Interaction of proteins containing dibasic motifs with COPI: Regulation of COPI trafficking

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ABSTRACT:

Eukaryotic life is defined by the ability of eukaryotic cells to form membrane bound compartments for specialized cellular processes. The protein transport machinery that carries cargo between these compartments is highly conserved and highlights the essential nature of cellular trafficking. Coatomer (COPI)-coated vesicles mediate membrane trafficking in the early secretory pathway. COPI vesicles transport cargo within the Golgi apparatus and from the Golgi and the endoplasmic reticulum (ER) Golgi intermediate compartment (ERGIC) to the ER. We now demonstrate that the COPI-binding protein Scy1-like 1 (Scyl1), a member of the Scy1-like family of catalytically inactive protein kinases oligomerizes through centrally located HEAT repeats and uses a C-terminal RKXX-COO^- motif to interact directly with the appendage domain of γ2-COP. Through a distinct site, Scyl1 interacts selectively with class II Arfs, notably Arf4, thus linking class II Arfs to γ2-bearing COPI subcomplexes. VPS-53 is a member of the Golgi-associated retrograde protein (GARP) complex, a tethering complex located at the *trans*-Golgi network and responsible for tethering vesicles derived from the endosomal system. We show that VPS-53 contains a canonical KKRL-COO^- dibasic COPI binding site and binds the γ-COP subunit of COPI. Interestingly, Scyl1 is the gene product lost in the muscle deficient (*mdf*) mouse and mutations in GARP are responsible for the phenotype of the wobbler (*wr*) mouse. Therefore, the data links two separate mouse models of neurodegeneration via interactions with the COPI system and thus strongly implicates retrograde cellular transport in neuronal survival.

RESUMÉ:

Chez les eucaryotes, les membranes compartimentalisent et organisent les différents processus cellulaires. La machinerie de transport entre les différents compartiments intracellulaires est très bien conservée d'un point de vue évolutif. Les vésicules de type COPI sont responsables du transport intracellulaire dans l'appareil sécrétoire. Les vésicules COPI transportent les différents cargo dans l'appareil de Golgi jusqu'au reticulum endoplasmique et son compartiment intermédiaire (ERGIC). Nous avons découvert que la proteine Scy1-like 1 (Scyl1), membre de la famille de proteines kinases inactives Scyl-like, oligomérize grâce à ses répétitions centrales HEAT et utilise son motif RXXX-COO^- situé en C-terminal pour interagir directement avec le domaine d'appendage de la protéine γ2-COP. Scyl1 interagit également de facon sélective avec les protéines Arf de classe II, incluant Arf4, mettant ainsi en évidence la relation entre les protéines Arf de classe II et les complexes γ2-COPI. VPS-53 est associée au complexe protéique rétrograde de l'appareil de Golgi (GARP), complexe qui prend en charge les vésicules dérivées du système endosomal. Nous avons demontré que VPS-53 contient un site dibasique KKRL-COO^- pour la liaison avec la sous-unité γ-COP de COPI. La protéine Scyl1 est absente chez la souris muscle deficient mouse (*mdf*) et les mutations au niveau du complexe GARP sont responsables du phénotype observé chez la souris wobbler (*wr*). Les données présentées dans cet ouvrage font donc le lien entre deux modèles murins de neurodégénération et le complexe COPI suggèrant ainsi un role essentiel pour le transport rétrograde dans la survie neuronale.

TABLE OF CONTENTS:

ABBREVIATIONS:

adaptor protein (AP)

ADP-Ribosylation Factor (Arf)

Amyotrophic lateral sclerosis (ALS)

Arf-related protein (Arl)

Brefeldin A (BFA)

Clathrin Heavy Chain (CHC)

coatomer (COP)

electron microscopy (EM)

endoplasmic reticulum (ER)

endoplasmic reticulum exit site (ERES)

endoplasmic reticulum Golgi intermediate compartment (ERGIC)

Glutathione S-Transferase (GST)

Golgi associated gamma ear Arf binding (GGA)

Golgi associated retrograde protein (GARP)

green fluorescent protein (GFP)

GTPase activating protein (GAP)

guanine nucleotide exchange factor (GEF)

guanosine diphosphate (GDP)

guanosine triphosphate (GTP)

KDEL-Receptor (KDELR)

last eukaryotic common ancestor (LECA)

mass spectrometry (MS)

Muscle deficient (*mdf*)

Sodium dodecyl sulfate (SDS)

Soluble NSF Attachment Protein Receptor (SNARE)

Trans-Golgi Network (TGN)

vacuolar protein sorting (VPS)

wobbler (*wr*)

LIST OF TABLES AND FIGURES:

Figure 3.2: GARP member VPS-53 binds to the γ-COP appendage domain.

Figure 3.3: Mutations in the dibasic motif of VPS-53 result in a loss of binding to the γ1-COP appendage domain.

CONTRIBUTION OF AUTHORS:

Chapters 1&4: Jason Hamlin wrote the introduction and discussion chapters of this thesis.

Chapter 2: Jason Hamlin performed the majority of the biochemical and microscopy experiments and wrote the paper in conjunction with Peter McPherson and advice from Paul Melançon. Lena Schroeder performed super-resolution microscopy experiments. Maryam Fotouhi, Martine Girard and Nathan Summerfeldt provided superior technical support and advice for bio-affinity experiments. Maria Ioannou performed live cell imaging. Hatem Dokainish performed gel-filtration experiments.

Chapter 3: Jason Hamlin performed the majority of experiments with technical support from Maryam Fotouhi. Manuscript written by Jason Hamlin and Peter McPherson.

DEDICATION:

This thesis is dedicated to my family and also my coworkers at the Montréal Neurological Institute. I would also like to thank my supervisors, past and present: Dr. Silvia T. Cardona (University of Manitoba) and Dr. Peter S. McPherson (McGill University). Thank you for giving me an opportunity and for always encouraging me to succeed.

CHAPTER 1: INTRODUCTION

Eukaryotes have become one of the most successful forms of cellular life on earth. The word eu- (*well, good*) karyote (*kernel, seed*) refers to the membrane bound nucleus in a plant, animal, fungus or protist cell observed under a microscope. And it is largely due to the membranous enclosure of cellular processes and enzymatic reactions in the eukaryotic cell that has allowed it to evolve into multicellular organisms and complex plant and animal life. The transport and regulation underlying cellular compartmentalization is a general theme for this thesis.

In one sense, membrane compartmentalization is necessary to separate the harsh, external (now oxidizing) environment from DNA. Multiple layers of compartmentalization, beginning with the plasma membrane, endosomes, Golgi, and endoplasmic reticulum (ER)/nuclear envelope the genes from the external environment. This enclosure quite suits the genetic material (as viewed separately from the cell as a whole) of a eukaryote. Firstly, the genes are within a membrane bound nucleus. The nucleus is itself connected to and bound by the ER, which contains a different protein content. The ER itself contacts every aspect of the cell through direct and indirect ER-membrane manipulations. The centrality of compartmentalization (Smart et al., 2014) is evidenced by the high degree of conservation between core-sets of trafficking genes among eukarya (Hirst et al., 2014; Mast et al., 2014; Wideman et al., 2014).

The introductory chapter will provide an overview of membrane trafficking with a focus on coated-vesicle carriers, specifically coatomer I (COPI). In chapter 1.1, I will provide an overview of membrane trafficking in general, using examples from the most understood trafficking system, clathrin. In Chapter 1.2, an overview of the early secretory system will be provided, and a detailed summary of the current understanding of COPI transport. Chapter 1.3

will discuss small GTPases that regulate membrane trafficking, and in more detail the Arf family of GTPase proteins that regulate COPI. Chapter 1.4 will overview our understanding of Scyl1, a COPI binding protein that is mutated in neurodegenerative disease.

1.1 Compartmentalization is the result of membrane trafficking

Membrane compartmentalization is maintained by a diverse set of trafficking proteins. These include proteins that identify and bind cargo on the membrane, membrane deforming proteins that help to form vesicles (Chapter 1.1.1), actin and tubulin binding proteins that help to physically move the vesicle from the membrane of origin to the target membrane (Chapter 1.1.2), tethers that help to identify the target (Chapter 1.1.3) and SNAREs and associated factors that drive vesicle fusion with the target membrane (Chapter 1.1.4). All of these processes are highly regulated to ensure compartmental identity and purposeful traffic flow. This regulation occurs in many cases via the actions of small GTPases, particularly in the Rab and Arf families.

1.1.1 Budding

One of the first steps in vesicle trafficking is budding. In this process, the lipid bilayer is distorted away from the planer lipid surface by the combined action of coat proteins, lipid binding proteins and lipid modifying enzymes. During this event, cargo is concentrated into the lumen of the vesicle by interacting with the adaptor layer of the coat complex.

For example, in the clathrin system functioning at the plasma membrane, the adaptor protein 2 (AP-2) binds to phosphatidylinositol 4,5 *bis*-phosphate (PtdIns(4,5)P_2)-rich patches of membrane on the cytosolic leaflet and recruits ENTH-domain containing proteins (Legendre-Guillemin et al., 2004) and BAR domain-containing proteins (Tebar et al., 1996), amongst others, which help to induce and stabilize the outward membrane curvature. AP-2 also interacts

with clathrin, and a clathrin coat then assembles, which contributes to the force required to curve the membrane. Therefore, the adaptin layer of the coat is responsible for the regulation of clathrin-coat forming protein assemblies at the stage of vesicle budding. Finally, the membrane scission protein dynamin performs the final step of the budding process, pinching off the formed vesicle from the donor membrane. The assembly of the clathrin vesicle, or any transport carrier does not occur in isolation but is tightly associated with the transport of cargo. The interaction between cargo and the regulatory adaptin layer (or homologous layer) in the vesicle coat is therefore of critical importance.

1.1.2 Vesicle transport

The primary method for how vectoral movement is achieved is that once a vesicle has been formed it is directed to its destination along microtubules or the actin cytoskeleton. To a large degree, this is under the control of Rab proteins (Caviston and Holzbaur, 2006), which link vesicles to the appropriate cytoskeletal motor complex, allowing for movement in the proper direction. For example, Rabs may link the vesicles to kinesin or dynein motors for plus-ended microtubule directed or minus-ended microtubule directed transport, respectively, or to myosin for transport on actin. For example: in yeast Ypt31 (Rab11) recruits myosin for secretory transport. Activated Ypt31 interacts directly with the globular tail domain of Myo2, thus recruiting the motor as an effector. Ypt31 positive vesicles are then transported from the trans-Golgi to growing areas of the cell surface for exocytosis (Lipatova et al., 2008). Rab7, helps recruit dynein for late-endosome to lysosome transport (Jordens et al., 2001). Rab6 interacts with minus-end microtubule motor protein dynein (Wanschers et al., 2008). Rab6 preferentially interacts with the motor in the GTP-bound activated form, thus the motor is an effector molecule of the regulatory GTPase.

1.1.3 Tethering

Initial contact between a vesicle and the acceptor membrane is accomplished by tethering factors. There are a wide variety of proteins that form tethering complexes that function at various locations in the cell. There are two main types: Multi-subunit tethers and extended coiled-coil containing tethers. For the latter, the tethering complex proteins contain long coiled-coil domains, which are found in a wide variety of protein complexes (intermediate filaments, transcription factors). . Coiled-coil domain containing proteins (intertwined alpha-helix, (Pauling et al., 1951)), can extend from the acceptor membrane many nanometers into the cytosol, making them well suited to "grab" incoming vesicles. Specific protein-protein interactions then occur between the incoming vesicle and the tether protein that ensure specificity. The tethering complex may also link to the vesicle fusion machinery, the final step in vesicle transport.

The Golgi contains well-studied examples of long coiled-coil tethers called the Golgins. Golgins are characterized by their tendency to form insoluble complexes and were included in what was termed the Golgi "matrix". Two examples of Golgins include p115, a component necessary for intra-Golgi transport (Waters et al., 1992) and GM130, which regulates the fusion of vesicles at the *cis*-Golgi (Barr et al., 1998).

Tethering factors themselves are recruited by regulatory GTPases to their sites of action. The Golgin p115 is a homo-dimer that binds Rab1 as an effector at the N-terminal globular head (Allan et al., 2000), and binds to GM130 and giantin at the C-terminus. A structural analysis of p115 shows that 12 armadillo-like (ARM) repeats form an extended alpha-coil and that residues in the first repeat are necessary for Rab1 binding (An et al., 2009). Thus, p115 is recruited via its N-terminus to active Rab1 at the *cis*-Golgi. Whether other factors such as cargo or coat proteins recruit tethering complexes to the membrane is not known.

The second main type of tethering factor is the multi-subunit tether. One example of a multi-subunit tethering complex is the Golgi Associated Retrograde Protein (GARP) complex, which is localized to the *trans*-Golgi network (TGN) (Conibear and Stevens, 2000). The GARP complex consists of four proteins in a stable equimolar tetrameric complex (Chapter 3), and knock-out of any member of GARP results in a build-up of endosome-derived vesicles unable to fuse with the TGN (Perez-Victoria et al., 2008).

1.1.4 Fusion

The final step in successful vesicle transport is fusion of the membrane bilayer with the acceptor membrane. This is accomplished by sets of proteins called SNARE proteins. SNARE proteins are present on both the incoming vesicle (v-SNAREs) and on the target membrane (t-SNAREs). When complementary pairs of SNAREs engage, their tight conformational engagement (coiled-coil bonds) overcomes the energy required to merge the two lipid bilayers (Hu et al., 2003). SNARES are subsequently disengaged in an ATP-dependent manner by the ATPase NSF (Block et al., 1988).

1.2 Trafficking in the early secretory pathway

One area where membrane trafficking functions in the transport of thousands of distinct proteins is in the early secretory pathway. In this section, an example of soluble and transmembrane cargo that traffic through this pathway will be provided. The role of two major vesicle coat systems, COPI and COPII in trafficking in the early secretory pathway will be discussed.

Proteins destined for the secretory pathway begin their synthesis on free ribosomes. The nascent polypeptide contains an N-terminal hydrophobic leader sequence, which binds the signal

recognition particle, temporarily halting translation and directing the nascent protein/ribosome complex to the ER (Walter and Johnson, 1994). Once at the ER, translation is reinitiated and the protein is threaded through the translocon, a pore-forming protein complex in the ER membrane, and is inserted into the lumen of the ER.

The environment of the ER allows for protein folding and glycosylation. When transmembrane or luminal proteins are properly folded and glycosylated, they are ready to exit the ER. Often the transmembrane proteins contain a diacidic or diphenylalanine motif in their cytoplasmic tails, which is then bound by COPII. For example, the lectin transporter ERGIC-53, which cycles between the ER and *cis*-Golgi in the early secretory pathway, contains a C-terminal diphenylalanine motif necessary for efficient packaging into COPII vesicles (Nufer et al., 2003).

COPII itself is composed of an inner, cargo-binding heterodimer of Sec23 and Sec24 and an outer coat composed of Sec13/Sec31. COPII is greatly enriched at ER exit sites and COPII vesicle biogenesis is the first membrane trafficking step for nearly all cargo that exits the ER in the secretory pathway (Barlowe et al., 1994). Initially, the small cytosolic GTPase Sar1 is activated by Sec12 (Weissman et al., 2001), an ER localized transmembrane GEF (guanine nucleotide exchange factor). Sar1 then recruits the Sec23/24 heterodimer, which binds to cargo motifs exposed on the cytosolic tail of the cargo or cargo adapter due for ER export (Miller et al., 2002).

Once the vesicles have budded they lose their COPII coats and fuse with stable structures called ERGICs (endoplasmic reticulum Golgi intermediate compartment). Alternatively, the vesicles undergo fusion to generate new ERGIC. There are roughly 200-500 ERGICs present in a typical HeLa cell or Fibroblast in cell culture (Schindler et al., 1993). They are biochemically distinct from the ER with a luminal pH of ~7.1 compared to the 7.4 of ER (Palokangas et al.,

1998). The ERGIC is located adjacent to the ERES and is therefore distributed throughout the cell wherever the ER is located (Itin et al., 1995). Cargo-containing ERGICs then engage the microtubule cytoskeleton for transfer of material between each other (by vesicles, or ERGIC fusion via tubular-vesicular structures) and mature towards the *cis*-side of the Golgi (Presley et al., 1997).

Cargo that has reached the *cis*-Golgi then moves through the Golgi stacks. There are two major models of transport within the Golgi: a Cisternal Maturation Model and an Anterograde Vesicle Trafficking model. According to the cisternal maturation model, the *cis* side of the Golgi retains its lumen contents but is physically moved and transformed and becomes the *trans* side of the Golgi as vesicles from more anterograde portions of the Golgi are trafficked in a retrograde manner.

In the anterograde vesicle trafficking model, cargo moves through the stationary stacks of the Golgi (from *cis* to *trans*) in COPI vesicles. One problem with this hypothesis is that some cargo, such as pro-collagen are actually larger than the 75 nm diameter of a vesicle. In either model, the cargo are exposed to the resident Golgi enzymes that are responsible for post-translational modification (glycosylation) of the anterograde directed cargo as they proceed to the *trans*-cisternae of the Golgi, and eventually appear, fully mature, at the *trans*-Golgi network (TGN). These two models are not mutually exclusive, and there is evidence to support a hybrid Anterograde vesicle-Maturation model of Golgi function. Much of the data provided in this work (Chapter 2) examines COPI during its role in retrograde vesicle transport, and does not argue for or against any functional role for COPI during anterograde transport.

The primary transport vesicles in the early secretory pathway are COPI (coatomer I) vesicles. COPI is a multi-subunit vesicle forming transport complex (discussed in detail below,

Sections 1.2.1 - 1.2.3). These 70-80 nm diameter coatomer-coated transport units are centered at the Golgi apparatus and carry resident enzymes to their appropriate position within the Golgi. COPI is the main retrograde transport route in the early secretory pathway.

1.2.1 COPI-mediated membrane trafficking

To maintain homeostasis in any cellular trafficking system, as cargo moves forward in an anterograde direction it needs to be balanced by retrograde flow. For example, after vesicles and transmembrane cargo receptors have successfully delivered cargo from the ER through the stacks of the Golgi to the TGN, the membranes and cargo-adapters need to be recycled in a retrograde flow to the ER. This is accomplished primarily by COPI (Waters et al., 1991).

The general steps in assembling a COPI vesicle are as follows. First, Arf-GDP is activated via the actions of Arf GEFs, which drive the small GTPase into a GTP-bound state recruiting it to the membrane. Arf that has been recruited to the membrane and activated then recruits the COPI coat complex. Fully formed COPI vesicles bud from the membrane with the action of membrane inserted Arf proteins (Figure 1.1).

EM analysis of COPI vesicles generated by *in vitro* budding reactions on naked liposomes demonstrates that the minimal machinery to bud a COPI vesicle of approximately ~75 nm is the COPI heptamer and the small G-protein Arf (Chapter 1.3) in its GTP bound form (Orci et al., 1993; Palmer et al., 1993). Although the minimal requirements are not nearly as efficient when COPI cargo is included (see Chapter 4).

1.2.2 Components of COPI

The COPI coat was originally described as a 700 kDa heptamer of α, β, γ, δ, ζ, β^1, and ε COP in an equimolar ratio. However, biochemical dissociation of the COPI heptameric structure in high salt has shown that COPI can be separated into two stable subcomplexes named the F-subcomplex (β, γ, δ, ζ COP) and B-subcomplex (α, β^1 and ε COP), (Orci et al., 1993). Overall, there is great similarity between the F/B-subcomplexes of COPI and the components of the clathrin coat. For example, clathrin takes the form of a triskelia in which three molecules of clathrin heavy chain assemble in a three–legged structure. Interestingly, the B-Subcomplex of COPI takes on a three-legged structure that is very similar to clathrin triskelia. For a more detailed comparison of COPI and clathrin see Chapter 1.2.3.

1.2.3 Protein recognition motifs and cargo of the COPI system

The fact that short motifs confer protein localization to the ER was first discovered using a viral protein system. The cytoplasmic tail of the adenoviral E3/19K protein is necessary for the ER localization of the protein, as a construct with a small C-terminal cytoplasmic deletion re-localizes to the plasma membrane. The converse is true; adding the C-terminus of E3/19K to a non-ER localized transmembrane protein confers ER localization, specifically, CD4 or CD8-E3/19K chimeras re-localize from the cell surface to the ER (Paabo et al., 1987). Additionally, chimeras of CD8 with the human protein UDPGT25, normally localized to the ER, re-localized the CD8 chimera to the ER (Nilsson et al., 1989). These experiments conclusively showed the C-terminus contains necessary and sufficient information for ER retention.

The information in the cytoplasmic segment of these proteins was found to be stored in two lysine residues at the -3 and -4 position to the c-terminus of the protein (Jackson et al.,

1990). These sequences generally have the characteristic KKXX-COO^- or KXKXX-COO^-. Mutations and substitutions that put the lysines out of this strict context result in loss of ER retention. Proteins that both purposely recycle between the ER and Golgi via COPII/COPI such as ERGIC-53 and p24 family proteins and resident ER proteins contain these motifs.

Soluble ER proteins that escape in COPII vesicles may be returned to the ER by the KDEL receptor. These soluble escapees contain a C-terminal KDEL motif (Munro and Pelham, 1987), which binds the luminal domain of the type 1 transmembrane KDEL receptor (Lewis and Pelham, 1990). Interestingly, pathogenic bacteria have managed to evolve mechanisms to use this motif when co-opting the eukaryotic cellular machinery. Subunit A of cholera toxin contains a C-terminal KDEL-COO^- motif, and uses the KDEL-receptor to gain entry into the ER from where it translocates into the cytosol. The cytoplasmic domain of KDEL receptor itself contains a dibasic motif, and binds COPI. Thus soluble KDEL-motif containing proteins are returned to the ER via COPI.

A recurring question is *where* does the dilysine motif of these cargo bind to coatomer? In clathrin-mediated endocytosis, the μ2 subunit of AP-2 has been shown to bind to tyrosine based sorting motifs such as the one contained in the cytoplasmic tail of the transferrin receptor (Hirst et al., 2001). The homologous subunit to μ2 in COPI is ζ-COP. ζ-COP has not been reported to be a cargo binding subunit. One complication in these studies is that COPI exists as two large and stable subcomplexes, F and B described above, making mapping a binding site to an individual protein more difficult. It does appear however that COPI has two binding sites for dilysine based sorting signals (Hudson and Draper, 1997). One is in the appendage domain of the γ-subunit in the F-subcomplex (Harter and Wieland, 1998), the second site is in the B-subcomplex (Ma and Goldberg, 2013). The ability for COPI to interact with multiple cargo

motifs through at least two distinct locations in the complex is intriguing and opens the possibility for multiple layers of regulation or modes of vesicle formation.

1.2.4 Comparison of the COPI and Clathrin coat systems

The ability to bind and deform membrane, leading to the formation of transport vesicles, is a fundamental ability of all eukaryotes. Indeed, all coat complexes in the cell are evolutionarily related to a single membrane-deforming ancestor that was present well before the Last Eukaryotic Common Ancestor (LECA) (Koumandou et al., 2013). This includes the well-studied clathrin and adaptin coat protein complex and the COPI and COPII complexes. Clathrin proteins form trimeric vertices, similar to the B-subcomplex of COPI. However, the evolutionary divergence of clathrin compared to the B-subcomplex of COPI is such that primary sequence homology algorithms initially did not detect significant similarity between the proteins. Thus, until recently there has been some controversy regarding the evolutionary relationship of the clathrin/AP-2 complexes with COPI. However, structure-based homology searches have now been successfully used to define these relationships and to identify new coat protein complexes in mammalian cells and other cells (Hirst et al., 2014).

The COPI F-subcomplex and AP-2 each contain four proteins (γ,β,δ,ζ COP) and (α,β2,μ2,σ2 AP-2). The large subunits, γ-COP and β-COP each contain a trunk and appendage domain, similar in structure to that of α-AP-2 and β2-AP-2 (Alisaraie and Rouiller, 2012) and have diverged from a common adaptin ancestor. Multiple interactions via the appendage domain of α-AP-2 regulate the assembly of clathrin vesicles (Owen et al., 1999), but none have been described for the analogous γ-COPI.

1.3 Arf proteins regulate COPI trafficking

ADP-ribosylation factor (Arf) was originally identified as a protein required for the efficient ADP-ribosylation, through the actions of the cholera toxin subunit B, of the regulatory subunit G_S of adenylate cyclase (Kahn and Gilman, 1984). Subsequently, Arf was found to be a GTP-binding protein, and it was determined that Arf-GTP, not Arf-GDP was required for cholera toxins effects (Kahn and Gilman, 1986). A GTPase is a protein that binds GTP and hydrolyzes the γ-phosphate group resulting in GDP and phosphate. The GTPase adopts differing conformations and binding partners depending upon its nucleotide binding status and thus GTPases generally act as molecular switches. Regulatory membrane trafficking GTPases can be divided in to the large GTPases (dynamin and related) and small GTPases. The large GTPase dynamin functions in the scission step of clathrin mediated endocytosis and acts as a direct mechanochemical enzyme rather than a switch.

The small GTPase Ras superfamily (Tetlow and Tamanoi, 2013) contains the Arf/Arl/Sar1, Ran, Rho and Rab groups and contains over 150 members. Generally, Ras controls many aspects of cellular proliferation, Ran controls import into the nucleus, Rho and the highly related Rac and Cdc42 control cell structure and morphology through regulation of actin and Rab/Arf/Arl/Sar1 control vesicle trafficking. There is also much overlap between their primary identified functions. Among the vesicle trafficking group, Rabs play a regulatory role (Hutagalung and Novick, 2011), recruiting effectors to remodel membranes and control vesicle trafficking between almost all endomembranes and Arf/Arl1/Sar1 regulate trafficking but also participate in the vesicle scission step (Pucadyil and Schmid, 2009), similar to dynamin.

The Arfs have similarities and differences with the other small GTPase proteins that regulate vesicle assembly. They are of similar size to the Rab proteins, roughly 20 kDa, and are associated with specific intracellular locations. In the active, GTP-bound state they recruit an array of effector proteins (Cherfils, 2014) that help to identify and organize vesicle assembly as well as modify the local lipid environment. For example, Arfs associate with COPI in a 2:1 molar ratio and are critical determinants in the recruitment of COPI coats to the membrane (Palmer et al., 1993). Uniquely, Arfs contain an N-terminal amphipathic helix and when the Arf is in the active, GTP-bound state, the helix inserts into the membrane, inducing positive curvature and promoting vesicle formation (Popoff et al., 2011). Arfs were the first of the family of small GTPases to diverge from the ancestral large membrane associated GTPases (Kahn et al., 2005).

In humans the Arf family of GTPases consists of 5 members, Arf1 and Arf3 (class I), Arf4 and Arf5 (class II) and Arf6 (class III). Class I and class II Arfs regulate COPI trafficking whereas Arf6, the only class III member, regulates clathrin trafficking through membrane recruitment of AP-2 containing complexes. Additionally, there are over 20 Arf-related proteins (Arl), which have been implicated in vesicle trafficking primarily at the Golgi. Arl-8 has been proposed to regulate vesicle trafficking from the soma to the synapse in neurons (Klassen et al., 2010).

Among the Arfs, Arf1 and Arf4 are highly homologous. Yet it is known that Arf1 is localized predominantly to the Golgi and Arf4 is localized predominantly to the ERGIC. The mechanisms that guide this distinct subcellular localization are unknown (Chun et al., 2008; Duijsings et al., 2009). It has been shown that Arf1-GDP is recruited in an initial step to membranes by the cytosolic tail of p23 (Gommel et al., 2001) and also by the ER-Golgi SNARE,

membrin (Honda et al., 2005). A Class II Arf receptor remains undiscovered (Chun et al., 2008), and it is predicted to be ERGIC localized and membrane associated in the presence of the fungal toxin brefeldin A (BFA), which inhibits several classes of Arf GEFs (see section 1.3.3).

1.3.1 Mechanism of Arf1 and COPI

Recent crystallographic studies show that Arf1 binds directly to the trunk domains of β-COP and γ-COP (Yu et al., 2012). Active Arf1 is tightly inserted into the membrane via the N-terminal amphipathic helix and an N-terminal myristoylation. The switch region of Arf1 binds select residues in the barrel of the trunk domain of the COPI proteins. It is in this fashion that the COPI complex is recruited to the membrane. In a sense, COPI achieves membrane binding with the same protein-subunits as the AP-2 complex, except instead of binding PtdIns(4,5)P_2 directly as α-adaptin does, γ-COPI binds the membrane-inserted Arf. Additionally, one Arf protein binding each β-COP and γ-COP explains the 2:1 stoichiometry of Arf and COPI on purified COPI vesicles.

1.3.2 Arf GEFs and GAPs

Much like the well-studied Rab family members, Arfs are activated by GEFs, which catalyze nucleotide exchange by binding to the GTPase in the GDP-bound conformation and dislodging the GDP. GTP loading of Arf results in a conformational change that exposes the N-term amphipathic helix and causes tight membrane association. At the membrane, active Arfs function to recruit effectors of membrane transport including directly binding COPI via γ-COP and β-COP (Yu et al., 2012) as well as lipid modifying enzymes, while the Arf protein itself helps to promote membrane curvature (Pucadyil and Schmid, 2009).

Arf GEFs control when and where Arf proteins are activated. The catalytic region of all ArfGEFs is Sec7 domain, a 200 amino-acid region that stabilizes the nucleotide-binding pocket on Arf (Goldberg, 1998), greatly lowering the affinity of bound-GDP. The domain architecture between Arf GEFs outside of the Sec7 domain is more variable, allowing for diversity in localization and regulation.

The fungal toxin BFA was found to inhibit Arf-mediated COPI transport. Treatment of a cell with micromolar amounts of BFA results in a complete loss of membrane associated Arf and COPI and a disintegration of the Golgi complex (Pelham, 1991). A crystal structure revealed that BFA stabilizes the Arf-GDP/Sec7 complex (Mossessova et al., 2003), aborting GTP cycling. There are several Arf GEFs that associate with the Golgi, including BIG1/2 at the TGN and GBF1 at the *cis*-Golgi and ERGIC.

GTPase activating proteins (GAPs) generally have two functions, first, to catalyze the hydrolysis of the GTPase bound GTP, and second to stabilize the GDP in the nucleotide binding pocket. For example, RasGAP uses a glutamic acid to coordinate the addition of water to break

the γ-phosphate bond (Scheffzek et al., 1997). As well, RasGAP has a conserved arginine residue that helps to stabilise Ras-GDP (Ahmadian et al., 1997). Arf GAPS all contain a 140 amino acid ArfGAP domain, and like Arf GEFs, contain multiple modules and motifs outside of the catalytic domain to ensure correct targeting and subcellular localization (Gillingham and Munro, 2007). Functionally, Arf GAPs control distinct activities. For example, uncoating of COPI vesicles is initiated with Arf GAPs since the COPI coat binds exclusively to GTP-bound Arfs and ArfGAP2 and ArfGAP3 have been shown to be essential for Golgi COPI coat disassembly (Kartberg et al, 2010). ArfGAP1 contains an amphipathic lipid packing sensor (ALPS) domain, which preferentially binds to curved membranes, and multiple studies have shown that Arf-GTP hydrolysis occurs on a curved COPI vesicle, which results in release of Arf and COPI to the cytosol. ArfGAP1 and ArfGAP2 appear to have overlapping Arf substrates, and it is therefore possible that specificity of Arf-deactivation (occurring in the cytosol and uncoating the vesicle) is less important than Arf-activation, which requires the correct membrane localization.

1.3.3 Arf Cascade and Rab/Arf crosstalk

Another way small GTPase proteins ensure correct compartment identity and directionality of transport is through GEF cascades (Zerial and McBride, 2001). Rab proteins have been well documented to have both GEFs and GAPs as effectors. By recruiting a GEF for a downstream Rab, the upstream Rab facilitates the activation of the next Rab in the transport sequence. The newly activated Rab recruits a GAP for the upstream Rab, thus deactivating it and assuring directionality of information flow. An ArfGEF cascade has been proposed where the GBF1-activated class II Arfs4/5 bind BIG1 as an effector at the *trans*-Golgi (Lowery et al., 2013).

There is also crosstalk between the Arf and Rab pathways. In the endosomal system, Rab35 and Arf6 antagonistically control cargo recycling to the cell surface (Allaire et al., 2013). Activated Rab35 supresses the activation of Arf6 by recruiting the Arf6 GAP ACAP1. Active Arf6 recruits the Rab35 GAP TBCD1B. Together, this double negative feedback loop leads to tight regulation of the two GTPases, ensuring that when one is active, the other is inactive.

1.4 Scyl1 is a COPI accessory protein

Through work of a previous Ph.D. graduate student in the McPherson laboratory it was demonstrated that the protein Scyl1, a member of the Scy1-like family of catalytically inactive protein kinases functions in retrograde COPI-mediated intracellular transport (Burman et al., 2008; Burman et al., 2010). Scyl1 contains a conserved but catalytically inactive N-terminal kinase-like domain (aa 14-264), 5 HEAT repeats (344-427, 501-537), a proline rich domain (589-619), followed by a variable C-terminus that contains a short coiled-coil region and a C-terminal RKLD-COO^- motif, a dibasic COPI binding motif (Burman et al, 2008). Scyl1 localizes to the *cis*-Golgi and ERGIC.

1.4.1 Domains and motifs of Scyl1

The N-terminal kinase domain of Scyl1 is non-functional, as it is missing several residues common to all functional kinases (Liu et al, 2000) and is therefore a pseudokinase. Further study has confirmed the kinase domain to be inactive as it is unable to bind ATP, as measured by calorimetry. The related CVAK104 (Scyl2) has been shown to have kinase activity under certain substrate conditions (Conner & Schmid, 2005). However, since the Scyl1 pseudokinase domain is present in all species that have Scyl1 (all metazoans) it must still have a function, possibly in

phosphoprotein regulation. Alternatively, the non-catalytic globular kinase domain could act as a protein scaffold, as has been reported for many pseudokinase domain containing proteins.

HEAT repeats are approximately 40 amino acids and form antiparallel alpha helices mediating protein-protein interactions (Groves and Barford, 1999). Three HEAT repeats are located in Scyl1 in two clusters after the kinase domain. Scyl1-GFP constructs lacking the kinase domain or the C-terminal dibasic COPI motif localize to the Golgi (Burman, unpublished observations). Following the HEAT repeats is a variable region, and human Scyl1 contains at least two splice variants that contain alternate truncations at the C-terminus of the protein (Kato et al, 2002).

An alternative splice variant of Scyl1 previously termed NTKL (N-terminal kinase like), was shown to localize to the centrosome during mitosis (Kato et al, 2002). Scyl1 has been shown to bind the GTPase Ran in a GTP specific manner, and a role for Scyl1 in nuclear tRNA export has been proposed (Chafe & Mangroo, 2010). However the biological significance of these findings remains unclear.

Amino acids 755-798 of Scyl1 encode for a coiled-coil domain. Coiled-coil structures are composed of two or more alpha helices that form a super-helix. Coiled-coil proteins often form dimers. The coiled-coil domain of Scyl1 is much shorter than that of a typical Golgin (Malsam et al., 2005), at only about 50 amino acids. Interestingly, when projected as a helical wheel, Scyl1 coiled-coil appears to contain an amphipathic helix. The C-terminus of Scyl1 binds COPI in both functional (Burman et al., 2008) and crystallographic assays (Ma and Goldberg, 2013). Knockdown of Scyl1 also blocks efficient COPI mediated retrograde traffic (Burman et al., 2008). Therefore, it appears Scyl1 contains the properties of a COPI accessory protein.

1.4.2 Scyl1 is mutated in the neurodegenerative *mdf* mouse model

The *mdf* mouse was originally discovered as a spontaneous mutant at the Jackson laboratories in the 1970s. The strain was bred and the embryos frozen to await further studies. In 1994, the first histochemical characterization of the *mdf* mouse was performed. It was found that the hind-limb atrophy that occurs in the mouse is neurogenic (Blot 1994), which was confirmed 20 years later in a genetic Scyl1 knockout study (Pelletier 2013).

The key feature of the *mdf* mouse is the neuromuscular degeneration of the hindlimbs at 4-6 weeks of age. This rapid and progressive degeneration results in the mouse having to pull itself along with the forelimbs in the advanced stages of the disease. One study showed cerebellar involvement with degradation of the Perkinje cells of the cerebellum (Schmidt et al., 2007). The same study also described gait ataxia and tremor in the *mdf* mouse. However the genetic knockout failed to replicate the ataxic phenotype therefore the ataxia may be something specific to the *mdf* mouse or the fashion in which the *mdf* mouse is different from the Scyl1 (-/-) knockout mouse. Additionally, the Scyl1 (-/-) mouse was found to contain TDP-43 positive inclusions (Pelletier et al., 2012), a hallmark of neurodegenerative disease.

The locus of the gene product lost in the *mdf* mouse was found to be on chromosome 19 and was mapped to the Scyl1 gene. A missense mutation likely causes mRNA degradation as there is no detectible Scyl1 mRNA transcript in the *mdf* mouse. The presence of the unstable (and degraded) transcript in the *mdf* mouse compared to an absence of the transcript altogether in the Scyl1 (-/-) mouse may be part of the reason for the difference in cerebellar and ataxic phenotypes.

There are several examples where dysfunctions in cellular membrane traffic can cause neurodegenerative disease. The ALS2 gene encodes the protein Alsin which functions as a GEF for Rab5 at the early endosomes (Topp et al, 2004), and is mutated in a recessive form of amyotrophic lateral sclerosis (Yang et al, 2001). The wobbler (*wr*) mouse suffers from spinal muscular atrophy (Andrews et al, 1974) and is caused by mutations in VPS-54. Together VPS-51, VPS-52, VPS-53 and VPS-54 form a tethering complex at the Golgi that accepts endosome derived vesicles (Conibear & Stevens, 2000) (see Chapter 3).

The fact that defects in intracellular trafficking are fatal in certain cell subtypes and not others may be due the fact that larger cells may be generally more reliant on membrane trafficking due to their size. Alternatively, neurons may be more reliant specifically on certain signalling pathways required for their individual function and survival. These signalling pathways are highly regulated by intracellular trafficking and likely depend on evolved trafficking accessory factors to achieve this specialization.

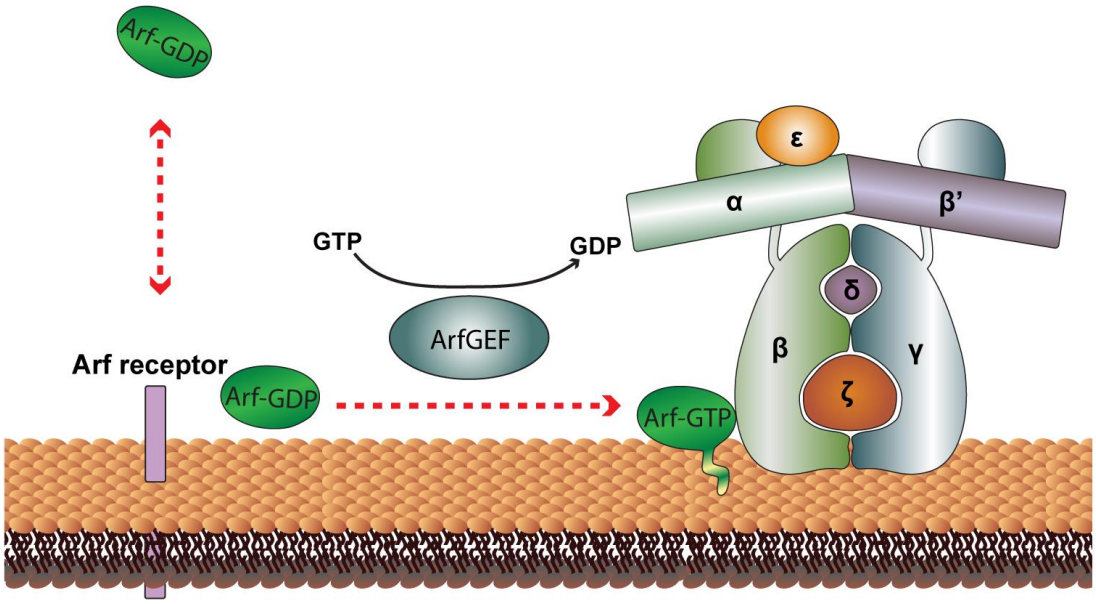

Figure 1.1 COPI complex schemata and recruitment to the membrane. The general steps in assembling a COPI vesicle. Initially, cytosolic Arf-GDP recruited to the membrane and is activated via the actions of Arf GEFs, which drive the small GTPase into a GTP-bound state. Arf undergoes a conformational change, notably Arf-GTP inserts an amphipathic helix into the membrane. Arf-GTP recruits the COPI coat complex. Fully formed COPI vesicles bud from the membrane with the action of membrane-inserted Arf proteins.

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PREFACE TO CHAPTER 2: Scyl1 scaffolds class II Arfs to selective subcomplexes of coatomer via the γ-COP appendage domain

Scyl1 was originally identified as a pseudokinase of 105 kDa and was named p105 (Liu et al., 2000). Later, analysis by the McPherson laboratory revealed that Scyl1 had a COPI-binding site and regulated COPI trafficking (Burman et al., 2008). Interestingly, mutations in the gene encoding Scyl1 are responsible for the muscle deficient (*mdf*) mouse (Schmidt et al., 2007). In our published results in Chapter 2, we sought to further understand the molecular mechanisms and regulatory pathways that underlie Scyl1 functioning in COPI transport.

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Scyl1 scaffolds class II Arfs to selective subcomplexes of coatomer via the γ-COP appendage domain

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Running title: Scyl1 scaffolds Arf4 and γ-coatomer

Keywords: Coatomer, Arf4, Scyl1, γ-COP, ERGIC-53, tubulation, early secretory pathway

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Summary

Coatomer (COPI)-coated vesicles mediate membrane trafficking in the early secretory pathway. There are at least three subclasses of COPI coats and two classes of Arf GTPases that couple COPI coat proteins to membranes. Whether mechanisms exist to link specific Arfs to selective COPI subcomplexes is unknown. We now demonstrate that Scy1-like 1 (Scyl1), a member of the Scy1-like family of catalytically inactive protein kinases oligomerizes through centrally located HEAT repeats and uses a C-terminal RKXX-COO^- motif to interact directly with the appendage domain of γ2-COP. Through a distinct site, Scyl1 interacts selectively with class II Arfs, notably Arf4, thus linking class II Arfs to γ2-bearing COPI subcomplexes. Therefore, Scyl1 functions as a scaffold for key components of COPI coats and disruption of the Scyl1 scaffolding function causes tubulation of the ER/Golgi intermediate compartment and *cis*-Golgi, similar to that observed following loss of Arf and Arf-GEF function. Our data reveal Scyl1 as a key organizer of a selective subset of the COPI machinery.

Introduction

COPI-coated vesicles carry cargo between the stacks of the Golgi apparatus and in a retrograde direction from the *cis*-Golgi and the endoplasmic reticulum (ER)/Golgi intermediate compartment (ERGIC) to the ER (Waters et al., 1991; Orci et al., 1997). Scyl1, a member of the Scy1-like family of catalytically inactive protein kinases was recently identified to function in retrograde COPI-mediated intracellular transport (Burman et al., 2008; Burman et al., 2010). Importantly, Scyl1 has also been identified as the gene product lost in an animal model of motor neuron disease, the muscle deficient (*mdf*) mouse (Blot et al., 1995; Schmidt et al., 2007). Homozygous recessive *mdf* mice develop a waddling gait, tremor and hind-limb paralysis at 4-8 weeks of age with neurogenic atrophy (Blot et al., 1995). Moreover, a recently developed Scyl1 knock out mouse displays motor neuron degeneration similar to *mdf* mice, and Scyl1$^{-/-}$ pathology is associated with TDP-43-positive cytoplasmic inclusions in motor neurons (Pelletier et al., 2012), a hallmark of motor neuron diseases such as ALS (Neumann et al., 2006). Thus, it is vital to develop a better understanding of the role of Scyl1 in membrane trafficking.

One key group of small GTPases involved in membrane trafficking are the Arfs, consisting in humans of class I (Arf1/3), class II (Arf4/5) and class III (Arf6), with class I and II Arfs functioning in COPI-mediated membrane trafficking (D'Souza-Schorey and Chavrier, 2006). Arfs are recruited to the membranes of the Golgi and the ERGIC in a GDP-bound form (Gommel et al., 2001; Honda et al., 2005; Manolea et al., 2010) where they are activated (entering a GTP-bound form) through the actions of GEFs (guanine nucleotide exchange factors). Activated Arfs bind COPI proteins, recruiting COPI coats to the membrane (Donaldson et al., 1992). Arf activation also induces release of an N-terminal amphipathic helix that inserts into the membrane (Antonny et al., 1997), aiding in membrane curvature and driving the fission

of fully-formed vesicles (Beck et al., 2008). Whereas class I Arfs function predominantly at the Golgi apparatus and have been relatively well studied, it is only recently that class II Arfs have been linked to COPI vesicle formation (Popoff et al., 2011). The site of action of class II Arfs remains uncertain, with indications that they function at the ERGIC (Chun et al., 2008) and at the *trans*-Golgi network (Deretic et al., 2005; Lowery et al., 2013).

COPI vesicles form at multiple cellular sites and are implicated in a variety of transport pathways including vesicle and tubule formation for both retrograde and anterograde carriers in the secretory pathway (Waters et al., 1991; Yang et al., 2011). The COPI coat is composed of 7 proteins organized into a heterotrimeric B subcomplex, composed of αβ'ε-COP, and a heterotetrameric F subcomplex, composed of γβζδ-COP. Isoforms of γ- and ζ-COP lead to further heterogeneity with $\gamma_1\beta\zeta_1\delta$-, $\gamma_1\beta\zeta_2\delta$-, and $\gamma_2\beta\zeta_1\delta$-COP variants (Moelleken et al., 2007). It has been proposed that heterogeneity of the γβζδ subcomplex helps achieve the functional heterogeneity observed in COPI trafficking (Wegmann et al., 2004; Moelleken et al., 2007). However, the functional roles of the individual γβζδ-COP subcomplexes and their linkage to other aspects of the COPI trafficking machinery such as Arfs and the αβ'ε subcomplex have not been defined.

We previously demonstrated that knock down of Scyl1 disrupts trafficking of selective cargo from the *cis*-Golgi to the ER (Burman et al., 2008). To better define the mechanism of action of Scyl1, we now use GST-Scyl1 to affinity select binding partners from tissue extracts, followed by mass spectrometry-based protein identification. This analysis identifies Arf4 and we establish that Scyl1 binds specifically to class II Arfs. Arf4 and COPI use non-overlapping binding sites on Scyl1, thus Scyl1 is well placed to couple COPI and Arf4 on ERGIC membranes. Remarkably, Scyl1 uses a C-terminal RKXX-COO^- motif to interact directly with

the appendage domain of γ2-COP and it associates predominantly with γ2-bearing COPI subcomplexes. Therefore, Scyl1 scaffolds class II Arfs to a γ2-COP-positive population of COPI and disruption of this scaffolding function by overexpression or knock down of Scyl1 causes ERGIC tubulation, similar to what is seen following disruption of Arf4 and Arf1 (Szul et al., 2007; Ben-Tekaya et al., 2010). Together, our studies indicate a role for Scyl1 in COPI-mediated retrograde transport in the early secretory pathway and implicate Scyl1 function in a γ2-COP and Arf4 regulated system.

Results

Scyl1 interacts specifically with class II Arfs

In a screen to identify interaction partners for Scyl1, we used GST-full-length Scyl1 in affinity-selection assays. As expected, mass spectrometry analysis of affinity-selected proteins revealed components of COPI coats. We also detected the small GTPase Arf4, which along with Arf5 is member of the class II family of Arfs, recently demonstrated to function in COPI vesicle formation (Popoff et al., 2011). To further examine the specificity of the Arf4 interaction we used affinity-selection assays with Western blots. Due to the unavailability of Arf isoform specific antibodies, we used constructs encoding C-terminally tagged fluorescent Arf proteins, which are widely used for the study of Arfs (Chun et al., 2008; Duijsings et al., 2009; Jain et al., 2012; Kudelko et al., 2012). Scyl1 binds specifically to class II Arfs, with a preference for Arf4, but does not bind class I Arfs (Figure 1A,B). Both GFP-tagged forms of Arf4 and Arf1 bind well to the GAT domain of the GGA3 protein (supplemental Figure 1), which is known to bind to both class I and class II Arfs (Dell'Angelica et al., 2000; Takatsu et al., 2002), indicating that the Arf-GFP constructs are not simply misfolded and further highlighting the specificity of Scyl1 for class II Arfs.

Like other GTPases, Arfs cycle between active, GTP-bound and inactive, GDP-bound configurations. In the active form, Arfs bind effectors such as coat proteins and lipid modifying enzymes, bringing them to the membrane. We sought to determine if Scyl1 is an Arf4 effector, that is, whether it would preferentially bind GTP-loaded Arf4. Cells were transfected with wild-type Arf4-GFP, Arf4-T31N-GFP (inactive) (Chun et al., 2008), Arf4-T48N-GFP (inactive), or Arf4-Q71L-GFP (GTP-locked) (Lowery et al., 2013). Alternatively, lysates from cells transfected with wild-type Arf4-GFP were treated with GMPP(NH)P, a non-hydrolyzable GTP

analogue, or with aluminum fluoride (AlF_4), which acts as a γ-phosphate GTP-mimetic by coordinating GDP in the nucleotide-binding pocket of heterotrimeric G proteins (Bigay et al., 1987) and Arfs (Taylor et al., 1992). Interestingly, affinity-selection assays reveal that GST-Scyl1 interacts with both GDP- and GTP-bound forms of Arf4 (Figure 1C). Quantification of the ratio of starting material to affinity-selected material reveals no significant difference in Scyl1 binding to the wild-type, active, or inactive forms of Arf4-GFP (Figure 1D). Thus, unlike most Arf-binding proteins, Scyl1 displays no nucleotide specificity in its interaction with the GTPase.

We next sought to determine if Scyl1 and Arf4 co-localize. HeLa cells were transfected with various Arf-GFP isoforms (Arf1, Arf3, Arf4, Arf5) for 18-22 hours, fixed and stained for endogenous Scyl1 by indirect immunofluorescence. Given the lack of Arf isoform specific antibodies, Arf-GFP constructs have been widely used to examine the localization of Arfs (Vasudevan et al., 1998; Chun et al., 2008; Manolea et al., 2010). Scyl1 co-localizes with Arf4 and Arf5, especially in distributed, cytoplasmic punctae (Figure 1G,H). There is less co-localization with Arf1 and Arf3 at peripheral punctae, and these two GTPases are found primarily at the Golgi (Figure 1E,F). Together, the data in figure 1 indicate that Scyl1 interacts with class II Arfs, notably Arf4.

Scyl1 co-localizes with Arf4 and COPI at the ERGIC

We next sought to better define the compartment where Scyl1 and Arf4 co-localize. The peripheral Scyl1-positive Arf4 punctae are also positive for ERGIC-53, a marker of the ERGIC and for β-COP (Figure 2A,B). Thus, Scyl1, Arf4 and COPI interact biochemically and co-localize at the ERGIC. We next utilized super resolution microscopy to better characterize the nature of the peripheral Scyl1 punctae. Interestingly, STED microscopy analysis of Scyl1 reveals that it localizes to a subset of COPI structures, with Scyl1 decorating a large portion of the

roughly 250-500 nm structures (arrowheads, Figure 2C), which are slightly larger in size than ERGIC structures (Klumperman et al., 1998). Yet Scyl1 is absent from other COPI-positive structures (arrows, Figure 2C). Therefore, Scyl1 interacts with a subset of COPI coats in structures morphologically similar to the ERGIC.

Residues on the opposite side of the nucleotide-binding pocket of Arf4 mediate Scyl1 interaction

Despite the high degree of amino acid conservation between human Arf4 and Arf5 (only 18 non-identical residues, bold; Figure 3A), Scyl1 binding to Arf4 is roughly three times greater than to Arf5 (Figure 1B). Thus, surface residues on Arf4 unique to this isoform are likely to contribute to the interaction. Using the available Arf5 crystal structure (PDB-2B6H) we identified non-identical residues that are surface exposed (bold and shaded, Figure 3A,B). We then mutated Arf4 to the Arf5 residue in these positions, generating Arf4-GFP LV112QE, Arf4-GFP AI135PV and Arf4-GFP N150S. GST-Scyl1 was incubated with cell lysates containing Arf4-GFP, Arf5-GFP or the Arf4-GFP mutants and proteins interacting specifically with Scyl1 were identified by Western blot (Figure 3C). GST-Scyl1 interaction with Arf4-LV112QE and Arf4-AI135PV are reduced to a level similar to that seen with Arf5, while the Arf4-N150S mutant interacts with GST-Scyl1 at a level not significantly different than the Arf4 wild-type (Figure 3D). Thus, select residues on the surface of Arf4 opposite to the nucleotide-binding pocket are critical for the interaction of the GTPase with Scyl1 (Figure 3B). These data are consistent with the observation that Arf4 interacts with Scyl1 in a manner independent of the nucleotide-bound state (Figure 1C,D).

Arfs interact directly with COPI (Zhao et al., 1997) and we thus questioned if Scyl1 interacts with Arf4 indirectly, via a complex with COPI. Scyl1 binds COPI primarily through a

dibasic cargo-like motif (RKLD-COO^-) at the extreme C-terminus (Ma and Goldberg, 2013) with additional contributions coming from a C-terminal coiled-coil domain (Burman et al., 2008). We thus took advantage of the Scyl1 truncation mutant, GST-Scyl1 1-750 (Figure 3E), which does not bind COPI (Burman et al., 2008) to test the potential involvement of COPI in mediating the interaction between Scyl1 and Arf4. While full-length GST-Scyl1 (1-806) interacts with both Arf4-GFP and endogenous β-COP from Arf4-GFP transfected cell lysates, GST-Scyl1 1-750 retains full Arf4 interaction but shows no β-COP binding (Figure 3F). However, since it is known that Arfs interact with COPI it is possible that COPI will show a small degree of binding to Scyl1 1-750 mediated by the bound Arf4. To test this possibility we co-expressed myc-γ2-COP with Arf4-GFP or GFP alone and repeated the pull down with wild-type Scyl1 and the 1-750 mutant. Under these conditions, we detect binding of γ2-COP to Scyl1 1-750 only in the presence of Arf4-GFP (supplemental Figure 2). These data indicate that the interaction of Scyl1 with Arf4 is not indirect via COPI, that COPI and Arf4 engage different sites on Scyl1, and that Arf4 can bind COPI when engaged with Scyl1. Coupled with the observation that Scyl1 binds to Arf4 on the opposite side of the nucleotide-binding pocket and that the nucleotide-binding region of Arf interacts with coatomer (Yu et al., 2012), these data suggest that Scyl1, COPI and Arf4 form a tripartite complex that would help organize COPI coats on the ERGIC membrane.

Scyl1 oligomerizes through centrally located HEAT repeats

Epitope-tagged Scyl1 expressed in COS-7 cells runs at roughly 270 kDa on SDS-PAGE following cross-linking (Kato et al., 2002). While Scyl1 may be cross-linked to other proteins in this experiment, given that it is overexpressed, likely to higher levels than its binding partners, this data is consistent with Scyl1 forming a trimer (Scyl1 has a molecular mass of approximately 90 kDa) (Kato et al., 2002). Gel filtration chromatography on soluble extracts of HEK-293T cells

overexpressing Flag-Scyl1 reveals a peak of Scyl1 (asterisk, Figure 4A) corresponding to a native molecular mass of a globular 412 kDa protein (calculated based on known globular markers, indicated by arrows). Thus, the migration of native Scyl1 is consistent with a pentamer, assuming a fully globular structure and that additional interacting proteins are not contributing to the size of the native protein. The migration of Scyl1 is greater than the heterotetrameric AP-2 complex (γ-adaptin, Figure 4A), composed of 2 ~110 kDa subunits and ~50 and 20 kDa subunits, but is smaller than clathrin triskelia (3 ~170 kDa subunits with lengthy non-globular regions). We suggest that Flag-Scyl1, with a globular N-terminal kinase domain and a partially unstructured C-terminal region oligomerizes into structures ranging from trimers to pentamers.

HEAT repeats mediate protein-protein interactions and act as scaffolds in protein complexes (Groves and Barford, 1999). We therefore hypothesized that the HEAT repeats of Scyl1 are responsible for oligomerization. To test this, we performed a series of co-immunoprecipitations using Flag and GFP-tagged Scyl1 constructs. Cells were transfected with GFP only, GFP-Scyl1 342-538 (GFP-HEAT), or full-length GFP-Scyl1 and co-transfected with full-length Flag-Scyl1, Flag-Scyl1 349-806, and Flag-Scyl1 547-806. Immunoprecipitation of full-length GFP-Scyl1 or GFP-HEAT with anti-GFP antibodies leads to co-immunoprecipitation of Flag-Scyl1 (Figure 4B,C). The kinase-like deletion construct (Flag-Scyl1 349-806) co-immunoprecipitates to the same extent as full-length Flag-Scyl1 but a construct lacking the kinase-like domain and the HEAT repeats (Flag-Scyl1 547-806) does not co-immunoprecipitate (Figure 4B,C). These data demonstrate that the region of Scyl1 encoding the HEAT repeats mediate the co-immunoprecipitation and therefore homo-oligomerization of Scyl1.

Scyl1 interacts preferentially with γ2-coatomer

There are multiple pools of COPI vesicles containing different heterotetrameric F subcomplexes composed of different isoforms of γ- and ζ-COP, specifically $\gamma_1\beta\zeta_1\delta$-, $\gamma_1\beta\zeta_2\delta$-, and $\gamma_2\beta\zeta_1\delta$-COP variants (Wegmann et al., 2004). Since Scyl1 interacts specifically with class II Arfs, (Figure 1A), we wondered if Scyl1 links the class II Arf regulatory circuit to an isoform specific pool of coatomer vesicles. To test this hypothesis, we transfected HEK-293T cells with GFP-Scyl1, then immunoprecipitated GFP (Figure 5A). The tagged protein is required as our endogenous Scyl1 antibody was raised against the C-terminus containing the COPI-binding motif. As expected, the GFP-Scyl1 immunoprecipitate was positive for β-COP (Figure 5B). Intriguingly, GFP-Scyl1 co-immunoprecipitates endogenous γ2-COP but γ1-COP is co-immunoprecipitated to a lesser degree (Figure 5B). This suggests that Scyl1 interacts with the heterotetrameric γβζδ-COP F subcomplex, and more specifically with the $\gamma_2\beta\zeta_1\delta$-COP variant. The γβζδ-COP subcomplex is structurally similar to the AP-2 complex of clathrin-coated vesicles with γ-COP most similar to α-adaptin (Hoffman et al., 2003). The appendage domain of α-adaptin interacts with various accessory proteins regulating the formation of clathrin-coated vesicles. We hypothesized that the appendage domain of γ2-COP mediates the interaction of Scyl1 with COPI coats and as such, confers specificity for the $\gamma_2\beta\zeta_1\delta$-COP variant. We thus used GST-tagged γ1-appendage and γ2-appendage in affinity-selection assays with lysates from cells expressing Flag-Scyl1 (Figure 5C). Interestingly, Scyl1 binds to the γ-appendage domains with a preference for γ2 versus γ1 (Figure 5C). The appendage domains and Flag-Scyl1 are readily detectable by ponceau S staining with no other proteins at comparable levels, supporting that the interaction is direct (Figure 5C). We next used Flag-Scyl1 constructs to map the interaction with the γ2-appendage. HEK-293T cells were transfected with Flag-Scyl1 (349-806), a kinase-like domain deletion construct, Flag-Scyl1 (547-806), which removes the kinase-like domain and the

HEAT repeats necessary for oligomerization (Figure 5E), or Flag-Scyl1 full-length (RK→AA) mutant, which reduces coatomer binding (Burman et al., 2008; Ma and Goldberg, 2013). GST-γ2-appendage binds the kinase deletion construct with the same enrichment as full-length Scyl1 (Figure 5D,E). Flag-Scyl1 (547-806) also binds but with less affinity than that of full-length protein. The Flag-Scyl1 full-length (RK→AA) mutant does not bind the GST-γ2-appendage (Figure 5D,E). Thus, Scyl1 uses the RKXX-COO^- motif to bind directly to the γ2-appendage domain with stronger binding when the protein contains HEAT repeats and is presumably oligomerized. It thus appears that Scyl1 links class II Arfs in an oligomeric form to a γ2-coatomer-positive pool of COPI.

Disruption of Scyl1 scaffolding function induces tubulation of the ERGIC

Overexpression of Scyl1 should alter the stoichiometry of the scaffolding complex with the potential to alter membrane budding at the *cis*-Golgi and/or ERGIC. We utilized live cell imaging to study the effect of the expression of Scyl1 on ERGIC dynamics. In HeLa cells transfected with YFP-ERGIC-53 and low levels of mCherry, the YFP signal was seen in punctae, with highly dynamic YFP-positive tubules extending from the punctae and moving towards the centre of the cell, towards the periphery, or laterally between YFP punctae at roughly the same distance from the nucleus (Figure 6A, Movie 1.avi). The observed behaviour of YFP-ERGIC-53 is similar to previous descriptions of tagged ERGIC-53 (Appenzeller-Herzog and Hauri, 2006). In sharp contrast, in cells expressing mCherry-Scyl1, the YFP-ERGIC-53 signal is significantly disrupted (Figure 6B, Movie 2.avi). Expression of fluorescent tagged-Scyl1 often causes the formation of fluorescent cytoplasmic inclusions, in addition to the fainter, fluorescent-positive signal similar to the distribution of fixed, endogenous Scyl1. YFP-ERGIC-53-positive tubules interconnect juxta-nuclear and peripheral punctae. These tubules are mostly static, remaining

connected between punctae over several minutes and occasionally breaking off or forming new tubules. Also, we observe that mCherry-Scyl1 appears to be absent from tubules and is associated predominantly with the punctae.

We performed quantification on fixed cells revealing that there are more and longer ERGIC-53 tubules in GFP-Scyl1 expressing cells than GFP only expressing cells (Figure 6C,D). Interestingly we also observe an altered morphology of the *cis*-Golgi as marked by GM130. GM130 staining appears dispersed and the Golgi generally appears larger, consistent with the expanded Golgi observed by EM analysis in Scyl1 knock down cells (Burman et al., 2010). Moreover, GM130-positive tubules emanate from the juxtanuclear Golgi region toward the periphery. Tubules are more numerous and longer in GFP-Scyl1 expressing cells than GFP only expressing cells (Figure 6E,F). Knock down of Scyl1 also induces tubulation as determined by staining for endogenous ERGIC-53 (supplemental Figure 3). This corroborates a screen in which disruption of the early secretory pathway was examined following knock down of putative kinases. The study revealed that Scyl1 knock down caused tubulation of the ERGIC (Farhan et al., 2010). Thus, disruption of the scaffolding function of Scyl1 by overexpression or loss of function leads to alterations in vesicle budding at the ERGIC and the *cis*-Golgi with the formation of abnormally long and static tubules.

Discussion

We originally identified the pseudo-kinase Scyl1 as a COPI-interacting protein that functions in COPI-mediated retrograde trafficking (Burman et al., 2008). Scyl1 interacts with COPI through a C-terminal RKXX-COO^- dibasic motif, similar to many other COPI-binding proteins (Nilsson et al., 1989; Jackson et al., 1993). Pseudo-kinases containing multiple protein domains often act as scaffolds. Here we demonstrate that Scyl1 uses the dibasic motif to bind directly to the appendage domain of γ-COP with an isoform preference for γ2-COP. Moreover, Scyl1 forms oligomers and through a site distinct from the COPI binding motif, interacts with Arf4 but not with the class I Arfs. Thus, Scyl1 links an Arf4-regulated pathway to a specific class of COPI vesicles.

The specificity of Scyl1 for class II Arfs is interesting given the high degree of sequence homology between class II Arfs and other Arf isoforms. Most studies of Arf function have focused on Arf1 and Arf6 and much less are known regarding the function of class II Arfs. Arf4 $^{-/-}$ mice are embryonic lethal whereas Arf4 heterozygotes have a neurological phenotype characterized by a reduction in the number of dendritic spines in the dentate gyrus, with subsequent deficiencies in related behavioural tasks (Jain et al., 2012). The effect this plays in development or neuronal survival has not been tested but since *mdf* mice also have a severe neurological deficit with motor neuron degeneration (Blot et al., 1995) and Arf4 and Scyl1 interact biochemically, we speculate that there is an underlying relationship between the two phenotypes.

GDP-bound Arfs are cytosolic and the myristoylated N-terminal amphipathic helix is necessarily shielded from the aqueous environment by packing against the globular protein (Liu et al., 2010). It is thought that Arfs are first recruited to membranes in a GDP-bound form by a membrane-

associated receptor where they are subsequently activated by ArfGEFs. For example, Arf1-GDP is recruited to membranes by the cytosolic domain of p23 (Gommel et al., 2001) or by the ER-Golgi SNARE, membrin (Honda et al., 2005). A class II Arf receptor has not been described (Chun et al., 2008) but is predicted to retain association with ERGIC/Golgi membranes in the presence of BFA and to interact with class II Arfs in their GDP-bound form. Although Scyl1 has these properties (Burman et al., 2008 and this study) and thus fits many criteria of a class II Arf receptor, we have been unable to directly demonstrate an influence of Scyl1 knock down on the membrane recruitment of Arf4-GFP and full resolution of this potential role awaits further study.

The Scyl1 C-terminal RKLD-COO^- sequence was recently co-crystalized with β'-COP (Ma and Goldberg, 2013), a subunit of the heterotrimeric αβ'ε-COP B subcomplex that forms the outer shell of COPI vesicles. We find that Scyl1 interacts with the appendage of γ2-COP, a component of the heterotetrameric $\gamma_2\beta\zeta_1\delta$-COP F subcomplex, and that this interaction is also mediated by the RKLD-COO^- sequence since mutation to AALD-COO^- abolishes the interaction. The multiple binding sites for Scyl1 on COPI were previously predicted, as a Scyl1 C-terminal peptide competes a p24α2/COPI interaction but a p24α2 dibasic peptide does not interfere with the Scyl1/COPI interaction (Burman et al., 2008). Through the centrally located HEAT repeats, Scyl1 oligomerizes. This creates a scaffold with three to five RKLD-COO^- sequences and as such, Scyl1 may not simply organize COPI coats on the membrane but could also contribute to biochemically linking αβ'ε- and $\gamma_2\beta\zeta_1\delta$-COP subcomplexes and/or linking the $\gamma_2\beta\zeta_1\delta$-COP subcomplex to other $\gamma_2\beta\zeta_1\delta$-COP subcomplexes, via the γ_2-appendage. The ability to rapidly polymerize and depolymerize is a central feature of vesicle coat proteins and Scyl1 oligomers likely have an important role in promoting COPI coat polymerization, perhaps in different configurations (Faini et al., 2012).

Knock down of Scyl1 was previously shown to cause tubulation of the ERGIC (Farhan et al., 2010). Here, we confirm that Scyl1 knock down induces ERGIC tubulation. Moreover, we demonstrate that overexpression of fluorescent-tagged Scyl1, which generates Scyl1-positive inclusions that likely sequester binding partners including endogenous Scyl1, Arf4 and coatomer induces robust tubulation of both the ERGIC and the *cis*-Golgi. Considering that Arf4 is sufficient for Arf-dependent COPI vesicle formation (Popoff et al., 2011), we speculate the observed tubulation upon disruption of Scyl1 function results from an impairment in the ability of COPI cargo carriers to vesiculate and bud from the membrane. We previously observed that knock down of Scyl1 regulates Golgi morphology (Burman et al., 2010) and that phenotype is therefore most likely due to Golgi tubulation.

Together, our data biochemically link through Scyl1, multiple early secretory tubulation phenotypes and further our understanding of COPI regulation. Since Scyl1 is the gene product lost in the *mdf* mouse, these findings expand our knowledge into the pathways and potential mechanisms underlying selective motor neuron death.

Materials and methods

Constructs and proteins

Bacterially expressed GST-Scyl1 constructs were cloned into pGEX-4T-1 (Clontech) and expressed in *E. coli* BL21. Mammalian expressed tagged Scyl1 constructs were PCR amplified from mouse Scyl1 DNA (Burman et al., 2008) into N-terminally tagged pCMV-Tag2B, pEGFP-C2 or mCherry-C2 (mCherry inserted into AgeI/BsrGI digested pEGFPC2). Arf1-GFP, Arf3-GFP, Arf4-GFP, Arf5-GFP in pEGFP-N1 were previously described (Chun et al., 2008). Arf4 mutants were generated from wild type human Arf4 using PCR mutagenesis and cloned into pEGFP-N1. Human GST- γ1 and GST- γ2 appendage (Moelleken et al., 2007) were expressed in *E. coli* BL21. Mouse myc-γ2-COP was expressed in pcDNA3.1. All plasmids were verified by sequencing.

Antibodies and Reagents

Affinity-purified rabbit polyclonal antibody was raised against the C-terminal amino acids (KKTTKGPMKLGARKLD-COO^-) of mouse Scyl1. Polyclonal antibodies against γ1-COP and γ2-COP were kindly provided by Dr. Felix Wieland (Heidelberg University). Affinity-purified rabbit polyclonal antibody was raised against human clathrin heavy chain (amino acids QEHLQLQNLGINPANIGFS). Polyclonal antibody against GFP and monoclonal antibody against Flag epitope (M2) were from Invitrogen (Carlsbad, CA) and Sigma (St. Louis, MO), respectively. Monoclonal antibodies against β-COP and GFP (used to verify polyclonal GFP immunoprecipitation) were obtained from AbCam (Cambridge, MA). Monoclonal antibodies against GAPDH, ERGIC-53 and GM130 were from Santa Cruz Biotechnology Inc. (Santa Cruz,

CA), ENZO Life Sciences Inc. (Farmingdale, NY), and BD Labs (Franklin Lakes, NJ), respectively. HeLa cells were transfected for localization and tubulation assays with JetPrime reagent (Polyplus Transfection; Illkirch, France). Alexa Fluor conjugated 488, 568 and 647 mouse and rabbit antibodies used for indirect immunofluorescence were obtained from Abcam.

Affinity selection and co-immunoprecipitation assays

For GST-fusion proteins, bacteria were lysed in PBS, 1mM DTT, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml aprotinin, and 0.5 µg/ml leupeptin (protease inhibitors), pH 7.4. For GST-GGA3 pulldowns, bacteria were lysed in 50mM TRIS, 100mM NaCl, 10mM $MgCl_2$, 0.1% SDS, 0.5% DOC, 1% Triton, 5% glycerol, protease inhibitors, pH 7.5. For affinity-selection assays, HEK-293T cells were collected in lysis buffer (20 mM HEPES, pH 7.4, protease inhibitors, 1mM $MgCl_2$, 30 nM Okadaic acid, 5 mM Sodium pyrophosphate, 1mM DTT) and Triton X-100 was added to 1% final concentration. Following 10 min rocking at 4 °C, the extracts were spun at 205,000 X *g* for 15 min to remove insoluble material. Aliquots of 1 ml were incubated for 1 hour at room temperature with GST or GST-Scyl1 fusion proteins pre-coupled to glutathione-Sepharose beads (GE Healthcare). Beads were subsequently washed three times with lysis buffer with 1% Triton X-100 and analyzed by SDS-PAGE. For nucleotide-specificity assays, 50 µM Aluminum Fluoride or 50 µM GMPP(NH)P was added to cell lysis buffer as indicated. Bands specifically interacting with GST-Scyl1 fusion proteins were cut and analyzed by tandem mass spectrometry (MS) or were processed for Western blot.

Gel filtration chromatography

HEK-293T cells were transfected with Flag-Scyl1 by the calcium phosphate method and incubated 48 hours. Plates of cells (2 x 15 cm) were collected in 5ml lysis buffer and lysates were immediately run on a pre-calibrated Hiprep 26/60 Sephacryl S-300 HR column (GE Healthcare). Fractions were collected, run on SDS-PAGE gel and analyzed for anti-Flag and the indicated proteins as internal controls by Western blot.

Cellular imaging

For fixed cell confocal imaging, cells were grown on poly-L-lysine–coated coverslips were washed in PBS and then fixed for 20 minutes in 4% paraformaldehyde at 4°C. After fixation, cells were permeabilized with 0.2% Triton X-100 in PBS for 2 minutes and processed for immunofluorescence with the appropriate primary and secondary antibodies in PBS + 0.01% BSA. Images were obtained using a Zeiss (Thornwood, NY) 710 Laser Scanning Confocal Microscope. Single slice confocal projections were obtained for each channel in the same focal plane and saved as LSM files before processing.

For STED imaging, NRK cells were fixed in 4% paraformaldehyde and permeabilized with 0.3% NP40 + 0.1% Triton X-100. After immunolabeling, cells were post-fixed in 3% paraformaldehyde + 0.1% gluteraldehyde and then embedded using Ultra Bed Low Viscosity Epoxy Kit (EMS). Cells were then cut into 80 nm thin sections using a Leica microtome and DiATOME Ultra 45 Diamond Knife. Sections were dried onto 1.5 coverglass, mounted in Mowiol, and imaged using a Leica TCS STED microscope featuring a pulsed diode laser (PDL 800-B, PicoQuant) emitting at 640nm (~8-65µW) for excitation and a Ti:Sapphire laser (Mai Tai, Spectra Physics) for depletion (~130mW). ATTO647N labeled samples were depleted at 770nm.

For live cell imaging, HeLa cells were plated at 30% confluence on 35 mm plates (MatTek Corporation; Ashland, MA) with glass coverslips in 2 ml DMEM + 10% serum. Cells were transfected with mCherry constructs and YFP-ERGIC-53, and the plates were incubated 20-22 hours. Live-cell imaging was performed using an Axio Observer Z1 microscope equipped with an epifluorescent plan-apochromat 40x oil objective (NA=1.4), Definite Focus system and an AxioCam MR3 camera (Zeiss). Cells were kept at 37°C in 5% CO_2 using the Incubation System S (Pecon, Germany). YFP and mCherry were illuminated using 470 nm and 591 nm laser lines respectively, from a Colibri.2 illumination source (Zeiss). Acquisition and analysis was performed using the ZEN 11.0 software (Zeiss), while movies were made using ImageJ 1.43m.

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Author Contributions

Conceived and designed the experiments: JNRH, PM, PSM. Performed the experiments: JNRH, LS, MF, HD, MSI, MG, NS. Wrote the paper: JNRH, PSM.

Competing Interests

The authors declare that they have no conflict of interest.

Figure legends

Figure 1. Scyl1 interacts with class II Arfs. (A) GST-tagged full-length Scyl1 or GST alone were incubated with HEK-293T cell lysates expressing Arf1-GFP, Arf3-GFP, Arf4-GFP or Arf5-GFP and specifically bound proteins were processed for Western blot with antibodies against GFP. An aliquot of the cell lysate (Input) equal to $1/20^{th}$ of that added to the beads was processed in parallel. **(B)** The signal in the GST-Scyl1 lane relative to the signal in the Input lane for blots as in A was determined (relative bound) for each Arf protein. Bars represent mean ± s.e.m. and statistical analysis employed a one-way ANOVA, NS = not significant. *** = $P < 0.001$. **(C)** GST-tagged full-length Scyl1 or GST alone were incubated with HEK-293T cell lysates expressing wild-type Arf4-GFP, Arf4-T31N-GFP (inactive) or Arf4-T48N-GFP (inactive), Arf4-Q71L-GFP, which is a GTP loaded (hydrolysis deficient) mutation or Arf4-GFP treated with aluminium fluoride or GMPP(NH)P to mimic a GTP-loaded conformation and specifically bound proteins were processed for Western blot with antibodies against GFP. An aliquot of the cell lysate (Input) equal to $1/20^{th}$ of that added to the beads was processed in parallel. **(D)** The signal in the GST-Scyl1 lane relative to the signal in the Input lane for blots as in A was determined (relative bound) for each Arf protein in C. **(E-H)** HeLa cells were

transfected with various Arf-GFP constructs for 18 hours. The cells were subsequently fixed and processed for GFP fluorescence and for indirect immunofluorescence with antibody recognizing endogenous Scyl1. Insets show co-localization at peripheral punctae, as indicated by arrowheads. Scale bars 15 μm, 2.5 μm (inset).

Figure 2. Scyl1 co-localizes with Arf4 at the ERGIC. (A/B) HeLa cells were transfected with Arf4-GFP for 18 hours. The cells were subsequently fixed and processed for GFP fluorescence and for indirect immunofluorescence with antibodies against the indicated proteins. Triple merge shows Scyl1/Arf4-GFP co-localization with β-COP and ERGIC-53. Insets show co-localization at peripheral punctae, as indicated by arrowheads. Scale bars 15 μm, 2.5 μm (inset). **(C)** Scyl1 decorates a subset of β-COP structures. NRK cells were grown to sub-confluency, fixed, and processed for immunofluorescence with antibodies against the indicated proteins. β'-COP was imaged by confocal microscopy 488 nm as a marker for COPI, and Scyl1 was imaged by STED microscopy, 647nm. Arrowheads indicate co-localizing regions. Arrows indicate regions of COPI absent of Scyl1. Scale bars 3 μm, 1 μm (magnification).

Figure 3. Surface residues on Arf4 opposite the switch regions are necessary for the interaction with Scyl1. (A) Alignment of the amino acid sequence of human Arf4 and Arf5. Switch regions are boxed. Non-identical residues are in boldface type. Surface residues selected for mutation are boxed in grey and indicated by initial residue number. (**B)** Structure of human Arf5-GDP, with the nucleotide binding pocket and surface residues selected for Arf4 point mutations indicated by arrows. **(C)** GST-tagged full-length Scyl1 or GST alone were incubated with HEK-293T cell lysates expressing Arf4-GFP, Arf5-GFP or Arf4-GFP containing the indicated mutations and specifically bound proteins were processed for Western blot with antibodies against GFP. An aliquot of the cell lysate (Input) equal to $1/20^{th}$ of that added to the

beads was processed in parallel. **(D)** The signal in the GST-Scyl1 lane relative to the signal in the Input lane for blots as in C was determined (relative bound) for each Arf4 construct. Bars represent mean ± s.e.m. and statistical analysis employed a one-way ANOVA, NS = not significant, * = $P < 0.05$. **(E)** Schemata of Scyl1 and Scyl1 residues 1-750 (deletion of the COPI binding region). **(F)** GST-tagged full-length Scyl1 (1-806), GST-Scyl1 deletion mutant (1-750), or GST alone were incubated with HEK-293T lysates expressing Arf4-GFP.

Figure 4. Scyl1 forms oligomers via centrally located HEAT repeats (A) Soluble lysates were prepared from HEK-293T cells expressing Flag-Scyl1 and subjected to gel filtration chromatography. Fractions were collected and analyzed by Western blot for mouse anti-Flag or indicated proteins. The migratory positions of globular markers are indicated by arrows. The peak of Scyl1 elution occurs at a fraction equivalent to a 412 kDa globular marker. **(B)** HEK-293T cell lysates expressing GFP, GFP-Scyl1-HEAT (342-538) or GFP-Scyl1-full-length (FL) and co-expressing Flag-Scyl1 (1-806), Flag-Scyl1 (349-806) or Flag-Scyl1 (547-806) were incubated with protein-A Sepharose beads coupled to rabbit anti-GFP. Specifically bound proteins were analyzed by Western blot with antibodies against GFP and Flag. Starting material (SM) represents $1/20^{th}$ of the input used for the selection experiment. **(C)** Schemata of Scyl1 depicting the deletion constructs used in (B), and the ability of Scyl1 to bind itself indicating oligomerization.

Figure 5. Scyl1 interacts with γ2-coatomer appendage domain (A) HEK-293T cell lysates expressing GFP-Scyl1 were incubated with protein-A Sepharose beads coupled to rabbit anti-GFP. Arrows indicate the successful immunoprecipitation of GFP-Scyl1 as measured by ponçeau S staining. **(B)** Specifically bound proteins in (A) were analyzed by Western blot for the indicated proteins. Input represents $1/20^{th}$ of the material used for the selection experiment. **(C)**

GST-tagged γ1-appendage, GST-tagged γ2-appendage or GST alone were incubated with HEK-293T cell lysates expressing Flag-Scyl1 full-length. Specifically bound Flag-Scyl1 was detected by ponçeau S staining. **(D)** GST-tagged γ2-appendage or GST alone were incubated with HEK-293T lysates expressing the indicated Flag-Scyl1 constructs. Specifically bound Flag-Scyl1 was analyzed by Western blot. Input represents $1/20^{th}$ of the material used for the selection experiment. (**E)** Schemata of Scyl1 constructs used in (D,E) with the COPI affinity indicated by plus marks.

Figure 6. Overexpression of Scyl1 tubulates ERGIC-53 containing membranes in living cells. (A) HeLa cells were plated on coverslips on 35 mm dishes (MatTek) and transfected with mCherry and YFP-ERGIC-53 for 18-22h. Cells were imaged over the course of 5 minutes and a representative field is shown. The top two panels are the first frames of the movie (Movie 1.avi) and the bottom panels are a 5X magnified view of the box in the top panel of the YFP-ERGIC-53 channel at the indicated time points. **(B)** HeLa cells were plated on coverslips on 35 mm dishes (MatTek) and transfected with mCherry-Scyl1 and YFP-ERGIC-53 for 18-22h. Cells were imaged over the course of 5 minutes and a representative field is shown. The top two panels are the first frames of the movie (Movie 2.avi) and the bottom panels are a 5X magnified view of the box in the top panel of the YFP-ERGIC-53 channel at the indicated time points. Scale bars 10 μm for the top panels, 2 μm for the bottom panels. **(C)** Average number of endogenous ERGIC-53 tubules per fixed cell expressing GFP only (N=15) or GFP-Scyl1 (N=16) was quantified using Image J 1.45 (NIH) measure function. **(D)** Average ERGIC-53 tubule length was quantified as in (C). Error bars represent plus or minus SEM. **(E)** Average number of endogenous GM130 tubules per cell expressing GFP only (N=14) or GFP-Scyl1 (N=15) was

quantified as in (C). **(F)** Average GM130 tubule length was quantified as in (C). Error bars represent plus or minus s.e.m.

Supplemental Figure 1. Arf-GFP constructs interact with GST-GGA3-GAT domain. GST-tagged full-length GGA3-GAT domain or GST alone were incubated with HEK-293T cell lysates expressing Arf1-GFP or Arf4-GFP and specifically bound proteins were processed for Western blot with antibodies against GFP. An aliquot of the cell lysate (Input) equal to 1/20th of that added to the beads was processed in parallel.

Supplemental Figure 2. Scyl1-Arf4-COPI form a tri-partite complex. GST-tagged full-length Scyl1, GST-Scyl1 (1-750) or GST alone were incubated with HEK-293T cell lysates co-expressing GFP and myc-γ2-COP or Arf4-GFP and myc-γ2-COP and specifically bound proteins were processed for Western blot with antibodies against the indicated proteins. An aliquot of the cell lysate (Input) equal to 1/20th of that added to the beads was processed in parallel.

Supplemental Figure 3. Knock down of Scyl1 causes tubulation of the ERGIC. (A) HeLa cells were transfected with siRNA targeting Scyl1 or with non-specific (mock) siRNA for 72h. The cells were mixed and reseeded onto the same coverslips for 24h, fixed and processed for indirect immunofluorescence with antibodies against the indicated proteins. Bottom panel magnification of boxed region. Arrowheads indicate tubulation of endogenous ERGIC-53. Scale bars, 20 μm, 5 μm (magnification). **(B)** Cells analyzed in A were harvested in parallel and processed for western blot with an antibody against Scyl1 and Clathrin heavy chain (CHC).

Movie 1. Live cell imaging of YFP-ERGIC-53 in cells expressing mCherry alone. Movie corresponding to Figure 6A.

Movie 2. Live cell imaging of YFP-ERGIC-53 in cells expressing mCherry-Scyl1. Movie corresponding to Figure 6B.

Figure 1)

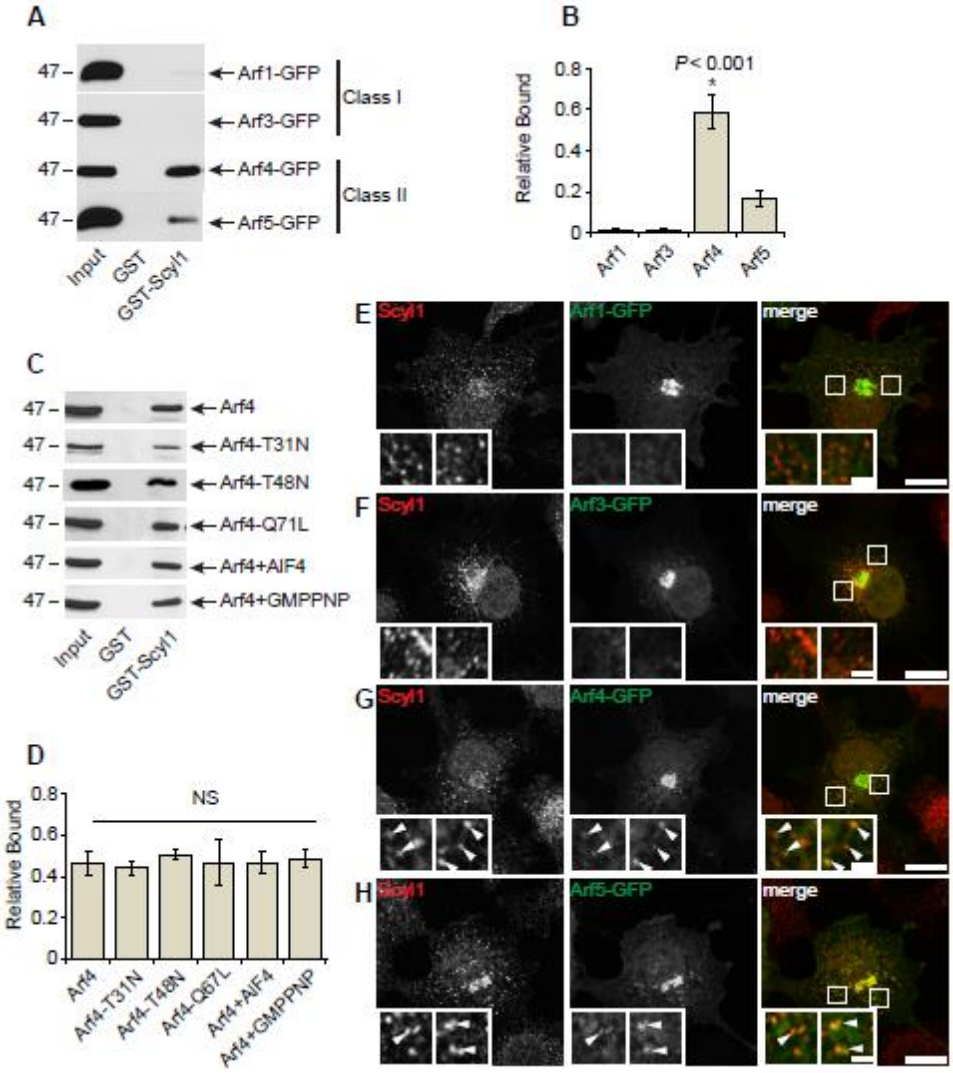

A
47 –
Arf1-GFP
Arf3-GFP
Arf4-GFP
Arf5-GFP
Class I
Class II
Input
GST
GST-Scyl1
B
Relative Bound
P< 0.001
*
0.8
0.6
0.4
0.2
0
Arf1
Arf3
Arf4
Arf5
C
Arf4
Arf4-T31N
Arf4-T48N
Arf4-Q71L
Arf4+AlF4
Arf4+GMPPNP
D
NS
Arf4-T31N
Arf4-T48N
Arf4-Q67L
Arf4+AlF4
Arf4+GMPPNP
E
F
G
H
Scyl1
Arf1-GFP
Arf3-GFP
Arf4-GFP
Arf5-GFP
merge

Figure 2)

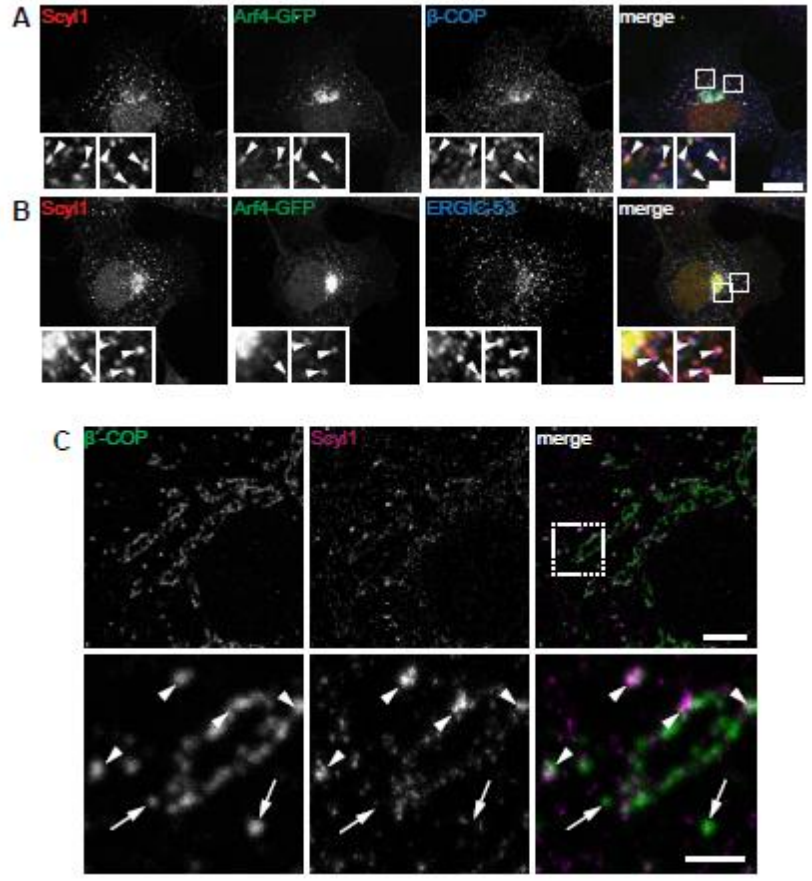
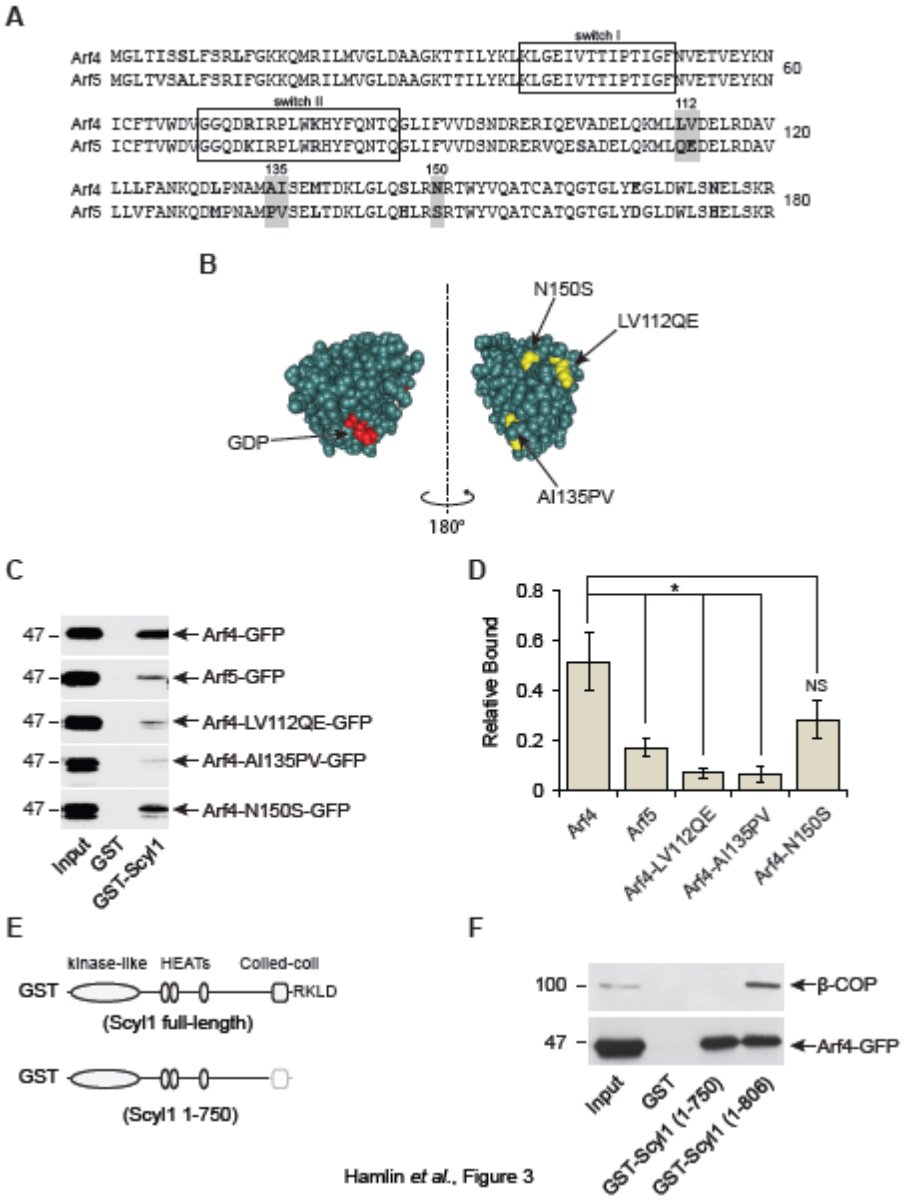
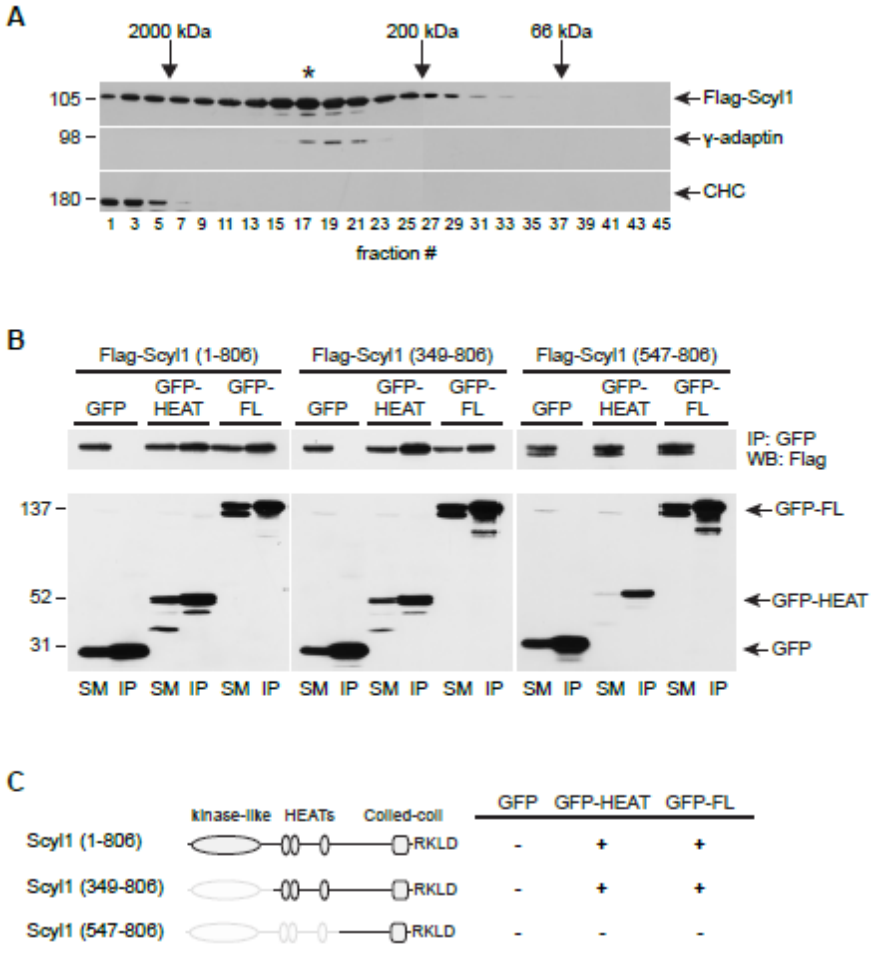

A
Scyl1
Arf4-GFP
β-COP
merge
B
Scyl1
Arf4-GFP
ERGIC-53
merge
C
β-COP
Scyl1
merge

Figure 3)



Hamlin *et al.*, Figure 3

Figure 4)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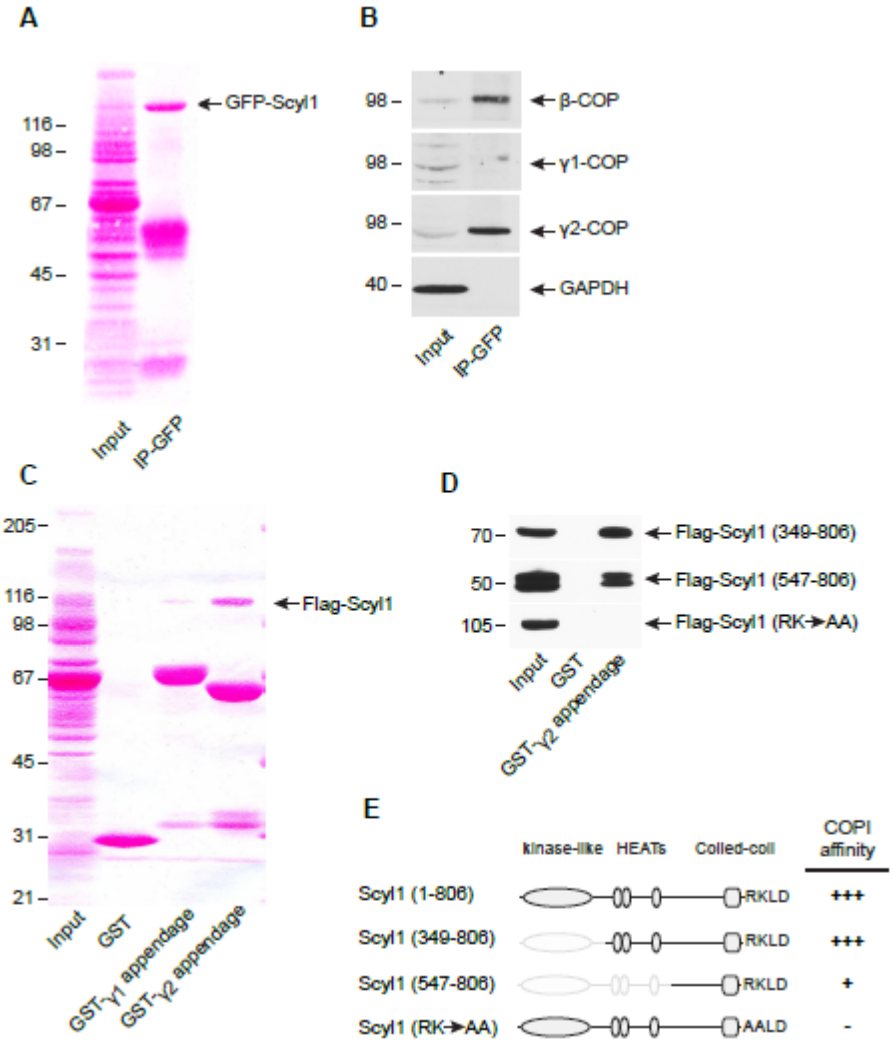
2000 kDa
200 kDa
66 kDa
105
98
180
Flag-Scyl1
γ-adaptin
CHC
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45
fraction #
B
Flag-Scyl1 (1-806)
Flag-Scyl1 (349-806)
Flag-Scyl1 (547-806)
GFP
GFP-HEAT
GFP-FL
IP: GFP
WB: Flag
137
52
31
GFP-FL
GFP-HEAT
GFP
SM IP SM IP SM IP SM IP SM IP SM IP SM IP SM IP SM IP
C
kinase-like
HEATs
Coiled-coil
GFP
GFP-HEAT
GFP-FL
Scyl1 (1-806)
Scyl1 (349-806)
Scyl1 (547-806)
RKLD
- + +
- + +
- - -

Figure 5)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98 –
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B
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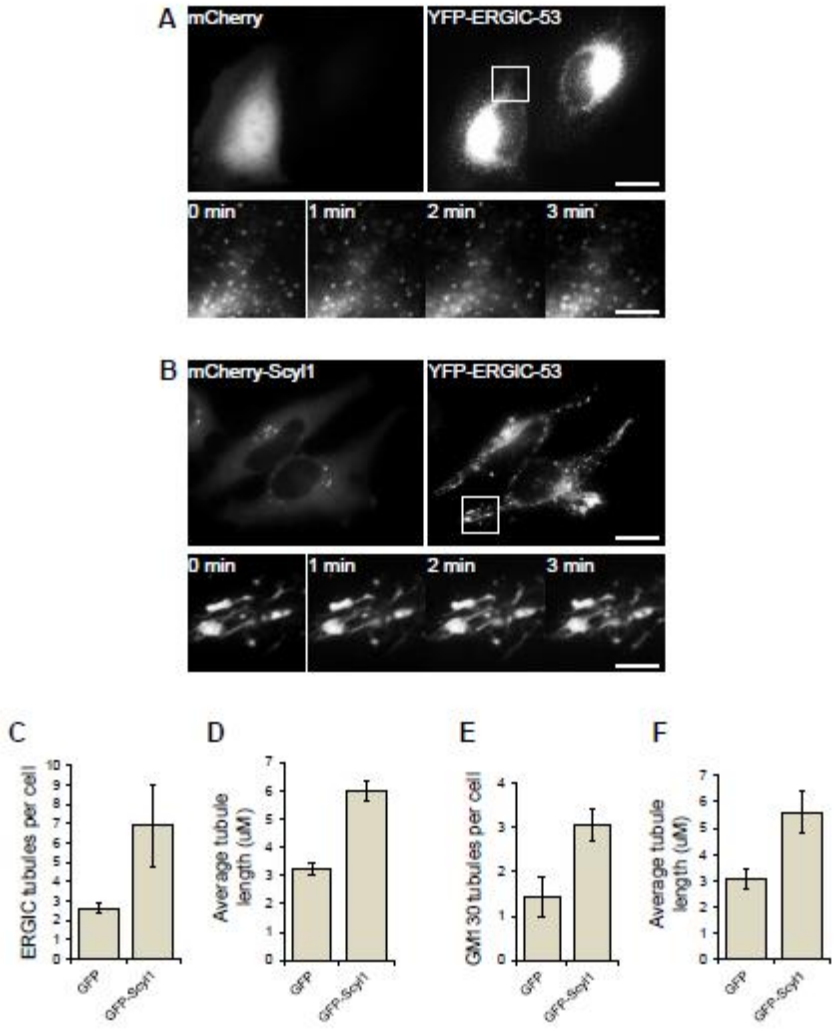
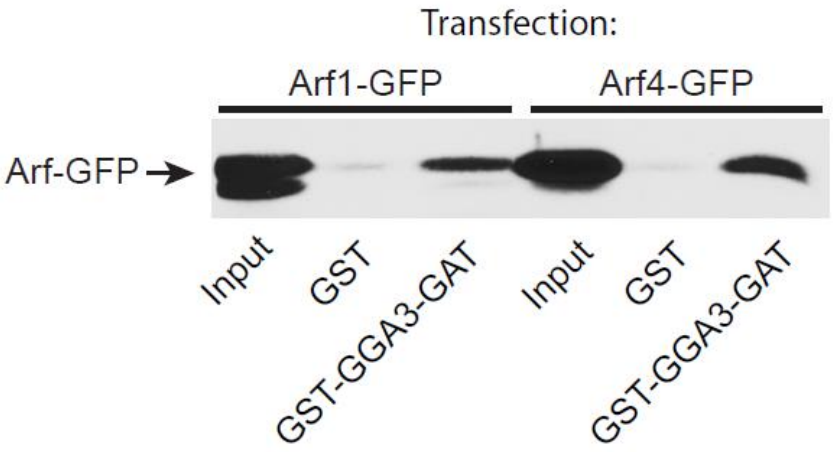
β-COP
98 –
γ1-COP
98 –
γ2-COP
40 –
GAPDH
Input
IP-GFP
C
205 –
116 –
98 –
67 –
45 –
31 –
21 –
Flag-Scyl1
Input
GST
GST-γ1 appendage
GST-γ2 appendage
D
70 –
Flag-Scyl1 (349-806)
50 –
Flag-Scyl1 (547-806)
105 –
Flag-Scyl1 (RK→AA)
Input
GST
GST-γ2 appendage
E
kinase-like
HEATs
Coiled-coil
COPI affinity
Scyl1 (1-806)
RKLD
+++
Scyl1 (349-806)
RKLD
+++
Scyl1 (547-806)
RKLD
+
Scyl1 (RK→AA)
AALD
-

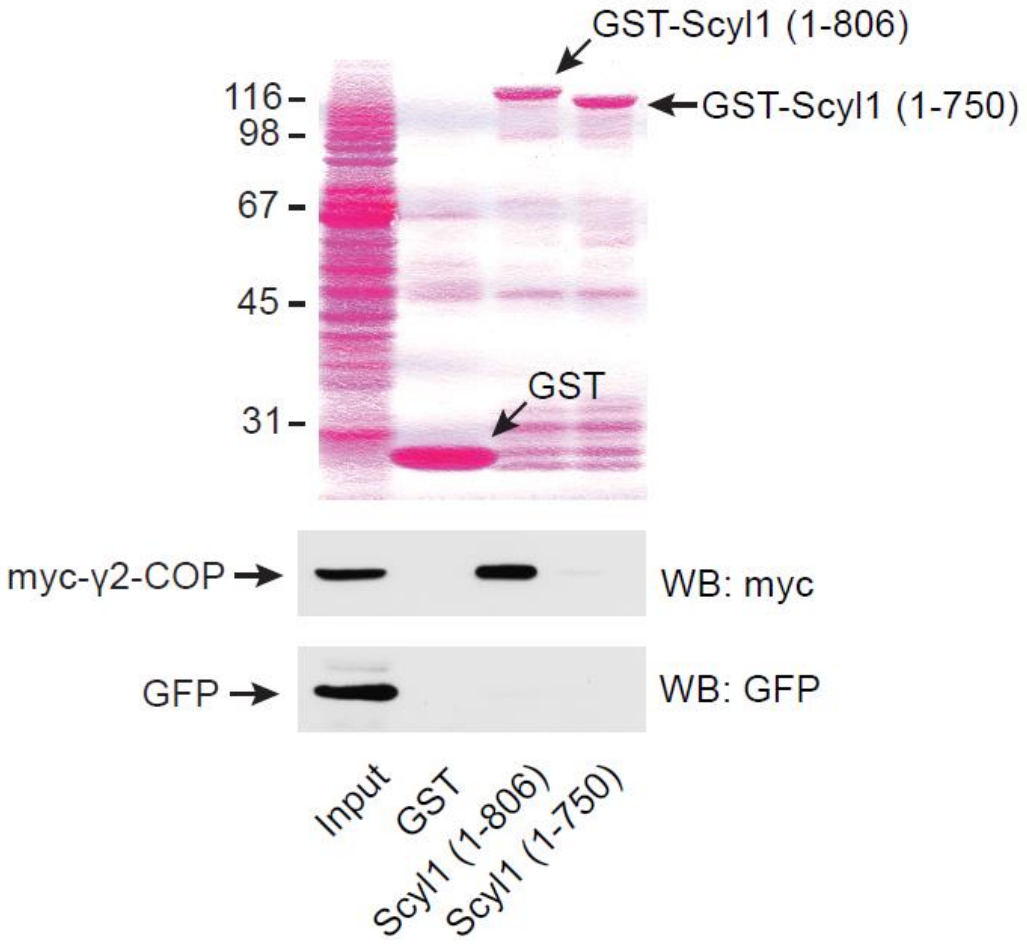
Figure 6)


A
mCherry
YFP-ERGIC-53
0 min
1 min
2 min
3 min
B
mCherry-Scyl1
YFP-ERGIC-53
0 min
1 min
2 min
3 min
C
ERGIC tubules per cell
GFP
GFP-Scyl1
D
Average tubule length (uM)
GFP
GFP-Scyl1
E
GM130 tubules per cell
GFP
GFP-Scyl1
F
Average tubule length (uM)
GFP
GFP-Scyl1

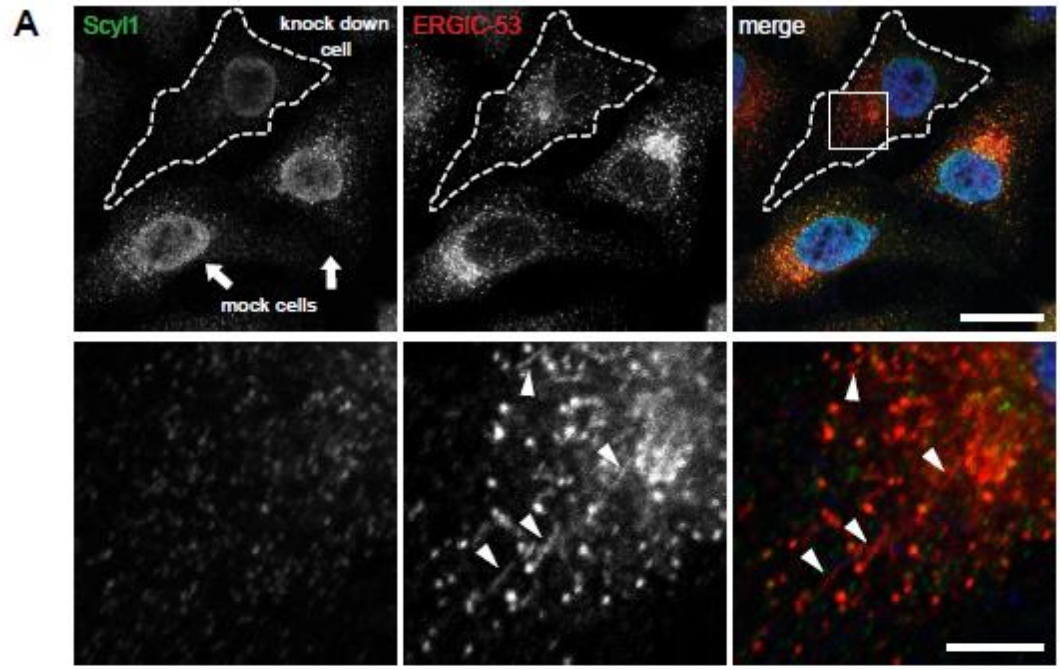
Supplemental Figure 1)



Supplemental Figure 2)



Supplemental Figure 3)



Movie 1)

Movie1.avi

Movie 2)

Movie2.avi

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PREFACE TO CHAPTER 3: The Golgi-associated retrograde protein (GARP) complex member VPS-53 contains a dibasic COPI binding motif

The findings presented in Chapter 2, revealing that Scyl1 directly binds the γ2-appendage of COPI (Hamlin et al., 2014) were interesting for a number of reasons. First, we had mapped at least one location on COPI where Scyl1 binds. Second, it led us to question the ability of the γ-appendage to bind *other* dibasic-motif containing proteins. Do other proteins display an isoform preference for the γ1-COP or the γ2-COP appendage? Is the γ-appendage a general platform for interfacing with the COPI regulatory molecules or cargo? The data here and published elsewhere (Harter and Wieland, 1998; Ma and Goldberg, 2013) suggests that there are indeed multiple sites on COPI that interface with both cargo and regulatory proteins. We investigate one candidate protein, VPS-53 that binds the γ-appendage of COPI. Interestingly, VPS-53 is also part of a protein complex that is mutated in a neurodegenerative disease model (Liewen et al., 2005).

Chapter 3 is a manuscript in preparation for publication.

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The Golgi-associated retrograde protein complex (GARP) member VPS-53 contains a dibasic COPI binding motif

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Running title: GARP member VPS-53 binds COPI

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SUMMARY

Coatomer (COPI) coated vesicles are the primary form of vesicular transport in the early secretory pathway. COPI has been found to control retrograde transport from the Golgi and the endoplasmic reticulum Golgi intermediate compartment (ERGIC) to the ER and Golgi inter-cisternal transport. VPS-53 is a member of the Golgi associated retrograde protein complex (GARP), a tethering complex located at the *trans*-Golgi network and responsible for tethering vesicles derived from the endosomal system. Here, we show that VPS-53 contains a canonical KKRL-COO^- dibasic COPI binding site and binds the γ-COP subunit of COPI. Lysine to alanine substitutions abolished binding to COPI. Interestingly, mutations in GARP are responsible for the phenotype of the neurodegenerative wobbler (*wr*) mouse. This data implicates GARP and the *trans*-Golgi network as an interface between the endosomal and early secretory pathways and provides additional data implicating COPI in neuronal degeneration.

INTRODUCTION

COPI coat complexes drive the formation of vesicles in the early secretory pathway. COPI consists of two stable subcomplexes, the trimeric clathrin-like B-subcomplex, composed of α-COP, β'-COP and ε-COP and the tetrameric AP-2-like F-subcomplex, composed of β-COP, γ-COP, δ-COP and ζ-COP (Chapter 1.2.1). The γ-COP subunit has significant homology with the α-adaptin subunit of AP-2 and the proteins share a membrane-adjacent trunk domain and an appendage domain connected by a linker. While the appendage domain of α-adaptin is well characterized and binds to multiple proteins that regulate the assembly of clathrin-coated vesicles, such a characterization has not been reported for γ-COP in COPI vesicular transport.

COPI binds transmembrane cargo containing a C-terminal dibasic motif with the residues KXKXX-COO^- or KKXX-COO^- (Nilsson et al., 1989; Nilsson and Warren, 1994). COPI also binds the cytosolic protein Scyl1 via an RKXX-COO^- motif (Burman et al., 2008). Peptides bearing each of these motifs, including that from Scyl1, have been crystalized in a complex with the terminal domain of the β'-COP subunit of the COPI B-subcomplex (Ma and Goldberg, 2013). KKXX-COO^- of the p24 proteins, a major component of COPI vesicles, has been reported to bind to the γ-COP appendage domain in the F-subcomplex (Harter and Wieland, 1998). Indeed, several reports suggest that there are multiple binding sites on COPI for dibasic-motif containing cargo and regulatory molecules (Burman et al., 2008; Hudson and Draper, 1997). We recently reported that Scyl1, which contains the C-terminal dibasic RKXX-COO^- motif, interacts directly with the γ-COP appendage domain (Hamlin et al., 2014)(Chapter 2). Therefore, Scyl1 contains at least two binding sites on COPI, one on the β'-COP terminal domain in the B-subcomplex and one on the F-subcomplex γ-COP appendage domain (Hamlin et al., 2014).

Notably, γ-COP contains two isoforms, γ1-COP and γ2-COP and while both isoforms are roughly equally expressed in cells, Scyl1 preferentially interacts with the γ2-COP isoform (Hamlin et al., 2014). We wondered if the ability to bind the γ-COP appendage domain in an isoform specific manner was unique to Scyl1. Additionally, we sought to examine if Scyl1 was a unique binding partner for the appendage of γ-COP or, if like the appendage of α-adaptin, multiple proteins bind to the γ-COP appendage to regulate COPI trafficking. To this end we used the γ1-COP and γ2-COP appendage domains as GST-fusion proteins and performed affinity selection assays with rat brain lysate. We then subjected the affinity-purified proteins to mass spectrometry analysis. As expected, Scyl1 was identified, with a preference in binding to the γ2-COP appendage. Interestingly, we also identified VPS-53, a member of the tetrameric VPS-(51-54) Golgi-associated retrograde protein (GARP) complex, a TGN vesicle-tethering complex.

Vesicular trafficking is highly regulated at multiple steps. Incoming vesicles must be correctly identified by the acceptor membrane to which they will fuse. This is primarily accomplished by tethering complexes, large, extended protein complexes which extend into the cytosol to make initial contact with vesicles (Hutagalung and Novick, 2011). Specificity is established at least in part by Rabs, such that a specific Rab on a vesicle binds selectively to a component of the tethering complex, but additionally, the tether itself may be membrane associated via an additional Rab. Thus, the extended tether introduces Rab polarity into the trafficking pathway. For example, the GRIP domain of multiple Golgi tethers interacts with Rab6 (Barr, 1999) and Arf-related related proteins (Sinka et al., 2008). Finally, tethering complexes link incoming vesicles (and importantly their cargo) with the vesicle fusion machinery such as SNARE proteins.

The GARP complex is a multi-subunit tethering complex localized to the *trans*-Golgi network (TGN) (Conibear and Stevens, 2000). The GARP complex consists of four proteins in a stable 1:1:1:1 complex, and knock-out of any member of the complex results in a build-up of endosome derived vesicles unable to fuse with the TGN (Perez-Victoria et al., 2008). The result is the cell cannot recycle lysosomal hydrolase export-receptors, such as the Mannose-6 phosphate receptor. Lysosomal hydrolases are released to the extracellular medium, and the lysosomes increase in size and number, presumably from an inability to degrade incoming material, and a homeostatic disruption results.

Much of what has been learned about the GARP complex has been derived from studies in yeast. In yeast, Ypt6 (equivalent to mammalian Rab6) binds to the Golgi SNARE protein Tlg1p (Siniossoglou and Pelham, 2001). Rab6 interacts with all GARP components (VPS-51/52/53/54). Deletion of Ypt6 is not lethal, leading only to a minor defect in trafficking. Also, the GARP complex is structurally related to other tethering complexes in the cell. It is related to the COG complex and the exocyst complex (Whyte and Munro, 2001), both of which have tethering functions in the Golgi/endosomal system.

Our data reveal that the γ-COP appendage interacts with a di-lysine binding motif at the C-terminus of the GARP member VPS-53. Mutations in the binding site result in a complete loss of the interaction, suggesting an absolute requirement of the -3 and -4 position lysines of VPS-53 to bind the γ-COP appendage domain. Moreover, it reveals an unexpected link between the COPI system in the early secretory system and the GARP tethering complex at the TGN. By interacting with specific sets of incoming vesicles, GARP helps to organize the complex responsibility of the *trans*-Golgi as a sorting hub between multiple cellular trafficking pathways.

RESULTS

Scyl1 uses a dibasic motif to bind the γ-COP appendage domain. As γ-COP is structurally and evolutionarily related to α-adaptin subunit of the adaptin/clathrin complex, and the α-adaptin appendage binds multiple accessory proteins (McPherson and Ritter, 2005), we predicted that multiple proteins interact with the γ-COP appendage. To test this, we expressed the γ-1 and γ-2 appendage as GST-tagged fusion proteins. Fusion proteins were immobilized on glutathione-Sepharose beads and mixed with detergent solubilized rat brain extracts. Affinity purified proteins were run on an SDS-gradient gel and stained with Coomassie blue (Figure 1). Fusion proteins were incubated in the presence (lanes 6-8) or absence (lanes 2-4) of tissue lysate to control for proteins that resulted from degradation of the fusion protein and/or bacterial contamination. Initially, we sought to identify both dibasic-motif containing proteins as well as isoform specific partners, thus running both GST-γ1-COP appendage and GST-γ2-COP appendage pull-downs in parallel. As shown in the figure, multiple specific bands are apparent. Thus to avoid missing a potential interaction-target, the entire content of each lane (Figure 1, lanes 6,7,8) was cut and subjected to mass spectrometry, and the resulting output was analyzed using the Scaffold 4.2.1 software (Proteome Software, Virginia).

Candidate proteins for interacting with the γ1-COP appendage domain and γ2-COP appendage domain are listed in Tables 1 and 2. The name of the protein identified in the bioinformatics screen is listed in column one. Proteins that were identified in the GST only lane were excluded from the list. Next, the total number of peptides identified are listed in the second column. Scyl1 was identified as a binding partner for γ1-COP appendage domain and γ2-COP appendage domain, with almost twice as many peptides identified for the γ2 isoform, as expected (Hamlin et al., 2014).

Candidate proteins listed in Table 1 and 2 were selected based on having a C-terminal sequence that matched or was related to the COP-binding motif consensus. For example, the ADP/ATP translocase was identified as a binding partner for the γ1-COP appendage domain with the same number of peptides as Scyl1 (Table 1). Additionally, this protein contains a perfect COPI interaction motif (YDEIKKYV-COO^-). However, since this protein is a mitochondrial membrane protein, and is likely escorted to the mitochondria by chaperones, it is unlikely that this motif is ever presented in a true biological context to the γ-COP appendage (communication E. Shoubridge), and we thus excluded it from further analysis.

Another protein that was a candidate was the Atlastin-1 GTPase. Atlastins are ER resident transmembrane proteins required for maintaining the reticular formation of the ER (Rismanchi et al., 2008). Mutations in Atlastin are a cause of spastic paraplegia (SPG3A). The C-terminal sequence of Atlastin also matches the COPI-binding motif consensus (EQPEKKKI-COO^-). As Atlastin is an ER resident it would make sense that Atlastin proteins that escape from the ER should be retrieved by the COPI system, possibly by interacting with the γ-COP appendage. However, upon further analysis using western blot with Atlastin specific antibodies, we could not reproduce the interaction (data not shown).

One protein that appeared in both lists was VPS-53, a member of the GARP complex. This was interesting because GARP functions in vesicular trafficking at the Golgi, and COPI functions within the Golgi itself. Also, VPS-53 peptides were detected in both the γ1-COP and γ2-COP analysis. The C-terminus of VPS-53 has the amino acid sequence (EKLIKKRL-COO^-), a match for a COPI-binding motif.

Remarkably, in there were also peptides for VPS-52 for the γ1-COP-appendage pulldowns. The GARP complex members VPS 51-54 exist in an equimolar complex. Presumably, a COPI interaction with the KKRL-COO^- of VPS-53 would pull-down the complex.

We first used anti-VPS-53 antibodies (J. Bonifacino, NIH) to further test the specificity of the interaction. Indeed, from both 293T cell lysates (Figure 3A) and rat brain lysate, (Figure 3B), a specific band at the expected size of 80 kDa is detected. Both the γ1-COP appendage and γ2-COP appendage appear to bind VPS-53 equally well in these conditions. Therefore, Scyl1 remains the only known protein that displays γ2-COP isoform specificity.

COPI dilysine motifs rely upon two lysines at the -3 and -4 or -3 and -5 position (Jackson et al., 1990; Nilsson et al., 1989). Mutation of these charged residues to neutral charged residues such as a lysine to alanine substitution result in the COPI-motif containing protein to escape the early secretory pathway due to a loss of COPI binding. To further test that it is indeed the two lysines at the -3 and -4 position of VPS-53 that mediated binding to COPI, we generated FLAG-tagged constructs containing the wild-type VPS-53 sequence and containing VPS-53 with (EKLI<u>KK</u>RL-COO^-) mutated to (EKLI<u>AA</u>RL-COO^-). Constructs were expressed in 293T cells, The FLAG-VPS-53 wild-type was able to bind to the γ1-appendage with same affinity as the endogenous protein (Figure 3A). Therefore the construct is likely expressed and folded properly in the cell. Next we expressed FLAG-VPS-53 containing the -3,-4 KK to AA mutation. Incredibly, the interaction with the γ-COP appendage domain was totally lost with this construct. Therefore, VPS-53 binds to COPI exclusively using a dilysine cargo-like motif.

DISCUSSION

There are several possible explanations for the observation that VPS-53 binds COPI. First, VPS-53 could simply be a COPI cargo, as it binds γ-COP with a COPI cargo dilysine motif. While both transmembrane cargo and other cytosolic proteins (Scyl1) bind to COPI, most reports indicate that transmembrane cargo binds to β'-COP and α-COP (Ma and Goldberg, 2013) that is present at the membrane-adjacent vertices of the B-subcomplex cage (Jackson, 2014). As well, one dilysine motif containing transmembrane protein was reported to bind to the γ-COP appendage domain (Harter and Wieland, 1998). Scyl1 on the other hand, binds to both the COPI B-subcomplex (Ma and Goldberg, 2013) and the F-subcomplex through the γ2-COP-appendage domain (Hamlin et al., 2014). Despite Scyl1 containing a COPI dibasic motif (Burman et al., 2008) it is unlikely that Scyl1 is a *bona fide* COPI cargo because it cycles between the membrane and cytosol (Burman et al., 2010) and thus has no need to be transported by COPI. Likewise VPS-53 does not cycle in the early secretory pathway; it alternates between the cytosol and membrane association at the TGN (Conibear and Stevens, 2000).

Second, VPS-53 may be recruited to the membrane by associating with late-Golgi COPI. As the Golgi is heavily enriched in COPI, it would be efficient for various tethers to take advantage of this rich pool of membrane associated COPI at the Golgi. However, VPS-53 is localized to the TGN (Bonifacino and Hierro, 2011), where the primary vesicular carriers are formed by clathrin and AP-1. Additionally, Rab proteins are well known to recruit tethering proteins as effectors (Cao et al., 1998; Christoforidis et al., 1999). The GARP complex itself is known to be recruited to the membrane by active Rab6 (Siniossoglou and Pelham, 2001). Therefore it seems unlikely that membrane-associated COPI recruits GARP to the Golgi membrane via VPS-53.

Third, it is possible that anterograde directed COPI vesicles use GARP as a tethering complex to engage the TGN. COPI vesicles travel in both anterograde and retrograde directions in the early secretory pathway (Orci et al., 1993; Orci et al., 2000; Pellett et al., 2013). In one hybrid Golgi model, the *cis*, *medial*, and *trans*-Golgi undergo cisternal maturation and COPI retrograde transport, while COPI vesicular transport is the primary anterograde transport between the *trans*-Golgi and the TGN.

As the TGN is the most anterograde destination for anterograde COPI vesicles, only vesicles filled with secretory cargo, (and devoid of retrograde cargo) would be suitable for tethering. On a hypothetical *trans*-Golgi to TGN anterograde vesicle the COPI F-subcomplex and the COPI B-subcomplex should have retrograde dilysine-binding sites *empty*. Perhaps this availability of the dilysine binding site on COPI is the information used by VPS-53/GARP to correctly identify an anterograde COPI vesicle. It is not clear how exposed/available the C-terminus KKRL-COO^- motif would be to participate in any COPI mediated vesicle docking.

Vesicle tethering proteins are located at essentially every location in the cell and are the first contact between the incoming vesicle and target membrane before vesicle fusion. In general, there are two types of tethers, long coiled-coil proteins and multi-subunit complexes. As a multi-subunit complex, GARP appears able to tether multiple sets of incoming vesicles through unique binding sites within individual GARP members.

MATERIALS AND METHODS

Constructs and proteins

Human GST- γ1-COP-appendage and GST-γ2-COP-appendage (Moelleken et al., 2007) were expressed in *E. coli* BL21. Human FLAG-VPS-53 wild-type and FLAG-VPS-53 (C-terminal mutation KKRL-COO^- to AARL-COO^-) were expressed in pCMVTAG3B in HEK293T cell culture, and cell lysates were used for affinity selection. All plasmids were verified by sequencing.

Affinity selection and co-immunoprecipitation assays

Monoclonal antibody against Flag epitope (M2) was from Invitrogen (Carlsbad, CA). For GST-fusion proteins, bacteria were lysed in PBS, 1mM DTT, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml aprotinin, and 0.5 µg/ml leupeptin (protease inhibitors), pH 7.4. For GST-GGA3 pulldowns, bacteria were lysed in 50mM TRIS, 100mM NaCl, 10mM $MgCl_2$, 0.1% SDS, 0.5% DOC, 1% Triton, 5% glycerol, protease inhibitors, pH 7.5. For affinity-selection assays, HEK-293T cells were collected in lysis buffer (20 mM HEPES, pH 7.4, protease inhibitors, 1mM $MgCl_2$, 30 nM Okadaic acid, 5 mM Sodium pyrophosphate, 1mM DTT) and Triton X-100 was added to 1% final concentration. Following 10 min rocking at 4 °C, the extracts were spun at 205,000 X *g* for 15 min to remove insoluble material. Aliquots of 1 ml were incubated for 1 hour at room temperature with GST or GST-γ1-COP-appendage or GST-γ2-COP-appendage fusion proteins pre-coupled to glutathione-Sepharose beads (GE Healthcare). Beads were subsequently washed three times with lysis buffer with 1% Triton X-100 and analyzed by SDS-PAGE. Bands specifically interacting with GST-Scyl1 fusion proteins were cut and analyzed by tandem mass spectrometry (MS) or were processed for Western blot.

Table 1. Proteins identified as dibasic-motif interaction candidates for the γ1-COP appendage. GST, GST-γ1-COP appendage domain were expressed in bacteria, immobilized on glutathione-Sepharose beads and applied to rat brain lysate. Specifically bound proteins were analyzed by mass spectrometry.

Name	Total Peptides	C-term sequence
Scyl1	50	RKLD-COO$^-$
ADP/ATP translocase	50	YDEIKKYV-COO$^-$
GIT1	10	REKKQ-COO$^-$
neurofibromin-like	8	RNSIKKIV-COO$^-$
Synaptotagmin	7	MLAVKK-COO$^-$
VPS-53	12	EKLIKKRL-COO$^-$
VPS-52	6	VELKKHKPNF-COO$^-$
Septin-7	7	NKKKGKIF-COO$^-$
Prohibitin	4	SLIKGKK-COO$^-$
Spectrin betachain	14	FSFFKKNK-COO$^-$
Atlastin-1	4	EQPEKKKI-COO$^-$

Table 2. Proteins identified as dibasic-motif interaction candidates for the γ2-COP appendage. GST, GST-γ2-COP appendage domain were expressed in bacteria, immoblizied on sepharose beads and applied to rat brain lysate. Specifically bound proteins were analyzed by mass spectrometry.

Name	Total Peptides	C-term sequence
Scyl1	99	RKLD-COO^-
ADP/ATP translocase	53	YDEIKKYV-COO^-
78K glucose regulated protein precurser	24	DTSEKDEL-COO^-
Succinyl CoA ligase	9	EFEKRKML-COO^-
Estradiol 17-beta dehydrogenase	3	YLKKTKKN-COO^-
VPS-53	3	LEKLIKKRL-COO^-
OGDH protein	1	DLDAFKKFS-COO^-
Thioredoxin related transmembrane protein 2	6	PDGENKKDK-COO^-

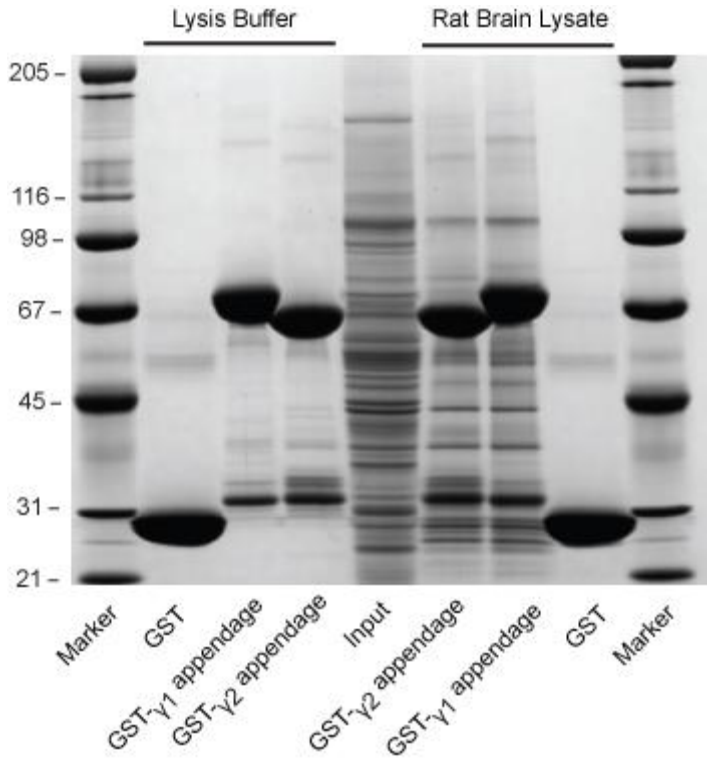

Figure 1. Coomassie stained SDS-PAGE gel of the GST-pulldown to be analyzed by mass spectrometry. The γ1-COP appendage domain, the γ2-COP appendage domain or GST only was incubated with lysis buffer (lanes 2-4) or rat brain lysate (lanes 6-8). Specifically bound proteins were sent for mass spectrometry.

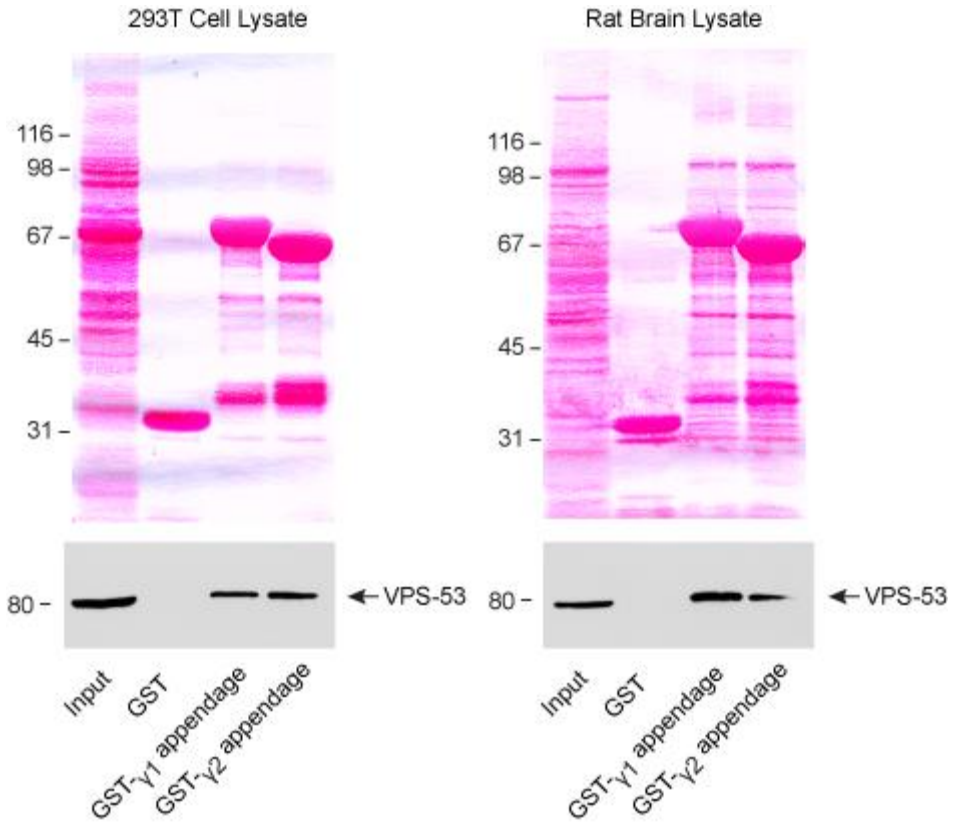

Figure 2. GARP member VPS-53 binds to the γ-COP appendage domain. 293T cell lysates and rat brain lysates were applied to immobilized GST-tagged γ1-COP and γ2-COP appendage domains or GST alone then run on SDS-PAGE. Membranes were probed with anti-VPS-53 antibody. Input = 5% of the material used in the affinity selection experiment.

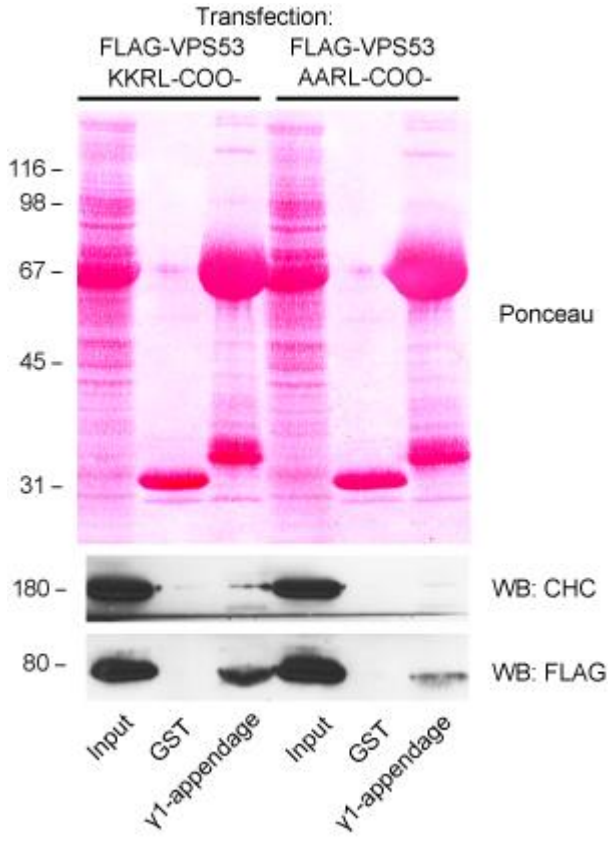

Figure 3. Mutations in the dibasic motif of VPS-53 result in a loss of binding to the γ1-COP appendage domain. FLAG-VPS-53 was expressed in 293T cells, and cell lysates were applied to immobilized GST-tagged γ1-COP appendage then run on SDS-PAGE. Membranes were probed with the indicated antibodies. CHC = Clathrin Heavy Chain. Input = 5% of the material used in the affinity selection experiment.

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Chapter 4: DISCUSSION

Membrane trafficking is central to eukaryotic life. The regulation required to control membrane trafficking is complex and involves contributions from multiple cellular components. These include global cellular signals, extracellular events, transmembrane and soluble cargo in need of transport, small GTPase regulatory molecules, vesicle coat and adaptor proteins and accessory regulatory molecules. This complexity is due to the fact that trafficking involves: both a source membrane and a destination membrane (Chapter 1.2.1-1.2.3); the movement of a myriad of cargo; and bidirectional movement of these cargo between a vast array of membrane-bound compartments.

This complexity requires multiple accessory regulatory molecules in addition to the core membrane deforming and cargo binding components. In this chapter, I propose two specific functions of the COPI accessory regulatory molecule Scyl1: In chapter 4.1 I discuss the current data that shows Scyl1 is a scaffold protein, bringing together multiple components of the COPI machinery and in chapter 4.2, I propose a new concept that Scyl1 may function as a "*pseudo*-cargo" (termed within) that supports the rapid and efficient formation of COPI vesicles in the absence of *bona fide* cargo. Independent of these data, chapter 4.3 shows that COPI is a potential link between two nearly identical mouse models of neurodegeneration, the *mdf* and *wr* mouse. Thus, these data directly implicate a retrograde transport deficit in neurodegeneration and this knowledge will lead to further research towards treating patients living with neurodegenerative disease.

4.1 Scyl1 is a scaffolding protein

Protein scaffolds contain multiple binding regions on distinct areas of the scaffold, allowing for simultaneous interaction with multiple proteins. For example, Ste5p functions as a scaffold for multiple components of the MAPK cascade in yeast (Printen and Sprague, 1994), greatly increasing the efficiency of the original cell surface activation to the final required output (Elion, 2001). This is achieved by bringing the multiple MAPK kinases in the cascade into the same location and proper orientation to phosphorylate each other. On the same note, by controlling the localization of their binding partners, scaffold proteins can increase signalling fidelity by decreasing the likelihood of incorrect protein-protein interactions or enzyme substrate relationships.

New data presented in this thesis (Chapter 2) demonstrates that Scyl1 is a scaffold protein. As mentioned, scaffolding proteins are identified by their ability to bind multiple other proteins but also usually do not contain their own enzymatic activity. In this sense, the primary purpose of the scaffolding proteins is to bring other proteins together to interact. The specificity and efficiency of molecule-molecule interactions is greatly increased by increasing local concentrations of molecules that operate together. Scyl1 interacts with: 1) itself, through oligomerization via the HEAT repeats, 2) Class II Arfs, via a COPI independent N-terminal region, 3) COPI via an RKLD-COO^- motif. Therefore, Scyl1 meets the most basic requirement of scaffolding proteins: interfacing with multiple proteins.

Discs large 1 protein (Dlg1), well known for its necessity in regulating cell polarity (Roberts et al., 2012) and cellular immunity (Humphries et al., 2012), is a protein scaffold with multiple domains connecting multiple cellular components. Both Dlg1 and Scyl1 have pseudokinase domains. While their enzymatic function is missing, pseudokinase domains may

also play a part in scaffolding if they retain the ability to bind pseudo-substrates or another specific protein. The kinase domain of Dlg1 is catalytically inactive, yet has specific binding partners. Attempts to find the specific binding partners of the pseudokinase domain of Scyl1 have not been successful (data not shown), although it remains possible that this is the region responsible for binding Class II Arfs including Arf4.

Additionally, Dlg1 oligomerizes in solution, similar to Scyl1, and functions in membrane trafficking, in this case in the clathrin pathway (Walch, 2013). Functioning in both exocytic and endocytic pathways, Dlg1 appears to link AP-1 to KIF5A for subsequent engagement with the exocyst complex. Whether or not Scyl1 functions as a link between the coat machinery and motor proteins remains to be determined. Pull-downs using the pseudokinase domain of Scyl1 reveal an interaction with tubulin, even after partial purification (data not shown), and it is very possible that like the scaffold Dlg1, Scyl1 links coat machinery to transport machinery.

The fact that Scyl1 oligomerizes via the HEAT repeats may not just organize COPI or other transport material, but may generate a *new* binding surface to stabilize or recruit proteins involved in retrograde COPI transport. HEAT repeats generate large binding surfaces to produce platforms for protein-protein interactions (Groves and Barford, 1999). The number of repeats and a variation in select residues offer the possibility for highly selective protein interaction modes. For example, multiple proteins involved in chromatin regulation contain HEAT repeats responsible for organising chromosome dynamics (Neuwald and Hirano, 2000). Data in Chapter 2 shows that the HEAT repeats are required for oligomerization of Scyl. Using the HEAT repeats as bait, we were also able to pull-down full-length Scyl1, as well as a faint but reproducible signal for GBF1 (data not shown), the primary guanine-exchange factor for Arfs in the early secretory pathway (Claude et al., 1999). Thus, while in a stable oligomerized state and bound to

COPI, Scyl1 may be recruiting both Class II Arfs, and the GEFs upon which they act, to facilitate retrograde transport in the early secretory pathway.

4.2 Is Scyl1 a *pseudo*-cargo required for the efficient vesiculation of COPI vesicles?

The *in vivo* requirements for COPI vesicle formation were originally described to be Arf and Coatomer along with purified and salt-washed Golgi membrane. Since the Golgi was salt washed, there were likely multiple additional unidentified residual components facilitating vesiculation. Studies using artificial liposomes however showed that Arf and COPI alone can form vesicles (Spang et al., 1998), but only with very specific lipid requirements, conditions which are not reflected *in vivo*. What were the additional components? Transmembrane proteins (which would remain inserted in the salt-washed Golgi membranes in the earlier experiments) are one candidate that would still have been present. In fact, the addition of KKXX-COO^- containing transmembrane-constructs to COPI and Arf liposome mixtures potentiated vesicle assembly (Bremser et al., 1999). Thus, transmembrane dibasic containing proteins are not just passive cargo but seem to be an essential part of COPI vesicle formation *in vivo*.

If dibasic proteins are essential for COPI vesicle formation, it is not clear what happens in situations where there is an imbalance in anterograde and retrograde flux and thus no readily available dibasic containing protein to support COPI formation. It is not unreasonable to hypothesize, because the ERGIC and *cis*-Golgi membrane are fractured into many pieces throughout the cell, they may contain an unequal distribution of dibasic cargo, and thus some may be unable to efficiently form COPI vesicles. A perfect candidate to fill this gap would be a cytosolic protein, which freely samples the Golgi and ERGIC membrane for partially complete COPI buds and simply facilitates the efficient formation by providing the necessary dibasic motif

adjacent to the membrane in a trimeric-pentameric form presented just like a transmembrane cargo protein.

Here, we define a *pseudo*-cargo as a protein that contains features of a transmembrane cargo, including a cargo-binding motif and self-oligomerization, but importantly, is found in both cytosolic and membrane-associated fractions within the cell. Therefore, in this hypothesis we term Scyl1 a COPI *pseudo*-cargo.

4.2.1 Cargo and *pseudo*-cargo oligomerization and COPI transport

At the cell surface, receptor tyrosine kinases convey their extracellular ligand binding status across the plasma membrane to the cytosol through dimerization. For example, the epithelial growth factor receptor (EGFR) undergoes structural changes at the dimer interface upon growth factor binding, promoting dimerization (Dawson et al., 2005). Upon dimerization the kinase is activated, and the required signal pathways become activated.

In the early secretory pathway, the transmembrane lectin export-receptor ERGIC-53 contains both a diphenylalanine motif and a dilysine motif. ERGIC-53 is shuttled between the ER and the ERGIC as it exports glycosylated proteins such as the blood clotting factor VIII (Cunningham et al., 2003). When binding glycosylated ligand cargo in the ER lumen, ERGIC-53 undergoes oligomerization, likely into hexamers. Upon ligand-bound ERGIC-53 arrival at the ERGIC, a pH sensitive histidine in the ligand-binding pocket becomes protonated, promoting the release of the cargo (Appenzeller et al., 1999).

Another family of export-receptors that cycle in the early secretory pathway are the p24 proteins. Due to their abundance on COPI vesicles, they were originally thought to be necessary for transport. Mutations in p24 members did not cause any major transport phenotypes in yeast,

however it is indispensable in the mouse, as mutation of the mammalian homologue is embryonic lethal at early stages (Denzel et al., 2000). It was later shown that p24 is involved in exporting GPI-linked proteins from the ER (Takida et al., 2008). Interestingly, p24 proteins form homo-dimers and hetero-dimers (Jenne et al., 2002) as they cycle in the early secretory pathway.

Whether or not there are strict rules as to if receptor oligomerization communicates *directionality* of transport for COPI, as opposed to *readiness* of transport is unclear. The fact that Scyl1 readily and stably oligomerizes, and is not a transmembrane protein, shows that it may be assisting into organizing other transmembrane KK-type cargo to be transported in a retrograde manner. In this sense, oligomerization of Scyl1 can be directly compared to the oligomerization of receptor. Dimerization or oligomerization status of transmembrane proteins is an efficient way to communicate ligand-binding information. As such, oligomerization of Scyl1 might help organize nascent COPI vesicles towards budding and transport (Figure 4.1).

4.2.2 Topology of the membrane-adjacent coiled-coil of Scyl1

Scyl1 contains a short predicted coiled-coil region between residues 755 and 800 at the C-terminus of the protein. Deletion of the COPI binding dibasic motif leads to a residual binding of Scyl1 to COPI (Burman 2008), and removal of the coil completely removes COPI binding. Interestingly, projected as a helical-wheel, the region appears amphipathic, with a negative cluster of charges to one side. Thus, the coil may be partially inserting into the membrane. This would put the RKLD-COO^- motif almost directly adjacent to the membrane. At this distance, it would appear to COPI like a transmembrane dibasic-cargo protein, which extend about 10 amino acids into the cytosol via the transmembrane segment. Together, this data supports the *pseudo*-cargo hypothesis for Scyl1.

As well, the coiled-coil could be weakly binding to COPI or to another vesicle budding related component. Potentially it could be recruiting v-SNARE proteins that must be incorporated into Scyl1 associated vesicles to ensure proper fusion with the ER. Further work utilizing exclusively the coiled-coil region should be performed to determine the function of this part of Scyl1.

4.2.3 Scyl1 regulates a sub-population of COPI vesicles

COPI vesicles are primarily regulated by the Arf family of small GTPases. The Arf proteins are divided into three classes based on sequence similarity (see Chapter 1.3), Class I (Arf 1-3), Class II (Arf 4,5) and Class III (Arf 6). Class I and II Arfs function at the Golgi and the early secretory pathway. Work in this thesis (Chapter 2) shows that Scyl1 is scaffolding the COPI vesicle to an Arf4 regulated network. Since knock-down of Arf4 results in loss of COPI retrograde transport (Volpicelli-Daley et al., 2005) the work here further confirms that Arf4 functions in a retrograde COPI pathway. Arf4 itself has been shown to work with lipid modifying enzymes to support vesicle formation (Ben-Tekaya et al., 2010), and is also crucial for mammalian development (Follit et al., 2014; Jain et al., 2012).

COPI vesicles themselves consist of a stable heptamer of α, β, ε (coat forming) and β', γ, δ and ζ (adaptin like) proteins. Additionally, the ζ and γ COPI each have two isoforms, and vesicle types encoding with $\gamma_1\beta\zeta_1\delta$-, $\gamma_1\beta\zeta_2\delta$-, and $\gamma_2\beta\zeta_1\delta$-COP variants. Scyl1 preferentially interacts with the γ_2 isoform of γ-COP. What is the purpose for COPI subunits having different isoforms? It is possible that different γ-COPI isoforms impart different coatomer localizations (Moelleken et al., 2007).

COPI has been shown to adopt multiple conformations (Faini et al., 2012). For what purpose is unknown, but certain conformations might promote certain pathways, motor protein associations or affinity towards other factors to the vesicle that would confer specificity to the function required. It is possible that a Scyl1 containing COPI vesicle might preferentially adopt a certain conformation.

Work by the Wieland group shows that different types of cargo interface with different regions of COPI. While all transmembrane COPI cargo contain a dibasic C-terminal motif, COPI distinguishes between cargo that deliberately cycle between the ER and the Golgi, and ER resident proteins. Proteins that cycle in the early secretory pathway such as the ERGIC-53 lectin export receptor and the p24 family (GPI-linked export receptors) bind to COPI via the γ-appendage of the F-Subcomplex and importantly, also require dimerization to bind COPI (Bethune et al., 2006). On the other hand, the ER resident proteins were unable to bind the γ-appendage, which have been previously shown to interact with the B-Subcomplex (Eugster et al., 2004). However, some reports have shown dibasic cargo that cycle, do bind to the B-subcomplex (Ma and Goldberg, 2013). What is clear is that there are multiple binding sites on COPI for dibasic C-terminal motifs. Much further study will be required to determine the rules and regulation that control the association between COPI and the cargo that is transported.

Similar to cargo cycling in the early secretory pathway Scyl1 binds directly to the γ-appendage, and forms stable trimers, it thus may organize COPI coats headed in a retrograde direction (and thus stabilize ER export-receptors, unbound from their ligand) from the ERGIC and Golgi. This is consistent with the fact that knock-down of Scyl1 leads to an impairment of retrograde dilysine tagged VSVG protein (Burman et al., 2008).

4.3 Mutations in proteins that bind COPI link the wobbler and muscle deficient mouse

The wobbler mouse (*wr*) is caused by mutations in the GARP member VPS-54, and is an animal model for ALS (Moser et al., 2013). The *mdf* mouse (Chapter 1.5) is most similar to the *wr* mouse and is also a model for ALS. The similar phenotype between the two mouse models suggests that there is potentially a similar underlying mechanism between them. It has been suspected that because VPS-54/GARP is a part of a retrograde pathway, that the *wr* mouse phenotype is the result of an impairment in retrograde trafficking. The work presented in this thesis now clearly links both *mdf* and *wr* disease models to the COPI pathway: mutations in COPI binding proteins Scyl1 and VPS-53 both lead to neurodegenerative disease in the mouse.

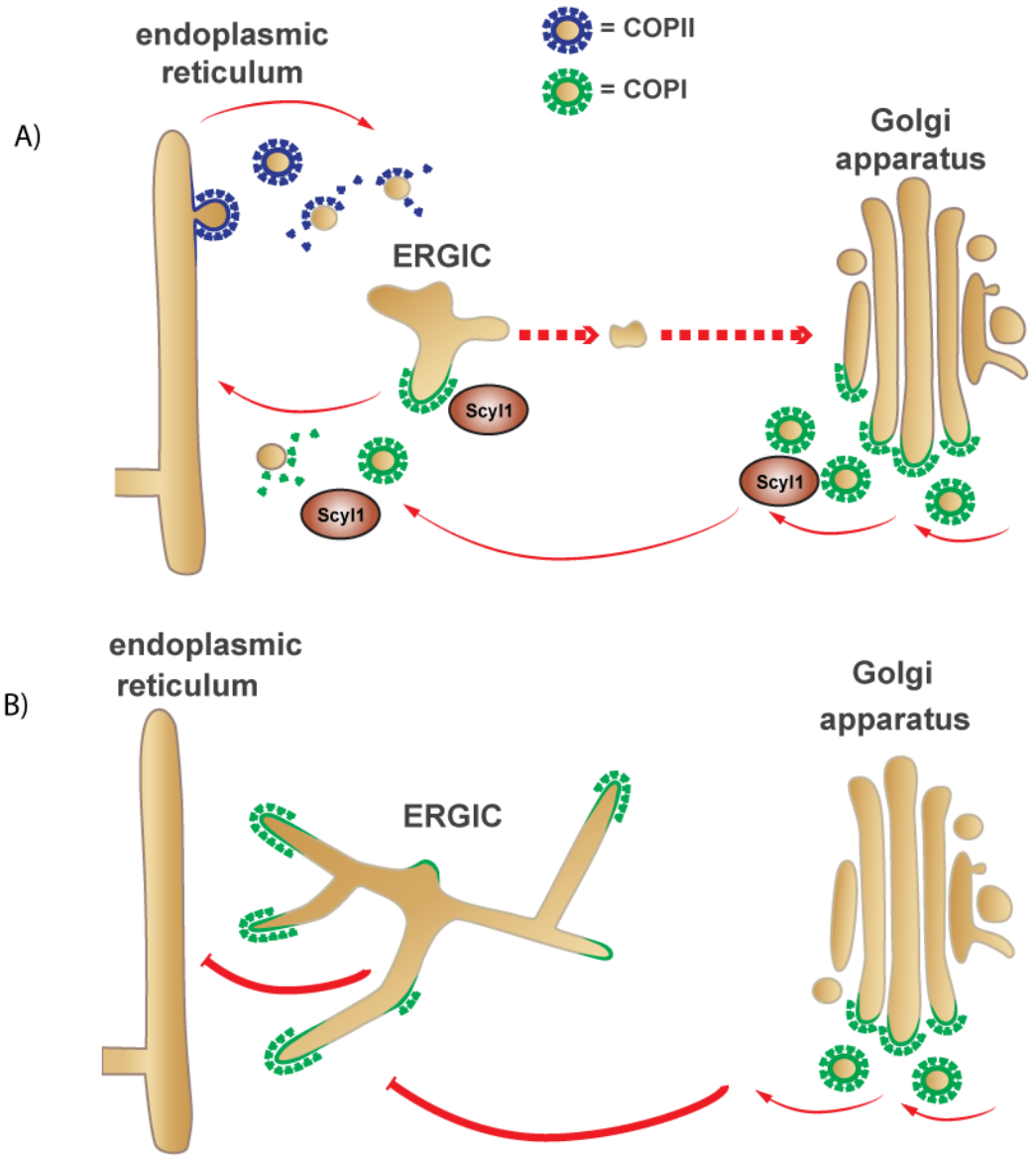

Figure 4.1 Depletion of Scyl1 results in a tubulation of the ERGIC. A) Schemata of the early secretory pathway in a normal functioning system. COPII vesicles (blue) bud from ER exit sites (ERES), shed their coats and undergo homotypic fusion or fuse with ERGICs. COPI vesicles (green) recycle cargo from the Golgi and the ERGIC to the ER. B) COPI retrograde transport is disrupted under conditions of Scyl1 depletion. The ERGIC becomes tubulated, likely as a loss of scission of budding COPI vesicles.

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