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Ceramide-1-Phosphate: A Novel Regulator of Golgi Fragmentation, Golgi-ER Vesicle Trafficking, and *Anaplasma phagocytophilum* Pathogenesis

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by

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of the requirements for the degree of
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List of Abbreviations

C1P	Ceramide-1-phosphate
CERK	Ceramide Kinase
CPTP	C1P transport protein
ASMase	Acid sphingomyelinase
HGA	Human granulocytic anaplasmosis
RC	Reticulate form
DC	Dense cord form
ApV	*A.phagoctyophilum* occupied vacuole
TGN	Trans Golgi network
CG	Cis Golgi
GRASP55	Golgi reassembly stacking protein 55
GRASP65	Golgi reassembly stacking protein 65
ER	Endoplasmic reticulum
COPI	Coatomer protein complex I
Cdc42	Cell division control protein 42
PKCα	Protein kinase α
JNK	c-Jun N-terminal kinase
AA	Arachidonic acid
cPLA2	Cytosolic phospholipase A2

Abstract

Anaplasma phagocytophilum, the etiologic agent of human granulocytic anaplasmosis (HGA), is a tick transmitted obligate intracellular bacterium that infects granulocytes, bone marrow progenitor cells and endothelial cells. HGA exhibits symptoms of febrile illness including fever, chills, headache, malaise, leukopenia and elevated liver enzymes which are treatable by antibiotics. However, elderly and immunocompromised patients are at a greater risk of developing fatal complications. Inside the host cell *A.phagocytophilum* resides in host cell derived vacuole called *A.phagocytophilum* occupied vacuole (ApV) where it undergoes its biphasic lifecycle. Being an intracellular pathogen, *A.phagocytophilum* relies heavily on nutrient acquisition from host cell and to obtain these nutrients they manipulate host Golgi-Endoplasmic reticulum (ER) vesicle trafficking system to reroute the nutrients to their ApV lumen. Since most obligate intracellular bacteria cannot synthesize their own sphingolipids, they uptake sphingolipids from host cell to promote their virulence and growth. Interestingly *A.phagocytophilum* is known to hijack ceramide rich trans Golgi (TGN) vesicles and inhibiting the delivery of these vesicles to the ApV lumen inhibits bacterial growth and impedes the generation of it infectious DC progeny.

Ceramide, the precursor of ceramide-1-phosphate (C1P), is at the center of sphingolipid biosynthetic pathway.C1P, a potent regulator of cell proliferation, migration and invasion, and inflammatory cytokine production, is generated at the TGN through phosphorylation of ceramide by ceramide kinase (CERK). A C1P transport protein (CPTP) then transports the lipid to the plasma membrane or other organelles where it potentially undergoes a catabolic process. Therefore, inhibiting CPTP increases endogenous C1P levels and triggers Golgi fragmentation, a phenomenon that is also observed in *A.phagocytophilum* infected cells. However, the mechanism by which C1P induces Golgi fragmentation is not known. The role of C1P is well

characterized in cancer biology, inflammatory diseases and wound healing; but little is known about its implication in microbial pathogenesis. Interestingly, Carlyon lab has shown that inhibiting ceramide generation by downregulation of acid sphingomyelinase (ASMase) arrests *A.phagocytophilum* growth both in in vitro and in vivo. Since TGN resident ceramide derived sphingolipids are crucial for *A.phagocytophilum* pathogenesis, hence we hypothesized that *A.phagocytophilum* elevates C1P levels in the TGN, increase in C1P induces Golgi fragmentation and disrupts Golgi-ER retrograde vesicle trafficking to promote anterograde traffic of TGN vesicles to ApV lumen and subsequently promote *A.phagocytophilum* growth. We have the following aims for this project 1) determine if CERK derived C1P is required for optimal *A.phagocytophilum* infection; 2) elucidate the mechanism by which C1P regulates Golgi-ER retrograde vesicle trafficking and Golgi fragmentation.

Here we show that *A.phagocytophilum* infected cells have higher C1P levels compared to non-infected cells and decreasing C1P levels by pharmacological inhibition and genetic ablation of CERK reduces *A.phagocytophilum* infection, disrupts bacterium's biphasic lifecycle, and blocks *A.phagocytophilum* induced Golgi fragmentation. Conversely, increasing C1P levels reverses these effects and also facilitates ApV maturation. We further corroborated our result in vivo as CERK knockout mice (CERK -/-) were resistant to *A.phagocytophilum* infection. Our results also demonstrate that increasing C1P levels blocks Golgi-ER retrograde vesicle trafficking which is inversed by CERK inhibition. Our data further suggests that C1P stimulates Golgi fragmentation by inducing activation of protein kinase Cα (PKCα) followed by translocation of Cdc42 to the cellular membranes and its subsequent activation, which in turn activates c-Jun kinase (JNK). Consequently, PKCα and JNK triggers phosphorylation of Golgi restacking protein 55 (GRASP 55) and Golgi restacking protein 65 (GRASP 65) respectively, thereby inducing Golgi fragmentation.

Our project demonstrates the significance of CERK derived C1P in *A.phagocytophilum* pathogenesis and these findings recognize C1P as a potential target for non-antibiotic based

treatment for *A.phagocytophilum* infection and also for other antibiotic resistant intracellular bacterial and other forms of microbial infections that depends on sphingolipid parasitism. Determining the role C1P in the maintenance of Golgi morphology lays the foundation to explore targeting CERK derived C1P in therapeutics of a plethora of diseases that is linked to Golgi fragmentation including Alzheimer's, Huntington's, Amyotrophic lateral sclerosis (AS) and various forms of cancer including lung, breast and pancreatic cancers.

Chapter 1: Introduction

1.1 Anaplasma phagocytophilum

A.phagocytophilum is a Gram negative, obligate intracellular bacterium that is the causative agent of human granulocytic anaplasmosis (HGA) (1, 2). HGA is an *Ixodes scapularis* tick transmitted infection which is often confused with Lyme disease (3). Clinical manifestation of HGA ranges from being asymptomatic to exhibiting symptoms of febrile illness including fever, chills, headache malaise; distinct laboratory prognosis includes leukopenia, thrombocytopenia and elevated levels of liver enzymes (4). Nevertheless HGA can develop fatal outcomes in elderly and immunocompromised patients, and also in case of delayed antibiotic treatment (5). Additionally HGA can also be transmitted perinatally and in some cases pose severe threat to the mother's health (5). In most cases, HGA patients are treated with the antibiotic Doxycycline (3).

A.phagocytophilum predominantly infects neutrophils, bone-marrow progenitor cells and endothelial cells (6, 7). *A.phagocytophilum* invasion of neutrophils and bone-marrow progenitor cells is mediated by the interaction of *A.phagocytophilum* surface protein OmpA, Asp14 and AipA with Sialyl Lewix x (sLex) capped P-selectin glycoprotein ligand 1 (PSGL-1) receptors on host cell (6, 8, 9 ,10). However, *A.phagocytophilum* invades endothelial cells by a PSGL-1 independent pathway, endothelial cell invasion is mediated by the interaction of B2 with lipid raft resident glycophosphatidylinositol (GPI)-anchored protein and caeiolin-1 (7, 11)

Inside the host cell *A.phagocytophilum* resides in host cell derived vacuole called the *A.phagocytophilum* occupied (ApV) which provides it a safe niche by preventing its fusion with lysosome and NADPH oxidase carrying secretory vesicles (6, 12). Inside the ApV, *A.phagocytophilum* completes its biphasic lifecycle involving the transition from infectious dense cored (DC) to non-infectious reticulate (RC) form and then transforms back to the DC form (13).

A.phagocytophilum invades the cell in DC form, 4 to 8 hours after invasion it transitions to RC form and replicates in RC form between 8 to 24 hours, finally between 28 to 32 hours it converts back to DC form to disseminate from the cell and start the next cycle of infection (13, 14) Nevertheless, to complete this lifecycle *A.phagocytophilum* requires nutrients from host cell (15).

Due to the small genome size, *A.phagocytophilum* is auxotrophic for many metabolites and therefore relies mostly on host cell derived nutrients (16, 17). Concordantly, *A.phagocytophilum* has evolved various mechanisms to hijack nutrients from host cell; such as inducing autophagy in host cell to obtain amino acids from the autophagosomes (18). Furthermore, *A.phagocytophilum* manipulates host cell vesicle trafficking mechanism to subvert the nutrient rich vesicles to ApV lumen to fulfill their nutritional virulence needs (19, 20). Most intracellular bacteria lack lipid biogenesis component and therefore predominantly rely on hijacking lipids from host cell (21). Lipids are essential for bacterial pathogenesis; they regulate bacterial invasion in host cell, provide structural component for bacterial membrane and are essential for bacterial multiplication and survival in host cell (21, 22). Sphingolipids, an important class of bioactive lipids, are crucial for bacterial pathogenesis and promote bacterial invasion, survival and replication in host, especially in cases of *Chlamydia, Neisseria, Pseudomonas*, *Legionella* and *Salmonella* infections (23, 24, 25, 26). In recent years, Carlyon lab has demonstrated that *A.phagocytophilum* parasitizes host sphingolipid and subsequently disrupts sphingolipid metabolism to complete it lifecycle in host cell (19, 27). They reported that various forms of sphingolipid subspecies such as ceramide, sphingomyelin and hexyl sphingolipid were found in DC organisms collected from the media from infected cells. (19) Additionally, inhibition of Acid sphingomyelinase (ASMase), the enzyme that catabolizes sphingomyelin to generate ceramide, hinders *A.phagocytophilum* growth (27). Indeed sphingolipids are pertinent for *A.phagocytophilum* infection and could be used as a target to develop non-antibiotic based therapeutics for *A.phagocytophilum* and other antibiotic resistant bacterial infections.

1.2 The Golgi apparatus

The Golgi is an important membrane-bound organelle that is composed of flatten disc-shape cisternae that are connected together in a ribbon like structure and is localized near the perinuclear area (28). The Golgi has three distinct sub compartment, the *cis*- Golgi (CG) the *medial*-Golgi and the *trans*-Golgi network (TGN) (28). The Golgi acts as the protein and lipid modification and sorting center of the cell; newly synthesized proteins and lipids are transported to the Golgi apparatus where they go through post translational modification and sorted in vesicles to be either sent to other organelles or secretory proteins are secreted out of the cells (29). Therefore, the Golgi apparatus is crucial for regulating various cellular signaling processes and maintain cellular homeostasis and consequently its structural integrity is pivotal for its functions (30). Concordantly, fragmentation and dispersion of the Golgi apparatus is linked to a plethora of diseases including diverse forms of cancers, various neurological conditions such as Alzheimer's, Huntington's and Parkinson's, and a number of microbial pathogenesis including bacterial and viral infections (29, 31, 32, 33) . Additionally, Golgi fragmentation is a requisite for cell division and one of the key check points for cells entering the G2 phase during mitosis (34). Consequently, inhibiting Golgi fragmentation during cell division leads to cell cycle arrest (35). There are a number of protein kinases, Golgi matrix proteins and microtubule protein that are responsible for maintaining Golgi morphology and its function (36, 37). Golgi Reassembly Stacking Protein, GRASP55 and GRASP65 are two key proteins that are essential for Golgi stacking and their depletion and phosphorylation leads to Golgi unstacking and subsequent Golgi fragmentation (38) (39). GRASP55 is predominantly localized in the TGN whereas GRASP65 is localized in CG (38). GRASPs form dimers and are localized between the cisternae, anchoring and tethering the membranes to ensure proper stacking of the compartments (40). During the onset of mitosis, GRASPs are phosphorylated by cyclin dependent kinase (Cdk1) or polo like kinase 1 (Plk-1) which disrupts the oligomer formation and subsequently triggers Golgi fragmentation (41). Post

mitosis protein phosphatase (PP2A) dephosphorylates GRASPs protein so that they can stack the Golgi cisternae, hence forming the compact stacked Golgi structure (42).

1.3 The Golgi -ER vesicle trafficking

Vesicle trafficking is a major mode of communication between membrane bound organelles of eukaryotic cells and crucial for protein sorting and modification, transport of proteins and lipids to their appropriate target organelles and release of secretory proteins and lipids (43). Vesicle trafficking is intricately regulated by coat proteins, tethering factors and soluble N-ethylmaleimide (SNAREs) proteins (44). Coat proteins modulate vesicle budding followed by tethering factors mediated tethering of vesicles to their target membranes (45, 46). Finally v-SNAREs on the vesicle and t-SNAREs on the target membrane allow the docking and fusion of vesicles to their target compartment (47). ER is the site of protein synthesis where newly synthesized protein are packaged in coat protein II (COPII) coated structures and exported to the Golgi via anterograde trafficking (48). In the Golgi, newly synthesized proteins, undergo post translational modification like glucosylation, sulfation, phosphorylation and proteolysis (49, 50) Next, the secretory proteins are packaged into coat protein complex 1 (COPI) and exported to the plasma membrane whereas the misfolded proteins and proteins and lipids from endocytic pathways are packaged into COPI vesicles at the Golgi and recycled back to the ER via retrograde trafficking (51). Obstructing Golgi-ER vesicle trafficking pathway can lead to accumulation of misfolded proteins in the cells, aberrant protein and lipid localization in organelles and hinder the release of secretory proteins (44). Concordantly, defective vesicle trafficking system is associated with a number of diseases including, microbial infections, various forms of cancers and neurological disorders.(52)

1.4 Sphingolipid metabolism

Sphingolipids are bioactive signaling molecules that are characterized by their eighteen carbon amino-alcohol backbone and they are essential for important cellular functions such as cell proliferation, cell differentiation, cellular growth, cell migration and invasion, apoptosis and senescence(53). The first sphingolipids were isolated from the brain in the late 19th century by Thudicum, who introduced the term “sphingosine” after the Greek mythical creature Sphinx because of it enigmatic structure (54). The chemical structure of sphingosine, the major building block of all sphingolipids, was elucidated in the 20th century and it was not until the mid 1980 that the biological functions of sphingosine, sphingosine-1-phosphate (S1P) and ceramide were identified (54).

Ceramide is the at the core of sphingolipid biosynthetic pathway, regulating important cellular processes like cell cycle arrest, apoptosis, senescence and stress response (53). Ceramide can be generated either by the de novo synthesis or salvage pathway. The de novo synthesis takes place at the endoplasmic reticulum (ER) by condensation of amino acid serine and palmitoyl-CoA by serine palmitoyl transferase (SPT) to form 3-ketosphinganine; followed by reduction of 3-ketosphinganine to sphingosine which is then acylated by ceramide synthase (CerS) to produce dihydroceramide which is then desaturated by desaturase (DEGS) to yield ceramide (55) . There are six CerS enzymes and each one has their specificity for a particular acyl CoA chain length (56). CerS1 is responsible for the synthesis of C18 ceramide, CerS2 synthesizes C20 and C26 ceramide, CerS3 is responsible for C22 and C26 ceramide synthesis, CerS4 is responsible for C18-C20, CerS5 synthesizes C16 ceramide and CerS6 is responsible for C14 ceramide synthesis. Once synthesized in the ER, ceramide is either converted to sphingosine by ceramidase (CDase), which is then phosphorylated by sphingosine kinase (SphK) to generate sphingosine-1-phosphate (S1P); or ceramide is transported to the Golgi apparatus by ceramide transport protein (CERT) or by vesicular trafficking, where it is phosphorylated by ceramide kinase (CERK) to generate ceramide-1-phosphate (C1P), or used to produce

sphingomyelin by sphingomyelin synthase (SMS) (55, 57, 58). The salvage pathway generates ceramide form sphingomyelinase (SM) mediated hydrolysis of sphingomyelin into ceramide and phosphocholine and the salvage pathway involves the generation of ceramide from S1P hydrolysis (55) (Fig 1). Due to its dynamic function in a number of major cell signaling pathways, aberrant sphingolipid metabolism is linked to several diseases including neurodegenerative disorders such as Alzheimer's, metabolic disorder like Neiman Pickman disease, various forms of cancers, cardiovascular diseases and various form bacterial infections such as *Chlamydia, Legionella, Anaplasma* and *Pseudomonas* infections (57, 26, 59, 23, 60)

1.5 Ceramide -1-phosphate (C1P)

Ceramide-1-phosphate is a bioactive sphingolipid that regulates key cellular processes such as cell proliferation, cell survival, macrophage migration and inflammatory eicosanoid production. Being a key regulator of various crucial cell signaling pathways, C1P is associated with a plethora of diseases such as various forms of neurological conditions, wound healing and various forms of cancers (58). In mammalian cell, C1P is produced at the TGN by ATP dependent phosphorylation of ceramide by CERK (61) (62). C1P is then transported by a C1P transport protein (CPTP) to the plasma membrane or other organelles where it undergoes a catabolic process (63) Therefore, CPTP downregulation causes C1P accumulation in the cell, consequently increasing inflammatory eicosanoid production and C1P accumulation has also been reported to induce autophagy (63, 64) C1P regulates inflammatory response by mediating eicosanoid production (65). Eicosanoids are class of lipids that includes prostaglandins, prostacyclin, leukotrienes and thromboxane that are essential for inducing inflammatory responses (66). Previously, our lab has shown that C1P regulates pro-inflammatory eicosanoid production by interacting with the C2 domain of cytosolic phospholipase A2 alpha (cPLA 2α) and inducing its translocation from the cytosol to the cellular membranes of the Golgi, subsequently activating cPLA 2α (67). cPLA 2α activation induces the release of arachidonic acid (AA) from the

cellular membranes which is then used by cyclooxygenase (COX-2) to synthesize prostaglandin (68). Since C1P modulates important stages of wound healing including cell proliferation and migration and inflammatory eicosanoid production, hence it is pertinent for wound healing physiology (69, 70).

C1P is a crucial component of cancer biology as it promotes cell proliferation, invasion and migration of cancer cells(71). Gomez-Munoz and colleagues have shown that exogenous C1P treatment stimulates cell proliferation in fibroblast and macrophages by inducing translocation of protein kinase Cα (PKCα) from cytosolic to membrane fraction and thereby inducing PKCα activation (72). Concomitantly, Nakamura and colleague have shown that C1P activates cPLA 2α to induce AA release through PKCα dependent pathway (73). Additionally, C1P is mitogenic as it stimulates DNA synthesis and cell proliferation by inducing phosphorylation and subsequent activation of extracellularly regulated kinases 1 and 2 (ERK 1 / 2) and c-Jun *N*-terminal kinase (JNK) (74). Indeed, because of its pleotropic nature, C1P is a crucial lipid in cell signaling pathways and thus its metabolism is linked to various diseases.

1.6 Hypothesis and aims

Previous research by Chalfant and Carlyon lab manifest a link between sphingolipid and *A.phagocytophilum* pathogenesis by showing that sphingolipid rich TGN vesicles are being trafficked to the ApV lumen and these vesicles are essential for the generation of *A.phagocytophilum* infectious DC progeny. Nonetheless, the implication of C1P in *A.phagocytophilum* infection is not known. Additionally both increased level of C1P and *A.phagocytophilum* infection stimulate Golgi fragmentation; which has been shown to facilitate *A.phagocytophilum* growth. Nevertheless the mechanism by which C1P triggers Golgi fragmentation is unknown. Since *A.phagocytophilum* hijacks ceramide rich vesicles from the TGN, the site where C1P is synthesized, and concurrently stimulates Golgi fragmentation, hence we hypothesize that *A.phagocytophilum* infection elevates CERK derived C1P levels which perturbs

Golgi-ER retrograde trafficking, subsequently inducing Golgi fragmentation, to promote anterograde trafficking of TGN vesicles to ApV to enhance *A.phagocytophilum* infection.

The following are the research aims for this project :

1) determine if CERK derived C1P is required for optimal *A.phagocytophilum* infection and ApV maturation.

2) elucidate the mechanism by which C1P regulates Golgi-ER vesicle trafficking and Golgi fragmentation.

1.7 Significance

HGA, the 2nd most common tickborne disease in the US and widespread in Europe and Asia, can also transmitted by blood transfusion and perinatally (5, 75). It was first identified as human disease in 1990, and ever since then HGA cases have increased steadily with a dramatic increase between 2013-2017, yet it remains underreported(76). HGA is a febrile illness with 36% hospitalization rate but it can be fatal if antibiotic treatment is delayed and it develops severe complications in elderly and immunocompromised patients (76). Currently Doxycycline is the only drug used to treat HGA patients (75). Our research shows how targeting the nutritional virulence aspect of the bacteria can arrest *A.phagocytophilum* growth. Nutritional acquisition from host cell by manipulation of host cell cellular processes and metabolism is one of the most fundamental aspect of microbial pathogenesis (17, 77) Most obligate intracellular pathogens (*A.phagocytophilum*, *Coxiella burnetiid, Chalmydia* and *Ehrlichia*) hijack host derived sphingolipids that is essential for them to cycle between noninfectious and infectious form, thus promoting bacterial growth (23, 26, 78). Here, for the first time, we report that CERK derived C1P is elevated in *A.phagocytophilum* infection and C1P promotes bacterial replication both in vitro and in vivo. We further demonstrate that C1P is crucial for ApV formation and maturation. We further discern the underlying mechanism by which C1P induces Golgi fragmentation and

regulates Golgi-ER retrograde vesicle trafficking. Therefore, deciphering the role of C1P in *A.phagocytophilum* pathogenesis will provide insight for developing novel non-antibiotic based therapeutics for *A.phagocytophilum* infection and also other bacterial infections that relies on sphingolipid acquisition form host cell to satisfy their nutritional virulence requirement.

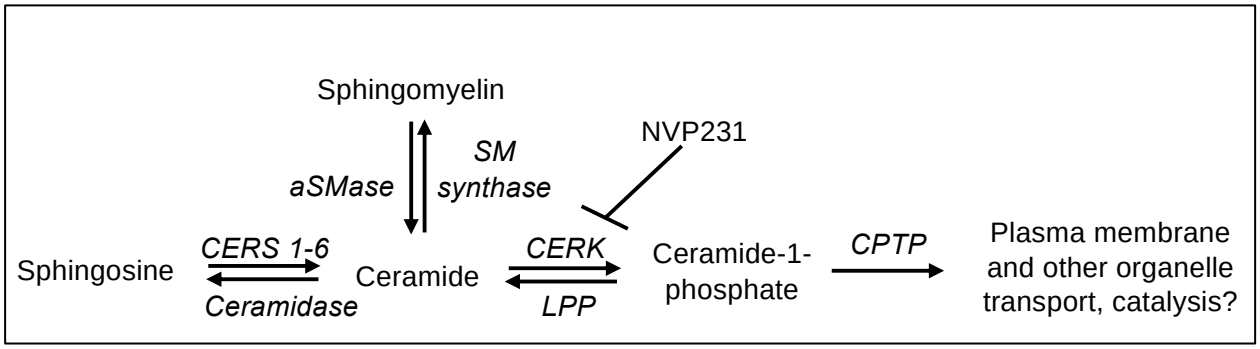

Fig 1: Schematic demonstrating sphingolipid metabolism.

Ceramide is at the center of sphingolipid metabolism and can be generated either by de novo synthesis or by the sphingomyelinase pathway which involves the breakdown of sphingomyelin by ASMase to generate ceramide. Ceramide is phosphorylated by ceramide kinase (CERK) in an ATP dependent manner to generate C1P.C1P is then transported by CPTP to the plasma membrane and other organelles where it is possibly catabolized by lipid phosphatases (LPPs). NVP231 is a commonly used small molecule CERK inhibitor and thus used for reducing endogenous C1P levels.

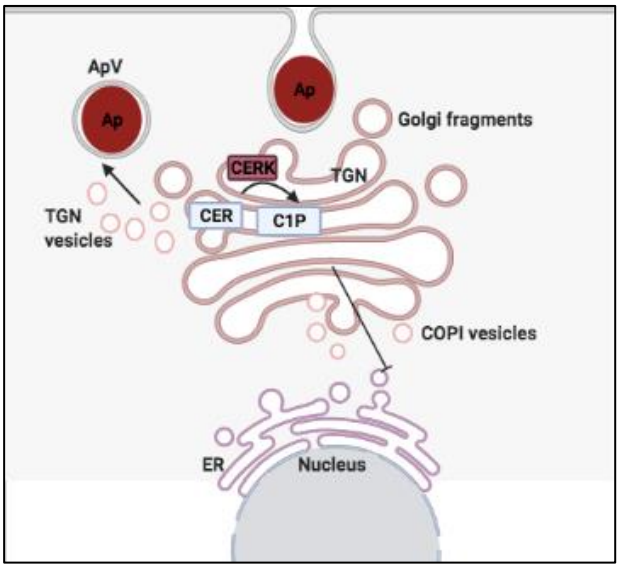

Fig 2: Hypothesis.

A.phagocytophilum infection elevates CERK derived C1P levels which perturbs Golgi-ER retrograde trafficking, subsequently inducing Golgi fragmentation, to promote anterograde trafficking of TGN vesicles to ApV lumen to enhance *A.phagocytophilum* infection.

Chapter 2: Determine if C1P is required for optimal *A.phagocytophilum* infection

2.1 Introduction

Parasitizing host cell derived sphingolipids is a common phenomenon in intracellular bacterial pathogenesis as sphingolipids facilitate their invasion in host cell, provide structural support to their membranes, promote their replication and survival in host cell and facilitate the development of pathogen occupied vacuole (POV) (23, 79). There are several Gram negative bacteria, including *P.aeruginosa*, *E.coli*, *C.trachomatis* and *N.gonnorhea* that activates host cell ASMase to generate ceramides from sphingomyelinase, which in turn assists with bacterial invasion in host cell, promote bacterial replication and stimulates cytokine release and inflammatory response (80). Intriguingly, some sphingolipid subspecies such as ceramide, sphingomyelin and monohexyl sphingolipid were detected in *A.phagocytophilum* DC organisms collected from the media of the infected cells. Furthermore, recently Carlyon lab has shown that inhibiting the generation of ceramide by ASMase downregulation hinders *A.phagocytophilum* growth and impedes its lifecycle by arresting the transition from RC to DC form (27). They also showed that ASMase knockout mice were resistant to *A.phagocytophilum* infection (27). Ceramide is the precursor of C1P; nevertheless the role of C1P in *A.phagocytophilum* pathogenesis is not known. *A.phagocytophilum* is known to hijack ceramide rich TGN vesicle from the host cell which promotes their growth and consequently induces Golgi fragmentation, which is also a manifestation of increased C1P levels in the cells (19, 63, 64). Since C1P is a sphingolipid that is mostly synthesized and localized in the TGN, we investigated the function of C1P in *A.phagocytophilum* growth and pathogenesis (62). Collectively, our data demonstrate that *A.phagocytophilum* infection induces an increase in CERK derived C1P levels in host cell and decreasing C1P levels by CERK downregulation inhibits bacterial growth both in vitro and in vivo.

Additionally, elevating C1P levels by CPTP downregulation further enhances *A.phagocytophilum* growth, augments *A.phagocytophilum* induced Golgi fragmentation and stimulates ApV maturation.

2.1.1 Hypothesis and aim

We hypothesize that *A.phagocytophilum* induces an increase in CERK derived C1P in host cell and manipulating C1P levels modulate bacterial load. Aim: 1) determine if CERK derived C1P is required for optimal *A.phagocytophilum* infection and promote ApV maturation.

2.2 Results

2.2.1 C1P downregulation arrests A.phagocytophilum induced Golgi fragmentation

Previously it was shown that perturbing ceramide generation by ASMase inhibition reduces *A.phagocytophilum* infection by obstructing the conversion from replicative RC to infectious DC form (27). As C1P is synthesized in the TGN by CERK mediated phosphorylation of ceramide and disrupting the transport of ceramide rich TGN vesicles inhibits *A.phagocytophilum* replication, we examined if there was a change in C1P levels in infected versus uninfected cells and manipulated the C1P levels by using small molecular CERK inhibitor NVP231(19) . This study was conducted in two different cell types that *A.phagocytophilum* can infect in vitro, Human Leukemia cell (HL-60s) and Rhesus monkey endothelial cells (RF/6A) (7, 11, 81) . Ultra-high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) analyses showed that infected HL-60 and RF/6A cells had a remarkable increase in the levels of multiple chain lengths of C1P: D-e-$C_{14:0}$, and D-e-$C_{16:0}$ C1P compared to uninfected cells. Conversely, the C1P levels were reduced in the infected cells by NVP231 mediated CERK inhibition (Fig 4A-B) . Since both C1P upregulation and *A.phagocytophilum* infection stimulate Golgi fragmentation, hence we examined the implication

of C1P in *A.phagocytophilum* stimulated Golgi fragmentation in HL-60 and RF/6A cells. Uninfected HL-60 and RF/6A cells had intact Golgi whereas infected cells had fragmented Golgi (Fig 4C-D). Counterintuitively, NVP231 mediated CERK inhibition blocked *A.phagocytophilum* induced Golgi fragmentation and also reduced the Golgi area compared to control (DMSO) treated infected cells (Fig 4E-F).

2.2.2 C1P downregulation by CERK inhibition reduces A.phagocytophilum load

Truchan and colleagues have reported that *A.phagocytophilum* triggered Golgi fragmentation is dependent on bacterial load, thus we investigated the effect of C1P downregulation on bacterial load (19). C1P levels were reduced by NVP231 mediated CERK inhibition (82). qPCR analyses of bacterial 16S rRNA expression showed that CERK inhibition reduced bacterial DNA load by approximately 98% compared to control (DMSO) treated cell (Fig 5A-B). Next, we investigated the effect CERK downregulation on *A.phagocytophilum* conversion from non-infectious RC to infectious DC form by examining the gene expression of DC specific marker aph1235 at three different time points; 24hrs, 28 hrs and 32 hrs. In congruent to previous studies, our results show that aph1235 expression increased over the course of time in control cells but its expression decreased notably in NVP231 treated cells (Fig 5C-D). To further corroborate the effect of C1P downregulation on *A.phagocytophilum* bacterial load, we genetically ablated CERK in RF/6A cells using siRNA. siRNA mediated silencing of CERK also showed a significant decrease in *A.phagocytophilum* 16S rRNA expression (Fig 5E). We used only RF/6A cells for this experiment as HL-60 cells have very low siRNA transfection efficiency. Therefore, collectively our data show that decreasing C1P inhibits *A.phagocytophilum* infection by preventing the generation of its infectious DC progeny. We further validated these results in vivo by injecting CERK-/- knock mice and wild type (WT) mice with *A.phagocytophilum* DC organisms and measuring the aph16S rRNA gene expression. In the WT mice aph16S rRNA expression increased over time with the bacterial load reaching to the highest on day 12 and then uniformly

decreasing to almost undetectable amount on day 28; however, aph16S rRNA was almost undetectable in CERK -/- mice on any day (Fig 5F). Thereby suggesting that genetically ablated CERK -/- mice are resistant to *A.phagocytophilum* infection.

2.2.3 Increasing C1P enhances A.phagocytophilum induced Golgi fragmentation, increases the bacterial load and promotes ApV maturation

Next we examined the effect of increasing endogenous C1P levels in *A.phagocytophilum* induced Golgi fragmentation and infection. To elevate C1P levels we transfected the cells with siRNA against CPTP (siCPTP) and the knockdown was confirmed by qPCR analyses of CPTP gene expression (Fig 6A). Also, lipidomic analyses showed that siCPTP treated cells had elevated levels D-e-$C_{14:0}$, and D-e-$C_{16:0}$ chain length C1P compared to control non-target siRNA (siNT) treated cells (Fig 6B). In congruent to previous studies C1P elevation stimulated Golgi fragmentation in uninfected RF/6A cells and it further promoted *A.phagocytophilum* induced Golgi fragmentation as infected siCPTP treated cells had higher percentage of cells with fragmented Golgi compared to control (siNT) infected cells (Fig 6C-D). Next we investigated the effect of increasing C1P levels on *A.phagocytophilum* bacterial load, qPCR analyses of siCPTP treated cells showed a prominent increase in the aph16S rRNA expression compared to siNT treated cells (Fig 6E). Furthermore, C1P upregulation increased the number of ApVs in the cells compared to control cells and also increased ApV area suggesting that C1P also enhances ApV maturation (Fig 6F-G). Take together our data suggest that i)C1P is essential for *A.phagocytophilum* induced Golgi fragmentation ii) C1P promotes *A.phagocytophilum* infection and modulates the generation of *A.phagocytophilum* infectious progeny iii) and facilitates ApV formation.

2.3 Discussion

Indeed, CERK derived C1P is crucial for *A.phagocytophilum* stimulated Golgi fragmentation, bacterial infection, completion of its biphasic lifecycle and formation of ApV. Collectively, our data shows that *A.phagocytophilum* infection induces a major increase in CERK derived C1P levels and concordantly decreasing C1P levels by CERK inhibition abrogates *A.phagocytophilum* infection. Furthermore, NVP231 mediated CERK inhibition also disrupts the conversion from non-infectious RC form to infectious DC form. These results are recapitulated in vivo as *A.phagocytophilum* fails to infect CERK -/- mice. Further, C1P is critical for *A.phagocytophilum* induced Golgi fragmentation as CERK inhibition arrests *A.phagocytophilum* triggered Golgi fragmentation. Conversely, increasing C1P levels by siCPTP inhibition further promotes *A.phagocytophilum* induced Golgi fragmentation, increases bacterial load and enhances ApV maturation. Therefore, here we show that C1P plays a pivotal role in *A.phagocytophilum* pathogenesis and contributes to its nutritional virulence needs. Thus our study recognizes C1P and thereby CERK as a potential target to develop drugs to treat *A.phagocytophilum* infection. Additionally, it also provides insight on exploring the role of C1P in the pathogenesis of other more fatal and multi-drug resistant bacterial infections like *O.tsutsugamushi*, *N.gonorrhea*, *P.aeruginosa, A.baumannii, K.pneumonia.*

2.4 Material and Method

2.4.1 Cultivation of uninfected and Infected cells

HL-60 and *A.phagocytophilum* (NCH-1 strain) infected HL-60 cells were gifts from Dr. Carlyon's lab, Virginia Commonwealth University. HL-60 cells were cultured in Isocove Modified Dulbecco (IMDM) media supplemented with 10% fetal bovine serum (FBS). RF/6A cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine, 4.5g of D-glucose,

100mg of sodium pyruvate and supplemented with 10% FBS, 1X nonessential amino acid and 15mM of HEPES. All the cells were cultured at 37℃ with 5% CO_2.

2.4.2 Preparation of host cell free DC organisms and Infection Assays

To isolate DC organisms, 90% infected HL-60 cells were centrifuged at 5200Xg for 15 mins, the supernatant was saved and the pellet was discarded. The supernatant was sonicated using the Misonix S 4000 sonicate at an amplitude of 30amps for pulsed 8 seconds at 8 seconds interval 3 times. Next its was centrifuged at 1000Xg for 5 minutes and the supernatant was saved and pellet discarded. This was repeated 3 times. Next the supernatant was centrifuged at 5200Xg for 5 minutes and the pellet now only contained the *A.phagocytophilum* DC organisms and hence the pellet was saved and used for the following experiments.

Uninfected HL-60 and RF/6A cells were pretreated with 300nm and 400nm respectively of NVP231 or 0.001% of DMSO for 1 hour and then infected with *A.phagocytophilum* DC organisms. The DC organisms were harvested using the procedure described above.

2.4.3 RNA extraction and qPCR analyses

RNA was extracted using Qiagen RNeasy Extraction kit following the manufacturer instructions. Next the RNA was converted to cDNA using Applied Biosystem cDNA Reverse transcription kit with Rnase inhibitor as per manufacturer's instruction. The cDNA was then used qPCR analyses using Applied Biosystem SYBR Green Master mix. The cDNA was diluted to 1:10 for aph16S rRNA and aph1235 gene expression and 1:100 to measure actin gene expression. 0.5μL of 10μM forward and reverse primers were mixed with 9 μL of SYBR Green mastermix which then mixed with 10 μL of diluted cDNA, hence the total PCR reaction was 20 μL. It was then run on the qPCR using the following running method: 95℃ for 10 minutes, 95℃ for 15 seconds and 60℃ for minutes for 40 cycles.

Table 1: qPCR primers from *A.phagocyophilum* gene analyses

Name	Sequence (5'-3')
aph16S F'	TGTAGGCGGTTCGGTAAGTTAAAG
aph16S R'	GCACTCATCGTTTACAGCGTG
aph 1235 F'	CGTGAAGAAATTTCTACAGCTGTGCC
aph 1235 R'	CTAACCTTGGGTCGATGCCACAAT

2.4.4 Immunofluorescence microscopy

Cells were fixed on the slide with 4% paraformaldehyde and then permeabilized with 100X Triton solutions. Then the cells were blocked with 10% goat serum solution for 30 minutes. Next the cell were incubated with primary antibody, TGN46 (NBP1-49643 , 1:1000) for 1 hour 30 minutes, after incubation the cells were washed with PBS 3 times and stained with secondary Alexa Fluor 555 antibody for 1 hour. Next the cells were mounted with Antifade Mount with DAPI to stain the nucleus and imaged on the Keyence microscope at the magnification of 100X.

2.4.5 Sphingolipid extraction

Cell pellets were resuspended and vortexed in LC/MS grade water followed by addition of Internal standards and then vortex and sonicating the samples to break apart the cell pellets. Next we added LC/MS grade methanol chloroform in 2:1 ratio and sonicated the samples. The samples were placed in the water bath shake at 48℃ for 6 hours at 30 RPM. Next the samples were sonicated and then centrifuged for 10 minutes at 4000RPM. Then the organic solvent was transferred to glass vial and dried down for 2.5 hours in SpeedVac Concentrator. Next the sample was resuspended in 500μL methanol, sonicated and the centrifuged at 4000 RPM for 1 minute. The aqueous layer transferred to autosampler vials for UPLC-ESI-MS/MS analysis . The samples were analyzed in the UPLC-ESI-MS/MS using the protocol described in (83)

2.4.5 Mouse study

The CERK knockout mice (CERK -/-) were generated by breeding the C57BL/6J CERK conditional knockout mice (CKO), which have loxP site introduced in intron 5 and intron 6, with C57BL/6J Cre recombinase mice, which results in deletion on exon 6, causing a frameshift and termination on exon 8, hence deleting the kinase domain (Fig 3). The knockout mice were identified by genotyping of mice genomic DNA. The DNA was extracted from the ear punch using Accustart II genotyping kit, followed by PCR reaction using the following primer sequence, P1 (5'-ATATCTCACGTACTGACGGTGGG-3') and P2 (5'-CCTGTTTCACTATCCAGGTTACGG-3') using the following PCR cycle protocol: 94°C for 1 min, 59°C for 1 min, and 72°C for 2 min for 33 cycles. The PCR product was analyzed by running them on 2% agarose gel, CERK WT mice had PCR product at 220bp and CERK-/- had PCR product at 550bp (84)

Two groups of 5-6 male and female 5-6 weeks old CERK-/- and WT mice were injected interperitoneally with 10^8 DC organisms as described in (85). Blood was collected from the saphenous vein on days 4,8,12,16 and 28 post injection. DNA was isolated from the collected blood using DNeasy Blood and Tissue Kit (Qiagen) and subjected to qPCR analyses. 50ng DNA template was mixed with Applied Biosystem SYBR green mastermix and aph16S rRNA forward and reverse primers and ran on the following qPCR thermal cycling protocol, 98℃ for 2 minutes followed by 40 cycles of 98℃ for 5 seconds and 60℃ for 30 seconds. Mice were euthanized on day 28.

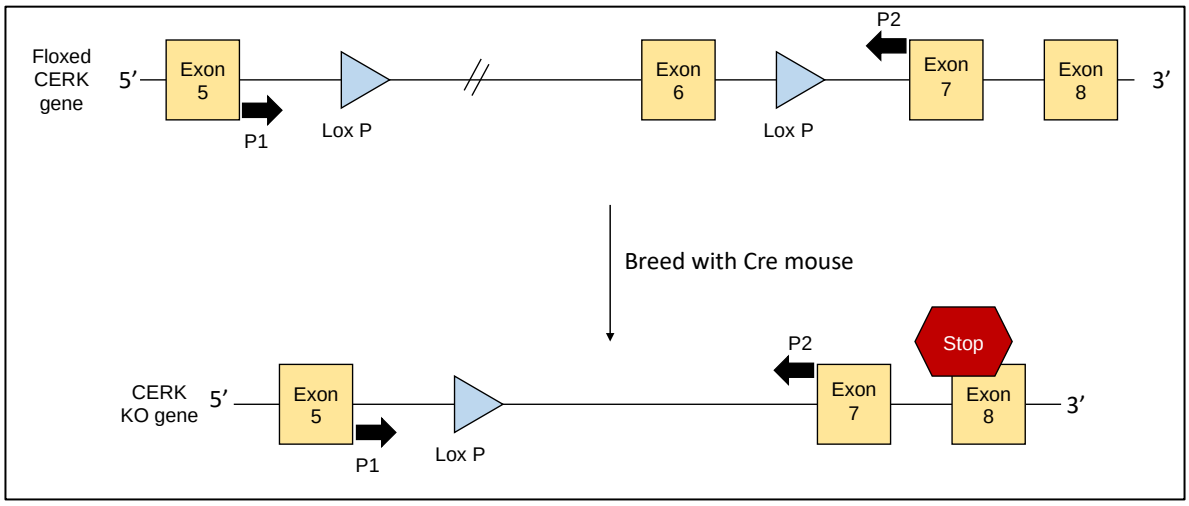

Fig 3: Generation of CERK KO mice.

C57BL/6J CERK floxed mice were bred with C57BL/6J Cre recombinase mice to generate CERK KO mice that has exon 6 deleted.

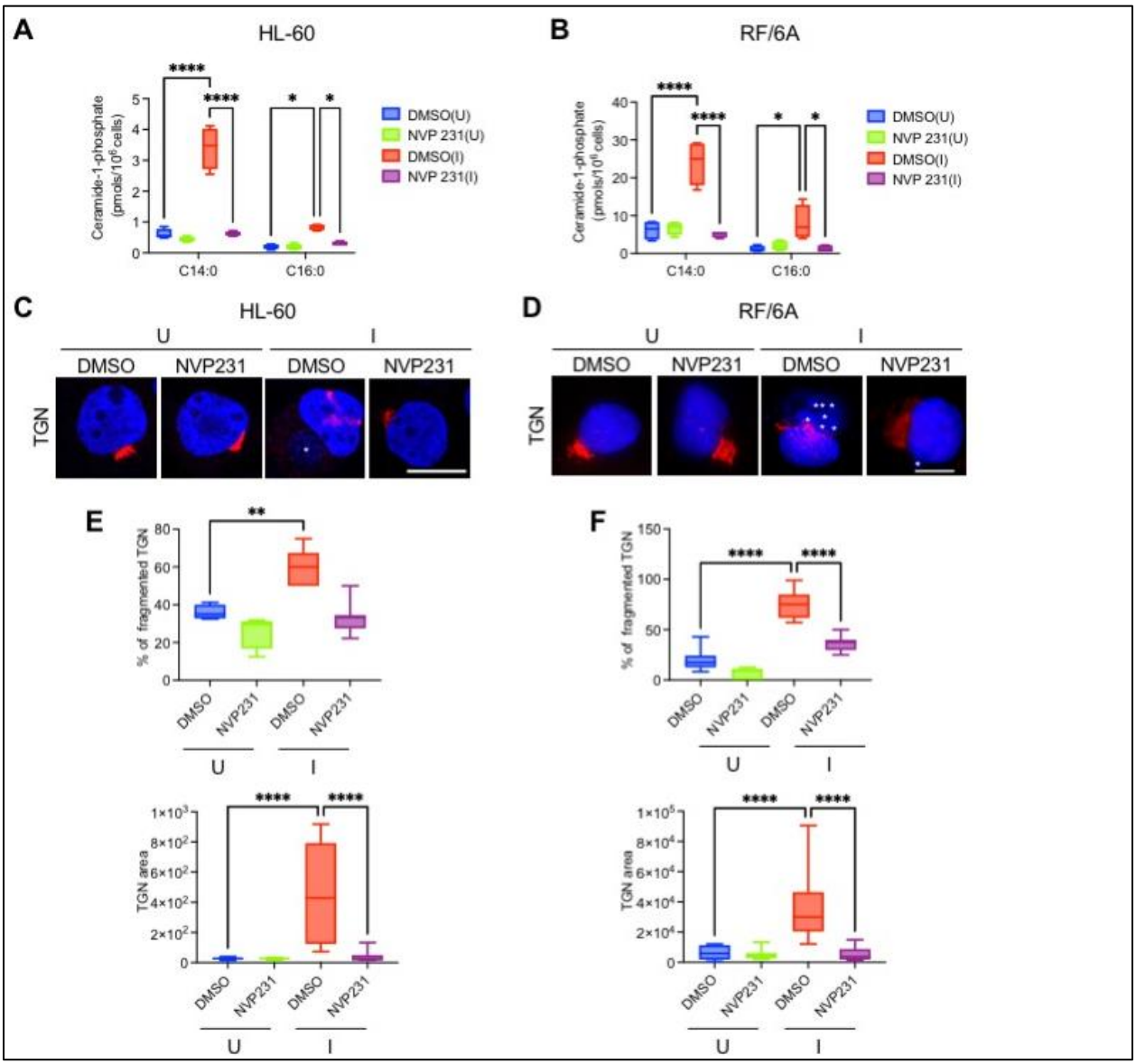

Fig 4: C1P promotes *A.phagocytophilum* induced Golgi fragmentation.

A and B. C1P analyses in uninfected and *A.phagocytophilum* infected HL-60 and RF/6A cells. HL-60 and RF/6A cells were pretreated with 300nM and 400nM of NVP231 respectively or 0.001% of DMSO for one hour and then infected with *A.phagocytophilum* DC organisms. 24 hours post infection the cells were harvested and C1P levels were analyzed by UPLC-ESI-MS/MS analyses. n=4. The samples were compared by Two-way ANOVA followed by Tukey test. C and D. Golgi morphology of uninfected and infected, DMSO and NVP231 treated cells HL-60 and RF/6A cells. The DMSO or NVP231 treated uninfected (U) and infected (I) cells were fixed on the

slide using 4% paraformaldehyde and then stained with TGN antibody TGN46 and the nucleus was stained with DAPI. "*" represents ApVs. The cells were imaged on the Keyence microscope at a magnification of 100X. Scale bar 10μm. E and F. The Golgi fragmentation was quantified by calculating the percentage of cells with fragmented Golgi and measuring Golgi area using ImageJ. n=4. The samples were compared by One-way ANOVA followed by Tukey test. *P<0.05, **P<0.01; ***P<0.001;****P<0.0001.

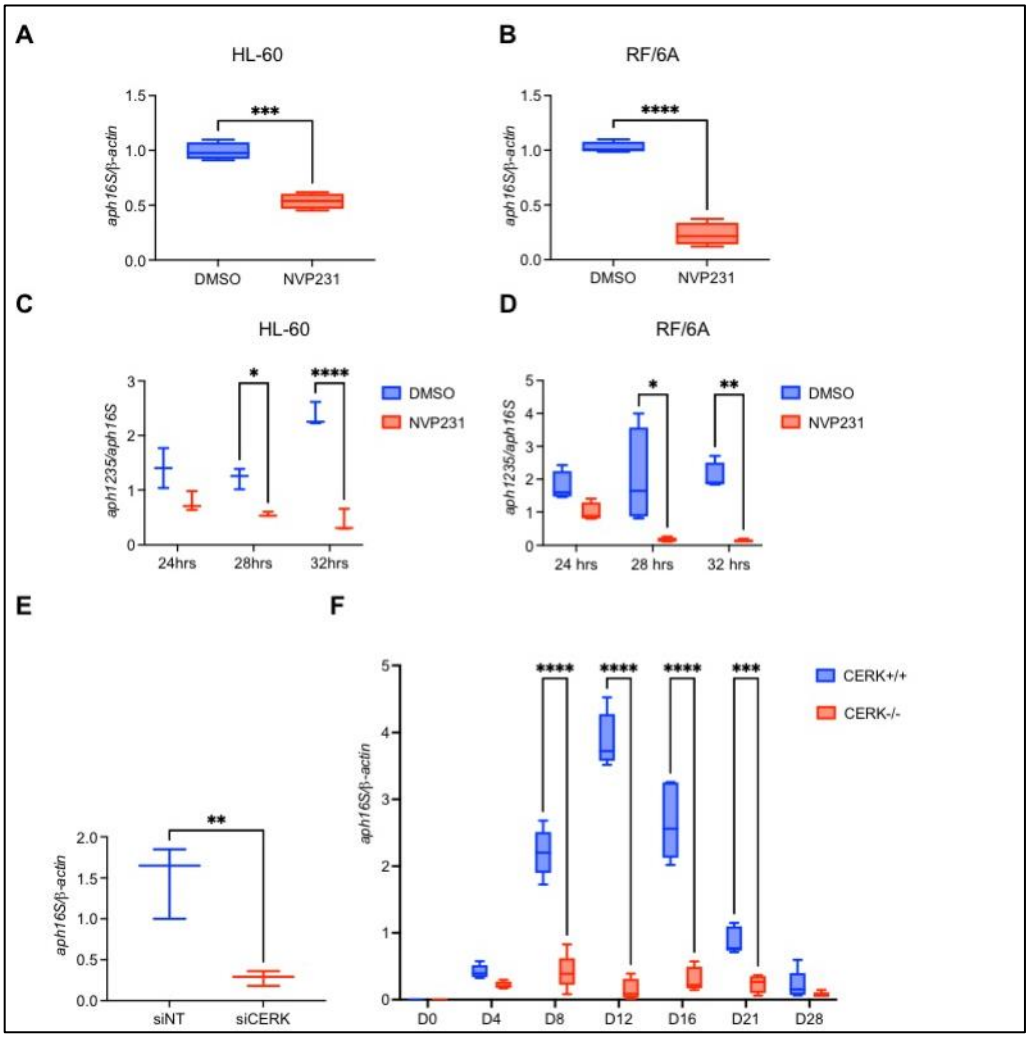

Fig 5: Downregulating C1P levels by CERK inhibition inhibits *A.phagocytophilum* infection and arrests its transition from non-infectious RC to infectious DC form.

A and B. HL-60 and RF/6A cells were pretreated with 300nm and 400nm of NVP231 respectively or 0.001% DMSO and the infected with *A.phagocytophilum* DC organisms. 24 hours post

infection, bacterial load was measured by qPCR analyses of *A.phagocytophilum* 16S rRNA gene expression. 16S rRNA expression was normalized to $\beta - actin$ expression by $2^{-\Delta\Delta Ct}$ method. n=3 for HL-60 cells and n=4 for RF/6A cells. C and D. The NVP231 treated infected cells were harvested 24, 28 and 32 hours post infection and aph1235 gene expression was measured by qPCR analyses. aph1235 expression was normalized to aph16S RNA expression by $2^{-\Delta\Delta Ct}$ method. n=3 for H-60 cells, n=4 for RF/6A cells. E. siRNA mediated CERK inhibition blocks *A.phagocytophilum* infection in RF/6A cells. RF/6A cells were transfected with non-target siRNA (siNT) or siRNA against CERK (siCERK) and 48 hours post siRNA transfection the cells infected *A.phagocytophilum* DC organisms . 24 hours post infection, bacterial load was determined by qPCR analyses bacterial 16S rRNA expression which was normalized to $\beta - actin$ expression by $2^{-\Delta\Delta Ct}$ method. n=3. The samples were compared by One-way ANOVA followed by Tukey test. F. CERK -/- mice are resistant to *A.phagocytophilum* infection. 5 to 6 weeks WT(CERK +/+) and CERK-/- were interperitoneally injected with 10^8 *A. phagocytophilum* DC organisms, blood was collected from the saphenous vein on Days 4, 8, 12, 16, 21 and 28 post injection and bacterial load was examined by qPCR analyses of aph16S rRNA expression. The data is a representation of 2 independent experiments conducted with 4 to 5 male and female mice per group. The samples were compared by Two-way ANOVA with Tukey comparison. *P<0.05, **P<0.01; ***P<0.001;****P<0.0001.

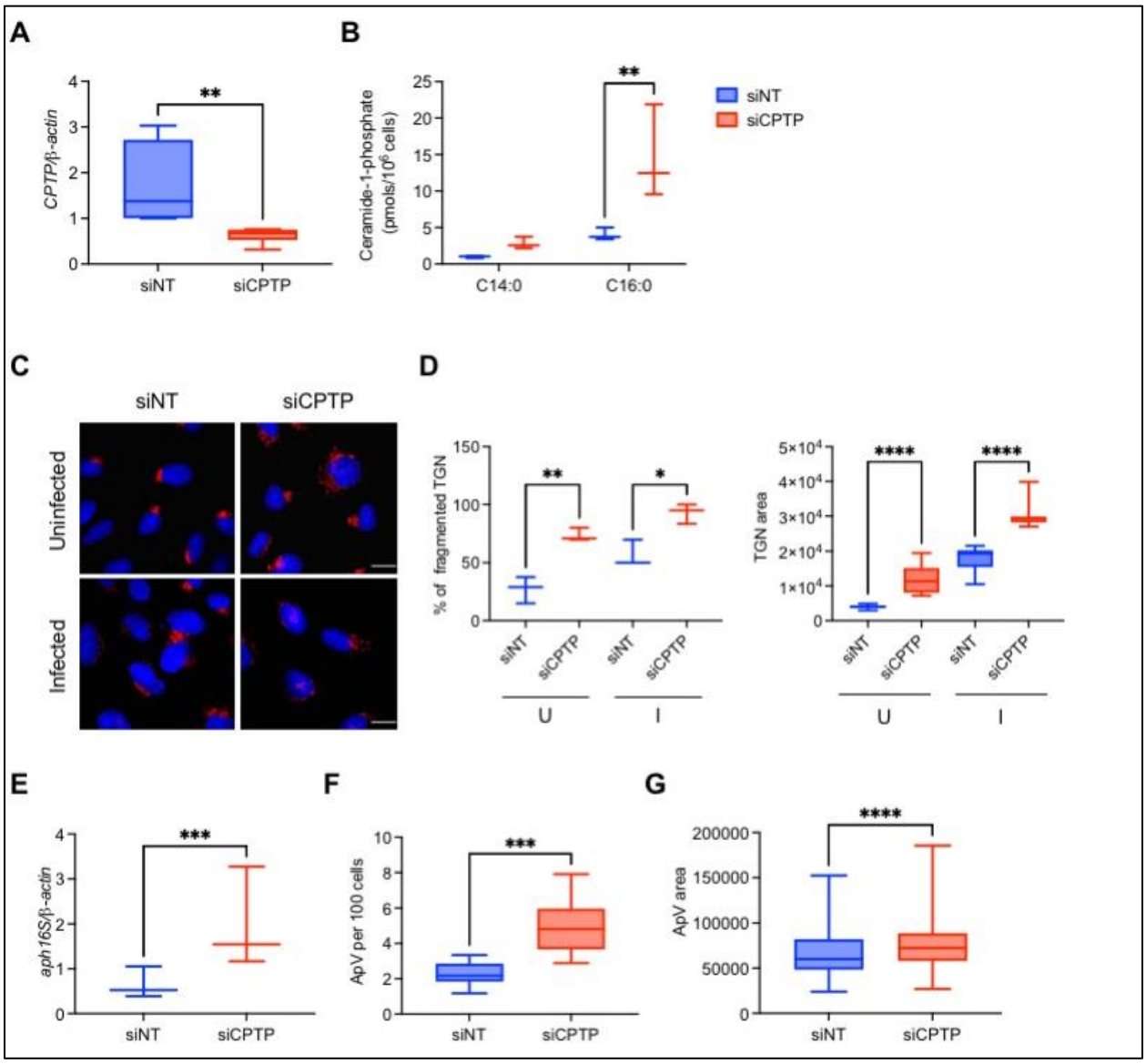

Fig 6: Elevating C1P levels by CPTP inhibition regulates *A.phagocytophilum* induced Golgi fragmentation, increases bacterial load and promotes ApV maturation.

A and B. RF/6A cells were transfected with non-target siRNA (siRNA) or siRNA against CPTP (siCPTP) and 48 hours post transfection the cells were infected with *A.phagocytophilum* DC organisms for another 24 hours. A. 72 hours post siRNA transfection, the knockdown was confirmed by qPCR analyses of CPTP gene expression which was normalized to $\beta - actin$ expression by $2^{-\Delta\Delta Ct}$ method. n=3. The samples were compared by One-way ANOVA followed by Tukey test. B Increase in C1P levels were examined by UPLC-ESI-MS/MS analyses of siNT

and siCPTP transfected cells 72 hours after siRNA transfection. n=3. The sampled were compares by Two-way ANOVA followed by Tukey test. C and D. Elevating C1P levels further augments *A.phagocytophilum* induced Golgi fragmentation. siNT and siCPTP treated uninfected and infected cells were fixed on slides with 4% paraformaldehyde, the trans-Golgi (TGN) was stained with TGN46 antibody and the nucleus was stained with DAPI. The cells were then imaged on the Keyence microscope at a magnification of 100X, scale bar= $10\mu m.$ n=3. D. Golgi fragmentation was quantified by calculating the percentage of cells with fragmented Golgi and measuring the trans-Golgi (TGN) area using ImageJ. The samples were compared by One-way ANOVA followed by Tukey test . E-G. Increasing C1P levels by CPTP downregulation increases bacterial infection and promotes ApV maturation. E. RF/6A cells were transfected with siNT or siCPTP and then infected with *A.phagocytophilum* DC organisms. 24 hours post infection, bacterial load was measured by qPCR analyses of bacterial 16S rRNA expression which was normalized to $\beta - actin$ expression by $2^{-\Delta\Delta Ct}$ method. n=3. F. The number of ApVs per cell were counted from the immunofluorescence microscopy figures in panel C. G. ApV area was measured using ImageJ. The samples were compared by One-way ANOVA followed by t-test. *P<0.05, **P<0.01; ***P<0.001;****P<0.0001.

Chapter 3: Elucidate the mechanism by which C1P induces Golgi fragmentation

3.1 Introduction

3.1.1 CPTP, C1P and Cell Division Control Protein 42 (Cdc42)

CPTP is a member of glycolipid transfer protein (GLTP) that shuttles C1P between membranes and possibly to other organelles (86). Downregulating CPTP increases C1P levels in the cell and consequently stimulates proinflammatory eicosanoid production in the cell (63). Additionally, elevating C1P by CPTP inhibition has shown to induce autophagy dependent inflammasome modulated cytokine release and triggers Golgi fragmentation (64) In this study, we elucidate the mechanism by which CPTP mediated C1P upregulation triggers Golgi fragmentation.

C1P is known to promote cancer cell migration and invasion by activating RhoA GTPase (87). Rho GTPases are a class of versatile proteins that regulate cell migration, cell polarity vesicular trafficking by modulating cytoskeleton rearrangement and are potent regulators of Golgi morphology(88). Rho GTPase cycles between inactive GDP-bound form to active GTP-bound form, and to interact with downstream target proteins and to participate in signaling pathways they have to be in their GTP-bound form(88) Additionally, active GTP-bound Rho GTPases are mainly localized to cellular membranes (89). Cell division control protein (Cdc42), a key regulator of cell polarity and cell migration, is a member of small Rho GTPases that is primarily localized in the Golgi (90). Cdc42 modulate several fundamental cellular functions including cytoskeletal rearrangement, cell cycle progression, transcription, translation, Golgi morphology and vesicle trafficking (91). Cdc42 is critical for COPI vesicle trafficking, active

membrane localized GTP-bound Cdc42 is known to inhibit the retrograde trafficking of COPI vesicles to the endoplasmic reticulum while promoting the anterograde trafficking of COPI vesicles to the cellular membrane (92).Membrane bound GTP-Cdc42 interacts with microtubule protein dynein and subsequently inhibits the interaction of COPI with dynein, thereby disrupting the trafficking of COPI vesicle to the ER (93). Interestingly, ceramide is known to regulate cell polarity by inducing translocation of Cdc42 from the membrane to the cytosol (94). Like C1P, Cdc42 is also pertinent for cell migration during wound healing stages, yet there is no link elicited between Cdc42 and C1P (69, 95) Since C1P induces Golgi fragmentation, a manifestation of perturbed Golgi-ER retrograde trafficking; we predict that C1P regulates Golgi morphology and Golgi-ER retrograde trafficking by inducing translocation of Cdc42 to cellular membranes and its subsequent activation.

3.1.2 The link between C1P, c-Jun N-terminal kinase (JNK) and protein kinase C-α (PKC α)

C1P is a multifunctional sphingolipid and one of its key function is to promote cell proliferation (96). It has been shown by Gomes-Munoz and colleagues that exogenously added C1P stimulates cell proliferation by activation of c-Jun N-terminal kinase (JNK) (74). JNK are kinases that are activated by proinflammatory and mitogenic signals which in turn phosphorylates and activates transcriptional factors, subsequently promoting cell proliferation (97) Furthermore, C1P induces cell proliferation in fibroblast and macrophages by activation of protein kinase C-α (PKCα) (72). PKCα belongs to the serine threonine protein kinase C family which is activated by lipid second messenger diacylglycerol(DAG) and calcium ion and its activation promotes cell proliferation by activating downstream transcription factors (98). Interestingly, PKCα controls AA release via $cPLA_2α$ activation, a phenomenon that is also regulated by C1P in a calcium ion (Ca^{2+}) dependent manner (99, 100). Interestingly, both JNK and PKCα activation are known to trigger Golgi fragmentation via phosphorylation of GRASP65 and GRASP55 respectively (101, 102). Golgi fragmentation induced by GRASP phosphorylation is a hallmark of cells undergoing mitosis,

another cellular process regulated by C1P (41, 65) . However, the implication of C1P in PKCα and JNK mediated GRASP55 and GRASP65 phosphorylation is not known. Interestingly Cdc42 is known to be an upstream regulator PKCα and JNK and promote their activation, and also regulates Golgi morphology (103, 90). Therefore, we examined if C1P induced Golgi fragmentation is regulated by Cdc42 mediated activation of PKCα and JNK which in turn induces GRASP phosphorylation and subsequent Golgi fragmentation.

3.1.3 Hypothesis and aims

We hypothesized that elevating C1P disrupts Golgi-ER retrograde trafficking of COPI vesicle and triggers Golgi fragmentation via Cdc42/PKCα/JNK mediated phosphorylation of GRASPs. Aim of this chapter: elucidate a mechanism by which C1P stimulates Golgi fragmentation and regulate Golgi-ER retrograde vesicle trafficking.

3.2 Results

3.2.1 CERK derived C1P is a general regulator of Golgi fragmentation

Previously, our lab has shown that increasing C1P by CPTP inhibition induces Golgi fragmentation, therefore we manipulated C1P levels to assess if CERK inhibition can block C1P induced Golgi fragmentation (63, 64). To explore the mechanism by which C1P triggers Golgi fragmentation, we have chosen human umbilical vascular endothelial cells (HUVECs) as we can genetically modify CERK and CPTP in HUVECs, also *A.phagocytophilum* infect human endothelial cells in vivo. We manipulated C1P levels by siRNA mediated knockdown of CPTP (siCPTP), CERK (siCERK) and as a rescue experiment we performed simultaneous knockdown of CERK and C1P (siCERK/siCPTP). In congruent to previous data in RF/6A cells, downregulating CPTP increased D-e-$C_{14:0}$, D-e-$C_{16:0}$, D-e-$C_{24:0}$, and D-e-$C_{24:1}$ C1P levels notably compared to non-target (siNT) treated cells, whereas siCERK knockdown reduced C1P levels

and as expected siCERK/CPTP had no significant effect on C1P levels (Fig 7A). The siRNA knockdowns were further confirmed by qPCR analyses of CPTP and CERK gene expression (Fig 7B). Next we examined the effect of manipulating C1P levels on Golgi fragmentation. Increasing C1P by CPTP downregulating triggered fragmentation of the cis and trans Golgi while CERK downregulation tended to compact the Golgi apparatus maintaining the intact structure. Accordingly, simultaneous CERK and CPTP downregulation had no significant effect on Golgi morphology, suggesting CERK inhibition impairs C1P induced Golgi fragmentation (Fig 7C-E) .

3.2.2 Increasing C1P levels disrupts Golgi-ER retrograde vesicular trafficking of COPI vesicles

Since Golgi fragmentation is a hallmark of disrupted Golgi-ER retrograde trafficking, we examined the effect of C1P on Golgi-ER retrograde vesicle trafficking (104) HEK293T and HUVEC cells were co-transfected with plasmid encoding for GFP tagged COPI cargo protein vesicular stomatitis virus G (pGFP-VSVG) and siNT or siCPTP or siCERK or siCERK/CPTP. At 32℃, GFP-VSVG was primarily localized in the Golgi and not in the ER (Fig 8A and 8C and 9A and 9C). At 40℃, the GFP VSVG moved out of the Golgi in the siNT, siCPTP, siCERK and siCERK/CPTP treated cells and moved to the ER (Fig 8D and 8F, 9D and 9F). However, siCPTP cells GFP-VSG got trapped in the trans Golgi and failed to move to the ER. Thereby, suggesting that increasing C1P levels blocks retrograde trafficking of COPI vesicles from the Golgi to the ER and entraps COPI vesicle in the Golgi. However, our data also suggest that C1P induced Golgi fragmentation is not linked to its effect on Golgi-ER vesicle trafficking as we have seen Golgi fragmentation at 37℃ when the movement of GFP-VSVG is not compromised.

3.2.3 C1P induces Golgi fragmentation by stimulating GRASP phosphorylation

Since our data demonstrated that C1P induced Golgi fragmentation is not caused by impaired Golgi-ER retrograde vesicular trafficking, studies were under taken to determine how C1P induces Golgi fragmentation. C1P is known to promote mitosis and consequent cell

proliferation (105). GRASP phosphorylation and subsequent Golgi fragmentation is fundamental for mitosis (35). At the onset of mitosis, GRASPs are phosphorylated by Cdk1 and pol like kinase 1 (Plk 1) which impairs the oligomerization of GRASPs, hence perturbing the stacking of the Golgi cisternae and consequently inducing Golgi fragmentation (40, 36). Post mitosis, GRASPs are dephosphorylated by protein phosphatase 2A (PP2A) which leads to their re-oligomerization and consequent Golgi stacking (40). Interestingly, C1P is known to promote cell proliferation by activating Cdk1 whereas ceramide accumulation induces PP2A activation (106, 107). Therefore, we examined the effect of C1P manipulation on GRASP phosphorylation. Elevating C1P levels by CPTP downregulation induced phosphorylation of both GRASP 55 and GRASP 65 which was blocked by CERK inhibition (Fig 10A-B). Accordingly, the rescue experiment of co-knockdown of CERK and CPTP had no significant effect on GRASP phosphorylation. We also examined whether increasing endogenous C1P was linked to cell proliferation. By performing a cell proliferation assay we showed that CPTP inhibition promoted cell proliferation which was impaired dramatically by CERK downregulation and the rescue experiment had no significant effect on cell growth (Fig 10C). To further assess if C1P triggers Golgi fragmentation by GRASP phosphorylation, we transfected the cells with plasmid encoding for either wild type CPTP (WT CPTP) or dominant negative CPTP (K60A) and or WT GRASP phospho-resistant GRASP55(T225A,T249A). The K60 residue is on the surface cavity of the CPTP protein that interacts with the C1P phosphate head group (63). Therefore mutating it impairs its interaction with C1P and as a result C1P is not transported to the plasma membrane and subsequently C1P levels increases in the cell(63) . Our data showed dominant negative CPTP (K60A) stimulated Golgi fragmentation in cells overexpressing WT GRASP55. In contrast, overexpression of dominant negative CPTP (K60A) failed to induce Golgi fragmentation in phospho-resistant GRASP55(T225A,T249A) overexpressed cells (Fig 10D). As C1P is generated mostly in the TGN and TGN derived vesicles are crucial for *A.phagocytophilum* infection, thus we examined only the TGN localized GRASP55 in this study. Furthermore, our data also showed that cells transfected

with phospho-resistant GRASP55(T225A,T249A) mutant had a decreased cell size and smaller nucleus compared to cells transfected with WT GRASP55. Therefore, our data indicates that C1P causes Golgi fragmentation via phosphorylation of GRASP and this effect is also linked to enhanced cell proliferation.

3.2.4 C1P stimulates translocation of Cdc42 from the cytosol to the cellular membranes and regulates Golgi fragmentation by a Cdc42 mediated pathway.

Once we showed that C1P induces Golgi fragmentation via GRASP phosphorylation, we investigated the factors upstream of GRASP that is mediated by C1P to cause this phenotype. Cdc42, a key modulator of cell migration, is known to regulate Golgi morphology and cellular vesicle trafficking (93, 108). Interestingly, ceramide regulates cellular localization of Cdc42 and thus its activation(109). Therefore, to assess if C1P effects Cdc42 cellular localization, we conducted a subcellular fractionation on siNT, siCPTP, siCERK and siCERK/siCPTP treated cells and examined Cdc42 expression in the membrane extract where it is found in its active GTP-bound form and in the cytosolic fraction where it is found in inactive GDP-bound form. Our data showed that increasing C1P induced the translocation of Cdc42 from the cytoplasmic to the membrane fraction that was blocked by CERK inhibition and also the rescue experiment involving siCERK/CPTP knockdown (Fig 11A). To examine if this was linked to Golgi fragmentation, we performed a siRNA mediated knocked down of Cdc42 and rescue experiment involving co-knockdown of siCdc42 and siCPTP (siCdc42/siCPTP) (Fig 11B) and assessed the Golgi morphology of these cells. Cdc42 ablation had no effect on Golgi fragmentation but in fact Cdc42 downregulation decreased the size of the Golgi area. Additionally, in the siCdc42/siCPTP knockdown cells C1P failed to trigger Golgi fragmentation (Fig 11C). Taken together, our data suggest that C1P regulates Golgi morphology by a Cdc42 mediated pathway and Cdc42 downregulation disrupts C1P induced Golgi fragmentation.

3.2.5 C1P induced PKCα and JNK activation play a key role in C1P induced Golgi fragmentation.

Adding exogenous C1P to cells has been shown to promote cellular proliferation in cancer cells and macrophages via PKCα and JNK activation (72, 74).Hence we examined how increasing endogenous C1P by CPTP inhibition effected PKCα and JNK activation. Our results showed that augmenting C1P levels CPTP inhibition induced phosphorylation of both PKCα and JNK and their subsequent activation which was abrogated remarkably by CERK inhibition and siCERK/CPTP treatment (Fig 12A). As both PKCα and JNK are involved in the maintenance of Golgi morphology, we assessed the effect of C1P manipulation on PKCα and JNK induced Golgi fragmentation. In concordant with previous data, CPTP downregulation triggered Golgi fragmentation where as PKCα inhibitor, G**Ö**6976 and JNK inhibitor SP600125 treatment had no significant effect on Golgi morphology. In cells co treated with siCPTP and G**Ö**6976 or SP600125, the cells had intact Golgi, suggesting that PKCα and JNK inhibition arrests C1P induced fragmentation (Fig 12B-C).

3.2.6 C1P regulates Golgi fragmentation via Cdc42/ PKCα/JNK mediated phosphorylation of GRASP protein

Previously, it has been reported that Cdc42 promotes cell proliferation via JNK activation, which can also be activated by PKCα (103, 110). Since some studies have reported Cdc42 to be an upstream regulator of PKCα, we explored the implication of Cdc42/PKCα/JNK signaling pathway in C1P induced Golgi fragmentation (111). To examine this signaling pathway, we investigated the effect of Cdc42 downregulation on PKCα and JNK activation. In congruent to previous data, CPTP ablation induced PKCα and JNK activation but Cdc42 downregulation blocked it. However, in siCdc42/siCPTP treated cells, phos-PKCα expression was not downregulated to the same extend as in the siCdc42 treated cells, thus suggesting that Cdc42 depletion cannot completely inhibit C1P regulated PKCα activation. Therefore our data suggest

that either Cdc42 directly activates JNK, subverting PKCα; or PKCα is possibly upstream of Cdc42, which is in agreement with other reports that suggest PKCα activates Cdc42 (111). Counterintuitively, C1P elevation by CPTP inhibition failed to induce JNK activation in siCdc42 downregulated cells (siCdc42/siCPTP), thereby suggesting that C1P modulates JNK activation through a Cdc42 signaling pathway (Fig 13A). As PKCα has been known to be upstream of JNK, thus we assessed if C1P induces JNK activation through a PKCα pathway. Our data showed that PKCα inhibition by G**Ö**6976 treatment blocked JNK activation and also arrested C1P induced JNK activation (Fig 13B) To further corroborate that PKCα is upstream of JNK, we inhibited JNK and co-treated the cells with siCPTP and JNK inhibitor SP600125 and examined its effect on PKCα activation. JNK inhibition has no significant effect on C1P stimulated PKCα activation. (Fig 13C) Indeed, PKCα is upstream of Cdc42 which in turn activates JNK. Since PKCα and JNK are known to induce GRASP55 and GRASP65 phosphorylation respectively, hence we examined whether C1P induces GRASP phosphorylation via PKCα/Cdc42/ JNK mediated pathway. Our data showed that Cdc42 inhibition arrested GRASP55 and GRASP65 phosphorylation and C1P elevation failed to induce their phosphorylation in Cdc42 inhibited cells (Fig 13D). In congruent to previous reports PKCα and JNK inhibition had no significant effect on GRASP phosphorylation and increasing C1P levels failed to induce GRASP phosphorylation in PKCα and JNK inhibited cells (Fig 13DE-F). Therefore, our data suggests that indeed C1P promotes GRASP phosphorylation and subsequent Golgi fragmentation by activating the PKCα/Cdc42/JNK signaling pathway

3.2.7 The role of Cdc42, PKCα and JNK in A.phagocytophilum pathogenesis

Cdc42 plays a crucial role in bacterial pathogenesis, regulating its invasion and the trafficking of COPI vesicles to the pathogen occupied vacuole (112). As C1P, which is augmented in the *A.phagocytophilum* infected cells, regulates Cdc42 translocation to the cellular membrane, we examine the effect of Cdc42 on *A.phagocytophilum* induced Golgi fragmentation, bacterial

load and ApV maturation. Downregulating Cdc42 reduced *A.phagocytophilum* induced Golgi fragmentation only by ~10% (Fig 14A-B). The effect of Cdc42 downregulation on bacterial load was determined by examining the protein expression of bacterial outer membrane protein p44 and by performing qPCR analyses of bacterial aph16S rRNA expression. Our data showed the Cdc42 downregulation had no significant effect on bacterial load (Fig 14C-D). However, downregulating Cdc42 reduced the expression of ApV protein APH0032 and also decreased ApV area, thereby suggesting Cdc42 knockdown effects ApV maturation (Fig 14C and 14E).

PKCα plays a pivotal role in the pathogenesis of intracellular bacterial infection as it facilitates bacterial infection by regulating its invasion and survival inside host cell (113). Additionally bacterial infections are also known to trigger PKCα activation in host cell. Nonetheless, the implication of PKCα in *A.phagocytophilum* infection is not known. Concomitantly, bacterial infections are known to also activate the JNK signaling pathway and JNK-/- mice have been shown to be resistant to *A.phagocytophilum* infection (114, 115). Since our data indicate that C1P, a promoter of *A.phagocytophilum* infection, induces Golgi fragmentation via a PKCα and JNK mediated pathway, thus we examined the role these two proteins in *A.phagocytophilum* infection. *A.phagocytophilum* stimulated the activation of PKCα and JNK which was repudiated by NVP231 mediated CERK downregulation(Fig 15A). Further, qPCR analyses of *A.phagocytophilum* aph16S rRNA expression showed that PKCα and JNK inhibition arrested *A.phagocytophilum* infection (Fig 15B-C). Indeed, C1P promotes *A.phagocytophilum* infection by activating PKCα and JNK signaling pathway.

3.3 Discussion

Previously our lab has shown that increasing C1P by CPTP inhibition induced Golgi fragmentation; however the underlying mechanism was not known (63, 64) Herein, we show that C1P promotes GRASP phosphorylation via PKCα/Cdc42/JNK signaling pathway, subsequently

induces Golgi fragmentation. Our data shows that increasing endogenous C1P causes fragmentation of the Golgi apparatus which is repudiated by CERK inhibition, elevated C1P also fails to induce Golgi fragmentation in CERK downregulated cells. Additionally, CERK inhibition has shown to enhance the Golgi stacking and further compacts the size of the Golgi. Indeed C1P is crucial modulator of Golgi morphology. As Golgi fragmentation is known to be a ramification of disrupted Golgi-ER vesicle trafficking, we examined the effect of C1P on COPI vesicle trafficking. Increasing C1P arrested the trafficking of COPI vesicle from the Golgi to the ER and entraps it in the TGN. However, as Golgi fragmentation is observed at both 32℃ and 37℃ where COPI trafficking is not disrupted, we conclude that C1P induced Golgi fragmentation is not linked to disrupted Golgi-ER retrograde trafficking of COPI vesicle. Therefore, studies were undertaken to elucidate the other signaling pathway by which C1P regulates Golgi morphology. GRASPs mediated Golgi fragmentation is essential for mitosis and thus an important regulator of cell proliferation. Previously it has been shown that adding exogenous C1P to cancer cells stimulates proliferation of cancer cells and macrophages (65). Hence, we examined if C1P enhances Golgi fragmentation. Our study delineates that elevating C1P stimulates phosphorylation of GRASP55 and GRASP65 and also stimulates cell proliferation in HUVECs both of which are arrested by CERK inhibition. Furthermore, elevated C1P fails to stimulate Golgi fragmentation in cells overexpressing phospho-resistant GRASP55 mutant protein. Collectively, this indicates that indeed C1P modulates Golgi fragmentation via phosphorylation of GRASP proteins.

Golgi localized Rho protein, Cdc42 modulates the trafficking of COPI vesicle trafficking and thus Golgi morphology (92). As ceramide regulates the cellular localization of Cdc42, therefore we examined the effect of C1P on Cdc42 cellular localization and activation. Increasing C1P stimulates the translocation of Cdc42 from the cytosol to the cellular membrane which is revoked by CERK inhibition. Concordantly, Cdc42 downregulation inhibits C1P triggered Golgi fragmentation, thus suggesting that C1P regulates Golgi fragmentation via Cdc42 signaling pathway. Since Cdc42 is known to be an upstream regulator of PKCα and JNK, which are also

essential regulators GRASP phosphorylation and subsequent Golgi fragmentation; we investigated the implication of PKCα and JNK in C1P regulated signaling pathway that stimulates GRASP phosphorylation. In congruent to previous reports, increasing C1P induces phosphorylation and subsequent activation of both PKCα and JNK which is rescinded by CERK downregulation. Additionally, PKCα and JNK inhibition blocks C1P triggered Golgi fragmentation. Next we show the involvement of Cdc42 in PKCα and JNK activation. Sole Cdc42 downregulation causes a dramatic inhibition of PKCα activation but Cdc42 downregulation doesn't have a major effect on C1P induced PKCα activation . Therefore, our data suggest that C1P is possibly activating PKCα by a Cdc42 independent pathway or PKCα is upstream of Cdc42 in this C1P regulated signaling pathway. This is also in coherent with some other studies that reported PKCα regulates Cdc42 activation. Indeed the relationship between PKCα and Cdc42 varies between cell types. In concordant to previous reports, our data shows that C1P regulates JNK activation via Cdc42 mediated pathway as its depletion perturbs C1P induced JNK activation. Moreover, JNK inhibition has no effect on C1P induced PKCα activation, suggesting that PKCα is also upstream of JNK. Indeed our data strongly demonstrates that C1P induces PKCα activation, which regulates Cdc42 translocation to cellular membrane and its activation, which in turn induces JNK activation. Lastly, we show that Cdc42, PKCα and JNK inhibition disrupts C1P stimulated GRASP55 and GRASP65 phosphorylation. Collectively, our data suggest that C1P regulates Golgi morphology in HUVECs by activating PKCα/Cdc42 /JNK pathway which in turn causes GRASP phosphorylation, subsequently triggering Golgi fragmentation.

Next we examined if C1P mediated PKCα/Cdc42/JNK has any effect on *A.phagocytophilum* infection. Our data illustrates that downregulating Cdc42 reduces Golgi fragmentation only by 10% and has no significant effect on *A.phagocytophilum* load. However, Cdc42 downregulation reduces ApV area and therefore hinders ApV maturation. Next we show that *A.phagocytophilum* induces PKCα and JNK activation which is abated by NVP231 mediated CERK inhibition. Concomitantly, PKCα and JNK inhibits *A.phagocytophilum* infection. Therefore

C1P promotes *A.phagocytophilum* infection by inducing PKCα and JNK activation. Nonetheless, due to the lack of effect of Cdc42 downregulation bacterial load, we predict that C1P activates PKCα which then directly activates JNK, subverting Cdc42 in RF/6A cells.

3.4 Methods

3.4.1 Cell Culture, siRNA and plasmid transfection

The following cell lines were used for this study:

Table 2: Cell lines used for this study .

Cells	Vendor	Catalog Number	RRID number
HUVEC	Lonza	CC-2935	CVCL_A4BB
HL-60	ATCC	CCL-240	CVCL_0002
RF/6A	ATCC	CRL-1780	CVCL_4552
HEK293T	ATCC	CRL-4051	CVCL_X491

HUVECs were purchased from Lonza and cultured in Endothelial cell growth medium-2 (EGM-2) supplement with bullet kit form Lonza. HEK293Ts were cultured in low glucose DMEM media supplemented 10% FBS and 1% penicillin and streptomycin. All the cells were grown in 5% CO_2 at 37℃.

Sequence specific CERK, CPTP and Cdc42 siRNA were purchased from Dharmacon. HUVECs were transfected with siRNA using Dharmafect 4 transfection reagent and HEK293Ts were transfected with Dharmafect 1 transfection reagent . GFP-VSVG plasmid was obtained from Addgene and 500ng of the plasmid was transfected in HUVECs and HEK293Ts with Lipofectamine LTX with PLUS reagent from Thermofisher using the manufacturer's instructions.

3.4.2 Antibodies for western blotting and immunofluorescence microscopy

The following antibodies were used for Western blotting experiments: Cdc42 (1:500, Cell signalling, #2462), Phospho-SAPK/JNK (1;500, Thr138.Try185) (Cell signaling, #81E11),

SAPK/JNK (1:500, Cell signalling, #9252), Phospho-PKCα(1:500, Cell signaling, #9375S), PKCα antibody (1:500, Cell signaling, 2056S), GRASP 65 antibody (1:500, Novus biological, # NBP2-75516) and GRASP55 (1:400, Santa Curz , #sc-271840). The Western blotting experiments were conducted using the protocol described in (116)

The following antibodies were used for immunofluorescence microscopy; TGN46, (1:100, Novus biologicals,NBP1-49643), GM130 (1:100,Novus biological, NBP2-53420), Calreticulin (1:50, Thermofisher Scientific, #PA3-900).

3.4.3 RNA extraction and qPCR analyses

RNA was extracted using Qiagen RNeasy Extraction kit following the manufacturer instructions. It was then converted to cDNA using Applied Biosystem cDNA Reverse transcription kit with Rnase inhibitor as per manufacturer's instruction. The cDNA was then used qPCR analyses using Applied Biosystem Taqman master. The cDNA was diluted to 1:10 for CERK and CPTP gene expression and 1:100 for actin. Taqman probed 1μL CERK or CPTP or β −actin primers are mixed with 9 μL of Taqman Mastermix which is then mixed with 10 μL of diluted cDNA and ran on the qPCR on the following running protocol: 95℃ for 10mins, 95℃ for 15 seconds and 60℃ for 1 minute for 40 cycles. CERK and CPTP gene expression was normalized to β −actin using the $2^{-\Delta\Delta CT}$ method.

3.4.4 Immunoprecipitation assay (IP)

The cell lysate was diluted in IP buffer (20nM Tris (pH7.5), 150mM NaCl, 1mM EDTA, 1% Triton X-100, 1mM Na_3VO to give a protein amount of 400ng and a total volume of 400μL. 10 μL of anti-phosphoserine agarose antibody or anti phospho-threonine agarose antibody was added to the diluted cell lysate and incubated at 4℃ overnight with gentle rocking. It was then centrifuged at 14000Xg for 1 minute, supernatant discarded and the pellet was washed 5 times

with 500 μL of IP buffer. The pellet was resuspended in 2X Laemmli buffer with BME incubated at 98℃ for 10 minutes. The samples was then microcentrifuge and loaded in 10% SDS gel for Western blotting analyses.

3.4.5 Cell proliferation assay

HUVECs (2X10^3) were plated on a six well plate and allowed to grow for 0-4 days. The cells fixed with methanol and stained with 0.05% of crystal violet solution. The crystal violet stained was the extracted with methanol and the light absorbance was measured using a plate reader at 590nm wavelength.

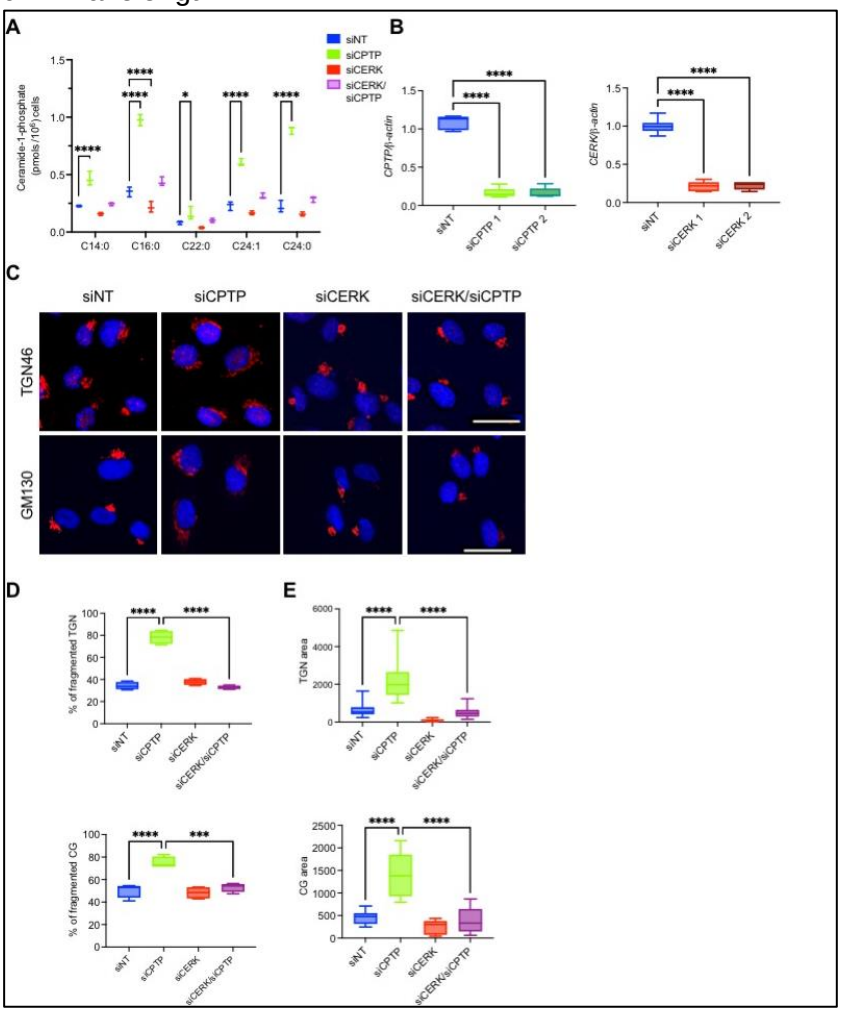

Figure 7: CERK derived C1P is a general regulator of Golgi fragmentation.

A. C1P levels were modulated by downregulating CPTP and CERK in HUVECs. HUVECs were transfected with on-target siRNA (siNT) , siRNA against CPTP(siCPTP), CERK (siCERK) and co-transfected with CERK and CPTP siRNA (siCERK/siCPTP) for 72 hours. C1P levels were analyzed by UPLC-ESI-MS/MS analyses. n=3. The samples were compared by Two-way ANOVA followed by Tukey test. B. CPTP and CERK downregulation were further compared by qPCR analyses of CPTP and CERK gene expression which was normalized to β-*actin* using $2^{-\Delta\Delta Ct}$ method. n=3. The samples were compared by One-way ANOVA followed by t-test. C. Increasing C1P induces Golgi fragmentation which is arrested by CERK inhibition. siNT, siCPTP, siCERK and siCERK/siCPTP transfected cells were fixed on slides with 4% paraformaldehyde and stained with TGN antibody (TGN46) or cis-Golgi antibody (GM130) and the nucleus was stained with DAPI. The cells were images on Keyence microscope at a magnification 40X, scale bar 20μm. D and E. Golgi fragmentation was quantified by calculating the percentage of cells with fragmented Golgi and measuring trans-Golgi (TGN) and cis-Golgi (CG) area using ImageJ. n=3. The samples were compared by One-Way ANOVA followed by Tukey test. *P<0.05, **P<0.01; ***P<0.001;****P<0.0001

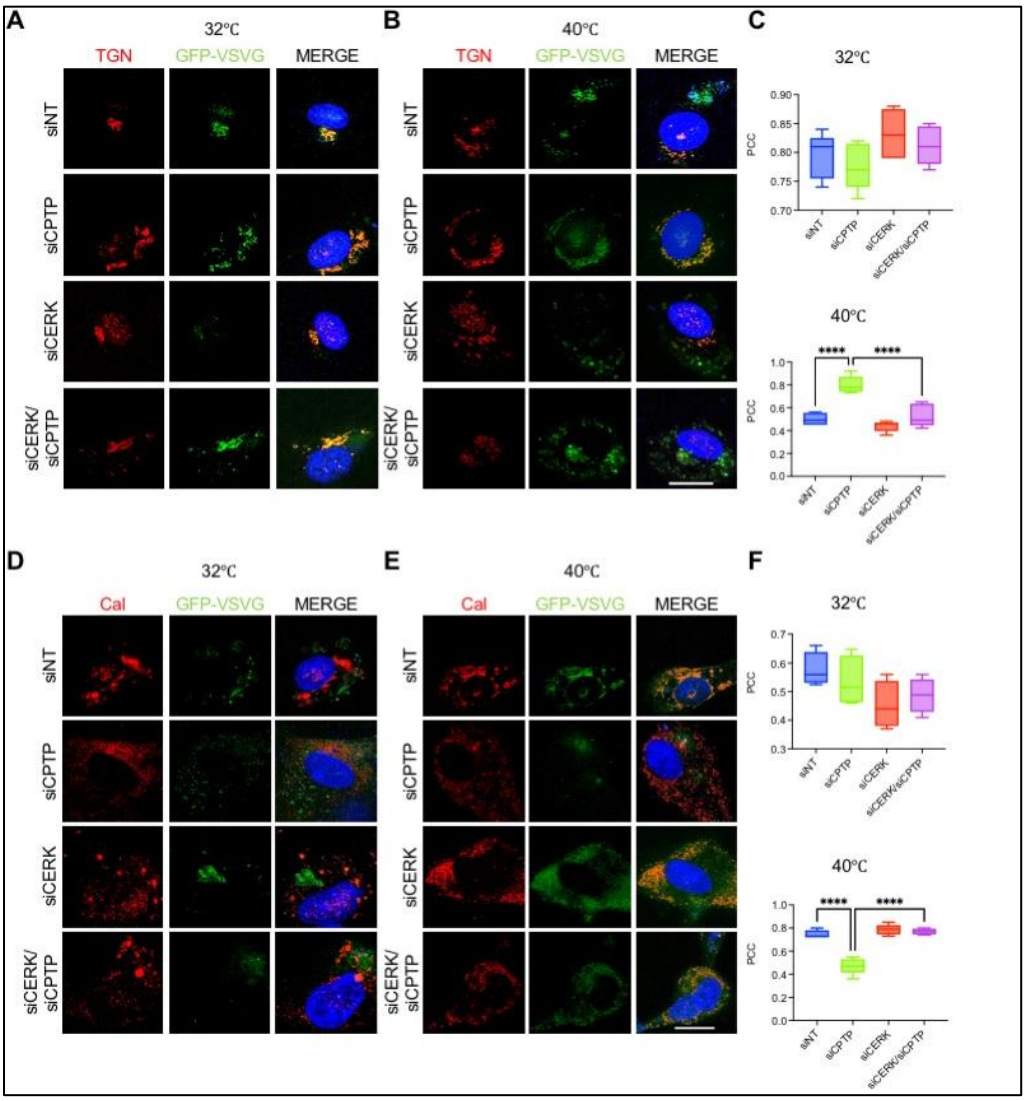

Figure 8: C1P regulates Golgi-ER retrograde trafficking in HUVECs.

A-B, D-E. HUVECs were co-transfected with plasmid encoding for GFP-VSVG or siNT,siCPTP, siCERK or siCERK/siCPTP. 72 hours post transfection the cells were incubated at 32℃ overnight and then at 40℃ for 3 hours. The cells were then fixed on slides and stained with TGN antibody (TGN46), GFP antibody, the ER specific antibody (calreticulin) and the nucleus was stained with DAPI. The cells were then imaged on the Keyence microscope at a magnification 40X, scale bar 20μm.n=3. C and F The colocalization of GFP-VSVG with the ER or the TGN marker was calculated by measuring Pearson's correlation coefficient (PCC) using the ImageJ. The samples

were compared by One-way ANOVA followed by Tukey test. *P<0.05, **P<0.01; ***P<0.001;****P<0.0001

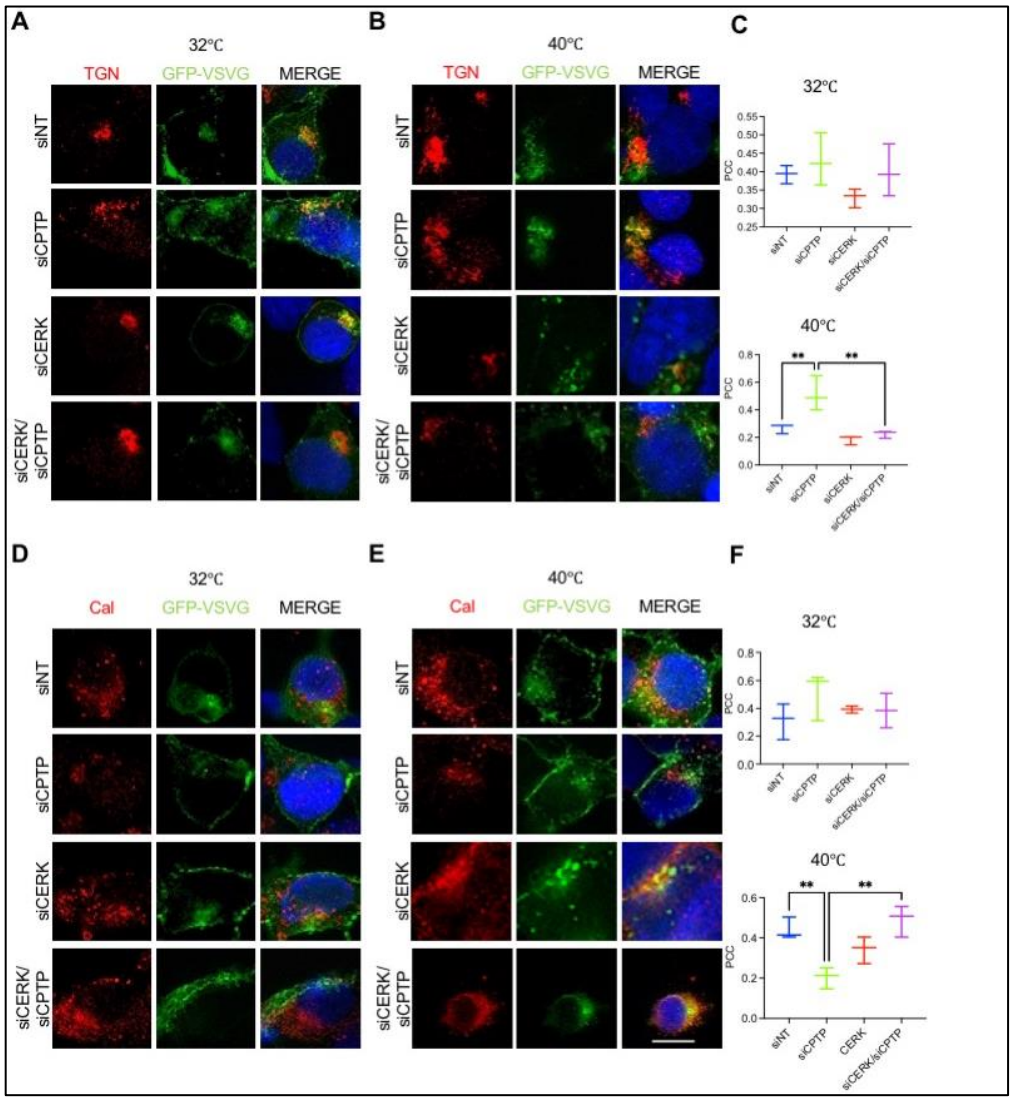

Figure 9: C1P regulates Golgi-ER retrograde trafficking in HEK293T.

A-B, D-E. HEK293T were co-transfected with plasmid encoding for GFP-VSVG or siNT,siCPTP, siCERK or siCERK/siCPTP. 72 hours post transfection the cells were incubated at 32℃ overnight and then at 40℃ for 3 hours. The cells were then fixed on slides and stained with TGN antibody (TGN46), GFP antibody, the ER specific antibody (calreticulin) and the nucleus was stained with DAPI. The cells were then imaged on the Keyence microscope at a magnification 40X, scale bar

20μm.n=3. C and F The colocalization of GFP-VSVG with the ER or the TGN marker was calculated by measuring Pearson's correlation coefficient (PCC) using the ImageJ. The samples were compared by One-way ANOVA followed by Tukey test. *P<0.05, **P<0.01; ***P<0.001;****P<0.0001

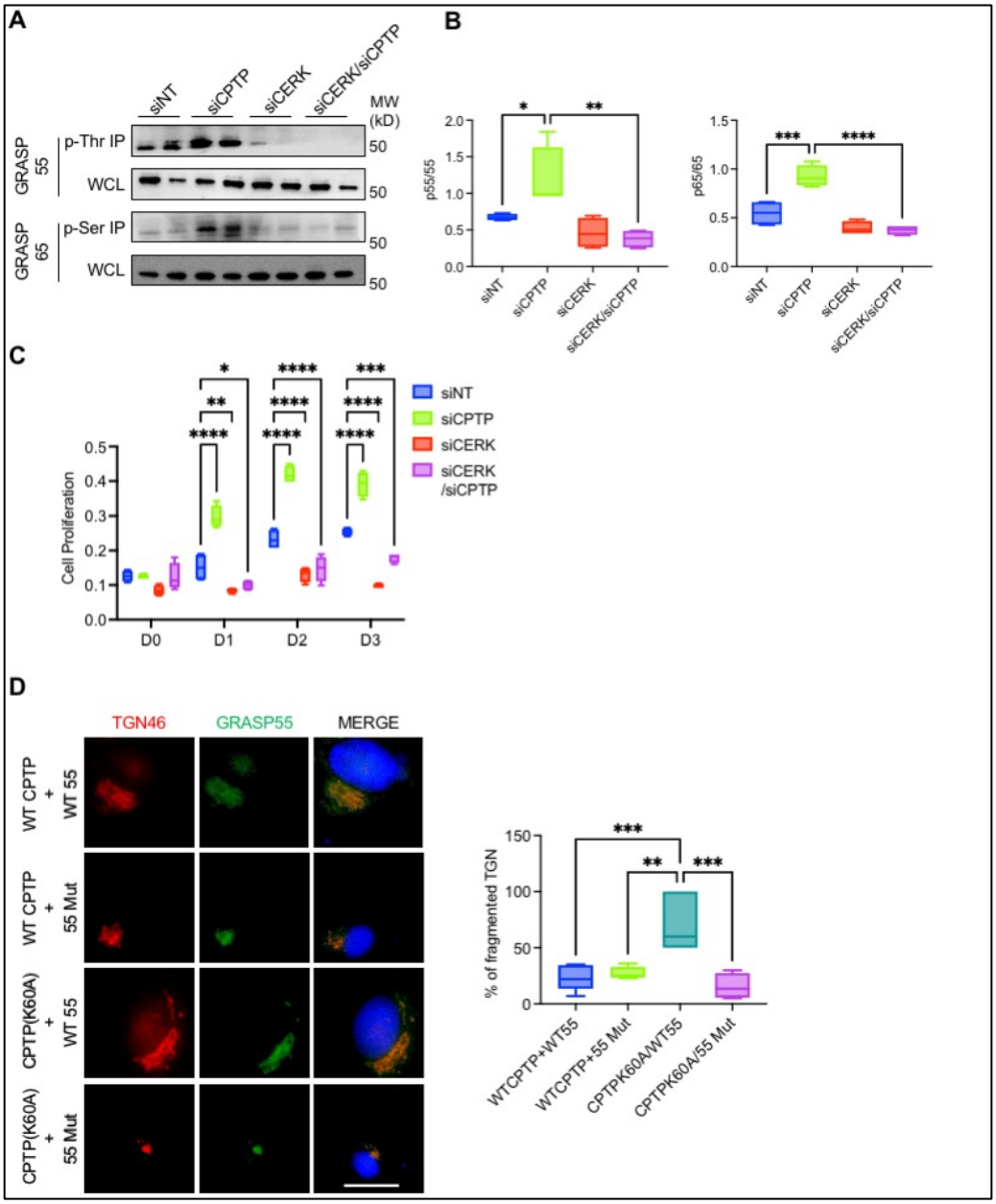

Fig 10: C1P induces Golgi morphology by promoting GRASP phosphorylation.

A. HUVECs were transfected with siNT, siCPTP, siCERK and siCERK/siCPTP and 72 hours post siRNA transfection the cells were harvested and immunoprecipitation assay was performed on the cell lysate. The cell lysate was incubated with agarose beads conjugated with phospho-threonine for GRASP55 or phospho-serine for GRASP65. The immunoprecipitated phospho-protein samples were analyzed for GRASP55 and GRASP65 expression by Western blotting analyses. B. The phospho-GRASP55 and phosph-GRASP65 were normalized to total levels of GRASP55 and GRASP65 respectively in the whole cell lysate (WCL) using ImageJ densitometry. n=4, The samples were compared by One-way ANOVA followed by Tukey test. C. B. C1P manipulation effects cells proliferation. C. Cell proliferation assay was performed on siNT, siCPTP, siCERK and siCERK/CPTP treated cells. n=4. The samples were compared by One-way ANOVA followed by Tukey test. D. Increasing C1P fails in to induce Golgi fragmentation in cells overexpressing phospho-resistant GRASP55(T225A,T249A) mutant (55Mut). Cells were transfected with WT CPTP, CPTP(K60A), WT GRASP55 and GRASP55(T225A,T249A) mutant (55 Mut) and 72 hours post transfection the cells were fixed on the slide and stained with TGN and GFP antibody and the nucleus was stained with DAPI. The cells were the imaged on the Keyence microscope at a magnification of 40X, scale bar 20μm. n=4. Golgi fragmentation was quantified by calculating percentage of cells with fragmented Golgi. The samples were compared by One-way ANOVA followed by Tukey test. *P<0.05, **P<0.01; ***P<0.001;****P<0.0001

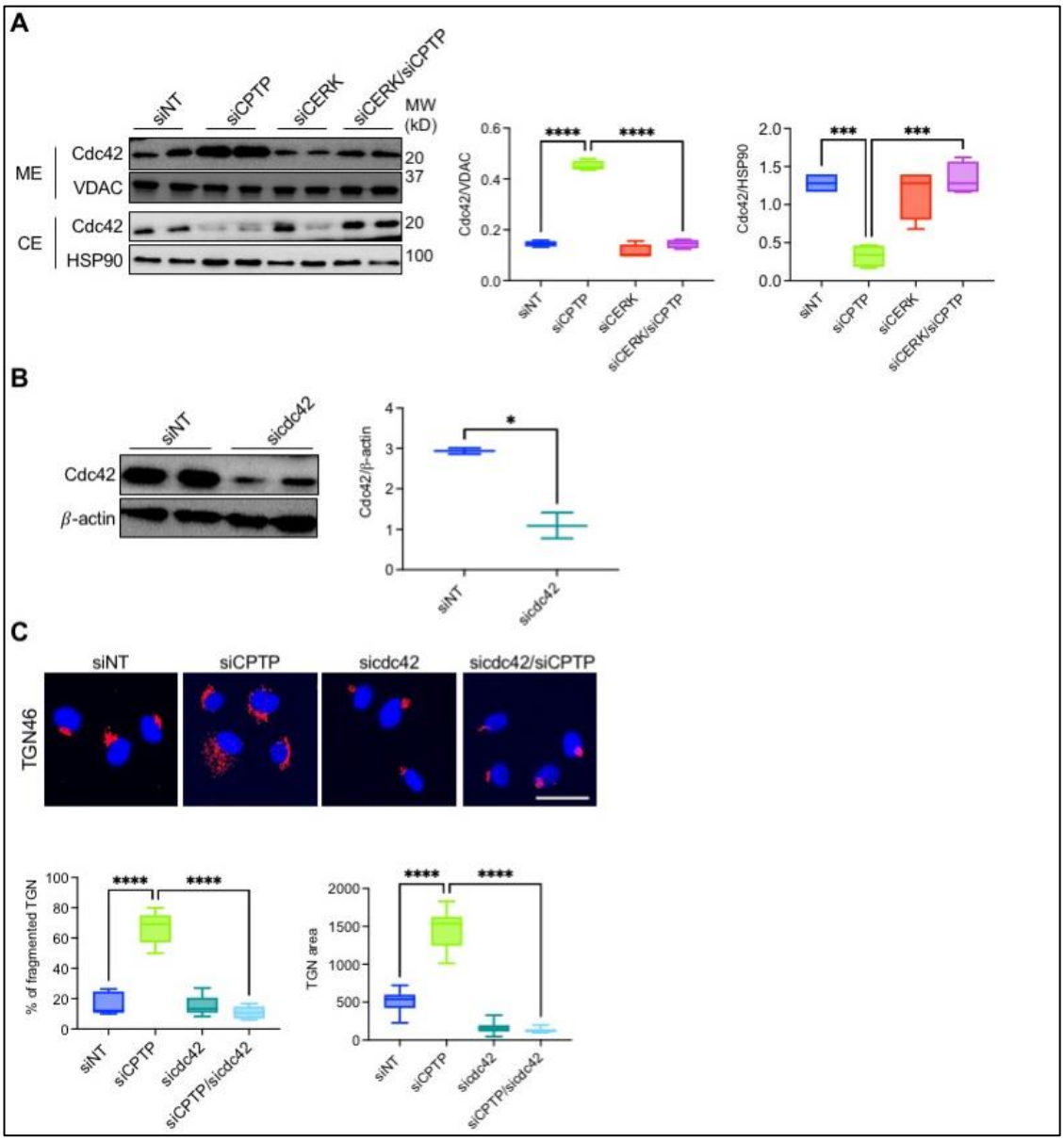

Fig 11: C1P stimulates translocation of Cdc42 from the cytosol to the cellular membranes and regulates Golgi fragmentation by a Cdc42 mediated pathway.

A. HUVECs were transfected with siNT, siCPTP, siCERK and siCERK/siCPTP and 72 hours post siRNA transfection the cells were harvested and subcellular fractionation was performed on the cell lysate. Cdc42 expression in the membrane extract (ME) and cytoplasmic extract (CE) was analyzed by Western blotting analyses. Cdc42 expression in the membrane and cytoplasmic extract were normalized to VDAC and HSP90 respectively using ImageJ densitometry. n=4. The samples were compared by One-way ANOVA followed by Tukey test B and C. Cdc42

downregulation arrests C1P induced Golgi fragmentation. B. HUVECs were transfected with siRNA against Cdc42 (siCdc42) and 72 hours post siRNA transfection, the knockdown was assessed by Western blotting analyses. Cdc42 expression was normalized to β – actin by ImageJ densitometry. C. siNT, siCPTP, siCdc42 and siCd42/siCPTP treated cells were fixed on the slide and the TGN and nucleus was stained with TGN 46 and DAPI respectively. The cells were imaged on Keyence microscope at a magnification 0f 40X, scale bar 20μm. n=4. Golgi fragmentation was quantified by calculating percentage of cells with fragmented Golgi and measuring TGN measuring ImageJ. The samples were compared by One-way ANOVA followed by Tukey test. *P<0.05, **P<0.01; ***P<0.001;****P<0.0001

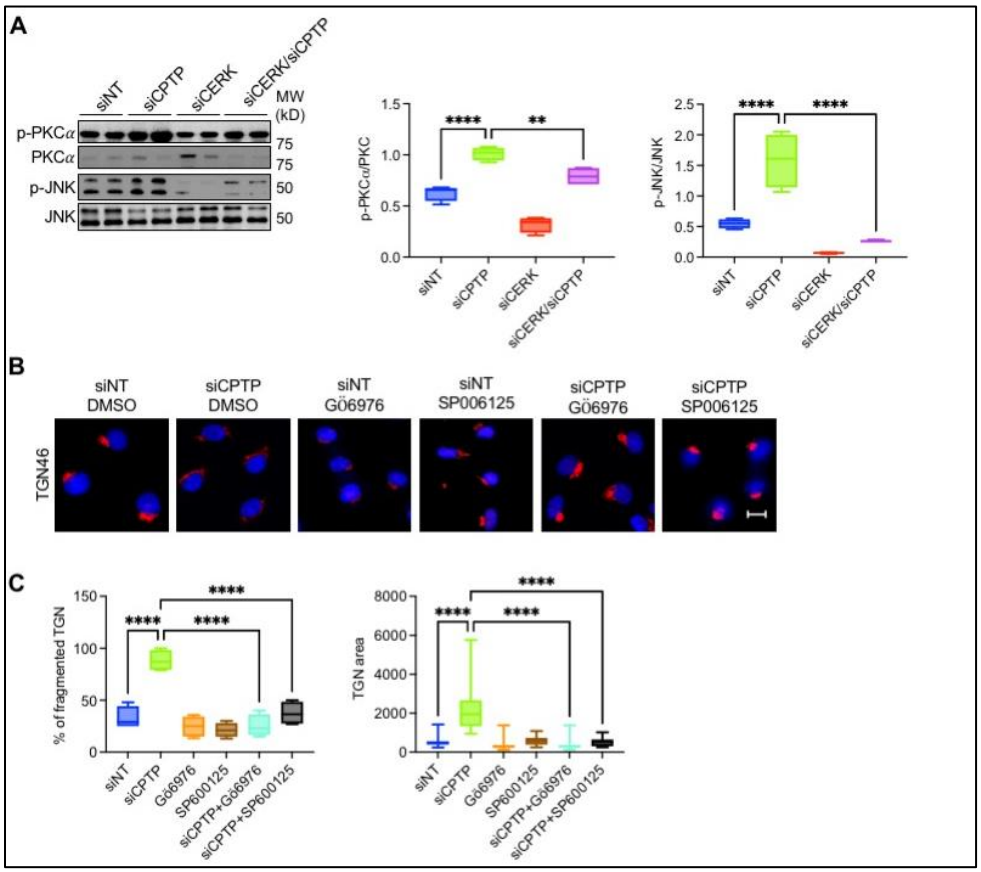

Fig 12: C1P induced PKCα and JNK activation play a key role in C1P induced Golgi fragmentation.

A. Elevating C1P levels induces PKCα and JNK phosphorylation. siNT, siCPTP, siCERK and siCERK/siCPTP treated cells were harvested 72 hours after siRNA transfection and phospho-PKCα (p- PKCα) and phospho-JNK (p-JNK) expression was detected by Western blot analyses. phospho-PKCα (p- PKCα) and phospho-JNK (p-JNK) were normalized to total PKCα and JNK levels by ImageJ densitometry. n=4. The samples were compared by One-way ANOVA followed by Tukey test. B. Inhibiting PKCα and JNK activation blocks C1P induced Golgi fragmentation. HUVECs were transfected with siNT and siCPTP for 72 hours and $3\mu M$ and $25\mu M$ of PKCα(G$\ddot{\mathbf{O}}$6976) and JNK (SP006125) inhibitor respectively for 24 hours. The cells were then fixed on a slide and stained with TGN antibody TGN46 and the nucleus was stained with DAPI. The cells were imaged on Keyence microscope at a magnification of 40X, scale bar $20\mu m$. n=4. Golgi fragmentation was quantified by calculating percentage of cells with fragmented Golgi and measuring TGN area using ImageJ. The samples were compared by One-way ANOVA followed by Tukey test. *P<0.05, **P<0.01; ***P<0.001;****P<0.0001

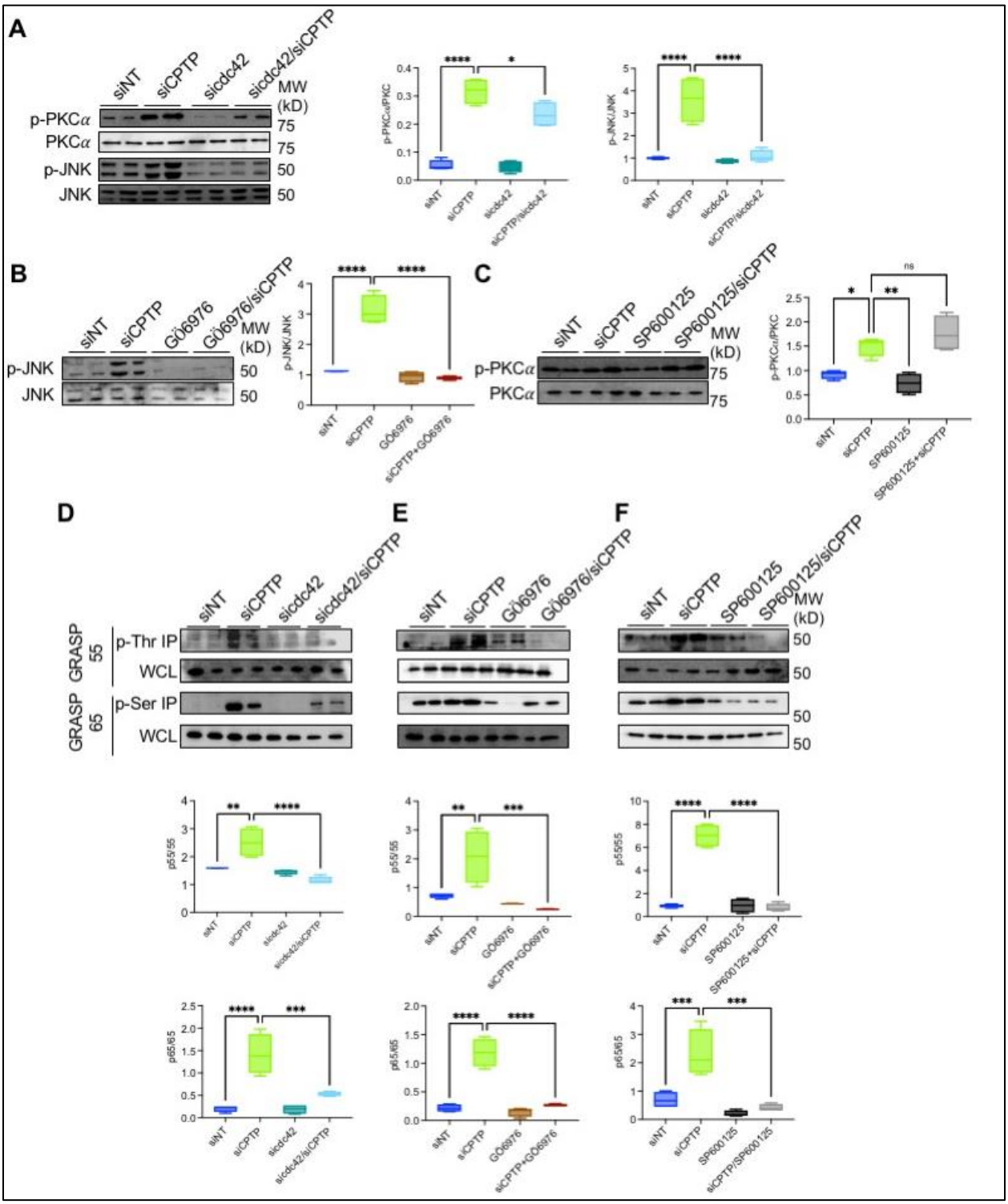

Fig 13: C1P regulates GRASP phosphorylation by PKCα/Cdc42/JNK mediated phosphorylation of GRASP55 and GRASP65 protein.

A. p- PKCα and p-JNK expression in siNT, siCPTP, siCdc42 and siCdc42/siCPTP treated cells were examined by Western blotting. p-PKCα and p-JNK expression were normalized to total level of PKCα and JNK expression respectively using ImageJ densitometry. n=4. The samples were compared using One-way ANOVA followed by Tukey test. p-JNK expression in siNT, siCPTP, G**Ö**6976 and G**Ö**6976/siCPTP treated cells were examined by Western blotting. p-JNK expression was normalized to JNK using ImageJ densitometry. n=4. The samples were compared using One-way ANOVA followed by Tukey test. p- PKCα expression in siNT, siCPTP, SP600125 and SP600125/siCPTP treated cells were examined by Western blotting. . p- PKCα expression was normalized to PKCα using ImageJ densitometry. n=4. The samples were compared using One-way ANOVA followed by Tukey test. D-F. Inhibition of PKC$\boldsymbol{\alpha}$/Cdc42/JNK signaling pathway disrupts C1P induced GRASP55 and GRASP65 phosphorylation. Cell lysate from siNT, siCdc42, siCPTP, G**Ö**6976, SP600125, G**Ö**6976/CPTP and SP600125/siCPTP treated cells were subjected to immunoprecipitation using phospho-serine (p-Ser) antibody and phospho-threonine (p-thr) antibody for GRASP65 and GRASP55 respectively. The pulled down protein was analyzed by Western blot analyses using GRASP65 and GRASP55 antibody. p-GRASP55 (P55) and p-GRAS65 (p65) expression was normalized to GRASP55 and GRASP65 respectively using ImageJ. The samples were compared by performing One-Way ANOVA followed by Tukey test. *P<0.05, **P<0.01; ***P<0.001;****P<0.0001.

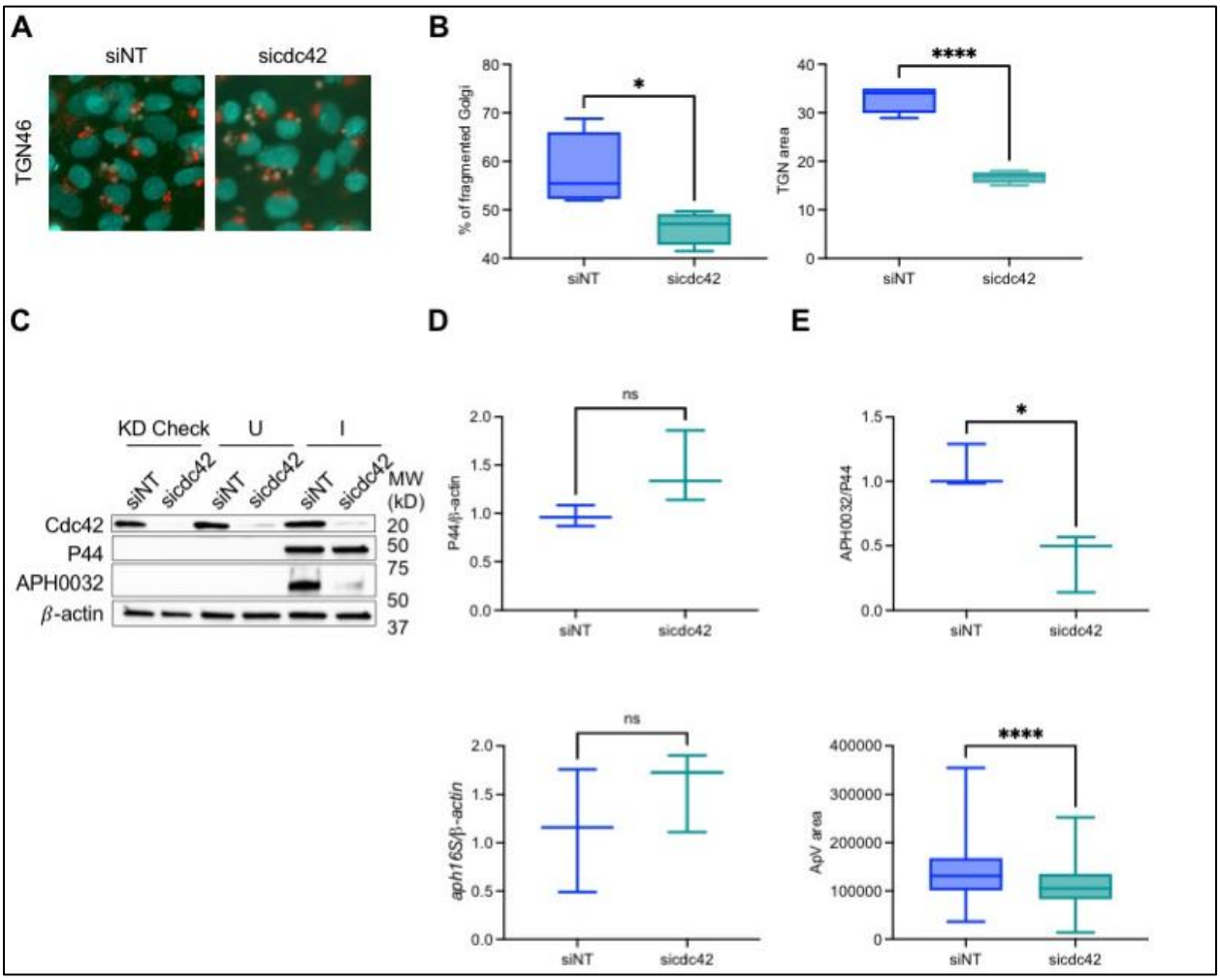

Fig 14: Cdc42 downregulation impairs ApV maturation.

A and B. Cdc42 downregulation blocks *A.phagocytophilum* induced Golgi fragmentation. RF/6A cells were transfected with siNT and siCdc42 and 48 hours post transfection the cells were infected with *A.phagocytophilum* DC organisms for 24 hours. The cells were then fixed on a slide and stained with TGN antibody TGN46 and the nucleus was stained with DAPI. The cells were imaged on the Keyence microscope at a magnification of 40X, scale bar 20μm. n=4. Golgi fragmentation was quantified by calculating percentage of cells with fragmented Golgi and measuring TGN area using ImageJ. The samples were compared by One-way ANOVA followed by Tukey test. C-E. Cdc42 downregulation has no effect on bacterial load but hinder ApV maturation. Western blot analyses of bacterial P44 and APH0032 protein expression. D. Bacterial load was determined by normalizing P44 protein expression to $\beta - actin$ using

ImageJ densitometry and qPCR analyses of aph16S rRNA expression that was normalized to $\beta\,actin$ using the $2^{-\Delta\Delta Ct}$ method. E ApV matuaration was analyzed by normalizing the protein expression of APH0032 to P44 using densitometry and measuring the ApV area using Image J. n=3. The samples were compared by One-way ANOVA followed by Tukey test. P*P<0.05, **P<0.01; ***P<0.001;****P<0.0001

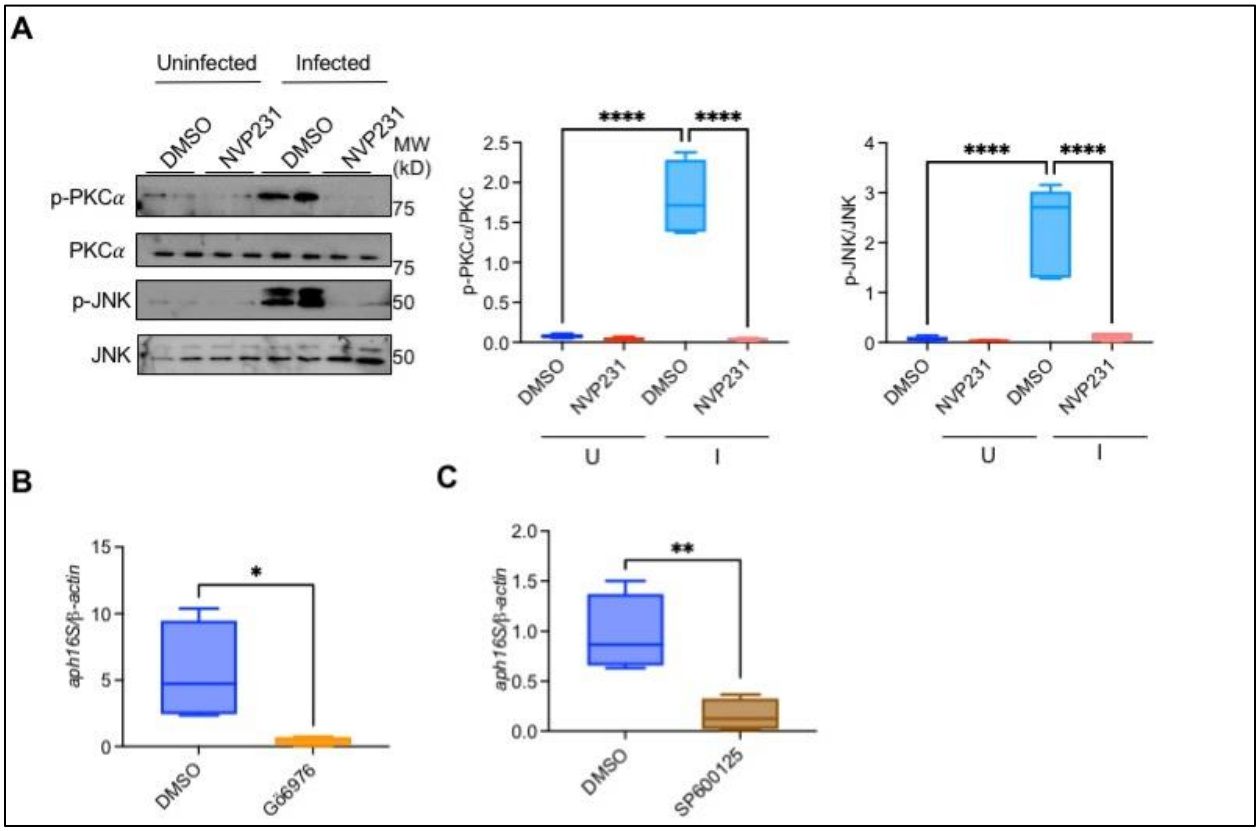

Fig 15: *A.phagocytophilum* infection induces JNK and PKCα activation via a C1P dependent pathway that modulates the bacteria load.

A. *A.phagocytophilum* infection induces PKC$\boldsymbol{\alpha}$ and JNK activation which is blocked by C1P downregulation. RF/6A cells were pretreated with 0,001% DMSO or 400nm NVP231 for one hour and then infected with *A.phagocytophilum* DC organisms. 24hours post infections, P-PKC$\boldsymbol{\alpha}$ and P-JNK expression was analyzed by Western blot analyses. P-PKC$\boldsymbol{\alpha}$ and P-JNK expression were normalized to PKC$\boldsymbol{\alpha}$ and JNK respectively by ImageJ densitometry. n=4. The samples were

compared by One-way ANOVA followed by Tukey test. . B and C. Inhibiting PKCα and JNK activation reduces *A.phagocytophilum* load. Cells were pretreated with 3μM and 25μM of PKCα(G$\mathbf{\ddot{O}}$6976) and JNK (SP006125) inhibitor respectively for 1 hour and then infected with *A.phagocytophilum* DC organisms . Bacterial load was determined by qPCR analyses aph16S rRNA expression that was normalized to β-*actin* using the $2^{-\Delta\Delta Ct}$ method. n=4. The samples were compared by One-Way ANOVA followed by Tukey test. *P<0.05, **P<0.01; ***P<0.001;****P<0.0001.

Chapter 4: Final Discussion

This study demonstrates that CERK derived C1P promotes the generation of *A.phagocytophilum* infectious progeny, thereby promoting bacterial infection and also regulates ApV maturation. This study further delineates that C1P is a crucial regulator of Golgi morphology and modulates Golgi fragmentation via Cdc42/ PKCα/JNK mediated signaling pathway. Indeed C1P induces Golgi fragmentation in *A.phagocytophilum* infected cells to promote the trafficking of sphingolipid rich TGN vesicles to the ApV lumen, subsequently satisfying the nutritional virulence of the pathogen. Intracellular bacteria are known to parasitize host cell derived sphingolipids to promote their colonization in host cell (79). Nevertheless, the implication of C1P in bacterial pathogenesis is not known. Here we show that *A.phagocytophilum* infected cells have elevated C1P levels compared to non-infected cells. Decreasing C1P levels by NVP231 mediated CERK inhibition arrests *A.phagocytophilum* induced Golgi fragmentation and represses *A.phagocytophilum* infection by perturbing the transition from non-infectious RC form to infectious DC from. This was also recapitulated in-vivo as *A.phagocytophilum* failed to infect CERK -/- mouse. Additionally increasing C1P by CPTP inhibition in *A.phagocytophilum* infected cells further augments *A.phagcytophlium* induced fragmentation and also facilitates ApV maturation.

Previously, our lab has shown that C1P is crucial for Golgi morphology, here we explain the mechanism by which C1P regulates Golgi morphology. In concordant to previous reports, our data demonstrates that increasing C1P by CPTP inhibition induces Golgi fragmentation which is blocked by CERK inhibition. As previous reports have demonstrated that Golgi fragmentation is triggered by inhibition of Golgi-ER retrograde vesicle trafficking, we examined the effected of C1P on Golgi-ER retrograde of COPI vesicle (117). However, our results are incoherent with the previous study as C1P induced Golgi fragmentation is observed even at

37℃, when COPI retrograde trafficking is not disrupted. Nonetheless C1P blocks the trafficking of COPI vesicle from the Golgi to the ER in CPTP downregulates cells under heat stress which is reversed by CERK downregulation. However, as C1P induced Golgi fragmentation is observed at 32℃ and 37℃ we conclude that C1P induced Golgi fragmentation is independent of C1P's effect on Golgi-ER retrograde vesicle trafficking. UV radiation resistance associated gene protein (UVRAG) is an important regulator of Golgi-ER retrograde trafficking and the C2 domain of UVRAG has C1P binding consensus sequence (118, 119). Therefore we predict that C1P could be regulating Golgi-ER retrograde trafficking by sequestration of UVRAG to the Golgi membrane. However, this area requires further research.

Furthermore, our study elucidates that C1P regulates Golgi morphology by triggering the activation of PKCα/Cdc42/JNK signaling pathway that leads to GRASP phosphorylation and subsequent Golgi fragmentation. Increasing C1P stimulates the phosphorylation of GRASP55 and GRASP65 which in turn causes Golgi fragmentation and consequently promote cell proliferation. Additionally, elevated C1P levels fail to induce Golgi fragmentation in cells overexpressing phospho-resistant GRASP55 mutant. C1P induces translocation of Cdc42 from cytoplasmic to the Golgi membranes where it is found in its GTP-bound form and abating Cdc42 perturbs C1P stimulated Golgi fragmentation. Additionally, C1P induces activation of PKCα and JNK and concomitantly, inhibiting them blocks C1P induced Golgi fragmentation. Indeed, increasing C1P triggers a signaling cascade that activates PKCα, followed by Cdc42 and JNK activation, which in turn facilitates GRASP phosphorylation subsequent Golgi fragmentation.

C1P induced PKCα, Cdc42 and JNK activation also plays an important role *A.phagocytophilum* pathogenesis. Inhibiting Cdc42 arrested *A.phagocytophilum* induced Golgi fragmentation in RF/6A cells only by 12% compared to more than 50% reduction in Golgi fragmentation in HUVECs. Additionally Cdc42 inhibition has no effect bacterial load but disrupts ApV maturation. Therefore, although Cdc42 as an effect on ApV maturation but C1P induces Golgi fragmentation in *A.phagocytophilum* infected cells by a Cdc42 independent signaling

pathway. PKCα and JNK are activated in *A.phagocytophilum* infection and NVP231 mediated CERK downregulation represses their activation in infected cells and inhibiting PKCα and JNK reduces *A.phagocytophilum* infection; hence C1P induced PKCα and JNK activation is crucial for *A.phagocytophilum* growth.

Comprehensively, this study shows that CERK derived C1P is crucial for *A.phagocytophilum* pathogenesis. Furthermore, this study discerns that C1P is an upstream activator of PKCα, Cdc42 and JNK and illustrates the mechanism which C1P induces Golgi fragmentation. Our study paves the way for further research in exploring the role of C1P in bacterial pathogenesis and evaluating it as a potential target to develop non-antibiotic based therapeutics for multi-drug resistant bacterial infections. Since Golgi fragmentation is a hallmark of a plethora of neurological disorders such as Alzheimer's, ALS and Huntington disease, therefore this study also paves the foundation of exploring the implication of C1P in such disease pathologies.

4.1 Limitations of this study and future directions

Our study couldn't identify the underlying reason of how *A.phagocytophilum* infection increases CERK derived C1P production in host cells. We predict that *A.phagocytophilum* infection stimulates ASMase activation which increases ceramide levels in the cell, which subsequently gets phosphorylated by CERK to generate C1P. Hence to test this hypothesis , we would first examine if *A.phagocytophilum* induces activation of ASMase to generate ceramide. Previous studies have suggested that bacterial invasion and bacterial toxins triggers the activation of ASMase, which in turn catalyzed the conversion of sphingomyelin into ceramide (80, 120). Interestingly, Carlyon lab has reported that inhibiting ASMase arrests *A.phagocytophilum* infection and disrupts the generation of infectious DC progeny (27) . Therefore, using western blotting analyses we would examine the effect of *A.phagocytophilum* infection on ASMase

activation. Next, to determine the effect of ASMase on C1P levels, we would inhibit ASMase in infected cells using pharmacological inhibitor Desipramine and examine C1P levels by lipidomic analyses. Furthermore we could not determine how disrupting GRASP phosphorylation would effect *A.phagocytophilum* bacterial load. Due to the low transfection efficiency of RF/6A cells we could not transfect these cells with phospho-resistant GRASP55(T225A,T249A) mutant and examine its effect on bacterial growth and ApV maturation.

Additionally it was not clear from our studies if PKCα is upstream of Cdc42 in C1P mediated GRASP phosphorylation signaling pathway. To examine the role of PKCα in C1P mediated Cdc42 translocation and subsequent activation, we would co-treat the cells with siCPTP and PKCα inhibitor , G**Ö**6976 and using subcellular fractionation assess its effect on translocation of Cdc42 to the cellular membranes. Furthermore it is still not clear how C1P stimulates PKCα activation. Like cPLA$_2\alpha$, PKCα is activated by increased levels of Ca^{2+} and has a lipid binding C2 domain. Therefore, using surface plasmon resonance assay (SPR) it can determined if C1P interacts to PKCα to induces its activation .

Furthermore the effect of C1P stimulated translocation of Cdc42 to the cellular membranes on Golgi-ER retrograde trafficking of COPI vesicle remains unknown. Since membrane localized Cdc42 is known to inhibit the trafficking of COPI vesicles from the Golgi to the ER, we would determine the involvement of this on C1P mediated disruption of Golgi-ER retrograde trafficking (93). To investigate this we would co-transfect the cells with siCPTP and siCdc42 (siCdc42/C1P) and examine if Cdc42 downregulation can arrest the trafficking of GFP-VSVG containing COPI vesicles from the ER to the Golgi.

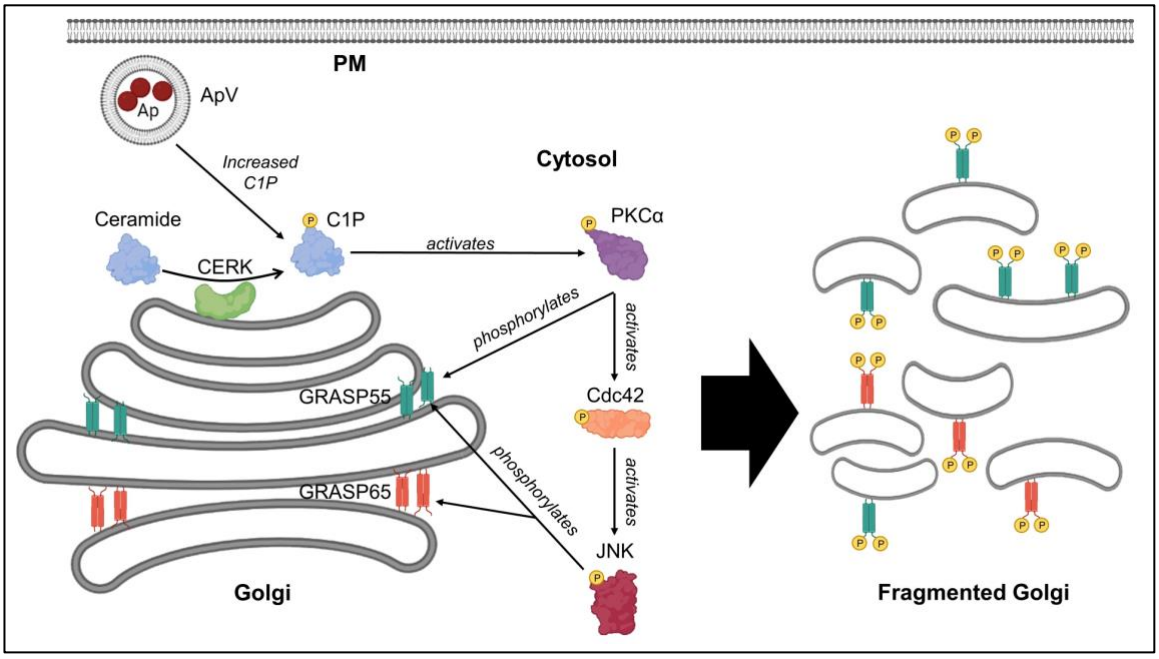

Fig 16: Schematic representation of C1P regulated PKCα/Cdc42/JNK signaling pathway that results in GRASPs phosphorylation and subsequent Golgi fragmentation.

A.phagocytophilum induces CERK derived C1P formation in host cell. Elevated C1P levels induces activation of PKCα which in turns causes translocation of Cdc42 from the cytoplasmic fraction to the cellular membrane and subsequently induces Cdc42 activation. Membrane bound Cdc42 disrupts retrograde trafficking of COPI vesicles from the ER to the Golgi and also induces JNK activation. PKCα and JNK activation stimulates GRASP55 and GRASP65 phosphorylation, thereby triggering Golgi fragmentation. The image was created on biorenders.com.

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Appendix 1: IACUC approval letter

UNIVERSITY OF SOUTH FLORIDA
USF RESEARCH & INNOVATION

RESEARCH INTEGRITY & COMPLIANCE
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

MEMORANDUM

TO:	Charles Chalfant,
FROM:	Farah Moulvi, MSPH, IACUC Coordinator Institutional Animal Care & Use Committee Research Integrity & Compliance
DATE:	9/1/2020
PROJECT TITLE:	The roles of CERK and CPTP in A. phagocytophilum pathogenesis The roles of ceramide and its derivatives in A. phagocytophilum pathogenesis
FUNDING SOURCE:	Federal government or major agency that awards grants based on peer-reviewed proposals (NIH, NSF, DOD, AHA, ACS, etc.) NIH National Institutes of Health; NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
IACUC PROTOCOL #:	R IS00008220
PROTOCOL STATUS:	**APPROVED**

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC **APPROVED** your request to use the following animals in your **protocol for a one-year period beginning 9/1/2020:**

Mouse: C57BL/6-CERK -/- (Male, Female, 8-12 weeks)	292
Mouse: C57Bl/6- Control (Male, female, 8-12 weeks)	292
Mouse: C57/Bl6 CPTP -/- (Male, Female 8-12 weeks)	292
Mouse: C57/Blk6 CPTPtran (Male, Female, 8-12 weeks)	292

Please take note of the following:

• **IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system.** After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

• **All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification.** Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application**.**

• **All costs invoiced to a grant account must be allocable to the purpose of the grant.** Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons