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Fission yeast mod5p regulates polarized growth through anchoring of tea1p at cell tips

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Microtubules have a central role in eukaryotic cell polarity[1], in part through interactions between microtubule end-binding proteins and the cell cortex[2,3]. In the fission yeast *Schizosaccharomyces pombe*, microtubules and the polarity modulator tea1p maintain cylindrical cell shape and strictly antipodal cell growth[4–7]. The tea1p protein is transported to cell tips by association with growing microtubule plus ends[8]; once at cell tips, tea1p releases from microtubule ends and associates with the cell cortex, where it coordinates polarized growth[4,6]. Here we describe a cortical protein, mod5p, that regulates the dynamic behaviour of tea1p. In *mod5Δ* cells, tea1p is efficiently transported on microtubules to cell tips but fails to anchor properly at the cortex and thus fails to accumulate to normal levels. mod5p contains a signal for carboxy-terminal prenylation and in wild-type cells is associated with the plasma membrane at cell tips. However, in *tea1Δ* cells, although mod5p remains localized to the plasma membrane, mod5p is no longer restricted to the cell tips. We propose that tea1p and mod5p act in a positive-feedback loop in the microtubule-mediated regulation of cell polarity.

From an insertional mutagenesis screen designed to identify non-essential genes regulating cell polarity in fission yeast (H.A.S., unpublished observations), we identified four genes whose loss-of-function phenotype resembles that of a *tea1Δ* strain, which forms bent or branched cells after a variety of stresses[4,5] (see Supplementary Information). One of these genes was *tea1*$^+$ itself (Fig. 1a), and two of the others were previously identified genes that are known to affect microtubule organization and consequently the normal delivery of tea1p to cell tips (*tea2*$^+$, *tip1*$^+$ (refs 9, 10)). The fourth gene, which we termed *mod5*$^+$ (for morphology defective 5), has not previously been characterized and is identified as open reading frame SPBC530.04 in the *S. pombe* genome (http://www.sanger.ac.uk/cgi-bin/yeastpub/pombe_chr_status). Deletion of the complete open reading frame of *mod5*$^+$ (*mod5Δ*) did not affect cell viability and yielded the same mutant phenotype as the original insertion mutant (Fig. 1b).

In immunofluorescence experiments with wild-type cells, tea1p was present both at cell tips and at the ends of microtubules (Fig. 1c,

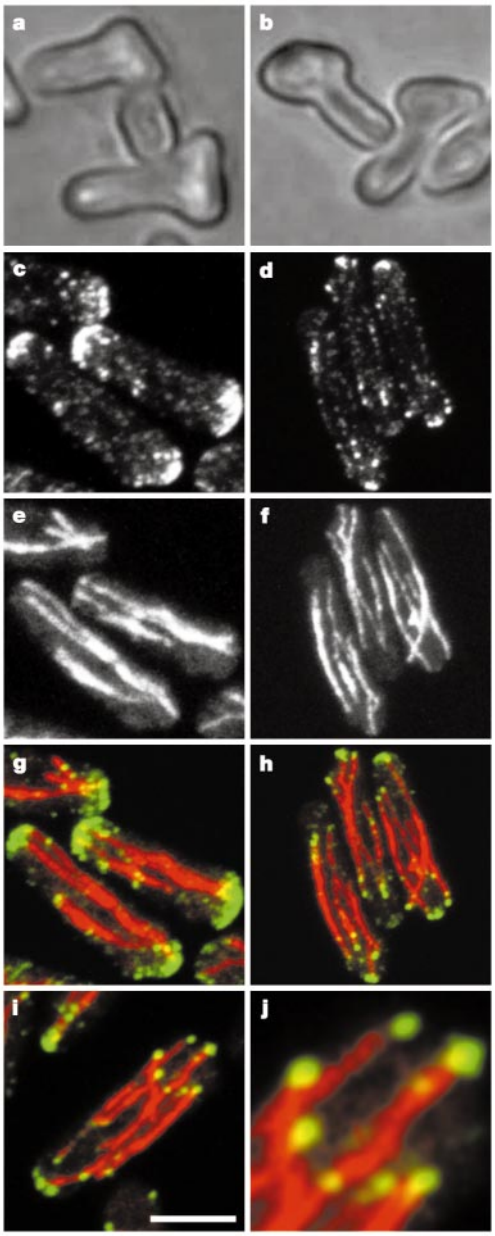

Figure 1 *mod5Δ* cells fail to localize tea1p at cell ends. **a**, **b**, Phenotype of *tea1Δ* cells (**a**) and *mod5Δ* cells (**b**) on solid medium 4 h after refeeding. Wild-type cells are uniformly cylindrical (see, for example, **c**) **c**, **d**, Anti-tea1p staining in wild-type cells (**c**) and *mod5Δ* cells (**d**). **e**, **f**, Anti-tubulin staining in wild-type cells (**e**) and *mod5Δ* cells (**f**). **g**, **h**, Merged images of **c** and **e**, and **d** and **f**, respectively. tea1p accumulates at cell tips in wild-type cells but is restricted to microtubule ends in *mod5Δ* cells. **i**, *mod5Δ* cell stained for tubulin and tea1p, showing tea1p on microtubule ends. **j**, Enlargement of the cell tip shown in **i**. Scale bar, 5 μm (**a**–**i**) and 1 μm (**j**).

e, g)[4]. Strikingly, in *mod5Δ* cells, although tea1p remained associated with microtubule ends, it no longer accumulated to high levels at cell tips (Fig. 1d, f, h–j; see also Supplementary Information), and 91% of tea1p spots observed at cell tips in *mod5Δ* cells were associated with a microtubule end ($n = 613$ spots). Immunoblotting experiments confirmed that tea1p levels are not altered in *mod5Δ* cells (data not shown). Microtubule organization was also generally similar between wild-type and *mod5Δ* cells (Fig. 1e, f), although a small percentage of *mod5Δ* cells contained a microtubule curling around the cell tip[4,8] (see Supplementary Information).

Because tea1p is normally targeted to cell ends by microtubules[4,8], we further investigated the role of mod5p in the cortical localization of tea1p by disrupting microtubules during polarity re-establishment experiments, in which cells are first grown to stationary phase and then diluted into fresh medium. This procedure increases the penetrance of polarity mutant phenotypes during the first cell cycle after dilution into fresh medium (ref. 9 and our unpublished observations) and was performed both in the presence and in the absence of the fungal microtubule inhibitor 2-methyl benzimidazolylcarbamate (MBC). Within 3 hours of being returned to fresh medium, wild-type cells not treated with the drug re-established a growth polarity axis, with tea1p uniformly decorating cell tips (Fig. 2a). By contrast, in *mod5Δ* cells not treated with drug, tea1p was found only at microtubule ends and did not accumulate at cell tips (Fig. 2b). It has been reported that the localization of tea1p at cell tips is strongly dependent on microtubules[4]. However, in wild-type cells treated with MBC during polarity re-establishment, at least one cell tip was able to accumulate significant levels of tea1p, despite the presence of only very short microtubule remnants (Fig. 2c). In contrast, *mod5Δ* cells exhibited nearly no detectable tea1p at cell tips after treatment with MBC (Fig. 2d; see Supplementary Information). We conclude that a major defect associated with the loss of mod5p is a failure to retain tea1p at cell tips, and that this is likely to be independent of the microtubule-based targeting of tea1p.

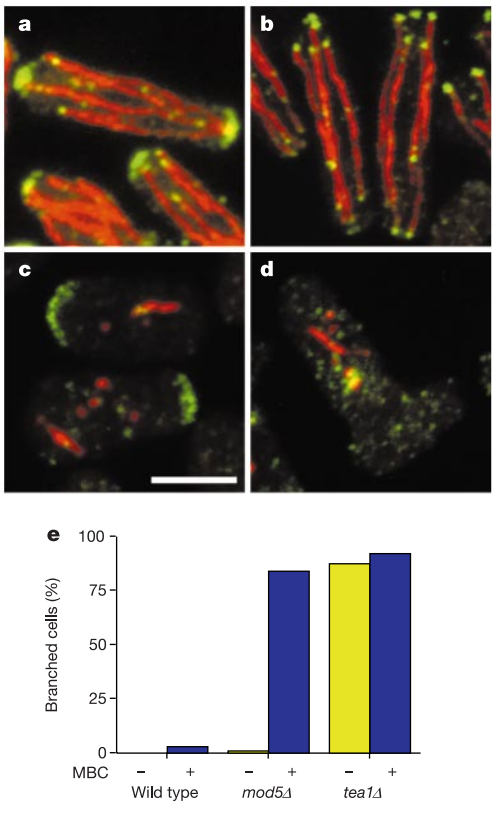

Figure 2 Microtubule-independent targeting of tea1p to the cortex depends on mod5p. Cells are stained for tea1p (green) and tubulin (red). **a**, **b**, Wild-type cells (**a**) and *mod5Δ* (**b**) cells 3 h after release to growth in the presence of dimethyl sulphoxide (control). **c**, **d**, Wild-type cells (**c**) and *mod5Δ* cells (**d**) 3 h after release to growth in the presence of 50 μg ml^{-1} MBC. Scale bar, 5 μm. **e**, Percentage of cells forming branches in wild-type, *mod5Δ* and *tea1Δ* cells 3 h after release to growth, in the absence and in the presence of MBC. The percentages shown in the first three columns are 0%, 3% and 1%, respectively.

These results also indicate that the targeting of tea1p to cell tips might be a two-step process in which microtubule-dependent delivery is followed by a microtubule-independent, but mod5p-dependent, cortical anchoring mechanism. We speculate that, in the absence of microtubules, diffusion of tea1p might allow its eventual cortical association, provided that mod5p is present. This interpretation is supported by the frequency of abnormal, branched cells observed in polarity re-establishment experiments (Fig. 2e). In *tea1Δ* cells a high frequency of cell branching was seen both in the absence and in the presence of MBC (88% and 92% of cells, respectively; $n = 200$ cells). In comparison, very few wild-type cells formed branches under either condition (0% and 3%, respectively). However, in *mod5Δ* cells the frequency of branched cells increased markedly in the presence of MBC, from 1% to 84%. We interpret these data to indicate, first, that the absence of tea1p from cell tips during polarity re-establishment leads to a high frequency of branching; second, that in *mod5Δ* cells not treated with the drug, the small amount of tea1p present at cell tips in association with microtubule ends might often be sufficient for normal polarized growth; and last, that in MBC-treated *mod5Δ* cells, the further loss of this small amount of microtubule-associated tea1p produces a phenocopy of *tea1Δ*.

To determine whether mod5p might have a role not only in the anchoring of tea1p at the cortex but also in the microtubule-based transport of tea1p, we examined live cells expressing green fluorescent protein (GFP)-tagged derivatives of tea1p and α-tubulin (atb2p, the non-essential α-tubulin in *S. pombe*[11–14] (K.E.S., unpublished observations)). In cells expressing GFP–tubulin at near-endogenous levels, no significant differences in microtubule dynamics were apparent between wild-type and *mod5Δ* cells (data not shown). The localization of tea1p–GFP in live wild-type and *mod5Δ* cells was similar to that of endogenous tea1p in fixed cells (Fig. 3a, b; see also Supplementary Information). Time-lapse videomicroscopy of wild-type cells revealed small particles of tea1p–GFP originating in the medial perinuclear region of the cell and translocating towards the cell tips at 2.43 ± 0.40 μm min^{-1} (mean ± s.d.; $n = 27$ particles; Fig. 3a), where they were then retained, as described recently[8]. In *mod5Δ* cells, tea1p–GFP particles moved from the nuclear periphery towards the cell tips at similar rates (2.69 ± 0.55 μm min^{-1}; $n = 27$ particles; Fig. 3b) but upon reaching cell tips either moved away from the cortex and/or disassembled, which is consistent with observations of fixed time-points (see Supplementary Information).

Three other differences in the properties of tea1p–GFP particles were also apparent in *mod5Δ* cells. First, *mod5Δ* cells contained 6.52 ± 0.98 tea1p–GFP particles per cell (mean ± s.d.; $n = 116$ cells), compared with 2.73 ± 1.24 in wild-type cells ($n = 131$ cells). In addition, tea1p–GFP particles in *mod5Δ* cells were 2–3-fold brighter than in wild-type cells (see Supplementary Information). Moreover, in many instances several tea1p–GFP particles seemed to move in a single stream towards the cell tips (Fig. 3b; see also Supplementary Information). The increased number and intensity of particles can be explained in part by the fact that *mod5Δ* mutants might contain a larger free cytoplasmic pool of tea1p–GFP because it is no longer sequestered at cell tips. In wild-type cells, tea1p–GFP particles translocating towards cell tips have been shown to be specifically associated with the plus ends of growing microtubule bundles[8]. The streaming behaviour of tea1p–GFP particles observed in *mod5Δ* cells indicated that several particles might be tracking

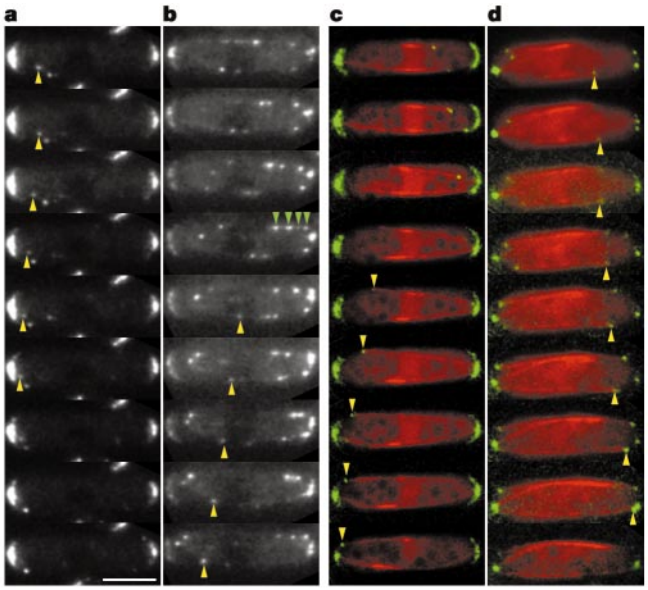

Figure 3 tea1p is transported on microtubule ends to cell tips in *mod5Δ* cells. **a**, **b**, Time lapse series of tea1p–GFP in wild-type cells (**a**) and *mod5Δ* cells (**b**). Images in **a** and **b** were taken at 10-s intervals. Yellow arrowheads indicate tea1p–GFP particles moving towards cell tips. In **b**, green arrows indicate tea1p–GFP spots moving in a stream. **c**, **d**, Double-labelled time-lapse series of tea1p-YFP (green) and CFP-atb2p (red) in wild-type cells (**c**) and *mod5Δ* cells (**d**). Images in **c** and **d** were taken at 15-s intervals. QuickTime movie files of this figure are provided in Supplementary Information. Scale bar, 5 μm.

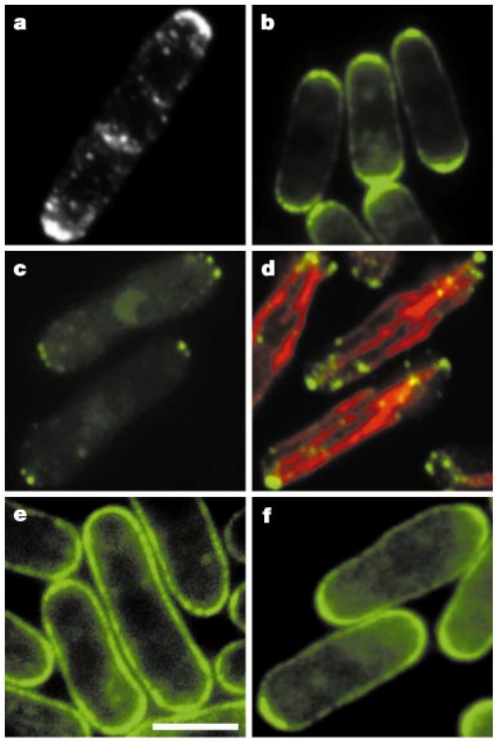

Figure 4 mod5p localization to cell tips depends on a functional *CaaX* sequence and on tea1p. **a**, Anti-mod5p immunofluorescence in wild-type cells. **b**, Localization of GFP–mod5p in wild-type cells (expressed from the *nmt81* promoter, replacing endogenous expression, about 3-fold higher than in the wild type). **c**, Localization of GFP–mod5ΔCaaXp (expressed from the *nmt41* promoter, replacing endogenous expression). **d**, Expression of mod5ΔCaaXp mimics the phenotype of *mod5Δ*. *mod5ΔCaaX* cells stained with antibodies against tea1p (green) and tubulin (red). **e**, Localization of GFP–mod5p in *tea1Δ* cells. **f**, Localization of GFP–mod5p in *tea3Δ* cells. Scale bar, 5 μm.

along the length of a single microtubule bundle rather than being uniquely associated with microtubule ends. However, by imaging tubulin tagged with cyan fluorescent protein (CFP) together with tea1p tagged with yellow fluorescent protein (YFP) we found tea1p–YFP particles present only at the ends of microtubules in both wild-type and *mod5Δ* cells (Fig. 3c, d; see also Supplementary Information). These results indicate that long microtubule bundles in *mod5Δ* cells might contain some shorter individual microtubules whose ends might not be detectable with current imaging methods. Alternatively, these microtubule ends might exist in wild-type cells as well, but might not normally be occupied by tea1p unless the free cytoplasmic pool is increased, as in *mod5Δ* cells. In any case, we do not find evidence for a fundamentally different mode of tea1p transport in *mod5Δ* cells.

To determine whether mod5p acts locally to retain tea1p at cell tips, we investigated its intracellular distribution. Immunostaining of wild-type cells with anti-mod5p antibodies showed mod5p at cell tips (Fig. 4a; see also Supplementary Information), and this was confirmed in live cells expressing amino-terminally tagged GFP–mod5p (Fig. 4b). GFP–mod5p localization to cell tips was partly affected by acute disruption of microtubules with MBC (see Supplementary Information) but was unaffected by disruption of actin filaments with latrunculin B (data not shown). The last four amino acids of the predicted mod5p-coding sequence are a consensus signal for C-terminal prenylation (CaaX, where C is cysteine, a is an aliphatic amino acid and X is any amino acid); mod5p is therefore predicted to be membrane associated[15]. We tested the functional significance of this motif both by deleting the last four amino acids of mod5p (mod5ΔCaaXp) and by mutating the putative prenylated cysteine residue to serine (mod5C519Sp); in both cases the normal localization of mod5p was disrupted (Fig. 4c; data not shown). Mod5ΔCaaXp appeared in a small number of spots throughout the cell, which were typically found near cell tips (Fig. 4c), and in the *mod5ΔCaaX* mutant the localization of tea1p was defective (Fig. 4d). These results indicate that the proper targeting of mod5p to the plasma membrane at cell tips might be essential for its function and that a normal microtubule distribution might contribute to its localization.

What *trans*-acting factors might be necessary for localizing mod5p at cell tips? We found that in *tea1Δ* cells, GFP–mod5p is no longer restricted to the cell tips but instead spreads out across the entire plasma membrane (Fig. 4e). tea1p is thought to be important for the cell-tip localization of several other effector proteins involved in polarized growth, including tip1p, tea2p, tea3p, pom1p and bud6p[9,10,16–18]. In *tip1Δ* cells, in which short microtubules lead to defects in tea1p targeting to cell tips[10], cortical GFP–mod5p was similarly mislocalized, but in *tea2-1* mutants, which have a similar microtubule defect, GFP–mod5p localization was nearly normal (see Supplementary Information), indicating that the role of tip1p in mod5p localization might extend beyond the regulation of microtubule dynamics. *tea3Δ* cells showed a slight spreading of GFP–mod5p away from cell tips (Fig. 4f; see also Supplementary Information), indicating that tea3p might also contribute to restricting mod5p to cell tips (see below). In *pom1Δ* and *bud6Δ* strains we observed normal GFP–mod5p localization (see Supplementary Information), indicating that they might not be directly involved in the tea1p-mediated regulation of mod5p localization.

Collectively, these results suggest a model of fission yeast cell polarity regulation involving a positive-feedback loop in which cortically localized mod5p at cell tips promotes the anchoring of microtubule-delivered tea1p. Correctly anchored tea1p then acts reciprocally to prevent mod5p from spreading out across the plasma membrane, and this spatial restriction of mod5p ultimately leads to

the proper subsequent anchoring of additional tea1p (see Supplementary Information for model). So far we have not been able to demonstrate a biochemical association between mod5p and tea1p, but mod5p is extracted from the cell cortex only under partly denaturing conditions (H.A.S., unpublished observations). In a two-hybrid screen we have identified an interaction between mod5p and tea3p (H.A.S., unpublished observations), which is related in structure to tea1p and interacts with tea1p by two-hybrid analysis, although this has not yet been confirmed biochemically[16]. This indicates that mod5p and tea1p might physically interact only indirectly, possibly linked by mutual interactions with tea3p. Recently it has been shown that the deletion of a coiled-coil region at the C terminus of tea1p prevents it from anchoring at the cell cortex[8]; thus, an interaction between mod5p and tea1p might occur through this region of tea1p.

We suggest that the tea1p–mod5p system provides spatially selective anchoring of tea1p at cell tips in the context of dynamic targeting. If tea1p were simply deposited at the cortex after associating with the plus ends of dynamic microtubules, the partly stochastic nature of the orientation of microtubule growth would leave open the possibility of mistargeting, and thus only low-fidelity positioning of tea1p. However, we would argue that this is held in check by the localization interdependence between tea1p and its cortical anchoring factor, mod5p. Interactions between plus-end-associated microtubule-binding proteins and cortical or plasma-membrane proteins, such as the association of EB1 family proteins with adenomatous polyposis coli (APC) protein (or Kar9p in budding yeast)[19–22], or of CLIP-170 family proteins with CLIP-associated proteins (CLASPs) and Ras GTPase-activating-protein-like protein (IQGAP1) (refs 23, 24), are thought to be important in a variety of microtubule-based eukaryotic cell behaviours, including mitotic spindle and/or nuclear positioning, and directed cell migration (see ref. 3 for references). It is thought that in most instances the purpose of these interactions is to regulate microtubule dynamics and/or the attachment of microtubules to subcellular structures[2,3,25,26]. In contrast, the primary role of cortical mod5p might be to increase the fidelity of the positioning of microtubule-targeted tea1p in the cell, thus ensuring that the correct identity of the polarized cell tip is continuously asserted. In conjunction with the selective stabilization of microtubule dynamic instability[27], spatially selective anchoring of microtubule-targeted proteins might be a more general principle regulating cell polarity. □

Methods

Yeast methods

S. pombe methods were as described[28]. Mutant cells were identified by starving cells on rich medium plates (YE5S) for 2 days, replica-plating them to fresh plates, and examining their cell shape after 4 h at 32 °C. Deletion of *mod5*$^+$ and GFP-tagging of mod5p and tea1p were performed by polymerase chain reaction (PCR)-based gene targeting with the *kanMX* selectable marker[29]; strains and primers used are listed in Supplementary Information. The C terminus of tea1p was tagged with GFP or YFP. Because of the CaaX box at the C terminus of mod5p, the N terminus of mod5p was tagged with GFP, and GFP–mod5p was expressed under the control of the weak *nmt81* promoter. Before analysis, expression of GFP–mod5p was induced for 2 days at 32 °C in medium lacking thiamine. The *nmt81* promoter drives the expression of GFP–mod5p to a concentration about threefold that of the endogenous protein (see Supplementary Information; additional data not shown). Deletion of the C-terminal *CaaX* box and mutation of C519S in *mod5* were achieved by PCR-based integration with a *ura4*$^+$ selectable marker[29], and confirmed by sequencing. Because steady-state expression concentrations of these mutant mod5p proteins from the endogenous *mod5*$^+$ promoter are lower than in wild-type cells, expression was restored to wild-type levels by replacing the endogenous promoter with the *nmt41* promoter (data not shown).

In polarity re-establishment experiments, strains were grown to stationary phase in YE5S liquid medium for 3 days at 25 °C before dilution 1:20 into fresh medium at 32 °C, in the presence or absence of 50 μg ml^{-1} MBC[9]. Cells were fixed with formaldehyde and branching was scored by light microscopy.

Antibodies

Anti-tea1p antibodies were raised in sheep against His_6-tea1p (ref. 4). Anti-tea1p antiserum gave only background staining in *tea1Δ* cells (see Supplementary Information), and was used at a dilution of 1:1,000. TAT1 anti-tubulin hybridoma supernatant[30] was a gift from K. Gull, University of Manchester, UK, and used at a dilution of 1:30. Anti-mod5p antibodies were raised in sheep against a GST fusion protein containing amino acids 28–426 of mod5p. The IgG fraction from anti-mod5p antiserum was purified on Protein G–agarose, and used at a dilution of 1:100. Alexa-conjugated fluorescent secondary antibodies (Molecular Probes) were used for immunofluorescence.

Microscopy

For immunostaining with anti-tea1p and anti-tubulin antibodies, cells were harvested by filtration and fixed in methanol at −80 °C for more than 10 min before being processed essentially as described[5]. Twelve Z-sections at 0.3-μm spacing were captured with a Leica TCS-SP confocal microscope and projected onto a single plane. Because neither mod5p nor GFP–mod5p could be preserved by methanol or aldehyde fixation methods (data not shown), for immunostaining with anti-mod5p antibodies, cells were harvested by filtration and fixed overnight in 10% trichloroacetic acid at 4 °C (ref. 16), then processed as above. Anti-mod5p immunofluorescence images were collected with a Nikon TE300 microscope equipped with Chroma filters, a PiFoc piezofocus device (Physik Instrumente), and a $CoolSnap_{HQ}$ camera (Roper Scientific), controlled by MetaMorph software (Universal Imaging). Seven Z-sections at 0.2-μm spacing were projected onto a single plane. Live-cell imaging of GFP, YFP or CFP was performed on the Nikon TE300 microscope. For time-lapse acquisition of tea1p–GFP, three Z-sections at 0.5-μm spacing were collected with 600-ms exposure time, at 10-s intervals, with appropriate neutral density filters. For time lapse acquisition of cells doubly labelled with tea1p–YFP and CFP–tubulin, two Z-sections at 0.6-μm spacing were collected with exposure times of 1,250 ms for YFP and 1,000 ms for CFP, at 15-s intervals, with neutral density filters. CFP–atb2p was expressed from a plasmid (pRL72 (ref. 18), a gift from F. Chang, Columbia University, USA) under control of the *nmt1* promoter induced for 20–24 h at 25 °C in 50 nM thiamine.

For measurements of the number of tea1p–GFP particles per cell, particles were counted from Z-stacks containing the entire cell volume; cell tips (terminal 10% of cell length) were not included.

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Sphingolipid signalling in *Arabidopsis* guard cells involves heterotrimeric G proteins

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In animals, the sphingolipid metabolite sphingosine-1-phosphate (S1P) functions as both an intracellular messenger and an extracellular ligand for G-protein-coupled receptors of the S1P receptor family, regulating diverse biological processes ranging from cell proliferation to apoptosis[1–3]. Recently, it was discovered in plants that S1P is a signalling molecule involved in abscisic acid (ABA) regulation of guard cell turgor[4]. Here we report that the enzyme responsible for S1P production, sphingosine kinase (SphK), is activated by ABA in *Arabidopsis thaliana*, and is involved in both ABA inhibition of stomatal opening and promotion of stomatal closure. Consistent with this observation, inhibition of SphK attenuates ABA regulation of guard cell inward K^+ channels and slow anion channels, which are involved in the regulation of stomatal pore size. Surprisingly, S1P regulates stomatal apertures and guard cell ion channel activities in wild-type plants, but not in knockout lines of the sole prototypical heterotrimeric G-protein α-subunit gene, *GPA1* (refs 5–8). Our results implicate heterotrimeric G proteins as downstream elements in the S1P signalling pathway that mediates ABA regulation of stomatal function, and suggest that the interplay between S1P and heterotrimeric G proteins represents an evolutionarily conserved signalling mechanism.

In higher plants, stomata form pores on leaf surfaces that regulate both uptake of CO_2 for photosynthesis and loss of water vapour during transpiration. The plant hormone ABA regulates stomatal pore size by mediating turgor changes in the guard cell pair surrounding the pore. These changes involve alterations in ion channel activities, which represent the final effectors of these processes[9–11]. Recently, S1P was shown to stimulate stomatal closure and Ca^{2+} mobilization in *Commelina communis* guard cells[4]. In addition, ABA promotion of stomatal closure in this species was partially attenuated by DL-*threo*-dihydrosphingosine (DL-*threo*-DHS)[4], a competitive inhibitor of mammalian SphKs[12]. Nevertheless, little is known about the cellular mechanisms that regulate S1P action in plant cells[13,14].

We used DL-*threo*-DHS and *N,N*-dimethylsphingosine (DMS), another potent inhibitor of mammalian SphKs[12], to investigate whether SphK might be involved in ABA-mediated changes in stomatal apertures in *A. thaliana*. DL-*threo*-DHS or DMS alone did not affect stomatal apertures (Fig. 1a, d). However, in epidermal peels that had been pre-treated with DL-*threo*-DHS or DMS, the effect of ABA on both stomatal opening and stomatal closure was significantly attenuated (Fig. 1a, d). Because inhibition of stomatal opening by ABA involves the inhibition of inwardly rectifying K^+ channels[10,11], we next assessed the effect of DMS on inward K^+ currents in *A. thaliana* guard cell protoplasts (GCPs)[6,15]. ABA inhibition of inward K^+ currents was abolished by pre-treating GCPs with DMS (Fig. 1b, c). Because ABA also promotes stomatal closure by activating slow anion channels[6,11,16], we next tested whether the effect of ABA on slow anion channels was altered by DMS. ABA activation of slow anion channel activity was partially inhibited by pre-treating GCPs with DMS (Fig. 1e, f). Together, these results suggest that ABA regulation of stomatal apertures and guard cell ion channel activities is transduced, at least in part, through SphK.

SphK activity has yet to be demonstrated in plants, although this enzyme has been characterized in animals[1] and yeast[17]. A related kinase activity able to phosphorylate D-*erythro*-dihydrosphingosine (D-*erythro*-DHS), which is structurally similar to D-*erythro*-sphingosine (Sph) except for the absence of the *trans*-4,5 double bond, has been reported in *Zea mays*[18]. A recombinant long-chain sphingoid base kinase (AtLCBK1) from *A. thaliana* was also shown to phosphorylate D-*erythro*-DHS[19]. Here, we show by direct assays of sphingoid base phosphorylation[20] that SphK activity is present in lysates prepared from leaves, mesophyll cell protoplasts (MCPs), or GCPs of *A. thaliana* (Fig. 2a). We found that Sph, a low abundance, naturally occurring sphingoid base isomer in *A. thaliana* leaves[21], was the best substrate among various exogenous sphingoid bases tested (Fig. 2b). Of the known inhibitors of mammalian SphKs, DL-*threo*-DHS was a substrate for *A. thaliana* SphK, whereas DMS was not (Fig. 2b). Furthermore, we found that DMS inhibited the activity of *A. thaliana* SphK in a concentration-dependent manner (Fig. 2c). Although a higher concentration of DMS was required for inhibition *in vitro*, such differences between the *in vitro* and *in vivo* concentrations are similar to those reported for other inhibitors used. For example, the phospholipase C (PLC) inhibitor U-73122 required an 80-fold higher concentration than was effective *in vivo* to inhibit guard cell PLC activity *in vitro*[22]. These results, together with those from Fig. 1, support the involvement of SphK in the regulation of guard cell responses to ABA.

We next tested the ability of ABA to modulate S1P levels *in vivo* by measuring the formation of [^{3}H]S1P from [^{3}H]Sph. Treatment of GCPs and MCPs with ABA resulted in a rapid and transient increase in [^{3}H]S1P formation, reaching a maximum after 2 min of ABA treatment (Fig. 2d, e). As expected, pre-treatment of MCPs with DMS blocked the ABA-induced increases in [^{3}H]S1P formation (data not shown). We next assayed SphK activity *in vitro* from protein extracts of MCPs that had been treated with ABA for various times *in vivo*. We found that stimulation of MCPs with ABA rapidly and transiently increased SphK activity (Fig. 2f), with a time course

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