Phosphoinositide-mediated clathrin adaptor progression at the trans-Golgi network

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Clathrin-coated vesicles mediate endocytosis and transport between the trans-Golgi network (TGN) and endosomes in eukaryotic cells. Clathrin adaptors play central roles in coat assembly, interacting with clathrin, cargo and membranes. Two main types of clathrin adaptor act in TGN–endosome traffic: GGA proteins and the AP-1 complex. Here we characterize the relationship between GGA proteins, AP-1 and other TGN clathrin adaptors using live-cell and super-resolution microscopy in yeast. We present evidence that GGA proteins and AP-1 are recruited sequentially in two waves of coat assembly at the TGN. Mutations that decrease phosphatidylinositol 4-phosphate (PtdIns(4)P) levels at the TGN slow or uncouple AP-1 coat assembly from GGA coat assembly. Conversely, enhanced PtdIns(4)P synthesis shortens the time between adaptor waves. Gga2p binds directly to the TGN PtdIns(4)K-kinase Pik1p and contributes to Pik1p recruitment. These results identify a PtdIns(4)P-based mechanism for regulating progressive assembly of adaptor-specific clathrin coats at the TGN.

The last subcompartment of the Golgi complex, the TGN, sorts proteins into distinct transport carriers that are targeted to different destinations, including the plasma membrane and the endosome–lysosome system. A major class of TGN-derived transport carriers are clathrin-coated vesicles (CCVs), which select cargo for delivery to endosomes. CCVs form through the concerted action of three types of highly conserved proteins: clathrin, which forms the outer coat scaffold; adaptors, which link clathrin to membranes by binding to clathrin, phosphoinositides and/or cargo proteins; and accessory proteins, which contribute to coat assembly, membrane invagination, scission and uncoating.

The principal adaptors that participate in TGN CCV formation are GGA proteins, epsin-related proteins and the heterotetrameric AP-1 complex (β1, γ1, µ1 and σ1; refs 1,2). Yeast cells express AP-1, two GGA proteins (Gga1p and Gga2p) and two Golgi-localized epsin-related proteins (Ent3p and Ent5p; ref. 2). Physical and genetic interaction studies indicate that these adaptors are part of an extended clathrin-based network in which AP-1 and GGA proteins seem to constitute distinct network hubs.

The relationship between AP-1 and Gga proteins during TGN CCV formation is uncertain. Both proteins seem to rely on similar sets of low-affinity (low micromolar dissociation constant) multivalent interactions for recruitment to the TGN, including binding to the activated form of the ADP-ribosylation factor 1 (ARF1) GTPase, PtdIns(4)P and cargo1,9. However, whether the adaptors act sequentially, in parallel or in distinct pathways remains unresolved, and the extent of co-localization has varied in different studies1. Here we have taken advantage of the dispersed nature of Golgi cisternae in the yeast Saccharomyces cerevisiae to assess the distribution and relative dynamics of fluorescently tagged AP-1 and Gga proteins expressed at endogenous levels. Our results provide evidence for sequential waves of adaptor-specific coat assembly, coupled by synthesis of PtdIns(4)P.

RESULTS

Gga2p and AP-1 assemble sequentially

Movies of cells expressing clathrin coat proteins fused to GFP or mRFP were acquired by spinning-disc confocal microscopy. Clathrin coat protein fusions, expressed from the normal chromosomal loci, localized as heterogeneous puncta throughout the cell, similar to patterns observed by immunofluorescence microscopy of fixed cells6,10. Fluorescently tagged clathrin heavy chain (Chc1p–mRFP) localized as transient puncta at the plasma membrane and at internal sites (Supplementary Movie S1) as observed by others1, normally persisting for 2 min or less. Internal clathrin foci grew to relatively large sizes (0.3–1.5 μm), assumed irregular and often changing shapes, and moved in random directions. TGN clathrin adaptors formed puncta with characteristics of the internal clathrin structures (Fig. 1).

The relative dynamics of specific protein pairs were assessed in two-colour movies of single optical sections by tracking individual puncta. In cells expressing Gga2p–mRFP and B1–GFP (β1 subunit of AP-1), Gga2p foci appeared first, increased in intensity, and then...

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Figure 1 Sequential assembly of clathrin adaptors. (a–f) Pairs of clathrin coat proteins were assessed in two-colour movies of single optical sections (a,c–f) or in three dimensions (b) by tracking puncta that remained separate from other puncta throughout their lifetimes and were present for at least seven frames (~240 frames). The top left image in a and c–f shows a merged image of live cells co-expressing GFP- and mRFP-tagged versions of the indicated clathrin adaptors; the arrowhead indicates a puncta selected for the kymograph below. Scale bars, 1 μm. The top left image in b shows an image of five optical sections along the z axis that were imaged at 0.3 μm intervals. One z-stack was collected every 2.1 s. Scale bar, 2 μm. The bottom panel in a–f shows a three-channel kymograph (merged, mRFP and GFP) of the selected puncta; the time to acquire one image pair was 1.2 s. Every other image pair is shown in the kymograph. The graphs show the normalized level of GFP and mRFP fluorescence intensity in the puncta as a function of time. (a) GPy3109. (b) GPy4974. (c) GPy3954. (d) GPy3912. (e) GPy3962. (f) GPy3900.

became AP-1 positive as the Gga2p intensity declined (Fig. 1a and Supplementary Movie S2). A total of 98% of Gga2p puncta transitioned to AP-1 (n = 101, 38 cells). Conversely, 95.8% of AP-1 puncta were preceded by the appearance of Gga2p (n = 142, 51 cells). In contrast, imaging of cells expressing clathrin light chain (GFP–Clc1p) and heavy chain (Chc1p–mRFP) revealed complete co-localization and coincident profiles, as expected for subunits of the same protein complex (Supplementary Fig. S1a). We presume that declines in coat protein fluorescence intensity reflect budding of CCVs. Influenently, we observed release of smaller puncta, but were unable to effectively track budding events.

The Gga2p to AP-1 sequence was also observed by three-dimensional live-cell imaging where optical sections were collected along the z axis over time (Fig. 1b), indicating that the appearance of puncta in single optical sections probably represents coat assembly rather than movement of pre-existing coats into the focal plane. On the basis of better temporal and spatial resolution and reduced photobleaching, subsequent analyses were carried out with single optical sections.

We determined the times between peak intensities of Gga2p–mRFP and β1–GFP in puncta (peak-to-peak time). Gga2p–mRFP fluorescence intensity peaked 10.4 s ± 0.55 s before AP-1 reached peak fluorescence intensity (Table 1, row 1, and Supplementary Fig. S1b). Similar results were obtained when the σ1 subunit was tagged with GFP and compared with Gga2p–mRFP (L.D., unpublished observations).

Chemically fixed cells were imaged by structured illumination microscopy (SIM), a super-resolution technique. Gga2p–GFP or β1–GFP was localized as clusters of ~100–200 nm structures, consistent with the size expected for individual or closely spaced coated pits (Fig. 2a,b). This indicates that puncta observed by confocal microscopy can be composed of multiple individual coats below the resolution limit of the microscope. In SIM images of cells expressing both Gga2p–mRFP and β1–GFP, most of the Gga2p structures were adjacent to, but distinct from, the β1 structures (Fig. 2e). Our data provide evidence that the consecutive fluorescence peaks of Gga2p and AP-1 represent a wave of semi-synchronous Gga2p coat formation followed by a wave of AP-1 coats. Multiple coated structures in each fluorescent puncta probably accounts for the heterogeneity in peak-to-peak values.

Two waves of clathrin adaptor assembly

Ent3p binds to Gga2p and depends on Gga2p for localization. Ent3p and Gga2p fluorescence intensities peaked simultaneously (Fig. 1c and Table 1, row 2, and Supplementary Fig. S1c and Movie S3). Moreover,
Table 1 Dynamics of clathrin adaptors, phosphoinositide binding reporters and Golgi markers at the TGN.

| Row | Mutations | Strain | Labelled proteins | Number of puncta (n) in total cells (c) | Peak-to-peak time (s) |
|-----|-----------|--------|-------------------|----------------------------------------|----------------------|
| 1   | Wild type | Gga2–mRFP β1–GFP | 102 ± 59           | 0.10 ± 0.55                          |
| 2   | Wild type | Gga2–mRFP Ent3–GFP | 91 ± 38            | 0.01 ± 0.16                          |
| 3   | Wild type | Ent3–GFP β1–mRFP | 30 ± 21            | 1.0 ± 1.40                           |
| 4   | Wild type | Ent3–GFP Ent5–mRFP | 99 ± 52            | 0.8 ± 0.70                           |
| 5   | Wild type | Gga2–mRFP Ent5–GFP | 91 ± 46            | 0.8 ± 0.14                           |
| 6   | Wild type | Ent5–GFP β1–mRFP | 58 ± 30            | 1.3 ± 0.16                           |
| 7   | Wild type | Gga2–mRFP Sec7–mRFP | 97 ± 25           | 0.29 ± 0.39                         |
| 8   | Wild type | Sec7–mRFP β1–GFP | 76 ± 48            | 1.0 ± 1.18                           |
| 9   | Wild type | Gga2–mRFP Chc1–mRFP | 95 ± 49           | 1.3 ± 0.4                           |
| 10  | Wild type | Chc1–mRFP β1–GFP | 59 ± 34            | 8.0 ± 0.7                           |
| 11  | gga1Δ gga2Δ | Chc1–mRFP β1–GFP | 71 ± 36            | 0.79 ± 0.73                         |
| 12  | β1Δ (ap2Δ) | Chc1–mRFP Gga2–GFP | 22 ± 40           | 0.9 ± 0.86                          |
| 13  | Wild type | Sec7–mRFP β1–GFP | 76 ± 48            | 1.0 ± 1.18                          |
| 14  | gga1Δ gga2Δ | Sec7–mRFP β1–GFP | 49 ± 25            | 2.6 ± 1.9                           |
| 15  | Wild type | Gga2–mRFP β1–GFP | 102 ± 59           | 1.0 ± 0.55                          |
| 16  | arf1Δ | Gga2–mRFP β1–GFP | 47 ± 41            | 2.0 ± 2.7                           |
| 17  | Wild type | Gga2–mRFP GFP–PmGSH1 | 104 ± 52         | 5.3 ± 0.4                           |
| 18  | Wild type | GFP–PmGSH1 β1–mRFP | 87 ± 52            | 4.1 ± 0.4                           |
| 19  | Wild type | Sec7–mRFP GFP–PmGSH1 | 103 ± 61         | 3.8 ± 0.54                          |
| 20  | gga1Δ gga2Δ | Sec7–mRFP GFP–PmGSH1 | 117 ± 68       | 1.1 ± 0.86                          |
| 21  | arf1Δ | Sec7–mRFP GFP–PmGSH1 | 45 ± 33            | 1.1 ± 0.2                           |
| 22  | Wild type | Gga2–mRFP GFP–PmGSH1 | 104 ± 52        | 5.3 ± 0.6                           |
| 23  | GPD–PIK1 GPD–FQ1 | Gga2–mRFP β1–GFP | 48 ± 27            | 0.0 ± 0.81                          |
| 24  | Wild type | Gga2–mRFP β1–GFP | 102 ± 59           | 1.0 ± 0.55                          |
| 25  | GPD–PIK1 GPD–FQ1 | Gga2–mRFP β1–GFP | 67 ± 35            | 5.7 ± 0.77                          |
| 26  | Wild type | Ent3–GFP Ent5–mRFP | 99 ± 52            | 8.4 ± 0.70                          |
| 27  | GPD–PIK1 GPD–FQ1 | Ent3–GFP Ent5–mRFP | 30 ± 17            | 3.9 ± 1.5                           |
| 28  | Wild type | Sec7–mRFP GFP–PK1 | 75 ± 47            | 4.1 ± 0.6                           |
| 29  | gga1Δ gga2Δ | Sec7–mRFP GFP–PK1 | 61 ± 36            | 1.2 ± 1.3                           |
| 30  | Wild type | Frq1–GFP Sec7–mRFP | 55 ± 38            | 1.5 ± 0.8                           |
| 31  | gga1Δ gga2Δ | Frq1–GFP Sec7–mRFP | 73 ± 48            | 0.4 ± 0.9                           |

Haploid cells expressing the indicated fluorescent proteins were imaged using live-cell microscopy. Multiple puncta from each strain were analysed for changes in fluorescence intensity of the indicated proteins over time. The time between the points of peak fluorescence intensity for each protein was determined and the mean peak-to-peak time was calculated for all of the analysed puncta in a given strain. The data are presented as the mean ± s.e.m. for each strain. The order of tagged proteins in each row represents the order of assembly. Some rows are repeated to facilitate comparison (1, 15, 24, 4, 26, 8, 13, 17, 22). Light blue shading separates groups that share a common wild type control.

Ent3p localized as 100–200 nm puncta that substantially co-localized with Gga2p by SIM (Fig. 2c,f). In comparison with AP-1 (either β1 (Table 1, row 3) or σ1 (L.D., unpublished observations)), Ent3p peaked on average 10.2 s earlier, similarly to Gga2p. Little co-localization between Ent3p and AP-1 was evident by SIM (Supplementary Fig. S2a).

Ent5p binds clathrin, Gga2p and AP-1 (refs 4, 6). Genetic interactions indicate that Ent5p function is more important for AP-1-mediated transport4. Ent5p appeared as puncta after Ent3p (Fig. 1d): 98.2% of Ent5p puncta became positive for Ent5p (n = 114, 38 cells) and almost all Ent5p puncta derived from Ent3p puncta (98.5%, n = 67, 23 cells). The peak-to-peak time between Ent3p and Ent5p was 8.4 s (Table 1, row 4), slightly shorter than that observed between Gga2p/Ent3p and AP-1. This difference can be attributed to a small population (~20%) of puncta in which Ent5p intensity peaked close to that of Ent3p (Supplementary Fig. S1d), and a main population that peaked 9.8 s after Ent3p. Similar results were obtained with Gga2p–mRFP and Ent5p–GFP (Fig. 1e and Table 1, row 5, and Supplementary Movie S4). In accord with these findings, most but not all Ent5p peaked coincidentally with AP-1 (Fig. 1f and Table 1, row 6, and Supplementary Fig. S1e). By SIM, 100–200 nm Ent5p puncta infrequently co-localized with Gga2p and more commonly overlapped with β1 (Fig. 2d,g and Supplementary Fig. S2d).

Together, our results reveal two waves of adaptor assembly: Gga2p and Ent3p assemble first, peaking along with a minor fraction of Ent5p, followed 10 s later by a spatially distinct peak of AP-1 assembly and most of Ent5p. These relationships correspond well with known physical and genetic interactions4.

### Sequential adaptor assembly occurs at the TGN

Sec7p, an ARF GTPase nucleotide exchange factor, is a TGN peripheral membrane protein15–15. Sec7p–mRFP and Gga2p–GFP fluorescence profiles were coincident (Fig. 3a and Table 1, row 7), providing evidence that the main site of Gga2p assembly is the TGN. Sec7p–mRFP peaked 10.4 s before β1–GFP, the same time separation observed between Gga2p and AP-1 (Fig. 3b and Table 1, rows 1 and 8). A total of 95.4% of Sec7p–mRFP puncta became positive for AP-1 (n = 154, 68 cells) and 94.4% of AP-1 puncta arose from Sec7p puncta (n = 108, 53 cells). These data indicate that the process of sequential clathrin adaptor assembly originates at the TGN.

### Clathrin and AP-1 dynamics depend on Gga proteins

Clathrin assembly, monitored with Chc1p–mRFP, peaked at about the same time as Gga2p (Fig. 3d and Table 1, row 9). In static images of cells, 56% of Gga2p co-localized with Chc1p and significant overlap was observed by SIM, as expected for assembly of individual Gga2p-containing clathrin coats (Fig. 2h). In cells expressing Chc1p–mRFP and β1–GFP, the peak of Chc1p preceded the AP-1 peak by 8 s (Table 1, row 10), consistent with the relative timing of Gga2p and AP-1 assembly. However, in contrast to Gga2p, substantial clathrin fluorescence signal persisted through the AP-1 peak (Fig. 3c). AP-1 co-localization with...
Chc1p in static images was lower than that observed for Gga2p; 44\% \quad (n = 75 \text{ cells}) \quad \text{versus} \quad 56\% \quad (n = 99 \text{ cells}), \quad P = 0.002. \quad \text{Similarly, the overlap between AP-1 and clathrin was less apparent by SIM, indicating that at steady state clathrin is preferentially associated with Gga2p-enriched coats (Supplementary Fig. S2b).}

In contrast to wild-type cells, cells lacking Gga proteins exhibited Chc1p peak intensity at virtually the same time as AP-1 (0.79 s; Fig. 3f and Table 1, row 11) and co-localization between AP-1 and clathrin increased from 44\% \quad (n = 75 \text{ cells}) \quad \text{to} \quad 58\% \quad (n = 78 \text{ cells}), \quad P = 0.001.

Similar results were obtained in cells lacking Ent3p and Gga proteins (L.D., unpublished observations). As expected from these findings, SIM analysis of gga1Δ gga2Δ cells revealed greater co-localization between clathrin and AP-1 (Supplementary Fig. S2c). Thus, Gga proteins seem to establish the initial timing and localization of clathrin assembly at the TGN. Consistent with this interpretation, inactivation of AP-1 (β1Δ) did not alter the relative timing of Gga2p and Chc1p assembly, except to eliminate the shoulder of clathrin fluorescence signal that corresponds to the peak of AP-1 in wild-type cells (Table 1, row 12, and Supplementary Fig. S3).

In gga1Δ gga2Δ cells, AP-1 assembly relative to Sec7p–mRFP was delayed by ≈2.5-fold when compared with wild-type cells (Fig. 3b,c and Table 1, rows 13 and 14). Thus, the timing of AP-1 assembly depends on Gga proteins, providing evidence that AP-1 recruitment is coupled to prior assembly of Gga/clathrin coats.

**Arf1p influences adaptor dynamics**

Arf GTPases are associated with Gga- and AP-1-mediated protein transport from the TGN (ref. 1). There are two Golgi-localized Arf proteins in yeast, Arf1p and Arf2p. Both yeast Gga proteins bind to Arf–GTP but this interaction is not absolutely required for Gga protein localization and function\(^7\). In contrast, AP-1 localization is more dependent on Arf–GTP (ref. 7). Adaptor dynamics were investigated in cells carrying a deletion of ARF1, which is expressed at ten times the level of ARF2 (arf1Δ arf2Δ cells are inviable\(^6\)). Although Golgi elements coalesce into a limited number of large structures in arf1Δ cells\(^15\), there was a clear progression from Gga2p to AP-1 (Fig. 4a and Supplementary Movie S5). However, AP-1 peak fluorescence signal was delayed by twofold when compared with wild-type cells (Table 1, rows 15 and 16, and Supplementary Fig. S1f).

**Temporal modulation of PtdIns(4)P levels by Gga and Arf1 proteins**

PtdIns(4)P binds to, and promotes localization of, Gga proteins and AP-1 in mammalian cells\(^16,19\). A similar low-affinity interaction between PtdIns(4)P and Gga protein occurs in yeast\(^20\). To monitor PtdIns(4)P levels, we used GFP fused to the PtdIns(4)P-binding PH domain of Osh1p (GFP–PH\(^{OSH1}\); ref. 21). GFP–PH\(^{OSH1}\) reached maximum intensity 5.3 s after the peak of Gga2p and 4.1 s before the peak of AP-1 (Table 1, rows 17 and 18). Consistent with this sequence, GFP–PH\(^{OSH1}\) also peaked after Sec7p–mRFP (Fig. 4b and Table 1, row 19). In gga1Δ gga2Δ cells, GFP–PH\(^{OSH1}\) exhibited a ≈3-fold delay in maximum recruitment when compared with Sec7p–mRFP (Fig. 4c and Table 1, rows 19 and 20). Similarly, GFP–PH\(^{OSH1}\) recruitment was slowed in arf1Δ cells (Fig. 4d and Table 1, row 21), known to have reduced PtdIns(4)P levels\(^22\). Thus, PtdIns(4)P increases at the TGN after recruitment of Sec7p and Gga2p and seems to peak before the maximum levels of AP-1. As for AP-1, the normal kinetics of PtdIns(4)P accumulation depends on Gga and Arf1 proteins.

**Depletion of PtdIns(4)P inhibits sequential assembly of clathrin adaptors**

PtdIns(4)P levels at the Golgi were lowered using a temperature-sensitive allele of PIK1 (pik1-83\(^\circ\)), encoding the PtdIns(4)-kinase associated with Golgi function in yeast\(^22,23\). At the permissive...
temperature, GFP–PH\textsuperscript{OSH1} exhibited a wild-type localization pattern. After 30 min at 37°C, the reporter was primarily cytoplasmic (Supplementary Fig. S4a–d), indicative of reduced PtdIns(4)P levels. There was no significant effect of Pik1p inactivation on localization of a PtdIns(3)P reporter, GFP–FYVE (Supplementary Fig. S4e–h).

In pik1\textsuperscript{83} cells shifted to 37°C, Gga2p and Ent3p maintained punctate co-localization (Fig. 5a,b). By comparison, a significant fraction of Ent5p was redistributed to the cytoplasm (Fig. 5c,d). Under the same conditions, AP-1 assembled, although little AP-1 was recruited to Gga2p puncta (Fig. 5a,b). By comparison, a significant fraction of Ent5p was redistributed to the cytoplasm (Fig. 5c,d). Under these conditions, we observed significantly higher Gga2p/AP-1 co-localization and a twofold reduction in the number of Ent5p-positive Golgi membranes.

**Pik1p overexpression increases the rate of adaptor progression**

To elevate PtdIns(4)P levels, we used strong constitutive promoters to drive expression of PIK1 and FRQ1, an adaptor necessary for Pik1p localization\textsuperscript{29}. In these cells, GFP–PH\textsuperscript{OSH1} peaked simultaneously with Gga2p, about 5 s earlier than in wild-type cells, providing evidence for increased rates of PtdIns(4)P synthesis at the TGN (Table 1, rows 22 and 23). Under these conditions, we observed significantly higher Gga2p/AP-1 co-localization and a twofold reduction in the peak-to-peak separation (Fig. 6a,d and Table 1, rows 22 and 23, and Supplementary Fig. S1g). Similar results were obtained with Ent3p and Gga2p were not altered (Fig. 6c,d). The diametric effects of elevating or reducing PtdIns(4)P levels identify this phosphoinositide as a critical factor controlling TGN clathrin adaptor progression.

To assess the fidelity of clathrin-mediated transport between the TGN and endosomes in Pik1p/Frq1p-overexpressing cells, maturation

Figure 3 Adaptor and clathrin dynamics at the TGN. (a–f) Panels presenting the indicated proteins as in Fig. 1. Scale bars, 2 μm. The time to acquire one image pair was 1.1–1.3 s. (a) GPY4933. (b) GPY4934. (c) GPY4935. (d) GPY4931. (e) GPY4932. (f) GPY4936.
of the mating pheromone α-factor was evaluated. Phenomere maturation is initiated in the TGN by the Kex2p protease, which relies on clathrin-, AP-1- and Gga-dependent cycling between the TGN and endosomes for localization\(^5,27–29\). Compared with wild-type cells, Pik1p/Frq1p-overexpressing cells exhibited partial α-factor maturation defects (8.6 ± 1.3% (n = 4) precursor forms in Pik1p/Frq1p-overexpressing cells versus 3.2 ± 1.2% (n = 4) in wild-type cells (P < 0.01); Fig. 6e). This result provides evidence that clathrin-mediated TGN localization of Kex2p is perturbed in Pik1p/Frq1p-overexpressing cells.

We also observed a defect in glycosylation of carboxypeptidase Y (CPY). The core oligosaccharides added to CPY in the endoplasmic reticulum (p1 CPY) are extended in the Golgi apparatus (p2 CPY). p2 CPY is proteolytically matured in the vacuole (mCPY). The Golgi α1–3 mannosyltransferase Mnn1p adds the final sugars to generate p2 CPY (ref. 30). Localization of Mnn1p to the TGN, as for Kex2p, depends on clathrin\(^19\). In pulse-chase experiments, p2 and mCPY in Pik1p/Frq1p-overexpressing cells were slightly smaller than in wild-type cells (Fig. 6f,g and Supplementary Fig. S6a,b). The p1 forms in the two strains were identical (Fig. 6f) and endoglycosidase H treatment eliminated the difference between mCPY species (Fig. 6g), indicating that the smaller sizes of p2 and mCPY in Pik1p/Frq1p-overexpressing cells are due to incomplete glycosylation in the Golgi. The defects in α-factor maturation and CPY glycosylation indicate that increased synthesis of PtdIns(4)P and shortened adaptor progression times are associated with compromised clathrin-mediated TGN–endosome traffic, probably because precocious AP-1 assembly sorts Kex2p and Mnn1p away from their substrates in the TGN.

**Gga2p acts in Pik1p recruitment and directly binds Pik1p**

In wild-type cells, Frq1p–GFP peaked at nearly the same time as Sec7p–mRFP whereas GFP–Pik1p reached maximum levels 4.1 s later, similar to the peak of PtdIns(4)P (Fig. 7a and Table 1, rows 19, 28 and 30). In gga1Δ gga2Δ cells, Pik1p recruitment was delayed, similarly to PtdIns(4)P levels, by threefold when compared with Sec7p (12.6 s; Fig. 7b and Table 1, rows 28 and 29). The profile of Frq1p was not altered (Table 1, rows 30 and 31). Pik1p and Frq1p in cell extracts bound to full-length Gga2p (Fig. 7c and Supplementary Fig. S6c,d). Moreover, Pik1p from cell extracts interacted specifically with the Gga2p VHS domain (Fig. 7d and Supplementary Fig. S6e) and the Gga2p VHS domain bound directly to a recombinant Pik1p fragment (Fig. 7e and Supplementary Fig. S6f). Together these results provide evidence that direct physical interactions between Gga proteins and Pik1p contribute to Pik1p recruitment to the TGN.

**DISCUSSION**

Our results demonstrate two sequential waves of clathrin coat assembly that originate at the TGN and are distinguished by adaptor type. Gga2p, Ent3p and a minor population of Ent5p assemble in the first wave. AP-1 and most Ent5p are recruited in the second wave. The relative timing of AP-1-enriched coat assembly is dependent on Gga proteins and Arf1p, and the progression between coat types is controlled by PtdIns(4)P. This coupled progression of adaptorspecific clathrin coat formation reveals a hitherto unrecognized process of TGN maturation.

The relationship between Gga and AP-1 adaptor function has not been clearly defined. Although the two adaptors share a number of interaction partners, including Arf, PtdIns(4)P and clathrin, they recognize different cargo sorting signals and certain accessory proteins, and only partially co-localize in static images\(^14,9\). We observed that the major populations of Gga2p and AP-1 were separated in time and space. These results indicate that most clathrin coats forming at the TGN consist primarily of one or the other type of adaptor, and so would be enriched with the corresponding cargo selectivity. This organization provides a simple mechanism to generate CCVs at the TGN targeted to different compartments based on adaptor-directed incorporation of targeting/fusion proteins. In accord with this view, Gga proteins recruit Ent3p, which in

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**ARTICLES**

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Figure 5 Depletion of PtdIns(4)P alters localization of AP-1 and Ent5p. (a–f) Representative still images from live cells of GGA2–mRFP ENT3–GFP pik1-83ts (GPY4940; a,b), GGA2–mRFP ENT5–GFP pik1-83ts (GPY4941; c,d) and GGA2–mRFP β1–GFP pik1-83ts (GPY4942; e,f) incubated at 24 °C (a,c,e) or shifted for 30 min to 37 °C (b,d,f). (g) The top panel shows still images from live cells of GPY4942 shifted to 37 °C for 30 min. The white arrowhead highlights puncta in the kymograph below. Scale bars, 2 µm. The bottom panel shows a three-channel kymograph of the selected puncta; the time to acquire one image pair was 1.2 s. Every other image pair is shown in the kymograph. (h) Gga2p–mRFP co-localization with β1–GFP (GPY4942) or Ent3–GFP (GPY4940) was quantified in pik1-83ts cells at 24 °C (light grey bars) or after a shift to 37 °C for 30 min (dark grey bars). Error bars, s.e.m.; n, number of events; ***, P < 0.001; NS, not significant (two-tailed t-test).

Our results indicate that PtdIns(4)P is a key regulator of the TGN adaptor assembly sequence. Changes in PtdIns(4)P levels that alter adaptor progression are accompanied by defects associated with adaptor function. For example, in combination with pik1ts alleles that lower PtdIns(4)P levels, deletions of Gga proteins or AP-1 subunits result in synthetic growth and/or partial α-factor maturation defects (L.D. and G.C., unpublished observations). Importantly, increased PtdIns(4)P synthesis due to Pik1p/Frq1p overexpression also leads to incomplete α-factor maturation and CPY glycosylation. These findings support the view that adaptor progression contributes to optimal function of clathrin-mediated traffic from the TGN.

The effects of PtdIns(4)P on adaptor assembly are likely to be, at least in part, direct. Mammalian AP-1 binds PtdIns(4)P and the residues in the mammalian AP-1 γ subunit necessary for phosphoinositide binding are conserved in the yeast protein19. Ent5p contains an amino-terminal domain homologous to phosphoinositide-binding ANTH domains. Binding of Ent5p (and Ent3p) to PtdIns(3)P and PtdIns(3,5)P2 has been reported36–38; however, the specificity of this interaction has been questioned39. Our findings are most consistent with a primary role for PtdIns(4)P in Ent5p localization in vivo.

Similarly to AP-1, Gga2p binds to PtdIns(4)P and Arf1p through low-affinity interactions that cooperate to enhance Gga2p membrane association20. However, Gga2p does not absolutely require Arf interaction for localization10 and our findings indicate that acute
PtdIns(4)P reduction does not markedly alter Gga2p recruitment. Thus, our in vivo analyses reveal differential dependencies of Gga2p and AP-1 on shared interaction partners, providing a basis for the observed temporal separation of adaptor assembly. Adaptor-specific interactions may also contribute to the spatial and temporal contribution of Pik1p-generated PtdIns(4)P and Arf1p to Gga2p localization, constitute a positive feedback pathway to drive PtdIns(4)P accumulation at the TGN (Fig. 7g). Thus, our model posits a regulatory network converging on Pik1p to generate a temporal gradient of PtdIns(4)P that controls adaptor progression.

PtdIns(4)P-coupled progression of Gga2p- to AP-1-enriched coats represents a previously unrecognized maturation process at the TGN (Fig. 7f and Supplementary Fig. S7). Although there are some differences in localization mechanisms of yeast and mammalian...
GGA proteins and AP-1, the strong conservation of binding partners, including ARF and PtdIns(4)P, favours the view that a similar progression occurs in mammalian cells. Supporting this possibility, mammalian GGA proteins often localize with a more compact perinuclear distribution than AP-1, and by immuno-electron microscopy are more prevalent than AP-1 on uncoated TGN membranes, consistent with assembly of GGA proteins at an earlier TGN stage than AP-1 (refs 35,41,42).

A PtdIns(3)P-controlled maturation process occurs in the endocytic pathway at early endosomes\(^1\) that, although mechanistically distinct, is analogous to what we describe for the TGN. Taken together, these findings reveal phosphoinositide-based maturation as a mechanism that allows temporal subspecialization within major organelles in both the secretory and endocytic pathways.

**METHODS**

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology

*Note: Supplementary Information is available on the Nature Cell Biology website*

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**AUTHOR CONTRIBUTIONS**

L.D. and G.S.P. conceived the experiments. L.D. carried out all microscopy, protein interaction and CPY experiments. G.C. carried out the \(\alpha\)-factor experiments, and L.D. and G.S.P. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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1. Traub, L. M. Common principles in clathrin-mediated sorting at the Golgi and the plasma membrane. *Biochim. Biophys. Acta* **1744**, 415–437 (2005).

2. Duncan, M. C. & Payne, G. S. ENTH/ANTH domains expand to the Golgi. *Trends Cell Biol.* **13**, 211–215 (2003).
24. Strahl, T., Hama, H., DeWald, D. B. & Thorner, J. Yeast phosphatidylinositol 4-kinase, Pik1, has essential roles at the Golgi and in the nucleus. J. Cell Biol. 171, 967–979 (2005).
25. Schu, P. V. et al. Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. Science 260, 88–91 (1993).
26. Hendrickx, K. B., Wang, B. G., Schnieders, E. A. & Thorner, J. Yeast homologue of neuronal frequenin is a regulator of phosphatidylinositol-4-kinase. Nat. Cell Biol. 1, 234–241 (1999).
27. Fuller, R. S., Sterne, R. E. & Thorner, J. Enzymes required for yeast prohormone processing. Annu. Rev. Physiol. 50, 345–362 (1988).
28. Payne, G. S. & Schekman, R. Clathrin—a role in the intracellular retention of a Golgi membrane protein. Science 245, 1358–1365 (1989).
29. Phan, H. L. et al. The Saccharomyces cerevisiae APS1 gene encodes a homolog of the small subunit of the mammalian clathrin AP-1 complex: evidence for functional interaction with clathrin at the Golgi complex. EMBO J. 13, 1706–1717 (1994).
30. Dean, N. Asparagine-linked glycosylation in the yeast Golgi. Biochim. Biophys. Acta 1426, 309–322 (1999).
31. Graham, T. R., Seeger, M., Payne, G. S., MacKay, V. L. & Emm, S. D. Clathrin-dependent localization of vps13 mammalian clathrin to the Golgi complex. J. Cell Biol. 127, 667–678 (1994).
32. Black, M. W. & Pelham, H. R. A selective transport route from Golgi to late endosomes that requires the yeast GGA proteins. J. Cell Biol. 151, 587–600 (2000).
33. Chidambaram, S., Zimmermann, J. & von Mollard, G. F. ENTH domain proteins are cargo adaptors for multiple SNARE proteins at the TGN endosome. J. Cell Sci. 121, 329–338 (2008).
34. Wang, J. et al. Epsin N-terminal homology domains bind on opposite sides of two SNAREs. Proc. Natl Acad. Sci. USA 108, 12277–12282 (2011).
35. Doray, B., Ghosh, P., Griffith, J., Geuze, H. J. & Kornfeld, S. Cooperation of GGAs and AP-1 in packaging MPRs at the trans-Golgi network. Science 297, 1700–1703 (2002).
36. Chidambaram, S., Mullers, N., Wiederhold, K., Haucke, V. & von Mollard, G. F. Specific interaction between SNAREs and epsin N-terminal homology (ENTH) domains of epsin-related proteins in trans-Golgi network to endosome transport. J. Biol. Chem. 279, 4175–4179 (2004).
37. Eugster, A. et al. Ent5p is required with Ent3p and Vps27p for ubiquitin-dependent protein sorting into the multivesicular body. Mol. Biol. Cell 15, 3031–3041 (2004).
38. Friant, S. et al. Ent3p is a PtdIns(3,5)P2 effecter required for protein sorting to the multivesicular body. Dev. Cell 5, 499–511 (2003).
39. Narayan, K. & Lemmon, M. A. Determining selectivity of phosphoinositide-binding domains. Methods 39, 122–133 (2006).
40. Singer-Krueger, B. et al. Yeast and human Ysl2p/Mon2 interact with Gga adaptors and mediate their subcellular distribution. EMBO J. 27, 1423–1435 (2008).
41. Hirst, J. et al. A family of proteins with γ-adaptin and WHS domains that facilitate trafficking between the trans-Golgi network and the vacuole/lysosome. J. Cell Biol. 149, 67–80 (2000).
42. Poussu, A., Lohi, O. & Lehto, V. P. Vear, a novel Golgi-associated protein with VHS and γ-adaptin 'ear' domains. J. Biol. Chem. 275, 1716–1723 (2000).
43. Žoric, R. et al. A phosphoinositide switch controls the maturation and signaling properties of APPL endosomes. Cell 136, 1110–1121 (2009).
METHODS

Media and strains. The strains used in this study are listed in Supplementary Table S1 (refs 4,7,22,44,45). Yeast strains were grown in standard rich medium (YPD) or synthetic dextrose medium (SD) with the appropriate supplements. Fluorescent tags and deletions were introduced at endogenous loci using standard PCR-based homologous recombination. All tagged genes were fully functional as assessed by growth of cells harbouring a tagged gene in a genetic background where deletion of the gene causes severe growth defects.

Except where noted here, all strains were generated from diploid cells by mating, sporulation and isolation of haploid spores. Strains expressing GFP–PIK1(550–760) under the control of the PHO5 promoter were generated by integrating pGFP–PHO5 (ref. 26) into the locus of haploid cells11. FVY and GFP-expressing strains were obtained by transformation of haploid cells with pRS316–FVYE–GFP. Strains expressing PIK1p and FLPp were generated by integrating the glucose phosphate dehydrogenase promoter at the 5′ end of both PIK1 and FRQ1 (ref. 46) and then carrying out the appropriate crosses. The VPS34 gene disruption was generated by replacing the first 2190 base pairs of the coding region with TRP1 in diploid cells, which were then sporulated for isolation of haploids. All integrations were confirmed by PCR. All primers used for integrations and deletions are described in Supplementary Table S2.

Plasmids. pFA6a–2×GFP(π)S–HIS3MX6 was generated using primers 5′-gccggccgctcgagctcgaatattgctcagtaaagaggaagttcatgcgc-3′ and 5′-gatccgctCGTACGGCCTCCTCGGGACTCAGATCTATGGCCTCCTCCGAAGTCAAGAGTTCATGCGC-3′ to amplify GFP fragment from pFA6a–2XGFP(π)S–HIS3MX6 (ref. 47), introducing restriction sites for Sall and PacI. The PCR product was then treated with Sall and PacI and introduced into the same sites in front of GFP(π)S on pFA6a–GFP(π)S–HIS3MX6 to create pFA6a–2×GFP(π)S–HIS3MX6. The construction was confirmed by sequencing. pFA6a–2×mRFP–TRP1 was generated using primers 5′-aattcagccgctcgagctcgaatattgctcagtaaagaggaagttcatgcgc-3′ and 5′-agatcgtgaagagagtgcctcgagctcgaatattgctcagtaaagaggaagttcatgcgc-3′ to amplify mRFP fragment from pFA6a–mRFP–TRP1 (ref. 48), introducing restriction sites for SgrAl and Ascl. The PCR product was then treated with SgrAl and Ascl and introduced into the same sites at the end of mRFP on pFA6a–mRFP–TRP1 to create pFA6a–2×mRFP–TRP1. The construct was confirmed by sequencing. To generate pRS316–GFP–FVYE, a BamH1 fragment encompassing GFP–FVYE from pGFP–FVYE–HIS3MX6–URA3 (ref. 49) was inserted into the BamH1 sites of pRS316 (ref. 50). To generate pPK1–GFP(π)S–PIK1, a BamH1 and EcoRI fragment from pFA6a–natNT2 (Euroscarf, accession number p30346) was ligated into pBlueScript KS(+) to generate pB–natNT2. Then, PK1 PCR fragments containing nucleotides −550 to −331, digested with SacI and NotI, and nucleotides −330 to +96, digested with EcoRI, were inserted into the respective sites of pB–natNT2 to generate pB–natNT2–PK1(−550 + 97). The EcoRI fragment was checked by sequencing. Finally, a PCR fragment containing GFP from pGFP–FVYE and nucleotides coding for an 8-amino-acid linker insertion at the 3′ end was cloned into an existing NsiI site at the ATG start site of PIK1 to generate pB–natNT2–GFP(π)S–PIK1. To integrate into the PK1 genomic locus, pB–natNT2–GFP(π)S–PIK1 was cut with SacI and EcoRV before transformation. To generate pGExTt1–Gga2(1–169), pGExTt1–Gga2(170–336), pGExTt1–Gga2(337–385), the relevant regions of Gga2 were amplified from Bacteria were lysed in PBS at pH 7.4 and 30 mM imidazole for Pik1 fragments), with a protease inhibitor mixture (Roche Diagnostics GmbH) by sonication. The resulting lysates were cleared by centrifugation for 20 min at 12,000 × g. Glutathione–Sepharose (Gga2 fragments) or Ni-NTA beads (Pik1 fragment or His6–Gga2) were added to the supernatant and allowed to bind for 1 h at 4 °C. GST fusions bound to glutathione were used directly. The bound Pik1 fragment or His6–Gga2 was washed four times with PBS, then three times with 50 mM HEPES at pH 7.4, 50 mM NaCl and 300 mM imidazole, and then eluted in 50 mM HEPES at pH 7.4, 50 mM NaCl and 300 mM imidazole. His6–Gga2 and BSA were coupled to CNBr-activated Sepharose following the manufacturer’s instructions (GE Healthcare). Proteins were analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting with anti-HA antibody (Covance) at a 1:1,000 dilution.

For affinity binding, yeast cells were grown to mid-logarithmic phase in YPD medium. A total of 15 × 10^9 cells per sample were converted to spheroplasts by resuspension in a final volume of 1 ml of lysis buffer: 100 mM MES–NaOH at pH 6.5, 1.5 mM MgCl2, 2 mM CaCl2, 0.2 mM dithiothreitol, 2 mM NaN3, containing 1% Triton X-100 and a protease inhibitor mixture2. The extract was clarified by centrifugation for 30 min at 16,000g at 4 °C. The supernatant was incubated for 30 min in the presence of Protein A Sepharose (Amersham Pharmacia Biotech) followed by centrifugation for 20 × 16,000g. The resulting supernatant was brought to 1 ml in lysis buffer and BSA-conjugated or His6–Gga2-conjugated Sepharose was added. After incubation for 1.5 h at 4 °C, the resin was washed twice in lysis buffer, then twice in lysis buffer without Triton X-100, and eluted with sample buffer at 100 °C for 3 min (2% SDS, 10% glycerol, 62.4 mM Tris–HCl at pH 6.8, 0.1 mg/ml bromphenol blue and 0.4% β-mercaptoethanol). Proteins were analysed by SDS–PAGE and immunoblotting with anti-GFP (Scantibodies, 1:1,000)
or anti-HA (Covance, 1:1,000) antibody and goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad). Yeast lysates for affinity binding experiments with N-terminal GST fusions of Gga2 were carried out as described above except that yeast cells were lysed in 50 mM HEPES, 50 mM NaCl and 1% Triton X-100. Beads containing GST alone or GST–Gga2 fragments were incubated with the yeast lysate for 2 h at 4 °C. Washing and elution of the beads were carried out as described above.

Direct binding experiments were carried out by incubating purified Pik1(80–760)–His6 with GST– or GST–Gga2p(VHS)-bound glutathione–Sepharose in 50 mM HEPES at pH 7.4, 50 mM NaCl and 300 mM imidazole for 1 h at 4 °C. Washing and elution were carried out as described above. To detect Pik1(80–760)–His6, penta His monoclonal antibody (Qiagen) was used for immunoblot analysis (1:1,000 dilution).

44. Robinson, J. S., Kilonsky, D. J., Banta, L. M. & Emr, S. D. Protein sorting in Saccharomyces cerevisiae: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. Mol. Cell Biol. 8, 4936–4948 (1988).

45. Yeung, B. G., Phan, H. L. & Payne, G. S. Adaptor complex-independent clathrin function in yeast. Mol. Biol. Cell 10, 3643–3659 (1999).

46. Janke, C. et al. A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. Yeast 21, 947–962 (2004).

47. Longtine, M. S. et al. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast 14, 953–961 (1998).

48. Huh, W. K. et al. Global analysis of protein localization in budding yeast. Nature 425, 686–691 (2003).

49. Burd, C. G. & Emr, S. D. Phosphatidylinositol(3)-phosphate signaling mediated by specific binding to RING FYVE domains. Mol. Cell 2, 157–162 (1998).

50. Sikorski, R. S. & Hieter, P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122, 19–27 (1989).

51. Vowels, J. J. & Payne, G. S. A role for the luminal domain in Golgi localization of the Saccharomyces cerevisiae guanosine diphosphatase. Mol. Biol. Cell 9, 1351–1365 (1998).
Figure S1 GFP-Clc1p Chc1p-mRFP colocalize in living yeast cells and the distribution of peak to peak fluorescence times. (a) Colocalization of GFP-Clc1p and Chc1p-mRFP. Upper panel: Merged image of cells expressing GFP-Clc1p Chc1p-mRFP (GPY3252); arrowhead indicates puncta selected for kymograph in the bottom panel. Time to acquire one image pair was 1.2s. Scale bar = 2μm. Bottom panel: three channel kymograph (merged, mRFP and GFP) of the selected puncta. Every other image pair is shown in the kymograph. Graph: normalized level of GFP and mRFP fluorescence intensity in the puncta as a function of time. (b-g) Distribution of peak to peak fluorescence times. Selected histograms of the time between peaks of fluorescent intensity for the indicated adaptors used to calculate average peak-to-peak times in Table 1. Events are binned in 3 second intervals. Strains used are: (b) GPY3109 (c) GPY3954 (d) GPY3912 (e) GPY3900 (f) GPY4937 (g) GPY4943.
**Figure S2** Structured illumination microscopy of clathrin and clathrin adaptors. (a) GPY3974 (b) GPY4932 (c) GPY4946 (d) GPY4963 cells were imaged by structured illumination microscopy. Maximum projection image is shown. Inset in C contains (left to right) the GFP, mRFP and merged maximum image projection for the puncta in the white box. Scale bar = 400 nm.
**Figure S3** Deletion of the β1 subunit of AP-1 does not affect the relative assembly kinetics of Gga2p and Chc1p. **Upper panel:** Merged image of cells expressing Chc1p-mRFP and Gga2p-GFP in a β1Δ strain (GPY4975); arrowhead indicates puncta selected for kymograph in the bottom panel. **Scale bar = 2μm.** **Bottom panel:** three channel kymograph (merged, mRFP and GFP) of the selected puncta; time to acquire one image pair was 1.2s. Every other image pair is shown in the kymograph. **Graph:** normalized level of GFP and mRFP fluorescence intensity in the puncta as a function of time.
**Figure S4** Inactivation of Pik1p decreases PtdIns(4)P but not PtdIns(3)P levels. Still images were acquired from wild-type or pik1-83ts cells grown at 24°C or shifted to 37°C for 30 minutes. Representative still images of the following strains: (a, b) GFP-PH<sup>OSH1</sup> GGA2-mRFP (GPY4948), (c, d) GFP-PH<sup>OSH1</sup> GGA2-mRFP pik1-83<sup>ts</sup> (GPY4947), (e, f) GFP-FYVE GGA2-mRFP (GPY4949), (g, h) GFP-FYVE GGA2-mRFP pik1-83<sup>ts</sup> (GPY4950). Scale bar = 2 μm.
Figure S5 Clathrin adaptor localization in lipid kinase mutants. Wild-type (pRS316-PIK1; GPY4976) or kinase-inactive (pRS316-pik1D918A; GPY4977) Pik1p were expressed from low copy plasmids in pik1-83ts cells. Colocalization between Gga2p-mRFP and β1-GFP at 37°C is shown where (n) = number of cells. Error bars show S.E.M (p<< 0.001). (b, c) Representative still images of (b) GFP-FYVE GGA2-mRFP vps34Δ (GPY4951); (c) GFP-PHOSH1 GGA2-mRFP vps34Δ (GPY 4952); GFP-PHOSH1 GGA2-mRFP (GPY4948). Scale bar = 2 µm. (d, e) Upper panel: merged image of live cells co-expressing the indicated GFP- and mRFP-tagged intracellular clathrin adaptors; arrowhead indicates puncta selected for kymograph in the bottom panel. Scale bar = 1 µm. Bottom panel: three channel kymograph (merged, mRFP and GFP) of the selected puncta; time to acquire one image pair was between 1.1-1.3 s. Every other image pair is shown in the kymograph. Graph: normalized level of GFP and mRFP fluorescence intensity in the puncta as a function of time. (d) ENT3-GFP ENT5-mRFP vps34Δ (GPY4953) and (e) β1-GFP GGA2-mRFP vps34Δ (GPY4954).
**Figure S6** Complete autoradiographs and immunoblots used in Figures 6 and 7. Regions of interest are boxed.
Sequential adaptor-enriched clathrin-coated vesicle formation at the TGN. Initially, low PtdIns(4)P concentrations favor recruitment of Gga proteins and assembly of Gga-enriched ccv without significant AP-1 recruitment. This stage could manifest as a red (Gga2p-mRFP) focus in live cell images and as individual, closely spaced red puncta in SIM images. As PtdIns(4)P levels increase Gga ccv mature and concomitantly AP-1 is recruited, initiating formation of distinct AP-1-enriched ccv. This intermediate stage would manifest as a yellow focus (Gga2p-mRFP and β1-GFP overlapping) in live cell images and adjacent, spatially distinct red and green puncta by SIM. The spatial separation of Gga and AP-1 signals in SIM images reflects formation of individual ccv that are enriched in one or the other adaptor. Finally, the temporal separation of Gga and AP-1 recruitment would lead to a last stage in which Gga ccv have budded and no additional Gga recruitment occurs (likely due in part to the depletion of Gga-dependent cargo collected into the Gga-enriched ccv). As a consequence, only the maturing AP-1-enriched ccv would remain, manifesting as a green focus in live cell images and closely spaced green puncta by SIM.
Supplementary Movie Legends

**Movie S1** Clathrin dynamics. Time-lapse movie of Chc1p-mRFP in live cells (GPY3100-20D). Interval between frames is 1.5 s. Playback rate is 7 frames per second.

**Movie S2** Transition from Gga2-mRFP to β1-GFP (AP-1). Merged time-lapse movie depicts the kinetics of Gga2-mRFP (red) and β1-GFP (green) fluorescence at selected puncta (arrows) in GPY3109 cells. Interval between frames is 1.3 s. Playback rate is 7 frames per second.

**Movie S3** Ent3p-GFP and Gga2p-mRFP assemble together. Merged time-lapse movie depicts the kinetics of Gga2p-mRFP (red) and Ent3p-GFP (green) fluorescence at a selected puncta (arrow) in GPY3954 cells. Interval between frames is 1.2 s. Playback rate is 7 frames per second.

**Movie S4** Transition from Gga2p-mRFP to Ent5-GFP. The merged time-lapse image depicts the kinetics of Gga2p-mRFP (red) and Ent5p-GFP (green) fluorescence at selected puncta (arrows) in GPY3962 cells. Interval between frames is 1.2 s. Playback rate is 7 frames per second.

**Movie S5** Transition of Gga2p-mRFP to β1-GFP at the TGN in arf1Δ cells. The merged time-lapse image depicts the kinetics of Gga2p-mRFP (red) and β1-GFP (green) fluorescence at selected TGN puncta (arrow) in arf1Δ cells (GPY4937). Interval between frames is 1.2 s. Playback rate is 7 frames per second.

**Movie S6** Uncoupling of Gga2p-mRFP and β1-GFP localization in pik1-83ts cells. The merged time-lapse image depicts the kinetics of Gga2p-mRFP (red) and β1-GFP (green) fluorescence at selected puncta (arrows) in pik1-ts83 cells (GPY4942) shifted for 30 min. at 37°C. Interval between frames is 1.2 s. Playback rate is 7 frames per second.

**Movie S7** Transition of Ent3p-GFP to Ent5p-mRFP in vps34Δ cells. The merged time-lapse image depicts the kinetics of Ent3p-GFP (green) and Ent5p-mRFP (red) fluorescence at selected puncta (arrows) in vps34Δ cells (GPY4953). Interval between frames is 1.2 s. Playback rate is 7 frames per second.
Supplementary Table Legends.

**Table S1** Strains used in this study. All strains were derived from SEY6210 or GPY404 as indicated in the table. Only differences from the parental genotypes are indicated.

**Table S2** Primers used in this study.

Supplementary References

4. Costaguta, G., Duncan, M.C., Fernandez, G.E., Huang, G.H. & Payne, G.S. Distinct roles for TGN/endosome epsin-like adaptors Ent3p and Ent5p. Mol Biol Cell 17, 3907-3920 (2006).
7. Fernandez, G.E. & Payne, G.S. Laa1p, a conserved AP-1 accessory protein important for AP-1 localization in yeast. Mol Biol Cell 17, 3304-3317 (2006).
22. Audhya, A., Foti, M. & Emr, S.D. Distinct roles for the yeast phosphatidylinositol 4-kinases, Stt4p and Pik1p, in secretion, cell growth, and organelle membrane dynamics. Mol Biol Cell 11, 2673-2689 (2000).
44. Robinson, J.S., Klionsky, D.J., Banta, L.M. & Emr, S.D. Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases. Mol Cell Biol 8, 4936-4948 (1988).
45. Yeung, B.G., Phan, H.L. & Payne, G.S. Adaptor complex-independent clathrin function in yeast. Mol Biol Cell 10, 3643-3659. (1999).