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14. ABSTRACT About 25% of breast cancers arise from the overexpression of ErbB2, which increases its cell surface level on mammary cells to result in enhanced mitogenic signaling. Recent evidence suggests that endocytic recycling contributes to the high surface level of ErbB2, but unlike all other currently known intracellular transport pathways, the conventional mechanism of protein sorting by coat proteins is thought not to play a significant role in endocytic recycling. However, in light of emerging evidence that this prevailing view might not be correct, we had originally proposed to gain insight into whether ErbB2 recycling follows the conventional mechanism of cargo sorting by testing whether it possessed recycling sorting signal(s), which are recognized by distinct coat proteins for sorting into different transport pathways. Since then, we have identified a novel clathrin coat complex that contains ACAP1 to mediate all examples of recycling that we have examined. This finding has led us to revise our originally proposed experiments, and we show that a pool of internalized ErbB2 colocalize and interact with the novel clathrin coat complex that we have recently identified for endocytic recycling. Significantly, our findings also suggest a more direct approach in the future to study whether ErbB2 recycling modulates its signaling.					
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INTRODUCTION

ErbB2 belongs to a family of surface transmembrane receptor tyrosine kinases whose activation drives intracellular mitogenic signaling. Its overexpression leads to tumor transformation that accounts for 25% of human breast cancers. A prevailing paradigm has been that surface ErbB2 is a static pool. Thus, therapies against ErbB2-driven tumors, such as monoclonal antibodies against ErbB2 and the antibiotic geldanamycin, are thought to be effective, because they enhance ErbB2 internalization to decrease its surface level. A recent study has reversed this prevailing paradigm by showing that the high surface distribution of ErbB2 is maintained mainly by its efficient endocytic recycling rather than its slowed internalization¹, leading to the prospect that the mitogenic signaling by surface ErbB2 may be decreased by inhibiting its endocytic recycling.

In this respect, even though accumulated studies on intracellular transport have led to the generalization that families of key transport factors mediate highly conserved mechanistic steps that include vesicle formation followed by vesicle docking and fusion², an apparent exception has been the endocytic recycling pathway, as coat proteins that recognize sorting signals on cargo proteins for their sorting into distinct transport pathways do not appear to play a significant role in endocytic recycling³. This has led to the proposal that endocytic recycling uses alternate lipid-based mechanisms to sort recycling proteins³. However, we had pointed out in our original proposal (done in an anonymous way as required by the Concept Award guidelines) that our laboratory had found at that time that the recycling of transferrin receptor (TfR), which had been the best model system to elucidate molecular mechanisms of recycling³, to use a recycling sorting signal⁴. Thus, we had proposed in our original application to gain insight into whether the conventional mechanism of cargo sorting by coat proteins applies to ErbB2 recycling by determining whether it possesses recycling sorting signal(s). Experimentally, we proposed two major tasks: i) identify a critical region in the ErbB2 cytoplasmic domain that mediates its endocytic recycling, and ii) identify critical residue(s) in the ErbB2 cytoplasmic domain that mediates this endocytic.

BODY

Since the initial proposal, we have made a significant advancement in our understanding of how endocytic recycling is achieved by the identification of a novel clathrin coat complex that mediates endocytic recycling for all well characterized examples of endocytic recycling⁵. The reason why this finding predicts that ErbB2 recycling will also use this coat complex is that coat proteins represent the core machinery by which transport from different intracellular compartments is initiated. They achieve this role by two well-characterized functions: i) deformation of compartmental membrane in forming transport vesicles and ii) cargo sorting that entails direct interaction with cargo proteins for their packaging into nascent vesicles². The ADP-Ribosylation Factor (ARF) family of small GTPases regulates the recruitment of coat proteins from cytosol to membrane to initiate vesicle formation. Their GTPase cycle is regulated by guanine nucleotide exchange factors (GEFs) that activate ARFs and GTPase-activating proteins (GAPs) that deactivate ARFs. 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, but also as their effectors by being core components of coat complexes^{6,7}.

Exploring whether other GAPs for ARF members may exhibit a similar behavior, we previously identified ACAP1, a GAP for ARF6⁸, to possess a novel function in cargo sorting by recognizing sorting signals in the cytoplasmic domain of the transferrin receptor (TfR) for its endocytic recycling⁴. Extending this finding substantially, we have recently found that ACAP1 is part of a novel clathrin coat complex for all well characterized examples of endocytic recycling⁵. Thus, in light of this finding, we have revised our original experimental tasks for ErbB2 recycling, which were meant to be the first step in gaining insight into this possibility, to pursue more direct evidence that ErbB2 recycling indeed follows the conventional mechanism of cargo sorting through coat proteins recognizing sorting signals. First, we find that the internalized pool of ErbB2 show significant colocalization with ACAP1 by confocal microscopy (see Figure 1 in Supporting Data section below). Second, we have found that this internalized pool interacts physically with ACAP1 through a co-precipitation approach of cellular lysates (Figure 2).

Thus, in this final report, we consider that the work which has identified the novel ACAP1-containing clathrin coat complex to represent essentially an alternate approach that has provided a more direct way of showing that ErbB2 recycling involves cargo sorting by coat proteins. A manuscript that describes our identification of this novel coat complex is enclosed. We have submitted this paper to the Journal of Cell Biology, and have received positive reviews. Recently, we have just finished work on a revised paper that addresses critical comments from these reviews. Thus, this revised paper is being sent back to the journal for a final decision.

KEY RESEARCH ACCOMPLISHMENTS

1. We have identified a novel clathrin coat complex that is predicted to be involved in sorting all cargo proteins transport from the recycling endosome to the plasma membrane.
2. We also show that ErbB2 is likely to use this novel coat complex for its recycling.

REPORTABLE OUTCOMES

A manuscript that describes the novel clathrin coat complex involved in endocytic recycling is enclosed.

CONCLUSIONS

We have identified a novel clathrin coat complex that contains ACAP1 to mediate all examples of endocytic recycling, and provide evidence that it is also involved in ErbB2 recycling. In the future, we will pursue studies that are intended to disrupt the ability of this novel coat complex to recognize ErbB2 at the recycling endosome, and then determine whether ErbB2-mediated signaling is altered.

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APPENDICES

One enclosed manuscript: Li, J. et al. An ACAP1-Containing Clathrin Coat Complex for Endocytic Recycling. *Manuscript submitted* (2007).

SUPPORTING DATA

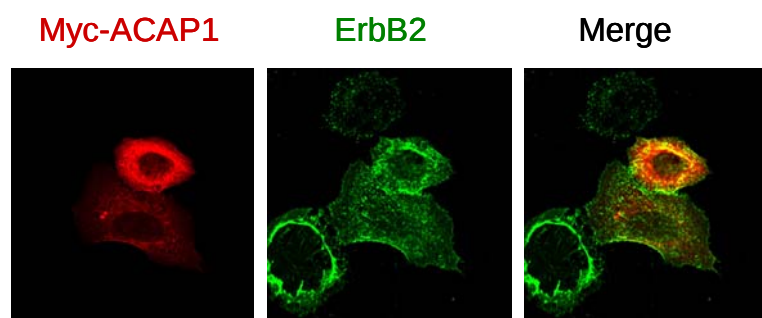


Figure 1. An internalized pool of surface ErbB2 shows colocalization with ACAP1. Antibody-bound surface ErbB2 is allowed to internalize in SKBr3 cells transfected with Myc-ACAP1, and then analyzed by confocal microscopy for ACAP1 (red) and antibody-bound ErbB2 (green).

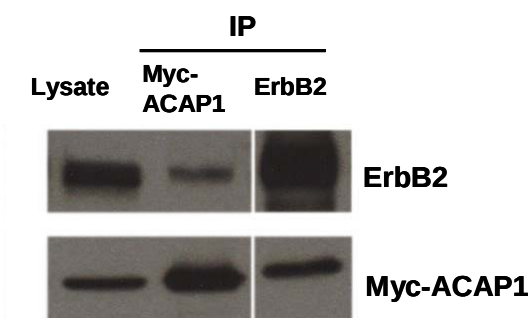


Figure 2. An internalized pool of surface ErbB2 interacts with ACAP1. Antibody-bound surface ErbB2 is allowed to internalize in SKBr3 cells transfected with Myc-ACAP1. Cells were then lysed followed by immunoprecipitation and blotting as indicated.

An ACAP1-Containing Clathrin Coat Complex for Endocytic Recycling

(Revision of JCB #200608033R)

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ABSTRACT

Whether coat proteins play a wide-spread 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 physiologic settings, stimulation-dependent recycling of integrin that is critical for cell migration, and insulin-stimulated recycling of glucose transporter type 4 (glut4) that 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 physiologic 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: i) deformation of compartmental membrane in forming transport vesicles and ii) 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 endoplasmic reticulum (ER) and the Golgi complex (Bonifacino and Glick, 2004; Kirchhausen, 2000).

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 (GEFs) 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 (Cukierman et al., 1995; Yoshihisa et al., 1993), 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 have previously identified ACAP1, 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 a significant 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 significant 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 non-polarized cells, for which evidence for a recycling sorting 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 AP1 adaptor, which contains the u1B subunit (Folsch et al., 1999), to mediate polarized TfR recycling to the basolateral surface of the plasma membrane (Rodriguez-Boulau 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 that 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 that 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 compartment(s) 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 also 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 physiologic 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 small interfering RNA (siRNA) inhibited endocytic recycling of both TfR (Dai et al., 2004) and integrin (Li et al., 2005), we were surprised to find initially that overexpression of ACAP1 also inhibited the recycling of both TfR (Fig 1A) and integrin (Fig 1B). Inhibition of integrin recycling was further confirmed by a quantitative biochemical recycling assay (Sup Fig 1A), as we had done previously (Li et al., 2005; Powelka et al., 2004). 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 1A) or integrin (Fig 1B and Sup Fig 1A) recycling. Moreover, internalization of surface TfR (Fig 1A) and integrin (Fig 1B) 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) mutant (Sup Fig 1B), and also endocytic transport to the lysosome, by examining the epidermal growth factor receptor (EGFR) upon ligand binding at the cell surface (Sup Fig 1C).

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 (Sup Fig 2A) and integrin (Sup Fig 2B) 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 electron microscopy (EM) (Fig 1C). As control, overexpression of other GAPs for ARF6 did not exhibit a similar effect (as example, see ACAP2 overexpression in Fig 1D). Moreover, overexpression of the catalytic dead mutant of ACAP1 induced a similar membrane coating (Fig 1E). Thus, as coat complexes need to cycle dynamically on and off their target membrane to accomplish 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 significant labeling for subunits of AP1, AP2, AP3, AP4, COPI, COPII, and GGAs; however, we detected labeling for the clathrin heavy chain (CHC) (data not shown). 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 2A). 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 co-precipitated with CHC using lysates derived from cells that overexpressed ACAP1, and in support of our hypothesis above that overexpressed ACAP1 locked a coat onto membrane to inhibit endocytic recycling, we found that ACAP1 could be co-precipitated with CHC even when cells were first permeabilized to allow leakage of their cytosol before being subjected to the co-precipitation procedure (Fig 2B). Moreover, ACAP1 and CHC could interact directly, as assessed by a pulldown approach in which ACAP1 as a GST-fusion protein was bound to beads and then incubated with purified soluble clathrin triskelia (Fig 2C).

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. While we had 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 by examining integrin recycling, as its internalization to the perinuclear recycling endosome was

largely unaffected by siRNA against CHC (Fig 2D). In contrast, we found that integrin recycling was inhibited (Fig 2E).

Both ACAP1 and clathrin also participate in glut4 recycling

We next sought to confirm these findings by additional approaches that would examine ACAP1 under more physiologic 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 physiologic 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 significant colocalization with endogenous CHC (Fig 3A).

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 significant colocalization with its internal pool under the basal (no insulin) condition (Fig 3A). The specificity of the observed staining for endogenous ACAP1 was confirmed by siRNA against ACAP1 (Sup Fig 3A). As sortilin (Shi and Kandror, 2005) and syntaxin6

(Shewan et al., 2003) had been shown to have some colocalization with internal glut4 previously, we also examined these two proteins and found that ACAP1 showed significant colocalization with sortilin and to a lesser extent with syntaxin6 (Fig 3A). Moreover, providing further support for our hypothesis above 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 (Sup Fig 3B). In contrast, permeabilization of HeLa cells with overexpressed ACAP1 retained a compact perinuclear distribution (Sup Fig 3C), 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. Whereas it was readily visualized in the differentiated (adipocyte) state (Fig 3A), this staining was difficult to detect in the undifferentiated (fibroblast) state (Sup Fig 3D). In contrast, staining for sortilin was not similarly affected (Sup Fig 3E). 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 (Sup Fig 3F), 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 physiologic usage of ACAP1, we next sought to further define the mechanistic relationship between ACAP1 and the clathrin triskelion in these cells, using glut4 recycling as the context.

Initially, we found by quantitative microscopy that siRNA against ACAP1 substantially inhibited the insulin-induced redistribution of internal glut4 to the cell surface (Fig 3B), as

previously described (Zeigerer et al., 2002). This result was further confirmed by an assay for the cellular uptake of glucose (Fig 3C), which had been shown previously 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 3C). Further specificity for the siRNA approach was reflected by the silencing of ACAP1 not having a significant effect on the level of CHC, ARF6, and select signaling proteins (Sup Fig 4A). Moreover, we found that the internalization of glut4 to its internal perinuclear location at the basal condition was not significantly affected (Sup Fig 4B). Silencing CHC, we found that glut4 recycling was also markedly inhibited (Fig 3D). Notably, siRNA against CHC did not prevent the accumulation of internal glut4 in the basal condition (Sup Fig 4C), which was consistent with surface glut4 previously shown to internalize by both clathrin and non-clathrin 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 5-fold reduction (Fig 4A). In cells treated with siRNA against CHC, we found that endogenous ACAP1 also had reduced colocalization with glut4, with quantitation again revealing about a 5-fold reduction (Fig 4B). In contrast, siRNA against

neither ACAP1 nor CHC had a significant effect on the colocalization of glut4 with sortilin (Sup Fig 5A). Similarly, the colocalization of glut4 and syntaxin6 was not affected significantly by either siRNA treatment (Sup Fig 5B).

Second, we found that ACAP1 associated with CHC in differentiated adipocytes through a co-immunoprecipitation approach (Fig 4C). As specificity, other transport factors known to participate in glut4 recycling, such as Rab11 (Zeigerer et al., 2002), Exo70 (Inoue et al., 2003) and TC10 (Chiang et al., 2001), did not associate with this complex (Fig 4C). In contrast, we detected a complex of glut4 with both ACAP1 and CHC (Fig 4D), for which the specificity of the immunoprecipitating antibody was verified (Fig 4E). Notably however, whereas we had found previously that the association of ACAP1 with integrin β 1 was stimulation-dependent (Li et al., 2005), insulin-stimulation did not enhance the association of glut4 with either ACAP1 or CHC (Figs 4D and 4E).

Third, because cargo sorting by coat proteins involves their direct interaction with cargo proteins (Bonifacino and Glick, 2004), we next examined whether, and potentially how, the ACAP1-containing clathrin coat complex interacted with glut4. Glut4 is a multi-spanning transmembrane protein that contains three significant cytoplasmic domains, at the amino terminus, at the carboxy terminus, and also having a middle cytoplasmic loop (Watson and Pessin, 2006). Thus, we appended each domain to GST and initially examined which domain(s) bound directly to soluble ACAP1 in a pulldown assay, as previously done (Dai et al., 2004; Li et al., 2005). ACAP1 was found to interact directly with the middle cytoplasmic loop, while the clathrin triskelion showed no significant binding to any of the domains examined (Fig 5A). We

also assessed the relationship between ACAP1 and the CHC in binding to the middle domain of glut4. Whereas 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 5B). Thus, these results suggested that ACAP1 acted like AP adaptors in bridging an interaction between cargo tails and clathrin triskelion.

We also determined residue(s) 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 6A). 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 6B). Subsequently, more detailed analysis of these regions by alanine scanning mutagenesis revealed a requirement for two basic residues (KR) in one region (Fig 6B). 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 6B). Focusing on residues where these mild reductions were detected, we found that a more extensive replacement with alanines at this sub-region 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 6B). When all these mutations were introduced into the entire middle domain of glut4, we found that its binding to ACAP1 became reduced (Fig 6C). Confirming this result in the context of adipocytes using a co-precipitation approach, we also found that mutations introduced in the context of the entire chimeric glut4 construct also led to reduced association with ACAP1 (Fig 6D). Importantly, this mutant construct also exhibited reduced

recycling in response to insulin (Fig 6E). 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 had 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 significant colocalization with ACAP1, CHC, and also with internal glut4 at the basal condition (Fig 7A). Remarkably, like endogenous ACAP1, we also found that endogenous ARF6 was also difficult to visualize in undifferentiated 3T3-L1 cells (Sup Fig 6A). Moreover, consistent with the general paradigm that ARFs acting 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 7-fold reduction for both cases (Fig 7B). In contrast, the fraction of ARF6 that colocalized with internal glut4 was not significantly altered by the knockdown of either ACAP1 or CHC (Fig 7C). Confirming these results, we also found that silencing ARF6 disrupted the physical association that we had seen between glut4 and the novel clathrin coat complex, to a similar degree as that seen for silencing CHC (Fig 7D and Sup Fig 6B).

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 8A). In contrast, this knockdown had no significant effect on the accumulation of glut4 internally (Sup Fig 6C). Second, we found that silencing

ARF6 reduced glucose uptake into adipocytes to a similar extent as that seen for silencing either ACAP1 or CHC (Fig 8B). 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 8C).

Finally, as GAP activity acts mechanistically upstream of ARF small GTPases while 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, and thereby obscuring our ability previously (in Sup Figs 2A and 2B) 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 physiologic 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 significant divergence with the human ACAP1, we knocked down endogenous ACAP1 in the mouse adipocytes and then stably transfected the human forms. By this approach, we found that the catalytic-dead mutant did not restore glucose uptake to a similar level as that seen for the wild-

type form (Fig 8D). 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 lower level of ACAP1 than that seen for the endogenous situation (Sup Fig 6D). 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 morphologic 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 physiologic 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 found previously 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, while 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 due to 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 physiologic 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 significant in 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 physiologic 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 significant for endocytic recycling.

Elucidating a 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 significantly 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, whereas we have found previously that stimulatory signaling regulates the role of ACAP1 in the cargo sorting of recycling integrin (Li et al., 2005), we find 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. Notably, this finding is consistent with a previous conclusion that the formation of glut4 vesicles likely occurs largely independent of insulin stimulation (Xu and Kandror, 2002).

In this respect, another notable observation is that the redistribution of sortilin from a fraction that likely represented its distribution in vesicular membrane to that on compartmental membrane upon the perturbation of ARF6, ACAP1, or CHC mirrors that of glut4 (seen in Fig 8C). 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 recycling 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 to those that we have 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 residue(s), while 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 that bind recognize 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 have 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

Alexa594-labeled Tf was obtained from Molecular Probes (Eugene, OR). Draq5 to stain DNA was obtained from Biostatus (Leicestershire, UK). EGF, insulin and other chemicals (unless specified) were obtained from Sigma (St. Louis, MO). ³H-2-deoxy-D-glucose was obtained from Perkin Elmer (Wellesley, MA). HeLa and TRVb1 cells were cultured as previously described (Dai et al., 2004). 3T3-L1 fibroblast was obtained and cultured as described in the instruction (ATCC, Rockville, MD). 3T3-L1 cells stably expressing Myc-Glut4 or both Myc-Glut4 and Sortilin-Myc-6xHis were cultured as previously described (Shi et al., 2005). The differentiation of 3T3-L1 was performed as previously described (Shi et al., 2005). Purified proteins that have been previously described include: clathrin triskelia (Matsui and Kirchhausen, 1990) and 6xHis-tagged ACAP1 (Dai et al., 2004).

Antibodies

The following antibodies have been described previously (Dai et al., 2004; Jackson et al., 2000; Li et al., 2005; Powelka et al., 2004): mouse TS2/16 against β 1 integrin, mouse DM1 α against α -tubulin, mouse 9E10 against the Myc epitope, mouse M2 against the Flag epitope, mouse 15E6 against the C-terminal hemagglutinin (HA) epitope, rabbit anti-ACAP1, rabbit anti-cellubrevin, rabbit anti-Arf6, rabbit anti-Lamp1, mouse M3A5 against β -COP, rabbit anti-Akt, and secondary antibodies conjugated to Cy2 or Cy3.

Additional antibodies acquired for the current study include: mouse anti-6xHis epitope and mouse HA.11 against the HA epitope (Covance Research Products, Berkeley, CA), mouse TD.1

and X22 against CHC (ATCC, Rockville, MD), mouse anti-syntaxin 6 (BD Biosciences, San Jose, CA), rabbit anti-Rab11 (Zymed Laboratories, San Francisco, CA), mouse anti-phospho-Akt (Ser473) (Cell Signaling, Beverly, MA), mouse 4G10 against phosphor-Tyr (Upstate, Charlottesville, VA), rabbit anti-clathrin (from S. Corvera, University of Massachusetts Medical Center, Worcester, MA), mouse anti-EGFR (from H. Band, Northwestern University Medical Center, Evanston, IL), rabbit anti-ERK and phosphor-ERK (from J. Blenis, Harvard Medical School, Boston, MA), rabbit anti-Exo70 (from Patrick Brennwald, University of North Carolina), and rabbit anti-glut4 (from Samuel Cushman, NIH, Bethesda, MD).

Plasmids and transfections

The following plasmids were used: Flag-tagged human ACAP1 wild-type and catalytic dead mutant (R448Q), and ACAP2 (Jackson et al., 2000). Myc-tagged human ACAP1 (Li et al., 2005), HA-Glut4-GFP (from T. McGraw, Cornell University Medical School, New York, NY), GFP-tagged VSVG-ts045 (from J. Lippincott-Schwartz, NIH, 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 carboxy terminus of GST, the coding sequences of ACAP1 or Glut4 cytoplasmic domains were amplified by PCR. ACAP1 was then subcloned into the BamH1 and EcoRI sites of pGEX-4T-3 vector (Amersham Pharmacia Biotech, Piscataway, NJ), while the Glut4 constructs were subcloned into the EcoRI and Not I sites. While 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, LA Jolla, CA).

Transient transfections were performed using Fugene 6 (Roche Biochemicals, Indianapolis, IN) 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, or Myc-tagged human ACAP1 wild-type, or Flag-tagged human ACAP1 R448Q were generated by transient transfection with selection in 1 mg/ml of G418 (LifeTechnologies, Inc., Gaithersburg, MD), and then maintained in 0.2 mg/ml of G418.

SiRNA

SiRNAs against the sequences (CGACATCATGGAATTCGTA, TAAGGACCCTGTAACCGTG and AGACGTATCTCGACATATT) for mouse ACAP1 (nucleotides 558-576, 903-921 and 2129-2147 respectively), and the sequence (GAGCTGCACCGCATTATCA) for human and mouse Arf6 [nucleotides 304-322, (Balana et al., 2005)] were obtained (Dharmacon, Chicago, IL). SiRNAs against the sequence (GCAATGAGCTGTTTGAAGA) for human and mouse CHC [nucleotides 3182-3200, (Huang et al., 2004)], and also scrambled sequences as control were obtained (Ambion, Austin, TX). Transfection of siRNAs was achieved by using Oligofectamine (Invitrogen, Carlsbad, CA) for HeLa cells, and by using the DeliverX Plus delivery kit (Panomics, Fremont, CA) 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 downregulation of surface EGFR through endosomes to the lysosome (Lill et al., 2000). The biochemical assay for integrin β 1 recycling was done as previously described (Li et al., 2005).

Co-precipitation studies on whole cell lysates were performed as previously described (Aoe et al., 1997). Pulldown 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 minutes following by PBS wash at 4°C for 5 minutes.

Cellular uptake of glucose was performed as described previously (Shi and Kandror, 2005).

Microscopy techniques

Localization studies by immunofluorescence microscopy and laser confocal microscopy were performed as previously described (Dai et al., 2004). Quantitation studies on images derived from confocal microscopy were performed through Adobe Photoshop CS and NIH image analysis software packages, Image J (v. 1.37a), using colocalization threshold plug-in (developed by Wright Cell Imaging Facility, Toronto, Canada).

Immunogold electron microscopy was performed as previously described (Aoe et al., 1997).

Subcellular fractionation

The experiment was performed essentially as previously described (Shi and Kandror, 2005). Briefly, 3T3-L1 adipocytes were homogenized and then subjected to centrifugation at 2,000 x g for 10 min at 4 °C to remove nuclei and cell debris. The resulting supernatant was subjected

centrifugation at 16,000 x g for 20 min at 4 °C to obtain pellet that contains compartmental membrane and supernatant that contains cytosol and vesicular membrane.

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FIGURE LEGENDS

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 then assessed for the uptake and recycling of Alexa594-conjugated Tf by immunofluorescence microscopy, ACAPs (green) and Tf (red); bar, 15 μ 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 (green) and integrin (red); bar 10 μ m.
- (C/D/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 then fixed for immunogold EM using anti-Flag antibody; bar, 200 nm.

Figure 2. Endosomal clathrin associates with ACAP1 and is required for integrin recycling.

- (A) Endogenous CHC show partial colocalization with transfected ACAP1. HeLa cells were transiently transfected with GFP-ACAP1, and then examined by immunofluorescence microscopy for ACAP1 (green) and CHC (red); bar, 10 μ m.

(B) Clathrin interacts with ACAP1. HeLa cells were transiently transfected with ACAP1-Myc, either permeabilized (to release cytosol) or not, and then 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 then incubated with soluble clathrin triskelia for a pulldown experiment. CHC was detected by immunoblotting for CHC while 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 (red) and clathrin (green); bar, 10 μ m.

(E) Knocking down CHC inhibits integrin recycling. A biochemical assay for integrin recycling was performed with graph showing the level of recycling integrin remaining internal at times indicated.

Figure 3. Both ACAP1 and clathrin participates 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: ACAP1 (green) and CHC (red) [Top row], HA-glut4-GFP (green) and CHC (red) [2nd row], HA-glut4-GFP (green) and ACAP1 (pseudo-red) [3rd row], sortilin-6xHis (green) and ACAP1 (red) [4th row], and ACAP1 (green) and Syntaxin 6 (red) [bottom row]; bar, 10 μ m. Note that staining for ACAP1, CHC, and syntaxin6 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 ten 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 then 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 above (in B) was performed, except cells were treated with siRNA against CHC.

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 then examined at the basal condition by confocal microscopy for glut4 (green) and CHC (red); bar, 10 μ m. Ten cells were also randomly selected and then quantified for the degree of colocalization between glut4 and 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 above (in A) was performed, except cells treated with siRNA

against CHC and the degree of colocalization between glut4 (green) and ACAP1 (red) was examined.

(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 then immunoblotting for proteins as indicated. TC10 was detected by transfecting cells with HA-TC10.

(D) Both ACAP1 and CHC associate with glut4. Differentiated 3T3-L1 cells stably expressing Myc-glut4 were analyzed in a co-precipitation experiment as described above (in C).

(E) Specificity controls for immunoprecipitating Myc-glut4. Above experiment (in D) was expanded to include additional controls as indicated.

Figure 5. Dissecting out 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 then incubated with either soluble ACAP1 or clathrin triskelia. Bound soluble components were detected by immunoblotting, while GST proteins were detected by coomassie-staining.

(B) ACAP1 bridges the binding of clathrin triskelia to glut4. The same pulldown experiment was performed as above (in A), except soluble proteins were incubated sequentially as indicated. Note that unbound soluble proteins after the first incubation were removed prior to the second incubation.

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 then incubated with ACAP1 in pulldown experiments. Truncation mutants were initially screened (left), followed by alanine-scanning mutagenesis of two regions identified to bind ACAP1 (middle and right). ACAP1 is detected by immunoblotting while 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. Similar experiments as described above (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 positions 23, 24, and 50-54 in the middle cytoplasmic domain, as defined in A, to alanines) was transfected into differentiated 3T3-L1 cells. Cell lysates were then 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 mediates its binding to ACAP1 represent recycling sorting signals. Wild-type or mutant form of HA-glut4-GFP (as described above in D) were transfected into differentiated 3T3-L1 cells and then 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.

Figure 7. Regulation of the novel clathrin coat complex by ARF6

(A) Endogenous ARF6 shows significant colocalization with endogenous ACAP1 and CHC, and also 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: ARF6 (green) and CHC (red) [top row], ARF6 (green) and ACAP1 (red) [middle row], and ARF6 (red) and glut4 (green) [bottom row]; bar, 10 um.

(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 (pseudo-red); bar, 10 um. 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 then examined at the basal condition by confocal microscopy for glut4 (green) and ARF6 (red); bar, 10 um. 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.

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 then 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 ten 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 then 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 then subjected to medium-speed 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 then assayed for the cellular uptake of glucose as described above (B).

SUPPLEMENTAL FIGURE LEGEND

Figure 1. Further characterization of ACAP1 overexpression.

(A) Overexpression of ACAP1 but not ACAP2 inhibits integrin recycling, as assessed by a quantitative recycling assay. HeLa cells were transiently transfected with Flag-ACAP1 or Flag-ACAP2, and then starved overnight. Antibody against integrin β 1 was added to the cell surface and then allowed to accumulate internally for 2 hours at the recycling endosome. A biochemical recycling assay was then performed after stimulating cells with serum, and then quantifying for the level of internal β 1 at times indicated, after surface stripping of antibody bound to those β 1 that had been externalized. This level was then calculated as a percentage of that prior to stimulation. The mean from three independent experiments is shown with standard error.

(B) Overexpression of ACAP1 does not inhibit the transport of VSVG-ts045 from the ER through the Golgi complex to the plasma membrane. HeLa cells were transiently co-transfected with Flag-ACAP1 and VSVG-ts045-GFP. Cells were then incubated at the non-permissive temperature for 2 hours to accumulate the VSVG at the ER, followed by shift to the permissive temperature for times indicated, and then examined by immunofluorescence microscopy for ACAP1 (red) and VSVG (green); bar, 10 μ m.

(C) Overexpression of ACAP1 does not inhibit the transport of surface EGFR through endosomal compartments to the lysosome. HeLa cells were transiently transfected with Flag-ACAP1. Cells were then stimulated with EGF for times indicated, and then examined by immunofluorescence microscopy for ACAP1 (green) and EGFR (red) [upper two rows]. At 30 minutes [bottom row], much of the EGFR has reached the lysosome, as assessed by confocal microscopy for ACAP1 (green), EGFR (red) and Lamp1 (pseudo-colored blue); bar, 10 μ m.

Figure 2. Overexpression of ACAP1 induces coated membrane structures and inhibits endocytic recycling in a manner that is independent of its GAP catalytic activity.

(A) Overexpression of a catalytic dead point mutant of ACAP1 inhibits TfR recycling. The same morphologic assay for TfR recycling as described above (Fig 1A) was performed, except cells were transiently transfected with Flag-ACAP1 (R448Q); bar, 15 μ m.

(B) Overexpression of a catalytic dead point mutant of ACAP1 inhibits integrin recycling. HeLa cells were transiently transfected with Flag-ACAP1 (R448Q) or mock transfected, and then starved overnight. Antibody against integrin β 1 was added to the cell surface and then allowed to accumulate internally for 2 hours at the recycling endosome. A biochemical recycling assay was then performed after stimulating cells with serum, and then quantifying for the level of internal β 1 at times indicated, after surface stripping of antibody bound to those β 1 that had been externalized. This level was then calculated as a percentage of that prior to stimulation. The mean from three independent experiments is shown with standard error.

Figure 3. Further characterizations of ACAP1.

(A) Specificity of antibody against ACAP1 as assessed by immunofluorescence microscopy that detects a reduction in its staining upon silencing ACAP1 in differentiated 3T3-L1 cells.

(B) Endogenous ACAP1 and CHC in differentiated 3T3-L1 cells lose their membrane localization upon cell permeabilization. Cells under the basal condition were either permeabilized or not prior to cell fixation. Cells were then examined by confocal microscopy for HA-glut4-GFP (green) and ACAP1 (pseudo-red) [top row], or HA-glut4-GFP (green) and CHC (red) [bottom row]; bar, 10 μ m.

(C) Overexpressed ACAP1 maintains a membrane distribution in HeLa cells that have been permeabilized to release cytosolic proteins. Cells were transiently transfected with Flag-ACAP1, and then either fixed immediately or first permeabilized and then fixed. Cells from either condition were then examined by immunofluorescence microscopy for ACAP1; bar, 10 μ m.

(D) Endogenous ACAP1 cannot be readily visualized by microscopy in undifferentiated 3T3-L1 cells. Confocal microscopy was used to compare staining for endogenous ACAP1 (green) in cells as indicated. Undifferentiated cells were also additionally stained for DNA by Draq5 (pseudo-blue) to mark cells in the analysis; bar, 10 μ m.

(E) Sortilin can be detected regardless of the differentiation state of 3T3-L1 adipocytes. Cells that stably expressed both myc-glut and sortilin-myc-6xhis were examined by confocal microscopy for ACAP1 (red) and sortilin (green) under the differentiation conditions noted.

(F) Endogenous ACAP1 in 3T3-L1 cells can be readily detected by immunoblotting regardless of the differentiation state. [Top] Cell lysates from conditions indicated were immunoblotted for proteins as indicated. [Bottom] Specificity of the rabbit antibody generated against ACAP1 was demonstrated by comparing with immunoblotting using pre-immune serum.

Figure 4. Characterizing effects of knocking down ACAP1 or CHC in adipocytes.

(A) Knocking down ACAP1 does not have a significant effect on the levels of CHC, ARF6, and select signaling proteins. Cell lysates derived from differentiated 3T3-L1 cells that had been treated with different siRNA conditions as noted were analyzed by immunoblotting using antibodies against proteins as indicated.

(B/C) Glut4 in differentiated 3T3-L1 cells still accumulates internally upon siRNA against either ACAP1 or CHC. Differentiated 3T3-L1 cells that stably expressed HA-glut4-GFP were treated with siRNA against ACAP1 (B) or CHC (C), and then examined by immunofluorescence microscopy for glut4; bar, 10 μ m.

Figure 5. The relative distribution of glut4 with either sortilin or syntaxin6 upon perturbation of the novel clathrin coat complex.

(A) The degree of colocalization between glut4 and sortilin is not affected significantly by siRNA against either ACAP1 or CHC. [Left] Differentiated 3T3-L1 adipocytes that stably expressed both myc-glut4 and sortilin-myc-6xhis were treated with siRNAs as indicated, and then examined by confocal microscopy for glut4 (red) and sortilin (green). [Right] Ten cells were randomly selected and then quantified for the degree of colocalization. The mean with standard error is shown.

(B) The degree of colocalization between glut4 and syntaxin6 is not affected significantly by siRNA against either ACAP1 or CHC. Same experiments as described above (in A) were performed, except colocalization of glut4 with syntaxin6 is assessed.

Figure 6. Further characterization of ARF6 in adipocytes.

(A) Endogenous ARF6 cannot be visualized by microscopy in undifferentiated 3T3-L1 cells. Confocal microscopy was used to compare staining for endogenous ARF6 (red) in cells as indicated; bar, 10 μ m. Undifferentiated cells were also additionally stained for DNA by Draq5 (pseudo-blue) to mark nuclei of cells.

(B) Quantitation of the association between ACAP1 and glut4 upon siRNA against either CHC or ARF6. The co-precipitation experiment described in Figure 7D was performed in three

different times, and then quantified for the level of association between ACAP1 and glut4. The mean with standard error is shown.

(C) Glut4 in differentiated 3T3-L1 cells still accumulates internally upon siRNA against ARF6. Differentiated 3T3-L1 cells that stably expressed HA-glut4-GFP were treated with siRNA against ARF6, and then examined by immunofluorescence microscopy for glut4; bar, 10 μ m.

(D) Stable expression of different forms of ACAP1 achieves a significantly lower level than that seen for endogenous ACAP1 in differentiated 3T3-L1 cells. Cell lysates derived from cells stably transfected with different forms of ACAP1 as noted were analyzed by immunoblotting using the anti-ACAP1 antibody. Note that transfected forms are tagged and thus appear with higher apparent molecular mass.

Figure 1

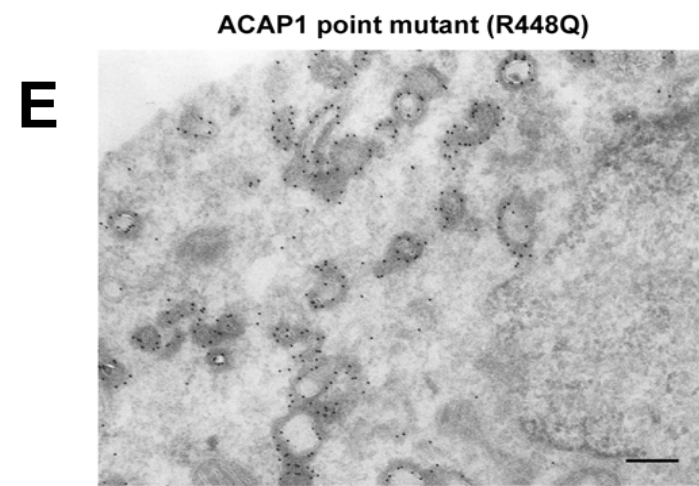
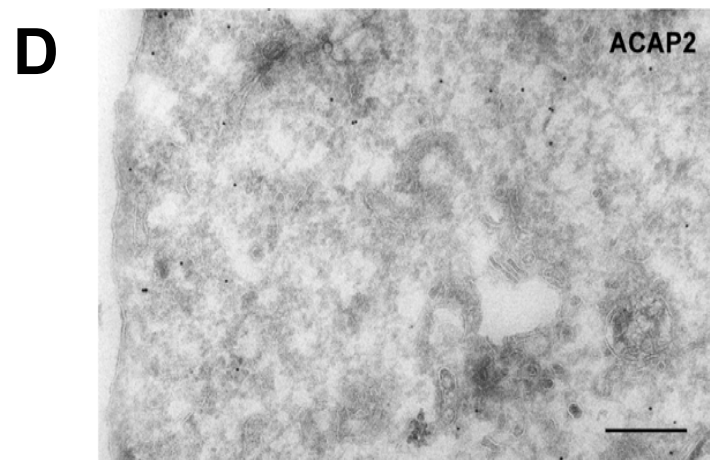
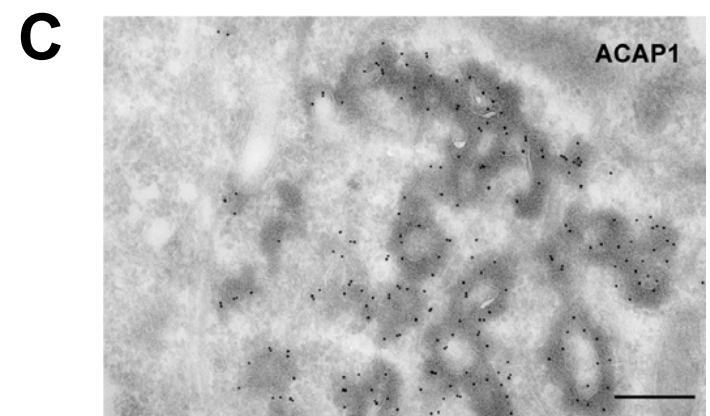
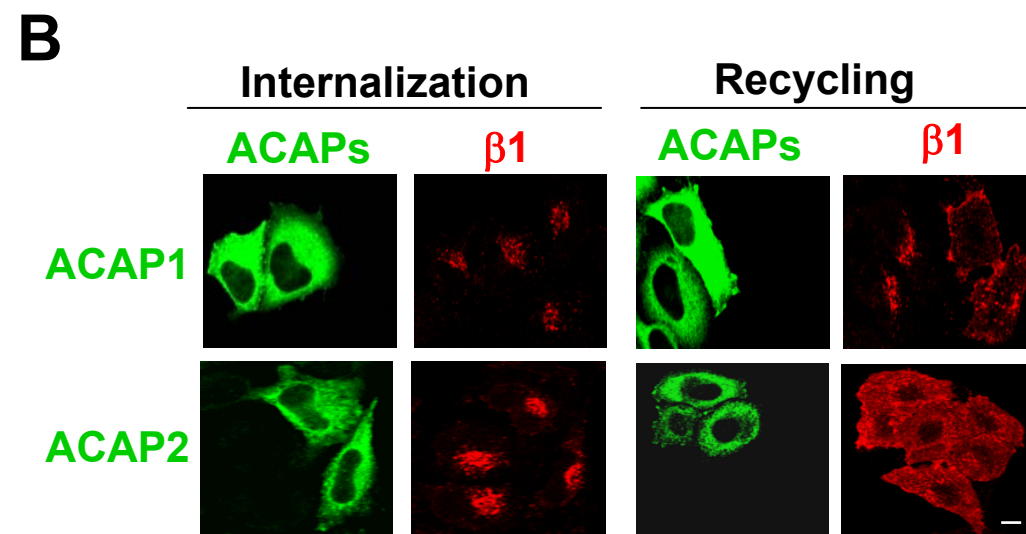
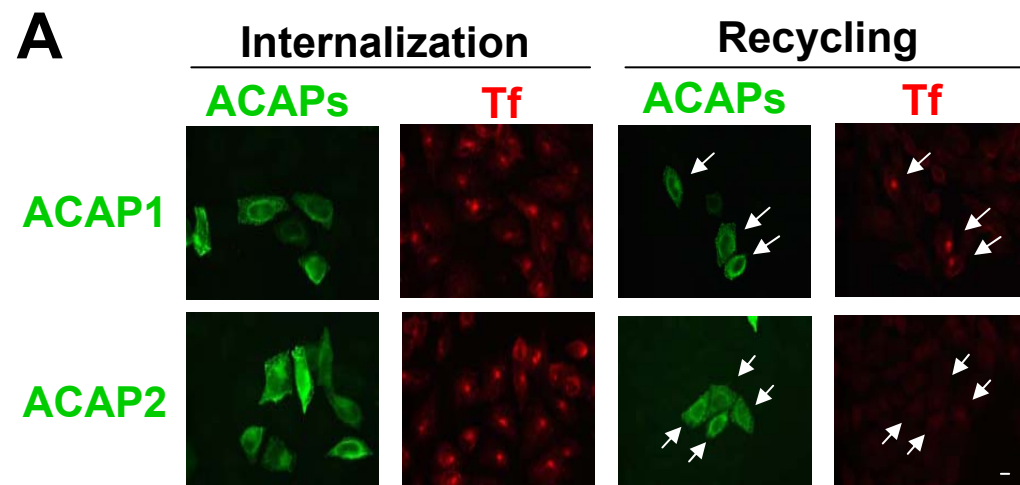
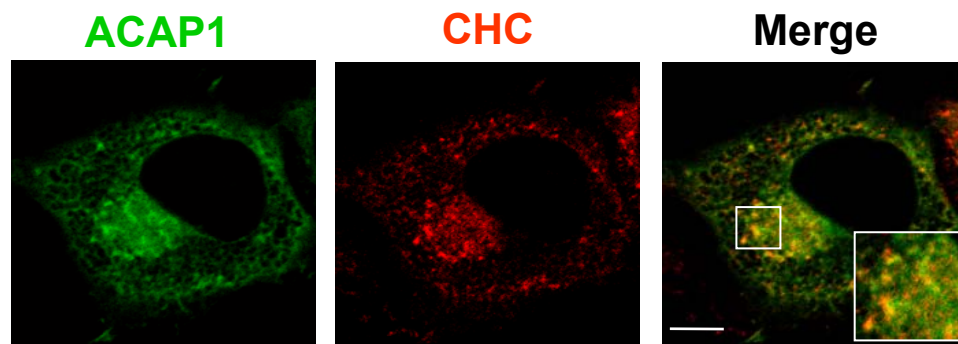
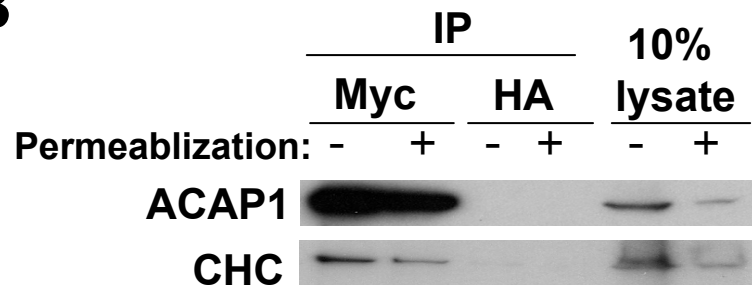


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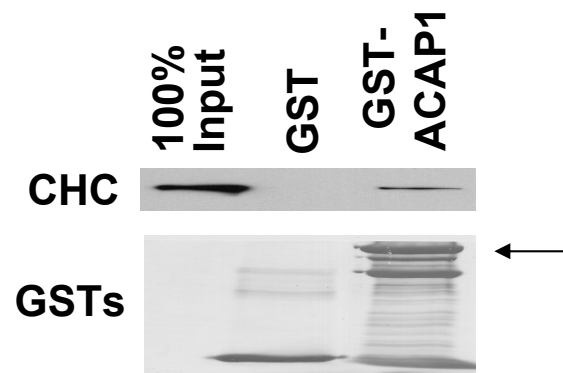
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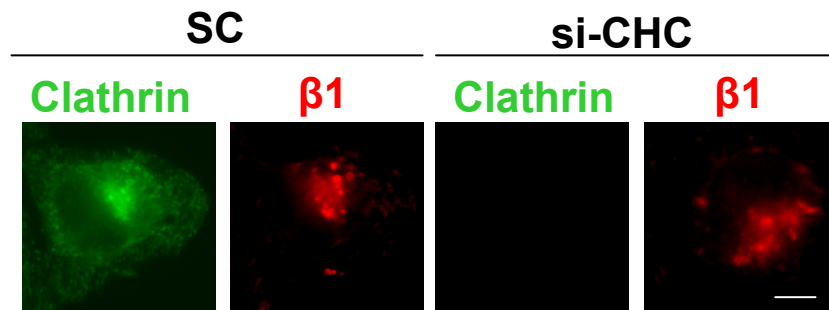
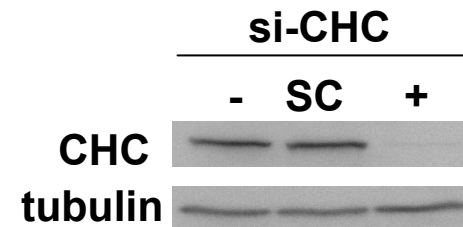
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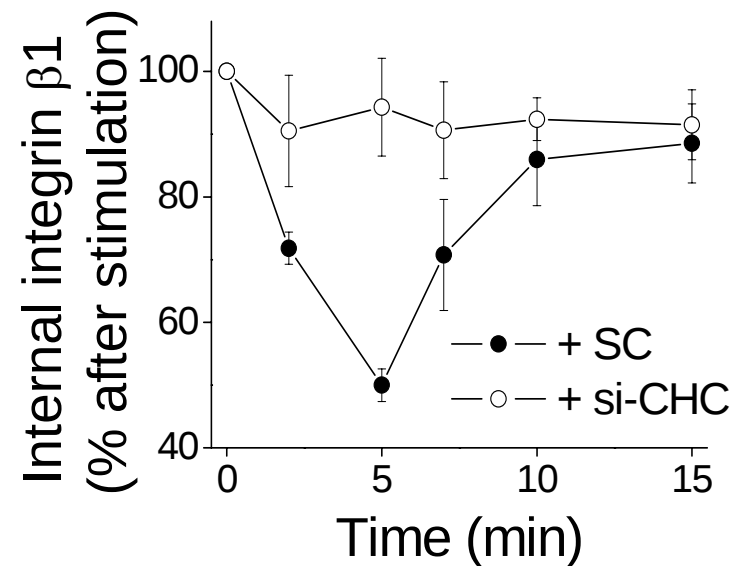
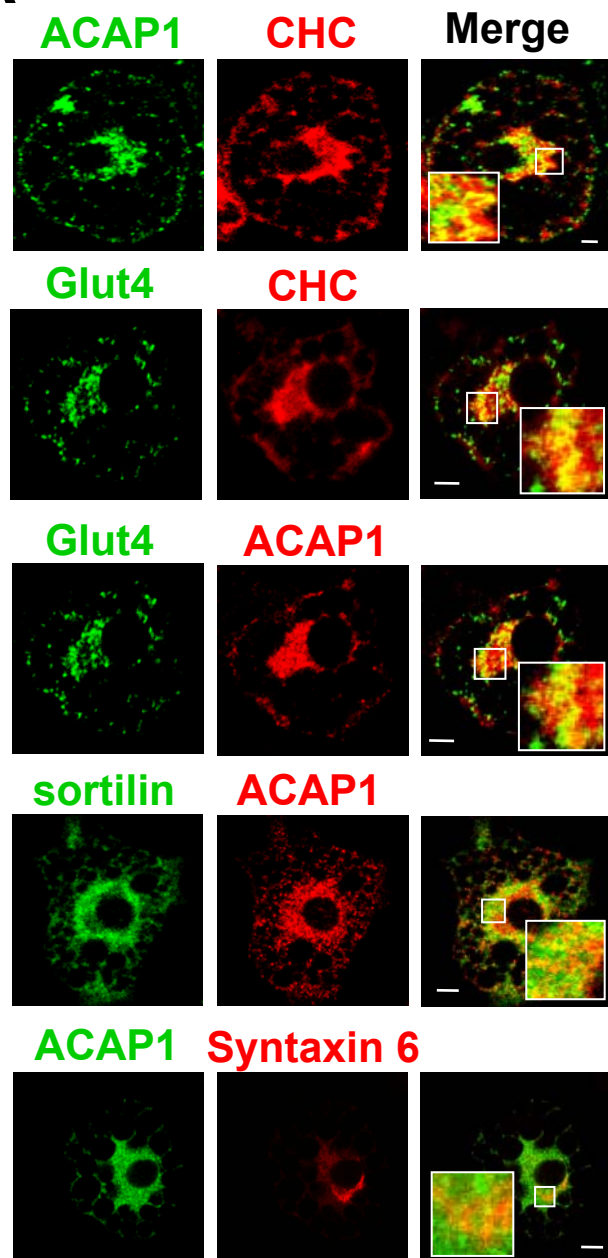
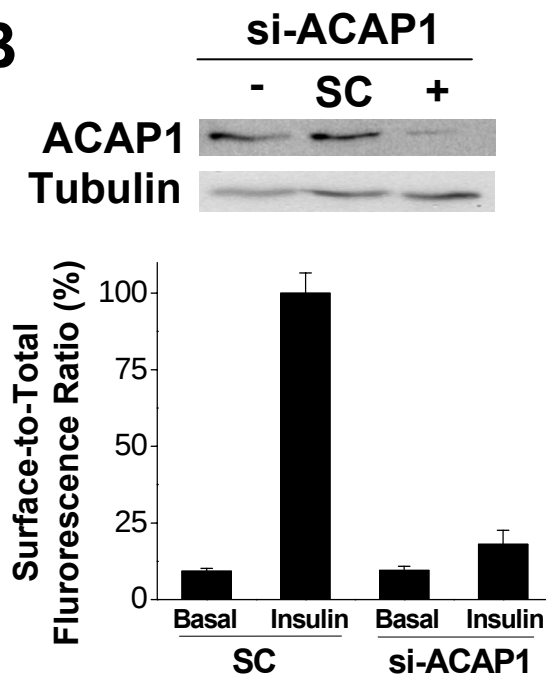


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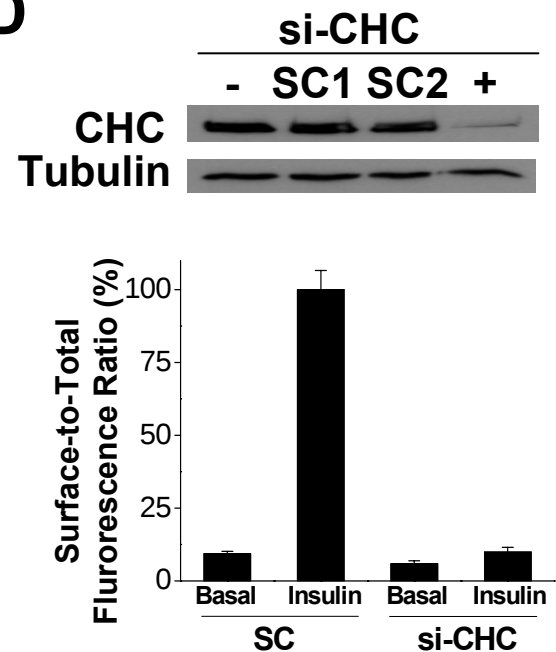
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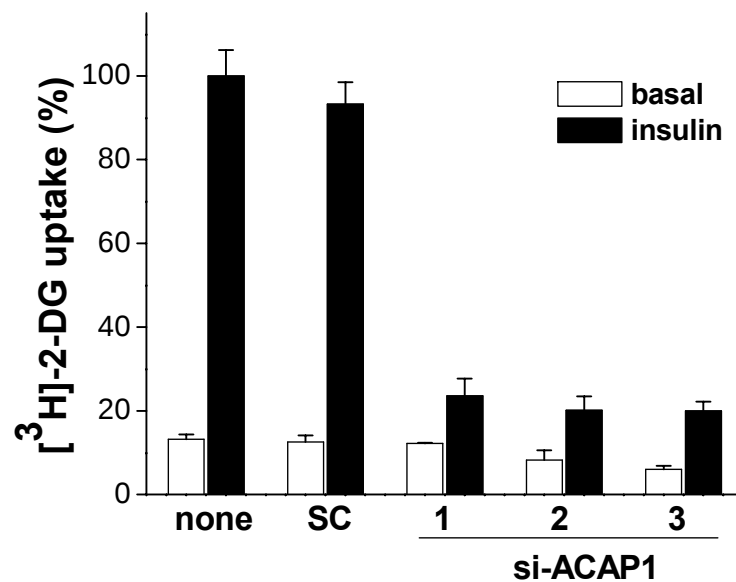


Figure 4

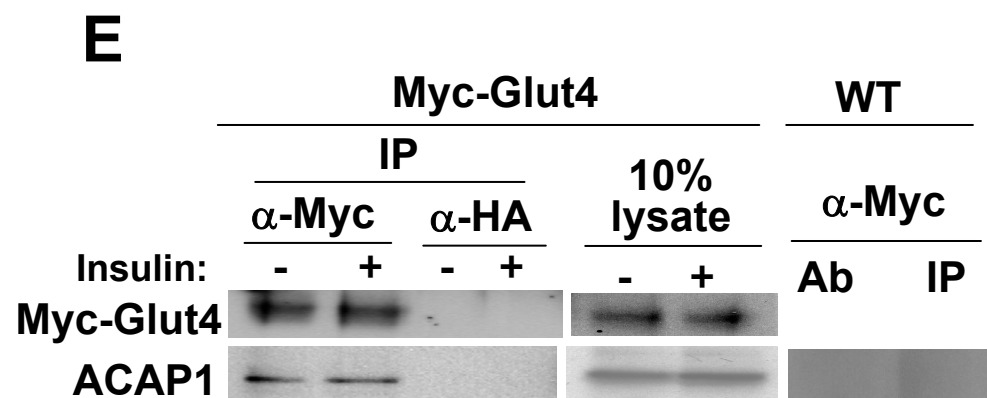
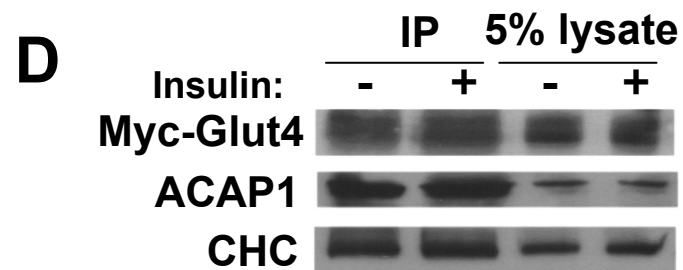
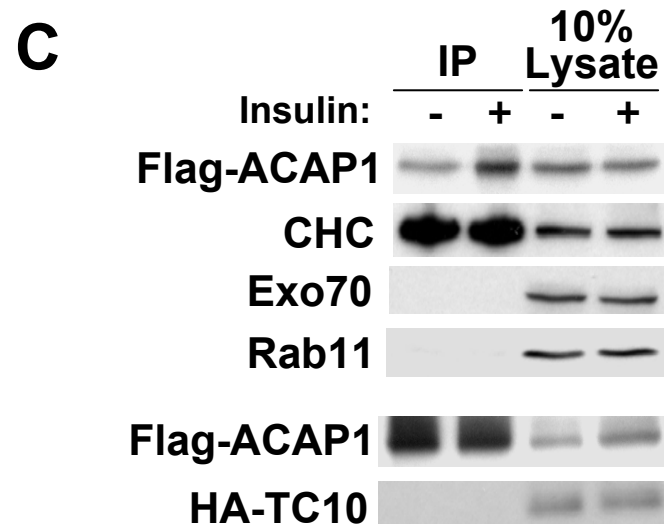
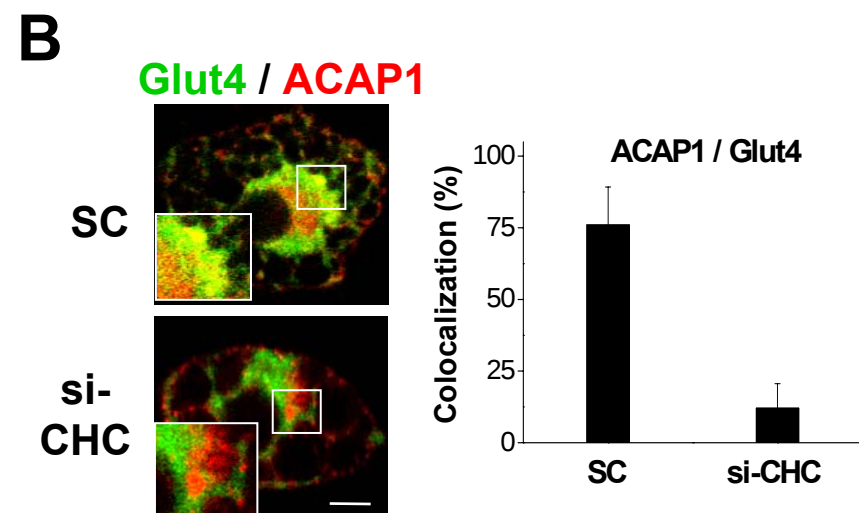
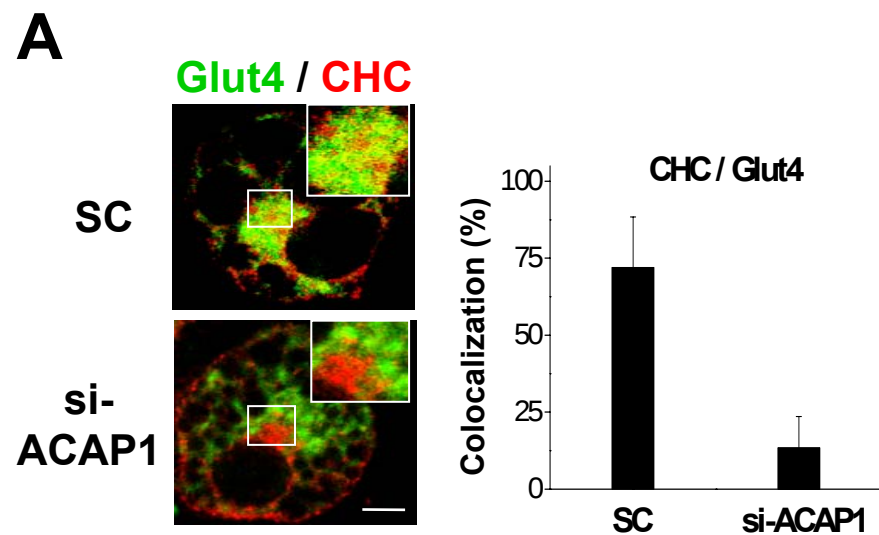
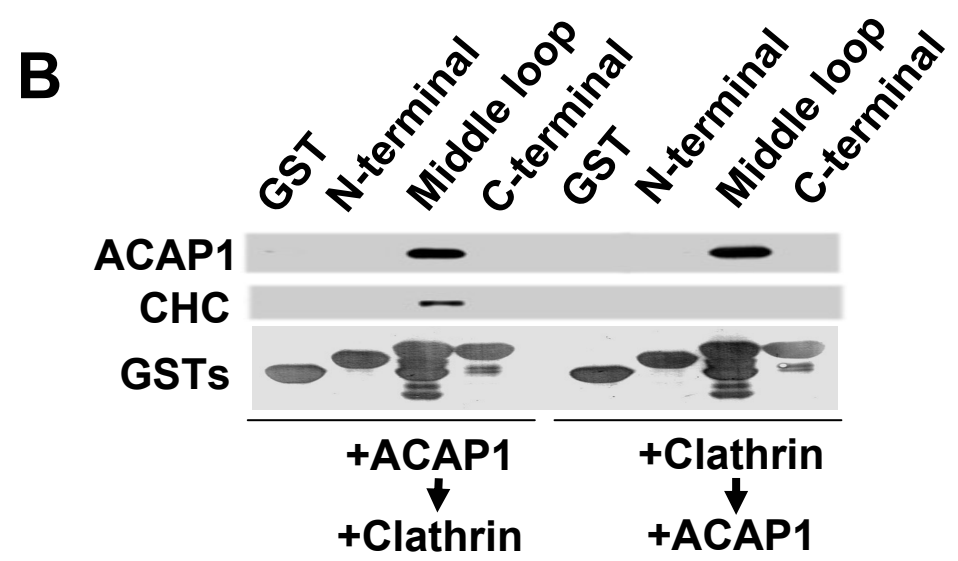
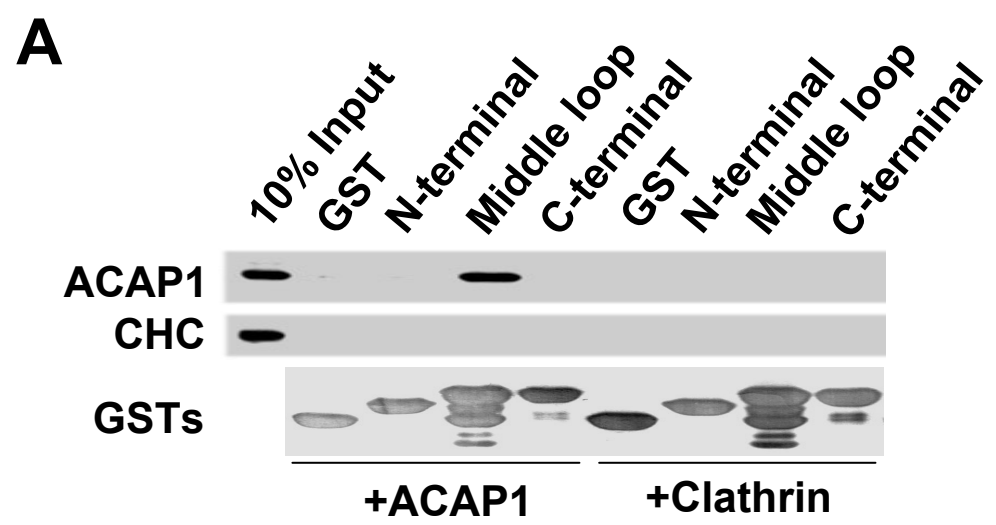


Figure 5



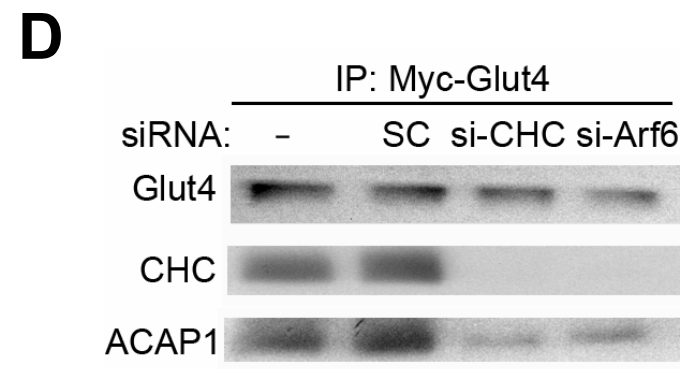
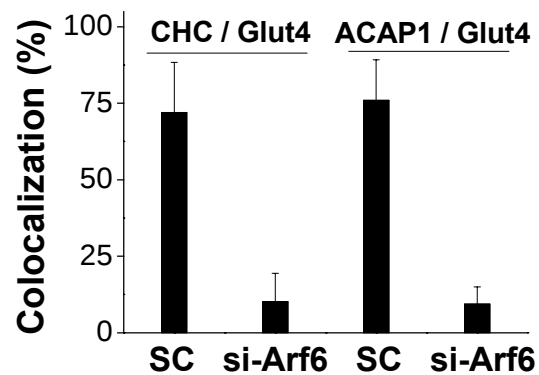
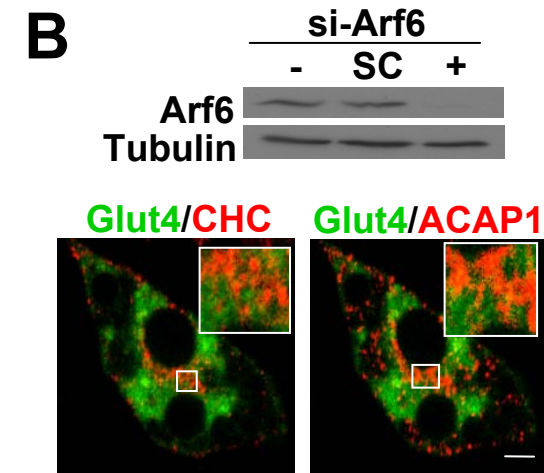
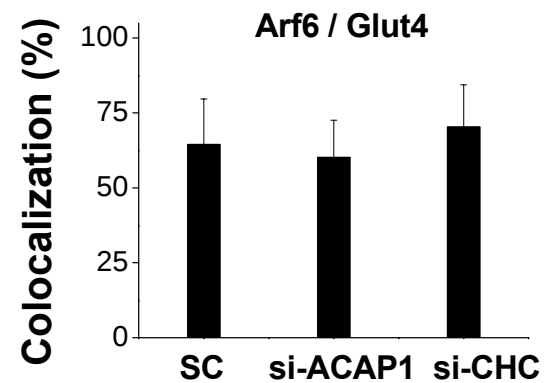
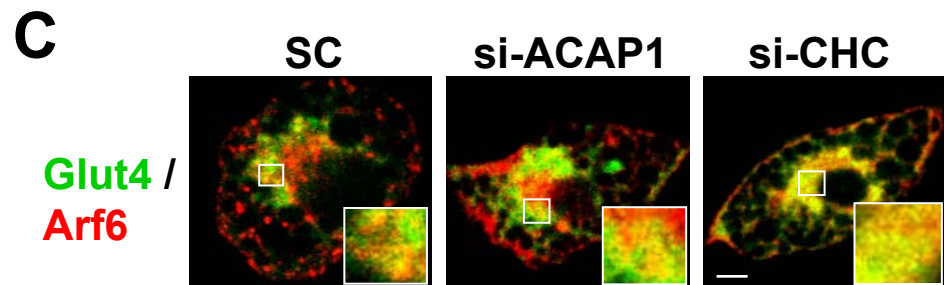
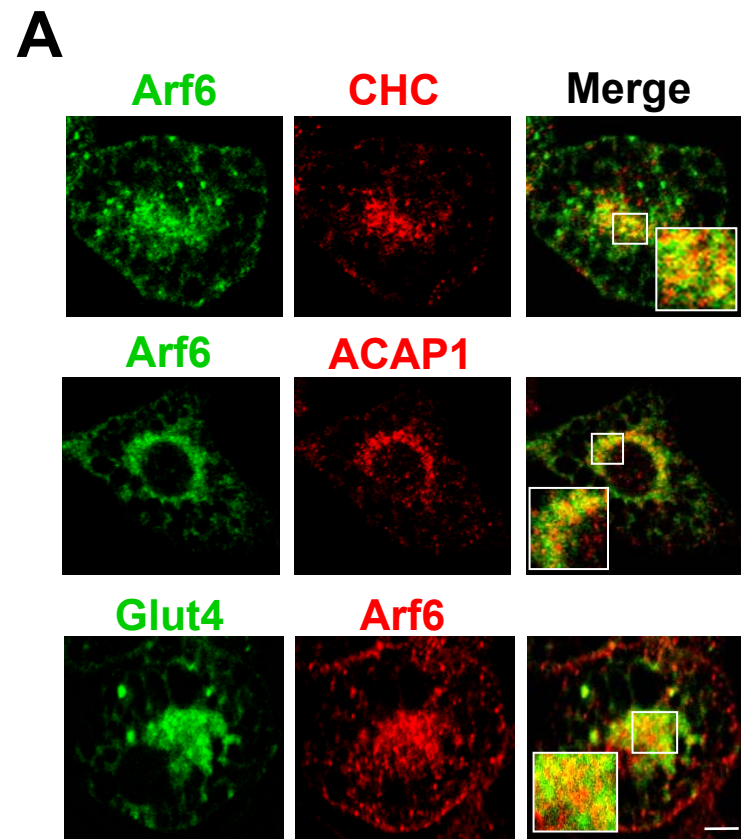
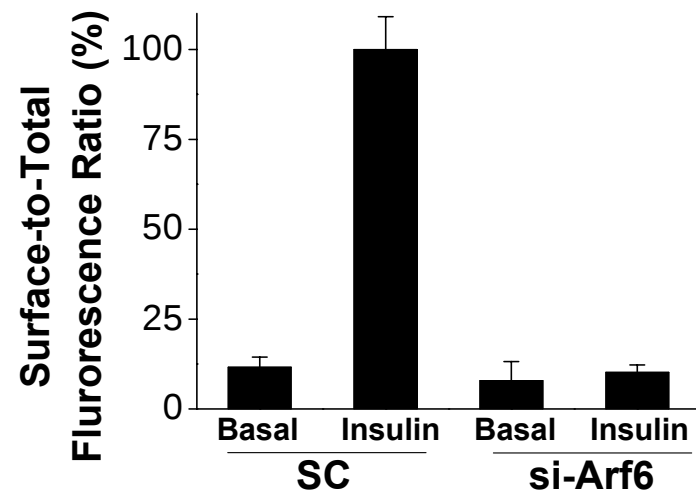
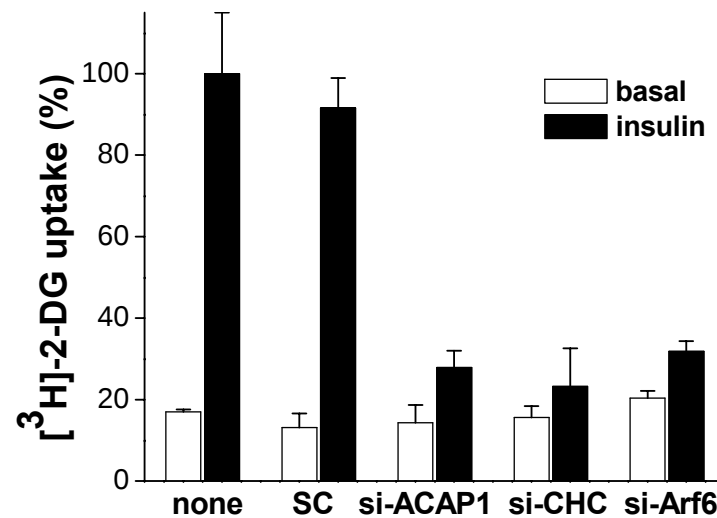


Figure 8

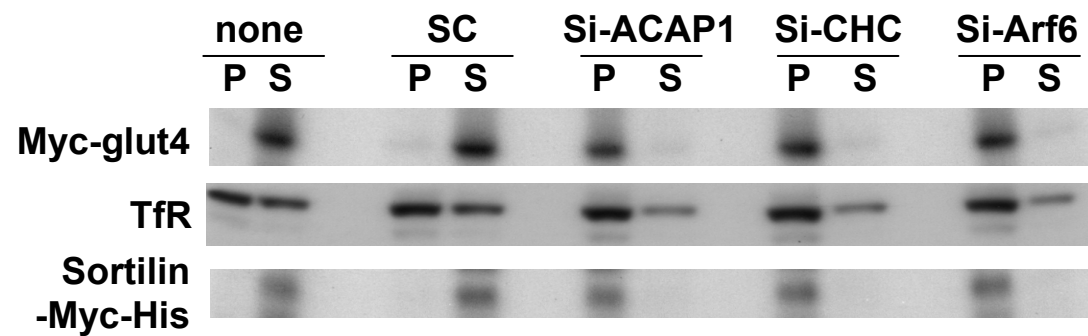
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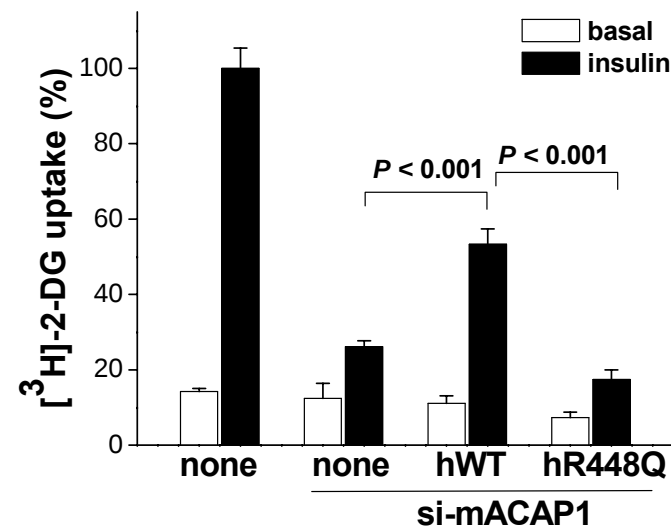
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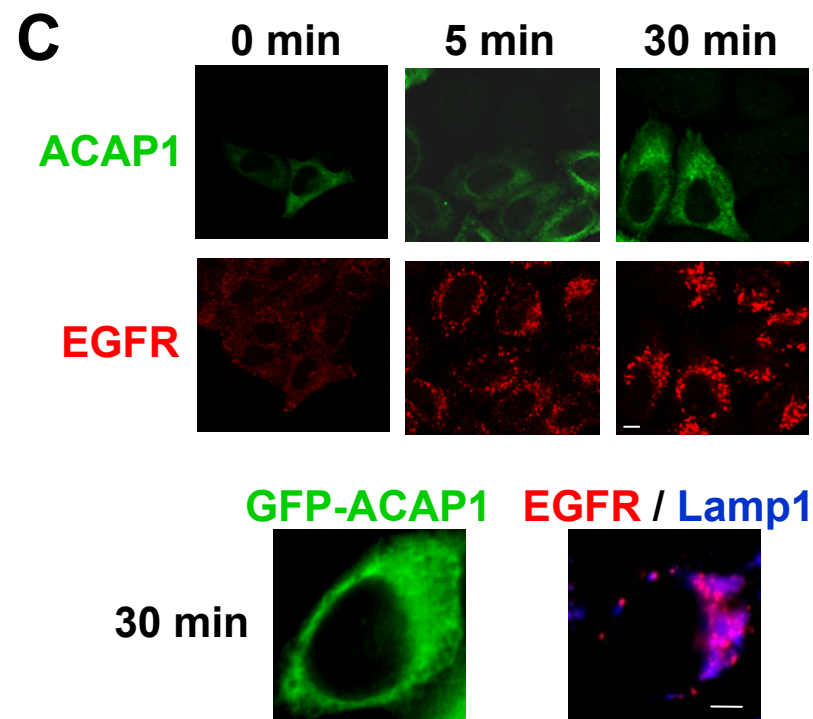
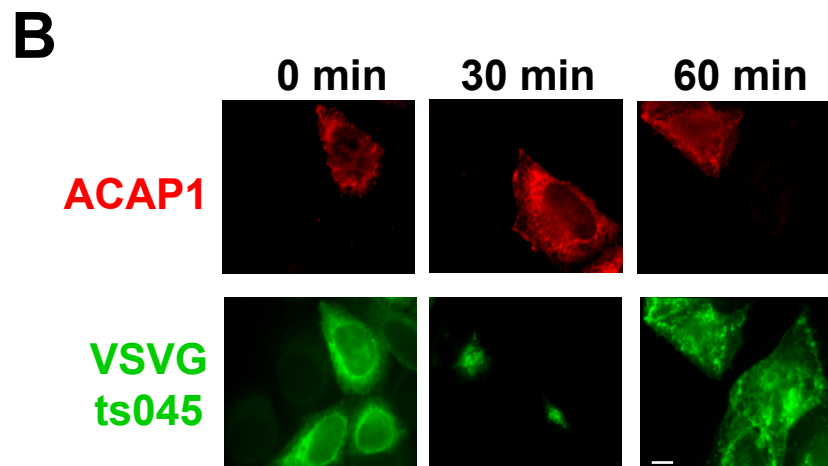
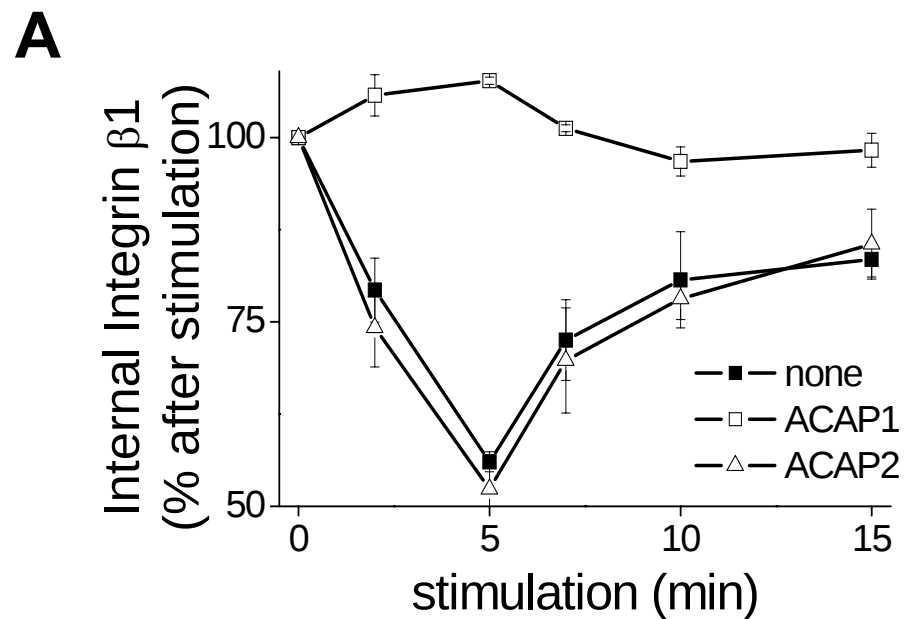
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D



Supplementary Figure 1



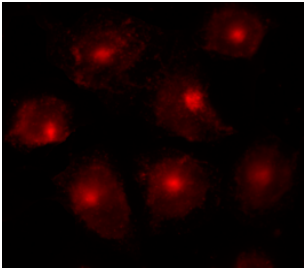
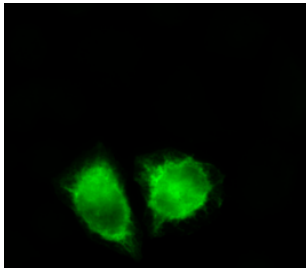
Supplementary Figure 2

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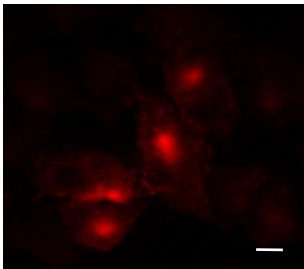
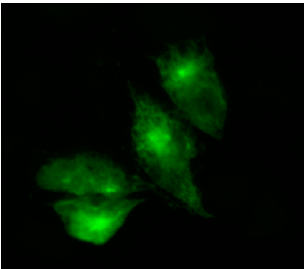
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R448Q

Tf

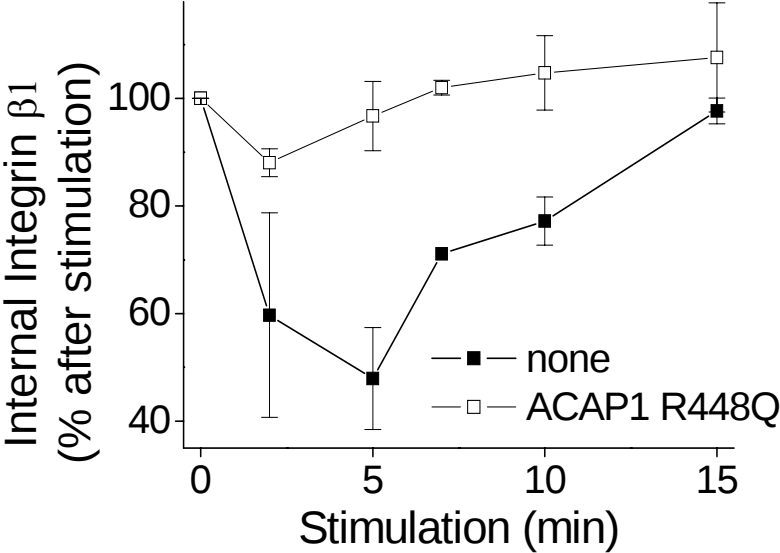
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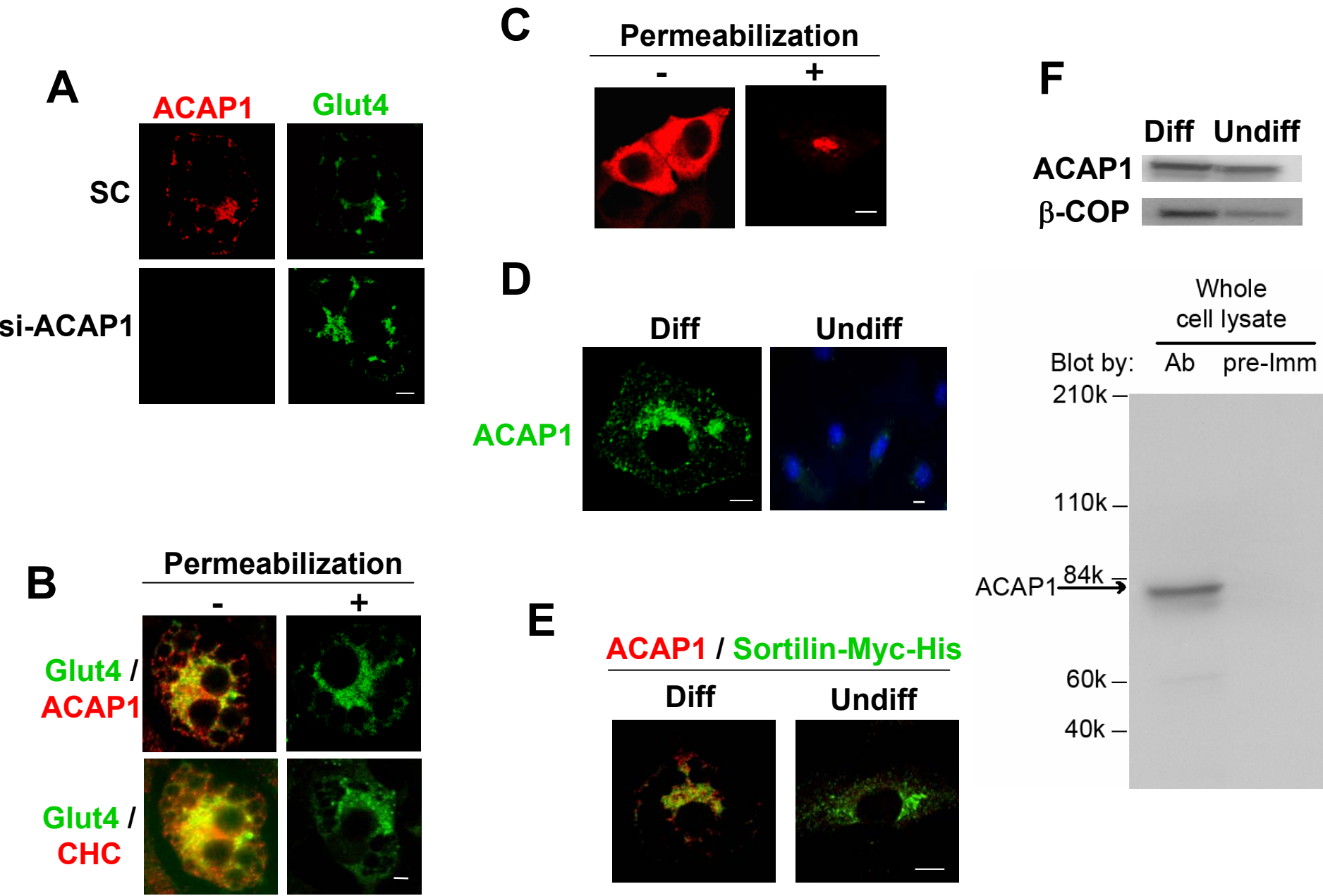
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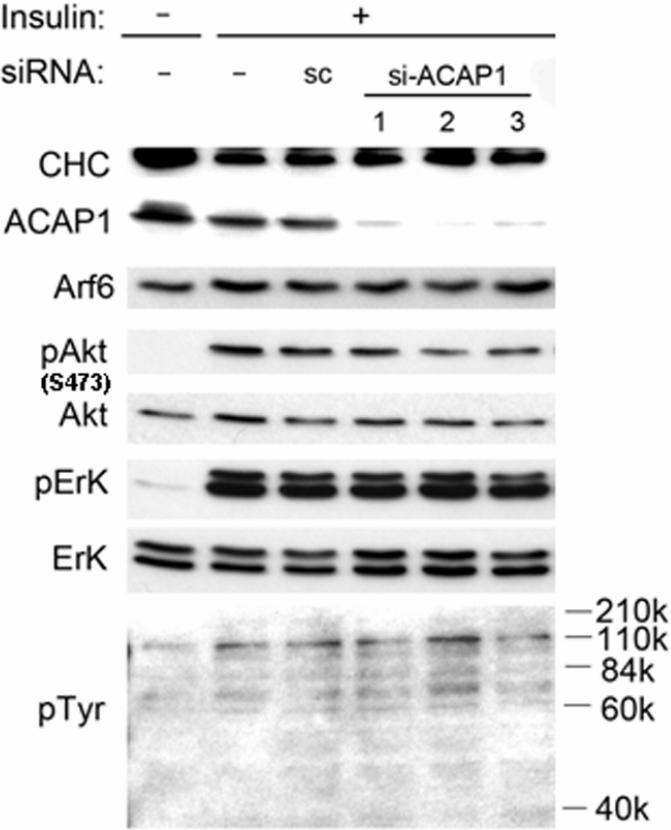


Supplementary Figure 3



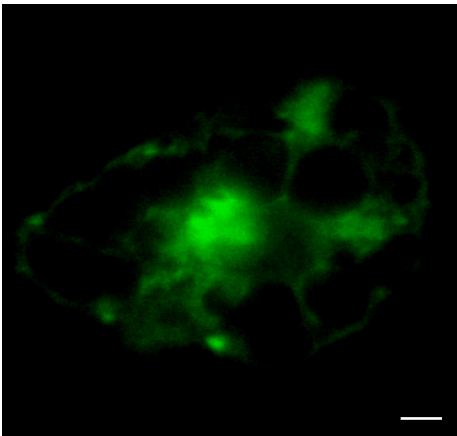
Supplementary Figure 4

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B

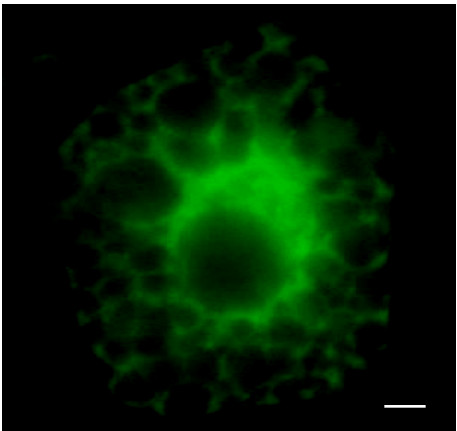
Glut4



si-ACAP1

C

Glut4

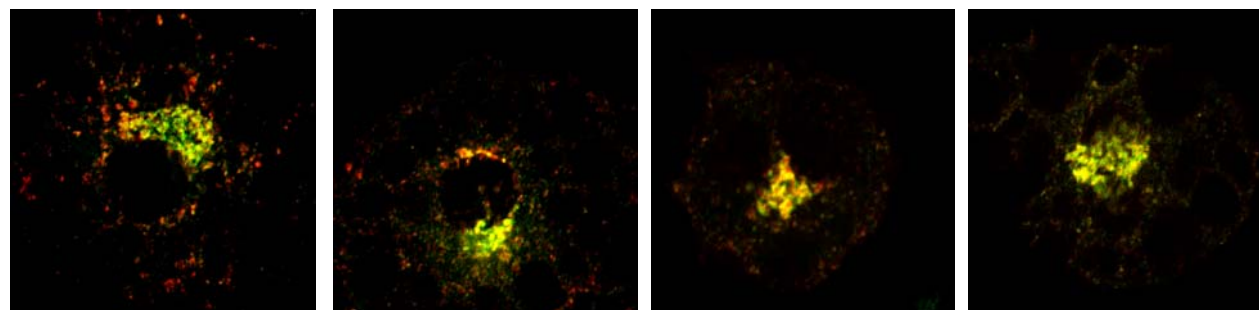


si-CHC

Supplementary Figure 5

A

Glut4 / sortilin-Myc-His

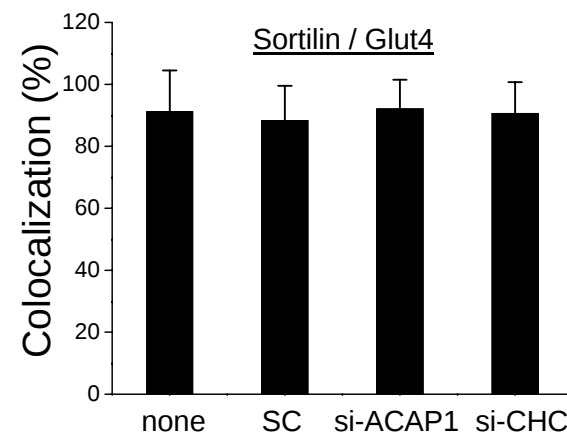


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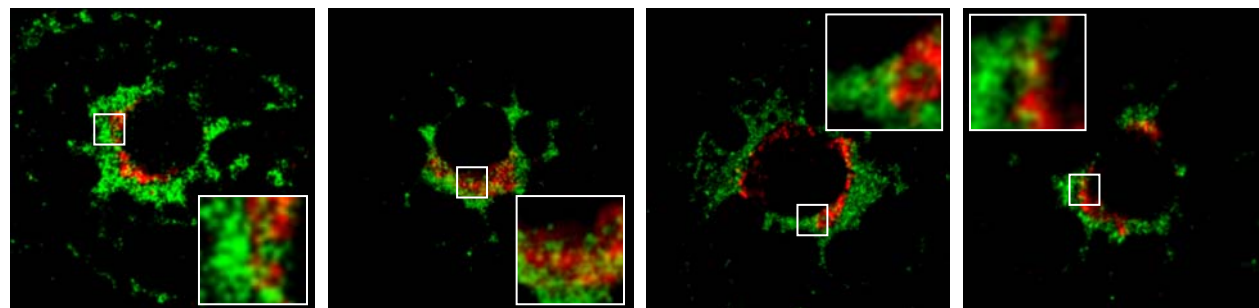
+ si-ACAP1

+ si-CHC



B

Syntaxin 6 / Glut4

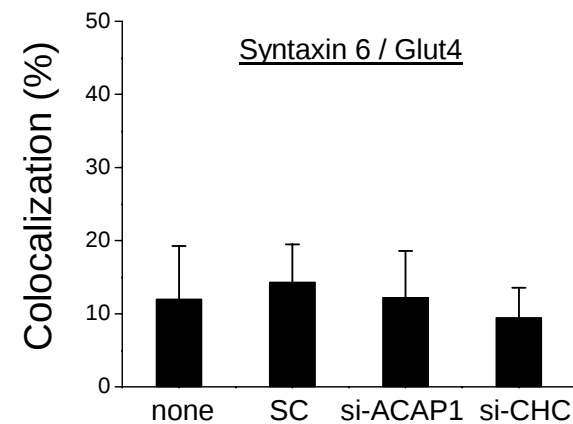


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Supplementary Figure 6

