition (Fig. 7 A). Remarkably, like endogenous ACAP1, we also found that endogenous ARF6 was also difficult to visualize in undifferentiated 3T3-L1 cells (Fig. S5 A, available at <http://www.jcb.org/cgi/content/full/jcb.200608033/DC1>). Moreover, consistent with the general paradigm that ARFs acts upstream of coat complexes (Donaldson and Jackson, 2000; Nie et al., 2003), we found that both endogenous CHC and ACAP1 showed reduced colocalization with Glut4 in differentiated 3T3-L1 cells treated with siRNA against ARF6, with quantitation revealing about a fivefold reduction for both cases (Fig. 7 B). In contrast, the fraction of ARF6 that colocalized with internal Glut4 was not considerably altered by the knockdown of either ACAP1 or CHC (Fig. 7 C). Confirming these results, we also found that silencing ARF6 disrupted the physical association between Glut4 and components of the novel clathrin coat complex (Fig. 7 D and Fig. S5 B).

We also sought more direct evidence that ARF6 played a role in Glut4 recycling. First, the insulin-induced redistribution of internal Glut4 to the plasma membrane was dramatically reduced upon siRNA against ARF6 (Fig. 8 A). In contrast, this knockdown had no considerable effect on the accumulation of Glut4 internally (Fig. S5 C). Second, we found that silencing ARF6 reduced glucose uptake into adipocytes to an extent similar to that seen for silencing either ACAP1 or CHC (Fig. 8 B). Third, a biochemical fractionation approach had been used previously to track the formation of Glut4 vesicles from endosomal membrane, whereby Glut4 in vesicular membrane was distinguished from that on compartmental membrane by velocity sedimentation of cell homogenate (Shi and Kandror, 2005). Using this approach, we found that knocking down ACAP1, CHC, or ARF6 all redistributed Glut4, sortilin, and TfR (to a lesser extent) from a fraction that reflected its distribution in vesicular membrane to that in compartmental membrane (Fig. 8 C).

Finally, as GAP activity acts mechanistically upstream of ARF small GTPases, whereas coat complexes act downstream

as their major effectors (Donaldson and Jackson, 2000; Nie et al., 2003), we considered a likely possibility that ACAP1 overexpression in HeLa cells had perturbed the ability of ACAP1 to act as coat component, thereby obscuring our ability previously (Fig. S1, D and E) to determine whether its GAP activity also played a role in endocytic recycling. To overcome this hurdle, we noted that the stable transfection of ACAP1 did not have a similar effect as its transient transfection (Li et al., 2005), likely because the former approach is known to express proteins at a more physiological level. Thus, to examine whether the GAP activity of ACAP1 played a role in Glut4 recycling, we sought to replace endogenous wild-type ACAP1 with the catalytic-dead mutant form by stable transfection. Moreover, as the sequence in mouse ACAP1 targeted by the siRNA showed considerable divergence with the human ACAP1, we knocked down endogenous ACAP1 in the mouse adipocytes and then stably transfected the human forms. Using this approach, we found that the catalytic-dead mutant did not restore glucose uptake to a level similar to that seen for the wild-type form (Fig. 8 D).



**Figure 7. Regulation of the novel clathrin coat complex by ARF6.** (A) Endogenous ARF6 shows considerable colocalization with endogenous ACAP1 and CHC and with internal Glut4 in differentiated 3T3-L1 cells. Differentiated 3T3-L1 cells were examined under the basal condition by confocal microscopy for the following combinations: (top) ARF6 (green) and CHC (red), (middle) ARF6 (green) and ACAP1 (red), and (bottom) ARF6 (red) and Glut4 (green). Bar, 10  $\mu$ m. (B) Knocking down ARF6 reduces the colocalization of both ACAP1 and CHC with internal Glut4. Differentiated 3T3-L1 cells stably expressing HA-Glut4-GFP were treated with siRNA against ARF6. (top) Cells were blotted for proteins as indicated. (bottom) Cells were also examined at the basal condition by confocal microscopy for Glut4 (green) and CHC (red), or Glut4 (green) and ACAP1 (pseudocolored). Bar, 10  $\mu$ m. The degree of colocalization derived from examining 10 randomly selected cells was also quantified. (C) Knocking down either ACAP1 or CHC does not affect the colocalization of ARF6 and internal Glut4. Differentiated 3T3-L1 cells stably expressing HA-Glut4-GFP were treated with siRNA against either ACAP1 or CHC and examined at the basal condition by confocal microscopy for Glut4 (green) and ARF6 (red). Bar, 10  $\mu$ m. The degree of colocalization derived from examining 10 randomly selected cells was also quantified. (D) Knocking down either CHC or ARF6 disrupts the association of Glut4 with ACAP1 and CHC. Cell lysates derived from differentiated 3T3-L1 cells stably expressing Myc-Glut4 were immunoprecipitated using the anti-Myc antibody followed by immunoblotting for proteins as indicated.



Moreover, providing an explanation for why expression of the human wild-type form did not restore glucose uptake to the control condition (when no silencing was achieved), we found that its stable expression resulted in a lower level of ACAP1 than that seen for the endogenous situation (Fig. S5 D). Thus, we concluded that the GAP activity of ACAP1 also played a role in Glut4 recycling.

## Discussion

We provide evidence that ACAP1 is part of a novel clathrin coat complex that mediates endocytic recycling. Initial insight came from studies on HeLa cells. However, because endosomal ACAP1 could not be readily visualized in these cells by morphological techniques unless ACAP1 was overexpressed, we subsequently pursued further studies in differentiated 3T3-L1 adipocytes in which endogenous endosomal ACAP1 was readily detectable. This situation has allowed us to take additional experimental approaches in a more physiological context to provide further evidence that ACAP1 functions as part of a novel clathrin coat complex in endocytic recycling.

### A coat complex important for endocytic recycling

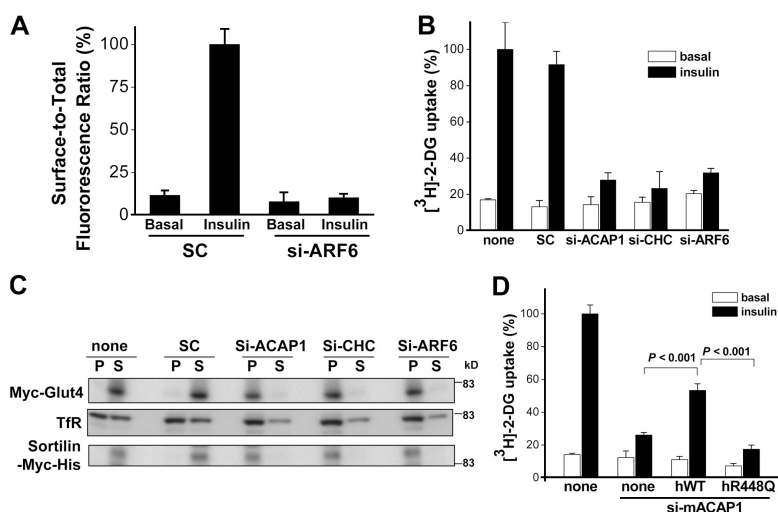
An initial puzzling observation was that transient overexpression of ACAP1 in HeLa cells inhibited the recycling of both TfR and integrin, as we had previously found that siRNA against ACAP1 also inhibited these recycling events (Dai et al., 2004; Li et al., 2005). Providing an explanation, we find that the acute overexpression of ACAP1 in HeLa cells stabilizes a coat onto endosomal membrane. In this setting, EM examination also reveals coated membrane structures that are pleomorphic, with some appearing characteristic of transport vesicles, with others being less spherical, suggesting that they are coated endosomal compartments. Thus, we propose that vesicle formation from endosomal compartments is being perturbed by ACAP1 overexpression,

because incompletely assembled coat complexes are formed as a result of the limiting level of endogenous clathrin.

A clathrin coating had been previously visualized on endosomal membrane by EM examination (Stoorvogel et al., 1996) and was suggested to participate in endocytic recycling (van Dam and Stoorvogel, 2002). However, its composition, regulation, and physiological significance has remained obscure, particularly as the prevailing view has been that the conventional mechanism of cargo sorting by coat proteins is unlikely to be important for endocytic recycling (Maxfield and McGraw, 2004). These issues are addressed in the current study, as we have found that a novel ACAP1-containing clathrin coat complex that is regulated by ARF6 functions in two key physiological settings that require endocytic recycling, integrin recycling critical for cell migration (Jones et al., 2006), and Glut4 recycling critical for glucose homeostasis (Bryant et al., 2002; Ishiki and Klip, 2005; Watson and Pessin, 2006). Altogether, these results point to a need to reconsider the current paradigm that conventional mechanism of cargo sorting through coat proteins is unlikely to be important for endocytic recycling.

### Elucidating key mechanistic steps of Glut4 recycling

Regulated endocytic recycling of Glut4 is considered a key mechanism by which insulin regulates glucose (Bryant et al., 2002; Ishiki and Klip, 2005; Watson and Pessin, 2006). Thus far, the only mechanism suggested for the initial step of this transport itinerary involves TUG, which has been proposed to retain Glut4 in an insulin-dependent manner (Bogan et al., 2003). We have now considerably expanded a mechanistic understanding of the early step in Glut4 recycling by identifying an ACAP1-containing clathrin coat complex for this process, as coat complexes are involved in coupling vesicle formation with cargo sorting (Bonifacino and Glick, 2004). However, although we previously found that stimulatory signaling regulates the role of ACAP1 in the cargo sorting of recycling integrin (Li et al., 2005), we find



**Figure 8. Characterizing the novel coat complex regulated by ARF6 in Glut4 recycling.** (A) Knocking down ARF6 inhibits insulin-stimulated redistribution of Glut4. Differentiated 3T3-L1 cells transfected with HA-Glut4-GFP were treated with different siRNA conditions as noted and assayed for Glut4 translocation by measuring the level of surface Glut4 (tracked by HA antibody binding to unpermeabilized cells) when normalized to total Glut4 (indicated by GFP intensity), comparing basal versus stimulated condition. The mean from examination of 10 randomly selected cells is shown with standard error. (B) Silencing ACAP1, CHC, or ARF6 leads to similar levels of reduction in the cellular uptake of glucose. Differentiated 3T3-L1 cells were treated with different siRNA conditions as noted and assayed for the uptake of radioactive 2-deoxy-glucose. The mean from three independent experiments is shown with standard error. (C) Silencing ACAP1, CHC, or ARF6 redistributes Glut4, sortilin, and TfR from vesicular membranes to compartmental membranes. Differentiated 3T3-L1 cells stably expressing both myc-Glut4 and sortilin-myc-his were homogenized and subjected to centrifugation to derive pellet that contains compartmental membrane and supernatant that contains vesicular membrane and cytosol. Both fractions were immunoblotted for endosomal proteins as indicated to reveal their relative distribution in compartmental and vesicular membranes. (D) GAP activity of ACAP1 plays a role in Glut4 recycling. 3T3-L1 cells were stably transfected with either human wild-type or mutant (R448Q) ACAP1. After differentiation, cells were silenced for endogenous mouse ACAP1 and assayed for the cellular uptake of glucose as described in B.

blotted for endosomal proteins as indicated to reveal their relative distribution in compartmental and vesicular membranes. (D) GAP activity of ACAP1 plays a role in Glut4 recycling. 3T3-L1 cells were stably transfected with either human wild-type or mutant (R448Q) ACAP1. After differentiation, cells were silenced for endogenous mouse ACAP1 and assayed for the cellular uptake of glucose as described in B.

in the current study that insulin stimulation does not enhance the association of ACAP1 with Glut4, suggesting that cargo sorting of Glut4 by ACAP1 is not similarly targeted by insulin stimulation.

Another notable observation is that the redistribution of sortilin from a fraction that likely represented its distribution in vesicular membrane to that in compartmental membrane upon the perturbation of ARF6, ACAP1, or CHC mirrors that of Glut4 (Fig. 8 C). This result further supports a recent proposal that sortilin plays an important role in the biogenesis of Glut4 vesicles by being a key cargo protein of these vesicles (Shi and Kandror, 2005). We also note that the same perturbations affect the relative distributions of TfR to a lesser extent. We suspect that a likely reason for why some portion of TfR still exists in the fraction that likely represents vesicular membrane is that TfR recycling is known to occur from both the sorting and the recycling endosome (Maxfield and McGraw, 2004). Thus, as ACAP1 mediates TfR recycling only from the latter compartment (Dai et al., 2004), one would predict that TfR can still be transported in vesicles that recycle from the sorting endosome.

Our results also shed insight into how cargo sorting of Glut4 is achieved by the novel clathrin coat complex. Notably, there is a striking similarity between key residues in Glut4 that represent recycling sorting signals as compared with those that we previously elucidated in TfR (Dai et al., 2004). In both cases, ACAP1 binds to two distinct regions within the cytoplasmic domain. Moreover, one region shares similarity in positively charged residues, whereas the other region shares similarity in predominantly hydrophobic residues. In considering how ACAP1 can recognize such diverse sequences on cargo proteins, one possibility is suggested by studies on Sec23p, the GAP for Sar1p in COPII transport, which has revealed multiple domains in recognizing distinct sorting signals on cargo proteins (Miller et al., 2003). Thus, it will be interesting to determine in the future whether a similar explanation applies to cargo sorting by ACAP1.

Finally, we note that Glut4 recycling in adipocytes is thought to involve a specialized transport pathway that is distinct from a “generic” recycling pathway, as tracked by TfR recycling (Watson and Pessin, 2006). Thus, as we found previously that ACAP1 also mediates TfR recycling (Dai et al., 2004), how can the same coat complex seemingly mediate two distinct populations of transport vesicles? One possibility is suggested by recent insights into the clathrin AP2 complex. Not only have accessory proteins been identified that couple distinct subsets of cargo proteins with the core AP2 clathrin complex (Owen et al., 2004), but this differential coupling is also implicated to form different populations of transport vesicles (Puthenveedu and von Zastrow, 2006). Thus, a future goal will be to determine whether the novel ACAP1-containing clathrin coat complex that we propose to have identified will couple to distinct accessory proteins in forming multiple populations of transport vesicles.

## Materials and methods

### Reagents and cells

Alexa 594-labeled transferrin was obtained from Invitrogen. Draq5 to stain DNA was obtained from Biostatus. EGF, insulin, and other chemicals (unless specified otherwise) were obtained from Sigma-Aldrich. <sup>3</sup>H-2-deoxy-D-glucose was obtained from PerkinElmer. HeLa and TRVb1 cells were cultured

as previously described (Dai et al., 2004). 3T3-L1 fibroblast was obtained and cultured following the manufacturer's instructions (American Type Culture Collection). 3T3-L1 cells stably expressing Myc-Glut4 or both Myc-Glut4 and sortilin-Myc-6xHis were cultured as previously described (Shi and Kandror, 2005). The differentiation of 3T3-L1 was performed as previously described (Shi and Kandror, 2005). Purified proteins that have been previously described are clathrin triskelia (obtained from W. Boll, Harvard Medical School, Boston, MA) and 6xHis-tagged ACAP1 (Dai et al., 2004).

### Antibodies

The following antibodies were described previously (Jackson et al., 2000; Dai et al., 2004; Powelka et al., 2004; Li et al., 2005): mouse TS2/16 against  $\beta$ 1 integrin, mouse DM1 $\alpha$  against  $\alpha$ -tubulin, mouse 9E10 against the Myc epitope, mouse M2 against the Flag epitope, mouse 15E6 against the C-terminal HA epitope, rabbit anti-ACAP1, rabbit anti-cellubrevin, rabbit anti-ARF6, rabbit anti-Lamp1, mouse M3A5 against  $\beta$ -COP, rabbit anti-Akt, and secondary antibodies conjugated to Cy2, Cy3, or Cy5.

Additional antibodies acquired for the current study include the following: mouse anti-6xHis epitope and mouse HA.11 against the HA epitope (Covance Research Products), mouse TD.1 and X22 against CHC (American Type Culture Collection), mouse anti-syntaxin 6 (BD Biosciences), rabbit anti-Rab11 (Zymed Laboratories), mouse anti-phospho-Akt (Ser473; Cell Signaling), mouse 4G10 against phosphor-Tyr (Upstate Biotechnology), rabbit anti-clathrin (obtained from S. Corvera, University of Massachusetts Medical Center, Worcester, MA), mouse anti-EGFR (obtained from H. Band, Northwestern University Medical Center, Evanston, IL), rabbit anti-ERK and phosphor-ERK (obtained from J. Blenis, Harvard Medical School, Boston, MA), rabbit anti-Exo70 (obtained from P. Brennwald, University of North Carolina at Chapel Hill, Chapel Hill, NC), and rabbit anti-Glut4 (obtained from S. Cushman, National Institutes of Health, Bethesda, MD).

### Plasmids and transfections

The following plasmids were used: Flag-tagged human ACAP1 wild-type and catalytic-dead mutant (R448Q), ACAP2 (Jackson et al., 2000), Myc-tagged human ACAP1 (Li et al., 2005), HA-Glut4-GFP (obtained from T. McGraw, Cornell University Medical School, New York, NY), and GFP-tagged VSVG-ts045 (obtained from J. Lippincott-Schwartz, National Institutes of Health, Bethesda, MD). GFP-tagged ACAP1 in pEGFP-C1 was generated by subcloning the coding sequence of ACAP1. To append ACAP1 or Glut4 cytoplasmic domains to the C terminus of GST, the coding sequences of ACAP1 or Glut4 cytoplasmic domains were amplified by PCR. ACAP1 was then subcloned into the BamHI and EcoRI sites of pGEX-4T-3 vector (GE Healthcare), whereas the Glut4 constructs were subcloned into the EcoRI and NotI sites. Although GST fusion constructs of Glut4 expressed well using the bacterial system, GST-ACAP1 did not. Thus, we subsequently transferred it into the NotI site of pVL1392 for baculovirus expression. Point mutants of Glut4 were generated by using QuikChange II XL site-directed mutagenesis kit (Stratagene).

Transient transfections were performed using Fugene 6 (Roche Biochemicals) for HeLa cells or electroporation as previously described (Bose et al., 2001) for differentiated 3T3-L1. 3T3-L1 cell lines that stably express HA-Glut4-GFP, Myc-tagged human ACAP1 wild type, or Flag-tagged human ACAP1 R448Q were generated by transient transfection with selection in 1 mg/ml G418 (Life Technologies) and maintained in 0.2 mg/ml G418.

### siRNA

siRNAs against the sequences CGACATCATGGAATTCGTA, TAAGGACCCTGTAACCGTG, and AGACGTATCTCGACATAIT for mouse ACAP1 (nucleotides 558–576, 903–921, and 2129–2147, respectively), and the sequence GAGCTGCACCGCATTATCA for human and mouse ARF6 (nucleotides 304–322; Balaña et al., 2005) were obtained (Dharmacon). siRNAs against the sequence GCAATGAGCTGTTGAAGA for human and mouse CHC (nucleotides 3182–3200; Huang et al., 2004) and scrambled sequences as control were obtained (Ambion). Transfection of siRNAs was achieved by using Oligofectamine (Invitrogen) for HeLa cells, and by using the DeliverX Plus delivery kit (Panomics) for 3T3-L1 adipocytes.

### Assays

The different transport assays used have been described previously: insulin-stimulated redistribution of HA-Glut4-GFP (Zeigerer et al., 2002), TfR recycling (Johnson et al., 2001), integrin recycling (Powelka et al., 2004), transport of VSVG-ts045 through the secretory pathway (Presley et al., 1997), and down-regulation of surface EGFR through endosomes to the lysosome (Lill et al., 2000). The biochemical assay for integrin  $\beta$ 1 recycling was done as previously described (Li et al., 2005).

Coprecipitation studies on whole cell lysates were performed as previously described [Aoe et al., 1997]. Pull-down assays using GST fusion proteins were performed as previously described [Yang et al., 2002]. Cell permeabilization studies were done by treating intact cells with 0.2% saponin in PBS at 4°C for 5 min followed by PBS wash at 4°C for 5 min. Cellular uptake of glucose was performed as described previously [Shi and Kandror, 2005].

### Microscopy techniques

Localization studies by laser confocal microscopy were performed as previously described [Dai et al., 2004]. In brief, images were acquired on an inverted microscope (TE2000; Nikon) with C1 confocal system and the Plan Achromat 40× oil (NA 1.00) and 60× oil (NA 1.40) objective lenses using EZ-C1 software (Nikon) at room temperature. Quantitation studies on images derived from confocal microscopy were performed through Photoshop CS (Adobe) and NIH image analysis software packages, Image J (v. 1.37a), using colocalization threshold plug-in (developed by the Wright Cell Imaging Facility, Toronto, Canada). Immunogold EM was performed as previously described [Aoe et al., 1997].

### Subcellular fractionation

The experiment was performed essentially as previously described [Shi and Kandror, 2005]. In brief, 3T3-L1 adipocytes were homogenized and subjected to centrifugation at 2,000 g for 10 min at 4°C to remove nuclei and cell debris. The resulting supernatant was subjected centrifugation at 16,000 g for 20 min at 4°C to obtain pellet that contains compartmental membrane and supernatant that contains cytosol and vesicular membrane.

### Online supplemental material

Fig. S1 shows further characterization of ACAP1 overexpression. Fig. S2 presents further characterizations of ACAP1 localization and the specificity of ACAP1 antibody. Fig. S3 shows the effects of knocking down ACAP1 or CHC in adipocytes. Fig. S4 presents the relative distribution of Glut4 with either sortilin or syntaxin 6 upon perturbation of the novel clathrin coat complex. Fig. S5 shows further characterization of ARF6 in adipocytes. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200608033/DC1>.

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