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# 1 The Mitochondrial Translocase of the Inner Membrane PaTim54 is involved in

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# 2 Defense Response and Longevity in Podospora anserina

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- 15 Running title: PaTim54 controls Defense Response and Longevity in P. anserina

16

#### 17 Abstract

18 Fungi are very successful microorganisms capable of colonizing virtually any ecological niche where they must constantly cope with competitors including fungi, bacteria and nematodes. We have 19 20 shown previously that the ascomycete Podopora anserina exhibits Hyphal Interference (HI), an antagonistic response triggered by direct contact of competing fungal hyphae. When challenged with 21 22 Penicillium chrysogenum, P. anserina produces hydrogen peroxide at the confrontation and kills the 23 hyphae of *P. chrysogenum*. Here, we report the characterization of the *PDC*<sup>2218</sup> mutant affected in HI. When challenged with *P. chrysogenum*, the *PDC*<sup>2218</sup> mutant produces a massive oxidative burst at the 24 25 confrontation. However, this increased production of hydrogen peroxide is not correlated to 26 increased cell death in P. chrysogenum. Hence, the oxidative burst and cell death in the challenger are uncoupled in PDC<sup>2218</sup>. The gene affected in PDC<sup>2218</sup> is PaTim54, encoding the homologue of the 27 budding yeast mitochondrial inner membrane import machinery component Tim54p. We show that 28 PaTim54 is essential in P. anserina and that the phenotypes displayed by the PDC<sup>2218</sup> mutant, 29 renamed PaTim54<sup>2218</sup>, are the consequence of a drastic reduction in the expression of PaTim54. 30 Among these pleiotropic phenotypes, PDC<sup>2218</sup>-PaTim54<sup>2218</sup>- displays increased lifespan, a phenotype 31 32 in line with the observed mitochondrial defects in the mutant.

#### 34 1. Introduction

35 Fungi are very successful microorganisms capable of colonizing virtually any ecological niche where 36 they must constantly cope with competitors such as other fungi, bacteria, nematodes (Boddy, 2000; 37 Boddy and Hiscox, 2016; Crowther et al., 2012). Fungi are often engaged in multiple kinds of biotic 38 interactions ranging from symbiotic to antagonistic (DeVay, 1956). In particular, saprotrophic fungi 39 that compete for nutrients have developed strategies to avoid spreading of competitors. Antibiosis, 40 the most famous and the most studied antagonistic interaction is viewed as a long-distance process that mainly relies on the capacity of fungi to constitutively produce and secrete toxic secondary 41 42 metabolites (Macheleidt et al., 2016). Hyphal interference (HI) and mycoparasitism are on the 43 contrary responses engaged after direct contact with challengers. Triggering such antagonistic 44 interactions implies that fungi must be endowed with a non-self-recognition system to adapt the 45 nature of the interaction with the competitor (Silar, 2012; Uehling et al., 2017). Non-self-recognition 46 has been studied in Vegetative Incompatibility (VI) observed in both ascomycete model systems 47 Neurospora crassa and Podospora anserina but this phenomenon involves competitors from the 48 same species (Glass et al., 2000; Saupe et al., 2000). On the contrary, HI (and mycoparasitism) takes 49 place between competitors from different species. HI was first investigated by Ikediugwu and 50 Websters who demonstrated that the basidiomycete Coprinus heptemerus kills hyphal cells of several ascomycetes competitors at the confrontation between thalli (Ikediugwu, 1976; Ikediugwu et al., 51 52 1970; Ikediugwu and Webster, 1970). Although in both VI and HI, the eventual outcome is the death 53 of fungal cells, the major discrepancy between VI and HI is the oxidative burst observed in HI only 54 (Silar, 2005). Indeed, when P. anserina is challenged by Penicillium chrysogenum, hydrogen peroxide accumulates on the thallus of P. anserina at the confrontation and it has been shown that cells of P. 55 56 chrysogenum in contact with those of *P. anserina* are killed. This latter feature of HI resembles the 57 oxidative burst taking place during innate immune response that kills or limit the spreading of invading pathogens in both plants and animals (Iles and Forman, 2002; Liu and He, 2016; Marino et 58 al., 2012). This analogy with the innate immune response of higher Eukaryotes was further 59

60 strengthened when it was shown in *P. anserina* that HI requires the NADPH oxidase (Nox) PaNox1, the homologue of mammalian NOX2/gp91<sup>phox</sup> and of plant Rboh O<sub>2</sub>-producing enzymes (Silar, 2005). 61 62 Indeed, when the PaNox1<sup>-</sup> mutant is challenged with P. chrysogenum, the accumulation of hydrogen peroxide on the mutant thallus on the one hand and cell death in P. chrysogenum on the other hand 63 64 are decreased at the confrontation of both mycelia. Noteworthy, three Nox isoforms are found in 65 fungi, Nox1/A, Nox2/B and Nox3/C. While the role of Nox3/C remains unknown, Nox1/A and/or 66 Nox2/B regulate an astonishing number of processes such as cellulose degradation, anastomosis (cell fusion), sexual reproduction, ascospore germination, cytoskeleton remodeling, symbiosis and 67 pathogeny (Aguirre et al., 2005; Brun et al., 2009; Scott, 2015; Scott and Eaton, 2008; Tudzynski et 68 al., 2012). However, only PaNox1 regulates HI (Brun et al., 2009; Silar, 2005). 69

In *P. anserina*, *IDC*<sup>343</sup>, the mutant of *PaNox1* was isolated in a screen designed to identify mutants of 70 71 Crippled Growth (CG), an epigenetically triggered mycelial degeneration observed when P. anserina 72 is grown on specific media (Haedens et al., 2005; Silar et al., 1999). Several Impaired Development of CG (IDC) mutants proved to be pleiotropic mutants defective in processes such as HI. In particular, 73 74 mutants of the PaNox1 complex or of the Cell Wall Integrity pathway (CWI) PaMpk1 MAP Kinase 75 pathway, are IDC mutants that exert similar HI defects as PaNox1 (Brun et al., 2009; Lacaze et al., 76 2015; Silar, 2005). Not only IDC mutants have been isolated in the original screens for CG mutants: 77 Promote Development of CG (PDC) mutants were also isolated and tested for HI. Amongst these IDC and PDC mutants, the PDC<sup>2218</sup> mutant showed unusual phenotype for HI. When challenged with P. 78 79 chrysogenum, the PDC<sup>2218</sup> mutant produces a massive oxidative burst at the confrontation. However, this increased production of hydrogen peroxide by the PDC<sup>2218</sup> mutant is not correlated to increased 80 cell death in P. chrysogenum (Silar, 2012). In this mutant, recognition of P. chrysogenum likely occurs 81 82 but the triggered response appears abnormal. Especially, the oxidative burst and cell death in the 83 challenger are uncoupled, which strengthen the previous assumption that hydrogen peroxide has a signaling role rather than a toxic one (Brun et al., 2009; Silar, 2005). With the aim to get insights into 84 the link between the oxidative burst in P. anserina and HI-induced cell death in the challenger, the 85

identification of the gene affected in PDC<sup>2218</sup> was undertaken. We first proceeded to positional 86 cloning of the mutation and we identified a deletion spanning 1560 bp in the mutant genome. Even 87 88 though the deletion disrupts the 5' part of the PaNip30 putative CDS as well as part of the 3' UTR (UnTranslated Region) of the neighboring PaCdk5 putative gene, our investigations show that none 89 90 of these genes are important for HI. Indeed, by a combination of different approaches: functional 91 complementation, gene deletion, genetic analyses and gene expression data, we show here that the gene important for creating the HI phenotype in PDC<sup>2218</sup> is PaTim54, the gene encoding the 92 homologue of the budding yeast mitochondria inner membrane carrier Tim54p (Hwang et al., 2007; 93 Kerscher et al., 1997; Kurz et al., 1999). Surprisingly, this gene is located more than 1000 bp away 94 95 from the deletion. We show that PaTim54 is essential in P. anserina and that the phenotypes displayed by the hypomorphic PDC<sup>2218</sup> mutant (renamed PaTim54<sup>2218</sup>) are the consequence of a 96 drastic reduction in the expression of PaTim54. 97

Contrary to many filamentous fungi, *P. anserina* has a limited vegetative growth phase that terminates by a the senescence of the cells at the thallus apex (Rizet, 1953a). For decades, this fungus has been a successful model system in studying aging and especially the effects of mitochondrial disorders in this degenerative process (Lorin et al., 2006; Osiewacz et al., 2010). In this paper, we show that the *PaTim54*<sup>2218</sup> mutation increases longevity and alters the respiratory chain, mitochondrial DNA (mtDNA) nucleoids as well as mitochondria morphology.

#### 104 2. Materials and methods

#### 105 **2.1. Strains, culture conditions and DNA preparations**

All the strains used in this study (Table 1) derived from the "S" (big S) wild-type strain that was used for sequencing the *P. anserina* genome (Espagne et al., 2008). The genome sequence and EST derived from the S strain are available at http://podospora.i2bc.paris-saclay.fr/. The polymorphic *P. comata* strain T was used for positional cloning of the *PDC*<sup>2218</sup> mutation (Boucher et al., 2017). The *PDC*<sup>2218</sup> (*PaTim54*<sup>2218</sup>) and the *PaNox1*<sup>-</sup> (*IDC*<sup>343</sup>) strains were first described in (Haedens et al., 2005); the 111 *DPaMpk1* in (Kicka et al., 2006) and the *mito-GFP* strain in (Sellem et al., 2007). All the deletions by 112 homologous recombination have been performed in strains inactivated for Pamus51, the gene 113 encoding the helicase of the NHEJ repair system, enhancing homologous recombination in these 114 strains (El-Khoury et al., 2008). Standard culture conditions, media and genetic methods for P. 115 ansering are reported in http://podospora.i2bc.paris-saclay.fr/; the M2 standard medium is a 116 medium in which carbon is supplied as dextrin, and nitrogen as urea. The methods used for nucleic 117 acid extraction and manipulation have been described (Lecellier and Silar, 1994). Transformation of 118 P. anserina protoplasts was carried out as described previously (Debuchy and Brygoo, 1985).

#### 119 2.2. Deletion of PaCdk5

120 The PaCdk5 (Pa 6 9000) predicted CDS was deleted by a split-marker strategy (Lalucque et al., 121 2012). To construct the deletion cassette of the entire PaCdk5 coding sequence, a hygromycin B 122 resistance (hygR) marker was fused by PCR with the upstream or the downstream sequence of the 123 PaCdk5 gene (Fig. S1). 1063 bp upstream and 1009 bp downstream of PaCdk5 were amplified from the wild-type genomic DNA with primers 9000 1 and 9000 2 for the upstream region and 9000 10 124 125 and 9000 11 for the downstream region (Table S1). Primers 9000 2 and 9000 10 contained 126 additional bases allowing fusion with the hygR cassette. The hygR cassette was amplified using 127 primers 9000\_HYGF/9000\_HYGR with pBC-hyg plasmid as template (Silar, 1995). In a second step, 128 two amplification reactions were performed using primers 9000\_1/HY and YG/9000\_11 to obtain the 129 fusion of two PCR products, the upstream (5') region/hygR and hygR/downstream (3') region. The 130 mixture of these two PCR products was used to transform a  $\Delta mus51::phleoR$  strain (El-Khoury et al., 131 2008) All the transformants showed a similar mutant phenotype (Figure 1). For twelve of them, 132 integration of the deletion K7 was checked by PCR using primers 9000\_5/HygL for the 5' flanking 133 sequences and HygR/214-6bis for the 3' flanking sequences. One Δmus51::phleoR ΔPaCdk5::hygR transformant was crossed with the wild type and homokaryotic [phleoS hygR] ΔPaCdk5::hygR 134 progeny was selected for further studies and analyzed by Southern blotting to confirm deletion of 135 136 PaCdk5 (Figure S1).

The *PaNip30* (*Pa\_6\_9010*) predicted CDS was deleted by a PCR-based strategy (Lalucque et al., 2012). 138 To construct the deletion cassette of the entire PaNip30 coding sequence, a nourseothricin 139 140 resistance (nouR) marker was fused by PCR with the upstream or the downstream sequence of the 141 PaNip30 gene (Figure S1). 514 bp upstream and 484 bp downstream of PaNip30 were amplified from 142 wild-type genomic DNA with primers 9010\_1 and 9010\_2 for the upstream region and primers 143 9010\_5 and 9010\_6 for the downstream region (Table S1). Primers 9010\_2 and 9010\_5 contained 144 additional bases allowing fusion with the nouR cassette. The nouR cassette was amplified using 145 primers 9010\_MKF/9010\_MKR from pBC-nour plasmid (Lalucque et al., 2012). In a second step, two 146 amplification reactions were performed using 9010\_1/9010\_MKR and 9010\_MKF /9010\_6 primers 147 pairs to obtain the fusion of two PCR products, the upstream (5') region/nouR and 148 nouR/downstream (3') region. The mixture of these two PCR products was used to transform a 149 Amus51::phleoR strain (El-Khoury et al., 2008). All the transformants exerted a wild-type phenotype 150 (Figure 1). For twelve of them, integration of the deletion K7 was checked by PCR using primers 151 9000\_10/validMK5' for the 5' flanking sequences and validMK3'/10\_20\_2R for the 3' flanking 152 sequences. One Amus51::phleoR APaNip30::nouR transformant was crossed with the wild type and homokaryotic [phleoS nouR] *DPaNip30::nouR* progeny was selected for further studies and analyz 153

ed by Southern blotting to confirm deletion of *PaNip30* (Figure S1).

#### 155 **2.4. Double deletion of** *PaCdk5* and *PaNip30*

The 5' hygR and 3'hyR split-marker deletion cassettes designed to target *PaCdk5* (described above) have been introduced into a purified [phleoR nouR]  $\Delta mus51::phleoR \Delta PaNip30::nouR$  homokaryotic strain by transformation. All The [hygR] transformants exerted a [ $\Delta PaCdk5$ ] mutant phenotype (Figure 1). Six transformants were selected and integration of the hygR cassette was checked by PCR using primers 9000\_5/HygL for the 5' flanking sequences and HygR/validMK5' for the whole intergenic region in between *PaCdk5* and *PaNip30* deleted CDS as the HygR primer hybridises in the 162 hygR deletion cassette of  $\Delta PaCdk5::hygR$  and validMK5' hybridises in the nouR deletion cassette of 163  $\Delta PaNip30::nouR$ . Three  $\Delta mus51::phleoR \Delta PaCdk5::hygR \Delta PaNip30::nouR$  transformants were crossed 164 with the wild type and homokaryotic [phleoS hygR nouR] progeny were selected for further studies. 165 Proper deletion of both genes was verified by Southern blotting (Figure S1) and the PCR product 166 corresponding to the amplification of the intergenic region (using primers HygR/validMK5') was 167 sequenced, further confirming the integrity of the intergenic space (data not shown).

#### 168 2.5. Deletion of PaTim54

169 The PaTim54 (Pa\_6\_9020) predicted CDS was deleted by a split-marker strategy (Lalucque et al., 170 2012). To construct the deletion cassette of the entire PaTim54 coding sequence, a hygromycin B 171 resistance (hygR) marker was fused by PCR with the upstream or the downstream sequence of the 172 PaTim54 gene (Figure S1). 1102 bp upstream and 832 bp downstream of PaTim54 were amplified 173 from the wild-type genomic DNA with primers 9020\_3 and 9020\_4 for the upstream region and 174 9020\_1 and 9000\_2 for the downstream region (Table S1). Primers 9020\_2 and 9020\_3 contained additional bases allowing fusion with the hygR cassette. The hygR cassette was amplified using 175 176 primers 9020\_HYGF/9020\_HYGR from pBC-hyg plasmid as previously described. In a second step, 177 two amplification reactions were performed using primers 9020\_1/HY and YG/9020\_4 to obtain the 178 fusion of two PCR products, the upstream (5') region/hygR and hygR/downstream (3') region. The 179 mixture of these two PCR products was used to transform a Δmus51::su8-1 strain (El-Khoury et al., 180 2008, p.). All the transformants exerted wild-type phenotype (data not shown). For twelve of them, integration of the deletion K7 was checked by PCR using primers HyGR/9020\_5 for the 5' flanking 181 182 sequences and 9010\_2F/HygL for the 3' flanking sequences. Two *Amus51::su8-1 APaTim54::hygR* 183 transformants bearing PCR-verified hygR deletion cassette were crossed with the wild type. 184 However, no homokaryotic [hygR] progeny was obtained. Further evidences argued that these two primary transformants were heterokaryotic and that deletion of *PaTim54* is lethal (see results). 185

186 **2.6. Plasmids** 

187 The pBC-hyg and pBC-phleo have been described in (Silar, 1995). Cosmids of the *P. anserina* genomic 188 library ref: 27C11, 31C04, 8H08, 11G02, 46G03, 1B05, 44C04, 3H09, 8C03, 38F08, 36C09, 39C12 and 189 12F01 bearing an hygR selection marker have been used for positional cloning. Plasmids of the P. 190 anserina genomic library ref: 342AG09 (bearing the Pa\_6\_8980 CDS), 340DH08 (Pa\_6\_8990 CDS), 191 224BE12 (Pa\_6\_8991 CDS), 328CD12 (Pa\_6\_9000 CDS) renamed pPaCdk5, 193BG01 (Pa\_6\_9010 CDS) renamed pPaNip30, 434CF12 (Pa\_6\_9020 CDS) renamed pPaTim54 and 142DC07 (Pa\_6\_9030 192 193 CDS) have been used for positional cloning. The pCDNA.2 plasmid backbone does not bear selection 194 marker in *P. anserina*. The average size of the insert is 3 kbp (Espagne et al., 2008).

#### 195 2.7. Constructions

2.7.1. pBC-hyg-PaCdk5: The pPaCdk5 plasmid was digested by the *Not*I and *Spe*I restriction enzymes.
It generated a 3632 bp fragment bearing the 1023 bp of *PaCdk5* predicted CDS plus the 1536 bp
upstream and the 1073 bp downstream of *PaCdk5* predicted CDS. This insert was subcloned into *Not1/Spe*I-digested pBC-hyg generating the pBC-hyg-PaCdk5 plasmid.

2.7.2. pBC-hyg-PaTim54 and pBC-phleo-PaTim54: The pPaTim54 plasmid was digested by the *Spel* and *Xhol* restriction enzymes. It generated a 3135 bp fragment bearing a 1450 bp encompassing
 *pPaTim54* predicted CDS (1386 bp) and one intron (64 bp) plus the 1019 bp upstream and the 666 bp
 downstream of *PaTim54* predicted CDS. This insert was subcloned into *Spel/Xhol*-digested pBC-hyg or
 pBC-phleo, generating the pBC-hyg-PaTim54 and the pBC-phleo-PaTim54 plasmids respectively.

205 2.8. PCR based complementation of *PaTim54<sup>2218</sup>*: The *PaCdk5<sup>PCR</sup>* PCR fragment encompassing the
152 base pairs upstream of the *PaCdk5* CDS as well as the 139 base pairs downstream of the CDS was
207 generated from wild-type genomic DNA using primers 9000\_1F/9000\_4R (Table S1) (GoTaq,
208 Promega). The *PaTim54<sup>PCR</sup>* PCR fragment encompassing the 504 base pairs upstream of *PaTim54* CDS,
209 the *PaTim54* CDS as well as the 182 base pairs downstream of the CDS was generated from wild-type
210 genomic DNA using primers 9020\_6F/9020\_5R (Table S1). Each PCR fragment was co-transformed
211 with pBC-hyg into *PaTim54<sup>2218</sup>* with a 3:1.

#### 212 2.9. RNA isolation and RT-qPCR

213 Vegetative cultures for RNA preparation were performed on Petri dishes containing standard M2 214 minimal medium and covered with a cellophane sheet. These cultures were inoculated with 6 215 implants from mat - wild-type strain or mutants. Dishes were placed at 27°C under standard light and 216 were removed from the incubation room at 48 h. Biological materials were collected and flash-frozen 217 in liquid nitrogen. Total RNA of P. anserina was extracted using the RNeasy Plant Mini Kit (cat#74904, 218 Qiagen, Hilden, Germany), including a grinding process using a tissue lyser II (QIAGEN) and a DNase 219 treatment on column. The total RNA quality was checked on gel after standard electrophoresis and 220 quantity was determined by using a Nanodrop spectrophotometer (Nanodrop Technologies). 4 µg of 221 total RNA was used for the RT; reverse transcription was performed with Superscript III or RevertAid 222 (Thermo Fisher Scientific). The 9000RTF and 9000RTR primers for the qPCR on Pa\_6\_9000/PaCdk5 223 are located within the CDS at position +723 and +922 respectively. The 9020RTF and 9020RTR 224 primers for the qPCR on Pa\_6\_9020/PaTim54 are located at position +62 (overlapping the single 225 encoded intron) and position +359 respectively. To quantify the genes with introns, all primers were 226 designed against two consecutive exons (Table S5), and an NRT (Not Reverse Transcribed) control 227 was performed on a pool of replicates to ensure that the Cq was above the Cq obtained from 228 corresponding reverse transcribed RNAs. Each RT-qPCR experiment contained at least three biological replicates (n = 6 for the  $PaTim54^{2218}$  mutant) (Table S6 and S7), and each biological 229 230 replicate was analyzed with three technical triplicates. Normalization genes were selected from a 231 pool of 7 housekeeping genes using geNorm (Table S6) (Vandesompele et al., 2002). RT-qPCR 232 normalization according to the  $\Delta\Delta$ Cq method (Pfaffl et al., 2002), standard error and 95% confidence 233 interval calculations, and statistical analyses were performed using REST 2009 software (Qiagen, Hilden, Germany). Table S8 shows fold-changes and standard errors. Genes were defined as 234 235 downregulated in the mutant strain if the ratio of their transcript level in the mutant strain compared 236 with that in the wild-type strain showed a fold change > 0 and < 1 and with a p-value of < 0.05. On 237 the other hand, genes were defined as up-regulated in the mutant strain if the ratio of their transcript level in the mutant strain compared with that in the wild-type strain showed a > 1 fold change and with a p-value < 0.05. Ratios with a 95 % confidence interval, including 1, were not considered significant (du Prel et al., 2009).

#### 241 **2.10.** DNA isolation and qPCR for mtDNA quantification

242 DNA was prepared as mentioned before (Lecellier and Silar, 1994). An additionnal RNAse step was 243 added requiring a second phenol-chloroform extraction step before final DNA precipitation. For the 244 senDNA assay (Figure 7B), DNA from the senescent apex of a WT strain grown 15 days on M2 245 medium was extracted. The mitochondrial DNA was quantified with primers targeted to NAD4 gene 246 and normalized to three nuclear loci corresponding to genes PaHMG8, PaHMG9 and MFM. 247 Importantly, NAD4 was chosen as the target because its chromosomal location is away from the main 248 chromosomal rearrangement leading to senDNA (Cummings et al., 1990). Each qPCR experiment 249 contained four biological replicates and each biological replicate was analyzed with three technical 250 replicates. The mitochondrial copy number normalization was performed according to the  $\Delta\Delta$ Cq 251 method (Pfaffl et al., 2002) by using nuclear loci normalization genes. Standard error and 95% 252 confidence interval calculations, and statistical analyses were performed using REST 2009 software 253 (Qiagen, Hilden, Germany) (Pfaffl et al., 2002). Table S10 shows fold-changes and standard errors.

### 254 2.11. Microscopic analyses

255 Microscopic observations were made on 3 to 5 days old mycelia grown directly on agar. One cm<sup>2</sup> agar 256 plugs were deposited onto a coverslip and mounted in water supplemented with DAPI (4',6-257 Diamidine-2'-phenylindole dihydrochloride ; Sigma-Aldrich) 0.1 µg.mL<sup>-1</sup>. For pictures of confrontation 258 between P. anserina and P. chrysogenum, plates were co-inoculated, incubated 3 days at 27°C and 259 agar plugs at the confrontation between both species were cut and mounted as previously described 260 but in water. Pictures were taken with an inverted microscope Leica DMI6000; sCMOS camera; LED 261 EL6000; dichroic filters XF131 (DAPI) and Qmaxgreen (GFP); Imagoseine Imaging Facility: 262 https://imagoseine.ijm.fr/676/accueil.htm . Pictures were analyzed with Fiji (Schindelin et al., 2012). 263 Diameter of nucleoids and of mitochondria was manually determined on undeconvoluted 2D images 264 using the Fiji software. The size (diameter) of 20 nucleoids (n=20) and the diameter of 20 265 mitochondria (n=20) has been determined on two to five representative fields of view (pictures) 266 depending on the genotype analyzed. Indeed, since the number of nucleoids and of mitochondria 267 was lower in the +, mito-GFP and the PaTim54<sup>2218</sup> pBC-phleo-PaTim54 mito-GFP strains than in the PaTim54<sup>2218</sup> mito-GFP strain, more samples (pictures) were required to count the 20 nucleoids and 268 269 the 20 mitochondria in both former strains. GFP fluorescence intensities in the mito-GFP control 270 strain and in the PaTim54<sup>2218</sup> mito-GFP strain were determined using the Fiji software as followed: 271 The maximum fluorescence intensity for five different transversal cuts along hyphae in teen different 272 samples (pictures) was measured using the "plot profile" function of the Fiji software. For every 273 measure, the background fluorescence intensity was subtracted. The different samples were 274 illuminated in the same conditions with the same exposure time. A t-test on the global distributions 275 (n=50) was performed.

### 276 2.12. Hyphal Interference assay

277 Hyphal Interference assays were performed as described in (Silar, 2005) and adapted from (Munkres, 278 1990). Plates were incubated in parallel and in duplicate 3 days at 27°C on standard M2 medium. P. 279 anserina and P. chrysogenum were co-inoculated at an approximate distance of 1.2 cm. For both DAB 280 (diaminobenzidine, Sigma Aldrich) as well as Blue Evans (Sigma Aldrich) staining procedures, the 281 solution was spread with a glass rod to ensure homogenous staining of thalli. For DAB staining (peroxide detection), plates were flooded in 100mM potassium phosphate buffer pH 6.9, 0.9 g.L<sup>-1</sup> 282 283 DAB and incubated 30 min at 27°C. The DAB staining solution was then discarded and pictures were 284 taken 3-6h later. For Blue Evans staining (cell death assay), plates were flooded in 0.1% Evans Blue in 285 water, incubated for 10 min and extensively rinsed with distilled water before pictures were taken.

#### 286 2.13. Longevity assay

287 For each analyzed genotype, triplicate cultures from three different freshly germinated homokaryotic 288 ascospores of each mating type were incubated on standard M2 medium in longevity tubes (30 cm 289 long) at 27°C. These ascospores were obtained from crossings of the wild type S strain with itself, with PaTim54<sup>2218</sup> and with PaTim54<sup>2218</sup> pBC-phleo-PaTim54. To minimize inheritance of the 290 291 senescence factor, the wild type S strain was used as female. For this purpose, the "male" implants 292 were removed from the crossings plates before ascospores projections. Total growth distance after 293 191 days incubation was measured. t-test statistical analysis was performed to compare the growth 294 distance mean of every tested genotypes. Note that measurements of *mat*- and *mat*+ strains of each 295 genotype were pooled together for t-test analysis making a pool of 18 measures per genotype.

#### 296 2.14. Mycelium fragmentation

A small plug of mycelium grown on agar plate is grinded is 500 μL sterile water in a FastPrep (500 rpm; 20 sec). The mixture is poured on an agar plate and individual hyphae are harvested under binocular and transferred on standard M2 medium. Homokaryosis is determined by testing the mating type of the regenerated thalli.

#### 301 2.15 Senescent DNA (senDNA) assay

The senDNA assay was performed on the DNA samples used for the qPCR-based mtDNA quantification. DNA from the following genotypes: *WT, PaTim54*<sup>2218</sup> and *PaTim54*<sup>2218</sup> *pBC-phleo-PaTim54* (two of the four DNA replicates of each genotype were analyzed) as well as DNA sample from an aged *WT* culture were digested by *Haelll*, ran on a 0.8% agarose gel and stained with Ethidium Bromide. SenDNA appear on the gel as intense and discrete bands in aged cultures only (Lorin et al., 2001). Molecular Weight marker: ThermoFisher scientific Generuler<sup>TM</sup> 1 Kb DNA ladder.

308

309 **3. Results** 

### 310 **3.1. The** *PDC*<sup>2218</sup> **mutant**

The PDC<sup>2218</sup> strain (all the strains used and generated in this study are reported Table 1) was isolated 311 312 in a screen designed to identify mutants of Crippled Growth (CG), an epigenetically-regulated 313 degeneration observed when P. anserina is grown on specific media (Haedens et al., 2005). The "Promote Development of Crippled Growth" PDC<sup>2218</sup> was isolated as a "weak" PDC mutant because 314 315 the severity of the degeneration observed on M2 standard medium was moderate. Additionally, reversion of the degeneration was not clear (Haedens et al., 2005). Compared to the wild type, when 316 grown on M2 standard medium at 27°C, the PDC<sup>2218</sup> mutant grew slower, exhibited a darker green 317 318 pigmentation and was almost devoid of aerial hyphae (Figure 1 and 2). This vegetative phenotype allowed us to characterize the PDC<sup>2218</sup> mutation during positional cloning of the mutation. In 319 addition, PDC<sup>2218</sup> showed temperature sensitivity. The mutant grew much slower than the wild type 320 321 at 18°C and did not grow at all at 37°C (Figure 2B).

322 We have previously shown that when P. anserina was challenged with P. chrysogenum, the Hyphal 323 Interference outcome is an oxidative burst on the thallus of P. anserina at the confrontation (revealed by DAB staining) as well as cell death in Penicillium chrysogenum (revealed by Trypan blue 324 325 or Evans blue staining) (Silar, 2005). Remarkably, the PDC<sup>2218</sup> mutant exhibited a modified Hyphal 326 Interference (HI) when confronted with P. chrysogenum with higher DAB precipitation and decreased 327 Evans blue staining (Figure 1) (Silar, 2012). Hence, in the mutant, a huge oxidative burst was triggered during HI but this was not correlated to increased cell death in the competitor (i.e., P. 328 chrysogenum). On the contrary, P. chrysogenum cell death was reduced. 329

### 330 **3.2.** Positional cloning of the *PDC*<sup>2218</sup> mutation

Previous studies determined that the  $PDC^{2218}$  mutation had a Second Division Segregation (SDS) frequency of 85%, indicating that it was located in the distal region of the arm of one of the seven chromosomes of *P. anserina* (Haedens et al., 2005). By crossing the  $PDC^{2218}$  mutant with different strains bearing mutations in distal parts of chromosomes, we did find a genetic linkage (d=7 cM) between the  $PDC^{2218}$  mutation and the  $\Delta PaTrx2$  deletion (Malagnac et al., 2007) (Table S2). In

agreement with the 85% SDS of the PaTrx2 gene, this result indicated that the PDC<sup>2218</sup> mutation was 336 337 located close to PaTrx2 on the left arm distal part of chromosome six. Note that we finally localized the PDC<sup>2218</sup> mutation about 93 kbp from the PaTrx2 gene (Figure 3) (see below). We continued 338 positional cloning of the mutation by crossing the PDC<sup>2218</sup> mutant (generated in the big S strain) with 339 340 the highly polymorphic strain T of the Podospora comata species (Boucher et al., 2017). We then analyzed co-segregation of polymorphic genetic markers and the PDC<sup>2218</sup> mutation in the progeny. 341 This allowed us to narrow down the location of the PDC<sup>2218</sup> mutation to a 370,822 bp interval 342 343 delimited by the 214-1 and Yeti-1 molecular markers (Figure 3). Thirteen cosmids of the P. anserina genome bank (Espagne et al., 2008) bearing wild-type sequences as well as the hygromycin B 344 resistance gene and overlapping the candidate interval were transformed into the PDC<sup>2218</sup> mutant 345 346 (see materials and methods). Only transformation with the 11G02 cosmid gave 12 "wild-typelooking" [WT] (from now on, phenotypes are indicated in square brackets) out of 34 [hygR] 347 transformants, suggesting that 11G02 carried the wild-type allele of the gene mutated in PDC<sup>2218</sup>. It 348 also indicated that the PDC<sup>2218</sup> mutation was recessive. The 22 non-complementing transformants 349 350 likely resulted from the heterologous integration of the cosmid and fortuitous inactivation of the 351 complementing sequence. Transformation with the partially overlapping 31C04 cosmid did not provide any complemented transformant. This led us to conclude that the PDC<sup>2218</sup> mutation was in 352 353 the non-overlapping part of the sequence carried by the 11G02 cosmid, allowing us to narrow down 354 the candidate interval for the mutation (Figure 3).

Seven predicted CDS were encoded in this interval. We selected seven plasmids of the *P. anserina* genome bank corresponding to the seven genes, each plasmid bearing a single predicted CDS (see materials and methods). Since the pCDNA.2 backbone of the bank's plasmids has no fungal selective marker, co-transformation of each plasmid along with empty pBC-hyg plasmid conferring resistance to hygromycin B was performed. Complementation of the mutant phenotype was observed both when wild-type  $Pa_6_{9000}$  (pPaCdk5 plasmid) or  $Pa_6_{9020}$  CDS (pPaTim54 plasmid) were introduced into  $PDC^{2218}$  (Figure 3). The  $Pa_6_{9000}$  predicted CDS encodes Pho85/PaCdk5, the *P.* 

362 anserina homologue of the yeast Cyclin Dependent Kinase 5 (Huang et al., 2007) and Pa\_6\_9020 363 encodes the homologue of the yeast mitochondrial Translocase of the Inner Membrane protein Tim54p, PaTim54 (Figure S2) (Kerscher et al., 1997). A total of 15 [WT] out of 137 [hygR] 364 365 transformants were obtained with the plasmid carrying PaCdk5 and 44 [WT] out of 49 [hygR] 366 transformants were obtained with the plasmid carrying *PaTim54*. On the contrary, co-transformation with the five other selected plasmids or empty pBC-hyg plasmid alone did not promote recovery of 367 368 [WT] transformants. Noteworthy, co-transformation with the plasmid bearing the predicted PaNip30 369 CDS (Pa\_6\_9010), encoding the Nip30 homologue protein of unknown function (Mammalian Gene 370 Collection (MGC) Program Team, 2002), located between PaCdk5 and PaTim54 on the chromosome 371 did not result in any complementation (0 [WT]/ 35 [hygR]). These results were confirmed by cotransformation experiments performed using PCR fragments consisting of PaCdk5 wild-type CDS 372 (PaCdk5<sup>PCR</sup>) or of PaTim54's one (PaTim54<sup>PCR</sup>) with pBC-hyg: 4 [WT] out of 50 [hygR] transformants 373 for PaCdk5<sup>PCR</sup> and 88 [WT] out of 97 [hygR] transformants for PaTim54<sup>PCR</sup> were observed (see 374 375 materials and methods and Figure 3). With the aim to rule out any artefactual effect due to co-376 transformation, we generated the pBC-hyg-PaCdk5 and pBC-hyg-PaTim54 plasmids by cloning the 377 PaCdk5 and the PaTim54 predicted CDS from pPaCdk5 and pPaTim54 into pBC-hyg respectively (see 378 materials and methods and Figure 3). In agreement with the previous experiments, complementation of PDC<sup>2218</sup> was observed with both pBC-hyg-PaCdk5 (15 [WT]/ 50 [hygR]) and pBC-hyg-PaTim54 (47 379 380 [WT]/ 50 [hygR]).

For the sake of simplicity, the *PDC*<sup>2218</sup> mutation was renamed *PaTim54*<sup>2218</sup> in the following parts of the manuscript as *PaTim54* proved to be the gene affected in *PDC*<sup>2218</sup> (see below). Five *PaTim54*<sup>2218</sup> *pBC-hyg-Cdk5* as well as five *PaTim54*<sup>2218</sup> *pBC-hyg-PaTim54* independent transformants presenting wild-type vegetative growth were tested for HI. Every transformants showed wild-type HI indicating that the HI default was complemented as well. Representative result for one of the [WT] transformants tested is shown Figure 1. This transformant of the *PaTim54*<sup>2218</sup> *pBC-hyg-Cdk5* genotype was annotated "*cis*" (see below for further explanations). Unexpectedly, taken together, 388 these above complementation experiments did not allow us to determine which of *PaCdk5* or 389 *PaTim54* was affected in the *PaTim54*<sup>2218</sup> mutant.

### 390 **3.3.** Molecular characterization of the *PaTim54*<sup>2218</sup> mutation

391 Both PaCdk5 and PaTim54 genes were amplified by PCR in PaTim54<sup>2218</sup> and sequenced. In addition to 392 the CDS per se, sequencing included 458 bp upstream and 139 bp downstream of PaCdk5 CDS as well 393 as 372 bp upstream and 223 bp downstream of PaTim54 CDS. Surprisingly, sequencing of both genes 394 did not reveal any mutation. With the aim to further investigate the sequence of the region between 395 PaCdk5 and PaTim54, we attempted PCR-based chromosome walking within the 3157 bp separating 396 both CDS (Figure 3). Strikingly, PCR amplification of the region between PaCdk5 and PaNip30 (using 397 primers 9000 3F/9010 3R binding in each CDS respectively) was about 1600 bp shorter than expected in the PaTim54<sup>2218</sup> mutant, strongly suggesting a deletion in the mutant's genome. 398 399 Sequencing of this PCR product confirmed that 1560 bp were lacking in *PaTim54*<sup>2218</sup>. This deletion 400 removed the first 140 bp of PaNip30 CDS as well as 1420 out of the 1856 bp of the intergenic region 401 between PaCdk5 and PaNip30. Analysis of P. anserina ESTs (Espagne et al., 2008) showed that the 402 PaCdk5 gene is transcribed and that it bears a long 3' UTR overlapping the observed deletion in the 403 mutant (Figure 3). However, this deletion did not affect the transcription level of PaCdk5, which was 404 similar to the one of PaCdk5 in the wild type (Figure 4). This result, together with the absence of 405 mutations in the PaCdk5 predicted CDS in the PaTim54<sup>2218</sup> mutant pointed to a role of PaTim54 in the specific phenotype of the *PaTim54*<sup>2218</sup> mutant. 406

### 407 **3.4.** *PaCdk5* and *PaNip30* loss-of-functions are not responsible for the *PaTim54*<sup>2218</sup> phenotypes

With the aim to test the role of *PaCdk5* and *PaNip30* in *P. anserina*, we <u>K</u>nocked <u>Out</u> (K.O) *PaCdk5* and *PaNip30* separately and we also generated the double  $\Delta PaCdk5 \Delta PaNip30$  K-O strain (see materials and methods). *PaCdk5* CDS was replaced by a hygromycin B resistance cassette while *PaNip30* CDS was replaced by a nourseothricin resistance (nouR) cassette (Figure S1). While the  $\Delta PaNip30$  strain was strictly identical to the wild type, mycelium in the  $\Delta PaCdk5$  as well as in the 413 double  $\Delta PaCdk5 \Delta PaNip30$  mutant strains was slightly more pigmented and with less aerial hyphae 414 (Figure 1). Importantly, the vegetative mutant phenotype of  $\Delta PaCdk5$  and of  $\Delta PaCdk5$   $\Delta PaNip30$  was clearly distinguishable from the one of *PaTim54*<sup>2218</sup>. Wild-type vegetative phenotype was restored (3 415 416 [WT] out of 12 [phleoR]) when  $\Delta PaCdk5$  was co-transformed with pPaCdk5 and pBC-phleo 417 (conferring resistance to phleomycin) together but not when transforming pBC-phleo alone (0 [WT] 418 out of 15 [phleoR]). Complementation of  $\Delta PaCdk5$  in the  $\Delta PaCdk5$   $\Delta PaNip30$  double mutant was 419 achieved by crossing the  $\Delta PaCdk5 \Delta PaNip30$  double mutant with a [WT] PaTim54<sup>2218</sup> pPaCdk5 pBC-420 phleo transformant. Phenotypic analyses of the progeny showed that the pPaCdk5 and the pBC-phleo transgenes were co-integrated in the genome as often observed in P. anserina (Razanamparany and 421 422 Bégueret, 1988) and that the  $\Delta PaCdk5 \Delta PaNip30 pPaCdk5 pBC-phleo$  progeny presented wild-type 423 phenotype. These data argued that only deletion of PaCdk5 accounted for the mutant vegetative 424 phenotype in  $\Delta PaCdk5$  as well as in  $\Delta PaCdk5 \Delta PaNip30$ . HI was as in the wild type for the three K.O 425 strains tested: ΔPaCdk5, ΔPaNip30 and ΔPaCdk5 ΔPaNip30 (Figure 1). In conclusion, PaNip30 has no 426 role in the processes studied here and PaCdk5 loss-of-function did not account for the PaTim54<sup>2218</sup> 427 phenotypes.

#### 428 3.5. PaCdk5 does not trans-complement PaTim54<sup>2218</sup>

The absence of mutation in PaCdk5 CDS, its unaffected expression in PaTim54<sup>2218</sup> as well as the 429 430 phenotype of  $\Delta PaCdk5$  were in sharp contrast to the observed complementation of  $PaTim54^{2218}$ 431 when this latter was transformed with PaCdk5 wild-type copy bearing transgenes. This led us to 432 hypothesize that the integration sites of the transgenes might account for this discrepancy. We thus investigated genetic linkage between the pBC-hyg-PaCdk5 transgenes and the PaTim54<sup>2218</sup> mutation 433 in three of the "complemented" [WT] PaTim54<sup>2218</sup> pBC-hyg-PaCdk5 mentioned before by crossing 434 435 them with the wild type (Table 2). Strikingly, no meiotic recombination was observed between the *pBC-hyg-PaCdk5* integrated transgenes and the *PaTim54*<sup>2218</sup> mutation in the three crossings. The  $\chi^2$ 436 437 statistical analysis of the F1 progeny phenotypes distribution argued that for the three parental transformants analyzed, the pBC-hyg-PaCdk5 transgenes were genetically linked to the PaTim54<sup>2218</sup> 438

439 mutation (P<10<sup>-2</sup>). Similar genetical analyses revealed that in the [WT, hygR] transformants co-440 transformed with PaCdk5<sup>PCR</sup> and pBC-hyg, both elements co-integrated and were genetically linked to PaTim54<sup>2218</sup> mutation (data not shown). Altogether, this indicated that the PaCdk5 bearing 441 transgenes could "complement" PaTim54<sup>2218</sup> only when inserted in cis close to the PaTim54<sup>2218</sup> locus 442 (Figure 1). This result was intriguing for bona fide functional complementation relies on "in trans" 443 rescue of the loss-of-function of a gene by its wild-type copy. We hence addressed the question 444 whether pPaCdk5 could *trans*-complement *PaTim54*<sup>2218</sup>. In a first step, three independent [WT] 445 446 complemented  $\Delta PaCdk5$  pPaCdk5 pBC-phleo strains were crossed with the wild type (Table 3). In the 447 progeny of the three crossings, [hygR phleoR] spores presented a [WT] phenotype while the [hygR 448 phleoS] progeny conserved a mutant [ΔPaCdk5] phenotype. In addition, equal distribution of the four 449 different phenotypic categories showed genetic independence between both loci, leading us to 450 conclude to ectopic chromosomal co-integration of both *pPaCdk5* and *pBC-phleo* ( $\chi^2$  analysis: P>0.05). We then tested if these transgenes could *trans*-complement *PaTim54*<sup>2218</sup> by crossing one  $\Delta PaCdk5$ 451 452 pPaCdk5 pBC-phleo F1 progeny from each parent transgenic line with PaTim54<sup>2218</sup> (Table 4). Strikingly, none of the PaTim54<sup>2218</sup> pPaCdk5 pBC-phleo progeny genotype presented a wild-type 453 phenotype (annotated PaTim54<sup>2218</sup> pPaCdk5 pBC-phleo-trans in Figure 1). This demonstrated that 454 wild-type copy of PaCdk5 did not trans-complement PaTim54<sup>2218</sup> although the pPaCdk5 transgenes 455 456 were functional.

457 Inversely, we wondered whether some *pPaCdk5* transgenes in "non-complemented" [PaTim54<sup>2218</sup>] 458 PaTim54<sup>2218</sup> pPaCdk5 pBC-phleo transformants were actually functional but that they did not complement PaTim54<sup>2218</sup> because they were ectopically integrated. To this aim, we crossed two 459 460 independent non complemented [PaTim54<sup>2218</sup>] PaTim54<sup>2218</sup> pPaCdk5 pBC-phleo transformants to ΔPaCdk5 (Table S3). Progeny analysis for both transformants showed that, first, pPaCdk5 and pBC-461 phleo were ectopically co-integrated (no genetic linkage between the PaCdk5/PaTim54<sup>2218</sup> locus and 462 the transgenes integration sites;  $\chi^2$  analysis: P>0.05) and second that the *pPaCdk5* transgenes 463 previously shown as unable to complement PaTim54<sup>2218</sup> did complement  $\Delta PaCdk5$ . Indeed, in both 464

465 crossings, the  $\Delta PaCdk5 \ pPaCdk5 \ pBC-phleo$  progeny presented wild-type phenotype. In addition, one 466 [PaTim54<sup>2218</sup>] PaTim54<sup>2218</sup> pPaCdk5 pBC-phleo transformant was crossed to the double  $\Delta PaCdk5$ 467  $\Delta PaNip30$  mutant (Table S4). In the progeny, the  $\Delta PaCdk5 \ \Delta PaNip30 \ pPaCdk5 \ pBC-phleo$  descendants 468 showed wild-type phenotype, demonstrating as mentioned before that only PaCdk5 loss-of-function 469 was responsible for the [pPaCdk5] mutant phenotype in  $\Delta PaCdk5 \ \Delta PaNip30$ .

470 Altogether, these data argued that the *PaTim54*<sup>2218</sup> mutation was not functionally *trans*-471 complemented by wild-type *pPaCdk5* but rather genetically *cis*-suppressed by genetically linked 472 *pPaCdk5*-bearing transgenes (Figure 1). Importantly, the *PaCdk5*<sup>PCR</sup> fragment allowing *cis*-suppression 473 of *PaTim54*<sup>2218</sup> mutation in the co-transformation experiments mentioned above does not span the 474 *PaTim54*<sup>2218</sup> deletion (Figure 3).

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#### 476 **3.6.** *PaTim54* is the gene affected in *PaTim54*<sup>2218</sup>

Hence, the most likely candidate gene for being altered in *PaTim54*<sup>2218</sup> remained *PaTim54*. Similarly 477 478 as what we did for the pBC-hyg-PaCdk5 transgenes, we first tested the genetic linkage between the pBC-hyg-PaTim54 integrated transgenes and the PaTim54<sup>2218</sup> locus in three independent [WT] 479 PaTim54<sup>2218</sup> pBC-hyg-PaTim54 complemented transformants by crossing them with the wild type. As 480 reported in Table 5, [hygS] progeny showed [*PaTim54*<sup>2218</sup>] mutant phenotype while all the [hygR] 481 482 descendants showed wild-type phenotype. First, this confirmed the capacity of the pBC-hyg-PaTim54 transgenes analyzed to complement *PaTim54*<sup>2218</sup>. Second,  $\chi^2$  statistical analysis of the F1 progeny 483 phenotypes distribution determined that in every transformants, the pBC-hyg-PaTim54 inserted 484 transgene and the PaTim54<sup>2218</sup> mutation were genetically independent (P>0.05). This led us to 485 conclude that PaTim54 wild-type copy did functionally trans-complement PaTim54<sup>2218</sup>. Altogether, 486 these data showed that *PaTim54* was the gene affected in *PaTim54*<sup>2218</sup>. 487

488 3.7. PaTim54 is essential in P. anserina

489 First identified in S. cerevisiae as an essential gene by Kerscher et al. (Kerscher et al., 1997), other 490 groups eventually obtained viable tim54 deleted strains (Hwang et al., 2007; Kovermann et al., 2002). 491 We attempted to delete PaTim54 to functionally assess PaTim54 loss-of-function. A Amus51 strain 492 (inactivated for the NHEJ repair system; see materials and methods), was transformed with a 493 hygromycin B-resistance cassette designed to delete PaTim54 and we obtained 40 [hygR] transformants with no mutant vegetative phenotype. For twelve of these [hygR] transformants, 494 495 integration of the deletion cassette was tested and validated by PCR (see materials and methods). 496 Four of these validated transformants were crossed with the wild type to obtain pure homokaryotic 497 ΔPaTim54 (devoid of Δmus51) in the F1 progeny. In P. anserina, two kinds of ascospores can be 498 found in asci. In a minority of asci, small homokaryotic ascospores can be recovered (along with large 499 self-fertile dikaryotic ascospores), while the vast majority of asci only present four large mat+/mat-500 self-fertile dikaryotic ascospores. None of the four crossings yielded homokaryotic [hygR]  $\Delta PaTim54$ 501 progeny. However, second division segregation (SDS) asci containing 4 [hygR] dikaryotic ascospores 502 could be recovered. These asci contained self-fertile dikaryotic ascospores of the mat+/mat-503  $\Delta PaTim 54/+$  genotype that gave rise to wild-type growing mycelium. We then harvested 504 homokaryotic progeny from two different self-fertile dikaryotic mat+/mat- ΔPaTim54/+ strains. For 505 the first strain, only 18 [hygS] out of 36 ascospores germinated and for the second strain, 14 [hygS]/ 506 36 harvested ascospores germinated. All together, these data suggested that  $\Delta PaTim 54$  deletion was 507 lethal and/or impaired for ascospore germination, and both phenotypes being recessive.

Since homokaryotic  $\Delta PaTim54$  could not be obtained, we could not verify the strain by Southern blotting but we validated *PaTim54* loss-of-function by complementation. To this purpose, we generated a pBC-phleo-PaTim54 plasmid by subcloning wild-type *PaTim54* from pPaTim54 into pBCphleo. In a first step, this pBC-phleo-PaTim54 plasmid was transformed into *PaTim54*<sup>2218</sup> and we obtained 46 [WT] complemented out of 54 [phleoR] transformants. We assumed that in these complemented transformants, the *pBC-phleo-PaTim54* transgenes were functional. We then transferred the *pBC-phleo-PaTim54* transgenes in a  $\Delta PaTim54$  strain in two steps. First, we used the

515 [hygR] mat+/mat-  $\Delta PaTim54/+$  self-fertile dikaryotic ascospores, (see above) to determine the 516 mating type of the nucleus carrying the *DPaTim54* deletion. To this purpose, we crossed one 517  $mat+/mat-\Delta PaTim54/+$  with both the mat+ and the mat- wild-type strains. As shown in Table 6, only 518 crossing with the *mat*- wild type partner yielded SDS asci with four  $\Delta PaTim54/+$  spores, arguing that 519 in the analyzed mat+/mat-  $\Delta PaTim54/+$  strains, the  $\Delta PaTim54$  deletion was carried by the mat+ 520 nucleus (Table 6). Then, we crossed the [hygR] mat+/mat- $\Delta PaTim54/+$  strain carrying the  $\Delta PaTim54$ deletion in the mat+ nucleus with a [WT phleoR] mat- PaTim54<sup>2218</sup> pBC-phleo-PaTim54 strain (Table 521 522 7). In the progeny, we harvested [WT hygR phleoR] viable homokaryotic  $\Delta PaTim54$  pBC-phleo-PaTim54 as well as viable dikaryotic ΔPaTim54/ΔPaTim54 pBC-phleo-PaTim54/+ descendants. 523 Notably, the  $\chi^2$  statistical analysis did not indicate genetic linkage between *PaTim54* and the different 524 525 pBC-phleo-PaTim54 transgenes studied (P>0.05). When tested for HI, the ΔPaTim54 pBC-phleo-526 PaTim54 progeny was comparable to the wild type (Figure 1). These data confirmed that wild-type 527 PaTim54 fully trans-complemented ΔPaTim54. Finally, to test whether PaTim54 was essential in P. 528 anserina, we proceeded to mycelium fragmentation of one ΔPaTim54/ΔPaTim54 pBC-phleo-529 PaTim54/+ dikaryotic strain (see materials and methods). After mycelium regeneration, we 530 recovered sixty [HygR phleoR]  $\Delta PaTim54/\Delta PaTim54$  pBC-phleo-PaTim54/+ dikaryotic thalli, fourteen 531 homokaryotic [hygR phleoR] ΔPaTim54 pBC-phleo-PaTim54 thalli but no homokaryotic [hygR phleoS] 532  $\Delta PaTim 54$ . This latter result strongly argued that deletion of PaTim 54 was lethal and more 533 specifically that *PaTim54* was essential for mycelial growth in *P. anserina*.

#### 534 **3.8.** *PaTim54* gene expression is affected in *PaTim54*<sup>2218</sup>

Because the sequence of the *PaTim54* CDS was not altered in *PaTim54*<sup>2218</sup>, we hypothesized that its expression may be affected in the mutant. To address this question, we tested *PaTim54* gene expression by RT-qPCR. Strikingly, *PaTim54* mRNA level was less than 10% of the wild-type level in *PaTim54*<sup>2218</sup> as well as in *PaTim54*<sup>2218</sup> *pPaCDk5 pBC-phleo-trans*, two strains harboring [PaTim54<sup>2218</sup>] mutant phenotype (Figure 4). On the contrary, *PaTim54* gene expression was like the wild type in the other strains tested except in the complemented *PaTim54*<sup>2218</sup> *pBC-phleo-PaTim54* strain, where 541 PaTim54 expression fold-change was 1.9 compared to wild type, suggesting that overexpression of PaTim54 in this strain restored wild-type phenotype. Interestingly, PaTim54 expression was reduced 542 to 23 % of the wild-type level in the PaTim54<sup>2218</sup> pBC-hyg-PaCdk5-cis transformant although this 543 544 strain exhibited a wild-type phenotype. In contrast, PaTim54 expression in  $\Delta PaCdk5$ , in  $\Delta PaNip30$  and in the double  $\Delta PaCdk5 \Delta PaNip30$  K-O strains was not statistically different from the wild type 545 suggesting that the gene replacements operated in these strains did not disturb *PaTim54* expression. 546 547 It is worthy to note that PaCdk5 gene expression was not down-regulated either in PaTim54<sup>2218</sup> pBCphleo-PaTim54, in PaTim54<sup>2218</sup> pPaCdk5 pBC-phleo-trans or in PaTim54<sup>2218</sup> pBC-hyg-PaCdk5-cis. 548 Altogether, these data strongly argued that only very low PaTim54 gene expression level was 549 responsible for the pleiotropic [PaTim54<sup>2218</sup>] mutant phenotypes. 550

#### 551 **3.9.** *PaMpk1* regulates *PaTim54*<sup>2218</sup>-triggered oxidative burst

552 As shown in Figure 1, we could detect a massive oxidative burst at the confrontation between PaTim54<sup>2218</sup> and P. chrysogenum during HI. However, cell death at the confrontation in P. 553 chrysogenum revealed by Evans blue staining was abolished compared to that when the wild type 554 555 was challenged with P. chrysogenum. The PaMpk1 MAP Kinase pathway as well as the PaNox1 556 complex are important regulators of HI in P. anserina. Indeed, the oxidative burst along with cell 557 death in *P. chrysogenum* at the confrontation are both abolished when  $\Delta PaMpk1$  and  $PaNox1^{-1}$ mutants are challenged (Figure 1) (Silar, 2005). In order to determine whether PaMpk1 and PaNox1 558 could play a role in PaTim54<sup>2218</sup>-triggered HI, we constructed the PaTim54<sup>2218</sup> ΔPaMpk1 as well as the 559 *PaTim54<sup>2218</sup> PaNox1<sup>-</sup>* double mutants. Interestingly, mycelium in both double mutant strains neither 560 looked alike PaTim54<sup>2218</sup>, nor ΔPaMpk1 or PaNox1 parental strains. On the contrary, mycelium in 561 562 both double mutant strains was scarce (Figure 1). This observation that mycelium in both double 563 mutant strains was more severely affected than in the single suggested a synergistic interaction between  $PaTim54^{2218}$  and  $\Delta PaMpk1$  as well as between  $PaTim54^{2218}$  and  $PaNox1^{-}$  regarding 564 vegetative growth. With the aim to assay HI in PaTim54<sup>2218</sup> ΔPaMpk1 and PaTim54<sup>2218</sup> PaNox1<sup>-</sup> 565 566 double mutants, we challenged both strains with P. chrysogenum. We observed that cell death in P.

chrysogenum at the confrontation with PaTim54<sup>2218</sup>, PaNox1<sup>-</sup>, and ΔPaMpk1 single mutants was 567 568 abolished as previously shown (Figure 1). The result was similar for PaTim54<sup>2218</sup>  $\Delta$ PaMpk1 and PaTim54<sup>2218</sup> PaNox1<sup>-</sup> double mutants. DAB staining was slightly decreased in PaTim54<sup>2218</sup> PaNox1<sup>-</sup> 569 double mutant while it was strongly reduced in PaTim54<sup>2218</sup> ΔMpk1 double mutant compared to in 570 the wild type. Hence, ΔPaMpk1 mutation was clearly epistatic over PaTim54<sup>2218</sup> mutation, while 571 *PaNox1*<sup>-</sup> mutation was only partially epistatic over the *PaTim54*<sup>2218</sup> mutation. Since vegetative growth 572 573 in both double mutants was similarly affected, both showing scarce and flat mycelium (Figure 1), the 574 fact that DAB staining in PaTim54<sup>2218</sup> PaNox1<sup>-</sup> when challenged with P. chrysogenum was just slightly decreased argued that drastic DAB staining loss observed in PaTim54<sup>2218</sup> ΔMpk1 when challenged 575 576 with P. chrysogenum was unlikely due to reduced mycelium density or growth defects. This led us to 577 conclude to a major role of the PaMpk1 MAPK, in contrast to the PaNox1 NADPH oxidase that may have a limited role, in regulating the massive oxidative burst triggered in PaTim54<sup>2218</sup>. 578

#### 579 **3.10.** *PaTim54*<sup>2218</sup> mutation affects the mitochondrial network

With the aim to investigate the mitochondrial network in *PaTim54*<sup>2218</sup> when grown on standard 580 medium but also when challenged with P. chrysogenum, we constructed a PaTim54<sup>2218</sup> mito-GFP 581 582 (Sellem et al., 2007). In yeast, Tim54p is involved in the import of proteins of the inner membrane 583 and it has been shown that null mutation of tim54 does not affect import of soluble matrix proteins 584 (Kerscher et al., 1997). Accordingly, GFP fluorescence intensities measured in the PaTim54<sup>2218</sup> mito-585 GFP strain were not statistically different than in the mito-GFP control strain arguing for a normal import of the soluble matrix mito-GFP reporter protein in PaTim54<sup>2218</sup> mutant background (Figure 586 587 S3). To standardize our cytological analyses, we focused on hyphae of the growing edge of the 588 thallus. Indeed, mitochondrial morphology is highly dynamic, with mitochondria forming long 589 longitudinal tubes in the apical hyphae (thread-like network) to shorter tubes and finally rounded 590 mitochondria in the central part of the thallus (data not shown). We thus analyzed GFP tagging pattern at the edge of the thallus in the PaTim54<sup>2218</sup> mito-GFP tagged strain and we compared it to 591 the control strain bearing only the *mito-GFP* transgene as well as to the complemented *PaTim54*<sup>2218</sup> 592

593 pBC-phleo-PaTim54 mito-GFP strain. Green fluorescence pattern in the control strain as well as in the 594 complemented PaTim54<sup>2218</sup> pBC-phleo-PaTim54 mito-GFP strain exhibited a thread-like 595 mitochondrial network formed of highly tubular and easily trackable mitochondria (Figure 5A). On the contrary, GFP tagging pattern in PaTim54<sup>2218</sup> mito-GFP was much more intricate. Mitochondria in 596 597 the mutant looked shorter, thinner, and somehow packed, as if filling the cytoplasm. They did not 598 form long tubular structures as in the control and in the complemented strain. In addition to the size 599 difference, we also determined that their diameter was significantly smaller ( $334 \pm 37$  nm; t-test: 600 P<10<sup>-9</sup>) in PaTim54<sup>2218</sup> compared to the control (456  $\pm$  52 nm) and the complemented strain (418  $\pm$  60 601 nm; t-test: P<10<sup>-5</sup>).

602 We also analyzed the mitochondrial network at the edge of the thallus when P. anserina was challenged with *P. chrysogenum*. For both the *mito-GFP* control strain as well as for *PaTim54*<sup>2218</sup> 603 604 mito-GFP, tagging pattern in hyphae in close contact to P. chrysogenum was not different from the 605 tagging pattern in hyphae of the growing edge without challenge (Figure 6). This indicated that 606 challenging with P. chrysogenum did not significantly affect P. anserina mitochondrial network at the 607 confrontation. However, we often observed small lateral branching hyphae that swelled in 608 PaTim54<sup>2218</sup> (Figure 6). When laterally branching hyphae were observed in the control, they exhibited 609 standard morphology but no swelling. This latter observation indicated that the challenge with P. 610 chrysogenum did induce swelling of small growing hyphae specifically in PaTim54<sup>2218</sup>.

# 611 **3.11.** *PaTim54*<sup>2218</sup> mutation affects nucleoids

In *S. cerevisiae*, it has been shown that the number of nucleoids, the structure where the mitochondrial DNA (mtDNA) is found, was decreased in the *tim54* mutant strains (Hwang et al., 2007). In order to assay mtDNA stability in *P. anserina*, we quantified mtDNA by qPCR and we cytologically analyzed the distribution of nucleoids on young mycelium. Samples were stained with DAPI and the number of nucleoids in 10 µm length hyphal segments was quantified (Figure 5A). Surprisingly, the number of nucleoids in *PaTim54*<sup>2218</sup> *mito-GFP* was dramatically increased compared

to the mito-GFP control (32 ± 9 in PaTim54<sup>2218</sup> vs. 9 ±3 in the control; t-test: P<10<sup>-9</sup>) and to the 618 619 PaTim54<sup>2218</sup> pBC-phleo-PaTim54 mito-GFP complemented strain (12 ± 4; t-test<10<sup>-8</sup>) (Figure 5B). Interestingly, these nucleoids were significantly smaller in *PaTim54*<sup>2218</sup> mito-GFP than in the control 620 (313 ± 72 nm in PaTim54<sup>2218</sup> vs. 429 ± 75 nm in the control; t-test: P<10<sup>-4</sup>) and in the complemented 621 622 strain (416  $\pm$  53 nm; t-test<10<sup>-4</sup>) (Figure 5C). These observations suggested that the nucleoids in PaTim54<sup>2218</sup> mito-GFP were somehow disorganized and likely fragmented. However, these data did 623 not allow us to conclude on the stability of the mtDNA in PaTim54<sup>2218</sup>. To this aim, the ratio of 624 625 mtDNA versus nuclear DNA in young mycelium was measured by qPCR in PaTim54<sup>2218</sup>, PaTim54<sup>2218</sup> pBC-phleo-PaTim54 and the wild type (Figure 7A). The level of mtDNA was similar in the three tested 626 627 strains, leading us to conclude that the important accumulation of nucleoids of small size in PaTim54<sup>2218</sup> was indicative of disorganization and/or fragmentation of nucleoids but not of increased 628 629 mtDNA level. Importantly, we determined that senescent DNA (senDNA) was absent in the DNA 630 samples used for the mtDNA level quantification (Figure 7B), which is in agreement with the 631 paradigm that senDNA is absent in young mycelium (Osiewacz et al., 2010). This suggested that senDNA did not account for disorganized and/or fragmented nucleoids observed in PaTim54<sup>2218</sup> 632 633 young mycelium and this indicated that senDNA did not alter mtDNA level quantifications. Finally, the fact that the number of nucleoids, their size as well as the quantity of mtDNA in the PaTim54<sup>2218</sup> 634 635 pBC-phleo-PaTim54 complemented strain were similar to the wild type, emphasized that all the defects evidenced above in *PaTim54*<sup>2218</sup> are due to the *PaTim54*<sup>2218</sup> hypomorphic allele. 636

#### 637 **3.12.** *PaTim54<sup>2218</sup>* mutation affects the *PaAOX* gene expression level and longevity in *P. anserina*

*P. anserina* is a potent model system to study aging (Marcou, 1961; Osiewacz et al., 2010). Indeed, contrary to many filamentous fungi, *P. anserina* vegetative growth is limited to about 10 cm (about 15 to 20 days of growth) on standard M2 medium and at standard temperature (27°C). This phenomenon is known as senescence (Rizet, 1953b). To study senescence in the wild type, in *PaTim54*<sup>2218</sup> as well as in the *PaTim54*<sup>2218</sup> *pBC-phleo-PaTim54* complemented strain, we analyzed the growth distance of mycelium cultures from freshly germinated spores (Figure 8). In these longevity

assays, all the cultures of the wild type as well as of the PaTim54<sup>2218</sup> pBC-phleo-PaTim54 644 complemented strains died after an average of 10 cm of growth, corresponding to about 15-20 days 645 of longevity. On the contrary, 12/18 cultures of the PaTim54<sup>2218</sup> genotype and corresponding to three 646 647 replicates from three single mat+ ascospores and one single mat- ascospore were still growing after 648 191 days of incubation for a total growth distance of approximately 1 m, a longevity never observed in WT. Statistical analysis supported the difference observed between the PaTim54<sup>2218</sup> strain and the 649 WT or the PaTim54<sup>2218</sup> pBC-phleo-PaTim54 complemented strains (Figure 8; P<4.10<sup>-8</sup>). Worth is to 650 651 note that these cultures were still growing when measurements were stopped. Remarkably, the three replicates from two individual mat + PaTim54<sup>2218</sup> spores underwent senescence after an 652 653 average of 22 and 25 cm growth distance respectively. This indicated that senescence was not completely abolished in *PaTim54*<sup>2218</sup>. Nevertheless, these longevity assays demonstrated that 654 PaTim54<sup>2218</sup> behaved as a long-lived mutant and that PaTim54<sup>2218</sup> hypomorphic allele was 655 656 responsible for increased longevity.

657 P. anserina is an obligate aerobe fungus in which respiratory mutants lacking Cytochrome c oxidase 658 activity (E.g PaCOX1 and PaCOX5 mutants) are nonetheless viable but they astonishingly show 659 strikingly increased lifespan. In such mutants, overexpression of the alternative oxidase encoding 660 gene PaAOX has been shown to compensate for the lack of Cytochrome c oxidase activity and the 661 exclusive use of the alternative respiration pathway that produces less reactive oxygen species leads to increased lifespan (Dufour et al., 2000; Lorin et al., 2001; Sellem et al., 2007). In order to test 662 663 whether the Cytochrome c respiratory pathway was disturbed in PaTim54<sup>2218</sup>, we quantified the expression of the PaAOX gene by RT-qPCR. Strikingly, strains harboring [PaTim54<sup>2218</sup>] phenotypes, 664 665 namely PaTim54<sup>2218</sup> and PaTim54<sup>2218</sup> pPaCdk5 pBc-phleo-trans, exhibited potent overexpression of the PaAOX gene, the hallmark of Cytochrome c respiratory pathway mutants (Figure 4). In particular, 666 the *PaTim54*<sup>2218</sup> mutant showed a *PaAOX* gene expression fold-change of ten compared to wild type. 667 In the other strains tested and especially in the [WT] PaTim54<sup>2218</sup> pBC-phleo-PaTim54 complemented 668

strain, *PaAOX* gene expression was like in the wild type. This last result strongly suggested that the
 *PaTim54<sup>2218</sup>* mutation impaired the respiratory chain.

### 671 4. Discussion

## 672 **4.1.** *The PDC*<sup>2218</sup> mutant and Hyphal Interference

673 Microbial communities are complex ecosystems where interplay between fungi, bacteria and 674 invertebrates such as nematodes are of major importance (Crowther et al., 2012; De Boer et al., 675 2005). Interactions can be of different kinds and in this paper we focused on Hyphal Interference 676 (HI), a defense response shown by filamentous fungi when their hyphae directly contact a competitor 677 fungus. This implies that fungi capable of HI i) can sense the competitor's hyphae, ii) recognize them 678 as non-self and iii) are endowed with an appropriate downstream signaling pathway and effectors 679 required to trigger the HI response. The ascomycete fungus P. anserina is able to display a HI 680 response when challenged with various fungal competitors; however the basidiomycete Coprinopsis 681 cinera is able to kill P. anserina hyphae, while P. anserina is able to kill P. chrysogenum (Silar, 2005). 682 Non-self-recognition not only in *P. anserina* but more generally in the fungal kingdom is largely 683 unknown. First involved in cell death during Vegetative Incompatibility in P. anserina and in N. 684 crassa, Nucleotide Oligomerization Domain (NOD)-like Receptors (NLRs) could play the role of 685 Pattern Recognition Receptors (PRRs) in fungi (Bidard et al., 2013; Lamacchia et al., 2016; Paoletti, 686 2016; Uehling et al., 2017). Nowadays, only Het-C in N. crassa, "the host", has been shown as a true 687 PRR able to bind the PhcA protein from Pseudomonas syringae, the "pathogen", and to trigger cell 688 death in the host (Wichmann et al., 2008). How P.anserina recognizes non-self-hyphae is an open 689 question. Similarly, it is not clear how cell death in the challenger, here *P. chrysogenum*, is triggered. 690 Fungi are able to produce many kinds of toxic compounds in order to kill their competitors. Among 691 the list of active antifungal compounds able to trigger hyphal death are secondary metabolites, 692 volatile organic compounds, small secreted polypeptides and secreted lytic enzymes (Silar, 2012). A 693 burst of Reactive Oxygen Species (ROS) is generated on the thallus of *P. anserina* when challenged by

694 different fungal species. However, these ROS are unlikely directly responsible for P. chrysogenum cell 695 death. Indeed, the PDC<sup>2218</sup> mutant analyzed here shows an increased oxidative burst and triggers less 696 death in the competitor. On the same line, we have previously shown that the ROS produced by the 697 PaNox1 NADPH oxidase are involved in signaling rather than in killing during HI (Brun et al., 2009). 698 Interestingly, epistasis analysis shows that PaNox1 only plays a minor role in the oxidative burst displayed by PDC<sup>2218</sup>. The additional sources of these ROS remain to be identified. Epistasis data 699 indicate that their production in PDC<sup>2218</sup> is under the control of the CWI PaMpk1 MAP kinase 700 701 pathway.

702 We want to stress the point that Nox1 and the Mpk1 MAP Kinase pathway control virulence in 703 mycoparasitic Trichoderma sp. developed for biocontrol of fungal plant pathogens as well 704 (Druzhinina et al., 2011; Karlsson et al., 2017). For instance, the Nox1 NADPH oxidase of Trichoderma 705 harzanium and the ROS it produces are important in the biocontrol of the phytopathogen Phytium 706 ultimum (Montero-Barrientos et al., 2011). In the closely related species Trichoderma virens, the 707 TmkA (=Tvk1) and TmkB MAP kinases, homologous to PaMpk2 and PaMpk1 respectively, play key 708 roles in the mycoparasitic activity against Sclerotium rolfsii, Rhizoctonia solani and Phytium spp. 709 (Kumar et al., 2010; Mukherjee et al., 2003). HI and mycoparasitism may be closely related processes 710 relying on a common set of regulatory pathways.

### 4.2. The *PDC*<sup>2218</sup> phenotypes result from transcriptional down-regulation of *PaTim54*

Following classical genetic cartography and positional cloning procedures, we characterized a 1560 bp deletion as responsible for the pleiotropic phenotypes in the  $PDC^{2218}$  mutant. This deletion identified in  $PDC^{2218}$  genome removes the 5' end of PaNip30, a putative gene with unknown function, together with most of the putative 3'UTR of PaCdk5, the homologue of the yeast cyclin-dependentkinase *Cdk5*. Several arguments immediately ruled out the possibility that PaNip30 was the gene affected in  $PDC^{2218}$ . Additionally, several strong arguments, especially complementation of  $PDC^{2218}$ with a wild-type copy of PaCdk5, suggested that PaCdk5 might be the gene affected in  $PDC^{2218}$ .

719 However, targeted gene deletion and cis- versus trans-complementation and functional 720 complementation experiments supported by Mendelian genetic analyses pointed to PaTim54 as the only gene affected in PDC<sup>2218</sup>. Although PaCdk5 deletion points to a role of this putative cyclin in 721 722 aerial hyphae production as well as mycelial pigmentation in P. anserina as well, we want to emphasize that *PaTim54* fully restores wild-type phenotypes in *PaTim54*<sup>2218</sup> mutants (*i.e.*, aerial 723 hyphae, pigmentation, growth rate, thermosensitivity, longevity, mitochondrial defects and hyphal 724 Interference) arguing that only *PaTim54* function is altered in *PaTim54*<sup>2218</sup> mutants. We eventually 725 show that PaTim54 is essential in P. anserina and that PaTim54<sup>2218</sup>, the allele present in PDC<sup>2218</sup>, is 726 hypomorphic and triggers low level of expression of PaTim54. There is no evidence to explain the 727 728 down-regulation in PaTim54 gene expression or the cis-suppressing effect of PaCdk5 bearing 729 transgenes. Further investigations including genome sequencing of several *cis*-suppressed [WT] PaTim54<sup>2218</sup> pBC-hyg-Cdk5 transformants will be required to understand this cis-suppression effect of 730 PaCdk5 bearing transgenes. However; since transformation of PaTim54<sup>2218</sup> with PaCdk5<sup>PCR</sup>, a PCR 731 732 fragment not overlapping the PaTim54<sup>2218</sup> deletion, leads to cis-suppression as well, we assume that 733 cis-suppression by PaCdk5 bearing transgenes does not rely on the comeback of missing regulatory 734 sequences controlling PaTim54 expression and deleted in PaTim54<sup>2218</sup>.

735

736 4.3. PaTim54, mitochondria and defense response

The *PaTim54* gene encodes the orthologue of the yeast mitochondrial Translocase of the inner Membrane Tim54p (Kerscher et al., 1997). PaTim54 protein shares 60% and 40% identity with *N. crassa* and *S. cerevisiae* Tim54p proteins, respectively. Noteworthy, no other homologue has been found in the *P. anserina* genome. Similarly to the yeast *tim54* mutants, *PaTim54<sup>2218</sup>* shows slow growth at cold temperature (18°C) and thermal sensitivity at 37°C. However, while the yeast Tim54p has no major role in mitochondrial morphology, *PaTim54<sup>2218</sup>* mutation promotes significant mitochondrial fission. Indeed, cytological analysis of mitochondria in living cells shows that the thread-like mitochondrial network is altered in *PaTim54*<sup>2218</sup>. This mitochondrial morphology change in *PaTim54*<sup>2218</sup> is reminiscent of increased mitochondrial fission and/or decreased mitochondrial fusion. In addition in the *tim54* yeast mutants, the number of nucleoids is decreased (Hwang et al., 2007). Although we have observed that the number of nucleoids is dramatically increased in *PaTim54*<sup>2218</sup>, mtDNA level remains unchanged. Hence, the *PaTim54*<sup>2218</sup> allele seems to promote the disorganization and fragmentation of nucleoids but not mtDNA degradation in *P. anserina*.

750 A growing body of evidence links mitochondria to immunity. In humans, several immune syndromes 751 are now considered as "likely" due to primary mitochondrial disorders like the Barth Syndrome or the 752 Cartilage Hair Hypoplasia. It becomes clear that mitochondria participate in RLR signaling (an antiviral 753 response based on RLR-Retinoic acid Like Receptor), antibacterial immunity and inflammation 754 (Walker et al., 2014; West et al., 2011). Noteworthy, both the mitochondrial ROS production as well 755 as the capacity of mitochondria to fuse are two important phenomena involved in RLR signaling and 756 inflammation, respectively. Signaling responses to non-self through mitochondria appears thus 757 conserved during evolution. We show here that the P. anserina mitochondrial network is not 758 modified in hyphae in close contact with hyphae of competing fungus during HI. However, we show 759 that PaTim54 function is required for the down-regulation of the oxidative burst during HI and to 760 trigger hyphal cell death in the challenging fungus. Hence, like the PaMpk1 and the PaNox1 signaling 761 pathways, mitochondria appear as important actors of HI. Further studies will be required to 762 understand how mitochondria and the PaMpk1 pathway interact in the regulation of the HI response 763 in P. anserina.

764 4.4. PaTim54, mitochondria and aging

Mitochondria play a major role in aging in a wide range of eukaryotes as well. This organelle is known to provide the cell with ATP through the respiratory chain. However, the byproducts of this process, namely the ROS, are able to damage cellular components such as proteins, nucleic acids and lipids. This damaging capacity of mitochondria-produced ROS is well known as the "Mitochondrial Free

769 Radical Theory of Aging" (Harman, 1972). However, ROS production is not anymore considered as 770 the only way mitochondria impact cell longevity. Evidences show that several regulatory pathways 771 merging in a quality control system in mitochondria influence lifespan as well (Osiewacz, 2011; 772 Osiewacz et al., 2010; Osiewacz and Bernhardt, 2013). Briefly, this quality control includes ROS 773 scavenging, mtDNA maintenance, protein-repair, -refolding and -degradation, mitochondria shaping 774 (fusion vs. fission) and mitophagy (Fischer et al., 2012). In yeast, Tim54p is an integral protein of the 775 mitochondrial inner membrane with two predicted transmembrane domains. Tim54p participates in 776 at least two distinct complexes. On the one hand, it associates with the voltage dependent channel 777 protein Tim22p responsible for the import of integral membrane proteins. Although the role of 778 Tim54p in the complex remains unclear, Tim54p per se has been shown to be required for the 779 insertion/import of the Tim22p translocase, the ATP/ADP carrier Aac1p and the i-AAA protease 780 Yme1p, three intrinsic proteins of the mitochondrial inner membrane (Bolender et al., 2008; Kerscher 781 et al., 1997; Kovermann et al., 2002; Kurz et al., 1999). On the other hand, yeast Tim54p directly 782 interacts with the i-AAA-protease Yme1p (Hwang et al., 2007). Tim54p is absolutely required to 783 stabilize the 1M Da high molecular weight Yme1p complexes as well as for the protease activity of 784 the complex. Tim54p therefore plays a pivotal role in the import of proteins into mitochondria as well as in their degradation (Hwang et al., 2007). Both roles may account for *PaTim54*<sup>2218</sup> phenotypes. 785 We have shown that the PaTim54<sup>2218</sup> mutation increases lifespan, a feature present in many 786 787 mitochondrial mutants. We have also demonstrated that mitochondrial morphology as well as 788 nucleoids organization are affected in the mutant. Noteworthy in *PaTim54*<sup>2218</sup>, the increased lifespan 789 may not be correlated to a decrease in mitochondrial fission. Finally, as supported by PaAOX gene overexpression in PaTim54<sup>2218</sup> which is characteristic of lack of Cytochrome c oxidase function 790 791 (Dufour et al., 2000; Lorin et al., 2001; Sellem et al., 2007), insufficient PaTim54 gene expression may 792 affect the respiratory chain function by disturbing import of its integral components (Kerscher et al., 793 1997; Kovermann et al., 2002). It has been shown that Yme1p plays a major role in shaping 794 mitochondrial morphology and in mtDNA stability (Campbell et al., 1994). N. crassa IAP-1 and P. 795 anserina PaIAP, both orthologues of Yme1p, have previously been studied (Klanner et al., 2001; Weil 796 et al., 2011). As in yeast, IAP-1 and PaIAP are involved in proteolysis of misfolded proteins, 797 participating in protein quality control (Osiewacz, 2011; Osiewacz et al., 2010). The N. crassa iap-1 798 mutant strains show slow growth on non-fermentable carbon sources at high-temperature (40°C) 799 and mutations in PaIAP leads to increased lifespan at normal temperature in P. anserina. Because of the similar lifespan increase in PaTim54<sup>2218</sup> and in PaIAP mutants, it is tempting to speculate that 800 both gene products may also work together in P. anserina. However, both mutant strains exhibit 801 802 some differences in their phenotypes. For instance, PaTim54 but not PaIAP is essential in P. anserina. Furthermore, the hypomorphic PaTim54<sup>2218</sup> allele is thermosensitive in contrast to knocking-out 803 804 PalAP that slightly increases longevity at 37°C (Weil et al., 2011). This may be accounted for by a 805 major role of Tim54p in protein import. Further studies of the role of PaIAP in Hyphal Interference as well as thorough studies of the double PaTim54<sup>2218</sup> ΔPaIAP mutant in comparison with both single 806 PaTim54<sup>2218</sup> and  $\Delta$ PaIAP mutants should give new insights into the role of both integral proteins of 807 808 the mitochondrial inner membrane.

### 809 5. Conclusion

The characterization of the gene actually affected in the *PDC*<sup>2218</sup> mutant has not been straightforward and *P. anserina* amenability for genetic analysis greatly helped us to differentiate "false positive" complementation (of *PaTim54*<sup>2218</sup> by *PaCdk5*) from true trans-complementation of *PaTim54*<sup>2218</sup> by *PaTim54*, thus evidencing that *PaTim54* was the actual gene whose decreased expression triggers the HI phenotypes. *In fine*, the study of the *PaTim54*<sup>2218</sup> hypomorphic mutant gave us the opportunity to get insights into the functions of *PaTim54* in relation with defense and aging, although this gene is essential in *P. anserina*.

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### 820 Conflict of interest:

821 The authors declare that there are no conflicts of interest neither commercial affiliations.

822

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# **Tables:**

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# 1052 Table 1: strains and genotypes

genotype	Detailed genotype	resistance markers	reference	
wild type	big S	-	(Rizet, 1953b)	
Δmus51	∆mus51::su8-1	-	(Lambou et al., 2008)	
Δmus51	∆mus51::phleoR	phleoR	(El-Khoury et al., 2008)	
mito-GFP	mito-GFP::hygR	hygR	(Sellem et al., 2007)	
ΔPaMpk1	∆PaMpk1::hygR	hygR	(Kicka et al., 2006)	
PaNox1 <sup>-</sup>	<i>IDC</i> <sup>343</sup>	-	(Malagnac et al., 2004)	
ΔPaTrx2	∆PaTrx2::phleoR	phleoR	(Malagnac et al., 2007)	
PaTim54 <sup>2218</sup>	PDC <sup>2218</sup>	-	(Haedens et al., 2005)	
			This study	
ΔPaCdk5	$\Delta PaCdk5::hygR$	hygR		
ΔPaNip30	∆PaNip30::nouR	nouR		
ΔPaTim54*	∆PaTim54::hygR	hygR		
$\Delta PaCdk5 \Delta PaNip30$		hygR nouR		
ΔPaCdk5 pPaCdk5 pBC-phleo		hygR phleoR		
ΔPaCdk5 ΔPaNip30 pPaCdk5 pBC-phleo	hygR phleoR nouR			
mat+/mat- ΔPaTim54/+		hygR		
ΔPaTim54 pBC-phleo-PaTim54		hygR phleoR		
PaTim54 <sup>2218</sup> ΔPaMpk1		hygR		
PaTim54 <sup>2218</sup> PaNox1 <sup>-</sup> PaTim54 <sup>2218</sup> pPaCdk5 pBC-phleo		- phleoR		
PaTim54 <sup>2218</sup> pBC-hyg-Cdk5		hygR		
PaTim54 <sup>2218</sup> PaCdk5 <sup>PCR</sup> pBC-hyg		hygR		
PaTim54 <sup>2218</sup> pBC-hyg-PaTim54		hygR		
PaTim54 <sup>2218</sup> pBC-phleo-PaTim54		phleoR		
PaTim54 <sup>2218</sup> PaTim54 <sup>PCR</sup> pBC-hyg	hygR			
PaTim54 <sup>2218</sup> mito-GFP		hygR		
PaTim54 <sup>2218</sup> pBC-phleo-PaTim54 mito-GFP		phleoR hygR		

1053 \*: homokaryotic lethal

# 1055 Table 2: PaTim54<sup>2218</sup> pBC-hyg-PaCdk5 X WT

PaTim54 <sup>2218</sup> pBC-hyg-PaCdk5 X WT	phenotypes			
	[WT hygR]	[WT hygS]	[PaTim54 <sup>2218</sup> hygS]	Genetic linkage : $\chi^2$ test
2	21	15	0	Yes: d<2.7cM : P<10 <sup>-2</sup>
3	21	27	0	Yes: d<2.0cM : P<10 <sup>-9</sup>
4	22	23	0	Yes: d<2.1cM : P<10 <sup>-4</sup>
transformant number	PaTim54 <sup>2218</sup> , pBC-hyg-PaCdk5	+,+	PaTim54 <sup>2218</sup> , +	
	ge	enotypes		

# 1057 Table 3: ΔPaCdk5 pPaCdk5 pBC-phleo X WT

ΔPaCdk5 {pPaCdk5 pBC phleo} X		phenotype	les		
WT	[hygR phleoR]	[hygS phleoS]	[hygS phleoR]	[hygR phleoS]	
2	6 [WT]	15 [WT]	9 [WT]	12 [ΔPaCdk5]	
6	9 [WT]	12 [WT]	10 [WT]	13 [ΔPaCdk5]	
7	11 [WT]	14 [WT]	10 [WT]	13 [ΔPaCdk5]	
transformant number	ΔPaCdk5, pPaCdk5 pBC-phleo	+, +	+, pPaCdk5 pBC-phleo	ΔPaCdk5, +	
	genotypes				

# 1059 Table 4: ΔPaCdk5 pPaCdk5 pBC-phleo X PaTim54<sup>2218</sup>

ΔPaCdk5 {pPaCdk5 pBC phleo} X	phenotypes			
PaTim54 <sup>2218</sup>	[hygR phleoR]	[hygS phleoS]	[hygS phleoR]	[hygR phleoS]
2 (F1)	7 [WT]	9 [PaTim54 <sup>2218</sup> ]	7 [PaTim54 <sup>2218</sup> ]	17 [ΔPaCdk5]
6 (F1)	9 [WT]	16 [PaTim54 <sup>2218</sup> ]	8 [PaTim54 <sup>2218</sup> ]	10 [ΔPaCdk5]
7 (F1)	11 [WT]	8 [PaTim54 <sup>2218</sup> ]	11 [PaTim54 <sup>2218</sup> ]	4 [ΔPaCdk5]
transformant number	ΔPaCdk5,	PaTim54 <sup>2218</sup> , +	PaTim54 <sup>2218</sup> ,	ΔPaCdk5, +
	pPaCdk5 pBC-phleo		pPaCdk5 pBC-phleo	
	genotypes			

## 1062

# 1063 Table 5: PaTim54<sup>2218</sup> pBC-hyg-PaTim54 X WT



1064

### 1065 Table 6: tetrad analysis of mat+/mat- ΔPaTim54/+ X WT mat- or mat+

∆PaTim54/+ X WT	crossing with mat -		crossing with mat +			
	phenotype	genotype	number	phenotype	genotype	number
FDS asci	2 [†]	$\Delta PaTim54/\Delta PaTim54$	3			
	2 [hygS]	+/+		4 [hygS]	+/+	8
SDS asci	4 [hygR]	∆PaTim54/+	7			

1066

# 1067 Table 7: mat- PaTim54<sup>2218</sup> pBC-phleo-PaTim54 X mat+/mat- ΔPaTim54/+

	phenotypes			
mat- PaTim54 <sup>2218</sup> pBC-phleo-PaTim54 X	[hygS phleoR]	[hygR phleoS]	[hygR phleoR]	[hygS phleoS]
mat+/mat- ΔPaTim54/ +	21 [WT]	0	4 [WT]	8 [PaTim54 <sup>2218</sup> ]
-	PaTim54 <sup>2218</sup> ,	∆PaTim54, +	ΔPaTim54,	PaTim54 <sup>2218</sup> , +
_	pBC-phleo-PaTim54		pBC-phleo-PaTim54	

genotypes

1068

1070 Figures captions:

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1072 Figure 1: Phenotypes of the different strains and Hyphal Interference assays (HI). A) vegetative 1073 growth of the different strains on standard M2 medium (first column); DAB staining (second column); 1074 Evans Blue staining (third column). Scale bars: 1 cm. B) schematic of the Hyphal Interference assay: 1075 The P. anserina tested strains are co-inoculated with three Penicillium chrysogenum (P. c) inocula. 1076 The oxidative burst as well as cell death revealed by DAB staining (brownish patch) and Evans blue 1077 staining (blue crescent) respectively are performed after 3 days on different plates incubated in parallel. C) results of the HI assays for the different genotypes tested. EB: Evans blue; DAB: 1078 1079 diaminobenzidin; semi-quantitative staining scoring -: no significant staining; +, ++, +++: staining of 1080 increasing intensity.

1081

Figure 2: Growth rate on M2 standard medium. A) Growth rate at 27°C, the standard temperature.  $WT: 6.6 \pm 0.5 \text{ mm/day}; PaTim54^{2218} (PDC^{2218}): 5.5 \pm 0.5 \text{ mm/day}; PaTim54^{2218} pBC-phleo-PaTim54 :$   $6.4 \pm 0.3 \text{ mm/day}. *: n=18; t-test P<10^{-6}; * for PaTim54^{2218} is the t-test probability in comparison with$  WT. B) relative growth rate of  $PaTim54^{2218}$  and  $PaTim54^{2218}$  pBC-phleo-PaTim54 strains at different temperatures. No growth for  $PaTim54^{2218}$  at 37°C. The result at 27°C is from the experiment in A). \* for  $PaTim54^{2218}$  is the t-test probability in comparison with WT. Relative growth rate values for  $PaTim54^{2218}$  pBC-phleo-PaTim54 are never significantly different to WT.

1089

Figure 3: Chromosome map of the *PaTim54<sup>2218</sup>* (*PDC<sup>2218</sup>*) locus. Upper part, diagram of the whole chromosome 6 (K6). Both polymorphic markers *214-1*, *yeti1*, the *PaTrx2* gene and the mutation *PDC<sup>2218</sup>* renamed *PaTim54<sup>2218</sup>* (\*) are reported and the distances are indicated in kbp. The regions covered by both 11G02 and 31C04 cosmids are indicated. Lower part, map of the *PaTim54<sup>2218</sup>* locus.

1094 The CDS of the three investigated genes PaCdk5 (Pa 6 9000), PaNip30 (Pa 6 9010) and PaTim54 1095 (Pa\_6\_9020) are represented. The 1560 pb deletion identified in the PaTim54<sup>2218</sup> mutant is indicated 1096 by the corresponding dashed line. This deletion removes the first 140 bp of PaNip30 CDS. The region 1097 covered by the PaCdk5 ESTs is indicated by the corresponding dashed line. The wild-type genomic 1098 DNA fragments which have been used for complementation experiments, and encompassing *PaCdk5*, 1099 PaNip30 or PaTim54 CDSs are represented. Sizes of the CDSs upstream sequences are indicated as 1100 negative starting from the start codon and size of CDSs downstream sequences are indicated as 1101 positive starting from the stop codon. bp: base pairs.

1102

1103 Figure 4: PaCdk5, PaTim54 and PaAOX genes expression level. Expression analysis by RT-qPCR of 1104 PaCdk5 (Pa\_6\_9000), PaTim54 (Pa\_6\_9020) and PaAOX (Pa\_3\_1710) in strains of interest. 1105 Genotypes are indicated on the vertical axis. Horizontal axis indicates the decimal logarithm of fold 1106 change (FC) in transcript detection relative to the wild-type strain (see Table S6 and S7 for Cq and 1107 Table S8 for numerical values of FC). Normalization genes selected with geNorm (Vandesompele et 1108 al., 2002) were AS1, GPD and UBC, with an average stability value M= 0.346 and  $V_{3/4}$  = 0.093 (see 1109 Table S6). Bars show standard errors and stars (\*) indicate FC with a p-value < 0.05 (see Table S8 for 1110 numerical values of standard errors). FCs with a 95 % confidence interval including the ratio 1 or very 1111 close to 1 were not considered as significant, even if the p-value is below the cut-off (du Prel et al., 1112 2009).

1113

Figure 5: Mitochondria and nucleoids in *PaTim54<sup>2218</sup>*. A) Epifluorescence microscopy photographs of
strains carrying the mito-GFP reporter. In these strains, the Green Fluorescent Protein is imported
into mitochondria and visualized in the green channel. Genotypes: *WT :+, mito-GFP*; *PaTim54<sup>2218</sup> :PaTim54<sup>2218</sup>, mito-GFP* and *PaTim54<sup>2218</sup> pBC-phleo-PaTim54 : PaTim54<sup>2218</sup> pBC-phleo-PaTim54 mitoGFP. DAPI stains nucleoids (mtDNA) and is visualized in the blue channel. Scale bar: 5 µm. B)* DAPI

tagged nucleoids have been counted in 10 μm-long hyphal segments (n=20) for every genotypes. C)
and D) The size (diameter) of 20 nucleoids (n=20) and the diameter of 20 mitochondria (n=20) have
been determined on two to five representative fields of view (pictures) depending on the genotype
analyzed (see Mat & Met). T-test P values are indicated in each panel.

1123

Figure 6: Mitochondria of *P. anserina* during HI. Z series photographs of hyphae of *P. anserina* (*P. a*)
and *Penicillium chrysogenum* (*P. c*) in confrontation. The mitochondrial network is highlighted by the *mito-GFP* reporter gene present in both the control *mito-GFP* and the *PaTim54<sup>2218</sup> mito-GFP* strains.
First column, DIC; second column, GFP fluorescence. Arrow heads indicate budding branching hyphae
specifically observed in *PaTim54<sup>2218</sup>* but not in the control. Scale bar= 10µm.

1129

1130 Figure 7: mtDNA level quantification and senDNA analysis. A) Copy number analysis by qPCR of 1131 **NAD4** in strains of interest. Genotypes are indicated on the vertical axis. Horizontal axis indicates 1132 fold change (FC) in copy number detection relative to the wild-type strain (see Table S9 for Cq and 1133 Table S10 for numerical values of FC). Three nuclear loci were used for normalization (Table S9). Bars 1134 show standard errors and stars (\*) indicate FC with a p-value < 0.05 (see Table S5 for numerical 1135 values of standard errors). B) Senescent DNA (senDNA) analysis. DNA was digested by HaeIII and 1136 stained with Ethidium Bromide. Two DNA sample replicates (1 and 2) -used for the mtDNA level 1137 quantification- per genotype were analyzed. MW, Molecular Weight marker. Bands sizes are indicated in base pairs (bp). Wtsen, genomic DNA extracted from senescent WT strain. \* Senescent 1138 DNA. Senescent DNA was not detected in the DNA samples used for the qPCR-based mtDNA 1139 quantification experiment. 1140

- 1142 Figure 8: Longevity assay. Total growth distance after 191 days incubation for triplicate cultures of
- 1143 three individual freshly germinated homokaryotic ascospores of both mating types for each analyzed
- 1144 genotype. In grey, dead cultures before day 191; in black, alive and still growing cultures at day 191.
- 1145 Every dead cultures eventually presented senescence on their thallus. (\*): P values <0.05 for the t-
- 1146 tests performed between the *PaTim54<sup>2218</sup>* strain and the *WT* (P<4.10<sup>-8</sup>) or the *PaTim54<sup>2218</sup>* pBC-phleo-
- 1147 *PaTim54* complemented strain (P<4.10<sup>-8</sup>).

# Figure 1:

Α		DAB	Evans Blue		B
~	*			WT	
		X		PaTim54 <sup>2218</sup> (PDC <sup>2218</sup> )	
C. or the second		-		ΔPaCdk5	(
1	ø			ΔPaNip30	
			0	ΔPaCdk5 ΔPaNip30	
	(0)			PaTim54 <sup>2218</sup> pBC-phleo-PaTim54	
A A A A A A A A A A A A A A A A A A A			-	PaTim54 <sup>2218</sup> pPaCdk5 pBC-phleo-tr	ans
				PaTim54 <sup>2218</sup> pBC-hyg-PaCdk5-cis	
	•			PaNox1	
	*	X	Pat	PaTim54 <sup>2218</sup> PaNox1	
and a second	0			Pa∆Mpk1	
				PaTim54 <sup>2218</sup> ∆PaMpk1	L



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С
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+	+
+++	-
+	+
+	+
+	+
+	+
+++	-
+	+
-	-
+++	-
-	-
+	-
	+ ++ + + + + + + +

Figure 2:



Figure 3:









Figure 5:





Figure 7:



Figure 8:



### **Supplemental Informations:**

Table S1\_S5 : genetics analyses data and primers tables.

**Table\_S6.** Cq for the housekeeping genes and identification of the normalization genes with geNorm [1]. Cq for *PaTim54* (Pa\_6\_9020).

**Table\_S7**. Cq for *AS1* (Pa\_1\_16650), *GPD* (Pa\_3\_5110), *UBC* (Pa\_4\_7790), *PaAOX* (Pa\_3\_1710) and *PaCdk5* (Pa\_6\_9000).

**Table\_S8.** Fold changes and standard errors for *PaCdk5* (Pa\_6\_9000), *PaTim54* (Pa\_6\_9020) and *PaAOX* (Pa\_3\_1710).

Table\_S9. Cq for the quantification of mitochondrial DNA.

 Table\_S10.
 Fold changes and standard errors for mitochondrial DNA quantification.

**Figure S1:** ΔPaCdk5, ΔPaNip30, ΔPaCdk5 ΔPaNip30 ΔPaTim54 and deletions.

Figure S2: Protein sequence alignment of PaTim54 from P. anserina, NcTim54 from N. crassa and

ScTim54 from Saccharomyces cerevisiae.

Figure S3: Measures of Green Fluorescence maximum intensities in hyphae of the *mito-GFP* control

strain and of the *PaTim54*<sup>2218</sup> *mito-GFP* strain.

