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

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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, 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 significant decrease in H3K56 acetylation levels, as detected by semiquantitative 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.

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). During sexual development of *S. macrospora*, ASF1 has been shown
78 to be essential for achieving fertility, and Δasf1 mutants arrest their life cycle at the protoperithecia
79 stage (6). Effects on DNA methylation (9) and histone modification (10) have also been described in *S.*
80 *macrospora asf1* deletion mutants. Since the structure of ASF1 is well characterized and no domains
81 have been detected that would allow it to carry out such enzymatic reactions by itself (8), functions in
82 DNA methylation and histone modification are likely to be carried out by interaction partners. Studies
83 in *S. cerevisae* showed a loss of H3K56ac histone acetylation in *asf1* deletion mutants, and the enzyme
84 identified as responsible for this modification was shown to be the HAT (histone acetyltransferase)
85 Rtt109 (11). Indeed, an interaction between Asf1, Rtt109, Vps75 and the target histone H3 has been
86 demonstrated in yeast, and it seems likely that acetylation occurs during the interaction of these
87 proteins (12). Therefore, the loss of H3K56ac in *S. cerevisae* Δasf1 strains appears to be caused by
88 disruption of the complex that allows Rtt109 to act on its target. The dependence of Rtt109 on
89 interaction partners is one of its distinguishing features, as *in vitro* experiments with the *S. cerevisiae*
90 proteins have shown that the HAT alone is unable to acetylate histones and only functions in the
91 presence of at least one of its interaction partners Asf1 or Vps75, while the presence of both is required
92 for full activity (11). Rtt109 is exclusively found in fungi (13) and is responsible for modifying specific
93 sites on histone H3, namely K9, K27 and K56, as clearly demonstrated in yeast (14, 15). In addition to
94 its role in histone acetylation, Rtt109 has been shown to be involved in a variety of processes in fungal
95 model organisms. In *Fusarium graminearum*, it has been shown to be involved in perithecia

96 morphogenesis, ascospore formation, conidiation and host plant infection (16). In *Aspergillus flavus* it
97 is important for aflatoxin synthesis, virulence and growth (17), whereas *Aspergillus fumigatus* requires
98 it for normal development and DNA damage response, as well as virulence (18). In *Neurospora crassa*,
99 a close relative of *S. macrospora*, RTT109 has been shown to be necessary for the production of small
100 RNAs (19). Therefore, RTT109 can be considered a major factor in many fungal models, suggesting its
101 importance in fungi in general, while the fact that is only found in this group of organisms might make
102 it an interesting target for anti-fungal drugs.
103 The histone acetylations generated by RTT109 are closely associated with newly synthesized histones
104 and play a crucial role in the assembly of nucleosomes during DNA replication and repair processes
105 (14). The involvement of H3K56ac in DNA damage repair, and thus the dependence of this process on
106 Rtt109, has been demonstrated in *S. cerevisae rtt109* deletion mutants, which lack H3K56ac and are
107 sensitive to the DNA double-strand break inducer methyl methanesulfonate (MMS) and the DNA
108 replication inhibitor hydroxyurea (HU) (20). A similar relationship between ASF1, RTT109 and H3K56ac
109 might exist in *S. macrospora*, since *asf1* deletion strains of this fungus show sensitivity to MMS and a
110 reduction in H3K56ac (10). Therefore, we hypothesized that RTT109 might be the link between ASF1,
111 histone acetylation and DNA damage repair in *S. macrospora* and chose SMAC_05078, the *S.*
112 *macrospora* RTT109 homologue, as a target for functional characterization.
113 Another potential candidate, which might contribute to the reduced genomic stability of *asf1* mutants,
114 could be the checkpoint kinase Rad53. Rad53 was shown to be important for DNA damage protection
115 under the influence of MMS and HU in *S. cerevisiae* (21). *S. cerevisiae* Rad53 is known to physically and
116 functionally interact with Asf1, and the activity of Rad53 is tightly regulated by its phosphorylation
117 state. Hypophosphorylated Rad53 is known to be bound to Asf1 and maintained in an inactive state,
118 while phosphorylation and release from the complex is a sign of active Rad53 (22). Deletion of *asf1* in
119 budding yeast causes defects in DNA damage recovery and may be due to insufficient inactivation of
120 Rad53 (23). In addition, *asf1* deletion mutants show similar sensitivities to MMS and HU as *rad53*
121 deletion mutants (22). Rad53 is the functional equivalent of checkpoint kinases Chk2 in humans and
122 PRD-4 in the filamentous fungus *Neurospora crassa*, since the corresponding genes can complement
123 an *S. cerevisiae* Δrad53 mutant (24, 25). The *S. macrospora* ortholog to PRD-4/Chk2 is SMAC_00634
124 (called CHK2 hereafter), which was chosen for analysis to shed more light on the chromatin modifier
125 network that contributes to the DNA damage protection functions of ASF1 in *S. macrospora*.
126 In this work, we created deletion mutants of *rtt109* and *chk2*, and analyzed their vegetative growth
127 and potential for sexual development. We also performed genotoxic stress assays to check for
128 similarities to *asf1* mutants. Furthermore, we determined the subcellular localization of the RTT109
129 protein by fluorescence microscopy and estimated the global amount of H3K56Ac in Δrtt109 by

130 semiquantitative western blots. We discuss our findings in the context of the roles of chromatin
131 modifiers during DNA damage protection and regulation of complex multicellular development.
132
133
134 **Materials and Methods**
135
136 **Strains, crosses and growth conditions**
137 Strains utilized in this study can be found in Table S1 in File S1. These strains were cultivated at a
138 temperature of 25 °C on either solid or liquid cornmeal medium (BMM) or complete medium (CM),
139 following previously established protocols (26) (27). In order to facilitate genetic crosses, the spore
140 color mutant fus was employed as a partner strain, enabling the identification of recombinant asci (3).
141 Previously described methods were used to carry out the transformation of *S. macrospora* (27).
142
143 **Cloning procedures, oligonucleotides and plasmids**
144 The oligonucleotides required for generating plasmids and conducting integration tests have been
145 provided in Table S2 in File S1. Additionally, all the plasmids utilized in this study are listed in Table S3
146 in File S1. The plasmid containing the deletion sequence for *rtt109* was generated through yeast
147 recombinant cloning (28). For assembling the deletion plasmid for *chk2,* and the complementation
148 plasmids for *rtt109* and *chk2*, Golden Gate cloning methodology was employed (29, 30).
149
150 **Generation of *rtt109* and *SMAC_00634* deletion mutants and complementation strains**
151 For conducting gene deletions, homologous recombination was utilized following established
152 procedures (31). In brief, a deletion cassette was constructed, comprising a selection marker in the
153 form of a hygromycin phosphotransferase gene under control of the constitutive P*gpd* promoter and
154 T*trpC* terminator from *Aspergillus nidulans* (32), flanked by upstream and downstream regions of the
155 target gene. This cassette was then cloned into a vector and transformed into the *S. macrospora* Δku70
156 strain after restriction and gel elution. Since KU70 is necessary for the ectopic integration of DNA, using
157 a *ku70* defective strain facilitates homologous recombination (31). Transformants that exhibited
158 resistance to hygromycin were selected and subsequent crosses were performed with the spore color
159 mutant fus, since most *S. macrospora* transformants are heterokaryotic. Ascospores from such crosses
160 were isolated to obtain homokaryotic deletion mutants and to enable the elimination of the Δku70
161 background. The resulting strains were verified through PCR. To complement the *rtt109* and *chk2*
162 mutants, the assembled plasmid containing the respective gene fused to an eGFP tag, controlled by a
163 P*gpd* promoter, was introduced into the corresponding strains via transformation. Ascospores were

164 isolated from the transformants that reached fertility, allowing the acquisition of homokaryotic strains
165 carrying the complementation construct.
166
167 **Growth tests and genotoxic stress assays**
168 Growth tests were performed on BMM media and the growth front was documented every 24 hours.
169 All tests were performed with three biological replicates. Statistical evaluation was conducted using a
170 Student's t-test. Phenotypic characterization involved observing and documenting the growth and
171 fertility of the strains on BMM plates using a Stemi 2000-C stereomicroscope (Zeiss). To assess the
172 resistance against methyl methanesulfonate (MMS), all strains were inoculated on BMM plates
173 supplemented with 0.007% (v/v) MMS, and the growth was observed and documented over a period
174 of 4 days. Similarly, resistance against hydroxyurea (HU) was evaluated on BMM plates containing 8
175 mM HU using the same approach.
176
177 **Microscopic analysis**
178 To investigate the subcellular localization of RTT109-EGFP fusion proteins, the strains were cultivated
179 on glass slides along with strains expressing H3-mRFP fusion proteins, as previously described (33).
180 Heterokaryotic strains can therefore express both fluorescence-labelled proteins (34). Subsequently,
181 light and fluorescence microscopy were performed using an AxioImager microscope (Zeiss), equipped
182 with a Photometrix Cool SnapHQ camera (Roper Scientific). EGFP fluorescence was detected using a
183 Chroma filter set 41017 (HQ470/40, HQ525/50, Q495lp), while mRFP fluorescence was detected using
184 set 49008 (EG560/40x, ET630/75 m, T585lp). The acquired images were further processed using
185 MetaMorph software (Molecular Devices).
186
187 **Semiquantitative analysis of H3K56ac**
188 To evaluate differences in the global levels of H3K56ac between the Δrtt109 strains and the wild type,
189 a semi-quantitative western blot analysis was conducted using antibodies specific to this particular
190 modification. The wild type strain SN1693, the Δrtt109 strain S60, and the corresponding
191 complementation strain S60K1 were grown in liquid BMM medium (26) at 27 °C for 4 days. The
192 mycelium was harvested by filtration, washed with PPP (27) and subsequently frozen in liquid nitrogen
193 before being ground to a fine powder. Protein extraction was performed by mixing the powder with
194 extraction buffer (50 mM Tris/HCl pH 7.5, 250 mM NaCl, 0.05 % NP-40, 0,3 % (v / v) Protease Inhibitor
195 Cocktail Set IV (Calbiochem)). After 20 min centrifugation, the protein concentrations were determined
196 using Bradford assays (35). Equal amounts of proteins were separated by SDS gel electrophoresis, and
197 transferred onto a PVDF membrane through western blotting. A H3K56ac-specific antibody (Active
198 Motif #39082) was used to detect the respective bands. The band intensities were compared using a

199 ChemiDoc XRS+ system with Image Lab 4.0 software. To normalize the results for variations in global
200 protein levels, a Coomassie gel run simultaneously served as control. Five biological replicates were
201 performed, and statistical significance was determined using a Student's t-test.
202
203
204 **Results**
205
206 **Deletion of *rtt109* in *S. macrospora* leads to sterility and impairs vegetative growth**
207 For the functional characterization of the histone acetyltransferase RTT109 in *S. macrospora*, a *ku70*
208 deletion strain was transformed using a deletion cassette designed for the *rtt109* sequence. The
209 deletion cassette consisted of a hygromycin phosphotransferase gene flanked by sequences
210 homologous to the *rtt109* gene, enabling the replacement of the target gene with a resistance marker.
211 Primary transformants that exhibited resistance to hygromycin were selected and subsequently
212 crossed in order to obtain homokaryotic deletion mutants without the Δku70 background. The deletion
213 of *rtt109* in the resulting strains was verified by PCR with primers designed to amplify a part of the
214 gene of interest. The correct integration of the deletion cassette was verified by PCR (Figure S1 in File
215 S1). Throughout the generation of the deletion strains, primary transformants did not exhibit any
216 noticeable defects in sexual development and demonstrated the ability to achieve fertility. Since
217 primary transformants are heterokaryotic in most cases, effects of gene deletions are often not directly
218 obvious and sometimes appear only in homokaryotic strains. Indeed, the homokaryotic Δrtt109 strains
219 were completely sterile (Table S4 in File S1). Microscopic examination confirmed a block in the sexual
220 development of Δrtt109 at the stage of young protoperithecia (Figure 1). While the ascogonia and early
221 protoperithecia were formed normally and at the expected time, no further structures of sexual
222 development were observed. Even after a week, there was no developmental progression beyond that
223 stage. The developmental effects of *rtt109* deletions in *S. macrospora* appeared to be even more
224 severe than in other ascomycete model organisms such as *F. graminearum*, where a reduction in
225 ascospore formation and aberrant perithecia have been previously documented (16). *rtt109* fused with
226 a C-terminal eGFP tag was reintroduced into the validated deletion strains through ectopic integration
227 to conduct complementation analysis and perform localization studies using fluorescence microscopy.
228 Complementation strains regained fertility and were able to grow wild type-like sexual structures
229 (Figure 1).
230 Deletion of *rtt109* also inhibited the vegetative growth rate of the respective mutants. Since RTT109 is
231 suspected to be an interaction partner of ASF1, we documented the growth of *rtt109* deletion mutants,
232 the respective complementation strain, and *asf1* deletion mutants to compare them to each other and
233 to the wild type (Figure 2). Deletion of *rtt109* caused a visible reduction in vegetative growth rate,

234 which was very similar to that of *asf1* deletion mutants, although the density of Δrtt109 mycelium
235 appeared higher and more wild type-like. The growth rate of *rtt109* deletion strains was fully restored
236 to wild type levels by reintroduction of the *rtt109* gene. We quantified this observation by measuring
237 the progress of the growth front in 5 biological replicates and detected a reduction of vegetative
238 growth speed of around 40 % in Δrtt109 and Δasf1 strains (Figure 3). The impairment of vegetative
239 growth in *S. macrospora* Δrtt109 is consistent with results in *F. graminearum*, where corresponding
240 deletion mutants were also reported to grow 40% slower than the wild type (16). This effect was
241 completely reversed by complementation of the *rtt109* mutant and the respective strains exhibited a
242 growth speed comparable to the wild type (Figure 3). In summary, the findings indicate that *rtt109*
243 plays a crucial role in the sexual development of *S. macrospora* and is also significant for vegetative
244 growth. The phenotype of *rtt109* deletion mutants appears highly similar to that of *asf1* deletion
245 mutants, suggesting that indeed both work in the same pathway.
246
247 ***S. macrospora* Δrtt109 and Δasf1 react similarly to methyl methanesulfonate and hydroxyurea**
248 In previous work, we discovered a sensitivity of *S. macrospora* Δasf1 strains to the DNA damaging agent
249 MMS as well as a reduction in global H3K56ac levels in ∆asf1 (10). The histone acetyltransferase
250 RTT109 interacts with the chromatin modifier ASF1 in *Candida albicans* (12) and is involved in various
251 mechanisms that ensure DNA stability during replication. Additionally, RTT109 is known as the primary
252 enzyme responsible for H3K56 acetylation in *S. cerevisiae* (14). In the case of *A. fumigatus*, deletion of
253 *rtt109* led to heightened sensitivity to DNA damaging agents such as MMS and HU (18). MMS induces
254 DNA methyl adducts, which can cause double-strand breaks during replication (36), while HU inhibits
255 the ribonucleotide reductase and leads to replication arrest by affecting dNTP supply (37). In our study,
256 we exposed *S. macrospora* Δrtt109, the corresponding complementation strain, an *asf1* deletion
257 mutant, and the wild type to MMS and HU. We found that the deletion of *rtt109* resulted in severe
258 sensitivity to MMS, comparable to the sensitivity exhibited by the *asf1* mutant (Figure 4). While the
259 wild type and complementation strains were able to grow relatively normally on BMM media
260 containing MMS, the mutants failed to grow at all. Strikingly, we did not observe any significant effect
261 of HU on the mutants (Figure 5). These observations contrast with the effects of *rtt109* deletions in *S.*
262 *cerevisae* (14) and *A. fumigatus* (18), where *rtt109* mutants are sensitive to MMS as well as HU,
263 suggesting the presence of additional factors in *S. macrospora* that respond to such specific stress
264 conditions.
265
266 ***S. macrospora* RTT109 is localized in the nucleus**
267 Given that RTT109 is described as a histone acetyltransferase, its targets are expected to be found
268 within the nuclear compartment of the cell. Consequently, it is necessary for RTT109 to localize within

269 the nucleus. Our complementation experiments with *rtt109* fused to an eGFP tag restored the
270 phenotype of the deletion mutant, so a correct localization of the fusion protein was expected. To
271 analyze the localization of RTT109, we employed fluorescence microscopy on the complementation
272 strains. These strains were cultivated alongside a marker strain that expressed histone H3 fused to an
273 mRFP tag. Since histones are primarily localized in the nucleus, this setup enabled us to perform co-
274 localization analysis in the resulting heterokaryons (33). Through the detection of both green and red
275 fluorescence in the same cellular compartment, we confirmed the co-localization of RTT109 and
276 histone H3 (Figure 6). Based on these findings, we can conclude that RTT109 in *S. macrospora* is indeed
277 localized within the nucleus.
278
279 **The *rtt109* deletion severely reduces H3K56Ac levels in *S. macrospora***
280 RTT109 is widely recognized as the primary, and possibly exclusive, histone acetyltransferase
281 responsible for the acetylation of H3K56 in fungal organisms (13, 14, 20). This specific histone
282 modification has been demonstrated to play a crucial role in the cellular response to DNA damage
283 induced by MMS (38). Therefore, we analyzed the levels of H3K56ac in the *S. macrospora* Δrtt109
284 mutant. To assess changes in the levels of global H3K56ac, we conducted semiquantitative western
285 blot analysis in the *S. macrospora* Δrtt109 deletion mutant and compared the results to the wild type
286 and the complemented mutant. Our findings revealed a significant reduction in the overall abundance
287 of this histone modification, with levels reaching only approximately 6 % of those observed in the wild
288 type strain (Figure 7, Figure S2 in File S1). The complementation strain exhibited a complete restoration
289 of H3K56ac production, indicating the successful reinstatement of normal acetylation. Our results
290 differ from previous studies conducted in yeast, which reported a complete depletion of K56
291 acetylation following the deletion of *rtt109* (39). Intriguingly, we observed a similar effect on H3K56ac
292 levels in *asf1* deletion mutants (10), despite the essential role of ASF1 in the establishment of this
293 modification in *S. cerevisiae* (40). These results might suggest the presence of an alternative pathway
294 or potential persistence of H3K56ac in *S. macrospora*, despite the deletion of *rtt109* or *asf1*.
295
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 S3 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 3B 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* ∆rad53 mutant and

304 are therefore functionally equivalent to Rad53 (24, 25). Rad53 plays a critical role in maintaining
305 genomic stability and regulating cell cycle arrests during DNA damage repair, as well as histone
306 recycling in *S. cerevisiae* (41).
307 In yeast, the interaction between Asf1 and Rad53 is well documented, with Asf1 controlling Rad53 by
308 binding hypophosphorylated, inactive Rad53 and ensuring recovery from DNA damage repair (23). 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 S4 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 (24).
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. However, under hydroxyurea stress, the deletion of *chk2*
327 had a clear effect, unlike the deletion of *asf1* (Figure 11). Reintroduction of *chk2* into *S. macrospora*
328 Δchk2 strains complemented the HU sensitivity phenotype (Table S5 in File S1). While all strains initially
329 exhibited slow growth under HU stress without significant differences within the first 48 hours, Δchk2
330 mutants ceased growing after 48 h. In contrast, the wild type and *asf1* deletion strain proved to be
331 resistant, consistent with previous observations comparing them to Δrtt109 strains. These results
332 suggest that CHK2 plays a critical role in the DNA damage response pathway specific to coping with
333 HU-induced stress, independent of ASF1 or RTT109.
334
335
336 **Discussion**
337

338 **RTT109 might be necessary for large parts of the functions of ASF1 during DNA damage protection**
339 **and sexual development**
340 In this study, we investigated the role of two potential partners of the histone chaperone ASF1 during
341 DNA damage protection and sexual development in the ascomycete *S. macrospora*. Our findings
342 suggest that the histone acetyltransferase RTT109 has significant functions under normal and DNA
343 damage stress conditions. Deletion of *rtt109* resulted in strains that exhibited similarities to *asf1*
344 deletions in terms of vegetative growth, sexual development, histone acetylation, and DNA damage
345 response. These observations suggest a close relationship between ASF1 and RTT109 in these
346 processes, as supported by their documented interactions in other fungal models like *C. albicans* and
347 *S. cerevisae* (12). The similarities between the respective deletion strains described raise the
348 hypothesis that the severe phenotype observed in *S. macrospora* Δasf1 may be the consequence of
349 the lack of interaction with RTT109. Studies in budding yeast have shown that Rtt109 relies on Asf1 to
350 obtain histone H3 for acetylating K56 and the absence of Asf1 may nullify the functions of Rtt109 (44).
351 In previous work, we demonstrated that sexual development in *S. macrospora* depends on the ability
352 of ASF1 to bind histones and hypothesized that it acts as scaffold for H3-H4 interactions with other
353 chromatin modifiers (10). The absence of such a scaffold might impair the ability of RTT109 to perform
354 its functions in histone acetylation, which could cause the severe developmental defects in *asf1*
355 deletion mutants. Thus, a reduction in H3K56ac may be the underlying issue in *S. macrospora* Δasf1
356 and proper acetylation of H3 may be crucial for sexual development. However, given the wide range
357 of putative and proven functions of ASF1 (45), it may be challenging to pinpoint its role during *S.*
358 *macrospora* development to a single interaction partner and its effect on H3K56ac. Interestingly, the
359 DNA damage response functions of ASF1 do not seem to depend on its interaction with histones in the
360 same way as sexual development, although ASF1 variants unable to bind histones showed a similar
361 reduction of H3K56ac as full deletion mutants (10). However, our results on the sensitivity of *rtt109*
362 deletion mutants to MMS show a dependence on RTT109 for DNA damage protection and therefore
363 probably for the establishment of H3K56ac. More sensitive assays such as ChIP-seq are needed to fully
364 quantify the potential differences in H3K56ac levels between *S. macrospora* Δasf1 and Δrtt109, and
365 we hypothesize that deletion of the HAT causes an even greater decrease in acetylation levels than the
366 loss of a putative scaffold. Other chromatin modifiers may be able to provide the low level activation
367 for RTT109 to perform at least basic functions in *S. macrospora asf1* mutants, causing a potentially
368 higher H3K56ac level in Δasf1. In *S. cerevisiae*, Rtt109 is known to interact not only with Asf1 but also
369 with another histone chaperone, Vps75, which also binds H3 and H4 (13). Although a fully functional
370 Asf1-Vps75-Rtt109 complex is known to be necessary for establishing correct acetylation patterns in
371 budding yeast (46), the mere presence of ASF1, even without histone binding ability, might be
372 sufficient to enable lower-level activity of RTT109, providing some form of DNA damage protection.

373 Therefore, the severe developmental defects observed in *S. macrospora* Δasf1, persistent when
374 expressing ASF1 variants unable to bind histones, may have roots not only in the misregulation of
375 developmental processes, but also in the accumulation of underlying DNA damage events,
376 compromising the respective strains as a whole. Such DNA damage accumulation might be too
377 insignificant to turn up during a sensitivity assay, but could potentially lead to disturbances during
378 tightly regulated processes, such as sexual development and fruiting body formation. While this
379 hypothesis requires further investigation, such as interaction studies between RTT109, ASF1 and
380 VPS75, the role of RTT109 during sexual development and the DNA damage response in *S. macrospora*
381 appears to be quite fundamental. As the primary facilitator of H3K56ac in fungi (14), RTT109 is clearly
382 essential for survival under MMS conditions, which can induce double-strand breaks during replication
383 (36). Furthermore, it is crucial during the formation of complex sexual structures, either by providing
384 the necessary genomic stability for such processes or by ensuring the correct transcription of
385 important genes through the establishment of proper acetylation patterns. Surprisingly, the deletion
386 of *rtt109* did not increase the sensitivity of *S. macrospora* to hydroxyurea, despite its well-known DNA
387 damaging properties that cause replication fork arrest by disrupting dNTP availability (37). In contrast,
388 other fungal model organisms like *A. fumigatus* and *S. cerevisiae* have been shown to exhibit
389 heightened sensitivity to HU when *rtt109* is deleted (18, 47). Interestingly, the total abolishment of
390 H3K56ac that results from a *rtt109* deletion appears to be specific to budding yeast, as *A. fumigatus*
391 H3K56 acetylation levels drop as significantly as those in *S. macrospora* (18), but low amounts of this
392 modification are detectable in both organisms. This might be an indicator of alternative H3K56ac
393 establishment pathways in multicellular fungi.
394
395 **CHK2 is not essential for sexual development, but provides resistance to genotoxic stress**
396 **independent of ASF1**
397 The second putative partner of ASF1 during DNA damage protection we investigated was CHK2. CHK2
398 is the ortholog of *N. crassa* PRD-4, which can complement a ∆rad53 mutant with respect to its function
399 in DNA damage response in *S. cerevisiae* (24). Deletion of *chk2* did not result in any noticeable defects
400 in vegetative growth or developmental processes, suggesting its negligible role in the formation of
401 fruiting bodies. Furthermore, the mutants lacking *chk2* did not exhibit increased sensitivity to MMS-
402 induced DNA damage stress, unlike the highly sensitive *asf1* and *rtt109* mutants. This indicates that
403 CHK2 operates in a distinct damage response pathway from ASF1 and RTT109 under these specific
404 conditions. However, we discovered a role for CHK2 in response to a different type of genotoxic stress,
405 as the mutants showed sensitivity to the replication fork stressor hydroxyurea, while the *asf1* and
406 *rtt109* mutants did not display such sensitivity. This suggests that CHK2 is involved in a genomic
407 protection system that functions independently of ASF1. Notably, this observation appears to be

408 specific for *S. macrospora*, as an *S. cerevisiae* Δasf1 mutant is sensitive to hydroxyurea (48). The precise
409 functions of CHK2 and its relationship with ASF1 in its broader chromatin modification network are
410 more challenging to elucidate, given the specific roles fulfilled by homologous checkpoint kinases like
411 PRD-4 or functional equivalents like RAD53 in their respective native organisms. However, it can be
412 inferred that any connection between ASF1 and CHK2 in DNA damage protection and sexual
413 development is of minor importance. Additionally, the diverse functions of ASF1 in *S. macrospora* do
414 not seem to include an active role in the response to hydroxyurea-induced stress.
415
416 In conclusion, our study revealed an essential role of the histone acetyltransferase RTT109 during
417 sexual development, vegetative growth, histone modification and genotoxic stress response in the
418 ascomycete *S. macrospora*. The phenotypic aberrations in *rtt109* deletion strains closely resemble
419 those previously observed in *asf1* deletion mutants, indicating a strong correlation between the
420 functions of ASF1 and RTT109. CHK2, another potential component of the ASF1-mediated chromatin
421 modifier network, appears to be unrelated to these processes but is instead involved in a distinct DNA
422 damage response system that operates independently of ASF1.
423
424
425 **Data Availability Statement**
426 Strains and plasmids are available upon request. The authors affirm that all data necessary for
427 confirming the conclusions of the article are present within the article, figures, and tables.
428
429
430 **Acknowledgements**
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434
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567 **Figures**

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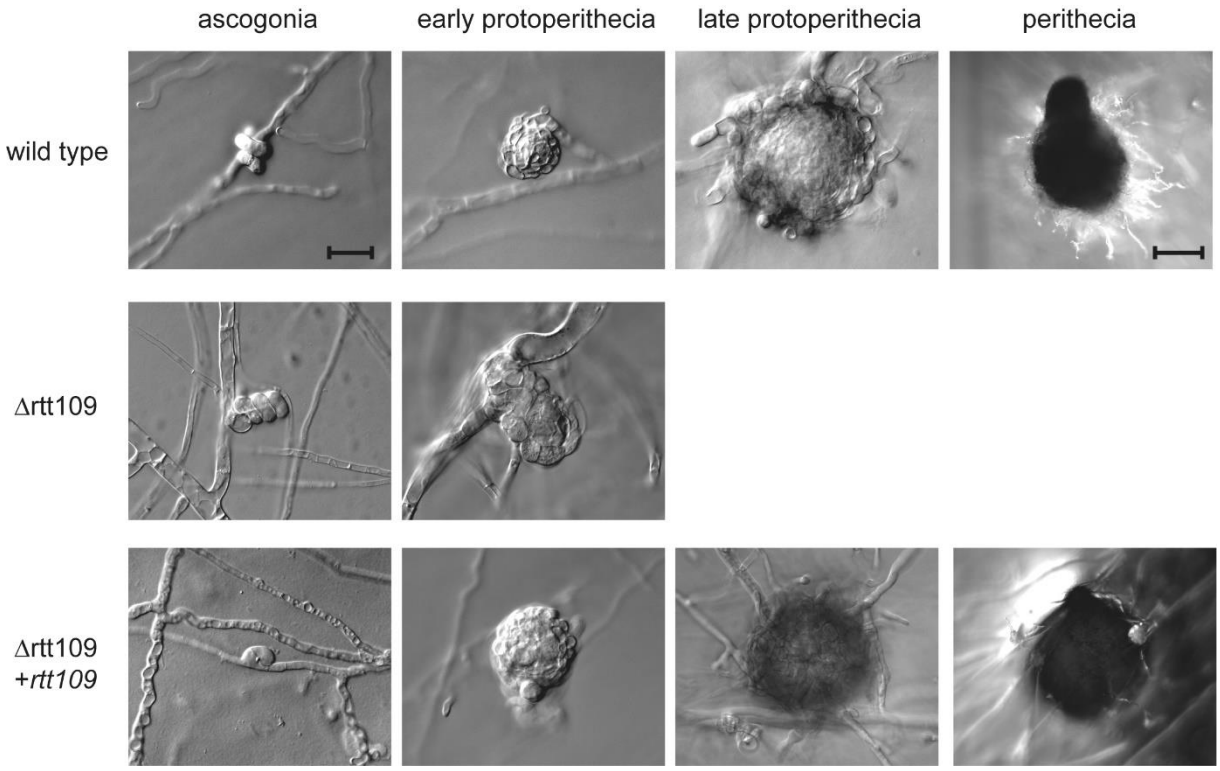

571

572 **Figure 1:** Development of *rtt109* deletion mutants compared to wild type and complementation
573 strains. *S. macrospora* strains lacking the *rtt109* gene were found to exhibit impaired development
574 with a block at the stage of early protoperithecia formation. Upon reintroduction of *rtt109*, normal life
575 cycle progression was fully restored, as evidenced by the formation of late, melanized protoperithecia
576 and the development of fully formed perithecia indistinguishable from those of the wild type strain.
577 The scale bar for ascogonia and protoperithecia represents 20 µm, while the scale bar for perithecia
578 represents 100 µm.

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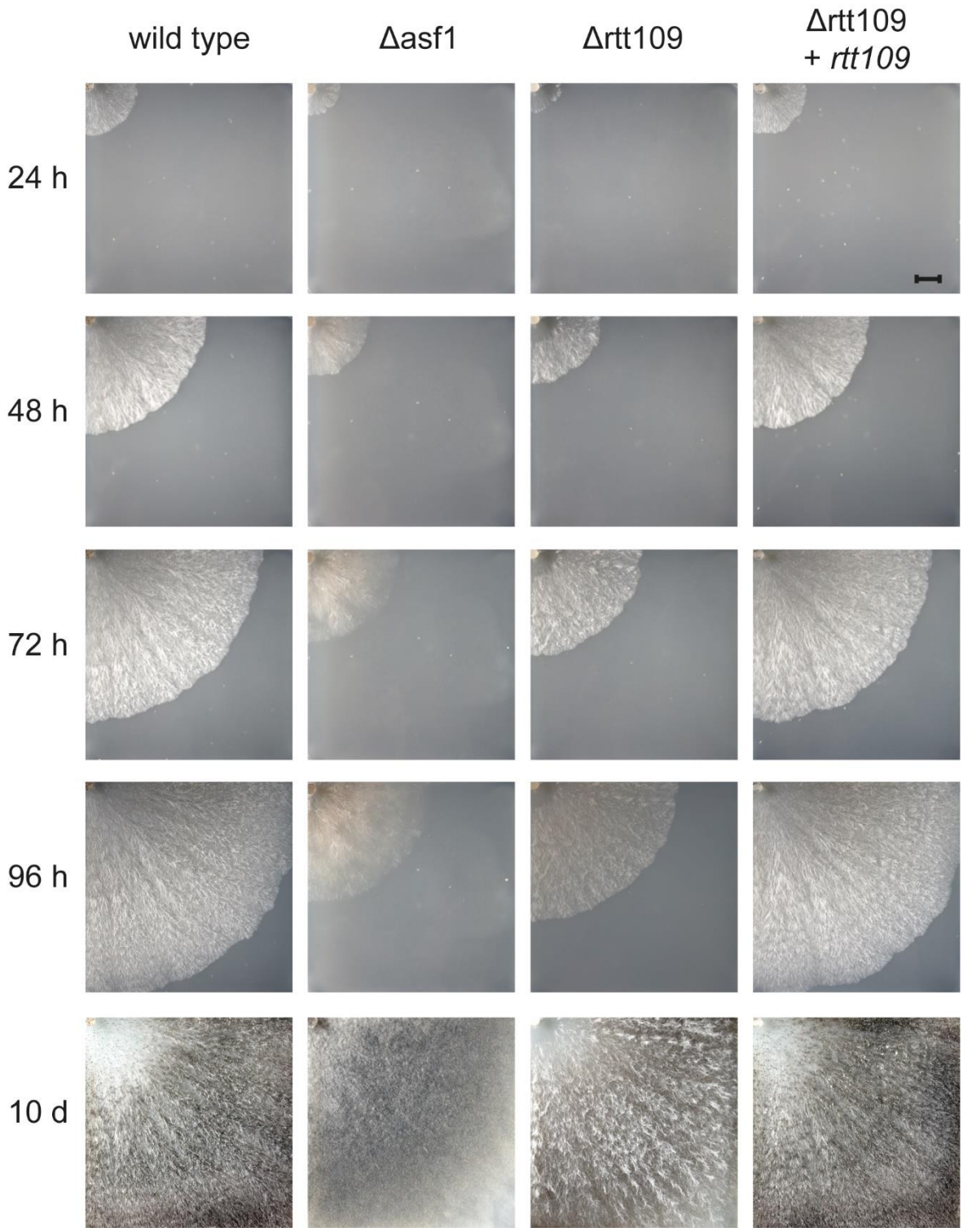


585

586 **Figure 2.** Comparison of vegetative growth of *rtt109* deletion and complementation strains with wild
587 type and *asf1* deletion strains. Mycelial spread was observed over a period of 96 h and a final time
588 after 10 d. While the wild type almost covered the entire plate in 96 h, *S. macrospora* Δrtt109 was
589 significantly slower and grew as slowly as Δasf1 strains. This effect was reversed by reintroduction of
590 *rtt109* in the *rtt109* deletion mutant, complementing the growth defect of the mutant. A visible
591 difference between Δrtt109 and Δasf1 was the density of the mycelium. *S. macrospora* Δrtt109
592 appeared to grow as densely as the wild type, while Δasf1 appeared to be thinner overall. Scale bar
593 represent 1 cm.

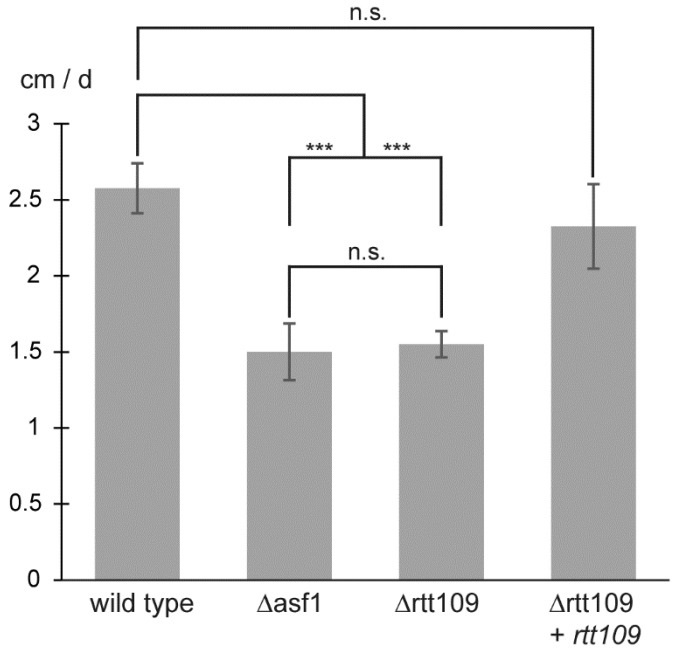
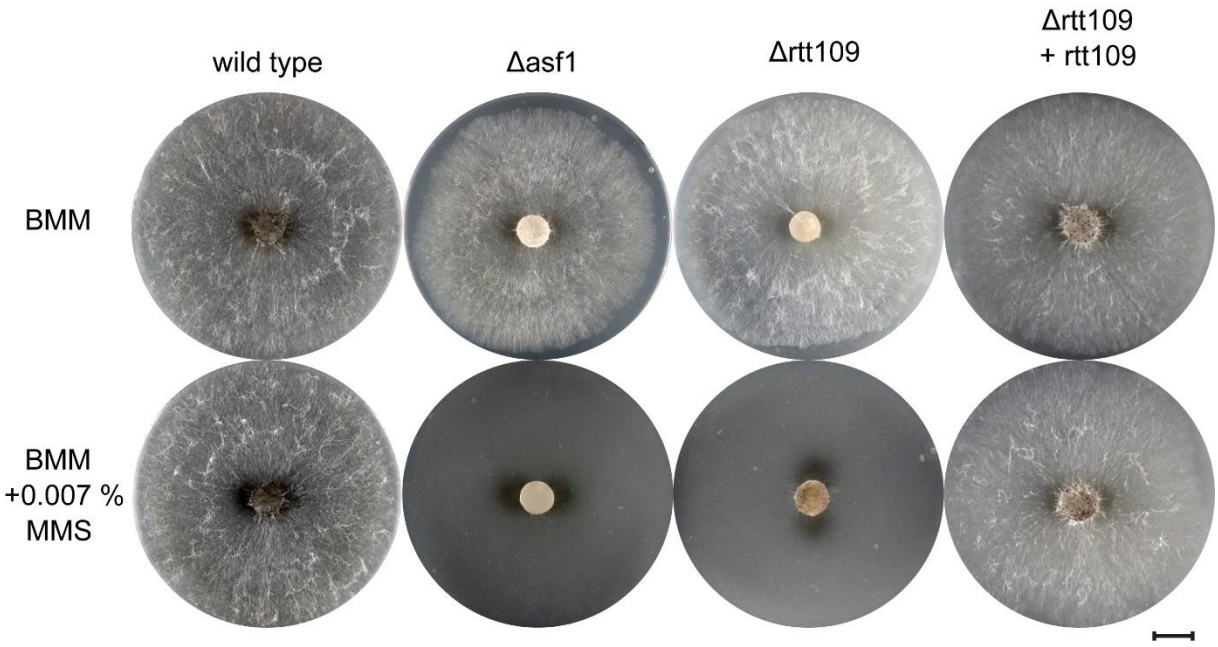


Figure 3. Quantification of the vegetative growth rates of *S. macrospora* Δrtt109, the respective complementation strain, Δasf1 and the wild type. While the wild type grew at about 2.5 cm per day, deletion of *rtt109* or *asf1* resulted in a significant decrease in growth rate to about 1.5 cm per day. Complementation of *rtt109* mutants restored the growth rate to wild type levels. Quantification was performed for 5 independent replicates and significance was assessed by Student's t-test. *** = p-value < 0.001, n.s. = p-value > 0.05.



605

606 **Figure 4.** MMS sensitivity test for *S. macrospora* Δrtt109. The sensitivity of *S. macrospora rtt109*
607 deletion mutants to the genotoxic compound MMS was observed after 4 days of growth on BMM
608 media and BMM media supplemented with 0.007 % MMS. While all strains exhibited normal growth
609 on BMM media, the addition of MMS completely halted the growth of the *rtt109* and *asf1* deletion
610 mutants. When *rtt109* was reintroduced into Δrtt109, its ability to survive under MMS stress was
611 restored. The scale bar provided represents 1 cm.

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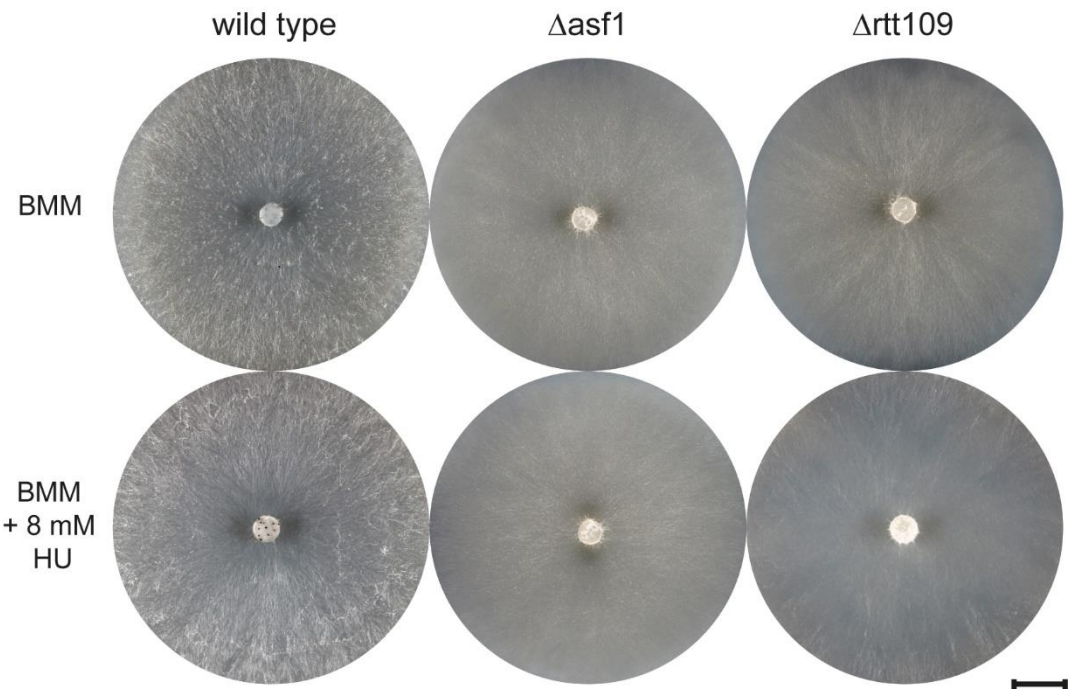

614

615 **Figure 5. HU sensitivity test for *S. macrospora* Δrtt109.** After a 4-day incubation on BMM media
616 supplemented with 8 mM hydroxyurea, the sensitivity of *rtt109* deletion mutants was assessed. *S.*
617 *macrospora* Δrtt109 exhibited no visible sensitivity to this DNA damaging agent, resembling the
618 resistance observed in Δasf1. Scale bar represents 1 cm.

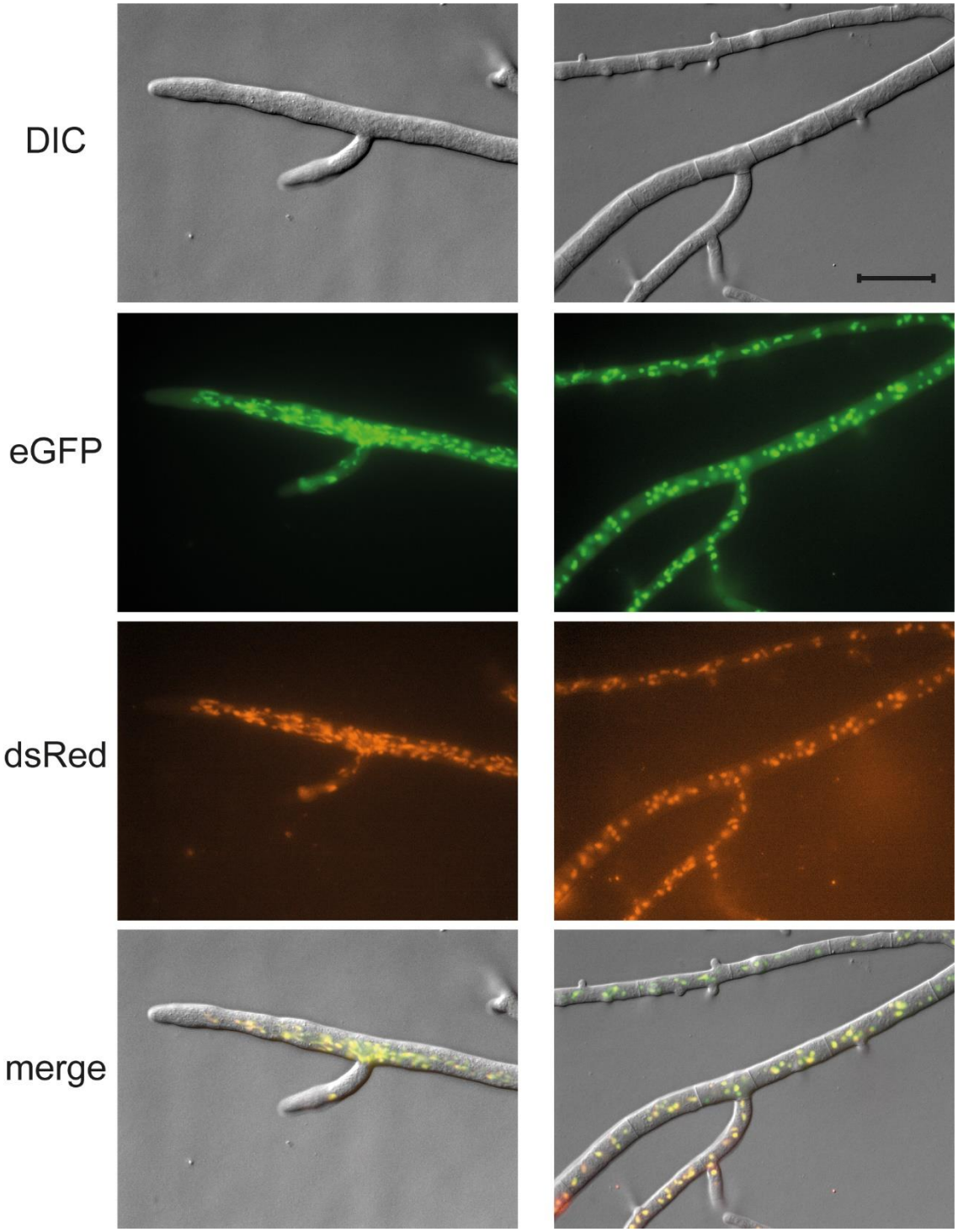

Figure 6. Localization analysis of RTT109 by fluorescence microscopy. RTT109 was expressed as a fusion protein with an eGFP tag and cultivated together with a strain expressing histone H3 fused to an mRFP tag. The visible colocalization of the green and red fluorescence indicates a nuclear localization of RTT109. Scale bar represents 20 µm.

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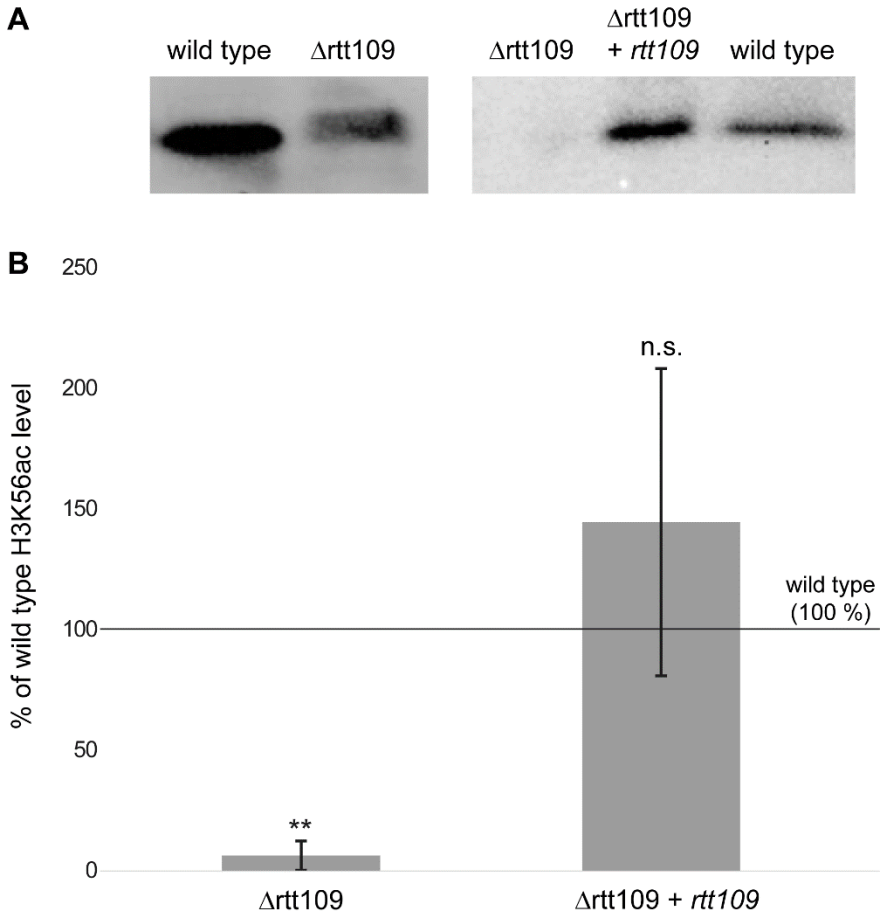

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628 **Figure 7.** Semiquantitative assessment of H3K56ac levels in *S. macrospora* Δrtt109.
629 **A.** The level of global H3K56ac was determined by comparing equal amounts of whole protein extracts
630 from the wild type, Δrtt109, and the respective complementation strain by SDS-Page separation and
631 western blotting with H3K56ac-specific antibodies. Uncropped blots and the corresponding Coomassie
632 gels are shown in Figure S2 in File S1.
633 **B.** Band intensity in Western blots was measured densitometrically and the ratio of mutant and
634 complemented strains to the wild type was calculated. Normalization was done by comparison with a
635 Coomassie gel run in parallel. The H3K56ac levels in the wild type strain were set at 100%. The deletion
636 mutant showed a significant reduction in global H3K56ac levels, reaching around 6% of the wild type
637 level. This reduction was restored upon complementation. The quantification was based on data from
638 five independent biological replicates and the significance was confirmed using a Student's T-test. * =
639 p-value < 0.05, ** = p-value < 0.01, n.s. = p-value > 0.05.

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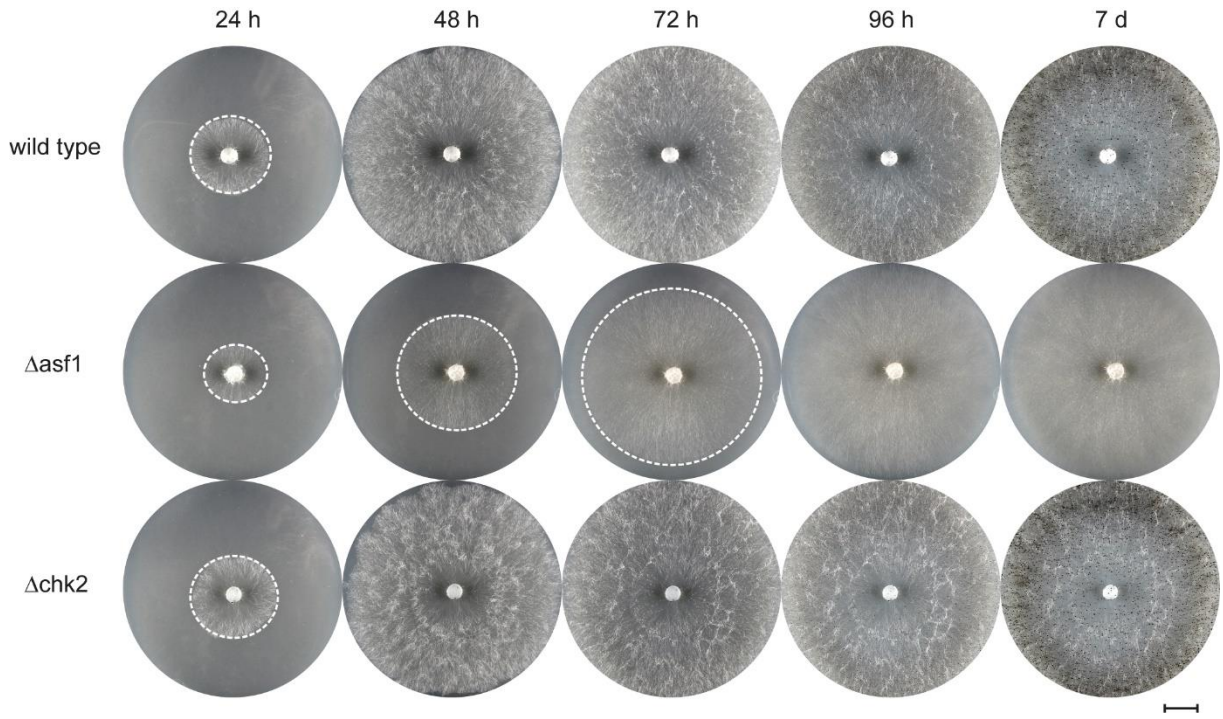

Figure 8: Growth comparison of *S. macrospora* Δchk2 with Δasf1 and the wild type. The growth and overall characteristics of the strains were monitored for 96 hours, with a final observation after 7 days on BMM media. There were no noticeable differences between the *chk2* deletion mutant and the wild 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. Scale bar represents 1 cm.

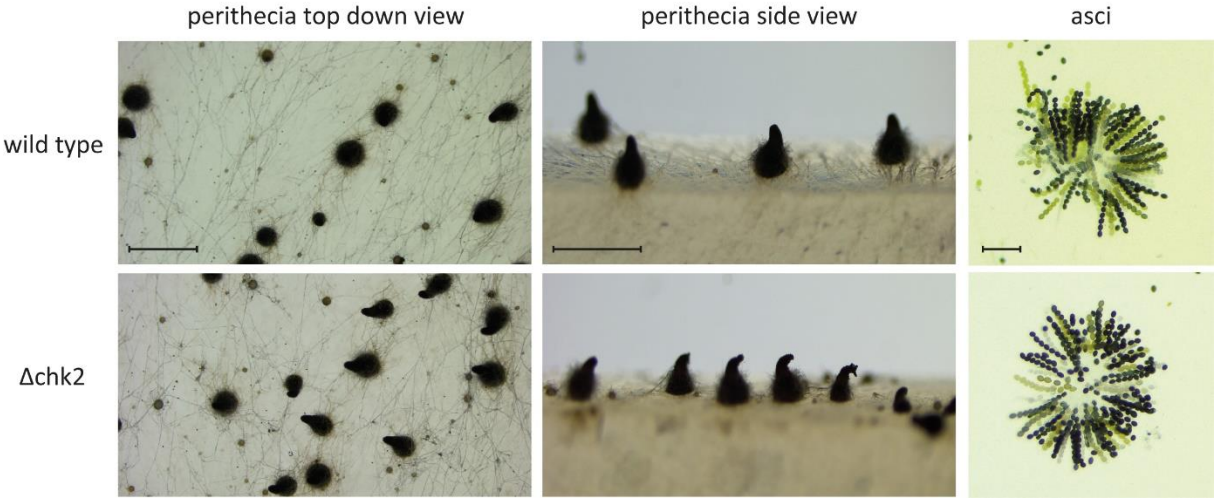

Figure 9: Morphological comparison of sexual structures generated by *S. macrospora* Δchk2 with those of the wild type. The *chk2* deletion mutant demonstrated the ability to undergo the complete life cycle of *S. macrospora* without any impairments. The fruiting bodies produced by the mutant exhibited 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.

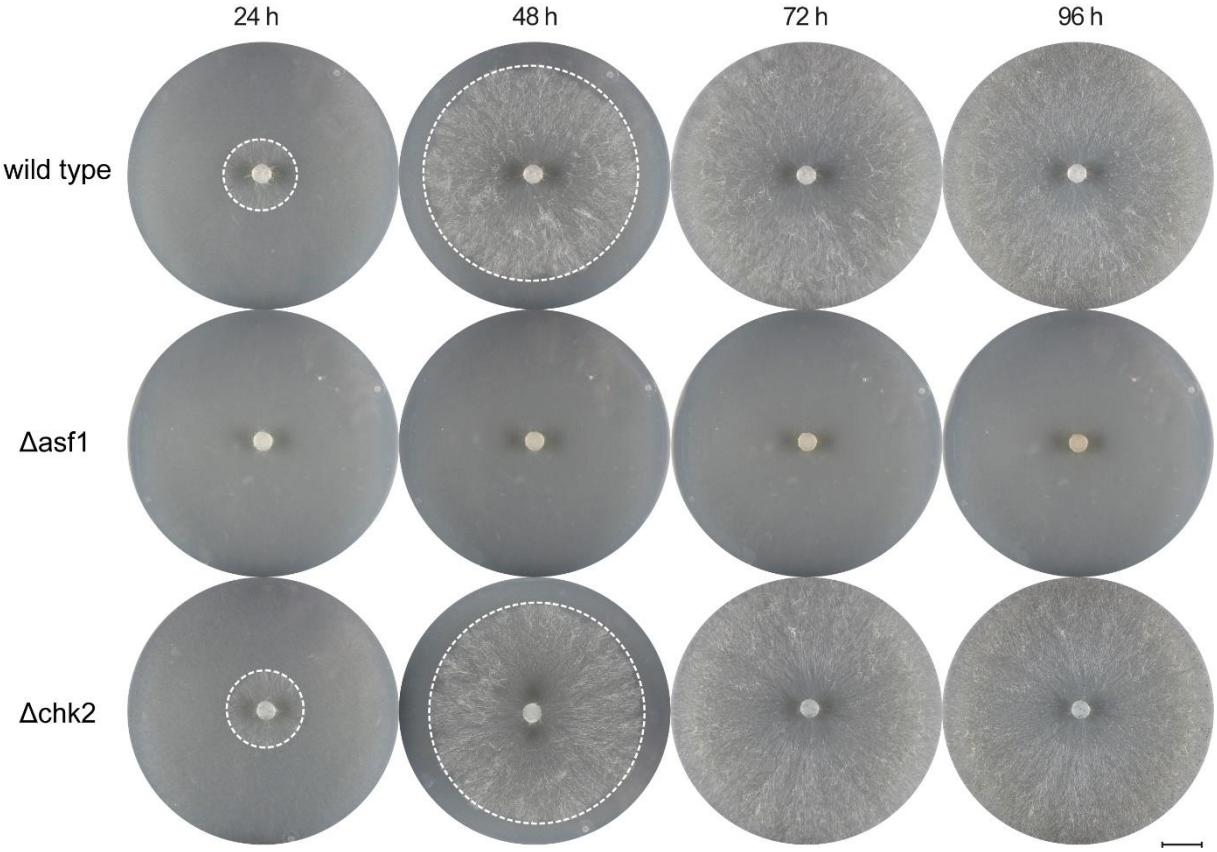

Figure 10: MMS sensitivity test for *S. macrospora* Δchk2. The growth of the strains was monitored on BMM media supplemented with 0.007% MMS for a duration of 96 hours. The *S. macrospora* Δchk2 strain did not display increased sensitivity to the DNA damaging agent compared to the wild type strain. Consistent with previous observations, the *asf1* deletion mutant exhibited a high level of sensitivity to MMS. Scale bar represents 1 cm.

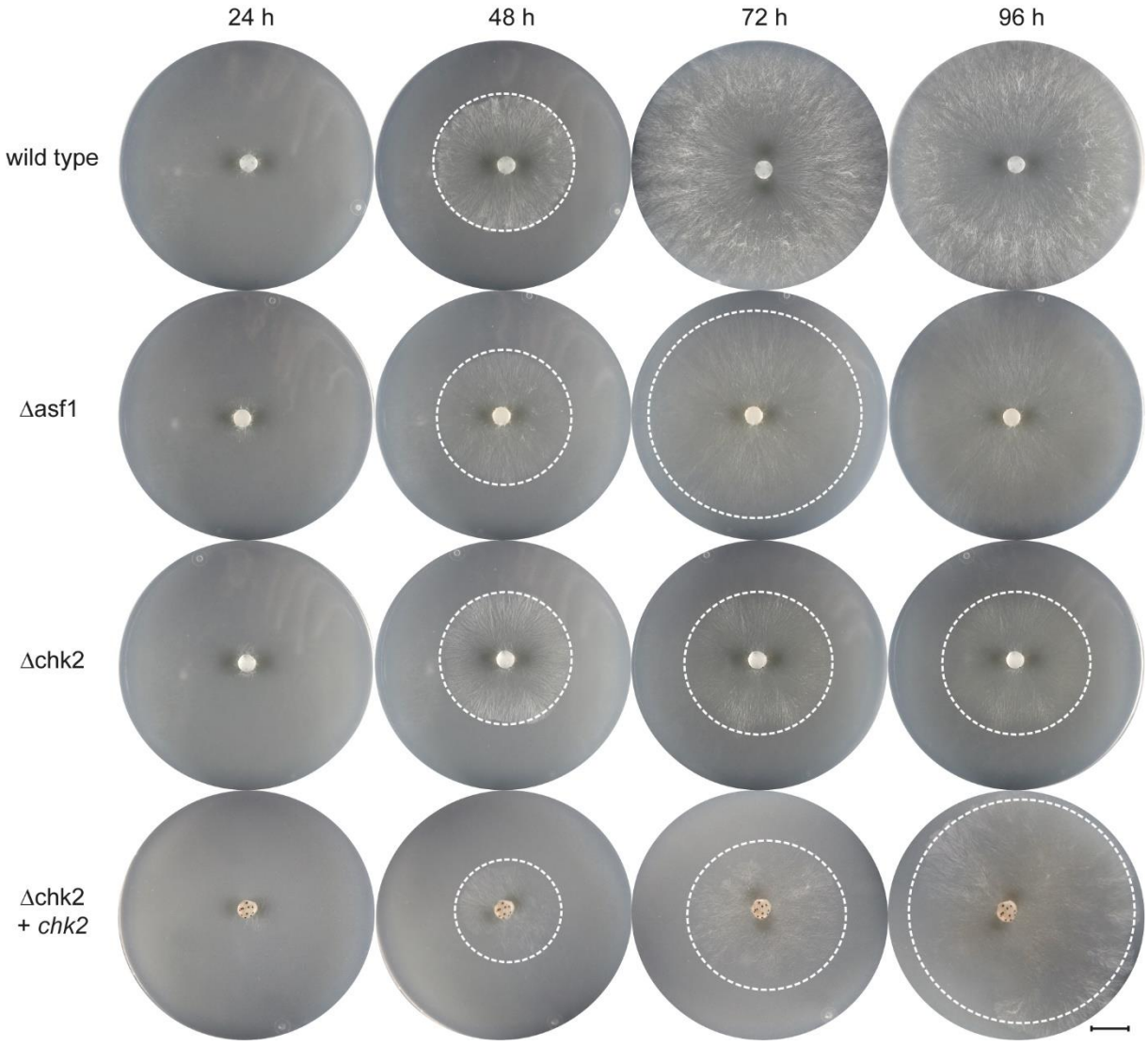

Figure 11: HU sensitivity test for *S. macrospora* Δchk2. The growth of the strains was observed on BMM media supplemented with 8 mM hydroxyurea for a period of 96 hours. In contrast to the observations with MMS, the *chk2* deletion mutant exhibited sensitivity to the DNA damaging agent hydroxyurea. 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. Scale bar represents 1 cm.