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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23 running head: chromatin-associated proteins in fungi

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Abstract

We performed a functional analysis of two potential partners of ASF1, a highly conserved histone chaperone that plays a crucial role in the sexual development and DNA damage resistance in the ascomycete *Sordaria macrospora*. ASF1 is known to be involved in nucleosome assembly and disassembly, binding histones H3 and H4 during transcription, replication and DNA repair and has direct and indirect roles in histone recycling and modification as well as DNA methylation, acting as a chromatin modifier hub for a large network of chromatin-associated proteins. Here, we functionally characterized two of these proteins, RTT109 and CHK2. RTT109 is a fungal-specific histone acetyltransferase, while CHK2 is an ortholog to PRD-4, a checkpoint kinase of *Neurospora crassa* that performs similar cell cycle checkpoint functions as yeast RAD53. Through the generation and characterization of deletion mutants, we discovered striking similarities between RTT109 and ASF1 in terms of their contributions to sexual development, histone acetylation and protection against DNA damage. Phenotypic observations revealed a developmental arrest at the same stage in Δrtt109 and Δasf1 strains, accompanied by a loss of H3K56 acetylation, as detected by western blot analysis. Deletion mutants of *rtt109* and *asf1* are sensitive to the DNA damaging agent MMS (methylmethane sulfonate), but not HU (hydroxyurea). In contrast, *chk2* mutants are fertile and resistant to MMS, but not HU. Our findings suggest a close functional association between ASF1 and RTT109 in the context of development, histone modification and DNA damage response, while indicating a role for CHK2 in separate pathways of the DNA damage response.

Article summary

In the filamentous fungus *Sordaria macrospora*, the conserved histone chaperone ASF1, which interacts with histones H3 and H4, was previously shown to be required for multicellular development and DNA damage response. Here, we have analyzed two additional chromatin-associated proteins. *rtt109* encodes a histone acetyltransferase, and deletion of the gene in *S. macrospora* results in a phenotype similar to that of a Δasf1 mutant, whereas *chk2* is involved in different aspects of the DNA damage response, but not in development.

Introduction

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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 Δasf1 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. cerevisae* 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. cerevisae* Δasf1

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. cerevisae 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* Δrad53 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 Δrtt109 by

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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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Materials and Methods

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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).

143

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).

150

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 P*gpd* promoter and

155 T*trpC* 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* Δku70

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 Δku70

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 P*gpd* promoter, was introduced into the corresponding strains via transformation. Ascospores were

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165 isolated from the transformants that reached fertility, allowing the acquisition of homokaryotic strains

166 carrying the complementation construct.

167

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.

177

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).

187

Western blot analysis

189 To evaluate differences in the global levels of H3K56ac between the Δrtt109 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 Δrtt109 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

203

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 Δku70 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 Δrtt109 strains were found to be sterile (Table S4 in File S1). Microscopic examination confirmed a block in the sexual development of Δrtt109 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 Δrtt109 mycelium appeared higher and more wild type-like. The growth rate of *rtt109* deletion strains was fully restored

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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 Δrtt109 and Δasf1 strains (Figure 3). The impairment of

238   vegetative growth in *S. macrospora* Δrtt109 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 Δasf1 and Δrtt109

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.

250

251   **S. macrospora Δrtt109 and Δasf1 react similarly to methyl methanesulfonate and hydroxyurea**

252   In previous work, we discovered a sensitivity of *S. macrospora* Δasf1 strains to the DNA damaging agent

253   MMS as well as a reduction in global H3K56ac levels in Δasf1 (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* Δrtt109, 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   *cerevisae* (15) and *A. fumigatus* (19), where *rtt109* mutants are sensitive to MMS as well as HU,

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

271

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

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* Δrtt109
290 mutant. To assess changes in the levels of global H3K56ac, we conducted western blot analysis in the
291 *S. macrospora* Δrtt109 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.

295

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* Δrad53 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 Δchk2 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*.

318

319   ***S. macrospora* Δchk2 shows the opposite reaction to MMS and HU as Δrtt109 and Δasf1**

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 Δchk2 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 Δchk2 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* Δchk2 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, Δchk2 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 Δrtt109 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.

342

343

344 **Discussion**

345

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. cerevisae* (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* Δasf1 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* Δasf1 and proper acetylation of H3 may be crucial for sexual development. While

371 overexpression experiments with *rtt109* in *S. macrospora* Δasf1 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

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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* Δasf1 and Δrtt109, 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* Δasf1, 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).

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

The second putative partner of ASF1 during DNA damage protection we investigated was CHK2. CHK2 is the ortholog of *N. crassa* PRD-4, which can complement a Δrad53 mutant with respect to its function in DNA damage response in *S. cerevisiae* (25). Deletion of *chk2* did not result in any noticeable defects in vegetative growth or developmental processes, suggesting its negligible role in the formation of fruiting bodies. Furthermore, the mutants lacking *chk2* did not exhibit increased sensitivity to MMS-induced DNA damage stress, unlike the highly sensitive *asf1* and *rtt109* mutants. This indicates that CHK2 operates in a distinct damage response pathway from ASF1 and RTT109 under these specific conditions. However, we discovered a role for CHK2 in response to a different type of genotoxic stress, as the mutants showed sensitivity to the replication fork stressor hydroxyurea, while the *asf1* and *rtt109* mutants did not display such sensitivity. The budding yeast equivalent, Rad53, is known to be important for restarting inhibited replication forks (49), so it is reasonable to assume that the replication fork stalling caused by hydroxyurea cannot be efficiently rescued in the absence of Rad53, or in this case CHK2 in *S. macrospora*. Since Δasf1 strains are not inhibited under hydroxyurea stress, this suggests that CHK2 is involved in a genomic protection system that functions independently of ASF1. Notably, this observation appears to be specific for *S. macrospora*, as an *S. cerevisiae* Δasf1 mutant is sensitive to hydroxyurea (50). The precise functions of CHK2 and its relationship with ASF1 in its broader chromatin modification network are more challenging to elucidate, given the specific roles fulfilled by homologous checkpoint kinases like PRD-4 or functional equivalents like RAD53 in their respective native organisms. However, it can be inferred that any connection between ASF1 and CHK2 in DNA damage protection and sexual development is of minor importance. Additionally, the diverse functions of ASF1 in *S. macrospora* do not seem to include an active role in the response to hydroxyurea-induced stress.

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

Data Availability Statement

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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.

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454 **References**

455 1.   Teichert I, Pöggeler S, Nowrousian M. 2020. *Sordaria macrospora*: 25 years as a model
456   organism for studying the molecular mechanisms of fruiting body development. Appl
457   Microbiol Biotechnol 104:3691-3704.
458 2.   Lord KM, Read ND. 2011. Perithecium morphogenesis in *Sordaria macrospora*. Fungal Genet
459   Biol 48:388-99.
460 3.   Kück U, Pöggeler S, Nowrousian M, Nolting N, Teichert I. 2009. *Sordaria macrospora*, a Model
461   System for Fungal Development, p 17-39 doi:10.1007/978-3-642-00286-1_2.
462 4.   Nowrousian M. 2018. Genomics and transcriptomics to study fruiting body development: An
463   update. Fungal Biology Reviews 32:231-235.
464 5.   Blank-Landeshammer B, Teichert I, Märker R, Nowrousian M, Kück U, Sickmann A. 2019.
465   Combination of proteogenomics with peptide *de novo* sequencing identifies new genes and
466   hidden posttranscriptional modifications. mBio 10:e02367-19.
467 6.   Gesing S, Schindler D, Fränzel B, Wolters D, Nowrousian M. 2012. The histone chaperone ASF1
468   is essential for sexual development in the filamentous fungus *Sordaria macrospora*. Mol
469   Microbiol 84:748-65.
470 7.   Yin X, Zhou M, Zhang L, Fu Y, Xu M, Wang X, Cui Z, Gao Z, Li M, Dong Y, Feng H, Ma S, Chen C.
471   2022. Histone chaperone ASF1A accelerates chronic myeloid leukemia blast crisis by activating
472   Notch signaling. Cell Death Dis 13:842.
473 8.   English CM, Adkins MW, Carson JJ, Churchill ME, Tyler JK. 2006. Structural basis for the histone
474   chaperone activity of Asf1. Cell 127:495-508.
475 9.   De Koning L, Corpet A, Haber JE, Almouzni G. 2007. Histone chaperones: an escort network
476   regulating histone traffic. Nat Struct Mol Biol 14:997-1007.
477 10. Schumacher DI, Lütkenhaus R, Altegoer F, Teichert I, Kück U, Nowrousian M. 2018. The
478   transcription factor PRO44 and the histone chaperone ASF1 regulate distinct aspects of
479   multicellular development in the filamentous fungus *Sordaria macrospora*. BMC Genetics
480   19:112.
481 11. Breuer J, Busche T, Kalinowski J, Nowrousian M. 2023. Histone binding of ASF1 is required for
482   fruiting body development but not for genome stability in the filamentous fungus *Sordaria
483   macrospora.* mBio (online early) https://doi.org/10.1128/mbio.02896-23.
484 12. Cote JM, Kuo YM, Henry RA, Scherman H, Krzizike DD, Andrews AJ. 2019. Two factor
485   authentication: Asf1 mediates crosstalk between H3 K14 and K56 acetylation. Nucleic Acids
486   Res 47:7380-7391.
487 13. Lercher L, Danilenko N, Kirkpatrick J, Carlomagno T. 2018. Structural characterization of the
488   Asf1-Rtt109 interaction and its role in histone acetylation. Nucleic Acids Res 46:2279-2289.

489   14.   Tsubota T, Berndsen CE, Erkmann JA, Smith CL, Yang L, Freitas MA, Denu JM, Kaufman PD.
490   2007. Histone H3-K56 acetylation is catalyzed by histone chaperone-dependent complexes.
491   Mol Cell 25:703-12.
492   15.   Han J, Zhou H, Horazdovsky B, Zhang K, Xu RM, Zhang Z. 2007. Rtt109 acetylates histone H3
493   lysine 56 and functions in DNA replication. Science 315:653-5.
494   16.   Tang Y, Holbert MA, Delgoshaie N, Wurtele H, Guillemette B, Meeth K, Yuan H, Drogaris P, Lee
495   EH, Durette C, Thibault P, Verreault A, Cole PA, Marmorstein R. 2011. Structure of the Rtt109-
496   AcCoA/Vps75 complex and implications for chaperone-mediated histone acetylation.
497   Structure 19:221-31.
498   17.   Kong X, van Diepeningen AD, van der Lee TAJ, Waalwijk C, Xu J, Xu J, Zhang H, Chen W, Feng J.
499   2018. The Fusarium graminearum Histone Acetyltransferases Are Important for
500   Morphogenesis, DON Biosynthesis, and Pathogenicity. Front Microbiol 9:654.
501   18.   Sun R, Wen M, Wu L, Lan H, Yuan J, Wang S. 2021. The Fungi-specific histone Acetyltransferase
502   Rtt109 mediates morphogenesis, Aflatoxin synthesis and pathogenicity in Aspergillus flavus by
503   acetylating H3K9. IMA Fungus 12:9.
504   19.   Zhang Y, Fan J, Ye J, Lu L. 2021. The fungal-specific histone acetyltransferase Rtt109 regulates
505   development, DNA damage response, and virulence in *Aspergillus fumigatus*. Mol Microbiol
506   115:1191-1206.
507   20.   Zhang Z, Yang Q, Sun G, Chen S, He Q, Li S, Liu Y. 2014. Histone H3K56 acetylation is required
508   for quelling-induced small RNA production through its role in homologous recombination. J
509   Biol Chem 289:9365-71.
510   21.   Driscoll R, Hudson A, Jackson SP. 2007. Yeast Rtt109 promotes genome stability by acetylating
511   histone H3 on lysine 56. Science 315:649-52.
512   22.   McClure AW, Diffley JFX. 2021. Rad53 checkpoint kinase regulation of DNA replication fork rate
513   via Mrc1 phosphorylation. eLife 10:e69726.
514   23.   Emili A, Schieltz DM, Yates JR, Hartwell LH. 2001. Dynamic Interaction of DNA Damage
515   Checkpoint Protein Rad53 with Chromatin Assembly Factor Asf1. Molecular Cell 7:13-20.
516   24.   Tsabar M, Waterman DP, Aguilar F, Katsnelson L, Eapen VV, Memisoglu G, Haber JE. 2016. Asf1
517   facilitates dephosphorylation of Rad53 after DNA double-strand break repair. Genes Dev
518   30:1211-24.
519   25.   Pregueiro AM, Liu Q, Baker CL, Dunlap JC, Loros JJ. 2006. The *Neurospora* Checkpoint Kinase
520   2: A Regulatory Link Between the Circadian and Cell Cycles. Science 313:644-649.
521   26.   Matsuoka S, Huang M, Elledge SJ. 1998. Linkage of ATM to cell cycle regulation by the Chk2
522   protein kinase. Science 282:1893-1897.
523   27.   Esser K. Cryptogams : cyanobacteria, algae, fungi, lichens : textbook and practical guide, p. *In*
524   (ed),
525   28.   Walz M, Kück U. 1995. Transformation of *Sordaria macrospora* to hygromycin B resistance:
526   characterization of transformants by electrophoretic karyotyping and tetrad analysis. Curr
527   Genet 29:88-95.
528   29.   Oldenburg KR, Vo KT, Michaelis S, Paddon C. 1997. Recombination-mediated PCR-directed
529   plasmid construction *in vivo* in yeast. Nucleic Acids Res 25:451-2.
530   30.   Terfrüchte M, Joehnk B, Fajardo-Somera R, Braus GH, Riquelme M, Schipper K, Feldbrügge M.
531   2014. Establishing a versatile Golden Gate cloning system for genetic engineering in fungi.
532   Fungal Genet Biol 62:1-10.
533   31.   Dahlmann T, Terfehr D, Becker K, Teichert I. 2021. Golden Gate vectors for efficient gene fusion
534   and gene deletion in diverse filamentous fungi. Curr Genet 67:317-330.
535   32.   Pöggeler S, Kück U. 2006. Highly efficient generation of signal transduction knockout mutants
536   using a fungal strain deficient in the mammalian *ku70* ortholog. Gene 378:1-10.
537   33.   Punt PJ, Dingemanse MA, Kuyvenhoven A, Soede RD, Pouwels PH, van den Hondel CA. 1990.
538   Functional elements in the promoter region of the *Aspergillus nidulans* gpdA gene encoding
539   glyceraldehyde-3-phosphate dehydrogenase. Gene 93:101-9.

34. Schmidt S, Märker R, Ramšak B, Beier-Rosberger AM, Teichert I, Kück U. 2020. Crosstalk Between Pheromone Signaling and NADPH Oxidase Complexes Coordinates Fungal Developmental Processes. Frontiers in Microbiology 11.

35. Engh I, Würtz C, Witzel-Schlömp K, Zhang Hai Y, Hoff B, Nowrousian M, Rottensteiner H, Kück U. 2007. The WW Domain Protein PRO40 Is Required for Fungal Fertility and Associates with Woronin Bodies. Eukaryotic Cell 6:831-843.

36. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248-254.

37. Han J, Zhou H, Li Z, Xu R-M, Zhang Z. 2007. Acetylation of Lysine 56 of Histone H3 Catalyzed by RTT109 and Regulated by ASF1 Is Required for Replisome Integrity*. Journal of Biological Chemistry 282:28587-28596.

38. Ensminger M, Iloff L, Ebel C, Nikolova T, Kaina B, Löbrich M. 2014. DNA breaks and chromosomal aberrations arise when replication meets base excision repair. J Cell Biol 206:29-43.

39. Koç A, Wheeler LJ, Mathews CK, Merrill GF. 2004. Hydroxyurea arrests DNA replication by a mechanism that preserves basal dNTP pools. J Biol Chem 279:223-30.

40. Wurtele H, Kaiser GS, Bacal J, St-Hilaire E, Lee EH, Tsao S, Dorn J, Maddox P, Lisby M, Pasero P, Verreault A. 2012. Histone H3 lysine 56 acetylation and the response to DNA replication fork damage. Mol Cell Biol 32:154-72.

41. Gunjan A, Verreault A. 2003. A Rad53 kinase-dependent surveillance mechanism that regulates histone protein levels in S. cerevisiae. Cell 115:537-49.

42. Pellicioli A, Lucca C, Liberi G, Marini F, Lopes M, Plevani P, Romano A, Di Fiore PP, Foiani M. 1999. Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase. The EMBO Journal 18:6561-6572.

43. Wakabayashi M, Ishii C, Inoue H, Tanaka S. 2008. Genetic analysis of CHK1 and CHK2 homologues revealed a unique cross talk between ATM and ATR pathways in *Neurospora crassa*. DNA Repair (Amst) 7:1951-61.

44. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-3402.

45. Zhang L, Serra-Cardona A, Zhou H, Wang M, Yang N, Zhang Z, Xu RM. 2018. Multisite Substrate Recognition in Asf1-Dependent Acetylation of Histone H3 K56 by Rtt109. Cell 174:818-830.e11.

46. Mousson F, Ochsenbein F, Mann C. 2007. The histone chaperone Asf1 at the crossroads of chromatin and DNA checkpoint pathways. Chromosoma 116:79-93.

47. Adkins MW, Carson JJ, English CM, Ramey CJ, Tyler JK. 2007. The Histone Chaperone Anti-silencing Function 1 Stimulates the Acetylation of Newly Synthesized Histone H3 in S-phase*. Journal of Biological Chemistry 282:1334-1340.

48. Cañas JC, García-Rubio ML, García A, Antequera F, Gómez-González B, Aguilera A. 2022. A role for the *Saccharomyces cerevisiae* Rtt109 histone acetyltransferase in R-loop homeostasis and associated genome instability. Genetics 222:iyac108.

49. Szyjka SJ, Aparicio JG, Viggiani CJ, Knott S, Xu W, Tavaré S, Aparicio OM. 2008. Rad53 regulates replication fork restart after DNA damage in *Saccharomyces cerevisiae*. Genes Dev 22:1906-20.

50. Lin L-j, Minard LV, Johnston GC, Singer RA, Schultz MC. 2010. Asf1 Can Promote Trimethylation of H3 K36 by Set2. Molecular and Cellular Biology 30:1116-1129.
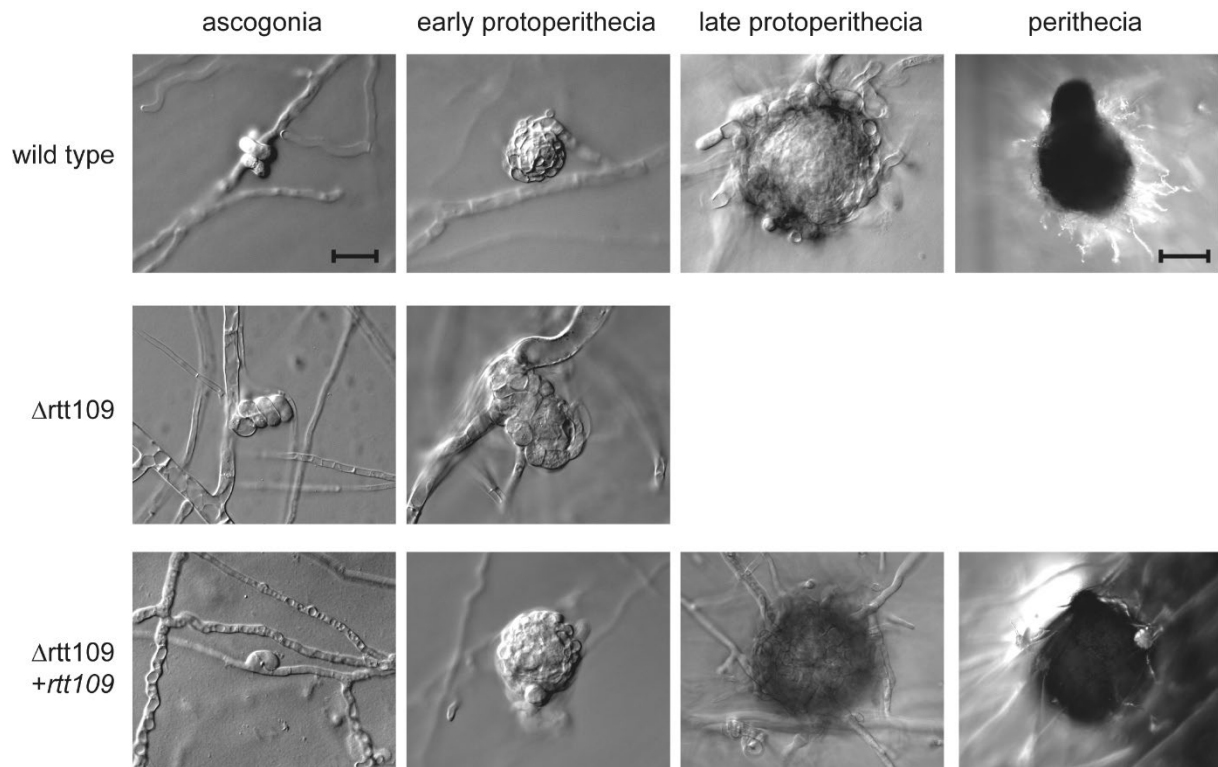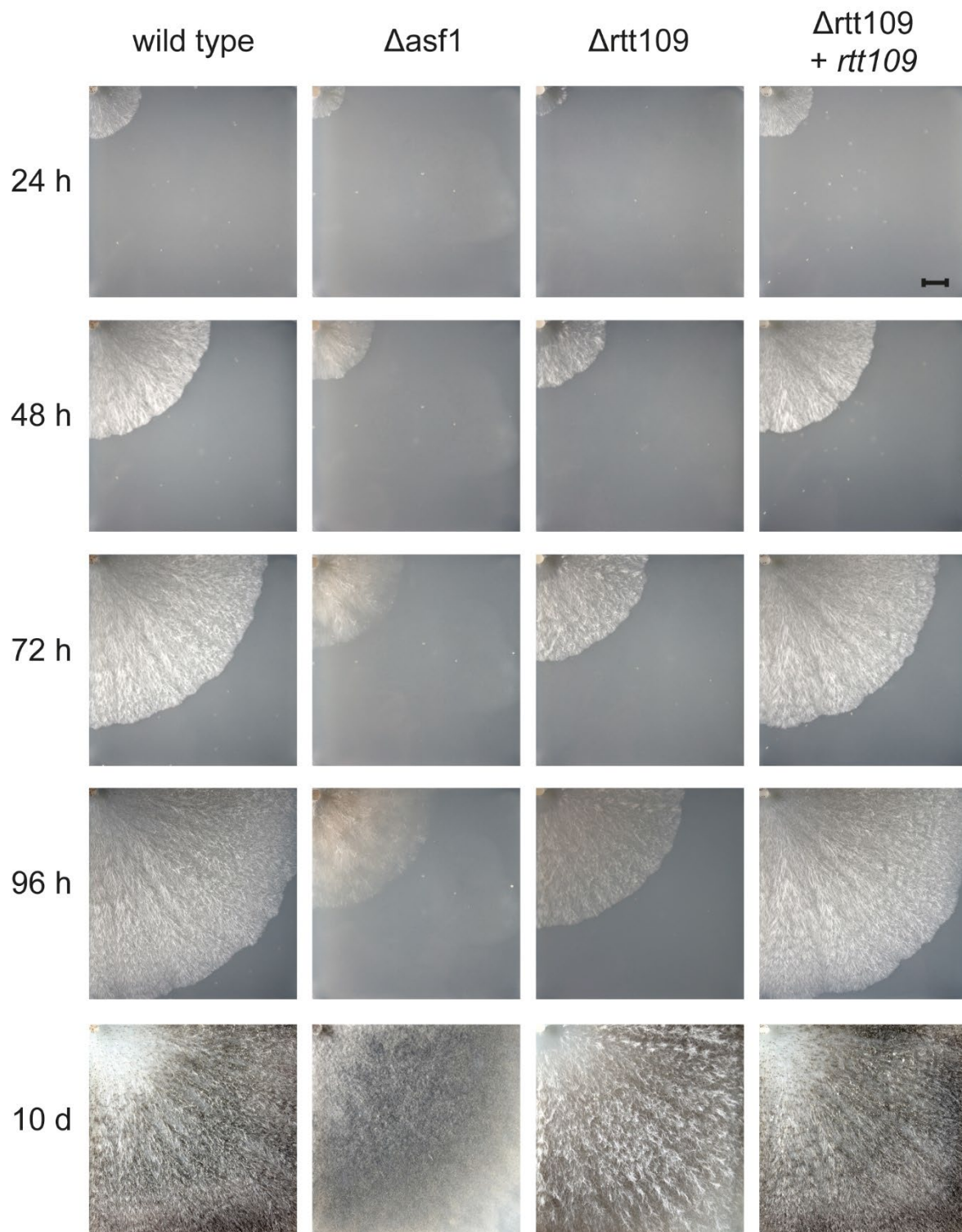
Figure 2. Comparison of vegetative growth of *rtt109* deletion and complementation strains with wild type and *asf1* deletion strains. Mycelial spread was observed over a period of 96 h and a final time after 10 d. While the wild type almost covered the entire plate in 96 h, *S. macrospora* Δrtt109 was significantly slower and grew as slowly as Δasf1 strains. This effect was reversed by reintroduction of *rtt109* in the *rtt109* deletion mutant, complementing the growth defect of the mutant. A visible difference between Δrtt109 and Δasf1 was the density of the mycelium. *S. macrospora* Δrtt109 appeared to grow as densely as the wild type, while Δasf1 appeared to be thinner overall. Scale bar represent 1 cm.

Daily vegetative growth rate

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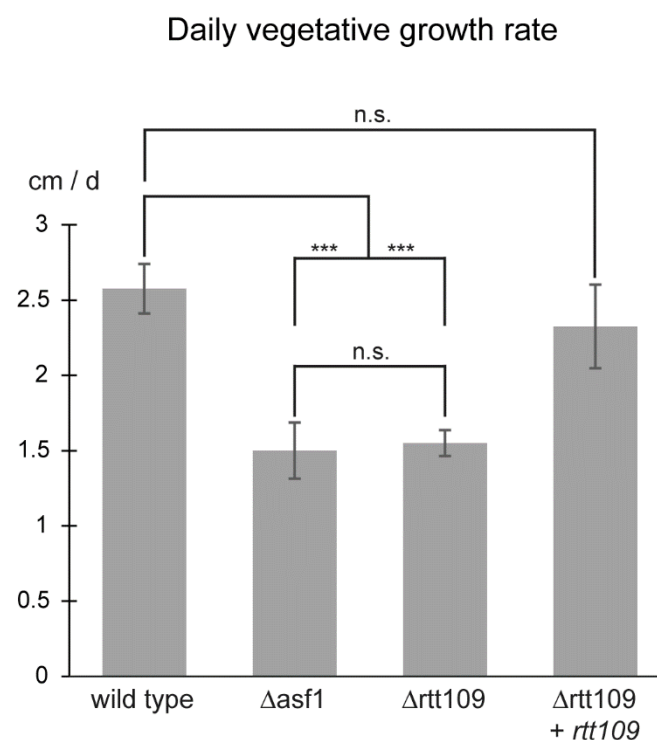
610   **Figure 3.** Quantification of the vegetative growth rates of *S. macrospora* Δrtt109, the respective
611   complementation strain, Δasf1 and the wild type. While the wild type grew at about 2.5 cm per day,
612   deletion of *rtt109* or *asf1* resulted in a significant decrease in growth rate 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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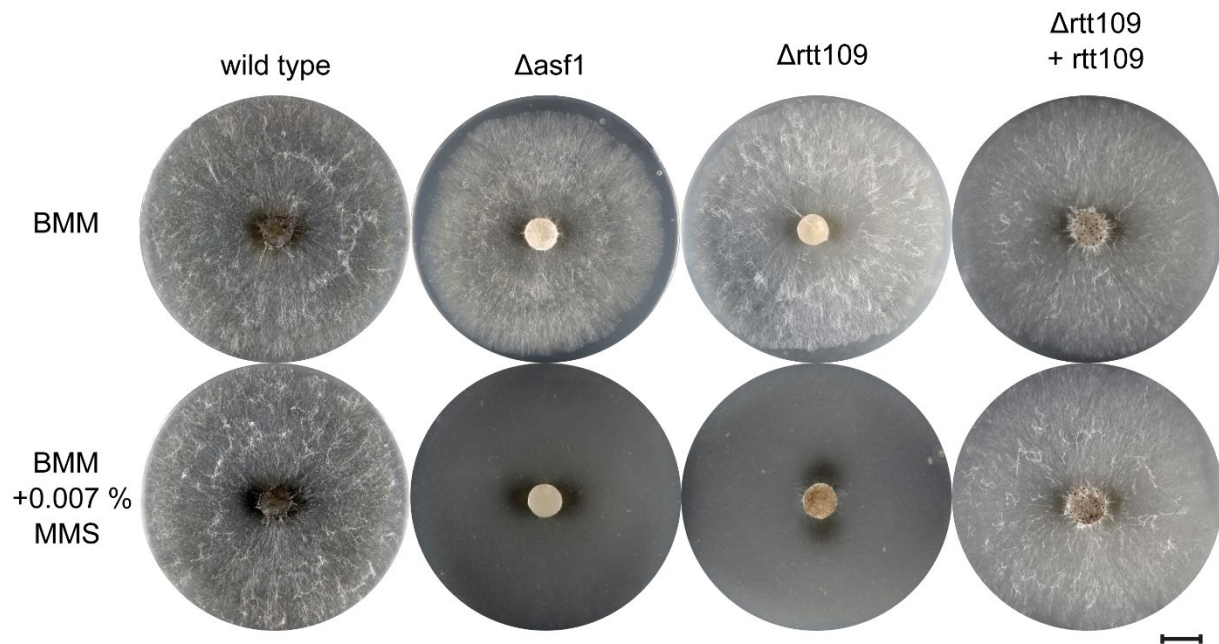
Figure 4. MMS sensitivity test for *S. macrospora* Δrtt109. The sensitivity of *S. macrospora rtt109* deletion mutants to the genotoxic compound MMS was observed after 4 days of growth on BMM media and BMM media supplemented with 0.007 % MMS. The addition of MMS completely halted the growth of the *rtt109* and *asf1* deletion mutants. When *rtt109* was reintroduced into Δrtt109, its ability to survive under MMS stress was restored. The scale bar provided represents 1 cm.
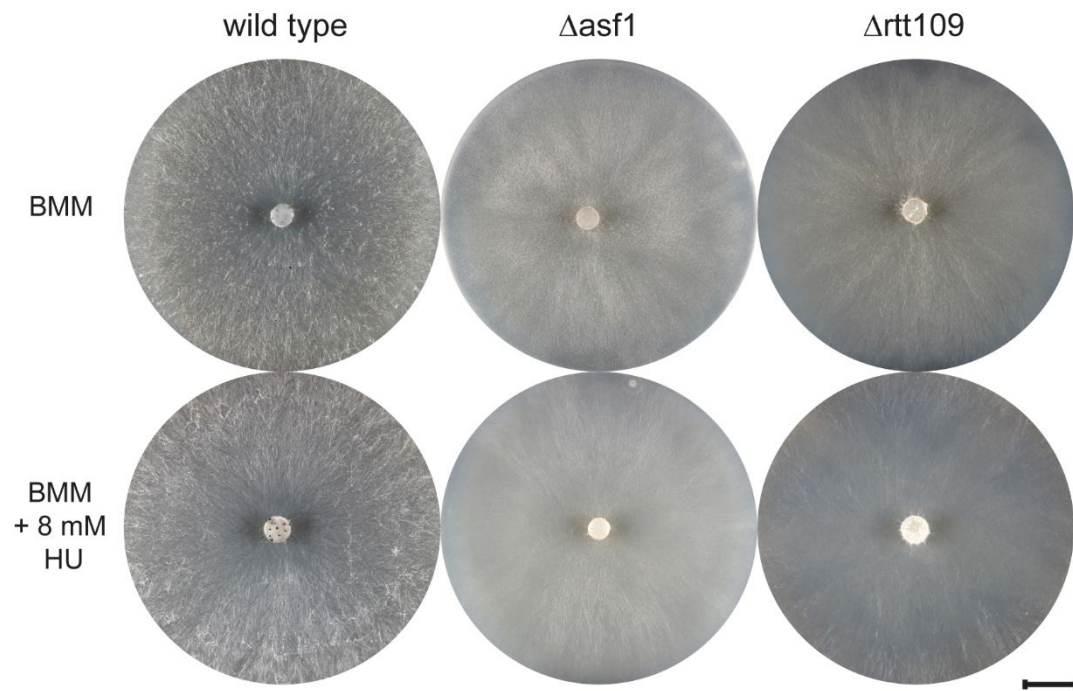
Figure 5. HU sensitivity test for *S. macrospora* Δrtt109. After a 4-day incubation on BMM media supplemented with 8 mM hydroxyurea, the sensitivity of *rtt109* deletion mutants was assessed. *S. macrospora* Δrtt109 exhibited no visible sensitivity to this DNA damaging agent, resembling the resistance observed in Δasf1. Scale bar represents 1 cm.

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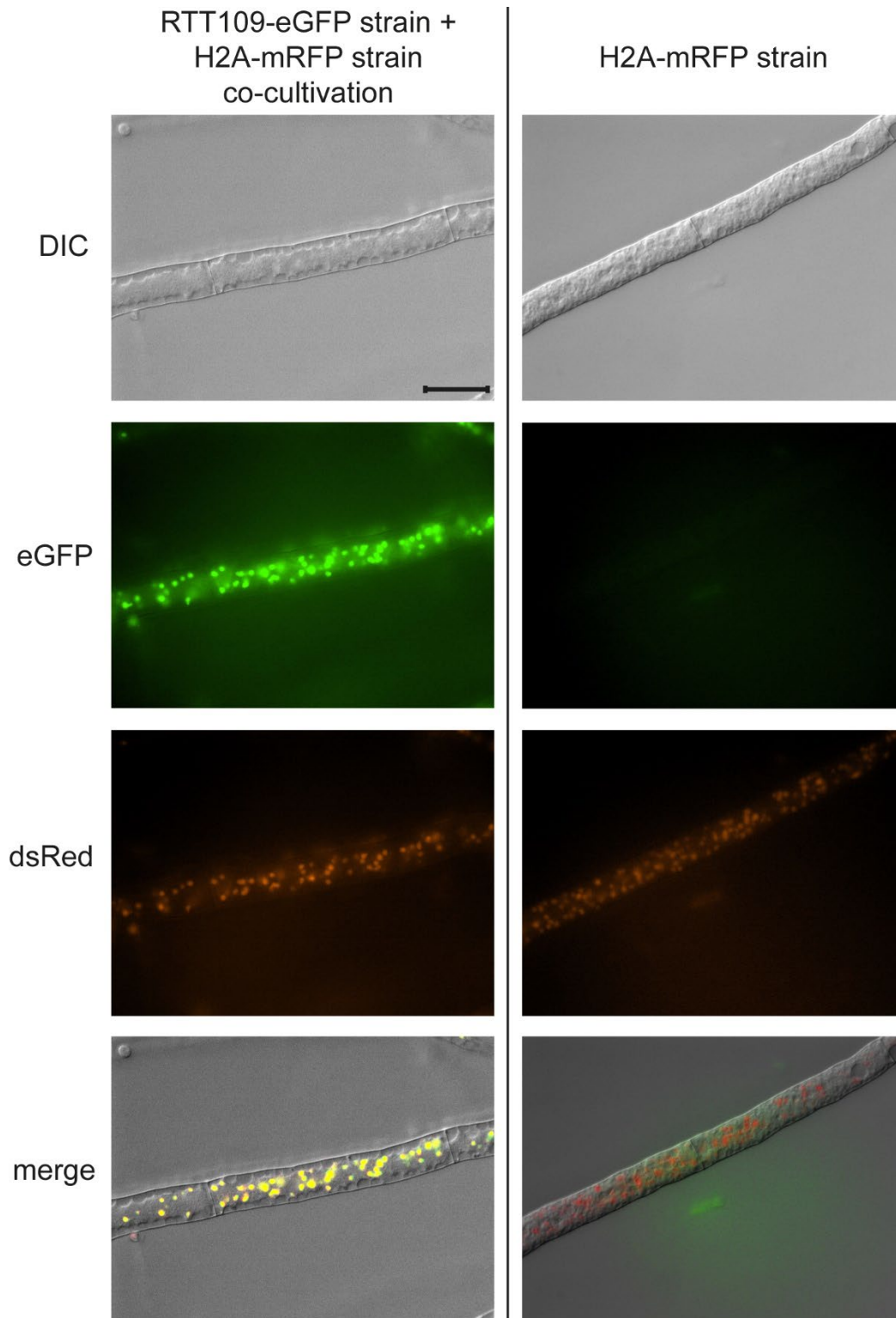
Figure 6. Localization analysis of RTT109 by fluorescence microscopy. RTT109 was expressed as a fusion protein with an eGFP tag and cultivated together with a strain expressing histone H3 fused to an mRFP tag. The visible colocalization of the green and red fluorescence indicates a nuclear localization of RTT109. No green autofluorescence was detectable in the reference strain expressing only H2A-mRFP. Scale bar represents 20 μm.
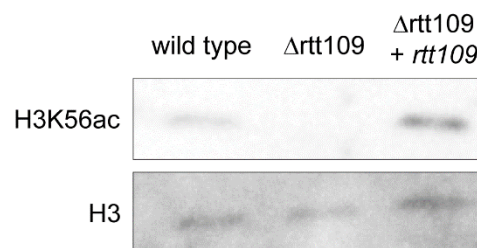
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Figure 7. Assessment of H3K56ac levels in *S. macrospora* Δrtt109.

The level of global H3K56ac was determined by comparing equal amounts of whole protein extracts from the wild type, Δrtt109, and the respective complementation strain by SDS-Page separation and western blotting with H3K56ac-specific antibodies. H3 antibodies were used to assess equal loading and comparable amounts of histone 3 in the protein extracts. In three biological replicates, no signal for H3K56ac was detectable in Δrtt109 strains. This effect was complemented by reintroduction of *rtt109* in the deletion mutant. Uncropped blots and the corresponding Coomassie gels are shown in Figure S3 in File S1.
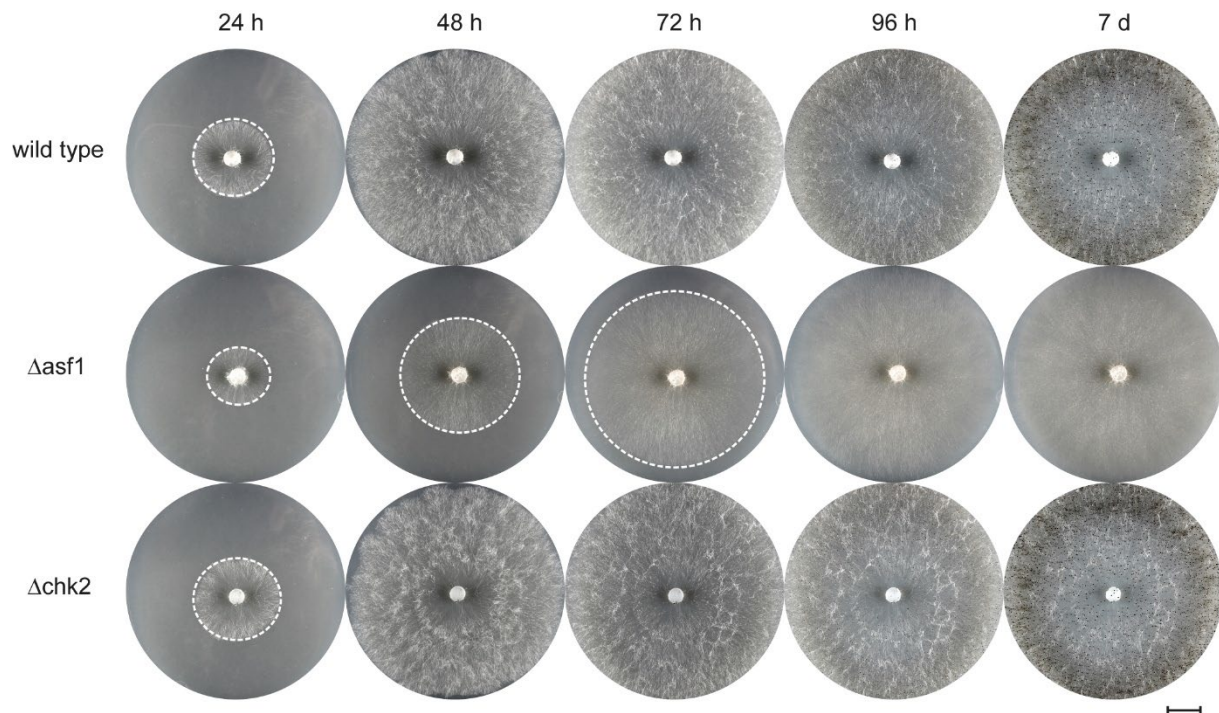
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Figure 8: Growth comparison of *S. macrospora* Δchk2 with Δasf1 and the wild type. The growth and overall characteristics of the strains were monitored for 96 hours, with a final observation after 7 days on BMM media. There were no noticeable differences between the *chk2* deletion mutant and the wild type strain. Both strains exhibited similar growth rates and developed visible perithecia. The Δasf1 strain displayed significantly slower growth and failed to produce perithecia throughout the observation period. White dashed circles indicate the growth front. Scale bar represents 1 cm.
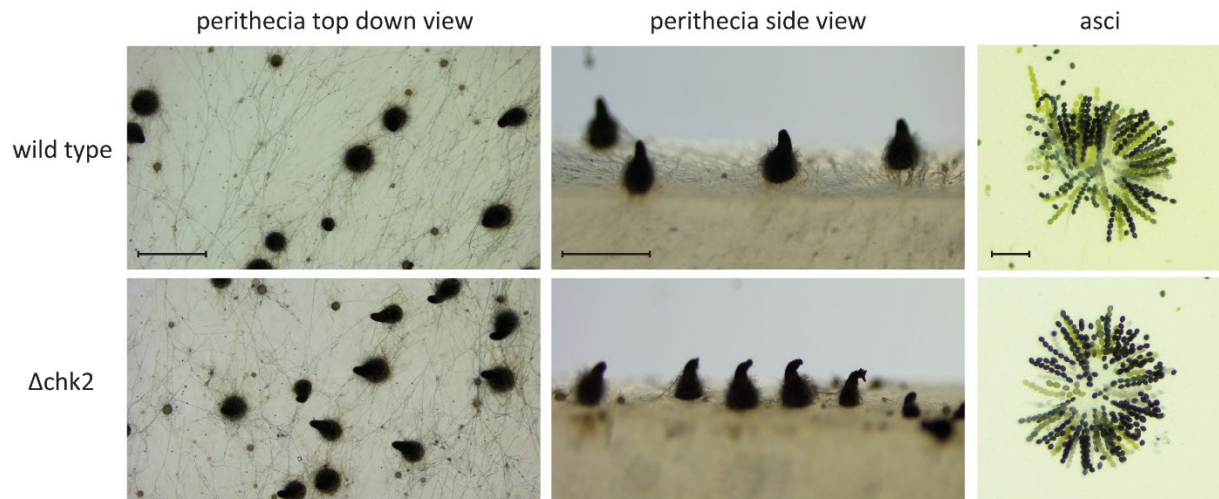
| perithecia top down view | perithecia side view | asci |

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Figure 9: Morphological comparison of sexual structures generated by *S. macrospora* Δchk2 with those of the wild type. The *chk2* deletion mutant demonstrated the ability to undergo the complete life cycle of *S. macrospora* without any impairments. The fruiting bodies produced by the mutant exhibited no abnormalities in comparison to the wild type strain. The perithecia were formed and positioned in a normal manner and the overall morphology of the perithecia did not display any visible defects. Δchk2 strains generated wild type-like asci. Scale bars represent 1 mm for top down view, 500 µm for side view and 100 µm for asci.
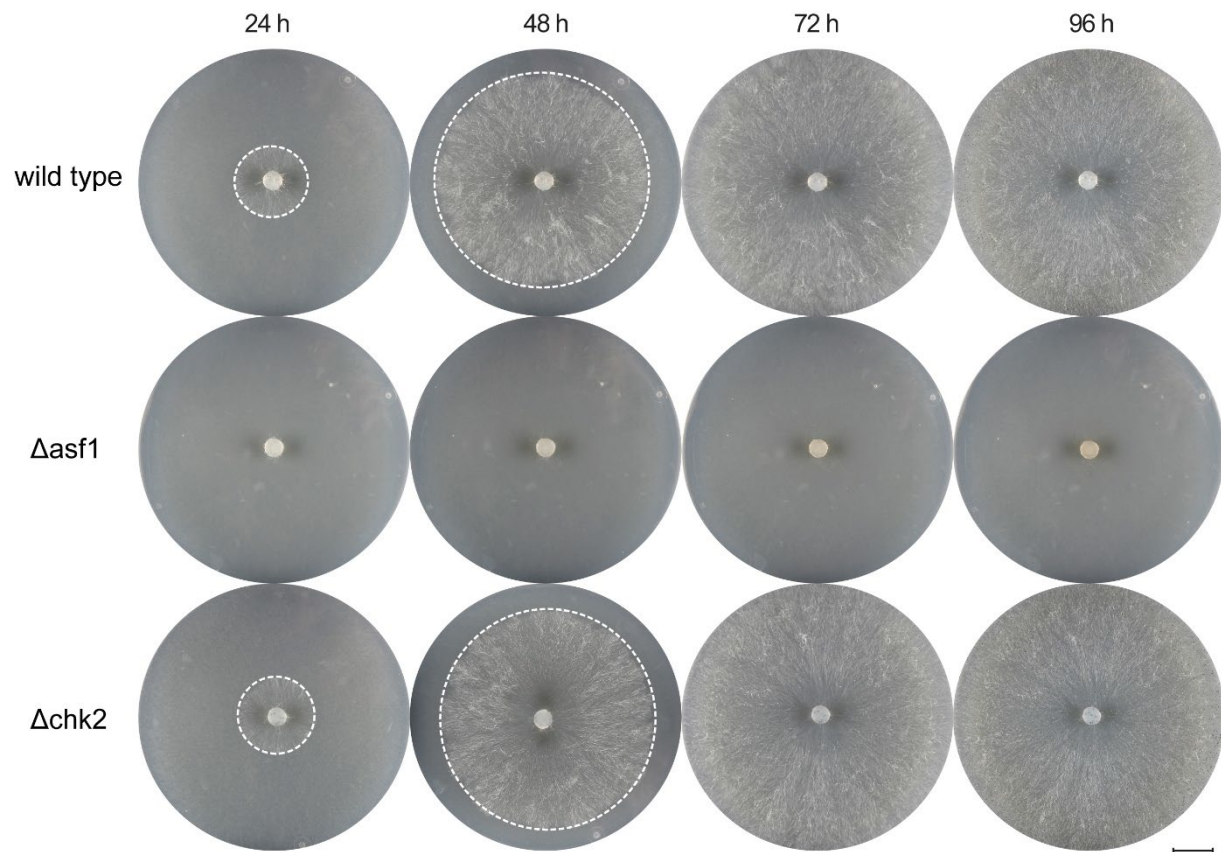
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Figure 10: MMS sensitivity test for *S. macrospora* Δchk2. The growth of the strains was monitored on BMM media supplemented with 0.007% MMS for a duration of 96 hours. The *S. macrospora* Δchk2 strain did not display increased sensitivity to the DNA damaging agent compared to the wild type strain. Consistent with previous observations, the *asf1* deletion mutant exhibited a high level of sensitivity to MMS. White dashed circles indicate the growth front. Scale bar represents 1 cm.
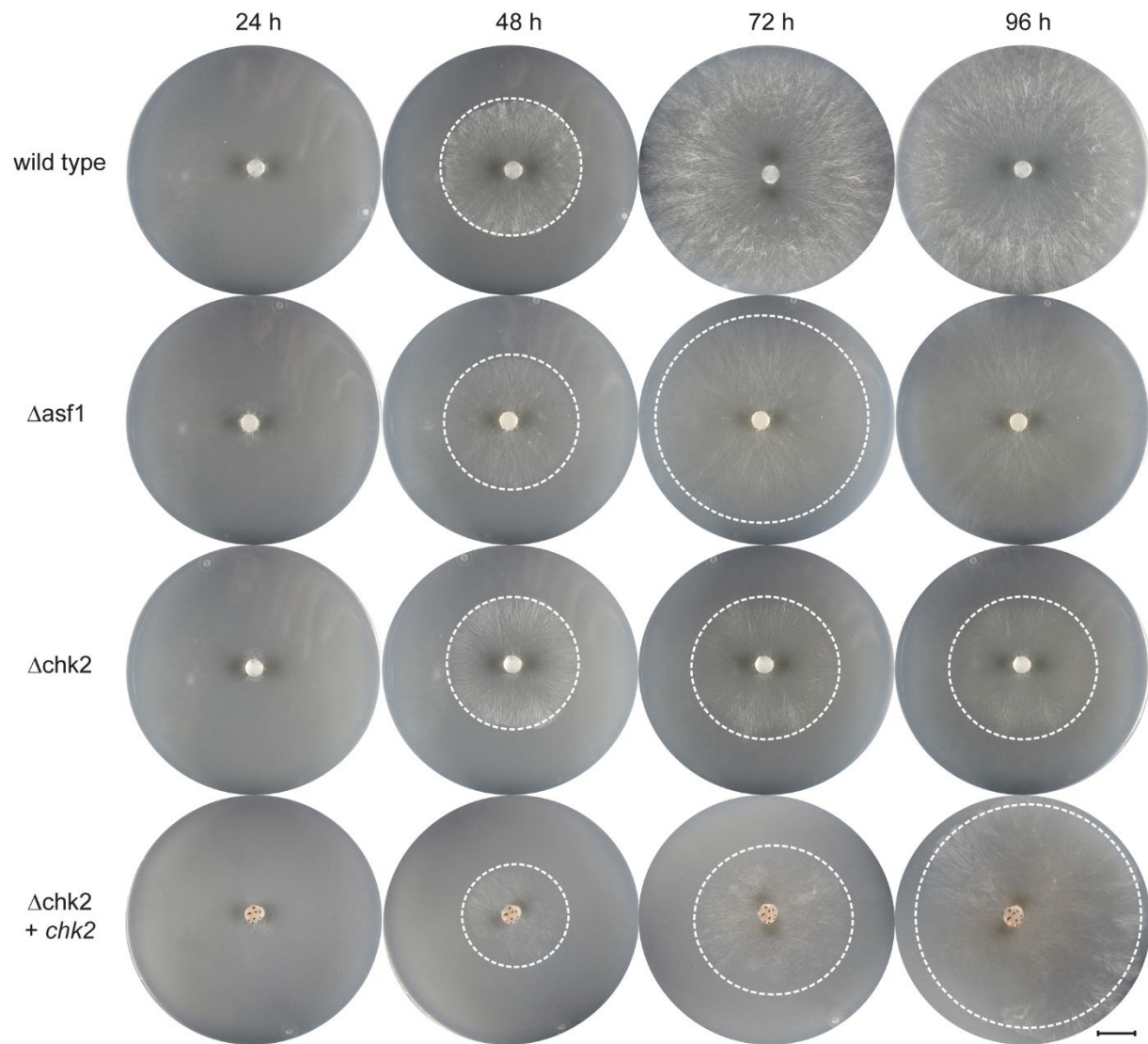
Figure 11: HU sensitivity test for *S. macrospora* Δchk2. The growth of the strains was observed on BMM media supplemented with 8 mM hydroxyurea for a period of 96 hours. In contrast to the observations with MMS, the *chk2* deletion mutant exhibited sensitivity to the DNA damaging agent hydroxyurea. While Δchk2 strains appeared to progress normally for the first 48 hours, they stopped growing after that time. Reintroduction of the *chk2* gene restored HU resistance. In contrast, the wild type and Δasf1 strains continued to grow under the same conditions. White dashed circles indicate the growth front. Scale bar represents 1 cm.