Chemical-genetic disruption of clathrin function spares adaptor complex 3–dependent endosome vesicle biogenesis

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INTRODUCTION

Exocytic and endocytic compartments exchange components and maintain their composition by means of carriers, some of which are vesicles (Bonifacino and Glick, 2004; De Matteis and Luini, 2011). The generation of vesicles and the selective loading of proteins and lipids into them require diverse monomeric and heteromeric cytosolic coats. Among the latter, heterotetrameric adaptor complexes AP-1–5 specify distinct trafficking routes. AP-1–5 adaptors comprise four subunits of various sizes: two large subunits, β1-5 and α, γ, δ, ε, or ζ; one medium-size, µ1-5 subunit; and a small, α1-5 subunit. All of these heterotetrameric adaptor complexes share the ability to recognize specific sorting signals in the cytosolic domains of membrane proteins. Some adaptors have in common the capacity to bind phosphoinositides, GTPases, and proteins that act as modules to connect a nascent vesicle to diverse machineries. These include, but are not limited to, membrane deformation, the cytoskeleton, or the recognition of specialized cargoes. In contrast, these adaptor complexes differ in their ability to bind clathrin-coated vesicles (Kirchhausen, 2000; Bonifacino and Traub, 2003; Bonifacino and Glick, 2004; Robinson, 2004; Hirst et al., 2011; McMahon and Boucrot, 2011).

Clathrin heavy-chain and the associated clathrin light-chain polypeptides assemble into triskelia, which are the structural building blocks of clathrin cages. These cages encase and help to concentrate adaptors with their bound membrane cargoes and other associated factors in the transition from a clathrin-coated membrane pit into a clathrin-coated vesicle bud. AP-1 and AP-2 bind to the...
clathrin heavy chain via the appendages of β adaptins (Kirchhausen, 2000; Bonifacino and Traub, 2003; Bonifacino and Glick, 2004; Robinson, 2004; Hirst et al., 2011; McMahon and Boucrot, 2011; Willox and Royle, 2012). Extensive biochemical, structural, and functional evidence indicates that clathrin association with AP-1 and AP-2 is necessary for vesicle formation by these adaptors (Kirchhausen, 2000; Bonifacino and Traub, 2003; Bonifacino and Glick, 2004; Robinson, 2004; Hirst et al., 2011; McMahon and Boucrot, 2011; Willox and Royle, 2012). In contrast, adaptor complexes AP-4 and AP-5 do not cofractionate with clathrin-coated vesicles and may function independently of clathrin, although more research on these complexes is needed to establish certainty (Hirst et al., 1999, 2013; Borner et al., 2006, 2012). A more thoroughly studied adaptor, the AP-3 complex, has a debatable relationship with clathrin. A functional equivalence for AP-3 with AP-1 or AP-2 has been inferred based on biochemical interactions and colocalization of AP-3 with clathrin. Nonetheless, the precise functional role of clathrin in AP-3 vesicle generation has been a matter of dispute since AP-3’s discovery (Dell’Angelica et al., 1997, 1998; Faundez et al., 1997, 1998; Shi et al., 1998; Blondeau et al., 2004; Peden et al., 2004; Theos et al., 2005; Borner et al., 2006; Salazar et al., 2009; Kural et al., 2012).

AP-3 generates vesicles that traffic membrane proteins from endosomes to lysosomes, lysosome-related organelles, or synaptic vesicles. Defects in this vesicle formation machinery translate into the type 2 Hermansky–Pudlak syndrome in humans, a disease that shares a similar constellation of phenotypes with AP-3–null mice and flies, the most prominent of these being pigment dilution (Wei, 2006; Wei and Li, 2012; Danglot and Galli, 2007; Newell-Litwa et al., 2007; Raposo and Marks, 2007; Raposo et al., 2007). In this study, we comprehensively explore the role of clathrin in AP-3–dependent vesicle budding, using rapid chemical-genetic perturbation of clathrin function. We recapitulate key biochemical and colocalization findings by others. However, despite a clathrin and AP-3 interaction, AP-3–dependent budding from endosomes is resistant to rapid clathrin inhibition in whole-cell assays. We propose that the interaction between clathrin and AP-3 at endosomes fulfills functions distinct from the canonical role of clathrin in AP-1– and AP-2–dependent vesicle formation.

RESULTS

We sought to test the function of clathrin interactions with the adaptor complex AP-3, using chronic and acute chemical-genetic perturbations of clathrin function. We hypothesized that if AP-3 vesicle budding from endosomes requires clathrin, as is the case for AP-1 and AP-2, then chronic and acute clathrin perturbation should similarly affect the content of these three adaptors in clathrin-coated vesicle fractions. We used short hairpin RNA (shRNA) down-regulation of clathrin heavy chain as a mean to chronically perturb clathrin. Acute interference of clathrin function was achieved with a clathrin light-chain chimera carrying in tandem an mCherry tag and an FKBP oligomerization domain (mCh-FKBP-CLC). Induction of mCh-FKBP-CLC quaternary structures impairs clathrin function in minutes after addition of the bivalent FKBP-binding agent AP20187 by trapping clathrin polyopeptides and their associated molecules on membranes (Moskowitz et al., 2003; Deborde et al., 2008; Zatic et al., 2011). This rapid onset of effects is in contrast with clathrin heavy-chain down-regulation by shRNA, which takes several days to attain clathrin function inhibition.

Clathrin and AP-3 interactions have been documented by means of in vitro pull-downs with the hinge-ear domain of AP-3 β3 subunits, as well as by AP-3 enrichment in clathrin-coated vesicles (Dell’Angelica et al., 1998; Blondeau et al., 2004; Borner et al., 2006; Salazar et al., 2009). Under knockdown conditions, clathrin vesicle formation is impaired, leading to reduced content of proteins copurifying with clathrin-coated vesicles including AP-3 subunits (Borner et al., 2006). We revisited these criteria in HEK293 cell and PC12 cells expressing mCh-FKBP-CLC to validate this recombinant tool. We used shRNA-mediated clathrin heavy-chain knockdown in HEK cells, followed by clathrin-coated vesicle isolation, to determine whether AP-3 adaptor subunits could be considered components of clathrin-coated vesicles in cells transiently expressing mCh-FKBP-CLC. Equal protein loads from control knockdown and clathrin heavy-chain down-regulated clathrin-coated vesicle fractions were analyzed by SDS–PAGE and Western blot (Figure 1, A and B). Clathrin heavy chain was enriched in clathrin-coated vesicle fractions (Figure 1A, compare lanes 1 and 3, asterisk), and the content of clathrin heavy chain and other fraction polypeptides decreased after clathrin heavy-chain down-regulation (Figure 1A, compare lanes 3 and 4). In addition, mCh-FKBP-CLC and adaptor complexes AP-1–3 were enriched in clathrin-coated vesicle fractions (Figure 1B, compare lanes 1 and 6), and their presence in clathrin-coated vesicle enriched fractions was similarly sensitive to shRNA clathrin heavy-chain down-regulation (Figure 1B, compare lanes 6 and 12). AP-3 content in clathrin-coated vesicle enriched fractions was insensitive to down-regulation of BLOC-1 complexes, a major AP-3 interactor (Supplemental Figure S1; Di Pietro et al., 2006; Salazar et al., 2009; Gokhale et al., 2012; Lee et al., 2012). These results are compatible with the hypothesis that a subset of clathrin-coated vesicles may contain the adaptor complex AP-3.

We further tested the possible association of AP-3 with clathrin by communomagnetic precipitations. We performed cross-linking with the homo-bifunctional cross-linker dithiobis-succinimidyl propionate (DSP) in intact cells (Lomant and Fairbanks, 1976; Zatic et al., 2010). Our rationale was to chemically stabilize putative clathrin–AP-3 associations in intact cells and use stringent isolation conditions to prevent cell-free clathrin–AP-3 associations after cell solubilization. mCh-FKBP-CLC HEK293 cells were treated in the absence or presence of DSP, and detergent cell extracts were incubated with magnetic beads containing antibody directed against clathrin light chain (Figure 1C), clathrin heavy chain (Figure 1D), or AP-3 β (Figure 1E). Isolated immunocomplexes were analyzed by Western blot. Clathrin light-chain immunocomplexes coisolated with both clathrin heavy chain and the AP-3 adaptor subunit β3 (Figure 1C, lane 3). Similarly, isolation of clathrin heavy-chain immunocomplexes precipitated both clathrin light chain and the AP-3 adaptor subunit β3, the latter only in the presence of DSP cross-linker (Figure 1D, compare lanes 5 and 6). In addition, the association between clathrin chains and AP-3 could be detected in reverse magnetic precipitations where AP-3 was isolated with clathrin–AP-3 interactions were also detected in PC12 neuroendocrine cells stably expressing mCh-FKBP-CLC (Figure 1F). We included PC12 cells in our study because of their extensively characterized AP-3 vesicle biogenesis pathway (Faundez et al., 1997, 1998; Faundez and Kelly, 2000; Shi et al., 1998). The selectivity of clathrin and AP-3 coprecipitations in HEK293 cells and PC12 cells was examined with either control iso-type-matched antibodies or peptide antigen competition (Figure 1, C and D, and E and F, respectively). These results suggest that a subset of clathrin-coated vesicles contains the adaptor complex AP-3 at steady state.
Our biochemical results suggested a low extent of overlap between clathrin and AP-3 polypeptides. Our goal was to quantitatively assess the extent to which clathrin and AP-3 associate in PC12 cells expressing mCherry-FKBP-CLC at steady state, in particular in early endosome compartments. Thus we performed indirect immunofluorescence experiments with high-resolution deconvolution microscopy or superresolution structured illumination microscopy (Figures 2 and 3, Supplemental Figures S2–S4, and Supplemental Movie S1). Polypeptides were detected with antibodies against AP-3 δ, clathrin light chain, or mCherry. We previously demonstrated that mCh-FKBP-CLC occupies nearly all clathrin heavy chain–positive structures in HEK293 cells (Zlatic et al., 2011). Similarly, mCh-FKBP-CLC and clathrin heavy-chain signals extensively overlapped in PC12 cells, as revealed by deconvolution microscopy (74 ± 8.6% of all clathrin heavy-chain pixels present in a cell, n = 10; data not shown), validating recombinant clathrin light chain as a marker of clathrin coats in this neuroendocrine cell. In contrast, only 10–20% of all clathrin-positive structures present in a PC12 cell overlapped with AP-3 pixels (Figures 2A and 3 and Supplemental Figure S2). Structures for which the AP-3 and clathrin overlapped in images...
acquired using deconvolution microscopy were imaged using superresolution structured illumination microscopy (SIM). SIM provides a theoretical doubling of spatial resolution above wide-field deconvolution (~120 nm, x-y; 300 nm, z). Analysis of SIM images showed that the overlap of clathrin and AP-3 remained (SIM, Figure 2, B and C). Arrows draw attention to the same discrete puncta in A and B. Dotted lines outline area of colocalization. Scale bar, 0.5 μm. (C2) A 45° rotation of C1 around a vertical axis. See Supplemental Movie S1.

In the following experiments, we specifically focused on early endosomal compartments since these endosomes bud a biochemically and pharmacologically tractable microvesicle population generated by an AP-3–dependent mechanism (Faundez et al., 1997, 1998; Faundez and Kelly, 2000; Clift-O’Grady et al., 1998; Shi et al., 1998). To examine the subcellular distribution of clathrin and AP-3 adaptor in endosomes, we determine the extent of AP-3 signal overlap with clathrin in endosomes identified with the early endosome marker EEA1 (Figure 3). We performed triple indirect immunofluorescence experiments with antibodies against AP-3 δ, the mCherry tag in mCh-FKBP-CLC, and EEA1 using high-resolution deconvolution microscopy. AP-3 and CLC colocalized in early endosomes (Figure 3). Moreover, the extent of AP-3–CLC signal overlap significantly increased from 20.7 ± 7.1% (average ± SEM) to 25.9 ± 6.6% after trapping clathrin chains on membranes by the addition of AP20187 (Figure 3, A–C). This increased overlap after AP20187 occurred at the expense of an accumulation of clathrin polypeptides on endosomes (Figure 3, A, B, and D) rather than an increase of AP-3 on these organelles (Figure 3, A, B, and E). We further tested the presence of AP-3 and clathrin overlapping domains in endosomes by transiently expressing an enhanced green fluorescent protein (EGFP)–tagged Rab5 Q79L mutant in PC12 cells stably expressing mCh-FKBP-CLC (Supplemental Figures S3 and S4). Molecules were detected in fixed cells using antibodies against EGFP, mCherry, and AP-3 δ. The EGFP-tagged Rab5 Q79L mutant allowed us to unequivocally identify early endosomes for quantitative analysis of AP-3 and clathrin subcellular overlap. Rab5 Q79L–positive endosomes were identified by their enlarged EGFP perimeter (Supplemental Figure S3). In these endosomes, we determined the percentage of their perimeter occupied by either clathrin or AP-3. mCh-FKBP-CLC covered 35 ± 5.2% of the EGFP-Rab5 Q79L–labeled perimeter (Supplemental Figure S4A, column 1). Similarly, AP-3 was present in 30.5 ± 5.9% of the EGFP-labeled endosome limiting membrane (Supplemental Figure S4A, column 3), the extent of which was not significantly modified by the addition of AP20187 (Supplemental Figure S4A, column 4). Of importance, a fraction of the entire AP-3 signal present in the endosome perimeter overlapped with mCh-FKBP-CLC (15.6 ± 3.8%; Supplemental Figure S4A, column 5). This result is in good agreement with previous quantitative electron immunomicroscopy data indicating that not all AP-3 budding profiles in early endosomes are decorated with clathrin in cell types other than PC12 cells (Pedersen et al., 2004; Theos et al., 2005). These results indicate that a pool of AP-3 overlaps with clathrin light chain in early endosomes, the site of origin for AP-3 microvesicles in PC12 cells.

PC12 cells generate clathrin-coated vesicles from the plasma membrane by a mechanism that is AP-2 and clathrin dependent yet brefeldin A insensitive (Figure 4, step 1). These clathrin-coated vesicles feed to early endosomes, where synaptic-like microvesicles (SLMs) bud by a mechanism that instead requires AP-3 and ARF1. This AP-3 budding pathway is sensitive to brefeldin A (Figure 4, step 2; Faundez et al., 1997, 1998; Faundez and Kelly, 2000; Shi et al., 1998). Synaptic membrane proteins sorted into these SLMs, such as SV2 or VAMP7, sequentially use these two vesicle-generation steps, whose vesicle products can be distinguished by their distinctive sedimentation properties (Figure 4). This allows selective isolation of clathrin-coated vesicles and SLMVs. We took advantage of features distinguishing these pathways to selectively test the requirement of clathrin in the budding of AP-2 and AP-3 vesicles in PC12 cells stably expressing mCh-FKBP-CLC. We first asked whether the content of adaptor subunits cosedimenting with clathrin-coated vesicles was sensitive to the acute inhibition of clathrin function in mCh-FKBP-CLC–expressing PC12 cells. Our hypothesis predicted that if AP-3 vesicle budding requires clathrin, then acute clathrin perturbation should similarly affect the content of adaptors in clathrin-coated vesicle fractions. Stably transfected mCh-FKBP-CLC PC12 cells were treated for 2 h with vehicle control or AP20187 to perturb clathrin function. We determined the global effectiveness of AP20187 treatment by the redistribution of clathrin and AP-3 toward the perinuclear area after drug incubation (Supplemental Figure S2), the accumulation of clathrin in EEA1-positive endosomes (Figure 3), and a reduction in endosome perimeter after drug addition (Supplemental Figure S4D, and data not shown; Debord et al., 2008; Zlatic et al., 2011). Clathrin-coated vesicle–enriched fractions were prepared from vehicle and AP20187–treated cells and fraction contents analyzed by SDS–PAGE and Western blot (Figure 5A). AP20187 selectively decreased a major polypeptide of ~180 kDa in clathrin-coated vesicle fractions resolved by SDS–PAGE. We confirmed this band as clathrin heavy chain by immunoblot (Figure 5, A and B).
Concurrent with clathrin heavy chain depletion, we detected decreased content of endogenous clathrin light chain and recombinant mCh-FKBP-CLC in clathrin-coated vesicle–enriched fractions (Figure 5C, compare lanes 6 and 12). Of importance, the content of AP-1 γ adaptin and AP-2 α adaptin were similarly reduced in clathrin-coated vesicle fractions isolated from AP20187-treated cells (Figure 5, B and C, compare lanes 1 and 2, and 6 and 12, respectively, and D). Clathrin-coated vesicle cargoes synaptobrevin 2, synaptophysin, and VAMP7 (Blondeau et al., 2004; Takamori et al., 2006) were reduced after AP20187 treatment, a further indication that plasma membrane clathrin-coated vesicle formation was impaired by mCh-FKBP-CLC oligomerization (Figure 5C, compare lanes 6 and 12, and D). Strikingly, even though treatment with AP20187 impaired the formation of AP-1 and AP-2 clathrin-coated vesicles, we observed an increase in the levels of AP-3 in clathrin-coated vesicle enriched–fractions as determined by immunoblot with antibodies against β3, 8, and σ3 subunits (Figure 5, B and C, compare lanes 1 and 2, and 6 and 12, respectively, and D). These results demonstrate that clathrin-coated vesicle formation by AP-1– and AP-2–dependent mechanisms is robustly impaired by acute clathrin oligomerization in PC12 cells expressing mCh-FKBP-CLC. Our findings suggest, however, that a putative clathrin–AP-3 interaction differs from other clathrin–adaptor interactions after acute clathrin function perturbation. This differential behavior of AP-3 content in clathrin-coated vesicle fractions after AP20187 treatment raises the question of whether it also reflects a differential requirement of clathrin in endosome AP-3 vesicle generation.

To test the function of clathrin interactions with the adaptor complex AP-3, we took advantage of PC12 cells expressing mCh-FKBP-CLC and the pharmacological sensitivity of AP-3 vesicle budding to brefeldin A. The sequential organization of AP-2 and AP-3 budding steps has so far precluded a direct test of clathrin’s role in endosome SLMV formation using long-term clathrin perturbations such as shRNA or expression of clathrin heavy-chain recombinant fragments (Figure 4). Therefore we used pharmacological epistasis between the brefeldin A and AP20187 blocks in mCh-FKBP-CLC–expressing PC12 cells to selectively assess a role of clathrin in AP-3 vesicle generation. Brefeldin A completely and reversibly inhibits SLMV formation by AP-3 while sparing the generation of AP-2 vesicles (Figure 4, steps 1 and 2; Faundez et al., 1997; Shi et al., 1998). If the AP-3 budding step requires clathrin, we reasoned that after a SLMV brefeldin A block, a brefeldin A washout in the presence of AP20187 should prevent de novo SLMV generation. In contrast, if clathrin is dispensable for AP-3 vesicle budding, then a brefeldin A washout in the presence of AP20187 should lead to a complete recovery of SLMV formation. We used VAMP7 and SV2 to track SLMV presence and their formation after pharmacological blockages. We selected these two transmembrane proteins because they are enriched in SLMV and mostly localize to endosome compartments at steady state in cells of the neural lineage (Newell-Litwa et al., 2009). In addition, VAMP7 is an AP-3 cargo that directly binds AP-3 (Martinez-Arca et al., 2003; Kent et al., 2012). mCh-FKBP-CLC–expressing PC12 cells were incubated in the presence of vehicle or brefeldin A for 2 h, and SLMVs were isolated by differential centrifugation followed by glycerol velocity sedimentation. Glycerol velocity gradients resolve SLMVs by size as a peak in the middle of the gradient (Figure 6, fractions 8–10; Clift-O’Grady et al., 1998). Brefeldin A decreased SLMVs as determined by VAMP7 and SV2 gradient content (Figure 6, A–C and A1–C1, open circles). This brefeldin A block was released after brefeldin A washout and incubation of cells in ethanol vehicle control for additional 2 h (Figure 6, B and B1, black circles). Of note, continuous incubation of cells in AP20187 instead of vehicle for 2 h had no effect on the reversibility of the brefeldin A washout (Figure 6, C and C1, black circles). This outcome was not due to either a reversal of the brefeldin A block after 4 h of incubation (Supplemental Figure S5) or a redistribution of mCh-FKBP-CLC chains away from endosomes after AP20187. In fact, AP20187 incubation neither modified clathrin or AP-3 nor changed AP-3-clathrin light-chain

FIGURE 3: Analysis of clathrin light-chain and AP-3 δ colocalization in PC12-cell early endosomes. (A, B) High-resolution deconvolution microscopy of PC12 cells incubated in the presence of vehicle or AP20187 and triple labeled with antibodies against AP-3 δ, the mCherry tag in mCh-FKBP-CLC, and EEA1. Scale bar, 5 μm. (C–E) Probability plots of the extent of pixel overlap among marker pairs (Y axis) in cells treated with vehicle (blue traces) and AP20187 (red traces). The p values were determined using the Kolmogorov–Smirnov test. Vehicle, n = 203 endosomes from 55 cells acquired from three biological replicates. AP20187, n = 203 endosomes from 68 cells acquired from three biological replicates.
overlap in the limiting membrane of enlarged early endosomes (Supplemental Figure S4A, compare even and odd columns) over a wide range of clathrin content per endosome and endosome size (Supplemental Figure S4, B and C). This observation is consistent with the described AP20187-dependent trapping of clathrin polypeptides on membranes (Moskowitz et al., 2003; Deborde et al., 2008; Zlatic et al., 2011). These results demonstrate that the biochemical interaction between AP-3 and clathrin chains found in PC12 cells is dispensable for the budding of AP-3-derived SLMV and are in agreement with the previous contention that AP-3 complexes carrying mutations in the β3 putative clathrin binding domain are functional.

The dispensability of clathrin function for AP-3–dependent SLMV formation suggests that interactions between AP-3 and clathrin detected in PC12 cells and other cells types may not follow a canonical association mechanism between the ear domain of the β subunit of the adaptor and clathrin terminal domain (Wilcox and Royle, 2012). To test this hypothesis, we focused on fibroblasts carrying the β3A clathrin-binding sequence (β3A clathrin box). These mutations included discrete changes in β3A or β3A mutations abating putative clathrin-binding determinants (clathrin box) in the β3A hinge-ear domain to test whether an AP-3–clathrin interaction is sensitive to mutagenesis of the β3 clathrin box. These mutations included discrete changes in the β3A clathrin-binding sequence 817SLLDL822 (β3A817AAA), a deletion of the 817SLLDL822 clathrin box (β3A807-831), and a truncation of the entire β3A ear domain (β3A807Stop). With the exception of β3A807Stop, all other β3A clathrin box mutants rescued a missorting phenotype in Ap3b+/-/- cells (Peden et al., 2002). We performed DSP cross-linking in intact cells followed by immunomagnetic isolation of AP-3 complexes with δ antibodies. The δ adaptin antibodies precipitated δ3 subunits from Ap3b+/-/- and notably low levels of clathrin in the presence of DSP but not BLOC-1 subunits detected with antibodies against palladin (Figure 7, compare lanes 1 and 2). These δ-3 polypeptides correspond to the reported δ-3 adaptor hemicomplexes described in Ap3b+/-/- cells (Peden et al., 2002). We used the BLOC-1 complex, an AP-3 interactor, and...
Experimentally, we defined the levels of α3 adaptin precipitated by δ antibodies as indications of reassembled AP-3 complexes with different recombinant β3A subunits (Di Pietro et al., 2006; Salazar et al., 2006, 2009; Gokhale et al., 2012). The content of clathrin precipitating with δ adaptin antibodies increased in all Ap3b1+/−/+ cells where AP-3 complexes were reassembled by reexpression of recombinant β3A chains (Figure 7, compare lane 2 with lanes 4, 6, 8, and 10). Of importance, this increase in clathrin coprecipitation with reassembled AP-3 complexes was not sensitive to any of the mutations ablating putative clathrin-binding determinants in the β3A hinge-ear domain. These results indicate that the β3A hinge-ear domain is not necessary for clathrin interactions with cross-linked AP-3 complexes. Our results suggest that AP-3 diverges from the model by which AP-1 and AP-2 adaptors bind to clathrin cages for vesicle biogenesis via the C-terminus of β adaptins with the terminal domain of clathrin-heavy chain (Leamon and Traub, 2012).

**DISCUSSION**

We explored the role of clathrin in AP-3-dependent vesicle budding by means of long-term clathrin shRNA down-regulation and rapid chemical-genetic perturbation of clathrin function. Down-regulation of clathrin heavy chain has been used to assess whether components enriched in clathrin-coated vesicle fractions truly reside in these organelles. This approach has been validated in HeLa cells using quantitative mass spectrometry alone or in combination with this observation as evidence of clathrin dispensability in AP-3 SLMV budding from endosomes. We examined alternative interpretations that could also explain our brefeldin A-AP20187 pharmacological epistasis results. We asked 1) whether AP-3 interacts and colocalizes with mCh-FKBP-CLC chimera does not prevent the formation of AP-3 vesicles from endosomes after a brefeldin A block. We interpreted our findings in PC12 cells indicate that acute perturbation of clathrin with a mCh-FKBP-CLC chimera does not prevent the formation of AP-3 vesicles from endosomes. This conclusion is also consistent with our past studies in cell-free vesicle reconstitution experiments. We estimated, however, that a role of this clathrin isoform is a remote option. Rodent cells do not express chromosome 22 clathrin, and our functional assessment of a clathrin-AP-3 interaction was conducted in rat PC12 cells (Wakeham et al., 2005). Thus a simple interpretation of our data is that the clathrin interaction with AP-3 is not required for vesicle budding from endosomes. This conclusion is in line with previous studies in which AP-3 function is restored in β3 mutant cells by expression of recombinant β3 chains carrying a defective clathrin-binding motif defined in vitro and presumed to be unique in AP-3 (Peden et al., 2002). The present conclusion is also consistent with our past studies in cell-free vesicle reconstitution experiments. Cell-free AP-3 budding is resistant to cytosolic clathrin depletion. Moreover, de novo vesicle formation from endosomes is principal component analysis of multiple datasets (Borner et al., 2006, 2012). AP-1 and AP-2 faithfully cosegregate with clathrin-coated vesicles in both approaches. These assays provide different answers, however, as to whether AP-3 resides in clathrin-coated vesicles (Borner et al., 2006, 2012). Comparison of control and clathrin-knockdown cells by quantitative mass spectrometry suggests that AP-3 would reside in these organelles, a result that we recapitulated here. However, similar to principal component analysis of clathrin-coated vesicle fractions, we also observed a discrepancy in cosedimentation of AP-3 with clathrin-coated vesicle fractions. In our case, there is a drastic difference between chronic and acute clathrin function perturbation despite consistent behavior by AP-1 and AP-2 adaptors. This suggests that AP-3 cosediments with clathrin-coated vesicles but in membranous organelles distinct from these caged vesicles. Second, it implies that the sensitivity of AP-3 to long-term clathrin RNA interference results from indirect effects of chronic perturbations in the endocytic pathway. The “anomalous” AP-3 behavior prompted us to reevaluate whether AP-3 indeed requires clathrin to generate vesicles and whether clathrin–AP-3 interactions follow principles similar to those by adaptors AP-1 and AP-2.

Our findings in PC12 cells indicate that acute perturbation of clathrin with a mCh-FKBP-CLC chimera does not prevent the formation of AP-3 vesicles from endosomes after a brefeldin A block. We interpreted

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**FIGURE 6:** Acute perturbation of clathrin function does not prohibit synaptic-like microvesicle formation after release of a brefeldin A block. PC12 cells stably expressing mCh-FKBP-CLC were treated for 2 h with brefeldin A (BFA) or vehicle control (EtOH) at 37°C. After this 2-h treatment, BFA-treated cells were either transferred to 4°C (BFA) or extensively washed at 4°C and then incubated with AP21087 (BFA-AP20187) or vehicle control (BFA-EtOH) for additional 2 h at 37°C. SLMVs sediment to the middle of 5–20% glycerol velocity gradients. Fractions from this gradient were loaded onto SDS–polyacrylamide gels for Western blot and densitometry quantifications. Immunoblot (IB) for SV2 and VAMP7 was used to track SLMVs. (A, A') Densitometry quantification of fraction signal per total signal in BFA-treated cells (white circles) as compared with EtOH-only treated cells (black circles). (B, B') Densitometry quantification of fraction signal per total signal in BFA-EtOH–treated cells (black circles). (C, C') Densitometry quantification of fraction signal per total signal in BFA-treated cells (white circles) as compared with BFA-AP20187–treated cells (black circles). (D, E) Western blot of VAMP7 and SV2, respectively, from EtOH–, BFA–, BFA-EtOH–, and BFA-AP20187–treated cells.
in coprecipitated clathrin. This association is specific, as experiments possess low levels of interactions or the mode of binding between AP-3 and clathrin would differ.

Models predict that the stoichiometry of AP-3 and clathrin interactions with clathrin cages. We indirectly reported motor that binds to clathrin cages directly. In contrast, motors that bind to AP-1 and AP-3 adaptors directly (Nakagawa et al., 2011; Gokhale et al., 2012). Although clathrin binds preponderantly to the C-terminus of β adaptin with the terminal domain of clathrin heavy chain (Edeling et al., 2006; Knuehl et al., 2006). However, the functional relevance of the appendage domain of β adaptin has been tested, and it is dispensable (Motley et al., 2006). In fact, the δ adaptin down-regulation phenotype can be fully rescued by expression of α adaptin lacking its appendage domain (Motley et al., 2006). In contrast, the δ allele Ap3d1<sup>807-831</sup>, which lacks the appendage domain of δ adaptin, possesses phenotypes indicating that the δ appendage is not dispensable (Kantheti et al., 2003). Collectively, the evidence supports our contention that clathrin-AP-3 interactions differ from those between clathrin and the adaptor complexes AP-1 and AP-2.

A question that arises from our data concerns the extent to which the functionality of a clathrin–AP-3 association observed in PC12 SLMV budding from endosomes reflects other putative AP-3–dependent budding events. Nearly 40% of all AP-3 immunoreactivity in cells is present in endosomes (Peden et al., 2004; Theos et al., 2005). A similar amount of AP-3, however, associates to tubules/vesicles not identified as endosomes, and 4–9% associates with the trans-Golgi network (Peden et al., 2004; Theos et al., 2005). This leaves open the possibility that there may be additional modalities of AP-3-dependent budding distinct from vesicle generation from endosomes, such as the formation of large, dense secretory vesicles from the Golgi complex (Asensio et al., 2010), where AP-3 and clathrin may interact in a productive manner.

**MATERIALS AND METHODS**

**Antibodies and constructs**

Anti-clathrin heavy chain was purchased from EMD Millipore (Billerica, MA; CP45, clone: X22) and BD Transduction Laboratories (Billerica, MA; CP45, clone: CON.1). Anti–adaptin γ (610385), and EEA1 (610456) were purchased from BD Transduction Laboratories. The AP3δ 4A4, c-mer 9E10, and SV2 10H antibodies, developed by Andrew Peden, J. Michael Bishop, and Kathleen M. Buckley, respectively, were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Department of Iowa City, IA). Anti-synaptophysin was purchased from EMD Millipore (MAB5258, clone: SY38). Anti-AP3β (13384-1-AP) and anti-pallidin (10891-1-AP) were purchased from Protein Tech Group (Chicago, IL). Anti-mCherry/DsRed was purchased from Clontech (Mountain View, CA; 632496). Anti-GFP was purchased from AVES Labs (Tigard, OR; GFP-mCherry/DsRed). Anti–clathrin heavy chain was purchased from EMD Millipore (AB9884 and Covance (Berkeley, CA; γ871 AAA), a deletion of the clathrin box in Ap3b (p33A<sub>A</sub> 807-831), or a truncation of Ap3b eliminating the entire ear domain containing the clathrin box (p33A<sub>A</sub> <sub>Stop</sub>) were treated with the cross-linker DSP. Lysates were subjected to coimmunomagnetic isolations directed against AP-3 δ (lanes 1–10). All rescue lysates from DSP-treated cells show increased coisolation of clathrin heavy chain (immunoblot [IB]; clathrin lanes 4, 6, 8, and 10) compared with unrescued lysate treated with DSP (IB, clathrin lane 2). Input represents 5% of immunomagnetic isolation load (lanes 11–15).

If clathrin is unessential for AP-3 vesicle budding, then what roles may clathrin play? We postulate that an interaction between AP-3 and clathrin reflects a scaffolding role such as Hrs-clathrin patches, clathrin-sorting nexin interactions, or clathrin organization of the kinesin microtubules (Raiborg et al., 2001; McGough andullen, 2012; Royle, 2012). Alternatively, clathrin binding to AP-3 could be a mechanism to deliver clathrin directionally by motors either in the absence or presence of vesicle membranes. Although we know of motors that bind to AP-1 and AP-3 adaptors directly (Nakagawa et al., 2000; Azavedo et al., 2009; Delevoye et al., 2009), there is no reported motor that binds to clathrin cages directly. In contrast, clathrin binds to dynemin by means of an intervening protein, bicaudal D. This motor–clathrin association is necessary for normal neurrotransmission in Drosophila (Li et al., 2010). These nonbudding models predict that the stoichiometry of AP-3 and clathrin interactions or the mode of binding between AP-3 and clathrin would differ from AP-1 and AP-2 interactions with clathrin cages. We indirectly tested the second prediction in fibroblasts carrying the β3A mutation pearl (Ap3b<sup>1<sub>pe/pe</sub></sup>; Peden et al., 2002). These Ap3b<sup>1<sub>pe/pe</sub></sup> cells possess low levels of δ-ε3 adaptor hemicomplexes that we found coprecipitated clathrin. This association is specific, as experiments in mChopa3δ1<sup>im/mim</sup> cells, which lack δ adaptin, are free of clathrin in these cross-linked precipitates, thus excluding spurious binding of clathrin to antibody–magnetic beads complexes. An equally striking finding is the observation that Ap3b<sup>1<sub>pe/pe</sub></sup> rescued with a recombinant β3A carrying a truncation of the entire β3A hinge-ear domain (β3A<sub>R07</sub>) still coprecipitates clathrin. These observations are consistent with a model by which either δ-ε3 adaptor hemicomplexes or AP-3 complexes lacking the ear domain associate to clathrin, directly or indirectly. This would represent a departure of the model by which canonical AP-1 and AP-2 adaptors productively engage clathrin cages for vesicle biogenesis via the C-terminus of β adaptin with the terminal domain of clathrin heavy chain (Edeling et al., 2006; Knuhehl et al., 2006; Schmid et al., 2006; Lemmon and Traub, 2012). Although clathrin binds preponderantly to the C-terminus of β2 adaptin, two-hybrid evidence suggests a putative interaction between clathrin and the C-terminal domain of α adaptin (Knuhehl et al., 2006). However, the functional relevance of the appendage domain of α adaptin has been tested, and it is dispensable (Motley et al., 2006). In fact, the α adaptin down-regulation phenotype can be fully rescued by expression of α adaptin lacking its appendage domain (Motley et al., 2006). In contrast, the δ allele Ap3d1<sup>807-831</sup>, which lacks the appendage domain of δ adaptin, possesses phenotypes indicating that the δ appendage is not dispensable (Kantheti et al., 2003). Collectively, the evidence supports our contention that clathrin-AP-3 interactions differ from those between clathrin and the adaptor complexes AP-1 and AP-2.

FIGURE 7: The clathrin-binding box of AP-3 β1 is dispensable for AP-3–clathrin coisolation. Pearl (Ap3b<sup>1<sub>pe/pe</sub></sup>) fibroblasts or Pearl fibroblasts rescued with full-length Ap3b1 (p33A), Ap3b1 containing a triple-alanine mutation in the clathrin box (p33A<sub>AAA</sub>), a deletion of the clathrin box in Ap3b1 (p33A<sub>A</sub> 807-831), or a truncation of Ap3b1 eliminating the entire ear domain containing the clathrin box (p33A<sub>A</sub> <sub>Stop</sub>) were treated with the cross-linker DSP. Lysates were subjected to coimmunomagnetic isolations directed against AP-3 δ (lanes 1–10). All rescue lysates from DSP-treated cells show increased coisolation of clathrin heavy chain (immunoblot [IB]; clathrin lanes 4, 6, 8, and 10) compared with unrescued lysate treated with DSP (IB, clathrin lane 2). Input represents 5% of immunomagnetic isolation load (lanes 11–15).
Peptide directed against the AP3 δ SA4 antibody antigen binding site (AQQVDIEEMPNALPSSDEEKPDPYRA) was obtained from the Emory University Microchemical Facility (Atlanta, GA) and Invitrogen (EvoQuest Team, Carlsbad, CA).

Clathrin heavy chain (RHS39799577067) and mutated (RHS397998822549) pLKO.1 shRNA constructs were purchased from Open Biosystems (Huntsville, AL). The constructs mCherry-FKBP-CLC and EGFP-Rab5-Q79L have been described ( Craiga et al., 2008; Zlatica et al., 2011).

Cell culture, transfection, and infection

HEK293 and SH-SY5Y cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM (HyClone, Logan, UT) containing 10% fetal bovine serum (HyClone) and 100 μg/ml penicillin and streptomycin (HyClone) and PC12 cells (American Type Culture Collection) cultured in DMEM containing 10% donor equine serum (HyClone), 5% fetal bovine serum, and 100 μg/ml penicillin and streptomycin were incubated at 37°C and 10% CO₂. Generation and culture of Ap3b<sup>−/−</sup> cells or Ap3b<sup>1+/−</sup> rescued with either recombinant wild-type β3A or β3A mutations ablating putative clathrin-binding determinants was as described (Pedena et al., 2002).

For transient transfections, HEK293 cells were transfected in wells of six-well plates were incubated with 1 μg of DNA in OptiMEM (Life Technologies, Grand Island, NY) containing 0.5% Lipofectamine 2000 (Invitrogen) for 4 h, followed by incubation with culture media. For stable transfections, PC12 cells were transfected as described in transient transfections, followed by 0.2 mg/ml G418 (Invitrogen) drug selection. Selected cells were serially diluted, and clonal populations were maintained in PC12 culture media supplemented with 0.1 mg/ml G418.

For lentiviral infection, lentivirus containing shRNA constructs were prepared by the Emory University Viral Vector Core. Antibiotic-free culture medium containing 1 μl of high-titer lentivirus was injected with HEK293 or SH-SY5Y cells for 24 h, followed by incubation for 6 d with culture medium supplemented with 0.1 mg/ml selection drug, hygromycin B (Invitrogen), in 10-cm culture plates.

Acute clathrin perturbation and clathrin-coated vesicle fractionation

HEK293 or PC12 cells expressing the mCh-FKBP-CLC construct were grown to confluence. Cells were incubated for 2 h at culture conditions in medium supplemented with 50 nM AP20187 (B/B Homodimerizer 635060; Clontech) or 0.05% ethanol vehicle control (Zlatica et al., 2011). HEK293 cells infected with lentivirus containing clathrin heavy-chain shRNA constructs and transiently expressing mCh-FKBP-CLC, SH-SY5Y cells infected with lentivirus containing clathrin or mutated shRNA constructs, and PC12 cells stably expressing mCh-FKBP-CLC with acute clathrin perturbation were prepared for clathrin-coated vesicle fractionation as previously described (Girard et al., 2004). Fractions were analyzed by either Western blot or Coomassie staining of 4–20% Criterion polyacrylamide gels (Bio-Rad, Hercules, CA).

DSP cross-linking and immunomagnetic precipitation

HEK293 and PC12 cells expressing mCh-FKBP-CLC were prepared for DSP cross-linking, followed by immunomagnetic precipitation as previously described (Salazar et al., 2009; Zlatica et al., 2010, 2011; Gokhale et al., 2012; Perez-Conrero et al., 2012). The resulting isolated proteins were analyzed by Western blot of 4–20% Criterion polyacrylamide gels.

Brefeldin A/AP20187 treatment followed by synaptic-like microvesicle fractionation

PC12 cells expressing mCh-FKBP-CLC were grown to confluence in 10-cm plates, moved to an ice bath at 4°C, and washed three times in ice-cold phosphate-buffered saline (PBS)/1 mM MgCl₂/0.1 mM CaCl₂. Sequential drug-treated cells were incubated with warm DMEM supplemented with 10 mg/ml brefeldin A (Epicentre Bio-technologies, Madison, WI) for 2 h at 37°C. Plates were then moved to an ice bath at 4°C and washed for 5 min with ice-cold DMEM three times. Cells were then incubated with ice-cold DMEM supplemented with either 50 nM AP20187 or 0.05% ethanol vehicle control for 15 min on an ice bath at 4°C to allow for drug equilibration. Cells were then moved to 37°C water bath for 10 min to quickly warm media and then to 37°C incubator for 2 h. Single drug-treated cells were incubated with warm DMEM supplemented with 10 mg/ml brefeldin A or 1% ethanol vehicle control for 2 or 4 h at 37°C. After incubation, cells were placed directly on an ice bath at 4°C and washed two times with ice-cold PBS/1 mM MgCl₂/0.1 mM CaCl₂ with subsequent synaptic-like microvesicle fractionation.

Synaptic-like microvesicle fractionation was performed as previously described (Faundez et al., 1997; Faundez and Kelly, 2000; Clift-O’Grady et al., 1998). Briefly, cells were lifted in intracellular buffer (38 mM potassium aspartate, 38 mM potassium glutamate, 38 mM potassium gluconate, 20 mM (N-morpholino)propanesulfonic acid–KOH, pH 7.2, 5 mM reduced glutathione, 5 mM sodium carbonate, 2.5 mM magnesium sulfate, 2 mM ethylene glycol tetraacetic acid) and sedimented for 5 min at 800 × g at 4°C in a tabletop centrifuge. Cell pellet was suspended in 300 μl of Intracellular buffer supplemented with Complete antiprotease (Roche, Indianapolis, IN). Homogenate was prepared with a 12-μm clearance cell cracker with intracellular buffer supplemented with Complete antiprotease at 4°C. Equal protein load from homogenate was centrifuged at 27,000 × g for 35 min at 4°C in a Sorval SS34 rotor (Thermo Scientific, Waltham, MA) to generate the first supernatant (S1) and pellet (P1). S1 was centrifuged at 218,000 × g for 75 min at 4°C in a Beckman SW55 rotor (Beckman Coulter, Palo Alto, CA). Aliquots of the glycerol gradient after centrifugation were analyzed by Western blot on 4–20% Criterion polyacrylamide gels (Bio-Rad).

Immunofluorescence, microscopy, and analysis

coverslips were prepared as previously described (Craiga et al., 2008; Zlatica et al., 2011). Cells were seeded to Matrigel-coated (BD Bioscience, San Jose, CA) coverslips. Cells were washed twice in PBS/1 mM MgCl₂/0.1 mM CaCl₂ and fixed with 4% paraformaldehyde in PBS. Cells were blocked, permeabilized, and probed with primary and secondary antibody in block buffer (PBS containing 15% donor equine serum [HyClone], 2% bovine serum albumin [HyClone], 1% fish gelatin [Sigma-Aldrich, St. Louis, MO], and 0.02% saponin [Sigma-Aldrich]). After secondary antibody probing, cells were washed twice in block buffer and once with PBS supplemented with 1 mM MgCl₂ and 0.1 mM CaCl₂ and mounted on slides with Gelvatol.

Deconvolution microscopy was carried out as previously described on a 200M inverted microscope (Carl Zeiss, Thornwood, NY) and Sedat filter set (Chroma Technology, Rockingham, UT) run with a multilambda, sequential capture, wide-field, three-dimensional microscopy system from SlideBook 4.0 OS X software (Intelligent
Imaging Innovations, Denver, CO; Craig et al., 2008; Newell-Litwa et al., 2009; Zlatic et al., 2011). Samples were imaged with a 100×/1.40 numerical aperture (NA) oil differential interference contrast objective (Carl Zeiss) and captured with a scientific-grade, cooled, charge-coupled device (CCD) CoolSNap HQ camera (Photometrics, Tucson, AZ) run with an ORCA-ER chip (Hamamatsu Photonics, Hamamatsu, Japan). Out-of-focus light was removed using a nearest-neighbor constrained iterative deconvolution algorithm with Gaussian smoothing.

SIM microscopy was carried out using a Nikon N-SIM microscopy system on an Eclipse Ti inverted microscope run with Nikon Elements software (Nikon, Melville, NY). The samples were imaged with a 100–/1.49 NA objective and an iXon DU897 electron-multiplying CCD camera (Andor Technology, Belfast, Northern Ireland). Wide-field images were acquired with a mercury lamp and the appropriate filters: 480/30-nm excitation, 535/40-nm emission (Alexa 488) or 540/25-nm excitation, 692/68-nm emission (Alexa 555). Wide-field images were deconvolved with Huygens software (Scientific Volume Imaging, Hilversum, Netherlands). SIM images were acquired with laser excitation and emission filters, 488-nm excitation, 520/40-nm emission (Alexa 488) and 561-nm excitation, 640/40-nm emission (Alexa 555). Images were acquired in three-dimensional SIM mode (for each SIM image 15 images with five different planes of three different angular orientations of illumination were collected), and z-stacks were collected for each image. SIM images were processed with Nikon Elements software.

Three-dimensional surface reconstruction was carried out using Imaris 6.3.1 software (Bitplane, St. Paul, MN) from SIM and deconvolution image z-stacks. Whole-cell and profile colocalization, profile perimeter, and total fluorophore pixel content were determined from three z-plane focal slices per region of interest and carried out with MetaMorph 6.1 software (Universal Imaging, Sunnyvale, CA) with manual thresholding.

Statistical analysis

Experimental conditions were compared using Synergy Kaleida-Graph, version 4.1.3 (Reading, PA), or StatPlus Mac Built5.6.0pre/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 the single lines extending from the box.

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