Oscillatory recruitment of signaling proteins to cell tips promotes coordinated behavior during cell fusion

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Cell-cell communication is essential for coordinating physiological responses in multicellular organisms and is required for various developmental processes, including cell migration, differentiation, and fusion. To facilitate communication, functional differences are usually required between interacting cells, which can be established either genetically or developmentally. However, genetically identical cells in the same developmental state are also capable of communicating, but must avoid self-stimulation. We hypothesized that such cells must alternate their physiological state between signal sending and receiving to allow recognition and behavioral changes. To test this hypothesis, we studied cell communication in the filamentous fungus *Neurospora crassa*, a simple and experimentally amenable model system. In *N. crassa*, germinating asexual spores (germlings) of identical genotype chemotropically sense others in close proximity, show attraction-mediated directed growth, and ultimately undergo cell fusion. Here, we report that two proteins required for cell fusion, a MAP kinase (MAK-2) and a protein of unknown molecular function (SO), exhibit rapid oscillatory recruitment to the plasma membranes of interacting germlings undergoing chemotropic interactions via directed growth. Using an inhibitable MAK-2 variant, we show that MAK-2 kinase activity is required both for chemotropic interactions and for oscillation of MAK-2 and SO to opposing cell tips. Thus, *N. crassa* germlings undergoing chemotropic interactions rapidly alternate between two different physiological states, associated with signal delivery and response. Such spatiotemporal coordination of signaling allows genetically identical and developmentally equivalent cells to avoid self-stimulation and to coordinate their behavior to achieve the beneficial physiological outcome of cell fusion.

chemotropism | MAPK | *Neurospora*

Communication between cells is essential for the development and survival of multicellular organisms, as well as populations of unicellular microbes. Cell-cell signaling is often based on functional differences between communicating partners, such that one cell sends a signal, while another cell perceives the signal and reacts with an appropriate cellular response. The identity as signal sender or receiver can be established either genetically or developmentally. For example, during muscle development in *Drosophila*, founder cells attract and fuse with fusion-competent myoblasts (1), which are genetically the same, but in different developmental lineages. Alternatively, the unicellular yeast, *Saccharomyces cerevisiae*, uses gene conversion and silencing to create genetic differences at the mating type locus, which results in differential production of proteins that specify cell type and are required for mating cell fusion (2). Communication also occurs between cells that are both genetically identical and in developmentally equivalent states. Macrophages communicate through diffusible growth factors and cytokines to undergo cell fusions that are necessary for the formation of multinucleate osteoclasts and giant cells (3). A likely prerequisite for macrophage fusion is their mutual recognition as “self,” which may be promoted by clonal expansion before fusion (4).

In microbes, individual cells can also synchronize their behavior to perform tasks that are not possible for individuals. For example, the social amoebae *Dictyostelium discoideum* uses waves of cAMP secretion to relay information about population density, resulting in chemotaxis along a concentration gradient (5). The human fungal pathogen, *Candida albicans* uses quorum-sensing molecules such as farnesol and tyrosol to regulate morphological transitions and biofilm development (6, 7). In filamentous ascomycete fungi, diffusible compounds of unknown structure mediate chemotropic interactions between germinating asexual spores (germlings), which result in cell fusions that aid in colony establishment (8–10).

In the filamentous fungus *Neurospora crassa*, chemotropic interactions between germlings are associated with the formation of specialized hyphae called conidial anastomosis tubes (CATs) (Fig. 1 *A* and *B*) (11). During colony establishment, CAT interactions and resulting cell fusions between germlings are thought to allow the young colony to act as an interconnected coordinated individual (9). *N. crassa* has a large number of genetic, molecular, and cell biology tools available (12) and is thus an excellent model to explore mechanisms of signaling between genetically identical and physiologically equivalent cells. Specifically, we hypothesized that germlings must alternate between signal sending and signal receiving to avoid responding to their own outgoing signals (self-stimulation) and thus be able to undergo productive chemotropic interactions.

A large number of mutants have been identified in *N. crassa* that either impair or impede germling fusion (9, 10). In particular, fusion between clonal *N. crassa* germlings requires mitogen-activated protein kinase (MAPK) signaling (11, 13, 14). In animals, plants and microbes, MAPK cascades can be activated in response to a variety of extracellular signals, resulting in cellular changes in transcription, translation, cell cycle progression, cytoskeleton organization, and morphogenesis (15). Specificity of MAPK signaling requires a highly coordinated spatiotemporal interplay between upstream activators and downstream targets. Although MAP kinase cascades are among the best-understood signal transduction pathways, many questions remain concerning their cellular dynamics, in particular, how their subcellular localization modulates signaling specificity and intensity (16).

MAPK modules generally consist of three MAP kinases. The MAPK cascade required for germling fusion in *N. crassa* consists of a MAPK, MAK-2, a MAPK kinase, STE-7, and a MAPK kinase kinase, NRC-1 (13). These three kinases are homologous to those in the pheromone response pathway of *S. cerevisiae*, Fus3p, Ste7p, and Ste11p, respectively. In *S. cerevisiae*, mating requires interac-

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Fig. 1. MAK-2 localizes to CAT tips involved in chemotrophic interactions. (A) Wild-type *N. crassa* conidial germling pairs of identical genotype undergoing chemotrophic attraction. (B) Attachment and fusion of the tips (arrow) shown in panel A (20 min later). (Scale bar, 10 μ m.) (C) Localization of MAK-2-GFP to the CAT tip (arrow) in a germling (*his-3::Pccg1 mak-2-gfp Δmak-2*) undergoing chemotrophic attraction. (Scale bar, 2 μ m.) (D) Western blot showing full length MAK-2-GFP protein ($\approx$ 67 kDa) produced in the *his-3::mak-2-gfp; Δmak-2* (AF-P611) and *his-3::Pccg1 mak-2-gfp; Δmak-2* (AF-M512) strains. Strain A9 (*his-3::Pccg1 gfp*) shows the approximately 26-kDa GFP protein. (E) Complementation of macroscopic phenotype of the Δ *mak-2* mutant with *Pccg1 mak-2-gfp* allele.

tions between genetically differentiated cells of opposite mating type, α and α , which communicate via mating-type specific peptide pheromones and receptors (2). In *N. crassa*, pheromones and pheromone receptors are required for mating, but not for chemotrophic interactions or cell fusion between germlings (17, 18).

Mutations in a gene of unknown function, called soft (*so*), also result in strains unable to undergo chemotrophic interactions and vegetative cell fusion, although mating cell fusion is unaffected (19). The *so* gene is conserved in the genomes of filamentous ascomycete fungi, but absent from unicellular fungi such as *S. cerevisiae* and *Schizosaccharomyces pombe*. Here we report the dynamic oscillation of MAK-2 and SO to CAT tips during chemotrophic growth and fusion of *N. crassa* germlings. The recruitment of MAK-2 and SO to CAT tips switches between fusion partners in a highly coordinated manner. These observations indicate that fusion partners alternate between two physiological states, involving signal sending and receiving. This exquisite signaling system allows genetically and developmentally identical cells to coordinate their behavior temporally and spatially to achieve mutual attraction and fusion, while avoiding self-stimulation.

Results

The MAP Kinase MAK-2 Shows Oscillatory Recruitment to Fusion Tips.

Mutations in the MAPK gene *mak-2* result in strains that are blind; *mak-2* mutants fail to undergo chemotrophic interactions and vegetative cell fusion, even when interacting with wild-type cells (11, 13). To determine the spatiotemporal localization dynamics of MAK-2 during chemotrophic interactions, we performed live-cell imaging on genetically identical germlings con-

Fig. 2. SO and MAK-2 oscillate to opposing CAT tips during chemotrophic interactions. (A) Time course of MAK-2-GFP localization in *his-3::Pccg1 mak-2-gfp Δmak-2* germling pairs. Graphic representation of MAK-2-GFP localization to CAT tips of two cells undergoing chemotrophic attraction. T1 = CAT tip one, T2 = CAT tip two. y axis shows the ratio of relative fluorescence intensity (R.F.I.) in the interacting zone as compared to background. x axis shows time (min). (B) Time course of SO-GFP localization to CATs in *his-3::Pccg1 so-gfp Δso* germling fusion pairs. Graphic representation of SO-GFP localization to CAT tips of fusion pairs over time. T1 = CAT tip one, T2 = CAT tip two. y axis and x axis identical to (A). (C) Time course of MAK-2-GFP and dsRED-SO localization in germling pairs [(*his-3::Pccg1 mak-2-gfp; Δmak-2*) + (*his-3::Pccg1 dsRED-so Δso*)]. Graph shows coordinated oscillation of MAK-2-GFP and dsRED-SO localization in one CAT tip of a germling pair. Fig. S2 shows MAK-2-GFP and dsRED-SO oscillation in both CAT tips. y axis and x axis identical to (A). (Scale bar, 2 μ m.)

taining a functional GFP tagged MAK-2 protein. The *mak-2* gene was expressed under the control of its native promoter or, alternatively, under the regulation of the *ccg-1* promoter (*Pccg-1*) (20). The functionality of MAK-2-GFP fusion proteins was determined by western analysis and complementation of a Δ *mak-2* mutant (Fig. 1 D and E). In germlings that showed no chemotrophic interactions, MAK-2-GFP was evenly dispersed in the cytoplasm and also localized to nuclei (Fig. 1C; see also Fig. 3A). In contrast, in cell pairs undergoing chemotrophic interactions, MAK-2 accumulated in discrete complexes at the cell cortex when cells were $<15 \mu$ m apart. These complexes of approximately 300 nm were concentrated at cortex regions closest to the partner cell (Figs. 1C and 2A). To address whether these complexes were membrane bound, we assessed colocalization of MAK-2-GFP with the membrane-selective dye FM4-64 (21) in *mak-2-gfp* germling pairs. The MAK-2-GFP particles were closely associated with the plasma membrane (Fig. 3C and Fig. S1). Colocalization of MAK-2-GFP with FM4-64 was not detected, even though FM4-64-labeled intracellular membrane bound organelles were observed.

Time course analyses revealed that apical accumulation of MAK-2 occurred in only one cell of a fusion pair; the partner cell showed diffuse cytoplasmic distribution (Figs. 1C and 2A). After 3–5 min, the apical MAK-2 signal dispersed to the cytoplasm, while in the partner cell, MAK-2 accumulated in discrete particles at the CAT tip. Analysis of chemotrophic interactions in over approximately 100 germling pairs showed that robust oscillatory recruitment of MAK-2 to CAT tips repeated up to six times before physical contact (Fig. 2A and Movie S1). The tempo

Fig. 3. MAK-2 and SO localization during germling fusion. (A) MAK-2-GFP and H1-dsRED colocalize in nuclei of a germling pair [(*his-3::Pccg1 mak-2-gfp*; $\Delta mak-2$) + *his-3::H1-dsRED*] during chemotropic interactions (arrow). (B) SO is excluded from nuclei in a germling pair [(*his-3::Pccg1 so-gfp Δso*) + *his-3::H1-dsRED*]; arrow marks a nucleus. Asterisk shows region of chemotropic attraction. (C) MAK-2-GFP localized to CATs tips is not bounded by membrane. *his-3::Pccg1 mak-2-gfp*; $\Delta mak-2$ strain labeled with membrane-selective dye FM4-64 (red). The MAK-2-GFP particles (green; arrow) are either associated with the plasma membrane or free within the cytoplasm. Other organelles are membrane-bound (asterisk). (D) A *his-3::Pccg1 so-gfp Δso* strain labeled with FM4-64 shows localization of SO-GFP to CAT tips (green; arrows), but not colocalization with membrane bound structures (red). (E) MAK-2-GFP and dsRED-S) localization at cell fusion point after contact between with germlings [(*his-3::Pccg1 mak-2-gfp*; $\Delta mak-2$) + (*his-3::Pccg1 dsRED-so Δso*)]. dsRED-SO delocalizes from the fusion point after contact. (F) MAK-2-GFP localization around the fusion pore as it enlarges. Z-stack of optical sections of the cells shown in 3E. The axis of stack rotation (relative to Fig. 3E) is marked by an arrow. dsRED-SO is sequestered within vacuoles passing through the fusion pore. (Scale bar, 2 μm .)

of the oscillation of MAK-2 from one CAT tip to the other remained similar (3–6 min) throughout the chemotropic interactions. Quantification of the relative signal intensity at the CAT tips revealed that formation and dispersal of complexes at the cell periphery was highly coordinated between the two fusion partners. High signal intensity in one cell correlated with low signal intensity in the second cell. Occasionally, interactions between three cells were observed. In these cases, two of the three cells synchronized their oscillation of MAK-2-GFP to the cell cortex and interacted in unison with the partner cell.

Localization of MAK-2 to the cell cortex occurred when germlings were approximately 15 μm apart and was initially dispersed around the CAT tip. As chemotropic growth continued, localization of MAK-2 became concentrated at the cell cortex in the region closest to the partner cell (Fig. 2A and Fig. S2). Thus, oscillation and signaling showed reinforcement, resulting in concentrated localization of MAK-2 at CAT tips.

The SO Protein Shows Similar Localization Dynamics to MAK-2. After observing the unexpected localization dynamics of MAK-2, we assessed the subcellular localization of SO. Strains containing a deletion of *so* show some CAT formation, but fail to undergo chemotropic interactions with a wild-type partner cell (19). These observations suggest that *so* mutants can initiate chemotropic interactions, but fail to maintain the signaling required for

continued directed growth. SO contains a conserved WW domain, indicating its potential for protein–protein interactions. In other systems, proteins containing WW domains have been linked with signaling to the membrane cytoskeleton (22). In conidial germlings not involved in chemotropic interactions, functional SO-GFP showed cytoplasmic and vacuolar fluorescence, consistent with earlier reports on the localization of SO in mature hyphae (23). However, unlike MAK-2, SO is completely excluded from nuclei (Fig. 3B and Fig. S3).

Remarkably similar to MAK-2, in germling pairs undergoing chemotropic attraction, SO-GFP formed particulate complexes, approximately 300 nm in diameter, that accumulated at CAT tips. We evaluated whether SO-GFP complexes were membrane-bound by assessing colocalization of FM4-64 and SO-GFP during chemotropic interactions between germlings (Fig. 3D and Fig. S1). As observed with MAK-2, SO particles were closely associated with the plasma membrane, but were not membrane bound. As neither MAK-2 nor SO have a membrane localization signal, these observations suggest that both proteins may be tethered to the plasma membrane via protein-protein interactions.

As with MAK-2, SO localization to CAT tips was dynamic, such that high signal intensity of SO in one CAT tip was correlated with low signal intensity of SO at the CAT tip of the fusion partner (Fig. 2*B* and [Movie S2](#)). SO exhibited oscillatory recruitment to CAT tips with indistinguishable dynamics to that of MAK-2 (recruitment alternated between cells with a period of 6–12 min) (Fig. 2*B* and [Fig. S2](#)). The localization of SO to the cell cortex was dispersed when germling fusion pairs were distant, but underwent reinforcement, such that SO localization became concentrated at the site of future cell contact and pore formation.

Protein Synthesis Is Required for Reinforcement of MAK-2 and SO Oscillation at CAT Tips, But Not for Its Maintenance. The rapid apical accumulation and dispersion cycles of MAK-2 and SO to CAT tips suggested their recruitment from elsewhere in the cell, rather than their de novo synthesis. To test this hypothesis, we analyzed the localization of both proteins during chemotropic growth in germlings treated with cycloheximide, which blocks protein synthesis. We first assessed whether the localization/oscillation of MAK-2 or SO to CAT tips in germling pairs undergoing robust chemotropic interactions ($\approx 2 \mu\text{m}$ apart) was affected by treatment with cycloheximide. As shown in Fig. S4, oscillation of MAK-2 and SO, as well as chemotropic interactions, were apparently unaffected by inhibition of protein synthesis over a 30 min time course. By contrast, germlings pairs that were distant ($>8 \mu\text{m}$), but showed some localization of MAK-2 and SO to CAT tips, failed to undergo reinforcement and thus robust MAK-2 or SO oscillation. In these germlings, the MAK-2/SO signals either disappeared from the CAT tips completely or showed delocalization around the cell cortex (Fig. S4). Chemotropic growth was also not apparent in these germling pairs after treatment with cycloheximide, although growth continued during the approximately 30 min time course. These data suggest that protein synthesis is required to initiate signaling and chemotropic interactions, perhaps via synthesis of proteins that interact with MAK-2 and SO, but is not required when robust oscillation of these proteins is occurring during chemotropic interactions and directed growth.

SO and MAK-2 Show Opposing Oscillatory Recruitment to CAT Tips. As both SO and MAK-2 exhibit similar dynamic localization to CAT tips, we ascertained whether SO and MAK-2 colocalized, and/or if their localization dynamics were coordinated. Live-cell imaging of a heterokaryotic strain that produced dsRED-SO and MAK-2-GFP revealed that SO and MAK-2 did not colocalize, but showed remarkably well-coordinated opposing recruitment to CAT tips (Fig. 2C and Fig. S2). When MAK-2 accumulated at one CAT tip, SO accumulated at the CAT tip of its fusion

partner. The highest tip signal intensity of one protein coincided with the lowest tip signal intensity of the other, and was always out of phase with the opposing CAT tip (Movie S3). These data suggest that coordination between MAK-2 and SO localization may act as a marker for a cell in the “signal sending” mode versus in a “signal perceiving” mode. This system would prevent self-stimulation that would otherwise occur via simultaneous production and reception of chemotropic signals.

The coordinated oscillation of MAK-2 and SO to CAT tips was observed until germlings physically touched. At contact, MAK-2 and SO concentrated at the point where cells adhered to each other (Fig. 3E). However, SO delocalized from the cell cortex to the cytoplasm/vacuoles after contact, while MAK-2 was part of a ring-like structure that expanded laterally around the fusion pore (Fig. 3F and Movie S1). MAK-2 remained localized in a ring even when organelles, such as vacuoles traveled through the pore, for example those containing dsRED-SO. These observations suggest that MAK-2 may also have a late function in germling fusion, including during membrane merger and fusion pore formation.

Functional MAK-2 Is Required for Coordinated Cell Behavior and Oscillatory Recruitment of SO. Signal transduction through MAPK cascades requires kinase activity; changing a specific amino acid in the ATP binding site of a MAPK renders it sensitive to inhibition by ATP analogues such as 1NM-PP1, but does not affect kinase activity in the absence of inhibitor (24). We constructed an inhibitable variant of MAK-2, introducing the analogous mutation of glutamine 100 to glycine 100. In the absence of the inhibitor 1NM-PP1, strains and germlings containing the *mak-2^{Q100G}* allele [Δ *mak-2 (mak-2^{Q100G})*] showed wild-type growth and fused normally (Fig. S5). In the presence of inhibitor, the Δ *mak-2 (mak-2^{Q100G})* strain showed a mutant *mak-2* phenotype; chemotropic interactions and fusion were not observed, consistent with inactivation of MAK-2 kinase activity. Wild-type cells showed no fusion deficiency in the presence of 1NM-PP1, demonstrating that the inhibitor is specific (Fig. S6). To assess whether kinase activity of MAK-2 is required for the oscillation of MAK-2-GFP in a partner cell, chemotropic interactions were studied between Δ *mak-2 (mak-2^{Q100G})* and Δ *mak-2 (mak-2-gfp)* germling pairs. Although normal oscillation of MAK-2-GFP to CAT tips was observed in Δ *mak-2 (mak-2-gfp)* cells before addition of inhibitor (Fig. S6), localization of MAK-2-GFP to the cell cortex of CAT tips was abolished after the addition of 1NM-PP1 (Fig. 4A). Chemotropic interactions of both cells also ceased after inhibitor addition. These results indicate that MAK-2 kinase activity in one cell is required for the recruitment of MAK-2 to the CAT tip of its fusion partner (i.e., MAK-2 recruitment is not cell autonomous).

To determine whether SO localization in a fusion partner also depended on MAK-2 kinase activity, SO-GFP localization dynamics were evaluated in Δ *mak-2 (mak-2^{Q100G})* and Δ *so (so-gfp)* germling pairs undergoing chemotropic interactions. In contrast to MAK-2-GFP, the inhibition of MAK-2 kinase activity in Δ *mak-2 (mak-2^{Q100G})* germlings resulted in the stabilization of SO-GFP in the partner cell in punctate complexes, which delocalized around the cell cortex over time (Fig. 4B and Fig. S6). Thus, MAK-2 kinase activity in one cell was required for the delocalization of SO from the CAT tip of its fusion partner.

To determine whether MAK-2 kinase activity is required for the recruitment of SO to the CAT tip within the same cell, fusion between germlings containing both *mak-2^{Q100G}* and *so-gfp* (Δ *mak-2 (mak-2^{Q100G}); so-gfp*) was evaluated. Soon after addition of 1NM-PP1, both fusion partners accumulated punctate SO complexes, which de-localized from CAT tips over time (Fig. 4C). Thus, the recruitment of SO to CAT tips did not require MAK-2 kinase activity, but MAK-2 kinase activity was required for the dispersion of SO complexes from the cell cortex to the

Fig. 4. Inhibition of MAK-2 kinase activity disrupts both MAK-2 and SO oscillation during chemotropic interactions. (A) Inhibition of MAK-2 kinase function by addition of 1NM-PP1 to (*his-3::Pccg1 mak-2^{Q100G}; Δmak-2*) germlings causes cessation of MAK-2-GFP oscillation in a partner cell (*his-3::Pccg1 mak-2-gfp; Δmak-2*). 1NM-PP1 added after 0 min time point. Note lack of MAK-2 oscillation in graphic representation of MAK-2-GFP localization to CAT tip over time. y axis shows the ratio of relative fluorescence intensity (R.F.I.) in the interacting zone as compared to background; x axis shows time. (B) Inhibition of MAK-2 kinase function in one cell (*his-3::Pccg1 mak-2^{Q100G}; Δmak-2*) causes a cessation of SO-GFP oscillation in the CAT of a partner cell (*his-3::Pccg1 so-gfp Δso*). 1NM-PP1 added after 0 min time point. Note lack of SO oscillation in graphic representation of SO-GFP localization in CAT tips after addition of 1NM-PP1. x axis and y axis as in (A). (C) Inhibition of MAK-2 kinase activity in [*his-3::Pccg1 mak-2^{Q100G}; Δmak-2 (so-gfp)*] germling pairs results in accumulation of the particulate SO-GFP complexes in both cells, which gradually lose polarity and spread around the cell cortex. 1NM-PP1 added after the 0 min time point. Graphic representation of SO-GFP localization to both CAT tips after the addition of 1NM-PP1. Y and x axis are as in (A). (Scale bar, 2 μ m.)

cytoplasm. These data imply that functional MAK-2 is required to coordinate the molecular dynamics of SO. However, SO may not be a direct target of MAK-2 as SO phosphorylation during germling fusion has not been detected, suggesting that the dispersion of SO from CAT tips may require an indirect signal from MAK-2. Together these data indicate that MAK-2 and SO are functionally related and their activity is interdependent both between and within cells undergoing chemotropic interactions required for cell fusion.

Discussion

Cell-cell communication is necessary for coordinating the physiological responses required for groups of cells to perform tasks that are not possible for individuals. By assessing spatiotemporal localization of MAK-2 and SO during chemotropic interactions and cell fusion in *N. crassa*, we identified a previously undescribed mode of cell-to-cell signaling. The coordinated transient recruitment of MAK-2 and SO to the plasma membrane of germlings undergoing chemotropic growth indicates that fusion

known that mutations in the pheromone genes and adenylate cyclase (involved in cAMP production) do not abrogate germling fusion or chemotropic interactions (11, 17). It is likely that the signaling molecule(s) that mediate germling fusion are species-specific, since interactions between species rarely or never occur (38). These observations further suggest that filamentous fungi use a host of diffusible molecules to orchestrate colony establishment, vegetative growth, sporulation, sexual development, and interaction with other species in the environment. Further studies will elucidate the chemical language of these organisms and its role in their communication and development.

Materials and Methods

Molecular techniques, including SO-GFP strain construction, were previously described (23). GFP alleles were expressed under the control of the native or *cgc1* promoters (20, 23). The SO-GFP strain used was AF-SoT8 and the MAK-2-GFP strain was AF-M512. AF-M512 contains the *mak-2* coding region cloned into pMF272 (20) using XbaI/PacI and integrated at the *his-3* locus in AF-M621 (*his-3*; Δ *mak-2* A). Table S1 shows all strains constructed and used in this study.

To construct the *mak-2*^{Q100G} allele, the homologous codon (CAG; Q100 in *N. crassa*) mutated in *FUS3* (24) was changed to GGG (G) using fusion PCR. The *mak-2*^{Q100G} allele was cloned into pMF272 using EcoRI/XbaI and transformed into AF-M621, producing strain MAL1. To express SO-GFP in the *mak-2*^{Q100G} background, *Pccg1 so-gfp* was cut from pSO8 (23) using NotI/EcoRI, cloned into pBCphleo (FGSC) and transformed into MAL1 (*his-3::Pccg1 mak-2*^{Q100G};

Δ *mak-2*). The construction of the CR73-1 (*his-3::Pccg1 hH1-dsRED rid-1* A) has been described (39).

Germlings were grown in Vogel's medium (40) for microscopy. For each image, four to six focal planes 0.2 μ m apart were recorded and were later projected into a single image. For time-lapse sequences, images were collected every 15–18 s. At each time point, two to three focal planes 0.2 μ m apart were recorded; these were later projected into a single image. Vacuoles were stained with 5 μ M CMAC for 30 min, and membranes were stained with 0.1 μ M FM4-64 for 3 h, before imaging. For experiments using 1NM-PP1 and cycloheximide, cells were grown in a timelapse chamber (Ibidi) in Vogel's liquid media. Either 20 μ M 1NM-PP1 or 4 μ M cycloheximide was added during imaging, as indicated. Microscopy was performed on a DeltaVision Spectris DV4 deconvolution microscope (Applied Precision Instruments) or on a BioRad Radiance 2000 confocal microscope. SVI Huygens Professional and Bitplane Imapris were used for image processing. For signal semiquantification, average pixel intensity was measured in a defined cellular region of interest surrounding the interaction zone and compared with an identically sized cellular region not involved in the interaction. Two or three pairs of interacting germlings were measured per experiment; the graphs each show one example from each experiment.

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