Extracellular cell wall $\beta(1,3)$glucan is required to couple septation to actomyosin ring contraction

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Cytokinesis has been extensively studied in different models, but the role of the extracellular cell wall is less understood. Here we studied this process in fission yeast. The essential protein Bgs4 synthesizes the main cell wall $\beta(1,3)$glucan. We show that Bgs4-derived $\beta(1,3)$glucan is required for correct and stable actomyosin ring positioning in the cell middle, before the start of septum formation and anchorage to the cell wall. Consequently, $\beta(1,3)$glucan loss generated ring sliding, oblique positioned rings and septa, misdirected septum synthesis indicative of relaxed rings, and uncoupling between a fast ring and membrane ingestion and slow septum synthesis, suggesting that cytokinesis can progress with defective septum pushing and/or ring pulling forces. Moreover, Bgs4-derived $\beta(1,3)$glucan is essential for secondary septum formation and correct primary septum completion. Therefore, our results show that extracellular $\beta(1,3)$glucan is required for cytokinesis to connect the cell wall with the plasma membrane and for contractile ring function, as proposed for the equivalent extracellular matrix in animal cells.

Introduction

Cytokinesis is a critical process for cell integrity and is very well conserved from animal to fungal cells. All require coordinated contractile actomyosin ring (CAR) closure and plasma membrane (PM) extension. Fungal cytokinesis requires the additional synthesis of a special division wall termed septum, strictly coupled to CAR contraction and PM extension (Pollard, 2010; Balasubramanian et al., 2012). The septum is a three-layered structure of a middle primary septum (PS) flanked by a secondary septum (SS) on each side. The Schizosaccharomyces pombe septum grows by simultaneous synthesis of both PS and SS. The last step of cytokinesis is cell separation by controlled cell wall and PS degradation. Correct septum formation and especially cell separation are critical processes for cell integrity and survival (Cabib et al., 2001; Sipiczki, 2007; Cortés et al., 2012).

The fission yeast cell wall contains different essential glucans, but no chitin has been detected (Pérez and Ribas, 2004). Branched $\beta(1,6)$glucan is located in the cell wall and SS; minor linear $\beta(1,3)$glucan (L-BG) is located mainly in the PS and some in the cell wall; and major branched $\beta(1,3)$glucan (B-BG) and $\alpha(1,3)$glucan are located in the cell wall and both PS and SS (Humbel et al., 2001; Cortés et al., 2007; Cortés et al., 2012). L-BG is a special glucan necessary, but not sufficient, for PS formation that interacts with high affinity with the fluorescent Calcofluor white (CW) in S. pombe (Cortés et al., 2007). B-BG and $\alpha(1,3)$glucan are essential for cell shape and integrity (Ribas et al., 1991; Hochstenbach et al., 1998; Katayama et al., 1999; Cortés et al., 2005, 2012). $\alpha(1,3)$glucan is essential for the PS adhesion strength needed to support the internal pressure during cell separation (Cortés et al., 2012). However, the B-BG functions for cell wall and septum structure and integrity remain unknown.

S. pombe contains four essential integral PM glucan synthases (GS) that localize to the CAR, septum, and growing poles. Bgs1 and Ags1 appear simultaneously at the division site before septum synthesis, whereas Bgs4 localizes after septum

Abbreviations used in this paper: B-BG, branched $\beta(1,3)$glucan; CAR, contractile actomyosin ring; CW, Calcofluor white; DR, dense ring; GS, glucan synthase; L-BG, linear $\beta(1,3)$glucan; MD, matériel dense; MM, minimal medium; MTD, matériel triangulaire dense; OE, old end; PM, plasma membrane; PS, primary septum; SS, secondary septum; TEM, Transmission electron microscopy; WT, wild type.
initiation. Bgs1 is responsible for the L-BG and PS synthesis; and Ags1 is responsible for the α(1,3)glucan and SS synthesis and the PS adhesion strength. The function of Bgs3 remains unknown (Cortés et al., 2002, 2005, 2007, 2012; Liu et al., 2002; Martín et al., 2003).

Bgs4 and Ags1 are essential for cell integrity during polarized growth and mainly cytokinesis (Cortés et al., 2005, 2012). Bgs4 is responsible for the cell wall B-BG synthesis and the major β(1,3)GS activity. Bgs4 is also responsible for the resistance to specific β(1,3)GS inhibitors (Ribas et al., 1991; Castro et al., 1995; Cortés et al., 2005; Martíns et al., 2011).

In this work, in addition to our findings concerning the essential Bgs4 B-BG functions for the cell wall and septum structure and integrity, we show for the first time that extracellular B-BG is important for CAR positioning in the cell middle. Furthermore, B-BG plays a role in coupling septum synthesis to CAR contraction and PM extension. Our findings reveal important similarities between the function of B-BG in connecting cell wall to CAR and determining intracellular functions of cytokinesis and an analogous role suggested for the ECM (functional equivalent of the cell wall) in animal cells (Xu and Vogel, 2011).

### Results

**Bgs4 is essential for cell integrity mainly during cytokinesis**

To study the essential functions of β(1,3)GS Bgs4, a bgs4Δ strain containing integrated bgs4Δ regulated by the 81X version (highest repression level) of the thiamine-repressible nmt1' promoter, was made (see Materials and methods). The 81X-bgs4Δ strain showed a uniform repression phenotype of cell lysis. Cell growth arrested after 8 h of bgs4Δ repression and sorbitol delayed growth arrest to 12 h (Fig. S1 A, arrow). Thus, sorbitol was selected to study stronger bgs4Δ repression defects in cells that would otherwise be dead. Cell lysis in the presence of sorbitol started at 8 h (Fig. S1, B and D [arrows]), earlier than cell growth arrest was detected, and reached 50% at 12 h. Cell lysis without sorbitol also started earlier than cell growth arrest, at 5–6 h (unpublished data). Coincident with the cell lysis, the increase in cell number also arrested at 8 h (Fig. S1 C, arrow). This discrepancy between absorbance arrest and start of cell lysis was also detected with α(1,3)GS ags1' repression (Cortés et al., 2012). This could be because of additional absorbance of lysed cells, released cytoplasmic material, or changes in cell shape, density, cell wall thickness, or cell wall composition. Most of the lysis occurred at the septum and very little at the poles (Fig. S1 D). In agreement with previous data from bgs4Δ point mutants (Ribas et al., 1991), the Bgs4-depleted cells showed a considerable decrease in total cell wall β-glucan (56%) and the corresponding compensatory increase in α-glucan (Table 1 and Fig. S1 E).

The Bgs4 amount was analyzed during Bgs4 depletion. Compared with other essential GS, the native expression of Bgs4 is by far the most abundant: 44-fold Bgs1, 9-fold Bgs3, and 6-fold Ags1 (Fig. S1 F). During bgs4Δ repression, Bgs4 decreased rapidly to 20% at 8 h, when lysis started, and at 12 h it was just 5% of the native level (Fig. S1 G). Therefore, a fivefold Bgs4 reduction is critical for cell wall and septum formation and cell integrity. Also, GFP-Bgs4 localization during repression decreased quickly; at 8 h it was not detected at the poles and at 10 h it was not detected at the septum (Fig. S1 H).

**Bgs4 is required for correct PS completion**

To know the Bgs4 function in septum formation precisely, the septation defects caused by Bgs4 absence were analyzed. The septation percentage increased after 8 h of bgs4Δ repression, when cell lysis started, reaching twice the amount of septa at 12 h. This increase was detected only considering live cells. When lysed cells were included (total cells), the septation index was unaffected (Fig. 1 A, left). CW specifically stains the PS (Cortés et al., 2007). CW staining showed a considerable increase in open septa and concomitant decrease in complete septa, generated during the period of lysis induction (Fig. 1 A, right).

To confirm the Bgs4 importance in septum completion, the septa were observed simultaneously by CW staining and GFP-Bgs1 localization. Surprisingly, many CW-stained incomplete septa appeared complete by GFP-Bgs1 (Fig. 1 B, arrowheads). The imbalance between open and complete CW-stained septa was not observed with GFP-Bgs1; instead, the amounts of open and complete septa were unaltered (Fig. 1 C, left). This uncoupling of CW-stained open septa occurred in both growing and complete GFP-Bgs1 septa (Fig. 1 C, right). Thus, Bgs4 absence causes a specific delay in CW-stained PS closure rather than in general cytokinesis completion.

To check if the GFP-Bgs1 advance and closure observed in CW open septa was a process common to all septum components, CW staining and localization of septum membrane Bgs1 and Bgs3 or the CAR components myosin II regulatory chain

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**Table 1. Incorporation of [14C]glucose into cell wall polysaccharides during Bgs4 depletion**

| Strain     | Thiamine* | Cell wall | α-Glucan* | β-Glucan* | Galactomannan* |
|------------|-----------|-----------|-----------|-----------|----------------|
| Control    | 0 [on]    | 25.0 ± 2.3| 22.2 ± 1.9| 62.7 ± 1.4| 15.1 ± 0.7     |
| 81X-bgs4*  | 6 [off]   | 22.1 ± 1.4| 33.8 ± 4.0| 52.4 ± 3.2| 13.8 ± 0.8     |
| 81X-bgs4*  | 8 [off]   | 24.0 ± 1.2| 40.1 ± 5.1| 43.2 ± 6.7| 15.8 ± 1.7     |
| 81X-bgs4*  | 10 [off]  | 24.0 ± 1.2| 40.1 ± 5.1| 43.2 ± 6.7| 15.8 ± 1.7     |

*Early log-phase bgs4Δ-repressed cell cultures were grown in MM + 1.2 M sorbitol for 0, 6, 8, and 10 h in the presence of thiamine (bgs4Δ-repressed conditions). 

**C** incorporation was added 6 h before harvesting. 

Percentage of incorporation of [14C]glucose = cpm incorporated per fraction x 100/total cpm incorporated. Values are the means and SDs calculated from three independent experiments. 

Values are percentages of the corresponding polysaccharide in the cell wall.
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confirmed that the CAR stays attached to the PM when the CW-stained PS is uncoupled (Fig. 1 E, brackets). A similar PS defect was observed in the Bgs4-defective cwg1-1 and cwg1-2 mutants (Fig. S5 A, see last section of Results). This defect was not permanent; at longer times the CW-stained PS was completed (see Fig. 4 below). This shows that Bgs4 is specifically required in the last stages of PS formation.

Bgs4 β(1,3)glucan is needed for correct and stable CAR positioning in the cell middle

To investigate other possible Bgs4 functions, the different cytokinesis steps were analyzed after 8 h of bgs4+ repression, when cell lysis started, ensuring the presence of mild Bgs4 absence phenotypes and the absence of compensatory mechanisms,

Figure 1. Bgs4 is required for PS completion but not for general septum closure. (A) Absence of Bgs4 promotes a septation increase in live but not in total cells (left). Bgs4 absence produces an increase in CW-stained open septa and concomitant decrease in complete septa (right). Arrow shows start of septum defects and cell lysis. Cells were grown in MM with thiamine (+T; repressed) and 1.2 M sorbitol (S). (B) Bgs4 absence causes a defect in CW-stained but not in GFP-Bgs1 septum completion (arrowheads). Cells were grown in MM+S+T for 10 h. (C) Bgs4 absence generates an increase in CW-stained open PS (arrow), not detected in GFP-Bgs1 septa (left). Defective open PS are detected in both advanced (with respect to the CW signal) and complete GFP-Bgs1 septa (right). Cells were grown as in A. Error bars indicate SD. (D) The defect in open PS does not correspond to a general defect in septum synthesis or CAR contraction. Progress (bracket) of WT PS coincides with that of septum membrane (Bgs1, Bgs3, and Psy1) and CAR (Rlc1) proteins but not in the absence of Bgs4, in which the CAR stays attached to the PM, whereas the PS formation is uncoupled and delayed. Cells were grown as in B. The number of experiments and cells or septa analyzed is shown in each case. Bars: [cells] 5 µm; [septum details] 1 µm.
which were induced at longer times (unpublished data). The absence of Bgs4 generated oblique positioned nodes, CAR, and septa in the cell middle (Fig. 2, A–C, dotted lines). Transmission electron microscopy (TEM) confirmed the formation of oblique septa (Fig. 2 D, arrows). Oblique CAR and septa (Rlc1, Cdc15, Bgs1, Bgs3, and Ags1) were quantified during Bgs4 depletion (Fig. 2 E). Maximal amount of oblique CAR (25%) was higher than that of septa (20%), suggesting that some oblique CAR could slide along the PM and correct their position to perpendicular in the cell middle, before septum synthesis initiation and which were induced at longer times (unpublished data). The absence of Bgs4 generated oblique positioned nodes, CAR, and septa in the cell middle (Fig. 2, A–C, dotted lines). Transmission electron microscopy (TEM) confirmed the formation of oblique septa (Fig. 2 D, arrows). Oblique CAR and septa (Rlc1, Cdc15, Bgs1, Bgs3, and Ags1) were quantified during Bgs4 depletion (Fig. 2 E). Maximal amount of oblique CAR (25%) was higher than that of septa (20%), suggesting that some oblique CAR could slide along the PM and correct their position to perpendicular in the cell middle, before septum synthesis initiation and which were induced at longer times (unpublished data). The absence of Bgs4 generated oblique positioned nodes, CAR, and septa in the cell middle (Fig. 2, A–C, dotted lines). Transmission electron microscopy (TEM) confirmed the formation of oblique septa (Fig. 2 D, arrows). Oblique CAR and septa (Rlc1, Cdc15, Bgs1, Bgs3, and Ags1) were quantified during Bgs4 depletion (Fig. 2 E). Maximal amount of oblique CAR (25%) was higher than that of septa (20%), suggesting that some oblique CAR could slide along the PM and correct their position to perpendicular in the cell middle, before septum synthesis initiation and
anchorage of the septum to the cell wall. The oblique septa (71 oblique septa; n = 6) showed a displacement of the septum base with respect to the perpendicular division plane by 0.28 ± 0.2 µm (0.04 ± 0.01 µm in 42 WT cells; n = 2) and of the septum center with respect to the cell middle by 0.23 ± 0.1 µm (0.04 ± 0.01 µm in WT). Therefore, the oblique CAR and septa can be positioned in a 0.56-µm middle region, always located in the cell cortex surrounding the nucleus (Rlc1-GFP Hht1-RFP; unpublished data).

To examine the CAR formation and dynamics in the absence of Bgs4 before septum initiation, when CAR position is fixed by the septum anchorage to the cell wall, time-lapses of CAR (Rlc1) and septum (CW staining) formation were performed. Many cells (31%, 11/35) formed a stable oblique CAR and the ensuing oblique septum (Fig. 2 F, dotted lines; and Video 1). In some cases the nodes also condensed in an oblique fashion (Fig. 2, F [right] and G [left]). Interestingly, many oblique CAR (23%, 8/35) slid along the PM to perpendicular position in the cell middle before septation start and some perpendicular CAR also slid along the PM until septum initiation (Fig. 2 G, arrows and dotted lines; and Video 2). The same defect in oblique CAR and septum positioning was observed in the cwg1-1 and cwg1-2 mutants (Fig. 2 H, Fig. S3 A, Video 3, and Fig. S5 B, see last section of Results). This shows that Bgs4 is required for proper and stable CAR positioning in the cell middle before septum initiation and anchorage to the cell wall. However, Bgs4 appears in the cell middle after septum initiation (Cortés et al., 2005), indicating that the CAR defects are not caused directly by Bgs4 absence, but by the lack of cell wall B-BG, owing to the Bgs4 defect in previous cycles.

**Bgs4 and its β(1,3)glucan are required for correct straight CAR constriction**

Absence of Bgs4 led to 20–25% misdirected CAR and septa (Fig. 3, A–D, arrows). A similar defect of misdirected CAR and septa was observed in cwg1-1 and cwg1-2 mutants (Fig. S5 C, see last section of Results), being more abundant in cwg1-1; therefore, it was selected for time-lapse study (Fig. 3 E, Fig. S3 B, and Video 4). The analysis showed that misdirected ingestion could appear and be corrected throughout septum progression, indicating that this defect is independent of the septation stage. In addition, some misdirected edges showed the opposite direction, whereas in others both edges pointed in the same wrong direction (Fig. 3 E and Fig. S3 B, arrows). Detailed CW staining showed wavy PS (Fig. 3 E, bottom right), indicative of multiple minor changes in septum synthesis direction that could not be detected by CAR and PM ingression analysis. TEM confirmed the formation of misdirected septa (Fig. 3 F, arrows). These data suggest the formation of a relaxed CAR unable to provide the tensile force needed for a straight growing septum and a weak flexible septum lacking the rigidity needed for a straight septum structure. In addition, some septa showed simultaneous misdirected progression and defective CW-stained PS, suggesting that ingestion can progress with defective PS and CAR forces (Fig. 3 E, Fig. S3 B, and Video 4). However, all misdirected septa were progressively corrected until septum completion, indicating some CAR force.

**Bgs4 is required for coupling septum growth to CAR contraction and PM extension**

Septation in the absence of Bgs4 was analyzed by TEM. In WT it started with the annular rudiment, a tiny PM invagination of PS, followed by a small septum that already had the three-layered structure of PS flanked by a SS. The septum grew perpendicular to the cell wall, forming a straight rigid structure, and simultaneously the PS drilled into the wall flanked by matériel triangulaire dense (MTD; triangular dense material; Fig. 3 F, left). In contrast, Bgs4 absence caused oblique, twisted, and misdirected septa, suggesting weak CAR and PS structures (Fig. 3 F, right, arrows). The SS was absent and the PS only connected to the wall by a thin PS covered by diffuse matériel dense (MD; dense material). Interestingly, as the PS progressed, it formed a very thin PS end structure indicative of defective PS synthesis in the last stages of septum formation (Fig. 3 F, right).

Detailed TEM analysis of WT septum showed that PS and PM edges always stayed in close contact (Fig. 3 G, left), indicating that septum growth stays coupled to PM extension and hence to CAR contraction (Fig. 3 H, left). Surprisingly, in the absence of Bgs4, the thinner PS edge (Fig. 3 G, right, arrowheads) appeared clearly detached and delayed from the PM edge, which formed a sharp tip extension with no PS material inside (Fig. 3 G, right, arrows). Similarly, a more advanced septum closure showed a growing PS but complete PM extension (Fig. 4 F), indicating that septum growth was uncoupled and delayed regarding PM extension and CAR contraction (Fig. 3 H, right). This shows that Bgs4 and/or its B-BG play a role in coupling septum synthesis to PM and CAR progression.

The uncoupling of CAR contraction from septum synthesis was analyzed by time-lapse. The start of CAR contraction and septum synthesis (t = 0) was located precisely to the time before the first image when a CW-stained PS signal was detected (Fig. 4 A, blue arrowheads; t = 2). In WT cells, the CAR coincided with the edge of the growing PS throughout the contraction process, indicating that CAR contraction and septum synthesis are coordinated (Fig. 4 A and Video 5). In the absence of Bgs4, CAR contraction progressed more slowly than in WT, and septum synthesis advanced much more slowly, delayed and uncoupled from CAR contraction (Fig. 4 A, Fig. S4 A, and Video 6). The analysis of 26 WT and 34 Bgs4-depleted time-lapses showed no significant differences in CAR assembly and maturation. However, Bgs4 absence promoted a 4-min increase (121% of WT) in CAR contraction and an 8.5-min increase (144%) in uncoupled septum synthesis (Fig. 4 B).

Kymographs showed that Bgs4 and its B-BG are critical for the early steps of CAR contraction and septum formation. WT kymograph showed uniform and coincident CAR contraction and PS formation (Fig. 4, C and D). The kymographs of Bgs4 absence showed strong defects in initial CAR contraction and PS formation and a clear delay in PS formation during and after CAR contraction (Fig. 4, C and D; and Fig. S4, B and C). The start of CAR contraction was severely compromised during a 6–8-min period, after which contraction proceeded even faster than in WT. The initial slow CAR contraction was coupled to a defective slow and asymmetric PS synthesis start,
Figure 3. 

Bgs4 and its β(1,3)glucan are required for correct straight CAR constriction and for coupling septum synthesis to CAR contraction and PM extension. (A–D) Bgs4 absence causes misdirected (arrows) CAR (A and C; Rlc1 and Cdc15) and septa (B and C; Bgs1, Bgs3, Psy1, and CW staining). Cells were grown as in Fig. 2 A. (D) Percentage of misdirected CAR and septa. Error bars indicate SD. (E) The defect in Bgs4 function (shown in the cwg1-1 mutant) produces similar misdirected CAR contraction and septum progression (arrows). During misdirected ingression, the CAR (Rlc1) stays attached to the PM.
as observed by the reduced CW staining on one side (Fig. 4, C and E; and Fig. S4 B, white arrows). TEM showed that the asymmetry was coincident with bent or twisted starting septa (Fig. 4 E, black arrows), suggesting that Bgs4 B-BG strengthens the starting septum and that the CAR force is not sufficient to maintain it straight. After this, PS synthesis uncoupled from CAR contraction, starting a delay that increased along septation. The 6-min delay in septum completion (t = 22–28) coincided with TEM observations of growing PS but complete PM extension (Fig. 4 F).

The analysis of WT CAR contraction and PS synthesis rates showed a coincident and constant rate (95 nm/min) throughout the process (Fig. 4 G). However, Bgs4 absence caused an initial slow CAR contraction and septation rate, sevenfold slower than in WT (15 nm/min in both cases). Interestingly, after this defect the CAR contraction rate increased to 127% faster than WT (120 nm/min). The PS synthesis rate also increased, but only to 76% of WT (72 nm/min), confirming the defective and delayed PS synthesis (Fig. 4 G). This suggests that in WT, CAR and PM ingression rates are restricted by the synthesis rate of the attached septum.

**Bgs4 β(1,3)glucan confers the rigidity needed for a straight septum**

In the absence of Bgs4, the septa presented important morphological and structural defects. Many septa had a curved shape (Fig. 5 A) and defects in PS maturation of CW-stained PS retracted from the cell wall (Fig. 5 B, left, arrowheads and arrows). TEM confirmed the retraction of mature PS, leaving a space filled by new MD (Fig. 5 B, right, arrows). Similar defects were observed in the *cwg1-1* and *cwg1-2* mutants (Fig. S5 D, see last section of Results).

To study the formation mechanism of curved septa, time-lapses of septum formation were performed. WT cells formed a rigid straight septum that, after maturation (11 min), was slowly and symmetrically degraded by its PS to permit sister cell separation (Fig. 5 C, top; and Video 7). In the absence of Bgs4, maturation of some septa extended for a long period (60 min), during which the septa displayed an oscillating curved shape, probably because of changes in internal pressure between sister cells. The lower pressure cell reacted by increasing its internal pressure, pushing the flexible septum and changing its curvature to the opposite cell (Fig. 5 C, middle and bottom, arrow; Fig. S4 D, arrow; and Video 8). In some cases the pressure transmitted by the pushing septum caused pole lysis in the opposite cell (Fig. 5 C, middle; and Fig. S4 D), or the cells equilibrated their internal pressure, returning to a straight septum and causing lysis at the septum region in both cells (Fig. 5 C, bottom). When no lysis occurred, both cells alternated the septum curvature for at least four waving cycles (Fig. 5 D, arrows; and Video 9).

This shows that Bgs4 B-BG is necessary to confer the rigidity needed for a straight septum to support the changes in internal pressure between sister cells.

**Bgs4 is responsible for the SS formation**

Because of the importance of Bgs4 in septum maturation and structure, complete septa were analyzed in detail by TEM. WT complete septa showed a clear transition to thicker septa in a maturation process of a second round of SS synthesis. In addition, the PS appeared well anchored into the cell wall (Fig. 6 A, left). However, in the absence of Bgs4 the septa showed a total absence of SS during septum formation (see Fig. 3 F) and the whole maturation process (Fig. 6 A, right). Although the SS contains different polysaccharides, this shows that Bgs4 B-BG is responsible for the SS formation. Bgs4 is also required for PS completion (see above for Figs. 1 and 4).

As a result, the initial complete septa showed a large middle region of defective very thin PS, which during septum maturation was gradually disappearing by addition of new PS material (Fig. 6 A, right).

Detailed TEM study of the PS showed the WT annular rudiments containing a PS formed at the wall surface. During septum growth and maturation, the PS drilled progressively to reach the middle of the cell wall (Fig. 6 B, top, arrows). However, in the absence of Bgs4 the PS was formed with only a very thin and twisted PS base reaching the wall surface. Then, during septum maturation the PS never drilled into the wall but gradually retracted from it, leaving a spaced filled by amorphous MD (Fig. 6 B, bottom, arrows). This shows that Bgs4 is not only required for PS completion but also for the integrity of the PS base and its anchorage into the wall.

**Bgs4 is essential for cell integrity at the start of cell separation**

The main defect of Bgs4 absence is the lysis and cytoplasm release after septum completion. Lysis occurred in either one or both sister cells, appearing in one single cell earlier than in both cells (Fig. 7, A [arrows] and B). After lysis in one cell, the surviving sister cell initiated a new cell growth cycle, the wall carcass of the dead cell sometimes remaining attached. At later times, the lysis appeared in both cells, probably because of an increase in cell wall weakness (Fig. 7 A). Cell growth is mainly monopolar and therefore most of the new defective wall is located at one pole. After septum synthesis, lysis occurs only on the side of defective cell wall. In the next cell cycle, the defective wall increases, generating after septation the lysis in both cells (Fig. 7 C). Similar cell lysis defects were observed in the *cwg1-1* and *cwg1-2* mutants (Fig. S5 E, see last section of Results).
Figure 4. Bgs4 absence causes a slower septum formation, uncoupled from an altered and faster CAR contraction. (A and B) WT CAR contraction and PS synthesis are coordinated, whereas Bgs4 absence produces a slower CAR contraction and much slower PS growth. (A) Cells were grown as in Fig. 2 A and imaged by time-lapse. Arrowhead shows first PS signal detected (t = 2), locating in the previous image (t = 0) the simultaneous start of PS synthesis and coupled CAR contraction. (B) The time of CAR assembly, maturation, and contraction and of PS formation was quantified. (C–G) WT CAR contraction and septation are uniform and coincident. In Bgs4 absence, initial CAR contraction and PS formation are strongly impaired (red bracket), but ensuing CAR contraction proceeds faster than in WT and uncoupled from a slower PS formation. (C) Kymographs of the time-lapses of A. (D) Scheme of CAR and PS progression in the kymographs. White arrows show asymmetric septum synthesis start. Arrowhead shows end of CAR contraction (green) and PS formation (blue). Rectangle marks interval (t = 22–26) of growing PS but complete CAR contraction. (E) The slow CAR contraction is coincident with a defective PS synthesis start (arrows). Cells were grown as in A. (F) Detail of defective growing PS uncoupled from complete PM extension. (G) Rates of CAR contraction and PS synthesis (2–6, 6–12, and 12–18 min). Error bars indicate SD. Bars, 1 µm.
Figure 5. Bgs4 \( \beta(1,3) \) glucan confers the septum strength needed for a rigid straight septum. (A and B) Absence of Bgs4 generates curved (A) and defective septa with no CW-stained PS at the septum base (B). Details of PS (arrows) retracted from the cell wall (B, right). Cells were grown as in Fig. 1 B. Arrows, cell wall; arrowheads, CW-stained PS base. Error bars indicate SD. (C and D) Bgs4 absence generates flexible curved septa, oscillating according to the changes in internal pressure between sister cells. Cells were grown in MM+S+T for 9 h and visualized by time-lapse. (C) WT rigid straight septum formation and progressive cell separation (top). Flexible curved septa with two oscillating cycles (arrow), ending with cell lysis in one (middle) or both cells (bottom). (D) Kymograph of a flexible curved septum with four waving cycles (arrow). Bars: [cells] 5 µm; [septum details] 1 µm.
To know the precise timing of cell lysis, the process was analyzed by time-lapse. After septum completion and maturation, an abrupt cell wall and PM rupture released the cytoplasmic material from either one or both cells (Fig. 7 D, arrow; Fig. S4 E, arrow; and Video 1). No cell separation was detected, indicating that lysis occurred just after septum maturation and before or at the start of cell separation.

To know the structure and/or process that fails, leading to lysis, septum maturation and cell lysis were studied by TEM (Fig. 7, E and F). In the WT after SS thickening, a new ring structure of dense material spanning from the PS and MTD to the wall surface appeared. We termed this structure dense ring (DR; Fig. 7 E, left). Cell separation started by controlled DR degradation, followed by gradual PS degradation, leaving a residual MTD called fuscannel (Johnson et al., 1973). The turgor pressure led the released SS to adopt the most stable spherical conformation (Fig. 7 E, left). In the absence of Bgs4, cell separation started with an uncontrolled cell wall degradation, probably because of a thick diffuse DR, leaving the PM exposed to the medium (Fig. 7 E, right). The exposed PM could not counteract the high internal pressure, causing the PM rupture and release of cytoplasm (Fig. 7 F, arrows). In no case was PS degradation detected, indicating that lysis occurs during cell wall degradation and before or at the start of PS degradation. These results show that Bgs4 is required for a correct septum–cell wall structure and to protect the cell from an excess of wall degradation at the start of cell separation.

Bgs4 is required to maintain the cell wall thickness and integrity during polarized growth

The pole lysis generated by Bgs4 absence occurred in both interphase and septated cells (Fig. 8 A, arrows), indicating a weakness of the pole wall that persists during cytokinesis. Interphase pole lysis was examined by CW staining and GFP-Bgs1 localization, which permitted discrimination of the growing poles. The lysis occurred at the old end (OE) during both monopolar and bipolar growth (Fig. 8, B [arrows] and C). The OE was located as the pole distant from the fission scar (Fig. 8 B). The OE supports most of the cell elongation, and hence the cell wall defect at this pole is stronger. The OE weakness also results in lysis after septation, probably because of the increase in the turgor pressure of the healthiest cell, transmitting the pressure to the sister cell, which will eventually lyse (Fig. 8 D, red arrowheads).

WT cells examined by TEM showed a uniform wall at both poles (Fig. 8 E). The absence of Bgs4 generated a very thin cell wall at the pole region (Fig. 8 F, red arrows). The thinness of the tip wall was restricted by its capacity to maintain the cell integrity. As a result, a thinner tip wall could not support the cell internal pressure, resulting in the cell wall and PM rupture and cytoplasm release (Fig. 8 F, right, red arrows).

The aforementioned cytokinesis and cell integrity defects were examined in the Bgs4-defective cgw1-1 and cgw1-2
point mutants. As expected, loss of Bgs4 function led to phenotypes similar to those of Bgs4 absence (Fig. S5, A–E). Because other GS collaborate in SS, PS, and cell wall synthesis, the possible suppression of defects by overproduction of other GS was analyzed in both Bgs4-depleted and -defective strains. In no case did the overproduction of any other GS suppress
the phenotypes promoted by the Bgs4 B-BG absence, and it even increased the lethality of cgw1-1 and cgw1-2 mutants (Fig. S5 F). This suggests that no other glucan can compensate the defect because of B-BG absence. However, the fact that compensatory mechanisms were able to form remedial SS and stable survivors (unpublished data) suggests that a specific induction and/or repression level of combined GS could compensate the lethal Bgs4 B-BG absence.

**Discussion**

CAR contraction is a well conserved process in fungi and animal cells (Bathe and Chang, 2010; Lee et al., 2012). How fungal CAR constrictions are coordinated with the late cytokinesis steps of septum synthesis and how the syntheses cooperate to assemble the septum have just started to be understood (Sipiczki, 2007; Cortés et al., 2012). Fission yeast contains different essential GS with specific roles in cytokinesis. However, the role of Bgs4 in the cell wall and septum remains unknown (Cortés et al., 2005). We have studied the septation process of *S. pombe* in depth and found interesting remedial septation mechanisms for cell survival (Fig. 9 A). Cell separation is critical and hence the result will differ, depending on the septation mechanism (Fig. 9 B).

**Bgs4 β(1,3)glucan is necessary for correct CAR positioning in the cell middle and for coupling septum synthesis to PM extension and CAR contraction**

Our data show for the first time that extracellular B-BG has a role in the CAR function. In the absence of B-BG the CAR is often oblique assembled, sliding in the cell middle until septum...
Bgs4 couples septation to ring contraction

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Figure 9. Models of the septation process and alternative septations of fission yeast. (A) WT septation (top). Simultaneous coordinated synthesis (arrow) of PS (perpendicular to cell wall) and SS (parallel to PS) form a three-layered septum. Septum maturation proceeds by PS anchorage into the cell wall (yellow arrow) and a second round of SS synthesis (middle). Septation in Bgs1 absence. The SS is synthesized parallel to the cell wall. The septum grows by successive parallel SS depositions (orange arrow). The MTD changes to a septum medial position, forming a dotted line of MD in the SS layers (Cortés et al., 2007). (bottom) Septation in Bgs4 absence. CAR and septum are oblique positioned in the cell middle. The septum grows as a weak twisted and misdirected PS (wavy arrows) that is delayed and uncoupled from CAR and PM ingression (red arrows). After septum completion, the defective middle region is repaired with new PS (orange arrows) and the PS is retracted from the cell wall. (B) WT cell separation (top). Controlled cell wall DR and PS degradation (arrow) and the osmotic pressure that curves the SS to the stable conformation ensure a safe separation. In Bgs1 absence, there is no cell separation (middle). Cell separation and lysis in Bgs4 absence (bottom). Uncontrolled cell wall DR degradation (arrow) leaves the PM exposed to the medium. Then the turgor pressure generates the PM rupture and cytoplasm release. F, fuscannel; FS, fission scar; Pr, turgor pressure. Bars, 1 μm.

synthesis initiation fixes CAR and septum position to the cell wall. We discarded a Bgs4 defect because it is not present during CAR positioning and sliding, appearing after septum initiation (Cortés et al., 2005). However, the cell wall B-BG absence generated in previous cycles is a defect present during CAR formation and sliding. CAR sliding has been described in the combined absence of microtubules or anillin-like Mid1 function with the absence of septum synthesis start (Pardo and Nurse, 2003; Huang et al., 2008). In these cases the defect was caused in the CAR, whereas in our case the defect is caused by cell wall B-BG absence. Supporting our finding of the important role of cell wall B-BG in CAR function, spherical protoplasts devoid of cell wall show unstable CAR sliding toward the poles (Mishra et al., 2012), whereas spherical mutants with cell wall form stable CAR and septa (Chang et al., 1994; Verde et al., 1995; Sipiczki et al., 2000).

Despite our knowledge of cytokinesis, little is known about how extracellular signals communicate with intracellular events. The extracellular cell wall must be connected to the PM and CAR, and here we show that B-BG is crucial for this interaction. As B-BG is external, the CAR must interact with B-BG through PM proteins connecting both sides. Bgs and Ags1 proteins cannot be candidates for such an interaction because all appear in the middle much later than the time of CAR defects. Candidates could be the scaffold protein anillin, G protein–coupled receptors, or ECM proteins, as suggested in animal cells (Almonacid and Paoletti, 2010; Hynes and Naba, 2012; Liu et al., 2012; Lu et al., 2012; Rincón and Paoletti, 2012; Zhang et al., 2012). In fact, proteins and polysaccharides of the animal ECM (functional equivalent of fungal cell wall) have been shown to be critical for cytokinesis (Hwang et al., 2003; Mizuguchi et al., 2003; Olson et al., 2006; Izumikawa et al., 2010; Jordan et al., 2011; Xu and Vogel, 2011). However, how the ECM is coupled to the CAR and how it affects the CAR function remain largely unknown.

Our results show a surprising separation between slower PS synthesis and faster CAR and PM ingression, indicating that the pushing force of PS synthesis is not necessary for CAR and
PM progression (Johnson et al., 2005). More surprisingly, the contraction rate of uncoupled CAR and PM is faster than normal, suggesting that in normal coupled cytokinesis the synthesis rate of attached PS restricts CAR and PM ingression rates. Moreover, our data show that septation can progress with a defective CAR pulling force. In the absence of B-BG, the initial septa are twisted and bent and larger septa appear misdirected, indicating a relaxed CAR without the tensile pulling force necessary to maintain the growing septum straight. These results suggest that cytokinesis can progress and be completed without or with a defective pushing force of septum synthesis and/or pulling force of CAR contraction, with the help of PM extension by the addition of membrane vesicles. In fact, it has been described that cytokinesis can be completed with defective CAR, but to date this has not been reported with delayed PS deposition (Pollard, 2010; Balasubramanian et al., 2012; Proctor et al., 2012).

It has been suggested that septum synthesis provides the primary force for the last cytokinesis steps based on the fact that in LatA-treated cells, septum synthesis can continue and be completed without the CAR (Proctor et al., 2012). However, only septa above 50% in length progress and complete septation, and the progression rate decreases to 30%, thus suggesting an important role for the CAR in the overall septum ingression process. In addition, it is possible that in these LatA-treated cells other CAR components more stable than actin could maintain a residual CAR that might help in the final septum progression (Naqvi et al., 1999; Wu et al., 2003). In Bgs4-depleted cells, the CAR structure is not altered but Bgs4 B-BG and septum are, indicating that when the CAR is intact the septum pushing force is not so critical. However, the misdirected CAR and PM progression suggests that not only the PS pushing force but also the CAR contraction force is defective, unable to maintain a straight septum progression. In these conditions, the PM and CAR ingression rate increased to 127% of WT, suggesting that when detached from the PS, the PM advance proceeds faster, whereas the attached CAR cannot constrict at similar rate, resulting in a larger and relaxed misdirected CAR structure, in agreement with the proposed low contractile force of the CAR (Proctor et al., 2012). That the septa are always completed indicates that the CAR constriction force is still able to finally correct the misdirected progression. In the absence of B-BG, the septa present a very sharp PM tip, suggesting that if some material is deposited between PM and PS the septum pushing force would be limited. In addition, the observation of septa with advancing PS but complete PM with no space for deposited material (Fig. 4 F) suggests that the PM can progress without pushing septum material. Collectively, these results suggest that both CAR and septum synthesis collaborate in a normal septum formation. Septum progression is necessary to complete a septum wall structure, and the CAR is essential for septation start and at least first-half progression and as a safety closing belt mechanism for a straight progression.

Previously, we have found interesting the convergent similarities between the structure and function of fission yeast PS and plant cell plate during cytokinesis (Cortés et al., 2007, 2012). Our current observations reveal important convergent similarities between fungal and animal cytokinesis, with a fungal cell wall B-BG required for connecting cell wall and CAR during cytokinesis and ECM polysaccharides required for animal cytokinesis (White and Bednarek, 2003; Mizuguchi et al., 2003; Olson et al., 2006; Izumikawa et al., 2010), suggesting that the extracellular cell wall is an evolutionarily highly conserved component of eukaryotic cytokinesis.

**Bgs4 is essential for SS formation and PS completion**

We found that Bgs4 B-BG is responsible for SS assembly (Fig. 9 A). A similar function has been described for Ags1 (Cortés et al., 2012), indicating that both B-BG and α-glucan are necessary but not sufficient for SS formation. Analysis of revertant protoplasts suggests that the primary wall formation step is the assembly of β-glucan microfibrils, whereas α-glucan might be involved in glucan bundle formation (Osumi et al., 1989; Konomi et al., 2003). In fact, α-glucan is required for adhesion (Cortés et al., 2012). Also, Bgs4 or Ags1 absence causes the induction of remedial SS and cell survival (Cortés et al., 2012; unpublished data). However, remedial SS appear later in the absence of Bgs4, suggesting different compensatory mechanisms. In fact, Rho1 GTPase regulates both α- and β-glucan synthesis, whereas Rho2 mainly regulates α-glucan synthesis (Pérez and Cansado, 2010; Perez and Rincón, 2010). Evaluation of the compensatory mechanisms will require further analysis of Ags1 and Bgs subunits.

We show that although the L-BG is responsible for the PS structure, it needs the participation of B-BG. Absence of Bgs4 causes PS thinning in the last stages of PS synthesis, indicating that B-BG is needed to reinforce the growing PS edge. In this sense, Bgs4 absence generates flexible oscillating septa. This flexibility is not caused by the absence of SS because it is not observed in the absence of Ags1 (Cortés et al., 2012), indicating that B-BG specifically confers the septum rigidity. Similarly to Saccharomyces cerevisiae chitin, S. pombe B-BG could provide the septum rigidity by binding to L-BG or α-glucan (Hartland et al., 1994). Indeed, it has been suggested that L-BG association with B-BG or α-glucan is critical for PS assembly (Cortés et al., 2007).

**Bgs4 is essential for cell integrity at the start of cell separation**

*S. pombe* is an excellent model for cell integrity studies because of its critical cell separation process (Ronceró and Sánchez, 2010; Cortés et al., 2012). Cell separation starts by controlled cell wall degradation and the DR could be important for this process (Fig. 9 B). L-BG has been shown to be essential for cell separation (Cortés et al., 2007). Thus, the DR could be a structure rich in L-BG, directing the degradation to this structure, whereas B-BG could accumulate around the DR to protect the cell wall. Ags1-depleted cells also display cell lysis during cell separation (Cortés et al., 2012). It will be interesting to evaluate whether Bgs4 and Ags1 cooperate in safe cell separation.

**Materials and methods**

**Strains and culture conditions**

The S. pombe strains used in this study are listed in Table S1. bgs4Δ::ura4* p81XH-bgs4Δ* strain 498 (his3+ selection) has been described previously (Cortés et al., 2005). This strain contains bgs4Δ* expressed under the control of the 81X version (low expression) of the thiamine-repressible nmt1* promoter (Moreno et al., 2000).
81X-bgs4 strain 1288 (Leu+, Ura-, and His+) contains the bgs4::ura4 deletion and a single p81X-GFP-bgs4 copy integrated adjacent to the his3Δ1 locus. This strain was generated from strain bgs4::ura4 p81X-bgs4 by continuous growth in minimal medium (MM) with histidine, colony isolation, and selection of clones that maintain the His+ phenotype. Next, the selected clones were analyzed by genetic cross with strain 285 (WT, Leu+, Ura+, and His+) and tetrads analysis. Clones that segregated both His+ and 2His−:2His−, indicative of 81X-bgs4 integration, were selected. Finally, these clones were analyzed by genetic cross with strain 420 (WT, Leu+, Ura+, and His+) and tetrads analysis. The clones that showed a 4His−:4His− segregation, indicative of a correct 81X-bgs4 integration adjacent to the his3Δ1 locus, were selected. All the 81X-bgs4 clones exhibited a strong lethal phenotype to the presence of thiamine (repressed conditions) and a WT phenotype in its absence (induced conditions). Other 81X-bgs4 strains were made from strain 1288 either by tetrads dissection or by random sporulation analysis by selecting against the corresponding parental auxotrophies. 81X-GFP-bgs4 strain 1493 (Leu+, Ura+, His+, and Ade−) contains the bgs4::ura4 deletion and a single p81X-GFP-bgs4 copy integrated adjacent to the his3Δ1 locus. This strain was made following a protocol similar to that described for strain 81X-bgs4.

GFP-bgs1+, GFP-bgs3+, GFP-bgs4+, and ags1−GFP strains 520, 1217, 561, and 3166, respectively, have already been described (Cortés et al., 2002, 2005, 2007, 2012). These strains contain the bgs1::ura4, bgs3::ura4, bgs4::ura4, and ags1−3 UTR::ags1+3 UTR::ura4 deletions and an integrated copy of Smal-cut pJK-GFP-bgs1+, Pac-cut pJK-GFP-bgs3+, Stu-cut pJK-GFP-bgs4+, and Age-cut pJK-ags1− (Leu+ selection), which direct their integrations at the Smal site of the bgs1+ promoter sequence (nt−748) adjacent to bgs1::ura4, the Pac site of the bgs3+ promoter sequence (nt−1857) adjacent to bgs3::ura4, the Stul site of the bgs4+ promoter sequence (nt−1320) adjacent to bgs4::ura4, and the Age site of the ags1− coding sequence (nt6025) in ags1−3 UTR::ags1+3 UTR::ura4, respectively. 2xGFP-bgs1+ strain 1731 was made as described for the GFP-bgs1+ strain (Cortés et al., 2002, 2004) and contains an integrated copy of Smal-cut pJK-2xGFP-bgs1+ strain (Leu+ selection), which directs its integration at the Smal site adjacent to bgs1::ura4, at position −748 of the bgs1+ promoter sequence. Likewise, 2xRFP-bgs1 strain 1780 contains an integrated copy of Smal-cut pJK-2xRFP-bgs1+ strain (Tomo et al., 2005) at position −748 of the bgs1+ promoter sequence. 2xRFP-bgs1+ GFP-bgs3+ strain 3332 was made by a genetic cross between strains 1780 (2xRFP-bgs1+, Leu+, Ura+, and His+) and 1217 (GFP-bgs3+, Leu+, Ura+, and His+), tetrad dissection, and analysis of RFP-bgs1+ and GFP-bgs3 localizations. 2xGFP-bgs1+ 81X-bgs4 strain 2300 was made by a genetic cross between strains 1731 (2xGFP-bgs1+, Leu+, Ura+, His+, and Ade−) and 1366 (81X-bgs4, Leu+, Ura+, His+, and Ade−) and tetrads analysis. 81X-bgs4 strain 2850 contains an integrated copy of SmaI-cut pJK-81X-bgs4+ strain 1857 adjacent to the SmaI site of the bgs4+ ORF, 5.9-kb HindIII–EcoRI fragment, respectively. Similarly, pAL-2xGFP contains a 9.7-kb SpeI–SpeI bgs4+ fragment and an 11.2-kb EcoRI–NruI fragment, respectively (Cortés et al., 2007, 2012). These multicopy plasmids contain the corresponding ORF and its native promoter sequence, and were used for overexpression studies.

Plasmids 41X-bgs1+, 41X-bgs3+, 41X-bgs4+, and 41X-ags1+ are the thymidine-repressible plasmid pJK-41XL (Moreno et al., 2000) with the 5.2-kb bgs1+ ORF, 5.5-kb bgs3+ ORF, 5.9-kb bgs4+ ORF, and 7.2-kb ags1− ORF sequence, respectively. Induced and repressed expression levels of the 41X plasmids are 10−50-fold higher than those of the 81X version and 5–20-fold lower than those of the 3X version. Higher expression levels of these ORFs with the 3X version is toxic for the cell. Therefore, the 41X plasmid was selected for overexpression studies at both induced and repressed expression levels.

Immunoblot analysis

Early logarithmic cells (106 cells) expressing the different tagged proteins were harvested (1,500 g, 5 min, 4°C), washed twice with cold buffer (1 mM EDTA and 1.2 mM sorbitol, 4°C), suspended in 100 µl of lysate buffer (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 150 mM NaCl, 1% Triton X-100 containing 1 mM phenylmethylsulfonyl fluoride, and 2 µg/ml aprotinin, leupeptin, and pepstatin), and broken with glass beads (FastPrep FP1200, 3 x 15 s, speed of 5.5 [MP Biomedicals; Thermo Fisher Scientific]). The total cell extracts were collected by bottom-tube perforation and centrifugation (3,000 g, 20 s, 4°C). The cell extracts were resuspended, centrifuged (2,000 g, 5 min, 4°C), and homogenized again in the same supernatant. This step improved the disgregation and immunodetection of membrane proteins from total cell extracts. Finally, samples were diluted with 2x loading buffer (2x is 100 mM Tris-HCl, pH 6.8, 2% SDS, 2% mercaptoethanol, 25 mM EDTA, 20% glycerol, and 0.05% bromophenol blue) and stored at −80°C. The protein concentration of the loading samples was quantified by the Bradford assay (Bio-Rad Laboratories). Samples (80 µg of total protein) were subjected to 3–8% SDS–PAGE (21,000 V, 60 min, 4°C), transferred to nitrocellulose membranes (EMD Millipore), and probed with monoclonal JL-8 anti-GFP (1:2,000; Living colors; Takara Bio Inc.) or monoclonal B-5-1-2 anti-a-tubulin (1:10,000; Sigma-Aldrich) antibodies. Immunodetection was performed with an anti–mouse horseradish peroxidase–conjugated antibody and the ECL detection kit (GE Healthcare). Protein amounts were quantified by chemiluminescence with a ChemiDoc XRS System (Bio-Rad Laboratories) and Quantity One software (Bio-Rad Laboratories). Total cell extracts and Western blots were repeated from three times to five times for each experiment. The data from the Western blots were quantified and the mean values of the relative amount of each protein were calculated from three to five independent total cell extracts.
Glucose incorporation in the cell wall poly saccharides

Labeling and fractionation of cell wall poly saccharides were performed essentially as described previously (Cortés et al., 2012). Early log-phase cells incubated in MM + 1.2 M sorbitol were diluted with the same medium (with 20 µg/ml thiamine for cells repressing bgs4) and supplemented with D-[1-14C]glucose [6 µCi/ml; Hartmann Analytic] 4 h before harvesting. The cells (early log-phase) were collected at the indicated times. Total glucose incorporation was monitored by measuring radioactivity in trichloroacetic acid-insoluble material. Cells were harvested, supplemented with unlabeled cells as the carrier, washed twice with 1 mM EDTA, and broken with glass beads (FastPrep FP120, 3 × 20 s and speed of 6.0). Cell walls were purified by repeated washing and differential centrifugation (once with 1 mM EDTA, twice with 5 M NaCl, and twice with 1 mM EDTA) at 1,500 g for 5 min. Purified cell walls were heated at 96°C for 20 min and [14C]glucose incorporation into the cell wall was monitored.

Cell wall samples were extracted with 6% NaOH for 60 min at 80°C and neutralized with acetic acid. Precipitation of the galactomannan fraction from the neutralized supernatant was performed with FeCl3’s reagent by adding 5 mg of unlabeled yeast mannan as the carrier (stock of 50 mg/ml in water (Sigma-Aldrich), as described previously (Algranati et al., 1996). FeCl3’s reagent was freshly prepared for each experiment by adding one volume of reagent B (3.5% CuSO4) to one volume of reagent A (17.3% potassium sodium tartrate dissolved in 12.5% KOH). In brief, four volumes of FeCl3’s reagent were added to the samples, mixed, and left overnight at 4°C. Precipitated galactomannan was washed by a centrifugation at 1,500 g for 10 min, the pellets were washed with FeCl3’s reagent and solubilized in 20–40 µl of 6N HCl. Then, 100 µl of 50 mM Tris-HCl, pH 7.5, was added and the radioactivity of the solutions was measured in a scintillation counter (Beckman Coulter) and considered the galactomannan fraction.

Other samples of cell wall suspension were incubated with Zymolyase 100T (25 µg of enzyme and 1/20 volume of cell wall suspension; AMS Biotechnology) in 50 mM citrate-phosphate buffer, pH 5.6, for 24 h at 37°C. Samples without enzyme were included as a control. After incubation, samples were centrifuged and washed with the same buffer. One ml of 10% trichloroacetic acid was added to the pellets, filtered in Whatman GF/C glass fiber filters (2 x 2 ml of 10% trichloroacetic acid and 2 ml of ethanol), and their radioactivity levels were measured in a scintillation counter. The Zymolyase 100T pellets were considered the β-glucan fraction, and the supernatants were the β-glucan-plus-galactomannan fraction. β-Glucan was calculated as radioactivity remaining after subtraction of galactomannan and β-glucan from total cell wall incorporation. All determinations were performed in duplicate, and data for each strain were calculated from three independent cultures.

Microscopy techniques

For cell wall staining, early log-phase cells grown in MM + 1.2 M sorbitol (MM+S), with 20 µg/ml thiamine for cells repressing bgs4) at 30°C or in YES at 25°C and shifted to 37°C for 5–7 h (thermosensitive mutant) cells were concentrated (1,000 g, 1 min) by centrifugation directly by adding a solution of CW (50 µg/ml final concentration [Blankophor; Blankophor GmbH & Co.]; Fluorescent Brightener 28 [Sigma-Aldrich]) to the sample and using the appropriate filter. GFP and RFP were directly visualized from early log-phase cells by using the appropriate filters. Images were obtained with a fluorescence microscope (DM RXA; Leica), a PL APO 63×/1.32 oil PH3 objective, a digital camera (DFC350FX; Leica), and CW4000 CytoFISH software (Leica). Images were processed with Photoshop CS2 software (Adobe). All the analyses were repeated in three to five independent experiments and representative images of the analyzed phenotype were indistinctly selected from the experiments. Graphical data were calculated from four independent experiments. The number of experiments and of total cells analyzed is shown in each case.

For time-lapse imaging, 0.3–0.6 ml of log-phase cells grown in MM+S (with 20 µg/ml thiamine for cells repressing bgs4) at 30°C or in YES at 25°C and shifted to 37°C for 5–6 h were collected by low-speed centrifugation (1,000 g, 1 min), resuspended in 300 µl of the same media containing CW (5 µg/ml final concentration), and placed in a well in a µ-Slide 8 well [8082-1-Uncoated, Ibidi] previously coated with 10 µl of Poly-L-lysine (1.39%; Sigma-Aldrich). Time-lapse experiments were made at 30°C (MM+S) or 37°C (YES) by acquiring epifluorescence and/or phase-contrast cell images in single planes and 1 × 1 binning on an inverted microscope [IX71; Olympus] equipped with a PlanApo 100×/1.40 IX70 objective and a Personal DeltaVision system (Applied Precision). Images were captured using CoolSnap HQ2 monochrome camera (Photometrics) and SoftWoRx 5.5 imaging software (Applied Precision). In some cases, time-lapse images were restored and corrected by 3D Deconvolution (conservative ratio; 10 iterations and medium noise filtering) through SoftWoRx imaging software. Subsequently, images were processed (color, brightness, contrast, and/or sharpness) with ImageJ (National Institutes of Health) and Photoshop CS2 software. All the time-lapses were repeated in two to three independent experiments. The data were calculated from two to three independent experiments. The number of experiments and of total cells analyzed is shown in each case.

TEM

Early log-phase cells were fixed with 2% glutaraldehyde EM (GA; Electron Microscopy Science) in 50 mM phosphate buffer, pH 7.2, and 150 mM NaCl (PBS) for 2 h at 4°C, post-fixed with 2% potassium permanganate for 1 h at room temperature, dehydrated in acetone, and embedded in epoxy resin. Ultrathin sections were examined with a Jem-1010 electron microscope (Jeol).

Online supplemental material

Fig. S1 shows that absence of Bgs4 promotes cell lysis and cytoplasm release during both cytokinesis and polarized growth. Fig. S2 shows that Bgs4 is required for CW-stained PS completion but not for CAR contraction and septum PM closure. Fig. S3 shows that Bgs4 and its β(1,3)glucan are required for correct and stable CAR positioning in the cell middle and for correct straight CAR constriction and septum ingression. Fig. S4 shows that Bgs4 is essential for coupling septum formation to CAR contraction, to confer the septum rigidity, and to protect the cell integrity at the start of cell separation. Fig. S5 shows that the Bgs4-defective cwg1-1 and cwg1-2 mutants show defects in pseudohyphae in the absence of Bgs4. The defects cannot be compensated by any other GS. Video 1 shows the formation of an oblique CAR and septum of two dividing Bgs4-depleted cells. Video 2 shows the formation of an altered CAR but normal septum of two dividing Bgs4-depleted cells. Video 3 shows the formation of an oblique CAR and septum of four cells expressing the cwg1-2 mutation of bgs4. Video 4 shows the formation of a misdirected CAR and septum of five cells expressing the cwg1-1 mutation of bgs4. Video 5 shows the coordinated CAR contraction and septum formation of a WT cell. Video 6 shows the uncoupling between CAR contraction and the slower septum formation of three Bgs4-depleted cells. Video 7 shows the formation of a rigid and straight septum and the progressive cell separation of two WT cells. Videos 8 and 9 show the formation of a flexible and oscillating curved septum with two and four waving cycles, respectively, of four Bgs4-depleted cells. Video 10 shows the cell lysis and release of cytoplasm from either one or both sister cells at the start of cell separation of three dividing Bgs4-depleted cells. Table S1 lists the fission yeast strains used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201304132/DC1.

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