Involvement of the Rho–mDia1 pathway in the regulation of Golgi complex architecture and dynamics

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ABSTRACT In mammalian cells, the Golgi apparatus is a ribbon-like, compact structure composed of multiple membrane stacks connected by tubular bridges. Microtubules are known to be important to Golgi integrity, but the role of the actin cytoskeleton in the maintenance of Golgi architecture remains unclear. Here we show that an increase in Rho activity, either by treatment of cells with lysophosphatidic acid or by expression of constitutively active mutants, resulted in pronounced fragmentation of the Golgi complex into ministacks. Golgi dispersion required the involvement of mDia1 formin, a downstream target of Rho and a potent activator of actin polymerization; moreover, constitutively active mDia1, in and of itself, was sufficient for Golgi dispersion. The dispersion process was accompanied by formation of dynamic F-actin patches in the Golgi area. Experiments with cytoskeletal inhibitors (e.g., latrunculin B, blebbistatin, and Taxol) revealed that actin polymerization, myosin-II–driven contractility, and microtubule-based intracellular movement were all involved in the process of Golgi dispersion induced by Rho–mDia1 activation. Live imaging of Golgi recovery revealed that fusion of the small Golgi stacks into larger compartments was repressed in cells with active mDia1. Furthermore, the formation of Rab6-positive transport vesicles derived from the Golgi complex was enhanced upon activation of the Rho–mDia1 pathway. Transient localization of mDia1 to Rab6-positive vesicles was detected in cells expressing active RhoA. Thus, the Rho–mDia1 pathway is involved in regulation of the Golgi structure, affecting remodeling of Golgi membranes.

INTRODUCTION Organization of Golgi components into a single complex localized in the perinuclear cell area in the proximity of the centrosome is a characteristic feature of mammalian cells (Thyberg and Moskalewski, 1999; Glick and Nakano, 2009; Sutterlin and Colanzi, 2010). How such an organization is developed and maintained, however, is poorly understood. Several factors are known to be integral to Golgi complex organization. The matrix proteins GRASP65, GRASP55, GM130, and GMAP210 are believed to tether Golgi cisternae to each other and keep them together (Ramirez and Lowe, 2009; Sengupta et al., 2009). In addition, the cytoskeleton plays an important role in the maintenance of Golgi architecture and its proper positioning in the cell.

The role of microtubules has been studied extensively for decades (for review see Thyberg and Moskalewski, 1999; Sutterlin and Colanzi, 2010). Microtubule depolymerization by means of nocodazole and other related drugs leads to rapid deterioration of the Golgi structure and the appearance of newly formed Golgi ministacks at endoplasmic reticulum (ER) exit sites scattered throughout the cell (Cole et al., 1996; Storrie et al., 1998; Thyberg and Moskalewski, 1999). Recovery of the Golgi following drug removal depends on the directional, retrograde movement of these ministacks along microtubules, their...
accumulation in the pericentrosomal area, and their subsequent fusion into ribbons (Thyberg and Moskalewski, 1999; Miller et al., 2009). Both radial microtubule arrays nucleated by the centrosome and microtubules nucleated or stabilized by the Golgi elements themselves (Chabin-Brion et al., 2001; Efimov et al., 2007; Hoppeler-Lebel et al., 2007; Rivero et al., 2009) participate in the recovery of the Golgi ribbon structure (Hoppeler-Lebel et al., 2007; Miller et al., 2009). In some cell types treated with the microtubule-stabilizing drug Taxol (Schiff and Horwitz, 1980), remodeling of the Golgi ribbons also occurs (Wehland et al., 1983; Hoshino et al., 1997); however, this process is slower and leads to a lower degree of fragmentation than Golgi dispersion induced by microtubule depolymerization.

Movement of Golgi elements along microtubules depends on microtubule-based molecular motors. Among these are cytoplasmic dynein (Corthesy-Theulaz et al., 1992; Burkhardt, 1998; Thyberg and Moskalewski, 1999; Allan et al., 2002) and several kinesins (Echard et al., 1998; Xu et al., 2002; Stauber et al., 2006; Gupta et al., 2008). The dynactin molecular complex, linking the microtubule motors with various cargos, including Golgi membrane elements (Schroer, 2004), was shown to be required for the maintenance of the Golgi architecture (Burkhardt et al., 1997; Burkhardt, 1998).

In addition to microtubules, the actin cytoskeleton seems to affect Golgi architecture and position. Structural information concerning the association of actin filaments with Golgi membranes is limited, although immuno–electron microscopy revealed β- and γ-actin at the Golgi-associated COPI-coated buds (Valderrama et al., 2000), and short filaments decorated with the tropomyosin isofrom Tm5NM-2 were detected at the budding zones on the ends of Golgi cisternae (Percival et al., 2004). Other studies suggest that the Golgi membrane might be surrounded by a spectrin–actin network, similar to that underlying the erythrocyte membrane (Beck and Nelson, 1998; Holleran and Holzbaur, 1998; De Matteis and Morrow, 2000; Kang et al., 2009).

A number of agents that affect the polymerization status of actin filaments were shown to perturb Golgi morphology and integrity. In particular, in several types of cultured cells, actin depolymerization by means of C2 botulinum toxin, cytochalasin D, or latrunculin B led to compaction of the Golgi and an apparent reduction in its projected area (Valderrama et al., 1998; Lazaro-Dieguez et al., 2006). Electron microscopy studies revealed the swelling of Golgi cisternae in such cells (Lazaro-Dieguez et al., 2006). This effect seems to be cell-type specific, since in cells of neural origin, latrunculin and cytochalasin produced dispersion of the Golgi complex rather than its compaction (Camer et al., 2003; Rosso et al., 2004).

Both myosin-driven actin movement and actin polymerization can affect Golgi organization and dynamics. Myosin VI localizes to the Golgi and is essential to normal Golgi morphology (Warner et al., 2003; Sahlender et al., 2005). Myosin 18 interacts with the Golgi membrane phospholipid phosphatidylinositol-4-phosphate via the GOLPH3 linker and controls the flattened shape of Golgi cisternae (Dippold et al., 2009). Myosin II is associated with Golgi membranes via interaction with Rab6, a Golgi-specific G-protein, and is involved in Golgi membrane fission (Miserey-Lenkei et al., 2010). Myosin 1b was recently shown to promote the formation of post-Golgi carriers (Almeida et al., 2011).

Experimental manipulations with several proteins that regulate the dynamics of actin filaments also produced structural and functional alterations in the Golgi apparatus. Both activation and knockdown of the actin-depolymerizing factor ADF/cofilin produce specific changes in Golgi-mediated secretion and trafficking events (Salvarezza et al., 2009; von Blume et al., 2009). A major regulator of cofilin, LIM kinase 1, was shown to be localized at the Golgi (Foletta et al., 2004) and affect its dynamics via cofilin phosphorylation (Rosso et al., 2004; Salvarezza et al., 2009). Actin filament nucleation by means of the Arp2/3 complex may also control Golgi organization and function. Indeed, the WHAMM protein (WASP homologue associated with actin, membranes, and microtubules), a novel actin nucleation-promoting factor that activates the Arp2/3 complex, was shown to associate with Golgi membranes and regulate Golgi architecture and ER-to-Golgi transport (Campellone et al., 2008).

Formin-family proteins stimulate both nucleation and elongation of actin filaments (Chhabra and Higgs, 2007; Goode and Eck, 2007; Chesarone et al., 2010). In particular, mammalian Diaphanosus-related formin 1 (mDia1), a direct target of small GTPase Rho (Watanabe et al., 1997), is a potent activator of actin polymerization in vitro (Li and Higgs, 2003). The Diaphanosus-related formins, and specifically mDia1, were shown to be involved in a variety of in vivo functions (for review see Narumiya et al., 2009; Chesarone et al., 2010), which include regulation of both cell polarity and intracellular trafficking of vesicles and organelles (Magdalena et al., 2003; Fernandez-Borja et al., 2005; Minin et al., 2006; Yamana et al., 2006; Wallar et al., 2007; Shi et al., 2009).

In the present study, we address the effects of mDia1 and its activator, RhoA, on the architecture and dynamics of the Golgi apparatus. We found that activation of the Rho–mDia1 pathway indeed induced marked reorganization of the Golgi, which depends on the actin cytoskeleton and can be greatly enhanced by the microtubule-stabilizing drug Taxol. We present evidence that Rho–mDia1 is involved in regulating the fusion of the Golgi membranes and formation of Rab6-positive Golgi-derived transport carriers and plays a critical role in Golgi complex integrity.

RESULTS

Activation of RhoA induces dispersion of the Golgi complex

In HeLa JW cells (Paran et al., 2006), expression of a constitutively active mutant of RhoA (RhoA-V14) produces a marked alteration in Golgi complex organization.

Labeling of Golgi with the trans-Golgi marker GalT–yellow fluorescent protein (YFP) (YFP fused to the N-terminus of β-galactosyltransferase) revealed disruption of the Golgi ribbon structure into smaller elements dispersed from the narrow perinuclear area over the entire central part of the cell (Figure 1A, top). A similar effect was observed using markers of medial Golgi (mannosidase II–green fluorescent protein [GFP]) or cis-Golgi (endo genous p115, Grasp65) (see Figures 3 and 6 later and Supplemental Figures S1–S3). The active form of RhoB also triggered Golgi dispersion, whereas another small GTPase, Rac, did not produce any effect on Golgi morphology (unpublished data).

Lysophosphatidic acid (LPA) treatment is known to rapidly activate Rho (Ren et al., 1999); its activation made it possible to track the dynamics of Golgi reorganization. Experiments with LPA stimulation were performed in serum-free medium (Ren et al., 1999); serum starvation by itself did not decrease the Golgi compactness (Figure 1, C and D). Time-lapse filming of control cells showed that Golgi elements were mobile, but overall ribbon organization remained generally unchanged throughout the observation period (Supplemental Movie S1). Time-lapse filming of LPA-treated cells revealed the dispersion and centrifugal movement of the Golgi elements. In less than 2 h, the ribbons underwent fragmentation into smaller elements that dispersed radially outward from the cell center (Supplemental Movie S2 and Figure 1B, top).

The extent of fragmentation and dispersion of the Golgi was similar to that produced by expression of constitutively active Rho (Figure 1A, top). The degree of Golgi dispersion was quantified
Rho effector. To this end, we examined whether constitutively active RhoA or LPA treatment would affect Golgi organization in mDia1 knockdown cells (Figure 1). A HeLa JW cell line stably expressing a vector encoding shRNA for mDia1 (Carramusa et al., 2007) was used in these experiments. The level of mDia1 expression in these cells decreased more than 90%, as revealed by Western blotting (Figure 1E). We found that neither transfection with active RhoA nor treatment with LPA led to significant dispersion of the Golgi in mDia1-depleted cells (Figure 1, A and B, bottom). Our measurements revealed that Golgi compactness in mDia1 knockdown cells treated with LPA or transfected with constitutively active Rho did not differ from that in control cells (Figure 1, C and D). Of note, in mDia1 knockdown cells with nonstimulated RhoA, a slight increase in compactness above control levels was detected. In line with these results, we found that RhoA activation led to the enrichment of mDia1-GFP in the cell area occupied by the Golgi complex (Supplemental Figure S1).

Active mDia1 induces Golgi dispersion in an actin polymerization– and myosin II–dependent manner

Golgi dispersion in LPA-treated cells is accompanied by the transient appearance of F-actin patches in the proximity of Golgi elements, as revealed by live imaging of mCherry-LifeAct–labeled cells (Supplemental Movie S3). To determine whether the effect of active mDia1 on Golgi integrity depends on actin polymerization, we treated cells with the actin polymerization inhibitor latrunculin B (Morton et al., 2000) and observed that such treatment reverses the effect of mDia1 on Golgi dispersion (Figure 2). Transfection of cells with a constitutively active truncated construct of mDia1 known as mDia1ΔN3 (Watanabe et al., 1999) produced the apparent dispersion of Golgi to an even more pronounced degree than that induced by Rho activation (Figure 2A, left; also see Figure 6A later). Latrunculin increased Golgi compactness, in agreement with previous results (Lazaro-Dieguez et al., 2006) (Figure 2B), whereas mDia1ΔN3 significantly decreased this morphometric parameter (Figure 2B). In cells expressing mDia1ΔN3, a gradual increase in LatB concentration reduced the mDia1 effect, returning Golgi compactness to control levels at 10 μM (Figure 2B). Taken together, these findings show that Rho-induced Golgi fragmentation is mediated by activation of mDia1; moreover, this effect depends on actin polymerization.

Similar to mDia1, myosin II activity is controlled by Rho (Vicente-Manzanares et al., 2009). Cell treatment with blebbistatin, an
This process requires de novo formation of numerous Golgi ministacks at the cell periphery, presumably at ER exit sites (Cole et al., 1996; Storrie et al., 1998) (Supplemental Movie S4). We found that microtubule depolymerization leads to pronounced Golgi dispersion not only in control cells, but also in mDia1-depleted cells and in cells with active mDia1. This finding enabled us to study how manipulations with Rho and mDia1 affect the recovery of dispersed Golgi following nocodazole removal.

Live imaging of Golgi recovery revealed that this is a two-stage process (Figure 3A and Supplemental Movies S5 and S6). The first stage comprises the rapid centripetal movement of Golgi fragments, leading to their concentration in the perinuclear area. In the second stage, the small fragments coalesce, or “fuse,” forming large, ribbon-like structures. We characterized the extent of Golgi complex recovery by measuring the average size (projected area) of individual Golgi fragments and the average number of such fragments per cell (Figure 3B).

The rate of fusion between Golgi elements during the second stage of recovery differed, depending on mDia1 and RhoA status. The fusion rate was maximal in mDia1 knockdown cells and eventually led to the efficient and rapid recovery of the Golgi in such cells (Figure 3A and Supplemental Movie S5). Control cells displayed a somewhat slower fusion rate (Figure 3A and Supplemental Movie S5), whereas the fusion of Golgi elements in cells expressing active Rho or active mDia1 was inefficient (Figure 3A, Supplemental Figure S3, and Supplemental Movie S6). These conclusions were supported by a rapid decrease in number and increase in size of Golgi elements in both mDia1-knockdown cells and in control cells (Figure 3B). At the same time, the number and size of Golgi fragments changed much more slowly in cells expressing active RhoA or active mDia1 (Figure 3, B and C), even though these particles were concentrated in the central part of the cell (Figure 3A, Supplemental Figure S3, and Supplemental Movie S6). These results suggest the involvement of Rho–mDia1 signaling during the fusion of Golgi elements into ribbon-like structures. Cells treated with latrunculin demonstrated slightly more efficient fusion of Golgi elements, in a manner similar to mDia1 knockdown (Figure 3C and Supplemental Figure S3).

Effects of Taxol on Rho-mDia1–mediated Golgi dispersion

We next studied the effect of the microtubule-stabilizing drug Taxol (Schiff and Horwitz, 1980) on Golgi reorganization induced by activation of the RhoA–mDia1 pathway. Surprisingly, Taxol treatment strongly enhanced the Golgi dispersion induced by either
Agreement with previous studies (Schiff and Horwitz, 1980). Live imaging of cells with labeled trans-Golgi and microtubules revealed the kinetics of microtubule-dependent Golgi dispersion mDia1ΔN3 or RhoA-V14 (Figures 4 and 5, Supplemental Movie S7, and unpublished data). Incubation of cells with Taxol for 3 h led to formation of prominent microtubule bundles (Figures 4 and 5), in agreement with previous studies (Schiff and Horwitz, 1980). Live imaging of cells with labeled trans-Golgi and microtubules revealed the kinetics of microtubule-dependent Golgi dispersion.
Rho–mDia1 pathway remodels Golgi complex

Rho–mDia1 activation led to the production of ministacks rather than the separation of the Golgi complex into individual cisternae.

The Rho–mDia1 pathway is involved in the formation of Rab6-positive, Golgi-derived vesicles

The small GTPases Rab6A and Rab6A′ localize to the trans-Golgi network and mark Golgi-derived exocytic carriers, as well as vesicles involved in Golgi-to-ER retrograde transport (Martínez et al., 1997; Girod et al., 1999; White et al., 1999; Del Nery et al., 2006; Grigoriev et al., 2007). To gain deeper insights into the functional role of the Rab6A–mDia1 pathway in Golgi dynamics, we examined the effects of RhoA activation and mDia1 knockdown on the generation of Rab6A-positive transport carriers (Figure 7 and Supplemental Movies S8 and S9). We found that RhoA activation significantly increased the abundance of such vesicles in comparison to control cells (Figure 7, A and B, and Supplemental Movie S8), whereas mDia1 knockdown completely abolished this increase (Figure 7, A and B, and Supplemental Movie S9). Of note, the Rab6A-positive tubular extensions radiating from the Golgi, as well as elongated tubular cytoplasmic vesicles, were more prominent in mDia1 knockdown cells, suggesting a fission defect (Supplemental Movie S9). Active RhoA did not appear to decrease the fraction of these Rab6A-positive tubular elements in mDia1-depleted cells (Supplemental Movie S9).

To determine the mode of action of mDia1 in formation of Rab6-positive vesicles, we used spinning disk confocal microscopy to study the colocalization of GFP-mDia1 and Cherry-Rab6A′ in cells expressing active RhoA (RhoA-V14). We found that both vesicular and tubular Rab6A′-positive structures often colocalize with small mDia1-positive patches (Figure 8). This colocalization event was very transient (no more than 30 s); however, such events could be seen in almost every frame (Figure 8). We found no colocalization of mDia1 and Rab6A′ in cells that did not express RhoA-V14 (unpublished data). Taken together, these results suggest that RhoA promotes the formation of Rab6-positive vesicles via activation of mDia1, which then transiently colocalizes with these structures.

Because Rab6-positive carriers are involved in exocytosis (Grigoriev et al., 2007), we checked whether mDia1 depletion or activation would affect the exocytosis of a membrane glycoprotein, temperature-sensitive vesicular stomatitis virus glycoprotein (VSVG). We did not detect, however, any differences in VSVG membrane delivery among control cells, cells expressing mDia1ΔN3, and mDia1-knockdown cells (Supplemental Figure S4).
FIGURE 5: Golgi dispersion induced by active mDia1 is enhanced by Taxol in an actin- and myosin II-dependent manner. HeLa JW cells transfected with GalT-YFP Golgi marker (green) alone (con) or together with active mDia1 (mDia1ΔN3) were either left untreated or incubated for 3 h with the microtubule-stabilizing drug Taxol (24 μM) alone (Taxol), with Taxol in combination with blebbistatin (50 μM) (Taxol + Bleb), or with Taxol and latrunculin B (2 μM) (Taxol + LatB). Microtubules (red) and F-actin (black and white photos) were visualized in the same cells by staining with antibody against tubulin and with phalloidin, respectively. Note that Taxol treatment does not induce significant Golgi dispersion in and of itself but strongly stimulates it in cells expressing active mDia1. Both inhibition of actin polymerization by latrunculin B and inhibition of myosin II activity by blebbistatin abolished this effect. Scale bar, 10 μm.

DISCUSSION

The major finding of this study is the discovery of the role of the Rho–mDia1 pathway in the modulation of Golgi architecture. This was demonstrated by experiments showing that constitutively active RhoA, as well as activation of Rho by LPA, appears to fragment the Golgi into ministacks; moreover, such fragmentation can be abolished by mDia1 knockdown. Expression of the active form of mDia1 also leads to similar Golgi fragmentation. The Rho–mDia1 pathway was also shown to be involved in the production of Rab6-positive, Golgi-derived transport vesicles. What are the mechanisms underlying the regulation of Golgi architecture through the Rho–mDia1 pathway? In search of mDia1 involvement in the local regulation of Golgi membrane sculpting, we examined the dynamics of mDia1 localization vis-à-vis the Golgi structures. We found that upon Rho activation, an mDia1-enriched "cloud" overlaps the Golgi complex. Transient F-actin patches visualized by means of the mCherry-LifeAct were also detected in this area. More definite colocalization of mDia1 and Golgi elements was found in Rab6-positive vesicular and tubular structures.

Furthermore, we determined that the effects of Rho and mDia1 on Golgi architecture depend on actin polymerization and can be abolished by treatment of cells with latrunculin. The enhanced dispersion of the Golgi in our experiments was also inhibited by blebbistatin, indicating myosin II involvement. The mDia1-dependent actin polymerization may, in principle, modify the hypothetical actin–spectrin coat surrounding the Golgi membrane (Beck and Nelson, 1998; Holleran and Holzbaur, 1998), thus affecting the membrane’s shape and mechanical characteristics.

Our experiments with Taxol suggest that Rho-mDia1–induced Golgi fragmentation may also depend on the interactions of Golgi membranes with microtubules. Like other formins, mDia1 could, in principle, interact with microtubules either directly or via microtubule-associated proteins (Bershadsky et al., 2006; Bartolini and Gundersen, 2010). Our observations of Golgi fragmentation dynamics in Taxol-treated cells are consistent with the notion that mDia1 might also modify such interactions. Thus, it appears that the Rho–mDia1 pathway controls the actin- and myosin II–mediated processes underlying the shaping and sculpting of Golgi membranes and may coordinate these processes with microtubule-based intracellular movements of the Golgi elements.

Membrane fusion and fission constitute the most basic processes underlying reorganization of complex membrane structures (Luini et al., 2008; Kozlov et al., 2010). Our data suggest that membrane fusion is the main mDia1-dependent process responsible for remodeling the shape of the Golgi complex. Constitutively active mDia1, as well as activation of Rho, inhibits fusion of Golgi fragments, preventing formation of Golgi ribbons in the process of recovery following nocodazole removal. During this process, knockdown of mDia1 somewhat promotes fusion of Golgi fragments.

In addition to suppressing Golgi membrane fusion, there exists some evidence that the Rho–mDia1 pathway helps to trigger fission events. In particular, the formation of Rab6-positive carriers may depend on membrane fission (Miserey-Lenkei et al., 2010). Constitutively active RhoA was shown to augment the production of such vesicles, the majority of which displayed a spherical morphology. In contrast, mDia1 knockdown enhanced the fraction of tubular, Rab6-positive carriers in our experiments. These results, together with localization of mDia1 to the Rab6-positive membrane structures, are consistent with the possible involvement of mDia1 in Golgi membrane fission; however, the molecular and physical mechanisms underlying mDia1-dependent membrane remodeling remain elusive.
Several recent studies (Salvarezza et al., 2009; von Blume et al., 2009; Miserey-Lenkei et al., 2010) demonstrated the involvement of other Rho-controlled, actin-associated regulatory and effector proteins in the functioning of the Golgi complex. Nonmuscle myosin II is regulated by RhoA via activation of Rho-associated kinase (ROCK) (Vicente-Manzanares et al., 2009). More recently, it was shown that myosin II interacts directly with Rab6 and plays an important role in the formation of Rab6-positive, Golgi-derived transport carriers (Miserey-Lenkei et al., 2010). This finding resembles the effect of the Rho–mDia1 pathway on the formation of the Rab6-positive vesicles found in our study. Our data suggest that RhoA-induced Golgi fragmentation and dispersion depend on both mDia1-driven actin polymerization and myosin II activity.

Another actin-related effector protein apparently involved in Golgi function is the actin depolymerizing protein ADF/cofilin (Rosso et al., 2004; Salvarezza et al., 2009; von Blume et al., 2009). Like mDia1, ADF/cofilin is regulated by a Rho-dependent pathway; thus, active Rho, via ROCK, activates LIM kinases (LIMK1 and LIMK2), which in turn phosphorylate and inactivate cofilin (Bernard, 2007; Bernstein and Bamburg, 2010). The LIMK1–cofilin pathway has been shown to participate in fission regulation (Salvarezza et al., 2009). Finally, another Rho-binding formin, DAAM1, was recently shown to be involved in the regulation of Golgi positioning and perhaps architecture (Ang et al., 2010).

Thus, mDia1 regulates Golgi architecture and dynamics in a Rho-dependent manner, perhaps in concert with other Rho effectors controlling actin polymerization and contractility. Elucidation of the precise molecular and physical mechanisms underlying mDia1 function in these processes is a challenging subject for future study.

MATERIALS AND METHODS

Chemicals and reagents
Nocodazole, latrunculin B, paclitaxel (Taxol), and LPA were purchased from Sigma-Aldrich (St. Louis, MO); blebbistatin from Calbiochem (Merck Eurolab, Darmstadt, Germany); and fibronectin from Biological Industries (Beit-Haemek, Israel).

HeLa JW cells (Paran et al., 2006) and HeLa JW cells stably transfected with cherry α-tubulin (kindly provided by Y. Paran and B. Geiger, Weizmann Institute of Science, Rehovot, Israel) were cultured in DMEM (Rhenium, Jerusalem, Israel) supplemented with 10% fetal calf serum (Biological Industries), l-glutamine, and penicillin–streptomycin solution (Sigma-Aldrich). Trypsin-EDTA (Biological Industries) was used to subculture the cells. Transfections of HeLa JW cells were performed in 36-mm dishes with jetPEI (Polyplus-transfection SA, Illkirch, France) according to the manufacturer’s instructions.

Plasmids
The mDia1-Flag, mDia1-GFP, mDia1ΔN3-GFP, and mDia1ΔN3-Flag (Watanabe et al., 1999; Higashida et al., 2004) were kindly provided by S. Narumiya (Kyoto University Faculty of Medicine, Kyoto, Japan). For preparation of monomeric red fluorescent protein (mRFP)–mDia1ΔN3, mRFP was amplified from pRSET-B-RFP plasmid and with taxol, still preserve joint cis and trans markers. Scale bar, 10 μm. (B) Transmission electron microscopy of control cells and cells expressing mDia1ΔN3. Arrows indicate the Golgi stacks; n, nucleus. Scale bar, 500 nm. Golgi elements in mDia1ΔN3 cells preserved a stacked structure.
sequence from bases 707-725 (GCATGAGATCATTCGCTGC) was synthesized, annealed and cloned in pSuper plasmids (Brummelkamp et al., 2002). The plasmids produced were then verified by DNA sequencing.

The HeLa JW siDia1 stable cell line was produced by transfection with the pSuper-retro-shDia1 plasmid. Selection was carried out in the presence of 0.4 μg/ml puromycin (Sigma-Aldrich) for 10 d. Western blot analysis of cell lysates showed an ∼90% reduction in mDia1 protein in the stably transfected cells. For mDia1 detection, mouse monoclonal antibody (mAb) against p140mDia1 (BD Biosciences, Heidelberg, Germany) was used at a 1:500 dilution in phosphate-buffered saline (PBS). Western blotting of α-tubulin (with the anti-α-tubulin antibody DM1A; Sigma-Aldrich) was used for loading control. Quantification of Western blot signals was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

Knockdown of mDia1

Knockdown of mDia1 was performed using pSuper Dia1-shRNA (shDia1) as well as pSuper-retro-Dia1-shRNA vectors, as described previously (Carramusa et al., 2007). Briefly, a small interfering oligonucleotide specific for human Dia1 and corresponding to its sequence from bases 707-725 (GCATGAGATCATTCGCTGC) was synthesized, annealed and cloned in pSuper plasmids (Brummelkamp et al., 2002). The plasmids produced were then verified by DNA sequencing.

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Videomicroscopy

Cells transfected with the plasmids listed were replated on fibronectin-coated, glass-bottomed dishes (MatTek Corporation, Ashland, MA) 6 h after transfection and placed on the microscope stage 24-36 h later. Images were recorded on an Olympus IX71 inverted fluorescence microscope equipped with a temperature and CO$_2$ control unit (Life Imaging Services, Reinach, Switzerland). Objectives used were Olympus plan ApoN 60×1.42 NA or Olympus 100×1.3 NA UplanFI. Images were filtered using the Unsharp Mask plug-in of Image J and converted to movies.

Image analysis

Vesicle density was measured using the ImageJ Analyze Particles plug-in applied to the polygon drawn around the cell. Before analysis, images were convolved and thresholded for better vesicle segmentation.

Golgi morphology was quantified using an index of circularity or compactness defined as $4\pi \times \text{Area}/\Sigma \text{Perimeter}^2$ (Bard et al., 2003), where Area represents the total Golgi projected area and $\Sigma \text{Perimeter}$ is the sum of the perimeters of all Golgi fragments. All of the parameters were measured per cell. The compactness index approaches the maximal value of 1 for the most compact shape, namely, a circle. At least 30 cells were taken for each measurement.

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