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Regulation of lipid signaling at the Golgi by the lipid phosphatases hSAC1 and OCRL1

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Summary

Phosphoinositides are key lipid signaling molecules present in membranes of all eukaryotes. Different species of phosphoinositides serve as membrane signposts at distinct cellular compartments. This asymmetric distribution of phosphoinositides is achieved by the presence of an elaborate set of lipid kinases and phosphatases operating at specific organelle membranes. The lipid phosphatase SAC1 is found in both endoplasmic reticulum (ER) and Golgi. Similar to yeast Sac1p, human SAC1 (hSAC1) is the major phosphatidylinositol-4-phosphatase (PI(4)P-phosphatase). Distinct localization of hSAC1 in both ER and Golgi membranes suggests that this PI(4)P-phosphatase has compartment specific roles in regulating steady state distribution of PI(4)P in these organelles. OCRL1 is a Golgi and endosomal localized PI(4,5)P₂ 5-phosphatase that is implicated in a severe X-linked disease, Lowe syndrome, which is characterized by congenital cataracts, Fanconi syndrome and mental retardation. How mutations in OCRL1 cause Lowe syndrome is unknown. The functional analysis of hSAC1 and OCRL1 in regulating Golgi PI(4)P and PI(4,5)P₂ is the main focus of this work.

Confocal immunofluorescence and immuno-electron microscopy (immuno-EM) show that PI(4)P and hSAC1 form an opposing gradient in the Golgi. hSAC1 is highly enriched at Golgi cisternal membranes while PI(4)P is concentrated at the *trans*-Golgi network (TGN) where cargo proteins are packaged and exported. Golgi enzymes such as N-acetylglucosaminyltransferase I (NacT1) and mannosidase II (ManII) are preferentially found in these PI(4)P-depleted areas. siRNA-mediated knock-down of hSAC1 leads to accumulation of PI(4)P at Golgi, plasma membrane and endosomal like structures and causes mislocalization of ManII and NacT1. This data suggests that SAC1 establishes PI(4)P-depleted Golgi regions that are important for proper localization and recycling of Golgi resident enzymes. Conversely, depletion of OCRL1 does not disturb Golgi morphology or induce mislocalization of Golgi resident enzymes. However, bulk secretion is inhibited in OCRL1 depleted cells. The OCRL1-b splice variant populates TGN and early endosomal compartment whereas the OCRL1-a splice variant containing an extra 8 amino acid acidic cluster is found only in a subset of late endosomal/lysosomal membranes. This distinct localization of OCRL1 splice variants indicates that each isoform might regulate different trafficking routes by regulating PI(4,5)P₂ levels at these compartments.

Together, the results show that hSAC1 and OCRL1 establish distinct phosphoinositide-specific domains within the Golgi that are instrumental for segregation of anterograde trafficking from the recycling of resident Golgi enzymes.

Zusammenfassung

Phosphoinositide sind Lipidsignale mit Schlüsselfunktion, die in den Membranen aller Eukaryoten vorkommen. Die verschiedenen Klassen von Phosphoinositiden dienen als spezifische Wegweiser an den Membranen zellulärer Kompartimente. Die asymmetrische Verteilung der Phosphoinositide wird durch ein komplexes Netzwerk von Lipidkinasen und -phosphatasen gewährleistet, die jeweils spezifisch an den Organellmembranen arbeiten. Die Lipidphosphatase Sac1 befindet sich sowohl im Endoplasmatischen Retikulum (ER) als auch im Golgi-Apparat. Konserviert von der Hefe zum Mensch, ist Sac1 die wichtigste Phosphatidylinositol 4-Phosphatase (PI(4)P-Phosphatase). Die Lokalisation von hSAC1 an ER- und Golgimembranen legt nahe, dass dieses Enzym kompartimentspezifische Rollen in der Regulierung der Verteilung von PI(4)P in diesen Organellen hat. OCRL1 ist eine PI(4,5)P₂ 5-Phosphatase am Golgi und in Endosomen. Mutationen in OCRL1 verursachen das Lowe Syndrom, eine schwere X-chromosomal vererbte Krankheit. Das Lowe-Syndrom zeichnet sich durch congenitale Katarakte, schwere Nierenschäden und geistige Behinderung aus. Wie Mutationen in OCRL1 das Lowe-Syndrom verursachen, ist unbekannt. Die funktionelle Analyse der Regulierung von PI(4)P und PI(4,5)P₂ am Golgi durch hSAC1 und OCRL1 ist das Thema dieser Arbeit.

Konfocale Immunfluoreszenz und Immuno-Elektronenmikroskopie zeigen, dass PI(4)P und hSAC1 entgegengesetzte Gradienten im Golgi bilden. hSAC1 ist in den Membranen der Golgizisternen angereichert, während PI(4)P im Trans-Golgi-Netzwerk (TGN) konzentriert ist. Im TGN werden sekretorische Proteine auf dem Transportweg verpackt und exportiert, Golgienzyme wie N-Acetylglucosaminyltransferase I (NacT1) und Mannosidase II (ManII) befinden sich bevorzugt in Golgiregionen mit geringem PI(4)P Gehalt. Depletierung von hSAC1 mittels siRNA führte zur Akkumulation von PI(4)P im Golgi, an der Plasmamembran und in endosmalen Strukturen und führte zur Mislokalisierung von ManII und NacT1. Dieses Ergebnis legt nahe, dass SAC1 am Golgi Regionen mit geringem PI(4)P-Gehalt herstellt, welche für die korrekte Lokalisation und das Recycling Golgi-residenter Enzyme wichtig sind. Depletierung von OCRL1 mittels RNAi verändert jedoch weder die Morphologie des Golgi, noch führt sie zur Mislokalisierung Golgi-residenter Enzyme. Allerdings hemmt die Entfernung von OCRL1 die zelluläre Proteinsekretion. OCRL1 kommt in zwei Spleissvarianten vor: OCRL1-a und OCRL1-b. Die OCRL1-b Variante kommt im TGN und in frühen Endosomen vor, wohingegen die Variante OCRL1-a, welche zusätzlich ein Sequenzmotif aus 8 hauptsächlich sauren Aminosäuren enthält, nur in einer bestimmten Population von späten Endosomen/ Lysosomen vor. Die spezifische Lokalisation von OCRL1 könnte bedeuten, dass die Isoformen jeweils spezifische Transportwege regulieren, indem sie die PI(4,5)P₂ Level in ihren Kompartimenten anpassen.

Zusammengenommen zeigen diese Resultate, dass hSAC1 und OCRL1 spezifische Phosphoinositidomänen im Golgi herstellen, die für die Trennung von Recycling von Golgi-residenten Proteinen und anterogradem Transport von sekretorischen Proteinen unerlässlich sind.

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1 Abbreviations

α	anti/ alpha
Amp	ampicilin
APS	amonium persulfate
aa	amino acid
ATP	adenosinetriphosphate
bp	base pair
CFP	cyan fluorescent protein
DAG	diacylglycerol
Da	Dalton
ddH ₂ O	distilled water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylendiaminetetracetatic acid
ER	endoplasmic reticulum
g	gram
GalT	galactosyltransferase
GFP	green fluorescent protein
hrs	hours
HEPES	4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
kb	kilobase
kDa	kilo Dalton
L	liter
LB	Luria Betrani broth
M	molar
mM	milimolar
mA	miliampere
mg	miligram
μ Ci	microcurie
μ l	microliter
μ g	microgram
μ M	micromolar
min	minute
ml	mililiter
nM	nanomolar
nm	nanometer
OD	optical density
ON	overnight
ONC	overnight culture
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PM	plasma membrane

pmol	picomol
PMSF	phenylmethylsulphonylfluoride
PtdIns	phosphatidylinositol
PI	phosphoinositides
PI(4)P	phosphatidylinositol-4-phosphate
PtdIns(3)P	Phosphatidylinositol(3)phosphate
PtdIns(4)P	Phosphatidylinositol(4)phosphate
PtdIns(5)P	Phosphatidylinositol(5)phosphate
PtdIns(3,4)P2	Phosphatidylinositol(3,4)bisphosphate
PtdIns(4,5)P2	Phosphatidylinositol(4,5)bisphosphate
PtdIns(3,5)P2	Phosphatidylinositol(3,5)bisphosphate
PtdIns(3,4,5)P3	Phosphatidylinositol(3,4,5)trisphosphate
RFP	red fluorescent protein
rpm	revolutions per minute
RT	room temperature
s	second
SAC	suppressor of actin mutants
SDS	sodium dodecyl sulfate
TAE	tris/acetate/EDTA
TCA	2,2,2-trichloroacetic acid
TEMED	N,N,N',N'-tetramethyldiamine
TGN	trans-Golgi-network
Tris	tris(hydroxymethyl)-aminomethane
Tryp	trypsin
TX-100	triton-X-100
Unit	unit of enzyme activity.
V	volt
WT	wildtype
YFP	yellow fluorescent protein

2 Introduction

2.1: Biological roles of phosphoinositides and their modifying enzymes

Eukaryotic cells possess an elaborate system of membrane enclosed compartments that are characterized by distinct sets of proteins and lipids. Each cellular compartment must maintain its unique function, molecular composition, and luminal environment. To achieve this diversity of structure and purpose, the membranes of these organelles must be kept separate for the most part, else they might rapidly fuse and become homogenous. These membrane compartments are, however, interconnected via exchange of materials using different transport vesicles or tubular-vesicular carriers (Bonifacino and Glick 2004). To achieve successful and specific inter-organelle material exchange, recognition of target membranes by transport vesicles is vital. Different classes of proteins have been found as main players in the formation of these transport vesicles/carriers, but advances in the field of membrane trafficking have recognized the active participation of membrane lipids in all transport events. In fact, lipid bilayers, the building blocks of all biological membranes contain embedded signals that are composed of different classes of phospholipids. One such important class of lipid-based signals are the phosphoinositides (PIs) (Toker 2002)

2.1.1: Phosphoinositides: lipid signaling in eukaryotic membranes

Phosphoinositides are phosphorylated derivatives of phosphatidylinositol (PtdIns) found throughout the eukaryotic kingdom. They are composed of a *myo*-inositol sugar moiety attached to diacylglycerol (DAG) via a phosphodiester bond. The unique property of these phospholipids is contributed by the inositol headgroup that can be reversibly phosphorylated at different positions (3, 4, 5-OH). The interplay between the kinases and phosphatases generates seven different species of phosphoinositides (Tolias and Cantley 1999; see Figure 2.0).

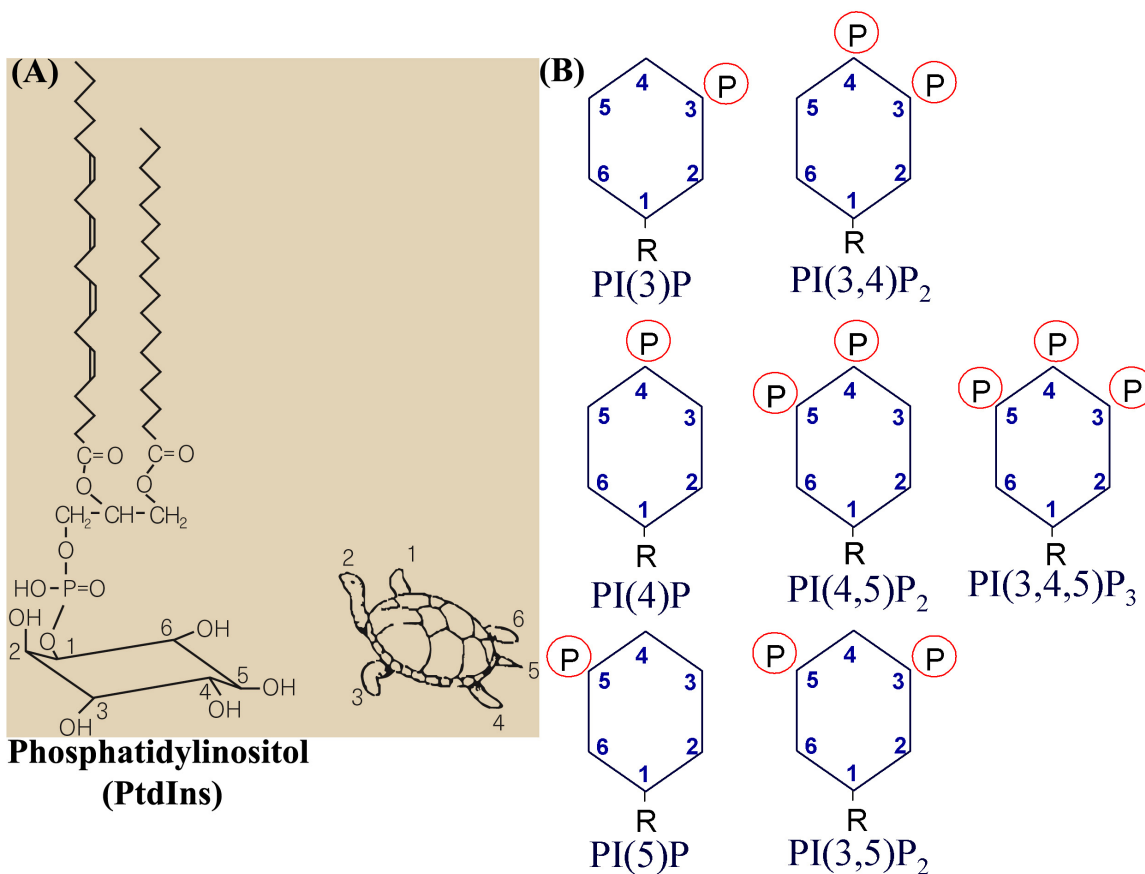


Figure 2.0:(A) Structure of phosphatidylinositol (PtdIns) and its phosphorylated derivatives, phosphoinositides (B)

These inositol-containing glycerophospholipids are among the most versatile of regulatory molecules, with strikingly diverse roles in cell signaling, membrane transport, actin cytoskeleton rearrangement, cell development, cell survival, and transcriptional regulation (Toker 2002). Each phosphoinositide species possesses unique functions at different cellular compartments. They mark and contribute to membrane identity of distinct cellular organelles (Cockcroft and De Matteis 2001; De Matteis and Godi 2004). For example, phosphatidylinositol-3-phosphate (PI(3)P) populates the endosomal systems (at the early endosomes (EE)), phagosomes (Ph) and the internal vesicles of multivesicular bodies (MVB) (Figure 2.1), while the phosphatidylinositol-3,5-bisphosphate (PI(3,5)P₂) is created at the MVBs or late endosomal compartment by the presence of the PIK-FYVE kinase. On the other hand, phosphatidylinositol-4-phosphate (PI(4)P) is enriched at the Golgi apparatus while phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) is found predominantly at the plasma membrane. Upon agonist mediated cell

stimulation, phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P₃) is transiently produced by the PI 3-kinases from PI(4,5)P₂ at the D-3 position of the *myo*-inositol ring. Figure 2.1 shows the intracellular “phosphoinositides map”. Phosphoinositides (for example PI(4,5)P₂, PI(4)P and inositol polyphosphates) are also present in the nucleus. However, the functions of individual nuclear phosphoinositides are unclear. They have been implicated in regulation of gene expression, mRNA export, DNA repair and telomerase maintenance (Gonzales and Anderson 2006; York 2006).

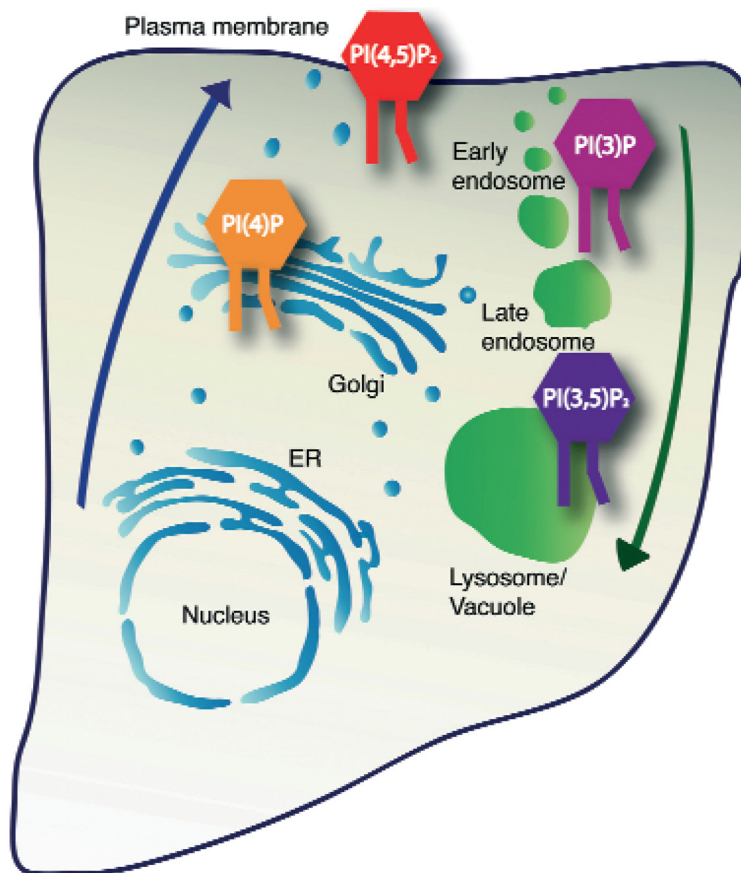


Figure 2.1: Spatially restricted pools of phosphoinositides at different cellular organelles. (Adapted from Mayinger P., 2006)

PI(4)P is highly enriched at the Golgi, PI(4,5)P₂ at the plasma membrane and the 3-phosphorylated species of phosphoinositides are located at the endosomal systems.

2.1.2: Phosphoinositide interacting factors

Spatially restricted generation of PIs at different organellar membranes serve as an ideal platform for the recruitment of distinct signaling complexes (Hurley and Meyer 2001). Phosphoinositides orchestrate diverse signaling and cellular events by recruiting diverse classes of proteins, containing specific phosphoinositide binding modules, to execute their functions (Lemmon 2003).

The pleckstrin homology domain (PH-domain) was originally identified in 1993 (Haslam, Koide et al. 1993; Mayer, Ren et al. 1993) as a stretch of 100-120 amino acids appearing twice in the platelet protein pleckstrin (Tyers, Rachubinski et al. 1988; Tyers, Haslam et al. 1989). PH-domain was initially found to interact with PI(3,4,5)P₃ and PI(3,4)P₂ in a group of proteins that are involved in PI 3-kinase-mediated signaling events. Protein kinase B/Akt, 3-phosphoinositide-dependent protein kinase 1 (PKD1) and the Bruton's tyrosine kinase (BTK) are examples of PH-domain containing proteins that bind to 3-phosphorylated inositol lipids. In subsequent studies, many PH-domains were found to bind a broad class of PIs and other membrane determinants such as the Arf GTPases. The abundance of PH-domains in the human genome (approximately 250 PH domains in human proteome) suggests that they are important lipid interacting domains with diverse functions depending on their binding specificities to different phosphoinositides and other membrane determinants (Yu, Mendrola et al. 2004).

The PH-domain of PLC- δ is targeted to the plasma membrane specifically via interaction with PI(4,5)P₂. A GFP-fusion protein of PLC- δ 's PH domain can detect only the plasma membrane pool of PI(4,5)P₂ but not PI(4,5)P₂ at the Golgi (Varnai and Balla 1998). The PH-domain of FAPP1 (phosphatidylinositol-four-phosphate adaptor protein 1) was found to bind PI(4)P in earlier studies (Dowler, Currie et al. 2000). However, it has been shown recently by Godi A. et al. that the Fapp1-PH domain binds to activated Arf1 in addition to PI(4)P at the *trans*-Golgi network (TGN) (Godi, Di Campli et al. 2004). Both membrane determinants (PI(4)P and GTP-bound Arf1) are crucial for proper targeting of Fapp1 to TGN. This is an example of a dual-key mechanism for protein recruitment to specific membrane compartments (Itoh and De Camilli 2004). Other PI(4)P-binding proteins found till date are adaptor protein 1 (AP1) (Wang, Wang et al. 2003), epsinR, oxysterol binding protein (OSBP) (Levine and Munro 1998), ceramide

transfer protein (CERT) (Kumagai, Yasuda et al. 2005) and Fapp2 (Godi, Di Campli et al. 2004). Even though these proteins are all targeted to the TGN via interaction with PI(4)P, with or without GTP-bound Arf1, they operate at distinct transport steps from the TGN. Fapp2, OSBP and CERT share two interesting features: N-terminal PH-domains that bind to PI(4)P with high affinity and C-terminal lipid-transfer domains with distinctive lipid specificities. The lipid-transfer domain of OSBP binds to oxysterol/cholesterol, CERT binds ceramide, and FAPP2 binds to glycolipids. These recent findings open new avenues in research focusing on non-vesicular lipid transport between organellar membranes (Maxfield and Mondal 2006).

Other phosphoinositide binding modules that operate at different organelles such as the endosomes and the plasma membrane include FYVE (Fab1p, YOTB, Vac1p and EEA1) (Burd and Emr 1998; Kutateladze, Ogburn et al. 1999) and PX (phox homology) domains (Simonsen and Stenmark 2001; Xu, Seet et al. 2001; Seet and Hong 2006). PI(3)P is generated at the endosomal membranes by the class III PI3-kinases, Vps34 (Herman and Emr 1990). PI(3)P is found at the membrane of the early endosomes, internal vesicles of multivesicular endosomes in mammals, and vacuolar endosomes in yeasts. Most FYVE and PX domains bind to PI(3)P specifically. The FYVE domain was first identified in four proteins: Fab1p, YOTB, Vac1p and EEA1. It contains 60-70 amino acids and folds into a zinc finger-like structure. FYVE domain binds to PI(3)P-containing membranes with very high affinity and requires membrane insertion. EEA1, Hrs, Rabenosyn-5 and other proteins operating at the endosomal systems contain the membrane targeting FYVE domain.

The PX domain was discovered in 1996 and is named after the phagocyte oxidase that provides a defense against microorganisms (Ponting 1996). PX domain is composed of 120 amino acids. The most common ligand for this module is PI(3)P, although some PX domains bind to PI(3,4)P₂ and PI(4,5)P₂ (Xu, Seet et al. 2001). PX domain of Vam7 was the first to be ascribed a specific recognition of PI(3)P (Cheever, Sato et al. 2001). This protein-lipid interaction is attributed to a pair of highly conserved basic motifs, RR ϕ (Arg-Arg-Tyr/Phe residues). Other PX domain containing proteins that are recruited to endosomal membranes include the sorting proteins nexin 3 and Vps5.

The Epsin N-terminal Homology (ENTH) domain was originally identified as an NH₂-terminal sequence of epsin 1 and several other proteins that had known or putative roles in endocytosis (Rosenthal, Chen et al. 1999) (Kay, Yamabhai et al. 1999). The ENTH domains were known to bind to PI(4,5)P₂ with high specificity. They are present in Epsin, AP180 and CALM (Clathrin Assembly Lymphoid Myeloid leukemia) proteins. These ENTH domains undergrid the invaginating plasma membrane through PI(4,5)P₂ interactions, and the clathrin coat connects to this scaffold. Recent structural data shows that proteins related to AP180 and CALM have an N-terminal domain with a distinct fold. This domain is named the ANTH domain (AP180 N-terminal Homology domain). Both domains bind PI(4,5)P₂ via different binding modes (Ford, Mills et al. 2002) (Ford, Pearse et al. 2001) Proteins that contain ENTH/ANTH domains are found to contain other recognition motifs for other types of protein-protein interaction modules. This is particularly important for assembly of protein complexes on membranes.

2.1.3: Organelle identity and membrane signposts defined by phosphoinositides

Distinct sets of enzymatic activities at different intracellular locations define the “functional identity” of each intracellular organelle. Recent progress in studying mechanisms of intracellular transport systems has defined a new concept explaining organelle membrane identity based on the presence of specific peripheral membrane proteins (membrane traffic machinery), activated forms of GTPases (including the GTP-exchange factors (GEF) and GTPase-activating proteins (GAP)), and lipids (Munro 2002; Munro 2004; Behnia and Munro 2005).

Although phosphoinositides are minor constituents of membrane lipids (typically 10% of the total membrane lipids in cellular membranes), phosphoinositides are a particularly important class of lipids that contribute to organelle identity. They are short-lived lipid signposts that direct effector proteins to specific membrane sites where cellular functions are carried out. Organelle identity is crucial for accurate recognition of transport vesicles traveling from one cellular compartment to another.

As illustrated in Figure 2.1, the membrane signposts expressed by individual cellular compartments are critical for a functional secretory pathway. The endoplasmic reticulum (ER) is the site of synthesis for phosphatidylinositol (the precursor of other

phosphoinositides) and there are no known or observed phosphoinositide species that accumulate at this compartment. However, all the other cellular compartments throughout the secretory pathway contain specific sets of phosphoinositides functioning in recognition of transport machineries that carry cargos to their final destinations. Figure 2.2 shows examples of phosphoinositide binding module (PIBM)-containing proteins (Overduin, Cheever et al. 2001).

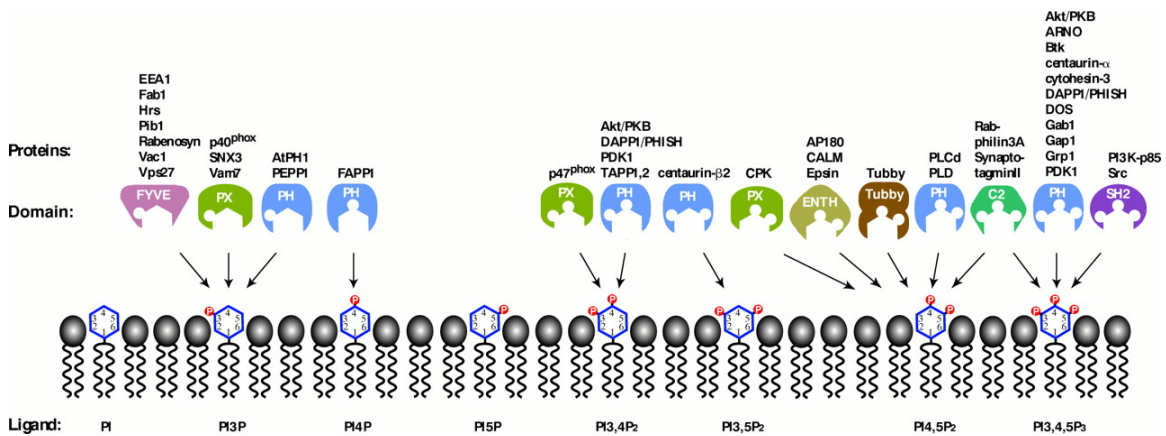


Figure 2.2: Phosphoinositide binding modules in effector proteins

Signaling domains that have been demonstrated to bind phosphoinositides are shown as colored shapes with schematic binding site. Arrows indicate the preferred ligand of the given signaling domain, and proteins that contain the given signaling domain are listed in corresponding colored shape. (Adapted from Overduin et al., 2001)

The importance of organelle membrane identity is further demonstrated by subversion of “original” membrane identity by various pathogenic organisms (Nguyen and Pieters 2005) such as *Salmonella*, *Listeria*, *Mycobacterim tuberculosis* and *Shigella*. These pathogens use their own secretion systems to transfer virulence factors into host cells for perturbations of membrane identity. Membrane transport and fusion events are particularly affected in this matter since proper trafficking and fusion reactions require recognitions of the right donor and acceptor membrane compartments.

2.1.4: Compartmentalized distribution of phosphoinositides generated by phosphoinositide kinases and phosphatases

To achieve differential distribution of phosphoinositides at intracellular membranes, every organelle is equipped with an array of phosphoinositide kinases and phosphatases (De Matteis and Godi 2004) as shown in Figure 2.3

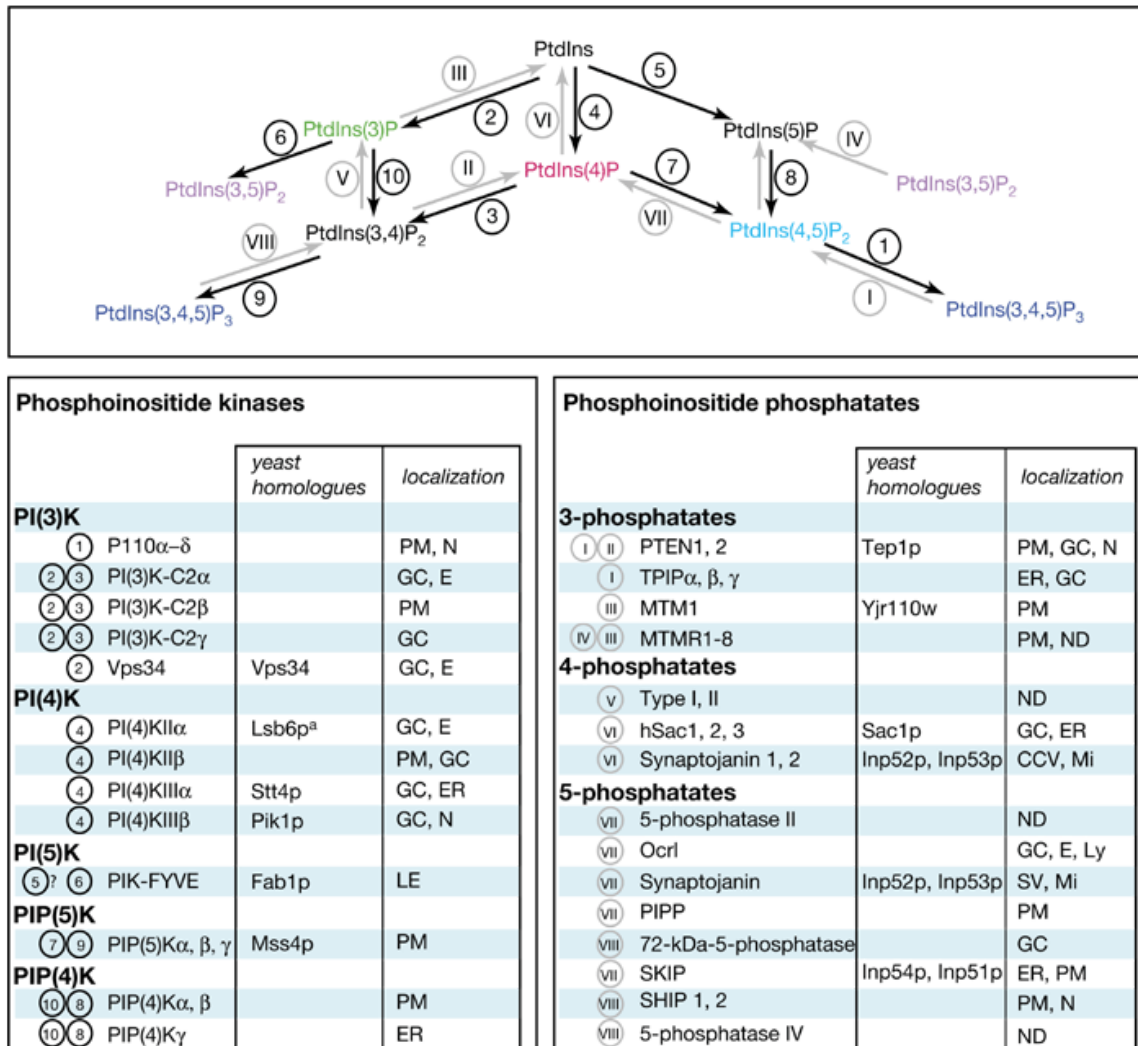


Figure 2.3: Phosphoinositide kinases and phosphatases and their intracellular localization

The phosphorylation/dephosphorylation cycles of phosphoinositides are catalyzed by distinct classes of phosphoinositide kinases and phosphatases. The reactions catalyzed by the kinases are in black arrows while the grey arrows indicate the phosphatase activities. Abbreviations: GC, Golgi complex; PM, plasma membrane; ER, endoplasmic reticulum; N, nucleus; E, endosomes; LE, late endosomes; Ly, lysosomes; SV, synaptic vesicles; CCV, clathrin-coated vesicles; Mi, mitochondria; ND, not determine. (Adapted from De Matteis M.A. and Godi A., 2004)

2.1.4.1: Phosphoinositide 3-kinases (PI 3-kinases)

The phosphoinositide kinases have been subjects of intensive studies due to their pivotal role in generating lipid signals. The phosphoinositide 3-kinases (PI 3-kinases) and its lipid products have been at the central stage of research interests due to their functions in cell proliferation, migration and transformation (Leevers, Vanhaesebroeck et al. 1999). The PI 3-kinases are classified into three different classes based on their domain structures, catalytic activities and modes of regulation (Fruman, Meyers et al. 1998). The class I PI 3-kinases are further subclassified into classes IA and IB with regard to their distinct regulatory subunits (50-85kD for class IA; p101 for class IB) and isotypes of p110 catalytic subunits (p110 α , p110 β and p110 δ). The main product of class I PI 3-kinases is PI(3,4,5)P₃ although they are capable of phosphorylating PtdIns, PI(4)P and PI(4,5)P₂. PI(3,4,5)P₃ is an important signal for cell proliferation and survival.

The class II PI 3-kinases (α , β and γ) are larger proteins that catalyze the phosphorylation of PtdIns and PI(4)P (but not PI(4,5)P₂) *in vitro*. Their C2 domain can interact with phospholipid substrate in a calcium dependent manner (Rizo and Sudhof, 1998). The PI3KII α and PI3KII γ have been shown to be associated with the Golgi complex (Ono, Nakagawa et al. 1998; Gaidarov, Smith et al. 2001; Kihara, Kabeya et al. 2001). The PI3KII α binds clathrin. Overexpression of this kinase induces the redistribution of the mannose 6-phosphate receptor (M6PR) from the TGN to the cell periphery and inhibits endocytosis (Gaidarov, Smith et al. 2001)

The class III PI 3-kinases can only catalyze the phosphorylation of PtdIns to PI(3)P. In yeast, there is only one PI 3-kinase, Vps34, which belongs to class III. All eukaryotes investigated so far have a Vps34p homologue. Recruitment of Vps34p to cellular membranes is mediated by interaction with a serine/threonine kinase, Vps15p (Stack, Herman et al. 1993; Stack, DeWald et al. 1995).

2.1.4.2: Phosphoinositide 4-kinases (PI 4-kinases)

The formation of PI(4)P is catalyzed by phosphatidylinositol 4-kinases using PtdIns as precursor (Balla and Balla 2006). PI(4)P was initially known as the precursor for the generation of PI(4,5)P₂ and PI(3,4,5)P₃. Only very recently, PI(4)P is recognized to be a unique lipid signal that regulates many cellular processes such as anterograde traffic from the Golgi (Hama, Schnieders et al. 1999; Schorr, Then et al. 2001).

There are 4 distinct classes of PI 4-kinases in mammals while yeasts possess three PI 4-kinases. In general, the PI 4-kinases are classified into type II and type III based on their biochemical properties. The mammalian type II PI 4-kinases are the PI4KII α and PI4KII β . Yeast has only a type II PI 4-kinase, Lsb6p (Han, Audhya et al. 2002; Shelton, Barylko et al. 2003). Lsb6p is a non-essential gene and contributes for a minor fraction of total PI 4-kinase activity in yeast. The PI4KII α is tightly associated with membranes and found at the Golgi and endocytic compartment while PI4KII β is mostly cytosolic and is recruited to the plasma membrane where it is activated by Rac1 (Wei, Sun et al. 2002).

Two isoforms of type III PI 4-kinases have been cloned in mammals: the PI4KIII α and its yeast homologue Stt4p (Yoshida, Ohya et al. 1994); the PI4KIII β and its homologue, Pik1p in yeast. PI4KIII α has been found at the plasma membrane and ER (Wong, Meyers et al. 1997) while the PI4KIII β has been localized to the Golgi complex (Godi, Pertile et al. 1999). However, yeast Pik1p has been found in the nucleus besides its Golgi localization. The PI4KIII β and PI4KII α are among the best studied PI4Ks in mammals. Both kinases are important in generating different pools of PI(4)P at the Golgi to recruit different adaptor proteins for anterograde trafficking (Wang, Wang et al. 2003; Godi, Di Campli et al. 2004). Both Pik1p and Stt4p are essential genes in yeast. Pik1p has similar functions to PI4KIII β at the Golgi (it generates PI(4)P pools that are important in forward trafficking, and maintenance of Golgi structure) while Stt4p regulates different pools of PI(4)P at the plasma membrane (for regulation of actin cytoskeleton and cell wall integrity) (Audhya, Foti et al. 2000)

2.1.4.3: Phosphatidylinositol-phosphate Kinases (PIP-kinases)

The phosphatidylinositol-phosphate kinases utilize single or double-phosphorylated inositol lipids as substrates. There are PIP 5-kinases and PIP 4-kinases which function in different paths of membrane trafficking.

The PIP 5-kinases were originally purified as activities that generate PI(4,5)P₂ from PI(4)P. Based on sequence homology, the PIP5-kinases are grouped into type I and type II. The Type II kinases phosphorylate the D-4 but not D-5 position. Thus, PIP 5-kinase II proteins are PIP 4-kinases. However, both group of kinases do phosphorylate the D-3 position of the inositol head group. There are three human PIP 5-kinases (PIP5KI α , PIP5KI β and PIP5KI γ) while budding yeast has Mss4p and Fab1p. Mss4p is localized to the plasma membrane where it plays a role in making PI(4,5)P₂ from PI(4)P (Homma, Terui et al. 1998). Mss4p contains a nuclear localization signal. It is activated at the plasma membrane through a regulated transport between the nucleus and the plasma membrane (Audhya and Emr 2003). The mouse ortholog of Fab1p is PIK-fyve. Both enzymes are important in generating PI(3,5)P₂ from PI(3)P and operate in endosomal systems (Gary, Wurmser et al. 1998; Odorizzi, Babst et al. 1998).

2.1.4.4: Phosphoinositide phosphatases

The consumption of phosphoinositides (signal termination) by different classes of phosphatases is critical for membrane homeostasis and cell physiology as testified by various human disease that are caused by mutations in different PI-phosphatases (Pendaries, Tronchere et al. 2003).

2.1.4.4.1: Phosphoinositide 3-phosphatases (PI 3-phosphatases)

The PI 3-phosphatases consist of phosphatase and tensin homolog deleted on chromosome ten (PTEN), TPIP, myotubularin (MTM) and myotubularin-related proteins (MTMRs). PTEN is one of the most studied PI-phosphatases till date. Other names given to this phosphatase include MMAC1 (mutated in multiple advanced cancers 1) and TEP1 (transforming growth factor β -regulated and epithelial cell-enriched phosphatase). PTEN was first identified as a tumor-suppressor gene located at chromosome 10q23.3, where mutations in this region caused high-grade glioblastomas, prostate and breast cancers (Li,

Yen et al. 1997). PTEN is a dual-specificity phosphatase towards protein substrates and phosphoinositides. Its tumor suppressor role is due to its ability to down-regulate Akt/PKB mediated cell proliferation and cell survival signals mediated by PI(3,4,5)P₃ (Maehama and Dixon 1998; Cantley and Neel 1999). PTEN is localized to the plasma membrane. This localization is controlled by multiple phosphorylation sites of PTEN at its C-terminus. The PTEN homologue in yeast, Tep1p was found to link phosphoinositide signaling and sporulation (Heymont, Berenfeld et al. 2000). However, a recent study showed that Tep1p has no PI(3)P 3-phosphatase activity *in vivo* (Rodriguez-Escudero, Roelants et al. 2005).

The MTM and MTMRs are PI 3-phosphatases that dephosphorylate PI(3)P and PI(3,5)P₂. Mutations in this class of phosphatases have been linked to human diseases such as myotubular myopathy and Charcot-Marie-Tooth syndrome (Blondeau, Laporte et al. 2000; Taylor, Maehama et al. 2000; Azzedine, Bolino et al. 2003). Ymr1p is the yeast homologue of MTM. Ymr1p plays an important role in the endocytic compartment in functions such as vacuolar protein sorting and maintenance of a functional endosomal system (Parrish, Stefan et al. 2004).

2.1.4.4.2: Inositol polyphosphate 5-phosphatases

The inositol polyphosphate 5-phosphatases comprise a large family of proteins that hydrolyze the 5-phosphate from the inositol ring of both the inositol phosphates and/or the PIs. They are classified into type I and type II 5-phosphatases according to their enzymatic activities. Only the type II 5-phosphatases can hydrolyze phosphatidylinositol phosphates. The type II phosphatases have an additional type II phosphatase domain. The existence of additional regulatory domains in type II 5-phosphatases is used to further classify them into SHIPs (SH2 domain-containing inositol 5-phosphatases) and GIPs (GAP domain-containing inositol 5-phosphatases) (Hughes, Cooke et al. 2000). SHIP1 and SHIP2 are examples of the SHIPs that can dephosphorylate PI(3,4,5)P₃ to PI(3,4)P₂ by removing the 5-phosphate. Mutations in SHIP1 have been shown to contribute to the development of acute leukemia and chemotherapy resistance. On the other hand, SHIP2 is linked to type 2 diabetes and obesity (Dyson, Kong et al. 2005). OCRL1 is an example of GIPs and will be discussed in detail in Section 2.3.4.3.

2.1.4.4.3: SAC domain-containing phosphatases

The SAC domain-containing phosphatases were the first identified in yeast (Guo, Stolz et al. 1999). The founding member of SAC domain phosphatases, Sac1p, was found initially as a suppressor of actin mutation, *act1-1^{ts}* (Cleves, Novick et al. 1989) (Novick, Osmond et al. 1989) giving rise to the name of the phosphatase. It was found that mutations in SAC1 could bypass the cellular requirement for the yeast phosphatidylinositol/phosphatidylcholine transfer protein, SEC14p (Cleves, Novick et al. 1989).

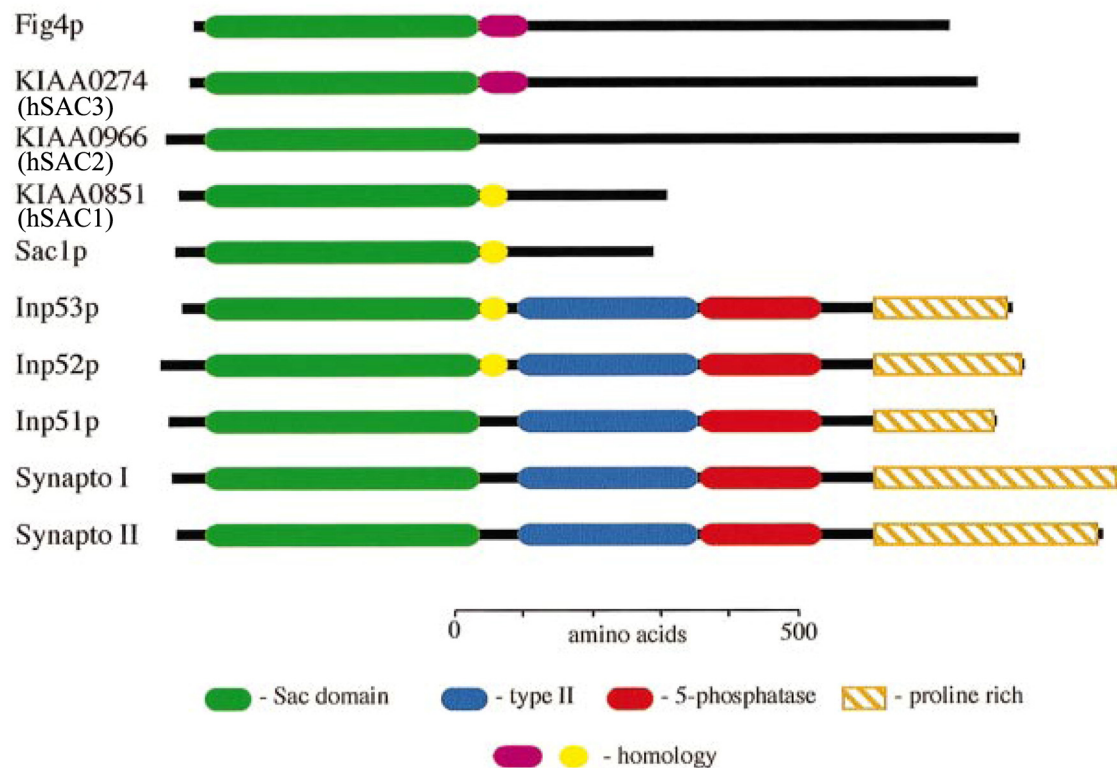


Figure 2.4: Members of the SAC domain-containing family of phosphatases.

The SAC domain is found in two classes of protein: the SAC domain-containing phosphatidylinositol phosphate 5-phosphatase (SCIPs) such as human synptojanins and the related yeast proteins Inp51p, Inp52p and Inp53p. The SCIPs contained a type II 5-phosphatase domain and a proline-rich region at the C-terminus. The second class of SAC domain-containing phosphatases are represented by yeast Sac1p and Fig4p and human hSAC1(KIAA0851), hSAC2(KIAA0966) and hSAC3(KIAA0274). (Adapted from Hughes W.E. et al., 2000)

The SAC domain is a 400 amino acid region that exhibits phosphatidylinositol polyphosphate phosphatase activity. It contains a highly conserved CX₅R(T/S) motif which is also present in many metal-independent protein and inositide polyphosphate phosphatases. The phosphatase signature motif CX₅R(T/S) was first identified in protein tyrosine phosphatase (PTP) (Maehama, Taylor et al. 2000). Both PTEN and MTM1-family of inositol-phosphate phosphatases were initially classified as PTPs due to the presence of this motif although their preferred substrates are phosphoinositides.

The first indication of a phosphatase activity associated with the SAC domain was found during work characterizing inhibitors of mammalian PI(4,5)P₂-dependent phospholipase D (PLD). Synaptojanin was identified as one of the other proteins able to inhibit the activity of PLD. This is attributed to the 5-phosphatase catalytic activity of synaptojanin that hydrolyzes PI(4,5)P₂ required for PLD activity (Chung, Sekiya et al. 1997). Further study carried out by Guo et al. revealed that the Inp52p and Inp53p of yeast synaptojanin homologues also possess a second phosphatase activity that is contributed by the SAC-domain (Guo, Stolz et al. 1999). The SAC-domain was found to exhibit a broader-specificity towards PI(3)P, PI(4)P and PI(3,5)P₂. However, PI(3,4)P₂, PI(4,5)P₂ and phosphoinositide species with adjacent phosphate groups are not substrates of the Sac phosphatase domain.

As shown in Figure 2.4, the SAC domain phosphatases fall into two main classes (Hughes, Cooke et al. 2000). The first category contains the SAC domain-containing phosphatidylinositol phosphate 5-phosphatases (SCIPs), that include synaptojanin, sjl1, sjl2 and sjl3 (Inp51p, Inp52p and Inp53p) (discuss in Section 2.1.4.4.4). The second category of SAC domain-containing phosphatases consists of proteins with an N-terminal SAC domain with no other recognizable domains. Sac1p and Fig4p in yeast and the mammalian SAC1 phosphatases (rSAC1, hSAC1, hSAC2 and hSAC3) are members of this phosphatase family.

2.1.4.4.4: SAC domain-containing inositol 5-phosphatases (SCIPs)

The type II 5-phosphatases that contain an N-terminal SAC domain are designated as SCIPs (SAC domain-containing inositol 5-phosphatases). Synaptojanins and their yeast homologues, Inp51p, Inp52p and Inp53p, are members of the SCIPs. Synaptojanin 1 is one of the most studied SCIPs in mammals and its function in synaptic vesicle recycling is well established ((McPherson, Garcia et al. 1996; Cremona and De Camilli 2001). Synaptojanin 1 plays important roles in clathrin-mediated endocytosis of synaptic vesicles by regulating the PI(4,5)P₂ levels at these specialized cells. It contains 3 main domains: the N-terminal SAC phosphatase domain, the 5-phosphatase domain and a C-terminal proline-rich domain. The proline-rich domain is proposed to be a targeting domain for synaptojanin 1 via protein-protein interactions. Synaptojanin 1 knockout mice have elevated cellular PI(4,5)P₂ levels and show accumulation of clathrin coated vesicles. Synaptojanin 2 was cloned shortly after the discovery of synaptojanin 1 as a presynaptic inositol-5-phosphatase (Nemoto Y. et al., 1997). Synaptojanin 2 shows ubiquitous expression and is localized differently from synaptojanin 1. Synaptojanin 2 appears to function at an earlier step in clathrin-mediated endocytosis (Kiss et al., 2002; Rusk et al. 2003).

SCIPs are also found in *Saccharomyces cerevisiae* where they are considered functional homologs of mammalian synaptojanins. They are therefore named Sjl1, Sjl2 and Sjl3 or Inp51p, Inp52p and Inp53p. Deletion of all three yeast synaptojanin like proteins causes lethality. This lethality is a consequence of a dramatic increase of PI(4,5)P₂ levels (Stefan, Audhya et al. 2002). The other observed phenotypes in Sjl knockouts are defective actin cytoskeleton, endocytosis and protein sorting from the Golgi to the vacuole. The yeast synaptojanins had also been found to be important in cellular events that require clathrin and have been shown to interact genetically with clathrin (Bensen, Costaguta et al. 2000).

The other members of SAC domain-containing proteins are Fig4p and Sac1p which contain only the N-terminal SAC domain without the 5-phosphatase domain (Figure 2.4). Fig4p was identified in a screen for pheromone-induced genes (FIG) (Erdman, Lin et al. 1998). The SAC domain of Fig4p is not able to complement a Sac1p deficiency. Steady state phosphoinositide levels of a Δ fig4p yeast mutant are comparable

to wild type yeast (Gary, Sato et al. 2002). Fig4p has no transmembrane domain and its recruitment to the vacuolar membrane needs assistance from other proteins (Rudge, Anderson et al. 2004). Fig4p is found to be a PI(3,5)P₂ specific phosphatase *in vitro* and regulates the MVB-associated PI(3,5)P₂ made by the Fab1p PI(3)P 5-kinase (Rudge, Anderson et al. 2004). Fig4p orthologues in human have been found but the functions of this phosphatase in mammals are unknown (Erdman, Lin et al. 1998). The yeast Sac1p and the mammalian SAC1 phosphatases will be discussed in Section 2.3.4.1 and Section 2.3.4.2.

2.2: Phosphoinositides and membrane trafficking

Over the last 2 decades, we have witnessed tremendous advances in identification of the molecular machinery that drives and controls the inter-organellar transport of proteins and lipids. However, the transport machinery that had been characterized and studied mainly consisted of protein factors. Membrane lipids were thought to be passive components of biological membranes and subjected to deformation by the action of coat proteins and small GTPases. The importance of membrane lipid composition in the formation and consumption of transport vesicles has only been recognized recently. Phosphatidic acid (PtdOH), diacylglycerol (DAG) and phosphoinositides (PIs) are found to be essential for vesicular transport (Simonsen, Wurmser et al. 2001) (Odorizzi, Babst et al. 2000).

The ER is the only organelle found so far that is devoid of any phosphoinositide species at steady state. The function of inositol lipids in this compartment is unclear. However, there is evidence that accumulation of PI(4)P at the ER negatively regulates its cellular functions (Kochendorfer, Then et al. 1999; Konrad, Schlecker et al. 2002). A very recent finding shows that eEF1A is a direct activator of PI4KIII β (Jeganathan and Lee 2006). Ectopic expression of eEF1A2 increases activity of PI4KIII β and cellular PI(4)P abundance.

The 3-phosphorylated inositol lipids was found to play important roles in regulating membrane trafficking and protein sorting at the endosomal and vacuolar compartments. Emr and colleagues have demonstrated that Vps34p (a PI 3-kinase) is required for normal and efficient routing of proteins from the Golgi to the vacuole (Schu,

1993). Vps34p is recruited to the Golgi and is activated by the Vps15p kinase (Stack, Herman et al. 1993). This Vps34/Vps150 complex is conserved from yeast to human since studies with human version of Vps34 recapitulate this activation circuit (Panaretos C. et al. 1997). The functionally conserved Vps34 PI 3-kinase generates pools of PI(3)P which serve as spatial cue for recruitment of effector proteins for TGN-endosomal sorting. PI(3,5)P₂ is another lipid signal found at the multivesicular bodies (MVB). It is generated via the sequential actions of PI 3-kinase and the PI(3)P 5-kinase. In yeast, Fab1p (the only PI(3)P 5-kinase) is recruited to the endosomal membranes via its FYVE domain through specific binding to PI(3)P. Fab1p is not an essential gene in *S.cerevisiae*, however deletion of this gene causes abnormal vacuolar sorting and morphology (Gary, Wurmser et al. 1998) (Odorizzi, Babst et al. 1998). Thus, PI(3,5)P₂ regulates the internalization of endosomal multivesicular bodies into the vacuole lumen for subsequent degradation. Furthermore, the synthesis of PI(3,5)P₂ is accelerated during osmotic stress (Dove, Cooke et al. 1997). Taken together, PI(3,5)P₂ may generally function in regulating lysosomal homeostasis and in formation of specialized MVBs involved in antigenic processing and presentation (Katzmann, Odorizzi et al. 2002; Gillooly, Raiborg et al. 2003).

PI(4,5)P₂'s functions in regulating membrane trafficking (Martin 2001) were first shown in reconstituted regulated dense core granules in permeabilized neuroendocrine cells by Martin and colleagues (Hay and Martin 1993; Hay, Fisette et al. 1995) They found that phosphatidylinositol transfer protein (PITP) synergizes with a PI(4)P-5 kinase in ATP-dependent dense core granules in regulated exocytosis. Other studies show that a resident granule PI 4-kinase is essential for stimulated secretion (Wiedemann, Schafer et al. 1998). The granule associated with the PI(4,5)P₂ pool was shown to be important in recruiting protein effectors that regulate the subsequent Ca²⁺-dependent fusion of dense core granules to the plasma membrane. The effector protein that was identified, called CAPS (calcium-dependent activator protein for secretion), bound to PI(4,5)P₂ via its PH domain. Other effector proteins that are recruited by PI(4,5)P₂ and involved in regulated secretory pathway include synaptotagmin and rabphilin. However, binding of these proteins to PI(4,5)P₂ is via their C2 domains in the presence of Ca²⁺. PI(4,5)P₂ function in the endocytic pathway is established in clathrin-mediated endocytosis which involves

adaptor protein AP-2 in clathrin coat assembly in a PI(4,5)P₂ dependent manner (Jost, Simpson et al. 1998; Gaidarov and Keen 1999). The GTPase, dynamin, is recruited by PI(4,5)P₂ for scission of clathrin-coated vesicles. PI(4,5)P₂ also plays essential roles in modulating channel function such as the TRP channel family (Hilgemann, Feng et al. 2001).

Besides the plasma membrane, a minor pool of PI(4,5)P₂ was shown to reside at the Golgi complex and play a role in secretion. The Golgi localized phospholipase D (PLD) which hydrolyzes phosphatidylcholine (PtdCho) to phosphatidic acid (PtdOH) and choline has been implicated in mammalian Golgi function. PI(4,5)P₂ is required for efficient catalytic activity of PLD. However, the small GTPase Arf was also shown to stimulate the activity of PLD. PtdOH which is generated by PLD from PtdCho activates PI(4)P 5-kinase. An interesting study which linked Arf and the generation of Golgi PI(4,5)P₂ was performed by the laboratory of De Matteis (Godi, Pertile et al. 1999). Arf stimulates a PLD-dependent synthesis of PI(4,5)P₂ on Golgi membranes by recruiting a specific PI 4-kinase (Pik1 β).

PI(4)P has been long known to be the precursor for synthesis of PI(4,5)P₂ catalyzed by the type I PIP 5-kinases (Section 2.1.4.3). However, PI(4)P has emerged as important signaling molecule at the Golgi complex and in the nucleus. PI(4)P in the nucleus functions as precursor of nuclear PI(4,5)P₂ while nuclear PI(4,5)P₂ is further metabolized by a nuclear localized PLC to generate different inositol polyphosphates via the inositol polyphosphate kinases (IPKs) and the inositol pyrophosphate synthases (IPSSs). The function of PI(4)P at the Golgi will be discussed in Section 2.3.3.

2.3: Biogenesis of the Golgi apparatus

The Golgi apparatus was first described by Camelio Golgi in 1898 when he investigated the structure of nerve cells of the spinal ganglia (Golgi 1898). However, the existence of this organelle was doubted for more than 50 years until additional pieces of data were obtained using the new techniques at that time, such as electron microscopy (EM). The hallmarks of the Golgi apparatus as observed in a thin-sectioned cell under EM appeared as a set of flattened, closed membranes (cisternae) which are stacked one upon the other. The cisternae were found to be in close proximity to the nucleus.

Besides morphological observations of the Golgi apparatus, the Palade's lab had performed pulse-chased experiments using radioactive amino acids such as [³H]leucine, autoradiography and electron microscopy in pancreatic exocrine cells (Jamieson and Palade 1967). These experiments shed light in defining the existence of the Golgi complex and confirmed its functions at the secretory pathway. In fact, the Golgi complex is the heart of the secretory pathway and plays essential roles in modifying lipids and proteins that are made in the endoplasmic reticulum.

Following the huge controversy about the existence of the Golgi apparatus, cell biologists are currently having fierce debates about how this organelle functions within the secretory pathway, and whether it is an autonomous organelle or dependent on membranes from the ER. It is still under debate which model(s) is/are describing best how intra-Golgi transport is achieved (Orci, Amherdt et al. 2000 ; Marsh and Howell 2002).

The cisternae maturation/progression model and the vesicular transport/stable compartment model are two major important hypotheses that have been proposed. Both models start from a common base where the newly synthesized proteins and lipids are packaged in COPII vesicles for transport out of the ER. Vesicles and tubules (Vesicular tubular cluster) are generated from the COPII budding events at the ER in both models. The vesicular transport model proposed that the Golgi complex is a stationary, stable compartment. The vesicles are the dynamic elements that are used to transport cargo between the static Golgi cisternae. In contrast, each Golgi membrane/cisternae functions as bulk carrier for cargo transport across the organelle in the cisternae maturation model. New cisternae are formed with each round of cargo transport. The cis or entry cisternae are formed by ATP-dependent fusion of vesicles and tubules derived from the endoplasmic reticulum (which then form the VTC). The maturation of each cisternae is accomplished by addition of the Golgi proteins via retrograde vesicles. The cis cisternae becomes medial and finally trans following a progressive series of maturation steps.

Most recently, a percolating vesicle model, a combination of both cisternae maturation and vesicular transport models was suggested (Orci, Ravazzola et al. 2000; Pelham and Rothman 2000). Based on this model, there are two populations of COPI vesicles. One COPI vesicle population would mediate the fast transport of anterograde

cargo between all cisternae in a bi-directional random walk as suggested by the even distribution of GS28 over the Golgi apparatus (Orci, Stannnes et al. 1997; Orci, Ravazzola et al. 2000). Directional transport is achieved due to the steady state entry of biosynthetic cargo at the *cis*-Golgi and its exit at the *trans*-Golgi. . The second population of COPI vesicles mediate the recycling of enzymes and materials back to ER. The percolating vesicle model also integrates a slow anterograde transport pathway mediated by cisternae progression/maturation that accounts for cargo that are too large for COPI vesicles and explains the different transport rates observed.

The continuity-based model was suggested very recently based on electron tomography studies. A cargo induced effect on the formation of tubular inter-cisternae connections was reported in these studies (Mironov, Beznoussenko et al. 2001; Marsh 2005). The continuity-based model suggests that transport of cargo is mediated through polymorphic carriers that have the ability to bridge adjacent cisternae in a process resembling ER-Golgi intermediate compartment (ERGIC) to cis-Golgi cargo transport. Cisternae maintenance including Golgi enzyme recycling are accomplished by tubules that would only connect subsequent cisternae. The roles of COPI vesicles would solely be the control of inter-cisternal fusion events via active uptake of SNARE proteins into COPI vesicles and the establishment of Golgi morphology.

However, regardless of which models that were described above, COPI retrograde transport plays a role in maintaining Golgi homeostasis and recycling of Golgi resident enzymes.

2.3.1: Mechanisms of Golgi enzyme subcompartmentalisation

The Golgi complex is not only the hub of the secretory pathway but it bears the most vital role in protein and lipid modifications. These post-translational modifications are important in modulating the functions and stability of proteins and lipids before they are delivered to their final destinations within or outside of the cell. Glycosylation, the most common and complex form of post-translational modification is performed by a set of glycosyltransferases that are positioned among the Golgi cisternae in a defined manner. The relative positions of glycosyltransferases are important in governing the structure and repertoire of the sugar branches that are expressed on the cell surface. The large diversity

of carbohydrate structures is produced by over 200 different glycosyltransferases situated within the individual Golgi stacks. These carbohydrates play an important role in cell-cell communication and development of multicellular organisms. This is exemplified by a large number of human diseases linked to mutations in glycosylenzymes (Freeze 2006; Ohtsubo and Marth 2006).

The membrane-bound glycosyltransferases are the main Golgi resident proteins that function for O-linked and N-linked glycosylations. Many of them have been cloned and sequenced. They share little amino acid sequence similarity. However, they are all type II transmembrane proteins and share a common domain structure. They possess a single hydrophobic (single anchor) domain, a luminal stem region and a large C-terminal catalytic domain.

One of the more challenging questions in cell biology is that how Golgi resident glycosyltransferases are localized and distributed throughout the Golgi cisternae in a highly organized manner. In view of a massive flow of proteins and lipids passing through the Golgi complex from the ER, it is unclear how modification, retention and secretion happens within the Golgi at such amazing rates and with high precision. Clearly, sorting and retention of resident Golgi proteins are important for the maintenance of a functional Golgi.

Mechanisms to localize a distinct set of glycosyl-enzymes in Golgi cisternae have been studied extensively and there are few hypotheses/models proposed from studies in different laboratories. The initial studies were focused on identification of a targeting signal responsible for the localization of several integral membrane proteins to the Golgi stacks. A common strategy used was to analyze the localization of hybrid molecules containing limited sequences derived from Golgi glycosyltransferases or viral glycoproteins known to be specifically retained in the Golgi complex. Localization of these fusion proteins revealed that sequences within the transmembrane domains can specify Golgi localization (Munro 1991; Nilsson, Lucocq et al. 1991; Swift and Machamer 1991; Aoki, Lee et al. 1992; Tang, Wong et al. 1992; Teasdale, D'Agostaro et al. 1992; Wong, Low et al. 1992). Munro S., and Colley et al. have also found that sequences flanking the transmembrane domain play an auxiliary role in mediating Golgi localization. Collectively, these data suggest that the transmembrane domain plays a

central role in the targeting and localization of resident Golgi proteins. In contrast, a number of studies have shown that alterations at the transmembrane domain can be made without abolishing Golgi retention (Munro 1991; Colley, Lee et al. 1992). Study from Burket J. et al. has demonstrated that all three domains of N-acetylglucosaminyltransferase I contribute to its medial Golgi retention (Burke, Pettitt et al. 1992; Burke, Pettitt et al. 1994).

It has been proposed that retention of resident Golgi proteins involves protein oligomerization within the environment of the Golgi compartment (Machamer 1991; Nilsson, Lucocq et al. 1991). This is based on a finding that the glycosyltransferases exist predominantly as high molecular weight aggregates after detergent extraction (Bendiak and Schachter 1987). Experiments carried out by Weisz et al., and Nilsson et al. further support the idea of aggregation model of Golgi retention (Nilsson, Pypaert et al. 1993; Weisz, Swift et al. 1993).

The aggregation model and membrane thickness model (selective partitioning of Golgi enzymes into Golgi cisternae based on the thickness of the membrane which is equivalent to the length of the transmembrane domain of the protein) are models based on a static Golgi (vesicular transport model). In contrast, the cisternae maturation model would predict that the Golgi resident enzymes traveled backward via COPI vesicles. In this scenario, COPI vesicles should be enriched for Golgi resident enzymes while cargos remain in the cisternae which are the main anterograde carriers. Localization of Golgi glycosyltransferases requires active partitioning of resident proteins into COPI vesicles rather than an anchoring mechanism. Both Klumperman and Nilsson laboratories have provided evidence for the concentration of Golgi enzymes in COPI vesicles (Lanoix, Ouwendijk et al. 1999; Lanoix, Ouwendijk et al. 2001; Martinez-Menarguez, Prekeris et al. 2001). The first group found that the Golgi enzyme mannosidase II is present in COPI vesicles with a concentration of 1.5-fold higher than in the cisternae by quantitative immuno-electromicroscopy. The Nilsson group showed that COPI vesicles contain Golgi enzymes at a concentration that is up to ten times higher than that found in the cisternae.

Recent studies using live cell imaging and fluorescent-tagged Golgi proteins (including the glycosyltransferases, scaffold, coat proteins and intirant proteins) revealed that the Golgi complex is a highly dynamic structure (Ward, Polishchuk et al. 2001). No

class of Golgi proteins is stably associated with the Golgi, instead localization of proteins at Golgi membranes depends on ongoing traffic between the Golgi and other organelles (Bos, Wraight et al. 1993; Reaves, Horn et al. 1993; Ward, Polishchuk et al. 2001). Yet, the residency time of different classes of Golgi associated proteins varies enormously. While Golgi processing enzymes have long half-lives at the Golgi, cargo proteins and cargo receptors spend relatively little time at this organelle (Ward, Polishchuk et al. 2001; Presley, Ward et al. 2002). It has therefore been recognized that the Golgi must contain functionally and spatially distinct domains that differ by protein and lipid composition.

Study from laboratory of Wieland provided evidence for a lipid segregation in Golgi membranes, as indicated a significant reduction of sphingomyelin and cholesterol content in COPI-coated vesicles compared with their donor/parental membranes (Brugger, Sandhoff et al. 2000).

Another example of the existence of different functional domains at the TGN defined by membrane lipids was demonstrated and proposed by Godi A. et al (Godi, Di Campli et al. 2004). The recruitment and targeting of the Fapp proteins, OSBP and CERT to the Golgi apparatus is dependent on binding of their N-terminal PH-domains to PI(4)P and Arf but with distinctive lipid-binding properties that may selectively target them to separate TGN domains (Godi, Di Campli et al. 2004). Fapp2 contains a glycolipidtransfer domain, OSBP possesses an oxysterol-binding domain while CERT binds to ceramide via its STAR-T domain. This allow generations of different lipid environments where these different proteins are bound or recruited.

2.3.2: Phosphoinositides at the Golgi Complex

As other organellar membranes, the Golgi complex is composed of a unique set of proteins and lipids. The Golgi apparatus is a highly polarized organelle where each membrane cisterna is occupied by different processing enzymes to modify cargo and lipids. The sequence of reactions for protein modification is important to generate functionally matured protein or lipid cargo.

Phosphatidylinositol-4-phosphate (PI(4)P) is concentrated at the Golgi apparatus. At steady state, the Golgi can be labeled with PI(4)P-binding probes such as the FAPP1-PH domain fused with green fluorescence protein. PI(4)P is generated by PI 4-kinases

that are recruited to the Golgi membranes. There are two main PI 4-kinases at the Golgi: PI4KII α and PI4KIII β . These two kinases are responsible for generating different pools of PI(4)P at the Golgi (Weixel, Blumental-Perry et al. 2005) that are recognized by different effectors. The PI(4)P pools made by the PI4KIII β are regulated by Arf1 through activation of this kinase. Besides Arf1, NCS-1 also stimulates the activity of PI4KIII β (yeast homolog of frequenin). The PI4KIII β created PI(4)P recruits the Fapp proteins to TGN exit sites (Godi, Di Campli et al. 2004). Depletion of PI4KIII β and the Fapp proteins caused accumulation of cargo and formation of long-tubular vesicular structures at the TGN. Thus, Fapps are essential elements of a PI(4)P- and Arf-regulated machinery that controls the generation of constitutive carriers destined to plasma membrane. The Fapp proteins represent members of a superfamily of TGN-associated proteins with a common targeting mechanism (based on PI(4)P and Arf1) but with distinctive lipid binding properties that may target them to separate TGN domains.

On the other hand, PI4KII α creates PI(4)P pools that are important for recruiting the adaptor protein 1 (AP1), which functions in clathrin-mediated trafficking pathways (Wang, Wang et al. 2003). Knock-down of this kinase led to inhibition of cargo exit out of the Golgi and caused fragmentation of this organelle. However, this defect could be rescued by supplying either exogenous PI(4)P or PI(4,5)P₂. Recently, PI4KII α has been implicated in regulating the endosomal trafficking and the degradation of the EGF receptor (Minogue, Waugh et al. 2006). Inactivation of PI4KII α using RNAi or the monoclonal antibody 4C5G causes accumulation of ligand-bound EGFR in sub-plasma membrane compartment. Furthermore, lysosomal degradation of activated EGFR was impaired in PI4KII α siRNA treated cells.

In budding yeasts, the function of PI(4)P in anterograde trafficking has been demonstrated in cells lacking the Golgi localized Pik1p. *S. cerevisiae* has only three PI 4-kinases and each localizes to different cellular compartments. Stt4p localizes to ER and PM while Pik1p is found at the Golgi and the nucleus. LSB6, the only type II PI 4-kinase in yeast has been shown to populate both the vacuolar and the plasma membrane. Null mutants of LSB6 have no detectable phenotype. In contrast, both Pik1p and Stt4p are essential for yeast viability. Stt4p and Pik1p create distinct PI(4)P pools that are non-exchangeable with distinct functions (Audhya, Foti et al. 2000). The PI(4)P pools made

by the Pik1p at the Golgi are important in regulation of endocytosis, secretion and vacuolar morphology whereas the Stt4p generated PI4P pools are implicated in the regulation of cytoskeleton and cell wall integrity.

The function of PI(4,5)P₂ in the Golgi is less well characterized compared to its functions at the plasma membrane. Attempts to visualize PI(4,5)P₂ at this compartment using fluorescence microscopy have not been successful. Evidence that suggests the presence of this phospholipids at the Golgi membranes came from studies conducted by the Shields' laboratory (Siddhanta, Backer et al. 2000; Sweeney, Siddhanta et al. 2002). The localization of PI(4,5)P₂ in the Golgi complex was visualized and quantified using electron microscopy in cells expressing the PLC δ PH domain (Watt, Kular et al. 2002). PI(4,5)P₂ has been shown to be important in control of ER-GC transport, in the formation of post-Golgi transport carriers, and in maintenance of Golgi structure. Spectrin binds to PI(4,5)P₂ and is responsible for maintaining structural integrity of this organelle (Siddhanta, Radulescu et al. 2003). Dynamin-based machineries is also regulated by PI(4,5)P₂ and is crucial for release of transport carriers from the TGN. Stimulation of PLD activity by PI(4,5)P₂ is important for trafficking out of the Golgi through control of phosphatidic acid (Freyberg, Siddhanta et al. 2003).

2.3.3: Golgi-localized phosphoinositide phosphatases:

Recruitment of distinct PI 4-kinases to Golgi complex is essential to generate distinct PI(4)P pools for binding of different effector proteins. The phosphoinositide phosphatases are equally important for maintaining the proper function of this organelle by terminating the PI(4)P and PI(4,5)P₂ signals. However, the phosphoinositide phosphatases at the Golgi complex are less well characterized compared to the PI 4-kinases. Till date, the type II 5-phosphatase, OCRL1 (Oculocerebrorenal of Lowe) and a PI(4)P-phosphatase, hSAC1 (KIAA080851) were the only known phosphoinositide phosphatases at the Golgi complex. The function of hSAC1 and OCRL1 in Golgi apparatus is the main theme of this work and will be discussed in details.

2.3.3.1: Yeast Sac1p, the founding member of the SAC domain phosphatases

Since its discovery as a phosphoinositide phosphatase, the cellular functions of Sac1p in yeasts have been studied in considerable extent in comparison to its mammalian counterparts. Sac1p is a type II transmembrane protein localizes to both ER and Golgi. The cytosolic N-terminal of Sac1p is anchored to cellular membranes via its C-terminal transmembrane domains. The SAC domain is located at the N-terminal and is capable of dephosphorylate PI(3)P, PI(4)P and PI(3,5)P₂ (Konrad, Schlecker et al. 2002). Sac1p null mutants accumulated eight to ten folds of PI(4)P, 1.5 folds of PI(3)P, a slightly elevated PI(3,5)P₂ level and four folds decrease of PI(4,5)P₂. Thus, Sac1p is the main phosphoinositide-4 phosphatase in yeasts and it plays pivotal role in controlling cellular PI(4)P levels at the ER and Golgi (Mayinger, Bankaitis et al. 1995; Schorr, Then et al. 2001; Tahirovic, Schorr et al. 2005).

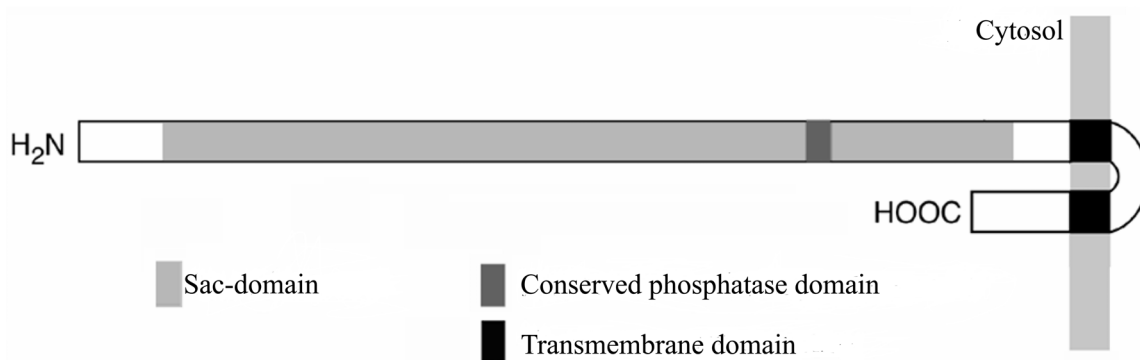


Figure 2.5: Domain structure of yeast Sac1p

Yeast Sac1p is a type II transmembrane PI(4)P phosphatase which contains a conserved phosphatase motif and two putative transmembrane domains. (Adapted from Konrad et al., 2002)

Similar to mammalian Golgi, PI(4)P is also concentrated at this organelle in yeast. Sac1p was shown to antagonize Pik1p (homolog of mammalian PI4KIII β) function in the Golgi by terminating PI(4)P signal generated by Pik1p (Schorr, Then et al. 2001). Deletion of Sac1p leads to excessive anterograde transport of chitin synthases, thus causes specific cell wall defects. Sac1p also play pivotal role in regulating other PI(4)P pools made by Stt4p (the PI4KII α homolog in mammals). This regulation is essential for normal endocytosis and vacuole morphology (Tahirovic, Schorr et al. 2005). In a very recent study, Sac1p was shown to be the main player in controlling the level of PI(4)P at the Golgi in response to nutrients availability and cell growth condition (Faulhammer,

Konrad et al. 2005). During exponential cell growth, PI(4)P is needed for secretion. Sac1p is mainly found in the ER during active cell growth. To slow down secretion during starvation, Sac1p is relocated to the Golgi where it terminates PI(4)P signal at this organelle to slow down anterograde trafficking. In addition, the fraction of Sac1p in ER is required for efficient dolichol oligosaccharide biosynthesis (Faulhammer, Konrad et al. 2005).

The function of Sac1p in ER was shown to be important in regulating PI(3)P and PI(4)P at this compartment. Retention of Sac1p in the ER stimulates ATP transport to the ER lumen (Konrad, Schlecker et al. 2002). Sac1p is needed in the ER to mediate ATP transport into this compartment for protein translocation (Mayinger, Bankaitis et al. 1995). The function of mammalian Sac1 in ER remains unknown.

Sac1p is conserved from yeast to mammals. Other studies of Sac1p homologs have been pursued in plant *Arabidopsis thaliana*, *Drosophila melanogaster* and in higher eukaryotes (rat and human). The *A. thaliana* consists of three Sac1 genes (AtSAC1a, AtSAC1b and AtSAC1c) which are functional homologs of yeast Sac1p due to its ability to rescue cold sensitivity and inositol auxotroph in *sac1-null* yeast mutant (Despres, Bouissonnie et al. 2003). They are found to localize at ER membranes and displayed overlapping expression patterns. Study from Zhong et al. demonstrated that the AtSAC genes are essential for normal cell morphogenesis, cell wall synthesis and in salt stress response (Zhong, Burk et al. 2005). In contrast, Sac1p homolog in fruit fly has been shown to play vital role in embryonic development (Wei, Sanny et al. 2003).

2.3.3.2: The mammalian SAC1 phosphatases

The rat SAC1(rSAC1) is the first Sac1p counterpart in mammalian cells characterized in 2000 (Nemoto, Kearns et al. 2000). Similar to Sac1p, the rSAC1 was shown to exhibit phosphoinositide phosphatase activity towards 3-phosphate, 4-phosphate and 3,5-bisphosphate. Expression of rSAC1 in $\Delta sac1$ yeast strains complements a wide phenotypes associated with Sac1p insufficiency. Both Sac1p and rSAC1 are integral membrane lipid phosphatases at the ER and the Golgi complex (Nemoto, Kearns et al. 2000; Konrad, Schlecker et al. 2002). rSAC1 is ubiquitously expressed but highly enriched in cerebellar Purkinje cells.

The *Homo sapiens* SAC1 homolog, hSAC1 was shown to behave as the yeast Sac1p in terms of substrate specificity and topology. Using specific antibodies against hSAC1, the endogenous hSAC1 was found to populate both ER and Golgi compartments. Different from Sac1p, hSAC1 contains a leucine zipper motif and it interacts with members of the coatamer I (COPI) (Rohde, Cheong et al. 2003). The hSAC1 gene (EST KIAA0851) maps to the C3CER1 segment of chromosome 3p21.3, which is found to be deleted commonly in SCID-derived tumors. Phosphatase inactive mutant of hSAC1 was found to accumulate in the Golgi and failed to bind COP1 despite of an intact putative COP1 interactive motif at the C-terminal. Thus, the localization of hSAC1 in both ER and Golgi is relied on functional phosphatase activity in addition to COP1 interaction via its C-terminal K(X)KXX motif (Rohde, Cheong et al. 2003). The domain structure of hSAC1 is shown in Figure 2.6.

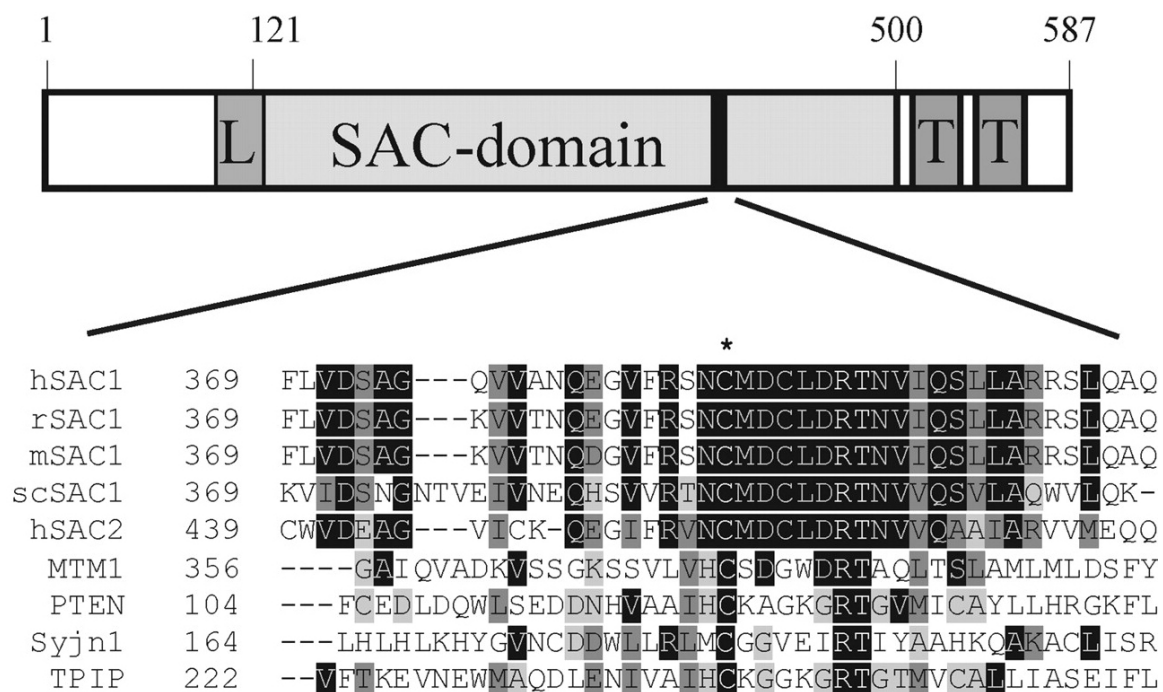


Figure 2.6: Domain structure of hSAC1

hSAC1 is also a type II transmembrane protein with a large N-terminal domain containing the SAC-domain (aa 121-500), a putative leucine zipper (L) motif (aa 98-126), and two transmembrane domains (T) at the C-terminus (aa 512-543; aa 550-569). The phosphatase signature motif CX5R(T/S) (aa 389-396) is indicated by a black bar. (Adapted from Rohde et al., 2003)

hSAC2 (KIAA0966) and hSAC3 (KIAA0274) are two other SAC-domain containing phosphatases in human that have not been characterized in details. The hSAC2 functions as an inositol polyphosphate 5-phosphatase. It has a different substrate specificity than human and rSAC1. It can dephosphorylate PI(4,5)P₂ and PI(3,4,5)P₃. However, its preferred substrate is PI(4,5)P₂ (Minagawa, Ijuin et al. 2001). The enzymatic activity and function of hSAC3 is currently unknown.

2.3.3.3: The PI(4,5)P₂ 5-phosphatase OCRL1

OCRL1 (Oculocerebrorenal of Lowe) is a phosphoinositide phosphatase that was found to be mutated in Lowe syndrome patients (Attree, Olivos et al. 1992). Lowe Syndrome is a rare X-linked disease that displays a broad spectrum of symptoms. Congenital cataracts, mental retardation and Fanconi syndrome of the proximal renal tubules are among the hallmarks of this progressive degenerative disease. It has been known for almost 2 decades that pathogenesis of Lowe Syndrome is due to the deficiency of PI(4,5)P₂ 5-phosphatase activity of this Golgi localized lipid phosphatase. Kidney proximal tubule cells derived from Lowe patients showed accumulation of PI(4,5)P₂ and abnormal actin cytoskeleton (Suchy and Nussbaum 2002). However, it is currently unknown how mutations in this lipid phosphatase can cause such tissue specific defects since OCRL1 is ubiquitously expressed. Interestingly, knockout mice of OCRL1 displayed none of the described manifestations of the disease (Janne, Suchy et al. 1998). OCRL1 deficiency was found to be complemented in mice by *Inpp5b*, another inositol polyphosphate 5-phosphatase.

OCRL1 has been localized predominantly to the Golgi (Olivosglander, Janne et al. 1995; Suchy, Olivosglander et al. 1995) but a lysosomal localization has also been found (Zhang, Hartz et al. 1998). Very recently, this phosphatase has been shown to populate the endosomal membranes (Ungewickell, Ward et al. 2004; Choudhury, Diao et al. 2005). The C-terminal Rho-GAP domain of OCRL1 was found to bind activated GTP bound form of Rac small GTPase (Rho GTPase family) (Faucherre, Desbois et al. 2003). It is believed that this interaction contributes to the steady state localization of OCRL1 at the TGN. However, upon stimulation of epidermal growth factor OCRL1 translocates from

TGN to plasma membrane and concentrates in membrane ruffles (Faucherre, Desbois et al. 2003).

Recent studies that showed interaction of OCRL1 with clathrin and defects in clathrin-mediated transport between the TGN and the endosomes raised the possibility that trafficking between these compartments causes defects in the tissues affected with Lowe syndrome. Figure 2.7 shows domain structure of OCRL1. Tissue-specific alternative splicing gives rise to two splice variants of OCRL1, namely OCRL1 isoform a and isoform b. Interestingly, the only difference between these two isoforms is the presence of eight amino acid insertion in the longer isoform a (Figure 2.7). Both isoforms are expressed in all tissues but with the shorter version being most abundant (except in brain). Brain tissues expressed only isoform a (the longer version of OCRL1). The importance of different OCRL1 splice variants is unclear at present.

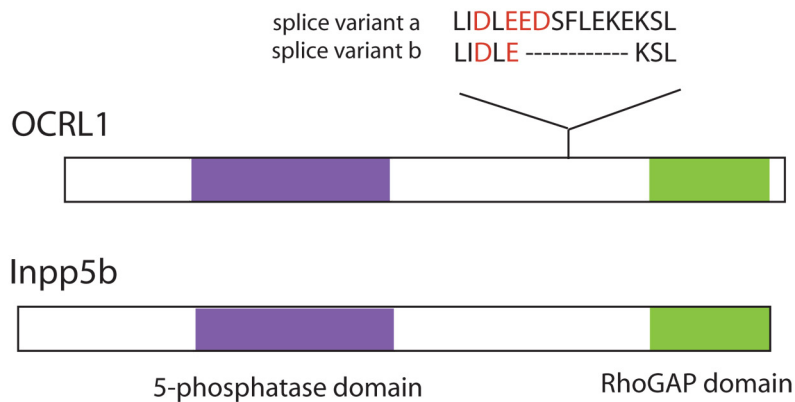


Figure 2.7: Domain structures of OCRL1 and Inpp5b

Humans and mice contain two 5-phosphatases with a Rho GAP-like domain, oculocerebrorenal syndrome of Lowe 1 (OCRL1) and Inpp5b. OCRL1 has two splice variants, termed a and b, that contained or lack an eight amino acid insertion immediately adjacent to the LIDLE clathrin-binding motif. In brain, only the a isoform is expressed, while b form is the major form in other tissues. (Adapted from Lowe M., 2005)

2.4: Aims of this work

The scope of this work is to study the regulation of phosphoinositides (PI(4)P and PI(4,5)P₂) at the Golgi complex by two distinct lipid phosphatases, hSAC1 and OCRL1. The function of PI 4-kinases in generating PI(4)P for maintenance of Golgi function and structural integrity is well accepted (Godi, Pertile et al. 1999; Wang, Wang et al. 2003; Godi, Di Campli et al. 2004). However, functional relevance of Golgi-localized PI-phosphatases is unknown. The fact that mutations in lipid phosphatases have led to various malacies in humans highlights the importance of studying compartment specific roles of lipid phosphatases.

hSAC1 is the main PI(4)P-phosphatase in the ER and Golgi while OCRL1 is the main PI(4,5)P₂ 5-phosphatase at the Golgi and endosomal membranes. The interplay between these two phosphatases in terminating PI(4)P and PI(4,5)P₂ signals at this compartment was investigated.

hSAC1 is the only integral membrane lipid phosphatase found in the Golgi and ER. Interestingly, hSAC1 contains the putative COPI interaction motif (KXXXX) at its C-terminal and the active phosphatase domain has been shown to be essential for sufficient recruitment/interaction with COPI (Rohde, Cheong et al. 2003). Since PI(4)P is an anterograde trafficking signal which recruits different transport machineries at the TGN, it is plausible that hSAC1 (a PI(4)P-phosphatase) is important to turn off PI(4)P signal for regulating Golgi function. We hypothesize a possible function of Golgi phosphoinositides in orchestrating functionally distinct regions/subdomains in the Golgi that segregates anterograde and retrograde trafficking within this organelle.

RNA interference (RNAi) was used to silent the expression of hSAC1 and OCRL1 in standard tissue culture cells. Functional analysis related to Golgi morphology, anterograde trafficking from the Golgi and lipid analysis were performed in hSAC1 and OCRL1-depleted cells independantly. Distinct pools of Golgi phosphoinositides were localized, visualized and analyzed using specific antibodies and GFP-fusion proteins that bind PI(4)P specifically. In addition, we analyze the distribution of hSAC1 and OCRL1 at the electron microscopy (EM) level to identify possible functional subdomains that are maintained by hSAC1 and OCRL1 within this organelle.

3 Results

3.1: Cellular localization of hSAC1

3.1.1: Localization of endogenous hSAC1

S.cerevisiae Sac1p was shown to localize to both ER and Golgi membranes (Whitters, Cleves et al. 1993; Foti, Audhya et al. 2001; Konrad, Schlecker et al. 2002). The cellular localization of rSac1 phosphatase had only been shown using indirect immunofluorescence against the Myc-tagged full length and the N-terminal portion of rSAC1 (amino acid 1-520) (Nemoto, Kearns et al. 2000). rSAC1 was found only in the ER. It was not clear whether hSAC1 resides only in the ER only or in both ER and Golgi as in yeast Sac1p. Therefore, we looked at the localization of endogenous hSAC1 using a specific antiserum (#69). In scanning confocal microscopy, hSAC1 was found in both ER and Golgi compartments as determined by colocalization with ER (Sec61 α) and Golgi (p58) markers. The GFP-hSAC1 fusion protein is also found in both cellular compartments as the endogenous hSAC1 (Rohde, Cheong et al. 2003).

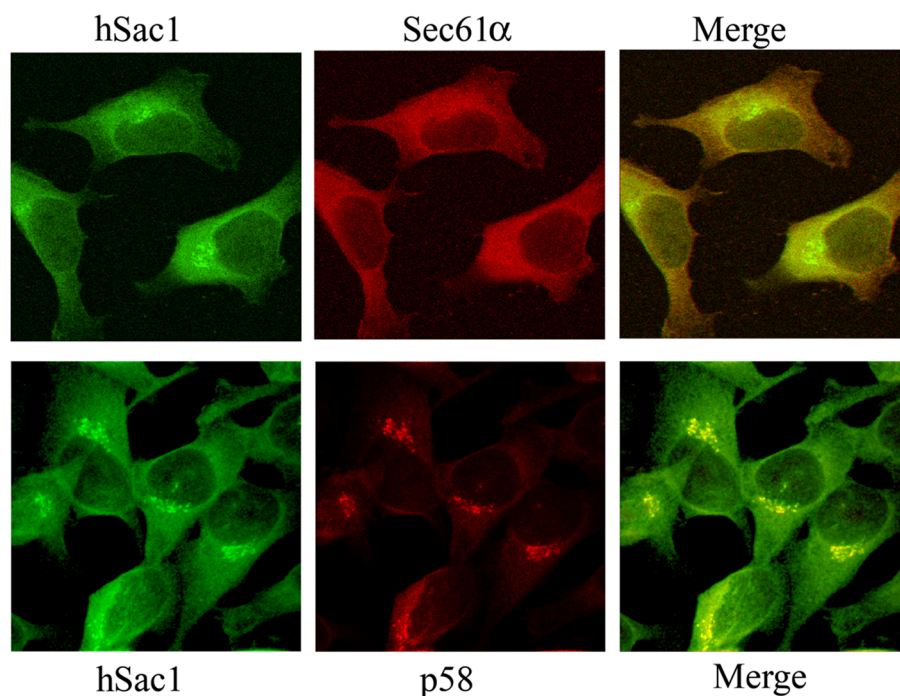


Figure 3.1: Endogenous hSAC1 localized to both ER and Golgi compartments (Rohde et al., 2003).

HeLa cells were fixed with 4% formaldehyde, permeabilized using 0.4% TritonX and stained with either an ER marker, Sec61 α or a Golgi marker, p58k.

3.1.2: The C-terminus of hSAC1 localizes to the ER

Similar to yeast Sac1p, hSAC1 contains two potential transmembrane domains at its C-terminus (amino acid 521-543; amino acid 550-569) (Rohde, Cheong et al. 2003)(Introduction Section 2.3.3.2). HeLa cells transfected with constructs in which the entire N-terminal cytosolic domain of hSAC1 was deleted were found to be restricted to the ER (Figure 3.2). This indicates that the N-terminal portion of hSAC1 is important for Golgi localization of this phosphatase.

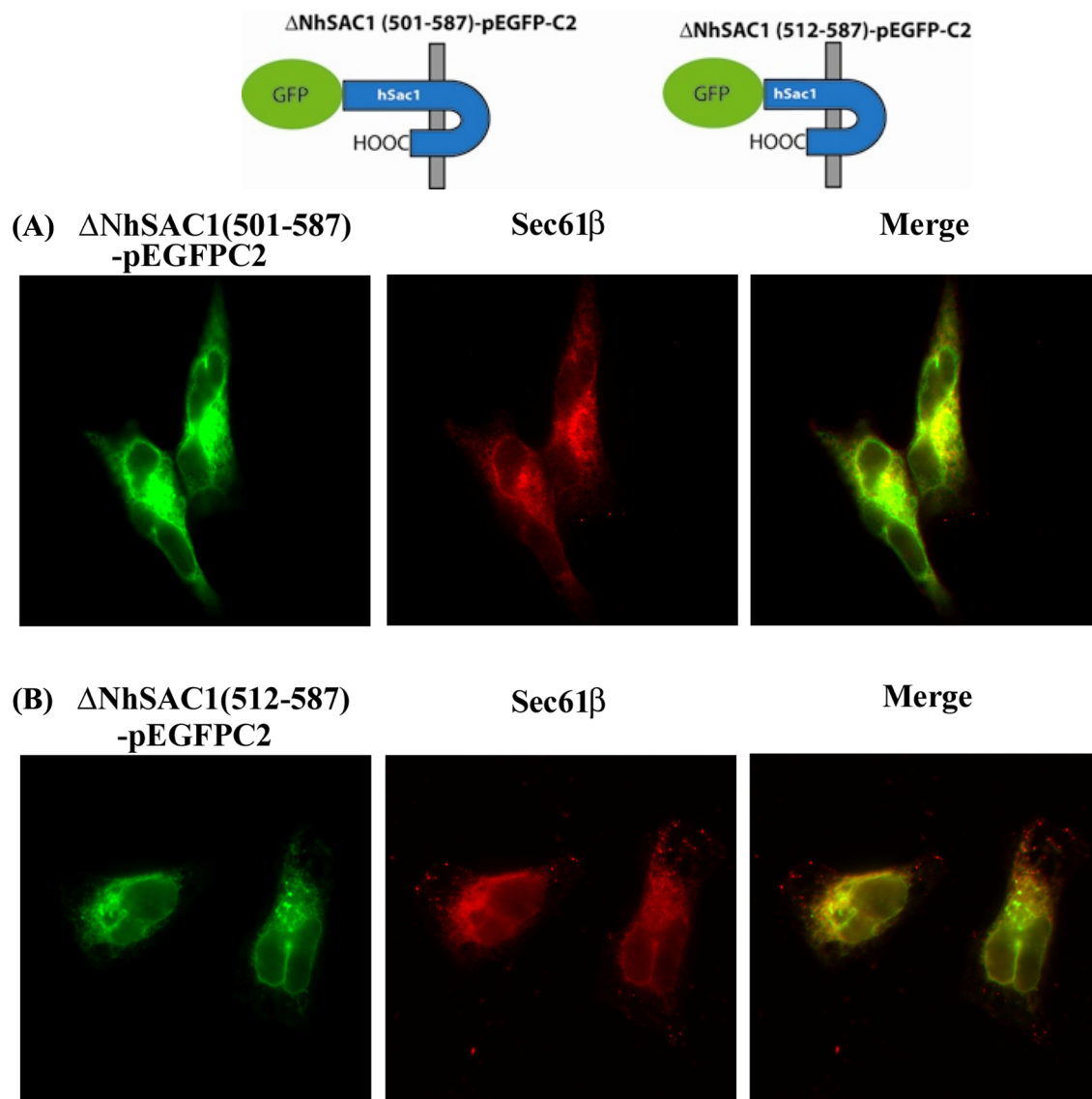


Figure 3.2: The C-terminus of hSAC1 localized to the ER: (A) and (B): Δ NhSAC1(501-587)pEGFPC2 and Δ NhSAC1(512-587)pEGFPC2 expressed in HeLa cells. Both N-terminal mutants localized to the ER as shown with ER marker, Sec61 β

3.1.3: A significant portion of hSAC1 is found at the Golgi complex

At steady state, hSAC1 is found in both ER and Golgi. Yeast Sac1p was shown to relocate to the Golgi complex during starvation to regulate secretion in response to growth conditions (Faulhammer et al., 2005). The shuttling mechanism is conserved from yeast Sac1p to hSAC1 (Blagoveschenskaya A. et al., 2006 manuscript submitted). However, a significant portion of hSAC1 localized to the Golgi at all times (regardless of growth condition). This indicates that the Golgi population of hSAC1 might have a constitutive function at this compartment. Furthermore, PI(4)P was reported to be concentrated at the Golgi complex in both yeast and mammals. Thus, it was interesting to investigate the function of the PI(4)P-phosphatase, hSAC1, at the Golgi in regulating compartment specific PI(4)P signals.

We first looked at the Golgi distribution of endogenous hSAC1 and GFP-hSAC1 in NIH-3T3 cells by co-localization studies with different Golgi markers (Figure 3.3). hSAC1 was found to be distributed throughout the *cis* to the *trans*-Golgi as shown by colocalization with *cis*, *medial* and *trans*-Golgi markers in scanning confocal microscopy (Figure 3.3). However, we found no complete overlap between hSAC1 and any individual Golgi marker. Therefore, we performed immunogold-labeling and electron microscopy to study the sub-Golgi distribution of hSAC1 in collaboration with Prof. Judith Klumperman (Utrecht, Netherlands). The GFP-hSAC1 was found to be concentrated at cisternal Golgi membranes in low expressing Hela cells (Figure 3.4) while Fapp1-PH domain (a PI(4)P-binding probe) was located at the TGN (Godi et al., 2004).

3.1.4: Detection of Golgi PI(4)P using the Fapp1-PH domain and PI(4)P antibody

The intracellular localization of different phosphoinositide species can be studied using fluorescently labeled phosphoinositide binding domains (Downes, Gray et al. 2005). The discovery of many specific phosphoinositide binding modules had led to advances in visualizing phosphoinositide pools and an understanding of their spatial and temporal dynamics in live cells (Varnai and Balla 2006). The Fapp1-PH domain is one of the most studied PI-binding modules which was initially found to bind PI(4)P only (Dowler, Currie et al. 2000).

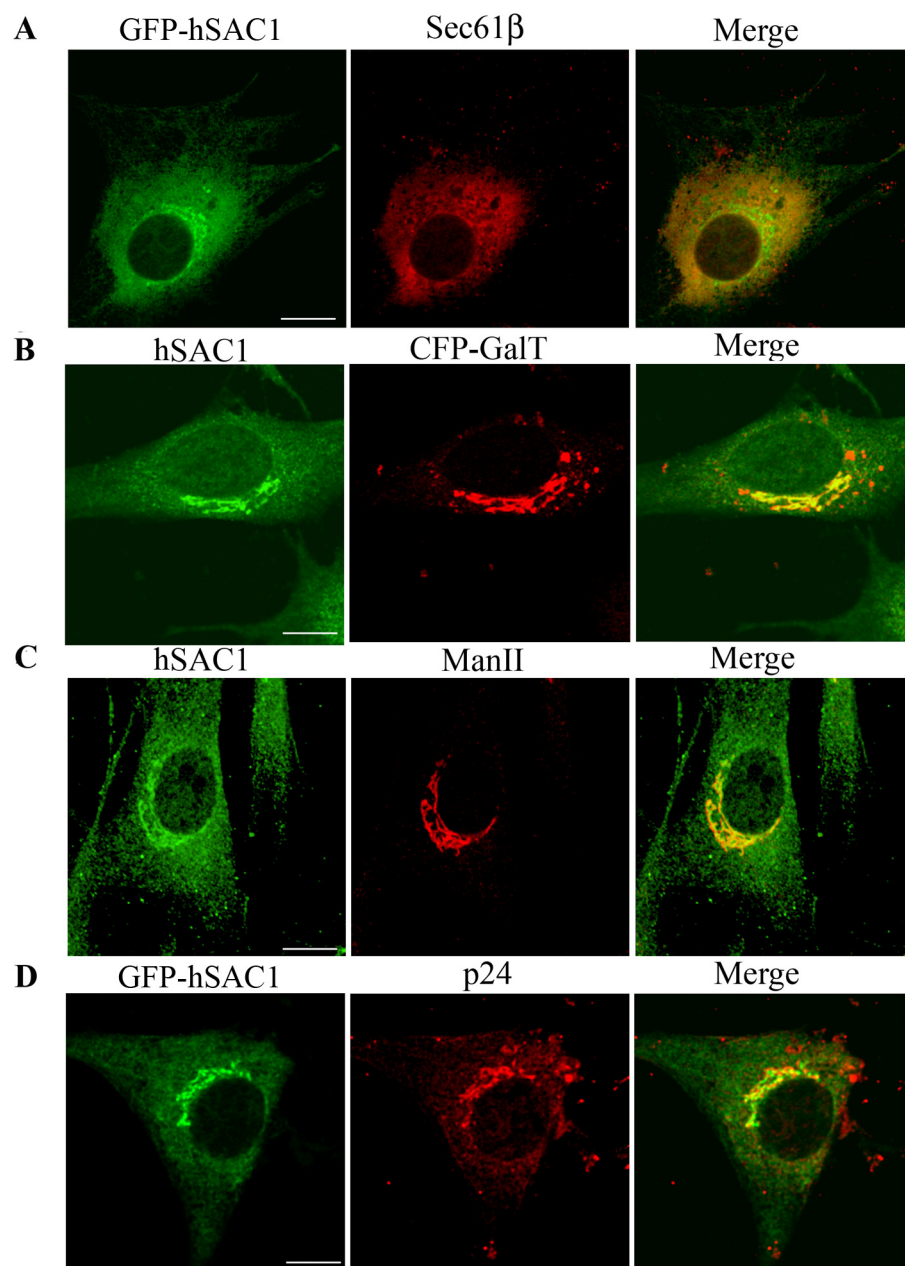


Figure 3.3: Golgi population of hSAC1

(A): GFP-hSAC1 showed both ER and Golgi localization in NIH-3T3 cells

(B): hSAC1 colocalized well with the trans Golgi marker, GalT-CFP, medial Golgi marker, mannosidase II (C) and cis Golgi marker, p24 (D).

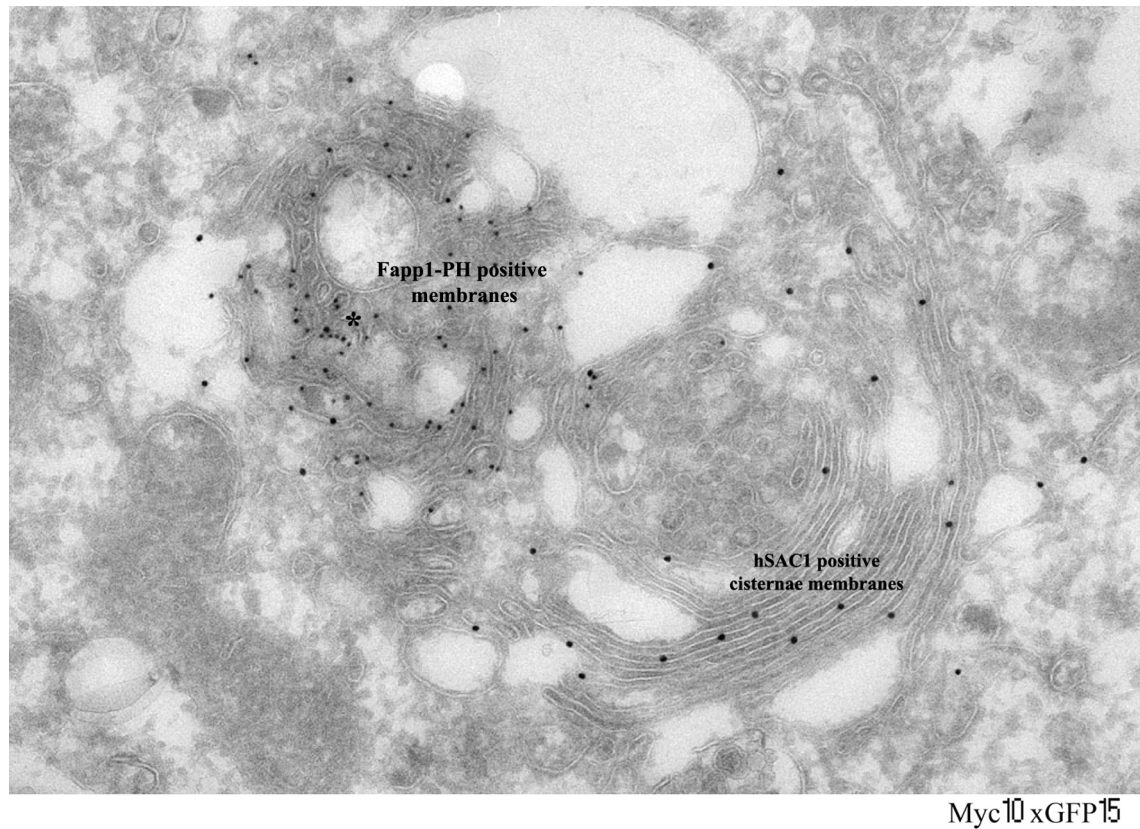


Figure 3.4: Majority of hSAC1 was found at cisternal Golgi membranes while the Fapp1-PH domain was located at the TGN.

Hela cells co-expressing GFP-hSAC1 and Fapp1-PH-Myc were fixed (according to Section 5.2.4.8) and processed for immunogold-labeling (collaboration with Klumperman's lab). GFP-hSAC1 positive membranes were devoid of Fapp1-PH-Myc. Asterisk marked showed a limited co-localization of GFP-hSAC1 and Fapp1-PH-Myc.

However, recent studies by the De Matteis laboratory have shown that Fapp1-PH domain binds not only to PI(4)P, but also to the small GTPase Arf1 (Godi et al, 2004). To further confirm that the Fapp1-PH-RFP stained regions represent the PI(4)P enriched Golgi regions, we compared the localization of both GFP-tagged and RFP-tagged Fapp1-PH domains with a specific PI(4)P antibody. Figure 3.5 shows that both GFP and RFP-tagged PH-domains of Fapp1 label the same Golgi regions. However, the GFP-tagged probe gave a higher background of diffuse cytosolic staining.

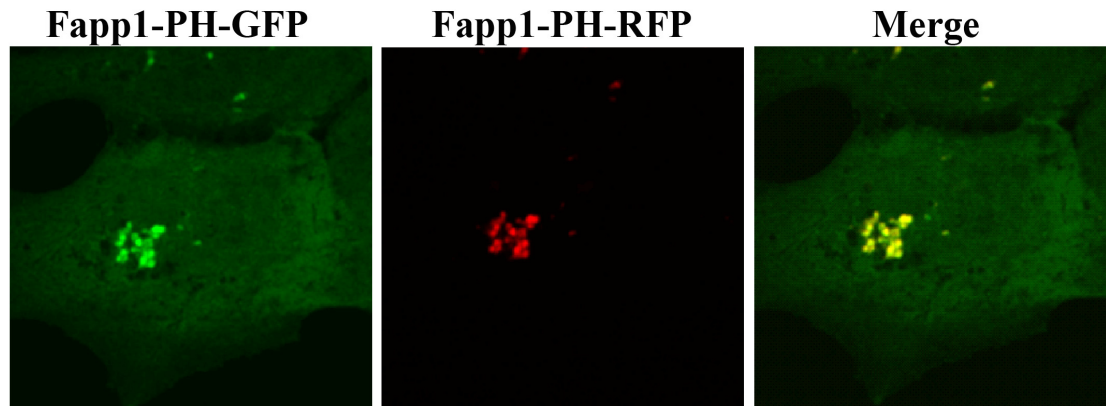


Figure 3.5: Both GFP and RFP-tagged Fapp1-PH domains localized to the same Golgi regions

The Fapp1-PH domain was fused with either GFP or RFP. Both fusion proteins localized to the perinuclear structures that resembled the Golgi complex where PI(4)P is highly enriched.

In addition, we found that most regions positive for Fapp1-PH staining were also stained by the PI(4)P antibody (Figure 3.6). However, there are non-overlapping regions contributed by the antibody and the Fapp1-PH domain. This suggests that there are additional PI(4)P pools in the Golgi that are not recognized by the Fapp1-PH domain. However, in both approaches to localize Golgi PI(4)P pools, we were able to confirm that hSAC1 is found in regions that are devoid of PI(4)P.

3.1.5: hSAC1 creates low PI(4)P regions in the Golgi

PI(4)P is the main phosphoinositide species at the Golgi complex in both yeast and mammals (De Matteis, Di Campli et al. 2005; Weixel, Blumental-Perry et al. 2005). It is well established that the Golgi associated PI 4-kinases play important roles in generating Golgi PI(4)P pools (Balla and Balla 2006). However, it remains largely unknown how the Golgi resident lipid phosphatase, hSAC1, contributes to the regulation of PI(4)P at the Golgi. Because PI(4)P is essential for anterograde transport carrier formation, we hypothesized that hSAC1 phosphatase activity may be required for terminating PI(4)P signals at this compartment.

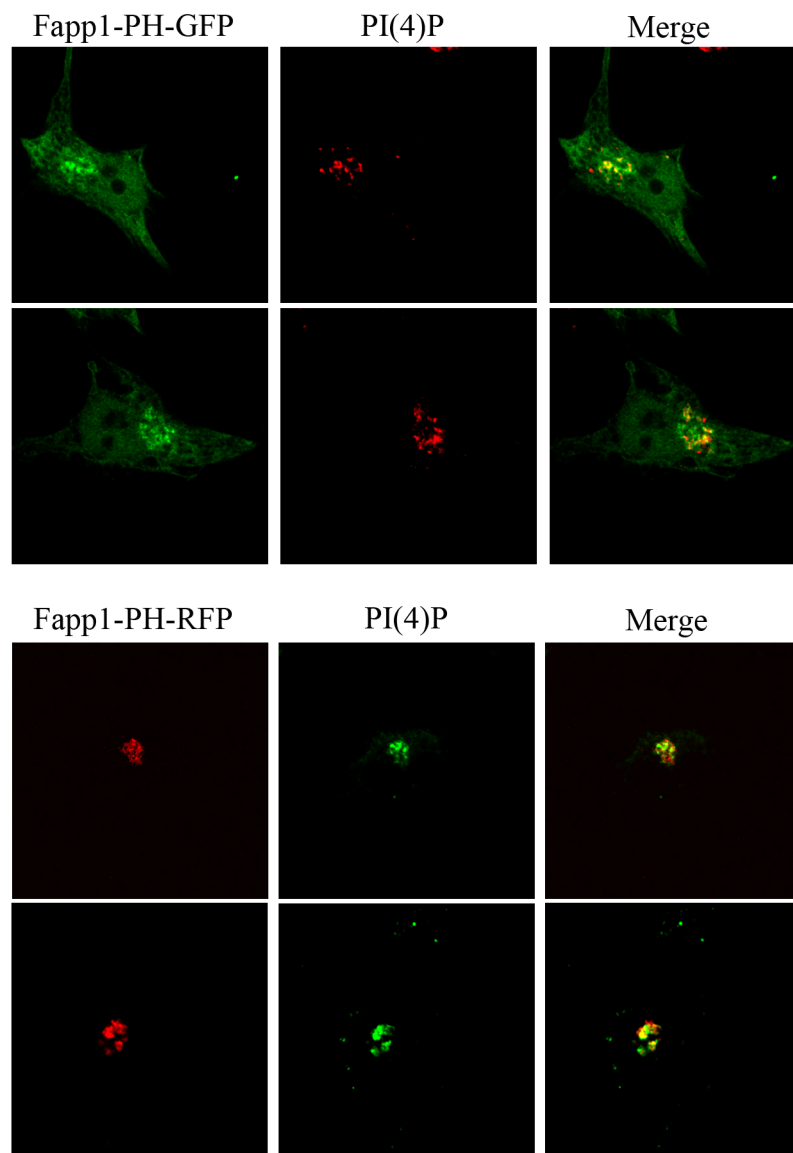


Figure 3.6: PI(4)P staining by Fapp1-PH domain and PI(4)P antibody

HeLa cells expressing either the GFP (Top panel) or RFP-tagged Fapp1-PH domain (lower panel) were stained with PI(4)P antibody and viewed by scanning confocal microscopy. Both Fapp-PH domain and PI(4)P staining colocalized well but there were PI(4)P antibody stained regions that were not stained by the Fapp1-PH domain. This indicates that there are different pools of PI(4)P that are not recognized by this PI(4)P-binding module.

We therefore compared the distribution of PI(4)P with the localization of hSAC1. To this end, we used a specific PI(4)P-binding probe made of the Fapp1-PH domain fused with RFP. hSAC1 was found enriched in Golgi regions that were not stained by the Fapp1-PH-RFP, usually in adjacent structures (Figure 3.7). To ensure that the Fapp1-PH-RFP signals we observed represented PI(4)P independent of Arf1, we performed another co-localization study of hSAC1 and PI(4)P using a specific PI(4)P antibody (Figure 3.8). Both results suggested that PI(4)P and hSac1 localized to distinct regions within the Golgi.

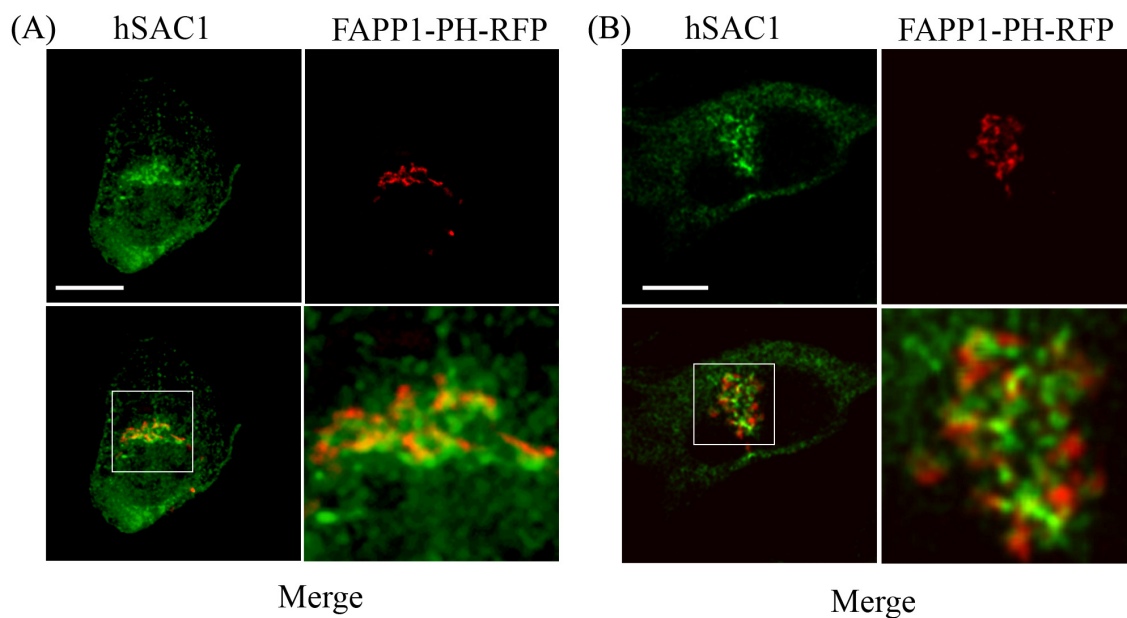


Figure 3.7: hSac1 localizes to PI(4)P-depleted Golgi regions.

- (A) NIH3T3 cells expressing FAPP1-PH-RFP (red) were stained with antibodies against hSac1 (green) and examined by confocal immunofluorescence microscopy. The boxed area is enlarged in the adjacent panel. The Scale bars represent 15 μ m
- (B) HeLa cells expressing FAPP1-PH-RFP (red) were stained with antibodies against hSac1 (green) and examined by confocal immunofluorescence microscopy. The boxed area is enlarged in the adjacent panel. The Scale bars represent 10 μ m .

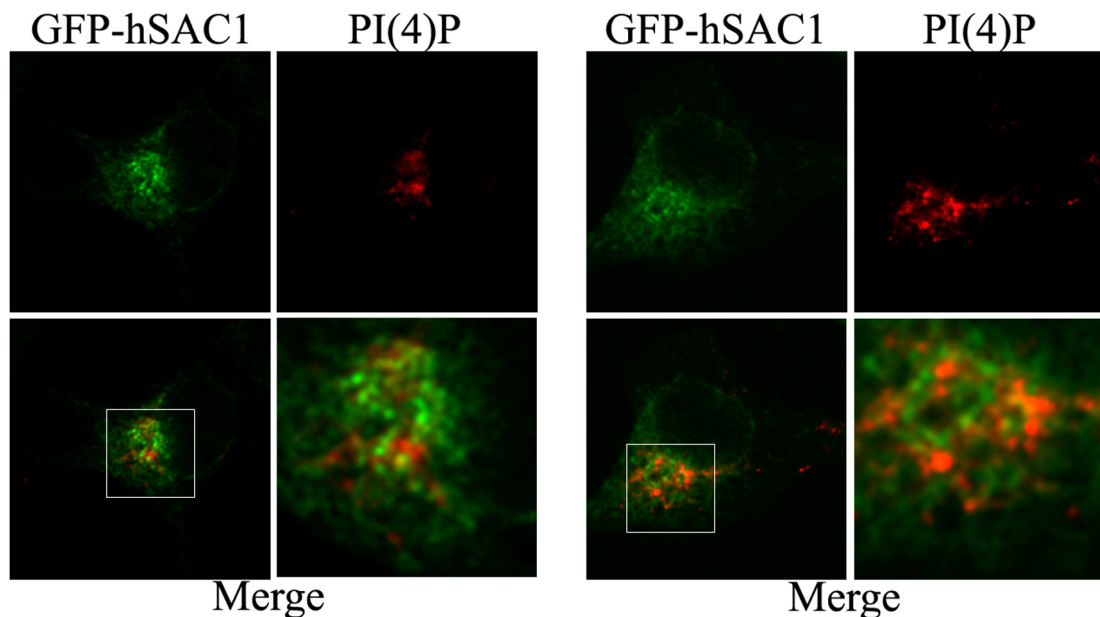


Figure 3.8: PI(4)P and hSAC1 defined different Golgi regions

HeLa cells expressing GFP-hSAC1 were stained with a specific PI(4)P antibody. hSAC1 localizes to Golgi regions where PI(4)P is not present.

To demonstrate that the phosphatase activity of hSAC1 is responsible for creating the PI(4)P-depleted regions in the Golgi, we examined the localization of a phosphatase-dead mutant of hSAC1 (GFP-hSAC1-C/S) in relation to Fapp1-PH-RFP and PI(4)P antibody staining. In contrast to wild type hSAC1, the GFP-hSAC1-C/S mutant was found to colocalize significantly with the PI(4)P-binding probe, Fapp1-PH-RFP (Figure 3.9A) and the PI(4)P antibody (Figure 3.9B). This result supports the idea that the phosphatase activity of hSAC1 is important for maintaining low PI(4)P Golgi regions.

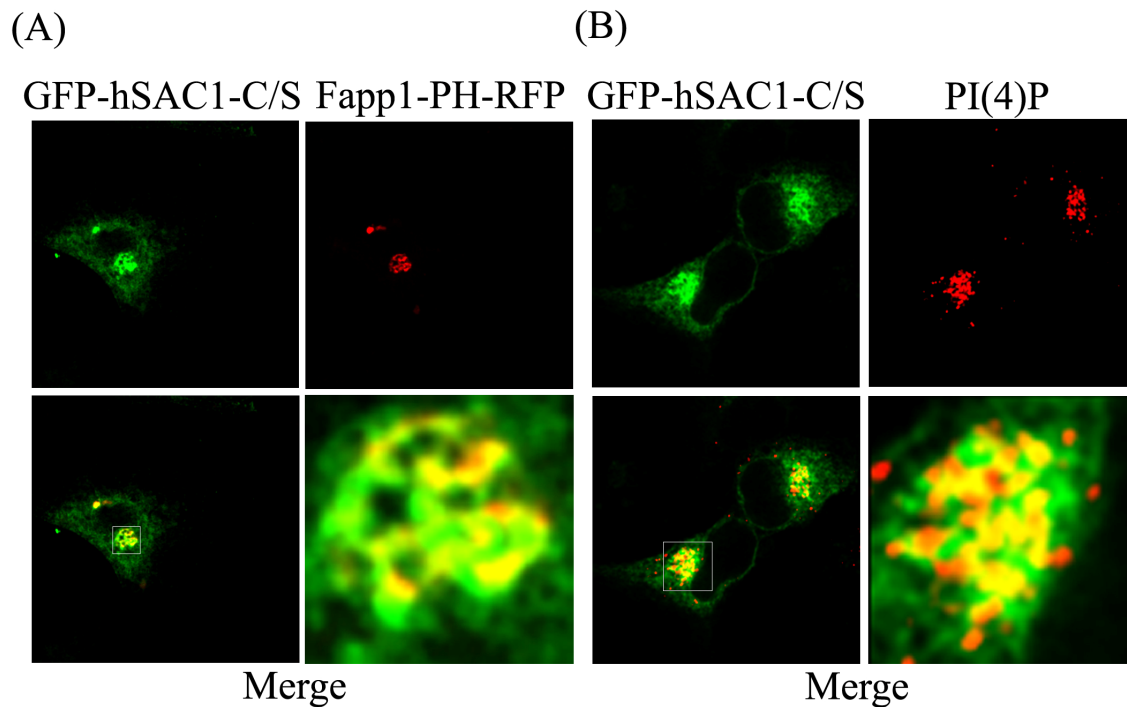


Figure 3.9: PI(4)P-phosphatase activity of hSAC1 is responsible for generating PI4P-depleted regions in the Golgi.

(A) HeLa cells expressing both GFP-hSAC1-C/S and Fapp1-PH-RFP were fixed with 4% formaldehyde and examined under scanning confocal microscopy.

(B) PI(4)P antibody staining was performed in HeLa cells expressing GFP-hSAC1-C/S after fixation with 2% formaldehyde and permeabilized with 10 μ g/ml digitonin in 100mM glycine PBS. In both attempts, GFP-hSAC1-C/S was found to localize to high PI(4)P regions

3.1.6: Immunogold labeling and electron microscopy studies of Golgi subdomains populated by PI(4)P and hSAC1

The striking staining pattern of hSAC1 adjacent to the Fapp1-PH-RFP stained structures suggests that the Golgi complex may be organized into functional regions that are defined by the amount of PI(4)P. PI(4)P was shown to be concentrated at the TGN by a detailed analysis of intra-Golgi distribution of the Fapp1-PH domain using immunogold EM analysis (Godi et al., 2004). To answer the question of whether hSAC1 creates low PI(4)P regions within the same Golgi cisternae, we analyzed the distribution of Fapp1-PH domain and hSAC1 in HeLa cells by immunogold-EM (in collaboration with Judith Klumperman's lab).

In low expressing HeLa cells, there were limited positive cisternae where both hSAC1 and Fapp1-PH were present (Figure 3.10). However, hSAC1 and Fapp1-PH domains were found in the same Golgi membranes when both fusion proteins were expressed at high levels (Figure 3.11). Interestingly, high level expression of Fapp1-PH domain caused membrane cluster formation that contained low amounts of hSAC1 (Figure 3.12). However, high expression of hSAC1 also induced membrane networks that were distinct from Fapp1-PH-induced membranes (Figure 3.12).

These data clearly show that hSAC1 is largely present at Golgi regions different from where Fapp1-PH-RFP is concentrated. The opposing gradient of PI(4)P (highly enriched at TGN) and the PI(4)P-phosphatase hSAC1 (enriched in cisternal membranes) in the Golgi indicates that hSAC1 is needed at this compartment to maintain PI(4)P-depleted Golgi regions. hSAC1 enrichment at Golgi cisternal membranes might contribute to functional organization of this organelle.

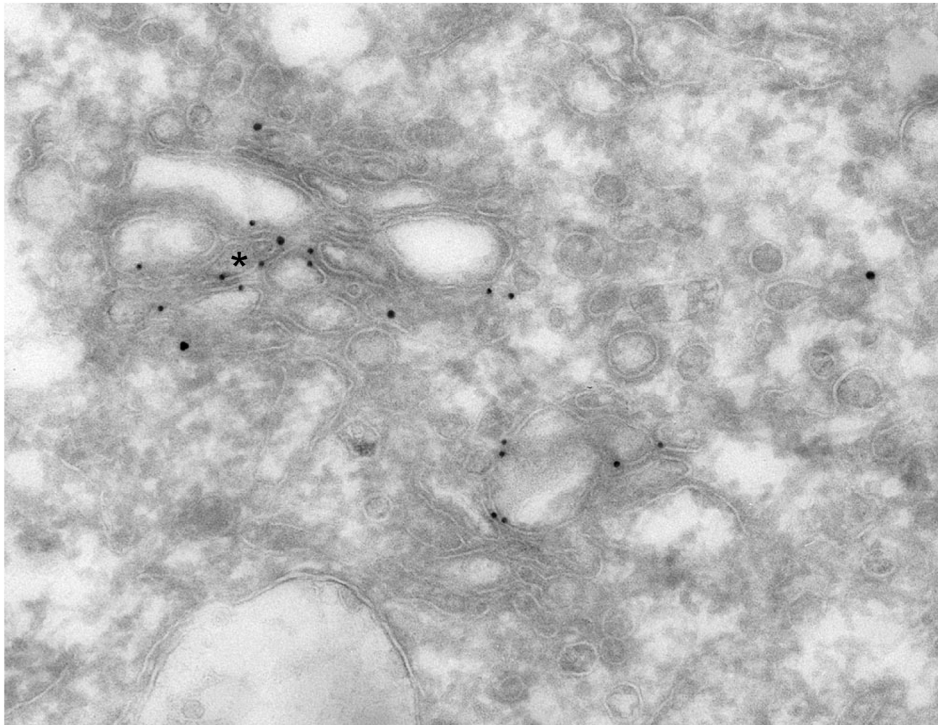


Figure 3.10: hSAC1 and Fapp1-PH domain showed limited colocalization in low expression HeLa cells
HeLa cells transfected with GFP-hSAC1 and Myc-Fapp1-PH-RFP were fixed according to the EM fixation protocol described in section 5.2.4.8. GFP-hSAC1 is marked by the presence of 15nm-gold particle while the 10nm-gold particles represent Fapp1-PH domain. The asterisk marks a subdomain of Golgi membranes where both hSAC1 and Fapp1-PH domain are present.

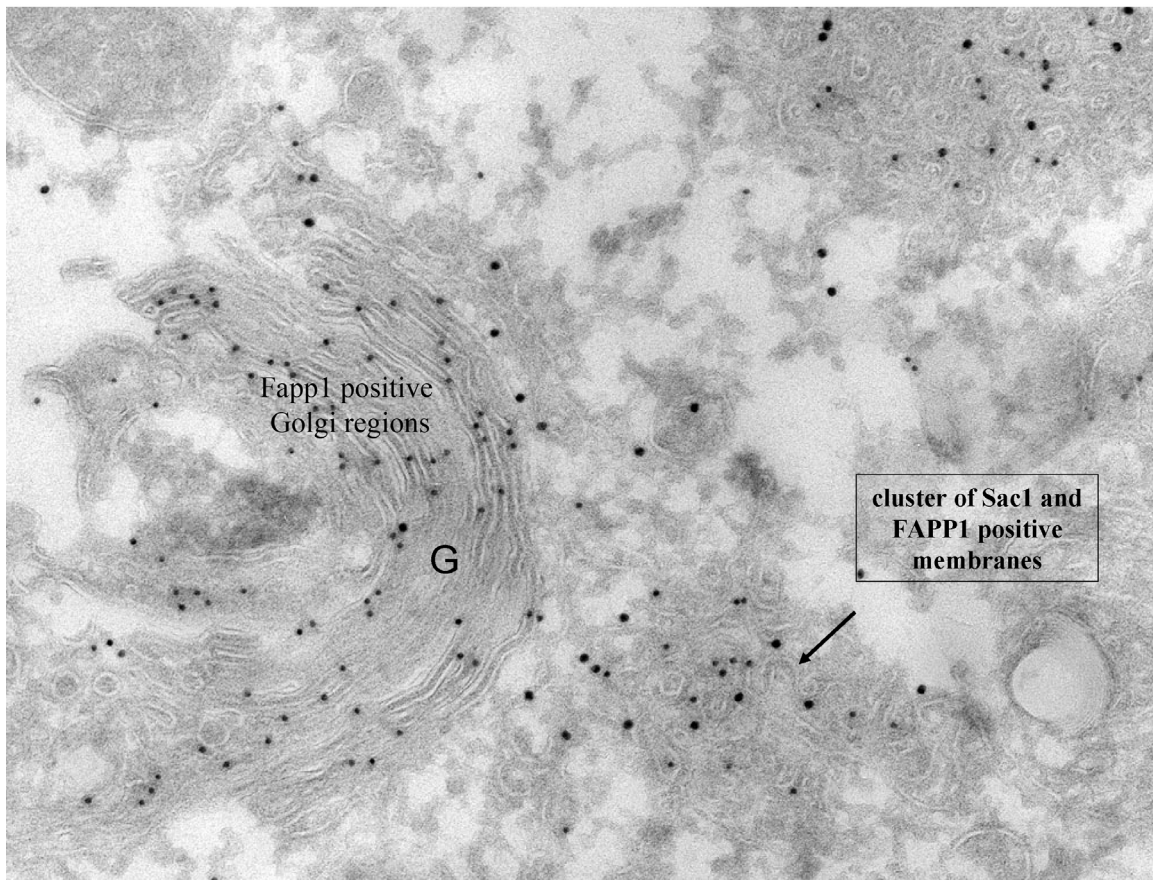
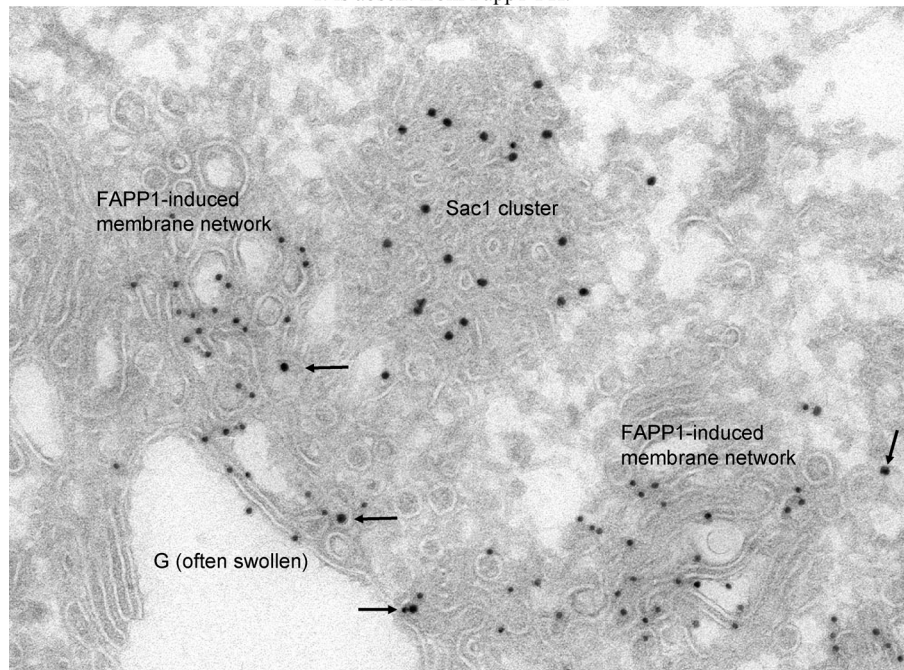


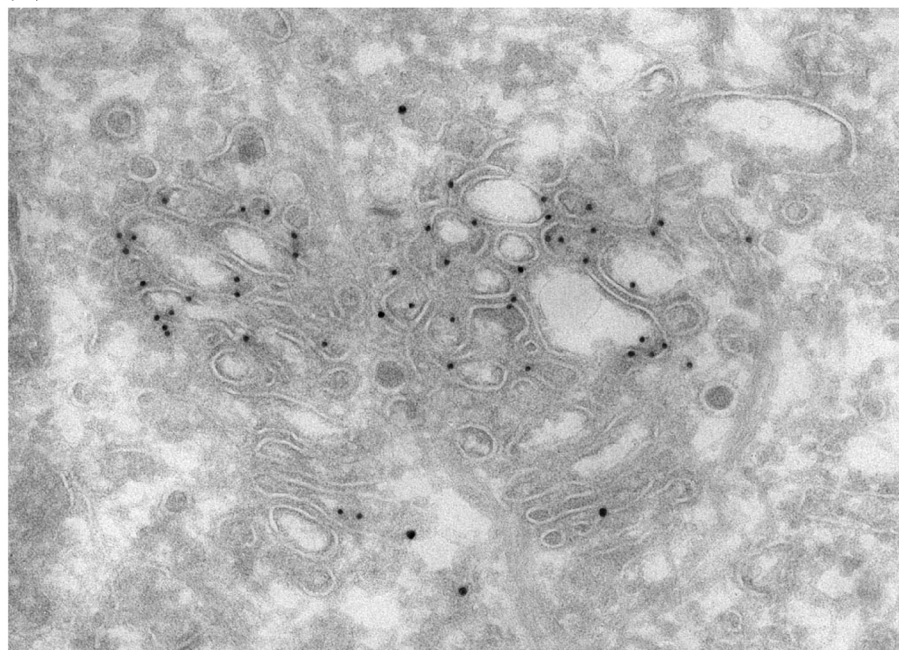
Figure 3.11: Fapp1-PH-Myc and GFP-hSAC1 were located at different Golgi regions except when Fapp1-PH-Myc was expressed in high level.

G: Golgi; Fapp1 positive regions that were devoid of hSAC1(left); a cluster of hSAC1 and Fapp1 positive membranes (right)

- (A) High level expression of hSAC1 induced membrane network that is distinct from Fapp1-induced network. It is absent from Fapp1-PH.



- (B) Localization of Fapp1-PH-Myc and hSAC1-GFP in Hela cells



Myc10 and GFP15

Figure 3.12: High expression of Fapp1-PH-Myc induced membrane networks that contained hSAC1
 (A) High level expression of Fapp1-PH-Myc and GFP-hSAC1 induced formation of distinct membrane networks. Fapp1-PH-Myc induced membrane networks contained low amount of hSAC1(B) Fapp1-PH-Myc and GFP-hSAC1 localized to the same membrane.

3.2: Depletion of hSAC1 using vector-based RNAi and synthetic siRNAs

RNAi (RNA interference) is a conserved cellular defense mechanism that is used by multicellular organisms against viral invasion, transposon expansion and post-transcriptional regulation (Tuschl, Zamore et al. 1999; Zamore 2002). It has been widely used for functional gene studies in plants, *C.elegans* and *D.melagogaster* but its applications in mammalian cell lines have only become successful with the discovery of sequence-specific inhibition of target mRNA by using short, synthetic RNA duplexes of 19-25bp (Elbashir, Harborth et al. 2001; Elbashir, Lendeckel et al. 2001; Elbashir, Harborth et al. 2002). However, this potent gene suppression technique using synthetic RNA is transient. To circumvent this problem, Brummelkamp et al. developed a vector-based siRNA expression system which allows stable expression of siRNA in mammalian cells over long periods of time (Brummelkamp, Bernards et al. 2002). Expression of short hairpin RNA (shRNA) via plasmid utilizes RNA polymerase III-based promoters. shRNAs are then processed into siRNAs by Dicer to induce gene silencing via the RNAi pathway (Wu, Wu et al. 2005).

To silence the expression of hSAC1, five different RNAi constructs were generated using the IMGENEX pSuppressor system as described in section 5.2.1.6 . The potency of knocking down endogenous hSAC1 was tested by Western blotting using a specific antiserum against hSAC1 (#69). In addition, expression of GFP-hSAC1 was compared under both control and hSAC1 knock down conditions. The control RNAi construct was made by creating 5 point mutations to the original sequence of construct I, which showed the highest potency in silencing hSAC1 expression.

As shown in Figure 3.13, construct I and II show the best suppression of GFP-hSAC1 and endogenous hSAC1. Construct I was used in subsequent experiments. Synthetic siRNA of the same sequence as construct I was used in parallel to the vector-based RNAi.

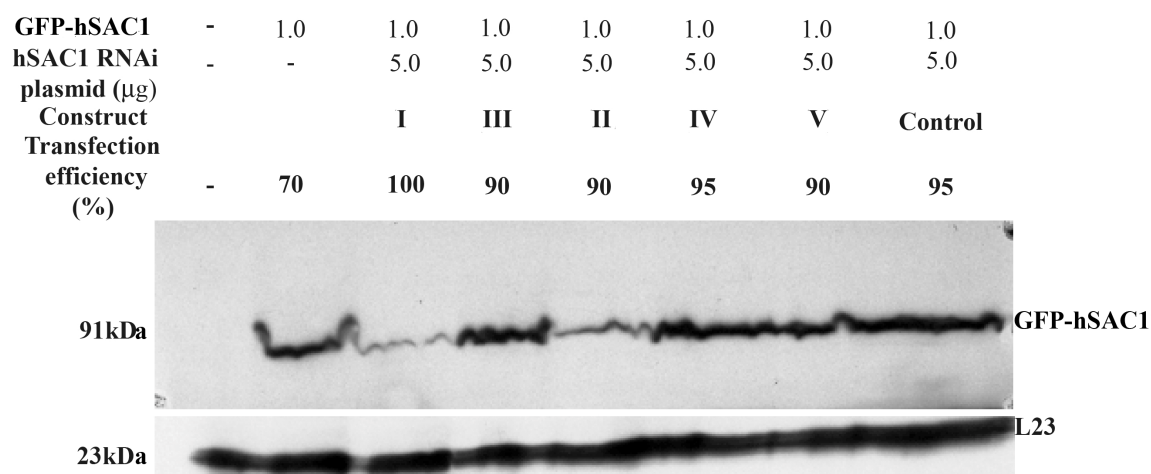


Figure 3.13: Silencing of GFP-hSAC1 expression using vector-based RNAi

Construct I-V were generated by cloning of oligonucleotides corresponding to hSAC1 siRNA sequences identified by using guide lines listed in (Harborth, 2003 ; <http://www.rockefeller.edu/labheads/tuschl/sirna.html>) . HeLa cells were transfected with RNAi constructs together with a plasmid encoding GFP-hSAC1. Depletion of GFP-hSAC1 was determined using Western blot and antibodies against GFP and a loading control L23 protein. Beta-gal assay was used to measure transfection efficiency.

Interestingly, endogenous hSAC1 appeared to be more stable than the ectopically expressed GFP- hSAC1 as shown in Figure 3.14 (and data not shown). The silencing effect could be detected 48hrs post-transfection for the GFP-hSAC1 but the endogenous hSAC1 depletion was observed only at 72hrs post-transfection and persisted till 120 hrs post-transfection. All the assays for studying the function of hSAC1 in HeLa cells depleted of hSAC1 were performed between 72hrs to 96 hrs post-transfection.

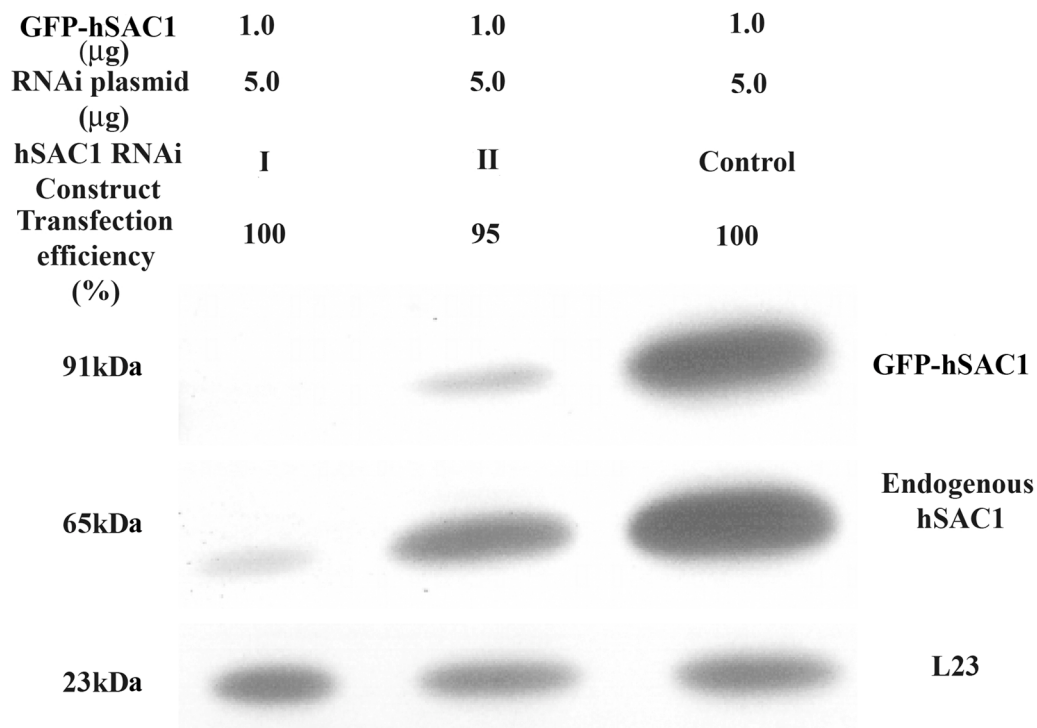


Figure 3.14: Knock down of GFP-hSAC1 and endogenous hSAC1 using Vector-based RNAi .

Construct (I) and (II) were used to deplete hSAC1. HeLa cells were transfected with both RNAi plasmids and hSAC1-GFP. Transfection efficiency was assayed using beta-gal assay.

In addition, efficiency of GFP-hSAC1 depletion was determined by examining the fluorescent GFP signals in HeLa cells co-transfected with GFP-hSAC1 as shown in Figure 3.15. Both results from Western blot (WB) and immunofluorescence (IF) showed that construct I was more potent than construct II in depleting hSAC1 levels. The RNAi silencing of hSAC1 using this vector-based method is specific because there was no reduction of hSAC1 in a control construct which contained 5 point mutations in the siRNA sequence of construct I.

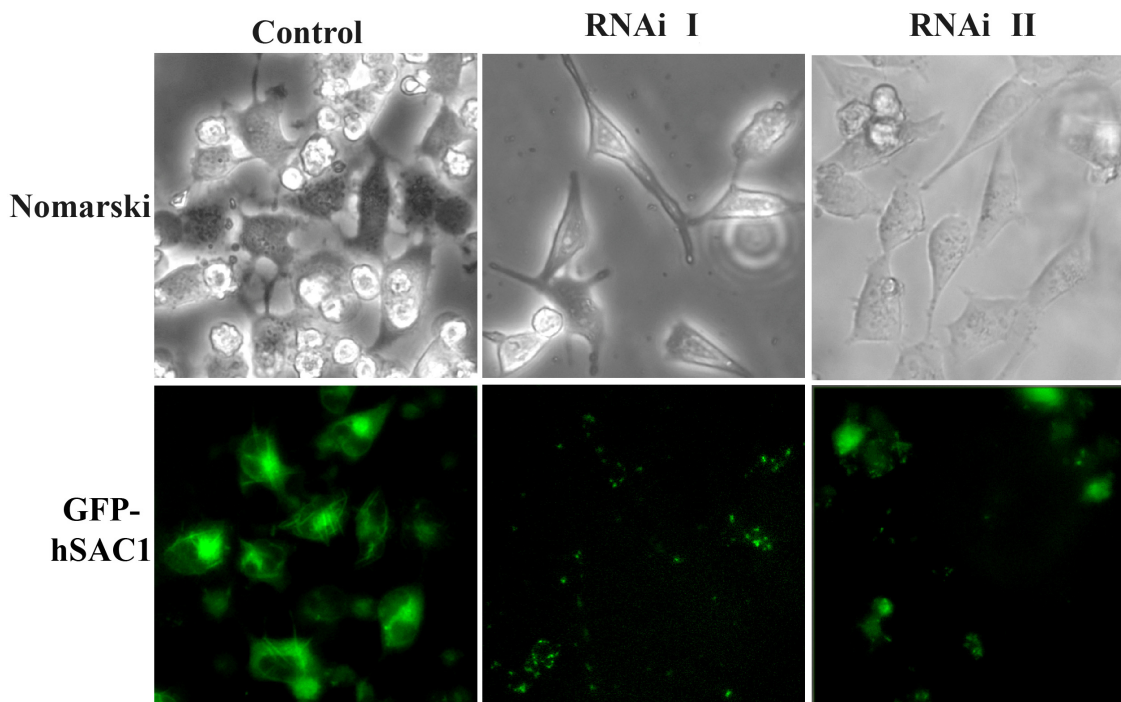


Figure 3.15: Fluorescence micrographs showing depletion of GFP-hSAC1 using plasmids that expressed siRNAs against hSAC1. HeLa cells transfected with different hSAC1 RNAi or control plasmids plus GFP-hSAC1 were fixed with 4% formaldehyde 72hrs post-transfections and viewed under the fluorescence microscope.

HeLa cells were used in all hSAC1 knock-down experiments because it is a well transfected standard tissue culture cell line and with defined intracellular structures that can be viewed after immunostaining under standard fluorescence microscope or laser scanning confocal microscope.

3.2.1: HPLC analysis of HeLa cells depleted of hSAC1

PI(4)P is the major lipid substrate of the hSac1 phosphatase. To analyze the total cellular phosphoinositide levels in particularly PI(4)P levels that are regulated by hSAC1, we knocked down hSac1 expression and did HPLC analysis. Depletion of hSac1 in HeLa cells led to a moderate increase in total cellular PI(4)P levels but had no significant effect in PI(4,5)P₂ level (Figure 3.16).

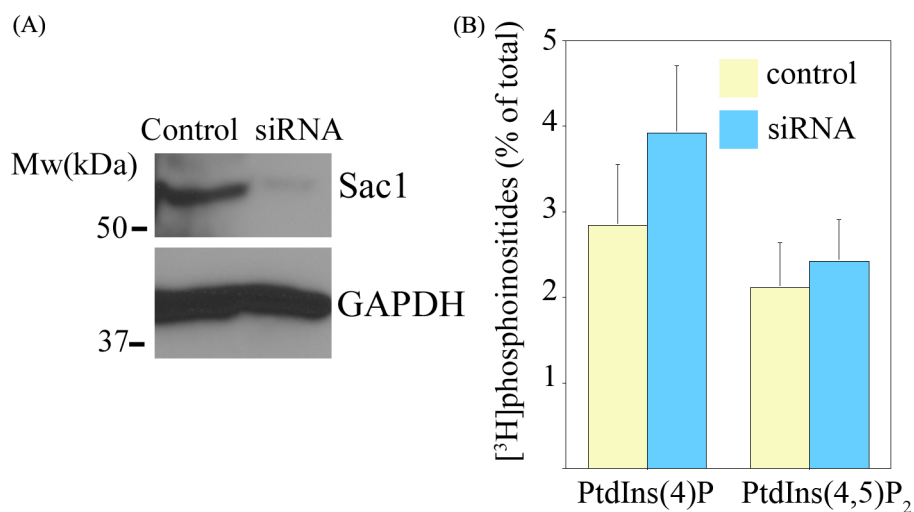


Figure 3.16: HPLC analysis of HeLa cells expressing hSAC1 RNAi and Control plasmids.

(A) HeLa cells depleted of hSAC1 were labeled with 10 μ Ci/ml of [³H]-myo-inositol to steady state for 12 hours. Lipids were extracted, deacylated and analyzed using HPLC.

(B) HPLC analysis of phosphoinositides in HeLa cells treated with hSAC1 siRNA.

This analysis showed that hSAC1 is a PI(4)P-phosphatase. Although changes in total PI(4)P levels is not as drastic as in the yeast Sac1p null mutants, local changes of PI(4)P pools in hSAC1 knock down cells might be sufficient in affecting compartment specific function. Therefore, we examined local changes of PI(4)P in hSAC1 depleted cells by PI(4)P antibody staining.

3.2.2: Elevated levels of PI(4)P in hSAC1-depleted cells are found at the plasma membrane and vesicular like structures

The increased levels of PI(4)P detected by HPLC were also supported by immunofluorescence studies of PI(4)P staining using a specific antibody in hSAC1 knock down cells. Under control conditions, PI(4)P was confined to the Golgi. In contrast, hSAC1-depleted cells showed a highly increased signal of PI(4)P and additional PI(4)P staining of other cellular compartments besides the Golgi. The plasma membrane and cytosolic vesicular structures were stained positive with PI(4)P antibody (Figure 3.17). This is in line with a recent report which shows that Sac1p null mutants have accumulation of PI(4)P in the endosomal compartment, ER and the plasma membrane (Tahirovic et al., 2005). These findings strongly support the idea that hSAC1 is a spatial regulator for restricting PI(4)P to specific Golgi regions.

In addition to accumulation of PI(4)P at Golgi membranes and other cellular compartments, N-acetylglucosaminyltransferase I (NacT1) was found to mislocalize in hSAC1-depleted cells (Figure 3.17). NacT1GFP was found to localize to the cell periphery in addition to diffuse, punctate vesicle-like structures. In control cells, NacT1GFP was only found at the Golgi compartment.

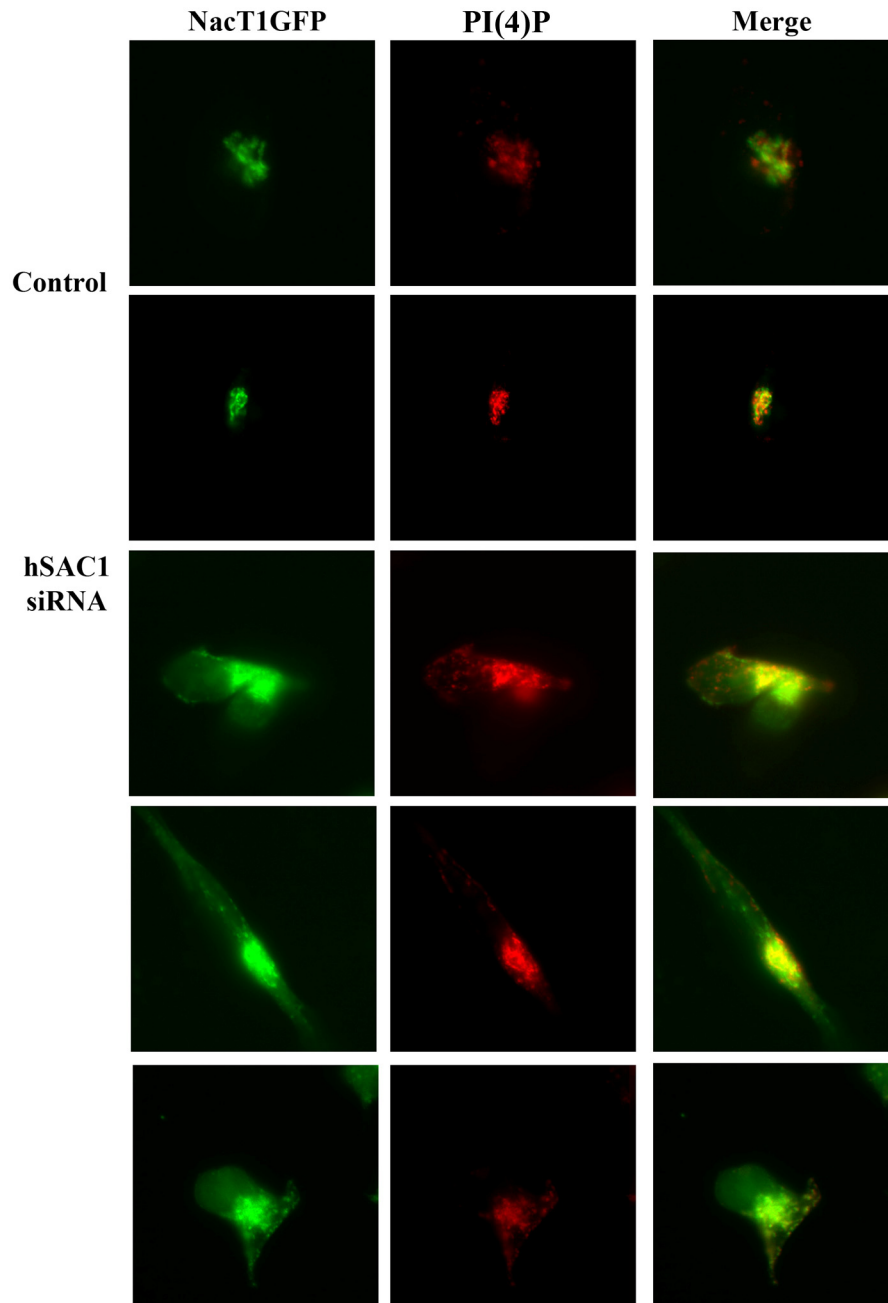


Figure 3.17: Depletion of hSAC1 abolished the spatially restricted distribution of PI(4)P at Golgi membranes.

Hela cells treated with control or hSAC1 siRNAs were co-transfected with NacT1GFP as transfection marker. Cells were fixed with 2% formaldehyde 72hrs post-transfection and permeabilized using digitonin as described in section 5.2.4.3. PI(4)P staining was restricted at Golgi membranes in control cells while hSAC1-depleted cells showed extensive PI(4)P staining at Golgi, plasma membrane and vesicle-like structures.

3.2.3: Golgi resident enzymes are preferentially located at low PI(4)P Golgi regions

The observation that PI(4)P staining did not colocalize with NacT1GFP suggests that NacT1GFP or other Golgi resident enzymes might be preferentially located at low PI(4)P Golgi regions. To test this hypothesis, we examined the localization of Golgi resident enzymes in relation to PI(4)P. We further examined the localization of two Golgi resident enzymes, mannosidase II (ManII) and NacT1 with PI(4)P in Hela cells. Cells were fixed with 2% formaldehyde, stained with a PI(4)P antibody and examined under the scanning confocal microscope. We found that both enzymes localized to Golgi regions that were devoid of PI(4)P (Figure 3.18). This staining pattern is reminiscent of that of our hSAC1 and PI(4)P co-localization studies.

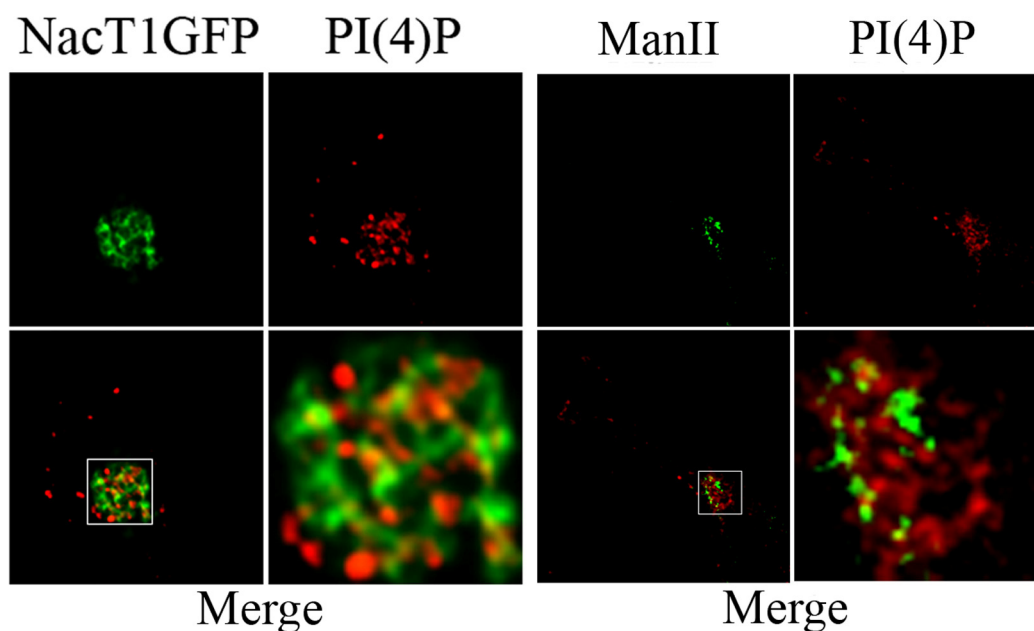


Figure 3.18: Golgi resident enzymes NacT1GFP and Man II are found in PI(4)P depleted Golgi regions

Hela cells transfected with NacT1GFP or non-transfect cells were fixed with 2% formaldehyde, permeabilized with digitonin and stained with PI(4)P antibody. Cells were examined under a scanning confocal microscope.

3.2.4: Depletion of hSAC1 causes mislocalization of Golgi resident enzymes and aberrant Golgi morphology

PI(4)P is important for maintaining functional and structural integrity of the Golgi complex. This was shown by depletion of PI 4-kinases which causes Golgi fragmentation and inhibition of anterograde transport (Godi et al., 2004; Wang et al., 2003). hSAC1 is the only known PI(4)P-phosphatase in the Golgi. The observation that ManII and NacT1 are preferentially localized to PI(4)P-depleted Golgi regions suggests that the low PI(4)P Golgi regions defined by the presence of hSAC1 may be important for the recycling of Golgi resident enzymes, while cargo that is exported from the Golgi is concentrated at high PI(4)P Golgi regions. Furthermore, we had shown that hSAC1 interacts with COPI (Rohde et al., 2003) and this interaction is also dependent on its phosphatase activity. Thus, an active PI(4)P-phosphatase hSAC1 may be essential in generating low PI(4)P Golgi regions that are important for recycling of resident Golgi enzymes via the COPI vesicles.

Therefore, we examined Golgi morphology by immunostaining and fluorescence microscopy in HeLa cells depleted of hSAC1 using different Golgi markers (TGN46, ManII and NacT1GFP). HeLa cells treated with either control or hSAC1 siRNAs were fixed and stained with specific antibody against ManII and TGN46 (Figure 3.20). For NacT1, we co-transfected a GFP-fusion protein of NacT1 together with hSAC1 siRNA or control RNAs and co-stained with ManII antibody. We found that both ManII and NacT1 were mislocalized in hSAC1-depleted cells. Both enzymes were found at the cell periphery and diffuse vesicular structures instead of a normal perinuclear dense structure (Figure 3.19). In most cases, cells that showed cell surface localization of both enzymes also had remnants of Golgi-like staining.

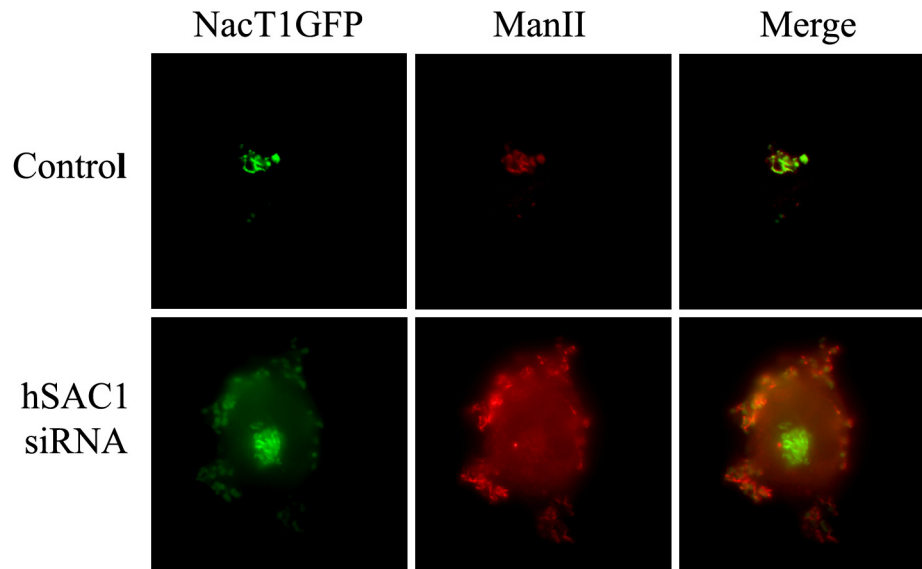


Figure 3.19: Depletion of hSAC1 causes mislocalization of Man II and NacT1.

Hela cells transfected with control or hSAC1 siRNAs and NacT1GFP were fixed with 4% formaldehyde and stained with ManII antibody. ManII and NacT1GFP showed perinuclear dense structures in control cells but were mislocalized to the cell periphery in hSAC1 depleted cells.

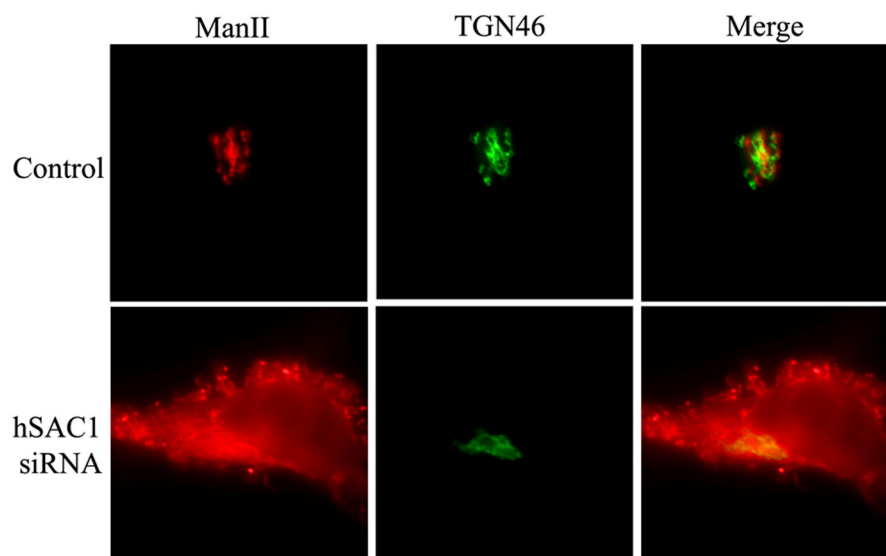


Figure 3.20: Depletion of hSAC1 causes mislocalization of Man II

Hela cells transfected with control or hSAC1 siRNAs were fixed with 4% formaldehyde and stained with Man II and TGN46 antibodies. ManII and TGN46 showed perinuclear dense structures in control cells but ManII was mislocalized to the cell periphery and vesicular, tubular-like structures in hSAC1 depleted cells. TGN46 staining was indistinguishable in both control and knock down cells.

3.2.5: Mislocalization of ManII and NacT1 in hSAC1-depleted cells could be rescued by ectopic expression of RNAi resistant wild type hSAC1

To further show that the mislocalization of Man II and NacT1 is hSAC1 RNAi specific and dependent on the phosphatase activity of hSAC1, we generated two specific RNAi-resistant hSAC1 constructs by introducing silent mutations in the nucleotide sequence targeted by the RNAi construct. Figure 3.21 and Figure 3.22 show immunofluorescence in HeLa cells expressing either the RNAi resistant wild type hSAC1 (GFP-hSAC1*) or the RNAi resistant phosphatase dead mutant (GFP-hSAC1-C/S*). Expression of the GFP-hSAC1* rescue plasmid in siRNA treated HeLa cells could rescue the mislocalization phenotype while the mutagenized phosphatase dead plasmid did not rescue. ManII was found localized at the Golgi in knock down cells expressing the GFP-hSAC1* rescue plasmid. On the other hand, ManII was still mislocalized at the cell periphery and in punctate diffused structures in hSAC1 siRNA treated cells expressing the RNAi resistant phosphatase-dead mutant (GFP-hSAC1-C/S*). This further demonstrated that the mislocalization of resident Golgi enzymes was dependent on the phosphatase activity of hSAC1.

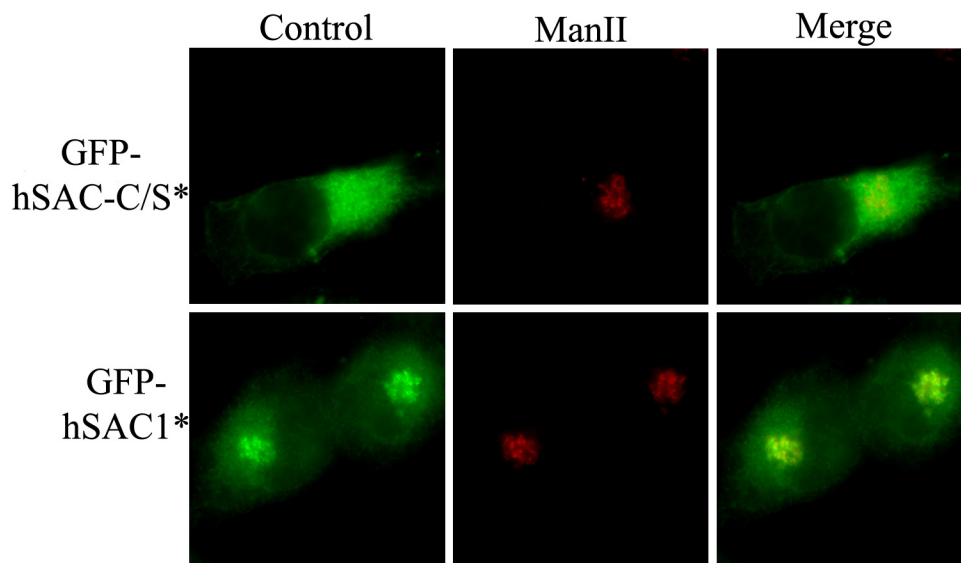


Figure 3.21: Localization of ManII in control cells transfected with either RNAi resistant plasmid GFP-hSAC1* or GFP-hSAC1-C/S*

HeLa cells were transfected with control siRNAs and plasmids encoding either the GFP-hSAC1* (mutagenized rescue plasmid) or GFP-hSAC1-C/S* (mutagenized phosphatase-dead hSAC1). Cells were fixed and stained with ManII 72hrs post-transfection and examined under the fluorescence microscope.

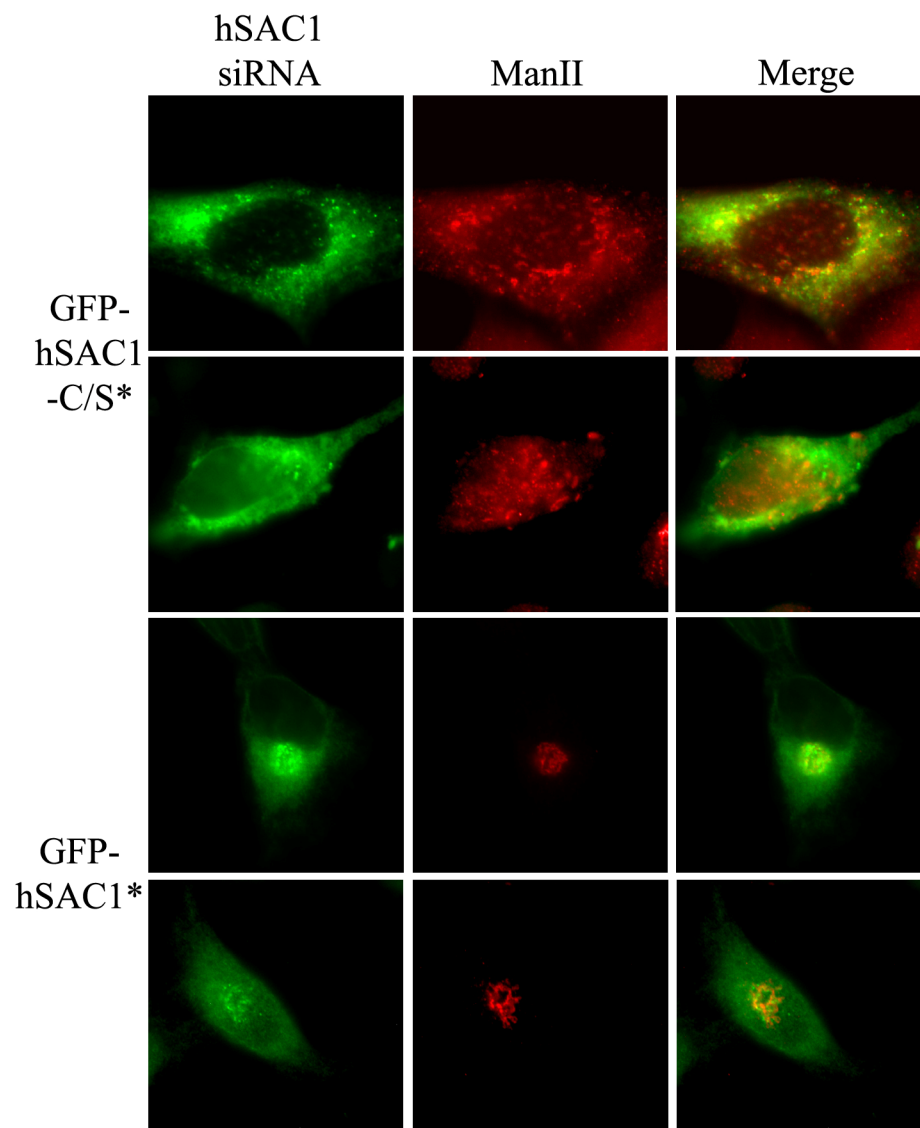


Figure 3.22: Mislocalization of ManII could be rescued by an ectopically expressed RNAi resistant wild type hSAC1 (GFP-hSAC1*)

Hela cells were transfected with hSAC1 siRNA or control together with either GFP, GFP-hSAC1* or GFP-hSAC1-C/S* and stained with ManII antibody. ManII was found to mislocalize to the cell periphery and punctate structures except in cells co-expressing GFP-hSAC1*

3.2.6: Cell surface biotinylation of NacT1GFP in hSAC1-depleted cells

Cell surface localization of NacT1 in hSAC1 RNAi cells was independently confirmed by surface biotinylation and streptavidin pull-down of NacT1GFP (Figure 3.22). HeLa cells were transfected with control or hSAC1 siRNAs together with NacT1GFP. In addition, we were able to show that cell surface expression of NacT1GFP in hSAC1-depleted cells could be abolished by ectopic expression of the RNAi resistant wild type hSAC1 (Figure 3.22, panel 4) but not the RNAi resistant phosphatase-dead mutant (GFP-hSAC1-C/S*, Figure 3.22, panel 3). The amount of detected biotinylated NacT1GFP was less in HeLa cells co-expressing the non-rescue GFP-hSAC1-C/S* and hSAC1 siRNA (panel 3). This was due to reduced expression of NacT1GFP (less input NacT1GFP in 3, 4 compared to 1 and 2). In conclusion, the cell surface localization of resident Golgi enzyme, NacT1GFP was a consequence of hSAC1 depletion.

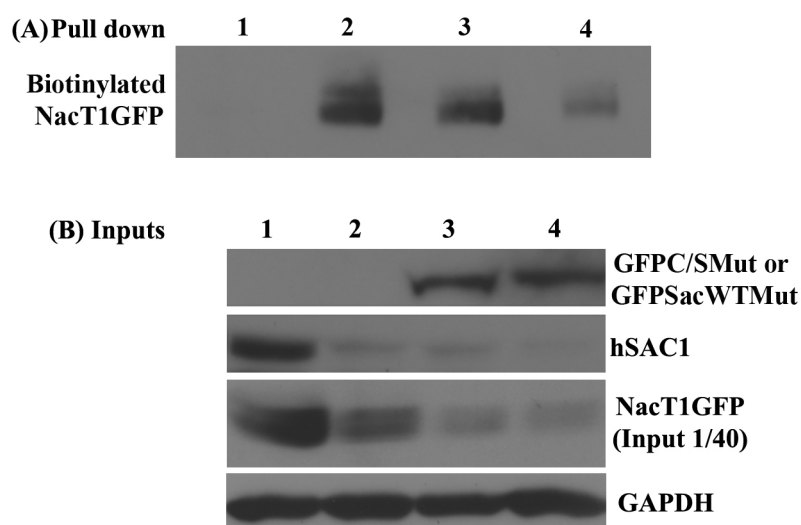


Figure 3.23: Cell surface biotinylation of NacT1GFP in HeLa cells depleted of hSAC1

- 1: HeLa cells transfected with control siRNAs and NacT1GFP
 - 2: HeLa cells transfected with hSAC1 siRNAs and NacT1GFP
 - 3: HeLa cells transfected with hSAC1 siRNAs, NacT1GFP and RNAi resistant phosphatase dead mutant, GFP-hSAC1-C/S* plasmid
 - 4: HeLa cells transfected with hSAC1 siRNAs, NacT1GFP and RNAi resistant wild type hSAC1, GFP-hSac1* plasmid.
- A. Biotinylated NacT1GFP detected using an antibody against GFP after pulldown via biotin
- B. Endogenous hSAC1 depletion by RNAi while the RNAi resistant GFP-hSAC1C/S* and GFP-Sac1* were detected using aGFP antibody. Total input of NacT1GFP (1/40 of total) detected using a GFP antibody. GAPDH was used as loading control.

3.2.7: Aberrant Golgi morphology and mislocalization of Golgi enzymes are not a consequence of apoptosis

Cell surface localization of Golgi resident enzymes, ManII and NacT1 was dependent on hSAC1 depletion. In addition to cell surface staining of both enzymes, the vesicular-diffuse staining of both enzymes in hSAC1-depleted cells resembled Golgi fragmentation during apoptosis. To rule out the possibility that the aberrant Golgi phenotype observed in hSAC1 knock down cells was a consequence of apoptosis, we carried out apoptosis tests in HeLa cells depleted of hSAC1.

We first examined nucleus integrity by DAPI staining in control and hSAC1 knock down cells. NacT1GFP was co-transfected with control or hSAC1 siRNAs as markers for transfected cells. In addition, we monitored the mislocalization phenotype in comparison with nuclear DAPI staining. We found no difference in DAPI staining for hSAC1-depleted cells compared to control cells. The nucleus was still intact even when NacT1GFP was found at the cell periphery or in vesicular structures in hSAC1 knock down cells (Figure 3.23).

In addition, we looked at an early apoptosis marker in hSAC1 siRNA treated cells. Poly(ADP-ribose) polymerase (PARP) is one of the first substrate cleaved by the caspases during onset of apoptosis (Soldani, Lazze et al. 2001; Soldani and Scovassi 2002). PARP synthesizes ADP-ribose using NAD in response to DNA strand breaks and is involved in many genomic processes such as DNA base excision repair, DNA replication, and transcription (Schreiber, Dantzer et al. 2006). Figure 3.24 shows Western blot analysis of HeLa cell lysates from hSAC1 siRNA or control transfected cells and probe with a specific antibody against PARP. Full-length PARP (116kDa) was cleaved into a 85kDa band and a 26kDa band (that was not detected by the monoclonal antibody) in HeLa cells treated with an apoptosis inducer, staurosporine (a kinase inhibitor). No cleavage of PARP was observed in hSAC1 knock down cells or in control cells. This result further showed that the observed Golgi phenotype in hSAC1-depleted cells was not caused by apoptosis.

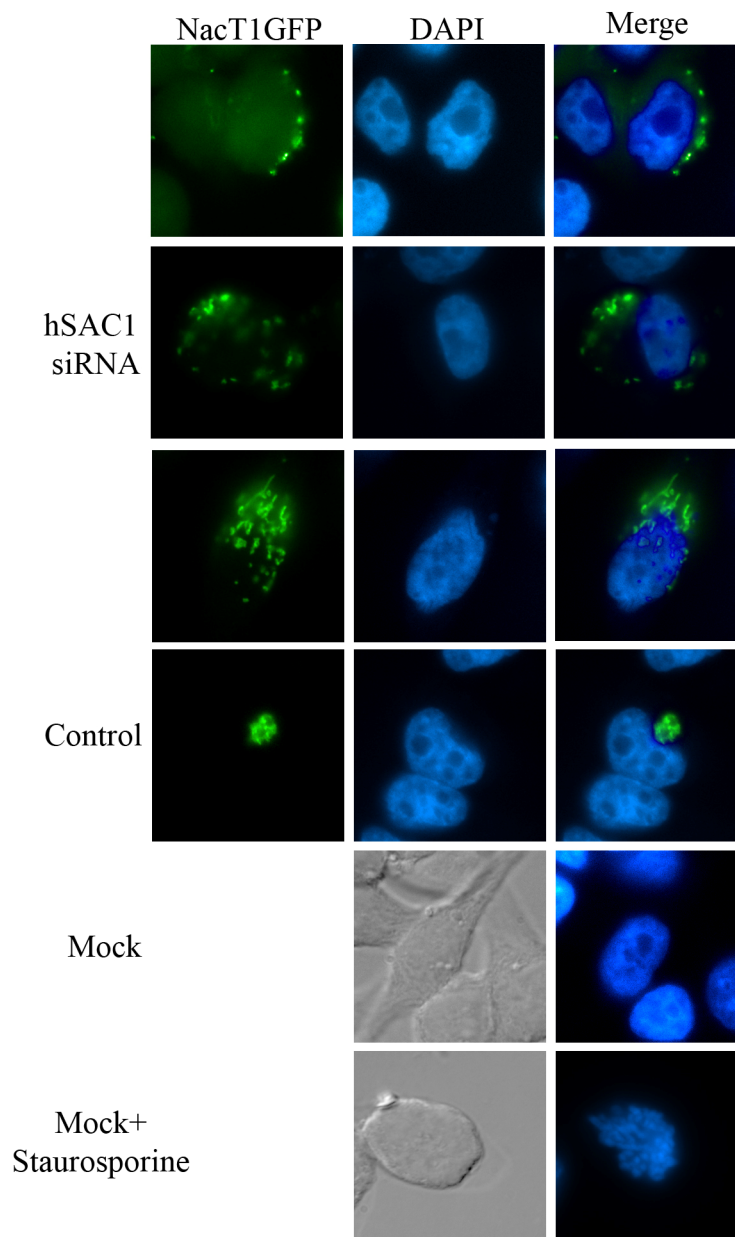


Figure 3.24: Depletion of hSAC1 causes mislocalization of NacT1GFP but does not cause apoptosis.

Hela cells were co-transfected with hSAC1 siRNA/control RNAs together with NacT1GFP, and stained with DAPI to monitor nuclear integrity. Only staurosporine treated mock transfected cells showed nuclear disintegration.

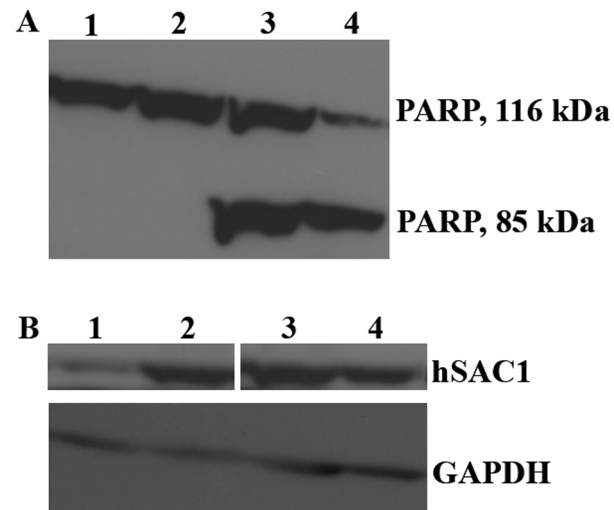


Figure 3.25: Analyzing PARP cleavage in HeLa cells treated with hSAC1 siRNA

1: HeLa cells treated with hSAC1 siRNA

2: HeLa cells treated with control siRNA

3: HeLa cells treated with control siRNA and staurosporine

4: HeLa cells treated with staurosporine

PARP cleavage was observed only in HeLa cells treated with apoptosis inducer, staurosporine.

Depletion of hSAC1 does not cause apoptosis.

3.2.8: Measuring bulk secretion and MHC-I trafficking in hSAC1-depleted HeLa cells

To study the consequences of hSAC1 depletion in secretion, a bulk secretion assay which measured the whole secretory cargo traverse through the secretory pathway was employed. To determine trafficking of proteins out of the TGN, HeLa cells depleted with hSAC1 were pulse-labeled with [³⁵S]-Methionine/Cystein and post-Golgi trafficking was blocked by imposing a reversible low temperature (18 °C) block on Golgi trafficking. Appearance of [³⁵S]-labeled proteins in the culture medium was monitored after shifting the temperature back to 37 °C. However, knock down of hSAC1 did not change bulk Golgi secretion (Figure 3.26)

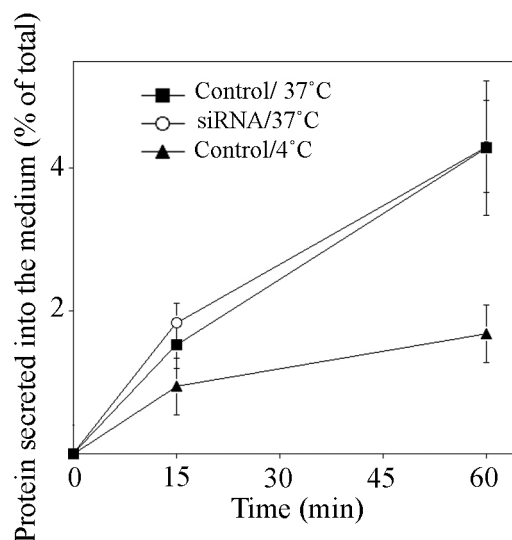


Figure 3.26: Depletion of hSAC1 did not affect anterograde bulk secretion

Hela cells transfected with either hSAC1 siRNA or control RNA were pulse labeled with [^{35}S]-Methionine/Cystein and incubated at 18°C for 3 hrs. The [^{35}S]-labeled proteins in the culture medium was monitored after shifting the temperature back to 37°C.

The bulk secretion result in hSAC1 siRNA treated cells was somewhat surprising. We anticipated that anterograde trafficking might be affected since PI(4)P was shown to be important for forward trafficking from the TGN to the plasma membrane (Godi, Di Campli et al. 2004; De Matteis, Di Campli et al. 2005). Thus, we further investigated anterograde transport from TGN to plasma membrane by monitoring a specific cargo, the Major Histocompatibility Complex Class I molecules (MHC-I). Anterograde transport of MHC-I was determined by cell surface biotinylated MHC-I normalized to the total cellular MHC-I. To this end, Hela cells treated with either control or hSAC1 siRNA were pulse-labeled with ^{35}S -Methionine/Cystein for 15 min at 37°C and incubated at 18°C for 3 hours (temperature block to accumulate labeled MHC-I at the TGN). Cells were chased at 37°C at various time points, cell surface MHC-I was biotinylated and total MHC-I was immunoprecipitated using a specific MHC-I antibody. The biotinylated MHC-I was then precipitated using streptavidin-agarose beads. We found that MHC-I trafficking was slightly affected in hSAC1-depleted cells (Figure 3.27). We reasoned that depletion of hSAC1 only affected MHC-I trafficking but not bulk secretion. Perhaps hSAC1 phosphatase activity was only important for trafficking of specific cargo proteins,

such as the highly glycosylated protein MHC-I. Whether there are glycosylation defects in hSAC1 knock down cells is currently under investigation.

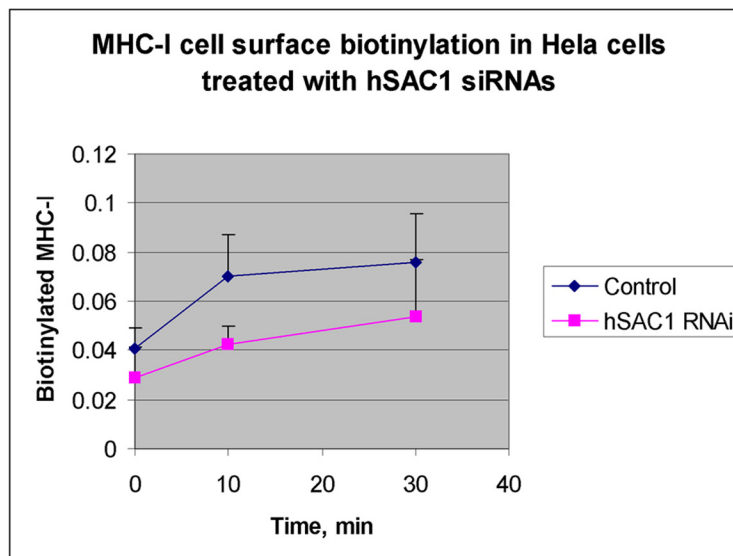


Figure 3.27: MHC-I surface biotinylation in HeLa cells treated with hSAC1 siRNAs

Control or hSAC1 siRNA treated cells were pulse-labeled for 15 min at 37°C followed by a reversible low temperature block of Golgi trafficking at 18°C. Cells were then shifted back to 37°C and cell surface biotinylation of MHC-I was carried out at different time points after temperature block release (0, 10 and 30 min). Biotinylated MHC-I and total MHC-I were quantified and expressed as biotinylated MHC-I over total MHC-I.

3.3: Splice variants of OCRL1

OCRL1 has two splice variants: isoform a (OCRL1-a) and isoform b (OCRL1-b). OCRL1-a contains an additional 8 amino acids. This stretch is adjacent to the clathrin binding motif that is present in both isoforms (Lowe 2005)(Introduction Figure 2.7). The functional relevance of OCRL1 splice variants is unknown. To study the cellular localization and function of OCRL1 splice variants, we generated antiserum that recognized both isoforms and one that was immunoreactive to OCRL1-a only. Figure 3.28 shows localization of GFP-OCRL1-b and OCRL1-a using the isoform specific antibody in COS7 cells. Interestingly, both isoforms showed distinct localization. The short version OCRL1-b localized to the Golgi (in particularly TGN,(Choudhury, Diao et al. 2005) and some punctate staining while OCRL1-a showed punctate staining that was reminiscent of endosomal/lysosomal compartments.

To characterize these punctate structures, we used different endosomal markers and performed immunofluorescence studies. OCRL1-b has been shown to localize to the early endosomes in addition to the TGN localization (Ungewickell, Ward et al. 2004; Choudhury, Diao et al. 2005). We could confirm that OCRL1-b populates the early endosomal membranes besides TGN (Figure 3.29). The punctate structures in GFP-OCRL1-b showed clear colocalization with EEA1(Figure 3.29) but not with the late/lysosomal marker, lamp2 (Figure 3.30). Conversely, OCRL1-a was found only at the late endosomal/lysosomal compartment as determined by colocalization study using lamp2 (Figure 3.31). However, the punctate structures stained by the OCRL1-a specific antibody did not overlap completely with lamp2 (Figure 3.31). This indicates that OCRL1-a might be present on other sub-endosomal/lysosomal compartments that were not stained by Lamp2. We attribute this differential localization of OCRL1-a and OCRL1-b to the 8 amino acid acidic cluster that is present in OCRL1-a. Our results suggest that there are distinct endosomal pools of OCRL1 that might play an important role in regulating phosphoinositide levels at these compartments. Even though the preferred substrate of OCRL1 is PI(4,5)P₂, OCRL1 also exhibits activity towards PI(3,4,5)P₃ and PI(3,5)P₂ (Zhang, Jefferson et al. 1995; Schmid, Wise et al. 2004) which are the main phosphoinositides at the endosomal membranes. However, more experiments will be needed to characterize the function of the OCRL1 isoforms at their distinct endosomal compartments.

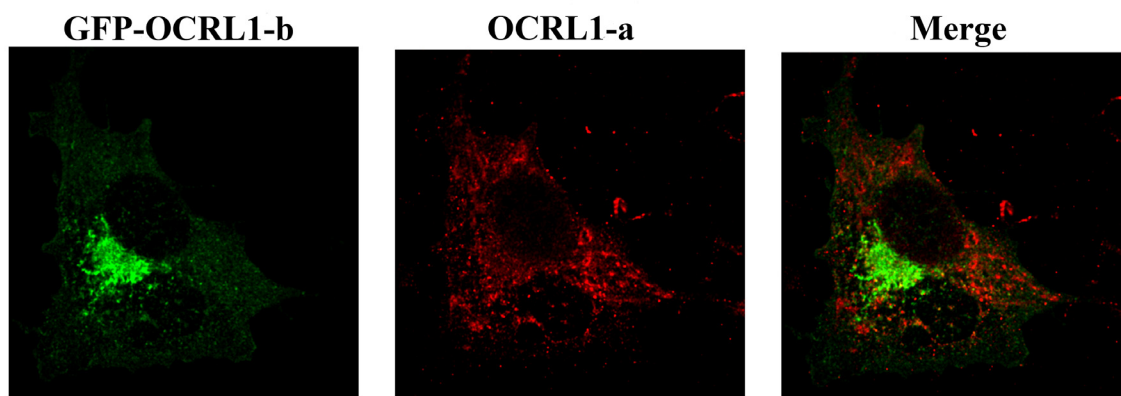


Figure 3.28: Colocalization study of OCRL1 isoform b and OCRL1 isoform a.

Cos7 cells transfected with GFP-tagged OCRL isoform b were stained with isoform a specific antibody. Isoform b is mainly localized to the Golgi (and some punctate structures) while isoform a is found at punctate like structures reminiscent of endosomal/lysosomal compartments

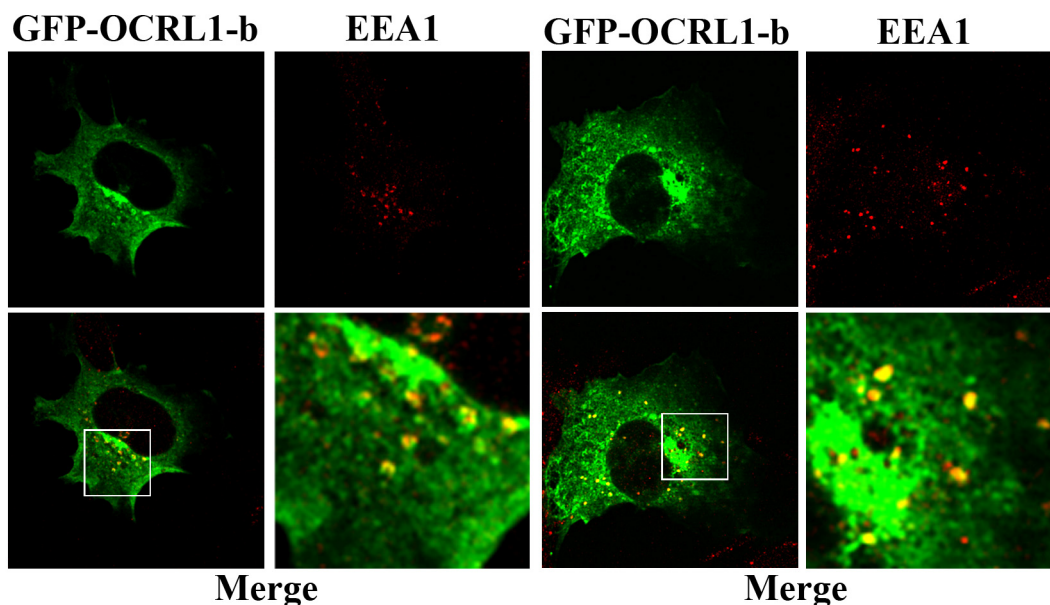


Figure 3.29: GFP-OCRL1-b is found at early endosomes in addition to the TGN

Cos7 cells expressing GFP-OCRL1-b were fixed with 4% formaldehyde and stained with an early endosomal marker, EEA1. The OCRL1 isoform b was found predominantly at the TGN with punctate structures that were stained positive for EEA1.

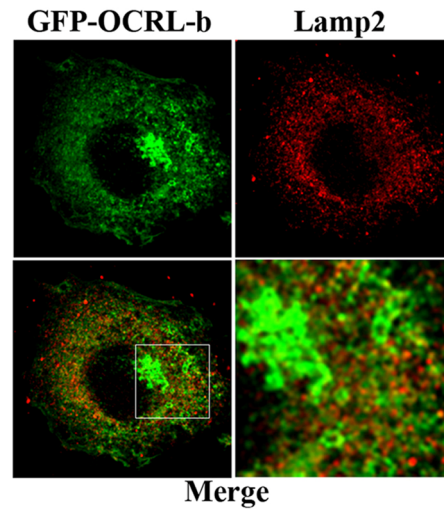


Figure 3.30: OCRL1 isoform b is not present at the late endosomal/lysosomal compartment
Cos7 cells expressing GFP-OCRL1-b were stained with Lamp2 antibody and view under laser scanning confocal microscope. The punctate structures of GFP-OCRL1-b do not stained positive with Lamp2.

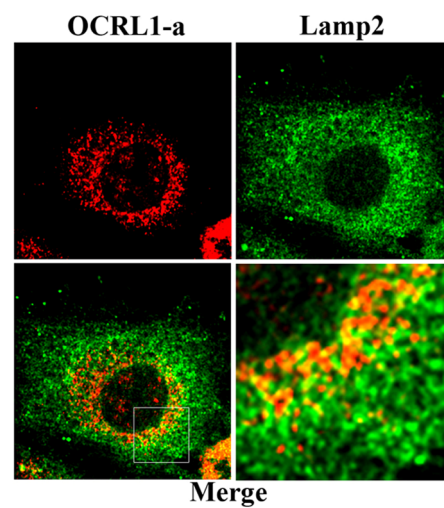


Figure 3.31: OCRL1 Isoform a is found in subpopulation of late endosomal/lysosomal membranes that are stained positive with Lamp2
Cos7 cells were fixed with 4% formaldehyde and stained with antibodies against OCRL1 isoform a and a late endosomal/lysosomal marker, lamp2. The punctate structures stained by the isoform a specific antibody showed partial colocalization with lamp2.

In addition, we also compared the localization of GFP-OCRL1-b expressed in COS7 cells with an antibody that recognized both isoforms of OCRL1 (Figure 3.32). As predicted, both GFP-OCRL1-b and the antibody staining colocalized very well at the TGN but showed no colocalization between the isoform specific endosomal population of OCRL1.

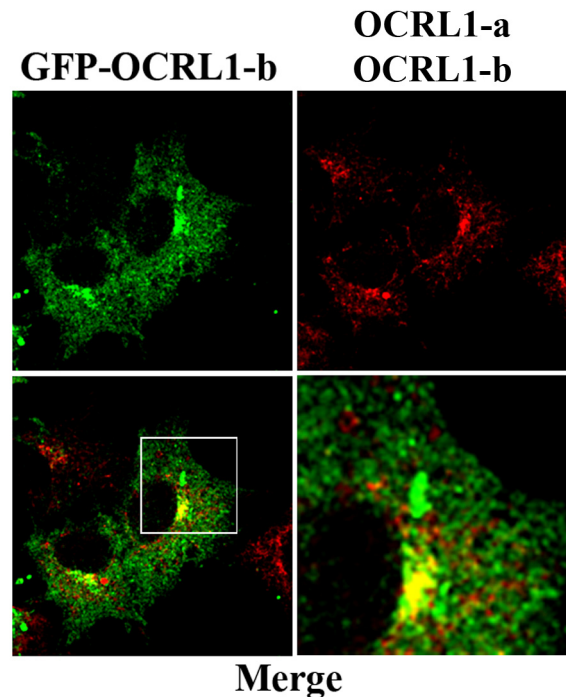


Figure 3.32: Antibody staining using antiserum that recognized both isoforms of OCRL1 showed colocalization at the Golgi-like structure but not the punctate endosomal like structures.

Cos7 cells transfected with GFP-OCRL1-b were stained with a specific antibody that recognized both isoforms of OCRL1. The antibody staining colocalized well with the Golgi population of GFP-OCRL1-b but there were punctate like structures of isoform a (red) that were not present in isoform-b.

3.3.1: Investigation of the cellular function of Golgi localized OCRL1-b

How PI(4,5)P₂ at the Golgi complex is regulated by lipid phosphatases is unclear. The fact that the steady state level of Golgi PI(4,5)P₂ is 9 folds lower than that of the plasma membrane PI(4,5)P₂ (Watt, Kular et al. 2002) suggests that PI(4,5)P₂ at this compartment is highly dynamic. OCRL1-b is the only Golgi-localized PI(4,5)P₂ 5-phosphatase known so far. Thus, it is a prime candidate for studying the dynamics of PI(4,5)P₂ regulation at the Golgi. We started our studies by investigating the cellular localization of OCRL1 in relation to hSAC1. The aim was to examine whether OCRL1 generated PI(4)P pools from PI(4,5)P₂ that are further metabolized by hSAC1 or whether these two phosphatases were in distinct subdomains in the Golgi with different functions.

Consistent with previous studies (Dressman, Olivos-Glander et al. 2000; Choudhury, Diao et al. 2005), we found that GFP-OCRL1-b localized to the TGN as shown in Figure 3.33.

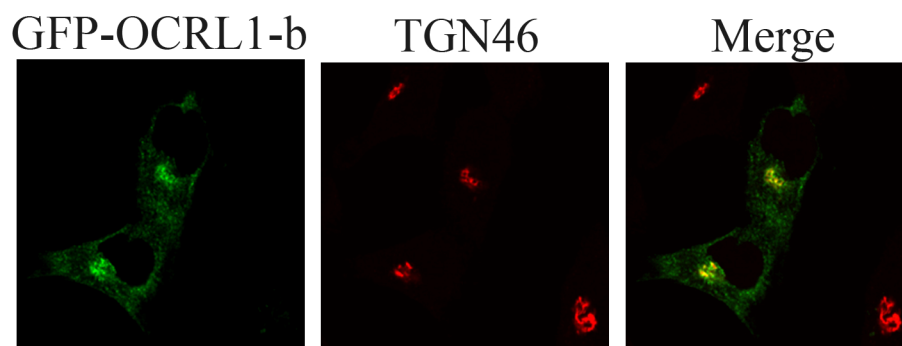


Figure 3.33: GFP-OCRL1-b is localized to the TGN

Cos7 cells expressing isoform b of GFP-tagged OCRL were immunostained with a TGN marker, TGN46.

When we examined OCRL1-b and hSAC1 distribution in the Golgi, we found no or very little overlap between the two phosphatases. OCRL1-b was found to populate regions in the Golgi that were distinct from where hSAC1 resided (Figure 3.34).

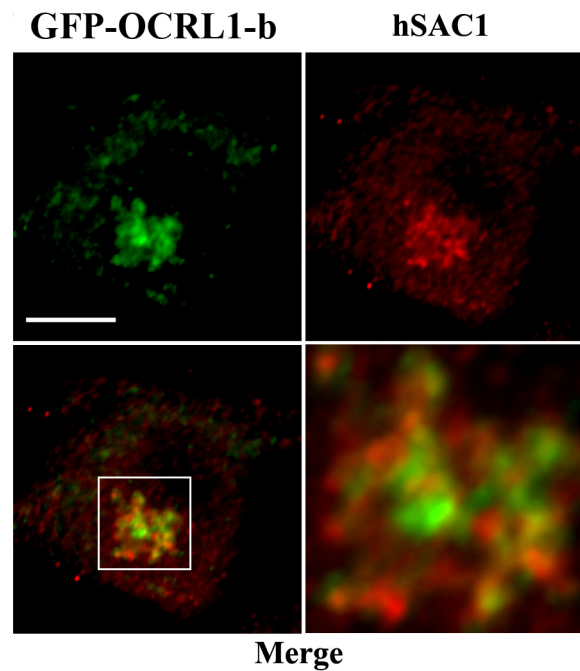


Figure 3.34: OCRL1 and hSAC1 populate distinct Golgi regions

Confocal immunofluorescence of hSAC1(red) and OCRL1(green) in HeLa cells. Both phosphatases did not colocalize.

Furthermore, when we examined PI(4)P staining using Fapp1-PH-RFP and PI(4)P antibody in HeLa cells expressing GFP-OCRL1-b, this PI(4,5)P₂ 5-phosphatase co-localized almost completely with FAPP1-PH-RFP and PI(4)P antibody staining (Figure 3.35), indicating that the localization of hSAC1 and OCRL1 to distinct Golgi regions was mutually exclusive and thus suggesting that formation of PI(4)P and of PI(4,5)P₂-depleted regions occurred at distinct sites.

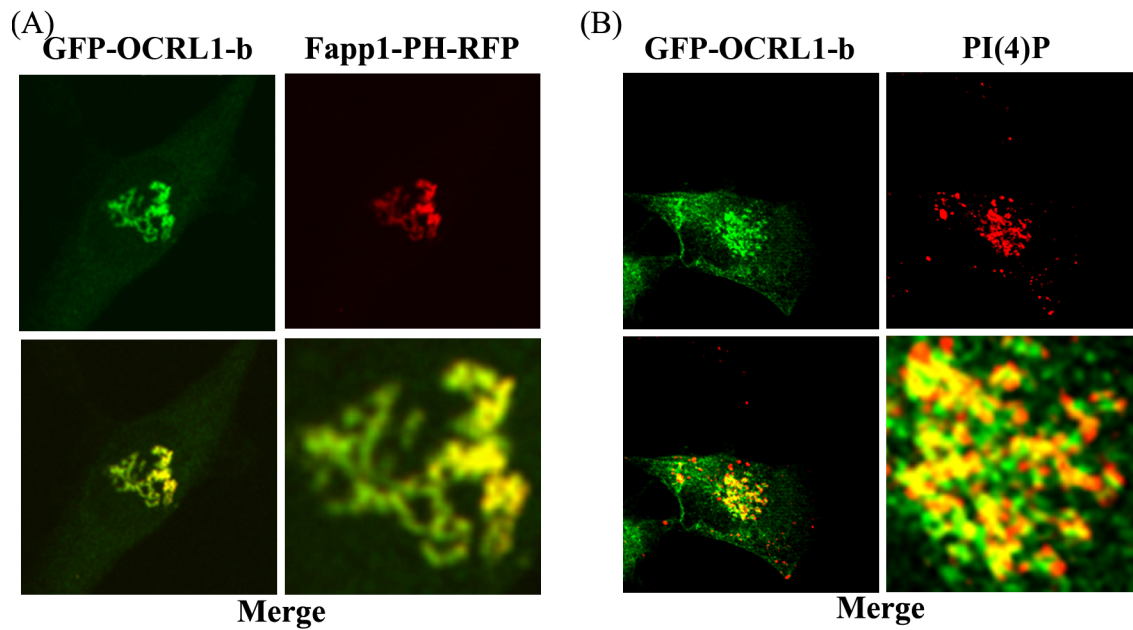
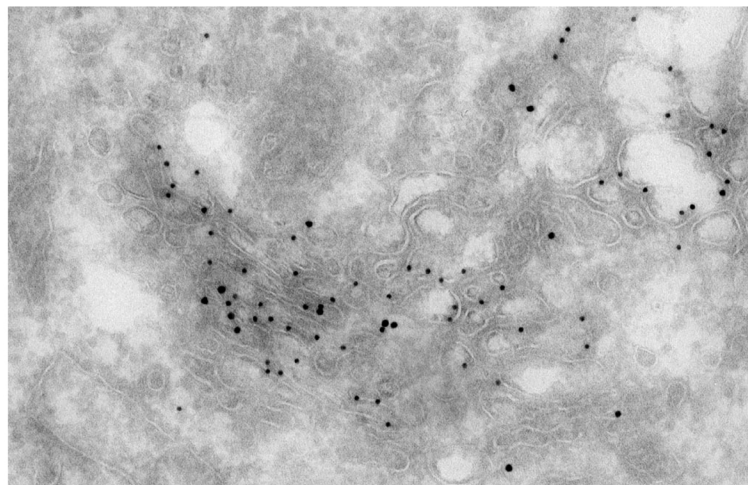


Figure 3.35: OCRL1 is found in PI(4)P positive Golgi regions

(A) OCRL1 was found in high PI(4)P Golgi regions. Different from hSAC1, OCRL1 co-localized almost completely with PI(4)P-binding domain, Fapp1-PH-RFP. This finding suggests that both phosphatases are located at different Golgi subcompartments.

(B) PI(4)P-specific antibody was used to detect PI(4)P in HeLa cells expressing GFP-OCRL1-b. GFP-OCRL1-b colocalized well with PI(4)P.



Fapp1-PH-Myc and GFP-OCRL1-b
Myc10 x GFP15

Figure 3.36: Electron micrograph showing GFP-OCRL1-b and Fapp1-PH-Myc labeled by immunogold.

HeLa cells co-expressing GFP-OCRL1-b and Fapp1-PH-Myc were fixed according to Section 5.2.4.8. Cells were processed for immunogold labeling and electron microscopy (collaboration with Klumperman's lab)

3.3.2: Depletion of OCRL1 using vector-based RNAi

To study the function of Golgi localized PI(4,5)P₂ 5-phosphatase OCRL1, three different RNAi constructs and a control plasmid containing four point mutations were generated. All three constructs were tested and showed successful knock down of OCRL1 in HEK293 cells, which have a high level of expression of this phosphatase. Construct OCRL1-A and OCRL1-C show equal potency in depleting OCRL1 (Figure 3.36). All subsequent experiments were performed using construct A. In general, depletion of OCRL1 can be observed between 48 hrs-72 hrs post-transfections. All experiments were carried out between 60 hrs to 72 hrs post-transfections.

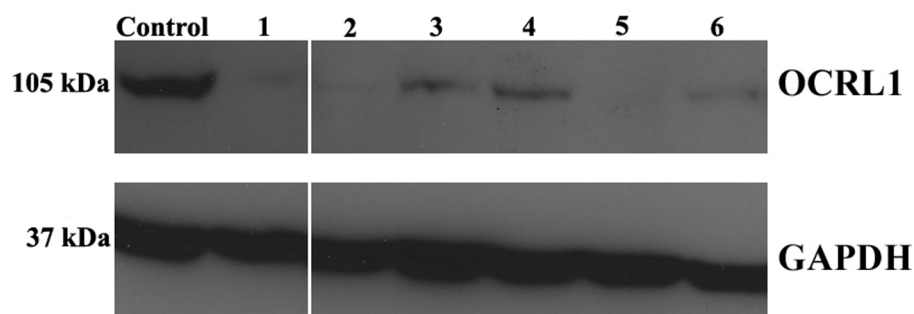


Figure 3.37: Depletion of OCRL1 by Vector-based RNAi

HEK293 cells were transfected with plasmids encoding siRNAs targeted to OCRL1 and a control plasmid that expressed siRNAs which contained 4 point mutations. Lane 1 & 2: RNAi construct A; lane 3 & 4: RNAi construct B and lane 5&6: RNAi construct C. GAPDH was used as a loading control.

We also monitored the extend of OCRL1 depletion by examining the GFP signal in HEK293 cells co-transfected with GFP-OCRL1 and either control or RNAi plasmids. In control plasmid transfected cells, the GFP signal was observed but not in OCRL1 RNAi plasmid A and B transfected cells (Figure 3.37).

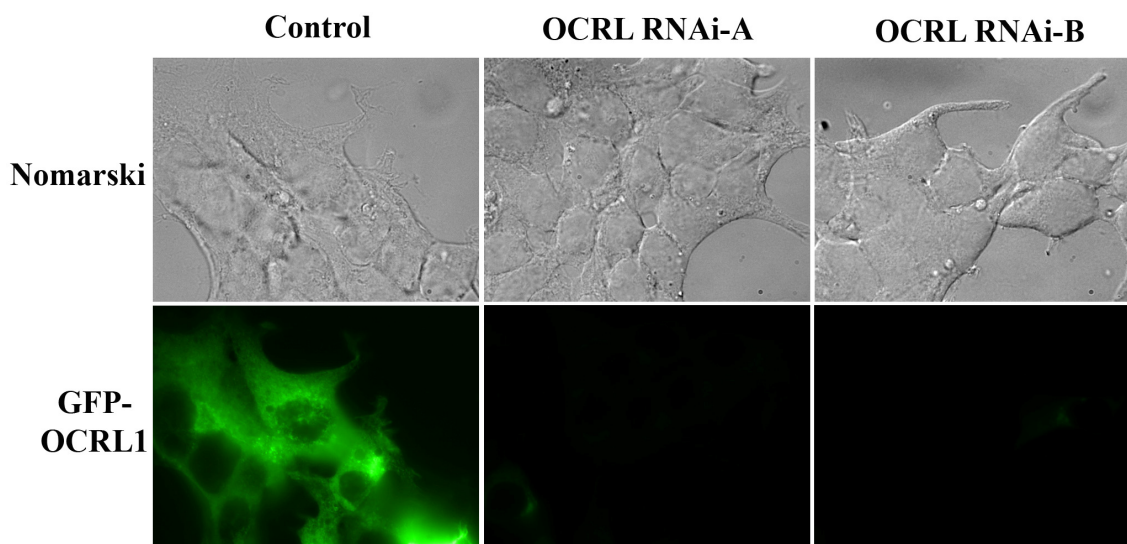


Figure 3.38: Depletion of OCRL1-GFP in HEK293 cells

HEK293 cells transfected with OCRL1-GFP and plasmids expressing either the control or siRNAs directed to OCRL1. Cells were fixed in 4% formaldehyde and viewed under fluorescence microscope.

3.3.3: HPLC analysis of HEK293 cells-depleted of OCRL1

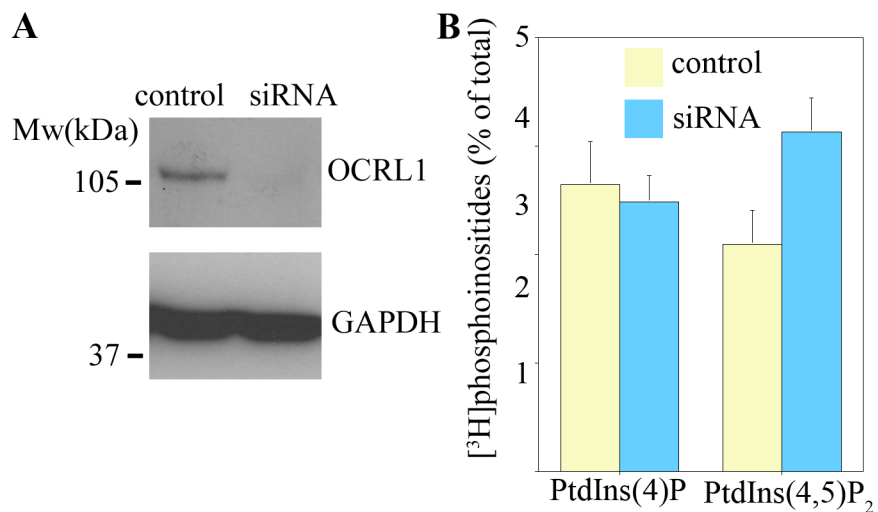


Figure 3.39: Depletion of OCRL1 causes accumulation of PI(4,5)P₂

(A) Depletion of OCRL1 using RNAi in HEK293 cells. Cells were labeled with 10 μ Ci/ml of [³H]-myo-inositol to steady state for 12 hours. Lipids were extracted, deacylated and analyzed using HPLC.

(B) HPLC analysis of phosphoinositides in HEK293 cells treated with OCRL1 siRNA. OCRL1-depleted cells showed accumulation of PI(4,5)P₂ but no significant effect on PI(4)P level.

The main substrate of OCRL1 is PI(4,5)P₂ (Zhang, Jefferson et al. 1995). Cell lines from Lowe syndrome patients exhibited accumulation of PI(4,5)P₂ (Zhang, Hartz et al. 1998). To examine the cellular levels of PI(4,5)P₂ in OCRL1-depleted cells, we performed HPLC analysis in HEK293 cells depleted of OCRL1 (Figure 3.38). OCRL1 knock-down lead to elevated cellular PtdIns(4,5)P₂, similar to the levels determined in cells from a Lowe syndrome patient. Thus, depletion of OCRL1 in tissue culture cells using RNAi could phenocopy the absence/dysfunction of OCRL1 .

3.3.4: Depletion of OCRL1 does not cause alterations in Golgi morphology

To address the question whether depletion of the Golgi localized PI(4,5)P₂ 5-phosphatase causes aberrant Golgi morphology and mislocalization of Golgi enzymes, we looked at the localization of NacT1 and ManII in HeLa cells depleted of OCRL1 (Figure 3.39). NacT1 and ManII in OCRL1 siRNA treated cells showed similar perinuclear localization as in control cells. Thus, knock down of OCRL1 does not affect Golgi morphology.

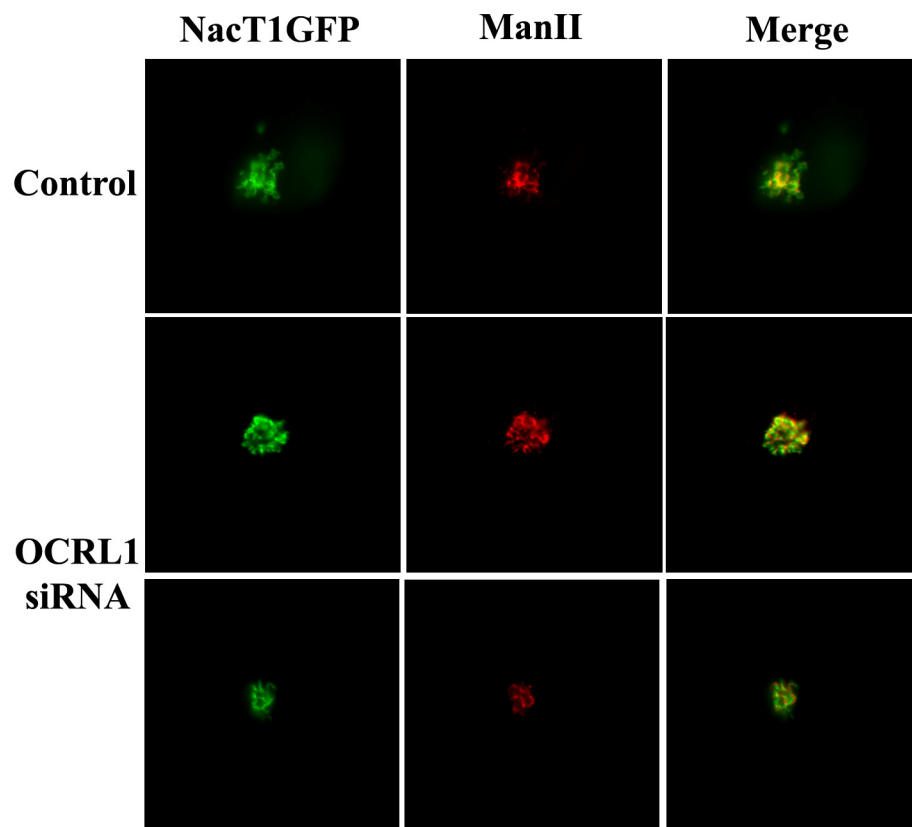


Figure 3.39: Depletion of OCRL1 does not cause aberrant Golgi morphology

HeLa cells transfected with NacT1GFP and OCRL1 siRNA or control RNA were fixed with 4% formaldehyde and stained with ManII antibody. Both OCRL1 siRNA and control cells show similar staining of both Golgi resident enzymes.

3.3.5: Depletion of OCRL1 causes defect in bulk secretion

The striking overlap between cellular OCRL1 and PI(4)P distribution suggests that OCRL1 may play a role in maintaining high PI(4)P Golgi regions by metabolizing PI(4,5)P₂ to PI(4)P. PI(4)P is an anterograde trafficking signpost at the Golgi, thus it was interesting to examine whether bulk anterograde trafficking was affected by depletion of OCRL1. Bulk anterograde trafficking was carried out in OCRL1-depleted HEK293 cells as described in Section 3.2.9 and Section 5.2.4.7.1.

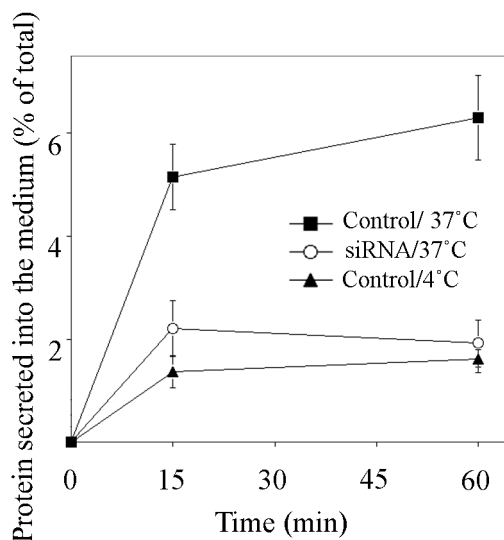


Figure 3.40: Bulk secretion was inhibited in HEK293 cells-depleted of OCRL1

HEK293 cells were transfected with plasmids for expressing siRNAs directed against Ocr11 or control RNAs. Three days after transfection cells were pulse labeled with [³⁵S]Met/Cys, incubated at 18°C for 3h and then shifted to 37°C. At the indicated times, radio-labeled proteins in the culture supernatants and inside the cells were precipitated, collected on filters and quantified by scintillation counting. As an additional control, the secretion assay was performed 4°C. The depicted data are means +/- SD from triplicates derived in three independent sets of experiments.

We found that knock down of OCRL1 caused defect in bulk anterograde trafficking. This indicates that the PI(4,5)P₂ 5-phosphatase activity of OCRL1 is important for maintaining constitutive secretion from TGN to the plasma membrane.

4 Discussion

In this study, I investigated the functions of two lipid phosphatases, hSAC1 and OCRL1 at the Golgi complex. hSAC1 is a PI(4)P-phosphatase localized to both the ER and Golgi (Rohde et al., 2003), while OCRL1 is a PI(4,5)P₂ 5-phosphatase found at the TGN and endosomes (Ungewickell, Ward et al. 2004; Choudhury, Diao et al. 2005). My work was focused on the functions of both phosphatases at the Golgi complex and how compartment-specific pools of PI(4)P and PI(4,5)P₂ at this organelle are regulated.

Recent work by different laboratories has established that PI(4)P is the main phosphoinositide species at the Golgi (De Matteis and Godi 2004; De Matteis, Di Campli et al. 2005) where it contributes to the maintenance of structural and functional integrity of this organelle (Godi, Pertile et al. 1999; Wang, Wang et al. 2003; Godi, Di Campli et al. 2004). In addition, PI(4)P at the Golgi complex serves as an organelle specific signal for recruitment of cellular machineries such as the adaptor proteins involved in membrane trafficking. The functions of PI 4-kinases in generating these PI(4)P pools have been studied in considerable detail (Balla 1998; Balla and Balla 2006). These PI 4-kinases are either tightly associated with the Golgi membranes (PI4KII α) or recruited to the Golgi by activation of the small GTPase, Arf1(PI4KIII β). In contrast, the function of lipid phosphatases at this compartment has remained unclear. The fact that many human diseases are linked to mutations in phosphatases highlights the importance of phosphatases in antagonizing or terminating signals at the proper space and time. Mutations in OCRL1 cause a severe X-linked disease, Lowe syndrome. The tissue specific manifestations of Lowe syndrome (congenital cataracts, Fanconi syndrome and mental retardation) suggest that tight regulation of PI(4,5)P₂ by this 5-phosphatase is critical for the proper function of specific tissues. To elucidate the etiology of Lowe syndrome, it is necessary to study the compartment-specific functions of OCRL1 and to understand how dysfunction of this phosphatase affects PI(4,5)P₂-regulated cellular events such as membrane trafficking. Although hSAC1 has not been linked to any known human diseases, this phosphatase is mapped to the C3CER1-segment on chromosome 3 (3p21.3) that is commonly eliminated in SCID-derived tumors (Rohde et al., 2003). This 1.4Mb chromosome segment that contains 19 active genes with possible tumor-suppressing properties (Kiss, Yang et al. 2002). The very recent finding that SAC1

shuttles between ER and Golgi in response to growth conditions (Faulhammer F. et al., 2005, Blagoveschenskaya A. et al., 2006 manuscript submitted) indicates that SAC1 phosphatase might be essential for regulating cell growth. To further characterize lipid signaling at the Golgi, it was important to study the function of hSAC1 and OCRL1, in particular their compartment specific roles.

RNA interference (RNAi) was employed to silence the expression of hSAC1 and OCRL1 in tissue culture cells followed by different functional assays to address compartment-specific roles of both phosphatases. We started our studies by generating different plasmid DNA constructs that express small interfering RNAs (siRNAs). Although selection of different siRNA sequences was done according to stringent guidelines (Harworth et al., 2003), the potency of gene suppression varied to great extent. However, in both attempts to deplete hSAC1 and OCRL1 using RNAi, I successfully generated two or more effective silencing constructs for each phosphatase.

4.1: hSAC1 is a PI(4)P-phosphatase found in both ER and Golgi

The ER localization of SAC1 phosphatase has been established in both yeast and mammals. However, the extent of Golgi localization of SAC1 phosphatase was not clear. In rats, SAC1 was found at ER membranes only by indirect immunofluorescence studies (Nemoto et al., 2000). However, our data clearly shows that the endogenous hSAC1 is found in both ER and Golgi membranes (Rohde et al., 2003). This is also supported by recent publications that yeast Sac1p is present at the Golgi in addition to the ER (Schorr et al, 2001; Faulhammer et al., 2005). Interestingly, expression of N-terminal truncated version of hSAC1 showed restricted ER localization. Thus, the N-terminal cytosolic domain of hSAC1 (which contains the SAC-phosphatase domain and the leucine zipper motif) is essential for Golgi targeting. The mechanism of how cellular distribution of hSAC1 is regulated remains unclear. In yeast, the interaction between Sac1p and Dpm1p (an ER-specific enzyme, dolicholphosphatemannose synthase) is essential for ER localization of Sac1p and efficient oligosaccharide biosynthesis during exponential growth conditions (Faulhammer et al. 2005). In contrast to yeast Sac1p, the ER localization of mammalian Sac1 is mediated by a canonical coatomer complex I (COPI)-binding motif missing in the *S.cerevisiae* Sac1p (Rohde et al., 2003). Furthermore, the

dynamics of ER and Golgi localization of hSAC1 was shown to be dependent on its phosphatase activity and its efficiency in COPI binding (Rohde et al., 2003). Mutating the dilysine motif at the C-terminal KEKID COPI-binding site of hSAC1 (hSAC1-K2A mutant) led to Golgi accumulation of hSAC1. When we compared the Golgi localization of the hSAC1-K2A mutant with wild type hSAC1, both wild type and hSAC1-K2A mutant localized to different Golgi regions (data not shown). This finding supports the idea that active retrieval of hSAC1 is important for steady state ER and Golgi distribution of this lipid phosphatase. Interestingly, mutations that eliminate PI(4)P-phosphatase activity of hSAC1 (hSAC1-C/S mutant) also result in an accumulation of this phosphatase at Golgi membranes though the putative COPI binding motif (KEKID) is present in the phosphatase-dead mutant. This observation suggests that efficient COPI interaction is coupled to lipid phosphatase activity. Furthermore, hSAC1 also relocates from the ER to the Golgi complex in response to serum starvation (Blogoveschenskaya A. et al., 2006 manuscript submitted). However, regardless of growth conditions (quiescence or normal growth condition), there is a significant portion of hSAC1 that is always present at the Golgi apparatus. This indicates that hSAC1 has important “house-keeping” or constitutive functions at the Golgi complex.

Confocal immunofluorescence studies of hSAC1 and different Golgi markers clearly show that hSAC1 is distributed throughout the Golgi complex. hSAC1 co-localizes equally well with the *cis*- (p24), medial- (mannosidase II), and *trans*-Golgi (galactosyltransferase (GalT) and TGN46) markers. However, further studies using immunogold-labeling and electron microscopy demonstrate that hSAC1 and PI(4)P localize to different Golgi regions. hSAC1 was found mainly at the *cis*- and *medial* Golgi cisternae while the PI(4)P probe Fapp1-PH localize to the TGN (Godi et al., 2004). Only under conditions where the Fapp1-PH domain is highly overexpressed, some hSAC1 is found to be localized at the same cisternae membrane. Overall, however, overexpression of Fapp1-PH domain causes formation of aberrant membrane networks that are distinct from hSAC1 positive membranes. The ability of Fapp1-PH domain to induce formation of membrane network could be due to overexpressed Fapp1-PH domain bound to PI(4)P in a dominant negative way that titrates out Golgi PI(4)P pools. Since PI(4)P is needed for anterograde trafficking, accumulation of vesicular-tubular membranes due to

inhibition of forward trafficking might lead to formation of these membrane network. In fact, Godi A. et al. found that cells with high expression of Fapp1-PH domain showed a marked delay in transport of ts045-VSV-G from the TGN to the plasma membrane (Godi et al., 2004). Taken together, these findings indicate that the Golgi apparatus is polarized by an opposing gradient of PI(4)P at the TGN and a concentration of hSAC1 PI(4)P-phosphatase at the cisternal Golgi compartments.

4.2: hSAC1-defined functional PI(4)P-depleted regions in the Golgi that are important for recycling of resident Golgi enzymes

Several reports have demonstrated that PI(4)P functions as an anterograde trafficking signal at the Golgi complex (Hama, Schnieders et al. 1999; Schorr, Then et al. 2001; Wang, Wang et al. 2003; Godi, Di Campli et al. 2004). Recruitment of adaptor proteins such as the adaptor protein 1 (AP1) is mediated by the PI(4)P generated by PI4KII α (Wang et al., 2003). In addition, formation of large pleiomorphic carriers (LPCs) at the TGN is facilitated by the recruitment of Fapp proteins via PI(4)P synthesized by PI4KIII β (Godi, 2004). These PI(4)P pools at the Golgi are also controlled by hSAC1 phosphatase. This study provides evidence that hSAC1 is not only required for temporal termination of PI(4)P but it is also a spatial regulator that functions in maintaining regions in the Golgi that are devoid of PI(4)P. We found that hSAC1 is responsible for creating PI(4)P-depleted Golgi regions that are important for segregating anterograde transport from retrograde trafficking of Golgi resident enzymes. PI(4)P defines Golgi regions active in secretory cargo trafficking. *Cis*- and *medial*- Golgi enzymes are excluded from these areas, thus preventing their anterograde escape to the cell periphery. However, there has to be some close contact between PI(4)P-enriched and PI(4)P-depleted domains because cargo passing through the Golgi has to have access to the processing enzymes.

The distinctive feature of hSAC1 depleted cells is mislocalization of Golgi resident enzymes such as ManII and NacT1 to the cell periphery. Besides cell surface localization, both enzymes are found in diffused-vesicular like structures. These could be internalized cell surface localized enzymes to endosomal compartments. In most cases, when we observe cell surface staining of ManII and NacT1, the Golgi is still intact as

determine by co-staining with another Golgi marker, TGN46 and p58k (data not shown). This observation is distinct from that of depletion of the Golgi localized PI4- kinases. The PI4KII α depleted cells show expanded Golgi with TGN46 staining is more punctate and or tubular compared to control cells. Overexpression of a dominant negative kinase dead mutant of PI4KIII β (D656A) causes a disorganized, filamentous/punctate Golgi structure as stained by a Golgi marker, giantin.

A spatially restricted PI(4)P gradient in the Golgi (highly concentrated at the TGN but not in other Golgi cisternae) is achieved by local recruitment of the PI 4-kinases and the presence of the PI(4)P-phosphatase, hSAC1 at this compartment. This is important to ensure that PI(4)P is only highly concentrated in regions where anterograde transport of cargo takes place, such as at the TGN, where the main sorting events and transport carrier formation are carried out. Furthermore, evidence suggests that there are different functional subdomains at the TGN that are orchestrated by PI(4)P and other phospholipids (Godi A., 2004). Fapp2, OSBP and CERT each contain a common Golgi targeting N-terminal PH-domain that targets them to the PI(4)P-enriched TGN while their distinctive lipid-binding properties enable a differential localization of these proteins to separate TGN domains. Thus, protein targeting via recognition of different lipid signals that are created at specific membranes may contribute significantly to how different transport events or cellular processes can be separated (for example, anterograde transport versus retrograde transport for Golgi enzyme recycling) or integrated (for example, recruitment of Fapps at the TGN coupled to formation of transport carriers and forward transport via microtubules).

The idea of functional membrane domains defined by differential localization of kinases and phosphatases is consistent with previous reports (Martin, 1997). A recent study by Tahirovic et al. had demonstrated that yeast Sac1p is required for confining PI(4)P-dependent reactions to specific intracellular membrane compartments (Tahirovic et al., 2005). In *S.cerevisiae*, a *sac1 null mutant* showed accumulation of PI(4)P at different intracellular membranes, which are normally devoid of PI(4)P. Such deregulation of intracellular PI(4)P pools leads to defects in vacuolar morphology and endosomal trafficking (Tahirovic et al., 2005). Similarly, in hSAC1-depleted cells, we find accumulation of PI(4)P. Although the accumulation of PI(4)P in hSAC1 siRNA

treated cells is less dramatic compared to the yeast null mutant, it is sufficient for causing mislocalization of Golgi resident enzymes and aberrant Golgi morphology. Intracellular localization of PI(4)P, detected by specific antibodies in both control and hSAC1-depleted cells reveal differences in terms of signal strength and compartments stained by the antibody. We find PI(4)P to be accumulated at the Golgi, plasma membrane and vesicular-like structures in hSAC1-depleted cells while the control cells display only a defined PI(4)P staining to Golgi-like structures.

To directly show that PI(4)P-phosphatase activity of hSAC1 is responsible for maintaining PI(4)P-depleted Golgi regions, we compare PI(4)P staining in wild type and phosphatase-dead mutant hSAC1-C/S. We found a drastic difference in co-localization studies. The active PI(4)P-phosphatase hSAC1 was localized to PI(4)P-depleted Golgi regions. Conversely, the phosphatase-dead hSAC1-C/S mutant co-localized almost completely with PI(4)P. This strongly suggests that the lipid phosphatase activity of hSAC1 is needed to create PI(4)P-depleted Golgi regions. The importance of a functionally active hSAC1 could be further demonstrated in subsequent rescue experiments (cell surface biotinylation of NacT1GFP and immunofluorescence staining of ManII in hSAC1-depleted cells). Only the ectopically expressed RNAi-resistant wild type hSAC1 could reverse the aberrant Golgi structure and mislocalization phenotype of ManII and NacT1. The RNAi-resistant phosphatase dead mutant, GFP-hSAC1-C/S*, did not rescue this phenotype.

How resident Golgi enzymes are partitioned and maintain their proper localization in Golgi cisternae remains a fundamental question in cell biology. Efforts to identify common targeting motifs in different Golgi resident enzymes have not been successful in explaining the asymmetric distribution of glycosyltransferases (Colley, 1997; Colley, 1997). The oligomerization/aggregation model and membrane thickness model, which are based on vesicular transport static model, have only partially explained how retention of Golgi glycosyltransferases might happen. In contrast, the maturation model which predicts Golgi as a highly dynamic entity proposes that active retrieval of resident Golgi enzymes by COPI vesicles is fundamental for keeping the Golgi glycosyltransferases in place. Regardless of the intra-Golgi trafficking models, both vesicular and maturation models suggest a role of COPI retrograde vesicles in maintenance of Golgi homeostasis

and retrieval of Golgi resident enzymes. We propose that Golgi phosphoinositides (in particular PI(4)P) play a fundamental role in organizing this organelle. The fact that large amounts of COPI interact with hSAC1 (Rohde et al., 2003), indicates that COPI-mediated retrieval of Golgi resident enzymes might preferentially occur at low PI(4)P Golgi regions created by hSAC1. The escape of resident Golgi enzymes from the Golgi to the cell periphery in hSAC1 knock down cells supports this idea. Thus, it is possible that the phosphatase activity of hSAC1 in depleting PI(4)P at specific Golgi regions is coupled to the formation of COPI retrograde vesicles. To further test this hypothesis, one would have to study the dynamics of fluorescently labeled COPI vesicles in cells depleted of hSAC1 using live cell imaging technique. In addition, the localization of COPI in hSAC1-depleted cells could be studied via immunostaining and fluorescent microscopy in addition to biochemical approaches such as cell fractionation. Analyzing phosphoinositide profiles of COPI vesicles in hSAC1-depleted cells might shed light on the possible role of lipid microdomains in the formation of COPI vesicles.

Figure 4.1 shows a schematic illustration of lipid phosphatase hSAC1 in maintaining PI(4)P-depleted Golgi regions for proper recycling of Golgi resident enzymes such as the Man II and NacT1.

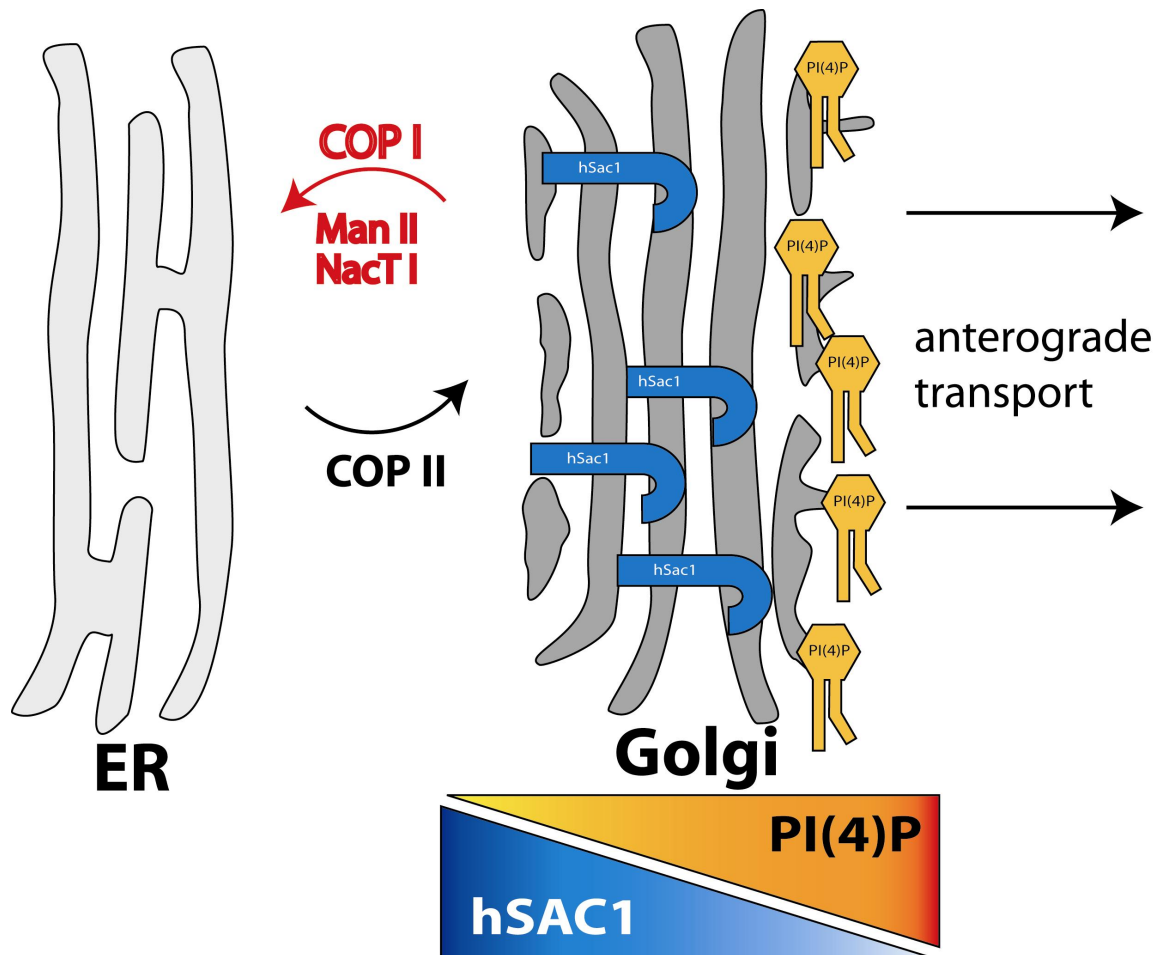


Figure 4.1: hSAC1 creates PI(4)P-depleted Golgi regions which are important for retrieval of Golgi resident enzymes such as mannosidase II (ManII) and N-acetylglucosaminyltransferase I (NacT1)
 COPII vesicles mediate transport of cargo from ER to Golgi while COPI vesicles are important for retrieval of Golgi resident enzymes. hSAC1 is concentrated at the Golgi cisternae while PI(4)P (anterograde transport signal) is highly enriched at the TGN. The opposing gradient of PI(4)P and hSAC1 is vital for segregating anterograde and retrograde transport events.

4.3: The PI(4,5)P₂ 5-phosphatase OCRL1 and its splice variants

The interest in studying the function of OCRL1 has been intensified since the discovery of a direct link between the Lowe syndrome and mutations of a PI(4,5)P₂ 5-phosphatase (Attree et al, 1992). How mutations in OCRL1 cause progressive multiple tissue dysfunctions in Lowe syndrome patients remains unclear. A failure to phenocopy Lowe syndrome in OCRL1 knockout mice (Janne, Suchy et al. 1998) further emphasizes the importance of studying the compartment specific function of this lipid phosphatase in tissue culture cells in order to dissect the intracellular molecular events governed by OCRL1.

OCRL1 has two tissue-specific splice variants, designated as isoform-a (OCRL1-a) and -b (OCRL1-b) (Lowe, 2005). Both isoforms are ubiquitously expressed with OCRL1-b being the predominant isoform expressed in all tissues with the exception of the brain. Interestingly, brain cells only express the longer version. The difference between these two isoforms is the presence of an additional 8 amino acids in OCRL1-a. These 8 amino acids form an acidic cluster that might contain specific binding motifs for other proteins (Introduction, Figure 2.7). This acidic cluster resembles phosphofurin acidic cluster sorting proteins (PACs) or Golgi-localizing, gamma-adaptin ear homology domain Arf-binding proteins (GGAs) binding sites. To study the isoform-specific function of OCRL1, we generated antibodies that recognize only OCRL1-a and examined intracellular localization of OCRL1-a in comparison to OCRL1-b. We found that OCRL1-b (which lacks the 8 amino acid sequence) was localized predominantly at the TGN and early endosomes as shown by co-localization studies using a TGN marker, TGN46 and an early endosome marker, EEA1. This is consistent with studies that demonstrate that OCRL1 associates with clathrin-positive endosomes that contain mannose-6-phosphate receptors (M6PRs) and transferrin (Tf) (Ungewickell et al., 2004; Choudhury et al., 2005). In contrast, OCRL1-a showed distinct localization from OCRL1-b. Immunofluorescence studies using antibody specific to OCRL1-a showed that the isoform-a was absent from perinuclear Golgi but localized to punctate structures. These structures did not colocalize with EEA1 but showed partial overlap with a late endosomal/lysosomal marker, lamp2. Although the lysosomal localization of OCRL1 has been reported (Zhang, Hartz et al. 1998), isoform specific localization of OCRL1 is novel.

The distinct localization of OCRL1 splice variants indicates that both isoforms might regulate different trafficking routes and have compartment specific functions. However, different splice variants of OCRL1 may be involved in different sorting steps involving clathrin-binding because both isoforms contain the clathrin-binding site LIDLE. Much work is needed to study the splice variant- specific function of OCRL1.

The Golgi localization of OCRL1 had been firmly established in different cell lines (Suchy, Olivosglander et al. 1995; Dressman, Olivos-Glander et al. 2000). To determine whether turnover of Golgi PI(4,5)P₂ by OCRL1 is coupled to PI(4)P metabolism by the PI(4)P-phosphatase hSAC1, we first localized both enzymes by immunostaining and confocal fluorescence microscopy study. We found that hSAC1 and OCRL1 populate different regions of the Golgi. OCRL1 was found preferentially in hSAC1-free Golgi regions that were highly enriched with PI(4)P, as determined by co-localization studies using Fapp1-PH domain and PI(4)P antibody. In addition, we silenced the expression of OCRL1 by RNAi and found that OCRL1 depletion had no effect on Golgi morphology or caused a mislocalization phenotype as observed in hSAC1-depleted cells. In contrast, knock down of OCRL1 caused defects in bulk anterograde secretion. Thus, hSAC1 and OCRL1 seem to play distinct roles in maintaining Golgi functions. The turnover of PI(4,5)P₂ at the Golgi complex is highly dynamic. Visualizing PI(4,5)P₂ using specific PI(4,5)P₂ binding domains fused with GFP or via a specific PI(4,5)P₂ antibody could not localize this phospholipid at the Golgi. Therefore, OCRL1 5-phosphatase activity might be required for converting the PI(4,5)P₂ signal to PI(4)P signal which is required for anterograde traffic.

The main unresolved question regarding Lowe syndrome is how an ubiquitously expressed lipid phosphatase could lead to tissue specific manifestations of complex phenotypes. More importantly, what is the functional relevance of the OCRL1 splice variants. To address this, one possibility is to selectively knock down the different isoforms of OCRL1 using RNAi and study intracellular trafficking events that might be governed by OCRL1. It is plausible that specialized cells in affected organs such as the lens epithelia cells, or the kidney proximal tubule cells require a functional OCRL1 for efficient and proper trafficking and recycling of cell surface membrane proteins. Elucidation of the function of OCRL1 splice variants in kidney proximal tubule cells is

attractive since one of the hallmarks of Lowe syndrome is Fanconi syndrome, which is a defect in solute and protein reabsorption in the proximal tubule (Lowe C.U., et al., 1952, Nussbaum R. et al., 2001). Recent finding of a Dent's disease patient with a mutation in OCRL1 further strengthens the notion that defective endosomal transport of transporter/channel might be involved in the development of progressive kidney failure (Hoopes, Shrimpton et al. 2005). Dent's disease is typically caused by defective endosomal chloride channel, CLC-5. Investigation in membrane trafficking, particularly clathrin-mediated transport from TGN to endosomal membranes, using fluorescent-tagged kidney transporters such as the CLC-5 and other transporters in proximal tubule cells may shed light on how dysfunction of OCRL1 causes Fanconi syndrome, one of the manifested symptoms in Lowe patients.

In conclusion, hSAC1 and OCRL1 are two functionally distinct lipid phosphatases that play important roles in regulating phosphoinositides at the Golgi complex. The separation of PI(4)P and PI(4,5)P₂ pools at Golgi membranes appears to be an important mechanism for organizing structurally and functionally distinct Golgi regions. Interplay between these two phosphatases tightly and spatially regulate the levels of both PI(4)P and PI(4,5)P₂. hSAC1 and OCRL1 establish separate phosphoinositide-specific domains within the Golgi, that are instrumental for segregation of anterograde trafficking from the recycling of resident Golgi enzymes.

5 Materials and Methods

5.1: Materials

5.1.1: Materials used in Heidelberg

All standard chemicals and reagents were purchased from either Merck (Darmstadt, Germany) or Sigma-aldrich Chemie GmbH (Deisenhofen, Germany). Nitrocellulose-Filter (BA85), Whatman 3MM from Schleicher and Schuell GmbH (Dassel, Germany). Acylamide solution from National Diagnostics (Atlanta/GA USA); prestained protein marker (broad range) from New England Biolabs GmbH (Schwalbach/Taunus, Germany); TEMED and skim milk powder from Fluka (Neu-Ulm, Germany). X-OMAT AR film from Kodak (Rochester, USA). Other kits used were Nucleobond AX100-2000 from Macherey-Nagel (Dueren, Germany); QIAquick PCR Purification Kit, QIAquick Gel Extraction Kit and QIAprep Spin Mini Prep Kit from Qiagen (Hilden, Germany). Tissue culture medium, Dulbecco's Minimum Essential Media (DMEM), fetal bovine serum (FBS), ampicilin and Lipofectamine/Plus transfection reagent were purchased from Invitrogen.

5.1.2: Materials used in Portland

All standard chemicals and reagents were purchased from either Sigma-Aldrich (Saint Louis, MO, USA), Fischer Scientific (Hampton, NH, USA), ISC Bioexpress (Kaysville, UT, USA) or VWR (West Chester, PA, USA). QIAquick PCR Purification Kit, QIAquick Gel Extraction Kit and QIAprep Spin Mini Prep Kit from QIAGEN (Valencia, CA, USA); GenElute Plasmid Miniprep and Maxiprep Kits from Sigma-Aldrich (Saint Louis, MO, USA); X-OMAT AR Filme from Kodak (Rochester, USA); GeneMate BlueAutoradiography Film from ISC Bioexpress (Kaysville, UT, USA); Nitrocellulose-filter (BA85) and Whatman 3MM from Fischer Scientific, EcoLume and EcoLight(+) scintillation liquid from MP Biomedicals; EZ-Link Sulfo-NHS-LC-Biotin (PIERCE) (Rockford, USA). ^3H -Thymidine, ^3H -*myo*-inositol, ^{35}S -Methionine/Cystein were from Perkin Elmer (Wellesley, MA, USA). Cell culture media DMEM, Trypsin-EDTA, Dulbecco's phosphate buffer saline (D-PBS) were from Sigma-Aldrich; OPTI-MEM Reduced-Serum Media, Lipofectamine/Plus and Lipofectamine 2000 from Invitrogen; FBS and New Born Calf Serum from Hyclone, Fischer Scientific.

5.1.3: Primers and oligonucleotides

Heidelberg:

Primers and oligos were synthesized by MWG Biotech (Ebersberg, Germany) and Biospring (Frankfurt, Germany).

Portland:

All oligos and primers were purchased from Operon (Huntsville, AL, USA).

Sequencing primers for TOPO-TA cloning:

PCR_Sac1a (Forward):

5'- GCCAGTGTGCTGGAATTCGCCCTT – 3'

PCR_Sac1b (Reverse):

5'- TGATGGATATCTGCAGAATTCGCC – 3'

For the generation of Δ NhSAC1(501-587)pEGFPC₂ and Δ NhSAC1(512-587)pEGFPC₂ N-terminal deletion mutants:

Sac1a:

5'- TGC AGA TCT ATT CAG TGG ATG AAT TAG AAT – 3'

Sac1b:

5'- TGC AGA TCT TAA GTG TTC CAA GGG ACT GG – 3'

Sac1c:

5'- GGA CTG CAG TCA GTC TAT CTT TTC TTT CTG G – 3'

Oligos for the generation of RNAi resistant plasmids:

GFP-hSAC1*:

GFP-hSAC1*forward:

5'- GGCAAACCAGGAAGGGGTCTTCCGGAGTAACTGCATGGATTGTC -3'

GFP-hSAC1* reverse:

5'- GACAATCCATGCAGTTACTCCGGAAGACCCCTTCCTGGTTTGCC -3'

GFP-hSAC1-C/S*:

GFP-hSAC1-C/S* forward:

5'- GGCAAACCAGGAAGGGGTCTTCCGGAGTAACAGCATGGATTGTC-3'

GFP-hSAC1-C/S* reverse:

5'- GACAATCCATGCTGTTACTCCGGAAGACCCCTTCCTGGTTTGCC-3'

Primer for sequencing of the mutagenized GFP-hSAC1* and GFP-hSAC1-C/S*:

5'- CCATAAGGAATGTAAAAATATGAGATG-3'

Primer for sequencing of the RNAi constructs:

5'-AAT ACG TGA CGT AGA AAG TA-3'

Oligos for the generation RNAi constructs:

hSAC1 RNAi plasmids:

Construct I:

5'-TCGAGGGCGTGTTCCGAAGCAATTTCAAGAGAAATTGCTTCGGA
ACACGCCTTTTT-3'

5'- CTAGAAAAGGGCGTGTTCCGAAGCAATTTCTCTTGAAAATTGCTT
CGGAACACGCCC-3'

Construct II:

5'-TCG AGC TTC AGA GAC TAG GAG TTT TTC AAG AGA AAA CTC CTA GTC
TCT GAA GTT TTT-3'

5'-CTA GAA AAA CTT CAG AGA CTA GGA GTT TTC TCT TGA AAA ACT CCT
AGT CTC TGA AGC-3'

hSAC1 RNAi Control plasmid:

5'-TCG AGA GCG TGG TCC GAA GCC ATT TTC AAG AGA AAT GGC TTC GGA
CCA CGC TTT TTT-3'

5'-CTA GAA AAA AGC GTG GTC CGA AGC CAT TTC TCT TGA AAA TGG CTT
CGG ACC ACG CTC-3'

OCRL1 RNAi constructs:

Construct A:

5'-TCGAGGATAGTGTACGCATCATGGTTCAAGAGACCATGATGCGTACAC
TATCTTTTT-3'

5'-CTAGAAAAAGATAGTGTACGCATCATGGTCTCTTGAACCATGATGCGT
ACACTATCC-3'

Construct B:

5'-TCGAGGACAGACTCCAAGTGACCGTTCAAGAGACGGTCACTTGGAGTC
TGTCTTTTT-3'

5'-CTAGAAAAAGACAGACTCCAAGTGACCGTCTCTTGAACGGTCACTTGG
AGTCTGTCC-3'

Construct C:

5'-TCGAGGCAGACGATCTCGGGCTCCTTCAAGAGAGGAGCCCGAGATCGT
CTGCTTTTT-3'

5'-CTAGAAAAAGCAGACGATCTCGGGCTCCTCTCTTGAAGGAGCCCGAGA
TCGTCTGCC-3'

Control OCRL1 construct:

5'-TCGAGCATCGTGTCCGCATCACGGTTCAAGAGACCGTGATGCGGACAC
GATGTTTTT-3'

5'-CTAGAAAAACATCGTGTCCGCATCACGGTCTCTTGAACCGTGATGCGG
ACACGATGC-3'

siRNAs sequences:

OCRL1 siRNA duplex (siOA):

Forward:

5'- GAU AGU GUA CGC AUC AUG G tt -3'

Reverse complement :

5'- C CAU GAU GCG UAC ACU AUC tt -3'

OCRL1 Control siRNA duplex (siOCtr):

Forward :

5'- CAU CGU GUC CGC AUC ACG G tt -3'

Reverse complement :

5'- C CGU GAU GCG GAC ACG ATG tt -3'

hSAC1 siRNA duplex (siA):

Forward :

5'- GGC GUG UUC CGA AGC AAU U tt -3'

Reverse complement :

5'- A AUU GCU UCG GAA CAC GCC tt -3'

hSAC1 Control siRNA duplex (siCtr):

Forward :

5'- AGC GUG GUC CGA AGC CAU U tt -3'

Reverse complement :

5'- A AUG GCU UCG GAC CAC GCU tt -3'

5.1.4: Plasmids and constructs

Vectors:

pCDNA3.0 (Invitrogen):

Eukaryotic expression vector for constitutive expression; contains CMV promoter and T7 promoter, neomycin resistance cassette

pCRII_TOPO (Invitrogen):

TOPO-cloning vector for "TA-cloning" of PCR products, contains T7 promoter and SP6 promoter.

pEGFPC1 and pEGFPC2 (Clontech):

pEGFPC1 and pEGFPC2 each encode the green fluorescence protein GFPmut1 variant which contains the double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr. Genes cloned into the multiple cloning sites (MCS) will be expressed as fusions to the C-terminus of EGFP if they are in the same reading frame as EGFP. A Neomycin resistance cassette (Neo^R), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5 and polyadenylation signals from Herpes simplex virus thymidine (HSV TK)kinase gene allows stably transfected eukaryotic cells to be selected for using G418.

pSuppressorNeo (from Gene Suppressor System; IMGENEX Corporation) :

Linearized plasmid vector (digested with Sall and XbaI) for cloning of the annealed oligos containing an 5'-XhoI (forward strand oligo) and an XbaI overhang of the 5'-reverse oligo. It contains a U6 promoter for expression of the siRNAs. Contained a Neomycin/Kanamycin resistance cassette (Neo/Kan^R)

GFP-hSAC1:

Full length wild type hSAC1 was obtained from pYEX_BX_hSAC1wt (Rohde et al., 2003) using BamHI restriction digest and cloned in frame into pEGFPC1. This resulted in an N-terminally tagged GFP-hSAC1.

GFP-hSAC1-C/S:

GFP-tagged N-terminally hSAC1 phosphatase dead mutant (Rohde et al., 2003)

 Δ hSAC1(501-587)pEGFPC2

hSAC1 N-terminal-deleted mutant fused with GFP at its N-terminus. Primer Sac1a and Sac1c were used in PCR to generate a 314bp fragment. PCR fragments were cloned into pCRII_TOPO vector and subcloned into pEGFPC2 using BglIII and PstI restriction sites.

Δ NhSAC1(512-587)pEGFPC2

N-terminal deletion mutant of hSAC1. Primer Sac1b + Sac1c were used in PCR to generate a 254bp fragment. Plasmid was generated as described in **Δ NhSAC1(501-587)pEGFPC2**

GFP-OCRL1-b

A kind gift from Martin Lowe (Choudhury, Diao et al. 2005)

pBB3:

Plasmid containing the 5'-end of the RedStar2 sequence fused with the Myc-Fapp1-PH domain and cloned into pcDNA3.0(-) (generated by Ben Brankatschk)

pBB2:

Plasmid containing the 5'-end of the GFP sequence fused with the Myc-Fapp1-PH domain and cloned into pcDNA3.0(-) (generated by Ben Brankatschk)

NacT1GFP:

Amino acid (1-103) of NacT1-Myc-GFP cloned into PCNG2 (a kind gift from Dave Shima)

hSAC1 RNAi resistance plasmids:

Both GFP-hSAC1* and GFP-hSAC1-C/S* were generated from the original template plasmids: GFP-hSAC1 and GFP-hSAC1-C/S using the QuickChange II Site-directed Mutagenesis kit (Stratagene).

pECFP-Golgi (human beta 1,4-galactosyltransferase) (CLONTECH):

Encodes a fusion protein consisting of enhanced cyan fluorescent protein (ECFP) and a sequence encoding the N-terminal 81 amino acid of human beta 1,4-galactosyltransferase.

5.1.5:Peptides used for generating hSAC1 antibody**Table 5.1: Peptide sequence for generating hSAC1 antibody**

Peptide used	Peptide sequence
hSAC1A (#66)	C QLQDNKTFLAML
hSAC1 B (#67)	C DGFQRHFDSQVI
hSAC1 C (#68)	C TRTGKRTHLGLI
hSAC1 D (#69)	C NGKDFVDAPRLVQKEKID

5.1.6 : Enzymes and kits**Heidelberg :**

Restriction enzymes, alkaline phosphatase and T4-DNA ligase and the corresponding buffers were purchased from New England Biolabs GmbH (Schwalbach/Taunus, Germany) or Roche Diagnostics GmbH (Mannheim, Germany), Taq polymerase was from TaKaRa (Shiga, Japan).

Portland:

Restriction enzymes, alkaline phosphatase, T4-DNA ligase, Taq polymerase and the corresponding buffers were purchased from New England Biolabs (Beverly, MA, USA) or Roche Diagnostics GmbH (Indianapolis, IN, USA); 100bp-DNA marker from New England Biolabs and 1kb-DNA marker from ISC Bioexpress. Kits used were Gene Suppressor System (IMGENEX Corporation) (Cat#: IMG-800); Mini and Maxipreps kits (SIGMA); TOPO TA Cloning Kit (Invitrogen);Quick Change Mutagenesis (Qiagen); QIAquick Gel Extraction kit (Qiagen); Phosphatase, alkaline (calf intestine) (Roche); T4 Phage Ligase (Roche); ECL solution (Milipore); ProLong Antifade Kit (P-7481) (Molecular Probes)

5.1.7: Cell culture media, transfection reagents and cell lines

Dulbecco's Minimum Essential Media (DMEM), L-glutamine, DMEM (Cystein/Methionine free), Fetal Bovine Serum and New Born Calf Serum were from Hyclone; Gentamycine, Trypsine/EDTA and D-PBS (-Ca²⁺ &-Mg²⁺) were purchased from SIGMA. OPTI-MEM Reduced Serum Media, Lipofectamine/ Plus reagent and Lipofectamine 2000 were purchased from Invitrogen.

Tissue culture cell lines used :**(1) HeLa cells**

Human cervical cancer cell line containing the E6 and E7 genes of the human papillomavirus 18 integrated into the genome.

(2) HEK293 cells

HEK 293 cells were generated by transformation of human embryonic kidney cell cultures (hence HEK) with sheared adenovirus 5 DNA.

(3) COS7 cells

Cercopithecus aethiops kidney cell line, transformed with SV40 large T antigen

(3) NIH-3T3 cells

Mouse fibroblast cell line

5.1.8: Fixatives used for immunogold EM sample preparation

(1) 8% EM grade glutaraldehyde from Polyscience (Cat.00216)

(2) 10% paraformaldehyde freshly prepared from paraformaldehyde (Sigma P1213)

Preparation of 10% stock solution of paraformaldehyde in aqua dest:

10 g paraformaldehyde were added to 75 ml aqua-dest (demi-water). Place the solution "au bain marie" (in another beaker with hot water) on a combined stirrer/heater in the hood. The temperature of the water bath should not exceed 60°C. The solution was kept at 55 °C to 58 °C under constant stirring until all paraformaldehyde had dissolved. Add 0.1 M NaOH to solution to get the final pH of the fixative to pH 7.0. Aqua dest was added to an end volume of 100 ml and filtered using whatman paper on a funnel and finally stored in aliquots in the -20 °C freezer. Upon thawing the solution would remain white until cleared in hot. If it did not get clear, it was not used.

Stock-solution of 0.2 M phosphate buffer:

Buffer A: $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O} = 27.6 \text{ g}$ in 1000 ml aqua dest

Buffer B: $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O} = 53.65 \text{ g}$ or

$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O} = 35.6 \text{ g}$ or

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O} = 71.7 \text{ g}$ in 1000 ml aqua dest.

Add 19 ml buffer A to 81 ml buffer B to get 0.2 M phosphate buffer, pH7.4. Check pH and if necessary add more of the appropriate buffer to get pH7.4

5.1.9: Antibodies**5.1.9.1: Primary antibodies****Table 5.2: Primary antibodies used and their tested applications**

Antibodies	Raise in	dilution	source
α -hSac1(#69)	Rabbit	IF 1:100 WB 1:7500	Self-made in ZMBH
α -PI4P	Mouse	IF 1:100-1:250	Echelon
α -Mannosidase II	Rabbit	IF 1:1000 WB 1:500	Complex Carbohydrate Centre University of Geogia
α -Mannosidase II	Mouse	IF 1:100	Chemicon
α -TGN46	Sheep	IF 1:1000	Serotec
α -p24 "Elfriede"	Rabbit	IF 1:200	Felix-Wieland, BZH, Heidelberg.
p58k (clone 58k-9)	Mouse	IF 1:100	SIGMA
α -Sec61 β	Rabbit	IF 1:100 WB 1:10 000	Benhard Dobberstein, ZMBH, Heidelberg

α -OCRL1	Mouse	IF 1:50 WB 1:400	Sharon F. Suchy, NIH
α -OCRL1 isoform a	Rabbit	WB 1:10000 IF 1:50	Tailer-made; GeneMed
α -OCRL1 isoform a & b	Guinea Pig	WB 1:500 IF 1:50	
L23a	Rabbit	WB: 1: 5000 IP: 1:50	Benhard Dobberstein, ZMBH, Heidelberg
Sec61 α	Goat	WB 1:100-1:1000 IF 1:100	SantaCruz
GFP (FL)	Rabbit	WB 1:1000	Santa Cruz
GFP	Rabbit	WB 1: 10 000	Dirk Gorlich,ZMBH, Heidelberg
GAPDH	Mouse	WB 1: 100 000	Abcam
MHC-I	Mouse Clone W6/32	IP 1:500 WB 1:5000	Klaus Früh, VGTI,OHSU West campus, Portland, OR
PARP	Mouse Clone C2-10	WB 1.5000	BD Biosciences Pharmingen

5.1.9.2: All secondary antibodies (coupled to fluorophore or HRP-coupled) were purchased from Jackson Immunoresearch.

5.1.10: Common buffer stocks and solutions

- (1) 1x SDS-PAGE running buffer
1.44% glycine, 0.3% Tris-base, 0.1% SDS
- (2) Ponceau S
0.2% ponceau S in 3% Trichloroacetic acid (TCA)
- (3) 20 X PBS
160g NaCl, 4g KCl, 28.8g Na₂HPO₄, 4.8g KH₂PO₄
- (4) 1x TAE buffer
-40mM Tris acetate, 2mM EDTA, pH adjusted to 7.8 using (1N HCl or 1N NaOH to adjust)
- (5) 6x DNA loading buffer
-0.25% bromophenol blue, 0.25% Xylen Cynol blue, 30% glycerol in H₂O
- (6) LB media
-1% (w/v) peptone, 0.5% (w/v) yeast extract, 170mM NaCl and pH adjusted to pH7.0 using 5N NaOH
- (7) LB/Amp
100µg/ml ampicillin in 1L of LB
- (8) LB/Kan
50µg/ml kanamycin in 1L of LB
- (9) SOB
2% (w/v) trypton, 0.5% yeast extract , 10mM NaCl, 10mM MgSO₄, 10mM MgCl₂ and 2.5mM KCl. pH was adjusted to pH7.0 using 5N NaOH.

5.2: Methods**5.2.1: Methods in Molecular Biology**

Extraction and purification of plasmid DNA from *E.coli* were performed according to the manufacturer's instructions (SIGMA Maxiprep and Miniprep kits, Portland; Nucleobond AX100-2000). All the standard methods of molecular biology that were not described in this chapter were performed according to (Sambrook, Fritsch et al. 1989).

5.2.1.1: Ethanol precipitation of plasmid DNA

1/10 volume of 3M Sodium acetate, pH 5.2 and 3 volumes of 100% Ethanol were added to DNA. Solutions were mixed and incubated in -20°C freezer either overnight or 1-2 hours before centrifugation at 13,000g for 10min. The resulting DNA pellet was washed 2 times with 70% Ethanol. The pellet was then air dried for 5 min and resuspended in water or TE buffer. The DNAs were stored at -20°C.

5.2.1.2: Agarose gel electrophoresis

To separate DNA fragments, 0.8%-2% agarose gels were used depending on the fragment size. Agarose was dissolved in 1 X TAE buffer by using a microwave oven. 1µg/ml ethidium bromide was added to the agarose solution, which was poured into a gel chamber. DNA samples were mixed with 6x loading buffer in a ratio of 1:5 before loading onto the agarose gel. Electrophoresis was performed in 1 x TAE buffer at 40-60mAs.

5.2.1.3: DNA Restriction Digests

For analytical restriction digest of plasmid DNA, 10 units of appropriate restriction enzymes were used. 50 units of restriction enzymes were used for preparative digestions with starting DNA materials between 5-10 µg.

For cloning of DNA fragments into desired expression vectors, the insert and backbone DNAs were digested with the appropriate restriction enzymes for 3 hours or overnight at 37°C. The DNA fragments were separated by gel electrophoresis and purified via QIAquick gel extraction kit according to the manufacturer's instructions.

5.2.1.4: Alkaline phosphatase treatment

Before ligating the digested and gel purified DNA fragments, the vector backbone was treated with approx. 5units of Antarctic Phosphatase for 15 min (blunt ends) or 1 hour at 37°C. The phosphatase was deactivated at 65°C for 5 min.

5.2.1.5: Ligation

Ligation was performed using 1 Unit of T4 ligase (Roche) in 10-20 μ l reaction. Different ratios of vector:insert were set up [1:5, 1:7 and 1:10 (v/v) e.g. 1 μ l Vector:5 μ l Insert] and incubated overnight at 16°C. The ligation mixture was desalted by using nitrocellulose membrane (Milipore) floats on water for 30 min before transforming into electrocompetent bacteria cells.

For cloning of PCR fragments into vector backbones, the TOPO-TA cloning was done according to the manufacturer's instructions. Taq Polymerase generated PCR fragments contained single deoxyadenosine overhangs at the 3'-end (dA-3') while the linearized pCRII vector possessed an overhanging deoxythymidine residue (dT-3'). This allowed efficient annealing of the PCR fragment to the vector, catalyzed by Topoisomerase I which was covalently bound to the vector. For subcloning of the PCR fragments into different mammalian expression vectors, appropriate restriction digests were performed and fragments were purified as described above.

5.2.1.6: Cloning of RNAi plasmid

The RNAi constructs were generated using the Gene Suppressor System (IMGENEX Corporation). Oligos containing a 5'-XhoI site (forward), a 5'-XbaI site (reverse complement strand), and inverted repeats of the targeting sequence flanked by a 6bp spacer were annealed according to the manufacturer's instructions. The annealed oligos were ligated into linearized pSuppressorNeo vector. The ligation product was then transformed into competent cells as described in 5.2.1.9.

5.2.1.7a: Polymerase Chain Reaction (PCR)

For cloning of hSAC1 C-terminal mutants (3-pEGFPC2; 5-pEGFPC2), PCR was used to amplify the last 254bp and 314bp of the C-terminal of hSAC1. PCR was performed using a mixture of Taq and Pfu polymerases. PCR was set up in 50 μ l reaction mixtures as follows:

50-100ng/ μ l template DNA (hSAC1wtGFP)

25pmol/ μ l 5'- and 3'- primers each

10mM dNTPs (2mM dNTPs stock)

3.2.1.7b: Site-directed mutagenesis

To generate RNAi resistant plasmid, GFP-hSAC1* and GFP-hSAC1-C/S*, PCRs were performed using the *PfuUltra* high-fidelity (HF) DNA polymerase (2.5U/ μ l), dNTP mix and 10x reaction buffer supplied by the manufacturer (Stratagene). GFP-hSAC1 and GFP-hSAC1-C/S were used as templates together with the mutagenesis primers listed in 5.1.3. Sample reactions were prepared as follow:

5 μ l 10 X reaction buffer

X μ l (5-50ng) of dsDNA template

X μ l (125ng) of oligonucleotide forward primer

X μ l (125ng) of oligonucleotide reverse primer

1 μ l of dNTP mix

ddH₂O to final volume of 50 μ l then add 1 μ l of *PfuUltra* HF DNA polymerase

PCRs were performed as follows:

Segment	Cycles	Temperature	Time
1	1	95°C	30s
2	2-18	95°C	30s
		55°C	1min
		68°C	2min/kb of plasmid length

The resulting PCR products were digested with 1 μ l of DpnI restriction enzyme (10U/ μ l) for 1 hour at 37°C to digest the parental supercoiled dsDNA. This was followed by transformation of XL1-Blue Supercompetent cells/ One shot Top 10 cells via chemical transformation (5.2.1.9).

5.2.1.8: Making of electrocompetent cells

Electrocompetent *E.coli* cells were made from DH5- α bacteria (Clontech). A 5ml DH5- α bacteria culture was set up in SOB media and incubated overnight at 37°C. The 5ml precultured bacteria were added into 500ml SOB the next day and further incubated at 37°C until they reached an $OD_{600nm} = 0.4$. Cells were pelleted by centrifugation at 3500 rpm, 4°C. The resulting pellet was washed 3 times with ice cold 1.4% Glycerol and finally resuspended in 2-3 volumes of 15% Glycerol. Cells were aliquoted (120 μ l) in 1.5ml tubes and snap frozen using liquid nitrogen. They were then stored at -80°C. The competent bacteria cells were electroporated using an electroporator at the setting of 2.5kV, 200 Ω and 25 μ F. If the time constant was less than 4.6, the cells were washed again with 1.4% Glycerol before storage in the freezer.

5.2.1.9: Transformation of competent cells

For electroporation of electrocompetent cells, 5-10 μ l of desalted ligation mixture were added to 60 μ l cell suspension and transferred to a prechilled 4mm-gap cuvette. Cells were electroporated at 2.3-2.5kV, 200 Ω , 25 μ F and 250 μ l of LB or SOC media were added to cells. After 1 hour incubation at 37°C in a shaker, cells were plated on either LB Ampicillin or Kanamycin plates and incubated overnight at 37°C. For transformations using chemically competent cells (One shot Top 10 cells, Invitrogen), cells were thawed on ice and the ligation mixture was added to 50 μ l of competent cells. Cells were incubated on ice for 30 min before heat-shock at 42°C for 45s. 200-250 μ l of SOC media was added to cells, which were then incubated for 1 hour at 37°C, 220rpm.

5.2.2: Methods in tissue culture

5.2.2.1: Cultivation and passaging of mammalian cells

All cell lines were cultivated using standard tissue culture techniques (according to instructions/ protocols at the ATCC website: <http://www.atcc.org/common/technicalInfo/TechLit.cfm>). Experimental work with mammalian cell lines was performed in sterile laboratory. Cells were cultured in 5% CO₂, 95% humidify water-jet humidified incubator 37°C.

Cells were passaged when they reach confluency between 75-95%. To passage or seed cells for experiments, 0.5ml-3ml of trypsin-EDTA (SIGMA) was added to cells after 1x wash with D-PBS (without calcium/magnesium). After a brief incubation at 37°C, serum-containing media was added to cells and cells were detached from plates/flasks by trituration. For experiments, 15µl of cell suspension were counted in a counting chamber (Haemocytometer) and triple values were used to calculate the mean.

5.2.2.2: Freezing and thawing cells

To preserve cell lines, cells were cultured in a flask until 95% confluency. After trypsinization, cells were centrifuged at 1200 rpm and resuspended in 2 ml DMEM containing 10% DMSO. 1ml of this suspension was added to each 2ml cryovial (NUNC) and stored at -80°C overnight before long term storage in a liquid nitrogen tank.

To recover frozen stock, cells were hand warmed and thawed by warming the cyovials in a 37°C water bath. The thawed cell suspension was added to a flask containing 10ml of media (at least 10 x volume of the frozen stock). Media was changed after cells were attached.

5.2.2.3: Transfections using Lipofectamine Plus and Lipofectamine 2000

Cells were seeded one day before transfection at a density between 2.2×10^5 to 10.0×10^5 depending on the size of the dishes used for each experiment. Plasmid DNAs or siRNAs and transfection reagents (Lipofectamine/Plus or Lipofectamine 2000) were diluted in OPTI-MEM reduced serum media in eppendorf tubes according to the manufacturer's instructions. After incubation for 20-25min at room temperature, the complexes were added to cells in antibiotic free DMEM or OPTI-MEM. In the case of OPTI-MEM, the media was changed 3-6 hours after incubation with serum containing DMEM. Cells were assayed at least one or two days post-transfection (for expression of GFP-tagged markers in immunofluorescence studies). For experiments using hSAC1 or OCRL1 knock down cells, the experiments were only performed on day 3 or day 4 post-transfection.

5.2.3: Expression and analysis of proteins in tissue culture cells

5.2.3.1: Preparation of cell lysates

In general, two methods were used to prepare cell lysates for SDS-PAGE. For pulse-chase and time course experiments (in which immunoprecipitations were performed), cells were lysed in 0.5ml (for 6cm-dish) or 1ml (for 10cm-dish) of RIPA buffer (1% NP40, 1% Sodium deoxycholate, 150mM NaCl, 50mM Tris pH 8.0). After 10-15 min incubation on ice, cells were collected in a 1.5ml eppendorf tubes and centrifuged at $13,000 \times g$ for 10 min at 4°C . Supernatants were transferred to new tubes and immunoprecipitations were performed.

Alternatively, cells were trypsinized and 1ml of cell suspension was used. After centrifugation at $1200-2000 \times g$ for 2 min, supernatants were discarded and cell pellets were resuspended in 2 x LSB and boiled for 5 min. Following 3 min of centrifugation at $13,000 \times g$ supernatants were loaded onto an SDS-PAGE gel.

5.2.3.2: SDS Gel Electrophoresis (SDS-PAGE)

To separate proteins, SDS-gel electrophoresis was employed according to the method of Laemli. 4% stacking and 10% separating gel were used. Polyacrylamide gels were made according to the table below:

	4% stacking gel	10% separating gel
Water (ml)	3	11.2
2M Tris-HCl, pH 8.8 (ml)	-	5
0.5M Tris-HCl, pH 6.5 (ml)	1.25	-
37% Polyacrylamide (ml)	0.67	8.3
10% SDS (μ l)	50	250
10% Ammonium Persulfate (μ l)	50	200
TEMED (μ l)	5	20

Protein samples were denatured in Laemli sample buffer (LSB) at 95°C for 5 min before they were loaded on SDS-gel. SDS-PAGE was performed at 50-60mA under ventilation.

To make 50ml of	2x LSB	5x LSB
1M Tris-HCl, pH=6,8 (ml)	6	15
Glycerol (ml)	10	25
Beta-Mercaptoethanol (ml)	5	10
Bromophenol blue (mg)	1	2
SDS (g)	2	5
ddH ₂ O	29	-

5.2.3.3: Western blot

After proteins were separated via SDS-PAGE, they were transferred onto nitrocellulose membrane by Western blotting. A Semi-dry blotting apparatus was used and Western blotting was set up as follows:

(from Below/on the surface of the apparatus):

3 Whatman papers soaked in buffer A2 (300mM Tris, 20%(v/v) Methanol)

3 Whatman papers soaked in buffer A1 (30mM Tris, 20% (v/v) Methanol)

nitrocellulose soaked in buffer A1 (30mM Tris, 20% (v/v) Methanol)

SDS-PAGE Gel equilibrated in buffer K (25m Tris, 40mM 6-aminohexanoic acid, 20% (v/v) Methanol, 0.01% SDS)

Finally 3 Whatman papers in Buffer K (25m Tris, 40mM 6-aminohexanoic acid, 20% (v/v) Methanol, 0.01% SDS) on top of the gel.

(Top)

Western blotting was performed for 1h at 297 mA followed by Ponceau S staining (to visualize protein bands on a nitrocellulose membrane). For documentation, the Ponceau S stained nitrocellulose was photocopied. The nitrocellulose was then washed with PBS/T followed by 1hour blocking in 10% milk/PBST. In some cases, blocking was done overnight at 4°C.

To detect specific proteins on the membrane, specific antibody against the protein of interest diluted in 2% milk/PBST was added to the membrane and incubated either at room temperature for 1 hour or overnight at 4°C on a shaker. The membrane was washed for 30 min in PBS/T (6 x 5 min PBS/T washes) and further incubated with respective secondary antibodies coupled to horse-radish peroxidase (HRP) in 2% milk/PBST. After 6 x 5 min PBS/T washes, the membrane was incubated with ECL solution (Millipore) for 5 min and specific protein bands were detected on KODAK film in dark room.

5.2.3.4: Immunoprecipitation

Cell lysates were prepared as Section 5.2.3.1. The lysates were incubated either overnight or 1-4 hours at 4°C with 50% agarose beads/PBS on a rotisserie. After preclearance, lysates were incubated with antibodies overnight in the cold room. Either 50% protein A/PBS or 50% protein G agarose/PBS was added to lysates (depending on the source of the primary antibody) and incubated for 1 hour at 4°C. The agarose beads were washed twice with RIPA buffer (1% NP40, 1% Sodium deoxycholate, 150mM NaCl, 50mM Tris pH 8.0) before addition of 2 x LSB. Samples were boiled at 95°C for 5 min and subjected to SDS-PAGE.

5.2.3.5: Generation of specific antiserum against hSAC1

Rabbit polyclonal antibodies against hSAC1 (#69) were generated by the assistance of animal facilities in the ZMBH, Heidelberg. Peptides were made by Peptide Specialty Laboratories GmbH, Heidelberg. The peptide sequences were listed in Table (5.1). Briefly, all 4 peptides were synthesized with a Cysteine at the N-terminus. Inject Mariculture Keyhole Limpet Hemocyanin (PIERCE) (Cat#: 77600) was coupled to these peptides and used for immunization. 4 rabbits were immunized:

Rabbit #66: Peptide hSAC1 A

Rabbit #67: Peptide hSAC1 B

Rabbit #68: Peptide hSAC1 C

Rabbit #69: Peptide hSAC1 D

5.2.4 : Cell biological, biochemical and molecular biology functional analysis

5.2.4.1: Immunofluorescence

Cells were seeded on round coverslips (Carolina) either after transfections (day one post-transfection) or a day before immunostaining. Before addition of fixative, cells were washed 2-3 times with PBS. Cells were fixed by 4% paraformaldehyde/PBS or 4% formaldehyde/PBS for 15 min at RT. After PBS washes (3-4 times), cells were incubated with 100mM Glycin/PBS or 50mM NH₄Cl/PBS for 5min and permeabilized using 0.1%-0.4% TritonX/PBS for 3 min. This was followed by 5 washes with PBS and cells were incubated with 1% BSA/PBS for at least 30min – 1hour at RT. Primary antibodies were

added to cells and incubated for 1 hour at RT (4°C for overnight incubation). Cells were incubated with secondary antibodies after 5 x 5 min PBS washes. Incubation was carried out at RT for 1 hour. For mounting of samples, cells were washed 5 x 5 min with PBS and rinsed with water before they were mounted. ProLong Antifade Kit (P-7481) (Molecular Probes) was employed to mount samples. Both primary and secondary antibodies were diluted in 1% BSA/PBS between 1:100-1:1000 dilution depending on the affinity of the antibodies. In general, all secondary antibodies were used in 1:100 dilutions.

5.2.4.2: Alternative protocol for immunostaining

After 15-20 min fixation with 4% paraformaldehyde/PBS, cells were washed twice and permeabilized using 0.2% Saponin/PBS for 30 min. Cells were then incubated with blocking solution (0.2% Saponin, 5% Gelatin in PBS) for 30 min to 1 hour. Both primary and secondary antibodies were diluted in blocking solution before added to cells. Antibody incubation periods were described as above (5.2.4.1)

5.2.4.3: Staining of phosphoinositides using specific antibodies

Localization of different phosphoinositide pools can be visualized either by ectopic expression of specific phosphoinositide binding domains fused with GFP or GFP variants or by specific antibodies against different species of phosphoinositides. To study PI(4)P at the Golgi membranes, expression of a Fapp1-PH domain fused with GFP and RFP was used. 24-48 hours post-transfection, cells were fixed and visualized under a fluorescence microscopy. In colocalization studies, cells were permeabilized as described in (5.2.4.1).

For staining of PI(4)P, cells were fixed with 2% Formaldehyde/PBS for 10 min at RT. After 3 washes with PBS, 10µg/ml digitonin in 100mM Glycine/PBS was used to permeabilize cells. After 5 min incubation at RT, cells were washed (3-5 times) with PBS and 1% BSA/PBS was added to cells for blocking. PI4P antibody was diluted in 1% BSA/PBS (1.100-1.200) and incubated overnight at 4°C. Secondary antibody incubation was as described in (5.2.4.1).

5.2.4.4: Immunofluorescence Microscopy

siRNA treated cells were trypsinized and reseeded on glass coverslips. Immunofluorescence was performed as described in Rohde H.M. et al, 2003. In brief, cells were fixed with 4% Paraformaldehyde, incubated with 0.1M Glycine, and permeabilized with 0.4% Triton X-100. Cells were incubated with respective primary and secondary antibodies diluted in PBS containing 1% bovine serum albumin.

Cells were examined using a Nikon Eclipse E800-microscope equipped with a CoolSNAP HQ-Camera from Photometrics (Tuscan, AZ, USA). Confocal micrographs were taken by an Olympus BX51-Microscope using an Olympus PlanApo 60 x Oil immersion objective. Images were analyzed using Metamorph Image software (for fluorescence micrographs) and Image J (Confocal micrographs).

5.2.4.5: Lipid extraction and HPLC analysis

Cells were labeled with 10 μ Ci/ml [³H]-*myo*-inositol in inositol-free media (ICN) for 48 hours before they were harvested. After washing with ice-cold PBS, 1ml of ice-cold 0.5M perchloric acid was added to cells and the cells were scraped into an eppendorf tube. The resulting pellet was washed once with ice-cold 0.5M perchloric acid, To solubilise lipids in the pellet, 750 μ l of methanol:chloroform:HCl (80:40:1 v/v) was added and vortexed briefly (1min). After incubation at room temperature for 30 min, 250 μ l of chloroform and 450 μ l of 0.1M HCl were added and centrifuged for 1min to separate the phases. The lower organic phase was transferred to a new tube and 50 μ l of 1M NH₄OH/Methanol was added to the upper aqueous phase to neutralize it. The aqueous phase was re-extracted twice with 450 μ l of synthetic lower phase, [Methanol: Chloroform: HCl, 80/40/1]: Chloroform: 0.1M HCl, 3/1/1.8 v/v. The pooled organic phase was washed with 5 volumes of 2M KCl, and dried in a speedvac.

Dried lipid pellet was deacylated by adding 200 μ l of methyamine reagent and incubated in a sealed tube at 53°C for 30min. The mixture was dried in a speedvac and the dried lipid was re-dissolved in 500 μ l of distilled water and 600 μ l butan-1-ol: petroleum ether: ethyl formate (H₂O-saturated), 20/4/1 v/v, vortexed and centrifuged for 1min. The upper phase was taken off and this step was repeated once with the lower

phase, followed by final extraction with petroleum ether: ethyl formate, 4/1 v/v. The lower phase was dried in a speedvac and the pellet was resuspended with 160µl of 10mM (NH₄)₄HPO₄. HPLC analysis of glycerophosphoinositols was carried out on a Jasco HPLC system equipped with an LB 508 Radioflow detector (Barthold, Bad Wildbach, Germany). The following gradient for elution of the HPLC column was used: buffer A contained distilled water, buffer B contained 1M (NH₄)₄HPO₄ (pH3.8). The gradient was run at 0% buffer B for 10 min and increased to 25% buffer B over 60min and 20 min at 0% buffer B. The flow rate was 1ml/min.

5.2.4.6: Cell Surface Biotinylation of NacT1GFP in hSAC1 siRNA treated cells

Hela cells were plated in 10cm-dishes one day before transfection in antibiotic free media. Cells were cotransfected with plasmid expressing NacT1GFP and RNAi resistant GFP-hSAC1* or GFP-hSAC1-C/S* together with siRNA directed against hSAC1 or a control siRNA using Lipofectamine 2000. 72 hours post-transfection, cells were washed 3 times with PBS and incubated with 0.3-0.5mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce) for 30 min at 4°C. 100mM Glycine/PBS²⁺ were used to quench for 5 min. Cells were lysed with 1ml of RIPA buffer supplemented with protease inhibitor cocktails (Roche) at 4°C for 10 min. Supernatants were incubated with agarose/PBS for 4 hours at 4°C before addition of streptavidin agarose. The samples (Streptavidin-Biotin lysates) were incubated overnight at 4°C. After two washes with RIPA buffer, the samples were boiled for 5 min in 2x Laemli sample buffer. The samples were then separated on a 10% SDS-PAGE gel and analysed by Western blotting.

5.2.4.7: Secretion assays

5.2.4.7.1: Bulk secretion assay

Cells were starved in Met/Cys free media for 30 min and then pulse labeled with 30µCi of [³⁵S]-Met/Cys (S35-EXPRESS from Amersham) per well of a 6-well plate for 10 min at 37°C. Incorporation of radioactivity into proteins was stopped by placing the cultures on ice and by 3 washes with ice-cold full DMEM (with Met/Cys plus spacing serum). Subsequently the cells were shifted to 18°C for 3 hours, washed with 5%BSA/PBS²⁺ followed by 2 washes with PBS²⁺ then shifted back to 37°C. To

determine the kinetics of secretion, culture supernatants were collected at different times. [³⁵S]-labeled proteins were precipitated by 10% TCA, collected on filters (GF/C filters, Whatman) and quantified by scintillation counting. To determine total incorporation of [³⁵S]-Met/Cys into cellular proteins, the cells were lysed in RIPA buffer supplemented with protease inhibitor (Roche) for 10 min. Supernatants were collected, TCA treated and processed as above. For controls, cells remained incubated on ice before supernatants were collected and cells were lysed and analysed.

5.2.4.7.2: [³⁵S]-Met/Cys pulse-chase and cell surface biotinylation of MHC-I molecules

Cells were starved in DMEM lacking Methionine and Cystein for 30 min at 37°C. 200µCi/ dish ³⁵S-Met/Cys were added to cells and incubated for 15 min. After 3 washes with cold DMEM, cells were incubated at 18°C for 3 hours in DMEM supplemented with 10mM Hepes to accumulate MHC-I molecules at the TGN. To start the chase, warm DMEM was added to each dish (except the 0 min dishes of control and knock-down cells) and incubated for 10 and 30 min. Cells were washed twice with PBS before lysis using RIPA supplemented with protease inhibitor cocktails (Roche). After preclearance using agarose beads, anti-MHC-I antibodies were added to lysates and incubated overnight at 4°C and subsequently 1 hour with protein G agarose beads. The beads were washed twice in RIPA buffer before 100µl of denaturing buffer (2% Sodium Deoxycholate, 2% NP40, and 5% SDS in PBS). were added and boiled for 5 min. Ice-cold PBS was added to each sample and the lysates were transferred into new tubes for overnight incubation with Streptavidin beads. The samples were then washed twice with RIPA buffer and denatured in 2 x Laemli sample buffer for SDS-PAGE. The gel was stained and dried, and exposed to a phosphoimager.

5.2.4.7.3: Staining and Fixing of SDS-PAGE gels

SDS-PAGE gels were incubated with Coomassie brilliant blue staining solutions (25% isopropanol, 10% acetic acid [glacial] and Coomassie brilliant blue R250) for 30 min at room temperature and destained in 10% acetic acid. The gels were then dried and exposed to a phosphoimager.

5.2.4.8: ImmunoGold EM (in collaboration with Prof. Dr. Judith Klumperman's lab)**5.2.4.8.1: Sample preparation for Immunogold labeling and electronmicroscopy**

Hela cells were plated in 10cm-dishes in 75-90% confluency one day before transfections. Single or double transfections were performed using Lipofectamine/Plus reagents. Cells were transfected either with only GFP-hSac1 construct or together with Fapp1-PH-RFP-Myc. For double-staining of hSAC1 and OCRL1, hSAC1-pCMV3TagMyc and GFP-OCRL1 constructs were used. Cells were fixed 24 hours post-transfections using either fixation protocol A or B as follows:

Fixation protocol A:

Cells in 10ml of culture medium were fixed by gently adding 10ml of 4% paraformaldehyde (diluted from 10% paraformaldehyde in ddH₂O) and incubated for 10 min at RT. The fixative-medium in dishes was replaced by 4% paraformaldehyde diluted in 0.1M phosphate buffer. Samples were stored at 4°C before they were sent for immunogold labeling.

Fixation protocol B:

An equal volume of Fixative A3 (4% paraformaldehyde, 0.4% Glutaraldehyde in H₂O) was added to cells containing cell culture medium. Fixation with Fixative A3 was done at RT for 10 min and Fixative B was added to cells for either 2 hours at RT or overnight at 4°C. After rinsing fixed cells with 0.1M Phosphate buffer, 1% paraformaldehyde in 0.1M phosphate buffer, pH7.4 were added to cells and samples were stored at 4°C.

The individual dishes were filled with appropriate fixatives, sealed well using parafilm and covered with plastic foam bags before sending out to Prof. Dr. Judith Klumperman's lab.

5.2.4.9: Apoptosis tests in Hela cells treated with hSAC1 siRNAs**(a) DAPI staining**

Hela cells treated with hSAC1 siRNA or control RNA were fixed with 4% formaldehyde and stained with 1µg/ml DAPI solution in the dark for 30min after 3 washes with PBS. Cells were mounted and viewed under a fluorescence microscope.

(b) PARP cleavage and WB

hSAC1-depleted cells or control cells were washed 3 times in PBS, trypsinized and cell suspensions were collected in eppendorf tubes. After 2 min centrifugation at 2 x 1000g rpm, cell pellets were collected and lysed in 2 x Laemli sample buffer (LSB). Lysed cells in LSB were boiled for 5 min and the supernatants were loaded on a SDS-PAGE gel after 5 min centrifugation at 14 x 1000g to removed cell debris. To check for PARP cleavage, a specific PARP antibody was used to detect PARP on a WB.

6 Literature

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