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# Functional studies of the mammalian Sac1 phosphoinositide phosphatase

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## Introduction

PIPs are phosphorylated derivatives of phosphatidylinositol (PtdIns) that serve primary intracellular roles: (i) in the specification of dedicated membrane microdomains that organize signal transduction processes, (ii) as co-factors for the regulated activities of proteins, and (iii) as precursors for second messengers such as diacylglycerol and soluble inositol phosphates (Fruman *et al*., 1998; Martin, 1998; Waselle *et al*., 2005; Di Paolo and De Camilli, 2006). In part, this diversification of function reflects the chemical diversity afforded by the poly-hydroxylated inositol headgroup. Mammalian cells express seven distinct PIP species -- phosphatidylinositol 3-phosphate (PtdIns-3-P), PtdIns-4-P, PtdIns-5-P, phosphatidylinositol 3,5-bisphosphate (PtdIns-3,5-$P_2$), PtdIns-4,5-$P_2$, PtdIns-3,4-$P_2$, and phosphatidylinositol 3,4,5-trisphosphate (PtdIns-3,4,5-$P_3$) --each of which interfaces with specific downstream effectors. Tight regulation of each of these PIP levels is essential to cellular processes that include vesicular trafficking, apoptosis, metabolism, actin reorganization, cell proliferation and cell growth (Fruman *et al*., 1998; Martin, 1998). Yeast do not synthesize PtdIns-3,4-$P_2$ or PtdIns-3,4,5-$P_3$ and, indeed, 3-OH PIPs are nonessential for yeast viability although these play important homeostatic functions (Fruman *et al*., 1998; Martin, 1998).

PtdIns generally constitutes less than 15% of the total cellular phospholipids in eukaryotic cells and PIPs are usually less abundant in terms of mass by an order of magnitude. PtdIns-4-P and PtdIns-4,5-$P_2$ are the major PIP species in mammalian cells --representing ~90% of total PIP mass. By comparison, PtdIns-3-P and PtdIns-5-P represent only ~0.25% of total PIP mass in mammalian cells (Rameh *et al*., 1997; Fruman *et al*., 1998; Martin, 1998; Waselle *et al*., 2005; Di Paolo and De Camilli, 2006).

Individual PIP species exhibit specific signaling capabilities and exhibit specific subcellular localizations that help define organelle identity. With regard to the latter set of functions, PtdIns-3-P and derived lipid species are mainly distributed in endosomal organelles, PtdIns-4-P is presented predominantly in Golgi membranes, PtdIns-4,5-$P_2$ and PtdIns-3,4,5-$P_3$, are minor phospholipids and enriched in the inner leaflet of the plasma membrane, and PtdIns-3,4,5-$P_3$ may also accumulate on endomembranes following growth factor receptor activation. Moreover, the lateral distribution of specific PIPs within the two-dimensional space of a given membrane is also heterogeneous (Shisheva, 2001; De Matteis and Godi, 2004; Waselle *et al*., 2005; Di Paolo and De Camilli, 2006). The presence of nuclear PIPs has also

been documented, and these may fuel an autonomous nuclear soluble inositol phosphate cycle that regulates gene transcription, mRNA processing and mRNA export from the nucleus (De Matteis and Godi, 2004; Waselle *et al*., 2005; Di Paolo and De Camilli, 2006).

PIP metabolism requires a highly coordinated balance between the activities of lipid kinases that generate PIPs and the activities of lipid phosphatases and phospholipases that degrade them. PIPs are subject to robust phosphatase-mediated turnover through via dephosphorylation at the 3-OH, 4-OH and 5-OH positions of the inositol ring. PtdIns-4-P and PtdIns-4,5-$P_2$ are the major PIP species in mammalian cells, and represent 90% of total cellular phosphorylated PIPs. (Fruman *et al*., 1998; Martin, 1998; Whisstock *et al*., 2002; Waselle *et al*., 2005; Di Paolo and De Camilli, 2006). As will be discussed below, homeostasis of the 4-OH PIPs is strongly influenced by the action of Sac1 phosphatases. However, phosphatases of the Sac1 family play important roles in regulating the degradation of the 3-OH PIPs as well. Compromised activity of such phosphatases dedicated to the 3-OH arm of the PIP pathway (e.g. the tumor suppressor PTEN) has devastating consequences for higher eukaryotes (Li *et al*., 1997; Simpson and Parsons, 2001). PIP phosphatases, while still poorly studied as a group, are increasingly subjects of intense research effort – in part because of their newly recognized roles as tumor suppressors. The integral membrane protein, Sac1, is a prototypical member of a major class of such lipid phosphatases. Yeast Sac1 (ySac1) has been broadly studied and Sac1 loss of function (LOF) in yeast causes a wide array of phenotypes such as cold sensitivity, inositol auxotrophy, and “bypass Sec14p”. However, the mammalian homologs of Sac1 are not well explored. At present, mammalian cell systems and mouse knockout models provide powerful systems for analysis of the mammalian Sac1 functions *in vivo*, and the mechanisms by which these functions are executed. Herein, we review the functional involvement of Sac1 phosphatases in regulating PIP metabolism, the functional properties of ySac1, and recent progress in deciphering Sac1 function in mammals.

## Materials and Methods

The experimental procedures and reagents employed herein are described in Cleves et al. (1989), Whitters et al., 1993; Guo et al., 1999; Rivas et al., 1999 ; Kochendörfer et al. (1999), Nemoto et al. (2000), Li et al (2002), Rohde et al. (2003), and Liu et al. (2008).

## Results and Discussion

### The Yeast Sac1 PIP Phosphatase

The Sac1 phosphatases are the prime subjects of this review. These enzymes not only represent a major class of PIP phosphatases, but these have the signature feature of being integral membrane proteins (Cleves et al., 1989; Whitters et al., 1993). The first member of the Sac1 family of phosphatases was identified in yeast by two independent genetic screens searching for modifiers of actin cytoskeleton defects and of *trans*-Golgi network exocytic failure caused by inactivation of the major yeast phosphatidylinositol (PtdIns)/phosphatidylcholine (PtdCho) transfer protein, respectively (Novick *et al*., 1989). Sac1 was demonstrated to be an integral membrane protein that localized to the endoplasmic reticulum (ER) and Golgi membranes in yeast and in mammalian cells (Xie *et al*., 1998; Nemoto *et al*., 2000). It consists of a 300-amino acid catalytic domain, designated the *SAC1*-like domain, and the catalytic domain is disposed to the cytosol (Cleves et al., 1989; Whitters et al., 1993). The catalytic SAC1 domain is common to other phosphoinositide phosphatases such as PTEN (Maehama *et al*., 2001), synaptojanins (Cremona *et al*., 1999) and yeast synaptojanin-like proteins (Srinivasan et al., 1997; Stolz et al., 1998). A highly conserved $CX_5R(T/S)$ motif almost certainly represents the core catalytic motif of the Sac1 domain – given that mutations in this motif eliminate catalytic activity (Nemoto et al., 2000, Rohde et al., 2003) and this motif is a signature of metal-independent phosphatases (Hughes, 2001). Sac1 is anchored to membranes by two C-terminal

transmembrane domains such that the C-terminus of the protein is also disposed to the cytosol (Konrad *et al*., 2002).

Based on genetic data, Sac1 was proposed to negatively regulate PIP signaling (Cleves et al., 1991; Whitters et al., 1993), and this was demonstrated to be directly true when Shuling Guo in John York's laboratory demonstrated Sac1 domains are PIP phosphatase domains (Guo et al., 1999). Biochemically, ySac1 catalyzes dephosphorylation of PtdIns-3-P, PtdIns-4-P, and PtdIns-3,5-$P_2$ to PtdIns *in vitro* and *in vivo* but, interestingly, is unable to utilize PtdIns-4,5-$P_2$ as substrate in either context (Guo *et al*., 1999; Rivas *et al*., 1999; Hughes *et al*., 2000). Moreover, ySac1 represents a major pathway for PtdIns-4-P degradation *in vivo*. Genetic ablation of ySac1 activity results in a nearly 10-fold increase in the steady-state levels of PtdIns-4-P with little effect on PtdIns-4,5-$P_2$ and far more modest increases in the steady-state levels of the 3-OH PIP species (Guo *et al*., 1999; Rivas *et al*., 1999; Hughes *et al*., 2000). Paradoxically, and a point of discussion revisited below, ySac1 degrades a PtdIns-4-P pool that is produced apparently exclusively by the plasma membrane-localized Stt4 PtdIns 4-OH kinase – one of three PtdIns 4-OH kinases in this organism (Nemoto *et al*., 2000; Foti *et al*., 2001).

The single *SAC1* gene in yeast is not essential for cell viability. Rather, *sac1* nullizygous alleles (*sac1Δ*) evoke a wide array of phenotypes, such as alterations in the actin cytoskeleton, cold sensitivity for growth, a curious inositol auxotrophy independent of the ability of Sac1-deficient yeast to produce their own inositol *de novo*, a 'bypass Sec14'phenotype where Sac1-insufficient yeast are able to survive the normally lethal consequences of loss of function of the major PtdIns/PtdCho transfer protein of this organism (Sec14), compromised cell integrity at alkaline pH, and deranged neutral lipid metabolism (Cleves et al., 1989; Novick et al., 1989; Whitters et al., 1993; Boyum and Guidotti, 1997; Kearns et al., 1997; Rivas et al., 1999). Moreover, ySac1 deficiencies somehow interfere with ATP uptake into the ER lumen resulting in compromised ER protein quality control systems (Kochendorfer *et al*., 1999).

ySac1 is proposed to be retained in the yeast ER through a direct interaction of its COOH-terminal region with a very abundant ER-localized integral membrane protein dolicholphosphate-mannose synthase (Dpm1; Faulhammer *et al*., 2005). Its interaction with Dpm1 is regulated by cell growth conditions. The interaction is detected only during exponential cell division – it is apparently lost when cells are challenged with limited nutrient conditions. Under such suboptimal growth conditions, ySac1 accumulates in Golgi membranes (Faulhammer *et al*., 2005). This cell-growth controlled switch of ySac1 between the ER and the Golgi provides reciprocal control of PtdIns-4-P levels at these organelles (Faulhammer *et al*., 2005). As PtdIns-4-P helps promote anterograde transport of secretory proteins from the Golgi system to the plasma membrane (Walch-Solimena and Novick, 1999; Wang *et al*., 2003; Godi *et al*., 2004), and yeast exocytic capacity is maximized during exponential growth (Finger and Novick, 1998), such a redistribution of Sac1 may coordinate secretory capacity with larger aspects of yeast cell physiology.

## Biochemical and Cell Biological Features of Mammalian Sac1 Proteins

Homologs of the ySac1 PIP phosphatase are disseminated throughout the eukaryotic kingdom as evidenced by inspection of insect, plant, and mammalian genomes (Figure 2). Interestingly, mammalian genomes encode only a single protein that closely resembles ySac1 in its molecular architecture. As expected, the rat Sac1 (rSac1), human Sac1 (hSac1) and murine Sac1 (mSac1) all exhibit the expected PIP phosphatase activities --with PtdIns-3-P, PtdIns-4-P, and PtdIns-3,5-$P_2$ as primary substrates – and these mammalian Sac1 proteins share the $CX_5R$(T/S) catalytic motif with ySac1 (Nemoto *et al*., 2000). The mammalian proteins exhibit a molecular mass of ca. 65-kDa, similar to ySac1, are integral membrane proteins of the ER and Golgi, and exhibit the same membrane topology as ySac1 (Figure 2). Moreover, heterologous

complementation analyses demonstrate that individual expression of each of these mammalian proteins rescues the pleiotropic phenotypes that are signatures of *sac1Δ* alleles.

The intracellular distribution of the Sac1 PIP phosphatase in the ER and Golgi of mammalian cells recapitulates aspects of what is observed in yeast, but with some unanticipated twists. The first interesting aspect is that hSac1 (and other mammalian versions – see below) contain a C-terminal KXKXX motif that serves as binding site for the coatomer (COP1) complex. This COPI interaction motif is a unique feature of mammalian Sac1s as it is not conserved in yeast, plant or *Drosophila* Sac1 proteins (Figure 2). This divergence reflects the differing mechanisms for ER retention of mammalian Sac1 enzymes. Compromise of the KXKXX motif abolishes retrieval of hSac1 from the Golgi back to the ER, and provokes accumulation of hSac1 in Golgi membranes (Rohde *et al*., 2003). Interestingly, the phosphatase activity of hSac1 is also apparently required for its interaction with the COPI complex. A "catalytic-dead" hSac1-C/S mutant (where Cys289 residue of the $CX_5R(T/S)$ core catalytic motif is replaced with Ser) is incapable of interacting with the COPI complex, and is incompetent for retrieval back to the ER – an intact KXKXX motif notwithstanding (Rohde *et al*., 2003). The mechanism by which the core catalytic motif regulates interaction with COPI is unclear, and this result suggests that the phosphatase activity of hSac1 may function as a switch to adjust its interaction with the COPI complex, thereby influencing hSac1 distribution in the ER and Golgi as a function of catalytic cycle.

Why the strategic adjustment in how mammalian Sac1 is retrieved from the Golgi system back to the ER relative to the Sac1 enzymes of other eukaryotes? Blagoveshchenskaya et al. (2008) report that, in quiescent mammalian cells, hSac1 is mainly localized in the Golgi complex – perhaps to downregulate Golgi PtdIns-4-P levels. Escape to the Golgi complex from the ER is achieved via oligomerization of hSac1 and subsequent recruitment of the coat protein II (COPII) complex (Blagoveshchenskaya *et al*., 2008). In the presence of growth factors, the p38 mitogen-activated kinase (MAPK) pathway induces dissociation of Sac1 oligomers -- thereby 'activating' the KXKXX complex to render hSac1 as competent cargo for COPI-mediated retrograde traffic from the Golgi to the ER. The proposed consequence of this ER retrieval is to increase the Golgi PhtdIns-4-P pool which helps promote protein export from the Golgi to the plasma membrane (Blagoveshchenskaya *et al*., 2008). This study suggests a link between growth factor signaling and lipid signaling at the Golgi complex. Thus, although yeast and mammalian cells use distinct mechanisms to control Sac1 PIP phosphatase distribution between ER and Golgi membranes, these widely divergent organisms appear to use similar strategies for coupling Sac1 localization to the regulation of anterograde transport activities from the Golgi system.

## Functional Analyses of Mammalian Sac1

Present interpretations of the work of Rohde et al (2003) and Blagoveshchenskaya et al (2008) hold, as principle thesis, that ER retention of Sac1 archives the enzyme in the ER and restrict its effects on Golgi function. Yet, the observed Sac1 redistribution between ER and Golgi membranes (and the corresponding interpretation of those data), is not coupled to any direct functional readout. One is left with the principal question of what do these redistribution phenomena truly mean for the mammalian cell? In that regard, mammalian Sac1 proteins remain poorly studied from the intracellular and organismal perspectives. This lack of information is made more striking by the fact that mammalian Sac1 proteins, while ubiquitously expressed (also in embryonic stem cells, Figure 3), nonetheless show interesting tissue distributions – e.g. particularly high expression is detected in cerebellum, hippocampus, and heart (Figure 3, Nemoto *et al*., 2000). From the perspective of the vertebrate organism, does mammalian Sac1 play any interesting tissue-specific functions? From the standpoint of individual cells, does Sac1 play a significant role in integrating growth factor signals with Golgi

exocytic activity? From that perspective, a difficulty with the simple Golgi PtdIns-4-P control model is presented by existing information regarding ySac1. This enzyme only degrades the PtdIns-4-P pool generated by the plasma membrane-localized ySac1 – not the PtdIns-4-P pool generated by the Pik1 PtdIns 4-OH kinase which is generally though to be the enzyme responsible for the PtdIns-4-P pool relevant to membrane trafficking from the Golgi complex (see above). If such a PtdIns-4-P pool specificity also holds true in mammalian cells (and it is not known whether this is in fact the case), then a revision of how we think Stt4- and Pik1-kinases act on the Golgi complex is in order. Finally, if the "ER-archive" model is generally true, this level of control likely represents a fine-tuning mechanism. This interpretation is based on the demonstration that a chimeric Sac1 protein, where the catalytic domain is tethered to an ER resident protein (Sec61), and is unable to shuttle between ER and Golgi membranes like the native Sac1 enzyme, is nonetheless able to functionally substitute for ySac1 *in vivo* (Rivas et al., 1999). The cumulative data raise the question: do Sac1 enzymes play more direct roles in ER or nuclear envelope function? As discussed below, emerging evidence suggests this is likely so – at least in the case of the mammalian Sac1 enzymes.

Meaningful study of mammalian Sac1 function requires transgenic mouse approaches, and such approaches have recently provided new insights. Gene trap experiments demonstrate that genetic ablation of the single murine *SAC1* gene results in a recessive (and fully penetrant) pre-implantation lethality -- most $sac1^{0/0}$ progeny failing to progress past E3.5 – and $sac1^{0/0}$ cells cannot be generated by selection from heterozygous ES cells (Liu et al., 2008). These collective data indicate the Sac1 PIP phosphatase plays an essential housekeeping role in mammalian cells. No obvious phenotypes derive from $SAC1^{+/0}$ haplo-insufficiency, however, nor are $sac1^{0/0}$ sperm or eggs deficient in any obvious way. Thus, 50% reductions in intracellular Sac1 load do not threaten obvious functional thresholds (Liu et al., 2008). As described below, the mechanisms for how Sac1 insufficiencies cause cell death are now being clarified, and functional rescue experiments demonstrate essential Sac1 functions require both PIP phosphatase activity and proper COP1-dependent retrieval of the enzyme back to the mammalian ER.

## Mammalian Sac1 and Golgi Membrane Organization

The intracellular localization of mammalian Sac1 enzymes to the ER and Golgi systems suggested these as likely points of failure under conditions of Sac1 insufficiency. Indeed, one striking intracellular phenotype associated with hSac1 depletion (by siRNA methods) in HeLa or HEK293 cells is a dramatic structural derangement of the Golgi membranes that is registered across the entire Golgi stack (Liu *et al*., 2008). A consistent dispersal of *cis*-, *medial*-, and *trans*-Golgi compartments from the typical compact structure to dispersed arrangements is observed. These phenotypes are further classified as 'moderately dispersed' or 'severely dispersed' as a function of magnitude of disorganization (Figure 4), and these phenotypic gradations presumably reflect the extent to which Sac1 levels have been diminished in those cells. Because Sac1-deficient cells exhibit significant reductions in viability, it was formally possible that disorganization of the Golgi system was an indirect consequence of activating apoptotic pathways. This is not the case, however, as evidenced by the fact that treatment with the pan-caspase inhibitor Z-VAD-fmk does not eliminate either the Golgi membrane derangements, or the reduced cell viability, evoked by hSac1-depletion (Liu *et al*., 2008).

With regard to intracellular membrane disorganization, the effect is apparently limited to the Golgi system as the gross morphologies of lysosomes, endosomes, mitochondria, and ER are not affected in hSac1-depleted cells. As discussed in more detail below, these Golgi structural phenotypes reflect 'on-target' effects that report the consequences of Sac1-deficiency as evidenced by the demonstration that the compromised viability and Golgi disorganization

phenotypes are both rescued by expression of silencing-resistant mSac1 transgenes in these human cell lines.

Structural compromise of Golgi membranes is often associated with defects in Golgi secretory function. For example, depletion of the peripheral Golgi protein Nir2 compromises Golgi structure, and Nir2 depletion also inhibits protein export from the TGN (Litvak *et al*., 2005). Considering that: (i) Sac1 is a major PtdIns-4-P phosphatase in cells that plays an important role in PtdIns-4-P turnover (Nemoto *et al*., 2000; Foti *et al*., 2001; Schorr *et al*., 2001), and (ii) PtdIns-4-P is an essential regulator for anterograde transport of secretory proteins from the Golgi system, it seemed likely that mammalian Sac1 deficiency compromises protein trafficking through this organelle. However, several lines of evidence indicate that both rate and efficiency of transport of a variety of cargo to, through, and from the disorganized Golgi system is not impaired in Sac1-depletion cells (Liu *et al*., 2008).

What are the effects of Sac1 depletion on PIP homeostasis in mammalian cells? Analyses of [$^3$H]-inositol labeled PIP species report modest (30%) increases in PtdIns-4-P in hSac1-depleted cells – an effect that is almost certainly an underestimate because it is an averaging measurement and only partial Sac1 deficiencies are being scored. In imaging experiments where individual cells with severely dispersed Golgi membranes can be analyzed individually, localization of endogenous TGN-associated PtdIns-4-P binding proteins (such as FAPP1, FAPP2 and Orp9), or other peripheral Golgi proteins (βCOP and PITPβ), is not disturbed under conditions of hSac1 deficiency that lead to severe Golgi dispersal phenotypes (Liu *et al*., 2008). Taken together, these data indicate that, unlike the case in yeast (Li et al., 2002), mammalian Sac1 deficiencies do not evoke redistribution of Golgi-associated PIP binding proteins to inappropriate intracellular locations (presumably as a result of ectopic accumulation of PtdIns-4-P in those locations). With the caveat that the effects of genuine Sac1 nullizygosity are not monitored in these experiments, the data suggest mammalian Sac1 may not represent as major a pathway for PtdIns-4-P degradation in mammalian cells ySac1 is in yeast. How Sac1 deficiency influences Golgi membrane organization remains to be elucidated, and represents an interesting question for future work.

## Mammalian Sac1 and Mitotic Spindle Organization

That mammalian Sac1 executes essential housekeeping functions is demonstrated not only by the pre-implantation lethality associated with *sac1* nullizygosity in mice, but also by the compromised viability of mammalian cells depleted for Sac1 protein (Liu et al., 2008). Fluorescence-activated cell sorting analyses demonstrate Sac1 depletion results in difficulties in progression through the G2/M phase of the cell cycle. This defect is accompanied by a significant (10-fold) increase, as assessed by α-tubulin staining, in the incidence of abnormal multipolar spindles in mitotic hSac1-depleted cells (Liu *et al*., 2008). Interestingly, the ectopic spindles are mechanically active and generate sufficient force to drive aberrant segregation of chromosomal material, as indicated by 4,6- diamidino-2-phenylindole (DAPI) staining of DNA at each spindle (Figure 5, Liu *et al*., 2008). Again, this mitotic phenotype is not rescued by challenge of Sac1-deficient mammalian cells with the pan-caspase inhibitor Z-VAD-fmk.

One plausible mechanism for generating multipolar spindles is the deregulation of centrosome duplication which further induces MT nucleation at ectopic centrosomes (Khodjakov and Rieder, 1999; Hinchcliffe and Sluder, 2001). However, Sac1-depleted mitotic cells present only two spindle poles which contain the centrosome marker, Centrin-2. This fidelity of centrosome duplication is observed even in cases where as many as five ectopic spindle poles are observed. Immunofluorescence analyses further demonstrate that γ-tubulin foci, which identify the γ-tubulin ring complex (γTuRC) that nucleates spindle MT assembly (Moudjou et al., 1996), mark each of the ectopic spindle asters in cells with multipolar spindles. These data indicate centrosome duplication occurs normally in hSac1-deficient cells with multipolar

spindles, and that the γTuRC is responsible for assembly of extra spindle poles in these cells. These findings are consistent with previous *in vitro* studies that demonstrate γTuRC is required, but not sufficient, for the formation of a functional centrosome (Martin et al. 1998; Moritz et al. 1998). Time-lapse video microscopy indicates the multipolar spindles of Sac1-deficient HeLa cells fail to resolve into bipolar spindles, and that such cells effectively fail to exit mitosis (Liu et al., 2008). Such catastrophic failure of organizing normal mechanically active spindles can cause chromosomal nondisjunction events (Wong and Stearns, 2003). This suggests an attractive mechanism for why Sac1-depleted mammalian cells fail to efficiently progress through G2/M and ultimately expire.

## Functional Properties of Mammalian Sac1

The Golgi morphology and mitotic spindle phenotypes provide robust readouts for functional complementation experiments where the effects of expressing silencing-resistant versions of mammalian Sac1 on these phenotypes can be monitored. These experiments employ Sac1-depleted HeLa cells expressing murine Sac1 variants whose expression is recalcitrant to the siRNAs used to silence *hSAC1* expression (Liu et al., 2008). Both the Golgi and multipolar spindle phenotypes are rescued by expression of wild-type mSac1, but not by the "PIP phosphatase-dead" $mSac1^{D391N}$. In a genetic version of a biochemical dose-response experiment, expression of mSac1 variants with partial PIP phosphatase activity (e.g mSac1R480H) results in partial rescue of both phenotypes. Finally, expression of the $mSac1^{AEAID}$ mutant that is incompetent for COPI binding and retrieval from the Golgi complex to the ER fails to rescue the Golgi morphological phenotype or the defects in mitotic spindle organization (Liu et al., 2008). These complementation experiments demonstrate that proper Golgi membrane and mitotic spindle organization require Sac1 PIP phosphatase activities and, in both cases, localization of Sac1 PIP phosphatase activity to the ER is a functional requirement.

## Sac1, The Golgi Complex, and Mitotic Spindle Organization

What is the relationship, if any, between the Golgi and spindle pole phenotypes of Sac1-deficient mammalian cells? The physical proximity of the Golgi apparatus with the centrosome is consistent with a functional linkage between these two organelles (Sütterlin *et al*., 2005; Kodani and Sütterlin, 2008). Indeed, several recent reports show that Golgi-associated proteins are involved in regulation of cell cycle progression related events. Specifically, a Golgi associated protein GRASP-65 plays an as yet undefined role in spindle dynamics (Sütterlin *et al*., 2005), and another (GM130) is involved in regulation of centrosome morphology, position and function during interphase (Kodani and Sütterlin, 2008). Are the mitotic spindle defects of Sac1-deficient mammalian cells a secondary effect of Golgi membrane disorganization? That remains a viable possibility. However, the Sac1-depletion effects on organization of the mitotic apparatus are not the result of secondary depletion of GRASP-65 or GM130 as both proteins are present in apparently normal amounts in Sac1-insufficent cells. Moreover, ectopic spindles in GRASP-65-deficient cells are not mechanically active, in contrast to those of Sac1-depleted mammalian cells (Liu *et al*., 2008). Thus, the mechanisms appear different.

Given that Sac1 is an integral membrane protein of the ER and the Golgi, and that ER localization is essential for Sac1 function in mammalian cells, possibilities for ER/nuclear functions for the Sac1 PIP phosphatase seem most likely. In this regard, PtdIns and PIP levels influence DNA synthesis, cell proliferation, and cell cycle progression (York and Majerus, 1994; Rubbini *et al*., 1997; Albi and Viola Magni, 2004). Although a direct connection between PIP species and regulation of γTRC organization during cell cycle progression remains to be demonstrated, these data suggest this to be an interesting avenue for future research. Novel functions for Sac1-mediated regulation of nuclear PIP metabolism must also be considered. The nuclear envelope is continuous with the ER, and it is possible that Sac1 phosphatases may

localize in the ER to regulate nuclear PIP signal during cell cycle progression. It is now an interesting question as to what intracellular locations are compatible with Sac1 function and which ones are not.

## Summary

The Sac1 PIP phosphatase is an enigmatic enzyme in that it occupies an intracellular location (ER) that is not normally associated with PIP signaling. Yet, genetic experiments in mice and silencing experiments in cultured cells report an essential housekeeping function for this protein. Detailed cellular analyses report maintenance of proper organization of the Golgi system, and of the mitotic spindle apparatus, are compromised when Sac1 functional thresholds are breached. While the Golgi derangements do not obviously affect protein transport through the organelle, the mitotic defects result in defects in progression though the G2/M stage of the cell cycle. Finally, both the catalytic PIP phosphatase activity, and its ability to be recycled back to the ER, represent essential functional features of the Sac1 enzyme.

We expect that current insights for Sac1 will set the blueprint for future analyses of its functions. Many questions remain to be answered in this field: does Sac1 have important roles in ER and plasma membranes connections since ySac1 only degrades the plasma membrane localized PtdIns 4-OH kinase Stt4 generated PtdIns-4-P? Does the ER-localized Sac1 play critical roles in regulating nuclear PIP signaling since ER is continuous with the nuclear envelope, and does it specifically happen in certain stage during cell cycle progression? Does anchoring Sac1-catalytic domain to other cellular membranes affect its function and what effects will be generated by mislocalizing the Sac1 catalytic domain to exotic membrane locations? Does the Golgi dispersion phenotype herald a crosstalk of the organelle with the mitotic apparatus (i.e. does Golgi disorganization provide ectopic nucleation sites for the γ-tubulin ring complex)? Clearly, there is much to be learned regarding the biological functions of Sac1-like lipid phosphatases, and we anticipate the discoveries yet to come will rival those derived from studies of the kinases – both in impact and in scope.

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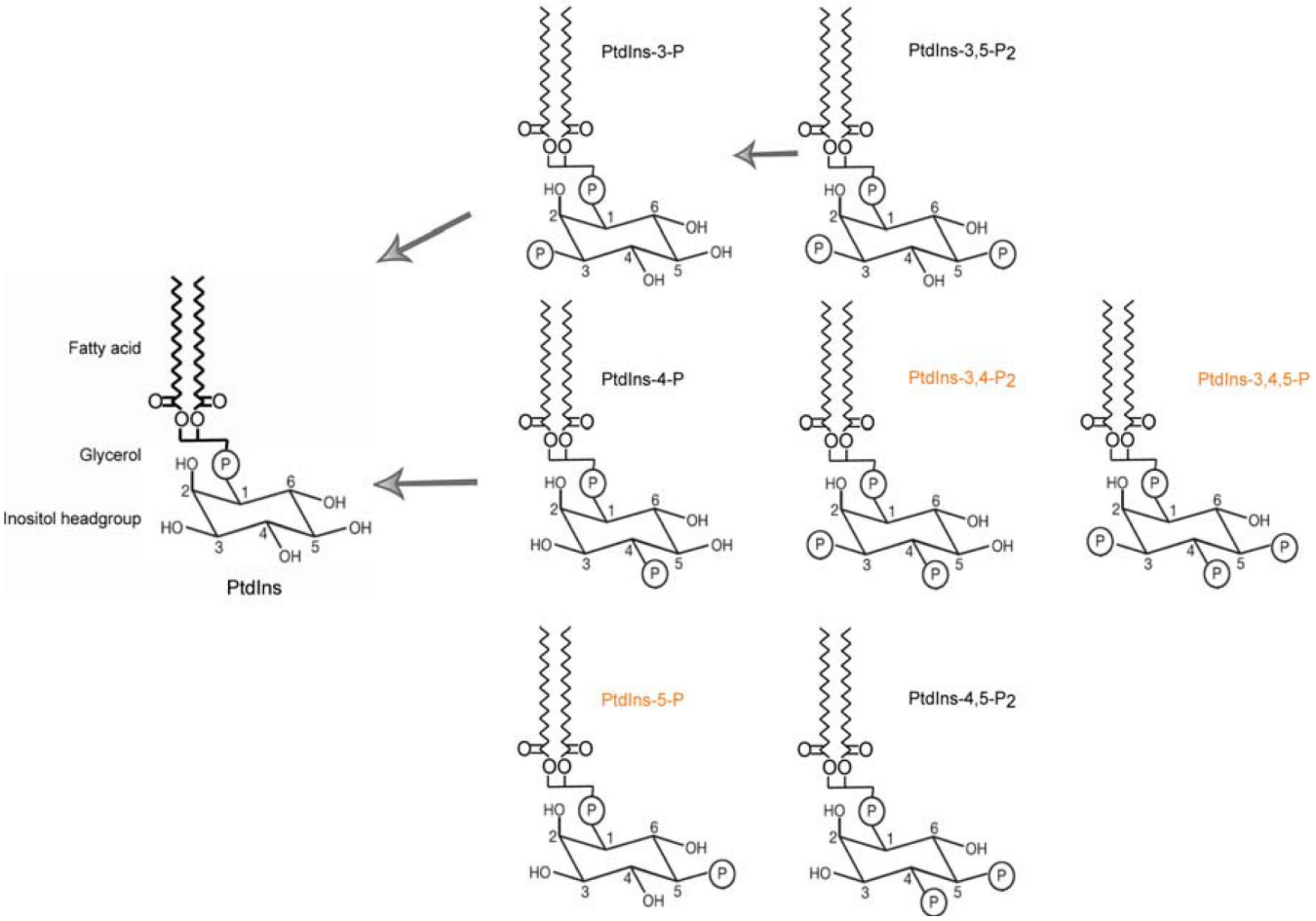


**Figure 1.**

Cellular PIP species and Sac1-mediated dephosphorylation. The chemical structures of PtdIns and its seven phosphorylated derivatives are shown. The PtdIns inositol headgroup, glycerol backbone and fatty acyl chains are illustrated. Mammals synthesize all seven phosphoinositides, although PtdIns-5-P is undetectable in several mammalian cell types (Kent, 1995; Fruman *et al.*, 1998; Shisheva, 2001; Di Paolo and De Camilli, 2006), and those shown in red are not present in yeast. Black arrows indicate Sac1 PIP phosphatase-catalyzed dephosphorylation reactions.

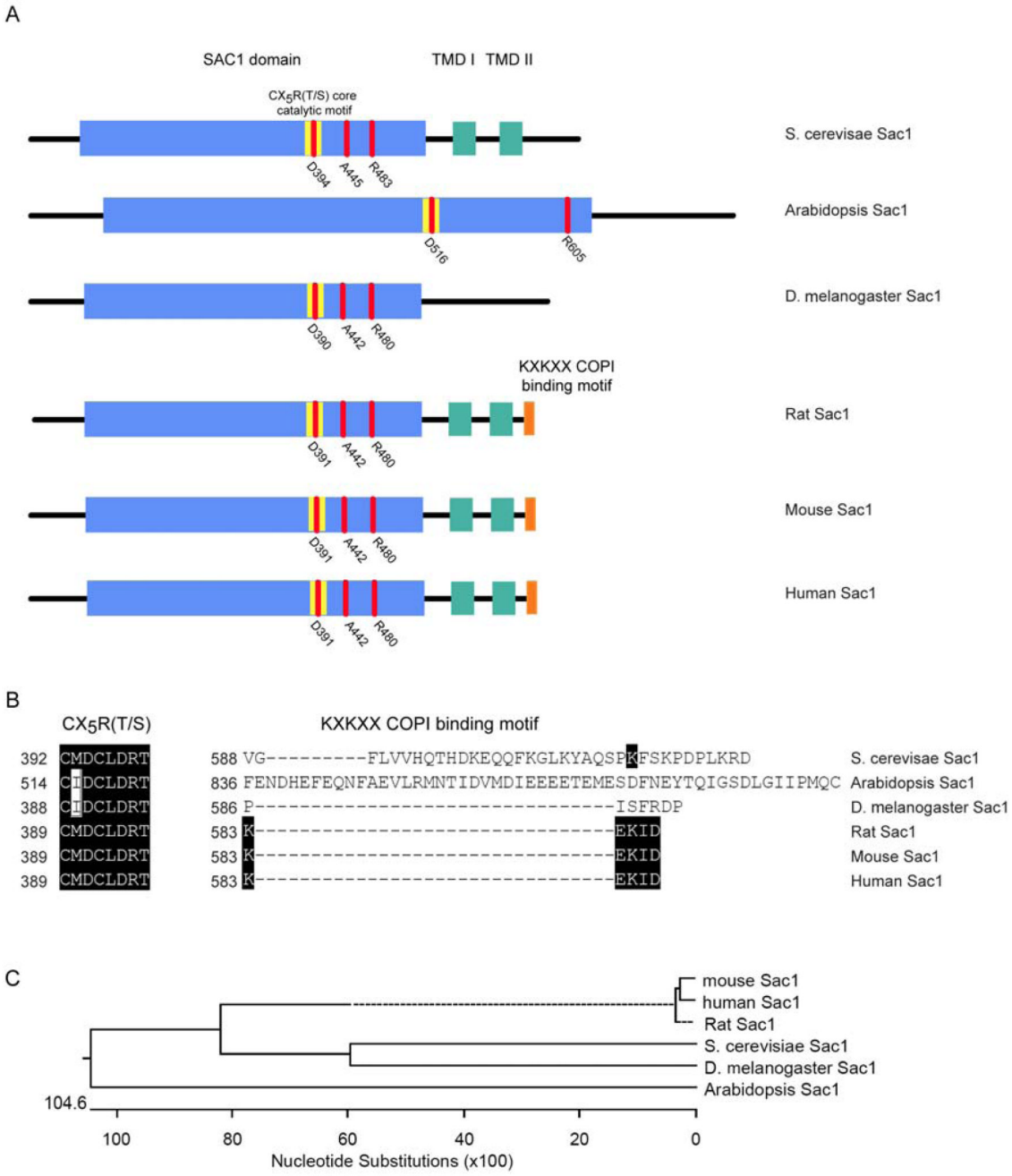


**Figure 2.**

Domain and primary structure of Sac1 protein family. **A** Diagrams of the domain organization of Sac1 proteins. Domains and motifs of interest are highlighted by colored boxes. Blue boxes show the Sac1 catalytic domain, yellow boxes show the $CX_5R(T/S)$ core catalytic motif, green boxes show the two transmembrane domains (TMD I and TMD II respectively), and the orange boxes show the KXKXX COPI binding motif. Three key amino acids within the Sac1 domain known to affect catalytic activity (Rivas et al., 1999; Liu et al., 2008) are indicated in red. **B** Sequence alignment of the core catalytic domains and the KXKXX COPI binding motifs of the Sac1 protein family. The $CX_5R(T/S)$ core catalytic domain of Sac1 is highly conserved in yeast, higher plants, *Drosophila*, and mammals, whereas the KXKXX COPI binding motif is

unique to mammalian Sac1 proteins. Identical amino acids are highlighted by black boxes. **C**. Phylogenetic tree of the Sac1 protein family.

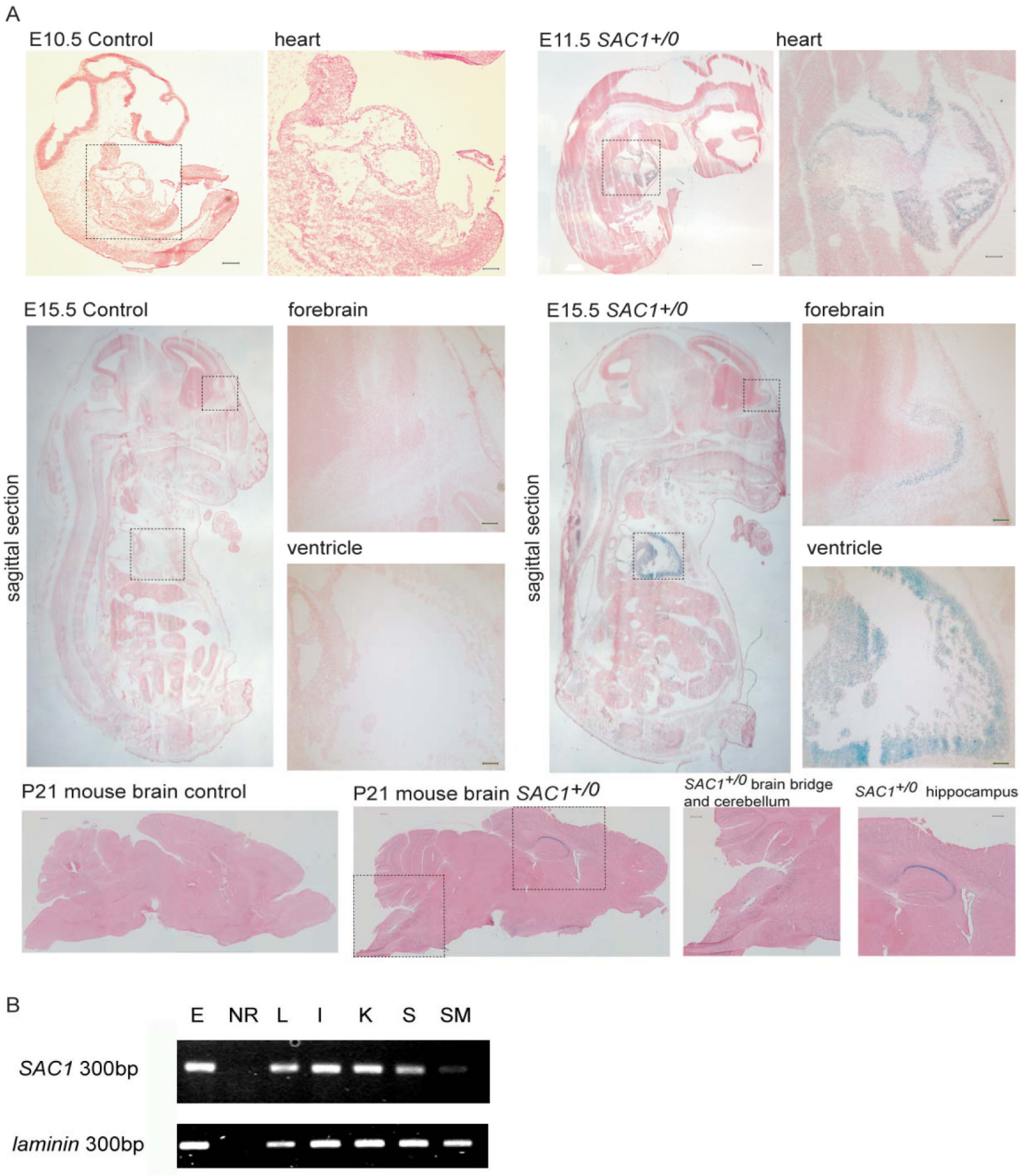


**Figure 3.**

Expression of *mSAC1* in the developing mouse. **(A)** *mSAC1* transcriptional expression patterns are indicated by X-gal staining in E10.5 and E11.5 $Sac1^{+/0}$ embryos. Moderate *mSAC1* expression was detected in the heart. In E15.5 $Sac1^{+/0}$ embryos, ubiquitous *mSAC1* expression was detected with particularly robust expression in the ventricle and the forebrain. p21 brains exhibited ubiquitous *mSAC1* expression that was particularly robust in the hippocampus, the cerebellum, and the brain stem. Scale bar, 100 μm. **(B)** Total mRNA from various mouse tissues and ES cells was purified, and RT-PCR amplification was used to estimate *mSAC1* expression. E, ES cells; NR, no RNA control; L, liver; I, intestine; K, kidney; S, stomach; SM, skeletal muscle.

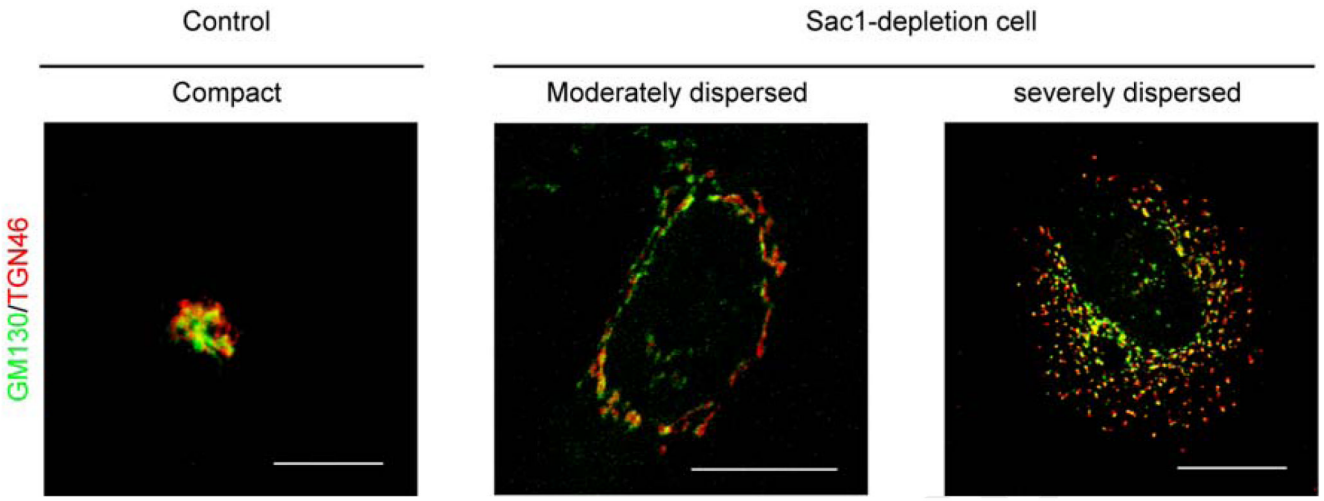


**Figure 4.**

hSac1 deficiency results in defective Golgi morphology in human cells. The TGN marker TGN46 is stained red, and the *cis*-Golgi marker GM130 is in green. The left panel shows a representative Golgi system of HeLa cells challenged with an irrelevant siRNA. The middle and right panels show the two types of Golgi dispersion in human Sac1-deficient cells. Based on the severity of dispersion, these are classified as moderately and severely dispersed, respectively. Moderately dispersed Golgi are defined by reticulation and relaxation of Golgi membranes, and severely dispersed Golgi are defined by extensive fragmentation of the Golgi system. Bar, 20 μm.

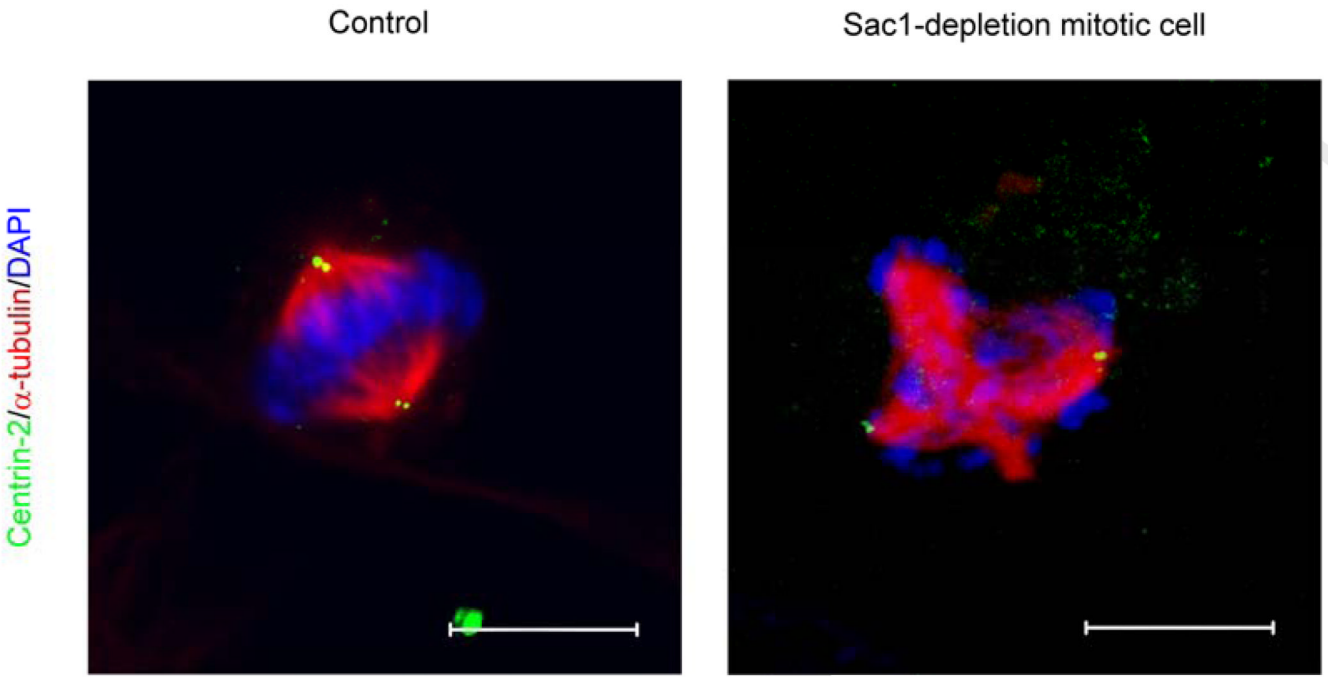


**Figure 5.**

Disorganization of mitotic spindles and aberrant distribution of chromosomal material in Sac1 deficient mammalian cells. Immunofluorescence images of control and a Sac1-depeleted mitotic cells are shown. The spindle marker α-tubulin, the centrosome marker centrin-2, and DNA (stained with DAPI) are rendered red, green and blue, respectively). The control mitotic cell in the left panel presents the typical two spindle poles, each containing a centrosome, with the chromosomes evenly distributed by the force of those two spindles. The right panel shows a *SAC1* siRNA treated cell presenting multiple ectopic spindle asters so that four spindle poles are evident. Each spindle pole generates sufficient force to pull chromosomes in an inappropriate manner. Only two centrosomes are observed, however. Bar, 10 μm.