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COMMENTARIES

High-content analysis of Rab protein function at the ER-Golgi interface

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ABSTRACT. The Rab family of small GTPases play fundamental roles in the regulation of trafficking pathways between intracellular membranes in eukaryotic cells. In this short commentary we highlight a recent high-content screening study that investigates the roles of Rab proteins in retrograde trafficking from the Golgi complex to the endoplasmic reticulum, and we discuss how the findings of this work and other literature might influence our thoughts on how the architecture of the Golgi complex is regulated.

KEYWORDS. ER-Golgi interface, Golgi morphology, high-content screening, Rab proteins, retrograde traffic

THE RAB PROTEINS AT THE ER-GOLGI INTERFACE

The Rab GTPases are a large family of small GTP-binding proteins that regulate distinct aspects of the endomembrane system, including vesicle budding, uncoating, motility, fusion, membrane organization and identity, often through the recruitment of effectors such as vesicle tethers, SNAREs, membrane and motor proteins. Rab proteins accomplish their functions by switching between an inactive GDP-bound and an active GTP-bound form, which determines their ability to bind effectors. In their inactive state, Rabs are bound to a molecule of GDP dissociation inhibitor (GDI),

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which keeps them in a soluble cytoplasmic state. The switch of Rab proteins from the GDP-bound state to the GTP state is mediated by Rab-specific guanine nucleotide exchange factors (GEFs), and GTP hydrolysis is enhanced by GTPase-activating proteins (GAPs). In addition, so-called GDI displacement factors (GDFs) have been proposed to play a role in selectively removing Rabs from GDI and help position the Rab at the appropriate membrane.[1,2]

Around one third of the ~60 members of the Rab GTPase family found in human cells have been associated with either the endoplasmic reticulum (ER) and Golgi complex, or the membrane intermediates at their interface (**Fig. 1**), of which only a handful have been reasonably well characterized.[3] The most prominent members are Rab1, Rab2, and Rab6 variants; where Rab1 and Rab2 isoforms regulate bi-directional ER-Golgi trafficking and Rab6 isoforms regulate intra-, early- and post-Golgi trafficking.[4,5] Other less-characterized Rabs have been also been associated with the functionality of the Golgi complex, for example Rab33b, Rab34, Rab41 and Rab43 have all been linked with intra-Golgi traffic and to a certain extent Golgi organization. Several Rab proteins (e.g. Rab8 isoforms, Rab10, Rab11a, Rab14, and Rab31) function at the *trans*-Golgi network (TGN), orchestrating trafficking events to or from the endosomal system.[6,7] At the ER, close to a dozen Rab proteins (**Fig. 1**) perform a diversity of functions, such as organelle maintenance, membrane tubulation, and control of the lipid environment.[8,9]

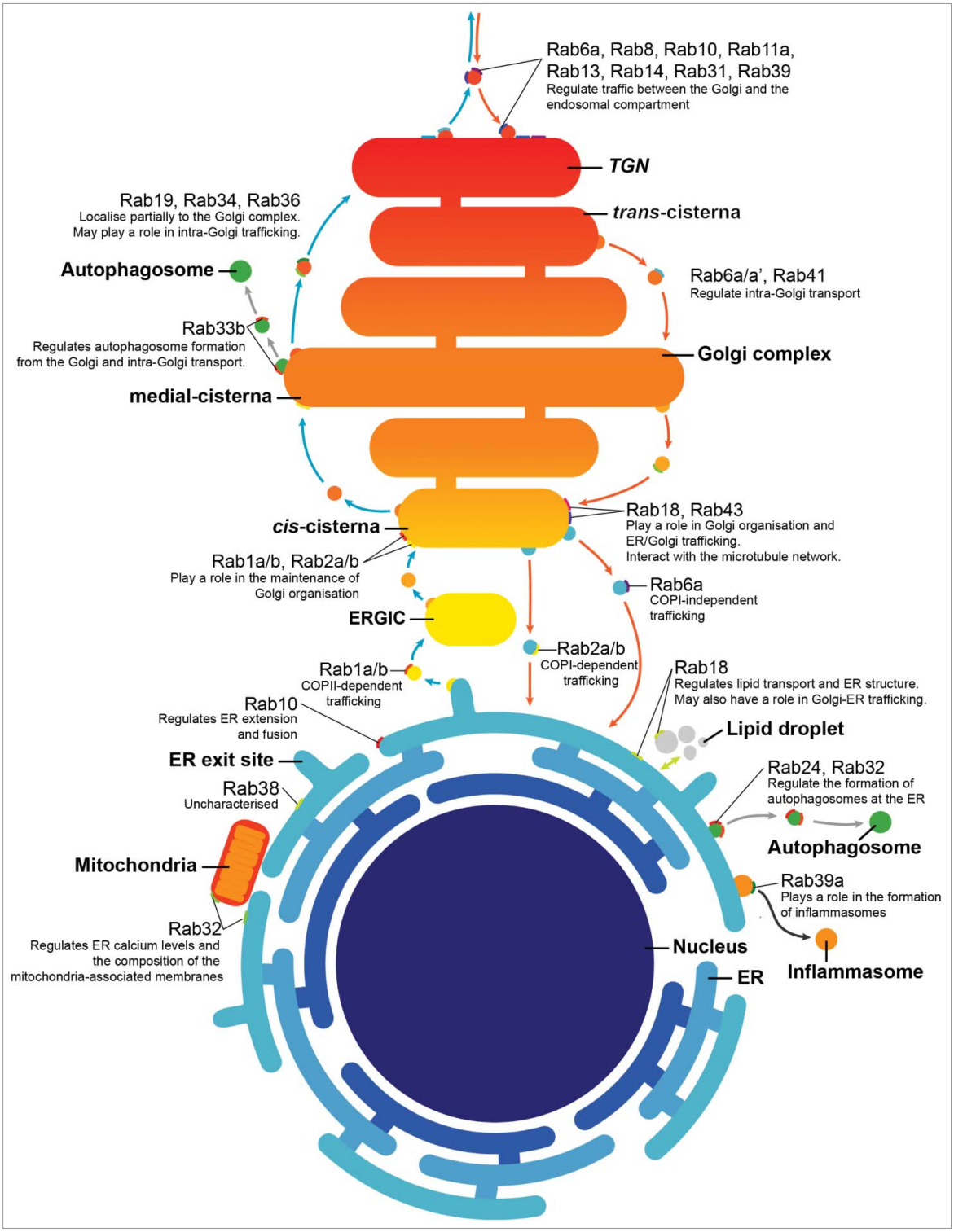
The fact that such a variety different Rab proteins are found in this part of the endomembrane system suggests that there also must be numerous and complex molecular mechanisms in place to ensure their functionality and maintain the local membrane architecture. Surprisingly, despite the field of Rab protein biology being relatively advanced, we are still adding to the list of Rab proteins functioning at the ER-Golgi interface. For example, in the last 3 years, Rab10 and Rab18 have been associated with the regulation of ER structure and membrane tubule morphology. Complexes containing Rab10 have been found to regulate ER extension and fusion in association with microtubules,[10] while Rab18 in conjunction with the Rab3GAP complex was observed to be required to maintain normal ER structure.[11] Of additional note is that in recent years the ER and Golgi complex have become associated with functions beyond their conventional roles in protein and lipid synthesis and transport. One notable example would be a role for these organelles in autophagy.[12-14] Despite the fact that these 2 sets of membranes engage in such a variety of tasks, all involving continual loss and gain of material, remarkably they are able to retain their unique morphologies in the cell. Clearly therefore, there is still much to discover about the ER-Golgi dynamic and how Rab proteins contribute to events here.

GOLGI ORGANIZATION VS. *TRANSPORT*

As the Golgi is effectively the meeting point between the secretory pathway and the endosomal system, maintenance of the morphology of this organelle at steady-state must at least partially be a consequence of a highly regulated balance between the amount of membrane that is internalised at the cell surface and that leaving the ER. Any significant imbalance between these trafficking pathways would be expected to affect the structure and therefore function of the Golgi; similarly alterations in the organization of the Golgi would be expected to result in the alteration of traffic rates in and out of the organelle. Therefore, it seems highly likely that the 2 processes of membrane traffic and organelle maintenance are tightly linked.

Several Rab proteins have been shown to participate in membrane traffic and organelle maintenance, such as Rab1, which regulates COPI-coat mediated transport between the ER and Golgi through associated interactions with key effectors such as GBF1. In parallel, it can interact with and recruit the *cis*-Golgi matrix proteins GM130 and giantin, both found to be crucial for the regulation of Golgi structure and the tethering of coated vesicles.[15-17] Similarly, Rab2 also promotes the recruitment of the COPI coat complex to the Golgi membrane and

FIGURE 1. Schematic of the Golgi-ER interface illustrating the localization, function and transport pathways of the Rab proteins according to the literature. Blue and red arrows depict anterograde and retrograde traffic, respectively.

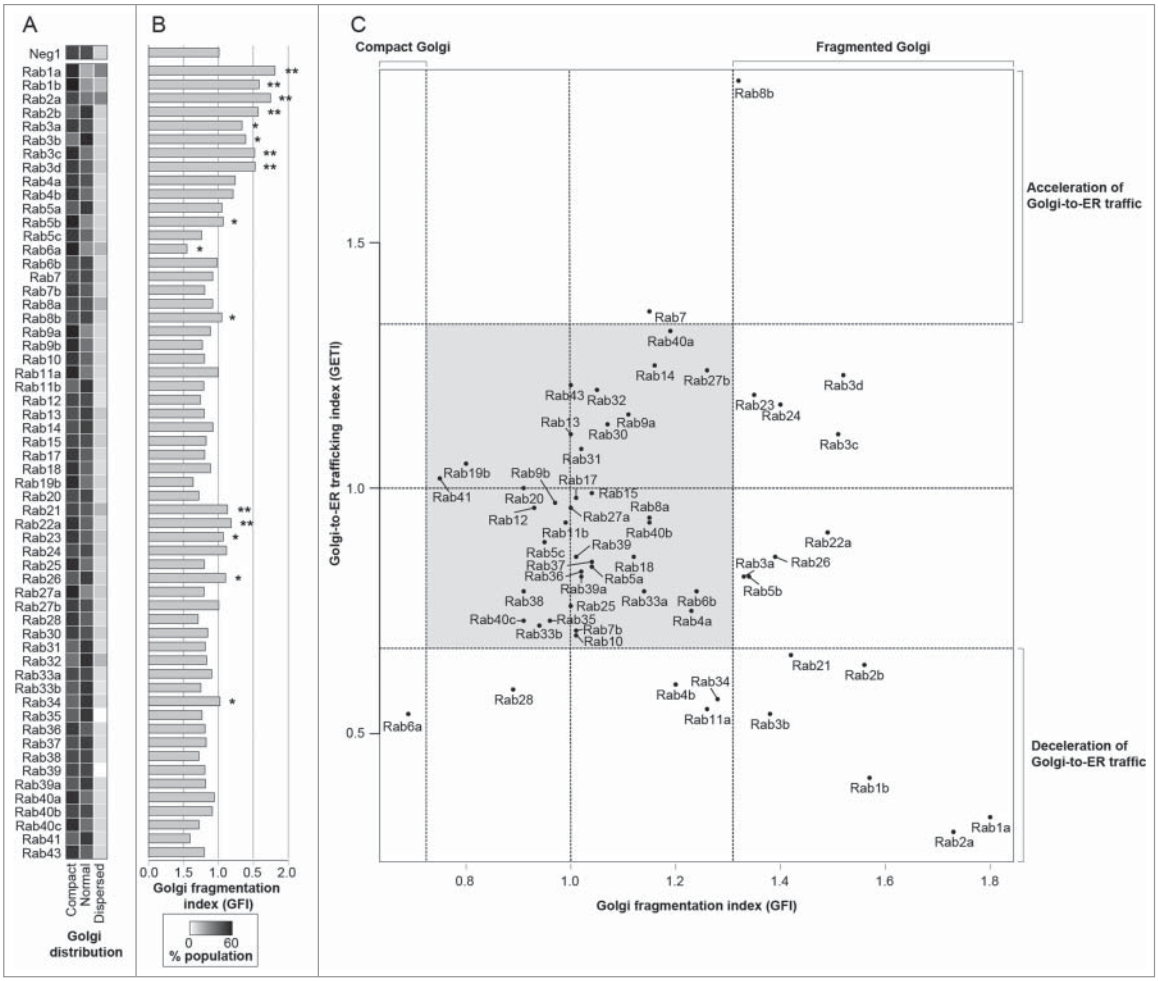


it interacts with GM130 and golgin-45, 2 regulators of Golgi structure.[8,18-20] These examples clearly highlight the linkage between a role for Rab proteins in organelle structure and trafficking, but when one considers the extensive number of Rab proteins localizing to these membranes, it seems likely that other family members will be involved. In order to further investigate this, we have recently reported the effects of systematic protein depletion of the Rab protein family in the context of transport pathways exiting the Golgi complex, particularly toward the ER.[21] This study provides the first systematic view of the key Rab molecules operating at the ER-Golgi interface with new implications for how Golgi architecture is maintained.

Using a high-content microscopy-based approach, we systematically assessed and ranked proteins involved in Golgi-to-ER retrograde transport in cultured mammalian cells. The screen was performed using a cell line stably expressing a GFP-tagged Golgi enzyme, which was treated with brefeldin A (BFA) to stimulate the production of Golgi-to-ER carriers. This allowed us to carry out a population analysis of the cellular response to the BFA treatment, specifically to determine the percentage of cells retaining an intact Golgi complex at each time point after treatment. Loss of this organelle under these conditions effectively represents the rate of transport carrier formation and movement of Golgi residents to the ER, a process which has an absolute requirement for membrane traffic machinery molecules such as Rab proteins. Logarithmic transformation of the data obtained at each time point allowed us to apply a linear model and extract a slope value. The value obtained for a population of cells treated with negative control non-silencing small interfering RNA (siRNA; Neg siRNA), was used to normalize all subsequent experiments giving us a Golgi-to-ER trafficking index (GETI). A GETI value of 1 is considered to represent the Golgi-to-ER trafficking kinetics of cells in control conditions, while values smaller or larger than 1, represent the inhibition or acceleration of this transport step.[21] Using the same image dataset we were able to extract various morphological and texture features describing Golgi complex organization after siRNA treatment, but prior to BFA addition. The texture features were utilized to classify the cell population into 3 categories (normal, compact and dispersed) based on their respective Golgi patterns (**Fig. 2A**), while at the same time Golgi fragments for each cell were counted to quantify the extent of disruption of the organelle. The resulting mean number of Golgi fragments per cell and per well were normalized to the values determined from cells treated with the negative control siRNAs on a plate-by-plate basis, which allowed the calculation of a 'Golgi fragmentation index' (GFI). GFI values greater than 1.0 indicate Golgi fragmentation whereas values smaller than 1.0 indicate Golgi compaction (**Fig. 2B**) (The image analysis pipeline is presented in more detail in[22]).

This quantitative image analysis routine revealed strong Golgi fragmentation and dispersion phenotypes in cells depleted of Rab1 and Rab2 variants. Rab1a and Rab2a were found to cause the most significant Golgi fragmentation with GFI values of 1.80 and 1.73 respectively. The depletion also affected the distribution of the Golgi, with a large percentage of cells in the population exhibiting a dispersed distribution (29% and 28%, respectively) when compared with the negative control (8%). On the other end of the spectrum, Rab6a was found to have the lowest GFI value of 0.68, with 52% of the cells exhibiting a compacted distribution of the Golgi. These parallel experiments allowed us to rank the proteins with respect to their influence on Golgi structure as denoted by the GFI values, both in terms of fragmentation and compaction. The candidates were then further divided into strong and weak effectors, based on the statistical significance of their effect on the Golgi, when compared to cells treated with negative control siRNAs. Specifically, depletions with a p-value of 0.05 to 0.10 were denoted as weak regulators, whereas depletions that had a p-value smaller than 0.05 were considered strong regulators. In total, 8 Rab proteins (Rab1a, Rab2a, Rab1b, Rab2b, Rab3d, Rab3c, Rab22a, and Rab21) were identified as strong disruptors (and therefore likely regulators) of Golgi

FIGURE 2. The effects of Rab protein depletion on Golgi organization and retrograde traffic in HeLa cells. (**A**) The heat map indicates the type of Golgi morphology observed in a population of cells depleted for a specific Rab protein. Texture features were utilized to classify the cell population into 3 categories (normal, compact and fragmented) based on their respective Golgi patterns. The results are presented as percentage of the population. (**B**) The number of Golgi fragments per cell was measured and a Golgi fragmentation index (GFI) value was calculated for each condition as described in.[22] Asterisks indicate p-values; * (<0 .10 to 0.05), and ** (<0 .05). (**C**) Graphical representation of the output of the 2 assays carried out in cells systematically depleted for each Rab protein, correlating GFI and Golgi-to-ER trafficking index (GETI) values. Eight proteins displayed effects on both Golgi structure and Golgi-to-ER trafficking. (**A-C**) Results are presented as means from 3 independent experiments.



structure and 6 Rab proteins (Rab24, Rab26, Rab3b, Rab23, Rab5b, and Rab3a) as weak disruptors. We also identified one Rab protein (Rab6a) causing a Golgi compaction phenotype.

These corresponding experiments therefore allowed us to directly compare the effect of Rab protein depletion on Golgi organization and transport, through the direct comparison of the GETI and GFI values. Correlation between effects on Golgi structure and retrograde transport out of the Golgi was observed for many of the identified regulators, with Rab1, Rab2, Rab3b, Rab6a, Rab8b and Rab21 being particularly prominent (**Fig. 2C**). Perhaps unsurprisingly, the primary Rabs associated with the

ER-Golgi interface, Rab1 and Rab2, were found in this group of strongest regulators of both Golgi organization and transport. As discussed above, Rab1 and Rab2 variants have long been associated with the regulation of anterograde and COPI-dependent retrograde traffic, in addition to linking to Golgi matrix proteins. Depletion of the COPI-independent retrograde pathway regulator Rab6a also inhibited Golgi-to-ER traffic in our assay, as well as inducing a compact Golgi phenotype, similar to that previously described.[23-25] This GTPase has been shown to interact with various Golgi proteins but one of its binding partners, myosin-II, is particularly interesting, since it provides a link between the Rab protein and the actin cytoskeleton.[26] Depletion of this myosin using siRNAs also induces a compact Golgi in cells (Galea & Simpson, unpublished observations), a phenotype that has been observed by others on perturbation of the actin network.[27,28] Although Rab6a is a fundamental driver of membrane flow from Golgi cisternae (it is highly visible on the carriers themselves), these observations suggest a wider role in maintenance of Golgi architecture perhaps through the actin cytoskeleton.

Interestingly, the depletion of Rab3 and Rab21 also resulted in the alteration of Golgi structure and inhibition of Golgi-to-ER traffic. This was unexpected, as other studies have suggested that these Rab family members are primarily localized to membranes distal from the ER-Golgi interface. The individual down-regulation of the 4 Rab3 isoforms revealed that Rab3a and Rab3d induced strong Golgi fragmentation, whereas Rab3b strongly inhibited Golgi-to-ER transport along with a mild fragmentation of the organelle. At first glance, these results are not easily explained, especially with regard to their influence on retrograde trafficking, as Rab3 isoforms have been associated principally with the regulation of secretory vesicles and to a certain extent endosomal compartments.[29,30] These GTPases are relatively poorly characterized and most of the studies examining their regulatory roles have been carried out in neurons and endocrine cells, in which they are enriched.[31] However, recent reports have linked Rab3 with multiple effectors which themselves function either in Golgi organization or Golgi-to-ER trafficking. Particularly interesting are 2 effectors, the growth-arrest-specific gene 8 (GAS8), a microtubule-binding protein found on Golgi membranes,[32] and the Rab3GAP complex (Rab3GAP1/2) shown to be required for the recruitment of Rab18 and vesicle-associated membrane protein (VAMP) associated protein B (VAPB) to the ER membrane and ER-Golgi interface, respectively, where both proteins are needed for the organization,[11,33] lipid control and trafficking activities[34-37] of the 2 organelles. Knowledge of these interactions allows us to postulate that the depletion of Rab3 isoforms might have an upstream effect on early secretory pathway membranes, either indirectly by causing an imbalance in trafficking to or from the TGN, or directly through a change in recruitment kinetics of these Rab3 effectors recently found at the ER-Golgi interface. Another consideration is how Rab3 levels may influence other Rabs on nearby membranes. The downregulation of any Rab protein in a network can result in an increased concentration of its effectors in a soluble pool. This might have various consequences, including up- or down-regulation of proteins in complementary networks[38] to compensate for the imbalance, the disruption of Rab cascades due to higher levels of GAPs or GEFs common to other Rab proteins leading to uncontrolled budding or fusion of vesicles,[9,39] or in the case of the 2 effectors described above, misregulation of the lipid environment at the ER and Golgi. Altogether, Rab3 isoforms and their interactors seem likely to be playing a wider role in organization of the ER-Golgi interface than previously appreciated.

The second endosomal GTPase identified, Rab21, predominantly localizes to the early endocytic pathway, although a small population residing on the Golgi stacks has been visualized in immuno-electron microscopy studies.[40] This Rab protein has been implicated in numerous functions, including the trafficking of integrins and receptors responsible for cell adhesion, migration, cell polarity and cytokinesis.[41,42] However, it has also been shown to interact with various actin and

microtubule cytoskeleton regulators, which in turn coordinate the transport of intracellular vesicles.[43-45] The Rab21 GEF, Varp, has been shown to interact with the TGN-localized golgin p230,[43] a protein important for Golgi positioning and possibly dynein-dynactin directed transport.[46] These interactions therefore implicate Rab21 in a role regulating the cytoskeletal organization of the Golgi, in addition to its established trafficking role at the TGN and endosomes.

Our results also revealed certain Rab protein depletions that disrupted only one process (either trafficking or Golgi architecture) without having an effect on the other. For example, a number of Golgi-associated Rab proteins identified in the Golgi-to-ER trafficking screen, namely Rab10, Rab11a and Rab34, each showed no detectable effect on Golgi morphology when depleted. Rab10 and Rab11a have been shown to mainly reside on various endosome populations, however in both cases a small proportion has been reported to be present on juxta-nuclear Golgi membranes[47] and, as described above, Rab10 has been recently associated with ER membranes.[10] We observed fluorescently-tagged Rab10 and Rab11a on Golgi-derived carriers induced in the presence of BFA, suggesting that they might play a more direct role in transport than previously appreciated. This will clearly need further in depth analysis to understand its significance. Rab34 localizes throughout the Golgi stack, both on Golgi cisternae and on a population of Golgi-associated vesicles, including *cis*-Golgi-derived transport carriers.[48] Previous protein depletion studies have shown that Rab34 is required for anterograde intra-Golgi transport of GFP-tagged vesicular stomatitis virus glycoprotein (VSV-G), a model secretory cargo molecule, but that its depletion has no effect on Golgi-to-TGN transport.[48] Why the depletion of Rab34 inhibits Golgi-to-ER traffic is also unclear at this stage. One possibility is that by disrupting intra-Golgi traffic, there would be a consequential change in the intra-Golgi distribution of certain membrane-associated molecular machinery (for example SNAREs and tethering factors) that would need to be assembled on retrograde-destined carriers prior to their departure from the Golgi. If this was the case, it highlights an impressive regulatory mechanism to govern the fidelity of membrane traffic events from this organelle.

CONCLUSION

A huge number of contributions over the years allow us now to appreciate that several Rab proteins, in particular Rab1, Rab2, Rab8, Rab6, Rab18, Rab33b and Rab43 are fundamental for normal Golgi function in mammalian cells.[18,49,50] A number of these proteins seemingly play a wider role in linking organelle morphology with trafficking at the ER-Golgi interface. Cellular depletions of either Rab1a/b, Rab2a/b or Rab6a all exhibit a strong influence on Golgi organization as well as Golgi-export, while comparatively few Rab proteins known to function in trafficking in or out of the TGN affect organelle structure when depleted. This suggests that membrane flux at the ER-Golgi interface, rather than at the Golgi/TGN-endosome interface, plays a more critical role in governing the morphology of this central organelle. Alterations in trafficking of specific lipids or structurally important membrane proteins supplied from the ER are undoubtedly part of the explanation for many of the observed effects on these membranes, but further systematic approaches are needed if we are to fully understand how the internal architecture of the cell is maintained.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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