REGULATION OF SUBCELLULAR LOCALIZATION OF LIPID PHOSPHATASE SAC1

By

JINZHI WANG

A THESIS/DISSERTATION

Presented to the Department of Cell & Developmental Biology
and the Oregon Health & Science University
School of Medicine

in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

May 2013

School of Medicine
Oregon Health & Science University
CERTIFICATE OF APPROVAL

This is to certify that the PhD dissertation

[JINZHI WANG]

has been approved

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Mentor/Advisor

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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my mentor, Dr. Peter Mayinger, for his constant guidance and support that make it possible for me to finish this dissertation.

I would like to extend my great appreciation to all of my committee members, Chair, Dr. Linda Musil, Dr. Caroline Enns, Dr. Peter Barr-Gillespie, Dr. William Skach and Dr. Show-Ling Shyng for their precious time and invaluable suggestions and encouragements during the past several years.

I would like to thank Dr. Vivek Bhalla (Stanford University) for providing me 14-3-3 plasmids and Dr. Gary Banker (OHSU) for Mito-TagRFP plasmid. Their gifts saved me a lot of time. No words can express my gratitude.

I am again particularly grateful for the assistance given by Dr. Caroline Enns. She provided not only transferrin receptor 2 plasmid but also her valuable and constructive suggestions during the planning and development of my research as well as during my manuscript preparations.

I would like to thank Dr. Teresa Nicolson (OHSU) and all members from her laboratory for sharing their reagents. I am especially thankful to Dr. Weike Mo. He trained me how to use their laser scanning confocal microscope.

I am thankful to the past and current Mayinger lab members, Hailan Piao, Milene Vallejo, Damian Lee and Anastasia Blagoveshchenskaya for sharing laboratory duties with me and for enjoying non-scientific time with me.

Finally, I wish to thank my wife, Juxing Chen, my parents, and parents-in-law for all the support and encouragement throughout my graduate study.

ABBREVIATIONS

AANAT: arylalkylamine–serotonin N-acetyltransferase

AAV: Adeno-associated virus

ACT1: actin

AMPK: AMP-activated protein kinase

AP-1: Adaptor protein complexes-1

Arf1: ADP-ribosylation factor 1

ATP: adenosine triphosphate

BAD: Bcl2 antagonist of cell death

BAX: Bcl-2-like protein 4, Apoptosis regulator BAX

CALM: Clathrin Assembly Lymphoid Myeloid

CERT: ceramide transport protein

Cdc25: Cell division cycle 25

ChREBP: Carbohydrate response element-binding protein

CI-M6PR: cation-independent mannose-6-phosphate receptor

CKII: Casien kinase II

Co-IP: co-immunoprecipitation

COP-I: coatomer complex-I

COP-II: coatomer complex-II

CVB3: coxsackievirus B3

DMEM: Dulbecco's modified Eagle medium

EBBS: Earle's Balanced Salt Solution

EEA1: Early Endosome Antigen 1

EGFP: Enhanced Green Fluorescent Protein

EM: electron microscope

ENTH: the Epsin N-terminal homology

Epsin: EPS-15-interacting protein

EpsinR: epsin-related protein

ER: endoplasmic reticulum

ERGIC-53: The ER-Golgi intermediate compartment

ExoS: exoenzyme S

FBS: fetal bovine serum

FAPP1/2: phosphoinositol 4-phosphate adaptor protein -1/2

FERM: Band 4.1 (F), Ezrin (E), Radixin (R), Moesin (M)

FYVE: Fab 1, YOTB, Vac 1, and EEA1 domain

GFP: Green Fluorescent Protein

GGA: Golgi-localised, γ-ear containing, ADP ribosylation factor-binding

GlcNAc6ST-1: N-acetylglucosamine 6-O-sulfotransferases 1

GM130: cis-Golgi Matrix Protein 130kDa

GnTI: β-1,2-N-acetylglucosaminyltransferase I

GOLPH3: Golgi phosphoprotein-3

GP Ibα: platelet glycoprotein Ibalpha

GRASP65: Golgi ReAssembly Stacking Protein 65kDa

GST: glutathione-Sepharose

GTs: Golgi glycosyltransferases

HCV: hepatitis C virus

IBV: infectious bronchitis virus

IP: immunoprecipitates

IPTG: isopropylthio-β-galactoside

JAK1: Janus Kinase 1

KCNK3: potassium channel subfamily K member 3

Lsb6: LAS17-binding protein 6, a PI4 kinase

MAP kinases: Mitogen-activated protein kinases

NCS-1: neuronal calcium sensor-1

OCRL1, oculocerebrorenal syndrome of Lowe 1

OSBP: oxysterol binding protein

PACS-2: phosphofurin acidic cluster sorting protein-2

PAR4: proteinase-activated receptors 4

PKA: protein kinase A

P-loop: phosphate-binding loop

PVDF: polyvinylidene difluoride

PBS: phosphate buffer solution

PCR: polymerase chain reaction

PDGF: platelet-derived growth factor

PH: pleckstrin homology

PI: phosphatidylinositol

PI4P: phosphatidylinositol-4-phosphate

PI3K: PI 3-kinase

PI4K: PI 4-kinases

PI4KIIα: type IIα PI 4-kinases

PI4KIIβ: type IIβ PI 4-kinase

PI4KIIIα: type IIIα PI 4-kinases

PI4KIIIβ: type IIIβ PI 4-kinase

PI3P: phosphatidylinositol 3-phosphate

PI4P: phosphatidylinositol 4-phosphate

PI5P: phosphatidylinositol 5-phosphate

PI(3,4)P_2: phosphatidylinositol 3,4-phosphate

PI(3,5)P_2: phosphatidylinositol 3,5-phosphate

PI(4,5)P_2: phosphatidylinositol 4,5-phosphate

PI(3,4,5)P_3: phosphatidylinositol 3,4,5-phosphate

PM: plasma membrane

PV: poliovirus

PX: phox homology

Rac: RAC-alpha serine/threonine-protein kinase, AKT1/PKB

RACK1: Receptor for Activated C Kinase 1

SAC1: Suppressor of actin mutations 1

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

sec6: an essential subunit of the exocyst complex

sec9: t-SNARE protein, homolog of SNAP-25

sec14: phosphatidylinositol/phosphatidylcholine transfer protein

SKL: serine-lysine-leucine,

TASK-1: TWIK-related acid-sensitive K(+) channel 1

TFEB: transcription factor EB

TfR2: transferrin receptor 2

TGN: trans-Golgi network

TM1: The first transmembrane domain of human SAC1

TM2: the second transmembrane domain of human SAC1

TLR: Toll-like receptors

TRAF: tumor necrosis factor receptor-associated factor

TS(ts): temperature-sensitive

UPR: unfolded protein response

VAPB: VAMP-associated protein B

Vps74: vacuole protein sorting 74

HIGHLIGHTS

- Two distinct regions (TM1 and cytoplasmic N-terminal domain) in SAC1 (Suppressor of actin mutations 1) control its Golgi distribution
- A potential oligomerization motif resides within the N-terminal cytoplasmic region adjacent to SAC1-TM1
- SAC1, even the Golgi targeted SAC1-K2A, may localize at the cis/medial/trans Golgi, absent from the trans-Golgi network (TGN)
- 14-3-3 proteins are novel SAC1 interacting partners and may regulate ER exit of human SAC1

SUMMARY

At steady state, human lipid phosphatase SAC1 continuously cycles between the ER and cisternal Golgi compartments, which is crucial for the spatial regulation of phosphatidylinositol-4-phosphate (PI4P) at both organelles. In proliferating mammalian cells, a canonical di-lysine motif (583-KEKID-587) at the C-terminus of Sac1 is required for coatomer complex-I (COP-I)-binding and continuous retrieval to the ER. A COP-I binding deficient mutant SAC1-K2A, in which lysines are substituted by alanines, accumulates at the Golgi. However, how the distribution of Sac1 within the Golgi is controlled remains uncertain and the mechanism responsible for Golgi retention of this mutant is unknown. Currently, whether SAC1-K2A mutant localizes at the TGN seems controversial. And it was unclear how ER exit and anterograde transport of SAC1 is regulated. Here, by utilizing the Golgi-targeted SAC1-K2A mutant as a model, we investigated the Golgi retention signal of the human SAC1. We showed that the first of the two transmembrane regions in human SAC1 (TM1) is sufficient for Golgi localization.

In addition, we determined that the N-terminal cytoplasmic domain of SAC1 also promotes Golgi localization, even when TM1 is mutated. We conclude that the distribution of SAC1 within the Golgi is controlled via both passive TM1 length-dependent partitioning and a retention mechanism that requires the N-terminal cytoplasmic region. We also identified a potential oligomerization motif within the region adjacent to SAC1-TM1, independent of the N-terminal leucine zipper motif. We found that BFA treatment causes the SAC1-K2A mutant to redistribute into the ER, implying that even the Golgi targeted SAC1-K2A mutant may localize at the cis/medial/trans Golgi instead of the TGN at steady state.

Moreover, we found that 14-3-3 proteins are novel SAC1 interacting partners. We showed that binding of 14-3-3 to SAC1 through recognizing a classic mode II-like 14-3-3-binding motif (145-RLSNTSP-151) in the cytoplasmic N-terminal domain of human SAC1. A deletion mutant of SAC1, SAC1(Δ145-151), which lacks the 14-3-3-binding motif, no longer co-immunoprecipitates with 14-3-3 proteins. When over-expressed, all 14-3-3 isoforms, with the exception of 14-3-3ζ, can co-immunoprecipitate with SAC1. A SAC1-S147A/T149A/S150A mutant shows much reduced interaction with 14-3-3, indicating that phosphorylation may be involved in regulating the interaction between SAC1 and 14-3-3. The GFP-SAC1 (Δ145-151) mutant shows a greatly compromised ability to traffic out of the ER compared to wild-type GFP-SAC1. This finding suggests that binding of 14-3-3 may regulate the ER to Golgi transport of lipid phosphatase SAC1.

CHAPTER ONE

INTRODUCTION

1. Phosphoinositides

Phosphoinositides are recently characterized as versatile membrane signaling molecules. Phosphoinositides, which are located at the cytosolic leaflet of the membrane bilayer, are very minor elements of membranes, less than 1% of total lipids, yet they have been reported to play critical roles in signal transduction, membrane trafficking as well as cytoskeleton remodeling [1, 2]. Phosphoinositides are phosphorylated derivatives of phosphatidylinositol [3]. Addition of a phosphate group to phosphatidylinositol (PI) can occur at three positions of the D-3, D-4 and D-5 of the inositol ring, which creates seven species of phosphoinositides [2-4]. These are phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 4-phosphate (PI4P), phosphatidylinositol 5-phosphate (PI5P), phosphatidylinositol 3,4-phosphate ($PI(3,4)P_2$), phosphatidylinositol 3,5-phosphate ($PI(3,5)P_2$), phosphatidylinositol 4,5-phosphate ($PI(4,5)P_2$) and phosphatidylinositol 3,4,5-phosphate ($PI(3,4,5)P_3$) [2, 5] (see Figure 1). Currently, there is a "lipid code" hypothesis, which proposes that distinct phosphoinositide lipids mark individual cellular membranes to enable sequential regulation of the complexity of membrane trafficking reaction and allow for the spatial and temporal signaling in each of the cellular membranes [6]. For instance, the plasma membrane is enriched in $PI(4,5)P_2$, Golgi membranes are adorned with PI4P, endosomal membranes are decorated with PI3P, and lysosomal membranes contain $PI(3,5)P_2$ [5, 7]. Therefore, phosphoinositides are landmarks of distinct subcellular membranes.

The function of phosphoinositides apparently depends on their ability to recruit specific effectors [1, 2]. Phosphoinositides can directly transport signalling effects through the binding of their phosphorylated head groups to effectors [3]. These effectors are proteins that have phosphoinositide-binding modules [8]. Well-characterized phosphoinositide-binding modules include PH domains (e.g., FAPP1/2) [9-11]; ENTH domain (e.g., Epsin) [12]; FYVE domain (e.g., EEA1) [13]; PX domain (e.g., PI3K) [9, 14]; FERM domain (e.g., JAK1) [15] and CALM domain (e.g., AP180) [16, 17]. These modules can be found in cytosolic proteins or cytosolic domains of membrane proteins. Thus, phosphoinositides can regulate the function of integral membrane proteins or recruit cytosolic signalling components and play multiple functions.

2. PI4P function at the Golgi

PI4P is mainly concentrated at the Golgi membranes, especially at the trans-Golgi network (TGN) and functions there. Recruitment of effectors at the Golgi is coordinated by PI4P and activated Arf1 via a coincidence detection mechanism [18]. Among the seven phosphoinositides, PI4P is one of the most abundant [1]. However, the function of PI4P has not been studied as intensively as the function of $P(4,5)P_2$ in PI3K/AKT signalling. PI4P has long been considered only as a precursor for $PI(4,5)P_2$ [2, 19]. Besides functioning as a precursor, recently several lines of evidence suggested that PI4P plays crucial roles in such as membrane trafficking and cell signaling [20]. Golgi PI4P has been shown to be vital in secretion [21]. The importance of Golgi PI4P in both vesicular membrane trafficking and non-vesicular lipid transport is well documented. The role of PI4P in membrane trafficking relies on its ability to recruit clathrin adaptors. For example, PI4P can recruit AP-1 [22], GGA proteins [23] and EpsinR [24]. PI4P is also involved in lipid transport via

recruiting lipid-transfer proteins. For example, PI4P can recruit the oxysterol binding protein OSBP [25], the ceramide transport protein CERT [26] and the phosphoinositol 4-phosphate adaptor protein-1/2 (FAPP1/2) [25, 27]. Another PI4P effector is Golgi phosphoprotein-3 (GOLPH3), a homolog of yeast Vps74. GOLPH3 binds to both PI4P and MYO18A conveying a tensile force at the TGN membrane, which is required for efficient membrane budding [28]. GOLPH3 was also found to be an oncogene, which may link PI4P to human cancer [29]. Membrane microenvironments enriched in PI4P have recently been found to serve as replication platforms required for a number of RNA viruses, such as hepatitis C virus (HCV) [30-34] coxsackievirus B3 (CVB3), poliovirus (PV) [34], and Aichi virus [35]. These are achieved by selectively recruiting host PI4Ks (PI4KIIIβ or PI4KIII α) to create and enlarge PI4P-enriched replication platforms [36, 37]. Furthermore, PI4P at the Golgi is required for COP-II vesicles to fuse with the Golgi membrane through modulating SNARE complex formation at the fusion stage [38]. In conclusion, these distinct functions of PI4P rely on recruiting proteins to specific Golgi membrane locations. The distribution of PI4P within the Golgi complex is critical for such functions and discrete Golgi PI4P pools are tightly modulated by specific PI kinases and phosphatases [2].

3. PI4P metabolism

Like all the other phosphoinositides, PI4P metabolism is regulated in a spatio-temporal manner [19]. This regulation of PI4P is enabled by different PI4P kinases and phosphatases, whose subcellular localizations and membrane associated catalytic activities are regulated both in yeast and in mammalians (see Figure 2A, B) [3, 39, 40]. Synthesis of PI4P is controlled by the compartment-specific PI 4-kinases. There are three PI 4-kinases

(PI4Ks) in yeast: Pik1, Stt4 and Lsb6 [3, 39]. Pik1, a type III PI 4-kinase, is the sole Golgi associated PI4K required for biosynthesis of PI4P in the Golgi compartment. The Golgi pool of PI4P synthesized by Pik1 is critical for vesicular Golgi-to-PM trafficking [41, 42]. Pik1 can also relocate to the nucleus when nutrients are scarce, although the nuclear function of Pik1 has not been resolved [43-46]. Stt4, another yeast type III PI4K, localizes to the PM, where it is responsible for producing distinct PM pool of PI4P [42, 47]. Stt4 has no clear roles in secretion but may regulate the vacuole morphology and the actin cytoskeleton [48]. Yeast Lsb6, a type II PI4K, localizes to the PM and the vacuolar membrane and may regulate endosome motility [49-51]. Both Pik1 and Stt4 are vital for producing PI4P. Ablation of either Stt4 or Pik1 is lethal in yeast [42]. When shifted to the non-permissive temperature, a temperature-sensitive (ts) double mutant, $stt4^{ts}pik1^{ts}$, lost 90% of total cellular PI4P in less than 1 hr [42]. Over-expression of Pik1 cannot compensate for deletion of Stt4 and vice versa [52]. In conclusion, Pik1 and Stt4 are main yeast PI4-kinases, which regulate spatial-temporal PI4P signaling pathways at distinct subcellular locations in yeast.

In mammals, four PI 4-kinases have been identified (see table 1). Two of them are type III PI 4-kinase isoforms (PI4KIIIα and PI4KIIIβ), which can be inhibited by wortmannin and LY 294002 at high micromolar concentrations probably due to homolog to the PI 3-kinase (PI3K) family members [17]. The other two mammalian PI 4-kinases are type II PI 4-kinases (PI4KIIα and PI4KIIβ), which can be inhibited by low micromolar concentrations of adenosine but are insensitive to wortmannin and LY 294002 [17, 53]. PI4KIIα and PI4KIIβ are highly conserved with a molecular weight about 55-kDa [54, 55], whereas PI4KIIIα and PI4KIIIβ are relatively larger with molecular weight 230 kDa and

92 kDa respectively [56, 57]. All four mammalian PI 4-kinases create the same PI4P lipid. Although the subcellular localizations of PI 4-kinases are still somewhat controversial, it is generally accepted that PI4KIIα, a homolog of yeast Lsb6, and PI4KIIIβ, a homolog of yeast Pik1, mainly localize to the Golgi [3, 17]. PI4KIIα is predominately targeted to the trans-Golgi network (TGN) via palmitoylation mediated by a conserved 174-CCPCC-178 motif within its catalytic domain [54, 58], while PI4KIIIβ is recruited to the Golgi membrane depending on ADP-ribosylation factor 1 (Arf1) [59] or on neuronal calcium sensor-1 (NCS-1) [60, 61]. It was suggested that the basal levels of PI4P might be generated by PI4KIIα while specific pool of PI4P could be the regulated product of PI4KIIIβ at the Golgi [62]. Moreover, PI4KIIα is also found at endosomes [51]. PI4KIIIβ, like its homolog Pik1, has been shown to cycle between cytoplasm and nucleus [63]. PI4KIIβ is mainly cytosolic in spite of its preserved CCPCC palmitoylation motif, but it can be recruited to the PM upon PDGF stimulation or by constitutive activation of Rac [64]. PI4KIIIα is predominantly found at the ER [65]. Electron microscopic analysis indicates that PI4KIIIα may associate with multivesicular bodies and mitochondria [66]. In summary, different localization of each of mammalian PI4-kinases creates distinct PI4P pool and regulates corresponding specific signals.

Turnover of PI4P is modulated by PI4P phosphatases [67]. The major PI4P phosphatase in yeast is Sac1p, which is an originating member of the Sac phosphatase domain family protein [67]. The purpose of my thesis is to understand how the subcellular localization of human PI4P phosphatase SAC1, a homolog of yeast Sac1p, is regulated.

4. The Sac phosphatase domain family

The Sac phosphatase domain proteins constitute an important family of lipid

phosphatases. These proteins have a common catalytical motif $CX_5R(T/S)$ discovered in many protein tyrosine phosphatases and lipid phosphatases [67]. This signature motif has a P-loop structure [67, 68]. Members of this family are evolutionarily conserved from yeast to mammals [68]. These proteins include mammalian SAC1, SAC2, SAC3, synaptojanin and its variants as well as yeast Sac1p, Fig4p, and the three dual specificity synaptojanin-like phosphatases Sjl1/Inp51p, Sjl2/Inp52p and Sjl3/Inp53p [67, 69-71]. Interestingly, one of the members, Inp51p, exhibits no phosphatase activity because the cysteine, arginine and threonine/serine residues within its $CX_5R(T/S)$ motif are missing [70, 72, 73]. In conclusion, Sac domain phosphatase family is composed of important enzymes, which can hydrolyze different species of phosphoinositides [67].

5. Lipid phosphatase Sac1

Sac1p was originally discovered in 1989 [74, 75], but was characterized as an inositide phosphatase a decade later when synaptojanin, a Sac phosphatase domain containing protein, was identified as a major lipid phosphatase [71]. Sac1 family proteins from yeast to mammalians are evolutionarily conserved and represent important phosphoinositide phosphatases (see Figure 3). The yeast *SAC1* gene was originally identified in screening temperature sensitive mutations that could suppress defects in *ACT1* (actin) mutants and was named Sac1 (Suppressor of actin mutation 1) [74, 75]. Sac1 mutants were also found to suppress mutations in several genes encoding factors involved in anterograde trafficking such as defects in *SEC14* (phosphatidylinositol/phosphatidylcholine transfer protein) gene as well as *SEC6* (an essential subunit of the exocyst complex) and *SEC9* (t-SNARE protein, homolog of SNAP-25) [74]. Human gene encoding lipid phosphatase SAC1 is *SACM1L* (or *KIAA0851*) [76]. The *SACM1L* gene is located in chromosome 3 in the human

genome at the position of p21.3. Human SAC1 protein (UniProtKB entry No. Q9NTJ5) consists of 587 amino acid residues with a calculated molecular weight of 67kDa (see Figure 4A, B). Yeast Sac1p (UniProtKB entry No. P32368) is composed of 623 amino acid residues with a longer cytoplasmic C-terminus compared to human SAC1. Biochemical analyses showed that both yeast and mammalian Sac1 are integral membrane proteins containing two transmembrane domains near the C-terminus [77, 78]. Both its large N-terminal domain (1-520) and its short 18-residue C-terminal domain (570-587) of human SAC1 face the cytoplasm. The first transmembrane domain (TM1) and the second transmembrane domain (TM2) of human SAC1 are separated by a putative 6-residue luminal region (543-DTWTET-549), which may form a hairpin structure when inserted into membrane bilayers (see Figure. 4A and B). This feature of Sac1 makes it a unique member of Sac phosphatase domain family in which most of them are cytosolic proteins, while Sac1 is an integral membrane protein.

Currently, there is no atomic structure available for mammalian SAC1. Structure of yeast Sac1p N-terminal cytoplasmic domain (residues 1–503) has been resolved by X-ray crystallography at 2.0Å resolution resolution [68] (see Figure 5). The cytoplasmic N-terminus of Sac1p can be grouped into three distinct subdomains: a SacN domain (residues 1–182), a catalytic domain (residues 183-461) with the CX5R(T/S) signature motif and a potential unstructured domain (residues 462-521) [68]. The striking features of the structure are a unique conformation of the catalytic P-loop and a large positively charged groove at the catalytic region [68]. The potential unstructured domain has been proposed to allow enzymatic activity in trans at the ER and other membranes contact sites [68, 79 79] (see Figure 6). Previous studies have demonstrated that human SAC1 can

form oligomers [76] and further research revealed that a N-terminal leucine zipper motif in human SAC1 is required for oligomerization during serum starvation [80].

The Sac1 phosphatase domain at the cytoplasmic N-terminus of SAC1 is an around 500 amino acid region characterized by the catalytic motif $CX_5R(T/S)$ [67, 70]. *In vitro* experiments have demonstrated that substrate specificity of yeast Sac1p phosphatase is obscure [67]. It can hydrolyze phosphate from any of monophosphoinositides PI3P, PI4P and PI5P as well as biphosphoinositides $PI(3,5)P_2$. But Sac1p cannot hydrolyze inositol lipids with adjacent phosphates such as $PI(3,4)P_2$, PI(4,5)P and $PI(3,4,5)P_3$. Only PI3P, PI4P and $PI(3,5)P_2$ can be efficiently dephosphorylated by Sac1 phosphatase [67, 69, 72]. However, genomic deletion of the yeast *SAC1* gene increases intracellular PI4P levels by twelve-fold but no significant increase of other phosphoinositide levels, suggesting that Sac1p is a predominant PI4P lipid phosphatase *in vivo* [81, 82]. Yeast Sac1 phosphatase has been reported to be an allosteric enzyme [83]. Binding of anionic phospholipids (PI or phosphatidylserine) to the positively charged groove of Sac1 may result in conformational changes of the catalytic P-loop and regulate the enzymatic activity of membrane associated Sac1 [83]. In conclusion, Sac1 is a phosphatase that is responsible for PI4P turnover.

Sac1 appears to be a housekeeping gene in that it plays crucial physiological functions [71, 84]. Deletion of yeast *SAC1* gene in yeast is not lethal but *sac1* mutants show pleiotropic phenotypes including abnormal actin cytoskeleton [48], defect in ATP transport into the ER [85], constitutively-activated unfolded protein response (UPR) [86], multi drug sensitivity [87, 88], inositol auxotrophy [77, 89], disruption of vacuole morphology and accumulation of lipid droplets [48, 77]. In plants, mutation of SAC1 has been shown to alter

cell morphogenesis, cell wall synthesis as well as actin organization [90]. However, knockdown of *SAC1* in mice and ablation of Sac1 in Drosophila create lethal phenotypes, implying that Sac1 in these two species could be vital for their organ development [91, 92]. Sac1 insufficiencies cause disruption of mammalian Golgi membranes and result in multiple active spindles [91]. Moreover, a recent study from our laboratory showed that downregulation of human SAC1 by siRNA impedes glycosylation fidelity and causes Golgi-specific defects in N- and O-linked glycosylation [93]. In conclusion, these phenotypes from Sac1 insufficiencies or Sac1 loss-of-function correlate with subcellular localization of Sac1 and its role in the spatial-temporal regulation of the PI4P lipid pools at distinct cellular membranes [7, 79, 91].

6. Subcellular distribution of Sac1

Studies in mammals have shown that lipid phosphatase SAC1 is located at the ER and Golgi and is responsible for PI4P dephosphorylation at both compartments [7, 77]. This ER/Golgi-localization of SAC1 is in agreement with the subcellular distribution of yeast Sac1p [77]. Compartmental localization of yeast Sac1p phosphatase correlates with the processes that it regulates [94]. Golgi-localized Sac1p participates in the regulation of Golgi-to-PM trafficking, while ER-localized Sac1p may be involved in such functions as ATP uptake [85, 94] and ER-based secretion [67]. Localization of yeast Sac1p is modulated by growth conditions and MAP kinase signaling [95, 96]. Glucose starvation has been shown to promote Golgi accumulation of yeast Sac1p [96]. Interaction with ER protein Dpm1p confers ER retention of yeast Sac1p [46, 96]. At steady state, both yeast Sac1p and human SAC1 are predominately localized at the ER. Human SAC1 continually cycles between the ER and the Golgi. This is achieved by the presence of a di-lysine retrieval motif (583-

KEKID-587) at the extreme C-terminus of human SAC1 [76, 80]. In proliferating cells, Sac1 is localized at both ER and cisternal Golgi but is absent from the TGN [93]. This distribution of Sac1 is critical for maintaining a polarized localization of PI4P at the TGN [93]. During serum starvation and in quiescent cells, Sac1 accumulates at the TGN, which reduces Golgi PI4P levels and slows anterograde trafficking [80] (see Figure 7). All mammalian Sac1 orthologs contain two distinct sequence motifs that are required for shuttling between ER and Golgi. The di-lysine motif at the C terminus of Sac1 is critical for interaction with the COP-I machinery and for retrieval to the ER [76, 80]. The leucine zipper at the cytoplasmic N-terminal domain promotes oligomerization of Sac1 is a prerequisite for Golgi accumulation [76, 80]. Yeast Sac1p lacks both the classic di-lysine retrieval motif and the leucine zipper motif. How the ER/Golgi localiztion of yeast Sac1p is regulated is unclear.

Although the steady-state subcellular localization of SAC1 to the Golgi is known, the retention signals have not been explored. Moreover, the molecular mechanism that modulates ER to Golgi anterograde transport of SAC1 remains to be discovered. These are central questions of my thesis.

7. ER-to-Golgi transport

The ER and the Golgi are two most important endomembrane organelles of the early secretory pathway [97]. Entry into the ER is the starting point for most newly synthesized secretory soluble and membrane proteins [98]. The ER provides not only docking sites for translational ribosomes but also an environment for protein folding and post-translational modification of nascent chains [98]. Once passed the ER quality control, newly synthesized membrane proteins destinated to post-ER compartments are exported from the ER to the

Golgi via the secretory pathway [99, 100]. The ER to Golgi anterograde trafficking is mediated by the coat protein complex-II (COP-II). COP-II, consisting of Sar1, Sec23/24 and Sec13/31, is responsible for cargo sorting and vesicle budding at the ER [101]. Several distinct ER export motifs in the cytoplasmic tails of membrane cargo proteins were found to be required for efficient ER exit, such as the di-acidic DXE motif in VSVG protein [102], the LXXL/ME motif in certain SNAREs [103] and a di-phenylalanine (FF) motif in ERGIC-53 [104] and p24 proteins [105, 106]. Sec24 subunit can directly bind to transmembrane cargo proteins and recruit them into the emerging vesicles. Two isoforms, Sec24a and Sec24b, have been shown to bind directly to the DXE motif [102] and the LXXL/ME motif respectively [103]. The Golgi to ER retrograde traffic is regulated by the coat protein complex-I (COP-I). The COP-I coat is composed of preformed cytoplasmic heptameric (α, β, β', γ, δ, ε, ζ) complexes [107]. COP-I can directly bind to di-lysine motifs and redistribute proteins containing these motifs from the Golgi to the ER [107, 108]. The first lysine at -3 position are definitely required for β'-COP binding and it is critical that the lysine (-3) must be separated two residues apart from the C-terminal carbonyl groups (-COOH) in KKXX and KXKXX motifs [107, 108]. In conclusion, ER-to-Golgi bi-directional traffic is regulated by the classic COP-II and COP-I coatomers and multiple cargo recruitment mechanisms are involved [98, 109, 110] (see Figure 8).

To maintain ER homeostasis, ER resident proteins that define ER structural and functional attributes must be separated from the cargo proteins and retained in the ER [98]. ER retention is accomplished in two ways [98]: (i) exclusion from entering transport vesicles and (ii) rapid retrieval of "escaped" residents via retrieval motifs. Classic ER retrieval motifs have been identified such as a H/KDEL sequence at the C-terminus of

soluble ER resident proteins [111] and a dibasic motif (either di-lysine or di-arginine) in transmembrane proteins [112]. Di-lysine motifs (either KKXX or KXKXX, X can be any amino acids) must locate at the extreme cytoplasmic C-terminus of membrane proteins and specific position requirements of these lysines must be met such as -3 and -4 for KKXX motif and -3 and -5 for KXKXX motif [107, 112, 113]. The destination of the secretory proteins after the ER is the Golgi apparatus. The anterograde transport vesicles that leave the endoplasmic reticulum are delivered to the cis face of the Golgi apparatus, where they fuse with the Golgi membrane [98].

8. Golgi apparatus and Golgi retention

The Golgi apparatus, arranged in a cis to medial to trans-Golgi to the TGN ordered manner, is a central sorting organelle [114, 115]. The Golgi is not only responsible for guiding cargo proteins entry from the ER but also for sorting cargo proteins to their different endomembranes [115]. Moreover, The Golgi carries out a variety of post-translational modifications [114, 115]. A number of important post-translational modifications have been functionally attributed to Golgi resident enzymes [116]. For example, Golgi glycosyltransferases (GTs) must be appropriately localized along the cis-Golgi to TGN axis to ensure sequential modification of proteins passing the Golgi compartments [116].

How the Golgi maintains subcompartment-specific distribution of Golgi enzymes was intensively studied in the 1990s. However, unlike sequence-specific target signals, such as the classic ER retention/retrieval motif (KKXX or KXKXX) [107] or the peroxisomal targeting signal (serine-lysine-leucine; SKL) at the extreme C-terminus of some peroxisomal proteins [117], no common Golgi retention sequence motif has been identified [115]. Most of our current knowledge of Golgi retention is from studies on GTs

and glycosidases [116]. Interestingly, all these proteins are type II membrane proteins that have short cytoplasmic N-termini followed by a membrane-spanning domain and then a large catalytic luminal domain [115, 116]. Golgi retention signals have been mostly found to be located within a relatively short region of these proteins consisting of the cytoplasmic tail, the transmembrane domain, and the adjacent luminal region [118-120].

Currently, two mechanisms have been proposed for Golgi retention signals, the bilayer thickness model and the oligomerization model [115]. The bilayer thickness model postulates that retention relies on the length of a membrane-spanning domain and the thickness of the membrane of the Golgi complex [115]. A recent bioinformatics investigation supports the idea that along the secretory pathway from the ER to PM, the length of the transmembrane domains of resident proteins varies, with 20 residues in Golgi and 24 residues in TGN and PM in vertebrates [121]. Correspondingly, transmembrane domains have been shown to play key roles in retaining Golgi proteins [122]. For example, β-galactoside alpha 2,6-sialyltransferase has a 17-residue transmembrane domain, which is sufficient for its Golgi retention [123]. Moreover, the transmembrane domain of bovine β 1,4-galactosyltransferase is key for its Golgi localization and removal of 18 of the 24 residues from its cytoplasmic domain did not disrupt the Golgi localization [124]. The oligomerization model proposes that retention is achieved by forming oligomers, too big to enter transport vesicles. Hassinen et al has shown that four N-glycosylation enzymes, namely, β-1,2-N-acetylglucosaminyltransferase I, β-1,2-N-acetylglucosaminyltransferase II, 1,4-galactosyltransferase I, and alpha-2,6-sialyltransferase I, form not only Golgi-localized homodimers, but also Golgi subcompartmental and functional specific heterodimers [125]. For example, the medial

Golgi enzyme β-1,2-N-acetylglucosaminyltransferase I can heterodimerize with medial Golgi enzyme β-1,2-N-acetylglucosaminyltransferase II, and the trans-Golgi enzyme 1,4-galactosyltransferase I can heterodimerize trans-Golgi enzyme alpha-2,6-sialyltransferase I [125].

Although, in many Golgi resident proteins, transmembrane domains are key for Golgi retention, no specific signals within transmembrane domains were identified [115]. However, cysteine within transmembrane domains may mediate dimerization by forming disulfide bridges, which may contribute to Golgi retention in some cases. For example, mutation of a cysteine residue in the transmembrane domain of β-1,4-galactosyltransferase prevents dimer formation and disrupts its Golgi localization [126, 127]. In contrast, two cysteine residues within the stem and transmembrane of N-acetylglucosamine 6-O-sulfotransferases 1 (GlcNAc6ST-1) domain were found to be critical for its dimerization, yet disruption of the dimer by mutagenesis did not change its localization [128]. Thus, in some cases, cysteines in the transmembrane domain could be a factor affecting Golgi retention but not always.

Besides transmembrane domains (TMs), cytoplasmic domains or luminal domains have also been reported to be required for Golgi retention. For example, the necessary and sufficient Golgi localization signal of uukuniemi virus membrane glycoprotein G1 locates within its cytoplasmic tail [9, 129]. And the critical signal for oligomerization and Golgi localization of N-acetylglucosaminyltransferase V lies in the luminal stem region [130]. Occasionally, a combination of a transmembrane domain and a cytoplasmic domain (or luminal domain) may be the key for Golgi retention [131]. For example, the Golgi localization of plant β-1, 2-xylosyltransferase is dependent on both its cytoplasmic

and transmembrane domain [131]. And all three domains of β-1,2-N-acetylglucosaminyltransferase I (GnTI) has been shown to significantly contribute to its Golgi localization [132]. Moreover, interaction with some Golgi proteins may be involved in Golgi retention. Very interestingly, the yeast PI4P binding protein Vps74p, a homolog of mammalian GOLPH3, has been reported to be required for correct subregional localization of a number of GTs [133, 134]. Lack of Vps74p results in mislocalization and subsequent degradation of these GTs in vacuole [133]. Thus, although TMs have been proved to key players in Golgi retention, regions outside of TM are also important and sometimes are sufficient to confer Golgi retention. Potential Golgi retention models were summarized in a recent review by Banfield [115] (see Figure 9).

Retention of TGN proteins is different from retention of cis/medial/trans Golgi proteins [122]. TGN proteins are continuously recycled from the PM or from endosomes. In many instances, the cytoplasmic domain carries the TGN localization signal [135]. For example, the TGN localization signal of TGN38 includes the YQRL motif in its cytoplasmic domain [136]. Brefeldin A (BFA) treatment causes a dramatic effect on the Golgi morphology and induces a rapid redistribution of Golgi proteins (cis-, medial-, and trans-Golgi markers) into the ER [137-139]. However, Several lines of evidence suggest that the TGN proteins do not redistribute into the ER. Instead, TGN proteins mix with endosomes during BFA treatment [140, 141]. Thus, the fungal metabolite brefeldin A (BFA) treatment can distinguish the TGN proteins from the cis/medial/trans Golgi resident proteins [142].

At steady state, human SAC1 shuttles between the ER and the Golgi compartments. When COP-I binding is inactivated, SAC1-K2A mutant accumulates at

the Golgi [80], whether SAC1-K2A resides at the cis/medial/trans Golgi or TGN is controversial and needs to be clarified [80, 91]. Moreover, the molecular mechanism that regulates ER to Golgi transport of SAC1 is unclear. Mass spectrometric analysis from our previous studies showed that 14-3-3σ was co-immunoprecipitated with Flag-SAC1 in the COS7 cells, suggesting it could be a new regulator of human SAC1. However, the physiological function of 14-3-3 interactions with SAC1 is unknown.

9. 14-3-3 basics

14-3-3 proteins are highly conserved and ubiquitously expressed in eukaryotic cells [143]. 14-3-3 was first identified in 1967 as an abundant acidic, cytosolic protein [144]. 14-3-3 was named after its chromatographic elution and migration profile because it eluted in the 14th fraction during DEAE-cellulose chromatography and was found on position 3.3 following starch-gel electrophoresis [144]. There are multiple genes encoding 14-3-3 proteins in different organisms [145]. For example, yeast has two 14-3-3 isoforms [146, 147], human and mouse each have seven isoforms [148], while higher plants have as many as 15 isoforms [149]. Mammalians have seven 14-3-3 isoforms (named beta (β), epsilon (ε), zeta (ζ), eta (η), theta (θ), gamma (γ) and sigma (σ)). They are composed of around 250 amino acid residues. In detail, the β isoform consists of 246 amino acids; γ has 247 residues; η has 246 residues; ζ has 245 residues; θ has 245 residues; σ has 248 residues and ε is the largest one and has 255 residues [145]. Therefore, mammalian 14-3-3 proteins have a molecular weight of around 30 kDa but these proteins predominantly form heteordimers or homodimers with a dimeric molecular mass of about 60kDa in the eukaryotic cells. As an evolutionarily highly conserved family of proteins, 14-3-3 proteins are thereafter identified involving in multiple cellular functions [148, 150, 151]. The

14-3-3 family proteins not only share identity at the amino acid level but also harbor a highly conserved structure [152].

10. 14-3-3 structure and action modes

Crystal structures of all seven human 14-3-3 isoforms coupled with phosphopeptides or nonphosphopeptides have been resolved (see Figure. 10) [152]. All known 14-3-3 crystal structures are from homodimers including both liganded [153-155] and non-liganded versions [156, 157]. In general, the resolved crystal structures of 14-3-3 isoforms reveal cup-shaped dimeric structures [148]. Each monomer of 14-3-3 proteins comprises 9 alpha helixes (α1-α9) [148]. The first four helices α1-α4 are responsible for forming the dimer. Helices α5-α9 arrange into an amphipathic and highly conserved peptide-binding groove. A striking feature of the concaved surface of the peptide-binding groove is the highly conserved positively charged cluster consisting of one lysine and two arginines (Lys49, Arg56, Arg127 as numbered in 14-3-3ζ) [158]. These core positive residues play a critical role in binding to the phosphate group of the phosphopeptide ligand proteins [152]. In conclusion, each 14-3-3 dimer carries two separate binding sites [153]. These two binding sites can be found in one ligand or in two independent ligands and this feature diversifies the regulation of the interactions of 14-3-3 with their ligands [152].

Based on 14-3-3 dimeric structural traits, currently three molecular modes, clamping, scaffolding and masking of 14-3-3 actions were proposed [159-161]. Clamping means that binding of 14-3-3 changes the catalytic activity or other properties of the ligand by stabilizing a certain conformation. A typical example is the allosteric clamping of AANAT (arylalkylamine–serotonin N-acetyltransferase) by 14-3-3, which holds the phosphorylated enzyme in an active conformation [155]. Scaffolding tethers molecules

together and serves as backbones for complex assembly. For instance, the scaffolding protein 14-3-3ζ directly binds to another scaffolding protein RACK1 (Receptor for Activated C Kinase 1) [162]. Masking postulates that binding of 14-3-3 prevents additional association of other proteins. For example, binding of 14-3-3 to human κ-opioid receptor (hKOPR) masks the adjacent ER retention signal and prevents COP-I coatomer binding, resulting in efficient plasma membrane delivery of hKOPR [163]. As a result, binding of 14-3-3 proteins to their ligands has diverse biological functions and consequences [164].

11. 14-3-3 function

Over two hundred of 14-3-3 interacting proteins have been reported [165]. 14-3-3 proteins regulate almost all primary biological processes such as signal transduction (e.g., 14-3-3 binds to Raf-1, a key component of signal pathway that regulates mitogenesis and differentiation [166]; and 14-3-3 mediates p85 binding and activates of PI3K/Akt pathway [167, 168]); apoptosis (e.g.,14-3-3ζ traps BAD in cytosol and prevents translocation of BAD-BAX to mitochondrial and protects stress-induced cell death [169], and 14-3-3 binding to PACS-2 is released by death ligand TRAIL-induced dephosphorylation at the site of Ser437 in PACS-2, which permits PACS-2 to intercede Bid translocation to mitochondria [170]); transcriptional regulation (e.g., 14-3-3 proteins bind to transcription factor EB (TFEB) and retain it in the cytoplasm) [168]; metabolism (e.g., 14-3-3 proteins are involved in regulation of cellular energy metabolism through regulating the AMP-activated protein kinase (AMPK) signaling) [171]; cell-cycle control (e.g., 14-3-3 binding to Cdc25b and Cdc25C, 14-3-3 binding to the S323 site on Cdc25B directly inhibiting the activity of Cdc25B. 14-3-3 binding to Cdc25C lowers the G2 checkpoint arrest) [172-175]. Taken together, depending on its ligand protein, binding of 14-3-3 affects multiple

signaling pathways that determine cell fate [164].

12. 14-3-3-binding motifs

Studies on the binding of 14-3-3 to Raf-1 result in the discovery of 14-3-3 as one of the class of phosphoserine/threonine sequence-specific binding protein modules [166, 176-178]. Binding of all 14-3-3 isoforms to their ligands (substrates or interacting proteins) mostly occurs via consensus amino acid sequence-specific interactions [148]. Two well-defined motifs are mode I 14-3-3 binding site, which has the consensus sequence RXXpS/pTXP, and mode II 14-3-3 binding site, which has the consensus sequence RXSXpS/pTXP, (where pS/pT stands for the phospho-serine or phospho-threonine and X can be any residues except cysteine) [158, 176, 179]. Most of 14-3-3 binding proteins have the mode I RXXpS/pTXP motif, but the proline residue at the position of +2 may not be strictly required in more than one-half of the 14-3-3-binding sites [180]. Mode II motifs RXSXpS/pTXP are prominent in plant 14-3-3-binding proteins [180]. Besides these two classic binding motifs, a mode III motif with a consensus of C-terminal sequence RXXpS/pTX-COOH has been identified by genetic screening of a random peptide library [181-183]. The founding signal for this mode III motif is a C-terminal sequence RGRSWTY-COOH that was previously reported [184]. Phosphoserine or phosphothreonine in the motif of RXXpS/pTX-COOH is required for 14-3-3 binding and an arginine (R) residue at the position of -5 was shown to enhance binding affinity [182, 183]. There are also other phosphorylation-dependent 14-3-3 binding motifs, for example, the YpTV motif. This non-classic 14-3-3 binding motif (YpTV) was found at the C-terminus of plant plasma membrane protein H^+-ATPase. Deletion or substitution of these three residues 946-YTV-948 in the H^+-ATPase abrogates 14-3-3 association [185]. Phosphorylation at the

position of Thr-947 is required for interaction with 14-3-3 [185-188]. In summary, 14-3-3 binding to its ligand mainly through sequence specific motif containing phosphorylated serine/threonine site.

Although phosphorylation of ligand proteins greatly enhances the affinity of 14-3-3 binding to these sites, the 14-3-3 proteins also interact with unphosphorylated proteins. These examples include the *Pseudomonas aeruginosa* virulence factor exoenzyme S (ExoS) (binding motif: DALDL) [189, 190], p190RhoGEF (binding motif: IQAIQNL) [191], and R18 peptide (core motif: WLDLE) [192]. An additional example of a nonphosphorylation-dependent 14-3-3 binding protein is the adhesion receptor, platelet glycoprotein Ibalpha (GP Ibα). Gu et al. [193] found that 14-3-3 recognizes the SGHSL motif at the C-terminus of GP Ibα. Because sequence specific phosphoserine peptides can inhibit the binding of 14-3-3 to unphosphorylated ligands [194], it is possible that both types of ligands utilize the same ligand-binding site on 14-3-3. Thus, regardless of whether 14-3-3 binding sites in target proteins are phosphorylation-dependent or phosphorylation-independent, they seem to bind to the same binding pocket (the amphipathic groove) in 14-3-3 proteins as the classic mode I and mode II motifs [154].

13. 14-3-3 inhibitors

To suppress the function of 14-3-3, sequence specific peptides from 14-3-3 binding ligands were found to competitively disrupt 14-3-3-ligand interactions [154, 158, 176]. Thereafter, in the effort to therapeutically interfere with 14-3-3 functions, some molecules were developed as potent 14-3-3 inhibitors that can regulate 14-3-3-ligand interactions in vitro. For example, R18 peptide (PHCVPRDLSWLDLEANMCLP), an unphosphorylated peptide with high affinity to all the 14-3-3 isoforms, has been shown to

block formation of 14-3-3-Raf-1 complex and is used widely in *in vitro* competitive inhibition experiments [195]. Difopein, a dimeric version of R18 peptide, can also effectively block the ability of 14-3-3 binding to target proteins [196, 197]. Recently, FOBISIN (FOurteen-three-three BInding Small molecule INhibitor) 101 was identified as a potent 14-3-3 inhibitor and can efficiently block the binding of 14-3-3γ to the pS259-Raf-1 peptide and the activity of 14-3-3 isoforms to stimulate ADP ribosyltransferase of exoenzyme S (ExoS) [198]. A more recent lysine-specific molecular tweezer was also reported to inhibit binding of 14-3-3 proteins to a phosphorylated target protein C-Raf and an unphosphorylated ExoS, suggesting a new technique to modulate 14-3-3 protein-protein interactions and 14-3-3 protein function [199]. In sum, with advances in understanding the physiological role of 14-3-3, more and more 14-3-3 inhibitors could be developed.

However, these small molecules usually function as general 14-3-3 inhibitors. For example, the R18 peptide can bind to all 14-3-3 isoforms with a comparable affinity [164]. Under such circumstances, to determine the specific role of 14-3-3 specific isoform, RNA interference techniques to inhibit different combinations of 14-3-3 genes are still not bad approaches [164].

14. Isoform-specific interaction

The highly conserved structure and shared identity at the amino acid level of the seven human 14-3-3 isoforms suggests redundant functions [152]. Consistently, some 14-3-3 interacting proteins show no specificity in their interaction with 14-3-3 isoforms. For example, apoptosis signal regulating kinase 1 (ASK-1) [200] and Tuberin [201] interact with all seven 14-3-3 isoforms. However, growing evidence suggest some specific roles of 14-

3-3 isoforms [202]. Certain 14-3-3 isoform-specific interactions have also been reported [203, 204]. For example, CDC25B strongly interacts with 14-3-3η, β and ζ proteins, but much less with 14-3-3ε and θ [205]. Moreover, the molecular scaffold, KSR1 (kinase suppressor of Ras 1), exhibited the highest binding affinity to 14-3-3γ and η, lower affinity to β, θ and ζ isoforms, and no apparent binding to the ε and σ isoform [206], showing isoform-specific interactions. Thus, some isoform-specific 14-3-3-ligand interactions complicate physiological functions of 14-3-3 proteins [161].

15. 14-3-3 proteins in membrane protein trafficking

Recently, 14-3-3 has also been reported to regulate membrane protein trafficking [182, 187, 204, 207]. Binding of 14-3-3 proteins to many transmembrane proteins was suggested to regulate forward transport of these proteins from the ER to the PM [159, 160, 208, 209]. Interestingly, these membrane proteins share the common feature of containing both a 14-3-3 binding motif and an ER retention signal, usually a di-arginine motif (RXR) [159, 160]. Binding of 14-3-3 was proposed to allow efficient surface expression of these membrane proteins by probing either their multimeric assembly [204] or by modulating ER exit through masking the ER retention signal [208, 209].

Several types of evidence suggest that 14-3-3-mediated ER export of membrane proteins is achieved by interfering with dibasic motif-mediated retention [160, 209]. A typical example of such a membrane protein is the potassium channel KCNK3/TASK-1 [210-212]. The COP-I interacting motif (dibasic amino acids) is found at the proximal cytosolic N-terminus of KCNK3, whereas a 14-3-3-binding motif (-RRSSPV) is located at the C-terminus of KCNK3. Binding of 14-3-3 to KCNK3 is phosphorylation-dependent [210, 212]. O'Kelly et al. [210] confirmed the interaction of COP-I with KCNK3 using mutagenesis and

sequential co-immunoprecipitation assays and they found that there are two pools of KCNK3 proteins (one pool of KCNK3 interacts with COP-I, the other pool of KCNK3 interacts with 14-3-3), which indicates that the binding of KCNK3 to COP-I and 14-3-3 is mutually exclusive. Therefore, phosphorylation-dependent recruitment of 14-3-3 to KCNK3 promotes cell surface expression of the channel, whereas the unphosphorylated KCNK3 is retrieved back by COP-I [210].

Another example is the membrane protein human κ-opioid receptor (hKOPR) [163]. The putative 14-3-3 binding motif (354-RQSTS-358) and the ER retention motif (359-RVR-361) in the hKOPR C-terminal tail are very close to each other. Li et al. [163] showed that mutation in the ER retention motif (359-RVR-361) of hKOPR reduced binding of COP-I to hKOPR and abrogated 14-3-3ζ-mediated regulation of hKOPR. Both knockdown of 14-3-3ζ by siRNA and usage of scavenger protein pGpLI-R18 showed compromised surface expression of the hKOPR in stable N2A-FLAG-hKOPR cells, suggesting that 14-3-3ζ promotes forward transport of the hKOPR by overcoming RVR motif mediated ER retention [163].

An additional example is proteinase-activated receptors 4 (PAR4). Cunningham et al found that PAR4 was largely retained in the ER via an ER retention motif 183-RAR-185 located within its intracellular loop-2. Mutation of 183-RAR-185 to 183-AAA-185 allowed efficient plasma membrane expression of PAR4 [213]. When PAR4 and PAR2, another family member of proteinase-activated receptors, were co-expressed in NCTC cells, heterodimerization between PAR2 and PAR4 has been shown to facilitate surface delivery of PAR4 through recruiting 14-3-3ζ and effective disruption of COP-I binding, indicating that binding of 14-3-3 to PAR2 and PAR4 dimer promotes anterograde

trafficking of the assembled complex [213]. In summary, accumulating evidence suggests that 14-3-3 plays critical role in forward transport of a number of membrane proteins, in which binding of 14-3-3 promotes their surface expression and reduces their ER localization.

Lipid phosphatase SAC1 is an ER/Golgi localized integral membrane protein, containing a canonical di-lysine ER retention signal (583-KEKID-587) at its extreme C-terminus. At steady state, SAC1 constantly shuttles between the ER and the Golgi compartments. Here, we found that SAC1 also possesses a potential 14-3-3 binding motif in its cytoplasmic N-terminus. We therefore speculated that binding of 14-3-3 to SAC1 might interfere with the classic KXKXX COP-I coatomer-binding motif (KEKID in SAC1) and regulate anterograde ER to Golgi trafficking of SAC1. We examined this hypothesis and also investigated the molecular basics that regulate 14-3-3-SAC1 interactions in chapter three.

Moreover, despite considerable progress have been made on Sac1-mediated PI4P metabolism in multicellular organisms and on subcellular localization of Sac1 at the ER/Golgi [79, 80, 91], how the Golgi subregional distribution of Sac1 is regulated remains obscure. In chapter two, we utilized the COP-I binding deficient, Golgi targeted mutant, SAC1-K2A, as a model and investigated the signals that control the Golgi distribution of SAC1. Surprisingly, we found that even the Golgi targeted SAC1-K2A mutant might localize at the cis/medial/trans Golgi, instead of the TGN.

FIGURES AND FIGURE LEGENDS

Figure 1

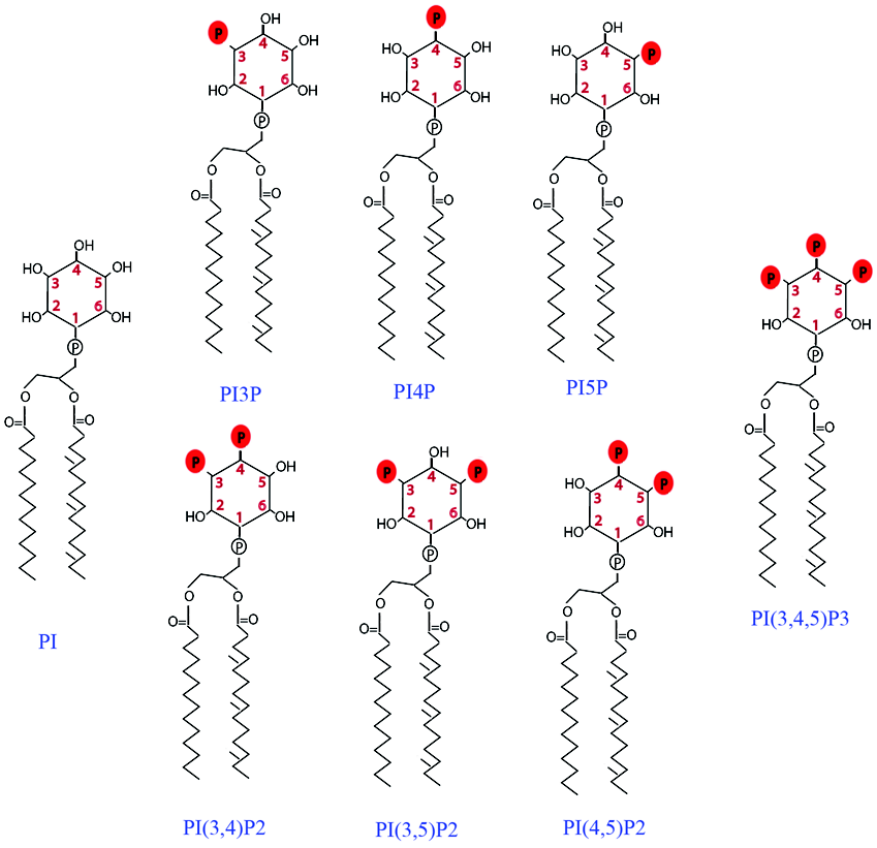
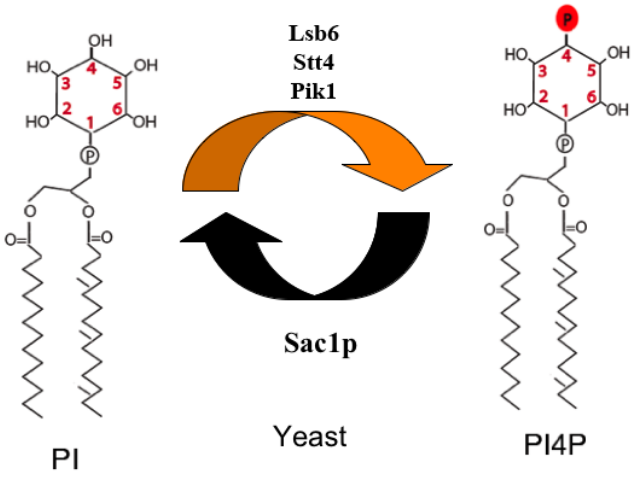


Figure 1. Phosphatidylinositol (PI) and the seven phosphoinositides. The glycerophospholipid phosphatidylinositol (PI) is composed of a hydrophobic diacyglycerol backbone linked to a polar inositol headgroup. The headgroup can be phosphorylated at the D3, D4 and D5 positions by various kinases to create seven phosphoinositides.

Figure 2

A.



B.

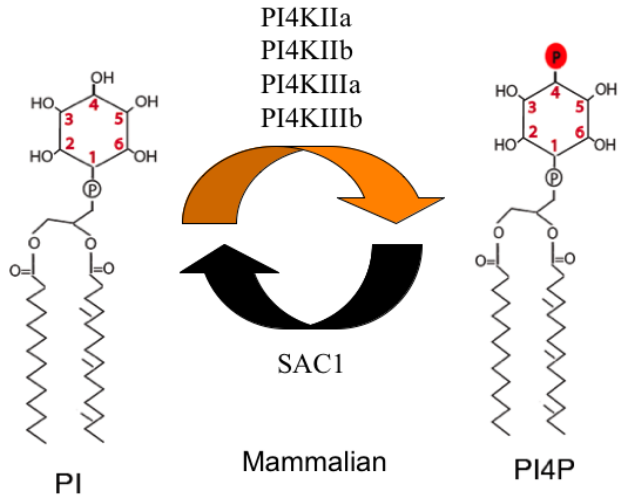


Figure 2. PI 4-kinases and PI4P phosphatases in yeast (A) and mammalian (B). There are three PI4 Kinases (Pik1, Stt4 and Lsb6) in charge of generating D4 phosphorylated phosphoinositides (PI4P) in yeast (B). There are four PI 4-Kinases (PI4KIIα, PI4KIIβ, PI4KIIIα and PI4KIIIβ) in mammalian in charge of generating D4 phosphorylated phosphoinositides (PI4P) (A). Sac1p and SAC1 are the sole PI4P phosphatase in yeast and in human respectively.

Table 1 Distinctive feature of mammalian PI4-kinase isoform

Features	**PI4KIIα**	**PI4KIIβ**	**PI4KIIIα**	**PI4KIIIβ**
Gene	*PI4KIIA*	*PI4KIIB*	*PI4KIIIA*	*PI4KIIIB*
MW	54 kDa	55 kDa	230 kDa	92 kDa
Yeast homolog	Lsb6p	Lsb6p	Stt4p	Pik1p
Wortmannin	Insensitive	Insensitive	IC_{50} 50-300 nM	IC_{50} 50-300nM
LY-294002	Insensitive	Insensitive	IC_{50} 50-100 mM	IC_{50} 50-100mM
Adenosine	Micromolar	Micromolar	Insensitive	Insensitive
[Subcellular localization] and function	[TGN] Recruit adaptors AP-1 and GGA; TGN-PM transport	[PM] Recruit to PM in response to PDGF and Rac	[ER] Resupply of PM PI4*P*	[Golgi] Golgi-to-PM traffic
	[Late endosome] Interact with AP-3; regulates degradation of EGFR		[Mitochondria] Not known	[Nucleus] Not known

Figure 3

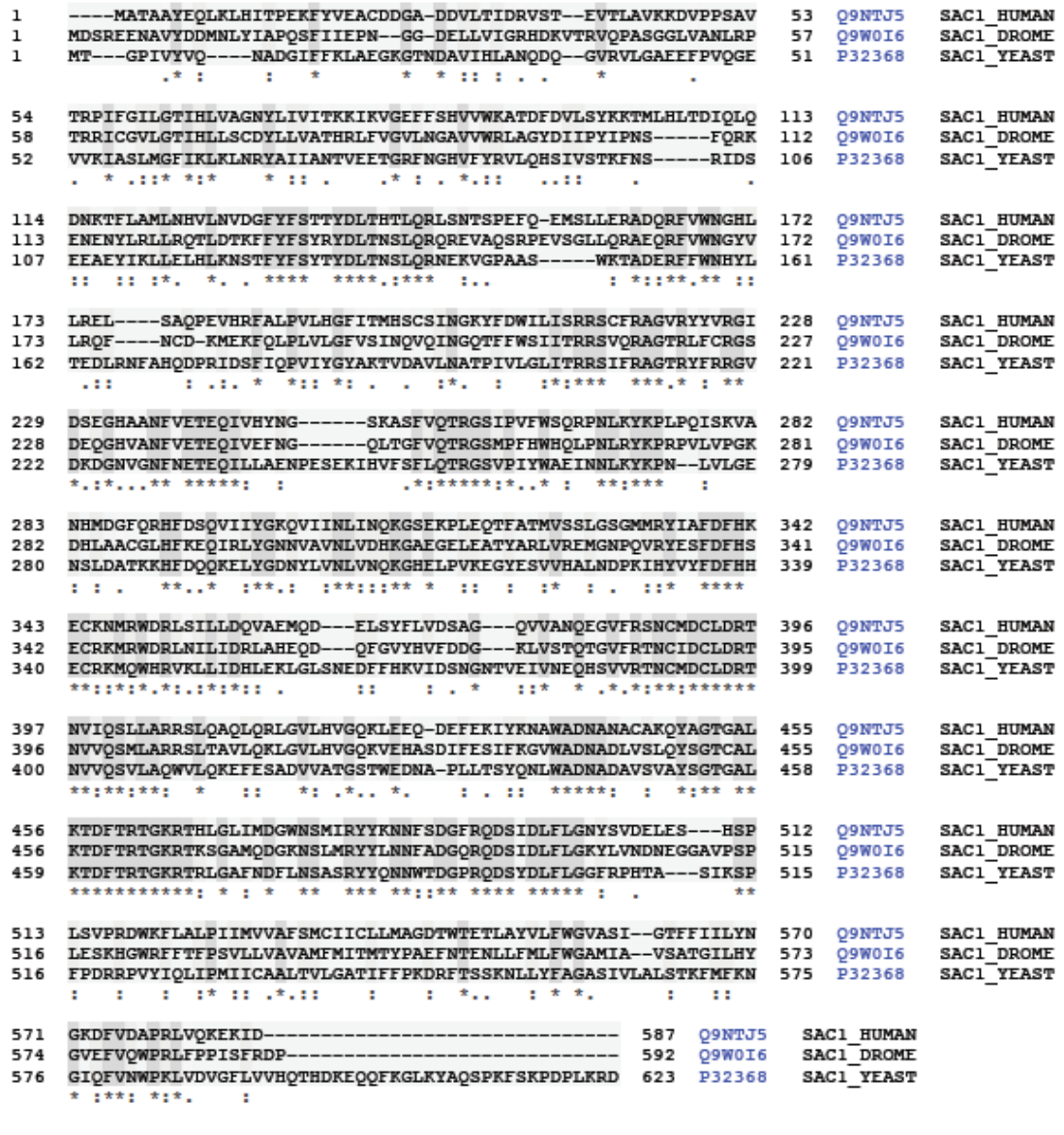


Figure 3. Sequence alignments of the Sac1 phosphatases. The protein sequences of human (UniProtKB entry: Q9NTJ5), fruit fly (UniProtKB entry: Q9W0I6) and baker's yeast (UniProtKB entry: P32368) were aligned by ClustalW2, a general multiple sequence alignment program for DNA or proteins. Asterisk (*) indicates conserved amino acid residues; Colon (:) indicates strongly similar residues; Period (.) indicates weakly similar residues.

Figure 4

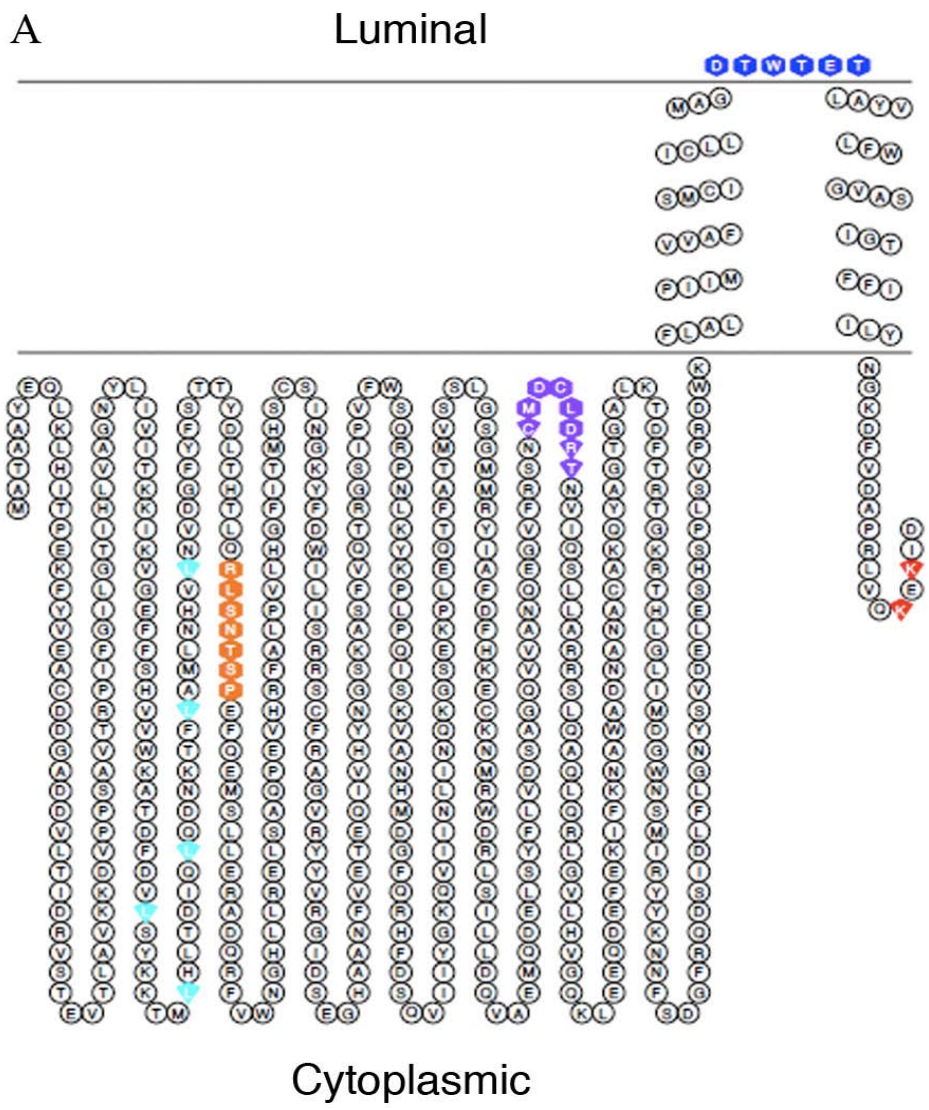


Figure 4

B

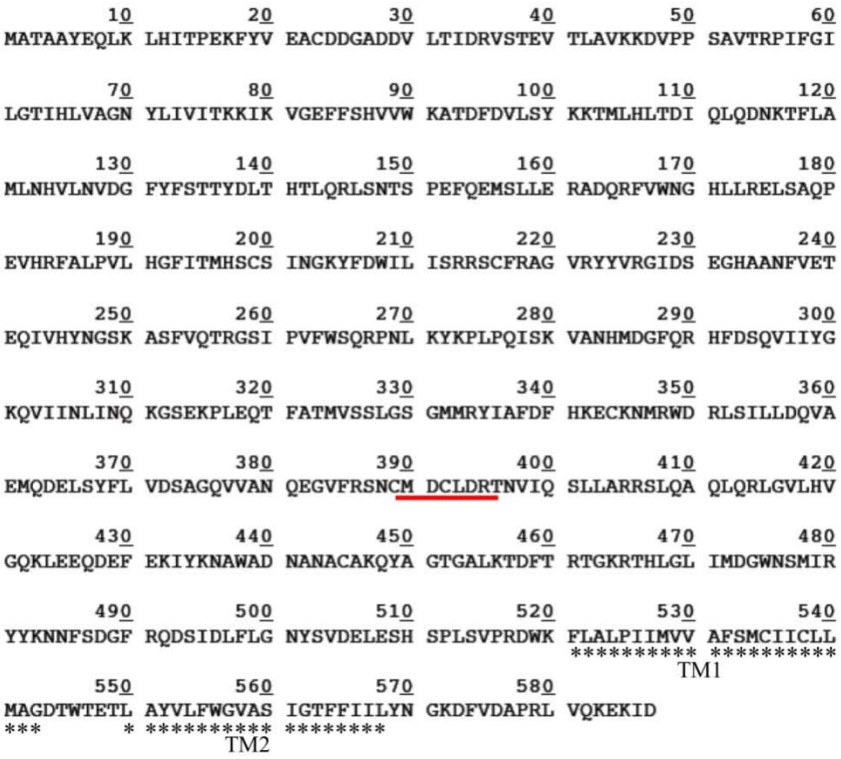


Figure 4. 2D topological diagram (A) and protein sequence of human SAC1 (B). SAC1 (UniProtKB entry: Q9NTJ5) is composed of 587 amino acid residues. SAC1 has two transmembrane domains near its C-terminus. Both the N-terminus and the C-terminus of SAC1 face cytoplasm. Important motifs are highlighted: Red, di-lysine retrieval motif (583-KEKID-587); blue: short luminal domain (544-DTWTET-549); purple: CX5R(S/T) catalytic motif (CMDCLDRT); orange: potential mode II 14-3-3 binding site; cyan: Leucine zipper motif. This 2D topological diagram of human SAC1 is generated using the TOPO2 program (http://www.sacs.ucsf.edu/TOPO2/) (A) and the sequence of human SAC1 is numbered using the UniProt identifiers. The catalytic motif (CMDCLDRT) is underlined by red, and the star label indicates two transmembrane domains (TM1 and TM2) of human SAC1 (B).

Figure 5

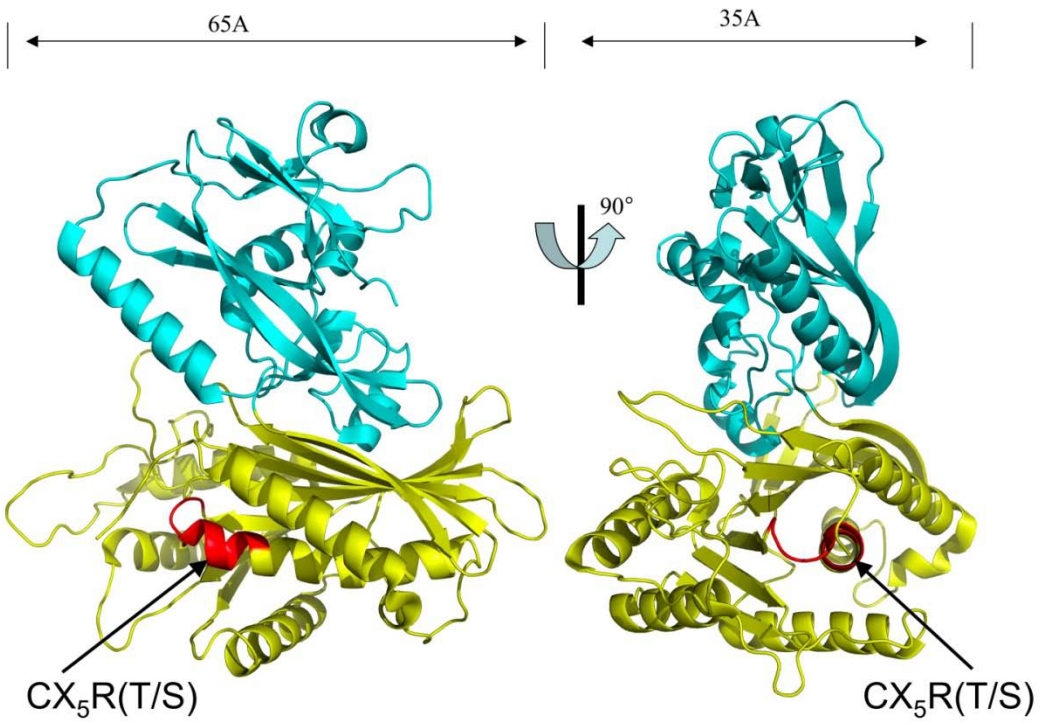


Figure 5. Ribbon diagram of the structure of yeast Sac1p phosphatase. (A) Overall crystal structure of cytoplasmic N-terminus of yeast Sac1p(1-503) reveals a SacN domain (blue) and a catalytic domain (yellow). The proposed unstructured domain is not shown here. The catalytic CX5R(T/S) motif (P-loop) is indicated in red. This structural diagram of Sac1p (PDB ID: 3LWT) is generated using the PyMol software. Figure is derived from Manford et al [68]

Figure 6

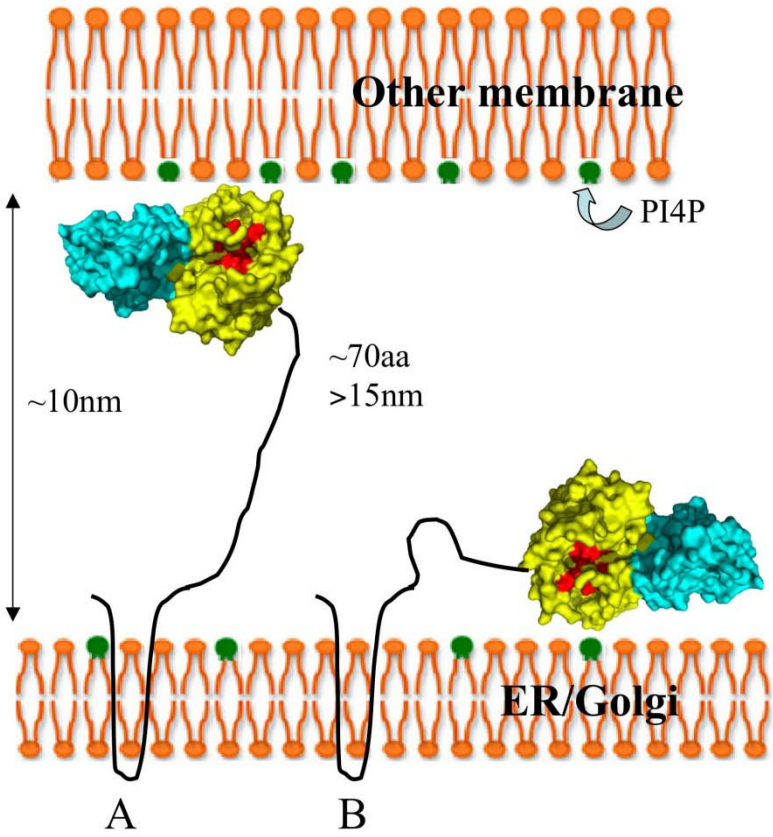


Figure 6. Cis-/trans-functional model of Sac1 at the membrane interface. The SacN sub-domain is colored with cyan and the catalytic sub-domain with yellow and the catalytic site P-loop is showing in red. Both SacN domain and catalytic domain dock on membrane surface probably through the polarized charge. The long unstructured linker between the catalytic domain and the first transmembrane domain allows Sac1 to hydrolyze PI4P either in a trans- (A) or a cis- (B) functional mode. The surface structural diagram of Sac1p (PDB ID: 3LWT) is generated using the PyMol software. This model is derived from Manford et al [68].

Figure 7

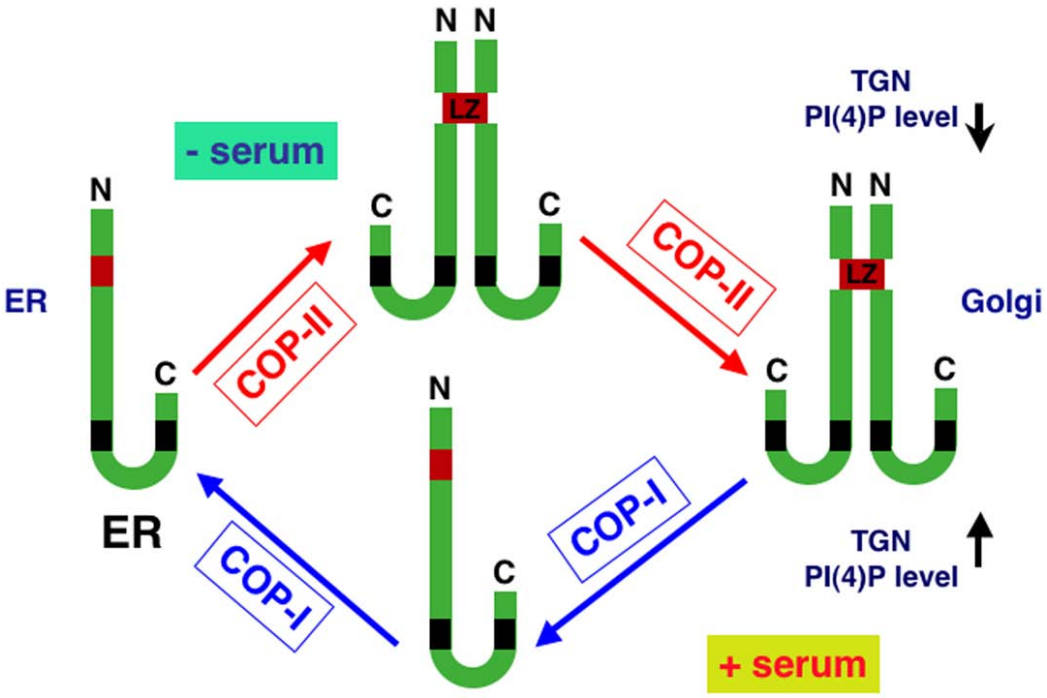


Figure 7. Human lipid phosphatase SAC1 shuttles between the ER and Golgi. In proliferating cells, Sac1 localizes at both ER and cisternal Golgi regions but is absent from the trans-Golgi network (TGN). The polarized distribution of Sac1 is critical for maintaining a steep PI4P gradient at the Golgi complex with the majority of PI4P confined to the TGN. During serum starvation and in quiescent cells Sac1 accumulates at the TGN, which reduces Golgi PI4P levels and slows anterograde trafficking. LZ, Leucine Zipper motif.

Figure 8

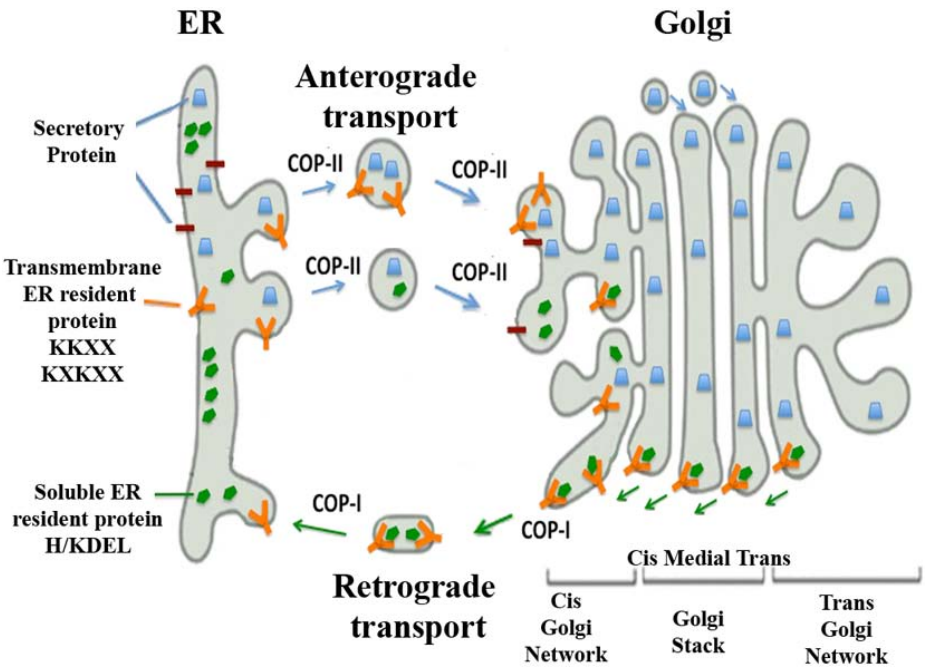


Figure 8. Bi-directional ER-to-Golgi transport. The anterograde transport of soluble and membrane secretory protein is mediated by COP-II coat complex while the retrograde retrieval is performed by COP-I coat complex. Those ER resident membrane proteins can be retrieved back to ER by COP-I via directly recognizing the KKXX or KXKXX retrieval motif. Soluble proteins with H/KDEL retention signal will be returned back to ER via the assistance of the KDEL receptor to carry them in COP-I-coated transport vesicles. Figure is derived from Alberts B, Johnson A, Lewis J, et al. the Molecular Biology of the Cell. 4th edition.

Figure 9

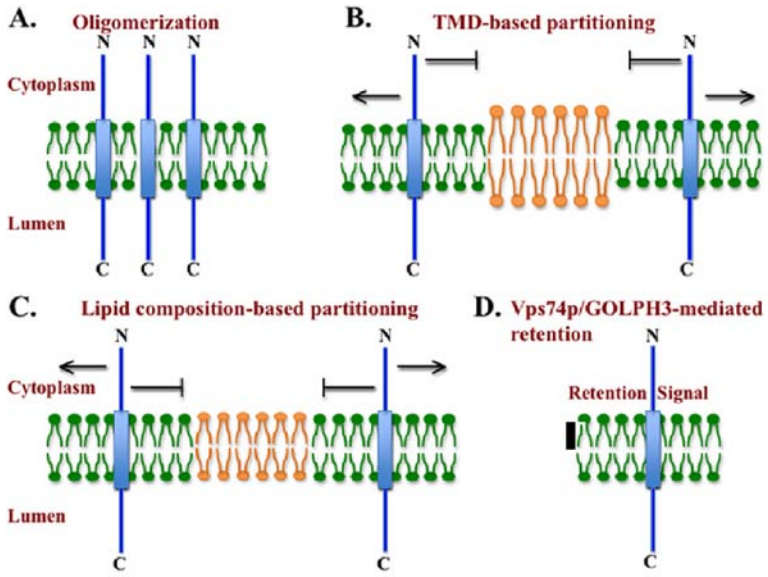


Figure 9. Simplified Golgi retention models. Only diagram of type II membrane protein is shown for clarify. A. The oligomerization mechanism. Oligomers can be formed through either cytoplasmic domain or stem region or the transmembrane domain. B. Transmembrane domain (TMD)-based partitioning mechanism. The ER and Golgi proteins usually have shorter transmembrane domain and cannot fit with thicker membrane bilayer such as TGN and PM, resulting in Golgi retention. C. Lipid composition-mediated Golgi partitioning. A gradient of sterols (cholesterol specifically) may exist in membranes of the secretory pathway and might explain this mode of retention. And ratios of lipids, particularly the ratio of glycerophospholipids to sphingolipids are increasing from the cis to the trans-Golgi, is the reason for observed Golgi protein. D. GOLPH3/VPS74 mediated retention. Loss of Vps74p in yeast results in mislocalization of several glycosyltransferases [133]. Vps74p/GOLPH3 was proposed to function as a late-Golgi resident glycosyltransferase sorting receptor for recruiting COP-I coat complex [133, 214]. Figure is derived from Banfield [115].

Figure 10

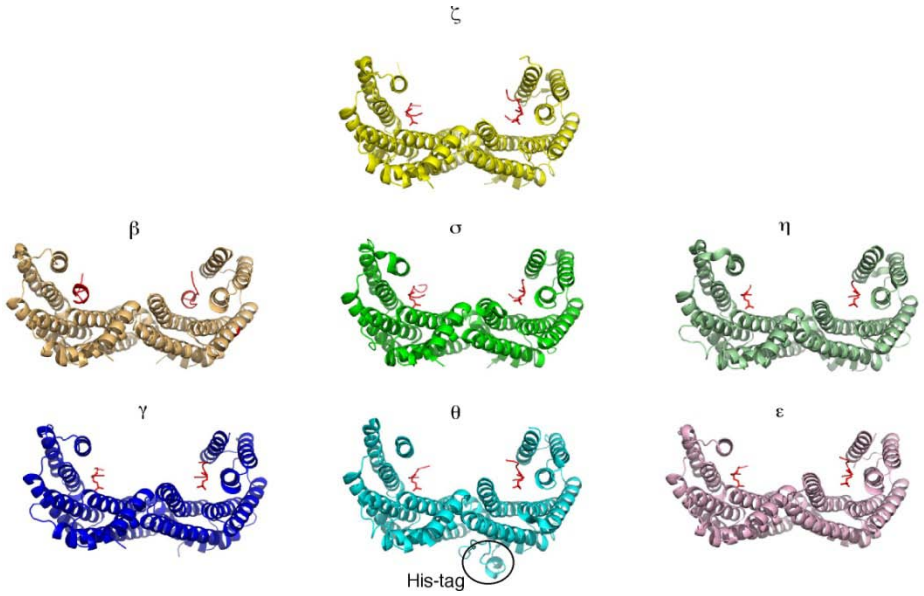


Figure 10. Crystal structures of 14-3-3 isoforms liganded with peptides. A side view of each isoform is shown: Yellow, Zeta (ζ) (PDB ID: 1QJB), liganded with mode 1 phosphopeptide RSHpSYPA; Lightorange, beta (β) (PDB ID: 2C23), liganded with a non-phosphorylated peptide (421-GLLDALDLASK-431) from exoenzyme S; Green, Sigma (σ) (PDB ID: 1YWT), liganded with a mode 1 phosphopeptide MARSHpSYPA; Palegreen, eta (η) (PDB ID: 2C74), liganded with a phosphopeptide RRQRpSAP; Blue, gamma (γ) (PDB ID: 2B05), liganded with a phosphopeptide RAIpSLP; Cyan, theta (θ) (PDB ID: 2BTP), liganded with a phosphopeptide RQRpSAP (Note: an N-terminal His tag was not removed and was indicated with a circle) and pink, epsilon (ε) (PDB ID: 2BR9), liganded with a phosphopeptide RRQRpSAP. pS denotes the phosphoserine residue. The liganded peptides are shown all the same color in red. Images are produced using PyMol software.

CHAPTER TWO

TWO DISTINCT REGIONS IN LIPID PHOSPHATASE SAC1 CONTROL ITS DISTRIBUTION WITHIN THE GOLGI

Jinzhi Wang[†], Juxing Chen[†], Caroline A. Enns[†], and Peter Mayinger[*1]

*Division of Nephrology & Hypertension and †The Department of Cell & Developmental Biology, Oregon Health & Science University, Portland, OR 97239

Short title: Golgi localization of SAC1 lipid phosphatase

[1]Corresponding author: Peter Mayinger, Division of Nephrology & Hypertension, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239 Tel.: 503-418-4012; Fax: 503-494-5330; email: mayinger@ohsu.edu

Note: manuscript was submitted to PLOS one

1. ABSTRACT

The lipid phosphatase Sac1 cycles between endoplasmic reticulum (ER) and cisternal Golgi compartments. In proliferating mammalian cells, a canonical dilysine motif at the C-terminus of Sac1 is required for coatomer complex-I (COP-I)-binding and continuous retrieval to the ER. Serum starvation triggers accumulation of Sac1 at the Golgi. The mechanism responsible for Golgi retention of Sac1 is unknown. Here we show that the first of the two transmembrane regions in human SAC1 (TM1) can function in Golgi localization. A minimal construct containing only TM1 and the adjacent flanking sequences is concentrated at the Golgi. Transplanting TM1 into transferrin receptor 2 (TfR2) induces Golgi accumulation of this normally post-Golgi protein, indicating that TM1 is sufficient for Golgi localization. In addition, we determined that the N-terminal

cytoplasmic domain of SAC1 also promotes Golgi localization, even when TM1 is mutated or absent. We conclude that the distribution of SAC1 within the Golgi is controlled via both the length of TM1 dependent passive partitioning and a retention mechanism that requires the N-terminal cytoplasmic region.

2. INTRODUCTION

The lipid phosphatase Sac1 is an evolutionarily conserved regulator of phosphatidylinositol-4-phosphate (PI4P) and plays vital roles at ER/plasma membrane contact sites and in Golgi organization and trafficking [79, 80, 91]. Sac1 is an integral membrane protein containing a large N-terminal cytoplasmic domain and two transmembrane regions near its C terminus [94]. The crystal structure of the N-terminal catalytic domain of yeast Sac1p was resolved, but the arrangement of the two transmembrane regions and their role in Sac1p localization has not been determined [68]. In mammals, Sac1 cycles continuously between endoplasmic (ER) and Golgi compartments via the canonical trafficking mechanisms involving COP-I and COP-II [98]. In proliferating cells, Sac1 localizes to both ER and cisternal Golgi regions but is absent from the trans-Golgi network (TGN) [93]. The polarized distribution of Sac1 is critical for maintaining a steep PI4P gradient at the Golgi complex with the majority of PI4P confined to the TGN [93]. During serum starvation and in quiescent cells Sac1 accumulates at the TGN, which reduces Golgi PI4P levels and slows anterograde trafficking [80]. All mammalian Sac1 orthologs contain two distinct sequence motifs that are required for shuttling between ER and Golgi. A di-lysine motif at the C terminus of Sac1 is essential for interaction with the COP-I machinery and for retrieval to the ER [76, 80]. A leucine repeat

region at the cytoplasmic N-terminal domain promotes oligomerization of Sac1 during serum starvation, which is a prerequisite for Golgi accumulation [76, 80]. However, how the distribution of Sac1 within the Golgi is controlled remains unclear.

The Golgi apparatus is a central sorting organelle guiding soluble and membrane-bound proteins arriving from the ER to their different endomembrane compartments. Passage through the Golgi is also required for post-translational processing of secretory proteins [115]. The mechanisms responsible for the characteristic intra-Golgi distribution of resident enzymes that are required for glycosylation and other modifications are not well established. Unlike the classic retrieval motifs that control steady state localization of ER proteins, no common Golgi sorting sequence has been found [115, 215]. Two mechanisms have been proposed for Golgi retention, commonly referred to as the oligomerization and the bilayer thickness model [115, 216]. The former proposes that retention of Golgi enzymes is achieved by forming large oligomers, which prevent sorting into anterograde transport vesicles [217, 218]. The latter postulates that retention relies on the length of membrane-spanning domains and the membrane bilayer thickness of the individual Golgi sections [217].

In this study, we determined that two distinct regions within human SAC1 function in Golgi retention. We found that a minimal construct containing only the first of the two transmembrane segments of SAC1 is efficiently concentrated at the Golgi. In addition, the large N-terminal cytosolic region of SAC1 was independently sufficient for Golgi localization. These results suggest that Golgi distribution of SAC1 is controlled via both membrane domain-dependent partitioning and protein-protein interactions.

3. MATERIAL AND METHODS

3.1 Cell culture and antibodies

HeLa cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (Thermo scientific), 1 mM glutamine, 1 mM sodium pyruvate (Invitrogen). Cells were incubated in 5% CO_2 humidified incubator at 37°C. Anti-GM130 mouse mAb was purchased from BD Biosciences (catalog number: 610822). Anti-GRASP65 rabbit polyclonal sera were purchased from Novus Biological (catalog number: NBP1-57592). Anti-flag M2 mouse mAb was purchased from Sigma (catalog number: F1804). Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 546-conjugated goat anti-mouse IgG were purchased from Molecular Probes (catalog numbers: A-11008 and A-21143).

3.2 Plasmids

Plasmids pGFP-SAC1, pGFP-SAC1-K2A, pGFP-SAC1(512-587) and pGFP-SAC1(501-587) were described previously [80]. Plasmid pMito-TagRFP is a gift from Dr. Gary Banker (OHSU). To construct pTaqRFP-peroxisome, a cDNA encoding TagRFP with additional nucleotides encoding peroxisomal targeting signal 1 (PTS1) (Ser-Lys-Leu) at its extreme C-terminus was amplified by PCR and subcloned into vector pCMV-3Tag-1A with BamHI and SalI. To generate pGFP-SAC1(478-549) and pGFP-SAC1(478-587)-K2A constructs, corresponding cDNAs were amplified by PCR and digested with BamHI and SalI, ligated into pEGFP-C1 vector digested with BglII and SalI by utilizing the isoschizomers BamHI and BglII. To construct pGFP-SAC1(501-549) and pGFP-SAC1(512-549), cDNA fragments encoding GFP-SAC1(512-549) and GFP-SAC1(501-549) were amplified by PCR using pGFP-SAC1(512-587) or pGFP-SAC1(501-587) as

templates respectively. The PCR products were digested with BamHI and SalI and subcloned into vector pCMV-3Tag-1A. To generate pGFP-TfR2(73-801) construct, human TfR2 cDNA encoding amino acid 73-801 was amplified by PCR and cloned into expression vector pEGFP-C2 by utilizing EcoRI and SalI restriction sites.

The SAC1 chimerical constructs containing the transmembrane domain of TfR2 (TMtfr2), pGFP-SAC1(TMtfr2)-K2A and pGFP-SAC1(478-549)TMtfr2, were generated by overlap extension PCR. The nucleotide sequence encoding TM1 of SAC1 was replaced with the sequence encoding the entire 24-residue TMtfr2. The PCR fragments were digested with BamHI and SalI and ligated into pEGFP-C1 as described above. Similarly, a TfR2 chimera containing the TM1 region of SAC1, GFP-TfR2(73-801)TM1, was generated by overlap extension PCR. The nucleotide sequence encoding TMtfr2 was substituted with the sequence encoding the entire 23-residue TM1 of SAC1. The cDNA was digested with EcoRI and SalI and ligated into pEGFP-C2 as described above.

To construct pFlag-SAC1(1-549) and pFlag-SAC1(1-549)TMtfr2, corresponding cDNA fragments were amplified by PCR using GFP-SAC1 and GFP-SAC1(TMtfr2)-K2A as templates respectively, and then subcloned into vector pCMV-3Tag-1A with BamHI and SalI. To construct pGFP-SAC1(152-549)TMtfr2 and pGFP-SAC1(152-549), pFlag-SAC1(1-549)TMtfr2 and pFlag-SAC1(1-549) were double digested with EcoRI and SalI and the resulting fragments of SAC1 were subcloned into vector pEGFP-C2 by utilizing the same restriction sites. All constructs were verified by sequencing and primer sequences are available upon request (see table 2). (GenBank accession numbers: TfR2, BC142630.1; SAC1, NM014016.3).

3.3 Mutagenesis

The plasmid pGFP-SAC1(1-518) was constructed by introducing two adjacent stop codons by site-directed mutagenesis using pGFP-SAC1 as a template. pGFP-SAC1(478-549)-E2A, pGFP-SAC1(478-549)C2S, pGFP-SAC1(478-549)ins3L, pGFP-SAC1-K2Ains3L were generated by mutagenesis using primers containing the desired mutations. QuikChange II XL Site-Directed Mutagenesis Kit was purchased from Agilent Technologies (catalog number: 200521). Primers were designed by utilizing online QuikChange Primer Design Software (https://www.genomics.agilent.com/). Experiments were performed according to manufacturer's instructions.

3.4 Immunofluorescence and confocal microscopy

Transient transfections were performed using the Lipofectamine2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. For confocal microscopy, 1.5 x 10^5 HeLa cells were plated into each well of a 6-well plate (Corning) containing glass coverslips and allowed to attach for 18-20 h at 37°C. The cells were transfected with 1 µg of DNA mixed with 3 µl Lipofectamine2000 and incubated for 24 h at 37°C.

24-hour after transfection, HeLa cells were washed three times with PBS and fixed with 4% paraformaldehyde in PBS for 15 min. Cells were treated with blocking & permeabilization buffer (PBS, 2% normal goat serum, 0.5% Triton X-100) for 30 min. The cells were then incubated with antibody diluted in PBS for 1 h. Primary antibodies used were anti-GM130 mouse mAb (2.5µg/ml final concentration), anti-flag M2 mouse mAb (1:500) and anti-GRASP65 rabbit polyclonal sera (1:200). Cells were washed four times with washing buffer (PBS, 0.1% Triton X-100, 0.2% BSA) and incubated with secondary antibody Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500) or Alexa

Fluor 546-conjugated goat anti-mouse IgG (1:500). Cells were then washed again four times with washing buffer. Coverslips were mounted onto microscope slides using Fluoromount-G (SouthernBiotech) and visualized on a Zeiss LSM 700 laser-scanning confocal microscope with a 63× 1.4 NA Plan-Apochromat objective lens. Excitation wavelengths of 488 nm (GFP; Alexa-488) or 555 nm (Alexa-546) were used. Images were analyzed using ZEN 2009 software. The depicted images are typical representatives of cells expressing a particular construct. All localization experiments were repeated at least twice.

3.5 Immunoprecipitation and Western blot

After transfection for 48 hrs, HeLa cells were washed twice with ice-cold PBS and lysed on ice in immunoprecipitation NET-T buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.4, 1% Triton X-100 with or without 5% glycerol) supplemented with 1xComplete Protease Inhibitor Cocktail (Roche Diagnostic, Indianapolis, IN). Cell lysates were centrifuged at 13,000rpm for 30 min to remove the cell debris and the supernatant was pre-cleared with either Protein A (Zymed, San Francisco, CA) or immobilized Protein G (PIERCE, Rockford, IL) beads for 1 hour at 4°C. Flag-tagged SAC1 and mutant proteins were then immunoprecipitated using anti-flag M2 antibody by overnight end-over-end rotation at 4°C. Next morning, SAC1 proteins were immunoprecipitated using mouse monoclonal anti-flag M2 antibody and protein G-Sepharose (Amersham Bioscience, USA). Cell lysates (10% of total) and immunoprecipitates were analyzed on western blots using indicated antibodies. After centrifugation, the beads were washed four times with NET-T buffer. All the samples were boiled in SDS sample buffer for 5 minutes and resolved by 12% SDS-PAGE. Proteins were transferred to nitrocellulose or PVDF

(polyvinylidene difluoride) membranes, and immuno-detected using mouse monoclonal M2 anti-flag (1:1,000) or anti-GFP antibody (1:1000).

4. RESULTS

4.1 Identification of Golgi localization domains in SAC1

The human phosphoinositide phosphatase SAC1 is an integral membrane protein with two transmembrane domains near the C-terminus (Figure 11A) [77, 94]. Consistent with our previous studies [80], transiently expressed GFP-SAC1 is mainly localized at the ER but also shows colocalization with the Golgi marker GM130 (Figure 11B). In contrast, GFP-SAC1-K2A, in which the two lysines in the C-terminal retrieval motif were replaced by alanines, quantitatively accumulates at the Golgi apparatus (Figure 11C). To map the regions in SAC1 that are required for Golgi targeting, a series of GFP and Flag-tagged truncation mutants were constructed (Figure 11A). The intracellular localization of these SAC1 mutants was analyzed by confocal microscopy and compared with either endogenous GM130 or endogenous GRASP65 as Golgi markers. To examine if the large cytoplasmic N-terminal domain contains a Golgi retention signal, GFP-SAC1 (1-518) was constructed, in which the entire cytoplasmic N-terminal domain was fused to GFP. GFP-SAC1(1-518) displayed cytoplasmic distribution but also showed some localization at the Golgi (Figure 11D), suggesting that the N-terminal domain itself may have a low affinity for the Golgi. However, this domain was not required for efficient Golgi retention because the mutant GFP-SAC1(478-587)-K2A, in which most of the cytosolic N-terminal domain was deleted, was quantitatively localized to the Golgi (Figure 11E). Furthermore, the truncation mutant Flag-SAC1(1-549), which contains the entire

cytoplasmic N-terminal domain, the transmembrane domain TM1, and six amino acids predicted to face the lumen, also showed robust Golgi localization (Figure 11F). These results suggested that the region from amino acid 478 to 549, which contains TM1 and short flanking sequences, was sufficient for Golgi retention. To test this idea, the GFP-tagged construct GFP-SAC1(478-549), containing only this region, was generated. Immunofluorescence microscopy showed that GFP-SAC1(478-549) displayed strong colocalization with the Golgi marker GM130 (Figure 11G). GFP-SAC1(478-549) contains only one of the two SAC1 transmembrane domains and is a bona-fide tail-anchored membrane protein, but showed no mis-targeting to other organelles such as mitochondria or peroxisomes (Figure 12). Taken together, these data indicate that the short TM1 construct contains all the elements that are needed for efficient Golgi retention.

4.2 The cytoplasmic region adjacent to TM1 is required for ER export

GFP-SAC1(478-549) contains a short 43 amino acid stretch that is part of the N-terminal cytoplasmic domain and six amino acids at the C-terminus that are predicted to face the ER and Golgi lumen. To further narrow down the sequence required for Golgi retention, a construct GFP-SAC1(478-543) was generated, in which the potential luminal region 544-DTWTET-549 was removed. GFP-SAC1(478-543) showed the same strong co-localization with Golgi marker GM130 as GFP-SAC1(478-549) (Figure 13A,B), suggesting that this short stretch in the SAC1 sequence is not required for Golgi retention. To examine if the adjoining N-terminal cytoplasmic region adjacent to TM1 is required for Golgi retention, pGFP-SAC1(501-549) and pGFP-SAC1(512-549) were constructed. Fluorescence microscopy showed that GFP-SAC1(501-549) localizes at both the Golgi and the ER (Figure 13C). In contrast, GFP-SAC1(512-549) is largely restricted to the ER

compartment (Figure 13D). This result suggested that the cytoplasmic region that flanks TM1 might be required for efficient trafficking of SAC1 from the ER. This region contains the sequence 505-DELE-508, which resembles the classical COP-II binding motif DXE [102]. However, a GFP-SAC1(478-549)-E2A mutant showed no ER retention (Figure 14) and it remains unclear whether and how this portion of the protein is required for SAC1 trafficking.

4.3 Replacing TM1 with TMtfr2 disrupts Golgi localization of GFP-SAC1(478-549)

To directly test whether TM1 is necessary for Golgi localization of SAC1(478-549), the chimeric construct GFP-SAC1(478-549)TMtfr2 was generated, in which TM1 was replaced with the 24-residue transmembrane domain of TfR2 (TMtfr2). TfR2 is an 801-residue type II membrane protein with a small cytoplasmic domain (amino acids 1-80), a putative 24-residue TM domain (amino acids 81-104) and a large ectodomain (amino acids 105-801). At steady state, wild-type TfR2 localizes mainly to the plasma membrane and intracellular endosomes [219, 220]. HeLa cells were transfected with plasmids expressing either GFP-SAC1(478-549) or GFP-SAC1(478-549)TMtfr2 and analyzed by confocal fluorescence microscopy. GFP-SAC1(478-549) colocalized strongly with GM130 as expected (Figure 15A). In contrast, significant amounts of the GFP-SAC1 (478-549)TMtfr2 chimeric protein were detected at the plasma membrane (Figure 15B). This result confirms that SAC1-TM1 plays a critical role in Golgi retention of GFP-SAC1(478-549).

4.4 Inserting TM1 of SAC1 into TfR2 redirects this protein to the Golgi

In order to determine if the TM1 of SAC1 is sufficient for promoting Golgi association, we replaced the transmembrane domain of TfR2 with TM1. To achieve a clear and

distinguishing localization phenotype, a GFP-TfR2(73-801) mutant was generated in which most of the cytoplasmic domain of TfR2 containing the YQRV endocytic motif was removed. The corresponding chimera GFP-TfR2(73-801)TM1 was also constructed (Figure 16A). GFP-TfR2(73-801) showed plasma membrane localization in transfected cells when analyzed by fluorescence microscopy (Figure 16B). In contrast, GFP-TfR2(73-801)TM1 was retained predominantly at the Golgi and no cell surface staining was detectable (Figure 16C). These data suggested that the TM1 region of SAC1 is sufficient to direct an unrelated membrane protein to the Golgi.

4.5 Lengthening of TM1 triggers PM accumulation of GFP-SAC1(478-549)

Sequence alignment of the membrane-spanning domains of resident Golgi proteins show no sequence homology and no particular motif required for retention has been identified [221, 222]. However, in some cases cysteine residues within transmembrane domains play a role in Golgi retention. For example, cysteine residue in the transmembrane domains of β-1,4-galactosyltransferase is required for Golgi localization and dimerization [126, 127]. In contrast, cysteine residues in the transmembrane domains of alpha 2,6-sialyltransferase [223] and N-acetylglucosamine 6-O-sulfotransferases 1 (GlcNAc6ST-1) [128] were found to be critical for its dimerization, but abolishment of dimer formation by mutagenesis did not lead to disruption of their Golgi localization. There are two cysteine residues present within TM1 of human SAC1 (FLALPIIMVVAFSMCIICLLMAG). To test whether these residues are critical for Golgi retention, they were mutated to serines. However, the corresponding GFP-SAC1(478-549)C2S mutant showed the same strong colocalization with Golgi marker GM130 as the

unsubstituted GFP-SAC1(478-549) and no cell surface labeling was detected (Figure 17A,B), demonstrating that the cysteines are not critical for Golgi localization.

According to the bilayer thickness model for Golgi retention, the length of transmembrane segments in resident Golgi proteins is the key determinant for localization. To examine if increasing the length of TM1 interferes with its capability to promote Golgi localization, three leucine residues were inserted into SAC1-TM1 converting it into a 26-residue-long hydrophobic domain (FLALPIIMVVAFSMCII CLLLLLMAG, inserted leucines are underlined). The elongated TM1 mutant, GFP-SAC1(478-549)ins3L, showed accumulation at the plasma membrane and reduced Golgi staining by fluorescence microscopy (Figure 17C). These data further support the idea that TM1 functions in the localization of SAC1 and indicate that the length of this domain is critical for proper Golgi partitioning.

4.6 The N-terminal cytoplasmic domain of SAC1 contributes Golgi localization

Because a truncated version of Sac1 comprising only the N-terminal cytoplasmic domain showed partial colocalization with Golgi markers (Figure 11D), we reasoned that this portion of the protein might independently promote Golgi targeting. To test this idea, we generated a construct in which the entire N-terminal region of Sac1 was fused to TMtfr2. The Flag-SAC1(1-549)TMtfr2 chimera efficiently accumulated at the Golgi and no plasma membrane localization was observed (Figure 18A). This result therefore suggests that the N-terminal region of Sac1 promotes Golgi targeting independently from the TM1-specific mechanism. Based on the structure of yeast Sac1p, the N-terminal region is composed of three distinct domains, the SacN domain (1-186), the catalytic domain (187-462) and an unstructured region (462-521). Our attempts to analyze these individual

domains for their potential roles in Golgi targeting were unsuccessful because the relevant truncated constructs GFP-SAC1(152-549) and GFP-SAC1(152-549)TMtfr2 formed aggregates and we were therefore unable to perform localization analyses (data not shown).

To examine the relevance of TM1 in the context of full-length SAC1, we replaced TM1 with either TMtfr2 or with the elongated TM1 that contains three additional leucines (ins3L). The GFP-SAC1 (TMtfr2)-K2A protein accumulated in the ER and was not present at Golgi membranes (Figure 18B). In contrast, the mutant GFP-SAC1ins3L-K2A localized to the Golgi where it was efficiently retained because no escape to the plasma membrane could be observed (Figure 18C). The TM1 region of SAC1 is therefore not strictly required for Golgi localization of the full-length SAC1 protein that lacks the COP-I retrieval motif. However, TM1 may play additional roles in the proper folding and arrangement of the two transmembrane segments, which is a prerequisite for ER exit.

4.7 A potential oligomerization domain is located at the adjacent region of TM1

A cysteine residue in β-1,4-galactosyltransferase is important for both its Golgi localization and dimerization [126, 127]. Although two cysteine residues within the stem and transmembrane of N-acetylglucosamine 6-O-sulfotransferases 1 (GlcNAc6ST-1) domain were found to be critical for its dimerization, disruption of the dimer by mutagenesis did not change its localization [128]. To investigate whether the cysteine residues in the SAC1-TM1 might be involved in dimerization, we performed co-immunoprecipitation assays to test whether Flag-SAC1(1-549) can pull down GFP-SAC1(478-549). HeLa cells were co-transiently transfected with the plasmid combinations as indicated, and then lysed and subjected to co-immunoprecipitation assays with anti-flag M2 antibody. The

immunoprecipitates and cell lysates were examined by immunoblotting with anti-flag M2 and anti-GFP antibodies. Surprisingly, Flag-SAC1(1-549) was able to recover GFP-SAC1(478-549), but not GFP-SAC1(501-587) (Figure 19A). These data suggest that the potential oligomerization domain of SAC1 may lie within the region from 478 to 501 of SAC1 instead of SAC1-TM1. To test this hypothesis, we examined whether Flag-SAC1(1-549) can pull down the chimeric GFP-SAC1(479-549)TMtfr2 and whether full length Flag-SAC1 can immunoprecipitate full-length chimeric mutant GFP-SAC1(TMtfr2)-K2A. Our results showed that Flag-SAC1(1-549) was able to recovery almost the same level of GFP-SAC1(478-549) as its corresponding chimeric mutant GFP-SAC1(478-549)TMtfr2. Similarly, full length Flag-SAC1 can co-immunoprecipitate with GFP-SAC1 as well as its corresponding chimeric mutant GFP-SAC1(TMtfr2)-K2A with no apparent difference (Figure 19B). These data suggested that replacement of SAC1-TM1 with TMtfr2 did not disrupt oligomerization. Moreover, sequence alignment revealed that primary sequences in the region of human SAC1(478-501) are as conserved as other regions across Sac1 family members (Figure 19C). Taken together, a potential oligomerization domain of SAC1 may reside within the cytoplasmic adjacent region of SAC1-TM1, most likely within the amino acid sequence of 478 to 501.

4.8 BFA treatment relocates GFP-SAC1-K2A and GFP-SAC1(478-549) to the ER

Brefeldin A (BFA) treatment can inhibit protein secretion and lead to the fusion of Golgi apparatus into the ER [137-139]. In contrast, BFA causes the TGN to mix with the endosomal system instead of the ER [140]. In order to distinguish whether SAC1 localizes at the TGN or cis/medial/trans Golgi, we performed BFA treatment experiments. HeLa cells transiently trasfected with GFP-SAC1-K2A or GFP-SAC1(478-549) were treated with or

without 10 μg/ml BFA for 30min. Intracellular localization of SAC1 constructs was analyzed by indirect immunofluorescence with anti-GM130 antibody. GM130 itself served as an endogenous control for BFA treatment. We found that in untreated cells both GFP-SAC1-K2A (Figure 20A) and GFP-SAC1 (478-549) (Figure 20C) are localized at the Golgi revealed by colocalization with cis-Golgi marker GM130. However, BFA treatment resulted in the redistribution of GFP-SAC1-K2A (Figure 20B) and GFP-SAC1(478-549) (Figure 20D) and endogenous GM130 into the ER, suggesting that GFP-SAC1-K2A and GFP-SAC1(478-549) could be localized to the cis/medial/trans Golgi but not to the TGN.

5. CONCLUSIONS

We have identified two distinct regions within human SAC1 that can function in Golgi retention. Either the first transmembrane domain (SAC1-TM1) or the large N-terminal cytosolic region of SAC1 was independently sufficient for Golgi localization. Moreover, we found that the cytoplasmic adjacent region of SAC1-TM1 might be an additional oligomerization domain. Further, the vast majority of SAC1-K2A mutant was found to localize at the cis/medial/trans Golgi instead of trans-Golgi network (TGN) at least at steady state.

6. DISCUSSION

Mammalian Sac1 orthologs have several unique properties related to their tightly controlled intracellular localization. In proliferating cells, Sac1 displays a specific distribution between the ER and the cisternal Golgi, which is essential for spatial control

of PI4P [93]. Serum starvation induces quantitative accumulation of Sac1 at the Golgi but this translocation is promptly reversed upon growth factor stimulation [80]. Previous studies have shown that a canonical C-terminal di-lysine motif, present in all mammalian Sac1 orthologues, is required for steady state distribution at the ER [76, 80]. In this study, we show that one of the two transmembrane domains in human SAC1 is sufficient for partitioning into the Golgi. The relevance of a transmembrane segment for Golgi localization is not unprecedented. For example, the first of three membrane-spanning domains of M1 glycoprotein of avian coronavirus was found to be sufficient to retain this protein in the cis-Golgi, When the membrane-spanning domain of M1 glycoprotein was transplanted into the VSVG protein, which can be efficiently delivered to the plasma membrane, the resulting chimera was retained in the Golgi [224]. Furthermore, the single transmembrane domains in many Golgi glycosylation enzymes are key determinants of their specific localization at the Golgi [76]. How transmembrane domains mediate Golgi retention is not entirely clear. Two mechanisms have been proposed, based either on oligomerization of Golgi enzymes into large complexes or on length of TM segment of membrane proteins. SAC1 can form oligomers, and oligomerization promoted by serum starvation coincides with Golgi accumulation [80]. However, the data presented here show that a minimal construct encompassing only transmembrane region TM1 plus short flanking regions efficiently accumulates at the Golgi. Furthermore, transplanting the TM1 sequence without its flanking regions into TfR2 was sufficient to redirect this protein to the Golgi. Our experiments also show that lengthening of TM1 significantly reduced Golgi retention and induced plasma membrane localization. Based on these results, we propose that bilayer thickness may contribute to the precise distribution of Sac1 within the Golgi.

Based on the bilayer thickness model, relatively short transmembrane domains are thought to correlate with Golgi retention [122, 123]. A recent bioinformatics investigation used comprehensive comparisons of transmembrane domains of integral membrane proteins to show that the transmembrane length of resident membrane proteins varies along the secretory pathway [121]. The mean hydrophobic length of transmembrane domains in vertebrate ER and Golgi proteins is 20.6 residues, whereas TGN membrane proteins have a mean value of 24.8 hydrophobic residues [121]. The predicted length of TM1 is 23 amino acids, which is longer than the common transmembrane spanning domains of cisternal Golgi enzymes [121]. It is therefore tempting to speculate that the specific dimensions of TM1 allow SAC1 to reach the TGN, which may be physiologically important because the phosphatase is responsible for downregulation of TGN PI4P levels during serum starvation.

An important question is whether steady state localization of human SAC1, especially the Golgi-targeted mutant SAC1-K2A, is located at the TGN or cis/medial/trans Golgi. Given the importance of PI4P in anterograde trafficking from the TGN, the presence of Sac1 in the TGN is paradoxical [91]. In this study, I used the cis-Golgi marker GM130 and medial-Golgi marker GRASP65 but no TGN marker. Of interest, brefeldin A (BFA) treatment resulted in redistribution of GFP-SAC1-K2A to the ER, suggesting that even the Golgi-targeted mutant SAC1-K2A could localize at the cis/medial/trans Golgi instead of the TGN [140]. Previous confocal immunofluorescence microscopy studies showed that SAC1 proteins did localize at the TGN because they co-localized with the TGN marker TGN46 [80, 91]. These controversial results need to be tested by using high-resolution microscope like electron microscopy (EM). In deed, EM

analysis revealed that wild type SAC1 is absent from the TGN, thus confining PI(4)P at the TGN [93]. If SAC1-K2A has no chance to reach the TGN, then TGN secretion will not be greatly affected by over-expression of SAC1-K2A at least at steady state.

There is evidence showing that even the Golgi-targeted SAC1-K2A mutant may not function at the TGN. Dr. Bankaitis laboratory (University of North Carolina) has established a HeLa cell line stably expressing SAC1-GFP (3-fold than endogenous level) [91]. This stable cell line was unintentionally generated with a GFP tag at the C-terminus of human SAC1, which disrupted the di-lysine motif (583-KEKID-587), and therefore, it is a counterpart to SAC1-K2A mutant instead of wild type SAC1. Surprisingly, they did not observe any obvious defect in the trafficking of VSVG along the secretory pathway using this stable cell line, implying that the TGN PI4P level is not affected by the stably over-expressed SAC1-GFP [91]. However, studies from Dr. Balla lab (National Institutes of Health) showed that acute PI4P depletion achieved by using rapamycin-induced TGN-targeted Sac1 phosphatase could rapidly eliminate TGN PI4P, terminate the formation of transport vesicles from the Golgi and block the transports of both the VSVG and the cation-independent mannose 6-phosphate receptor (CI-M6PR) [225]. Taken together, these data suggest that Golgi-localized SAC1-K2A mutant may not targeted to the TGN and may have limited roles in depleting TGN PI4P.

Nonetheless, serum starvation seems to cause TGN accumulation of human SAC1 [80]. Then, how serum starvation releases the cis/medial/trans Golgi retention of human SAC1 and subsequently results in TGN PI4P depletion needs further investigation.

A specific distribution of human SAC1 within the Golgi is required to establish a cis-to-trans PI4P gradient in proliferating cells [93]. The N-terminal cytoplasmic region of SAC1 may play multiple roles in this process. Oligomerization of this domain is a key determinant in controlling ER exit or interaction with COP-I because oligomerization-deficient SAC1 mutants remain concentrated at the ER during serum starvation [98]. The presented data suggest that the N-terminal region of SAC1 plays an additional role in the targeting of SAC1 to the Golgi. In yeast, the N-terminal domain of Sac1p interacts with the peripheral Golgi protein Vps74, a PI4P effector required for Golgi localization of glycosyltransferases [226]. Whether GOLPH3, the human homolog of Vps74 [28], interacts with SAC1 has not been examined. However, deletion of Vps74 in yeast does not abrogate Golgi localization of Sac1p and there is evidence that additional integral Golgi membrane proteins are involved in Sac1p localization [46].

In summary, we have identified two specific regions in human SAC1 that are independently involved in Golgi targeting. Combined with our previous studies, these results support a multi-step mechanism controlling distribution of SAC1 at the Golgi. During proliferation, SAC1 partitions into the cisternal Golgi but is continuously retrieved to the ER by interacting with retrograde COP-I complexes. The first transmembrane segment TM1 is sufficient to bring about Golgi localization but only when the COP-I sorting motif is absent. Because Golgi partitioning via transmembrane domains is a passive process an additional retention mechanism must be in place to establish the steady state distribution of SAC1 at the cisternal Golgi in proliferating cells. Our data show that the N-terminal domain of SAC1 plays an important role in this process. This view is supported by our previous work showing that elimination of the N-

terminal domain of SAC1 triggers ER accumulation if the C-terminal COP-I binding motif is intact. When ER retrieval is turned off during serum starvation, SAC1 behaves like a resident Golgi enzyme and TM1 may then direct the protein to Golgi regions with appropriate membrane thickness resulting in the turnover of PI4P at these sites.

7. FIGURES AND FIGURE LEGENDS

Figure 11

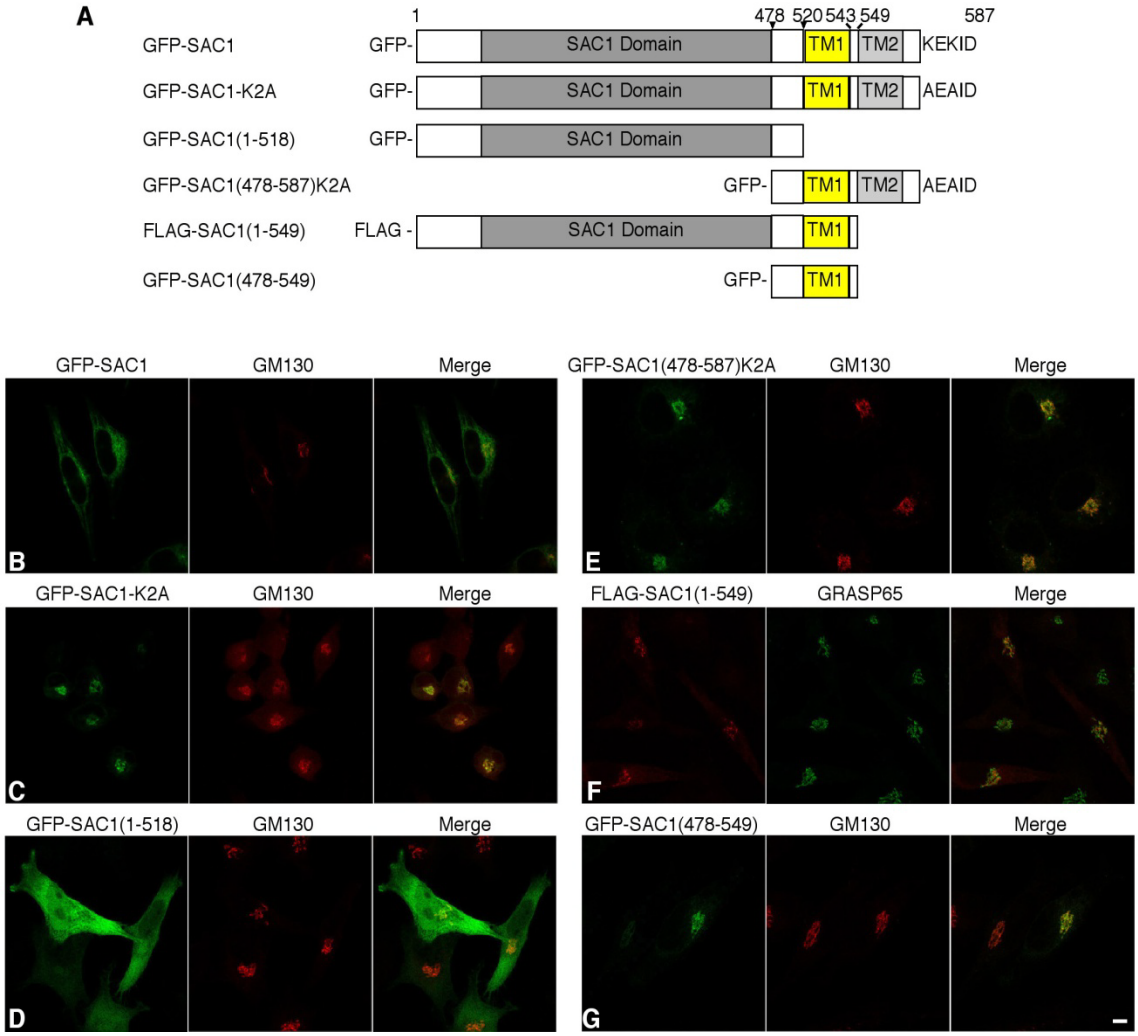


Figure 11. Mapping the Golgi retention motif in SAC1. (**A**) Overview of truncated SAC1 constructs. (**B-G**) HeLa cells were transfected with the indicated Flag-tagged SAC1 (red) or GFP- constructs (green), costained with anti-GM130 (red) or anti-GRASP65 antibodies (green) and analyzed by confocal immunofluorescence microscopy. (**B**) GFP-SAC1; (**C**) GFP-SAC1-K2A; (**D**) GFP-SAC1(1-518); (**E**) GFP-SAC1(478-587)-K2A; (**F**) Flag-SAC1(1-549); (**G**) GFP-SAC1(478-549). Scale bar, 50 µm.

Figure 12

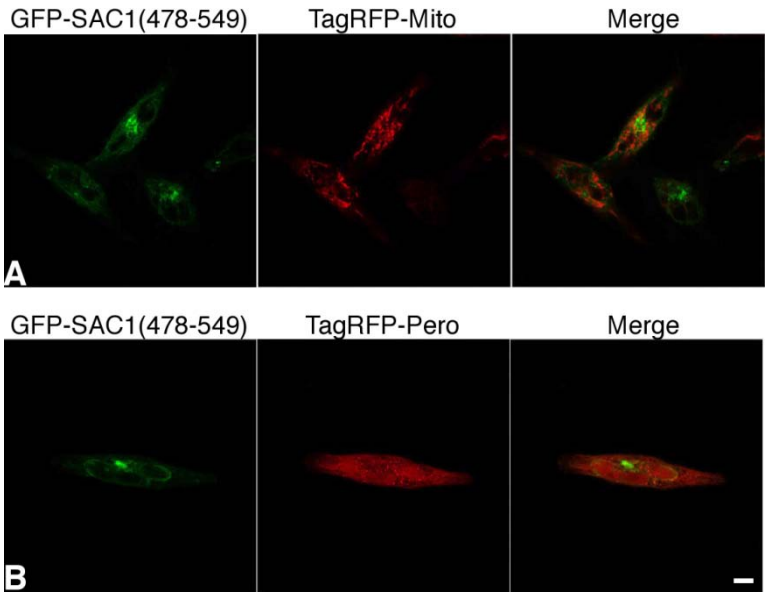


Figure 12. No mistargeting of GFP-SAC1(478-549) to mitochondrial and peroxisomal membranes. HeLa cells were cotransfected with GFP-SAC1(478-549) (green) and pTagRFP-Mito (red) to visualize mitochondria (**A**) or pTagRFP-Pero (red) to visualize peroxisomes (**B**). Localization of the expressed proteins was analyzed by confocal fluorescence microscopy. Scale bar, 50 µm.

Figure 13

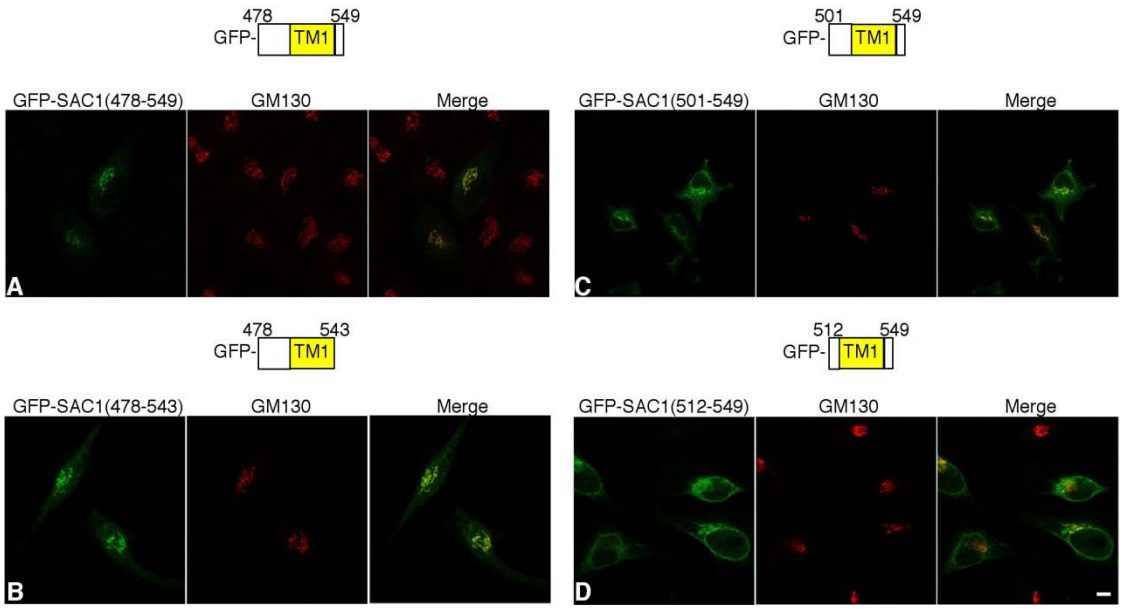


Figure 13. The cytoplasmic flanking region of TM1 is required for ER export. HeLa cells were transfected with the indicated GFP-tagged SAC1 constructs (green), costained with anti-GM130 antibodies (red) and analyzed by confocal immunofluorescence microscopy. (**A**) SAC1(478-549); (**B**) GFP-SAC1(478-543); (**C**) GFP-SAC1(501-549); (**D**) GFP-SAC1(512-549). Scale bar, 50 µm.

Figure 14

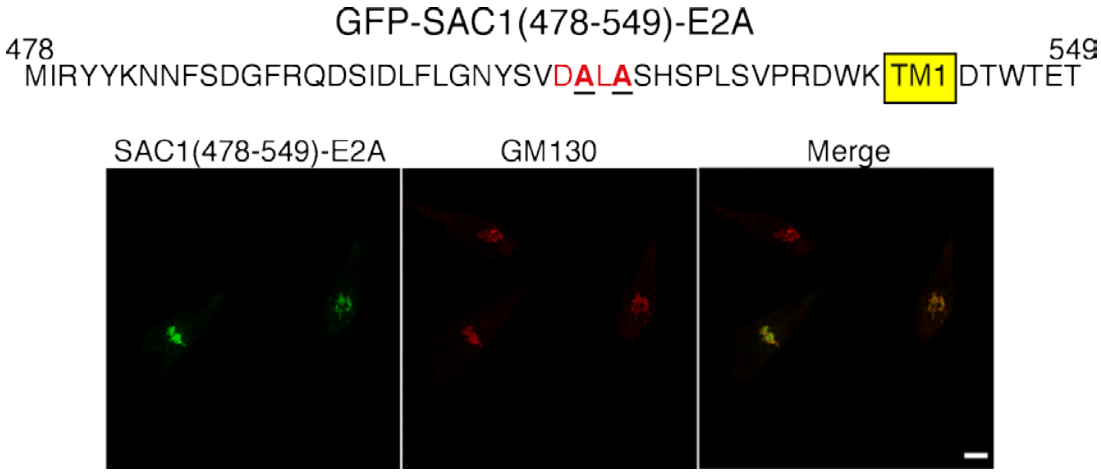


Figure 14. The 505-DELE-508 sequence adjacent to TM1 is not required for ER export. HeLa cells were transfected with GFP-SAC1(478-549)-E2A (green), costained with anti-GM130 antibodies (red) and analyzed by confocal immunofluorescence microscopy. Scale bar, 50 µm.

Figure 15

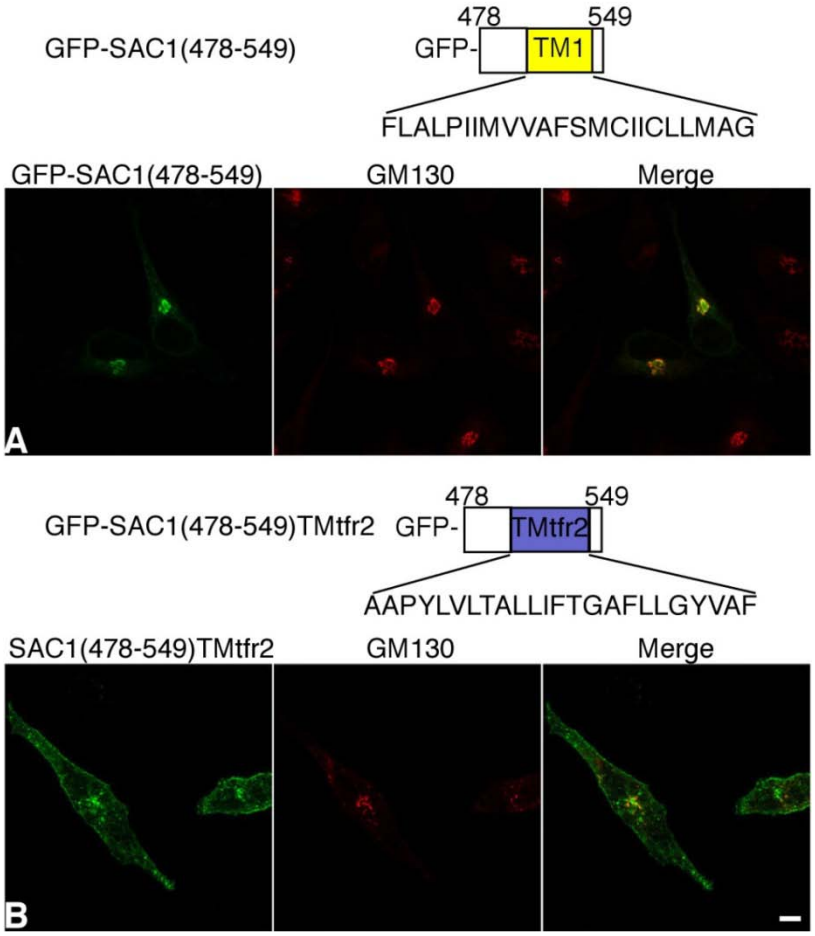


Figure 15. Replacing TM1 with the transmembrane domain of TfR2 results in loss of Golgi retention. HeLa cells were transfected with the indicated GFP-tagged SAC1 constructs (green), costained with anti-GM130 antibodies (red) and analyzed by confocal immunofluorescence microscopy. (**A**) GFP-SAC1(478-549); (**B**) SAC1(478-549)TMtfr2. Scale bar, 50 µm.

Figure 16

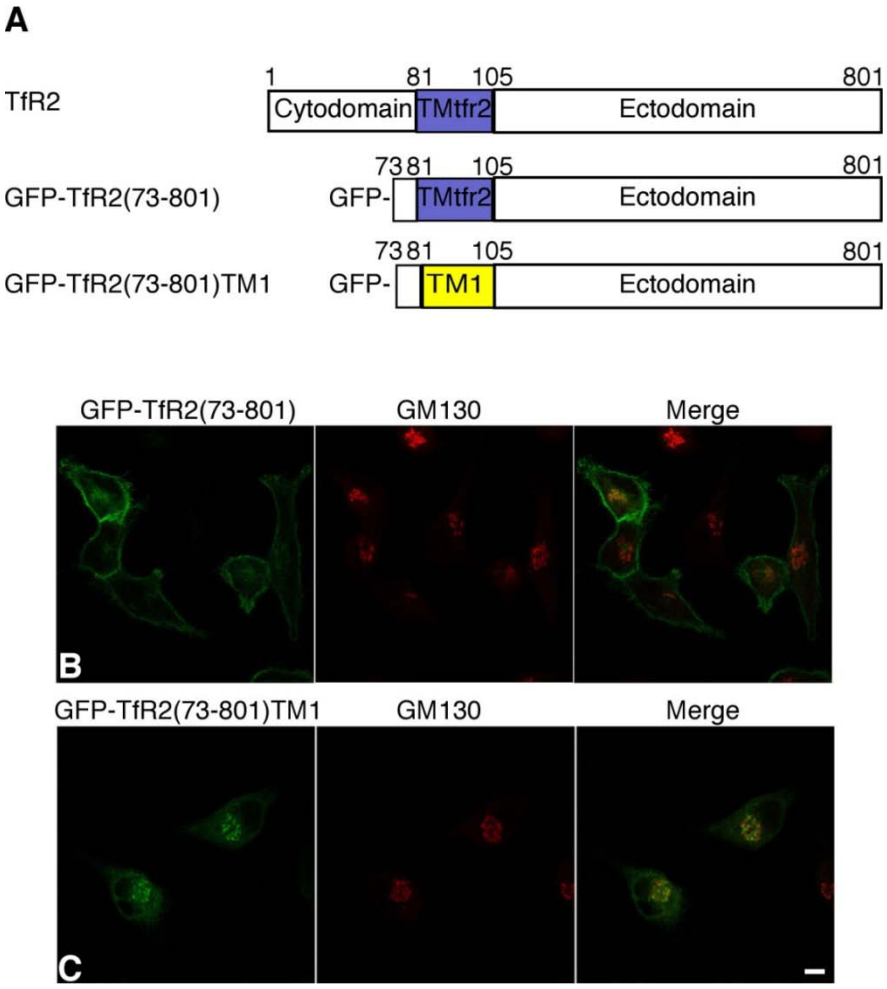


Figure 16. Insertion of TM1 induces Golgi retention of TfR2. HeLa cells were transfected with the indicated GFP-tagged TfR2 constructs that lack the N-terminal endocytic sorting motif (green), costained with anti-GM130 antibodies (red) and analyzed by confocal immunofluorescence microscopy. (**A**) GFP-TfR2(73-801); (**B**) GFP-TfR2(73-801)TM1. Scale bar, 50 µm.

Figure 17

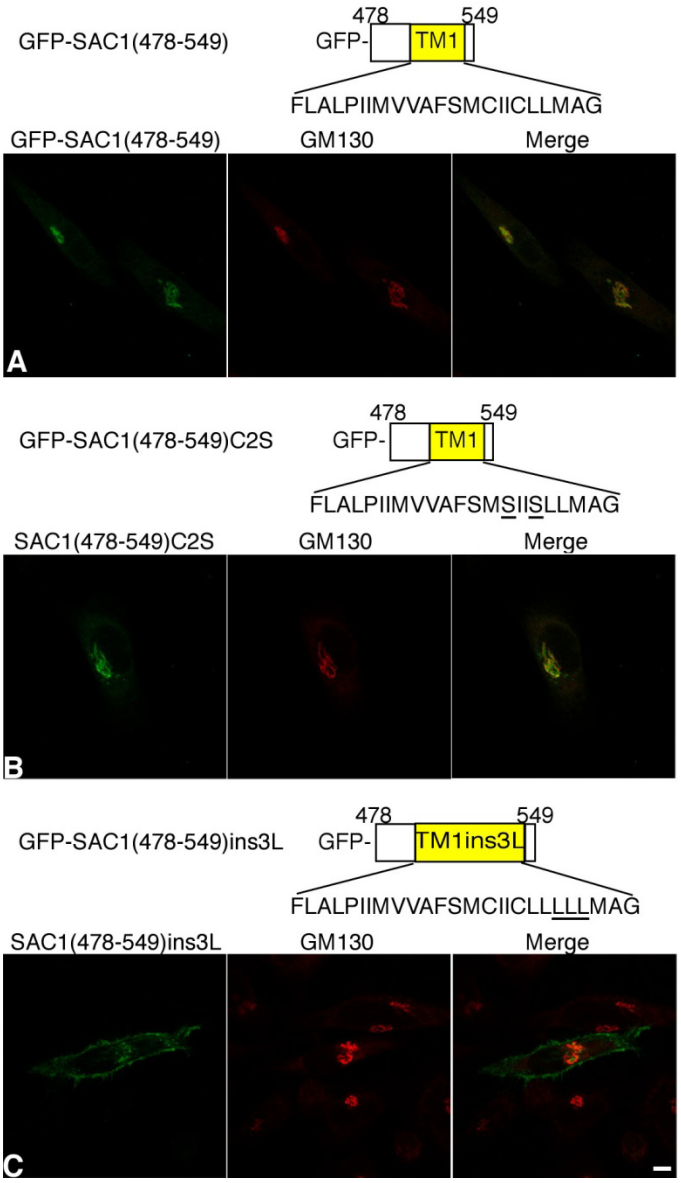


Figure 17. Lengthening TM1 by inserting three leucine residues results in a loss of Golgi retention. HeLa cells were transfected with the indicated GFP-tagged SAC1 constructs (green), costained with anti-GM130 antibodies (red) and analyzed by confocal immunofluorescence microscopy. (**A**) GFP-SAC1(478-549); (**B**) GFP-SAC1(478-549)C2S; (**C**) GFP-SAC1(478-549)ins3L. Scale bar, 50 µm.

Figure 18

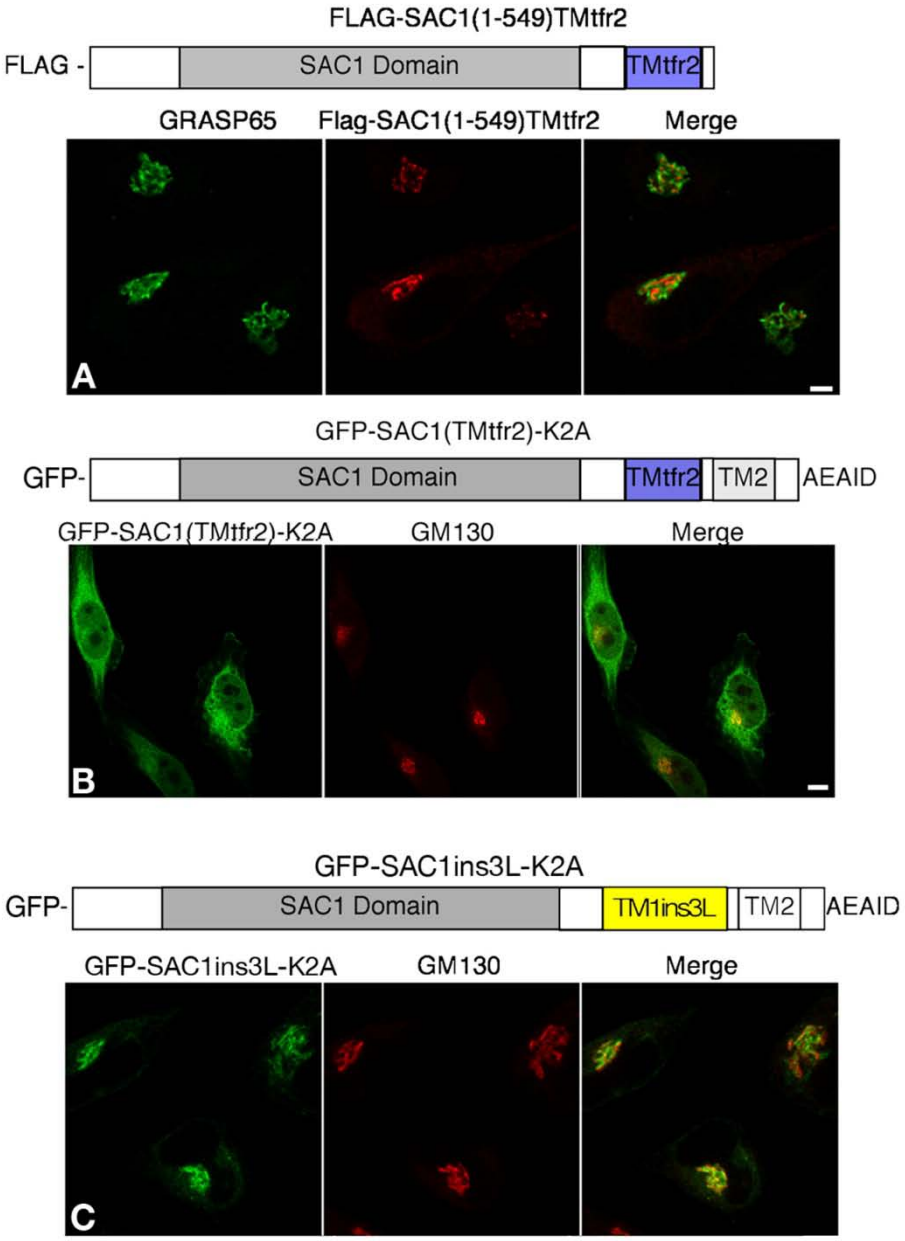


Figure 18. The N-terminal domain of SAC1 promotes Golgi retention. HeLa cells were transfected with the indicated SAC1 constructs, costained with anti-GM130 antibodies (red) and analyzed by confocal immunofluorescence microscopy. (**A**) Flag-SAC1(1-549)TMtfr2; (**B**) GFP-SAC1(TMtfr2)-K2A; *C*, GFP-SAC1ins3L-K2A. Scale bar, 50 µm.

Figure 19

A.

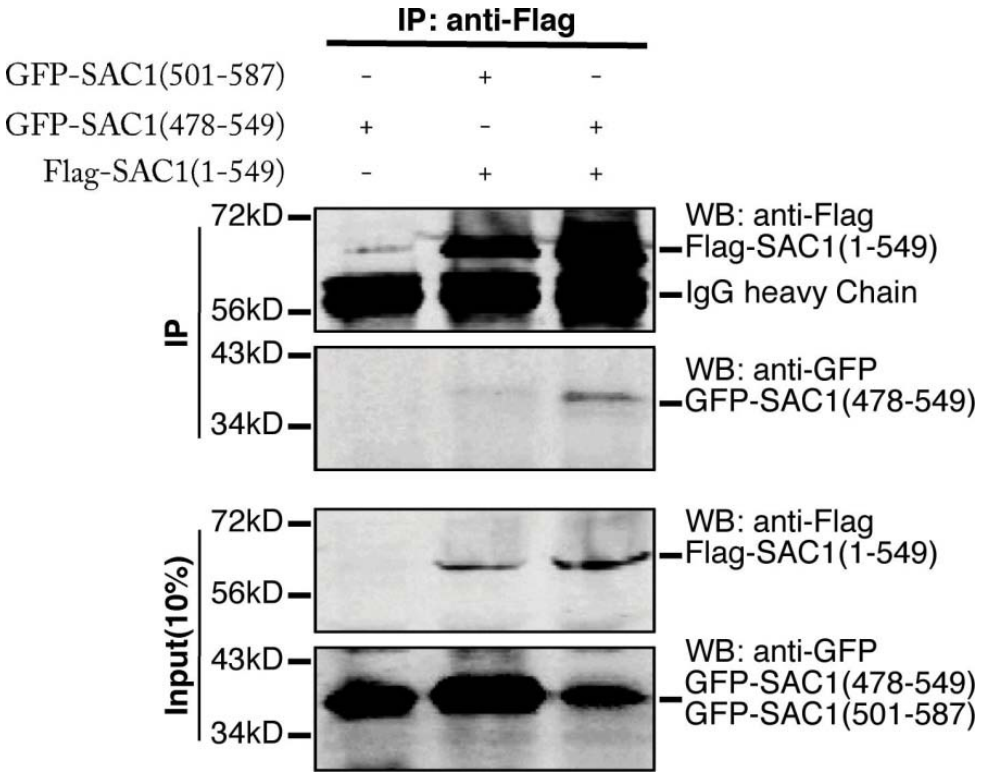
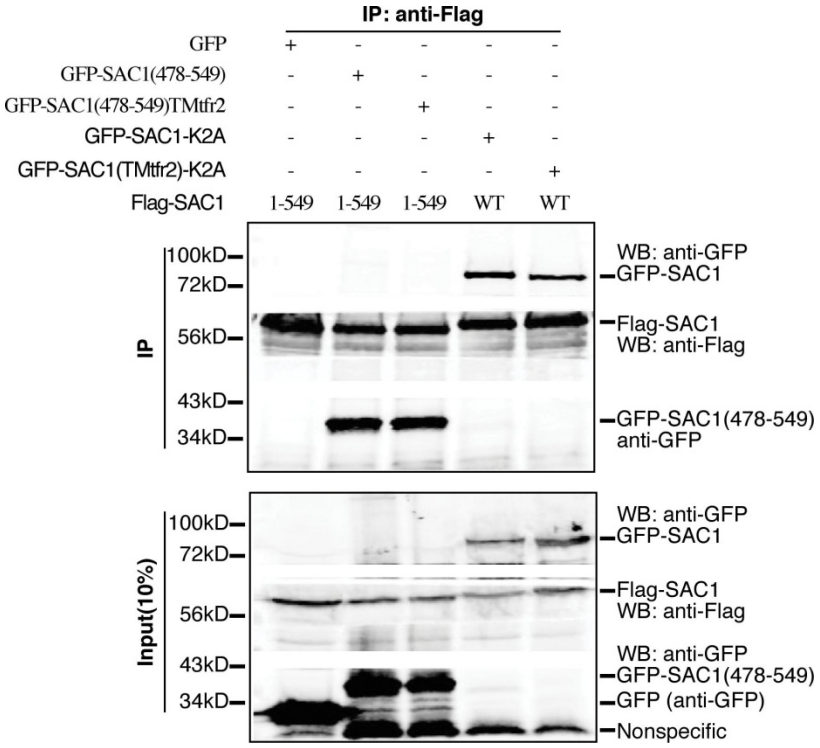

IP: anti-Flag
GFP-SAC1(501-587) - + -
GFP-SAC1(478-549) + - +
Flag-SAC1(1-549) - + +
IP
72kD
56kD
43kD
34kD
WB: anti-Flag
Flag-SAC1(1-549)
IgG heavy Chain
WB: anti-GFP
GFP-SAC1(478-549)
Input(10%)
72kD
56kD
43kD
34kD
WB: anti-Flag
Flag-SAC1(1-549)
WB: anti-GFP
GFP-SAC1(478-549)
GFP-SAC1(501-587)

Figure 19

B.


IP: anti-Flag
GFP + - - - -
GFP-SAC1(478-549) - + - - -
GFP-SAC1(478-549)TMtfr2 - - + - -
GFP-SAC1-K2A - - - + -
GFP-SAC1(TMtfr2)-K2A - - - - +
Flag-SAC1 1-549 1-549 1-549 WT WT
IP
100kD
72kD
56kD
43kD
34kD
WB: anti-GFP
GFP-SAC1
Flag-SAC1
WB: anti-Flag
GFP-SAC1(478-549)
anti-GFP
Input(10%)
100kD
72kD
56kD
43kD
34kD
WB: anti-GFP
GFP-SAC1
Flag-SAC1
WB: anti-Flag
WB: anti-GFP
GFP-SAC1(478-549)
GFP (anti-GFP)
Nonspecific

C.

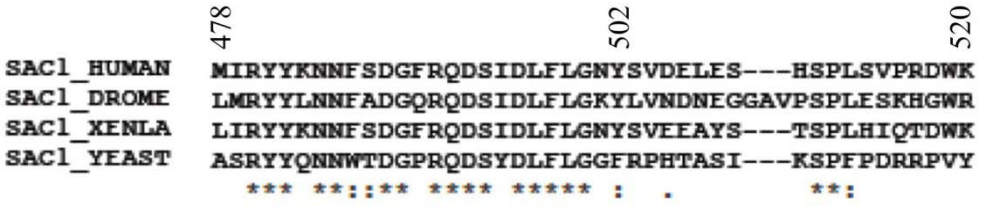

478
502
520
SAC1_HUMAN MIRYYKNNFSDGFRQDSIDLFLGNYSVDELES---HSPLSVPRDWK
SAC1_DROME LMRYYLNNFADGQRQDSIDLFLGKYLVNDNEGGAVPSPLESKHGWR
SAC1_XENLA LIRYYKNNFSDGFRQDSIDLFLGNYSVEEAYS---TSPLHIQTDWK
SAC1_YEAST ASRYYQNNWTDGPRQDSYDLFLGGFRPHTASI---KSPFPDRRPVY

Figure 19. A potential oligomerization domain is located at the adjacent region of SAC1-TM1. (A) Flag-SAC1(1-549) immunoprecipitates GFP-SAC1(478-549) but not GFP-SAC1(501-587). HeLa cells plated in 10cm plates were co-transiently transfected with the combinations of control vector pCMV-3Tag-1A and GFP-SAC1(478-549); Flag-SAC1(1-549) and GFP-SAC1(501-587); and Flag-SAC1(1-549) and GFP-SAC1(478-549) (each plasmid 3μg) plasmids as indicated, The cells were lysed and cell lysates were recovered by immunoprecipitation using an anti-flag M2 antibody. Cell lysates and the IPs were analyzed by western blotting using anti-flag M2 (1:000) and anti-GFP antibody (1:1000). (B) SAC1-TM1 is not responsible for oligomerization. HeLa cells were co-transiently transfected with the combinations of pFlag-SAC1(1-549) and vector pEGFP-C1; pFlag-SAC1(1-549) and vector pGFP-SAC1; pFlag-SAC1(1-549) and vector pGFP-SAC1(478-549)TMtfr2; pFlag-SAC1 (3μg) and pGFP-SAC1-K2A (3μg); and pFlag-SAC1 (3μg) and pGFP-SAC1(TMtfr2)-K2A (3μg) plasmids as indicated, The cells were lysed and cell lysates were recovered by immunoprecipitation using an anti-flag M2 antibody. Cell lysates and the IPs were analyzed by western blotting using anti-flag M2 (1:000) and anti-GFP antibody (1:1000). (C) Sequence alignment indicates highly homologous in the region from amino acid 478 to 501 (numbered as in human SAC1). The amino acid sequences of SAC1(478-520) adjacent to TM1 of human SAC1 protein are aligned with *Zebrafish* (DANDR), *Xenopus laevis* (XENLA), and *Yeast* sequences. The sequence alignments were generated using ClustalW2 program.

Figure 20

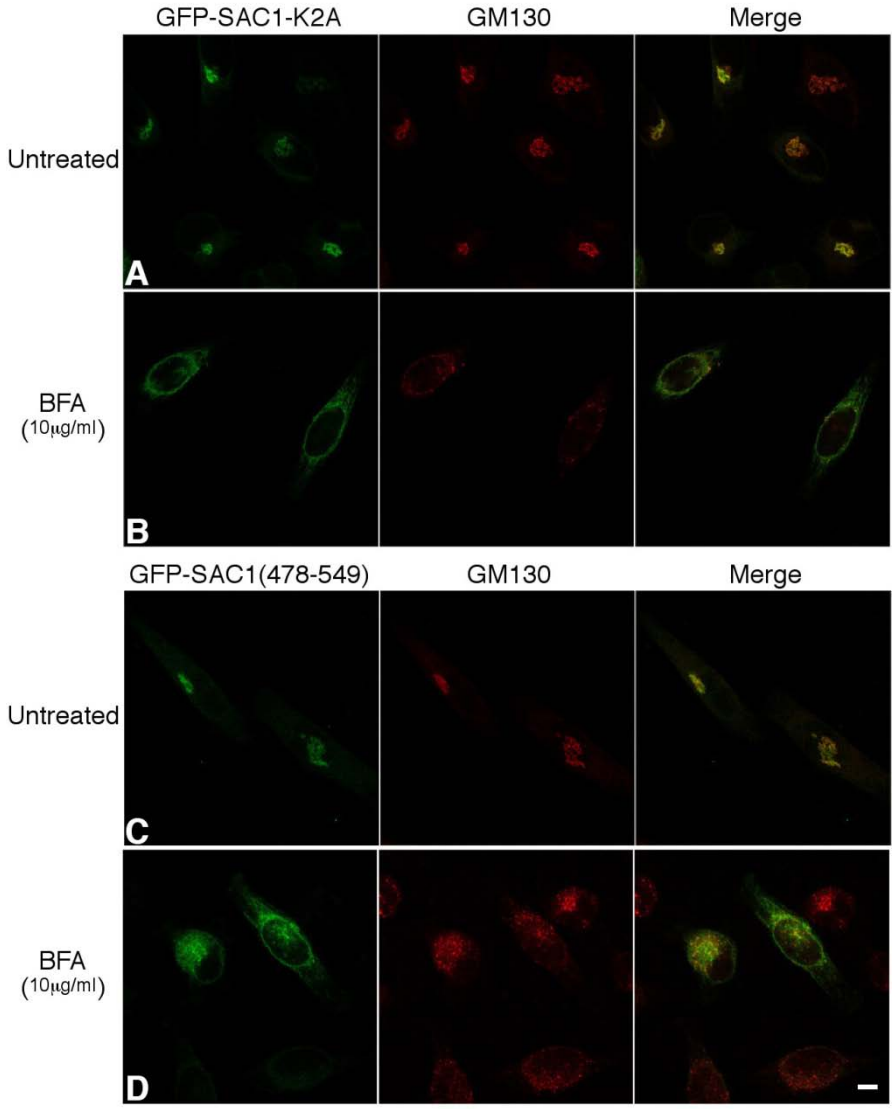


Figure 20. Brefeldin A (BFA) treatment relocates GFP-SAC1-K2A and GFP-SAC1(478-549) to the ER compartment. HeLa cells were transiently transfected with GFP-SAC1-K2A (**A** and **B**) (green) or GFP-SAC1(478-549)(**C** and **D**) (green) for 24 hours. The cells were then untreated (A and C) or treated (B and D) with 10μg/ml of BFA for 30 minutes. Cells were fixed and probed with anti-GM130 (red). Intracellular localization of constructs was analyzed by indirect immunofluorescence with GM130 as endogenous control. Scale bar, 50μm

CHAPTER THREE

14-3-3 PROTEINS REGULATE ER EXIT OF LIPID PHOSPHATASE SAC1

Jinzhi Wang† and Peter Mayinger[*1]

*Division of Nephrology & Hypertension and †The Department of Cell & Developmental Biology, Oregon Health & Science University, Portland, OR 97239

Short title: 14-3-3 interacts with lipid phosphatase SAC1

Note: this work is not published.

1. ABSTRACT

At steady state, human lipid phosphatase SAC1 (Suppressor of actin mutations 1) continuously cycles between the ER and Golgi, which is crucial for the spatial regulation of phosphatidylinositol-4-phosphate (PI4P) at both organelles. A classic KXKXX retrieval motif present at the C-terminus of SAC1 suffices to maintain ER retrieval from post ER compartments. However, it was unclear how ER exit and anterograde transport of SAC1 is regulated. Here, we show that 14-3-3 proteins bind to SAC1 through recognizing a classic mode II-like 14-3-3-binding motif (145-RLSNTSP-151) in the cytoplasmic N-terminal domain of human SAC1. A deletion mutant of SAC1, SAC1(Δ145-151), which lacks the 14-3-3-binding motif, cannot coimmunoprecipite with 14-3-3 proteins. When over-expressed, all 14-3-3 isoforms, with the exception of 14-3-3ζ, can co-immunoprecipitate with SAC1. A consensus phosphorylation site (threonine 149) in the putative 14-3-3-binding motif is not critical because the SAC1-T149A mutant that eliminates the consencus sequence does not abrogate 14-3-3 association. However, a

SAC1-S147A/T149A/S150A mutant shows much reduced interaction with 14-3-3, indicating that phosphorylation may be involved in regulating the interaction between SAC1 and 14-3-3. Moreover, the GFP-SAC1 (Δ145-151) mutant shows a greatly compromised ability to traffic out of the ER compared to wild-type GFP-SAC1. This finding suggests that binding of 14-3-3 may regulate the ER to Golgi transport of lipid phosphatase SAC1.

2. INTRODUCTION

The lipid phosphatase Sac1, an originating member of Sac phosphatase domain protein family, is an evolutionary conserved phosphatidylinositol-4-phosphate (PI4P) metabolic enzyme [76-78, 80]. Sac1 is an integral membrane protein containing a large N-terminal cytoplasmic domain and two transmembrane regions close to its C terminus with a short C-terminal domain also facing the cytosol [94]. Crystal struture of cytoplasmic N-terminal domain of yeast Sac1p reveals that the N-terminal 500 amino acid residues region contains a SacN domain, a catalytic domain and an unstructured region, but the function relevance of these domains is not known [68]. The unstructured region consisting of about 70 amino acid residues has been proposed to allow trans-action of Sac1 to regulate the PI4P on other membranes [68]. Correspondingly, Sac1 was reported to play vital roles at ER/plasma membrane contact sites by regulating the PM PI4P level [79]. At steady state, SAC1 continuously cycles between the ER and Golgi, which is important to mop up the inappropriately generated PI4P at the improper Golgi membranes, and to maintain a steep PI4P gradient in the early secretory pathway from the ER to the Golgi compartment, with PI4P mainly concentrated at the TGN [80, 91, 93]. Although the N-terminal leucine zipper

motif-mediated oligomerization has been reported to promote Golgi accumulation of human SAC1 during serum starvation [76, 80], the molecular mechanism that regulates ER to Golgi trafficking of human SAC1 remains unclear.

14-3-3 family proteins are highly conserved and ubiquitously expressed in eukaryotic cells [143]. They are small, acidic cytosolic proteins with monomeric molecular weight about 30kD [143]. 14-3-3 proteins exist in homodimers or heterodimers in nature [143]. They are seven 14-3-3 isoforms in human and mice [145]. The crystal structure of 14-3-3 isoforms revealed that each monomer of 14-3-3 dimer has a large negatively charged groove. Therefore, every 14-3-3 dimer has two binding sites [152]. Binding of 14-3-3 to ligands depends on specific phosphoserine/threonine-containing motifs in the ligand proteins, and the dimeric nature of 14-3-3 allows them to function as intramolecular and intermolecular bridges. As cytosolic adaptor proteins, 14-3-3 proteins have been suggested to take part in multiple signaling processes with many biologic consequences [152]. Several hundred of 14-3-3 interacting proteins have been identified [165]. 14-3-3 proteins regulate almost all primary biological processes such as signal transduction [166-168, 227]; apoptosis [169, 170]; transcriptional regulation [168]; metabolism [171]; cell-cycle control [172-175]. Moreover, a number of membrane protein have been found to be 14-3-3 interacting proteins, binding of 14-3-3 to them controls the surface transport of these transmembrane proteins [159, 160, 207, 228].

In this study, we showed that 14-3-3 proteins are novel human SAC1 interacting partners. We suggested that 14-3-3 binds to SAC1 through recognizing a consensus mode II-like 14-3-3 binding site (145-RLSNTSP-151) located in the SacN domain of SAC1. Deletion of this binding site in SAC1 results in failure of co-immunoprecipitation of 14-

3-3 proteins with SAC1. We hypothesize that binding of 14-3-3 may regulate ER exit of lipid phosphatase SAC1 by probing the correctly assembled SAC1 oligomers. Consistently, the 14-3-3 binding deficient SAC1 mutant showed compromised traffic out of ER compared with wild type SAC1 protein.

3. MATERIAL AND METHODS

3.1 Cell culture and antibodies

HeLa cells (ATCC number CCL-2) and COS7 cells (ATCC number CRL-1651) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, USA). Hep3B cells (ATCC Number: HB-8064) were obtained from Dr. Caroline Enns lab at Oregon Health Science University (OHSU). Hep3B and the derived stable cells Hep3B/Flag-SAC1 were cultured in the Minimum essential medium (Eagle) supplemented with 2 mM L-glutamine and Earle's Balanced Salt Solution (EBSS) adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate and 10% FBS. Anti-flag M2 agarose was obtained from Sigma (USA). Anti-flag M2 mouse monoclonal antibody was purchased from Sigma (catalog number: F1804). The polyclonal anti-GFP, N-terminal rabbit antibody was purchased from Sigma (catalog number: G1544). Mouse monoclonal anti-myc-tag antibody (9E10) was purchased from Sigma (USA) (catalog number: F1804). Mouse 12CA5 monoclonal anti-HA11 antibody was purchased from Abcam (USA) (catalog number: ab16918). Anti-14-3-3 pan antibody was purchased from Santa Cruz Biotechnology (USA) (catalog number: sc-13959). Secondary antibodies IRDye 800CW Goat (polyclonal) anti-Mouse IgG (H+L), (catalog number: 926-32210) and

IRDye 680 Conjugated Goat (polyclonal) anti-Rabbit IgG (H+L), were purchased from Rockland Immunochemicals (catalog number: 926-32221)

3.2 Transfections

Transient transfections were performed using the Lipofectamine 2000 transfection reagent (catalog number: 11668-019) (Invitrogen) according to the manufacturer's protocol. For confocal microscopy, 1.5 x 10^5 COS-7 cells were seeded into each well of a 6-well plate (Corning) containing glass coverslips and allowed to attach for 18-20 hours at 37°C. The cells were transfected with 0.5 μg of DNA mixed with 1.5 μl Lipofectamine2000 with a ratio of Lipofectamine2000/DNA=3/1 and incubated for 24 h at 37°C. For co-immunoprecipitation, HeLa or COS-7 cells were plated in the 10cm dishes and grow to 80-90% confluence sixteen hours prior to the transfection experiment. The DNA mixture of pCMV3Tag-1A-SAC1 (5μg) and each of the pMD-HA-14-3-3 isoforms (2.5μg each) were prepared with 10μl Lipofectamine 2000 at a ratio of Lipofectamine 2000/DNA=4/3 in all experiments. The overnight culture medium was removed by vacuum aspiration. 1ml of transfection mixture was then directly added into 10cm dishes. After one-hour incubation, the transfection mixture was replaced with normal culture medium (DMEM plus 10% FBS) and cells were allowed to grow for another 48 hours. Transfected cells were then processed for immunoprecipitation, immunoblotting or immunostaining as indicated.

3.3 Establishment of stable cell line Hep3B/Flag-SAC1

Hep3B cells were plated into a 35mm-dish (Corning) and allowed to attach to the surface overnight at 37°C. Next morning, transfection of Hep3B cells with pCMV3Tag-1A-SAC1 (1 μg of DNA mixed with 3 μl Lipofectamine 2000) was performed using

Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). 72 hours later, cells were selected by 400 μg/ml G418 (Geneticin) and the culture medium was replaced with fresh MEM medium supplemented with 400 μg/ml G418 every 4 days. After about 3 weeks of selection, the stable clones expressing pCMV3Tag-1A-SAC1 were recloned to obtain a pure cell line. Established clones were confirmed by gel electrophoresis and Western blot analysis with anti-flag M2 antibody. Subsequently characterized stable cells were preserved in minimal essential medium containing with 10% fetal bovine serum and 400 μg/ml G418 and stored at -80°C.

3.4 Plasmids and site-directed mutagenesis

The plasmids pEGFP-C1-SAC1 and pEGFP-C1-SAC1-K2A were described previously [80]. pcDNA3-myc-14-3-3σ was a gift from Dr. Gary Thomas lab(OHSU). Plasmids encoding HA-tagged mouse 14-3-3 isoforms were kindly provided by Dr. Vivek Bhalla at the Stanford University. There are pMD-HA-14-3-3β, pMD-HA-14-3-3γ, pMD-HA-14-3-3δ, pMD-HA-14-3-3σ, pMD-HA-14-3-3θ, pMD-HA-14-3-3ζ and pMD-HA-14-3-3ε. To construct pCMV3Tag-1A-SAC1 and pCMV3Tag-1A-SAC1-K2A, the cDNA fragments of Sac1 was amplified by PCR using pEGFP-C1-SAC1 as template. The cDNA was then digested with BamHI and SalI and subcloned into the pCMV3Tag-1A, which is a N-terminal tagging vector with three copies of Flag tags, cleaved by the same restriction enzyme sites. Deletion mutants such as pCMV3Tag-1A-SAC1(Δ145-151) and pEGFP-C1-SAC1(Δ145-151) and point-mutation mutants pCMV3Tag-1A-SAC1(S147A), pCMV3Tag-1A-SAC1(T149A), pCMV3Tag-1A-SAC1(S147A/T149A) and pCMV3Tag-1A-SAC1(S147A/T149A/S150A) were generated by site-directed mutagenesis. Primers containing the desired mutations were designed by utilizing online Quikchange Primer

Design Software (https://www.genomics.agilent.com/). QuikChange II XL Site-Directed Mutagenesis Kit was purchased from Agilent Technologies (catalog number: 200521). Experiments were performed according to manufacturer's instructions. All the constructs have been confirmed by sequencing and available upon request (see table 2).

3.5 Immunoprecipitation and Western blot

After transfection for 48 hrs, COS-7 cells were washed twice with ice-cold PBS and lysed on ice in immunoprecipitation NET-T buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.4, 1% Triton X-100 with or without 5% glycerol) supplemented with 1xComplete Protease Inhibitor Cocktail (Roche Diagnostic, Indianapolis, IN). Cell lysates were centrifuged at 13,000rpm for 30 min to remove the cell debris and the supernatant was pre-cleared with either Protein A (Zymed, San Francisco, CA) or immobilized Protein G (PIERCE, Rockford, IL) beads for 1 hour at 4°C. HA or Myc-tagged 14-3-3 proteins were immunoprecipitated using mouse monoclonal anti-HA11 or anti-myc (9E10) antibody and protein G-Sepharose (Amersham Bioscience, USA). Flag-tagged SAC1 and mutant proteins were immunoprecipitated using anti-flag M2-agarose by overnight end-over-end rotation at 4°C. Cell lysates (10% of total) and immunoprecipitates were analyzed on western blots using indicated antibodies. After centrifugation, the beads were washed four times with NET-T buffer. All the samples were boiled in SDS sample buffer for 5 minutes and resolved by 12% SDS-PAGE. Proteins were transferred to nitrocellulose or PVDF (polyvinylidene difluoride) membranes, and immuno-detected using mouse monoclonal anti-HA (1:1,000), monoclonal anti-myc (1:1,000), M2 anti-flag (1:1,000), or anti-GFP antibody (1:1000).

3.6 Indirect immunofluorescence microscopy

Transient transfections were performed using the Lipofectamine2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. For confocal microscopy, 1.5 x 10^5 COS-7 cells were plated into each well of a 6-well plate (Corning) containing glass coverslips and allowed to attach for 18-20 h at 37°C. The cells were transfected with 1 µg of DNA mixed with 3 µl Lipofectamine2000 and incubated for 24 h at 37°C. 24-hour after transfection, COS-7 cells were washed three times with PBS and fixed with 4% paraformaldehyde in PBS for 15 min. Cells were treated with blocking & permeabilization buffer (PBS, 2% normal goat serum, 0.5% Triton X-100) for 30 min. The cells were then incubated with primary antibody, anti-GM130 mouse mAb (2.5µg/ml final concentration) diluted in PBS for 1 hr. Cells were washed four times with washing buffer (PBS, 0.1% Triton X-100, 0.2% BSA) and incubated with secondary antibody Alexa Fluor 546-conjugated goat anti-mouse IgG (1:500). Cells were then washed again four times with washing buffer. Coverslips were mounted onto microscope slides using Fluoromount-G (SouthernBiotech) and visualized on a Zeiss LSM 700 laser-scanning confocal microscope with a 63× 1.4 NA Plan-Apochromat objective lens. Excitation wavelengths of 488 nm (GFP; Alexa-488) or 555 nm (Alexa-546) were used. Images were analyzed using ZEN 2009 software. The depicted images are typical representatives of cells expressing a particular construct. All localization experiments were repeated at least twice.

4. RESULTS

4.1 14-3-3σ is a new interacting partner for lipid phosphatase SAC1

By mass spectrometric analysis, we previously found that 14-3-3σ could be precipitated

with human lipid phosphatase SAC1 from COS-7 cells infected with AAV (Adeno-associated virus) expressing Flag-tagged SAC1 (data not shown). To verify this interaction, COS-7 cells were co-transfected with plasmids pEGFP-C1-SAC1 and pcDNA3-Myc-14-3-3σ. After 48 hrs, cells expressing amino-terminal Myc-tagged 14-3-3σ and GFP-tagged SAC1 were lysed and subjected to co-immunoprecipitation assays using anti-myc antibodies. Myc-14-3-3σ pulled down GFP-SAC1 whereas the controls did not recover any GFP-SAC1 protein (Figure 21), indicating that the intracellular interaction between 14-3-3 and SAC1 is specific. Taken together, these data demonstrate that full length SAC1 interacts with 14-3-3 proteins, suggesting that 14-3-3σ could be a new regulator of human lipid phosphatase SAC1.

4.2 Binding of 14-3-3σ to SAC1 via recognizing a classic mode II-like motif

The binding of 14-3-3 to its ligands usually depends on specific amino acid sequences in target proteins [148]. Two main interacting motifs are consensus mode I motif (RXXpS/pTXP) [176] and mode II motif (RXSXpS/pTXP) [158]. We employed the online MiniMotif Miner application (http://mnm.engr.uconn.edu/MNM/SMSSearchServlet) to predict short motifs in human SAC1 [229, 230]. We identified a potential 14-3-3 binding motif in human lipid phosphatase SAC1 protein at position 145 of SAC1 (145-RLSNTSP-151) (Figure 22A). This N-terminal site exhibits highly similarity to the consensus mode II 14-3-3-binding motif (RXSXpSXP). To explore whether this presumed region composed of amino acids 145-151 of SAC1 acts as a 14-3-3-binding site, we generated a deletion mutant, pcDNA3-Flag-SAC1(Δ145-151), in which the entire putative region (145-RLSNTSP-151) was removed by mutagenesis. WT (Flag-SAC1) or deletion mutant (Flag-SAC1(Δ145-151)) were co-transfected with Myc-tagged 14-3-3σ

in COS-7 cells. Cells were lysed and subjected to co-immunoprecipitation assays with anti-flag antibody. The immunoprecipitates (IP) and cell lysates were examined by western blotting with anti-flag M2 and anti-myc antibodies. Myc-14-3-3 was able to be co-immunoprecipitated with Flag-tagged wild-type (WT) SAC1 protein, but not the deletion mutant protein Flag-SAC1(Δ145-151) (Figure 22B). These data indicate that the region of amino acids 145–151 in human SAC1 is necessary for its binding to 14-3-3σ. Therefore, we suggest that 14-3-3 might bind to lipid phosphatase SAC1 through recognizing amino acid RLSNTSP sequence of SAC1.

4.3 Effects of 14-3-3 on the ER export of SAC1

14-3-3 proteins are well known to regulate ER exit of various surface expression membrane proteins such as the potassium channels KCNK3 [210] and human κ-opioid receptor [163]. These membrane proteins share an exceptional common feature that containing a 14-3-3 binding motif and an ER retention signal, often a di-arginine motif [159]. Binding of 14-3-3 was proposed to allow efficient surface expression of these membrane proteins by probing the multimeric assembly [204] and modulating ER exit by masking the ER retention signal [159, 160]. Of interest, lipid phosphatase SAC1 possesses a 14-3-3 binding motif in its cytoplasmic N-terminus and contains a canonical di-lysine ER retrieval/retention signal in its far C-terminus. At steady state, SAC1 constantly shuttles between the ER and cis-Golgi compartments. The canonical di-lysine motif at the C-terminus of Sac1 is vital for COP-I binding and retrograde traffic to the ER [76, 80]. We therefore speculated that 14-3-3 binding to SAC1 might interfere with the classic KXKXX COP-I binding motif (583-KEKID-587 in SAC1) and regulate anterograde ER to Golgi transport of SAC1. To examine this hypothesis, we generated GFP-SAC1(Δ145-

151) mutant, which is a GFP-tagged version of SAC1 with the 14-3-3-binding motif removed. COS-7 cells were transfected with plasmids expressing either GFP-SAC1 or GFP-SAC1(Δ145-151) and treated with or without serum. When we analyzed the subcellular localization of this mutant by immunofluorescent microscopy, we found that GFP-SAC1(Δ145-151) displayed much less colocalization with cis-Golgi marker GM130 than wild type GFP-SAC1 (Compare Figure 23A, C with Figure 23B, D). Even under serum starvation conditions, which have been reported to trigger Golgi accumulation of SAC1, we did not find significantly high level of localization of SAC1(Δ145-151) at the Golgi compared to wild type SAC1 (Figure 23C and D). The compromised ER exit of GFP-SAC1(Δ145-151) suggested that 14-3-3 proteins might play a role in anterograde transport of lipid phosphatase SAC1 from the ER to Golgi.

However, ER confined localization of GFP-SAC1(Δ145-151) could simply due to misfolding of this deletion mutant protein. Dr. Blagoveshchenskaya in our laboratory have examined the phosphatase activity of mutant Flag-SAC1(Δ145-151) and found that Flag-SAC1(Δ145-151) mutant is enzymatically inactive compared to the WT(Flag-SAC1) (data not shown), raising a high chance of misfolding of this deletion mutant, but only when we excluded the possibility that 14-3-3 was not required for regulating the phosphatase activity of lipid phosphatase SAC1. Misfolding of this deletion mutant SAC1(Δ145-151) may also void our claim that 145-RLSNTSP-151 is the region involving in 14-3-3-SAC1 interaction. Therefore, cautions should be taken here and further studies should be performed to examine whether this mutant is misfolded or not.

4.4 Isoform-specific binding of 14-3-3 to human SAC1

The mammalian 14-3-3 proteins are highly acidic and ubiquitously expressed proteins [148,

[151]. There are seven mammalian 14-3-3 isoforms (β, γ, ε, σ, ζ, η and θ) in human and mice [231]. In many cases, the 14-3-3 ligands show certain preference for particular isoforms of 14-3-3 [203-205, 232]. To determine whether SAC1 interacts preferentially with a specific 14-3-3 isoform, we carried out co-immunoprecipitation analysis using the established stable cell line of Hep3B/Flag-SAC1, which constitutively expresses a Flag-tagged SAC1 (Figure 24A). Hep3B/Flag-SAC1 cells were transfected with plasmids expressing specific 14-3-3 isoforms. Protein complexes were precipitated with anti-flag M2 antibody. Western blot analysis revealed that SAC1 associates with all isoforms but only very weakly with the ζ isoform. However, although repeated pull-downs showed variable binding of 14-3-3 isoforms, the γ isoforms consistently bound very strongly to SAC1 (Figure 24B), indicating a possible preference of SAC1 for interacting with this isoform.

4.5 S147A/T149A/S150A mutant shows greatly reduced interaction with 14-3-3

The principal function of 14-3-3 is to bind phosphoserine/threonine-containing motifs such as the consensus mode I motif (RSXpSXP), mode II (RXSXpSXP) and mode III SWTY motif [148, 204]. Next, we examined whether 14-3-3 binding to SAC1 is phosphorylation-dependent. Inspection of the amino acid sequence RLSNTSP of SAC1 revealed that there are three potential serine/threonine phosphorylation sites (S147, T149 and S150) in that region.

Initially, we generated two mutants by site-directed mutagenesis, pCMV3Tag-1A-SAC1(T149A) in which threonine T149 was changed to alanine, and pCMV3Tag-1A-SAC1(S147A) in which serine S147 was exchanged to alanine. We simultaneously constructed a Golgi-localized pCMV3Tag-1A-SAC1-K2A mutant, in which the two

lysines in the C-terminal retrieval motif were replaced by alanines. COS-7 cells were transiently cotransfected with plasmid pMD-HA-14-3-3γ and empty vector, Flag-tagged SAC1 or Flag-tagged SAC1-K2A. Cells were subjected to co-immunoprecipitation assays with anti-flag M2 antibody and analyzed by western blot analysis. The results showed that both the single alanine substitution mutants SAC1(T149A) and SAC1(S147A) precipitated the same level of 14-3-3 protein as the wild type Flag-SAC1 and the K2A mutant (Figure 25A). These results showed that the single alanine replacement does not abolish the association of 14-3-3 with SAC1.

Secondly, we generated a double alanine substitution mutant pCMV3Tag-1A-SAC1(S147A/T149A) in which 145-RLSNTSP-151 was mutated to 145-RLANASP-151. However, co-immunoprecipitation results showed that pulldown of 14-3-3γ displayed no significant reduction in copurifying the S147A/T149A mutant compared to amounts of pulled-down wild type SAC1 (Figure 25B).

Finally, we generated a triple alanine substitution mutant, pCMV3Tag-1A-SAC1(S147A/T149A/S150A), in which 145-RLSNTSP-151 was mutated to 145-RLANAAP-151. The SAC1(S147A/T149A/S150A) mutant showed greatly decreased recovery of 14-3-3 from the precipitates compared to wild type SAC1 (Figure 25C). These results suggest that binding of 14-3-3 to SAC1 may be regulated by multiple phosphorylations in the mode II-like 14-3-3 binding motif. Single phosphorylated serine or threonine in this region may be sufficient to recruit 14-3-3 proteins.

5. DISCUSSION

Lipid phosphatase SAC1 (Suppressor of actin mutations 1) continuously shuttles between

the ER and Golgi, which are crucial for the spatial regulation of phosphatidylinositol-4-phosphate (PI4P) at both organelles [7, 80]. At steady state, human SAC1 is predominantly localized at the ER, which is achieved by a classic KXKXX retrieval motif present in the C-terminal of SAC1 [76, 80]. COP-I coatomer has been demonstrated to directly bind to this di-lysine motif (584-KEKID-587) in SAC1, which is sufficient to maintain ER retrieval of SAC1 from post ER compartments [76, 80]. However, it was unknown how ER exit and forward transport of SAC1 is coordinated. The mammalian 14-3-3 family proteins are highly conserved and ubiquitously expressed in eukaryotic cells [233]. 14-3-3 proteins interact with various proteins and regulate a wide range of biological functions [148, 234]. Here, we found that 14-3-3 proteins interact with human SAC1. We investigated the molecular basics on how 14-3-3 proteins bind to SAC1 and suggested a possible role for 14-3-3 in regulating anterograde ER to Golgi trafficking of human SAC1.

5.1 The molecular basis of SAC1 and 14-3-3 interactions

Relying on online motif-prediction software, we have identified a potential 14-3-3 binding site (145-RLSNTSP-151) in human SAC1. Based on the known crystal structure of yeast Sac1p, this motif is located at the cytoplasmic SacN domain [68]. We showed that removal of this short region of amino acids in SAC1 abolishes its association with 14-3-3. We therefore suggest that 14-3-3 binding to SAC1 occurs through recognizing this motif (145-RLSNTSP-151). However, we have not ruled out whether this deletion mutant is correctly folded or not. Sequence specific peptides from 14-3-3 binding ligands have been proved to competitively disrupt 14-3-3 protein-ligand interactions [154, 158, 176]. To further understand the 14-3-3-SAC1 interaction, we have tried to express and purify recombinant GST-tagged or His-tagged SAC1 and 14-3-3 proteins from bacterial

BL21(DE3) so that we could utilize the *in vitro* peptide competition assay to further characterize whether this is the site in SAC1 for 14-3-3-SAC1 interaction or not. Unfortunately, only recombinant 14-3-3 proteins have been successfully expressed and purified (see Appendices Figure 26). Recombinant SAC1 proteins either completely form inclusion bodies or could not be expressed in E.coli (see Appendices Figure 27). Therefore, it is not feasible to use this strategy to examine the interaction of 14-3-3 with SAC1 *in vitro*.

Although the highly conserved structure and shared identity at the amino acid level of the seven human 14-3-3 isoforms suggest their redundant functions [152], certain 14-3-3 isoform-specific interactions with their ligands have been reported [203, 204]. Here we found that, with the exception of 14-3-3ζ, all 14-3-3 isoforms interact with human SAC1 but 14-3-3γ shows consistently high level of recovery from the coimmunoprecipitation with SAC1. C-terminal amino acid residues of 14-3-3 isoforms are not as conserved as the region that forms the peptide binding groove [152]. They are believed to be the most variable region among the 14-3-3 isoforms [152]. C-terminal stretch of 14-3-3 was reported as an auto-inhibitory domain by occupying the ligand-binding groove and has been suggested playing a critical role in the regulation of 14-3-3-ligand binding [235, 236]. 14-3-3ζ displays a slightly structural difference with a distinct extended C-terminus from other 14-3-3 isoforms (see Figure 10). We have no clue whether this slightly structural difference of 14-3-3ζ accounts for the much lower level of pulldown of this isoform by human SAC1 or not.

It is generally known that interactions between 14-3-3 proteins and their ligands involve in phosphorylation of classic 14-3-3 binding motifs [148]. The primary function of

14-3-3 proteins is to bind phospho-serine/threonine-containing motifs in a sequence-specific manner, such as the well-characterized consensus mode I motif (RSXpSXP), mode II (RXSXpSXP) and mode III SWTY motif [148, 204]. The amino acid sequence of RLSNTSP in SAC1 closely resembles the consensus mode II 14-3-3 binding motif. Examination of the amino acid sequence RLSNTSP further identified three potential phosphoserine/threonine sites in the 14-3-3-binding motif of SAC1. Among them, by prediction, Ser-147 (S147) is a potential Casein Kinase I (CKI) and/or PKA (protein kinase A) consensus phosphorylation site; Thr-149 (T149) is a potential Casein kinase II (CKII) consensus phosphorylation site; Ser-150 (S150) is a potential p38 MAPK phosphorylation site. The putative motif RLSNTSP bears high resemblance to the canonical mode II 14-3-3 binding motif, yet mutating the likely core phosphorylation sites T149 and S147 in this motif did not affect interaction with 14-3-3 proteins. However, elimination of all three phosphorylation sites in the RLSNTSP motif showed greatly reduced recovery of 14-3-3 from the immunoprecipitates. These results imply that binding of 14-3-3 to SAC1 may be regulated by multiple phosphorylations in the mode II-like 14-3-3 binding motif. One of phosphorylated serine or threonine in this region is sufficient to allow binding of 14-3-3 to human SAC1. To date, there is no evidence showing that human SAC1 is phosphorylated at these sites. Therefore, how 14-3-3 proteins binding to SAC1 is regulated awaits further studies.

5.2 The effect of 14-3-3 binding to SAC1

14-3-3 proteins have been implicated in regulating ER exit of various plasma membrane proteins such as the potassium channel KCNK3 [210, 211] and human κ-opioid receptor [163]. These membrane proteins share the common feature of containing a 14-3-3 binding motif

and possessing an ER retention signal. Binding of 14-3-3 was proposed to allow efficient surface expression of these membrane proteins by probing the multimeric assembly, modulating ER exit or by masking the ER retention signal [159, 181, 207, 228]. The 14-3-3 binding motif in SAC1 is situated near its cytoplasmic N-terminus whereas a canonical di-lysine ER retention signals located at its C-terminus. This arrangement of 14-3-3 binding motif and COP-I retention motif in SAC1 is very similar to that of potassium channel KCNK3, in which 14-3-3 binding motif and COP-I retention motif are placed at C-terminus and N-terminus respectively [210]. At steady state, SAC1 constantly shuttles between ER and cis-Golgi compartments. The canonical di-lysine motif at the C-terminus of Sac1 is vital for coatomer complex-I (COP-I)-binding and returning to the ER. Serum starvation has been reported to provoke accumulation of SAC1 at the Golgi. We therefore speculate that 14-3-3 binding to SAC1 may interfere with classic KXKXX COP-I coatomer-binding motif (KEKID in SAC1) and regulate anterograde ER to Golgi transport of SAC1. Examination of subcellular localization of SAC1(Δ145-151) mutant by confocal microscopy revealed that even serum starvations couldn't trigger this mutant out of the ER compared with the SAC1 wild type. We suggest that, like other membrane proteins that have a 14-3-3 binding site and an ER retention signal, 14-3-3 binding to SAC1 regulates anterograde trafficking of SAC1 from the ER to the Golgi.

In summary, we discovered that 14-3-3 proteins are novel interacting partners of human lipid phosphatase SAC1. We present that 14-3-3 binding to SAC1 occurs likely via a classic mode II-like motif (145-RLSNTSP-151) at the cytoplasmic N-terminus in SAC1. Isoform-specific interaction analysis indicated that most isoforms with exception of 14-3-3ζ bind to human SAC1. Regulation of 14-3-3 binding to SAC1 may be

controlled by multiple phosphorylations as only substitution of all potential phsophorylation sites within the mode II-like motif resulted in a strong reduction in 14-3-3 binding. Fluorescence microscopy indicated that the SAC1(Δ145-151) mutant displays greatly compromised trafficking out of the ER, which suggests that 14-3-3 might regulate anterograde ER to Golgi trafficking of lipid phosphatase SAC1.

6. FIGURES AND FIGURE LEGENDS

Figure 21

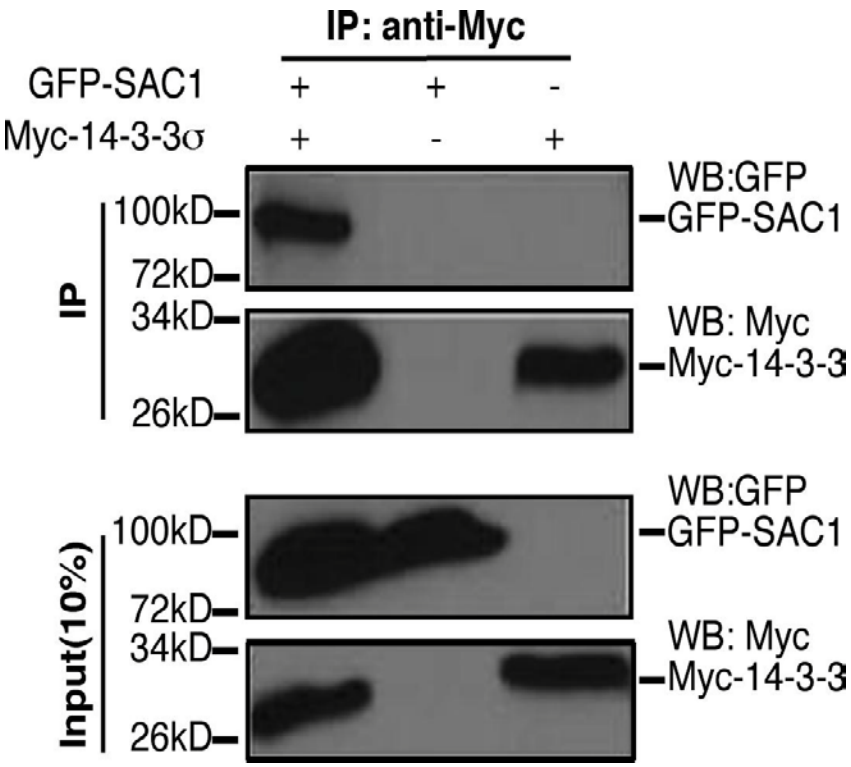
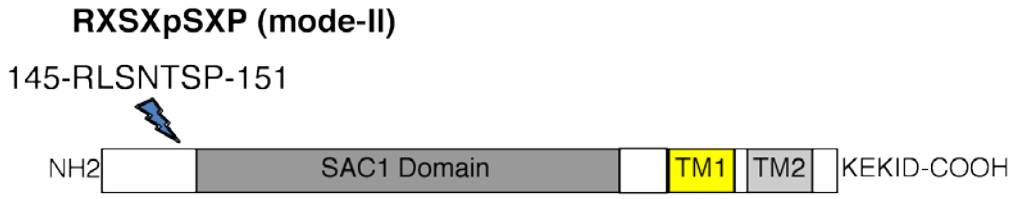


Figure 21. 14-3-3σ is a novel human SAC1 binding protein. COS-7 cells were transiently co-transfected with 2.5μg of pcDNA3-Myc-14-3-3σ and 5μg of pEGFP-C1-SAC1. The cells were lysed and subjected to Co-IP assays. Myc-14-3-3σ in the cell lysates was recovered by immunoprecipitation with the anti-myc (9E10). Cell lysates and the IPs were analyzed by western blotting with anti-myc (1:1000) and the anti-GFP antibody (1:1000).

Figure 22

A.



B.

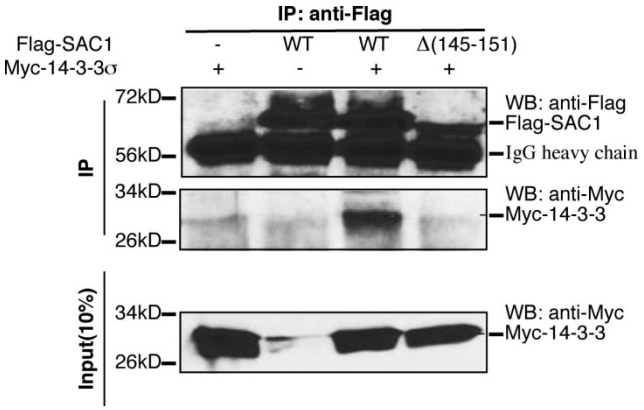


Figure 22. 14-3-3σ associates with human SAC1 via a classic mode 2-like motif. (A) A schematic representation of human lipid phosphatase SAC1 with two transmembrane domains at its C-terminus. SAC1 domain: SAC1 phosphatase catalytic domain. The arrow indicates location of the putative 14-3-3 binding site (145-RLSNTSP-151) in SAC1. (B) Deletion of amino acid RLSNTSP sequence in SAC1 abolishes binding of 14-3-3 to SAC1. COS-7 cells were co-transfected with plasmid pCMV3Tag-1A-SAC1, which expresses Flag-tagged SAC1, and plasmid pcDNA3-Myc-14-3-3σ, which expresses Myc-tagged 14-3-3σ. After 48 hrs, the pre-cleared cell lysates were incubated with anti-flag M2 antibody overnight and purified using protein G sepharose. The proteins precipitated by anti-flag M2 antibody were subjected to SDS-PAGE and Western blot analysis.

Figure 23

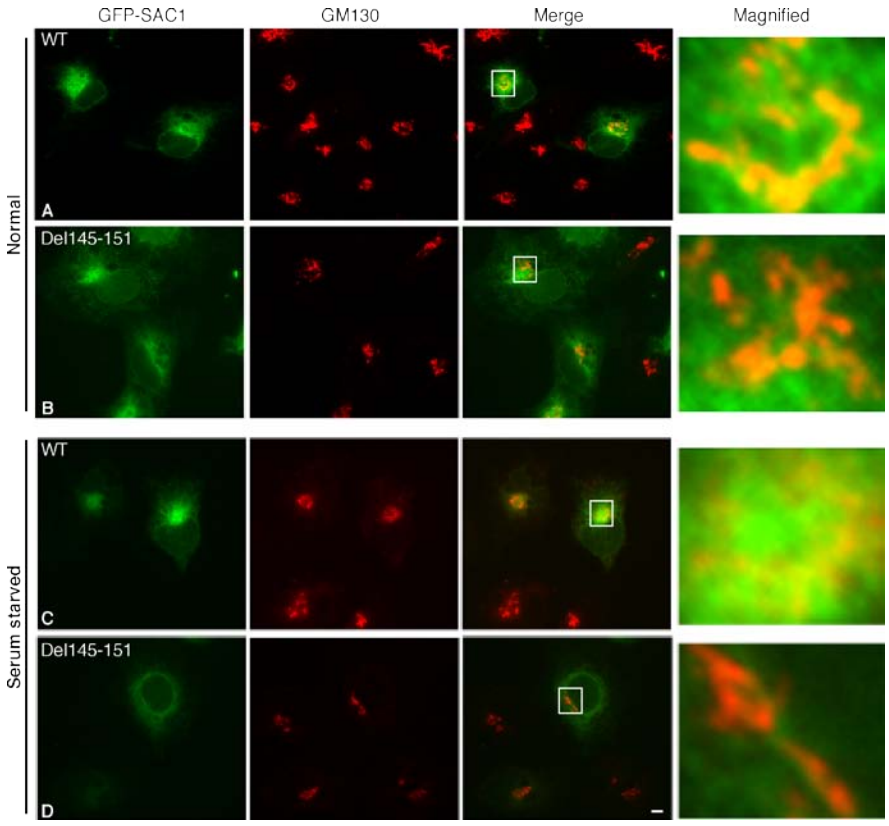
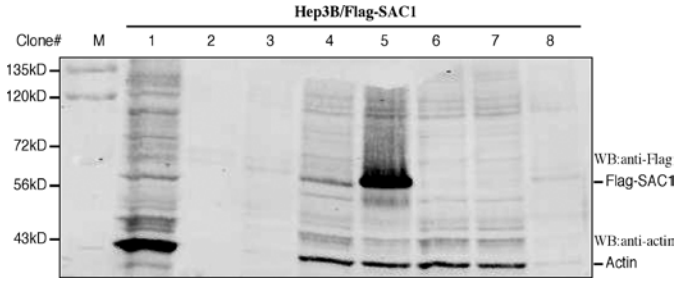


Figure 23. Effects of 14-3-3 on the ER export of SAC1. COS-7 cells were transiently transfected with plasmids pEGFP-SAC1 (A and C) and pEGFP-SAC1(Δ145-151) (B and D). After 24 hrs the cells were subjected to culture media containing 10% FBS serum (A and B) or to serum-depleted medium (0.2% FBS)(C and D) for 48 hrs. The cells were then fixed and co-stained with anti-GM130 antibody (red). Subcellular localization of wild type (WT) GFP-SAC1 (green) and deletion mutant GFP-SAC1(Δ145-151) (green) was analyzed by confocal immunofluorescence microscopy. Scale bar, 50μm. Magnification (x180) Note: del145-151 means Δ145-151 in this figure.

Figure 24

A.


Hep3B/Flag-SAC1
Clone#
M
1
2
3
4
5
6
7
8
135kD
120kD
72kD
56kD
43kD
WB:anti-Flag
Flag-SAC1
WB:anti-actin
Actin

B.

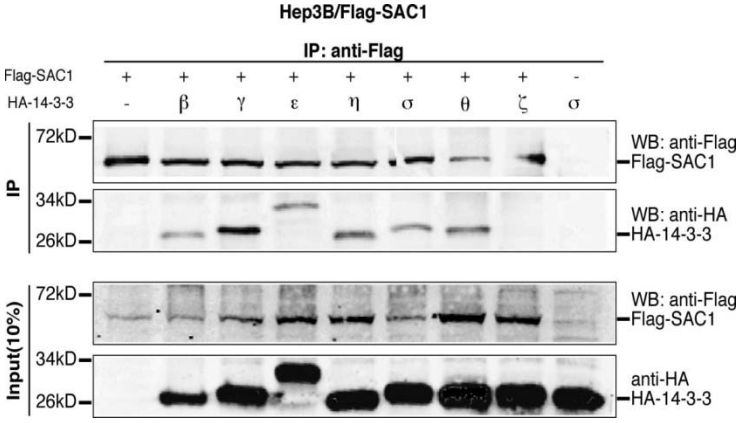
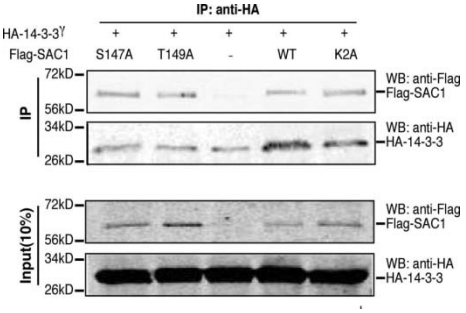

Hep3B/Flag-SAC1
IP: anti-Flag
Flag-SAC1
HA-14-3-3
β
γ
ε
η
σ
θ
ζ
IP
72kD
34kD
26kD
WB: anti-Flag
Flag-SAC1
WB: anti-HA
HA-14-3-3
Input(10%)
anti-HA

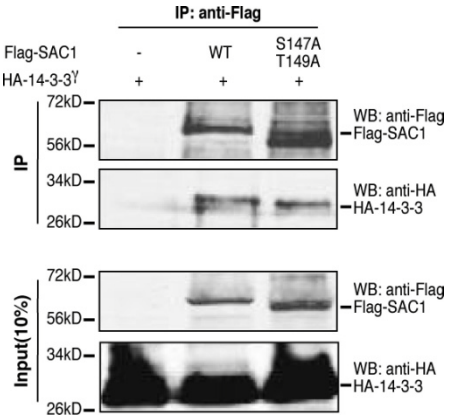
Figure 24. Interaction of 14-3-3 isoforms with human SAC1. (A) Establishment of Hep3B/Flag-SAC1 stable cell line. Hep3B cells were transfected with pCMV3Tag-1A-SAC1 (1 μg of DNA mixed with 3 μl Lipofectamine 2000) by using Lipofectamine 2000. After 72 hrs, transfected cells were selected using 400 μg/ml G418 (Geneticin). Established clones were screened by gel electrophoresis and subsequent western blot analysis. Results showed that one clone (#5) out of 8 expresses Flag-SAC1 when probed with anti-flag M2 antibody and anti-beta actin antibody (loading control). The #5 stable clone expressing Flag-SAC1 was picked and re-cloned to secure a pure cell line. Experiments for probing isoform-specific interactions of 14-3-3 with SAC1 were conducted by utilizing this stable cell line (#5); (B) All isoforms interact with SAC1 other than 14-3-3ζ isoform. Hep3B/Flag-SAC1 cells plated in 10cm plates were transiently transfected with 5μg of empty vector, pMD-HA-14-3-3β, pMD-HA-14-3-3γ, pMD-HA-14-3-3ε, pMD-HA-14-3-3η, pMD-HA-14-3-3σ, pMD-HA-14-3-3θ and pMD-HA-14-3-3ζ respectively. Meanwhile, Hep3B cells were transiently transfected with pMD-HA-14-3-3σ as a negative control. The cells were lysed and cell lysates were used for immunoprecipitation with the anti-flag M2 antibody. Cell lysates and the IPs were analyzed by western blotting analysis with anti-flag M2 (1:000) and the anti-HA11 antibody (1:1000).

Figure 25

A.



B.



C.

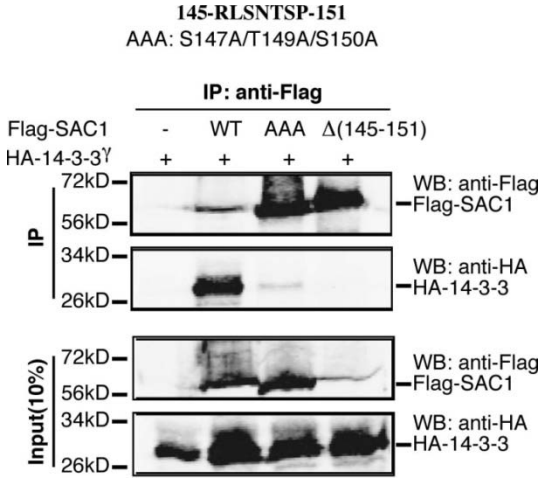


Figure 25. Phosphorylation-dependent binding of 14-3-3 to human SAC1.

(A) Single alanine substitution mutants S147A and T149A did not disrupt 14-3-3 binding to SAC1. COS-7 cells plated in 10cm plates were co-transiently transfected with pMD-HA-14-3-3γ (2.5μg) and corresponding SAC1 plasmids as indicated, The cells were lysed and cell lysates were recovered by immunoprecipitation using an anti-flag M2 antibody. Cell lysates and the IPs were analyzed by western blotting using anti-flag M2 (1:000) and anti-HA11 antibody (1:1000). (B) Double alanine substitution mutant SAC1(S147A/T149A) showed no reduction of association with 14-3-3 protein. COS-7 cells plated in 10cm plates were transiently co-transfected with plasmids as indicated combinations. Co-IP assays and subsequent western blot analysis were conducted as described above. (C) Triple alanine mutant SAC1(S147A/T149A/S150A) showed reduced binding of 14-3-3 proteins. COS-7 cells plated in 10cm plates were transiently cotransfected with plasmids as indicated. Co-IP assays and subsequent western blot were conducted as described above.

CHAPTER FOUR

FUTURE DIRECTIONS

In the chapter two, we identified two distinct regions (the first transmembrane domain (TM1) and cytoplasmic N-terminal domain) of human lipid phosphatase SAC1 are sufficient for its Golgi retention. Despite our discoveries, however, a number of questions remain unanswered regarding the exact mechanism and regulation of SAC1 retention in the Golgi. Those questions suggest the following future directions.

1) My data suggest that 43 amino acid residues (478-520) in the cytoplasmic region adjacent to the SAC1-TM1 could be potential oligomerization domain. However, the critical residues in this region that responsible for the oligomerization are unknown and whether and how this oligomerization of human SAC1 is regulated is unanswered. Also, we do not know whether oligomerization of human SAC1 occurs at the ER, Golgi, or both. Finally, we have not investigated whether oligomerization of human SAC1 affects its catalytic activity or whether SAC1 is a dimer or a multimer. Sequence alignment of the cytoplasmic region of SAC1 from amino acid 478 to 520 shows high homology among the Sac1 family proteins from yeast to human (see Figure 19C). Could this mean that this region is a universal oligomerization domain among the SAC1 family members? This will be interesting for yeast Sac1p, which has no conserved leucine zipper motif.

2) We showed that GFP-SAC1(512-549) mutant displayed the ER confined localization and could not get out of the ER when we tried to narrow down the cytoplasmic Golgi retention signal of the minimal construct GFP-SAC1(478-549), suggesting a potential ER export signal present in the adjacent region of SAC1-TM1. The sequence 505-DELE-508 in SAC1 has proved not to be an ER export signal, I

hypothesize that oligomerization of SAC1 could serve as an ER export signal in such that correctly assembled SAC1 oligomers may promote ER exit. In agreement with this hypothesis, previous studies from our laboratory have shown that yeast GFP-Sac1(Δ494-504) deletion mutant strictly localizes at the ER [46]. Does this data suggest that the region between residues 494 and 502 in the yeast Sac1p may function as an ER export signal? Consistent with the yeast experiments, my co-IP assay revealed that the human Flag-SAC1(1-549) protein could recover much more GFP-SAC1(478-549) than GFP-SAC1(501-587), suggesting that the critical region for oligomerization of SAC1 is located at 478-501, but it needs to be tested.

3) We have not yet identified the signal at the cytoplasmic N-terminal domain of SAC1 that confers sufficient Golgi retention of SAC1. According to the known Golgi retention mechanisms, oligomerization of SAC1 mediated either by leucine zipper motif or by the TM1 adjacent region (478-520) or both could contribute to Golgi retention of SAC1. Another possibility is that the recently identified PI4P binding protein GOLPH3, a homolog of yeast Vps74, may be responsible for sufficient Golgi retention of human SAC1. Yeast Vps74 has been reported to be vital for properly maintaining Golgi sub-region distribution of some glycosylation enzymes [133]. Furthermore, a yeast two-hybrid assay confirmed that Vps74 could directly interact with Sac1p via the cytoplasmic N-terminal domain and retained Sac1p at the cis-Golgi and medial-Golgi in yeast [226]. However, whether GOLPH3 interacts with human SAC1 is unknown.

4) One important question is whether steady state localization of human SAC1, especially the Golgi-targeted mutant SAC1-K2A, is located at the TGN or cis/medial/trans Golgi. In this study, I used the cis-Golgi marker GM130 and medial-

Golgi marker GRASP65. However, I did not use any TGN marker. Interestingly, brefeldin A (BFA) treatment resulted in redistribution of GFP-SAC1-K2A to the ER, suggesting that even the Golgi-targeted mutant SAC1-K2A could localize at the cis/medial/trans Golgi instead of TGN because BFA treatment normally results in TGN fusion with the endosomal system, not the ER [140]. Previous confocal immunofluorescence microscopy studies showed that SAC1 proteins do localize at the TGN because they co-localized with the TGN marker TGN46. These controversial results need to be resolved by using high-resolution electron microscopy (EM) and quantitative biochemical analysis.

5) Recent studies have shown that plus-strand RNA viruses could hijack host PI4P kinases to expand the PI4P enriched membranes at the Golgi in order to enlarge their replication platforms [37]. What happens to PI4P phosphatase SAC1 during these processes is not known. Is there simply too much PI4P for SAC1 to handle? Alternatively, could SAC1 phosphatase activity be inhibited by certain mechanism like binding of a viral protein? I will examine my hypothesis that non-structural NS5A protein of Hepatitis C virus (HCV) regulates SAC1 enzymatic activity through directly interacting with VAMP-associated protein (VAPB) protein, which has recently identified to interact with SAC1 and control phosphoinositide metabolism [79, 237, 238]. Moreover, it has been shown that depletion of the Golgi-specific PI4P pool by SAC1-K2A mutant leads to reduction in HCV secretion [239]. Based on my BFA treatment data, the Golgi-targeted SAC1-K2A localizes at the cis/medial/trans Golgi, not the TGN. Could thus be possible that to release the brake of cis/medial/trans Golgi retention for SAC1-K2A so that it can reach TGN will make it a more potent antagonist for viral secretion?

For the 14-3-3 stories described in the chapter three, there are more questions than answers. Future studies should focus on the following questions:

1) We found that deletion mutant SAC1(Δ145-151), in which the potential 14-3-3-binding motif is removed, showed compromised trafficking out of the ER compared with wild type SAC1. This ER-restricted steady state localization of SAC1(Δ145-151) could be explained in at least three ways. Firstly, the deletion of the potential 14-3-3-binding motif could affect a COP-II mediated anterograde trafficking. Secondly, the deletion could cause the speed-up of COP-I mediated retrograde retrieval. Finally, SAC1(Δ145-151) could be simply misfolded. To test all these hypotheses, the wild type SAC1 and SAC1(Δ145-151) proteins tagged with the green-to-red photoswitchable fluorescent protein Dendra2 would be instrumental. The advantage of these constructs is that they can be used to measure half-life of both the Dendra2-SAC1 and Dendra2-SAC1(Δ145-151), as well as to track the SAC1 movement.

2) Investigation of human SAC1 binding to different isoforms of 14-3-3 revealed that the 14-3-3γ isoform consistently shows higher binding affinity to human SAC1 than other isoforms. Could this mean that 14-3-3γ is the main isoform of 14-3-3 responsible for SAC1 trafficking? An experiment in which 14-3-3γ would be knocked down by siRNA *in vivo* would answer this question.

3) Compared to the wild type SAC1, the triple alanine mutant, SAC1(S147A/T149A/S150A), which lacks all potential phosphorylation sites, showed reduced association with 14-3-3. Combined with the SAC1 single or double alanine mutants tested, this result might suggest that binding of 14-3-3 to SAC1 is regulated by phosphorylation at multi-sites. Next experiments to continue with this project would

include testing the hypothesis that phosphorylation on SAC1 occurs within the mode II-like 14-3-3 binding motif (145-RLSNTSP-151). I can utilize mass spectrometry to identify SAC1 phosphorylation and isolate phosphorylated peptides from human SAC1. Once phosphorylated sites within 145-RLSNTSP-151 are confirmed, existing kinase specific phosphorylation prediction tools, such as NetPhos 2.0 [240] and Scansite [241], will be used to predict the potential kinase(s) that can phosphorylate serine (S) sites and threonine site (T) within this 14-3-3 binding motif (145-RLSNTSP-151). Then, kinase selective inhibitors or dominant negative mutants will be used to decipher what are the signaling pathways that regulate the phosphorylation of SAC1 within its 14-3-3 binding site.

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APPENDICES

Table 2. Plasmid information

Plasmids	Primer pairs (5'-3')	Cutting sites
pcDNA3-TfR2(TM1)	gcggcagcaggacggaggttcctggctttgcctattatcatggttgttgccttttcaatgt gcattatctgtttgcttatggctggtcgagggtcctgccaggcg	BamHI EcoRI
	cgcctggcaggaccctcgaccagccataagcaaacagataatgcacattgaaaagg caacaaccatgataataggcaaagccaggaacctccgtcctgctgccgc	
pCMV-3Tag-1A-TaqRFP-peroxisome	catgtcgacctattacagcttactaagtttgtgccccag	BamHI SalI
	catggatccatggtgtctaagggcgaag	
pGFP-SAC1(478-549)	catggatccatgatacgatattataag	BamHI SalI
	catgtcgacttattatgtttctgtccaagtgtc	
pGFP-SAC1(478-587)-K2A	catggatccatgatacgatattataag	BamHI SalI
	catgtcgactcagtctatcgcttctgcctggaccagtctggg	
pGFP-SAC1(501-549)	atggatccatggtgagcaagggcgag	BamHI SalI
	catgtcgacttattatgtttctgtccaagtgtc	
pGFP-SAC1(512-549)	atggatccatggtgagcaagggcgag	BamHI SalI
	catgtcgacttattatgtttctgtccaagtgtc	
pGFP-TfR2(73-801)	atgaattcaacctcattccctgggc	EcoRI SalI
	gagtcgacttatcagaagttgttatcaatg	
pGFP-SAC1(TMtfr2)-K2A	ccaagggactggaaagctgccccctacctggtcctgacggccctgctgatcttcactg gggccttcctactgggctacgtcgccttcgacacttggacagaa	BamHI SalI
	ttctgtccaagtgtcgaaggcgacgtagcccagtaggaaggccccagtgaagatcag cagggccgtcaggaccaggtagggggcagctttccagtcccttgg	
pGFP-SAC1(478-549)TMtfr2	catggatccatgatacgatattataag	BamHI SalI
	catgtcgacttattatgtttctgtccaagtgtc	
pEGFP-C2-TfR2 (73-801)TM1	atgaattcaacctcattccctgggc	EcoRI SalI
	gagtcgacttatcagaagttgttatcaatg	
pCMV-3Tag-1A-SAC1 (1-549)	catggatccgcgacggcggcctacgag	BamHI SalI
	catggatccatgatacgatattataag	
pCMV-3Tag-1A-SAC1 (1-549)TMtfr2	ccaagggactggaaagctgccccctacctggtcctgacggccctgctgatcttcactg gggccttcctactgggctacgtcgccttcgacacttggacagaa	BamHI SalI
	ttctgtccaagtgtcgaaggcgacgtagcccagtaggaaggccccagtgaagatcag cagggccgtcaggaccaggtagggggcagctttccagtcccttgg	

pGFP-SAC1 (1-518)	taagtgttccaagggactgataattcctggctttgcctaata	BamHI
	tattaggcaaagccaggaattatcagtcccttggaacactta	
pGFP-SAC1(478-549)-E2A	cttggaaactattcagtggatgcattagcatctcatagtccttaagtgtt	BamHI
	aacacttaaaggactatgagatgctaatgcatccactgaatagtttccaag	SalI
pGFP-SAC1(478-549)C2S	gttgccttttcaatgtccattatctctttgcttatggctggtgacac	BamHI
	gtgtcaccagccataagcaaagagataatggacattgaaaaggcaac	SalI
pGFP-SAC1(478-549)ins3L	catggatccatgatacgatattataag	BamHI
	catgtcgacttattatgtttctgtccaagtgtc	SalI
pGFP-SAC1-K2Ains3L	attatctgtttgcttctcctactgatggctggtgacact	BamHI
	agtgtcaccagccatcagtaggagaagcaaacagataat	
pCMV3Tag-1A-SAC1	catggatccgcgacggcggcctacgagca	BamHI
	catgtcgacttatcagtctatcttttctttc	SalI
pCMV3Tag-1A-SAC1-K2A	catggatccgcgacggcggcctacgagca	BamHI
	catgtcgactcagtctatcgcttctgcctggaccagtctggg	SalI
pCMV3Tag-1A-SAC1 (Δ145-151)	atttgacccatactttgcaggaattccaagaaatgagtct	BamHI
	agactcatttcttggaattcctgcaaagtatgggtcaaat	SalI
pEGFP-C1-SAC1 (Δ145-151)	atttgacccatactttgcaggaattccaagaaatgagtct	BamHI
	agactcatttcttggaattcctgcaaagtatgggtcaaat	
pCMV3Tag-1A-SAC1 (S147A)	ctttgcagcggctagccaacactagtcc	BamHI
	ggactagtgttggctagccgctgcaaag	SalI
pCMV3Tag-1A-SAC1 (T149A)	cagcggctatccaacgctagtcctgaattcc	BamHI
	ggaattcaggactagcgttggatagccgctg	SalI
pCMV3Tag-1A-SAC1(S147A/T149A)	ccatactttgcagcggctagacaacgctagtcctgaattccaaga	BamHI
	tcttggaattcaggactagcgttgtctagccgctgcaaagtatgg	SalI
pCMV3Tag-1A-SAC1(S147A/T149A/S150A)	gacccatactttgcagcggctagccaacgctgctcctgaattccaagaaatg	BamHI
	catttcttggaattcaggagcagcgttggctagccgctgcaaagtatgggtc	SalI
pET28a-SAC1(1-504)	catggatccgcgacggcggcctacgag	BamHI
	catgtcgacttaccactgaatagtttccaag	EcoRI
pGEX-4T-1-SAC1(1-458)	catggatccgcgacggcggcctacgag	BamHI
	catgtcgacttagtcagtcttcaaggcaccag	EcoRI
pGEX-4T-1-SAC1(1-504)	catggatccgcgacggcggcctacgag	BamHI
	catgtcgacttaccactgaatagtttccaag	EcoRI

pEGFP-C2-SAC1(152-549)TMtfr2	enzymatic digestion	EcoRI SalI
pEGFP-C2-SAC1(152-549)	enzymatic digestion	EcoRI SalI
pET28a-14-3-3episilon	enzymatic digestion	BamHI EcoRI
pGEX-4T-1-14-3-3sigma	enzymatic digestion	BamHI EcoRI

Figure 26

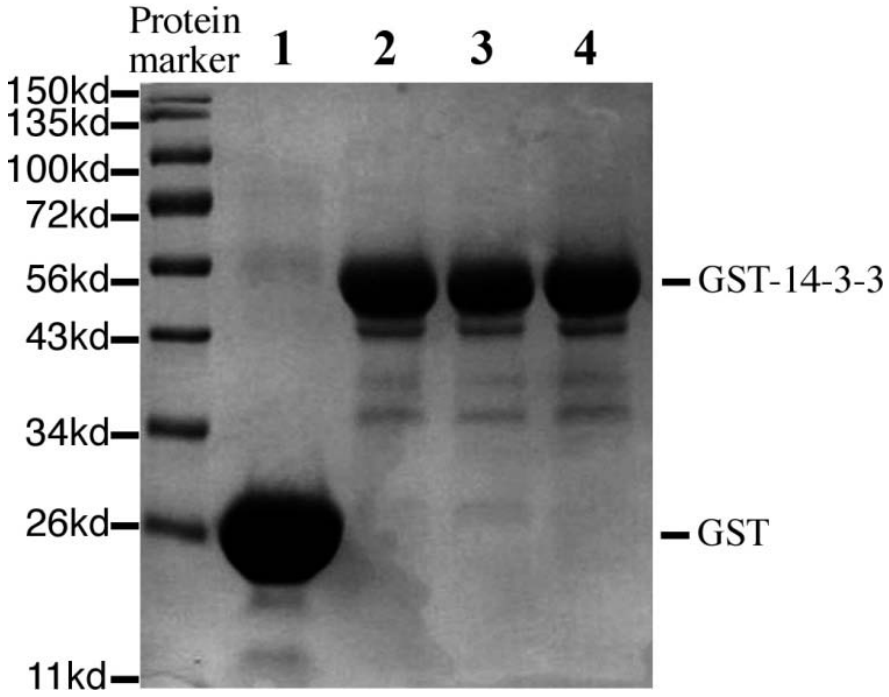

Figure 26. Expression and purification of GST and GST-14-3-3σ in E.coli.

Constructs of pGEX-4T-1 and pGEX-4T-1-14-3-3σ were transformed into bacterial BL21(DE3). Expression of recombinant proteins was induced by IPTG at 30°C for 3hrs. Cells were precipitated and lysed. Recombinant proteins were purified from the supernatent. Samples of purified proteins were resolved on SDS-PAGE gel and transferred to PVDF membrane and stained with Panceau S. 1-recombinant GST protein; 2-4, recombinant GST-14-3-3σ

Figure 27

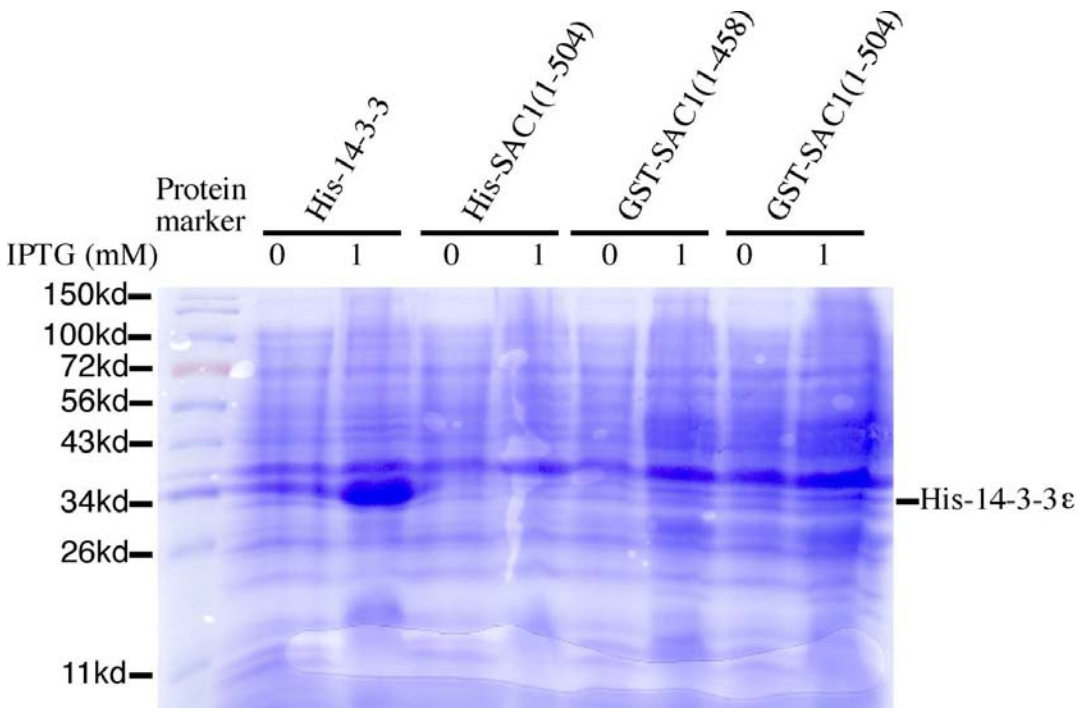

Figure 27. Expression of recombimant GST- or His-tagged SAC1 and 14-3-3 proteins in E.coli. Constructs of pET-28a-14-3-3ε, pET28a-SAC1(1-504), pGEX-4T-1-SAC1(1-458) and pGEX-4T-1-SAC1(1-504) were transformed into bacterial BL21(DE3). Expression of recombinant proteins was induced by IPTG at 30°C for 3hrs. Cells were precipitated and lysed. Recombinant proteins were purified from the supernatent. Samples of recombinant proteins from the supernatent were resolved on SDS-PAGE gel and analyzed by Coomassie staining. Only recombinant 14-3-3 proteins were successfully expressed in E.coli. No soluble recombinant SAC1 proteins can be detected.