

Cell migration and division in amoeboid-like fission yeast

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Summary

Yeast cells are non-motile and are encased in a cell wall that supports high internal turgor pressure. The cell wall is also essential for cellular morphogenesis and cell division. Here, we report unexpected morphogenetic changes in a *Schizosaccharomyces pombe* mutant defective in cell wall biogenesis. These cells form dynamic cytoplasmic protrusions caused by internal turgor pressure and also exhibit amoeboid-like cell migration resulting from repeated protrusive cycles. The cytokinetic ring responsible for cell division in wild-type yeast often fails in these cells; however, they were still able to divide using a ring-independent alternative mechanism relying on extrusion of the cell body through a hole in the cell wall. This mechanism of cell division may resemble an ancestral mode of division in the absence of

cytokinetic machinery. Our findings highlight how a single gene change can lead to the emergence of different modes of cell growth, migration and division.

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Introduction

Cellular morphogenesis requires polarization of the cytoskeleton and proper positioning of the cell division plane (Drubin and Nelson, 1996; Guertin et al., 2002). Fission yeast *Schizosaccharomyces pombe* are rod-shaped cells that grow by tip extension and divide by medial fission (Mitchison and Nurse, 1985). The spatial control of cell polarity and division in *S. pombe* makes this yeast a convenient model to study morphogenesis (Chang and Martin, 2009; Hayles and Nurse, 2001). Similar to other yeasts and fungi, *S. pombe* cells are surrounded by a cell wall, an extracellular matrix-like structure made of polysaccharides that allows the yeast cells to support the turgor pressure (Harold, 2002; Kopecká et al., 1995). Cell wall is a key regulator of cellular morphogenesis, and enzymatic removal of the cell wall results in rounded cells (protoplasts) unable to organize polarized growth zones and failing to divide (Osumi et al., 1989).

Free-living eukaryotic cells lacking a cell wall, such as amoebas, usually counteract turgor pressure by means of cortical actin cytoskeleton that generates a tension-resistant actomyosin cortex directly underlying the plasma membrane (Stockem et al., 1982). While such cells are unable to generate permanent rigid cell shapes, they, similarly to yeast and fungi that remodel the cell wall at the growth zones, rely on local weakening of the actomyosin cortex to allow cell expansion. In amoebas, this results in pseudopodium formation and movement (Webb and Horwitz, 2003) and in yeasts and fungi, produces polarized cell growth (Chang and Martin, 2009).

Actin polarization at the growth zones and proper function of the actomyosin division ring in *S. pombe* both rely on cell wall remodeling, resulting in tip growth and division septum assembly,

respectively (Mulvihill et al., 2006; Santos et al., 2005). During tip growth, cell wall remodeling enzymes are transported in a polarized manner to the sites of growth to locally modify the cell wall and allow for its expansion partly driven by turgor pressure (Cortés et al., 2005; Cortés et al., 2002). The wall, in turn, is necessary for polarized growth zones to develop (Osumi et al., 1989). Thus, polarized cell growth, which involves addition of new membrane at growth sites, generates the characteristic cylindrical shape of fission yeast (Harold, 1990; Minc et al., 2009). Cell division in fission yeast, as in most eukaryotic cells, depends on an actomyosin ring (Marks et al., 1986). Ring contraction is coordinated with synthesis of new cell wall behind the closing ring, coupling actomyosin contraction to septum assembly. Thus, cell wall is involved in establishing and maintaining cell shape and also regulates cell division (Kobori et al., 1994; Madden and Snyder, 1998).

To probe the functions of the cell wall we analyzed cells lacking *pck2* gene (Toda et al., 1993). *pck2* encodes for one of the two protein kinase C homologues in *S. pombe* and is required for the activation of key enzymes that synthesize the β -1,3-glucan, a major structural component of the fission yeast cell wall that forms a fibrillary network responsible for its mechanical strength (Kobori et al., 1994; Kopecká et al., 1995; Osumi et al., 1998; Toda et al., 1993), and also regulates α -glucan biosynthesis (Calonge et al., 2000). We find that weak-walled *pck2* Δ cells are unable to establish and maintain cylindrical shape and, unexpectedly, form cytoplasmic protrusions apparently caused by cell wall rupture due to internal turgor pressure. Strikingly, multiple cycles of protrusion result in efficient cell migration.

Moreover, protrusion events also allow cells to overcome failure of conventional cytokinesis and to divide using a novel mechanism relying on protrusion formation. Our data reveal how changes in a single gene can lead to the emergence of new morphogenetic properties and the generation of cellular movement in a non-motile yeast cell.

Results

Generation of fission yeast cells defective in cell wall biogenesis
When fission yeast cells are treated with lytic enzymes that digest the cell wall, they become rounded protoplasts (Osumi et al., 1989). Upon removal of the lytic enzymes, these protoplasts regenerate a new cell wall and re-form their rod shape. To interfere with cell wall biogenesis, we used *pck2Δ* cells. *pck2Δ* cells maintain functional cell wall during normal growth, but are unable to fully recover from protoplasting and only reassemble a weak or partial cell wall, which does not stain for β -1,3-glucans. These cells exhibit abnormal rounded cell shapes (Kobori et al., 1994) (see experimental design in supplementary material Fig. S1). When grown in osmotically stabilizing media, these *pck2Δ* cells after protoplast recovery (which we will refer to as “*RP-pck2Δ* cells”) epigenetically maintain abnormal morphology for many generations.

RP-pck2Δ cells form cytoplasmic protrusions

To investigate how cell wall defects in *RP-pck2Δ* cells affect cell morphogenesis, we used time-lapse microscopy. We found that these cells often formed cytoplasmic protrusions, in which the cell appeared to slowly “flow out” from a hole in the cell wall. Protrusions were seen in 80% of cells ($n=50$ cells) and their initiation required cells to reach a certain minimal volume (Fig. 1A; supplementary material Fig. S2A,B; Movie 1). Most cells (34/42 cells) formed a single protrusion at one time

(Fig. 1B). The frequency of protrusion formation was between 0 to 2 events per cell cycle and they were observed at all cell cycle stages (Fig. 1C). Protrusions were not explained by cell growth, as their volume increase rate was faster than the rate of cell growth (Fig. 1D). Consistent with this, plasma membrane detached from the cell wall at the rear as the front of the cell protruded (Fig. 1E, time 120 min; supplementary material Movie 2). As fungal cells possess high internal turgor pressure (Bastmeyer et al., 2002; Harold, 2002; Minc et al., 2009), we tested whether this pressure drives protrusion events, by changing osmolarity of the medium. When we increased the effective turgor pressure by lowering sorbitol levels in the medium, the rate of protrusion growth increased (Fig. 1D,F). Protrusions did not depend on the nature of the osmostabilizer used, and also formed when sorbitol was replaced for polyethylene glycol or sucrose (data not shown). Similar protrusions were seen in wild-type fission yeast cells when the cell wall was digested with lytic enzymes, although the rates of protrusion in these cases were a magnitude faster (supplementary material Fig. S3A,B; Movie 3). It is likely that such increased rate would result from a total lack of cell wall at the plasma membrane of the protrusion, whereas in *RP-pck2Δ* cells some cell wall is likely present around the protrusion. Thus, our results suggest that protrusions are caused by internal turgor pressure forcing cellular contents out of a hole in the cell wall.

Protrusion formation required the presence of at least some cell wall, since cells in the continuous presence of the lytic enzyme exhibited no protrusions (Fig. 1B). F-actin disassembly by latrunculin A (LatA) (Ayscough et al., 1997) or inhibition of protein secretion with Brefeldin A (Klausner et al., 1992) completely abolished cell protrusions, while depolymerization of interphase microtubules had little effect (Fig. 1B). Thus, protrusion formation requires an intact actin cytoskeleton

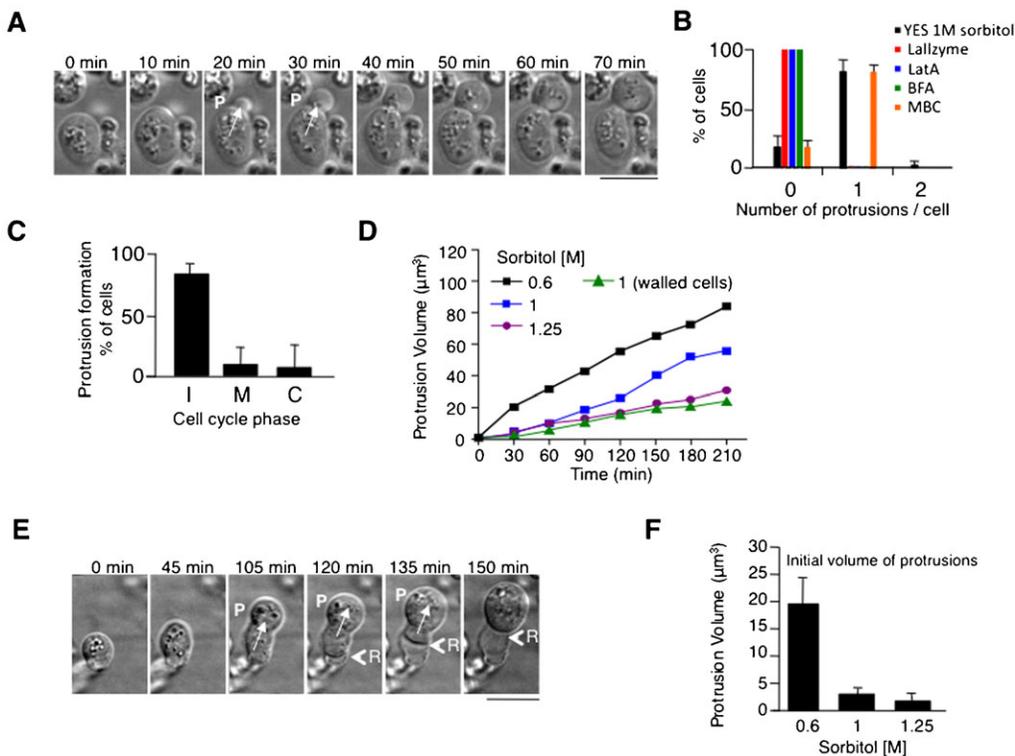


Fig. 1. *pck2Δ* cells form cellular protrusions. (A) Time-lapse DIC images of *RP-pck2Δ* cells forming protrusions in 1 M Sorbitol. P marks appearance of a protrusion. (B) Percentage of cells with the indicated number of protrusions in the indicated conditions. (C) Cell cycle stage of cells initiating a protrusion. Frequency of protrusion appearance at different stages of the cell cycle (I interphase, M mitosis and C cytokinesis). (D) Protrusion volume increase in protruding *RP-pck2Δ*. Volume increase during growth of walled *pck2Δ* in 1 M Sorbitol is also shown. (E) Time-lapse DIC images of a protruding *RP-pck2Δ* cell showing the retraction (R) of the cell body as the protrusion expands. (F) Average protrusion volume in the first video frame where protrusion is visible in *RP-pck2Δ* cells at the indicated osmolarity. Scale bars: 5 μm .

and/or membrane secretion. This could reflect a requirement for cell growth or for cell wall remodeling, which are dependent on actin and secretory pathways.

RP-pck2Δ cells exhibit cell migration

Strikingly, microscopic observation over many hours revealed amoeboid-like movement of *RP-pck2Δ* cells on agar pads (Fig. 2A; supplementary material Movie 4) (Webb and Horwitz, 2003; Yanai et al., 1996). Cells were observed to move across the agar for distances exceeding many cell lengths. Cell movement was also observed in cells attached to the glass surface in liquid medium, proving that movement is not a consequence of physical constraint imposed by the cover slip in agar pads (supplementary material Movie 5). The movement occurred at an average rate of $0.062 \pm 0.01 \mu\text{m min}^{-1}$ (in 1 M Sorbitol). The formation of a new protrusion was followed by an increase in velocity of cell movement (Fig. 2B). The migration rate was significantly higher ($0.11 \pm 0.01 \mu\text{m min}^{-1}$) at low osmolarity (0.6 M sorbitol) and was reduced ($0.046 \pm 0.002 \mu\text{m min}^{-1}$) at high osmolarity (1.25 M sorbitol) (Fig. 2C), suggesting that movement depends on turgor pressure. Like many motile cells, including amoebas and macrophages (Harshey, 2003; Kirfel et al., 2004; Uchida and Yumura, 2004), migrating *RP-pck2Δ* left a

trail of materials behind them, presumably cell wall fragments (Fig. 2A, time 19.5 h, arrowheads). Thus, cell migration of *RP-pck2Δ* may be a result of repeated cycles of protrusion driven by internal turgor pressure, accompanied by weak cell wall rupture and repair.

Actomyosin-independent division in protruding *RP-pck2Δ* cells
During cell migration we observed that defective walled *RP-pck2Δ* exhibited an abnormal mode of cell division. In fission yeast, cell division normally involves the assembly and contraction of an actomyosin ring accompanied by the formation of the division cell wall septum (Krapp et al., 2004). Surprisingly, *RP-pck2Δ* cells frequently divided without the characteristic division septum of yeast cells (Fig. 3A,B, Fig. 2A; supplementary material Movie 6) in a manner uncoordinated with nuclear division (Fig. 3B,C). In contrast, mitotic spindles and nuclear division were apparently normal in *RP-pck2Δ* cells (Fig. 3B).

Consistent with a recent report (Mishra et al., 2012), the observation of actomyosin rings in these cells showed lateral ring sliding during assembly or contraction followed by ring collapse and division failure in most cells (67/78 cells) (supplementary material Movie 7). The sliding of these non-functional actomyosin

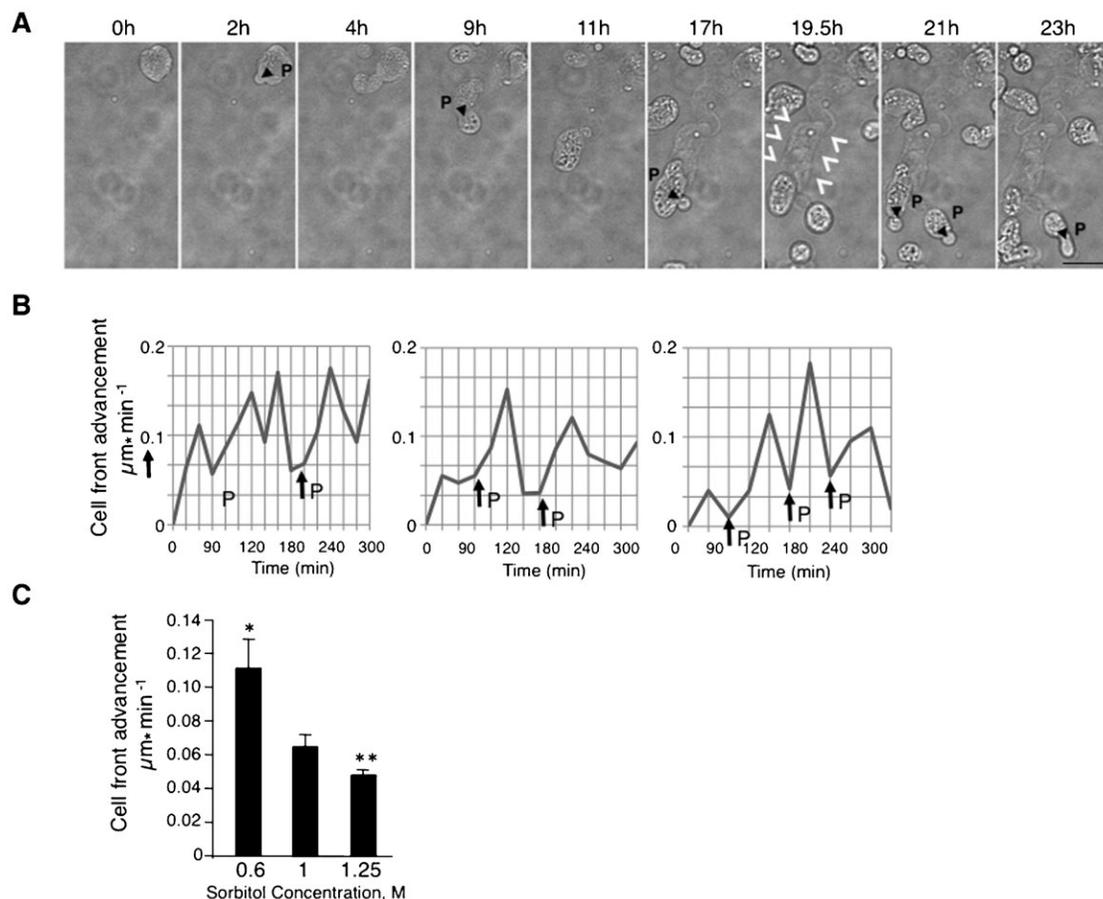


Fig. 2. Cell migration of *RP-pck2Δ*. (A) Time-lapse bright-field images of protruding *RP-pck2Δ* cells. Arrows indicate appearance of cell protrusions (P). Arrowhead denotes the presence of a trail of cell material left behind during cell movement. Scale bar: 5 μm . (B) Increase in the rate of cell front advancement upon protrusion formation. Velocity averages over 30 minute timespan are shown during migration of protruding *pck2Δ* cells grown in 1 M Sorbitol. P indicates the protrusion appearance. (C) Average velocity of protruding *pck2Δ* cells grown at each of the indicated osmolarities. Error bars show SEM. The differences between rates of movement at different osmolarities (0.6 M vs 1 M and 1 M vs 1.25 M sorbitol) are statistically significant, indicated by asterisks ($P < 0.0001$).

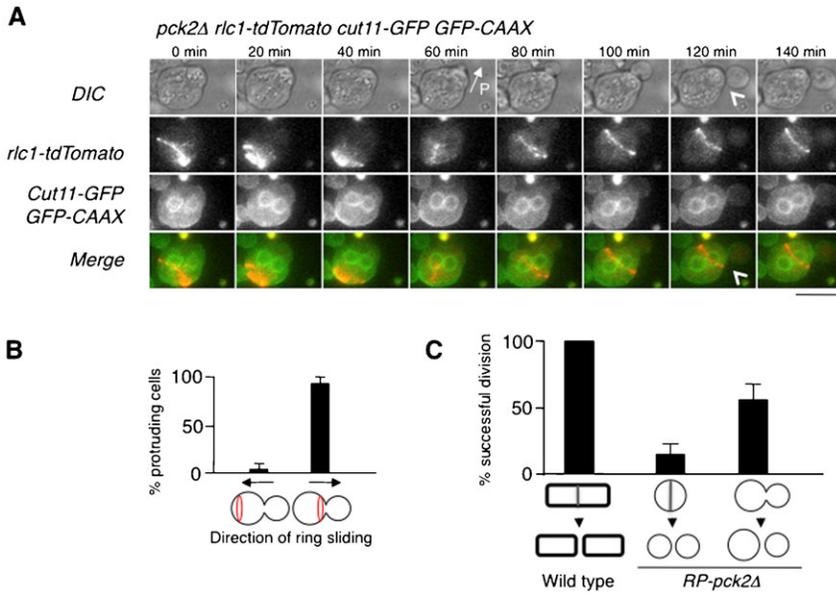


Fig. 4. Actomyosin ring fails to divide most of *RP-pck2Δ* cells. (A) Cell separation independent of the actomyosin ring. Time lapse images of protruding *pck2Δ* cells expressing CAAX-GFP, Rlc1-tdTomato and Cut11-GFP as markers of the plasma membrane, actomyosin ring and nuclear envelope, respectively. Maximum z projections are shown. Scale bar: 5 μ m. (B) Frequency of ring sliding towards the protrusion and away from it. (C) Percentage of walled and *RP-pck2Δ* cells undergoing successful divisions.

Thus, our data suggest that *RP-pck2Δ* cells can divide using an alternative mechanism that relies on cell protrusion through a small hole in the cell wall.

Discussion

In this work we induced aspects of amoeboid-like cell migration and actomyosin ring-independent cytokinesis by weakening the cell wall in fission yeast cells. Cells with defective cell wall were generated by deleting the gene encoding the protein kinase C homologue, *pck2*, required for the synthesis of key components that provide the mechanical strength to the cell wall (Kobori et al., 1994; Kopecká et al., 1995). After enzymatic digestion of the cell wall, *pck2Δ* cells cannot fully regenerate and assemble a defective cell wall. In these cells, we observed protrusions resembling mammalian cell blebs. We interpret this as evidence of local rupture of the cell wall that leads to membrane being pushed through the hole. This interpretation is supported by the increase in the frequency of protrusion events and of

the protrusion expansion rate at high intracellular turgor pressure. An intact actin cytoskeleton and/or membrane secretion is also required for protrusion formation.

Unexpectedly, repetitive cycles of protrusion formation resulted in cell migration. Cells moved over many cell lengths, usually in a single direction. A protrusion, or expansion of the cell forward, is always accompanied by detachment and retraction of the cytoplasm from the wall at the rear, resulting in an effective movement of the cell. Formation of a protrusion was frequently followed by an appearance of a new protrusion on its surface. This is likely a result of partial cell wall being formed at the surface of the expanding protrusion. It would explain the slow rate of protrusion expansion in *RP-pck2Δ* compared to that in the presence of cell wall digesting enzymes. Thus, migration in these cells may be a result of repeated cycles of protrusion driven by internal turgor pressure and accompanied by weak cell wall rupture and repair.

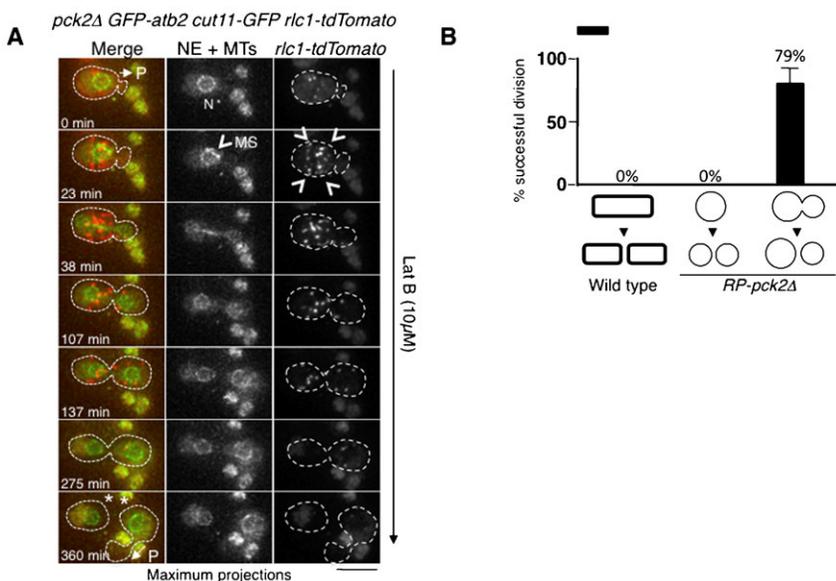


Fig. 5. Cell division in the absence of actomyosin ring in *RP-pck2Δ* cells. (A) Time-lapse images of protruding *pck2Δ* cells expressing GFP-Atb2, Rlc1-tdTomato and Cut11-GFP as markers of the mitotic spindle, actomyosin ring and nuclear envelope, respectively. Cells were treated with 10 μ M of LatB and recorded in multiple focal planes every 7.5 minutes. Maximum z-projections of representative time points are shown. P indicates the formation of a protrusion at time 0 and time 360 min, the asterisk denotes cell separation and N indicates the position of the nuclei. Arrowheads denote the myosin spots at the cell cortex indicative of an unassembled actomyosin ring. MS indicates the formation of the mitotic spindle at time 23 min. Dashed line highlights the cell border. Scale bar: 5 μ m. (B) Percentage of *pck2Δ* and *RP-pck2Δ* cells undergoing successful divisions in the presence of 10 μ M of LatB.

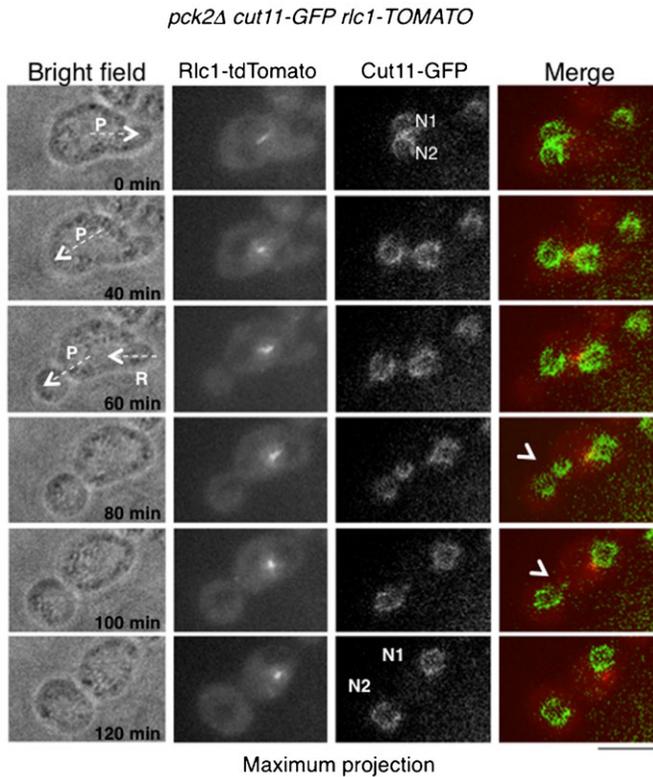


Fig. 6. Nuclear segregation driven by protrusion formation. Protruding *RP-pck2Δ* cell expressing Rlc1-tdTomato and Cut11-GFP as markers of the actomyosin ring and nuclear envelope (NE) were recorded in multiple focal planes every 20 minutes. Maximum z projections of fluorescence and single sections of bright field images of representative time points are shown. P and arrow at time 0 min and 40 minutes indicate the formation of a protrusion. R at time 60 minutes denotes the retraction of a previously formed protrusion when the second one appeared. N1 and N2 show the two nuclei present in the cell. Arrowheads indicate deformation of the nucleus when it is passing through the neck toward the protrusion. Scale bar: 5 μ m.

During amoeboid movement, local disruption of the actomyosin cortex results in cell expansion and the formation of pseudopodia. The amoeba cortex and the yeast cell wall are both locally modified or softened to allow cell expansion, which results in pseudopodia formation and in fungal growth, respectively (Chang and Martin, 2009; Harold, 2002; Stockem et al., 1982). In fungi, the overall cell volume increases as the cell grows while in amoebas, actomyosin contraction of the cortex at the rear leads to cell body translocation (Webb and Horwitz, 2003; Yoshida and Soldati, 2006). In *RP-pck2Δ*, we see a distinct phase of protrusion expansion, followed by the detachment of membrane at the rear. In animal cells, membrane detachment from the actin cortex, or local rupture of actin meshwork, induces the formation of cell protrusions or blebs (Charras et al., 2005; Diz-Muñoz et al., 2010; Tinevez et al., 2009). Blebbing induced by hydrostatic pressure has been proposed to be the driving force for movement of amoeboid (Langridge and Kay, 2006; Yanai et al., 1996; Yoshida and Soldati, 2006), embryonic cells (Blaser et al., 2006; Jaglarz and Howard, 1995) and also for tumor cell invasion (Charras and Paluch, 2008; Friedl and Wolf, 2003; Sahai and Marshall, 2003; Wolf et al., 2003).

Our findings demonstrate how a non-motile yeast cell can be transformed into an amoeboid form by weakening the cell wall,

giving credence to the old idea that fungi may indeed be “amoebas in a tube” (Heath and Steinberg, 1999; Reinhardt, 1892).

It has previously been reported that cells deprived of cell wall fail to undergo cell division (Jochová et al., 1991). More recently, it has been shown that actomyosin rings in protoplasts slide sideways (Mishra et al., 2012). Consistent with this, our time-lapse experiments also show ring sliding in *RP-pck2Δ* cells, leading to the failure of septum assembly. Since round cells containing normal cell wall (*orb6-25* and *scd1Δ*) are able to complete cell division and create a septum in most of the cases (Bernal et al., 2012; our unpublished data), it is possible that cell wall plays a role in anchoring the ring and preventing sliding. Importantly, this sliding was always directed towards the protrusion, suggesting that in addition to cell geometry (Mishra et al., 2012) and secretion (Pardo and Nurse, 2003), other factors such as flow of membrane and/or cytoplasm into the protrusion may contribute to directional ring sliding.

Surprisingly, after the failure of cytokinesis in *RP-pck2Δ* cells, the ring reassembled in a fraction of cells, similar to observations made in wild-type protoplasts suggesting the existence of a checkpoint-like control monitoring completion of cytokinesis (Mishra et al., 2012). This control could be novel or could partially overlap with mechanisms protecting the cell from partial damage to the ring (Le Goff et al., 1999; Liu et al., 2000).

Unexpectedly, in many cells cytoplasmic protrusion detached from the “mother cell”, generating two independent cellular compartments with no visible septum between them. Cell separation occurs at the narrow neck at the site of protrusion formation by a mechanism that is independent of the actomyosin ring and cell cycle stage. This conclusion is supported by our observations that cell separation occurs in cells with no visible actomyosin ring, in cells in which the ring is assembled in another part of the cell at the moment of cell separation, and in also cells treated with Latrunculin B, at a concentration that disassembles the ring. We speculate that membrane pinching and cell separation could result from cell membrane fusion, new membrane addition by vesicular transport, or from membrane breakage at the neck.

In the absence of nuclear segregation, cell separation would not be sufficient to overcome the failure of cytokinesis and to allow *RP-pck2Δ* cell proliferation. However, in many cells one of the nuclei moved into the protrusion, generating a functional daughter cell with normal ploidy. The nuclear segregation into the new compartment often occurred independently of mitotic spindle elongation and might be a consequence of cytoplasmic flow into the protrusion. We speculate that this division may be similar to the behavior of vesicles divided *in vitro* by extrusion when they are mechanically passed through a pore (Hanczyc et al., 2003; Mayer et al., 1986). This mode of cytokinesis by cell extrusion, independent of cytokinetic machinery, may resemble an ancestral mechanism of cell division in the absence of a contractile actomyosin ring. A similar mechanism of *ftsZ*-independent division, based on extrusion, has also been shown in bacteria lacking cell wall (Leaver et al., 2009). Interestingly, amoeboid slime mold cells are able to divide without myosin II or a discrete contractile ring (Neujahr et al., 1997; Zang et al., 1997).

Changes in cell wall may have underlain the evolution of different fungal forms. For instance, the pressure-driven protrusion events similar to the ones we describe could lead to

the evolution of budding morphogenesis (Hartwell et al., 1974; Marchant and Smith, 1968). These findings potentially provide insights into the evolutionary relationships between walled cells such as fungi and more amoeboid forms, and how changes in cell wall metabolism and turgor pressure can lead to the evolution of different modes of cell growth, migration and division.

Materials and Methods

S. pombe strains, plasmids and media

The *S. pombe* strains used in this study are as follows: PPG42.10: h+ pck2:kan leu1-32 ura4-D18 (from Pilar Perez), RD716: h+ pck2:kan cut11-GFP:Ura4 Rlc1-TOMATO:Nat pREP3X-GFP-atb2, RD814: h+ pck2:kan cut11-GFP:ura4 Rlc1-TOMATO:Nat pREP3X-GFP-CAAX, RD696: h+ pck2:kan Rlc1-TOMATO:Nat pREP3X-GFP-atb2. Plasmid pREP3X-GFP-CAAX was constructed by cloning a PCR fragment encoding the last 19 amino acids from Cdc42p (Miller and Johnson, 1994) fused to the C-terminus of the green fluorescent protein (GFP). Cells were grown in YES or in MM-Leu when carrying plasmids and transferred to YES medium for 1 hour before forming protoplasts.

Protoplast formation and protoplast recovery

To form protoplasts, cells were washed with SCS buffer and incubated with 0.1 g/ml Lallzyme MMX in SCS buffer (20 mM sodium citrate [pH 5.8] 0.6 M–1.5 M Sorbitol) until 100% of cells were converted to protoplasts. Time of incubation ranged from 8 minutes at 36°C to 12 min at 30°C. Protoplasts were harvested by centrifugation, inoculated into YES containing 1 M sorbitol (regeneration medium) (Kobori et al., 1989), and allowed to regenerate at 30°C for 4–6 hours.

Microscopy and image analysis

Microscopy was performed at 25°C with either an upright wide-field fluorescence microscope or a delta vision confocal fluorescence microscope. Typically, confocal stacks were made of 12 z sections spaced by 0.5 μm taken with 0.5 s exposure, except Rlc1-TOMATO (0.1 s exposure). Images were acquired with MetaMorph software (Molecular Devices) and processed and analyzed with MetaMorph and Image J (<http://imagej.nih.gov/ij>). Movies of protoplast formation were taken at 30°C.

Pharmacological inhibitors and drugs

Methyl-2-benzimidazole carbamate (MBC, Aldrich) was used at a final concentration of 50 μg/ml from a 100× stock solution made fresh in DMSO. Latrunculin A (LatA, Sigma) was used at a final concentration of 100 μM from a 200× stock in DMSO. Brefeldin A (BFA, Sigma) was used at a final concentration of 20 μg/ml from a 100× stock in ethanol. Latrunculin B (LatB, Sigma) was used at a final concentration of 10 μM from a 200× stock in DMSO. In the data presented, these drugs were added to the media 10 min prior to start of observation.

Data quantification

The cell trajectories and velocity were measured by manually tracking the leading edge of each cell/protrusion using the Manual Tracking plugin of ImageJ. Protrusion volumes were approximated as spheric volumes based on the diameter of the protrusion. Divisions were recorded as successful when two compartments were generated from one. Rate of ring contraction of walled cells ($n=8$) and RP-*pck2Δ* cells ($n=8$) calculated by measuring ring diameter over time and calculating averages from the total contraction time.

Statistical analyses

For determination of the statistical significance between two groups, the Student's t-test was used. Probability values ($P<0.05$) were considered to be statistically significant. Values depicted are means ± s.e.m.

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Competing Interests

The authors have no competing interests to declare.

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