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1	Deletion of the epigenetic regulator GcnE in <i>Aspergillus niger</i> FGSC
2	A1279 activates the production of multiple polyketide metabolites
3	
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23	Declarations of interest: none.
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25 ABSTRACT

Epigenetic modification is an important regulatory mechanism in the biosynthesis of 26 27 secondary metabolites in Aspergillus species, which have been considered to be the treasure trove of new bioactive secondary metabolites. In this study, we reported that deletion of the 28 29 epigenetic regulator gcnE, a histone acetyltransferase in the SAGA/ADA complex, resulted in the production of 12 polyketide secondary metabolites in A. niger FGSC A1279, which 30 was previously not known to produce toxins or secondary metabolites. Chemical workup and 31 structural elucidation by 1D/2D NMR and high resolution electrospray ionization mass (HR-32 ESIMS) yielded the novel compound nigerpyrone (1) and five known compounds: 33 carbonarone A (2), pestalamide A (3), funalenone (4), aurasperone E (5), and aurasperone A 34 (6). Based on chemical information and the literature, the biosynthetic gene clusters of 35 36 funalenone (4), aurasperone E (5), and aurasperone A (6) were located on chromosomes of A. niger FGSC A1279. This study found that inactivation of GcnE activated the production of 37 38 secondary metabolites in A. niger. The biosynthetic pathway for nigerpyrone and its derivatives was identified and characterized via gene knockout and complementation 39 experiments. A biosynthetic model of this group of pyran-based fungal metabolites was 40 proposed. 41

42 Keywords

43 Histone acetyltransferase GcnE, Secondary metabolite, Epigenetic regulator, Polyketide,
44 Nigerpyrone

45 **1. Introduction**

The genus *Aspergillus* is a well-known producer of secondary metabolites (SMs) including polyketides, non-ribosomal peptides, indole terpenes and terpenes (Varga et al., 2003; Bok et al., 2006; Sanchez et al., 2012), which are important resources for new drug discovery (Rossano et al., 1999; Fisch et al., 2009). For example, bioinformatic analysis of four

available genomes of A. niger species (NRRL3, ATCC 9029) (Baker, 2006), ATCC1015 50 (Andersen et al., 2011), CBS513.88 (Pel et al., 2007), and SH2 (Yin et al., 2014), indicates 51 that A. niger encodes at least 81 putative SM biosynthetic gene clusters (Inglis et al., 2013) 52 for 42 polyketides (PKS), 33 non-ribosomal peptides (NRPS), four PKS-NRPS hybrids and 53 two indole alkaloids. The large number of putative SM biosynthetic gene clusters suggests 54 that A. niger has the potential to produce abundant SM products. However, most of these 55 56 putative gene clusters are silent in standard laboratory cultivation conditions (Fisch et al., 2009), and only limited SM products have been characterized from A. niger (Tanaka et al., 57 58 1966; Hiort et al., 2004; Serra et al., 2005; Nielsen et al., 2009; Sorensen et al., 2009; Chiang et al., 2011). 59

Epigenetic modification is an important regulatory mechanism in the biosynthesis of SM products in *Aspergilli* (Fisch et al., 2009), which could activate SM gene clusters and induce new SM products by changing the chromosomal region of SM gene clusters from a heterochromatic to a euchromatic state via histone demethylation and acetylation (Bayram et al., 2008; Fisch et al., 2009). For example, 10 novel SM compounds are induced by adding the epigenetic modification reagent 5-azacytidine (5-AZA) to *A. niger* ATCC1015 cultures (Fisch et al., 2009).

Spt-Ada-Gcn5-acetyltransferase (SAGA/ADA) complex is an epigenetic regulator in fungal secondary metabolism (Wu and Yu, 2015). GcnE (the Gcn5 homolog in *Aspergilli*) is a histone acetyltransferase (HAT) in SAGA/ADA complex that might regulate secondary metabolism by histone modification (Baker and Grant, 2007; Brakhage, 2013). Previous work illustrated that GcnE participates in increasing the acetylation level of histone H3 lysine K9 in *Aspergilli* (Reyes-Dominguez et al., 2008), and affects the biosynthesis of secondary metabolites. For example, *A. flavus* $\Delta gcnE$ mutant does not produce aflatoxin (Lan et al.,

2016). Deletion of the *gcnE* gene in *A. nidulans* decreases the production of orsellinic acid,
sterigmatocystin, penicillin and terrequinone (Nutzmann et al., 2011).

A. niger strain FGSC A1279 (*kusA*::DR-*amdS*-DR, *pyrG*⁻), a derivative of industrial strain NRRL3 (ATCC9029) that is used for gluconic acid production (Baker, 2006; Yuan et al., 2008; Carvalho et al., 2010; Arentshorst et al., 2015), is an SM-silent strain and has zero level of SM production. The low background of SM production in FGSC A1279 makes it ideal for investigating the role of epigenetic regulators on SM production. Moreover, genome sequences of NRRL3 (ATCC 9029) (Baker, 2006) and FGSC A1279 (Wang et al., 2017) are available for detailed gene information of the host strain.

In this study, we reported that deletion of the epigenetic regulator gcnE, a histone 83 acetyltransferase in the SAGA/ADA complex, resulted in the production of 12 polyketide 84 secondary metabolites in A. niger FGSC A1279. Chemical workup and structural elucidation 85 by 1D/2D NMR and high resolution electrospray ionization mass (HR-ESIMS) yielded the 86 novel compound nigerpyrone (1) and five known compounds: carbonarone A (2), 87 pestalamide A (3), funalenone (4), aurasperone E (5), and aurasperone A (6). Based on 88 chemical information and literature searches, the biosynthetic gene clusters of funalenone (4), 89 aurasperone E (5), and aurasperone A (6) were located on the chromosomes of A. niger 90 FGSC A1279. This study showed that inactivation of GcnE activated the production of toxins 91 92 and metabolites in A. niger. This is distinct from the role of GcnE in previous studies, 93 suggesting that the role of GcnE may have diverse effects on regulating SMs among different Aspergilli. The biosynthetic pathway of nigerpyrone and its derivatives was identified via 94 gene knockout and complementation experiments, and a biosynthetic model of this group of 95 96 pyran-based fungal metabolites was proposed.

97 **2. Material and methods**

98 2.1. Strains and culture conditions

All strains used in this study are in Table S1. Strains were maintained on potato dextrose 99 agar (PDA) medium (20 g dextrose, 15 g agar, infusion from 200 g potatoes per 1 L medium, 100 pH6.0) at 30 °C for spore harvest. For genetic transformation, CD medium (0.3% NaNO₃, 0.2% 101 KCl, 0.05% MgSO₄·7H₂O, 0.001% FeSO₄·7H₂O, 0.1% K₂HPO₄, 2.0% glucose, pH5.5) was 102 used. WATM, YES, and CYA media were used for SM profile analysis. WATM medium 103 was composed of 2.0 g/l yeast extract, 3.0 g/l peptone, 2.0 g/l dextrose, 30.0 g/l sucrose, 5.0 104 105 g/l corn steep solids, 2.0 g/l NaNO₃, 1.0 g/l K₂HPO₄·3H₂O, 0.5 g/l MgSO₄, 0.2 g/l KCl, 0.01 g/l FeSO4·7H₂O, pH7.0. YES medium was composed of 20 g/l yeast extract, 150 g/l sucrose, 106 107 pH 6.0. CYA medium was prepared on the basis of CD medium by adding 5 g/l yeast extract and 1 ml/l trace metal solution (1% ZnSO₄·7H₂O, 0.5% CuSO₄·5H₂O). For solid cultivation, 108 15 g/l agar was added. 109

An A. niger gcnE deletion mutant ($\Delta gcnE$) was constructed according to the method 110 developed by Szewczyk et al. (Szewczyk et al., 2006), based on homologous recombination 111 using a PCR amplified deletion cassette containing the upstream flanking arm (1.4 kb) of 112 gcnE, the selection marker pyrG (orotidine-5'-phosphate decarboxylase) and the downstream 113 flanking arm of gcnE. The pyrG gene was used for auxotrophic selection. Uridine 114 prototrophic transformants were selected and confirmed by PCR amplification (Fig. S1). 115 Primers for deletion cassette construction and transformant identification are listed in Table 116 S2. When required, 10 mM uridine was added to medium to maintain auxotrophy. The SM 117 backbone gene epaA was knocked out using the same method (Table S3). 118

119 2.2. Cultivation of A. niger $\Delta gcnE$ mutant for secondary metabolite collection

To collect secondary metabolites, the *A. niger* $\Delta gcnE$ mutant was cultivated for 7 days at 25 °C in the dark on 10 Petri dishes with WATM medium. Cultures were extracted with ethyl acetate (EtOAc) plus 1% formic acid for 24 hours. The crude extract was filtered and dried on a rotary evaporator at 38 °C.

124 2.3. SPE separation of A. niger \triangle gcnE crude SM extract

A C18 solid-phase extraction (SPE) column (SPE C18-E Giga Tube 20 g/60 ml, Strata, 125 Phenomenex, USA) was used to separate A. niger $\Delta gcnE$ crude extracts. The SPE stationary 126 phase was conditioned by sequential washing with 60 ml 100% methanol and 240 ml 100% 127 deionized water. A. niger $\Delta gcnE$ crude extract (4.0 g) was chromatographed with four 128 subfractions (480 ml each) generated stepwise from deionized water to methanol: 25% 129 130 methanol, 50% methanol, 100% methanol, and 100% methanol plus 0.1% trifluoroacetic acid (TFA). Subfraction 2 (50% methanol, 50 mg) and 3 (100% methanol, 410 mg) were further 131 132 purified by semi-preparative HPLC (semi-prep HPLC).

133 2.4. Semi-prep HPLC fractionation

Semi-prep HPLC was used to purify subfractions obtained by SPE separation. A semi-prep 134 HPLC column ACE C18-HL (250 mm × 10 mm i.d., Advanced Chromatography 135 Technologies, UK) was connected to an Agilent 1200 series binary pump and monitored by 136 an Agilent photodiode array detector. Detection was at 230, 254, 280, and 410 nm. Flow rate 137 was 1.5-2.0 ml/min. Solvent A consists of 95% H₂O, 5% methanol, and 0.05% TFA. Solvent 138 B was 100% acetonitrile. Separations were done at room temperature (20-25°C). Optimal 139 separation conditions were determined using analytical systems and then conditions were 140 adapted to the semi-preparative scale. 141

142 2.5. LC/HR-ESIMS assay of A. niger secondary metabolites

For LC/HR-ESIMS analysis, each semi-prep HPLC subfraction was dissolved in
MeOH:Milli-Q H₂O (9:1), and centrifuged at 10,000 rpm for 10 min. LC/HR-ESIMS analysis
was conducted as follows. High resolution mass spectrometric data were obtained using an
LTQ XL/LTQ Orbitrap Discovery MS system (Thermo Fisher Scientific, Waltham, MA,
USA) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA
autosampler, and Accela pump, C18 SunFire 150 × 4.6 mm Waters). Conditions used were:

149 capillary voltage 45 V, capillary temperature 320°C, auxiliary gas flow rate 10-20 arbitrary 150 units, sheath gas flow rate 40-50 arbitrary units, spray voltage 4.5 kV, and mass range 100-151 2000 amu (maximum resolution 30,000). For LC/HR-ESIMS, a Waters SunFire C18 152 analytical HPLC column (5 μ m, 4.6 × 150 mm) was used with mobile phase of 0-100% 153 MeOH over 30 min at a flow rate of 1 ml/min. LC/HR-ESIMS data were analyzed by 154 Xcalibur software followed by dereplication using Antibase2012 database.

155 2.6. NMR characterization of A. niger secondary metabolites

NMR spectra were used to elucidate the structure of A. niger secondary metabolites. ¹H 156 NMR, ¹³C NMR, COSY, HSQC and HMBC spectra were recorded on a Bruker Daltonics 157 Advance III HD 400 NMR spectrometer, using deuterated solvent methanol-d₄ (Cambridge 158 Isotopes Laboratories, USA). Chemical shift values were reported in parts per million (ppm) 159 with tetramethylsilane (TMS) as the external standard. Coupling constants were recorded in 160 Hertz (Hz). When higher field was needed, NMR spectra were recorded on a Bruker 161 Daltonics Advance III HD 600 NMR. NMR data were analyzed using MestReNova 9.0.1 162 software and ACD/I-Lab (http://ilab.cds.rsc.org/?cdsrdr=1). 163

164 2.7. Characterization of putative biosynthetic gene clusters for A. niger secondary 165 metabolites by gene knockout

Gene knockout of putative cluster genes for identified A. niger secondary metabolites was 166 done based on homologous recombination using a PCR-amplified deletion cassette 167 containing the upstream and downstream flanking arms of the target gene and the ptrA 168 (pyrithiamine (PT) resistance) selection marker. Primers for constructing deletion cassettes 169 are listed in Table S3. 0.5 µg/ml pyrithiamine (PT) was used to screen transformants. The 170 171 host strain for gene knockout was the A. niger FGSC A1279 $\Delta gcnE$ mutant. Gene deletion transformants were confirmed by PCR amplification using internal primers (Table S3 and Fig. 172 S1). 173

174 **3. Results and discussion**

175 3.1. Chemical diversity of A. niger FGSC A1279 ΔgcnE mutant

176 To investigate the effect of gcnE on A. niger secondary metabolism, we constructed a $\Delta gcnE$ mutant and a gcnE complementation (gcnE-com) strain using homologous 177 recombination (Fig. S1). Three types of solid media (WATM, CYA and YES) commonly 178 used for fungal secondary metabolite production were chosen (Frisvad and Thrane, 1987; 179 Rank et al., 2012) to investigate metabolite changes in the $\Delta gcnE$ mutant and gcnE-com 180 strain. Crude extract from cultures was subjected to LC/HR-ESIMS analysis. No compounds 181 were produced in A. niger FGSC A1279 cultures. Metabolite profiling by LC/HR-ESIMS 182 analysis demonstrated that multiple SMs were produced in all of three $\Delta gcnE$ mutant cultures 183 184 and the SM profile of gcnE-com strain was similar with the wild strain (Fig. 1), suggesting 185 that gcnE deletion activated the synthesis of SM products in A. niger. It has been reported that, in Aspergilli, histone acetylation usually correlates with transcriptional activation and 186 187 chromatin rearrangement (Baker and Grant, 2007; Reyes-Dominguez et al., 2008). The gcnE gene is an epigenetic regulator that is a key histone acetyltransferase in the SAGA/ADA 188 complex. In primary metabolism, GcnE increases the acetylation level of the proline 189 utilization cluster, as shown by chromatin immunoprecipitation in A. nidulans (Reyes-190 Dominguez et al., 2008). However, the role of GcnE might vary for different secondary 191 metabolite gene clusters in fungi. For example, inactivation of *gcnE* abolishes the production 192 of aflatoxin in A. flavus (Lan et al., 2016) and reduces the production of four known 193 metabolites (orsellinic acid, sterigmatocystin, penicillin and terrequinone) in A. nidulans, 194 suggesting that *gcnE* is required for transcription activation in these two *Aspergillus* strains 195 (Nutzmann et al., 2011). However, recent genome-wide ChIP-seq experiments revealed that 196 most SM cluster genes in A. nidulans are poorly decorated with activating heterochromatic 197 marks (histone demethylation and acetylation), even under inducing conditions (Connolly et 198

al., 2013; Gacek-Matthews et al., 2016), implying that gcnE may not be required for 199 transcriptional activation of certain SM gene clusters. Therefore, GcnE may have diverse 200 effects on regulating SMs among different Aspergilli. This result was in accordance with the 201 effects of histone deacetylase HdaA in A. nidulans. Deletion of HdaA induces the 202 biosynthesis of sterigmatocystin, penicillin, and norsolorinic acid in A. nidulans, but does not 203 change the expression of terraquinone A (Shwab et al., 2007). A similar phenomenon was 204 205 found in the plant pathogen Fusarium graminaerum. Deletion of heterochromatin protein 1 (hep1) in F. graminaerum leads to the activation of the aurofusarin gene cluster while 206 207 repressed the deoxynivalenol cluster (Gacek and Strauss, 2012).

208 3.2. Dereplication of newly produced compounds in A. niger ∆gcnE mutant

To further analyze the chemical diversity in the $\Delta gcnE$ mutant, we used WATM medium 209 since more SM products are likely to be produced on this medium (Fig. 1). Dereplication 210 using the Antibase database (H, 2012) led to the identification of 11 SM products in the A. 211 *niger* $\Delta gcnE$ mutant (Fig. 2 and Table S4), all of which were known fungal metabolites, 212 including 9 compounds discovered in *Aspergilli*. The ion peak with t_R (7.84) min was likely 213 to be carbonarone A, originally isolated from the fungal strain A. carbonarius WZ-4-11 214 (Zhang et al., 2007). Funalenone ($t_R = 8.42 \text{ min}$) belongs to polyketide compound group of 215 phenalenones which have diverse structures and biological activities (Gao et al., 2016). The 216 biosynthetic gene cluster of funalenones has been fully characterized (Gao et al., 2016). 217 218 Pestalamide A may have been eluted at t_R (10.10 min), which was isolated from fungal strain Pestalotiopsis theae W148 (Ding et al., 2008). The highly toxic metabolites fumonisin B2 (t_R 219 = 10.94 min) and B4 (t_R = 11.92 min) were detected in the A. niger $\Delta gcnE$ mutant. A. niger 220 221 NRRL3 (ATCC9029), the starting strain of FGSC A1279, is generally regarded as safe (GRAS) in the industry and should not produce any toxins or toxic compounds. This is 222 particularly true for A. niger FGSC A1279 which is used for gluconic acid production (Baker, 223

224 2006). These results indicate that deletion of *gcnE* had an epigenetic impact on its SM 225 production. Five polyketide derivatives (dimeric naphthopyrone family), aurasperone E ($t_R =$ 226 13.70 min), fonsecinone B ($t_R = 14.50$ min), isoaurasperone A ($t_R = 14.77$ min), aurasperone 227 A ($t_R = 15.66$ min) and asperpyrone C ($t_R = 16.33$ min) were also found in the extracts of the 228 *A. niger* $\Delta gcnE$ mutant (Fig. 2). Taken together, these results indicated that the newly 229 identified compounds were likely to be polyketide metabolites.

230 3.3. Structural elucidation by HR-ESIMS and 1D/2D NMR

To verify the identity of the newly emerged metabolites in the $\Delta gcnE$ mutant, the strain was cultivated on WATM medium at 2-L scale and crude extract was subjected to chemical workup and semi-prep HPLC purification, yielding six pure compounds **1** (3.7 mg), **2** (1.8 mg), **3** (7.8 mg), **4** (9.2 mg), **5** (1.5 mg), and **6** (3.0 mg).

The molecular formula of nigerpyrone (1) was established as $C_{12}H_{10}O_2$ by HR-ESIMS 235 (187.07 [M+H]⁺). Dereplication using the Antibase database suggested that this might be a 236 new compound. The molecular formula suggested eight degrees of unsaturation. The ¹³C and 237 HSQC NMR data of 1 in CD₃OD (Fig. S2) revealed one benzene/aromatic ring (δ_{C} 128.38-238 136.30 ppm), one carbonyl (δ_{C} 178.14 ppm), and four olefinic carbons (δ_{C} 117.36-117.52 239 ppm and 158.45-169.48 ppm), consistent with the presence of five double bonds and one ring 240 system. This analysis accounted for seven double bonds, suggesting the presence of an 241 additional ring in the structure of **1**. Analysis of the ¹H-¹H COSY spectrum revealed two 242 contiguous spin systems, one consisting of H-10 through H-14, and the other consisting of H-243 2 to H-3 (Fig. 3 and Fig. S2). The connection of these two spin systems at C-6 was evident by 244 the HMBC correlation of H-5 and H-8 to C-6 (Fig. 3), which was further confirmed by the 245 correlation of H-8 to C-5 in the HMBC spectrum. The connection of methylene to the 246 benzene ring was confirmed by the HMBC correlation of H-8 to C-9 (Fig. 3). The structure 247 was further confirmed by comparing ¹³C experimental data with published data (Dai et al., 248

2007) and predicted ¹³C NMR data (Elyashberg et al., 2010) (Fig. S2). The resulting r^2 value 249 of 0.9992 indicated that the proposed structure was correct (Bremser, 1978; Elyashberg et al., 250 2010). Inspection of the ¹H, ¹³C, and HR-ESIMS data of compounds **2** and **3** (Fig. S3 and S4) 251 indicated that both 2 and 3 were known compounds (Zhang et al., 2007; Ding et al., 2008): 252 carbonarone A (2) was isolated from A. carbonarius WZ-4-11 (Zhang et al., 2007) and 253 pestalamide A (3) was isolated from *Pestalotiopsis theae* W148 (Ding et al., 2008). 254 Comparison of ¹H and ¹³C NMR spectra of compounds **1**, **2** and **3** (Fig. S2, S3 and S4) 255 confirmed the structure of the new compound 1. 256

Inspection of the ¹H, ¹³C and HR-ESIMS data of compounds **4** (Fig. S5), **5** (Fig. S6) and **6** (Fig. S7), indicated that they were known. Compound 4 is produced by *A. niger* FO-5904) (Inokoshi et al., 1999), 5 by *A. niger* CMI-IMI 205879 (Priestap, 1984), and 6 by *A. fonsecaeus* NRRL 67, O 16-1 (Priestap, 1984).

261 3.4. Putative biosynthetic gene clusters of compounds from the $\Delta gcnE$ mutant

Homologous BLAST search and comparison of the literature allowed the identification of 262 the putative biosynthetic gene clusters of known compounds discovered from the $\Delta gcnE$ 263 mutant (Table S5). Genes for synthesizing funalenone (4) were identified in Penicillium 264 herquei (Gao et al., 2016). By homologous search against the annotated genome of A. niger 265 CBS513.88, we identified five homologous genes in A. niger responsible for the biosynthesis 266 of funalenone (Fig. S8 and Table S5). The literature (Chiang et al., 2010) indicated that 267 268 monodic typhenone might be the precursor of aurasperone E(5), aurasperone A(6) and other derivatives such as fonsecinone B, isoaurasperone A, and asperpyrone C. In A. nidulans, the 269 key biosynthetic enzyme of monodictyphenone is proposed to be a non-reducing polyketide 270 271 synthase (NR-PKS) *mdpG* (Chiang et al., 2010). We therefore performed a homologous search of the A. niger genome using mdpG as the reference query, and identified an open 272 reading frame (ORF) An11g07310 located in contig 11, annotated as a NR-PKS with 273

multiple domains of KS-AT-PT-PP (Table S5). Other proposed biosynthetic genes involved in the biosynthesis of **5** and **6** were not located in the close proximity of An11g07310 and were scattered in other genomic loci. However, genes for secondary metabolites are generally tightly clustered.

278 3.5. Characterization of key biosynthetic genes for pestalamide A

Inspection of the structures of 1-3 led to speculation that their precursors contain one 279 280 phenylacetate and two acetate units, suggesting that compounds 1-3 may be synthesized via a polyketide biosynthetic pathway. The incorporation of phenylacetate in fungal secondary 281 282 metabolism is also observed in the biosynthesis of the β -lactam antibiotic penicillin G in Penicillium chrysogenum (Koetsier et al., 2009). In vitro assays show that the gene product 283 PhIB is a phenylacetyl-CoA ligase responsible for the activation of phenylacetate into 284 phenylacetyl-SCoA (Koetsier et al., 2009). We performed a homolog search of PhIB in A. 285 niger, and identified ORF An09g01820, annotated as a ferulate: CoA ligase (AMP-forming) 286 (Fig. 4A and Table S6), with moderate sequence identity (35%) to PhlB. 287

Analysis of genes in proximity to An09g01820 (epaB) identified a candidate NR-PKS gene 288 cluster (epa) spanning a 23.9-kb genomic region (Fig. 4A and Table S6). The epa cluster 289 possesses six ORFs (Table S6). In silico analysis indicated that epaA (An09g01860) encodes 290 a typical Clade III NR-PKS (2,617 amino acids in length) with a domain organization of 291 SAT-KS-AT-PT-ACP-Met-R domains, which shares high amino acid sequence identity (97%) 292 293 with AzaA polyketide synthase (2,599 amino acids in length) in the biosynthesis of azaphilones from A. niger ATCC 1015 (Zabala et al., 2012). The gene product EpaC is 294 annotated as an acyl-CoA transferase. Adjacent to epaA, there are three genes, orf1-3, 295 296 encoding oxidoreductase, 3-hydroxybenzoate 4-hydroxylase, and salicylate hydroxylase 297 (Table S6).

To determine if the epa cluster was responsible for synthesizing pestalamide A (3), we 298 knocked out the PKS gene (An09g01860, epaA) by homologous recombination (Table S3 299 and Fig. S1). Inactivation of the epaA gene abolished the production of 1-3 (Fig. 4B), and 300 complementation strain (epaA-com) could produce compound 1-3 (Fig. S1 and Fig. 4B), 301 suggesting that the identified biosynthetic genes were responsible for the synthesis of 1-3. We 302 also performed cell-free extract experiments, in which all biosynthetic enzymes and cofactors 303 304 were available for the biosynthesis of 3, to test if 1 and 2 were the biosynthetic precursors of 3 (data not shown). Feeding purified 1 and 2 into cell-free extracts of the $\Delta gcnE$ mutant did 305 306 not produce 3, suggesting that 1 and 2 were by-products in the biosynthesis of 3. Based on the genetic and cell-free extract experiments, a biosynthetic model for 1-3 is proposed in Fig. 307 4C. We propose that the biosynthesis of 1-3 begins with the polyketide assembly by EpaA to 308 309 form phenylacetyl triketide precursor from successive condensation of two malonyl-CoA, presumably with one phenylacetyl-CoA starter unit. For the nigerpyrone (1) biosynthesis, the 310 reactive polyketide chain is released as an aldehyde (8) through the R-domain, in a manner 311 similar to the previously characterized 3-methylorcinaldehyde synthase in Acremonium 312 strictum (Bailey et al., 2007). The cyclization and dehydration of 8 may create nigerpyrone 313 (1). This nonenzymatic rearrangement to form pyrone moiety from aldehyde was also 314 observed in the plant metabolites, arabidopyl derivatives (Weng et al., 2012). For the 315 biosynthesis of 2 and 3, an extra methyl group will be added through the C-methyltransferase 316 317 domain, followed by the reduction to generate 8a, which undergoes oxidation and transamination to produce the intermediate 9. The candidate gene products for this series of 318 biotransformation could be orf1-3 (Table S6). The cyclization of 9 produces carbonarone A 319 320 (2). We propose that EpaC (An09g01800), an acyl-CoA transferase, could catalyse the transfer of 2-methylsuccinyl-CoA, a common intermediate in the ethylmalonyl-CoA pathway 321 (Erb et al., 2009), to generate the final product pestalamide A (3). 322

323 **4. Conclusions**

In conclusion, we confirmed that deletion of gcnE resulted in the production of 12 324 polyketide metabolites in an A. niger FGSC A1279 $\Delta gcnE$ mutant. Chemical workup of 325 organic extracts of the culture broth from the $\Delta gcnE$ mutant identified six pure compounds, 326 including one new compound nigerpyrone (1) and five known fungal polyketide metabolites 327 (2-6). The structures of these compounds were confirmed by HR-ESIMS and 1D/2D NMR. 328 Finally, the biosynthetic gene cluster for 1-3 was verified via gene knockout and 329 complementation, and a biosynthetic model of this group of pyran-based fungal metabolites 330 was proposed. To our best knowledge, this was the first time to confirm that the inactivation 331 of gcnE resulted in activating the biosynthesis of polyketide metabolites in A. niger FGSC 332 333 A1279.

334

335 **Declarations of interest**

336 None.

337

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346 **References**

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492 Figures



Figure 1. SM profile of *A. niger***FGSC A1279** *AgcnE* **mutant cultivated on different media.** Blue curve, $\Delta gcnE$ mutant; red curve, wild strain; green curve, gcnEcomplementation strain (*gcnE*-com). To collect secondary metabolites, *A. niger* strains were cultivated for 7 days at 25 °C in the dark using different media. The crude extract was dissolved in MeOH:Milli-Q H₂O (9:1) and centrifuged at 10,000 rpm for 10 min, before LC/MS analysis.



Figure 2. SM products detected in *A. niger ΔgcnE* mutant cultivated on WATM medium. *A. niger* ΔgcnE mutant was cultivated for 7 days at 25 °C in the dark on WATM medium.
High resolution mass spectrometric data were obtained using an LTQ XL/LTQ Orbitrap
Discovery MS system coupled to a Thermo Instruments HPLC system. LC/MS data were
analyzed by Xcalibur software with Antibase2012 database. Compounds that were purified
by chemical workup and characterized by spectroscopic analysis were marked in bold.

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Figure 3. Secondary metabolites isolated and characterized by HR-ESIMS and 1D/2D
 NMR in *A. niger* Δ*gcnE* mutant. *A. niger* Δ*gcnE* mutant was cultivated on WATM medium.
 ¹H NMR, ¹³C NMR, COSY, HSQC and HMBC spectra were recorded on a Bruker Daltonics

Advance III HD 400 NMR spectrometer, using deuterated solvent methanol-d4. Chemical shift values were reported in parts per million (ppm) with tetramethylsilane (TMS) as the external standard. When higher field was needed, NMR spectra were recorded on a Bruker Daltonics Advance III HD 600 NMR. NMR data were analyzed using MestReNova 9.0.1 software and ACD/I-Lab (http://ilab.cds.rsc.org/?cdsrdr=1). Compound structures were drawn using ChemBioDraw software. Chemical formulas and exact masses are marked for each compound. Dereplication data are in online supplemental file (Fig. S2-S7).



Figure 4. Characterization of the biosynthetic cluster for pestalamide A. (A) Putative gene cluster of compounds 1-3, which was identified by the homologous BLAST search of *P*. *chrysogenum* PhIB gene. (B) LC/MS detection of compounds 1-3 in gene knockout and complementation strains. Retention time for purified compounds 1-3 was determined by LC/MS. *A. niger* strains were cultivated on WATM medium. (C) Proposed model of the biosynthesis of nigerpyrone (1), carbonarone A (2) and pestalamide A (3).

526 Supplemental materials

- 527 **Table S1.** Strains and plasmids used in this study
- 528 Table S2. Primers used for *gcnE* deletion-cassette construction and transformant
 529 identification
- **Table S3.** Primers used to knock out *epaA* (An09g01860) and verify mutants
- **Table S4.** Putative compounds detected in *A. niger* FGSC A1279 $\Delta gcnE$ mutant based on
- 532 LC/MS data and Antibase database
- **Table S5.** Putative biosynthetic gene clusters for known compounds detected in the $\Delta gcnE$ mutant
- 535 **Table S6.** Putative biosynthetic gene cluster for pestalamide A.

- **Figure S1.** Design of deletion cassettes, complementation cassettes, and PCR verification of
- 538 *A. niger* mutants ($\Delta gcnE$, $\Delta gcnE\Delta epaA$, gcnE complementation and epaA complementation).
- 539 Figure S2. LC/MS and NMR data for the novel compound nigerpyrone
- 540 Figure S3. LC/MS and NMR data of compound carbonarone A
- 541 Figure S4. LC/MS and NMR data of compound pestalamide A
- 542 Figure S5. LC/MS and NMR data of compound funalenone
- 543 Figure S6. LC/MS and NMR data of compound aurasperone E
- 544 Figure S7. LC/MS and NMR data of compound aurasperone A
- 545 **Figure S8.** Putative biosynthetic gene clusters of the known compounds in the $\Delta gcnE$ mutant