A role for Sar1 and ARF1 GTPases during Golgi biogenesis in the protozoan parasite Trypanosoma brucei

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ABSTRACT A single Golgi stack is duplicated and partitioned into two daughter cells during the cell cycle of the protozoan parasite Trypanosoma brucei. The source of components required to generate the new Golgi and the mechanism by which it forms are poorly understood. Using photoactivatable GFP, we show that the existing Golgi supplies components directly to the newly forming Golgi in both intact and semipermeabilized cells. The movement of a putative glycosyltransferase, GntB, requires the Sar1 and ARF1 GTPases in intact cells. In addition, we show that transfer of GntB from the existing Golgi to the new Golgi can be recapitulated in semipermeabilized cells and is sensitive to the GTP analogue GDPγS. We suggest that the existing Golgi is a key source of components required to form the new Golgi and that this process is regulated by small GTPases.

INTRODUCTION Compartmentalization of specific cellular processes within membrane-bound organelles is a unique feature of eukaryotic cells. In a manner analogous to the duplication and segregation of genetic material, these membrane-bound organelles undergo biogenesis during the cell cycle and partitioning during cell division, thereby ensuring passage through successive generations. The Golgi is one such organelle specialized in the modification and sorting of macromolecules (such as lipids and proteins) destined for the plasma membrane and other cellular locations. The Golgi has a complex architecture comprising stacks of cisternae and a scaffold protein matrix organized into functionally distinct compartments. Transiting cargoes are modified by sequential arrays of glycosyltransferases and glycosidases brought together by circulating COPI vesicles (Glick and Nakano, 2009). How such an organelle comprising functionally distinct compartments can be duplicated and inherited is an intriguing question (Warren, 1993; Lowe and Barr, 2007).

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Abbreviations used: GntB, UDP-GlcNAc:Thr/Ser mucin-type polypeptide alpha-GlcNAc-transferase; GRASP, Golgi reassembly stacking protein.

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Secretory cargoes are synthesized and modified in the endoplasmic reticulum (ER) and then concentrated and packaged into COPII-coated vesicles at ER exit sites to be transported to the Golgi for further modification (Gomez-Navarro and Miller, 2016). The key regulator of COPII coated vesicle formation is the small GTPase Sar1, which is activated and recruited to the ER membrane by the guanine nucleotide exchange factor (GEF) Sec12 (Nakano and Muramatsu, 1989; Barlowe and Schekman, 1993; Bielli et al., 2005). Sar1-GTP in turn recruits the inner shell of the COPII coat, the Sec23/24 heterodimer, followed by the Sec13/31 heterotetramer, which associates with the coat to form the outer shell. This increases the GTP hydrolysis activity of Sar1, thereby inducing coat polymerization followed by vesicle scission (Lederkremer et al., 2001; Bi et al., 2002; Fath et al., 2007). GTP hydrolysis by Sar1-GTP, its displacement by the TRAPPI complex, and subsequent Sec23 phosphorylation result in uncoating of COPII-coated vesicles (Lord et al., 2011). Other tethering complexes, including Rab GTPases and soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) proteins, then mediate fusion of uncoated vesicles with cis-Golgi membranes (Malsam and Sollner, 2011; Gillingham and Munro, 2016).

Movement and modification of secretory cargo in the Golgi require both anterograde and retrograde movement of COPI-coated vesicles, although whether the cargo and/or enzymes are transported by these vesicles is a matter of debate (Pfeffer, 2010; Glick and Luini, 2011; Morriswood and Warren, 2013). Similar to COPII-coated vesicles, the formation of COPI-coated vesicles is regulated.
by a small GTPase termed ARF1 (Kahn and Gilman, 1986; Serafini et al., 1991). Activation of ARF1 by its GEFs results in direct binding of ARF1-GTP to the Golgi membrane through a myristoylated N-terminal amphipathic helix, which in turn recruits a heptameric coatomer protein complex (α, β, β′, γ, δ, ε/COP; Waters et al., 1993; Palmer et al., 1993; Hara-Kuge et al., 1994; Franco et al., 1996; Antonny et al., 1997). A conformational change in the coatomer induces coat polymerization, followed by vesicle fission. The GTP hydrolysis activity of ARF1 is facilitated by ARFGAP proteins and is believed to uncoat the COPI vesicles (Tanigawa et al., 1993). GTP hydrolysis by ARF1 is also required for efficient and selective cargo uptake, although the exact mechanism is not well understood (Nickel et al., 1998; Malsam et al., 1999).

In interphase mammalian cells, several hundred Golgi stacks are connected to each other in a ribbon-like structure close to the nucleus and often the centrosome (Nakamura et al., 2012). The number of Golgi stacks increases as cells progress through the cell cycle, and these stacks are fragmented, dispersed throughout the cytoplasm during mitosis, and reassembled during telophase and cytokinesis (Wang and Seemann, 2011). Precisely how Golgi stacks are duplicated is unclear, in large part due to the large number of Golgi stacks in mammalian cells and the fact that they are subsumed in a ribbon-like structure. To circumvent this problem, simpler organisms such as the yeast *Pichia pastoris* and protozoan parasites such as *Toxoplasma gondii* and *Trypanosoma brucei* have been used to study Golgi biogenesis (He, 2007). *T. brucei* cells have a single Golgi and single copies of organelles such as the basal body, kinetoplast, and flagellum. The new Golgi forms at a fixed distance (~1 μm) from the old Golgi and next to the new ER exit site. The new Golgi is readily distinguished from the old because it grows toward the posterior end of the cell, next to the flagellar pocket (from which the flagellum emerges and runs toward the anterior end of the cell; He et al., 2004). Using fluorescent protein–tagged markers, it has been determined that Golgi components arrive sequentially to the newly forming Golgi (Ho et al., 2006). The Golgi matrix protein TbGRASP and the putative glycosyltransferase GntB were found arrive early, followed by the COPI-coated vesicle component Tbc-COP and, finally, a cargo marker (the procyclin signal sequence attached to yellow fluorescent protein [YFP]). Recruitment of this cargo marker indicates that the new Golgi is functional for transport.

Photobleaching experiments suggested that the old Golgi might be the source of the putative glycosyltransferase, GntB (He et al., 2004). Repetitively photobleaching the area between the old and new Golgi reduced the appearance of GntB in the new Golgi, suggesting that it was being transported from the old Golgi. This evidence was indirect, however, and conflicted with work on *P. pastoris*, in which the new Golgi appeared to arise from the ER at random sites (Bevis et al., 2002). To extend these results in *T. brucei*, we devised a direct method to understand the mechanism of Golgi biogenesis using photoactivatable green fluorescent protein (PAGFP)–tagged Golgi components. We also examined the role played by the Sar1 and ARF GTPases in the movement of GntB from the old to the new Golgi and, in addition, established a semi-permeabilized cell system to permit biochemical manipulation of this process.

**RESULTS**

A photoactivation-based assay to study Golgi biogenesis in *T. brucei*

In *T. brucei*, the new Golgi forms next to the old Golgi ~2 h after cytokinesis (He et al., 2004; Figure 1A). To identify and track old Golgi components during Golgi biogenesis, a photoactivatable system was established in which a Golgi component was fused to PAGFP, which is visible at 488 nm only when it has been photoactivated by 405-nm laser irradiation (Patterson and Lippincott-Schwartz, 2002; Figure 1B). As a reference marker for both the new and old Golgi, the Golgi matrix marker TbGRASP was fused to mCherry and stably expressed together with the PAGFP-tagged marker in

**FIGURE 1:** Photoactivatable assay to study Golgi biogenesis in *T. brucei*. (A) Schematic representation of Golgi duplication in *T. brucei*. (B) Schematic representation of the photoactivation assay in *T. brucei* cells undergoing Golgi biogenesis. (C) Cells stably expressing TbGRASP-mCherry and GntB-PAGFP were fixed and stained using an anti-GFP antibody to detect GntB-PAGFP (green); TbGRASP-mCherry was visualized directly (red); and DNA was visualized using DAPI (blue). Bar, 2 μm. (D) Western blotting of whole-cell lysates from parental 427 cells or those expressing TbGRASP-mCherry and GntB-PAGFP fractionated using 15% SDS–PAGE and blotted for GntB-PAGFP (anti-GFP antibody) and TbGRASP-mCherry (anti-mCherry antibody). The observed migration matches the expected sizes of GntB-PAGFP (33 kDa) and TbGRASP-mCherry (80 kDa). Anti-BIP antibody was used as a loading control.
The Golgi enzyme GntB moves from the old Golgi to the new Golgi

To determine whether the old Golgi provides components to the newly forming Golgi, we fused the putative Golgi enzyme GntB to PAGFP. Because Golgi enzymes are localized to the Golgi by their membrane-spanning domains and flanking regions (Munro, 1991; Nilsson et al., 1991, 1996), we fused PAGFP to an N-terminal fragment of GntB that contains its predicted spanning domain and flanking cytoplasmic regions. This truncated form of GntB localized to the Golgi (Figure 1C), as shown by the generation of a stable cell line expressing both GntB-PAGFP and the reference Golgi marker TbGRASP-mCherry. Both colocalized by immunofluorescence microscopy (Figure 1C), and both migrated as single protein bands at the expected positions after SDS–PAGE and Western blotting (Figure 1D).

To investigate whether GntB-PAGFP moves from the old Golgi to the newly forming Golgi, we determined the cellular positions of the old and new Golgi using the reference marker TbGRASP-mCherry (Figure 2A, top, and Supplemental Movie Figure 1A). The region occupied by the old Golgi, marked by TbGRASP-mCherry, was irradiated with 405-nm light to photoactivate the GntB-PAGFP, and its fluorescence intensity was measured over 30 min during Golgi biogenesis by time-lapse microscopy (Figure 2A and Supplemental Movie Figure 1B). GntB moved to the new Golgi during Golgi biogenesis, as judged by the decrease of signal in the old Golgi and a corresponding increase of signal in the new Golgi ($t_{1/2} = 2.9$ min). Steady state was reached within 10 min, when both the old and the new Golgi had approximately equivalent amounts of fluorescence (Figure 2B). These results show that GntB-PAGFP moves from the old Golgi to the new Golgi and may also move from the new to the old Golgi (see Discussion).

To determine whether the decrease in signal in the old Golgi was related to Golgi duplication, we photoactivated Golgi-localized GntB-PAGFP in cells early in the cell cycle before Golgi duplication had begun and followed its movement over 30 min (Figure 2C). Photoactivated GntB-PAGFP remained in the single Golgi and did not accumulate elsewhere in the cell (Figure 2, C and D). These results suggest that the loss of photoactivated GntB-PAGFP from the old Golgi and its accumulation elsewhere depend on ongoing Golgi duplication, and the old Golgi likely provides components to the new Golgi during Golgi biogenesis.
Dominant-negative forms of the GTPases Sar1 and ARF1 inhibit transport of the Golgi enzyme GntB during biogenesis

To understand how components from the old Golgi are transferred to the new Golgi, we focused on small GTPases because these enzymes regulate vesicular trafficking and the formation of coated vesicles (Pucadyil and Schmid, 2009). The GTPase ARF1 regulates the formation of COPI-coated vesicles in many organisms (Gillingham and Munro, 2007; Donaldson and Jackson, 2011; Jackson and Bouvet, 2014). A T. brucei homologue of ARF1 (TbARF1) was previously identified that is 86% similar and 74% identical to the human ARF1 protein (Field, 2005; Price, 2005; Supplemental Figure S1A). TbARF1 was shown to localize to the Golgi by immunofluorescence microscopy (Price et al., 2007). A dominant-negative mutant of the ARF1 GTPase (ARF1 [Q71L]) that is restricted to the GTP-bound form inhibits cargo uptake into COPI-coated vesicles in mammalian cells (Pepperkok et al., 2000). A TbARF1 mutated at the corresponding residue was generated (TbARF1 [Q71L]; Supplemental Figure S1A) and used to investigate whether COPI-coated vesicles might mediate transfer of GntB.

Because overexpression of TbARF1 [Q71L] is toxic to T. brucei cells (Price et al., 2007), a stable cell line in which TbARF1 [Q71L] expression could be induced by addition of doxycycline was generated (Supplemental Figure S1). Expression of TbARF1 [Q71L] was monitored over an 8-h time period by Western blotting using an antibody that recognizes the 3× Ty1 tag at the C-terminus of TbARF1 [Q71L] (Supplemental Figure S1B). Microinjection of ARF1 [Q71L] into mammalian cells was previously shown to reduce mobility of the COPI coat component ε-COP at the Golgi region, using fluorescence recovery after photobleaching (FRAP; Presley et al., 2002). To test whether the TbARF1 [Q71L] mutant also affects COPI dynamics, we performed FRAP experiments in T. brucei cells expressing monomeric EGFP (mEGFP)–tagged Tbε-COP (Supplemental Figure S1, C and D) after induction of TbARF1 [wild-type] or [Q71L] (Supplemental Figure S1, E–H). FRAP analysis revealed that the mEGFP–Tbε-COP signal recovered rapidly in uninduced cells ($t_{1/2} = 9$ s) or cells in which TbARF1 [wild-type] expression was induced ($t_{1/2} = 15$ s; Supplemental Figure S1, E and F). In contrast, no significant recovery of the mEGFP–Tbε-COP signal was observed within 15 min after induction of TbARF1 [Q71L] (Supplemental Figure S1, G and H). This indicates that TbARF1 [Q71L] reduces the mobility of mEGFP–Tbε-COP and thus behaves in a manner similar to that of its mammalian homologue.

The effect of TbARF1 [Q71L] on GntB transfer was measured using cells stably expressing TbGRASP-mCherry, GntB-PAGFP, and inducible TbARF1 [Q71L]. GntB-PAGFP was then photoactivated in the old Golgi in cells undergoing Golgi duplication in the absence or presence of TbARF1 [Q71L], the latter induced using doxycycline for 4 h (Figure 3A). Images of these photoactivated cells were acquired immediately and after 30 min, at which time point the levels of GntB-PAGFP signal in the new Golgi had reached steady state (Figure 2B). The signal was quantified in the old and new Golgi at time point 0 (time of photoactivation) and 30 min (Figure 3B), and the signal was normalized to the fluorescence intensity in the old Golgi immediately after photoactivation. After 30 min in the absence of TbARF1 [Q71L] expression, the normalized fluorescence intensity in the new Golgi increased from 5 to 60% (median). In the presence of TbARF1 [Q71L] expression, the normalized...
fluorescence intensity in the new Golgi from 4 to only 15% (median). This suggests that disrupting TbARF1 function leads to inhibition of transport from the old to the new Golgi, suggesting a role for the GTPase TbARF1 in Golgi biogenesis.

We then asked whether the COPII vesicle system that mediates trafficking from the ER to the Golgi has a role in Golgi biogenesis. A T. brucei homologue of Sar1 (TbSar1), the small GTPase that regulates COPII-coated vesicle formation, was previously identified (Field, 2005; Sevova and Bangs, 2009). The TbSar1 protein shares 66% sequence similarity and 48% identity with human Sar1a (Supplemental Figure S2A). In mammalian and yeast cells, the Sar1 dominant-negative mutant Sar1a [H79G] ([H77L] in yeast) has been shown to inhibit uncoating of COPII vesicles and inhibit ER-to-Golgi transport (Pepperkok et al., 1998; Saito et al., 1998). According to the alignment of T. brucei and human Sar1, the histidine at position 79 corresponds to histidine at position 74 in T. brucei Sar1 (Supplemental Figure S2A). This histidine was therefore mutated to glycine to generate a putative dominant-negative mutant form of TbSar1, and a Ty1 tag was fused to its C-terminus (Supplemental Figure S2). The functionality of this mutant on ER export was tested using a previously described secretory marker, the procyclin signal sequence (ss) fused to a fluorescent protein (Ho et al., 2006). A stable cell line was generated overexpressing PagFP fused to this signal sequence (ss-PagFP) and expressing TbSar1 [wild-type] or the [H74G] mutant in an inducible manner (Supplemental Figure S2, B and C). On expression of the mutant but not wild-type TbSar1, ss-PagFP accumulated at the nuclear envelope and ER, as shown by immunofluorescence microscopy (Supplemental Figure S2D). Intracellular levels of ss-PagFP increased in the presence of mutant TbSar1 [H74G], as analyzed by Western blotting (Supplemental Figure S2E). These results show that TbSar1 [H74G] inhibits ER export in T. brucei cells in a dominant-negative manner.

A cell line was then generated that expresses the TbSar1 [H74G] mutant in a doxycycline-inducible manner together with stable expression of TbGRASP-mCherry and GntB-PagFP (Figure 3C). GntB-PagFP localized to the old Golgi was photoactivated in the presence of TbSar1 [H74G], and its accumulation in the new Golgi was determined after 30 min (Figure 3C); the signal was normalized to the fluorescence intensity in the old Golgi immediately after photoactivation. After 30 min in the absence of TbSar1 [H74G] expression, the normalized fluorescence intensity in the new Golgi increased from 4 to 54% (median; Figure 3D). In the presence of TbSar1 [H74G] expression, the normalized fluorescence intensity in the new Golgi increased from 7 to only 15% (median; Figure 3D). Together, these results suggest that both ARF1 and Sar1 GTPases are involved in transporting material from the old to the new Golgi.

Because ARF1 regulates COPI vesicle formation, the finding that TbARF1 [Q71L] expression inhibits GntB transfer from one Golgi to another raised the possibility that GntB is carried by COPI-coated vesicles. However, GntB transfer from the old to the new Golgi is also inhibited by TbSar1 [H74G] expression, which blocks ER export. This suggests that either GntB is transferred directly to the new Golgi via the ER or another factor must be transported from the ER to enable COPI vesicles to transfer GntB directly from the old Golgi to the new. To determine whether TbSar1 [H74G] expression affects the association and dynamics of COPI coat components in the Golgi membranes, we established stable cell lines expressing mEGFP-Tb-COP and inducible TbSar1-Ty1 [H74G]. Seven (-) TbSar1-Ty1 [H74G] and eight (+) TbSar1-Ty1 [H74G] independent experiments. Bar, 2 µm.

**FIGURE 4:** Mobility of the COPI coat component, Tb-COP, in the Golgi region in the presence of the TbSar1 H74G mutant. (A, B) Parental 29.13 cells or cells stably expressing mEGFP-Tb-COP and inducible TbSar1-Ty1 [wild-type] or [H74G] were either (A) fractionated by 15% SDS-PAGE followed by anti-GFP antibody to detect mEGFP-Tb-COP (expected size 61 kDa) and anti-BIP antibody as a loading control, or (B) fixed and stained using an anti-Ty1 antibody to detect TbSar1-Ty1 [wild-type] or [H74G], mEGFP-Tb-COP was visualized directly. Blue, DAPI; green, mEGFP-Tb-COP; red = TbSar1-Ty1 [wild-type] or [H74G]. (C) Cells stably expressing mEGFP-Tb-COP and inducible TbSar1-Ty1 [wild-type] were immobilized on a glass-bottom 96-well plate and photobleached using a 488-nm laser over the old Golgi area (open red circle). The recovery after photobleaching was followed by time-lapse microscopy (closed arrowheads; time interval 20 s, total time 15 min). Time 0 min represents the first image after photobleaching. (D) The fluorescence intensity in the photobleached area was quantified and normalized to the prebleach fluorescence intensity and plotted vs. time as the mean ± SD. Five (-) TbSar1-Ty1 [wild-type] and six (+) TbSar1-Ty1 [wild-type] independent experiments. (E, F) Similar experiments and analysis were carried out as for C and D, using cells stably expressing mEGFP-Tb-COP and inducible TbSar1-Ty1 [H74G]. Seven (-) TbSar1-Ty1 [H74G] and eight (+) TbSar1-Ty1 [H74G] independent experiments. Bar, 2 µm.
Golgi recovered rapidly in the absence (t_{1/2} = 13 s) or presence (t_{1/2} = 20 s) of TbSar1 [wild-type] or in the absence of TbSar1 [H74G] (t_{1/2} = 14 s). In contrast, the recovery of mEGFP–Tbe-COP in the old Golgi was reduced sevenfold in the presence of TbSar1 [H74G] (t_{1/2} = 97 s; Figure 4, C–F). Similar results were obtained for FRAP analysis of the new Golgi. mEGFP–Tbe-COP in the new Golgi recovered rapidly in the absence (t_{1/2} = 11 s) or presence (t_{1/2} = 13 s) of TbSar1 [wild-type] or in the absence of TbSar1[H74G] (t_{1/2} = 13 s) but was reduced fivefold in the presence of TbSar1[H74G] (t_{1/2} = 62 s). This suggests that TbSar1[H74G] expression inhibits COPⅠ vesicle trafficking in both new and old Golgi and is consistent with the idea that these vesicles transport Golgi components directly between the old Golgi and the new.

A semipermeabilized system to study the mechanism of Golgi biogenesis

To be able to directly manipulate cells undergoing Golgi biogenesis, we established a system in which living cells were treated with digitonin (Figure 5A). This treatment selectively permeabilizes the plasma membrane while leaving internal membranes intact and has been used to permeabilize cells for studying processes such as nuclear transport and nuclear envelope breakdown (Adam et al., 1990; Mühlhäusser and Kutay, 2007). We combined this with our photocytoskeletal assay in order to perturb Golgi biogenesis.

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We first wanted to determine the concentration of digitonin sufficient to permeabilize the plasma membrane without affecting the integrity of internal membranes. For this, we treated cells stably expressing PAGFP as a cytoplasmic marker with increasing concentrations of digitonin and fractionated them into soluble (cytosolic) and sedimentable (membrane) fractions by centrifugation (Figure 5B). The fate of the cytoplasmic PAGFP and the ER luminal marker BIP were monitored by Western blotting using anti-GFP and anti-BIP antibodies, respectively. In cells not treated with detergent, all detectable PAGFP and BIP were found in the pellet fraction, whereas in cells treated with 1% Triton X-100, which solubilizes all membranes, both PAGFP and BIP were in the soluble supernatant fraction. In contrast, cells treated with 40 μg/ml digitonin for 10 min at room temperature retained BIP entirely in the membrane fraction, whereas PAGFP was found in the cytosolic fraction, suggesting that the plasma membrane had been successfully permeabilized but ER membranes remained intact.

Using this concentration of digitonin, we refined the time required to permeabilize the plasma membrane using our microscopy-based assay (Figure 5C). Cells stably expressing cytoplasmic mEGFP and attached to glass-bottomed plates by gentle centrifugation were treated with 40 μg/ml digitonin, and the release of mEGFP was monitored over time (Figure 5C). In the presence of 40 μg/ml digitonin, the mEGFP signal disappeared completely within 5 min. These conditions were therefore used to generate semipermeabilized cells in all subsequent experiments.

We next tested whether transport of Golgi components could be visualized in semipermeabilized cells that had been supplemented with cytosolic extract (Figure 6A). Cytosolic extract was prepared by collecting untransfected cells via gentle centrifugation, lysing the cells by freeze-thawing, and then fractionating the lysate by high-speed centrifugation. The supernatant fraction was then added back to digitonin-permeabilized cells expressing GntB-PAGFP and TbrGRASP-mCherry (Figure 6A and Supplemental Movie Figure 2). GntB-PAGFP in the old Golgi was then photoactivated, and this photoactivated population was followed over a 30-min time period. As in intact cells (Figure 2B and Supplemental Movie Figure 2), the Golgi enzyme GntB-PAGFP translocated to the new Golgi during biogenesis to the extent of ~25–40% of the total fluorescence, although at a reduced rate (Figure 6B). In the absence of cytosol, transfer of GntB from the old to the new Golgi was inhibited (Supplemental Figure S3), suggesting that cytosolic components are required for this transfer.

GTPases inhibit translocation of GntB from the old Golgi to the new Golgi

To generalize the role of small GTPases in Golgi biogenesis and as an alternative method to overexpressing GTPase dominant-negative mutants, we tested the effect of the slowly hydrolyzable GTP analogue GTPγS on Golgi biogenesis in our semipermeabilized system.
Golgi increased from 6% at 0 min to 27% at 60 min. In contrast, preincubation with 50, 100, or 500 μM GTPγS almost completely abolished GntB-PAGFP translocation. This result indicates that GTP hydrolysis and therefore the activity of GTPases are required to transport material from the old to the new Golgi during biogenesis.

**DISCUSSION**

In this study, we provided direct evidence that the existing Golgi in *T. brucei* can act as a source of components for the newly forming Golgi. To do this, we established a photoactivatable assay in which a subpopulation of Golgi components residing in the old Golgi was visualized by photoactivation and their movement followed by time-lapse microscopy. These photoactivation experiments, using the putative Golgi enzyme GntB fused to PAGFP, indicated that GntB is transferred from the old Golgi to the new, suggesting that the old Golgi provides at least some components to the new Golgi during biogenesis. The signal at the old and new Golgi reached steady state at ∼50% of the initial photoactivated signal ∼10 min after photoactivation. This raises the possibility that transfer is bidirectional, such that, at steady state, the flux of GntB from old to new is matched by the flux from new to old. Such bidirectional transfer might help ensure that both old and new Golgi reach the same size. When the new Golgi is small, the flux from old to new would be greater than that from new to old, assuming that the flux depends on the Golgi membrane surface area. Hence the new Golgi would grow. Once they reached the same size, the fluxes would be the same. To test this idea, we carried out photoactivation of the new Golgi, but the results were inconclusive for technical reasons because the small size of the new Golgi meant that the signal after photoactivation was difficult to detect. Our earlier work also implicated the Sec16 scaffold protein at COPII budding sites as a determinant of Golgi size (Sealey-Cardona et al., 2014). It will be important to determine how this might be coordinated with bidirectional transfer between old and new Golgi.

To gain insight into the mechanism of transfer, we focused on small GTPases, key regulators of vesicular trafficking, and specifically the Sar1 and ARF1 GTPases. The transfer of photoactivated GntB-PAGFP from old to new Golgi was first monitored in the presence of a GTP-locked dominant-negative mutant of TbARF1, TbARF1 [Q71L]. The efficacy of this mutant was tested using FRAP experiments, which showed that it behaved similarly to its mammalian counterpart. The accumulation of GntB-PAGFP in the new Golgi was lower (15 vs. 60% over 30 min) in the presence of TbARF1 [Q71L], indicating that TbARF1 [Q71L] inhibits the transfer of GntB-PAGFP to semipermeabilized cells expressing TbGRASP-mCherry and GntB-PAGFP were incubated with increasing concentrations of GTPγS for 20 min (Figure 6C). GntB-PAGFP was photostimulated in the old Golgi and imaged at 0 and 60 min. The signal in the old and the new Golgi was then quantified at both time points (Figure 6D), and the signal in the old and the new Golgi was normalized to the fluorescence intensity in the old Golgi immediately after photoactivation. In the absence of GTPγS, the normalized fluorescence intensity in the new Golgi increased from 6% at 0 min to 27% at 60 min. In contrast, preincubation with 50, 100, or 500 μM GTPγS almost completely abolished GntB-PAGFP translocation. This result indicates that GTP hydrolysis and therefore the activity of GTPases are required to transport material from the old to the new Golgi during biogenesis.
the new Golgi. Given that ARF1 is a regulator of COPI-coated vesicle formation, this result suggests that COPI vesicles might act as carriers of GntB-PAGFP from the old to the new Golgi.

The GTPase Sar1 is required for the budding of COPII vesicles from ER exit sites. A dominant-negative mutant, TbSar1 [H74G], was constructed based on the mammalian protein and validated using a secretory marker, which accumulated in the ER and nuclear envelope, showing that it inhibited ER-to-Golgi transport. Inducible expression of this mutant TbSar1 had a similar effect as expression of T苞ARF1 [Q71L], resulting in a reduction in transfer (15 vs. 54% over 30 min) of GntB-PAGFP from the old to the new Golgi. This in turn implicates the ER in Golgi biogenesis.

Two possibilities can be envisioned to explain this effect of TbSar1 [H74G]. The first is that the movement of GntB from the old to the new Golgi is indirect, via the ER, such that GntB first moves from the old Golgi back to the ER by retrograde transport and then through the ER to the new ER exit site, from which it is delivered to the new Golgi. This latter step would be blocked by expression of TbSar1 [H74G]. Movement of Golgi enzymes through the ER has been used to explain Golgi biogenesis in mammalian cells, although whether it occurs is still debated (Villeneuve et al., 2017, and references therein). Furthermore, it is not clear whether these results can be applied to T. brucei because the Golgi does not undergo the extensive morphological transformations during the cell cycle seen in mammalian cells. The only relevant experiments are those in which the region between the old and new Golgi was repetitively photo-bleached, which would include any ER connecting the two. Although this had no significant effect on the transfer of GntB, more work is needed to confirm and extend these results (He et al., 2004).

A second possibility to explain the effect of TbSar1 [H74G] on Golgi biogenesis is that this inhibition prevents the delivery of components from the ER to the old Golgi that are needed for COPI vesicle trafficking of GntB from the old to the new. Such components could include membrane lipids, given that the budding of COPI vesicles results in loss of membrane surface area that would need to be replenished, as well as membrane proteins such as SNAREs and golgin tethers. Consistent with this interpretation, FRAP analysis of mEGFP-Tb-COP showed significantly reduced mobility of this COPI coat component in the old Golgi region in the presence of TbSar1 [H74G], suggesting that TbSar1 [H74G] affects COPI trafficking. Future work will aim to identify these components.

A semipermeabilized cell assay was established to facilitate experimental manipulation of Golgi duplication. Cells were treated with digitonin under conditions that allowed almost complete escape of cytoplasmic contents but preserved internal membranes. Photoactivation of GntB-PAGFP in the old Golgi resulted in transfer to the new Golgi, albeit at a slower rate and to a lesser extent than in intact cells. This transfer required added cytosol and was sensitive to GTPyS, arguing that we have, for the first time, reconstituted some elements of Golgi biogenesis in a permeabilized cell system.

Taking the results together, this study shows that the old Golgi is a direct source of at least some components during Golgi duplication in T. brucei and that the small GTPases TbARF1 and TbSar1 are crucial to this process. In addition, cytosolic factors, possibly additional GTPases, are required to transfer these components.

**MATERIALS AND METHODS**

**DNA constructs and cell lines**

To generate stable cell lines overexpressing TbGRASP-mCherry and GntB-PAGFP, the mCherry coding sequence was cloned into the pXS2-TbGRASP-GFP plasmid (He et al., 2004) in which GFP was removed by Nhel and BamHI restriction digest; the PAGFP coding sequence was cloned into pXS2-GntB (1–56 aa)-YFP plasmid in which YFP was removed by Nhel and BamHI restriction digest. The GntB-PAGFP coding sequence was then cloned into the pHDI034 vector. Plasmids (30 μg/transfection) were linearized with Nsil and transfected into 5 × 10⁷ 427 Lister strain T. brucei cells by electroporation. Cells containing the transgenes were selected using 10 μg/ml blasticidin (TbGRASP-mCherry) or 1 μg/ml puromycin (GntB-PAGFP). Clonal cell lines were generated by serial dilution and screened by Western blotting and immunofluorescence microscopy.

To generate stable cell lines in which inducible overexpression of GTPase dominant-negative mutants, TbARF1 and TbSar1 coding sequences were amplified from 427 genomic DNA and cloned into a pLew100 vector with either a 3×-Ty1 tag or a single-Ty1 tag. Mutations were introduced by site-directed mutagenesis using PfuUltra High-Fidelity DNA polymerase and the primers CATGTTGGGATGT-CTTGGTCTAGATGTTTCTCGCTGT and ACAGCCGAC-GAAGAACATCTAGACCACCAAAATCATCCACATG for TbARF1 and GACTATTGATATGGGTGGCGGTCTGGAAGCGCGTCGCCT and AGGCAGACGCGCTTCCAGACGGCACCACATCATATACGT for TbSar1. Plasmids containing the desired mutations were then linearized by NotI and transfected into the T. brucei 29.13 cell line, a derivative of the T. brucei 427 strain that expresses the tetracycline repressor protein and T7 RNA polymerase. Stably transfected clones were selected using 5 μg/ml phleomycin and screened after induction of expression with 5 ng/ml doxycycline for 8 h by Western blotting and immunofluorescence microscopy.

To generate stable cell lines in which an endogenous allele of Tbe-COP was replaced with mEGFP–Tbe-COP, a pCR4Blunt construct containing 500 base pairs of the 5′ untranslated region of Tbe-COP followed by the blasticidin resistance gene, tubulin intergenic region, mEGFP coding sequence, and 500 base pairs of Tbx-COP was excised by NotI and transfected into the T. brucei 29.13 cell line, a derivative of the T. brucei 427 strain that expresses the tetracycline repressor protein and T7 RNA polymerase. Stably transfected clones were selected using 10 μg/ml blasticidin. Clonal cell lines were generated by serial dilution and screened by Western blotting and immunofluorescence microscopy.

All cell lines were grown at 27°C in SDM-79 medium (Invitrogen) supplemented with 1× penicillin/streptomycin (Thermo Fisher), 1× l-glutamine (Thermo Fisher), 7.5 mg/l hemin (Sigma-Aldrich), and 10% fetal bovine serum (for 427 cells from Sigma-Aldrich, for 29.13 cells from Clontech; Tet system approved).

**Antibodies and Western blotting**

Anti-GFP was described previously (He et al., 2004). Anti-BIP and anti-Ty1 antibodies were kind gifts of J. D. Bangs (University at Buffalo, SUNY) and K. Gull (University of Oxford, UK), respectively. Anti-m-Cherry (Living Colors) antibody was purchased from Takara Clontech.

For Western blotting, lysates from 2 × 10⁷ cells (unless otherwise indicated) were separated by SDS–PAGE and then transferred to a nitrocellulose membrane. Primary antibodies and horseradish peroxidase (HRP)–coupled secondary antibodies were diluted in phosphate-buffered saline (PBS), 5% (wt/vol) milk powder, and 0.1% (vol/vol) Tween-20. HRP-coupled secondary antibodies were detected using ECL reagents (Thermo Fisher) and either Kodak Biomax MS films or a Bio-Rad Chemidoc Touch system.

**Immunofluorescence microscopy**

Cells were spun down at 3800 × g for 1 min at 4°C, washed once with PBS, and attached to glass coverslips by centrifugation at 1800 × g for 5 s. Attached cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 10 min at room temperature.

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and then further fixed and permeabilized with ice-cold 100% methanol (Sigma) for 5 min at -20°C, followed by five brief PBS washes. Fixed cells were then blocked with 3% bovine serum albumin (Sigma-Aldrich) in PBS and incubated with the primary antibody and then a secondary antibody conjugated to Alexa 488 or 568 dyes (Thermo Fisher). Coverslips were then mounted on glass slides in Fluoromount G supplemented with 4',6-diamidino-2-phenylindole (DAPI; Electron Microscopy Sciences) mounting medium. Mounted coverslips were imaged using Visiview software (Vistron Systems) installed on a Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss) equipped with an alpha-Plan-Apochromat 100×/1.46 Oil DIC RMS objective, PCO 1600 camera, and Lumencor SOLA 6-LCR-SB light source.

**Live-cell imaging, photoactivation assay, and FRAP experiments**

For live-cell imaging, cells were either embedded in low-melting agarose or spun down on 96-well glass-bottomed plates when additional treatments were required. Low-melting agarose plates were prepared by melting agarose (Agarose Type VII; Sigma-Aldrich) in sterile MilliQ H₂O (8%), diluted in phenol red-free MEM and conditioned MEM to 0.8%, and distributed into 17 × 35 mm Petri dishes. Plates were stored at 4°C. Before live-cell imaging, cells were washed twice with phenol red-free conditioned MEM, resuspended, and added drop by drop to the low-melting agarose plates. The excess liquid was let to dry for 1–2 min until cells were immobilized. A rectangular piece of agarose was removed, mounted on a four-well LabTek chambered coverglass (Thermo Fisher) upside down with cells facing the coverglass, and sealed with high-viscosity silicone (Bayer).

All photoactivation experiments were performed using a Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss) equipped with a FRAP unit, a Yokogawa CSU-X1 Nipkow spinning-disk unit, a Roper CoolSNAP HQ2 charge-coupled device camera, and a Plan-Apochromat 63×/1.4 Oil objective. Photoactivation was carried out using a 405-nm laser line (0.01% transmission, 5 ms/pixel) and VisiFRAP unit, and acquisition of time-lapse images was performed using 488- and 568-nm laser lines (30% transmission). The photoactivation area was selected using Visiview software. The mean fluorescence intensity of the old and new Golgi regions was quantified using ImageJ (http://rsbweb.nih.gov/ij/) and then normalized to values at time point 0.

**Preparation of cytosol from *T. brucei* cells by a freeze–thaw method**

Approximately 3 × 10⁸ untransfected strain 427 *T. brucei* cells were centrifuged at 1800 × g for 5 min at room temperature, and the supernatant medium was removed. Cells were washed twice in 1 ml of ice-cold wash buffer I (20 mM 1,4-piperazinediethanesulfonic acid [PIPES], pH 6.9, 110 mM KOAc, 10 mM Mg(OAc)₂, 1× protease inhibitor cocktail without EDTA [Roche]) and then once in 1 ml of ice-cold wash buffer II (200 mM sucrose, 20 mM PIPES, pH 6.9, 50 mM KOAc, 10 mM Mg(OAc)₂, 1× protease inhibitor cocktail without EDTA [Roche], 1 mM dithiothreitol, 0.5 mM ethylene glycol tetraacetic acid) by resuspension and centrifugation at 3800 × g for 1 min at 4°C. The cell pellet was then immersed in liquid N₂ and thawed three times, followed by centrifugation at 20,000 × g for 40 min at 4°C. The supernatant cytosolic extract was then diluted to ∼5 mg/ml in ice-cold wash buffer II and centrifuged again at 11,000 × g for 1 min at 4°C and supplemented with a 1× energy mix (0.25 mM ATP, 0.25 mM GTP, 25 μg/ml creatine kinase, 5 mM creatine phosphate, 2.5 mM sucrose) before use.

**Digitonin permeabilization assay**

*T. brucei* cells (1.8 × 10⁷) were centrifuged at 3824 × g for 1 min at 4°C and washed once with 1 ml of ice-cold wash buffer I. The pellet was resuspended in 900 μl of wash buffer II, and 40 μl of this suspension was transferred to a single well of a 96-well glass-bottom plate and centrifuged for 5 s at 400 × g. The supernatant was replaced with 40 μl of digitonin mix (wash buffer II supplemented with 40 μg/ml digitonin and 1× energy mix [see earlier description]), incubated for 5 min at room temperature, and then removed. The semipermeabilized cells were washed with cytosol once to remove residual digitonin. Cytosolic extract preincubated with or without inhibitors was added to the cells, and photoactivation and time-lapse imaging were then performed. In GTPyS experiments, GTP was omitted from all buffers and the energy mix. Digitonin was purchased from Calbiochem and prepared according to the manufacturer’s instructions.

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