The yeast LATS/Ndr kinase Cbk1 regulates growth via Golgi-dependent glycosylation and secretion

Cornelia Kurischko¹, Venkata K. Kuravi¹, Nattha Wannissorn¹, Pavel A. Nazarov¹, Michelle Husain², Chao Zhang³, Kevan M. Shokat³, J. Michael McCaffery² and Francis C. Luca¹

¹Department of Animal Biology and the Mari Lowe Center for Comparative Oncology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104

²Integrated Imaging Center, Johns Hopkins University, Baltimore, MD 21218

³Howard Hughes Medical Institute & Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA 94143

Correspondence should be addressed to Francis C. Luca <u>fluca@vet.upenn.edu</u> Tel. (215) 573-5664; Fax. (215) 573-5188;

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Abbreviations: RAM, regulation of Ace2 and morphogenesis; GEF, guanine nucleotide exchange factor; ER, endoplasmic reticulum; EM, electron microscopy; SNARE, soluble NSF attachment receptor; COG, conserved oligomeric Golgi complex

ABSTRACT

Saccharomyces cerevisiae Cbk1 is a LATS/Ndr protein kinase and a downstream component of the RAM (regulation of Ace2 and morphogenesis) signaling network. Cbk1 and the RAM network are required for cellular morphogenesis, cell separation and maintenance of cell integrity. Here we examine the phenotypes of conditional *cbk1* mutants to determine the essential function of Cbk1. Cbk1 inhibition severely disrupts growth, protein secretion and triggers the Swe1-dependent morphogenesis checkpoint. Cbk1 inhibition also delays the polarity establishment of the exocytosis regulators Rab-GTPase Sec4 and its exchange factor Sec2, but does not interfere with actin polarity establishment. Cbk1 binds to and phosphorylates Sec2, suggesting that it regulates Sec4-dependent exocytosis. Intriguingly, Cbk1 inhibition causes a >30% decrease in post-Golgi vesicle accumulation in late secretion mutants, indicating that Cbk1 also functions upstream of Sec2-Sec4, perhaps at the level of the Golgi. In agreement, conditional cbk1 mutants mislocalize the cis-Golai mannosyltransferase Och1, are hypersensitive to the aminoglycoside hygromycin B and exhibit diminished invertase and Sim1 glycosylation. Significantly, the conditional lethality and hygromycin B sensitivity of *cbk1* mutants are suppressed by moderate overexpression of several Golgi mannosyltransferases. These data suggest that an important function for Cbk1 and the RAM signaling network is to regulate growth and secretion via Golgi and Sec2/Sec4dependent processes.

INTRODUCTION

Cell polarity is a common cellular feature among eukaryotic cells that is characterized by asymmetry in cell shape, protein distribution and cellular function (Nelson, 2003; Pruyne *et al.*, 2004). The establishment and maintenance of polarized growth are critical for the development and function of specialized cells, tissues and organs. Polarized growth is particularly important for the development and function of neuronal cells, which can reach several meters in length, and for epithelial cells, which regulate homeostasis and maintain physical barriers between biological compartments (Arimura and Kaibuchi, 2005; Dow and Humbert, 2007; Tang, 2008). Dynamic changes in cell polarity are essential for cell migration during cell and tissue development. Polarized growth and morphogenesis are also important for yeast and filamentous fungi development (Harris, 2006).

Saccharomyces cerevisiae is an excellent model organism for studying conserved mechanisms of polarized growth and cell division. *S. cerevisiae* cells undergo polarized growth during cell division, mating and pseudohyphae formation (Pruyne *et al.*, 2004). Many of the evolutionarily conserved elements of cell growth and proliferation were first identified and characterized in yeast (Pruyne *et al.*, 2004). Yeast polarity establishment involves the recruitment and activation of Cdc42 GTPase to a cortical landmark. Cdc42 regulates several polarity processes, including septin assembly and actin cytoskeleton polarization (Park and Bi, 2007). Polarized actin cables are necessary for delivering organelles and secretory vesicles to the cortical sites of polarized growth, (see (Pruyne *et al.*, 2004; Park and Bi, 2007) for review). Defects in yeast polarity establishment, vesicle trafficking or exocytosis can yield a variety of phenotypes ranging from minor morphological aberrations to severe growth inhibition and cellular lysis.

Intracellular trafficking pathways are important for polarized growth (Cole and Fowler, 2006). The endoplasmic reticulum (ER) and the Golgi apparatus are essential for the synthesis, post-translational modification and trafficking of secretory proteins and vesicles (Brennwald and Rossi, 2007). Plasma membrane and cell wall proteins are synthesized in the ER and transported to the Golgi apparatus, where they are commonly modified by glycosylation (Jigami, 2008). Cargo-bearing vesicles from the Golgi are delivered to endosomes, vacuoles or the plasma membrane. Vesicle sorting, trafficking and fusion are mediated by numerous GTPases and vesicle tethering complexes (Whyte and Munro, 2002; Lundquist, 2006; Park and Bi, 2007). Similarly, Golgi structure and function are maintained by retrograde and intra-Golgi trafficking pathways, which are also controlled by Rab family

GTPases and membrane docking complexes (Oka and Krieger, 2005). Retrograde trafficking mechanisms are essential for retrieval of ER and Golgi enzymes and maintenance of organelle function. Membrane docking complexes cooperate with SNARE proteins to mediate membrane fusion (Whyte and Munro, 2001, 2002; Bruinsma *et al.*, 2004; Hsu *et al.*, 2004; Oka and Krieger, 2005; Zolov and Lupashin, 2005). ER and Golgi trafficking defects often compromise protein glycosylation, cell wall biogenesis and cell integrity.

In yeast, many post-Golgi secretory vesicles are delivered to the sites of polarized growth along actin cables in a myosin V dependent fashion (Pruyne *et al.*, 2004; Lowe and Barr, 2007). Both vesicle delivery and docking to the plasma membrane are regulated by the Rab GTPase Sec4 and its GEF Sec2 (Nair *et al.*, 1990; Guo *et al.*, 1999). Sec4 and Sec2 associate with post-Golgi secretory vesicles and are required for myosin V-dependent transport along actin cables to the sites of polarized growth. In addition Sec4 stimulates the assembly of the exocyst, an eight-subunit vesicle tethering complex on the plasma membrane (Salminen and Novick, 1987; Guo *et al.*, 1999; Hsu *et al.*, 2004), which mediates membrane fusion and exocytosis via an interaction with SNARE proteins (Hsu *et al.*, 2004). Sec4 function is dependent on the guanyl nucleotide exchange factor (GEF) Sec2, which converts inactive Sec4-GDP to active Sec4-GTP (Walch-Solimena *et al.*, 1997; Elkind *et al.*, 2000). Sec2 is regulated by phosphorylation in vivo, however the kinase(s) responsible for Sec2 phosphorylation has not been identified (Elkind *et al.*, 2000). Two hybrid data suggest Sec2 might be regulated by Cbk1 kinase, a component of the conserved *S. cerevisiae* <u>R</u>egulation of <u>A</u>ce2 and polarized <u>Morphogenesis</u> (RAM) signaling network (Racki *et al.*, 2000).

Cbk1 kinase and the RAM signaling network are important for polarized growth, differential gene expression and maintenance of cell integrity (Dorland *et al.*, 2000; Racki *et al.*, 2000; Bidlingmaier *et al.*, 2001; Colman-Lerner *et al.*, 2001; Weiss *et al.*, 2002; Nelson *et al.*, 2003; Kurischko *et al.*, 2005). RAM is comprised of two kinases, Cbk1 and Kic1, and several associated proteins, Mob2, Hym1, Tao3 and Sog2 (Nelson *et al.*, 2003). Cbk1 is a member of the Lats/NDR/Dbf2 kinase family, which includes the mammalian and *Drosophila* Lats/warts tumor suppressor, *S. pombe* Orb6 and *C. elegans* Sax1 (Xu *et al.*, 1995; Verde *et al.*, 1998; Geng *et al.*, 2000; Racki *et al.*, 2000; Zallen *et al.*, 2000; Hergovich *et al.*, 2003; Emoto *et al.*, 2004; He *et al.*, 2005; Hergovich *et al.*, 2006). Deletion of *S. cerevisiae* CBK1 or other RAM genes causes cell morphology defects, cellular lysis and death (Sullivan *et al.*, 1998; Du and Novick, 2002; Kurischko *et al.*, 2005). Dosage suppressors of *cbk1* and other RAM mutants

include genes expressing cell wall components, suggesting that Cbk1 and RAM maintain cell integrity by controlling cell wall biosynthesis (Du and Novick, 2002; Vink *et al.*, 2002; Kurischko *et al.*, 2005). The lethality of RAM gene deletions, designated *ram* Δ , is also suppressed by mutations in the enigmatic *SSD1* gene, which encodes an RNA binding protein of unknown function (Sutton *et al.*, 1991; Du and Novick, 2002; Jorgensen *et al.*, 2002). In the absence of *SSD1*, *ram* Δ cells are spherical in morphology, fail to form robust mating projections and fail to degrade the septum following cytokinesis (Nelson *et al.*, 2003). The latter phenotype is due to misregulation of Ace2 transcription factor (Racki *et al.*, 2000; Colman-Lerner *et al.*, 2001; Weiss *et al.*, 2002).

Cbk1 and RAM proteins localize to sites of polarized growth (similarly to Sec2 and Sec4 proteins), which include the bud cortex during bud emergence and growth, the tips of mating projections and the bud neck region during mitotic exit (Bidlingmaier *et al.*, 2001; Colman-Lerner *et al.*, 2001; Du and Novick, 2002; Weiss *et al.*, 2002; Nelson *et al.*, 2003). In addition, a fraction of Cbk1 and Mob2, which associate as a complex, co-localizes with Ace2 transcription factor in the daughter cell nucleus during mitotic exit (Colman-Lerner *et al.*, 2001; Weiss *et al.*, 2002). Ace2 activity and localization are dependent on Cbk1 kinase activity, however Ace2 is not essential for viability, cell morphogenesis or maintenance of cell integrity (Dohrmann *et al.*, 1992; O'Conallain *et al.*, 1998; Bidlingmaier *et al.*, 2001; Weiss *et al.*, 2002; Kurischko *et al.*, 2005). These data indicate that Cbk1 and the RAM network maintain cell integrity and control polarized growth independently of Ace2.

The mechanism for Cbk1 and the RAM network in regulating polarized growth and maintenance of cell integrity is not known. To gain insight into the essential function of the RAM network, we investigated the consequence of Cbk1 inhibition in vivo. Here, we present evidence that Cbk1 and the RAM signaling network maintain cell integrity and control polarized growth via regulating Golgi function and exocytosis. In light of the conservation of Cbk1 and other RAM proteins among eukaryotes, these findings suggest universal mechanisms for RAM in regulating cell integrity and cell polarity.

MATERIAL AND METHODS Yeast strains and plasmid construction

The yeast strains and relevant genotypes used in this study are listed in **Table 1**. All strains from our laboratory and from the deletion consortium (available from Open Biosystems) are isogenic S288c derivatives. *S. cerevisiae* strains were maintained and grown using standard conditions (Guthrie and Fink, 1991). The *cbk1-as* allele (Weiss *et al.*, 2002) was subcloned into the integrative vector pRS403. The plasmid was linearized in the *CBK1* promoter and integrated upstream of *cbk1* Δ in the heterozygous diploid strain FLY1509. The diploid was sporulated and dissected to obtain FLY2084 (MAT*a cbk1-as::HIS3::cbk1* Δ *::KANMX*). Strain FLY2531 was constructed by transforming FLY2084 with pRC556 (SEC4-GFP) (Schott *et al.*, 2002). The temperature sensitive allele *cbk1-8* was generated by random PCR mutagenesis of the DNA encoding the kinase domain (amino acids 351-672), as described (Luca and Winey, 1998), using the following oligonucleotide primers: forward agaagatttccacactgtga and reverse ttgtctgattgtattccaat. DNA sequence analysis reveals that *cbk1-8* has point mutations that cause two amino acid substitutions in the ORF (E430V T550A). *cbk1-8* was subcloned into pRS403 and integrated into FLY1509, as described above, to yield FLY2661.

The plasmid pMAL-SEC2 (encoding full length MBP-Sec2) was provided by Ruth Collins (Cornell University). Derivative plasmids encoding MBP tagged Sec2⁴⁵¹⁻⁷⁵⁹, Sec2¹⁻⁵⁰⁸, Sec2¹⁻²⁰⁴, and Sec2²⁰⁵⁻⁴⁵⁰ were constructed by PCR amplifying and subcloning the corresponding *SEC2* fragments using combinations of the following forward and reverse oligos: FLO350 (forward) ggggaattcgatgcttctgaggaagcaaa; FLO354 (forward) : aaactgcagttgctgttcctgggcatcat; FLO383 (forward): ggggaattcgaagttggaattggaattggaattggaattggaagcaaagtg (reverse): aaactgcagtgaagaaattg FLO352 (reverse): aaactgcagtgaagaattg; FLO353 (reverse): aaactgcagtccaatttttgaagaaattg; FLO353 (reverse): ggggaattcacaaaaaataagccaaagat.

Dosage suppressor screen. *cbk1-8* cells were transformed with an ordered array of high copy plasmids containing overlapping fragments of yeast genomic DNA (Open Biosystems) (Jones *et al.*, 2008) and screened for growth at 34°C. Some relevant dosage suppressors are listed in **Supplement Table S1** and were confirmed by PCR and by re-transformation. The remaining suppressors from this screen will be reported in a separate manuscript.

Cbk1-as inhibition in vivo

Cells were synchronized in G1 by treatment with alpha factor or in G0, as described (Weiss and Winey, 1996; Ayscough *et al.*, 1997). Cells were released from G1 or G0 block at a cell density of 5x10⁶ cells/ml and 5uM of the kinase inhibitor 4-Amino-1-tert-butyl-3-(1'-naphthyl)pyrazolo[3,4-d]pyrimidine (1NA-PP1) (Bishop *et al.*, 2000) was added at 0 or 30 min after release. At least 200 cells were counted per time point to determine the percentage of budded cells.

Electrophoresis and immunoblot methods

Standard SDS-PAGE and immunoblotting were conducted as described (Laemmli, 1970; Towbin *et al.*, 1979). Radiolabeled gels were analyzed with a STORM phosphorimager (Molecular Dynamics). Immunoblots were probed with various primary antibodies followed by alkaline phosphatase conjugated anti-mouse or anti-rabbit IgG secondary antibodies (Promega). Immunoblots were processed for ECF (Amersham) and scanned on a STORM phosphorimager (Molecular Dynamics). Mouse monoclonal anti-Myc was obtained from Covance; monoclonal anti-GFP was obtained from Roche; Rabbit polyclonal anti-ADH (alcohol dehydrogenase) was obtained from Abcam.

Secretion assays

Bgl2 secretion assays were conducted as described (Adamo *et al.*, 1999; He *et al.*, 2007). Briefly, cells were grown to mid-log phase and shifted to 22°C or 37°C for 2.5 hours. Cells were collected and treated with 0.2 mg/ml zymolyase (USB) supplemented with 2.5 U/ml chitinase (Sigma) for 40-60 min to yield 100% spheroplasts, as determined by microscopy. The internal (cellular) and external (solubilized cell wall) fractions were separated by centrifugation at 2000 rpm for 5 min and the pellet (internal fractions) and supernatant (external fractions) were subjected to SDS-PAGE and immunoblot analysis. Immunoblots were probed with anti-Bgl2 antibody (He *et al.*, 2007), processed for ECF (Amersham) and analyzed on a STORM phosphorimager (Molecular Dynamics).

Invertase secretion assays were conducted as described (Adamo *et al.*, 1999; He *et al.*, 2007). Cells were grown to mid log phase in YPD medium (2% glucose), and shifted to experimental conditions (22° or 37°C) for two hours in low glucose medium (YPD containing 0.1% glucose). Samples were collected at t = 0 and after two hours in low glucose medium. Invertase activity was determined using a colorimetric assay to measure glucose released from sucrose (Adamo *et al.*, 1999; He *et al.*, 2007). The ratio of secreted invertase / total invertase

activity was calculated using the following formula: (external invertase activity at T = 2h - external invertase activity at T = 0) / (external invertase activity at 2h - external invertase activity at T = 0) + (internal invertase activity at 2h – internal invertase activity at T = 0) (He *et al.*, 2007).

The degree of invertase glycosylation was determined in strains expressing Myc tagged invertase (Suc2-Myc) as described (Click *et al.*, 2002). Briefly, invertase expression was induced by transferring to low glucose medium (0.1% glucose) for 2.5 h. Total protein was extracted by vortexing cells in protein sample buffer containing glass beads and loaded onto 7.5% polyacrylamide gels, immunoblotted and probed with anti-Myc antibody (9E10; Covance).

Media secretion assays were performed as described (Gaynor and Emr, 1997). A logarithmic culture of asynchronous growing cells was incubated in methionine and cysteine deficient medium containing 10 uCi ³⁵S-methionine/cysteine (Trans label, ICN) per 1 OD⁶⁰⁰ of cells for 30 min at 37°C. Cells were chased for 45 min with medium containing 125 mM methionine and 25 mM cysteine at 37°C and protein synthesis/secretion was halted by adding NaF and NaN₃ to a final concentration of 20mM. Cells were pelleted and conditioned medium was collected. The secreted proteins were precipitated with 10% TCA. Proteins were visualized by SDS-PAGE analysis followed by autoradiography.

In vivo pulse labeling experiments of carboxypeptidase Y (CPY) were done as described (Franzusoff and Schekman, 1989). Logarithmically growing cells were collected, washed once and resuspended in 3 ml of methionine and cysteine deficient medium at a concentration of ~5 x 10⁷ cells/ml. The cells were pre-incubated for 30 min at 37°C and then labeled with 300 uCi ³⁵S-Met (ICN) for 10 min at 37°C. The cells were chased for 5 and 15 min in chase solution (5% yeast extract, 125 mM Met, 25 mM Cys) and the cells were precipitated with TCA. CPY was immunoprecipitated with a rabbit polyclonal CPY antibody (Abcam) and analyzed by 10% SDS-PAGE followed by autoradiography.

Light microscopy and cytology

Fluorescent and DIC microscopy was carried out as described (Luca *et al.*, 2001). FACS analysis was conducted as in (Weiss and Winey, 1996). Cells were treated with Alexa⁵⁹⁴- conjugated concanavalin A (Molecular Probes) and FM4-64 (Molecular Probes) as described (Lew and Reed, 1993; Vida and Emr, 1995). Where designated, cells were treated with Latrunculin A (Molecular Probes) to disrupt the actin cytoskeleton, as described (Ayscough *et al.*, 1997). FM4-64 uptake experiments were conducted as previously described (Vida and

Emr, 1995; Wang *et al.*, 1996). Vacuoles were also visualized by incubating cells with 100uM CellTracker Blue CMAC (Molecular Probes/Invitrogen). Cells expressing Och1-GFP were methanol/acetone fixed prior to imaging as described (Guo *et al.*, 2001).

Electron microscopy and quantification

Conventional transmission electron microscopy experiments were carried out as previously described (Rieder *et al.*, 1996). For quantification of secretory vesicles, 25 images from each experimental parameter were acquired on an FEI Tecnai 12 TWIN electron microscope equipped with a SIS Megaview III wide-angle camera yielding a total *n* of 50 cells/parameter. The numbers of vesicles in the bud and mother were tabulated utilizing the 'touch-counting' function in iTEM Olympus Soft Imaging Solutions v. 5.0 software. Subsequently, the numbers of vesicles in the bud and mother from each sample type were entered into Microsoft Excel. The averages and standard deviations were calculated using the function feature and the resulting data, including averages and SD, were transferred to Graph Pad Prism4. The bargraphs were created with the calculated Standard Error of Mean (SEM).

In vitro kinase assay and affinity precipitation experiments.

Cbk1 kinase assays were performed as in (Weiss *et al.*, 2002). MBP and MBP-Sec2 substrates were expressed in *E. coli* from pMAL2 (New England Biolab) and pMAL2-Sec2 vectors (gifts from Dr. Ruth Collins, Cornell University) and purified according to the manufacturer's protocol (New England Biolab). The affinity precipitation experiments with MBP and MBP-Sec2 immobilized on amylose-agarose were carried out as described (Rahl *et al.*, 2005) with slight modifications. Approximately 0.2 mg of yeast cell extract containing Cbk1-Myc was incubated for 30 min with 50ul of immobilized amylose resin containing approximately 2 mg/ml of MBP or MBP-Sec2. The amylose-agarose pellet was washed 3X in lysis buffer containing 1% NP-40 and protease inhibitors, 3X in TBS containing 500mM NaCl and 1mM EDTA, and resuspended in protein sample buffer for immunoblot analysis. Immunoprecipitation of epitope-tagged yeast proteins and immunoblots were conducted as described (Weiss *et al.*, 2002; Kurischko *et al.*, 2005) with monoclonal anti-Myc (9E10; Covance) or anti-HA antibodies (12CA5; Covance).

RESULTS

Cbk1 is required for bud emergence.

To determine the essential function of Cbk1 kinase and the RAM signaling network, we investigated the phenotypes of conditional cbk1 mutants, cbk1-as and cbk1-8. The analog sensitive allele *cbk1-as* encodes a kinase (Cbk1-as) that is specifically inhibited by the drug 1NA-PP1 (Weiss et al., 2002), while cbk1-8 is a recessive conditional loss-of-function allele that causes lethality at restrictive temperature (34° or 37°C). In the absence of 1NA-PP1, *cbk1-as* cells are indistinguishable from wild type cells. However, in the presence of 1NA-PP1, *cbk1-as* cells display severe growth and morphology defects (Figure 1). When Cbk1-as was inhibited in G0 or G1 synchronized *cbk1-as* cells, bud emergence was severely delayed in comparison to mock-treated cbk1-as cells or 1NA-PP1-treated wild type cells (Figure 1A and Supplement Figure S1). Conditional *cbk1*-8 cells displayed similar bud delays when shifted to restrictive temperature (data not shown). Typically, it took over 4 hours for 50% of 1NA-PP1treated cbk1-as cells to form buds following release from G0 or G1. In contrast, 50% of 1NA-PP1-treated wild type cells or mock-treated *cbk1-as* cells initiated buds within 60-90 min after G0 or G1 release. The phenotypes of Cbk1-as inhibition were similar regardless of whether cells were synchronized by nutrient deprivation (G0) or by treatment with mating pheromone, which arrests cells in G1 at Start (Figure 1A and Supplement Figure S1).

Upon continuous exposure to 1NA-PP1, many *cbk1-as* cells eventually developed buds, however they commonly exhibited morphology and lysis defects (**Supplement Figure S2**). Surviving cells developed abnormally wide bud necks (mother-daughter cell junctions), were spherical in morphology and displayed cell separation defects. The number of colony forming units of *cbk1-as* cultures remained stable for up to 8 hours in 1NA-PP1 (**Supplement Figure S2**), suggesting that the overall viability of the population remained constant. In contrast, *cbk1-8* cells displayed a precipitous loss of viability when maintained at restrictive temperature for greater than 2 hours (**Supplement Figure S2**). Similarly, expression of catalytically inactive Cbk1 (Cbk1-KD) failed to rescue the lethality of *cbk1* Δ cells (data not shown). These data indicate that Cbk1 kinase activity is necessary for bud emergence and maintenance of cell integrity and suggest that some in vivo Cbk1-as kinase activity persists in the presence of 1NA-PP1.

Cbk1 inhibition triggers the G2 morphogenesis checkpoint.

Delayed bud emergence could be caused by a defect in cell cycle progression. Thus, we monitored DNA content in 1NA-PP1 treated *cbk1-as* cells to determine if Cbk1 inhibition interferes with S phase entry. Intriguingly, Cbk1 inhibition during G1 led to a G2 cell cycle arrest, as determined by FACS and microscopic analyses (**Figure 1B**). 1NA-PP1 treated *cbk1-as* cells arrested uniformly as unbudded cells with 2N DNA content and single nuclei. We observed similar results with *cbk1-8* cells (data not shown), although a partial cell separation defect at permissive temperature (22°C) hampered flow cytometric analysis (see **Supplement Figure S2**). These data indicate that Cbk1 kinase activity is critical for bud emergence and polarized growth, but is not essential for cell cycle entry or for G1 and S phase progression.

The G2 arrest in 1NA-PP1-treated *cbk1-as* cells could be the consequence of a cell cycle checkpoint or could reflect a role for Cbk1 in M phase entry. Polarized growth defects often activate the morphogenesis checkpoint, which induces G2 arrest by inhibiting mitotic Cdk1 activation (McMillan *et al.*, 1998; Lew, 2003). To test if Cbk1 inhibition leads to morphogenesis checkpoint activation, we assayed DNA content, chromosome segregation and nuclear number in *cbk1-as swe1* Δ double mutant cells, which lack Swe1 kinase, the central component of the morphogenesis checkpoint. In the absence of 1NA-PP1, *cbk1-as swe1* Δ cells were indistinguishable from wild type, *swe1* Δ or *cbk1-as* single mutant cells (data not shown). Significantly, Cbk1 inhibition failed to induce a G2 arrest in *cbk1-as swe1* Δ cells, but instead caused a severe loss of viability, preceded by a modest increase in bi-nucleate cells (**Figure 1B;** and unpublished data). These data indicate that diminished Cbk1 activity leads to morphogenesis checkpoint activation and that the morphogenesis checkpoint is essential for maintaining cell viability when RAM signaling is compromised.

Cbk1 inhibition causes a modest delay in S phase entry.

A previous study suggested that at least one RAM component is required for G1 progression (Bogomolnaya *et al.*, 2004). In agreement, FACS analysis of synchronized *cbk1-as* cells consistently revealed a modest delay in S phase initiation upon Cbk1 inhibition (**Figure 1B**, compare the histograms of *cbk1-as* cells at 1h; and **Supplement Figure S3**). The modest G1 delay was not diminished in *swe1* Δ cells, indicating that it was not caused by the morphogenesis checkpoint (**Figure 1C**). These data suggest that Cbk1 and RAM have a non-essential role in G1 progression. Nevertheless, the apparent G1 delay upon Cbk1 inhibition is not long enough to account for the >2 hour delay in bud emergence.

It is well established that the lethality of $cbk1\Delta$ and other $ram\Delta$ mutations is suppressed by truncation or deletion of the poorly understood *SSD1* gene (Du and Novick, 2002; Jorgensen *et al.*, 2002; Kurischko *et al.*, 2005). We investigated whether *SSD1* mutations ($ssd1\Delta$ or ssd1-d) rescue the bud and cell cycle delays of conditional cbk1 mutants. We found that Cbk1 inhibition in ssd1-d cells caused a very brief (10-15') delay in bud emergence and S phase initiation, but did not trigger a G2 arrest or cause a loss of viability (**Figure 1C, and unpublished data**). The apparent G1 delay in cbk1-as ssd1-d cells was Swe1-independent and did not occur in mock-treated cbk1-as ssd1-d cells or 1NA-PP1 treated wild type cells (**Figure 1B, C**; and unpublished data). Thus, in addition to playing a major role in bud emergence (**Figure 1A**), Cbk1 and RAM have a non-essential role in G1 progression or S phase initiation that is independent of Ssd1 or the Swe1 morphogenesis checkpoint.

Cbk1 is required for bud growth.

To address whether Cbk1 kinase activity is required for bud growth after bud emergence, we measured bud size in 1NA-PP1-treated *cbk1-as* cells at various times after release from G0 or G1 arrest (see Materials and Methods). When Cbk1-as was inhibited in late G1/early S phase (after ~50% of the cells formed small buds), bud size remained constant compared to mock treated *cbk1-as* cells (**Figure 2A**). Similarly, the percentage of unbudded and small budded cells remained relatively constant for over an hour (**Supplement Figure S4**). The size of mother cells also remained constant upon Cbk1 inhibition (data not shown). Thus, Cbk1 kinase activity is required for both apical (bud emergence) and isotropic growth (bud and mother cell growth).

Cbk1 is required for secretion.

The significant delay in bud emergence and growth in conditional *cbk1* mutants could reflect a role for the RAM network in cell wall remodeling and/or plasma membrane expansion. To test if Cbk1 kinase inhibition interferes with cell wall deposition, we pulse-labeled *cbk1-as* cells with fluorescent Concanavalin A (Alexa⁵⁹⁴-ConA), a lectin that binds cell wall glycoproteins (**Figure 2B**). After ConA washout, new cell wall deposition during polarized growth causes the development of a ConA-free zone on the cell surface (Lew and Reed, 1993; Pruyne *et al.*, 2004). Most mock-treated *cbk1-as* cells displayed unlabeled buds within 30 minutes of ConA washout, as observed in wild type cells. In contrast, nearly all of the buds in

1NA-PP1 treated *cbk1-as* cells displayed prominent fluorescent ConA-labeling, suggesting that Cbk1 kinase activity is essential for the polarized secretion of cell wall proteins.

We also tested if Cbk1 is required for the periplasmic secretion of well-characterized markers for the two major classes of post-Golgi vesicles, invertase and Bgl2 endo-beta-1,3-glucanase (Harsay and Bretscher, 1995). We first measured the ratio of secreted invertase activity to total invertase activity in *cbk1-8*, wild type cells and a conditional exocytosis mutant (*exo84-121*) at 22° and 37°C. The amount of secreted invertase activity was diminished by ~50% in *cbk1-8* cells at 37°C in comparison to similarly treated wild type cells (**Figure 3A**). Secreted invertase activity of *cbk1-8* cells was similar to that of the exocyst mutant *exo84-121*. Interestingly, both *cbk1-8* and *exo84-121* mutants were moderately impaired for invertase secretion at 22°C. These data are consistent with a role for Cbk1 in secretion.

We monitored Bgl2 secretion in conditional *cbk1* mutants by analyzing the relative amounts of Bgl2 in internal (cytoplasmic) and external (periplasmic space/cell wall) fractions of yeast cells. Bgl2 is rapidly secreted in wild type cells, so it is almost exclusively present in the external fraction at 22°C and 37°C (**Figure 3B**) (Adamo *et al.*, 1999; He *et al.*, 2007). When *cbk1-8* and *exo84-121* cells were grown at 22°C, most Bgl2 was present in the external fraction of cells (the removed cell wall fraction). In contrast, when *cbk1-8* and *exo84-121* cells were shifted to 37°C, a significant amount of Bgl2 remained in the internal fraction of cells. Immunoblots of total cell extracts indicate that total Bgl2 level remain the same in all experimental conditions (data not shown). These data indicate that Cbk1 is required for efficient secretion of Bgl2 and invertase.

In wild type yeast, most secreted proteins remain in the periplasmic space and contribute to cell wall biogenesis, however pulse-labeling experiments revealed that some glycosylated proteins are secreted beyond the cell wall and into the medium (Adamo *et al.*, 1999). To determine if Cbk1 is required for protein secretion into the medium, we conducted media secretion assays with ³⁵S-methionine labeled cells. Wild type cells secreted a reproducible pattern of ³⁵S-labeled proteins into the medium (**Figure 3C**) (Gaynor and Emr, 1997; Schmitz *et al.*, 2008). One of the most prominent secreted proteins in the medium was previously identified as Hsp150 (Gaynor and Emr, 1997). We observed that the overall level of most ³⁵S-labeled proteins in the medium of *cbk1-8* cells was diminished at 37°C, with the exception of Hsp150 (**Figure 3C**). In addition, there were intriguing differences in the electrophoretic patterns of secreted proteins in *cbk1-8* cells that are discussed below. Collectively, these data suggest that Cbk1 is necessary for the efficient secretion of multiple,

but not all, cargos.

Cbk1 inhibition does not disrupt the establishment of actin cytoskeleton polarity.

Polarized secretion and bud emergence are mediated by the actin cytoskeleton, which is organized in cables and cortical actin patches (Pruyne *et al.*, 2004). Actin cables polarize at the incipient bud site and serve as tracks for the myosin-dependent transport of secretory vesicles to the plasma membrane, whereas cortical actin patches polarize to the incipient bud site and mediate endocytosis. Establishment and maintenance of actin cable and patch polarity are dependent on the rho-like GTPase Cdc42 and septins, the latter of which assemble into an hour-glass shaped ring at the mother-daughter cell junction and serve as scaffolds for the actin cytoskeleton and other proteins (Longtine and Bi, 2003; Pruyne *et al.*, 2004; Versele and Thorner, 2005).

To determine if Cbk1 regulates the establishment or maintenance of actin cytoskeleton polarity, we monitored actin cable and patch associated proteins Abp140 and Abp1 in synchronized *cbk1-as* cells (**Figure 4**), as in (Huckaba *et al.*, 2004). Notably, Cbk1-as inhibition did not prevent the establishment of actin cable associated Abp140-GFP or actin patch protein Abp1-GFP polarity to the incipient bud site (**Figure 4A, B, and D**). Polarization of both proteins occurred with similar kinetics in 1NA-PP1 and mock-treated *cbk1-as* cells. Cbk1 inhibition also did not interfere with Cdc42 GTPase or septin (Cdc3) localization (data not shown). These data indicate that the severe delay in bud emergence in drug-treated *cbk1-as* cells is not caused by gross disruption of the actin cytoskeleton organization.

Delayed bud emergence and growth might reflect a problem with the delivery of secretory vesicles and other cargo to the incipient bud site. Myosin V, encoded by *MYO2*, is the principle motor for actin-dependent organelle and post-Golgi vesicle transport (Pruyne *et al.*, 2004). In wild type cells, myosin V accumulates at the incipient bud site prior to bud emergence (Lillie and Brown, 1994). We monitored Myo2-GFP in synchronized *cbk1-as* cells to determine if Cbk1 is necessary for myosin V function or localization. We observed no detectable delay in the polarized localization of myosin V to the incipient bud site upon Cbk1 inhibition (**Figure 4C and D**). Thus, the delayed bud emergence in *cbk1* mutants is not caused by gross defects in actin polarity or myosin V activity.

Cbk1 regulates Sec2 and Sec4 localization.

Polarized growth and secretion are dependent on the Rab GTPase Sec4 and its guanyl nucleotide exchange factor Sec2 (Walworth *et al.*, 1989; Nair *et al.*, 1990). GTP-bound Sec4 stimulates the polarized delivery of post-Golgi secretory vesicles to the plasma membrane and associates with Sec15, a component of the exocyst vesicle tethering complex (Salminen and Novick, 1989; Walch-Solimena *et al.*, 1997; Guo *et al.*, 1999). GTP-bound Sec4 also contributes to the assembly of the exocyst complex, which tethers secretory vesicles to the plasma membrane and facilitates membrane fusion via interactions with SNARE proteins (Guo *et al.*, 1999; Hsu *et al.*, 2004). In wild type cells, Sec2 and Sec4 associate with vesicles and concentrate at the bud cortex via myosin V-dependent transport (Walch-Solimena *et al.*, 1997). In the absence of Sec2 or Sec4-GTP activity, many post-Golgi secretory vesicles accumulate in the cytoplasm because they are not delivered to the sites of polarized growth and are not tethered to the plasma membrane by the exocyst complex (Walworth *et al.*, 1989; Walch-Solimena *et al.*, 2000).

We monitored Sec2-GFP and Sec4-GFP localization in G0 block and released *cbk1-as* cells to determine if Cbk1 controls Sec2 or Sec4 function during polarized secretion. When Cbk1 was inhibited upon G0 release, Sec2 and Sec4 recruitment to the incipient bud site was severely delayed (**Figure 5**). In contrast, Cbk1 localization was not impaired in conditional *sec2-41* mutants at restrictive temperature, indicating that Cbk1 influences Sec2-Sec4 localization but not vice versa (**Figure 6C**). Intriguingly, polarized Cbk1 localization is abolished by disruption of the actin cytoskeleton with latrunculin A or by disruption of myosin V, indicating that the Cbk1 polarity establishment requires actin and myosin V, but is independent of Sec2-associated secretory vesicles (**Figure 6A and B**). These data support the hypothesis that Cbk1 regulates polarized secretion, at least in part, via Sec2 and Sec4. Alternatively, Cbk1 might influence Sec2 and Sec4 localization by regulating an upstream function, such as ER or Golgi trafficking.

Cbk1 binds to and phosphorylates Sec2.

Since Cbk1 is required for the establishment of Sec2 and Sec4 localization in G0 synchronized cells, it seemed plausible that Cbk1 regulates Sec2 or Sec4 directly. In support, all three proteins localize similarly in vivo (Novick and Brennwald, 1993; Walch-Solimena *et al.*, 1997; Weiss *et al.*, 2002) and two-hybrid experiments suggest that Cbk1 binds Sec2 (Racki *et al.*, 2000). Furthermore, like conditional *cbk1* mutants, conditional *sec2-41* cells arrest in G2 at

the Swe1-dependent morphogenesis checkpoint when shifted to 34°C (**Figure 7**). In the absence of Swe1, *sec2-41* cells do not arrest in G2 and thus accumulate as binucleate cells.

To further explore the interactions between Cbk1 and Sec2, we conducted affinity precipitation experiments with recombinant Sec2 and epitope-tagged Cbk1 from yeast cell extracts. In agreement with previous two-hybrid data, recombinant full length Sec2 specifically precipitated physiologically expressed Cbk1 and its regulatory subunit Mob2 (**Figure 8A** and unpublished data). We also found that Cbk1 binds the N-terminal half (amino acids 1-508) of Sec2, but does not efficiently bind the 1-204 amino acid region, which contains the GEF domain, or the 205-450 amino acid or C-terminal regions of Sec2 (**Fig 8A**). Moreover, recombinant Sec2 precipitates Cbk1 from extracts of $mob2\Delta$ cells, indicating that Sec2 can bind catalytically inactive Cbk1 (data not shown). Co-precipitation experiments with yeast extracts confirm the Cbk1 and Sec2 interaction (data not shown). These data support the model that Cbk1 regulates secretion via Sec2.

Sec2 is phosphorylated in vivo, however the kinase responsible for its phosphorylation has not been identified (Elkind *et al.*, 2000). To test if Cbk1 phosphorylates Sec2, we employed in vitro Cbk1 kinase assays using recombinant MBP-Sec2 fusion proteins as substrate (**Figure 8B and Supplement Figure S5**). As previously demonstrated (Weiss *et al.*, 2002; Nelson *et al.*, 2003), immunoprecipitated Cbk1 phosphorylates itself and the exogenous substrate histone H1 in vitro. Immunoprecipitated Cbk1 also phosphorylated MBP-Sec2 but not MBP (maltose binding protein MalE) in vitro. In the absence of the Cbk1 regulatory subunit Mob2, Cbk1 kinase activity is greatly diminished (Weiss *et al.*, 2002). Significantly, Cbk1 from *mob2* Δ cells failed to phosphorylate Sec2-MBP in vitro, confirming that Sec2 phosphorylation in vitro is dependent on active Cbk1 (**Figure 8B**). In addition, Cbk1 did not efficiently phosphorylate N-terminal or C-terminal truncated forms of Sec2 (Sec2¹⁻⁵⁰⁸, Sec2¹⁻²⁰⁴ or Sec2²⁰⁵⁻⁴⁵⁰) in vitro, suggesting that native structure of Sec2 is important for Cbk1-dependent phosphorylation (data not shown). These data suggest Cbk1 and Sec2 are functionally related and support the model that Cbk1 controls Sec2 function.

Cbk1 is required for post-Golgi vesicle formation.

If Cbk1 regulates secretion and bud emergence exclusively via Sec2 or Sec4, then Cbk1 inhibition should cause an accumulation of post-Golgi vesicles, as shown for conditional *sec2* and *sec4* mutants. Alternatively, if Cbk1 functions upstream of Sec2 or Sec4, then Cbk1 inhibition might interfere with post-Golgi vesicle accumulation in conditional *sec2* or *sec4*

mutants. Accordingly, we analyzed the phenotypes of conditional *cbk1-as* and *cbk1-8* cells by conventional electron microscopy (EM). Significantly, Cbk1 inactivation in *cbk1-as* or in conditional *cbk1-8* cells did not cause an accumulation of vesicles (**Figure 9 and** data not shown). EM morphological analysis did not reveal any obvious structural cell wall defects in *cbk1-as* and *cbk1-8* cells. However, both *cbk1-as* and *cbk1-8* cells are sensitive to Calcofluor White, indicating that they have elevated levels of cell wall chitin. The lack of vesicle accumulation in *cbk1* mutants indicates that Cbk1 does not mediate bud growth or secretion exclusively via Sec2 or Sec4 and suggests that Cbk1 has multiple roles in the secretory pathway.

If Cbk1 functions prior to Sec2-Sec4 in the secretory pathway, then Cbk1 inhibition might prevent or diminish the accumulation of post-Golgi secretory vesicles in conditional *sec2* or exocyst mutants. Accordingly, we synchronized *cbk1-as sec2-41* and *cbk1-as sec6-4* double mutant cells and allowed them to bud (and enter S phase) prior to inhibiting Cbk1 for 30 minutes. We then shifted the cells to 37° C for 15 min to inactivate Sec2 or Sec6 (a component of the exocyst tethering complex) and quantified the average number of secretory vesicles per EM section. Strikingly, Cbk1 inhibition diminished vesicle accumulation in *sec2-41* mutants by approximately ~35% at 37°C (**Fig 9**). Cbk1 inhibition also causes a ~20% reduction in secretory vesicles in *sec2-41* cells at 22°C. We observed a similar reduction in secretory vesicle accumulation in *sec6-4* cells at 37°C (**Supplement Figure S8**). These data indicate that Cbk1 is required for the efficient formation of post-Golgi secretory vesicles and implicate RAM signaling in regulating endoplasmic reticulum (ER) or Golgi trafficking functions.

Cbk1 mutants are hypersensitive to hygromycin B and display glycosylation defects, suggesting a role in Golgi trafficking.

If Cbk1 and the RAM network are required for Golgi function, then conditional Cbk1 mutants should display glycosylation defects. Golgi trafficking and glycosylation mutants are often hypersensitive to the drug hygromycin B (Dean, 1995; Dean and Poster, 1996). We therefore assayed for hygromycin B sensitivity and observed that *cbk1-8* cell growth was severely impaired on medium containing 50 ug/ml hygromycin B at 22°C. The *cbk1-8* cells were as sensitive to hygromycin B as several severe Golgi trafficking mutants, including the conserved oligomeric Golgi (COG) complex mutants *cog1* Δ and *cog8* Δ . In contrast, wild type and *sec2-41* cells were resistant to 50 ug/ml hygromycin B (**Figure 10A**). Likewise, *ssd1* Δ and *ace2* Δ cells were not sensitive to hygromycin B (data not shown). The enhanced hygromycin

B sensitivity of *cbk1-8* cells is consistent with the model that Cbk1 function is important for glycosylation. The lack of hygromycin B sensitivity for *sec2-41*, *ssd1* Δ and *ace2* Δ cells suggest that the hygromycin B sensitive phenotype of *cbk1* cells is not simply caused by misregulation of Sec2, Ssd1 or Ace2 transcription factor.

To further explore the role of Cbk1 in secretion and/or Golgi function, we assayed for glycosylation of several secreted cargos. ER or Golgi trafficking mutants often disrupt the glycosylation or processing of cargos, such as Carboxypeptidase Y (CPY). In wild type cells, CPY is synthesized and modified in the ER as preproenzyme (P1) and is further glycosylated in the Golgi to yield a P2 form, which is subsequently transported to the vacuole where it is proteolytically processed to the mature form (M). ER to Golgi trafficking or glycosylation mutants cause an accumulation of the P1 or P2 forms of CPY (Gaynor and Emr, 1997; Nothwehr *et al.*, 2000). We pulse-labeled *cbk1-8* cells with ³⁵S-Met at 37°C and analyzed CPY by autoradiography and observed no detectable defect in CPY processing (**Figure 10B**). In contrast, CPY processing was incomplete in *vps1* Δ and *cog1* Δ cells, which are defective in Golgi to vacuole trafficking and intra-Golgi trafficking, respectively. These data indicate that Cbk1 inhibition does not globally inhibit ER to Golgi trafficking, glycosylation or Golgi to vacuole trafficking.

It is possible that Cbk1 controls the trafficking and/or glycosylation of a subset of cargos. Thus, we investigated whether Cbk1 kinase is required for invertase glycosylation. In wild type cells, invertase is glycosylated in the Golgi, which causes it to migrate as a characteristic smear on protein gels. In Golgi trafficking mutants, such as $cog1\Delta$, invertase is hypoglycosylated and thus migrates more rapidly on protein gels ((Gaynor and Emr, 1997; Whyte and Munro, 2001) and Figure 10C). Immunoblot analysis demonstrates that total invertase levels are diminished in *cbk1-8* cells (Figure 10C). More significantly, the electrophoretic mobility of invertase is reproducibly aberrant in cbk1-8 cells at 22°C than in similarly prepared wild type cells (Figure 10C, see brackets). When *cbk1-8* cells were grown at 22°C, invertase migrates slightly faster on protein gels than invertase from wild type cells, suggesting that invertase is moderately hypoglycosylated in *cbk1* mutants. Curiously, the overall electrophoretic pattern of invertase from *cbk1-8* cells at 37°C was not as dramatically affected. Nevertheless, these results are consistent with the hygromycin B sensitivity phenotype of *cbk1-8* cells at 22°C and suggest that Cbk1 is required for the glycosylation of some proteins.

Many proteins involved in cell wall biosynthesis are glycosylated in the Golgi. The cell

wall protein Sim1 was identified as a dosage suppressor of *cbk1-8* mutants (see below) and *ram* Δ mutants (Du and Novick, 2002; Kurischko *et al.*, 2005). Sim1 is a glycosylated protein that migrates much slower on protein gels than predicted from its amino acid composition, which is 48 kD (Velours *et al.*, 2002). In wild type cells Sim1-CFP migrates as ~127 kD smear on one-dimensional protein gels (**Figure 10D**). Impaired Golgi function in *cog1* Δ cells leads to increased electrophoretic mobility of Sim1-CFP, due to impaired glycosylation (**Figure 10**). To determine if Cbk1 is required for Sim1 glycosylation, we assayed the electrophoretic mobility of Sim1-CFP migrates faster in *cbk1-8* cells at 37°C, than in similarly treated wild type cells or in *cbk1-8* cells at 22°C (**Figure 10D, see brackets**). Moreover, a small amount of Sim1-CFP migrates as a discrete ~83kD band in *cbk1-8* cells at 37°C. There are differences in the electrophoretic patterns of Sim1-CFP from *cbk1-8* and *cog1* Δ cells, indicating that Cbk1 does not function exclusively via Cog1. Nevertheless, these data strongly suggest that Cbk1 kinase is required for proper Sim1 and invertase glycosylation and support the model that Cbk1 and the RAM network regulate Golgi trafficking and/or sorting.

Media secretion assays support a role for Cbk1 in glycosylation. To investigate if Cbk1 is required for the glycosylation of other secreted proteins, we analyzed the electrophoretic mobilities of proteins that are secreted into the medium. As noted above, the overall level of protein secretion (with the exception of Hsp150) was diminished in *cbk1-8* cells at 37°C (Figure 3C). In addition, at least three unidentified secreted proteins appear to migrate more rapidly on protein gels of *cbk1-8* cells, suggesting that they are hypo-glycoslyated (see brackets in Fig 3C). Hsp150 glycosylation appears normal in *cbk1-8* cells. By comparison, the Golgi mutant *cog1* Δ does not efficiently secrete or glycosylate most cargos (Figure 3C). Hsp150 from *cog1* Δ cells is hypo-glycosylated and migrates more rapidly on protein gels. These data suggest that Cbk1 is required for efficient glycosylation and secretion of a subset of cargos and support the model that Cbk1 regulates Golgi trafficking. Nevertheless, definitive proof that the relevant differences in patterns of secreted proteins are caused by aberrant Cbk1-dependent glycosylation, and not by differences in the overall content of secreted proteins, will require identification of the proteins. Thus far, the low abundance of the secreted proteins has impeded their purification and identification.

We conducted parallel media secretion assays with $ace2\Delta$ and ssd1-d cells to determine if differences in Cbk1-dependent glycosylation or secretion require the Cbk1-

regulated transcription factor Ace2 or Ssd1. The protein mobility of all but one secreted protein from *ace2* Δ cells and all proteins in *ssd1-d* cells appear normal (compare protein patterns to that of wild type cells), suggesting that glycosylation and Golgi trafficking are not dependent on Ace2 or Ssd1. Strikingly, both *cbk1-8* and *ace2* Δ cells failed to secrete a ~125 kD protein that is only second in prominence to Hsp150 (**Fig 3C arrow**), suggesting that the expression or secretion of the protein is dependent on Ace2 transcription. The identity of the 125 kD protein is unknown, however we have established that it is not the Ace2-regulated Dse4/Eng1 gluconase or Cts1 chitinase (**Supplement Figure S6**).

The overall pattern of secreted proteins from $cbk1\Delta ssd1-d$ cells was nearly identical to that of ssd1-d and wild type cells, suggesting that, in the absence of functional Ssd1, Cbk1 is dispensable for secretion or glycosylation of most cargos. There were two notable differences in the pattern of secreted proteins from $cbk1\Delta ssd1-d$ cells and ssd1-d cells. First, the secretion (or expression) of the Ace2-dependent 125 kD band was diminished in $cbk1\Delta ssd1-d$ cells, which is consistent with the hypothesis that the 125 kD protein's expression is Ace2-dependent. Second, one of the glycosylated proteins from $cbk1\Delta ssd1-d$ cells appears to migrate as two bands on protein gels, suggesting that its glycosylation (or other post-translational modification) is diminished in the absence of Cbk1 (Figure 3C, see arrowheads). This observation suggests that at least one protein is glycosylated in a Cbk1-dependent and Ssd1-independent fashion. Nevertheless, in the absence of functional Ssd1, Cbk1 is dispensable for the glycosylation and secretion of other cargos.

Cbk1 is not required for FM4-64 uptake.

A reduction in Golgi trafficking or exocytosis could be caused by defects in endocytosis or membrane recycling. Thus, we treated *cbk1-as* cells with the fluorescent endocytic dye FM4-64 to determine if Cbk1 inhibition interferes with endocytosis. Wild type cells and untreated *cbk1-as* cells readily take up FM4-64, as detected by prominent labeling of endocytic vesicles and vacuoles (**Figure 11**). 1NA-PP1-treated *cbk1-as* cells also internalized FM4-64, but displayed aberrant vacuole morphology. The majority of inhibited *cbk1-as* cells contained smaller vacuole structures. The FM4-64 structures co-localized with the vacuole dye CellTracker Blue CMAC (Invitrogen) in both *cbk1-as* and wild type cells. The smaller vacuole structures in *cbk1* cells were more definitively observed upon hypertonic expansion. These data indicate that Cbk1 kinase activity is not essential for endocytosis, but demonstrate that Cbk1 is required for normal vacuolar morphology. Early and late Golgi trafficking defects

cause similar vacuole morphology defects (Lafourcade *et al.*, 2004). Thus, these findings are consistent with a role for Cbk1 in trafficking.

Genetic interactions support a role for Cbk1 in regulating Golgi function.

To gain insight to the essential function of Cbk1, we screened a yeast DNA library for dosage (high copy) suppressors of the temperature sensitive phenotypes of *cbk1-8* mutants. The complete set of dosage suppressors will be reported in a separate manuscript. Several dosage suppressors were previously identified as suppressors of *cbk1* Δ lethality (Du and Novick, 2002; Kurischko *et al.*, 2005). These include genes involved in cell wall biogenesis (*CCW12, SIM1, SRL1*). Significantly, we also discovered that several *cbk1-8* dosage suppressors encode Golgi localized mannosyltransferases or regulators, Mnn1, Mnn4, Mnn6, Mnn9, Ktr2, Pmt1, and Pmt3 (**Figure 12, Supplement Table S2**). Each of the mannosyltransferase plasmids also suppressed the hygromycin B sensitivity of *cbk1-8* mutants. These data support a role for Cbk1 in Golgi regulation and suggest that the cell growth and cell integrity defects of *cbk1-8* cells derive from Golgi misregulation. Moderate mannosyltransferase overexpression may ameliorate the effects of Golgi misregulation in *cbk1-8* cells by restoring or elevating the glycosylation of Golgi-derived cargos.

Mutations in functionally related genes often cause slow growth or death when combined with each other (Kaiser and Schekman, 1990; Finger and Novick, 2000; Schuldiner *et al.*, 2005). Thus, we also investigated the genetic relationships between *CBK1* and several genes involved in vesicle trafficking. We discovered that *cbk1-8* is lethal in combination with *sec2-41, cog1* Δ , *ypt6* Δ and *sec16-2* (**Figure S7**). Cog1 and Ypt6 mediate Golgi trafficking and Sec16 is involved in ER to Golgi trafficking. These data are consistent with the finding that a conditional RAM mutant (*tao3*) is lethal in *gyp1* Δ strains, which lack a nonessential cis-Golgi localized GAP for Rab-GTPases (Du and Novick, 2002). Intriguingly, late secretory mutants, such as *sec2-41* and exocyst mutants are not commonly lethal in combination with Golgi trafficking or glycosylation mutants and vice versa (Finger and Novick, 2000). These genetic interactions support the model that Cbk1 and the RAM network are required for multiple steps in secretion, including: 1) an early role in mediating Golgi trafficking and 2) a later role in Sec2-dependent trafficking and secretion.

Och1 mannosyltransferase mislocalizes in *cbk1-8* cells.

To investigate whether Golgi trafficking is disrupted in *cbk1-8* cells, we monitored the localization of Och1 mannosyltransferase, a common marker for the cis-Golgi (Gaynor *et al.*, 1994). In wild type cells at 22° and 35°C, Och1 localizes to cis-Golgi vesicles, which are evident as numerous cytoplasmic puncta (**Figure 12**). In contrast, Och1-GFP localized to smaller, more dispersed puncta in *cbk1-8* cells at 35°C (**Figure 12**). The more dispersed Och1 distribution in *cbk1-8* cells was similar to, although less pronounced than that described for *cog* mutants (Bruinsma *et al.*, 2004), which are deficient in Golgi enzyme retrieval. We also observed that the Och1-GFP fluorescence is brighter in *cbk1-8* cells than wild type cells, which was expected because Och1 expression is elevated in RAM and several cell wall biosynthesis mutants (Yamamoto and Jigami, 2002; Nelson *et al.*, 2003). Together, the hypo-glycosylation and Och1 mislocalization phenotypes of *cbk1* mutants may implicate Cbk1 in Golgi enzyme retrieval.

DISCUSSION

Mutations in the RAM signaling network were previously shown to cause morphogenesis and cellular lysis defects, however the specific roles of the RAM network had not been determined (Du and Novick, 2002; Vink *et al.*, 2002; Nelson *et al.*, 2003; Kurischko *et al.*, 2005). This study clearly establishes that the *S. cerevisiae* Lats/NDR kinase Cbk1 is required for Golgi function and secretion, thereby revealing a previously unknown function of the RAM signaling network. In support, we establish that Cbk1 kinase activity is essential for bud emergence and cell growth independently of actin polarity establishment. Cbk1 inactivation disrupts Och1 mannosyltransferase localization, invertase and Sim1 glycosylation, efficient post-Golgi vesicle formation and secretion of several proteins. In addition to Golgi regulation, it is likely that Cbk1 kinase also regulates Sec2, since Cbk1 binds to and phosphorylates Sec2 in vitro and is necessary for Sec2 and Rab GTPase Sec4 recruitment to the incipient bud site. The sum of these data suggest that Cbk1 and the RAM network regulate growth and maintain cell integrity by controlling Golgi and Sec2-Sec4 functions.

Cbk1 and Golgi function. Several lines of evidence implicate Cbk1 in regulating Golgi function. First, *cbk1* mutants display enhanced sensitivity to hygromycin B, which is a phenotype that strongly correlates with diminished or aberrant glycosylation (Dean, 1995; Dean and Poster, 1996). Moreover, glycosylation of invertase, the cell wall protein Sim1 and

several secreted proteins is significantly diminished in conditional *cbk1* mutants. Significantly, Cbk1 inhibition disrupts the localization of the cis-Golgi enzyme Och1 and reduces the amount of trans-Golgi-derived vesicles in late secretory mutants by 20-35%. Genetic interactions, such as the identification of several mannosyltransferases genes as *cbk1-8* dosage suppressors, also support a role for Cbk1 in regulating Golgi function. We propose that mannosyltransferase overexpression rescues the temperature sensitivity and hygromycin B sensitivity of *cbk1-8* mutants by compensating for the diminished glycosylation of Golgi-derived cargos.

The Golgi-related phenotypes of conditional *cbk1* mutants may reflect direct mechanisms for Cbk1 in regulating intra-Golgi trafficking, recycling or structure. For example, Cbk1 may regulate the Golgi via direct phosphorylation of Golgi trafficking mediators, such as those that regulate the retrieval of Golgi enzymes required for glycosylation and/or secretion. Indeed, other protein kinases, such as Cdk1, were shown to directly regulate the Golgi structure (Draviam *et al.*, 2001; Preisinger and Barr, 2005). The hypoglycosylation phenotypes of *cbk1* mutants might reflect decreased Golgi enzyme activity caused by protein mislocalization, aberrant intra-Golgi trafficking or alterations in the luminal environment of the Golgi (de Graffenried and Bertozzi, 2004; Schmitz *et al.*, 2008). In support, *cbk1* mutants and the Golgi trafficking *cog* mutants both cause similar Och1 mislocalization phenotypes. Thus, Cbk1 might regulate Golgi enzyme retrieval mechanisms. In principle, some of the mutant *cbk1* phenotypes could be caused by defective ER function or trafficking, however unimpaired CPY processing activities in *cbk1* mutants strongly argue against a role for Cbk1 in ER-mediated glycosylation, ER to Golgi or Golgi to vacuole trafficking.

It is possible that Cbk1 controls Golgi function indirectly. For example, Cbk1 might regulate the expression of glycosylation mediators and/or Golgi trafficking proteins. Indeed, several Golgi mannosyltransferases and associated co-factors are transcriptionally regulated (Igual *et al.*, 1996; Jigami, 2008). While this indirect mechanism is formally possible, the Cbk1-dependent Ace2 transcription factor cannot be responsible for the Golgi-related phenotypes of *cbk1* mutants, since deletion of *ACE*2 does not cause cellular lysis, hygromycin B sensitivity or detectable hypoglycosylation defects. Nevertheless, Cbk1 might control Golgi enzyme expression independently of Ace2. Indeed, Och1 mannosyltransferase expression is induced by HOG pathway inactivation and is enhanced in RAM deletion mutants, likely as a consequence of cell wall integrity pathway activation (Nelson *et al.*, 2003; Narang *et al.*, 2008).

Nonetheless, Och1 induction alone cannot account for the diminished glycosylation of invertase and Sim1 in conditional *cbk1* mutants.

Interactions between Cbk1 and Sec2 suggest a post-Golgi function for Cbk1 in secretion. The relationship between Cbk1 and Sec2 suggests that Cbk1 regulates bud emergence and secretion, at least in part, via the Rab GTPase Sec4 during post-Golgi trafficking and secretion. Sec2 associates with trans-Golgi derived secretory vesicles and mediates the guanyl nucleotide exchange to yield active Sec4-GTP, which stimulates vesicle trafficking to the plasma membrane and contributes to exocyst assembly. We demonstrated that Cbk1 binds to and phosphorylates full length Sec2 in vitro. Notably, Cbk1 binds Sec2 within the N-terminal ~500 amino acid region (Sec2¹⁻⁵⁰⁸), but does not efficiently bind a smaller fragment (Sec2¹⁻²⁰⁴) that contains the GEF domain. Currently, the Cbk1-dependent phosphorylation sites of Sec2 are unknown. In vivo labeling experiments (analyzed on one-dimensional gels) did not reveal any significant differences in Sec2 phosphorylation levels from wild type and *cbk1-8* cells (data not shown), however modest but functionally relevant changes in phosphorylation might be obscured if Sec2 is phosphorylated by multiple kinases. The synthetic lethality of *cbk1-8* and *sec2-41* alleles is also consistent with a cooperative function for Cbk1 and Sec2 in growth control (**Supplement Figure S7**).

The Cbk1-Sec2 interactions support the model that Cbk1 directly regulates the late secretory pathway. Our data indicate that Cbk1 kinase is necessary for the recruitment of Sec2 and Sec4 to the incipient bud site in synchronized cells. Although Sec2 and Sec4 are critical for exocyst assembly and protein secretion (He *et al.*, 2007), the primary consequence of Sec2-Sec4 misregulation in *cbk1* mutants is not known. The apparent Sec2-Sec4 mislocalization and secretion defects of *cbk1* mutants could be caused by diminished secretory vesicle formation or by misregulation may be masked by an earlier defect in Golgi function or secretory vesicle formation. Further insight to the purpose of the Cbk1-Sec2 interactions will come from the identification and functional analysis of Sec2 phosphorylations. Nevertheless, taken together with the apparent Golgi phenotypes, the Cbk1-Sec2 interactions suggest that Cbk1 regulates multiple steps in trafficking that are critical for cell growth and maintenance of cell integrity.

Many of the *cbk1* mutant phenotypes, including Swe1 checkpoint activation, could be caused by diminished Golgi or diminished Sec2-Sec4 function. Some Golgi mutants exhibit enhanced hygromycin B sensitivity, diminish glycosylation activity and trigger the morphogenesis checkpoint, just as observed for *cbk1* mutants (Mondesert and Reed, 1996; Mondesert *et al.*, 1997). Conditional *sec2* mutants also trigger the Swe1 morphogenesis checkpoint activation (**Figure 7**). Thus, we speculate that loss of Cbk1 or RAM activity ultimately causes aberrant cell wall biosynthesis via misregulation of both Golgi and Sec2-Sec4, thereby leading to checkpoint activation and diminished cell integrity.

Functional relationship between RAM and Ssd1. Curiously, the lethality of CBK1 and RAM gene deletions is suppressed by loss-of-function mutations in the enigmatic SSD1 gene, which encodes a conserved RNA binding protein of unknown function (Sutton et al., 1991; Costigan et al., 1992; Du and Novick, 2002; Jorgensen et al., 2002). Although it is probable that Cbk1 has a modest role in Golgi regulation in the absence of Ssd1 (see Figure **3C**, *cbk1* Δ *ssd1* Δ lane), Cbk1 is not essential for Sec2 function, bud emergence or maintenance of cell integrity in *ssd1*^Δ cells (Racki *et al.*, 2000; Bidlingmaier *et al.*, 2001; Weiss et al., 2002; Nelson et al., 2003; Kurischko et al., 2005). Moreover, ssd1^Δ cells do not exhibit secretion or hypoglycosylation defects. Nevertheless, $ssd1\Delta$ and ssd1-d loss-of-function mutations share synthetic lethal or enhanced phenotypes with mutations in a variety of genes encoding trafficking proteins, such as Rab and Ras family GTPase and COG proteins (Li and Warner, 1998; Rosenwald et al., 2002; Collins et al., 2007). We speculate that Ssd1 functions as an inhibitor of Cbk1-dependent trafficking and secretion events and that Cbk1, in turn, negatively regulates Ssd1. It is probable that Cbk1-dependent growth control also involves the regulation of Ssd1-dependent RNA functions. In support, Cbk1 binds Ssd1 by two-hybrid Moreover, the RAM network protein Tao3 was recently shown to be (Racki *et al.*, 2000). involved in small nucleolar RNA maturation and localization (Qiu et al., 2008). Further work is necessary to elucidate the mechanism of Ssd1 function with regard to Cbk1 and RAM network function.

Given the conservation of RAM network proteins and vesicle trafficking regulators, our data suggest a conserved role for Cbk1-related kinases in regulating Golgi trafficking and secretion. In agreement, mutations in *S. pombe orb6* (*CBK1*) and other RAM orthologs halt cellular growth and cause a dramatic loss of polarized growth (Verde *et al.*, 1998; Hirata *et al.*,

2002; Hou *et al.*, 2003; Huang *et al.*, 2005; Kanai *et al.*, 2005). Moreover, *Drosophila* and *C. elegans* NDR mutations cause morphogenic and developmental defects in a variety of cell types, including neuronal tissues where NDR mutations cause neuronal tiling defects (Geng *et al.*, 2000; Zallen *et al.*, 2000; Emoto *et al.*, 2004; Gallegos and Bargmann, 2004). These phenotypes could be caused by compromised Golgi, Rab GTPase or exocyst function. In support, Rab GTPases regulate vesicle trafficking and influence polarized morphogenesis and development of a variety of cell types (Stenmark and Olkkonen, 2001). Furthermore, the evolutionary conserved eight-subunit exocyst complex was shown to regulate post-Golgi vesicle transport and to localize to regions of membrane expansion, including growth cones and the tips of growing developing neurons (Hsu *et al.*, 2004). Thus, we suggest that a conserved function for Cbk1-related kinases among eukaryotic cells is to regulate cell growth and development via modulating Golgi function and secretion.

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Table 1. Yeast strains

Strain	Genotype
BY4741	MATa ura3 Δ 0 his3 Δ 0 leu2 Δ 0 met15 Δ 0
FLY869	MATa CBK1-GFP::KANMX ssd1-d
FLY905	MATa CBK1-myc::KANMX ssd1-d
FLY906	MATa CBK1-myc::KANMX mob2∆::HIS3 ssd1-d
FLY1050	MATa SIM1-CFP::KANMX ssd1-d
FLY1509	MATa/x cbk1∆::KANMX/CBK1
FLY1632	MAT <i>x ace2</i> Δ:: <i>KANMX</i>
FLY2084	MATa cbk1-as2::HIS3::cbk1_::KANMX
FLY2086	MATx cbk1-as2::HIS3::cbk1_::KANMX
FLY2184	MATx ssd1_::NATMX
FLY2204	MATa cbk1-as2::HIS3::cbk1_::KANMX ssd1-d
FLY2236	MATa cbk1-as2::HIS3::cbk1\Delta::KANMX swe1Δ::KANMX
FLY2288	MATa CBK1-myc::KANMX SSD1-HA::HIS3
FLY2293	MATa cbk1\[]:KANMX ssd1\[]:NATMX
FLY2339	MATa ABP1-GFP::KANMX cbk1-as2::HIS3-cbk1∆::KANMX
FLY2373	MATa/x SEC2-GFP::KANMX/ SEC2-GFP::KANMX_cbk1-as2::HIS3::cbk1∆::KANMX/cbk1- as2::HIS3::cbk1∆::KANMX
FLY2386	MATa/x ABP140-GFP::KANMX/ ABP140-GFP::KANMX cbk1-as2::HIS3-cbk1∆::KANMX/ cbk1-as2::HIS3- cbk1∆::KANMX
FLY2389	MATa sec2-41 swe1::KANMX
FLY2452	MATx SEC2-myc::KANMX CBK1-HA::HIS3
FLY2485	MATa sec2-41 CBK1-GFP::KANMX
FLY2489	MATa MYO2-GFP::KANMX cbk1-as2::HIS3::cbk1∆::KANMX
FLY2531	MATa cbk1-as2::HIS3-cbk1∆::KANMX [pSEC4-GFP]
FLY2535	MATa CBK1-GFP::KANMX MYO2::HIS3
FLY2539	MATa CBK1-GFP::KANMX myo2-66::HIS3
FLY2574	MAT <i>x bgl2</i> Δ:: <i>KANMX</i>
FLY2575	MATx vps1_::KANMX
FLY2661	MAT <i>a cbk1-8::HIS3-cbk1</i> Δ::KANMX
FLY2704	MATa/x cbk1-8::HIS3::cbk1\Delta::KANMX/ cbk1-8::HIS3::cbk1Δ::KANMX SEC2-GFP::KANMX/ SEC2-GFP::KANMX
FLY2764	MAT <i>x cog1</i> Δ:: <i>KANMX</i>
FLY2765	MAT <i>x cog8∆::KANMX</i>
FLY2766	MAT <i>x ypt6</i> _::KANMX
FLY2567	MATa SUC2-Myc:KANMX
FLY2789	MATa cbk1-as::HIS3::cbk1_::KANMX sec6-4 SSD1
FLY2792	MATa cbk1-as::HIS3::cbk1_::KANMX sec2-41 SSD1
FLY2802	MATx SUC2-Myc:KANMX cog1
FLY2847	MATa/x SIM1-CFP::KANMX/ SIM1-CFP::KANMX cbk1-8::HIS3-cbk1∆::KANMX/ cbk1-8::HIS3-cbk1∆::KANMX
FLY2848	MATa/x SUC2-myc::KANMX/ SUC2-myc::KANMX cbk1-8::HIS3::cbk1∆::KANMX/ cbk1-8::HIS3::cbk1∆::KANMX
FLY2866	MAT <i>x dse4</i> Δ::KANMX
FLY2867	MATa DSE4-TAP::HIS6MX

 FLY2865
 MATx SIM-CFP::KANMX cog1∆::KANMX

 FLY2885
 MATa CTS1-TAP::HIS6MX

 FLY2886
 MATx cts1∆::KANMX

 FLY2922
 MATa cbk1-8::HIS3::cbk1∆::KANMX OCH1-GFP::URA3

 GY2479
 MATa exo84-121

 JLY284
 MATx OCH1-GFP::URA3

 NY416
 MATx sec16-2

 NY770
 MATx sec2-41

note: MATx = MAT α

FIGURE LEGENDS

Figure 1. Cbk1 kinase inhibition interferes with bud emergence and induces a Swe1dependent cell cycle delay. (A) Cbk1 inhibition delays bud emergence. *cbk1-as* (FLY2084) and wild type cells (BY4741), were released from G1 arrest and placed into fresh medium with 0um (DMSO), 5uM, 10uM 1NA-PP1. The percentage of budded cells was plotted over time (n>200 cells). (B) Cbk1 inhibition induces a Swe1-dependent G2 arrest. *cbk1-as* and *cbk1-as swe1* Δ (FLY2236) cells were released from G0 into medium +/- 5uM 1NA-PP1 and at designated time points samples were processed for FACS to reveal the relative DNA content in the cell population. 4 hours after release from G0 block, 0% and 11% of mock and drug treated *cbk1-as swe1* Δ cells contained two nuclei. T0 = time of 1NA-PP1 addition. Note the modest delay in S phase initiation. See supplement Figure S3 for data from a similar experiment. (C) Cbk1 inhibition induces a modest Ssd1-independent delay in bud emergence. *cbk1-as ssd1-d* cells (FLY2204) were synchronized in G1 and released into medium +/- 5 um 1NA-PP1. The percentage of buds was scored over time (n>200). Similar phenotypes were observed with *cbk1* Δ *ssd1* Δ cells (data not shown).

Figure 2. Cbk1 kinase activity is required for bud growth. (A) Bud length was measured in synchronized cells upon Cbk1 inhibition. G1 synchronized *cbk1-as* cells (FLY2084) were incubated until ~50% of the cells formed small buds. At that time (T=0), 0um (DMSO) or 10uM 1NA-PP1 was added and samples were taken and fixed in 3.7% formaldehyde at designated time points. (B) Cbk1 kinase inhibition prevents cell wall deposition. Asynchronously growing *cbk1-as* cells were treated with ConA-Alexa⁵⁹⁴ and transferred into ConA-free medium +/- 5um 1NA-PP1 for 60 minutes.

Figure 3. Cbk1 is required for secretion of invertase, Bgl2 endo-beta-1,3-glucanase and other proteins.

(A) Invertase secretion is diminished in conditional *cbk1-8* cells. The ratio of secreted invertase / total invertase is plotted for wild type cells (BY4741), *cbk1-8* cells (FLY2661) and the conditional exocyst mutant *exo84-121* (GY2479), see Methods.

(B) The cell wall protein Bgl2 accumulates inside *cbk1-8* and *exo84-121* cells at 37°C. Top panel: immunoblot of Bgl2 in the internal fraction (cytoplasmic) of wild type, *cbk1-8* and *exo84-121* cells shifted to restrictive temperature. Middle panel: immunoblot of Bgl2 from the external

(solubulized cell wall) fraction of cells. Bottom panel: Immunoblot of the internal fractions was stripped and re-probed with anti-ADH (Abcam) as a protein loading. $bg/2\Delta$ cells (FLY2574) were used as a negative control.

(C) Autoradiogram of media secretion assays for wild type (BY4741), *cbk1-8* (FLY2704), *cog1* Δ (FLY2764), *ace2* Δ (FLY1632), *ssd1* Δ (FLY2184) and *cbk1* Δ *ssd1* Δ (FLY2293) cells. Proteins secreted into the media of ³⁵S-methionine labeled cells were TCA precipitated, separated on protein gels, and processed for autoradiography. The brackets denote several proteins that appear to be hypoglycosylated in *cbk1-8* cells. Hsp150 is noted and the arrowhead below Hsp150 points to a protein whose expression and/or secretion is dependent on Cbk1 and Ace2. The two arrows on the right point to a protein that appears to split into two bands in *cbk1* Δ *ssd1* Δ double mutant cells.

Figure 4. Cbk1 kinase inhibition does not interfere with actin cytoskeleton polarization.

(A) G0 synchronized *cbk1-as* cells expressing the actin patch associated Abp1-GFP (FLY2339), (B) the actin cable associated Abp140-GFP (FLY2386) or (C) type V myosin Myo2-GFP (FLY2489) were released into medium +/- 5um 1NA-PP1. The percentage of budded cells with polarized GFP was scored over time (---- 0um 1NA-PP1; - - 5uM 1NA-PP1). 1NA-PP1 was added 30 min after G0 release. T0 = time of 1NA-PP1 addition. (D) Representative images of 1NA-PP1 treated cells from A-C at T= 60 min.

Figure 5. Cbk1 kinase inhibition delays Sec2 and Sec4 localization to the incipient bud site. *cbk1-as* cells expressing Sec2-GFP and GFP-Sec4 (FLY2373 and FLY2531) were released from G0. After 30 min of release, 0um or 5uM 1NA-PP1 was added (T=0) and the percentage of cells with polarized Sec2 and Sec4 was scored over time (— 0um 1NA-PP1; - - 5uM 1NA-PP1). Representative images of cells are shown for the T= 60 min time points.

Figure 6. Cbk1p localization is dependent on actin and myosin V, but is Sec2 independent. (A) Cbk1 kinase localization is dependent on the actin cytoskeleton. Logarithmically growing Cbk1-GFP cells (FLY869) were treated with Latrunculin A (LatA) for 30 min (middle panel). Almost all Cbk1-GFP disappears from the cortex of buds, bud neck and the nucleus. Upon LatA washout (right panel), Cbk1-GFP reappears on the cell cortex and bud neck within 30 min. (B) Cbk1 polarity establishment is dependent on myosin V (Myo2). (C) Cbk1 polarity establishment is independent of Sec2. G0 synchronized *CBK1*-

GFP myo2-66 (FLY2539), *CBK1-GFP* (FLY2535), and *CBK1-GFP sec2-41* cells (FLY2485) were released into 36°C medium and the percentage of cells with polarized Cbk1-GFP was scored over time. Samples were methanol/acetone fixed at designated time points and observed by fluorescence microscopy. Representative images of cells at T= 60 min are shown on the right.

Figure 7. Conditional sec2 mutants induce the Swe1-dependent checkpoint. (A) G0 synchronized *sec2-41* (NY770) and *sec2-41 swe1* Δ cells (FLY2389) were transferred to fresh medium at 22°C and 34°C. Samples were fixed and processed for FACS analysis. *sec2-41 SWE1*+ cells arrest in G2 (with post S phase DNA content and unsegregated chromatin) at 34°C. (B) After 4h at 34°C, 0% of the sec2-41 SWE1 cells are binucleate. By 4 hours at 34°C almost all *sec2-41 swe1* Δ cells remain unbudded and 45% are binucleate (n>200).

Figure 8. Cbk1 binds and phosphorylates Sec2. (A) Immunoblot showing the results of Sec2 affinity precipitation experiments with MBP (maltose binding protein/malE) fusion proteins. Recombinant MBP-Sec2¹⁻⁷⁵⁹ and MBP-Sec2¹⁻⁵⁰⁸ precipitate Cbk1-Myc from yeast cell extracts (from FLY2288). MBP-Sec2⁴⁵¹⁻⁷⁵⁹, MBP-Sec2¹⁻²⁰⁴ and MBP-Sec2²⁰⁵⁻⁴⁵⁰ do not efficiently precipitate Cbk1-Myc. Amylose-resin (beads) and MPB (not shown) do not precipitate Cbk1-Myc. Similar results were obtained with extracts of *mob2 ssd1-d cells* (data not shown). (B) Autoradiograph of Cbk1 kinase assay using MBP-Sec2¹⁻⁷⁵⁹, MBP or histone H1 as substrate. The uncropped image and corresponding Coomassie stained gel is shown in Supplemental Figure S5. Cbk1-Myc was immuno-precipitated from *MOB2*+ or *mob2* cells (FLY905, FLY906).

Figure 9. Cbk1 inhibition diminishes secretory vesicle accumulation in sec2-41 cells.

cbk1-as sec2-41 cells (FLY2792) were synchronized in early S phase by G0 block and release. When ~50% of the cells developed small buds, the cells were incubated at 22°C for 30' +/- 1NA-PP1. The cells were then shifted to 37°C for 15' (to inactivate Sec2) and samples were collected for EM. (A-D) representative EM figures of *cbk1-as sec2-41* cells. (n=nucleus; v=vacuole; arrows indicate vesicles.) (E) The average number of secretory vesicles per EM section of *cbk1-as sec2-41* cells is plotted. The data are compiled from two independent experiments.

Figure 10. Cbk1 inhibition causes glycosylation defects.

(A) *cbk1-8* cells are sensitive to Hygromycin B. Ten-fold dilution series of cells were spotted onto plates containing 50 ug/ml Hygromycin B. Strains used are BY4741, FLY2661, NY770, FLY2765 and FLY2766.

(B) Carboxypeptidase Y (CPY) is glycosylated and processed normally in *cbk1-8* cells at 37°C. Wild type, *cbk1-8*, *cog1* Δ and *vps1* Δ cells were incubated and pulse labeled with ³⁵S-Met at 37°C. ³⁵S- labeled CPY was immunoprecipitated and analyzed on 10% SDS-PAGE gels. The radiolabeled gels were digitized by a STORM phosphorimager.

(C) Immunoblot of Myc-tagged invertase shows that invertase is hypoglycosylated in *cbk1-8* cells at 22°C. Note that invertase levels are diminished at 37°C. (D) Immunoblot of Sim1-CFP shows Sim1 hypoglycosylation in *cbk1-8* cells at 37°C. Wild type, *cbk1-8* and *cog1* Δ cells expressing SIM1-CFP tag were incubated at 22°C or 37°C for 2.5 hrs. Immunoblots were probed with anti-GFP antibody. Brackets denote the major range of electrophoretic mobilities for invertase and Sim1. The yeast strains used for A-C are BY4741, FLY2661, NY770, FLY2764, FLY2765, FLY2766, FLY2704, FLY2575, FLY2567, FLY2848, FLY2802, FLY1050, FLY2847 and FLY2865.

Figure 11. Cbk1 kinase inhibition causes aberrant vacuole morphology.

(A) Cbk1-kinase inhibition does not prevent FM4-64 (red) uptake, but causes the aberrant accumulation of many small vacuoles. The cells were treated with the vacuole dye CellTracker blue (CMAC) to confirm that FM4-64 is delivered to the vacuoles.

(B) Cells were treated in water for 1h after FM4-64 labeling to increase vacuole size.

Figure 12. Cbk1 kinase and Golgi mannosyltransferases are functionally linked.

(A) The conditional lethality of *cbk1-8* mutants is suppressed by mannosyltransferase overexpression. Ten-fold dilution series of *cbk1-8* cells containing empty vector (pGP654) or dosage suppressors plasmids were spotted onto YPD plates and incubated at the designated temperatures. Cells were also spotted onto YPD plates containing 50ug/ml Hygromycin B and incubated at 22°C. The suppressor plasmids are detailed in Supplement Table S1. (B) Och1 mannosyltransferase localization is aberrant in *cbk1-8* cells. Och1-GFP was monitored in wild

type and *cbk1*-8 cells (JLY284, FLY2922). Cells were grown at 22°C and, where designated, transferred to 35°C for 1 hour. Each image was obtained from a single optical plane.

Supplemental figures

Figure S1 Cbk1 inhibition delays bud emergence in G0 synchronized cells. *cbk1-as* (FLY2084) cells were synchronized in G0 and released into medium +/- 5uM 1NA-PP1. The number of buds was scored over 2 hours.

Figure S2 Viability and morphologies of conditional *cbk1* mutants. The viabilities of *cbk1-as* cells +/- 1NA-PP1 (top left) and *cbk1-8* cells at 22°C and 37°C (bottom left) were determined by counting the number of colony forming units over 8 hours. Representative DIC images of the cells after 8 hours in 1NA-PP1 or at 37°C are shown on the right. Note that most of the *cbk1-8* cells have lysed by 8 hours at 37°C.

Figure S3 Cbk1 inhibition in synchronized *cbk1-as SSD1* and *cbk1-as ssd1-d* cells causes a modest S phase delay. (S3-A) This is a duplicate experiment for Fig1 B.

Figure S4 Cbk1 inhibition delays growth. *cbk1-as* cells (FLY2084) were synchronized in G0 and released to fresh medium until ~40% of cells formed small buds (time 0). The culture was split in two and treated with either DMSO (untreated) or 1NA-PP1. Samples were fixed at designated intervals. The percent of unbudded, small budded and large budded cells were plotted over time. Note that by 60' the DMSO cells already lost synchrony.

Figure S5 Uncropped immunoblot and corresponding Coomassie Blue stained gel for Figure 4A, which demonstrate that Cbk1 phosphorylates Sec2 in vitro.

Figure S6 Media secretion assays with wild type, *dse4* Δ , Dse4-TAP, *cts1* Δ and Cts1-TAP cells. The ~125 kD Cbk1 and Ace2-dependent protein (arrowhead) is present in *dse4* Δ and *cts1* Δ cells and does not appear larger in molecular weight in Dse4-TAP or Cts1-TAP cells. Thus, the 125 kD Cbk1 and Ace2-dependent protein cannot be Dse4 or Cts1. The yeast strains used in this experiment are BY4741, FLY2704, FLY2866, FLY2867, FLY2885 and FLY2886.

Figure S7. *cbk1-8* is lethal or causes severe growth defects when combined with trafficking mutants *sec2-41*, *sec16-2*, *cog1* Δ and *ypt6* Δ . Top panel: tetrads from a cross between wild

type cells (BY4741) and a *cbk1-8 ypt6* Δ double mutant, which was obtained from the cross between *cbk1-8* and *ypt6* Δ cells (FLY2661, FLY2766). Some *cbk1-8 ypt6* Δ clones were viable but exhibit severe slow growth phenotypes.

Table: Synthetic interactions between *cbk1-8* and the trafficking mutants *sec2-41, sec16-2,* $cog1\Delta$ and $ypt6\Delta$. The significance of missing or under-represented double mutants was determined by the Chi-square test. Clones of *cbk1-8 cog1*\Delta and *cbk1-8 ypt6*\Delta double mutants that were alive were backcrossed to wild type parental strains (BY4741or BY4742) and the segregation patterns strongly support synthetic lethality or synthetic growth interactions. Strains used for the crosses were FLY2661, NY770, NY416, FLY2764, FLY2765, FLY2766.

Figure S8. Cbk1 inhibition diminishes secretory vesicle accumulation in sec6-2 cells.

cbk1-as sec6-2 cells (FLY2789) were synchronized in early S phase by G0 block and release. When ~50% of the cells developed small buds, the cells were incubated at 22°C for 30' +/-1NA-PP1. The cells were then shifted to 37°C for 15' (to inactivate Sec6) and samples were collected for EM. (A-D) EM images of representative *cbk1-as sec6-2* cells. (n=nucleus; v=vacuole; arrows indicate vesicles). (E) The average number of secretory vesicles per EM section of *cbk1-as sec6-2* cells is plotted.







В























-1NA-PP1

+1NA-PP1

В





-1NA-PP1

+1NA-PP1

Α

-LatA +LatA LatA wash-out







Figure 7







Figure 8





Figure 9



Α











В DMSO





Β

Och1-GFP localization

