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1 **Kin1 is a plasma membrane-associated kinase which regulates the cell surface in fission**
2 **yeast**

3

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21 **Running title:** Morphogenesis and cell integrity in *S. pombe*

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23 MAPK signalling

24 **Abbreviations:** 1NM-PP1, 4-Amino-1-*tert*-butyl-3-(1'-naphthylmethyl)pyrazolo[3,4-
25 d]pyrimidine; BFA, Brefeldin A; CWI, Cell Wall Integrity; DMSO, dimethyl sulfoxide.

1 **ABSTRACT**

2 Cell morphogenesis is a complex process that depends on cytoskeleton and membrane
3 organization, intracellular signalling and vesicular trafficking. The rod shape of the fission
4 yeast *Schizosaccharomyces pombe* and the availability of powerful genetic tools make this
5 species an excellent model to study cell morphology. Here we have investigated the function
6 of the conserved Kin1 kinase. Kin1-GFP associates dynamically with the plasma membrane at
7 sites of active cell surface remodeling and is present in the membrane fraction. *Kin1Δ* null
8 cells show severe defects in cell wall structure and are unable to maintain a rod shape. To
9 explore Kin1 primary function, we constructed an ATP analog-sensitive allele *kin1-as1*. Kin1
10 inhibition primarily promotes delocalization of plasma membrane-associated markers of
11 actively growing cell surface regions. Kin1 itself is depolarized and its mobility is strongly
12 reduced. Subsequently amorphous cell wall material accumulates at the cell surface, a
13 phenotype that is dependent on vesicular trafficking, and the Cell Wall Integrity (CWI)
14 Mitogen Activated Protein Kinase (MAPK) pathway is activated. Deletion of CWI MAPK
15 components reduces *kin1Δ* hypersensitivity to stresses such as those induced by Calcofluor
16 white and SDS. We propose that Kin1 is required for a tight link between the plasma
17 membrane and the cell wall.

1 INTRODUCTION

2 The control of cell morphology is necessary for the correct execution of many cellular
3 functions as well as for morphogenetic transitions during the life cycle. In metazoan
4 organisms cell morphogenesis involves diverse mechanisms including cell adhesion,
5 migration and proliferation. In addition, establishment and maintenance of cell morphology
6 requires a complex coordination between cytoskeleton and plasma membrane organization,
7 cell polarity and intracellular signalling cascades. Unicellular eukaryotes such as yeast
8 constitute excellent model organisms to identify and study the regulatory mechanisms of cell
9 morphogenesis.

10 Among yeasts, the fission yeast *Schizosaccharomyces pombe* is a powerful model
11 because cells exhibit a strict rod shape during their vegetative life cycle. The cell diameter
12 remains constant and cells extend at only one cell end during early G2 but at both cell ends
13 later in G2. This switch to a bipolar growth pattern is called “New End Take Off” (NETO).
14 During interphase, microtubules (MTs) are organized along the main cell axis as longitudinal
15 bundles with an anti-parallel configuration. The MT plus ends extend towards the cell ends
16 where they contribute to the delivery of polarity factors at the cortex whereas the minus ends
17 are in close contact with the nucleus (for reviews, La Carbona *et al.*, 2006; Sawin and Tran,
18 2006). During polarized growth, cell wall remodeling resumes in the cell ends where F-actin
19 cables and patches are polymerized. The F-actin cytoskeleton shows a spatial and temporal
20 relationship with proteins of the endocytic machinery (Galletta and Cooper, 2009). For3 is a
21 formin responsible for the nucleation of interphase F-actin cables onto which exocytic
22 vesicles are delivered (Feierbach and Chang, 2001). At mitotic onset, F-actin is reorganized
23 with several other proteins as a contractile ring in the middle of the cell and cytoplasmic
24 microtubules are depolymerized while a mitotic spindle is assembled within the nucleus.

1 At the plasma membrane, vesicular trafficking involves a balance between exocytosis
2 and endocytosis. Several proteins regulate this balance. Molecular motors such as the type V
3 myosin Myo4 are required for targeting of vesicles and cell wall-synthesizing enzymes to the
4 plasma membrane along F-actin cables (Feierbach and Chang, 2001; Motegi *et al.*, 2001;
5 Mulvihill *et al.*, 2006; Win *et al.*, 2001). Sla2 is a transmembrane protein that regulates
6 endocytosis and F-actin organization and controls plasma membrane internalization
7 (Castagnetti *et al.*, 2005; Iwaki *et al.*, 2004). The *psy1* gene encodes a syntaxin 1 homolog, a
8 component of the docking/fusion system t-soluble N-ethylmaleimide-sensitive factor
9 attachment protein receptor (SNARE), and is involved in sporulation mechanisms. Psy1 is
10 essential for vegetative growth and is localized at the plasma membrane during the vegetative
11 cell cycle, including the invaginating membranes during septum synthesis (Nakamura *et al.*,
12 2001).

13 Fission yeast cell shape also depends on the composition and the polarized synthesis
14 of the cell wall, a complex, rigid and dynamic polysaccharide structure that overlays the
15 plasma membrane (Perez and Ribas, 2004). This structure is composed of cross-linked
16 polysaccharides and glycoproteins. An essential function of the cell wall is to provide
17 mechanoresistance to abrupt changes in environmental conditions. Cell wall synthesis and
18 remodeling during the cell cycle are tightly regulated and require oriented intracellular
19 trafficking to target cell wall-synthesizing enzymes to polarized growth sites. As a
20 consequence, cell wall structure could be affected by mutants for functions in transport,
21 trafficking, glycosylation or cell cycle control. In *S. pombe*, various glucan synthases have
22 been identified. For example, Ags1 is necessary for α 1,3-glucan synthesis (Hochstenbach *et al.*,
23 1998; Katayama *et al.*, 1999). Bgs1, Bgs2, Bgs3 and Bgs4 are the β 1,3-glucan synthases
24 involved, respectively, in septum formation (Cortes *et al.*, 2002; Liu *et al.*, 2002), cell
25 sporulation (Martin *et al.*, 2000), elongation (Martin *et al.*, 2003) and cell wall growth and

1 prevention of cell lysis during cytokinesis (Cortes *et al.*, 2005). The stability of Bgs1 at the
2 plasma membrane depends on the regulatory factor Cfh3 (Sharifmoghadam and Valdivieso,
3 2009).

4 Fission yeast morphology mutants have been isolated using genetic screens. Molecular
5 cloning of the corresponding genes has revealed distinct regulatory mechanisms involving cell
6 wall assembly, F-actin polarization, polarity factors and interphase microtubule functions.
7 Some mutants such as those of the *orb* class are unable to establish a rod shape whereas
8 another group called *tea* mutants are defective in restricting opposite growth zones (for a
9 review, Hayles and Nurse, 2001). Yeast mutants defective in general cell wall metabolism
10 also show extensive cell shape defects (for a review see Ishiguro, 1998).

11 Mitogen Activated Protein Kinase (MAPK) signalling cascades convert a stimulus
12 detected at the cell surface (after a change in environmental conditions) to an intracellular
13 signal to promote an adapted cellular response (Waskiewicz and Cooper, 1995). MAPK
14 cascades are composed of a core kinase module which includes a MAPK kinase kinase
15 (MAPKKK), a MAPK kinase (MAPKK) and a MAPK. The MAPK pathway is activated by
16 upstream regulators such as protein kinases, small Rho GTPases and transmembrane protein
17 sensors. Activation of MAPK promotes a transcriptional response as well as cytoplasmic
18 responses such as cytoskeletal reorganization and cell cycle delay. In fission yeast, three
19 MAPK signalling cascades have been described. These include the mating pheromone-
20 responsive Spk1 pathway, the stress-activated protein kinase (SAPK) Sty1 pathway and the
21 Cell Wall Integrity (CWI) Pmk1 pathway. When the cell wall is damaged, for instance by
22 chemicals or by abrupt modification of the osmotic pressure, the CWI MAPK pathway is
23 activated (Barba *et al.*, 2008; Levin, 2005). The fission yeast CWI pathway is composed of
24 the MAPKKK Mkh1, the MAPKK Pek1 and the MAPK Pmk1 (Loewith *et al.*, 2000; Madrid
25 *et al.*, 2006; Sengar *et al.*, 1997; Sugiura *et al.*, 1999; Toda *et al.*, 1996; Zaitsevskaya-Carter

1 and Cooper, 1997). Ultimately, the transcription factor Atf1 is a major downstream Pmk1
2 target for stress response (Takada *et al.*, 2007). Pmk1 activity is also dependent on the cell
3 cycle and peaks at the time of cell separation during cytokinesis (Madrid *et al.*, 2007). Fission
4 yeast CWI components are detected within the cytoplasm and the nucleus throughout the cell
5 cycle and at the septum during cell division (Madrid *et al.*, 2006). This pathway is partially
6 activated by Pck2, a protein kinase C, and the small GTPase Rho2 (Barba *et al.*, 2008; Ma *et*
7 *al.*, 2006). Various molecular mechanisms for CWI MAPK regulation have been reported in
8 fission yeast, including MAPK phosphatases, mRNA stability, phosphorylation-dependent
9 inhibition and the Sty1/Spc1 SAPK pathway (Madrid *et al.*, 2007; Sugiura *et al.*, 1998;
10 Sugiura *et al.*, 1999; Sugiura *et al.*, 2003). The fission yeast Pck1 and Pck2 protein kinase C
11 homologs also regulate cell wall integrity but this function may be partially independent of
12 the CWI MAPK pathway (Barba *et al.*, 2008; Toda *et al.*, 1996).

13 The fission yeast *kin1* gene encodes an evolutionarily conserved serine/threonine
14 protein kinase of the KIN1/PAR-1/MARK family (for a review, Tassan and Le Goff, 2004).
15 Studies using disrupted *kin1::LEU2* (Levin and Bishop, 1990) or complete null *kin1Δ*
16 (Drewes and Nurse, 2003; La Carbona *et al.*, 2004) alleles reported a role for Kin1 in
17 maintenance of a regular rod shape. However, cells were still able to establish a longitudinal
18 axis. A systematic characterization of the phenotypes of non-essential kinase deletion strains
19 (Bimbo *et al.*, 2005) revealed specific stress sensitivities for the *kin1* mutant, including
20 sensitivity to excess chloride ion and SDS. Kin1 mutant cells show an abnormally enlarged
21 new cell end, initially referred to as the “ice-cream cone” phenotype (Levin and Bishop,
22 1990). In addition, Kin1 is involved in cell separation, interphase F-actin polarization and
23 nuclear centering, and is important for completion of cytokinesis in a specific set of polarity
24 mutants (Cadou *et al.*, 2009; La Carbona and Le Goff, 2006). The Kin1 kinase associates
25 dynamically with the cell cortex at the cell ends during interphase in an F-actin and

1 microtubule independent manner. Kin1 also colocalizes with the contractile ring at mitosis
2 and on both sides of the septum during cytokinesis (Cadou *et al.*, 2009).

3 Here we have investigated the role of Kin1 in the regulation of the cell surface. Kin1-
4 GFP dynamically associates with the plasma membrane at sites of active cell wall growth.
5 Kin1 is present in the sterol-rich fraction of membranes and its polarized localization is
6 dependent on intact vesicular trafficking. We show that *kin1Δ* cells exhibit a thicker cell wall
7 than wild type cells with delocalized β-glucan-containing deposits on the lateral cortex. To
8 characterize the primary function of Kin1, we produced *kin1-as1*, an ATP analog-sensitive
9 allele of Kin1. We show that inactivation of Kin1 triggers a rapid delocalization of plasma
10 membrane markers of actively growing cell surface regions, including Kin1 itself. This
11 phenotype is followed by the formation of localized Cell Wall Deposits (CWD). CWD
12 formation is suppressed by perturbation of membrane trafficking but it is exacerbated in a
13 *sla2* endocytic mutant. Inhibition of Kin1 also leads to the activation of the CWI MAPK
14 module that ultimately exacerbates CWD formation. Consistent with abnormalities in the cell
15 wall, *kin1Δ* cells are hypersensitive to various stresses. This sensitivity is alleviated by
16 deletion of either Pck2 or any of the core CWI MAPK components. Cell Wall Deposits are
17 partially dependent on a hyperactivated CWI MAPK pathway. We propose that the dynamic
18 association of Kin1 with the plasma membrane is required for a robust and tight link between
19 plasma membrane and the cell wall during vegetative growth. This function contributes to
20 proper cell morphogenesis and tolerance to stress.

21

22 **RESULTS**

23

24 **1) Kin1 is a plasma membrane associated protein kinase**

1 We have previously shown that Kin1 exhibits a cell-cycle regulated and polarized localization
2 at the cell cortex. Kin1 accumulates at the cell ends during interphase and is a component of
3 the Contractile Actomyosin Ring (CAR) at mitosis. At septation, Kin1 is present on both sides
4 of the septum, corresponding to new ends of the presumptive daughter cells (Cadou *et al.*,
5 2009).

6 Here, we have examined how Kin1 interacts with the cortex using sub-cellular
7 fractionation. Integral plasma membrane GFP-Bgs4 was used as a control (Cortes *et al.*, 2002;
8 Liu *et al.*, 2002). We observed that Kin1, like GFP-Bgs4, is present in the insoluble fraction
9 after ultracentrifugation of a preclearing protoplast lysate obtained under non-denaturing
10 conditions (Fig. 1A), indicating that the main pool of Kin1 is present in the membrane
11 fraction. Next, using different extraction buffers, we observed that Kin1 is completely
12 solubilized by 2% SDS, consistent with an interaction with membranes (Fig. 1B).
13 Interestingly, Kin1 is not solubilized by 1% Triton X-100 at 4°C, suggesting that the major
14 pool of Kin1 is present in the Triton X-100-insoluble lipid raft fraction (Fig. 1B). It is not
15 solubilised by 0.5 M NaCl, 0.1 M Na₂CO₃ pH 11 or 2 M urea, so we can rule out the
16 possibility that the major part of Kin1 is adsorbed to the plasma membrane by weak
17 interactions (Liu *et al.*, 2002), i.e., it is not a peripheral membrane protein. A very small pool
18 of Kin1 seems to be solubilized by 0.5 M NaCl, suggesting that different Kin1 sub-
19 populations may be present in the plasma membrane.

20 These data show that Kin1 is anchored in the membrane sterol-rich fraction. Kin1 does
21 not exhibit transmembrane domains or canonical lipid anchoring amino acid motifs (e.g., GPI
22 anchor, CAAX box), the membrane association of Kin1 may be mediated by an as-yet-
23 unidentified post-translational modification.

24 A possible link between sub-cellular fractionation (Fig. 1, A and B) and GFP-tagged
25 localization data (Cadou *et al.*, 2009) is an association of Kin1 with sites of active membrane

1 flux at the plasma membrane where sterol-rich domains are polarized. To test this hypothesis,
2 Kin1-GFP localization was assayed in cells after treatment with the ER-to-Golgi vesicular
3 traffic inhibitor Brefeldin A (BFA, Fig. 1C). BFA treatment depolarized the sterol-rich
4 domains detected by filipin, a histochemical stain for membrane sterols, as described by
5 Wachtler *et al.* (2003). We observed that Kin1-GFP is delocalized over the entire plasma
6 membrane in BFA treated cells. This suggests that Kin1-GFP polarization requires intact
7 vesicular traffic and/or polarized sterol-rich domains.

8

9 **2) Kin1 is required for proper cell wall structure**

10 It has been shown that *kin1Δ* cells have abnormal morphologies including “ice cream cone”
11 shaped cells and irregular cell outlines (Drewes and Nurse, 2003; La Carbona *et al.*, 2004;
12 Levin and Bishop, 1990). In addition, *kin1Δ* cells are hypersensitive to treatment with cell
13 wall degrading enzymes (Levin and Bishop, 1990). As shown in Fig. 2A, $77\pm 6.5\%$ of *kin1Δ*
14 cells ($n > 400$) show deposition of extra cell wall material on the cortex detected by methyl
15 blue (beta-glucan staining), especially in the central region (hereafter collectively referred to
16 as Cell Wall Deposits or CWD). This phenotype is exacerbated by incubating cells 5 hours at
17 high temperature (37°C , Fig. 2A; $89.5\pm 2.5\%$ of cells contain CWD). Biochemical
18 measurements consistently show a higher content of both alpha and beta glucans in the cell
19 wall of *kin1Δ* cells compared to wild type cells (P. Perez, pers. comm.).

20 Because CWD in *kin1Δ* cells are predominantly located in the central region close to
21 the septum synthesis site, we examined whether their presence might be correlated with
22 septum synthesis. *Kin1Δ* was outcrossed to *sid4-SAI*, a thermosensitive mutant of a
23 component of the Septation Initiation Network (SIN) that fails to septate at 37°C
24 (Balasubramanian *et al.*, 1998). CWD were detected in the central region of *kin1Δ sid4-SAI*
25 mutant cells (Fig. 2B), indicating that CWD are unlikely to correspond to excess septal

1 material. Similar results were observed with other SIN mutants including *cdc7-24* and *cdc11-*
2 *123* (data not shown). We also detected CWD in G1-arrested *kin1Δ cdc10-V50* or G2-arrested
3 *kin1Δ cdc25-22* cells (Fig. 2B), indicating that CWD can also form in interphase cells.

4 The *kin1Δ* cell wall was also examined by Transmission Electron Microscopy (TEM,
5 Fig. 2C). This revealed a thicker cell wall and two distinct cell wall structure defects: (a) a
6 multilayered structure on the outer face of the cell wall, and (b) the presence of cell wall
7 depositions (called amorphous depositions), with distinct electron density between the cell wall
8 and the plasma membrane (Fig. 2C).

9 Taken together, these data suggest that Kin1 deletion promotes the localized synthesis
10 of extra cell wall material (CWD), the presence of which is probably responsible for the
11 defects in cell morphology.

12

13 **3) Using chemical genetic inhibition to study Kin1 function**

14 To further address the primary function of Kin1 in cell wall synthesis and membrane
15 dynamics, we used the chemical genetic approach developed by K. Shokat and colleagues
16 (Bishop *et al.*, 2000). This strategy is based on site-directed mutagenesis of the ATP binding
17 pocket in the catalytic domain which makes the kinase uniquely sensitive to the bulky
18 nonhydrolyzable ATP analog 4-amino-1-*tert*-butyl-3-(1'-naphthylmethyl) pyrazolo [3,4-*d*]
19 pyrimidine (hereafter abbreviated as 1NM-PP1). First, it was determined by comparison with
20 other protein kinases (Bishop *et al.*, 2000) that to enlarge the ATP binding pocket of Kin1 we
21 should introduce an F-to-G mutation at position 220 in the Kin1 Open Reading Frame (Fig.
22 3A). The resulting mutated kinase was named Kin1-as1 (for analog sensitive 1). Expression of
23 Kin1-as1 from a multicopy plasmid rescued the *kin1Δ* phenotype (data not shown), indicating
24 that Kin1-as1 is functional. Next, the wild type *kin1* ORF was replaced by a *kin1-as1* version
25 (see Material and Methods). The resulting strain, *kin1-as1*, exhibited a wild type phenotype

1 indicating that the F220G mutation does not modify Kin1-as1 activity when expressed from
2 the *kin1* locus. Furthermore, a GFP tag was inserted at the C-terminus of the Kin1-as1 ORF as
3 described in Cadou *et al.* (2009). Expression levels of Kin1, Kin1-GFP and Kin1-as1-GFP
4 were very similar under normal growth conditions (supplementary Fig. S1).

5 The *kin1-as1* inhibitory phenotype was studied using increasing doses of the ATP
6 analog 1NM-PP1. We observed that septum positions were rapidly modified with low doses
7 of 1NM-PP1 (asterisk, Fig. 3B; supplementary Fig. S2), confirming our previous results using
8 a repressible allele of Kin1 (Cadou *et al.*, 2009; La Carbona and Le Goff, 2006). Thus,
9 inhibition of Kin1 kinase activity leads primarily to an off-center division site. Interestingly,
10 we also observed the formation of localized CWD stained by methyl blue. This material was
11 present as discrete patches in the ends of $22.2 \pm 8.9\%$ of early G2 cells or on the lateral cortex
12 of $10.4 \pm 3.5\%$ of late G2 cells (arrowheads, Fig. 3B). This phenotype is reminiscent of the cell
13 wall defects observed in *kin1* Δ cells. 1NM-PP1-mediated CWD formation was studied further
14 in ATP analog-treated *kin1-as1* cells by TEM. We observed that CWD correspond to
15 amorphous material deposited between the plasma membrane and the structured cell wall
16 layers (TEM, Fig. 3B). Thus, inhibition of Kin1-as1 promoted localized accumulation of cell
17 wall material. Furthermore, the percentage of cells containing CWD increased with increasing
18 1NM-PP1 concentrations (histogram, Fig. 3B).

19 In 1NM-PP1-containing medium, the Kin1-as1-GFP protein is dispersed in a broad
20 region at the cell end (Fig. 3C; compare also Kin1-as1-GFP signals in DMSO or 1NM-PP1
21 treated cells prior to the bleaching in the FRAP experiment, Fig. 3D). Thus, the kinase activity
22 of Kin1 is required for the maintenance of its polarized state but not for its cortical
23 localization. Remarkably, Kin1-as1-GFP signal colocalizes with CWD (supplementary Fig.
24 S3).

1 Fluorescence Recovery After Photobleaching (FRAP) allows one to monitor protein
2 mobility in live cells (Reits and Neefjes, 2001). Here, Kin1-as1-GFP recovery was measured
3 after laser illumination of a cell end (see Material & Methods). In the absence of 1NM-PP1,
4 kinetics of recovery were indistinguishable between Kin1-GFP and Kin1-as1-GFP (data not
5 shown). In cells treated with DMSO only, Kin1-as1-GFP exhibited a high mobility (the
6 mobile fraction is ~ 60%) at the cell end (Fig. 3D). In parallel, the single type IV membrane-
7 spanning protein GFP-Psy1 was almost immobile under identical FRAP conditions.
8 Interestingly, in cells treated with 1NM-PP1, Kin1-as1-GFP protein showed a marked
9 reduction in mobility. This suggests that the kinase activity of Kin1 contributes to its mobility.

10

11 **4) Kin1 is required for plasma membrane organization**

12 Cell wall structure dynamics depend on the polarization of the growth machinery at the cell
13 ends and at the division site (septum). Active cell wall synthesis sites are intimately linked
14 with polarized F-actin structures, trafficking and sterol-rich plasma membrane domains
15 (Wachtler *et al.*, 2003). We previously showed that *kin1Δ* cells exhibit a depolarized
16 interphase F-actin cytoskeleton on the lateral cortex (La Carbona and Le Goff, 2006). Here,
17 we have monitored localization of the sterol-rich domains in the plasma membrane using the
18 fluorescent dye filipin. In contrast to wild-type cells, *kin1Δ* cells show depolarized sterol-rich
19 domains on the entire plasma membrane (Fig. 4A).

20 TEM studies did not reveal accumulation of vesicles in the cytoplasm (Fig. 2C),
21 suggesting that general secretion might not be defective in *kin1Δ* cells. We therefore
22 examined the localization of the fission yeast t-SNARE syntaxin homolog GFP-Psy1, a
23 component of the late secretion SNARE complex. In *kin1Δ* cells, GFP-Psy1 was detected on
24 the overall plasma membrane, as in wild type cells, suggesting that Kin1 does not regulate
25 GFP-Psy1 plasma membrane targeting (Fig. 4B). However, we observed that GFP-Psy1

1 accumulated on the lateral cortex (arrowheads, Fig. 4B), indicating that Kin1 affects the
2 distribution of GFP-Psy1 within the plasma membrane.

3 Polarization of plasma membrane sterol-rich domains (and cell wall synthesis) is
4 dependent on efficient intracellular trafficking. In yeast, the amphiphilic fluorescent dye FM4-
5 64 is taken up by endocytosis and transported to the vacuolar membrane. When living *kin1Δ*
6 cells incorporate the FM4-64 dye (Fig. 4C), stained vacuoles exhibit morphology defects.
7 Vacuoles seemed strikingly smaller and more numerous in *kin1Δ* cells compared to wild type
8 cells, suggesting a defect in vacuole biogenesis. Time-lapse studies revealed that initial
9 incorporation of FM4-64 was not affected in *kin1Δ* cells but that later the signal of FM4-64
10 was more diffuse than in wild type cells (supplementary Fig. S4).

11 During vegetative growth, polarized cell wall synthesis is dependent on the
12 localization of transmembrane beta-glucan synthase holoenzymes at cell wall remodeling sites
13 (Cortes *et al.*, 2002; Cortes *et al.*, 2005; Cortes *et al.*, 2007; Liu *et al.*, 2002; Martin *et al.*,
14 2003). We therefore monitored the localization of GFP-tagged vegetative beta-glucan
15 synthase catalytic subunits Bgs1, Bgs3 and Bgs4. *Kin1Δ* cells were not defective in polarized
16 localization of GFP-Bgs3 (supplementary Fig. S5). By contrast, GFP-Bgs1 and GFP-Bgs4
17 signals were not only observed at cell wall remodeling sites but could also be detected on the
18 lateral cortex of *kin1Δ* cells (Fig. 4D; supplementary Fig. S5). In addition, Western blot
19 analysis revealed that expression of Bgs1 and Bgs4, but not Bgs3, was significantly
20 upregulated in the absence of Kin1 (supplementary Fig. S6). Thus, Kin1 regulates Bgs1 and
21 Bgs4 expression as well as their polarization at the plasma membrane.

22 If in the *kin1Δ* mutant Bgs1 is more stable at the plasma membrane, the *kin1Δ*
23 mutation should lower the sensitivity to temperature of *cps1-191*, a thermosensitive allele of
24 *bgs1*. Indeed, we observed growth rescue at 32°C and 37°C in the *kin1Δ cps1-191* double
25 mutant compared to the single *cps1-191* mutant (Fig. 4E).

1 To characterize the primary function of Kin1 at the plasma membrane, we used the
2 rapid inactivation of Kin1-as1 mediated by 1NM-PP1. We observed that sterol-rich domains
3 are depolarized when 1NM-PP1 is added to the culture medium (Fig. 5A). Since overall
4 endocytosis is not affected in the *kin1* Δ mutant, one explanation for the increased stability of
5 the beta-glucan synthase Bgs1 at the plasma membrane is that a specific regulator of the
6 endocytosis of Bgs1 is altered. Cfh3 is a protein that colocalizes and co-immunoprecipitates
7 with Bgs1 and regulates its stability at the plasma membrane (Sharifmoghadam and
8 Valdivieso, 2009). To test if the early phenotype of Kin1 inactivation affects Cfh3 regulation,
9 we followed GFP-Cfh3 in a *kin1-as1* background. We observed that the cell-cycle regulated
10 distribution of GFP-Cfh3 was perturbed in 1NM-PP1-treated cells. In particular, GFP-Cfh3
11 was strikingly depolarized at the plasma membrane compared to control cells (Fig. 5B).
12 Delocalized Cfh3-GFP coincided with sites of CWD formation (supplementary Fig. S3).
13 Moreover, 1NM-PP1-treatment of *GFP-Psy1 kin1-as1* cells also showed that GFP-Psy1
14 localization was perturbed and that GFP-Psy1 accumulates at sites of CWD formation
15 (supplementary Fig. S3). These observations link the role of Kin1 in proper localization of
16 GFP-Cfh3 and GFP-Psy1 with cell wall regulation.

17 Next, we determined the kinetics of GFP-Cfh3 depolarization and CWD formation by
18 analyzing samples in the fluorescence microscope during a time-course experiment following
19 inhibition of Kin1-as1 with 1NM-PP1 (Fig. 5C). The results show clearly that depolarization
20 of GFP-Cfh3 is nearly complete after 60 minutes and precedes the accumulation of cell wall
21 material in CWD. This demonstrates that the primary function of Kin1 resides in plasma
22 membrane organization and suggests that CWD is a consequence of the disruption of this
23 primary function. In parallel, activation of the Cell Wall Integrity Pmk1 MAP kinase was
24 determined. Pmk1 basal phosphorylation was detected at different time points by employing
25 an anti-phospho p42/p44 antibody as described earlier (Madrid *et al.*, 2006). Pmk1 activation

1 was only obvious after 90 minutes (Fig. 5D), supporting the idea that this phenotype may be a
2 consequence of CWD formation.

3 In summary, our data suggest that Kin1 function is primarily required for proper
4 polarization of sterol-rich domains and proper localization of regulators of cell wall synthesis
5 at the plasma membrane. They suggest further that the initial effect of inhibition of Kin1 is
6 depolarization of the cell wall synthesis machinery, which then leads to CWD. Presence of
7 CWD then triggers the CWI pathway which further exacerbates the formation of CWD.

8

9 **5) A possible role for Kin1 in maintenance of a robust link between plasma membrane** 10 **and the cell wall during vegetative growth**

11 A possible explanation for the generation of CWD could be a localized invagination of the
12 plasma membrane due to a modification of the mechanical properties of the lipid bilayer. This
13 could result from a loss of a robust and tight link between the cell wall and the plasma
14 membrane at the cell surface. The enlarged periplasmic space could then be filled by
15 amorphous cell wall material (Figs. 2 and 3). How could the plasma membrane invaginate
16 into the cytoplasm? One possibility is that a defect in the balance between exocytic and
17 endocytic vesicles plays a role.

18 It is unlikely that Kin1 function regulates exocytic vesicle fusion because no
19 accumulation of large vesicles is observed by TEM in *kin1Δ* cells or during Kin1-as1
20 inhibition. Following our hypothesis, we reasoned that inhibition of active vesicular
21 trafficking would suppress CWD formation since cell wall material is targeted to the cell
22 cortex by secretion machinery. Indeed, *kin1-as1* cells pre-treated with BFA, an inhibitor of
23 ER to Golgi transport, showed a complete suppression of CWD formation after 1NM-PP1
24 addition (Fig. 6A). By comparison, CWD were observed in 39.2% of control cells. However,
25 cells treated with both BFA and 1NM-PP1 still showed asymmetric septa, demonstrating that

1 1NM-PP1 is able to inactivate Kin1-as1 in the presence of BFA and strongly suggesting that
2 nuclear mispositioning and CWD formation are distinct phenotypes. Consistently, no CWD
3 were observed in an ATP analog-treated *kin1-as1 for3Δ* mutant, where no interphase F-actin
4 cables are nucleated (data not shown). Similarly, a strong reduction of CWD (6.4% compared
5 to 45.7% in a *myo4⁺* background) was observed in a 1NM-PP1-treated *kin1-as1 myo4Δ*
6 mutant, where exocytic vesicle transport was disturbed (Fig. 6B). A corollary to our
7 hypothesis is that an inhibition of the endocytosis process should stimulate CWD formation.
8 To test this, we used the *sla2Δ* strain which is deleted for the End4/Sla2 transmembrane
9 protein. Interestingly, control *sla2Δ* cells stained with methyl blue dye showed discrete
10 punctate staining on the lateral cortex (Fig. 6B). This indicates that the *sla2Δ* mutation itself
11 promotes formation of localized CWD, consistent with an imbalance between endocytosis and
12 exocytosis. Moreover, when Kin1-as1 was inhibited by 1NM-PP1, a dramatic increase in both
13 the number and intensity of CWD was observed (Fig. 6B). Thus, the endocytosis defect
14 exacerbates kin1-as1-mediated CWD formation.

15 Next, we monitored Kin1-GFP localization in the mutants *for3Δ*, *myo4Δ* and *sla2Δ*
16 (data not shown). Kin1-GFP was not strictly accumulated at cell ends in any of these
17 situations. We conclude that vesicular trafficking defects (either exocytosis or endocytosis)
18 promote depolarization of Kin1-GFP around the cell cortex, even though Kin1-GFP remains
19 at the plasma membrane. We also monitored Kin1-GFP mobility by FRAP in *for3Δ*, *myo4Δ*,
20 and *sla2Δ* mutants and in BFA-treated cells. Only BFA alters Kin1-GFP mobility and that
21 only moderately (Supplementary Fig. S7). Since BFA is a strong inhibitor of general
22 membrane trafficking and polarization of sterol-rich domains, this observation suggests that
23 the altered mobility of Kin1-GFP in this situation may be due to broad BFA-mediated
24 perturbation of plasma membrane organization.

25

1 **6) The Pmk1 MAP kinase pathway affects CWD formation in *kin1Δ* cells.**

2 In yeast cells, cell wall integrity (CWI) MAPK module is involved in the sensing of and
3 response to perturbations in the cell wall and the plasma membrane (Levin, 2005; Barba *et al.*,
4 2008). In *S. pombe* the cell integrity MAP kinase module is composed of MAPKKK Mkh1,
5 MAPKK Pek1 and MAPK Pmk1, the key element of the pathway. The protein kinase C
6 ortholog Pck2 and the small Rho GTPase Rho2 act upstream of the Mkh1-Pek1-Pmk1
7 cascade (Ma *et al.*, 2006; Barba *et al.*, 2008), and they are responsible for Pmk1 activation in
8 response to several stresses such as hypertonic or hypotonic shock (Barba *et al.*, 2008). The
9 presence of altered cell walls in the *kin1Δ* mutant suggested that Pmk1 activity might be
10 deregulated in these cells. CWD formation was monitored in a *kin1Δ pmk1Δ* double mutant.
11 As shown in Table 2 and supplementary Fig. S8, deletion of *pmk1*⁺ reduced the number of
12 CWD in *kin1Δ* cells. Identical results were obtained with deletions of *mkh1*⁺ and *pek1*⁺ (data
13 not shown). Similarly, CWD formation was partially abolished in *kin1-as1* cells disrupted in
14 either *pmk1*⁺, *pek1*⁺ or *mkh1*⁺, and treated with 1NM-PP1 (data not shown). Partial
15 suppression of CWD was also observed in *kin1Δ pck2Δ* cells, indicating that Pck2 is also
16 involved in the formation of CWD (Table 2; supplementary Fig. S8). The fact that *pck2Δ*
17 suppresses CWD more strongly than *pmk1Δ* suggests that Pck2 is also involved in CWD
18 formation independently of the Pmk1 pathway. This result is not surprising since Pck2
19 regulates cell wall integrity independently of the CWI cascade (Toda *et al.*, 1996; Barba *et al.*,
20 2008). Moreover, CWD in *kin1Δ* cells were not markedly affected by deletion of *rho2*⁺ (Table
21 2; supplementary Fig. S8), suggesting that the Pmk1 pathway may branch at the Rho2 level.
22 On the other hand, Pmk1 hyperactivation elicited by deletion of the dual specificity
23 phosphatase *pmp1*⁺ (Sugiura *et al.*, 1998) significantly increased the number of CWD in
24 *kin1Δ* cells (Table 2; supplementary Fig. S8).

1 We also tested the possible role of Pck1, a Pck2 homolog, in CWD formation. Pck1
2 negatively regulates cell wall integrity and the Pmk1 pathway, but its function remains
3 obscure (Arellano *et al.*, 1999; Barba *et al.*, 2008). A *kin1Δ pck1Δ* double mutant showed
4 extensive CWD (Table 2; supplementary Fig. S8) and severe growth defects, supporting the
5 existence of synthetic interaction between the single mutants.

6 The above results suggested the existence of a specific link between Pmk1 activity and
7 CWD formation in *kin1Δ* cells. To further explore this hypothesis, we first determined Pmk1
8 basal phosphorylation in growing cells from the above mutants by employing the anti-
9 phospho p42/44 antibody. As can be seen in Fig. 7A, Pmk1 was hyperphosphorylated in the
10 *kin1Δ* mutant. Importantly, the increase in Pmk1 phosphorylation elicited by *kin1⁺* deletion
11 was abolished in *kin1Δ pck2Δ* cells but not in the *kin1Δ rho2Δ* mutant (Fig. 7A). Moreover,
12 basal Pmk1 phosphorylation was higher in either *kin1Δ pmp1Δ* or *kin1Δ pck1Δ* cells than in
13 their respective single mutant counterparts (Fig. 7A).

14 In *S. pombe*, calcineurin and Pmk1 play antagonistic roles in chloride homeostasis, and
15 Pmk1 hyperactivation leads to strong sensitivity to this anion (Sugiura *et al.*, 1998). Pmk1
16 hyperphosphorylated cells lacking *kin1⁺* showed an evident growth inhibition in YES medium
17 supplemented with 0.2 M MgCl₂ (Fig. 7B), and this phenotype was rescued by additional
18 deletion of *pmk1⁺* or *pck2⁺* genes (Fig. 7B). Interestingly, disruption of either *pck1⁺* or *pmp1⁺*
19 in a *kin1Δ* background clearly increased cell sensitivity to chloride anions as compared to
20 single *kin1Δ*, *pck1Δ* or *pmp1Δ* parental strains (Fig. 7B).

21 As a whole, our results strongly suggest that in fission yeast: (i) Kin1 operates at the
22 plasma membrane and its function influences Pmk1 activity *via* Pck2 but in a Rho2-
23 independent fashion; (ii) hyperactivation of the Pmk1 MAPK pathway is partially responsible
24 for the production of CWD in the absence of Kin1 kinase; and (iii) other Pmk1-independent
25 mechanisms are also important in CWD formation.

1 The cell wall is essential for determination of cell shape and also for
2 mechanoresistance to environmental changes. Since lack of Kin1 function alters cell wall
3 organization, *kin1Δ* cells might behave differently from wild type cells under stress
4 conditions.

5 Congruent with a strong cell separation defect at high temperature (Levin and Bishop,
6 1990), *kin1Δ* cells show reduced growth at 37°C (data not shown). We monitored the effect of
7 plasma membrane stress and cell wall damaging agents on *kin1Δ* cell growth to show that
8 these cells are extremely sensitive to stress induced by low doses of SDS and to the cell wall
9 damaging agent Calcofluor white (Fig. 7C), but not to the beta-glucan synthase inhibitor
10 casprofungin (data not shown). Thus, our data indicate that Kin1 function is required for cell
11 tolerance to specific stress conditions, and confirm those obtained by Bimbo *et al.* (2005)
12 during a systematic study of non-essential kinase deletions. Importantly, deletion of *pmk1⁺* or
13 *pck2⁺* alleviated the hypersensitivity of *kin1Δ* cells to either SDS or Calcofluor white (Fig.
14 7C; data not shown). Thus, our results indicate that Kin1 function is required for resistance to
15 thermal and cell wall stresses, and that Pmk1 activity is involved in this response.

16

17 **7) SICS and CWD**

18 Recently, Robertson and Hagan have described the formation of Stress-Induced Calcofluor
19 Structures (SICS) upon treatment of fission yeast cells with either 1.2 M Sorbitol or 0.6 M
20 KCl (Robertson and Hagan, 2008). During the course of this study, we have observed that
21 treatment of cells with 0.01% SDS can also promote SICS (supplementary Fig. S9).

22 SICS appear to be very similar to the CWD described in the present work. We
23 therefore examined whether Kin1 is required for SICS formation. Incubation in Sorbitol or
24 SDS exacerbated cell wall deposits in *kin1Δ* cells (supplementary Fig. S9). In addition, *kin1-*
25 *as1* cells treated with both 1NM-PP1 and Sorbitol or SDS showed additive effects on

1 accumulation of cell wall material compared to 1NM-PP1-treated control cells (data not
2 shown). These observations strongly suggest that Kin1 is dispensable for SICS formation.

3

4 **DISCUSSION**

5 Proper regulation of cell morphogenesis is essential for eukaryotic cells to carry out their
6 functions, divide and adapt to environmental changes. Here, we have demonstrated a role for
7 the fission yeast Kin1 kinase in controlling cell surface organization.

8

9 **Kin1 associates dynamically with the plasma membrane**

10 Fission yeast Kin1 is structurally related to the evolutionary conserved PAR-1/MARK protein
11 kinase family (Tassan and Le Goff, 2004). These are plasma membrane proteins, as
12 demonstrated by sub-cellular fractionation experiments in budding yeast and plasma
13 membrane localization in metazoan systems (Chartrain *et al.*, 2006; Elbert *et al.*, 2005; Hurov
14 *et al.*, 2004; Tibbetts *et al.*, 1994; Vaccari *et al.*, 2005). Fission yeast Kin1-GFP is detected at
15 the plasma membrane and accumulates in actively growing cell surface regions. Kin1-GFP
16 signal does not overlap exactly with methyl blue. Kin1 is probably present on the cytoplasmic
17 face of the plasma membrane and thus in a slightly different position from the cell wall. A
18 similar conclusion has been reached for the budding yeast homologs Kin1p and Kin2p
19 (Tibbetts *et al.*, 1994). Moreover, our results consistently show that Kin1 associates with the
20 Triton X-100-insoluble membrane fraction, suggesting that it is preferentially located in
21 sterol-rich lipid rafts. However, *in silico* analysis of the Kin1 ORF does not reveal any known
22 membrane association domains such as FYVE, PH, CAAX box, GPI anchoring motifs or
23 transmembrane domains. Moreover, it is not detached from the membrane by 0.5M NaCl or
24 2M urea, indicating that this is not simply a peripheral membrane protein. This suggests that

1 Kin1 may be anchored in the lipid membrane by an as-yet-unidentified post-translational
2 modification.

3
4 Our FRAP studies reveal that Kin1 interaction with the plasma membrane is highly
5 dynamic compared to a membrane spanning protein. This high mobility is evidently not
6 influenced by plasma membrane viscosity, but we show that it does require Kin1 kinase
7 activity, suggesting a dynamic interaction with a putative membrane protein substrate. This is
8 a puzzling observation considering that Kin1 associates with the membrane fraction. An
9 alternative possibility is that inhibition of Kin1-as1 rapidly alters plasma membrane properties
10 and this may eventually modify Kin1 dynamics. It will be necessary to decipher the
11 underlying molecular mechanism regulating the association of Kin1 with the membrane to
12 address this issue.

13

14 **A role for Kin1 in regulating the cell surface**

15 Budding yeast Kin1 homologs, Kin1p and Kin2p, have been isolated as multicopy
16 suppressors of several mutants acting at different levels of the secretory pathway, including
17 the *cdc42-6* and *rho3-V51* alleles. Kin1p and Kin2p have been shown to interact with and
18 regulate phosphorylation of proteins acting at the final stage of exocytosis such as the t-
19 SNARE Sec9p. Epistatic data suggest a role for Kin1p and Kin2p downstream of *CDC42* and
20 *RHO3* and upstream of vesicle fusion (Elbert *et al.*, 2005).

21 In contrast to budding yeast, *sec9* is essential in fission yeast (Nakamura *et al.*, 2005)
22 and vesicular trafficking mutants in fission yeast remain poorly characterized (Takegawa *et*
23 *al.*, 2003). Exocytic mutants such as *rho3Δ* or the exocyst component *sec8-1* have been
24 described which accumulate vesicles about 100 nm in diameter, indicating a defect in vesicle
25 fusion with the plasma membrane (Wang *et al.*, 2002; Wang *et al.*, 2003). However, we did

1 not detect such vesicles in *kin1Δ* by TEM. In addition, we were unable to detect suppression
2 of these mutants by Kin1 overexpression in fission yeast (data not shown). Growth of *kin1Δ*
3 *rho3Δ* cells is severely reduced at 25°C and inhibited above that temperature. Electron
4 microscopy studies show that *kin1Δ rho3Δ* cells exhibit tremendous cell wall accumulation at
5 36°C (unpublished results), suggesting that Rho3 and Kin1 act in nonredundant pathways to
6 regulate the cell surface. Thus, budding and fission yeast Kin1 homologs may control a late
7 step in plasma membrane organization, such as membrane recycling, even though molecular
8 mechanisms may differ. In higher eukaryotes, mammalian Par-1 may regulate exocytosis
9 although Par-1 does not interact with the Sec9 SNAP25 homolog (cited in Elbert *et al.*, 2005).

10 The early defect of Kin1 loss-of-function impinges on several aspects of plasma
11 membrane organization such as localization of t-SNARE and beta-glucan synthases and
12 polarization of sterol-rich domains. With regard to Kin1 localization, our data are consistent
13 with a primary role for Kin1 in maintenance of polarized actively growing cell surface regions
14 in the plasma membrane. We propose that Kin1 deletion or inhibition may promote localized
15 loss of cohesiveness between the plasma membrane and the cell wall. Exocytic and cell-wall
16 regulators are concentrated at these sites and thus the periplasmic space formed by this loss of
17 cohesiveness would be rapidly filled up by amorphous cell wall deposits. A possible role for
18 phosphorylation catalyzed by the Kin1 kinase might therefore be the recycling of proteins
19 transiently present at the plasma membrane, including Kin1 itself and cell wall synthesizing
20 enzymes. In conclusion, Kin1 function would be required to ensure a robust and tight link
21 between the plasma membrane and the cell wall during vegetative growth.

22

23 **Kin1 inhibition causes plasma membrane depolarization which leads to cell wall damage**
24 **and hyperactivation of the CWI Pmk1 pathway**

1 Cell wall damage and plasma membrane stretch are detected and relayed by transmembrane
2 protein sensors connected with the cell wall via post-translational modifications. Following
3 detection, the CWI MAPK pathway plays a major role in intracellular signalling to promote
4 an adapted response (Barba *et al.*, 2008; Levin, 2005). We propose that *kin1* deletion or
5 inhibition mimics continuous cell wall or plasma membrane stress. In *kin1* Δ cells, the beta-
6 glucan synthase Bgs1 is more stable at the plasma membrane than in wild type cells. Cfh3 is a
7 specific regulator of Bgs1 endocytosis. Time-course experiments consistently show that the
8 primary effect of Kin1-as1 inhibition is the rapid depolarization of Cfh3 and this may impair
9 Bgs1 endocytosis. Accumulation of Cell Wall Deposits (CWD) is not detected until later and
10 Pmk1 activation is detected even later, suggesting that CWI pathway is activated as a
11 consequence of the plasma membrane and cell wall defects. Production of empty periplasmic
12 spaces as a result of Kin1 inhibition may lead to a cellular response involving a rapid
13 compensation by synthesis of amorphous cell wall material. Kin1 inhibition may also trigger
14 (either directly or indirectly *via* induction of CWD) a permanent signal of cell surface injury,
15 activating the CWI pathway and leading to more CWD than would result from Kin1
16 inhibition alone. Inhibition of CWI signalling suppresses CWD formation, indicating that
17 CWI contributes to such a cell wall response. However, this suppression is only partial,
18 suggesting that other molecular pathways are also involved. Sty1 MAPK, the core component
19 of the Stress-Activated Protein Kinase (SAPK) pathway in fission yeast, may also participate
20 in CWD formation. However, *kin1* Δ *sty1* Δ cells did not show any significant change in the
21 number of CWD compared to *kin1* Δ alone (data not shown).

22

23 **Kin1, Cell Wall Integrity and Sensitivity to Stress**

24 The presence of CWD may be detrimental to the cell's capacity to resist specific stresses,
25 specifically those which challenge cell wall plasticity. Thus, *kin1* Δ cells are hypersensitive to

1 several stress conditions, including upward shifts in osmotic pressure and temperature. *Kin1Δ*
2 cells have a higher content of beta- and alpha-glucan (P. Perez, pers. com.) and we have
3 shown that the expression of Bgs1 and Bgs4 is upregulated in these cells. This is consistent
4 with *kin1Δ* cells being insensitive to caspofungin, an inhibitor of cell-wall synthesizing
5 enzymes. Inhibition of the CWI pathway reduces cell wall thickening and contributes to the
6 alleviation of stress sensitivity.

7 The function of fission yeast Pck1 is largely unknown, but its physical interaction with
8 Rho GTPases and genetic interactions with *pck2Δ* and other cell wall regulating genes suggest
9 a function in cell wall integrity (Arellano *et al.*, 1999; Calonge *et al.*, 2000; Ma *et al.*, 2006;
10 Toda *et al.*, 1996). Our work reveals a strong synthetic interaction between *kin1Δ* and *pck1Δ*.
11 In double mutant cells, growth and cell polarity are severely compromised. We conclude that
12 Kin1 is required for the maintenance of cell integrity in the absence of Pck1 whereas the
13 *kin1Δ pck2Δ* mutant shows phenotypic suppression. Thus, the *kin1Δ* mutation could be useful
14 in discriminating the distinct functions of these protein kinase C homologs.

15

16 **SICS versus CWD: an identical consequence of two unrelated causes?**

17 It is tempting to speculate that SICS and CWD are the same consequence of plasma
18 membrane disruption, occurring when wild type cells are subjected to stress or when Kin1 is
19 inhibited under normal growth conditions, respectively. For example, we have suggested that
20 formation of CWD in the absence of Kin1 activity may be due to loss of cohesiveness
21 between the cell wall and plasma membrane and subsequent accumulation of amorphous cell
22 wall material. A similar loss of cohesiveness could result from hypertonic treatment (in the
23 case of 1.2 M sorbitol or 0.6 M KCl) or damage to the plasma membrane (in the case of SDS).
24 However, SICS are not dependent on the CWI pathway, whereas CWD are partially

1 dependent on that pathway. This suggests that SICS and CWD may correspond to different
2 cellular defects that only appear to have similar outcomes.

3

4 **Kin1 cooperates with Sla2-dependent membrane internalization and acts downstream of** 5 **Myo4 and For3**

6 A mutation like *sla2* Δ that alters endocytosis promotes accumulation of cell wall material at
7 the plasma membrane (this study; Ge *et al.*, 2005), perhaps due to inhibition of membrane
8 internalization. Fluorescent FM4-64 uptake suggests that Kin1 has no significant role in
9 endocytosis itself but inhibition of endocytosis *via* the *sla2* Δ mutation exacerbates *kin1-as1*-
10 mediated CWD formation. This and other observations support the notion that inability of
11 cells to properly regulate membrane internalization promotes the accumulation of cell wall
12 material between a layered cell wall and the plasma membrane.

13 Vesicles are transported *via* F-actin cables to sites of polarized growth by molecular
14 motors such as the type V myosin Myo4 (Feierbach and Chang, 2001; Motegi *et al.*, 2001;
15 Mulvihill *et al.*, 2006; Win *et al.*, 2001). In accordance with a Kin1 function in membrane
16 recycling at the cell cortex, we show that inhibition of general vesicle targeting to the plasma
17 membrane by deletion of *myo4*, by BFA-mediated interruption of intracellular trafficking or
18 by inhibition of the formation of F-actin cables through *for3* deletion in the *kin1-as1*
19 background, suppresses the CWD phenotype usually associated with Kin1 inhibition. Myo4
20 has been shown to target the beta-glucan synthase Bgs1 protein to sites of polarized growth
21 (Mulvihill *et al.*, 2006). Here we show that Kin1 is not involved in targeting Bgs1, nor its
22 regulator Cfh3, to the plasma membrane but in their restriction to polarized growth sites.
23 Rather than regulating exocytic and/or endocytic flux *per se*, Kin1 appears to regulate the
24 balance between these processes, both spatially and dynamically.

25

1 **EXPERIMENTAL PROCEDURES**

2

3 ***S. pombe* strains, media and reagents**

4 *S. pombe* strains used in this study and their genotypes are listed in Table 1. Media (EMM and
5 YES) and genetic methods were as described (Moreno *et al.*, 1991). Cells were grown at
6 25°C, 30°C or 37°C for 5h when stated. Genetic crosses and sporulation were performed in
7 EMM agar plates with 1/10 limiting nitrogen source NH₄Cl. Tetrad dissection was performed
8 with a Singer MSM system (Somerset, UK) and genotypes of interest were selected by
9 appropriate replica plating. GFP-Psy1-expressing strains were derived from *leu1-32 h-* (WT)
10 or *kin1::kanMX6 leu1-32 h- (kin1Δ)* cells transformed with the pTN381 (Psy1 promoter-GFP-
11 Psy1) plasmid (a kind gift from T. Nakamura, Osaka, Japan) integrated at the *leu1* locus. For
12 nonstandard growth conditions, cells were grown in liquid media containing 0.01% Sodium-
13 Dodecyl-Sulfate (SDS), 1.2 M Sorbitol, or 50 μg/ml Brefeldin A (diluted in ethanol, Sigma).
14 For stress induction conditions, EMM- or YES-based agar plates contained 0.1M or 0.2M
15 MgCl₂, 0.005% SDS, or 0.5 mg/ml Calcofluor white. Exponentially growing cells of different
16 strains were diluted and 10⁵, 10⁴, 10³, 10² cells were spotted for 3-5 days at 30°C. The ATP-
17 analog 4-Amino-1-*tert*-butyl-3-(1'-naphthylmethyl)pyrazolo[3,4-d]pyrimidine (Bishop *et al.*,
18 2000), abbreviated as 1NM-PP1, was synthesized as described in Dischinger *et al.* (2008),
19 prepared as a 25 mM stock solution in DMSO and stored at -20°C.

20

21 **Mutagenesis and integration of *kin1-as1***

22 Mutagenesis of phenylalanine to glycine at position 220 in the Kin1 ORF was performed
23 using a “QuickChange Multi Site-Directed Mutagenesis” kit (Stratagene) and the
24 pREP41GFP-Kin1 plasmid as a DNA matrix (La Carbona *et al.*, 2004). The resulting
25 pREP41GFP-kin1-as1 (F220G) plasmid was fully sequenced and transformed into

1 *kin1::kanMX6 leu1-32 (kin1Δ)* cells. Expression of the mutated Kin1-as1 protein fully
2 rescued all tested *kin1Δ* phenotypes (cell morphology, zymolyase and salt resistance,
3 septation efficiency), indicating that the mutated Kin1-as1 protein was functional (data not
4 shown). The mutagenized ORF was amplified by PCR and transformed into *kin1::LEU2*
5 disrupted cells (Levin and Bishop, 1990). Kin1-as1 integrants were selected by growth on
6 EMM agar plates containing 1 M KCl that did not allow *kin1Δ* growth. Leucine auxotrophy
7 due to loss of the *LEU2* marker confirmed integration at the *kin1* locus. The *kin1* ORF was
8 further modified by homologous recombination using the Gly5-GFP-kanMX cassette as
9 described (Bahler *et al.*, 1998; Cadou *et al.*, 2009). G418-resistant colonies were selected and
10 the *kin1-as1-GFP* ORF was fully sequenced on the genome. Inhibition of Kin1-as1 was
11 performed using 1NM-PP1 diluted from a 25 mM stock solution in DMSO. Except when
12 otherwise stated, cells were incubated 2h with 20 μM 1NM-PP1 at 30°C.

13

14 **Western blotting**

15 A 50 ml cell culture was harvested by centrifugation at 3,000 rpm, washed in 20 ml ice-cold
16 PBS and then in 10 ml ice-cold STOP buffer (10 mM EDTA, 150 mM NaCl, 50 mM NaF,
17 0.05% NaN₃). Dry cell pellets were stored at -70°C. Lysis was carried out by vortexing cells
18 in 200 μl of lysis buffer (10% glycerol, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% nonidet
19 P-40, 15 mM imidazole, Roche protease inhibitor cocktail) supplemented with 2% SDS and
20 0.3 g of glass beads (Sigma). The cell lysate was then centrifuged for 10 min. at 13,000 rpm at
21 4°C and the supernatant was diluted with 3X Laemmli loading buffer. Proteins were separated
22 by SDS-PAGE and transferred onto nitrocellulose or PVDF Immobilon-P membranes.
23 Membranes were blotted in PBS/5% milk/0.1% Tween 20. Rabbit polyclonal anti-Kin1 (La
24 Carbona *et al.*, 2004) and mouse monoclonal anti-GFP (Roche) were used at 1:3000 and
25 1:1000 dilutions, respectively. For a loading control, mouse monoclonal anti-PSTAIR (anti-

1 Cdc2, Sigma) was used at 1:5000 dilution. Secondary antibodies were conjugated to either
2 alkaline phosphatase or horseradish peroxidase and revealed by ECF/Storm or West DURA
3 (Pierce), respectively. Activated Pmk1 was detected in total cell extracts using a rabbit anti-
4 p42/44 antiphospho-antibody at 1:1000 dilution and the ECL system as described (Madrid *et*
5 *al.*, 2006).

6

7 **Sub-cellular fractionation**

8 Sub-cellular fractionation was performed using 200 ml of exponentially growing cells. Cells
9 were incubated for 10 min. in RB buffer (1.2 M Sorbitol, 30 mM beta-mercaptoethanol, 50
10 mM Tris pH 8.5) and washed twice in SP buffer (1.2 M Sorbitol, 50 mM NaH₂PO₄ pH 5.8).
11 Cells were then incubated 1h45 at 30°C in SP buffer containing 10 mg/ml Lysing enzymes
12 (Sigma). The resulting spheroplasts were checked by phase contrast microscopy and spun at
13 3,000 rpm for 5 min. at 4°C. Spheroplasts were resuspended in 250 µl of L buffer (365 mM
14 sucrose, 20 mM MOPS pH7.4, Roche protease inhibitor cocktail) and lysed under
15 nondenaturing conditions using a Dounce homogenizer until 90% lysis efficiency was
16 achieved. Cellular debris was removed by centrifugation at 3,000 rpm for 5 min. at 4°C. The
17 cell lysate was then spun at 100,000 g for 1h at 4°C and the pellet and supernatant
18 corresponded to the membrane and cytoplasmic fractions, respectively. The membrane
19 fraction was resuspended in TNE buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM
20 EDTA, Roche protease inhibitor cocktail) and different aliquots were pelleted at 100,000 g for
21 30 min. at 4°C and resuspended in TNE buffer, TNE+0.5 M NaCl, TNE+0.1 M Na₂CO₃
22 pH11, TNE+2 M urea, TNE+1% Triton X-100, and TNE+2% SDS. After 30 min. on a
23 rotating wheel and further centrifugation at 100,000 g for 30 min. at 4°C, supernatants and
24 pellets were analyzed by Western blot.

25

1 **Microscopy techniques**

2 Cells were observed after reaching the exponential growth phase. For time-lapse video
3 microscopy, 2 μ l of cells were mounted on 2% EMM agarose pads. GFP movies were
4 captured using a spinning disk Nikon TE2000 microscope with a 100x 1.45 NA PlanApo and
5 a HQ2 Roper camera. Incorporation of FM4-64 (Molecular Probes) was recorded in live cells
6 (1 image per 10 min) on agarose pads. Sterol-rich domains were detected using 5 μ g/ml filipin
7 (Sigma) and a DMRXA Leica microscope equipped with a neutral filter. For cell wall and
8 septum detection, cells were fixed with 4% formaldehyde (Sigma) for 30 min., washed in PBS
9 and stained with 0.5 mg/ml methyl blue (Sigma). Cells were observed using a DMRXA Leica
10 microscope with a 100x 1.45 NA PlanApo and a CoolSNAP ES camera. Cell lengths were
11 measured by ImageJ software. Colocalization experiments between cell wall (stained by
12 methyl blue) and GFP tagged membrane proteins and Fluorescence Recovery After
13 Photobleaching (FRAP) were performed on a spinning disc Nikon TE2000 microscope and
14 used the Metamorph software. For FRAP, GFP signals were recorded every second and the
15 bleach was applied for 0.5 sec. Cells were then observed every second for 2 min. Normalized
16 FRAP data were calculated by the ImageJ software. For Transmission Electron Microscopy,
17 cells were stained with potassium permanganate. Images were captured by a Jeol Jem-1010
18 (Peabody, MA).

19

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6

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3

1 **Table 1: *S. pombe* strains used in this study.**

2

strain	Genotype	Reference
XLG009	<i>h- cdc10-V50 leu1-32</i>	(Reymond <i>et al.</i> , 1992)
XLG029	<i>h- leu1-32</i>	Lab stock
XLG031	<i>h- cdc25-22 leu1-32 ura4-D18</i>	(Russel and Nurse, 1986)
XLG046	<i>h- kin1::kanR cdc25-22 leu1-32</i>	(La Carbona and Le Goff, 2006)
XLG053	<i>h- leu1-32 kin1::kanMX6</i>	(La Carbona <i>et al.</i> , 2004)
XLG092	<i>h- kin1::kanR cdc10V50 leu1-32</i>	This study
FM401	<i>h- myo4::ura4+ ade6-M216 leu1-32 ura4-D18</i>	(Motegi <i>et al.</i> , 2001)
PPG3847	<i>h- cps1-191 ura4-D18</i>	(Liu <i>et al.</i> , 1999)
PPG165	<i>h- pck1::ura4+ leu1-32 ura4-D18</i>	(Arellano <i>et al.</i> , 1999)
PPG166	<i>h- pck2::LEU2 leu1-32</i>	(Arellano <i>et al.</i> , 1999)
XLG431	<i>h+ kin1::kanR pck1::ura4+ leu1-32 ura4-D18</i>	This study
XLG454	<i>h- kin1::kanR pck2::kanR leu1-32 ura4-D18</i>	This study
XLG485	<i>h+ kin1::kanR pmk1::ura4+ leu1-32 ura4-D18</i>	This study
XLG510	<i>h- sid4-SA1 leu1-32</i>	(Balasubramanian <i>et al.</i> , 1998)
XLG520	<i>h+ kin1::kanR sid4-SA1</i>	This study
#519	<i>h- his3-D1 ura4-D18 leu1-32 bgs1::ura4+ P_{bgs1+}::GFP-bgs1⁺:leu1⁺</i>	(Cortes <i>et al.</i> , 2005)
#1216	<i>h- his3-D1 ura4-D18 leu1-32 bgs3::ura4+ P_{bgs3+}::GFP-bgs3⁺:leu1⁺</i>	(Cortes <i>et al.</i> , 2005)
#561	<i>h- his3-D1 ura4-D18 leu1-32 bgs4::ura4+ Pbgs4+::GFP-bgs4+:leu1+</i>	(Cortes <i>et al.</i> , 2005)
XLG535	<i>h- kin1::kanR bgs1::ura4+ P_{bgs1+}::GFP-bgs1⁺:leu1⁺ leu1-32 ura4-D18</i>	This study
XLG536	<i>h- kin1::kanR bgs3::ura4+ P_{bgs3+}::GFP-bgs3⁺:leu1⁺ leu1-32 ura4-D18</i>	This study
XLG537	<i>h- kin1::kanR bgs4::ura4+ P_{bgs4+}::GFP-bgs4⁺:leu1⁺ leu1-32 ura4-D18</i>	This study
TP319-13C	<i>h- pmk1::ura4+ ura4-D18</i>	(Toda <i>et al.</i> , 1996)
XLG572	<i>h- kin1::Gly5-GFP-kanR leu1-32 ura4-D18</i>	(Cadou <i>et al.</i> , 2009)
XLG595	<i>h- kin1-as1::Gly5-GFP-kanR ade6-704 leu1-32 ura4-294</i>	This study
XLG602	<i>h+ rho2::kanR ade6- ura4-D18 leu1-32</i>	(Ma <i>et al.</i> , 2006)
XLG605	<i>h+ pmp1::kanR ade6- leu1-32 ura4-D18</i>	(Sugiura <i>et al.</i> , 1998)
XLG614	<i>h- kin1::kanR rho2::kanR leu1-32 ura4-D18</i>	This study
XLG615	<i>h- pmp1::kanR kin1::kanR ade6- leu1-32</i>	This study
XLG620	<i>h+ kin1-as1::Gly5-GFP-kanR sla2::kanR ade6- leu1-32 ura4-</i>	This study
XLG679	<i>h- leu1:P_{psyl}- GFP-psyl</i>	(Nakamura <i>et al.</i> , 2001)
XLG680	<i>h- kin1::kanR leu1:P_{psyl}- GFP-psyl</i>	This study
XLG700	<i>h- kin1-as1::Gly5-GFP-kanR myo4::ura4+ ade6- leu1-32 ura4-</i>	This study
XLG709	<i>h- kin1-as1 leu1:P_{psyl}- GFP-psyl ade6- leu1-32 ura4-</i>	This study
XLG717	<i>h- kin1::kanR cps1-191</i>	This study
XLG724	<i>h- kin1-as1 GFP-Cfh3:leu1 ade6- leu1-32 ura4-</i>	This study

1 **Table 2: Mutations in components of the Cell Wall Integrity pathway modulate *kin1Δ*-**
2 **dependent formation of Cell Wall Deposits.** The indicated strains were cultured to mid log
3 phase at 25°C and stained with methyl blue. Percentage of CWD containing cells (mean±SD,
4 n>400) is indicated.

5

strain	% of cell with CWD
WT	None
<i>kin1Δ</i>	77 ± 6.5
<i>kin1Δ pmk1Δ</i>	39.4 ± 2.2
<i>kin1Δ pck2Δ</i>	17 ± 5.3
<i>kin1Δ rho2Δ</i>	66.1 ± 0.5
<i>kin1Δ pck1Δ</i>	91.6 ± 7.8
<i>kin1Δ pmp1Δ</i>	92 ± 0.9

6

1 **FIGURE LEGENDS**

2 **Fig. 1.** Kin1 associates with sterol-rich domains of the plasma membrane and its polarization
3 depends on intact vesicular traffic. A) Kin1 and GFP-Bgs4 localization were determined by
4 sub-cellular fractionation in *GFP-Bgs4* cells and Western blot analysis using anti-Kin1 (upper
5 panels) and anti-GFP (lower panels) antibodies, respectively. Total cell extracts (CE) of
6 exponentially growing GFP-Bgs4 protoplasts were prepared under nondenaturing conditions.
7 Cytosol and membrane fractions were separated by ultracentrifugation. B) Membrane
8 fractions were further separated in buffers containing either 0.5 M NaCl, 100 mM Na₂CO₃ pH
9 11, 2 M urea, 1% Triton X-100 at 4°C and 2% SDS. C) Brefeldin A (BFA) treatment
10 depolarizes Kin1-GFP on the plasma membrane. *Kin1-GFP* cells were treated for 1h with 100
11 µg/ml of BFA. Polarization of lipid rafts was assayed by filipin dye as a control for BFA
12 efficiency. Bar, 5 µm.

13
14 **Fig. 2.** Kin1 is required for morphogenesis and cell wall synthesis regulation. A) Wild type
15 (WT) and *kin1Δ* cells were cultured to mid-log phase, fixed at 25°C or after 5h at 37°C and
16 beta-glucans were stained with methyl blue. Arrowheads show the presence of Cell Wall
17 Deposits (CWD) on the lateral cortex. Percentage of CWD containing cells (mean±SD,
18 n>400) are indicated in the top right for *kin1Δ* mutants. Bar, 2 µm. B) *cdc10-V50*, *cdc25-22*,
19 and *sid4-SAI* mutations (G1 arrest, G2 arrest and septum inhibition, respectively) were
20 combined with *kin1Δ* and compared to *kin1⁺* cells (WT). Exponentially growing cells were
21 shifted to 37°C for 4h and stained with methyl blue. CWD are shown by arrowheads. Bar, 2
22 µm. C) Cell wall structures observed by Transmission Electron Microscopy. Left: the wild
23 type (WT) cell shows cell wall (cw), nucleus (n), plasma membrane (pm), septum (s). Right:
24 *kin1Δ* cells showing two different Cell Wall defects: (a) multiple layers of cell wall, (b)

1 amorphous cell wall material (am, white arrow) between the plasma membrane and the cell
2 wall. Insets show 2X magnifications of the cell surface. Bars, 1 μm .
3
4 **Fig. 3.** Phenotypic characterization of the inhibition of an ATP analog-sensitive Kin1-as1
5 kinase. A) Identification of the F220G analog-sensitive (as1) mutation in Kin1 ORF by
6 sequence alignment with Calmodulin Kinase and Cyclin dependent kinase Cdk2 (according to
7 Bishop *et al.*, 2000). B) Effect of Kin1-as1 inhibition on the cell wall and septum: *kin1-as1*
8 cells were incubated either with DMSO or 20 μM 1NM-PP1 for 2h at 30°C. In the upper
9 panels, cells were stained with methyl blue for fluorescence microscopy (Bar, 2 μm). The
10 asterisk shows an asymmetric septum and arrowheads show CWD. In the middle panels, cells
11 were processed for TEM. Arrows show amorphous cell wall material (am), cell wall (cw),
12 plasma membrane (pm) (Bars, 1 μm). An inset depicts the distinctive nature of the electron
13 density of the cell wall material in the TEM image (Bar, 0.5 μm). The histogram on the lower
14 panel indicates the percentage of *kin1-as1* cells with CWD following incubation with various
15 doses of 1NM-PP1 for 2h at 30°C. C) Localization of Kin1-as1-GFP in cells incubated either
16 with DMSO or 20 μM 1NM-PP1 for 2h at 30°C. Asterisks depict Kin1-as1-GFP signals on
17 the lateral cortex. The right panel shows a part of the middle image with depolarized Kin1-
18 as1-GFP on the cortex. Bar, 2 μm . D) Mobility of Kin1-as1-GFP protein in the cell end
19 analyzed by Fluorescence Recovery After Photobleaching (FRAP). *Kin1-as1* cells were
20 treated either with DMSO or 20 μM 1NM-PP1 for 2h at 30°C. Fluorescence of Kin1-as1-GFP
21 in representative cells is shown before the laser bleach (pre), during the bleach (bleach) and at
22 50 and 100 sec after the bleach (t50 and t100). The bleached areas are indicated by white
23 squares. Bar, 5 μm . The graph on the right shows the mean normalized fluorescence intensity
24 in the cell end plotted over time (n=10 for each condition). The arrow indicates the bleach.

1 The membrane spanning protein GFP-Psy1 has been processed under identical FRAP
2 conditions as a control.

3
4 **Fig. 4.** Kin1 is required for plasma membrane organization and endocytosis. A) Wild type
5 (WT) and *kin1* Δ cells were stained with filipin. B) GFP-Psy1 localization was detected in
6 exponentially growing WT and *kin1* Δ cells at 25°C. Arrowheads show accumulation of GFP-
7 Psy1 on the lateral cortex. C) FM4-64 (8 μ M) incorporation into WT or *kin1* Δ cells was
8 monitored after 1h. D) Localization of GFP-Bgs1 in exponentially growing cells at 25°C was
9 examined in WT and *kin1* Δ strains. Arrowheads denote delocalized GFP-Bgs1 signals. Bars, 2
10 μ m in all micrographs. E) *Kin1* Δ rescues *cps1-191* (*bgs1*^{ts}) sensitivity to heat. Growth of
11 indicated strains was monitored by serial dilutions on YES medium at different temperatures.

12
13 **Fig. 5.** *Kin1-as1* inhibition initially alters growth area polarization and subsequently cell wall
14 structure and Pmk1 activation. *Kin1-as1* cells were treated with either DMSO or 20 μ M
15 1NM-PP1 for 2h at 30°C. A) Cells were stained with filipin to detect sterol-rich domains
16 (Bar, 2 μ m), B) GFP-Cfh3 (Bgs1 complex) was localized during different stages of the cell
17 cycle. Arrowheads denote depolarized GFP-Cfh3 on the lateral cortex (Bar, 5 μ m), C)
18 Kinetics of CWD formation and GFP-Cfh3 depolarization at the cortex. *Kin1-as1 GFP-Cfh3*
19 cells were incubated with 20 μ M 1NM-PP1 and samples were collected at the indicated times.
20 Cfh3 localization was detected by its GFP signal and CWD by methyl blue staining. The
21 percentage of cells in the population is indicated (n>100). D) Pmk1 activation was monitored
22 by western blot. Its activated form was detected using the anti-phospho MAPK antibody
23 (p42/44) in whole cell extracts. Cdc2 was used as a loading control.

24

1 **Fig. 6.** Vesicular trafficking modulates CWD formation. A) Exponentially growing *kin1-as1*
2 cells were treated with Ethanol or Brefeldin A (BFA) for 1h and then DMSO or 20 μ M 1NM-
3 PP1 was added for 2h at 30°C. B) Strains of the indicated genotypes were incubated either
4 with DMSO or 20 μ M 1NM-PP1 for 2h at 30°C. Arrowheads show CWD. Samples were
5 stained with methyl blue. Bars, 2 μ m.

6
7 **Fig. 7.** The Cell Wall Integrity MAPK pathway is constitutively activated in *kin1 Δ* cells and
8 contributes to CWD formation. A) Analysis of CWI MAPK pathway activation in wild type
9 (WT) and indicated mutant cells by Western blot. Whole cell extracts were prepared and
10 Pmk1 activation was monitored using the anti-phospho MAPK antibody (p42/44). Cdc2 was
11 used as a loading control. (B, C) The indicated strains were cultured to mid log phase and
12 growth was assayed by 10-fold serial dilutions on B) YES control plates or YES plates
13 containing 0.1 M or 0.2 M MgCl₂, and C) EMM control plates or EMM plates containing the
14 cell wall damaging agents SDS or Calcofluor white (Cw).