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Clathrin-dependent mechanisms modulate the subcellular distribution of class C Vps/HOPS tether subunits in polarized and nonpolarized cells

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ABSTRACT Coats define the composition of carriers budding from organelles. In addition, coats interact with membrane tethers required for vesicular fusion. The yeast AP-3 (Adaptor Protein Complex 3) coat and the class C Vps/HOPS (HOmotypic fusion and Protein Sorting) tether follow this model as their interaction occurs at the carrier fusion step. Here we show that mammalian Vps class C/HOPS subunits and clathrin interact and that acute perturbation of clathrin function disrupts the endosomal distribution of Vps class C/HOPS tethers in HEK293T and polarized neuronal cells. Vps class C/HOPS subunits and clathrin exist in complex with either AP-3 or hepatocyte growth factor receptor substrate (Hrs). Moreover, Vps class C/HOPS proteins cofractionate with clathrin-coated vesicles, which are devoid of Hrs. Expression of FK506 binding protein (FKBP)–clathrin light chain chimeras, to inhibit clathrin membrane association dynamics, increased Vps class C/HOPS subunit content in rab5 endosomal compartments. Additionally, Vps class C/HOPS subunits were concentrated at tips of neuronal processes, and their delivery was impaired by expression of FKBP–clathrin chimeras and AP20187 incubation. These data support a model in which Vps class C/HOPS subunits incorporate into clathrin-coated endosomal domains and carriers in mammalian cells. We propose that vesicular (AP-3) and nonvesicular (Hrs) clathrin mechanisms segregate class C Vps/HOPS tethers to organelles and domains of mammalian cells bearing complex architectures.

INTRODUCTION Membranous organelles maintain their steady-state structural and functional identity despite the continuous flow of protein and lipids through them. Flow is dictated by departing carriers budding off organelles and incoming vesicles fusing with their targets. Thus fidelity in vesicle budding and fusion is central to generating and maintaining organelle identity. Coats and adaptors specify vesicle-budding events by selecting cargoes loaded into vesicle carriers. In contrast, Soluble NSF Attachment Protein REceptors (SNAREs), rabs, and tethers determine vesicle fusion fidelity (Bonifacino and Glick, 2004). Vesicular and nonvesicular mechanisms regulate flow of macromolecules between endosomes in mammals. Flow between compartments along the endocytic pathway occurs by vesicle-mediated mechanisms (Stoorvogel et al., 1996; Peden et al., 2004), and the processes of tubule-mediated transfer of cargoes (Delevoye et al., 2009), kiss-and-run (Bright et al., 2005), and endosome maturation (Stoorvogel et al., 1991; Rink et al., 2005; Poteryaev et al., 2010). Here we show that the coat clathrin interacts with endosomal tethers involved in endosome maturation and fusion (class C Vps/HOPS subunits) by means of vesicular (clathrin and the clathrin adaptor AP-3 [Adaptor Protein Complex 3]) and nonvesicular mechanisms (clathrin and hepatocyte growth factor receptor substrate, Hrs).
Mammalian endosomes possess clathrin domains in the form of budding profiles that contain AP-1/AP-3 clathrin adaptors (Stoorvogel et al., 1996; Peden et al., 2004; Theos et al., 2005) or as flat lattices defined by the presence of Hrs (Raiborg et al., 2002, 2006). Of these coats, buds containing AP-3 and clathrin mature into vesicles that transport their contents between early endosomes and late endosomal/lysosomal compartments (Di Pietro and Dell’Angelica, 2005; Dell’Angelica, 2009). In contrast, Saccharomyces cerevisiae generates AP-3 vesicles at the Golgi complex destined for the lysosomal equivalent, the vacuole (Odorizzi et al., 1998). This process is independent of clathrin in yeast (Seeeger and Payne, 1992; Anand et al., 2009). Mutation of the human clathrin adaptor AP-3 causes Hermansky Pudlak Syndrome (HPS), in which lysosome-related organelles such as melanosomes, dense-platelet granules, and lamellar bodies are defective. HPS patients exhibit pigment dilution, bleeding diathesis, and pulmonary fibrosis, and this pathology is directly attributable to defects in vesicular biogenesis (Di Pietro and Dell’Angelica, 2005; Dell’Angelica, 2009). HPS phenotypes are triggered in mice when any one of 15 genes is defective. These 15 gene products assemble into protein complexes: AP-3, BLOC-1 to BLOC-3 (biogenesis of lysosome-related organelles complex), and tethering complexes constituted by class C Vps subunits (Li et al., 2004). Vps33a mutant mice possess a phenotype similar to other HPS mutants, which might be explained by interactions of Vps33a with other HPS complexes (Suzuki et al., 2003). Whether these genetic similarities underlie associations of Vps33a with HPS complexes, such as AP-3, into common complexes is presently unknown. This hypothesis is supported, however, by genetic and biochemical interactions between yeast class C Vps proteins and AP-3 (Anand et al., 2009; Angers and Merz, 2009; Salazar et al., 2009).

The mammalian class C Vps protein Vps33a/b assembles into a core complex of class C Vps proteins including Vps11, Vps16, and Vps18. This core associates with Vps39 and Vps41 to form the homotypic fusion and Protein Sorting (HOPS) complex (Kim et al., 2001; Nickerson et al., 2009; Zhu et al., 2009). The class C Vps core complex could also associate with Vps8/KIAA0804 and Vps3/TGFBRAP1 to constitute a hypothetical mammalian CORVET complex, a molecular species so far only documented in yeast (Peplowska et al., 2007; Markgraf et al., 2009). Class C core subunits and accessory HOPS subunits are critical for fusion events at the vacuole, a lysosome equivalent in the yeast S. cerevisiae. In this organism, HOPS orchestrates interactions with the GTPase ypt7, a rab7 ortholog, SNAREs, and, recently, AP-3 to achieve selective membrane fusion (Ostrowicz et al., 2008; Angers and Merz, 2009; Nickerson et al., 2009; Wickner, 2010). Association of AP-3 and HOPS occurs at the vesicle fusion step with the vacuole when incoming Golgi-derived AP-3–coated vesicles reach the vacuole, the compartment where HOPS complexes reside in yeast (Angers and Merz, 2009).

Here we show that mammalian Vps class C/HOPS subunits coprecipitated and/or colocalized with the endosomal coats clathrin, AP-3, and Hrs. Isolated clathrin-coated vesicles had Vps class C/HOPS subunits on them, yet they were depleted of Hrs. We also found that acute perturbation of clathrin dynamics on and off membranes using FBPl–clathrin light chain (CLC) chimeras (Moskowitz et al., 2003; Deborde et al., 2008) altered the distribution of class C Vps/HOPS proteins in two different types of mammalian cells. HEK293T cells with reduced clathrin function had elevated levels of Vps class C proteins in rab5-positive endosomes, suggesting that clathrin dynamics at vesicular AP-3– or nonvesicular Hrs-positive endosomal domains regulate Vps class C protein content on early endosomes. We hypothesized that coats in endosomal tether-coat complexes could contribute to polarized distribution of tethers, such as the HOPS complex. In support of this idea, differentiated neuroendocrine cells accumulated Vps class C/HOPS subunits at the growing tip of processes, where they were concentrated with clathrin. Targeting of Vps class C/HOPS subunits from cell bodies to neurites was sensitive to clathrin function perturbation. Our data suggest that vertebrate cells differ from yeast in that the vertebrates engage in novel interactions between class C Vps–containing tethers and coats, perhaps for specialized demands such as polarized targeting in neuroendocrine and epithelial cells.

**RESULTS**

**Subunits of the Vps class C/HOPS tethering complexes associate with clathrin–AP-3 adaptor subunits**

We initially explored molecular interactions between tethering complexes and coats taking advantage of protein complexes affected in HPS. Mouse mutations on genes encoding the class C-Vps protein Vps33a or AP-3 subunits trigger a phenotype that recapitulates HPS (Suzuki et al., 2003; Li et al., 2004), suggesting that AP-3 and complexes containing Vps33a act on a common pathway. Consistent with this interpretation, subunits of the tethering HOPS complex interact with AP-3 in both S. cerevisiae (Angers and Merz, 2009) and mammalian cells (Salazar et al., 2005, 2009). Despite this similarity, in mammalian cells, but not yeast, AP-3 shows interactions with clathrin (Seeeger and Payne, 1992; Dell’Angelica et al., 1998; Anand et al., 2009). This observation suggests that Vps class C/HOPS tethers interact with coats differently in vertebrates as compared with yeast.

We tested whether all four class C Vps proteins, class C Vps33a–b isoforms, and the two HOPS-specific subunits associated with AP-3 and clathrin complexes isolated from HEK293T cells. As described in Materials and Methods, we used in vivo cross-linking with dithiobisuccinimidyl propionate (DSP) coupled to immunofinity chromatography with monoclonal antibodies (mAbs) against the AP-3 δ subunit or clathrin chains. DSP treatment decreases immunoreactivity of AP-3 δ subunit detected with a mAb against δ adaptin (see Materials and Methods). Vps class C and HOPS subunits present in AP-3 or clathrin immune complexes were detected with antibodies either against endogenously expressed Vps33b or tags engineered in recombinantly expressed subunits (Vps11, Vps16, Vps18, Vps33a, Vps33b, Vps39, and Vps41). We expressed tagged recombinant proteins because available class C Vps or HOPS subunit antibodies failed to detect endogenous proteins in multiple cell types, including HEK293T cells (our unpublished data).

We first determined whether phenotypic similarities between mice carrying mutations in either the class C Vps protein Vps33a or AP-3 subunits were predictors of a biochemical interaction between Vps33a and AP-3. Recombinantly expressed Vps33a selectively co-precipitated with AP-3 immunocomplexes (Figure 1A, compare lanes 4 and 6). The association of Vps33 isoforms with AP-3 δ subunit or clathrin chains. DSP treatment decreases immunoreactivity of AP-3 δ subunit detected with a mAb against δ adaptin (see Materials and Methods). Vps class C and HOPS subunits present in AP-3 or clathrin immune complexes were detected with antibodies either against endogenously expressed Vps33b or tags engineered in recombinantly expressed subunits (Vps11, Vps16, Vps18, Vps33a, Vps33b, Vps39, and Vps41). We expressed tagged recombinant proteins because available class C Vps or HOPS subunit antibodies failed to detect endogenous proteins in multiple cell types, including HEK293T cells (our unpublished data).

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FIGURE 1: Association of class C Vps/HOPS subunits with the adaptor complex AP-3. HEK293T cells or cell lines expressing recombinant class C Vps/HOPS subunits (A–G) were left untreated (B–G, odd lanes) or treated (B–G, even lanes) with DSP at 4°C. Detergent-soluble cell extracts were incubated with sheep anti–mouse IgG-coated magnetic beads as follows: (A) Extracts of DSP-treated HEK293T cells transfected with Vps33A (odd lanes) or Vps33b (even lanes) incubated with magnetic beads decorated with antibody directed against AP-3 δ (lanes 3–6). The peptide used to raise the AP-3 δ mAb was added in excess during the immunoprecipitation to determine the specificity of signals detected by immunoblot (lanes 3 and 4). (B) Extracts of HEK293T cells (lanes 3, 4, 9, and 10) and cells expressing Vps18–Myc (lanes 1, 2, and 5–8) were incubated with anti–mouse IgG magnetic beads either lacking mouse antibody (lanes 5 and 6, No antibody) or linked to mouse mAb directed against AP-3 δ (lanes 7–10). (C) Extracts of HEK293T cells (lanes 3, 4, 7, 8, 11, and 12) and cells expressing Vps16–HA (lanes 1, 2, 5, 6, 9, and 10) were incubated with anti–mouse IgG magnetic beads decorated with antibody directed against AP-3 δ (lanes 5–12). The peptide used to raise the AP-3 δ mAb was used as in A (lanes 5–8). (D) Extracts of HEK293T cells (lanes 5, 6, 11, 12, 17, and 18) cells expressing Vps11–HA (lanes 3, 4, 9, 10, 15, and 16), or cells expressing Vps39–GFP (E and F lanes 5–8). AP-3 δ peptide competition was performed as in A (lanes 5 and 6). (E and F) Extracts of HEK293T cells (E and F lanes 3, 4, 7, 8, 11, and 12) and cells expressing Vps39–GFP (E lanes 1, 2, 5, 6, 9, and 10) or cells expressing Vps41–Myc (F lanes 1, 2, 5, 6, 9, and 10) were incubated with anti–mouse IgG magnetic beads bound to AP-3 δ antibodies (E and F lanes 5–12). AP-3 δ peptide competition was performed as in A (E and F lanes 5–8). (G) Cells expressing both Vps16–HA and Vps41–Myc were incubated with anti–mouse IgG magnetic beads only (lanes 3 and 4) or beads decorated with mAbs directed against: AP-3 δ subunit (lanes 5–8, AP-3 δ) or AP-1 γ subunit (lanes 9 and 10, AP-1 γ). Specificity of association with AP-3 complexes was determined by peptide competition as in A (lanes 5 and 6). Immune complexes were resolved by SDS–PAGE, and their composition was assessed by immunoblot with antibodies against AP-3 δ, AP-3 β, AP-1 γ, CHC, endogenous Vps33b, Myc, GFP, or HA tags. Arrows in B, D, E, and F depict specific bands, and other bands correspond to background sheep or mouse IgG from beads or residual signal from previous immunoblot probings. Arrowhead in AP-1 γ blot strip depicts sheep or mouse IgG from beads or residual signal from previous immunoblot probing. All experiments were performed at least twice in stably or transiently expressing cells. For A–G, inputs represent 5%.
First, cross-linked AP-3 complexes were immunoaffinity purified with AP-3-δ antibodies, yet none of these proteins were detected with beads alone (Figure 1B, compare lanes 5 and 6 with 7–10). Pretreating beads displaying δ-antibodies with the δ-peptide abolished binding of AP-3 subunits, clathrin, and all Vps subunits to these beads (Figure 1A, compare lanes 3 and 4 with lanes 5 and 6; Figure 1, C, E, and F, compare lanes 6 and 8 with 10 and 12; Figure 1D, compare lanes 8, 10, and 12 with lanes 14, 16, and 18). Abolished binding indicates that class C Vps and HOPS subunits interact with the beads by binding to intact AP-3 and not via some nonspecific mechanism. As a control, we showed that AP-1 complexes were not cross-linked to Vps33b, Vps16-HA (hemagglutinin), or Vps41-Myc, showing that the association of class C Vps and HOPS subunits with AP-3 was selective (Figure 1G). AP-3-Vps subunit associations could result simply from abundantly expressed tagged proteins in endosomal compartments. To test this hypothesis, we asked whether the Vps33b-interacting protein Spe39 coisolates with AP-3 and clathrin complexes. We selected Spe39 because we previously demonstrated that Spe39 defines a subclass of Vps class C/HOPS complexes (Zhu et al., 2009). In fact, Spe39 is present in a discrete pool of the total Vps33b and other class C protein as determined by quantitative immunomicroscopy (Zhu et al., 2009). Importantly, endogenous Spe39 does not colocalize with AP-3 or CLCs (Supplemental Figure 1, A and B). Consistent with our immunomicroscopy, neither endogenous nor recombinant green fluorescent protein (GFP)-tagged Spe39 coprecipitated with AP-3 immunocomplexes (Supplemental Figure 1, C and D). These results argue against nonselective DSP cross-linking of recombinantly expressed proteins to AP-3.

Others have proposed that the HOPS subunit Vps41 can associate with AP-3 as a clathrin-like coat (Rehling et al., 1999; Darsow et al., 2001). Consequently, we next looked for association of clathrin with Vps class C/HOPS complexes (Zhu et al., 2009). In fact, Spe39 is present in a discrete pool of the total Vps33b and other class C protein as determined by quantitative immunomicroscopy (Zhu et al., 2009). Importantly, endogenous Spe39 does not colocalize with AP-3 or CLCs (Supplemental Figure 1, A and B). Consistent with our immunomicroscopy, neither endogenous nor recombinant green fluorescent protein (GFP)-tagged Spe39 coprecipitated with AP-3 immunocomplexes (Supplemental Figure 1, C and D). These results argue against nonselective DSP cross-linking of recombinantly expressed proteins to AP-3.

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Vps class C and HOPS subunits are present in clathrin-coated organelles

Vps class C and HOPS subunits association with clathrin–AP-3 and clathrin–Hrs predicts that these Vps proteins should be present in
domains of early endosomes as well as clathrin-coated vesicle carriers. The presence of Vps class C and HOPS subunits in early endosomes and clathrin-coated carriers would be consistent with the observation that AP-3, AP-3-clathrin budding profiles, clathrin–Hrs flat coats, Vps class C, and HOPS subunits are found in early endosomal compartments (Raiborg et al., 2002, 2006; May 15, 2011).

**FIGURE 2:** CHC and CLC associate with class C Vps/HOPS subunits. (A) Extracts of DSP-treated HEK293T cells transfected with Vps33A or Vps33b were incubated with magnetic beads decorated with antibody directed against control IgG (lane 2) or CLC (lane 3). Immunocomplexes were resolved by SDS–PAGE, and contents were analyzed by immunoblot with antibodies against AP-3 (δ). AP-3 δ immunoblot with antibodies against AP-3 δ cells. Immunocomplexes were resolved by SDS–PAGE, and contents were analyzed by antibodies (lanes 8). SV2 and SY38 antibodies recognize neuronal antigens absent in HEK293T control mouse IgG (mAb SV2 [top two panels] and SY38 [bottom two panels]), lane 6) were eluted under native conditions using excess δ decorated with AP-3 δ beads in lane 6. Clarified cell extracts were first immunoprecipitated with magnetic beads expressing Vps41–Myc were treated in the absence (lanes 1, 3, and 5) or presence of DSP (odd lanes 3 and 4). Cross-linked AP-3 complexes bound to beads in lane 6 were eluted under native conditions using δ antigenic peptide. These eluted cross-linked AP-3 complexes were subjected to a second round of immunoprecipitations with control mouse IgG (mAb SV2 [top two panels] and SY38 [bottom two panels]), lane 7) or CLC antibodies (lane 8). SV2 and SY38 antibodies recognize neuronal antigens absent in HEK293T cells. Immunocomplexes were resolved by SDS–PAGE, and contents were analyzed by immunoblot with antibodies against AP-3 δ and Myc epitope. Arrows mark specific bands for AP-3 δ and Vps41–Myc. Note the different background bands obtained with the two different negative control antibodies during the second round of immunoprecipitation. Input represents 5%. (D) Interaction of Vps class C/HOPS subunits with clathrin is not affected by down-regulation of AP-3. HEK293T cells stably expressing Vps41–Myc were infected with lentiviruses encoding nontargeting shRNA (Scr, lanes 1, 3, and 5) or shRNA targeting AP-3 δ (lanes 2, 4, and 6). After 6 d, cells were incubated in the presence of DSP (lanes 1–6) and homogenized. Detergent cell extracts were immunoprecipitated with magnetic beads decorated with control mouse IgG (monoclonal SY38, lanes 3 and 4), or antibodies against CLCs (lanes 5 and 6). Immunocomplexes were resolved by SDS–PAGE, and contents were analyzed by immunoblot with antibodies against AP-3 β, AP3 δ, CHC, Myc epitope, Vps33b, and β actin. Lanes 1 and 2 correspond to immunoblot with antibodies against AP-3 β, AP3 δ, CHC, Myc epitope, Vps33b, and β actin. Lanes 1 and 2 correspond to homogenates run on different gels to assess efficiency of down-regulation. (E) Vps class C/HOPS subunits interaction with AP-3 β, AP3 δ, CHC, Myc epitope, Vps33b, and β actin. Lanes 1 and 2 correspond to homogenates run on different gels to assess efficiency of down-regulation. (F) Vps33b associates with clathrin–Hrs coats. HEK293T cells (lanes 1–6) or transiently transfected with Hrs–Myc (lanes 1–6) were treated in the absence (odd lanes) or presence of DSP (even lanes). Detergent-soluble cell extracts were immunoprecipitated with magnetic beads decorated with AP-3 δ antibodies (lanes 3–10) either in the absence (lanes 7–10) or presence of an excess of δ antigenic peptide as a control (lanes 3–6). Immunocomplexes were resolved by SDS–PAGE, and contents were analyzed by immunoblot with antibodies against AP-3 δ, CHC, Myc epitope, Vps33b, and β actin. Lanes 1 and 2 correspond to homogenates run on different gels to assess efficiency of down-regulation. (F) Vps33b associates with clathrin–Hrs coats. HEK293T cells (lanes 1–6) or transiently transfected with Hrs–Myc (lanes 1–6) were treated in the absence (odd lanes) or presence of DSP (even lanes). Detergent-soluble cell extracts were immunoprecipitated with magnetic beads decorated with AP-3 δ antibodies (lanes 3–10) either in the absence (lanes 7–10) or presence of an excess of δ antigenic peptide as a control (lanes 3–6). Immunocomplexes were resolved by SDS–PAGE, and contents were analyzed by immunoblot with antibodies against AP-3 δ, CHC, Myc epitope, Vps33b, and β actin. Lanes 1 and 2 correspond to homogenates run on different gels to assess efficiency of down-regulation. (G) Vps class C/HOPS subunits interaction with AP-3 β, AP3 δ, CHC, Myc epitope, Vps33b, and β actin. Lanes 1 and 2 correspond to homogenates run on different gels to assess efficiency of down-regulation. (H) Vps class C/HOPS subunits interaction with AP-3 β, AP3 δ, CHC, Myc epitope, Vps33b, and β actin. Lanes 1 and 2 correspond to homogenates run on different gels to assess efficiency of down-regulation.
Peden et al., 2004; Richardson et al., 2004; Theos et al., 2005). We
determined the subcellular distribution of Vps class C and HOPS
subunits by immunofluorescence using quantitative Delta deconvo-
lution microscopy. We used antibodies against coats (clathrin, AP-1 γ,
and AP-3 δ) and either the early endosome marker, rab5, or the
late endosomal markers rab7, rab7b, and LAMP-1. Our rationale for
including the late endosomal marker rab7b is that its depletion se-
lectively increases the protein levels of AP-3 (Propida et al., 2010).
We focused on endogenously expressed Vps33b and used Vps16-
HA to further validate Vps33b results. In addition, we analyzed the
subcellular localization of the HOPS subunits Vps39–GFP and
Vps41–Myc in clathrin-positive compartments and endosomes.
Endogenous Vps33b partially overlapped with puncta positive for
AP-3 (Figure 3, B and C) and/or clathrin (Figure 3, E and F). Although
partial, of these two coats, fluorescent signal overlap was more pro-
nounced between Vps33b and clathrin than Vps33b with AP-3. One
third of all Vps33b-positive pixels were positive for CHC (Figure 3, E,
F, and J). Similar to Vps33b, Vps16-HA was preferentially found in
puncta positive for CHC (Figure 3, G and J) or AP-3 δ (Figure 3H),
where coats signals partially overlapped with class C Vps subunits.
In contrast to CHC localization, Vps33b and Vps16-HA signals mini-
mally overlapped with AP-1 γ (Figure 3, I and J).
Vps33b was found in rab5 endosomes, in which we observed a
partial overlap of fluorescent signals (Figure 3, A and J). In contrast,
Vps33b was undetectable in late endosomes defined by rab7 and
LAMP-1 staining (Figure 3J). Vps33b overlapped with rab7b (Figure
3, D and J), however, at levels similar to those of AP-3 (Figure 3J).
Rab7b organelles were also positive for clathrin and the late endo-
osomal SNARE vesicle-associated membrane protein 7 (VAMP7;
Figure 3J). Another class C Vps protein, Vps16–HA, showed a similar
pattern of endosomal localization compared with Vps33b (Figure 3J).
Analysis of the distribution of the HOPS subunits Vps41–Myc
and Vps39–GFP revealed that the signal overlap with coats and en-
dosomal markers was far less pronounced as compared with the
class C Vps proteins Vps33b/Vps16–HA. Nonetheless, the two
HOPS-specific Vps subunits, Vps41–Myc and Vps39–GFP, were lo-
calized to rab5- and CHC-positive compartments (Figure 3J).
The partial overlap between coats, Vps class C, and HOPS pro-
teins suggests that domains of endosomes, rather than the whole
organelle, contain these factors. We tested this hypothesis by en-
larging rab5 compartments expressing the GTPase-deficient
rab5Q79L. This tool has been successfully used to enlarge en-
dosomes facilitating the identification of domains in the limiting mem-
brane of endosomes by high-resolution optical microscopy (Raiborg
et al., 2002, 2006; Craigie et al., 2008). We determined the distribu-
tion of coats, HOPS subunits, and class C Vps proteins by deconvo-
lution immunofluorescence microscopy and volume rendering of
digitally reconstructed endosomal organelles (Figure 4 and Supple-
mental Movies 1 and 2). Single optical sections revealed that endog-
ogenous Vps33b, Vps16–HA, and Vps41–Myc were present in discrete
domains that partially overlapped either with CHC (Supplemental Movie 1) or AP-3 δ (Supple-
mental Movie 2) at the limiting membrane of enlarged endosomes.
We isolated clathrin-coated vesicles from HEK293T cells or
HEK293T cells stably expressing either Vps41–Myc or Vps16–HA
and asked whether class C Vps/HOPS proteins were present in these
organelles (Figure 5 and Supplemental Figure 2). Clathrin-coated
vesicle isolation was monitored by the enrichment of CHC detected
by Coomassie Blue dye (Figure 5A) or by immunoblot with antibod-
ies CHC, AP-1 γ, AP-2 α, and AP-3 δ subunits in clathrin-coated
vesicle fractions (Figure 5B and Supplemental Figure 2, compare
lanes 1 and 6). Clathrin-coated vesicles contained class C Vps pro-
teins Vps33b and Vps16–HA and the HOPS subunit Vps41–Myc
(Figure 5B and Supplemental Figure 2, compare lanes 1 and 6).
Of these Vps proteins, Vps41–Myc and Vps16–HA were clearly en-
riched in clathrin-coated vesicle fractions. We isolated clathrin-
coated vesicles from control or CHC RNAi–treated cells to define
whether Vps class C and HOPS subunits cosedimenting with clathrin-
coated vesicles correspond to true components of these carriers or
just contaminants present on these membranes (Borner et al., 2006).
Coomassie Blue staining revealed that clathrin-coated vesicle frac-
tions isolated from CHC down-regulated cells decreased the con-
tent of several polypeptides, including CHC (Figure 5A, asterisks).
Down-regulation of CHC precluded formation of clathrin-coated
vesicles as determined by the absence or decreased levels of adap-
tor subunits from clathrin-coated vesicles (Figure 5B; Supplemental
Figure 2, compare lanes 6 and 6′; Figure 5C). Importantly, the levels
of Vps33b, Vps16–HA, or Vps41–Myc were significantly reduced from
clathrin-coated vesicles isolated from CHC RNAi–treated cells
indicating that Vps33b, Vps16–HA, and Vps41–Myc specifically re-
side in clathrin-coated vesicles (Figure 5B; Supplemental Figure 2,
compare lanes 6 and 6′; Figure 5C). Contamination of clathrin-
coated vesicle fractions with early endosomes markers such as
EEA1, rabaptin5, and Hrs was negligible (Figure 5B). Therefore
these results indicate that clathrin, Vps class C, and HOPS subunits
coalesce both in coated domains of early endosomes as well as
clathrin-coated vesicle carriers.

Acute perturbation of clathrin alters Vps class C/HOPS
subunit subcellular distribution
We evaluated the effect of CHC shRNA on the distribution of Vps
class C/HOPS subunits in rab5- and rab7b-positive endosomes.
CHC down-regulation, which is achieved within several days of
shRNA treatment, did not alter the distribution of Vps class C/HOPS
subunits in rab5 or rab7b compartments, suggesting compensatory mechanisms controlling Vps class C/HOPS subunit
subcellular distribution (unpublished data). To overcome possible
compensatory mechanisms, we acutely perturbed clathrin function
by using a chemical/genetic approach that uses chimeric CLCs car-
rying an oligomerization module, a modified FKBP 12 domain
(Moskowitz et al., 2003; Deborde et al., 2008). FKBP domains
selectively oligomerize upon addition of the cell-permeant bivalent
chemical AP20187. CLC oligomerization that follows incubation
with AP20187 halts vesicle formation from donor organelles, such as
plasma membrane or trans-Golgi network by “freezing” clathrin
ontosomes and aborted budding profiles (Moskowitz et al.,
2003; Deborde et al., 2008). Thus we used FKBP–CLC chimeras to
trap clathrin at membranes. Tagged FKBP–CLC chimeras incorpo-
rated into 60.7 ± 18.5% (n = 30) of all CHC-positive organelles
in HEK293T cells as determined by deconvolution microscopy
(Supplemental Figure 3A). Like endogenous CLC (Figure 2), chimeric
mCherry-FKBP–CLC specifically coprecipitated class C Vps/HOPS
subunits (Supplemental Figure 3D). Cell treatment with AP20187
for 2 h induced enlargement of CHC-tagged-FKBP–CLC dual-la-
beled organelles and the accumulation of endogenous and re-
combiant clathrin chains at the perinuclear region (Supplemental
Figure 3, B and C). The perinuclear accumulation of CHC in
Clathrin associates with class C Vps/HOPS subunits

FIGURE 3: Vps class C/HOPS are present in AP-3, clathrin, Rab5, and Rab7b-compartments. HEK293T cells were fixed, and either triple (A–F) or double (G–I) labeled for indirect immunofluorescence with antibodies against the following antigens: Vps33b, rab5, and AP-3 δ (A–C); Vps33b, rab7b, and CHC (D–F); HA epitopes to detect recombinantly expressed Vps16-HA (G–I), and either CHC (G), AP-3 δ (H), or AP-1 γ (I). Cells were imaged by Delta deconvolution fluorescence microscopy. Bar depicts 5 μm; images to the right are 300% magnifications of white-boxed inserts.

(J) Quantitative analysis of Vps class C/HOPS subcellular distribution. HEK293T cells or those expressing recombinant Vps16–HA, Vps39–GFP, or Vps41–Myc were labeled with combinations of antibodies against endogenous Vps33b or tags engineered in the Vps class C/HOPS subunits Vps16, Vps39, Vps41; the coats CHC, AP-1 γ, AP-3 δ; the early endosome marker rab5; the late endosome markers VAMP7, rab7, rab7b, and LAMP-1. Cells were imaged by Delta deconvolution fluorescence microscopy, and the percentage of overlapping pixels of different antigen combinations was determined using Metamorph. The number of images analyzed appears in parentheses, and quantifications were obtained in at least three independent experiments.
HEK293T cells indicates that clathrin-dependent processes are rapidly inhibited by FKBP–CLC chimeras (Moskowitz et al., 2003; Deborde et al., 2008).

We next determined the effect of chemically perturbing clathrin function in HEK293T- and NGF-differentiated PC12 cells on Vps class C/HOPS subunits (Figures 6–9 and Supplemental Movies 3–6). We determined the speed of the AP20187-induced Vps class C redistribution by in vivo, time-lapsed confocal microscopy. mCherry-FKBP-CLC– and Vps18-GFP–expressing cells were imaged 25 min before drug addition and for 2 h in the presence of AP20187. mCherry-FKBP-CLC oligomerization induced by AP20187 quickly caused an accumulation of recombinant clathrin and Vps18–GFP in the perinuclear region. Concomitantly, Vps18–GFP organelles distributed in the cell periphery grew in number and size (Figure 6C and Supplemental Movie 5 and 6). Vps18–GFP redistributed in <30 min after AP20187 addition and reached a plateau by 60 min. Addition of either AP20187 to cells expressing solely Vps18–GFP (Figure 6A and Supplemental Movie 3) or ethanol vehicle to mCherry-FKBP-CLC and Vps18-GFP doubly expressing cells (Figure 6B and Supplemental Movie 4) did not alter the subcellular distribution of either fluorescently tagged protein. These results demonstrate a swift rearrangement of class C Vps

FIGURE 4: Vps class C/HOPS subunits localize to discrete domains of enlarged early endosomes. HEK293T cells transiently expressing recombinant rab5Q79L–GFP alone (A) or rab5Q79L–GFP plus either Vps16–HA (B) or Vps41–Myc (C) were fixed and triple labeled for indirect immunofluorescence with antibodies against GFP (A–C) plus: (A) antibodies against Vps33b and CHC, (B) antibodies against HA epitope plus either AP-3 δ or CHC, or (C) antibodies against Myc epitope plus either AP-3 δ or CHC. Cells were imaged by Delta deconvolution fluorescence microscopy. Bar depicts 5 μm; images to the right are 300% magnifications of insets. Supplementary Movies 1 and 2 depicts a tridimensional rendering of enlarged endosomes shown in panel C.
proteins by acute chemical/genetic inhibition of clathrin-coated vesicle formation.

The effects of AP20187-induced perturbation of clathrin function were not just restricted to Vps18–GFP. Analysis of images from fixed specimens showed that drug addition caused redistribution of endogenous Vps33b, Vps18–GFP, or Vps41–Myc into larger puncta preferentially found at the perinuclear region as was seen with Vps18–GFP during live imaging (Figure 7). This change in Vps class C/HOPS subunits distribution occurred concomitantly with an increased colocalization of Vps class C/HOPS subunits with either CHC or mCherry-FKBP–CLC (Figure 7A). Importantly, AP20187 increased the colocalization of clathrin chains with AP-3 as well as rab5 and rab7b, suggesting that AP20187 similarly traps clathrin chains on both endosomes (Figure 7, B and C). These results are consistent with the presence of clathrin in AP-3–, rab5–, and rab7b-positive compartments, as was observed at steady state in HEK293T cells (Figure 3J).

Vps class C/HOPS subunit binding to both clathrin–AP-3 and clathrin–Hrs coats suggest that Vps class C/HOPS subunit accumulation should be preferentially observed in early endosomes as compared with other intracellular compartments, such as late endosomes or the trans-Golgi network. To test this hypothesis, we analyzed the effects of AP20187 on the colocalization of rab5, rab7b, and AP-1 with CHC and/or endogenous Vps33b in mCherry-FKBP–CLC–expressing cells using deconvolution microscopy. The amount of CHC-positive puncta that were also positive for Vps33b doubled from 12.3 ± 5.2% (n = 120) to 20.8 ± 11.9% after drug addition (n = 120, p < 0.0001, Wilcoxon–Mann–Whitney test; Figure 8). AP20187 increased the colocalization of Vps33b with rab5 from 10.2 ± 5% to 20.6 ± 6.9% (n = 60, p < 0.0001, Wilcoxon–Mann–Whitney test; Figure 8; Supplemental Figure 4, compare A and B). In contrast, colocalization between rab7b and Vps33b modestly decreased after 2 h of clathrin perturbation (Figure 8; Supplemental Figure 4, compare C and D). Vps33b colocalization with clathrin in rab5-positive endosomes was selective because overlap between Vps33b and AP-1 remained at background levels before or after AP20187 incubation (Supplemental Figure 5 and Figure 8). Although AP20187 induced clathrin recruitment to rab5– and rab7b-positive compartments to a similar extent (Figure 8), the preferential association of Vps33b to rab5-positive organelles after drug-induced clathrin perturbation supports the hypothesis that clathrin coats selectively regulate the localization of class C Vps proteins to early endosomes.

Clathrin and Vps class C proteins are targeted to neuronal processes by clathrin-dependent mechanisms

We tested whether class C Vps proteins present in clathrin-coated organelles undergo directional delivery in polarized cells. To this end, we used human cortical neurons HCN-1A, a cell type that extends processes (Ronnett et al., 1990), as well as NGF-differentiated PC12 cells. HCN-1A cells were double-labeled with antibodies against endogenous Vps33b and CHC, and cells were imaged by high-resolution Delta deconvolution microscopy (Figure 9A). Vps33b was concentrated at the tip of neuronal processes where it preferentially colocalized with clathrin. This polarized distribution of a class C Vps/HOPS subunit was also observed by in vivo imaging of NGF-differentiated PC12 cells expressing recombinant mCherry-FKBP–CLC with either Vps39–GFP (unpublished data) or Vps18–GFP (Figure 9B).
We predicted that if class C Vps proteins associate with clathrin-positive organelles generated at the cell body for subsequent delivery to neurites, then inhibition of clathrin-dependent mechanisms by AP20187 should lead to a progressive decrease of mCherry-FKBP-CLC and Vps18-GFP fluorescent signals in the proximal segment of neurites. NGF-differentiated PC12 cells expressing mCherry-FKBP-CLC and Vps18-GFP were treated with vehicle or with AP20187 and continuously imaged by confocal
microscopy for 2 h. The integrated fluorescence intensity per volume unit was measured in the proximal third of the neurite (Figure 9B, arrows). Addition of AP20187 decreased CLC and Vps18 fluorescence in the proximal segment of neurites after 2 h (Figure 9B). Quantification of CLC and Vps18 fluorescence intensity revealed a progressive reduction per unit of proximal neurite volume after AP20187 incubation (Figure 9C, open circles). This decrease was not observed in vehicle-treated cells (Figure 9C, closed circles). Changes in fluorescence intensity per voxel induced by drug incubation are due to neither changes in neurite length nor diameter. These results indicate that Vps18–GFP delivery to neurites of polarized neuroendocrine cells is sensitive to acute perturbation of clathrin function.

**DISCUSSION**

Mouse mutants in subunits of HPS protein complexes and Vps33a, a Vps class C tether complex subunit, share phenotypes. The shared phenotypes suggest that associations between Vps33a and other HPS protein complexes participate in the same pathway, delivering membrane proteins from early endosomes to late endosomes/lysosomes and lysosome-related organelles (Suzuki et al., 2003; Li et al., 2004). Defects in this route trigger HPS in humans (Li et al., 2004; Di Pietro and Dell’Angelia, 2005). Similarly, *S. cerevisiae* orthologues of mCherry-FKBP–CLC and Vps41–Myc were probed with antibodies against mCherry and Myc epitopes. Note the redistribution of clathrin chains and Vps class C/HOPS subunits. (B) HEK293T cells mock transfected (−) or expressing mCherry-FKBP–CLC (+) were treated in the presence of ethanol vehicle (−) or AP20187 (+) for 2 h. Cells were stained with antibodies against CHC and one of the following antigens: GFP, to detect exogenously expressed Vps18–GFP; Myc, to detect exogenously expressed Vps41–Myc; or Vps33b to detect the endogenous protein. (C) HEK293T cells expressing mCherry-FKBP–CLC (+) were treated in the presence of ethanol vehicle (−) or AP20187 (+) for 2 h. Cells were stained with antibodies against mCherry and one of the following: AP-3 δ, rab5, rab7b, or CHC. (B and C) Percentage of overlapping pixels between different antigen combinations was determined using Metamorph. Numbers depicted in parentheses denote numbers of analyzed images obtained from at least three independent experiments. NS, not significant; * p < 0.0001, ** p < 0.003, *** p < 0.0004, Wilcoxon–Mann–Whitney Rank Sum Test.
Vps class C proteins and AP-3 subunits participate in the delivery of cargoes to the vacuole. Genetic and/or biochemical interactions between AP-3 and HOPS subunits in yeast and vertebrates provide mechanistic insight into the molecular organization of this genetic pathway (Angers and Merz, 2009, 2010; Nickerson et al., 2009; Lazar et al., 2009). Our results are the first evidence of vertebrate Vps class C/HOPS proteins interacting with the early endosomal coats clathrin and AP-3 and the clathrin-binding scaffold Hrs. Moreover, we provide biochemical evidence of an interaction between Vps33a and AP-3 predicted from the phenotypic similarities between Vps33a- and AP-3-deficient mice. Clathrin and AP-3 highlight fundamental differences in the way that yeast and mammalian Vps class C and coats interact. In contrast with yeast AP-3, the mammalian ortholog of this adaptor complex interacts with clathrin (Seeger and Payne, 1992; Dell’Angelica et al., 1998; Anand et al., 2009). These observations suggest differences in the biochemical and functional architecture of mechanisms controlled by vertebrate Vps class C/HOPS-containing tethers and coats. Mammalian class C Vps proteins (Vps11, 16, 18, and 33a-b) and the HOPS-specific subunits Vps39 and Vps41 establish specific interactions with clathrin chains (Figure 2). We identified Vps class C/HOPS proteins in isolated clathrin-coated carriers, clathrin-positive domains of rab5-positive early endosomes, and rab7b-containing endosomes (Figures 3–5).

The association of clathrin with Vps class C/HOPS proteins has at least two modalities: one occurring with the AP-3 complex and another in which Hrs participates. Clathrin–AP-3–Vps class C/HOPS and clathrin–Hrs–Vps class C/HOPS associations suggest vesicular and nonvesicular mechanisms controlling Vps class C/HOPS subunit subcellular distribution along the endocytic pathway, respectively. We focused on the association between Vps class C/HOPS subunits and clathrin chains because quantitative fluorescence microscopy indicated a greater degree of overlap between Vps33b/Vps16 and CHC than with AP-3 (Figure 3J). The functionality of Vps class C/HOPS protein–clathrin interactions was demonstrated by acute perturbation of clathrin function (Moskowitz et al., 2003; Deborde et al., 2008). Chimeric CLC carrying the oligomerization module FKBP and AP20187 treatment rapidly redistributed CHC, class C Vps proteins (Vps18, Vps33b), and HOPS subunits (Vps39, Vps41) to organelles distributed throughout the cytoplasm and the perinuclear region (Figures 6–8). Quantitative immunofluorescence microscopy revealed that, upon clathrin function perturbation, Vps33b content preferentially increased in rab5-positive over either rab7b-containing endosomes or AP-1–positive organelles, such as the trans-Golgi (Figure 8 and Supplemental Figures 4 and 5). These findings support a model whereby clathrin-dependent mechanisms acutely define the subcellular distribution of Vps class C/HOPS-containing tethers. We directly tested this model in human cortical neurons and in NGF-differentiated PC12 cells, two polarized cellular models. We observed enrichment of class C Vps/HOPS subunits and clathrin at the tip of neurites (Figure 9A). Moreover, acute perturbation of clathrin function led to a progressive depletion of Vps18–GFP and mCherry-FKBP–CLC fluorescence as the result of two concomitant processes: (1) inhibition of newly formed Vps18–clathrin–positive organelles entering the proximal neurite from the cell body plus (2) Vps18–clathrin–positive organelles already present in the neurite proximal segment at the time of drug addition progressively moving downstream toward the neurite tip.

Clathrin, AP-3, and Vps41 form a tripartite complex (Figure 2C). The association of Vps class C/HOPS subunits with clathrin, however, is independent of AP-3 expression levels (Figure 2D). Conversely, association of AP-3 and class C Vps/HOPS proteins is independent of clathrin expression (Figure 2E). These independent associations suggest that Vps class C/HOPS subunits establish multipronged interactions with clathrin and adaptors, such as AP-3 and/or that other clathrin-interacting molecules in early endosomes may mediate clathrin–Vps class C/HOPS subunit associations. We identified the early endosomal clathrin–Hrs flat coat participating in clathrin–Vps class C subunit interactions. Endogenous Hrs as well as recombinant Hrs coprecipitated endogenous clathrin and vps33b. Hrs and AP-3 mechanisms are likely independent because AP-3 is present in clathrin-coated vesicles yet Hrs is excluded from these coated carriers (Figure 5B). Therefore we propose that vesicular and nonvesicular clathrin–Vps class C/HOPS protein complexes regulate the subcellular distribution of class C Vps/HOPS subunits along the endocytic route. Structural predictions point to the presence of CHC homology domains in S. cerevisiae Vps 11, 18, 39, and 41, raising the possibility of multipronged associations between components of the coat and Vps class C/HOPS subunits (Darsow et al., 2001; Nickerson et al., 2009). Irrespective of whether multiple clathrin-binding molecules, such as AP-3 and Hrs, vesicular or nonvesicular mechanisms, or multipronged interactions between coats and Vps tethers exists, however, the redistribution of Vps class C/HOPS subunits upon of acute perturbation of clathrin function indicates that
Clathrin associates with class C Vps/HOPS subunits

Clathrin associates with class C Vps/HOPS subunits in early endosomes. Alternatively, FKBP-CLC/AP20187 could possibly cause promiscuous recruitment of cytoplasmic Vps class C/HOPS protein pools to membranes by oligomerized FKBP–CLC. We do not favor this alternative hypothesis, however, because we do not detect an increased association of Vps33b to rab7b- or AP-1–positive organelles (Figure 8; Supplemental Figures 4 and 5).

AP-3 budding occurs mainly, if not exclusively, from transferrin receptor–positive endosomes (Peden et al., 2004; Theos et al., 2005; Craige et al., 2008). Quantitative electron microscopy indicates that half of these AP-3 budding profiles possess clathrin on them (Peden et al., 2004; Theos et al., 2005). Perhaps, the presence of clathrin allows coats and Vps class C/HOPS subunits to be recruited at early stages in the vesicle life cycle. In the absence of clathrin, tethers and coats could undergo late interactions at target organelles, as proposed previously for yeast (Angers and Merz, 2009, 2010). Early inclusion of Vps class C/HOPS tethers into clathrin-coated carriers could serve a role for long-range delivery of tethers to polarized domains in mammalian cells. This hypothesis is supported by our findings that endogenous Vps33b and recombinantly expressed Vps18 and Vps39 display polarized distribution in the tip

significant pools of Vps class C/HOPS tethers are under control of clathrin-dependent mechanisms.

Angers and Merz have put forward an attractive model in which interactions between a tether, HOPS, and the coat AP-3 mediate docking of vesicles with the vacuole in S. cerevisiae, a process that culminates with fusion of membranes. Donor compartment (Golgj) and incoming AP-3–coated vesicles are devoid of HOPS complex in this model. HOPS complexes reside in the acceptor vacuolar compartments where coat and tether encounters occur to facilitate vesicle consumption (Angers and Merz, 2009, 2010). Our data suggest that, in addition to this mechanism, mammalian Vps class C/HOPS tethers are included in clathrin- and clathrin-AP-3–coated carriers, suggesting a coat-dependent mechanism for delivering Vps class C/HOPS tethers. Perturbing clathrin function with FKBP-CLC/AP20187 caused an increase in the number and size of organelles positive for Vps class C/HOPS subunits and trapped Vps33b in rab5-positive endosomes (Figures 6–8; Supplemental Figure 4). We attribute these effects to inhibition of clathrin-coated vesicle budding, consistent with published data (Moskowitz et al., 2003; Deborde et al., 2008) and possibly to changes in the dynamics of flat clathrin–Hrs coats in early endosomes. Alternatively, FKBP-CLC/AP20187 could possibly cause promiscuous recruitment of cytoplasmic Vps class C/HOPS protein pools to membranes by oligomerized FKBP–CLC. We do not favor this alternative hypothesis, however, because we do not detect an increased association of Vps33b to rab7b- or AP-1–positive organelles (Figure 8; Supplemental Figures 4 and 5).

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FIGURE 9: Polarized distribution of clathrin and Vps class C proteins in neuronal cells. (A) Human cortical neuronal cells, HCN-1, were fixed and processed for indirect immunofluorescence microscopy. Cells were double labeled with antibodies against endogenous Vps33b and CHC and imaged by high-resolution Delta deconvolution microscopy as previously described. HCN-1 cells that spontaneously extend processes in culture were imaged. (B) PC12 cells expressing recombinant mCherry-FKBP–CLC and Vps18–GFP were differentiated with NGF to induce process extension. Cells were imaged by live time-lapse confocal microscopy in the presence of 50 nM AP20187. Images of cells at time 0 and 120 min after drug addition are presented in (B). Panels depict images pseudocolored using the ICA LUT from Image J. Arrows mark the segment of the neurite where fluorescence intensities were measured over time. (C) Depicts a quantitative analysis of fluorescence intensity per voxel for mCherry-FKBP–CLC and Vps18–GFP. Neurite volume was measured in the proximal third of neurites as indicated by the arrows in (B). Closed circles correspond to cells imaged in the presence of ethanol vehicle (0.05% vol/vol), and open circles correspond to cells imaged in the presence of AP20187 (50 nM). Addition of either ethanol or AP20187 is marked by an arrow and corresponds to time 0. All data were normalized to the fluorescence intensity at time 0. Fluorescence intensities for mCherry-FKBP–CLC or Vps18–GFP at 60, 90, and 120 min after drug addition are significantly different from those from ethanol-treated cells (p < 0.00015, Wilcoxon–Mann–Whitney Rank Sum Test). Bar represents 20 μm.
of neurites in human cortical neurons or differentiated PC12 cells (Figure 9). An alternative yet nonexistent model is that Vps class C/HOPS subunits could play a role in cargo selection either through indirect effects of Vps proteins on cargo recognition by coats or by direct association of class C Vps/HOPS proteins with SNAREs and non-SNARE membrane proteins. Support for this interpretation was obtained recently when ema/CLEC16A, which is a lectin-type membrane protein, was shown to directly bind to Vps16A in Drosophila melanogaster (Kim et al., 2010).

At least four modalities of content delivery between stages of the endocytic route have been documented: vesicle-mediated (Stoorvogel et al., 1996; Peden et al., 2004), tubule-mediated transfer of cargo (Delevoye et al., 2009), kiss-and-run (Bright et al., 2005), and endosome maturation (Stoorvogel et al., 1991; Rink et al., 2005; Poteryaev et al., 2010). Endosome maturation is kinetically defined by the conversion over time of the same endosome from a rab5- to a rab7-decorated compartment (Stoorvogel et al., 1991; Rink et al., 2005; Poteryaev et al., 2010). This process depends on a switch mechanism in which a later acquisition of HOPS subunits promotes rab7 activation by interactors. This process depends on a switch mechanism in which a later acquisition of HOPS subunits promotes rab7 activation by interactors (Figure 3), suggesting that Vps class C/HOPS subunit binding may be short-lived on them. In contrast, class C Vps/HOPS associates to rab7b compartments. Rab7b has been implicated in delivery of Toll-like receptors from the cell surface to lysosomes in macrophages as well as in retrograde transport between endosomes and Golgi complex in HeLa cells (Wang et al., 2007; Progida et al., 2010). The presence of clathrin in rab7b-positive compartments and the observation that down-regulation of rab7b increases the expression level of AP-3 (Progida et al., 2010) suggest a role of rab7b in vesicle-mediated transport between early endosomes and late endosomes/lysosomes or in a specialized retrograde transport between endosomes and the Golgi complex. Irrespective of whether rab7b-clathrin compartments represent vesicles, endosomes, or a combination thereof, our findings suggest that maturation of clathrin-coated membranes in transit among endosomal compartments could occur.

S. cerevisiae class C Vps proteins (Vps11, 16, 18, and 33) form a core that incorporates into CORVET and HOPS complexes (Peplowska et al., 2007; Nickerson et al., 2009; Ostrowicz et al., 2010; Wickner, 2010). Vps8 and Vps3 constitute the CORVET core whereas Vps39 and Vps41 establish specific interactions with the core defining the HOPS complex (Peplowska et al., 2007; Markgraf et al., 2009; Ostrowicz et al., 2010). The organization and subcellular localization of HOPS has been partially characterized in metazoans (Kim et al., 2001; Richardson et al., 2004; Zhu et al., 2009; Cullinan et al., 2010). In contrast, metazoan CORVET has not been studied. Although putative human orthologues of CORVET subunits, Vps8/KIAA0804 and Vps3/TGFBRA1, are present in databases, it remains unknown whether these gene products assemble with class C Vps proteins to form a mammalian CORVET complex. It is formally possible that mammalian class C Vps proteins, such as Vps33b and Vps16, identified in clathrin-containing organelles by biochemical and immunolocalization studies, may be part of both CORVET and HOPS complexes. The presence of class C Vps subunits in both complexes might explain why there was a relatively higher colocalization between Vps33b/Vps16 (found in both CORVET and HOPS) and clathrin as compared with Vps41, Vps39, and clathrin (found only in HOPS; Figure 3). If this were the case, our data suggest that perturbing clathrin function affects Vps class C proteins along rab5 and rab7b compartments (Figure 8) by stalling maturation from a CORVET to a HOPS-positive-clathrin–coated vesicle or endosome.

Our results demonstrate a unique functional architecture of mechanisms controlled by vertebrate Vps class C/HOPS–containing tethers and clathrin coats. We postulate that clathrin-dependent mechanisms provide long-range and directional delivery of class C Vps/HOPS tethers to organelles and/or specialized domains of mammalian cells bearing complex architectures.

MATERIALS AND METHODS

Antibodies

The following antibodies were used in this study: polyclonal anti-myc (A19010SA) and anti-HA (A190108A; Bethyl Laboratories, Montgomery, TX); polyclonal anti-GFP (cat# GFP-1020; Aves Labs, Tigard, OR, and cat#132002, Synaptic Systems, Göttingen, Germany); monoclonal anti-Lamp H4A3, anti-SV2, and anti-AP38 SA4 (Developmental Studies Hybridoma Bank, Iowa City, IA); monoclonal anti-rab7 ab50533 (Abcam, Cambridge, MA); monoclonal anti-rab7b clone 383 (Abnova, Taipei, Taiwan); monoclonal anti-rab5, anti–AP1γ adaptin, anti–rabaptin 5, and anti-EEA1 (610724, A36120, 610676, and 610456; BD Bioscience Transduction Laboratories, Pasadena, CA); monoclonal anti–CLC X22 (Calbiochem, San Diego, CA); monoclonal anti–CLC CON.1 (MMS423P; Covance, Berkeley, CA); polyclonal anti–mCherry dsRed (632496; Clontech, Mountain View, CA); monoclonal anti–AP2α adaptin and anti–actin (A4325 and AS441; Sigma, St. Louis, MO); polyclonal anti–AP3β1 (13384–1-AP; ProteinTech Group, Chicago, IL); monoclonal anti–GFP 3E6 (A11120; Molecular Probes, Eugene, OR); monoclonal anti–HRS A-5 (Enzo Life Sciences, Plymouth Meeting, PA); anti–synaptophysin clone SY38 (Millipore, Billerica, MA); and monoclonal anti–transferrin receptor H68.4 (136800; Zymed Laboratories, San Francisco, CA). Anti-VAMP7 was a gift from Andrew Peden (Department of Clinical Biochemistry, University of Cambridge, UK). Immunofluorescence secondary antibodies were Alexa Fluor 488, 555, 568, or 647 anti–rabbit, rat, chicken, or isotype specific mouse IgG (Molecular Probes). The secondary antibodies used on Western blots were horseradish peroxidase-goat anti-mouse or anti-rabbit (626420 and G21234; Invitrogen, Carlsbad, CA).

Plasmids, oligos, and peptides

Plasmids encoding C-terminally tagged murine Vps11–HA and Vps16–HA, and N-terminally tagged GFP-murine Vps18 and GFP–Vps39 were gifts from Robert Piper (Department of Molecular Physiology and Biophysics, University of Iowa, Iowa City, IA). Vps18–myc from Liping Wang (Human Nutrition Research Center, UC Davis, CA), N-terminally tagged myc–HRS from Harold Stenmark (Institute for Cancer Research; Oslo University Hospital) (Raiborg et al., 2002), and N-terminally tagged GFP–Rab5Q79L from Laura Volpicelli (University of Pennsylvania) (Volpicelli et al., 2001). Spe39–enhanced GFP and HA-tagged Vps33a and Vps33b have been described (Zhu et al., 2009).

C-terminal Vps41–myc was created from human Vps41 cDNA clone (cat# SC111791; Origene, Rockville, MD) and the primers 5′-caccatgcggcaagacagagag and 3′-ttggagatgaaaaaagaacaaaaactt-attctgaagatcgttag using PCR. The PCR product was cloned into

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TOPO vector pCDNA 3.1 (Invitrogen), following the manufacturer’s directions. The TOPO vector DNA was subsequently cut with en-
zymes BamHI and EcoRV, and the Vps41-myc fragment was sub-
cloned into pIREShyg3. The resulting clone was confirmed as error-
free by DNA sequencing.

An N-terminally tagged HA–FKBP–CLC construct was the gift of Enrique Rodriguez-Boulan (Deborde et al., 2008). This con-
struct was modified by replacing the HA tag with a fluores-
cent mCherry tag as follows. We designed PCR primers comple-
mentary to mCherry that were flanked by Nhel and BglII
3′(5′ GCTAGCATTGGTAGGAAAGGCC and 3′ AGATCTCTTTGTA-
AGCTCTGTCATGC) sites to allow cloning from a pmCherry vector (Clontech) into the TOPO TA 2.1 vector (Invitrogen). The TOPO TA 2.1 mCherry vector and the HA-FKBP–CLC PCR prod-
cut were each digested with Nhel and BglII. Appropriately sized DNA bands were identified on an agarose gel, extracted, and li-
gated to create mCherry-FKBP–CLC. Coding sequence was veri-
fied as error-free by DNA sequencing. The ARGENT Regulated Homodimerization Kit containing FKBP plasmids and the di-
erization drug AP20187 was purchased from ARIAD (www.ariad.com; Cambridge; MA).

All siRNA oligos were purchased from Dharmacon (Lafayette, CO). The siCONTROL Non-Targeting siRNA Pool #1 (D0012061305) was used for control knockdown. siGENOME human CLTC, NM_004859 siRNA was used for CHC knockdown (D00401002) sense: GCAAGAUGCCUGUUGAGGAAU, antisense: 5’ pCUU-
CAAAAGCUAUCUUGCUU. siRNA oligos were transfected as de-
scribed later in the text. AP3δ (RHS4533-NM_003938) and CHC (RHS3979957067) shRNA in a pLKO.1 vector for lentiviral infection were obtained from Open Biosystems (Huntsville, AL). Control shRNA in a pLKO.1 vector was obtained from Addgene (vector 1864; Cam-
bridge, MA).

The peptide against the epitope for AP3δ SA4 (AQQVDVITEEM-
PenalPSDDEDKPDNPYRA) (Salazar et al., 2009) was purchased from the Emory Microchemical Facility (Atlanta, GA) and Invitrogen (EvoQuest Team, Carlsbad, CA).

Cell culture, transfection, and lentivirus infection
HEK293T and HCN-1A cells (American Type Culture Collection [ATCC], Manassas, VA) in DMEM (HyClone, Logan, UT) supple-
mented with 10% fetal bovine serum (FBS) (HyClone) and 100 μg/ml penicillin and streptomycin (HyClone) and 100 μg/ml penicillin and streptomycin (HyClone) were incubated at 37°C with 10% CO2.

For recombinant DNA expression, HEK293T cells were transfected in six-well dishes with 0.5–2.0 μg DNA in 0.25% Lipofectamine 2000 (Invitrogen) diluted in Opti-Mem (Life Tech-
nologies, Grand Island, NY). Cells were transfected for 4 h fol-
lowed by incubation in either culture medium alone or culture
medium containing the selection drug(s) G418 (1 μg/ml) and/or hygromycin (0.1 μg/ml) for the maintenance of stable cell lines. PC12 cells were transfected by nucleofection with 3 μg of DNA using Amxam Cell Line Nucleofector Kit V (cat# VCA-1003; Lonza Walkersville, Koeln, Germany, www.lonza.com), and were plated on Matrigel-coated glass-bottom culture dishes (Matek, Ashland, MA) in PC12 culture medium supplemented with 100 ng/ml NGF 2.5S (murine, natural) (cat# 13257-019; Invitrogen). PC12 cells were differentiated for 48–72 h at 37°C with 10% CO2 For siRNA knockdown, HEK293T cells were transfected in six-well dishes with 50 nM oligo for 4 h, incubated for 20 h in culture medium, and transfected a second time with 50 nM oligo for 4 h followed
by incubation for 3 d in culture medium. For lentiviral infection, HEK293T cells seeded in 10-cm plates were infected with 1 μl of high-titer lentivirus containing the shRNA constructs mentioned earlier in the text. Following a 24-h infection, cells were incubated for up to 6 d in culture medium supplemented with 4 μg/ml puromycin for selection. The Emory Neuroscience NINDS Viral Vector Core Facilities prepared all high-titer lentiviruses.

Cross-linking and immunoprecipitation
Cross-linking was performed as previously described using DSP (Craigie et al., 2008; Salazar et al., 2009; Zlatic et al., 2010). DSP is a homobifunctional reversible and cell-permeable cross-linker with a 12-A spacer arm that stabilizes labile protein interactions (Lomant and Fairbanks, 1976). Plates of confluent HEK293T cells were placed on ice, washed twice with ice-cold phosphate-buffered saline (PBS)/1 mM MgCl2/0.1 mM CaCl2, and then incubated with 1 mM DSP (cat# 22585; Thermo Scientific, Rockford, IL) or dimethyl sulfoxide control in PBS/1 mM MgCl2/0.1 mM CaCl2 for 2 h on ice. DSP was then quenched with 25 mM TRIS, pH 7.4, followed by two rinses with ice-
cold PBS/1 mM MgCl2/0.1 mM CaCl2. Cells were lysed in Buffer A (150 mM NaCl, 10 mM HEPES, 1 mM EGTA, 0.1 mM MgCl2) + 0.5% Triton X-100 by incubation for 30 min at 4°C. Any remaining cellular debris was scraped from plates and centrifuged at 16,000 × g for 10 min. The supernatant was collected, diluted to 1 μg/ml in Buffer A + 0.5% Triton X-100, and incubated with Dynal immunomagnetic precipitation beads (Dynal, Oslo, Norway) in the absence or pres-
ence of a 10 μM SA4 peptide competitor. Beads and lysate were incubated at 4°C for 2 h and washed six times with Buffer A contain-
ing 0.1% Triton X-100 to remove nonspecifically bound material. The material that remained bound after these washes was then eluted by treatment with either the peptide antigen or SDS–PAGE sample buffer followed by incubation at 75°C for 5 min. Immunoprecip-
itated material was analyzed on SDS–PAGE Western blot.

Despite reduced detection of δ in SA4 immunoprecipitates, all other AP-3 subunits are readily detectable in these immunocom-
plexes (Salazar et al., 2009). Decreased detection of AP-3 δ by the SA4 mAb in immunoblots after DSP cross-linking likely reflects a chemical modification of the SA4 epitope by the cross-linking agent. The lysine and arginine present in this peptide are susceptible to modification by DSP. The SA4 antibody is used sequentially in im-
munoprecipitation and immunoblot, thus effectively magnifying the difference between (–) and (+) DSP samples.

Immunolocalization, microscopy, and quantification
Coverslips were prepared as previously described (Faundez et al., 1997). Cells were seeded onto Matrigel (BD Bioscience, San Jose, CA)-coated glass coverslips, washed twice in PBS/1 mM MgCl2/0.1 mM CaCl2, and fixed using 4% paraformaldehyde in PBS. Cells were then permeabilized and blocked with 0.02% sa-
ponin (Sigma), 15% horse serum (HyClone), 2% bovine serum album-
in, and 1% fish skin gelatin (Sigma) in PBS. Blocked and permea-
bilized cells were incubated with the primary and secondary antibodies described earlier in the text and were mounted with Gelvatol onto slides. Fixed-cell confocal microscopy was performed on fixed cells as described (Deborde et al., 2008) using an Axiovert 100M microscope (Carl Zeiss, Thornwood, NY) with Argon/HeNe (488/543) laser excitation. Images were captured using a Plan APO-
chromat 63x/1.4 oil DIC objective, BP 505–550/LP 560 filter set, and LSM 510 3.2.0.104 software (Carl Zeiss). Deconvolution microscopy was performed as described (Deborde et al., 2008) with a 200M in-
verted microscope using 63x/1.4 and 100x/1.4 oil DIC objectives (Carl Zeiss) and a Sedat filter set. Images were collected using a

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scientific grade cooled charge-coupled Cool-Snap HQ camera with ORCA-ER chip on a multichannel, wide-field, three-dimensional microscopy system using Slidebook 4.0 OS X software (Intelligent Imaging Innovations, Denver, CO). Out-of-focus light was removed with a constrained iterative deconvolution algorithm (Swedlow et al., 1997). Confocal and deconvolution images were processed and analyzed using LSM Image Browser 4.0.0.157 (Carl Zeiss), Meta-morph software version 6.1 (Universal Imaging, Sunnyvale, CA), and Imaris 6.3.1 software (Bitplane, St. Paul, MN). Colocalization and puncta size were determined from three consecutive z-series focal planes per image. All channels were thresholded individually. Percent colocalization was determined by calculation of pixel area containing fluorescent signals from two channels per total pixel area for a single fluorophore. Puncta size was determined by integrated morphometric analysis of total pixel area within puncta as determined by image thresholding.

Clathrin-coated vesicle isolation
HEK293T or HEK293T cells stably expressing tagged HOPS or class C Vps proteins were used to prepare clathrin-coated vesicles as described previously (alternate protocol 2 in Girard et al., 2004). Briefly, cells were grown to confluence and washed twice with PBS/1 mM MgCl2/0.1 mM CaCl2. Cells were lifted in CCV buffer (100 mM MES, 1.0 mM EGTA, 0.5 mM MgCl2, pH 6.5), transferred to a Potter Elvehjem glass-tesfon homogenizer, and homogenized for 10 strokes at 1500 rpm on a Tri-R Stir-R variable speed laboratory motor (Model S63C; Tri-R Instruments, Rockville Center, NY). Homogenate was centrifuged in an SS-34 fixed-angle rotor for 20 min at 17,000 × g, 4°C. The resulting supernatant was further centrifuged in a type 40 fixed-angle rotor for 60 min at 56,000 × g, 4°C. The resulting pellet was resuspended, homoge- nized, and transferred to polyallomer centrifuge tubes (Beckman, Palo Alto, CA). The sample was then underlaid with D2O-sucrose solution (8% sucrose, 100 mM MES, 1.0 mM EGTA, 0.5 mM MgCl2, D2O) (D2O cat# 364312-10G; Sigma) and was centrifuged in a SW-55 swing-bucket rotor for 2 h at 116,000 × g, 4°C. This final centrifugation step was repeated and the pellet was resuspended and aliquoted along with reserves from previous fractionation steps and was run on SDS–PAGE gels for Western blotting or Coomasie stain.

Acute clathrin perturbation
HEK293T or HEK293T cells stably expressing tagged HOPS or class C Vps proteins and/or transiently expressing tagged FKBP–CLC were incubated for 2 h at 37°C, 10% CO2 in culture medium supplemented with 50 nM AP20187 (ARIAD) or 0.05% ethanol vehicle control. Culture dishes were then placed on ice and processed for either immuno-locallization or cross-linking followed by immunoprecipitation.

Live cell imaging
HEK293T or PC12 cells expressing Vps18–GFP and/or mCherry–FKBP–CLC were grown on Matrigel-coated glass-bottom culture dishes (Matek). Imaging medium consisted of Hank’s balanced salt solution minus phenol red and NaHCO3 (Sigma) and was centrifuged in a type 40 fixed-angle rotor for 60 min at 17,000 × g, 4°C. The resulting supernatant was transferred to a Potter Elvehjem glass–teflon homogenizer, and homogenized for 10 strokes at 1500 rpm on a Tri-R Stir-R variable speed laboratory motor (Model S63C; Tri-R Instruments, Rockville Center, NY). Homogenate was centrifuged in an SS-34 fixed-angle rotor for 20 min at 17,000 × g, 4°C. The resulting supernatant was further centrifuged in a type 40 fixed-angle rotor for 60 min at 56,000 × g, 4°C. The resulting pellet was resuspended, homoge- nized, and transferred to polyallomer centrifuge tubes (Beckman, Palo Alto, CA). The sample was then underlaid with D2O-sucrose solution (8% sucrose, 100 mM MES, 1.0 mM EGTA, 0.5 mM MgCl2, D2O) (D2O cat# 364312-10G; Sigma) and was centrifuged in a SW-55 swing-bucket rotor for 2 h at 116,000 × g, 4°C. This final centrifugation step was repeated and the pellet was resuspended and aliquoted along with reserves from previous fractionation steps and was run on SDS–PAGE gels for Western blotting or Coomasie stain.

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Statistical analysis
Experimental conditions were compared with the nonparametric Wilcoxon–Mann–Whitney Sum Test using Synergy Kaleida-Graph v4.03 (Reading, PA) or StatPlus Mac Built 5.6.0pre/Universal (AnalystSoft, Vancouver, Canada). Data are presented as boxplots displaying the four quartiles of the data, with the “box” comprising the two middle quartiles, separated by the median. The upper and lower quartiles are represented by single lines extending from the box. Circles correspond to outlier points defined by the statistical software as beyond the upper or lower quartile plus 1.5 times the value of the 2–3 interquartile distance.

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