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# Accumulation of specific sterol precursors targets a MAP kinase cascade mediating cell–cell recognition and fusion

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**Sterols are vital components of eukaryotic cell membranes. Defects in sterol biosynthesis, which result in the accumulation of precursor molecules, are commonly associated with cellular disorders and disease. However, the effects of these sterol precursors on the metabolism, signaling, and behavior of cells are only poorly understood. In this study, we show that the accumulation of only ergosterol precursors with a conjugated double bond in their aliphatic side chain specifically disrupts cell–cell communication and fusion in the fungus *Neurospora crassa*. Genetically identical germinating spores of this fungus undergo cell–cell fusion, thereby forming a highly interconnected supracellular network during colony initiation. Before fusion, the cells use an unusual signaling mechanism that involves the coordinated and alternating switching between signal sending and receiving states of the two fusion partners. Accumulation of only ergosterol precursors with a conjugated double bond in their aliphatic side chain disrupts this coordinated cell–cell communication and suppresses cell fusion. These specific sterol precursors target a single ERK-like mitogen-activated protein (MAP) kinase (MAK-1)-signaling cascade, whereas a second MAP kinase pathway (MAK-2), which is also involved in cell fusion, is unaffected. These observations indicate that a minor specific change in sterol structure can exert a strong detrimental effect on a key signaling pathway of the cell, resulting in the absence of cell fusion.**

sterol biosynthesis | ergosterol | MAP kinase signaling | cell fusion | *Neurospora crassa*

**S**terols are essential constituents of eukaryotic cell membranes. They feature prominently in the structure and function of the lipid bilayer by maintaining its microfluidic state. In addition, sterols and their specific interactions with proteins and other lipids are critical for the formation of membrane subdomains, mediating a plethora of biological processes, including cell polarization, signal transduction, subcellular sorting, and pathogen defense (1, 2).

Sterol biosynthesis deficiencies, and more specifically the accumulation of intermediates of the biosynthesis pathway, are commonly associated with cellular malfunctioning and disease. Examples include innate, inheritable diseases (e.g., Smith–Lemli–Opitz syndrome or desmosterolosis), neurodegenerative disorders (e.g., Alzheimer’s, Huntington’s, or Parkinson’s disease), and inflammation-induced disorders (e.g., primary cicatricial alopecia) (3–6). In plants, sterol biosynthesis deficiencies result in developmental defects, and in fungi the lack of the main membrane sterol ergosterol causes resistance against important antifungal drugs of the polyene group (7, 8). The accumulation of a specific ergosterol precursor with a conjugated double bond in its side chain results in cell mating defects in baker’s yeast, emphasizing the importance of the structure–function relationship of membrane sterols (9).

Although the detrimental effects of deficient sterol biosynthesis and the accumulation of sterol precursors become increasingly

evident, the molecular consequences of these defects remain poorly understood. In particular, it is unclear whether the observed deficiencies are caused by an unspecific malfunctioning of the cell membrane affecting numerous molecular processes or by specific effects on individual biochemical or signaling pathways.

In recent years, *Neurospora crassa* has become widely appreciated as the primary model for studies on cell–cell fusion and related membrane-associated signaling events in filamentous fungi (10). Genetically identical germinating spores of this fungus undergo fusion, thereby forming a supracellular network, which develops further into the interconnected mycelial colony. Before fusion, cell pairs use a unique mode of communication, in which the two partners switch between signal sending and receiving in a highly coordinated, alternating manner, thereby establishing a cell–cell dialogue that orchestrates the cell pairs’ growing toward and fusing with each other. These switches between signal sending and receiving involve the highly dynamic assembly and disassembly of distinct membrane-associated protein complexes, containing either the mitogen-activated protein (MAP) kinase (MAK-2)-signaling module or the SO (soft) protein, which associates with a second ERK1/2-like MAP kinase pathway, the MAK-1 cascade, in filamentous fungi (11, 12).

In this study, we have analyzed how the accumulation of structurally different ergosterol precursors influences this highly

## Significance

**Deficiencies in sterol biosynthesis resulting in the accumulation of precursor sterol molecules are commonly associated with cellular malfunctioning and disease, including neurodegenerative and inflammatory disorders. However, the molecular and cellular consequences of the aberrant accumulation of sterol precursors are not understood. In particular, it is unclear whether specific biochemical or signaling pathways are targeted by the precursors and to what extent their specific structures contribute to their disruptive effects. Here we show that the accumulation of ergosterol precursors specifically targets a conserved ERK MAP kinase pathway that mediates fungal cell–cell communication and fusion. This effect is only caused by precursors with a conjugated double bond in their aliphatic side chain, indicating specific structure–function relationships in the mechanism of action.**

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The authors declare no conflict of interest.

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coordinated cell–cell signaling mechanism. We show that specifically the presence of precursors carrying a conjugated double bond in their aliphatic side chain results in defects in cell–cell communication and fusion. These defects can be pinpointed to the MAK-1 MAP kinase cascade, indicating that the buildup of certain sterols causes very specific rather than general effects on the mechanism of self-signaling.

## Results

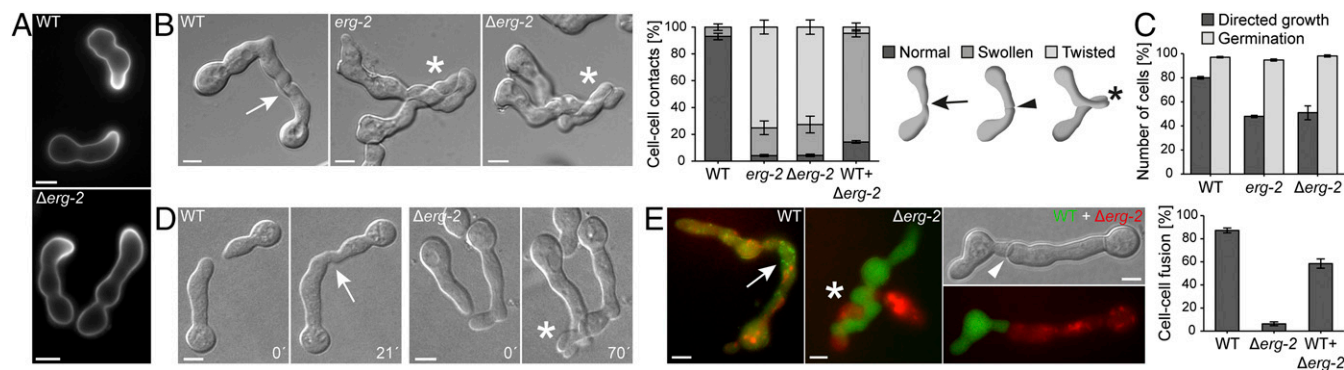
**Ergosterol-2 Deficiencies Result in Cell Fusion Defects.** Similar to most polar growing cells, germ tube tips of *N. crassa* possess a characteristic apical sterol-rich membrane domain (Fig. 1A). Because the MAK-2 and SO protein complexes mediating cell–cell communication and fusion specifically associate with this membrane sector, we set out to test the contribution of ergosterol to these processes. Ergosterol biosynthesis mutants were analyzed by light microscopy for defects in germling communication and fusion. In an *erg-2* (*ergosterol-2*) mutant (FGSC 2723), interacting cell tips frequently failed to arrest growth after physical contact and curled around each other, giving rise to corkscrew-like structures (Fig. 1B). Although more than 60 *N. crassa* mutants affected in germling fusion have been identified so far, a comparable phenotype has not yet been described (13). The gene *erg-2* (NCU01333) is homologous to *erg4* of *Saccharomyces cerevisiae* and encodes a sterol C-24(28) reductase, mediating the last step of the predicted ergosterol biosynthesis pathway in *N. crassa* (Fig. 2A). Sequencing of the mutant allele identified a nonsense mutation at position 1523 (G > A), shortening the encoded protein by 77 aa residues. To test the effect of a complete loss of *erg-2*, a  $\Delta$ *erg-2* gene knockout mutant was analyzed. The macroscopic appearance of  $\Delta$ *erg-2* cultures was normal, although the linear hyphal extension rate was significantly reduced (Fig. S1A). Quantitative analyses revealed two defects related to germling fusion. First, the directed growth of cell pairs toward each other within a population was reduced by about 30% compared with WT (Fig. 1C). In contrast to the predominantly bidirectional tip-to-tip WT interactions, germ tube attraction was increasingly unidirectional in the mutant, and cells met in a tip-to-side mode (Fig. S2A). Second, interacting  $\Delta$ *erg-2* cells failed to arrest growth after contact and formed the unique convoluted structures observed in the classical mutant (Fig. 1B and D and Movies S1 and S2). In these cell pairs, fusion was reduced by about 90% (Fig. 1E). The mutant phenotype was fully complemented by

reintroduction of an N-terminally GFP-tagged ERG-2 construct, confirming that it was fully caused by the lack of *erg-2* (Fig. S1C and E). A C-terminal tagged version complemented only partially, suggesting that the presence of GFP at this terminus impairs ERG-2 functions. Both GFP-tagged constructs localized to the perinuclear endoplasmic reticulum, consistent with the predicted site of ergosterol biosynthesis (Fig. S1D) (14). Together, these data indicate that the C terminus of ERG-2 is important for its functions. The failure of *erg-2* fusion pairs to arrest growth suggests that the cells are unable to recognize and to react to their physical contact. In  $\Delta$ *erg-2*/WT pairings, cell–cell recognition and growth arrest was normal, indicating a cell-autonomous phenotype. However, cell merger within these heterotypic pairs was still significantly impaired and the cell contact zones commonly appeared swollen (Fig. 1B and E and Fig. S2B).

So far, most described germling fusion mutants were also affected in fusion between hyphae within the mature mycelium, when tested (13). Consistent with this notion, hyphal fusion pairs of  $\Delta$ *erg-2* also exhibited the growth arrest failure and hyphae twisted around each other (Fig. S1B). In contrast, the sexual interaction between mating partners was unaffected (Fig. S3A and B), indicating that  $\Delta$ *erg-2* defects are specific for vegetative fusion events. Fruiting body development following fertilization and sexual spore formation were, however, significantly impaired in  $\Delta$ *erg-2*, indicating postfertilization functions for normal ergosterol biosynthesis (Fig. S3C–F).

### **$\Delta$ erg-2-Like Phenotypes Fully Correlate with the Presence of a Conjugated Double Bond in the Side Chain of the Accumulating Sterols.**

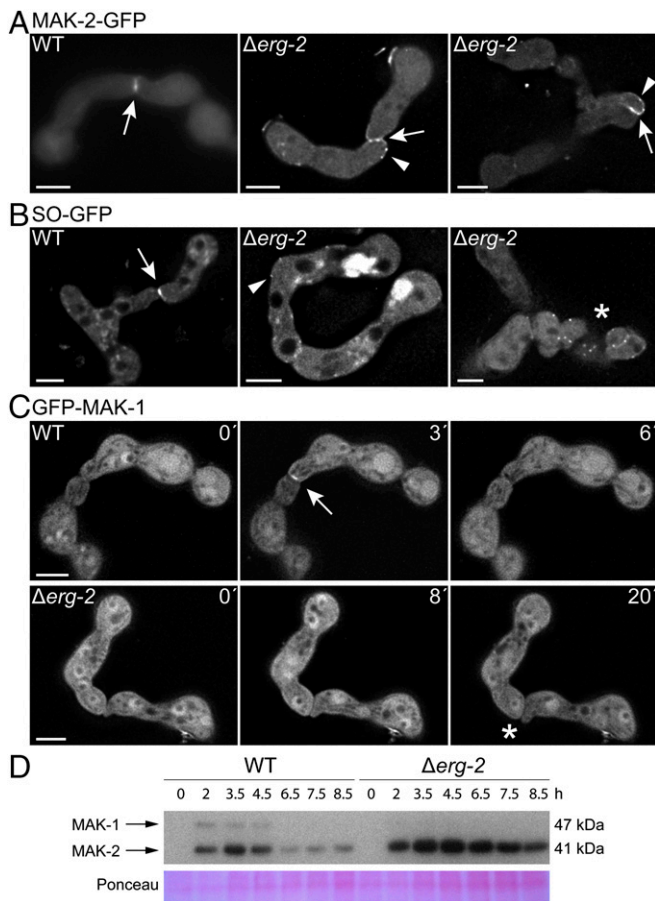
Sterol profiling revealed that ergosterol, the main sterol of the WT, is absent in the  $\Delta$ *erg-2* mutant. It instead accumulates the precursor ergosta-5,7,22,24(28)-tetraenol (Fig. 2B and C). The only structural difference between this intermediate and ergosterol is an additional double bond within its aliphatic side chain. The overall sterol amount of WT and mutant are, however, comparable (Fig. 2D), and formation of the sterol-rich apical domain is unaffected in the mutant (Fig. 1A). These observations raised the question of whether the observed  $\Delta$ *erg-2* defects are either caused by the absence of the end product ergosterol or by the accumulation of the precursor. We therefore analyzed germling fusion in eight additional single, double, and triple ergosterol biosynthesis mutants and determined their respective sterol profiles. The *erg-10a* and *erg-10b* mutants still accumulated ergosterol, suggesting redundant functions of the



**Fig. 1.** Mutation of *erg-2* disturbs cell–cell interactions and fusion. (A) Sterol distribution visualized by filipin staining. (B) (Left) *erg-2* and  $\Delta$ *erg-2* mutant cells twist around each other after physical contact (asterisks). (Right) Quantification of types of interaction following contact in WT, mutant, and mixed pairings. (C) Mutation of *erg-2* significantly reduces the number of germlings involved in directed growth toward each other within a cell population. (D) Whereas WT germlings arrest directed growth after the cells have touched (arrow),  $\Delta$ *erg-2* germlings continue to grow (asterisk, contact site). Images are stills from Movies S1 and S2, respectively. (E) Fusion of WT germlings expressing GFP and mCherry results in mixing of the cytoplasm (arrow).  $\Delta$ *erg-2* pairs frequently fail to fuse after contact (asterisk). In WT/ $\Delta$ *erg-2* germling pairings contact sites appear swollen (arrowhead) and the fusion frequency is increased compared with mutant pairs. All error bars represent SDs of three independent experiments. The number of cells or cell pairs per replicate ranged from 50 to 140. (All scale bars, 5  $\mu$ m.)







**Fig. 4.** SO and the MAK-1 MAP kinase are not recruited to the cell-cell contact point in  $\Delta erg-2$ . (A) (Left and Center) In WT and  $\Delta erg-2$  cell pairs MAK-2 concentrates at the site of cell-cell contact (arrows). (Right) In the mutant the kinase localizes to the continuously growing tips (arrowheads). (B) (Right) WT cells that strongly focus SO-GFP at their touching cell tips (arrow). (Center and Left)  $\Delta erg-2$  (N2-49) germlings fail to cluster SO after physical contact (arrowhead) and during subsequent growth (asterisk). (C) GFP-MAK-1 is transiently recruited to the fusion point (arrow) in WT pairings (Top) but does not accumulate at the contact zone (asterisk) of  $\Delta erg-2$  pairs (Bottom). (D) Immunoblot analysis testing the phosphorylation of the MAP kinases MAK-1 and MAK-2 in WT and  $\Delta erg-2$  germlings. (All scale bars, 5  $\mu$ m.)

of the cytoskeleton and localization of the polarity factor BEM-1 were normal in  $\Delta erg-2$ , indicating that polarized growth is unaffected in the mutant (Fig. S5 A and B). Taken together, these observations indicate that although the mutant can undergo tropic interactions, albeit with decreased efficiency, it remains locked in the cellular program of directed growth, which is not terminated upon cell-cell contact.

In contrast to MAK-2 dynamics, the recruitment of the SO protein was highly affected during the tropic interactions of  $\Delta erg-2$  germlings, although the overall amount of SO within the cell was comparable in WT and mutant (Fig. S6B). Only few aggregates of the protein formed at the plasma membrane, and these complexes were not concentrated at the cell tip but appeared at seemingly random locations across the cell cortex (Fig. 3B). Nevertheless, this aberrant complex formation still alternated with MAK-2 recruitment, indicating that the coordination between the two fusing cells remains generally unaffected (Fig. S5C). After cell-cell contact, however, SO did not concentrate at the contact zone, as is typical for WT (Fig. 4B). In heterotypic WT/ $\Delta erg-2$  fusion pairs, SO recruitment did not improve in the mutant cell

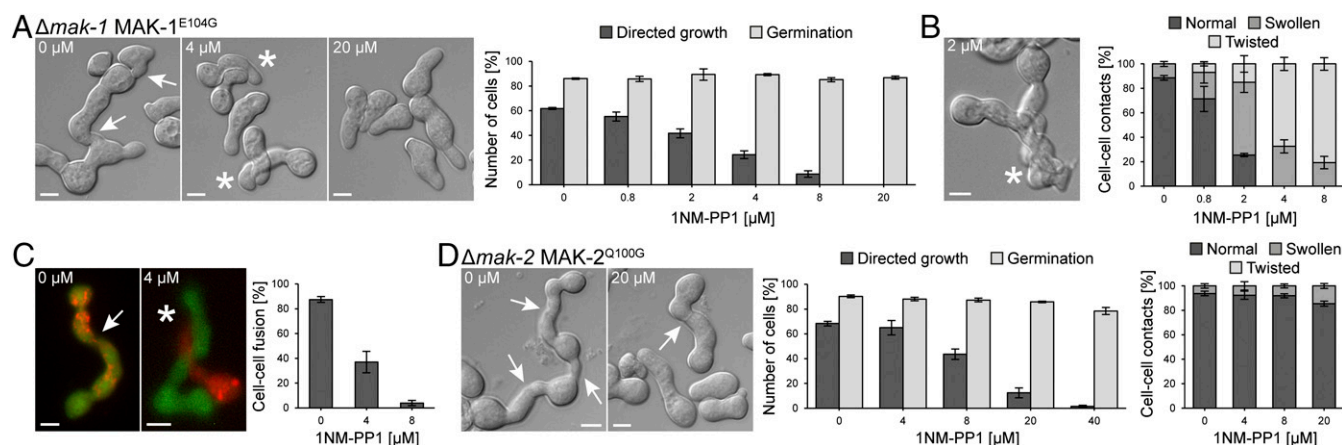
and worsened in the WT partner, consistent with the notion that the cell dialogue mechanism requires two fully functional partners (Fig. S2 D and E). In conclusion, MAK-2 signaling is not affected in the presence of the sterol precursor, whereas SO dynamics are. In addition to its role in cell fusion, SO aggregates at septal pores in injured hyphae. This function is unaffected in  $\Delta erg-2$  (Fig. S6A), highlighting the specific effects of sterol precursor accumulation on cell-cell communication and fusion.

**The Cell-Cell Communication and Fusion Defects Are Caused by Deficiencies in MAK-1 MAP Kinase Signaling.** A recent study in *Sordaria macrospora*, a close relative of *N. crassa*, identified the SO homolog PRO40 as a scaffolding protein of the MAP kinase MAK-1 cell wall integrity pathway (12). We confirmed the physical interaction of SO and the two upstream kinases of the MAK-1 module for *N. crassa* by yeast two-hybrid analysis (Fig. S7A). In addition,  $\Delta mak-1$  strains of *N. crassa* exhibit no cell-cell interactions related to fusion, as observed for the  $\Delta so$  mutant (15, 16). We therefore analyzed the dynamics of GFP-tagged MAK-1 in WT and  $\Delta erg-2$ . During tropic growth of WT germlings, no SO-like recruitment of MAK-1 to the cell tips occurred. However, as soon as the cells touched, the kinase accumulated at the contact zone (Fig. 4C and Movie S3), where it colocalized with SO and remained during fusion pore formation (Fig. S7B).  $\Delta erg-2$  fusion pairs failed to recruit MAK-1 after cell contact, further corroborating our notion that  $\Delta erg-2$  cells fail to switch their cellular programming toward cell fusion after physical contact (Fig. 4C). Consistent with these localization data, the MAK-1 phosphorylation level was significantly reduced in the mutant compared with the WT reference strain during the germling fusion period (Fig. 4D). In contrast, MAK-1 activation in response to  $H_2O_2$  stress was comparable in both isolates (Fig. S6C), indicating that specifically the fusion-related functions of MAK-1 are affected in  $\Delta erg-2$ .

These observations raised the question of whether the lack of MAK-1 recruitment and activation is a consequence or the cause of the  $\Delta erg-2$  defects. To address this issue, we combined chemical inhibition with molecular genetics. By site-directed mutagenesis, the gatekeeper residue of the MAK-1 ATP binding pocket was replaced by a glycine residue (E104G) by site-directed mutagenesis, rendering the kinase sensitive to the inhibitor 1NM-PP1. Expression of the mutated  $mak-1^{E104G}$  kinase allele in the  $\Delta mak-1$  mutant fully complemented the phenotype in the absence of the inhibitor (Fig. S8A). The MAK-1<sup>E104G</sup>-GFP fusion protein also exhibited WT-like subcellular dynamics during germling fusion (Fig. S9). In contrast, when germlings grew in the presence of 20  $\mu$ M 1NM-PP1 the phenotype of the inhibitable mutant was comparable to  $\Delta mak-1$  (Fig. 5A and Fig. S8B). A strain carrying a WT allele showed no defects under the same conditions (Fig. S8). Together, these data indicate that 1NM-PP1 specifically and efficiently inhibits MAK-1<sup>E104G</sup>. Inhibition at different stages of the germling interaction indicated that MAK-1 functions are essential for induction and maintenance of tropic growth, but also for fusion pore formation after cell-cell contact (Fig. S9). With decreasing inhibitor concentrations the cell interaction rate rose in an almost linear manner. Inhibitor concentrations between 0.8 and 8  $\mu$ M caused an increasing number of germling pairs to exhibit the unique  $\Delta erg-2$ -like phenotype of twisting germ tubes (Fig. 5A and B). In these pairs, cell merger was also reduced comparable to  $\Delta erg-2$  (Fig. 5C). As a control the same tests were conducted with a  $\Delta mak-2$  strain carrying the inhibitable variant MAK-2<sup>Q100G</sup>. Consistent with the MAK-2 function during cell-cell communication, germling interactions were inhibited by 1NM-PP1 in a dose-dependent manner (Fig. 5D and Fig. S8G). However,  $\Delta erg-2$ -like germ tube twists were never observed.

To test whether the lack of MAK-1 activity influences ergosterol biosynthesis in a qualitative or quantitative way, the sterol profiles and amounts were determined for  $\Delta mak-1$  and the WT.





**Fig. 5.** Inhibition of MAK-1 phenocopies the specific defects of  $\Delta erg-2$ . (A) (Left)  $\Delta mak-1$  cells expressing MAK-1<sup>E104G</sup> are sensitive to 1NM-PP1. Germlings interact normally in the absence of the inhibitor (arrows). Directed growth decreases in a dose-dependent manner in the presence of 1NM-PP1 and unusual cell-cell contacts appear (asterisks). (Right) Quantitative analysis. (B) (Left) An intermediate concentration of 1NM-PP1 results in the  $\Delta erg-2$ -like twisting of germ tubes (asterisk). (Right) Quantitative analysis. (C) Inhibition of MAK-1 reduces cell fusion in a dose-dependent manner. In the absence of 1NM-PP1, GFP- and dsRED-expressing cells form normal cell-cell contacts (arrow) and fuse. In presence of the inhibitor cell-cell fusion is reduced. (D) Interactions of  $\Delta mak-2$  cells expressing MAK-2<sup>Q100G</sup> are reduced in the presence of 1NM-PP1. Growth arrest is, however, normal. (Left) DIC (differential interference contrast). (Center) Quantification of directed growth. (Right) Quantification of contact types. All error bars represent SDs of three independent experiments. The number of cells or cell pairs per replicate ranged from 50 to 150. (All scale bars, 5  $\mu$ m.)

No significant differences were detected (Fig. S10A). Together, these results indicate that the specific inhibition of MAK-1 is sufficient to fully recapitulate the unique phenotypic  $\Delta erg-2$  defects observed during germling fusion.

Because the SO protein was identified as an interaction partner of the MAK-1 MAP kinase module and its plasma membrane recruitment is deficient in  $\Delta erg-2$ , we reasoned that a partial inhibition of SO might also result in the observed phenotype. Because no enzymatic function of SO is known, we repressed the expression of the *so* gene by putting it under control of the copper repressible promoter *Picu-1* (17). Full repression resulted in  $\Delta so$ -like phenotypes, whereas partial repression again produced  $\Delta erg-2$ -like deficiencies (Fig. S10 B–E). We conclude that both cell-cell communication and cell-cell fusion defects of mutants accumulating sterols with a conjugated double bond in the aliphatic side chain can be fully attributed to deficiencies in MAK-1 MAP kinase signaling.

## Discussion

In this study, we show that the accumulation of sterol precursors results in deficiencies in MAP kinase signaling. This effect is, however, highly specific. It is only caused by sterol molecules carrying a conjugated double bond in their aliphatic side chain and affects specifically certain functions of the MAK-1 cascade. This specificity is underlined by the observation that the general development of the respective mutants is only little affected, the plasma membrane at their cell tips is still enriched in sterols, and other membrane-associated processes, such as the localization of polarity factors or recruitment of the MAK-2 MAP kinase, are normal. The sterol precursors therefore obviously substitute most of the general ergosterol functions. These findings raise the question of how the altered membrane composition specifically affects MAK-1 signaling. We hypothesize that the precursors disturb membrane subdomain formation. Sterols play important roles in establishing these subdomains, which are involved in various signaling processes, including MAP kinase signaling. In mammals, the MAP kinase ERK module assembles together with its upstream activator Ras in nanoclusters at the membrane. Mathematical modeling indicated that decreases in Ras cluster formation result in reduced signaling (18). Different membrane-associated proteins are discussed as potential sensors and upstream

activating factors of the MAK-1 module (19, 20). The exact functional relationships are, however, so far not understood. We hypothesize that clustering of such upstream factors might also be essential for full MAK-1 activation. Interestingly, stress-induced activation of MAK-1 is not affected in  $\Delta erg-2$ , suggesting that the upstream components mediating cell-cell interactions and the stress response differ, which further highlights the specificity of the precursor's effect.

Our findings raise the question as to why the conjugated double bond is having a destructive effect. We consider two—mutually not exclusive—hypotheses. First, the presence of a conjugated double bond in the sterol side chain, which is oriented adjacent to the fatty acid tails of phospholipids, results in its increased rigidity, which might lead to a tighter packaging of sterols and membrane lipids, thereby reducing the fluidity of the membrane (9). This in turn could prevent the efficient formation of microdomains or protein nanoclusters involved in MAK-1 activation. Second, the conjugated double bond should be prone to oxidation, which would severely disturb the molecule structure, as shown for oxidation products of cholesterol precursors (21). As a consequence domain formation might also be deficient. At the tips of growing fusion hyphae, reactive oxygen species are strongly accumulating and seem to play a role in cell-cell signaling (22). This oxidizing environment might render fusion tips specifically prone to the oxidation of the sterol precursor. However, when determining the sterol profiles, the expected oxidation products were not detected; they might, however, be unstable and/or are only produced in small amounts.

Our data indicate that MAK-1 activity is involved in two processes of the cell fusion reaction. First, it is required to initiate and maintain the tropic interaction of the fusion cells, and second, it mediates cell-cell recognition upon physical contact. Both functions are affected in  $\Delta erg-2$ , however to a different extent. Although the tropic interactions still occur, albeit in a reduced frequency, contact recognition is mostly abolished. A potential explanation could be different modes of MAK-1 activation in the two processes. Our localization data indicate that the activation during tropic growth occurs mainly in the cytoplasm, whereas contact sensing involves plasma membrane recruitment of the kinase. In general, the subcellular localization of MAP kinase activation can influence its output. Whereas activation of MAK-1 homologous ERK in

the cytoplasm of mammalian cells occurs in a dosage-dependent, linear manner, activation at the membrane is more switch-like and even low signal intensities result in maximum output (23). Our MAK-1 inhibition experiments revealed that inhibitor concentrations that still allow tropic interactions of *N. crassa* cells fully block growth arrest after their physical contact. We therefore hypothesize that the directed growth relies on lower MAK-1 activation levels achieved in the cytoplasm, whereas cell–cell contact recognition requires a rapid and extensive activation, involving recruitment to the plasma membrane. Future challenges include a clearer understanding of these different modes of MAK-1 activation, including the identification of the upstream activating factors.

In summary, our data identified very specific effects of certain sterol precursors on specific functions of an individual MAP kinase pathway. MAP kinase signaling cascades are highly conserved in eukaryotic organisms. It will therefore be of great interest to test whether deficiencies in other organisms that are caused by or correlate with the accumulation of specific sterol precursors also include MAP kinase signaling deficiencies. Elucidating the exact relationship between sterol structure and the activity of individual, specific signaling pathways will be a future challenge furthering our general understanding of membrane-associated signal transduction processes and their role in growth, development, and disease.

## Materials and Methods

A detailed description of the materials and methods used in this study is provided in *SI Materials and Methods*. Strains used in this study are listed in Table S1. Mutants were constructed via transformation (24) and/or crossing (25), purified into homokaryotic strains, and confirmed via genotyping with

specific oligonucleotides (Table S2). Fungal cultures were routinely grown on Vogel's minimal medium (MM) (26).

Germling interaction and fusion assays were conducted as described previously (27). Directed growth between germinating spores was calculated from microscopic images with comparable cell densities. Cell–cell contact sites were classified as normal (narrow contact surface), swollen (broadened contact surface), or twisted (germ tubes unable to arrest growth). Quantitative cell–cell fusion assays were performed as described before (28).

For live-cell imaging, samples were analyzed by fluorescence or deconvolution microscopy (27). Membrane sterols were stained with a solution of 100  $\mu\text{g}/\text{mL}$  of filipin III in 1% (vol/vol) DMSO.

To construct an ATP-analog-sensitive variant of MAK-1, the gatekeeper amino acid residue E104 was mutated into glycine (29). For the dose-dependent inhibition of MAP kinase activities, agar blocks were cut after 2 h of incubation and treated with 0.8–40  $\mu\text{M}$  of 1NM-PP1 or 0.2% (vol/vol) of DMSO as a control. After incubation for an additional 2 h in a humidity chamber at 30 °C, samples were analyzed for directed growth, cell–cell contacts, and fusion.

Immunoblot analysis of phosphorylated levels of MAP kinases in interacting germlings was performed as described in ref. 30.

For sterol extraction and analysis, mycelia were harvested from shaking cultures. In short, sterols were obtained from the biomasses by saponification with alcoholic KOH solutions and subsequent extraction of the samples with *n*-hexane. The organic phases were derivatized with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and analyzed by GC/MS.

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