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RESEARCH ARTICLE

A high-content screening microscopy approach to dissect the role of Rab proteins in Golgi-to-ER retrograde trafficking

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ABSTRACT

Here, we describe a high-content microscopy-based screen that allowed us to systematically assess and rank proteins involved in Golgi-to-endoplasmic reticulum (ER) retrograde transport in mammalian cells. Using a cell line stably expressing a GFP-tagged Golgi enzyme, we used brefeldin A treatment to stimulate the production of Golgi-to-ER carriers and then quantitatively analysed populations of cells for changes in this trafficking event. Systematic RNA interference (RNAi)-based depletion of 58 Rab GTPase proteins and 12 Rab accessory proteins of the PRAF, YIPF and YIF protein families revealed that nine of these were strong regulators. In addition to demonstrating roles for Rab1a, Rab1b, Rab2a, and Rab6a or Rab6a′ in this transport step, we also identified Rab10 and Rab11a as playing a role and being physically present on a proportion of the Golgi-to-ER tubular intermediates. Combinatorial depletions of Rab proteins also revealed previously undescribed functional co-operation and physical co-occurrence between several Rab proteins. Our approach therefore provides a novel and robust strategy for a more complete investigation of the molecular components required to regulate Golgi-to-ER transport in mammalian cells.

KEY WORDS: Rab proteins, High-content screening, Retrograde traffic

INTRODUCTION

In eukaryotic cells, the secretory pathway is responsible for the biogenesis and correct intracellular distribution of a wide range of proteins, complex carbohydrates and lipids. At the heart of the secretory pathway is the Golgi complex, which not only serves as the first major destination for newly synthesised secretory cargo leaving the endoplasmic reticulum (ER), but also is important in receiving material from endocytic compartments. The Golgi complex is therefore a highly dynamic organelle, requiring highly regulated membrane traffic processes in order to maintain its size, shape and physical composition.

Bi-directional membrane traffic between the ER and the Golgi complex occurs through similar general mechanisms. In both cases, membrane carriers form on the donor organelle, followed by budding, transport and then tethering to and fusion with the target organelle. Distinct machineries facilitate the formation of the anterograde and retrograde transport carriers, with a key role of this machinery being cargo selection and maintenance of the fidelity of traffic. Cytoplasmic coat protein complexes are known to play a significant role in this regard, with coat protein complex II (COPII) driving cargo export from the ER, and coat protein complex I (COPI) facilitating transport from the Golgi back to the ER (reviewed by Lorente-Rodriguez and Barlowe, 2011). This scenario was further complicated by the discovery that Golgi enzymes, and also certain protein toxins passing through the entire retrograde pathway from cell surface to ER, can traffic from the Golgi complex to the ER independently of COPI function (Girod et al., 1999; White et al., 1999). Although the regulation mechanism of this so-called COPI-independent pathway is still poorly characterised, it nevertheless suggests that a wider range of transport carriers operate from the Golgi than simply those involving the COPI coat.

Additional control of membrane traffic is provided by the Rab GTPases, a family of small GTP-binding proteins that further specify membrane identity and vesicle budding, uncoating, motility and fusion through the recruitment of a wide variety of effector proteins. Rab proteins switch between an inactive GDP-bound and an active GTP-bound form, which in turn determines their ability to bind to relevant membranes and interacting proteins, termed the 'membrome' (Gurkan et al., 2005). GDP–GTP exchange is mediated by Rab-specific guanine-nucleotide-exchange factors (GEFs), GTP hydrolysis is enhanced by GTPase-activating proteins (GAPs) (Barr and Lambright, 2010) and GDP dissociation inhibitor (GDI)-displacement factors (GDFs) have been proposed to play a role in selectively removing Rabs from GDIs and helping position the Rab at the appropriate membrane (Moya et al., 1993; Sivars et al., 2003). In humans there are at least 60 Rabs, and the crosstalk between multiple Rabs through shared effectors, or effectors that recruit selective Rab activators, has been shown to be important for the spatiotemporal regulation of vesicle traffic (Pfeffer and Aivazian, 2004; Sannerud et al., 2003; Vetter and Wittinghofer, 2001).

Various large-scale approaches have been applied to catalogue the key residents of the Golgi complex, including biochemical isolation and proteomics (Wu et al., 2000), and, more recently, microscopy-based organelle morphology studies (Chia et al., 2012). We and others have previously used RNA interference (RNAi) screening to identify factors important for the transit of secretory cargo through this organelle (Bard et al., 2006; Simpson et al., 2012; Wendler et al., 2010); however, to date, there have been no systematic studies focusing on the cargo exit from this organelle, particularly in the retrograde direction towards the ER. Possible reasons for this are the existence of relatively few well established markers for this pathway, the difficulty to study it in isolation from the anterograde step and the slow rate of Golgi enzyme recycling (Cole et al., 1998; Storrie et al., 1998). One way in which some of these shortcomings can be overcome is through the use of the fungal metabolite brefeldin A (BFA). Addition of this drug stabilises the complex between the GDP-bound form of ARF1 (the small GTPase required to activate COPI assembly on the Golgi membrane) and

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Received 22 December 2014; Accepted 18 May 2015

Journal of Cell Science (2015) 128, 2339-2349 doi:10.1242/jcs.167973

GEFs containing a Sec7 domain. This prevents ARF1 activation resulting in loss of COPI from the Golgi membranes and collapse of the organelle into the ER, typically within 30 min of application. This redistribution of Golgi markers to the ER is accompanied by a dramatic tubulation of Golgi membranes (Doms et al., 1989; Klausner et al., 1992; Lippincott-Schwartz et al., 1990; Peyroche et al., 1999).

The wide use of BFA also has served to shift the paradigm that all intracellular transport events are vesicular in nature. Studies of post-Golgi traffic using light and electron microscopy in non-perturbed cells have revealed the presence of extensive tubular membranes (Luini et al., 2008; Trucco et al., 2004), and we have previously shown that as many as 20% of the membranes involved in transport steps between the ER and Golgi complex are tubular (Simpson et al., 2006). Combining these observations with the fact that both the cis and trans regions of the Golgi complex are also composed of extensive membrane tubule networks, the evidence is strong that membrane tubule formation is an inherent property of this organelle. Use of BFA therefore, could be considered a method to simply highlight and enhance tubule synthesis, effectively stimulating Golgi-to-ER trafficking events.

The challenge is to design a robust and quantitative methodological approach capable of systematically identifying the protein machinery associated with this transport step. Traditional microscopy methods only permit low throughput of samples, with results being determined from a relatively small cell population. Therefore, in this study, we have used high-content screening (HCS) microscopy in conjunction with RNAi to quantitatively probe the relevance of all Rab GTPases to the formation of Golgi-derived transport carriers and their transfer of cargo to the ER. Our experimental design allows both accurate population analysis from a high number of cells, and also the ability to test individual genes and gene combinations in a highly parallel and quantitative manner for their relevance to this pathway.

RESULTS

Golgi export trafficking assay

In order to systematically identify molecules that play a regulatory role in retrograde transport from the Golgi to the ER we developed an automated microscopy assay based on the localisation of a fluorescently tagged marker of the Golgi complex. Specifically, we utilised a previously described HeLa cell line stably expressing GFP-tagged N-acetylgalactosaminyltransferase-2 (GalNAc-T2, also known as GALNT2) (Storrie et al., 1998). To stimulate the formation of Golgi-derived carriers, we treated these cells with BFA for increasing lengths of time. We observed a complete redistribution of the enzyme to the ER after 30 min of treatment, as shown by colocalisation with the ER shaping protein REEP-5 (Voeltz et al., 2006) (supplementary material Fig. S1A). By contrast, little colocalisation was found between GalNAc-T2 and the trans-Golgi network (TGN) marker TGN46 (also known as TGOLN2), which has been shown to redistribute into the endosomal compartment under these conditions (Chege and Pfeffer, 1990) (supplementary material Fig. S1). In order to provide a robust and quantitative measure of this trafficking event, we used an automated fluorescence microscope to capture images of cells under these conditions (Fig. 1A), which were then subjected to automated image analysis using HCS software (see Materials and Methods for details) (Galea and Simpson, 2013). This allowed us to carry out a population analysis of the cellular response to BFA treatment specifically to determine the percentage of cells retaining an intact Golgi complex at each time point (Fig. 1B). Loss of this organelle under these conditions effectively represents the rate of transport carrier formation and movement of GalNAc-T2 cargo to the ER. We observed that 49% of the population displayed a redistribution of the Golgi marker within 5 min of BFA treatment, with an exponential loss of the Golgi across the entire period of study. Repeating this experiment three times, each with four technical replicates, revealed high reproducibility in the data obtained (supplementary material Fig. S2). 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 small interfering RNA (siRNA; Neg siRNA), was used to normalise all subsequent experiments giving us a Golgi-to-ER trafficking index (GETI). We consider the kinetics of this experiment to represent control conditions, and therefore assigned it a GETI value of 1.0 (Fig. 1B). The GETI of non-transfected cells was found to be not significantly different from that determined from cells transfected with negative control siRNAs (data not shown). To demonstrate the validity of this approach, we next treated cells with siRNAs targeting Sec22b, a protein believed to be the sole functional SNARE on COPI-coated transport carriers, playing a role in their docking and fusion with the ER (Burri et al., 2003; Dilcher et al., 2003; Lewis et al., 1997). As expected, there was a marked increase, compared to the negative control, in the number of cells retaining an intact Golgi at all time points after BFA treatment (Fig. 1C), resulting in a GETI value of 0.47 (Fig. 1D). This indicates that under these conditions trafficking between these organelles has been slowed, and demonstrates the validity of this approach in terms of being able to identify factors regulating Golgi-to-ER traffic.

Rab GTPase screen

We next applied the above strategy to analyse the role of Rab family proteins in Golgi-to-ER trafficking, as to date no systematic investigation has been carried out. Using the experimental approach shown in Fig. 1 we studied BFA-induced retrograde traffic in cells depleted for 58 Rab proteins, and 12 accessory proteins, including three members of the prenylated Rab acceptor domain family (PRAF), seven members of the Yip1 domain family (YIPF) and two members of Yip-interacting factor family (YIF). Initial visual inspection of these 70 time-course experiments revealed that depletion of several of the targets led to a noticeable increase in retention of intact Golgi in the presence of BFA (Fig. 2A). Automated quantification of the images allowed us to calculate a GETI value for each siRNA treatment (Fig. 2B), and therefore rank all these proteins with respect to their influence on Golgi redistribution (Fig. 2C). The candidates were then further divided into strong and weak effectors, based on the statistical significance of their strength of inhibition, as compared to cells treated with negative control siRNA. In total, we identified 11 Rab proteins and one Rab accessory protein as being strong inhibitors of Golgi-to-ER retrograde traffic and five Rab proteins as weak inhibitors (Fig. 2C). Downregulation of Rab2a was found to have the most significant impact on Golgi-to-ER redistribution, with Rab1a and Rab1b having the next strongest effect. Depletion of Rab6a and the Rab6a splice variant Rab6a′ (hereafter denoted Rab6a/a′), previously described regulators of COPI-independent trafficking, also strongly reduced Golgi-to-ER redistribution, as did other Golgi-associated Rab proteins (Rab2b, Rab11a and Rab34). Four Rab proteins that are known to be mostly associated with endosomal membranes, Rab28, Rab21, Rab3b and Rab4b, also showed a significant inhibitory effect on this transport step. In

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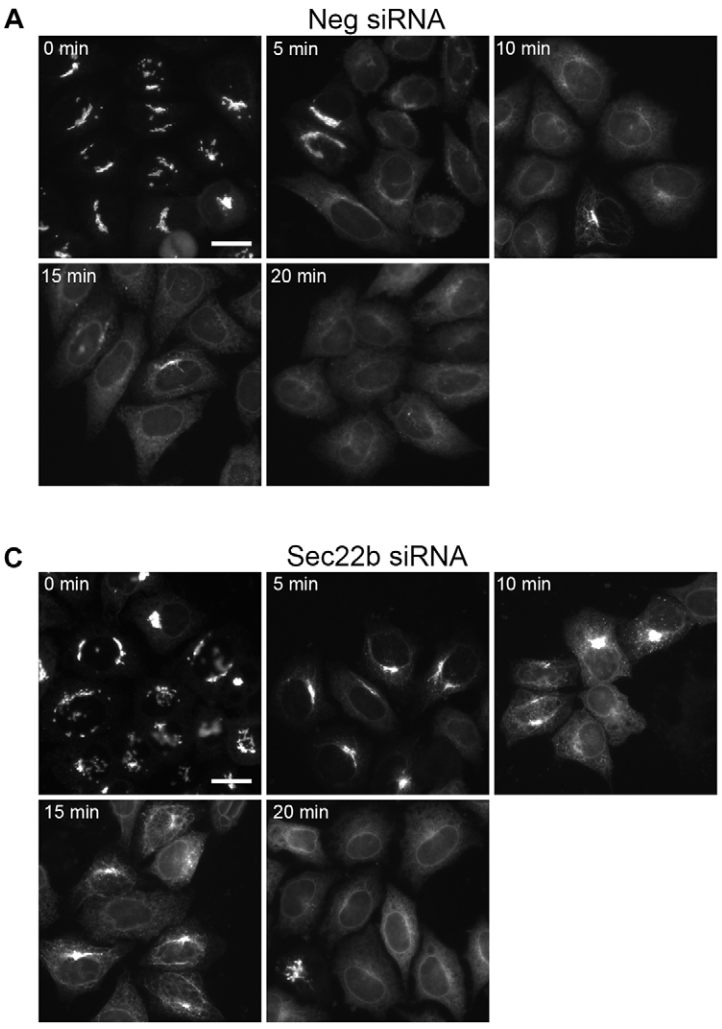

Fig. 1. Golgi-to-ER retrograde transport assay design. (A) Representative images of GalNAc-T2–GFP-expressing HeLa cells transfected with negative control (Neg) siRNAs and treated with 10 µg/ml BFA for increasing lengths of time. Cells were fixed at the time points indicated. (B) Quantification of the proportion of cells retaining an intact Golgi complex at each time point, and its log-transformed graph, allowing calculation of a Golgi-to-ER trafficking index (GETI). (C) Cells transfected with siRNAs targeting Sec22b were treated as in A. (D) Quantification of the GETI for Sec22b-depleted cells. The deceleration of retrograde trafficking is indicated by a reduction of the GETI value from 1 to 0.47. Scale bars: 40 µm.

addition to the Rab proteins, one accessory protein, Praf2, also ranked as strong inhibitor (Fig. 2C). The depletion of Rab8b and Yif1a showed a marked acceleration in Golgi-to-ER trafficking (Fig. 2C), although these were not considered further.

Our initial screen was performed using a pool of sequences containing four siRNAs against each target. Although such pools are powerful in terms of their gene coverage, they carry the higher risk that if one or more sequences in each pool show off-target effects or inefficient knockdown, false positive or indeed false negative results might be recorded. Therefore, in order to confirm and validate the hits obtained in our primary screen, we tested whether the phenotypes of the strong inhibitors and one of the weak inhibitors (Rab10) could also be reproduced by individual siRNAs, with sequences independent from those present in the pooled library. Testing of two sequences against each target revealed that for five of the strong inhibitors, a significant reduction in Golgi-to-ER transport could be recorded with both siRNA sequences, whereas for Rab1b, Rab3b, Rab6a/a′, Rab10, Rab11a and Rab21 a reduction in the transport step was observed in the presence of only one of the two sequences (Fig. 3A). Only the transport phenotype in cells depleted for Rab28 was not validated in these experiments. The knockdown efficiency of the candidates was verified by real-time quantitative-PCR (qPCR) (Fig. 3B). These experiments showed a knockdown efficiency of greater than 60%, and typically 80%, for all the siRNAs tested, with the exception of those targeting Rab2b, Rab3b and Rab4b. In these three cases, despite testing several qPCR primer pairs, the corresponding mRNAs could not be detected. Other large scale transcriptome and proteome (Nagaraj et al., 2011) studies of HeLa cells have suggested that these three Rab isoforms exist in HeLa cells, albeit at low levels. However, given that we were unable to reliably detect them in our HeLa cells they were excluded from subsequent experiments.

Cargo and Rab co-occurrence analysis

Although our phenotypic assay for Golgi-to-ER traffic defines the core Rab machinery, it does not directly report on whether these Rab proteins are physically associated with the transport carriers themselves. In order to address this, we transfected the GalNAc-T2–GFP-expressing HeLa cells with mCherry–Rab constructs. From the nine candidates obtained from the primary and validation screens, we selected the eight Rab proteins that gave strong Golgi redistribution phenotypes and for which we were able to generate a fluorescent fusion construct. The mCherry–Rab34 construct that we prepared, on transfection into cells consistently mislocalised to the nucleus, so was not considered in subsequent studies. Each construct was transfected into cells and was allowed to express for 20 h prior to treatment with BFA for 4 min (Fig. 4A). This time point was sufficient to stimulate the formation of Golgi-derived tubular carriers, but insufficient to induce the complete redistribution of the Golgi. Following confocal microscopy

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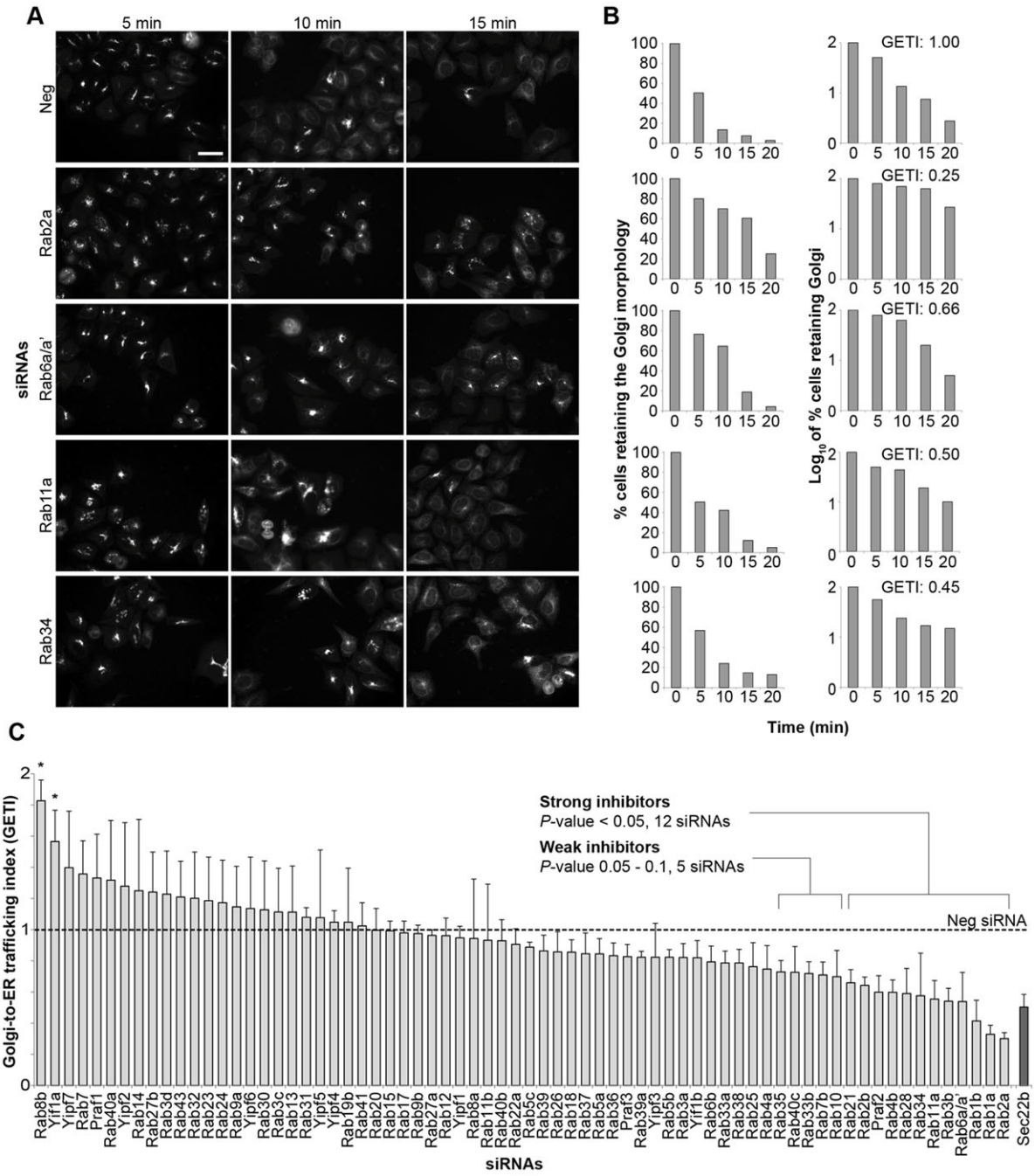

Fig. 2. RNAi screen for Rab proteins involved in Golgi-to-ER retrograde transport. GalNAc-T2–GFP-expressing HeLa cells were transfected with siRNAs as indicated, treated with 10 µg/ml BFA for increasing lengths of time, and then fixed. (A) Representative images of cells depleted for four different Rab proteins and treated with BFA for the times indicated. Scale bar: 40 µm. (B) Quantification of the proportion of cells retaining an intact Golgi complex at each time point and its log-transformed graph, allowing calculation of a GETI. (C) GETI values obtained for each of the 58 Rab and 12 Rab-accessory proteins screened. Hits were selected based on the statistical difference between the GETI values determined from cells treated with negative control siRNAs (Neg) and those treated with siRNAs targeting each gene; targets showing a $P<0.05$ were classified as strong inhibitors, whereas targets with a P-value between 0.05 and 0.10 were classified as weak inhibitors of retrograde traffic. Results are presented as mean±s.e.m. from three independent experiments. Asterisks denote strong Golgi-to-ER traffic accelerators ($P<0.05$).

imaging, we quantified in randomly selected individual cells the proportion of GalNAc-T2 cargo-containing tubules that were decorated with Rab proteins and vice versa, by manually identifying each tubular carrier and calculating its intensity profile in both channels (Fig. 4B). This analysis revealed that ~60% of all Golgi cargo carriers were coated with Rab1 variants, and more than 75% of them with Rab2a (Fig. 4C), emphasising the importance of these Rab proteins in the export from the Golgi complex. In addition, we observed that high proportions (56%) of carriers were coated with Rab6a, suggesting that COPI-independent transport mechanisms were also being induced in our assay. Of the other Rab proteins tested, Rab10 and Rab11a were found to be present on only a minor fraction (<5%) of the tubular carriers, whereas Rab21 was found to be completely absent. Similar results were obtained when we interrogated the proportion of Rab-protein-positive tubular carriers that were positive for cargo (Fig. 4C).

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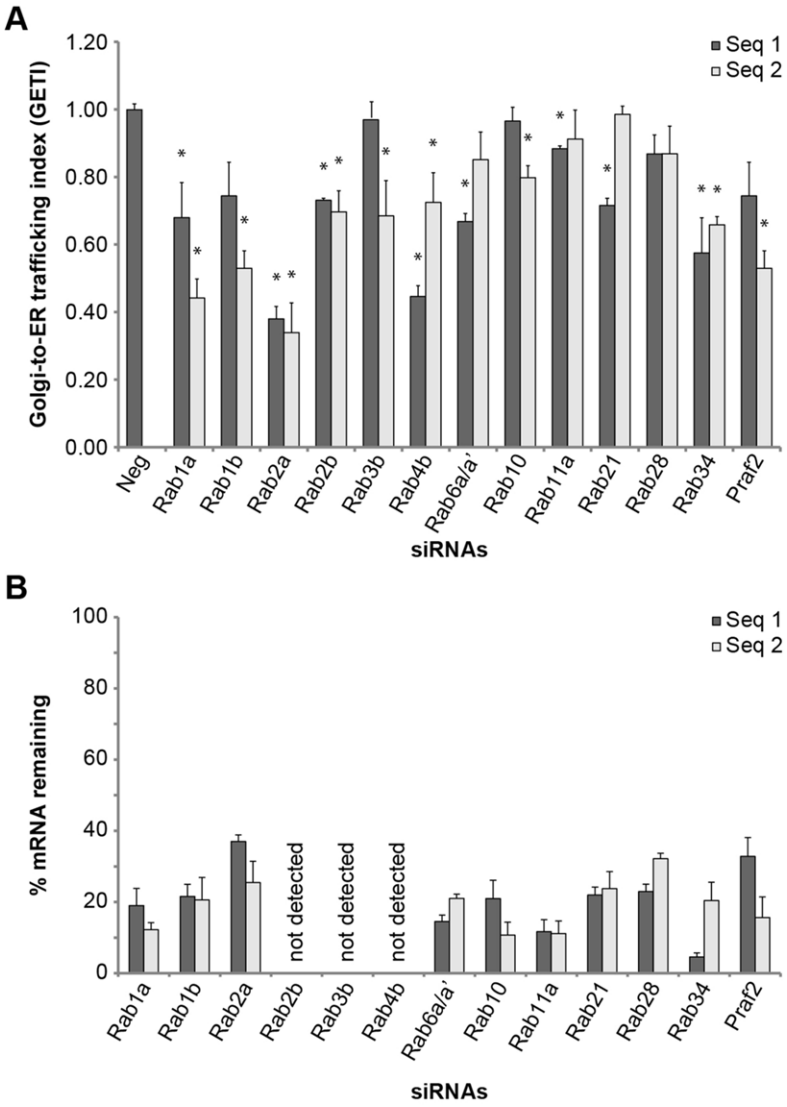

Fig. 3. Validation of the hits obtained from the primary screen. (A) GalNAc-T2–GFP-expressing HeLa cells were separately transfected with two independent siRNA sequences (Seq1 and Seq2) targeting each individual hit, followed by BFA treatment and analysis as described for Fig. 1. GETI values for each condition were determined. Results are presented as mean±s.e.m. from three independent experiments. *P<0.05, indicating siRNAs sequences considered to have a significant effect on Golgi-to-ER traffic. (B) Validation of the efficiency of the siRNA knockdown at the mRNA level by real-time qPCR. Bars indicate relative mRNA levels remaining in cells after 48 h of siRNA transfection compared to cells with negative control (Neg) siRNA. Results are presented as mean±s.d. of three independent experiments each containing three replicates.

Synergy between Rab proteins

Our approach has identified the Rab proteins associated with Golgi-derived cargo-containing carriers, but does not specifically address whether these Rab proteins are operating independently from one another in parallel pathways, or in a synergistic manner on the same pathway. In an attempt to resolve this question, we focussed on the validated candidates, and used siRNAs to downregulate them in a pairwise fashion. We selected a BFA treatment time of 15 min, as in previous experiments this was to be the most indicative time point for identifying strong regulators. 'Hits' were selected based on whether an additional 10% or more of cells in the population retained an intact Golgi complex when compared to the results from the single siRNA treatments. A heat map showing the percentage increase in inhibition of retrograde transport, compared to the single siRNA treatment, was generated, indicating the potential synergy between Rab proteins (Fig. 5A). Rab1 isoforms (Rab1a and Rab1b) showed the greatest synergistic effect with other Rab proteins, in particular Rab6a/a′. Depletions of Praf2, in combination with Rab11a or Rab21 also produced a strong synergistic phenotype.

Our functional data strongly suggest that there is a high degree of synergy between certain Rab proteins in the regulation of transport events between the Golgi complex and the ER. Based on the results shown in Fig. 5A, we investigated whether multiple Rab proteins were present on the same tubular membrane structures. The majority of the tubular carriers containing GalNAc-T2–GFP were decorated with either Rab1, Rab2 or Rab6a isoforms, so we initially tested combinations of these Rab proteins (GFP- and mCherry-tagged) in co-occurrence experiments similar to those described previously (Fig. 5B,C). These experiments revealed that a large fraction (>70%) of the tubular carriers contained a combination of both Rab1 isoforms, and that Rab1b was also frequently observed on structures decorated with Rab2a (>90%), Rab6a (>60%) and Rab11a (>20%) (Fig. 5D). Interestingly, a high frequency of tubule co-occurrence was also seen for Rab1b and Rab6a. By contrast, Rab6a-positive tubules seldom contained Rab10 (<5%), and we were unable to detect tubules containing Rab6a and Rab11a.

Functional assay for retrograde transport

In order to extend the results from our BFA phenotypic assay, we next analysed the transport of a well-characterised exogenous cargo that traffics between the Golgi complex and ER. Cells treated with either negative control siRNAs, or siRNAs targeting our candidate genes, were incubated with fluorescently labelled Shiga-like toxin-1 B subunit (SLTxB) for 4 h, and then imaged. In the Neg siRNA-treated cells, the toxin was found to be largely distributed between the Golgi complex and the ER. Presence of the toxin in the nuclear envelope was

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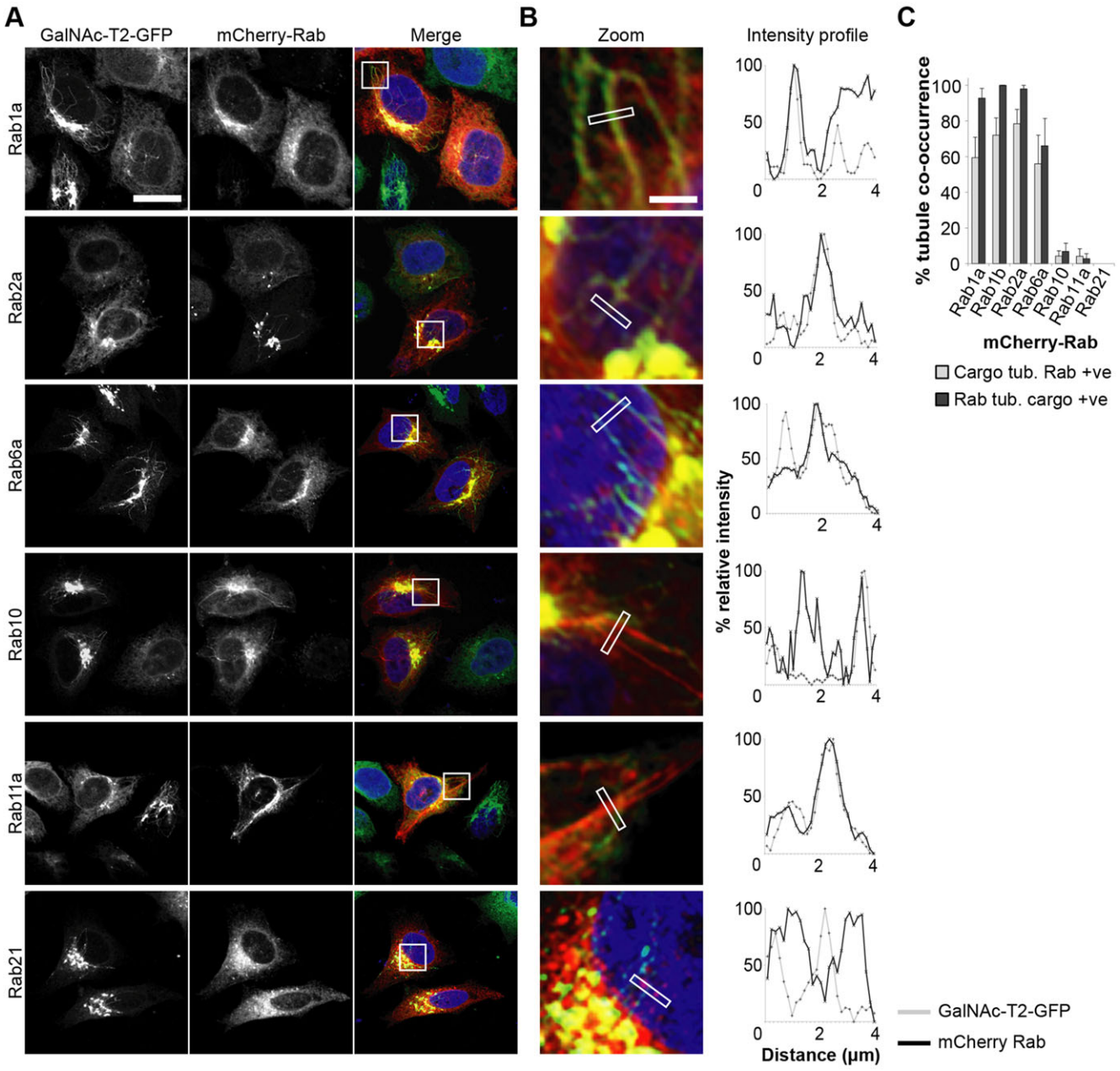

Fig. 4. Co-occurrence between Rab proteins and GalNAc-T2–GFP cargo. GalNAc-T2–GFP-expressing HeLa cells were transfected with constructs encoding mCherry-tagged Rab proteins and treated with 10 µg/ml BFA for 4 min. (A) Representative images of cells expressing mCherry–Rab proteins. In the merged image the GalNAc-T2-GFP is shown in green, the mCherry–Rab in red, and the nucleus in blue. Scale bar: 20 µm. (B) Magnified image of the area marked in A. Scale bar: 4 µm. Fluorescence intensity profiles of the areas marked are shown on the right. (C) Quantification of the percentage of GalNAc-T2-positive tubules co-occurring with the various mCherry–Rabs. Results are presented as mean±s.e.m. of a minimum of 20 tubular carriers analysed per experimental condition.

used as an indicator of ER delivery (Girod et al., 1999), and in Neg-treated cells this phenotype was scored in almost 90% of the cells (Fig. 6). Depletion of the COPI-independent pathway regulator Rab6a/a′ resulted in the most dramatic reduction of the SLTxB delivery to the ER, with less than 30% of cells showing this phenotype. Depletion of Rab1a and Rab1b also significantly decrease toxin delivery to the ER, but downregulation of the other Rab proteins tested only caused a mild reduction in SLTxB trafficking.

DISCUSSION

Despite a relatively large number of systematic studies addressing the mechanisms of anterograde transport between the ER and Golgi complex (Bard et al., 2006; Simpson et al., 2012; Wendler et al., 2010), comparatively little attention has been paid to the molecular nature of the retrograde pathway. One likely reason is that this pathway is difficult to study in isolation from forward trafficking. Nevertheless, it is clear that it serves an important role in both recycling of anterograde machinery molecules back to the ER for further rounds of secretory transport and also for the quality control of Golgi residents. Early studies have established that endogenous Golgi enzymes, for example galactosyltransferase, routinely pass through the ER as part of their normal life cycle (Cole et al., 1998). Further study of this pathway has been facilitated using various tagged GalNAc-T2 constructs, which have shown that the recycling

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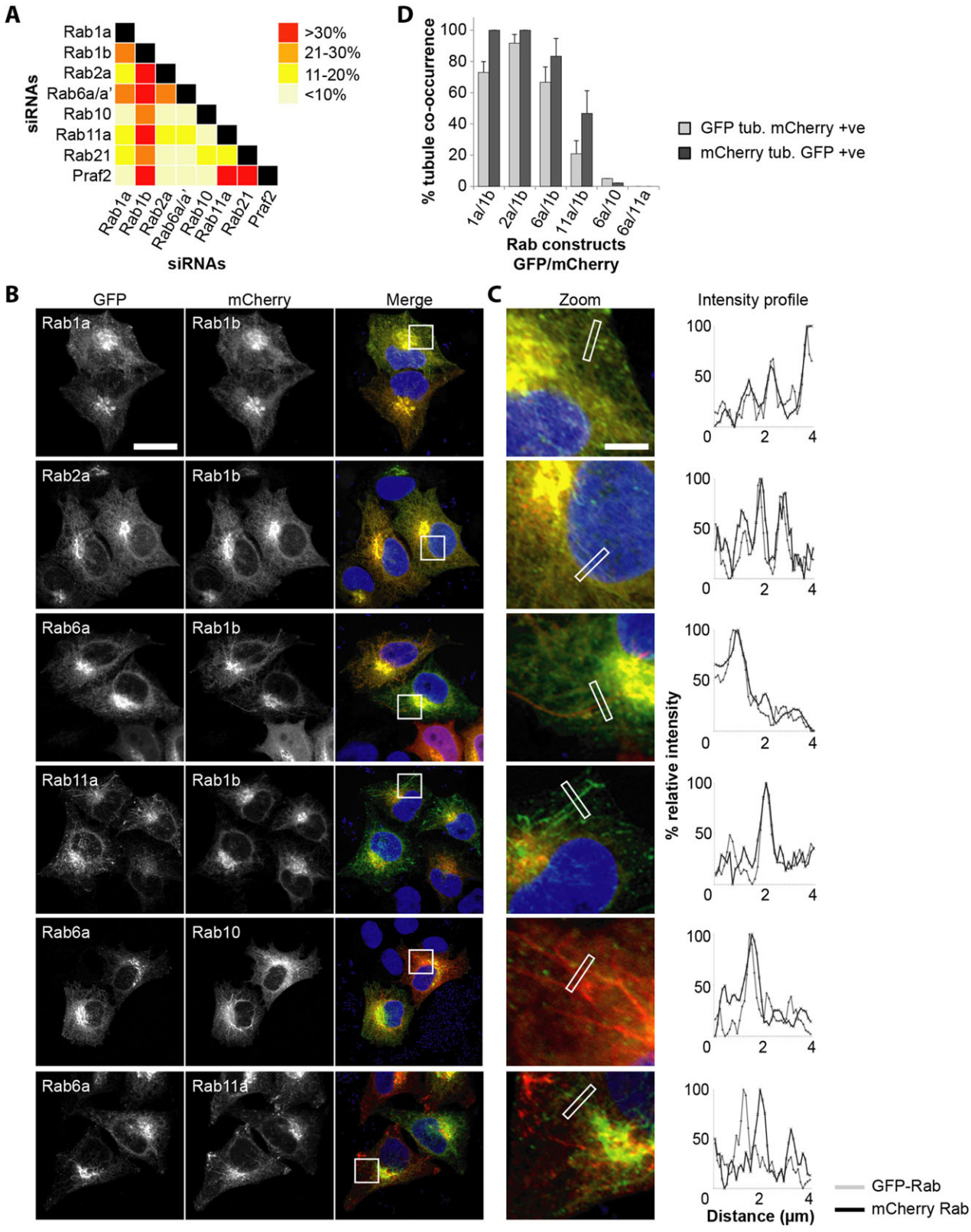

Fig. 5. Co-operativity of Rab function. (A) Pairwise depletion of selected Rabs previously identified as playing a role in Golgi-to-ER traffic. GalNAc-T2–GFP-expressing HeLa cells were treated with BFA for 15 min and then fixed, stained and imaged as in the primary screen. The percentage of cells in the population still retaining an intact Golgi complex was measured. The heat map indicates the percentage increase in inhibition of retrograde transport compared to the single siRNA treatments. Results shown represent the means of four independent experiments; a minimum of 40 cells were analysed per experiment. (B) Representative images of cells co-expressing GFP– and mCherry–Rab proteins. In the merged image, the GFP–Rab is shown in green, the mCherry–Rab in red, and the nucleus in blue. Scale bar: 20 µm. (C) Magnified image of area marked in B. Scale bar: 4 µm. Fluorescence intensity profiles of the areas marked are shown on the right. (D) Quantification of the co-occurrence of membrane tubules decorated with GFP- and mCherry-tagged Rab proteins. Results are presented as mean±s.e.m. of a minimum of 20 tubular carriers analysed per experimental condition.

Journal of Cell Science

Journal of Cell Science (2015) 128, 2339-2349 doi:10.1242/jcs.167973

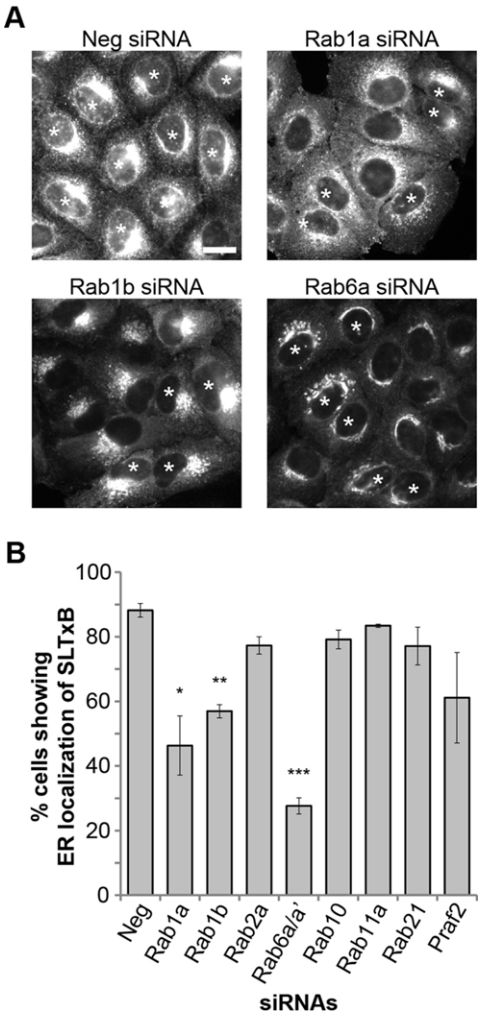

Fig. 6. Retrograde transport of SLTxB-Cy3. HeLa cells were transfected with siRNAs as indicated, incubated with 1.5 µg/ml SLTxB–Cy3 for 4 h and then fixed. (A) Representative images showing the distribution of the toxin in cells depleted for various Rab proteins. Asterisks denote cells showing toxin in the nuclear envelope. Scale bar: 20 µm. (B) Quantification of the proportion of cells showing nuclear envelope (representative of ER) localisation of SLTxB. Results are presented as mean±s.e.m. from three independent experiments; a minimum of 100 cells were analysed per experiment. *P=0.01–0.05, **P=0.001–0.01, ***P<0.001.

of this protein between the Golgi complex and ER is slow, with an apparent half-time of ∼1.5–2 h (Storrie et al., 1998). In order to stimulate the rapid production of Golgi-derived transport carriers, we treated a stable cell line expressing GalNAc-T2–GFP with BFA, as this treatment has been described to cause the redistribution of resident enzymes of the cis-, medial and trans-Golgi to the ER (Chege and Pfeffer, 1990; Lippincott-Schwartz et al., 1989). Consistent with this, we observed a strong colocalisation of our Golgi marker with the ER-resident protein REEP-5 after 30 min of treatment. In contrast, very little colocalisation of GalNAc-T2 was seen with the TGN marker TGN46, strongly suggesting that under these conditions we were specifically observing a Golgi-to-ER trafficking event. BFA works by targeting Sec7-domain-containing GEFs, such as GBF1, which mediate the formation of transport carriers through recruitment of the COPI coat complex (Peyroche et al., 1999). Stimulation of Golgi export pathways by BFA has been previously used in the context of understanding trafficking in the early secretory pathway (Sciaky et al., 1997), in addition to being used to identify key retrograde traffic regulators, such as phosphatidylinositol transfer proteins (Carvou et al., 2010) and diacylglycerol (Fernandez-Ulibarri et al., 2007).

Based on this knowledge we developed an RNAi-based high-content assay and automated image analysis approach to systematically analyse Rab GTPase involvement in Golgi-to-ER traffic. By acquiring image data at multiple time points after BFA addition, we were able to quantitatively analyse the kinetics of Golgi redistribution across large cell populations, rather than just examining a small number of individual cells at a single end-point. The downregulation of 11 Rab proteins and one Rab accessory protein resulted in the inhibition of Golgi-to-ER traffic, whereas depletion of two proteins, Rab8b and Yif1a, caused an acceleration of the process. The reason for this acceleration phenotype is unclear, although one hypothesis would be that these proteins play an inhibitory role in Golgi export and therefore their depletion effectively removes this control step, resulting in a small acceleration in traffic. Further detailed characterisation would be required to validate this hypothesis.

Early work identified Rab1 and Rab2 isoforms as being the primary Rab proteins associated with the ER–Golgi interface (Plutner et al., 1991; Tisdale et al., 1992), and in our screen these Rab proteins were all found in the group of strongest regulators of the Golgi-to-ER step. Rab1 plays a role in anterograde traffic through the recruitment of various effectors involved in the budding and tethering of COPII vesicles (Allan et al., 2000; Moyer et al., 2001; Weide et al., 2001), whereas Rab2 has been shown to be primarily localised to ER–Golgi vesicular–tubular intermediates and actively participate in COPI recruitment (Alvarez et al., 2003; Tisdale et al., 2009). These studies have long suggested that the primary function of Rab2 is in COPI-mediated Golgi-to-ER transport, and our functional experiments now add strong support to this. Other studies have also suggested roles for Rab33b (Starr et al., 2010; Valsdottir et al., 2001), Rab18, Rab43 (Dejgaard et al., 2008) and Rab30 (Kelly et al., 2012) at the ER–Golgi interface. Of these Rab proteins, our screen only confirmed a role for Rab33b in Golgi-to-ER traffic, suggesting that these other candidates play roles outside of this specific trafficking event, for example in anterograde traffic or organelle morphology maintenance (Sandoval and Simmen, 2012). Rab6 variants are also known to play a role in the transfer of certain cargo types from the Golgi to the ER, predominantly in the form of tubular carriers that lack the COPI coat (Heffernan and Simpson, 2014). This GTPase is present as two splice variants, denoted as Rab6a and Rab6a′, varying from each other by three amino acids. Various studies have suggested that the two proteins exhibit different functional properties and act sequentially to regulate retrograde membrane traffic from endosomes to the Golgi complex and from the Golgi complex to the ER (Echard et al., 2000; Mallard et al., 2002). The siRNAs used in the present study targeted both variants, and resulted in a strong inhibition of Golgi redistribution and inhibition of SLTxB transfer to the ER, thereby highlighting its role in transfer of cargo from the Golgi to the ER, and that our assay captures retrograde trafficking events that are independent of COPI function.

A number of Rab proteins identified in this work have no known functional role in the retrograde transport step. Rab10, Rab11a and Rab21 have been shown to mainly reside on various endosome populations; however, in each case a small proportion has been reported to be present on juxta-nuclear Golgi membranes (Babbey et al., 2006; Landry et al., 2009; Simpson et al., 2004; Ullrich et al., 1993). None of these Rab proteins, however, showed phenotypes as strong as Rab2a, the most potent regulator, suggesting that their involvement in trafficking might be minor, although still detectable by our assay.

Journal of Cell Science (2015) 128, 2339-2349 doi:10.1242/jcs.167973

Praf2 was the only Rab accessory protein identified and validated as a hit. The function of this protein remains elusive, although it has been reported to localise to the Golgi complex, ER and intermediate compartment (Liang and Li, 2000; Ruggiero et al., 2008; Schweneker et al., 2005). Other PRAF family members might play a role in displacing Rab proteins from GDI as part of their activation cycle (Figueroa et al., 2001; Maier et al., 2009), although this activity has not specifically been demonstrated for this particular family member. Interestingly reduced Praf2 protein levels have been linked to improved cell survival, mediated through interactions with Bcl-xL (Vento et al., 2010), providing an intriguing link between trafficking and cell death processes.

Our functional experiments define for the first time, in a systematic manner, the Rab proteins required for Golgi-to-ER transport. To probe whether these proteins are physically present on the carriers themselves, we carried out a careful analysis of confocal images of GalNAc-T2-cargo-containing tubules, revealing that the vast majority were positive for either Rab1, Rab2 or Rab6a/a′. We did not detect carriers containing Rab21, therefore suggesting that the role of this Rab protein is more likely to be at the level of carrier formation, rather than transport itself. We did, however, detect a small, but reproducible, number of carriers containing either Rab10 or Rab11a. Rab10 has long been associated with dynamics of endosomes (Babbey et al., 2006); however, very recently a new function in regulating ER structure and membrane tubule morphology has been reported (English and Voeltz, 2013). This elegant paper proposes that complexes containing Rab10 regulate ER extension and fusion in conjunction with microtubules. Our observations that occasional Rab10-coated carriers exist in cells, and that Rab10 depletion reduces Golgi-to-ER transport, are consistent with a role for this Rab in membrane tubule dynamics in this part of the cell. Rab11a was also found in our experiments to be involved in Golgi-to-ER traffic. This protein predominantly localises to recycling endosomes, although some studies have indicated that a pool might localise to Golgi membranes (Ullrich et al., 1996; Urbé et al., 1993), likely through interactions with phosphatidylinositol 4-kinase β (de Graaf et al., 2004). Although Rab11a has also been shown to share interactors with Rab6a, further suggesting strong interplay between these molecules (Miserey-Lenkei et al., 2007), we found that these two Rab proteins do not co-occur on the same carriers, an observation consistent with a previous *in vivo* interaction study on these two proteins (Miserey-Lenkei et al., 2007). We did find, however, that depletion of Rab11a in conjunction with Rab1 or Rab2a resulted in a further decrease of Golgi-to-ER transport, when compared to the depletion of each Rab alone. This result would be suggestive of these Rab proteins working in a co-operative manner, possibly through cascades in which effectors such as GEFs might be shared (McDonold and Fromme, 2014). The strongest co-operation between Rab proteins with respect to Golgi-to-ER transport, perhaps unsurprisingly, involved Rab1 isoforms. Strong levels of co-operation were seen between Rab1b and Rab2a, and between Rab1b and Rab6a/a′, with the majority of tubular carriers being decorated with these pairs of Rab proteins. Our functional assay studying SLTxB transfer to ER further supports important roles for Rab1 isoforms and Rab6a/a′ in the retrograde pathway. Our experiments do not provide the imaging resolution to establish whether these Rab proteins occupy the same areas of tubular membranes, or whether they form distinct micro-domains, as is seen, for example, on early endosomes (Sönnichsen et al., 2000). Nevertheless, this study reports for the first time clear functional data implicating these Rab proteins in retrograde traffic between the Golgi complex and the ER.

In summary, we present a novel quantitative imaging approach to systematically identify proteins with a role in retrograde traffic between the Golgi and ER, and applied this technology to the Rab family of small GTPases. Not only were we able to confirm previously published roles for Rab6a/a′ in this transport step, but we provide clear data for Rab1a, Rab1b and Rab2a as being regulators of Golgi-to-ER transport, in addition to their established role in anterograde traffic. Importantly, the sensitivity of our assay allowed us to propose additional Rab proteins with a role in this transport step. These studies therefore pave the way for a more complete investigation of the molecular components required to regulate Golgi-to-ER transport in mammalian cells.

MATERIALS AND METHODS

Cell culture

Wild-type HeLa cells (ATCC CCL2) were grown in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (PAA Laboratories) and 1% glutamine (Life Technologies). HeLa cells stably expressing GFP-tagged GalNAc-T2 (Storrie et al., 1998) were cultured as above in the presence of 0.5 mg/ml G-418 sulphate (Life Technologies).

Solid phase reverse transfection

Transfection-ready optical quality polystyrene plates were prepared based on a previously published protocol (Erfle et al., 2008), and adapted for a 384-well format (Galea and Simpson, 2013). A 'SMARTpool' siRNA library (Dharmacon) was designed to contain four sequences against each target gene (58 Rabs, three members of the PRAF, two members of the YIF and seven members of the YIPF families) including a positive control, Sec22b, and a negative control (Neg). Validation experiments were carried out using two individual siRNA sequences (Ambion). Briefly, 50 nM of each siRNA pool were combined with 0.125 µl of Lipofectamine2000 (Life Technologies), mixed with gelatin and fibronectin (Sigma-Aldrich) to increase adherence of the complex and then arrayed into 15 identical 384-well plates (Galea and Simpson, 2013). For double siRNA transfections, 50 nM of each sequence was used. Following drying in a rotary evaporating centrifuge, the plates were stored desiccated at room temperature until needed.

Golgi-to-ER trafficking assay

The GalNAc-T2-GFP HeLa cells were seeded into the transfection-ready 384-well plates and after 48 h cells were treated with 10 µg/ml BFA (Sigma-Aldrich) for 5, 10, 15 or 20 min at 37°C. Following this, cells were fixed with 3% PFA and nuclei were stained with 0.2 µg/ml Hoechst 33342. A total of 25 fields of view per well were acquired sequentially with a 40×, 0.6 NA Luc Plan FLN objective on a Scan^R fully automated wide-field microscope (Olympus) containing standard filter sets.

Quantification of Golgi-to-ER trafficking assay

Images were analysed using Columbus HCS Analysis software (Version 2.4.1 PerkinElmer). Individual cells were segmented, and morphological and intensity measurements were calculated in order to remove apoptotic and mitotic cells from the analysis. The intensity profile of each cell in the GFP (Golgi) channel was extracted, and, based on this, a linear classifier was set-up to distinguish the cells with a high maximum intensity (indicating retention of an intact Golgi) from those with a low maximum intensity (indicating Golgi redistribution). The percentage of cells in each sub-population was then determined from the linear classifier. The linear classifier algorithm was trained using cells treated with Neg siRNAs and positive control siRNAs (Sec22b), with typically a 50% reduction in maximum intensity of the signal being classified as Golgi redistribution. The kinetics of this event (indicative of retrograde traffic) was obtained by logarithmically transforming the redistribution data obtained from the five time points (0, 5, 10, 15 and 20 min) following BFA treatment. A linear model was applied to the dataset, and the generated slope was normalised against the slope obtained from cells treated with Neg siRNAs in order to obtain a Golgi-to-ER trafficking index (GETI).

A Student's *t*-test was performed comparing the data between the target gene and the negative control. Results are presented as mean±s.e.m. from three independent experiments.

Total RNA extraction, cDNA synthesis and real-time quantitative PCR

HeLa cells were seeded in 12-well plates and transfected with 10 pmol of Silencer Select siRNA (Ambion) using Oligofectamine (Life Technologies) according to the manufacturer's instructions. Cells were transfected with siRNAs for 48 h in all experiments. Total RNA from cells, was purified using the Invisorb spin cell RNA mini kit (STRATEC Molecular) as recommended by the supplier. RNA concentrations were determined using a NanoDrop1000 (Thermo Scientific). cDNA synthesis was performed with 500 ng of total RNA using the high capacity cDNA reverse transcription kit (Life Technologies) according to the supplier's instructions. Real-time qPCR was performed using Fast SYBR® green PCR MasterMix (Life Technologies) in a 7500 FAST real-time PCR system (Life Technologies). A twentieth of the cDNA reaction was used as a template for the reaction and 200 nM of each primer (supplementary material Table S1) was used. All samples were run in triplicates. Results were obtained using the $-\Delta Ct$ method, with mRNA levels from specific siRNA-treated cells being normalised to those found in cells treated with non-silencing (Neg) siRNAs.

Co-occurrence assays

Wild-type and stably transfected GalNAc-T2–GFP-expressing HeLa cells were seeded on glass coverslips and were transfected with DNA using FuGENE6 (Promega) according to the manufacturer's instructions. Cells were treated with 10 µg/ml of BFA for 4 min at 37°C followed by fixation and staining.

Confocal image acquisition and quantification

Confocal images (1024×1024 pixels) were acquired with an Olympus FV1000 confocal microscope equipped with a 60×, 1.35 NA oil immersion objective. A minimum of five cells were imaged for each construct tested. Images were analysed using ImageJ (NIH), and tubular carriers were manually identified and counted. In order to measure the co-occurrence of two proteins, the percentage relative intensity of each channel was calculated using the plot profile tool. Only relative intensities of at least 50% of the peak maximum intensity value were considered as representing structures containing co-occurring signals. A minimum of 30 tubular carriers were analysed per sample. For immunostainings, cells were fixed in methanol (Fisher Scientific) for 4 min at −20°C. Cells were immunostained for TGN46 (Bio-Rad Laboratories) and REEP-5 (Proteintech) as markers of the TGN and the ER, respectively.

Shiga-like toxin-1 B subunit functional assay

Recombinant wild-type SLTxB was purified as described previously (Girod et al., 1999). The purified B-chain was labelled with Cy3 according to the manufacturer's instructions (Amersham, GE Healthcare). HeLa cells were seeded on glass coverslips and transfected for 48 h with siRNAs as described above. The cells were subsequently incubated on ice with 1.5 µg/ml SLTxB in DMEM without FBS for 30 min and then washed twice with cold PBS to remove unbound toxin followed by incubation for 4 h at 37°C with DMEM supplemented with 10% FBS. Cells were fixed in PFA and nuclei were stained. Images were acquired on a Leica DMI6000B inverted fluorescence microscope with a 40×, 1.25 NA oil immersion objective. A minimum of 100 cells were analysed per siRNA treatment, and the proportion of cells showing SLTxB in the nuclear envelope was recorded as described previously (Girod et al., 1999). A Student's *t*-test was performed comparing the data between the target gene and the negative control. Results are presented as mean±s.e.m. from three independent experiments.

Acknowledgements

We acknowledge Dr Vasanth Singan for preparation of the mCherry–Rab constructs.

Competing interests

The authors declare no competing or financial interests.

Author contributions

G.G. and J.C.S. conceived and designed the experiments; G.G., A.P. and E.D.O. prepared and tested the RNAi library; G.G. performed the experiments; G.G., M.G.B. and J.C.S. interpreted the results and prepared the article.

Funding

This work was funded by a Principal Investigator (PI) grant from Science Foundation Ireland (SFI) [grant number 09/IN.1/B2604 to J.C.S.]. This work was carried out in the UCD Cell Screening Laboratory, supported by a grant from the UCD College of Science.

Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.167973/-/DC1

References

Allan, B. B., Moyer, B. D. and Balch, W. E. (2000). Rab1 recruitment of p115 into a cis-SNARE complex: programming budding COPII vesicles for fusion. *Science* **289**, 444-448.

Alvarez, C., Garcia-Mata, R., Brandon, E. and Sztul, E. (2003). COPI recruitment is modulated by a Rab1b-dependent mechanism. *Mol. Biol. Cell* **14**, 2116-2127.

Babbey, C. M., Ahktar, N., Wang, E., Chen, C. C.-H., Grant, B. D. and Dunn, K. W. (2006). Rab10 regulates membrane transport through early endosomes of polarized Madin-Darby canine kidney cells. *Mol. Biol. Cell* **17**, 3156-3175.

Bard, F., Casano, L., Mallabiabarrena, A., Wallace, E., Saito, K., Kitayama, H., Guizzunti, G., Hu, Y., Wendler, F., DasGupta, R. et al. (2006). Functional genomics reveals genes involved in protein secretion and Golgi organization. *Nature* **439**, 604-607.

Barr, F. and Lambright, D. G. (2010). Rab GEFs and GAPs. *Curr. Opin. Cell Biol.* **22**, 461-470.

Burri, L., Varlamov, O., Doege, C. A., Hofmann, K., Beilharz, T., Rothman, J. E., Sollner, T. H. and Lithgow, T. (2003). A SNARE required for retrograde transport to the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* **100**, 9873-9877.

Carvou, N., Holic, R., Li, M., Futter, C., Skippen, A. and Cockcroft, S. (2010). Phosphatidylinositol- and phosphatidylcholine-transfer activity of PITPbeta is essential for COPI-mediated retrograde transport from the Golgi to the endoplasmic reticulum. *J. Cell Sci.* **123**, 1262-1273.

Chege, N. W. and Pfeffer, S. R. (1990). Compartmentation of the Golgi complex: brefeldin-A distinguishes trans-Golgi cisternae from the trans-Golgi network. *J. Cell Biol.* **111**, 893-899.

Chia, J., Goh, G., Racine, V., Ng, S., Kumar, P. and Bard, F. (2012). RNAi screening reveals a large signaling network controlling the Golgi apparatus in human cells. *Mol. Syst. Biol.* **8**, 629.

Cole, N. B., Ellenberg, J., Song, J., DiEuliis, D. and Lippincott-Schwartz, J. (1998). Retrograde transport of Golgi-localized proteins to the ER. *J. Cell Biol.* **140**, 1-15.

de Graaf, P., Zwart, W. T., van Dijken, R. A. J., Deneka, M., Schulz, T. K. F., Geijsen, N., Coffer, P. J., Gadella, B. M., Verkleij, A. J., van der Sluijs, P. et al. (2004). Phosphatidylinositol 4-kinasebeta is critical for functional association of rab11 with the Golgi complex. *Mol. Biol. Cell* **15**, 2038-2047.

Dejgaard, S. Y., Murshid, A., Erman, A., Kizilay, O., Verbich, D., Lodge, R., Dejgaard, K., Ly-Hartig, T. B. N., Pepperkok, R., Simpson, J. C. et al. (2008). Rab18 and Rab43 have key roles in ER-Golgi trafficking. *J. Cell Sci.* **121**, 2768-2781.

Dilcher, M., Veith, B., Chidambaram, S., Hartmann, E., Schmitt, H. D. and Fischer von Mollard, G. (2003). Use1p is a yeast SNARE protein required for retrograde traffic to the ER. *EMBO J.* **22**, 3664-3674.

Doms, R. W., Russ, G. and Yewdell, J. W. (1989). Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. *J. Cell Biol.* **109**, 61-72.

Echard, A., Opdam, F. J. M., de Leeuw, H. J. P. C., Jollivet, F., Savelkoul, P., Hendriks, W., Voorberg, J., Goud, B. and Fransen, J. A. M. (2000). Alternative splicing of the human Rab6A gene generates two close but functionally different isoforms. *Mol. Biol. Cell* **11**, 3819-3833.

English, A. R. and Voeltz, G. K. (2013). Rab10 GTPase regulates ER dynamics and morphology. *Nat. Cell Biol.* **15**, 169-178.

Erfle, H., Neumann, B., Rogers, P., Bulkescher, J., Ellenberg, J. and Pepperkok, R. (2008). Work flow for multiplexing siRNA assays by solid-phase reverse transfection in multiwell plates. *J. Biomol. Screen.* **13**, 575-580.

Fernandez-Ulibarri, I., Vilella, M., Lazaro-Dieguez, F., Sarri, E., Martinez, S. E., Jimenez, N., Claro, E., Merida, I., Burger, K. N. J. and Egea, G. (2007). Diacylglycerol is required for the formation of COPI vesicles in the Golgi-to-ER transport pathway. *Mol. Biol. Cell* **18**, 3250-3263.

Figueroa, C., Taylor, J. and Vojtek, A. B. (2001). Prenylated Rab acceptor protein is a receptor for prenylated small GTPases. *J. Biol. Chem.* **276**, 28219-28225.

Galea, G. and Simpson, J. C. (2013). High-content screening and analysis of the Golgi complex. *Methods Cell Biol.* **118**, 281-295.

Girod, A., Storrie, B., Simpson, J. C., Johannes, L., Goud, B., Roberts, L. M., Lord, J. M., Nilsson, T. and Pepperkok, R. (1999). Evidence for a COP-I-independent transport route from the Golgi complex to the endoplasmic reticulum. *Nat. Cell Biol.* **1**, 423-430.

Gurkan, C., Lapp, H., Alory, C., Su, A. I., Hogenesch, J. B. and Balch, W. E. (2005). Large-scale profiling of Rab GTPase trafficking networks: the membrome. *Mol. Biol. Cell* **16**, 3847-3864.
Heffernan, L. F. and Simpson, J. C. (2014). The trials and tubule-ations of Rab6 involvement in Golgi-to-ER retrograde transport. *Biochem. Soc. Trans* **42**, 1453-1459.
Kelly, E. E., Giordano, F., Horgan, C. P., Jollivet, F., Raposo, G. and McCaffrey, M. W. (2012). Rab30 is required for the morphological integrity of the Golgi apparatus. *Biol. Cell* **104**, 84-101.
Klausner, R. D., Donaldson, J. G. and Lippincott-Schwartz, J. (1992). Brefeldin A: insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* **116**, 1071-1080.
Landry, M.-C., Sicotte, A., Champagne, C. and Lavoie, J. N. (2009). Regulation of cell death by recycling endosomes and golgi membrane dynamics via a pathway involving Src-family kinases, Cdc42 and Rab11a. *Mol. Biol. Cell* **20**, 4091-4106.
Lewis, M. J., Rayner, J. C. and Pelham, H. R. B. (1997). A novel SNARE complex implicated in vesicle fusion with the endoplasmic reticulum. *EMBO J.* **16**, 3017-3024.
Liang, Z. and Li, G. (2000). Mouse prenylated Rab acceptor is a novel Golgi membrane protein. *Biochem. Biophys. Res. Commun.* **275**, 509-516.
Lippincott-Schwartz, J., Yuan, L. C., Bonifacino, J. S. and Klausner, R. D. (1989). Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell* **56**, 801-813.
Lippincott-Schwartz, J., Donaldson, J. G., Schweizer, A., Berger, E. G., Hauri, H.-P., Yuan, L. C. and Klausner, R. D. (1990). Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. *Cell* **60**, 821-836.
Lorente-Rodriguez, A. and Barlowe, C. (2011). Entry and exit mechanisms at the cis-face of the Golgi complex. *Cold Spring Harb. Perspect. Biol.* **3**, a005207.
Luini, A., Mironov, A. A., Polishchuk, E. V. and Polishchuk, R. S. (2008). Morphogenesis of post-Golgi transport carriers. *Histochem. Cell Biol.* **129**, 153-161.
Maier, S., Reiterer, V., Ruggiero, A. M., Rothstein, J. D., Thomas, S., Dahm, R., Sitte, H. H. and Farhan, H. (2009). GTRAP3-18 serves as a negative regulator of Rab1 in protein transport and neuronal differentiation. *J. Cell. Mol. Med.* **13**, 114-124.
Mallard, F., Tang, B. L., Galli, T., Tenza, D., Saint-Pol, A., Yue, X., Antony, C., Hong, W., Goud, B. and Johannes, L. (2002). Early/recycling endosomes-to-TGN transport involves two SNARE complexes and a Rab6 isoform. *J. Cell Biol.* **156**, 653-664.
McDonold, C. M. and Fromme, J. C. (2014). Four GTPases differentially regulate the Sec7 Arf-GEF to direct traffic at the trans-golgi network. *Dev. Cell* **30**, 759-767.
Miserey-Lenkei, S., Waharte, F., Boulet, A., Cuif, M.-H., Tenza, D., El Marjou, A., Raposo, G., Salamero, J., Héliot, L., Goud, B. et al. (2007). Rab6-interacting protein 1 links Rab6 and Rab11 function. *Traffic* **8**, 1385-1403.
Moya, M., Roberts, D. and Novick, P. (1993). DSS4-1 is a dominant suppressor of sec4-8 that encodes a nucleotide exchange protein that aids Sec4p function. *Nature* **361**, 460-463.
Moyer, B. D., Allan, B. B. and Balch, W. E. (2001). Rab1 interaction with a GM130 effector complex regulates COPII vesicle cis-Golgi tethering. *Traffic* **2**, 268-276.
Nagaraj, N., Wisniewski, J. R., Geiger, T., Cox, J., Kircher, M., Kelso, J., Pääbo, S. and Mann, M. (2011). Deep proteome and transcriptome mapping of a human cancer cell line. *Mol. Syst. Biol.* **7**, 548.
Peyroche, A., Antonny, B., Robineau, S., Acker, J., Cherfils, J. and Jackson, C. L. (1999). Brefeldin A acts to stabilize an abortive ARF–GDP–Sec7 domain protein complex: involvement of specific residues of the Sec7 domain. *Mol. Cell* **3**, 275-285.
Pfeffer, S. and Aivazian, D. (2004). Targeting Rab GTPases to distinct membrane compartments. *Nat. Rev. Mol. Cell Biol.* **5**, 886-896.
Plutner, H., Cox, A. D., Pind, S., Khosravi-Far, R., Bourne, J. R., Schwaninger, R., Der, C. J. and Balch, W. E. (1991). Rab1b regulates vesicular transport between the endoplasmic reticulum and successive Golgi compartments. *J. Cell Biol.* **115**, 31-43.
Ruggiero, A. M., Liu, Y., Vidensky, S., Maier, S., Jung, E., Farhan, H., Robinson, M. B., Sitte, H. H. and Rothstein, J. D. (2008). The endoplasmic reticulum exit of glutamate transporter is regulated by the inducible mammalian Yip6b/GTRAP3-18 protein. *J. Biol. Chem.* **283**, 6175-6183.
Sandoval, C. O. and Simmen, T. (2012). Rab proteins of the endoplasmic reticulum: functions and interactors. *Biochem. Soc. Trans.* **40**, 1426-1432.
Sannerud, R., Saraste, J. and Goud, B. (2003). Retrograde traffic in the biosynthetic-secretory route: pathways and machinery. *Curr. Opin. Cell Biol.* **15**, 438-445.
Schweneker, M., Bachmann, A. S. and Moelling, K. (2005). JM4 is a four-transmembrane protein binding to the CCR5 receptor. *FEBS Lett.* **579**, 1751-1758.
Sciaky, N., Presley, J., Smith, C., Zaal, K. J. M., Cole, N., Moreira, J. E., Terasaki, M., Siggia, E. and Lippincott-Schwartz, J. (1997). Golgi tubule traffic and the effects of brefeldin A visualized in living cells. *J. Cell Biol.* **139**, 1137-1155.
Simpson, J. C., Griffiths, G., Wessling-Resnick, M., Fransen, J. A. M., Bennett, H. and Jones, A. T. (2004). A role for the small GTPase Rab21 in the early endocytic pathway. *J. Cell Sci.* **117**, 6297-6311.
Simpson, J. C., Nilsson, T. and Pepperkok, R. (2006). Biogenesis of tubular ER-to-Golgi transport intermediates. *Mol. Biol. Cell* **17**, 723-737.
Simpson, J. C., Joggerst, B., Laketa, V., Verissimo, F., Cetin, C., Erfle, H., Bexiga, M. G., Singan, V. R., Hériché, J.-K., Neumann, B. et al. (2012). Genome-wide RNAi screening identifies human proteins with a regulatory function in the early secretory pathway. *Nat. Cell Biol.* **14**, 764-774.
Sivars, U., Aivazian, D. and Pfeffer, S. R. (2003). Yip3 catalyses the dissociation of endosomal Rab–GDI complexes. *Nature* **425**, 856-859.
Sönnichsen, B., De Renzis, S., Nielsen, E., Rietdorf, J. and Zerial, M. (2000). Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. *J. Cell Biol.* **149**, 901-914.
Starr, T., Sun, Y., Wilkins, N. and Storrie, B. (2010). Rab33b and Rab6 are functionally overlapping regulators of Golgi homeostasis and trafficking. *Traffic* **11**, 626-636.
Storrie, B., White, J., Röttger, S., Stelzer, E. H. K., Suganuma, T. and Nilsson, T. (1998). Recycling of Golgi-resident Glycosyltransferases through the ER Reveals a Novel Pathway and Provides an Explanation for Nocodazole-induced Golgi Scattering. *J. Cell Biol.* **143**, 1505-1521.
Tisdale, E. J., Bourne, J. R., Khosravi-Far, R., Der, C. J. and Balch, W. E. (1992). GTP-binding mutants of rab1 and rab2 are potent inhibitors of vesicular transport from the endoplasmic reticulum to the Golgi complex. *J. Cell Biol.* **119**, 749-761.
Tisdale, E. J., Azizi, F. and Artalejo, C. R. (2009). Rab2 utilizes glyceraldehyde-3-phosphate dehydrogenase and protein kinase C{iota} to associate with microtubules and to recruit dynein. *J. Biol. Chem.* **284**, 5876-5884.
Trucco, A., Polishchuk, R. S., Martella, O., Di Pentima, A., Fusella, A., Di Giandomenico, D., San Pietro, E., Beznoussenko, G. V., Polishchuk, E. V., Baldassarre, M. et al. (2004). Secretory traffic triggers the formation of tubular continuities across Golgi sub-compartments. *Nat. Cell Biol.* **6**, 1071-1081.
Ullrich, O., Stenmark, H., Alexandrov, K., Huber, L. A., Kaibuchi, K., Sasaki, T., Takai, Y. and Zerial, M. (1993). Rab GDP dissociation inhibitor as a general regulator for the membrane association of rab proteins. *J. Biol. Chem.* **268**, 18143-18150.
Ullrich, O., Reinsch, S., Urbé, S., Zerial, M. and Parton, R. G. (1996). Rab11 regulates recycling through the pericentriolar recycling endosome. *J. Cell Biol.* **135**, 913-924.
Urbé, S., Huber, L. A., Zerial, M., Tooze, S. A. and Parton, R. G. (1993). Rab11, a small GTPase associated with both constitutive and regulated secretory pathways in PC12 cells. *FEBS Lett.* **334**, 175-182.
Valsdottir, R., Hashimoto, H., Ashman, K., Koda, T., Storrie, B. and Nilsson, T. (2001). Identification of rabaptin-5, rabex-5, and GM130 as putative effectors of rab33b, a regulator of retrograde traffic between the Golgi apparatus and ER. *FEBS Lett.* **508**, 201-209.
Vento, M. T., Zazzu, V., Loffreda, A., Cross, J. R., Downward, J., Stoppelli, M. P. and Iaccarino, I. (2010). Praf2 is a novel Bcl-xL/Bcl-2 interacting protein with the ability to modulate survival of cancer cells. *PLoS ONE* **5**, e15636.
Vetter, I. R. and Wittinghofer, A. (2001). The guanine nucleotide-binding switch in three dimensions. *Science* **294**, 1299-1304.
Voeltz, G. K., Prinz, W. A., Shibata, Y., Rist, J. M. and Rapoport, T. A. (2006). A class of membrane proteins shaping the tubular endoplasmic reticulum. *Cell* **124**, 573-586.
Weide, T., Bayer, M., Köster, M., Siebrasse, J.-P., Peters, R. and Barnekow, A. (2001). The Golgi matrix protein GM130: a specific interacting partner of the small GTPase rab1b. *EMBO Rep.* **2**, 336-341.
Wendler, F., Gillingham, A. K., Sinka, R., Rosa-Ferreira, C., Gordon, D. E., Franch-Marro, X., Peden, A. A., Vincent, J.-P. and Munro, S. (2010). A genome-wide RNA interference screen identifies two novel components of the metazoan secretory pathway. *EMBO J.* **29**, 304-314.
White, J., Johannes, L., Mallard, F., Girod, A., Grill, S., Reinsch, S., Keller, P., Tzschaschel, B., Echard, A., Goud, B. et al. (1999). Rab6 coordinates a novel Golgi to ER retrograde transport pathway in live cells. *J. Cell Biol.* **147**, 743-760.
Wu, C. C., Yates, J. R., Neville, M. C. and Howell, K. E. (2000). Proteomic analysis of two functional states of the golgi complex in mammary epithelial cells. *Traffic* **1**, 769-782.