A HIGH CONTENT SCREENING MICROSCOPY APPROACH TO DISSECT THE MECHANISM OF GOLGI-TO-ER RETROGRADE TRAFFIC

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University College Dublin

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The Golgi complex is the central sorting and processing station of the secretory or anterograde pathway. Nascent proteins leaving the endoplasmic reticulum (ER) are first received and collected into an ER-Golgi intermediate compartment, and then if destined for secretion are subsequently transported through the Golgi cisternae to the trans-Golgi network (TGN), during which time a wide range of post-translational modifications occur. The proteins received at the TGN, are selectively sorted and packaged into distinct carrier vesicles that are sorted and transported to either the plasma membrane or endosomal/lysosomal compartments. For certain proteins and lipids however, they may need to be selectively retained in specific Golgi cisternae, or in some cases returned to the ER by active transport. In the face of an incessant flow of material passing through it, the Golgi must maintain its structural and functional integrity. Although much is known about the machinery regulating the anterograde pathway, comparatively little is known about the retrograde route back to the ER, and how the Golgi regulates its structure.

In this work, two novel quantitative imaging approaches were devised to systematically identify proteins with a role in regulation of Golgi morphology and retrograde traffic from the Golgi to the ER. Utilising RNA interference (RNAi) technology and fluorescence microscopy coupled to the high throughput capabilities of high content screening a protocol to systematically probe gene function on a large scale and extract single cell multi-parametric data was developed. HeLa cells stably expressing a GFP-tagged Golgi enzyme GalNAc-T2 were used as a model to investigate the proteins regulating Golgi structure, and the metabolite brefeldin A was used as method to induce retrograde traffic from the Golgi to the ER.

The newly established approaches were first validated with a test set of targets. The first experiments targeted 70 proteins, composed primarily of the Rab GTPases and several Rab-accessory proteins, while a second larger test set of targets comprised 352 proteins associated with cytoskeleton structure, function and regulation. Systematic depletion of the GTPases confirmed the previously established role for Rab6a in this pathway, and provided clear data for involvement of Rab1a, Rab1b and Rab2a as regulators of Golgi-to-ER transport, in addition to their known role in anterograde traffic.
Rab10 and Rab11a were identified as potential novel regulators of this transport step and localisation studies showed their physical presence on a proportion of the Golgi-to-ER tubular intermediates. In addition, combinatorial depletions of Rab proteins also revealed previously undescribed functional co-operation and physical co-occurrence between several Rabs on the transport intermediates. An intricate interplay between the actin and microtubule cytoskeletal network in controlling Golgi structure and Golgi-to-ER trafficking was determined from the screen of cytoskeletal components. Not only did it confirm previously published roles for cytoplasmic dynein complex 1 as a regulator of both structure and trafficking, but it also identified several novel regulators of the two processes, for example Cdc42, Myo18a and Myh9.

A genome-wide RNAi screen was then performed, allowing the interrogation of a total of 21,585 proteins, and thereby providing a global analysis of gene function associated with the two processes. This screen revealed that more than 10% of the genome encodes proteins that can be linked to the function of the Golgi complex and confirmed previously described organelle structure regulatory proteins along with multiple novel regulatory proteins and trafficking associated complexes. Network analysis revealed that the regulation of the structure of the Golgi complex occurs at multiple levels, which together with the well-established core machinery, involves cross-talk between small GTP-binding protein regulation, actin and microtubule cytoskeleton organisation and membrane proteins of the ER. Approximately 14% of the genome could be linked to retrograde trafficking. Consistent with the literature, the crucial role of COPI-dependent and COPI-independent trafficking machinery was observed. Roles for the TRAPP and COG tethering complex as regulators of Golgi-to-ER transport were also identified. The results obtained further suggest that ER structural organisation plays a role in the delivery of the Golgi transport carriers to the ER. In addition to the core machinery associated with Golgi-to-ER redistribution, the screen also revealed roles for multiple GTPases, phosphatases and kinases in controlling the dynamics of this trafficking step.

Finally, combined analysis of the two screens revealed an intricate interplay between Golgi structure organisation and retrograde traffic. Network analysis revealed key links between Golgi structure organisation, retrograde trafficking, small GTP-binding protein regulation, protein tethering complexes and cytoskeletal organisation. These results provide the most comprehensive assessment of genes associated with Golgi structure, organisation and retrograde pathway function in mammalian cells to date.
ACKNOWLEDGMENTS

In these last four years I had the opportunity of crossing paths with many wonderful people that helped me survive and enjoy my PhD. Here I take the opportunity to thank the people that supported and aided me through this adventure.

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Finally, I thank my parents for their love and encouragement and without whom, I wouldn’t have embarked in this voyage of learning.
STATEMENT OF AUTHORSHIP

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree stated on the Title Page, and I have not obtained a degree elsewhere on the basis of the research presented in this submitted work.
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AP-(1-5)</td>
<td>Clathrin associated adaptor proteins (1 to 5)</td>
</tr>
<tr>
<td>ARF1</td>
<td>ADP-ribosylation factor 1</td>
</tr>
<tr>
<td>ARFGAP</td>
<td>Arf GTPase Activating Protein</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>BICD</td>
<td>Bicaudal-D</td>
</tr>
<tr>
<td>CATCHR</td>
<td>Complexes associated with tethering containing helical rods</td>
</tr>
<tr>
<td>CD</td>
<td>Cytoplasmic dynein</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CLT-A/B/C</td>
<td>Clathrin light chain A/B/C</td>
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<td>COG</td>
<td>Conserved oligomeric Golgi</td>
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<tr>
<td>COPI</td>
<td>Coat protein I</td>
</tr>
<tr>
<td>COPII</td>
<td>Coat protein II</td>
</tr>
<tr>
<td>CORVET</td>
<td>Class C core vacuole/endosome tethering</td>
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<tr>
<td>C-terminal</td>
<td>Carboxyl-terminal</td>
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<tr>
<td>CTx</td>
<td>Cholera toxin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
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<tr>
<td>DRC</td>
<td>Dynein regulatory complex</td>
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<tr>
<td>DSL1</td>
<td>Dependent on Sly1-20</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>ERGIC</td>
<td>ER-Golgi intermediate compartment</td>
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<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
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<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<tr>
<td>GalNAc-T2</td>
<td>N-acetylgalactosaminyltransferase-2</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating proteins</td>
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<tr>
<td>GARP</td>
<td>Golgi-associated retrograde protein</td>
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<tr>
<td>GBF1</td>
<td>Golgi-localised Brefeldin A-resistant factor 1</td>
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<td>GDI</td>
<td>GDP-dissociation inhibitor</td>
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<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>GETI</td>
<td>Golgi-to-ER trafficking index</td>
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<td>GFI</td>
<td>Golgi fragmentation index</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GO</td>
<td>Gene ontology</td>
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<tr>
<td>GOLPH3</td>
<td>Golgi phosphoprotein 3</td>
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<tr>
<td>GRASP</td>
<td>Golgi Reassembly And Stacking Protein</td>
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<td>HCS</td>
<td>High content screening</td>
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<td>HOPS</td>
<td>Homotypic fusion and vacuole protein sorting complex</td>
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<td>HTS</td>
<td>High throughput screening</td>
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<tr>
<td>IC</td>
<td>Intermediate compartment</td>
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<tr>
<td>INCENP</td>
<td>Inner centromere protein</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical aperture</td>
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<tr>
<td>NEG</td>
<td>Negative control siRNA</td>
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<td>NSF</td>
<td>N-ethylmaleimide-sensitive factor</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amine-terminal</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PE</td>
<td>Pseudomonas exotoxin</td>
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<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PM</td>
<td>Plasma membrane</td>
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<td>PPIN</td>
<td>Protein-Protein interaction network</td>
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<tr>
<td>PRAF</td>
<td>Prenylated Rab acceptor domain family</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SM</td>
<td>Sec1/Munc18-like</td>
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<tr>
<td>SNAP</td>
<td>Synaptosomal-associated protein</td>
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<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>STX</td>
<td>Syntaxin</td>
</tr>
<tr>
<td>STx</td>
<td>Shiga toxin</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>TRAPP</td>
<td>Transport protein particle</td>
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<tr>
<td>t-SNARE</td>
<td>Target membrane localised SNARE</td>
</tr>
<tr>
<td>VAMP</td>
<td>Vesicle associated membrane protein</td>
</tr>
<tr>
<td>VpsC</td>
<td>Vacuolar protein sorting class C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>v-SNARE</td>
<td>Vesicle localised SNARE</td>
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<tr>
<td>VSVG</td>
<td>Vesicular stomatitis virus G</td>
</tr>
<tr>
<td>VTC</td>
<td>Vacuolar-transporter chaperone</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>YIF</td>
<td>Yip interacting factor family</td>
</tr>
<tr>
<td>YIPF</td>
<td>Yip1 domain family</td>
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CHAPTER 1 - INTRODUCTION
1.1. The endomembrane system

Eukaryotic cells have evolved a complex array of membrane-bound compartments to allow biochemical reactions to be carried out with the highest specificity and efficiency. The organelles making up the endomembrane system are in constant flux, being generated and maintained while continuous exchange of protein and lipid components occurs. This trafficking of molecules throughout the system is very highly regulated, and is coordinated by various regulatory molecules to ensure that cargo is directed to the appropriate destination and the organelle identity is retained (Palade, 1975; Porter et al., 1945).

In the endomembrane system, the biosynthetic or secretory pathway delivers newly synthesised molecules from the endoplasmic reticulum (ER) to the plasma membrane (PM), the extracellular space and various other intracellular compartments (Palade, 1975). At each step, this forward traffic is counterbalanced by a retrograde recycling pathway. This recycles various proteins and lipids that function in the sorting of secretory cargo or the formation of transport intermediates, and retrieves residents of upstream organelles. This retrograde pathway ultimately links the PM with the ER (Farquhar, 1983; Munro and Pelham, 1987; Sleight and Pagano, 1984) (Fig. 1.1).

Figure 1.1. Model of organisation and trafficking in the endomembrane system of mammalian cells. An overview of a generic mammalian cell endomembrane system with the major compartments and trafficking pathways shown. Arrows represent the major trafficking routes and their direction. Red and blue arrows represent the anterograde and retrograde/endocytic pathways, respectively. Coat complexes are coloured to illustrate their participation in their respective transport step; COPII in green, COPI in blue and clathrin in yellow.
In the secretory pathway, the Golgi complex can be considered as the central sorting and processing station (Rothman, 1981). Nascent proteins from the ER are first received and collected into an ER-Golgi intermediate compartment (ERGIC). Subsequently secretory material is transported through the Golgi cisternae to the TGN, during which time many post-translational modifications occur. The proteins received at the TGN are selectively sorted and packaged into various vesicular and tubular carriers that are destined either for the PM or endosomal compartments (Palade, 1975). During this transit, many proteins are selectively retained in specific Golgi cisternae or returned to the ER by active transport. In the face of an incessant flow of material passing through it, the Golgi must maintain its structural integrity (Munro and Pelham, 1987).

1.2. The Golgi complex

The mammalian Golgi complex is the most recognisable landmark in the eukaryotic cell, composed of four to seven closely stacked membrane-bound cisternae and positioned to form a juxtanuclear ribbon structure. The outermost stacks are flanked by a loose network of vesicles and tubules, an indication of the large traffic of incoming and outgoing cargo associated with the organelle (Farquhar and Palade, 1981). The disk-shaped cisternae have evolved a narrow centre and dilated lateral rims, providing an internal environment with a low area/volume ratio to favour interactions between the enzymatic machinery and their cargo (Ladinsky et al., 1999).

The Golgi is not homogeneous in composition, and many of the resident proteins show a polarised distribution, largely dependent on the local lipids of the cisternae, protein-protein interactions and the amino acid composition and length of their transmembrane and cytoplasmic tail domains (Nilsson et al., 1993; Rabouille et al., 1995). This distribution is essential for a multitude of processes that the organelle performs; from trafficking, where molecular machinery for tethering, fusion and membrane curvature are needed at the extremities of the organelle; to the regulation of glycoprotein and glycolipid biosynthesis, where the correct distribution of specific enzymes ensures accurate glycosylation of proteins and lipids (Farquhar and Hauri, 1997; Opat et al., 2001).

The organised morphology of the Golgi is believed to be supported by the action of the Golgi matrix, various cytoskeletal elements and the correct balance in the bidirectional flow of membranes and proteins into and out of it. As such, any changes in flux through this organelle are likely to cause a change in its appearance and function. The structural organisation of the Golgi and associated trafficking pathways therefore
ideally need to be studied in tandem in order to generate a more comprehensive understanding of its function.

1.2.1. Golgi matrix

The Golgi matrix is an inter-cisternal meshwork made of protein components (Slusarewicz et al., 1994) that have been proposed to be integral for the organisation and stacking of the Golgi membranes. Its function still remains to be fully defined, although roles in regulating trafficking have been attributed to several protein components of the matrix. Two protein families have been shown to function and localise to the matrix, namely the Golgi Reassembly and Stacking Proteins (GRASPs) and the golgin families of proteins.

The GRASP family is composed of two proteins, termed GRASP55 and GRASP65 that have been shown to have distinct localisations. GRASP65 is found on the cis-Golgi cisternae (Barr et al., 1997) and GRASP55 is predominantly at the medial Golgi (Shorter et al., 1999). These two proteins have been shown to participate in the stacking of cisternae, supporting their role as Golgi stacking factors (Xiang and Wang, 2010).

The golgin, coiled coil family proteins also localise to distinct regions throughout the Golgi, where they are usually peripherally associated with the cytoplasmic face of the Golgi membrane (Barr et al., 2001; Wong and Munro, 2014). Their predominant function appears to be in the tethering or long-range attachment of membranes, a process coordinated by their interaction with Rab GTPases (Valsdottir et al., 2001; Weide et al., 2001). The reason for this interaction is yet to be clearly defined, although in cases such as for p115 and BICD, this binding is required for the recruitment of the golgins to the Golgi membrane (Allan et al., 2000; Short et al., 2002). Therefore, it has been suggested that the role of the Rabs is to trigger particular conformational changes in the golgins, and perhaps facilitating increased specificity during membrane tethering (Rosing et al., 2007; Short et al., 2002).

The two Golgi matrix protein families also interact with each other. GRASP55 is a specific binding partner of the medial-Golgi localised golgin-45. Disruption of this complex by the depletion of golgin-45 results in the dispersal of the Golgi apparatus and inhibition of protein transport (Short et al., 2001). GRASP65 is an adaptor for the golgin GM130, and this interaction is necessary for both proteins to localise to Golgi membranes (Barr et al., 1998). GRASP65 has also been implicated in trafficking, through its interaction with
p24 proteins, a family of cargo receptors involved in recycling between the ER and Golgi (Barr et al., 2001).

Golgins help maintain the Golgi structure through their interaction with and regulation of the cytoskeletal network. At the cis-Golgi, the golgin GMAP-210 has been reported to bind microtubules, and centrosomes (Infante et al., 1999). This microtubule binding protein is recruited to the Golgi membrane through its interaction with the small GTPase ADP ribosylation factor-1 (Arf1) (Cardenas et al., 2009; Gillingham et al., 2004; Rios et al., 1994). There it recruits γ-tubulin-containing complexes to the Golgi, linking the organelle to the centrosome, and through the action of AKAP450 (recruited by GM130), stabilises the Golgi ribbon structure (Rios et al., 1994; Rivero et al., 2009). At the trans-Golgi side, the golgin Gcc185 recruits the CLASP family of microtubule-binding proteins for the same function (Efimov et al., 2007). The Golgi complex therefore acts as a second microtubule organising organelle through microtubule nucleation and stabilisation (Chabin-Brion et al., 2001).

1.2.2. Cytoskeletal control of Golgi structure and trafficking

The organisation of the Golgi ribbon therefore is highly dependent on the microtubule and actin cytoskeleton networks (Egea et al., 2013; Papanikou and Glick, 2014; Yadav and Linstedt, 2011). Microtubule network depolymerisation through the action of nocodazole, results in the scattering of the Golgi throughout the cell as mini-stacks, which re-cluster in the cell centre following the reassembly of the network (Ho et al., 1989). Additionally, actin depolymerisation through the action of various toxins such as cytochalasins, latrunculins, jasplakinolide and botulinum toxins, results in a compacted Golgi morphology, through the loss and conjoining of the Golgi stacks (di Campli et al., 1999; Valderrama et al., 1998). Such depolymerisation phenotypes highlight the intricate and finely balanced ‘tug of war’ between the two cytoskeletal networks in order to keep the organelle in balance.

The position of the ribbon and transport into or out of the Golgi is determined by the action of molecular motors. In particular, the position of the Golgi in the centre of non-polarised cell is maintained by an array of minus-end-directed microtubule motors. Cytoplasmic dynein (CD) 1, a large motor complex is one such example. Inhibition of CD-1 and CD-2 function through overexpression of one or more of its subunits results in a Golgi scattered throughout the cell, mimicking the effect of microtubule depolymerisation (Burkhardt et al., 1997; Vaisberg et al., 1996). These phenotypes were also observed
upon disruption of the interaction between CD-1 and its two regulators dynactin (DCTN) and NUDE-like (NDEL1) (Karki and Holzbaur, 1999; Liang et al., 2004; Schroer, 2004). CD-1 has also been associated with both anterograde and retrograde trafficking (Chen et al., 2005; Hoogenraad et al., 2001; Matanis et al., 2002; Presley et al., 1997), along with kinesin-2 which has been shown to play a role in COPI-dependent recycling between the Golgi complex and ER (Stauber et al., 2006).

The Golgi also relies on the actin cytoskeleton to support its functions and position. This cytoskeletal network has been mostly associated with the budding of coated transport vesicles (Lázaro-Díéquez et al., 2007; Valderrama et al., 2000; Valderrama et al., 2001), although recent discoveries would indicate that the actin network has a structural role at the Golgi. Depletions of multiple Golgi localising, actin binding proteins such as Arp2/3, WASP homologue associated with actin, membranes, and microtubules (WHAMM) (Campellone et al., 2008) and cortactin (Kirkbride et al., 2012) have mimicked the actin depolymerisation phenotype. Also, myosin motors such as myosin18A (MYO18A) and Myosin-II have been implicated in the structural organisation of the Golgi. The F-actin motor protein MYO18A localises to distal Golgi membranes, where it binds to Golgi phosphoprotein 3 (GOLPH3). The complex has been shown to connect the Golgi apparatus to F-actin to provide a tensile force required for efficient tubule and vesicle formation (Dippold et al., 2009; Taft et al., 2013). Myosin-II is recruited to the Golgi membrane through interactions with the golgin giantin and Rab6a (Rosing et al., 2007; Valente et al., 2010). This myosin and GTPase form a complex which facilitates myosin-II localisation to Golgi membranes and in turn controls the fission of anterograde and retrograde Rab6 transport carriers (Miserey-Lenkei et al., 2010; Rosing et al., 2007). The role and function of cytoskeletal components in Golgi structure maintenance and trafficking is further discussed in Chapter 4.

1.2.3. Mechanisms of trafficking through the Golgi

There are currently at least three models of how transport through the Golgi occurs: a) an anterograde vesicular transport model, b) a cisternal maturation model, and c) a rapid-partitioning model. The anterograde vesicular trafficking model states that all the resident Golgi enzymes have an allocated fixed position in the organelle, and that cargo moves from cisterna to cisterna by anterograde transport (Orci et al., 2000; Ostermann et al., 1993) (Fig. 1.2A). The cisternal maturation model claims that the Golgi is in a continuous dynamic change, whereby the cisternae mature from the cis- to the
trans-Golgi. During this maturation process the cargo stays within the same cisterna as the resident proteins and enzymes move in a retrograde direction (Glick et al., 1997; Glick and Luini, 2011; Glick and Nakano, 2009) (Fig. 1.2B). The cisternal maturation model accounts better for the transport of larger molecules, in particular pro-collagen, which has been shown to form large aggregates that remain in the cisternae as they transverse the Golgi stack (Beznoussenko et al., 2014; Bonfanti et al., 1998; Clermont et al., 1993; Mironov et al., 2001). The model also fits better with experimental data showing that purified intra-Golgi vesicles are in some cases enriched with glycosylation enzymes, rather than cargo (Lanoix et al., 1999; Martinez-Menarguez et al., 2001; Rabouille et al., 1995).

However, the cisternal maturation model does not fit with all of the experimental data, particularly with regard to the predicted lag period between the entry of cargo molecules in the Golgi and their exit. In fact, experiments done on the transport kinetics of the temperature-sensitive GFP-VSVG-ts045G model cargo marker revealed that the cargo protein passes through the Golgi stacks at a faster rate than predicted by the model, with no apparent lag. The study showed that incoming cargo molecules rapidly mix with those already in the system and exit from it, in no particular order (Patterson et al., 2008). Given these results, a new model for intra-Golgi transport was developed to better explain the dynamics in action; termed the rapid-partitioning model.

The model postulates that the Golgi is a continuous system, with spatially distinct compartments, maintained by a lipid gradient across the Golgi and through regulated fission and fusion events (Lippincott-Schwartz and Phair, 2010; Patterson et al., 2008; Sharpe et al., 2010) (Fig. 1.2C). The model as yet does not account for the existence of discrete cisternae and distinct Golgi compartments (Glick and Luini, 2011), but is currently the model that fits best with experimental data available (Jackson, 2009).

The proposed intra-Golgi transport models are not mutually exclusive and may occur simultaneously. Although the exact dynamics of intra-Golgi transport still have to be defined, what is certain is that all models are dependent on the recycling of proteins through the action of membrane fusion proteins, coat protein complexes, vesicle tethering proteins and other trafficking machinery (Glick and Luini, 2011; Jackson, 2009)
Figure 1.2. Models for intra-Golgi trafficking. Cargo synthesised in the ER and transported through the secretory pathway is shown in grey; Golgi processing enzymes are shown in green. (A) Anterograde vesicular model. Secretory cargo travels from the ER to the intermediate compartment (IC), the Golgi and finally to the PM in distinct carriers. The Golgi cisternae are stable and biochemically distinct; resident Golgi proteins have a fixed position, as cargo proteins flows through the Golgi. (B) Cisternal maturation model. Cargo in the IC, received from the ER, moves forward as a result of the maturation of the compartment into a later one; while Golgi enzymes move in the opposite direction through vesicles travelling from a mature compartment to a younger one. (C) Rapid-partitioning model. Cargo arriving from the ER equilibrates across the stack via inter-cisternal continuities. Secretory cargo is partitioned dynamically between domains, which contain resident Golgi proteins, and export regions. These various domains are established by segregation of different lipid classes. Secretory cargo can exit the Golgi at every level of the stack.
1.3. **Golgi/ER membrane traffic**

Bi-directional membrane traffic between the ER and the Golgi complex occurs via 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 IC and Golgi back to the ER. A second, relatively uncharacterised Rab6a-mediated, COPI-independent retrograde pathway also exists, and is utilised by cycling glycosylation enzymes to cycle back to the ER (Brandizzi and Barlowe, 2013; Spang, 2013).

The mechanism of transport from a donor compartment to an acceptor membrane is conserved and can be summarised in 7 major steps (Fig. 1.3). (1) The initiation of a trafficking step starts with the binding of specific cargo to receptors present in the donor membrane. The activated receptors initiate a cascade reaction starting from (2) the activation and recruitment of a GTPase (Sar1 or Arf1) to the donor membrane, followed by the formation of a coat protein complex cage for the budding of the lipid bilayer. (3) Followed, by scission and (4) uncoating of the curved membrane to create a nascent vesicle or transport carrier. (5) The fully formed carrier is then transported towards its destination by cytoskeletal motors, where tethering complexes bring the vesicle in close proximity to the acceptor membrane and initiate (6) docking and (7) fusion of opposing membranes through the formation of fusogenic soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes. All these major membrane trafficking steps are regulated by Rab GTPases and their effectors (Bonifacino and Glick, 2004; Brandizzi and Barlowe, 2013; Spang, 2013). The machinery required for the trafficking step between the Golgi and the ER will be explained in more detail in the following sections, starting with the Rab GTPases.
The mechanisms for exchange of membranes and proteins between two cellular compartments can be summarised in seven major steps: (1) Initiation or binding of specific cargo to receptors; (2) budding of the lipid bilayer through the recruitment of a coat protein complex; (3) scission to create a nascent carrier; (4) uncoating of the carrier; (5) transport of the carrier and tethering to the acceptor membrane, followed by (7) docking of, (8) and fusion of opposing membranes. Figure taken from Bonifacino and Glick (2004).

### 1.3.1. Rab GTPases

The Rab (Ras-like in rat brain) GTPases are a large family of small GTP-binding proteins that help specify membrane identity and vesicle budding, uncoating, motility and fusion through the recruitment of a wide variety of effector proteins. The family is composed of over 60 members in human cells, of which 20 are associated with the Golgi membranes (Gilchrist et al., 2006; Stenmark, 2009)(JCS Lab, unpublished work). Rab proteins switch between an inactive GDP-bound and an active GTP-bound form, which in turn determines their ability to bind effectors. In their inactive state, Rabs are bound to a molecule of GDP dissociation inhibitor (GDI), which keeps them in a soluble cytoplasmic state (Garrett et al., 1994; Sasaki et al., 1990; Shapiro and Pfeffer, 1995; Shisheva et al., 1999; Ullrich et al., 1993). The activation of the Rab proteins from a GDP state to a GTP state is mediated by Rab-specific guanine nucleotide exchange factors (GEFs) (Burstein and Macara, 1992; Burton and De Camilli, 1994; Burton et al., 1994), and GTP hydrolysis is enhanced by GTPase-activating proteins (GAPs) (Barr and Lambright, 2010; Becker et al., 1991; Gibbs et al., 1988; Trahey and McCormick, 1987). 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 (Moya et al., 1993; Pfeffer and Aivazian, 2004; Sivars et al., 2003).
Many Rabs are known to regulate distinct aspects of trafficking mechanisms, from vesicle budding (Carroll et al., 2001), uncoating (Semerdjieva et al., 2008), motility (Roland et al., 2007; Wu et al., 2002) and fusion (Simonsen et al., 1998; Stenmark et al., 1994), often by the recruitment of vesicle tethers, SNAREs and motor proteins (Fig. 1.4.). Due to the crucial regulatory role of the Golgi complex in trafficking, a large number of Rabs function on these membranes. The most prominent members are Rab1, Rab2, and Rab6 variants; where Rab1 and Rab2 regulate Golgi/ER trafficking (Beard et al., 2005; Monetta et al., 2007; Rosing et al., 2007; Short et al., 2001; Tisdale et al., 1992; Wu et al., 1998) and Rab6 regulates intra, early and post-Golgi trafficking (Antony et al., 1992; Del Nery et al., 2006; Girod et al., 1999; White et al., 1999). Other less-characterised Rabs such as Rab18 (Carpanini et al., 2014; Dejgaard et al., 2008), Rab33b (Valsdottir et al., 2001; Zheng et al., 1998), Rab40 (Lee et al., 2007; Rodriguez-Gabin et al., 2004), and Rab43 (Dejgaard et al., 2008; Haas et al., 2007) have also been linked with the Golgi membranes and its associated trafficking. The role of these Rab GTPases is further discussed in Chapter 4.
Figure 1.4. Rab GTPase functions in membrane trafficking. Rab family proteins regulate various steps of transport, namely: (a) cargo sorting through the recruitment of receptors into a budding carrier; (b) uncoating through the recruitment of phosphoinositide (PI) kinases or phosphatases that alter the PI composition of the transport carrier; (c) transport through the recruitment of motor adaptors or by binding directly to motors; (d) tethering by the recruitment of tethering factors or complexes that bring the carrier in close proximity to the acceptor membrane and assist in SNARE complex formation and (e) membrane fusion. Figure taken from Stenmark (2009).

1.3.2. SNAREs, membrane fusion machinery

The fusion of transport carriers with their target membranes is fundamental for intracellular membrane trafficking and is driven by the assembly of functional soluble SNARE complexes (Hong, 2005; Söllner et al., 1993). The SNARE protein family is composed of at least 36 members, all localised to specific membrane compartments (Hong, 2005). All SNAREs are membrane-associated proteins that contain characteristic SNARE domains; heptad repeats ~60-70 amino acids in length that are predicted to form coiled-coils (Fasshauer et al., 1998; Weimbs et al., 1997). Functionally, SNARE proteins
can be classified as v-SNAREs or t-SNAREs according to their presence on vesicles or target membranes, respectively. The vesicle localising v-SNAREs usually contain a single SNARE motif which binds to the either two to three domains present on t-SNAREs. Upon pairing of the two classes of SNAREs, a SNARE-pin is formed, causing two membranes to fuse together (Fukuda et al., 2000; Hong, 2005; Sutton et al., 1998; Weber et al., 1998).

Structurally, SNAREs are also classified as Q or R types based on the conservation of a particular amino acid (either glutamine or arginine) in the SNARE domain (Fasshauer et al., 1998). SNARE pairing usually occurs in a 3Q:1R ratio (Fasshauer et al., 1998), where most R-SNAREs act as v-SNAREs and Q-SNAREs act as t-SNAREs. However, there are exceptions; the R-SNAREs, Ykt6 and Sec22B act as part of the t-SNARE sub-complexes, whereas GS15, Bet1, and Slt1 are Q-SNAREs that function as v-SNAREs (Hong and Lev, 2014). The Q-SNAREs are segregated into four major subfamilies (Qa, Qb, Qbc, and Qc) based on the amino acid sequence homologies of their SNARE domains. A t-SNARE is generally assembled from one heavy chain and two light chains of SNARE domains. The two light chains can come from one or two proteins. Qa-SNAREs are considered as the heavy chain components, with key members being many of the syntaxins (1, 2, 4, 5, 7, 13, 16 and 18), whereas Qb and Qc-SNAREs are considered as light chain components. Examples of Qb-SNAREs are Vti1a, Vti1b, GS27, and GS28; while examples of Qc-SNAREs are Stx6, Stx10, and Bet1. The fourth group, Qbc SNAREs, lack a transmembrane sequence and possess two SNARE domains, an example of which is SNAP-25 (Fukuda et al., 2000). The R-SNAREs include Sec22B and the vesicle associated membrane proteins (VAMPs) (Fukuda et al., 2000; Hong, 2005; Hong and Lev, 2014; Jahn and Scheller, 2006; Sutton et al., 1998).

SNAREs have been shown to form multiple distinct complexes, through the combinatory use of the various members of the Q-, and R-SNAREs; with the function and the localisation of the complex determined by its components. SNARE proteins are not specific to one single complex but can be incorporated in several complexes. For example Stx5 has been found to be part of three complexes: a) an early anterograde trafficking complex, composed of Stx5, GS27, Bet1, and Sec22B for the fusion of ER derived COPII vesicles to the ERGIC (Zhang et al., 1999; Zhang et al., 1997); b) a late anterograde trafficking complex, composed of Stx5, GS28, Bet1 and Ykt6, for the fusion of ER derived carriers to the Golgi (Shorter et al., 2002; Zhang and Hong, 2001); and c) an intra-Golgi and early Golgi trafficking complex, composed of Stx5, GS28, GS15, and Ykt6 (Parlati et al., 2002; Xu et al., 2002a) (Fig. 1.5).
To date, several SNARE complexes that drive the fusion of membranes have been identified, although many are still to be fully characterised. Such a case is the SNARE complex involved with the retrograde transport of carriers derived by COPI complex. The v-SNARE, Sec22B has been shown to be incorporated in the COPI derived vesicle (Lewis et al., 1997), while the ER located t-SNARE complex is thought to be composed of Stx18 (Hirose et al., 2004; Nakajima et al., 2004), Slt1/Use1 (Dilcher et al., 2003) and Sec20/BNIP1 (Burri et al., 2003; Verrier et al., 2008) (Fig. 1.5), although not enough evidence has been shown to confirm this.

The "SNARE hypothesis" states that SNAREs form the minimal machinery needed to fuse two membranes together and the proteins themselves confer specificity to the fusion of transport vesicles with their target membranes (Bock et al., 2001; Scales et al., 2000; Sollner et al., 1993). The specificity of the SNAREs is a subject of controversy, conflicting results have shown these complexes may be highly specific (Fukuda et al., 2000; Izawa et al., 2012; Parlati et al., 2002) but also may be promiscuous on occasions (Tsui and Banfield, 2000; Yang et al., 1999). Numerous studies have now shown that membrane-fusion specificity is largely dependent on SNARE proteins, because different SNARE-pins can catalyse distinct fusion events (Furukawa and Mima, 2014; Hong and Lev, 2014; Izawa et al., 2012; Wong and Munro, 2014). This controversy led to the discovery of many regulators that play either positive or negative roles in the control of complex formation and SNARE specificity. The localisation and activity of SNAREs is also affected by post-translational modifications (Fukasawa et al., 2004; Nagy et al., 2004; Veit et al., 1996).

Among the regulators of SNAREs, the Sec1/Munc18-like (SM) proteins, Rab GTPases, golgins and the COG tethering complex have been shown to regulate the fusion process between the Golgi and ER. SM proteins are a small family of soluble, arc-shaped proteins, essential for fusion (Bracher et al., 2000). These proteins associate with SNARES in three different conformations to assist in the SNARE-pin assembly (Laufman et al., 2009). There are at least seven mammalian members of the SM protein family: Munc18-1, Munc18-2, Munc18-3, Vps33A, Vps33B, Vps45, and Sly1 (Hong, 2005; Malsam et al., 2008). In addition to their interactions with SNAREs, SM proteins also interact with tethering complexes. For example, the SM protein Sly1 interacts with SNARE proteins (Stx5 and Stx18) (Kosodo et al., 2002; Peng and Gallwitz, 2002; Williams et al., 2004) and the COG complex (COG4) in the ER to Golgi pathway (Laufman et al., 2009).
1.3.3. Multi-subunit tethering complexes

The multi-subunit tethering complexes can be classified into two groups: a) the Rab effectors and SNARE interactors, DSL-1, COG complex, GARP and Exocyst complexes; and b) the Rab GEFs, TRAPP and the VpsC complexes. Of these, the two CATCHR (complexes associated with tethering containing helical rods) complexes, DSL1 and COG, along with the TRAPP complex are known to function at the Golgi complex and ER/Golgi interface (Fig. 1.5).

1.3.3.1. DSL1 complex

In yeast, the ER localising DSL1 (dependent on Sly1-20) complex consists of three subunits, Dsl1 (Vanrheenen et al., 2001), Dsl3/Sec39 (Sweet and Pelham, 1993), and Tip20 (Kraynack et al., 2005; Mnaimneh et al., 2004). The complex has been shown to serve as an acceptor of COPI derived carriers through its interaction with the three Q-SNAREs of the ER, Use1, Ufe1 and Sec20 (Andag et al., 2001; Kraynack et al., 2005; Reilly et al., 2001; Ren et al., 2009; Tripathi et al., 2009). In yeast, the complex has been
shown to also interact with the Rab GTPase Ypt1 (homologue of mammalian Rab1) as part of its tethering function (Kamena et al., 2008).

The DSL1 complex is evolutionarily conserved in mammalian cells and is called the NRZ complex based on its subunit names, NAG (Neuroblastoma-associated gene; homologue of Sec39p), RINT-1 (RAD50-interacting protein; homologue of Tip20p), and ZW10 (Zeste White-10; homologue of Dsl1) (Aoki et al., 2009; Hirose et al., 2004; Tripathi et al., 2009). The NRZ complex subunits have been implicated in membrane trafficking (Hatsuzawa et al., 2000; Hirose et al., 2004), cell cycle (Williams et al., 1992; Xiao et al., 2001) and also autophagy regulation. The exact mechanism by which the tethering complex functions is yet to be defined, but similar to the yeast counterpart the NRZ complex is associated with the ER SNAREs Stx18 (Aoki et al., 2009; Hatsuzawa et al., 2000; Hirose et al., 2004) and BNIP1 (Nakajima et al., 2004; Uemura et al., 2009). The complex has been suggested to have a role in ER SNARE complex assembly (Diefenbacher et al., 2011; Kraynack et al., 2005) and in catalysing the binding of the COPI v-SNARE Sec22B with its ER t-SNARE binding targets (Aoki et al., 2008).

1.3.3.2. COG complex

The conserved oligomeric Golgi (COG) complex functions as a vesicular tether during retrograde intra-Golgi trafficking, by bringing vesicles close to a specific Golgi cisterna and promoting SNARE-mediated fusion (Zolov and Lupashin, 2005). The complex is made of eight subunits, named Cog1-8 (Kingsley et al., 1986; Ram et al., 2002; Suvorova et al., 2002; Ungar et al., 2002; Whyte and Munro, 2001); separated into two sub-complexes named Lobe A (Cog1-4) and Lobe B (Cog5-8) bridged by the interaction of Cog1 and Cog8 (Fotso et al., 2005; Ungar et al., 2005). The complex is particularly important for the tethering of COPI vesicles containing glycosylation enzymes, and defects in COG subunits lead to improper glycosylation of secretory proteins (Kingsley et al., 1986; Shestakova et al., 2006).

The complex primarily localises to the tips and rims of the Golgi cisternae and their associated vesicles, on both the cis- and trans-Golgi network faces (Vasile et al., 2006). The complex is found both in soluble and in its octameric form, but can also be stable in different arrangements, include Lobe A and Lobe B sub-complexes (Willett et al., 2013b). The COG complex has been shown to interact with a variety of trafficking machinery, including SNAREs, SNARE-interacting proteins, Rabs, coiled-coil tethers, vesicular coats and molecular motors, to regulate intra-Golgi trafficking. The complex interacts with
various SNARE complexes functioning in intra-Golgi and trans-Golgi trafficking, namely Cog4 with Stx5 (Shestakova et al., 2007; Sohda et al., 2010), Cog6 with Stx5, Stx6, GS27 and SNAP29 (Kudlyk et al., 2013; Laufman et al., 2011; Willett et al., 2013a); and Cog8 with Stx5, GS27 and Stx16 (Laufman et al., 2013; Willett et al., 2013a).

To date, no interactions between COGs and ERGIC SNAREs have been reported. Multiple COG-Rab interactions were shown in yeast two hybrid screens, these include Rab1a/b, Rab2, Rab6a/b, and Rab10 amongst others (Miller et al., 2013; Sohda et al., 2010). The interaction between the COGs and the GTPases was shown to be stronger with the GTP-bound Rabs than the GDP version suggesting that the COG complex is an effector of these Rabs (Miller et al., 2013). Various other yeast two hybrid screens supported the central regulatory role of this complex through its interactions with various golgins, such as p115, GM130, and giantin (Miller et al., 2013; Sohda et al., 2010). The complex also interacts with coat protein complex subunits, specifically with the COPI component, β-COP (Miller et al., 2013) and the clathrin adaptor protein Ap3b1 (Kristensen et al., 2012), suggesting that the complex might also be involved in either coat protein recruitment or vesicle budding.

1.3.3.3. TRAPP complex

The transport protein particle (TRAPP) tethering complex is arranged into three different conformations: TRAPPI, TRAPPII and TRAPPIII; each complex performing a distinct function. In yeast, there are eleven TRAPP subunits, seven of which are the common core for all three conformations. TRAPPI is required for ER-to-Golgi transport (Sacher et al., 1998), TRAPPII is required at the TGN (Cai et al., 2005) and TRAPPIII is required for autophagy (Lynch-Day et al., 2010).

In mammals, all yeast TRAPP components are conserved, with the addition of subunits not found in yeast; the components are TrappC-1, 2, 2L, 3, 4, 5, 6A, 6B, 8, 9, 10, 11, and 12. Subunits TRAPPC9 and TRAPPC10 are unique to the TRAPPII complex (Yamasaki et al., 2009; Zong et al., 2012); while TRAPPC8, TRAPPC11, TRAPPC12 and TRAPPC13 (Scrivens et al., 2011) are unique to the TRAPPIII complex (Brunet and Sacher, 2014). The function of the three mammalian TRAPP complexes is thought to be similar to their yeast counterparts, although it is still not yet fully defined. In yeast the TRAPPI complex tethers COPII vesicles at the ERGIC, where most of the TRAPP subunits localise, and where it is a GEF for Ypt1 (homologue of Rab1) (Kim et al., 2006; Lord et al., 2011; Sacher et al., 2001). TRAPPII is also a GEF for Ypt1, Ypt31/32 (the
homologue of Rab11) (Morozova et al., 2006); it has been suggested that the complex functions at early Golgi membranes where it interacts with COPI coat components to tether intra-Golgi carriers, both in yeast and mammalian cells (Sciorra et al., 2005; Yamasaki et al., 2009). The TRAPPIII complex has been shown to localise to the early and late Golgi compartments, where it plays a role in autophagy; the complex is also an Ypt1 GEF and binds COPII coat subunits to initiate phagophore formation as part of the autophagy pathway (Shirahama-Noda et al., 2013; Tan et al., 2013; Wang et al., 2014).

1.3.4. COPII transport carriers

Newly synthesised proteins and lipids in the ER, destined to be exported to different intra-membrane compartments of the endomembrane system or the extracellular space, have to be correctly assembled before transitioning to the Golgi. Proteins to be exported are gathered at ER exit sites (ERES), which are responsible for the assembly of the transport vesicles that carry the newly synthesised proteins to their destination. ERES are enriched in COPII and large multi-domain Sec16 proteins required for export site assembly and function (Novick et al., 1980; Supek et al., 2002). Localisation of COPII activity into these micro-domains helps to maintain a crucial concentration of COPII proteins and enables the efficient recycling of these coat proteins as they are recruited back to the membrane for use in subsequent rounds of COPII budding (Bannykh et al., 1996; Orci et al., 1991; Tang et al., 2005).

The COPII-derived carriers are responsible for anterograde transport from the ER to the ERGIC/cis-Golgi compartment. The process of COPII vesicle budding is initiated by activation of Sar1-GDP by the GEF Sec12, which is a type II ER intrinsic membrane protein (Barlowe and Schekman, 1993). The binding of Sar1 to the ER membranes through an amino-terminal α-helix, triggers the recruitment of the two COPII inner layer subunits Sec23 and Sec24 (Kuehn and Schekman, 1997; Saito et al., 1998; Weissman et al., 2001). Sar1-GTP binds directly to the Sec23 portion of the heterodimer (Hicke and Schekman, 1989; Kaiser and Schekman, 1990); while Sec24 binds to and selects specific cargo for packaging into the Golgi-destined carriers including the various SNAREs involved in ER-to-Golgi transport (Barlowe et al., 1994; Miller et al., 2003; Mossessova et al., 2003a). This is followed by the recruitment of the heterotetramer Sec13-Sec31 to the pre-budding complex of Sar1-GTP-Sec23/Sec24 to provide the complete outer layer of the coat. The Sec13-Sec31 complex cross-link and drive membrane deformation to form COPII vesicles (Saito-Nakano and Nakano, 2000; Salama et al., 1997). During or after the
formation of the vesicle, the GAP activity of Sec23 stimulates the hydrolysis of Sar1-GTP, uncoating the transport carrier and releasing the coat subunits for further cycles (Futai et al., 2004) (Fig. 1.6).

The size of the COPII vesicles can be adapted to the bulk of the cargo. Several lines of evidence indicate that the COPII cage is flexible, which would explain the existence of different isoforms of most of the COPII subunits (Fath et al., 2007; Miller and Schekman, 2013). These different isoforms are Sar1 (A and B), Sec23 (A and B), Sec24 (A, B, C, and D), and Sec31 (A and B). There is only one type of Sec13 (Dudognon et al., 2004; Salama et al., 1997; Stankewich et al., 2006).

**Figure 1.6. COPII coat complex formation.** Graphical representation of the COPII machinery (left panel) and the mechanisms of its assembly; starting from the activation and recruitment of the GTPase Sar1, coordinated by its GEF, the ER resident protein Sec12, which produces the GTP-bound form of SAR1 (top panel). Sar1 recruits Sec23-Sec24 heterodimers onto the ER membrane. Sec23 binds to Sar1 while Sec24 recruits the protein cargo destined in the vesicle (middle panel). The coat complex is complete, when Sec13-Sec31 heterodimers polymerise on the underlying Sar1-Sec23-Sec24-cargo complexes, initiating membrane budding (bottom panel). Figure taken from Brandizzi and Barlowe (2013).
1.3.5. COPI derived transport carriers

COPI derived transport carriers are responsible for intra-Golgi trafficking and the cycling of cargo that carries a KDEL, KKXX or KKKXX dilyasine motifs, to the ER. Cargo carrying one of these motifs binds either to the coat complex subunits or to receptors (such as KDEL receptor) present at cis-Golgi membranes, to initiate the budding process (Cosson and Letourneur, 1994; Johannes et al., 1997; Letourneur et al., 1994; Martinez et al., 1994). COPI vesicle formation is initiated by the recruitment of the small GTPase ADP-ribosylation factor 1 (Arf1) to the Golgi membrane in a GTP-dependent manner (Donaldson et al., 1992; Franco et al., 1996; Kahn et al., 1992) (Fig. 1.7). Arf1 is recruited by a family of oligomerising cargo receptor proteins, generically named the p24 family, and then the GTP-loading is catalysed by Golgi-localised Brefeldin A-resistant factor 1 (Gbf1), a large Sec7 domain-containing GEF (Aguiler-Romero et al., 2008; Beck et al., 2008; Bethune et al., 2006a; Fiedler et al., 1996; Peyroche et al., 1999).

![Figure 1.7. COPI coat complex formation.](image)

Figure 1.7. COPI coat complex formation. Graphical representation of the COPI machinery (left panel) and the mechanisms of their assembly. COPI coat formation is initiated by the recruitment of Arf1 to the Golgi membrane by its GEF containing a conserved Sec7 domain (top panel). The active GTPase recruits the coatomer complex to the membranes (middle panel), which in turn interact with motifs on cytosolic domains of membrane cargo and mediate cargo incorporation into nascent COPI vesicles (bottom panel). Figure taken from Brandizzi and Barlowe (2013).
Following nucleotide exchange, Arf1 undergoes a conformational change, leading to the exposure of a myristoylated N-terminal amphipathic helix that provides stable membrane anchorage as well as initiating membrane deformation which starts forming the vesicle bud (D’Souza-Schorey and Chavrier, 2006; Franco et al., 1996). The membrane bound activated Arf1 recruits the pre-assembled coatamer to the membrane. The coatamer consisting of the following 7 subunits: α-, β-, β’-, γ-, δ-, ε- and ζ-COP, with two main sub-complexes: the γ-, δ-, β-, ζ-COP tetrameric complex, which constitutes the inner layer core, and the α-, β’, ε-COP trimeric complex, which forms the outer layer of the COPI coat (Beck et al., 2009; Bethune et al., 2006b; Duden et al., 1991; Harrison-Lavoie et al., 1993; Malhotra et al., 1989; Schekman and Orci, 1996; Serafini et al., 1991b; Stenbeck et al., 1993; Waters et al., 1991) (Fig. 1.7). Uncoating of COPI vesicles, a necessary step prior to fusion with the target membrane, is triggered and controlled by Arf GTPase Activating Proteins (ArfGAP), incorporated in the COPI vesicle, which catalyses the hydrolysis of the Arf-bound GTP (Malsam et al., 1999; Tanigawa et al., 1993).

Early studies on the drug brefeldin A (BFA) highlighted the importance of Arf1 and the COPI coat complex for the maintenance of Golgi structure and trafficking. This highly specific drug, targets the interaction between Arf1 and its guanine nucleotide exchange factors, by forming a GEF-Arf1-GDP intermediate (Mossessova et al., 2003b; Peyroche et al., 1999; Renault et al., 2003). This prevents Arf1 from being activated, lack of COPI recruitment to the membrane, and results in the collapse of the organelle into the ER typically within a few minutes of application. This redistribution of Golgi markers to the ER is accompanied by a dramatic tubulation of Golgi membranes which can be reversed upon washout of the drug. The effects of ARF1 inactivation highlight the delicate balance of Golgi organisation. ARF1 has been shown to bind and recruit various effectors, essential for the maintenance of the Golgi organisation, including COPI itself, clathrin associated adaptor proteins (AP-1), lipid recruiting enzymes and membrane tethers. Therefore, the loss of ARF1 activity leads to a cascade event, through the imbalance of the lipid and protein environment in the Golgi resulting in loss of structure (Doms et al., 1989; Donaldson et al., 1990; Lippincott-Schwartz et al., 1990; Miller et al., 1992; Rios et al., 1994; Sciaky et al., 1997).
1.3.6. COPI-independent transport carriers

Along with COPI-dependent retrograde transport, another pathway exists to recycle proteins from the Golgi to ER; seemingly independent of both Arf1 and coatomer complex presence. The COPI-independent recycling pathway has been shown to be responsible for the trafficking of the *Shigella dysenteriae* Shiga toxin and *Escherichia coli* Shiga-like toxin (both lacking a KDEL retrieval sequence) and Golgi-resident glycosylation enzymes to the ER, in a Rab6a-dependent manner (Girod et al., 1999; White et al., 1999). Relatively little is known about the regulation and machinery of this pathway, partly due to the involvement of Rab6a in other trafficking processes namely COPI- and clathrin-independent trafficking steps in the Golgi (Storrie et al., 2012).

The role of Rab6a has been under scrutiny, to determine the effectors unique to the COPI-independent transport and the mechanisms by which these carriers form (reviewed by Heffernan and Simpson (2014)). To date, only the molecular motor dynein-dynactin complex (Short et al., 2002; Wanschers et al., 2008), along with three of its accessory proteins have been associated with this pathway, namely the two coiled-coil proteins BICD1/2 (Hoogenraad et al., 2001; Matanis et al., 2002) and “idling” dynein protein LIS1/PAFAH1B1 (Yamada et al., 2013). Recently, a non-progressive myosin motor has been implicated with this pathway, myosin-II. Rab6a has been shown to interact with the golgin giantin to recruit the myosin, to mediate fission and formation of ER-destined vesicles in a COPI-independent manner (Miserey-Lenkei et al., 2010; Rosing et al., 2007). Other candidates have been suggested such as KIF20A/Rabkinesin6 but this seems to be highly unlikely since the motor protein is barely expressed in interphase cells. The protein is present 10-fold higher in prophase cells where it localises to the mid-zone of the spindle during anaphase and to the cleavage furrow and midbody during telophase, (Fontijn et al., 2001; Hill et al., 2000) leaving its interphase function unclear. The mechanics and the effectors of this pathway are still unknown.

1.4. Mapping the retrograde system: protein toxins and future therapeutics

A number of bacterial and plant species have evolved common strategies in order to defend themselves and ensure their survival. Some of these strategies have taken the form of producing protein toxins, designed to bind to the cell surface and subsequently utilise the trafficking machinery in the target cell to reach their site of action, as part of the cell killing mechanism. Prominent members of this group of cytotoxins include the bacterial exotoxins, Shiga toxin (STx), Cholera toxin (CTx) and the plant protein ricin; all
causing life threatening diarrhoea and vomiting among other symptoms (Finkelstein and LoSpalluto, 1969; Kimberg et al., 1971; Paton and Paton, 1998; Tarr et al., 2005; Trofa et al., 1999); affecting millions of individuals and causing more than a million deaths per year (Beddoe et al., 2010).

The majority of the cytotoxins use similar strategies for their internalisation. For example, STx binds to a glycolipid globotriaosylceramide (Gb3) receptor on the PM, the complex of the two is internalised through both clathrin-dependent and independent endocytosis (Falguieres et al., 2001; Sandvig et al., 2010; Sandvig et al., 1989). Many toxins move through the endosomal compartments to the TGN, although a large proportion accumulates in lysosomes, which is seemingly rendered ineffective for cell killing. Transport from the TGN to the ER varies depending on the toxin; some travel retrogradely from the TGN to the cis-Golgi to the ER, while others seem to by-pass the Golgi and go directly to the ER. CTx and STx traffic from the Golgi to the ER using different paths: CTx contains a KDEL motif, and therefore a portion of it at least is transported in a COPI-dependent manner (Chen et al., 2002; Majoul et al., 1998); whereas STx has been shown to use a Rab6A-dependent COPI-independent pathway; in fact this toxin was the model cargo used to discover this trafficking route (Girod et al., 1999; Sandvig and van Deurs, 2002; Spooner et al., 2006; White et al., 1999).

Over the last two decades, the transport pathways of these cytotoxins have been under extensive study, not only for the development of vaccines and therapeutics to stop and cure the effects caused by these cytotoxins, but also to understand the basic molecular machinery regulating retrograde traffic. The advances made through these discoveries are leading to the development of a promising new therapeutic approach based on the transport of these cytotoxins. A series of new experimental drugs blocking trafficking are under developed for the treatment of infections caused by toxin-producing bacteria (Stechmann et al., 2010); while new drug delivery vector systems are being tested based on the trafficking mechanisms of these toxins (Mukhopadhyay and Linstedt, 2013). For these reasons alone, greater understanding of the molecular regulation of retrograde traffic is essential.

1.5. **High content screening: Systematic approach to study trafficking**

Over the past 40 years, our understanding of the cell and the biochemistry of protein and lipid transport has increased exponentially. Starting from the first vesicular trafficking model devised by Palade (Palade, 1975), various approaches have been used
to understand the fundamentals of membrane trafficking. Systematic biochemical approaches have led to the identification of the mechanisms needed for vesicle formation and fusion at the Golgi complex, most notably the COPI coat protein complex, and its wider regulators (Balch et al., 1984; Malhotra et al.; Pfeffer and Rothman, 1987). Genetic screens in yeast have elucidated the function of the ‘SEC’ proteins and their integral role in regulating several membrane trafficking pathways (Kaiser and Schekman, 1990; Novick et al., 1980; Novick and Schekman, 1979). These ground-breaking studies are just a few examples of approaches that have led to the discovery and understanding of the core machinery needed for membrane trafficking; but there are still many questions left unanswered.

Advances in fluorescence microscopy and fluorescent tags provide additional possibilities to study the living mammalian cell as a complete system. The advent and development of high-throughput screening (HTS) and high-content screening (HCS) technologies has further pushed the boundaries of what is possible in terms of large-scale microscopy studies. The incorporation of these technologies with the latest advances in genome sequencing, RNAi and chemical libraries provide the opportunity of dissecting cellular process, reproducibly on a genome-wide level in a relatively short time, a prospect which was impossible until a few years ago (Taylor, 2010).

HCS incorporates automation with the spatial and temporal subcellular information coming from fluorescence microscopy (Taylor, 2010), providing the necessary tools needed to deliver high volumes of robust quantitative image data useful for fundamental biomolecular research. This technology has already been applied extensively in understanding basic cellular processes such as apoptosis (Giuliano et al., 1997), anterograde trafficking (Bard et al., 2006; Liebel et al., 2003; Simpson et al., 2012; Starkuviene et al., 2004), Golgi structure maintenance (Chia et al., 2012), toxin trafficking (Moreau et al., 2011) and endosomal regulation and trafficking (Breusegem and Seaman, 2014) amongst others. The history and advances of HCS are further discussed in Chapter 3.
1.6. **Objective of study**

The hypothesis of this study is that due to the complexity of the membranes in the early secretory pathway, and the fact that they have the capacity to undergo massive structural changes, numerous families and classes of proteins will be necessary for the proper regulation of Golgi structure maintenance and Golgi-to-ER trafficking. As such, disturbance of any proteins that play a role in these processes would be expected to either visually change the morphology of the Golgi or affect the rate of trafficking out of this organelle. Using carefully designed HCS RNAi microscopy assays, novel regulators of membrane dynamics between the Golgi and the ER should be able to be identified.

The overall aim of the work presented here is to design and apply a high-throughput cell biology method to systematically probe the regulators of Golgi-to-ER traffic and Golgi morphology, thereby providing the first genome-wide screens of these two cell functions. The specific objectives of the work were:

a) to design and validate two HCS assays for the identification of Golgi morphology and Golgi-to-ER regulators (Chapters 3 and 4);

b) to identify comprehensive lists of molecules functionally associated with Golgi morphology and Golgi-to-ER transport (Chapter 5); and

c) to begin to characterise a discrete number of novel retrograde trafficking-associated proteins, including identification of their sub-cellular localisation and interaction networks (Chapters 4 and 5+).
CHAPTER 2 - MATERIALS AND METHODS
2.1 Cell culture

Wild-type HeLa cells (human cervical cancer cell line, ATCC CCL2) were routinely cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies) supplemented with 10 % heat inactivated foetal bovine serum (FBS) (PAA Laboratories) and 1 % glutamine (Life Technologies) in 10 cm dishes (Corning) at 37 ˚C in a humidified atmosphere of 5 % CO₂/ 95 % air. HeLa cells stably expressing Golgi stack enzyme N-acetylgalactosaminyltransferase-2 (GalNAc-T2) fused to the green fluorescent protein (GFP) (Storrie et al., 1998) were maintained in DMEM supplemented with 10 % FBS in the presence of 0.50 mg/ml G-418 sulphate (Life Technologies). Cells were routinely sub-cultured 1:5 on reaching confluency; by first rinsing the dish with 0.25 % trypsin-EDTA solution (Life Technologies) to remove dead cell debris and remaining growth medium, followed by an incubation step with the latter solution until cells detached. Cells were used from passages 1 to 5, after which they were discarded.

2.2 siRNA transfection

2.2.1 Solid phase reverse transfection

All solid phase reverse transfection knockdown experiments were performed in 384-well plates (Viewplate-384 FTC, PerkinElmer) and all liquid dispensing for plate preparation for the genome-wide screens were performed using a Microlab STAR (Hamilton Robotics). The protocol used for the solid-phase transfection was modified from a previously published protocol used in a 96-well format (Erfle et al., 2008; Galea and Simpson, 2013).

The Rab pilot screen was carried out using a ‘SMARTpool’ siRNA library (Dharmacon/ GE healthcare) designed to contain four non-overlapping sequences against each target gene (appendix B1). The cytoskeleton pilot screen was carried out using a pool of two sequences against each of the 352 target genes (Ambion/ Life Technologies) (appendix B2). The genome-wide screens were carried out using the Silencer® Select Human Genome Library V4 siRNA set (Ambion/ Life Technologies), containing three unique, and non-overlapping siRNAs for each of the 21,585 human targets. Validation experiments were carried out using two individual siRNA sequences (Ambion/ Life Technologies) (appendix B3).
The solid phase reverse transfection was performed using 50 nM siRNA diluted in Opti-MEM® (Life Technologies), 0.125 µL Lipofectamine® 2000 (Life Technologies), 3.4 mM sucrose (Sigma-Aldrich), and 0.26 µL 0.1 % fibronectin solution from human plasma (Sigma-Aldrich) / gelatine powder from bovine skin (Sigma-Aldrich) solution per well in a volume of 10.25 µL. When cells were transfected with more than one siRNA, 50 nM of each siRNA was used without changing the concentration of the other reagents.

Using the liquid handling robot, 3.8 µL of the Opti-MEM/sucrose/Lipofectamine 2000 solution (master mix: 0.205 g of sucrose in 1.5 mL Opti-MEM and 1.6 mL Lipofectamine 2000) was added to each well of a 384-well plate, followed by the addition of 3 µL of each siRNA (6 µM) and then incubated for 20 minutes at room temperature. Meantime, 8.75 µL of fibronectin were added to 2.5 mL of gelatine solution (0.032 g of gelatine in 40 mL nuclease-free water (Fisher Scientific). After the incubation period, 4.16 µL of the gelatine/fibronectin solution was added to the transfection mixture. An aliquot of 8.5 µL of the transfection mixture (containing the siRNA) was transferred in a new 384-well plate previously filled with 166 µL of nuclease-free water. The solution was mixed thoroughly and distributed (10.25 µL per well) to the corresponding wells of the final 384-well plates. Up to 15 replicate plates were prepared at this step. The plates were dried down for 6 h at 37 °C using the centrifugal vacuum concentrator, miVac Quattro (Genevac). Plates were stored in sealed containers containing desiccating agents.

HeLa GalNAc-T2-GFP cells were seeded at a concentration of 1000 cells per well in a total volume of 40 µL complete DMEM supplemented with 0.50 mg/ml G-418 sulphate, into the transfection 384-well plates prepared as described above. To ensure uniform distribution of cells in each well, an automated cell dispenser, Multidrop 384 Reagent Dispenser (Thermo Fisher Scientific) was used. The cells were subsequently incubated with the siRNAs for a total of 48 h at 37 °C in a humidified atmosphere of 5 % CO2/ 95 % air prior to treatment and fixation.

2.2.2 Forward transfection

Cells plated in 12-well plates (Corning) with a confluency of 20 % were transfected with Silencer Select siRNAs using Oligofectamine (Life Technologies). Briefly, 0.33 µL of a 30 µM siRNA stock and 1 µL of Oligofectamine were diluted in 90 µL and 9 µL of Opti-MEM respectively and incubated for 7 minutes at room temperature. When cells were transfected with more than one siRNA, the same volume (0.33 µL) of each was used without changing the amount of Oligofectamine. After that, diluted siRNA and
Oligofectamine were mixed together and incubated for a further 20 minutes at room temperature to allow the formation of the transfection complexes. Transfection complexes were added to the cells, previously washed with serum-free DMEM, in a drop-wise manner and cells were then incubated at 37 °C for 4 h. The final FBS concentration of media was brought up to 10 % with the addition of DMEM containing 30 % FBS and incubation proceeded for a further 44 h.

2.3 Total RNA extraction, cDNA synthesis and quantitative Real-Time PCR

Total RNA from HeLa cells treated as described above was isolated using an Invisorb Spin Cell RNA mini kit (Invitek) as recommended by the supplier. Briefly, cells were lysed directly in the plate and the lysate was then transferred to a DNA-binding spin filter. 70 % molecular biology grade ethanol (Sigma-Aldrich) was added to the flow-through and this was applied to an RNA-binding spin filter. After several washing steps, RNA was eluted in 40 µL of elution buffer. RNA concentration was determined using NanoDrop3000 (Thermo Fisher Scientific) and then stored at -20 °C until further use.

Complementary DNA (cDNA) synthesis was performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer’s instructions. Briefly, 500 ng of RNA were reverse-transcribed using random hexamer primers. The reaction was carried out in a Mastercycler Pro (Eppendorf) and the cycling program used was the following: 25 °C, 10 minutes → 37 °C, 120 minutes → 85 °C, 5 minutes. When the cycling program was complete samples were kept at 10 °C.

Real-time PCR was performed using Power SYBR green PCR MasterMix (Applied Biosystems/ Life Technologies). Briefly, 1/20 of the cDNA reaction was used as template for the reaction and 200 nM of each gene-specific primer was used. Quantitative real-time PCR was performed in a 7500 FAST real-time PCR system (Applied Biosystems) using the following cycling program: 50 °C, 2 minutes → 95 °C, 10 minutes → (95 °C, 15 seconds → 60 °C, 1 minute) × 40. A melting curve stage was also included in every run in order to assess the synthesis of the specific PCR product. All samples were run in quadruplicate, along with samples of cells treated with siRNAs with no cognate target, Neg siRNA. Results were obtained using the –ΔCt method.
2.4 DNA transfection

Cells (20 x10^3) plated in a 12-well plate containing glass coverslips were transfected with a DNA plasmid using FuGENE® 6 (Promega). Briefly, 0.75 µL of FuGENE 6 and 50 µL of Opti-MEM were mixed and incubated for 5 minutes at room temperature and then added to 0.25 µg of the DNA plasmid. When cells were transfected with more than one DNA plasmid, 0.25 µg of each plasmid were used without changing the concentration of the other reagents. The transfection mixture was then incubated for 30 minutes at room temperature. After incubation the transfection mixture was added in a drop-wise manner to the cells, which were incubated for 20 h at 37 °C.

2.5 Golgi-to-ER trafficking assay

HeLa GalNAc-T2-GFP cells previously transfected were incubated with 10 µg/mL of BFA in DMEM, (Sigma-Aldrich) at 37˚C. Following the addition of the drug, the cells were fixed at various time points (5, 10, 15, 20 minutes) with 3% paraformaldehyde (PFA) (Sigma-Aldrich) in 200 mM phosphate-buffered saline solution (PBS) (Sigma-Aldrich, pH 7.4, 100 µM MgCl₂ and 100 µM CaCl₂) for 20 minutes at room temperature. Nuclei were stained using Hoechst 33342 (Sigma-Aldrich) in PBS at a final concentration of 0.2 µg/mL for 30 minutes. Following fixation and staining, the cells were washed three times in PBS and stored in PBS at 4 °C.

2.6 Golgi morphology assay

HeLa GalNAc-T2-GFP cells previously transfected with siRNA for 48 h were fixed with a 3% PFA solution for 20 minutes at room temperature. Nuclei were stained using Hoechst 33342 in PBS at a concentration of 0.2 µg/mL for 30 minutes. Following fixation and staining, the cells were washed three times in PBS and stored in PBS at 4 °C.

2.7 Image acquisition

2.7.1 Automated image acquisition

The automated image acquisition was carried out using an Olympus Scan^R automated wide-field microscope. For the Golgi morphology assay, twenty-five fields of
view per well were acquired sequentially with a 40x/ 0.6 NA LucPlanFLN objective (Olympus). In the case of the Golgi-to-ER trafficking genome-wide screen, nine fields of view per well were acquired with a 20x/ 0.45 NA LucPlanFLN objective (Olympus). Two channels were acquired per field of view, namely a Hoechst 33342 channel and a GFP channel.

2.7.2 Laser scanning confocal image acquisition and quantification

Confocal images (1024*1024 pixels; dwell time of 12.5 µs) were acquired with an Olympus FV1000 confocal microscope equipped with a 60x/ 1.35NA UPlanSAPO oil immersion objective (Olympus). Sequential scanning mode and Kalman averaging of 3 times were used in all cases. Excitation wavelengths of 488 nm, 559 nm and 635 nm were used for GFP, mCherry/Cy3 and Cy5 fluorophores, respectively.

In the co-occurrence assay, a minimum of 5 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 PFA and permeabilised using 0.2% Triton X-100 detergent (Sigma-Aldrich) in PBS for 5 min. Cells were immunostained for TGN46 (Bio-Rad Laboratories) and CLIMP-63 (Enzo Life Sciences) as markers of the TGN and the reticular ER, respectively. The anti-sheep Cy3-conjugated and anti-mouse Cy5-conjugated secondary antibodies were used (Jackson ImmunoResearch Laboratories).

2.8 Image analysis and data formatting

Images acquired with the Olympus Scan^R automated microscope were analysed using Columbus™ software (PerkinElmer). The software was used to segment individual cells from the raw image data; the nucleus was segmented using the nuclear stain, while the cytoplasm was segmented using the GFP channel through the background fluorescence of the tagged protein in the ER; although the majority of the fluorescence of the GalNAc-T2-GFP was concentrated at the Golgi complex, the small amount of
fluorescence in the ER was sufficient to accurately identify the cytoplasm boundary for each cell. Morphological and intensity measurements were then extracted from the segmented cells to distinguish between healthy and apoptotic or dividing cells that would skew the analysis. Cells close to the border of the field of view were also removed from the analysis.

2.8.1 Golgi morphology assay

Upon the completion of the segmentation and population selection, the Golgi complex was segmented; using the fluorescence emitted by the GFP tagged protein. A variety of measurements were then taken: total area, fragment area, number of fragments per cell and the percentage population showing high level of fragmentation.

For each plate the mean number of Golgi fragments per cell was used to rank the effect of the depletion of each gene on Golgi morphology. The screen data was normalised plate by plate based on the negative control, to allow comparison and combination of data from the plates across the screen. This was done by the division of each sample value by the mean of the negative control (siNeg) (Detailed image analysis protocols and R-script for data handling are found in Appendix A3).

2.8.2 Golgi-to-ER trafficking assay

Upon the completion of the segmentation and population selection, the intensity profile of each cell was measured to determine the maximum and mean intensity of the GFP tagged protein in the cytoplasm. Cells which maintained normal Golgi morphology showed a higher maximum intensity and a higher mean intensity in the cytoplasm compared to cells that underwent redistribution of the Golgi complex. A linear classifier was used to select cells into populations based on these particular intensity features. In this case, cells that retained the Golgi morphology in the positive control wells from those which underwent redistribution in the negative control wells were selected and used as a training set (containing 10 cells per group) for the linear classifier. The linear classifier then used the features associated with this population of cells to classify the state of Golgi across all cells in the well and ultimately in the entire plate. This analysis identified the percentage population of the cells that retained normal Golgi morphology and therefore a
quantified the effect of protein depletion on the kinetics of Golgi-to-ER trafficking induced by BFA.

Through the combination of all the time points obtained through the BFA treatment, the kinetics of the retrograde trafficking step was calculated. A 'phenoprint' for each down-regulated target was created, showing the percentage population of cells that retained Golgi morphology over the time of BFA treatment. These values were logarithmically transformed to achieve a symmetrical distributed data set; a linear model was then applied to the phenoprint to determine the slope (the rate of redistribution), therefore providing an index of the Golgi-to-ER trafficking. This index was normalised plate by plate based on the negative control (siNeg), through the division of each index by the mean of the negative control index. An average of the normalised values of the replicates was made in order to obtain a Golgi-to-ER trafficking index (GETI) and this was used to rank the various genes based on their effects (Detailed image analysis protocols and R-script for data handling are found in Appendix A4).

2.9 Bioinformatics

Protein-protein interaction network analysis was carried out using STRING database (Jensen et al., 2009) and layout was manually modified using Cytoscape (Killcoyne et al., 2009; Shannon et al., 2003). Cytoscape and DAVID Bioinformatics Resources 6.7 were used for the annotation of gene attributes using Gene Ontology (GO). Functional and cellular component annotation clustering was done using DAVID (Huang da et al., 2009; Huang et al., 2008).

2.10 Statistical Analysis

All statistical analyses including t-test, heat maps, and clustering were performed using R 3.02 (www.r-project.org).
CHAPTER 3 - ASSAY DESIGN AND OPTIMISATION

3.1. Introduction

Traditionally, biochemical and genetic approaches have been the mainstay for determining the function and properties of proteins within cells. Although these techniques have been invaluable in terms of describing the intimate details about a particular protein under study, in many cases they do not provide contextual ‘in cell’ data. By contrast, fluorescence microscopy of intact cells is a technique that can provide this type of information, although it potentially suffers from being low throughput and only qualitative. For these reasons, conventional fluorescence microscopy has not widely been used in large-scale systematic studies of cell function, or in drug screens for particular phenotypes.

HTS typically employing a simple colorimetric readout from an entire well, has however been used for several decades as a tool in industry for early stage drug discovery (Pereira and Williams, 2007). With increasing demand for more information, a progression in this technology was clearly needed. Through the 1990s, significant advances were being made in genome sequencing, chemical compound libraries were expanding at a dramatic rate, and there was a general move towards understanding cell function in a systematic way. HCS was therefore a natural evolution from HTS, as it incorporated the automation, speed and efficiency of HTS, with the spatial and temporal subcellular information coming from wide-field fluorescence microscopy, and latterly confocal microscopy (Taylor, 2010). HCS has thus been able to position itself as a technology to deliver high volumes of robust quantitative image data useful for both drug discovery and fundamental biomolecular research.

The term ‘HCS’ was first brought to prominence by work of Dr. Lansing Taylor and colleagues in 1997 (Giuliano et al., 1997). In this study the group performed two automated screens to measure drug induced transport of the human glucocorticoid receptor and also drug induced apoptosis. These pioneering screens provided single cell data for a variety of cellular measurements, including nuclear size and shape changes, nuclear DNA content, mitochondrial potential, and actin cytoskeletal rearrangement, all in a single series of experiments, thereby significantly extending the capability of HTS at that time. Since then, a large number of automated imaging platforms have been developed (Starkuviene and Pepperkok, 2007), making this technology, and the ability to rapidly generate tens of thousands of images, more widely accessible. Indeed the large volumes of quantitative data that can potentially be extracted from fluorescence images of individual cells also present new challenges. The mining and storage of data is becoming an issue with the increase in size and the complexity of the screens (Niederlein et al.,
HCS relies on the fact that all cells, in all wells across an entire screen, are imaged and analysed in an identical manner. Of equal importance is that the software required to perform the analysis must be flexible, such that it can be easily tuned to various cell types and assays requiring a different read-out. Open-source platforms, such as Cell-Profiler from the Broad Institute, have therefore been developed to provide tools by which a variety of biological questions can be quantitatively addressed (Carpenter et al., 2006; Eliceiri et al., 2012).

The Golgi complex is a unique and central organelle in the secretory pathway. The physiological state of this organelle is directly dependent on the bidirectional flow of membranes and proteins into and out of it, and as such any changes in flux through it are likely to cause a change in its appearance. Over the last decade, a small number of systematic studies have probed the mechanisms that maintain this organelle, initially attempting to identify its core residents (Gilchrist et al., 2006; Rabouille et al., 1995; Slusarewicz et al., 1994; Wu et al., 2000a). The majority of the research assessing trafficking through the Golgi complex has focused on anterograde transport; with comparatively few large-scale studies looking into the mechanisms regulating its physical maintenance or cargo exit, particularly in the retrograde direction towards the ER. Possible reasons for this are that there are relatively few well characterised markers for this pathway, that it is difficult to study in isolation from the anterograde step, and certainly in terms of Golgi enzyme quality control and recycling it is slow; the recycling of proteins between the two organelles, taking up to 2 h (Cole et al., 1998; Storrie et al., 2000; Storrie et al., 1998). The ever changing nature of the Golgi complex has deterred studies on a genome-wide scale. The structural plasticity of this organelle is evident when following its cycle through mitosis, during which it disassembles and subsequently reassembles into a functional unit upon completion of cell division (Lippincott-Schwartz et al., 2000; Lowe and Barr, 2007; Tang et al., 2008; Zaal et al., 1999). Likewise, perturbations to the cell such as stress responses and apoptosis have also been shown to affect the morphology of this central organelle (Bankaitis et al., 2012; Lippincott-Schwartz et al., 2000), making the study of its maintenance and regulation a very difficult one.

One of the earliest attempts to use automated imaging in an HCS format to study Golgi complex function and transport was reported in 2003. The group of Rainer Pepperkok described the development of an automated microscope and associated assays, by which protein secretion and the integrity of the Golgi complex could be addressed (Liebel et al., 2003). This system was then applied to examine the effects of overexpression of 100 fluorescently-tagged proteins in these two assays (Starkuviene et
al., 2004). Specifically, a temperature sensitive fluorescently-tagged molecule (VSV-G-tsO45), a variant of the VSV-G, was used as a secretory cargo marker. This transmembrane protein has the feature of accumulating in the ER at 39.5 °C, but when induced to fold by reducing the temperature to 32 ºC it is rapidly transported through the secretory pathway, via the Golgi complex to the PM, where an antibody recognising an external epitope can detect it. The premise of this assay therefore, is that overexpression of any proteins associated with this pathway, including those physically located at the Golgi complex, might interfere with the delivery of this cargo to the cell surface. By incorporating an immunostaining for a Golgi marker (for example the *cis*-Golgi matrix protein GM130), the physical state of the Golgi complex itself was also assessed (Starkuviene et al., 2004).

More recently, RNA interference (RNAi) strategies, including at a genome-wide scale, have become the methodology of choice for probing protein function at and transport through the Golgi complex. The majority of these studies have so far concentrated on first identifying proteins associated with secretion, and then analysing Golgi complex morphology as a secondary screen. The first such study employed a chemiluminescence approach using horseradish peroxidase fused to a signal sequence as a measure of secretion (Bard et al., 2006). From this primary screen, 130 candidates were depleted by RNAi and the appearance of the Golgi complex (as judged by mannosidase II-GFP) was assessed. Although the effects on the Golgi complex were only manually annotated, the images that were presented would be highly amenable to automated HCS analysis. A subsequent secretion screen, also carried out in Drosophila S2 cells, followed a similar approach, but on this occasion GM130 was used as the marker to assess Golgi complex physiology (Wendler et al., 2010). The only genome-wide screen for secretion in human cells was reported recently by our lab (Simpson et al., 2012). Although again the focus of this work was secretion, in this case the secondary analysis of the Golgi complex was semi-automated, with the effect of depletion of more than 550 genes on GM130 immunostaining assessed.

All the studies described thus far have only assessed the Golgi complex in the context of its role in protein secretion, and at best in a semi-automated manner. Recently however, an RNAi screen targeting all kinases and phosphatases was carried out to specifically classify changes to Golgi complex morphology and thereby identify key regulators of its maintenance and architecture. The study used three different Golgi sub-compartment markers (*Helix pomatia* lectin as a *cis*- marker, over-expressed mannosidase II-GFP as a medial marker, and immunostained TGN46 as a *trans* and
TGN marker), and applied a Support Vector Machine (SVM) machine learning approach to automatically identify and classify nine distinct Golgi complex phenotypes (Chia et al., 2012).

The only genome-wide screen to have probed the Golgi-to-ER retrograde trafficking step, albeit only indirectly, was also carried out by the Bard lab. In this study two protein toxins, ricin and *Pseudomonas* exotoxin (PE) were used to study the mechanisms involved in the internalisation and trafficking of these toxins which follow the retrograde transport pathway from endosomes through the Golgi to the ER, and ultimately translocate into the cytosol where they inhibit protein synthesis (Moreau et al., 2011). The study employed a chemiluminescence approach using HeLa cells stably expressing a luciferase with a short half-life as a marker for protein synthesis. Upon delivery of the protein to the cytosol the toxin inhibits translation and luciferase production, while knockdown of a gene important for intoxication rescued the luciferase signal in toxin-treated cells. The small interfering RNA screen identified 178 regulators of ricin or PE intoxication and showed that there was only a 13% overlap in the genes required for the activity of the two toxins. Furthermore this study also showed that the transport of ricin, but not PE, is dependent on Golgi complex integrity, further confirming the intricate system in place for retrograde trafficking and the importance of the Golgi in orchestrating membrane trafficking (Moreau et al., 2011).

It is clear that there remains a large knowledge gap with respect to the molecular machinery and mechanisms in place to control and maintain Golgi architecture and regulate the Golgi-to-ER retrograde trafficking step. There is a need to study these processes not only in a systematic manner, but also on a genome-wide scale in order to fully appreciate and understand the complexities of the pathways and networks involved. Although HCS is only now emerging as a mainstream cell biology tool in the academic environment, the initial signs are that it will become an increasingly important method for systematically dissecting a wide range of cellular processes and functions. In this chapter, a robust method that can be used to systematically and quantitatively analyse the morphology of the Golgi complex, and study the kinetics of Golgi-to-ER retrograde transport from very large numbers of cells in a highly parallel manner is described. This chapter also details the steps taken in optimising the HCS pipeline of the assay itself, to the solid phase reverse transfection, image analysis and all the steps required for the completion of these two genome-wide screens.
3.2. Results

3.2.1. Assay concept and design

The protocol presented addresses two biological questions; namely in a model human cell line, which genes play a role in a) maintenance of Golgi complex morphology, and b) regulation of the Golgi-to-ER retrograde transport step. In order to do this, the genome was systematically probed by RNAi, using siRNAs targeting every gene in turn. The HCS pipeline is composed of a series of steps, as follows (Fig. 3.1). Multi-well optical quality imaging plates were first prepared by addition of a transfection mixture containing the siRNA of interest; consisting of three unique non-overlapping siRNAs for each of the 21,585 human targets. These were dried down, and stored in this highly stable format until needed (Fig. 3.1A). The human genome-wide siRNA library used contained 21,585 targets, which were prepared on a total of 62 384-well plates.

HeLa cells were used throughout the study, the model cell line was selected based on merits of being one of the easiest, fastest growing cell lines and its extensive characterisation, having its proteome, transcriptome and genome mapped (Landry et al., 2013; Nagaraj et al., 2011; Wu et al., 2008), making it an ideal candidate for a pioneering project like this one; albeit further validation and characterisation of novel regulators has to be done in a non-tumoral cell lines. Specifically, the previously described HeLa cell line stably expressing the GFP-tagged Golgi marker N-acetylgalactosaminytransferase-2 (GalNAc-T2) was utilised for this project (Storrie et al., 1998). This was selected over other options such as antibody staining to reduce the costs across a large screen and also to minimise the post-processing time of the plates prior to image acquisition. Electron microscopy studies of GalNAc-T2 tagged with the VSV epitope have shown the enzyme to localise throughout the Golgi complex with a slightly polarised distribution towards the trans-cisternae, with an approximately ten-fold concentration excess in the Golgi stacks compared to associated tubular-vesicular membrane structures such as the ERGIC compartment and the TGN (Rottger et al., 1998). The GFP tagged protein has been shown to have a similar localisation to the VSV epitope tagged protein, therefore indicating it to be a bona fide marker for the Golgi stack (Storrie et al., 1998).

The cells were seeded at a concentration of 1000 cells into each well of the plate in a semi-automated manner; optimised to reach a confluency of 80% after 48 h. Maximum reproducibility of cell number and distribution in the well was achieved by counting cells with an automated counting device and then seeding them using a rapid distribution system. The cells were then incubated with the siRNAs for a total of 48 h prior treatment and/or fixation. For the Golgi-to-ER assay the cells were then treated with BFA
and incubated for various lengths of time, fixed, and the nuclei counter-stained in order to provide a consistent marker for the autofocus in the imaging step (Fig. 3.1B). The cells were then imaged on an automated screening microscope, with every image across the entire screen being acquired in an identical manner (Fig. 3.1C). The image data were stored, duplicated, and transferred to the image analysis server (Fig. 3.1D). Finally the images were analysed in such a way that a quantitative measure of the Golgi complex morphology and/or the redistribution of the GFP-tagged Golgi marker into the ER, was made for every cell in every well across the screen (Fig. 3.1E).

**Figure 3.1. High content screening pipeline.** (A) Solid phase reverse transfection plates were prepared in a 384-well plate format using a liquid handling robot. The plates were dried and stored until ready to use. (B) HeLa GalNAc-T2-GFP cells were plated at a density of 1,000 cells per well in the 384-well plate and incubated for 48 h. Cells were treated when appropriate and then fixed with 3% PFA, and the nuclei counter-stained with Hoechst 33342. (C) Images from every well are acquired using an automated fluorescence microscope. (D) Image data are then duplicated and stored on a server, which is accessed by the (E) image analysis software, which segments the cells and extracts the relevant features. The image data and extracted features are then linked through a database, which also allows the data to be visualised.
3.2.2. Solid-phase reverse transfection

In order to systematically down-regulate proteins in a reproducible and high throughput manner, solid-phase transfection has been proposed as being superior to conventional liquid-based forward transfection for several reasons (Erfle et al., 2007; Erfle et al., 2008). Firstly, the plate preparation (including at the level of an entire genome-wide library) can be done relatively quickly, meaning that the reagents are all treated and “aged” to a similar degree. Secondly, the plate preparation can be easily programmed on a liquid handling robot, making all wells highly comparable. Thirdly, siRNA transfection efficiencies tend to be higher than those obtained using liquid forward transfection (Erfle et al., 2007). Finally, the plates can be stored under desiccated conditions for extensive periods of time following fabrication, meaning that experiments can be timed with the availability of the HCS imaging system. The protocol used for the solid-phase transfection was taken and modified from a previously published protocol used in a 96-well format (Erfle et al., 2008). The genome screen was carried out in 384-well plates, to reduce the reagent costs and allow the siRNA library to be prepared on a smaller number of plates.

To optimise the experimental conditions and efficiency of the transfection, plates with siRNAs targeting the inner centomere protein (INCENP), which has a known role in mitosis and a clear cellular phenotype on depletion, were produced. INCENP is part of the chromosomal passenger complex together with Aurora-B kinase, survivin and borealin (Ruchaud et al., 2007a), and on its depletion cells show multi-lobed nuclei due to segregation defects (Fig. 3.2A). This entire protein complex plays a critical role in cell division, and the absence of any one component disrupts mitosis and the stability of the other members of the complex (Ruchaud et al., 2007b). Using a range of siRNA amounts and Lipofectamine 2000 volumes (both detailed below), phenotypic penetrance (defined as the percentage of cells showing multi-lobed nuclei) was determined across the cell population for each condition, and compared with the results against cells transfected with negative control (Neg) siRNAs (sequences with no cognate mRNA target) (Fig 3.2 B-C).
Figure 3.2. Solid phase reverse transfection optimisation. The efficiency of reverse transfection was tested using siRNAs targeting the inner centromere protein (INCENP). (A) Depletion of this protein results in HeLa cells containing multi-lobed nuclei. Scale bar: 20 µm. (B) To optimise the transfection conditions, 6 different siRNA concentrations combined with 0.150 µL of Lipofectamine 2000 were tested. After 48 h of siRNA transfection the cells were fixed and the number of multi- and single-lobed nucleated cells was counted. Typically less than 25% of the cells in the Neg-treated control showed multi-lobed nuclei, whereas when cells where treated with INCENP siRNA concentrations of 50 or 41.7 nM over 80% of the population showed this phenotype. (C) The two optimal siRNA concentrations determined from the previous experiments were then tested again, but now in the context of a range of transfection reagent volumes. The highest INCENP phenotype penetrance (ca. 85%) was found at a siRNA: Lipofectamine 2000 ratio of 50 nM: 0.125 µL. Results are presented as means and s.e.m. from 3 independent experiments.

Starting from a scaled down version of the published protocol (Erfle et al., 2008) we tested different ratios of siRNA:Lipofectamine 2000 by following the INCENP phenotype penetrance through the population of imaged cells. To optimise the transfection conditions of the protocol, 6 different siRNA concentrations (125, 80, 62.5, 50, 41.7 and 5.8 nM) combined with 0.150 µL of Lipofectamine 2000, a lipid-based transfection reagent, were tested. After 48 h of siRNA transfection, the cells were fixed and the numbers of multi-lobed and single-nucleated cells were counted. The quantification was carried out using an image analysis routine, measuring the nucleus area and roundness, based on which a linear classifier was set up to identify the two populations. As shown in Fig. 3.2B, in cells that were not treated with siRNA or Lipofectamine 2000, less than 25% of the population showed cells with multi-lobed nuclei, while INCENP phenotype penetrance of over 80% was observed in cells treated with
siRNA concentrations of 50 and 41.7 nM. These siRNA concentrations (41.7 and 50 nM) were tested against 8 different Lipofectamine 2000 volumes (0.2, 0.175, 0.150, 0.125, 0.1, 0.075, 0.05 and 0.025 µL), to reduce the concentration of the transfection reagent used; so to achieve a maximal reduction in the amount of gene expression, but at the same time reducing to a minimum the toxicity that the transfection procedure might cause the cells. As shown in Fig. 3.2C, in untreated cells ca. 22% of the population showed a multi-lobed phenotype, whereas the highest INCENP phenotype penetrance was observed in cells treated with a siRNA concentration of 50 nM and 0.125 µL of Lipofectamine 2000, with a phenotype penetrance of ca. 90%. These optimisation experiments revealed that there was no correlation between high siRNA concentrations and large Lipofectamine 2000 volumes with respect to the INCENP phenotype penetrance. The optimal conditions were found to be a concentration of 50 nM siRNA transfected using 0.125 µL of Lipofectamine per well of a 384-well plate.

3.2.3. Assays

3.2.3.1. Golgi-to-ER trafficking assay

In order to systematically identify molecules that play a regulatory role in transport out of the Golgi, and particularly in the retrograde direction towards the ER, an automated microscopy assay based on the localisation of the Golgi protein GalNAc-T2 was developed. To stimulate and accelerate the formation of Golgi-derived carriers to the ER, cells were treated with BFA for increasing lengths of time. The drug BFA has been shown to cause the redistribution of resident enzymes of the cis-, medial and trans-Golgi to the ER, whereas the TGN is a distinct compartment fuses with the endosomal compartment (Chege and Pfeffer, 1990). 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 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 an extensive presence of
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.

To stimulate the formation of Golgi-derived carriers we treated HeLa GalNAc-T2 cells in multi-well plates with 10 µg/ml of BFA and then fixed them at increasing time points (0, 5, 10, 15, 20 min) following treatment (Fig. 3.3A). After staining of the nuclei, an automated fluorescence microscope was used to capture images from each well, and these were then subjected to automated image analysis using HCS software (Detailed protocols can be found in 3.2.4. and appendix A). This allowed population analysis of the cellular response to BFA treatment to be carried out, specifically to determine the percentage of cells retaining an intact Golgi complex at each time point (Fig. 3.3B). Loss of this organelle under these conditions effectively represents the rate of transport carrier formation and movement of GalNAc-T2 cargo to the ER. Under these conditions, 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. Logarithmic transformation of the data obtained at each time point allowed a linear model to be applied to the redistribution profile and therefore the extraction of a slope value. The value obtained for a population of cells treated with Neg siRNAs, was used to normalise all subsequent experiments giving us a Golgi-to-ER Trafficking Index (GETI). This value represents the control conditions, and we therefore assigned it a GETI value of 1.0 (Fig. 3.3B). The GETI of non-transfected cells was found to be not significantly different to that determined from cells transfected with Neg siRNAs.

Next, cells treated with siRNAs targeting Sec22b were examined, 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, resulting in a GETI value of 0.47 (Fig. 3.3D). 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.

Figure 3.3. Representative results from Golgi morphology and Golgi-to-ER assays. (A) Representative images of HeLa GalNAc-T2-GFP cells transfected with negative control siRNAs (Neg) and treated with 10 μg/mL BFA for increasing lengths of time. Cells were fixed at the time points indicated. Scale bars: 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 Golgi-to-ER trafficking index (GETI). (C) Cells transfected with siRNAs targeting Sec22b were treated as above. (D) Quantification of GETI for Sec22b-depleted cells. The deceleration of retrograde trafficking is indicated by a reduction of the GETI value from 1 to 0.47. Cells untreated with BFA (0 min) and transfected with the positive control (C) showed a marked disruption of the Golgi ribbon when compared to the (A) negative control. These cells are indicated with an arrow. The disruption of the Golgi morphology was quantified to provide a ratio of the increase or decrease fragmentation level to the negative control, giving a Golgi Fragmentation Index (GFI).
3.2.3.2. Golgi morphology assay

Based on the pilot experiments described above establishing an automated assay to measure Golgi-to-ER transport, it seemed logical to extend a similar approach to analyse in parallel the morphological state of the Golgi. Cells were transfected with siRNA by solid phase reverse transfection for 48 h. Upon finishing the incubation period the cells were fixed, and the nuclei counter-stained. Following staining, an automated fluorescence microscope was used to capture images from each well, and these were then subjected to automated image analysis using HCS software. This analysis allowed us to carry out a population analysis to obtain a quantitative measure of the Golgi complex size and fragment numbers (Detailed protocols can be found in 3.2.4. and appendix A).

To validate this approach we tested this assay using the positive control from the Golgi-to-ER assay, the Golgi SNARE Sec22b, which on depletion was also observed to cause a marked change in Golgi morphology prior to BFA treatment (Fig. 3.3D). HeLa GalNAc-T2 cells were incubated with siRNAs against Neg siRNAs, or the positive control (Sec22b) for 48 h. Following incubation the cells were fixed and imaged. The resulting images were analysed using the image analysis pipeline described below to provide a ratio of the increase or decrease fragmentation level to the negative control, giving a Golgi Fragmentation Index (GFI).

3.2.3.3. Selection of positive controls

In order to select additional positive controls suitable for both the Golgi morphology and Golgi-to-ER retrograde screens, a literature search was conducted to identify regulators that might be implicated in both mechanisms. A short list of the 16 protein candidates was generated included molecules associated with COPI vesicle formation (Arf1, and coatomer subunits COPB1, and COPB2) (Serafini et al., 1991a), cargo recruitment to the COPI vesicle (KDELREN1) (Lewis and Pelham, 1990), SNARE and SNAP proteins associated with the fusion and docking implicated with Golgi/ER trafficking (GOSR1, Stx5, Stx16, Sec22b, Vamp4, Vti1A and Ykt6) (Xu et al., 2000; Xu et al., 2002a), two Rab proteins associated with Golgi maintenance and Golgi-to-ER trafficking (Rab33b and Rab6a, respectively) (White et al., 1999; Zheng et al., 1998) and a kinesin protein (Kif20a), which has been shown to bind specifically to Rab6 and has been implicated in Rab6 trafficking (Echard et al., 2001). In addition, a relatively less well characterised GTPase Arf1 was tested, as it has been shown to localise to trans-Golgi cisternae where its function is presumed to be similar to other Arfs and Rabs, recruiting
specific effectors. Depletion and over-expression of Arl1 activity has been shown to affect both Golgi structure and recycling from endosomes (Lowe et al., 1996; Yoshino et al., 2003). Finally, the protein CtBP1, also known as BARS, was also tested as it has recently been associated with COPI-coated vesicle formation and basolaterally directed post and early-Golgi carrier formation (Valente et al., 2013; Yang et al., 2005).

**Figure 3.4. Selection of positive controls.** A selection of proteins previously described as being involved with Golgi morphology maintenance and/or Golgi-to-ER trafficking were tested to determine the effect of their depletion in the relevant assays. HeLa GalNAc-T2-GFP cells were transfected with either non-silencing negative control siRNAs (Neg), or single siRNA sequences targeting the 16 proteins. The cells were incubated for 48 h with the siRNAs, followed by treatment with BFA and then fixed at increasing time points (0, 5, 10, 15, 20 min) following treatment. (A) The images obtained from the various time points were analysed and a GETI value was obtained for each target. The depletion of Arf1, Arl1, COPB2, Sec22b, Stx16, Stx5, Vamp4 and Ykt6, showed a marked decrease in Golgi-to-ER distribution. (B) The images obtained from the initial time point (0 min) were analysed and a GFI value was obtained for each target. (A) Only depletion of Rab6a, Sec22b, and Stx16 showed a statistically significant difference in GFI compared to negative controls. Results are presented as means and s.e.m. from 3 independent experiments. Asterisks indicating p-values; * (<0.10 to 0.05), and ** (<0.05).

The 16 genes were down-regulated using reverse transfection initially using a single sequence for each target in the GalNAc-T2-GFP cell line. The cells were incubated
for 48 h with the siRNAs, treated with BFA and then fixed at increasing time points (0, 5, 10, 15, 20 min) following treatment. After fixation, the cells were stained, washed and imaged using an automated wide field system. The GFI and GETI were obtained for the 16 knockdowns (Fig 3.4).

The depletion of the 16 positive control candidates revealed that 12 had a statistically significant inhibitory effect on the Golgi-to-ER trafficking step. The knock-down of the three physical components of the COPI coat, Arf1, COPB1, and COPB2, resulted in a marked slowdown in retrograde kinetics with GETI values of 0.59, 0.36 and 0.33 respectively, although in the case of the COPI coatomer components, COPB1 and COPB2, we also observed a high rate of cell loss compared to our negative control, most likely due to the essential role of these two proteins in the functioning of the cell. The lower cell number seen on depletion of the two COPI coatomer components, seemed to lead to high variability between replicates. Depletion of the SNARE proteins Sec22b, Stx16, Stx5, Vamp4, and Ykt6 also resulted in a strong reduction in the kinetics of the retrograde step, with GETI values of 0.27, 0.35, 0.42, 0.69 and 0.60 respectively. The depletion of the COPI-independent transport regulator RAB6a also showed a marked decrease in this retrograde step (Fig. 3.4A).

Next, the 16 candidates were tested for their role in Golgi morphology regulation, revealing that only three proteins showed a statistically significant change in GFI compared to the negative control upon depletion; namely the two SNARE proteins, Sec22b and Stx16 and the Golgi GTPase Rab6a, with GFIs of 1.70, 1.61 and 1.25, respectively (Fig. 3.4B). Through manual inspection of the images, a disruption of the Golgi was visually confirmed in cells depleted for the three proteins, when compared with Neg-treated cells (Fig. 3.5A). These results would indicate that the two processes of Golgi morphology and Golgi-to-ER traffic regulation occur through different mechanisms, resulting in very few candidates showing a phenotype in both assays.

Through the combination of the results obtained from both experiments, 4 proteins were selected as optimal positive controls for both screens. These were Rab6a, Sec22b, Stx16, and Ykt6. Down-regulation of each of the four proteins showed a distinct slowdown in Golgi-to-ER trafficking, while Rab6a, Sec22b, and Stx16 down-regulation showed a fragmentation of the Golgi complex (Fig. 3.5A). Quantitative PCR was carried out to confirm the depletion of the mRNA in the transfected cells of the four selected positive controls (Fig. 3.5B).
Figure 3.5. Validation of the selected positive controls. (A) HeLa GalNAc-T2-GFP cells were transfected with either negative control siRNAs (Neg), or with siRNAs against the four proteins selected as positive controls, Rab6a, Sec22b, Stx16 and Ykt6. The cells were analysed to obtain GETI and GFI values and the effectiveness of their down-regulation as judged by RT–qPCR (B). Manual inspection of the images, and calculated GFI values, revealed a disruption in Golgi complex morphology compared to Neg in cells depleted for Rab6a, Sec22b, and Stx16. In all the depletions we observed a significant percentage of cells retaining their Golgi structure after treatment with BFA compared to the negative control.

3.2.4. Cell seeding, fixing and staining

In order to enhance reproducibility, cell seeding, fixing and staining were all performed in a semi-automated manner. A variety of manual and automated options were tested in order to determine which techniques would be more suitable for the efficiency of the screen while reducing cell loss to a minimum. In Fig. 3.6 the proportion of cell loss rate using various techniques after three PBS washes is compared to the efficiency of only one manual wash. In this optimisation process we tested manual washes using a multi-channel pipette, against fully automated ones using a plate washer and a liquid
handling robot; as a control the cells prior to the washes were imaged. A significant loss of cells using all methods was observed, particularly when using the plate washer and liquid handling robot, where in both cases more than half of the cells were lost. From this experiment we deduced that the optimal method is performing a manual wash using a multichannel pipette. Reduction in the number of PBS washes to one resulted in a cell loss of only ca. 10-15%.

**Figure 3.6. Comparison of the efficiency of different washing methods.** A variety of manual and automated options for cell washing were tested in order to determine the most appropriate method to retain high numbers of cells but that could still be applied in a systematic manner across the entire screen. HeLa cells growing in multi-well plates were first incubated with Hoechst 33342 for 5 minutes. Cell loss rates using various techniques after a number of PBS washes were then determined. Wash methods tested were manual washing using a multi-channel pipette, a fully automated plate washer, and a fully automated liquid handling (LiHa) robot. Control cell values were determined from imaging the cells prior to the washes. A significant loss of cells was observed using all methods, particularly when using the plate washer and liquid handling robot, where in both cases as many as half of the cells were lost.

Cells were passaged routinely and used only up to passage number 5, to minimise any form of variability in the behaviour of the cells. Typically, 1000 cells were seeded per well in a total volume of 40 µL of complete DMEM into the 384-well optical plates prepared as described earlier. Maximum reproducibility of cell number and distribution in the well was achieved by counting cells with an automated counting device (Cell Scepter) and then seeding using an automated dispensing system (MultiDrop 384 Reagent Dispenser). The cells were then incubated with the siRNAs for a total of 48 h prior to fixation. The cells were fixed using a semi-automated 96-channels pipetting system (VIAFLO) with 3% PFA in PBS for 20 min, washed with PBS, and stained with 0.2 µg/mL Hoechst 33342. Although these pipetting steps can be carried out manually, use of the VIAFLO ensured that all the wells were treated at a similar time point thereby increasing consistency. The proportion of cells lost using this approach was also found to be similar to that seen using manual pipetting. The cells were finally washed once for 5 min each time with PBS and held at 4 °C overnight. This final step aided the uniform uptake of the nuclear stain prior to imaging.
3.2.5. Image acquisition

Achieving the highest possible image quality is an essential aspect of the screen, as ultimately not every image will be manually examined. The choice of objective is critical and dependent on the subsequent analysis routine planned. For simple cell fluorescence intensity measurements, a 10x/0.4 NA objective is likely to be sufficient, meaning that a single field of view may contain in excess of 100 cells, and therefore perhaps no more than 2–4 fields of view per well need to be imaged. By contrast, when any advanced subcellular measurements are envisaged and resolution is important, a 40x objective should be used. In this scenario, no more than 25 cells may be present in a single field of view, and therefore upward of 20 fields of view may be required to obtain sufficient cells for the analysis. In the case of the Golgi morphology screen presented here, for image acquisition we used an Olympus Scan^R automated wide-field microscope, equipped with a 40x Luc Plan FLN 0.6 NA objective. The use of this air objective provided sufficient resolution to detect and quantify more than 50 individual Golgi fragments was possible. The acquisition of 20 fields of view per well and 2 colour channels (Hoechst 33342 and GalNAc-T2-GFP) using the 40x objective for a 384-well plate took an average time of 7 h. In the Golgi-to-ER trafficking screen a 20x Luc Plan FLN 0.45 NA objective was able to provide sufficient resolution to distinguish between cells that retained the Golgi and those which underwent redistribution. In this case the acquisition of 6 fields of view per well and 2 colour channels (Hoechst 33342 and GalNAc-T2-GFP) for a 384-well plate took an average time of 4 h while acquiring over a 100 cells per well.

3.2.6. Image analysis

The images acquired with the Olympus Scan^R automated wide-field microscope were analysed using Columbus analysis software (PerkinElmer). This software is modular in nature, such that routines can be easily tailored to specific assays; in this case the same initial cell segmentation and selection pipeline for the two assays was applied, and only the subsequent modules specific to the two assays were modified (Detailed protocols of the two image analysis routines are presented in appendix A1-2).

In order to obtain single cell data, Columbus was used to segment individual cells from the raw image data (Fig. 3.7A-C); the nuclei were segmented using the nuclear stain, while the cytoplasm was segmented using the GFP channel. Although the majority of the fluorescence of the GalNAc-T2-GFP is concentrated at the Golgi complex, the small amount of fluorescence in the ER (even in untreated cells) was sufficient to
accurately identify the cytoplasm boundary for each cell (Fig. 3.7D). Cells close to the border of the field of view were removed from the analysis (Fig. 3.7E). Morphological and intensity measurements were then extracted from the segmented cells to distinguish between healthy and apoptotic or dividing cells that would skew the analysis. This was done by quantifying the intensity of the nuclear stain, and measuring the nucleus and cell size (Fig. 3.7F). Cells that were undergoing apoptosis and cell division tended to have smaller, brighter nuclei and their cell area tended to be smaller than healthy cells.

### 3.2.6.1. Golgi-to-ER trafficking assay

In the Golgi-to-ER trafficking assay, a pipeline that would be able to distinguish between cells that retained the Golgi structure from those that underwent redistribution was built. This selection was based on the intensity profile of each cell. Each cell was measured to determine the maximum and mean intensity of the GFP tagged protein in the cytoplasm. Cells which maintained the Golgi structure showed a higher maximum and mean intensity in the cytoplasm than cells that underwent the redistribution of the Golgi complex. To reduce the volume of data generated by the image analysis routine, a linear classifier to select cells into populations based on these particular intensity features was applied, and this reduced the quantitative descriptors for each cell to a minimum. In this case, cells that retained the Golgi morphology (positive controls) from those which underwent redistribution (negative control) were selected and used as a ‘training’ set for the linear classifier (Fig. 3.7G). The linear classifier then used the features associated with this population of cells to classify the state of Golgi across all cells in the well and ultimately the entire plate (Fig. 3.7H-I). In this way, large volumes of highly complex image data became rapidly distilled down into a format that allowed genes influencing Golgi-to-ER redistribution to be identified and ranked.

The redistribution data for each time point were then merged in an automated manner through scripts written in R, a software environment for statistical computing and graphics (R-script can be found in appendix A4). The data handling script transformed the redistribution time series in a logarithmical manner to achieve a symmetrically distributed data set and to obtain a linear distribution of the data, followed by application of a linear model. Data sets were normalised plate by plate based on the negative control to remove systematic errors from the data and to allow comparison and combination of data from the plates across the screen. The script generated the average of the normalised values of the replicates.
Figure 3.7. Image analysis strategy for quantifying Golgi redistribution. (A-C) Images of HeLa GalNAC-T2-GFP cells following 48 h of transfection with siRNAs targeting SEC22B. Scale bar represents 20 µm. (D) The cytoplasm of each cell was segmented based on the fluorescence in the GFP channel. (E) Cells with a cytoplasm touching the border of the field of view were removed from the analysis (arrows). (F) Morphological and intensity measurements were then extracted from the segmented cells to distinguish between healthy, and apoptotic or dividing cells that would skew the analysis (arrows). The intensity profile of each cell was calculated to distinguish between cells which retained the Golgi structure, which tend to show higher maximum and mean intensity from those which underwent Golgi redistribution. (G) The cell population was manually inspected and cells showing either an intact Golgi or redistribution phenotypes were selected and used as a training set for the linear classifier. Cells showing an intact Golgi and which were used for training the linear classifier are marked with a green circle and cells showing a redistribution of the Golgi structure are marked in red (H). Intensity measurements were used to identify and classify the different phenotypes across the entire field of view. Automated identification of two populations based on the linear classifier. Cells with an intact Golgi are shown in green, and cells which underwent Golgi redistribution in red. (I) Graphical output from the linear classifier, depicting the threshold used to classify the two populations.
3.2.6.2. Golgi morphology assay

In order to quantify the disruption of the Golgi complex an image analysis routine to segment the Golgi complex (GalNAc-T2-GFP signal) was built (detailed protocol can be found in appendix A1). Through the quantification of the Golgi fragment number it was possible to detect whether the Golgi complex was fragmented, compacted or unaffected by the depletion of each protein under test. Similarly to the previously described image analysis routine, the data generated through the image analysis pipeline was inputted into R, followed by normalisation based on the negative control to remove systematic errors from the data and to allow comparison and combination of data from all plates across the screen. An average of the GFI values of the replicates was generated at the end of the data normalisation routine (R-script can be found in appendix A3).

3.3. Discussion and Conclusions

Traditionally, fluorescence microscopy has been long considered an appropriate method by which the general physiology and morphology of organelles can be studied. Despite the advent of super-resolution microscopy, light microscopy in general still suffers from fundamental limits in resolution. This is potentially problematic for the study of organelles such as the Golgi complex, as this organelle is highly compact in nature, yet proteomic techniques indicate that it is potentially ‘home’ to several hundred proteins (Au et al., 2007). In recent years there have been many elegant fluorescence microscopy techniques presented that can help further refine protein localisation to this organelle (for example (Dejgaard et al., 2007)). However, the limitation of such approaches is always that they are labour-intensive, and not compatible for use with high numbers of samples or cells. Therefore, although automated fluorescence imaging and analysis of the Golgi will not solve the problems associated with resolution, as a technique it does provide accessibility in terms of systematically studying the relevance of all genes to the function of this organelle. Similarly, systematic analysis of Golgi-to-ER trafficking has also not been analysed, likely because of its inaccessibility in terms of markers and slow physiological recycling between the two organelles. To date, high content screening on a genome-wide scale has been applied to study many fundamental processes in human cells; for example endocytosis (Collinet et al., 2010), secretion (Simpson et al., 2012) and cell division (Neumann et al., 2010); however not yet to the study of the physiology of this organelle and the retrograde transport associated with it.
HCS and high-content analysis are extremely powerful tools for the quantitative analysis of large volumes of image data. Although the infrastructure required for successful HCS experiments is both extensive and costly, it is a technique that can provide novel systems-level insight into complex biological questions. The protocols presented here provide a realistic approach for the systematic depletion of large numbers of target genes in a highly parallel manner, followed by treatment for the induction of retrograde transport and quantitative analysis of both the architecture of the Golgi complex and the kinetics of the Golgi-to-ER retrograde step. The output of this approach is presented in Chapters 4 and 5.
CHAPTER 4 - RAB AND CYTOSKELETAL-ASSOCIATED PROTEIN PILOT SCREENS

Some of the results presented in this chapter were published in: Galea G, et al. A high content screening microscopy approach to dissect the role of Rab proteins in Golgi-to-ER retrograde trafficking. JCS. 2015
4.1. Introduction

Following optimisation of the two HCS assays to measure Golgi morphology and Golgi-to-ER transport (Chapter 3); the next step was to validate the newly established assays and protocols with a test set of targets. The first experiments targeted 70 proteins, composed primarily of the Rab GTPases and several Rab-accessory proteins, while a second larger test set of targets comprised 352 proteins associated with cytoskeleton structure, function and regulation.

The Rab GTPases are a large family of small GTP-binding proteins that help 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 effectors. Rab function, including membrane association, is regulated through a wide set of interactors, previously termed the ‘membrome’ (Gurkan et al., 2005). In their inactive state Rabs are bound to a molecule of GDI, which keeps them in a soluble cytoplasmic state (Ullrich et al., 1993). GDP-GTP exchange is mediated by Rab-specific GEFs, and GTP hydrolysis is enhanced by GAPs (Barr and Lambright, 2010). In addition, so-called GDFs have been proposed to play a role in selectively removing Rabs from GDI and help 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 membrane traffic (Del Conte-Zerial et al., 2008; Pfeffer and Aivazian, 2004; Rink et al., 2005; Rojas et al., 2008; Sannerud et al., 2003; Vetter and Wittinghofer, 2001). To date the roles played by Rab proteins have typically been examined on a protein-by-protein basis, and no systematic investigation has been carried out with regard to their role in Golgi structure and retrograde Golgi-to-ER transport.

Trafficking at the ER-Golgi interface has long been recognised to be regulated by Rabs. In 1989, the yeast Rab Ypt1p was the first to be identified as an important regulator for transport to the Golgi complex (Bacon et al., 1989) and since then the mammalian homologues Rab1a and Rab1b have been identified and described as important regulators of COPI-mediated transport between the ER and Golgi (Plutner et al., 1991; Schwaninger et al., 1991; Tisdale et al., 1992) through associated interactions with key effectors such as GBF1 (Monetta et al., 2007). Rab1 has also been shown to interact with the cis-Golgi proteins, GM130 and giantin, coiled-coil golgin proteins that mediate the tethering of coated vesicles, and promote their fusion at the cis-Golgi complex (Beard et
al., 2005; Rosing et al., 2007; Wu et al., 1998). Similar to Rab1, Rab2 also promotes the recruitment of the COPI coat complex to the Golgi membrane through its interactions with GM130, golgin-45, and the protein kinase Cθ (Short et al., 2001; Tisdale, 2000). However, in contrast with Rab1, which mostly promotes anterograde transport, Rab2 has been suggested to play a more prominent role in retrograde trafficking from the ERGIC back to the ER (Tisdale and Balch, 1996). Rab1 and Rab2 are not the only regulators of the bidirectional trafficking between the ER and the Golgi complex, one of the best-characterised Rabs regulating ER/Golgi trafficking is Rab6a, which is present in two isoforms, Rab6a and Rab6a’. Both proteins stimulate trafficking of Golgi-localized glycosylation enzymes back to the ER and disperse the Golgi complex. However, it is only depletion or inactivation of Rab6a’ that blocks COPI-independent retrograde transport of Shiga toxin from the Golgi complex to the ER (Del Nery et al., 2006). Rab6 has been shown to work in conjunction with other Rabs, such as Rab33b, through common effector proteins such as GM130, rabaptin-5 and rabex-5 to direct this retrograde step (Starr et al., 2010; Valsdottir et al., 2001).

Additionally, several less-characterised Rabs (Rab18 and Rab43) have also been proposed to regulate these pathways. Rab43 seems to be involved in the regulation of Golgi structure as its inactivation lead to a striking Golgi redistribution into scattered punctate structures co-localising with ER exit sites, without blocking secretion (Dejgaard et al., 2008). It has been suggested that Rab18 acts in the same COPI-independent pathway as Rab6, playing a complementary role through the regulation of tethering or fusion (Dejgaard et al., 2008). Both Rab GTPases have also been closely associated with the microtubule cytoskeletal network; depletion of Rab18 leads to gross accumulations of neuro-filaments and disorganisation of the microtubule cytoskeleton (Carpanini et al., 2014), and Rab43 has been suggested to be a regulator of the dynein/dynactin complex, a key complex involved in the maintenance of Golgi structure that tethers the Golgi to the microtubules (Burkhardt et al., 1997; Dejgaard et al., 2008; Liu and Storrie, 2012). Rab18 has also been shown to be required for normal ER structure through its interaction with the Rab3GAP complex (Gerondopoulos et al., 2014). These observations suggest that such less well characterised Rabs are also likely to be important with respect to trafficking between the two organelles and maintenance of the Golgi structure, involving elaborate cascades of proteins, lipids and cytoskeletal components.

Maintenance of Golgi structure and position, and actual membrane transport between organelles, are both highly dependent upon the actin and microtubule cytoskeleton and their associate molecular motors. Microtubule depolymerisation leads to the scattering of the Golgi into mini-stacks throughout the cell, which re-cluster in the cell
centre following the reassembly of the microtubule network (Ho et al., 1989). Actin depolymerisation on the other hand, leads to a more compacted Golgi through the loss and conjoining of the Golgi stacks (Valderrama et al., 1998). These observations highlight the intricate ‘tug of war’ between the two cytoskeletal networks in order to keep the Golgi complex in balance. The position of the Golgi in the centre of non-polarised cell is maintained by microtubule motors such as CD-1, which is a large complex made up of multiple copies of 6 different subunits (Höök and Vallee, 2006). Inhibition of CD-1 function through overexpression of one or more of its components scatters the Golgi throughout the cell, mimicking the effect of microtubule depolymerisation (Burkhardt et al., 1997). Similar Golgi fragmentation phenotypes have been described for another dynein family member, cytoplasmic dynein-2 (CD-2) (Vaisberg et al., 1996) and also for the minus end-directed kinesin motor Kifc3, but only in cells depleted of cholesterol (Xu et al., 2002b). As the Golgi is effectively the meeting point between the secretory and endocytic pathways, the morphology of this organelle at a steady-state is also a result of a highly regulated balance between the amount of membrane that is constantly internalised at the cell surface and that leaving the ER. Any significant imbalance between trafficking pathways will affect the structure and therefore function of the Golgi. One likely explanation therefore for the Golgi scattering phenotype described above is that the perturbation of one of these pathways through affecting molecular motor function, either into or out of the Golgi complex, results in a reduced cycling of Golgi proteins and lipids between the Golgi and the ER and ultimately loss of membrane balance in this organelle (Cole et al., 1998). As such, any changes to levels of proteins that mediate trafficking pathways into or out of the Golgi complex will often cause fragmentation of this organelle, and this point forms the basis of the Golgi morphology assay described in this chapter.

The actual transport step between the ER and Golgi is thought mostly to rely on the microtubule network and its associated motor proteins. Anterograde transport from the ER to the Golgi complex is mediated by cytoplasmic dynein (Burkhardt et al., 1997), while the identity of the motors mediating transport in the retrograde direction remains unclear. The COPI-dependent pathway is at least partially driven by the heterotrimeric kinesin-2 motor (Stauber et al., 2006) although this may not be the unique retrograde motor protein as an interaction between COPI-coated vesicles and dynein-1 through the action of Cdc42 suggests that this minus end-directed motor also functions in the retrograde step (Chen et al., 2005). The COPI-independent pathway is less well characterised; the motor responsible for this movement has yet to be identified, but it is has been shown not to be kinesin-2 (Stauber et al., 2006). Other candidates have been suggested such as KIF20A/rabkinesin6 but this seems to be highly unlikely since the motor protein is barely...
expressed in interphase cells. The protein is present 10-fold higher in prophase cells where it localises to the mid-zone of the spindle during anaphase and to the cleavage furrow and midbody during telophase, (Fontijn et al., 2001; Hill et al., 2000) leaving its interphase function unclear. The dynein/dynactin-binding protein bicaudal-D1 (BICD1) was identified through a yeast two-hybrid screen for binding partners of Rab6a, providing a link to CD-1 for this pathway. BICD1 has been shown to enhance the recruitment of dynein/dynactin to Rab6a-containing vesicles and inhibition of the Rab6a-BICD1-CD-1 complex reduces microtubule minus-end directed movement of Rab6a vesicles (Hoogenraad et al., 2001; Matanis et al., 2002).

The actin cytoskeleton has an intricate network surrounding the Golgi, and several myosins, including myosin I/II/V/VI have been implicated in Golgi dynamics and vesicle budding (Allan et al., 2002). Of particular interest is myosin VI, a minus end-directed actin motor, which localises to the Golgi where through its binding with optineurin, where it interacts with Rab8 to regulate Golgi structure (Sahlender et al., 2005). The disruption of this Golgi localising complex has been shown to cause Golgi fragmentation and a severe reduction of transport from the Golgi to the PM, further underlining the complex network of proteins involved in the maintenance of this central organelle. The actin network has been closely linked with trafficking, both in terms of endocytic and secretory pathways, but particularly from the Golgi complex to the endosomes and PM (Engqvist-Goldstein and Drubin, 2003; Schuh, 2011), although recent work has shown that actin regulators have a role in the timing of budding and fission reactions, in regard to Golgi-to-ER retrograde transport (Petrosyan et al., 2012; Taft et al., 2013; Valente et al., 2010). Cdc42, a key regulator of actin dynamics, has been suggested to be crucial in ensuring vesicle budding is completed before vesicle translocation by inhibiting the recruitment of the dynein to COPI vesicles until its maturation (Chen et al., 2005; Hehnly et al., 2010). Rab6a, has been shown to interact with giantin to recruit myosin II to mediate fission and formation of an ER-destined vesicle (Miserey-Lenkei et al., 2010; Rosing et al., 2007); linking both COPI-mediated and independent trafficking with the actin cytoskeleton.

In summary, there is still much work to be done elucidating the exact nature of the Rabs, their effectors and cytoskeleton components, and specifically how they link the Golgi and the bi-directional trafficking. In this chapter the first systematic analysis of the Rab GTPases and an extended set of cytoskeletal proteins is interrogated with respect to elucidating the complex mechanism underlying the regulation of the Golgi complex and the Golgi-to-ER retrograde step.
4.2. Results

4.2.1. Rab GTPase protein set

To date no systematic investigation of Rab involvement in Golgi structure or the transport of retrograde-destined cargo has been carried out. In order to address this, 58 Rab proteins, and 12 Rab-accessory molecules, including 3 members of the prenylated Rab acceptor domain family (PRAF), 7 members of the Yip1 domain family (YIPF), and 2 members of Yip interacting factor family (YIF), were targeted. The assay was carried out in 384-well plate format as described in the previous chapter. In this ‘pilot screen’ a ‘SMARTpool’ RNAi library (Dharmacon) was used; it contained pools of four small interfering RNA (siRNA) sequences against each target gene, and wells containing the positive control, (siRNAs targeting Sec22b), the transfection control (siRNAs targeting INCENP), and negative control, Neg siRNA (Fig. 4.1A). Three technical replicates were carried out for the two assays.

4.2.1.1. Golgi morphology screen

The Rab GTPases and selected accessory molecules were first tested to investigate their role in the maintenance of Golgi structure. HeLa GalNAc-T2-GFP stably transfected cells were plated into solid-phase transfection-ready plates and incubated for 48 h. The cells were fixed, the nuclei stained, and then imaged using a HCS microscope. Using an automated image analysis routine (detail protocol can be found in Appendix A1), the Golgi fragments were counted for each cell, and the resulting mean number of Golgi fragments per cell and per well was normalised to the Neg siRNA data on a plate-by-plate basis. This allowed 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 (Full list of results can be found in appendix B).
Figure 4.1 RNAi screen of Rab proteins identifies Golgi morphology regulators. HeLa GalNAc-T2-GFP cells were transfected with siRNAs for 48 h, fixed and stained. The number of Golgi fragments per cell was measured and a Golgi fragmentation index (GFI) value was calculated for each condition as described in the Material and Methods. (A) Layout of 384 well-plate containing siRNAs targeting Rab GTPases, including positive and negative controls. (B) Representative images of 6 identified regulators, Rab1a, Rab1b, Rab2a, Rab2b, Rab3d, Rab2 displaying a fragmented Golgi phenotype, and 2 regulators, Rab6a and Praf1 showing a compact Golgi phenotype, in comparison to the negative control (Neg siRNA). Scale bar: 40 µm (C) Systematic analysis of the Rab proteins and the 12 Rab-accessory proteins on Golgi structure based on the GFI. Hits were selected based on a statistical difference between the Neg siRNA and the target gene; candidates that showed a p-value smaller than 0.05 were selected as strong regulators, while weak regulators showed a p-value between 0.05 and 0.10. Based on these criteria, 10 proteins were denoted as strong regulators while 8 proteins were defined as weak regulators of Golgi structure maintenance.
Initial manual inspection of the image data generated from the screen, highlighted the strong Golgi dispersion phenotypes in cells depleted of Rab1 and Rab2 variants, while a compact Golgi morphology was observed upon depletion of Rab6a and Praf1 (Fig. 4.1B). These observations were confirmed through our quantitative image analysis routine, in which depletion of Rab1a and Rab2a were found to cause the most significant Golgi fragmentation with GFI values of 1.80 and 1.73 respectively, while Rab6a and Praf1 were found to have the lowest GFI values of 0.69 and 0.68 respectively, indicating a compaction phenotype. These parallel experiments allowed us to rank the proteins with respect to their influence on Golgi structure, both in terms of fragmentation and compaction (Fig. 4.1C). 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, eight Rab proteins (Rab1a, Rab2a, Rab1b, Rab2b, Rab3d, Rab3c, Rab22a, and Rab21) and two Rab-accessory proteins (Yipf2, and Yipf1) were identified as strong disruptors of Golgi structure and 6 Rab proteins (Rab24, Rab26, Rab3b, Rab23, Rab5b, and Rab3a) as weak disruptors. We also identified one Rab protein (Rab6a) and one Rab-accessory protein (Praf1) causing a Golgi compaction phenotype (Fig. 4.1C).

4.2.1.2. Golgi-to-ER trafficking screen

The assay to systematically analyse Rab family proteins in Golgi-to-ER trafficking was next applied. Similarly to the previous screen, HeLa GalNAc-T2-GFP cells were first transfected for 48 h, but then treated with BFA to induce the retrograde redistribution of the GFP-tagged Golgi enzyme. Cells were fixed at increasing time points of BFA treatment (5, 10, 15 and 20 min), stained and imaged. Initial inspection of the image data revealed a number of Rab depletions in which a clear deceleration of Golgi-to-ER traffic could be observed (Fig. 4.2A). Using the image analysis routine described in the previous chapter, a population analysis of the cellular response to the BFA treatments was carried out to determine the percentage of cells retaining an intact Golgi complex at each time point (detail protocol can be found in Appendix A2). Loss of this organelle under these conditions effectively represents the rate of transport carrier formation and movement of GalNAc-T2 cargo into the ER. The kinetics of this retrograde traffic step were obtained, first by logarithmically transforming the redistribution data obtained from each of the five
time points to achieve a symmetrical and linear distributed data set, to which a linear model was applied.

Figure 4.2. RNAi screen of Rab proteins identifies Golgi-to-ER traffic regulators. HeLa GalNAc-T2-GFP 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 4 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 Golgi-to-ER trafficking index (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 (siNeg) and those treated with siRNAs targeting each gene; targets showing a p-value <0.05 were classified as strong inhibitors, while targets with a p-value between 0.05 and 0.10 were classified as weak inhibitors of retrograde traffic. Results are presented as means and s.e.m. from 3 independent experiments.
The slope of the graph was calculated and normalised per experiment based on the redistribution kinetics of the control (Neg siRNA), to obtain a Golgi-to-ER Trafficking Index (GETI) as described in the Material and Methods (Fig. 4.2B).

This systematic set of experiments allowed ranking of the proteins with respect to their influence on Golgi retrograde redistribution, both in terms of accelerating and decelerating the process (Fig. 4.2C). The candidates were then further divided into strong and weak effectors, based on the statistical significance of their inhibition, when compared to cells treated with negative control siRNAs. Specifically, proteins with a p-value of 0.05 to 0.10 were denoted as weak regulators, whereas proteins that had a p-value smaller than 0.05 were considered strong regulators. In total, eleven Rab proteins (Rab2a, Rab1a, Rab1b, Rab6a, Rab3b, Rab11a, Rab34, Rab28, Rab4b, Rab2b, and Rab21) and one Rab-accessory protein (Praf2) were identified as strong inhibitors of the Golgi-to-ER retrograde traffic, and 5 Rab proteins as weak inhibitors (Rab10, Rab7b, Rab33b, Rab40c, and Rab35) (Fig. 4.2C) (Full list of results can be found in appendix B).

Depletion of Rab2a was found to have the most significant impact on Golgi-to-ER redistribution, with Rab1a and Rab1b being the next most potent inhibitors, these three proteins showing a stronger phenotype than the positive control Sec22b. The previously described regulator of COPI-independent trafficking Rab6a also had a strong effect along another Golgi-localising protein, Rab34. Five largely endosomal associated GTPases Rab11a, Rab28, Rab21, Rab3b and Rab4b also showed a significant inhibition effect on this transport step (Fig. 4.2C). In addition to the Rab proteins, one accessory protein, Praf2, also ranked as a strong inhibitor. At the other end of the spectrum, depletions of Rab8b and Yif1a showed an acceleration of Golgi-to-ER trafficking (Fig. 4.2C).

The results from the two pilot screens were next compared with each other. A direct comparison of the GETI with the GFI values indicated that for a number of depletions there is a correlation between a strong Golgi fragmentation phenotype and inhibition of Golgi-to-ER traffic. Six Rab proteins were identified to be important regulators for both process, with depletions of Rab1 and Rab2 variants having the strongest impact, along with the two endosomal GTPases Rab21 and Rab3b. Depletion of Rab6a had an inhibiting effect on the retrograde step but caused a compact Golgi phenotype, whereas depletion of Rab8b accelerated the retrograde step but caused a fragmented Golgi phenotype (Fig. 4.3A).
Figure 4.3 Correlation study and validation of regulators identified from primary screen. (A) Graphical representation of the output of the two assays on the Rab GTPase dataset, correlating GFI and GETI values. Nine proteins displayed regulatory effects on both Golgi structure maintenance and Golgi-to-ER trafficking. (B) HeLa GalNAc-T2-GFP cells transfected for 48 h using two independent siRNA sequences targeting each individual hit obtained from the Golgi-to-ER assay. Cells were then treated with 10 µg/mL BFA and fixed at various time points (0, 5, 10, 15, 20 min) and imaged and analysed to obtain a GETI value. Hits with a p-value less than 0.05 were considered validated. Results are presented as means and s.e.m. from 3 independent experiments. * p-values <0.05, indicating siRNAs sequences considered to have a significant effect on Golgi-to-ER traffic.

The initial screen was performed using a pool of sequences, containing four siRNAs against each target. While such pools are powerful in terms of their gene coverage, they carry the higher risk that if one sequence in each pool shows off-target effects false positive result may be recorded. Therefore in order to validate the hits obtained in the primary screen, we tested whether the phenotypes of the ‘strong inhibitors’ and the strongest 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 revealed that for 5 of the ‘strong inhibitors’, a significant reduction in Golgi-to-ER transport could be recorded with both siRNA sequences, while for Rab1b, Rab3b, Rab6a, Rab10, Rab11a and Rab21 a reduction in the transport step was observed using one of the two sequences (Fig. 4.3B).

4.2.1.3. Cargo-Rab co-occurrence

These phenotypic assays begin to define the Rab machinery required for Golgi distribution, but do not directly report on whether the Rabs identified are physically associated with the transport carriers themselves. In order to address this, the HeLa GalNAc-T2-GFP cells were transfected with mCherry-Rab constructs and co-occurrence experiments were carried out (Fig. 4.4A). From the 12 hits obtained from the primary and validation screens Rab proteins that gave strong Golgi redistribution phenotypes, and for which mCherry-fusion constructs were available in the lab, were selected. Each construct was transfected into cells and allowed to express for 20 h prior to treatment with BFA for 4 min. 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 imaging, randomly selected individual cells were analysed to determine the proportion of GalNAc-T2 cargo containing tubules that were decorated with Rab proteins and vice versa. This was done by manually segmenting each tubular carrier and calculating the intensity profile of the tubule over the two channels (Fig. 4.4B). This analysis revealed that over 60% of all Golgi cargo carriers were coated with Rab1 variants and over 75% with Rab2 variants (Fig. 4.4C), emphasising the importance of these Rabs in export from the Golgi complex. In addition, high proportions (56%) of carriers were observed to be coated with Rab6a, suggestive that COPI-independent transport mechanisms were also being induced in this assay. Of the other Rabs tested, Rab10 and Rab11a were found to be present only in a minor fraction (<5%) of the cargo tubular carriers (Fig. 4.4A-C); whereas Rab3b, Rab4b and Rab21 were found to be completely absent. Similar results were obtained when quantifying the Rab positive tubule carriers which were positive for cargo (Fig. 4.4C). Slightly higher percentage co-occurrences were observed in this case, as Rab tubule carriers were harder to identify as a result of most of the Rab protein population being present as a soluble pool in the cytoplasm.
Figure 4.4. Co-occurrence between Rab proteins and GalNAc-T2-GFP cargo. HeLa GalNAc-T2-GFP cells were transfected with constructs encoding mCherry-tagged Rabs 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) Zoom of area marked in (A). Scale bar: 4 µm. Fluorescence intensity profiles of the areas marked in the image. (C) Quantification of the GalNAc-T2-positive tubules co-occurring with the various mCherry-Rabs. Results are presented as means and s.e.m. of a minimum of 20 tubular carriers analysed per experimental condition.
4.2.1.4. Rab cooperativity screen

The experiments so far identify the key Rabs involved in trafficking out of the Golgi complex, but do not specifically address whether these proteins are operating independently from one another in parallel pathways, or in a cooperative manner on the same pathway. In an attempt to resolve this question, and focussing on the validated inhibitors, a series of pairwise down-regulation experiments were designed. Similar to the primary screen, cells were transfected for 48 h and then treated with BFA for 15 min and then fixed, stained and imaged. A BFA treatment time of 15 min was selected, as in the previous experiments this showed to be the most indicative time point for identifying strong regulators. The resulting raw images were analysed with an image analysis routine to assess the percentage of the cell population retaining a Golgi complex. Each pairwise depletion was carried out eight times, and hits were selected based on whether an additional 10% of cells, compared to a single siRNA treatment, retained an intact Golgi complex. Based on these results, a heat map showing the reproducibility of any additive effect, and therefore potential cooperation between Rab proteins was generated (Fig. 4.5A). Analysis of this map revealed that Rab1 isoforms (Rab1a and Rab1b) had the greatest additive effect, indicating cooperativity on the same pathway. Interestingly, enhanced reduction of Golgi export was also seen in cells depleted for combinations of Rab1 isoforms with Rab3b or Rab11a.

4.2.1.5. Rab-Rab co-occurrence experiments

These functional data strongly suggest that there is a high degree of cooperativity between certain Rabs in the regulation of transport events out of the Golgi complex. Based on the results shown in Fig. 4.5A, experiments were designed to determine whether multiple Rabs could be visualised on the same tubular membrane structures. The previous experiments (Fig. 4.4) indicated that the majority of the tubular carriers carrying the GalNAc-T2-GFP were decorated with either Rab1, Rab2, or Rab6a, so initially combinations of these Rabs (GFP- and mCherry-tagged) were tested in co-occurrence experiments similar to those described previously. All GFP- and mCherry-Rab constructs were already available in the laboratory. Tubular carriers were manually identified and then quantifications of their intensity profiles were carried out over the two channels to provide an indication of Rab protein co-occurrence (Fig. 4.5B, C).
Figure 4.5. Co-operativity of Rab function. (A) Pairwise depletion of selected Rabs previously identified as playing a role in Golgi-to-ER traffic. HeLa GalNac-T2-GFP cells were treated with BFA for 15 min and then fixed, stained and imaged. The percentage of cells in the population still retaining an intact Golgi complex was measured, and hits were selected based on whether an additional 10% of cells retained an intact Golgi when compared to the single siRNA treatment. The heat map indicates the reproducibility of this phenotype across 8 independent experiments. (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) Zoom of area marked in (B). Scale bar: 4 µm. Fluorescence intensity profiles of the areas marked in the image. (D) Quantification of the co-occurrence of membrane tubules decorated with GFP- and mCherry Rabs. Results are presented as means and s.e.m. of a minimum of 20 tubular carriers analysed per experimental condition.
This analysis revealed that a large fraction (>70%) of tubular carriers contained a combination of the two Rab1 isoforms and that Rab1b in particular was observed to present on Rab2a- (>90%), Rab6a- (>60%) and Rab11a- (>20%) positive tubular carriers (Fig. 4.5C). By contrast, Rab6a-positive tubules had none (Rab11a) or low levels of Rab10 (<5%).

4.2.2. Cytoskeletal screen

To date no systematic investigation of involvement of the cytoskeleton and its extensive regulatory machinery has been investigated with respect to Golgi structure or Golgi-to-ER transport. To address this, 352 proteins associated with the actin, microtubule or intermediate filament cytoskeleton were selected (this target list was previously designed in the Simpson laboratory). The targets included Rho GTPases and their associated GEFs and GAPs, the integrin protein family, the kinesin motor protein family, the myosin motor family, dynein motor subunits, and various actin, microtubule and intermediate filament structural and accessory proteins (Appendix B). The assay was carried out in 384-well plate format as described in the previous chapter. In this pilot screen an RNAi library (Life Technologies/Ambion) was used containing two siRNA sequences against each target gene. The plate also included two transfection controls (INCENP and COPB) and wells containing negative control siRNAs (Neg siRNA). One technical replicate was assessed for the two assays (Fig. 4.6A).

4.2.2.1. Cytoskeletal Golgi morphology screen

The cytoskeletal dataset was first tested to investigate the role of these proteins in the maintenance of Golgi structure. HeLa GalNAc-T2-GFP cells were transfected in solid-phase format for 48 h, treated and analysed as described above (4.2.1.1). Hits were selected through a standard deviation cut-off based on the variation of the negative control. Proteins that ranked higher/lower than the two standard deviation cut-off where denoted as weak regulators, while proteins ranked higher/lower than the three standard deviation cut-off where marked as strong regulators. Based on these criteria, 14 strong regulators and 26 weak regulators showed a fragmented Golgi phenotype; while 5 strong regulators and 19 weak regulators showed a compact Golgi phenotype (Fig. 4.6B).
Figure 4.6. RNAi screen of cytoskeletal dataset identifies Golgi morphology regulators. HeLa GalNAc-T2-GFP cells were transfected with siRNAs for 48 h, fixed and stained. The number of Golgi fragments per cell was measured and a Golgi fragmentation index (GFI) value was calculated for each condition as described in the Material and Methods. (A) Layout of 384 well-plate containing the cytoskeletal dataset, including positive and negative controls. (B) Systematic analysis of the 352 cytoskeletal associated proteins on Golgi structure based on the GFI. Hits were selected through a standard deviation cut-off based on the variation of the negative control. Proteins that ranked higher/lower than the two three standard deviation cut-off where denoted as weak regulators, while proteins ranked higher/lower than the three standard deviation cut-off where marked as strong regulators. (C) Based on these criteria, 14 proteins were denoted as strong regulators causing strong Golgi fragmentation while 5 proteins were defined as strong regulators causing Golgi compaction.
Depletion of the kinesin motor subunit Kif2a (GFI 1.85) was found to have the most significant effect on Golgi fragmentation, along with nine other proteins associated with the microtubule network; including three members of the cytoplasmic dynein complex, Dyn1i2 (GFI 1.73), Dyn1i1 (GFI 1.47) and Dyn1h1 (GFI 1.46); the microtubule component Tubb2c (GFI 1.75) and the kinesin motor Kif1b (GFI 1.43). Two proteins associated with the actin cytoskeleton were identified, the non-muscle myosin heavy chain IIA (Myh9) (GFI 1.62) and the integrin Itga4 (GFI 1.42) (Fig. 4.6C) (Full list of results can be found in appendix B).

At the other end of the spectrum depletions of actin associated components showed the strongest compaction phenotype. The Rho GTPase activating protein 1, ArhGAP1 (GFI 0.56) was found to have the strongest impact, along with the integrin ItgaV (GFI 0.56), adducin 3, Add3 (GFI 0.59) and paxillin, Pxn (GFI 0.62). One protein associated with the microtubule network was identified as a strong regulator, namely the microtubule plus-end tracking protein Clasp2 (GFI 0.61) (Fig. 4.6C).

Bioinformatic analysis also confirmed the observed enrichment of microtubule network associated proteins in the 'hits' leading to a fragmented Golgi phenotype; and similarly in the actin network associated proteins causing a compact Golgi phenotype; using the list as a background for the enrichment analysis (Fig. 4.7A). Protein-protein interaction network (PPIN) analysis highlighted three networks in particular; one centred on the dynein microtubule motor complex, and two involving networks of actin-associated proteins (Fig. 4.7B). One of these latter networks was centred around Cdc42, and contained two Rho GTPase activating proteins, ArhGAP1, ArhGAP18, Rhof, a member of the Rho-GTPase family, the kinase Pik3cg and NCK adaptor protein 2 (Nck2). The second network contained members associated with focal adhesions and was composed of the integrins Itgb1, ItgaV, caveolin 2 (Cav2), two protein kinases Limk1, Mapk3, the focal adhesion proteins vinculin (Vlc), paxillin (Pxn), parvin (Parva), the muscle-specific filamin (Flnc) and talin 1 (TLN1) (Fig. 4.7B).
Figure 4.7. Functional enrichment and protein network analysis of Golgi morphology regulators. (A) Gene Ontology (GO) enrichment analysis of putative regulators causing Golgi fragmentation and compaction. GO enrichment was performed using DAVID. (B) Protein-protein interaction network of the putative regulators identified in the Golgi morphology assay. Red nodes depict regulators displaying Golgi fragmentation while blue nodes depict regulators displaying Golgi compaction.
4.2.2.2. Cytoskeletal Golgi-to-ER trafficking screen

The Golgi-to-ER trafficking assay (Chapter 3) was next applied to the cytoskeletal dataset. HeLa GalNAc-T2-GFP cells were transfected for 48 h, treated and analysed as described above (4.2.1.2). Hits were again selected through a standard deviation cut-off based on the variation of the negative control. Based on this ranking, 5 strong inhibitors and 43 weak inhibitors of Golgi-to-ER traffic were identified. Only 1 weak accelerator of this transport step was identified (Fig. 4.8A). Depletion of the integrin Itga2b showed the strongest inhibition of the retrograde trafficking step, followed by syndecan-4 (Sdc4) and myosin 18a (Myo18a) (Fig. 4.8B). At the other end of the spectrum the only identified weak accelerator of the retrograde step was found to be Kif1a (Fig. 4.8B). Manual inspection of the images revealed that cells depleted of the two integrins identified as hits, resulted in a reduced cell area along with the disruption of the Golgi structure (Fig. 4.8C).

Bioinformatic PPIN analysis of the hits obtained from the screen identified potentially 3 complexes inhibiting Golgi-to-ER trafficking; an integrin complex, a dynein complex and a Rho GTPase based complex. The latter is composed of three Rho GTPase activating protein, ArhGAP12, ArhGAP29, ArhGAP44; two Rho GTPases, Rhog and Rhoq; Rho-associated protein kinase Rock2; and the protein-tyrosine kinase ezrin (Ezr) (Fig. 4.9A) (Full list of results can be found in appendix B).

Finally, the correlation of the hits associated with the two screens was assessed. A direct comparison of the GETI with the GFI values indicated that there was no strong correlation between regulators of Golgi structure and Golgi-to-ER traffic. The only hits identified as regulators of both processes, were Itga4 and Sdc4 (Fig. 4.9B).
Figure 4.8. RNAi screen of cytoskeletal dataset identifies Golgi-to-ER traffic regulators. HeLa GalNAc-T2-GFP cells were transfected with siRNAs for 48 h and then treated with 10 µg/mL BFA and fixed at various time points (0, 5, 10, 15 and 20 min). The percentage population still retaining the Golgi complex was measured for each time point and a Golgi-to-ER trafficking index (GETI) value was calculated for each condition as described in the Materials and Methods. (A) Systematic analysis of the 352 cytoskeletal associated proteins on Golgi-to-ER transport based on the GETI. Hits were selected through a standard deviation cut-off based on the variation of the negative control. Proteins that ranked higher/lower than the two three standard deviation cut-off where denoted as weak regulators, while proteins ranked higher/lower than the three standard deviation cut-off where marked as strong regulators. (B) Based on these criteria, we identified 5 strong inhibitors, 43 weak inhibitors and 1 weak accelerator of Golgi-to-ER trafficking. (C) Representative images of 7 identified regulators, Itga2b, Sdc4, Myo18a, Itga3, ArchGAP17, Itgam and Kif1a, at 15 min treatment with BFA. Scale bar: 40 µm.
Figure 4.9. Correlation study and protein network analysis of Golgi-to-ER trafficking regulators. (A) Protein-protein interaction network of the putative regulators identified in the Golgi-to-ER trafficking assay. The size of the node depicts the strength of the phenotype, where the largest node represents the strongest inhibition of Golgi-to-ER trafficking. (B) Graphical representation of the output of the two assays on the cytoskeletal dataset, correlating GFI and GETI values. Two proteins displayed effects on both Golgi structure maintenance and Golgi-to-ER trafficking.
4.3. Discussion & Conclusions

The work presented in this chapter had two goals, firstly to establish and validate a robust methodology with which to assess and rank the regulatory effect of proteins on their role in Golgi structure and Golgi-to-ER retrograde trafficking; which to date no study has systematically investigated. Secondly, to use these assays to perform the first systematic investigation of two test datasets, the Rab GTPase proteins and a collection of cytoskeleton-associated proteins. The two assays were shown to have performed robustly and successfully identified several proteins that have previously been associated with Golgi structure and transport. Through manual inspection of the raw image data and comparison with the quantitative indexes generated, the image analysis routine performed well and reproducibly. In the following sections, the potential new regulators identified and their roles in Golgi maintenance and Golgi-to-ER traffic are discussed in detail.

4.3.1. Rab protein screen

The systematic depletion of 58 Rab proteins and 12 Rab-accessory proteins affirmed the important role of this GTPase family in the regulation of Golgi structure and of the Golgi-to-ER step. Interplay between structure and retrograde transport regulation was observed amongst the identified regulators, of which Rab1, Rab2, Rab3, Rab6a, Rab8b and Rab21 were shown to have a dual role. However, this was not always the case, protein depletions of Rab22a, Yipf1, Yipf2 and Praf1 disrupted the Golgi without altering the rate of retrograde trafficking, suggesting that there are various regulatory complexes maintaining the Golgi structure not tied with the retrograde step. A regulatory role for the endosomal GTPase Rab22a on Golgi assembly was previously shown by overexpression of the protein or its Q64L mutant (active GTP form), which induced Golgi fragmentation (Kauppi et al., 2002; Simpson et al., 2004; Weigert et al., 2004), similar to our gene depletion results. Similar phenotypes were also observed for two relatively uncharacterised Rab accessory proteins, Yipf1 and Yipf2, which localise to the early secretory pathway membranes (Lisauskas et al., 2012). Yipf2 has been shown to mildly inhibit BFA induced Golgi-to-ER traffic through over expression (Lisauskas et al., 2012), however, this phenotype was not detected in the screen, suggesting that the protein has a minor role in this trafficking step. Depletion of the transmembrane protein Praf1 induced loss of the Golgi ribbon and its compaction. The Rab-accessory protein Praf1 localises to the Golgi complex, post-Golgi vesicles, lipid rafts, endosomes, and the PM, where it interacts with the SNARE protein VAMP2 and Rab3a (Compton and Behrend, 2006).
precise function of this accessory protein is unknown but it has been proposed that it may function in the docking, fusion and sorting processes at the Golgi and PM (Compton and Behrend, 2006; Compton et al., 2009); suggesting that its depletion might lead to the inhibition of trafficking towards the PM, resulting in accumulation of proteins and lipids at the Golgi complex, and in turn, disruption of its structure.

A number of Rab proteins localising to the ER-Golgi interface were identified as regulators of both Golgi structure and the retrograde step. Early work identified Rab1 and Rab2 variants as being the primary Rabs associated with the ER-Golgi interface (Plutner et al., 1991; Tisdale et al., 1992), and in our screen these Rab proteins were both found in the group of strongest regulators for both processes. 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 the functional experiments described here now add strong support to this. The two GTPases have been also associated with Golgi structure maintenance through their interaction with the Golgi matrix components and cis-Golgi golgins, such as p115, GM130, golgin-45, and Grasp55, amongst others (Allan et al., 2000; Beard et al., 2005; Moyer et al., 2001; Seemann et al., 2000; Tisdale and Balch, 1996). Depletion or inhibition of one of these golgins has been shown to cause fragmentation of the organelle structure or even lead to the complete loss of Golgi structure and a redistribution of Golgi enzymes to the ER (Puthenveedu and Linstedt, 2001; Seemann et al., 2000; Short et al., 2001). 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 Rabs, our screen only confirmed a role for Rab33b in Golgi-to-ER transport, suggesting that these other candidates play roles outside of Golgi morphology maintenance and this specific trafficking event, for example in anterograde traffic (Sandoval and Simmen, 2012).

The COPI-independent regulator Rab6a (Girod et al., 1999; White et al., 1999) was also identified as a regulator of structure and transport, showing a strong inhibition of Golgi-to-ER traffic and a compaction of the Golgi structure. Rab6a not only regulates Golgi-to-ER transport but also intra-Golgi trafficking (Del Nery et al., 2006) and has been recently linked to the actin-binding protein myosin II (Miserey-Lenkei et al., 2010). The GTPase has been shown to bind directly to the coiled-coil motif of myosin II in a GTP-
dependent manner, being then recruited to Golgi membranes. Depletion or inhibition of myosin II results in long tubules that emanate from the Golgi complex and fail to undergo fission similar to a Rab6-depletion (Miserey-Lenkei et al., 2010); suggesting that Rab6a can interact with the actin cytoskeleton through this accessory protein. This, in turn, might explain the compact Golgi phenotype observed upon the depletion of this GTPase which is also characteristic of the depolymerisation of the actin network. The strong inhibition of Golgi redistribution through the knockdown of Rab6a, highlights its role in transfer of cargo from the Golgi to the ER, but also emphasises that this assay is able to capture retrograde trafficking events that are independent of COPI function.

Depletion of the two GTPases, Rab3 and Rab21 also led to the alteration of Golgi structure and inhibition of Golgi-to-ER traffic. Depletions of the four Rab3 isoforms revealed that Rab3c and Rab3d induce strong Golgi fragmentation while Rab3b strongly inhibits Golgi-to-ER transport along with a mild fragmentation of the organelle. Rab3b is relatively poorly characterised, although one report has indicated that in fibroblasts a pool is localised to Golgi membranes and that it may provide a link to microtubules via another protein, termed growth-arrest-specific gene 8 (GAS8) (Nishimura et al., 2008). The fact that Rab3b was not seen co-occurring with the GalNAc-T2 cargo suggests that although this Rab may be important for Golgi export, perhaps through recruitment of motors, it is not a constituent of the carriers themselves. Rab21 is also associated with microtubule regulation and predominantly localises to the early endocytic pathway, with a small population residing in the Golgi stacks (Egami and Araki, 2008; Simpson et al., 2004). This small GTPase associates with the cytoplasmic domain of α-integrin chains to regulate the endocytic and exocytic traffic of integrins and the cell adhesion complex (Pellinen et al., 2006). This is particularly interesting since multiple integrin proteins and cell adhesion components were identified as potential regulators for both Golgi structure and Golgi-to-ER trafficking, further suggesting that disruption of trafficking at one end of the endomembrane system can have repercussions in other trafficking pathways.

Conversely, a number of proteins identified in the Golgi-to-ER screen, showed no effect on Golgi morphology, namely Rab10, Rab11a, Rab34 and Praf2. 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 (Babbey et al., 2006; Landry et al., 2009; Ullrich et al., 1993). None of these Rabs however showed phenotypes as strong as Rab2a, the most potent regulator, suggesting that their involvement in trafficking may be minor, albeit still detectable by our assay. Rab34 localises throughout the Golgi stack, both on Golgi cisternae and on a
population of Golgi-associated vesicles, including on cis-Golgi-derived transport carriers (Goldenberg et al., 2007). Previous protein depletion studies have shown that Rab34 is required for intra-Golgi transport of VSVG-GFP but that its depletion had no effect on Golgi-to-TGN transport. Its main function is attributed to regulation of the lysosomal population (Goldenberg et al., 2007; Kasmaipour et al., 2012; Wang and Hong, 2002). Praf2, another potential regulator of Golgi-to-ER traffic identified in the screen, was the only accessory protein 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).

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 may play a role in displacing Rabs 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.

The 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 a careful analysis of confocal images of GalNAc-T2 cargo-containing tubules was carried out, revealing that the vast majority were positive for either Rab1, Rab2 or Rab6a. Carriers containing either Rab3b, Rab4a or Rab21 were not detected, therefore suggesting that the role of these Rab proteins is more likely to be at the level of carrier formation, rather than transport itself. However, a small, but reproducible, number of carriers containing either Rab10 or Rab11a were detected. 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 was reported (English and Voeltz, 2013). This elegant paper proposes that complexes containing Rab10 regulate ER extension and fusion in conjunction with microtubules. The observation 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 to be relevant to Golgi-to-ER traffic. This protein predominantly localises to recycling endosomes, although some studies have indicated that a pool may localise to Golgi
membranes (Ullrich et al., 1996; Urbe 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), these two Rab proteins did 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). However, 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 Rabs working in a co-operative manner, possibly through cascades in which effectors such as GEFs might be shared (McDonold and Fromme, 2014). The strongest cooperation between Rab proteins with respect to Golgi-to-ER transport was found, perhaps unsurprisingly, between Rab1a and Rab1b. Similar levels of co-operation were seen between Rab1b and Rab2a, with almost all tubular carriers being decorated with both proteins. These 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 on early endosomes for example (Sonnichsen et al., 2000). Nevertheless, this study suggests for the first time clear functional data implicating these Rabs in retrograde traffic between the Golgi complex and the ER.

4.3.2. Cytoskeletal screen

The systematic depletion of cytoskeleton-associated proteins revealed strong interplay between the actin and microtubule networks in terms of regulating Golgi structure and Golgi-to-ER trafficking. Protein enrichment analysis performed on the identified Golgi morphology hits revealed enrichments of microtubule and actin associated proteins in the identified genes involved in Golgi fragmentation and compaction, respectively. The enrichment analysis was carried using the cytoskeletal dataset as a background list. These results emphasise the critical role of these two networks in the maintenance of Golgi structure.

The microtubule network has for long been linked with membrane trafficking and in particular the Golgi complex, although increasing evidence shows the importance of the actin network in controlling Golgi-membrane-associated budding and fission reactions, through the action of key regulators of actin dynamics. Rho GTPases have long been associated with actin regulation, and a Cdc42 centred protein network was identified as a potential actin regulating complex of Golgi morphology. The depletion of Golgi localising
Cdc42 and five of its interacting partners showed similar Golgi compacting phenotypes. The Rho GTPase Cdc42 is recruited and activated at the Golgi (Erickson et al., 1996; Matas et al., 2004) to regulate protein transport from the Golgi to the ER (Luna et al., 2002) through its direct interaction with COPI. Cdc42 is also involved in regulating transport carrier formation and dynein motor-based microtubule translocation (Chen et al., 2005; Hehnly et al., 2010). The Cdc42 centred network was also found in the siRNA screen described here, and furthermore is associated with other networks in particular with a dynein motor complex, which has been shown to interact directly with Cdc42 and a cell adhesion network complex. These results thereby showcase the central role of this protein in coordinating the microtubule and actin cytoskeletal network to keep the Golgi structure in balance.

The previously described role of the cytoplasmic dynein complex as a Golgi morphology and retrograde transport regulator was confirmed by this siRNA screen. Depletion and overexpression of CD-1 and CD-2 components have been previously shown to cause disruption of the Golgi, mimicking the effects of depolymerisation of microtubules (Höök and Vallee, 2006; Vaughan and Vallee, 1995). CD-1 in particular, has also been linked with Golgi-to-ER trafficking both in a COPI-mediated and independent manner (Chen et al., 2005; Hoogenraad et al., 2001; Matanis et al., 2002). The minus end-directed motor protein has been shown to be recruited to COPI vesicles by Cdc42 (Chen et al., 2005) and to a Rab6a centred complex by BICD (Matanis et al., 2002).

Two myosin motor proteins were identified as inhibitors of the retrograde step and Golgi structure regulation, myosin18a (Myo18a), and Myh9. The F-actin non-progressive motor Myo18a is still poorly characterised, but recent studies link this protein with COPI mediated transport and actin regulation. This myosin molecule interacts with a Cdc42-binding kinase (MRCK), and the protein adaptor Lrap35a, to regulate F-actin dynamics (Tan et al., 2008). In addition, Myo18a binds to the COPI adaptor Golgi phosphoprotein 3 (Golph3) (Taft et al., 2013). The Myo18a-Golph3 complex connects the Golgi apparatus to F-actin to provide the tensile force required for efficient tubule and vesicle formation (Dippold et al., 2009; Eckert et al., 2014; Taft et al., 2013). These interactions between Myo18a and MRCK and Golph3 could suggest a regulatory role in COPI mediated transport for Myo18a. The second actin myosin identified is the myosin-II complex subunit, Myh9. Myosin-II is recruited to the Golgi membrane by its interaction with the golgin giantin and the COPI-independent regulator Rab6a through direct binding to the coiled-coil motif of the myosin in a GTP-dependent manner, thereby regulating the fission
and formation of Rab6a-positive COPI-independent carriers destined to the ER (Duran et al., 2003; Miserey-Lenkei et al., 2010; Rosing et al., 2007; Valente et al., 2010).

A cell adhesion complex composed primarily of integrins was found to affect both Golgi morphology and Golgi-to-ER transport. Depletion of components of this integrin centred network, resulted in a compact Golgi, which could be attributed to a decrease in cell size due to reduced cell-substrate binding. An inhibition of Golgi-to-ER trafficking was also recorded on depletion of components of this network. The significance of this result is far from clear, and merits further investigation, as the primary function of the integrin protein family is related to cell adhesion. One possible link however may be through Cdc42 which can be activated by integrins to regulate cell polarity in migrating astrocytes and in turn Golgi structure (Etienne-Manneville and Hall, 2001). This could link the integrin complex with Golgi structure maintenance and indirectly, through organelle disruption, to Golgi-to-ER transport.

Preliminary analysis of these screens indicates the complexity and the connections between Golgi structure maintenance and retrograde trafficking, and how these might link to multiple cytoskeletal components and several Rab and Rho GTPases. In depth characterisation of the putative regulators identified is now required to understand the molecular mechanism controlling these interlinked pathways.
CHAPTER 5 - GENOME-WIDE SCREEN FOR REGULATORS OF GOLGI MORPHOLOGY AND GOLGI-TO-ER TRAFFICKING.
5.1. Introduction

Since its identification in 1897 by the Italian physician Camillo Golgi, the central organelle that bears his name has been shown to be essential to a multitude of processes including the sorting of cargo, biosynthesis of complex glycans, and aspects of regulation of autophagy, apoptosis, mitosis, cell migration, signal transduction and the cytoskeleton. To accurately carry out these functions, the organisation and positioning of this organelle is crucial. The mammalian Golgi is composed of four to seven closely stacked membrane-bound cisternae, positioned to form a juxtanuclear ribbon structure. The outermost stacks are flanked by a loose network of vesicles and tubules; an indication of the large volume of incoming and outgoing cargo associated with the organelle. Being at the centre of anterograde and retrograde membrane trafficking pathways, the physiological state of the Golgi complex is directly dependent on the flow of membrane and protein into and out of it, and as such any changes in flux through it are likely to cause a change in its appearance and function. However, how the linkage between organisation and function is achieved remains relatively poorly understood.

In mammalian cells nascent proteins leaving the ER are first sequestered into a so-called ER-Golgi intermediate compartment (ERGIC). This compartment is thought to arise through the fusion of transport carriers derived from the ER (Saraste and Svensson, 1991; Schweizer et al., 1990). ERGIC structures are believed to mature, and the prevailing model is that these membranes ultimately become the first Golgi cisterna (Glick et al., 1997; Glick and Luini, 2011; Losev et al., 2006; Weiss and Nilsson, 2003). Cargo destined for secretion is transported in an anterograde direction through the cis- and medial Golgi cisternae to the trans-Golgi network (TGN), during which time many post-translational modifications occur, particularly focussed on changing the glycosylation profiles of the proteins passing through. The proteins received at the TGN, are selectively sorted and packaged into distinct carriers that are destined either for the plasma membrane or endosomal/lysosomal compartments. However, during this transit resident Golgi proteins need to be selectively retained in specific cisternae, and in some cases certain molecules (such as escaped ER-residents and Golgi enzymes requiring quality control) need to be returned to the ER in a retrograde direction (Lewis and Pelham, 1990).

This bi-directional membrane traffic between the ER and the Golgi occurs via similar general mechanisms. In both cases, a membrane-bound carrier forms 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 (Barlowe et al., 1994), and coat protein complex I (COPI) facilitating transport from the Golgi back to the ER (Lorente-Rodriguez and Barlowe, 2011). This seemingly simple scenario was complicated by the discovery that Golgi enzymes, and also certain protein toxins passing through the entire retrograde pathway from cell surface to ER, could 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 using the COPI coat.

Many questions remain relating to the mechanisms by which the organisation and functions of the Golgi are controlled. This is partly due to the lack of a systematic approach in studying this organelle, and also because of its highly dynamic nature. In this study the first two genome-wide RNAi screens in mammalian cells to investigate Golgi structure maintenance and retrograde Golgi-to-ER traffic are presented.

5.2. Results

The two genome-wide screens were performed in duplicate using a ‘Silencer Select’ Human Genome Library of siRNAs from Ambion/Life Technologies, which targets 21,585 human genes using a pool of three siRNAs per target. The siRNA library was prepared into 62 384-well plates, with each plate containing a set of negative controls (Neg siRNA), a transfection control (INCENP) to gauge the performance of the solid-phase transfection, and four positive assay controls; Stx16, Ykt6, Rab6a, and Sec22b (Fig. 5.1).

![Figure 5.1. Layout of solid-phase transfection plates used for the genome-wide screen. The genome-wide siRNA library was prepared in 384-well optical quality polystyrene plates. Each plate contained 4 wells of a negative control (Neg siRNA), and five positive controls to gauge the performance of the transfection and the assays.](image-url)

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5.2.1. Genome-wide screen to identify regulators of Golgi morphology

HeLa GalNAc-T2-GFP cells were seeded in prepared solid-phase transfection plates, incubated for 48 hours, fixed, stained and imaged. An image analysis routine was designed and applied to segment and measure the number of Golgi fragments per cell (Detail protocol provided in appendix A1), and normalised using the mean value obtained for the population of cells treated with negative control siRNAs (Neg); thus providing a Golgi fragmentation index (GFI) as described in the Material and Methods. Using the GFI, the phenotype of each protein depletion was quantified, ranked and compared over the whole genome (Fig. 5.2A) (Full list of results can be found in appendix B).

Analysis of the index revealed numerous genes with stronger phenotypes than the positive controls and also that there was a degree of variability in the performance of the positive controls across all plates of the screen (Fig. 5.2B). To identify putative regulators in a consistent manner therefore, ‘hit identification’ was set-up by considering the standard deviation of GFI values of the cells treated with Neg siRNAs. A cut-off of three times the average standard deviation of the negative control was used (Cut-off GFI: >1.21 / <0.79). Using this threshold, and through the removal of hits with a consistent low cell number (<40 cells), a total of 2,638 targets were initially identified as potential regulators of Golgi structure. The STRING database was used to annotate the selected hits, of which only 40% were shown to have interacting partners. Compiling these data together revealed a final list of 1,137 genes, sub-divided into two groups 635 genes which on down-regulation result in a Golgi fragmentation and 519 genes which resulted in the compaction of the Golgi (Fig. 5.2C).

In order to gain further information on the specific cellular compartments that these regulators function in, a protein enrichment analysis was performed for each phenotype group using the DAVID annotation database (www.david.abcc.ncifcrf.gov). To remove redundant gene ontology (GO) annotations with overlapping members, the terms were clustered, and an enrichment score was calculated based on the mean p-values for each annotation term in the cluster. An enrichment score of 1.3 is equivalent to a p-value of 0.05, and therefore deemed to be significant. Strong protein enrichments for GO terms relating to cytoskeletal components and transport mechanisms in and out of the Golgi were found for both phenotype groups. Enrichment of proteasome complex-associated proteins was observed in relation to the Golgi fragmentation phenotype, while a strong enrichment for proteins associated with ribosomes was detected in relation to the compact Golgi phenotype (Fig. 5.2D).
Figure 5.2. High content siRNA screen of Golgi morphology regulators. (A) Graphical overview of the effects of systematic depletion using a genome-wide siRNA library on Golgi morphology. Values are normalised to the negative control, Neg siRNA, to give a Golgi fragmentation index (GFI). Hit selection was based on three times the standard deviation of the Neg siRNA. (B) Graphical representation of the performance of the positive controls in the screen; depletions of Sec22b and Stx16 displayed marked Golgi fragmentation, while depletion of Rab6a displayed compaction of the Golgi in the cell population. (C) Analysis overview of the genome-wide screen, where from the 21,585 genes tested, 2,648 genes were found to affect the Golgi morphology on depletion. (D) Overview of the GO enrichment analysis of the identified regulators. Terms with enrichment scores equal to or higher than 1.13 were statistically significant.
In order to further understand the potential regulators of Golgi organisation identified in the screen, a bioinformatic analysis of the hundred strongest fragmentation hits was conducted, revealing a strong enrichment of endomembrane and cytoskeleton components. The strongest hits were the glycosylated ER membrane receptor SSR3, followed by the SNAP receptor NAPA, and the RAR-related orphan receptor C, RORC. Among the other strong Golgi fragmenters, multiple components of Golgi associated COG, COPII, SNARE, and TRAPP complex components were identified (Fig. 5.3A).

To further refine the potential involvement of these complexes in the regulation of Golgi structure, a protein-protein interaction network (PPIN) analysis of all Golgi fragmenting hits annotated with GO terms related to Golgi membranes and cytoskeletal components was carried out. The analysis confirmed the presence of multiple interacting

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**Figure 5.3. Analysis of the Golgi fragmentation hits.** (A) Graphical representation of GO cellular component analysis of the hundred strongest fragmentation hits. (B) PPIN of the fragmentation hits with GO annotations associated with the Golgi and cytoskeletal components. Red and yellow nodes indicate proteins associated with the Golgi and cytoskeleton, respectively; white nodes have no association; node size indicates the strength of the fragmentation phenotype. The network was constructed using the STRING database and the layout was manually modified using Cytoscape for easier interpretation. Proteins not connected to any other protein in the network were excluded.
protein complexes in the Golgi fragmentation phenotype group; namely components of cytoplasmic dynein, TRAPP, COG, SNARE, and COPII complexes (Fig. 5.3B).

Next, candidates causing compaction of the Golgi were analysed. This revealed a strong enrichment towards proteins related to transcription, with fewer hits associated with endomembrane and cytoskeletal systems. The depletion of the kinase, Map3k8 was found to have the strongest compaction phenotype, along with the ER resident protein Herpud2, and the respiratory chain enzyme, Pdss2 (Fig. 5.4A). PPIN analysis of the compaction hits associated with the Golgi and cytoskeletal components, revealed that multiple subunits of the COPI coat, cytoplasmic dynein, Rho and a glycosylation enzyme protein network resulted in a compact Golgi phenotype upon depletion (Fig. 5.4B).

Figure 5.4. Analysis of the Golgi compaction hits. (A) Graphical representation of GO cellular component analysis of the hundred strongest compaction hits. (B) PPIN of the Golgi compaction hits with GO annotations associated with the Golgi and cytoskeletal components. Red and yellow nodes indicate proteins associated with the Golgi and cytoskeleton, respectively; node size indicates the strength of the compaction phenotype. The network was constructed using the STRING database and the layout was manually modified using Cytoscape for easier interpretation. Proteins not connected to any other protein in the network were excluded.
The PPIN analysis revealed a large number of protein families and multi-subunit complexes associated with Golgi structure. These included the Golgi associated COG and TRAPP tethering complexes, the Rab and Rho GTPase families, the SNARE protein family, COPI, COPII and clathrin coat protein components, and the three cytoskeletal motor protein families, kinesins, myosins and dyneins.

Next, an expansion on the identified complexes and protein families identified in the PPIN analysis was carried out. Starting with the various classes of tethering complexes, it was observed that multiple subunits of the COG and TRAPP complexes displayed strong fragmentation phenotypes upon depletion, while other tethering complexes such as GARP, CORVET, Golgins and Exocyst did not significantly alter Golgi structure. Four COG components out of the eight known were identified as hits (Fig. 5.5A). A comparable Golgi fragmentation phenotype was observed in cells depleted of eight of the fourteen tested TRAPP subunits, of which three are unique to the TRAPP III complex (Trappc11, Trappc12, and Trappc13). The two subunits unique to the TRAPP II complex did not have any significant effect on Golgi structure (Fig. 5.5B).

Cytoskeletal networks have been shown to be essential for the proper maintenance of Golgi structure and position; although it remains unclear which molecular motors are involved in this mechanism. Systematic depletion of the dynein motor protein family revealed that multiple components of the cytoplasmic dynein complex I disrupted Golgi structure. Knockdown of the cytoplasmic dynein complex I components, heavy chain Dync1h1, intermediate chain Dync1i2, and the two light chains, Dynlt3 and Dynll2 displayed a strong Golgi fragmentation phenotype. The down-regulation of the cytoplasmic dynein complex II, heavy chain subunit Dync2h1, the Rab6a interacting dynein light chain Dynrb2, and two axonemal dynein components, Dnah10 and Dnal4 resulted in a compaction of Golgi structure. Comparable phenotypes were observed for the COPI-associated myosin proteins, Myo18a and Myo18b. Interestingly, the depletion of the class 3 myosins, Myo3a and Myo3b, resulted in contrasting phenotypes, with the latter displaying fragmentation. Marked Golgi fragmentation was measured for the two myosin regulatory light chains, Myl7 and Myl12a. Knockdown of two components of the Kinesin-1 complex, Klc2 and Kif5b, displayed a compact Golgi morphology, while a marked fragmentation of the organelle was measured for cells depleted of Kif1b and Kif23, the latter displayed multi-lobed nuclei (Fig 5.6).
Figure 5.5. Effects of the down-regulation of COG and TRAPP tethering complex subunits on Golgi structure. (A) Four of the COG complex components and (B) Eight TRAPP complex subunits (shown in bold) were identified as strong fragmentation inducers. Manual inspection of the raw image data revealed strong Golgi dispersal patterns in all COG component depletions, although this phenotype was only observed in a smaller subset of the population in components not identified as hits. Similar Golgi fragmentation patterns were observed for the depletion of the different TRAPP complex components. Scale bar: 20 µm.
GTPases have long been known to be essential in defining organelle identity, recruiting coat proteins and linking membranes to cytoskeletal networks. Down-regulation of 59 RAB GTPases revealed that 10 members of the family may act as regulators of Golgi organisation. The depletion of the ER regulator Rab18 was found to induce the strongest Golgi fragmentation effect, followed by the Golgi/ER trafficking regulators Rab1a and Rab2a. Depletions of several Rab GTPases associated with transport pathways between the Golgi and plasma membrane (Rab31, Rab26, and Rab9a) were
found to cause milder disruption to the Golgi ribbon. The ER localising Rab24 and the COPI-independent regulator Rab6a were found to show Golgi compaction upon knockdown, along with the endosomal proteins Rab14 and Rab11b (Fig. 5.7A). Depletion of the Rho family of GTPases revealed that the filopodia-associated proteins Rhou and Rhov have Golgi disruptive effects, while the plasma membrane-associated Rhof and Rac2 showed a Golgi compaction upon knockdown (Fig. 5.7B).

Figure 5.7. Effects of depletion of GTPases on Golgi structure. (A-B) Graphical representation of the GFI of RAB and RHO GTPases identified as hits (shown in bold). (C) Representative images of cells depleted of the strongest GTPase hits identified from the genome-wide screen. Scale bar: 20 µm.

Vesicle formation and membrane curvature is mediated by coat protein complexes, which not only direct transport in and out of the Golgi but also mediate intra-Golgi trafficking. SNARE proteins in turn mediate transport carrier fusion with the
receiving membrane. Down-regulation of COPI, COPII, and clathrin coat complex subunits, revealed that several components of the COPI coat protein (COPA, COPB2, COPZ1, COPB1, COPD/ARCN1 and Arf1) cause compaction of the Golgi (Fig. 5.8A). Manual inspection of the images showed that cells depleted for these proteins displayed a globular Golgi complex (Fig. 5.8C). One COPII (Sec31a) and COPI subunit (COPE) were found to cause Golgi fragmentation upon knockdown. Depletion of the 37 known SNARE proteins revealed that 8 members of the family caused strong fragmentation of the Golgi complex, while only one syntaxin, Stx1a, was found to cause Golgi compaction (Fig. 5.8B).

Figure 5.8. Effects of depletion of SNARE and coat protein subunits on Golgi structure. (A-B) Results from the depletion of 46 coat proteins components of the COPI, COPII and clathrin complexes and 37 SNARE family proteins. (C) Representative images of the strongest hits from the protein groups. Scale bar: 20 µm.
5.2.2. Genome-wide screen to identify regulators of Golgi-to-ER trafficking

The genome-wide screen for the Golgi-to-ER trafficking regulators was performed in duplicate using solid-phase-transfection plates prepared and laid out as described above (Fig. 5.1). HeLa GalNAc-T2-GFP cells were seeded in the prepared transfection plates, incubated for 48 h, treated with BFA for the appropriate time followed by fixation, staining and imaging. For each target gene, 3 plates were prepared, treated with BFA for 5, 10 or 15 min. The redistribution of the Golgi proteins into the ER was measured and the kinetics for each target gene was calculated and normalised per time series using the average redistribution kinetics of cells treated with the negative control, Neg siRNA; giving a Golgi-to-ER trafficking index (GETI) as described in the Material and Methods (detailed protocol can be found in appendix A2). Using this index, the phenotype strengths of the depletions were quantified, ranked, and compared over the whole genome (Fig. 5.9A) (Full list of results can be found in appendix B).

Initial inspection of the data revealed numerous genes giving stronger phenotypes than the strongest positive control, and similar to the Golgi morphology assay, a degree of variability in the performance of the positive controls was measured (Fig. 5.9B). A functional threshold to classify the strongest modulators of traffic using a cut-off of 3 times the standard deviation of cells treated with the negative control was initially set-up, but this was found to be too stringent, therefore a lower threshold of 2 times the standard deviation (Cut-off GETI: 1.21 / 0.79) was used. Using this threshold and through the removal of hits that had a consistent low cell number (<40 cells), identified total of 3,028 proteins were identified as potential regulators of Golgi-to-ER trafficking. These were mapped using GO, and bioinformatically analysed to determine their putative function (Fig 5.8C).

GO term cellular component enrichment analysis of the candidates revealed a significant difference in the GO terms associated with the identified inhibitors and accelerators of the retrograde step. In the case of the inhibitors, strong enrichments were observed for GO terms related to the Golgi complex, Golgi-associated vesicles and Golgi trafficking whereas low enrichment for any GO terms related with trafficking or organelle organisation were observed when investigating the accelerators, with the majority of the hits annotated with terms relating to transcription and ribosomal components (Fig. 5.9D).
Figure 5.9. High content siRNA screen for Golgi-to-ER trafficking regulators. (A) Graphical overview of the effects of systematic depletion using a genome-wide siRNA library on Golgi-to-ER trafficking; data were normalised to the negative control Neg siRNA, to give a Golgi to ER trafficking index (GETI). Hit selection was based on two times the standard deviation of the Neg siRNA. (B) Graphical representation of the performance of the positive controls in the screen; depletions of Sec22b and Ykt6 displayed the strongest inhibition of the retrograde step. (C) Procedure carried out for the analysis of the genome-wide screen, where from the 21,585 genes tested, 3,028 genes were found to affect the retrograde traffic on depletion. (D) Overview of the GO enrichment analysis of the putative regulators identified. Terms with enrichment scores equal to or higher than 1.13 were statistically significant.
Following the enrichment analysis, focus was placed on the inhibitors of Golgi-to-ER trafficking; in particular proteins with GO terms associated with the Golgi, Golgi associated trafficking and cytoskeletal components. This was followed by inspection of the top hundred inhibitors, revealing that various components previously described as regulators of Golgi/ER trafficking were identified as hits. The strongest of these was Sec1 family domain containing 1 (Scfd1/Sly1), followed by the previously identified regulator from the cytoskeletal screen, the integrin Itga3 and dynein light chain component Dynll2 (Fig. 5.10A).

![Figure 5.10. Analysis of inhibitors of Golgi-to-ER trafficking.](image)

(A) Graphical representation of GO cellular component analysis of the hundred strongest inhibitors. Proteins are annotated using GO terms. (B) PPIN analysis of the inhibitors with GO annotations associated with Golgi membranes and the cytoskeleton component. Nodes colour indicates the association of the proteins with its respective complex; node size indicates the strength of the inhibition of the retrograde step. The network was constructed using the STRING database and the layout was manually modified using Cytoscape for easier interpretation. Proteins not connected to any other protein in the network were excluded.
Several of the COPI, COPII, TRAPP, and COG cellular components also showed marked inhibition of Golgi-to-ER trafficking upon depletion, along with several cytoskeletal associated components, protein phosphatases and kinases (Fig. 5.10A). A PPIN analysis of the putative regulators with GO terms associated with the Golgi and trafficking associated with this organelle confirmed that the involvement of the SNARE family proteins, the COG and TRAPP tethering complexes and the two coat proteins COPI and COPII in this trafficking step and also revealed the importance of a clathrin coat-associated network and an ER membrane associated protein network (Fig. 5.10B).

Transport carriers are formed at different regions of the Golgi membrane through the action of several distinct coat proteins. For retrograde cargo, this process is thought to be largely driven through the multi-subunit cytoplasmic coat complex COPI, the less well characterised COPI-independent complex and a number of associated regulators. Aside from the main core machinery, little is known about the regulators and molecular mechanism involved in this trafficking step. Therefore the effects of depletion on secretory pathway-associated coat complexes, tethering complexes, GTPases and molecular motors were examined to determine their function and involvement in this membrane trafficking step. Down-regulation of COPI, COPII and clathrin coat protein complexes revealed that siRNAs targeting seven of the nine COPI coatomer subunits (COPB1, COPA, COPB2, COPZ1, COPG, COPD/ARCN1 and COPE) and two of the three clathrin chains (CLTA, CLTC) inhibited the retrograde step. Several clathrin adaptor proteins (Ap3m2, Ap4b1, Ap3b2, Ap3s2, and Ap3d1) were found to have similar phenotypes upon knockdown, along with two subunits of the COPII coat complex (Sar1a, and Sec24a) (Fig. 5.11A).
Individual cellular trafficking steps are also regulated through small GTPases of the Arf and Rab families, in addition to the SNARE fusion proteins, together termed the membrene. Down-regulation of the SNARE protein family showed eight members as transport inhibitors and three as accelerators. The depletion of Ykt6 was found to be the strongest inhibiting phenotype followed by Sec22b and Stx10 (Fig. 5.11B). Of the 59 RAB GTPases assessed, seven were inhibitors, of which the four strongest phenotypes were observed from the knockdown of Golgi/ER trafficking regulators, Rab2a, Rab1a, Rab18, and Rab1b, while no Rho GTPases were identified as regulators (Fig. 5.12A).
It has become apparent that the SNARE proteins, although central to the process of fusion, are often not the first point of contact between a vesicle and its target. Instead, a tethering process physically links the two before fusion occurs. Many factors that have an apparent role in tethering have been identified but none specifically for Golgi-to-ER trafficking; therefore an examination the depletion phenotypes of the five known tethering complexes, (COG, TRAPP, GARP, CORVET, Exocyst, NRZ) and the golgin family was carried out. This revealed that only depletions of COG and TRAPP complex subunits generally resulted in inhibition of traffic. Five of eight subunits of the COG complex (Fig. 5.13A) and eight of fourteen TRAPP complex subunits (Fig. 5.13B) showed a marked decrease in this trafficking step upon depletion.
Figure 5.13. Effects of depletion of COG and TRAPP tethering complex subunits on Golgi-to-ER trafficking. (A-B) Five of the COG complex components and nine TRAPP complex subunits (shown in bold) were identified to have a regulatory effect on the retrograde traffic upon knockdown. (B) Representative images showing the distribution of the Golgi in cells depleted of the strongest hits of the two groups after treatment with BFA for 15 min. Scale bar: 40 µm.

The cytoskeleton, composed of actin filaments, microtubules and intermediate filaments, regulates many processes in the cell including intracellular transport. Actin and microtubules are polarised structures, along which bidirectional transport of motor proteins occurs: myosins along actin and the dynein/dynactin complex and kinesins along microtubules. Down-regulation of the three motor protein families; dyneins, myosins and kinesins, revealed that the Kinesin-1 complex component Kif5a had the strongest inhibitory effect out of all the motor proteins, followed by the axonemal, light chain Dnal4 and the Golgi-localising Dymeclin, Dym. Rab6a interacting myosins, Myosin-II complex, heavy chain Myh9 and COPI interacting Myo18a were also shown to inhibit this trafficking step (Fig. 5.14)
The trafficking assay not only defined the machinery required for Golgi distribution, but also provided information with regard to the mechanisms and kinetics of the process. Four redistribution profiles were identified in the 1,137 Golgi-to-ER inhibitors. These profiles were identified through a K-means clustering analysis of the raw redistribution data (Fig. 5.15). The four trafficking profiles were denoted as: i) profile A representing a sigmoid redistribution; ii) profile B displaying linear redistribution of the Golgi marker; iii) profile C denoting an exponential redistribution; and iv) profile D showing a linear redistribution followed by a decrease in trafficking at the final time point (Fig. 5.15A).
Figure 5.15. Analysis of the redistribution profile of inhibitors of Golgi-to-ER trafficking. (A) Heat map depicting the redistribution profile of the identified hits clustered in four groups. (B) Graphical representation of GO enrichment analysis of the members of each group based on cellular and function component terms. (C) PPIN of the inhibitors, classified into the four groups (depicted by node colour). The network was constructed using the STRING database and the layout was manually modified using Cytoscape for easier interpretation. Proteins not connected to any other protein in the network were excluded.
A functional and component cellular enrichment analysis was carried out for each group using the 1,137 hits as the background list. Targets displaying a redistribution profile A were found to be enriched for terms relating to phosphorylation, while strong enrichment for several protein complex subunits implicated with transport and fusion of vesicles (Fig. 5.15B) were found for genes that displayed a linear redistribution (profile B) upon depletion. These included members of the Golgi associated TRAPP, SNARE and clathrin coat complexes, and also several ER membrane proteins and microtubule-associated components including several dynein components and the Rab GTPase Rab18 (Fig. 5.15C).

All but one of the coat complex components identified showed an exponential redistribution profile (profile C) along with a cell adhesion protein complex (Fig. 5.15B-C). The largest redistribution group composed of 433 genes had redistribution profile D. In this cluster, high protein enrichment was measured for components implicated with maintenance of the Golgi structure, particularly actin binding proteins including several components of the myosin complex and WD40 repeat proteins. Included in this cluster were the COPI-independent regulator Rab6a and several of its interacting proteins such as Kif5a, Myh9 and Chml (Fig. 5.15B-C).

5.2.3. Regulators of Golgi structure and transport

Analysis of the genome data was carried out to determine potential mechanisms involved in the maintenance of Golgi structure and the trafficking through it. The two genome-wide studies revealed that approximately 11% of the 21,585 tested targets displayed alterations to Golgi structure upon depletion, whereas 14% were found to have marked effects on Golgi-to-ER trafficking. The next step was to determine whether there was a correlation between retrograde transport and Golgi structure and if any of the potential regulators are involved in the two processes (Fig 5.16). Correlation analysis of the two screens revealed no significant relationship between retrograde trafficking and Golgi structure, although analysis of the list of common regulators revealed that a number of complex subunits are involved in both process. A strong enrichment towards proteins with GO terms associated with the Golgi complex, intra-Golgi vesicle mediated transport and post-Golgi transport was measured in depletions causing a fragmented Golgi phenotype and an inhibition of the retrograde step; among which several components of the COG, SNARE and TRAPP protein complexes were found to belong to this group (Fig. 5.13B).
Figure 5.16. Linking Golgi structure and trafficking. (A) Graphical representation of the GFI and GETI for each of the siRNAs tested; also showing the total number of putative hits identified for each phenotype and their overlap. (B) List of proteins that displayed effects on both Golgi-to-ER trafficking and Golgi structure upon knockdown. (D) Venn diagram depicting a comparison of the Golgi structure and Golgi-to-ER trafficking hits with the 1,131 strong secretion inhibitors from a genome-wide secretion screen (Simpson et al., 2012). (E) Comparison of the inhibitors of Golgi-to-ER trafficking with screens for ricin, PE and Shiga toxin trafficking inhibitors (Moreau et al., 2011). A total of 116 genes were identified in the toxin trafficking screen and matched with the retrograde trafficking hits identified.
Proteins annotated with GO terms relating to vesicle components and membrane budding were found to be strongly enriched in protein depletions causing a compact Golgi phenotype and an inhibition of Golgi-to-ER trafficking; several COPI complex and clathrin coat protein components were found to be part of this group (Fig. 5.13C). No significant functional protein enrichment was measured in association with the two other phenotypes (acceleration of the Golgi-to-ER trafficking and alterations of the Golgi structure), aside those associated with transcription.

Next, an analysis of the regulators identified from the two screens in context of two genome-wide screens for secretion regulators and toxin trafficking was carried out. In the first study (Simpson et al., 2012), a total of 1,131 secretion inhibitors (only the strong regulators were considered) were assessed and matched to the genes identified. Comparison with these data revealed an overlap between secretion regulators, Golgi structure and Golgi-to-ER trafficking. Two components of the COPI coat complex (ARCN1 and COPB2) were found to be common to the Golgi compacting, secretion and retrograde trafficking inhibitors, while Trappc5 and Rab18 were found to cause fragmentation of the Golgi and inhibit both secretory and retrograde traffic (Fig. 5.16D).

The role of the genes identified in the screen was next examined in the context of a recent toxin trafficking study. A total of 116 genes were found to inhibit toxin trafficking and match with the gene list used in this study (Moreau et al., 2011). A comparison of the inhibitors of ricin, Pseudomonas exotoxin (PE) and Shiga toxin trafficking (all of which require retrograde traffic to the ER) with the inhibitors of Golgi-to-ER trafficking was made, to look for overlap between these mechanisms. Only six genes were found to overlap with the trafficking of the toxins, namely Colec10, Atp1a3, Thbs2, Prdm10, Nbeal1 and Rap2a, whereas eight proteins were found to overlap with Golgi fragmentation regulators Spata2, MAST3, Aatk, Jph4, Nbeal1, Srebf2, C16orf62 and Prkd2 (Fig. 5.16E).
5.3. Discussion & Conclusions

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 Golgi structure maintenance, and the retrograde pathway associated with it; and no studies have addressed the link between Golgi structure and transport at a genome-level. Based on this, two genome-wide screens to systematically dissect the molecular machinery involved in both maintenance of Golgi morphology and trafficking from the Golgi complex to the ER were devised, to understand the mechanisms that control these two pathways and possible links between them.

5.3.1. Regulators of Golgi morphology

Due to its structural complexity and highly dynamic nature, understanding the mechanisms that regulate structure and position of the Golgi complex has been particularly elusive. Using an RNAi strategy a global analysis of gene function with respect to Golgi structure maintenance was carried out, and revealed that more than 10% of the genome encodes proteins that can be linked to this fundamental cellular component.

The properties of Golgi cisternae have been linked to the pathways of Golgi membrane traffic; the current predominant model is cisternal maturation, which postulates that Golgi cisternae form de novo and then progressively mature into TGN cisternae. According to this model, resident Golgi proteins recycle from older to younger cisternae, thereby staying within the organelle, while biosynthesis cargo moves forward (Losev et al., 2006; Matsuura-Tokita et al., 2006). Therefore it would be expected that disruption of traffic would lead to alteration of the organelle structure. This was confirmed by the identification of multiple complexes known to regulate trafficking, namely the COG, TRAPP, SNARE, COPI, and COPII, along with cell adhesion associated complexes.

The maintenance of Golgi structure and position has also been shown to be highly dependent upon the actin and microtubule cytoskeleton network, which was confirmed in the screen by protein enrichment analysis. Multiple motor complex components of the dynein, myosin and kinesin family proteins have been shown to alter the Golgi structure upon knockdown. Particularly of interest is the cytoplasmic dynein complex 1; depletion of several sub-units of this complex lead to strong Golgi
fragmentation. This multi-subunit motor complex has been previously implicated with Golgi structure maintenance where inhibition of dynein-1 function through overexpression of one or more of its components has been shown to scatter the Golgi throughout the cell (Burkhardt et al., 1997). The screen results would also indicate that multiple other molecular motors associated with Golgi/ER trafficking are implicated with Golgi organisation, perhaps indirectly. A few examples are the dynein light chain, Dynlrb1 which was found to interaction with Rab6a (Wanschers et al., 2008); Myo18a which interacts with Golph3 (Dippold et al., 2009; Taft et al., 2013), an interactor of the COPI complex (Eckert et al., 2014) and Kinesin-1 complex components (Klc2 and Kif5b) which have been shown to be required for the bidirectional motility of ER exit sites and efficient ER-to-Golgi transport (Gupta et al., 2008); all resulting in a compact Golgi morphology upon depletion.

The recruitment of peripheral membrane proteins, in particular small GTPases could be a key to understanding the connection between organisation and dynamics of this organelle. Multiple Rab proteins assigned key roles in regulation of protein trafficking were identified through the primary screen as regulators of Golgi organisation. The strongest fragmentation hit was the Golgi/ER and microtubule network and ER regulator Rab18 (Carpanini et al., 2014; Dejgaard et al., 2008; Gerondopoulos et al., 2014) followed by two other Golgi/ER trafficking regulators Rab1a and Rab2a (Tisdale et al., 1992; Tisdale and Jackson, 1998). Depletions of Rab31, Rab26, and Rab9b also lead to disruption of the Golgi ribbon. These three GTPases mediate transport to and from the plasma membrane through different pathways (Li et al., 2012; Rodriguez-Gabin et al., 2009; Zhang et al., 2014), suggesting that impairment of trafficking in or out of the Golgi causes alterations of Golgi structure, although it is not understood why in some cases this leads to fragmentation and in others compaction of Golgi shape. Rab14 was found to have the strongest Golgi compaction phenotype, followed by Rab11b and Rab6a. These three GTPases have been strongly linked with trafficking associated with the Golgi, where Rab14 was shown to regulate the transit of endocytosing GLUT4 through early endosomes towards the TGN (Reed et al., 2013) and Rab6a has been previously shown to be involved in Golgi-to-ER, TGN-to-endosomal, intra-Golgi trafficking (Girod et al., 1999; Reed et al., 2013; White et al., 1999).

Fusion of transport carriers at their destination membrane requires the coordinated action of Rab GTPases, tethering complexes and SNAREs. In this screen two tethering complexes, COG and TRAPP, were identified as Golgi organisation regulators. The golgin coiled-coil protein family, highly implicated with Golgi organisation, was not identified as a
key regulator; the explanation may be that the 48 hour siRNA-mediated gene knockdown was not sufficient to down-regulate the proteins appropriately to display a phenotype.

Depletion of the fourteen TRAPP complex subunits revealed that eight components specific to TRAPPI- and TRAPPIII-complexes caused Golgi fragmentation. The two TRAPPII-specific subunits Trappc9, and Trappc10 did not show any significant effects on the Golgi, contradicting previous work showing RNAi depletion of the two TRAPPII-subunits to show defects in intra-cellular transport and microtubule organisation leading to fragmentation of the Golgi (Cai et al., 2005; Zong et al., 2012). The three tethering TRAPP complexes have distinct functions; TRAPPI complex has been shown to tether COPII vesicles at the ERGIC (Cai et al., 2007; Sacher et al., 2001), TRAPP II has been suggested to mediate intra-Golgi trafficking through its interaction with the COPI coat components (Barrowman et al., 2000; Cox et al., 2007), whereas TRAPPIII has functions in anterograde transport at the Golgi and also regulates autophagy (Behrends et al., 2010; Lynch-Day et al., 2010; Scrivens et al., 2011).

Four of the eight COG complex subunits showed marked Golgi disruption upon depletion. The four subunits identified as hits compose one of the two lobes making the complex (COG1 to COG4 and COG5 to COG8) (Fotso et al., 2005; Ungar et al., 2005). Membrane-bound COGs are present in different arrangements, including the complete COG1-8 complex, lobe A and lobe B sub-complexes, and possibly other minor assemblies (Willett et al., 2013b); which could be an explanation for the number of components identified. The COG complex both physically and functionally interacts with many classes of molecules to mediate intra-Golgi trafficking, namely with SNAREs, SNARE-interacting proteins, Rabs, coiled-coil tethers, vesicular coats, and molecular motors (Zolov and Lupashin, 2005). In particular this complex has been shown to interact with the COPI coat in yeast (Suvorova et al., 2002); but unlike the COG complex, depletion of COPI coat protein subunits were found to lead to Golgi compaction. The down-regulation of coat complex components could result in a slow down or halt of the recycling of proteins between the Golgi and the ER (Cosson et al., 2002; Serafini et al., 1991a), and also a disruption of intra-Golgi transport (Lanoix et al., 1999; Martinez-Menarguez et al., 2001), leading to the reduction of the Golgi volume. Similarly, depletion of the other Golgi-to-ER regulator, Rab6a, was also found to give a comparable phenotype (Young et al., 2005) suggesting that disruption of this retrograde transport step leads to a reduction in size of the Golgi.

The final stage in vesicular/tubular trafficking is the fusion of transport carriers with their target membranes. The fundamental intracellular membrane trafficking process is
driven by an assembly of functional SNARE complexes. Depletion of the 37 SNARE proteins revealed that nine members of this family showed alteration in Golgi morphology. Three ER/Golgi SNAREs Sec22b (Joglekar and Hay, 2005), Stx18 (Lewis et al., 1997), and Ykt6 (Zhang and Hong, 2001) and four post-Golgi SNAREs Stx10, Vamp2, Vamp3 and Stx16 (Ganley et al., 2008; Hu et al., 2007; Mallard et al., 2002; Rowe et al., 2001) were identified as Golgi fragmentation regulators. The brain specific post-Golgi Stx1a was found to cause Golgi compaction upon depletion (Rowe et al., 2001; Rowe et al., 1999). The identified SNAREs would indicate that alteration of traffic, especially ones directed towards the Golgi leads to fragmentation of the organelle, probably due to an imbalance in the lipid and protein composition of the latter. While as observed with Stx1a, depletion of a SNARE protein, which functions in transport to the PM, can lead to Golgi compaction, which could be due to an accumulation of proteins at the organelle.

5.3.2. Regulators of Golgi-to-ER trafficking

The results of the Golgi organisation screen highlighted the intricate relationship between Golgi organisation and trafficking. These two mechanisms cannot be studied in isolation but have to be considered together for a correct understanding of the mechanisms in place. Anterograde traffic through the Golgi has been extensively studied but the counter-balancing retrograde flow is still relatively poorly understood. This genome-wide screen described here to identify Golgi-to-ER trafficking regulators was therefore conducted. This study is the first and most comprehensive assessment of genes associated with the retrograde pathway function in metazoans so far. The systematic knockdown of the 21,585 genes revealed that 14% of the genome encodes proteins that can be linked to this trafficking step.

The identified hits were classified into two groups, composed of accelerators and inhibitors of trafficking; cellular component enrichment analysis of the former revealed that a large proportion of the genes identified are associated with transcription and translation regulation, therefore this group was not considered any further. Strong enrichment for proteins associated with the Golgi, Golgi associated trafficking and cytoskeletal components were found for the retrograde inhibitors; many complexes such as COPI, COPII, TRAPP, COG, and SNARE complexes, which were previously described to be functioning between the early secretory step were identified (Burri et al., 2003; Spang, 2013; Willett et al., 2013b).
Transport between the two organelles is initiated through vesicle formation at the donor membrane; this crucial step is generally mediated through the action of district coat protein complexes. In the Golgi-to-ER trafficking step this process is thought to be largely mediated by the COPI coat complex (Serafini et al., 1991a), while a COPI-independent transport exists although no coat proteins have been associated with it. Data from the screen confirmed the function of COPI, and in particular that seven of the nine coatomer subunits inhibited retrograde transport upon depletion, but also revealed that two clathrin chains and several clathrin adapter proteins had marked inhibitory effects.

Coat proteins work in tandem with small GTPases, and SNARE fusion proteins for the recruitment, delivery, and fusion of transport carriers. Down-regulation of the Rab GTPases confirmed the function of the strongest hits presented in chapter 4, Rab1 and Rab2, Rab6a were shown to have strong inhibitor effects on the retrograde trafficking upon depletion. Additionally, to the pilot screen results (chapter 4) the microtubule regulator Rab18 was identified as a strong regulator of this trafficking step. This GTPase has been previously described to be linked to COPI-independent trafficking (Dejgaard et al., 2008).

Depletion of the SNARE proteins also highlighted the presence of multiple COPI-associated fusion proteins such as Ykt6 (Zhang and Hong, 2001) and Sec22b (Hay et al., 1997; Joglekar and Hay, 2005). Two of the identified SNARE proteins Stx5 and Stx6 were recently shown to interact with COG6, (Kudlyk et al., 2013; Laufman et al., 2011) a subunit of the COG tethering complex associated with Golgi organisation, intra-Golgi trafficking (Zolov and Lupashin, 2005) and potential tether for ER directed vesicle through its interaction with COPI (Suvorova et al., 2002). The COG complex was in turn identified as a key regulator, several subunits of this tethering complex have been shown to inhibit retrograde intra-Golgi trafficking (Fotso et al., 2005; Kudlyk et al., 2013; Sohda et al., 2010; Zolov and Lupashin, 2005) and this complex has been associated with Rab6a dependent, COPI-dependent trafficking for the intracellular delivery of Subtilase cytotoxin to the ER (Smith et al., 2009). A second tethering complex was identified from the primary screen, the TRAPP complex. Similar to the Golgi morphology screen, only components unique to the TRAPPI- and TRAPPIII- complexes were identified, with no significant inhibitory function been measured for Trappc9 and Trappc10, TRAPPII- components (Cox et al., 2007). Both identified complexes have been associated with COPII vesicle tethering with TRAPPI complex functioning at the ERGIC compartment (Cai et al., 2007; Sacher et al., 2001) and TRAPPIII at the Golgi (Scrivens et al., 2011). These novel results would
suggest that TRAPPI- and TRAPPIII- complexes may also function in Golgi-to-ER trafficking.

Anterograde transport from the ER to the Golgi complex is mediated by cytoplasmic dynein (Burkhardt et al., 1997), but the identity of the motors mediating transport in the retrograde direction remains unclear. The COPI-dependent pathway has been previously associated with heterotrimeric kinesin-2 (Stauber et al., 2006) and dynein-1 (Chen et al., 2005), while the motor responsible for COPI-independent movement has yet to be identified although it has been suggested that CD-1 and Kif20a might have a role (Heffernan and Simpson, 2014). In this screen, none of these motor proteins were identified, probably due to the stringent cut-offs in place for hit selection (CD1 and kinesin-2 were identified in the cytoskeletal pilot screen). The down-regulation of the three motor protein families; dyneins, myosins and kinesins, revealed that the kinesin-1 complex component Kif5a had the strongest inhibitory effect out of all the motor proteins. Kinesin-1 is a heterotetramer of two kinesin heavy chain (KHC) subunits (Kif5a, Kif5b, or Kif5c) and two kinesin light chain (Klc) subunits. The kinesin molecular motor was previously identified as a potential interacting partner to BICD2, a Rab6a protein linker. BICD2 has been shown to interact with the two kinesin-1 isoform Kif5a and Kif5b in HEK293 cells (Grigoriev et al., 2007). Kif5b has also been shown to localise with components of COPI coatomer upon expression of a mutant kinesin light chain 1 (Rahman et al., 1999), further suggesting a Golgi-to-ER role for this motor protein. Two myosin proteins linked with Golgi/ER trafficking were identified. First, the myosin Myo18a, linked to COPI carriers through its interaction with Golph3 (Taft et al., 2013). The complex of the two (Myo18a-Golph3) has been shown to connect the Golgi apparatus to F-actin to provide a tensile force required for efficient tubule and vesicle formation (Dippold et al., 2009; Taft et al., 2013). The second, Golgi localising myosin-II, has been suggest to mediate fission and formation of ER-destined vesicle through its interaction with Rab6a and giantin (Rosing et al., 2007).

The phenotypic assay not only defined the machinery required for Golgi-to-ER traffic but could also provide mechanistic details of the identified protein functions. Clustering of the raw kinetic data revealed four different redistribution profiles, denoting the different molecular machinery in place for the trafficking between the two organelles; such as coat proteins, tethering complexes and cytoskeletal components, amongst others. Each redistribution profile exhibited enrichments towards different molecular machinery, and further experiments will be needed to understand the significance of the proteins identified in each profile.
5.3.3. Regulators of Golgi structure and transport

The data from the two genome-wide screens reveal that there is just a small overlap between the two processes, but the genes exhibiting the dual role are clustered into complexes. The result would suggest that there are four key multi-subunit complexes that have dual regulatory effects, namely the COG, COPI, TRAPP, and SNARE complexes. Four key RAB GTPases were also identified to have this double role, Rab1a, Rab2a, Rab6a and Rab18, all previously associated with Golgi-to-ER trafficking, highlighting the important role of this trafficking step to the proper organisation of the Golgi.

A comparable overlap of proteins inhibiting secretion and Golgi structure was measured when comparing the Golgi organisation genomic data with the secretion screen (Simpson et al., 2012), with no complex being identified as having a dual role; this could be partly explained by the way the analysis was carried out, where only strong secretion inhibitors where considered, perhaps Golgi structure alteration has only a minor effect on this trafficking flow. The only proteins of interest found in common were the TRAPP subunit Trappc5 and the GTPase Rab18. The presence of the TRAPP subunit can be attributed to the role of the complex with COPII function (Cai et al., 2007), while a role for Rab18 role could be related to its connection to the microtubule network (Dejgaard et al., 2008). Components of the COPI complex were found to be common to all three screens, highlighting the previously described role of COPI as the coat protein complex mediating intra-Golgi trafficking (Antony et al., 1992; Seemann et al., 2000; Yang et al., 2011).

Similarly, comparison of the genomic data with protein toxin trafficking screens (Moreau et al., 2011), revealed only a very small amount of overlap; this is probably due to the small amount of hits validated in the toxin screen which amounted to only 116 genes, whose function is not only restricted to the early secretory step but throughout the endomembrane system. Therefore, it would be ideal if this assessment would be made with the complete primary screen list, which was not reported in that published work.

Overall, the two genome level screens have revealed that as much as 10% and 14% of the genome encodes for proteins related to Golgi structure and retrograde transport, respectively. The analysis carried out in this study only focused on less than 40% of the identified hits, which were found to be annotated and previously described; this leaves the door open for further characterisation of the remainder of the hits, for example initial determination of localisation, role and wider interaction network in the endomembrane system. The results of the screen not only confirmed previously published
roles for multiple complexes in this transport step, but also revealed potential new regulators for both Golgi organisation and retrograde trafficking. These studies therefore pave the way for a more complete investigation of the molecular components required to regulate Golgi structure and Golgi-to-ER transport in mammalian cells. The generation of these lists of hits is only the first step in this global study. Further significant work, needs to be carried out, starting with the validation of these hits through siRNA deconvolution and over-expression systems, ideally carried out in other cell lines. Only when a complete validated list of hits is obtained, studies on the function role of these proteins can be carried out. An approach that can be taken could be the characterisation of these hits through localisation and proteomic studies of their interacting partners, followed by an applied investigation on the function of these proteins on new cargo molecules such as cytotoxins.
CHAPTER 6 - CONCLUDING REMARKS AND FUTURE PERSPECTIVES
Significant advances have been made during recent years in defining the organisational principles of the endomembrane system. The central role of the Golgi complex in trafficking events has been under scrutiny, as well the mechanism by which it retains its complex structure. Analytical studies of this organelle have produced significant advances in our understanding of its function, although some aspects still elude our comprehension.

Traditionally, biochemical and genetic approaches have been the mainstay for determining the function and properties of organelles such as the Golgi. Although these techniques have been invaluable in terms of describing the intimate details of a particular protein under study, in many cases they do not provide contextual 'in cell' data. In more recent years a level of complexity surrounding this organelle has emerged with the discovery that the Golgi complex is involved in cellular processes other than protein and lipid modification and their onward trafficking such as apoptosis, autophagy and cytoskeleton regulation, amongst others (Hicks and Machamer, 2005; Mijaljica et al., 2006; Zhu and Kaverina, 2013). This leads to the conclusion that there is a requirement for larger-scale systematic studies of this central organelle. However, its integrated and dynamic nature makes it difficult to study in isolation, and single aspects of its trafficking are not easily studied without influencing other pathways. A relatively large number of systematic (genome-wide) studies addressing the mechanisms of anterograde transport between the ER and Golgi complex have been conducted (Bard et al., 2006; Simpson et al., 2012; Wendler et al., 2010), however to date there have been no large-scale studies focusing on cargo exit from this organelle, particularly in the retrograde direction towards the ER.

In order to address this, and to build on previous studies in the group relating to anterograde transport through this organelle (Simpson et al., 2012), two HCS assays were devised. These assays were designed to specifically identify regulators of Golgi structure and organisation, and the mechanisms that govern cargo exit from the organelle. HCS was considered as an ideal approach to achieve these objectives, as it incorporates the automation, speed and efficiency of HTS, with the spatial and temporal subcellular resolution coming from wide-field fluorescence microscopy. Critical to this study is the design of a robust method to systematically and quantitatively analyse the morphology of the Golgi complex, and assess the kinetics of Golgi-to-ER retrograde transport from very large numbers of cells in a reproducible and high throughput manner.

Using HCS as a platform, two new protocols were established using RNAi technology to probe gene function in these pathways and mechanisms under study. The
procedures were designed to be carried out using solid-phase reverse transfection plates in a 384-well plate format prepared using liquid handling robotics. HeLa cells were used for both screens; this model cell line was selected based on merits of being one of the easiest and fastest growing cell lines; having been extensively characterised; and having its proteome, transcriptome and genome mapped (Landry et al., 2013; Nagaraj et al., 2011; Wu et al., 2008). These features make it an ideal candidate for such screens, although inevitably further validation and characterisation of identified regulators is likely to be needed to be carried out in other cell types too. Specifically, a HeLa cell line stably expressing the GFP-tagged Golgi marker N-acetylgalactosaminytransferase-2 (GalNAc-T2) was utilised for this project, as this provided a robust and convenient reagent for monitoring the state of the Golgi complex (Storrie et al., 1998).

For the Golgi-to-ER trafficking assay the drug BFA was used to stimulate this trafficking event. Although this treatment may not be physiological, nonetheless it overcomes the time resolution and frequency of transport carrier synthesis difficulties associated with this trafficking step, and thereby provides an opportunity to define its regulatory machinery. An automated fluorescence microscope was used to capture images of cells under these conditions, which were then subjected to automated image analysis using HCS software. The image analysis routines were designed to segment cells individually and extract the relevant Golgi complex features and the cellular response to BFA treatment, specifically to determine the percentage of cells retaining an intact Golgi complex at each time point. Based on these figures, two indices assessing the Golgi structure and the kinetics of Golgi-to-ER redistribution were calculated, termed the Golgi fragmentation index (GFI) and Golgi-to-ER trafficking index (GETI).

The newly established protocols were initially trialled on two small test sets of proteins, composed of the Rab family of small GTPases and a cytoskeletal associated set. The systematic depletion of 58 Rab GTPases and 12 Rab-accessory proteins of the PRAF, YIPF and YIF protein families revealed 10 potential Golgi structure and 14 potential Golgi-to-ER regulators. The study confirmed previously published roles for Rab6a in this transport step (Girod et al., 1999; White et al., 1999) and provided clear data for Rab1a, Rab1b and Rab2a as being regulators of Golgi-to-ER transport, in addition to their established role in anterograde traffic (Plutner et al., 1991; Tisdale et al., 1992). The study also suggested two novel regulators for Golgi-to-ER transport, Rab10 and Rab11a, with localisation studies indicating that the two are physically present on a proportion of the Golgi-to-ER tubular intermediates. In addition, while combinatorial
depletions of Rab proteins revealed previously undescribed functional co-operation and physical co-occurrence between several Rabs.

The systematic depletion of the cytoskeletal pilot dataset revealed an intricate interplay between the actin and microtubule cytoskeletal network in controlling Golgi structure and Golgi-to-ER trafficking. The reported link between microtubule and actin networks with Golgi structure was confirmed in this study (Ho et al., 1989; Valderrama et al., 1998), whereby strong enrichments for microtubule or actin associated proteins were found in Golgi fragmentation and compaction phenotypes, respectively. The dual role of the CD-1 as a regulator of both structure and trafficking (Burkhardt et al., 1997; Chen et al., 2005) was confirmed through the identification of several of its components as both Golgi fragmenters and inhibitors of retrograde trafficking. A Cdc42 protein network was singled out as a novel Golgi structure regulator, with various members of the network also identified as trafficking inhibitors. Bioinformatic analysis of the results corroborated the central role of this Rho GTPase protein network, which was found to be linked to three other major Golgi altering protein network complexes, including a complex associated with cell adhesion, the dynein complex and myosins Myo18a and Myh9, both being linked to COPII-dependent and COPI-independent trafficking (Dippold et al., 2009; Miserey-Lenkei et al., 2010; Valente et al., 2010)

Work with these two test datasets demonstrated the robustness and sensitivity of the newly established assays and paved the way for an investigation on a genome-wide level of the molecular component requirements for structure and retrograde trafficking control in mammalian cells. The two large-scale studies represent the first and most comprehensive assessment of genes associated with Golgi structure and retrograde function in metazoans so far. The down-regulation of the 21,585 proteins through an RNAi strategy revealed that more than 10% of the genome encodes proteins that can be linked to this fundamental cellular component. Previously described organelle structure regulator complexes, COG (Kudlyk et al., 2013; Suvorova et al., 2002; Ungar et al., 2002), TRAPP (Barrowman et al., 2000; Sacher et al., 2001), SNARE (Zhang and Hong, 2001), the clathrin coat (Radulescu et al., 2007) and COPI coat (Chen et al., 2005; Wu et al., 2000b) complexes were confirmed in the study, as were multiple cytoskeletal network components, such as CD-1 (Burkhardt et al., 1997) and myosin-II complexes (Miserey-Lenkei et al., 2010; Valente et al., 2010), while new links between cell adhesion and Golgi structure have been identified, in particular for the cell adhesion complex family of integrins. Bioinformatic analyses revealed that the regulation of the organelle occurs at multiple levels. Alongside the well-established core machinery, it involves cross-talk with
small GTP-binding protein regulation, actin and microtubule cytoskeleton organisation and ER membrane proteins. The results of the Golgi organisation screen also highlighted the intricate relationship between Golgi organisation and trafficking and how these two mechanisms cannot be studied in isolation but have to be considered together for a correct understanding of the mechanisms in place.

The Golgi-to-ER genome-wide screen revealed that this trafficking step relies on different membrane trafficking complexes at multiple stages. Consistent with the well-established literature, the role of the COPI coat complex in this trafficking step was confirmed, along with certain SNARE family proteins. A potentially new role for the TRAPP tethering complex as regulators of Golgi-to-ER transport was suggested, in addition to their established role in anterograde traffic (Allan et al., 2000; Barrowman et al., 2000; Sacher et al., 2001). Interestingly, the data suggest that the clathrin coat complex might also have a role in this trafficking step, although further validation will need to be carried out to confirm this. The identification of the ERES protein, Sec16b, would also suggest that ER structural organisation has a crucial role in the delivery of the Golgi transport carriers (Watson et al., 2006). Alongside the core machinery associated with Golgi-to-ER redistribution, the screen also revealed the role of multiple GTPases, phosphatases and kinases in controlling the dynamics of this trafficking step. The redistribution data also provided mechanistic information for the candidates identified. Through the analysis of the redistribution profiles, four phenotypes were identified and found to be characterised by proteins performing different functions in the retrograde step. This analysis could provide valuable information for characterising undescribed hits identified in the screen.

The screens revealed that Golgi organisation and transport seem to rely on different molecular mechanisms. However, a small number of genes were found to have a dual role, with several components of four key multi-subunit complexes identified, namely COG, COPI, TRAPP, and SNARE complexes. Both Golgi structure and retrograde traffic were found to depend on a common set of Rab GTPases, namely Rab1a, Rab2a, Rab6a and Rab18, all localising to the Golgi and Golgi-ER interface, highlighting the important role of this trafficking step to the proper organisation of the Golgi. Comparison analysis done with a secretion inhibition screen (Simpson et al., 2012) revealed two common regulators, Rab18 and Trappc5, highlighting the importance of the GTPase as a microtubule regulator and the malleable nature of the TRAPP complex as these candidates were found to be involved in the three processes. By contrast, no distinctive overlap was measured when comparing the genomic data with a list of inhibitors of toxin
trafficking (Shiga, ricin and PE) (Moreau et al., 2011). Overall, the two genome-wide screens highlight the large knowledge gap with respect to the molecular machinery and mechanisms that there are in place to control and maintain Golgi architecture and regulate the Golgi-to-ER retrograde trafficking step.

The results presented here need to be interpreted with consideration to the limitations of the methodology applied. Advantages of RNAi include the high efficiency of gene knockdown and relative ease of being able systematically target every gene in the genome. This approach has already been applied to successfully address many significant questions relating to cell function, including cell division, secretion, and infection. However, RNAi approaches also have limitations. Many of these, relate to the design of the siRNA sequence and its precision in terms of recognising its correct mRNA target. Non-specific and off-target effects are well documented problems, particularly when pooled sequences are used, and also when screens are carried out on such a large scale. Such pools are powerful in terms of their gene coverage, but they carry the higher risk that if one or more sequences in each pool shows off-target effects or inefficient knockdown, false positive or indeed false negative results may be recorded. In addition, biological “artefacts” must also be taken in consideration; for example molecular redundancy of function in certain trafficking pathways, which would explain why some knockdowns failed to yield a phenotype.

Taking such limitations into account, it is clear that further characterisation of candidates genes is essential if they are to be confirmed as playing a role in either maintaining Golgi or regulating Golgi-to-ER transport. The approach adopted here was deconvolution of the siRNA pools allowing bona fide siRNA sequences correctly targeting cognate genes to be identified on an individual basis. Ongoing work would require the depletion levels of a number of candidates to be measured using qPCR, antibody staining, and/or Western blotting, thereby ultimately providing multiple strands of evidence that the phenotypes were specific. Rescue experiments and localisation studies could be used to further validate the roles of the identified candidates.

The challenge now is to understand how these protein networks identified work in synchrony to regulate the organisation of the organelle and how they control export trafficking events. The data presented here provide the basic information to address this. One next step would be to pin-point novel complexes of interest through the cross-referencing of the data obtained with other gene expression, localisation and functional databases, in order to narrow down the potential number of candidates. Individual genes
and their cognate protein can then be validated and characterised in detail, and their specific role in either Golgi organisation or Golgi related trafficking fully established.
Appendix A: Image analysis routine and data handling

Chapter 2 makes reference to two image analysis routines for the analysis of the Golgi morphology and Golgi-to-ER redistribution in Columbus image analysis software. Examples of the settings used and a representative screenshots for the two routines are provided in sections A1 and A2. The settings used in the image analysis routines were adjusted to compensate for small variations from plate to plate. The R scripts used for data handling are provided in sections A3 and A4.

A1 Golgi morphology image analysis routine

Images acquired with the Olympus Scan^R automated microscope were analysed using Columbus™ software (PerkinElmer). The software was used to segment individual cells from the raw image data; the nucleus was segmented using the nuclear stain (A1.1), while the cytoplasm was segmented using the GFP channel through the background fluorescence of the tagged protein in the ER (A1.2); although the majority of the fluorescence of the GalNAc-T2-GFP was concentrated at the Golgi complex, the small amount of fluorescence in the ER was sufficient to accurately identify the cytoplasm boundary for each cell. Morphological and intensity measurements were then extracted from the segmented cells to distinguish between healthy and apoptotic or dividing cells that would skew the analysis (A1.3-A1.6). Cells close to the border of the field of view were also removed from the analysis (A1.7).

Upon the completion of the segmentation and population selection, the Golgi complex was segmented (A1.8); using the fluorescence emitted by the GFP tagged protein. A variety of measurements were then taken: total area, fragment area, number of fragments per cell and the percentage population showing high level of fragmentation (A1.9). For each plate the mean number of Golgi fragments per cell was used to rank the effect of the depletion of each gene on Golgi morphology. The screen data was normalised plate by plate based on the negative control, to allow comparison and combination of data from the plates across the screen. This was done by the division of each sample value by the mean of the negative control (siNeg) (A3).
A1.1 Nuclei segmentation

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A1.2 Cytoplasm segmentation

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A1.4 Calculate intensity properties (Cell)

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A1.5 Calculate morphology properties (Nuclei)

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<tr>
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<th>Nuclei</th>
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</thead>
<tbody>
<tr>
<td>Region</td>
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</tr>
<tr>
<td>Method of detection</td>
<td>Standard</td>
</tr>
<tr>
<td>Measurements</td>
<td>Area and roundness</td>
</tr>
<tr>
<td>Output population</td>
<td>Morphology nuclei</td>
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A1.6 Calculate morphology properties (Cell)

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<tbody>
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<td>Method of detection</td>
<td>Standard</td>
</tr>
<tr>
<td>Measurements</td>
<td>Area and roundness</td>
</tr>
<tr>
<td>Output population</td>
<td>Morphology cell</td>
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A1.7 Select population (Remove border objects)

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<td>Region</td>
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<td>Selected RBO population</td>
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A1.8 Select population (Population filtering by size and intensity)

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<td>&lt;15,000</td>
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<td>Filter 3: Nucleus roundness</td>
<td>&gt;0.7</td>
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<tr>
<td>Filter 4: Nucleus mean intensity</td>
<td>&gt;1300</td>
</tr>
<tr>
<td>Filter 5: Nucleus mean intensity</td>
<td>&lt;2000</td>
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<tr>
<td>Filter 6: Cell area (px$^2$)</td>
<td>&gt;19,000</td>
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<td>Filter 7: Cell area (px$^2$)</td>
<td>&lt;50,000</td>
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A1.9 Find spots

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<td>Method of detection</td>
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<td>Detection sensitivity</td>
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<td>Splitting coefficient</td>
<td>0.80</td>
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<td>Output population</td>
<td>Golgi Fragments</td>
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A2 Golgi-to-ER redistribution image analysis routine

Upon the completion of the segmentation and population selection as carried out for previously (A2.1-A2.8), the intensity profile of each cell was measured to determine the maximum and mean intensity of the GFP tagged protein in the cytoplasm (A2.9). Cells which maintained normal Golgi morphology showed a higher maximum intensity and a higher mean intensity in the cytoplasm compared to cells that underwent redistribution of the Golgi complex. A linear classifier was used to select cells into populations based on these particular intensity features. In this case, cells that retained the Golgi morphology in the positive control wells from those which underwent redistribution in the negative control wells were selected and used as a training set (containing 10 cells per group) for the linear classifier (A2.10). The linear classifier then used the features associated with this population of cells to classify the state of Golgi across all cells in the well and ultimately in the entire plate. This analysis identified the percentage population of the cells that retained normal Golgi morphology and therefore quantified the effect of protein depletion on the kinetics of Golgi-to-ER trafficking induced by BFA.

Through the combination of all the time points obtained through the BFA treatment, the kinetics of the retrograde trafficking step was calculated. A 'phenoprint' for each down-regulated target was created, showing the percentage population of cells that retained Golgi morphology over the time of BFA treatment. These values were logarithmically transformed to achieve a symmetrical distributed data set; a linear model was then applied to the phenoprint to determine the slope (the rate of redistribution), therefore providing an index of the Golgi-to-ER trafficking. This index was normalised plate by plate based on the negative control (siNeg), through the division of each index by the mean of the negative control index. An average of the normalised values of the replicates was made in order to obtain a Golgi-to-ER trafficking index (GETI) and this was used to rank the various genes based on their effects (A4).
A2.1 Find nuclei

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A2.2 Find Cytoplasm

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A2.3 Calculate intensity properties (Nuclei)

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A2.4 Calculate intensity properties (Cell)

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A2.5 Calculate morphology properties (Nuclei)

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A2.6 Calculate morphology properties (Cell)

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A2.7 Select population (Remove border objects)

![Image](image_url)

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A2.8 Select population (Population filtering)

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<td>&gt;0.7</td>
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<td>&lt;2200</td>
</tr>
<tr>
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Output population: Working population

A2.9 Calculate intensity properties (Golgi)

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<td>Region</td>
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<tr>
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<tr>
<td>Measurements</td>
<td>Mean and max. intensity</td>
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<td>Output population</td>
<td>Intensity Golgi</td>
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A2.10 Select population (Linear classifier)

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<td>Classification measurements</td>
<td>Mean and max. intensity</td>
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<tr>
<td>Output population A</td>
<td>Intact Golgi</td>
</tr>
<tr>
<td>Output population B</td>
<td>Redistributed Golgi</td>
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# Load layout file (uni)
un <- read.csv(file.choose(), header=T, sep=";", fill=T)
u1 <- un[,5]
u2 <- un[,8]
u3 <- un[,9]
u4 <- un[,10]
uni <- data.frame(u1, u2, u3, u4)
colnames(uni) <- c("well", "refseq", "gsymb", "fullname")

# Load first replicate (rep1)
rep1 <- read.table(file.choose(), header=T, sep="t")
nrow(rep1)

# Extraction of cell no from rep1 (wp)
wp <- rep1[,4]

# Extraction of data from rep1 (pna)
pn <- rep1[,5]

# Extraction of siNEGs (negall1, negmean1, negall1a)
  neg1 <- pn[1]
  neg2 <- pn[13]
  neg3 <- pn[25]
  neg4 <- pn[37]
  negall1 <- c(neg1, neg2, neg3, neg4)
  negmean1 <- mean(negall1[1:2])
  negall1a <- data.frame(negall1, negmean1)

# Neg normalisation (ind1)
ind1 <- pn/negmean1

# Creation of file 1
rep1 <- data.frame(wp, pn, ind1)
colnames(rep1) <- c("wp (1)", "mean frag no (1)", "index (1)")

# Load first replicate (rep2)
rep2 <- read.table(file.choose(), header=T, sep="t")
nrow(rep2)

# Extraction of cell no from rep2 (wp)
wp <- rep2[,4]

# Extraction of data from rep2 (pna)
pn <- rep2[,11]

# Extraction of siNEGs (negall1, negmean1, negall1a)
  neg1 <- pn[1]
  neg2 <- pn[13]
  neg3 <- pn[25]
  neg4 <- pn[37]
  negall1 <- c(neg1, neg2, neg3, neg4)
  negmean1 <- mean(negall1[1:2])
  negall1a <- data.frame(negall1, negmean1)

# Neg normalisation (ind1)
ind2 <- pn/negmean1

# Creation of file 2
rep2 <- data.frame(wp, pn, ind2)
colnames(rep2) <- c("wp (2)", "mean frag no (2)", "index (2)")

# Average of index
indall <- data.frame(ind1, ind2)
indmean <- rowMeans(indall)
indall[is.na(indall)] <- 0

# t.test of index
pval <- apply(indall[, c(1, 2)], 1,
A4  Golgi-to-ER redistribution data handling R script

#Replicate 1:

#Load layout file (uni)
un<-read.csv(file.choose(),header=T, sep=".", fill=T)
u1<-un[,5]
u2<-un[,8]
u3<-un[,9]
u4<-un[,10]
uni<-data.frame(u1, u2, u3, u4)

#Load time point 0 (t0)
t0<-read.table(file.choose(),header=T,sep="\t")
nrow(t0)

#Load time point 5 (t5)
t5<-read.table(file.choose(),header=T,sep="\t")
nrow(t5)

#Load time point 10 (t10)
t10<-read.table(file.choose(),header=T,sep="\t")
nrow(t10)

#Load time point 15 (t15)
t15<-read.table(file.choose(),header=T,sep="\t")
nrow(t15)

#Extraction of plate ID name (pna)
pn5<-t5[,3]
pn10<-t10[,3]
pn15<-t15[,3]
pna<-data.frame(pn5, pn10, pn15)

#Extraction of phenotype (pt)
pt0<-t0[,12]
pt5<-t5[,20]
pt10<-t10[,20]
pt15<-t15[,20]
pt=matrix(c(pt0, pt5, pt10, pt15),
nrow=384,
col=4)

#Replacement of all 0 values with 0.0001
pt[pt == 0] <- 0.0001

#Log values (plog)
plog<-log(pt)

#Apply linear model to pheno-print (rt)
mylm<-function(data){
x<-c(0.5,10,15)
y<-as.vector(data)
d<-data.frame(x,y)
lm(y~x,d)$coefficients[2]
}
rt<-apply(plog,1,mylm)

#Extraction of siNEGs (negall1, negmean1, negall1a)
neg1<-rt[1]
neg2<-rt[13]
neg3<-rt[25]
neg4<-rt[37]
negall1<-c(neg1, neg2, neg3, neg4)
negmean1<-mean(negall1[1:4])
negall1a<-data.frame(negall1, negmean1)

# Neg normalisation (ind1, ct1)
ind1<-rt/negmean1
c1<-(1-sd(negall1/negmean1)^3)

# Extraction of cell population numbers (dt)
wp0<-t0[,12]
wp5<-t5[,12]
wp10<-t10[,12]
wp15<-t15[,12]
dt=matrix(
c(wp0, wp5, wp10, wp15),
nrow=384,
ncol=4)

# Creation of file 1
rep1<-data.frame(pna, uni, dt, pt, rt, ind1)
colnames(rep1)<-c("plate5", "plate10", "plate15", "well", "refseq", "gsymb", "fullname", "wn0", "wn5", "wn10", "wn15", "t0", "t5", "t10", "t15", "R", "I")

# second replicate
# Load time point 5 (t5)
t5<-read.table(file.choose(),header=T,sep="\t")
nrow(t5)
# Load time point 10 (t10)
t10<-read.table(file.choose(),header=T,sep="\t")
nrow(t10)
# Load time point 15 (t15)
t15<-read.table(file.choose(),header=T,sep="\t")
nrow(t15)

# Extraction of plate ID name (pna)
pn5<-t5[,3]
pn10<-t10[,3]
pn15<-t15[,3]
pna<-data.frame(pn5, pn10, pn15)

# Extraction of phenotype (pt)
pt0<-t0[,12]
pt5<-t5[,20]
pt10<-t10[,20]
pt15<-t15[,20]
pt=matrix(
c(pt0, pt5, pt10, pt15),
nrow=384,
ncol=4)

# Replacement of all 0 values with 0.0001
pt[pt == 0] <- 0.0001

# Log values (plog)
plog<-log(pt)

# Apply linear model to pheno-print (rt)

# Extraction of siNEGs (negall2, negmean2, negall2a)

# Extraction of siNEGs (negall2, negmean2, negall2a)
# Neg normalisation (ind2, ct2)
ct2<-(1-sd(negall2/negmean2)*3)
ind2<-rt/negmean2

# Extraction of cell population numbers (dt)
wp0<-t0[,12]
wp5<-t5[,12]
wp10<-t10[,12]
wp15<-t15[,12]
dt=matrix(c(wp0, wp5, wp10, wp15), nrow=384, ncol=4)

# Creation of file 2
rep2<-data.frame(pna, uni, dt, pt, rt, ind2)
colnames(rep2)<-c("plate5", "plate10", "plate15", "well", "refseq", "gsymb", "fullname", "wn0", "wn5", "wn10", "wn15", "t0", "t5", "t10", "t15", "R", "I")

# Average of index
indall<-data.frame(ind1, ind2)
indmean<-rowMeans(indall)

# t-test of index
pval<-apply(indall[, c(1, 2)], 1, function(temp) unlist(t.test(temp, alternative = c("two.sided")) [c("p.value")]))

# Creation of masterfile
rfulldata<-data.frame(rep1, rep2, indmean, pval)
cta<-mean(ct1 + ct2)/2
write.csv(rfulldata, "fulldata.csv")
write.csv(cta, "cutoff.csv")
Appendix B: siRNA libraries and result tables

Table B1.1. Rab associated proteins pilot screen
List of siRNA sequence targeting the Rab GTPase and Rab-accessory proteins (Tab1. gene_list). GFI and GETI values for the targets tested in the pilot screen (Tab2. primary_screen) and the GETI values from the hit validation process (Tab3. Validation).

Table B1.2. Cytoskeleton-associated proteins screen
List of siRNA sequence targeting the Cytoskeletal associated proteins (Tab1. gene_list). GFI and GETI values for the targets tested in the pilot screen (Tab2. primary_screen).

Table B1.3. Golgi morphology regulators genome-wide screen
GFI values for the targets tested in the genome-wide Golgi morphology screen after quality control for replicates, cell numbers (Tab1. Primary_screen). List of siRNAs which were disregarded due to insufficient cells remaining at the end of the experiment (Tab2. low_cell_no). List of siRNAs that led to a strong Golgi fragmentation phenotype upon transfection (Tab3. fragmentation hits). List of siRNAs that led to a strong Golgi compaction phenotype upon transfection (Tab4. compaction hits).

Table B1.4. Golgi-to-ER regulators genome-wide screen
GETI values for the targets tested in the genome-wide Golgi-to-ER trafficking screen after quality control for replicates, cell numbers (Tab1. Primary_screen). List of siRNAs which were disregarded due to insufficient cells remaining at the end of the experiment (Tab2. low_cell_no). List of siRNAs that led to a strong inhibition of retrograde trafficking upon transfection (Tab3. Inhibitors). List of siRNAs that led to acceleration of the retrograde step upon transfection (Tab4. accelerators). The groups of genes used for the analysis of the different protein families (Tab5. grouped genes). Clustering classification of the redistribution profiles (Tab6. GETI_clustering).
Appendix C: Permissions for reproduction of figures

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