Stress-regulated kinase pathways in the recovery of tip growth and microtubule dynamics following osmotic stress in *S. pombe*

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Summary

The cell-integrity and stress-response MAP kinase pathways (CIP and SRP, respectively) are stimulated by various environmental stresses. Ssp1 kinase modulates actin dynamics and is rapidly recruited to the plasma membrane following osmotic stress. Here, we show that osmotic stress arrested tip growth, induced the deposition of abnormal cell-wall deposits at tips and led to disassociation of F-actin foci from cell tips together with a reduction in the amount of F-actin in these foci. Osmotic stress also ‘froze’ the dynamics of interphase microtubule bundles, with microtubules remaining static for approximately 38 minutes (at 30°C) before fragmenting upon return to dynamic behaviour. The timing with which microtubules resumed dynamic behaviour relied upon SRP activation of Atf1-mediated transcription, but not on either CIP or Ssp1 signalling. Analysis of the recovery of tip growth showed that: (1) the timing of recovery was controlled by SRP-stimulated Atf1 transcription; (2) re-establishment of polarized tip growth was absolutely dependent upon SRP and partially dependent upon Ssp1 signalling; and (3) selection of the site for polarized tip extension required Ssp1 and the SRP-associated polarity factor Wsh3 (also known as Tea4). CIP signalling did not impact upon any aspect of recovery. The normal kinetics of tip growth following osmotic stress of *pol1.S402A/E* mutants established that SRP control over the resumption of tip growth after osmotic stress is distinct from its control of tip growth following heat or gravitational stresses.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/121/24/4055/DC1

Key words: *S. pombe*, Stress response, Sty1, Pmk1, Ssp1, Tea1

Introduction

Eukaryotic cells adapt to changes in their extracellular environment via the controlled induction of a spectrum of responses, many of which are mediated by conserved, three-tier, stress-activated mitogen-activated protein (MAP) kinase cascades. In mammalian cells these include the p38 and JNK pathways whereas in *Schizosaccharomyces pombe* the Sty1 (also known as Spc1) stress-response pathway (SRP) plays a major role and the Pmk1 (also known as Spm1) cell-integrity pathway (CIP) plays a less prominent role in maintaining homeostasis in response to changes in the cell environment (Madrid et al., 2007; Toda et al., 1996; Toone and Jones, 2004; Widmann et al., 1999; Zaitsevskaya-Carter and Cooper, 1997).

SRP activation promotes a wide range of responses, including the modulation of transcription of specific gene cohorts and inhibition of entry into mitosis in response to insults (Chen et al., 2003; Petersen and Hagan, 2005; Shiozaki and Russell, 1995; Toone and Jones, 2004). In addition to promoting a range of transcriptional responses to diverse stresses (Chen et al., 2003; Chen et al., 2008; Reiter et al., 2008), cells lacking the SRP MAP kinase Sty1 delay commitment to mitosis in unperturbed cell cycles and are unable to modulate their cell cycle in response to environmental cues (Millar et al., 1995; Petersen and Hagan, 2005; Petersen and Nurse, 2007; Shiozaki and Russell, 1995). There is crosstalk between SRP and CIP signalling, because SRP signalling has been reported to both promote and attenuate CIP signalling (Loewith et al., 2000; Madrid et al., 2007; Madrid et al., 2006; Toda et al., 1996; Zaitsevskaya-Carter and Cooper, 1997). CIP signalling is required for cytokinesis and to maintain cell integrity and vacuolar function in response to osmotic and hydrostatic pressure (Bone et al., 1998; George et al., 2007; Toda et al., 1996; Zaitsevskaya-Carter and Cooper, 1997). It is unclear how CIP signalling modulates the completion of cytokinesis; however, its action in concert with Pck2 and Pck1 suggests that it might influence the activity of α- and β-glucan synthases (Arellano et al., 1999; Calonge et al., 2000; Sengar et al., 1997; Tajadura et al., 2004; Toda et al., 1996).

*S. pombe* cells are cylindrical, grow by tip extension and divide by medial fission (Hayles and Nurse, 2001). Both of these processes require the actin cytoskeleton, with patches of filamentous (F)-actin localizing to cell tips (the sites of growth) during interphase and to the medial contractile ring (the site of fission) during cytokinesis (Marks and Hyams, 1985; Rupes et al., 2001). Immediately following cytokinesis, growth initiates at the ‘old’ end – that which existed before division. The ‘new’ end, which was created by division, does not grow until cells have reached a critical size threshold and entered G2 phase of the cell cycle (Castagnetti et al., 2007; Mitchison and Nurse, 1985), whereupon growth starts from the new end, a step referred to as new-end take off (NETO). Since growth from the old tip persists after NETO, NETO constitutes a rate change point (RCP), as the overall rate of growth is increased by the activation of this new site (Mitchison and Nurse, 1985). The restriction of new tip growth to G2 phase can be overcome by disruption of microtubules, but the requirement to achieve a minimal cell size cannot be overcome (Castagnetti et al., 2007).
Thus, size control influences both tip growth and cell-cycle commitment (Castagnetti et al., 2007; Fantes, 1977; Mitchison and Nurse, 1985; Rupes et al., 2001).

NETO is instigated at two levels: marking the new end as a site that is competent to grow and the turnover of the F-actin cytoskeleton to generate free actin monomers. The former is reliant upon the Tea1 polarity system (discussed below), whereas the latter was demonstrated by the promotion of bipolar growth in otherwise monopolar cdc10.129 G1-arrested cells by transient depolymerization of actin (Rupes et al., 1999). Ssp1 kinase promotes the disassembly of F-actin (Rupes et al., 1999). Ssp1 was identified as a suppressor of sts5 and pep1 lethals, as well as in a screen for mutations that confer sensitivity to low pH (Matsusaka et al., 1995; Rupes et al., 1999). Deletion of sspl blocks NETO and reduces sensitivity to the actin-monomer-sequestering drug Latrunculin A, whereas elevation of Ssp1 levels, similar to transient depolymerization of F-actin, promotes NETO in cdc10.129 cells. Ssp1 responds to osmotic stress. Exogenously expressed Ssp1 is recruited to the plasma membrane within a minute of stress. This recruitment to plasma membranes revealed crosstalk between Ssp1 and SRP signalling, because Ssp1 permanently associated with membranes of sty1Δ cells and recruitment was blocked by constitutive SRP signalling (Rupes et al., 1999).

Whereas the physical process of cell-tip extension is an actin-dependent process, the microtubule cytoskeleton governs where this tip extension will occur by delivering a number of polarity factors to the cell tips (Hayles and Nurse, 2001). The interphase microtubule cytoskeleton is composed of four to six bundles of microtubules that are generated from nucleation sites on the cell nucleus in the middle of the cell and extend from the central region towards the cell tips (Drummond and Cross, 2000; Hagan and Hyams, 1988; Hoog et al., 2007; Sawin et al., 2004; Tran et al., 2001; Zimmerman et al., 2004). These bundles are generated and maintained by MAPs such as Ase1 and microtubule motor proteins such as Klp2 (Loiodice et al., 2005; Yamashita et al., 2005; Carazo-Salas et al., 2005). Exploitation of one tubulin-GFP imaging system found no impact of the fission yeast CLASP homologue Peg1 upon interphase microtubule function (Bratman and Chang, 2007), whereas the exploitation of a different GFP-tubulin expression system revealed that the normal behaviour of microtubule bundles relies upon dynein and Peg1 (Grallert et al., 2006). Individual microtubules polymerize from the end of these bundles, invariably reaching the cell tips before depolymerizing (Drummond and Cross, 2000; Grallert et al., 2006; Hoog et al., 2007; Tran et al., 2001). This is in contact with the cell tip that promotes the deposition of polarity factors at cell tips (Feierbach and Chang, 2001; Martin et al., 2005; Martin et al., 2007; Snaith and Sawin, 2003). The Tea1 polarity factor forms a complex with a kinesin-related protein (Tea2), Wsh3 (also known as Tea4) and the fission yeast orthologues of the plus-end tracking proteins (+TIPs) EB1 and CLIP170 (Mal3 and Tip1, respectively) (Beinhauer et al., 1997; Bieging et al., 2007; Browning and Hackney, 2005; Browning et al., 1998; Brunner and Nurse, 2000; Martin et al., 2005; Tatebe et al., 2005). The +TIPs impart an inherent affinity for the growing tips of microtubules upon the complexes, whereas the Tea2 motor protein can translocate the complex along the microtubule lattice to the end of the microtubule (Browning et al., 2003). Upon arrival at cell tips, Tea1 associates with the membrane-associate CAAX-box protein Mod5 (Snaith and Sawin, 2003). The deposition of Tea1 and Wsh3 to the tips promotes the recruitment of Pom1 kinase, which in turn modulates the activity of the Rga3 GTPase-activating protein (GAP) function to promote Cdc42 activity (Tatebe et al., 2008). Activated Cdc42 stimulates For3/Bud6-mediated actin polymerization to promote tip growth (Martin et al., 2007). Thus, the association between Tea1, Bud6, the formin For3 and Wsh3 promotes the Cdc42-dependent polymerization of actin to maintain polarized tip growth (Feierbach and Chang, 2001; Glynn et al., 2001; Martin et al., 2005; Martin et al., 2007).

Wsh3 was identified through its affinity for both Tea1 and the Sty1-stress-pathway MAP kinase kinase kinase (MAPKKK) Win1 (Martin et al., 2005; Tatebe et al., 2005). Wsh3 recruits the fission yeast protein phosphatase 1, Dis2, to cell tips to modulate the cytoskeleton in response to stress (Alvarez-Tabares et al., 2007). Strains in which Wsh3 cannot recruit PPI to itself at cell tips fail to select the correct growth site following osmotic stress (Alvarez-Tabares et al., 2007; Tatebe et al., 2005).

Exposures to osmotic, heat or centrifugation stress have all led to a transient dispersal of F-actin patches from cell tips (Bao et al., 2001; Petersen and Hagan, 2005; Rupes et al., 1999; Soto et al., 2007). Following heat and centrifugation stress, phosphorylation of serine 402 of the fission yeast polo kinase, Plo1, is required for the recruitment of F-actin patches back to cell tips to promote a return to tip growth (Petersen and Hagan, 2005). It is not clear whether the recovery of tip growth following other forms of stress, such as osmotic stress, involves similar or distinct controls. Less is known about the impact of diverse stresses upon the microtubule cytoskeleton, except that microtubule dynamics freeze following osmotic stress (Tatebe et al., 2005). We now describe a previously uncharacterized consequence of exposure to osmotic stress – the deposition of cell-wall material at growing tips – and document the contributions of SRP, CIP and Sp1 signalling in restoring tip growth and microtubule dynamics following osmotic stress.

**Results**

Osmotic stress perturbs septation and induces Calcofluor-white foci at cell tips

To assess the impact of osmotic stress upon growth, an equal volume of pre-warmed medium containing 2.4 M sorbitol was added to cells that had been grown to early log phase in rich medium at 30°C before fixation with 3% formaldehyde and staining with 0.02 mg ml⁻¹ Calcofluor. Prior to stress, Calcofluor-white staining was as reported previously (Mitchison and Nurse, 1985); growing ends stained more brightly than non-growing ends, birth scars stained poorly and division septa gave very bright signals (Fig. 1A, 0 minutes). Three new patterns were induced by osmotic stress. At 1 hour after stress, bright spots of Calcofluor staining appeared at the cell tips (Fig. 1A,B; Fig. 2B). We shall refer to these bright dots as stress-induced Calcofluor structures (SICS). An hour later, SICS were seen further away from tips, towards the cell centre (inset in Fig. 1A, arrow). At 3 hours after the imposition of stress, long cells with one, two or three septa were present. Staining of F-actin and chromatin at this 180-minute time point showed that the two compartments on either side of the septum in such long cells underwent mitosis and formed actin rings between their anaphase nuclei (Fig. 1C, also see below). In addition, some sorbitol-treated cells that were completing cytokinesis did not completely break down their septal material (Fig. 2B, lower panel).

Since SICS had not been detected in previous studies of osmotic stress in *S. pombe* (Kawasaki et al., 2006), we investigated whether the higher concentrations of stain used in standard protocols (Moreno et al., 1991) obscured them. SICS were barely detectable when 2 mg ml⁻¹ of Calcofluor white was used (the concentration...
Osmotic stress of the S. pombe cytoskeleton

in standard protocols) (Fig. 1B), indicating that SICS are indeed masked by the higher generic fluorescence with stronger staining protocols.

We next stained cells with Calcofluor white at 30 minutes and at 1 hour after the imposition of other stresses that also stimulate the Sty1 and Pmk1 signalling (Madrid et al., 2006; Toone and Jones, 2004). SICS were only seen when osmotic stress was induced with either 1.2 M sorbitol or 0.6 M KCl, and not following any other form of stress (Fig. 1D,E).

Kinetic analysis of SICS staining

We assessed the kinetics of SICS with cells that showed the following features appeared in the culture: SICS at one or both tips, septum staining either with or without tip SICS staining, or cells with multiple septa. The number of cells in which both tips stained peaked 60 minutes after stress (Fig. 2A). By contrast, the frequency of cells that had SICS staining at one tip had a much broader profile and did not decline until 150 minutes after stress (Fig. 2A). Pre-NETO cells generally had SICS at only one tip, the growing tip (Fig. 1A, cell 1), suggesting that the patches were only induced at growing tips. Consistently, cells with SICS at both tips had passed NETO. Although some small pre-NETO cells had SICS at both ends (e.g. Fig. 1A, cell 2), we assume that these were the small number of cells that, at the time of stress, were completing cytokinesis and re-initiating old-end growth.

To test the hypothesis that SICS formed at actively growing tips, mutants that fail to execute NETO and so grow in a monopolar fashion were stained with Calcofluor following osmotic stress (Fig. 2C). In each case, dual-tip SICS staining was largely suppressed and cells in which a single tip stained predominated. We assume that the dual-tip staining arose from cells that were re-initiating growth from the old tip in the final stages of cytokinesis when the stress was applied.

The decrease in cells with dual-tip SICS staining was accompanied by an increase in the proportion of septating cells with SICS at their tips (Fig. 2B). Because this could be explained by the acceleration of mitotic commitment that occurs following osmotic stress (Millar et al., 1995; Shiozaki and Russell, 1995), we monitored the length of septating cells. Osmotic stress did indeed reduce the size of dividing cells, suggesting that cells that have SICS staining in addition to septum staining had committed to mitosis following SICS deposition at both tips (Fig. 3; Table 1). These plots of the lengths of septating cells had a bimodal distribution 150 minutes after exposure to stress, because a fraction of cells far exceeded the maximum length at division in wild-type cells (Fig. 3B). The proportion of the population that showed this increased size declined after a further 150 minutes, indicating that long cells with septa were a transient feature of the culture (Fig. 3C). In 78% of these long cells at the 150-minute time point, the nuclei on either side of the septum were undergoing mitosis (Fig. 1C). We assume that the...
stress disrupted an ongoing septation event in these cells so that they returned to tip growth in the next cell cycle without having degraded the primary septum that was being laid down at the time of stress. Activation of the septation machinery in the next cell cycle would then enable such cells to complete the aborted septation event, split in two and divide with normal cell length.

**Bright Calcofluor patches persist following resumption of tip growth**

The execution of NETO and mitosis in wild-type populations made it difficult to address the origins of the SICS ring staining that appeared 120 minutes after the stress (Fig. 1A). We therefore followed SICS induction by stressing cdc10.v50 cells that had been arrested in G1 prior to NETO by incubation at 36°C for 3.5 hours (Marks et al., 1992; Mitchison and Nurse, 1985). As with wild-type cells (Fig. 1A), SICS appeared at cell tips within 10 minutes of osmotic stress (Fig. 4A,B). After 50 minutes, SICS staining extended further back away from the tip and by 80 minutes the SICS staining formed clear rings behind normally stained cell tips (Fig. 4A,B). These data suggest that SICS are displaced from the cell tips by insertion of new cell-wall material at tips. To address this possibility we combined Calcofluor staining with the pulsed lectin approach of May and Mitchison (May and Mitchison, 1986) in which cells are isolated from the culture by filtration, stained with a fluorescent lectin marker and returned to liquid culture. Further cell growth inserts new, lectin-free cell-wall material at cell tips beyond a central belt of fluorescently labelled material. Thus, a dark cap at the tip of a fluorescent lectin jacket identifies cell-wall material that has been inserted after exposure to the lectin (Fig. 4C). Combining this pulsed lectin approach by coating cells in FITC lectin when the stress was applied, and fixing and staining with Calcofluor 180 minutes later confirmed that the ring of Calcofluor patches lay at the boundary between the fluorescent and dark zones in the lectin channel (Fig. 4C). We conclude that the rings of SICS that were distal to cell tips at later time points following osmotic stress arise because of outgrowth beyond sites at which the cell-wall composition has been permanently modified.

The pulsed lectin approach also enabled us to monitor the kinetics of tip growth following stress by plotting the frequency with which dark zones appear at cell tips against time. There was a delay of around an hour between the timing with which dark zones appeared in stressed cells versus the controls (Fig. 4D). The delay was the same whether the stress was induced by sorbitol or KCl (Fig. 4E).

**Osmotic stress induces the depolymerization of the F-actin cytoskeleton**

Since a polarized F-actin cytoskeleton promotes cell-tip extension (Marks and Hyams, 1985; Rupes et al., 2001), we stained cells with TRITC-phalloidin to monitor the integrity of the F-actin cytoskeleton following stress. At 30 minutes after osmotic stress, the intensity of the F-actin signal was radically reduced (Fig. 5E,H). Increasing the levels and contrast of the fluorescence signal uniformly in each image to approach that of unstressed cells revealed the displacement of F-actin patches from cell tips that was reported by Rupes et al. (Rupes et al., 1999; Bao et al., 2001) (Fig. 5F,I). This displacement is transient, with actin patches relocating to cell tips at later time points (Fig. 5I, asterisks). The kinetics with which F-actin patches disassociated from cell tips mirrored the appearance of bright foci of SICS at tips until the 40-minute time point, after
which the number of cells with delocalized F-actin patches declined (Fig. 5J).

### Osmotic stress freezes microtubule dynamics before disrupting microtubule bundles

Since the polarity with which the F-actin cytoskeleton directs growth is determined by the delivery of polarity factors to cell tips by microtubules (Feierbach and Chang, 2001), we assessed the impact of osmotic stress upon microtubule dynamics. We used an allele of the α-tubulin-encoding atb2 (also known as tub1) gene at the endogenous atb2 locus, in which the sequences encoding GFP were fused in frame to the atb2 ORF under the control of the thiamine-repressible nmt81 promoter (Garcia et al., 2001) in minimal medium (EMM2) [SICS appeared at cell tips in response to osmotic stress in EMM2 medium with identical kinetics to rich, yeast extract (YES) medium (supplementary material Fig. S1)].

As was observed for strains expressing higher levels of the fluorescent tubulin (Tatebe et al., 2005), osmotic stress arrested all dynamic behaviour in wild-type cells (Fig. 6A,B; supplementary material Movie 1). Moreover, cessation of microtubule dynamics occurred independently of SRP signalling, because microtubule dynamics were also arrested in cells lacking SRP signalling (Fig. 6C) and in cells with enhanced SRP signalling (Fig. 6D). The cessation of microtubule dynamics in wild-type cells was transient because dynamic behaviour returned 38±1.2 minutes after the imposition of stress (Fig. 6B; Table 2; supplementary material Movies 2 and 3). Resumption of dynamics was accompanied by the transient fragmentation of the microtubule bundles into a number of short microtubules that appeared at a variety of angles to the long axis of the cell (Fig. 6B, panel boxed in red; supplementary material Movie 2). Immunofluorescent localization of microtubules in fixed, sorbitol-treated wild-type cells revealed a similar disruption of bundles by osmotic stress in rich medium (supplementary material Fig. S2), indicating that the fragmentation observed by live-cell imaging is not an artifact arising from the use of GFP-tubulin to visualize microtubules.

### Scoring cells in which a microtubule bundle was aligned parallel

Arising from the use of GFP-tubulin to visualize microtubules, the fragmentation observed by live-cell imaging is not an artifact in rich medium (supplementary material Fig. S2), indicating that cells revealed a similar disruption of bundles by osmotic stress localization of microtubules in fixed, sorbitol-treated wild-type (supplementary material Movie 2). Immunofluorescent variety of angles to the long axis of the cell (Fig. 6B, panel boxed in red; supplementary material Movie 2). Immunofluorescent localization of microtubules in fixed, sorbitol-treated wild-type cells revealed a similar disruption of bundles by osmotic stress in rich medium (supplementary material Fig. S2), indicating that the fragmentation observed by live-cell imaging is not an artifact arising from the use of GFP-tubulin to visualize microtubules.

### Osmotic stress of the S. pombe cytoskeleton

#### Table 1. Osmotic stress accelerates commitment to mitosis

<table>
<thead>
<tr>
<th>Time post-sorbitol (minutes)</th>
<th>Cell length at division* (μm)</th>
<th>Peak bin† (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.1±0.8</td>
<td>11.5-11.75</td>
</tr>
<tr>
<td>150</td>
<td>15.4±4.6</td>
<td>11.25-11.5</td>
</tr>
<tr>
<td>180</td>
<td>12.5±3.7</td>
<td>10.25-10.5</td>
</tr>
</tbody>
</table>

*Cell length at division was measured for a minimum of 100 cells at each time point.
†Peak bins of the plots shown in Fig. 3.

#### Table 2. Microtubule dynamics following osmotic stress

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time before resumption of dynamic behavior following osmotic stress (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>37.8±1.2</td>
</tr>
<tr>
<td>pmk1.DΔ</td>
<td>38.5±0.9</td>
</tr>
<tr>
<td>ssp1.DΔ</td>
<td>37.3±3.5</td>
</tr>
<tr>
<td>sty1.DΔ</td>
<td>71.3±8.3*</td>
</tr>
<tr>
<td>wis1.DΔ</td>
<td>24.5±1.7</td>
</tr>
<tr>
<td>atf1.DΔ</td>
<td>69.5±7.1</td>
</tr>
<tr>
<td>srk1.DΔ</td>
<td>39.0±1.9</td>
</tr>
</tbody>
</table>

*n=8 for all strains examined.
*The resumption of microtubule dynamics in sty1.DΔ cultures was highly heterogeneous, with 72 out of 400 cells failing to recover dynamics up to 2.5 hours after osmotic stress. The calculations of recovery time in sty1.DΔ cultures therefore represent the time at which the first cell in the field of view exhibited a return to dynamic behaviour.

#### SRP signalling determines the duration of the arrest of microtubule dynamics

We next investigated whether SRP, CIP and Ssp1 signalling had any impact upon the resumption of microtubule dynamics following osmotic stress by monitoring microtubule dynamics in cells that lacked either the SRP MAPK Sty1 (sty1.DΔ cells), the CIP MAPK Pmk1 (pmk1.DΔ cells) or Ssp1 kinase (ssp1.DΔ). Whereas abolition of CIP or Ssp1 signalling did not influence the arrest or resumption of microtubule dynamics, resumption was greatly delayed by abolition of SRP signalling (Fig. 6C; Table 2; supplementary material Movies 3 and 4). Furthermore, the population was extremely heterogeneous with respect to the resumption of dynamic behaviour, because 81% of the cells failed to re-initiate dynamic behaviour for more than 2 hours after the imposition of stress (supplementary material Movies 3 and 4). We therefore asked whether enhancement of SRP signalling accelerated the return to dynamics by stressing a strain in which the MAPKK Wis1 (and therefore the SRP itself) was constitutively active (wis1.DΔ) (Shiozaki et al., 1998). wis1.DΔ significantly reduced the period of stasis from 38±1.2 to 24.5±1.69 minutes (Fig. 6C; Table 2; supplementary material Movie 3). We conclude that SRP, but not CIP or Ssp1, signalling controls the time at which microtubules return to dynamic behaviour following osmotic stress.
SRP signalling impacts cell responses in two principal ways: it either directly phosphorylates molecules to set in train a set of post-translational modifications that will instigate change – such as the activation of the Srk1 kinase (Lopez-Aviles et al., 2005; Lopez-Aviles et al., 2008) – or it activates the Atf1 transcription factor to alter transcription and modify the composition of the proteome (Shiozaki and Russell, 1996; Wilkinson et al., 1996). To address whether the requirement for SRP signalling for the timely restoration of microtubule dynamics arose from signalling to the downstream kinase Srk1 or from Atf1-mediated transcription, we monitored microtubule dynamics in cells from which either gene had been deleted.

Whereas microtubule dynamics resumed with wild-type kinetics in srk1Δ cells, the behaviour of microtubules in atf1Δ cells was indistinguishable from that in sty1Δ cells; dynamics resumed after a similar delay and the population displayed the same heterogeneity in resumption seen in sty1Δ cells (Table 2; supplementary material Movie 3). We conclude that SRP-stimulated transcription is required to promote rapid recovery of microtubule dynamics following osmotic stress.

Efficient recovery of tip growth following osmotic stress requires Sty1
We next investigated whether the loss of either of the three stress pathways influenced the resumption of tip growth. Deletion of ssp1Δ did not affect the kinetics of the arrest or resumption of growth (Fig. 7A); however, it did affect the polarity of this growth because cells often branched and the re-growing tips were fatter than normal (Fig. 7B).

Deletion of pmk1Δ did not alter the kinetics of SICS appearance; however, we noted an accumulation of cells with septa at 150 minutes after stress (Fig. 7C,D). Monitoring the frequency of cells with anaphase chromosomes indicated that this peak in septation index did not arise from a synchronization of cell cycle and so we conclude that it arises from an extension of the duration of septation (Fig. 7E). This is consistent with the compromised efficiency of septation in pmk1Δ cells (Toda et al., 1996; Zaitsevskaya-Carter and Cooper, 1997).

Deletion of sty1Δ did not alter the kinetics of SICS appearance, but did delay the resumption of cell-tip extension during recovery from the stress by around 90 minutes (Fig. 8A-C). In addition, SICS

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The association between Wsh3, the SRP MAPKKK Win1 and the extension following osmotic stress. Whereas deletion of Srk1 had no effect on the kinetics of stress 

If, by contrast, Sty1 influences tip growth by additional means, the profiles would be different. We therefore monitored tip growth following the application of osmotic stress to cells lacking either Wsh3 or the Wsh3 anchor and/or mediator Tea1.

The balance of the mitotic inducer Cdc2 also induced morphology defects at cell tips (Fig. 8F,G; supplementary material Fig. S3A). Importantly, however, these long, cdc2-arrested cells did not swell at their tips (Fig. 8F,G; supplementary material Fig. S3A). We conclude that the Sty1 MAPK cascade plays an important role in promoting both the timing and polarity recovery of tip growth following osmotic stress whereas deletion of Srk1 had no effect on the kinetics of recovery of tip growth, cells lacking Atf1 resumed growth with a similar delay as sty1Δ cells (Fig. 8C), suggesting that efficient recovery of growth is as a result of the Sty1-dependent modulation of gene transcription through Atf1.

Neither Tea1 nor Wsh3 are required for re-initiation of tip extension following osmotic stress

The association between Wsh3, the SRP MAPKKK Win1 and the branching of wsh3Δ cells following osmotic stress (Tatebe et al., 2005; Alvarez-Tabares et al., 2007) suggest that Wsh3 could be a major conduit through which Sty1 signalling could control the kinetics of tip-growth recovery. If this were to be the case, the response profile of wsh3Δ should mimic that of sty1Δ. If, by contrast, Sty1 influences tip growth by additional means, the profiles would be different. We therefore monitored tip growth following the application of osmotic stress to cells lacking either Wsh3 or the Wsh3 anchor and/or mediator Tea1.

Plots of the number of cells with SICS tip staining suggested that tea1Δ and wsh3Δ cells, similar to sty1Δ, delayed the timing of recovery (Fig. 9A,B). However, in both strains the stress also induced altered morphology in which cells selected a site at the cortex from which to extend other than the cell tips (Fig. 9C-F), i.e. they either branch (Fig. 9E) or, because neither of these strains undergo NETO and grow at only one tip, re-initiate growth from an end that was not growing previously (Fig. 9F) (Tatebe et al., 2005). When cells select these new branch sites there is no growth at the old tip, which harbours the SICS; thus, SICS tip staining does not decline, even though these cells have re-initiated tip growth (Fig. 9E). We therefore used the lectin pulse assay to find that there was, in fact, no delay in the re-establishment of tip growth following osmotic stress when either Tea1 or Wsh3 are absent (Fig. 9F,G). Furthermore, the distribution of either Tea1 or Wsh3 did not change upon osmotic stress, with both proteins remaining at cell tips in 100% of cells at every time point tested (Tea1, every 10 minutes for 60 minutes; Wsh3, every 3 minutes for 60 minutes; n = 200 for both strains) following the imposition of stress [e.g. Fig. 9H,I; cited as unpublished data in Tatebe et al. (Tatebe et al., 2005)]. As Sty1 plays a role in the re-establishment of tip growth following osmotic stress (Fig. 8), it was possible that these polarity proteins were retained at cell tips because of signalling from Sty1 that was stimulated by the stress; however, both Tea1 and Wsh3 were retained at cell tips in 100% of cells.
at every time point tested (Tea1, every 10 minutes for 60 minutes; Wsh3, every 3 minutes for 60 minutes; n=200 for both strains) in osmotically stressed \(\text{sty1.}\Delta\) cells. Thus, the timing over which the arrest and recovery of tip growth is controlled by Sty1 in response to osmotic stress does not appear to involve either Tea1 or Wsh3, even though Wsh3 is required to correctly select the site from which the growth should re-initiate (Alvarez-Tabares et al., 2007; Tatebe et al., 2005).

Distinct SRP-mediated responses control the recovery of tip growth following distinct stresses

As recovery of tip growth following heat, centrifugation or hydrostatic pressure is dependent upon phosphorylation of the polo kinase Plo1 on serine 402 (George et al., 2007; Petersen and Hagan, 2005), we investigated whether recovery from osmotic stress was influenced by mutation of serine 402 of Plo1 to alanine to block phosphorylation or to glutamic acid to mimic it. In either case, the kinetics with which tip growth resumed were indistinguishable from those of wild-type cells (Fig. 10). Thus, the re-initiation of tip growth by SRP signalling following osmotic stress requires a distinct set of controls to those used to re-start growth after heat and centrifugation stresses, even though SRP signalling is crucial in both cases.

**Discussion**

We have extended previous assessments of the impact of osmotic stress upon the cytoskeleton (Bao et al., 2001; Rupes et al., 1999; Tatebe et al., 2005) with the characterization of the kinetics with which microtubule dynamics and cell-tip growth resume following arrest in response to osmotic stress.

Whereas SRP signalling had no impact upon the previously reported osmotic-stress-induced arrest of dynamic behaviour (Tatebe et al., 2005), it did influence the timing with which dynamic behaviour resumed. Excessive SRP signalling accelerated the transition back to dynamic behaviour whereas abolition of signalling greatly delayed it. The impact was profound, as 81% of \(\text{sty1.}\Delta\) cells failed to resume any dynamic behaviour for up to 2.5 hours after the imposition of stress. The requirement for SRP signalling to re-establish microtubule dynamics was due to activation of the Atf1 transcription factor, as \(\text{atf1.}\Delta\) cells exhibited an identical delay and asynchronous resumption of dynamic behaviour as \(\text{sty1.}\Delta\) cells, even though all upstream SRP signalling remains intact in \(\text{atf1.}\Delta\) cells. SRP signalling to Atf1 alters the transcription of a large cohort of genes (Chen et al., 2003). The transcription of the genes belonging to the core environmental-stress response (CESR) are modulated in response to all stresses tested to date, whereas the levels of other transcripts are only modulated in response to particular stresses.
The only genes that are directly linked to cytoskeletal function in the CESR are the protein kinase *kin1*" and the formin *fus1*. Whereas the actin-binding proteins *app1*" and *wsp1*, the kinesin-like protein *tea2*" and the gene that encodes the homologue of the Dop1 protein that modulates morphogenesis in *Aspergillus nidulans* are de-repressed in unstressed *sty1Δ* cells, there is a notable absence of genes encoding cytoskeletal molecules from the genes whose transcript levels show significant alteration in response to osmotic stress (http://www.sanger.ac.uk/PostGenomics/S_pombe/projects/stress/) (Chen et al., 2003). It would therefore seem likely that the requirement for Atf1-mediated transcription to promote recovery of microtubule dynamics (and tip growth) is an indirect effect arising from alterations in cell physiology, such as changes that would counteract the osmotic pressure imposed by the presence of sorbitol in the medium. In this scenario the enhanced SRP signalling in *wis1.DD* cells invokes a physiological environment that accelerates a resumption of microtubule dynamics.

Osmotic stress induced the appearance of punctate Calcofluor-white staining deposits at actively growing tips. Upon the resumption of growth these SICS were displaced from the cell tip to form a ring structure around the cell wall. Similar results were obtained whether 0.6 M KCl or 1.2 M sorbitol was used to induce the stress. As the *bgs1*" gene product is responsible for generating the material that is stained strongly with Calcofluor white in septa (Cortes et al., 2007), stimulation of Bgs1 activity might be responsible for generating SICS. Consistently there was a progressive delay in the timing with which SICS appeared and a decrease in the number of SICS induced when cells were given a 10-minute pre-treatment or simultaneously treated with the (1-3)β-D glucan-synthase inhibitor aculeacin A (Miyata et al., 1981; Perez et al., 1983) (supplementary material Fig. S4). Given the close association between the functions of the Rho GTPases, the protein-kinase-C homologues Pck1 and Pck2, and α- and β-glucan synthases, it would seem likely that the appearance of the SICS involves the stimulation of Rho GTPases to promote protein-kinase-C activity (Arellano et al., 1999; Calonge et al., 2000; Tajadura et al., 2004). Whereas *pck1.Δ* or *pck2.Δ* cells still formed SICS with normal kinetics (supplementary material Fig. S4), simultaneous deletion of both molecules is lethal (Toda et al., 1993) and so cannot be used to address any functional redundancy between these molecules.

The bright septum staining during septation corresponds to a thickening and differentiation of the cell wall in preparation for cell separation (Duran and Perez, 2004), and so it would seem logical that the foci at the cell tip could arise from the repair of damage that was induced at the growing tip as membrane-fusion events were perturbed by the osmotic stress. Perhaps these structures cover weakened areas of the cell wall that would impose additional levels of stress on the cell.
of stress to the plasma membrane and hence to the cell below. The recruitment of the osmo-protective kinase Ssp1 to the cell cortex within a minute of osmotic stress certainly indicates a need for modification of events at the cell periphery after imposition of stress (Rupes et al., 1999). Interestingly, however, Ssp1 activity did not play any role in the generation of SICS, as the kinetics of their appearance and disappearance in *ssp1*Δ did not differ from that in wild-type cells.

Osmotic stress also perturbed septation in cells that were in the process of septating when the stress was applied, generating two compartments either side of a septum that re-initiated growth and eventually divided. Each compartment underwent anaphase with similar timing, suggesting that the decision to commit to mitosis was occurring with similar timing in each compartment. This is in contrast to mitotic controls in *sep* mutants, in which the two compartments either side of the septum undergo mitosis asynchronously (Grallert et al., 1999). This distinction suggests that the septation following osmotic shock is incomplete and that the two compartments share a common cytoplasm.

Although CIP signalling is activated by osmotic stress and is required for survival after exposure to osmotic stress (Madrid et al., 2006; Toda et al., 1996; Zaitsevskaya-Carter and Cooper, 1997), the cascade was not required to halt tip growth or promote the return to growth following stress. The more generic SRP signalling, however, did impact on both the timing and polarity of tip growth following recovery. Furthermore, SICS patterns formed away from
cell tips in sty1.Δ cells, highlighting differences in general cell-wall composition that could account for the higher Ssp1 and Pmk1 activities in sty1.Δ cells. It has previously been noted that sty1.Δ cells branch following heat shift (Tatebe et al., 2005). In this mode of abnormal growth, the selection of the site at which polarized growth will take place is defective and yet the actin-based tip extension at this site is unperturbed. In contrast to the linear (albeit branched) tip extension following heat shock, we found that recovery of sty1.Δ cells from osmotic stress was accompanied by diffuse, depolarized expansion of the cell tip, reminiscent of the ‘round-bottomed flask’ phenotype arising from treatment with the cell-wall inhibitor aculeacin A or the immediate consequence of shifting temperature-sensitive ‘orb’ mutants to the higher temperature (Hayles and Nurse, 2001; Miyata et al., 1981). This phenotype indicated that the cell-wall deposition and so presumably the underlying polarity of the actin cytoskeleton were perturbed by osmotic shock. Clearly, we cannot say whether the microtubule-based site selection was also perturbed by osmotic shock as it is epistatic to the polarity defect. Thus, the different stresses impose distinct challenges upon the growth machinery that are both addressed by the same pathway.

In addition to controlling the mode and location of tip growth following stress, the Sty1-dependent SRP modulates when the recovery will take place. We previously reported that the SRP-dependent phosphorylation of serine 402 of Plo1 promoted recovery of cell division and tip growth after exposure to heat and

Fig. 9. SICS, tip growth and cell morphology in sorbitol-treated cells lacking the polarity factors Tea1 or Wsh3. (A-G) Wild type, tea1.Δ and wsh3.Δ cells were either processed as described for Fig. 1A to determine the kinetics of the appearance of SICS at cell tips (A,B,E) and to analyze cell morphology (C,D,E), or processed with fluorescent lectin for the analysis of tip growth as described for Fig. 4C (F,G). The arrow in the right-hand panel of E indicates a wsh3.Δ cell with SICS at one tip (the end that was growing prior to the addition of sorbitol) that has re-initiated growth at a site on the cell cortex. (F) Quantification of the kinetics of the appearance of cells with dark tip regions in lectin-coated exponentially growing strains following sorbitol treatment. Note that tea1.Δ and wsh3.Δ cells re-initiate growth with the same kinetics as wild-type cells. (G) Pulsed lectin staining of wsh3.Δ cells. Top panel, Calcofluor; middle, lectin; lower, merged (lectin in green, Calcofluor in red). The highlighted cell (star) has SICS at one tip (the growing tip prior to the addition of sorbitol) and has re-initiated tip growth (dark non-lectin-stained region) at the opposite (previously non-growing) tip. (H) tea1.pkGFP cells were grown to mid-log phase in YES media, fixed and processed for anti-Pk immunofluorescence either immediately before stress (upper panel) or 20 minutes post-stress (lower panel). (I) Maximum projections of wsh3.tdTomato cells either immediately before stress (upper panel) or 21 minutes after the addition of sorbitol to a final concentration of 1.2 M (lower panel). Scale bars: 5 μm.
centrifugation stress (Petersen and Hagan, 2005). As osmotic stress causes a transient arrest of cell proliferation (Kawasaki et al., 2006) and we now find an impact on the recovery of tip growth, it was possible that phosphorylation of serine 402 could also mediate this Sty1-dependent recovery function. However, we saw no impact of mutating of serine 402 of plo1 kinase. Wild-type, plo1.S402A and plo1.S402E cells were grown and processed as described in Fig. 1A, and the appearance of SICS at cell tips was plotted as a function of time.

Materials and Methods

Materials, strains, cell culture and data analysis

The strains used are listed in supplementary material Table S1. Cells were cultured in YES or appropriately supplemented EMM2 minimal medium and standard fission yeast approaches were employed throughout (Moreno et al., 1991). Imaging of fixed cells employed a Zeiss Axioplan 2 microscope equipped with an ep-fluor, 100X, 1.45 NA, oil-immersion objective lens, a Quantix camera (Photometrics) and MetaMorph software (Molecular Devices). Photoshop CS2 (Adobe) was used for downstream image processing. Cell-length measurements were performed on at least 100 cells per time point using Imagel software (http://srb.info.nih.gov/ij/). All other analyses were performed on a minimum of 200 cells for each feature counted at each time point.

Stress treatments and Calcofluor staining

Stress treatments were adapted from those described by Chen et al. (Chen et al., 2003) Unless otherwise stated, cells were grown to a density of $4 \times 10^6$ cells ml$^{-1}$ in YES medium at 30°C. Cells were subjected to osmotic stress by the addition of an equal volume of pre-warmed YES containing either 2.4 M sorbitol or 1.2 M KCl. For other stresses, an equal volume of pre-warmed YES medium was added to a culture of cells at $4 \times 10^6$ cells ml$^{-1}$ and stress was then applied as follows: oxidative stress, $H_2O_2$ added to a final concentration of 2 mM; heavy-metal stress, CdSO$_4$ added to a final concentration of 0.5 mM; alkylating agent, methylmethane sulfonate (MMS) added to a final concentration of 0.02%; heat shock was achieved by transfer to a pre-warmed flask in a water bath at 39°C. For Calcofluor white staining, 100 µl 37% formicdehyde solution (Sigma, F1635) was added to 900 µl of culture. After 10 minutes at room temperature and two washes in phosphate buffered saline (PBS), cells were mounted in the stain. Unless otherwise stated, cells were mounted in PBS containing Calcofluor to a final concentration of 0.02 mg ml$^{-1}$.

Actin localization

Actin localization was performed as described (Marks and Hyams, 1985) with the following modification: TRITC-phalloloid (R415 from Molecular Probes; stock 6.6 µM in methanol) was diluted to a final concentration of 0.33 µM by removing the methanol from 10 µl stock solution using a DNA Speed Vac (Savant) and re-suspending the precipitate in 200 µl PM buffer.

Indirect immunofluorescence

The rabbit anti-V5 antibody (Bethyl Laboratories) was used to localize Tea1.PkGFP following standard formaldehyde fixation protocol (Hagan and Asycough, 2000). Microtubule localization using TAT1 (Woods et al., 1989) (a gift from Keith Gull, University of Oxford, Oxford, UK) employed formaldehyde/gluteraldehyde fixation (Hagan and Asycough, 2000).

FITC-lectin cell-growth assay

The cell-wall lectin-staining growth assay was adapted from May and Mitchison (May and Mitchison, 1986). Cells were grown to a density of $4 \times 10^6$ cells ml$^{-1}$ in YES medium, treated with an equal volume of medium (with or without sorbitol), and immediately recovered by filtration and washed in fluorescent lectin (fluorescein-labelled soybean agglutinin from gymcone max, catalogue number FL-1011; Vector Laboratories) as follows: control cells were washed in 0.02 mg ml$^{-1}$ lectin in PBS washed in YES and resuspended to a density of $2 \times 10^5$ cells ml$^{-1}$ in YES. Osmotically shocked cells were washed in a 0.1 mg ml$^{-1}$ lectin in PBS/1.2 M sorbitol or PBS/0.6 M KCl as appropriate, before washing in YES/1.2 M sorbitol or YES/0.6 M KCl and suspended at a density of $2 \times 10^6$ cells ml$^{-1}$ in the appropriate YES stress medium. Cells were isolated by filtration for all wash steps. Cells were imaged without fixation as described for fixed cells.

Live-cell imaging

For all live-cell imaging, cells were grown in filter-sterilized EMM2-supplemented media and then mounted in an Iwaki EZView 24-well glass-bottomed culture dish (with lectin; Sigma L2380; 1.5 mg/ml) in 500 µl filter-sterilized EMM2. Cells were osmotically stressed by the addition of an equal volume (i.e. 500 µl) of pre-warmed 2.4 M sorbitol in filter-sterilized EMM2. The distribution and dynamics of microtubules and Wsh3 following osmotic stress was analyzed by live-cell imaging using a Delavision Spectris system. For analysis of microtubule dynamics at high temporal resolution, 0.3-µm slices were captured with a single passage through a set of 25 z-slices at 10-second intervals for 8 minutes. Microtubule and Wsh3 distribution and dynamics were studied at lower temporal resolution by capturing 0.3-µm slices with a single passage through a set of 25 z-slices at 3-minute intervals for 90 minutes. Each of the z-stacks was compressed to a maximal projection. All image analysis used Imaris (Bitplane) software.

We thank Agnes Grallert for generating and analyzing the data shown in Table 2 and supplementary material Movies 3 and 4, for advice, and for useful discussions; Steve Bagley for technical support in the Paterson Advanced Imaging Facility; Keith Gull for TAT1 antibody; and Janet Quinn, Nic Jones, Paul Nurse, Takashi Toda, Ursula Fleig and Mohan Balasubramanian for strains. This work was supported by Cancer Research UK (CRUK) grant number C147/A6058.

References


**Supplementary Table 1: Strains used in this study**

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