Spatial landmarks regulate a Cdc42-dependent MAPK pathway to control differentiation and the response to positional compromise

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A fundamental problem in cell biology is to understand how spatial information is recognized and integrated into morphogenetic responses. Budding yeast undergoes differentiation to filamentous growth, which involves changes in cell polarity through mechanisms that remain obscure. Here we define a regulatory input where spatial landmarks (bud-site–selection proteins) regulate the MAPK pathway that controls filamentous growth (fMAPK pathway). The bud-site GTPase Rsr1p regulated the fMAPK pathway through Cdc24p, the guanine nucleotide exchange factor for the polarity establishment GTPase Cdc42p. Positional landmarks that direct Rsr1p to bud sites conditionally regulated the fMAPK pathway, corresponding to their roles in regulating bud-site selection. Therefore, cell differentiation is achieved in part by the reorganization of polarity at bud sites. In line with this conclusion, dynamic changes in budding pattern during filamentous growth induced corresponding changes in fMAPK activity. Intrinsic compromise of bud-site selection also impacted fMAPK activity. Therefore, a surveillance mechanism monitors spatial position in response to extrinsic and intrinsic stress and modulates the response through a differentiation MAPK pathway.

spatial cues | GTPase | polarity establishment | polar landmarks | MAPK

Positional information is critical for the establishment of polarity and the regulation of cell division. Spatial context is also important for many biological processes, including development, neuronal organization and guidance, directional motility, and cell differentiation. Positional information comes from proteins that mark the cell-surface and gradients of diffusible receptors, peptide ligands, and transcription factors (1–4). Evolutionarily conserved protein modules control cell polarity in eukaryotes (5, 6). In yeast, polarity is determined by cell type. Positional cues mark the poles of haploid and diploid cells, which are recognized by a core module composed of the bud-site GTPase Rsr1p (7), its guanine nucleotide exchange factor Bud5p (8, 9), and its GTPase activating protein Bud2p (10). Rsr1p in turn regulates the ubiquitous polarity establishment GTPase Cdc42p (11). Active (GTP-bound) Cdc42p associates with multiple effector proteins to initiate and maintain polarized growth at specific sites.

Cell polarity can be reorganized in response to extrinsic cues. Yeast cells can orient their axis of growth along pheromone gradients (12) and to the site of a wound (13). Cell polarity is also reorganized during filamentous/invasive/pseudohyphal growth, which occurs in response to nutrient limitation (glucose or nitrogen), and which results in the formation of branched chains of interconnected cells (14–16). Many fungal species undergo filamentous growth, and in some species of pathogenic microorganisms, filamentous growth is required for virulence (17). In yeast, the change in polarity during filamentous growth is striking in haploid cells, which switch from axial to distal-unipolar budding (15, 18). It is not clear how polarity is reorganized during filamentous growth, except that signal transduction pathways are involved and the same positional cues that regulate bud-site selection in diploid cells are also required for filamentous growth (19, 20).

Among the signaling pathways that regulate filamentous growth is an ERK-type MAPK pathway called the filamentous growth (fMAPK) pathway. MAPK pathways are evolutionarily conserved protein modules that regulate cell differentiation and stress responses in eukaryotes. The fMAPK pathway is regulated by the signaling mucin Msb2p (21), a cell-surface glycoprotein that is proteolytically processed and activated in glucose-limiting conditions (22, 23). At the plasma membrane (PM), Msb2p functions with transmembrane proteins Sho1p (21, 24, 25) and Opy2p (26–33). The transmembrane regulators connect (in some manner) to a cytosolic scaffold-type adaptor, Bem4p, that also regulates the fMAPK pathway (34–36). Msb2p and Bem4p associate with Cdc42p (21, 34) to promote its function in the fMAPK pathway (37, 38). Like many Rho GTPases (39–42), Cdc42p has multiple roles in regulating cell polarity and signaling. In the fMAPK pathway, Cdc42p regulates a protein kinase cascade composed of Ste20p (PAK), Ste11p (MAPKKK), Ste7p (MAPKK), and Kss1p (MAPK) (43, 44). Kss1p regulates a suite of transcription factors (45–47) that control the expression of target genes, whose products together with other proteins and pathways generate the filamentous cell type.

Here we report a new regulatory connection between bud-site–selection proteins and the fMAPK pathway. We show that the bud-site GTPase Rsr1p, together with positional landmarks, regulate the fMAPK pathway through the shared GTPase Cdc42p. This is a new role for bud-site–selection proteins in regulating MAPK signaling. Following-up on this discovery led to the identification of a surveillance mechanism, where positional cues provide information about spatial context to regulate the cellular response to extrinsic and intrinsic morphogenetic stress. In this way, cells monitor positional integrity before engaging in MAPK-dependent differentiation and other responses.

Significance

We identify a new role for bud-site–selection proteins outside of their established roles in regulating growth site determination, as components of a surveillance pathway that monitors spatial position during intrinsic and extrinsic morphogenetic stress and regulates a Cdc42p- and MAPK-dependent response.

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Results

Bud-Site GTPase Rsr1p Regulates the fMAPK Pathway. Rsr1p is an established regulator of bud-site selection that functions through Cdc42p (7, 10, 48). Rsr1p was also uncovered in a screen for Cdc42p-interacting proteins that impact fMAPK activity (34). To determine whether Rsr1p regulates the fMAPK pathway, the *rsr1*Δ mutant was examined in a strain background that undergoes filamentous/invasive growth (∑1278b) (14, 49), a fungal behavioral response that is controlled by the fMAPK pathway. In this background, the *rsr1*Δ mutant was defective for phosphorylation of the MAP kinase Kss1p (P~Kss1p) (Fig. 1*A*) and showed the same defect as a null mutant in the fMAPK pathway (*ste11*Δ). The *ste11*Δ, *ste12*Δ and *ste20*Δ mutants have equivalent phenotypes in filamentous growth assays and were used interchangeably. The *rsr1*Δ mutant was defective for invasive growth (Fig. 1*B*) by the plate-washing assay (15). The *rsr1*Δ mutant was also defective for expression of transcriptional targets of the fMAPK pathway, including *FRE-lacZ* (Fig. 1*C* and *SI Appendix*, Fig. S1*A*) (44), *FLO11* (see below), and *FUS1-HIS3* (*SI Appendix*, Fig. S1*B*), which in ∑1278b cells lacking an intact mating pathway (*ste4*) shows dependency on fMAPK regulators (21, 34).

The fMAPK pathway also regulates the response to defects in protein glycosylation (28, 50, 51). To determine whether Rsr1p is involved in this aspect of fMAPK regulation, a conditional glycosylation mutant, *pmi40-101*, whose glycosylation defect is suppressed by mannose (50), was examined. Rsr1p was required for fMAPK activity in the *pmi40-101* mutant experiencing glycosylation deficiency (Fig. 1*D* and *SI Appendix*, Fig. S1*C*). In this blot and other blots, total Kss1p levels can vary as a result of positive feedback, because *KSS1* is a transcriptional target of the fMAPK pathway (52). Together, the results show that Rsr1p positively regulates the fMAPK pathway.

Rsr1p Controls the fMAPK Pathway by Nucleotide Cycling and Interaction with the Guanine Nucleotide Exchange Factor Cdc24p. Rsr1p regulates bud-site selection through the Cdc42p module (7). To determine whether Rsr1p regulates the fMAPK pathway through Cdc42p, the amount of active Cdc42p (Cdc42p-GTP) in the cell was increased by disrupting *RGA1*, which encodes the main GTPase activating protein for Cdc42p in the fMAPK pathway (53, 54). The *rga1*Δ *rsr1*Δ double-mutant bypassed the fMAPK signaling defect of the *rsr1*Δ single-mutant (Fig. 2*A*), which indicates that Rsr1p functions at or above the level of Cdc42p in the fMAPK pathway. The *rga1*Δ mutant did not rescue the bud-site–selection defect of the *rsr1*Δ mutant (*SI Appendix*, Table S3). Thus, bypass occurs by raising Cdc42p-GTP levels, not restoring the bud-site–selection defect of *rsr1*Δ.

Rsr1p is a Ras-type GTPase that cycles between active (GTP-bound) and inactive (GDP-bound) conformations. In the GTP-bound conformation, Rsr1p interacts with effector proteins. A version of Rsr1p that fails to interact with effectors (T35A) (55–57) or versions that mimic the GDP- (K16N) or GTP-locked states (G12V) were defective for fMAPK activity (Fig. 2*B*). A GTP-locked version of Rsr1p might be expected to constitutively activate the fMAPK pathway. However, cells containing $Rsr1p^{G12V}$ have a bud-site–selection defect (*SI Appendix*, Table S3) that results from sequestering Cdc24p in the inactive state (7) and from its failure to concentrate at polarized sites (56). Thus, as for many GTPases, nucleotide cycling of Rsr1p is necessary for its function in the fMAPK pathway. The G12V, K16N, and T35A versions of Rsr1p have a dominant-negative phenotype, which induces a bud-site–selection defect in wild-type cells (55). The G12V, K16N, and T35A versions of Rsr1p also caused a defect in fMAPK activity in wild-type cells (Fig. 2*C*).

During bud-site selection, Rsr1p recruits Cdc24p to the PM (56, 58, 59). A version of Cdc24p that is constitutively anchored to the PM by myristoylation (Myr-Cdc24p) (34) bypassed the fMAPK signaling defect of the *rsr1*Δ mutant (Fig. 2*D*). Myr-Cdc24p also bypassed the signaling defect of the *bud4*Δ mutant (*SI Appendix*, Fig. S1*D*). Together, these results indicate that one function for bud-site–selection proteins in fMAPK regulation is PM recruitment of Cdc24p. Rsr1p also interacts with Cdc24p at bud sites (7, 58, 60, 61). To determine whether Rsr1p regulates the fMAPK pathway through interaction with Cdc24p, a version of Cdc24p was examined that at permissive temperatures cannot interact with Rsr1p (*cdc24-4* or G168D) (58). Cells harboring the *cdc24-4* allele showed reduced fMAPK pathway activity (Fig. 2*D*) [p*cdc24-4* (30 °C)]. Thus, Rsr1p interacts with and recruits Cdc24p to the PM to regulate the fMAPK pathway.

All of the versions of Rsr1p tested that were defective for bud-site selection were defective for fMAPK activity, which may indicate that bud-site selection itself is tied to fMAPK regulation. To test this possibility, a version of Rsr1p was examined that lacked the polybasic domain, which mediates homotypic interactions (56, 57, 62),

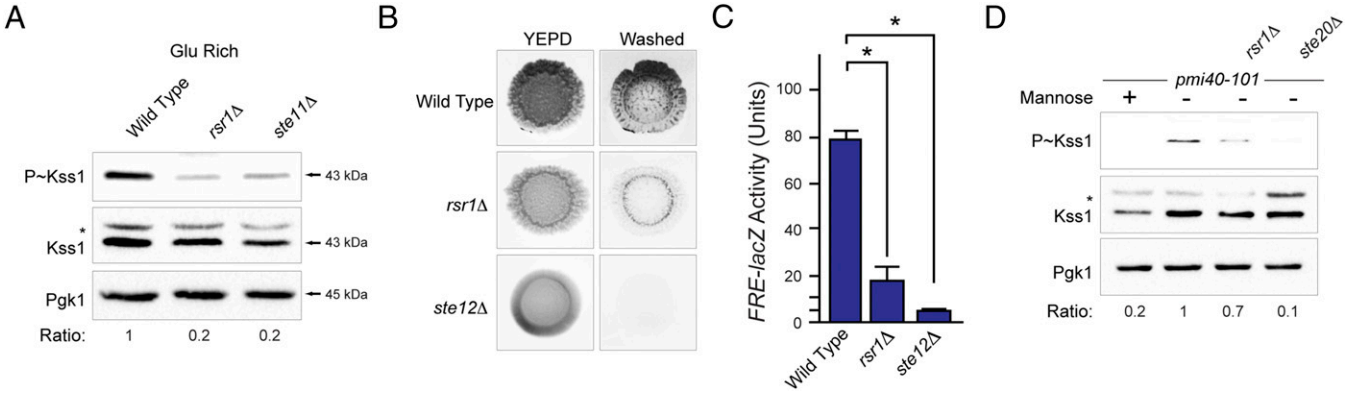

Fig. 1. Rsr1p regulates the fMAPK pathway. (*A*) Immunoblot analysis of P~Kss1p levels in wild-type cells and the *rsr1*Δ and *ste11*Δ mutants. Cells were grown to midlog phase in SD+AA (glucose-rich media). Cell extracts were examined by immnoblot analysis using p42/p44 antibodies (to detect P~Kss1p), Kss1p antibodies, and Pgk1p antibodies as a control for protein levels. Numbers indicate relative band intensity for P~Kss1p to total Kss1p (Ratio). Asterisk refers to a background band (102). (*B*) Plate-washing assay. Cells were grown for 96 h on YEPD medium. The plate was photographed, washed, and photographed again. (*C*) Expression of the *FRE-lacZ* reporter. Cells were grown to midlog phase in SD-URA medium to maintain selection for the plasmid and evaluated by β-galactosidase assays. β-Galactosidase assays were performed from independent cultures and are expressed in Miller Units. Error bars show differences between samples. *$P < 0.05$. (*D*) Strains harboring the *pmi40-101* mutation alone and with the indicated deletions were grown to midlog phase in YEPD or YEPD + 50 mM mannose. P~Kss1p levels were examined as in *A*.

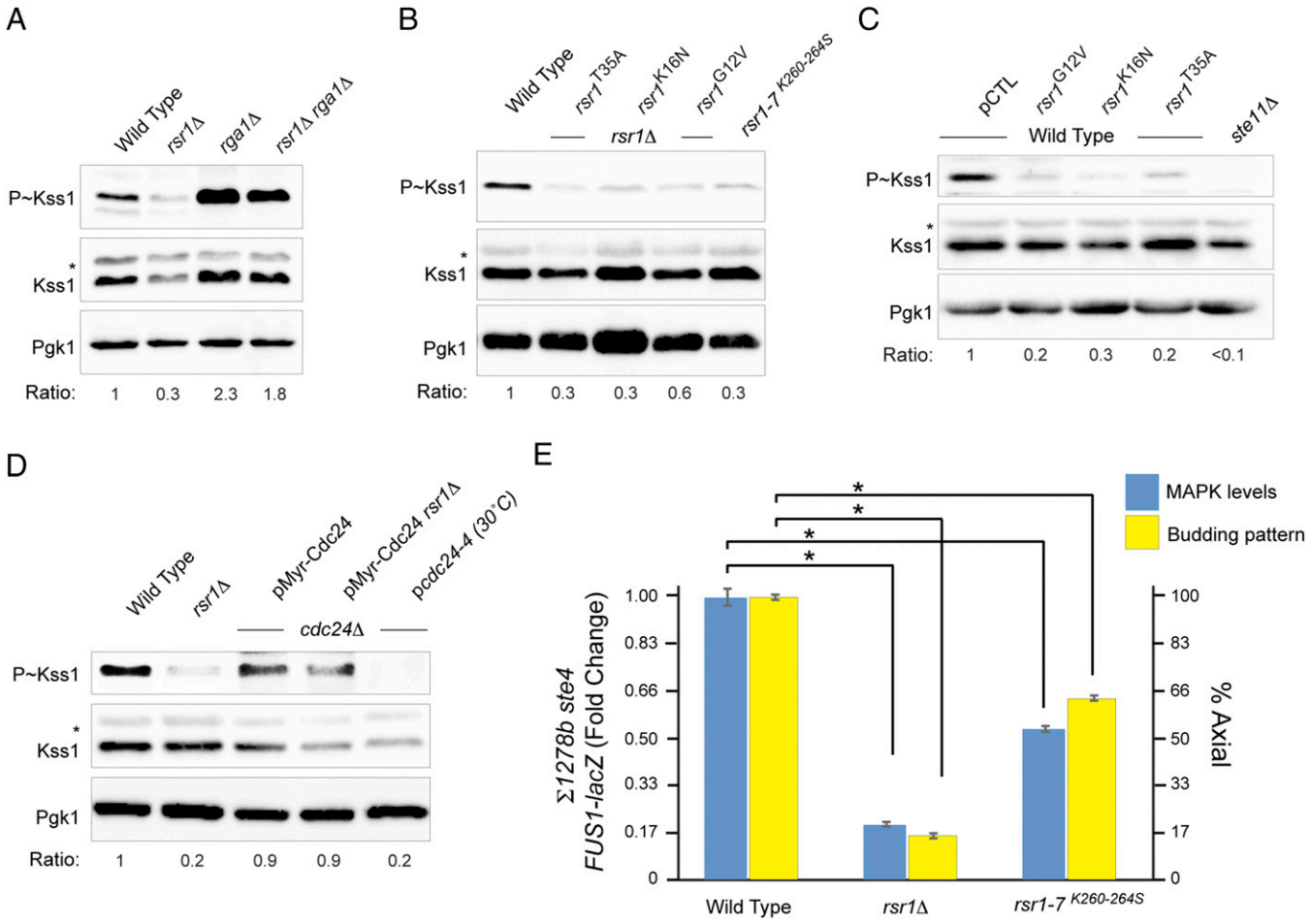

Fig. 2. Rsr1p regulates the fMAPK pathway through GTPase cycling and interaction with Cdc24p. (*A*) P~Kss1p levels were examined as in Fig. 1*A* for the strains indicated. Cells were grown to midlog phase in SD+AA. (*B*) P~Kss1p levels were examined as in Fig. 1*A*, except that cells were grown in SD-URA or SD-LEU media to maintain selection for plasmids harboring alleles of *RSR1*. (*C*) P~Kss1p levels were examined as in Fig. 1*A* in wild-type cells harboring the indicated *RSR1* alleles. Cells were grown to midlog phase in YEPD media. (*D*) P~Kss1p levels were examined as in Fig. 1*A* in the *cdc24::NAT* and *cdc24::NAT rsr1*Δ strains carrying YEp351-*Cdc24p-GFP* (wild-type), pRS425-*CDC24-4* (*cdc24-4*), and YEp351-*MYR-Cdc24p-GFP* (Myr-Cdc24p) plasmids. (*E*, *Left* axis) β-Galactosidase assays were performed as described in Fig. 1*C*. Wild-type values were set to 1. Other values were adjusted accordingly. The experiment was performed in triplicate. Error bars show the SD between trials. (*Right* axis) Axial budding expressed as a percentage was determined for wild-type cells, *rsr1*Δ, and *rsr1-7*$^{K260\text{-}264S}$ mutants grown to midlog phase in SD-URA medium. Budding pattern was determined by CFW staining. More than 200 cells were counted in independent trials. Error bars show the SD between trials. *$P < 0.01$.

and that was defective for fMAPK activity (Fig. 2*B*) (*rsr1-7*$^{K260\text{-}264S}$). The *rsr1-7* mutant has a conditional bud-site–selection defect (62). In our hands, the defect was less severe than reported (62), which might result from differences in growth conditions or strain backgrounds. The partial bud-site–selection defect of the *rsr1-7*$^{K260\text{-}264S}$ mutant (Fig. 2*E*, yellow bars) showed a corresponding defect in fMAPK activity (Fig. 2*E*, blue bars). These results, and results presented below show a correspondence between bud-site selection and fMAPK activity.

Axial Cues Regulate the fMAPK Pathway in Glucose-Rich Conditions. During bud-site selection, Rsr1p is recruited by positional landmarks to bud sites (11). We asked whether positional landmarks also regulate the fMAPK pathway. In glucose-rich conditions (2% glucose), haploids bud in an axial pattern (Fig. 3*A*) (63). A mutant lacking axial cues showed a defect in fMAPK activity in glucose-rich conditions (Fig. 3*B*, *axl2*Δ, blue bars), based on the expression of the fMAPK pathway target *FLO11* (64). The *axl2*Δ mutant showed the same defect as the core module (Fig. 3*B*, *rsr1*Δ, blue bars) and the same genetic suppression pattern as the *rsr1*Δ *rga1*Δ double-mutant (Fig. 3*C*; compare with Fig. 2*A* and *SI Appendix*, Table S3). Like *axl2*Δ, other axial mutants were also defective for fMAPK activity, based on P~Kss1p analysis and *FUS1* reporter activity (*SI Appendix*, Fig. S2).

Two additional experiments support the idea that axial cues regulate the fMAPK pathway. First, restoring axial budding to axial mutants by loss of multigenerational cortical marks (Rax proteins) (65–67) restored MAPK signaling (*SI Appendix*, Fig. S3 *A–D*, and Table S4). The Rax proteins might impact fMAPK through multiple mechanisms, as these proteins localize to the division site as well as the distal pole (65–67). Rax proteins have not been extensively studied in haploid cells. Consistent with their roles in regulating distal-pole budding in diploid cells, Rax1p and Rax2p regulated invasive growth (*SI Appendix*, Fig. S3*E*) and distal-pole budding of filamentous haploid cells (*SI Appendix*, Fig. S3*F*).

A second experiment supporting a role for axial cues in regulating fMAPK comes from analysis of separate functional domains on the Axl2p protein. In addition to its role in regulating bud-site selection, Axl2p also interacts with Cdc42p and plays a role in regulating septin organization. This role for Axl2p was uncovered by its ability to suppress the septin organization defects of an allele

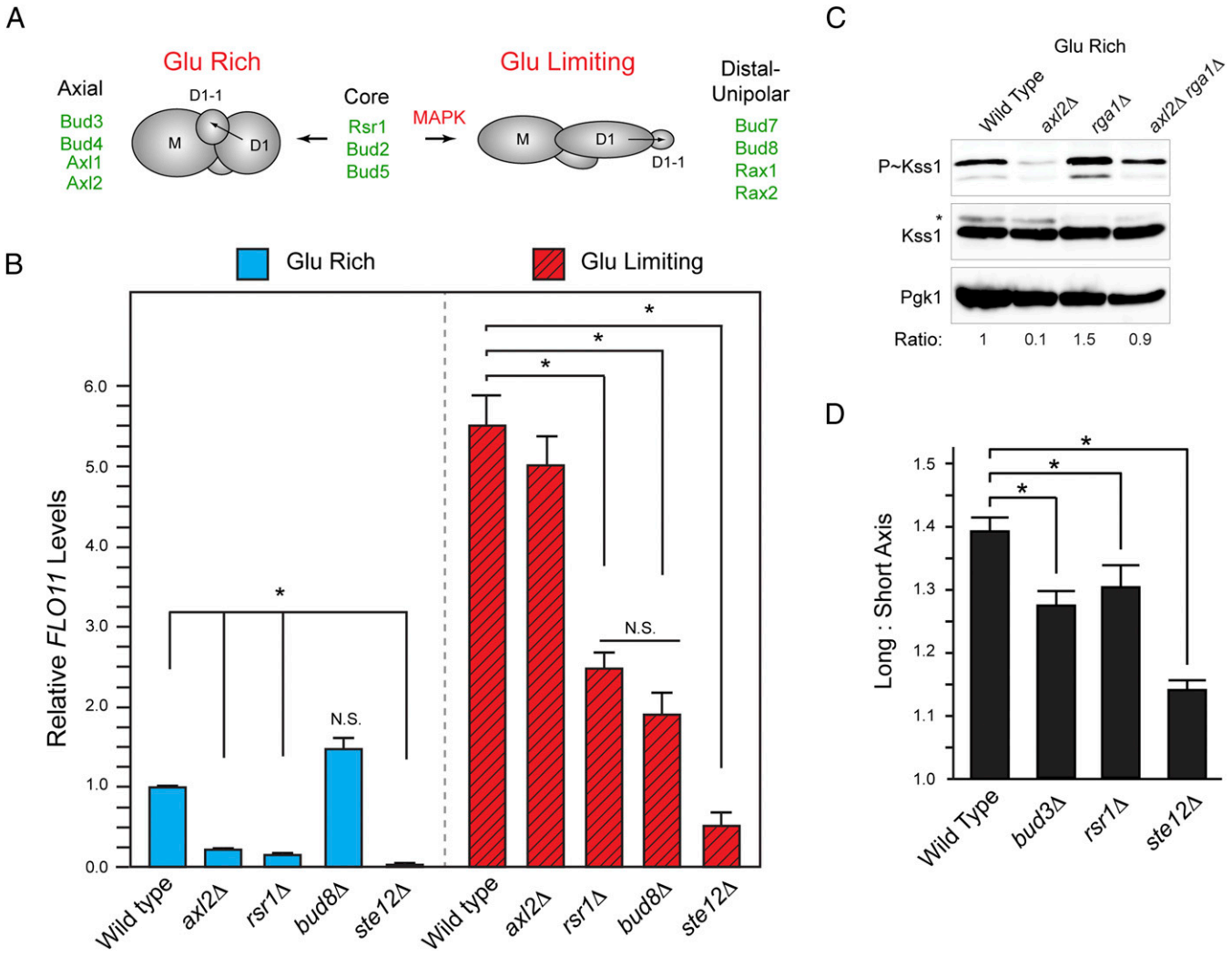

Fig. 3. Bud-site–selection proteins conditionally regulate the fMAPK pathway depending on glucose availability. (*A*) Budding pattern of haploid cells in glucose-rich and glucose-limiting conditions (Glu, glucose). Proteins required for axial budding, distal-unipolar budding, and the core module are shown. (*B*) Quantitative PCR (qPCR) analysis of *FLO11* expression (relative to *ACT1* levels) in the indicated mutants in glucose-rich (YEPD) and glucose-limiting (YEP-Gal) conditions. Assays were performed from independent cultures. Average values are shown. Error bars show the SD between trials. *$P < 0.01$. (*C*) P~Kss1p levels were examined as in Fig. 1*A* for the indicated strains. Cells were grown to midlog phase in YEPD medium. NS, not significant. (*D*) Ratio of the long-to-short axis in the indicated mutants. More than 50 cells were counted for each mutant. Cells were incubated in glucose-limiting media (YEP + 0.2% glucose). *$P < 0.001$.

of *CDC42* called *cdc42*^{V36G} (68). A version of Axl2p that is specifically defective for septin organization functioned in the fMAPK pathway (*SI Appendix*, Fig. S4 *A* and *B*) (p1-544, 641–725) (68, 69). By comparison, a version of Axl2p that is specifically defective for axial budding did not (*SI Appendix*, Fig. S4 *A* and *B*) (p1-544, 641–685). This version of Axl2p (p1-544, 641–685) lacks an interaction site for Bud4p but retains the ability to localize to the mother-bud neck (68). Therefore, the bud-site–selection function of Axl2p underlies its role in regulating the fMAPK pathway. These results reinforce the idea that axial cues regulate the fMAPK pathway.

Why do axial cues regulate fMAPK in an environment where cells do not normally undergo filamentous growth (Fig. 3*A*, Glu Rich)? Basal activity of the fMAPK pathway in glucose-rich conditions prepares cells for invasive growth (70). Specifically, at high and moderate glucose levels, cells express *FLO11*, which promotes adhesion during biofilm/mat formation (71) and contributes to the initiation of filamentous growth. As glucose levels decrease, cells become elongated through a mechanism that involves the polarisome (20) and a delay in the cell cycle (72, 73), although cells continue to bud axially (70). Axial cues (*bud3*Δ) and the core module (*rsr1*Δ) were required for cell elongation (Fig. 3*D*), which occurs in an fMAPK-dependent manner during filamentous growth (Fig. 3*D*) (*ste12*Δ) (72, 74). Axial cues were also required for the fMAPK response to protein glycosylation deficiency (*SI Appendix*, Fig. S4*C*). Therefore, axial cues regulate basal fMAPK activity in glucose-rich conditions to prepare cells for invasive growth and contribute to the diversity of MAPK-dependent responses, like the response to protein glycosylation deficiency.

Bud8p Regulates the fMAPK Pathway In Glucose-Limiting Conditions. Glucose depletion triggers invasive growth (18) and activates the fMAPK pathway (Fig. 3*B*, compare blue bar to red bar for wild-type) (31). In glucose-limiting conditions, haploid cells switch from axial to distal-unipolar budding by utilization of the distal-pole marker Bud8p (Fig. 3*A*) (20). Bud8p was required for *FLO11* expression in glucose-limiting conditions (Fig. 3*B*, compare wild-type to *bud8*Δ, red bars). Bud8p was also required for P~Kss1p activity (*SI Appendix*, Fig. S4*D*). Therefore, Bud8p regulates the fMAPK pathway in glucose-limiting conditions.

In glucose-rich conditions, Bud8p is not required for budding in haploid cells (63). Under this condition, Bud8p did not regulate the fMAPK pathway (Fig. 3*B*, *bud8*Δ, blue bars, and *SI Appendix*, Fig. S2). Similarly, axial cues, which do not regulate distal-pole budding under nutrient-limiting conditions (63), did not regulate *FLO11* expression in glucose-limiting conditions (Fig. 3*B*, *axl2*Δ, red bars). The core module is required for bud-site selection under all conditions (63) and regulated fMAPK signaling under all conditions tested (Fig. 3*B*, *rsr1*Δ, blue and red bars). Therefore, different positional landmarks regulate the fMAPK pathway in different nutrient states corresponding to their roles in regulating bud-site selection.

A Specific Input from the Rsr1p Branch Regulates the fMAPK Pathway. Msb2p and other proteins regulate Cdc42p in the fMAPK pathway (see Fig. 7, discussed below) (21, 22, 34). To define how inputs from the Msb2p and Rsr1p branches impact the fMAPK pathway, the *msb2*Δ and *rsr1*Δ single-mutants were compared with an *msb2*Δ *rsr1*Δ double-mutant and MAPK-null mutant (*ste11*Δ). In glucose-rich conditions, Msb2p and Rsr1p both regulated the fMAPK pathway (Fig. 4*A* and *SI Appendix*, Fig. S5*A*). In glucose-limiting conditions, *rsr1*Δ played a more minor role (Fig. 4*B*). This observation supports the data presented in Fig. 3*B*, which shows that axial and core mutants have a fivefold decrease in MAPK activity in glucose-rich conditions [Fig. 3*B*, blue bars, compare wild-type to *rsr1*Δ and *axl2*Δ (although the pathway is activated to a lower overall level)], compared with a ∼1.8-fold decrease seen in distal and core mutants under glucose-limiting conditions (Fig. 3*B*, red bars, compare wild-type to *rsr1*Δ and *bud8*Δ). Thus, bud-site–selection proteins play quantitatively different roles in regulating the fMAPK pathway under different conditions.

Msb2p is activated in glucose-limiting conditions by proteolytic processing (23), which may partially obviate the requirement for the Rsr1p branch. In support of this possibility, a hyperactive allele of *MSB2*, *MSB2*$^{\Delta 100-818}$ (21), bypassed the fMAPK signaling defect of the *rsr1*Δ mutant (Fig. 4*C* and *SI Appendix*, Fig. S5*B*). Similarly, a hyperactive version of Sho1p, Sho1p^{P120L} (22) also bypassed the fMAPK signaling defect of the *rsr1*Δ mutant (*SI Appendix*, Fig. S5*C*). Hyperactive versions of Msb2p and Sho1p also bypassed the signaling (*SI Appendix*, Fig. S5*D*) and invasive growth (*SI Appendix*, Fig. S5*E*) defects of the *axl2*Δ mutant. Therefore, the Rsr1p branch can be bypassed by activation of the Msb2p branch.

Like other signaling pathways, the fMAPK pathway shares components with other MAPK pathways, including the mating and high osmolarity glycerol response (HOG) pathways (75–78). Despite using common components, each MAPK pathway induces a specific response (52). In the mating pathway, Rsr1p does not regulate MAPK signaling but contributes to cell polarization [e.g., the formation of cells with mating projections or shmoos (79)]. Rsr1p and Gβγ-Far1p-Cdc24p have a redundant function in cell polarization during mating (79–81), and Rsr1p becomes essential for shmoo formation in cells with defective Gβγ-mediated chemotropism (80). We also found that Rsr1p did not regulate the mating pathway, based on sensitivity of cells to the mating pheromone α factor (Fig. 4*D*) and P∼MAPK analysis (*SI Appendix*, Fig. S6*A*). To determine whether Rsr1p regulates the HOG pathway, the *RSR1* gene was disrupted in cells lacking the redundant Sln1p branch (*ssk1*Δ) (82). The *rsr1*Δ *ssk1*Δ double-mutant showed

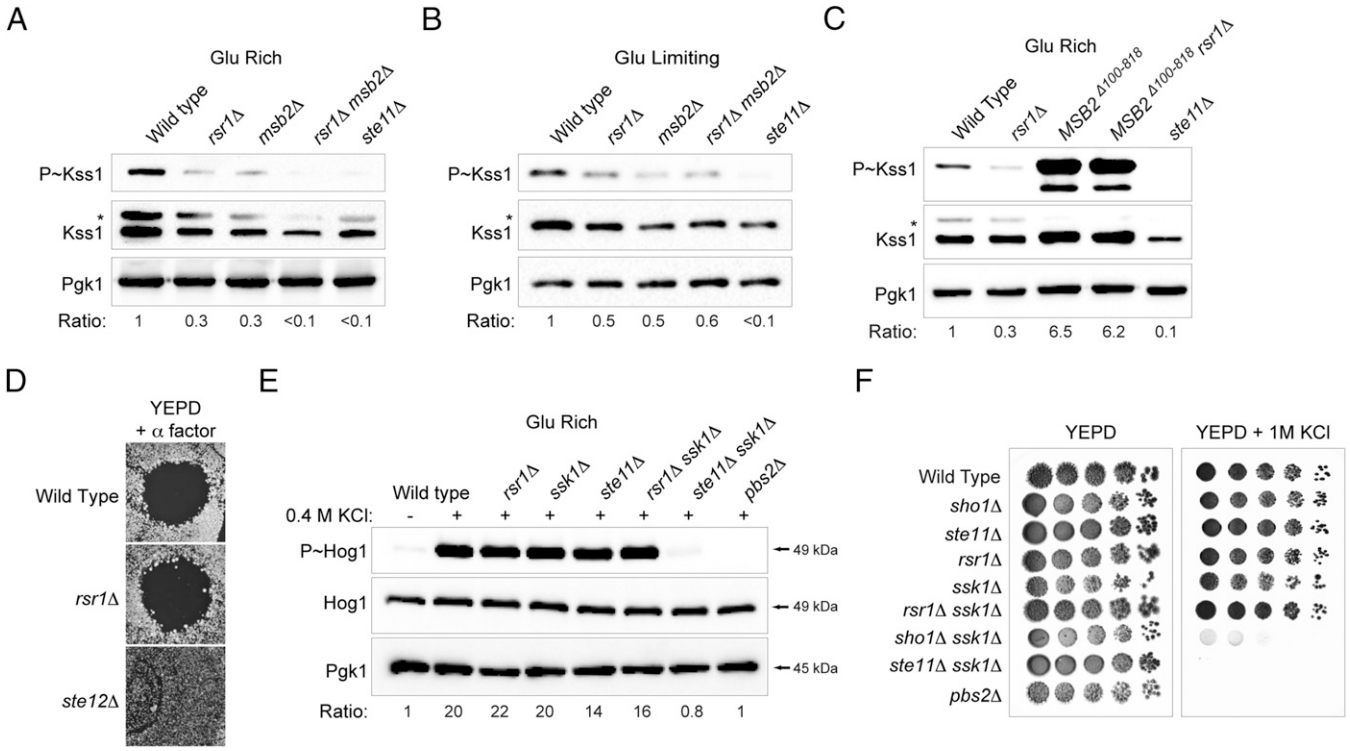

Fig. 4. Roles of the Rsr1p and Msb2p branches in regulating the fMAPK pathway and other MAPK pathways that share components. (*A*) P∼Kss1p levels were examined as in Fig. 1*A* for the strains indicated. Cells were grown in SD+AA medium to midlog phase. (*B*) P∼Kss1p levels were examined as in Fig. 1*A* for the strains indicated. Cells were induced in YEP-GAL medium to midlog phase. (*C*) P∼Kss1p analysis of Msb2$^{\Delta 100-818}$ signaling in wild-type cells and the *rsr1*Δ mutant. P∼Kss1p levels were examined as in Fig. 1*A*. Cells were grown in SD+AA medium to midlog phase. (*D*) Halo assays. Approximately equal concentrations of the indicated strains were spread onto YEPD media. Next, 5 μm of α-factor was spotted onto plates, which were incubated for 48 h at 30 °C. The experiment was performed four times and a typical example is shown. (*E*) P∼Hog1p levels in the indicated mutants. Cells were grown to midlog phase in YEPD. KCl was added to a final concentration of 0.4 M for 10 min. Cell extracts were evaluated by immunoblot analysis using antibodies that recognize P∼p38, Hog1p, and Pgk1p proteins. Ratio refers to P∼Hog1p to Hog1p levels. (*F*) Serial dilutions were spotted onto YEPD and YEPD + 1 M KCl for 3 d at 30 °C.

normal phosphorylation of the MAPK Hog1p in response to salt (P~Hog1p) (Fig. 4*E*, compare *rsr1*Δ *ssk1*Δ to *ste11*Δ *ssk1*Δ and *pbs2*Δ, which lacks the HOG MAPKK). Moreover, the *rsr1*Δ *ssk1*Δ mutant did not show a growth defect in high-salt medium (Fig. 4*F*). Thus, Rsr1p does not regulate the HOG pathway. In fact, basal cross-talk to the fMAPK pathway that occurs in the *pbs2*Δ mutant (24, 83), which requires fMAPK components, was also dependent on Rsr1p (*SI Appendix*, Fig. S6*B*). We also tested whether general defects in cell polarity might influence fMAPK activity. Mutants defective for polarized growth [e.g., polarisome mutants *bud6*Δ, *pea2*Δ, *spa2*Δ and *bni1*Δ (84)] or that exhibit hyperpolarized growth [*hsl1*Δ and *hsl7*Δ (85)] did not impact fMAPK (*SI Appendix*, Fig. S6*C*). Therefore, bud-site–selection proteins play a specific role in regulating the fMAPK pathway.

Yeast Cells Dynamically Orient Their Growth Site Based on Glucose Levels, Which Has a Corresponding Impact on fMAPK Activity. The fact that bud-site–selection proteins regulate the fMAPK pathway indicates that spatial/positional information itself may control aspects of the differentiation response. Except for the observation that prolonged nutrient starvation leads to loss of axial budding in haploids (63), the dynamics of nutrient-dependent changes in polarity in haploid cells has not been explored. To define the rate of polarity reorganization during filamentous growth, a two-fluorescent staining technique was used (86). Midlog phase cells shifted from glucose-rich (YEPD) to glucose-limiting [YEP-Galactose (Gal)] conditions switched from axial (>99%) to distal-unipolar budding (~50%) after 2.5 h (Fig. 5*A*, red square, and *SI Appendix*, Fig. S7). Cells shifted from YEP-Gal back to YEPD reverted to axial budding in a similar timespan (Fig. 5*A*, green triangle). Cells shifted from YEPD to YEPD in a mock experiment remained axial (Fig. 5*A*, yellow circle). Examples of the two-fluorescent staining technique are shown in Fig. 5*B*. Given that doubling time is ~2.5 h, these results indicate that yeast cells survey glucose availability and orient their axis of growth within a growth cycle.

Time-lapse microscopy showed that cells transferred from YEPD to YEP-GAL switched from axial to distal-unipolar budding in a single cycle (Movies S1 and S2). Most haploid cells bud distally in glucose-limited media, as evident by the single-cell invasive growth assay (Fig. 5*C*, *Upper*) (18). Filamentous cells

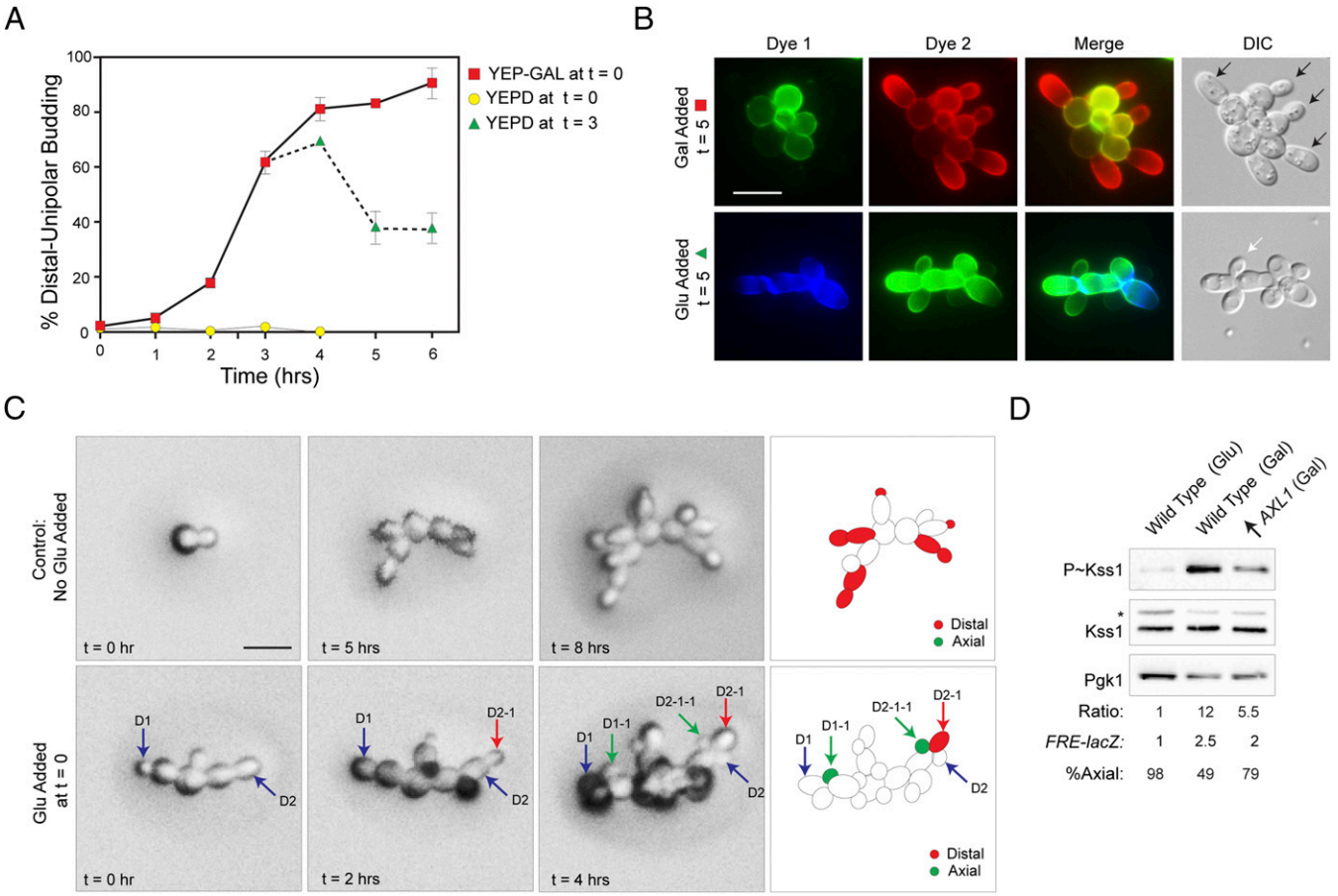

Fig. 5. Extrinsic changes in spatial position regulate changes in fMAPK activity. (*A*) Distal-unipolar budding (%) was determined in cells grown in different conditions. At time 0, midlog phase cells in YEPD were harvested, washed, and transferred to YEPD (yellow circle) or YEP-Gal (red square) medium. At the indicated times, cell aliquots were evaluated for distal-unipolar buds by two-fluorescent staining. For the switchback experiment, cells grown in YEP-Gal for 3 h were transferred to YEPD medium and evaluated for distal-pole budding (green triangle). At least 50 cells were counted for each experiment. Error bars show SD between separate trials. (*B*) Examples of FITC-ConA/TRITC-ConA double-labeling (*Upper*) and CFW/FITC-ConA double-labeling (*Lower*) of cells from the 5-h time point. Distal buds are marked with black arrows. The axial bud in the lower panel is marked with a white arrow. (Scale bar, 5 μm.) (*C*, *Upper*) Example of the budding pattern of filamentous cells by the single cell assay. (Scale bar, 10 μm). (*Lower*) Time course of budding pattern of filamentous cells exposed to glucose. Cell D1 budded at the proximal pole, D1-1. Cell D2 budded at the distal pole (D2-1). D2-1 budded at the proximal pole D2-1-1. Diagram at right illustrates the budding pattern. Red, distal-unipolar; green, axial. Pattern was confirmed by serial images taken in the plane of the *z* axis. (*D*) P~Kss1p levels were examined as in Fig. 1*A* for the strains indicated. Wild-type cells were grown in YEPD (Glu) or YEP-Gal (Gal). ↑*AXL1* refers to p*Gal-AXL1*. Cells were also evaluated for *FRE-lacZ* activity. β-Galactosidase assays were performed as described in Fig. 1*C*. Differences in *FRE-lacZ* activity are expressed as a ratio (wild-type set to 1), and differences between trials were <10%. More than 200 cells were counted for each strain to determine percent axial.

exposed to glucose switched to axial budding (Fig. 5*C*, *Lower*, D1 produced D1-1, and *SI Appendix*, Fig. S8). Not all cells switched (Fig. 5*C*, *Lower*, D2 produced D2-1, and *SI Appendix*, Fig. S8), but produced daughters that budded axially in the following cycle (Fig. 5*C*, D2-1 produced D2-1-1). Large cells typically retained the distal pattern, which may indicate that commitment to bud distally occurs at a specific point in the cell cycle. The switch in polarity is not a result of changes in the marks themselves, which are present under all conditions (*SI Appendix*, Fig. S9), but may be controlled by Axl1p, a cell-type specific protein that is required for axial budding (87, 88) whose levels are regulated by glucose availability (20).

Because budding pattern impacts fMAPK activity (Figs. 1–4), the switch in polarity in response to glucose availability may have a corresponding impact on MAPK signaling. In support of this possibility, cells forced to bud axially in glucose-limiting conditions (by overexpressing *AXL1*) (Fig. 5*D*, % Axial) showed reduced P~Kss1p levels (Fig. 5*D*) and *FRE-lacZ* activity (Fig. 5*D*, *FRE-lacZ*). These results suggest the possibility that the switch to axial budding by filamentous cells encountering a glucose-rich environment provides a mechanism for attenuation of the fMAPK pathway. Cell polarity reorganization can therefore dictate MAPK activity in response to an extrinsic cue.

Intrinsic Compromise of Bud-Site Selection Impacts fMAPK Activity. The above data indicate that bud-site–selection proteins may monitor the spatial position of the cell and regulate a MAPK-dependent response. In addition to extrinsic cues like glucose, budding pattern is tied to intrinsic processes such as transcription, cell cycle progression, cytoskeletal organization, phosphatidylinositol phosphate signaling, cytokinesis, and protein trafficking (89, 90). The above findings predict that mutants in these processes that confer bud-site–selection defects would show reduced fMAPK activity. This prediction is based on the correspondence between budding pattern and fMAPK activity in cells compromised for Rsr1p, Bud2p, Bud3p, Bud4p, Bud5p, Bud7p, Bud8p, Axl1p, Axl2p, and Rax function (Figs. 1–4 and *SI Appendix*, Figs. S2 and S3). To further test this possibility, a mutant was examined in which bud-site selection was compromised, in a process not otherwise connected to fMAPK regulation. At permissive temperatures, the septin mutant (*cdc12-6*) displays normal cytokinesis but has a bud-site–selection defect (Fig. 6*A*, % Axial), which may result from mis-localization of axial cues at the mother-bud neck (91). The bud-site–selection defect of the *cdc12*-6 mutant corresponded to a defect in fMAPK activity (Fig. 6*A*). Defects in phosphatidylinositol phosphate signaling, which also compromises bud-site selection, likewise compromised fMAPK signaling, which can account for a previous result from our laboratory (92). Therefore, intrinsic compromise of bud-site selection attenuates fMAPK activity.

What if the regulatory input by bud-site–selection proteins is ignored? To test this possibility, bypass of the signaling defect of bud-site–selection mutants was examined. Cells expressing $MSB2^{\Delta 100–818}$, which signals independent of the Rsr1p branch (Fig. 4*C* and *SI Appendix*, Fig. S5*B*), had irregular morphologies, including growth at multiple sites (Fig. 6*B*). This phenotype does not occur in wild-type cells because of singularity in budding (93, 94) but has been reported in cells expressing activated versions of Cdc42p (94, 95). Such cells showed localization of GFP-Bud8p at multiple sites (Fig. 6*C*) and had multiple septin rings (Fig. 6*C*), indicative of multiple mother-bud necks. Cells with elevated fMAPK activity, like $MSB2^{\Delta 100–818}$ or cells that overexpress the *MSB2* gene (*GAL-MSB2*), which also bypasses *rsr1*Δ, showed growth defects over multiple passages (Fig. 6*D*), which indicates that this growth pattern is not optimal for viability. Therefore, bypassing the regulatory input of the Rsr1p branch leads to growth and polarity defects.

Discussion

Bud-site–selection proteins are among the most intensively studied positional landmarks in eukaryotes, and the molecular basis for how they function is well understood (11). Bud-site–selection proteins

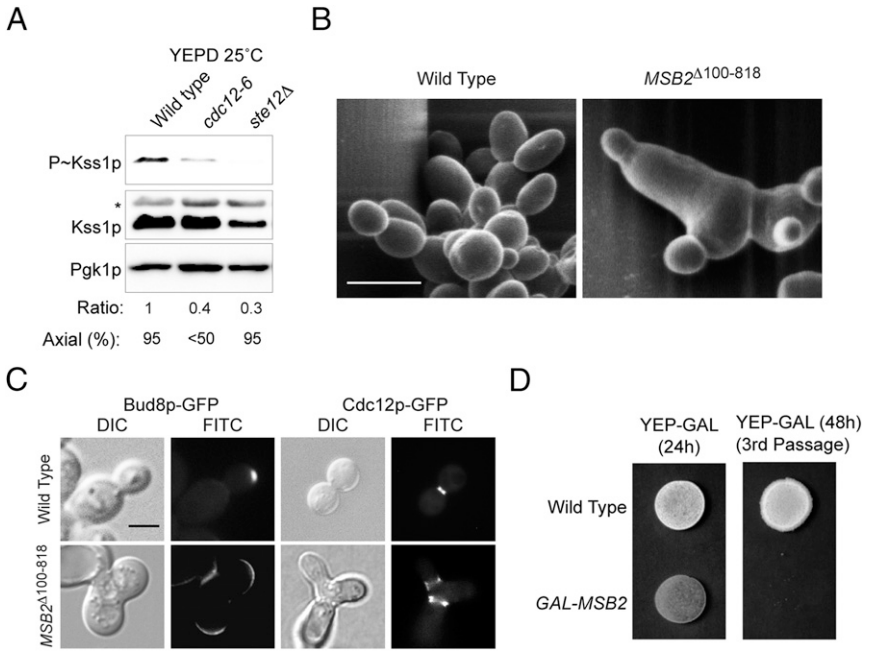

Fig. 6. Intrinsic compromise of spatial position impacts fMAPK activity. (*A*) P~Kss1p levels were examined as in Fig. 1*A* for the strains indicated. Budding pattern was determined by bud-scar staining and visual inspection. More than 200 cells were counted for the experiment. (*B*) Scanning electron micrographs of wild-type cells (*Left*) and cells with an activated fMAPK pathway (*Right*). (Scale bar, 5 μm.) (*C*) Localization of GFP-Bud8p and Cdc12p-GFP (103) in cells expressing $MSB2^{\Delta 100–818}$. (Scale bar, 5 μm.) (*D*) Growth defect of the indicated yeast strains spotted onto YEP-GAL for the times shown overexpressing *MSB2* over multiple passages.

are not known to function outside the budding pathway. Here we define a new role for bud-site–selection proteins as regulators of an ERK-type MAPK pathway. To our knowledge, this is the first connection between positional landmarks and MAPK regulation in yeast and may extend broadly to other systems. This connection does not arise from a moonlighting function of a particular bud-site–selection protein but involves the entire bud-site–selection machinery. We further identify a surveillance mechanism that allows cells to sense spatial information and control a Cdc42p- and MAPK-dependent response.

Spatial Cues Regulate the fMAPK Pathway. The discovery that bud-site–selection proteins regulate the fMAPK pathway builds on the understanding of how the fMAPK is regulated. Two regulatory branches converge on the Cdc42p module to regulate the fMAPK pathway: the Msb2p branch, which also contains Sho1p, Opy2p and Bem4p (21, 24, 26–34), and the Rsr1p branch, whose activity is governed by positional landmarks (Fig. 7).

A potentially trivial explanation is that bud-site–selection proteins elevate the level of Cdc42p-GTP in the fMAPK pathway. Indeed, Rsr1p interacts with Cdc24p and functions at the level of Cdc42p. Two observations, however, indicate that Rsr1p plays a specific role in fMAPK regulation. One is that Rsr1p exhibits a nonadditive input to the fMAPK pathway (Fig. 4*A*). Thus, the Rsr1p and Msb2p branches cooperate to transmit a signal to fMAPK. The second observation is that Rsr1p regulates the fMAPK pathway but not other MAPK pathways that share components (Fig. 4 *D–F* and *SI Appendix*, Fig. S6*A*). Bud-site–selection proteins may selectively regulate Cdc42p in the fMAPK pathway through Bem4p. Bem4p and Rsr1p interact, based on a two-hybrid screen for cell polarity regulators (Fig. 7, dashed line between Rsr1p and Bem4p) (96), and both proteins bind to Cdc24p. Rsr1p interacts with the CH domain of Cdc24p, which has an auto-inhibitory function (58), and Bem4p binds to the autoregulatory PH-like domain of Cdc24p (34). Thus, Bem4p and Rsr1p may cooperatively regulate Cdc42p by binding separate autoregulatory domains in Cdc24p. Alternatively, Rsr1p may initiate Cdc24p activation at bud sites early in the cell cycle that is sustained by Bem4p. The salient finding from this study is that spatial information is integrated into the fMAPK pathway through a shared GTPase module.

Bud-Site–Selection Proteins as Coincidence Detectors of Nutrient Status. We also show that positional landmarks conditionally regulate the fMAPK pathway in a manner that corresponds to their nutrient-dependent functions in bud-site selection. Glucose levels feed into the fMAPK pathway in two ways (Fig. 7). One is by changes in the glycosylation of Msb2p that occur under nutrient-limiting conditions, resulting in elevated processing of under-glycosylated Msb2p and fMAPK pathway activation (23). This mechanism involves the unfolded protein response. The other is by differential recognition of positional landmarks in different nutrient states. Axial position is an indicator of nutrient surplus, whereas distal-pole budding is an indicator of starvation.

At the distal pole, signaling and position-dependent budding are coordinated. Sho1p interacts with Bud8p (Fig. 7, dashed line between Bud8p and Sho1p) (22), and the proteins localize to the distal pole (70, 97). The fMAPK pathway and other pathways regulate Bud8p-dependent bud-site selection (20). The interaction between signaling and polarity proteins at the distal pole may cluster signaling machinery at the site where polarity reorganization occurs. It is plausible that other bud-site–selection proteins make specific contacts with fMAPK regulators to modulate the signaling response. Such interactions may contribute to a pathway-specific response.

Regulation of Spatial Integrity by a Surveillance Pathway. We show that a surveillance mechanism monitors spatial position and executes a MAPK-dependent response to extrinsic and intrinsic cues. As discussed above, extrinsic changes in glucose levels lead to quantitatively

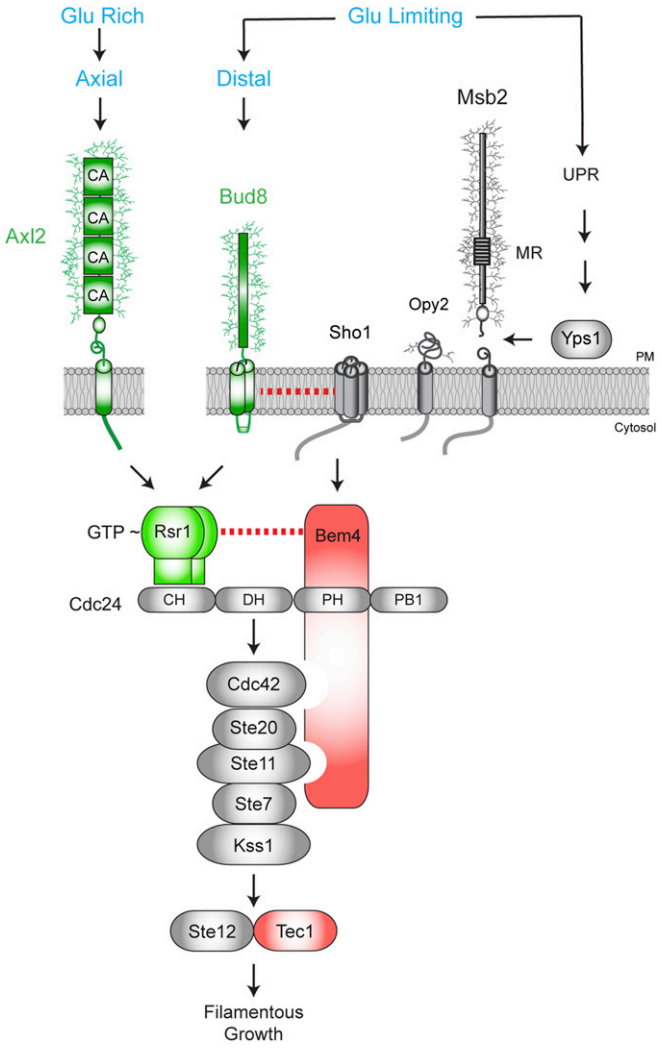

Fig. 7. Model of the fMAPK pathway with inputs from bud-site–selection proteins. In glucose-rich conditions, axial cues (Bud3p, Bud4p, Axl1p, and Axl2p; Axl2p is shown) regulate Cdc24p in the filamentous growth pathway through Rsr1p. Msb2p, Sho1p, Opy2p, and Bem4p also regulate the fMAPK pathway through Cdc42p. In glucose-limiting conditions, Bud8p regulates Rsr1p-dependent activation of the filamentous growth pathway. Glucose limitation also induces processing of Msb2p by Yps1p, which leads to Cdc24p activation through Sho1p and Bem4p. Msb2p, Bud8p, and Axl2p are glycosylated proteins; CA, cadherin-like repeats; MR, mucin repeats; UPR, unfolded protein response.

different signaling from axial and distal cues. We also show that intrinsic problems with bud-site selection, such as in mutants where bud-site selection is compromised, lead to attenuation of the fMAPK pathway. In this way, cells compromised for spatial integrity dampen MAPK signaling until the cell gets its bearings. The regulatory mechanism defined here may extend to other systems. Positional marks have been identified in filamentous fungal species (98) and have been shown to influence cell polarity and virulence in human (99, 100) and plant fungal pathogens (101). It may be interesting to define how such cues impact regulatory pathways to control pathogenic differentiation programs. Spatial cues may generally impact signaling pathways to remodel cell fate and mount responses to compromised positional integrity.

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