Coupling fission and exit of RAB6 vesicles at Golgi hotspots through kinesin-myosin interactions

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The actin and microtubule cytoskeletons play important roles in Golgi structure and function, but how they are connected remain poorly known. In this study, we investigated whether RAB6 GTPase, a Golgi-associated RAB involved in the regulation of several transport steps at the Golgi level, and two of its effectors, Myosin IIA and KIF20A participate in the coupling between actin and microtubule cytoskeleton. We have previously shown that RAB6–Myosin IIA interaction is critical for the fission of RAB6-positive transport carriers from Golgi/TGN membranes. Here we show that KIF20A is also involved in the fission process and serves to anchor RAB6 on Golgi/TGN membranes near microtubule nucleating sites. We provide evidence that the fission events occur at a limited number of hotspots sites. Our results suggest that coupling between actin and microtubule cytoskeletons driven by Myosin II and KIF20A ensures the spatial coordination between RAB6-positive vesicles fission from Golgi/TGN membranes and their exit along microtubules.

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The microtubule (MT) and actin cytoskeletons play important roles in Golgi structure and function. It is now well established that intact MT network and the minus-end MT dynein motor are required for maintaining the Golgi structure. MT depolymerization causes Golgi ribbon fragmentation and Golgi membranes redistribution near endoplasmic reticulum (ER) exit sites. In addition, cis- and trans-Golgi membranes can nucleate and polymerize MTs. This allows the dynamic association of the Golgi complex with the MTOC (MT organizing center) and polarized secretion of Golgi-derived transport carriers toward the leading edge of migrating cells.1 Actin depolymerization has less pronounced effect on Golgi structure, but leads to its compaction.2 Several studies have illustrated that actin and actin-binding proteins, including motors of the myosin family, regulate early events of transport biogenesis at the Golgi complex such as protein sorting and membrane fission.3–9

An important challenge is therefore to better understand how the actin and MT cytoskeletal systems are connected at the Golgi level. Some players have been identified. Connections can be established via the formin family proteins, such as mDia-1, formin-like/FMNL 1 and 2, and INF2 that are present on Golgi membranes.10–13 A WASP (Wiskott–Aldrich syndrome protein) homolog, WHAMM, directly interacts with MTs and was shown to play a role in the maintenance of

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**Fig. 1** GFP-RAB6-positive vesicles exit the Golgi at fission hotspots. a HeLa cells stably expressing GFP-RAB6 were imaged every second for 1 min (Supplementary Movie 1). Orange circles show the location of five fission hotspots. Details of each fission event for the five regions of hotspots are displayed. In each case, sequences of images are displayed at the top and a scheme of the events at the bottom. The Golgi region is highlighted by a white line. Vesicles exiting the Golgi are drawn and painted either yellow-green or orange-red. A sum of the events is shown at the end of each sequence. b Cells were treated for 45 min with para-nitro-blebbistatin. Then, para-nitro-blebbistatin was washed out. After recovery of normal GFP-RAB6 vesicles trafficking, the cells were imaged every second for 60 s (Supplementary Movie 3). A scheme of the images is displayed at the bottom. The Golgi regions are highlighted by a white line. Tubes are drawn in yellow. Regions where the GFP-RAB6-positive fission hotspots are found are drawn in red. Bars: 10 μm.
Golgi structure as well as in ER to Golgi transport\textsuperscript{14}. Other candidates are members of the Golgin family, such as the Dro- sophila golgin Lava lamp that interacts both with the dynein/ dynactin complex and spectrin\textsuperscript{15}, and the p230/golgin-245, shown to interact with MACF1, a giant protein that links MTs to the actin cytoskeleton\textsuperscript{16}.

In this study, we investigated how RAB GTPases, key regulators of intracellular transport and membrane trafficking, and molecular motors control the coupling between actin and MT cytoskeleton at the Golgi complex. One of the main functions of RAB GTPases is to recruit actin- or MT- based motors on transport carriers, allowing them to move along cytoskeletal tracks. This is the case for RAB6, the most abundant RAB at the Golgi that regulates several transport steps at the Golgi as well as Golgi homeostasis\textsuperscript{17–21}. Two closely related RAB6 isoforms, RAB6A and RAB6A\textsuperscript{′}, are present on Golgi/TGN membranes\textsuperscript{6,22}. In this manuscript, we will collectively call them RAB6. RAB6 was previously shown to directly or indirectly interact with several
motors, including KIF5B, KIF20A (also known as Rabkinesin-6/MKlp2), the dynein–dynactin complex (via Bicaudal-D), Myosin II and Myosin Va. However, it remains unknown whether RAB6 acts as a platform to couple actin- and MT-associated motors in order to coordinate the function of MTs and actin in Golgi function.

The interaction between RAB6 and Myosin II is critical for the fission of RAB6-positive transport carriers from Golgi/TGN membranes. Here, we show that KIF20A is also involved in the fission process. The coupling between actin and MT cytoskeleton driven by Myosin II and KIF20A ensures the spatial coordination of RAB6-positive vesicles formation at fission hotspots sites and their exit from Golgi/TGN membranes along MTs.

**Results**

**RAB6-positive vesicles exit the Golgi complex at fission hotspots.** We have previously shown that RAB6 and Myosin II are implicated in the fission of RAB6-positive transport carriers at the Golgi complex. The inhibition of this process leads to the formation of long membrane tubes connected to the Golgi complex. Detailed analysis of time-lapse microscopy of HeLa cells stably expressing GFP-RAB6 (Supplementary Fig. 1, Supplementary Movie 1) now revealed that RAB6-positive vesicles exit the Golgi complex in defined areas (Fig. 1a, Supplementary Fig. 1 and Supplementary Movie 1). We named them Golgi fission hotspots. A detailed illustration of Golgi fission hotspots for one Golgi is displayed in Fig. 1a. At the optical microscopy resolution, the Golgi fission hotspots are seen at the extremities rather than at the flatter regions of the Golgi. Over 60-s movies, we observed the existence of 6.4 ± 0.4 fission hotspots per Golgi (n = 13 Golgi) and measured a total of 12.5 ± 1.3 vesicles per min exiting the Golgi complex at fission hotspots (n = 13 Golgi). This indicates that 1–2 RAB6-positive vesicles exit the Golgi complex at fission hotspots per min (Fig. 1a). The formation of a vesicle usually takes 1 s (Fig. 1a; example in region #1, between time 3 and 4; example in region #5, between time 4 and 5). Movies performed on the same Golgi complex at 10-min interval revealed that the fission hotspots are stable over time (Supplementary Fig. 1, Supplementary Movie 2). If RAB6 vesicles exit the Golgi at preferential sites, one expects that these sites are similar to the ones where the membrane tubes are formed after inhibition of the fission process. To address this point, GFP-RAB6 cells were treated with the Myosin II inhibitor blebbistatin to inhibit fission and to allow the appearance of membrane tubes. Then, blebbistatin was washed out and the Golgi fission hotspots were localized using detailed analysis of time-lapse movies over a 60-s movie. The location of fission hotspots was similar to that of the RAB6-positive membrane tubes obtained after inhibition of the fission process (Fig. 1b, Supplementary Movie 3). Altogether, these results highlight the existence of RAB6-positive Golgi fission hotspots on the Golgi complex.

**KIF20A is involved in the fission process of RAB6-positive vesicles.** To decipher the molecular mechanisms of the fission process of RAB6-positive vesicles from Golgi membranes, we looked for proteins interacting with both Myosin II and RAB6. KIF20A, that we previously identified as a RAB6 effector present on Golgi membranes25,35, appeared as an interesting candidate since it was recently shown to directly interact with Myosin II.47,48

We originally proposed that KIF20A was responsible for the movement of RAB6-positive vesicles along MTs, since dominant negative mutants of KIF20A reduced trafficking25. Alternatively, KIF20A might be involved in the generation of RAB6 vesicles. Good evidence later showed that RAB6 vesicles move thanks to KIF 5B6.17. Consistent with these results, we found that KIF20A inhibition using paprotrain, a specific chemical inhibitor of KIF20A motor activity11, did not affect the speed of RAB6-positive vesicles (0.91 ± 0.04 µm/s in control, 0.89 ± 0.04 µm/s in paprotrain-treated cells, n = 47). To address the possible function of KIF20A in the regulation of RAB6 vesicles, we performed time-lapse video-microscopy experiments in HeLa cells stably expressing GFP-RAB6 following KIF20A inhibition with paprotrain or its depletion by short interfering RNA (siRNA) (Fig. 2a, Supplementary Fig. 2). Interestingly, in both cases, we observed the appearance of long tubules connected to the Golgi complex (Fig. 2a, Supplementary Fig. 2, Supplementary Movie 4) and a strong reduction in the number of RAB6-positive vesicles in the cytoplasm (Fig. 2b, Supplementary Fig. 2, Supplementary Movie 4). This phenotype, strikingly similar to the one observed after Myosin II inhibition or its depletion9, suggests that KIF20A is involved in the fission process of RAB6-positive vesicles from Golgi membranes. Fissions defects were also observed using a second inhibitor BKS0349 which is derived from paprotrain and displays higher affinity (Supplementary Fig. 2) or by over-expressing KIF20 constructs lacking the motor domain (Supplementary Fig. 3). To further confirm that the motor activity of KIF20A is required for the fission of RAB6-positive vesicles, we overexpressed at moderate levels (to avoid bundling of MTs) in control or KIF20A siRNA-treated cells, either wild-type or the K165A mutant of KIF20A (Supplementary Fig. 4). This conserved lysine in the P-loops of kinesins and myosins is absolutely required for nucleotide binding and motor activity.30,31 As expected, expression of GFP-KIF20A WT rescued the fission defects resulting from KIF20A depletion (Supplementary Fig. 4). In contrast, GFP-KIF20A-K165A expressing cells still displayed membrane tubules connected with the Golgi and a decreased number of vesicles in the cytoplasm (Supplementary Fig. 4). Interestingly, GFP-KIF20A-K165A by itself induced the appearance of Golgi-connected membrane tubules and fission defects, indicating that this construct acts as a dominant negative mutant (Supplementary Fig. 4). Altogether, these results demonstrate that the ATPase activity of KIF20A is required for the fission process of Rab6-positive vesicles.
Finally, as already reported after RAB6 or Myosin II depletion/inhibition, the trafficking of the secretory cargo VSV-G from the Golgi to the plasma membrane was impaired after KIF20A inhibition (Supplementary Fig. 2).

KIF20A, RAB6, and Myosin II are co-localized at Golgi fission hotspots. KIF20A can be visualized on fixed cells associated with the Golgi complex25 (Fig. 2c). The co-localization was more pronounced with the TGN marker TGN46 than with the cis-Golgi marker GM130 (Fig. 2c). We validated the Golgi localization of KIF20A using two different antibodies (Supplementary Fig. 5) as well as in a GFP-KIF20A cell line (Supplementary Fig. 5) and we showed that the Golgi signal decreased following KIF20A depletion by siRNA (Supplementary Fig. 5).

We performed a detailed analysis of the extent of co-localization between RAB6, Myosin II, and KIF20A on these Golgi fission hotspots. GFP-RAB6, Myosin II, and KIF20A were found to co-localize on dotted structures (Fig. 2d). On average, 6.3 ± 0.7 of these dots were found per Golgi (n = 21 Golgi). Following KIF20A inhibition with paprotrain, dots containing the three proteins were found at the base of membrane tubules, suggesting that these dots correspond to the sites of fission (Fig. 2e). To further confirm this hypothesis, we treated the cells with blebbistatin to induce the appearance of membrane tubules and then washed out the drug. As previously shown6, this leads to membrane fission along the tubules close to membrane bulges that are labeled by RAB6, cargos and F-actin. We observed an accumulation of KIF20A close to the membrane bulges, indicating the presence of KIF20A at the fission sites (Supplementary Fig. 5).

KIF20A displays two Myosin II binding sites. KIF20A was recently shown to directly interact with Myosin II27,28. The Myosin II binding site has been restricted to the last 60 amino acids of KIF20A and has been shown by co-immunoprecipitation experiments to be the main contributor in Myosin II binding28. Using yeast two-hybrid experiments, we confirmed this interaction and identified a second binding site corresponding to the KIF20A-529–665 fragment (Fig. 3a and Supplementary Fig. 6). This fragment was previously characterized as the RAB6 binding domain of KIF20A (RBD)25. The tail domain of Myosin II thus mediated this interaction along the membrane bulges, identifying the presence of KIF20A at the fission sites (Supplementary Fig. 2).
KIF20A recruits and/or stabilizes Myosin II at the Golgi complex. We then investigated whether KIF20A could be involved in the recruitment and/or stabilization of Myosin II at the Golgi complex. In order to quantify the amount of Myosin II on the Golgi, we used a rat cell line and a Myosin II-specific antibody, named AD7, whose specificity was previously validated. KIF20A depletion reduced by 35% the amount of Myosin II associated with the Golgi complex (Fig. 3c). Similar results were obtained using a Myosin light chain specific antibody (Supplementary Fig. 7). Altogether, the above results show that
both KIF20A and Myosin II act together in the fission of RAB6-positive vesicles from the Golgi complex.

**RAB6 is required for the Golgi association of KIF20A.** KIF20A interacts directly with RAB6. To further characterize this interaction, we solved the crystal structure of the complex between RAB6 and KIF20A (Fig. 4a-c). The RAB6:KIF20A structure reveals that the 603–645 residues of KIF20A represent the RBD. KIF20A-RBD is a dimer composed of two parallel alpha helices that form a right-handed coiled-coil additionally stabilized by an inter-helical cysteine bridge (Fig. 4a). Both RBD helices participate in RAB6 binding, two RAB6 molecules binding at opposite sides of the dimer (Fig. 4a-c). The RAB6:KIF20A interface is formed by complementary surfaces of the partners (Fig. 4b) that mediate hydrophobic and polar contacts between the molecules. KIF20A K629 and S630 residues are located in the central part of the RAB6-binding site (Fig. 4b, c) and form polar contacts with the partner (Fig. 4c). Prediction based on the structure indicates that substitution of these polar residues by bulky hydrophobic residues should substantially alter the structure indicates that substitution of these polar residues by bulky hydrophobic residues should substantially alter the structure of KIF20A, which then binds to Myosin II (see model on Fig. 6c and discussion).

**KIF20A limits RAB6 diffusion at the Golgi ensuring RAB6 localization at fission hotspots.** At the metaphase/anaphase transition, KIF20A was shown to stabilize the central spindle thanks to its additional C-terminal MT-binding domain and to promote the localization on MTs of several proteins, including Plk1 and Aurora B. A tempting hypothesis is that KIF20A fulfills a similar role on Golgi membranes to anchor RAB6 near MTs, which would favor the loading of RAB6 vesicles onto MTs.

To test this hypothesis, we performed FRAP (fluorescence recovery after photobleaching) experiments in HeLa cells stably expressing GFP-RAB6 (Fig. 5a, b, Supplementary Movie 5). After FRAP on Golgi extremities, a wave of RAB6 fluorescence coming from the core of the bleached area to the extremity of the Golgi was observed (Fig. 5b, Supplementary Movie 5). Following KIF20A inhibition with paprotrain, the wave of diffusion corresponding to RAB6 fluorescence was faster (Fig. 5c, Supplementary Movie 5) as exemplified at time 5 s. The half-time recovery after photobleaching was also reduced by 50% as compared to control cells (Fig. 5d, e), reflecting a higher mobility of GFP-RAB6 on Golgi membranes (Fig. 5d, e).

The above results indicate that KIF20A limits the diffusion of RAB6 molecules on Golgi membranes and confine them into membrane areas close to MTs.

**KIF20A co-localizes with Golgi-associated growing MTs on Golgi membranes.** After fixation from Golgi membranes, RAB6-positive vesicles are transported along MTs to the cell periphery. Golgi membranes can nucleate and polymerize MTs that serve in particular to sustain polarized secretion. We thus

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**Fig. 4** Definition of the RAB6-BD of KIF20A. RAB6 partially recruits KIF20A on the Golgi complex. RAB6 is required for KIF20A:Myosin II interaction. Relative binding affinities between RAB6, KIF20A, and Myosin II. a–e KIF20A-RAB6-binding domain (KIF20A-RBD) is a dimer composed of parallel helices that form a right-handed coiled-coil stabilized by an inter-helical cysteine bridge and two RAB6 molecules bind on opposite sides of the dimer. The RAB6:KIF20A interface makes hydrophobic and polar contacts between the molecules. b, in particular K629 and S631, are buried in the interface and involved in direct interactions at the center of the interface. d Yeast-two-hybrid interactions between the KIF20A-RBD:RAB6-529-665 fragment or KIF20A-RBD:RAB6-529-665-K626W-S631W fragment overexpressed in HeLa cells remained mostly cytosolic in contrast to the Golgi-localized wild-type form (Fig. 4e), demonstrating the importance of the RAB6:KIF20A direct interaction for KIF20A recruitment to Golgi. The mutations do not affect KIF20A-RBD dimerization and importantly its ability to interact with the tail domain of Myosin II (Fig. 4d). This suggests that RAB6 and Myosin II may bind to the KIF20A dimeric-coiled-coil (603–665) in different ways. Since this site has two RAB6-binding sites, one of them might be used for Myosin II binding rather than for RAB6 binding and the tripartite complexes between KIF20A, RAB6, and Myosin II can still be formed. Finally, depletion of RAB6 reduced by 40%, the amount of KIF20A associated with the Golgi complex (Fig. 4f), indicating that RAB6 contributes to the recruitment and/or the stabilization of KIF20A at the Golgi. Since the recruitment of KIF20A on the Golgi only partially depends on RAB6, it is likely that additional mechanisms are implicated or alternatively that the Golgi-associated pool of KIF20A is very stable.

To further understand this process, we tested whether RAB6 is required for KIF20A/Myosin II interaction. Co-immunoprecipitation experiments showed that the complex between KIF20A and Myosin II was lost in RAB6-depleted cells (Fig. 4g). Similar results were obtained in MEF cells derived from RAB6 conditional KO mice embryos and incubated in vitro with tamoxifen to deplete RAB6 (Supplementary Fig. 7).

We next measured the relative binding affinities of RAB6 for Myosin II, RAB6 for KIF20A, and KIF20A for Myosin II using a previously described solid-phase assay. These experiments revealed that RAB6 and Myosin II-1148-1652 bind to KIF20A-RBD-529-665 with similar affinities in the micromolar range (Fig. 4h). On the other hand, RAB6 binds to Myosin II-1148-1652 with around five times lower affinity (Fig. 4h). The fact that RAB6 and Myosin II display the highest affinities for KIF20A suggests that KIF20A is central for the interaction between RAB6 and Myosin II. A likely scenario is that RAB6 first recruits KIF20A, which then binds to Myosin II (see model on Fig. 6c and discussion).
wondered if KIF20A interacts with MTs that polymerize on Golgi membranes. First, we observed that after nocodazole treatment and washout, tubulin dots corresponding to reforming MTs co-localize with KIF20A (Fig. 6a). Second, Myosin II and RAB6 are found co-localized on peripheral dots with GCC185, a TGN Golgin known to recruit the MT + TIP CLASP proteins to the TGN, allowing the nucleation and formation of Golgi MTs (Fig. 6b). Quantification indicated that there are 7.51 ± 0.46 dots of triple co-localization per Golgi (n = 25 Golgi). Altogether, these results indicate that RAB6, KIF20A, and Myosin II are co-localized on dots corresponding to places of polymerization of MTs originating from Golgi membranes.

**Discussion**

The main finding of this study is the existence of preferential sites for the fission of RAB6-positive post-Golgi secretory vesicles on Golgi/TGN membranes. Hotspots for the fission of endocytic vesicles and ER-derived vesicles (ER-exit sites) as well as exocytic hotspots have been previously described at the plasma or ER.
membranes, but to our knowledge never on Golgi/TGN membranes.

We measured around six hotspots per Golgi. Remarkably, this number corresponds to the number of spots where RAB6, Myosin II, and KIF20A co-localize and to the number of membrane tubes observed after the inhibition of Myosin II ($n = 5$, ref. 6), that of KIF20A ($n = 5$, Fig. 2) or RAB6 depletion ($n = 4$, ref. 6). This suggests that a connection between actin and MT cytoskeletons established by an actin-based motor and a MT-based motor that interact with each other is necessary for the

![Diagram](image)

1. GFP-RAB6
2. MLC-mCherry
3. GCC185
4. GFP-RAB6
5. MLC-mCherry
6. GCC185
7. GFP-RAB6
8. MLC-mCherry
9. GCC185

Different proteins interacting at different locations.
generation of Golgi fission hotspots. It is likely however that additional mechanisms determine where and when hotspots can form. A tentative hypothesis is that some RAB6 molecules are concentrated in domains, maybe enriched in specific lipids such as PtdIns-4-phosphate. The existence of such domains has been postulated but not yet demonstrated. In addition, other fission promoting molecular complexes such as CIBP1-S/BARS, members of the 14-3-3 family, phosphoinositide kinase PI4KIIIβ, and lysophosphatidic acid acyltransferase δ have been identified. It will be important to test in future experiments whether KIF20A/RAB6/Myosin II complexes are physically or functionally related to these complexes. Another important issue will be to investigate the relationships between the budding and fission machineries of RAB6-positive vesicles. RAB6 depletion leads to the accumulation of budding profiles on Golgi membranes that are coated with clathrin and COPI. This suggests that the fission machinery driven by Myosin II and KIF20A is distinct from that required for budding.

The sequence of events that can be envisioned for the generation of fission hotspots is the following (Fig. 6c). RAB6 depletion leads to a decrease of Golgi-associated KIF20A, indicating that RAB6 participates, at least in part, to KIF20A recruitment on Golgi/TGN membranes. This would allow the clustering of RAB6 molecules and surrounding membranes to MTs. In support of this hypothesis, we showed that KIF20A inhibition increases the diffusion rate of RAB6 on Golgi/TGN membranes. Such a role for KIF20A is reminiscent of the one proposed during mitosis where KIF20A promotes the MT-dependent localization of Plk1, Aurora B, and Cdc14A to the central spindle. Myosin II would then be recruited by KIF20A and RAB6 and would drive the fission of RAB6-positive transport carriers from Golgi/TGN membranes. An alternative scenario is that Myosin II is first recruited and then interacts with KIF20A. This is however unlikely since depletion of KIF20A decreases the amount of Myosin II associated with Golgi/TGN membranes.

RAB6-positive transport vesicles move along MTs toward the cell periphery and the plasma membrane to convey secretory cargoes such as VSV-G or NPY. Whether the MTs used originate from MTOC or Golgi membranes is unknown, but our observations suggest that at least part of these MTs could originate from Golgi membranes. Indeed, KIF20A co-localizes with MTs that regrow from Golgi membranes. In addition, GCC185, a golgin involved in the formation of Golgi-associated MTs, could also be visualized on structures containing RAB6 and Myosin II. Importantly, the motor activity of KIF20A appears to be required for a normal fission process (Supplementary Figs. 2–4). As KIF20A is not the kinesin moving RAB6-positive vesicles along MTs (KIF5B is responsible for this, see refs. 6,17), two possibilities can be envisioned: the motor activity of KIF20A could be important to generate a level of membrane tension required for vesicle fission or alternatively it could be essential for the local organization of MTs to favor the right positioning of the fission machinery.

We previously showed that RAB6 controls the fission of its own transport carriers, which provides a mechanism for coordinating several events involved in the biogenesis of transport vesicles. Our study illustrates that in addition RAB6 could select a subset of MTs for transport of its own transport carriers. It will be interesting to see whether this is a unique mechanism or if other RAB GTPases that interact with actin and MT-based motors could function in the same way for the biogenesis of transport vesicles.

**Methods**

**Antibodies and reagents.** The following reagents were used: Paraformaldehyde (OPTOPHARMA Ltd), Y-27632 (Calbiochem), Papirotrain (Enzo Life Sciences). The following antibodies were used for biochemical experiments: Mouse anti-GM130 (BD Biosciences, #610822), sheep anti-TGN46 (ABD Serotec, AHP300), rabbit anti-KIF20A (ref. 25), Bethyl Laboratory, #A300-879A; proteintech, #15911-1-AP), goat anti-KIF20A (Santa Cruz Biotechnology, sc-104954), rabbit anti-RAB6 (ref. 47) and Santa Cruz Biotechnology, sc-310), human anti-Giantin (Racombinant antibody platform, Institut Curie, Paris, France); rabbit anti-myc (Santa Cruz Biotechnology, sc-1777), rabbit anti-Tubulin (Santa Cruz Biotechnology, sc-807), rabbit anti-HCDC45 (Bethyl Laboratory, A304-490A), human anti-β-tubulin (Recombiant antibody platform, Institut Curie), rabbit anti-GFP (Santa Cruz Biotechnology, sc-8454), rabbit anti-Plk1 (Cell Signaling Technology, #2161), rabbit anti-Aurora B (Cell Signaling Technology, #9296), rabbit anti-Cdc14A (Cell Signaling Technology, #4376), human anti-β-catenin (Cell Signaling Technology, #6194), mouse anti-poly-histidine antibody (Recombiant antibody platform, Institut Curie). The placem encapsulating the following fusion proteins were used: GFP-RAB6 (F. Perez, Institut Curie, mCherry-RAB6, MLC-mCherry (A.M. Lennon-Dumet, Institut Curie), GFP-KIF20A-AAC, GFP-KIF20A-S92-6655, GFP-KIF20A-S92-6875, GFP-KIF20A-25-687 (L. M. Yu, Institut Curie).

**Cell culture and transfection.** HeLa25 and Rat Clone 9 cells (ATCC #CRLL1439) were grown in DMEM medium (Gibco BRL) supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin, and 2 mM glutamine. Both cell lines were tested free for mycoplasma contamination. Cells were seeded onto 6-well plates, 18-, or 12-mm glass coverslips or Fluorodish and grown for 24 h before transfection. HeLa cells stably expressing GFP-RAB6A or GFP-KIF20A (KIF20A-BAC, a kind gift from A. Hyman, Dresden, Germany) were cultivated in the presence of 100 µg/ml geneticin (Gibco BRL). For expression of the constructs used in this study, HeLa cells were transfected using either the calcium phosphate precipitation method or X-tremeGENE9 (Roche) following the manufacturer’s instructions. For silencing experiments, HeLa and Rat Clone 9 cells were transfected with the corresponding siRNA once (in the case of RAB6A/V siRNAs) or twice at 24 h interval (in the case of KIF20A siRNAs) using HiPerFect (Qiagen) following the manufacturer’s instructions. For rescue experiments, cells were transfected with GFP-RAB6A or GFP-KIF20A-K165A 24 h after transfection with KIF20A siRNAs. Forty-eight hours after this second round of transfection, cells were proceeded for video-microscopy experiments. The N-terminal 25 amino acids truncated version of KIF20A was used because of better expression efficiency.

**RNA interference.** The sequences of the siRNAs used in this study are the following: human RAB6A/V (GACAUUCUUGAUCACCCAGA)48. These oligonucleotides were obtained from Sigma (Paris, France). For silencing human KIF20A, different sequences were used: one described in ref. 36 (obtained from Sigma) and specific SMART pools chemically synthesized by Dharmacon Research.
Inc. SiRNA targeting luciferase (CGUAGCCCAGAAUCUUUGA) was used as a control and was obtained from Sigma.

Yeast two-hybrid experiments. A yeast two-hybrid screen of a human placental cDNA library with mouse KIF20A-529-887 as a bait was performed by Hybrigenics SA (www.hybrigenics.com). Several clones corresponding to human non-muscle cDNA library with mouse KIF20A-529-887 as a bait was performed by Hybrigenics. Several clones corresponding to nucleotides 3984–4143 of the CDS of human Myosin IIA. Different sequences corresponding to human Myosin IIA have been amplified by PCR and inserted into the SmaI site of yeast two-hybrid pGADGH vector. Yeast two-hybrid experiments were performed as described in ref. 8 except that the LexA fusion proteins correspond to different domains of KIF20A described in ref. 8.

Construction of KIF20A mutants. For KIF20A-529-665-K629W-S631W mutants: single-point mutations were inserted in the mouse KIF20A sequence at position 1884, 1887, and 1893 using the QuickChange mutagenesis kit (Agilent). For mCherry-KIF20A-796-887: the 2388-2661 fragment of mouse KIF20A was amplified by PCR and inserted into a pOPIN-GFP vector (Addgene) allowing the mammalian expression of human Myosin II heavy chain gene have been isolated. These clones correspond to a protease inhibitor cocktail (Sigma). The following primary antibodies were used: human anti-Giantin (1:200, ref.50), mouse anti-GM130 (1:1000), mouse AD7 anti-myosin II (1:50 to 1:200, ref.33), rabbit anti-GCC185 (1:100), rabbit anti-KIF20A (1:2000 to 1:8000, ref.25), goat anti-GFP (1:2000), rabbit anti-MHC (Covance; 1:2000), rabbit anti-RAβ6 (Santa Cruz 1:1000 or ref. 47 1:2000), mouse anti-GFP (Roche; 1:1000), rabbit anti-KIF20A (Bethyl or A71425; 1:1000). Secondary Horseradish Peroxidase (HRP)-coupled antibodies were from Jackson Laboratories.

Immunofluorescence microscopy. HeLa or Rat Clone 9 cells grown on coverslips were fixed either in 4% paraformaldehyde (PFA) for 15 min at RT, in methanol (2 min, −20 °C) or in TCA (10% TCA 20 min at 4 °C, followed by a 3 min permeabilization in PBS, 0.1% Triton X-100). For mAD7 staining, cells were treated for 2 min with 0.1% Triton X-100 after fixation with PFA. Cells were then processed for immunofluorescence as previously described. The following primary antibodies were used: human anti-Giantin (1:200, ref. 50), mouse anti-GM130 (1:1000), mouse AD7 anti-myosin II (1:50 to 1:120, ref. 23), rabbit anti-GCC185 (1:100), rabbit anti-KIF20A (1:2000 to 1:8000), rabbit anti-MHC (1:200, Cell Signaling), human and rabbit anti-β tubulin (1:200, ref. 55), and sheep anti-TGN46 (1:1000). Fluorescently coupled secondary antibodies were obtained from Jackson. Coverslips were mounted in Mowiol and examined under a three-dimensional deconvolution microscopy (Leica DM-IRX2) with autofocus with a z-axis (Physik Instrument) and a 100 × 1.4NA-PL-APO objective lens for optical sectioning or a DMIRA Leica microscope with a 63× objective lens. Three-dimensional or one-dimensional multicolor image stacks were acquired using the Metamorph software (MDS) through a cooled CCD camera (Photometrics CoolSnap HQ). For deconvolution, cell images were acquired as described in ref. 8.

Time-lapse fluorescence microscopy. Transfected cells were grown either on glass bottom Fluorodish or on glass coverslips and transfected, just before observation, to custom-built aluminium microscope slide chambers (Ludin chamber, LIS). Time-lapse imaging was performed at 37 °C using a spinning-disk microscope mounted on an inverted motorized microscope (Nikon TE2000-U) through a 100 × 1.4NA PL-APO objective lens. The apparatus is composed of a Yokogawa CSU-X1 spinning disk unit, a Roper Scientific CoolSnap HQ2 CCD camera for image acquisition and Metamorph software (MDS) to control the setup. Acquisition parameters were 100 ms exposure for GFP channel and 100 ms for mCherry channel. Laser was set to 30% in each case. Images shown in figures correspond to the maximal intensity projection through the Z and performed with the ImageJ software (NIH Image). For FRAP analyses, HeLa cells stably expressing GFP-RAB6 were maintained in culture medium in glass bottom Fluorodish cell culture dishes and imaged on a similar spinning disk microscope equipped with FRAP head (Errol and Roper). Images were collected before bleaching of a rounded 30 pixels region of the Golgi apparatus every second for 5 s and after photo bleaching every 2 s for 20 s using the Metamorph software. After correction of the photo-bleaching due to acquisition, the background was subtracted. The intensity of fluorescence was then normalized and plotted on the graph.

Quantification of the number of transport carriers and membrane tubes. A total of 16–20 transfected cells were imaged for each condition. When cells were treated with drugs, quantifications were performed on the same cells, before and after drug treatment. The maximal intensity projection through the z axis of each image stack was done using the Image J software. The number of transport carriers (corresponding to transport carriers moving at least on three following snapshot) and tubes (connected to the Golgi complex) was manually counted using the cell counter macro from ImageJ (NIH Image).

Quantification of Myosin II and KIF20A Golgi-associated fluorescence intensity. Images of control or depleted cells were acquired using the same parameters, without automatic scaling and gain adjustment, avoiding saturated pixels. In all cases, the Golgi area was defined by the Giantin labeling, and the absolute intensity of Golgi-associated Myosin II or KIF20A in this area was measured (using Image J software). In the case of KIF20A depleted cells, since cells are multinucleated and have a bigger Golgi, the KIF20A Golgi-associated fluorescence intensity was normalized to the size of the Golgi.

Recombinant protein purification and solid-phase assays. GST fused to Myosin II-1148-1652 was expressed in the BL21 (DE3) strain of Escherichia coli after induction with 500 μM isopropyl-β-D-thiogalactopyranoside at 20 °C overnight. Cells were lysed in PBS, 2 mM β-mercaptoethanol, protease inhibitor and lysozyme by cell disruption. The GST fusion proteins were affinity-purified using Glutathione Sepharose 4B (GE Healthcare) and eluted with 50 mM Tris pH 8, 300 mM NaCl, 2 mM β-mercaptoethanol, 5% glycerol, and 10 mM reduced glutathione. Purified proteins were then dialyzed against a solution containing 20 mM Hepes pH 7.5, 100 mM NaCl, 5 mM MgCl2. GST fused to wild-type RAB6A was expressed in the BL21 codon plus (DE3)-RIL strain expressing RAB6A:GTP, and 0.4 mM GTP. A last step of affinity-purification using glutathione sepharose 4B at 20 °C overnight. Cells were lysed by cell disruption in 50 mM Hepes pH 7.5, 500 mM LiCl, 2 mM β-mercaptoethanol, 10 μM GTP, protease inhibitor, PMSF, and lysozyme by cell disruption in 50 mM Hepes pH 7.5, 500 mM LiCl, 2 mM β-mercaptoethanol, 10 μM GTP, and 15 mM reduced glutathione. Purified proteins were then dialyzed against a 2 L solution containing 25 mM Hepes pH 7.5, 50 mM NaCl, 2 mM β-mercaptoethanol, 2 mM MgCl2, 10 μM GTP. 6x His-Wild-type RAB6A was expressed in the BL21 codon plus (DE3)-RIL strain expressing RAB6A:GTP, 400 μM isopropyl-β-D-thiogalactopyranoside at 30 °C for 4 h. Cells were lysed by cell disruption in 50 mM Hepes pH 7.5, 500 mM LiCl, 2 mM β-mercaptoethanol, 1 mM MgCl2, 10 μM GTP, protease inhibitors, PMSF, and lysozyme. Proteins were affinity-purified using HitTrap Chelating HP (GE Healthcare) and eluted with 50 mM Hepes pH 7.5, 500 mM LiCl, 2 mM β-mercaptoethanol, 1 mM MgCl2, 10 μM GTP, 500 mM Imidazole. His-tags were removed by recombinant Tobacco Etch Virus (rTEV) protease cleavage during overnight dialysis in 50 mM Hepes pH 7.5, 100 mM NaCl, 2 mM β-mercaptoethanol, 1 mM MgCl2, 10 μM GTP. A last step of gel filtration with Superdex 75 16/600 (GE Healthcare) was performed with a buffer containing 25 mM Hepes pH 7.5, 50 mM NaCl, 1 mM MgCl2, 2 mM DTE, 10 μM GTP. 6x His-KIF20A-529-665 was purified as in ref. 85. GST-GFP was a kind gift of the recombinant antibody platform (Institut Curie).

Solid-phase assays were carried out as in ref. 85. Briefly, 96-well plates (Nunc) were coated with 10 μg of either GST-GFP, GST-Mycosin II-1148-1652, GST-RAB6A-GTP, and 6–40 μg recombinant 6xHis-RAB6B-GTP or 6xHis-KIF20A-529-665 proteins was used. Interactions were performed in a buffer containing 20 mM Hepes pH 7.5, 100 mM NaCl, and 5 mM MgCl2. Protein interaction was revealed by anti-6 × His antibodies (1:1000), using 100 μl TMD (BD Biosciences) as a chromogenic substrate at room temperature for 10 min. The reactions were stopped by the addition of sulphuric acid. Absorbance was measured at 450 nm in a plate reader.

Purification, crystallization, and structure determination of RAβ6:KIF20A-603–665 complex. The KIF20A fragment (603–665) was cloned in pGEXII plasmid, containing a N-terminal GST-tag followed by a rTEV protease cleavage site. RAβ6A-Q72L fragment (8–195) was cloned in a modified PACYC plasmid with an introduced rTEV cleavage site after the N-terminal His-tag. The complex was purified by Ni-NTA affinity chromatography using a QAF ion exchange column and a Q5/2–Q72L–8–195 in E. coli BL21 (DE3). The complex was purified using Glutathione Sepharose 4B in a batch mode. The complex was eluted by rTEV cleavage and
subsequently purified by Ni-NTA. Extra RAB6A-Q72L-8-195 was added to the purified complex mix to ensure saturation of KIF20A-RBD with the binding partners.

The complex at concentration of 10 mg/ml was crystalized in 100 mM Hepes pH 7.4, 200 mM ammonium sulfate, 24% (w/v) PEG 8 K, 2% (v/v) isopropanol in hanging drop geometry at 17°C. A 2A resolution data set was collected at the European Synchrotron Radiation Facility (ESRF) synchrotron (beamline ID29).

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Author contributions
S.M.L. carried out the experiments presented in Figs. 1, 2a,d,e; 3, 4d,g; 5, 6b. Supplementary Figs. 1–7 with the help of S.B.; H.B. the experiments in Fig. 4h and constructs in Supplementary Figs. 3, 4; A.D. the experiments in Figs. 2c, 6a; V.F. helped for microscopes development and image analysis; G.B. and O.P. determined the X-ray structure with the help of R.B.; O.P., G.B. and A.H. analysed the structure; C.B. and C.G. designed, synthesized and developed paprotrain and BKS0349; S.M.L., A.E., A.H., B.G., O.P. conceived the project; S.M.L., H.B., A.D., A.E., A.H., B.G., O.P. designed and interpreted the experiments; S.M.L., O.P., A.E., A.H., B.G. wrote the manuscript. S.M.L., H.B., O.P., S.B., A.D., V.F., C.G., C.B., A.H., A.E. and B.G. edited the manuscript. B.G and A.H. secured funding.

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