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 LPA or by expression of constitutively active mutants, resulted in pronounced fragmentation of the Golgi complex into mini-stacks. 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 is, however, poorly understood. Several factors are known to be integral to Golgi complex organization. The matrix proteins GRASP65, GRASP55, GM130, and GMAP210 are thought to tether Golgi cisternae to each other, and keep them together (Ramirez and Lowe, 2009; Sengupta et al., 2009). In addition, the cytoskeleton also 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 a 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 mini-stacks 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 mini-stacks 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 positioning. Structural information concerning the association of actin filaments with Golgi membranes is limited, although immunoelectron microscopy revealed β- and γ-actin at the Golgi-associated COPI-coated buds (Valderrama et al., 2000), and short filaments decorated with the tropomyosin isoform 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 (EM) 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 (Camera 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).

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 homolog 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 Diaphanous-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 Diaphanous-related formins, and specifically mDia1, were shown to be involved in a variety of in vivo functions [for a 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-YFP (YFP fused to the N-terminus of β-galactosyl-transferase), revealed disruption of the Golgi ribbon structure into smaller elements dispersed from the narrow perinuclear area over the entire central part of the cell (Fig. 1A, upper panel). A similar effect was observed using markers of medial-Golgi (mannosidase II-GFP), or cis-Golgi (endogenous p115, Grasp65) (see Figs. 3 and 6 below, and Supplementary Figs. 1-3). The active form of RhoB also triggered Golgi dispersion, while another small GTPase, Rac, did not produce any effect on Golgi morphology (data not shown).

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 re-organization. Experiments with LPA stimulation were performed in serum-free medium (Ren et al., 1999); serum-starvation by itself did not decrease the Golgi compactness (Fig. 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 (Supplementary Movie 1). Time-lapse filming of LPA-treated cells revealed the dispersion and centrifugal movement of the Golgi elements. In less than two hours, the ribbons underwent fragmentation into smaller elements that disperse radially outward from the cell center (Supplementary Movie 2 and Fig. 1B, upper panel).

The extent of fragmentation and dispersion of the Golgi was similar to that produced by expression of constitutively active Rho (Fig. 1A, upper panel). The
degree of Golgi dispersion was quantified using the index of “compactness” or “circularity” (Bard et al., 2003); the value of this index dropped twofold, both in cells treated with LPA, and in cells expressing constitutively active Rho (Fig. 1C, D).

**Effects of active RhoA on Golgi organization requires mDia1**

Since mDia1 is a well-known primary target of Rho, we investigated whether Rho-induced Golgi dispersion is mediated by this Rho effector. To this end, we examined whether constitutively active RhoA or LPA treatment would affect Golgi organization in mDia1 knockdown cells (Fig. 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 (Fig. 1E). We found that neither transfection with active RhoA, nor treatment with LPA, led to significant dispersion of the Golgi in mDia1-depleted cells (Fig 1A and B, lower panels). 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 (Fig. 1C and D). Of note, in mDia1 knockdown cells with non-stimulated 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 (Supplementary Fig. 1).

**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 (Supplementary Movie 3). 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 (Fig. 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 (Fig. 2A, left panel, and Fig 6A, below). Latrunculin increased Golgi compactness, in agreement with previous results (Lazaro-Dieguez et al., 2006) (Fig. 2A, upper right panel, and 2B), while mDia1ΔN3 significantly decreased this morphometric parameter (Fig. 2B). In cells expressing mDia1ΔN3, a gradual increase in LatB concentration reduced the mDia1 effect, returning Golgi compactness to control levels at 10 µM (Fig. 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 inhibitor of myosin II activity, produced some fragmentation of the Golgi complex (Supplementary Fig. 2A). Measurements of Golgi compactness revealed, however, that fragmentation induced by constitutively active mDia1 was significantly more pronounced (Supplementary Fig. 2B). Moreover, blebbistatin prevented the decrease of Golgi compactness induced by active mDia1 expression (Supplementary Fig. 2A and B). Thus, mDia1 functions in concert with myosin II, in the process of Rho-dependent Golgi fragmentation and dispersion.
Rho and mDia1 activation interfere with the fusion of Golgi elements recovering from nocodazole treatment

Depolymerization of microtubules by nocodazole treatment led to the pronounced disruption of the Golgi complex, and the appearance of numerous dispersed Golgi elements (Thyberg and Moskalewski, 1985, 1999). This process requires de novo formation of numerous Golgi mini-stacks at the cell periphery, presumably at ER exit sites (Cole et al., 1996; Storrie et al., 1998) and Supplementary Movie 4). 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 (Fig. 3A, and Supplementary Movies 5 and 6). 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 (Fig. 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 (Fig. 3A, and Supplementary Movie 5). Control cells displayed a somewhat slower fusion rate (Fig. 3A, and Supplementary Movie 5), while the fusion of Golgi elements in cells expressing active Rho or active mDia1 was inefficient (Fig.
3A, Supplementary Fig. 3, and Supplementary Movie 6). 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 (Fig. 3B). At the same time, the number and size of Golgi fragments changed much more slowly in cells expressing active RhoA or active mDia1 (Fig. 3B, C), even though these particles were concentrated in the central part of the cell (Fig. 3A, Supplementary Fig. 3, and Supplementary Movie 6). 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 (Fig. 3C and Supplementary Fig. 3).

**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 mDia1ΔN3 or by RhoA-V14 (Fig. 4, Fig. 5, Supplementary Movie 7, and data not shown). Incubation of cells with taxol for three hours led to formation of prominent microtubule bundles (Figs. 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 (Fig. 4 and Supplementary Movie 7). In taxol-treated cells that did not contain active mDia1, Golgi elements were concentrated near the ends of the microtubule bundles, usually in the cell center, and displayed essentially normal compact morphology (Fig. 4, and Supplementary Movie 7). In taxol-treated cells expressing the active form of mDia1, newly-formed microtubule bundles often moved from the center of the cell, to the
periphery (Fig. 4, and Supplementary Movie 7), along with the Golgi fragments associated with them. This process was accompanied by further fragmentation of Golgi elements (Fig. 4 and Supplementary Movie 7). In some cases, Golgi elements moved along microtubule bundles (Fig. 4 and Supplementary Movie 7). As a result, cells expressing active mDia1 that were incubated with taxol for three hours, displayed strong fragmentation of the Golgi complex, and dispersion of Golgi elements throughout the entire cell area (Figs. 4 and 5).

Enhanced Golgi dispersion induced by taxol in cells expressing active mDia1 can be prevented by simultaneous treatment of cells with latrunculin B, or with the myosin II inhibitor blebbistatin (Fig. 5). Thus, taxol treatment significantly enhanced Golgi fragmentation and dispersion induced by active mDia1, while inhibition of actin polymerization or myosin II-driven contractility suppressed both Golgi fragmentation and dispersion.

**Golgi fragmentation induced by active Rho and mDia1 leads to formation of Golgi mini-stacks**

To characterize the elements into which the Golgi complex is dispersed under conditions of Rho or mDia1 activation, we visualized the cis- and trans-Golgi compartment, using corresponding markers (the trans-Golgi marker, GalT-YFP, and the cis-Golgi marker, p115). As described in previous studies, cis- and trans- Golgi compartments are spatially separated, but adjacent to each other (Rothman, 1981; Glick and Nakano, 2009). Analysis of Golgi organization upon activation of Rho or mDia1 under various experimental conditions (Fig. 6) revealed that even the smallest fragments of Golgi contained both cis- and trans- compartments. In particular, the fragments resulting from maximal Rho-mDia1-mediated Golgi fragmentation (in cells
expressing active RhoA and treated with taxol) still consisted of spatially separated regions positive for cis- and trans- markers (Fig. 6A). The immunofluorescence observations were confirmed by transmission electron microscopy (TEM) of control cells, and cells expressing active mDia1. In both cases, Golgi stack structures were seen in the cells, even though in cells expressing mDia1ΔN3, they were smaller in size and slightly swollen (Fig. 6B). Thus, Golgi dispersion induced by Rho-mDia1 activation led to the production of mini-stacks, 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 exocytotic carriers, as well as vesicles involved in Golgi-to-ER retrograde transport (Martinez *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 RhoA-mDia1 pathway in Golgi dynamics, we examined the effects of RhoA activation and mDia1 knockdown on the generation of Rab6A-positive transport carriers (Fig. 7, and Supplementary Movies 8, 9). We found that Rho activation significantly increased the abundance of such vesicles, in comparison to control cells (Fig. 7A, B, and Supplementary Movie 8), while mDia1 knockdown completely abolished this increase (Fig. 7A, B, and Supplementary Movie 9). Notably, 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 (Supplementary Movie 9). Active RhoA did not
appear to decrease the fraction of these Rab6A-positive tubular elements in mDia1-depleted cells (Supplementary Movie 9).

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

Since Rab6-positive carriers are shown to be involved in exocytosis (Grigoriev et al., 2007) we have checked whether mDia1 depletion or activation would affect the exocytosis of a membrane glycoprotein, temperature-sensitive vesicular stomatitis virus glycoprotein (VSVG). We have not detected, however, any differences in VSVG membrane delivery between control cells, cells expressing mDia1ΔN3, and mDia1-knockdown cells (Supplementary Fig. 4).

**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, appear to fragment the Golgi into mini-stacks; 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 co-localization 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, inhibit 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. Non-muscle 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 depends 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 lysophosphatidic acid (LPA) were purchased from Sigma (Sigma, St. Louis, MO, USA); blebbistatin, from Calbiochem
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 Dulbecco’s Modified Eagle’s Medium (DMEM) (Rhenium Ltd., Jerusalem, Israel), supplemented with 10% fetal calf serum (FCS) (Biological Industries Israel), L-glutamine, and penicillin-streptomycin solution (Sigma). Trypsin-EDTA (Biological Industries Israel) 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 Dr. S. Narumiya (Kyoto University Faculty of Medicine, Kyoto, Japan). For preparation of mRFP-mDia1ΔN3, monomeric red fluorescent protein was amplified from pRSET-B-RFP plasmid and introduced into mDia1ΔN3-GFP using Eco47 III/Xho I sites, instead of GFP. Mannosidase II-GFP (Man II-GFP) was a gift from Dr. V. Malhotra (Center for Genomic Regulation, Barcelona, Spain). β-GalT-YFP was purchased from Clontech. RhoA-V14 was fused to a VSV tag, as previously described (Helfman et al., 1999). Rab6A-GFP was kindly provided by Prof. S. Lev (Weizmann Institute of Science), mCherry-Rab6A' was kindly provided by Dr F. Perez (Institut Curie, Paris, France); mCherry-LifeAct (Riedl et al., 2008) was kindly provided by Dr. R. Wedlich-Soldner (Max Planck Institute of Biochemistry, Martinsried, Germany).
Knockdown of mDia1

Knockdown of mDia1 was performed using pSuper Dia1-shRNA (shDia1) as well as pSuper-retro-Dia1-shRNA vectors, as described in our previous publication (Carramusa et al., 2007). Briefly, a small interfering oligonucleotide specific for human Dia1 and corresponding to its sequence from bases 707 to 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) for 10 days. Western blot analysis of cell lysates showed an ~90% reduction in mDia1 protein in the stably transfected cells. For mDia1 detection, mouse monoclonal antibody against p140mDia1 (BD Biosciences, Heidelberg, Germany) was used at a 1:500 dilution in PBS. Western blotting of α-tubulin (with the anti-α-tubulin antibody DM1A, Sigma) was used for loading control. Quantification of Western blot signals was performed using Image J software (NIH, USA, http://rsb.info.nih.gov/ij/).

Immunostaining and fluorescence microscopy

Following transfection, cells were plated on glass coverslips coated with fibronectin (20 µg/ml). Cells were then cultured for 24 hours, prior to treatment with drugs, and fixation.

For microtubule visualization, cells were fixed as previously described (Zilberman et al., 2009), and stained using an indirect immunofluorescence method with mouse monoclonal anti-α-tubulin antibody (clone DM1A, Sigma) and Cy3- or Cy5-conjugated
goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc., City, State, USA?). For mDia1, F-actin and Golgi visualization, cells were fixed with 3% paraformaldehyde in PBS for 20 min at 37°C, and then permeabilized with 0.25% Triton X-100 for 5 min. F-actin was stained with coumarin-phalloidin (Sigma); mDia1 was stained with the mouse mAb against p140mDia1 (BD Biosciences); and Golgi, with mouse anti-p115 or anti-Grasp65 antibodies (a gift from Prof. S. Lev).

Fluorescence images were captured with an Olympus IX71 inverted fluorescent microscope equipped with a CCD camera (Cool SNAP HQ, Photometrics, Tucson, AZ, USA), and controlled by a Delta Vision system (Applied Precision, Inc., Issaquah, WA, USA). A dichroic mirror, and excitation and emission filter wheels (Chroma Technology Corp., Rockingham, VT, USA) were adjusted for detection of FITC, DAPI, Rhodamine, and Cy-5.

Observations of the co-localization of GFP-mDia1 and Cherry-Rab6A’ were carried out using a PerkinElmer spinning-disk confocal microscope based on an inverted Olympus microscope IX81, equipped with a PL FL 100x1.4NA objective lens, Hamamatsu C9100-13 EMCCD camera for image acquisition, and Volocity software to control the setup (PerkinElmer, Waltham, MA, USA). Acquisition parameters were 100 msec exposure for the 488 channel, and 100 msec for the 561 channel. Lasers were set to 24% in each case. Images were converted into TIFF files by Image J software, and arranged into figures using Adobe Photoshop.

Transmission electron microscopy was performed as described in (Levenberg et al., 1998).

**Video Microscopy**

Cells transfected with the plasmids listed above were replated on fibronectin-coated glass-bottomed dishes (MatTek Corporation, Ashland, MA, USA) 6 hours following
transfection, and placed on the microscope stage 24-36 hours later. Images were recorded on an Olympus IX71 inverted fluorescence microscope equipped with a Temperature & CO₂ control unit (Life Imaging Services, Reinach, Switzerland; www.lis.ch/). Objectives used were Olympus plan ApoN 60x/1.42 NA, or Olympus 100x 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 Image J “analyze particles” plug-in, applied to the polygon drawn around the cell. Prior to analysis, images were convolved and thresholded, for better vesicle segmentation.

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

(Spell out all first names, the first time they are used)

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References


Figure Legends

Figure 1
Golgi dispersion induced by active RhoA is an mDia1-dependent process. (A and B) Control HeLa JW cells (con) and mDia1 knockdown cells (shDia1) – were either (A) transfected with constitutively active Rho (RhoA-V14) and fixed 24 hours later; or (B) treated with RhoA activator LPA (12 µM) in serum-free medium and filmed for 2 hours (see Supplementary Movie 2). (A) Golgi was visualized by transfection with trans-Golgi marker GalT-YFP (shown in green, see also the enlarged insets) and actin - by staining with phalloidin (red). RhoA-V14-transfected cells in (A) are marked by white asterisks. In (B) Golgi was visualized by ManII-GFP. Scale bars: (A) 10 µm, (B) 10 µm. (C, D) Degree of Golgi dispersion was quantified using a compactness (circularity) index calculated on the basis of morphometric measurements ((Bard et al., 2003) see also Materials and Methods). Bars represent compactness values ± SEM. P values were calculated according to the Student’s t-test. (C) Compactness values for control (con) and mDia1-knockdown (shDia1) cells treated, or not treated, with LPA were measured on fixed specimens stained with p115 cis-Golgi marker (D) Effect of RhoA-V14 on Golgi compactness in control (con) and Dia1-knockdown (shDia1) cells. (E) Western blot illustrating the depletion of mDia1 in HeLa JW cells expressing mDia1-targeted shRNA.

Figure 2
An active form of mDia1 (mDia1ΔN3) induces Golgi dispersion in an actin polymerization-dependent manner. (A) Cells were transfected with Golgi marker ManII-GFP shown in red (con), or co-transfected with ManII-GFP and constitutively
active mDia1 (mDia1ΔN3). Latrunculin B at 2 µM concentration was added 24 hours after transfection; cells were fixed 2 hours later. F-actin was visualized by phalloidin staining (shown in green). Scale bar: 10 µm. (B) Dispersion of Golgi induced by mDia1ΔN3 is gradually reduced in cells treated with ascending concentrations of latrunculin B. Complete return of the compactness value to control levels was observed in 10 µM latrunculin B-treated mDia1ΔN3-transfected cells. Error bars in (B) represent the standard error of mean (SEM) values.

Figure 3
Active Rho prevents fusion of Golgi elements into ribbon structures. (A) Cells expressing the Golgi marker ManII-GFP, alone or in combination with plasmids encoding shRNA against mDia1 or constitutively active Rho, were treated with nocodazole (2.5 µM) for 3 hours until the Golgi were completely dispersed. Nocodazole was then washed out, and the process of Golgi recovery was filmed in a time-lapse manner. Frames from the three time-lapse movies taken for cells of each type at indicated timepoints are shown in montage A (see Supplementary Movies 5 and 6). Scale bar: 10 µm; time: minutes. Note that even though Golgi elements move centripetally in all cases, efficient fusion of dispersed elements into ribbon structures occurs in control and mDia1 knockdown cells, but not in cells expressing active RhoA. Quantification of average size and number of Golgi elements at different time points after nocodazole removal are shown in graph (B). (C) Quantification of the effect of latrunculin and constitutively active mDia1 (mDia1ΔN3) on the dynamics of Golgi recovery after nocodazole treatment. The corresponding movie frames are shown in Supplementary Figure 3. The data are normalized to their initial value at the zero time-point. Particle size (in µm²) at the zero time-point (average ± SD): control
(1.3 ± 0.32), RhoV14 (1.3 ± 0.4), siDia1 (1.7 ± 0.4), mDia1ΔN3 (0.54 ± 0.14), LatB (0.54 ± 0.15). Particle number at the zero time-point (average ± SD): control (86 ± 15.8), RhoV14 (66 ± 12.27), siDia1 (64.4 ± 35.9), mDia1ΔN3 (106 ± 61.5), LatB (57.6 ± 25). For each cell type, 7-10 time-lapse movies were taken for this analysis. Error bars show standard deviation.

**Figure 4**
Dynamics of Golgi fragmentation and dispersion induced by taxol in active mDia1-expressing cells. Sequences from time-lapse movies illustrating the effect of taxol (24 µM) on microtubules (green) and Golgi (red) in a control cell (upper panel) and an mDia1ΔN3-expressing cell (lower panel). Microtubules and Golgi were visualized by transfection of cherry-α-tubulin and mannosidase II-GFP, respectively. Time after addition of taxol is shown in minutes. Note that taxol treatment leads to formation of prominent microtubule bundles in both control and mDia1-expressing cells. In control cells, Golgi membranes remain associated with the ends of these bundles, and do not undergo fragmentation. In mDia1ΔN3-transfected cells, a large Golgi fragment can move either together with moving bundles (arrows) or along such bundles (arrowheads). The process of fragmentation continues during the course of such movement (see Supplementary Movie 7). Scale bar: 5 µm.

**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 (shown in green) alone (con), or together with active mDia1 (mDia1ΔN3), were either left untreated, or incubated for 3 hours 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.

**Figure 6**

Dispersed Golgi elements preserve a mini-stack structure. (A) The Golgi compartments were labeled by cell transfection with the trans-Golgi marker GalT-YFP (green) and immunofluorescence staining with antibody to cis-Golgi marker, p115 (red). Non-fragmented Golgi in control or taxol-treated cells display typical cis- and trans-cisternae, forming the ribbon structure. Fragmented Golgi elements in RhoA-V14- or mDia1ΔN3-expressing cells, and even the smallest fragments in RhoA-V14-expressing cells treated with taxol, still preserve joint cis- and trans-markers. Scale bar: 10 µm. (B) TEM 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.

**Figure 7**

Production of Rab6-positive transport carriers is enhanced by active RhoA, and inhibited by mDia1 knockdown. (A) Rab6A-GFP was transiently expressed in control HeLa JW cells (con), in cells expressing active RhoA (RhoA-V14), in mDia1
knockdown cells (shDia1), and in mDia1 knockdown cells expressing active RhoA. Rab6A-GFP localizes to Golgi-derived vesicular and tubular carriers. Note the numerous vesicles in control cells expressing RhoA-V14, and the elongated (tubular) morphology of Rab6 membranes in mDia1 knockdown cells, with or without active RhoA (insets). Scale bars: 10 µm and 4 µm (insets). (B) The density of Rab6-positive carriers increases in control, but not in mDia1 knockdown cells expressing active RhoA. Error bars show standard deviation.

**Figure 8**

Co-localization of mDia1 and Rab6A’ in cells expressing constitutively active RhoA. Cells were triple-transfected with GFP-mDia1 (green), Cherry-Rab6A’ (red) and RhoA-V14-VSV (not shown). The co-localization regions in the merged images are colored yellow. The images of the same cell at two different time points are shown in upper and lower rows, respectively. Some sites of co-localization are indicated by arrowheads, and numbered. Insets on the right panel show magnified images corresponding to these co-localization events. Scale bars: 10 µm and 1.5 µm (insets).
**Supplementary Figures**

**Supplementary Figure 1**

Enrichment of mDia1 in the Golgi area induced by active RhoA. Cells co-transfected with RhoA-V14 and mDia1-GFP (green) were stained with cis-Golgi marker Grasp65 (red). Scale bar: 10 µm.

**Supplementary Figure 2**

Effect of blebbistatin on Golgi dispersion in control cells, and in cells expressing constitutively active mDia1. (A) Six hours following transfection with the Golgi marker mannosidase II-GFP alone (left panels), or together with constitutively active mDia1 (mDia1ΔN3) (right panels), cells were placed in either control medium (upper panels), or in a medium containing 50 mM blebbistatin (lower panels) and incubated for an additional 16 hours. After fixation, the cells were stained with TRITC-phalloidin to visualize the organization of F-actin. Scale bar 10 µm. (B) Bars represent compaction indices of Golgi complexes in control and treated cells. At least 30 cells were scored for each measurement. Error bars show standard deviation.

**Supplementary Figure 3**

Active mDia1 prevents fusion of Golgi elements into ribbon structures. Cells expressing the Golgi marker mannosidase II-GFP, alone or in combination with plasmid encoding mDia1ΔN3, were treated with nocodazole (2.5 µM) for 3 hours until the Golgi were completely dispersed. Nocodazole was then washed out, and the process of Golgi recovery was filmed in a time-lapse manner. Latrunculin (2 µM ) was added 1 hour before the nocodazole washout and was present in the medium throughout the recovery process. Scale bar: 10 µm; time: minutes.
Supplementary Figure 4
Effect of mDia1 activation and knockdown on the membrane delivery of VSVG glycoprotein. Control (con) cells, cells expressing active mDia1 (ΔN3), and cells with stable mDia1 knockdown (shDia) were transfected (or co-transfected) with a thermosensitive mutant of VSVG (VSVG-YFPts045). Cells were incubated at a non-permissive temperature (40°C) overnight, and at 32°C for an additional 2 hours, to enable the VSVG to approach the plasma membrane. Surface VSVG was detected by an anti-ectodomain antibody (Miserey-Lenkei et al., 2010). To calculate the ratio between surface and total VSVG, Z-stacks were taken and corresponding intensities were measured, using ImageJ software. Error bars show standard deviation.

Supplementary Movies

Movie 1: Golgi complex dynamics in HeLa JW cells. To visualize the Golgi, cells were transfected with mannosidaseII-GFP. Time-lapse: 1 frame per min; total duration 1 hour; acceleration 600 times. Arrow indicates the act of fusion of Golgi elements.

Movie 2: Activation of the Rho-mDia1 pathway by treatment with LPA leads to Golgi dispersion in control cells (upper panel), but not in mDia1-knockdown cells (lower panel). Golgi marker: mannosidase II-GFP. LPA was added to cells following serum starvation for 16 hours. Imaging began 5 min after the LPA application; time-lapse: 1 frame per min, total duration 2 hours, acceleration 600 times. Note the decrease in
size of the Golgi elements, and the increase in number and average mutual distance in cells treated with LPA. For still image, see Figure 1B.

**Movie 3:** Dynamics of actin and Golgi in cells treated with LPA to activate endogenous mDia1. Imaging began 5 min after the LPA application. Time-lapse: 1 frame per min, total duration 1 hour, acceleration 600 times. Golgi and F-actin were visualized by mannosidase II-GFP (shown in red) and mCherry-LifeAct (shown in green), respectively. Note the increase in number and intensity of actin aggregates in the proximity of the Golgi, at 10-15 min time points.

**Movie 4:** Microtubule disruption with nocodazole leads to rearrangement of the Golgi complex into smaller elements, localized throughout the cell area. Imaging began immediately following drug application. Time-lapse: 1 frame per min; total duration 2 hours; acceleration 600 times. Golgi was visualized with GalT-YFP. Note the complete disappearance of the central Golgi, and multiple instances of *de novo* formation of peripheral Golgi elements.

**Movie 5:** Dynamics of Golgi complex recovery following nocodazole washout: Comparison of control and mDia1 knockdown cells. Transient transfection of both types of cells with mannosidase II-GFP was used for Golgi visualization. Control cells (left panel) and shDia1 cells (right panel) were treated for 3 hours with nocodazole, until the Golgi complex was fully dispersed. Imaging began immediately after drug washout. Time lapse: 1 frame per min; total duration 3 hours, acceleration 600 times. For still image, see Figure 3.
Movie 6: Dynamics of Golgi complex recovery following nocodazole washout: Comparison of control cells and cells expressing active RhoA (RhoA-V14). Transient transfection of mannosidase II-GFP was used for Golgi visualization. Control cells (left panel) and RhoA-V14-expressing cells (right panel) were treated for 3 hours with nocodazole until the Golgi complex was fully dispersed. Imaging began immediately after drug washout. Time lapse: 1 frame per min; total duration 3 hours; acceleration 600 times. Note that in cells expressing active Rho, dispersed Golgi elements move towards the cell center, but fail to fuse into ribbons. For still image, see Figure 3.

Movie 7: Dynamics of the Golgi complex and microtubules in control and in mDia1ΔN3-expressing cells treated with taxol. HeLa JW cells stably expressing cherry-α-tubulin (shown in green) were transfected with the Golgi marker mannosidase II-GFP (shown in red), alone (control) or in combination with mDia1ΔN3. Imaging began immediately after the drug application. Time-lapse: 1 frame per 2 min; total duration 3 hours; acceleration 1,200 times. Taxol treatment induced formation of microtubule bundles in control (left panel) as well as in mDia1ΔN3-expressing cells (right panel). In control cells, the Golgi complex remained intact during the period of observation, while in cells expressing an active form of mDia1, Golgi elements move along the microtubule bundles (red arrow), or together with moving microtubule bundles (green arrow), which leads to further Golgi fragmentation and dispersion. For still image, see Figure 4.

Movie 8: Dynamics of Rab6A-GFP vesicles in control (left panel) and RhoA-V14 expressing cells (right panel). Time-lapse: 1 frame per sec; total duration 50 seconds; acceleration 10 times. Images are inverted, to facilitate visualization of tiny vesicular
and tubular structures. Note the increased number of Rab6 vesicles in cells expressing active RhoA. For still image, see Figure 7.

**Movie 9:** Dynamics of Rab6A-GFP vesicles in mDia1 knockdown cells (left panel) and in mDia1 knockdown cells expressing RhoA-V14 (right panel). Time-lapse: 1 frame per sec; total duration 50 seconds; acceleration 10 times. Images are inverted as in Supplementary Movie 7. Note the prominent Rab6-positive tubular extensions radiating from Golgi (black arrows) in mDia1-knockdown cells expressing, or not expressing, active RhoA. For still image, see Figure 7.