# Coatomer-bound Cdc42 regulates dynein recruitment to COPI vesicles

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Cytoskeletal dynamics at the Golgi apparatus are regulated in part through a binding interaction between the Golgi-vesicle coat protein, coatomer, and the regulatory GTP-binding protein Cdc42 (Wu, W.J., J.W. Erickson, R. Lin, and R.A. Cerione. 2000. *Nature.* 405:800–804; Fucini, R.V., J.L. Chen, C. Sharma, M.M. Kessels, and M. Stamnes. 2002. *Mol. Biol. Cell.* 13:621–631). The precise role of this complex has not been determined. We have analyzed the protein composition of Golgi-derived coat protomer I (COPI)–coated vesicles after activating or inhibiting signaling through coatomer-bound Cdc42. We show that Cdc42 has profound effects on the recruitment of dynein to COPI vesicles. Cdc42, when bound to coatomer, inhibits dynein binding to COPI vesicles whereas preventing the coatomer–Cdc42 interaction stimulates dynein binding. Dynein recruitment was found to involve actin dynamics and dynactin. Reclustering of nocodazole-dispersed Golgi stacks and microtubule/dynein-dependent ER-to-Golgi transport are both sensitive to disrupting Cdc42 mediated signaling. By contrast, dynein-independent transport to the Golgi complex is insensitive to mutant Cdc42. We propose a model for how proper temporal regulation of motor-based vesicle translocation could be coupled to the completion of vesicle formation.

## Introduction

The secretory and endocytic pathways rely on molecular motors and the cytoskeleton for the directed motility of transport intermediates (Allan et al., 2002; Engqvist-Goldstein and Drubin, 2003). The efficient use of actively motile vesicles must involve precise temporal and spatial regulation. For example, premature action of motor proteins could lead to the translocation of incompletely assembled transport vesicles. Delayed action of motors could lead to the accumulation of nascent vesicles at their sites of assembly. Cytoskeletal proteins interact with vesicle coat proteins or cargo proteins (Stamnes, 2002). Characterization of these interactions may help explain how motor proteins and cytoskeletal dynamics are connected to vesicular transport.

The Golgi complex is typically a compact structure near the microtubule organizing center (MTOC). Because of its limited distribution, trafficking to and from the Golgi may be especially dependent on the cytoskeleton. All three classes of motor proteins, dynein, kinesin and myosin, have been implicated in Golgi transport or positioning (Allan et al., 2002). Dynein is especially important for transport to the Golgi apparatus and for Golgi positioning at the MTOC (Thyberg and Moskalewski, 1999). Dynein and its adaptor, dynactin, have been found to associate with Golgi- and ER-derived vesicles (Fath et al., 1997; Watson et al., 2005).

Actin dynamics are also important for Golgi transport and morphology (Fucini et al., 2002; Luna et al., 2002; Duran et al., 2003; Carreno et al., 2004; Chen et al., 2004; Matas et al., 2004). The GTP-binding protein, Cdc42, regulates actin dynamics and is linked to several transport steps (Cerione, 2004). Cdc42 function at the Golgi is mediated through a binding interaction with the coat protomer I (COPI)–vesicle coat protein, coatomer (Wu et al., 2000; Fucini et al., 2002; Chen et al., 2004). A COOH-terminal dilysine motif on the putative cargo receptor, p23, competes with Cdc42 for a binding site on the γ-COP subunit of coatomer. This suggests a link between COPI vesicle assembly, cargo packaging, and the regulation of actin dynamics.

ADP-ribosylation factor (ARF) stimulates Arp2/3-dependent actin polymerization on Golgi membranes by recruiting coatomer and Cdc42 (Fucini et al., 2002; Chen et al., 2004). Cdc42 signaling leads to the assembly of a specific pool of actin that can be defined by its toxin sensitivity and by its specific

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Abbreviations used in this paper: ARF, ADP-ribosylation factor; COPI, coat protomer I; endoH, endoglycosidase H; IC, intermediate chain; MTOC, microtubule organizing center; VTC, vesiculotubular cluster.

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association with the actin-binding protein mAbp1 (Fucini et al., 2002). We report the unexpected finding that Cdc42 regulates the recruitment of the microtubule-dependent motor protein, dynein, to coatomer-coated membranes.

## Results and discussion

### Dynein is a Cdc42-sensitive component of Golgi vesicles

We analyzed the consequences of blocking Cdc42 recruitment to budding COPI vesicles using a peptide corresponding to the coatomer-binding motif of p23 (Wu et al., 2000; Fucini et al., 2002; Chen et al., 2004). Activating ARF with GTPγS in a cell-free Golgi-vesicle budding assay leads to COPI vesicle formation as indicated by the presence of coatomer in a vesicle-enriched fraction (Fig. 1, A and B). GTPγS also activates Cdc42 causing it to appear in the vesicle fraction (Fig. 1 B). As expected, the p23 peptide prevented Cdc42 from binding coatomer and appearing with the vesicles (Fig. 1 B). The levels of a high molecular weight protein was greatly increased in the presence of the peptide (Fig. 1 A). Subsequent analysis of tryptic fragments by mass spectrometry led to the identification of this protein as the heavy chain subunit of dynein.

Dynein is a multimeric microtubule-based motor protein composed of at least two heavy chains bound to intermediate chain (IC) and light chain subunits. Western blots of the Golgi-vesicle fractions confirmed that the dynein IC levels are increased when the coatomer–Cdc42 complex is disrupted by the p23 peptide (Fig. 1 B). A control peptide had no effect on dynein levels (unpublished data). Because both dynein heavy chain (Fig. 1 A) and IC (Fig. 1 B) behave similarly, it is likely that the entire dynein complex is present in this vesicle fraction.

Because the p23 peptide might have multiple effects, we used an independent approach to test the consequences of activating vesicle formation in the absence of coatomer-bound Cdc42. Constitutively active recombinant ARF1(Q71L) promoted coatomer assembly on the Golgi membranes, but did not lead to Cdc42 activation or recruitment (Fig. 1 C). Importantly, ARF1(Q71L) also stimulated the recruitment of dynein. Thus, two separate methods for stimulating coatomer assembly without Cdc42 led to increased dynein levels. The results indicate that coatomer-bound Cdc42 negatively regulates dynein binding to the vesicles.

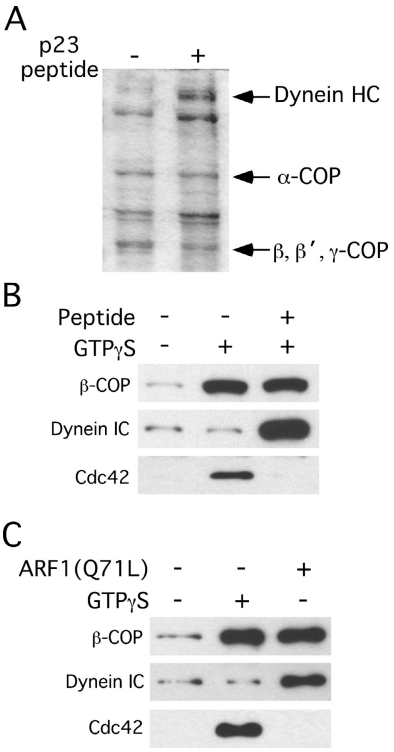


Figure 1. **Dynein is present on Golgi vesicles assembled without Cdc42.** (A) Shown is a Coomassie blue–stained gel of Golgi vesicle-enriched fractions obtained from budding incubations performed in the presence of 20 μM GTPγS with or without 250 μM p23 peptide as indicated. The identities of the coatomer subunits, α-COP, β-COP, β′-COP, and γ-COP were confirmed by Western blotting (not depicted). (B) Immunoblots of the vesicle-enriched fractions were probed with the indicated antibodies. (C) Shown is a blot of Golgi-binding assays performed in the presence of 20 μM GTPγS or 25 μg/ml of recombinant ARF1(Q71L) and probed as indicated.

### Recombinant Cdc42 blocks dynein recruitment

Multiple GTP-binding proteins are candidates to function in the secretory pathway or in motor protein recruitment (Hammer and Wu, 2002; Symons and Rusk, 2003). To test whether Cdc42 is sufficient to mediate the effects of GTPγS on dynein recruitment, we examined the effects of adding recombinant Cdc42 to the vesicle budding reaction in addition to ARF1(Q71L) (Fig. 2, A and B). Addition of the constitutively active Cdc42(Q61L) greatly decreased the amount of dynein in the vesicle fraction. Cdc42(Q61L) and GTPγS were found to reduce dynein levels to the same extent (Fig. 2 A) indicating that Cdc42 is the predominant GTP-binding protein that negatively regulates dynein recruitment in the budding reaction.

We tested whether the binding interaction between coatomer and Cdc42 is important by using a mutant form of Cdc42(Q61Lss) that is constitutively GTP-bound, activates multiple Cdc42 effectors, but fails to bind coatomer (Wu et al., 2000). Cdc42(Q61Lss) had no effect on dynein levels (Fig. 2 B), confirming a requirement for the binding interaction with coatomer. Cdc42(Q61L) is a GTPase-deficient mutant, whereas a second active form of Cdc42, (F28L), can complete the GTP binding/hydrolysis cycle. Cdc42(F28L) stimulates ER to Golgi transport whereas Cdc42(Q61L) blocks it (Wu et al., 2000). Thus, we anticipated that these two active forms of Cdc42 might have opposing effects on dynein recruitment to membranes. Indeed, Cdc42(F28L) did not affect dynein levels on the vesicles (Fig. 2 B).

We determined whether interfering with Cdc42 signaling had any effect on dynein distribution in whole cells. Cytoplasmic dynein is normally found in dispersed punctate structures that are concentrated in the perinuclear region (Fig. 2 C). In cells expressing Cdc42(Q61L), the perinuclear dynein becomes more diffuse or reduced and there is an increase in cell-surface dynein (Fig. 2 C). The ability of recombinant Cdc42(Q61L) to inhibit dynein binding and alter dynein distribution in cells

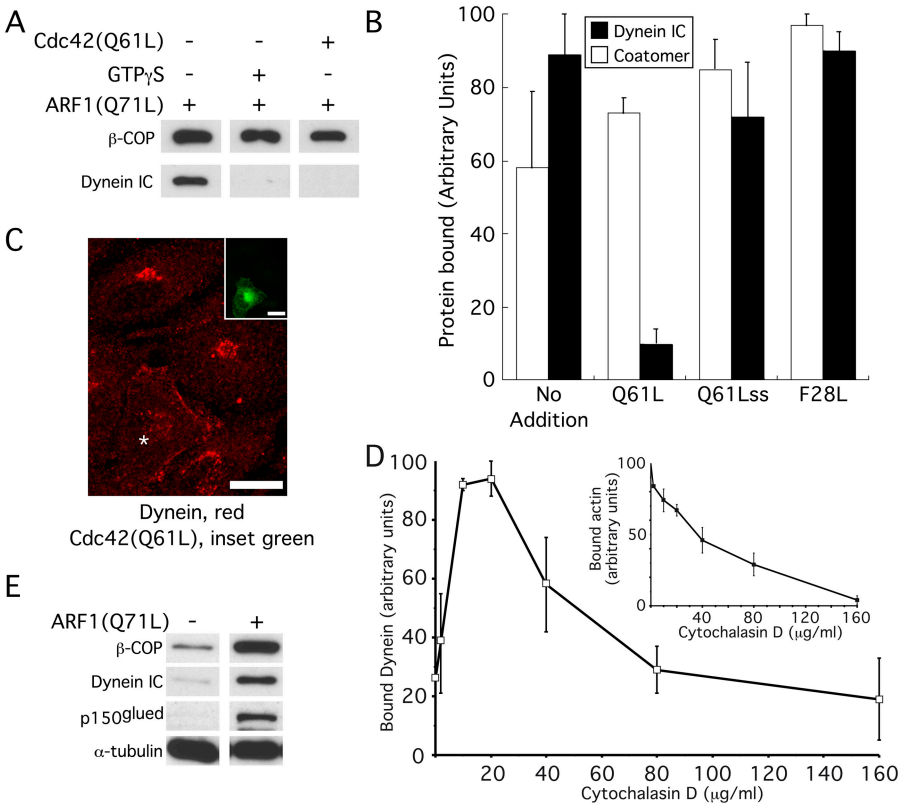


Figure 2. **Cdc42 and actin affect dynein localization.** (A) A Western blot of COPI-vesicle–enriched fractions isolated by flotation from Golgi-budding reactions was probed with the indicated antibodies. Incubations were performed in the presence of 25 μg/ml ARF1(Q71L), 20 μg/ml Cdc42(Q61L), and GTPγS as indicated. (B) Plotted are the average levels of dynein and coatomer found in the COPI-vesicle enriched fraction isolated as in A. 20 μg/ml of recombinant mutant Cdc42 proteins were added as indicated. The error bars represent the SEM ($n$ = 3). (C) NRK cells that had been transfected (asterisk) with GFP-Cdc42(Q61L) (inset image) were labeled with an antibody against the dynein light chain (red). Bars, 20 μm. (D) Golgi-binding assays were used to determine the levels of bound dynein and actin (inset, graph) at various concentrations of cytochalasin D. The error bars represent the SEM ($n$ = 3). (E) A Golgi-binding assay were performed adding ARF1(Q71L) when indicated and probed with the indicated antibodies.

supports the model that the coatomer–Cdc42 complex regulates dynein binding to membranes.

### Dynein recruitment is sensitive to actin dynamics

Cdc42 could affect dynein recruitment by regulating Arp2/3-dependent actin polymerization or alternatively through a parallel effector that is independent of actin dynamics. We used the actin toxin, cytochalasin D, to test whether disrupting actin dynamics affected dynein binding to the membrane. We found that cytochalasin D stimulated dynein binding but only at concentrations around 10–20 μg/ml (Fig. 2 D). In this range, overall levels of bound actin were only moderately affected (Fig. 2 D, inset). The sensitivity of dynein recruitment to low levels of actin toxins suggests that Cdc42 regulates dynein through a specific toxin-sensitive actin-based structure.

Because a binding interaction between spectrin and the dynactin complex has been implicated in dynein association with membranes (De Matteis and Morrow, 2000; Holleran et al., 2001), we hypothesized that high levels of cytochalasin D caused a more general disruption of actin and spectrin on the Golgi membrane that precluded dynein binding (Fig. 2 D). Dynactin has also been shown to associate with ER-derived COPII-coated vesicles (Watson et al., 2005). Thus, we investigated whether dynactin is involved in dynein binding to the Golgi membranes. The levels of the dynactin subunit, $p150^{glued}$, also increased when ARF(Q71L) is added to the Golgi-binding assay (Fig. 2 E). Tubulin levels on the Golgi are not affected by ARF1(Q71L) suggesting that dynein is not recruited through changes in microtubule dynamics (Fig. 2 E). Together, our results indicate that Cdc42-mediated changes in actin dynamics regulate dynactin-based recruitment of dynein motors to the membrane.

### Dynein associates with COPI vesicles in vitro and in whole cells

Although dynein associates with budding vesicles on the Golgi (Fath et al., 1997), it has not been implicated directly in COPI-vesicle–mediated trafficking. Hence, we tested whether COPI vesicles cofractionate with dynein upon flotation through an isopycnic sucrose gradient (Fig. 3 A). COPI vesicles fractionate with a buoyant density equivalent to ~42% sucrose (fractions 5–8). Addition of either GTPγS or ARF1(Q71L) activates COPI vesicle formation as indicated by the presence of coatomer in the center of the gradient (Fig. 3 A and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200501157/DC1). Dynein cofractionates with COPI vesicles when vesicle formation is activated by ARF1(Q71L). However, when ARF and Cdc42 are activated simultaneously with GTPγS, dynein remains in the bottom load fractions.

As an additional test that dynein is associated with COPI vesicles, as opposed to another vesicle type with a similar density, we performed immuno-isolation experiments with either anti-dynein (Fig. 3 B) or anti-coatomer (ζ-COP) antibodies (Fig. 3 C). Coprecipitation was observed when ARF1(Q71L) was used to activate vesicle budding but not with GTPγS. Sedimentation of vesicles at high speed (Fig. 3 B) or precipitation of β-COP (Fig. 3 C) confirmed that vesicle recovery was similar whether ARF1(Q71L) or GTPγS was included. The results provide strong evidence that COPI vesicles associate with dy-

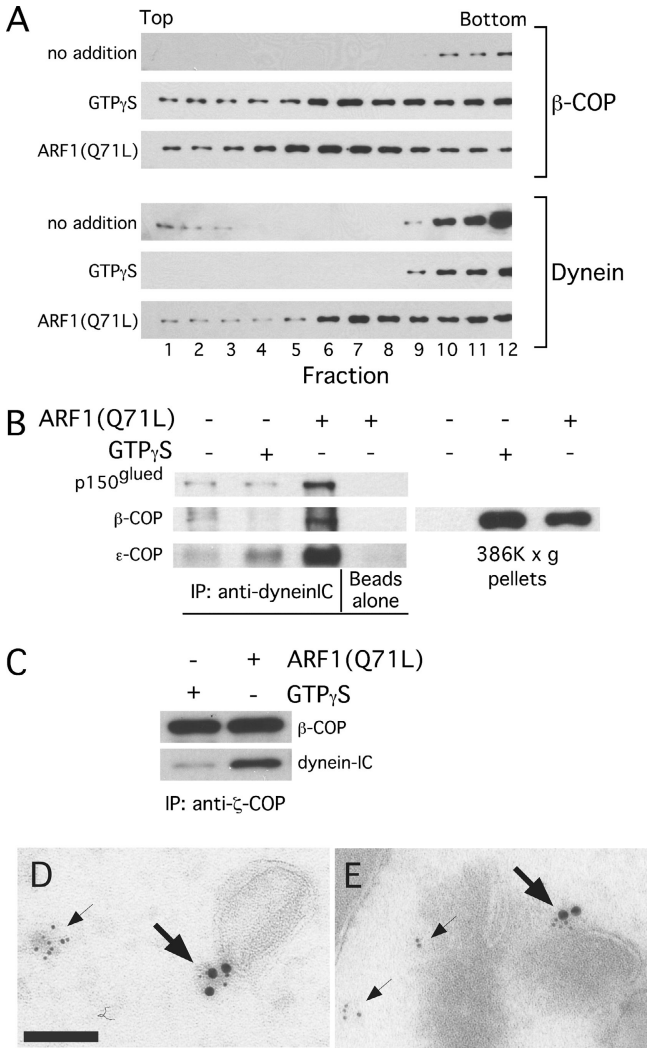


Figure 3. **Dynein is recruited to COPI vesicles.** (A) Vesicle extracts from budding reactions performed with GTPγS or ARF1(Q71L) were fractionated on a sucrose gradient. Shown are immunoblots of the fractions probed as indicated. (B) Shown is a blot of proteins precipitated with the anti-dynein IC antibody from a vesicle extract. Coatomer levels were determined using anti–ε-COP and anti–β-COP. Dynein levels were inferred using anti-p150$^{glued}$. The blot on the left indicates the total amount of COPI vesicles in the extract isolated by sedimentation. (C) Vesicles were precipitated with the anti–ζ-COP antibody as in B. The amounts of coatomer and dynein were determined by probing immunoblots with the appropriate antibodies. (D and E) Cryosections were taken from Vero cells and decorated with anti–ε-COP, large gold particles, and anti-dynein IC, small gold particles. The large arrows indicate structures labeled with both antibodies and the small arrows indicate structures labeled only with anti-dynein. Bar, 300 nm.

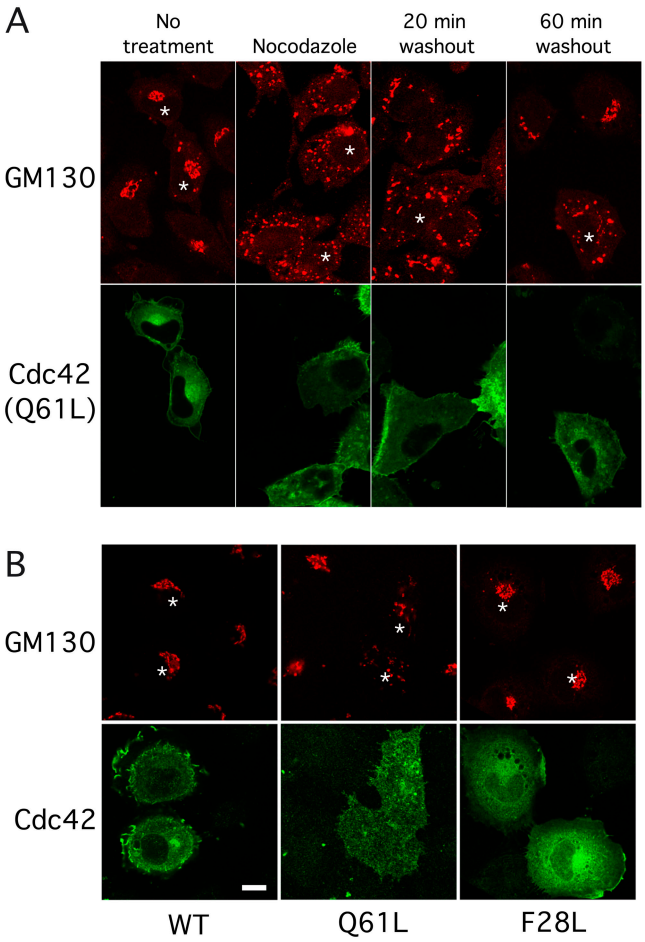


Figure 4. **Reclustering of Golgi membranes is sensitive to Cdc42 function.** (A) NRK cells were transfected with a plasmid for the expression of myc-Cdc42(Q61L). The cells were treated with nocodazole and washed for the indicated times. The Golgi were labeled using an anti-GM130 antibody (red). Transfected cells (asterisks) were identified using an anti-myc antibody (green). (B) Before nocodazole treatment, NRK cells were transfected (asterisks) with HA–wild-type Cdc42 (WT), myc-Cdc42(Q61L), or HA-Cdc42(F28L) as indicated (green). The cells were allowed to recover for 60 min after the nocodazole washout as in A. The Golgi apparatus was labeled with anti-GM130 (red). Bar, 10 μm.

nein predominantly when vesicle formation is activated by ARF1(Q71L).

To determine whether COPI vesicles associate with dynein in cells, we characterized the localization of coatomer and dynein within Vero cells using immunoelectron microscopy. Fig. 3 (D and E) shows examples of vesicle profiles that are positively labeled for both coatomer and dynein. 61% (48/79) of small 50–150-nm circular or pleiomorphic profiles that were decorated with two or more large gold particles (coatomer) also contained at least two small gold particles (dynein). Many structures were observed that were decorated only with the anti-dynein antibody (Fig. 3, D and E), which is consistent with dynein's role in the motility of a variety of structures. The results provide strong evidence that COPI vesicles interact with dynein both in vitro and in whole cells.

## Golgi clustering is disrupted by mutant Cdc42

Microtubules play an important role in Golgi localization within cells (Thyberg and Moskalewski, 1999). Depolymerizing microtubules with nocodazole leads to a dramatic dispersal of Golgi membranes. When nocodazole is removed, microtubules repolymerize and the dispersed Golgi recluster at the juxtanuclear MTOC via dynein-based translocation (Ho et al., 1989; Corthesy-Theulaz et al., 1992; Hafezparast et al., 2003). Because this processes involves dynein-based motility of coatomer-coated structures, we tested whether mutant Cdc42 affected it (Fig. 4).

We confirmed that Golgi membranes dispersed in NRK cells after a 2-h incubation with nocodazole (Fig. 4 A). In nontransfected cells, normal Golgi morphology was largely restored within 1 h after washing out the nocodazole (Fig. 4, A and B). In the presence of Cdc42(Q61L), the dispersed Golgi membranes coalesced into larger punctate structures, but they were not translocated back toward the nucleus (Fig. 4, A and B). Neither wild-type Cdc42 nor Cdc42(F28L) expression had any detectable effect on the ability of Golgi stacks to recluster in a juxtanuclear region (Fig. 4 B). When scored blindly, 79% (49/62) of Cdc42(Q61L)-transfected cells had dispersed Golgi membranes after the 1-h washout. By contrast, only 14% (9/66) of wild-type Cdc42-transfected cells, 16% (10/61) of the Cdc42(F28L)-transfected cells, and 9% (8/88) of nontransfected cells displayed dispersed Golgi. This is consistent with the results presented above indicating the Cdc42(Q61L) specifically disrupts dynein recruitment.

### Cdc42 affects ER-Golgi transport only in cells using dynein-mediated motility

Molecular motor proteins and actin dynamics may play multiple roles in trafficking between the Golgi and the ER (Allan et al., 2002; Luna et al., 2002; Matanis et al., 2002; Short et al., 2002; Stamnes, 2002; Duran et al., 2003). The translocation of vesiculotubular clusters (VTCs) from ER exit sites to the juxtanuclear Golgi complex is a trafficking step where coatomer (Aridor et al., 1995; Scales et al., 1997; Stephens et al., 2000; Presley et al., 2002), Cdc42 (Wu et al., 2000; Fucini et al., 2002), and dynein (Burkhardt et al., 1997; Presley et al., 1997) have each been implicated. As with Golgi positioning (Fig. 4), we anticipated that Cdc42 might regulate the dynein-based motility of coatomer-coated VTCs. Cdc42(Q61L) expression led to an increase in non-Golgi processed (endoglycosidase H [endoH]-sensitive) VSVG(ts045) after release from the ER (Fig. 5 A and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200501157/DC1) confirming the previous studies (Wu et al., 2000; Fucini et al., 2002). In Cdc42(Q61L)-transfected cells, VSVG appeared to be adjacent to SEC23-positive ER exit sites (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200501157/DC1). The results indicate that Cdc42(Q61L) expression leads to immobile VTCs.

Although translocation of VTCs to the Golgi normally relies on dynein and microtubules, cells that have adapted to nocodazole resume ER to Golgi transport in a microtubule-independent manner (Thyberg and Moskalewski, 1999). In this case, the Golgi membranes are found dispersed throughout the cell near ER-exit sites (Fig. S3 B). We found that endoH-sensitive VSVG was not observed in nocodazole-adapted cells expressing Cdc42(Q61L) indicating that ER-to-Golgi transport was no longer compromised (Fig. 5 A and Fig. S2). VSVG colocalized with the dispersed Golgi compartments in the Cdc42(Q61L)-transfected nocodazole-adapted cells (Fig. 5 B). VSVG and Golgi markers did not colocalize in Cdc42(Q61L) expressing cells without nocodazole. When another inhibitor, BAPTA-AM, is used to block ER-to-Golgi transport (Ahluwalia et al., 2001; Chen et al., 2002), VSVG is not transported to the Golgi even after adaptation to nocodazole (Fig. 5 C). These

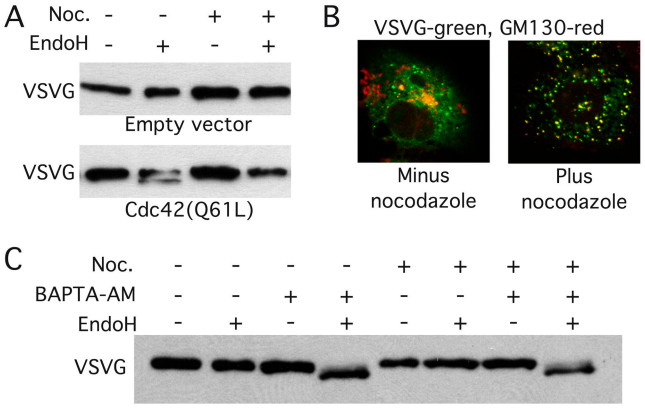


Figure 5. **Translocation of VTCs is sensitive to Cdc42 function.** (A) Vero cells were cotransfected with vectors expressing GFP-VSVG(ts045) and myc-tagged Cdc42(Q61L). VSVG was accumulated in the ER. Where indicated, 20 μM nocodazole was added for 6 h before incubating at 32°C for 15 min. The cells were lysed and digested with endoglycosidase H (EndoH) where indicated. VSVG levels were determined by Western blotting. (B) Cells expressing Cdc42(Q61L) and GFP-VSVG(ts045) were treated as in A then decorated with antiGM130. (C) Cells expressing GFP-VSVG(ts045) were treated with nocodazole (for 6 h) and either BAPTA-AM or DMSO (for 1 h) before the shift to 32°C for 15 min. The amount of endoH-sensitive VSVG was determined by Western blotting.

results suggest that Cdc42 signaling in the early secretory pathway is especially important during dynein-mediated translocation of the VTCs.

### The regulation of dynein-based vesicle motility

Our data suggest a model through which temporal regulation of dynein motors could be connected to transport vesicle coat assembly and cargo packaging. Early in vesicle formation, when cargo proteins are not yet concentrated, coatomer would be associated with Cdc42 thereby stimulating actin assembly but inhibiting dynein binding. As coatomer binds cargo proteins such as p23 and vesicle formation is completed, Cdc42 would dissociate from coatomer, halting actin polymerization and allowing dynein recruitment and motility. Given the emerging connections between vesicle formation and cytoskeletal function (Stamnes, 2002), it seems likely that related regulatory processes may be used by various trafficking steps within the cell.

## Materials and methods

#### Materials

Rat liver Golgi membranes and bovine-brain cytosol were isolated as described previously (Malhotra et al., 1989). Recombinant myristoylated ARF1(Q71L) was obtained by expression in *E. coli* (Ahluwalia et al., 2001). Nocodazole and anti–α-tubulin were obtained from Sigma-Aldrich. The following antibodies were used: anti-dynein IC 74.1 (Covance), anti-dynein LC E16 (Santa Cruz Biotechnology, Inc.), anti-p150$^{Glued}$ (BD Biosciences), and anti-GM130 (BD Biosciences). All other antibodies and materials were obtained as described previously (Fucini et al., 2000, 2002).

#### Golgi membrane binding and budding reactions

Golgi membrane binding and budding reactions were performed as described previously (Fucini et al., 2000, 2002; Chen et al., 2004). For the binding assays, the Golgi membranes were reisolated from the reaction by flotation (Fucini et al., 2000). In Fig. 1 (A and B), vesicles were enriched from the high salt supernatant by sedimentation through a sucrose cushion (Fucini et al., 2000) in order to facilitate large scale reactions needed for

identification. In all other cases, vesicles were purified by fractionating the high salt supernatant on a 5-ml 30–50% sucrose isopycnic gradient (Malhotra et al., 1989; Serafini et al., 1991; Ostermann et al., 1993). Fractions were recovered from the top and analyzed by Western blotting. Where indicated Western blot signals were quantified by densitometry.

**Immuno-isolation of COPI vesicles**

Budding incubations were performed as described above. The high salt supernatant containing vesicles was diluted twofold in 25 mM Hepes, pH 7.4, 2.5 mM magnesium acetate. Anti-dynein IC 74.1 or anti–ζ-COP antibody was used to saturate protein G agarose (Invitrogen). 20 μl of beads were added to 200 μl of supernatant and incubated for 1 h at 4°C with mixing. After washing three times in 25 mM Hepes, pH 7.4, 0.5 mM EDTA, 50 mM KCl, the beads were collected and the bound proteins were analyzed by Western blot.

**Immunoelectron microscopy**

Vero cells were fixed with 3% PFA plus 0.1% glutaraldehyde in 0.1 M PBS, pH 7.4. The cells were washed, pelleted in 9% gelatin in PBS, and infiltrated with 2.3 M sucrose in PBS. The pellet was frozen in liquid nitrogen and sectioned with a cryomicrotome. Thin sections, collected on grids, were blocked in 5% goat serum and incubated at RT for 60 min with a mixture of the primary antibodies (anti-dynein and anti–ε-COP). The grids were washed in PBS and immunolabeled with anti–mouse and anti–rabbit IgG coupled to gold (Electron Microscopy Sciences). Grids were washed, embedded in 0.3% uranyl acetate in 2% methyl cellulose, and examined using a microscope (model H-7000; Hitachi).

**Transfection and Golgi clustering assay**

NRK cells were transfected using lipofectamine (Invitrogen) with plasmids expressing wild-type Cdc42, Cdc42(Q61L), or Cdc42(F28L). For the Golgi clustering assay, NRK cells were exposed to nocodazole (20 μM) at 37°C for at least 2 h to scatter the Golgi. The cells were then washed with α-MEM media and incubated without nocodazole for the indicated times. Immunofluorescence was performed as described previously (Fucini et al., 2002). Images were acquired using a confocal microscope (model LSM-510; Carl Zeiss MicroImaging, Inc.) and a 63× objective (Carl Zeiss MicroImaging, Inc.) with an NA of 1.40.

**GFP-VSVG(ts045) transport assay**

Vero cells were cotransfected using lipofectamine (Invitrogen) with GFP-ts045-VSVG (Presley et al., 1997) and the Cdc42(Q61L) plasmid. Transfected cells were incubated for 14–16 h at the restrictive temperature (39.5°C) to accumulate VSVG protein in the ER. VSVG protein was released from the temperature block by switching the cells to 32°C media containing 10 μg/ml cycloheximide. The cells were incubated for 15 min at 32°C and lysed for Western blot analysis or processed for immunofluorescence. Nocodazole or BAPTA-AM was added at the indicated times by replacing the medium with medium containing 20 μM nocodazole or 50 μM BAPTA-AM plus 10 μg/ml cycloheximide. The treatment of VSVG protein with endoH (Calbiochem) was done according to the manufacturer's instructions.

**Mass spectrometry**

A vesicle-enriched pellet was obtained as described above from budding reactions performed in the presence of cytochalasin D. The gel-resolved high molecular weight band was digested with trypsin and the mixture was fractionated on a Poros 50 R2 RP micro-tip (Erdjument-Bromage et al., 1998). Resulting peptide pools were then analyzed by matrix-assisted laser desorption/ionization reflectron time-of-flight mass spectrometry using a Reflex III instrument obtained from Bruker Daltonics. Selected mass values were taken to search the protein nonredundant database (National Center for Biotechnology Information, Bethesda, MD) using the PeptideSearch (Mann et al., 1993) algorithm.

**Online supplemental material**

Fig. S1 shows the quantification of coatomer and dynein levels in vesicle fractions isolated by flotation as in Fig. 3 A. Fig. S2 shows the time course of ER-to-Golgi VSVG transport in the presence of nocodazole and/or Cdc42(Q61L). Fig. S3 shows the localization of VSVG and Golgi membranes relative to ER exit sites. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200501157/DC1.

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