

1 **Functional analysis of chromatin-associated proteins in *Sordaria macrospora* reveals similar**  
2 **roles for RTT109 and ASF1 in development and DNA damage response**

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30 **Abstract**

31 We performed a functional analysis of two potential partners of ASF1, a highly conserved histone  
32 chaperone that plays a crucial role in the sexual development and DNA damage resistance in the  
33 ascomycete *Sordaria macrospora*. ASF1 is known to be involved in nucleosome assembly and  
34 disassembly, binding histones H3 and H4 during transcription, replication and DNA repair and has  
35 direct and indirect roles in histone recycling and modification as well as DNA methylation, acting as a  
36 chromatin modifier hub for a large network of chromatin-associated proteins. Here, we functionally  
37 characterized two of these proteins, RTT109 and CHK2. RTT109 is a fungal-specific histone  
38 acetyltransferase, while CHK2 is an ortholog to PRD-4, a checkpoint kinase of *Neurospora crassa* that  
39 performs similar cell cycle checkpoint functions as yeast RAD53. Through the generation and  
40 characterization of deletion mutants, we discovered striking similarities between RTT109 and ASF1 in  
41 terms of their contributions to sexual development, histone acetylation and protection against DNA  
42 damage. Phenotypic observations revealed a developmental arrest at the same stage in  $\Delta rtt109$  and  
43  $\Delta asf1$  strains, accompanied by a loss of H3K56 acetylation, as detected by western blot analysis.  
44 Deletion mutants of *rtt109* and *asf1* are sensitive to the DNA damaging agent MMS (methylmethane  
45 sulfonate), but not HU (hydroxyurea). In contrast, *chk2* mutants are fertile and resistant to MMS, but  
46 not HU. Our findings suggest a close functional association between ASF1 and RTT109 in the context  
47 of development, histone modification and DNA damage response, while indicating a role for CHK2 in  
48 separate pathways of the DNA damage response.

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51 **Article summary**

52 In the filamentous fungus *Sordaria macrospora*, the conserved histone chaperone ASF1, which  
53 interacts with histones H3 and H4, was previously shown to be required for multicellular  
54 development and DNA damage response. Here, we have analyzed two additional chromatin-  
55 associated proteins. *rtt109* encodes a histone acetyltransferase, and deletion of the gene in *S.*  
56 *macrospora* results in a phenotype similar to that of a  $\Delta asf1$  mutant, whereas *chk2* is involved  
57 in different aspects of the DNA damage response, but not in development.

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## 61 Introduction

62 The fruiting bodies of ascomycetes are some of the most intricate structures of the fungal kingdom.  
63 While fruiting body formation represents a significant developmental process in the fungal life cycle,  
64 our understanding of the specific factors that regulate this differentiation remains limited. *Sordaria*  
65 *macrospora*, a homothallic ascomycete, has proven to be an excellent model to gain insight into the  
66 genetic background of sexual development and multicellular development in general (1). *S.*  
67 *macrospora* offers multiple advantages as a model organism, such as a very fast life cycle as it  
68 generates its complex fruiting bodies, the perithecia, in under 7 days under laboratory conditions (2).  
69 Multiple approaches, from the investigation of mutants from random mutagenesis programs (3) to  
70 transcriptomics-based reverse genetics (4), have been used to study the complex genetic network of  
71 perithecia formation. Such research has yielded a lot of information about developmental genes, some  
72 of which are known to be conserved in higher eukaryotes and are in cases like the components of the  
73 STRIPAK complex (5) or the histone chaperone ASF1 (6, 7) even relevant for human diseases. ASF1 is  
74 known to interact with histones H3 and H4, both individually and as a dimer. It is involved in histone  
75 assembly and disassembly and was the first histone chaperone discovered to be involved in these  
76 processes during DNA replication, repair and transcription and was long thought to be the only one  
77 involved in all three processes (8). While ASF1 facilitates histone transfer and binds non-DNA bound  
78 H3-H4, it is not incorporated into the nucleosomes (9). During sexual development of *S. macrospora*,  
79 ASF1 has been shown to be essential for achieving fertility, and  $\Delta$ asf1 mutants arrest their life cycle at  
80 the protoperithecia stage (6). Effects on DNA methylation (10) and histone modification (11) have also  
81 been described in *S. macrospora asf1* deletion mutants. Since the structure of ASF1 is well  
82 characterized and no domains have been detected that would allow it to carry out such enzymatic  
83 reactions by itself (8), functions in DNA methylation and histone modification are likely to be carried  
84 out by interaction partners. Studies in *S. cerevisiae* showed a loss of H3K56ac histone acetylation in  
85 *asf1* deletion mutants, and the enzyme identified as responsible for this modification was shown to be  
86 the HAT (histone acetyltransferase) Rtt109 (12). Indeed, an interaction between Asf1, Rtt109, Vps75  
87 and the target histone H3 has been demonstrated in yeast, and it seems likely that acetylation occurs  
88 during the interaction of these proteins (13). Therefore, the loss of H3K56ac in *S. cerevisiae*  $\Delta$ asf1  
89 strains appears to be caused by disruption of the complex that allows Rtt109 to act on its target. The  
90 dependence of Rtt109 on interaction partners is one of its distinguishing features, as *in vitro*  
91 experiments with the *S. cerevisiae* proteins have shown that the HAT alone is unable to acetylate  
92 histones and only functions in the presence of at least one of its interaction partners Asf1 or Vps75,  
93 while the presence of both is required for full activity (12). Rtt109 is exclusively found in fungi (14) and  
94 is responsible for modifying specific sites on histone H3, namely K9, K27 and K56, as clearly  
95 demonstrated in yeast (15, 16). In addition to its role in histone acetylation, Rtt109 has been shown to

96 be involved in a variety of processes in fungal model organisms. In *Fusarium graminearum*, it has been  
97 shown to be involved in perithecia morphogenesis, ascospore formation, conidiation and host plant  
98 infection (17). In *Aspergillus flavus* it is important for aflatoxin synthesis, virulence and growth (18),  
99 whereas *Aspergillus fumigatus* requires it for normal development and DNA damage response, as well  
100 as virulence (19). In *Neurospora crassa*, a close relative of *S. macrospora*, RTT109 has been shown to  
101 be necessary for the production of small RNAs (20). Therefore, RTT109 can be considered a major  
102 factor in many fungal models, suggesting its importance in fungi in general, while the fact that is only  
103 found in this group of organisms might make it an interesting target for anti-fungal drugs.

104 The histone acetylations generated by RTT109 are closely associated with newly synthesized histones  
105 and play a crucial role in the assembly of nucleosomes during DNA replication and repair processes  
106 (15). The involvement of H3K56ac in DNA damage repair, and thus the dependence of this process on  
107 Rtt109, has been demonstrated in *S. cerevisiae rtt109* deletion mutants, which lack H3K56ac and are  
108 sensitive to the DNA double-strand break inducer methyl methanesulfonate (MMS) and the DNA  
109 replication inhibitor hydroxyurea (HU) (21). A similar relationship between ASF1, RTT109 and H3K56ac  
110 might exist in *S. macrospora*, since *asf1* deletion strains of this fungus show sensitivity to MMS and a  
111 reduction in H3K56ac (11). Therefore, we hypothesized that RTT109 might be the link between ASF1,  
112 histone acetylation and DNA damage repair in *S. macrospora* and chose SMAC\_05078, the *S.*  
113 *macrospora* RTT109 homologue, as a target for functional characterization.

114 Another potential candidate, which might contribute to the reduced genomic stability of *asf1* mutants,  
115 could be the checkpoint kinase Rad53. Rad53 was shown to be important for DNA damage protection  
116 under the influence of MMS and HU in *S. cerevisiae* (22). *S. cerevisiae* Rad53 is known to physically and  
117 functionally interact with Asf1, and the activity of Rad53 is tightly regulated by its phosphorylation  
118 state. Hypophosphorylated Rad53 is known to be bound to Asf1 and maintained in an inactive state,  
119 while phosphorylation and release from the complex is a sign of active Rad53 (23). Deletion of *asf1* in  
120 budding yeast causes defects in DNA damage recovery and may be due to insufficient inactivation of  
121 Rad53 (24). In addition, *asf1* deletion mutants show similar sensitivities to MMS and HU as *rad53*  
122 deletion mutants (23). Rad53 is the functional equivalent of checkpoint kinases Chk2 in humans and  
123 PRD-4 in the filamentous fungus *Neurospora crassa*, since the corresponding genes can complement  
124 an *S. cerevisiae*  $\Delta$ rad53 mutant (25, 26). The *S. macrospora* ortholog to PRD-4/Chk2 is SMAC\_00634  
125 (called CHK2 hereafter), which was chosen for analysis to shed more light on the chromatin modifier  
126 network that contributes to the DNA damage protection functions of ASF1 in *S. macrospora*.

127 In this work, we created deletion mutants of *rtt109* and *chk2*, and analyzed their vegetative growth  
128 and potential for sexual development. We also performed genotoxic stress assays to check for  
129 similarities to *asf1* mutants. Furthermore, we determined the subcellular localization of the RTT109  
130 protein by fluorescence microscopy and estimated the global amount of H3K56Ac in  $\Delta$ rtt109 by

131 western blot analysis. We discuss our findings in the context of the roles of chromatin modifiers during  
132 DNA damage protection and regulation of complex multicellular development.

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## 135 **Materials and Methods**

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### 137 **Strains, crosses and growth conditions**

138 Strains utilized in this study can be found in Table S1 in File S1. These strains were cultivated at a  
139 temperature of 25 °C on either solid or liquid cornmeal medium (BMM) or complete medium (CM),  
140 following previously established protocols (27) (28). In order to facilitate genetic crosses, the spore  
141 color mutant *fus* was employed as a partner strain, enabling the identification of recombinant asci (3).  
142 Previously described methods were used to carry out the transformation of *S. macrospora* (28).

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### 144 **Cloning procedures, oligonucleotides and plasmids**

145 The oligonucleotides required for generating plasmids and conducting integration tests have been  
146 provided in Table S2 in File S1. Additionally, all the plasmids utilized in this study are listed in Table S3  
147 in File S1. The plasmid containing the deletion sequence for *rtt109* was generated through yeast  
148 recombinant cloning (29). For assembling the deletion plasmid for *chk2*, and the complementation  
149 plasmids for *rtt109* and *chk2*, Golden Gate cloning methodology was employed (30, 31).

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### 151 **Generation of *rtt109* and *SMAC\_00634* deletion mutants and complementation strains**

152 For conducting gene deletions, homologous recombination was utilized following established  
153 procedures (32). In brief, a deletion cassette was constructed, comprising a selection marker in the  
154 form of a hygromycin phosphotransferase gene under control of the constitutive *Pgpd* promoter and  
155 *TtrpC* terminator from *Aspergillus nidulans* (33), flanked by upstream and downstream regions of the  
156 target gene. This cassette was then cloned into a vector and transformed into the *S. macrospora*  $\Delta ku70$   
157 strain after restriction and gel elution. Since *KU70* is necessary for the ectopic integration of DNA, using  
158 a *ku70* defective strain facilitates homologous recombination (32). Transformants that exhibited  
159 resistance to hygromycin were selected and subsequent crosses were performed with the spore color  
160 mutant *fus*, since most *S. macrospora* transformants are heterokaryotic. Ascospores from such crosses  
161 were isolated to obtain homokaryotic deletion mutants and to enable the elimination of the  $\Delta ku70$   
162 background. The resulting strains were verified through PCR. To complement the *rtt109* and *chk2*  
163 mutants, the assembled plasmid containing the respective gene fused to an eGFP tag, controlled by a  
164 *Pgpd* promoter, was introduced into the corresponding strains via transformation. Ascospores were

165 isolated from the transformants that reached fertility, allowing the acquisition of homokaryotic strains  
166 carrying the complementation construct.

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### 168 **Growth tests and genotoxic stress assays**

169 Growth tests were performed on BMM media and the growth front was documented every 24 hours.  
170 All tests were performed with three biological replicates. Statistical evaluation was conducted using a  
171 Student's t-test. Phenotypic characterization involved observing and documenting the growth and  
172 fertility of the strains on BMM plates using a Stemi 2000-C stereomicroscope (Zeiss). To assess the  
173 resistance against methyl methanesulfonate (MMS), all strains were inoculated on BMM plates  
174 supplemented with 0.007% (v/v) MMS, and the growth was observed and documented over a period  
175 of 4 days. Similarly, resistance against hydroxyurea (HU) was evaluated on BMM plates containing 8  
176 mM HU using the same approach.

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### 178 **Microscopic analysis**

179 To investigate the subcellular localization of RTT109-EGFP fusion proteins, the strains were cultivated  
180 on glass slides along with strains expressing H3-mRFP fusion proteins, as previously described (34).  
181 Heterokaryotic strains can therefore express both fluorescence-labelled proteins (35). Subsequently,  
182 light and fluorescence microscopy were performed using an AxioImager microscope (Zeiss), equipped  
183 with a Photometrix Cool SnapHQ camera (Roper Scientific). EGFP fluorescence was detected using a  
184 Chroma filter set 41017 (HQ470/40, HQ525/50, Q495lp), while mRFP fluorescence was detected using  
185 set 49008 (EG560/40x, ET630/75 m, T585lp). The acquired images were further processed using  
186 MetaMorph software (Molecular Devices).

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### 188 **Western blot analysis**

189 To evaluate differences in the global levels of H3K56ac between the  $\Delta$ rtt109 strains and the wild type,  
190 a western blot analysis was conducted using antibodies specific to this particular modification. The  
191 wild type strain SN1693, the  $\Delta$ rtt109 strain S60, and the corresponding complementation strain S60K1  
192 were grown in liquid BMM medium (27) at 27 °C for 4 days. The mycelium was harvested by filtration,  
193 washed with PPP (28) and subsequently frozen in liquid nitrogen before being ground to a fine powder.  
194 Protein extraction was performed by mixing the powder with extraction buffer (50 mM Tris/HCl pH  
195 7.5, 250 mM NaCl, 0.05 % NP-40, 0,3 % (v / v) Protease Inhibitor Cocktail Set IV (Calbiochem)). After 20  
196 min centrifugation, the protein concentrations were determined using Bradford assays (36). Equal  
197 amounts of proteins were separated by SDS gel electrophoresis, and transferred onto a PVDF  
198 membrane through western blotting. H3K56ac-specific antibodies (Active Motif #39082) and H3-  
199 specific antibodies (Cell Signaling #9715) were used to detect the respective bands.

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## Results

### Deletion of *rtt109* in *S. macrospora* leads to sterility and impairs vegetative growth

For the functional characterization of the histone acetyltransferase RTT109 in *S. macrospora*, a *ku70* deletion strain was transformed using a deletion cassette designed for the *rtt109* sequence. The deletion cassette consisted of a hygromycin phosphotransferase gene flanked by sequences homologous to the *rtt109* gene, enabling the replacement of the target gene with a resistance marker. Primary transformants that exhibited resistance to hygromycin were selected and subsequently crossed in order to obtain homokaryotic deletion mutants without the  $\Delta ku70$  background. The deletion of *rtt109* in the resulting strains was verified by PCR with primers designed to amplify a part of the gene of interest. The correct integration of the deletion cassette was verified by PCR (Figure S1 in File S1). Throughout the generation of the deletion strains, primary transformants did not exhibit any noticeable defects in sexual development and demonstrated the ability to achieve fertility. Since primary transformants are heterokaryotic in most cases, effects of gene deletions are often not directly obvious and sometimes appear only in homokaryotic strains. To obtain homokaryons, the primary transformants were crossed with the spore color mutant *fus* and the resulting homokaryotic ascospores were isolated. Homokaryotic  $\Delta rtt109$  strains were found to be sterile (Table S4 in File S1). Microscopic examination confirmed a block in the sexual development of  $\Delta rtt109$  at the stage of young protoperithecia (Figure 1). While the ascogonia and early protoperithecia were formed normally and at the expected time, no further structures of sexual development were observed. Even after a week, there was no developmental progression beyond that stage. The developmental effects of *rtt109* deletions in *S. macrospora* appeared to be even more severe than in other ascomycete model organisms such as *F. graminearum*, where a reduction in ascospore formation and aberrant perithecia have been previously documented (17). *rtt109* fused with a C-terminal eGFP tag was reintroduced into the validated deletion strains through ectopic integration to conduct complementation analysis and perform localization studies using fluorescence microscopy. Complementation strains regained fertility and were able to grow wild type-like sexual structures (Figure 1).

Deletion of *rtt109* also inhibited the vegetative growth rate of the respective mutants. Since RTT109 is suspected to be an interaction partner of ASF1, we documented the growth of *rtt109* deletion mutants, the respective complementation strain, and *asf1* deletion mutants to compare them to each other and to the wild type (Figure 2). Deletion of *rtt109* caused a visible reduction in vegetative growth rate, which was very similar to that of *asf1* deletion mutants, although the density of  $\Delta rtt109$  mycelium appeared higher and more wild type-like. The growth rate of *rtt109* deletion strains was fully restored

235 to wild type levels by reintroduction of the *rtt109* gene (Figure 2). We quantified this observation by  
236 measuring the progress of the growth front in 5 biological replicates and detected a reduction of  
237 vegetative growth speed of around 40 % in  $\Delta$ rtt109 and  $\Delta$ asf1 strains (Figure 3). The impairment of  
238 vegetative growth in *S. macrospora*  $\Delta$ rtt109 is consistent with results in *F. graminearum*, where  
239 corresponding deletion mutants were also reported to grow 40% slower than the wild type (17). This  
240 effect was completely reversed by complementation of the *rtt109* mutant and the respective strains  
241 exhibited a growth speed comparable to the wild type (Figure 3). In summary, the findings indicate  
242 that *rtt109* plays a crucial role in the sexual development of *S. macrospora* and is also significant for  
243 vegetative growth. The phenotype of *rtt109* deletion mutants appears highly similar to that of *asf1*  
244 deletion mutants, suggesting that indeed both function in the same pathway.

245 To further assess whether *asf1* and *rtt109* act in a similar manner during development, or whether  
246 there is a combinatorial effect in the absence of both genes, we attempted to cross  $\Delta$ asf1 and  $\Delta$ rtt109  
247 strains to obtain double mutants. However, no fruiting bodies were formed in crosses of the two  
248 strains (Figure S2 in File S1). The inability of the mutants to cross with each other may be another  
249 indication that ASF1 and RTT109 act in a similar developmental pathway.

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### 251 ***S. macrospora* $\Delta$ rtt109 and $\Delta$ asf1 react similarly to methyl methanesulfonate and hydroxyurea**

252 In previous work, we discovered a sensitivity of *S. macrospora*  $\Delta$ asf1 strains to the DNA damaging agent  
253 MMS as well as a reduction in global H3K56ac levels in  $\Delta$ asf1 (11). The histone acetyltransferase  
254 RTT109 interacts with the chromatin modifier ASF1 in *Candida albicans* (13), *Schizosaccharomyces*  
255 *pombe* (37) and *S. cerevisiae* (14) and is involved in various mechanisms that ensure DNA stability  
256 during replication. Additionally, RTT109 is known as the primary enzyme responsible for H3K56  
257 acetylation in *S. cerevisiae* (15). In the case of *A. fumigatus*, deletion of *rtt109* led to heightened  
258 sensitivity to DNA damaging agents such as MMS and HU (19). MMS induces DNA methyl adducts,  
259 which can cause double-strand breaks during replication (38), while HU inhibits the ribonucleotide  
260 reductase and leads to replication arrest by affecting dNTP supply (39). In our study, we exposed *S.*  
261 *macrospora*  $\Delta$ rtt109, the corresponding complementation strain, an *asf1* deletion mutant, and the wild  
262 type to MMS and HU. We found that the deletion of *rtt109* resulted in severe sensitivity to MMS,  
263 comparable to the sensitivity exhibited by the *asf1* mutant (Figure 4). While the wild type and  
264 complementation strains were able to grow relatively normally on BMM media containing MMS, the  
265 mutants failed to grow at all. Strikingly, we did not observe increased sensitivity against HU for the  
266 mutant strains. While aerial hyphae production appeared somewhat reduced, all strains were able to  
267 grow under HU stress (Figure 5). These observations contrast with the effects of *rtt109* deletions in *S.*  
268 *cerevisiae* (15) and *A. fumigatus* (19), where *rtt109* mutants are sensitive to MMS as well as HU,



269 suggesting the presence of additional factors in *S. macrospora* that respond to such specific stress  
270 conditions.

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### 272 ***S. macrospora* RTT109 is localized in the nucleus**

273 Given that RTT109 is described as a histone acetyltransferase, its targets are expected to be found  
274 within the nuclear compartment of the cell. Consequently, it is necessary for RTT109 to localize within  
275 the nucleus. Our complementation experiments with *rtt109* fused to an eGFP tag restored the  
276 phenotype of the deletion mutant, so a correct localization of the fusion protein was expected. To  
277 analyze the localization of RTT109, we employed fluorescence microscopy on the complementation  
278 strains. These strains were cultivated alongside a marker strain that expressed histone H3 fused to an  
279 mRFP tag. Since histones are primarily localized in the nucleus, this setup enabled us to perform co-  
280 localization analysis in the resulting heterokaryons (34). Through the detection of both green and red  
281 fluorescence in the same cellular compartment, we confirmed the co-localization of RTT109 and  
282 histone H3 (Figure 6). Based on these findings, we can conclude that RTT109 in *S. macrospora* is indeed  
283 localized within the nucleus.

284

### 285 **The *rtt109* deletion leads to a loss of H3K56ac *S. macrospora***

286 RTT109 is widely recognized as the primary, and possibly exclusive, histone acetyltransferase  
287 responsible for the acetylation of H3K56 in fungal organisms (14, 15, 21). This specific histone  
288 modification has been demonstrated to play a crucial role in the cellular response to DNA damage  
289 induced by MMS (40). Therefore, we analyzed the levels of H3K56ac in the *S. macrospora*  $\Delta$ rtt109  
290 mutant. To assess changes in the levels of global H3K56ac, we conducted western blot analysis in the  
291 *S. macrospora*  $\Delta$ rtt109 deletion mutant and compared the results to the wild type and the  
292 complemented mutant. Our findings revealed a loss of this histone modification in the deletion mutant  
293 (Figure 7, Figure S3 in File S1). The complementation strain exhibited a complete restoration of  
294 H3K56ac production, indicating the successful reinstatement of normal acetylation.

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### 296 **Deletion of *chk2*, a functional equivalent of *rad53*, has no effect on sexual development**

297 The second potential partner for ASF1 during DNA damage protection we analyzed was the putative  
298 checkpoint kinase CHK2, which is orthologous to the checkpoint kinases PRD-4 from *N. crassa* and the  
299 human CHK2. It is the closest *S. macrospora* homolog to the *S. cerevisiae* checkpoint kinase Rad53  
300 (Figure S4 in File S1). In contrast to the analyzed filamentous fungi and animals, there is a second  
301 homolog in *S. cerevisiae*, Dun1, which is more similar to PRD-4 and CHK2 proteins from animals both  
302 in sequence and domain structure than Rad53 (Figure S4B in File S1). However, PRD-4 from *N. crassa*  
303 and the human CHK2 were both shown to be able to complement an *S. cerevisiae*  $\Delta$ rad53 mutant and

304 are therefore functionally equivalent to Rad53 (25, 26). Rad53 plays a critical role in maintaining  
305 genomic stability and regulating cell cycle arrest during DNA damage repair, as well as histone recycling  
306 in *S. cerevisiae* (41).

307 In yeast, the interaction between Asf1 and Rad53 is well documented, with Asf1 binding  
308 hypophosphorylated, inactive Rad53 and thus ensuring recovery from DNA damage repair (24). To  
309 investigate the role of CHK2 in *S. macrospora*, we deleted the *S. macrospora chk2* using homologous  
310 recombination. Hygromycin resistant primary transformants were crossed to obtain homokaryotic  
311 strains, and the deletion of *chk2* was confirmed by PCR (Figure S5 in File S1). Both primary  
312 transformants and homokaryotic deletion mutants showed normal growth and fruiting body  
313 development without any noticeable differences compared to the wild type. Vegetative growth  
314 appeared wild type-like (Figure 8) and  $\Delta$ chk2 strains achieved fertility within the expected timeframe.  
315 To further assess potential developmental defects, we examined the morphology of perithecia and  
316 asci in the mutant compared to the wild type and found no detectable differences (Figure 9). Thus, a  
317 deletion of *chk2* has no discernible effect on the development of *S. macrospora*.

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#### 319 ***S. macrospora* $\Delta$ chk2 shows the opposite reaction to MMS and HU as $\Delta$ rtt109 and $\Delta$ asf1**

320 Given the known involvement of RAD53 and its functional equivalent PRD-4 in DNA damage protection  
321 (42, 43), it was reasonable to expect a similar role for *S. macrospora* CHK2. Therefore, we conducted  
322 sensitivity tests with the genotoxic substances MMS and HU on the *S. macrospora chk2* deletion  
323 mutant. Surprisingly, the presence or absence of *chk2* did not have a noticeable impact on MMS  
324 resistance in *S. macrospora* (Figure 10), in contrast to findings with the *N. crassa* ortholog PRD-4 (25).  
325 This suggests that the relationship between ASF1 and CHK2 may not be significant in the context of  
326 MMS-induced stress in our model system. To evaluate the possibility that a CHK2 paralog might exist  
327 in *S. macrospora* that could substitute for CHK2 in the  $\Delta$ chk2 mutant and thus explain the lack of  
328 developmental phenotypes and the MMS resistance of the mutant, we performed BLASTP analysis (44)  
329 with CHK2. While several proteins showed partial similarity to CHK2, this similarity was restricted to  
330 the serine/threonine protein kinase domain, and none of the proteins contained the additional  
331 forkhead-associated domain that is present in CHK2 orthologs from *S. macrospora* and other fungi  
332 (Figures S4A, S6 and S7 in File S1). Thus, it appears unlikely that these putative kinases can substitute  
333 for CHK2 in the  $\Delta$ chk2 mutant.

334 In contrast to growth on MMS, under hydroxyurea stress, the deletion of *chk2* had a clear effect, unlike  
335 the deletion of *asf1* (Figure 11). Reintroduction of *chk2* into *S. macrospora*  $\Delta$ chk2 strains  
336 complemented the HU sensitivity phenotype (Table S5 in File S1). While all strains initially exhibited  
337 slow growth under HU stress without significant differences within the first 48 hours,  $\Delta$ chk2 mutants  
338 ceased growing after 48 h. In contrast, the wild type and *asf1* deletion strain proved to be resistant,

339 consistent with previous observations comparing them to  $\Delta rtt109$  strains. These results suggest that  
340 CHK2 plays a critical role in the DNA damage response pathway specific to coping with HU-induced  
341 stress, independent of ASF1 or RTT109.

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## 344 **Discussion**

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### 346 **RTT109 might be necessary for numerous functions of ASF1 during DNA damage protection and** 347 **sexual development**

348 In this study, we investigated the role of two potential partners of the histone chaperone ASF1 during  
349 DNA damage protection and sexual development in the ascomycete *S. macrospora*. Our findings  
350 suggest that the histone acetyltransferase RTT109 has significant functions under normal and DNA  
351 damage stress conditions. Deletion of *rtt109* resulted in strains that exhibited similarities to *asf1*  
352 deletions in terms of vegetative growth, sexual development, histone acetylation, and DNA damage  
353 response. These observations suggest a close relationship between ASF1 and RTT109 in these  
354 processes, as supported by their documented interactions in other fungal models like *C. albicans*, *S.*  
355 *pombe* and *S. cerevisiae* (13, 14, 37). *S. macrospora rtt109/asf1* double mutants may be useful to  
356 determine if the two proteins act in the same pathway. A phenotype similar to single mutants with  
357 respect to growth and development should be observed in double mutants if RTT109 and ASF1 act in  
358 a cooperative manner during these processes. However, the single mutants proved to be unable to  
359 form sexual structures in genetic crosses. This could be the result of an effect on sexual development  
360 shared by both deletion strains, or the result of stress levels too high to allow proper crossing. The  
361 similarities between the respective deletion strains described raise the hypothesis that the severe  
362 phenotype observed in *S. macrospora*  $\Delta asf1$  may be the consequence of the lack of interaction with  
363 RTT109. Studies in budding yeast have shown that Rtt109 relies on Asf1 to obtain histone H3 for  
364 acetylating K56 and the absence of Asf1 may nullify the functions of Rtt109 (45). In previous work, we  
365 demonstrated that sexual development in *S. macrospora* depends on the ability of ASF1 to bind  
366 histones and hypothesized that it acts as scaffold for H3-H4 interactions with other chromatin  
367 modifiers (11). The absence of such a scaffold might impair the ability of RTT109 to perform its  
368 functions in histone acetylation, which could cause the severe developmental defects in *asf1* deletion  
369 mutants. Thus, a reduction or improper positioning of H3K56ac may be the underlying issue in *S.*  
370 *macrospora*  $\Delta asf1$  and proper acetylation of H3 may be crucial for sexual development. While  
371 overexpression experiments with *rtt109* in *S. macrospora*  $\Delta asf1$  might provide information about the  
372 general level of H3K56ac necessary for development, positioning might still be problematic, and since  
373 ASF1 is thought to be the scaffold necessary for RTT109 to function at its full extent (12), higher RTT109

374 levels might not even correlate with higher H3K56ac levels in *asf1* deletion mutants. However, given  
375 the wide range of putative and proven functions of ASF1 (46), it may be challenging to pinpoint its role  
376 during *S. macrospora* development to a single interaction partner and its effect on H3K56ac.  
377 Interestingly, the DNA damage response functions of ASF1 do not seem to depend on its interaction  
378 with histones in the same way as sexual development, although ASF1 variants unable to bind histones  
379 showed a similar reduction of H3K56ac as full deletion mutants (11). However, our results on the  
380 sensitivity of *rtt109* deletion mutants to MMS show a dependence on RTT109 for DNA damage  
381 protection and therefore probably for the establishment of H3K56ac. More sensitive assays such as  
382 ChIP-seq are needed to fully quantify the potential differences in H3K56ac levels between *S.*  
383 *macrospora*  $\Delta asf1$  and  $\Delta rtt109$ , and our results indicate that deletion of the HAT causes an even greater  
384 decrease, or even complete loss in H3K56 acetylation levels than the loss of a putative scaffold. In *S.*  
385 *cerevisiae*, Rtt109 is known to interact not only with Asf1 but also with another histone chaperone,  
386 Vps75, which also binds H3 and H4 (14). Although a fully functional Asf1-Vps75-Rtt109 complex is  
387 known to be necessary for establishing correct acetylation patterns in budding yeast (47), the mere  
388 presence of ASF1, even without histone binding ability, might be sufficient to enable lower-level  
389 activity of RTT109, providing some form of DNA damage protection. Therefore, the severe  
390 developmental defects observed in *S. macrospora*  $\Delta asf1$ , persistent when expressing ASF1 variants  
391 unable to bind histones, may have roots not only in the misregulation of developmental processes, but  
392 also in the accumulation of underlying DNA damage events, compromising the respective strains as a  
393 whole. Such DNA damage accumulation might be too insignificant to turn up during a sensitivity assay,  
394 but could potentially lead to disturbances during tightly regulated processes, such as sexual  
395 development and fruiting body formation. While this hypothesis requires further investigation, such  
396 as interaction studies between RTT109, ASF1 and VPS75, the role of RTT109 during sexual  
397 development and the DNA damage response in *S. macrospora* appears to be quite fundamental. As  
398 the primary facilitator of H3K56ac in fungi (15), RTT109 is clearly essential for survival under MMS  
399 conditions, which can induce double-strand breaks during replication (38). Furthermore, RTT109 is  
400 crucial during the formation of complex sexual structures, either by providing the necessary genomic  
401 stability for such processes or by ensuring the correct transcription of important genes through the  
402 establishment of proper acetylation patterns. Surprisingly, the deletion of *rtt109* did not increase the  
403 sensitivity of *S. macrospora* to hydroxyurea, despite its well-known DNA damaging properties that  
404 cause replication fork arrest by disrupting dNTP availability (39). In contrast, other fungal model  
405 organisms like *A. fumigatus* and *S. cerevisiae* have been shown to exhibit heightened sensitivity to HU  
406 when *rtt109* is deleted (19, 48).

407

408

409 **CHK2 is not essential for sexual development, but provides resistance to genotoxic stress**  
410 **independent of ASF1**

411 The second putative partner of ASF1 during DNA damage protection we investigated was CHK2. CHK2  
412 is the ortholog of *N. crassa* PRD-4, which can complement a  $\Delta$ rad53 mutant with respect to its function  
413 in DNA damage response in *S. cerevisiae* (25). Deletion of *chk2* did not result in any noticeable defects  
414 in vegetative growth or developmental processes, suggesting its negligible role in the formation of  
415 fruiting bodies. Furthermore, the mutants lacking *chk2* did not exhibit increased sensitivity to MMS-  
416 induced DNA damage stress, unlike the highly sensitive *asf1* and *rtt109* mutants. This indicates that  
417 CHK2 operates in a distinct damage response pathway from ASF1 and RTT109 under these specific  
418 conditions. However, we discovered a role for CHK2 in response to a different type of genotoxic stress,  
419 as the mutants showed sensitivity to the replication fork stressor hydroxyurea, while the *asf1* and  
420 *rtt109* mutants did not display such sensitivity. The budding yeast equivalent, Rad53, is known to be  
421 important for restarting inhibited replication forks (49), so it is reasonable to assume that the  
422 replication fork stalling caused by hydroxyurea cannot be efficiently rescued in the absence of Rad53,  
423 or in this case CHK2 in *S. macrospora*. Since  $\Delta$ asf1 strains are not inhibited under hydroxyurea stress,  
424 this suggests that CHK2 is involved in a genomic protection system that functions independently of  
425 ASF1. Notably, this observation appears to be specific for *S. macrospora*, as an *S. cerevisiae*  $\Delta$ asf1  
426 mutant is sensitive to hydroxyurea (50). The precise functions of CHK2 and its relationship with ASF1  
427 in its broader chromatin modification network are more challenging to elucidate, given the specific  
428 roles fulfilled by homologous checkpoint kinases like PRD-4 or functional equivalents like RAD53 in  
429 their respective native organisms. However, it can be inferred that any connection between ASF1 and  
430 CHK2 in DNA damage protection and sexual development is of minor importance. Additionally, the  
431 diverse functions of ASF1 in *S. macrospora* do not seem to include an active role in the response to  
432 hydroxyurea-induced stress.

433  
434 In conclusion, our study revealed an essential role of the histone acetyltransferase RTT109 during  
435 sexual development, vegetative growth, histone modification and genotoxic stress response in the  
436 ascomycete *S. macrospora*. The phenotypic aberrations in *rtt109* deletion strains closely resemble  
437 those previously observed in *asf1* deletion mutants, indicating a strong correlation between the  
438 functions of ASF1 and RTT109. CHK2, another potential component of the ASF1-mediated chromatin  
439 modifier network, appears to be unrelated to these processes but is instead involved in a distinct DNA  
440 damage response system that operates independently of ASF1.

441

442

443 **Data Availability Statement**

444 Strains and plasmids are available upon request. The authors affirm that all data necessary for  
445 confirming the conclusions of the article are present within the article, figures, and tables.

446

447

#### 448 **Acknowledgements**

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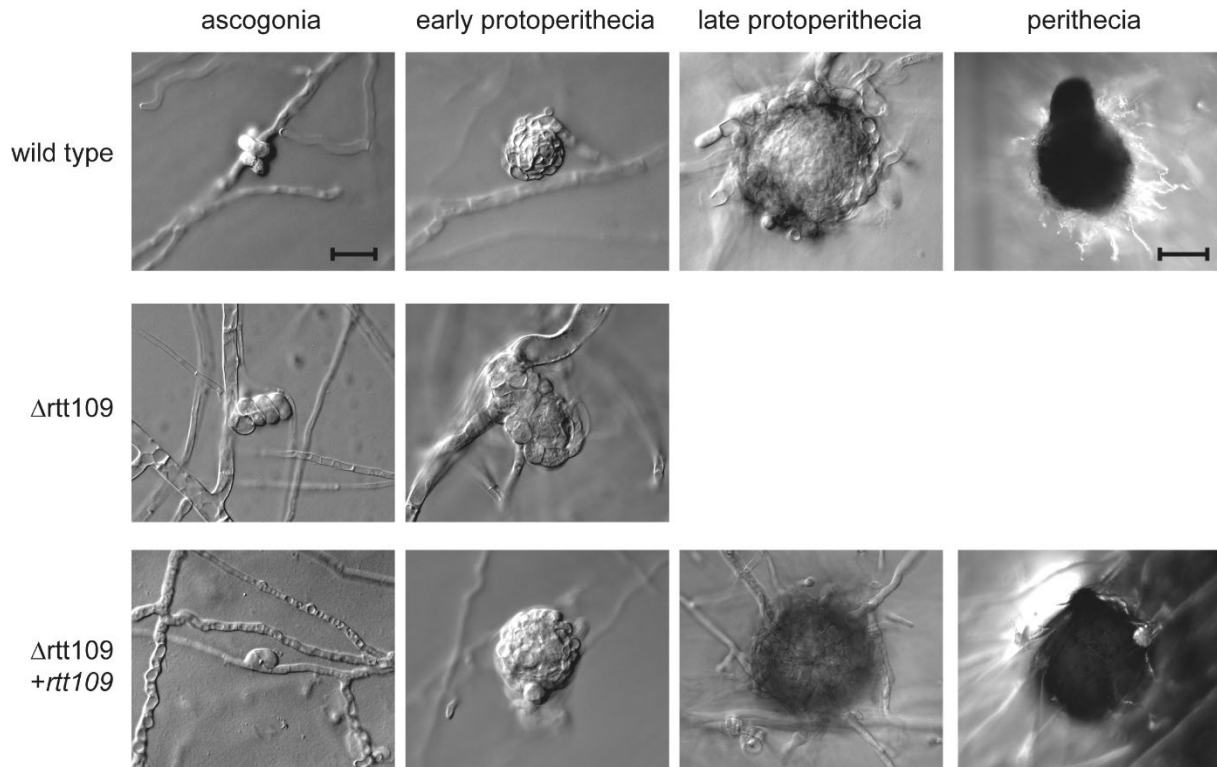
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585



586 **Figures**

587



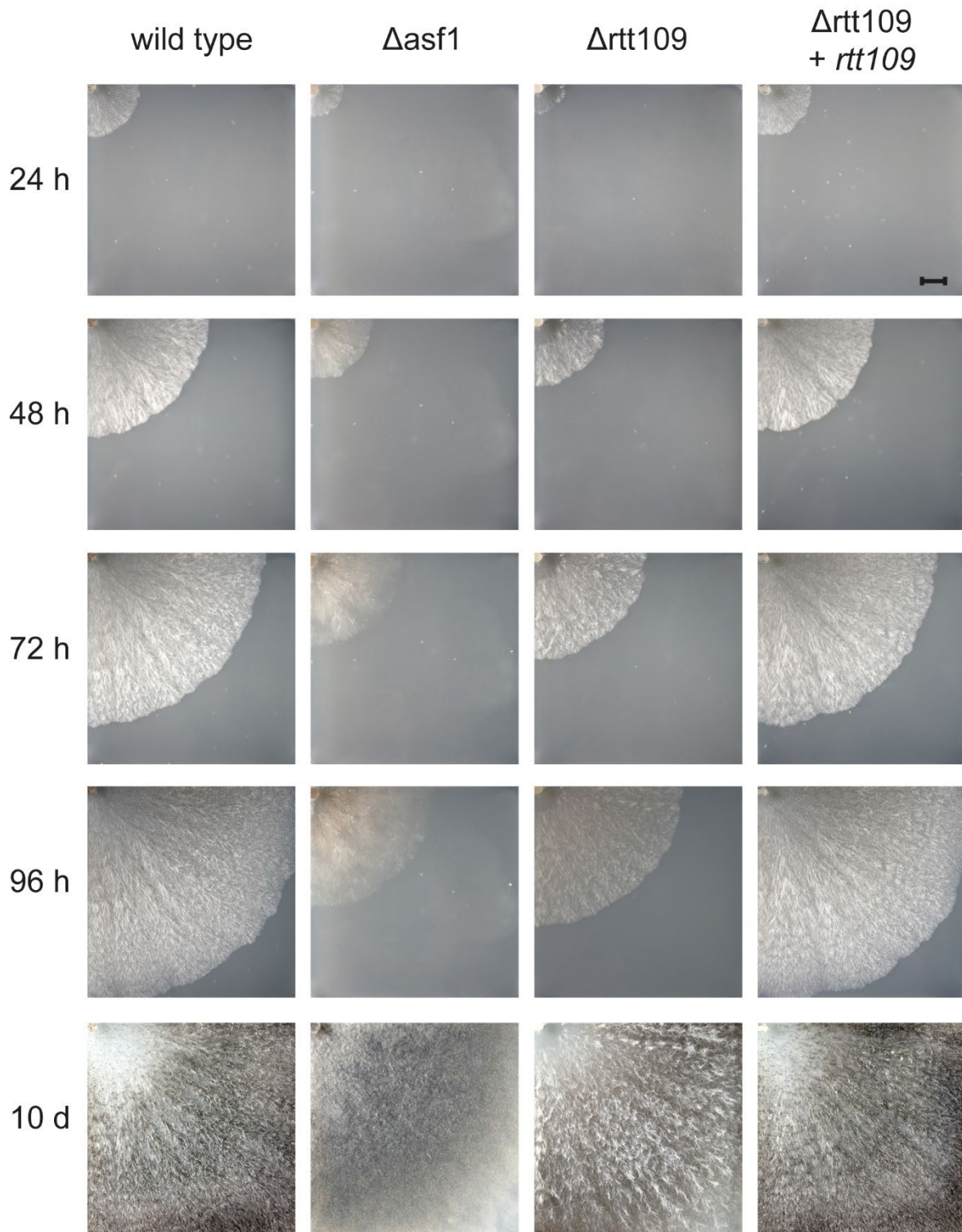
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590 **Figure 1:** Development of *rtt109* deletion mutants compared to wild type and complementation  
591 strains. *S. macrospora* strains lacking the *rtt109* gene were found to exhibit impaired development  
592 with a block at the stage of early protoperithecia formation. Upon reintroduction of *rtt109*, normal life  
593 cycle progression was fully restored, as evidenced by the formation of late, melanized protoperithecia  
594 and the development of fully formed perithecia indistinguishable from those of the wild type strain.  
595 The scale bar for ascogonia and protoperithecia represents 20  $\mu\text{m}$ , while the scale bar for perithecia  
596 represents 100  $\mu\text{m}$ .

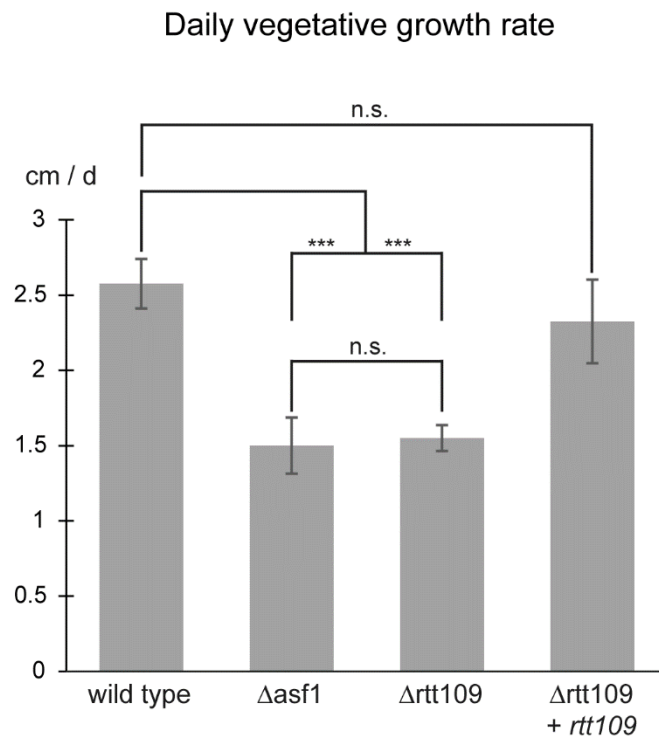
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600 **Figure 2.** Comparison of vegetative growth of *rtt109* deletion and complementation strains with wild  
601 type and *asf1* deletion strains. Mycelial spread was observed over a period of 96 h and a final time  
602 after 10 d. While the wild type almost covered the entire plate in 96 h, *S. macrospora*  $\Delta rtt109$  was  
603 significantly slower and grew as slowly as  $\Delta asf1$  strains. This effect was reversed by reintroduction of  
604 *rtt109* in the *rtt109* deletion mutant, complementing the growth defect of the mutant. A visible  
605 difference between  $\Delta rtt109$  and  $\Delta asf1$  was the density of the mycelium. *S. macrospora*  $\Delta rtt109$   
606 appeared to grow as densely as the wild type, while  $\Delta asf1$  appeared to be thinner overall. Scale bar  
607 represent 1 cm.



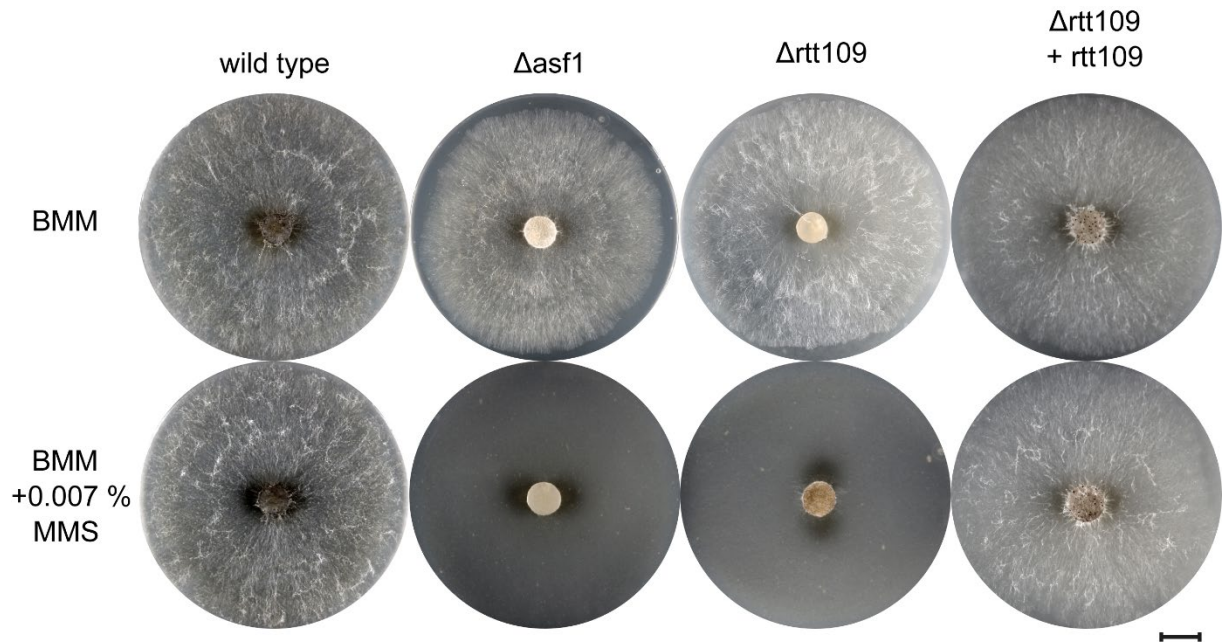
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610 **Figure 3.** Quantification of the vegetative growth rates of *S. macrospora*  $\Delta rtt109$ , the respective  
611 complementation strain,  $\Delta asf1$  and the wild type. While the wild type grew at about 2.5 cm per day,  
612 deletion of *rtt109* or *asf1* resulted in a significant decrease in growth rate to about 1.5 cm per day.  
613 Complementation of *rtt109* mutants restored the growth rate to wild type levels. Quantification was  
614 performed for 5 independent replicates and significance was assessed by Student's t-test. \*\*\* = p-  
615 value < 0.001, n.s. = p-value > 0.05.

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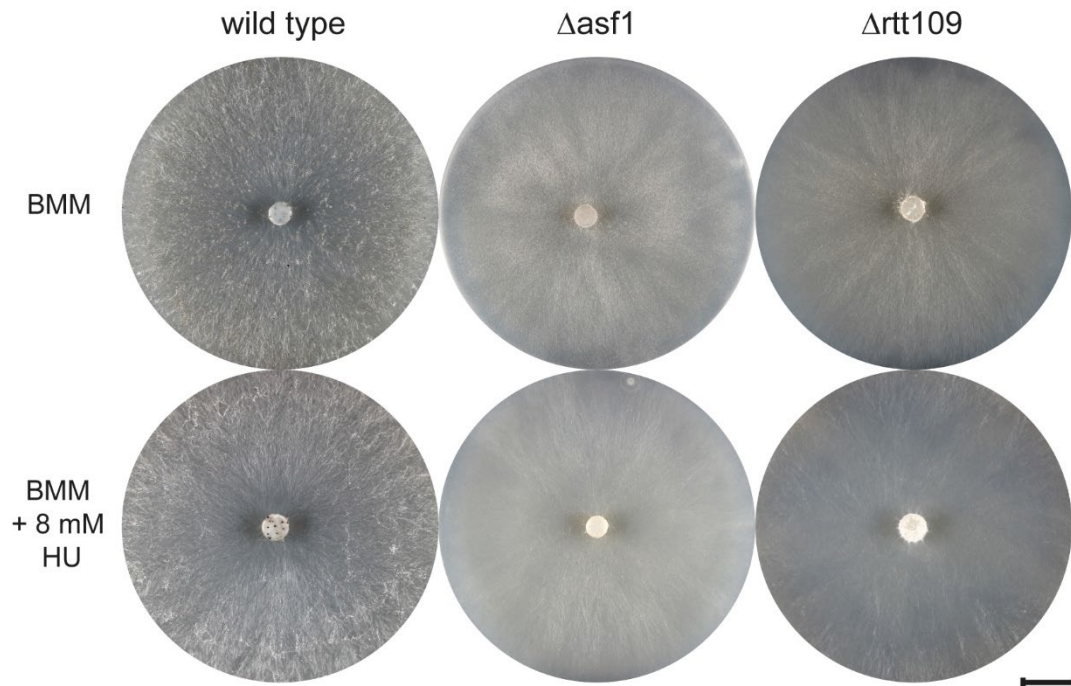


618

619 **Figure 4.** MMS sensitivity test for *S. macrospora*  $\Delta rtt109$ . The sensitivity of *S. macrospora* *rtt109*  
620 deletion mutants to the genotoxic compound MMS was observed after 4 days of growth on BMM  
621 media and BMM media supplemented with 0.007 % MMS. The addition of MMS completely halted the  
622 growth of the *rtt109* and *asf1* deletion mutants. When *rtt109* was reintroduced into  $\Delta rtt109$ , its ability  
623 to survive under MMS stress was restored. The scale bar provided represents 1 cm.

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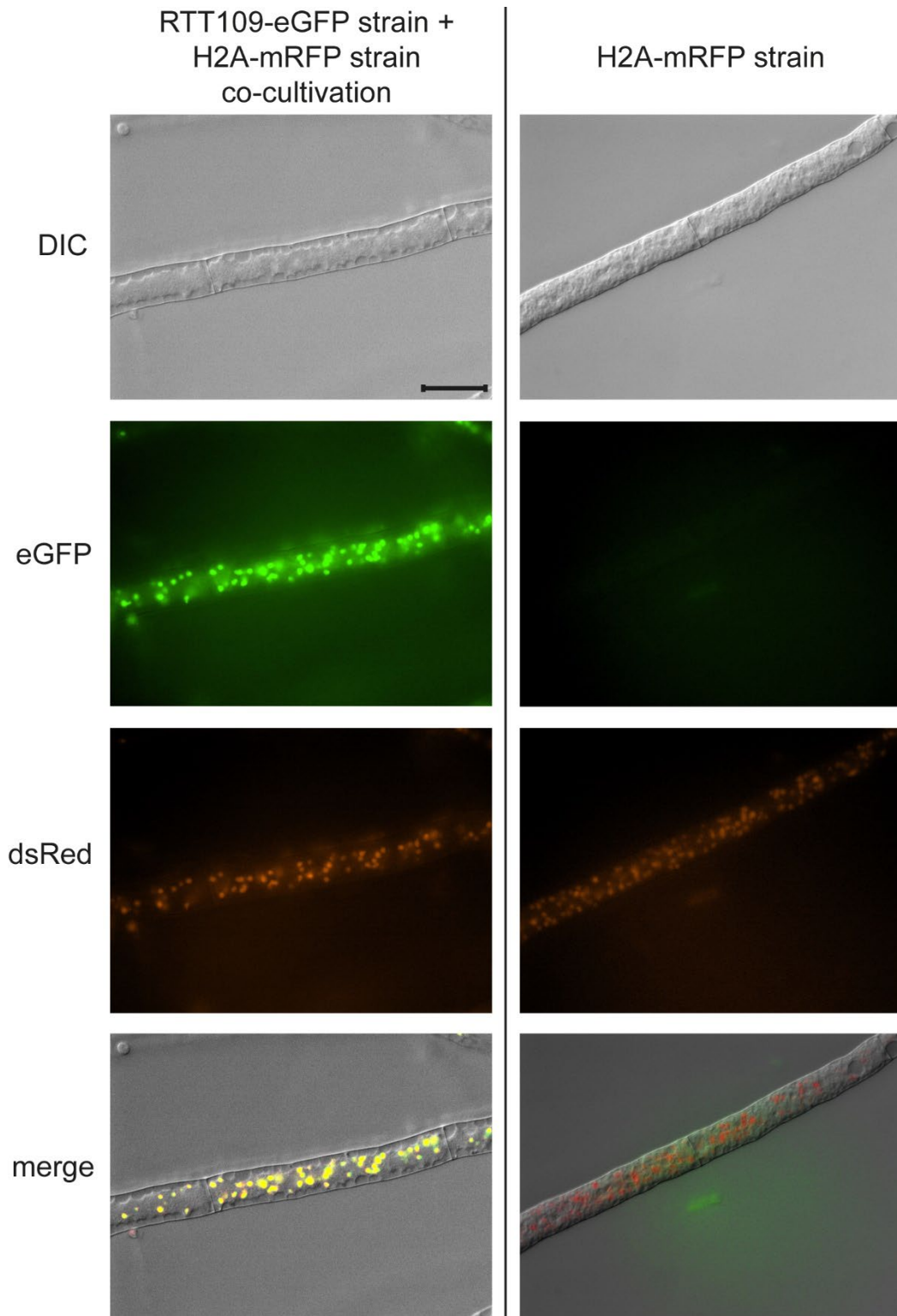


626

627 **Figure 5. HU sensitivity test for *S. macrospora*  $\Delta rtt109$ .** After a 4-day incubation on BMM media  
628 supplemented with 8 mM hydroxyurea, the sensitivity of *rtt109* deletion mutants was assessed. *S.*  
629 *macrospora*  $\Delta rtt109$  exhibited no visible sensitivity to this DNA damaging agent, resembling the  
630 resistance observed in  $\Delta asf1$ . Scale bar represents 1 cm.

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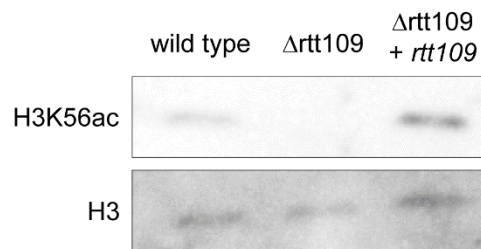
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634 **Figure 6.** Localization analysis of RTT109 by fluorescence microscopy. RTT109 was expressed as a fusion  
635 protein with an eGFP tag and cultivated together with a strain expressing histone H3 fused to an mRFP  
636 tag. The visible colocalization of the green and red fluorescence indicates a nuclear localization of  
637 RTT109. No green autofluorescence was detectable in the reference strain expressing only H2A-mRFP.  
638 Scale bar represents 20  $\mu\text{m}$ .

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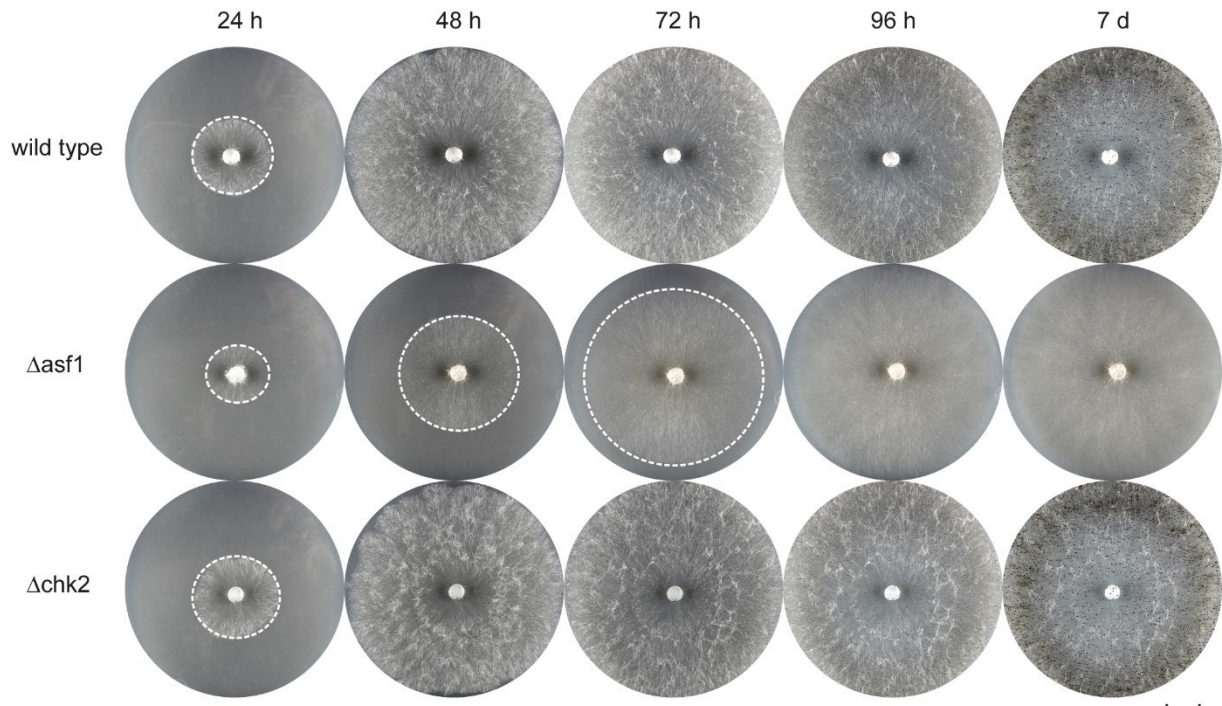
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641 **Figure 7.** Assessment of H3K56ac levels in *S. macrospora* Δrtt109.

642 The level of global H3K56ac was determined by comparing equal amounts of whole protein extracts  
643 from the wild type, Δrtt109, and the respective complementation strain by SDS-Page separation and  
644 western blotting with H3K56ac-specific antibodies. H3 antibodies were used to assess equal loading  
645 and comparable amounts of histone 3 in the protein extracts. In three biological replicates, no signal  
646 for H3K56ac was detectable in Δrtt109 strains. This effect was complemented by reintroduction of  
647 *rtt109* in the deletion mutant. Uncropped blots and the corresponding Coomassie gels are shown in  
648 Figure S3 in File S1.

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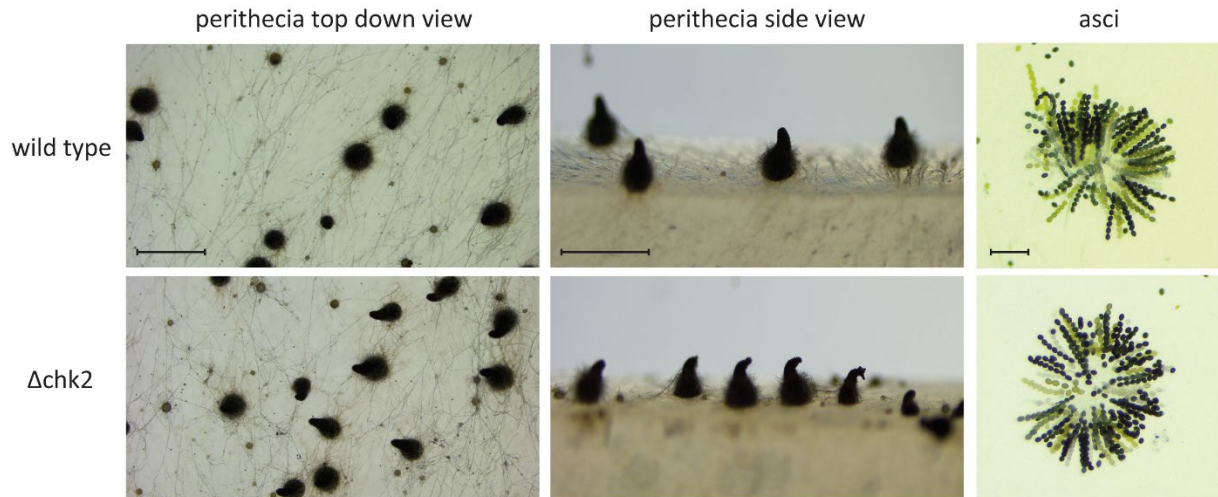
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652 **Figure 8: Growth comparison of *S. macrospora*  $\Delta chk2$  with  $\Delta asf1$  and the wild type.** The growth and  
653 overall characteristics of the strains were monitored for 96 hours, with a final observation after 7 days  
654 on BMM media. There were no noticeable differences between the *chk2* deletion mutant and the wild  
655 type strain. Both strains exhibited similar growth rates and developed visible perithecia. The  $\Delta asf1$   
656 strain displayed significantly slower growth and failed to produce perithecia throughout the  
657 observation period. White dashed circles indicate the growth front. Scale bar represents 1 cm.

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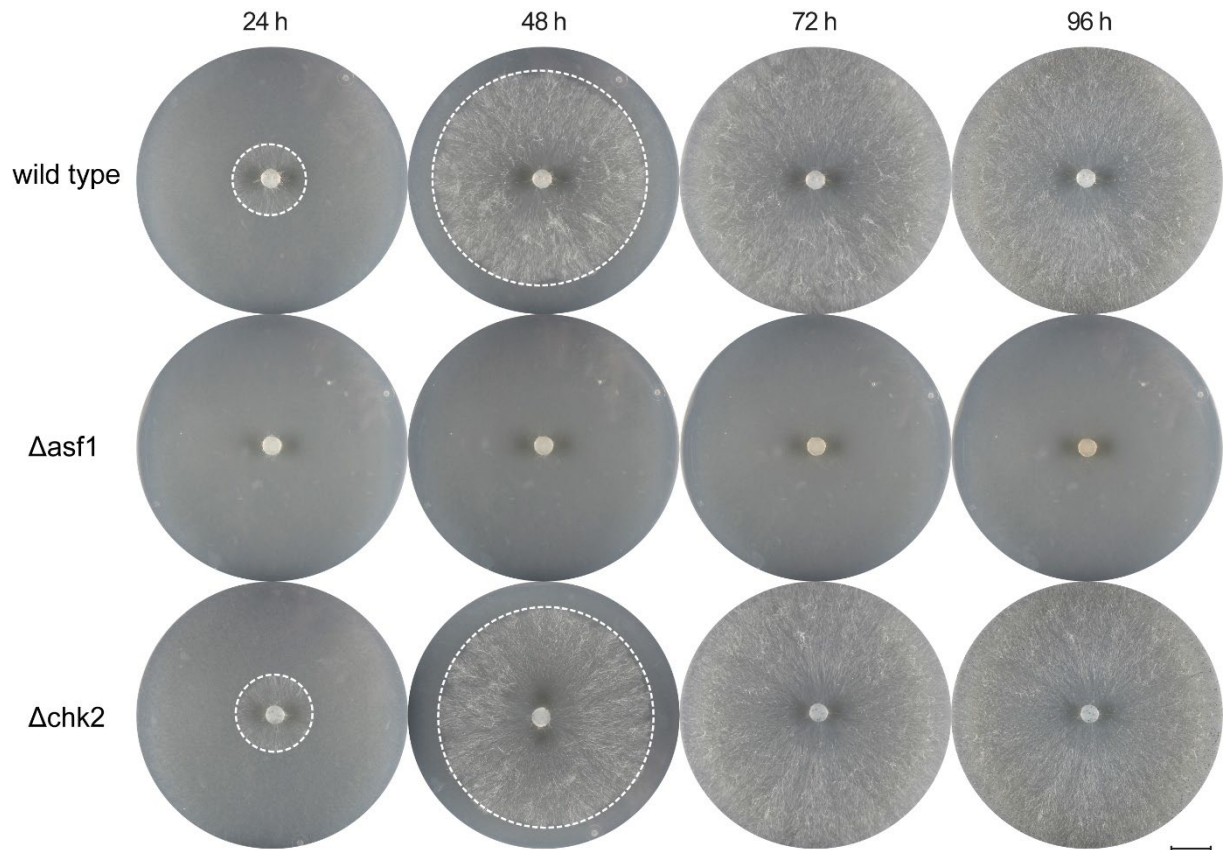


660

661 **Figure 9: Morphological comparison of sexual structures generated by *S. macrospora*  $\Delta$ chk2 with**  
662 **those of the wild type.** The *chk2* deletion mutant demonstrated the ability to undergo the complete  
663 life cycle of *S. macrospora* without any impairments. The fruiting bodies produced by the mutant  
664 exhibited no abnormalities in comparison to the wild type strain. The perithecia were formed and  
665 positioned in a normal manner and the overall morphology of the perithecia did not display any visible  
666 defects.  $\Delta$ chk2 strains generated wild type-like asci. Scale bars represent 1 mm for top down view, 500  
667  $\mu$ m for side view and 100  $\mu$ m for asci.

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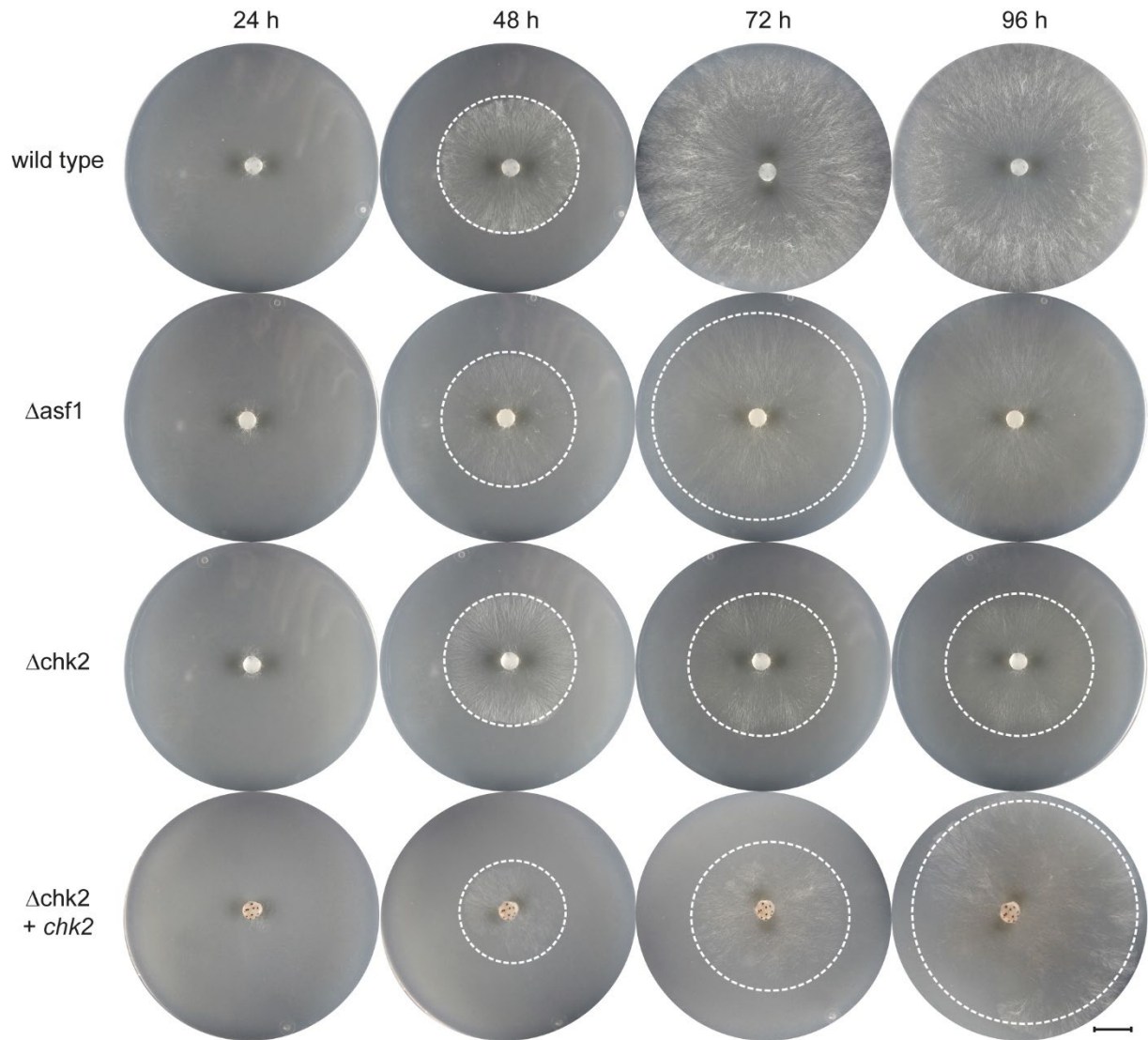


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671 **Figure 10: MMS sensitivity test for *S. macrospora*  $\Delta chk2$ .** The growth of the strains was monitored on  
672 BMM media supplemented with 0.007% MMS for a duration of 96 hours. The *S. macrospora*  $\Delta chk2$   
673 strain did not display increased sensitivity to the DNA damaging agent compared to the wild type  
674 strain. Consistent with previous observations, the *asf1* deletion mutant exhibited a high level of  
675 sensitivity to MMS. White dashed circles indicate the growth front. Scale bar represents 1 cm.

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679 **Figure 11: HU sensitivity test for *S. macrospora*  $\Delta chk2$ .** The growth of the strains was observed on  
680 BMM media supplemented with 8 mM hydroxyurea for a period of 96 hours. In contrast to the  
681 observations with MMS, the *chk2* deletion mutant exhibited sensitivity to the DNA damaging agent  
682 hydroxyurea. While  $\Delta chk2$  strains appeared to progress normally for the first 48 hours, they stopped  
683 growing after that time. Reintroduction of the *chk2* gene restored HU resistance. In contrast, the wild  
684 type and  $\Delta asf1$  strains continued to grow under the same conditions. White dashed circles indicate the  
685 growth front. Scale bar represents 1 cm.

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