Importance of MAP Kinases during Protoperithecial Morphogenesis in *Neurospora crassa*

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**Abstract**

In order to produce multicellular structures filamentous fungi combine various morphogenetic programs that are fundamentally different from those used by plants and animals. The perithecium, the female sexual fruitbody of *Neurospora crassa*, differentiates from the vegetative mycelium in distinct morphological stages, and represents one of the more complex multicellular structures produced by fungi. In this study we defined the stages of protoperithecial morphogenesis in the *N. crassa* wild type in greater detail than has previously been described; compared protoperithecial morphogenesis in gene-deletion mutants of all nine mitogen-activated protein (MAP) kinases conserved in *N. crassa*; confirmed that all three MAP kinase cascades are required for sexual development; and showed that the three different cascades each have distinctly different functions during this process. However, only MAP kinases equivalent to the budding yeast pheromone response and cell wall integrity pathways, but not the osmoregulatory pathway, were essential for vegetative cell fusion. Evidence was obtained for MAP kinase signaling cascades performing roles in extracellular matrix deposition, hyphal adhesion, and envelopment during the construction of fertile protoperithecia.

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**Introduction**

The perithecium is the female sexual reproductive organ, or fruitbody, of *Neurospora crassa* within which *ascospores*, the products of meiosis, are generated [1,2]. The perithecium is composed of at least 14 morphologically distinct cell-types [2,3], and is formed by various processes including: hyphal aggregation; adhesion; septation; branching; and cell differentiation. As a result of these processes, filamentous fungi achieve multicellularity in a way that is fundamentally different from that in plants or animals, with the important point being that fungal tissues and organs are formed from the growth, aggregation and differentiation of hyphae [2,4]. Fruitbody morphogenesis in *N. crassa* provides an excellent model system for the study of fungal multicellular development. Perithecium morphogenesis in *N. crassa* and other members of the Sordariomycetes (e.g. *Sordaria* spp., *Podospora* spp., *Gelasinospora* spp. and *Chaetomium* spp.) has three main stages: ascogonial, protoperithecial and perithecial, each involving the differentiation of several morphologically distinct cell-types [2-3]. The ascogonial stage of development is observed as a very small (5–20 µm in diameter) coiled hyphal branch, also described in *N. crassa* as a hyphal knot [2,3]. The protoperithecial stage is initiated by enveloping hyphae [3] that wrap around the ascogonium [3] to form an almost spherical, or more specifically subpherical, structure [5]. Initiation of the protoperithecial stage represents a key morphogenetic event, when differentiation of distinct, multicellular tissues commences [2]. Fruitbody expansion and internal differentiation lead to the mature ‘female’ protoperithecum. Protoperithecia of *N. crassa* can form one or more trichogynes. Each trichogyne is a specialized ‘female’ hypha that is required for non-self fusion with cells of the opposite mating-type. Trichogynes can grow to several hundred micrometers in length [6] and can form branches [7]. The peptide sex-pheromone [8,9] released by the ‘male’ triggers the homing response of ‘female’ trichogynes [6,7]. The fertilizing agent (spermatium) may be: an asexual spore, the conidium, of which there are three types in *N. crassa* (*macroconidia, microconidia* and *arthroconidia* [3]); a germinated ascospore (meiospore); or indeed any vegetative cell or hypha of the mating partner [10,11]. In *in heterothallic* species, such as *N. crassa*, fertilization by an opposite mating-type ‘male’ partner provides the necessary signal for the transition from the protoperithecial to the perithecial stage of fruitbody development [12]. In *homothallic*, self-fertile Sordariomycetes such as *Sordaria macrospora*, that lack any type of conidium or trichogyne, mating and hence, non-self fusion is not a requirement for progression to the perithecial stage [2,12,13,14,15]. However, certain members of the Sordariomycetes possess ascI with four ascospores and each ascospore contains nuclei of opposite mating-type. As a result, each ascospore behaves as if it were homothallic; this condition is termed secondary homothallism (or pseudohomothallism) [16,17,18]. In secondary homothallic species, such as
N. tetrasperma, mating involving trichogyne and spermatium fusion does not commonly occur [16,17,18,19].

Upon successful mating-cell fusion (plasmogamy) in N. crassa, the male nucleus travels through the trichogyne into the ascogonium [3] of the yet unfertilized protoperitheium. Arrival of the ‘male’ nucleus inside the ascogonium induces continued differentiation and further expansion of the protoperitheium. It also initiates the dikaryotic phase of the life cycle, which is restricted to the developing ascogenous hyphae [3] within the differentiating perithecium [11,20]. Increasing melanization of the perithecial wall cells ultimately leading to the almost-black mature peritheium, is a visual marker of continued sexual development upon fertilization [21]. In N. crassa, dikaryotic cells are generated and maintained by the formation of specialized hyphal compartments called croziers [22]. Recurrent cycles of crozier cell fusion generate binucleate cells subtending the tip cell of ascogenous hyphae [1,22,23], inside which subsequent nuclear fusion (karyogamy) yields a very short-lived diploid stage prior to meiosis, ascospore development and ascospore delimitation [24,25]. Whether crozier fusion can be considered a non-self fusion or a self-fusion process, i.e., regulated by genes expressed from both parent nuclei or from only one, remains unresolved.

Mitogen-activated protein (MAP) kinase phosphorylation cascades are highly conserved and well-characterized signaling pathways in eukaryotes [26,27,28]. In Saccharomyces cerevisiae, a total of 16 MAP kinases constitute five partially overlapping signaling pathways that are involved in regulating pheromone-induced mating, filamentous growth, cell wall modification and repair, responses to high osmolarity and ascospore wall assembly [26,29,30,31,32,33]. Three-tiered MAP kinase modules comprising orthologs to nine of the budding yeast MAP kinases, have been identified in N. crassa [34,35] and are regarded to be equivalent to the pheromone response (PR) pathway, the cell wall integrity (CWI) pathway, and the osmoregulatory (OS) pathway from budding yeast [26,32]. Gene-deletion mutants of the PR-MAP kinase pathway were amongst the first hyphal-fusion mutants characterized in N. crassa [36,37,30,39], and connections between their pleiotropic phenotype and defects in fruitbody morphogenesis have been previously recognized [23,40,41]. A model summarizing all nine MAP kinase components and their functions in N. crassa, including the cross-communication with other signaling pathways involved in filamentous growth and sexual morphogenesis, has recently been provided [41]. Hitherto, studies have documented that all nine MAP kinase mutants are unable to differentiate fertile perithelia, but the specific stages of development at which defects occur have remained mostly uncharacterized. Reports of defective sexual fruitbody development resulting from MAP kinase mutations have been made in Magnaporthe grisea [42], Fusarium graminearum [43], Podospora anserina [44,45,46], Cochliobolus heterosporus [47], and Aspergillus nidulans [48,49], but overall, detailed ultrastructural studies of these defects are lacking. Furthermore, neither has a direct connection between hyphal fusion and fruitbody morphogenesis been established.

This study, firstly analyzed the key morphogenetic stages comprising protoperithecial development in the N. crassa wild-type in greater detail than has been accomplished so far, secondly addressed the specific role of MAP kinases in this process, and thirdly asked the question: to what extent do defects in vegetative hyphal fusion (VHF) influence protoperithecial morphogenesis?

Materials and Methods

Media and culture conditions

Strains were maintained on solid (2% agar) or in liquid Vogel’s minimal medium (VMM) [50] with 2% sucrose using standard N. crassa cultivation techniques [11]. For Ignite selection (bar resistance gene), NH₄NO₃ was substituted by 0.5% (w/v) proline as alternative nitrogen source to increase the potency of Ignite selection at an effective final concentration of 400 µg/ml [51]. For hygromycin B selection (hph resistance gene) [52] or nourseothricin selection (nat1 resistance gene), [53] drugs were added at final concentrations of 200 µg/ml and 30 µg/ml, respectively. To induce the sexual cycle in N. crassa, strains were grown under nitrogen- and carbon-limiting conditions on solid synthetic crossing medium (SCM) [54] and low-sucrose (0.2% sucrose in dH₂O) agar (LSA), in most cases overlaid with cellophane. Development of conidial germings, including the quantification of conidial anastomosis tube (CAT)-mediated cell fusion, was assessed as described in detail previously [55,56].

Selection of gene-deletion mutants for morphogenetic analysis

Previous work on sexual development in N. crassa has used mutant strains generated by different methods and obtained from a variety of sources (references in Table 1). Therefore, we decided to verify earlier findings using gene-deletion strains (a.k.a. gene knock-out (KO) strain) exclusively generated by homologous recombination and obtained only from one source, in this case the Fungal Genetics Stock Center (FGSC, Kansas City, Missouri, USA) [57,58]. Our analysis included new gene-deletion strains that, due to updated annotation of the N. crassa genome or problems noted by the community with older strains, recently became available. Replacement strains used in this study were ∆nrc-1 FGSC18162, ∆os-4 FGSC18202, ∆os-5 FGSC18203, and ∆os-2 FGSC17933. Homokaryons of ∆nrc-1 FGSC18162 were generated through single spore propagation of asexual conidia (micro- and macroconidia) on hygromycin B selection medium.

Polymerase chain reaction (PCR) genotyping of N. crassa gene-deletion mutants

All KO strains used in this study (Table 2) were produced and verified by Southern blotting within the NIH Neurospora Genome Knock-Out Project [59]. The genotype of the deposited strains can be looked up in the regularly updated master spreadsheet of the Neurospora Genome Project: http://www.dartmouth.edu/~neurosporagenome/knockouts_completed.html. Additionally, the replacement of targeted open reading frames (ORF) by the hph-knock-out cassette was verified for each strain within this study. For this, genomic DNA was purified after phenol/chloroform extraction and analyzed by PCR (Figures S1 and S2). Specific primer pairs were used to probe for: (1) the absence of the target gene from its original locus; (2) the presence of the hph-KO cassette at this locus; (3) the absence of the target ORF from the whole genome; and (4) the presence or absence of mus51 and mus52 loci that could indicate whether the obtained gene-deletion mutant strain had been successfully recovered after back-crossing to the wild type. When reactions were performed as multiplex PCRs [60], an additional pair of oligonucleotides binding within the actin locus (NCU04173.3) was used as an internal positive control for each reaction. Table S1 lists all primers used for genotyping in this study.
Table 1. Stages of fruitbody development accomplished by \textit{N. crassa} cell-fusion mutants used as females in heterozygous crosses with the wild type.

| References | this study, [41,89,90] | this study, [41,89,90] | this study, [41,89,90] | this study, [40,41] | this study, [40,41] | this study, [40,41] | this study, [40,41] | this study, [40,41] |
|------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|
| Ascospore germination | | | | | | | | |
| Ascospore ejection | | | | | | | | |
| Neck/ostiole development | | | | | | | | |
| Ascospore production/mutation | | | | | | | | |
| Ascogenous hyphae | | | | | | | | |
| Crozier fusion | | | | | | | | |
| Perithecium differentiation | | | | | | | | |
| Nuclear transit/fertilization | | | | | | | | |
| Trichogyne homing and fusion | | | | | | | | |
| Fertilizable protoperithecium | | | | | | | | |
| Trichogyne emergence | | | | | | | | |
| Fruitbody expansion | | | | | | | | |
| ECM deposition/hyphal adhesion | | | | | | | | |
| Enveloping hyphae | | | | | | | | |
| Septation/branching | | | | | | | | |
| Ascogonial coil formation | | | | | | | | |

Tick marks indicate completed stages of sexual development. Empty boxes show developmental stages identified as being fully blocked. (MAP3K, MAP kinase kinase kinase; MAP2K, MAP kinase kinase; MAPK, MAP kinase).

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Assessment of female and male fertility

To evaluate female fertility, gene-deletion strains were inoculated onto SCM or LSA plates and incubated for 2–4 days at 25°C in constant light. In parallel, a wild type strain of opposite mating-type was cultured on standard VMM for 2–3 days at 30°C until sufficient conidia had developed. Protoperithecia, which usually developed after 3–4 days on the KO mycelium, were fertilized with opposite mating-type ‘male’ conidia from the wild type, either 'dry' or 'wet'. For dry-fertilization, male conidia were collected on agar plates and either evenly distributed onto the female mycelium by flooding, or applied as 5 μl droplets at defined positions. The same procedure was employed to test male fertility, except that the wild type was used as female partner and conidia of the opposite mating-type 'male' conidia from the wild type, either evenly distributed onto the female mycelium by exchanging the lid onto the Petri dish lid, by inverting the culture, and subsequently transferred onto the female mycelium by exchanging the lid onto the female culture plate and gently tapping off the male conidia.

In all crosses, development of protoperithecia and the differentiation into mature perithecia was monitored under a stereomicroscope for three weeks post-fertilization. The appearance of ascospores was defined as the determining feature of successful sexual reproduction. Ascospores were collected from the Petri dish lid, microscopically analyzed and then cultured on selection medium to assess viability. In crosses where perithecia appeared within that time period, but no ascospores could be recovered, the perithecia were cracked open, using dissecting needles, to evaluate ascus and ascospore development.

Evaluation of osmosensitive MAP kinase mutants

In order to phenotypically verify putative OS-MAP kinase mutants, fungal development was tested under salt stress (3% and 6% w/v NaCl) and in the presence of the phenylpyrrol fungicide fenpiclonil (1.5 or 4.5 μM). Conidial germination assays were performed as described earlier [55]. Radial colony extension rates were assessed on VMM agar plates, and if required, supplemented with salt or fungicide as described above. For this, inoculated plates were incubated overnight (~16 h) at 25°C. The next morning, margins of four randomly chosen radii were marked in each colony. In some cases, the plates were then transferred to 35°C and extension of the colony edges was marked every 2 h for a period of 8 h. In all cases, maximal extensions rates were measured and mean extension rates of duplicate samples were calculated (n = 8 for each tested condition).
Plasmid construction

The MAK-1-gfp expression plasmid pAL1-MAK-1 was constructed by first generating the GFP expression vector pAL1 through subcloning the gfp coding region from pMF272 [61] into pBARGRG1 [62] using BamHI/EcoRI restriction/ligation, and subsequently ligating the nac-l gene amplified from N. crassa wild type cDNA using oligonucleotides mak1_BamHI_fwd 5' -GATC-CTGATTCCGATGCTGGATCTCGTG-3' and mak1_XmaI_rev 5'-GATCCCGGGAATTCGATGCTGGATCTCGTG-3' (BamHI and Xmal restriction sites underlined) into BamHI/ Xmal linearized pAL1 in-frame to gfp. Plasmid pAL1-OS-2 for the expression of OS-2-gfp was generated by amplifying the coding region for os-2 from N. crassa wt cDNA using oligonucleotides os2_1_BamHI_fwd 5'-TTTCTCGAGCATGGATGCTGGATCTCGTG-3' and os2_2_GS_if_rv 5'-AGACAGCAGCCGAGC TTGCGGCGGAAACTCCTTC-3' (underlined are the 15 bp overlaps required for recombination), then amplifying the gfp coding region from pAL1-MAK-1 using oligonucleotides GS_gfp_if_rv 5'-CCGCGGAAAGCTGATGCGGACCCGAGAAGAGG-3' and mak2_GS_GFP_if_rv 5'-AGACAGCAGCCGCCTCTACAATCTCACATGCACTGCG-3', and the coding region of gfp amplified from pAL1-MAK-1 using oligonucleotides mak2_GS_GFP_if_rv 5'-ATTATAGGCGGCTGATGCTGGATCTCGTG-3' and GFP_if_EcoRV_rv 5'-AGCTCGGTGTTACGACGTTGACACGCACAAAGAGG-3' (BamHI restriction site underlined) into the (TIFF). for DIC microscopy, an inverted Nikon TE2000-U Eclipse widefield microscope (Nikon Instruments Europe BV, UK) equipped with Wollaston polarizer, prism and analyzer was used, along with a Nikon Plan Fluor 100×/1.4 N.A. DIC H oil immersion, Nikon Plan Apo 60×/1.2 N.A. DIC H water immersion, and Nikon Plan Fluor 20×/0.5 N.A. dry objectives fitted with the corresponding DIC lens slides. Images were acquired with Nikon ACT-1 software on a Nikon digital DXM1200F color camera and stored as TIFF. For widefield fluorescence microscopy, the same microscope and objectives were used with: a CoolLED pE-2 excitation system (http://www.cooled.com/); 470 nm LED array module with a Nikon B-2A filter for GFP imaging, and a 390 nm LED array module with a Nikon UV-2A for Calcofluor White (CFW) imaging. Image capture was with a Hamamatsu Orca-ER C4742-80 camera (Hamamatsu Photonics UK Ltd, Welwyn Garden City, UK) and MetaMorph software v7.6.0 (Molecular Devices LLC, Sunnyvale CA, USA, http://www.moleculardevices.com). Samples were prepared using the inverted-agar-block method [66] and CFW staining was as previously described [67]. Optical sectioning was performed with a P-721 PICOC Z objective focusing system connected to an E-625 PZT piezo servo controller (www.physikinstrumente.com) allowing rapid z-stack acquisition with 0.2 to 0.5 μm step size. Apart from basic brightness, contrast and display range adjustments using the ImageJ freeware platform (rsbweb.nih.gov/ij/) no further manipulation, such as deconvolution, was used to prepare the raw data for presentation.

Low-temperature scanning electron microscopy (LTSEM)

All samples for LTSEM were prepared and incubated in the same way as for other applications described previously [38], either on VMM agar plates (mature hyphal colonies and conidial germlings) or SCM or LSA plates (development of protoperithecia) overlaid with sterile cellophane (525 gauge uncoated Rayophane, A.A. Packaging, Preston, UK) to allow rapid sample preparation. At desired time points ~12 mm² cellophane rectangles carrying the specimens were cut out, adhered to the cryospecimen carrier (Gatan, Oxford, UK) with Tissue-Tek OCT compound (Sakura Finetek, Torrance, USA) and then immediately cryofixed by plunging into subcooled liquid nitrogen. The specimen carrier was transferred under low vacuum to the cold stage (~120°C) of a 4700II field-emission scanning electron microscope (Hitachi, Wokingham, UK). On the stage the samples were partially freeze-dried at ~80°C to remove surface ice by sublimation; cooled down to ~120°C; sputter-coated in a Gatan Alto 2500 cryopreparation system at ~180°C and coated with ~10 nm of 60:40 gold-palladium alloy (Testbourne Ltd., Basingstoke, UK) in an argon gas atmosphere. The specimen was examined at ~160°C with a beam accelerating voltage of 2 kV, a beam current of 10 μA, and working distances of 12–15 mm. Digital images were captured at a resolution of 2560×1920 pixels using in most cases the signal from the lower secondary electron detector, and saved as TIFF.
Results

Protoperithecial development in the *N. crassa* wild type

Ascogonial coil formation. An ascogonial coil initially differentiates as a branch from a compartment of a vegetative hypha in the sub-peripheral region of the colony. It usually emerges from a trunk hypha (the thickest hyphae of the sub-peripheral mycelium) [3] or one of its branches with a thickness of 5–10 μm (Figure 1A). This ascogonial branch, with similar hyphal diameter to its 'parent hypha', immediately coils around on itself and adheres to itself to form a tight helical structure (Figure 1B), where the pitch of the helix is equivalent to the width of the coiling hypha (Figures 1B, 2A and 2B). This is very similar to observations made in *S. macrospora* [2] and *S. humana* [68]. After the tip of the ascogonial coil had made a complete revolution, septa (dividing cross-walls) were being formed in the earlier part of the coil (arrowheads in Figure 1B). These septa were positioned approximately two-thirds of a revolution of the helix apart from the previous septa (Figures 2C and 2D). Branches emerged from the septated ascogonial compartments, but not from the ascogonial-tip cell (Figures 1C and 2E). These branches, in turn, enveloped the coil, ‘hugging’ its surface whilst following the grooves formed on the surface of the ascogonial coil between adjoining hyphae (Figures 1D and 2F). These enveloping hyphae, continue to septate, branch, and further wrap around the ascogonial-coil core, referred thereafter as the *ascogonium*. Enveloping hyphae are often narrower (<5 μm width) than the ascogonial-coil hypha. Residues of extracellular matrix (ECM) secretion, presumably required for the tight adhesion between the revolutions of the ascogonial coil to itself and between the coil and enveloping hyphae, are shown in enlarged views in Figures 1G and 1H.

Protoperithecial expansion. After enveloping hyphae which originated from the ascogonial compartments have enlarged the structure to a diameter of 20–30 μm, additional enveloping hyphae emerge, either as branches of the initial enveloping hyphae (Figure 1D) or from neighboring vegetative hyphae and envelop the young fruitbody further. ECM accumulates on the outer surface of the forming protoperitheium at this early stage of development, and eventually covers the whole structure evenly (Figure 1I). Usually, after the structure has expanded to a diameter of 40–50 μm and enveloping hyphae have established a compact casing around the ascogonium, the product of fruitbody expansion is a subspherical protoperitheium (Figure 1E).

Trichogyne emergence. Protoperithecial expansion in *N. crassa* usually arrests when the fruitbody has reached a diameter of 80–100 μm. At this stage, elongated trichogynes will have emerged (arrowhead in Figure 1F) resembling the endpoint of female-autonomous protoperithecum maturation. For the transition into peritheicum morphogenesis, non-self, mating cell-fusion is required. Figure 3 summarizes the main stages of sexual fruitbody development in *N. crassa* schematically. Table 3 provides an overview of the range of sizes observed throughout these main morphogenetic stages.

Genotypic verification of MAP kinase gene-deletion mutants

PCR-based genotyping confirmed targeted gene deletion in all MAP kinase mutants. PCR genotyping confirmed that the targeted open reading frames have been successfully exchanged for the *lpkH*-gene deletion cassette in all MAP kinase KO mutants used in this study (Figures S1 and S2). Residual presence of wild type genes indicated weak heterokaryotic background for three of the nine strains, however, this can be regarded as insignificant as it did not alter the dominant mutant phenotypes of these strains (for additional information please refer to Figure S1 and S2 legends.)

MAP kinase mutant phenotypes co-segregated with *hygromycin B* resistance. PR- and CWI-MAP kinase gene-deletion strains identical to those used in this study have previously been verified as being correct by back-crossing and co-segregation analyses [69]. Very recently, the same analysis has been conducted for all OS-MAP kinase mutants available from the FGSC by the same group, showing significant co-segregation of the mutant phenotype with the hygromycin B-resistance marker in the evaluated progeny of the ‘new’ as mutants: Δos-4 FGSC18202 (91%), Δos-5 FGSC18203 (95%) and Δos-2 FGSC17933 (100%) (ratio of co-segregation indicated as percentages). In contrast, the same mutant phenotype did not co-segregate in any of the ‘old’ (Δos-4, FGSC11479; Δos-5, FGSC11490 and Δos-2, FGSC11436) OS-MAP kinase mutant progeny (S. Free, pers. comm.). This data, taken together with our own PCR-based genotyping analyses (previous section) and the consistency of the mutant phenotypes within and between the MAP kinase cascades (see following sections), provide very strong evidence that the developmental phenotypes described in this study are exclusively caused by the targeted mutations in all of the nine MAP kinase gene-deletion strains used.

Vegetative morphogenetic defects in MAP kinase mutants

Colony phenotypes of MAP kinase gene-deletion mutants were distinct between the MAP kinase pathways, but conserved within each cascade. As reported earlier [40,41], CWI-MAP kinase (Δmek-1, Δmek-2 and Δmek-1) mutants showed rosette-like colony growth caused by areas of increased mycelial autolysis as typical features (Figure 4A). Mutants of the PR-MAP kinase pathway (Δos-1, Δos-2 and Δos-1) were characterized by short aerial hyphae and conidiation starting from the colony center (Figure 4B), which was also consistent with previous findings [36,37,41]. MAP kinase mutants from the OS-MAP kinase pathway displayed ‘sticky’ and intensively orange-colored macroconidiophores (conidia-bearing hyphae) or macroconidia, with increased macroconidiation typically occurring around the edge of the culture dish rather than in the colony center (Figure 4C). The morphological alterations during conidiogenesis in *os* mutants have previously been connected to conidial lysis [41,70,71]. We, however, did not observe this under our tested conditions. Hyphal lysis, including ‘bleeding’ of intensely orange colored droplets, nevertheless, did occur within the vegetative mycelium (data not shown), presumably from ruptured hyphal tips [72].

Defects during fruitbody morphogenesis in MAP kinase mutants

Deletion of MAP kinases exclusively affected female fertility. None of the tested MAP kinase mutants generated progeny when used as a female in heterozygous crosses with a wild-type male. Whereas, ascospores were produced in reciprocal and confrontation crosses (where the mutant effectively acted as male) suggesting that male fertility, i.e. the ability of cells of a particular strain to participate successfully in a non-self fusion event leading to fertilization of an opposite mating-type female, is uncoupled from the self-fusion defect.

Where available, both mating types of a particular gene-deletion strain were tested; however, mating-type dependent effects were never evident. Decreased ascospore viability was generally observed in the progeny recovered from heterozygous crosses involving MAP kinase mutant strains, as previously reported [23]. Nevertheless, the production of meiotic progeny involving a
mutant strain as male contributor unambiguously demonstrated successful completion of the sexual cycle despite the genetic defect. Impairment of ascospore germination (a.k.a. ascospore lethality) due to genetic defects of the mutant progeny is, by our definition, a problem associated with the subsequent vegetative growth phase.

Defects during protoperithecial morphogenesis were evident at the ultrastructural level. Assessment of female-autonomous fruitbody development by stereomicroscopy showed that six out of nine MAP kinase mutant-strains developed protoperithecial-like structures beyond the stage of ascogonial coils, the exceptions being the α mutants that did not initiate sexual development under the test conditions (Figure 5). In comparison to the wild type, which after 3–4 days post-inoculation formed abundant, quite regular and subspherical protoperithecia

Figure 1. Protoperithecial morphogenesis of *N. crassa* wild type. LTSEM of the main stages of protoperithecial development. (A) Two ascogonial coils differentiated from the vegetative mycelium of a two day-old culture. These two coils have formed on branches (bh) off the main arterial trunk hyphae (th). Some of the surrounding branches have fused with each other, they are therefore considered to be fusion hyphae (fh). Vegetative hyphal fusion is instrumental in the establishment of a fully co-operative interconnected mycelium. Scale bar, 50 μm. (B) Higher magnification of the ascogonial coil boxed in (A). On careful inspection a septum can be seen on the lower part of the coil (aligned with arrowheads). Scale bar, 5 μm. (C) A slightly expanded ascogonial coil again formed on a side branch of a trunk hypha, the coil is being wrapped around by enveloping hyphae. Scale bar, 20 μm. (D) A slightly later stage where enveloping hyphae (arrowheads) originating from the ascogonium have wrapped around the central ascogonial coil (ac). These enveloping hyphae exhibit septation and branching. The ‘parent hypha’ (ph) of the ascogonial coil can be clearly defined, and is separated from the developing fruitbody by a basal septum (bs). (E) The subepithelial shape of the protoperithecium becomes evident after additional enveloping hyphae have formed a protective casing around the ascogonium. Trunk hyphae (th), their branches (bh) and fusion hyphae (fh) can be clearly distinguished. Scale bar, 5 μm. (F) Mature protoperithecium, with visible ECM secretion ‘gluing’ enveloping hyphae together, and a trichogyne (arrowhead) emerging from its center. Scale bar, 20 μm. (G) Enlarged view of the boxed area in (D) showing ECM strands between hyphae (arrowhead). Scale bar, 2 μm. (H) Enlarged view of the boxed area in (B) showing ECM strands (arrowhead) between the tightly attaching revolutions of the ascogonial coil. Scale bar, 1 μm. (I) Enlarged view of the boxed area in (F) showing the surface hyphae of the protoperithecium evenly covered in ECM. Scale bar, 5 μm.

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that were 80–100 μm in diameter, the frequency, size and shape of developing, protoperithecial-like hyphal aggregates varied considerably between different mutants grown under identical conditions. Fertilization of the female mutant mycelium with opposite mating-type wild-type conidia did not trigger further fruitbody differentiation. More conclusive morphological details of these structures, however, were not discernible with this simple technique and led to the employment of LTSEM for more detailed microscopic analysis. Structural definition of the developed protoperithecial precursors was generally improved when strains were cultured on LSA compared to SCM. Furthermore, the more efficient suppression of conidiogenesis on LSA in comparison to SCM greatly facilitated observations and sample preparation. Special care was taken to ensure that any unusual morphological features or altered surface properties were not due to preparation artifacts, by having an identically prepared wild-type control on each cryospecimen carrier alongside the actual samples. The sublimation of surface ice during partial freeze-drying after cryofixation [73,74] was the key advantage over light microscopy techniques that allowed the true three-dimensional surface topology of the developing protoperitheium to be resolved (Figure 6). Key morphogenetic features typical for each of the mutants of the three MAP kinase cascades are described in the following sections.

**OS-MAP kinase pathway: excessive ECM secretion might prevent ascogonial coil formation.** The OS-MAP kinase pathway controls multiple cellular stress responses and, in sequential interaction with the CWI-MAP kinase pathway, is required for cell survival upon cell wall damage [73,76]. Commonly reported phenotypic defects in mutants of the three
The inability to grow on hyper-osmotic medium (e.g. 3% NaCl or LSA at 25°C) resulted in relatively small colonies in plates up to a diameter of 30 cm. The latter occurred on SCM or LSA at 25°C in plates up to a diameter of 30 cm.

**CWI-MAP kinase pathway: defects in hyphal aggregation and adhesion aborted protoperithecial maturation.** The CWI-MAP kinase pathway senses and responds to cell-wall stress during vegetative growth, and in response to a variety of other signals including pheromone-induced morphogenesis. Common phenotypes in mik-1, mek-1 and mak-1 mutants include altered cell walls, defects in cell-cell adhesion and increased autolysis [40,41,79].

Our analysis showed that CWI-MAP kinase mutants clearly progressed beyond ascogonial coil formation and expansion, but failed to form tightly packed protoperithecia through organized aggregation and adhesion of enveloping hyphae (Figures 6G–6L). Protoperithecial development terminated at a loosely coiled stage that aborted, and the hyphal aggregates formed were reabsorbed into the colony, presumably fostered by increased autolysis in these mutants (Figure 7I).

Another interesting observation was early-onset of fruitbody development at the leading edge of Δmik-1, Δmek-1, and Δmak-1 colonies in plates that had been centrally inoculated (Figure 7H). This contrasted with the N. crassa wild type that only underwent fruitbody formation once the centrally inoculated colony had reached the edge of the culture plate. The latter occurred on SCM or LSA at 25°C in plates up to a diameter of 30 cm.

**PR-MAP kinase pathway: activation of the PR-MAP kinase cascade occurred at the end stages of protoperithecial morphogenesis.** During yeast mating, the PR-MAP kinase pathway regulates chemotropic interaction of mating partners leading to non-self fusion and fertilization (reviewed in [80]). Mutants of the orthologous PR-MAP kinases NRC-1, MEK-2 and MAK-2 of *Neurospora* have been reported to progress further in fruitbody development than mutants of the other two MAP kinase cascades, but still remained female sterile [41]. This was confirmed by our ultrastructural analysis, showing that Δmik-1, Δmek-2 and Δmak-2 strains of *N. crassa* formed densely packed protoperithecia with evidence of hyphal adhesion and normal ECM deposition on the outside (Figure 6M–6R). These mutants, however, did not show signs of trichogyne differentiation, which would be required for subsequent non-self fusion with the male mating partner.

**Localization of MAP kinases during protoperithecial development**

Genetic complementation rescued sexual development in all three MAP kinase mutants. Wild-type morphology was restored by ectopic expression of OS-2-GFP, MAK-1-GFP and MAK-2-GFP in Δos-2 ([FGSC17933]), Δmek-1 ([FGSC11320]) and Δmak-2 ([FGSC11462]), respectively (Figure 8). In the rescued Δos-2 mutants, female sterility due to the lack of protoperithecia; and increased resistance to phenylpyrrole fungicides, such as fludioxonil or fenpiclonil [70,77,78].

Although the *N. crassa* Δos-4, Δos-5 and Δos-2 gene-deletion strains analyzed in this study showed most of the above mentioned defects, our analysis could not confirm the previously reported hyphal fusion defect [41] (Figure S3). We therefore sought other reasons for the absence of protoperithecia. Despite several attempts and testing various culture conditions, we were unable to find evidence of ascogonial coils in any of the three Δs mutants (Figures 6A–6F). An interesting observation made during the SEM studies was of extensive clusters of ECM depositions covering hyphal surfaces (Figures 7A and D) and macroconidiophores (Figures 7B and 7F) of the three Δs mutants, in a way not observed in the wild-type control samples (Figures 7E and 7G). Interestingly, detached conidia, however, were free of these surface depositions (Figure 7C).

Central OS-MAP kinase components in filamentous fungi include: the inability to grow on hyper-osmotic medium (e.g. >3% NaCl or 1 M sorbitol); hyphal lysis; increased pigmentation of macroco-

**Figure 3. Main stages of protoperithecial development.** The ascogonium forms as a specialized, coiled, hyphal branch from a ‘parent hypha’ of the vegetative mycelium. The coil expands, adheres to itself, septates and branches. It sends out more branches, which envelop it. Additional, enveloping hyphae from neighboring areas of the vegetative mycelium aggregate, reinforce and expand the protective casing around the ascogonium. Secretion of ECM is a precursor to hyphal adhesion during this process, which potentially also involves hyphal fusion. Continued fruitbody expansion, cellular differentiation through septation, branching and cell conglutination (conglutinate cells are those that have adhered to each other), melanization and emergence of the trichogyne mark the final stages of protoperitheciun maturation. Mating-cell fusion leading to fertilization and dikaryon formation mark the transition into perithecial development. Autonomous developmental stages of the protoperitheciun are highlighted with grey shading. Table 3 summarizes the range of sizes observed for these main developmental stages observed during protoperitheciun morphogenesis.

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Table 3. Size ranges of developmental stages during protoperithecium morphogenesis.

| Developmental stage                                      | Min. ø | Max. ø | Mean ø * |
|----------------------------------------------------------|---------|--------|----------|
| Trunk hypha                                              | 5 μm    | 10 μm  | 6 μm     |
| Ascogonial coil                                          | 9 μm    | 13 μm  | 12 μm    |
| Ascogonial coil with branches and enveloping hyphae      | 10 μm   | 24 μm  | 17 μm    |
| Subspherical protoperithecium with enveloping hyphae     | 24 μm   | 100 μm | 60 μm    |
| Mature protoperithecium                                  | 37 μm   | 100 μm | 74 μm    |

*Mean diameter were calculated from 100 individual measurements on 2–6 day old wild type cultures grown on LSA.

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Figure 4. Colony morphology of MAP kinase mutants. All MAP kinase mutants showed macroscopic colony phenotypes clearly distinct from the wild type and between the three MAP kinase pathways, but highly conserved within each cascade. (A) CWI-MAP kinase mutants (Δmik-1, Δmek-1 and Δmak-1) typically showed increased autolysis resulting in rosette-like colony growth, and slow colony extension even on nutrient rich media. (B) MAP kinase mutants of the PR pathway (Δnrc-1, Δmek-2 and Δmak-2) were characterized by short aerial hyphae and conidiation starting from the colony center. (C) Colony phenotypes of OS-MAP kinase mutants (Δos-4, Δos-5 and Δos-2) comprised reduced aerial hyphae in the colony center, elevated carotenoid biosynthesis and intense production of ‘sticky’ aerial hyphae and macroconidiophores were foremost at the plate edge. (D) Wild type controls, and the ‘old’ Δos-2 strain FGSC11436, which displayed a colony phenotype different to that of the genuine os mutants (see Figure S4 for a more detailed genotypic and phenotypic comparison between the two Δos-2 mutants FGSC11436 and FGSC17933).

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transformants the wild-type phenotypes that were recovered included: the formation of ascogonial coils that developed into mature protoperithecia; absence of excessive ECM secretion and hyphal lysis; and turgid hyphae with smooth surfaces (Figures 8A and 8B). The vegetative hyphal fusion defects of \( \text{D} \text{mak-1} \) [41] and \( \text{D} \text{mak-2} \) strains [36,37,41] were also fully recovered in their respective transformants (Figures 8C and 8E), as was their ability to complete protoperithecial development (Figures 8D and 8F).

When used as females in heterozygous crosses with the wild type, the rescued transformants of all three MAP kinase mutants successfully completed sexual development, producing viable ascospores. Notably, genetic complementation of \( \text{D} \text{os-2} \) FGSC11436 restored osmoresistance and fenpiclonil sensitivity, but not the hyphal fusion defect of this strain suggesting the hyphal fusion defect is uncoupled from the \( \text{os-2} \) deletion (Figure S4).

Figure 5. Protoperithecial development in MAP kinase gene-deletion mutants. In comparison to the wild type, which formed regular, subspherical protoperithecia 40–80 μm in diameter, only mutants of the PR- and CWI-MAP kinase cascades formed protoperithecial-like structures of similar appearance. These however, did vary in size, shape and degree of pigmentation and were not clearly discernable as protoperithecia even to an experienced microscopist using the stereomicroscopy technique shown here. It was these observations that warranted investigations using more powerful microscopic techniques, as used for Figures 1 and 6–8. Protoperithecial-like structures could not be observed in any of the newly generated OS-MAP kinase mutants. In contrast to the other os mutants, \( \Delta \text{os-2} \) FGSC11436 showed disorganized mycelial architecture, typical of hyphal fusion defects. The \( \Delta \text{nrc-1} \) strains generated from FGSC18162 by vegetative homokaryon selection (HS) showed no phenotypic differences compared to \( \Delta \text{nrc-1} \) FGSC11466. In order to calibrate the results, all strains were inoculated onto cellophane over LSA medium (and SCM for comparison), and incubated for 5–7 days at 25°C dependent on the rate of developmental of the mutant strain. By cutting out cellophane squares carrying mycelium the same samples as shown here were subsequently prepared for LTSEM. Finally, these female cultures were fertilized with opposite mating type conidia of the wild type to confirm female sterility. All scale bars, 50 μm.

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Figure 6. ECM and hyphal adhesion seem essential for the organized assembly of enveloping hyphae into protoperithecia. (OS)

Despite several attempts, ascogonial coils, let alone protoperithecial-like structures, could not be identified in mycelia of the three OS-MAP kinase mutants. Large areas of the mycelium were collapsed, indicating extensive lysis of vegetative hyphae. Hyphal loops (a.k.a. hyphal coils or lassoes), as
Optical sectioning of protoperithecia. Optical sectioning of fluorescently labelled protoperithecia revealed the tightly wound hyphal network of living fruitbodies (Figure 9). CFW staining of cell walls and expression of fluorescent fusion proteins within the cytoplasm facilitated optical sectioning of complete ascogonial coils and differentiated enveloping hyphae. The assembled multicellular structures, however, remained loose hyphal aggregations and ECM was absent, suggesting that hyphal adhesion was not sufficient to form subspherical protoperithecia. Scale bars: (A) 10 μm; (B, C, E) 50 μm; (D, F) 25 μm. (CWI) Δmek-1, Δmek-1 and Δmak-1 strains initiated ascogonial coils and differentiated enveloping hyphae. The assembled multicellular structures, however, remained loose hyphal aggregations and ECM was absent, suggesting that hyphal adhesion was not sufficient to form subspherical protoperithecia. Scale bars: (G) 10 μm; (H, I, K, L) 25 μm; (J) 50 μm. (PR) Δncr-1, Δmek-2 and Δmak-2 strains produced ECM, and hyphal aggregations resembled better-organized and more spherical 'early-stage' protoperithecia. Nevertheless, trichogynes have not been observed in these strains, and sexual development did not progress beyond this stage. Scale bars: (M–R) 25 μm.

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Figure 7. Excessive ECM deposition in OS-MAP kinase mutants and aborted fruitbody development in CWI-MAP kinase mutants. (A) All hyphal surfaces of Δos-2 (FGSC17933) were covered with punctate clusters of ECM depositions. Scale bar, 10 μm. Magnified view in inset; scale bar, 5 μm. (B) Macroconidiophores of Δos-2 were also heavily covered in ECM material. Scale bars, 20 μm. (C) Granular ECM depositions were not present on the surfaces of matured, detached macroconidia. Scale bar, 2 μm. (D) Higher magnification of the clustered ECM depositions on a mature hyphal surface of Δos-2. Scale bar, 2 μm. (E) Smooth surface of a mature hypha of the wild type control. Scale bar, 2 μm. (F) ECM-covered macroconidiophore of Δos-5. Scale bar, 20 μm. (G) Wild type macroconidiophore. Scale bar, 20 μm. (H) CWI-MAP kinase mutant strains displayed early-onset initiation of fruitbody development at the colony periphery. Inset shows a magnified view of the protoperithecial-like ‘hyphal knot’ formed only about 400 μm behind the leading colony edge of Δmk-1. Scale bar, 100 μm; in inset 10 μm. (I) Immature multicellular structures in the sub-periphery of Δmak-1 colonies aborted, then autolyzed and were subsequently reabsorbed into the mycelium, resulting in little evidence of any recognizable protoperithecial-like structures. Scale bar, 50 μm.

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neighboring vegetative hyphae (Figure 9D). A similar trend was observed for MAK-1-GFP distribution, although with overall weaker fluorescence intensities (Figure 9E). Although OS-2-GFP could not be detected in the rescued mutant fruitbodies, it could be detected in conidial germlings (A. Lichius, unpublished data).

**Discussion**

**Protoperithecial morphogenesis in the N. crassa wild type**

To provide a baseline comparator for the analyses of MAP kinase gene-deletion mutants we commenced with a detailed description of protoperithecial morphogenesis in the wild type using live-cell imaging and LTSEM. We conclude that this process can be conveniently divided into four main morphogenetic stages of hyphal differentiation, leading from the vegetative mycelium to the fertilizable protoperitheium. Notable key aspects highlighted by our analyses include: (1) the initial stage being the ascogonial coil, a tight-helical branch with outer dimensions not exceeding 15 μm in diameter, that forms the ascogonium in the center of the developing protoperitheium; (2) septation of the ascogonial coil is a precursor to the emergence of enveloping hyphae; (3) enveloping hyphae, which differentiate as branches of either the ascogonium or one of its neighboring compartments, enlarge the structure; (4) additional enwrapping by enveloping hyphae that may originate from the surrounding vegetative mycelium determine shape, and (5) regulated deposition of extracellular matrix involved in the tight adhesion of ascogonial coil and enveloping hyphae seals the subspherical encasing of the developing fruitbody.

We also take this opportunity to clarify that ascogonial coils in *N. crassa* and related ascomycetes are developmentally different to vegetative hyphal coils (a.k.a. hyphal lassoes). The formation of hyphal lassoes has been described by several authors, and interpreted to be abortive ascogonial coils or ‘pseudo-ascogonia’ in *N. crassa* [81,82], *N. tetrasperma* [83] and *S. macrospora* [84]. However, we are not aware of any evidence to support this interpretation in *N. crassa* as has been recently discussed for hyphal lassoes in *Aspergillus nidulans* [85].

The formation of a subspherical fruitbody from tubular hyphae can most efficiently be achieved by the combination of a tight coil and enwrapping branches. Hyphal lassoes are too loosely wound to generate the compact ascogonial coil observed in the center of developing protoperithecia.

**Gene deletions that impact on sexual progression mainly affected the female partner**

All gene-deletion mutants investigated in this project were blocked in sexual development only when used as females in heterozygous crosses with the wild type. Using conidia of mutant strains as the male fertilizing agent for opposite mating-type, wild-type females did not block sexual development and resulted in the successful production of ascospores. Exclusive female sterility is consistent with earlier findings [36,40,86] and confirms asymmetry of female and male function during mating in *N. crassa* [20]. Mutant strains that are highly male-fertile, but completely female-sterile, are not surprising, as the female function is more complex and thus presents a larger mutational target than the male function. Female function is therefore more easily lost through targeted gene-disruption than is male function [87]. Thus far, the only reported example of a *N. crassa* mutant that has been shown to be female and male sterile is the *Δprm-1* strain [68].

**MAP kinase mutants were defective at different stages of protoperithecial morphogenesis**

Light microscopy illustrated that six out of nine mutants in this investigation formed protoperithecum-like structures that did not mature when judged by size and degree of pigmentation compared with the wild type protoperitheium. For CWI-MAP kinase mutant strains this is the first report that protoperithecum-like structures develop to these advanced stages. Furthermore,
morphological differences between protoperithecium-like structures observed in CWI-MAP kinase and PR-MAP kinase mutants indicated that the deleted genes have different functions during protoperithecial morphogenesis.

Two essential characteristics of developmentally arrested mutant protoperithecia became evident: (1) enveloping hyphae, which were only arranged as loose knots with significant gaps between them, and (2) excess or absence of ECM deposition. Loose-knit protoperithecia were observed for the MAP kinase mutants of the CWI pathway. Furthermore, these mutants did not appear to deposit ECM, which markedly contrasted with MAP kinase mutants of the PR pathway and the wild type. MAP kinase mutants of the OS pathway produced excess ECM, which formed granulated clusters on all hyphal surfaces, accumulating in greater quantities on conidiophores. These latter MAP kinase mutants were characterized by the complete absence of developing fruitbodies, consistent with previous reports [89,90].

**Figure 9. Optical sectioning of developing protoperithecia.** Montages of selected optical sections through developing protoperithecia of the rescued \( \Delta \text{mak-2} \) strain expressing MAK-2-GFP (NCAL043). (A) The small dimensions of a late stage ascogonial coil are fully accessible to optical sectioning when labelled with CFW and MAK-2-GFP. Scale bar, 5 µm. (B) With increasing size, CFW dye is unable to penetrate the interior of the developing fruitbody, and consequently cannot be used to optically section the interior of the ascogonium. Fluorescently labelled MAK-2, however, allows visualization of the whole protoperithecium. Scale bar, 10 µm. (C) Optical sectioning of a mature protoperithecium reveals the complex and tightly wound hyphal network comprising this structure. Scale bar, 20 µm. (D) Middle section of a protoperithecium expressing MAK-2-GFP. The corresponding surface plot shows that fluorescence intensity peaks in the central core region, suggesting that MAK-2-GFP accumulates in the ascogonial coil tissue. (E) MAK-1-GFP fluorescence in the rescued \( \Delta \text{mak-1} \) strain (NCALD10) also peaked in the central ascogonium region, however, was not as pronounced as in the case of MAK-2. Scale bar, 10 µm. Movies S5, S6, S7, and S8 show full z-stacks of optically sectioned protoperithecia. doi:10.1371/journal.pone.0042565.g009
Controlled extracellular matrix deposition was crucial for adhesion of enveloping hyphae

Our results suggested that the organized assembly and adhesion of enveloping hyphae is essential for protoperithecial morphogenesis. If hyphal adhesion was possible to a small degree, protoperithecial development progressed further, compared to mutants where ECM deposition seemed to be deregulated. The inability of MAP kinase mutants of the OS pathway to initiate sexual development is potentially due to excessive ECM secretion on the hyphal surface, which could prevent efficient coiling and adhesion, and correlated with numerous collapsed and ‘bleeding’ hyphae, and ‘sticky’ conidiophores. MAP kinase mutants of the CWI pathway only formed loose hyphal aggregates that lacked ECM. These aggregates aborted and became quickly autolysed PR-MAP kinase mutants progressed furthest by forming compact, organized protoperithecial-like structures. However, emergence of trichogyne-like hyphae was not observed in PR-MAP kinase mutants, suggesting that fruitbody development stalled before a mature stage was reached even in these strains. This MAP kinase pathway-dependent differences in superficial ECM deposition led us to propose that regulated ECM deposition should be regarded as a defining stage of protoperithecial development in N. crassa. In addition, some of the ECM produced may play an important role for the formation of conglutinate cells and pseudoparenchymatous tissues [2,4,5].

Apical dominance might restrict sexual development and senescence in the colony sub-periphery

Another aspect that influenced fruitbody development and its analysis, respectively, was the early-onset of autolysis in the mycelia of CWI-MAP kinase mutants. This phenomenon has been reported for a number of homologous strains in different fungal species and is generally regarded as one determining feature of fungal cell-wall mutants [41,79,91]. Autolysis of immature protoperithecia occurred in the wild type, but much less frequently. Early autolysis resulted in very quick degradation of aborted protoperithecia in the center and sub-periphery of colonies of all CWI-MAP kinase mutants of N. crassa. As a likely consequence of this, intact protoperithecial aggregates could only be located and analyzed close to the colony edge, interestingly, even before the mycelium had reached the edge of the culture dish. In the wild type or other developmental mutants we studied, it was not common to observe protoperithecial formation commencing before tips of peripheral leading-hyphae had encountered the edge of the Petri dish. Premature entry into sexual development and early-onset autolysis of the initiated fruitbodies are both signs of accelerated senescence of these mutants. Wild type colonies can extend to considerable sizes before initiating protoperithecial development in response to physical confinement at the colony edge. This ‘edge-effect’ stimulus of fruitbody formation has been documented for species closely-related to N. crassa [92], including S. macrospora [84,93] and S. brevicollis [94], supporting the hypothesis that apical dominance of the leading-edge of the actively growing colony suppresses ascogonial coil initiation, and consequently delays aging in the sub-periphery of the wild type. The particular phenotype of increased senescence in CWI-MAP kinase mutants greatly impeded experimental analysis of fruitbody development, but provided one possible explanation why protoperithecial initials of these mutants had escaped detection by earlier investigators.

Trichogyne emergence concludes protoperithecial development

MAP kinase mutants of the PR pathway (meo-1, mek-2 and mak-2) have previously been reported to progress further in fruitbody development than mutants of the other two MAP kinase cascades [41]. This notion was confirmed by our analysis, which due to the absence of trichogyne-like hyphae, further suggests that the PR pathway might also act during the transition from protoperithecial to perithecial development. Thus, signaling through the PR pathway may not exclusively be involved in trichogyne homing and mating-cell fusion, but also in trichogyne initiation. As trichogynes are formed in the wild type in the absence of opposite mating-type pheromone, the involvement of a self-signaling molecule, which triggers morphogenetic transitions up until this point, is indicated. Crostalk between all three Neurospora MAP kinase pathways during regulation of female sexual development is very likely as suggested previously [40,41].

Taken together, we propose that deregulated MAP kinase signaling leads to the inability to develop protoperithecia in an organized manner, i.e. to assemble a tightly wound ascogonial coil with enveloping hyphae ‘hugging’ its surface through adhesion and regulated ECM deposition. Hyphal attachment following tip-growth arrest is a precursor to vegetative hyphal fusion (VHF) and might provide an important trigger for the activation of the fusion machinery [95]. The lack of contact-induced tip growth arrest and hyphal tip attachment is a commonly observed phenotype in VHF mutants [96], including CWI- and PR-MAP kinase gene-deletion strains. Therefore, the inability to attach and trigger the transition to the next morphogenetic stage may provide a functional connection between VHF and fruitbody development. In the event that certain developmental checkpoints are not reached, further fruitbody morphogenesis is aborted and the material becomes reabsorbed and recycled within the colony. This has important implications for the preparation and timing of experimental analysis of protoperithecial-defective strains.

The role of self-fusion during protoperithecial morphogenesis remains unclear

All nine MAP kinase mutants analyzed in this study were defective in sexual development, but only six were defective in vegetative hyphal self-fusion. The link between vegetative cell fusion and sexual development has repeatedly been made (e.g. [23,67,69]) but whether self-fusion events are involved in the formation of the fully functioning/co-operative multicellular structure of the protoperitheciun has not been established. Unfortunately, despite very careful observations, we were unable to clearly identify self-fusion connections within the developing protoperithecia. An obstacle for these analyses is that unless one can actually observe the fusion-process occurring, it is difficult to reliably differentiate a fusion event from a septation event inside the fruitbody. Our findings from optically sectioning early-stage protoperithecia suggest that MAK-2 and MAK-1 participate in differentiation processes inside the ascogenous tissue. Considering the importance and presence of both MAP kinases at germling fusion sites in N. crassa [96,97], this leaves the possibility for cell fusion to occur in the developing protoperithecia. OS-2, on the other hand, was not detected in ascogenous tissue, nor was it detected at advanced stages of fruitbody differentiation following ascogonial coil initiation. Fusion between dissected-out paraphyses (sterile hyphae that grow between asci) in perithecia has been observed in S. macrospora [2] and N. crassa (K.M. Lord & N.D. Read, unpublished results), and in ascogenous tissue cell-fusion occurs in croziers [22]. Combined with results of independent
studies that recently identified additional hyphal fusion mutants of \textit{N. crassa} with normal protoperithecial development \cite{69,90}, we can conclude that: (1) not all genes essential for VHF are required for protoperithecial development, and thus signaling processes regulating fusion in both processes are likely to be different, and (2) consequently, proteins that are essential in both processes might regulate different cellular events, not necessarily connected to cell fusion.

**Conclusions**

Our analysis indicates that MAP kinase gene deletions did not lead to the disruption of protoperithecial development at a conserved stage, but that blockage occurred at distinct stages dependent on the affected MAP kinase cascade. The loss of an individual MAP kinase could not be compensated within the MAP kinase signalling network, further supporting the notion that each cascade functions during a specific stage of protoperitheciun development and in a sequential manner. The morphogenetic phenotypes of the nine MAP kinase mutants in \textit{Neurospora} suggests that the OS and CWI pathways act upstream of the PR pathway. The successful genetic rescue of all three terminal MAP kinase mutants proved that signaling through all three cascades is essential for perithecial development. The finding that \textit{os} genes are dispensable for hyphal fusion, while essential for protoperithecial morphogenesis, complicates our attempts to understand whether hyphal fusion is required for fruitbody formation. However, this does not exclude the possibility that hyphal fusion is required, because fusion events inside the developing protoperitheciun are likely to be regulated differently than VHF in the mature mycelium.

Finally, this study highlights that MAP kinases play roles in some of the key processes involved in the early stages of multicellular development in fungi, particularly: extracellular matrix deposition; hyphal adhesion; and hyphal envelopment, during the construction of protoperithecia. These are fundamental features displayed by fungi achieving the multicellular state by hyphal aggregation. The evolution of fungi with multicellular differentiated tissues has been estimated to have occurred at least 500 million years ago, to have occurred independently in the Ascomycota and Basidiomycota \cite{99}, and possibly more than once in the Ascomycota \cite{100}. Indeed, the morphology of the perithecium has been shown from fossil evidence to be conserved for over 400 million years \cite{101,102}. In the future, it will be extremely important to determine the molecular basis of how fungi achieve multicellularity, in order that we can identify and analyse the key molecules (e.g. cell adhesion molecules) involved in this process. It will also be interesting to determine whether fungi share some of this molecular machinery with animals, from which they have been estimated to diverge 2,635 million years ago \cite{103}. The analysis of multicellular development in experimentally and genetically tractable fungi such as \textit{N. crassa} and \textit{S. macrospora} should provide useful models from which to gain significant insights into these processes in even more complex eukaryotes \cite{2}.

**Supporting Information**

**Figure S1** PCR-genotyping set up 1. (A) Schematic overview of the PCR set up initially used for genotypic verification of gene-deletion strains. (a) Simplified representation of the gene-deletion process by homologous recombination. The open reading frame (ORF) of the \textit{hph} cassette and the target gene ‘X’ are in antisense. As macroconidia, which were used for KO cassette transformation, are multinucleate, gene deletion does not necessarily occur in all nuclei of a single spore. This may result in heterokaryotic cells that contain both wild type and deletion mutant nuclei, therefore being hygromycin B resistant, but still expressing a wild type copy of the target gene X. (b) Presence of the target gene X in the genome of an individual strain was analyzed using one gene-specific primer positioned inside the wild type ORF (X\_300\_fw) paired up with a primer positioned inside the 3’ flank used for homologous recombination (3r\_X). In case, PCR 1 produces a fragment of the predicted size, the target gene is still present in that strain. The 3r\_X primer was used together with a matching forward primer (3f\_X) to amplify a fragment of the 3’-flank as internal PCR control (PCR 2). (c) Presence of the \textit{hph}-cassette in the target locus was analyzed with PCR 3, using the \textit{hph}-cassette-specific \textit{hph}\_300\_fw primer paired up with 3r\_X. Again as internal PCR control (PCR 4) \textit{hph}\_300\_rv was used together with \textit{hph}\_test\_fw to amplify a part of the \textit{hph} gene. Due to the poor binding capacities of \textit{hph}\_test\_fw, which consequently gave only very little product (all PCR 4 bands are rather weak), this approach was soon abandoned and replaced by the improved PCR genotyping set up 2 shown in Figure S2. Green arrows represent forward primers, red arrows represent reverse primers.

The \textit{hph}-cassette is oriented in antisense relative to the target gene locus. Therefore, in PCRs 3 two seemingly non-matching reverse primers were used. (B) Functionality of all genotyping primers was initially verified using wild type gDNA template. (C) According to the schematics in (A) presence of the \textit{hph}-cassette in the targeted loci could be confirmed for all six MAP kinase gene-deletion mutants of the PR-MAP kinase and CWI-MAP kinase pathway. The \textit{hph}-specific PCRs 4 did only result little product, if at all. However, functional presence of that gene is sufficiently backed up by the fact that all strains grew on 100 μg/ml hygromycin B. Extremely weak, residual wild type background could only be detected for \textit{Δnrc-1} and \textit{Δnuck-2}, but not for any of the other four kinase mutants. This residual wild-type background, however, was in no case sufficient to rescue the dominant mutant phenotype, which was identical amongst all three PR-MAP kinase mutants (Figure 4B). Furthermore, the wild type-like phenotype of the heterokaryotic \textit{Δnrc-1} FGSC11627 replacement strain switched to a mutant phenotype identical to that of \textit{Δnrc-1} FGSC11466 after the first generation of vegetative homokaryon purification in eight of ten isolated clones providing sufficient proof for their correctness (data not shown). (D) All three PR-MAP kinase mutants (\textit{Δnrc-1} FGSC11466, \textit{Δnuck-2} FGSC11524 and \textit{Δnuck-2} FGSC11462) have been confirmed to still carry the \textit{Δmus-51} deletion, whereas, all three CWI-MAP kinase mutants (\textit{Δwil-1} FGSC11326, \textit{Δnuck-1} FGSC11316 and \textit{Δnuck-2} FGSC11320) have been complemented in that gene after back-crossing. This is consistent with the genotypic annotation of these strains in the master spreadsheet maintained at the Dartmouth \textit{Neurospora} Genomics Project site. In \textit{Δnuck-2} FGSC11524 homokaryon purification has been accomplished not by back-crossing, but vegetatively through single spore isolation of uninuclear microconidia. Consequently, the \textit{Δmus-51} could not be complemented by the mating partner. Due to low viability of the progeny after back-crossing \textit{Δnrc-1} FGSC11466 and \textit{Δnuck-2} FGSC11428 strains had to be deposited as heterokaryons. To date, there are no reports that deletion of \textit{mus-51} or \textit{mus-52} in \textit{Neurospora}, or other \textit{ku70}- or \textit{ku80}-orthologous deletions in other fungi, have an influence on the phenotype, apart from their intended molecular effect of eliminating non-homologous end-joining recombination \cite{104}. Therefore, presence of these mutations is not expected to influence phenotypic analyses of this study in any way. (TIF)

**Figure S2** PCR-genotyping set up 2. (A) Schematic overview of the improved PCR set up used for genotypic verification of
gene-deletion strains. (a) Simplified representation of the gene-deletion process by homologous recombination. For detailed description please refer to Figure S1 legend. (b) Presence of the target gene X in an individual strain was analyzed using two gene-specific primers positioned inside the wild type ORF (X_1000 rv and X_500 fw) paired up with primers positioned outside of the 5’ and 3’ flank used for homologous recombination (X-5’200 fw and X-3’300 rv). If PCR 1 and PCR 2 produced amplicons of the predicted size, then a copy of the target gene was still present in that strain. (c) Presence of the hph-knock-out cassette in the target locus was analyzed with primers specific for the cassette (hph_800 fw and hph_300 rv), paired up with the same primers outside of both flanks as described before. Analogous to (b), products from PCR 3 and PCR 4 confirmed that the hph-cassette has been integrated exactly at the targeted location in the genome, and thus replaced the native gene. Amplification products from PCRs 1–4 have relative size differences of at least 200 bp to one another, in order to facilitate differentiation after gel-electrophoresis. Green arrows represent forward primers, red arrows represent reverse primers. The hph-KO cassette is oriented in antisense relative to the target gene locus. Therefore, in PCRs 3 and 4 two seemingly non-matching reverse primers were used. (B–D) Genotypic verification of os mutants. All primers were initially tested using wild type gDNA template (left column). In all ‘new’ Δos-2, Δos-4, and Δos-5 mutants the hph-KO cassette has replaced the targeted gene at its specific locus, confirming that the genes were correctly removed through homologous recombination. The ORFs of the targeted genes could not be detected somewhere else in the genomes of Δos-2 and Δos-4, respectively, whereas, in Δos-5 weak bands in PCRs 1, 2 and the os-5-specific amplicon indicate residual presence of the wild type locus, suggesting that this strain is still heterokaryotic. Nevertheless, this extremely weak wild-type background was not enough to alter the dominant mutant phenotype of strain Δos-5 FGSC18203, which notably was 100% consistent with that of Δos-4 FGSC18202 and Δos-2 FGSC17933 (Figure 4C). The presence of mus-52 was found in all three Δos mutant strains, whereas mus-51 was absent from Δos-4 and Δos-5 indicating that only Δos-2 has successfully been recovered from back-crossing. As outlined in the master spread-sheet of the Neurospora Genome Project, Δos-4 FGSC18202 and Δos-5 FGSC18203 have been homokaryon-purified vegetatively by isolation of hygromycin B-resistant microconidia, and thus Δmus-51 could not be complemented. However, apart from its intended molecular phenotype (eliminated non-homologous recombination) no further macro- or microscopic phenotypes are known to be caused by these mutations. A pair of primers amplifying a part of the actin locus was used as internal PCR controls, generating a 700 bp fragment in each reaction. Indicated PCRs 1 and 2 refer to the PCR set up shown in (A).

Figure S3 Phenotypic characterization of OS-MAP kinase mutants. A by-product of our morphogenetic analyses of protoperithecial development was the finding that vegetative hyphal fusion in the mature colony and between conidial germings of the OS-MAP kinase mutants Δos-4 FGSC18202, Δos-3 FGSC18203 and Δos-2 FGSC17933 mutants was functional and indistinguishable from the wild type. (A) VHF in the mature colony of all three os mutants (Δos-4, FGSC18202; Δos-5, FGSC18203 and Δos-2, FGSC17933) is indistinguishable from the wild type controls (mat A, FGSC2489 and mat a, FGSC2490). A fusion defect was only observed in the ‘old’ Δos-2 FGSC11436 strain. See Movies S1, S2, S3, and S4 for hyphal fusion phenotypes of these strains. (B) Fusion competency of the ‘new’ os mutants was furthermore confirmed in germling-fusion assays. Again, only FGSC11436 was found to be cell fusion defective. Arrowsheads indicate fusion connections. All scale bars, 10 μm. As this contradicted previous reports [41], we wanted to confirm the genuine phenotype of these strains by testing two additional, well-documented characteristics of OS-MAP kinase mutants: osmosensitivity and resistance to phenylpyrrole fungicides [70]. All three ‘new’ os mutant strains showed increased osmosensitivity under salt stress, and increased resistance against the fungicide fenpiclonil, confirming that they are genuine OS-MAP kinase mutants of N. crassa. Ectopic expression of OS-2-GFP restored osmotolerance and fungicide sensitivity in Δos-2 transformants (NCAL020-1 and -2). (C) Whereas, the wild type strains were able to grow in the presence of 6% NaCl colony extension in all three os mutants was significantly impaired already in the presence of 3% salt, and completely blocked with 6% NaCl in the medium. (D) Only the three os mutants were resistant to the fungicide, whereas resistance was lost in the genetically complemented Δos-2::OS-2-GFP strains. (E) In comparison to the VMM controls, CAT-mediated cell fusion was significantly reduced in the os mutants in the presence of 3% NaCl, which was recovered close to wild-type levels in the Δos-2::OS-2-GFP transformants (NCAL020-1 and -2). Germination fusion was effectively inhibited through 1.5 μM fenpiclonil in the wild type and rescued Δos-2::OS-2-GFP transformants, but unaffected in the three genuine os mutants. Taken together, these findings confirm that Δos-4, FGSC18202; Δos-5, FGSC18203 and Δos-2, FGSC17933 are genuine osmosensitive MAP kinase mutants, and that they are dispensable for cell fusion in N. crassa. (TIF)

Figure S4 Phenotypic and genotypic characterization of Δos-2 FGSC11436. (A) Differences in colony development, measured as radial colony extension, under salt and fungicide stress were compared between wild type, Δos-2 FGSC11436, Δos-2 FGSC17933 and corresponding genetically complemented transformants NCAL018 and NCAL020, respectively. The wild type was able to grow in the presence of 6% w/v NaCl in the medium, but unable to grow in the presence of the fungicide fenpiclonil (1.5 and 4.5 μM). This behavior was not altered through ectopic expression of OS-2-GFP in the wild type transformant strains NCAL016-1 and -2. Due to its hyphal fusion defect, colony development of Δos-2 strain FGSC11436 was slowed on the control medium. In addition, this strain showed increased sensitivity to salt stress and was unable to grow on medium supplemented with 6% NaCl. However, it was resistant to fenpiclonil. Osmosensitivity was rescued through ectopic expression of OS-2-GFP in the wild type transformant strains NCAL018-1 and -2, and its sensitivity to fenpiclonil was restored. With respect to osmosensitivity and fenpiclonil resistance, Δos-2 strain FGSC17933 and its corresponding OS-2-GFP transformants NCAL020-1 and -2, respectively, showed identical characteristics. (B) Normal hyphal morphology at the colony periphery of Δos-2 FGSC11436 under non-stress conditions and in the wild type in the presence of 6% salt. In the presence of 3% salt, both Δos-2 mutants show swollen hyphae and irregular growth pattern. Scale bars, 100 μm. (C) Multiplex PCR confirming that the native os-2 locus has been correctly exchanged for the hph-knock-out cassette. The 5’ os-2 signal is of the wrong size and thus most likely an unspcific product. Alternatively, this signal might indicate an unintended genetic alteration at the 5’-flank of the os-2 locus. Indicated PCRs 1–4 refer to the PCR set up shown in Figure S2. (D) Genetic complementation of Δos-2 FGSC11436 with os-2 gfp did not rescue the central-conidiation phenotype, nor did it rescue the
VHF defect of that strain. Collectively, the results strongly suggest that although the os-2 gene has been correctly deleted in FGSC11436, leading to increased osmosensitivity and fenpiclonil resistance, the cell fusion defect must be caused by an additional, unintended genetic alteration in that strain, and thus is not part of the genuine os-2 mutant phenotype.

Table S1 Oligonucleotides used for PCR-genotyping. Please refer to Figures S1 and S2 to deduce primer positions and pairing. (DOCX)

Movie S1 Time-courses of successful VHF in the ‘correct’ Δos mutants is easily revealed by cytoplasmic streaming through fusion connections. Movie S1 showing Δos-5 FGSC18203, Movie S2 showing Δos-4 FGSC18202, and Movie S3 showing Δos-2 FGSC17933. (MP4)

Movie S2 Time-courses of successful VHF in the ‘correct’ Δos mutants is easily revealed by cytoplasmic streaming through fusion connections. Movie S1 showing Δos-5 FGSC18203, Movie S2 showing Δos-4 FGSC18202, and Movie S3 showing Δos-2 FGSC17933. (MP4)

Movie S3 Time-courses of successful VHF in the ‘correct’ Δos mutants is easily revealed by cytoplasmic streaming through fusion connections. Movie S1 showing Δos-5 FGSC18203, Movie S2 showing Δos-4 FGSC18202, and Movie S3 showing Δos-2 FGSC17933. (MP4)

Movie S4 Time-course showing the fusion incompetence in the ‘wrong’ Δos-2 strain FGSC11436, whose fusion hyphae ignore each other, and lack the growth arrest response upon physical contact required to establish a fusion connection. (MP4)

Movie S5 Z-stack sequence of an optically sectioned late-stage ascosporic coil, expressing MAK-2-GFP, stained with the cell-wall marker dye CFW. (MP4)

Movie S6 Z-stack sequence of an optically sectioned early stage protoperithecium, expressing MAK-2-GFP, stained with the cell-wall marker dye CFW. (MP4)

Movie S7 Z-stack sequence of an optically sectioned mature protoperithecium, expressing MAK-2-GFP. (MP4)

Movie S8 Z-stack sequences of optically sectioned protoperithecium expressing MAK-2-GFP, which shows elevated accumulation in the center. (MP4)

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Author Contributions

Conceived and designed the experiments: AL KML CEJ RO PB. Performed the experiments: AL KML CEJ RO PB. Analyzed the data: AL KML RO PB. Contributed reagents/materials/analysis tools: CEJ NDR. Wrote the paper: AL KML.

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