INVESTIGATING NOVEL REGULATORS OF GOLGI MEMBRANE TUBULATION

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The Golgi complex serves as a vital organelle from which proteins and membrane lipids are modified, sorted, and trafficked to various destinations. Mutations that cause defects in structural maintenance or membrane trafficking at the Golgi are commonly linked to neurodegeneration, metabolic disease, and reproductive disorders. Both structural maintenance and membrane trafficking rely on cooperative efforts of coated vesicles and membrane tubules. Although extensive information is available for membrane coated vesicle traffic, knowledge of membrane tubules remains comparably deficient. Understanding the regulatory mechanisms behind membrane tubules may help elucidate how Golgi tubule biogenesis can respond to varying physiological stimuli such as increased secretory loads.

I utilized an siRNA library against all known and purported human kinases, or the kinome, in a high throughput, microscopy-based screen that identified proteins involved in Brefeldin A (BFA)-induced Golgi membrane tubulation. This screen successfully identified siRNAs that significantly inhibited or enhanced the effects of BFA-induced Golgi tubulation. Among the identified hits, I further characterized two inhibitory siRNA that targeted Protein-Associating with the Carboxyl-terminal domain of Ezrin (PACE1) and diacylglycerol kinase γ (DGK-γ), and determined that they play important roles in maintaining intact Golgi ribbon structures through regulating Golgi membrane tubule biogenesis. I found that these proteins also facilitate Golgi reassembly and anterograde membrane trafficking of both soluble and

transmembrane proteins, further buttressing the importance of membrane tubules in multiple, cellular processes.

The results of my studies will be useful not only in contributing to the understanding of existing regulatory mechanisms behind membrane tubulation but also in discovering novel mechanisms. Further studies on membrane tubulation will help shed light on physiological functions and possibly lead to translational advances in diseases that depend on membrane tubulation and/or trafficking.

BIOGRAPHICAL SKETCH

Kevin D. Ha was born in Sacramento, California. He attended the University of California, San Diego, where he obtained his B.S. in Molecular Biology and conducted undergraduate research studying the biochemical mechanisms underlying early onset of Alzheimer's disease. He subsequently worked as an associate research scientist with the Discovery Team within the Department of Cell Biology at Pfizer in La Jolla, California, investigating novel therapeutic antibodies against numerous cancers. While at Pfizer, Kevin harnessed a deep interest in cellular biology and advance technologies. He subsequently joined the Graduate Field of Biochemsitry, Molecular and Cell Biology in the department of Molecular Biology and Genetics at Cornell University in 2006. He later joined the laboratory of Dr. Bill Brown in 2007, where he studied Golgi membrane tubulation. He successfully defended his Ph.D. dissertation in 2012 and accepted a postdoctoral fellowship at the University of California, San Francisco, under the mentorship of Dr. Bin Liu, where he will resume translational studies on cancers, particularly discovering novel therapeutic antibodies via a phage display screen.

Kevin used to have numerous hobbies, including computers, computer software programming, web-design, photography, music production, turntablism, screenwriting, cinematography, weight lifting, fishing, following practically all sports, and cooking. The arrival of Kevin's two children, Atticus and Melia, saw the departure of his hobbies. His hobbies were quickly replaced with diaper-changing, spoon-feeding, book readings, stroller walks, picking up toys from the ground, and collecting spittle and drool on his clothing.

For my loving family, Samantha, Atticus, and Melia

For my supportive family, Eric, Tony, Ruby, Lisa, and my mom

But especially for my father and Davis

The winds carry the ashes peacefully to the skies

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ABBREVIATIONS

AA – Arachidonic acid

ACAT – acyl-CoA cholesterol acyltransferase

AGPAT – 1-acylglycerol-3-phosphate acyltransferase

AKAP – A-kinase anchoring protein

ARF – ADP-ribosylation factor

ATP – Adenosine triphosphate

AU – Arbitrary unit

BAR – Bin-amphiphysin-rvs

BARS – BFA-inhibited, ADP-ribosylated substrate

BFA – Brefeldin A

BIG2 – BFA-inhibited GEF 2

CERK – Ceramide kinase

CPT1 - Cholinephosphotransferase

CVAK104 – Coated vesicle-associated kinase of 104 kDa

DAG - Diacylglycerol

DGK – Diacylglycerol kinase

ER – Endoplasmic reticulum

ERC – Endosomal recycling compartment

ERES – Endoplasmic reticulum exit site

ERGIC – ER-Golgi-intermediate compartment

ERM – Ezrin, radixin, moesin

GalT – Galactosyltransferase

GEF – Guanine nucleotide exchange factor

GGA1 – Golgi-localized, γ-ear containing, ARF-binding

GFP – Green fluorescent protein

GUV – Giant unilamellar vesicle

L_d – Liquid disordered

L_o – Liquid ordered

LPA – Lysophosphatidic acid

LPAAT – Lysophosphatidic acid acyltransferase

LPAT – Lysophospholipid acyltransferase

LPC – Lysophosphatidylcholine

LPE - Lysophosphatidylethanolamine

LPL - Lysophospholipid

M6PR – Mannose6-phosphate receptor

MAFP – Methyl arachidonyl fluorophosphonate

ManII – Mannosidase II

MBOAT – Membrane bound O-acyltransferase

MT – Microtubule

MTOC – Microtubule organizing center

NLS – Nuclear localization sequence

PA – Phosphatidic acid

PACE1 – Protein-associating with the carboxyl-terminal domain of ezrin

PACOCF3: Palmitoyl trifluoromethylketone

PAFAH – Platelet activating factor acetylhydrolase

PC - Phosphatidylcholine

PIP – Phosphoinositide phosphate

PKC – Protein kinase C

PKD – Protein kinase D

PL - Phospholipid

PLA – Phospholipase A

PLAAp – PLA_2 activating protein peptide

PLD – Phospholipase D

PM – Plasma membrane

PS - Phosphatidylserine

SEM – Standard error of the mean

SM – Sphingomyelin

SMS – Sphingomyelin synthase

SNX – Sorting nexin

SRP – Signal recognition particle

SS – Signal sequence

SSHRP – Soluble secretable horseradish peroxidase

TGN – *trans*-Golgi network

VSVG – Vesicular stomatitis virus protein G

CHAPTER 1

Mechanisms of Membrane Trafficking[§]

[§] This chapter includes work published in full, with modifications, from Kevin D. Ha, Benjamin A. Clarke, and William J. Brown. Regulation of the Golgi complex by phospholipid remodeling enzymes. *Biochim Biophys Acta (BBA)*. (2012). The text is reprinted here with permission from the publisher, Elsevier.

1.1. Abstract

Higher eukaryotic cells possess numerous membrane-delimited compartments such as early and late endosomes, endosomal recycling compartment (ERC), the Golgi complex, the *trans*-Golgi network (TGN), endoplasmic reticulum (ER), and lysosomes, among others. These membranous compartments contain varying protein and lipid compositions that differ from each other and determine their physiological roles and functions. Eukaryotic cells utilize membrane trafficking to effectively sort and transport proteins and membranes between these membrane compartments in order to carry out secretion and endocytosis and to maintain their homeostatic compositions. Defects in membrane trafficking may yield an assortment of physiological diseases, including neurodegenerative and reproductive disorders. Membrane trafficking intermediates comprise both vesicles and membrane tubules. Both are similar in the respect that they participate in the trafficking of both protein and membrane cargo destined for diverse, intracellular destinations. However, membrane tubules differ from membrane vesicles with respect to their formation, regulation, and function. While extensive research has helped elucidate membrane vesicle biology in detail, comparable information is lacking on membrane tubule biology. Recent studies discovered important proteins, chiefly phospholipases and acyltransferases, to be involved in membrane tubulation in addition to establishing the importance of membrane tubules in a variety of physiological functions. Although these studies help clarify membrane tubule roles in membrane trafficking and Golgi structure maintenance, the biochemical regulation behind membrane tubulation remains largely uninvestigated. Here, I will provide a broad overview on membrane trafficking followed by a detailed review on phospholipid remodeling-based membrane tubulation.

1.2. Introduction

Cargo proteins and membranes destined for transport to organelles are synthesized in the ER, then transported to the Golgi complex where protein cargo undergo post-translational modifications such as glycosylation, and finally sorted into membrane carriers destined to specific target membranes. The Golgi complex is composed of stacks of flattened cisternae. Both the number of stacks and cisternae they contain vary between different organisms. Mammalian cells typically feature the Golgi complex as a single, long stack, called a Golgi ribbon, in a juxtanuclear position, although certain cell types such as neuronal dendrites utilize Golgi stacks positioned far from the nucleus, called Golgi outposts [1], while skeletal muscle myofibers display dispersed Golgi structures [2]. The cisternal Golgi element closest to the nucleus and ER is called the *cis*-Golgi cisterna while the opposite end of the stack is the *trans*-Golgi cisterna. At the *trans*-face of the Golgi complex, cisternae display higher plasticity and become tubular and vesicular. The TGN lies further *trans* from the Golgi stack and bears an extensive tubulovesicular morphology [3], and this morphology may be due to the gradual segregation of proteins and membranes destined for differing compartments via its consumption by coated vesicle and membrane tubule formation [2]. Similar to the TGN, the ER-Golgi-intermediate compartment (ERGIC) resides between the Golgi stack and the ER and also assumes a tubulovesicular cluster morphology [4].

A protein destined for secretion typically bears a hydrophobic signal sequence (SS) on its N-terminus, which is recognized by the signal-recognition particle (SRP) during nascent translation and then directs the peptide along with its translational machinery to the ER [5]. SRP arrests protein translation when it binds to the SS [6], but upon binding to the SRP receptor, it

dissociates and permits translation to resume with the ribosome now bound to a protein that directs nascent proteins across membranes, called the translocon [7]. Protein cargo is then sorted and packaged into COPII vesicles in specific sites within the ER called ER exit sites (ERESs), which then send protein and membrane cargo towards the ERGIC [8]. Interestingly, ERESs appear to have distinct cargo [9], further demonstrating that the ER, along with the Golgi, possesses sorting abilities. The ERGIC also serves to further sort protein cargo by separating proteins destined for the Golgi from proteins to be returned back to the ER [10-11].

1.3. Models of intra-Golgi trafficking

Once at the *cis*-Golgi cisterna, protein and membrane cargo make their way towards the *trans*-Golgi cisterna. The mechanism by which this occurs remains a heavily contentious issue. One early model proposed by Dr. George Palade, called the forward vesicular-trafficking or stable compartments model, is based on the idea that Golgi cisternae remain static while protein and membrane cargo must travel across the sequential cisternae of the Golgi stack by means of successive vesicle budding and fusion events that were assumed to move in an anterograde direction [12]. However, later biochemical, genetic, and cell biological studies refuted this model, leading to the more widely accepted model that COPI vesicles mediate retrograde trafficking through and from the Golgi [13]. For example, purified intra-Golgi COPI vesicles possess Golgi resident proteins and not cargo [14]. Furthermore, many organisms transport protein cargos that do not fit in conventional COPI vesicles, such as procollagen in mammalian fibroblasts [15-16].

This led to the proposal of a second model, called the Golgi maturation model, where *cis*-Golgi cisternae mature into *trans*-Golgi cisternae while Golgi resident proteins, such as glycosylation enzymes, are recycled back to *cis*-Golgi cisternae via COPI coated vesicles [17]. The Golgi maturation model is best supported by live-cell imaging studies which showed that early Golgi markers are replaced with later Golgi markers within the same Golgi element [18-19]. In the maturation model, incoming protein must undergo a lag phase after entering the Golgi *cis*-cisterna to allow time for retrograde, vesicular traffic of Golgi resident enzymes from mature *trans*-cisternae to arrive and modify the cargo proteins. However, later studies analyzing the kinetics of cargo transport in living cells using fluorescently-tagged proteins observed that proteins entering the Golgi did not exhibit any lag phase and instead, their rate of exit depending on their abundance within the Golgi [20].

This heralded a third model of intra-Golgi trafficking by the Lippincott-Schwartz laboratory, which postulates that both protein cargo and Golgi resident enzymes can move bidirectionally, both *cis*-to-*trans* and *trans*-to-*cis* within Golgi stacks. This model additionally depends on the sorting of lipids into different domains at each level of the Golgi and that proteins associate with specific lipid environments [21]. Some protein cargo at the *trans*-Golgi cisternae is packed into COPI vesicles destined for retrograde traffic [22], while anterograde cargo protein within the TGN is sorted and packaged into secretory vesicles for transport to endosomal compartments, such as the ERC [23-24]. Once at the ERC, proteins are further sorted to destinations such as the plasma membrane [25]. Although the Golgi complex has been considered a major hub for protein sorting, protein cargo can be sorted prior to arriving and/or after leaving the Golgi complex [2]. An example includes the endosomal recycling compartment, which sorts both endocytic and TGN-derived protein cargo to be recycled or

degraded [26]. Although the Golgi is considered the major hub for protein trafficking and sorting, some proteins are capable of bypassing entry into the Golgi during trafficking under "unconventional pathways" [27-29].

When scrutinizing the membrane carriers themselves, decades of research have established a firm understanding in the biochemical mechanisms behind coated vesicle biogenesis and functions [30]. In contrast, comparably little is known about membrane tubules [31].

1.4. Significance of membrane tubules

Membrane tubules have fascinated cell biologists since the discovery that a fungal metabolite, Brefeldin A (BFA), can readily induce the formation of tubules from the Golgi complex [32]. With technological advancements in both fluorescence and electron microscopy, it became clear that dynamic, endogenous, membrane tubules exist. They were observed to be originating from many organelles including the ER, ERGIC [33], Golgi [34-37], TGN [38], endosomes [39], and plasma membrane [40] in diverse organisms from yeast to humans.

Membrane tubules are significant in membrane trafficking as they originate from many organelles for trafficking routes (Figure 1.1). In the ER, maintenance of membrane tubules may serve as a structural feature although they may also facilitate ER protein sorting and trafficking [41]. Membrane tubules also emanate from the plasma membrane to form contacts between animal cells, providing a way for intercellular communication or facilitating the spread of pathogens [42]. Prominent membrane tubules also originate from the Golgi and are extremely dynamic, where they are able to grow, shrink, and detach to traverse across the cell [43-44].

Some of the first studies exploring the mechanism behind membrane tubule biogenesis began through analyzing BFA. Studies have shown that BFA acts as an ADP-ribosylation factor (ARF) GTPase exchange factor (GEF) inhibitor to disrupt coated vesicle formation, such as COPI and AP-1 clathrin-coated vesicles, at the Golgi complex [45-46]. This inhibition stimulates Golgi membrane tubules that traffic in a retrograde fashion along microtubules to fuse with the ER [47-49]. This initially led to speculation that membrane tubulation may simply be an artifact of failed coated vesicle formation such as COPI, COPII, and AP-1 clathrin [32]. Evidence against this possibility was provided by previous studies that have shown that RNAi knockdown of Golgi ARFs [50], some of their GEFs such as GBF1 [51], and AP-1 associated GGA1 (Golgi-localized, γ-ear containing, ARF-binding) [52] also induced membrane tubule formation. Likewise, another study showed that addition of low-ATP cytosol can also induce membrane tubules *in vitro* using isolated, intact Golgi membranes [53].

The observation that loss of coat proteins can induce membrane tubulation inferred that membrane tubule-based trafficking pathways might exist independently from coat proteins. Nevertheless, membrane tubulation and coat vesicles may not be mutually exclusive but can instead work in concert to facilitate membrane trafficking [54]. Some have even proposed that membrane tubules help increase Golgi fenestration, a widely observed morphology on the fringes of cisternae that helps to increase surface area to volume ratio and serve as precursors to membrane vesicles [55]. Both membrane tubulation and vesiculation require the preliminary step of inducing membrane curvature in order to form the vesicle or tubule membrane bud. Multiple mechanisms have been proposed to induce membrane curvature [56-59]. These mechanisms include changes to lipid structures within membrane bilayer leaflets, insertion of proteins into membrane bilayer or leaflet, and the formation of protein scaffolds alongside

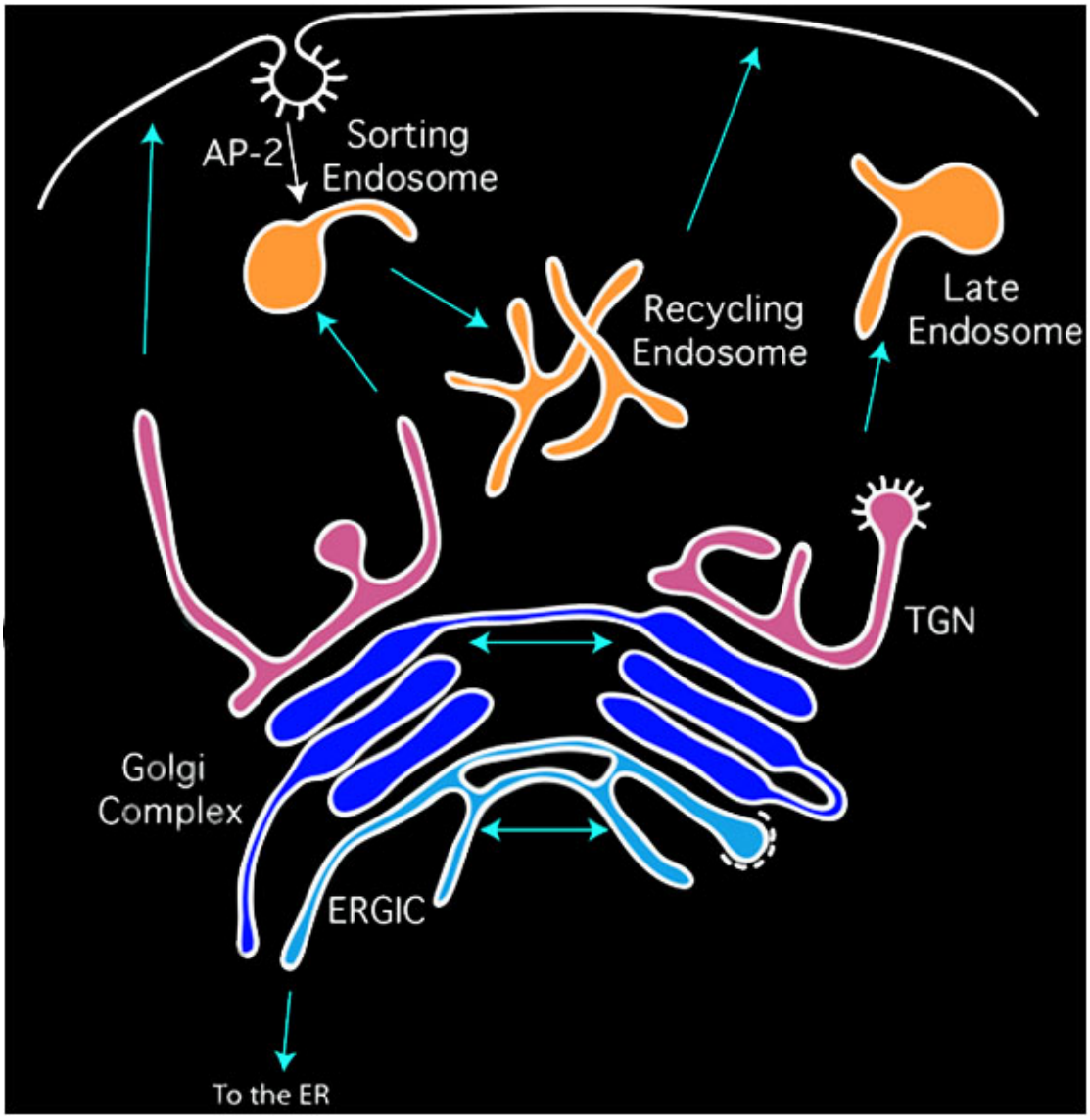


Figure 1.1. Illustration of membrane tubules participating in numerous trafficking routes.

membranes. We have a deep understanding on the involvement of proteins that insert into or bind to membranes to induce curvature [60], but we are only beginning to understand how lipid-remodeling enzymes function in membrane tubulation [31, 61-62].

1.5. Involvement of lipid-modifying enzymes in membrane tubulation

The importance of lipid changes within membranes is exemplified by the discovery that cytoplasmic phospholipases may play roles in inducing membrane tubulation to facilitate trafficking events, possibly by altering the physical structures of lipids in single leaflets of a membrane bilayer to induce membrane curvature, and thus membrane tubules [31]. For example, phospholipase A_2 (PLA_2) enzymes can alter phospholipid (PL) shapes by virtue of cleaving acyl chains from *sn*-2 positions of PLs to generate membrane tubules that facilitate membrane trafficking and structural homeostasis at the Golgi [34, 37]. Intra-Golgi membrane tubules may possibly permit bidirectional transport of both soluble and membrane-bound proteins [55]. Interestingly, modulation of PL shapes can also be catalyzed by lysophospholipid acyltransferases (LPATs) that carry out the reverse reaction of PLA_2s by transferring fatty acids onto lysophospholipids (LPLs), which subsequently contributes to trafficking and Golgi homeostasis [63-64]. Furthermore, PLAs and LPATs may provide both individual and concerted functions in extending, shrinking, and fission of membrane tubules to generate membrane vesicles.

The roles of lipid-modifying enzymes in membrane tubules are not limited within Golgi membranes as other studies showed evidence that phospholipases also induce membrane tubules among the ERGIC and endosomes [33, 39]. Although these phospholipases may bear a common

mechanism in generating membrane tubules, their distinct localizations may help generate membrane tubules of diverse origins to accommodate numerous membrane trafficking pathways.

1.6. Review of lipid-modifying enzymes in Golgi membrane dynamics

The mammalian Golgi complex is an iconic structure consisting of stacks of flattened membrane cisternae that are dynamically modified by the formation of coated vesicles and membrane tubules [58, 65-68]. In addition, the cisternal stacks are often bridged together by membrane tubules into large ribbon-like structures, often forming a single interconnected organelle [69-70]. The coated vesicles and membrane tubules mediate cargo import and export events, which by their combined activities help to shape the architecture and polarity of the Golgi complex, and contribute to bidirectional trafficking of cargo across the cisternal stacks. Although numerous structural, scaffold, fusion, and tethering proteins, e.g., COPI and AP-1 clathrin coats, GRASP, SNARE proteins, etc., have been shown to be crucial elements that regulate the functional organization of the Golgi complex, many fundamental questions about how the Golgi works remain unanswered, e.g., how secretory proteins move from the *cis* to the *trans* side of the Golgi stack [71]. Our lack of understanding is primarily due to the integrated and dynamic nature of the Golgi membranes, which makes it difficult to alter only a single aspect of Golgi trafficking without affecting every other. Recently, the dynamic functional organization of the mammalian Golgi complex has been reported to involve continual phospholipid remodeling by the opposing actions of phospholipase A (PLA) and lysophospholipid acyltransferase (LPAT) enzymes, a process in which acyl chains are continually removed and reincorporated (Figure 1.2) [62, 72]. In other words, dynamic

remodeling of Golgi membrane phospholipids is thought to play a role in shaping the structure of the Golgi complex itself. This review will focus on how these recently identified enzymes contribute to the dynamic nature of the mammalian Golgi complex.

In the 1960's Lands and colleagues established that after their synthesis, glycerol phospholipid acyl chains turnover in a cycle of deacylation and reacylation catalyzed by the opposing actions of PLA and LPAT enzymes, respectively, in what is now called the Lands cycle (Figure 1.2) [73-74]. The turnover of acyl chains serves to remodel phospholipids and thus alter their functional properties, although the full extent of the Lands cycle role in cells is unclear. Nevertheless, several functions of the Lands cycle have been established including the incorporation and release of arachidonic acid (AA) for the production of eicosanoids and prostaglandins [75], production of platelet activating factor (PAF) [76-78], and the incorporation of host-derived fatty acids into membrane phospholipids in *Giardia* and other intracellular parasites [79]. Here we describe recent studies which suggest that Lands cycle phospholipid remodeling might regulate the functional organization of the Golgi complex.

The mammalian Golgi complex, including the ER-Golgi-Intermediate Compartment (ERGIC) and *trans*-Golgi Network (TGN), produces types of membrane cargo transporters such as COPI and AP-1 clathrin-coated vesicles, regulated secretory granules, and constitutive secretory vesicles, whose function and biogenesis have been extensively studied (Figure 1.3) [23-24, 80-82]. For example, COPI coated vesicles mediate retrograde trafficking from the Golgi complex and ERGIC, whereas AP-1 coated vesicles mediate the sorting, packaging, and transport of newly synthesized lysosomal enzymes from the TGN to endosomes [80, 82-83]. Membrane tubules of uniform diameter (60-80 nm) have also been observed to emanate from different regions of the Golgi complex (Figure 1.3), but in contrast to coated vesicles, much less

is known about their functions, and indeed, even their existence or prevalence under normal conditions is a matter of debate [58, 71, 84]. For example, although the ERGIC and TGN exhibit extensive membrane tubulation, the cisternal membranes of the Golgi stack proper generally have few tubules. When viewed by 3-D tomographic reconstructions, membrane tubules are observed to link cisternal stacks into intact ribbons, but they are often not prominently seen in static images to extend out into the cytoplasm [58, 85-88]. Rather, Golgi membrane tubules are most readily observed when coated vesicle formation is inhibited or when GFP-tagged proteins are overexpressed for live cell imaging experiments [89-93], leading to concerns that they are only seen under unusual conditions. However, recent studies have revived the idea that COPI (and perhaps AP-1) coated vesicles are intimately linked to the formation of membrane tubules [54], and that membrane tubules can perform trafficking functions independent of coated vesicles, both of which utilize Golgi-associated PLA_2 and LPAT remodeling enzymes [62].

The relationship between Golgi complex coated vesicles and membrane tubules has puzzled cell biologists since the discovery of the remarkable properties of brefeldin A (BFA) [90, 93-96]. BFA is an inhibitor of guanine nucleotide exchange factors (GEFs) for certain ADP-ribosylation factor (ARF) GTPases [97-99], which prevents the recruitment of COPI coatomer and AP-1 subunits to Golgi and TGN membranes and the formation of COPI and AP-1 coated vesicles [97, 100-101]. As a consequence, Golgi and TGN membranes form extensive numbers of membrane tubules [90, 93, 95-96, 102]. Interestingly, BFA-induced membrane tubules move in the same directions as the coated vesicles that would normally form, i.e., retrograde back to the ER and anterograde from the TGN to endosomes. These studies suggested that BFA-induced membrane tubules are exaggerated forms of membrane protrusions that would normally be used to produce coated vesicles [94]. These original observations on the effects of

BFA were followed by a period when little progress was made on understanding the exact nature of Golgi membrane tubules. More recently, however, several studies have shown that RNAi knockdown of Golgi ARFs [50], some of their GEFs such as GBF1 [51], and AP-1-associated GGA1 (Golgi-localized, γ ear-containing, ADP ribosylation factor-binding) [103] also induced membrane tubule formation. These results are consistent with original ideas about the BFA effect, which are that membrane tubules reflect an inherent capacity of Golgi membranes to generate curvature, which if not used to make COPI or AP-1 coated vesicles, will continue to form abnormally long membrane tubules [94, 104]. Thus, the Golgi complex has an underlying COPI- and AP-1-independent mechanism capable of initiating membrane curvature, which recent studies report include PLA_2 enzymes that associate with the cytoplasmic side of Golgi membranes.

Although COPI vesicle formation has been extensively examined, there is no clear consensus on the exact molecular mechanisms [82, 105]. A variety of *in vitro* reconstitution studies have shown that COPI vesicles can be produced on artificial liposomes with purified coatomer subunits and ARF1-GTP [80, 105-107], indicating that these components represent the minimal machinery required for COPI vesicles biogenesis. In particular, ARF1-GTP, which is required for recruiting the coatomer subunits for vesicle budding, has also been reported to be sufficient to mediate the fission step for release of a free vesicle [107]. ARF1 could induce membrane fission by virtue of its energetically favorable interaction with positively curved membranes and its unfavorable interaction with the negatively curved bud neck, which makes resolution of the fission event energetically attractive [105]. In addition to this minimal system, a variety of other proteins have been reported to participate in COPI vesicle fission. For example, incubation with ARFGAP1, phospholipase D (PLD), and brefeldin A-ribosylated

substrate (BARS) have been shown stimulate the completion of membrane fission [108-112]. It is not clear how ARFGAP1 promotes budding or if ARFGAP1 is a stoichiometric component of the budded vesicle because it was recently reported that *in vitro* COPI vesiculation in the presence of GTP but lacking ARF GEFs is inhibited by enzymatic concentrations of ARFGAP1 [113]. The ARF1-GTP stimulated action of PLD has also been reported to contribute to vesicle fission by hydrolyzing phosphatidylcholine (PC) to phosphatidic acid (PA), which could aid in producing negative curvature and recruiting BARS at the bud neck [114-116]. Although BARS and PLD may not be required in the minimal machinery for COPI vesicle formation, they could enhance the efficiency of COPI budding *in vivo* (discussed further below).

1.7. PLA and LPAT enzymes remodel phospholipids and the Golgi complex

PLA enzymes cleave acyl chains from phospholipids to yield a lysophospholipid (LPL) and a free fatty acid (Figure 1.2). PLA enzymes are subdivided by their specificity to the *sn*-1 (PLA_1) and *sn*-2 (PLA_2) position of phospholipids; some cross-specificity has been observed [117]. Nine PLA_1 enzymes have been identified in mammals, six of which are extracellular proteins of the pancreatic lipase family; the remaining are intracellular lipases unrelated to pancreatic lipase [118]. The mammalian PLA_2 superfamily comprises 14 groups of enzymes (Group I, II, etc.), which are distinguished by their cellular locations, substrate specificities, and Ca^{2+} dependence [75]. The *sn*-2 position of phospholipids in mammalian cells can be enriched in AA, which plays critical roles in many signal transduction pathways ranging from inflammation to mitogenesis [119]. Indeed, the diverse PLA_2 superfamily plays important roles in myriad physiological processes such as reproduction, inflammation, heart disease, Alzheimer's disease, and cancer [120-122]. Although most current literature primarily cite PLA_2 enzymes for their roles in

inflammation, support for other functions is provided by recent studies showing that cytoplasmic PLA_2 enzymes regulate membrane trafficking.

The first indication that cytoplasmic PLA_2 enzymes play a role in vesicle and membrane tubule biogenesis came from studies of pharmacological PLA_2 inhibitors in cultured mammalian cells [62, 123-124]. PLA_2 inhibitors block intra-Golgi protein trafficking in cell-free reconstitution systems, thus implicating the requirement for PLA_2 activity at the Golgi complex [125]. More specifically, cytoplasmic PLA_2 enzymes appear to play critical roles in forming membrane tubules, as inhibitory PLA_2 drugs block BFA-induced membrane tubulation and trafficking in these organelles [124, 126]. Conversely, PLA_2 stimulating peptides melittin and a peptide from a mammalian protein containing a melittin homology domain, PLA_2 activating protein peptide (PLAAp), enhance cytosol-dependent Golgi membrane tubulation *in vitro* [127], possibly by embedding within the hydrocarbon region of membranes to facilitate PLA_2 activity. A potential link between the catalytic lipase activities of PLA_2 enzymes and membrane tubulation is supported by a hypothesis that the conversion of cylindrically-shaped phospholipids to conically-shaped products in local regions of a single leaflet of a lipid bilayer forces the membrane to adopt a curved structure, thus facilitating the formation of membrane tubules (Figure 1.2) [123]. Indeed, diverse lipid components alone can induce membrane curvature as observed in fluorescent, giant unilamellar vesicles [128].

In the context of the Lands cycle, LPATs oppose the action of PLA enzymes (Figure 1.2). LPATs catalyze the transfer of a fatty acid from an acyl-CoA donor to a LPL, most commonly at the *sn*-2 position, to yield a phospholipid. Mammalian LPATs comprise two gene families of transmembrane proteins, 1-acylglycerol-3-phosphate acyltransferase (AGPAT) and membrane

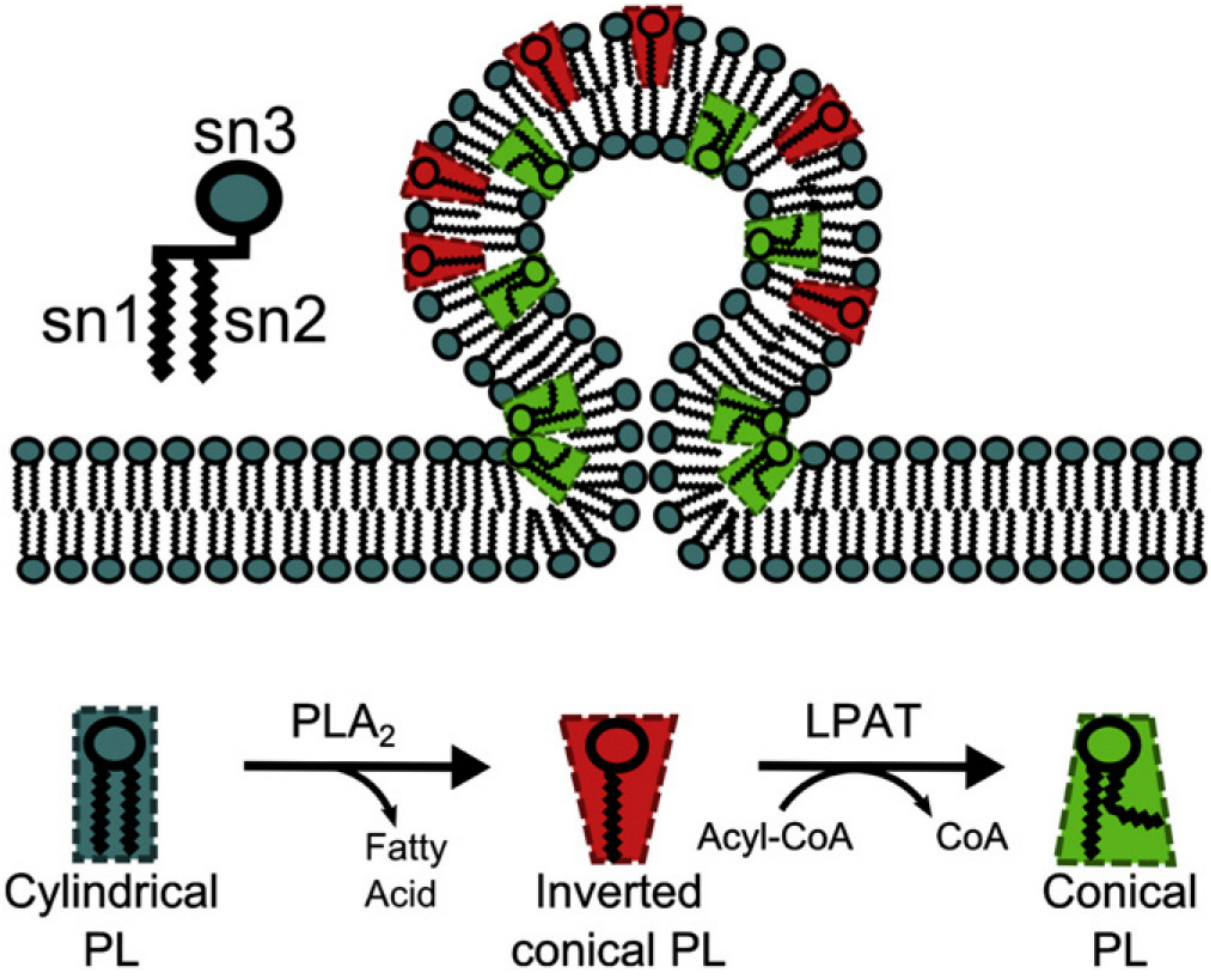


Figure 1.2. A simple model of Lands cycle phospholipid remodeling by PLA_2 and LPAT enzymes and how their phospholipid products can generate membrane curvature based on their physical shapes.

bound O-acyltransferases (MBOAT) [129-131]. The catalytic activities and substrate specificities of their gene products are not entirely characterized; however, 14 or more members are expected to have more than nominal LPAT activity. These enzymes are also categorized, and sometimes named, according to substrate preferences, e.g., enzymes that produce PA from lysophosphatidic acid (LPA) are referred to as LPAATs; enzymes that produce PC are referred to as LPCATs [132]. Most LPATs are ER or mitochondria localized; AGPAT3/LPAAT3 is exceptional and also localizes to the Golgi complex [63].

The individual activities of PLA and LPAT enzymes to remodel membrane lipids has been extensively studied in terms of lipid homeostasis, inflammation, and signal transduction. Emerging evidence, from multiple independent studies, support an additional role for lipid remodeling in membrane trafficking of the Golgi complex, as well as other organelles. The exact molecular mechanisms responsible for LPAT mediated trafficking are still unknown. One possibility is that the interconversion of phospholipids and LPLs confers negative and positive curvature respectively, directly to membranes. This curvature promotes the formation of tubular and/or vesicular membrane carriers (Figures 1.4 and 1.5) [59, 123, 128]. Another possibility is that compartment-specific PLA and LPAT activity produces a concentration gradient of lipid species that recruit effector proteins, such as COPI and clathrin coats (discussed further below). This recruitment model has many parallels to the extensively studied phosphoinositide phosphate (PIP) remodeling system [133]. It should be noted that lipid curvature and effector recruitment models are not mutually exclusive.

The localized synthesis, remodeling, and selective partitioning of phospholipids, as well as cholesterol, sphingolipids, and plasmalogens throughout the secretory system create tremendous potential for membrane heterogeneity and complexity [134]. The maintenance of a

gradient of lipid species between membrane compartments is crucial to and assisted by the selective partitioning of protein and lipid cargoes into anterograde- and retrograde-bound vesicles and membrane tubules [21]. Curved membranes tend to exclude sphingolipids and are enriched in phospholipids. For instance, *in vitro* reconstitution of COPI budding has shown that phase transition of phospholipid enriched domains is prevented at bud sites [106]. Phospholipid transfer proteins, flippases, PIPs, and, DAG also play important roles in the dynamic organization of the Golgi complex but are beyond the scope of this review [112, 133, 135-141].

Adding to this cast of phospholipid remodelers, the past two years have seen the identification of four specific cytoplasmic PLA and one integral membrane LPAT enzyme that play roles in the dynamic functional organization of the Golgi complex (Table 1.1). Although these enzymes can individually catalyze changes in membrane phospholipids, it has not yet been directly demonstrated that any pair of PLA and LPAT enzymes are functionally coupled in a Lands cycle of phospholipid remodeling. In any case, it is likely that these discoveries herald many more reports of PLA and LPAT enzyme involvement in Golgi membrane trafficking, and other compartments of the secretory and endocytic pathways.

1.7.1. PAFAHIb

One of the first PLA_2 members discovered to be directly involved in membrane tubulation and trafficking are the two members of Group VIII PLA_2s, also known as platelet activating factor acetylhydrolase (PAFAH) Ib 2 and 3. These two PLA_2 enzymes, called α1 (gene name PAFAHIb2) and α2 (gene name PAFAHIb3), are Ca^{2+}-independent and may form either homo- or hetero-dimers to form a catalytic pocket for the phospholipid substrate (Table) [142]. Both subunits within the dimer harbor a conserved GXSXG sequence and contribute

Class	Gene Name	Features
PLA Enzymes	PAFAHIb	Has two Ca^{2+}-independent PLA_2 subunits, potentially links membrane tubules to microtubules, affects Golgi structure and trafficking, also regulates endocytic recycling
	$cPLA_2\alpha$	Ca^{2+}-dependent PLA_2, maintains Golgi structure, provides intra-Golgi connections
	PLA2G6/$iPLA_2$-β	A cytoplasmic PLA_2 associated with the ERGIC and regulates it structure/function
	$iPLA_1\gamma$	A cytoplasmic PLA1 enzyme located to cis/medial Golgi cisternal and influences anterograde trafficking
LPATs	AGPAT3/LPAAT3	An integral membrane LPAT of both the ER and Golgi complex that regulates both Golgi structure and trafficking

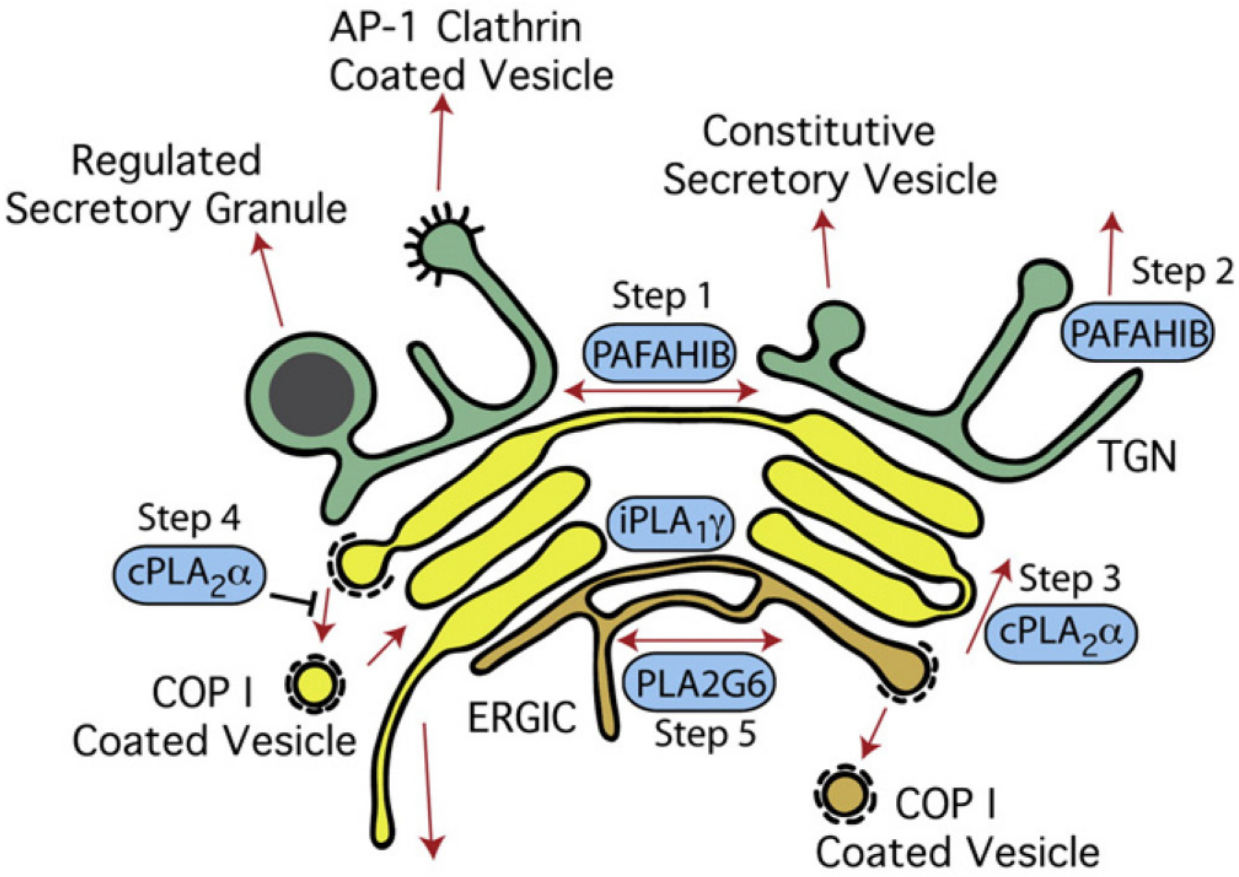
Table 1.1. Table of phospholipid remodeling enzymes at the Golgi complex

critical residues to the catalytic pocket, where the loss of the serine is sufficient to abolish the lipase activity [143]. The catalytic dimers also associate with a non-catalytic, regulatory β-subunit also known as Lis1 (gene name PAFAHIb1), which is the causative gene for a neurodegenerative disorder called Miller-Dieker lissencephaly [144]. Lis1 is better known for its role in regulating dynein-mediated, microtubule-dependent transport [145-146]. Lis1 appears to inhibit minus-end directed movement of dynein, and with the help of kinesin, the Lis1/dynein complex undergoes anterograde transport to the plus-end of microtubules, where the dynein-associated proteins, Nde1 or Ndel1, bind to the Lis1/dynein complex and mediate cargo transport back towards the minus ends of microtubules [147]. Nde1 and Ndel1 thus permit the compact accumulation of Lis1 in a juxtanuclear region where the minus ends of microtubules converge near centrosomes and indeed, mouse cells lacking Ndel1 show a cytoplasmic dispersed distribution of Lis1 [148]. Thus, Lis1 has dual, but not completely separate, roles in Golgi membrane dynamics via PAFAHIb-dependent phospholipid modification and dynein-mediated, microtubule-dependent transport.

Interestingly, the possible combinations of catalytic dimers between α1 and α2 exhibit different substrate specificities and are differentially regulated by Lis1 [149]. Differences between α1 and α2 are punctuated by the observation that in rats, α1 is only expressed in developing, neonatal neurons and α2 is conversely expressed in both neo- and post-natal stages and is not limited to neural tissues [150].

All three members of the PAFAHIb group contribute to the maintenance of Golgi architecture (Figure 1.3, steps 1 and 2) [34]. This was first shown when biochemical fractionation of bovine brain cytosol identified all three subunits as cofractionating with PLA_2 activity and Golgi membrane tubulation in an *in vitro* reconstitution assay. Importantly, purified

Figure 1.3. Membrane trafficking pathways from and within the mammalian Golgi complex. Several studies have recently reported that four cytoplasmic PLA enzymes are associated with the Golgi complex and regulate trafficking in various ways. As shown in Step 1, the cytoplasmic PLA_2 enzyme PAFAHIb induces membrane tubules from the Golgi complex that contribute to the formation of an intact ribbon structure. In addition, PAFAHIb also appears to influence anterograde trafficking fromthe TGN (Step 2). A second cytoplasmic enzyme, $cPLA_2\alpha$,mediates the formation of intra-cisternal membrane tubules that appear to facilitate anterograde transportwhen the Golgi complex receives a large bolus of secretory cargo (Step 3). In addition, $cPLA_2$ may also negatively regulate COPI coated vesicle formation (Step 4), perhaps by shifting the balance toward membrane tubule formation. A third cytoplasmic PLA_2, PLA2G6, was shown to mediate the formation of membrane tubules from the ERGIC, which may be important for connecting regions involved in COPI vesicle budding (Step 5). The final PLA enzyme recently found associated with ERGIC and Golgi membranes is $iPLA_1\gamma$, which appears to contribute to anterograde transport through the Golgi complex by an unknown mechanism.


AP-1 Clathrin
Coated Vesicle
Regulated
Secretory Granule
Constitutive
Secretory Vesicle
Step 1
PAFAHIB
Step 2
PAFAHIB
TGN
Step 4
cPLA2α
iPLA1γ
Step 3
cPLA2α
COP I
Coated Vesicle
ERGIC
PLA2G6
Step 5
COP I
Coated Vesicle

catalytically active, but not inactive α1 and α2 can induce Golgi membrane tubulation *in vitro*, and both α1 and α2 partially localize to the Golgi cisternae and the TGN *in vivo*. PAFAHIb α1 and α2 recruitment to Golgi membranes may be PIP dependent, as α1 appears to have a strong affinity for PI(4)P in protein-lipid overlay assays [151], and PI(4)P is known to be enriched within Golgi membranes [133]. Knockdown and over-expression studies demonstrated that both Lis1 binding and the PLA_2 activities of α1 and α2 are important for regulating Golgi structure and function. For example, knockdown of α1 and α2 caused the Golgi ribbon to fragment into separated mini-stacks, which could result from both loss of PLA_2 activity that is required to generate membrane tubules and Lis1-regulated, dynein-mediated movement of Golgi mini-stacks to the cell center [152-153]. This idea is supported by the finding that Golgi mini-stacks produced following Lis1 knockdown are still able to generate membrane tubules, whereas mini-stacks in α1/α2 knockdown cells cannot [34]. Likewise, the loss of either Nde1 or Ndel1 also leads to the dispersal of the Golgi complex [154]. Although it is tempting to hypothesize that a compact, juxtanuclear Golgi structure can be achieved by α1/α2 establishing a link between Golgi membrane tubules and Lis1/dynein/Nde1/Ndel1, other reports demonstrate that α1/α2 directly compete with Ndel1 and dynein for Lis1 binding (Figure 1.6) [155]. Indeed, Lis1 undergoes stark, conformational changes when it switches from a complex with a α2/α2 homodimer to one with Ndel1 [156].

In addition to regulating membrane tubulation from the Golgi complex, PAFAHIb has been shown to have similar activity on early endosomes and the endocytic recycling compartment [151]. Moreover, PAFAHIb contributes to efficient endocytic recycling of transferrin and transferrin receptors. Thus, PAFAHIb functions at multiple intracellular compartments [62].

The discovery that PAFAHIb regulates Golgi structure and function is somewhat surprising given that it has a very strong substrate preference for PAF, which has an acetyl group in the *sn*-2 position and is best known as a potent extracellular mediator of platelet activation and inflammation [157-158]. How then could PAFAHIb be involved in Golgi dynamics? First, PAFAHIb is a cytoplasmic enzyme, which due to its location is unavailable to hydrolyze extracellular PAF, and, moreover, mice knockout studies show that it is not the key enzyme responsible for turnover of extracellular PAF, a function most likely carried out by an unrelated, secreted PAFAH [157-160]. Second, PAFs possibly have other roles in cells because they are ubiquitously found in intracellular membranes, including those of *Saccharomyces cerevisiae* [161]. The relevant question for Golgi structure/function is how can PAFAHIb change membrane shape since PAF is structurally similar to LPLs? One possibility is that *in vivo* PAFAHIb hydrolyzes unidentified substrates. Another possibility is that PAF is nevertheless sufficiently different from LPLs to help generate outward membrane curvature when hydrolyzed on the cytoplasmic leaflet of Golgi membranes. Reconstitution experiments using defined phospholipid compositions will be required to definitively address these issues.

1.7.2. cPLA$_2$α

Another PLA$_2$ member, a cytoplasmic Group IV PLA$_2$ named cPLA$_2$α (gene name PLA2G4A) also exerts effects on membrane dynamics at the Golgi complex (Table). cPLA$_2$α is one of the most studied PLA$_2$ members for its roles in the generation of AA and its subsequent signaling pathways [162]. cPLA$_2$α possess a C2 domain that binds to intracellular Ca^{2+}, leading to its translocation from the cytosol to both ER and Golgi membranes [163-164], where it regulates both Golgi structure and membrane trafficking.

Similar to the membrane tubulating activities of PAFAHIb, San Pietro et al. found that cPLA$_2$α is capable of forming membrane tubules between Golgi cisternae [165], which provide direct continuities between *cis*, *medial*, and *trans* Golgi cisternae for anterograde intra-Golgi protein and membrane transport (Figure 1.3, Step 3) [20, 87-88]. These intra-cisternal membrane tubules are induced during times of rapidly increased secretory load [166], but it is unclear how stable they are and if they function under other conditions. Indeed, the same study goes on to demonstrate that cells from cPLA$_2$α knockout mice are still capable of transporting VSV from the ER through the Golgi complex to the plasma membrane but the protein becomes sequestered within the Golgi when PAFAHIb α1 is knocked down. This clearly establishes redundancy of multiple PLA$_2$ members in membrane trafficking. However, it remains possible that cPLA$_2$α may serve to provide distinct trafficking and sorting services than that of PAFAHIb, and this is demonstrated through experiments showing that cPLA$_2$α is specifically involved in transport of transmembrane junction proteins such as VE-cadherin, occludin, and claudin-5 from the Golgi complex to cell-cell contacts [167]. Also, in Purkinje neurons, cPLA$_2$α relies on Ca^{2+} influxes to translocate from the cytosol to the Golgi in order to regulate AMPA receptor levels at the plasma membrane [168]. Finally, other recent studies have provided evidence that cPLA$_2$, in essence, negatively regulates COPI coated vesicle formation (Figure 1.3, Step 4), likely by shifting the balance toward membrane tubule formation (discussed further below) [54].

Overexpression of cPLA$_2$α in kidney epithelial cells leads to fragmentation of the Golgi ribbon and prevents anterograde transport as protein cargo sequesters within the ER, while leaving the cytoskeletal and microtubule networks intact [169]. It is possible that membrane tubules formed by cPLA$_2$α may serve as precursors to membrane vesicles and this claim is buttressed by observations that cholesterol-induced vesiculation of the Golgi membranes requires

cPLA$_2$α recruitment to the Golgi and that this is inhibited by the cPLA$_2$ inhibitor, methyl arachidonyl fluorophosphonate (MAFP) [170]. Interestingly, this same report goes on to show that this vesiculation is independent of clathrin coat complexes and requires dynamin 1 and 2, thus supporting the view that vesicles can possibly be pinched off of nascent membrane tubules. The activation of cPLA$_2$α and its targeting to membranes have been extensively examined. cPLA$_2$α can be activated through three distinct phosphorylation sites by PKC, MAPK family member ERK1/2, and CaM kinase, but the phosphorylation of cPLA$_2$α does not always lead to AA release [171-173]. This observation clearly implicates cPLA$_2$α to be involved in other processes that do not involve liberating AA and may also explain a possible route in uncoupling the functions of releasing AA from Golgi phospholipids and of regulating Golgi structure. The relationship between phosphorylation and Ca^{2+}-binding in regulating cPLA$_2$α translocation to membranes remains unclear. However, one report notes that AA can possibly mimic Ca^{2+} ionophores in stimulating cPLA$_2$α translocation to ER but not Golgi membranes in a Ca^{2+}-independent manner [174]. The targeting of cPLA$_2$α to Golgi membranes may be similar to PAFAHIb in that PIPs play a facilitative role. Indeed, reports show that PI(4,5)P$_2$ enhances both cPLA$_2$α activity and membrane-binding affinity *in vitro* through interactions with cationic residues within the membrane-binding surface of the protein [175-176]. However, another study raises conflicting results by showing that PI(4,5)P$_2$ only enhances cPLA$_2$α activity and not its Ca^{2+}-dependent membrane binding affinity [177]. Incidentally, Golgi membranes contain PI(4,5)P$_2$ in addition to PI(4)P [178].

Both membrane tubulation and vesiculation may be facilitated through enhanced membrane fluidity, and PLA$_2$ activity may be involved in increasing membrane fluidity, as the addition of palmitoyl trifluoromethylketone (PACOCF3), a PLA$_2$ inhibitor, reduces membrane

fluidity in rat hippocampal tissue [179-180]. Enhanced membrane fluidity may facilitate lateral segregation of conically shaped LPLs generated by PLA_2 enzymes into regions of high curvature such as membrane tubules [181]. Membrane fluidity also facilitates the formation of lipid rafts as exemplified by the observation that PLA_2 activity is specifically responsible for the budding and fission of liquid ordered (L_o) phase rafts from liquid disordered (L_d) giant unilamellar vesicles (GUVs) [182]. This is potentially explained by the hypothesis that PLA_2 enzymes produce conically-shaped LPLs within L_o rafts, which in turn lead to the budding of rafts into membrane vesicles and/or tubules and is overall energetically favorable because the protrusion of L_o rafts into vesicles/tubules away from GUVs decrease the free energy that is confined within L_o/L_d phase boundary [182]. In another twist, lipid phases can additionally lead to inhibition of $cPLA_2\alpha$. One study shows that although sphingomyelin (SPH) cannot be hydrolyzed by $cPLA_2\alpha$, L_o phases enriched in SPH can bind to and sequester $cPLA_2\alpha$ from L_d phases that contain hydrolysable glycero-phospholipid substrates [183]. The same study showed that such inhibition is specifically due to sequestering $cPLA_2\alpha$ within phases apart from substrate phases because the addition of cholesterol or ceramide leads to the abolishment of separated phases and the reestablishment of $cPLA_2\alpha$ activity.

1.7.3. PLA2G6/iPLA$_2$-β

A Group VIA-2 PLA_2, PLA2G6/iPLA$_2$-β (also known as iPLA2B, PNPLA9), is a third PLA_2 member that regulates Golgi trafficking indirectly by regulating the ERGIC (Table) [33]. The ERGIC is an independent compartment located between the ER and the *cis*-face of the Golgi complex, facilitates bidirectional trafficking between the two organelles, and may serve as a precursor to the *cis* aspect of the Golgi stack [184-185]. The ERGIC is comprised of

tubulovesicular membrane structures and potentially serves roles in cargo protein quality, sorting, and concentration [184]. PLA2G6/iPLA$_2$-β has multiple splice variants, of which the full length variant, called L-iPLA$_2$, is catalytically active while its shorter splice variants, ankyrin-iPLA$_2$-1 and -2 are inactive but thought to negatively regulate L-iPLA$_2$ via forming oligomers through their ankyrin repeats [186-187]. PLA$_2$ involvement at the ERGIC is first implicated through the use of PLA$_2$ drug inhibitors that prevent the recycling of ERGIC-localized protein ERGIC-53 in addition to the formation of ERGIC membrane tubules [188]. Recent work shows that ARF1 and ARF4 knockdown can generate ERGIC membrane tubules that are dependent on PLA2G6/iPLA$_2$-β as knockdown of this protein also leads to inhibition of membrane tubule formation [33]. The same study noted that PLA2G6/iPLA$_2$-β-induced membrane tubules may be viable alternatives to vesicles in quickly and efficiently directing cargo between ERGIC clusters and cargo containing COPI vesicles (Figure 1.3, Step 5). ERGIC tubulation and mobility are also regulated by microtubules and their associated motor proteins and whether these tubule-inducing functions are independent or shared with PLA2G6/iPLA$_2$-β remains unknown [189].

Aside from basic cell biological interest in understanding how phospholipid modifying enzymes and membrane tubules influence intracellular membrane trafficking, recent studies have revealed that the function of these enzymes and membrane structures might be linked to pathological conditions. For example, mutations in human PLA2G6A gene (and in mouse models) cause a spectrum of autosomal recessive neurodegenerative disorders, characterized by severe cognitive and motor regression, now referred to as PLA2G6-Associated Neurodegeneration (PLAN) [190-193]. The connection between PLA2G6's function at the ERGIC and disease manifestations in PLAN is not understood but could involve specific defects in secretory trafficking.

1.7.4. iPLA$_1\gamma$

The final cytoplasmic PLA protein that has effects on the Golgi complex is a PLA$_1$ family member, iPLA$_1\gamma$ (gene name DDH2 or KIAA0725p) (Table) [194-195]. The other two cytoplasmic PLA$_1$ family members include iPLA$_1\alpha$ (PA-PLA$_1$) and iPLA$_1\beta$ (p125) [196]. While iPLA$_1\gamma$ shares a common characteristic with iPLA$_1\beta$ in that the overexpression of either results in structural disruption of both the ERGIC and the Golgi, only the overexpression of iPLA$_1\gamma$ lead to mislocalization of Golgi resident proteins such as p115 and GM130 [197]. One of the first iPLA$_1\gamma$ studies showed that the protein is localized to both Golgi and ERGIC membranes (Figure 1.3) [194]. The same study indicated that siRNA knockdown of iPLA$_1\gamma$ disrupted COPI- and Rab6-independent, i.e., BFA stimulated, Golgi-to-ER retrograde trafficking, whereas VSVG-ts045-GFP anterograde transport was unaffected. However, a more recent study conflicts with these results, finding that iPLA$_1\gamma$ knockdown did not affect Golgi-to-ER retrograde trafficking but did inhibit VSVG-ts045-GFP anterograde transport [195]. These discrepancies can be explained by the observation that the original knockdown studies had an unintended off-target knockdown of Rab6 [195], which regulates Golgi retrograde trafficking [198]. In mammals, the physiological roles of the PLA$_1$ family are unclear but it is known that iPLA$_1\alpha$ is highly expressed in brain and testis and thus possibly linked to neural development and spermatogenesis, similar to PAFAHIb [159-160, 199-200]. In addition, the molecular mechanisms by which iPLA$_1\gamma$ influences trafficking through the Golgi complex are unknown.

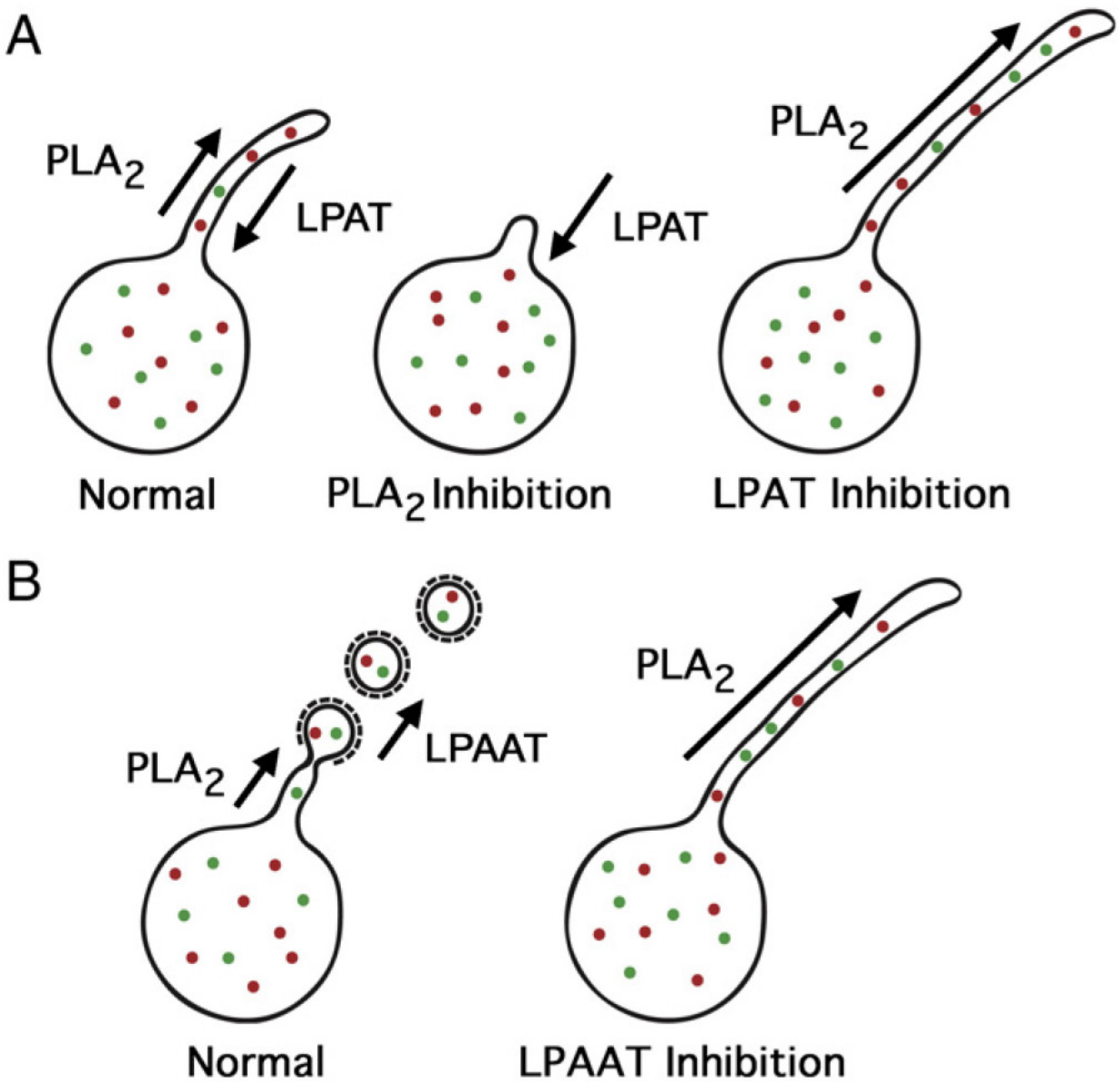
1.7.5. Lysophospholipid acyltransferases

The fruitful studies of PLA_2 enzymes in Golgi membrane trafficking stimulated an interest in LPAT activity as a potential mediator of Golgi membrane trafficking, perhaps by counteracting the tubulating activity of PLA_2 enzymes (Figure 1.4). Given that in model membrane systems PLA_2 and LPAT lipid products stabilize positive and negative curvature, respectively, and that PLA_2 activity stimulates membrane tubulation in cells, it was hypothesized that LPAT activity might prevent tubule formation in cells [123]. It had also been hypothesized that the negative curvature at the neck of a budding vesicle could be stabilized by LPAT lipid products [201]. Finally, it was suggested that cytoplasmic PLA_2 and LPAT enzymes could work in concert: cytoplasmic PLA_2 enzymes could promote outward curvature or budding that would subsequently be acted on LPATs to produce negative curvature to facilitate coated vesicle production [123].

The first two reports of LPAT activity being involved in membrane trafficking were from studies of BARS and endophilin [202-204]. BARS is a mediator of Golgi membrane tubule fission [110], and endophilin is an N-BAR domain containing protein that was recently shown to be required for uncoating of coated vesicles at the plasma membrane but not fission per se [205]. Both of these proteins were reported to have low levels of acyltransferase activity; however, this activity was later determined to be associated with LPAAT enzyme contaminants [206].

The first solid indication that LPATs were involved in membrane trafficking came from studies that utilized a powerful new tool, the small molecule CI-976. CI-976 (2,2-methyl-N-(2,4,6,-trimethoxyphenyl)dodecanamide) was originally synthesized as a fatty acid anilide derivative designed to mimic fatty acyl-CoA for inhibition of acyl-CoA:cholesterol acyltransferase (ACAT) [207-208]. It is a small hydrophobic, membrane permeant, and

Figure 1.4. Possible routes for the individual and concerted actions of PLA_2 and LPAT enzymes to influence the formation of membrane tubules and coated vesicles. (A) Membrane tubules could be generated and regulated by the opposing actions of PLA_2 and LPAT enzymes. Hydrolysis of membrane phospholipids by cytoplasmic PLA_2 enzymes could generate positive curvature inducing LPLs, ultimately resulting in membrane tubule formation. The LPLs could be reacylated by LPAT enzymes back to phospholipids, thus negatively regulating the formation of membrane tubules. **(B)** The hydrolytic activity of cytoplasmic PLA_2 enzymes could generate membrane tubules that are consumed through coated vesicle budding, which is facilitated by LPAAT activity for membrane fission. In this generic model, the coated vesicles could be either COPI vesicles budding from the ERGIC or AP-1 clathrin-coated vesicles budding from the TGN. The consequences of inhibition by PLA_2 and LPAT antagonists are shown, which have been supported by several studies [31, 62].


A
PLA2
LPAT
PLA2
LPAT
PLA2
Normal
PLA2 Inhibition
LPAT Inhibition
B
PLA2
LPAAT
PLA2
Normal
LPAAT Inhibition

competitive ACAT inhibitor [209]. While CI-976 possesses only weak inhibitor activity against ACAT, a screen for inhibition of Golgi associated LPAT activity showed strong inhibition of LPC and lysophosphatidylethanolamine (LPE) acyltransferase activities [210-213]. With this potent inhibitor, the study of LPAT enzymes in mammalian membrane trafficking immediately progressed. Most intriguingly, treatment of mammalian cells with CI-976 stimulated a remarkable enhancement of Golgi membrane tubulation and retrograde trafficking [211-212]. This tubulation activity was phenotypically similar to BFA induced tubulation, although comparatively delayed. Additionally, CI-976-induced Golgi tubulation was inhibited by concomitant PLA_2 inhibition, indicating for the first time that both PLA_2 and LPATs work in concert to regulate the dynamic structure of the Golgi complex [212]

Golgi-associated LPAT enzyme(s) could regulate membrane trafficking by one of, or a combination of, five mechanisms (Figures 1.4 and 1.5): 1) production of conical phospholipids that resist the high positive curvature present in tubules; 2) conversion of inverted-cone shaped LPLs that stabilize positive curvature to phospholipids that are less stabilizing; 3) production of conical phospholipids that stabilize the high negative curvature at the neck of budding vesicles; 4) production of phospholipids that recruit vesicle-budding factors; and, 5) production of phospholipids that affect lipid and protein partitioning. There is evidence for all of these mechanisms and deciphering the relative contribution of each is a major challenge in the field.

Further studies identified a PC- and PE-forming LPAT tightly associated with the Golgi complex [210]. The connection between this enzyme and CI-976 stimulated tubulation of the Golgi complex and Golgi-to-ER retrograde trafficking is unclear [212], and the exact molecular identity of the LPC/LPEAT activity is still unknown. In fact, recent studies strongly indicate that the observed CI-976 effect on the Golgi could be due to inhibition of LPAAT mediated acylation

of LPA to PA, which is catalyzed by AGPAT3/LPAAT3 (also called LPAATγ), a Golgi localized LPA specific acyltransferase (Table) [63].

AGPAT3/LPAAT3 was the first specific LPAT shown to be involved in the dynamic function of the Golgi complex. It is an integral membrane protein with two transmembrane domains and is found in both the Golgi complex and ER [214]. Overexpression of AGPAT3/LPAAT3 was shown to slow ERGIC-53 recycling from the cis-Golgi to the ER/ERGIC and to reduce BFA-stimulated membrane tubulation. Conversely, AGPAT3/LPAAT3 knockdown accelerated ERGIC-53 recycling. Importantly, overexpression of AGPAT3/LPAAT3 counteracted the tubule-inducing effects CI-976, suggesting that AGPAT3/LPAAT3 is a direct target of the drug. However, this result does not rule out the possibility that the CI-976-inhibitable Golgi LPC/LPEAT activity also contributes to Golgi membrane remodeling. For example, a Golgi LPC/LPEAT enzyme could come into play because AGPAT3/LPAAT3 generated PA can be hydrolyzed to DAG by PA phosphatase and then converted to PC or PE, followed by Lands Cycle remodeling via $cPLA_2\alpha$ (Figure 1.7). In addition, PC and PE can be generated via Golgi localized cholinephosphotransferase (CPT1) or a phosphoethanolamine transferase, respectively [215].

Like PLA_2 enzymes, LPATs are also involved in other secretory and endocytic trafficking steps. For example, CI-976 reversibly inhibited a very late step in COPII vesicle budding, resulting in the accumulation of secretory cargo at ER exit sites (ERESs) [64]. Interestingly, ERESs underwent Sar1p-dependent membrane tubulation in the presence of CI-976, suggesting the involvement of LPAT activity in the fission of COPII budding elements *in vivo*. In addition to secretory trafficking, CI-976 was also found to stimulate membrane tubules from endosomes and to inhibit recycling of transferrin and transferrin receptors from the

endocytic recycling compartment, suggesting a role for LPATs in the budding of vesicles from endosome tubules [211]. Currently, the precise LPATs involved in COPII budding or endocytic recycling have yet to be identified.

1.8. Integration of cPLA$_2$α and AGPAT3/LPAAT3 to Influence Golgi COPI Vesicle and Membrane Tubule Formation

The above studies on Golgi-associated PLA$_2$ and LPAT enzymes suggest an intimate association between the formation of membrane tubules and coated vesicles. Support for this idea came from previous studies showing that CI-976 inhibited COPI vesicle formation [110]. This association was recently directly tested by Hsu and colleagues who discovered that cPLA$_2$α and AGPAT3/LPAAT3 function to regulate the relative abundance of COPI vesicle and membrane tubule formation (Figure 1.5) [54]. Using purified Golgi complex membranes in an *in vitro* COPI budding assay, they first found that COPI coatomer subunits and ARF1 are required for both vesicle and membrane tubule formation. They surmised that ARF1 and coatomer initiates the budding events that become vesicles and membrane tubules. When examining endogenous cPLA$_2$α and AGPAT/LPAAT3 by immunofluorescence microscopy, they were shown to colocalize with endogenous coatomer proteins. The coatomer subunit a-COP was shown by immuno-EM to localize to the base and tip, but not the stem, of membrane tubules. In this assay, AGPAT3/LPAAT3 activity increased COPI vesicle formation, which was shown by the addition of CI-976 or AGPAT3/LPAAT3 antibody to budding reactions. The authors suspect that AGPAT3/LPAAT3-generated PA, a known recruiter of COPI coatomer subunits and BARS, a COPI vesicle fission protein [110], as well as a negative curve-inducing phospholipid [59,

216], are responsible for stimulating COPI coating of the nascent vesicle. In support of this idea, inhibition of AGPAT3/LPAAT3, by CI-976 or specific antibody, stimulated membrane tubulation at the expense of vesicle production. The final step of COPI vesicle fission required the conversion of PC to PA by PLD2, but not PLD1.

The addition of pharmacological PLA_2 inhibitors or anti-$cPLA_2\alpha$ antibody to cytosol in their reconstitution assay increased vesicle formation and concomitantly decreased tubule formation, which was reversed by the addition of purified $cPLA_2\alpha$. Their model posits that ARF1 and COPI coatomer recruitment initiates bud formation and that buds then mature into either vesicles or membrane tubules, depending on the balance of $cPLA_2\alpha$ and AGPAT3/LPAAT3 activity (Figure 1.3: Step 5, Figure 1.5B-E). It is still a matter of debate as to whether generation of curve-inducing PA or recruitment of effector proteins, is most important in AGPAT3/LPAAT3-mediated vesicle biogenesis. They further suggest that AGPAT3/LPAAT3 generated PA initiates negative membrane curvature but is insufficient for complete vesicle budding, a process that is finalized by the additional generation of PA by PLD2 (Figure 1.5F). The generation of PA by both enzymes could aid in the recruitment of the fission protein BARS [110]. Hsu and colleagues also assert that the biogenesis of Golgi membrane tubules, both intercisternal and intracisternal, which facilitate anterograde trafficking and Golgi ribbon formation respectively, are initiated as COPI buds (Figure 1.5D). These results provide important insights into how Lands cycle phospholipid remodeling might influence the dynamic behavior of Golgi membranes. Interestingly, however, this model of membrane curvature initiation solely by COPI/ARFs cannot explain enhanced Golgi membrane tubule formation by BFA or knockdown of ARF GEFs, and underscores the notion that cytoplasmic PLA_2 enzymes can induce membrane tubules on their own (Figure 1.5G). In addition, because COPI vesicle

Figure 1.5. Models for cPLA$_2$α and AGPAT3/LPAAT3 activities contributing to COPI vesicle and Golgi membrane tubule formation. **(A)** Flat membrane that will be modified by curve producing proteins; **(B)** Arf1 and coatomer binding deforms membranes, initiating the first steps of vesiculation or tubulation**; (B to C)** AGPAT3/LPAAT3 activity produces unsaturated PA, a lipid that resists positive curvature**; (B to D)** cPLA$_2$α activity produces LPC, a positive curvature stabilizing and tubule inducing lipid; **(C to E)** LPC stabilizes positive curvature of the bud and unsaturated PA stabilizes negative curvature at the bud neck; **(F)** The concerted PA production activities of AGPAT3/LPAAT3 and PLD2 at the bud neck help to recruit BARS and aide vesicle fission; **(G)** PLA$_2$ activity can induce membrane curvature and tubulation in the absence of coatomer.

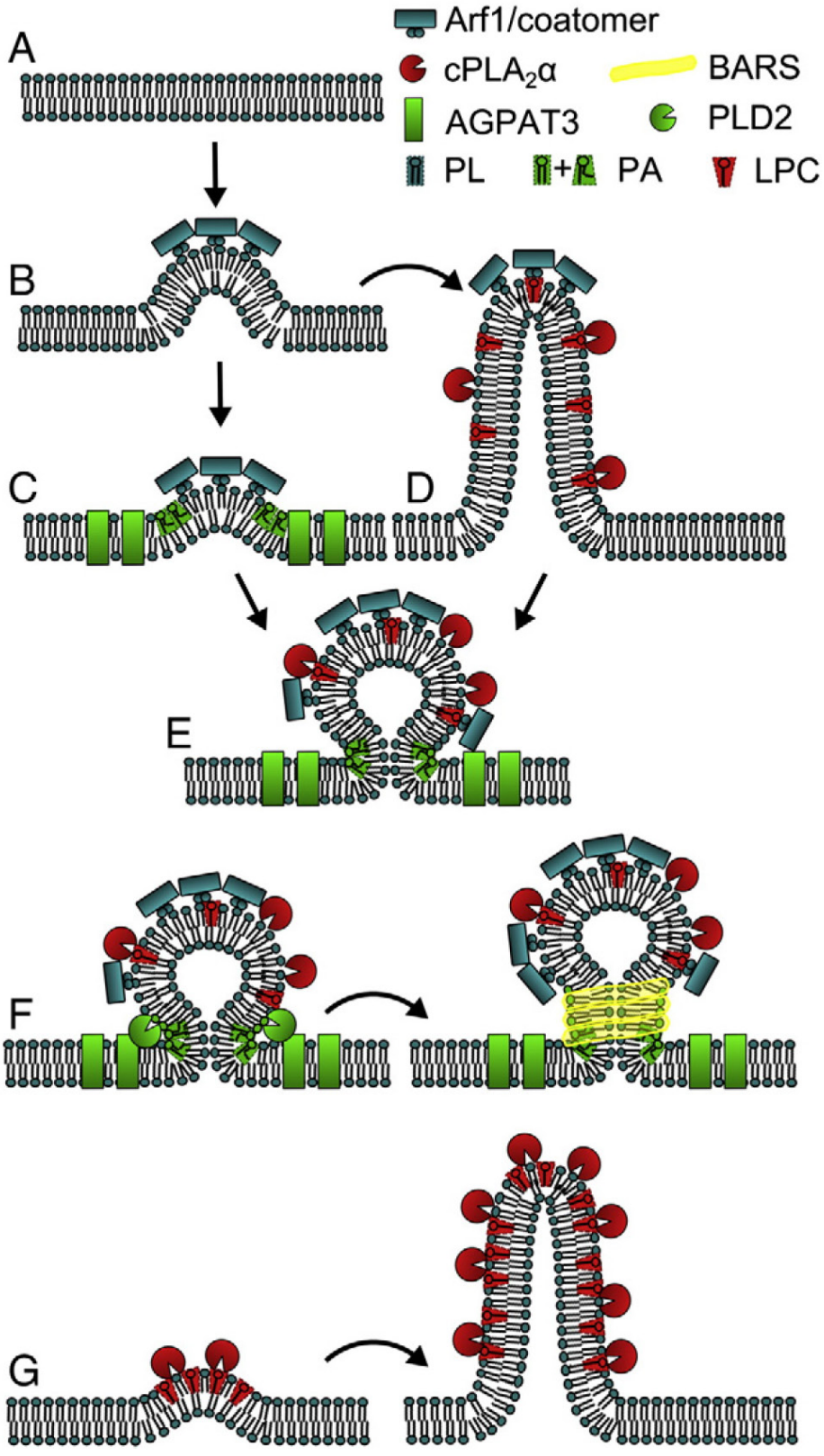
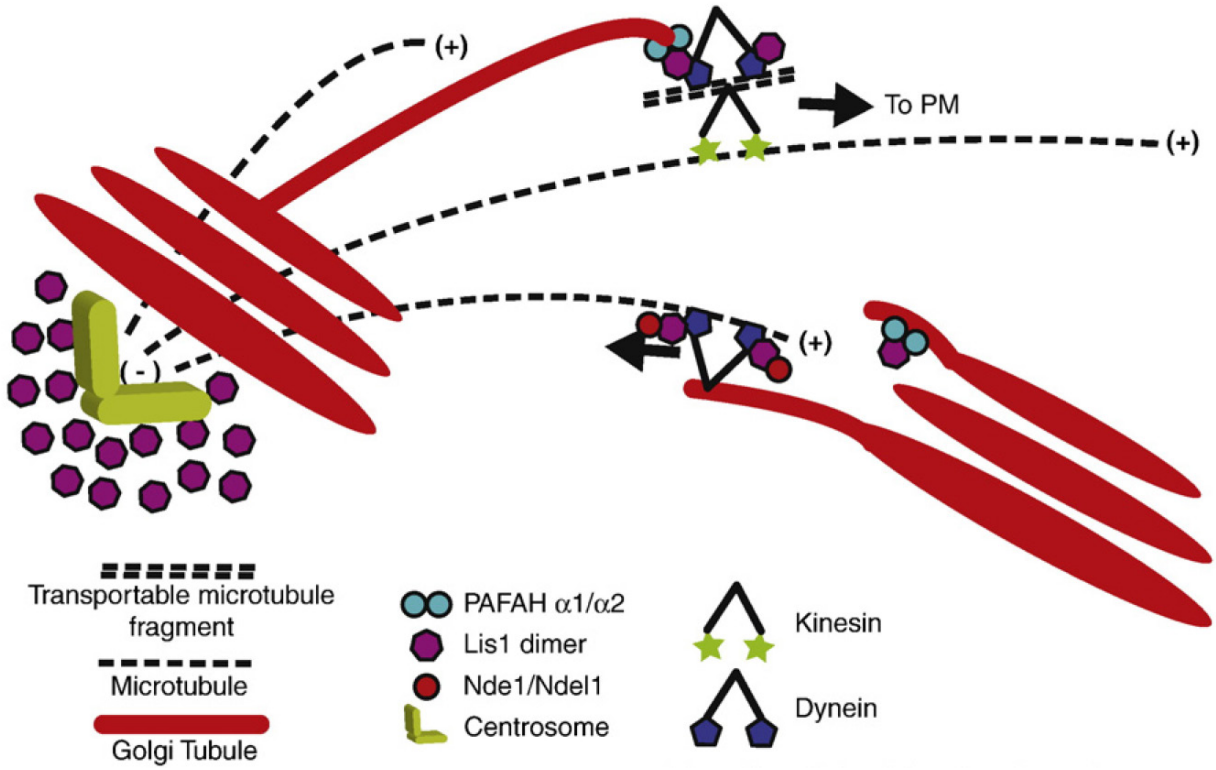

Arf1/coatomer
cPLA2α
BARS
AGPAT3
PLD2
PL
+
PA
LPC
A
B
C
D
E
F
G

Figure 1.6. Model integrating membrane curvature produced by PLA_2 activity and transport along microtubules. Cytoplasmic PLA_2 activity, perhaps that of PAFAHIb, aids in the formation of membrane tubules from the TGN in conjunction with kinesin and microtubules for transport to the plasma membrane (PM) (top membrane tubule). In addition, PAFAHIb α1 and α2 initiate outward membrane curvature to generate a membrane tubule (middle short membrane tubule), which can subsequently be pulled/extended along microtubules (MT) by Lis1 and Ndel interactions with dynein (bottom longer membrane tubule). These concerted activities may be able to carry the membrane tubule towards the minus end of microtubules, thus facilitating the coalescence of Golgi stacks near the centrosome. Adapted from Yamada et al., 2008.


(+)
To PM
(+)
(+)
(-)
Transportable microtubule fragment
Microtubule
Golgi Tubule
PAFAH α1/α2
Lis1 dimer
Nde1/Ndel1
Centrosome
Kinesin
Dynein

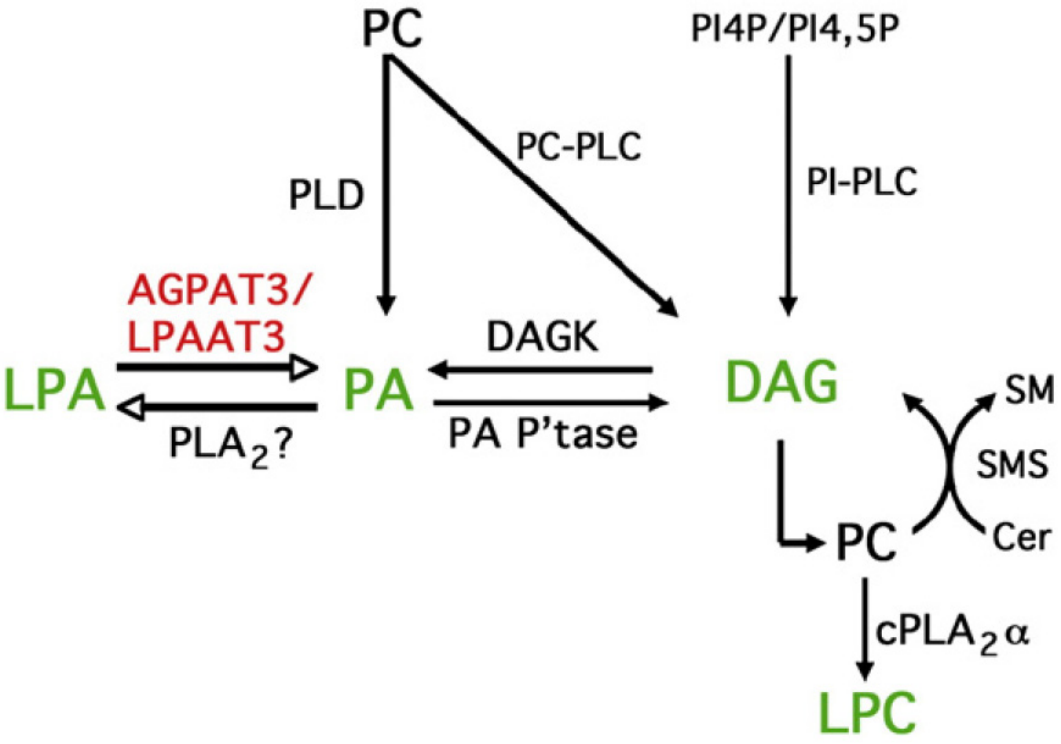
budding and fission can be reconstituted using minimal components coatomer and ARF-GTP [107], these phospholipid modifying enzymes likely function to enhance the efficiency of the COPI budding machinery.

The central roles of DAG and PA in Golgi membrane tubule and vesicle formation have been extensively examined, and their participation raises important points for the analysis of how lipid/phospholipid changes affect membrane trafficking. Both DAG and PA have been shown to be required for membrane tubule and vesicle formation from the Golgi and TGN [116, 217-220], likely by generating both curvature-altering phospholipids and platforms for effector protein binding. How the Golgi complex controls the numerous pathways that lead to and from PA and DAG is still a mystery (Figure 1.7). In addition, understanding how lipids/phospholipids can exert dramatic local effects in the face of rapid lateral diffusion and conversion to other products is a challenge. Nevertheless, it is becoming increasingly clear that remodeling of Golgi membrane phospholipids by PLA_2 and LPAT enzymes adds a new layer of complexity to an already complex organelle. Moreover, it is likely that the full complement of PLA and LPAT enzymes involved in secretory (and endocytic) trafficking has not yet been revealed.

1.9. Conclusions and future directions

Although the past decade of research revealed detailed information on the many facets of membrane trafficking, especially in coated vesicles, there is little information on how physiological stimuli such as growth factors, stress, and increased secretory loads can lead to signaling that subsequently affects membrane tubulation. Although membrane tubules and vesicles may work in a concerted manner, more studies are needed to delineate the importance of

Fig. 1.7. Enzymatic pathways, several involved in Lands cycle reactions, which lead to the modification and interconversion of lipid species. Lysophosphatidic acid (LPA); phosphatidic acid (PA); diacylglycerol (DAG); phosphatidylcholine (PC); lysophosphatidylcholine (LPC); sphingomyelin (SM); ceramide (Cer); phosphatidylinositol 4-phosphate (PI4P); phosphatidylinositol 4, 5-phosphate (PI4,5P); 1-acylglycerol-3-phosphate acyltransferase 3 (AGPAT3); lysophosphatidic acid acyltransferase 3 (LPAAT3); phospholipase A2 (PLA_2); diacylglycerol kinase (DAGK); phosphatidic acid phosphatase (PA P'tase); phospholipase D (PLD); PC specific phospholipase C (PC-PLC); phosphatidylinositol specific PLC (PIPLC); sphingomyelin synthase (SMS).


PC
PI4P/PI4,5P
PC-PLC
PLD
PI-PLC
AGPAT3/
LPAAT3
DAGK
LPA
PA
DAG
SM
SMS
PA P'tase
PLA2?
PC
Cer
cPLA2α
LPC

membrane tubules from membrane vesicles and to determine any unique roles for membrane tubules. It remains unclear whether membrane tubules actually possess different compositions of membrane-bound or soluble protein cargo when compared to membrane vesicles. It is also unknown whether membrane tubules have any advantages in efficiency when compared to membrane vesicles, although studies have suggested that tubule-based continuities within Golgi cisternae provide efficient means of transporting soluble and membrane cargo [2, 37]. As membrane tubules and vesicles may work cooperatively, studying one aspect without disrupting the other may be the most difficult obstacle to overcome. Further understanding membrane tubule biology will not only advance the field of membrane trafficking but may also uncover novel physiological functions with translational significance. Despite the wealth of information on numerous enzymes partaking in membrane tubulation, there is relatively little information on the temporal and spatial regulation in producing the proper amounts of membrane tubules. This study seeks to address this problem by identifying novel regulators of membrane tubulation, thus shedding light on its regulation.

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CHAPTER 2

Global Kinome Analysis Reveals PACE1 as a Novel Regulator of Golgi Membrane Tubulation

2.1. Abstract

Membrane tubules play important roles in numerous processes ranging from Golgi structural maintenance to membrane trafficking. Previous studies have identified important players in membrane tubulation, including lipid-remodeling enzymes such as PLAs and LPATs. However, it remains unclear how these lipid-remodeling enzymes or other proteins with membrane tubulating abilities can be regulated during physiological changes such as increased secretory loads or growth stimuli. Kinases are known regulators of both protein function and signal transduction and are thus likely suspects in regulating membrane tubule biogenesis. The present study analyzes the entire library of known and predicted human kinases, or the kinome, within the context of a screen designed to detect potential regulators of BFA-induced Golgi tubulation in HeLa cells. We then focused on further characterizing one inhibitory hit, PACE1 (Protein-Associating with the Carboxyl-terminal domain of Ezrin), in determining its roles in Golgi membrane tubulation and function. PACE1 co-localized with Golgi resident proteins such as galactosyltransferase tagged with GFP (GalT-GFP), mannosidase-II (ManII), GPP130, and the TGN protein, mannose6-phosphate receptor (M6PR). Knockdown of PACE1 inhibited BFA-induced Golgi tubulation, Golgi structural maintenance, Golgi ribbon reformation, and anterograde trafficking of both soluble and transmembrane protein cargo. All of these phenotypes were reversed upon overexpression with an RNAi-resistant construct of PACE tagged with myc (PACE1-myc). We conclude that PACE1 plays important roles in membrane tubulation at the Golgi and that these membrane tubules participate in a variety of physiological functions. The results of this study should help pave the way in understanding both existing and novel mechanisms behind membrane tubulation at the Golgi complex.

2.2. Introduction

The functional aspects and biochemical mechanisms of membrane tubules are being unraveled as numerous reports link them to roles in membrane trafficking [1]. A fundamental step in forming membrane tubules includes the formation of membrane curvature via three primary mechanisms, including changes to lipids within membrane leaflets, insertion of proteins into one leaflet, and the formation of protein scaffolds alongside membranes [2]. The importance of lipid changes within membranes is exemplified by the discovery that numerous cytoplasmic phospholipases play roles in inducing membrane tubulation to facilitate trafficking events, possibly by altering the physical structures of lipids in single leaflets of a membrane bilayer to induce membrane curvature, and thus membrane tubules [3]. For example, the phospholipase A_2 (PLA_2) enzymes can alter phospholipid (PL) shapes by virtue of cleaving acyl chains from *sn*-2 positions of PLs to spawn membrane tubules that facilitate membrane trafficking and structural homeostasis at the Golgi [4-5]. Interestingly, modulation of PL shapes can also be catalyzed by lysophospholipid acyltransferases (LPATs) that carry out the reverse reaction of PLA_2s by transferring fatty acids onto lysophospholipids (LPLs), which subsequently contributes to trafficking and Golgi homeostasis [6-7]. The roles of lipid-modifying enzymes in membrane tubules are not limited within Golgi membranes as other studies show evidence that phospholipases also induce membrane tubules among endosomes and the ER-Golgi intermediate compartment (ERGIC) [8-9]. Despite the wealth of information on numerous enzymes partaking in membrane tubulation, relatively little is known about how these proteins are temporally or spatially regulated to

produce the proper amounts of membrane tubules. Furthermore, novel proteins may exist to induce membrane tubules.

2.3. Results

2.3.1. siRNA screen for proteins that regulate membrane tubulation

Golgi membrane tubules are both ephemeral and highly dynamic, making them difficult to image at steady-state levels. Fortunately, BFA possesses two important characteristics in serving as an excellent model system for studying membrane tubule biogenesis. First, it induces abundant amounts of Golgi membrane tubules within several minutes in a reversible manner. Secondly, BFA-induced Golgi membrane tubulation ultimately results in the fusion of all Golgi cisternal membranes into the ER, resulting in complete loss of the traditionally compact, juxtanuclear Golgi structure. We exploited this second property of BFA to identify novel protein regulators of Golgi membrane tubulation. We hypothesized that protein inhibitors of BFA-induced membrane tubulation will slow down the loss of compact, juxtanuclear Golgi structures while protein-activators will hasten the loss of compact, juxtanuclear Golgi structures.

We utilized an siRNA library targeting all of the known human kinases, along with known pseudo-kinases and scaffold proteins, in a high-throughput, functional screen that analyzed the loss of compact, juxtanuclear Golgi structures upon treatment with BFA. HeLa cells stably expressing GFP-tagged galactosyltransferase (GalT-GFP), a resident of *trans* Golgi cisternae, were incubated in 96-well plates with siRNAs or controls and then treated with BFA to induce rapid tubule-mediated, retrograde transport of Golgi membranes to the ER. During this

process, the average GalT-GFP fluorescence per area significantly dims, as the ER possesses a surface area that is five-fold larger than that of the Golgi complex [10-11]. We then utilized automated image analysis software to quantify the loss of bright, juxtanuclear Golgi signal following BFA treatment (Figure 2.2). BFA treatment (5 μg/ml) leads to complete loss of compact, juxtanuclear Golgi structures within 20 minutes under the conditions of this assay (Figure 2.1A).

The siRNA library (Ambion) targets 719 gene products via three separate, unique siRNA oligos per gene. Utilizing 96-well plates, HeLa/GalT-GFP cells were first plated and then transfected with either 30 nM siRNA or water as negative controls. After 24 h, cells were treated with BFA (5 μg/ml for 10 min). Nuclei were stained with Hoechst and then imaged and quantitated via automation. Analysis software quantitated intact Golgi structures as determined by average GalT-GFP fluorescence per area exceeding an arbitrary threshold. The entire library was screened three independent times and the results were averaged, with potential screen hits determined as those that exceed a 20% threshold difference in GalT-GFP fluorescence when compared to untransfected, untreated cells. Since three separate siRNA oligos were available against each gene, potential screen hits were not necessarily ruled out in the case that only one siRNA exhibited a strong phenotype while the others did not. Among the 719 targeted genes within the library, 93 were identified as potential regulators of BFA-induced Golgi tubulation (Figure 2.1B). The majority of the hits comprise genes involved in lipid modification, cell cycle regulation, or have other/unknown functions, while a small portion of the hits are linked to membrane trafficking or the cytoskeleton (Figure 2.1C). Among these results, PACE1 (Protein-Associating with the Carboxyl-terminal domain of Ezrin) was identified as a potential hit, with a 25% inhibition of BFA-induced Golgi tubulation. Of the three siRNAs targeting PACE1, one

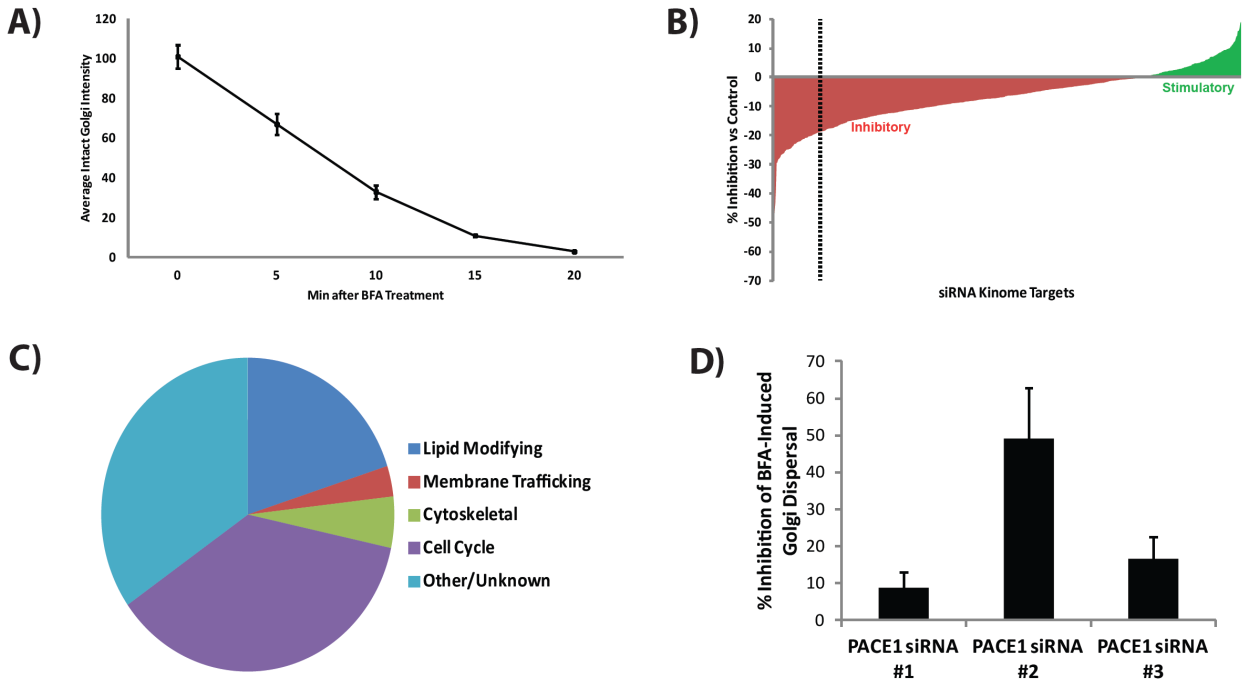


Figure 2.1. PACE1 knockdown inhibits BFA-induced Golgi dispersal. (A) HeLa cells stably expressing the *trans*-Golgi marker GalT-GFP undergo Golgi dispersal after 20' of 1 µg/ml BFA treatment and can be quantified via integrated granular intensity. Error bars represent SEM of n=3. **(B)** 726 human kinases were knocked down with 3 distinct siRNA oligos and their effects on BFA-induced Golgi dispersal were graphed as averaged values for each kinase target. ~12% kinase targets exceed an arbitrary threshold of 20% inhibition (dotted vertical line) relative to untransfected control cells. **(C)** Categorization of hits above the threshold. **(D)** Averaged quantitation of each PACE1 siRNA oligo on BFA-induced Golgi dispersal. Error bars indicate SEM of n=3. Bar graphs represent mean values.

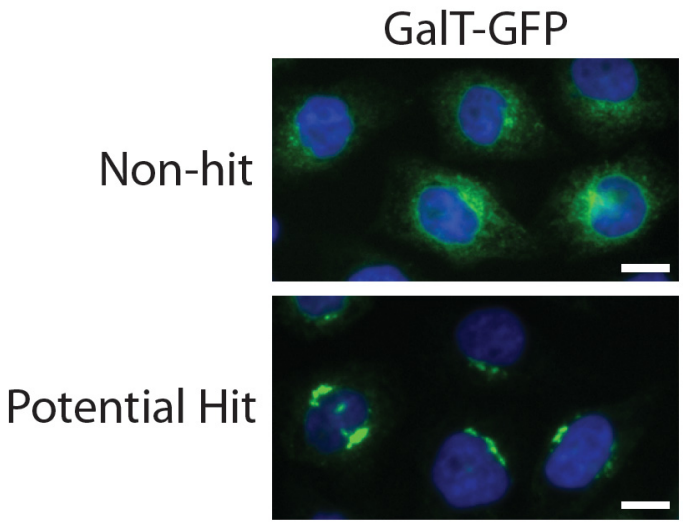
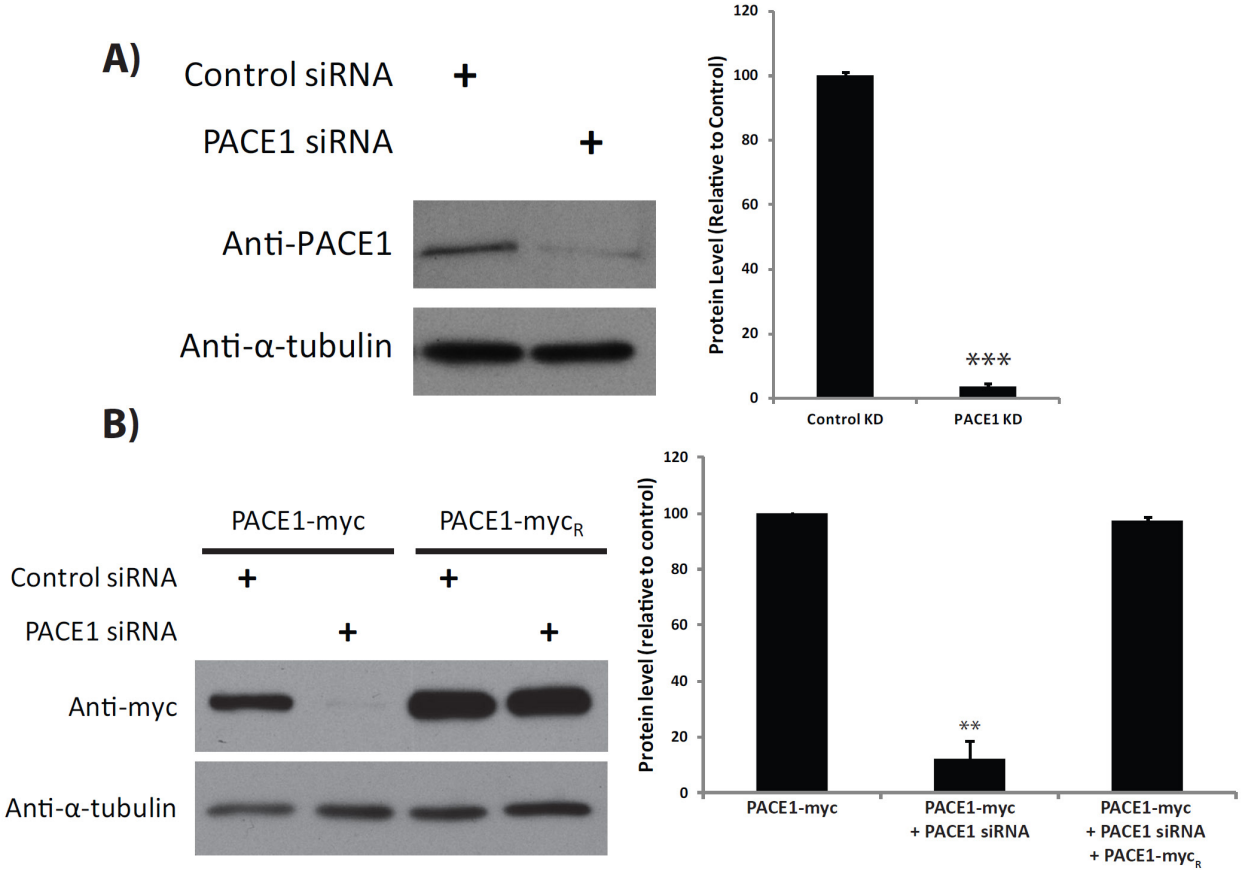


Figure 2.2. Potential hits inhibit BFA-induced Golgi dispersal. HeLa cells stably expressing the *trans*-Golgi marker GalT-GFP and stained for nuclei via Hoechst (blue) underwent Golgi dispersal upon BFA treatment (1 μg/ml) after 10'. Hits that inhibited this process yielded cells with intact, juxtanuclear structures while non-hits leave BFA effects intact, permitting intact, juxtanuclear Golgi structures to become absorbed into a dispersed, ER-like structure. Scale bar represents 10 μm.

siRNA alone inhibited BFA-induced Golgi tubulation by 49% ± 14% standard error of the mean (SEM) when compared to untransfected control (Figure 2.1D). Western analysis of whole cell HeLa lysates showed that a similar siRNA, but not from the library, reduced endogenous PACE1 protein levels *in vivo* by >95% (Figure 2.3A). Furthermore, this siRNA also reduced overexpressed, myc-tagged PACE1 levels by >90%, whereas rescue with a PACE1-myc construct, harboring two silent mutations to confer RNAi-resistance, completely restored PACE1-myc protein levels (Figure 2.3B).

PACE1 is also known as Scy1-like 3 (Scyl3) and is a member of the Scy1-like (Scyl) family of purported kinases. All three members of the Scyl family possess a conserved serine/threonine kinase domain along with HEAT domain repeats (Figure 2.4). Scy1 itself is a yeast protein with unknown functions. PACE1 is a ubiquitously expressed 742-residue protein that was originally indentified in a yeast 2-hybrid screen as an interacting protein with the C-terminal domain of Ezrin [12]. Ezrin is a member of the ERM (Ezrin, Radixin, Moesin) family that is involved in both facilitating interactions between the cytoplasmic domains of transmembrane proteins with filamentous actin and coordinating signal transduction from the plasma membrane into the cytoplasm [13-14]. The lone study that identified PACE1 revealed that the protein localizes to both the Golgi complex and plasma membrane ruffles, with N-terminal myristoylation required for localization to the former [12]. Additionally, the same study reported that PACE1 does not actually possess any true kinase activity, suggesting that it is a pseudo-kinase. Although PACE1's functions remain unknown, studies have implicated its two family members as important players vesicle-mediated membrane trafficking. For example, several studies provided evidence that Scyl1 regulates both Golgi morphology and COPI-mediated retrograde trafficking [15-16]. Scyl2, also known as CVAK104 (coated vesicle-

Figure 2.3. PACE1 knockdown reduces both endogenous PACE1 and transiently expressed PACE1-myc and can be rescued with an RNAi-resistant construct. (A) Western blot of HeLa cell lysates that have been transiently transfected with siRNA against control or PACE1 and then probed with anti-PACE1 antibody. Protein levels were quantified and normalized to α-tubulin expression levels. **(B)** HeLa cells were transfected with siRNA against either control or PACE1 and were additionally transfected with PACE1-myc or an RNAi-resistant construct, PACE1-mycR. Whole cell lysates were then analyzed via Western blotting and probed with anti-myc and anti-α-tubulin antibodies. ** and *** denote two-tailed P-value of <0.01 and <0.001, respectively, for n=3. Error bars indicate SEM of n=3. All bar graphs display mean values.


A)
Control siRNA +
PACE1 siRNA +
Anti-PACE1
Anti-α-tubulin
Protein Level (Relative to Control)
120
100
80
60
40
20
0
***
Control KD
PACE1 KD
B)
PACE1-myc
PACE1-myc$_R$
Control siRNA + +
PACE1 siRNA + +
Anti-myc
Anti-α-tubulin
Protein level (relative to control)
120
100
80
60
40
20
0
**
PACE1-myc
PACE1-myc + PACE1 siRNA
PACE1-myc + PACE1 siRNA + PACE1-myc$_R$

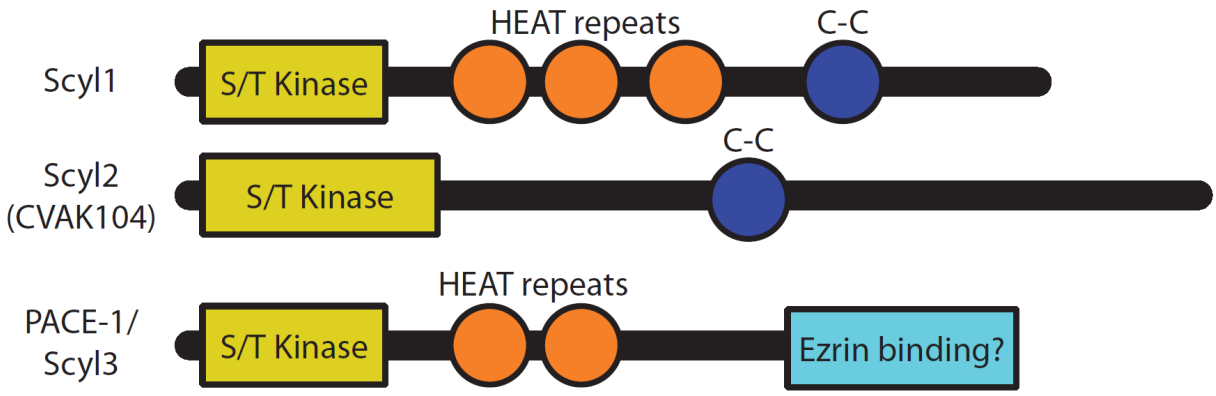


Figure 2.4. Scyl family members and their known protein domains. S/T kinase: serine/threonine kinase domain. C-C: coiled-coil domain.

associated kinase of 104 kDa), has been reported to actually possess kinase activity and is capable of regulating the β2-adaptin subunit of the AP-2 clathrin adaptor protein [17]. Scyl2 has also been linked to AP-1 and the regulation of clathrin-mediated SNARE sorting [18]. Furthermore, Scyl2 also strongly associated with the Golgi complex and can regulate trafficking between the TGN and the endosomal system [19].

2.3.2. PACE1 co-localizes with Golgi membrane markers

PACE1 has previously been observed to co-localize with the *trans*-Golgi resident protein, Golgin-97, in addition to plasma membrane ruffles within MCF-7 human breast cancer cells [12]. In HeLa cells, endogenous PACE1 immunofluorescence co-localized with GPP130, a *cis*-Golgi marker but very little was seen in the cell cortex (Figure 2.5A). HeLa cells overexpressing PACE1-myc show that the protein, as determined via anti-myc antibodies, co-localized with GPP130, a *cis*-Golgi marker, Mannosidase-II (Man II), a *medial*-Golgi marker, and partially co-localized with mannose6-phosphate receptor (M6PR), a *trans*-Golgi network (TGN) marker, and with membrane ruffles (Figure 2.5B).

2.3.3. PACE1 knockdown disrupts BFA-induced Golgi tubulation

After confirming that PACE1 localized to the Golgi complex, we next analyzed whether knockdown of endogenous PACE1 disrupts BFA-induced Golgi tubulation and its subsequent retrograde trafficking into the ER. HeLa/GalT-GFP cells were first transfected with either siRNA against PACE1 or scrambled siRNA as a negative control. After 24 h, cells were treated

with BFA for various periods of time. These experiments confirmed the siRNA screen results and further demonstrated that PACE1 knockdown significantly reduced the rate of BFA-induced Golgi loss (Figure 2.6A). To directly determine if PACE1 knockdown inhibits Golgi membrane tubule formation, we counted the number of cells with tubulated Golgi membranes. The results showed that PACE1 knockdown reduced membrane tubule formation by ~43% (Figure 2.6B). To show that these phenotypes are not due to off-target defects, a PACE1-myc fusion expression construct, which harbors two silent mutations to confer siRNA resistance, lead to a significant rescue of knockdown phenotypes by restoring intact Golgi structures and BFA-induced Golgi membrane tubules to more closely resemble control levels (Figure 2.7A & 2.7B).

2.3.4. PACE1 knockdown disrupts Golgi reformation after BFA washout

The effects of BFA have long been known to be reversible, which has been used as a model system for biogenesis of the Golgi complex [20]. After washing out BFA from cells, the Golgi undergoes the reformation of separate mini-stacks, which appear as punctate staining within the juxtanuclear region [21]. These mini-stacks then coalesce into an intact Golgi ribbon via PLA_2-dependent membrane tubules [22]. We thus sought to determine whether PACE1 may facilitate this step in forming Golgi membrane tubules from Golgi mini-stacks to restore an intact ribbon structure. HeLa/GalT-GFP cells were first transfected with either siRNA against PACE1 or a scrambled siRNA as a negative control. The next day, cells were treated with BFA (1 μg/ml) for 20 minutes to completely disperse the Golgi complex, and then subsequently washed free of the drug to permit Golgi reformation. PACE1 knockdown led to a significant disruption of Golgi ribbon reformation from mini-stacks, and this was most pronounced within 90 minutes

Figure 2.5. PACE1-myc co-localizes with Golgi markers. (A) HeLa cells incubated with antibodies against endogenous PACE1 and cis-Golgi marker. **(B)** HeLa cells transiently transfected with PACE1-myc and then probed with antibodies against myc epitope along with Golgi markers such as *cis*-Golgi marker GPP130, *medial*-Golgi marker mannosidase-II (ManII), and *trans*-Golgi network (TGN) marker mannose6-phosphate receptor (M6PR). Scale bar represents 10 μm.

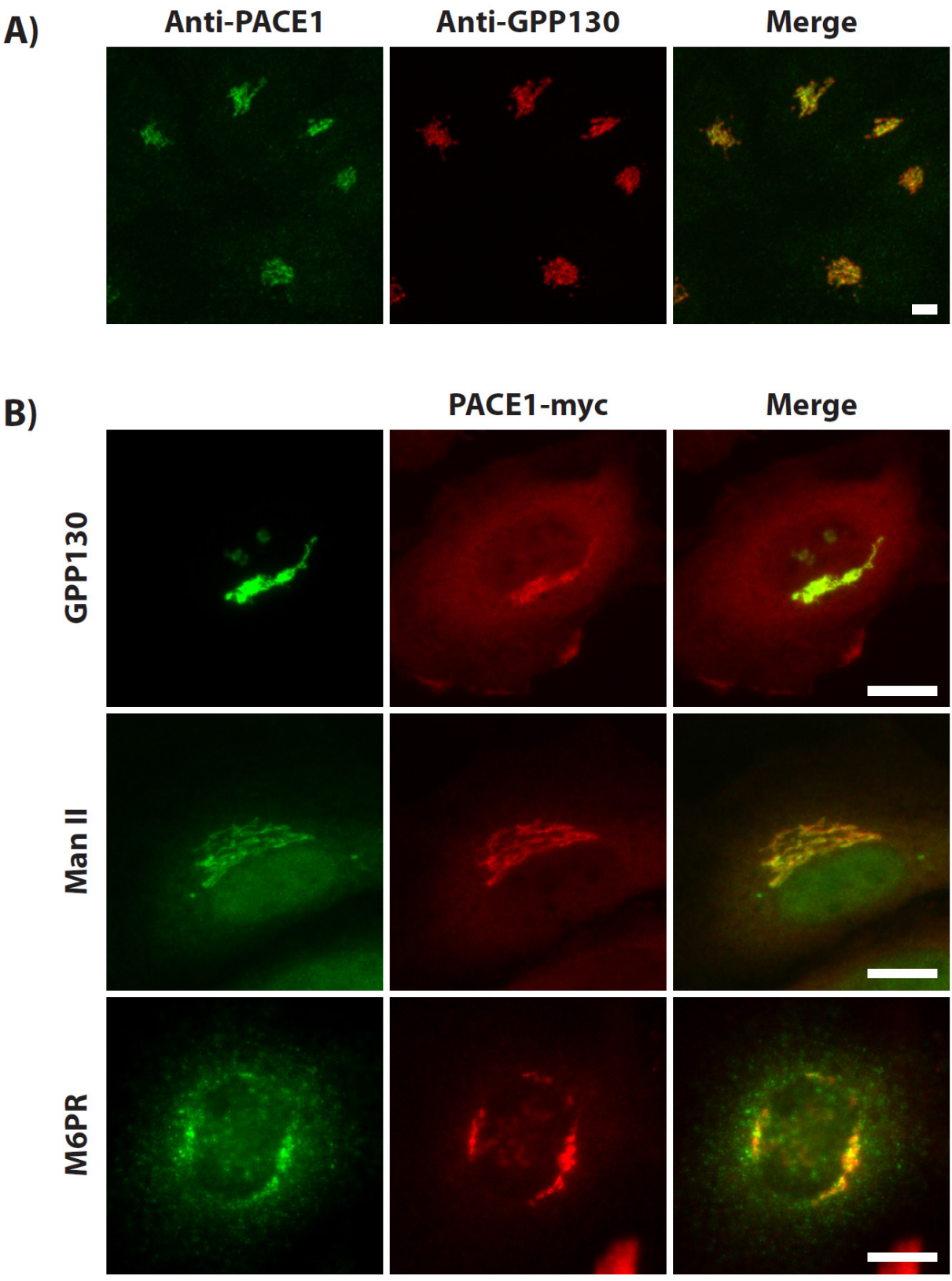
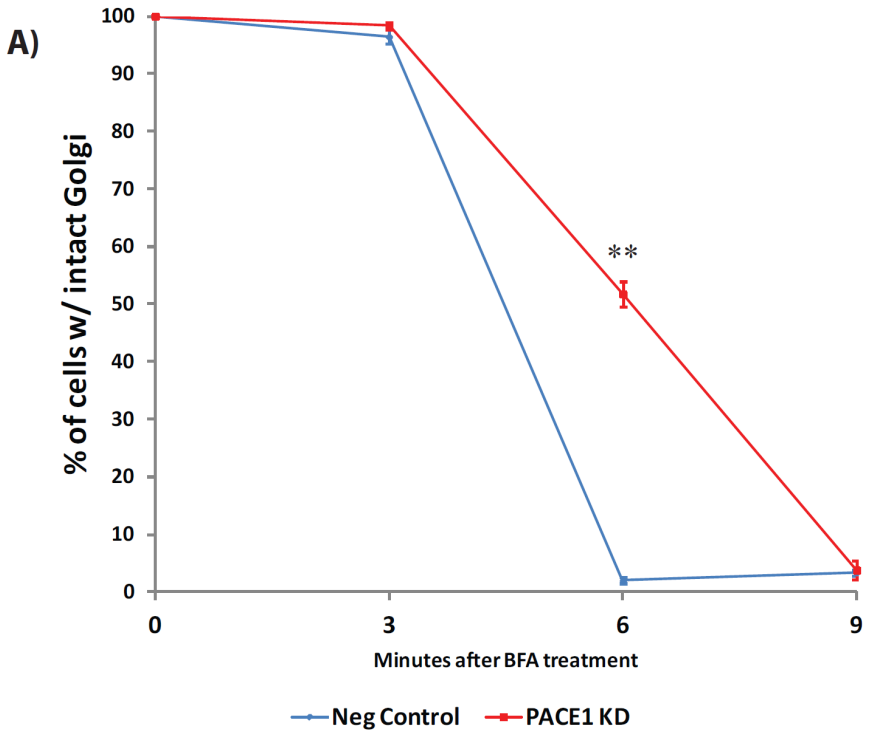

A)
Anti-PACE1
Anti-GPP130
Merge
B)
PACE1-myc
Merge
GPP130
Man II
M6PR

Figure 2.6. PACE1 knockdown delays Golgi dispersal and tubulation. HeLa cells stably expressing GalT-GFP were transfected with siRNA against control or PACE1 for 18 h and then treated with 1 μg/ml BFA over 9' and then quantitated for **(A)** loss of intact Golgi structures or **(B)** percentage of cells possessing tubulated Golgi structures (displaying >3 Golgi membrane tubules spanning the length of the Golgi). Two-tailed P-values of <0.01 for n=3 denoted by **. Error bars indicate SEM of n=3.


A)
% of cells w/ intact Golgi
100
90
80
70
60
50
40
30
20
10
0
**
0
3
6
9
Minutes after BFA treatment
Neg Control
PACE1 KD

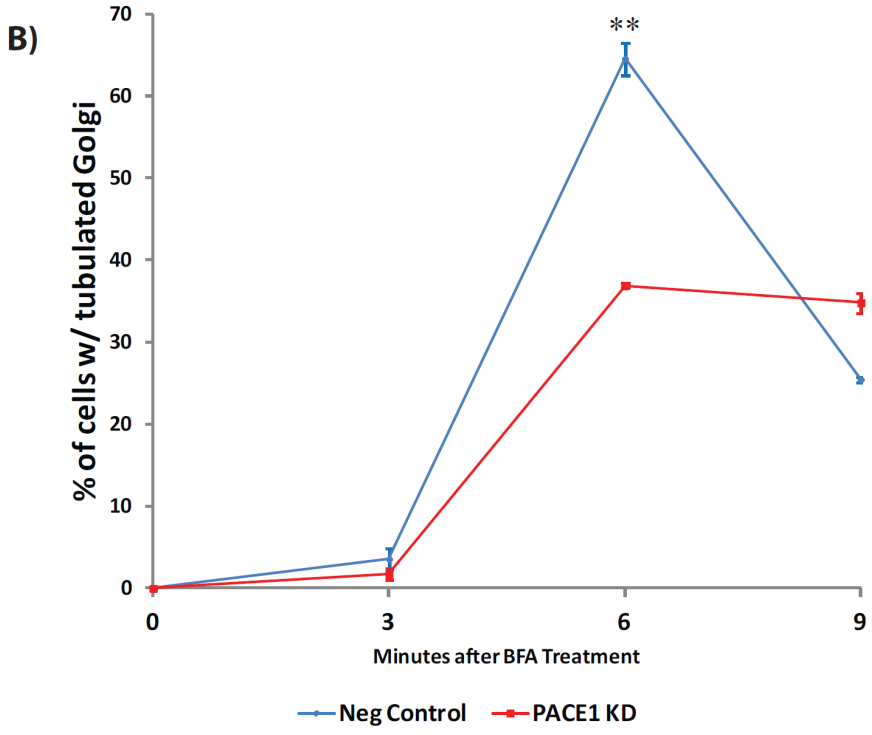
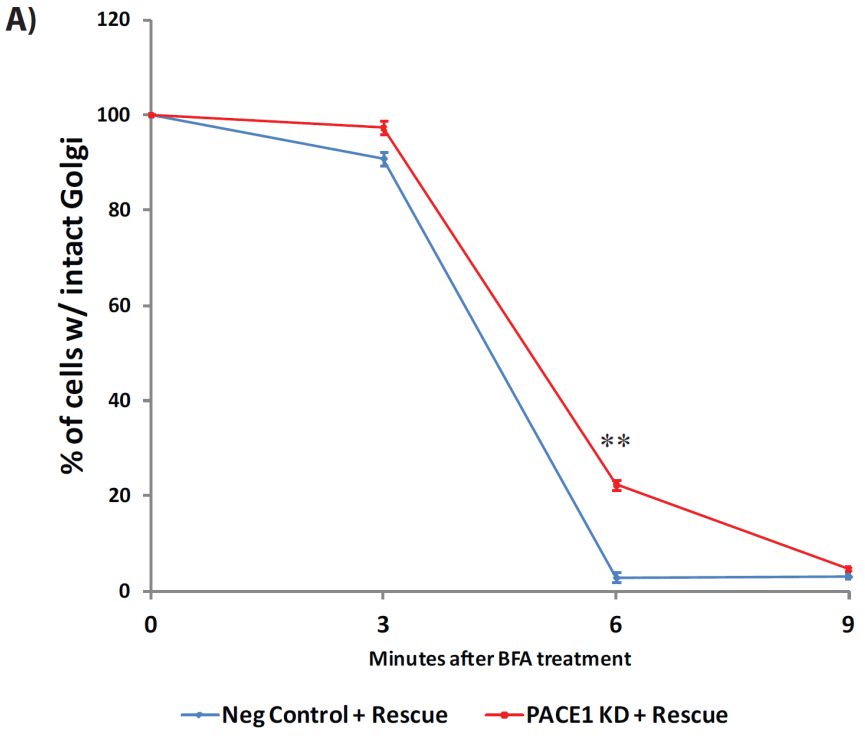
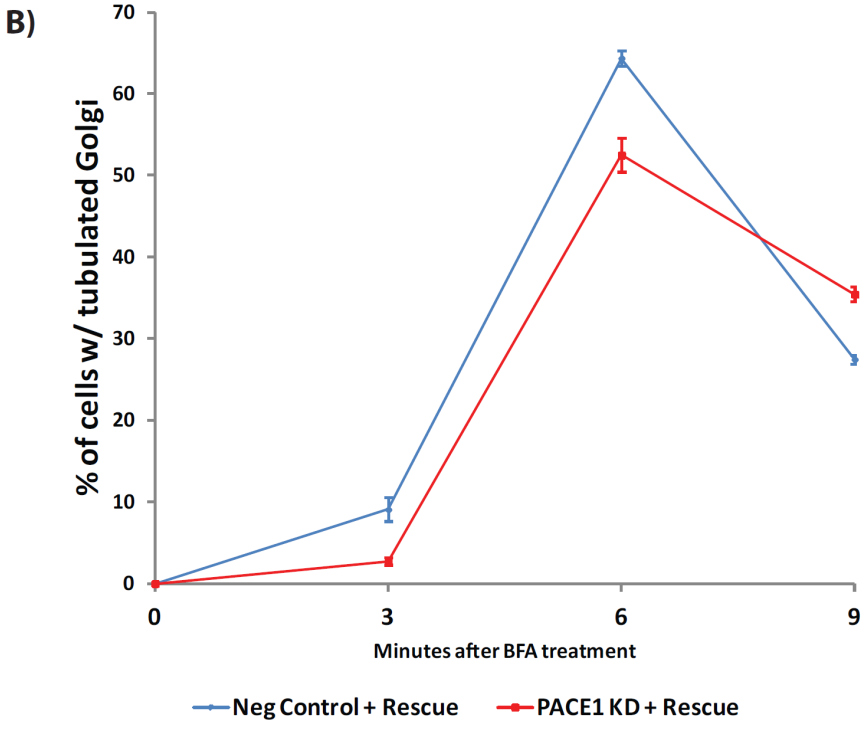

B)
% of cells w/ tubulated Golgi
70
60
50
40
30
20
10
0
**
0
3
6
9
Minutes after BFA Treatment
Neg Control
PACE1 KD

Figure 2.7. PACE1 rescue restores BFA-induced Golgi dispersal and tubulation. HeLa cells stably expressing GalT-GFP were transfected with siRNA against control or PACE1 along with empty pcDNA vector (control) or RNAi-resistant PACE1 (PACE1-myc$_R$) for 18 h. Cells were then treated with 1 μg/ml BFA over 9 min and then quantitated for (A) loss of intact Golgi structures or (B) percentage of cells possessing tubulated Golgi structures (displaying >3 Golgi membrane tubules spanning the length of the Golgi). Two-tailed P-values of <0.01 for n=3 denoted by **. Error bars indicate SEM of n=3.


A)
120
100
80
60
40
20
0
% of cells w/ intact Golgi
**
0
3
6
9
Minutes after BFA treatment
Neg Control + Rescue
PACE1 KD + Rescue


B)
70
60
50
40
30
20
10
0
% of cells w/ tubulated Golgi
0
3
6
9
Minutes after BFA treatment
Neg Control + Rescue
PACE1 KD + Rescue

(Figure 2.8A). Live-cell imaging revealed that PACE1 knockdown cells failed to reform intact Golgi ribbon upon BFA washout, when compared to control cells (Figure 2.8C). In control cells, about 13% ± 2% (SEM) possessed fragmented Golgi structures while PACE1 knockdown cells possessed about 84% ± 4% (SEM) fragmented Golgi structures (Figure 2.8B).

2.3.5. PACE1 knockdown fragments the Golgi

Membrane tubules are not only important for reformation of Golgi ribbon structures from mini-stacks but are also thought to be vital in the homeostatic maintenance of intercisternal Golgi connections [23]. For example, knocking down cytoplasmic PLA_2 enzymes that mediate Golgi tubule formation eventually leads to Golgi fragmentation into puncta-like mini-stacks [4-5]. Thus, we examined whether knocking down PACE1 disrupts maintenance of intact Golgi ribbons, leading to Golgi fragmentation. When HeLa cells were transfected with scrambled RNA as control, about 14% ± 2% (SEM) displayed a fragmented Golgi phenotype, characterized by possessing greater than 3 separate Golgi fragments (Figure 2.9). In contrast, cells transfected with PACE1 RNA resulted in a significant increase to 37% ± 5% (SEM) of cells harboring fragmented Golgi structures.

The fragmented Golgi ribbon appeared to be mini-stacks as each punctate-like structure contains Golgi markers such as GalT-GFP, a *trans*-Golgi marker, GPP130, a *cis*-Golgi marker, mannosidase-II (ManII), a *medial*-Golgi marker, and M6PR, a TGN marker (Figure 2.10). Golgi fragmentation has been previously linked to defects in cytoskeletal components, including microtubules, filamentous actin, and both microtubule- and actin-based motor complexes [24-27]. However, in PACE1 knockdown cells, both microtubule and actin cytoskeletal networks

largely appeared unaffected when compared to control cells (Figure 2.10) Furthermore, Golgi fragmentation induced by PACE1 knockdown did not appear to affect the localization of coat proteins such as β-COP or clathrin AP-1 adaptors from localization to Golgi mini-stacks, despite the Golgi undergoing significant fragmentation (Figure 2.10).

2.3.6. PACE1 knockdown disrupts membrane trafficking

Membrane tubules not only connect the Golgi complex together for structural integrity but are also important for anterograde and retrograde trafficking of protein-membrane cargo and in certain contexts, intercisternal tubules increase in abundance in response to secretory loads entering the Golgi complex [4-5, 23]. Therefore, we examined whether knocking down PACE1 leads to any phenotypic changes in anterograde trafficking of both soluble (secretory horseradish peroxidase, ssHRP) and membrane-bound (VSV-G) cargo. HeLa cells transfected with a scrambled control siRNA yielded a ssHRP secretion rate of 0.29 (A.U./h), while in contrast, PACE1 knockdown cells yielded a rate of 0.10 (A.U./h), which is ~65% slower (Figure 2.11A). Rescue of PACE with an RNAi-resistant PACE1-myc construct restored ssHRP secretion to control levels (Figure 2.11B).

Interestingly, transmembrane protein cargo may undergo anterograde trafficking via distinct mechanisms and routes than those of soluble cargo [28-29], and thus PACE1 knockdown could have differential effects on secretion. In addressing this possibility, an overexpression construct harboring the temperature-sensitive transmembrane protein, VSVG-tsO45-YFP, was transfected into HeLa cells. At 40ºC, VSVG-tsO45-YFP reversibly misfolds, leading to its sequestration within the ER. When shifted to 20ºC, it is able to correctly fold and traffick out of

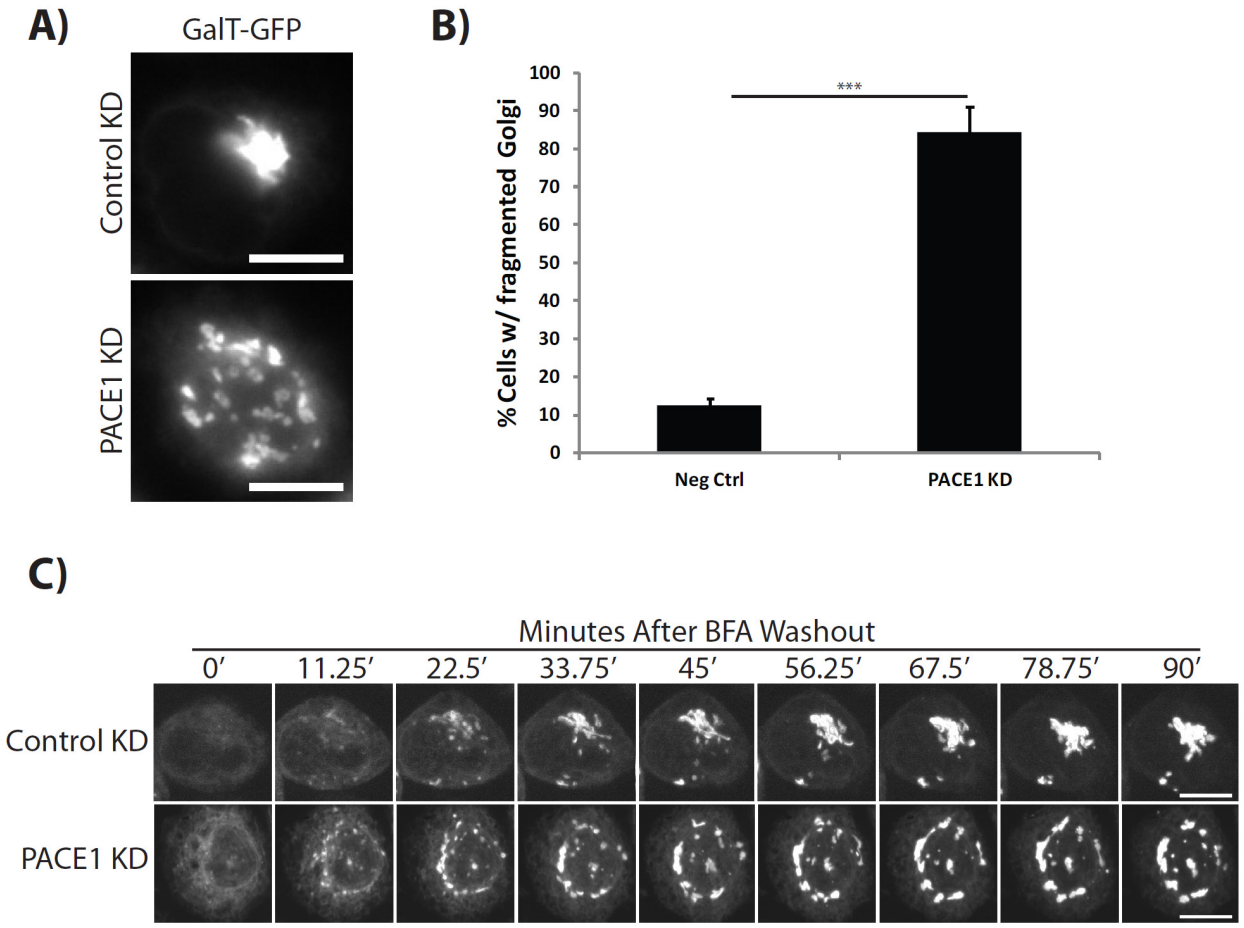


Figure 2.8. PACE1 knockdown prevents Golgi ribbon reformation. (A) HeLa cells stably expressing GalT-GFP were knocked down with either control or PACE1 siRNA, treated with BFA at 1 μg/ml for 20', washed of drugs, and then permitted to undergo Golgi ribbon recovery for 90'. Scale bar represents 10 μm. **(B)** Cells were quantitated for possessing fragmented Golgi structures (>4 Golgi fragments. Two-tailed P-value of <0.001 for n=3 is denoted by ***. Error bars indicate SEM of n=3. **(C)** Live-cell imaging of HeLa cells stably expressing GalT-GFP were treated with BFA as in (A) but then washed free of drug and permitted to recover in serum medium over 90' at 37ºC. Scale bar represents 10 μm.

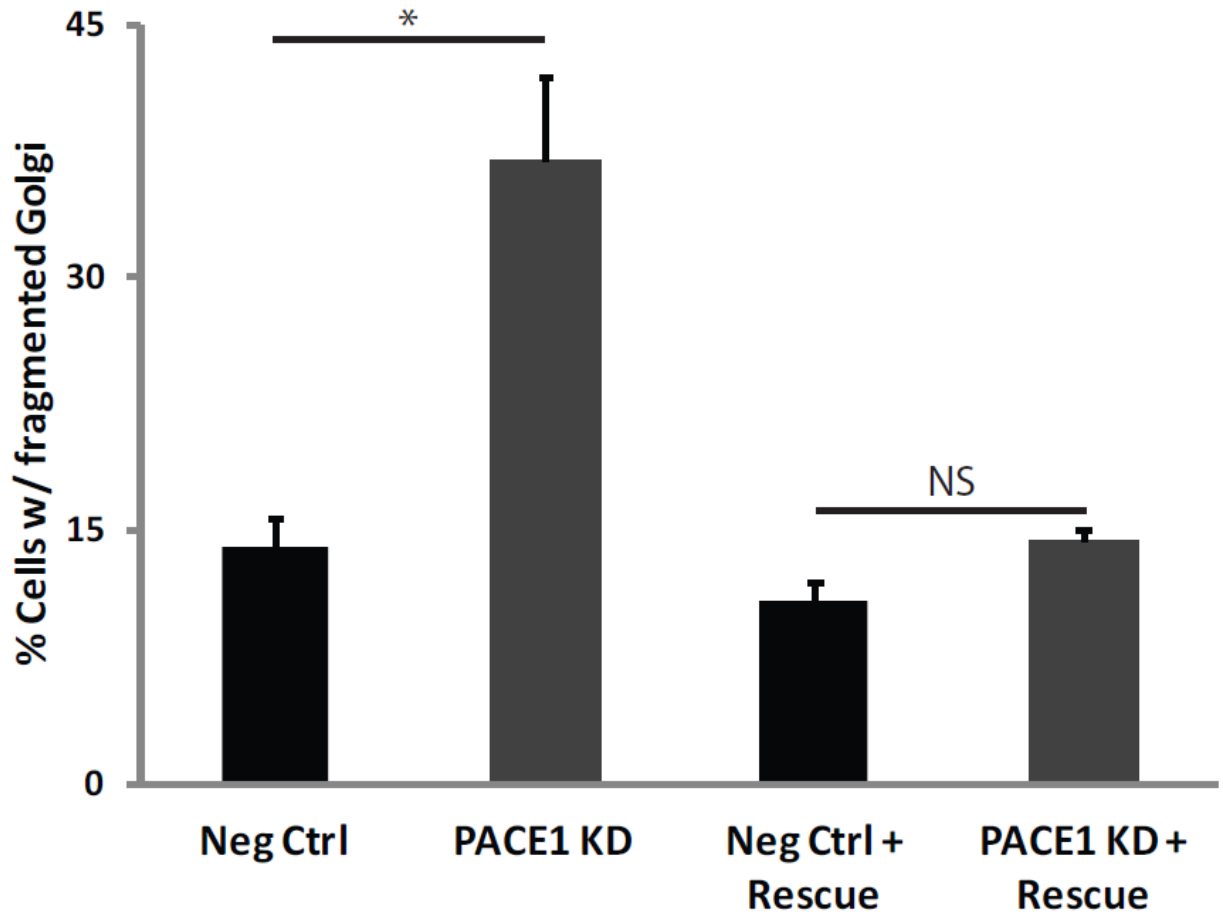


Figure 2.9. PACE1 knockdown leads to Golgi fragmentation. HeLa cells stably expressing GalT-GFP were knocked down with either control or PACE1 siRNA and quantitated for fragmented Golgi structures (>4 Golgi fragments). Two-tailed P-values of <0.05 or >0.05 are denoted by * or 'ns', respectively. Error bars indicate SEM of n=3.

Figure 2.10. Golgi fragments by PACE1 knockdown co-localize with Golgi markers. HeLa cells stably expressing *trans*-Golgi marker, GalT-GFP, where transiently transfected with siRNA against either control or PACE1. Cells were then labeled via immunofluorescence against Golgi markers, including *cis*-Golgi GPP130, *medial*-Golgi mannosidase-II (ManII), along with coatomer protein β-COP and cytoskeletal components α-tubulin and filamentous actin (F-actin). Scale bar represents 10 μm.

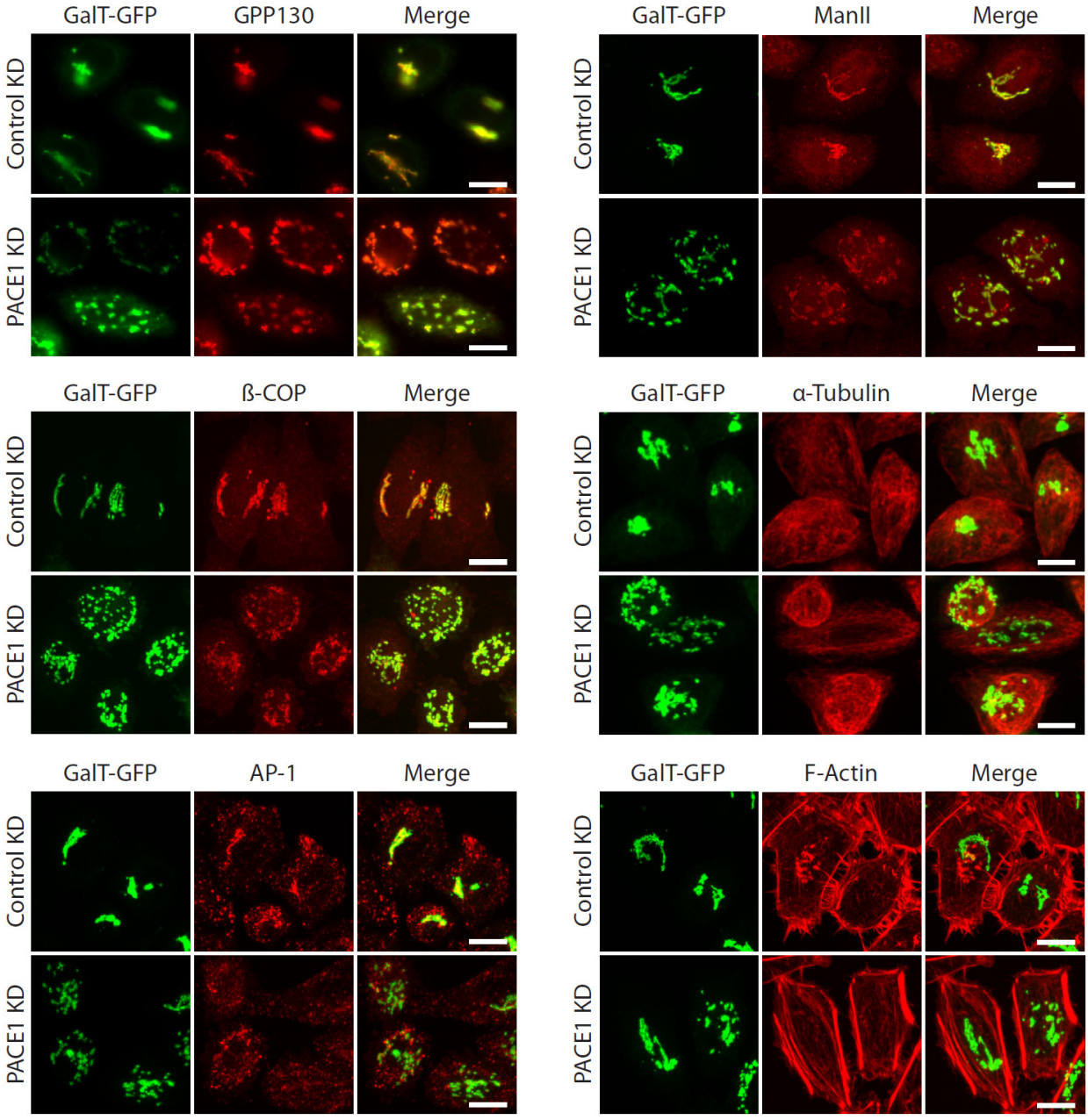
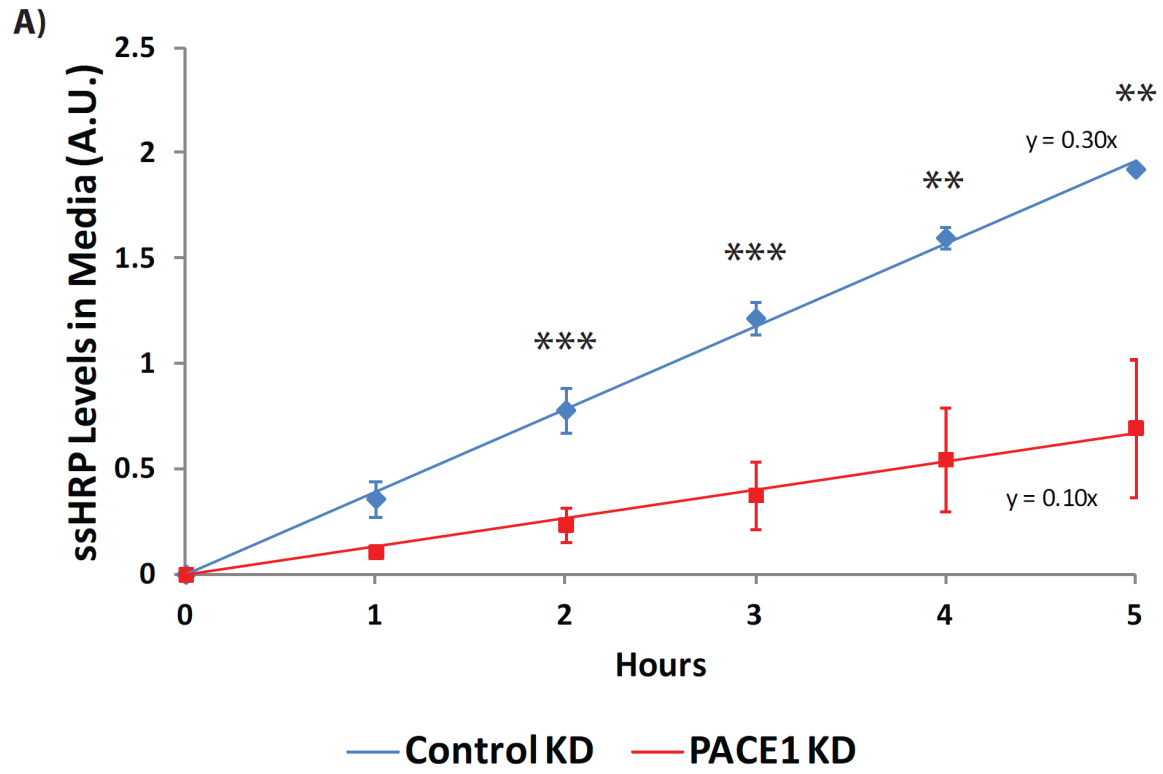
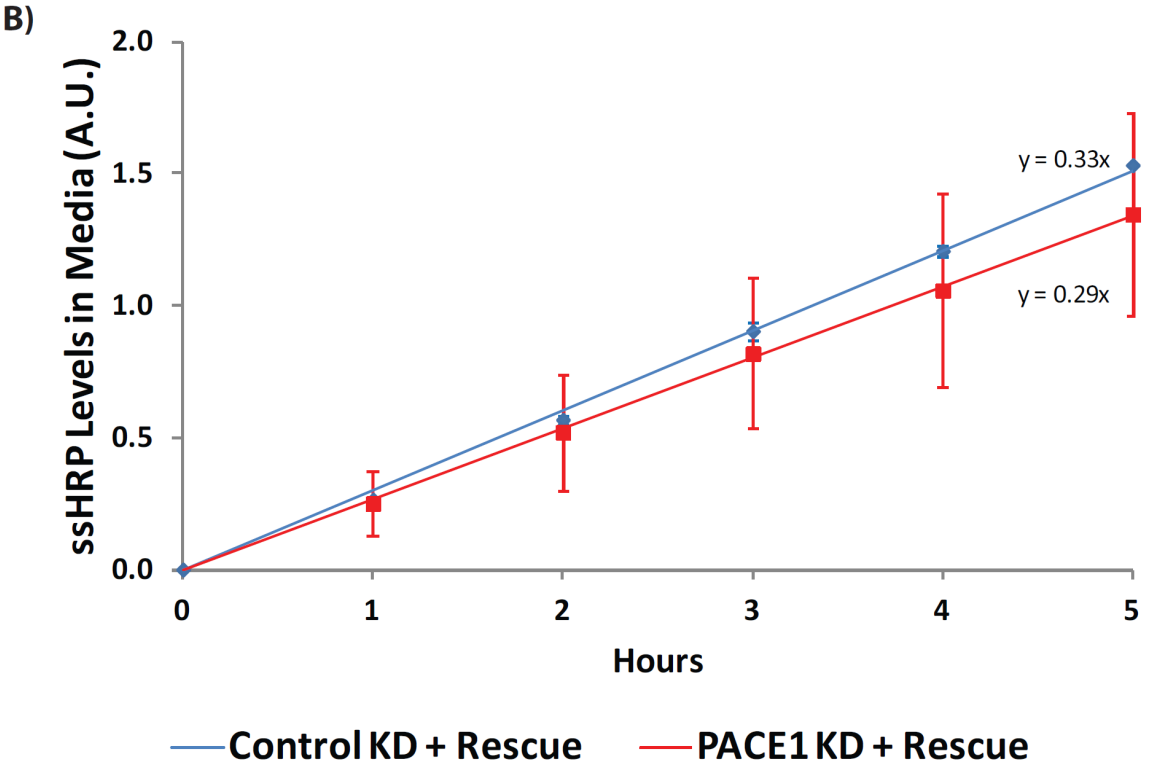

GalT-GFP
GPP130
Merge
Control KD
PACE1 KD
GalT-GFP
ManII
Merge
Control KD
PACE1 KD
GalT-GFP
ß-COP
Merge
Control KD
PACE1 KD
GalT-GFP
α-Tubulin
Merge
Control KD
PACE1 KD
GalT-GFP
AP-1
Merge
Control KD
PACE1 KD
GalT-GFP
F-Actin
Merge
Control KD
PACE1 KD

Figure 2.11. PACE1 knockdown disrupts ssHRP secretion. (A) HeLa cells knocked down with either control or PACE1 siRNA were transfected with ssHRP-FLAG and then assayed for ssHRP-FLAG secretion into the media. Equations represent simple linear regressions fitted to the data, with the y intercept set to zero. Two-tailed P-values of <0.01 and <0.001 are denoted by ** and ***, respectively. Error bars indicate SEM of n=3. **(B)** The same experiment was done along with rescue with empty pcDNA3 vector (control) or RNAi-resistant PACE1-myc.


A)
ssHRP Levels in Media (A.U.)
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
Hours
***
***
**
**
y = 0.30x
y = 0.10x
Control KD
PACE1 KD


B)
ssHRP Levels in Media (A.U.)
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
Hours
y = 0.33x
y = 0.29x
Control KD + Rescue
PACE1 KD + Rescue

the ER until it is blocked within the TGN, where a final temperature shift towards 32ºC leads to its eventual trafficking onto the plasma membrane (PM) in a synchronous fashion [30-31]. VSVG-ts045-YFP on the PM can be quantified via antibodies that specifically recognize extracellular epitopes of VSVG-ts045-YFP without having to permeablize the cells. While no surface VSVG-ts045-YFP is visible prior to a temperature shift to 32ºC, an abundant amount of ectopic VSVG-ts045-YFP can be detected 45 minutes after temperature shift (Figure 2.12). Knocking down PACE1 significantly reduced VSVG-ts045-YFP trafficking to the plasma membrane, rendering a conspicuously visible amount of the protein still present within the TGN when compared to control cells (Figure 2.13A). Using this system, cells transfected with control siRNA were able to transport VSVG-ts045-YFP to the PM at a rate of ~9.3 (A.U./15 min) while cells transfected with PACE1 siRNA yielded a rate of ~5.3 (A.U./15 min) of VSVG-ts045-YFP onto the PM, which is ~ 43% less than that of control cells (Figure 2.13B).

2.4. Discussion

Here we describe an siRNA screen of the human kinome in a high-throughput assay to identify regulators of Golgi membrane tubule formation and function. The screen utilized BFA because it is a potent inducer of membrane tubulation in mammalian cells, and we reasoned that proteins which influence BFA-stimulated tubule formation would also function at the Golgi under normal circumstances. Indeed, this approach was validated by finding that several proteins identified in this way, e.g., PACE1, help regulate the functional organization of the Golgi complex.

Figure 2.12. PACE1 knockdown disrupts VSVG-YFP trafficking. HeLa cells knocked down with either control or PACE1 siRNA were transfected with VSV -YFP and after 1.5 h of temperature block at 19.5ºC to accumulate VSVG-YFP at the TGN, cells were shifted to 32ºC to permit VSVG-YFP to traffic from the TGN to the plasma membrane. Surface VSVG was detected via anti-VSVG antibodies over non-permeablized cells. Scale bar represents 10 μm.

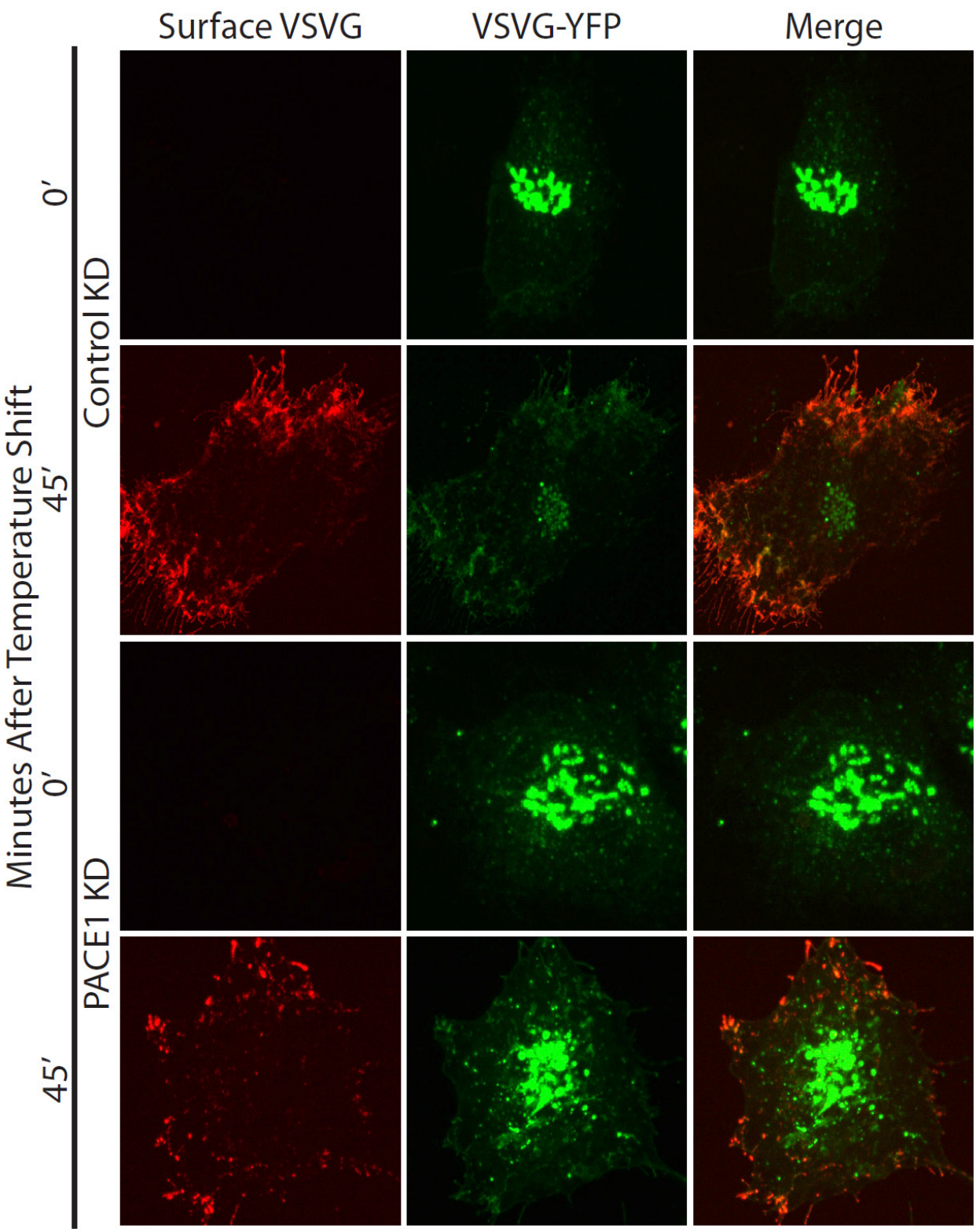
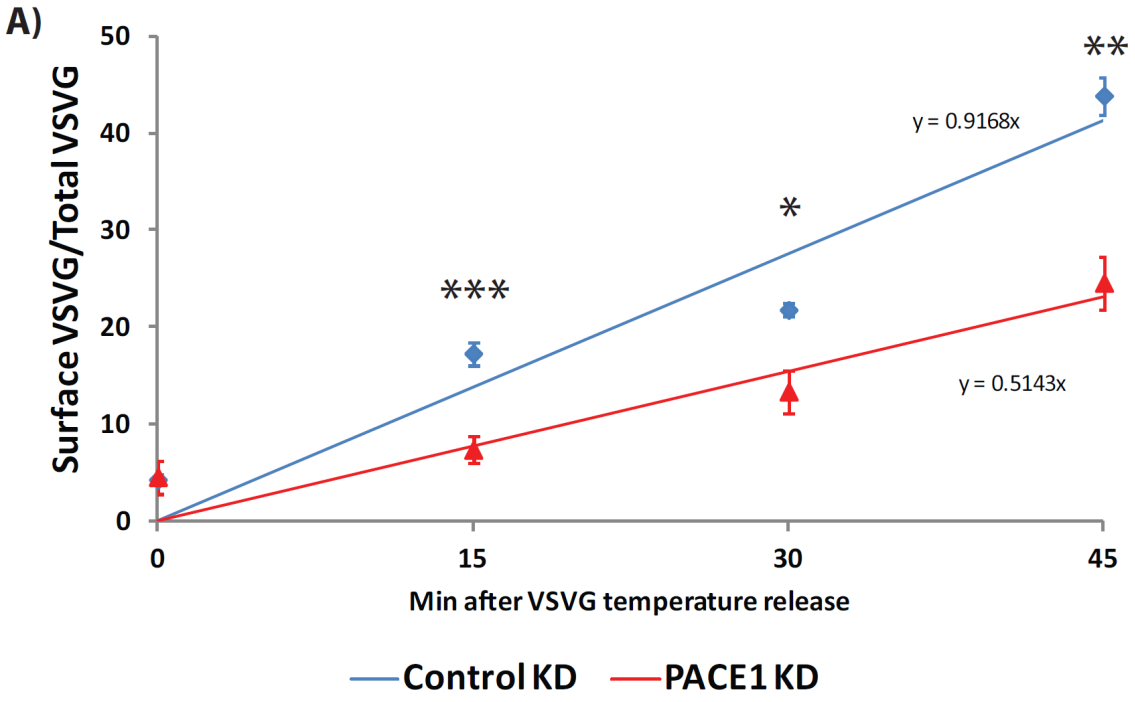
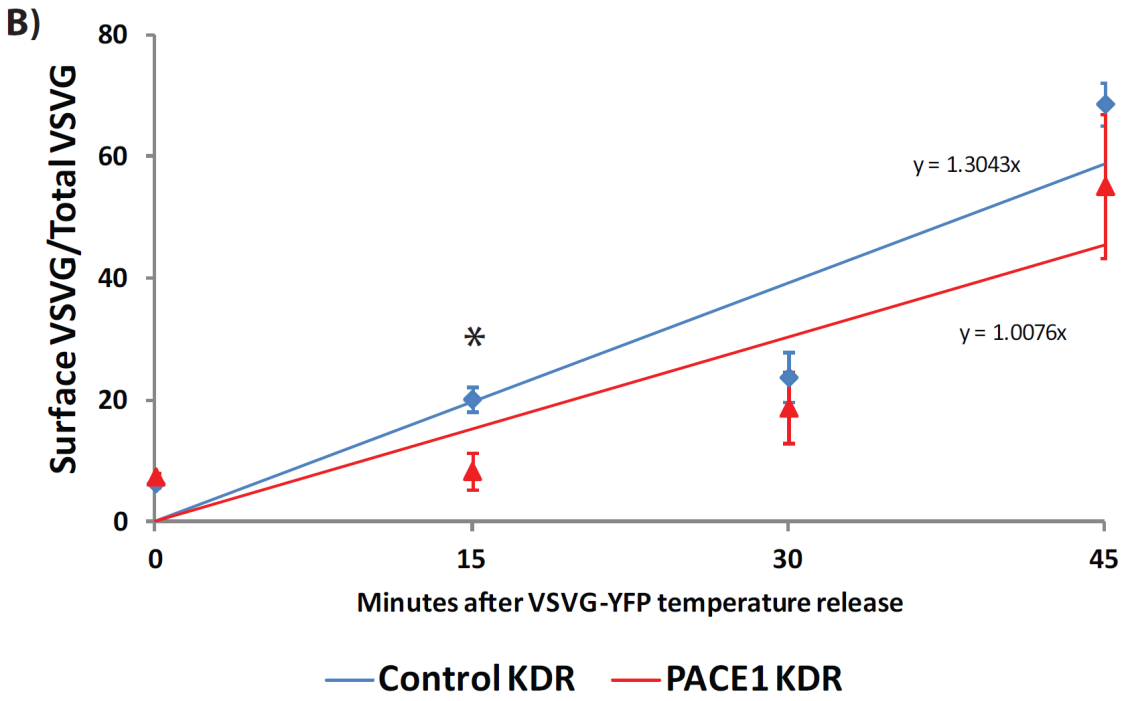

Surface VSVG
VSVG-YFP
Merge
Minutes After Temperature Shift
0′
45′
Control KD
PACE1 KD
0′
45′

Figure 2.13. PACE1 decreases VSVG-YFP secretion kinetics. HeLa cells knocked down with either control or PACE1 siRNA were transfected with VSV -YFP and after 1.5 h of temperature block at 19.5ºC to accumulate VSVG-YFP at the TGN, cells were shifted to 32ºC to permit VSVG-YFP to traffic from the TGN to the plasma membrane. Surface VSVG was detected via anti-VSVG antibodies over non-permeablized cells. **(A)** The ratios of fluorescent intensities of surface VSVG-YFP to total VSVG-YFP were quantitated and a simple linear regression was fitted to the data. Two-tailed P values within timepoints of <0.05, <0.01, and <0.001 are denoted by *, **, and ***, respectively. Error bars indicate SEM of n=3. **(B)** Same as (B) but with rescue via empty pcDNA3 vector (control) or RNAi-resistant PACE1-myc.


A)
Surface VSVG/Total VSVG
50
40
30
20
10
0
0
15
30
45
Min after VSVG temperature release
y = 0.9168x
y = 0.5143x
***
*
**
Control KD
PACE1 KD


B)
Surface VSVG/Total VSVG
80
60
40
20
0
0
15
30
45
Minutes after VSVG-YFP temperature release
y = 1.3043x
y = 1.0076x
*
Control KDR
PACE1 KDR

Although PACE1 was initially identified as a binding partner of ezrin, our results clearly show that its loss by siRNA knockdown results in several structural and functional changes to the Golgi complex. Whether PACE1 serves as a modulator or direct mediator of Golgi membrane tubulation is unclear. Our results showed that knocking down PACE1 does not completely block Golgi membrane tubulation, which supports the idea that PACE1 can serve as a modulator of Golgi membrane tubulation. However, it is possible that, although siRNA-mediated knockdown reduced PACE1 levels by ~90%, the remaining PACE1 is sufficient to mediate some Golgi function.

Interestingly, PACE1's other family members appear to play important but not widely recognized roles in Golgi function. Scyl2/CVAK104 was originally found to be a kinase associated with plasma membrane AP-2 clathrin-coated vesicles [17]; however, recent studies demonstrated that Scyl2/CVAK104 binds to clathrin at the TGN and contributes to AP-1 clathrin-coated vesicle formation for M6PR-mediated delivery of newly synthesized lysosomal enzymes to lysosomes [18-19]. In contrast, Scyl1 was recently shown to be required for COPI-dependent retrograde trafficking from the Golgi complex to the ER [16]. The molecular mechanisms by which PACE1 regulates Golgi structure and function are unknown, but given the functions of other family members, PACE1 may have some influence on coated vesicle formation. Additional studies will be required to dissect its specific role.

PACE1 itself may be regulated, and while little is known about the protein, it has been identified in two separate ubiquitinome studies to be the only member of the Scyl family to be ubiquitinated [32-33]. Its physiological roles would be fascinating to pursue, as its family member, Scyl1, was previously linked with neurodegenerative disorders in mice harboring loss-

of-function mutations in the protein [34]. Interestingly, the functions of Scyl1 are not completely confined within the Golgi, as it has been reported that Scyl1 is also involved in nuclear tRNA export as a regulator at the nuclear pore complex, which raises a possibility that Scyl family members may have diverse functions across multiple organelles [35]. Roles in nuclear transport may not be too surprising, considering that both Scyl1 and PACE1 possess HEAT domain repeats, which have been previously linked to nuclear transport [36]. Redundant, functional roles among Scyl family members are unclear, but it should be noted that Scyl1 knockdown shares a similar Golgi fragmentation phenotype as PACE1 knockdown [16].

The connection, if any, between PACE1's role in regulating Golgi membrane tubulation and its association with ezrin is unknown. Interestingly, the screen also identified LIMK2 as a potential regulator of BFA-induced Golgi membrane tubulation. It was previously reported that LIMK1, along with cofilin, positively regulates membrane tubules from the TGN in a filamentous actin-dependent manner [37]. It is tempting to speculate that LIMK2 may have effects on BFA-induced membrane tubulation, while LIMK1 was not identified in the screen as it primarily tubulates the TGN, which exhibits phenotypes different from those of the Golgi cisternae stack upon BFA treatment [38]. As PACE1 was originally discovered to interact with ezrin, which is involved in linking signal transduction between transmembrane receptors to the actin cytoskeleton, it will be interesting to determine whether the actin cytoskeleton may play important roles in generating membrane tubules [14]. Previous reports support this possibility by showing that membrane tubules can form in an actin-dependent fashion, without requiring microtubules [39-40].

Although this study narrowly focused on a single hit gleaned from the siRNA screen, many other intriguing hits may provide roles in Golgi membrane tubulation and/or Golgi membrane dynamics, including lipid-modifying kinases. Among these are diacylglycerol kinases (DAGKs). Previous reports already suggest that they may be required for the early formation of membrane tubules [41]. However, it remains difficult to definitely conclude how DAGKs may directly participate in forming membrane tubules. DAGKs modulate levels of both diacylglycerol (DAG) and phosphatidic acid (PA), resulting in downstream signal transduction pathways that encompass a diverse expanse of physiological functions [42]. Likewise, the DAGK-related ceramide kinase (CERK) was also identified as a potential regulator of Golgi membrane tubulation and has been observed to localize to the Golgi, and ceramide itself is thought to play important roles in deforming the membrane [43-44]. Other intriguing hits include the family of A-kinase anchoring proteins (AKAPs), which potentially serve to spatially and temporally compartmentalize signal transduction among subcellular domains [45]. Many AKAP proteins remain unstudied but interestingly, one such protein, BFA-inhibited GEF 2 (BIG2), possesses AKAP domains and partially localizes to the Golgi in a way where it may link the cyclic AMP and ARF pathways [46].

This study focused primarily on a single protein, PACE1, whose knockdown inhibited BFA-induced Golgi tubulation. However, the screen also identified proteins whose knockdown enhanced BFA-induced Golgi dispersal (thus, would be negative regulators of Golgi function). A majority of the activating hits correspond to proteins involved in cell cycle regulation or have unknown functions. Hits associated with cell cycle regulation may have unintended side-effects, but this group of putative negative regulators of Golgi function provide many opportunities for further study.

The siRNA screen described here has been very fruitful but several shortcomings should be noted. First, some targets may not have been effectively knocked down by the library siRNAs. Second, many targets of the siRNA library may be specific to certain cell lineages and not be expressed in HeLa cells. Third, our screen examined only BFA-induced membrane tubules of Golgi origin. Other organelles such as endosomes and the ERGIC have been observed to possess dynamic membrane tubules, which were not assayed here [1].

In conclusion, our results yielded both positive and negative regulators of BFA-induced Golgi membrane tubulation. Further studies into these hits may provide additional clues in unraveling the complex regulatory mechanisms behind Golgi membrane tubulation within membrane trafficking.

2.5. Materials and Methods

Reagents and plasmids. BFA and cycloheximide were purchased from Enzo Life Sciences, Inc. Both were dissolved in DMSO. BFA was used at 5 μg/ml for the siRNA screen and at 1 μg/ml for all other experiments. Mammalian expression plasmids for pcDNA3-PACE1-myc were obtained from Dr. A Bretscher (Cornell University). p-ssHRP-FLAG was a gift from Dr. V. Malhotra (Centre for Genomic Regulation, Barcelona, Spain). pIRES-ERGIC-53-GFP was a gift from Dr. R. Scheckman. pEGFP-N1/VSVG-ts045-YFP was a gift from Dr. B. Storrie (University of Arkansas).

Cell culture, transfection, and RNAi. Media and culture reagents were purchased from Invitrogen (Carlsbad, CA). Plastic culture vessels were purchased from Corning (Corning, NY).

Human epithelial (HeLa) cells were grown in modified Eagle's minimal essential medium (MEM) with 10% fetal bovine serum (Life Technologies, CA) at 37ºC, 95% humidity, and 5% CO_2. For incubations at 19.5ºC or 37ºC at atmospheric CO_2, MEM containing $1/10^{th}$ normal sodium bicarbonate levels was used to maintain pH levels. siRNA kinase library (Silencer® Human Kinase siRNA Library V2, AM80010V2, Ambion, Life Technologies, NY). Double-stranded RNA targeting human PACE1 were purchased from GenePharma Co., Ltd. (Shanghai, China) using sequences 5' – GGUUAAUAAAGCUGCCAAGuu – 3' (sense) and 3' - uuCCAAUUAUUUCGACGGUUC - 5' (anti-sense) for PACE1. Library or individual RNA were transfected with Lipofectamine RNAiMax or Lipofectamine2000 in Opti-MEM (Invitrogen, CA), respectively, at a final RNA concentration of 30 nM. Experiments were conducted at least 18 h after the initial RNA transfection. RNAi-resistant constructs of the above were generated using QuickChange II (Agilent Technologies) using two silent mutations within the target siRNA sequence (above).

High throughput siRNA screen. HeLa cells stably expressing GalT-GFP were plated in black 96-well plates with optical-grade clear bottoms (Molecular Devices, CA) at a density of 3,500 cells/well. 24 h later, the cells were transfected with RNA from the Silencer® siRNA kinase library using Lipofectamine RNAiMAX (Invitrogen, CA) in Opti-MEM (Invitrogen, CA). After 5 h of incubation with RNA, media over wells were replaced with MEM with 10% FBS. 18 h later, cells were treated with 5 μg/ml of BFA in serum-free MEM for 10 min at 37ºC, followed by fixation in 3.7% formaldehyde for 10 min at room temperature. After 3 washes with PBS, 0.1% Hoechst stain in PBS was added for 15 min at room temperature to stain for nuclei. After a final PBS wash, plates were sealed with parafilm and stored in PBS at 4ºC until further imaging

on an ImageXpress® Micro XL Widefield HCS System (Molecular Devices, CA) using a 20X air objective with the accompanying MetaXpress imaging software (Molecular Devices, CA).

High throughput imaging and quantitative analysis. MetaXpress software (Molecular Devices, CA) quantitated and imaged >300 cells per well. Granular intensity, defined as average GalT-GFP fluorescence intensity per unit of area, was quantitated per cell. Thresholds were set for minimum granular intensity to measure only intact, compact GalT-GFP structures while excluding dispersed, reticular GalT-GFP structures. The siRNA library was screened three separate times in addition to control (no RNA). Inhibitory hits were determined as possessing >20% average granular intensity when compared to control values while activating hits possessed <-15% average granular intensity when compared to control values. Hits were determined from both combined siRNAs and individual siRNA results.

Fluorescence microscopy and live-cell imaging. Cells were prepared as described previously (Bechler et al., 2010). Either GFP-tagged proteins or secondary antibodies conjugated to fluorescein isothiocyanate (FTIC), tetramethylrhodamine isothiocyanate (TRITC) were used at a dilution of 1:100. Coverslips were mounted over Vectashield (Vector Laboratories, CA) mounting media, sealed with clear nail polish, and stored at -20ºC until further imaging at room temperature. Wide-field epifluorescence was conducted on an Axioscope II (Carl Zeiss, Inc.) with Plan-Apo 40x/NA1.4 air objective mounted to a Orca II camera (Hamamatsu Photonics), utilizing Openlab software (PerkinElmer). Spinning disk confocal images were taken on an Eclipse TE2000-U (Nikon) with Plan-Apo 60x/NA1.4 oil objective with an Ultraview LCI (PerkinElmer), a 1394 ORCA-ER camera (Hamamatsu Photonics) and Ultraview imaging

software (PerkinElmer). Image analysis and Z-projections were conducted with ImageJ version 1.45s software. For live cell imaging, imaging was done at 37ºC using 35 mm tissue culture dishes with cover glass bottoms (World Precision Instruments, Inc., FL) over the Eclipse TE2000-U fitted with a custom-made housing and heater/blower equipped with a thermal regulator set to 37ºC.

HRP secretion assay. In ssHRP secretion experiments, 6 cm dishes of cells were first transfected with p-ssHRP-FLAG for 18 h. Then the dishes were washed and incubated with 2.5 ml MEM containing 10% FBS, where 50 µl media samples were collected every hour. ssHRP-FLAG levels in the media and lysates were assayed using 10 λ of media samples with 50 λ of the substrate, 3, 3', 5, 5'-tetramethylbenzidine (Sigma-Alrich). The reaction was stopped after 10' using 50 λ of 1 M HCl and the plates were quantitated using a SpectraMax Plus384 spectrophotometer (Molecular Devices, CA) with the absorption wavelength of 450nm. Data points were normalized to total HRP expression, as described previously [6].

Golgi phenotype assays. HeLa/GalT-GFP cells were categorized as fragmented as determined by possessing > 4 Golgi fragments. Cells were categorized as tubulated by possessing >3 Golgi membrane tubules. Cell were categorized as intact if the Golgi is neither fragmented, tubulated, or dispersed. Minimum of 100 cells per condition were counted; n = 3.

Cell lysates, antibodies, and immunoblotting. Whole cell lysates were prepared through scraping cells in the presence of RIPA buffer with complete protease inhibitor cocktail (Roche Applied Science, Germany), and then analyzed by SDS-PAGE and Western via PVDF membranes. Mouse anti-myc antibodies were diluted 1:1000 (Cell Signaling Technology, MA). Both rabbit and mouse HRP-conjugated secondary antibodies were diluted 1:20,000 (Jackson

ImmunoResearch, PA). Mouse hybridoma media against an extracellular, ectopic epitope of VSVG-ts045-YFP was a gift from Dr. G. Whittaker (Cornell University) and used without dilution. PACE1 antibody (SAB1407775, Sigma) was obtained from Sigma and used at 1:50 dilution for immunofluorescence and 1:150 dilution for Western blot. Anti-α-tubulin antibody (Sigma) was diluted at 1:10,000 for Western and 1:1000 for immunofluorescence. Anti-ManII and anti-M6PR were produced previously in-lab. Anti-GPP130 (PRB-144C, Covance) was diluted 1:800 for immunofluorescence. Alexafluor-conjugated Phalloidin was obtained as a gift from Dr. Anthon Bretscher (Cornell University). Anti-myc antibody (9E10, Cell Signaling) was diluted 1:1000 for Western blot and 1:100 for immunofluorescence.

VSVG-tsO45-YFP trafficking assay. HeLa cells in dishes with glass coverslips (Mediatech) were transfected with VSVG-ts045-YFP for 5 h and then incubated at 40ºC for 18 h. Media in dishes were then replaced with 20ºC 1/10th bicarb MEM with 10% FBS and cycloheximide (100 μg/ml) and dishes were incubated over 20ºC water bath in a 4ºC room for 1.5 h. Media in dishes were then replaced with 32ºC MEM with 10% FBS and dishes were incubated at 32ºC during collection of samples. Following fixation, coverslips were washed and then immunostained against an ectopically exposed epitope of VSVG without permeablizing cell membranes. The ratio of ectopically trafficked VSVG was determined as ectopic, anti-VSVG fluorescence divided by VSVG-YFP fluorescence using ImageJ version 1.45s software.

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Table 2.1. Annotated list of hits that inhibited BFA-induced Golgi dispersal. HeLa cells stably expressing GalT-GFP were treated with BFA (1 μg/ml) for 10' and then fixed and stained against nuclei with Hoechst. Cells were then quantitated for intact Golgi structures and either individual siRNA oligos or averaged results of all 3 oligos against a single target with averaged values of >20% more intact Golgi structures when compared to no-siRNA control cells were considered hits.

	Green highlighted genes denote that a single siRNA promoted BFA-induced Golgi tubulation by an average greather than 15% (n=3). All non-green genes show average values of all 3 siRNA oligos.
	Gray highlighted genes denote that the average inhibition falls below 15% when subtracting the SEM.

Gene	Avg	SEM	Gene description
AKAP8	-40.1	10.6	A kinase (PRKA) anchor protein 8
RPS6KL1	-34.2	10.9	ribosomal protein S6 kinase-like 1
MAP3K1	-30.1	3.1	mitogen-activated protein kinase kinase kinase 1
CaMKIINalpha	-29.0	16.0	calcium/calmodulin-dependent protein kinase II inhibitor 1
PRKCG	-26.8	5.3	protein kinase C, gamma
ADRBK1	-26.6	7.3	adrenergic, beta, receptor kinase 1
CALM2	-26.3	13.1	calmodulin 2 (phosphorylase kinase, delta)
CNKSR1	-25.5	6.5	connector enhancer of kinase suppressor of Ras 1
ATR	-25.2	8.2	ataxia telangiectasia and Rad3 related
SNARK	-22.4	2.8	NUAK family, SNF1-like kinase, 2
MAP3K6	-21.7	6.7	mitogen-activated protein kinase kinase kinase 6
MAP4K4	-21.7	1.9	mitogen-activated protein kinase kinase kinase kinase 4
NEK11	-21.4	2.1	NIMA (never in mitosis gene a)- related kinase 11
VRK1	-20.7	2.6	vaccinia related kinase 1
PTK9	-20.2	5.0	PTK9 protein tyrosine kinase 9
KIAA1446	-19.8	4.5	KIAA1446
TYK2	-19.0	2.6	tyrosine kinase 2
CSNK1D	-18.7	2.9	casein kinase 1, delta
CaMKIINalpha	-18.5	7.9	calcium/calmodulin-dependent protein kinase II inhibitor 1
MYO3B	-18.4	2.6	myosin IIIB
BTK	-18.4	4.3	Bruton agammaglobulinemia tyrosine kinase
CIB2	-18.2	3.7	calcium and integrin binding family member 2
LY6G5B	-17.8	1.7	lymphocyte antigen 6 complex, locus G5B
PIK3C2G	-17.0	3.9	phosphoinositide-3-kinase, class 2, gamma polypeptide
CHKB	-16.9	6.3	choline kinase beta
DLG3	-15.8	13.5	discs, large homolog 3 (neuroendocrine-dlg, Drosophila)
PTK9	-15.7	5.2	PTK9 protein tyrosine kinase 9
KIAA1765	-14.1	2.8	doublecortin and CaM kinase-like 3
LOC283846	-14.0	9.1	LOC283846
PIK3C2G	-14.0	6.7	phosphoinositide-3-kinase, class 2, gamma polypeptide
AKAP9	-13.3	12.0	A kinase (PRKA) anchor protein (yotiao) 9
MAP3K7	-12.4	4.8	mitogen-activated protein kinase kinase kinase 7
ROCK2	-12.1	4.2	Rho-associated, coiled-coil containing protein kinase 2
KIS	-11.9	3.6	U2AF homology motif (UHM) kinase 1
AKAP7	-11.5	8.4	A kinase (PRKA) anchor protein 7
DGKH	-11.0	3.7	diacylglycerol kinase, eta
STK24	-10.5	4.8	serine/threonine kinase 24 (STE20 homolog, yeast)

Table 2.2. Annotated list of hits that enhanced BFA-induced Golgi dispersal. HeLa cells stably expressing GalT-GFP were treated with BFA (1 μg/ml) for 10' and then fixed and stained against nuclei with Hoechst. Cells were then quantitated for intact Golgi structures and either individual siRNA oligos or averaged results of all 3 oligos against a single target with averaged values of >15% less intact Golgi structures when compared to no-siRNA control cells were considered hits.

	Green highlighted genes denote that a single siRNA promoted BFA-induced Golgi tubulation by an average greather than 15% (n=3). All non-green genes show average values of all 3 siRNA oligos.		
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BTK	-18.4	4.3	Bruton agammaglobulinemia tyrosine kinase
CIB2	-18.2	3.7	calcium and integrin binding family member 2
LY6G5B	-17.8	1.7	lymphocyte antigen 6 complex, locus G5B
PIK3C2G	-17.0	3.9	phosphoinositide-3-kinase, class 2, gamma polypeptide
CHKB	-16.9	6.3	choline kinase beta
DLG3	-15.8	13.5	discs, large homolog 3 (neuroendocrine-dlg, Drosophila)
PTK9	-15.7	5.2	PTK9 protein tyrosine kinase 9
KIAA1765	-14.1	2.8	doublecortin and CaM kinase-like 3
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DGKH	-11.0	3.7	diacylglycerol kinase, eta
STK24	-10.5	4.8	serine/threonine kinase 24 (STE20 homolog, yeast)

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CHAPTER 3

DAG Kinase (DGK)-γ Regulates Golgi Membrane Tubulation

3.1. Abstract

Membrane trafficking is a vital process in sorting and transporting protein and membrane cargo towards numerous membranous compartments within eukaryotic cells. This highly complicated process relies on many different proteins and lipid species in order to maintain constant intracellular traffic. Defects in this process yield numerous disease states, such as neurodegeneration. The most commonly observed trafficking vessels include coated vesicles and membrane tubules. In contrast to coated vesicles, membrane tubules remain relatively understudied but are prominent features of the mammalian Golgi complex. Although previous studies have established critical roles for membrane tubules in trafficking routes, the regulatory mechanism behind membrane tubulation is largely unknown. In this study, we analyzed the involvement of diacylglycerol kinases (DGKs) in membrane tubulation and found that they may participate in Golgi membrane tubule biogenesis. Furthermore, we observed that one specific isoform, DGK-γ, is important for membrane tubulation and its subsequent loss via siRNA knockdown leads to Golgi fragmentation, inhibition of anterograde trafficking of both soluble and transmembrane proteins, and disruption of Golgi ribbon formation. Further studies on DGKs may help shed light on their possible roles as novel regulators of both membrane tubulation and trafficking.

3.2. Introduction

Diacylglycerol (DAG) is phosphorylated by diacylglycerol kinases (DGKs) to yield phosphatidic acid (PA) (Figure 1.7). Both DAG and PA serve as active lipid second messengers

to influence a diverse array of functions in eukaryotic cells [1-2]. DAG is an uncharged lipid that partakes in numerous membrane trafficking roles through recruiting or activating proteins, with examples that include protein kinase C (PKC) at the Golgi complex [3], Munc13-1 at pre-synaptic vesicles [4], ARF GTPase activating protein (ARF GAP) at the Golgi [5], and protein kinase D (PKD) at the *trans*-Golgi network (TGN) [6]. On the other hand, PA has been reported to provide vital roles in membrane fusion during both vesicular secretion [7] and mitochondrion fusion [8], which is possibly attributed to its conically-shaped structure favoring negative membrane curvature [9]. While there has been significant progress in establishing roles for DAG and DGK in membrane trafficking, little is known about their roles in membrane tubulation.

The nine known isoforms of human DGKs are separated into five classes, depending on the shared protein domains (Table 3.1). Although most have unknown or uncharacterized functions, some isoforms have been partially characterized. For example, DGK-α translocates from a diffuse localization to membranous structures upon treatment with the DGK inhibitor R59022 [10] or to the nuclear matrix in rat thymocytes in a phosphorylation-free manner upon treatment with the mitogen concanavalin A [11].

Little information is available about the biochemical regulation of DGKs. For Type I DGKs, calcium is a known modulator. Calcium could serve to activate Type I DGKs through binding to the EF-hand motif, where the EF-hand motifs could be auto-inhibiting the DGKs [12]. Studies have shown certain lipid species, such as phosphatidylserine (PS) can activate Type I DGKs such as DGK-α, Type IV DGKs, and Type V DGK-θ [13] while inhibiting DGK-ε and Type II DGKs [14]. Other lipids such as sphingosine and phosphatidylinositol phosphates (PIPs) can also activate specific DGKs but not others [15]. The mechanisms by which these

	Type	Localization	Notable domains	Functions
DGK-α	I	TGN, endosomes, Golgi, PM, nucleus	Recoverin homology like, EF-hands, C1	Membrane ruffling, apoptosis, Src signal transduction, thymic regulation
DGK-β	I	Membrane structures		Unknown
DGK-γ	I	Nucleus, PM		Highly expressed in brains
DGK-δ	II	Nucleus	PH, C1	Supresses ER-to-Golgi traffic
DGK-η	II	Unknown		
DGK-κ	II	PM	EPAP repeat, PH, C1	
DGK-ε	III	Nucleus	C1,	Competitively inhibited by ceramide
DGK-ζ	IV	Nucleus, cytoplasm	C1, MARCKS, Ank repeat, PDZ-domain-binding	Rac regulator, inflammation, innate immunity, neurite formation, leptin signaling
DGK-ι	IV	Nucleus, cytoplasm		Unknown
DGK-θ	V	Membranes, nucleus	Proline rich, PH, Ras binding	Unknown

Table 3.1. Table of known diacylglycerol kinases, their localization, domains, and functions.

lipids regulate DGK activity remain unknown. Phosphorylation can also play a role in regulating DGKs, as studies have shown that DGK-α, DGK-δ, and DGK-θ, can be phosphorylated by PKC [16] and Src [17]. Phosphorylation of DGKs can have diverse consequences, as PKC-induced phosphorylation of DGK-δ leads to its removal from the PM while phosphorylation of DGK-ζ leads to both its removal from the nucleus and reduced catalytic activity [18-19].

In addition to regulation via phosphorylation and lipids, DGKs can possibly be regulated through protein-protein interactions. All DGKs additionally have C1 domains that closely mirror those of PKC [20] and studies have shown that at least in DGK-θ, the C1 domains may also contribute to kinase activity [21]. Interestingly, C1 domains do not necessarily bestow DAG-binding capabilities among the DGK isoforms, with studies showing that only DGK-β and DGK-γ could bind to DAG analogues such as phorbol esters via their C1 domains [22], while other isoforms utilize them to bind to other proteins [23]. Protein interactions with C1 domains may be more ubiquitous than previously thought, as seven DGK isoforms are capable of binding to β-arrestin [24], most likely through their C1 domains, as it is the only shared domain across all DGK members [25].

Within the scope of membrane trafficking, reports have shown that both DAG and PA may regulate this process, where PA is required for COPI vesicle fission [26] and DAG is required to recruit PKD to the TGN for membrane tubulation and subsequent trafficking to the PM [6, 27]. DAG has also been reported to be required for Golgi membrane tubule formation [28]. Evidence supporting that DGKs are involved in membrane trafficking was provided by previous observations that one particular DGK, DGK-ζ, interacts with sorting nexin 27 (SNX27) to help mediate endosomal recycling [29]. This is quite relevant to membrane tubulation as many sorting nexins are implicated in membrane tubules that are both clathrin-dependent and

independent within the PM and endosomes [30-33], albeit by bin-amphiphysin-rvs (BARs)-dependent mechanisms that involves protein oligomerization.

In a previous high throughput siRNA screen of the human kinome to identify novel regulators of Golgi membrane tubulation and function, we identified several members of the DGK family as strong hits (Chapter 2). We reasoned that DGKs could potentially play roles in membrane tubulation by virtue of their activity in generating PA from DAG, where PLAs, such as $iPLA_1\gamma$, can then cleave cylindrically-shaped PA into conically-shaped lysophosphatidic acid (LPA) to yield membrane tubules [34]. It is additionally possible that the local changes in DAG/PA levels induced by DGKs can facilitate membrane trafficking events.

3.3. Results

3.3.1. DGK-γ Knockdown Inhibits BFA-Induced Golgi Tubulation

To determine whether DGKs may play roles in Golgi membrane tubulation, siRNAs against eight isoforms of DGK (-α, -β, -γ, -δ, -ε, -ι, -θ, and -ζ) were transfected into HeLa cells stably expressing a GFP-fused *trans*-Golgi resident protein, galactosyltransferase (HeLa/GalT-GFP) and subsequently assayed for their effects on inhibiting BFA-induced Golgi dispersal. BFA serves as an excellent model for studying Golgi membrane tubules, as membrane tubules are both highly dynamic and elusive at steady-state levels. Additionally, BFA induces complete loss of intact, juxtanuclear Golgi structures as the result of its hyper-activation of membrane tubules, which subsequently traffic in a retrograde fashion to fuse with ER membranes [35]. As GalT-GFP trafficks from the Golgi complex to the ER, its average fluorescent intensity per area decreases because the mammalian ER possesses five-fold greater membrane surface area than

the Golgi complex [36-37]. We utilized three distinct siRNA oligos against each DGK gene in assessing their ability to affect BFA-induced Golgi loss. Interestingly, knockdown of all eight isoforms appears to hinder BFA-induced Golgi loss with a minimum of 20% inhibition when compared to untransfected control cells (Figure 3.1A). Among the eight isoforms, DGK-γ showed the strongest phenotype with three siRNAs averaging 46% ± 7.9% (standard error of mean, SEM) inhibition of BFA-induced Golgi loss. More specifically, one siRNA exhibited the greatest efficacy with 53.6% ± 14.5% (SEM) inhibition when compared to control cells (Figure 3.1B). Western blot analysis on whole-cell HeLa lysates confirmed that this siRNA reduced levels of transiently expressed GFP-DGK-γ in HeLa cells. Furthermore, transfection with an RNAi-resistant construct (GFP-DGK-γ_R) completely restored GFP-DGK-γ protein levels (Figure 3.2A). DGK-γ knockdown cells displayed ~90% reduced protein levels when compared to control, while overexpression of GFP- DGK-γ_R) yielded similar protein levels as control cells overexpressing GFP-DGK-γ without siRNA transfection (Figure 3.2B).

After observing that DGK-γ knockdown interferes with BFA-induced Golgi loss, we inferred that DGK-γ might be disrupting Golgi membrane tubulation, which BFA robustly promotes. To assess whether DGK-γ may be involved in this process, HeLa/GalT-GFP cells were transfected with siRNA against control (scrambled siRNA) or DGK-γ and then treated with BFA (1 μg/ml), followed by fixation after various time intervals. Within 6 minutes of BFA treatment, almost no control cells harbored intact non-tubulated Golgi structures (Figure 3.3A) but roughly 65% of cells harbored tubulated Golgi membranes (Figure 3.3B) with the remaining 35% cells displaying dispersed Golgi structures (data not shown). In contrast to control cells, DGK-γ knockdown cells displayed significantly more intact non-tubulated Golgi structures at 6

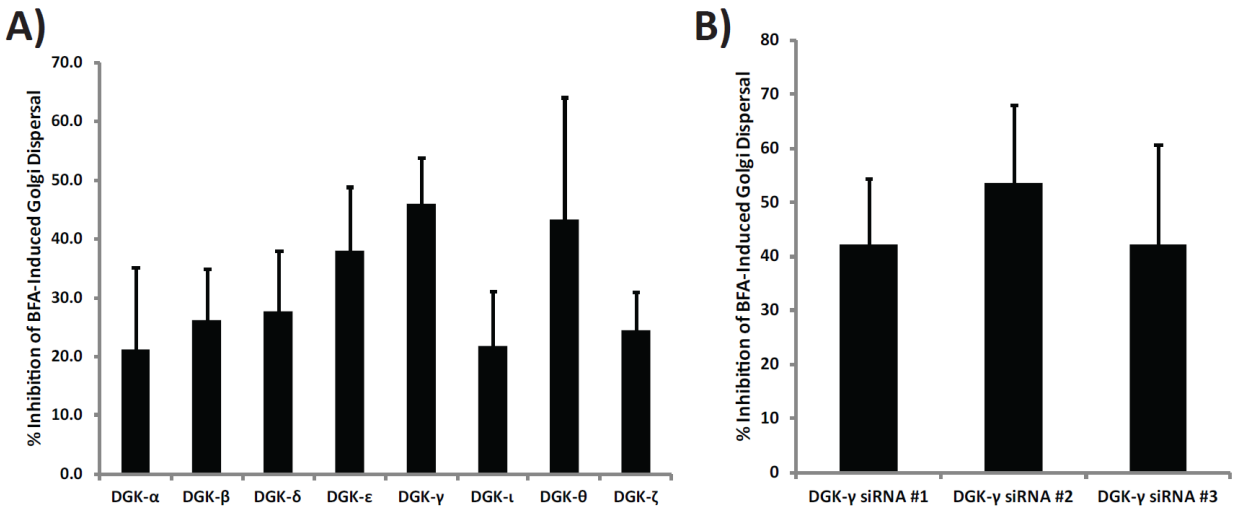


Figure 3.1. Diacylglycerol kinases inhibit BFA-induced Golgi dispersal. (A) HeLa cells stably expressing galactosyltransferase-GFP (GalT-GFP) were transfected with 3 separate and unique siRNAs against 8 different isoforms of DGKs and then treated with BFA (1 μg/ml) for 10' and then fixed. Cells with intact Golgi structures, determined by GalT-GFP fluorescence, were quantitated and averaged after pooling siRNA data against respective DGK isoforms. **(B)** Average inhibition of BFA-induced Golgi dispersal for individual siRNAs against DGK-γ are shown, extracted from same experiments in (A). Error bars represent SEM for n=3. Bar graphs represent mean values.

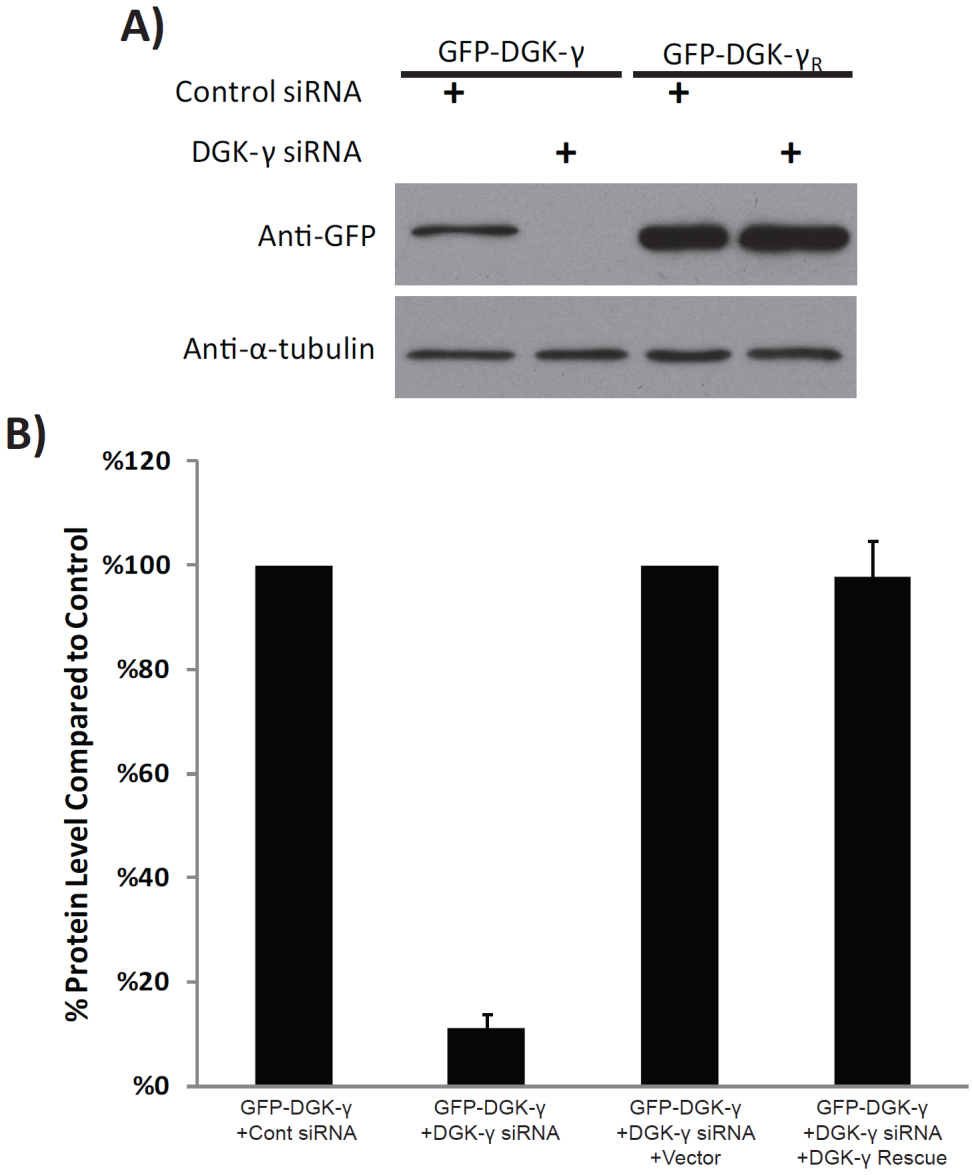


Figure 3.2. DGK-γ siRNA reduces overexpressed GFP-tagged DGK-γ. (A) Western blot analysis of whole cell HeLa lysates transfected with combinations of pEGFP-C3/DGK-γ (GFP-DGK-γ), RNAi-resistant construct (GFP-DGK-γ_R), control siRNA, and DGK-γ-siRNA. **(B)** Densitometry analysis of (A). Error bars indicate SEM for n = 3. Bar graphs represent mean values.

minutes, which was attributed to ~30% of the cells (Figure 3.3A). Similarly, DGK-γ knockdown decreased the number of cells containing tubulated Golgi structures at 6 minutes from 65% as seen in control cells down to ~35% (Figure 3.3B). DGK-γ knockdown clearly slowed down the effects of BFA-induced Golgi tubulation, and these defective phenotypes were significantly reversed upon overexpression of an RNAi-resistant construct that bears silent mutations within the siRNA target site, GFP-fused DGK-γ (GFP-DGK-γ_R) (Figures 3.4A, 3.4B).

3.3.2. DGK-γ Knockdown Disrupts Golgi Structure Homeostasis

After establishing potential roles for DGK-γ in Golgi membrane tubulation, we next sought to determine whether DGK-γ knockdown of Golgi membrane tubulation could affect the structural homeostasis of the Golgi complex. Previous reports have shown that Golgi membrane tubules are required for intra-cisternal continuities within the Golgi [38-39] and for the assembly of an intact Golgi cisternal ribbon from fragmented Golgi mini-stacks [40-41]. In the latter circumstance, BFA has proven to be a useful model system because it can be washed out from cells to readily induce the formation of Golgi mini-stacks from the ER [42], which subsequently form an intact Golgi ribbon in a PLA_2- and membrane tubule-dependent manner [39].

We therefore investigated whether DGK-γ knockdown could disrupt the reformation of an intact Golgi ribbon following BFA washout from cells. HeLa/GalT-GFP cells were transfected with siRNA against either control or DGK-γ and then treated with BFA (1 μg/ml) for 20 minutes. After washing the drug from the culture vessels, the cells underwent recovery and Golgi reformation over 90 minutes. Control cells were able to completely reform intact, juxtanuclear Golgi ribbon structures, but DGK-γ knockdown cells exhibited significant defects in Golgi ribbon reformation, showing fragmented Golgi mini-stacks in the form of scattered puncta

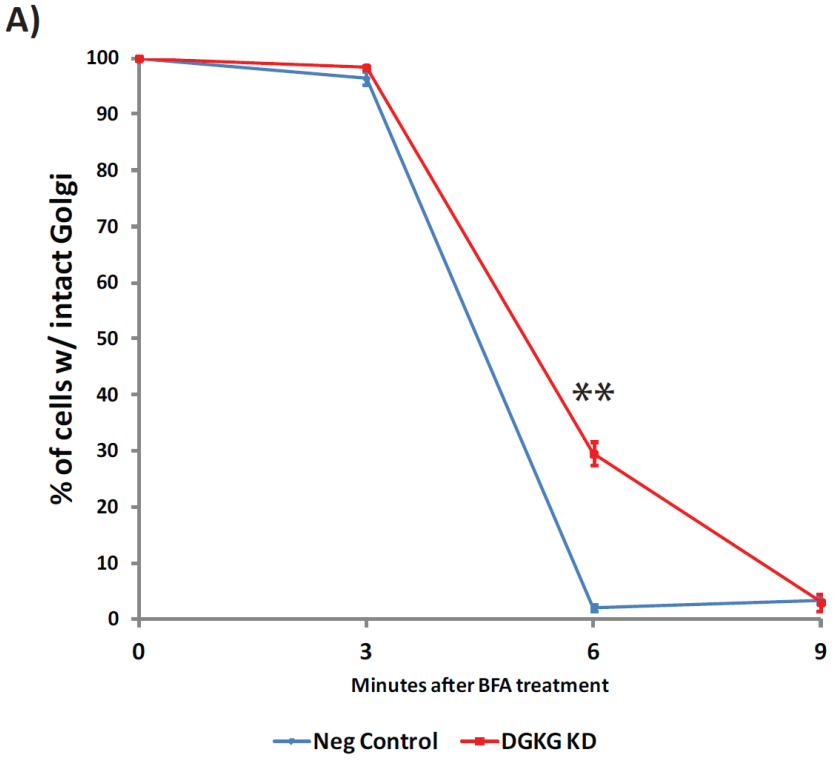
throughout the cell (Figure 3.5A). Knocking down DGK-γ significantly increased fragmented Golgi structures after BFA washout relative to control cells (Figure 3.5B). Time-lapse imaging clearly showed that Golgi mini-stacks were able to form for both control and DGK-γ knockdown cells but in DGK-γ cells, Golgi mini-stacks failed to coalesce into an intact Golgi ribbon structure (Figure 3.5D). Furthermore, DGK-γ knockdown interfered with Golgi structural homeostasis, as HeLa/GalT-GFP cells transfected with DGK-γ siRNA also exhibited a similar increase in Golgi fragmentation relative to control cells even without the use of BFA (Figure 3.5C).

The Golgi fragments induced by DGK-γ knockdown were confirmed to be mini-stacks, as the *trans*-Golgi marker, GalT-GFP, co-localized with both *cis*-Golgi marker GPP130 and *medial*-Golgi marker, ManII (Figure 3.6). Previous studies have shown that disruptions in cytoskeletal processes can lead to Golgi fragmentation, including microtubule depolymerization [43], filamentous actin (F-actin) polymerization [44], microtubule motor complexes [45], and myosin motors [46]. However, cytoskeletal defects in DGK-γ knockdown cells were excluded, as both microtubule- and actin-filaments appear normal when compared to control cells (Figure 3.6). Similarly, coat proteins such as β-COP and AP1 remain primarily co-localized within GalT-GFP labeled Golgi membranes regardless of DGK-γ knockdown (Figure 3.6).

3.3.3. DGK-γ Knockdown Disrupts Membrane Trafficking

Numerous studies have established a requirement for Golgi membrane tubules within the context of membrane trafficking, particularly the secretion of protein and membrane cargo passing through the Golgi cisternae stacks [47-48]. As our results implicated DGK-γ in Golgi membrane tubulation, we next explored whether DGK-γ knockdown leads to significant defects

Figure 3.3. DGK-γ knockdown inhibits BFA-induced Golgi membrane tubulation. (A) HeLa cells were transfected with siRNA against control or DGK-γ for 18 h and then treated with BFA for varying intervals. Cells were categorized under Golgi phenotypes corresponding to intact non-tubulated Golgi or **(B)** tubulated Golgi (as possessing >3 Golgi membrane tubules). Error bars indicate SEM of n=3. Two-tailed P-values of <0.01 are denoted by **.


A)
100
90
80
70
60
50
40
30
20
10
0
% of cells w/ intact Golgi
**
0
3
6
9
Minutes after BFA treatment
Neg Control
DGKG KD

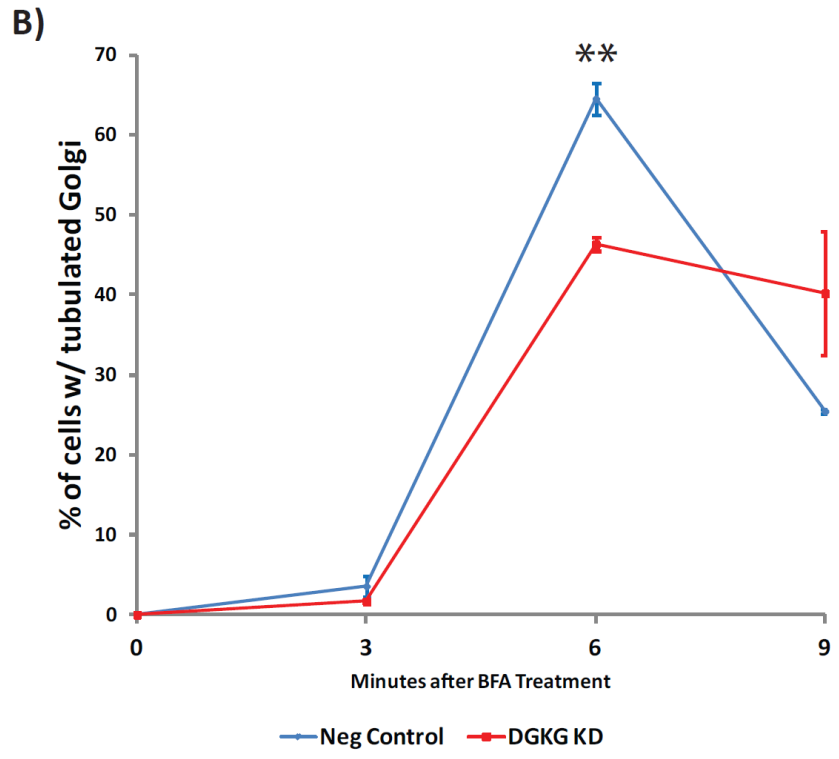
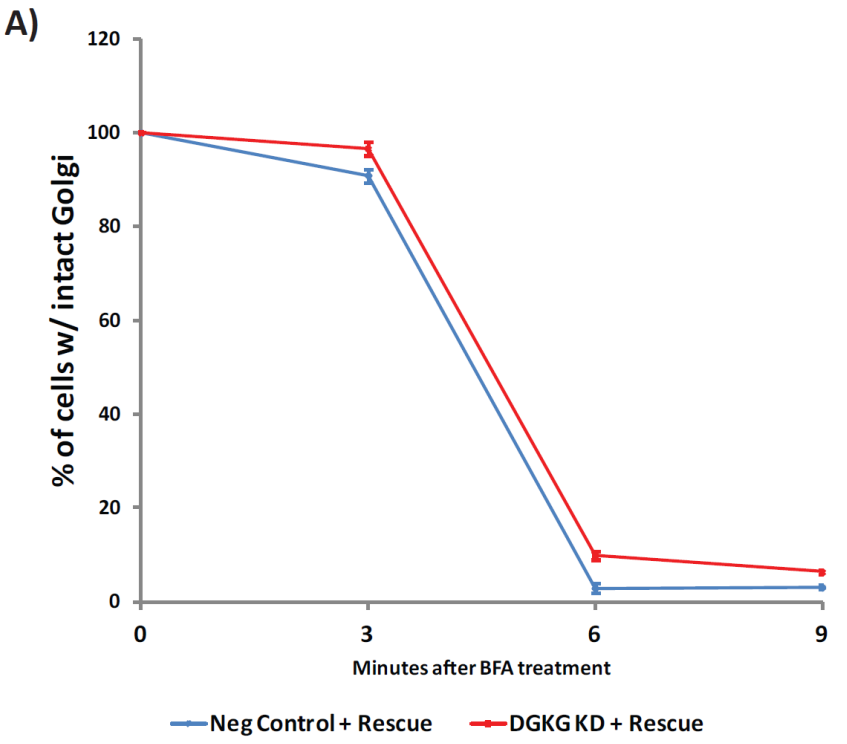
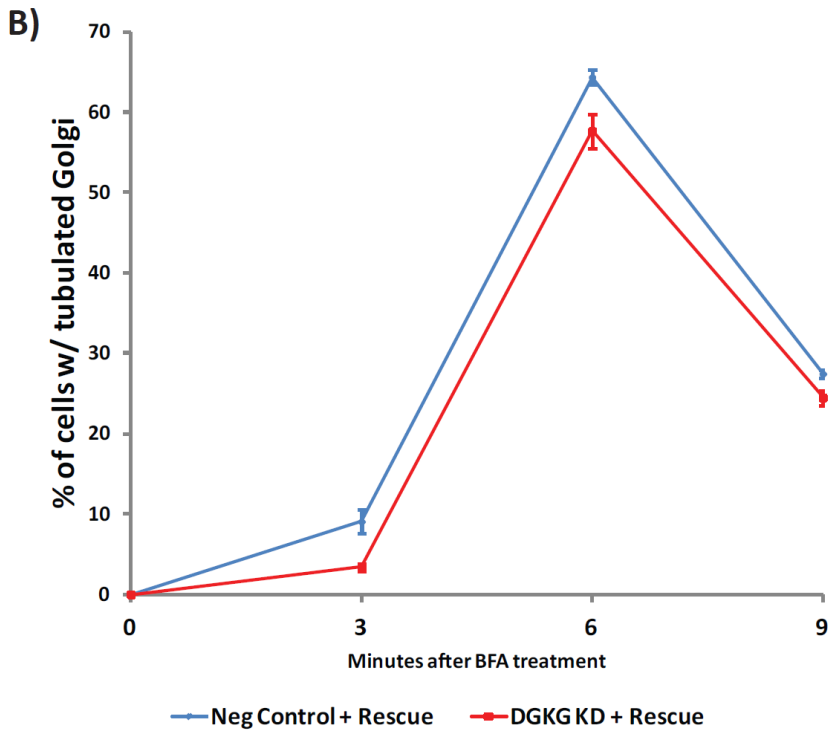

B)
70
60
50
40
30
20
10
0
% of cells w/ tubulated Golgi
**
0
3
6
9
Minutes after BFA Treatment
Neg Control
DGKG KD

Figure 3.4. DGK-γ rescue restores BFA-induced Golgi membrane tubulation in DGK-γ knockdown cells. A) HeLa cells were transfected with siRNA against control or DGK-γ and empty pEGFP vector or RNAi-resistant GFP-DGK-γ for 18 h. Cells were then treated with BFA for varying intervals. Cells were categorized under Golgi phenotypes corresponding to intact non-tubulated Golgi or (B) tubulated Golgi (as possessing >3 Golgi membrane tubules). Error bars indicate SEM of n=3. Two-tailed P-values of <0.01 are denoted by **.


A)
120
100
80
60
40
20
0
% of cells w/ intact Golgi
0
3
6
9
Minutes after BFA treatment
Neg Control + Rescue
DGKG KD + Rescue


B)
70
60
50
40
30
20
10
0
% of cells w/ tubulated Golgi
0
3
6
9
Minutes after BFA treatment
Neg Control + Rescue
DGKG KD + Rescue

in membrane trafficking from the Golgi. We therefore utilized an expression construct of secretory horseradish peroxidase (ssHRP), which is secreted from the cell while maintaining its catalytic activity to permit quantification of the protein within the media [49]. HeLa cells were transfected with ssHRP along with siRNA control or DGK-γ and were permitted to secrete ssHRP into fresh media. HeLa cells with DGK-γ knocked down exhibited significant decreases of ssHRP within the media over time, with secretion rates roughly 30% slower than control cells (Figure 3.7A). This defect in secretion was effectively restored through rescue with an overexpression construct of RNAi-resistant GFP-DGK-γ (Figure 3.7B).

Studies have additionally shown that Golgi membrane tubules serve prominent roles in membrane trafficking from the TGN, particularly cargo exit from the TGN destined for the plasma membrane and endosomes [50-51]. We next explored whether DGK-γ knockdown could impact membrane traffic exiting the TGN through the use of a temperature-sensitive mutant version of tsO45-VSVG-YFP (VSVG-YFP). HeLa cells were transfected with the construct for this protein along with siRNA against control or DGK-γ and then subjected to a series of aforementioned temperature blocks in order to gauge TGN-to-plasma membrane trafficking. Surface expressed VSVG levels were quantitated with the use of anti-VSVG antibodies on non-permeablized cells. After 45 minutes from the point of TGN release (shift from 20ºC to 32ºC), VSVG-YFP was present on the plasma membrane, as determined by labeling with anti-VSVG antibodies, for both control and DGK-γ knockdown cells (Figure 3.8). However, DGK-γ knockdown cells experienced a significant delay in trafficking VSVG-YFP from the TGN, as relatively large amounts of the protein were still visible within the TGN (Figure 3.8). VSVG-

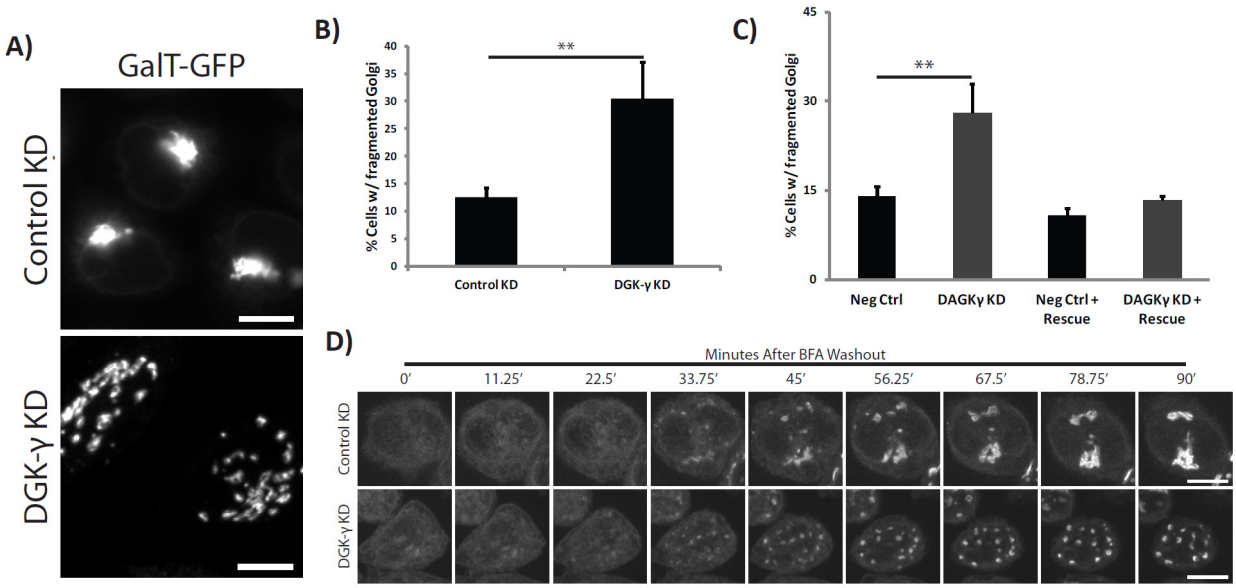
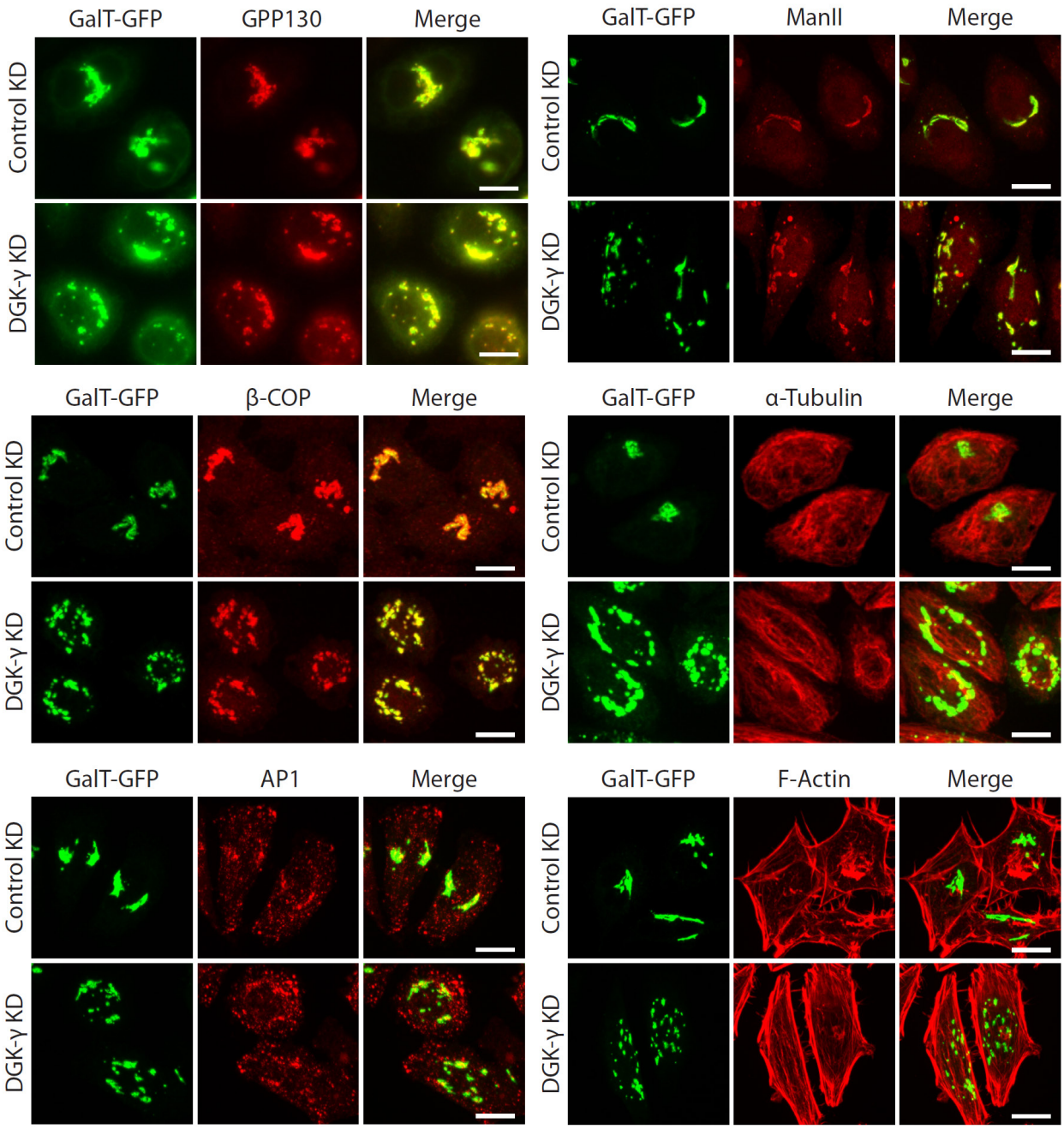


Figure 3.5. DGK-γ knockdown disrupts Golgi structure homeostasis. (A) HeLa cells stably expressing GalT-GFP were transfected with siRNA against either control or DGK-γ and then treated with BFA (1 μg/ml) for 20 min, followed by drug washout and recovery in serum medium for 90 min at 37ºC. Scale bar represents 10 μm. **(B)** Cells from (A) were quantified as containing fragmented Golgi structures, as determined by possessing >4 Golgi fragments per cell and displayed as percentage of entire populations. **(C)** HeLa cells stably expressing GalT-GFP were transfected with siRNA against control or DGK-γ for 18 h and then assayed for fragmented Golgi structures as in (B) but without any BFA usage. Error bars in (B) and (C) indicate SEM of n=3. Two-tailed P-values of <0.01 are denoted by **. **(D)** Live-cell imaging of cells as in (A), using a Z-axis projections of slices throughout whole cells. Scale bar represents 10 μm.

Figure 3.6. DGK-γ knockdown-induced Golgi fragments co-localize with Golgi markers and coat proteins, while the cytoskeleton remains intact. HeLa cells stably expressing the *trans*-Golgi marker, GalT-GFP, were transfected with siRNA against either control or DGK-γ for 18 h and then labeled with immunofluorescence against Golgi markers such as *cis*-Golgi GPP130, *medial*-Golgi mannosidase-II (ManII), and *trans*-Golgi network (TGN) marker mannose6-phosphate receptor (M6PR). Scale bar represents 10 μm.


GalT-GFP
GPP130
Merge
Control KD
DGK-γ KD
GalT-GFP
ManII
Merge
Control KD
DGK-γ KD
GalT-GFP
β-COP
Merge
Control KD
DGK-γ KD
GalT-GFP
α-Tubulin
Merge
Control KD
DGK-γ KD
GalT-GFP
AP1
Merge
Control KD
DGK-γ KD
GalT-GFP
F-Actin
Merge
Control KD
DGK-γ KD

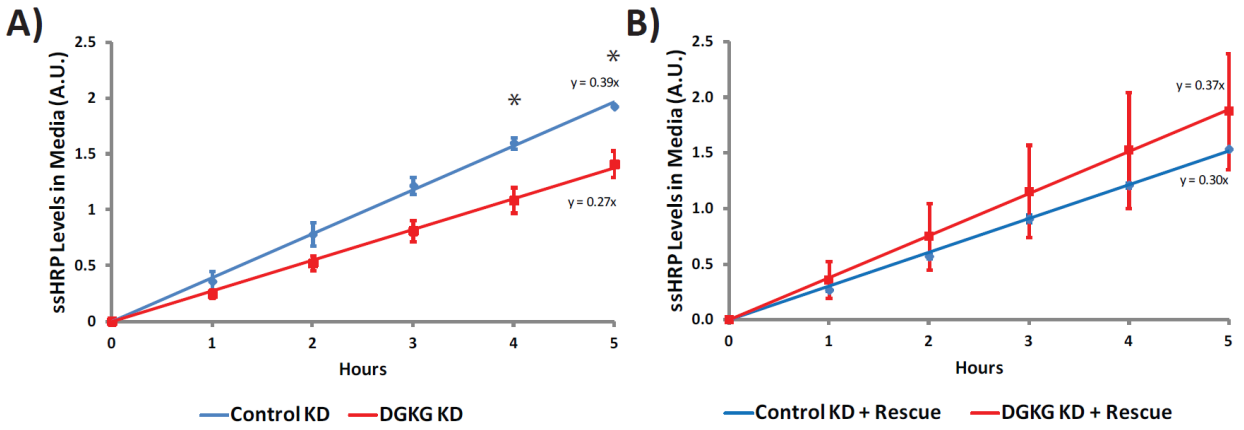


Figure 3.7. DGK-γ knockdown disrupts ssHRP anterograde trafficking. (A) Simple linear regression curves fitted to measured protein levels of ssHRP secreted into the media by HeLa cells transfected with siRNA against control or DGK-γ. Equations of regression curves are shown. Two-tailed P-values of <0.05 are indicated by *. **(B)** Same as in (A) but with rescue using empty pEGFP or RNAi-resistant GFP-DGK-γ for control and DGK-γ knockdown cells, respectively. Error bars indicate SEM of n=3. Data points represent mean values.

YFP secretion could be quantitated through the simple measurement of the ratio of fluorescence of ectopically labeled VSVG versus that of VSVG-YFP. Using this method, DGK-γ knockdown cells showed a significant delay in VSVG-YFP secretion, when compared to control cells, by 45 minutes after temperature release from the TGN (Figure 3.9).

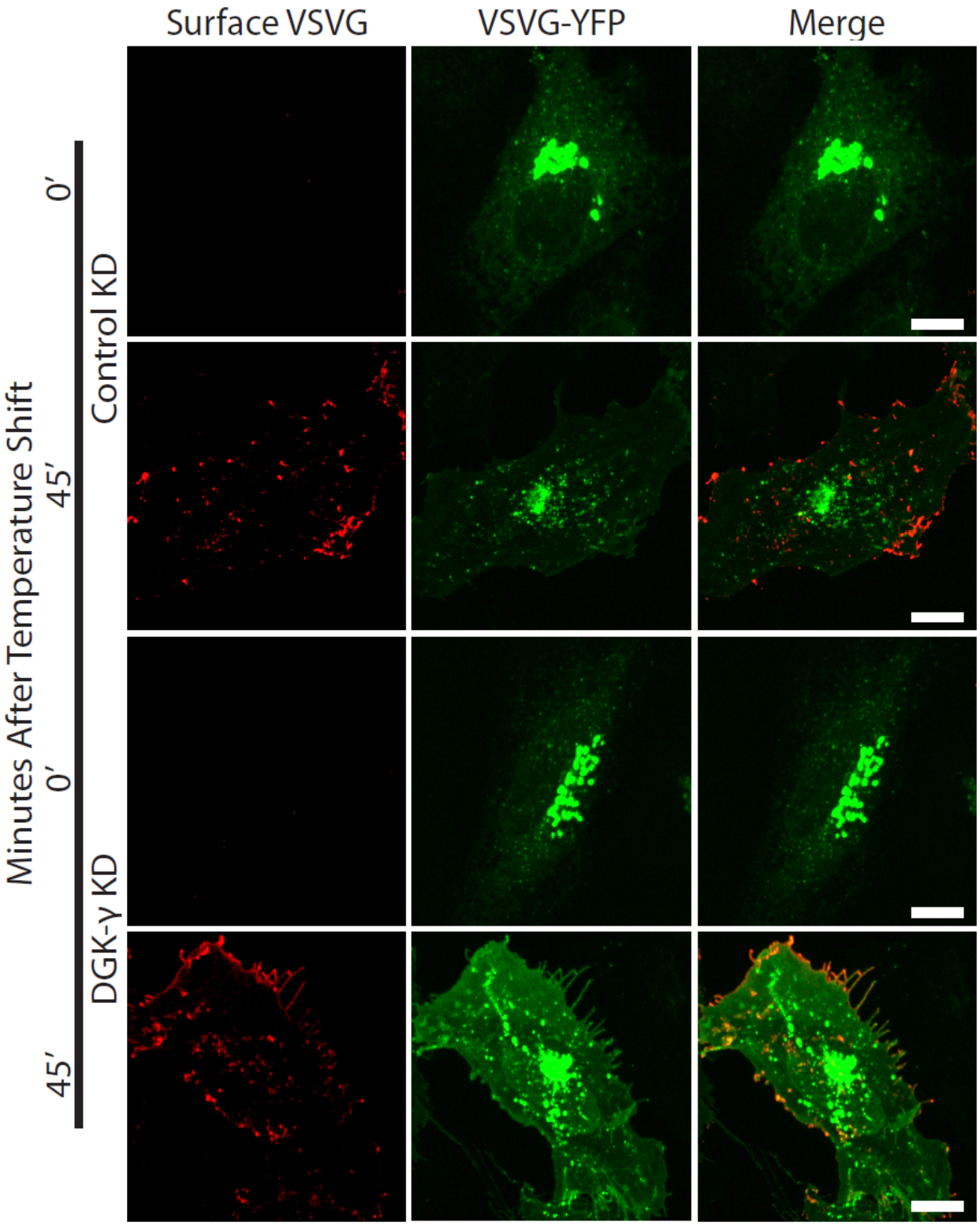
3.4. Discussion

In this study, we demonstrated that siRNAs against multiple DGKs inhibited BFA-induced Golgi dispersal. We then focused on a single member of this family, DGK-γ, and showed that knocking down DGK-γ expression resulted in Golgi fragmentation, Golgi reformation defects, and decreases in anterograde secretion of both soluble and transmembrane protein cargo, all possibly the result of its involvement in membrane tubulation. The results of the knockdown experiments are likely not due to unintended side effects as rescue via an RNAi-resistant, GFP-tagged DGK-γ construct restored all of these phenotypes.

Knockdown of DGK-γ did not yield complete inhibition of tubulation, which could be caused by or result from several factors. First, although knockdown was extensive (~90%), the remaining DGK-γ could provide enough enzymatic activity to influence membrane tubule formation. Second, other DGK isoforms may play redundant roles, which would be consistent with our siRNA screen results. And, third, DGK-γ may not directly induce membrane tubulation but instead serve as a modulator.

Interestingly, previous reports observed that DGK-γ was soluble but primarily associated with the cytoskeleton in Purkinje cells [52]. This conflicts with other reports proposing that the

Figure 3.8. DGK-γ knockdown decreases VSVG-YFP TGN to PM trafficking. HeLa cells were transfected with ts045-VSVG-YFP (VSVG-YFP) along with siRNA against either control or DGK-γ and then incubated at 40ºC to sequester VSVG-YFP within the ER. A temperature shift to 20ºC for 3 h blocks VSVG-YFP within the *trans*-Golgi network (TGN) and a temperature shift to 32ºC then permits VSVG-YFP to traffick to the plasma membrane (PM). All images show cells at 0 or 45 min at 32ºC. Scale bar represents 10 μm.


Surface VSVG
VSVG-YFP
Merge
Minutes After Temperature Shift
0′
45′
Control KD
0′
45′
DGK-γ KD

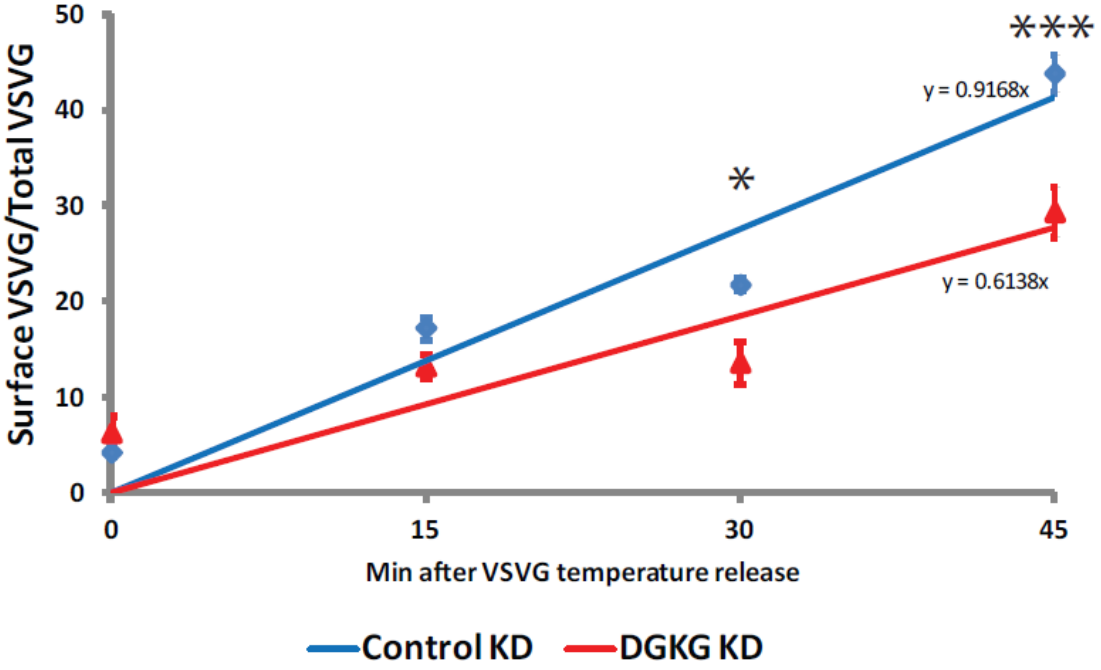


Figure 3.9. DGK-γ knockdown decreases VSVG-YFP secretion kinetics. HeLa cells were transfected with ts045-VSVG-YFP (VSVG-YFP) along with siRNA against control or DGK-γ and then incubated at 40ºC to sequester VSVG-YFP within the ER. Cells were then shifted to 20ºC for 3 h to accumulate VSVG-YFP into the *trans*-Golgi network (TGN), where a subsequent temperature shift to 32ºC allowed VSVG-YFP to traffick from the TGN to the plasma membrane (PM). VSVG on the plasma membrane was then detected using an anti-VSVG antibody which recognizes an ectopic epitope, and antibody incubations were carried out in non-permeablized cells. The mean ratio of anti-VSVG fluorescence over VSVG-YFP fluorescence were plotted alongside a simple linear regression curve fitted to the data. Equations represent slopes of regression curves. Error bars indicate SEM of n=3. Two-tailed P-values of <0.001 are denoted by ***.

entire C1 domain of DGK-γ acts as a nuclear localization sequence (NLS) [53]. However, this can be reconciled through differences between cell types.

Formulating a model for DGK-γ in membrane tubulation becomes quite complicated as both its lipid substrates and products may partake in membrane trafficking, and sometimes even in similar processes. For example, DAG and PA have been shown to be essential for fission of membranes from the TGN [6] and COPI vesicles from *cis*-Golgi cisternae [26], respectively, and both can induce membrane fusion events [7, 54]. Their redundant roles in membrane fission and fusion have been suggested to be the result of their strongly conical shapes [55]. Loss of DGK-γ could possibly decrease PA levels and therefore lower membrane tubule abundance. Numerous studies have focused on the roles of phospholipase D (PLD), which removes the choline head group from phosphatidylcholine (PC) to generate PA, and how the subsequent pool of PA is important for many membrane trafficking events [56]. Similarly, disruption of DGK-γ could conceivably yield similar phenotypes as PLD defects, as both would result in decreased PA levels. However, substrate availability for both PLD and DGK may be important in deciphering whether PA origin is significant and if so, PLD may possibly generate more PA than DGK, as PC makes up half of all Golgi membranes while DAG levels are very small [57]. More studies are required on DGKs in order to establish a clearer role for them within the scope of membrane trafficking and tubulation.

3.5. Materials and methods

Materials. BFA and cycloheximide were purchased from Enzo Life Sciences, Inc. Both were dissolved in DMSO. Mammalian expression plasmid for pEGFP-C3-GFP-DGKγ was obtained

from Harvard PlasmID. pSS-HRP-FLAG was a gift from Dr. V. Malhotra (Centre for Genomic Regulation, Barcelona, Spain). pIRES-ERGIC-53-GFP was a gift from Dr. R. Scheckman.

Cell culture, transfection, and RNAi. Media and culture reagents were purchased from Invitrogen (Carlsbad, CA). Plastic culture vessels were purchased from Corning (Corning, NY). Human epithelial (HeLa) cells were grown in modified Eagle's minimal essential medium (MEM) with 10% fetal bovine serum (Life Technologies, CA) at 37ºC, 95% humidity, and 5% CO_2. For incubations at 19.5ºC or 37ºC at atmospheric CO_2, MEM containing $1/10^{th}$ normal sodium bicarbonate levels was used to stabilize media pH levels.

siRNAs against all DGK isoforms were obtained from an siRNA kinase library (Silencer® Human Kinase siRNA Library V2, AM80010V2, Ambion, Life Technologies, NY). Additional double-stranded RNA targeting human DGKγ was purchased from GenePharma Co., Ltd. (Shanghai, China) using sequences 5' – GGGUGGGAGCCUCAAACAAtt – 3'. RNA was transfected with Lipofectamine RNAiMax or Lipofectamine2000 in Opti-MEM (Invitrogen, CA), respectively, at a final RNA concentration of 30 nM. Experiments were conducted 24 h after the initial RNA transfection. RNAi-resistant constructs of the above were generated using QuickChange II (Agilent Technologies) using two silent mutations within the target siRNA sequence (above).

Fluorescence microscopy and live-cell imaging. Cells were prepared as described previously (Bechler et al., 2010). Either GFP-tagged proteins or secondary antibodies conjugated to fluorescein isothiocyanate (FTIC), tetramethylrhodamine isothiocyanate (TRITC) were used at a dilution of 1:100. Coverslips were mounted over Vectashield (Vector Laboratories, CA)

mounting media and stored at -20ºC until further imaging at room temperature. Wide-field epifluorescence was conducted on an Axioscope II (Carl Zeiss, Inc.) with Plan-Apo 40x/NA1.4 air objective mounted to a Orca II camera (Hamamatsu Photonics), utilizing Openlab software (PerkinElmer). Spinning disk confocal images were taken on an Eclipse TE2000-U (Nikon) with Plan-Apo 60x/NA1.4 oil objective with an Ultraview LCI (PerkinElmer), a 1394 ORCA-ER camera (Hamamatsu Photonics) and Ultraview imaging software (PerkinElmer). For live cell imaging, imaging was done at 37ºC using 35 mm tissue culture dishes with cover glass bottoms (World Precision Instruments, Inc., FL) over the Eclipse TE2000-U fitted with a custom-made housing and heater/blower equipped with a thermal regulator set to 37ºC.

HRP secretion assay. In ssHRP secretion experiments, 6 cm dishes of cells were first transfected with p-ssHRP-FLAG for 24 h. Then the dishes were washed with and incubated with 2.5 ml MEM containing 10% FBS, where 50 ul media samples were collected every hour. ssHRP-FLAG levels in the media and lysates were assayed via 3, 3', 5, 5'-tetramethylbenzidine (Sigma-Alrich) and normalized to total HRP expression, as described previously [58].

Cell lysates, antibodies, and immunoblotting. Cells were lysed by scraping dishes in the presence of RIPA buffer with complete protease inhibitor cocktail (Roche Applied Science, Germany). Lysates were analyzed by SDS-PAGE, transferred onto PVDF membranes (Whatman Inc., United Kingdom). Rabbit antibodies against GFP were diluted 1:1000 and were a gift from Dr. A. Bretscher (Cornell University). HRP-conjugated secondary antibodies against rabbit and mouse were diluted 1:20,000 (Jackson ImmunoResearch, PA).

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APPENDIX 1

Identifying regulatory mechanisms of PAFAH Ib

A1.1. Introduction

Lipid remodeling enzymes such as phospholipases and lysophospholipid acyltransferases play important roles in generating membrane curvature to induce membrane tubulation at organelles such as the Golgi complex and endosomes. Although recent studies have discovered numerous lipid remodeling enzymes to be involved in membrane tubulation, no direct proof is available on how lipid remodeling enzymes can induce membrane tubulation strictly alone through their catalytic activity. One method in proving their involvement in membrane tubulation includes a reconstitution assay that utilizes purified proteins to establish the minimal machinery involved in membrane tubulation. This study examines the regulatory mechanisms behind one particular phospholipase, called platelet activating factor acetylhydrolase (PAFAH) Ib, to establish the ideal requirements for an *in vitro* reconstitution assay.

A1.2. Results

A1.2.1. PAFAH Ib α1 binds to PI3P and PI4P monolayers

PAFAH Ib was previously identified as an important mediator in membrane tubulation and trafficking at the Golgi and endosomes [1-2]. The exact mechanism by which PAFAH Ib α subunits are targeted to the Golgi or endosomes is unclear, but apparently it does not require their catalytic activities [2]. Phosphoinositides are possible suspects as previous studies showed that they play important roles in serving as lipid signposts for the Golgi and endosomes to facilitate membrane trafficking events [3-5]. More specifically, phosphatidylinositol-4-phosphate (PI4P) and phosphatidylinositol-3-phosphate (PI3P) are enriched within the Golgi

complex [6-7] and endosomes [4], respectively. The trafficking roles of PIPs may be more promiscuous than currently believed, as recent studies have linked phosphoinositide-3-kinase (PI3K) to secretion from the TGN as well [8]. The roles of PIPs in membrane tubulation is unclear, but one study reported that a Golgi protein, GOLPH3, binds to PI4P at the TGN to link it to an unconventional myosin and filamentous actin to facilitate membrane tubule and vesicle formation [9].

To investigate whether particular PIP species can recruit PAFAH Ib onto membranes, monolayers of PIPs were spotted onto nitrocellulose membranes, along with other lipids and cholesterol. Subsequent incubation with purified PAFAH Ib α subunits, followed by standard Western immunoblotting protocol, revealed that PAFAH Ib α1 reproducibly binds to both PI3P and PI4P (Figure A1.1).

A1.2.2. PAFAH Ib α1 binds to PI3P and PI4P liposomes

Although PAFAH Ib was capable of binding to PI3P and PI4P on nitrocellulose membranes (PIP strips), this did not reflect the physiological composition of either Golgi or endosomal membranes, both of which have large molar percentages of other lipids such as phosphatidylcholine (PC) [10]. To resolve this issue, unilamellar liposomes were formulated with PC:PS:PIP at a ratio of 80:20:0, 79:20:1, 75:20:5, and 70:20:10, where PIP species were increased at the expense of PC. After addition of purified hemagglutinin (HA)-tagged PAFAH Ib α1 or α2, liposomes were spun down, separated from the supernatant, and then subjected to Western blotting. Both α1-HA and α2-HA bound to PI3P and PI4P liposomes with an affinity that correlates with PIP molar percentage. Likewise, both α1 and α2 presence within the

supernatant decreased as increasing amounts bound to liposomes (Figure A1.2). This affinity is most likely specific, as both α1 and α2 failed to bind to liposomes containing PI(3,4)P_2 (Figure A1.3).

A1.2.3. PAFAH Ib α1 binds to other proteins

Previous studies showed that bovine brain cytosol (BBC) possessed Golgi tubulation activity [11] and that further biochemical fractionation of BBC yielded a final gel filtration (GF) fraction that had >5,400 fold enrichment in tubulation activity. Within this GF fraction, all three subunits of PAFAH Ib were detected via MALDI-TOF and Western blotting. However, not all proteins were able to be identified, including proteins between 66 and 45 kDa. Any combination of PAFAH Ib subunits is incapable of inducing Golgi membrane tubulation and requires a sub-threshold amount of BBC. This implied that PAFAH Ib α subunits required other proteins and/or cofactors present within the BBC. To identify potential protein partners, HeLa cells were transfected with a α1-HA and α2-HA constructs. After whole cell lysate preparation, the HA epitope was immunoprecipitated and then analyzed through SDS-PAGE and silver staining. The silver stained gel showed copious amounts of α1-HA protein along with heavy and light chains of the anti-HA antibody (Figure A1.4). Four specific bands were observed in the α1-HA lane, and two of these bands fall within the unidentified proteins range within the GF fraction. Thus, it is likely that the tubulating activity of the GF fraction is due to a complex formed by PAFAH Ib α1/α2 along with their bound protein partners.

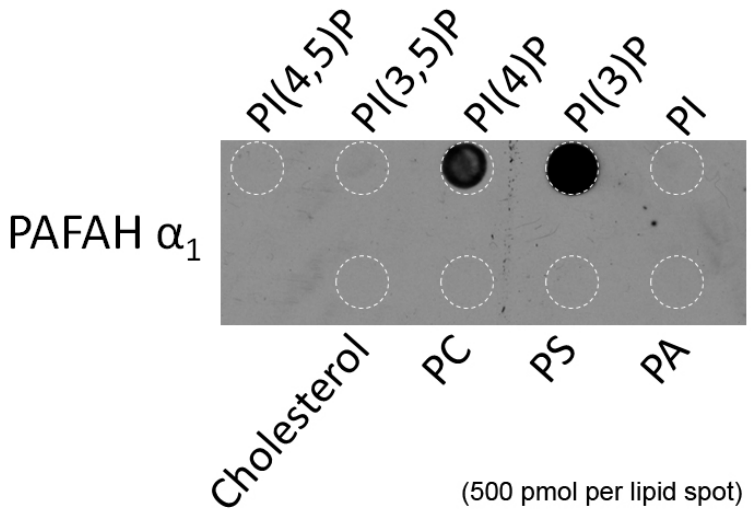

Figure A1.1. PAFAH Ib α1 specifically binds to PI4P and PI3P. 500 pmol of lipids or cholesterol were spotted onto nitrocellulose membranes and permitted to dry. The membrane was then incubated with purified PAFAH Ib α1 and processed using a standard Western blotting protocol. PAFAH Ib α1 was then detected using anti-α1 antibody. This figure was published by Bechler, M. E., Doody, A. M., Ha, K. D., Judson, B. L., Chen, I., Brown, W. J. "The phospholipase A enzyme complex PAFAH Ib mediates endosomal membrane tubule formation and trafficking," *Mol Cell Bio* (2011) 22:2348-59.

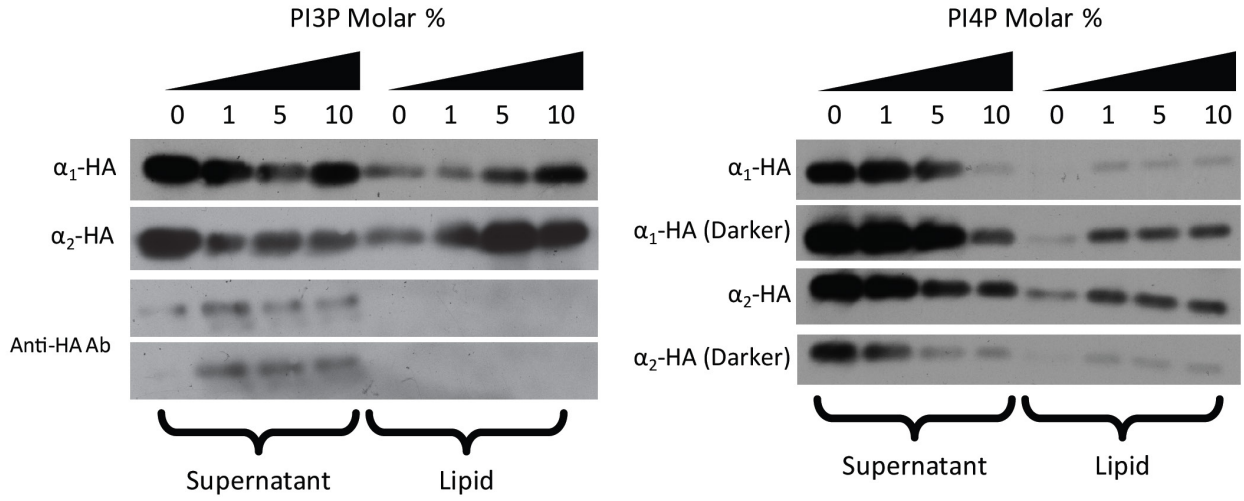

Figure A1.2. PAFAH Ib α1-HA and α2-HA specifically bind to PI3P- and PI4P-containing liposomes. Purified PAFAH Ib α1-HA and α2-HA were added to liposome mixtures containing PC:PS:PI3P/PI4P at 80:20:0, 79:20:1, 75:20:5, and 70:20:10 molar ratios. Liposomes were then pelleted, and both pellet and supernatant fractions were analyzed via SDS-PAGE/Western using anti-HA antibodies.

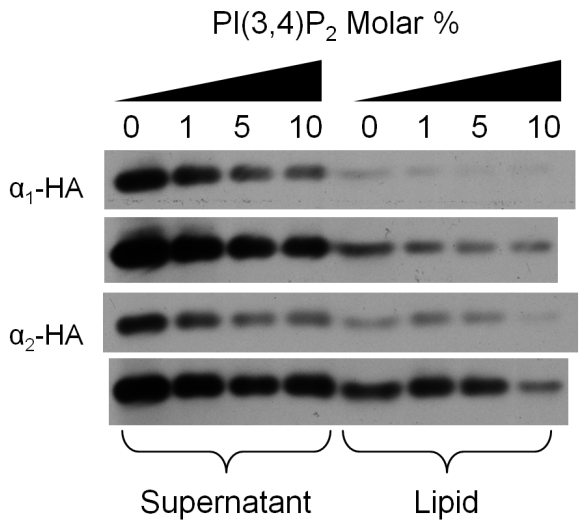

Figure A1.3. PAFAH Ib α1-HA and α2-HA do not bind specifically to PI(3,4)P_2. Purified PAFAH Ib α1-HA and α2-HA were added to liposome mixtures containing PC:PS:PI(3,4)P_2 at 80:20:0, 79:20:1, 75:20:5, and 70:20:10 molar ratios. Liposomes were then pelleted, and both pellet and supernatant fractions were analyzed via SDS-PAGE/Western using anti-HA antibodies.

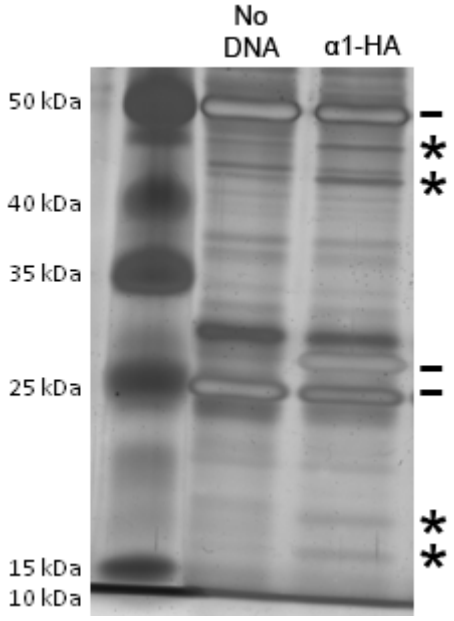

Figure A1.4. PAFAH Ib co-immunoprecipitates with additional proteins. HeLa cells were transfected with α1-HA and then whole cell lysates were prepared using RIPA buffer. Lysates were immunoprecipitated using anti-HA antibodies and then analyzed via SDS-PAGE and silver staining. Specific bands immunoprecipitated with α1-HA are denoted by *. Hyphens denote α1-HA (30 kDa), heavy and light chains of anti-HA antibodies (50 kDa and 25 kDa, respectively).

A1.3. Discussion

We showed that PAFAH Ib α1 bound strongly to PI3P and PI4P monolayers and that both α1 and α2 bound specifically to PI3P- and PI4P-containing liposomes. PI3P and PI4P are present in endosomes and the TGN, respectively, and may serve as recruiting lipids for PAFAH Ib α subunits. It remains unclear whether α1/α2 may be binding to these PIPs via their catalytic site or through some novel domain outside of the catalytic pocket. Additionally, α1-HA was shown to co-immunoprecipitate with at least four specific proteins, with at least two proteins possibly found within the GF fraction as well. Further studies are required in order to identify these proteins in order to decisively determine the minimal components required to establish an *in vitro* reconstitution assay for PAFAH Ib-based membrane tubulation.

A1.4. Materials and methods

Lipid-protein overlays (PIP strips/arrays). Nitrocellulose membranes (Amersham Hybond-C Extra RPN203E) were cut into appropriate sizes and then spotted with lipids or PIP containing diC16 acyl chains. Lipids were resuspended in chloroform and the resuspended into 1 mM stock solutions using spotting buffer, composed of chloroform:methanol (1:1) with 0.2% Ponceau S (Fluka 09276). 1 to 2 µl of diluted lipid was spotted onto the membrane. After drying the membrane, protein was added to 5% non-fat dry milk in tris-buffered saline with Tween-20 (TBS/T) o/n at 4ºC, followed by standard Western blotting protocols.

Liposome pulldown assay. Liposomes were prepared using standard protocols, using pulldown buffer containing 0.1 M NaCl, 20 mM HEPES, 12.5 mM EGTA, and 0.1 μg/ml fatty-acid free BSA with a final pH of 7.2. Liposomes were formulated with PC:PS:PIP at molar ratios from 80:20:0 to 70:20:10, where PS levels were unchanged. Liposomes were diluted to a final concentration of 1-2 mM and then 60 μl of lipids were incubated with 6 μg of purified, HA-tagged α1 or α2 for 30 min at RT. Lipids were spun down at 50,000 RPM. Supernatant fractions were collected and both the supernatant and pellet were resuspended with SDS sample buffer to equal volumes. Equal volumes of each were then loaded onto SDS-PAGE, followed by Western analysis using anti-HA antibodies.

Immunoprecipitation of proteins. HeLa cells were first transfected with α1-HA or α2-HA constructs and then whole cell lysates were prepared with ice-cold RIPA lysis buffer containing protease inhibitor cocktail (Roche). Lysates were then cleared of debris through centrifugation, followed by pre-clearing using both rabbit/mouse serum and Protein A/G slurry for 1 h on ice. After removing Protein A/G slurry, a second round of pre-clearing using only Protein A/G slurry was conducted for 1 h at 4ºC. Supernatant was then removed and incubated with anti-HA antibody for 3 h on ice, followed by capture using Protein A/G agarose for 1.5 h at 4ºC with gentle rocking. Agarose beads were then collected and processed for SDS samples and subsequent SDS-PAGE and silver staining using standard protocols.

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