The NDR Kinase Scaffold HYM1/MO25 Is Essential for MAK2 MAP Kinase Signaling in Neurospora crassa

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Abstract

Cell communication is essential for eukaryotic development, but our knowledge of molecules and mechanisms required for intercellular communication is fragmentary. In particular, the connection between signal sensing and regulation of cell polarity is poorly understood. In the filamentous ascomycete Neurospora crassa, germinating spores mutually attract each other and subsequently fuse. During these tropic interactions, the two communicating cells rapidly alternate between two different physiological states, probably associated with signal delivery and response. The MAK2 MAP kinase cascade mediates cell–cell signaling. Here, we show that the conserved scaffolding protein HYM1/MO25 controls the cell shape-regulating NDR kinase module as well as the signal-receiving MAP kinase cascade. HYM1 functions as an integral part of the COT1 NDR kinase complex to regulate the interaction with its upstream kinase POD6 and thereby COT1 activity. In addition, HYM1 interacts with NRC1, MEK2, and MAK2, the three kinases of the MAK2 MAP kinase cascade, and co-localizes with MAK2 at the apex of growing cells. During cell fusion, the three kinases of the MAP kinase module as well as HYM1 are recruited to the point of cell–cell contact. hym-1 mutants phenocopy all defects observed for MAK2 pathway mutants by abolishing MAK2 activity. An NRC1-MEK2 fusion protein reconstitutes MAK2 signaling in hym-1, while constitutive activation of NRC1 and MEK2 does not. These data identify HYM1 as a novel regulator of the NRC1-MEK2-MAK2 pathway, which may coordinate NDR and MAP kinase signaling during cell polarity and intercellular communication.

Introduction

Appropriate cellular responses to external and internal stimuli depend on the highly orchestrated activity of interconnected signaling cascades. Within these networks, individual proteins can function in more than just one pathway. This raises the question of signal fidelity and the avoidance of undesired crosstalk in response to a specific signal. One crucial level of control arises from the formation of discrete complexes involving scaffold proteins that bind multiple components of a given pathway [1,2]. By facilitating the transient spatio-temporal organization of the different signaling factors, scaffolds promote kinase-substrate interactions, or kinase activation by upstream components such as membrane bound receptors. Scaffold proteins may also actively participate in signal modulation, for example by recruiting opposing phosphatases to the complex [3–7].

The best-studied scaffold is the budding yeast protein Ste5p, which binds the three kinases Ste11p, Ste7p and Fus3p of the mitogen-activated protein kinase (MAP kinase) module of the pheromone response pathway [8,9]. Upon binding of pheromone to its transmembrane receptor, dissociation of the receptor-associated heterotrimeric G protein triggers the recruitment of Ste5p to the plasma membrane through its interaction with the free Gβγ dimer. This membrane association facilitates the phosphorylation of the MAP kinase kinase (MAPKKK) Ste11p by the membrane-associated p21-activated (PAK) kinase Ste20p and activates the mating pathway. The outcome of this activation includes cell cycle arrest, expression of mating specific genes, reorganization of the cytoskeleton and the control of cell fusion of the two mating partners. Although the mating MAP kinase cascade is highly conserved throughout evolution, the Ste5p scaffold is specific for budding yeast. Even close fungal relatives, such as Ashbya gossypii or Candida albicans lack obvious homologs.

Recent studies in Neurospora crassa indicate that a MAP kinase module homologous to the yeast pheromone response pathway is essential for the communication and subsequent fusion of vegetative cells. The three kinases involved are the MAPKKK NRC1, the MAPKK MEK2 and the MAP kinase MAK2 (homologous to budding yeast Ste11p, Ste7p, and Fus3p, respectively) [10–13]. Upstream activators of this pathway, including a postulated secreted signal and its cognate receptor are so far unknown. N. crassa and other filamentous fungi possess pheromone receptors, heterotrimeric G-proteins, and PAK kinases [14,15]. However, these upstream components of the yeast pheromone response pathway are dispensable for vegetative cell communication [16,17]. Cell-cell signaling and tropic growth of N. crassa germlings involves the unusual subcellular dynamics of the MAP kinase MAK2 and SOFT, a conserved, Pezizomycotina-specific protein of unknown molecular function. Both proteins are recruited to the plasma membrane of the tip region of...
Author Summary

Intercellular communication and cellular morphogenesis are essential for eukaryotic development. Our knowledge of molecules and mechanisms associated with these processes is, however, fragmentary. In particular, the molecular connection between signal sensing and regulation of cell polarity is poorly understood. Fungal hyphae share with neurons and pollen tubes the distinction of being amongst the most highly polarized cells in biology. The robust genetic tractability of filamentous fungi provides an unparalleled opportunity to determine common principles that underlie polarized growth and its regulation through cell communication. In *Neurospora crassa*, germinating spores mutually attract each other, establish physical contact through polarized tropic growth, and fuse. During this process, the cells rapidly alternate between two different physiological states, probably associated with signal delivery and response. Here, we show that the conserved scaffolding protein HYM1/MO25 interacts with the polarity and cell shape-regulating NDR kinase complex as well as a MAP kinase module, which is essential for cell communication during the tropic interaction. We propose that this dual use of a common regulator in both molecular complexes may represent an intriguing mechanism of linking the perception of external cues with the polarization machinery to coordinate communication and tropic growth of interacting cells.

Results

HYM1 functions as scaffold protein for the COT1 NDR kinase pathway

HYM1 interacts with and regulates morphogenetic NDR kinases in unicellular yeasts, but its function during highly polar filamentous growth is unclear. We identified the uncharacterized ORF NCU03576 as the *N. crassa* homolog of HYM1. The *N. crassa* protein matched HymA of *Aspergillus nidulans* and MO25 of *Schizosaccharomyces pombe* with E-values of 2e−142 and 5e−67, respectively. To test a potential involvement of HYM1 in the NDR complex we analyzed its interaction with COT1 and POD6 in yeast two-hybrid assays. Both tests rendered positive results (Figure 1A). To corroborate these interactions in *vivo* by immunoprecipitation experiments, we generated a strain that expressed functionally tagged versions of the three proteins. We modified the endogenous loci of cot-1 and pod-6 to encode for HA- and myc-tagged kinase genes, respectively, and fused this strain with an isolate that ectopically expressed GFP-tagged HYM1 at the *his-3* locus. The anti-GFP immunoprecipitation recovered both myc-COT1 and HA-POD6 from extracts of myc-cot-1;HA-pod-6 + hym-1-gfp::his-3, but not the myc-cot-1;HA-pod-6 control strain (Figure 1B). In order to test if HYM1 promotes COT1 function, the activity of the kinase was compared in wild type and Δhym-1. COT1 activity purified from Δhym-1 was reduced to 70% (SD = ±5; n = 5) of wild type level (Figure 1C). Expression of HYM1-GFP in Δhym-1 increased COT1 activity back to wild type level, confirming that the reduced kinase activity was due to the deletion of hym-1.

HYM1 might promote COT1 activation by connecting the NDR kinase with its activating kinase POD6. We predicted that in this case the interaction between COT1 and POD6 should be reduced or abolished in a Δhym-1 background. To test this hypothesis, we crossed myc-cot-1;HA-pod-6 with Δhym-1 to generate myc-cot-1;HA-pod-6Δhym-1. Reciprocal co-precipitation experiments between myc-COT1 and HA-POD6 revealed that the weak interaction between the two kinases observed in wild type was abolished in a Δhym-1 background (Figure 1D). Together, these data indicate that HYM1 functions as a scaffold for COT1 and POD6, thereby promoting COT1 activity.

In order to determine the subcellular localization of COT1 and HYM1, we modified both gene loci to express C-terminally GFP-tagged proteins. The generated strains displayed wild type characteristics, indicating the functionality of the two fusion proteins under the control of their endogenous promoters. COT1-GFP formed an apical membrane-associated crescent in growing hyphal tips and accumulated as a bright, discrete dot that partially overlapped with the distal region of the Spitzenkörper (Figure 1E). The Spitzenkörper is an apex-associated cluster of vesicles, cytoskeletal elements and polarity factors that serves as vesicle supply center and guides polar tip growth [40–42]. HYM1-GFP, in contrast, did not form this apical cap and fully co-localized with the Spitzenkörper (Figure 1E). The intensity of the apical localization was higher in COT1-GFP compared to HYM1-GFP, a difference
that was also reflected by a ca. 15-fold higher expression level of COT1 in comparison to HYM1 (Figure S1). Moreover, both proteins also labeled constricting septa and accumulated around the septal pore of mature septa (Videos S1, S2). Deletion of hym-1 did not alter the apical and septum localization of COT1-GFP (Figure 1E), indicating that the scaffold is dispensable for proper localization of the NDR kinase. In summary, these data establish HYM1 as a functional component of the COT1-POD6 kinase complex. However, in contrast to the situation in unicellular yeasts [27,28,43,44], HYM1 is not essential for MOR signaling in N. crassa. HYM1 is essential for MAK2 MAP kinase activity

Mutations in the cot-1, pod-6 or mob-2a/2b genes, which define the central components of the N. crassa MOR pathway, share characteristic defects, such as the inhibition of polar tip extension and the initiation of extension-arrested new tips along the entire cell [33–35,37,39]. In contrast, Δhym-1 did not display this barbed-wired appearance, but generated defects that were highly reminiscent of strains deficient in components of the mak-2 MAP kinase pathway [10,12,13,45]. Both, Δhym-1 and Δmak-2 mutants were characterized by a reduced hyphal growth rate (ca. 35% of wild type) and highly knobby cell morphology, stunted aerial mycelium formation and de-repressed conidiation. In addition, both mutants were unable to develop female sexual structures, called proteroperithecia (Figure 2A, 2B). Moreover, similar to MAK2 pathway mutants, germinating conidia of Δhym-1 or hyphal cells within an established Δhym-1 colony showed no mutual attraction and self-fusion (Figure 2C, 2D).

A similar cell fusion deficiency is described for strains defective in components of the MAK1 cell wall integrity MAP kinase pathway [12,46]. However, the relationship between the MAK1 and MAK2 MAP kinase modules during hyphal fusion is unresolved. To test if HYM1 might influence the activity of these signaling modules, we compared the phosphorylation status of the two MAP kinases in wild type and Δhym-1 by using phospho-specific antibodies against the activated proteins. In wild type cells, MAK1 and MAK2 displayed basal activities that can be
stimulated ca. 5- to 10-fold under conditions of oxidative stress [12,47]. MAK1 displayed wild type activities in non-stressed and H2O2-stimulated Dhym-1, but MAK2 activity was completely abolished in Dhym-1 (Figure 2E). Driven by these results, we asked if HYM1 interacted with the MAK2 pathway in vivo by performing co-precipitation experiments. We detected weak, but consistent interactions between myc-HYM1 and flag- and HA-tagged versions of three kinases of the MAK2 module (Figure 3A). However, the interaction between HA-MAK2 and its upstream kinase flag-MEK2, which was used as positive control, was stronger and stable after several washes with 300 mM NaCl, while the interactions between HYM1 and the three kinases were only detected in immunoprecipitations lacking the high salt washing steps (Figure S2). Moreover, yeast two-hybrid tests between HYM1 and the three kinases of the MAK2 MAP kinase cascade were negative (Figure 3B). In control experiments, we observed interactions between NRC1 and MEK2 and between MEK2 and MAK2, confirming the functionality of the yeast two-hybrid constructs and the physical interaction of the NRC1-MEK2-MAK2 cascade. In summary, HYM1 is essential for MAK2 activity, but appears to interact with the three MAP kinases only in an indirect manner, probably as part of a larger protein complex. In line with this hypothesis, we found that the PAK kinase STE20/NCU03894 physically interacted with HYM1 and with NRC1 in yeast two-hybrid assays. The second PAK kinase present in N. crassa, CLA4/NCU00406, which we used as control, did not interact with HYM1 or NRC1 (Figure 3B).

HYM1 is required for signal transduction through the NRC1-MEK2-MAK2 kinase cascade

Based on these results, HYM1 could function upstream of the MAK2 pathway or as a component of a scaffold complex that connects the three kinases of the MAP kinase cascade. To distinguish between these two possibilities we employed constitutive activated components of the MAK2 MAP kinase module. We hypothesized that this activation would complement Dhym-1 defects in the first case, while the activation should not be transferred to the downstream components in the latter one. First, we constructed a proline 448 to serine-substituted version of NRC1. Homologous mutations have previously been shown to confer constitutive activity of related fungal MAPKKKs [48,49].

Figure 2. Dhym-1 displays phenotypic characteristics of MAK2 MAP kinase pathway mutants. (A) Phenotypic characterization of Dhym-1, Δmak-2 and wild type regarding macroscopic morphology and conidiation pattern (left panel: growth in slants for 5 days on minimal medium), hyphal morphology (upper right panel; bar), and protoperithecia formation (lower right panel). (B) Production of conidiospores was quantified by counting conidia generated in slants grown at room temperature for 5 days (n = 5; standard deviations are indicated as bars). (C) HYM1 and MAK2 are required for vegetative cell fusion. Hyphal (left panel) and germling fusion events (right panel; fusion events are indicated by arrows) were assessed by light microscopy in wild type, Dhym-1 and Δmak-2 cultures. Cell fusion was not observed in Dhym-1 and Δmak-2. (D) Quantification of cell fusion competence and formation of forced heterokarya that grew on double-selective media supplemented with 150 μg/ml hygromycin and 20 μg/ml nourseothricin. 10⁶ hygromycin-resistant conidiospores of the indicated strains ("wild type" carried an ectopically integrated (EC) hygromycin-resistance cassette, while the hygromycin-resistance cassette was used to replace the two gene deletions in Dhym-1 and Δmak-2) were plated alone (second column) or in decreasing concentrations together with a second "wild type" strain that carried an ectopically integrated nourseothricin-resistance cassette (column 3–6). Column 1 indicated lack of growth of the "wild type" nat⁸ control strain. 1% sorbose was added to Vogels minimal medium to restrict radial growth of the forming colonies generated by forced heterokaryons after incubation for 3 days at room temperature. (E) HYM1 is required for MAK2 activity. Total soluble protein was extracted from the indicated strains grown in the presence or absence of 8 mM H2O2. Western blot analysis with anti-phospho-p42/p44 antibody detected activated MAK1 and MAK2, respectively. Equal loading was confirmed by re-probing the membrane with anti-tubulin antibody. A typical Western blot is shown. MAK1 and MAK2 activities from 5 independent experiments were quantified for the diagrams.

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Ectopic expression of flag-NRC1(P448S) in the N. crassa wild type and Δnrc-1 control strains increased MAK2 activity ca. 12-fold (Figure 4A; Table 1). When we expressed this construct in Δmek-2 as control, hyperactivation of MAK2 was blocked, confirming that the constitutive NRC1 signal is transmitted through the MAPK cascade. No MAK2 activity was detected when we expressed flag-NRC1(P448S) in Δhym-1. Analogous results were obtained, when we expressed a constitutive active version of MEK2 in these strains. Expression of MEK2(S212D;T216D) resulted in MAK2 hyperactivation in wild type and Δmek-2, but not in Δhym-1. Thus, HYM1 is required for signal transmission through the entire NRC1-MEK2-MAK2 MAP kinase cascade (Table 1).

Consistent with the observed MAK2 activities in these strains, expression of flag-NRC1(P448S) rescued the vegetative hyphal growth defects of Δnrc-1, but not of Δmek-2, while flag-MEK2(S212D;T216D) complemented Δmek-2 (Table 1; Figure S3). Interestingly, the two constitutive hyperactive kinase variants also increased the growth rate of Δhym-1, although we were unable to detect MAK2 activity in this strain. Thus, a basal signal transduction rate below the detection level of the p42/44 anti-ERK antibodies seems sufficient for sustained vegetative growth in the absence of HYM1. In contrast, the cell fusion defects of Δnrc-1, Δmek-2 and Δhym-1 were not complemented by flag-NRC1(P448S) or MEK2(S212D;T216D), suggesting that cell fusion requires signal based, adjustable MAP kinase activation.

To further support the finding that HYM1 promotes signal transduction through the MAP kinase module, we tested if the artificial tethering of two of the kinases rescued the Δhym-1 defects. A flag-MEK2-NRC1 fusion protein was generated and expressed in the different mutants. This construct rescued all defects of Δmek-2 and Δnrc-1 and resulted in wild type levels of MAK2 activity in both strains, confirming the functionality of the two fused kinases. Moreover, this construct resulted in wild type MAK2 activity levels when expressed in Δhym-1 and fully complemented the vegetative and developmental defects of Δhym-1 (Figure 4B, 4C). These data indicate that HYM1 functions as part of an essential adaptor complex that connects the components of the NRC1-MEK2-MAK2 cascade to allow signaling through the cell fusion MAP kinase pathway.

The three kinases of the MAK2 MAP kinase module and HYM1 are recruited to the contact zone of germling fusion pairs

The interactions of the three MAK2 pathway kinases and of the kinases with HYM1 suggest a similar subcellular localization of all proteins. We have recently shown that MAK2 is recruited to the tips of two communicating germlings in an oscillatory manner [13]. Its localization in mature hyphae or the subcellular dynamics of the two upstream kinases were unknown. We determined that MAK2-GFP expressed under the control of the ccg-1 promoter localized in the cytoplasm, was not excluded from the nuclei and accumulated in the Spitzenkörper of growing hyphal tips (Figure 5A). Moreover, MAK2 labeled constricting septa and the septal pore of mature septa (Figure 5A; Video S3). Thus, MAK2 displayed a localization pattern highly similar to the localization described for HYM1. Co-localization experiments employing MAK2-mCherry and HYM1-GFP fusion proteins confirmed the co-localization of both factors in the Spitzenkörper of growing hyphal tips and at septa (Figure 5B). To exclude that the used promoter and thus the ca. two-fold higher expression level of MAK2 affected its localization, we compared two strains that expressed MAK2-GFP under the control of the native and the ccg-1 promoter, but no differences in MAK2 localization were observed (Figures S1, S4). Functional MEK2-GFP and NRC1-GFP fusion proteins, expressed under the control of their native and the ccg-1 promoters, did not display any apical accumulation, but localized in the cytoplasm and accumulated around mature septal pores (Figure 5A). Moreover, both proteins were associated with constricting septa (Videos S4, S5). In contrast to MAK2, MEK2 and NRC1 were excluded from nuclei (Figure 5A). When we addressed the localization of the three kinases in a Δhym-1 background, we found that MAK2-GFP, but not the other two kinases, strongly accumulated in the nucleus in addition to the described localization at the hyphal tip and the septum (Figure 5C). These data indicate differential functions of
MAK2 and its upstream kinases NRC1 and MEK2 at the tips of vegetatively growing cells and suggest a function of HYM1 in regulating the nuclear versus cytosolic accumulation of MAK2.

Since MEK2, NRC1 and HYM1 are essential for germling fusion, we tested if GFP fusion constructs of these components displayed a dynamic localization to communicating germling tips, as described for MAK2 [13]. In germlings of strains expressing either one the three constructs under their native promoters barely any fluorescence was detectable. Therefore, strains carrying functional gfp fusion constructs under the control of the stronger cg-1 or tef-1 promoters were employed. MEK2-GFP showed subcellular dynamics comparable to MAK2 in communicating partner cells (Figure 6). Based on this observation and the physical interaction of the two kinases (Figure 3) we tested if the two proteins co-localized during the dynamic recruitment associated with tropic growth of fusing germlings, by employing GFP and mCherry tagged protein variants (Figure 7). While the majority of the protein aggregates that formed at the plasma membrane of communicating tips contained both kinases, we frequently observed spots containing only one of the two proteins. Together, these data indicate that MEK2 and MAK2 functionally interact at the plasma membrane of fusion tips, but that they appear to be independently recruited and/or released from these kinase complexes.

NRC1-GFP and HYM1-GFP could barely be detected in germlings carrying constructs controlled by the native or the cg-1 promoters. Expression of a functional nc-1-gfp construct under control of the tef-1 promoter [50] resulted in a weak, but detectable GFP signal in young germlings (note that in germlings Pog-1 is less active than Ptef-1). In rare cases, we observed oscillatory recruitment of the NRC1-GFP fusion protein to fusion tips, suggesting a similar subcellular dynamics of all three kinases of the MAK2 pathway (Figure 6, Figure S5). Expression of a tef-1-hym1-gfp construct resulted in detectable GFP signals, but plasma membrane recruitment and dynamics of HYM1-GFP in tropic growing cell tips could not be unambiguously determined. However, after germlings had established physical contact, HYM1 and NRC1 both clearly accumulated at the future fusion site, similar to MAK2 and MEK2, indicating that the three kinases as well as HYM1 share their highly dynamic subcellular localization during the process of germling fusion. In contrast, the NDR kinase COT1 stably localized to both tips of interacting germ tubes, which likely represents its general function in hyphal polarity establishment and maintenance (Figure 6).

Discussion

Establishing cell polarity and maintaining cellular asymmetry are essential properties that govern morphogenesis and the development of uni- and multicellular organisms. These processes require the sensing of subtle intra- or extracellular signals and the transduction of this information to various cellular outputs via intricate signaling networks. An emerging theme is that signaling cascades form molecular assemblies within cells. Their spatial organization is ensured by scaffolding proteins, which allow the compartmentalization of signaling pathways.

The findings reported in this study allow three significant conclusions. First, we show that COT1, POD6 and HYM1 physically interact, and the interaction of both kinases is dependent on HYM1. Moreover, HYM1 regulates the activity of COT1. Thus, we propose that HYM1 functions as scaffold of the COT1 NDR kinase pathway in N. crassa to bridge the two kinases of the MOR pathway. Intriguingly, and in contrast to the situation in unicellular fungi, Δhym-1 mutants do not develop the polarity defects characteristic for MOR pathway mutants in N. crassa. One possible interpretation of these data may be that the presence of HYM1 in the COT1 pathway is an evolutionary relic and not physiologically relevant. However, we consider this
hypothesis unlikely. We base this conclusion on the findings that [39]. The fact that the localization of COT1 in COT1 is required for the proper localization of the NDR kinase [39]. Moreover, Thr589 phosphorylation of dependent Thr589 phosphorylation of COT1 is required for full D may explain a phenotype that does not fully resemble the central MOR elements not display the typical hyperbranching defects characteristic for N. crassa Intriguingly, we found that also mutants in the uncharacterized fungal-specific protein with unknown function) may blur more scaffolds HYM1 and TAO3 (and potentially with SOG2 - a component of the MOR pathway in all ascomycete and basidiomycete fungi analyzed to date, [26,27,43,44], and it would be surprising, if N. crassa HYM1 would represent an exception.

Second, the co-precipitation of HYM1 with NRC1, MEK2 and MAK2, and the expression of constitutive-active NRC1 and MEK2 versions and of a MEK2-NRC1 fusion protein clearly demonstrate that HYM1 is critical for the organization of the MAK2 pathway. We also show that HYM1 co-localizes with MAK2 at the hyphal apex and with all three MAK2 pathway kinases at septa and the contact point of communicating fusion cells. However, our yeast two-hybrid data also suggest that HYM1 does not physically interact with any of the three kinases of the MAK2 cascade. Because animal MO25 functions as master regulator for multiple STE20-like kinases [32], we speculate that additional kinase(s) of this group may bridge HYM1 with the three MAK2, and the expression of constitutive-active NRC1 and MEK2 versions and of a MEK2-NRC1 fusion protein clearly demonstrate that HYM1 is critical for the organization of the MAK2 pathway. We also show that HYM1 co-localizes with MAK2 at the hyphal apex and with all three MAK2 pathway kinases at septa and the contact point of communicating fusion cells. However, our yeast two-hybrid data also suggest that HYM1 does not physically interact with any of the three kinases of the MAK2 cascade. Because animal MO25 functions as master regulator for multiple STE20-like kinases [32], we speculate that additional kinase(s) of this group may bridge HYM1 with the three kinases of the MAK2 module as part of a functional HYM1-kinase complex that organizes the MAK2 cascade. This hypothesis is supported by the physical interaction of HYM1 and NRC1 with the N. crassa PAK kinase STE20, but not the related PAK kinase CLA4 in our yeast two-hybrid experiments. Interestingly, Δste-20 is fusion competent and has only mild growth defects (data not shown), indicating that additional components must be part of the signal receiving machinery of the self-fusion pathway. Furthermore, our data indicate that self-signaling during germling fusion

### Table 1. Phenotypic characteristics of constitutive hyperactive NRC1 and MEK2 variants in the indicated strains.

|                  | his-3 | nrc-1:his-3 | nrc-1(P448S):his-3 | mek-2:his-3 | mek-2(DD):his-3 |
|------------------|-------|-------------|-------------------|-------------|-----------------|
| Growth rate*     | 3.8(±0.2) | 3.2(±0.1)  | 3.3(±0.2)         | 3.6(±0.2)  | 3.1(±0.1)       |
| Aerial mycelium  | wild type | wild type  | reduced           | wild type  | wild type       |
| Conidiation      | wild type | wild type  | reduced           | wild type  | wild type       |
| Protoperithecia  | wild type | wild type  | delayed           | wild type  | wild type       |
| Germling fusion  | wild type | wild type  | wild type         | wild type  | wild type       |
| MAK2 activity**  | 100% | 120% (±15%) | 1260% (±35%)     | 125% (±15%) | 1120% (±30%)    |

|                  | Δnrc-1:his-3 | Δnrc-1:nrc-1:his-3 | Δnrc-1:nrc-1(P448S):his-3 | / | / |
|------------------|-------------|-------------------|--------------------------|---|---|
| Growth rate      | 1.6(±0.2)   | 3.3(±0.1)         | 3.1(±0.2)                | / | / |
| Aerial mycelium  | no          | wild type         | reduced                  | / | / |
| Conidiation      | derepressed | wild type         | reduced                  | / | / |
| Protoperithecia  | no          | wild type         | very few                 | / | / |
| Germling fusion  | no          | wild type         | no                       | / | / |
| MAK2 activity    | 0%          | 115% (±25%)       | 1410% (±25%)             | / | / |

|                  | Δhym-1:his-3 | Δhym-1:nrc-1:his-3 | Δhym-1:nrc-1(P448S):his-3 | Δhym-1:mek-2:his-3 | Δhym-1:mek-2(DD):his-3 |
|------------------|-------------|-------------------|--------------------------|-------------------|-----------------------|
| Growth rate      | 1.4(±0.1)   | 1.4(±0.3)         | 2.9(±0.2)                | 1.4(±0.3)         | 2.8(±0.2)             |
| Aerial mycelium  | no          | no                | no                       | no                | no                     |
| Conidiation      | derepressed | derepressed       | derepressed              | derepressed       | derepressed           |
| Protoperithecia  | no          | no                | very few                 | no                | very few              |
| Germling fusion  | no          | no                | no                       | no                | no                     |
| MAK2 activity    | 0%          | 0%                | 0%                       | 0%                | 0%                     |

|                  | Δmek-2:his-3 | / | Δmek-2:nrc-1(P448S):his-3 | Δmek-2:mek-2:his-3 | Δmek-2:mek-2(DD):his-3 |
|------------------|-------------|---|--------------------------|-------------------|-----------------------|
| Growth rate      | 1.3(±0.1)   | / | 1.3(±0.3)                | 3.1(±0.3)         | 2.8(±0.4)             |
| Aerial mycelium  | no          | / | no                       | wild type         | reduced               |
| Conidiation      | derepressed | / | derepressed              | wild type         | reduced               |
| Protoperithecia  | no          | / | no                       | wild type         | very few              |
| Cell fusion      | no          | / | no                       | wild type         | no                     |
| MAK2 activity    | 0%          | / | 0%                       | 130% (±20%)       | 1090% (±35%)           |

*In cm/day.
**Basal MAK2 activity was determined as described in Material and Methods; MAK2 activity was calculated relative to wild type, whose activity was set to 100% (n=3).
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involves specific requirements for the regulation of the MAK2 module in addition to a general function of MAK2 in hyphal morphogenesis. Constitutive hyperactivation of NRC1 and MEK2 rescues the vegetative growth defects of MAK2 pathway mutants, but not their fusion defects. Together with our observation on the subcellular localization of MAK2 and its upstream kinases, this indicates that self-signaling, but not continuous hyphal growth requires a regulated on-off switch of the MAP kinase module.

Third, the individual components of the MAK2 kinase cascade display distinct localization behaviors. During self-communication and germling fusion, MEK2 and MAK2 show similar oscillating localization to the plasma membrane of interacting tips, but aggregates containing only one of the two kinases were frequently detected. The signal of the upstream kinase NRC1 was very weak and often close to the detection limit. Nevertheless, it appears to underlay a similar subcellular dynamics. This indicates that...
membrane-bound signaling complexes do not necessarily contain equimolar amounts of the three kinases required for self-communication, as also described for the homologous kinases of the yeast mating pathway [51]. Interestingly, MAK2 localizes in the cytosol and the nucleus, while MEK2 and NRC1 are exclusively cytosolic proteins. This suggests that only the MAP kinase shuttles between the two compartments. Moreover, MAK2 accumulates in the nucleus in Δhym-1 cells. This may indicate that HYM1 promotes cytosolic sequestration of MAK2. Alternatively, MAK2 in Akym-1 cells is inactive and may therefore accumulate in the nucleus. During vegetative growth of mature hyphae MAK2 accumulates in the Spitzknöpfen of the hyphal apex, while MEK2 and NRC1 are not enriched at the cell tips of hyphae. Nevertheless, all three kinases localize to the forming septum and around the mature septal pore. We have no explanation for the septum association of the MAK2 cascade, but an increasing array of signaling machinery is found to be associated with the septal pore in various filamentous fungi. Thus, this and other studies identify the septal pore as a potential signaling hub within the fungal cell (summarized in [42,52]). The different localization studies identify the septal pore as a potential signaling hub within the fungal cell (summarized in [42,52]). The different localization behaviors of the three MAP kinase components are also reflected by different protein abundances of the three kinases. MAK2 is expressed at ca. 10-fold higher level than MEK2 and NRC1 when the three proteins are expressed from their native promoters. The expression level of H YM1 is comparable to those of NRC1 and MEK2. The relative protein abundance might thus reflect the hierarchical order within the kinase cascade.

In summary, we conclude that HYM1 functions as scaffold of the COT1 NDR kinase complex and as essential regulator of the MAK2 MAP kinase cascade. We have previously identified a complex and interdependent genetic relationships between cot-1 and nrc-2 mutants [12]. Thus, we propose that this dual use of a common regulator in the two pathways may promote the coordination of intercellular communication, tropic tip growth and cell polarity. Further analysis is required to thoroughly test this intriguing hypothesis and to unravel this signaling network, which is essential for efficient fungal growth and adaptation.

Materials and Methods

Strains, media, and growth conditions

Strains used in this study are listed in Table S1 (see also [53]). General genetic procedures and media used in the handling of N. crassa are available through the Fungal Genetic Stock Center (www.fgsc.net).

Two-hybrid plasmids and methods

The Matchmaker Two-Hybrid system 3 (Clontech, USA) was used according to the manufacturer’s instructions. cDNA of genes of interest for two hybrid tests was amplified with primers listed in Table S2 spanning the coding region from start to stop codon as annotated by the N. crassa database (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html) and cloned either into the pGADT7 vector containing the GAL4 activation domain or into pGBK7 containing the DNA-binding domain. Fusion proteins were (co-) expressed in S. cerevisiae AH109 and potential interactions determined by growth tests on SD medium lacking the amino acids adenine, histidine, leucine and tryptophane.

Plasmid construction and fungal expression of tagged proteins

Modification of the endogenous cot-1 and hym-1 loci to allow expression of C-terminal GFP-tagged fusion proteins was achieved by PCR-amplification of the ORFs of both genes and 1 kb fragments of their 3’UTRs using genomic DNA with primers that incorporated XhoI and SalI/BamHI sites, respectively, at the ends. The fragments were cloned into the vector pGFP::hpl::loxP [54] and transformed in Δmus-52::barR;his-3 to ensure homologous recombination with the endogenous cot-1 and hym-1 loci. Transformants were backcrossed with wild type to remove the Δmus-52::barR mutation and the correct genotype was confirmed by Southern analysis.

To obtain strains for the subcellular localization of MEK2 and NRC1, plasmids containing either gfp- or mCherry-fusion constructs were built based on the described plasmid pMF272 [55]. The ORFs of nrc-1 and cot-1 were amplified by PCR as annotated by the N. crassa database (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html) using primers listed in Table S2 and introduced via XhoI/PacI or BamHI/PacI, respectively, into pMF272. Constructs containing nrc-1 and cot-1 with the native promoters were obtained by using a forward primer binding 1 kb upstream of the ORF and the reverse primers described for the ccg-1 constructs, as listed in Table S2, and integrated into pMF272 using NotI and PacI, thereby replacing the ccg-1 promoter. Plasmids containing the tef-1 promoter were constructed by amplifying the 0.9 kb promoter region from the described plasmid pAB621 [50] using primers listed in Table S2 containing a T to A replacement at position 38, thereby destroying a present XhoI restriction site. The fragment was inserted into pMF272 using NotI and XhoI, replacing the ccg-1 promoter. mCherry constructs were obtained by replacing sgfp by mCherry, using the restriction sites PacI and EcoRI. The final plasmids were transformed into his-3 and his-3;trp-1;his-3, respectively, and the transformants were selected on media lacking histidine. For co-localization experiments plasmids were additionally transformed into his-3;trp-1 and his-3;trp-1 and selected on media supplemented with nicotinic acid or tryptophane.

To allow expression of myc/flag/HA-tagged fusion proteins from the his-3 locus, the candidate ORFs were amplified with primers listed in Table S2 spanning the coding region from start to stop codon as annotated by the N. crassa database and cloned into the vectors pFLAGN1, pHAN1 or pCG1-3×MYC [54,56] to allow expression of N-terminally tagged flag-NRC1, flag-MEK2, HA-MAK2 and myc-HYM1. Constitutive-active versions of flag-NRC1 and flag-MEK2 were generated by site-directed mutagenesis according to the manufacturer’s instructions (Stratagene, USA). For generation of the flag-MEK2-NRC1 fusion construct, the ORF of nrc-1 was amplified by PCR and introduced via BamHI into vector pFLAGN1-NRC1. The final plasmids were transformed into his-3, his-3;trp-1 or trp-1;his-3, respectively, and were selected for complementation of the his-3 auxotrophy. Immunoprecipitation was performed with cell extracts from fused, heterokaryotic strains that were selected by their ability to grow on minimal media lacking supplements [37]. All fusion constructs were also expressed at the his-3 locus of the respective deletion mutant and tested for full complementation of the deletion mutant defects.
Microscopy

Low magnification documentation of fungal hyphae or colonies was performed as described [36] using an SZX16 stereomicroscope, equipped with a Colorview III camera and CellB imaging software (Olympus). Images were further processed using Photoshop CS2 (Adobe). Fluorescence microscopy was performed as described [57,58]. An inverted Axio Observer. Z (Zeiss) microscope equipped with a QuantEM 512SC camera (Photometrics) and the slidebook 5.0 software (Intelligent Imaging Innovations), or an Zeiss Axiohot 2 equipped with a pco pixelfly camera and a modified version of 4D microscopy software [59], programmed by Christian Hennig and Ralf Schnabel, were used for image acquisition. Images were deconvolved using SVI Huygens Essential software and further processed using ImageJ.

Protein methods

Liquid N. crassa cultures were grown at room temperature, harvested gently by filtration using a Buchner funnel and ground in liquid nitrogen. Immunoprecipitation and cot1 kinase activity assays were performed as described [37,39]. Monoclonal mouse α-HA (clone HA-7, Sigma Aldrich, Germany), monoclonal mouse α-FLAG M2 antibody (Sigma-Aldrich, Germany), monoclonal mouse α-myc (9E10, Santa Cruz, USA), monoclonal mouse α-GFP (Invitrogen GmbH, Germany) antibodies and GFP-Trap beads (ChromoTek, Germany) were used in this study.

Protein extraction for the analysis of the MAK2 phosphorylation status was performed as described in [47] with minor modifications. Briefly, the frozen mycelial powder was incubated in 95% ethanol at -20°C for ≥12 h, the supernatant removed after centrifugation and the pellet vacuum-dried in a SpeedVac concentrator (Thermo Fisher Scientific, USA). Extraction buffer (100 mM Tris pH 7.0, 1% (v/v) SDS; supplemented with 5 mM NaF, 1 mM PMSE, 1 mM Na2VO4, 25 mM β-glycerophosphate, 2 mM benzamidine, 2 mg/μl pepstatin A, 10 mg/μl aprotinin, 10 mg/μl leupeptin) was added, the samples mixed and incubated at 80°C for 5 min and the supernatant collected after centrifugation. After a second round of extraction, the supernatants pooled, subjected to another centrifugation step, and the protein concentration determined using a Nanodrop spectrophotometer (ND-1000, Peqlab, Germany). Sample volumes corresponding to 75 μg total protein per lane were subjected to SDS polyacrylamide gel electrophoresis and subsequent Western blotting using polyclonal rabbit α-Phospho-p42/44 MAPK (Cell Signaling Technology, Inc., USA) and goat α-rabbit IgG-HRP (Santa Cruz, USA) as primary and secondary antibodies, respectively. For quantification of MAK1 and MAK2 phosphorylation levels, exposed films were scanned at a resolution of 600 dpi and densitometry was performed on the resulting tiff-files employing the AIDA Image Analyzer (version 4.22; raytest Isopenmessgerate, Germany) in transmission mode. Intensity values [arbitrary units] measured within a region of interest of fixed size containing the MAK1 or MAK2 protein bands were corrected by subtraction of local background, normalized to the protein amount loaded and used for further evaluation.

Supporting Information

**Figure S1** Expression analysis of the used GFP fusion constructs under the control of the ccg-1 and their native promoters. (A) Anti-GFP Western blot of normalized cell extracts of strains expressing GFP fusion proteins under the control of the indicated promoters. (B) Quantification of the relative expression levels of the indicated proteins under the control of their native promoters. Protein levels are normalized to MAK2 abundance (n = 3). (C) Quantification of the relative expression levels of the indicated proteins under the control of the indicated promoters. Protein levels are normalized to ccg-1 promoter driven expression (n = 3).

**Figure S2** HYM1 has a weaker binding affinity to components of the MAK2 pathway than the kinases among each other. Co-immunoprecipitation experiments between HYM1 and MAK2 (A) and between MAK2 and MEK2 (B), in which the bound precipitates were either washed twice with lysing buffer (left panels) or three times in lysing buffer supplemented with 300 mM NaCl (right panels). Note that the interaction between HYM1 and MEK2, but not between MAK2 and MEK2 is abolished under the high salt washing conditions.

**Figure S3** Phenotypic characteristics of constitutive hyperactive NRC1 and MEK2 variants. Phenotypic characterization of the strains described in Table 1 regarding macroscopic morphology and conidiation pattern (left panel), germling fusion (upper right panel), and protoperithecia formation (lower right panel).

**Figure S4** The promoter strength does not influence the apical localization of MAK2-GFP. Localization of MAK2-GFP expressed under the control of the ccg-1 promoter (A) and its native promoter (B) resulted in comparable localization patterns at the hyphal tip and the septum.

**Figure S5** Oscillating recruitment of NRC1-GFP to the tips of interacting germings. NRC1-GFP is expressed under the control of the tef-1 promoter. Between the two time points shown, tip localization has switched between the two germings. Upper images: DIC, lower images: GFP Fluorescence.

**Table S1** Neospora crassa strains used in this study.

**Table S2** Primers used in this study. Restriction enzyme recognition sites are indicated in bold, lower case letters and mismatched nucleotides for insertion of mutations are depicted in italic, lower case letters.

**Video S1** Time-course of COT1-GFP localization during septum formation. COT1-GFP formed cortical rings at incipient septation sites that constricted during septum formation and accumulated around the septal pore of the completed septum (a) GFP channel; (b) FM4-64 channel; (c) merged. The plasma membrane was stained with FM4-64. Images were captured at 15 sec. intervals.

**Video S2** Time-course of HYM1-GFP localization during septum formation. HYM1-GFP formed cortical rings at incipient septation sites that constricted during septum formation and
accumulated around the septal pore of the completed septum (a) GFP channel; (b) FM4-64 channel; (c) merged. The plasma membrane was stained with FM4-64. Images were captured at 15 sec. intervals. (AVI)

**Video S3** Time-course of MAK2-GFP localization during septum formation. MAK2-GFP formed cortical rings at incipient septation sites that constricted during septum formation and accumulated around the septal pore of the completed septum (a) GFP channel; (b) FM4-64 channel; (c) merged. The plasma membrane was stained with FM4-64. Images were captured at 15 sec. intervals. (AVI)

**Video S4** Time-course of MEK2-GFP localization during septum formation. MEK2-GFP formed cortical rings at incipient septation sites that constricted during septum formation and accumulated around the septal pore of the completed septum (a) GFP channel; (b) FM4-64 channel; (c) merged. The plasma membrane was stained with FM4-64. Images were captured at 15 sec. intervals. (AVI)

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### Author Contributions

Conceived and designed the experiments: SS AF. Performed the experiments: AD JJ SM TS. Analyzed the data: AD JJ SM TS AF SS. Wrote the paper: AF SS.

Video S5 Time-course of NRC1-GFP localization during septum formation. NRC1-GFP formed cortical rings at incipient septation sites that constricted during septum formation and accumulated around the septal pore of the completed septum (a) GFP channel; (b) FM4-64 channel; (c) merged. The plasma membrane was stained with FM4-64. Images were captured at 15 sec. intervals. (AVI)
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